SUPPLMENTAL MATERIAL

Gene therapy for catecholaminergic polymorphic ventricular tachycardia by inhibition of Ca^{2+}/calmodulin-dependent kinase II

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EXPANDED METHODS

Mice

All procedures with mice were performed under protocols approved by the Boston Children's Hospital Institutional Animal Care and Use Committee. *Ryr2*$^{R176Q/+}$ CPVT mice were described previously[1]. The investigators performing mouse assays and data analysis were blinded to genotype and treatment group.

AAV production and administration

The AAV backbone plasmid pENN.AAV.cTNT.PI.eGFP.WPRE.rBG, (AAV-cTNT-GFP, Addgene plasmid # 105543) was a gift from James M. Wilson. We annealed AIP oligos (Table I online-only data supplement) and cloned the double stranded insert into AAV-cTNT-GFP using BsrGI and KpnI. Sequencing confirmed the proper insertion and frame with GFP. The backbone vector AAV-cTNT-GFP was used as a control. To produce AAV9-GFP-AIP and AAV9-GFP, one hundred and forty micrograms of AAV-ITR, 140 µg AAV9-Rep/Cap, and 320 µg pHelper (pAd-deltaF6, Penn Vector Core) plasmids were produced by Maxiprep (Invitrogen, K210017) and transfected into 10 15-cm plates of HEK293T cells using PEI transfection reagent (Polysciences, 23966-2). Sixty hours after transfection, cells were scraped off of plates, resuspended in lysis buffer (20 mM Tris, pH 8, 150 mM NaCl, 1 mM $MgCl_2$, 50 µg/ml benzonase) and lysed by three freeze-thaw cycles. AAV in cell culture medium was precipitated by PEG 8000 (VWR, 97061-100), resuspended in lysis buffer, and pooled with cell lysates. AAV was purified in a density gradient (Cosmo Bio USA, AXS-1114542) by ultracentrifugation (Beckman, XL-90) with a VTi-50 rotor and concentrated in phosphate-buffered saline (PBS) with 0.001% pluronic F68 (Invitrogen, 24040032) using a 100 kDa filter tube (Fisher Scientific, UFC910024). AAV titer was quantified by qPCR (primer sequences in Table II online-only data supplement) using a fragment of the TNT promoter DNA to make a standard curve.

AAV was injected into P3 pups subcutaneously with a total volume of less than 50 µl. Retro-orbital injection was performed to inject AAV into older animals with a volume of less than 250 µl per eye. AAV dosage was normalized based on body weight at both neonatal and adolescent stages. For neonatal studies the dosage range was from 5 x 10^9 viral genomes per gram body weight (vg/g) to 5 x 10^{11} vg/g. For the adolescent cohort, a dose of 1 x 10^{11} vg/g was used.

Demonstration of inhibition of cardiac CaMKII by AAV9-GFP-AIP

P3 mouse pups were injected with either AAV9-GFP or AAV9-GFP-AIP at a dose of 5 x 10^{11} vg/g as described. At P10, the animals were either administered saline or isoproterenol at 4mg/kg by intraperitoneal injection. After 5 minutes the animals were sacrificed, and the hearts immediately flash frozen in liquid nitrogen. Whole heart lysates were made by homogenization of heart tissue in lysis buffer (120mM NaCl, 40mM HEPES, 1mM EDTA, 40mM NaF, 10mM β-glycerophosphate, 0.3% CHAPS at pH 7.5, 1% Triton-X-100 and protease/phosphatase Inhibitor cocktail). Western blots were performed as described below and probed using antibodies against total CaMKII, pT286-CaMKII, and GAPDH (Table II in the online only Data Supplement).

Electrophysiology Studies

Animals underwent electrophysiology studies at 2-4 months of age. After anesthesia with isoflurane, sharp platinum needle electrodes (Grass) were inserted into each limb to obtain a lead I (right upper extremity to left upper extremity) and a lead II (right upper extremity to left lower extremity). We

performed a right jugular vein cutdown and inserted a 1.1 Fr octapolar catheter (ADInstruments) into the right ventricle. Surface ECG and intracardiac signals were digitized at 4 kS/s and recorded (PowerLab software, ADInstruments). Confirmation of proper catheter placement was performed by steady-rate pacing at 600 beats per minute (bpm) with a corresponding widening of the QRS on surface ECG. Next we administered isoproterenol (2 mg/kg) and epinephrine (3 mg/kg) intraperitoneally and recorded resulting arrhythmia for 3 minutes. More than three PVCs or a single couplet during the recording period was considered a positive response. We performed programmed ventricular stimulation using a digital stimulator (ADInstruments) at twice the capture threshold by delivering 8 stimuli (S1 x 8) at a rate 10% higher than the resting heart rate followed by two early extra-stimuli (S2 and S3). The coupling intervals were decreased by 10 msec intervals to 40 msec and then by 5 msec steps until either ventricular arrhythmia was induced or there was the loss of ventricular capture. If there was a loss of capture above 40 msec then the output was increased or the catheter was repositioned. Ventricular arrhythmia was defined at three or more induced ventricular beats. The duration of arrhythmia was measured from the last paced beat.

Isolation of murine ventricular cardiomyocytes.

Heterozygous knock-in *Ryr2*$^{R176Q/+}$ or WT mice and their wild-type littermates of either sex and 8–12 weeks-old were used for the isolation of ventricular cardiomyocytes. Ventricular cardiomyocytes were isolated by retrograde perfusion through the aorta using a standardized enzymatic digestion protocol [2] with minor modifications. Briefly, mice were injected with heparin before euthanasia. Hearts were isolated, perfused and digested with collagenase II (Worthington) using Langendorff apparatus at 37°C. Once the heart is properly dissociated, ventricles were removed from the cannula, gently rubbed by a pair of tweezers, and pipetted repetitively to collect isolated CMs. Cell suspension was passed through a 100 μm cell strainer to remove clumps and then centrifuged at 20x g. Cell pellet was gently resuspended in perfusion buffer containing 60 μM $CaCl_2$ and CMs were left to settle to the bottom by gravity. Intracellular calcium storage was gradually restored by transferring CMs to perfusion buffer containing increasing $CaCl_2$ (400 μM, 900 μM, 1.2 mM, respectively) every 10 min. Eventually CMs were collected by centrifugation at 20x g and resuspended in plating medium (MEM supplemented with 10% FBS and 10 mM butanedione monoxime (BDM)) and plated to laminin-coated coverslips. One hour later, plating medium was changed to warm culture medium (MEM supplemented with 100 μg/ml BSA and 10mM BDM). CMs were incubated with 4μM Rhod-2 for 30 min before imaging.

Immunofluorescent staining of cultured cells

Cultured iPSCs or differentiated iPSC-CMs were seeded on gelatin and Geltrex-coated glass coverslips. Cells were fixed by 4% paraformaldehyde for 15 minutes, washed with PBS, and then blocked and permeabilized with 5% donkey serum plus 0.2% Triton X-100 at 4°C overnight. Samples were then incubated with primary antibodies (Table II, online-only data supplement) for at least 3 hours at room temperature or overnight at 4°C. After washing, slides were incubated with Alexa Donkey secondary antibodies (Life Technologies). Imaging was performed on an Olympus FV3000 confocal microscope.

Histology

Tissues were fixed in 4% paraformaldehyde overnight and allowed to sink in 30% sucrose (typically 3-4 hours) prior to freezing in OCT. 8-10 μm thick cryosections were affixed to slides. Samples were permeabilized with 0.5% Triton X-100 and blocked overnight at 4°C with 4% donkey serum plus 1% BSA. Primary antibodies (Table II, online-only data supplement) were incubated with tissue sections for 4-6

hours at RT and washed with PBS prior to incubation with secondary antibodies or phalloidin. Tissue sections were imaged with the Keyence automated microscope.

Gene Expression

For western blot analysis, protein lysates were separated by SDS-PAGE using on 4–12% gradient gels (Invitrogen, Bolt gels, NW04122BOX) and transferred to a polyvinylidene difluoride (PVDF) membrane, using a semi-dry transfer method for 45 minutes at 15V. Membranes were blocked with 5% milk/TBST (Tris-buffered saline, 0.1% Tween-20) for one hour and incubated with primary antibodies (Table II in the online-only Data Supplement) either for three hours at room temperature or overnight at 4 degrees with agitation. Horseradish peroxidase (HRP)-conjugated secondary antibodies were probed for 1–2 h at room temperature, followed by four 15 min TBST washes. After adding Immobilon Western Chemiluminescent HRP Substrate (Millipore, WBKLS0500), chemiluminescence. Imaging and quantification were performed with a digital imager (ImageQuant LAS 4000, GE).

For analysis of phosphorylated and total phospholamban, whole heart lysates were normalized to 0.25mg/ml and loaded at a final concentration of 0.2mg/ml on the capillary western assay plate for the WES automated western machine (Protein Simple). Antibodies for phospholamban and phospho-phospholamban were optimized and loaded at dilutions of 1:500 and 1:2500 respectively. HRP-conjugated secondary antibodies were added per manufacturer's recommendations. Quantification and band-selection were performed by protein analysis software Compass (Protein Simple).

Total RNA was isolated using TRIzol (ThermoFisher). Quantification was performed by RT-qPCR using a Bio-Rad thermocycler with Sybr Green chemistry (iTaq, Bio-Rad). *Tbp* and *Hprt* was used as an internal control Primers are shown in Table I in the online-only Data Supplement. Relative mRNA expression was normalized to internal gene control levels (*Tbp or Hprt*) and the expression level in the heart for each animal (delta-delta).

Modified RNA production and transfection of adult cardiomyocytes

We synthesized the CN19o -P2A-mcherry construct (Genewiz, supplemental table III) and cloned it into vector pTEMPLZ-Luc carrying two UTRs that flank the gene-coding sequences[3]. We PCR amplified the polyA-containing PCR products as a template for *in-vitro* transcription (IVT). Using 1.6µg of purified tail PCR product and a combination of 3'-O-Me-m7G(5')ppp(5')G cap analog (New England Biolabs), psudouridine-5'-triphosphate (Trilink), ATP and GTP, we performed IVT with the MEGAscript T7 kit (Ambion) as per manufacturer's instructions. The RNA was then purified by MEGAclear Transcription Clean-Up kit (Ambion) and treated with phosphatase for 1 hour prior to final RNA purfication.

Transfection of isolated adult cardiomyocytes was performed using RNAiMax (Invitrogen). 5µl of 400ng/µl modRNA was diluted in 40µl of Opti-MEM and combined with equal volume of Opti-MEM containing RNAiMax. Mixture was incubated at room temperature for 5 minutes and 12.5µl of RNA-RNAiMax was added to each 0.5ml of culture medium in 48well plate. Adult cardiomyocytes were assayed 12-18 hours after transfection for transgene expression.

iPSC generation and iPSC-CM differentiation

Patients with a clinical diagnosis of CPVT and pathogenic RYR2 mutation consented to participate in this study under protocols approved by the Boston Children's Hospital Institutional Review Board (IRB). Peripheral blood mononuclear cells were isolated from a peripheral blood sample and reprogrammed to pluripotency using the CytoTune Sendai reprogramming kit (ThermoFisher). At least four reprogrammed colonies were selected and sequenced to confirm the presence of the reported clinical mutation(s).

These colonies were then stained for the pluripotency markers Oct4 and SSEA4 (Table II in the online-only Data Supplement) and karyotyped (Cell Line Genetics). All of the iPSC lines were maintained in mTeSR1 medium (STEMCELL Technologies) and passaged in versene solution (15040066, Thermo Fisher Scientific) every three to five days. Culture dishes were pre-coated with Geltrex (ThermoFisher), diluted 1:200.

After at least 20 passages, human iPSCs were seeded onto Geltrex-coated dishes at 150,000 cells/ml for iPSC differentiation to iPSC-CMs based on the timeline shown in Figure IV-A of the online-only data supplement. On day 3 of culture, mTeSR1 medium was removed, cells were rinsed once with PBS (without Ca^{2+} or Mg^{2+}), and cultured in Differentiation Medium (RPMI medium (11875093, Thermo Fisher Scientific) with B27 without insulin (A1895601, Thermo Fisher Scientific)) and containing 8-10 µM CHIR99021 (72054, STEMCELL Technologies). After 48 hours, the medium was changed to differentiation medium without CHIR99021. At differentiation day 3, cells were cultured in differentiation medium containing 5 µM IWR-1 (3532, Tocris). After 48 hours, cells were cultured in differentiation medium without IWR until day 15, with media changes every 2 days. At day 15, the cells were cultured in Selection Medium (Non-Glucose DMEM (11966025, Thermo Fisher Scientific) with 0.4 mM Lactate (# L7022, Sigma Aldrich) for 4 days to enrich for iPSC-CMs. To isolate single iPSC-CMs for Ca^{2+} imaging, cells were incubated in collagenase 1 (Sigma C-0130, 100 mg collagenase 1 in 50 ml PBS/20% FBS) for 45 minutes, followed by a 0.25% Trypsin incubation at 37°C for 5-10 mins. 50% FBS in DMEM with 50 µg/ml DNase I (# 260913, EMD Millipore) was used to stop trypsinization. The iPSC-CMs were suspended in Culture Medium (RPMI: DMEM 1:1, plus B27 without insulin) containing 5% FBS and 10 µM Y27632.

Ca^{2+} imaging of single cells and cell clusters

IPSC-CMs were seeded on 0.1% gelatin and Geltrex-coated coverslips. After 24 hours, cells were transduced with adenovirus that expresses the GCaMP6f-Junctin nanosensor (G6-JT). After 48 hours, the coverslips were placed in an imaging chamber (IonOptix) and a temperature of 36-37°C was maintained by using a mini-peristaltic pump to circulate extracellular buffer through an in-line heater regulated by a closed-loop controller. The samples were imaged on an Olympus FV3000R using line scan mode (2 msec/line, 15000 lines per recording). The scan line was positioned within individual iPSC-CMs through the longest dimension to maximize recording cell area. Extracellular buffer containing (in mM) NaCl 140, KCl 4, $MgCl_2$ 2, $CaCl_2$ 1.2, HEPES 10, Glucose 15, and sodium pyruvate 2, with pH of 7.4, was supplemented with 600 nM myristolated Autocamtide-2-related Inhibitory Peptide (SCP0001 Sigma) or 600nM myristolated PKA 14-22 inhibitory peptide (47648, Millipore) and or 1 µM isoproterenol. Drugs were circulated through the imaging chamber for at least 5 minutes prior to imaging.

Table 1. Oligonucleotides used in this study

Name	Description	Sequence
AIP-F	AIP cloning	GTACAAGAAGGCCCTGCATCGCCAGGAGGCCGTGGACTGTCTCTAAGGTAC
AIP-R	AIP cloning	CTTAGAGACAGTCCACGGCCTCCTGGCGATGCAGGGCCTTCTT
TNNT2-F	AAV titer	TCGGGATAAAAGCAGTCTGG
TNNT2-R	AAV titer	CCCAAGCTATTGTGTGGCCT
TBP-F	QPCR	GCCAGCTTCGGAGAGTTCTGGGATT
TBP-R	QPCR	CGGGCACGAAGTGCAATGGTCTTTA
GFP-F	QPCR	ACGTAAACGGCCACAAGTTC
GFP-R	QPCR	AAGTCGTGCTGCTTCATGTG
HPRT-F	QPCR	TCAGTCAACGGGGGACATAAA
HPRT-R	QPCR	GGGGCTGTACTGCTTAACCAG
AIP-F	QPCR	CTTCAAGATCCGCCACAACAT
AIP-R	QPCR	TTAGAGACAGTCCACGGCCTC

Table 2. Antibodies used in this study

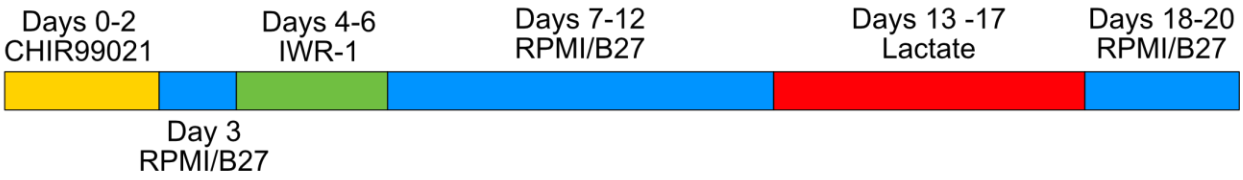
Antigen	Company	Cat. No.	Dilution	Application
OCT4	Santa Cruz	SC8628	1:200	IF
SSEA4	Millipore	MAB4304	1:200	IF
TNNI3	Abcam	ab56357	1:200	IF
RYR2	Abcam	ab2827	1:400	IF
GFP	Rockland	600-101-215	1:400	IF
NeuN	Abcam	ab177487	1:1000	IF
Phalloidin Alexa 647	ThermoFisher	A22287	1:400	IF
CaMKII	Abcam	ab134041	1:1000	WB
P-CaMKII (Thr286)	Cell Signaling	12716S	1:1000	WB
GAPDH	Santa Cruz	sc-25778	1:2000	WB
P-Phospholamban (Thr17)	Badrilla	A010-13	1:2500	WB
Phospholamban	Cell Signaling	14562S	1:500	WB

Table 3. Open reading frame sequence for modRNA production

Name	Sequence
Cn19o-P2A-mCherry	GACTCACTATAGGCTAGCCTCGAGAATTCACGCGGGCCGCCATGGCCAAGCGAGCCCC CAAGCTGGGCCAGATCGGCCGACAGAAGGCCGTGGACATCGAGGATGCTACTAACTTC AGCCTGCTGAAGCAGGCTGGCGACGTGGAGGAGAACCCTGGACCTGGTCTCCGGCCG ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTC AAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGC GAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGC CCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTAC GTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAA GTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTC CTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCT CCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGA TGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGG ACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGC AGCTGCCCGGCGCCTACAACGTCAATATCAAGTTGGACATCACCTCCCACAACGAGGAC TATACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGG ACGAGCTGTACAAGTAGGCTCGCGTGGTACCTCTAGAGTCGAC

SUPPLEMENTAL FIGURES

A



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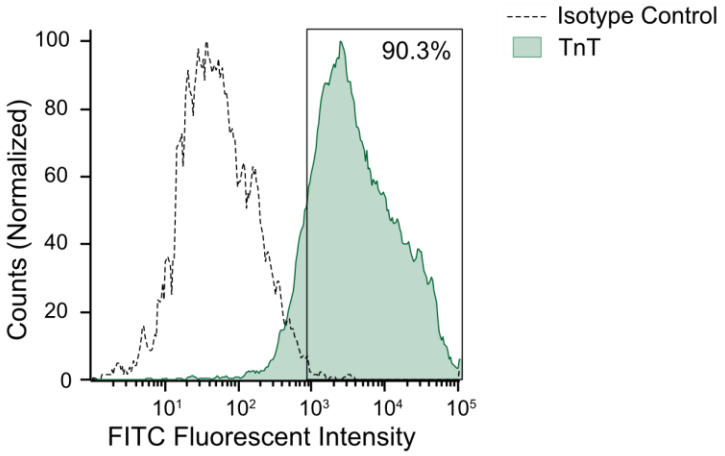
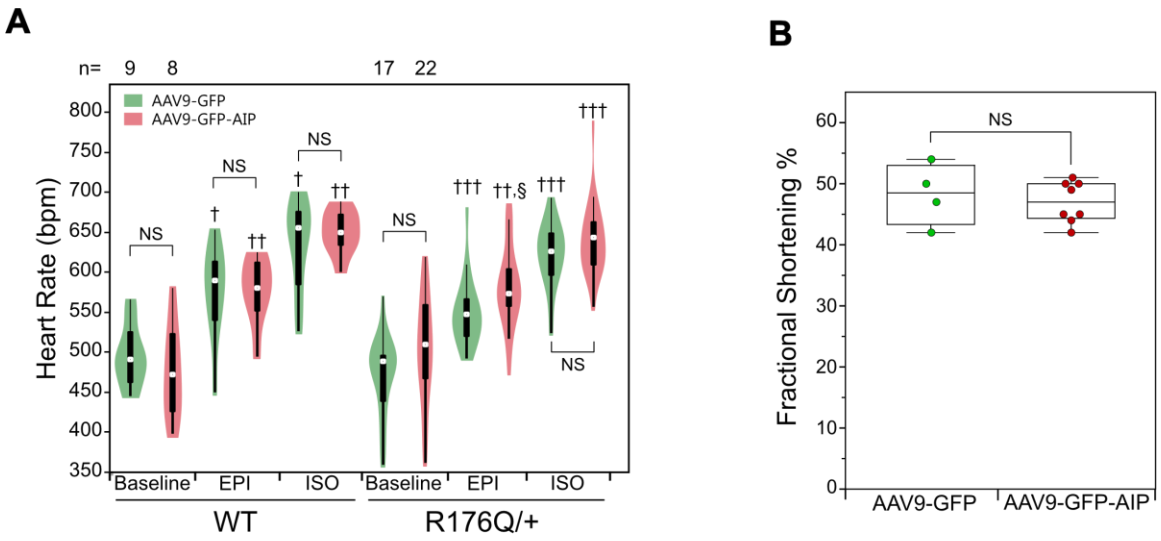
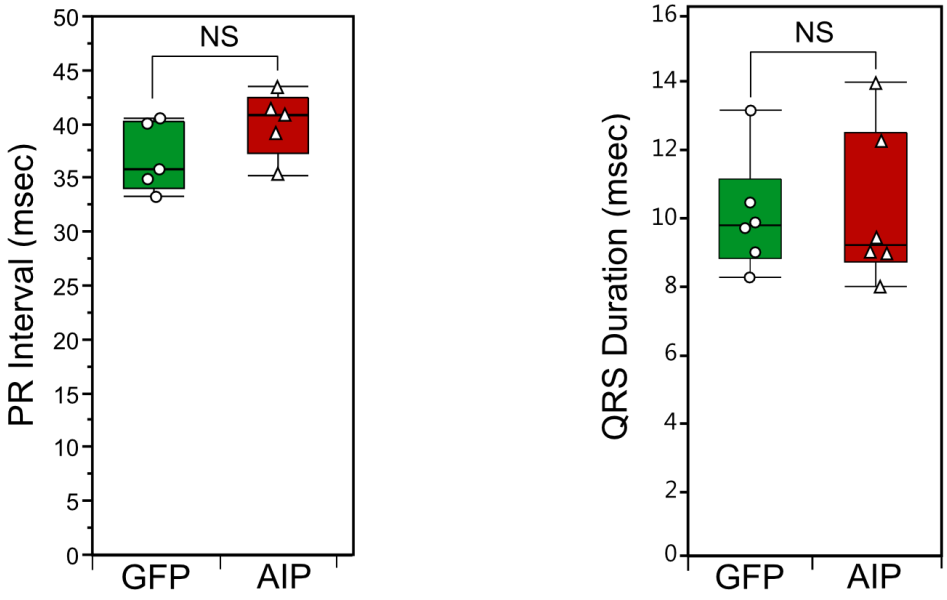


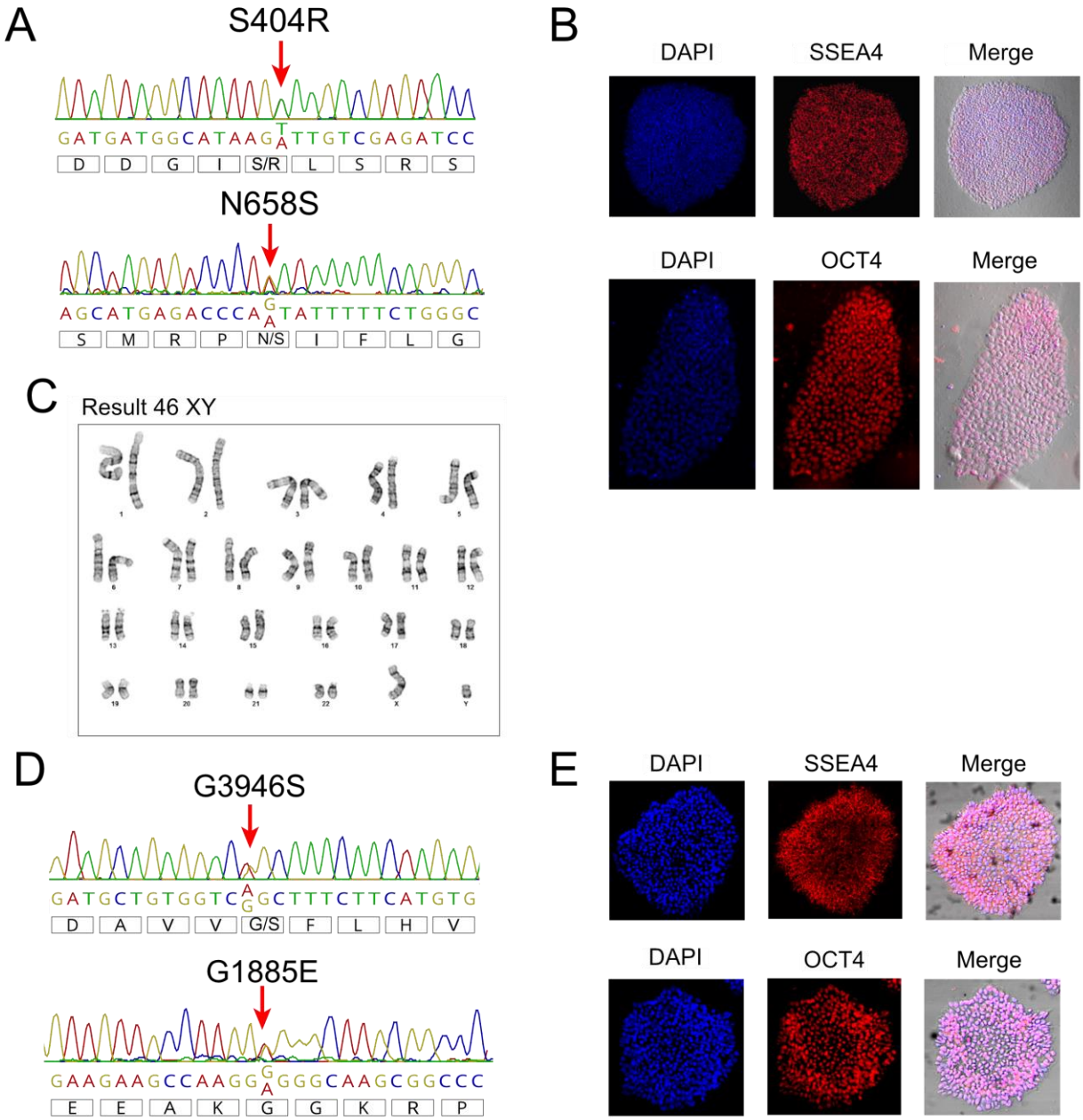
Supplemental Figure 1. Differentiation of iPSCs to iPSC-CMs. A. Schematic of differentiation protocol including lactate selection. **B.** Differentiated iPCS-CMs were isolated into a single cell suspension, fixed and stained with FITC-conjugated antibody against cardiac troponin-T (TnT). IPSC-CMs were analyzed by flow-cytometry. A representative result is shown with appropriate isotype control.



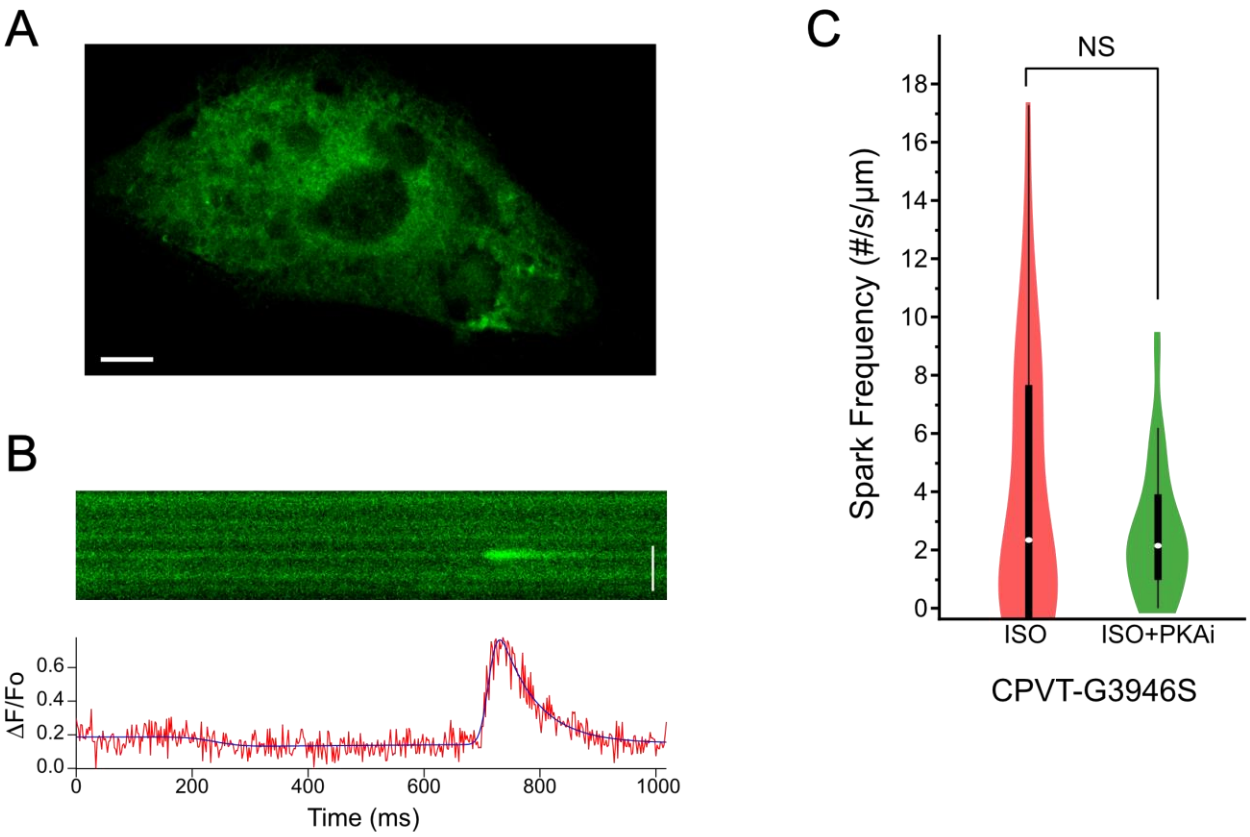
Supplemental Figure 2. A. Heart rate of mice with indicated genotype, AAV treatment, and adrenergic stimulation. In WT mice, heart rate was comparable between AAV9-GFP-AIP and AAV9-GFP treatment groups. In R176Q/+ mice, AAV9-GFP-AIP mice had slightly higher heart rate than AAV-GFP mice under epinephrine stimulation. This was not observed in the other conditions. N per group as listed above the graph. **B.** Echocardiography did not demonstrate a significance difference in systolic function of AAV9-GFP-AIP mice, compared to AAV-GFP. Significance testing by Steel-Dwass: †, EPI or ISO vs. baseline with corresponding virus. §, AVV9-GFP vs. AAV9-GFP-AIP with corresponding condition. †, §, $P<0.05$; ††, §§, $P<0.01$; †††, §§§, $P<0.001$. NS, non-significant P-value.



Supplemental Figure 3 - **Effect of AAV9-GFP-AIP on PR and QRS intervals.** No significant change in PR or QRS intervals in adolescent *RYR2*$^{R176Q/+}$ animals treated with AAV9-GFP-AIP. compared to AAV-GFP NS, non-significant P-value (Student's *t*-test).



Supplemental Figure 4 - Characterization of patient iPSCs. A-C. Characterization of iPSC line CPVT-S404R. Sequencing analysis of CPVT-S404R demonstrated the causative mutation (S404R) and a variant of unknown significance, N658S (A). Immunofluorescent staining of iPSC colonies for pluripotency markers OCT4 and SSE4 (B). CPVT-S404R iPSCs had a normal karyotype. **D-F.** Characterization of iPSC line CPVT-G3946S. Sequencing analysis of CPVT-G3946S demonstrated causative mutation (G3946S) and known modifying mutation (G1885E; Ref 3, online only Data Supplement) (D). Immunofluorescent staining of iPSC colonies for pluripotency markers OCT4 and SSE4 (E). CPVT-G3946S iPSCs had a normal karyotype (F).



Supplemental Figure 5 - Expression of Junctin-GCaMP6f (JT-G6F) in iPSC-CMs. **A.** Confocal micrograph of iPSC-CM expression JT-G6F. Scale bar = 20 μm. **B.** Confocal line scan at 500 Hz demonstrating single Ca^{2+} release event or "spark" with fitted line profile (lower panel). **C.** Quantification of spark frequency in CPVT iPSC-CMs treated with isoproterenol (ISO) and/or pre-incubation with a myristoylated PKA inhibitor (PKAi), did not demonstrate any significant change. Significance testing with Student's *t*-test. NS, non-significant P-value.

SUPPLEMENTAL REFERENCES

1. Kannankeril PJ, Mitchell BM, Goonasekera SA, Chelu MG, Zhang W, Sood S, Kearney DL, Danila CI, De Biasi M, Wehrens XHT, Pautler RG, Roden DM, Taffet GE, Dirksen RT, Anderson ME, Hamilton SL. Mice with the R176Q cardiac ryanodine receptor mutation exhibit catecholamine-induced ventricular tachycardia and cardiomyopathy. *Proc Natl Acad Sci U S A*. 2006; 103: 12179–12184.

2. Li D, Wu J, Bai Y, Zhao X, Liu L. Isolation and culture of adult mouse cardiomyocytes for cell signaling and in vitro cardiac hypertrophy. *J Vis Exp* 2014 May 21; 87: 51357-51365.

3. Zangi L, Lui KO, von Gise A, Ma Q, Ebina W, Ptaszek LM, Später D, Xu H, Tabebordbar M, Gorbatov R, Sena B, Nahrendorf M, Briscoe DM, Li RA, Wagers AJ, Rossi DJ, Pu WT, Chien KR. Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. *Nat Biotechnol*. 2013; 31: 898–907.