

# A mutation in calsequestrin, CASQ2<sup>D307H</sup>, impairs Sarcoplasmic Reticulum Ca<sup>2+</sup> handling and causes complex ventricular arrhythmias in mice

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## Abstract

**Objective:** A naturally-occurring mutation in cardiac calsequestrin (CASQ2) at amino acid 307 was discovered in a highly inbred family and hypothesized to cause Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT). The goal of this study was to establish a causal link between CASQ2<sup>D307H</sup> and the CPVT phenotype using an *in vivo* model.

**Methods and results:** Cardiac-specific expression of the CASQ2<sup>D307H</sup> transgene was achieved using the  $\alpha$ -MHC promoter. Multiple transgenic (TG) mouse lines expressing CASQ2<sup>D307H</sup> from 2- to 6-fold possess structurally normal hearts without any sign of hypertrophy. The hearts displayed normal ventricular function. Myocytes isolated from TG mice had diminished  $I_{Ca}$ -induced Ca<sup>2+</sup> transient amplitude and duration, as well as increased Ca<sup>2+</sup> spark frequency. These myocytes, when exposed to isoproterenol and caffeine, displayed disturbances in their rhythmic Ca<sup>2+</sup> oscillations and membrane potential, and delayed afterdepolarizations. ECG monitoring revealed that TG mice challenged with isoproterenol and caffeine developed complex ventricular arrhythmias, including non-sustained polymorphic ventricular tachycardia.

**Conclusions:** The findings of the present study demonstrate that expression of mutant CASQ2<sup>D307H</sup> in the mouse heart results in abnormal myocyte Ca<sup>2+</sup> handling and predisposes to complex ventricular arrhythmias similar to the CPVT phenotype observed in human patients.

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*This article is referred to in the Editorial by Bassani and Bassani (pages 7–9) in this issue.*

## 1. Introduction

CASQ2 is the most abundant Ca<sup>2+</sup> buffering protein present in the lumen of the cardiac sarcoplasmic reticulum

**Abbreviations:** SR, Sarcoplasmic reticulum; CASQ2, Calsequestrin; CPVT, Catecholaminergic polymorphic ventricular tachycardia; WT, Wild-type; TG, Transgenic; RyR2, Cardiac ryanodine receptor.

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(SR). It allows calcium to be stored at total concentrations of up to 20 mM, while the free concentration remains  $\sim 1$  mM [1–4]. It binds calcium with high capacity (40–50  $\text{Ca}^{2+}$ /CASQ2) and moderate affinity ( $k_d \sim 1$  mM) [1,3–5]. Increasingly, it has become apparent that CASQ2 might also play an important role in regulating the SR  $\text{Ca}^{2+}$  release [6,7]. Studies have suggested that CASQ2 can interact functionally with the cardiac ryanodine receptor (RyR2) *in vitro*, either by binding with the RyR2 directly or through its interactions with junctin and triadin [8–11]. It is hypothesized that this interaction allows CASQ2 to be localized to the terminal cisternae of the junctional SR (jSR) [8,10,12,13]. Also, it was proposed that through interaction with triadin, CASQ2 might be able to communicate with the RyR2 [6,14]. However, the nature of this interaction and its exact role in  $\text{Ca}^{2+}$  release remain to be understood.

The importance of CASQ2 is highlighted by the fact that mutations in the CASQ2 gene have been recently linked to ventricular arrhythmias and sudden cardiac death [15–17]. In particular, a naturally-occurring mutation in cardiac CASQ2 at amino acid 307 (CASQ2<sup>D307H</sup>) was discovered in seven Bedouin families [18,19], as a potential cause for Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) [19]. CPVT is a disease that is characterized by adrenergically-mediated ventricular tachycardia that can rapidly progress into ventricular fibrillation and sudden cardiac death [18]. The characteristic symptoms are recurrent syncope, seizures, or sudden death triggered by exercise or emotional stress, both of which raise the levels of catecholamines [18].

Sequencing of DNA obtained from CPVT patients showed that CASQ2 exon 9 had a G  $\rightarrow$  C substitution at nucleotide 1038 [19]. This substitution resulted in a change from an aspartate residue to a histidine at position 307 in CASQ2 [19]. This aspartate residue is invariant both in vertebrate and invertebrate CASQ [19]. Acute expression of this mutant protein in isolated adult rat cardiomyocytes by adenoviral gene transfer resulted in decreased SR  $\text{Ca}^{2+}$  storage,  $\text{Ca}^{2+}$  channel-gated  $\text{Ca}^{2+}$  release ( $\text{Ca}^{2+}$  transients) and local  $\text{Ca}^{2+}$  release events ( $\text{Ca}^{2+}$  sparks) [20]. In addition, the rhythmicity of the  $\text{Ca}^{2+}$  transients was disrupted in these myocytes upon catecholamine stimulation.

In the present study, we wished to determine the effect of CASQ2<sup>D307H</sup> on heart function by chronically expressing this mutant protein *in vivo*. This was accomplished by generating TG mouse lines that express CASQ2<sup>D307H</sup> in the heart. Transgenic expression of mutant protein does not lead to cardiac hypertrophy. Cardiomyocytes isolated from the TG mice displayed *in vitro* arrhythmias that are consistent with the CPVT phenotype. Most importantly, complex ventricular arrhythmias, including non-sustained ventricular tachycardia, are triggered in the hearts of TG mice when challenged with isoproterenol and caffeine. Our findings

in the TG mouse suggest that CASQ2<sup>D307H</sup> mutation can contribute to the CPVT phenotype in humans.

## 2. Materials and methods

### 2.1. Generation of TG mice expressing the CASQ2<sup>D307H</sup> mutant protein

The D307H mutation was introduced into the rat CASQ2 open reading frame, using PCR methodology. Once the D307  $\rightarrow$  H mutation was confirmed by DNA sequencing, the coding region of the mutant protein was sub-cloned into a vector containing the  $\alpha$ -MHC promoter as described previously [21–23]. TG mice (FVB/N) expressing mutant CASQ2<sup>D307H</sup> were generated by the TG core facility. This investigation conforms to the guide for Care and Use of laboratory animals published by the US national Institutes of Health (NIH publication # 85-23, revised 1996).

### 2.2. Western blotting analyses

Western blotting was performed following standard protocols [24].

### 2.3. Isolation of mouse myocytes and electrophysiological recordings

Myocytes were isolated using established protocols [25]. Transmembrane ionic currents were measured using whole-cell patch-clamp recordings, as described previously [26], with Axopatch 200B amplifier (Axon Instruments, USA) and pClamp-9 software.

### 2.4. Confocal $\text{Ca}^{2+}$ measurements

Intracellular  $\text{Ca}^{2+}$  imaging was performed by using an Olympus Fluoview 1000 laser scanning confocal microscope equipped with an Olympus 60  $\times$  1.4NA oil objective. Fluo-3 was excited by the 488-nm line of an argon-ion laser, and the fluorescence was acquired at wavelengths  $>510$  nm in the line-scan mode of the confocal system at the rate of 2 ms per scan.  $\text{Ca}^{2+}$  spark parameters were quantified with a detection/analysis computer algorithm [26].

### 2.5. ECG monitoring and induction of arrhythmia

ECG recordings were obtained from mice as previously described [27–29]. Mice over-expressing CSQ<sup>D307H</sup> ( $n=8$ ) and their wild-type littermates ( $n=8$ ) were anesthetized with isoflurane (1–1.5%) at minimum effective concentrations, and placed on a heating pad (Braintree Scientific, Inc.) to maintain normothermia. ECGs were recorded using a physiologic data acquisition system (MP 100, Biopac Systems) with a sampling rate of 2 kHz for  $84 \pm 17$  min. After baseline recording (10 min), 5 pairs of mice (5 WT and 5 TG: 2 pairs each from 2- and 4-fold lines, and 1 pair from

6-fold line) received 4 doses of isoproterenol (2 mg/kg IP) in 10–20 min intervals, the first 2 of which were combined with caffeine (120 mg/kg IP [30,31]). To ensure that the observed arrhythmias were adrenergically mediated, another group of

mice (3 pairs from 2-fold line) received incremental doses of isoproterenol alone every 10 min (cumulative dose of  $8.75 \pm 0.02$  mg/kg IP). Both TG and age-matched WT littermates were tested the same day using the same drug protocol.

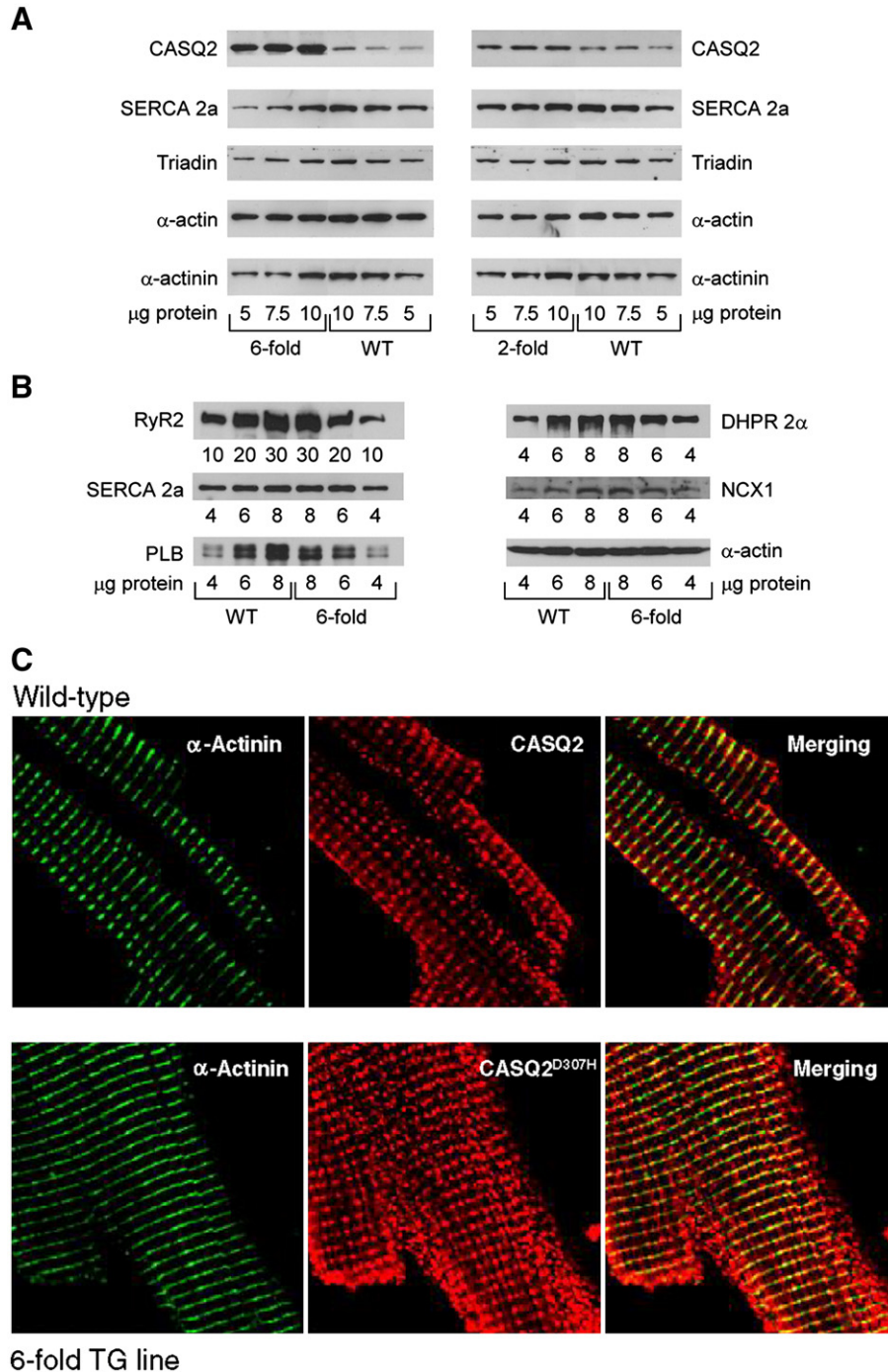


Fig. 1. (A–B) Expression of SR proteins in TG (2- and 6-fold CASQ2<sup>D307H</sup>) and WT hearts. (A) 5–10  $\mu$ g of whole heart homogenates (2- and 6-fold CASQ2<sup>D307H</sup>) were separated on 10% SDS-PAGE gels and probed with antibodies against CASQ2, SERCA 2a, triadin,  $\alpha$ -actin and  $\alpha$ -actinin. (B) 4–8  $\mu$ g (10–30  $\mu$ g for RyR2) of whole heart homogenates (6-fold CASQ2<sup>D307H</sup>) were separated on SDS-PAGE gels and probed with antibodies against RyR2 (4–20% gradient gel), SERCA 2a (10% gel), Phospholamban (PLB; 15% gel), L-type Calcium Channel (DHPR 2 $\alpha$ ; 10% gel) and Na<sup>+</sup>/Ca<sup>2+</sup> Exchanger (NCX1; 10% gel). The 120-kDa full length NCX1 protein is shown [44]. (C) CASQ2 Immunofluorescence in TG and WT myocytes. Mice ventricular cardiomyocytes from WT littermates and TG (6-fold CASQ2<sup>D307H</sup>) were immunostained with mouse anti- $\alpha$ -actinin (1:500) or rabbit anti-calsequestrin (1:200), followed by Alexa 488-conjugated goat anti-mouse (1:300) and Cy<sup>TM</sup>3-conjugated Donkey anti-rabbit (1:300 dilution) antibodies.

## 2.6. Statistical analysis

Cross tabulations with chi-square and yale correction factor or z-test were used as appropriate for categorical variables using a statistical software package (Sigmastat 2.03, Jandel Scientific).

## 3. Results

### 3.1. Generation and characterization of TG mice expressing CASQ2<sup>D307H</sup>

Cardiac-specific expression of the CASQ2<sup>D307H</sup> transgene was achieved using the  $\alpha$ -MHC promoter [21–23]. Out of five positive TG lines containing the transgene, three TG lines (F8, F17, and F21) were propagated for further characterization. Three- to six-month-old mice were used for the studies, except as indicated.

The level of CASQ2<sup>D307H</sup> protein expression in the three TG lines was determined by western blotting using an antibody specific for cardiac CASQ2 (Fig. 1A). Quantification of CASQ2 bands showed that the CASQ2<sup>D307H</sup> protein is expressed 2-, 4- and 6-fold in TG lines F21, F8 and F17, respectively (Fig. 1A and data not shown). These data confirmed that we obtained multiple TG lines with graded expression of the CASQ2<sup>D307H</sup> protein, thus allowing us to compare the effect of expressing different amounts of mutant protein. To determine whether expression of CASQ2<sup>D307H</sup> protein induced alterations in other SR proteins, we determined the levels of RyR2, L-type Calcium Channel (DHPR 2 $\alpha$ ), Na<sup>+</sup>/Ca<sup>2+</sup> Exchanger (NCX1), triadin, SERCA 2a and phospholamban (PLB) in the 4- and 6-fold

lines (Fig. 1B and data not shown). Quantification and correction for loading variations indicated that there were no significant changes in the expression levels of these proteins. Therefore 2- to 6-fold expression of CASQ2<sup>D307H</sup> does not alter the expression levels of related SR Ca<sup>2+</sup> transport proteins. For most of the studies described below we compared the 2-fold and 6-fold over-expressors.

### 3.2. TG cardiac myocytes show subtle structural changes

To determine whether the mutant CASQ2<sup>D307H</sup> was being properly localized to the junctional SR, confocal and electron microscopy (EM) were performed. Imaging of the stained cells revealed the expected patterns of fluorescence for CASQ2 and  $\alpha$ -actinin (Fig. 1C). The CASQ2 staining co-localized with the  $\alpha$ -actinin staining, consistent with the fact that the Z-line ( $\alpha$ -actinin) and junctional SR (CASQ2) are in close proximity to one another. The only difference between the TG and WT staining pattern was that the intensity of CASQ2 staining appeared brighter in the TG myocytes, which is consistent with the over-expression of CASQ2<sup>D307H</sup> in the 6-fold line.

The ultrastructure of myocytes expressing CASQ2<sup>D307H</sup> was very similar to that of WT littermate cells but subtle changes were observed. We observed variations in the size and internal configuration of the jSR cisternae associated with the transverse tubules. In WT myocardium, the jSR is typically in the form of flat cisternae with a dense content, due to calsequestrin, that is condensed into periodic densities (Fig. 2A). Most of the cisternae in the mutant hearts have the same general structure as WT ones (Fig. 2B and C). Some however, show a decrease in the dense content, a general loosening of the content disposition and

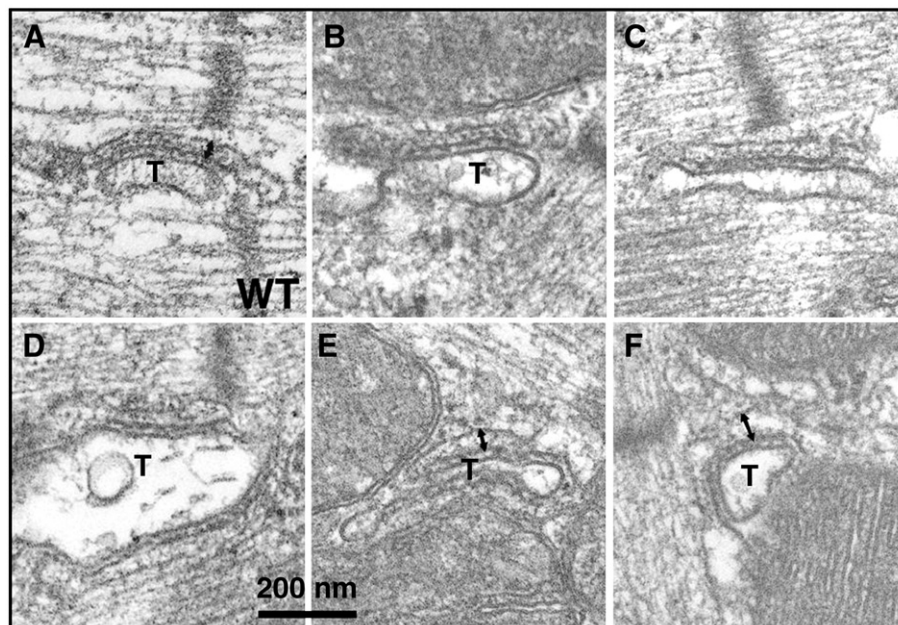


Fig. 2. jSR alterations in CASQ2<sup>D307H</sup> (6-fold) myocytes. (A) In WT myocardium, the jSR cisternae are narrow, they contain a condensed form of CASQ2 and are closely apposed to wide T tubule profiles (T). (B, C) Most of the jSR cisternae in the mutant myocytes are quite similar to those in WT. (D–F) Alterations of jSR in mutant myocytes include a looser appearance of the CASQ2 in the lumen and an increase in width of the cisternae. The double arrows in A, E and F indicate the widening of terminal cisternae in TG myocytes.

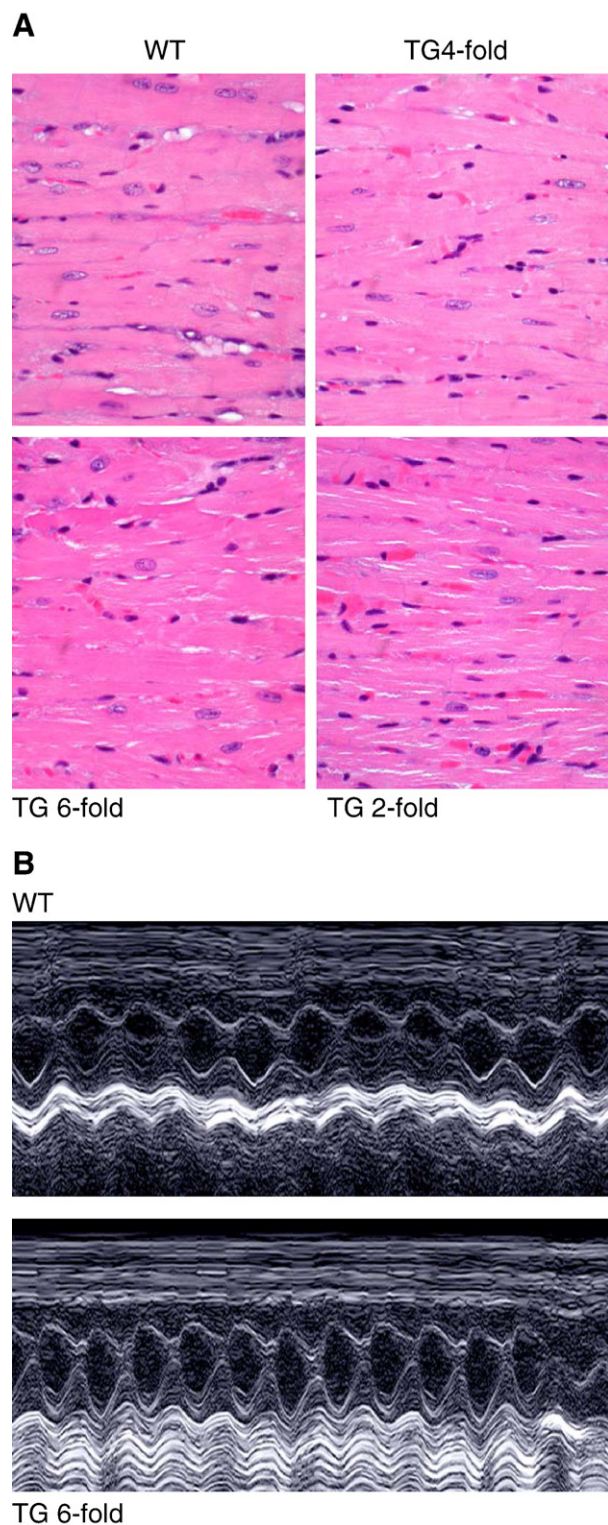


Fig. 3. TG hearts show normal morphology and function is unaltered. (A) Hearts from TG mice (~1 year old) were subjected to standard histological analysis by staining with hematoxylin/eosin. (B) Examples of M-mode echocardiography in 4–6 month old WT and TG mice (6-fold  $CASQ2^{D307H}$ ) showing normal cardiac chamber size and contractility with no evidence of structural abnormalities. Summary of data are shown in Table 1.

some widening of the cisternae (Fig. 2E and F). Some TG myocytes show evidence for a disturbance of T tubules. Normally, T tubules run transversely at the Z lines and have occasional longitudinal branches. In thin sections, each intermyofibrillar space has either none or a single T tubule profile. Some myocytes from  $CASQ2^{D307H}$  TG hearts show multiple T tubule profiles, indicating a convoluted tubule (data not shown).

### 3.3. TG hearts expressing $CASQ2^{D307H}$ show normal overall structure and contractility

We also wanted to make sure that expression of mutant  $CASQ2^{D307H}$  does not cause cardiac hypertrophy/pathology that may complicate our analysis. Several lines of evidence suggest that the TG hearts are essentially normal. First, there was no change in the heart weight-to-body weight ratios of TG ( $4.4 \pm 0.7$  mg/g) compared to WT ( $4.3 \pm 0.3$  mg/g) mice. Second, hearts from TG and WT littermates were subjected to standard histological analysis by staining with hematoxylin/eosin. These hearts appear normal and do not exhibit hypertrophy or fibrosis. Myocyte structure is similar in appearance in all hearts (Fig. 3A). Third, we examined the expression level of genes that are known markers of hypertrophy: atrial natriuretic factor (ANF),  $\beta$ -myosin heavy chain ( $\beta$ -MHC) and skeletal  $\alpha$ -actin by RT-PCR. These genes are typically re-activated under conditions of cardiac hypertrophy, but we did not see activation of these genes in TG hearts (data not shown).

M-mode echocardiography in WT (Fig. 3B, top panel) and TG (lower panel) mice showed normal cardiac chamber and contractility with no evidence of structural abnormalities. There were no differences in the left ventricular end systolic dimension (LV-ESD) and end diastolic dimension (LV-EDD) and fractional shortening (FS) in TG mice compared to their littermate controls (see Table 1).

### 3.4. SR $Ca^{2+}$ content and release

Calsequestrin is a  $Ca^{2+}$  storage protein and expression of mutant  $CASQ2^{D307H}$  could affect calcium stores and release properties. Therefore, we studied the calcium handling properties of myocytes isolated from TG and WT hearts. Caffeine applications (10 mM) were used to assess changes in

Table 1  
Echocardiographic analysis of WT and TG hearts

	WT (n=8)	TG (n=9)
LV-ESD (cm)	$0.15 \pm 0.02$	$0.14 \pm 0.01$
LV-EDD (cm)	$0.32 \pm 0.02$	$0.33 \pm 0.01$
ES-PW (cm)	$0.14 \pm 0.01$	$0.13 \pm 0.01$
ED-PW (cm)	$0.07 \pm 0.01$	$0.07 \pm 0.01$
FS%	$54 \pm 3.3$	$58.1 \pm 3.6$

Data shown represent mean  $\pm$  s.e.m.; left ventricular end systolic dimension (LV-ESD), end diastolic dimension (LV-EDD), posterior wall thickness at end systole and end diastole (ES-PW and ED-PW, respectively) and fractional shortening (FS), a surrogate of systolic function.

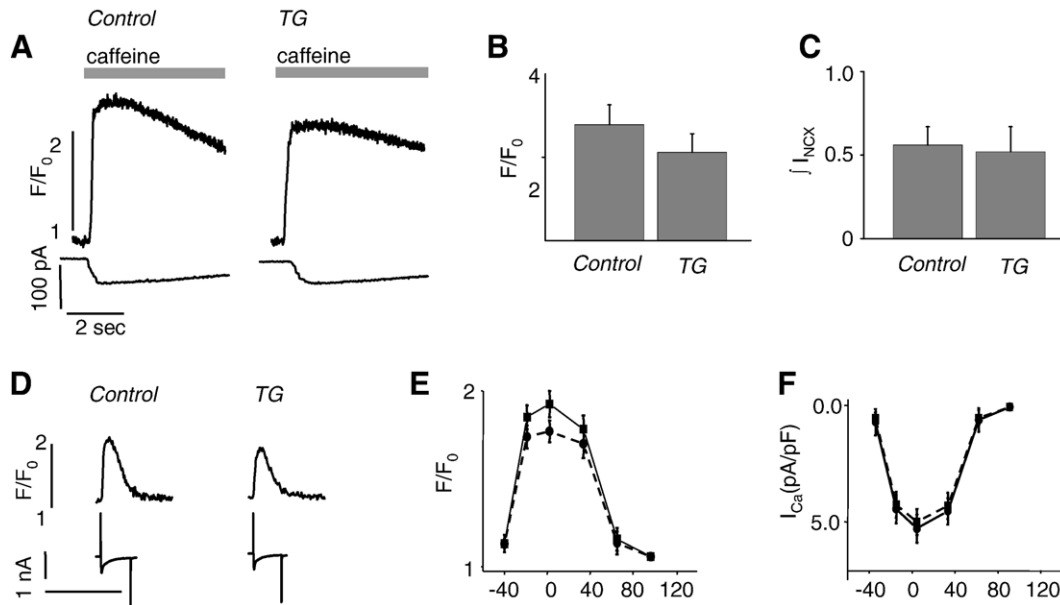


Fig. 4. Intracellular  $\text{Ca}^{2+}$  load,  $I_{\text{Ca}}$  and  $\text{Ca}^{2+}$  transients in WT and TG myocytes (6-fold  $\text{CASQ2}^{\text{D307H}}$ ). (A) Patch clamp recordings were used to measure  $\text{Ca}^{2+}$  transients (top traces) and NCX currents (bottom traces); pooled data for caffeine-induced  $\text{Ca}^{2+}$  transients (B) and NCX currents (C); (D) representative recordings of  $I_{\text{Ca}}$  (bottom traces) and calcium transients (top traces) evoked by depolarization steps from  $-50$  mV MP to  $0$  mV; voltage dependencies of  $\text{Ca}^{2+}$  transients (E) and  $I_{\text{Ca}}$  (F) in ventricular myocytes, isolated from WT (solid lines) and TG (6-fold  $\text{CASQ2}^{\text{D307H}}$ ) hearts (dashed lines).

the total SR  $\text{Ca}^{2+}$  content in each group of isolated myocytes. The relative amounts of  $\text{Ca}^{2+}$  released from the SR after caffeine administration were assessed from changes in both Fluo-3 fluorescence and  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchange current ( $I_{\text{NCX}}$ ) in myocytes dialyzed with the  $\text{Ca}^{2+}$  indicator Fluo-3 (Fig. 4A). Neither the caffeine-induced transients [ $2.39 \pm 0.24$  (WT) vs.  $2.06 \pm 0.22$  (TG)], nor the  $I_{\text{NCX}}$  currents [ $0.55 \pm 0.12$  (WT) vs.  $0.52 \pm 0.15$  (TG)] changed significantly (Fig. 4B–C).

### 3.5. $\text{Ca}^{2+}$ sparks, $\text{Ca}^{2+}$ transients and $I_{\text{Ca}}$

The effects of expressing mutant  $\text{CASQ2}^{\text{D307H}}$  on  $I_{\text{Ca}}$  and intracellular [ $\text{Ca}^{2+}$ ] transients in patch-clamped myocytes are illustrated in Fig. 4D–F. There were no apparent changes in the parameters of  $I_{\text{Ca}}$  in myocytes expressing  $\text{CASQ2}^{\text{D307H}}$  and cells from WT littermates (Fig. 4F, Table 2). The peak amplitude of  $I_{\text{Ca}}$  was nearly identical for all experimental groups of cells. In addition, the time course of  $I_{\text{Ca}}$  decay was similar (Table 2). Thus, expression of  $\text{CASQ2}^{\text{D307H}}$  did not change the characteristics of the  $\text{Ca}^{2+}$  trigger for  $\text{Ca}^{2+}$  release from the SR. However,  $\text{Ca}^{2+}$  transients from TG mice were smaller and had reduced  $\tau_{\text{decay}}$  compared to WT myocytes (Fig. 4D–E and Table 2). Measurement of spontaneous  $\text{Ca}^{2+}$

sparks in permeabilized myocytes from TG mice and their WT littermates showed a trend toward decreased  $\text{Ca}^{2+}$  spark amplitude and a significant increase in  $\text{Ca}^{2+}$  spark frequency (Supplemental data and Table 3).

### 3.6. $\text{Ca}^{2+}$ cycling in rhythmically paced myocytes

The effects of isoproterenol treatment ( $1 \mu\text{mol/L}$ ) on periodic  $\text{Ca}^{2+}$  transients in WT and TG myocytes are illustrated in Fig. 5. To induce arrhythmia in myocytes, we used a modified protocol [30] where the bath solution contains  $100 \mu\text{M}$  of caffeine. The myocytes were stimulated at  $1$  Hz, and membrane potential (MP) changes were recorded in the current-clamp mode. The exposure of WT myocytes to isoproterenol caused an increase in the amplitude of  $\text{Ca}^{2+}$  transients without any apparent disturbances in periodic  $\text{Ca}^{2+}$  cycling (Fig. 5A–B; results are representative of six myocytes). However, in TG myocytes expressing  $\text{CASQ2}^{\text{D307H}}$ , the amplitude of  $\text{Ca}^{2+}$  current-induced  $\text{Ca}^{2+}$  transients was reduced (Fig. 5A). The action potential duration between control and TG myocytes was not significantly different ( $\text{APD}_{90}$  is  $57 \pm 24$  for WT and  $45 \pm 19$  for TG animals). Isoproterenol administration in the presence of caffeine

Table 2  
Parameters of  $I_{\text{Ca}}$  and Ca transients

	$I_{\text{Ca}}$			Ca transients			N of cells
	Peak amplitude (pA/pF)	$\tau_{\text{fast}}$ (ms)	$\tau_{\text{slow}}$ (ms)	$F/F_0$	Rise time (ms)	$\tau_{\text{decay}}$ (ms)	
WT	$-4.88 \pm 0.55$	$15.7 \pm 4.7$	$76 \pm 25$	$1.96 \pm 0.16$	$28 \pm 4$	$320 \pm 16$	12 (7 animals)
TG	$-4.19 \pm 0.55$	$16.1 \pm 4.2$	$75 \pm 23$	$1.61 \pm 0.14^*$	$24 \pm 3$	$268 \pm 15^*$	9 (6 animals)

\*Significantly different at  $P < 0.05$ , compared with WT.

Table 3

Characteristics of spontaneous  $\text{Ca}^{2+}$  sparks recorded in permeabilized myocytes isolated from WT mouse hearts and TG mice expressing  $\text{CASQ2}^{\text{D307H}}$

	Amplitude $\Delta F/F_0$	Rise time, ms	HA width, $\mu\text{m}$	Frequency, $100 \mu\text{m}^{-1} \text{s}^{-1}$	N of sparks	N of cells	N of mice
WT	$0.78 \pm 0.01$	$8.0 \pm 0.1$	$2.20 \pm 0.02$	$7.7 \pm 0.5$	1152	80	6
TG	$0.75 \pm 0.01$	$8.7 \pm 0.1^*$	$2.24 \pm 0.02$	$10.2 \pm 0.8^*$	1077	62	3

\*Significantly different at  $P < 0.05$ , One way ANOVA.

caused profound disturbances in  $\text{Ca}^{2+}$  cycling manifested by extra-systolic, spontaneous  $\text{Ca}^{2+}$  transients. As seen in the line-scan images (Fig. 5B), spontaneous release usually originated locally and then propagated through the cell as a regenerative  $\text{Ca}^{2+}$  wave. Importantly, the membrane potential traces showed delayed afterdepolarizations (DADs) and the occasional generation of action potentials at the time of spontaneous  $\text{Ca}^{2+}$  transients. Similar results were obtained in four other myocytes. These results indicate that expression of  $\text{CASQ2}^{\text{D307H}}$  destabilizes the  $\text{Ca}^{2+}$  release mechanism, leading to spontaneous, premature discharges of SR  $\text{Ca}^{2+}$  stores in myocytes undergoing periodic pacing.

### 3.7. Complex ventricular arrhythmias are triggered in TG hearts by isoproterenol and caffeine

Continuous ECG monitoring prior to drug challenge did not reveal any significant spontaneous ventricular arrhythmias in WT mice. In contrast,  $\text{CASQ2}^{\text{D307H}}$  mice had more

spontaneous supraventricular ectopic beats at baseline than WT mice (cumulative numbers of observations were 378 vs. 8 in TG and WT mice, respectively,  $P < 0.001$ ).

After challenge with isoproterenol alone or in combination with caffeine, mutant  $\text{CASQ2}^{\text{D307H}}$  mice developed significantly more supraventricular and junctional ectopic beats compared to WT (cumulative numbers of observations for junctional ectopic beats were 2862 vs. 39 in TG and WT mice, respectively,  $P < 0.001$ ).  $\text{CASQ2}^{\text{D307H}}$  mice did not have an increased number of simple ventricular arrhythmias compared to WT (Fig. 6A). However, after drug challenge, the number of total complex ventricular arrhythmias was increased 4-fold ( $P < 0.001$ ) in  $\text{CASQ2}^{\text{D307H}}$  mice compared to WT mice (Fig. 6A). Furthermore, there was a significant increase ( $P < 0.05$ ) in the number of  $\text{CASQ2}^{\text{D307H}}$  mice that developed complex ventricular arrhythmias (Fig. 6B), including non-sustained ventricular tachycardia, compared to WT mice (Fig. 6C and D).

## 4. Discussion

CPVT is a potentially devastating condition that can lead to sudden cardiac death even in very young children after physical or emotional stress. CPVT can be caused by mutations in either the ryanodine receptor (RyR2) or the  $\text{CASQ2}$  gene [15,17,19,32–35]. There are several different RyR2 mutations that are linked to CPVT and they are all dominant, while there are fewer known  $\text{CASQ2}$  mutations and these are all recessive [15,17].

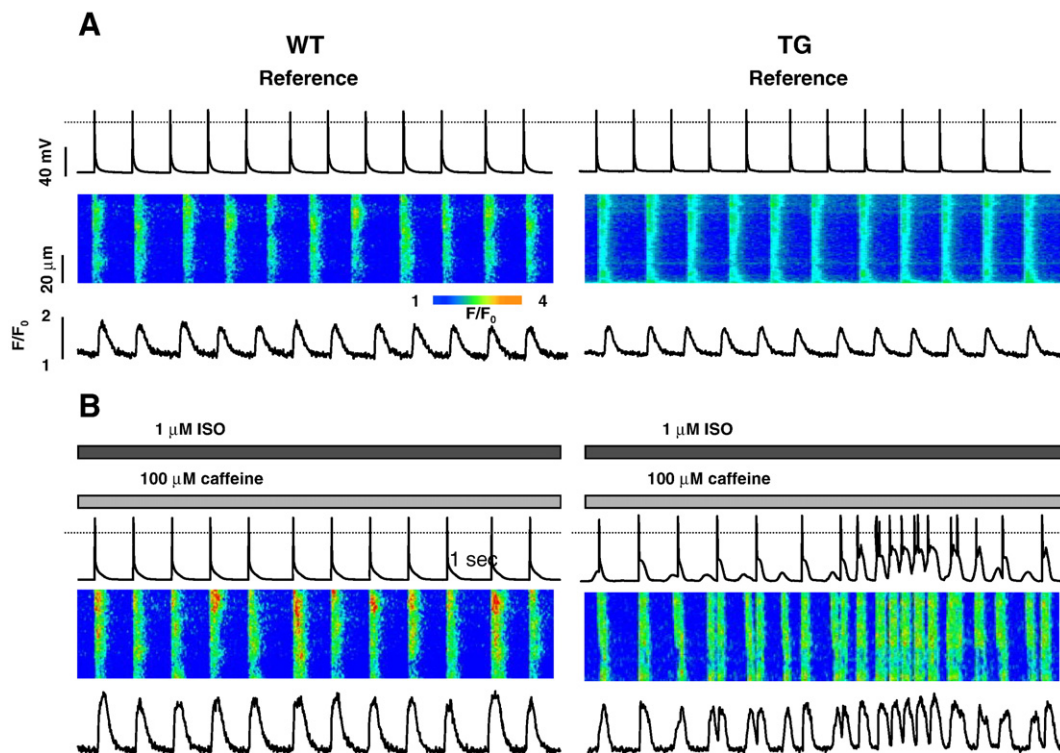


Fig. 5. TG myocytes (6-fold  $\text{CASQ2}^{\text{D307H}}$ ) show spontaneous  $\text{Ca}^{2+}$  transients. Recording of membrane potential (top traces), along with line-scan confocal images (middle traces) and temporal profiles (bottom traces) in myocytes before (A) and after superfusion (B) with  $1 \mu\text{M}$  of ISO and  $100 \mu\text{M}$  of caffeine.

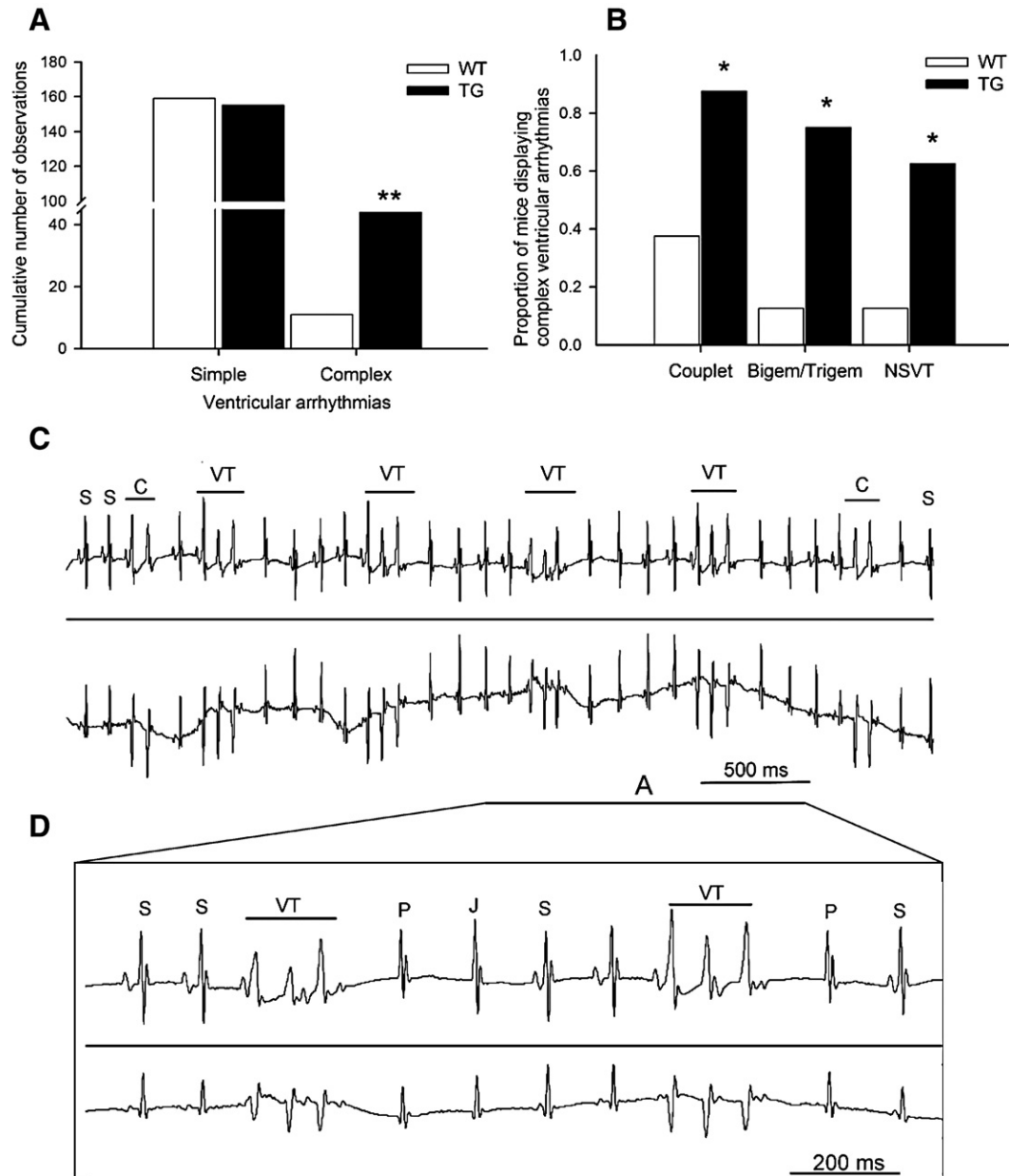


Fig. 6. Mice expressing mutant CASQ2<sup>D307</sup> displayed more complex forms of ventricular arrhythmia, including non-sustained ventricular tachycardia, compared to aged-matched WT mice. (A) Cumulative number of simple and complex ventricular arrhythmias in TG ( $n=8$ ) from 2-, 4- and 6-fold over-expression and age-matched WT ( $n=8$ ) mice. Under light anesthesia, mice were challenged with IP isoproterenol injection alone or in combination with caffeine injection. Simple ventricular arrhythmias included single isolated ventricular ectopic beats. Complex ventricular arrhythmias included: couplet, bigeminy, trigeminy and non-sustained ventricular tachycardia. \*\* $P<0.001$  TG vs. WT mice. (B) Higher proportion of mice expressing CASQ2<sup>D307H</sup> ( $n=8$ ), which displayed complex forms of ventricular arrhythmias compared to WT ( $n=8$ ), following treatment with isoproterenol alone or in combination with caffeine injection. \* $P<0.05$  TG vs. WT mice. (C) Representative ECG tracings (lead 1 top and lead 3 bottom) in a TG mouse (6-fold CASQ2<sup>D307H</sup>) displaying a run of non-sustained polymorphic ventricular tachycardias (NSVT). Please note the presence of 2 couplets before and after the run of NSVT, which can be considered as an attempted, but aborted VT. (D) Magnification of section A of the ECG tracings (lead 1 top and lead 3 bottom) displaying 2 non-sustained polymorphic ventricular tachycardias. Please note a junctional escape beat (i.e., compensatory pause) following each NSVT. S: sinus beat; P: compensatory pause; J: Junctional ectopic beat; VT: ventricular tachycardia.

In the present study, we chose to introduce increasing amounts of mutant CASQ2<sup>D307H</sup> to compete with the endogenous WT CASQ2 protein to determine its effect on Ca<sup>2+</sup> handling and heart function. Since the CASQ2<sup>D307H</sup> mutation is recessive, we predicted that low amounts of CASQ2<sup>D307H</sup> would not significantly affect heart function, while higher amounts of CASQ2<sup>D307H</sup> would out-compete the

endogenous WT CASQ2 and lead to ventricular arrhythmias. Our findings show that TG mice did not develop a significant amount of spontaneous ventricular arrhythmias under resting conditions. However, when challenged with catecholamines, the TG hearts developed a much higher level of complex ventricular arrhythmias compared to WT hearts (Fig. 6A). These complex VEBs included couplets, bigeminy, trigeminy,

as well as non-sustained ventricular tachycardia, both monomorphic and polymorphic, consistent with the CPVT phenotype observed in patients in that they are triggered by catecholamines. We observed arrhythmias in all 3 TG lines and did not observe a graded response between lines.

The predisposition to complex ventricular arrhythmias in our TG mice could be due to the D307H mutation itself and/or increased expression of CASQ2 in the heart. Recent studies showed that over-expression of WT mouse CASQ2 (20-fold) and canine CASQ2 (10-fold) in the mouse hearts can cause mild to severe cardiac hypertrophy and result in an increase in SR Ca<sup>2+</sup> storage capacity [36,37]. Despite an increase in Ca<sup>2+</sup> storage capacity, Ca<sup>2+</sup> release was severely compromised in these mice. These data suggested that excessive amounts of CASQ2 can also lead to abnormally high levels of Ca<sup>2+</sup> storage but reduced Ca<sup>2+</sup> release. In contrast, we did not observe cardiac hypertrophy or pathology by increasing CASQ2 levels from 2- to 6-fold in TG hearts. This was supported by several lines of evidence, including histological data showing normal morphology and structure. In addition, expression of CASQ2<sup>D307H</sup> did not cause an increase in SR Ca<sup>2+</sup> storage. These facts represent a striking difference between the CASQ2<sup>WT</sup> and CASQ2<sup>D307H</sup> expression models suggesting that the mutant protein behaves very differently from the WT protein.

Biochemical studies on purified CASQ2<sup>D307H</sup> mutant protein showed that the D307H mutation affects the Ca<sup>2+</sup>-dependent conformation of the protein and the ability of CASQ2 to interact with junctin and triadin [38]. An additional possibility is that the mutation may also affect the ability of CASQ2 to polymerize into long polymers that appear to be crucial to its buffering capacity [39,40]. Our findings suggest that the expression of CASQ2<sup>D307H</sup> destabilizes the Ca<sup>2+</sup> release mechanism, possibly by interfering with the CASQ2-triadin and CASQ2-junctin interactions that regulate RyR2 function [6], leading to spontaneous, premature discharges of SR Ca<sup>2+</sup> stores in myocytes undergoing periodic pacing. Indeed, the membrane potential traces of TG myocytes showed delayed afterdepolarizations (DADs) and the occasional generation of action potentials at the time of spontaneous Ca<sup>2+</sup> transients (Fig. 5). Increased spontaneous Ca<sup>2+</sup> release leads to the development of DADs through activation of depolarizing membrane currents via the Na–Ca exchange. These oscillations in membrane potential are thought to be the underlying cause of ventricular arrhythmias [20,41–43] and would also appear to be the mechanism underlying the complex ventricular arrhythmias observed in our TG mice.

In conclusion, the findings of the present study demonstrate that over-expression of CASQ2<sup>D307H</sup> in the mouse heart results in abnormal myocyte Ca<sup>2+</sup> handling and complex ventricular arrhythmias that could contribute to the CPVT phenotype observed in human patients. Our study provides important evidence to establish a link between a mutation in CASQ2 and complex ventricular arrhythmias that are consistent with the CPVT phenotype.

Limitations: a potential limitation associated with our methodology was the lack of evaluation of arrhythmias in conscious

mice. However, one of the main advantages of recording ECG in anesthetized mice is the ability to record from multiple leads to improve the diagnostic specificity, which can be technically very difficult with telemetric devices in conscious and exercising mice.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.cardiores.2007.03.002](https://doi.org/10.1016/j.cardiores.2007.03.002).

## References

- [1] Cala SE, Jones LR. Rapid purification of calsequestrin from cardiac and skeletal muscle sarcoplasmic reticulum vesicles by Ca<sup>2+</sup>-dependent elution from phenyl–sepharose. *J Biol Chem* 1983;258:11932–6.
- [2] Campbell KP, MacLennan DH, Jorgensen AO, Mintzer MC. Purification and characterization of calsequestrin from canine cardiac sarcoplasmic reticulum and identification of the 53,000 Da glycoprotein. *J Biol Chem* 1983;258:1104–97.
- [3] Scott BT, Simmerman HK, Collins JH, Nadal-Ginard B, Jones LR. Complete amino acid sequence of canine cardiac calsequestrin deduced by cDNA cloning. *J Biol Chem* 1988;263:8958–64.
- [4] Yano K, Zarain-Herzberg A. Sarcoplasmic reticulum calsequestrins: structural and functional properties. *Mol Cell Biochem* 1994;135:61–70.
- [5] Ostwald TJ, MacLennan DH, Dorrington KJ. Effects of cation binding on the conformation of calsequestrin and the high affinity calcium-binding protein of sarcoplasmic reticulum. *J Biol Chem* 1974;249:5867–71.
- [6] Györke I, Hester N, Jones LR, Györke S. The role of calsequestrin, triadin, and junctin in conferring cardiac ryanodine receptor responsiveness to luminal calcium. *Biophys J* 2004;86:2121–8.
- [7] Szegedi C, Sarkozi S, Herzog A, Jona I, Varsanyi M. Calsequestrin: more than ‘only’ a luminal Ca<sup>2+</sup> buffer inside the sarcoplasmic reticulum. *Biochem J* 1999;337:19–22.
- [8] Beard NA, Laver DR, Dulhunty AF. Calsequestrin and the calcium release channel of skeletal and cardiac muscle. *Prog Biophys Mol Biol* 2004;85:33–69.
- [9] Franzini-Armstrong C, Kenney LJ, Varriano-Marston E. The structure of calsequestrin in triads of vertebrate skeletal muscle: a deep-etch study. *J Cell Biol* 1987;105:49–56.
- [10] Jorgensen AO, Shen AC, Arnold W, McPherson PS, Campbell KP. The Ca<sup>2+</sup>-release channel/ryanodine receptor is localized in junctional and corbular sarcoplasmic reticulum in cardiac muscle. *J Cell Biol* 1993;120:969–80.
- [11] Kobayashi YM, Alseikhan BA, Jones LR. Localization and characterization of the calsequestrin-binding domain of triadin 1. Evidence for a charged beta-strand in mediating the protein–protein interaction. *J Biol Chem* 2000;275:17639–46.
- [12] Franzini-Armstrong C, Protasi F. Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. *Physiol Rev* 1997;77:629–99.
- [13] Zhang L, Kelley J, Schmeisser G, Kobayashi YM, Jones LR. Complex formation between junctin, triadin, calsequestrin, and the ryanodine receptor. Proteins of the cardiac junctional sarcoplasmic reticulum membrane. *J Biol Chem* 1997;272:23389–97.
- [14] Terentyev D, Cala SE, Houle TD, Viatchenko-Karpinski S, Györke I, Terentyeva R, et al. Triadin overexpression stimulates excitation–

- contraction coupling and increases predisposition to cellular arrhythmia in cardiac myocytes. *Circ Res* 2005;96:651–8.
- [15] di Barletta MR, Viatchenko-Karpinski S, Nori A, Memmi M, Terentyev D, Turcato F, et al. Clinical phenotype and functional characterization of CASQ2 mutations associated with catecholaminergic polymorphic ventricular tachycardia. *Circulation* 2006;114:1012–9.
- [16] Eldar M, Pras E, Lahat H. A missense mutation in the CASQ2 gene is associated with autosomal-recessive catecholamine-induced polymorphic ventricular tachycardia. *Trends Cardiovasc Med* 2003;13:148–51.
- [17] Terentyev D, Nori A, Santoro M, Viatchenko-Karpinski S, Kubalova Z, Györke I, et al. Abnormal interactions of calsequestrin with the ryanodine receptor calcium release channel complex linked to exercise-induced sudden cardiac death. *Circ Res* 2006;98:1151–8.
- [18] Lahat H, Eldar M, Levy-Nissenbaum E, Bahan T, Friedman E, Khoury A, et al. Autosomal recessive catecholamine- or exercise-induced polymorphic ventricular tachycardia: clinical features and assignment of the disease gene to chromosome 1p13–21. *Circulation* 2001;103:2822–7.
- [19] Lahat H, Pras E, Olender T, Avidan N, Ben Asher E, Man O, et al. A missense mutation in a highly conserved region of CASQ2 is associated with autosomal recessive catecholamine-induced polymorphic ventricular tachycardia in Bedouin families from Israel. *Am J Hum Genet* 2001;69:1378–84.
- [20] Viatchenko-Karpinski S, Terentyev D, Györke I, Terentyeva R, Volpe P, Priori SG, et al. Abnormal calcium signaling and sudden cardiac death associated with mutation of calsequestrin. *Circ Res* 2004;94:471–7.
- [21] Gulick J, Subramaniam A, Neumann J, Robbins J. Isolation and characterization of the mouse cardiac myosin heavy chain genes. *J Biol Chem* 1991;266:9180–5.
- [22] Subramaniam A, Jones WK, Gulick J, Wert S, Neumann J, Robbins J. Tissue-specific regulation of the alpha-myosin heavy chain gene promoter in transgenic mice. *J Biol Chem* 1991;266:24613–20.
- [23] Subramaniam A, Gulick J, Neumann J, Knotts S, Robbins J. Transgenic analysis of the thyroid-responsive elements in the alpha-cardiac myosin heavy chain gene promoter. *J Biol Chem* 1993;268:4331–6.
- [24] Babu GJ, Zheng Z, Natarajan P, Wheeler D, Janssen PM, Periasamy M. Overexpression of sarcolipin decreases myocyte contractility and calcium transient. *Cardiovasc Res* 2005;65:177–86.
- [25] Zhao W, Frank KF, Chu G, Gerst MJ, Schmidt AG, Ji Y, et al. Combined phospholamban ablation and SERCA1a overexpression result in a new hyperdynamic cardiac state. *Cardiovasc Res* 2003;57:71–81.
- [26] Terentyev D, Viatchenko-Karpinski S, Valdivia HH, Escobar AL, Györke S. Luminal  $Ca^{2+}$  controls termination and refractory behavior of  $Ca^{2+}$ -induced  $Ca^{2+}$  release in cardiac myocytes. *Circ Res* 2002;91:414–20.
- [27] Xu Y, Zhang Z, Timofeyev V, Sharma D, Xu D, Tuteja D, et al. The effects of intracellular  $Ca^{2+}$  on cardiac  $K^+$  channel expression and activity: novel insights from genetically altered mice. *J Physiol* 2005;562:745–58.
- [28] Chaves AA, Dech SJ, Nakayama T, Hamlin RL, Bauer JA, Carnes CA. Age and anesthetic effects on murine electrocardiography. *Life Sci* 2003;72:2401–12.
- [29] Okamoto Y, Chaves A, Chen J, Kelley R, Jones K, Weed HG, et al. Transgenic mice with cardiac-specific expression of activating transcription factor 3, a stress-inducible gene, have conduction abnormalities and contractile dysfunction. *Am J Pathol* 2001;159:639–50.
- [30] Cerrone M, Colombi B, Santoro M, di Barletta MR, Scelsi M, Villani L, et al. Bidirectional ventricular tachycardia and fibrillation elicited in a knock-in mouse model carrier of a mutation in the cardiac ryanodine receptor. *Circ Res* 2005;96:e77–82.
- [31] Liu N, Colombi B, Memmi M, Zissimopoulos S, Rizzi N, Negri S, et al. Arrhythmogenesis in catecholaminergic polymorphic ventricular tachycardia: insights from a RyR2 R4496C knock-in mouse model. *Circ Res* 2006;99:292–8.
- [32] Laitinen PJ, Brown KM, Piippo K, Swan H, Devaney JM, Brahmabhatt B, et al. Mutations of the cardiac ryanodine receptor (RyR2) gene in familial polymorphic ventricular tachycardia. *Circulation* 2001;103:485–90.
- [33] Laitinen PJ, Swan H, Kontula K. Molecular genetics of exercise-induced polymorphic ventricular tachycardia: identification of three novel cardiac ryanodine receptor mutations and two common calsequestrin 2 amino-acid polymorphisms. *Eur J Hum Genet* 2003;11:888–91.
- [34] Priori SG, Napolitano C, Tiso N, Memmi M, Vignati G, Bloise R, et al. Mutations in the cardiac ryanodine receptor gene (hRyR2) underlie catecholaminergic polymorphic ventricular tachycardia. *Circulation* 2001;103:196–200.
- [35] Priori SG, Napolitano C, Memmi M, Colombi B, Drago F, Gasparini M, et al. Clinical and molecular characterization of patients with catecholaminergic polymorphic ventricular tachycardia. *Circulation* 2002;106:69–74.
- [36] Jones LR, Suzuki YJ, Wang W, Kobayashi YM, Ramesh V, Franzini-Armstrong C, et al. Regulation of  $Ca^{2+}$  signaling in transgenic mouse cardiac myocytes overexpressing calsequestrin. *J Clin Invest* 1998;101:1385–93.
- [37] Sato Y, Ferguson DG, Sako H, Dorn GW, Kadambi VJ, Yatani A, et al. Cardiac-specific overexpression of mouse cardiac calsequestrin is associated with depressed cardiovascular function and hypertrophy in transgenic mice. *J Biol Chem* 1998;273:28470–7.
- [38] Houle TD, Ram ML, Cala SE. Calsequestrin mutant D307H exhibits depressed binding to its protein targets and a depressed response to calcium. *Cardiovasc Res* 2004;64:227–33.
- [39] Park H, Wu S, Dunker AK, Kang C. Polymerization of calsequestrin. Implications for  $Ca^{2+}$  regulation. *J Biol Chem* 2003;278:16176–82.
- [40] Wang S, Trumble WR, Liao H, Wesson CR, Dunker AK, Kang CH. Crystal structure of calsequestrin from rabbit skeletal muscle sarcoplasmic reticulum. *Nat Struct Biol* 1998;5:476–83.
- [41] Bers DM. Excitation–Contraction Coupling and Cardiac Contractile Force. 2nd ed. Dordrecht, The Netherlands: Kluwer Academic Publishers; 2001.
- [42] Cranfield PF, Aronson RS. Cardiac Arrhythmias: The Role of Triggered Activity and Other Mechanisms. Mount Kisco, NY: Futura Publishing Company, Inc; 1988.
- [43] Priori SG, Corr PB. Mechanisms underlying early and delayed afterdepolarizations induced by catecholamines. *Am J Physiol* 1990;258:H1796–805.
- [44] Philipson KD, Longoni S, Ward R. Purification of the cardiac  $Na^+Ca^{2+}$  exchange protein. *Biochim Biophys Acta* 1988;945:206–98.