The hERG R56Q$^{+/-}$ LQTS variant induces electrical instability in hiPSC-CMs that is rescued by the RPR260243 hERG activator

R. Venkateshappa[1], D.V. Hunter[1], P. Muralidharan[1], R.S. Nagalingam[1,2], G. Huen[1], S. Faizi[1], S. Luthra[1], E. Lin[1], Y.M. Cheng[1], J. Hughes[1], R. Khelifi[1], P, Dhunna[1], R. Johal[1], V. Sergeev[1], S. Shafaattalab[1], L.M. Julian[3], D.T. Poburko[1], Z. Laksman[4], G.F. Tibbits[1,2,5], T.W. Claydon[1]

Running title: Rescue of hERG R56Q arrhythmogenicity by RPR260243

Supplementary Material

Material and Methods

hiPSC culture

All experiments were performed using WiCell iPS(IMR90)-1 cells, kindly gifted by Dr. G.F. Tibbits, and maintained on Matrigel® (Corning, CLS356234) coated 6-well cell culture plates in mTeSR™Plus (STEMCELL Technologies, 100-0276) following the manufacturer's standard culturing procedures. Cells were passaged when they reached 80% confluency, approximately every 4 days: hiPSCs were dissociated to small aggregates using Versene (Gibco, 15040066) and light mechanical disruption and seeded at a split ratio of 1:20 in mTeSR™ Plus.

Generation of gene-edited cell lines

To perform CRISPR-Cas9 editing of hiPSCs, we used an approach described previously in Ran *et al.* 2013[1]. The pCCC vector, a variant of the pSpCas9(BB)-2A-GFP vector with the Cbh promoter exchanged for the full-length CAGGS promoter to increase efficiency in hiPSC[2], was kindly gifted by Dr. F. Lynn and used to express the R56Q sgRNA, Cas9, and GFP in hiPSC cells. The R56Q sgRNA design was performed using the CRISPR design tool from the Broad Institute at MIT (http://crispr.mit.edu/) and the guide with the closest PAM site to the hERG R56 target site was selected, synthesized, and inserted into the BbsI site of the pCCC vector as described in Ran *et al.* 2013. A 127 bp ssODN (synthesized by IDT as a 4 nmole Ultramer) complementary to the sense strand, with 38 bases (PAM-distal) and 89 bases (PAM-proximal) on either of the expected double stranded break site, was used to provide the template for homology directed repair and included the hERG c.167G>A (p.Arg56Gln mutation, as well as a silent mutation (c.174G>A) in the PAM site.

The day before transfection, hiPSCs were passaged into small clumps using Versene (Gibco, 15040066) and seeded at a density of 100,000-200,000 cells per well of a 24-well plate. The following day, 500 ng of pCCC-sgRNA vector and 10 pmol of the ssODN were transfected

into each well using Lipofectamine 3000 according to the manufacturer's guidelines. Media changes with fresh mTeSR Plus™ were performed 12 and 24 h after the transfection reagents were applied. Two days after transfection hiPSCs were dissociated into single cells using Accutase (STEMCELL Technologies, 07920) and FACS was used (BD FACSAria Fusion Cell Sorter) to select GFP-positive cells. Between 500-1,000 GFP-positive cells were plated into each well of a 6-well culture dish, which was coated with Matrigel® (Corning, CLS356234) and contained mTeSR™ Plus supplemented with penicillin-streptomycin (Life Technologies, 15140122) and CloneR™ (STEMCELL Technologies, 05888), to aid in the survival and growth of the individually seeded cells. After 48-72 h the media was changed, and every-other-day media changes were performed for the following 7-10 days until individual colonies could be isolated. Individual colonies were identified under a microscope and manually transferred into a 96-well plate for further growth and analysis.

A sample of cells from each clone were lysed using DirectPCR Lysis Reagent with Proteinase K Solution (Viagen, 301-C and 501-PK) and the lysate was used as the PCR template to amplify the region around the hERG R56 site. The PCR product was sent for Sanger sequencing (GENEWIZ, Azenta Life Sciences) to determine the genotype of the clones. Clones that were selected for further experiments were expanded and cryopreserved using mFreSR™ (STEMCELL Technologies, 05855). Off-target sites were predicted using CRISPOR (http://crispor.tefor.net/)[3]; the top three exonic off-targets sites in the ZNRF3, SUPT5H and SLC38 genes amplified by PCR and Sanger sequenced. No off-target mutations were identified in the clones.

Directed differentiation of hiPSCs to cardiomyocytes

Differentiation of the hiPSC clones was performed following the STEMdiff™ Ventricular Cardiomyocyte Differentiation Kit protocol (STEMCELL Technologies, 05010). Briefly the

hiPSC clones were seeded as small aggregates onto Matrigel®-coated 12-well plates with mTeSR™Plus and ROCK inhibitor 10 μM Y-27632 (Biogems, 1293823) at a density of 300,000-500,000 cells/well 48 h prior to the start of differentiation. Upon reaching 95% confluency, a series of timed media changes were performed according to the differentiation kit protocol and beating monolayers could be identified approximately 7-10 days after the start of differentiation.

Beating hiPSC-CMs monolayers were dissociated following procedures in the STEMdiff™ Cardiomyocyte Dissociation Kit (STEMCELL Technologies, 05025). Briefly, the beating monolayer was washed with Dulbecco's Phophate Buffered Saline (D-PBS, VWRL0119-0500) and treated with STEMdiff™ Cardiomyocyte Dissociation Medium for 7–10 min at 37°C and 5% CO_2. The dissociated cells were resuspended in STEMdiff™ Cardiomyocyte Support Medium at low enough density onto a 12 mm glass coverslip (VWR, 89015-725) coated with 10 μg/ml fibronectin (Gibco, PHE0023) to yield single, beating hiPSC-CMs. The media was changed to STEMdiff™ Cardiomyocyte Maintenance Medium after 24 h and was refreshed every 2 days. Electrophysiological and immunocytochemical analysis of the cells was performed 4-10 days after replating.

Cellular phenotyping of hiPSC-CM clones

hiPSC-CMs were fixed with 4% paraformaldehyde in D-PBS (Thermo Scientific, 28906), followed by a 0.1 % Triton X-100 (Thermo Scientific, 28314) in D-PBS solution permeabilization for 15 min each at room temperature. Coverslips were then blocked with 4% normal goat serum (MiliporeSigma, NS02L) in PBS for 1 h, PBS washed, and probed overnight at 4 °C with mouse monoclonal anti-cTnT (1:1000) (Invitrogen, MA5-12960) and rabbit monoclonal anti-α-actinin (Invitrogen, 710947). The following day the coverslips were incubated at room temperature for 2 h with secondary antibodies, Alexa Fluor 488-conjugated goat anti-mouse (1:1000) (Invitrogen,

A-11029) and Alexa Fluor 555-conjugated goat anti-rabbit (1:1000) (Invitrogen, A32732), and the nuclei were counterstained stained with Hoescht 33342 (Abcam, ab228551). The cells underwent a post-staining fixation with 4% paraformaldehyde solution for 5 min prior to the final PBS washes and mounting onto microscope slides with ProLong Glass Antifade Mountant (Thermo Scientific, P36980). Images were acquired on a Nikon Ti-E inverted epifluorescence microscope equipped with a Zyla 5.5 CMOS camera using a 20x Superfluor objective with a 1.5x multiplier. Fluorophores were excited with a Sutter Lambda XL light source and SmartShutter. Images were captured using Nikon Elements software (version 4.30, Nikon).

RNA from hiPSCs and hiPSC-CMs was isolated using the RNeasy Mini Kit (Qiagen, 74004) and cDNA was synthesized using the Qiagen QuantiTect Reverse Transcriptase Kit (Qiagen, 205311), both according to the manufacturer's guidelines. Primers were designed to target unique regions within pluripotency (NANOG, POU5F1, SOX2, C-MYC, KLF4) and cardiomyocyte-related (ACTN1, MYL2, KCNH2) genes as well as two reference genes (GJA1, GAPDH). Products were visualized on a 2% agarose gel to show qualitative levels of transcripts in the undifferentiated hiPSCs and the differentiated hiPSC-CMs.

The effect of the hERG R56Q CRISPR-Cas9 edit on relative membrane expression of hERG1a and hERG1b protein was assessed using western blot from whole cell lysates. Total protein from hiPSC-CM monolayers was isolated using RIPA lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (Thermo Fisher Scientific, 89900) containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich, PPC1010). The protein suspension was centrifuged at 14,000 rpm for 20 min at 4°C to remove the cell debris. A Bradford assay was used for the measurement of protein concentrations. For western blot analysis, 60 mg of total protein was loaded in each lane of 8 % SDS-polyacrlyamide gels under reducing

conditions along with PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific, 26620) and transferred into 0.45 mm Immobilon-P membrane PVDF (Millipore, IPVH85R). Blots were blocked with 5 % BSA or 5 % milk in TBST for 1 h at room temperature. For primary antibody detection, blots were probed with 1 % BSA or 1 % milk in TBST at 4 °C overnight, followed by washing three times with TBST for 10 mins and incubating with secondary antibody in TBST with 1 % non-fat milk for 1 h at room temperature. hERG transcripts and GAPDH loading control were detected by SuperSignal West Atto Ultimate Sensitivity chemiluminescent substrate (Thermo Fisher Scientific, A38554). Detected bands were quantified using ImageJ (NIH, USA) to measure relative band intensity. Antibodies used were: hERG1a rabbit monoclonal (Cell Signaling, D1Y2J, 1:1,000); hERG1b rabbit polyclonal (Enzolifesciences, ALX-215-051, 1:1,000); GAPDH mouse monoclonal (Proteintech, 60004-1-Ig, 1:20,000); Goat anti-Rabbit IgG HRP (Invitrogen, G-21234, 1:5,000); Goat anti-mouse IgG HRP (Invitrogen, G-21040, 1:5,000).

Electrophysiology

Electrophysiological recordings were performed using an Axon instruments 200B amplifier and Digidata 1440 interface. Signals were acquired at 10 kHz sampling frequency and low-pass filtered at 2 kHz from single beating cardiomyocytes using perforated patch clamp. A P-97 puller (Sutter Instruments) was used to fashion borosilicate glass capillaries with a tip resistance of 2.5-3 MΩ when filled with an internal solution containing (in mM): $CaCl_2$ 2, EGTA 5, HEPES 10, KCl 150, KOH, MgATP 5, NaCl 5, and supplemented with 140-170 μg/ml amphotericin B (pH 7.2 with KOH). Cells were continuously perfused at a rate of 2 ml/min with external solution containing (in mM): $CaCl_2$ 1.8, Glucose 15, HEPES 15, KCl 5.4, $MgCl_2$ 1, NaCl 150, Na-Pyruvate 1 (pH 7.4 using NaOH). All compounds were dissolved in DMSO, and working stocks were prepared in external solution containing <1% DMSO. All experiments were carried out at 37°C,

with the bath temperature monitored and maintained using a TC-344B Warner Instruments temperature controller equipped with a bath chamber thermistor, a heated platform, and an in-line perfusion heater.

To record I_{Kr}, cells were patched and monitored in current clamp mode for stable ventricular action potentials (diastolic potential <-60 mV) before 5 min superfusion with external solution containing 10 μM nifedipine to stop beating. I_{Kr} was then recorded during 2 s voltage clamp steps from -60 mV to +40 mV in 20 mV increments (holding potential was -40 mV) followed by a 3 s step to -40 mV to monitor deactivation. I_{Kr} was measured again following 5 min superfusion with 10 μM dofetilide and the dofetilide-sensitive I_{Kr} current was used for analysis.

The time course of dofetilide-sensitive deactivating IKr tail currents at -40 mV was fitted with a double exponential equation:

$$I = A_{fast}\exp(t/\tau_{fast}) + A_{slow}\exp(t/\tau_{slow}) + C$$

where A_{fast} and A_{slow} are the current amplitudes of the fast and slow components, τ_{fast} and τ_{slow} are the time constants of the fast and slow components, and C is the residual amplitude. To simplify comparison of deactivation kinetics[4,5], the weighted average tau was calculated as:

$$\tau_{weighted} = (A_{fast} \times \tau_{fast} + A_{slow} \times \tau_{slow}) / (A_{fast} + A_{slow})$$

To record action potentials, the threshold current injection was determined in each cell by stimulating cells with increasing current amplitude until a constant 1:1 response was observed. Following this, 1.5-2x this threshold current was employed during a range of programmed stimulation regimens designed using the Axon 200B clampex software. We utilized a 4 ms blanking pulse to eliminate artefacts associated with the 2 ms stimulation pulses. To record action potentials during standard S1-S2 paired stimulations, a train of eight S1 stimuli was applied with a constant cycle length before an S2 stimulus that was applied at varying coupling intervals from

500 to 110 ms. The dynamic stimulation procedure involved stimulating cells for 30 beats at progressively shorter basic cycle lengths (BCLs) from 500 ms to 167 ms.

Results

Figure S1. Cellular markers of hiPSC-CM differentiation

hiPSC clones maintained characteristic features of hiPSCs and were successfully differentiated into beating hiPSC-CMs monolayers that stained for cardiac troponin T (cTnT) and alpha-actinin (ACTN2) in both a monolayer and when plated as individual cells (Figure S1A). Although immature in nature, these hiPSC-CMs presented clear striation patterning of these cardiac sarcomeric markers. This is further highlighted by RT-PCR measures demonstrating that cardiomyocyte differentiation of the clones resulted in decreased expression of pluripotency transcripts and increased expression of cardiomyocyte transcripts (Figure S1B).

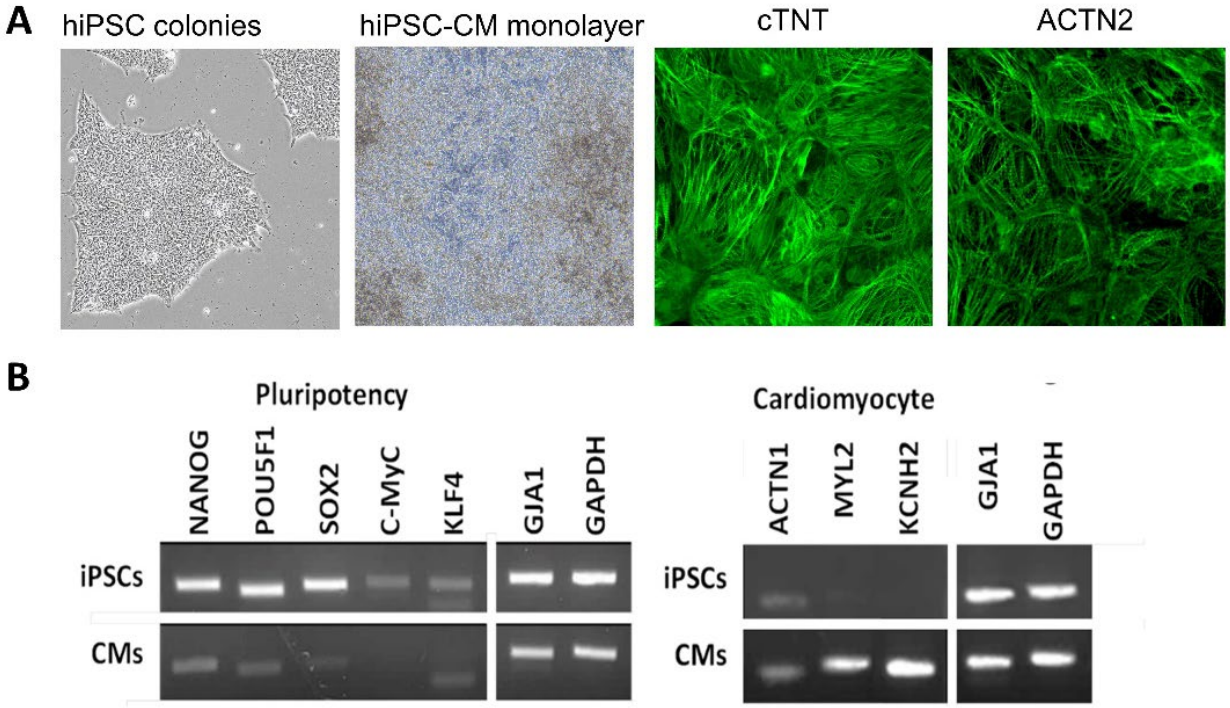
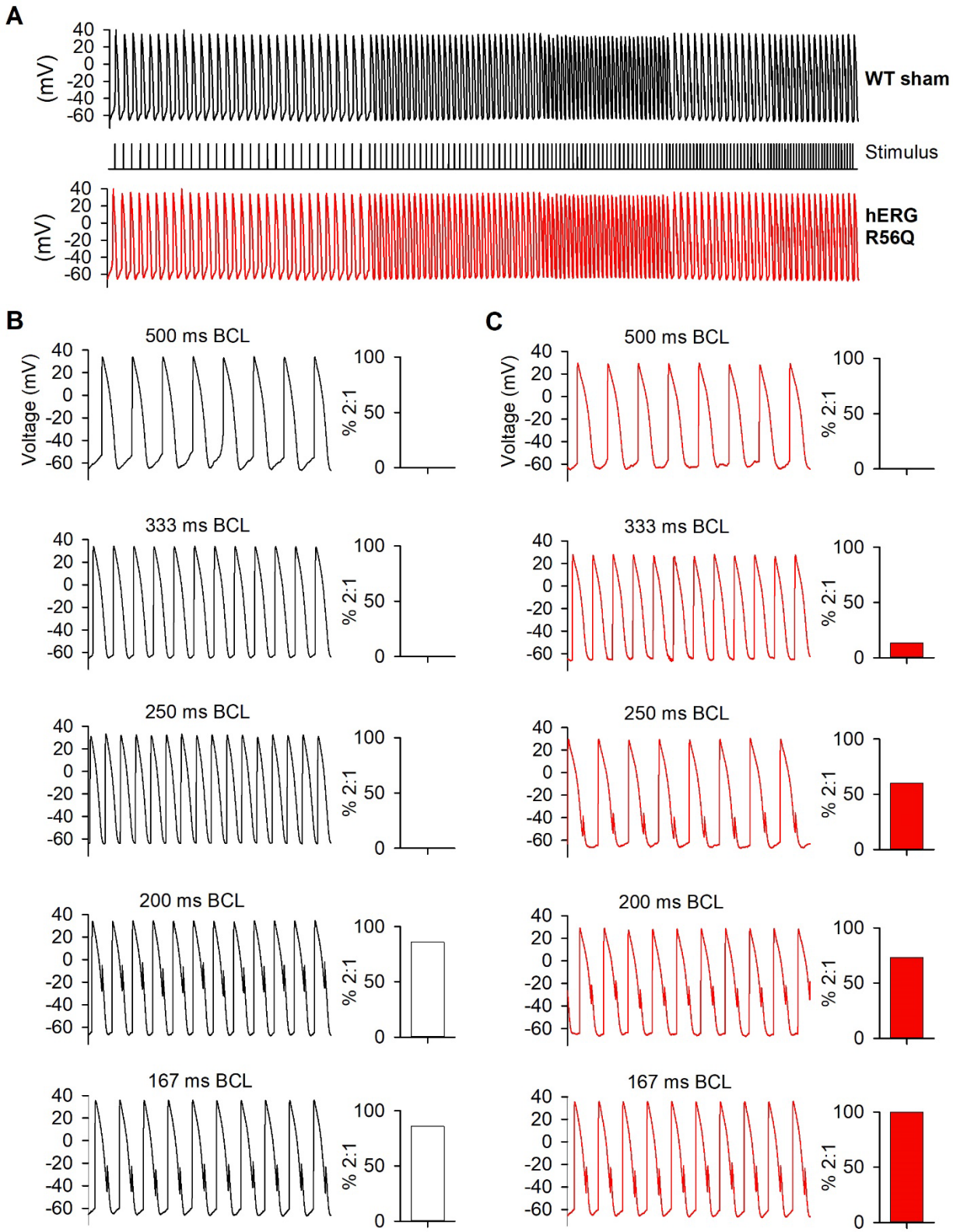


FIGURE S1

Figure S2. Action potential restitution properties of hiPSC-CMs during dynamic rate changes.

Figure S2 shows representative membrane potential recordings in response to a stimulus protocol designed to interrogate how action potentials adapt to dynamic changes in stimulus rate. Example membrane responses obtained from a WT sham cell and a hERG R56Q cell are shown. Figure S2A highlights the progressive action potential changes observed during 30 stimulations applied at progressively shorter cycle lengths from a BCL of 500 ms to 167 ms. Action potentials recorded during the final 5 s at each cycle length are shown on a slower time-base in Figure S2B-C. Cells demonstrated 1:1 capture of action potentials with stimulations applied at longer BCLs and progression to 2:1 capture at shorter BCLs. The percentage of cells displaying 2:1 capture at each BCL is shown as bar charts. We observed an increased incidence in the loss of 1:1 capture at shorter BCLs in hERG R56Q cells compared with WT sham cells. For example, at a BCL of 250 ms 1:1 capture was preserved in 100 % of WT sham cells, but only 40 % of hERG R56Q cells, with 60 % of the latter group demonstrating 2:1 capture at this BCL.

The relationship between APD_{90} and DI during rate changes was used to plot dynamic electrical restitution curves. The slope of the dynamic restitution curve was determined by fitting the APD_{90}-DI relationship with a sigmoidal function and the average maximum slopes are shown. We observed no remarkable differences across the conditions tested: maximum slope values were 2.0 ± 0.3 in WT sham (n=7), 1.7 ± 0.2 in WT sham with 10 μM RPR260243 (n=7), 2.1 ± 1.1 in hERG R56Q (n=15), and 2.3 ± 0.4 in hERG R56Q with 10 μM RPR260243 (n=15).



175

176 **FIGURE S2**

Table S1. Kinetics components of I_{Kr} deactivation in WT sham and hERG R56Q hiPSC-CMs.

Deactivation gating of hERG channels is often described as a weighted average of the time constants yielded from bi-exponential fits to current decay. However, additional information may be gleaned from description of the two phases of deactivation kinetics. Table S1 summarizes the time constants and relative amplitudes of fast and slow phases of deactivation recorded from WT sham and hERG R56Q hiPSC-CMs in the absence and presence of RPR260243. The hERG R56Q variant accelerated the time constant of the slow phase of deactivation (p=0.022) and tended to reduce its relative contribution, although the latter did not reach statistical significance. Application of RPR260243 increased the relative contribution of the slow component of deactivation (p=0.034) and slowed the time constant of the fast component (p=0.012).

	A_{fast}	τ_{fast} (ms)	A_{slow}	τ_{slow} (s)
WT sham	0.57 ± 0.08	267 ± 50	0.43 ± 0.08	3.13 ± 0.54
hERG R56Q$^{+/-}$	0.80 ± 0.07	158 ± 26	0.20 ± 0.07	1.02 ± 0.12*
hERG R56Q$^{+/-}$ + RPR260243	0.42 ± 0.11*	575 ± 151*	0.58 ± 0.11*	1.96 ± 0.52

*p<0.05

TABLE S1

References

1. Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* **8**, 2281–2308 (2013).
2. Krentz, N. A. J., Nian, C. & Lynn, F. C. TALEN/CRISPR-Mediated eGFP Knock-In Add-On at the OCT4 Locus Does Not Impact Differentiation of Human Embryonic Stem Cells towards Endoderm. *PLOS ONE* **9**, e114275 (2014).
3. Concordet, J.-P., Haeussler, M. & Haeussler. CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Res* **46**, W242–W245 (2018).
4. Shi, Y. P., Thouta, S., Cheng, Y. M. & Claydon, T. W. Extracellular protons accelerate hERG channel deactivation by destabilizing voltage sensor relaxation. *J Gen Physiol* **151**, 231–246 (2019).
5. Ng, C.-A. *et al.* Heterozygous KCNH2 variant phenotyping using Flp-In HEK293 and high-throughput automated patch clamp electrophysiology. *Biology Methods and Protocols* **6**, bpab003 (2021).