A Precision Medicine Approach to the Rescue of Function on Malignant Calmodulinopathic Long-QT Syndrome

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Rationale: Calmodulinopathies comprise a new category of potentially life-threatening genetic arrhythmia syndromes capable of producing severe long-QT syndrome (LQTS) with mutations involving CALM1, CALM2, or CALM3. The underlying basis of this form of LQTS is a disruption of Ca\(^{2+}\)/calmodulin (CaM)-dependent inactivation of L-type Ca\(^{2+}\) channels.

Objective: To gain insight into the mechanistic underpinnings of calmodulinopathies and devise new therapeutic strategies for the treatment of this form of LQTS.

Methods and Results: We generated and characterized the functional properties of induced pluripotent stem cell–derived cardiomyocytes from a patient with D130G-CALM2–mediated LQTS, thus creating a platform with which to devise and test novel therapeutic strategies. The patient-derived induced pluripotent stem cell–derived cardiomyocytes display (1) significantly prolonged action potentials, (2) disrupted Ca\(^{2+}\) cycling properties, and (3) diminished Ca\(^{2+}\)/CaM-dependent inactivation of L-type Ca\(^{2+}\) channels. Next, taking advantage of the fact that calmodulinopathy patients harbor a mutation in only 1 of 6 redundant CaM-encoding alleles, we devised a strategy using CRISPR interference to selectively suppress the mutant gene while sparing the wild-type counterparts. Indeed, suppression of CALM2 expression produced a functional rescue in induced pluripotent stem cell–derived cardiomyocytes with D130G-CALM2, as shown by the normalization of action potential duration and Ca\(^{2+}\)/CaM-dependent inactivation after treatment. Moreover, CRISPR interference can be designed to achieve selective knockdown of any of the 3 CALM genes, making it a generalizable therapeutic strategy for any calmodulinopathy.

Conclusions: Overall, this therapeutic strategy holds great promise for calmodulinopathy patients as it represents a generalizable intervention capable of specifically altering CaM expression and potentially attenuating LQTS-triggered cardiac events, thus initiating a path toward precision medicine. (Circ Res. 2017;120:39-48. DOI: 10.1161/CIRCRESAHA.116.309283.)

Key Words: action potential  ■ calmodulin  ■ induced pluripotent stem cells  ■ L-type calcium channels  ■ long-QT syndrome  ■ nucleotides

A n increasingly recognized group of patients experience diseases called calmodulinopathies, caused by missense mutations in calmodulin (CaM), a ubiquitous Ca\(^{2+}\) sensor vital to immune system, heart, and brain function. Calmodulinopathy patients often experience life-threatening cardiac arrhythmias associated with long-QT syndrome (LQTS), \(^{1-4}\) catecholaminergic polymorphic ventricular tachycardia, \(^{5,6}\) and idiopathic ventricular fibrillation. \(^{6}\) Their symptoms are often resistant to conventional therapy, suggesting alternate underlying disease mechanisms that require novel therapeutic strategies.

LQTS-associated CaM mutations are known to alter the Ca\(^{2+}\)/CaM-binding affinity, \(^{7}\) implicating a myriad of Ca\(^{2+}\)/CaM-binding partners as potential pathogenic elements. The L-type Ca\(^{2+}\) channel (LTCC), which plays a vital role in LQTS, represents one such target. \(^{8-10}\) In fact, LQTS-associated calmodulinopathy mutations (D96V, D130G, and F142L) mediate a decrease in Ca\(^{2+}\)/CaM-dependent inactivation (CDI) of LTCCs, a critical form of channel regulation. \(^{8}\) This would
result in the failure of calmodulinopathy-affected LTCCs to inactivate during the plateau of the cardiac action potential (AP) and is predicted to prolong the AP duration (APD), a cellular correlate of prolonged QT intervals identified on the ECG.10,11

There are 3 distinct CaM genes, CALM1 (chr14q31), CALM2 (chr2p21), and CALM3 (chr19q13), with 85% nucleotide sequence homology that encode for completely identical 149 amino acid CaM proteins. In all reported cases of LQTS-associated calmodulinopathies, the mutation occurs heterozygously in one of these 3 redundant CALM genes, that is, with only 1 out of 6 alleles harboring the mutation. Thus, only a small fraction of mutant CaM protein causes the severe phenotype. This large dominant negative effect may be rationalized by the known preassociation of Ca2+-free CaM to the LTCCs.10,12 In fact, the reduction of CDI because of mutant CaM expression corresponds to a highly nonlinear effect such that a relatively small amount of mutant CaM can significantly decrease CDI in HEK293 cells.10 However, this phenomenon remains to be substantiated in a cardiac system under conditions mimicking that of a calmodulinopathy patient. To this end, we generated induced pluripotent stem cells (iPSCs) from a patient harboring the p.D130G-CaM missense mutation,1 resulting from a single nucleotide substitution (c.389 A>G) within the CALM2 gene. Cardiomyocytes (CMs) differentiated from these cells (iPSC-CMs) offer an ideal platform for exploring the dominant negative effect of mutant CaM within a patient-specific genetic background and provide a model system with which to understand the pathogenesis and treatment options of CaM-mediated LQTS.

The nonlinear CDI effect in calmodulinopathies may also provide an opportunity for novel therapeutic interventions. Impaired repolarization resulting from a deficit of LTCC CDI exhibits a nonlinear threshold such that the fraction of channels harboring a CDI deficit can increase without overt electric dysfunction up to a critical threshold. At this point, addition of even a minute fraction of affected channels generates the substrate for flagrant arrhythmogenesis.8 Should this threshold behavior hold true, a relatively small decrease in mutant CaM could result in a significant increase in electric stability and thus lead to substantial clinical improvement. To this end, we exploit the precise genetic control of a variant of CRISPR/Cas9 technology, CRISPR interference (CRISPRi),13–15 to selectively downregulate mutant CaM expression without permanently altering the genome. Taking advantage of the fact that patients with calmodulinopathies harbor mutations in only 1 of 3 CALM genes, mutant CaM could be attenuated while largely sparing wild-type (WT) CaM. As a test bed for therapeutic development, we use our D130G-CaM–containing iPSC-CMs (iPSCD130G-CaM–CMs) because these cells are able to form a functional syncytium with the genetic background of the patient. Such a disease model readily permits application of CRISPRi to downregulate mutant CALM genes and enables analysis of the functional effects of such a manipulation.

In this study, we demonstrate that iPSC-CMs derived from a patient harboring the p.D130G-CaM missense mutation within CALM2 accurately recapitulate the cellular LQTS phenotype. Specifically, the iPSCD130G-CaM–CMs demonstrate prolonged APs, disrupted Ca2+ cycling, and diminished LTCC CDI, consistently across extended culture time. Having established a viable model system, we next use CRISPRi to selectively silence the expression of the CALM2 gene (both mutant and WT CALM2 alleles) and correlate this reduction with a functional rescue of the iPSCD130G-CaM–CMs. In particular, we have corrected fully the magnitude of CDI in these cells, resulting in normalization of the AP profile and therapeutic attenuation of the APD.

**Methods**

**Study Participant**
A p.D130G-CaM missense mutation secondary to c.389 A>G-CALM2 was identified previously in a young female patient with severe LQTS that was referred to the Windland Smith Rice Sudden Death Genomics Laboratory (M.J.A.) at Mayo Clinic, Rochester, MN, for research-based genetic testing.1 This study was approved by the Mayo Foundation Institutional Review Board, and informed consent was obtained.

**Generation of iPSCs**
Dermal fibroblasts were isolated from a punch skin biopsy obtained from the p.D130G-CaM–positive patient and expanded in DMEM containing 10% fetal bovine serum. These fibroblasts were reprogrammed using the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Invitrogen) according to manufacturer’s recommendations. Colonies were isolated as separate clones and characterized for pluripotency based on immunofluorescent staining (Online Figures I and II). Sanger sequencing of genomic DNA of each clone confirmed a heterozygous mutation c.389 A>G-CALM2. WT iPSCs used for control experiments were a generous gift from Dr Bruce Conklin.16 All cell lines were tested negative for mycoplasma.

**Cell Culture**
I iPSCs were cultured and differentiated in a feeder-free and xeno-free system using a modified protocol described previously.17 Briefly, iPSCs were cultured on Geltrux matrix (Gibco)–coated tissue culture plates and fed daily with Essential 8 medium (Gibco). When cells were ≈30% confluent, they were mechanically dissociated using 0.5 mmol/L EDTA in Dulbecco’s phosphate-buffered saline. For differentiation into cardiomyocytes, cells were dissociated and plated on fresh Geltrux matrix–coated plates. When confluent (day 0), media was exchanged with RPMI-1640 (Roswell Park Memorial Institute; Sigma-Aldrich) supplemented with B-27 (insulin) (Gibco) and 6 µmol/L CHIR99021. Cells were maintained in this media for the first 7 days, with medium exchange every 2 days. On day 3, 5 µmol/L

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**Nonstandard Abbreviations and Acronyms**

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>AP</td>
<td>action potential</td>
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<td>APD</td>
<td>action potential duration</td>
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<td>BFP</td>
<td>blue fluorescence protein</td>
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<td>CaM</td>
<td>calmodulin</td>
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<td>CaT</td>
<td>Ca2+ transients</td>
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<td>CDI</td>
<td>Ca2+/CaM-dependent inactivation</td>
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<td>CRISPRi</td>
<td>CRISPR interference</td>
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<td>gRNA</td>
<td>guide RNA</td>
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<td>iPSC</td>
<td>induced pluripotent stem cell</td>
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<td>iPSC-CM</td>
<td>induced pluripotent stem cell–derived cardiomyocyte</td>
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<td>KRAB</td>
<td>Krüppel-associated box</td>
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<td>LTCC</td>
<td>L-type Ca2+ channel</td>
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<td>LQTS</td>
<td>long-QT syndrome</td>
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<td>WT</td>
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IWR-1 (Inhibitor of Wnt Response) was added. On day 7, media was changed to RPMI-1640 with B-27 supplement (Gibco) and was exchanged every 2 days. Spontaneous contraction of iPSC-CMs was observed by day 12.

Twelve to 14 days post differentiation, sheets of contracting iPSC-CMs were dissociated using 0.05% trypsin-EDTA (Gibco). The isolated cells were preplated for 4 to 8 minutes on Geltrex-coated tissue culture plates to decrease the number of noncardiac cells, and the nonadherent cells were then plated on Geltrex matrix–coated glass coverslips at ~2.5×10^5 cells/cm^2 for electrophysiological studies and ~3×10^5 cells/cm^2 on plastic coverslips to create monolayers for imaging.

**CRISPR Construction, Transfection, and Transduction**

The lentiviral transfer vectors containing cDNA for enzymatically dead Cas9 fused with suppressor Krüppel-associated box (KRAB) and blue fluorescence protein (BFP; pHR-SFFV-dCas9-BFP-KRAB) was purchased from Addgene (plasmid #46911). BFP was replaced with monomeric red fluorescence protein (mRuby). The entire construct (dCas9-mRuby-KRAB) was then cloned via Gibson assembly (New England Biolabs) into the lentiviral vector pRRLsin18.cPPT.CMV.GFP.Wpre54.18 The lentiviral vector (pKL-U6gRNA(3s)-PGKpuro2ABFP) containing cDNA for the guide RNA (gRNA) backbone (driven by the human U6 promoter) and a BFP marker (driven by PGK promoter) was purchased from Addgene (plasmid #50946), and BFP was replaced with cyan fluorescence protein. E-CRISPR gRNA sequence prediction program19 was used to generate candidate gRNA sequences that selectively complement to the human CALM2 gene (Online Table I). The candidate gRNA sequences were then cloned into the aforementioned lentiviral transfer vector using Golden Gate assembly.

For gRNA screening, both dCas9-mRuby-KRAB and candidate gRNA were expressed in HEK293 cells by transfection with polyethylenimine.10 For iPSC-CM transduction, lentivirus was generated using Lenti-X-Concentrator (Clontech) according to the manufacturer’s recommendations and added to monolayers on day 21 post differentiation. Expression was confirmed by mRuby and cyan fluorescence protein visualization.

**MRNA Expression**

Total RNA was extracted 4 days post transfection in HEK293 cells and 8 to 9 days post transduction in iPSC-CMs using an RNeasy Kit (Qiagen). Complementary DNA was made using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantification of CALM mRNA levels was performed using quantitative real-time polymerase chain reaction with TaqMan gene expression assay (Applied Biosystems). CALM expression level was normalized to the expression level of a house-keeping gene GAPDH. Probe numbers are as follows: CALM1, Hs00300085_s1; CALM2, Hs00830212_s1; CALM3, Hs00968732_g1; and GAPDH, Hs00758991_g1.

**Electrophysiology**

Whole-cell recordings of iPSC-CMs were performed 28 to 30 days post differentiation at room temperature using an Axopatch 200B amplifier (Axon Instruments). Traces were low-pass filtered at 2 kHz and digitally sampled at 10 kHz. P/8 leak subtraction was used, with series resistances of 1 to 2 MΩ. Internal solutions contained (in mM/L) the following: CsMeSO₄, 114; CsCl, 5; MgCl₂, 1; MgATP, 4; HEPES (pH 7.3), 10; BAPTA, 10; and ryanodine, 0.005, at 295 mmol/L adjusted with CsMeSO₄. Seals were formed in Tyrode solution containing (in mM/L) the following: NaCl, 135; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 0.33; NaH₂PO₄, 0.33; HEPES, 5; and glucose, 5 (pH 7.4). After patch rupture, bath solution was switched to Ca²⁺– or Ba²⁺–containing solution.

\[
f_{50} = \left( \frac{r_{50/Ba} - r_{50/Ca}}{r_{50/Ca}} \right) / r_{50/Ba}
\]

where \( r_{50/Ba} \) and \( r_{50/Ca} \) are currents remaining after 50 ms with Ba²⁺– and Ca²⁺–containing external solution.

**Imaging**

Monolayers of iPSC-CMs expressing either a genetically encoded voltage or Ca²⁺– sensor (ASAP120 or GCaMP6f21, respectively, via lentiviral transduction) were paced using a custom field stimulation apparatus in RPMI-1640 medium with B-27 supplement. Expression efficiency of the genetically encoded sensors was assessed via flow cytometry (Online Figure III). At 30, 45, and 60 days post differentiation, green fluorescence was imaged with an Evolve 512 Delta camera at ≥190 frames per second, and the relative change in fluorescence signal was measured. The time from upstroke to 80% repolarization (APD₈₀) was used to index the APD while the magnitude of peak transient, time to peak, and decay time constant were used as metrics for calcium transients. All metrics were quantified using custom Matlab (Mathworks) scripts. For all experiments involving treatment with CRISPRi, control cells were recorded on the same day and were from the same culture, minimizing any culture-dependent variability of the cells.

**Statistical Analysis**

All parameters are shown as mean±SEM. Technical and biological replicates are indicated in the figure legend. The D’Agostino and Pearson omnibus normality test was used to confirm a normal distribution before application of the statistical test for comparison of means. Data that did not initially correspond to a normal distribution were lognormally transformed. The \( P \) test was used to compare variances, and a 2-sided Student \( t \) test (adjusted for unequal variances where applicable) was used to compare the difference in means across sample groups. Reported \( P \) values are from the 2-sided Student \( t \) test. Minimal sample size to ensure adequate power was determined as previously described.22

**Results**

**Proband Identification and Generation of Mutation-Harbor- ing iPSCs**

An increasing number of patients are being diagnosed with calmodulinopathies resulting from single heterozygous missense mutations within their CALM1, CALM2, or CALM3 genes. Here, we focus on a p.D130G-CaM mutation identified within CALM2 of a female infant with severe LQTS.1

The proband was born at term and noted to have bradycardia (Figure 1A). An ECG, recorded 12 hours after birth, revealed a QTc of 740 ms and 2:1 atrioventricular block (Figure 1A). She was treated with \( β \)-blockers, phenytoin, spironolactone, potassium, and placement of a single-chamber pacemaker within the first week of life. At 6 years of age, a single-chamber implantable cardioverter-defibrillator was implanted, and \( β \)-blocker therapy was continued. At 11 and 14 years of age, she experienced appropriate defibrillator discharges for ventricular fibrillation.

Next-generation whole-exome sequencing followed by CALM1, CALM2, and CALM3 gene-specific analysis identified a p.D130G-CaM mutation (c.389 A>G, CALM2) within the patient (Figure 1B). The mutation maps to an EF-hand within CaM (Figure 1C), and similar to other calmodulinopathies, causes a reduction in the \( Ca^{2+} \) binding affinity.2 To create a model system with which to understand the pathogenesis and treatment options for this type of CaM-mediated LQTS, multiple clones of iPSCs were generated from the patient’s skin biopsy. Two clones with normal karyotypes at passage 25 and expressing the pluripotency markers (Nanog, Oct4, and SSEA4) were selected (Online Figure I).
In addition, the ability to generate each of the 3 germ layers was confirmed by staining differentiated embryoid bodies for α-fetoprotein (endoderm), smooth muscle actin (mesoderm), and glial fibrillary acidic protein (ectoderm) and by analysis of teratoma formation (Online Figure II). Monolayers of iPSC-CMs (iPSCD130G-CaM-CMs) were then generated from these 2 clones of iPSCs.

To confirm that the background of these iPSCD130G-CaM-CMs was not significantly different from iPSCs derived from healthy individuals, we quantified the mRNA levels for multiple proteins that could potentially alter cardiac AP morphology and excitation–contraction coupling. Compared with WT iPSC-CMs (iPSCWT-CMs), we found no difference in the mRNA levels of CALM1, CALM2, CALM3, CACNA1C, KCNH2, NCX1, SCN5A, PLN, or SERCA2 (Online Figure IV). Only KCNQ1 and RYR2 seemed somewhat elevated in the iPSCD130G-CaM-CMs; however, this variation would not be expected to contribute to a LQT phenotype. Importantly, this validates our iPSCWT-CMs as a relevant control, despite the potential variability that can occur because of differing genetic backgrounds.23

Altered APs and Calcium Transients in iPSCD130G-CaM-CMs

Previous work has linked mutations in CaM with LQTS; however, direct evidence demonstrating AP prolongation because of the D130G-CaM mutation has yet to be shown in human cardiomyocytes. We, therefore, characterized the iPSCD130G-CaM-CMs to confirm that these cells exhibit prolonged APDs that typically underlie LQTS. To measure APDs, the monolayers were transduced with the genetically encoded voltage sensor ASAP1, which features rapid kinetics and stable long-term expression, allowing accurate APD measurements over multiple time points.20 The resultant APDs (Figure 1D and 1E) measured from WT iPSC-CMs (iPSC WT-CMs) were comparable to those previously reported (Online Table I).16,24 Under these same conditions, iPSC D130G-CaM-CMs exhibited dramatically longer APs and APDs (Figure 1G and 1J; red) compared with their WT counterparts (gray). This result could be observed at multiple pacing frequencies (Figure 1H and 1K), a feature associated with increased arrhythmogenic risk.25 Moreover, the phenotype was stable for long periods of time.
in culture, such that APDs measured at 30 days in culture were not significantly different than those measured after 45 or 60 days (Figure 1F, 1I, and 1L; Online Figure VI).

In addition to the electrical disturbance, dysfunctions in Ca\(^{2+}\) cycling are often associated with arrhythmogenesis in LQTS.\(^{26}\) As such, we examined the intracellular Ca\(^{2+}\) transients of iPSC-CMs using GCaMP6f, a genetically encoded Ca\(^{2+}\) sensor with a high signal-to-noise ratio and fast kinetics.\(^{21}\) Figure 2A shows the calcium transients (CaTs) from a monolayer of iPSC\(_{\text{WT}}\)-CMs paced at 0.25 Hz with the rise and decay kinetics comparable to those previously reported.\(^{27}\) However, monolayers of iPSC\(_{\text{D130G}}\)-CMs exhibit CaT amplitudes over 3 times larger than WT with slower rise and decay kinetics (Figures 2B through 2F), akin to the phenotype observed in CaM\(_{\text{D130G}}\)-overexpressing rodent myocytes.\(^{10,11}\) Likewise, these CaT effects were stable over time (Online Figure VII). Although sarcoplasmic reticulum content of the iPSC\(_{\text{D130G}}\)-CMs was not significantly different from the WT myocytes (Online Figure VIII), the trend was in the direction of increased sarcoplasmic reticulum Ca\(^{2+}\). Thus, the patient-derived iPSC\(_{\text{D130G}}\)-CMs recapitulate the LQTS phenotype, demonstrating significant proarrhythmic potential despite limited, native expression levels of CaM\(_{\text{D130G}}\).

**IPSC\(_{\text{D130G}}\)-CMs Exhibit Diminished CDI**

Previous studies have implicated the cardiac LTCC as a major contributor to the LQT phenotype in patients with CaM-mediated LQTS.\(^{10,11}\) In particular, the D130G mutation weakens the affinity of Ca\(^{2+}\) binding to CaM,\(^{2}\) resulting in a significant decrease in CDI when CaM\(_{\text{D130G}}\) is overexpressed in rodent myocytes.\(^{10,11}\) However, the relevance of these results remains to be established in human CMs with physiological levels of mutant CaM expression. We, therefore, examined the effect of the D130G mutation on LTCC CDI in patient-derived iPSC-CMs. To this end, we performed whole-cell patch clamp recordings of individual CMs. iPSC\(_{\text{D130G}}\)-CMs exhibited a rapid decay in their Ca\(^{2+}\) current in response to a 10-mV depolarizing step (Figure 3A, red). To isolate the extent of pure CDI, Ba\(^{2+}\), which binds poorly to CaM, was used as the charge carrier to gauge the extent of voltage-dependent inactivation within the same cell.\(^{10}\) CDI can be seen as the excess inactivation of the Ca\(^{2+}\) trace (Figure 3A; red) compared with the Ba\(^{2+}\) trace (black). Population data showing the average normalized peak Ba\(^{2+}\) currents as a function of voltage is shown in Figure 3B. For CDI quantification, we first measure the fraction of current remaining after 50 ms (\(r_{50}\)) for both the Ca\(^{2+}\) and Ba\(^{2+}\) currents. By plotting the \(r_{50}\) values as a function of voltage, a hallmark U-shaped relationship is observed with Ca\(^{2+}\) as the charge carrier (Figure 3C, red). The difference between the Ba\(^{2+}\) and Ca\(^{2+}\) \(r_{50}\) values at 10 mV, normalized by the Ba\(^{2+}\) \(r_{50}\) value, quantifies the extent of pure CDI (Figure 3C). Applying this same protocol to the iPSC\(_{\text{D130G}}\)-CMs reveals a profound attenuation in the kinetics and extent of CDI (Figures 3D and 3G) without altering the voltage activation profile (Figures 3E and 3H). Quantifying this result across voltages (Figures 3F and 3I) confirms a significant decrease in CDI (red; \(P<0.01\)), an effect that is maintained over time in culture (Online Figure IX). Importantly, this reduction of CDI is significant even in the iPSC\(_{\text{D130G}}\)-CM background, where the patient’s other 5 CALM alleles are WT. Thus, these iPSC\(_{\text{D130G}}\)-CMs not only provide a viable model system for this LQTS-associated calmodulinopathy but also suggest that the loss of LTCC CDI is a significant underlying mechanism leading to arrhythmogenesis in these patients.

To further bolster this LTCC-centric hypothesis, we examined the effects of other potential CaM targets that might contribute to the LQT phenotype of calmodulinopathy patients. To date, only 3 genetic forms of LQTS result from mutations within a channel known to be modulated by CaM. LQT1 results from loss-of-function mutations in KCNQ1, LQT3 results from gain-of-function mutations in SCN5A, and LQT8 is caused by inactivation altering mutations within CACNA1C, somewhat mirroring the LTCC effects described in this study. Of these forms of LQT, only LQT8 approaches the extreme APD prolongation seen in calmodulinopathy patients.\(^{28,29}\) To corroborate this in our model system, we mimicked the effect of each LQT mechanism pharmacologically. Consistent with clinical findings, enhancement of the current through the CaM-mediated LTCC\(_{\text{D130G}}\) is a significant underlying mechanism leading to arrhythmogenesis in these patients.
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harboring CaM_D130G was necessary to achieve significant arrhythmogenesis in the model cells. Importantly, the simulation predicted a threshold for the induction of electric instability precisely matching the expected levels of mutant CaM expression in calmodulinopathy patients based on reported CALM gene expression. Such a threshold highlights an important therapeutic principle; namely, only a small reduction in the expression levels of the mutant CaM may be needed to provide significant clinical benefit to patients.

Toward a New Therapeutic Strategy

Having confirmed a major role for LTCC CDI deficits in generating the LQT phenotype in this calmodulinopathy patient, we next considered the implications of this mechanism on a novel therapeutic intervention. As our results predict a significant functional benefit conferred by even a small shift in the expression of mutant versus WT CaM (Online Figure XI), we sought to reduce the fraction of mutant CaM expressed in patients with CaM-mediated LQTS. Because all 3 CALM genes encode for identical CaM proteins, we reasoned that we might be able to take advantage of the sequence variation at the nucleotide level. We, thus, used CRISPRi to decrease the transcription of the CALM2 alleles, both the WT and the D130G-containing CALM2 alleles.

The CRISPRi technology uses a short gRNA, which binds specifically to a target nucleotide sequence. By pairing this gRNA with a nuclease-dead Cas9 (dCas9) fused to suppressor KRAB, selective suppression of the target gene could be achieved. Our first step was, therefore, to optimize gRNA sequences capable of selectively targeting CALM2. Sequence optimization was first done in silico, followed by evaluation of the efficiency and specificity of each candidate gRNA in HEK293 cells via quantitative real-time polymerase chain reaction (Online Figures XII and Table II). We choose design 21 (Figure 4A) because this gRNA specifically reduced the expression of CALM2, without appreciable alteration of either CALM1 or CALM3.

Having identified a potential treatment strategy, we next sought to test this approach within our iPSC_D130G-CaM-CMs. Monolayers were lentivirally transduced with genes encoding dCas9-mRuby-KRAB and gRNA-cyan fluorescence protein, and expression of both constructs was confirmed by visualization of red and blue fluorescence, respectively. Compared with untreated iPSC_D130G-CaM-CMs, the CRISPRi-treated iPSC_D130G-CaM-CMs exhibited significantly lower levels of CALM2 mRNA with unaltered levels of CALM1 and CALM3 (Figure 4B) mRNA level with the overall reduction of total amount of CaM protein (Online Figure V). In addition, we probed the effect of treatment on multiple cardiac genes and found no significant change in the mRNA levels of CACNA1C, KCNQ1, KCNH2, SCN5A, RYR2, SERCA2, NCX1, or PLN (Online Figure IV). Having achieved a selective decrease in CALM2 transcription, we next tested whether
this reduction correlated with a functional effect within the iPSC_{D130G-CaM} CMs. Indeed, treatment of the monolayers resulted in a substantial shortening of the APDs in response to 0.5-Hz stimulation (Figure 4C, blue) compared with untreated monolayers (gray). This effect was consistent across multiple trials, resulting in a significant decrease in APD as compared with untreated iPSC_{D130G-CaM} CMs (Figure 4C, right), establishing CRISPRi as a robust and promising strategy for the treatment of CaM-mediated LQTS.

Having established functional rescue of the iPSC_{D130G-CaM} CM monolayers, we next considered the underlying mechanism. Our previous results suggest that APD prolongation of these cells stems from a CDI deficit of the LTCC (Figure 3). We, therefore, predicted that successful treatment of these cells should correspond to a correction (increase) in the CDI of the LTCCs. Indeed, treated iPSC_{D130G-CaM} CMs displayed significantly faster CDI (Figure 4D) as compared with untreated cells. In fact, CDI in the treated cells was nearly identical to that of iPSC_{WT} CMs (Figure 3). Thus, CRISPRi effectively reduced the expression of the mutant and WT CALM2 alleles, resulting in normalization of the APD and restoration of LTCC’s CDI mechanism.

**Generalization of the CRISPRi Strategy Across Calmodulinopathy Subtypes**

Beyond the proband described in this study, the CRISPRi treatment strategy is readily generalizable to any calmodulinopathy. In contrast to the classic CRISPR/Cas9 genome-editing technique where the gRNA sequence is tailored to an exact locus within the affected gene, CRISPRi targets the entire gene of interest itself, resulting in repression regardless of the specific base-pair alteration. This technique can, therefore, be adjusted to target CALM1 or CALM3 genes, providing efficacy across calmodulinopathy patient populations agnostic to the phenotype. We, thus, created gRNA sequences targeting each of the CALM genes (Figure 5A; Online Figure XII and Table II) and tested their efficacy in iPSC-CMs. Using iPSC_{WT} CMs, we are indeed able to specifically decrease the expression of either CALM1 (Figure 5B) or CALM3 (Figure 5C), thus providing a modular toolkit for the treatment of calmodulinopathies resulting from a mutation within any of the 3 CALM genes.

**Discussion**

iPSC_{D130G-CaM} CMs provide a good model system for investigating the underlying pathology of LQTS-associated...
calmodulinopathies. Two distinct iPSC\textsubscript{D130G-CaM}–CM clones each formed a stable contracting syncytium and exhibited prolonged APs, Ca\textsuperscript{2+} cycling disturbances, and diminished LTCC CDI across extended culture. Creation of this model system enabled the generation and testing of a new therapeutic strategy. Taking advantage of the genome targeting precision of CRISPRi, we were able to selectively and efficiently silence both the WT and the D130G-containing \textit{CALM} alleles, resulting in functional rescue of both LTCC CDI and cardiac AP morphology. This proof-of-principle therapy thus represents a first step toward a novel, targeted therapeutic design for calmodulinopathies.

Previous studies on the underlying mechanism of LQTS-associated calmodulinopathies have involved the overexpression of mutant CaM in rodent myocytes.\textsuperscript{10,11} Although such studies have implicated the LTCC\textsuperscript{10} and ruled out the Na\textsubscript{v}1.5 channel\textsuperscript{11} as major contributors to the LQTS phenotype of calmodulinopathy patients, they do not represent the native expression levels of mutant CaM. In particular, calmodulinopathy patients harbor a single heterozygous mutation in only 1 of 3 redundant \textit{CALM} genes. The ability of the resultant small fraction of mutant CaM protein to produce the severe phenotype seen in patients has been attributed to CaM preassociation to the LTCC.\textsuperscript{10}

In this context, a fraction of LTCCs prebound to mutant CaM will display diminished CDI, disrupting the precise tuning of the AP by Ca\textsuperscript{2+} influx. The profound prolongation of the APs and decreased CDI observed in the iPSC\textsubscript{D130G-CaM}–CMs corroborate just such a dominant negative effect. Moreover, the amelioration of the LQTS phenotype of the iPSC\textsubscript{D130G-CaM}–CMs via suppression of \textit{CALM}2 transcription firmly establishes this mutation as the causative genetic mechanism. However, this new mechanistic insight also presents a significant challenge to the treatment of these calmodulinopathy patients. The preassociation of LTCCs with both mutant and WT CaM makes selective targeting of the disrupted LTCCs nearly impossible. Thus, any treatment option for these patients must selectively target the mutant CaM before cytosolic expression and binding to the LTCC.

Fortunately, CRISPRi provides just such a therapeutic option. In fact, recent work demonstrates that CRISPRi is capable of robust gene knock down within both iPSCs and iPSC-derived cardiomyocytes, making this a highly attractive method that has already been validated for our model system. Moreover, this technique offers the advantages of selectivity, reversibility, and generalizability.\textsuperscript{13-15} That is, RNA transcription of specific mutation-containing \textit{CALM} genes can be repressed, without modifying the patient genome and risking permanent alteration of off-target or downstream elements.\textsuperscript{13,14} Furthermore, this technique is generalizable to any calmodulinopathy. Here, we present a simple therapeutic toolbox in which 3 gRNAs targeting \textit{CALM}1, \textit{CALM}2, or \textit{CALM}3 can be chosen to match any calmodulinopathy patient. Importantly, this means that although this study focused on the LQTS-associated calmodulinopathies, the therapy developed here should also be effective for the catecholaminergic polymorphic ventricular tachycardia– and idiopathic ventricular fibrillation–associated calmodulinopathies. Moreover, as expected, because of the widespread distribution of CaM, calmodulinopathy patients also exhibit extracardiac phenotypes including seizures and developmental delays.\textsuperscript{2} Importantly, the CRISPRi
toolkit should be effective on these noncardiac symptoms, as targeting of the CRISPRi can be adjusted to include any affected organ systems.

More broadly, this therapeutic principle could be applied to the treatment of any disease in which there is a redundancy of the affected gene. Thus, the CRISPRi strategy described here not only represents a promising new treatment option for calmodulinopathy patients but could also provide a generalizable strategy in the treatment of a variety of diseases. Fortuitously, development of CRISPR/Cas9 delivery into patients is already well underway,34 propelling the translation of these findings toward improving patients’ health and quality of life and in the case of patients with a LQTS- or catecholaminergic polymorphic ventricular tachycardia—or idiopathic ventricular fibrillation–associated calmodulinopathy, preventing sudden death in the young.

Acknowledgments

David T. Yue passed away on December 23, 2014. His mentorship, wisdom, and kindness are greatly missed. We thank Drs Zhaohui Ye and Linchao Cheng for help in conducting teratoma formation assays, Dr Stephen Eacker for his insight in optimizing the delivery of CRISPR into iPSCs, and Dr Peter Anderson for assistance with flow cytometry. We also thank Dr Leslie Tung for providing valuable advice and discussions and members of the Calcium Signals Laboratory for ongoing feedback.

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Disclosures

D.J. Tester receives royalties from Transgenomic; M.J. Ackerman is a consultant for Boston Scientific, Gilead Sciences, Medtronic, and St Jude Medical and receives royalties from Transgenomic. The other authors report no conflicts.

References

27. Hawong HS, Krysktal DO, Feaster TK, Sánchez-Freire V, Zhang J, Kamp TJ, Hong CC, Wu JC, Knollmann BC. Comparable calcium handling of

**Novelty and Significance**

**What Is Known?**

- Calmodulinopathies comprise a new category of life-threatening genetic cardiac arrhythmias caused by single heterozygous point mutations within the calcium sensor calmodulin (CaM).
- Calmodulinopathy mutations alter the Ca2+ binding affinity of CaM, implicating numerous Ca2+/CaM binding partners as potential pathogenic elements, including the L-type Ca2+ channel, which exhibits disrupted feedback regulation in the presence of mutant CaM when studied in a heterologous expression system.

**What New Information Does This Article Contribute?**

- This study develops a robust model system using induced pluripotent stem cells derived from a calmodulinopathy patient, which recapitulates the phenotype of the patients and provides a test bed for mechanistic understanding and therapeutic design.
- A mechanistic link between defective Ca2+ regulation of L-type Ca2+ channel and the calmodulinopathy phenotype is established.
- A therapeutic strategy based on CRISPR interference demonstrates restoration of the action potential in the calmodulinopathy induced pluripotent stem cell–derived cardiomyocytes.

Calmodulinopathies represent a growing class of severe cardiac arrhythmias, which are often resistant to conventional treatments. This disorder is associated with mutations that disrupt Ca2+ binding to CaM, a ubiquitous Ca2+ sensor molecule vital to heart and skeletal muscle contraction, memory, and immunologic responses. Here, we use induced pluripotent stem cell–derived cardiomyocytes derived from a recently identified calmodulinopathy patient with severe long-QT syndrome to create a model system suitable for examining calmodulinopathy pathogenesis and designing therapeutic interventions. Using this model system, we demonstrate a significant impact of the calmodulinopathy mutations on the Ca2+ regulation of L-type Ca2+ channels, establishing this channel as a major causative factor of the long-QT syndrome phenotype. Furthermore, application of CRISPR interference robustly suppresses the expression of the mutant CaM gene and produces a functional rescue of the calmodulinopathy phenotype, as evidenced by the restoration of the cardiac action potential morphology and L-type Ca2+ channel function. This therapeutic strategy is generalizable to any calmodulinopathy mutation; thus, it holds great promise for improving the health and quality of life of many calmodulinopathy patients.
A Precision Medicine Approach to the Rescue of Function on Malignant Calmodulinopathic Long-QT Syndrome

Worawan B. Limpitikul, Ivy E. Dick, David J. Tester, Nicole J. Boczek, Pattraranee Limphong, Wanjun Yang, Myoung Hyun Choi, Jennifer Babich, Deborah DiSilvestre, Ronald J. Kanter, Gordon F. Tomaselli, Michael J. Ackerman and David T. Yue

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Supplementary Material

A precision medicine approach to the rescue of function in malignant calmodulinopathic long QT syndrome

Limpitikul et al, Circulation Research

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1. Additional Data

1.1 Characterization of the iPSC clones

The iPSC clones generated in this study were derived from a skin biopsy from a patient harboring a p.D130G-CaM mutation residing in the *CALM2* gene. In order to ensure the quality of the generated iPSCs, the karyotype and pluripotency of the cells were fully characterized and validated (Online Figure I).

**Online Figure I. Assessment of karyotype and pluripotency markers for the iPSC clones.** A, Sanger sequencing of *CALM2* genomic DNA confirms c.389 A>G. B, Karyotyping results of both iPSC clones revealed normal size, shape, and number of chromosomes. Karyotyping was commercially performed by WiCell Cytogenetics. C, Immunohistochemistry demonstrated the presence of pluripotency markers: Nanog (red), Oct4 (green), and SSEA4 (red). Nuclei are counter-stained with DAPI (blue).
1.2 iPSC\textsubscript{D130G-CaM} clones are capable of differentiating into three germ layers

An important feature of iPSC lines is pluripotency; that is, the ability to differentiate into all three germ layers, endoderm, mesoderm, and ectoderm. We have confirmed the pluripotency of iPSC\textsubscript{D130G-CaM} clones both \textit{in vitro} and \textit{in vivo}. First, embryoid bodies were generated \textit{in vitro} and markers of three germ layers were labeled by immunohistochemistry (Online Figure II(A)). Next, the ability of iPSC clones to form teratomas \textit{in vivo} was assessed by injection of iPSCs into immunodeficient mice (Online Figure II(B)). The hematoxyline and eosin staining of the harvested teratomas revealed tissues from endodermal, mesodermal and ectodermal origins.

\begin{center}
\textbf{Online Figure II. Ability of iPSCs\textsubscript{D130G-CaM} to differentiate into all germ layers.} A, Immunostaining of differentiated embryoid bodies for the endoderm-specific \textalpha-fetoprotein (AFP, green), the mesoderm-specific smooth muscle actin (SMA, red), and the ectoderm-specific glial fibrillary acid protein (GFAP, green). Cells are counter-stained with DAPI for visualization of the nuclei (blue). B, Five-to-eight-week-old teratomas harvested from mice injected with undifferentiated iPSCs\textsubscript{D130G-CaM}. Tissue sections were stained with haematoxyline and eosin. All three germ layers, including gut-like structures (Endo: endoderm), immature bone and cartilage (Meso: mesoderm), and immature neural tube and neuroectoderm (Ecto: ectoderm), are identifiable in teratomas generated from both iPSCs\textsubscript{D130G-CaM} clones.
\end{center}
1.3 Expression of genetically encoded sensors

Expression of the voltage sensor ASAP1 and the calcium sensor GCaMP6f was assessed by flow cytometry 7-10 days after transduction (Online Figure III). A large fraction of cells expressed the ASAP sensor, while a smaller fraction expressed the GCaMP6f sensor. The expression of GCaMP6f was kept low so as to reduce the potential effect of the sensor on resting the Ca$^{2+}$ concentration.

**Online Figure III. Quantification of sensor expression.** A-B, Exemplar scatter and histogram plots of iPSCWT-CMs and iPSCD130G-CaM-CMs expressing ASAP1. C, Population data of ASAP1 expression level gauged by percentage of fluorescent cells in both iPSC-CM clones. D-E, Exemplar scatter and histogram plots of iPSCWT-CMs and iPSCD130G-CaM-CMs expressing GCaMP6f. F, Population data of GCaMP6f expression level gauged by % of fluorescent cells in both iPSC-CM clones indicating the percentage of iPSCWT-CMs which express GCaMP6f.
1.4 QPCR analysis of iPSC-CM mRNA levels

In this study, we utilized iPSC-CMs with different individual genetic backgrounds for control (WT) and calmodulinopathy cells. In order to ensure that these cells are an appropriate control, we examined the RNA profile of the iPSCWT-CMs as compared to the iPSCD130G-CaM-CMs, focusing on those proteins which might contribute to alterations in QT interval or calcium handling (Online Figure IV). Very little difference was found between the two iPSC-CM lines, with only a small elevation in KCNQ1 (Online Figure IV(E)) and RYR2 (Online Figure IV(H)) detected. Importantly, this difference cannot account for the LQT phenotype of these cells. In fact, increased levels of KCNQ1 would tend to decrease the QT interval. Moreover, treatment of the iPSCD130G-CaM-CMs did not alter the mRNA level for any protein tested, with the exception of the targeted CALM2 gene, indicating specificity of the treatment.

Online Figure IV. Analysis of the genetic background of WT, D130G and D130G treated cells. A, QPCR results indicating mRNA levels of multiple relevant proteins from iPSCWT-CMs (gray), iPSCD130G-CaM-CMs (red) and treated iPSCWT-CMs (Blue) (** p<0.01, *** P<0.001). Error bars indicate ± SEM. n indicates biological replicates.
1.5 Total calmodulin protein levels in iPSC-CMs

The total calmodulin protein levels in iPSC\textsubscript{WT}-CMs and iPSC\textsubscript{D130G-CaM}-CMs was assessed using western blot analysis (Online Figure V). No difference was seen between these two cell lines; however a small but statistically significant decrease in calmodulin protein was seen following treatment with CRISPRi, consistent with a decrease in \textit{CALM2} expression.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure_v.png}
\caption{Online Figure V. Assessment of calmodulin protein expression. A, Western blot of iPSC\textsubscript{WT}-CMs, iPSC\textsubscript{D130G-CaM}-CMs and treated iPSC\textsubscript{D130G-CaM}-CMs probed with anti-calmodulin. Samples were normalized to total protein as determined with a Coomassie stain (inverted intensity image shown at the bottom). B, Normalized intensity of the calmodulin protein bands. No difference in calmodulin levels were detected between iPSC\textsubscript{WT}-CMs (gray) and iPSC\textsubscript{D130G-CaM}-CMs (red), however treatment produced a small decrease in calmodulin protein (blue). * \textit{p}<0.05. Error bars indicate ± SEM.}
\end{figure}
1.6 The AP phenotype is stable at multiple time points

In the main text we demonstrated APD prolongation at multiple cycle lengths in iPSC$_{D130G-CaM}$-CMs 30 days post-differentiation (Figure 1D-L). The equivalent data was obtained 45 and 60 days post-differentiation (Online Figure VI) on the same population of monolayers in Figure 1, demonstrating that the phenotype of the iPSC$_{D130G-CaM}$-CMs is stable over time. Summary data for a cycle length of 2 seconds is displayed in main text Figures 1F, I, L.

Online Figure VI. Stability of iPSC-CM APs across multiple time points. A, Exemplar APs from iPSC$_{WT}$-CMs paced at 0.5 Hz, recorded via fluorescence imaging using ASAP1 45 days post-differentiation. Scale bar indicates percent change in fluorescence. B, Population data for average APDs at various cycle lengths (CL) for 45-day-old iPSC$_{WT}$-CMs (n=9). Each biological replicate (n) is an average value of 2 technical replicates/measurements for all data in this figure. Error bars indicate ± SEM throughout. C, Exemplar APs from 45-day-old iPSC$_{D130G-CaM}$-CMs (red). WT reproduced in gray. D, Average APD data for iPSC$_{D130G-CaM}$-CMs (red, n=7) across multiple pacing frequencies. WT reproduced in gray (*** p<0.001 compared to WT and corrected for unequal variance, both population normally distributed). E-F, Alternate D130G clone demonstrating the same result as C, D (n=9, *** p<0.001 compared to WT and corrected for unequal variance, both population normally distributed). G-L, Equivalent results were observed for iPSC$_{D130G-CaM}$-CMs 60 days post-differentiation. Format as in A-F. (n=7, 7, and 7 for WT, D130G clone#1, and D130G clone#2, respectively; (*** p<0.001 compared to WT and corrected for unequal variance, both populations normally distributed)
1.7 Disruption of Ca\(^{2+}\) cycling across multiple time points

In the main text we demonstrated significant disruption of Ca\(^{2+}\) cycling, with CaT amplitudes several fold larger in iPS\(_{D130G-CaM}\)-CMs as compared to their WT counterparts (Figures 2A-F). The data was collected 30 days post-differentiation, however comparable results could also be obtained 45 and 60 days post-differentiation from the same population of monolayers in Figure 2 (Online Figure VII), indicating a stable phenotype of the D130G harboring monolayers.

Online Figure VII. Stability of iPS-CM CaTs across multiple time points. A, Exemplar CaTs recorded from 45-day-old iPS\(_{WT}\)-CMs (WT) using GCaMP6f. Scale bar indicates percent change in fluorescence. B, Exemplar CaTs recorded from iPS\(_{D130G-CaM}\)-CMs (red) 45 days post-differentiation, as compared to WT (gray). C, Exemplar CaTs from an alternate D130G clone 45 days post-differentiation. D-F, Population data demonstrating larger amplitude and slower kinetics for both iPS\(_{D130G-CaM}\)-CMs clones compared to WT after 45 days in culture (red) (Peak, average peak fluorescence change; Tpeak, average time to peak; T\(_{\text{decay}}\), average decay time constant; n=5, 7, and 6 for WT, D130G clone #1, and D130G clone #2, respectively; *** p<0.001 compared to WT and corrected for unequal variance, both population normally distributed). Each biological replicate (n) is an average value of 2 technical replicates/measurements for all data in this figure. G-L, Equivalent results were seen for iPS\(_{D130G-CaM}\)-CMs 60 days post-differentiation. Format as in A-F. (n=5, 7, and 6 for WT, D130G clone #1, and D130G clone #2, respectively; *** p<0.001 compared to WT and corrected for unequal variance, both population normally distributed)
1.8 SR content effects in iPSC_{D130G-CaM-CMs}

Given the large effects of the calmodulinopathy mutation on CaTs, we examined the SR content of these cells via application of 5 mM caffeine. While the SR content did appear somewhat enhanced in the iPSC_{D130G-CaM-CMs} as compared to their WT counterparts (Online Figure VIII), this increase did not reach a level of statistical significance. This lack of a significant effect may be representative of the relatively immature nature of the iPSC-CMs\textsuperscript{6,7}.

![SR content graph](image)

**Online Figure VIII. SR content of iPSC_{D130G-CaM-CMs}**. No statistical difference was seen in the SR content of iPSC_{D130G-CaM-CMs} (red) as compared to iPSC_{WT-CMs} (gray). SR content was assessed by application of caffeine while imaging the Ca\textsuperscript{2+} sensitive dye Indo-1. Error bars indicate SEM across coverslips (biological replicates).
1.9 Disruption of Ca\textsuperscript{2+}/CaM-dependent inactivation (CDI) of LTCCs at the older differentiation age

In the main text we demonstrated significant disruption of CDI of LTCCs in iPSC\textsubscript{D130G-CaM}-CMs as compared to their WT counterparts (Figure 3). The data was collected 30 days post-differentiation, however comparable results could also be obtained 60 days post-differentiation (Online Figure IX), indicating a stable phenotype of the D130G harboring monolayers.

Online Figure IX. Stability of iPSC-CM LTCC CDI at later time point in culture. A, Exemplar whole-cell current recordings in Ca\textsuperscript{2+} (red) and Ba\textsuperscript{2+} (black) for iPSC\textsubscript{WT}-CMs 60 days post-differentiation. Ba\textsuperscript{2+} current is normalized to Ca\textsuperscript{2+} peak, scale bar corresponds to Ca\textsuperscript{2+}. B, Average normalized current and voltage relationship for iPSC\textsubscript{WT}-CMs (n=7, no technical replicate). Error bars indicate ± SEM throughout. C, Population data for Ca\textsuperscript{2+} (red) and Ba\textsuperscript{2+} (black) for iPSC\textsubscript{WT}-CMs (n=7, no technical replicate), where \( r_{50} \) quantifies the extent of current inactivation across voltages. Red arrow depicts extent of CDI (\( f_{50} \)) at 30-mV test potential here and throughout. D, Exemplar whole-cell current recordings in Ca\textsuperscript{2+} (red) and Ba\textsuperscript{2+} (black) for iPSC\textsubscript{D130G-CaM}-CMs 60 days post-differentiation. Ba\textsuperscript{2+} current is normalized to Ca\textsuperscript{2+} peak, scale bar corresponds to Ca\textsuperscript{2+}. E, There is no significant shift in the current voltage relationship for iPSC\textsubscript{D130G-CaM}-CMs (p>0.05) as compared to WT (B). F, Population data demonstrates a significant decrease in CDI for the iPSC\textsubscript{D130G-CaM}-CMs (n=8, ***p<0.001 compared to WT, corrected for unequal variance). G-I, Alternate D130G clone at 60 days post-differentiation demonstrating the same result as D-F (n=5, ***p<0.001 compared to WT, corrected for unequal variance).
1.10 A pharmacological study of LQT effects in iPSC-CMs

Of the various forms of LQT, only three correspond to disruptions in membrane channels known to associate with calmodulin: LQT1 (loss of function mutations in KCNQ1), LQT3 (gain-of-function mutations in SCN5A), and LQT8 (gain-of-function mutations in CACNA1C). We therefore utilized a pharmacological approach to demonstrate that each of these LQT phenotypes could be recapitulated within our iPSCWT-CMs. Application of the LTCC antagonist BayK 8644 more than doubled the $APD_{80}$ of iPSCWT-CMs (Online Figure X(A)). Such a profound effect on APD is matched in the clinical setting only by LQT8 (Timothy Syndrome) and LQT15 (calmodulinopathies) (Online Figure X(C)), supporting the supposition that the LQT seen in calmodulinopathy patients is due, in large part, to disruption of LTCC channels. Application of maximal doses of $I_{KS}$ blocker, chromanol 293B, or the NaV1.5 channel agonist, ATX II, produced moderate APD prolongation (Online Figure X(A,B)), corresponding to the clinical phenotype of LQT1 and 3 patients (Online Figure X(C)).

Online Figure X. Pharmacological study of APD prolongation in iPSCWT-CMs. A, Exemplar AP recordings of iPSCWT-CMs (gray) in the presence of 10 μmol/L BayK 8644 (left, black), 10μmol/L chromanol 293B (middle, black) or 0.5 μmol/L ATX II (right, black). B, Average $APD_{80}$ values measured for each drug. Error bars indicate ± SEM throughout. C, Comparison of reported QT intervals corresponding to LQT syndromes associated with channels known to interact with CaM.

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<th>Type</th>
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<td>LQT15</td>
<td>CALM2</td>
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A modeling study of calmodulinopathy CDI deficits

We have demonstrated a reduction in LTCC CDI underlying the LQTS phenotype of iPSC-D130G-CaM-CMs (Figure 3). However, it is not possible to rule out the contribution of other CaM targets to the overall phenotype. We therefore considered whether a CDI specific effect of CaM-D130G on the LTCC would be sufficient to produce a calmodulinopathy phenotype. To this end, we utilized a validated and publically available model of an adult mammalian ventricular myocyte (the Luo-Rudy model: LRd)\(^1-4\). With this \textit{in silico} method, we were able to selectively adjust the CDI of the LTCC channel to match the CDI data obtained for this mutation (Figure 3)\(^8\). In addition, this model provides insight into calmodulinopathy associated arrhythmias in an adult myocyte featuring intact EC coupling\(^2,6\). In consideration of the variable expression of mutant CaM expected among calmodulinopathy patients, the model was run with variable fractions of LTCC channels harboring CaM-D130G. The result was APD prolongation which was dependent on expression levels of CaM-D130G in a non-linear manner (Online Figure XI). At low levels of CaM-D130G expression, the APD increased monotonically, however a threshold for severe electrical instability was reached near a ratio of one in six LTCCs harboring a CaM-D130G versus CaM-WT. Importantly, this corresponds to the expected expression levels of mutant CaM among calmodulinopathy patients, where the cardiac expression of a single \textit{CALM} allele was determined to be 0.17 ± 0.02 (\textit{CALM1}), 0.12 ± 0.01 (\textit{CALM2}), and 0.21 ± 0.03 (\textit{CALM3}) based on the average expression levels from five different RNAseq databases\(^5\). Thus calmodulinopathy patients appear to lie directly in the vicinity of the threshold for APD instability, making them highly susceptible to cardiac arrhythmia.

Online Figure XI. Modeling of a non-linear dose dependence of APDs on mutant CaM expression. A, Average APDs determined from an adult mammalian ventricular myocyte model\(^1-4\), plotted as a function of the fraction of LTCCs harboring CaM-D130G (relative to the total number of LTCCs). Data is plotted ±SEM. The gray area indicates electrical instability in the modeled action potentials. The rose shading depicts the range of physiologically relevant expression levels for mutant CaM based on RNAseq databases for normal human gene expression levels\(^5\). B, Exemplar action potentials simulated at 1-Hz pacing corresponding to the colored points in A. Black: no CaM-D130G; Blue: 0.12 CaM-D130G; Red: 0.2 CaM-D130G.
1.12 GRNA screening

Over twenty different gRNAs targeted to \textit{CALM1}, \textit{CALM2} and \textit{CALM3} were screened (Online Figure XII). Design 21 (Kg116) was chosen for treatment of the \textit{CALM2} iPSCD130G-CaM-CMs (Figure 4) as this design demonstrated significant efficacy against \textit{CALM2}, and no appreciable effect against \textit{CALM1} or \textit{CALM3}. Similarly, design 2 was used for selective efficacy against \textit{CALM1} (Figure 5B), and design 8 was utilized for \textit{CALM3} (Figure 5C).

\textbf{Online Figure XII. GRNA screening.} QPCR results indicating mRNA levels of \textit{CALM1-3} in HEK293 cells after CRISPRi treatment using various candidate gRNA sequences. The dashed line at 1 indicates control cells treated with scrambled gRNA. Designs 18-21 (rose) caused a significant decrease in \textit{CALM2} mRNA (n=3, * p<0.05 compared to control) without altering \textit{CALM1} or \textit{CALM3}, while design 2 selectively decreased \textit{CALM1} (n=4, # p<0.05 compared to control) and design 8 selectively decreased \textit{CALM3} (n=6, $ p<0.05$ compared to control). Each biological replicate (n) is an average value of 3 technical replicates/measurements for all data in this figure.
Online Table I: Sequence of candidate gRNAs

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<td>Kg198</td>
<td>GTTTCTACCGCGTTGTCGG</td>
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<tr>
<td>Kg205</td>
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<td>Kg214</td>
<td>GCCGGCGACGTTGTCGG</td>
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<tr>
<td>Kg214R</td>
<td>GCCTGCGACAAACCGTCGC</td>
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<tr>
<td><strong>Target CALM3:</strong></td>
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<tr>
<td>Kg32</td>
<td>GGTCGGAGACAGCGGATCA</td>
</tr>
<tr>
<td>Kg106</td>
<td>GCTCTGAGCGGGCTAAGCG</td>
</tr>
<tr>
<td>Kg198</td>
<td>GTTTCTACCACCGTGTGTCGG</td>
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</table>
Online Table II: reported action potential duration in other control iPSC-CM lines

<table>
<thead>
<tr>
<th>$APD_{90}$ (ms)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>477</td>
<td>Du et al., 2015(^9)</td>
</tr>
<tr>
<td>548</td>
<td>Spencer et al., 2014(^10)</td>
</tr>
<tr>
<td>425</td>
<td>Scheel et al., 2014(^11)</td>
</tr>
<tr>
<td>340</td>
<td>Sinnecker et al., 2013(^12)</td>
</tr>
<tr>
<td>340</td>
<td>Lee et al., 2012(^13)</td>
</tr>
<tr>
<td>286</td>
<td>Lopez-Izquierdo et al., 2014(^14)</td>
</tr>
<tr>
<td>414</td>
<td>Ma et al., 2011(^15)</td>
</tr>
<tr>
<td>392</td>
<td>This study</td>
</tr>
</tbody>
</table>
2. Supplementary Methods

**Flow cytometry**

100 uL of $10^{11}$ PFU/ml of lentivirus containing either ASAP1 or GCaMP6f gene was added to a single well of a 24 well plate, with iPSC-CMs seeded at a density of $2.5 \times 10^5$ cells/well. Flow cytometry was done 7-10 days after the addition of virus using an Accuri C6 flow cytometer (BD Biosciences).

**Sequencing of CALM2 genomic DNA**

Genomic DNA was isolated from iPSCs using DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's protocol. A portion of CALM2 200 base pairs up and downstream from the c.389 A>G mutation was cloned using standard PCR (forward primer: 5'-AGGTGTCACTTGACTTTGGGA-3'; reverse primer: 5'-ATTTCAAGGGGAAGGGTCAGT-3'). Standard Sanger sequencing was performed on the PCR product using the aforementioned forward primer.

**Immunohistochemistry**

Standard fluorescent immunohistochemistry was performed on non-differentiated iPSCs and embryoid bodies. Briefly, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X, and stained with primary, secondary antibodies, and finally counter-stained with DAPI for nuclear visualization. Primary antibodies included (manufacturer, catalog number): polyclonal rabbit anti-Oct4 IgG (AbCaM, ab19857), polyclonal rabbit anti-nanog IgG (AbCaM, ab80892), monoclonal mouse anti-SSEA4 IgG (AbCaM, ab16287), polyclonal rabbit anti-glial fibrillary acidic protein Ig (Dako, Z0334), rabbit polyclonal anti-smooth muscle action IgG (AbCaM, ab5694), and mouse monoclonal anti-a-fetoprotein IgG1 (R&D Systems, MAB1368). Secondary antibodies (all purchased from Invitrogen) include (catalog number): 594 goat anti-mouse IgG (A-11020), 594 donkey anti-rabbit IgG (A-21207), 488 goat anti-mouse IgG (A-31620), and 488 donkey anti-rabbit IgG (A-21206).

**Embryoid body formation analysis**

Embryoid bodies (EBs) were formed using a previously described method. Briefly, iPSCs were enzymatically dissociated using Accutase (Sigma Aldrich) and transferred into low-attachment tissue culture plates filled with EB medium to allow clusters of cells grown in suspension. After 8 days, EBs were transferred onto gelatin-coated treated tissue culture plates. EBs were then collected for analysis after 25 days in culture. EB medium included 20% FBS in knockout DMEM (ThermoFisher Scientific, 10829018) supplemented with 1% L-glutamine, 1% non-essential amino acids, 1% penicillin-streptomycin, and 0.1 mmol/L $\beta$-mercapto ethanol.

**Teratoma formation**

Teratoma formation analysis was performed as previously described. Briefly, 5x10^6 of undifferentiated iPSCs were mixed with Matrigel (BD Biosciences) at a 1:1 ratio and injected intramuscularly into NOD/SCID IL2 receptor gamma chain knockout (NSG) mice. Teratomas were harvested 5-8 weeks post injection, paraffin-embedded, sectioned, and stained with hematoxylin and eosin.
**Western blot for CaM protein quantification**

IPSC-CMs were collected 8-9 days post transduction. Cells were lysed in a Tris-HCl based buffer and a western blot of the collected protein was performed as previously described\(^{18}\). All samples were run in duplicate on 12.5% Tris-HCl precast gels (Bio-Rad) in 25 mmol/L Tris, 192 mmol/L glycine, and 0.1% (wt/vol) SDS running buffer. Primary antibody incubations were performed overnight at 4°C using rabbit monoclonal anti-calmodulin antibody (Abcam, Cat. No. ab45689). Relative band densities were quantified using ImageJ software. The intensity of the bands was normalized by the total amount of protein obtained from a Coomassie stain.

**SR content measurement**

SR content was measured using the ratiometric calcium dye Indo-1 AM. Cells were loaded with 1\(\mu\)mol/L dye for 30 minutes at 37°C, and rinsed 3 times, followed by an additional 10 minutes incubation in Tyrodes at 37 °C to allow for de-esterification of Indo-1 AM. Cells were stimulated for several minutes by application of an electric field across the coverslip using a C-Pace electrical stimulator (Ion Optix). The bath solution was then exchanged for a Na\(^+\)-free Tyrode’s solution (Na\(^+\) was replaced with choline ion to minimize the action of Na-Ca exchanger) with 1.8 mmol/L Ca\(^{2+}\), and 10 mmol/L 2,3-butanedione monoxime. After baseline imaging, SR content was determined by the addition of 5 mmol/L caffeine. Fluorescence was measured using 340-nm excitation and 405- and 485-nm emission wavelengths. The intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) was calculated as 
\[
[Ca^{2+}] = K_{d/Indo} \cdot \beta \cdot (R - R_{min})/(R_{max} - R),
\]
where R is the ratio of fluorescence signal at 405 and 485 nm. \(K_{d/Indo}\) was 800 nmol/L\(^3\). \(R_{min}\) was determined to be 0.33 in a ~ 0 mmol/L Ca\(^{2+}\) Tyrode’s after 30 min incubation with BAPTA-AM. \(R_{max}\) was determined to be 20.85 in a Na\(^+\)-free Tyrode’s (Na\(^+\) was replaced with choline ion to minimize the action of Na-Ca exchanger) with 10 mmol/L Ca\(^{2+}\), 25 \(\mu\)mol/L digitonin and 10 mmol/L 2,3-butanedione monoxime. \(\beta\), as defined by the ratio of fluorescence signal at 485 nM under Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound conditions, was determined to be 0.85.

**Ventricular myocyte model (LRd model)**

The original LRd model\(^2\) was adjusted as previously described such that the duration and shape of the action potential was more similar to human\(^1\). Additional adjustments were made to the LTCC module, primarily substituting a CDI specific deficit expected for calmodulinopathy into a variable fraction of LTCC channels. Equations governing the final LTCC channel pools are as follows:

For the CDI gate (\(f_{CDI}\)):

\[
k_{on} = 4 \cdot 10^3 \text{ mM}^2\text{ms}^{-1} \quad (1)
\]

\[
k_{off} = 1 \cdot (K_{CDI})^2 \cdot k_{on} \text{ ms}^{-1} \quad (2)
\]

\[
df_{CDI} = k_{on} \cdot [Ca] \cdot f_{CDI} + k_{off} \cdot (1 - f_{CDI}) \quad (3)
\]

\[
K_{CDI} = 6.325e^{-4} \text{ mM}
\]
The activation gate ($d$), followed standard Hodgkin and Huxley equations:\(^\text{19}\):

$$dd = \frac{d^{\text{steady state}} - d}{\tau_d}$$ \hspace{1cm} (5)

$$d_1^{\text{steady state}} = \frac{1}{1 + e^{-(V + 14)/(25/2)}}$$ \hspace{1cm} (6)

$$d_2^{\text{steady state}} = \frac{1}{1 + e^{-(V + 14 - 15)/(25/1.5)}}$$ \hspace{1cm} (7)

$$d^{\text{steady state}} = d_1^{\text{steady state}} \cdot d_2^{\text{steady state}}$$ \hspace{1cm} (8)

$$\tau_d = 0.59 + \frac{5}{1 + e^{-3.052(V + 20) + 15}} \cdot \frac{1}{1 + e^{2.052(V + 20) - 75}}$$ \hspace{1cm} (9)

The voltage dependent inactivation gate ($f$):

$$df = \frac{f^{\text{steady state}} - f}{\tau_f}$$ \hspace{1cm} (10)

$$f^{\text{steady state}} = 1 - 0.4 \cdot (1 - \frac{1}{1 + e^{(V + 32)/8}} \cdot \frac{0.8}{1 + e^{(50 - V)/20}})$$ \hspace{1cm} (11)

$$\tau_f = \frac{30}{0.0197 \cdot e^{-0.0337(V + 10)^2 + 0.02}}$$ \hspace{1cm} (12)

With the gates set, the total current through the L-type channel, as well as the Na\(^+\)/Ca\(^{2+}\) exchange current and Ca\(^{2+}\) activated potassium channel was defined as:

$$i_{Ca,\text{Ltype}} = d \cdot f \cdot (f_{CDI} + P_{O}^{\text{modeCa}} (1 - f_{CDI})) \cdot i_{Ca}$$ \hspace{1cm} (13)

$$i_{Na/Ca} = d \cdot f \cdot (f_{CDI} + P_{O}^{\text{modeCa}} (1 - f_{CDI})) \cdot i_{Na}$$ \hspace{1cm} (14)

$$i_{K_{Ca}} = d \cdot f \cdot (f_{CDI} + P_{O}^{\text{modeCa}} (1 - f_{CDI})) \cdot i_{K}$$ \hspace{1cm} (15)

$$P_{O}^{\text{modeCa}} = 0.1$$
Where $P_{O}^{\text{modeCa}}$ represents the relative open probability of the L-type channel in mode $\text{Ca}^{2+}$ as compared to mode 1. $i_{Ca}$, $i_{Na}$ and $i_{K}$ represent the single channel open level as set by the GHK equation.

With the wild type channel in place, a second pool of LTCC channels were added to simulate the effect of CaM$_{D130G}$ on the channel. Properties of this pool of channels were made based on this study and on the previous biophysical characterization of these channels$^8$. This pool of channels was modeled as above, except for the following alterations:

\begin{align*}
    k_{on} &= 1000 \ast 4 \cdot 10^{3} \text{ mM}^2 \text{ms}^{-1} \\
    k_{off} &= 10^{-3} \cdot (K_{CDI})^2 \cdot k_{on} \text{ ms}^{-1} \\
    df_{CDI} &= k_{on} \cdot [Ca]^2 \cdot f_{CDI} + k_{off} \cdot (1 - f_{CDI}) \\
    P_{O}^{D130G,\text{modeCa}} &= 0.95
\end{align*}

All other equations in the model not mentioned here were identical to those described$^1$ and are entirely based on the original 2007 LRd model$^2$. 

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3. Supplementary References


