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Title: Don't skip a beat : studying cardiac rhythm in human pluripotent stem cell-derived cardiomyocytes

Issue Date: 2016-09-14

Chapter 4

A splice site mutation in *TECRL* is associated with inherited lethal arrhythmias in humans

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EMBO Mol Med 2016 (In Press).

Chapter content modified from the manuscript accepted for publication in *EMBO MOL MED* (2016), with the title “*TECRL*, a new life-threatening inherited arrhythmia gene associated with overlapping clinical features of both LQTS and CPVT”.

Abstract

Genetic causes of many familial arrhythmia syndromes remain elusive. In the current study, we performed whole-exome sequencing in members of a consanguineous Sudanese family diagnosed with exercise induced arrhythmias and identified a homozygous c.331+1G>A mutation in *TECRL*. To understand the consequences of this mutation, we generated human induced pluripotent stem cells (hiPSCs) from an affected individual (TECRL_{Hom}-hiPSCs), his heterozygous but clinically asymptomatic father (TECRL_{Het}-hiPSCs) and a healthy blood relative (CTRL-hiPSCs). Using hiPSC-derived cardiomyocytes (hiPSC-CMs), we showed that the *TECRL*c.331+1G>A mutation leads to skipping of exon3. Western blotting revealed decreased RYR2 and CASQ2 protein in TECRL_{Hom}-hiPSC-CMs and analysis of intracellular calcium ([Ca²⁺]_i) dynamics revealed smaller calcium transient amplitude as well as elevated diastolic calcium relative to CTRL-hiPSC-CMs. The rise of the [Ca²⁺]_i transient was also markedly slower and contained lower sarcoplasmic reticulum (SR) calcium stores as evidenced by the lower magnitude of caffeine-induced [Ca²⁺]_i transients. In addition, decay phase of the [Ca²⁺]_i transient was slower in TECRL_{Hom}-hiPSC-CMs due to decreased SERCA and NCX activities. Furthermore, TECRL_{Hom}-hiPSC-CMs demonstrated action potential (AP) prolongation compared with CTRL-hiPSC-CMs and *TECRL* knockdown in control human embryonic stem cell-derived CMs (hESC-CMs) also resulted in significantly longer APs. In addition, adrenergic stimulation by Noradrenaline (NA) significantly increased the propensity for triggered activity based on delayed after depolarizations (DADs) in TECRL_{Hom}-hiPSC-CMs. Interestingly, treatment of TECRL_{Hom}-hiPSC-CMs with the class Ic antiarrhythmic drug, flecainide significantly reduced the triggered activity in these cells. In summary, we demonstrate that a novel mutation in *TECRL* is associated with familial arrhythmias and that patient-specific hiPSC-CMs recapitulate salient features of the disease *in vitro*. These findings have implications for the diagnosis and treatment of inherited cardiac arrhythmias.

Introduction

Inherited arrhythmogenic diseases (IADs) are one of the prevalent causes of sudden cardiac death (SCD) in the young (Wilde *et al*, 2013). IADs can be classified into disorders with or without structural heart defects. The latter, comprised of channelopathies such as long-QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia (CPVT) are caused by mutations in genes encoding ion channel and calcium handling proteins that primarily affect the electrical activity of the heart (Schwartz *et al*, 2013).

Susceptibility to ventricular tachyarrhythmias in response to β -adrenergic stimulation is markedly increased in individuals diagnosed with CPVT, often leading to syncope and sudden death (Leenhardt *et al*, 2012). Autosomal dominant mutations in the cardiac ryanodine receptor *RYR2* (65% of all cases) and autosomal recessive mutations in the calcium-sequestering protein *CASQ2* (2-5% of all cases) have been identified to cause CPVT (Priori *et al*, 2001; Laitinen *et al*, 2001; Lahat *et al*, 2001; Postma *et al*, 2002). Mutations in *TRDN* (Roux-Buisson *et al*, 2012), a calcium release complex protein, and the calcium-binding protein *CALM1* (Nyegaard *et al*, 2012) have also been implicated in CPVT. A normal electrocardiogram (ECG) at rest and typical arrhythmias, including bi-directional and polymorphic ventricular tachycardia (VT), in response to catecholaminergic stress is observed in CPVT.

Although our understanding of genetic loci associated with IADs has markedly improved over the last few years, the genetic basis responsible for the clinical phenotype in a number of familial arrhythmias remains elusive (Schwartz *et al*, 2013). In 2007, an early-onset and highly malignant form of CPVT was reported in a consanguineous Arab family of Sudanese origin (Bhuiyan *et al*, 2007). Segregation analysis indicated an autosomal recessive mode of inheritance. Sequencing of the coding exons and exon-intron boundaries of *RYR2*, *CASQ2* and several other genes involved in cardiac excitation such as *KCNJ2*, *FKBP12.6*, *SCN5A*, *KCNH2*, *KCNQ1*, *KCNE1*, *KCNE2* and *NCX1* did not reveal any mutations. In the present study, we identified a G>A point mutation in the splice donor site of intron 3 on both alleles of *TECRL* gene (also known as *SRD5A2L2*) on chromosome 4 in the affected individuals of this family.

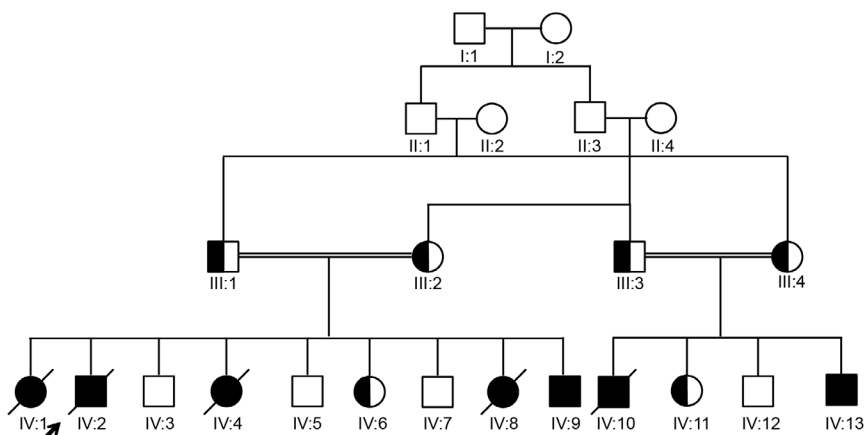
To date, all but two children who inherited the *TECRL*c.331+1G>A mutation in the affected family, died of a cardiac event during physical activity. To understand the functional consequence of this mutation and model the disease phenotype *in vitro*, we generated human induced pluripotent stem cells (hiPSCs) (Takahashi *et al*, 2007) from a 5 year-old symptomatic patient (referred to as *TECRL*_{Hom}-hiPSCs), his heterozygous (but clinically asymptomatic) father (*TECRL*_{Het}-hiPSCs) and a blood relative without the mutation (CTRL-hiPSCs). hiPSCs were differentiated into cardiomyocytes (CMs) and analyzed *in vitro*. Using these patient-derived hiPSC-CMs, we showed that the c.331+1G>A mutation in *TECRL* leads to skipping of exon 3. *TECRL*_{Hom}-hiPSC-CMs recapitulated aspects of the disease phenotype *in vitro* including increased susceptibility to triggered activity, which could be alleviated by flecainide.

Results

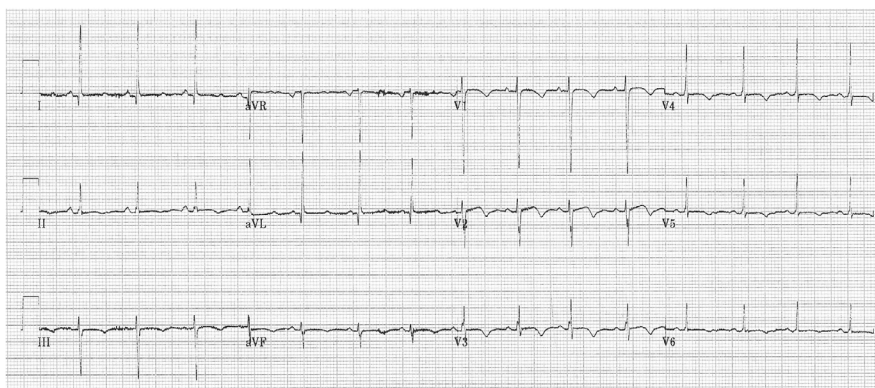
Clinical data analysis and Pedigrees

Previously, a large consanguineous family with two sub-families and several children affected by CPVT was reported (Bhuiyan *et al*, 2007). Parents are first-degree cousins and 7 of 13 children in the family (Fig. 1A) presented exertion-induced arrhythmias and/or sudden death during early childhood. In five of these children, an arrhythmic episode was fatal. Two children (IV:9 and IV:13) survived an arrhythmic attack.

A



B



C

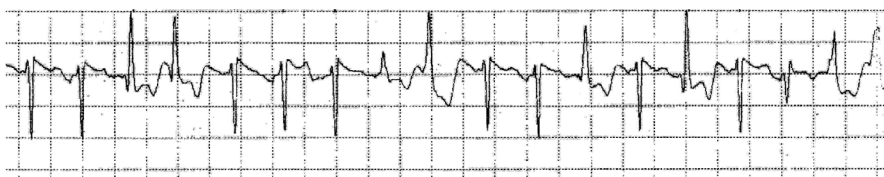


Figure 1. Familial arrhythmias in a consanguineous Sudanese family. (A) Pedigree of a family diagnosed with arrhythmias; Arrow indicates the proband, solid symbols represent family members affected by

arrhythmias who are also homozygous for mutations identified by exome sequencing. Crossed symbols indicate deceased individuals. Half-filled symbols correspond to individuals heterozygous for mutations identified by exome sequencing. **(B)** Electrocardiogram of subject IV:13 showing mild QTc prolongation. **(C)** ICD interrogation of subject IV:13 reveals an episode of VT.

Subject IV:13 is studied in this manuscript. At the age of 4, he developed cardiac arrest whilst running and was hospitalized. Single-channel ECG recording from the DC shock apparatus showed ventricular fibrillation and torsade de pointes (TdP), which was reversed to sinus rhythm following DC shock. ECG showed a QTc interval of 450 msec (Fig. 1B). He had a second attack whilst in the hospital but was in sinus rhythm in between the two attacks with a normal QRS axis, a QTc interval of 450 msec and no ST changes. He later received an implantable cardioverter de-fibrillator (ICD) and has had no further syncope or cardiac arrest. However, ICD interrogation revealed the occasional occurrence of a fast rhythm around 193 beats/min (cycle length 310msec) with polymorphic ventricular ectopy (Fig. 1C).

Clinical features of the other affected subjects are summarised in Table S1. IV:1, IV:2, IV:4 and IV:10 have been described earlier (Bhuiyan *et al*, 2007). Additional clinical details of IV:8, IV:9 and IV:10 are presented in the 'Appendix'.

Whole-exome sequencing and variant filtering

The presentation in this family (Fig. 1A) was compatible with an autosomal recessive inheritance pattern. To uncover the underlying genetic cause of SCD in this family, whole-exome sequencing was performed on genomic DNA obtained from two children, IV:2 and IV:10, who presented clinical symptoms and are first cousins (Fig. 1A). Genomic DNA from the parents of IV:2 (III:1 and III:2; Fig. 1A) who are clinically normal was also subjected to exome sequencing. On average, this yielded more than 81.5 million reads per sample, 87% of which could be mapped. The mean coverage of the target region was ~103-fold, with over 93% of target regions covered by ≥10 reads. In total 67,000-78,000 single nucleotide variants (SNVs) and 4,300-5,400 small insertion-deletions were identified in each of the individuals.

The disease-causing variants are under such strong negative selection that they are unlikely to be present in the population. Thus, we filtered out genetic variants representing single nucleotide polymorphisms (SNPs) or reported to have >1% minor allele frequency in exome and genome databases (public 1000Genomes and NHLBI Exome Sequencing projects, the CoLaus study and Vital-IT/SIB internal exome collection). We prioritized variants according to putative functionality and disease inheritance pattern. These filters resulted in 5 SNVs (Table 2), four on chromosome 4: 65-73Mb and one on chromosome 17:2.6Mb shared by IV:2 and IV:10 as homozygous and III:1 and III:2 as heterozygous according to disease inheritance pattern filter.

Validation and segregation of candidate variants

5 SNVs (Table S2) shared by IV:2 and IV:10 were further investigated in the family members by Sanger sequencing. Of these, an exon mutation in *ANKRD17*, NC_000004.11:g.73984490A>C (ENST00000358602:exon22:c.4103T>G) and a splice site mutation in *TECRL*, NC_000004.12:g.65194229C>T (NM_001010874:exon4:c.331+1G>A), were found to be homozygous in children who had presented with a clinical phenotype

(IV:2, IV:4, IV:8, IV:9, IV:10, IV:13; Fig. 1A). Parents were found to be heterozygous in both families. *ANKRD17*c.4103A>C and *TECRL*c.331+1G>A variants were not reported as SNPs in the general population (neither in dbSNP, 1000Genomes, 6500NHLBI, ExAC browser nor in our in-house CoLaus and Vital-IT/SIB databases) and indicated a substitution deficit and evolutionary conservation (phastCons-46way score of 0.498 and GERP++ score of 5.32 for *ANKRD17*; phastCons-46way score of 0.397 and GERP++ score of 5.23 for *TECRL*). Additionally, these mutations were not found in control DNA samples obtained from 72 Saudi Arabian subjects.

ANKRD17 has been reported to regulate cell cycle progression and is ubiquitously expressed in all human tissues (Deng *et al*, 2009). In mice, deletion of *Ankrd17* resulted in embryonic lethality due to compromised vascular maturation (Hou *et al*, 2009). The thin myocardium and poor ventricular trabeculation in *Ankrd17* knockout embryos were assumed to be secondary to the lack of circulating blood. Moreover, hearts isolated from *Ankrd17* null embryos remained beating indicating no conduction abnormalities. This suggested that a mutation in *ANKRD17* might not be causal to the CPVT phenotype in the patient family.

TECRL, on the other hand was identified in a genome-wide transcriptional profiling study on human embryonic stem cells (hESCs) differentiating to CMs *in vitro*. In the mouse embryo, its expression was restricted to the heart, with predominant expression in the inflow tract (Beqqali *et al*, 2006). Since the affected members of the CPVT family only displayed cardiac anomalies, we hypothesized that *TECRL* may be the gene responsible for their clinical phenotype.

TECRL is an endoplasmic reticulum (ER) protein expressed preferentially in the heart

Human *TECRL* maps to chromosome 4q13 and contains 12 exons (Appendix Fig. S1A). The open reading frame of *TECRL* encodes a 363 amino acid protein and predicted to contain a ubiquitin-like domain in the N-terminal half of the protein, three transmembrane segments as well as a 3-oxo-5- α steroid 4-dehydrogenase domain in the C-terminal half of the protein (Appendix Fig. S1B-C). *TECRL*c.331+1G>A mutation found in the Sudanese family is located in the splice donor site of intron 3 resulting in an internal deletion in the putative ubiquitin-like domain (Appendix Fig. S1A-C). Comparison of amino acid sequences revealed high degree of cross-species conservation (Appendix Fig. S2D), suggesting that *TECRL* might have an essential function in the heart.

To determine the spatio-temporal expression pattern of *Tecrl*, its expression in early mouse embryos was investigated by *in situ* hybridization. *Tecrl* was not expressed in the cardiac crescent at embryonic day E7.5 (Fig. 2A) but was observed at E8.5 in the entire heart with the strongest expression occurring in the developing inflow tract (ift) (Fig. 2B), especially in the left horn (Fig. 2C). At E9.5, *Tecrl* expression was still detectable in the atria and ventricles, albeit at lower levels whereas strong expression remained in the inflow tract (Fig. 2D). From E10 onwards, *Tecrl* was also expressed at low levels in somites, particularly in the myotome region, that gives rise to skeletal muscle (Fig. 2E-F). At E10.5, cardiac expression of *Tecrl* was no longer restricted to the inflow tract and its expression was maintained in the somites (Fig. 2G). At E14.5, *Tecrl* was expressed in the entire myocardium but not in the lungs, which served as negative control (Fig. 2H). In adult mice, reverse transcription quantitative polymerase chain

reaction (RT-qPCR) analyses showed that *Tecrl* expression was highest in the heart with very low to almost undetectable levels in brain, skeletal muscle, stomach, pancreas, liver, kidney, small intestine and uterus (Fig. 2I).

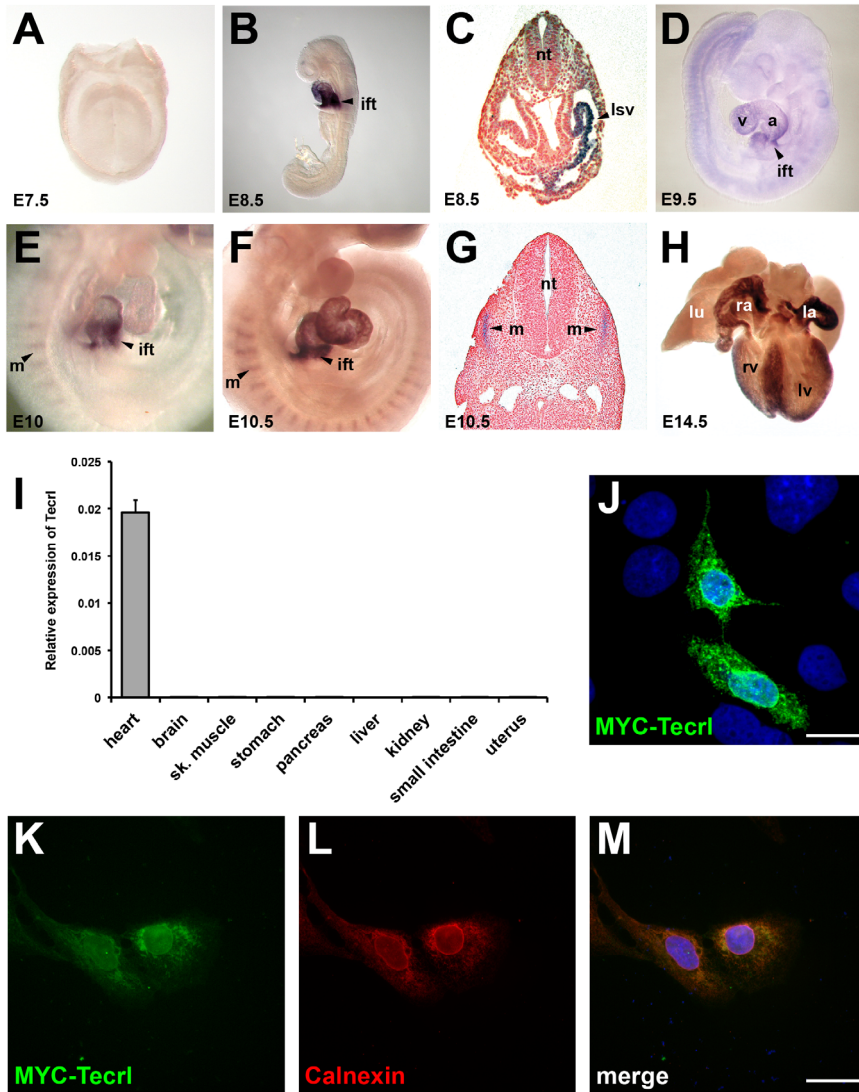


Figure 2. Spatio-temporal, tissue and subcellular expression of mouse *Tecrl*. Expression of *Tecrl* in mouse development is not seen in (A) cardiac crescent at E7.5 but is prominent (B) in the inflow tract (ift) region at E8.5, particularly (C) in the left sinus venosus (lsv). (D) At E9.5, expression is observed in all four chambers of the heart but is strongest in the inflow tract. (E-G) From E10 onwards, *Tecrl* is expressed in the somites. (H) At E14.5, *Tecrl* expression is present in the entire myocardium of the heart; [atrium (a), ventricle (v), neural tube (nt), lung (lu), right atrium (ra), left atrium (la), right ventricle (rv) and left ventricle (lv)]. (I) mRNA expression analysis of *Tecrl* by RT-qPCR in adult mouse tissues demonstrates preferential expression of *Tecrl* in the heart. (J) Localization of MYC-Tecrl in COS-1 cells. The accumulation of MYC-Tecrl is perinuclear consistent with it being localized to the ER. (K-M) Co-localization of MYC-Tecrl and the ER marker Calnexin in H10 cells. Nuclei stained with DAPI. Scale bar: 10 μ m.

We next investigated the cellular localization of Tecrl in COS-1 cells by immunofluorescence analysis which demonstrated that MYC epitope-tagged mouse Tecrl protein primarily resides in the ER (Fig. 2J), which was also confirmed in H10 cells by co-localization with Calnexin, an ER chaperone (Fig. 2K-M).

Collectively, these results demonstrated that *Tecrl* encodes an ER protein expressed preferentially in the heart and that it is evolutionarily conserved, suggesting an important role in the heart.

Derivation of patient-specific hiPSCs and differentiation to CMs

Skin biopsies were obtained from patient IV:13 carrying the homozygous *TECRL*c.331+1G>A mutation, his heterozygous father, III:3 and a family member, IV:7 who does not carry the mutation.

Figure 3. Generation of hiPSCs and differentiation to cardiomyocytes. (A) Skin fibroblasts (left) from IV:13 homozygous for the *TECRL*c.331+1G>A mutation were reprogrammed to hiPSCs (center), which expressed the pluripotency markers NANOG and SSEA4 (right). Scale bars: 100 μ m. (B) *TECRL*_{Hom} hiPSCs have a normal karyotype (left) and PluriTest demonstrates a high pluripotency score and low novelty score for all three hiPSC lines (right). (C) *TECRL*_{Hom}-hiPSCs generate derivatives of mesoderm (left), endoderm (center) and ectoderm (right). Scale bars: 25 μ m (D) DNA sequencing confirms *TECRL*c.331+1G>A in the affected family members. Control (left); Heterozygous (Center); Homozygous (right).

Dermal fibroblasts (Fig. 3A- left image) were reprogrammed to hiPSCs with Sendai virus vectors encoding the four transcription factors OCT4, SOX2, KLF4, and MYC. Colonies obtained after reprogramming (Fig. 3A- center image) morphologically resembled hESC colonies. hiPSC clones expressed the pluripotency markers, NANOG and SSEA4 (Fig 3A-right image; Appendix Fig. S2) and had a normal karyotype (Fig. 3B- left image; Appendix Fig. S2). One line per genotype was selected for further experiments. The transcriptional profile of the hiPSC-lines used in the study were analyzed by PluriTest (Müller *et al*, 2011), which showed a high pluripotency score and low novelty score as expected (Fig 3B; right image). The pluripotent nature of the hiPSCs was further confirmed by their ability to differentiate to derivatives of all three germ layers (Fig. 3C). Importantly, nucleotide sequence analysis confirmed the presence of a homozygous c.331+1G>A mutation in *TECRL*_{Hom}-hiPSCs (Fig 3D).

A mutation at the exon 3-intron 3 boundary of *TECRL* leads to exon 3 skipping

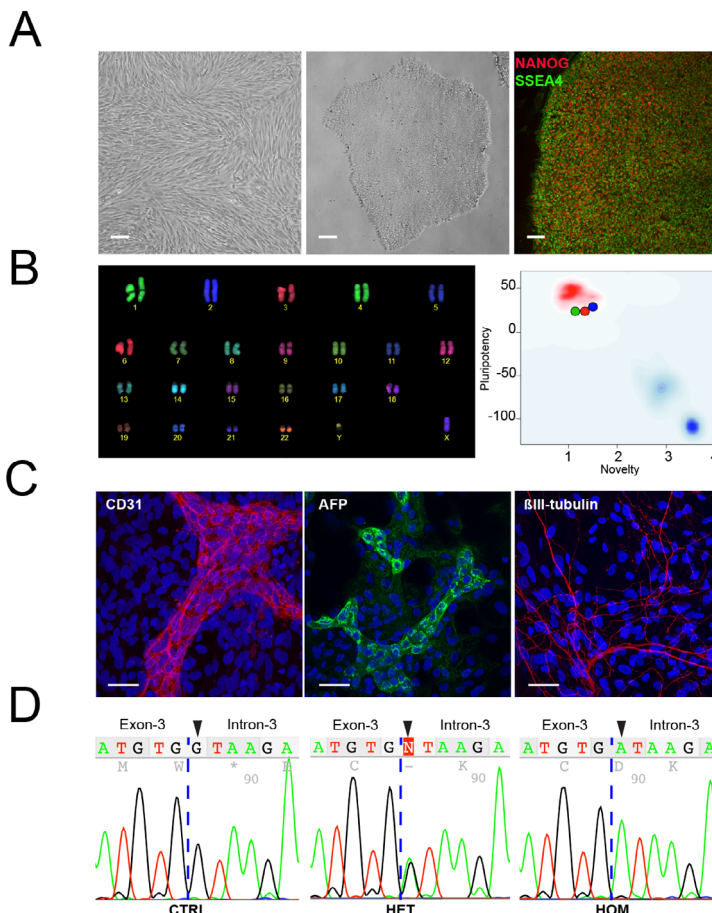
hiPSCs were differentiated to the cardiac lineage with a monolayer protocol (Fig. 4A) and all three lines produced similar percentages of CMs. Contractile areas were observed in culture at day 10 of differentiation. The structure and organization of sarcomeres in *TECRL*_{Hom}-hiPSC-CMs, *TECRL*_{Het}-hiPSC-CMs and CTRL-hiPSC-CMs was similar based on ACTN2 immunostaining (Fig. 4B).

Figure 4. Differentiating hiPSCs to cardiomyocytes (CMs). (A) Schematic of the protocol used for cardiac differentiation of hiPSCs. (B) ACTN2 immunostaining of CTRL-, *TECRL*_{Het}- and *TECRL*_{Hom}-hiPSC CMs. Scale bar: 25 μ m. (C) Analysis of RT-PCR products by gel electrophoresis. Amplification products of *TECRL* exons 2-4 from CTRL-, *TECRL*_{Het}- and *TECRL*_{Hom}-hiPSC-CMs (left) and coding sequence of *TECRL* from CTRL- and *TECRL*_{Hom}-hiPSC-CMs (right).

To determine the effect of the c.331+1G>A mutation in *TECRL*, cDNA was prepared from hiPSC-CMs and PCR analysis was performed with primers designed to target *TECRL* exons

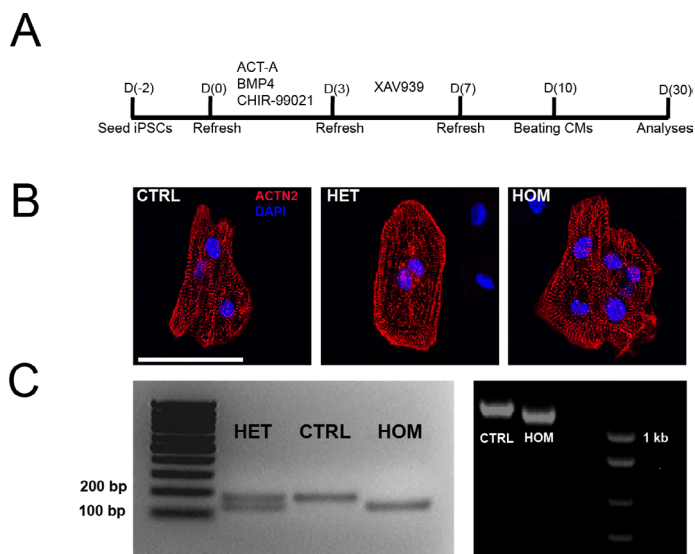
2-4. A 171 bp product is expected if the mRNA of *TECRL* contains exon 3 whereas a 126 bp product should be present when exon 3 is missing. Analysis of the amplicons from *TECRL*_{Het}-hiPSC-CMs clearly showed two transcripts, a longer product containing exon 3 and a shorter DNA fragment lacking exon 3 (Fig. 4C; left image). As expected, CTRL-hiPSC-CMs exclusively yielded the longer PCR product corresponding to 171 bps. On the contrary, PCR analysis of *TECRL*_{Hom}-hiPSC-CMs revealed a single product of 126 bps (Fig. 4C; left image), indicating that the c.331+1G>A mutation in the splice-donor site of *TECRL* intron 3 causes complete skipping of exon 3. Amplification of the entire *TECRL* coding region showed a shorter PCR product (1046 bp) of *TECRL* from *TECRL*_{Hom}-hiPSC-CMs compared to the 1091 bp amplicon of CTRL-hiPSC-CMs (Fig 4C; right image). Nucleotide sequencing analysis of the PCR product from *TECRL*_{Hom}-hiPSC-CMs confirmed deletion of 45 bps corresponding to exon 3.

*TECRL*_{Hom}-hiPSC-CMs exhibit abnormalities in calcium handling



Impaired calcium homeostasis underlies the pathophysiology of various IADs, particularly CPVT. To investigate whether the *TECRL*c.331+1G>A mutation has an effect on canonical

calcium handling proteins, we investigated their expression at the protein level by western blotting. There was a 52% reduction in RYR2 protein and 85% reduction in CASQ2 protein in TECRL_{HOM}-hiPSC-CMs, while SERCA2a, PLB, NCX and Ca_v1.2 protein levels were unaffected (Appendix Fig. S3).



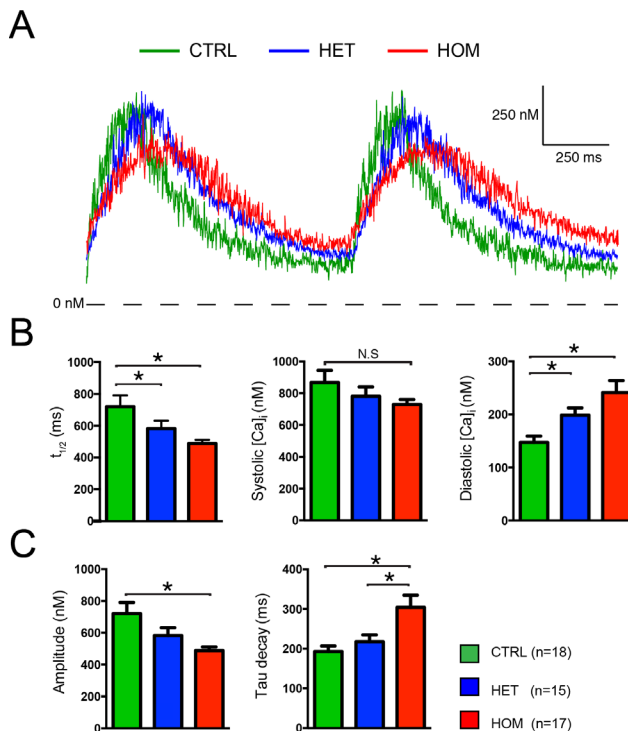


Figure 5. Whole-cell $[Ca^{2+}]_i$ transients in hiPSC-CMs. (A) Representative traces of $[Ca^{2+}]_i$ transients in Indo-1A loaded hiPSC-CMs paced at 1 Hz. (B) Time to reach 50% of $[Ca^{2+}]_i$ transient amplitude, $t_{1/2}$; $[Ca^{2+}]_i$ concentrations during systole and diastole (C) Amplitude and tau decay of the $[Ca^{2+}]_i$ transient; $n = 5-6$ cells each from three independent experiments; * $P < 0.05$, statistical significance was assessed with one-way ANOVA on ranks followed by Dunn's test in case failed normality or variance. Otherwise, one-way ANOVA followed by Student-Neuman-Keuls test was used;

Next, we studied intracellular calcium ($[Ca^{2+}]_i$) transients of $TECRL_{Hom}$ -hiPSC-CMs. Fig. 4A shows typical $[Ca^{2+}]_i$ transient recordings of CTRL-hiPSC-CMs, $TECRL_{Het}$ -hiPSC-CMs and $TECRL_{Hom}$ -hiPSC-CMs following 1-Hz electrical stimulation. A summary of average $[Ca^{2+}]_i$ characteristics is shown in Fig 4B-C. The $[Ca^{2+}]_i$ transient rose markedly slower in $TECRL_{Hom}$ -hiPSC-CMs than in CTRL-hiPSC-CMs (Fig. 5A) resulting in a significant difference in the time required to reach 50% of the $[Ca^{2+}]_i$ transient amplitude, $t_{1/2}$ (Fig. 5B). While there were no significant differences in systolic $[Ca^{2+}]_i$, the diastolic $[Ca^{2+}]_i$ was markedly higher in $TECRL_{Hom}$ -hiPSC-CMs compared with CTRL-hiPSC-CMs (Fig. 5B).

Consequently, the amplitude of the $[Ca^{2+}]_i$ transient was lower in $TECRL_{Hom}$ -hiPSC-CMs (Fig. 5C). Furthermore, the decay of the $[Ca^{2+}]_i$ transient was remarkably slower in $TECRL_{Hom}$ -hiPSC-CMs, as indicated by the significant increase in the time constant (tau) of decay (Fig. 5C).

To gain further insight into the observed differences in $[Ca^{2+}]_i$ transient properties of $TECRL_{Hom}$ -hiPSC-CMs and ascertain the cause of slower decay time, we measured the activity of sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA), Na^+ - Ca^{2+} exchanger (NCX) and the slow mechanisms (mitochondrial Ca^{2+} uniporter and sarcolemmal Ca^{2+} ATPase) in Ca^{2+} extrusion

from the cytoplasm by application of the RYR2 agonist, caffeine and the NCX1 blocker, NiCl_2 (Bassani & Bers, 1995; Díaz *et al*, 2004). 10 mM caffeine induced fast release of Ca^{2+} from the SR into the cytosol and SERCA activity (K_{SERCA}) was analyzed by comparing the rates of decay of spontaneous $[\text{Ca}^{2+}]_i$ transients (K_{sys}) and caffeine-evoked transients (K_{caff}). The role of NCX in Ca^{2+} removal from the cytoplasm was obtained through subtraction of the rate of decay of the caffeine-evoked $[\text{Ca}^{2+}]_i$ transients (K_{caff}) from that of caffeine-evoked $[\text{Ca}^{2+}]_i$ transients in the presence of 10 mM NiCl_2 ($K_{\text{caff+Ni}}$). Representative $[\text{Ca}^{2+}]_i$ transients of a CTRL-hiPSC-CM and $\text{TECRL}_{\text{Hom}}$ -hiPSC-CM are shown in Fig. 6A.

The amplitude of caffeine-evoked $[\text{Ca}^{2+}]_i$ transients in the presence of NiCl_2 was considerably lower in $\text{TECRL}_{\text{Hom}}$ -hiPSC-CMs compared with CTRL-hiPSC-CMs (Fig. 6B). However, fractional Ca^{2+} release, which is a measure of the amplitude of the normal systolic $[\text{Ca}^{2+}]_i$ transient as a fraction of the amplitude of caffeine-evoked $[\text{Ca}^{2+}]_i$ transient in the presence of NiCl_2 , did not differ significantly between the groups (Fig. 6B). K_{SERCA} and K_{NCX} were significantly lower in $\text{TECRL}_{\text{Hom}}$ -hiPSC-CMs compared with CTRL-hiPSC-CMs (Fig 6C). K_{slow} mechanisms indicated by the rate of Ca^{2+} transient decay in the presence of caffeine and NiCl_2 ($K_{\text{caff+Ni}}$) was not affected in $\text{TECRL}_{\text{Hom}}$ -hiPSC-CMs (Fig. 6C).

Although SERCA and NCX activities were lower in $\text{TECRL}_{\text{Hom}}$ -hiPSC-CMs, the relative contribution of SERCA, NCX, and slow mechanisms to $[\text{Ca}^{2+}]_i$ transient decay did not differ significantly between the groups (Fig. 6D). SERCA and NCX removed about 65-70% and 25-30% of the activator Ca^{2+} from the cytosol respectively, whereas the mitochondrial Ca^{2+} uptake and sarcolemmal Ca^{2+} -ATPase accounted for the removal of <8% of $[\text{Ca}^{2+}]_i$.

The $[\text{Ca}^{2+}]_i$ transient properties of $\text{TECRL}_{\text{Het}}$ -hiPSC-CMs were largely in between CTRL-hiPSC-CMs and $\text{TECRL}_{\text{Hom}}$ -hiPSC-CMs, suggesting a gene dosage dependency (Fig. 5B-C and 6B-D).

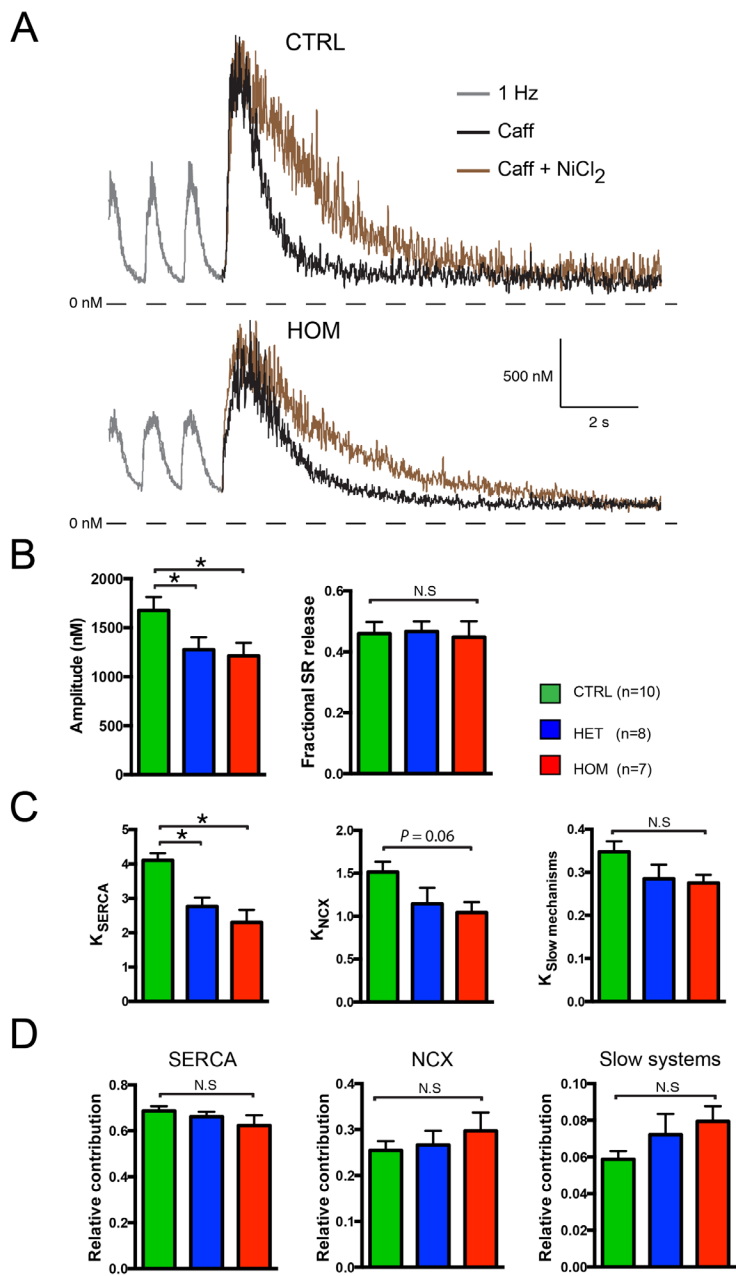


Figure 6. $[Ca^{2+}]_i$ extrusion mechanisms in hiPSC-CMs. **(A)** Representative traces of $[Ca^{2+}]_i$ transients in Indo-1 AM loaded hiPSC-CMs paced at 1 Hz, in the presence of caffeine (caff) or caffeine and $NiCl_2$. **(B)** Amplitude of $[Ca^{2+}]_i$ in the presence of caffeine and $NiCl_2$ and fractional SR release in hiPSC-CMs. **(C)** Rate constants of SERCA-, NCX- and slow mechanisms-based $[Ca^{2+}]_i$ decay in hiPSC-CMs. **(D)** Relative contribution of SERCA, NCX and slow mechanisms to $[Ca^{2+}]_i$ extrusion in hiPSC-CMs; Data information: n = 2-3 cells each from three independent experiments; * $P < 0.05$ statistical significance was assessed with one-way ANOVA on ranks followed by Dunn's test in case failed normality or variance. Otherwise, one-way ANOVA followed by Student-Neuman-Keuls test was used.

TECRL_{HOM}-hiPSC-CMs and hESC-CMs with *TECRL* knockdown exhibit prolonged APs

Increased diastolic $[Ca^{2+}]_i$ is believed to be a substrate for delayed afterdepolarizations (DADs) leading to triggered arrhythmias which maybe further aggravated by catecholaminergic stimulation. TECRL_{HOM}-hiPSC-CMs showed elevated diastolic $[Ca^{2+}]_i$ which prompted us to investigate their action potential (AP) properties. We also studied the AP parameters of hESC-CMs with *TECRL* knockdown in order to compare their electrophysiological phenotype to TECRL_{HOM}-hiPSC-CMs.

First, we studied the AP properties of TECRL_{HOM}-hiPSC-CMs (Fig. 7A). Representative APs and averaged AP parameters of hiPSC-CMs from CTRL, TECRL_{HET} and TECRL_{HOM} lines paced at 1-Hz are shown in Fig. 7B-C. Average resting membrane potential (RMP), maximum upstroke velocity (dV/dt_{max}), maximal AP amplitude (APA_{max}) and AP plateau amplitude (APA_{plat}) did not differ significantly between the three groups (Fig. 7C). However, APs of TECRL_{HOM}-hiPSC-CMs displayed marked prolongation of AP duration (APD) at 20% repolarization (APD₂₀) and there was a clear trend for an increase in APD at 50% (APD₅₀) and 90% (APD₉₀) repolarization compared to CTRL-hiPSC-CMs (Fig. 7C).

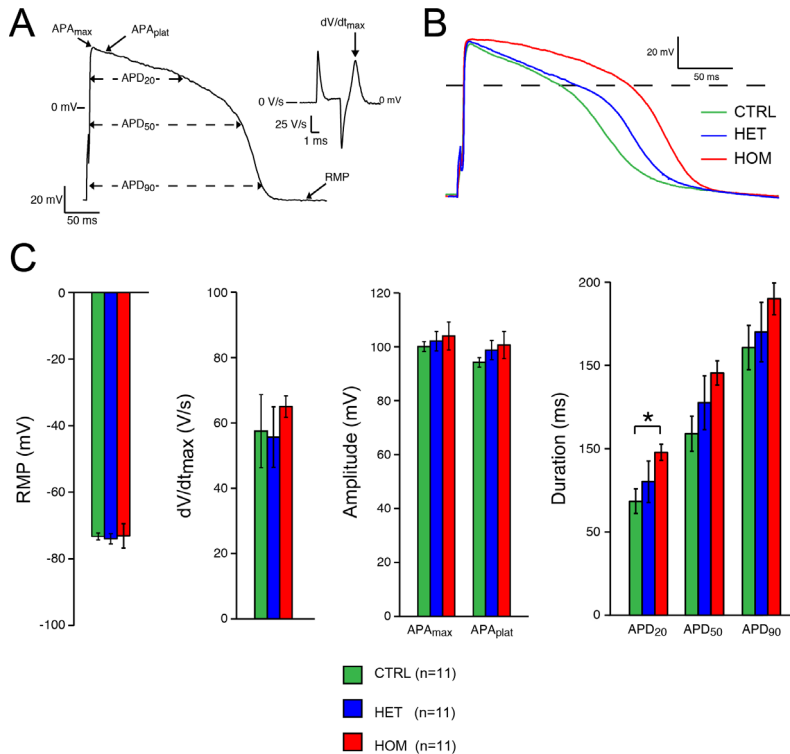


Figure 7. AP characteristics of TECRL-hiPSC-CMs. (A) AP illustrating the analysed parameters **(B)** Representative APs from control (CTRL), heterozygous (HET) and homozygous (HOM) CMs. **(C)** RMP, dV/dt_{max} , APA_{max} , APA_{plat} , APD₂₀, APD₅₀ and APD₉₀ of CTRL-, TECRL_{HET}- and TECRL_{HOM}-hiPSC-CMs; $n = 3-4$ cells each from three independent experiments; * $P < 0.05$ statistical significance was assessed with one-way ANOVA on ranks followed by Dunn's test in case failed normality or variance. Otherwise, one-way ANOVA followed by Student-Neuman-Keuls test was used; AP = action potential; APA_{max} = maximum AP

amplitude, APA_{plat} = AP plateau amplitude; APD_{20} , APD_{50} , and APD_{90} = AP duration at 20, 50, and 90% repolarization, respectively; dV/dt_{max} = maximum upstroke velocity; RMP = resting membrane potential.

Next, we performed lentiviral short hairpin (sh) RNA-mediated knockdown of *TECRL* in hESC-CMs to study their AP properties. Lentiviral vectors encoding five different *TECRL*-specific shRNAs (Appendix table S3) for *TECRL* were tested in hESC-CMs, two of which (*TECRL*-sh#3 and *TECRL*-sh#4) gave efficient knockdown as assessed by RT-qPCR. However, following puromycin selection, hESC-CMs treated with *TECRL*-sh#4 showed pronounced cytotoxicity (Appendix Fig. S4A). Therefore, *TECRL*-sh#3 (hereinafter referred to as sh*TECRL*) was selected for further experiments and transduction in hESC-CMs resulted in 70% reduction in *TECRL* mRNA level in comparison with cells exposed to a control vector (sh*CONTR*; Appendix Fig. S4B). hESC-CMs transduced with sh*CONTR* or sh*TECRL* maintained their cardiac phenotype and knockdown of *TECRL* in hESC-CMs did not affect *TNNT2* expression (Appendix Fig. S4B). AP parameters of sh*CONTR*- or sh*TECRL*-transduced hESC-CMs stimulated at 1-Hz were recorded and representative traces as well as averaged data are shown in Appendix Fig. S4C-D. RMP, dV/dt_{max} , APA_{max} and APA_{plat} did not differ significantly between the two experimental groups (Appendix Fig. S4D). However, hESC-CMs treated with sh*TECRL* displayed significantly prolonged APs as evidenced by the increase in APD_{20} , APD_{50} and APD_{90} values (Appendix Fig. S4D). In addition, western blotting for canonical calcium handling proteins revealed a 56% decrease in RYR2 protein and 18% decrease in CASQ2 in sh*TECRL*-treated hESC-CMs whilst SERCA2a, PLB, NCX1 and $Ca_v1.2$ protein levels were unaffected (Appendix Fig. S5E).

*TECRL*_{Hom}-hiPSC-CMs have an increased susceptibility to triggered activity

To evaluate the susceptibility of *TECRL*_{Hom}-hiPSC-CMs to triggered activity, we applied a fast pacing episode (3 Hz; 10 s), followed by a 10-s pause in the absence or presence of 10 nM noradrenaline (NA). Representative traces of CTRL-hiPSC-CMs and *TECRL*_{Hom}-hiPSC-CMs, in the absence and presence of NA are shown in Fig. 8A and 8C respectively. In the absence of NA, the last stimulated AP (arrow) is followed by APs, which seem to be due to diastolic depolarization rather than DADs (Fig. 8A) since the frequency of such “triggered” APs did not differ significantly between the experimental groups (Fig. 8B). Moreover, the prevalence of “triggered” and spontaneous APs (*i.e.* APs elicited without a fast pacing protocol) was also found to be similar in the absence of NA (Fig. 8B), which further suggests that the “triggered” APs in Fig. 8A are not due to DADs. However, in the presence of NA, “triggered” APs were abundantly present in *TECRL*_{Hom}-hiPSC-CMs compared with CTRL-hiPSC-CMs (Fig. 8C). The incidence of “triggered” APs was also significantly higher than that of spontaneous APs (Fig. 8D), indicating that the higher frequency of “triggered” APs in *TECRL*_{Hom}-hiPSC-CMs is due to DADs rather than spontaneous activity. Similar to what was observed for $[Ca^{2+}]_i$ transient properties, AP properties and susceptibility to triggered activity of *TECRL*_{Het}-hiPSC-CMs were largely in between those of CTRL-hiPSC-CMs and *TECRL*_{Hom}-hiPSC-CMs.

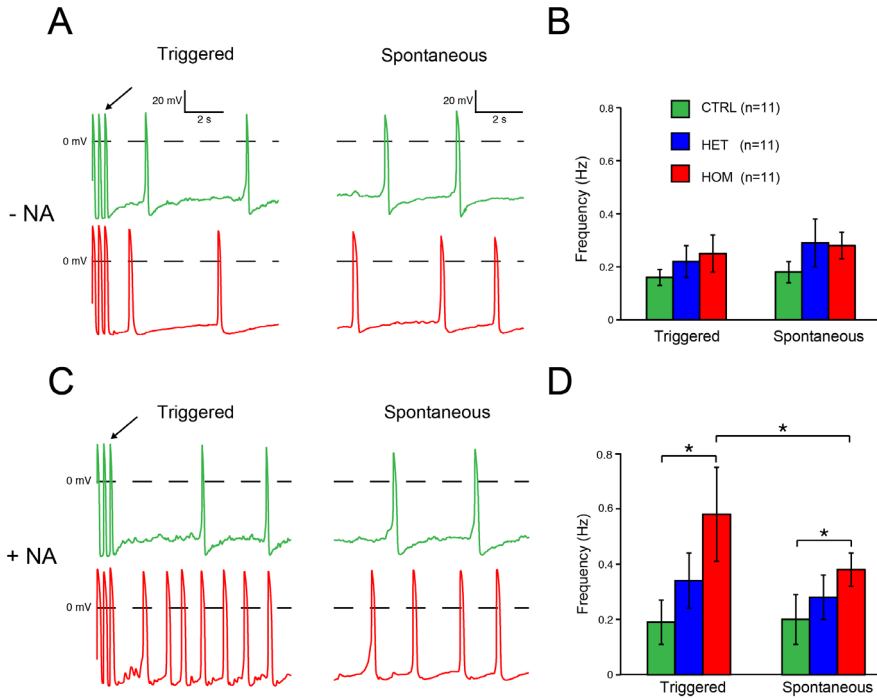


Figure 8. $TECRL_{Hom}$ -hiPSC-CMs demonstrate increased susceptibility to triggered activity in response to NA. (A) Representative AP traces and (B) averaged activity of triggered and spontaneous activity in hiPSC-CMs in the absence of NA. (C) Representative AP traces and (D) averaged activity of triggered and spontaneous activity in hiPSC-CMs in the presence of NA; $n = 3-4$ cells each from three independent experiments; * $P < 0.05$, statistical significance within a group before and after application of NA was assessed with paired t -test or Wilcoxon Signed Rank Test in case of failed normality; NA = Noradrenaline.

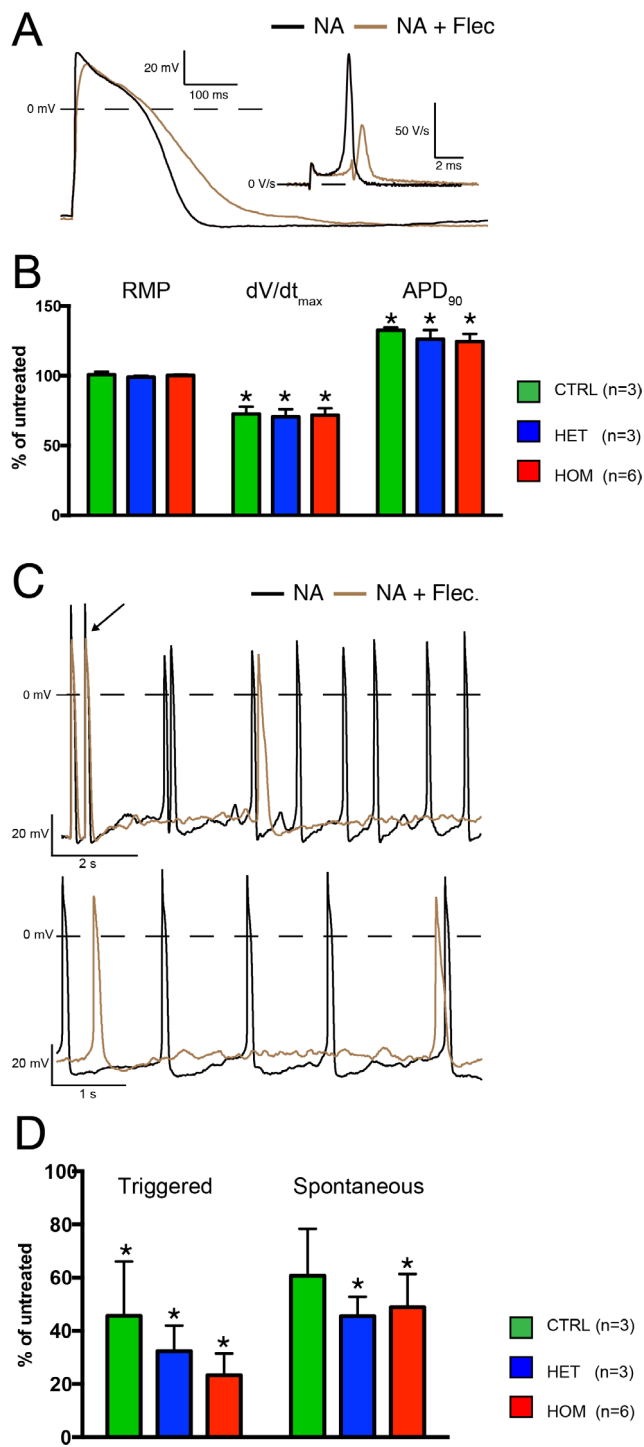
To evaluate the susceptibility of $TECRL_{Hom}$ -hiPSC-CMs to triggered activity, we applied a fast pacing episode (3 Hz; 10 s), followed by a 10-s pause in the absence or presence of 10 nM noradrenaline (NA). Representative traces of CTRL-hiPSC-CMs and $TECRL_{Hom}$ -hiPSC-CMs, in the absence and presence of NA are shown in Fig. 8A and 8C respectively. In the absence of NA, the last stimulated AP (arrow) is followed by APs, which seem to be due to diastolic depolarization rather than DADs (Fig. 8A) since the frequency of such “triggered” APs did not differ significantly between the experimental groups (Fig. 8B). Moreover, the prevalence of “triggered” and spontaneous APs (*i.e.* APs elicited without a fast pacing protocol) was also found to be similar in the absence of NA (Fig. 8B), which further suggests that the “triggered” APs in Fig. 8A are not due to DADs. However, in the presence of NA, “triggered” APs were abundantly present in $TECRL_{Hom}$ -hiPSC-CMs compared with CTRL-hiPSC-CMs (Fig. 8C). The incidence of “triggered” APs was also significantly higher than that of spontaneous APs (Fig. 8D), indicating that the higher frequency of “triggered” APs in $TECRL_{Hom}$ -hiPSC-CMs is due to DADs rather than spontaneous activity. Similar to what was observed for $[Ca^{2+}]_i$ transient properties, AP properties and susceptibility to triggered activity of $TECRL_{Het}$ -hiPSC-CMs were largely in between those of CTRL-hiPSC-CMs and $TECRL_{Hom}$ -hiPSC-CMs.

Effect of flecainide on hiPSC-CMs

Flecainide, a class Ic antiarrhythmic drug has been shown to be effective in CPVT patients, (Watanabe *et al*, 2009; van der Werf *et al*, 2011) although its precise mechanism of action has been a point of debate. To investigate whether flecainide has an effect on triggered activity of $TECRL_{Hom}$ -hiPSC-CMs, we administered 5 μ M of the drug and analyzed AP properties.

Fig. 9A shows typical APs of a $TECRL_{Hom}$ -hiPSC-CM in the absence and presence of flecainide. The average effects of flecainide on AP parameters in all three hiPSC-CM groups are summarized in Fig. 9B. Flecainide reduced the dV/dt_{max} and caused AP prolongation in all three groups without affecting the RMP (Fig. 9B). These effects did not differ significantly between the groups. Fig. 9C shows typical examples of triggered (top) and spontaneous (bottom) APs of a $TECRL_{Hom}$ -hiPSC-CM in the absence and presence of flecainide. The averaged effects of the drug on triggered and spontaneous activity in the three experimental groups are summarized in Fig. 9D. Whilst flecainide reduced the frequency of both triggered and spontaneous activity (Fig. 9D), these reductions were not significantly different between the three groups. However, the reduction in triggered activity was more pronounced than that of spontaneous activity in $TECRL_{Het}$ -hiPSC-CMs and $TECRL_{Hom}$ -hiPSC-CMs (Fig. 9D). These results suggest that a treatment regimen with flecainide might be effective in preventing arrhythmias in patients carrying the *TECRLc.331+1G>A* mutation.

Figure 9. Flecainide alleviates triggered activity in $TECRL_{Hom}$ -hiPSC-CMs. (A) Representative AP trace of a $TECRL_{Hom}$ -hiPSC-CM in the presence of NA alone or NA and flecainide. (B) Effect of flecainide on AP parameters of hiPSC-CMs. (C-D) Addition of 5 μ M of flecainide decreased the susceptibility to triggered activity in hiPSC-CMs. Note that the effect of flecainide on triggered activity of $TECRL_{Het}$ - and $TECRL_{Hom}$ -hiPSC-CMs is more pronounced than its effect on their spontaneous activity; n = 1-2 cells each from three independent experiments; *P<0.05, statistical significance within a group before and after application of flecainide was assessed with paired t-test; NA = noradrenaline, Flec = flecainide.



Discussion

VT is the most frequently reported (63%) form of cardiac rhythm at the time of SCD (Deo & Albert, 2012). However, in many cases, its etiology is unknown. In the current study, we investigated a consanguineous Sudanese family clinically diagnosed with exercise-emotion related arrhythmias (*i.e.* CPVT). Seven of 13 children in this family were homozygous for the disease-causing mutation and presented clinically with exercise-induced arrhythmias at a mean age of 7.6 years. Arrhythmias resulted in sudden death in 5 children. A previous study on this family described the clinical features and reported that chromosome 7 possibly contained the disease locus (Bhuiyan *et al.*, 2007). However, at the time of that study, CPVT was not established in subject IV:8 (IV:8 in the previous study) who was one year old and was therefore considered unaffected for analysis by homozygosity mapping. This factor along with the stringent settings applied in the prior study (disease allele frequency of 0.0001; penetrance of 0 for carriers/non-carriers and 0.99 for affected individuals) resulted in the disease locus being allocated to chromosome 7p14-22. Recently, subject IV:8 died suddenly whilst dancing. Following the identification of a novel homozygous c.331+1G>A*TECRL* mutation by exome sequencing and inclusion of IV:8 as an affected individual, *TECRL* located on chromosome 4 was identified as the disease-causing gene.

In this study, we also described a c.4103A>C mutation in *ANKRD17* on chromosome 4 found to be heterozygous in the parents and homozygous in the affected children. *Ankrd17* deficiency in mice causes vascular instability and the observed phenotype is similar to that of other gene-targeted mutants such as *Hey1/Hey2*, *Notch1*, *Tie2* and *EphrinB2* with predominant roles in vascular function (Hou *et al.*, 2009). Thus, we proposed that a mutation in this gene does not cause CPVT, which is primarily an electrical disorder of the heart. However, it is possible that the c.4103A>C *ANKRD17* mutation might have a role in congenital heart defects observed in two of seven affected children in the reported CPVT family. In mice, embryos lacking *Ankrd17* displayed arrested development of the endocardium, which is commonly associated with morphological defects in the heart.

TECRL was identified in a human genome-wide transcriptional analysis of hESCs differentiated to CMs. We showed, by *in situ* hybridization and RT-qPCR analysis of embryonic and adult mouse tissues that *Tecrl* is preferentially expressed in cardiac tissue, suggesting an important role for this gene in the heart. Reactome pathway analysis indicates a role for *TECRL* in fatty acid and lipid metabolism while gene ontology annotates its function as an enzyme involved in catalysis of redox reactions. By expressing Myc-*Tecrl* in H10 cells, we showed that *Tecrl* is localized to the ER, which is a critical site of protein, lipid and glucose metabolism as well as calcium homeostasis. In the adult heart, free fatty acids are the primary substrate for energy production at rest and abnormalities in fatty acid oxidation have been linked to arrhythmias (Bonnet *et al.*, 1999). Perturbations in physiological levels of lipids/fatty acids and metabolic function can have direct consequences on ion channels, including calcium handling proteins (Charnock, 1994; Boland & Drzewiecki, 2008; Barth & Tomaselli, 2009). Further studies are necessary to elucidate the role of *TECRL* in lipid metabolism and how mutations are linked to cardiac arrhythmias.

hiPSCs offer unprecedented opportunities to model cardiac diseases *in vitro* and are especially valuable when myocardial tissue of the patient tissue is not accessible to determine

the consequences of a particular mutation. Using hiPSCs, we demonstrated in this study that patient-derived TECRL_{Hom}-hiPSC-CMs lack exon3 of *TECRL* and recapitulate salient features of the disease phenotype *in vitro*. Skipping of exon3 resulted in a shorter mRNA transcript of *TECRL* in mutant CMs without affecting the downstream reading frame.

Altered calcium homeostasis is a hallmark of CPVT phenotype and mutations in RYR2 or CASQ2 have been identified to cause this disease. Interestingly, protein levels of RYR2 and CASQ2 were significantly downregulated in TECRL_{Hom}-hiPSC-CMs. We also found a smaller $[Ca^{2+}]_i$ transient amplitude in these CMs. Since Ca^{2+} concentration of the SR is a major regulator of the $[Ca^{2+}]_i$ transient amplitude, this finding suggests a lower SR Ca^{2+} content in TECRL_{Hom}-hiPSC-CMs. Indeed, caffeine-evoked transients in the presence of 10 mM NiCl₂, a measure of SR Ca^{2+} content, were significantly lower in TECRL_{Hom}-hiPSC-CMs, which could be associated with downregulation of RYR2.

Additionally, the diastolic calcium concentration was significantly higher in TECRL_{Hom}-hiPSC-CMs, which could explain their increased propensity to DADs upon adrenergic stimulation with NA. The increase in diastolic calcium in TECRL_{Hom}-hiPSC-CMs may in part be reasoned by the observed decrease in SERCA and NCX activity. Although, we did not observe a decrease in protein levels of SERCA2A and NCX in TECRL_{Hom}-hiPSC-CMs compared with CTRL-hiPSC-CMs, we noted a decrease in their activity by functional studies. It has been established that a number of post-translational modifications regulate the activity of ion channels, which could clarify the observed effects (Ruknudin *et al*, 2007; Stammers *et al*, 2015). Decreased NCX activity has been shown to reduce DAD amplitude resulting in fewer spontaneous APs (Bögeholz *et al*, 2015). However, in our model, despite a lower NCX activity in TECRL_{Hom}-hiPSC-CMs compared with CTRL-hiPSC-CMs, the increased diastolic calcium was sufficient to induce DADs and subsequent triggered APs.

We also assessed the relative contribution of SERCA, NCX and the slow mechanisms to calcium removal from the cytosol. In isolated adult ventricular CMs, the decline of the $[Ca^{2+}]_i$ transient is mainly due to reuptake of Ca^{2+} into the SR by SERCA and extrusion of Ca^{2+} via the sarcolemmal NCX, with a minor contribution of the slow mechanisms (Bers, 2006; Dibb *et al*, 2007). Our results correlate with this existing data suggesting that the Ca^{2+} transport systems in hiPSC-CMs are similar to those reported in adult human and animal CMs (Bers, 2000).

At the single-cell level, APs of TECRL_{Hom}-hiPSC-CMs showed an increase in APD₂₀. Although not significant, APD₅₀ and APD₉₀ were also prolonged, compatible with the borderline QTc prolongation in some of the patients homozygous for the *TECRL* mutation. Interestingly, hESC-CMs with *TECRL* knockdown also exhibited markedly prolonged APD₂₀, APD₅₀ and APD₉₀. Moreover, RYR2 downregulation at the protein level was also apparent in *TECRL* knockdown CMs. These findings suggest that *TECRL* is attributable to the phenotype seen in TECRL_{Hom}-hiPSC-CMs.

Finally, we demonstrated that the antiarrhythmic drug flecainide controlled the incidence of triggered APs in TECRL_{Hom}-hiPSC-CMs. Flecainide was first classified as a blocker of Na⁺ current (I_{Na}) and more recently, it has been shown to reduce exercise-induced arrhythmias in CPVT patients (Watanabe *et al*, 2009; van der Werf *et al*, 2011). In agreement with flecainide's effect as I_{Na} blocker, we observed a decrease in the AP upstroke velocity. We also observed

AP prolongation, as previously reported in isolated human cardiac tissue (Wang *et al*, 1990). The precise mechanisms by which flecainide exerts an antiarrhythmic effect is unclear, but has been proposed to be due to reduced opening of the RYR2 channels (Watanabe *et al*, 2009) or I_{Na} blockade resulting in decreased excitability (Liu *et al*, 2011; Bannister *et al*, 2015), or a combination of both (Watanabe *et al*, 2009). Although not conclusive, our results suggested that depression of the upstroke velocity of the AP leading to decreased excitability presumably contributes to the observed reduction in triggered activity. It is encouraging that flecainide reduced the incidence of triggered activity in $TECRL_{Hom}$ -hiPSC-CMs. However, some DADs were still observed emphasizing the need for additional or more effective drugs to prevent their occurrence in $TECRL_{Hom}$ -hiPSC-CMs. Moreover, the I_{Kr} blocking effect and consequential QT-prolonging effect of flecainide may potentially offset its beneficial effect on the triggered activity.

Taken together, the findings presented in this study have implications for clinical diagnosis and management of familial arrhythmia syndromes. CMs generated from patient-specific hiPSCs were valuable to study the effect of the c.331+1G>A mutation in *TECRL*. The apparent immature phenotype of hiPSC-CMs (for eg., APD<200 ms) compared with freshly isolated human adult ventricular CMs did not preclude their use in disease modeling. Screening for *TECRL* mutations could be offered to families with catecholamine-induced arrhythmias of autosomal recessive inheritance in which *CASQ2* (and *RYR2*) screening is negative.

Materials and Methods

Ethics statement

Signed informed consents were obtained from participating patients from the Sudanese family or their guardians, the study adhered to the Declaration of Helsinki and the research protocol was approved by the Al-Ain Medical District Human Research Ethics Committee, College of Medicine, United Arab Emirates (UAE) University. Study on hESCs and hiPSCs were performed in the Netherlands and their use was approved by the Medical Ethics Committee of Leiden University Medical Center (LUMC).

Clinical data analysis

Clinical data of family members (where possible), including baseline ECGs, exercise ECGs and 24-hour Holter recordings were collected and evaluated. Family members who experienced sudden cardiac death at young age or who demonstrated arrhythmias were considered affected.

Exome sequencing

Detailed methods are provided in the 'Appendix'.

Genomic DNA from individuals III:1, III:2, IV:2 and IV:10 (Fig. 1D) was extracted from peripheral blood lymphocytes and WES was carried out at the Beijing Genomics Institute (BGI; Shenzhen, China).

Reprogramming of primary fibroblasts to induced pluripotent stem cells

Primary fibroblasts from members of the affected family were isolated from skin biopsies with informed consent under protocols approved by the Al Ain Hospital Ethics committee for research, SEHA, Abu Dhabi, UAE. Low-passage skin fibroblasts were reprogrammed to induced pluripotent stem cells using non-integrating Sendai virus vectors encoding OCT4, SOX2, KLF4, and MYC as described previously (Zhang *et al*, 2014). Official identification of the cell lines used in this study are as follows: LUMC0046iTECRL (hiPSCs with heterozygous *TECRL*c.331+1G>A mutation); LUMC0047iCTRL (Control hiPSCs) and LUMC0048iTECRL (hiPSCs with homozygous *TECRL*c.331+1G>A mutation).

Of several hiPSC clones with embryonic stem cell morphology those showing robust expression of pluripotency markers, OCT-4, NANOG, SSEA4 and/or TRA-181, as analyzed by flow cytometry or by immunocytochemistry, were selected for further study. These hiPSC lines were karyotyped and subjected to a global assessment of pluripotency by PluriTest (Müller *et al*, 2011).

Maintenance of hiPSC lines and differentiation to CMs

hiPSCs lines were maintained in a feeder-free culture in mTESR1 medium (STEMCELL Technologies) on Matrigel (BD Biosciences) and passaged once a week with 1mg/ml Dispase (Gibco).

To induce cardiac differentiation, cells were seeded in a high density on Matrigel and supplemented with mTESR1 medium. Two days post-seeding, mTESR1 medium was replaced with BPEL medium (Ng *et al*, 2008) containing 20 ng/ml Activin-A (R&D systems),

20 ng/ml BMP4 (R&D systems) and 1.5 μ mol/L CHIR99021 (Axon Medchem). On day 3, the medium was replaced with BPEL medium containing 5 μ mol/L XAV939 (Tocris Biosciences). Cells received BPEL medium on day 7 and every 3-4 days thereafter. Beating CMs were first seen in culture at day 10.

AP measurements

hiPSC-CMs were dissociated to single cells at day 20 using TrypLE™ Select (Life Technologies) and plated on Matrigel coated coverslips. APs were recorded 10 days after dissociation with the amphotericin-perforated patch-clamp technique as described previously (Devalla *et al*, 2015) and further detailed in the 'Appendix'.

Ca²⁺ measurements

hiPSC-CMs were dissociated at day 20 and [Ca²⁺]_i transients were measured 10 days after dissociation in Indo-1 AM (Molecular Probes, Eugene, OR, USA) loaded cells as described before (van Borren *et al*, 2010; Verkerk *et al*, 2015) and elaborated in the 'Appendix'.

Statistics

Experiments with hiPSC-CMs were performed on cells obtained from $n \geq 3$ independent differentiations. Statistical analysis was carried out with SigmaStat 3.5 software and data are presented as mean $\pm$ standard error of the mean. Normality and equal variance assumptions were tested with the Kolmogorov-Smirnov and the Levene median test respectively. Groups were compared using one-way analysis of variance (ANOVA) followed by pairwise comparison using the Student-Newman-Keuls test or, in cases of failed normality and/or equal variance test, ANOVA based on ranks (Kruskal-Wallis test) followed by Dunn's test. $P < 0.05$ was considered statistically significant.

Acknowledgements

Funding for this study from the following sources is gratefully acknowledged: Netherlands Organization for health research and development- (ZonMw-TOP 40-00812-98-12086) to H.D.D and (ZonMw-MKMD-40-42600-98-036) to R.P; European Research Council advanced grant (STEMCARDIOVASC-323182) to C.L.M; The Leenaards Foundation, the Swiss Institute of Bioinformatics and the Swiss National Science Foundation (31003A-143914, 51RTP0_151019) grants to Z.K; Fondation Suisse de Cardiologie (No. 29283) to Z.A.B; The Netherlands CardioVascular Research Initiative, The Dutch Heart Foundation, Dutch Federation of University Medical Centers, The Netherlands Organization for Health Research and Development and the Royal Netherlands Academy of Sciences (CVON-PREDICT) to A.A.M.W.

The authors thank the following people for contributing to the research presented in this manuscript: Prof. Jacques S. Beckmann for initiating the next-generation sequence study of the Sudanese family and incorporating this family for exome sequencing; Dr Tom van Wezel (Department of Pathology, LUMC) for helpful discussions; M. Ohtaka, K. Nishimura, and M. Nakanishi (National Institute of Advanced Industrial Science and Technology, Japan) for providing the Sendai virus vectors; S. van de Pas and A. 't Jong from the LUMC hiPSC core facility for support with reprogramming; D. de Jong, K. Szuhai and H. Tanke (Department of Molecular Cell Biology, LUMC) for karyotyping, Verena Schwach (Department of Anatomy & Embryology, LUMC) for support with cell culture and Annemarie Kip (Department of Cardiology, LUMC) for help with the lentiviral vector production. The computations were performed at the Vital-IT Center (<http://www.vital-it.ch>) for high-performance computing of the SIB Swiss Institute of Bioinformatics. The authors are especially grateful to the patient families for tissue samples and their consent for the study.

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Appendix

Clinical data

In the current study, we investigated a consanguineous Sudanese family (Fig. 1A) clinically diagnosed with exercise-emotion related arrhythmias (*i.e.* catecholaminergic polymorphic VT [CPVT]). Seven of 13 children in this family were identified to carry a homozygous c.331+1G>A *TECRL* mutation and presented clinically with exercise-induced arrhythmias at a mean age of 7.6 years. Arrhythmias resulted in sudden death in five children.

Subject IV:8 was diagnosed at birth with a severe form of Tetralogy of Fallot (pulmonary atresia/ventricular septal defect [VSD]) and underwent cardiac surgery. In 2014, at age eight, she suffered cardiac arrest whilst dancing. She received two direct current (DC) shocks with an automated external defibrillator (AED) for VT from the emergency ambulance team and arrived at the hospital in a state of coma, where she was fully ventilated. She died in the hospital after six weeks from brain damage and chest infection.

Subject IV:9, aged 2.5 years, collapsed at home in August 2014 whilst running. He was resuscitated at the hospital but developed hypoxic ischemic encephalopathy and seizures, which were controlled by phenobarbital. He was ventilated in the intensive care unit (ICU) for six weeks. He survived but developed severe brain damage and quadriplegic cerebral palsy. He is currently on atenolol 12.5 mg daily. His ECG could not be recorded at the time of collapse for confirmation of VT. 48-h Holter monitoring showed sinus rhythm and frequent premature ventricular contractions.

Subject IV:10 had his first syncopal episode and seizures at age seven. He collapsed at home whilst running and died at eight years of age. His ECG showed a prolonged QTc of 480 ms. 24-h Holter record and exercise ECG also showed prolonged QTc of 470-480 ms. He was prescribed β -blockers (metoprolol 50 mg orally once a day) until death.

Segregation analysis and homozygosity mapping

A previous study on this family described the clinical features and also reported chromosome 7 as a possible homozygous locus in the affected individuals (Bhuiyan *et al*, 2007). However, at the time of that study, CPVT was not established in subject IV:8 (IV:8 in the previous study) who was one year old and was therefore considered unaffected for analysis by homozygosity mapping. This factor along with the stringent settings applied in the prior study (disease allele frequency of 0.0001; penetrance of zero for carriers/non-carriers and 0.99 for affected individuals) resulted in the recessive locus being allocated to chromosome 7p14-22. In 2014, subject IV:8 died suddenly whilst dancing and following the identification of a novel homozygous c.331+1G>A *TECRL* mutation on chromosome 4 by exome sequencing and inclusion of IV:8 as an affected individual, the consensus homozygous locus in all the affected individuals was identified as chromosome 4.

Materials and Methods

Exome capture, sequencing and bioinformatic analysis

Genomic DNA from individuals III:1, III:2, IV:2 and IV:10 (Fig. 1A) was extracted from peripheral blood lymphocytes. Three μ g of genomic DNA was randomly sheared into 150- to 200 bp fragments (Covaris, Woburn, MA). Adapters were ligated to both ends of the fragments, which were then purified by the Agencourt AMPure SPRI beads (Beckman Coulter, Brea, CA, USA). Fragments with an insert size of about 250 bps were excised. Extracted DNA was amplified by ligation-mediated polymerase chain reaction (LM-PCR), purified, and hybridized to Agilent's SureSelect biotinylated RNA library (BAITS, Agilent Technologies, Santa Clara, CA) for enrichment. Hybridized fragments were enriched using streptavidin-coated magnetic beads whereas non-hybridized fragments were washed out after 24 hrs. Quality control of captured LM-PCR products was performed using the Agilent 2100 Bioanalyzer. Each captured library was then loaded on the Illumina HiSeq2000 platform (Illumina, San Diego, CA) and high-throughput sequencing was performed to ensure that each sample meets the desired average sequencing depth and the sequences of each individual were generated as 90 bp paired-end reads. Raw image files were processed by Illumina CASAVA software, version 1.7 for base calling with default parameters.

Alignment and variant calling

Sequence reads were aligned to the human reference genome (UCSC hg19) using SOAPaligner, version 2.21 (Li *et al*, 2009a), and high-quality single nucleotide variants (SNVs) were detected using SOAPsnp software (Li *et al*, 2009b). For insertion/deletion (indel) variants, sequence reads were aligned with BWA (Li & Durbin, 2010) and indels detected using GATK (Van der Auwera *et al*, 2013). SOAPaligner (soap2.21) was used to align clean reads to the human reference genome with maximum 3 mismatches. Based on the results from SOAPaligner, software package SOAPsnp was used to assemble the consensus sequence and call genotypes in target regions. Quality control filtering of candidate single nucleotide polymorphisms (SNPs) was based on the following criteria: SNP quality is ≥ 20 , sequencing depth between 4 and 10^7 , estimated copy number ≤ 2 and the distance between 2 SNPs is ≥ 5 .

Variant prioritization and validation

Sudanese family: The resulting variants of each sequenced patient were annotated using ANNOVAR (Wang *et al*, 2010, release February 2013) with the following publicly available databases: dbSNP (Sherry *et al*, 2001, release 137), 1000Genomes (Genomes Project Consortium, 2010, release April 2012), 6500NHLBI (<http://evs.gs.washington.edu/EVS/>, release June 2012); CoLaus cohort (Firmann *et al*, 2008) and Swiss institute of Bioinformatics (Vital-IT/SIB internal database (372 exomes collection, release April 2013). Variants were filtered by putative functionality (either non-synonymous, stopgain, stoploss, splice site SNVs, or frameshift insertions/deletions) and disease inheritance pattern (homozygous in the 2 affected siblings and heterozygous in the parents). This resulted in identification of 5 SNVs (Appendix table S1), 4 on chromosome 4: 65-73Mb and 1 on chromosome 17:2.6Mb, all shared by IV:2 and IV:10 as homozygous and III:1 and III:2 as heterozygous following the expected disease inheritance pattern.

Prioritized variants were validated by amplification of the encompassing genomic regions and

subsequent Sanger sequencing. Primer sequences are available on request.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as described before (Nieto *et al*, 2006; van Eldik *et al*, 2011). Probe template primers were designed with the T3-promoter sequence (5'-ATACAATTAACCCTCACTAAAGGG-3') at the 5' end of the forward primer and T7-promoter sequence (5'-ATAGGTAATACGACTCACTATAGGGC-3') at the 5' end of the reverse primer. Digoxigenin-labeled probes were generated using the purified PCR product as a template for probe transcription with either T3 RNA polymerase or T7 RNA polymerase (Promega, Madison, WI). Forward and reverse probe template primers were: 5'-ACTGCCACAG-TCCACAATGA-3' and 5'-CCCCAATAAAATGCACAACC-3' for murine *Tecrl* probe 1 and 5'-CCCAGACACTTCCAGTTGGT-3' and 5'-CCTGGCAAATGTCAACACAC-3' for murine *Tecrl* probe 2. Images were taken on a Olympus SZX9 microscope (Olympus Life Science, Japan) coupled to a Leica DFC480 digital camera (Leica Microsystems, Germany).

Cell culture

COS-1 cells (fibroblast-like monkey kidney cells) were cultured in DMEM-F12 (Thermo Fisher Scientific, Waltham, MA) with 7.5% fetal bovine serum (FBS). H-10 cells (immortalized neonatal rat cardiomyocytes [CMs]) were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS.

For immunocytochemistry cells were plated on 0.1% gelatin-coated glass coverslips in a 12-well cell-culture plate. COS-1 and H10 cells were transiently transfected using Lipofectamine 2000 (Thermo Fisher Scientific) and analyzed 48 h after transfection.

Plasmid constructs

The coding sequence of mouse *Tecrl* with an amino-terminal c-myc epitope tag (MYC-*Tecrl*) was amplified by PCR from mouse E17.5 heart cDNA and inserted into pCRII-TOPO (Thermo Fisher Scientific). cDNA encoding mouse *Tecrl* with a N-terminal c-myc epitope were subcloned into the pcDNA3.1 expression vector (Thermo Fisher Scientific). All cloning products were confirmed by sequencing. Cloning primers were as follows.

MYC-*Tecrl* forward:

5'- caccATGgaacaaaaactcatctcagaagaggatctgTTCAAAGGCACAAGTCC-3'

Tecrl reverse:

5'- TTATAATATGAGTGAATTA-3'

Immunocytology

Cells were fixed in 2% paraformaldehyde for 30 minutes, washed 3 times in phosphate-buffered saline (PBS) and permeabilized in 0.1% Triton-X/PBS for 8 min. Aspecific binding sites were blocked by incubation of the cells in 4% normal goat serum (NGS) for 1 h at room temperature (RT). Next, cells were incubated with primary antibody in 4% NGS for 1 h at RT. Secondary antibody incubation occurred in 4% NGS for 1 h at RT. Nuclear staining was performed as a last step using DAPI (Molecular Probes, Eugene, OR). Coverslips were then mounted on glass slides with Mowiol (EMD Millipore, Billerica, MA) and images were captured using a Leica TCS SP8 confocal laser scanning microscope. Primary antibodies were as follows: mouse anti-c-Myc 1:50 (Santa Cruz Biotechnology, Dallas, TX), rabbit anti-calnexin 1:100 (Santa Cruz Biotechnology), mouse anti-sarcomeric α -actinin 1:800 (clone EA-53;

Sigma Aldrich, Zwijndrecht, the Netherlands). Alexa Fluor 488- and Alexa Fluor 647-conjugated secondary antibodies were used at a dilution of 1:250 (Thermo Fisher Scientific).

DNA isolation and sequencing

Genomic DNA was isolated from cultured fibroblasts and human induced pluripotent stem cell (hiPSC) colonies using standard protocols (Gentra Puregene Cell kit, Qiagen, Valencia, CA). Regions of interest in the *TECRL* gene was amplified by PCR using 30 ng genomic DNA as template. Purified fragments were sequenced with the following primer: 5'-GGTCTTCCCATGGAAACAGTTAG-3'.

Action potential (AP) measurements:

Coverslips containing hiPSC-CMs were put in a recording chamber on the stage of an inverted microscope and superfused with modified Tyrode's solution ($36 \pm 0.2^\circ\text{C}$) containing (in mM): NaCl 140, KCl 5.4, CaCl_2 1.8, MgCl_2 1.0, glucose 5.5, and HEPES 5.0; pH was adjusted to 7.4 with NaOH. Patch pipettes (borosilicate glass; resistance 2–3 M Ω) were filled with (in mM): K-gluconate 125, KCl 20, NaCl 10, amphotericin-B 0.22, HEPES 10, pH was adjusted to 7.2 with KOH. Signals were low-pass filtered (cut-off frequency 10 kHz) and digitized at 40 kHz. Potentials were corrected for the calculated liquid junction potential (Barry&Lynch, 1991). Data acquisition and analysis were accomplished using custom software.

The hiPSC-CMs of all three cell lines showed spontaneous beating upon visual inspection. We recorded spontaneous APs as well as overdrive paced APs at 1 Hz, elicited by 3-ms, $\approx 1.2\times$ threshold current pulses through the patch pipette. As depicted in Fig. 7A, we analyzed maximal diastolic potential (MDP), AP amplitude (APA_{max}), AP plateau amplitude (APA_{plat} - defined as the potential difference between MDP and the membrane potential 20 ms after the upstroke), maximal upstroke velocity (dV/dt_{max}) and AP duration (APD) at 20, 50 and 90% repolarization (APD_{20} , APD_{50} , and APD_{90} , respectively). Data from 10 consecutive APs were averaged. Susceptibility to triggered APs was tested by applying a 3-Hz pacing episode of 10-s followed by a 10-s pause in the absence or presence of 10 nM noradrenaline (NA) (Centrafarm, Etten-Leur, the Netherlands).

Ca^{2+} measurements

In short, hiPSC-CMs were loaded with 3.75 $\mu\text{mol/L}$ Indo-1 AM (Thermo Fisher Scientific for 30 min at 37°C in Tyrode's solution. Subsequently the cells were washed with the Tyrode's solution for 30 min at 37°C to remove excess indicator and allow its full de-esterification inside the cells. Dual wavelength emission (405-440)/(505-540) nm of Indo-1 AM, after excitation at 340 nm was recorded at 37°C in Tyrode's solution in which the CaCl_2 was elevated to 2.4 mM. Indo-1 AM is a ratiometric $[\text{Ca}^{2+}]_i$ indicator less prone to cell contractions and loss of dye, but may have an adverse effect on cell viability and rendered many previously spontaneous beating small cells quiescent (Lancaster *et al*, 2004). Following Indo-1 AM loading, hiPSC-CMs ceased to contract spontaneously but $[\text{Ca}^{2+}]_i$ transients could be elicited at 1 Hz using field stimulation. Signals were digitized at 1 kHz, filtered at 100 Hz, and corrected for background fluorescence before the I405/I505 ratio values were calculated. Data acquisition and analysis were accomplished using custom software.

$[\text{Ca}^{2+}]_i$ was calculated using the formula: $[\text{Ca}^{2+}]_i = \beta \cdot K_d (R - R_{\text{min}})/(R_{\text{max}} - R)$, where β is the ratio of maximal to minimal I_{505} (2.2) and K_d is the dissociation constant for Indo-1 AM which is 250

nmol/L at 37°C (data sheet for Indo-1 AM, Thermo Fisher Scientific), and $R_{\min}$ and $R_{\max}$ are the ratios at minimal and maximal calcium concentration. $R_{\max}$ was determined by blocking the Na^+/K^+ exchanger with 2 μM of Gramicidin (Sigma-Aldrich, St Louis, MO), which rapidly causes $[\text{Ca}^{2+}]_i$ overload. To determine $R_{\min}$, the cells were loaded with Indo-1 AM in calcium-free Tyrode's solution and also measured in the same solution. We obtained an averaged $R_{\min}$ value of 0.31 ± 0.04 (average $\pm$ standard error of the mean, $n=24$), and an averaged $R_{\max}$ value of 2.21 ± 0.24 ($n=24$).

We analyzed diastolic and systolic $[\text{Ca}^{2+}]_i$ concentrations, $[\text{Ca}^{2+}]_i$ transient amplitudes, speed of the rising phase of the $[\text{Ca}^{2+}]_i$ transient inferred by the time to reach 50% of the maximal $[\text{Ca}^{2+}]_i$ transient amplitude ($t_{1/2}$), and time constant (τ) of the $[\text{Ca}^{2+}]_i$ transient decay. The rates of decay of systolic as well as caffeine-induced $[\text{Ca}^{2+}]_i$ transients were obtained by fitting single exponential functions to the decay phase of the transients. The amplitude of $[\text{Ca}^{2+}]_i$ transients evoked by application of 10 mM caffeine and 10 mM NiCl_2 was taken as a measure of sarcoplasmic reticulum (SR) Ca^{2+} content (Bassani & Bers, 1995). The various mechanisms involved in Ca^{2+} extrusion from the cytoplasm were studied as described previously (Díaz et al, 2004; Walden et al, 2009). In short, the SR-dependent rate of Ca^{2+} uptake (SERCA activity; K_{SERCA}) was calculated by subtracting the decay rate constants of the caffeine (10 mM)-evoked Ca^{2+} transient (K_{caff}) from systolic Ca^{2+} transient decay rate constant (K_{sys}). The contribution of NCX to Ca^{2+} removal from the cytoplasm was deduced through subtraction of the Ca^{2+} decay of the caffeine-evoked Ca^{2+} transients (K_{caff}) from the rate of decay of the caffeine-evoked Ca^{2+} transients in the presence of 10 mM NiCl_2 ($K_{\text{caff+Ni}}$) to block the NCX. The decay rate constants of the caffeine-induced Ca^{2+} transients in the presence of 10 mM NiCl_2 were used for assessing the activity of the slow mechanisms (mitochondrial Ca^{2+} uptake and sarcolemmal Ca-ATPase) in $[\text{Ca}^{2+}]_i$ decay.

shRNA-mediated knockdown of *TECRL* expression

TECRL expression in human embryonic stem cell-derived CMs (hESC-CMs) was selectively inhibited using self-inactivating lentiviral vector (SIN-LV) particles encoding five different human *TECRL* gene-specific shRNAs. The shuttle constructs used to generate the SIN-LVs were extracted from the MISSION TRC shRNA library (Sigma-Aldrich, Appendix table S2). The negative control vector (sh*CONTR*) had the same genetic makeup, except that it contained the *Photinus pyralis* luciferase (Pp-Luc)-specific shRNA-coding sequence of plasmid SHC007 (Sigma-Aldrich) instead of a human *TECRL*-specific shRNA-coding sequence. SIN-LV particles were produced in 293T cells as described previously (Bingen et al, 2013). SIN-LV particles were concentrated by ultracentrifugation and subsequently suspended in PBS containing 1% bovine serum albumin fraction V (Sigma-Aldrich). SIN-LV suspensions were stored in 100 μL portions at -80°C until use.

The knockdown experiments were carried out in hESC-CMs. Day 18 cells were dissociated with 10x TrypLE (Thermo Fisher Scientific) and transduced with SIN-LV particles. At 12 hrs post-transduction, the inoculum was replaced by CM culture medium followed at 72 hrs post-transduction by CM culture medium containing 1 $\mu\text{g}/\text{ml}$ puromycin. This was replaced with CM culture medium three days later and knockdown efficiency of *TECRL* was assessed by reverse transcription-quantitative PCR 10 days post-transduction, using the two different primer sets listed in Appendix table S3. Of the five different shRNAs for *TECRL* that were tested, *TECRL-sh#3* was selected for further experiments based on knockdown efficiency and

cell viability. Further analysis of the effects of *TECRL*-knockdown in hESC-CMs by western blot and patch clamping was performed 10 days post-transduction.

Western blotting

Cells were lysed in ice-cold RIPA-buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.2% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with DTT, PMSF, NaF, Na_3VO_4 and cOmplete™, Mini protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were cleared by centrifuging at $10.000\times g$ for 15 min at 4°C. Western blotting was performed according to standard protocols.

Briefly, protein concentrations were determined using the Pierce™ BCA protein assay (Thermo Fisher Scientific) and proteins were resolved by Mini-PROTEAN TGX™ precast gels (Bio-Rad Laboratories, Veenendaal, the Netherlands). Proteins were subsequently transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories) using a Trans-Blot Turbo™ Transfer System (Bio-Rad Laboratories) or by overnight wet transfer for the RYR2 blot. PVDF membranes were incubated overnight at 4°C with the following primary antibodies: mouse anti-RYR2 clone C3-33 (1:3,000, Thermo Fisher Scientific), mouse anti-CASQ2 clone E-12 (1:1,000, Santa Cruz Biotechnology), rabbit anti-SERCA2a (1:5,000, Badrilla, Leeds, United Kingdom), mouse anti-PLB clone A1 (1:1000, Badrilla), mouse anti-NCX clone C2C12 (1:1000, Thermo Scientific), rabbit anti- $\text{Ca}_v1.2$ (1:200, Alomone Labs, Jerusalem, Israel), mouse anti-sarcomeric α -ACTN clone EA-53 (1:1,000, Sigma-Aldrich), mouse anti-GAPDH clone 6C5 (1:5,000, Fitzgerald Industries International, Acton, MA). Horseradish peroxidase-conjugated secondary antibodies (GE Healthcare Bio-Sciences, Pittsburgh, PA) were used for detection and were incubated for 1 h at RT. Western blots were developed with ECL prime western blotting detection reagent (GE Healthcare Bio-Sciences) and images were acquired using the ImageQuant LAS 4000 biomolecular imager (GE Healthcare Bio-Sciences). Densitometric analysis of western blots was performed using Image J software.

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Figures

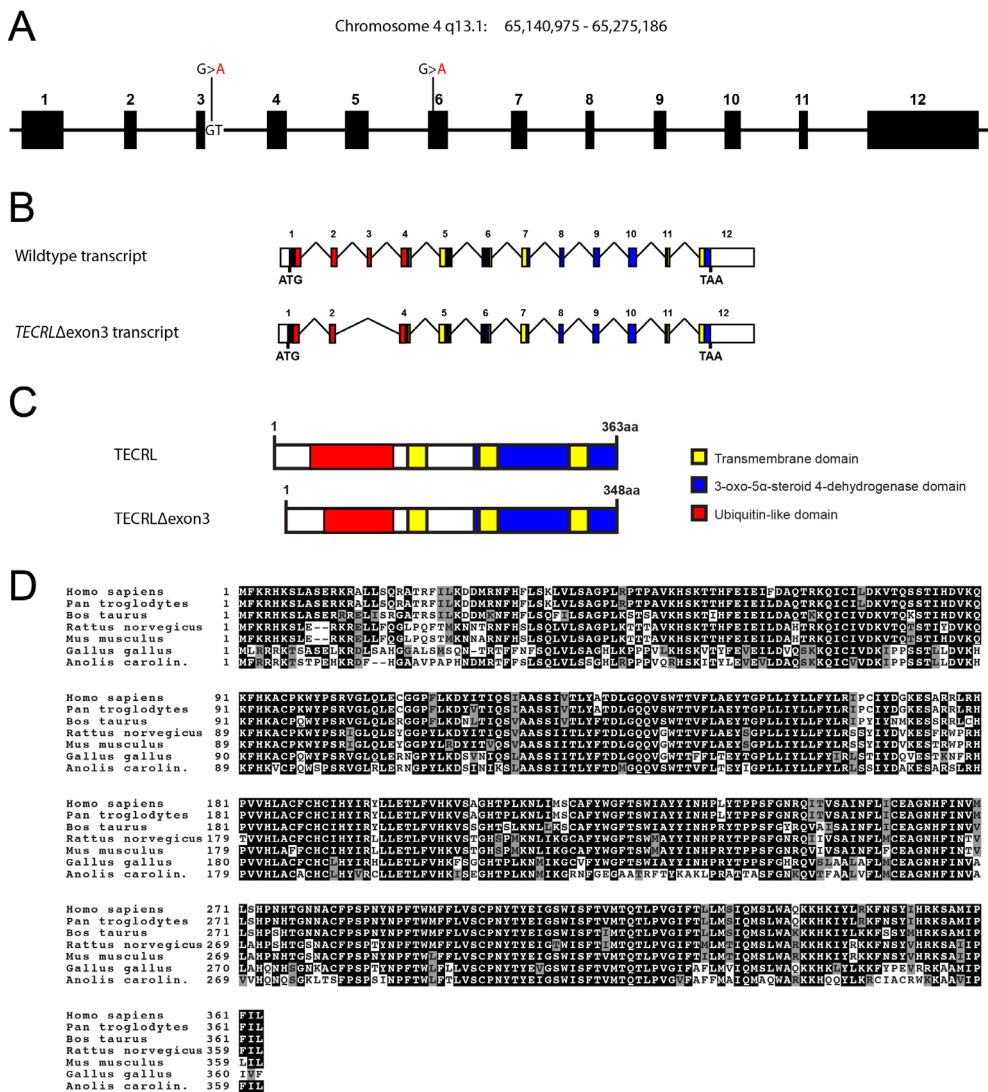
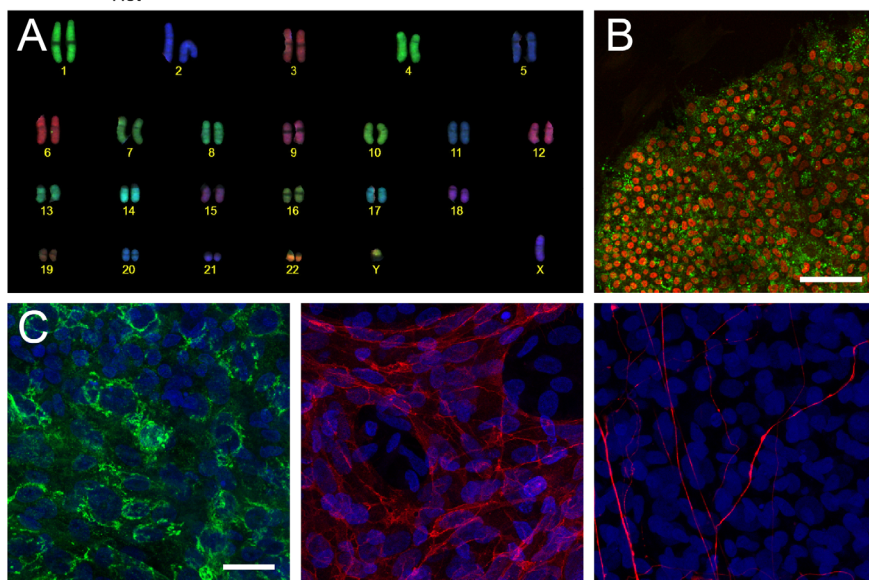


Figure S1. Genomic organization, evolutionary conservation and tissue expression of *TECRL*. (A) Human *TECRL* maps to chromosome 4q13 and contains 12 exons. (B) Genetic structure of wildtype *TECRL* and *TECRL*Δexon3. (C) The *TECRL* protein is predicted to contain a ubiquitin-like domain, 3 transmembrane domains and a 3-oxo-5-α-steroid 4-dehydrogenase domain. (D) Comparison of *Tecrl* amino acid sequences shows a high degree of cross-species conservation.

TECRL_{Het}-hiPSCs

CTRL-hiPSCs

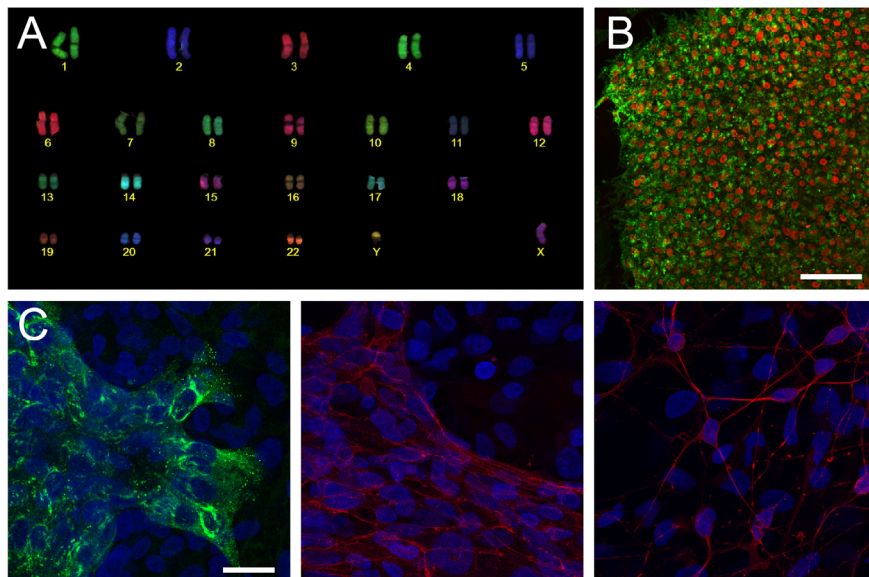


Figure S2. Characterization of TECRL_{HET} and CTRL hiPSCs. hiPSCs from both lines show (A) a normal karyotyp (B) express the pluripotency genes NANOG and SSEA4 (scale bars: 100 µm) and (C) differentiate to derivatives of endoderm (left), mesoderm (center) and ectoderm (right) (scale bars: 25 µm).

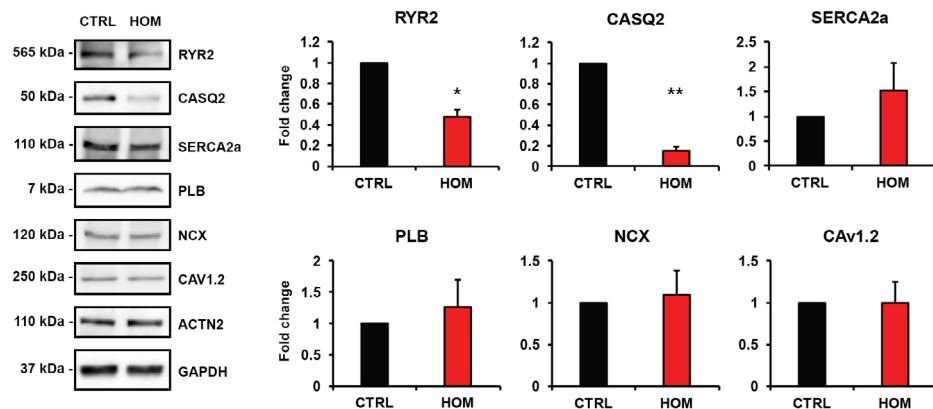


Figure S3. Protein levels of canonical calcium handling proteins in $\text{TECRL}_{\text{HOM}}$ -hiPSC-CMs. Representative western blots of calcium handling proteins in CTRL- and $\text{TECRL}_{\text{HOM}}$ -hiPSC-CMs (left panel) and quantification of protein levels by densitometric analysis. Graphs show the mean values of fold change relative to GAPDH and ACTN2 as internal controls; Western blots were quantified from results of three independent experiments; Mutant values represent fold change relative to that of controls, which was converted to 1. The standard error of the mean (s.e.m.) is shown by error bars. Statistically significant fold changes are marked * ($P<0.05$) and ** ($P<0.01$), using the one sample unpaired Student's *t*-test.

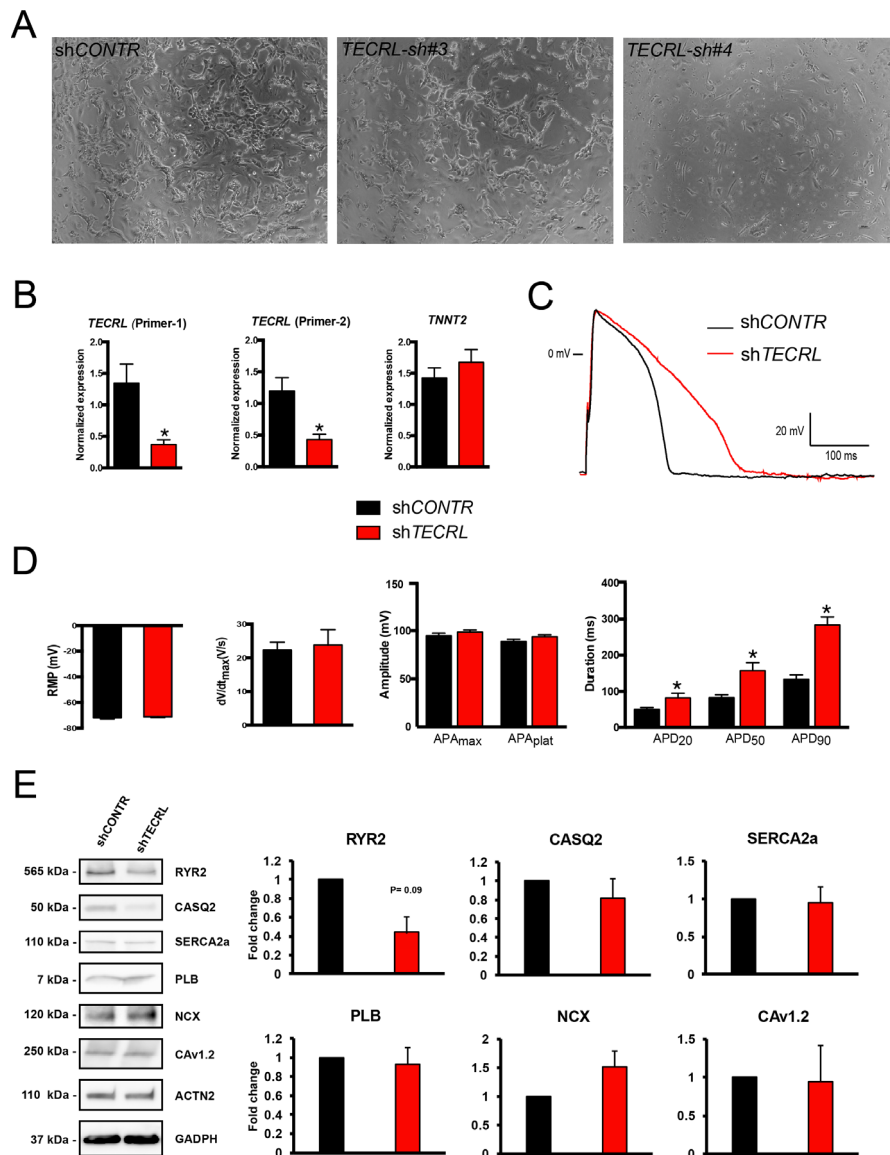


Figure S4. shRNA-mediated knockdown of *TECRL* in hESC-CMs. (A) hESC-CMs transduced with SIN-LVs encoding control shRNA (*shCONTR*) or with two different *TECRL*-specific shRNAs (*TECRL-sh#3*, hereinafter referred to as *shTECRL* and *TECRL-sh#4*) (B) mRNA expression of *TECRL* and *TNNT2* in hESC-CMs treated with *shCONTR* or *shTECRL*. (C) Representative APs and (D) averaged AP parameters RMP, dV/dt_{max} , APA_{max} , APA_{plat} and APD_{20} , APD_{50} and APD_{90} of *shCONTR*- and *shTECRL*-transduced hESC-CMs. (E) Representative western blots of calcium handling proteins in *shCONTR*- and *shTECRL*-transduced hESC-CMs (left panel) and quantification of protein levels by densitometric analysis; $n = 3-4$ cells each from three independent experiments for the electrophysiological measurements. * $P < 0.05$ unpaired *t*-test or Mann-Whitney Rank Sum Test. Western blots were quantified from results of three independent experiments. *shTECRL* values represent fold change relative to that of *shCONTR*, which was converted to 1. The s.e.m. is shown by error bars. Statistical significance was calculated using the one sample unpaired Student's *t*-test; AP = action potential; APA_{max} = maximum AP amplitude, APA_{plat} = AP plateau amplitude; APD_{20} , APD_{50} , and APD_{90} = AP duration at 20, 50, and 90% repolarization, respectively; dV/dt_{max} = maximum upstroke velocity; RMP = resting membrane potential.

Tables

Subject in current pedigree	Subject in Bhuiyan <i>et al</i> , 2007	Gender	Triggers for cardiac arrest	Age at first syncopal episode	Current condition	ECG	QTc (ms)	Other cardiac nomalies
IV:1	IV:1	F	Playing	10	Deceased	NA	NA	No
IV:2	IV:2	M	Skating	12	Deceased	Sinus rhythm	450-490 (Bhuiyan <i>et al</i> , 2007)	No
IV:4	IV:4	F	Playing	8	Deceased	Sinus rhythm	470-480 (Bhuiyan <i>et al</i> , 2007)	VSD/Infundibular stenosis
IV:8	IV:8	F	Dancing	8	Deceased	Sinus rhythm	460-470	VSD/Pulmonary atresia
IV:9	Not yet born	M	Running	2.5	Severe brain damage since 2014	Yes	420	No
IV:10	IV:9	M	Running	7	Deceased	Yes (Bhuiyan <i>et al</i> , 2007)	480 (Bhuiyan <i>et al</i> , 2007)	No
IV:13	Not yet born	M	Running	4	ICD implanted and no syncope since	Sinus rhythm , 1 ECG showing mild QT prolongation (Fig. 1B of this manuscript)	450 (Fig. 1B of this manuscript)	No

Table S1. Clinical features of affected family members of a consanguineous Sudanese family diagnosed with CPVT. Please see Fig. 1A for the pedigree

CHROMOSOME	POSITION GRCH37.P13	REF. ALLELE	ALT. ALLELE	GENE	ALIAS	AMINO ACID/SPLICE- SITE CHANGE	EXPRESSION	REFERENCE
CHR4	73984490	A	C	ANKRD17 (4q13.3)	KIAA0697	V1117G	Ubiquitous; Muscle, Heart, Lung, Brain, Kidney, Liver, Spleen (RT-PCR on human tissues)	Deng <i>et al</i> , 2009
CHR4	71096931	T	C	FDC-SP (4q13.3)	C4orf7	L7P	Strong expression in Trachea, Lymph node, Prostrate, Tonsil; Low expression in Thyroid and Stomach (RT- PCR on human tissues)	Marshall <i>et al</i> , 2002
CHR4	68456632	G	A	STAP1 (4q13.2)	BRDG1	M230I	Not detected in non- hematopoietic tissues such as heart (RT- PCR on mouse tissues)	Masuhara <i>et al</i> , 1999
CHR4	65194229	C	T	TECRL (4q13.1)	SRD5A2L2	Splice site; c.331+1	Highly enriched in the heart	Beqqali <i>et al</i> , 2006; This publication N/A
CHR17	2607763	C	T	CLUH (17p13.3)	KIAA0664	E28K	-	N/A

Table S2. SNVs identified by exome sequencing in the Sudanese family as heterozygous in the parents and homozygous in the clinically affected children

SHRNA	SEQUENCE	MISSION LIBRARY CLONE	MATCH REGION	MATCHING TRANSCRIPTS
<i>TECRL-SH#1</i>	GATTGGATCATGGATTAGTTT	TRCN0000158841	Coding sequence (CDS)	NM_001010874.4, XM_005265663.2, XM_005265662.3
<i>TECRL-SH#2</i>	GATGAGTATCCAGATGTCTTT	TRCN0000159663	CDS	NM_001010874.4, XM_005265663.2, XM_005265662.3
<i>TECRL-SH#3</i>	CCATTCAAAGTATTGCAGCTT	TRCN0000160461	CDS	NM_001010874.4, XM_005265664.2, XM_005265662.3, XM_005265665.2, XM_005265663.2
<i>TECRL-SH#4</i>	GCAGGACACACACCTTTGAAA	TRCN0000163528	CDS	NM_001010874.4, XM_005265665.2, XM_005265662.3, XM_005265664.2, XM_005265663.2
<i>TECRL-SH#5</i>	CCTAACACTTTGACAGGCCAA	TRCN0000164455	3'untranslated region (UTR)	NM_001010874.4, XM_005265662.3

Table S3. shRNA sequences to knockdown *TECRL* expression

TARGET	SEQUENCE
ARP (HOUSEKEEPING GENE)	F: CACCATTGAAATCCTGAGTGATGT R: TGACCAGCCCAAAGGAGAAG
GUSB (HOUSEKEEPING GENE)	F: CCAAGAGCCAGTTCCTCATCAA R: CACATCTGCATCACTTCTCTCTG
TECRL	<u>Primer pair-1</u> F: TCTGGATAAGGTGACACAATCA R: TCTAGCTGCAGACCAACTCG <u>Primer pair-2</u> F: TTGGCTTGCTTCTGTCATTG R: TGTGATTTCCTGTTTCCAA
TNNT2	F: TTCGACCTGCAGGAGAAGTT R: GCGGGTCTTGAGACTTTCT

Table S4. Primer sequences for RT-qPCR

