

Genetic Mosaicism in Calmodulinopathy

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SUPPLEMENTAL INFORMATION

ONLINE METHODS

Mutation discovery and detection of mosaicism

For Proband 1, Sanger sequencing of all exons and flanking intronic regions was performed of the following genes: *KCNE1*, *KCNE2*, *KCNQ1*, *KCNH2*, *SCN5A*, *SCN4B*, *CAV3*, *CACNA1C*, *CALM1*, *CALM2*, and *CALM3*. Targeted sequencing of the *CALM3* exon 5 and its flanking intronic regions was also performed in the parents of Proband 1. For Proband 2, clinical genetic testing revealed a *CALM3* mutation, which was confirmed by Sanger sequencing in the cardiogenetics research unit of University Hospital Lausanne. For Proband 3, genetic testing of *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1* and *KCNE2* using next-generation sequencing was negative. Subsequent Sanger sequencing of *CALM1*, *CALM2*, and *CALM3* was performed in the proband, and then targeted sequencing of *CALM1* was performed in the parents. For Proband 4, initial Sanger sequencing of *KCNQ1* and *KCNH2* performed at the Princess Al jawhara Center of Excellence in Research of Hereditary Disorders, King Abdulaziz University was negative for mutations. Exome sequencing of Proband 4 was performed in the Genome Centre, National University of Singapore using NimbleGen SeqCap EZ Exome Library 3.0 analyzed on an

Illumina HiSeq2500. Validation of suspected mutations discovered by exome sequencing was performed by the cardiogenetics research unit of University Hospital Lausanne.

Mosaicism was investigated in the family of Proband 1 using next-generation sequencing. Briefly, oligonucleotide primers (forward: CACTGCTGAGGGATGGTGAT; reverse: GCAGGGGAGTGTGAAGAGA) were used for PCR amplification of *CALM3* exons 5 and 6 from genomic DNA. The resulting 485 bp amplicon was fragmented with a Covaris E220 sonicator and prepared for sequencing using the NEXTflex™ Rapid DNA Sequencing system (Bioo Scientific, Austin TX), following the manufacturer's instructions. The resulting libraries were sequenced (2 x 100 bp paired-end) on a HiSeq 1500 instrument (Illumina, San Diego, CA). Base calling, read filtering, and demultiplexing were performed with the Illumina processing pipeline (CASAVA 1.8.2). Sequence read pairs were mapped to the human genome assembly GRh37/hg19 (build 37.2, February 2009) using NovoAlign (version 3.02.07). Variant calls were generated using the Genome Analysis Toolkit.

Mosaicism in the family of Proband 4 was investigated using four single molecule molecular inversion probes (smMIPs; sequences available upon request) that encompassed the pathogenic variant to a minimum of 50 discrete captures in triplicate. smMIP libraries were prepared as described previously,¹ and sequenced using a paired-end 150bp protocol on an Illumina MiniSeq system. Base calling and raw reads filtering were performed using the Illumina processing pipeline. Reads were mapped to the human genome (GRh37/hg19) using BWA (version 0.7.12) and processed using custom python scripts and pipelines.¹ Variants were called using GATK (version 3.7-0) and visualized using Integrated Genome Viewer (IGV; version 2.4.19).

All CaM mutations reported in this paper have been included in the International Calmodulinopathy registry as described by Crotti, et al.²

Measurement of Ca²⁺ binding affinity

The methods for producing wild-type and mutant recombinant CaM proteins, and measuring Ca²⁺ affinity were described previously.^{3,4} Briefly, Ca²⁺ ion binding to WT, E141K, and E141V CaM was assessed using standard fluorescence-based methods.⁵ Two-dimensional ¹⁵N-¹H nuclear magnetic resonance (NMR) spectroscopy was used to monitor structural perturbations in the mutated CaM protein, both in the absence and presence (25:1 or 75:1, Ca²⁺:CaM) of Ca²⁺.

Human iPSC culture and cardiomyocyte differentiation

Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) were derived as previously described.⁶ Briefly, hiPSCs were maintained in E8 medium on low-density (1:400) growth factor-reduced Matrigel (Corning, Corning, NY) and passaged every 4 days at 65-85% confluency. hiPSC were differentiated into cardiomyocytes using a GSK3B inhibitor and WNT inhibitor 2 days later. The culture medium used for cardiac differentiation and hiPSC-CM maintenance was CDM3.⁶ At differentiation day 22 to 26, cells were dissociated with Liberase TH (0.13 units/ml; Roche, Indianapolis, IN), then plated onto 15 mm glass coverslips coated with a 1:800 dilution of Cultrex basement membrane matrix (Trevigen, Gaithersburg, MD). Media was changed every 48 hours after plating.

Plasmids and transfections

Human *CALM1* and *CALM3* open reading frame cDNAs were synthesized by a commercial vendor (ATUM, Newark, CA), and subcloned into the pIRES2-eGFP vector (BD Biosciences,

Mountain View, CA). Site-directed mutagenesis was performed to generate *CALM1*-E141V and *CALM3*-E141K using the QuikChange system (Agilent Technologies, Santa Clara CA). Mutagenic primer sequences are available upon request. All constructs were sequenced to verify the engineered mutation and to exclude inadvertent polymerase or cloning errors. Transfection of iPSC-derived cardiomyocytes (4 - 14 days after plating) with WT or mutant CaM plasmids was performed using TransIT-LT1 reagent (Mirus Bio, Madison, WI) according to the supplier's protocol. Transfected Cells were used for electrophysiological experiments 36-72 hours post transfection. Green fluorescent cells were selected for patch-clamp recording.

Electrophysiology

Glass electrodes were pulled using a Model P-1000 micropipette puller (Sutter Instruments, Novato, CA) and flame-polished for a final resistance of 1-2 MΩ. Currents carried by Ca²⁺ or Ba²⁺ were recorded at room temperature in the whole-cell configuration of the patch clamp technique, filtered at 5 kHz and leak subtracted using a P/4 method. Data were acquired using an Axopatch 200B amplifier and Clampex 10.3 software (Molecular Devices, San Jose, CA). For whole-cell voltage-clamp recordings, the holding membrane potential was -80 mV and measurements were made at room temperature. Ca²⁺ current was recorded first followed by the measurement of Ba²⁺ current. The bath solution contained (in mM): 150 Tris, 10 glucose, 1 MgCl₂, and 10 CaCl₂ or 10 BaCl₂. The pH was adjusted to pH 7.4 with methanesulfonic acid, and the osmolality of the bath solution was adjusted to 340 mOsm/Kg with sucrose. The pipette solution contained (in mM): 135 CsCl, 10 EGTA, 1 MgCl₂, 10 HEPES, and 4 Mg-ATP with the pH adjusted to 7.3 with CsOH, and the osmolality was adjusted to 310 mOsm/Kg with sucrose.

For whole-cell current-clamp recordings, data were acquired using an Axopatch 700B amplifier and Clampex 10.3 software (Molecular Devices). Spontaneous action potentials were recorded at 31°C. The bath solution contained (in mM): 1.8 CaCl₂, 15 glucose, 15 HEPES, 5.4 KCl, 1 mM

MgCl₂, 150 NaCl, and 1 Na-pyruvate adjusted to pH 7.4 and an osmolality of 340 mOsm/Kg. The pipette solution contained (in mM): 2 CaCl₂, 5 EGTA, 10 HEPES, 150 KCl, 5 MgATP, and 5 NaCl adjusted to pH 7.2 and an osmolality of 320 mOsm/Kg.

Statistical analysis was performed with the SigmaPlot 12.5 software (Systat, San Jose, CA) using a 2-way analysis of variance (ANOVA) and Holm-Sidak multiple comparisons method.

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Supplemental Table S1 – Other variants in arrhythmia susceptibility genes or paralogs discovered by exome sequencing in the family of Proband 4.

Individual	Phenotype	<i>CALM3</i>	<i>SCN5A</i>	<i>ANK2</i>	<i>ANK3</i>	<i>AKAP9</i>
I.1	Unaffected	-	p.R18W / WT*	p.E1550Q / WT	p.P2490L / WT	-
I.2	Unaffected	-	-	-	p.P2490L / WT	p.V1790G / WT
II.3	Unaffected	-	-	p.E1550Q / WT	p.P2490L / p.P2490L	p.V1790G / WT
II.4	Affected	p.D130G / WT	-	p.E1550Q / WT	p.P2490L / WT	-
II.5	Affected	p.D130G / WT	p.R18W / WT	p.E1550Q / WT	p.P2490L / p.P2490L	p.V1790G / WT

* WT = wildtype allele; hyphens indicate homozygosity for wildtype allele.

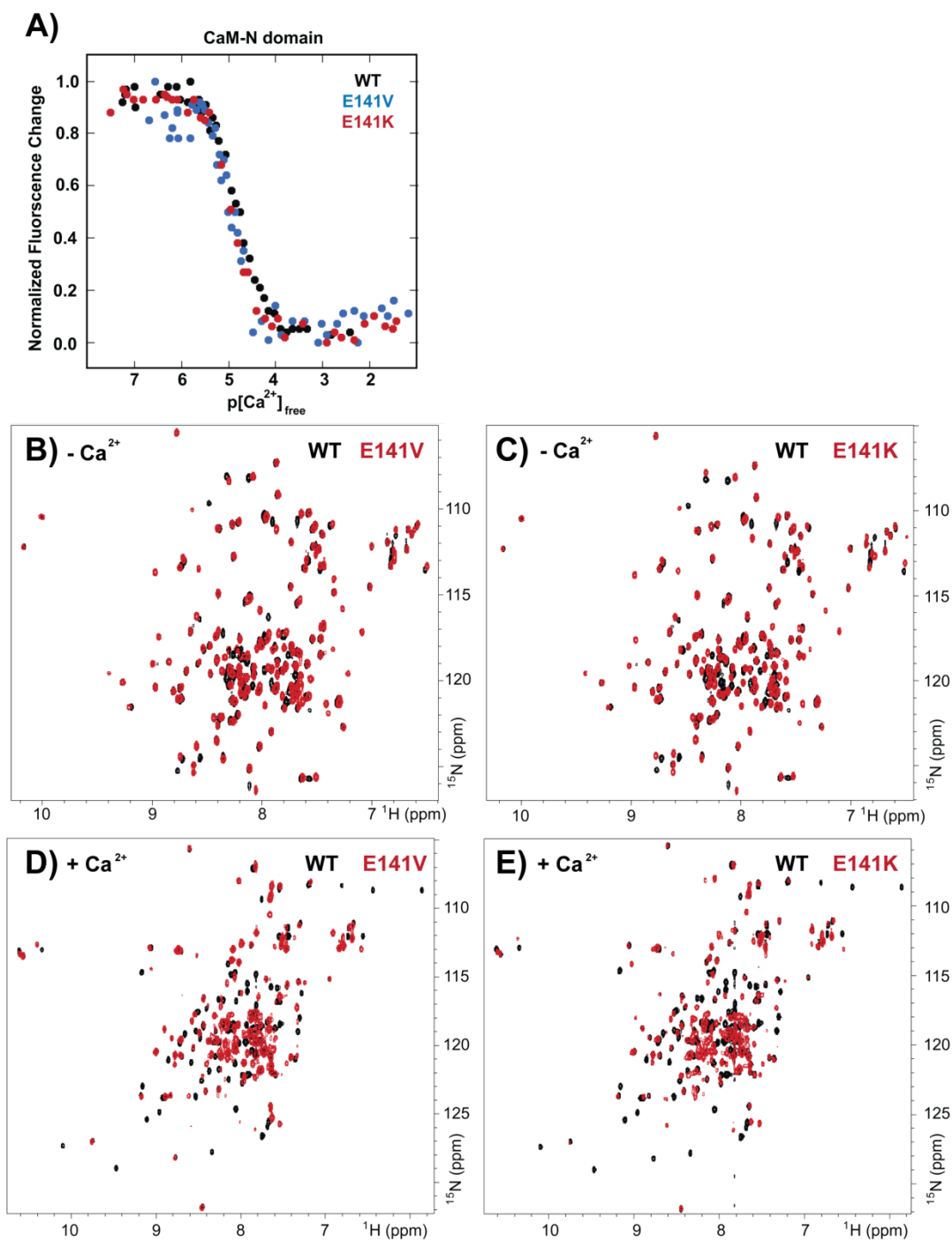


Fig. S1: CaM-N domain Ca^{2+} binding data and NMR of E141V and E141K CaM in the absence and presence of Ca^{2+} . **A.** Overlay of WT (*black*), E141K (*red*), and E141V (*blue*) normalized fluorescence of the CaM-N domain during a Ca^{2+} titration. Neither mutation causes a significant difference to the CaM-N domain Ca^{2+} binding affinity. Overlay of 2D ^{15}N - 1H HSQC NMR spectra. **B.** E141V apo CaM (*red*) and WT apo CaM (*black*) **C.** E141K apo CaM (*red*) and WT apo CaM (*black*) **D.** E141V Ca^{2+} CaM (*red*) and WT Ca^{2+} CaM (*black*) **E.** E141K Ca^{2+} CaM (*red*) and WT Ca^{2+} CaM (*black*).

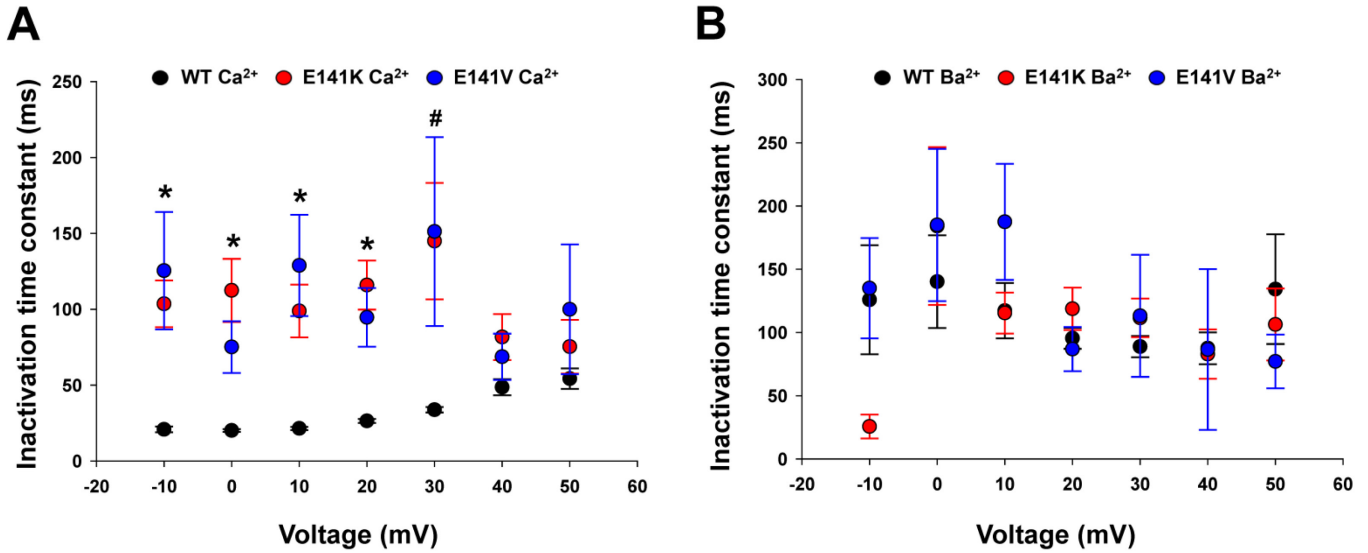


Fig. S2: Inactivation time constants of E141K and E141V.

A. Inactivation time constants determined by fitting Ca²⁺ current decay from the peak to 100 ms after the initiating voltage step at different test potentials. **B.** Inactivation time constants determined by fitting Ba²⁺ current decay from the peak to 100 ms after the initiating voltage step at different test potentials. Data symbols represent mean values of WT (n=17), E141K (n=10), and E141V (n=8), and error bars represent standard error of the mean (SEM) (*, $p < 0.05$, both mutants vs WT; #, $p < 0.05$, E141K vs WT).

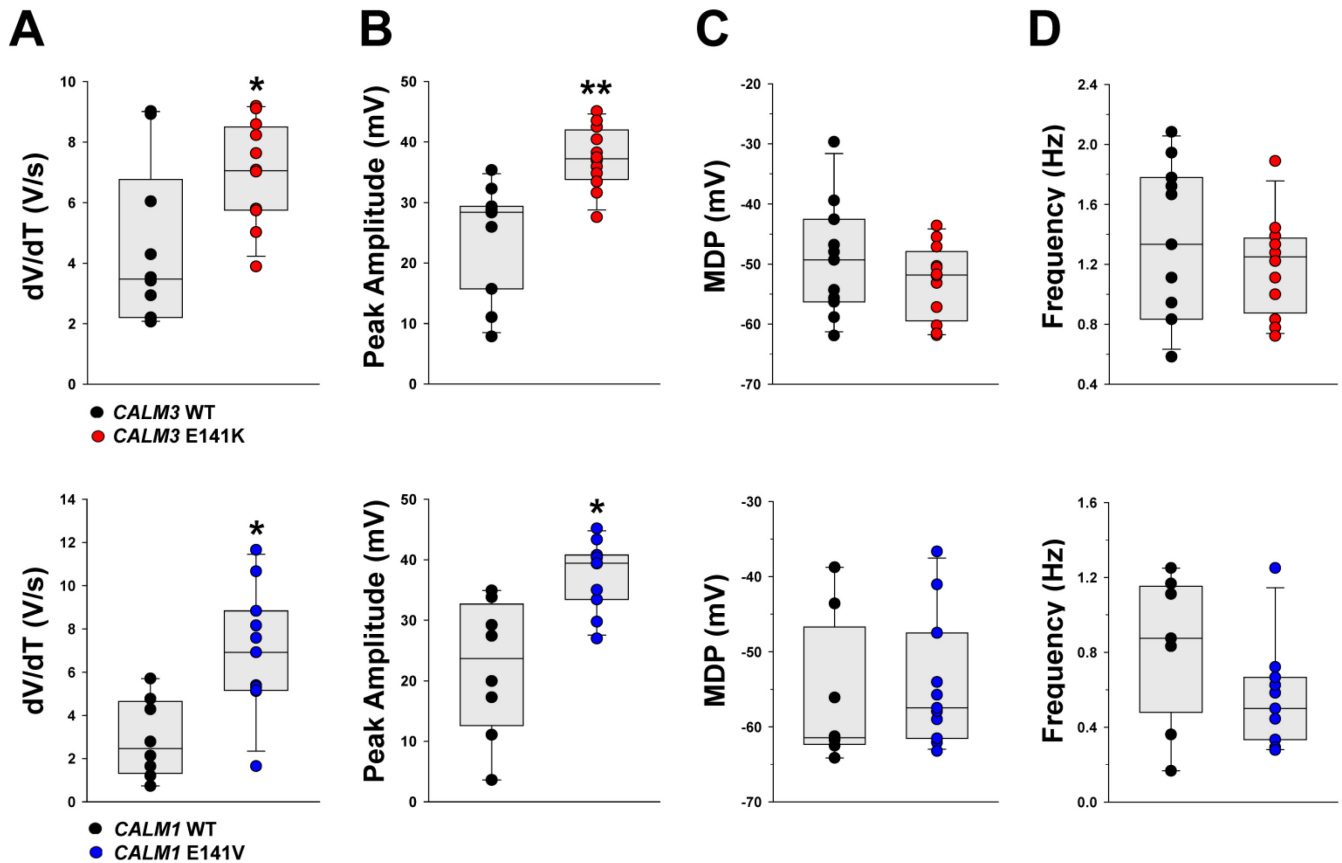


Fig. S3: Action potential characteristics of iPSC-CMs expressing E141K or E141V mutants.

A. The peak amplitude of spontaneous APs. (*, $p < 0.05$; **, $p < 0.001$ comparing mutant to WT). **B.** Box plots showing dV/dT values determined for spontaneous APs (*, $p < 0.05$, comparing mutant to WT). **C.** Box plots showing maximal diastolic potential (MDP) for iPSC-CMs expressing mutant or WT CaM. **D.** Box plots showing frequency of spontaneous action potentials determined for iPSC-CMs expressing either mutant or WT CaM. 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 5th percentiles, respectively. All data points are plotted (CALM3 WT, $n=11$; CALM3 E141K, $n=12$; CALM1 WT, $n=8$; CALM1 E141V, $n=9$).