SUPPLEMENTAL MATERIAL

Supplemental Methods

Heterologous expression of mutant and wild-type Ito channels

Heterologous expression of Kv4.3 and KChIP2 was accomplished by co-transfecting 0.5 μg of pIRES2-*KCND3*^{WT}-EGFP or pIRES2-*KCND3*^{V392I}-EGFP with 0.5 μg pIRES2-*KCNIP2*^{WT}-dsRed2 using 3 μl of Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) in Gibco® OPTI-MEM media (Invitrogen, Carlsbad, CA). Transfected TSA201cells exhibiting both green and red fluorescence at 24 hours post-transfection were selected for electrophysiological experiments.

Reprogramming from PBMCs

Patient-specific PBMCs were processed by the Mayo Clinic's Regenerative Medicine Biotrust facility and stored in liquid nitrogen until further use. A 37°C water bath was used for quick thawing of frozen PBMCs. The PBMCs were transferred immediately into cold base DMEM with 4.5g/L glucose, L-glutamine, and sodium pyruvate (Corning, 10-013-CV) and centrifuged at 1000 RPM for 5 min to dilute out freezing solution. Blood media was used to grow PBMCs for 3 to 7 days in a 5% CO_2, 37°C, and humidified incubator. Briefly, blood media is composed of IMDM (Gibco, 12440053), 20% KnockOut Serum Replacement (Gibco, 10828010), IGF-1 (Peprotech, 100-11), EPO (Peprotech, 100-64), IL-3 (Peprotech, 200-03), and hSCF (Peprotech, 300-07). DNA for transfection was prepared with pCXLE-hOCT3/4-shp53-F (OCT3/4) (Addgene, 27077), pCXLE-hSK (SOX2/KLF4) (Addgene, 27078), pCXLE-hUL (L-MYC) (Addgene, 27080), and PCXWB-EBNA1 (EBNA-1) (Addgene, 37624). The P3 Primary Cell 4D-Nucleofector X Kit (Lonza, V4XP-3024) and the Lonza 4D Nucleofector machine were used to reprogram the PBMCs. Afterwards, the PBMCs were placed in a 37°C, 5% CO_2, and

humidified incubator. Media was changed to 50:50 of Blood media and ES media on D2 after transfection. ES media is composed of KnockOut DMEM (Gibco, 10829018) with 20% KnockOut Serum Replacement. On D4, media was replenished with ES media only. On D7, media was changed to mTeSR1 (STEMCELL Technologies, 85850) only with 1% Antibiotic-Antimycotic (AA) (Gibco, 15240-062). mTeSR1 media was changed every other day until seeing visible iPSC colonies. Colonies were picked within 21 days post-infection with the Yamanaka factors. Two representative clones were further expanded for each line.

Mutation correction of KCND3-V392I using CRISPR/Cas9 technology

Genome editing of cell lines was generated under contract by Applied Stem Cell. The *KCND3 392I* mutation in KCND3-V392I-derived iPSC cells was corrected to V392 using the CRISPR/Cas9 technology. Briefly, two guide gRNAs were designed, validated in vivo and one candidate gRNA was chosen for genome editing on patient iPSC line based on specificity score, cutting efficiency, off-target profile. A single-stranded oligodeoxynucleotide (ssODN) was designed to be used as a repair template and silent mutations TCC>TCa and CTG>CTc in gRNA binding site were introduced to the ssODN to prevent re-cutting (**Supplemental Table I**). The KCND3 patient iPSC line was transfected with gRNA construct and ssODN using a Neon system and transfected iPSCs were subjected to puromycin selection. Single-cell colonies were picked for genotyping, and two clones with mutation correction were expanded for further studies. All sequences of gRNA and ssODN are listed in **Supplemental Table I**.

Karyotyping of iPSC lines

Karyotyping for each of the iPSC lines used was completed by Mayo Clinic's Cytogenetics Laboratory. All iPSC lines tested demonstrated normal karyotypes (**Supplemental Figure I**).

Differentiation of iPSCs into iPSC-cardiomyocytes (iPSC-CMs)

The iPSCs were cultured in mTeSR1 with 1% AA in 6-well plates (Falcon, 353046) pre-coated with Geltrex (Life Technologies, A1413302) and incubated at 37°C with 5% CO_2 and humidity. The mTeSR1 media was changed daily. At 85% confluence, iPSCs were disaggregated with ReLeSR (STEMCELL Technologies, 05872) and seeded into 24-well plates (Falcon, 3524), cultured in mTeSR1 with 1% AA, and allowed to grow for 3-4 days until 80-90% confluent. For differentiation, the culture medium was changed to RPMI 1640 with L-glutamine (Gibco, 11875-093) supplemented with B27 minus insulin (B27 (-)) (Gibco, A18956-01) and 1% AA containing 5 µM CHIR99021 (Sigma, SML1046) until D2. At D2, the medium was changed to RPMI 1640 with L-glutamine and B27 (-) with 1% AA, containing 5 µM IWP2 (Sigma, I0536) and incubated until D4. At D4, the medium was changed to RPMI 1640 with L-glutamine supplemented with B27 (-) with 1% AA and cells were maintained in this media until beating iPSC-CMs appeared, typically around D10 or D12. One week following the initial observation of beating, the media was changed, and the iPSC-CMs were maintained in DMEM with 4.5g/L glucose, L-glutamine, and sodium pyruvate (Corning, 10-0013-CV) supplemented with 2% FBS (Gibco, 10437-028) plus 1% AA.

Cardiomyocyte aggregate cultures were maintained in DMEM with 4.5g/L glucose, L-glutamine, and sodium pyruvate with 2% FBS and 1% AA. At differentiation day 10-15, lactate selection was initiated to select for cardiomyocytes. Briefly, Lactate Selection media includes RPMI with L-glutamine, without glucose (Gibco, 11879-020), L-ascorbic acid 2-phosphate (Sigma, A8960), rice-derived recombinant human albumin (Sigma, A9731), and DL-lactate (Sigma, L4263). Lactate selection media was used for 4-6 days; the culture was then changed to RPMI 1640 with GlutaMAX and HEPES (Gibco, 72400-047) plus B27 (-) for maintenance. At differentiation day

30-60, the iPSC-CMs were subjected to dissociation with a mixture of Liberase (Creative Biomart, NATE-0994) and DNase I (Invitrogen, 18047-019), followed by the use of TrypLE Express (Life Technologies, 12605-010), to obtain single-cell suspensions of CMs. These cells were added to 0.1% gelatin-coated glass coverslips (Neuvitro, GG-12-GELATIN) in a 24-well plate with RPMI 1640 with GlutaMAX and HEPES plus 20% FBS. Cells were then stored in a 5% CO_2, humidified incubator at 37°C.The next day, the media was changed to RPMI 1640 with GlutaMAX and HEPES plus B27 (-) for maintenance until use in patch clamp assays.

Induced pluripotent stem cell-derived cardiomyocyte cells (iPSC-CMs) culture

Following written informed consent for this Mayo Clinic IRB-approved study (09-006465), patient-specific induced pluripotent stem cells (iPSCs) were generated from peripheral blood mononuclear cells (PBMCs) derived from an 18-year-old male with the KCND3-V392I mutation. Reprogramming and differentiation of iPSCs protocol were described in supplemental material. Karyotyping for each of the iPSC lines used was completed by Mayo Clinic's Cytogenetics Laboratory. All iPSC lines tested demonstrated normal karyotypes. iPSC-CM aggregate cultures were maintained in DMEM media. At differentiation days of 30-60, the enriched iPSc-CMs were subjected to enzymatic dissociation to obtain single cell suspensions of cardiomyocytes. These cells were added to 0.1% gelatin coated glass coverslips maintained in DMEM media or RPMI 1640 with GlutaMAX and HEPES plus 20% FBS and stored in a 5% CO_2 incubator at 37^{o}C before use.

Electrophysiological Ito measurements and data analysis

Standard whole cell patch clamp technique was used to measure Ito Kv4.3-WTor Kv4.3-V392I plus KChIP2-WT currents from TSA201 cells and Ito currents from control or Kv4.3-V392I hiPSC-CMs at room temperature (22-24^{o}C) with the use of an Axopatch 200B amplifier,

Digidata 1440A and pClamp version 10 software (Axon Instruments, Foster City, CA). The extracellular (bath) solution contained (mmol/L): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, pH adjusted to 7.4 with NaOH. The pipette solution contained (mmol/L): 110 KCl, 31 KOH, 10 EDTA, 5.17 $CaCl_2$, 1.42 $MgCl_2$, 4 MgATP and 10 HEPES, pH adjusted to 7.2 with KOH following established protocols[1, 3]. Microelectrodes were pulled on a P-97 puller (Sutter Instruments, Novato, CA) and fire polished to a final resistance of 2-3 MΩ. Series resistance was compensated by 80-85%. Currents were filtered at 5 kHz and digitized at 10 kHz. Data were analyzed using Clampfit (Axon Instruments, Sunnyvale, CA), Excel (Microsoft, Redmond, WA), and graphed with Origin 2015 (OriginLab Corporation, Northampton, MA) or GraphPad Prism 8.3 (GraphPad Software, San Diego, CA).

Electrophysiological action potential measurement

Action potentials (APs) from healthy control or KCND3-V392I iPSC-CMs were recorded at room temperature (22-24^{o}C) using current clamp mode at a constant rate of 1 Hz through 5 ms depolarizing current injections of 300-500 pA with the use of an Axopatch 200B amplifier, Digidata 1440A and pClamp version 10.4 software. The extracellular (bath) solution contained (mmol/L): 150 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 1 Na-Pyruvate and 15 HEPES, pH adjusted to 7.4 with NaOH. The pipette solution contained (mmol/L): 150 KCl, 5 NaCl, 2 $CaCl_2$, 5 EGTA, 5 MgATP and 10 HEPES, pH adjusted to 7.2 with KOH. Data were analyzed using Clampfit, Excel (Microsoft, Redmond, WA), and graphed with GraphPad Prism 8.3 (GraphPad Software, San Diego, CA).

Microelectrode array (MEA) measurement

iPSC-CMs were dissociated and seeded at 50,000 cells per well on 48-well Biocircuit MEA plate (Axion BioSystems, Inc. M768-BIO-48) pre-coated with Matrigel. Cells were cultured in a

humidified incubator at 37^0C and 5% CO_2 for 7-10 days after dissociation, medium were changed every two days. 4-24 hours before starting experiment, the medium was changed and the MEA plate was placed in Maestro MEA device (Axion BioSystems, Inc.) with an automatically adjusted and controlled environment (37^0C and 5% CO_2), and equilibrated for 2-5 min. Testing compound was prepared fresh at 10x the final concentration in warmed medium and added to the testing well after removing 10% of the media volume. The local extracellular action potential (LEAP) assay induction was performed on half of the electrodes in certain number of wells for baseline recording using the AxIS Navigator software (Axion BioSystems, Inc), compound was then added to the same wells and LEAP induction was performed on another half of the electrodes for measurement of compound effect. Data analysis was done using Cardiac Analysis Tool (Axion BioSystems, Inc. Version 3.1.4)

Immunocytochemistry

iPSC-CMs were cultured on an 8-well chamber (Celltreat Scientific Product, 229168) and fixed with 4% paraformaldehyde for 10 minutes at room temperature (RT), washed 3 times with PBS, permeabilized with 0.2% Triton X-100/ PBS (PBST)/5% goat serum for 15 minutes at RT, washed 3 times with PBS and blocked with PBST/5% goat serum for 60 minutes at RT, then incubated with 1:100 dilution of primary antibody at RT overnight. The next day, cells were washed 15 mins x 3 times with PBST/5% goat serum, incubated with 1:250 dilution of secondary antibody at RT for 60 minutes, washed 15 mins x 3 times with PBST/5% goat serum and 3 times with PBS, and rinsed 3 times with milli-Q water. After air drying, cells were mounted in anti-fade medium with DAPI (Vector Laboratories, H-1000) and cover slip, the edges of slide were sealed with nail polish to prevent leaking. Confocal microscopy (LSM, 780, Zeiss) was used to obtain immunofluorescent images. All antibodies used are listed on **Supplemental Table II**.

For subsequent measurements, iPSC-CMs were selected in different random fields. The fluorescent intensity of single cardiomyocyte was measured on confocal microscopy by drawing spline contour around each cell.

Western blot

iPSC-CMs were lysed in RIPA buffer (EMD Millopore Corp., 20188) with Protease Inhibitor Cocktail (Sigma, P8340), kept on ice for 30 minutes, sonicated samples at a frequency of 20 kHz for 5 x 10 sec, and centrifuged at 14000 rpm for 10 minutes at 4^{o}C. Supernatant was removed to a new tube, concentration was measured using Pierce BCA Protein Assay Kit (ThermoFisher, 23225). 5 ug of protein was mixed with 2X loading buffer (950 ul Laemmli sample buffer + 50 ul 2-Mercaptoethanol) as 1:1 dilution, denatured at 95^{o}C for 10 minutes, and proteins were loaded on 4-15% TGX gel (Bio-Rad, 456-1083) and transferred onto PVDF membrane using Trans-Blot Turbo Transfer System (Bio-Rad, 1704150). Membrane was incubated with primary antibody diluted to 1:1000 in PBS-T with 5% Bovine Serum Albumin (BSA) overnight at 4^{o}C on shaker. The next day, the membrane was washed with TBS-T buffer for 3 x 15 minutes, and then was incubated in secondary antibody diluted to 1:2000 in PBS-T with 5% BSA for 1 hour at RT. The membrane was then washed in TBS-T for 3 x 15 minutes. Finally, membrane was incubated with SuperSignal™ West Pico PLUS chemiluminescent ECL substrate (ThermoFisher, 34577) and exposed to HyBlot CL autoradiography film (Denville Scientific Inc., E3012). Film was scanned and saved as TIF file, the relative expression of KCND3, KCNQ1, MLC2v, α-Actinin and cTnT to that of Vinculin was analyzed and quantified with Image J (National Institutes of Health, Bethesda, MD). All antibodies used are listed on **Supplemental Table II**.

Drug

Acacetin (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO at a concentration of 10 mM and diluted to 10 or 30 μM final concentration.

Statistical analysis

All data were expressed as mean ± SEM. Student t test was performed to determine statistical significance between two groups, un-paired nonparametric (Mann-Whitney) test was used to determine statistical significance of KCND3 protein expression between two groups, Z-score test was used to determine statistical significance of proportion of Phase I Notch between two groups, and paired t test was used to compare data collected before and after Acacetin. A $p<0.05$ was considered to be significant. All statistical analysis was carried out using GraphPad Prism 8.3 (GraphPad Software, San Diego, CA).

Supplemental Tables

Table I. List of sequences used for genome editing and sequencing (5'-3')

gRNA-#1	AATGACCAGGACGCCACTCA
gRNA-#2	CTGCTCCTTGAGTGGCGTCC
ssODN	GACATGGTGCCTAAGACGATTGCAGGGAAGATCTTCGGCTCCATCT GCTCATTGAGTGGCGTCCTCGTCATTGCCCTGCCAGTCCCTGTGATT GTTTCCAACTTTAGCCGGATTTA
Check-For	GCACATAAGGATCTTGTCTCTCTTG
Check-Rev	GAGGCCATCTGTGCAGTGATCCT

Table II. List of antibodies used for Western blot and Immunofluorescence

Primary antibodis	Manufacture	Catalog #	Host	Type	Dilution
KCND3	alomone labs	APC-017	Rabbit	Polyclonal	1:1000 (WB), 1:200 (IF)

a-Actinin	Sigma	A7811	Mouse	Monoclonal	1:200 (IF)
cTNT	abcam	ab45932	Rabbit	Polyclonal	1:200 (IF)
OCT4	ThermoFisher Scientific	PA5-27438	Rabbit	Polyclonal	1:200 (IF)
SSEA4	ThermoFisher Scientific	MA1-021	Mouse	Monoclonal	1:200 (IF)
Nanog	ThermoFisher Scientific	PA1-097	Rabbit	Polyclonal	1:200 (IF)
Tra-1-60	Santa Cruz	sc-21705	Mouse	Monoclonal	1:200 (IF)
Secondary antibodies	**Supplier**	**Cat #**	**Host**	**Type**	**Dilution**
Alexa Fluor 488	ThermoFisher Scientific	A-11008	Goat	Polyclonal	1:250 (IF)
Alexa Fluor 594	ThermoFisher Scientific	A-11005	Goat	Polyclonal	1:250 (IF)
Mouse IgG HRP-conjugated Antibody	R&D Systems	haf007	Goat	Polyclonal	1:2500 (WB)
Rabbit IgG HRP-conjugated Antibody	ThermoFisher Scientific	65-6120	Goat	Polyclonal	1:2500 (WB)

Table III. Patch clamp action potential parameters from isogenic control and KCND3-V392I-derived iPSC-CMs

	MDP (mV)	Amplitude (mV)	dv/dt max (mV/ms)	APD50 (ms)	APD90 (ms)

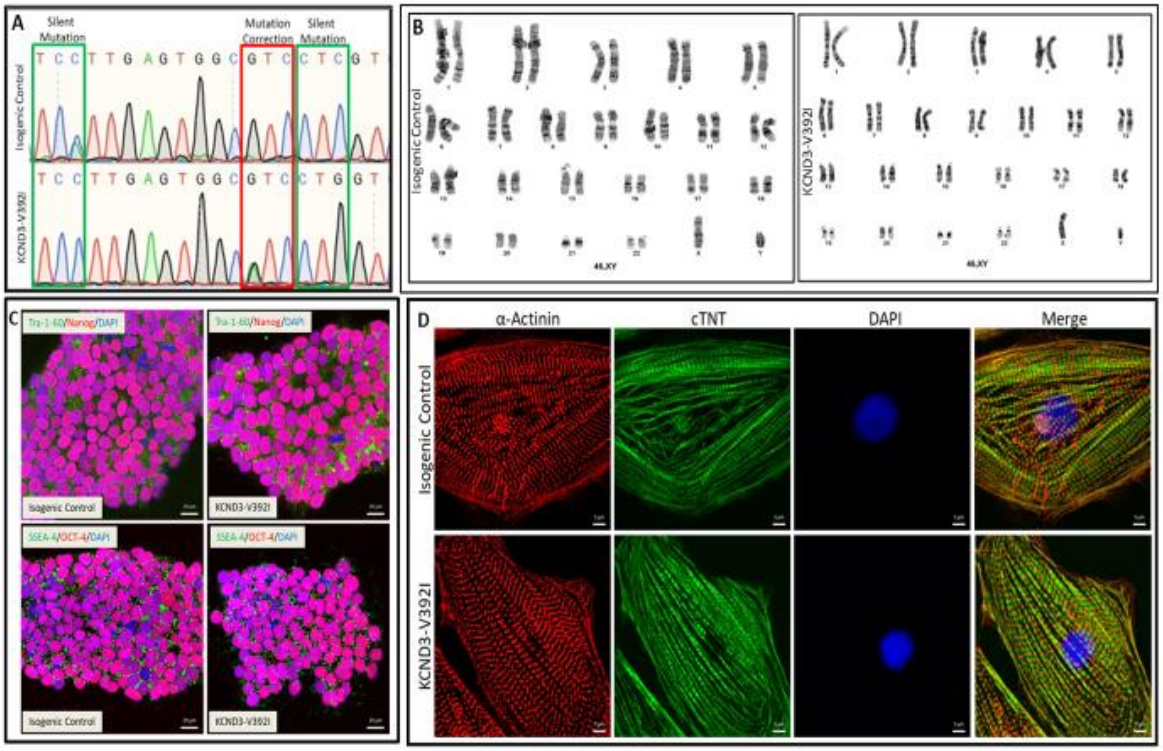
Isogenic control before Acacetin (n=10)	-54.7±3.2	96.1±3.3	29.6±6.1	288.2±31.2	350.2±34.9
Isogenic control after Acacetin (n=10)	-46.4±3.0*	84.9±5.2*	17.1±3.9*	272.0±36.9	330.9±38.4
KCND3-V392I before Acacetin (n=12)	-56.7±1.6	94.0±3.0	35.3±7.1	325.8±31.5	384.4±35.5
KCND3-V392I after Acacetin (n=12)	-47.9±3.4*	81.6±5.4*	23.1±6.1	294.3±33.2	360.3±39.0

*P<0.05 vs. before Acacetin

Table IV. MEA action potential parameters from KCND3-V392I-derived iPSC-CMs

	APD30 (ms)	APD50 (ms)	APD90 (ms)	APD90 Fridericia s (ms)
KCND3-V392I without Acacetin (n=110)	187.2±0.009	262.3±0.01	323.2±0.01	295.4±0.008
KCND3-V392I with Acacetin (n=118)	204.1±0.009	273.4±0.01	329.2±0.01	311.5±0.008

Supplemental Figure and Figure Legend



Supplemental Figure I. Generation of patient-specific iPSC-CMs Shown in panel A is sanger sequencing of isogenic control and KCND3-V392I iPSCs, shown in panel B are normal female karyotype of isogenic control and KCND3-V392I iPSCs. Shown in panel C are representative confocal images of undifferentiated patient-specific mutant and isogenic control iPSCs demonstrating four pluripotent markers (Tra-1-60, NANOG, SSEA4, and OCT4). Scale bars equal 20μm. Following cardiac differentiation, iPSC-derived cardiomyocyte maturity was confirmed with cardiac specific markers (α-actinin and cTnT) as shown in panel D.