

ORIGINAL ARTICLE

Genetic Mosaicism in Calmodulinopathy

BACKGROUND: CaM (calmodulin) mutations are associated with congenital arrhythmia susceptibility (calmodulinopathy) and are most often de novo. In this report, we sought to broaden the genotype-phenotype spectrum of calmodulinopathies with 2 novel calmodulin mutations and to investigate mosaicism in 2 affected families.

METHODS: CaM mutations were identified in 4 independent cases by DNA sequencing. Biochemical and electrophysiological studies were performed to determine functional consequences of each mutation.

RESULTS: Genetic studies identified 2 novel CaM variants (*CALM3*-E141K in 2 cases; *CALM1*-E141V) and one previously reported CaM pathogenic variant (*CALM3*-D130G) among 4 probands with shared clinical features of prolonged QTc interval (range 505–725 ms) and documented ventricular arrhythmia. A fatal outcome occurred for 2 of the cases. The parents of all probands were asymptomatic with normal QTc duration. However, 2 of the families had multiple affected offspring or multiple occurrences of intrauterine fetal demise. The mother from the family with recurrent intrauterine fetal demise exhibited the *CALM3*-E141K mutant allele in 25% of next-generation sequencing reads indicating somatic mosaicism, whereas *CALM3*-D130G was present in 6% of captured molecules of the paternal DNA sample, also indicating mosaicism. Two novel mutations (E141K and E141V) impaired Ca²⁺ binding affinity to the C-domain of CaM. Human-induced pluripotent stem cell-derived cardiomyocytes overexpressing mutant or wild-type CaM showed that both mutants impaired Ca²⁺-dependent inactivation of L-type Ca²⁺ channels and prolonged action potential duration.

CONCLUSIONS: We report 2 families with somatic mosaicism associated with arrhythmogenic calmodulinopathy, and demonstrate dysregulation of L-type Ca²⁺ channels by 2 novel CaM mutations affecting the same residue. Parental mosaicism should be suspected in families with unexplained fetal arrhythmia or fetal demise combined with a documented CaM mutation.

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Mutations in the Ca^{2+} sensing protein CaM (calmodulin) are associated with a spectrum of severe congenital arrhythmia susceptibility referred to as calmodulinopathy.^{1–10} A common clinical presentation associated with CaM mutations is long QT syndrome (LQTS), which significantly increases the likelihood of arrhythmic events and sudden cardiac death in the young. Other clinical presentations of calmodulinopathy have included catecholaminergic polymorphic ventricular tachycardia (CPVT), idiopathic ventricular fibrillation, and sudden unexplained death. Most CaM mutations identified to date have been classified as de novo because the mutation is not detectable in the biological parents.

CaM is a ubiquitously expressed protein that exhibits a high degree of evolutionary conservation.¹¹ There is also genomic redundancy within vertebrates as evidenced by the existence of 3 separate CaM genes (*CALM1*, *CALM2*, and *CALM3*) each encoding an identical 149 amino acid polypeptide.¹² CaM is composed of C-terminal and N-terminal domains, each with 2 Ca^{2+} binding EF-hand motifs, which are connected by a flexible linker helix.¹³ Most identified human CaM mutations affect residues within the third and fourth EF-hands in the C-domain, and most replace highly conserved residues essential for Ca^{2+} binding. Previous studies demonstrated that LQTS-associated CaM mutations cause impaired Ca^{2+} binding to the C-domain, dysregulated inactivation of L-type Ca^{2+} channels (LTCC), and prolongation of cardiac action potentials in a variety of cardiac cellular models.^{7,14,15} Elucidating genotype-phenotype correlations and a complete mutation spectrum are expected to improve diagnostic accuracy, and enable stratification of patients for the most appropriate and mechanistically guided therapy.

In this study, we report 2 novel mutations affecting the same amino acid residue in CaM encoded by either *CALM1* or *CALM3*. These cases provide further evidence of genotype-phenotype correlation in LQTS-associated calmodulinopathy with mutations that impair CaM Ca^{2+} binding affinity. Additionally, we report 2 families in which a pathogenic CaM mutation was inherited from a mosaic parent, which we believe are the first documented examples of mosaicism in calmodulinopathy. Recognizing mosaicism has important implications for family counseling in this disorder.

METHODS

All data, analytical methods, and study materials supporting this study are available from the corresponding author on reasonable request for purposes of reproducing the results or replicating the procedures.

The study was approved by institutional review committees, and the participants gave informed consent as follows. Genetic testing and review of medical records of Proband 1 were approved by the Local Ethics Committee of Virgen

de las Nieves University Hospital. Approval for investigating Proband 2 and 4 was obtained from the Unit of Biomedical Ethics Research Committee, King Abdulaziz University. The parents of Proband 3 gave written informed consent for molecular studies and research using a consent document approved by the IRCCS Istituto Auxologico Italiano.

A full description of the methods is provided in the [Data Supplement](#).

RESULTS

Clinical Presentations

Proband 1 (family A) was a Spanish female diagnosed with fetal sinus bradycardia at 16 weeks' gestation. ECG at birth demonstrated profound bradycardia (61 bpm) and 2:1 AV block with markedly prolonged QTc intervals (725 ms; Figure 1A). There were transient episodes of T-wave alternans (Figure 1B). Propranolol was administered at 3 mg/kg. The family history was negative for LQTS and diseases of cardiac conduction, and both parents had normal resting ECG traces (father, 410 ms; mother 422 ms). On the 23rd day of life, the proband had a cardiac arrest. The parents initiated CPR and promptly called emergency services. The initial ECG at the time of resuscitation showed VF, and an external shock (9 joules) restored sinus rhythm (Figure 1C). Treatment with mexiletine (13 mg/kg) and flecainide (5 mg/kg) was initiated, but then stopped when no changes in the QTc interval were observed.

An epicardial cardioverter defibrillator (ICD) was implanted. She experienced 2 appropriate ICD discharges at age 3 years while on propranolol. Echocardiogram demonstrated left atrial dilatation and findings suggestive of left ventricular noncompaction. At age 4 years, the proband developed symptomatic hypoglycemia, and propranolol dose was reduced to 2 mg/kg per day. However, at age 5 years, she had hypoglycemic coma and died. The parents later attempted to have more children. The mother reported 3 spontaneous miscarriages, and they voluntarily interrupted 2 other pregnancies because of fetal bradycardia in one case and spina bifida in the other (Figure 2A). They later conceived 2 healthy sons with normal ECG recordings. DNA from the interrupted pregnancies and the healthy sons was not available for genetic testing.

Proband 2 (family B) is a male offspring of unrelated parents from Saudi Arabia who had bradycardia (45–60 bpm) detected 12 hours after an uncomplicated birth. ECG demonstrated bradycardia and prolonged QTc intervals (660 ms; Figure 1D). He was initially treated with propranolol without change in the QTc duration. Approximately 10 weeks later, a pacemaker was implanted because of syncope and recurrent 2:1 AV block, and he was paced initially at 100 bpm. However, there was failure to capture paced beats attributed to prolonged refractory period. Pacing rate was lowered

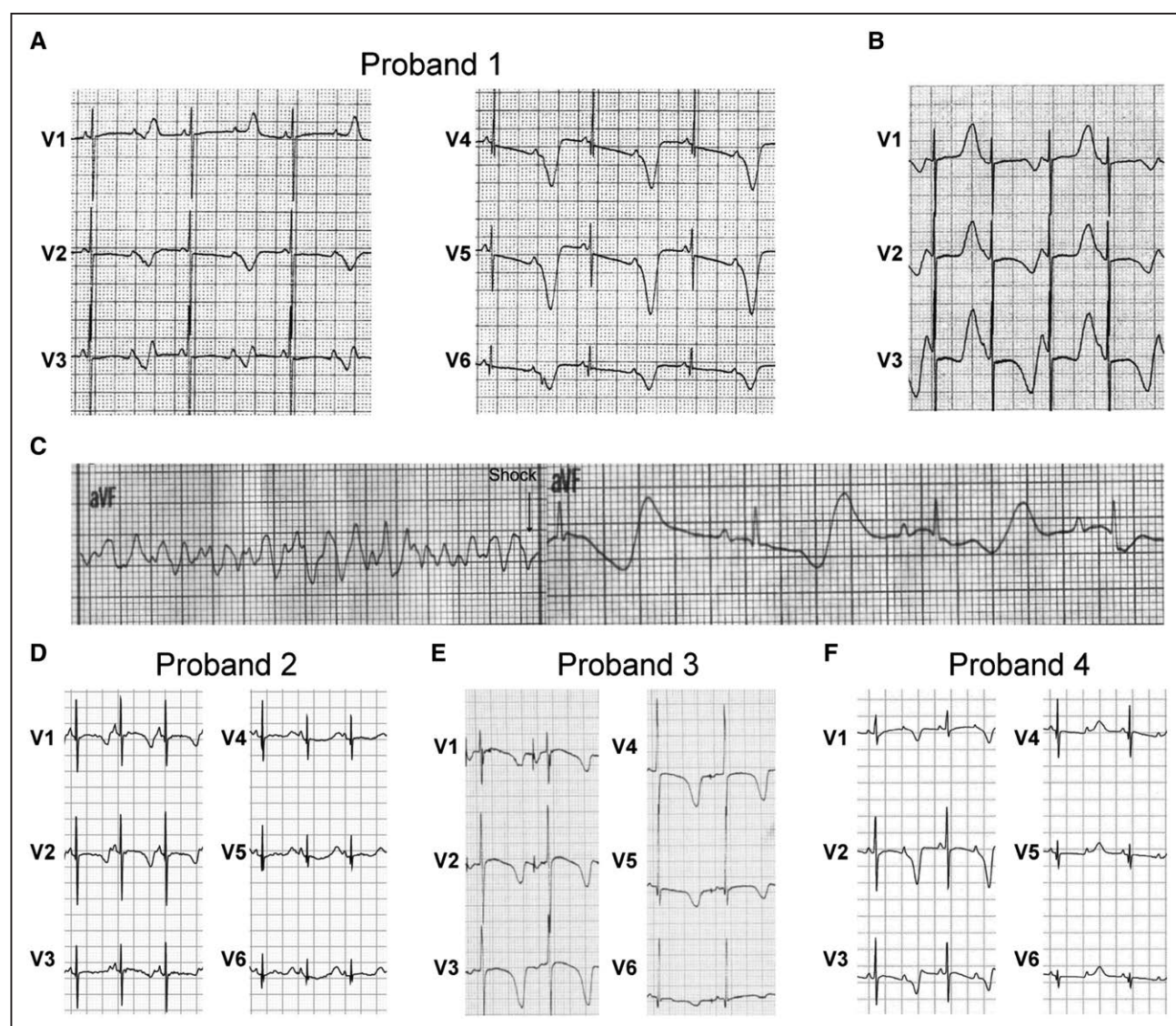


Figure 1. Abnormal electrocardiographic traces from probands.

A. Twelve-lead ECG traces obtained at birth for Proband 1 illustrating sinus bradycardia, a prolonged QTc interval (725 ms), and 2:1 AV block. **B.** An episode of T-wave alternans recorded from Proband 1 is illustrated. **C.** ECG rhythm during resuscitation of Proband 1 from ventricular fibrillation cardiac arrest on the 23rd day of life. **D.** ECG trace recorded at age 2 wk from Proband 2 showing a QTc of 660 ms. **E.** ECG trace recorded at age 18 mo from Proband 3 showing a QTc of 630 ms and inverted T waves throughout the precordial leads. **F.** ECG trace from Proband 4 recorded at age 4 days showing a QTc of 505 ms and 2:1 AV block.

to 60 bpm, propranolol dose was adjusted, and mexiletine was added with resumption of sinus rhythm at an intrinsic rate of 90 bpm. Echocardiogram showed normal systolic function. At 21 months of age, he was clinically well without episodes of syncope. Both parents and a nonidentical twin sister had no symptoms and normal ECGs, including normal QTc duration (Figure 2B).

Proband 3 (family C) is a 7-year-old Maltese boy who suffered a first syncopal episode at 28 days of life. An ECG performed at that time was remarkable for a QTc >500 ms, but the patient was not treated. At 18 months, he suffered a second syncopal episode while breastfeeding. A new ECG at that time demonstrated a QTc of 630 ms and deeply inverted T waves in the

precordial leads. LQTS was diagnosed, and propranolol therapy was started. Echocardiogram showed a structurally normal heart with good biventricular function. One month later, a single chamber ICD was implanted (Figure 1E). During the ensuing 6 months, he had 2 episodes of loss-of-consciousness with detection of ventricular tachycardia/ventricular fibrillation correctly treated with ICD shocks. For 3 months, mexiletine was added to propranolol therapy, but it was not tolerated and was stopped. At age 3 years, propranolol dose was increased to 3 mg/kg per day. Left cardiac sympathetic denervation was considered but deferred because the child was not having further events. The family history was negative for cardiac events, and the QTc of both parents was normal (Figure 2C).

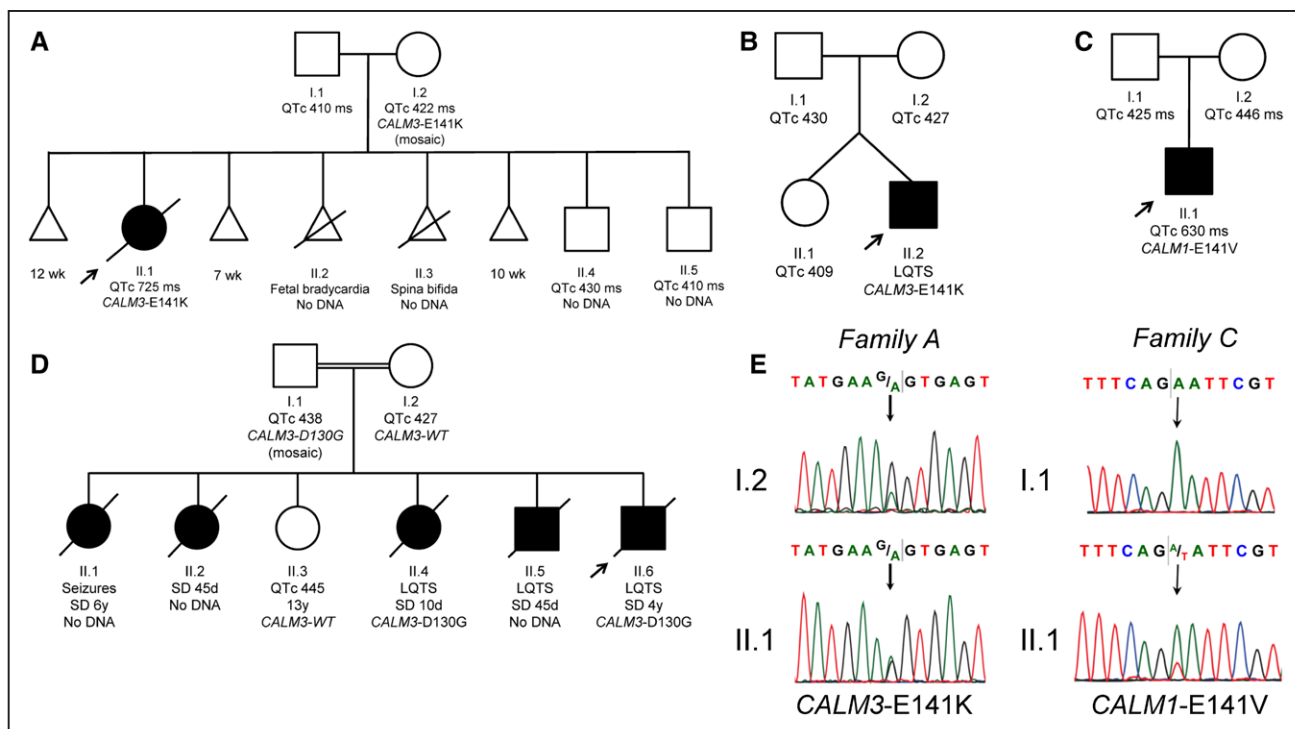


Figure 2. Pedigrees of calmodulinopathy families and discovery of novel CaM (calmodulin) mutations.

A–D, Pedigrees representing the family structures of Probands 1–4. Males are represented as square symbols, females as circles, miscarriages as triangles, and elective abortions as triangles with diagonal lines. The QTc or phenotype, and *CALM3* genotype are given below each pedigree symbol. In Family D, age of sudden death (SD) is given. Individuals unavailable for genotyping are labeled as No DNA. **E,** Nucleotide sequencing traces showing heterozygous mutations in *CALM3* for Proband 1 (Family A, II.1) and the mosaic mother (I.2), and heterozygous mutation in *CALM1* for Proband 2 (Family C, II.1) compared with a normal genotype in the father (I.1). LQTS indicates long QT syndrome.

Proband 4 (family D), the sixth child of consanguineous parents, presented with several episodes of self-terminating torsades de pointes during the first week of life. After hospitalization, he had multiple episodes of ventricular arrhythmia for which he received DC shocks and required mechanical ventilation. He was treated with high dose propranolol, magnesium, lidocaine, and later mexiletine. ECG recorded 4 days after birth demonstrated a prolonged QTc interval (505 ms; Figure 1F) with 2:1 AV block. A pacemaker was implanted during his second week of life, and he became clinically stable. He did well despite a persistent QTc >600 ms on a regimen of propranolol, mexiletine, and pacing at 60 bpm until age 4 years when he died suddenly.

Family history was notable for sudden death in four siblings (Figure 2D). The first sibling had a seizure disorder treated with anticonvulsants starting at age 3 years, then died suddenly at age 6 years. The second

child died suddenly at age 45 days. The third child was asymptomatic at age 13 years. The fourth child had fetal bradycardia and was delivered prematurely at 7 months gestation. After birth, the child exhibited LQTS with 2:1 AV block and died suddenly 10 days after birth following implantation of an ICD. The fifth child also had LQTS with 2:1 AV block detected after birth and died suddenly at age 45 days. There was no family history of deafness. The parents, who are closely related, were healthy with normal ECGs.

A summary of clinical and genetic features of the 4 cases is provided in Table 1.

Discovery of novel CaM Mutations and Parental Mosaicism

Genetic testing of Proband 1 for mutations in *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2*, *KCNJ8*, *CAV3*, and

Table. Clinical and Genetic Features of Probands With Calmodulinopathy

Proband	Mutation	Inheritance	QTc, ms	Sex	Age Onset, d	Phenotype
1	<i>CALM3</i> -E141K	mosaic	725	F	1	Fetal bradycardia, SCA, LQTS, 2:1 AV block
2	<i>CALM3</i> -E141K	de novo	660	M	1	Bradycardia, syncope, LQTS, 2:1 AV block
3	<i>CALM1</i> -E141V	de novo	630	M	28	Syncope, LQTS, VT/VF
4	<i>CALM3</i> -D130G	mosaic	505	M	4	VT/VF, LQTS, 2:1 AV block, SCD

AV indicates atrioventricular; LQTS, long QT syndrome; SCA, sudden cardiac arrest; SCD, sudden cardiac death; and VT/VF, ventricular tachycardia/fibrillation.

SCN4B was negative. Later testing of the 3 calmodulin genes demonstrated a novel heterozygous variant in *CALM3* (c.421G>A; p.Glu141Lys; E141K; Figure 2E). The variant affects the first nucleotide of *CALM3* codon 141 (GAG to AAG), which is the last base pair of exon 5. The variant was absent in the father and was not observed in the Genome Aggregation Database (gnomad.broadinstitute.org). However, the mother carried the *CALM3* variant in peripheral blood cells, but only in $\approx 25\%$ of next-generation sequencing reads, which is consistent with somatic mosaicism and presumed germline mosaicism due to transmission of the variant. The mother's basal and exercise ECG traces were normal, and she never experienced an arrhythmic event.

Proband 2 was also discovered to be heterozygous for *CALM3*-E141K. The variant was absent in both parents and his unaffected, nonidentical twin sister consistent with a de novo mutation. Genetic testing of Proband 3 revealed a novel heterozygous nonsynonymous variant in *CALM1* (c.422A>T; p.Glu141Val; E141V; Figure 2E), which was absent in both parents consistent with a de novo mutation. This variant affects the second nucleotide of *CALM1* codon 141 (GAA to GTA, which is the first base pair of exon 6). The variant was not observed in the Genome Aggregation Database.

Initial screening of *KCNQ1* and *KCNH2* genes in Proband 4 revealed no mutations. Subsequent exome sequencing demonstrated a previously reported heterozygous *CALM3* mutation (c.389A>G; p.Asp130Gly, D130G).¹⁶ This mutation was confirmed by Sanger sequencing and also demonstrated in the deceased fourth sibling, but neither parent was found to have the variant by exome sequencing (read depth at this nucleotide position was 43–46x). Other variants in other known arrhythmia susceptibility genes (*SCN5A*, *ANK2*, and *AKAP9*) or paralogs (*ANK3*) discovered by exome sequencing did not segregate with the phenotype (Table SI in the [Data Supplement](#)). Because of recurrence of this mutation in the family, we investigated the parents and healthy sibling for evidence of mosaicism using single-molecule molecular inversion probes,¹⁷ which offer the advantage over other next-generation sequencing approaches by use of a unique molecular identifier to detect mosaicism with greater sensitivity. We detected the c.389A>G variant in Proband 4 at constitutive levels (minor allele frequency 0.5), and this variant was absent in the mother and unaffected sister (II.3). However, we detected the c.389A>G variant in 3/84, 5/89, and 5/63 discrete triplicate captures (molecules) in paternal DNA, correlating with a minor allele frequency of 0.056 (range 0.036–0.079). This result is consistent with somatic mosaicism in the father. Due to transmission of this variant to the affected children, germline mosaicism is assumed.

Novel CaM Mutants Impair Ca²⁺ Binding Affinity

Amino acid sequence alignments illustrate that the novel mutations E141K and E141V affect a strictly conserved glutamate residue within the fourth Ca²⁺ binding EF-hand motif of the CaM C-domain (Figure 3A). Because the glutamate side chain directly chelates the Ca²⁺ ion in this site, we investigated whether E141K and E141V impair Ca²⁺ affinity using a previously described fluorescence assay. Inspection of raw Ca²⁺ binding data revealed diminished amplitude of fluorescence change for E141K and E141V relative to wild-type (WT) CaM, which suggests the mutated C-domain binds Ca²⁺ ions weakly (Figure 3B). The Ca²⁺ binding affinities (K_d) for WT, E141K, and E141V CaM C-domain determined from normalized binding data were 2.3 ± 0.2 $\mu\text{mol/L}$, 75 ± 7 $\mu\text{mol/L}$, and 54 ± 4 $\mu\text{mol/L}$, respectively (Figure 3C). There were no significant differences in the Ca²⁺ affinities of the CaM N-domain (Figure SIA in the [Data Supplement](#)). A previous report demonstrated that CaM-E141G also impairs C-domain Ca²⁺ binding affinity to a lesser extent (K_d = 27 ± 0.5 $\mu\text{mol/L}$).⁴

We investigated the conformational changes associated with Ca²⁺ binding in more detail for the E141V and E141K mutations. Two-dimensional 15N-1H nuclear magnetic resonance spectra show that in the absence of Ca²⁺, E141V and E141K protein backbones adopt nearly identical folded structures that are similar to WT apo-CaM (Figure SIB in the [Data Supplement](#)). In the presence of Ca²⁺, many of the signals arising from residues in the CaM C-domain are reduced in intensity or significantly shifted, indicating that Ca²⁺ binding is impaired in mutated proteins (Figure SIC and SID in the [Data Supplement](#)). Our results are consistent with previous studies of a mutation of the corresponding strictly conserved glutamate residue in position 12 of the EF-hand Ca²⁺ binding loop in calbindin D_{9k}, which showed that the mutated EF-hand is unable to attain the conformation of the WT EF-hand when a Ca²⁺ ion is fully engaged.¹⁸

Novel CaM Mutants Affect Ca²⁺-Dependent Inactivation of LTCC

A primary mechanism proposed for calmodulinopathy-associated LQTS is dysregulation of LTCC secondary to impaired Ca²⁺-dependent inactivation (CDI).^{7,14} To determine the effect of E141K and E141V on CDI, we recorded endogenous L-type Ca²⁺ currents in human induced pluripotent stem cell derived cardiomyocyte (iPSC-CM) transiently transfected with WT or mutant CaM. Figure 4A illustrates representative Ca²⁺ current traces evoked by 100 ms depolarizing voltage steps from -80 to 60 mV recorded from human iPSC-CM expressing WT or mutant CaM. There are obvious dif-

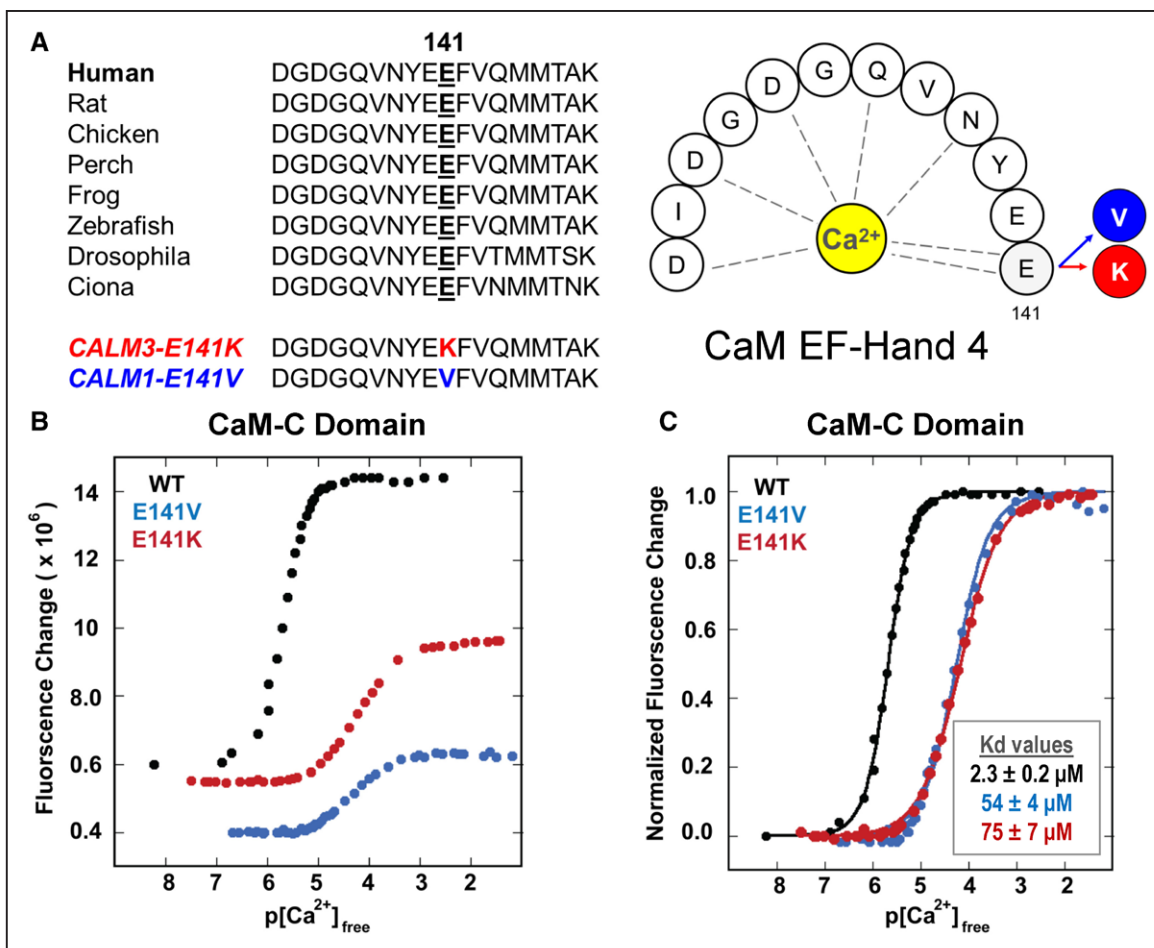


Figure 3. E141K and E141V reduce Ca^{2+} binding affinity of the calmodulin C-lobe.

A, Amino acid sequence alignments comparing a segment of the CaM (calmodulin) C-domain from different species (**left**) and a schematic of the fourth EF-hand motif showing the location of the E141K and E141V mutations (**right**). **B**, An overlay of wild-type (black), E141K (red), and E141V (blue) fluorescence change during Ca^{2+} titration for the CaM C-domain. **C**, Normalized fluorescence change for Ca^{2+} titration experiments. The Ca^{2+} affinities (K_d) for the CaM C-domain are provided in the inset.

ferences in the time course and extent of Ca^{2+} current decay over time indicating that channel inactivation is slower and less complete for both E141K and E141V expressing cells. Current-voltage plots further demonstrate significant differences in peak (Figure 4B) and late (Figure 4C) Ca^{2+} currents in cells expressing mutant compared with WT CaM.

Figure 5A illustrates averaged normalized Ca^{2+} and Ba^{2+} current traces recorded at +10 mV from cardiomyocytes transfected with WT or mutant CaM. The WT CaM-expressing cells exhibit discordant time courses of current decay between Ca^{2+} and Ba^{2+} current traces indicative of CDI, which is blunted substantially in cells transfected with E141K or E141V. Cells expressing either E141K or E141V exhibit significantly larger inactivation time constants than cells expressing WT CaM when Ca^{2+} , but not Ba^{2+} , is the permeating ion (Figure S2 in the [Data Supplement](#)). Impaired CDI is also evident in comparisons of Ca^{2+} current recorded at 100 ms (I_{end}) and normalized to the peak current (I_{peak}). Larger $I_{\text{end}}/I_{\text{peak}}$ ratios indicate impaired CDI. The $I_{\text{end}}/I_{\text{peak}}$ ratios

are significantly larger for E141K and E141V compared with WT expressing cells across all tested voltages (Figure 5B). These results indicate that E141K and E141V cause impaired CDI.

Effect of Novel CaM Mutants on the Cardiac Action Potential

We investigated the effect of E141K and E141V on action potential (AP) morphology and dynamics exhibited by recombinant CaM-transfected iPSC-CMs. Representative spontaneous action potential traces show overt differences in AP duration (APD) in cells expressing mutant CaM (Figure 6A). Specifically, the APD_{90} and APD_{50} were significantly longer for E141K and E141V transfected iPSC-CM compared with WT expressing cells (Figure 6B and 6C). Further, the proportional effect on APD_{50} was greater as reflected in a significantly smaller $\text{APD}_{90}/\text{APD}_{50}$ ratio for iPSC-CM transfected with mutant CaM compared with WT CaM-expressing cells (Figure 6D). This finding indicates prolongation

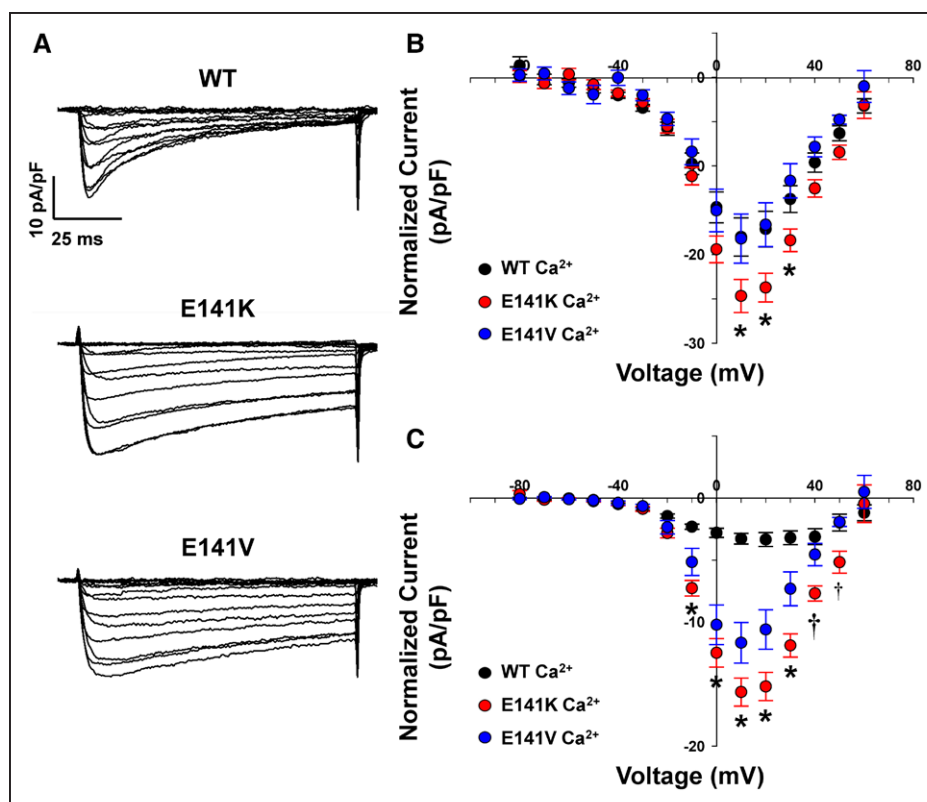


Figure 4. Effect of E141K and E141V CaM (calmodulin) on induced pluripotent stem cell derived cardiomyocyte (iPSC-CM) Ca^{2+} current.

A, Average traces of Ca^{2+} current recorded from iPSC-CMs transfected with CaM-WT (top), CaM-E141K (middle), or CaM-E141V (bottom). **B**, Current density vs voltage plot comparing the peak Ca^{2+} current of WT, E141K, and E141V expressing cells (* $P < 0.05$, E141K vs WT). **C**, Current density vs voltage plot comparing the late Ca^{2+} current recorded 100 ms after each voltage step (* $P < 0.05$, both mutants vs WT; † $P < 0.05$, E141K vs WT). Data symbols represent mean values of WT ($n = 17$), E141K ($n = 10$), and E141V ($n = 8$) expressing cells and error bars are SEM.

of phase 2 repolarization where L-type Ca^{2+} current is prominent. The peak AP amplitude and the rate of rise (dV/dT) were significantly different in cells expressing E141K or E141V compared with WT expressing cells (Figure S3A and S3B in the [Data Supplement](#)). There was no difference between groups in maximum diastolic potential (Figure S3C in the [Data Supplement](#)) or in the frequency of spontaneous AP firing (Figure S3D in the [Data Supplement](#)). These results demonstrate that both E141K and E141V evoke prolonged action potential duration consistent with a cellular arrhythmogenic substrate underlying clinical LQTS.

DISCUSSION

In this study, we report 2 novel CaM mutations that affect the same amino acid residue in CaM but occur in 2 different genes. Both *CALM3*-E141K and *CALM1*-E141V cause impaired C-domain Ca^{2+} binding in recombinant CaM proteins, and cause a pattern of LTCC dysfunction consistent with impaired CDI. This study provides further evidence of the effect of CaM dysfunction on human cardiomyocyte electrophysiology, and supports dysregulation of LTCC as a pathogenic mechanism in calmodulinopathy presenting with

LQTS. Importantly, we also report evidence of parental somatic mosaicism in the families with *CALM3*-E141K and *CALM3*-D130G, and these findings have important implications for genetic counseling in the setting of unexplained fetal demise or multiple sudden unexplained deaths of young children in families with a pathogenic CaM mutation.

Clinical Aspects of Calmodulinopathy-Associated LQTS

CaM mutations associated with severe forms of LQTS potentiate the risk of sudden cardiac death in the young.^{3,4,7,8,16} In a recent study, the average age at diagnosis of calmodulinopathy-associated LQTS was 0.8 years compared with 23 years in LQTS patients without CaM mutations.⁴ In that same study, the average QTc interval duration was significantly longer in CaM mutation-positive patients (676 ms) compared with CaM mutation-negative patients (514 ms), and the prevalence of sudden cardiac arrest associated with calmodulinopathy was also significantly higher.

The 2 novel mutations we discovered affect the same residue of CaM (E141), and a third mutation at this position (*CALM1*-E141G) was previously reported.⁴ All 3

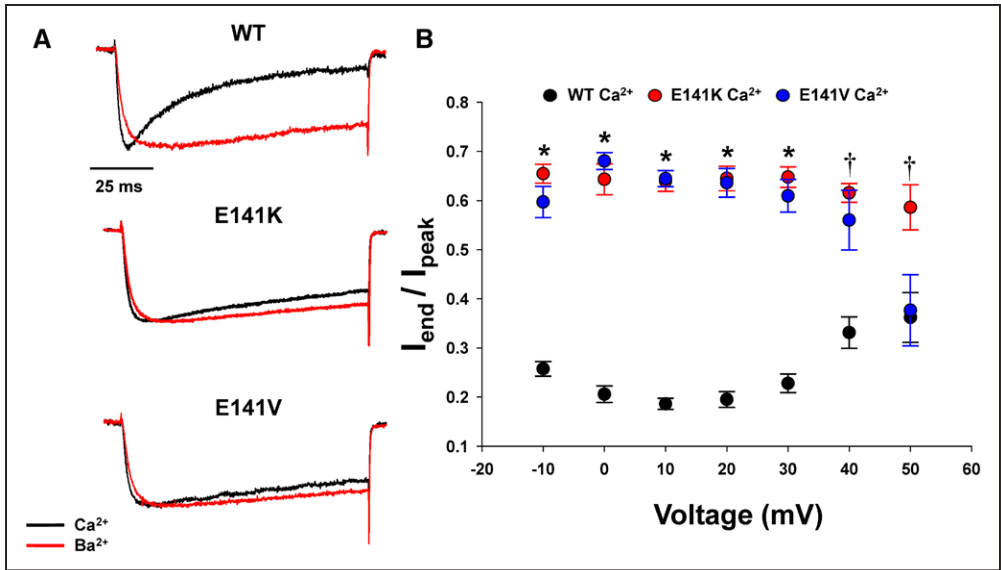


Figure 5. E141K and E141V impair Ca²⁺-dependent inactivation. **A**, Average traces of Ca²⁺ (black) and Ba²⁺ (red) current recorded at +10 mV from iPSC-CMs transfected with CaM-WT (top), CaM-E141K (middle), or CaM-E141V (bottom). **B**, Plots of I_{end}/I_{peak} for test voltages between -10 to +50 mV (**P*<0.05, both mutants vs WT; †*P*<0.05, E141K vs WT). Data symbols represent mean values of WT (n=17), E141K (n=10), and E141V (n=8) and error bars are SEM.

substitutions of residue E141 are associated with severe LQTS. In Proband 1 and 2, who were both carriers of CALM3-E141K, the severity of LQTS based on QTc duration was more severe than Proband 3 (CALM1-E141V) and the published case with CALM1-E141G. Conceivably, the E141K mutation displays a more severe clinical

phenotype than E141G or E141V because of side group charge differences in the mutant residue. Lysine substitution (E141K) introduces a positively charged residue that more severely disrupts Ca²⁺ binding affinity of the EF-hand motif compared to neutral glycine (E141G) and valine (E141V) substitutions (Figure 3), which can

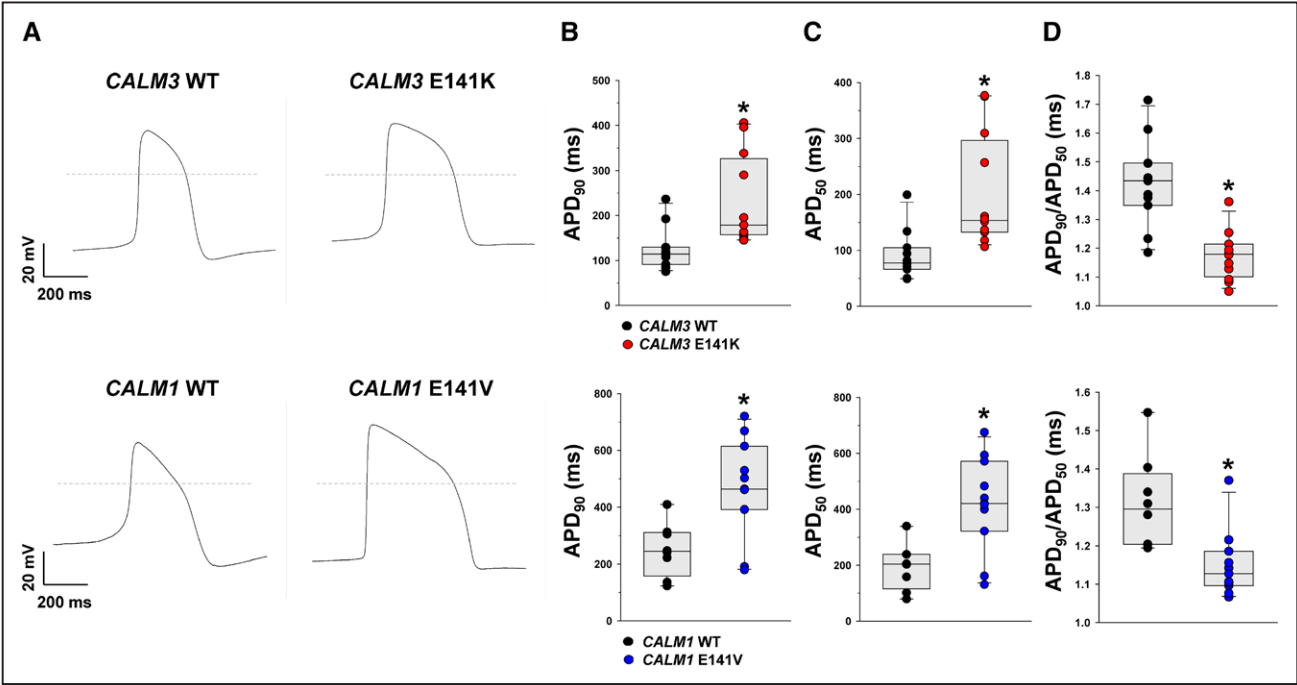


Figure 6. E141K and E141V prolong the action potential duration in induced pluripotent stem cell derived cardiomyocyte (iPSC-CM). **A**, Representative traces of action potentials recorded from human iPSC-CMs transfected with CALM3 WT or E141K (top), or with CALM1 WT or E141V (bottom). **B**, Box plots of action potential duration at 90% repolarization (APD₉₀) of spontaneous action potentials (Aps) in cells expressing E141K or E141V compared with WT expressing cells. The vertical height of each box plot represents the 25th to 75th percentile, the solid black line within the box marks the median, and the mean value is indicated by the dashed line with the box. Whiskers (error bars) above and below the box indicate the 95th and fifth percentiles, respectively. All data points are plotted. **C**, Box plots of action potential duration at 50% repolarization (APD₅₀) of spontaneous APs in cells expressing E141K or E141V compared with WT expressing cells. **D**, Box plots depicting ratios of APD₉₀ to APD₅₀ determined for spontaneous AP (**P*<0.05, comparing mutant to WT).

be attributed to the more significant change in electrostatic interactions driving the binding of Ca^{2+} ions.

A potential novel clinical aspect of Proband 1 was the occurrence of hypoglycemia, which was the eventual cause of death. Although hypoglycemia has been reported in association with propranolol therapy in children, it is conceivable that altered LTCC function in pancreatic β -cells as a consequence of the CaM mutation may have promoted hyperinsulinemia. A similar mechanism might explain hypoglycemia in Timothy syndrome,¹⁹ which is caused by gain-of-function Ca^{2+} channel mutations.^{20,21}

Proband 1 had documented left ventricle noncompaction, an anatomic abnormality of the ventricle wall observed in a variety of genetic and acquired conditions as well as occasionally in healthy persons with normal heart size and function.²² There have been 4 previous reports of calmodulinopathy accompanied by left ventricle noncompaction, all associated with *CALM2* mutations (D96V, D96G, D132E, and D132H).^{3,7,8,23} At the present time, there is insufficient evidence to suggest that left ventricle noncompaction is a direct or indirect consequence of CaM mutation. However, there have been prior associations with other genetic arrhythmia syndromes including CPVT associated with specific types of *RYR2* mutations (exon 3 deletion).^{24,25} Interestingly, in one case, left ventricle noncompaction developed subsequent to the worsening of cardiac arrhythmia.²⁵ It is conceivable that severe or progressive arrhythmic disorders associated with dysregulation of intracellular Ca^{2+} may predispose to this anomaly.

Cellular Aspects of Calmodulinopathy-Associated LQTS

In our study, we used human iPSC-CM as a cellular model to determine the effect of E141K and E141V mutants on LTCC function and action potential morphology. Human iPSC-CM express endogenous LTCC and may have advantages over heterologous cellular platforms that require transfection of multiple subunit-encoding plasmids. This cellular model allows us to record Ca^{2+} currents and study action potential characteristics of cells expressing CaM mutations with a single plasmid transfection. Using this system, we demonstrated impaired Ca^{2+} -dependent regulation of LTCC inactivation, consistent with the cellular mechanism proposed for calmodulinopathy-associated LQTS.^{7,14,15} Human iPSC-CM also have utility as a cellular model to assess potential therapeutic strategies including gene therapies and targeted antiarrhythmic drugs.^{15,26–28}

In addition to LTCC dysfunction, other cellular targets of CaM have been investigated to explain the pathogenesis of calmodulinopathies. CaM mutations associated with CPVT are known to deregulate the *RYR2* channel and cause arrhythmogenic spontaneous Ca^{2+}

release.^{9,29,30} Because our cases lacked overt features of CPVT, we did not pursue investigations of *RYR2* function. Additional evidence supporting CaM-mediated dysregulation of the cardiac sodium channel ($\text{Na}_v1.5$) and the small-conductance Ca^{2+} -activated potassium channel (SK) channel has also been presented.³¹ Enhanced $\text{Na}_v1.5$ late current was observed in heterologous cells transfected with the CaM mutant E141G,⁴ but prior studies in cultured murine cardiomyocytes did not demonstrate effects on sodium current.³²

Mosaicism in Calmodulinopathy

An important and unique aspect of our study was the demonstration of parental mosaicism in 2 families. Mosaicism is an established genetic phenomenon that can explain recurrent mutation-positive offspring of apparently mutation-negative parents, including in families with LQTS.³³ The demonstration of mosaicism in our study was accomplished by deep resequencing of the mutant gene using next generation sequencing technologies. In the family of Proband 1 (Figure 2A), a mosaic mother carried the pathogenic variant (*CALM3*-p.E141K) in $\approx 25\%$ of next generation sequencing reads of DNA from peripheral blood cells. A similar degree of germline mosaicism would be consistent with the observed recurrent intrauterine fetal demise secondary to fetal calmodulinopathy in this family. However, this level of mosaicism in the heart would likely give rise to overt disease, and we speculate that cardiomyocytes exhibit a lower level of mosaicism to explain the absence of a maternal phenotype. By contrast, the family of Proband 4 (Figure 2D) exhibits a high degree of recurrent cardiac arrhythmia among offspring that we presume is secondary to transmission of *CALM3*-p.D130G from the mosaic father, but in this case the proportion of mutation-positive next generation sequencing reads from peripheral blood was only $\approx 6\%$. This degree of somatic mosaicism seems at odds with the high rate of disease recurrence among offspring in the family of Proband 4 suggesting that the proportion of mutation-positive peripheral blood cells may not reflect the level of mosaicism in the father's germline. We can also speculate that either the mutation was selectively amplified in the father's germline as was demonstrated previously for *FGFR2* mutations, or that sperm carrying the mutation had a selective advantage for fertilization.^{34,35}

CONCLUSIONS

CaM mutations are associated with a diversity of heart rhythm disorders, including LQTS, CPVT, idiopathic ventricular tachycardia, and sudden death. We report novel mutations associated with the LQTS and sudden death phenotypes, and demonstrated parental somatic mosaicism in 2 families. Our findings expand the mutational

spectrum and add to the emerging genotype-phenotype correlation in this severe congenital arrhythmia syndrome.

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