SUPPLEMENTAL MATERIAL

A rare non-coding enhancer variant in *SCN5A* contributes to the high prevalence of Brugada syndrome in Thailand

Supplemental Methods

Genome sequencing data generation and processing.

Case and control samples were sequenced by the Illumina FastTrack service on HiSeqX with a PCR-free library. Fastq files were aligned to the GRCh37 reference build using BWA Mem 0.7.12[47] and converted to BAM using Samtools 1.3.1[48]. The data from the alignment and MarkIlluminaAdapters was merged with MergeBamAlignment and sorted with Sambamba 0.6.6[49]. Where a sample belonged to multiple read groups, the read groups were merged with sambamba merge. BAM files were split into chromosomes and each chromosome cleaned with Picard CleanSam, duplicates were removed with Sambamba markdup. Indels were realigned with GATK 3.5 RealignerTargetCreator and IndelRealigner. All chromosomes and non-chromosomes were merged back into one bam file with sambamba merge. Haplotypes were called with GATK HaplotypeCaller[50]. All GVCFs were merged into 10,000,000 base pair chunks with GATK3.8 CombineGVCFs and called with GenotypeGVCFs. All chunked VCFs were merged into chromosomes with GATK CombineVariants, and SNP and Indel variants were recalibrated with GATK VariantRecalibrator and ApplyRecalibration. The variants were annotated with SNPsift/SNPeff 4.1. The chromosomes were merged into one file with Picard. The pipeline was implemented in Snakemake[51].

Generation of hiPSC-derived cardiomyocytes

The parental PGP1 and edited A7 and C8 hiPSC lines were maintained in undifferentiated state in presence of mTeRS1 medium on Matrigel Matrix-coated plates until they reached 65-80% confluence. Differentiation of the parental PGP1 and edited A7 and C8 hiPSC lines into cardiomyocytes was performed as described by Maas *et al.*[21] without cardiomyocytes expansion and with the following modifications. Differentiation was started in RPMI 1640 medium containing 2% B27 supplement without insulin (both by Gibco/Thermo Fisher Scientific) (hereafter indicated as RPMI/B27−) and 6 µM CHIR99021 (Selleck Chemicals). After 24 hours a volume of RPMI/B27− medium without CHIR99021 equal to 2/3 of the original differentiation medium was added. On day 3 of differentiation 1/3 of the original volume of RPMI/B27− medium was further added to gradually dilute the CHIR99021 concentration. The differentiation protocol continued with 3-days treatment with 2 µM Wnt-C59 (MedChemExpress; Wnt signaling pathway inhibitor) in RPMI/B27− medium, without medium change. After Wnt signaling inhibition the culture was maintained until day 43 in RPMI/B27− medium alone, with medium change every 3-4 days. On day 44, a 7-8 days metabolic-selection that enriches the culture for cardiomyocytes was started by switching the medium to RPMI 1640 without glucose (Gibco/ThermoFisher Scientific) supplemented with 500 µg/mL bovine serum albumin (Sigma-Aldrich/Merck) and 8 mM Na-L-lactate (Sigma-Aldrich/Merck) with medium change every other day

(adapted from Tohyama *et al*.[52]). The culture was then switched to RPMI 1640/B27− for 1 day before proceeding to either perform RNA isolation or to dissociate the cells for electrophysiological analysis.

Preparation of hiPSC-CMs for single cell electrophysiological analysis

The hiPSC-CMs cultures were dissociated to obtain single cardiomyocytes as follows. The culture was incubated at room temperature for 7-10 minutes in a mixture (50/50) of Hanks Balanced Salt Solution (HBSS, Gibco/Thermo Fisher Scientific) without $CaCl_2$ and $MgCl_2$ and HBSS with $CaCl_2$ and $MgCl_2$. After HBSS mixture removal the culture was incubated for 15-20 minutes at 37°C in presence of TrypLE Select Enzyme 10x (Gibco/Thermo Fisher Scientific). The cells were then detached from the surface by pipetting, the enzyme solution was diluted with addition of RPMI/B27− medium and the cells in suspension were collected by centrifugation. The cell pellet was further dissociated in presence of Liberase TM Research Grade (Roche/Merck) as previously reported[53]. The hiPSC-CMs, seeded on Matrigel Matrix-coated cover glasses (VWR International GmbH) in RPMI 1640/B27− medium, were analysed 8-10 days after dissociation.

Generation and preparation of hiPSC-CMs for transfection experiments

The hiPSC line LUMC0099iCTRL#04 (generated by the iPSC core facility of Leiden University Medical Center and registered at LUMCi004-A · Cell Line · hPSCreg) was maintained in undifferentiated state in presence of mTeRS1 medium on Matrigel Matrix-coated plates, differentiation started when it reached 85-95% confluence. Cardiomyocyte differentiation was performed as described by Maas et al.[21] with the following modifications. Differentiation started in RPMI 1640 medium containing 2% B27 supplement without insulin (both by Gibco/Thermo Fisher Scientific) with the addition 213ug/ml of L-ascorbic acid 2-phospahte (Sigma-Aldrich/Merck) (hereafter indicated as RPMI/B27−) and 6 µM CHIR99021 (Selleck Chemicals) for 3 days followed by 3-days treatment with 2 µM Wnt-C59 (MedChemExpress; Wnt signaling pathway inhibitor) in RPMI/B27− medium. On day 8 of differentiation the medium was switched to RPMI 1640 containing 2% B27 supplement with insulin and without L-ascorbic acid 2-phospahte (hereafter indicated as RPMI 1640/B27+) until day 30, with medium change every 3-4 days. A 7-8 day metabolic-selection that enriches the culture for cardiomyocytes was then started by switching the medium to RPMI 1640 without glucose (Gibco/ThermoFisher Scientific) supplemented with 500 µg/mL bovine serum albumin (Sigma-Aldrich/Merck) and 8 mM Na-L-lactate (Sigma-Aldrich/Merck) with medium change every other day (adapted from Tohyama S. et al.[52]). The culture was then switched to RPMI 1640/B27+ for 1 day before proceeding to dissociation and reseeding for transfection experiments. Dissociation of hiPSC-cardiomyocytes culture was performed by incubation in presence of StemPro Accutase (Gibco/ThermoFisher Scientific) for 45minutes at 37^{o}C and 5%CO_2, followed by gentle dissociation by pipetting, dilution of the dissociation reagent with RPMI 1640/B27+ medium before centrifugation and resuspension of the hiPSC-cardiomyocytes pellet in RPMI 1640/B27+ medium. Cardiomyocytes were seeded at 10.5x10^4 cells/cm2 in RPMI 1640/B27+ medium and transfected 7 days later.

Cell Culture and Transfection Luciferase Assays

The RE5 fragment was cloned into a modified pGL2-Basic plasmid containing an SV40 minimal promoter and an adjusted multiple cloning site for in vitro analysis by a transfection luciferase assay. HEK-293 cells were grown in 24-well plates in DMEM (ThermoFisher Scientific, 31966-021) supplemented with 10% FBS (ThermoFisher Scientific, 10270-106) and Pen/Strep (ThermoFisher Scientific, 15070-063). hiPSC-CM cultures were grown as described above. HEK-293 cells were transfected using polyethylenimine 25 kDa (PEI, Brunschwig, 23966-2) at a 1:3 ratio (DNA:PEI). Standard transfections HEK-293 cells with 250 ng of reporter construct and 100 ng pcDNA3.1(+)- (ThermoFisher Scientific, V790-20) or 100 ng pcDNA3.1-MEF2C and/or -Myocardin per well. Standard transfections of hiPSC-CMs were carried out using ViaFect transfection reagent (Promega E4981) and 500 ng of reporter construct. 48 hours after transfection, cells were lysed using Renilla luciferase assay lysis buffer (Promega, E291A-C) and luciferase activity was measured. Luciferase measurements were performed using a GloMax Explorer (Promega, GM3500). During the measurement, 100 µL D-Luciferin (p.j.k, 102111) was injected (150 µL/second) followed by a 1 second delay and 5 seconds of measurement. Transfections were carried out at last four times and measured in duplicates. Statistics were performed using Student's unpaired t tests.

Supplemental Tables

Supplemental tables are available in the Excel File S1.

Table S1: Epigenetic datasets used (in Fig 1E).

Table S2: Primer sequences.

Table S3 - Details of the SCN5A coding variants detected in Brugada syndrome (BrS) cases from Thailand, separated into ultra-rare (gnomAD FAF < 0.00001) and low frequency (0.00001 < gnomAD FAF < 0.001) variants. Variants are described with respect to the ENST00000333535 transcript.

Table S4: The RE5 variant is not detected in the following genome sequencing datasets of individuals of Asian ancestry.

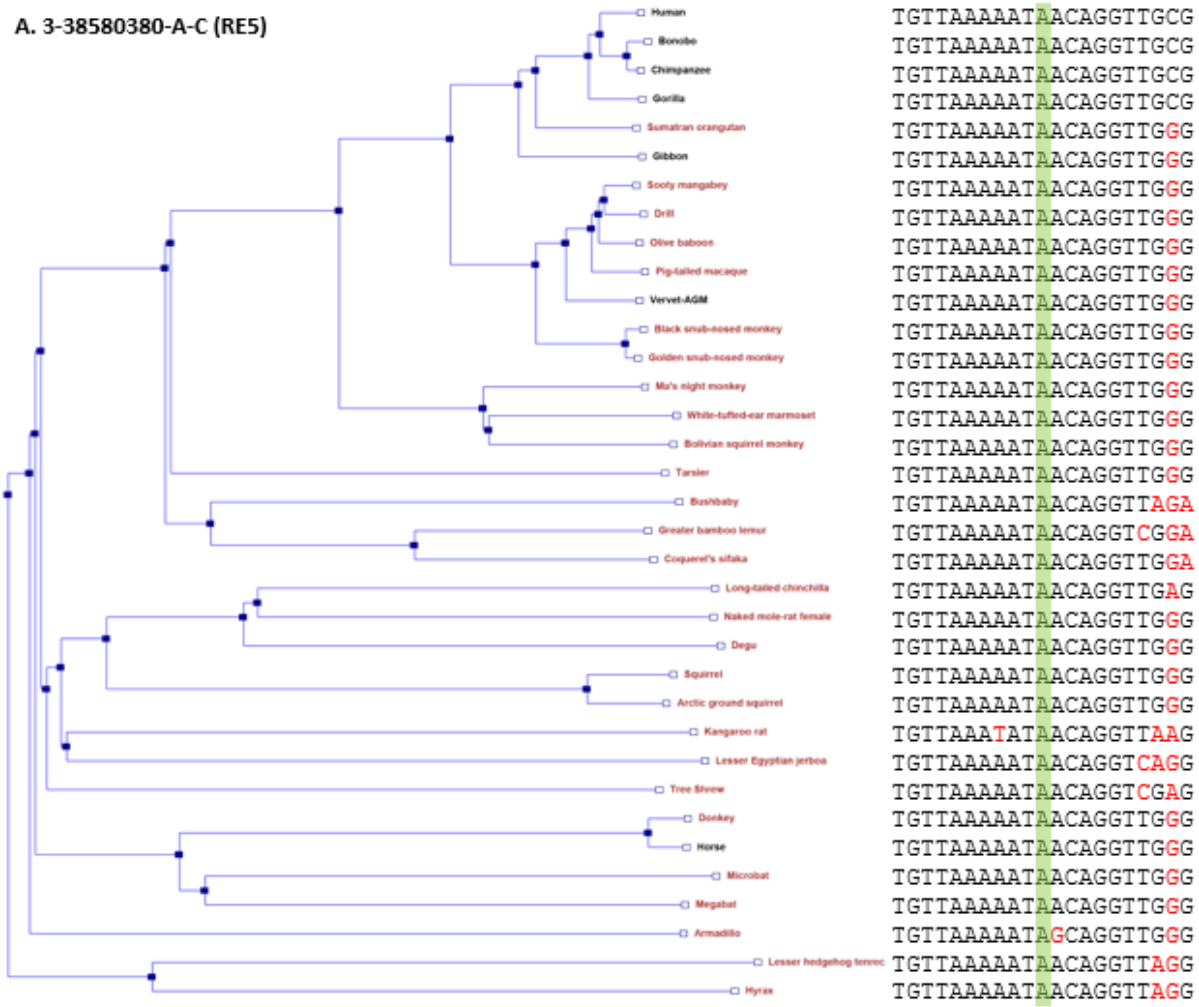
Table S5: Clinical characteristics of probands with BrS carrying RE5 variant. ECG – electrocardiogram; VF – ventricular fibrillation; VT – ventricular tachycardia.

Table S6: Results of transcription factor motif scanning analysis of the hs2177 enhancer sequence encompassing the RE5 variant, showing variant-dependent motif recognition sites and site detected in both wild type sequence and RE5 variant sequence.

Table S7: RNA sequencing results showing allele specific imbalance with reduced expression (from 1 to 0.66 = 34% reduction) of the SCN5A gene from the RE5 variant-carrying allele in cardiomyocytes from the A7 hiPSC line.

Table S8: Population minor allele frequencies in East and South Asian countries of the SCN5A p.Arg965Cys and RE5 variants. Shaded frequencies are those highlighted in Figure 4 - gnomAD South Asian data is used for India, Pakistan and Bangladesh and SG10K data for Malay and Chinese ancestry is used for Malaysia and China respectively. Note - for the SG10K v5.3 databases, any variant with AC between 1 and 5 is set to AC=5 and therefore the SG10K v5.3 Indian frequency for p.Arg965Cys (stated as AC=5) is not included here as the exact AC/AF is unknown.

Supplemental Figures



B. 3-38724980-T-C (RE2)

Human	CAATGG----------TATATGGTGAAAGTT
Bonobo	CTATGG--ins57bases--TATATGGTGAAAGTT
Chimpanzee	CTATGG--ins57bases--TATATGGTGAAAGTT
Gorilla	CAATGG----------TACATGGTGAAAGTT
Sumatran orangutan	CAATGG----------TATATGGTGAAAGTT
Gibbon	CAATGG----------TATATGGTGAAAGTT
Sooty mangabey	CAATGG----------TATATGATGAAAGTT
Drill	CAATGG----------TATATGATGAAAGTT
Olive baboon	CAATGG----------TATATGATGAAAGTT
Crab-eating macaque	CAATGG----------TATATGATGAAAGTT
Macaque	CAATGG----------TATATGATGAAAGTT
Pig-tailed macaque	CAATGG----------TATATGATGAAAGTT
Vervet-AGM	CAATGG----------TATATGATGAAAGTT
Black snub-nosed monkey	CAATGG----------TATATGATGAAAGTT
Golden snub-nosed monkey	CAATGG----------TATATGATGAAAGTT
Ma's night monkey	CGATGG----------CATGGTGAAAGTG
White-tufted-ear marmoset	CGATGG----------CATGGTAAAAGTG
Bolivian squirrel monkey	CAATGG----------CAGGGTGAAAGTG
Tarsier	CAATGG----------TATA--GAGACAGTT
Mouse Lemur	CCATGG----------CATGGTGAGAGCT
Greater bamboo lemur	TGATGG----------CATGGTGAAAGCT
Coquerel's sifaka	CAATG-----------ACATGATGAAAGCT
Bushbaby	TGATGG----------CATGGTAAAAGCT

C. 3-38719550-C-T (SCN10A-short promoter)

Human	CCCGCCACTACGCCCGGCTAA
White-tufted-ear marmoset	GCTGCCACTATGCCCAGCAAA
Bolivian squirrel monkey	GCTGCCACTATGCCCAGCAAT
Greater bamboo lemur	CTGGCCACCATAACCTGCAAA
Coquerel's sifaka	CGGGCCACCACATCCTGAGAA

D. 3-38683338-C-T (intergenic)

Human	TGCTAAATTGCCCAGGCTGTT
Bonobo	TGCTAAATTGCCCAGGCTGTT
Chimpanzee	TGCTAAATTGCCCAGGCTGTT
Gorilla	TGCTAAATTGCCCAGGCTGTT
Sumatran orangutan	TGCTAAATTGCCCAGGCTGTT
Gibbon	TGCTAAATTGCCCAGGCTGTT
Sooty mangabey	TGCTATATTGTCCAGGCTGTT
Drill	TGCTATATTGTCCAGGCTGTT
Olive baboon	TGCTATATTGTCCAGGCTGTT
Crab-eating macaque	TGCTATATTGTCCAGGCTGTT
Macaque	TGCTATATTGTCCAGGCTGTT
Pig-tailed macaque	TGCTATATTGTCCAGGCTGTT
Vervet-AGM	TGCTATATTGTCCAGGCTGTT
Black snub-nosed monkey	TGCTATATTGTCCAGGCTGTT
Golden snub-nosed monkey	TGCTATATTGTCCAGGCTGTT

Fig. S1 | Conservation across species for the affected bases of four candidate non-coding variants at the SCN5A/SCN10A locus. *Data is from Ensembl version v109 (Feb 2023), species with high quality assemblies are labelled in black and species with low quality assemblies in red. For each variant, species*

with orthologous alignments for the affected base and 10 flanking 5' and 3' bases are displayed. ***A.*** *For the RE5 3-38580380-A-C variant, all eutherian mammals are aligned (55/90 species with no alignment), with complete conservation across the variant base (shaded in green).* ***B.*** *For the RE2 3-38724980-T-C variant, the variant base (shaded in orange) is not fully conserved across primates (0/23 species with no alignment), with several insertion/deletion gaps in close proximity.* ***C.*** *For the SCN10A-short promoter 3-38719550-C-T variant, the variant base (shaded in orange) is not conserved across primates (18/23 species with no alignment).* ***D.*** *For the intergenic 3-38683338-C-T variant, the variant base (shaded in orange) is not conserved across primates (8/23 species with no alignment).*

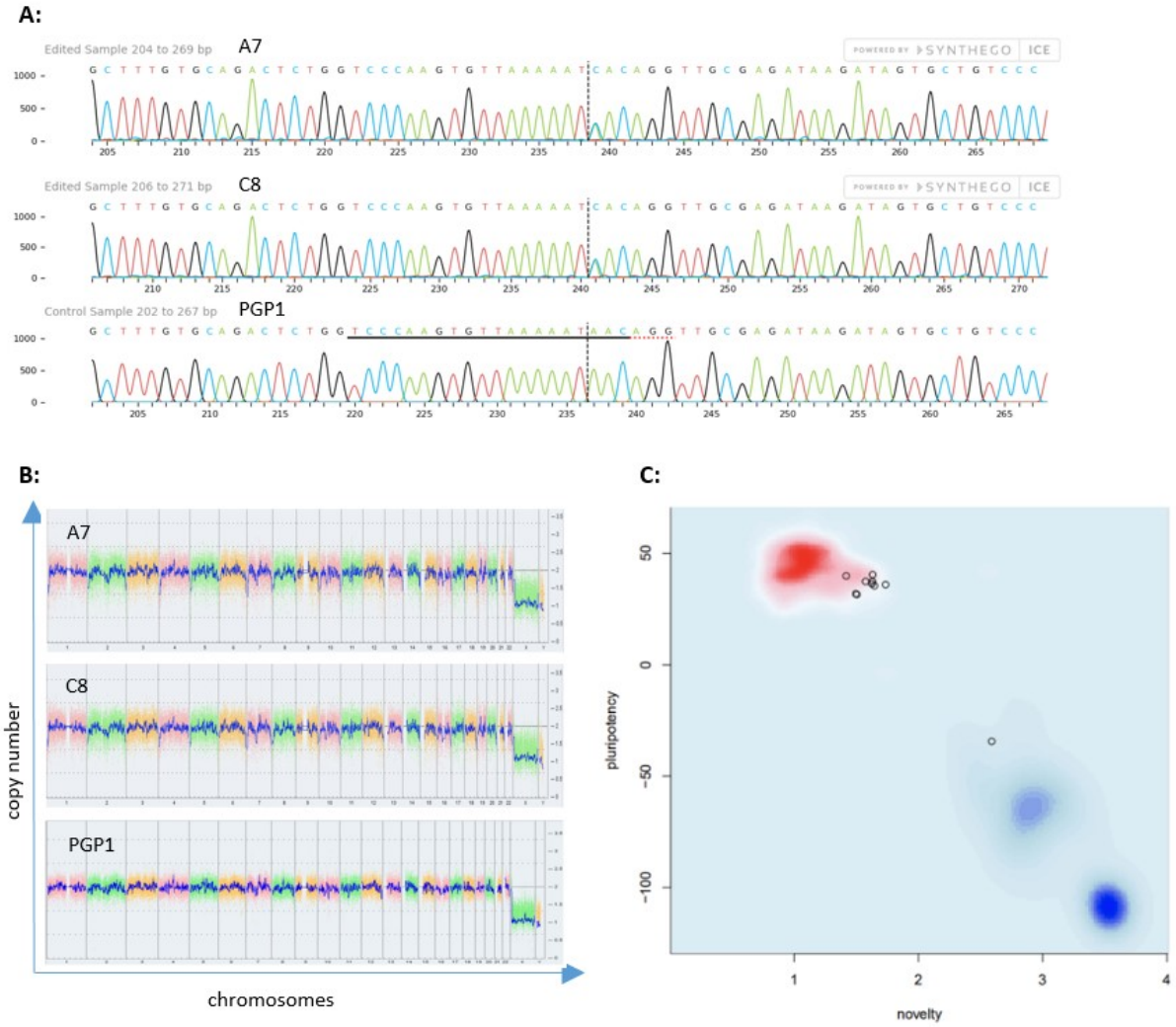


Fig. S2 | Quality control data on the edited hiPSC clones A7 and C8 carrying the heterozygous chr3-38580380-A-C variant. ***A****. Sanger sequencing chromatograms of edited and parental (PGP1) hiPSCs showing the introduction of the heterozygous A/C SNP in the A7 and C8 clones.* ***B****. Karyotype analysis by KaryoStat Assay (Applied Biosystems) indicated that genomic integrity is maintained in the edited clones.* ***C.*** *A Pluritest Assay (Thermo Scientific) performed on the edited A7 and C8 clones (bold circles) showed the similarity of the edited clones transcriptome to the reference set of >450 samples (red).*

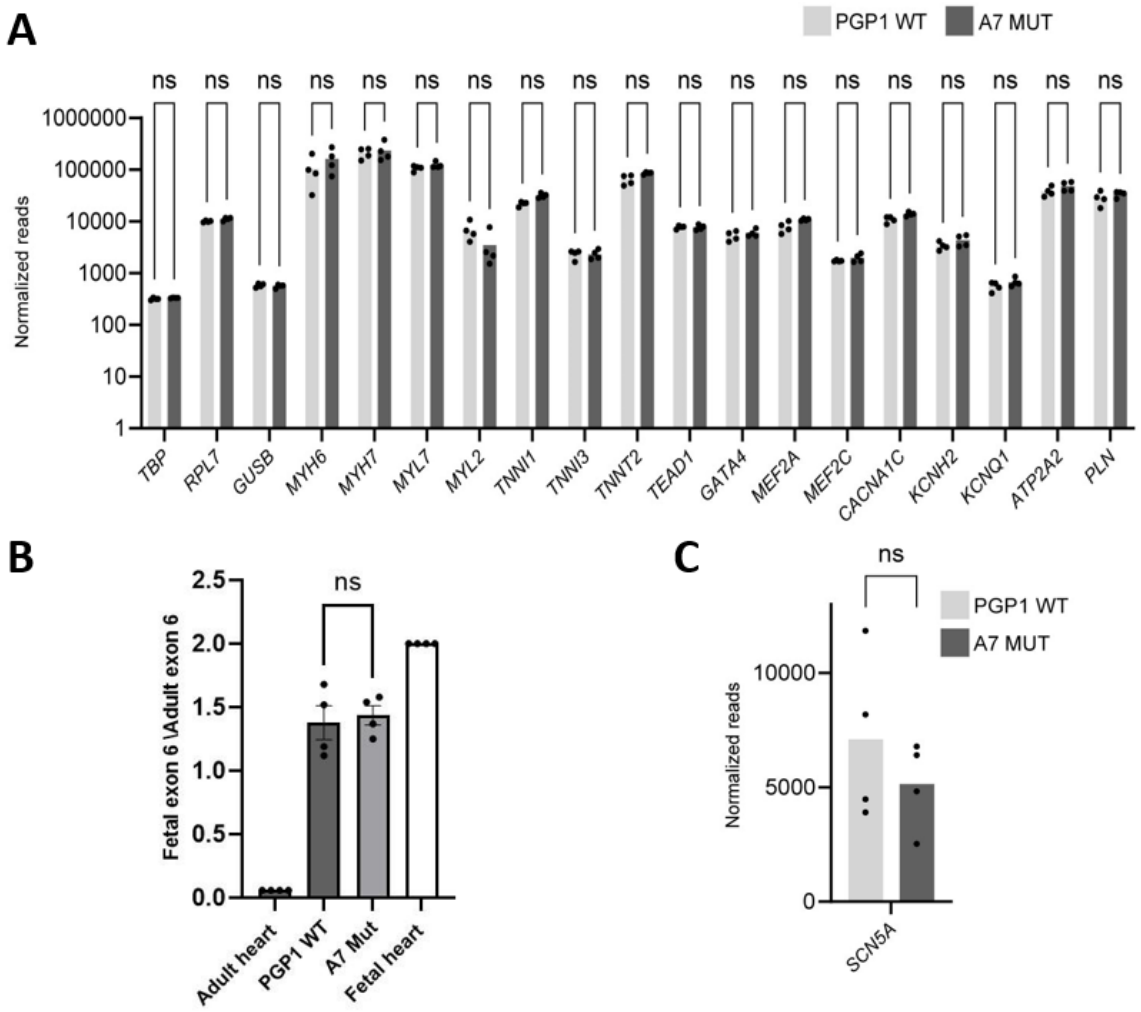


Fig. S3 | Quality control of cardiomyocyte differentiation and maturation using RNAseq data. A*. RNAseq data for cardiomyocyte differentiation and maturation–relevant genes in the wild type (PGP1) and RE5 variant edited (A7) lines, along with three housekeeping genes (TBP, RPL7, GUSB). Bulk RNAseq values are DESEQ2-normalized. The means and individual sample values are plotted for four differentiation batches of hiPSC-CMs from the PGP1 and A7 lines.* ***B****. The ratio of the "foetal" and "adult" SCN5A exon 6 reads (means +/- SEM from four differentiation batches) from DSEQ2-normalized RNAseq data from cardiomyocytes of the wild type (PGP1) and RE5 variant edited (A7) hiPSC lines as well as from publicly available reference samples (fetal_heart_UW_13w_GSM1059495 and adult_heart_GSM1698563). Using a non-parametric Mann- Whitney test, no significant difference was observed in the expression ratio between the two populations of cardiomyocytes indicating a comparable level of maturation.* ***C.*** *Normalised SCN5A expression between the wild type (PGP1) and RE5 variant edited (A7) hiPSC lines, demonstrating a 28% reduction in SCN5A expression in the RE5 samples, although this is non-significant due to the expected variability in cardiomyocyte composition and maturity between the lines.*

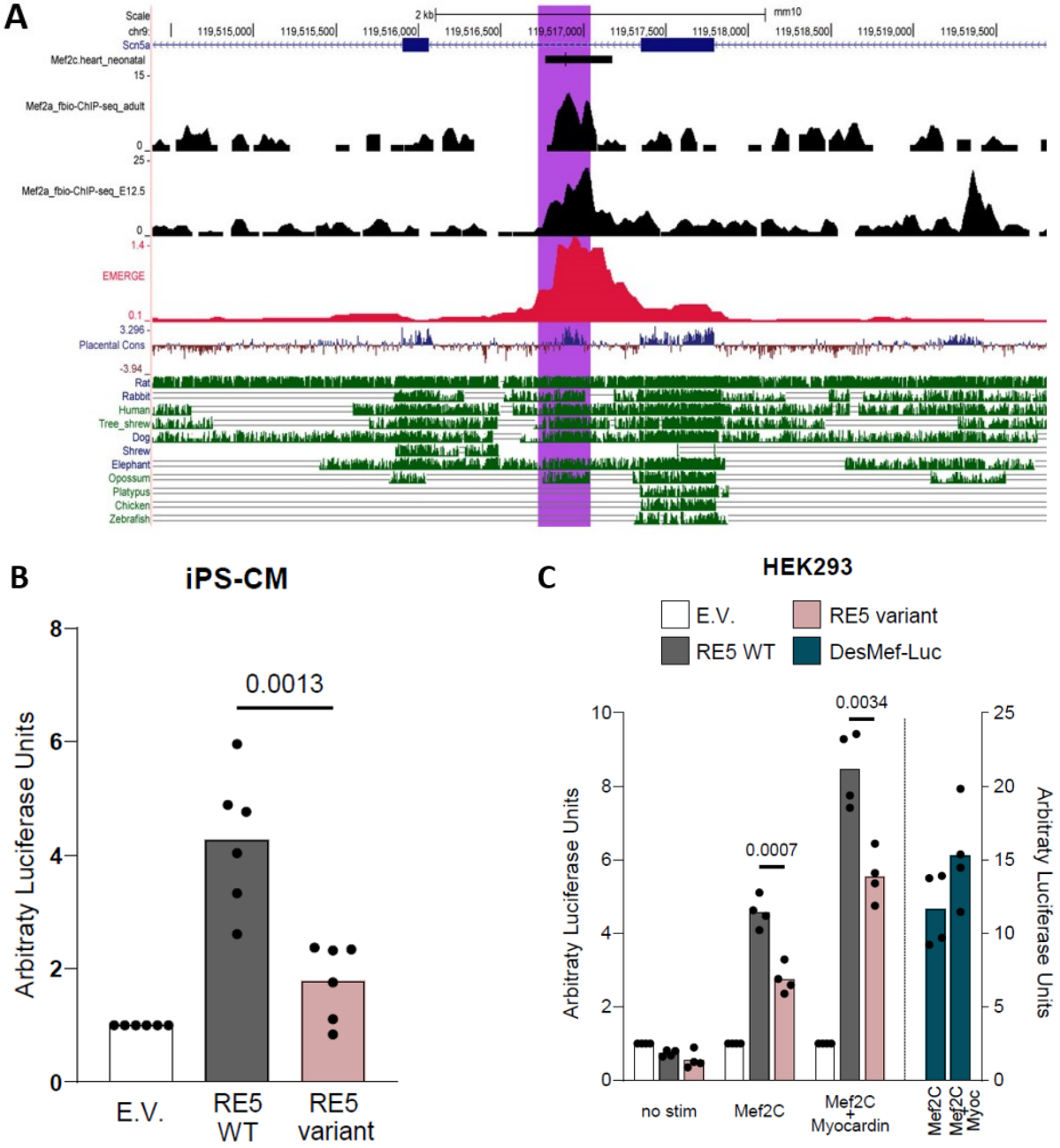


Fig. S4 | Loss of the predicted Mef2 binding motif in the mutant allele results in reduced gene expression. A*. Mef2 binding found within the highly conserved region within the RE5 enhancer. UCSC browser view of the mouse orthologue of the human RE5 region (purple) showing (bio)ChIP-seq for Mef2C and Mef2A datasets from neonatal, adult and embryonic cardiac tissue (black), EMERGE (red) and conservation tracks (green).* ***B****. Luciferase assay (n=6) shows enhancer activity differences between wild type (WT, grey) and RE5 variant (MUT, red) alleles transfected into hiPSC-CMs.* ***C****. Luciferase assay in HEK-293 cells (n=4) where transfection of Mef2C alone and Mef2C co-transfected with MYOCARDIN caused a statistically significant decrease in reporter activity for the RE5 variant compared to wild type (as normalized to vehicle, E.V., white). A fragment known to respond to Mef2 stimulation is included as a control (DesMef, blue)[54]. Statistical significance was determined using unpaired t-tests (p-values shown in figure).*

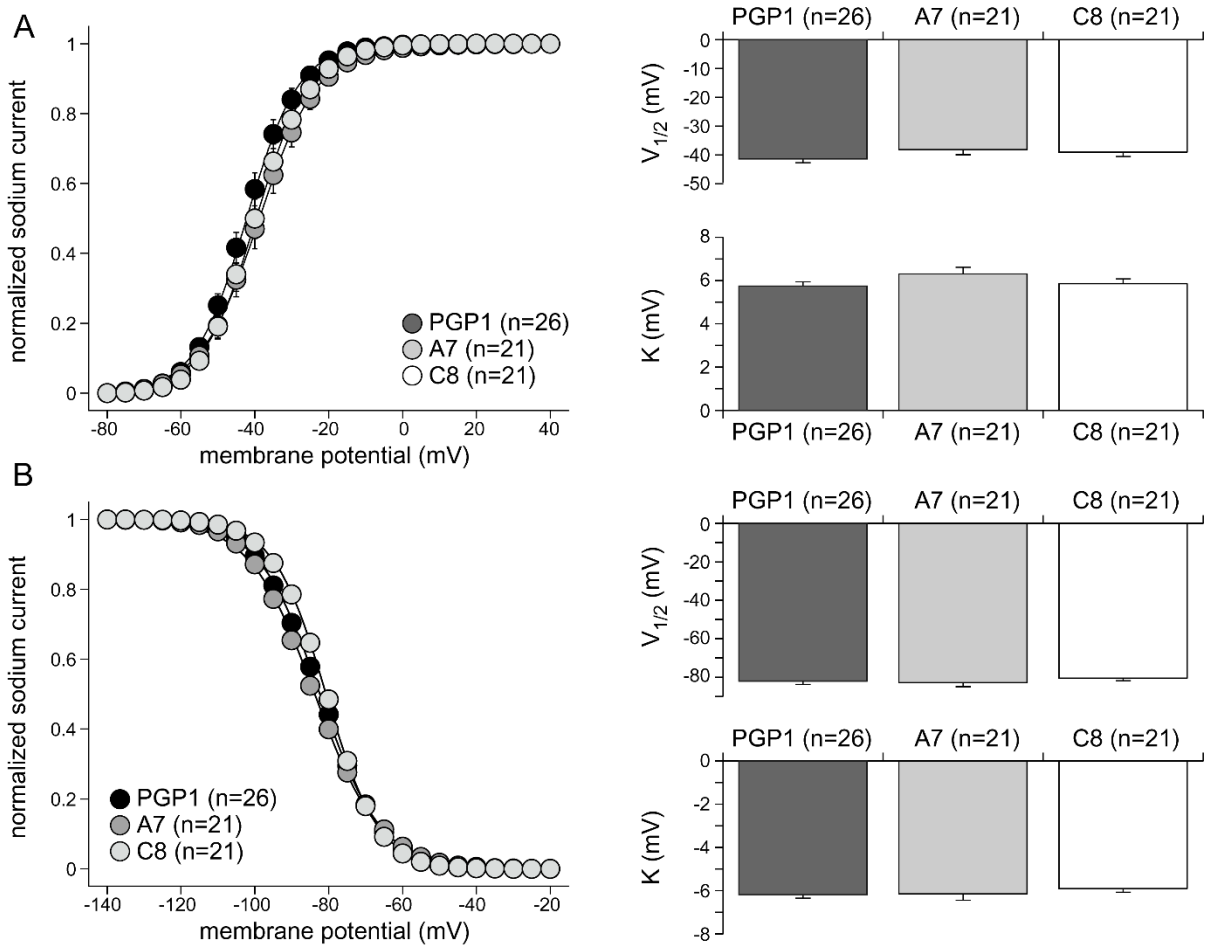


Fig. S5 | Sodium current (I_{Na}) characterization in control hiPSC-CMs (PGP1) and in hiPSC-CMs of the two RE5 variant lines (A7 and C8). A. *Average voltage dependence of activation curves (**left panel**) and average $V_{1/2}$ and k values (**right panels**). Solid lines represents the fitted Boltzmann function ($I/I_{max}=A/\{1.0+exp[(V_{1/2}-V)/k]\}$). B. Average voltage dependence of inactivation curves (**left panel**) and average $V_{1/2}$ and k values (**right panels**). Solid lines represents the fitted Boltzmann function ($I/I_{max}=A/\{1.0+exp[(V_{1/2}-V)/k]\}$).*

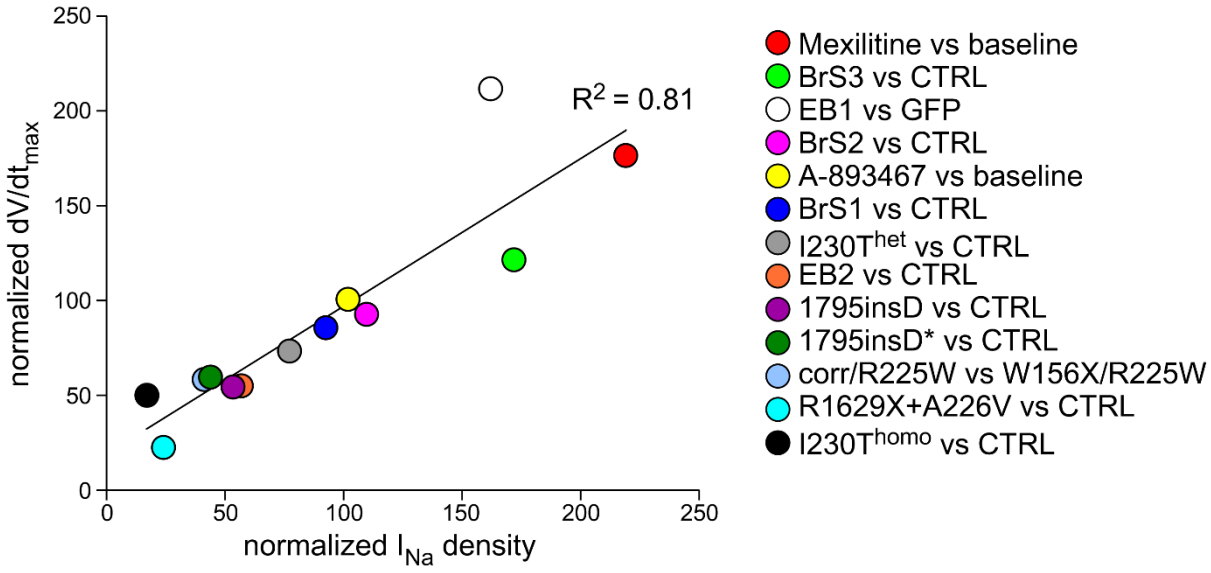


Fig. S6 | Relationship of normalized sodium current (I_{Na}) density and normalized maximal action potential upstroke velocity (dV/dt_{max}) in hiPSC-CMs. *Numbers are percentages and are calculated by dividing: drugs conditions vs baseline conditions, gene modulation vs GFP condition, or SCN5A mutation lines vs control (CTRL) lines. Solid line represents a linear fit (Y = 19.4 + 0.78X (R^2 = 0.81). Data are extracted from Nasilli et al., 2023 (Mexilitine vs baseline)[55]; Campostrini et al., 2023 (corr/R225W vs W156X/R2225W)[56]; Barc et al., 2022 (EB2 vs CTRL)[5]; Marchal et al., 2021 (EB1 vs GFP)[57]; Casini et al., 2019 (A-803467 vs baseline)[58]; Ma et al., 2018 (R1629X+A226V vs CTRL)[59]; Veerman et al., 2017 (I230Thet and I230Thomo vs CTRL)[60]; Portero et al., 2017 (1795insD vs CTRL)[61]; Veerman et al., 2016 (BrS1-3 and 1795insD* vs CTRL)[26].*