Unexpected Structural and Functional Consequences of the R33Q Homozygous Mutation in Cardiac Calsequestrin: A Complex Arrhythmogenic Cascade in a Knock In Mouse Model

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Abstract — Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmogenic disorder characterized by life threatening arrhythmias elicited by physical and emotional stress in young individuals. The recessive form of CPVT is associated with mutation in the cardiac calsequestrin gene (CASQ2). We engineered and characterized a homozygous CASQ2<sup>R33Q/R33Q</sup> mouse model that closely mimics the clinical phenotype of CPVT patients. CASQ2<sup>R33Q/R33Q</sup> mice develop bidirectional VT on exposure to environmental stress whereas CASQ2<sup>R33Q/R33Q</sup> myocytes show reduction of the sarcoplasmic reticulum (SR) calcium content, adrenergically mediated delayed (DADs) and early (EADs) afterdepolarizations leading to triggered activity. Furthermore triadin, junctin, and CASQ2-R33Q proteins are significantly decreased in knock-in mice despite normal levels of mRNA, whereas the ryanodine receptor (RyR2), calreticulin, phospholamban, and SERCA2a-ATPase are not changed. Trypsin digestion studies showed increased susceptibility to proteolysis of mutant CASQ2. Despite normal histology, CASQ2<sup>R33Q/R33Q</sup> hearts display ultrastructural changes such as disarray of junctional electron-dense material, referable to CASQ2 polymers, dilatation of junctional SR, yet normal total SR volume. Based on these observations, we propose that the phenotype of the CASQ2<sup>R33Q/R33Q</sup> CPVT mouse model is portrayed by an unexpected set of abnormalities including (1) reduced CASQ2 content, possibly attributable to increased degradation of CASQ2-R33Q, (2) reduction of SR calcium content, (3) dilatation of junctional SR, and (4) impaired clustering of mutant CASQ2. (Circ Res. 2008;103:298-306.)

Key Words: sudden death | genetics | calsequestrin | triggered activity | transgenic mice

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a genetically transmitted disease characterized by stress- or emotion-induced life-threatening arrhythmias occurring in the structurally intact heart. In 2001,1 we demonstrated that mutations in the cardiac ryanodine receptor cause the autosomal dominant form of CPVT and subsequently reported that approximately 50% of CPVT patients carry mutations in RyR2.2 In 2001, Lahat et al.3 showed that mutations in the cardiac calsequestrin gene (CASQ2) are responsible for the rare autosomal recessive form of the disease that accounts for approximately 3% of CPVT.4 The identification of the genes underlying CPVT has had implications that extend beyond those impacting clinical management of patients inasmuch as it stimulated fundamental research targeted to understand the links between intracellular calcium regulation and arrhythmogenesis. We recently developed a knock-in mouse model carrying the R4496C RyR2 mutation identified in the first genotyped CPVT family and demonstrated that the RyR2<sup>R4496C</sup> mice develop bidirectional and polymorphic VT similar to those observed in patients. In this model we also demonstrated the occurrence of delayed after depolarizations (DADs) induced by adrenergic stimulation in isolated myocytes from the heart of heterozygous mice, suggesting that arrhythmias are elicited by triggered activity. Recently 2 mutants CASQ2 knock-in mice models were developed by Song et al:7 the first strain carries the homozygous point mutation discovered by Lahat et al in the first recessive CPVT family (D307H), and the second strain carries a homozygous deletion <del>A9/E9</del>; in analogy with RyR2 mice,5 both models develop bidirectional-polymorphic...
VTs on sympathetic activation. Interestingly, the finding of CASQ2 reduction and calreticulin increase in both strains led to the hypothesis that reduction in CASQ2 activates a compensatory increase in calreticulin.\(^7\) Here we describe a novel knock-in mouse carrier of a CASQ2 point mutation at position 33 (R33Q); the characterization of the CASQ2\(^{R33Q/R33Q}\) mouse model provides information that shed new light on the complex pathogenesis of recessive CPVT.

**Materials and Methods**

Detailed methods for mouse generation, electrophysiological measurements, immunofluorescence, real-time PCR, microarray, protein and electron microscopy analysis are reported in the online supplement (available online at http://circres.ahajournals.org).

**Generation of Knock-In of R33Q CASQ2 in Mouse Model**

The knock-in strain was generated by homologous recombination of the targeting vector with 129SvJ embryonic stem cells genome. A 450-bp DNA segment encompassing 5' UTR and exon 1 of the CASQ2 was used to screen RPCI-21 PAC mouse genomic library. Positive clones were sequenced to define the structure of an 8.5-Kb region encompassing promoter, exon 1, and part of intron 1 of mouse CASQ2. This region was cloned in 3 parts and assembled into the targeting vector pFlrt (supplemental Figure I). The linearized targeting vector was electroporated into 129SvJ embryonic stem cells. The clone selected with G418 and gancyclovir was injected into targeting vector pFlrt (supplemental Figure I). The linearized targeting vector was electroporated into 129SvJ embryonic stem cells. The clone selected with G418 and gancyclovir was injected into targeted ES cells. This region was cloned in 3 parts and assembled into the targeting vector pFlrt (supplemental Figure I). The linearized targeting vector was electroporated into 129SvJ embryonic stem cells. The clone selected with G418 and gancyclovir was injected into targeted ES cells.

**In Vivo Phenotype**

In vivo ECG recording was performed using intraperitoneal devices (Data Sciences International). Surgery was performed under general anesthesia (Avertin 0.025 mg/kg) using a heating pad to keep body temperature at 37°C. Arrhythmias were defined according to Cerrone et al,\(^5\) transthoracic 2-dimensional, M-mode, and Doppler echocardiography. Positive clones were sequenced to define the structure of an 8.5-Kb region encompassing promoter, exon 1, and part of intron 1 of mouse CASQ2. This region was cloned in 3 parts and assembled into the targeting vector pFlrt (supplemental Figure I). The linearized targeting vector was electroporated into 129SvJ embryonic stem cells. The clone selected with G418 and gancyclovir was injected into targeted ES cells. This region was cloned in 3 parts and assembled into the targeting vector pFlrt (supplemental Figure I). The linearized targeting vector was electroporated into 129SvJ embryonic stem cells. The clone selected with G418 and gancyclovir was injected into targeted ES cells.

**Histology**

Hearts of 8-week-old mice (CASQ2\(^{WT/WT}\) n=2; CASQ2\(^{R33Q/WT}\) n=2; CASQ2\(^{R33Q/R33Q}\) n=2) were fixed by retrograde aortic perfusion (3.5% glutaraldehyde, 0.1 M Na-cacodylate buffer, pH 7.2). Specimens were processed as described in the online data supplements. Sections were examined with a Philips 410 Microscope (Fei Co.), equipped with a Hamamatsu C4742-95 digital imaging system (Advanced Microscopy Techniques).

**Immunocytochemistry**

Isolated ventricular myocytes were processed using an established protocol (see online data supplements). Coverslips were incubated with polyclonal anti-CASQ2 (Affinity Bioreagents, PA1-913) and electron microscopy analysis are reported in the online supplements (available online at http://circres.ahajournals.org).

**Electron Microscopy**

The hearts of CASQ2\(^{WT/WT}\) (n=2) and CASQ2\(^{R33Q/R33Q}\) (n=2) were fixed by retrograde aortic perfusion (3.5% glutaraldehyde, 0.1 M Na-cacodylate buffer, pH 7.2). Specimens were processed as described in the online data supplements. Sections were examined with a Philips 410 Microscope (Fei Co.), equipped with a Hamamatsu C4742-95 digital imaging system (Advanced Microscopy Techniques). The width of the lumen of junctional sarcoplasmic reticulum (jSR) was measured in electron micrographs taken at 135 000× magnification using Adobe Photoshop and a grid of perpendicular lines randomly drawn perpendicularly across the jSR.

**Patch Clamp Experiments in Isolated Ventricular Myocytes**

Isolated cardiac myocytes were isolated using an established enzymatic digestion protocol.\(^9\) Transmembrane action potentials and currents were recorded in whole cell configuration as previously described\(^\text{using a MultiClamp 700B amplifier (Axon Instruments)}.\) Only quiescent, Ca-tolerant, rod-shaped cells with resting potential ≤−80 mV were used. Myocytes were electrically stimulated with 3ms/1.5 to 2.5 nA depolarizing pulses. Data were corrected for liquid junction potential before analysis. Action potential duration (APD) was measured at 90% and 50% of repolarization (APD90 and APD50). Triggered activity was defined as an unstimulated action potential arising from a delayed afterdepolarization (DAD) or a early afterdepolarization (EAD). L-type calcium current (\(I_{\text{L Ca}}\)) was recorded with 200 ms depolarizing pulses from a holding potential of −40 mV, with 10 mV steps from −40 mV to +60 mV. Current/voltage (IV) curves were obtained 10 mV voltage steps (−40 mV to +60 mV) from a holding potential of −40 mV. Protocols to assess releasable SR Ca\(^{2+}\) content by integration of the sodium-calcium exchange current (NCX), steady-state inactivation (SSII), and steady state activation (SSA) curves are reported in the online supplements.

**Real-Time PCR**

Total RNA from the hearts of 8-week-old CASQ2\(^{R33Q/WT}\) (n=5) and CASQ2\(^{WT/WT}\) (n=5) was extracted (RNA Easy, Qiagen) and retro-transcribed with random examers (ThermoScript RT-PCR system-Invtranogen). Real Time quantification of targets genes (CASQ2, Triadin, Junctin, RyR2, SERCA2, Phospholamban and Calreticulin—list of primers provided in online supplements) and housekeeping reference transcript (GAPDH) was performed with CYBR, using the ABI PRISM 7000 detection system (Applied Biosystems). Relative gene expression was quantified as follows (from User Bulletin #2 for the ABI PRISM 7000): fold change = \(2^{-\Delta\Delta Ct}\) where ΔCt = Ct(sample)−Ct(referece) and Δ(ΔCt) = ΔCt(sample)−ΔCt(control). Ct is the fractional cycle number at which the fluorescence passes the fixed threshold. Data were analyzed with the comparative threshold cycle (\(Ct\)) relative-quantification method. Variance in fold change was calculated from genes mRNAs values (target gene) compared with control mRNAs (WT counterpart) and reference gene.

**Microarray Analysis**

Experiments were performed on total RNA from hearts of 8-week-old CASQ2\(^{R33Q/WT}\) (n=5) and CASQ2\(^{WT/WT}\) (n=5). Labeled cRNA probes were generated from total RNA using the GeneChip IVT Labeling Kit (Affymetrix). The cRNA was fragmented, biotinylated, and hybridized to Mouse Genome 430 2.0 Array chips, containing 14 000 mouse genes. Quality control and boxplot of raw intensities indicated the absence of outliers. Probe level was conv-erted to expression values using both the Robust Multi-array Average (RMA) procedure and the MA5.0 algorithms. Differentially expressed genes were identified using SAM by computing single-gene statistics and repeated permutations to determine correlation of expression level with the analyzed phenotypes. Supervised analyses was carried out to identify expression signatures of CASQ2\(^{R33Q/WT}\) samples as compared to CASQ2\(^{WT/WT}\).

**Protein Analysis**

Total homogenates and microsomal fractions were obtained using standard methods (online data supplements) from 8-week-old...
CASQ2WT/WT, CASQ2R33Q/WT, and CASQ2R33Q/R33Q mice. Western blot relied on the following primary antibodies: anti-CASQ (ABR, PA1-913), anti-Triadin (ABR, MA3-927), anti-RyR2 (ABR, MA3-916), anti-Phospholamban (ABR, MA3-922), anti-Calreticulin (Upstate), anti-GAPDH (Chemicon), anti-SERCA2 (Santa Cruz, SC-8094), and antijunctin (kind gift of Dr. D.H. Kim, Gwangju Institute of Science and Technology, Korea). Chemiluminescent detection was performed with substrate reagents by Pierce Biotechnology. Densitometric analysis was performed with Image for Windows software (V. Beta 4.0.2; Scion).

**Trypsin Digestion**

Recombinant CASQ2-WT and CASQ2-R33Q proteins, generated and purified as described,10 were digested by trypsin (CASQ2:trypsin=50:1 w/w; Sigma-Aldrich) in 20 mmol/L MOPS pH 7.2, 500 mmol/L KCl at 25°C for 30 minutes. Soyben trypsin inhibitor (inhibitor:trypsin=1:1 w/w; SERVA) halted the reaction and digested samples were electrophoresed on 10 to 17.5% polyacrylamide gradient gels. Slab gels were stained with Coomassie brilliant blue. Densitometric analysis was performed as described above.

![Figure 1. ECG recording showing the onset of bidirectional ventricular tachycardia in a conscious mouse during acoustic stimuli.](image-url)
Statistical Analysis

Statistical analysis was performed using SPSS v14 package. One-way ANOVA with Bonferroni posthoc analysis or Student t test were used. Western blot data were analyzed by densitometric analysis which retuned arbitrary units of band intensities. Chi square test (Fisher exact test) was used to compare difference of incidence of DADs/triggered activity. Data are expressed as mean±SD unless specified. Values of P<0.05 were considered statistically significant.

Results

Phenotype of CASQ2R33Q/R33Q Mice

No differences were observed in the duration of the pregnancy, delivery, size, and survival of litters among CASQ2WT/WT, CASQ2R33Q/WT, and CASQ2R33Q/R33Q mice. In the 3 groups of mice, (n=10 for each group) no differences were present in weight (CASQ2WT/WT 20.1±1.6 g; CASQ2R33Q/WT 20.5±1.9 g P=0.63 versus CASQ2WT/WT; CASQ2R33Q/R33Q 21.8±2.1 g P=0.06 versus CASQ2WT/WT) and in heart/body weight ratio (CASQ2WT/WT 5.5±0.6 mg/g; CASQ2R33Q/WT 5.6±0.8 mg/g P=0.9 versus CASQ2WT/WT; CASQ2R33Q/R33Q 6.0±1.2 mg/g; P=0.27 versus CASQ2WT/WT).

Echocardiographic evaluation was unremarkable in CASQ2R33Q/R33Q versus CASQ2WT/WT (see supplemental Table I). Likewise, all the ECG parameters were unremarkable.

Continuous ECG recording (n=7 per group, ages 8 weeks) documented the presence of spontaneous ventricular premature beats (VPBs) in 6/7 CASQ2R33Q/R33Q and in none of the CASQ2WT/WT or CASQ2R33Q/WT mice. Exposure to environmental stressors such as noise and physical contact induced both nSVT as sVT episodes in 6/7 CASQ2R33Q/R33Q mice but not in CASQ2WT/WT or CASQ2WT/R33Q mice (Figure 1). Duration of sustained VT ranged from 5 to 232 seconds.

Light Microscopy of Ventricular Tissue and CASQ2 Localization by Immunohistochemistry

Histological examination of cardiac specimens failed to demonstrate gross structural abnormalities (Figure 2A). CASQ2-R33Q and α-actinin colocalized at the Z-line both in homozygous CASQ2R33Q/R33Q and CASQ2WT/WT mice, as judged by immunofluorescence confocal microscopy (Figure 2B). The reduced fluorescence intensity of CASQ2R33Q/R33Q samples is consistent with the decreased amount of CASQ2-R33Q detected by Western blotting (see below).

Electron Microscopy

Electron microscopy of both CASQ2WT/WT and CASQ2R33Q/R33Q hearts showed that the SR forms junctions with T-tubules (dyads) in proximity of the Z line, as expected (Figure 3). The percentage of cell volume occupied by SR was identical: 2.9±0.9 for CASQ2WT/WT and 2.9±1.0 for CASQ2R33Q/R33Q mice (P=0.4; see supplemental Table II). The jSR cisternae of WT samples were narrow and flat (Figure 3A through 3C), whereas they were much wider (41±10 nm versus 26±4 nm; P<0.0001, see supplemental Table II) and had a more variable size in R33Q samples (Figure 3D through 3F).

The jSR of CASQ2WT/WT myocytes contained an electron-dense chain-like line, referable to condensed CASQ2 molecules, that runs parallel to the SR membrane (Figure 3A through 3C, and Figure 2A, Exemplificative ventricular sections (hematoxylin-eosin stain) of CASQ2WT/WT, CASQ2R33Q/WT, and CASQ2R33Q/R33Q (100×). Bars=10 μm. B, Ventricular cardiomyocytes from CASQ2WT/WT and CASQ2R33Q/R33Q immunostained with rabbit anti-CASQ and mouse anti-α-actinin antibodies. Bars=20 μm.
single arrows), and is confined to those junctional areas bearing feet, ie, the cytoplasmic domains of RyR2 (Figure 3A and 3D, multiple arrows)\(^1\). On the other hand, the \textit{chain-like} line of condensed CASQ2 was missing in \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes, and the SR lumen was either empty or contains some electron-dense material that is not clustered as in SR/T-tubule junctions of WT myocytes (Figure 3D through 3F).

**Electrophysiological Characterization of Cardiac Myocytes**

We compared the action potential duration (APD) of \textit{CASQ2}\textsuperscript{WT/WT}, \textit{CASQ2}\textsuperscript{R33Q}, and \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes at 1 or 5 Hz at 35°C. No significant differences were detected in the APD\(_{50}\), APD\(_{90}\), in the amplitude of AP, and in the resting potential among the 3 groups (Table). At 5Hz, 56% (18/32) \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes, but none of the \textit{CASQ2}\textsuperscript{WT/WT} and \textit{CASQ2}\textsuperscript{R33Q}, developed DADs (\(P<0.001\)) and (9/32) 28% \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes and none of the \textit{CASQ2}\textsuperscript{WT/WT} and \textit{CASQ2}\textsuperscript{R33Q} showed triggered activity (TA) (\(P=0.041\)). In the presence of epinephrine 200 nmol/L, no triggered activity was induced in \textit{CASQ2}\textsuperscript{WT/WT} myocytes (n=15) versus 8% \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes (1/12) (\(P=0.44\), versus \textit{CASQ2}\textsuperscript{WT/WT}); finally we perfused with 200 nmol/L epinephrine \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes (n=17) that failed to develop triggered activity in unstimulated settings and observed that 47% of such \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes (8/17) developed triggered activity at 5 Hz (\(P=0.003\), versus \textit{CASQ2}\textsuperscript{WT/WT}, \(P=0.043\), versus \textit{CASQ2}\textsuperscript{R33Q}). Interestingly, when paced at 1 Hz in the presence of epinephrine, 23.5% of \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes (4/17) developed EADs at a take off potential \(-50\) mV and \(-60\) mV (Figure 4).

No significant differences were observed in the \(I_{\text{calc}}\) current between \textit{CASQ2}\textsuperscript{WT/WT} and \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes either in absence or in the presence of epinephrine. The time course of \(I_{\text{calc}}\) decay was similar in the 2 groups. In the presence of 200 nmol/L epinephrine, SSA curves significantly shifted to left in both WT and R33Q cells with no difference between the 2 groups [\(V_1/2\): from \(-16.6\) mV to \(-21.2\) mV in \textit{CASQ2}\textsuperscript{WT/WT} myocytes (\(P=0.044\)) and from \(-15.3\) mV to \(-21.6\) mV in \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes (\(P<0.001\)). SSI curves did not shift significantly in the 2 groups leading to the conclusion that there was no significant difference of \(I_{\text{calc}}\) window current between \textit{CASQ2}\textsuperscript{WT/WT} and \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes in the presence of epinephrine. We assessed the total SR Ca\(^{2+}\) content of \textit{CASQ2}\textsuperscript{WT/WT} and \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes from the integral of \(I_{\text{NCX}}\) evoked by the application of caffeine and demonstrated that the SR Ca\(^{2+}\) content is decreased by 35% in \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes as compared with \textit{CASQ2}\textsuperscript{WT/WT} suggesting that the SR ability to store Ca\(^{2+}\) was significantly suppressed in \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes (Figure 5). There was no significant difference of the \(\tau\) of \(I_{\text{NCX}}\) between \textit{CASQ2}\textsuperscript{WT/WT} and \textit{CASQ2}\textsuperscript{R33Q/R33Q} (806±40 ms versus 760±66 ms, \(P=0.57\)), suggesting no increase of NCX function in \textit{CASQ2}\textsuperscript{R33Q/R33Q} myocytes.

**mRNA and Protein Levels in \textit{CASQ2}\textsuperscript{R33Q/R33Q}, \textit{CASQ2}\textsuperscript{R33Q}, and \textit{CASQ2}\textsuperscript{WT/WT} Mice**

Western blot of total heart homogenates and of microsomal fractions extracted from the heart of 8-week-old \textit{CASQ2}\textsuperscript{WT/WT}, \textit{CASQ2}\textsuperscript{R33Q/R33Q}, and \textit{CASQ2}\textsuperscript{R33Q/R33Q} mice revealed a dramatic decrease in the levels of CASQ2 protein (<50% of normal) in \textit{CASQ2}\textsuperscript{R33Q/R33Q} mice (Figure 6A; ANOVA \(P<0.001\)). Real-time PCR failed to demonstrate changes in the level of mRNA \textit{CASQ2}\textsuperscript{R33Q/R33Q} as compared to \textit{CASQ2}\textsuperscript{WT/WT}. \textit{CASQ2}-R33Q reduction could be accounted for by increased proteolysis: in vitro trypsin digestion of WT-CASQ2 and R33Q-

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Data are expressed as mean±SE. NA indicates data not available.
CASQ2 was performed to test this hypothesis (Figure 6B). Densitometric scans showed that about 75% of CASQ2-R33Q was digested versus 23% of CASQ2-WT, ie, mutant CASQ2 is more prone to trypsin digestion than CASQ2-WT (compare lane D with lane B).

Protein levels of RyR2, junctin, triadin, calreticulin, SERCA, and phospholamban were compared in CASQ2\(^{R33Q/R33Q}\) versus CASQ2\(^{R33Q/WT}\) and CASQ2\(^{WT/WT}\) hearts. Densitometric scans showed a 25% reduction of the calsequestrin-binding protein triadin in microsomal fractions from CASQ2\(^{R33Q/R33Q}\) hearts as compared to CASQ2\(^{WT/WT}\) hearts and a reduction of 70% of junctin. No changes were observed in the protein levels of RyR2, SERCA, calreticulin, and phospholamban in CASQ2\(^{R33Q/R33Q}\) hearts (Figure 6C). mRNA levels for RyR2, triadin, junctin, calreticulin, SERCA, and phospholamban extracted from CASQ2\(^{R33Q/R33Q}\) mice (n=5) were identical to those observed in CASQ2\(^{WT/WT}\) mice (n=5), as judged by real-time PCR. Similarly, we failed to detect differences in mRNA levels in 14 000 well characterized mouse genes in CASQ2\(^{R33Q/R33Q}\) hearts (n=5) versus CASQ2\(^{WT/WT}\) (n=5), using gene expression profiling microarrays (data not shown).

**Discussion**

CPVT is an inherited arrhythmogenic disorder characterized by syncopal events and sudden cardiac death, elicited by...
physical and emotional stress, that manifests in the pediatric age.\(^2\) Mutations in the cardiac ryanodine receptor (\(\text{RyR2}\)) and in the calsequestrin (\(\text{CASQ2}\)) genes are responsible for the autosomal dominant and recessive variants of CPVT, respectively. We recently reported the characterization of a knock-in mouse model carrier of the \(\text{RyR2}^{\text{R4496C}}\) mutation identified in the Italian family that led to the discovery of the genetic basis of autosomal dominant CPVT.\(^1\) Remarkably the knock-in mice have a phenotype that closely resembles clinical manifestations of CPVT patients.\(^5\) The extensive phenotypic characterization of the model showed that delayed after-depolarization and triggered activity represent the pivotal arrhythmogenic mechanism of CPVT.\(^6,12\)

In the present study, we report the characterization of a knock-in mouse homozygous carrier of the \(\text{CASQ2}^{\text{R33Q}}\) mutation that we identified in highly symptomatic CPVT patients\(^13\): the main objective of the present study is to derive information germane to the understanding of the pathophysiology of the autosomal recessive form of CPVT that remains controversial.\(^7,14\)

**Morphological Abnormalities and Contractile Function**

CPVT is classified as an inherited arrhythmogenic disorder that occurs in individuals with a structurally intact heart: so far, in fact, no functional or morphological cardiac abnormalities have been described in patients with autosomal recessive CPVT. The murine models of recessive CPVT present modest structural abnormalities: similarly to our \(\text{CASQ2}^{\text{R33Q/R33Q}}\) mice, the \(\text{CASQ2}^{\text{KO/KO}}\) mice present normal cardiac contractility. However, ultrastructural assessment of \(\text{CASQ2}^{\text{R33Q/R33Q}}\) mice showed that the total SR volume is unchanged, whereas the width of the jSR is doubled. It remains to be clarified whether such features might represent the initial stages of a cardiomiopathy or are mere adaptive phenomena. In addition, \(\text{CASQ2}^{-}\) appears not be properly clustered and this might also be related to the inability of R33Q to form Ca-dependent polymers\(^15\) at physiological intra-SR [Ca\(^{2+}\)].

**In Vivo and In Vitro Electrophysiological Profile**

In analogy with what observed in the \(\text{RyR2}^{\text{R4496C}}\) knock-in mouse model,\(^5,6\) the \(\text{CASQ2}^{\text{R33Q/R33Q}}\) mice develop polymorphic and
SR Calcium Content and Calsequestrin Depletion

To further investigate the mechanisms leading to arrhythmias in isolated R33Q cardiac myocytes, we measured the integral of $I_{\text{NCX}}$ evoked by the application of caffeine, and showed a substantial decrease as compared to what observed in WT cells. There is an apparent discrepancy between these data and those reported by Terentyev et al, showing that in rat isolated myocytes overexpressing CASQ2-R33Q on top of endogenous CASQ2-WT, $I_{\text{NCX}}$ is unchanged despite the reduction of SR free calcium. Terentyev et al linked the observed decrease in free calcium to the impairment of CASQ2 as act as the intraluminal calcium sensor of the RyR2 macromolecular complex and to the impairment of the physiological deactivation of the RyR2 at low intraluminal calcium levels. Furthermore, based on single channel analysis, we have shown that R33Q failed to inactivate RyR2 as intra-SR $[Ca^{2+}]$ decreased. Thus, despite the leaky RyR2, normal calcium transients were recorded because of the compensatory effect of the high calcium binding provided by overexpressed CASQ2-R33Q and endogenous CASQ2-WT. Because CASQ2 levels are markedly reduced in CASQ2$^{R33Q/R33Q}$ mice (Figure 6), the limitations of the model used by Terentyev et al, ie, CASQ2-R33Q overexpression in the presence of endogenous CASQ2-WT, have now become clear. Therefore, based on the present data, we propose that 2 consequences prompted by the mutation concur to the pathogenesis of the homozygous CASQ2$^{R33Q/R33Q}$ phenotype: (1) reduction of CASQ2 levels (likely attributable to posttranslational regulation leading to increased protein degradation, see below) and (2) impairment of RyR2 deactivation, as shown by Terentyev et al and Qin et al.

Despite a substantial reduction of CASQ2 seems to be central in recessive CPVT, it is still a matter of debate whether a threshold decrease of CASQ2 is required for eliciting the CPVT phenotype or whether even a minor loss of CASQ2 increases propensity to arrhythmias. At variance with data reported in the heterozygous knock out mouse model, most human heterozygous individuals carriers of premature truncations of CASQ2 are asymptomatic. We reported that all 9 carriers of the G112+5X mutation leading to a premature stop codon had no clinical signs of CPVT. Postma et al reported that 1 of 3 carriers of the heterozygous R33X mutation had arrhythmias, yet the clinical description of this individual is insufficient to support a causal link between the heterozygous mutation and the arrhythmias. It is possible that compensatory mechanisms to haploinsufficiency of CASQ2 are different in humans and mice, and human beings may have the ability to upregulate the expression of the wild-type allele thus attenuating the consequences of CASQ2 deficiency in the heterozygous state. In the presence of heterozygous missense mutations, the residual function of the 50% mutant CASQ2 may be adequate to prevent functional impairment, as seen in our CASQ2$^{R33Q/WT}$ mice that do not display reduction of CASQ2 levels and in analogy to the asymptomatic parents of the R33Q-linked CPVT patient.

Mechanisms Leading to Reduced CASQ2 in Recessive CPVT and Compensatory Responses

Recently Song et al showed that the CASQ2 reduction observed in CASQ2$^{G112+5X/H11001}$ mice is not paralleled by a reduction of CASQ2 mRNA, yet they did not provide any explanation to account for the protein reduction. In the CASQ2$^{R33Q/R33Q}$ model, we observed a CASQ2 reduction in the presence of normal levels of mRNA and reasoned that CASQ2-R33Q might be more susceptible to proteolysis: a trypsin digestion test supported the view that accelerated CASQ2 catabolism is likely to underlie the observed CASQ2 reduction. Exposure of new proteolytic sites nearby the N or C terminus of the CASQ2-R33Q protein might be taken as indication of misfolding and protein instability, as compared to CASQ2-WT. CASQ2-R33Q because of its higher conformational flexibility, as recently shown, might undergo accelerated in vivo degradation via proteasome, thus reducing effective CASQ2 content within the SR lumen. The relationships linking missense mutation, protein instability, and activation of posttranslational regulatory mechanisms are currently under investigation. A complementary mechanism for CASQ2-R33Q reduction might be attributable to inappropriate CASQ2 polymerization leading to defective SR retention, as suggested by Cala and coworkers.

In the CASQ2$^{G112+5X/H11001}$ and CASQ2$^{E99/\Delta E9}$ models, Song et al found an increase in calreticulin and RyR2, and thus considered this process as a compensatory response to reduced CASQ2. In our model, we failed to observe such an adaptive response, and thus we argue that the increase in calreticulin and RyR2 might be a mutation-specific finding and should not be regarded as the univocal response to CASQ2 decrease. In our model, we found, instead, a decrease in the levels of triadin and junctin, in the absence of mRNA reduction, as assessed by real-time PCR: interestingly, also the knock out CASQ2 model showed a similar reduction of
triadin and junctin, yet the mechanism underlying this response remains so far undefined.

To provide a comprehensive assessment of the heart adaptive response to CASQ2-R33Q, we performed microarray studies to define the gene expression profiling of the heart of CASQ2<sup>R33Q/R33Q</sup> mice versus that of their CASQ2<sup>WT/WT</sup> littermates. Unexpectedly, no changes in mRNA levels were identified in any of the 14,000 genes probed suggesting that the heart does not activate adaptive transcriptional responses to mutant R33Q CASQ2, at least by 8 weeks of age.

Conclusion
In light of the unexpected finding of the central role of CASQ2 decrease in the R33Q knock in mouse model, we now propose that CASQ2 reduction is one of the common pathogenetic mechanisms of autosomal recessive CPVT, as supported by the evidence that the CASQ2 knock out mice is a CPVT phenotype. We also suggest that the different missense mutations present in CPVT patients may be characterized by additional and specific functional abnormalities of CASQ2 (such as, CASQ2 polymerization impairment, altered calcium affinity, altered interaction with RyR2) leading to variable compensatory re-organization and fibrillation elicited in a knock-in mouse model carrier of a mutation in the cardiac ryanodine receptor (RyR2). Circ Res. 2005;96:e77–e82.

Disclosures
None.

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Unexpected Structural and Functional Consequences of the R33Q Homozygous Mutation in Cardiac Calsequestrin: A Complex Arrhythmogenic Cascade in a Knock In Mouse Model

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Methods

Generation of knock-in of R33Q CASQ2 in mouse model

The knock-in mouse model was generated by homologous recombination using genetic manipulation of embryonic stem cells. A PCR-amplified 450 bp DNA fragment from C57BL/6J mouse genome encompassing 5’ UTR and exon 1 of the CASQ2 gene (primers: F-CCATGATCTCTATTCTGGAGACTG; R-CCACCTTAAGAGTTTGCCCACAG) was used to screen a RPCI-21 PAC mouse genomic library (129S6/SvEvTac strain). Two out of 118 positive clones were sequenced to define the genomic structure of a 8.5 Kb region encompassing part of promoter, exon 1 and part of intron 1 of the mouse CASQ2 gene. By means of XbaI and BamHI digestion we obtained three fragments that were independently cloned into pSL1180 plasmids. Site-direct mutagenesis was performed in exon 1 to introduce the R33Q mutation in the targeted region (Quick Change Stratagene). Finally the three fragments were assembled into the targeting vector pFlrt: 5’arm (5.6 kb) encompassing promoter and exon 1 of CASQ2 PGK-Neo gene flanked by Frt sites was used for the selection with G418, a 3’arm (2.3 Kb) encompassing intron 1 of CASQ2 and HSV-TK cassette (outside the region of homology) for the selection with ganciclovir (Online Figure I). The targeting vector was linearized with NotI and transferred into 129Sv/J embryonic stem cells by electroporation (Telethon Core Facility for Conditional Mutagenesis, DIBIT San Raffaele, Italy). Positive and negative selections were performed using G418 and gancyclovir, respectively. Four hundred G-418 and ganciclovir resistant clones were screened for homologous recombination by southern blot using an external probe to the target sequence of homology. One out of five positive clones was injected into C57BL/6NCrl blastocysts which are transferred to pseudo-pregnant CD-1 females. We obtained two chimeric male mice (with 60-70% of chimerism) that were mated to
Wild-type (WT) C57BL/6NCrL female mice. Breeding produced 1/200 agouti mouse. 

CASQ2^{R33Q/WT}-Neo male mice were bred with transgene Flp female mice (from CFCM, DIBIT) expressing recombinase to remove the Neo selectable marker. Mating of two heterozygous animals resulted in the homozygous mutant mice. Mice genotype of the colony was determined by sequencing of DNA extracted from tail biopsy specimens (DNasy Tissue Kit, Qiagen).

Animals are maintained and bred at the Charles River Laboratories in Calco, Italy, and transferred to the Maugeri Foundation for characterization of the phenotype. Animals are maintained and studied according to the protocols approved by the Animal Care and Use committee at the Maugeri Foundation, University of Pavia.

Electrophysiological recordings in isolated ventricular myocytes

Ventricular myocytes were isolated using an established enzymatic digestion protocol yielding to 60-80% rod-shaped, calcium-tolerant myocytes. Within 8 hours from isolation, laminin-coated dishes containing isolated ventricular myocytes were mounted on the stage of an inverted microscope. The myocytes were bathed with the solution containing (mmol/L): 140 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES and 5 glucose, pH adjusted to 7.4 with NaOH at 35°C. Transmembrane potentials and currents were recorded in whole cell current mode as previously described using a MultiClamp 700B amplifier (Axon Instruments). All signals were acquired at 5 kHz (Digidata 1322A, Axon Instruments) and analyzed with the use of personal computer running pCLAMP version 9.2 software (Axon Instruments). Patch electrodes were pulled from borosilicate glass on a P-97 horizontal puller (Sutter Instruments). The electrodes had a resistance of 2–3 MΩ when filled with patch electrode solutions containing (mmol/L): 120 potassium aspartate, 20 KCl, 1 MgCl2, 4 Na2APT, 0.1 GTP, 10 HEPES, 10 glucose, pH adjusted to 7.2 with NaOH. Only quiescent, Ca-tolerant, rod-shaped cells with clear cross striations and a resting potential \( \leq -80 \text{ mV} \) were used for action potential recordings. Myocytes were electrically stimulated by intracellular current injection through patch electrodes using depolarizing pulses with duration of 3 ms and
amplitude of 1.5-2.5 nA. The liquid junction potential between pipette and bath solution was calculated with pCLAMP software and corrected after experiments. Action potential duration (APD) was measured at 90% and 50% of repolarization (APD$_{90}$ and APD$_{50}$). DADs were defined as phase 4 positive (depolarizing) deflections of the membrane potential. EADs were defined as positive (depolarizing) oscillations occurring during phase 2 or 3 of action potential. Triggered activity was defined as an unstimulated action potential developing from a DAD or EAD. L-type calcium current (ICa-L) was recorded in voltage-clamp mode with 200ms pulses from a holding potential of -40 mV, to different test potentials increased from -40 mV to +60 mV with 10mV steps. Pipettes (2 to 3 MΩ) were filled with (mmol/L) 80 Aspartate acid, 10 NaCl, 2 MgCl2, 70 CsOH, 40 CsCl, 10 EGTA, 2 Na2APT, 0.1 GTP, 10 HEPES, pH adjusted to 7.2 with CsOH. Myocytes were superfused with solution contained (mmol/L) : 140 NaCl, 4 CsCl, 1 CaCl2, 1 MgCl2, 10 HEPES, 5 glucose and 0.02 tetrodotoxin, pH adjusted to 7.4 with NaOH. Current/voltage (I/V) curves were obtained by applying voltage steps in 10 mV increments (−40 mV to +60 mV) from a holding potential of −40 mV. The steady-state inactivation (SSI) curves were determined by using a double-pulse protocol: conditioning pulse duration was 1 s and test pulse duration was 200 ms. Pulses were applied to test potential of +10 mV steps from various conditioning pulse level (from −70 mV to +30 mV). For steady-state activation (SSA) curves, the conductance ($g_{Ca}$) was calculated from the equation: $g_{Ca}=I_{Ca}/(V_{m}-V_{rev})$, where $I_{Ca}$ is the peak current elicited by depolarizing test pulse to various potentials and $V_{rev}$ is the reversal potential (obtained from the extrapolated I/V curves). Max $g_{Ca}$ is the maximum Ca$^{2+}$ conductance. The points for $g_{Ca}/max g_{Ca}$ were plotted against the membrane potential as a relative amplitude. To assess releasable SR Ca2+ content by integration of the sodium-calcium exchange current (NCX), cells were clamped at -40 mV and abruptly superfused with 10 mmol/L of caffeine after a train of conditioning pulses at 1 Hz to induce stable loading. Pipettes (1 to 1.5 MΩ) were filled with (mmol/L): 115 CsCH3O3S, 20 CsCl, 12 NaCl, 10 HEPES, 0.1 EGTA, and 5 MgCl2, pH adjusted to 7.2 with CsOH. The concentration of amphotericin B was 250 μg/mL. Myocytes were superfused with solution
contained (mmol/L): 140 NaCl, 4 CsCl, 1 CaCl2, 1 MgCl2, 10 HEPES, 0.03 Niflumate, 5 glucose, pH adjusted to 7.4 with NaOH.

Indirect immunofluorescent labelling
Twenty-four hours post isolation ventricular myocytes were fixed on coverslips in 4% paraformaldehyde for 15 minutes at room temperature. Coverslips were then washed in PBS with gentle shaking. Fixed cells were treated with 1% Triton X-100 1% BSA in PBS for 20 minutes at room temperature. All the cells were kept in blocking solution (10% BSA in PBS 1X) for 1 hour at 37°C. Coverslips were then incubated for 1 hour at 37°C with a polyclonal antibody against CASQ2 (Affinity Bioreagents, PA1-913) and monoclonal anti-α-actinin (Sigma A7811). After washing cells were incubated with goat anti-rabbit Alexa fluor 488 and goat anti-mouse Alexa fluor 633 secondary antibodies for 1 hour at 37°C. The cells were washed several times and mounted on slides with mounting medium (90% glycerol plus 2% DABCO), a photo bleaching inhibitor.

Confocal microscopy was performed with a TCS-SP digital scanning confocal microscope (Leica, Deerfield, IL) equipped with a 63 x/NA = 1.32 oil immersion objective using a 488-nm and 633-nm laser line for excitation of fluorophores (pinhole diameter at 1 µm).

Real Time PCR
Total RNA from the from hearts of 2-month-old CASQ2^{R33Q/R33Q} and CASQ2^{WT/WT} mice (5 animals in each group) was extracted using RNA Easy fibrous tissue midi kit (Qiagen). cDNA was obtained using random examer from 2.5 µg of RNA using ThermoScript RT-PCR system (Invitrogen). Real time PCR mix contained 3µl of cDNA were added in a 25µl total volume. Real Time quantification of mRNAs of the targets genes (CASQ2, Triadin, Junctin, RyR2, SERCA2, Phospholamban and Calreticulin) and of the reference gene (GAPDH) was performed with a CYBR Green 2X SensiMix One-Step Kits (Quntace cat. QT205-02), using the ABI PRISM 7000 detection system, from Applied Biosystems. The following primers were used (5’→3’): Calreticulin, forward -
GCTACGTGAAGCTGTTTCCGAG, reverse -ACATGAACCTTCTTGGTCCAG; Calsequestrin, forward -TTATCCAATCTCCCAGCAAC, reverse -CAGAAATGATGCAATGCCCA; Junctin, forward - ATTGCATTGCTGCGCTCT , reverse -CATCGCAGCTGGCAG; Ryanodine receptor, forward - CCTTTATAACCGGACTCGACG, reverse - TTGGCCCCATATGTGTGTTGATG; SERCA2a, forward - GCTGTTTTCGAGTTGAACC, reverse - GCATCATTCACACCACCATCACCAG; Triadin, forward - GGCTTCTTGTCACTGCTCTGAT, reverse - CAATCTTGGCAATGGAGCTTG; Phospholamban, forward - TGCCTTCCTGGCATAATGGA, reverse - GGTTCTCAGGATTCTGACGTGC; GAPDH, forward: -GTATGACTCCACTCACGGCAAA reverse: -TTCCCATTCTCGGCCTTG. Primers were synthetized (MWG, Biotech, Germany) and optimised for a final concentration of 900nM for targeted genes and 300nM for GAPDH. The following experimental run protocol was used: denaturation 95°C for 10 min and amplification, repeated 40 times, 95°C for 15sec, 60°C for 30 sec and 72°C for 30 sec. Specificity of RT-PCR products is documented with gel electrophoresys and resulted in a single product with the expected length. Melting curve analysis showed specific melting temperatures. Relative gene expression was quantified as follows: fold change= \( 2^{-\Delta(Ct)} \); where \( \Delta Ct = Ct_{target} - Ct_{reference} \) and \( \Delta(Ct)_{sample} = Ct_{sample} - Ct_{control} \). Ct is the fractional cycle number at which the fluorescence passes the fixed threshold. Data were analyzed with the comparative threshold cycle (Ct) relative-quantification method.

Variance in fold change was calculated from genes mRNAs values (target gene) compared with control mRNAs (the same genes from WT counterpart) and reference gene.

**Microarray analysis**

Total RNA from hearts of 2-month-old CASQ2\(^{R33Q/R33Q}\) and CASQ2\(^{WT/WT}\) mice (5 animals in each group) are extracted using RNA Easy fibrous tissue midi kit (Qiagen). Labeled cRNA probes were generated from total RNA samples (GeneChip IVT Labelling Kit, Affymetrix). Briefly, double-stranded cDNA was retrotranscribed from total RNA. After in vitro transcription, the cRNA was
fragmented, biotinylated cRNA and hybridized to Mouse Genome 430 2.0 Array chips, containing 14,000 mouse genes, using the Affymetrix Fluidics Station 400 according to the manufacturer’s instructions. Data quality control from microarray hybridization was carried out considering the MAS5.0 (Microarray Suite/Software, Affymetrix) control parameters after a global scaling at a target intensity of 100. Quality and control parameters as well as boxplot of raw intensities showed a high quality of the data set and the absence of outliers. Probe level data was converted to expression values using both the Robust Multi-array Average (RMA) procedure and the MAS5.0 algorithms. In the former case, PM values (Perfect Match) were background-adjusted, normalized using invariant set normalization and log transformation. In the latter case, intensity levels were normalized using the Global Scaling option to target value (i.e. TGT=100). Differentially expressed genes were identified using SAM \(^3\). Single-gene statistics were computed by measuring the strength of the relationship between gene expression and the response variability. By repeated permutations of the data we determined if the expression level of any genes was significantly related to the analyzed phenotypes. SAM routine also allowed controlling the False Discovery Rate (FDR), i.e. the proportion of falsely rejected null hypotheses among all rejected null hypotheses, by computing a q-value. The cutoff for significance was determined by a tuning parameter \(\Delta\), achieved by controlling the q-value or the false discovery rate for the gene list that includes that gene and all the most significant ones. Supervised analyses was carried out to identify expression signatures in log2 RMA data of \(\text{CASQ}^2\text{R33Q/R33Q}\) samples compared with \(\text{CASQ}^2\text{WT/WT}\). Results were reported as q-value; for every probeset q-value threshold to identify a differentially expressed gene was set at 10%.

**Protein analysis**

Hearts excised from \(\text{CASQ}^2\text{WT/WT}\), \(\text{CASQ}^2\text{R33Q/WT}\) and \(\text{CASQ}^2\text{R33Q/R33Q}\) 2 month old mice were washed in phosphate buffered saline (PBS) plus a complete protease inhibitors (Roche) then homogenized using a hand-held blender in lysis buffer containing (in mmol/L): 10 \(\text{Na}_2\text{Pipes}\), 0.3
sucrose, pH 7.4 plus a complete protease inhibitors. The homogenate was spun at 8000g for 15 min at 4°C and supernatant was collected and analyzed by western blot as total heart homogenate. Microsomal fraction was prepared as follows: hearts from two-months old \( \text{CASQ}^2_{\text{WT/WT}} \) and \( \text{CASQ}^2_{\text{R33Q/R33Q}} \) were homogenized in buffer containing 10 mmol/L Hepes and 20 KCl mmol/L with a mix of complete protease inhibitors. The homogenates were centrifuged three times at 650g for 10 min at 4°C. The supernatant was spun at 120000g for 90 min at 4°C. Pellets representing microsomal fractions were resuspended in a buffer containing 0.3 M sucrose, 5 mmol/L Imidazole with complete protease inhibitors, pH 7.4, and stored at -80°C. Protein concentrations were determined by the Lowry assay (Bio-Rad) and immunoblot analysis was performed in 4-12% or 10% polyacrylamide gels.

The following primary antibodies were used: rabbit anti-CASQ (ABR, PA1-913); mouse anti-Triadin (ABR, MA3-927); mouse anti-RyR2 (ABR, MA3-916); mouse anti-Phospholamban (ABR, MA3-922), anti-Calreticulin (Upstate) anti-SERCA2 (Santa Cruz , SC-8094), anti-junctin (kind gift of Dr. D.H. Kim, Gwangju Institute of Science and Technology, Korea) and mouse anti-GAPDH (Chemicon). HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (ABR SA1-100 and SAI-200) were used as secondary antibodies. In addition to GAPDH immunostaining, we used Red Ponceau staining to assure equal loading. Chemioluminescent detection was performed with Western blotting substrate reagent (Pierce Biotechnology). Densitometric analysis was performed with Image for Windows software (version Beta 4.0.2; Scion, Frederick, MD; www.scioncorp.com).

**Electron microscopy**

The hearts were fixed by perfusion through the aorta with 3.5% glutaraldehyde in 0.1 M Na\(^+\) Cacodylate buffer-pH 7.2; kept at room temperature for 1 hour and stored at 4°C. Small bundles of cells teased from the papillary muscles were then post-fixed in 2% OsO\(_4\) in NaCaCo buffer for 2 hr and block-stained in saturated uranyl acetate. After dehydration, specimens were embedded in an epoxy resin (Epon 812). Ultrathin sections were cut in a Leica Ultracut R microtome (Leica...
and double-stained with uranyl acetate and lead citrate. Sections were examined with a Philips 410 Microscope (Fei Co.), equipped with a Hamamatsu C4742-95 digital imaging system (Advanced Microscopy Techniques).

**Online Table I – Echocardiographic parameters**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>LVDd (mm)</th>
<th>LVDs (mm)</th>
<th>IVS (mm)</th>
<th>PW (mm)</th>
<th>FS (%)</th>
<th>LVm (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASQ2^{R33Q/R33Q}</td>
<td>3.4±0.11</td>
<td>1.7±0.07</td>
<td>0.9±0.03</td>
<td>0.9±0.02</td>
<td>51</td>
<td>119±4</td>
</tr>
<tr>
<td>CASQ2^{WT/WT}</td>
<td>3.2±0.10</td>
<td>1.5±0.08</td>
<td>0.9±0.04</td>
<td>0.9±0.03</td>
<td>54</td>
<td>99±6</td>
</tr>
<tr>
<td>p</td>
<td>0.382</td>
<td>0.115</td>
<td>0.611</td>
<td>0.175</td>
<td>0.079</td>
<td>0.076</td>
</tr>
</tbody>
</table>

Data are reported mean ± SE. LVDd = left ventricular diameter, diastolic; LVDS = left ventricular diameter, systolic; IVS = interventricular septum; PW = posterior wall; FS = fractional shortening; LVm = Left Ventricular Mass.
Online Table II. jSR width and total SR volumes in papillary myocytes

A) the width of jSR lumen in CSQ2R33Q/R33Q hearts is significantly larger, and more variable, than in CSQ2WT/WT. B) The percentage of fiber volume occupied by the SR in CSQ2WT/WT and in CSQ2R33Q/R33Q is not different.

<table>
<thead>
<tr>
<th></th>
<th>CSQ2WT/WT (mean ± SD)</th>
<th>CSQ2R33Q/R33Q (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A - width of jSR lumen</td>
<td>26 ± 4 nm (n= 139)*</td>
<td>41 ± 10 nm (n= 108)*</td>
</tr>
<tr>
<td>B - SR vol./total fiber vol, %</td>
<td>2.9 ± 0.9 (n= 107)#</td>
<td>2.9 ± 1.0 (n= 112)#</td>
</tr>
</tbody>
</table>

*n = No. of measurements, from 24 jSR profiles for each group. Student’s t test: P < 0.0001 (extremely significant).

#n = No. of micrographs, from 36 and 46 cells respectively in CSQ2WT/WT and CSQ2R33Q/R33Q mice. Student’s t test: P = 0.4.

Percentage of fiber volume occupied by the SR in CSQ2WT/WT (column A, two animals) and in CSQ2R33Q/R33Q (column B, two animals). Student’s t test: P = 0.4.
References


LEGEND

Online Figure I

Diagram showing the genomic structure of CASQ2 (upper panel A), the targeting vector (middle panel B), and the mouse genome after homologous recombination (lower panel C) with the sequence of mutation R33Q in heterozygosis and homozygosis.
A. Genomic Structure *CASQ2*

B. pFlrt-*CASQ2*-R33Q vector

C. Recombining *CASQ2*-R33Q-neo

**Online figure I**