Cardiovascular Research (2015) **108**, 299–311
doi:10.1093/cvr/cvv222

Ablation of HRC alleviates cardiac arrhythmia and improves abnormal Ca handling in CASQ2 knockout mice prone to CPVT

Bin Liu[1], Hsiang-Ting Ho[1], Lucia Brunello[1], Sathya D. Unudurthi[1], Qing Lou[1], Andriy E. Belevych[1], Lan Qian[1], Do Han Kim[2], Chunghee Cho[2], Paul M. L. Janssen[1], Thomas J. Hund[1], Bjorn C. Knollmann[3], Evangelia G. Kranias[4], and Sándor Györke[1]*

[1]Department of Physiology and Cell Biology, College of Medicine, and Davis Heart and Lung Research Institute, The Ohio State University, 473 W. 12th Avenue, Columbus, OH 43210, USA; [2]School of Life Sciences and Systems Biology Research Center, Gwangju Institute of Science and Technology (GIST), Gwangju, Korea; [3]Division of Clinical Pharmacology, Vanderbilt University Medical School, Nashville, TN 37232, USA; and [4]Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA

Received 18 May 2015; revised 14 September 2015; accepted 17 September 2015; online publish-ahead-of-print 26 September 2015

Time for primary review: 37 days

Aims Cardiac calsequestrin (CASQ2) and histidine-rich Ca-binding protein (HRC) are sarcoplasmic reticulum (SR) Ca-binding proteins that regulate SR Ca release in mammalian heart. Deletion of either CASQ2 or HRC results in relatively mild phenotypes characterized by preserved cardiac structure and function, although CASQ2 knockout (KO), or Cnull, shows increased arrhythmia burden under conditions of catecholaminergic stress. We hypothesized that given the apparent overlap of functions of CASQ2 and HRC, simultaneous ablation of both would deteriorate the cardiac phenotype compared with the single knockouts.

Methods and results In contrast to this expectation, double knockout (DKO) mice lacking both CASQ2 and HRC exhibited normal cardiac ejection fraction and ultrastructure. Moreover, the predisposition to catecholamine-dependent arrhythmia that characterizes the Cnull phenotype was alleviated in the DKO mice. At the myocyte level, DKO mice displayed Ca transients of normal amplitude; additionally, the frequency of spontaneous Ca waves and sparks in the presence of isoproterenol were decreased markedly compared with Cnull. Furthermore, restitution of SR Ca release was slowed in DKO myocytes compared with Cnull cells.

Conclusion Our results suggest that rather than being functionally redundant, CASQ2 and HRC modulate cardiac ryanodine receptor-mediated (RyR2) Ca release in an opposing manner. In particular, while CASQ2 stabilizes RyR2 rendering it refractory in the diastolic phase, HRC enhances RyR2 activity facilitating RyR2 recovery from refractoriness.

Keywords Excitation–contraction coupling • Ryanodine receptor • Catecholaminergic polymorphic ventricular tachycardia

1. Introduction

The process of excitation–contraction (EC) coupling that governs the contractile function of the heart is served by elaborate molecular machinery. A key protein involved in this process is the cardiac ryanodine receptor (RyR2) that forms a Ca release channel on the membrane of the sarcoplasmic reticulum (SR). The homotetrameric RyR2 is at the centre of a multi-molecular complex composed of ~15 proteins, including FK-506-binding protein (FKBP), calmodulin (CaM), and sorcin on the cytosolic side and triadin (TRD), junctin (JCN), calsequestrin (CASQ2), and histidine-rich Ca-binding protein (HRC) on the luminal side.[1] The precise functional roles of most of the components of the RyR2 complex and how they interact to regulate the release of Ca via RyR2s remain poorly understood.

CASQ2 and HRC are low-affinity Ca-binding proteins present in the cardiac SR.[2,3] CASQ2 is localized to the junctional SR (jSR) where it forms strings of polymers for sequestering Ca; CASQ2 also controls RyR2 activity via interactions with TRD and JCN.[3] Specifically, it has been suggested that CASQ2 inhibits RyR2 upon the decrease of SR luminal Ca that follows systolic Ca release, thereby rendering RyR2 refractory in the diastolic phase.[4,5] Human and mice missing functional CASQ2, while preserving normal cardiac structure and contractile performance, become vulnerable to catecholaminergic polymorphic ventricular tachycardia (CPVT),[6] a familial arrhythmogenic syndrome

* Corresponding author. Tel: +1 614 292 3969; fax: +1 614 292 4888, E-mail: sandor.gyorke@osumc.edu

manifested during exercise or emotional stress. At the cellular level, loss of CASQ2 increases the propensity towards spontaneous Ca sparks and waves.[4,6] This increase in diastolic Ca release has been attributed to impaired refractoriness of RyR2.[7] HRC is distributed across the entire SR space rather than being restricted to the jSR. As a low-affinity, Ca-binding protein, it also buffers SR Ca; moreover, HRC also interacts with TRD in a Ca-dependent manner to modulate RyR2 function.[2] Additionally, HRC binds to the SR Ca ATPase (SERCA2a) pump, thereby inhibiting Ca reuptake into the SR. Similar to the deletion of CASQ2, the deletion of HRC in mice does not result in substantial structural or functional changes in the heart, although HRC-deficient myocytes show increased frequency of Ca waves and sparks. Additionally, HRC ablation increases susceptibility to cardiac hypertrophy induced by pressure-overload or chronic isoproterenol (ISO) treatment in mice.[8,9] Moreover, a genetic variant of this protein is associated with ventricular arrhythmias in human patients with cardiomyopathy.[10] Thus, CASQ2 and HRC exhibit certain similarities in functional properties, and their deletion appears to result in comparable alterations in Ca handling and cardiac phenotype.

In this study, we examined the consequences of combined deletion of CASQ2 and HRC. Given the possible overlap of functions of these two SR Ca-binding proteins, one might expect that simultaneous deletion of both of them would markedly exacerbate arrhythmias and result in overall worsening of the cardiac phenotype compared with the single-protein knockouts. Rather than increasing arrhythmia vulnerability and exacerbating cardiac dysfunction, double CASQ2 × HRC knockout (DKO) mice demonstrated decreased arrhythmia vulnerability compared with the Cnull mouse. Our results suggest a novel mechanism of reciprocal regulation of RyR2 by CASQ2 and HRC, thereby alterations in Ca handling caused by the deletion of one of these proteins are partly offset by the deletion of the other.

2. Methods

For details regarding methods, refer to the Supplementary material online, *Methods*.

2.1 Ethical approval

All animal procedures were approved by The Ohio State University Institutional Animal Care and Use Committee. The study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011).

2.2 Generation of DKO mouse models

Cnull mice were crossbred with HRC KO (Hnull) mice to obtain DKO mice.[6,8]

2.3 Transthoracic echocardiography (Echo)

Mice were lightly anaesthetized. *In vivo* cardiac function was assessed using a Visualsonic Vevo 2100 imaging system. Data were analysed using VisualSonic Software.

2.4 Electrocardiographic recordings

2.4.1 Surface ECG

After the mice were lightly anaesthetized, ECG was recorded before and after the administration of catecholamines. ECG traces were analysed using LabChart 7 Pro (AD Instruments).

2.4.2 Telemetry

Mice were implanted with ETA-F10 radiotelemetres through surgery.[11] ECGs were recorded continuously from conscious mice. ECG traces were analysed using P3 Plus software (Ponemah).

2.5 Cardiomyocyte isolation and confocal Ca imaging

Mouse ventricular myocytes were isolated as previously described.[12,13] Confocal imaging was recorded using an Olympus Fluoview 1000 confocal microscope. The ventricular myocytes were loaded with ~8 μM Fluo-3 AM (Invitrogen, Carlsbad, CA, USA) for 25 min at room temperature, followed by 25 min of incubation in fresh Tyrode solution, to wash out the dye, and allow the de-esterification of the dye in the cells. Fluo-3 was excited with the 488 nm line of an argon laser, and emission was collected at 500–600 nm. Fluo-3 fluorescence was recorded in the line-scan mode of the confocal microscope. The myocytes were paced at 0.5, 2, or 4 Hz using extracellular platinum electrodes. To assess the SR Ca load, 20 mM caffeine was applied at the end of the experiments.

2.6 Transmission electron microscopy

Transmission electron microscopy (TEM) was performed on a FEI Tecnai G2 Spirit TEM. The quantification of SR was based on the established stereology point and intersection counting technique.[14]

2.7 Western blots

Quantitative western blots were performed as previously described.[13]

2.8 Cardiac muscles preparation

Mice were injected with heparin (Intraperitoneal or IP injection, 40 μL heparin sodium 10 000 U/mL, APP Pharmaceuticals, LLC, Schaumburg, IL, USA) 10 min before receiving anaesthesia (IP, 70 mg/kg, pentobarbital sodium, Lundbeck Inc., Deerfield, IL, USA). The hearts were rapidly excised and placed in a modified Krebs–Henseleit (KH) buffer, containing (in mM) 120 NaCl, 5 KCl, 2 $MgSO_4$, 1.2 NaH_2PO_4, 20 $NaHCO_3$, 0.25 Ca, and 10 glucose (pH 7.4), equilibrated with 95% O_2–5% CO_2. 2,3-Butanedione monoxime (BDM, 20 mM) was also added to the dissection buffer to minimize cutting injury.[15] Thin, uniform, non-branched trabeculae or small papillary muscles were carefully dissected from the right ventricle. The muscles were mounted between a force transducer and a micromanipulator as previously described.[16] Fresh KH buffer lacking BDM and containing elevated Ca concentration (1.5 mM) was used to perfuse the muscles. Muscles were stretched to an optimal length, i.e. where an increase in length led to approximately equal increases in both the resting and the active developed tension. It has been shown that this muscle length corresponds to a sarcomere length of around 2.2–2.3 μm.[17] Muscles were then allowed to stabilize for at least 15 min at a baseline pacing frequency of 4 Hz before starting the experimental protocol. All experiments were performed at 37°C. The developed tension was recorded with LabView software and normalized to the cross-sectional area of the muscle, to allow comparison between muscles of different dimensions.

2.9 Statistical analysis

Results are expressed as Mean ± SEM. Statistical significance was determined using one-way or two-way ANOVA followed by Tukey's *post hoc* test (nested ANOVA when appropriate) or unpaired Student's *t*-test. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1 Normal cardiac function was preserved in DKO mice

To examine the consequences of simultaneous deletion of the two SR Ca-binding proteins, CASQ2 and HRC, we crossbred Cnull mice and Hnull mice to generate CASQ2-HRC double KO mice. The absence of both CASQ2 and HRC did not precipitate premature mortality. *In vivo* cardiac function was assessed by Echo in DKO in comparison with the three other groups (WT, Cnull, and Hnull). Consistent with previous reports, 2-month-old Cnull and Hnull mice showed preserved cardiac ejection fraction (EF) compared with WT mice (62 ± 3, 65 ± 3, and 64 ± 2%, respectively, *Figure 1B*). EF in age-matched DKO mice was not significantly different from WT or the two single-protein KO mice (60 ± 3%) (*Figure 1B*). Furthermore, left ventricular end-diastolic dimensions (LVEDD) and end-systolic dimensions (LVESD) were similar among the groups (*Figure 1B*). The heart-weight to body-weight ratio for DKO showed a small but significant increase compared with WT (*Figure 1C*). However, DKO hearts exhibited no cellular hypertrophy (see Supplementary material online, *Figure S1*) and no obvious signs of ultrastructural remodelling such as myofibrillar disarray (*Figure 2A*). Thus, the combined deletion of CASQ2 and HRC resulted in only limited cardiac structural remodelling.

3.2 Arrhythmia vulnerability was alleviated in DKO mice compared with Cnull

Development of arrhythmia upon catecholamine infusion is a key feature of Cnull mice.[6] To assess the impact of combined deletion of CASQ2 and HRC on arrhythmia susceptibility, we recorded surface ECG in DKO mice and mice from the three control groups. Under baseline conditions (before catecholamine stimulation), all four groups had similar ECG characteristics except heart rate (HR) (see Supplementary material online, *Table S1*), which was significantly slowed in Cnull and DKO mice (407 ± 14 and 387 ± 18 bpm, respectively) compared with WT (486 ± 11 bpm).

Following catecholamine challenge, Cnull mice developed ventricular arrhythmias, including bigeminies (*Figure 1D*) and bidirectional ventricular tachycardia (VT) (*Figure 1D*), as previously described.[6] Under the same conditions, DKO mice displayed only occasional ventricular bigeminies (*Figure 1D*). Thus, the probability of developing ventricular arrhythmias was markedly decreased in DKO mice compared with Cnull mice. Neither WT nor Hnull mice displayed signs of ventricular arrhythmias. Catecholamine challenge increased HR in all experimental groups, and the extent of increase was similar among WT, DKO, and Cnull mice (30%), although smaller in Hnull mice (11%). These results suggest that sympathetic responsiveness was not altered in DKO mice.

Surface ECG studies could be influenced by the utilization of anaesthesia; thus, we performed additional telemetric ECG measurements in unanaesthetized DKO and Cnull mice. As shown in Supplementary material online, *Figure S2*, consistent with surface ECG recordings, the arrhythmic events (premature ventricular contraction or PVC and bigeminies) were substantially less pronounced in DKO mice than in Cnull mice. Cnull mice also displayed the bidirectional VT typical of CPVT (see Supplementary material online, *Figure S2C*). Taken together, both surface and telemetric ECG demonstrated that arrhythmia vulnerability was alleviated in DKO mice compared with Cnull mice.

3.3 DKO myocytes displayed frequency-dependent alterations in Ca handling that were alleviated by ISO

The impact of the deletion of CASQ2 and HRC on myocyte Ca handling was examined using confocal microscopy. The parameters of evoked Ca transients and spontaneous Ca waves (SCWs) were measured in field-stimulated myocytes (0.5 Hz) under baseline conditions and after exposure to ISO (100 nM). The SR Ca content was then measured by application of caffeine at the end of the experiment.

Consistent with previous reports,[6,8] ablation of either CASQ2 or HRC resulted in several changes in the parameters of pacing-induced Ca transients. In particular, Ca transient amplitude was increased and decay time shortened in Hnull (but not Cnull) myocytes compared with WT (*Figure 3B* and *C*). At the same time, SR Ca content was increased in Hnull and decreased in Cnull myocytes compared with WT myocytes, respectively (*Figure 3D*). Notably, in DKO myocytes, Ca handling was not further deteriorated compared with the single KOs. In fact, the Ca transient amplitude (*Figure 3B*) and SR Ca content (*Figure 3D*) in DKO myocytes were not different from WT. However, the rate of Ca transient decay was significantly slower in DKO myocytes compared with the other three groups (*Figure 3C*).

Considering our findings that SR Ca reuptake was slowed in DKO myocytes, we sought to examine the effect of stimulation frequency on Ca release. Although the Ca transient amplitude in DKO myocytes was similar to that in Cnull and WT at 0.5 Hz, at higher pacing frequencies of 2 Hz (*Figure 4A* and *C*) and 4 Hz (*Figure 4B* and *D*) Ca transient amplitude in DKO decreased progressively compared with the other groups.

Exposure to 100 nM ISO significantly increased the amplitude (*Figure 3B*) and shortened the decay time (*Figure 3C*) of Ca transients in all four groups, compared with the corresponding baseline condition. ISO also diminished the differences observed among the groups under baseline conditions, for both Ca transient amplitude and its decay time (*Figure 3B* and *C*). Additionally, ISO elevated the SR Ca content in all groups except Cnull myocytes (*Figure 3D*). The absence of an increase in the SR load in Cnull myocytes could be attributed to the occurrence of SCWs, which is expected to drain the SR of Ca (*Figure 3E*).

Collectively, these results show that while the Ca transient amplitude in DKO myocytes was preserved at a low pacing frequency (0.5 Hz), it became significantly reduced at higher pacing frequencies (2 and 4 Hz). These alterations in SR Ca release were alleviated by ISO.

3.4 Pro-arrhythmic Ca waves and Ca spark-mediated leak were alleviated in DKO myocytes compared with Cnull myocytes

Increased frequency of SCWs in the presence of ISO is a hallmark of CPVT at the cellular level. To examine the effect of combined deletion of CASQ2 and HRC on myocyte arrhythmic potential, we measured SCWs and sparks in DKO myocytes and cells from the three control groups. Consistent with previous results,[6,8] exposure to ISO resulted in a marked increase in SCWs frequency in Cnull myocytes and a more modest increase in SCWs in Hnull myocytes (*Figure 3E*). Notably, in DKO myocytes, SCWs frequency was significantly lower than in Cnull cells, although moderately higher than in Hnull cells (*Figure 3E*). Consistent with the results on SCWs frequency, Ca spark frequency in DKO myocytes was significantly lower than in Cnull cells, but higher than in WT and Hnull cells (*Figure 5A* and *B*). The amplitude of Ca

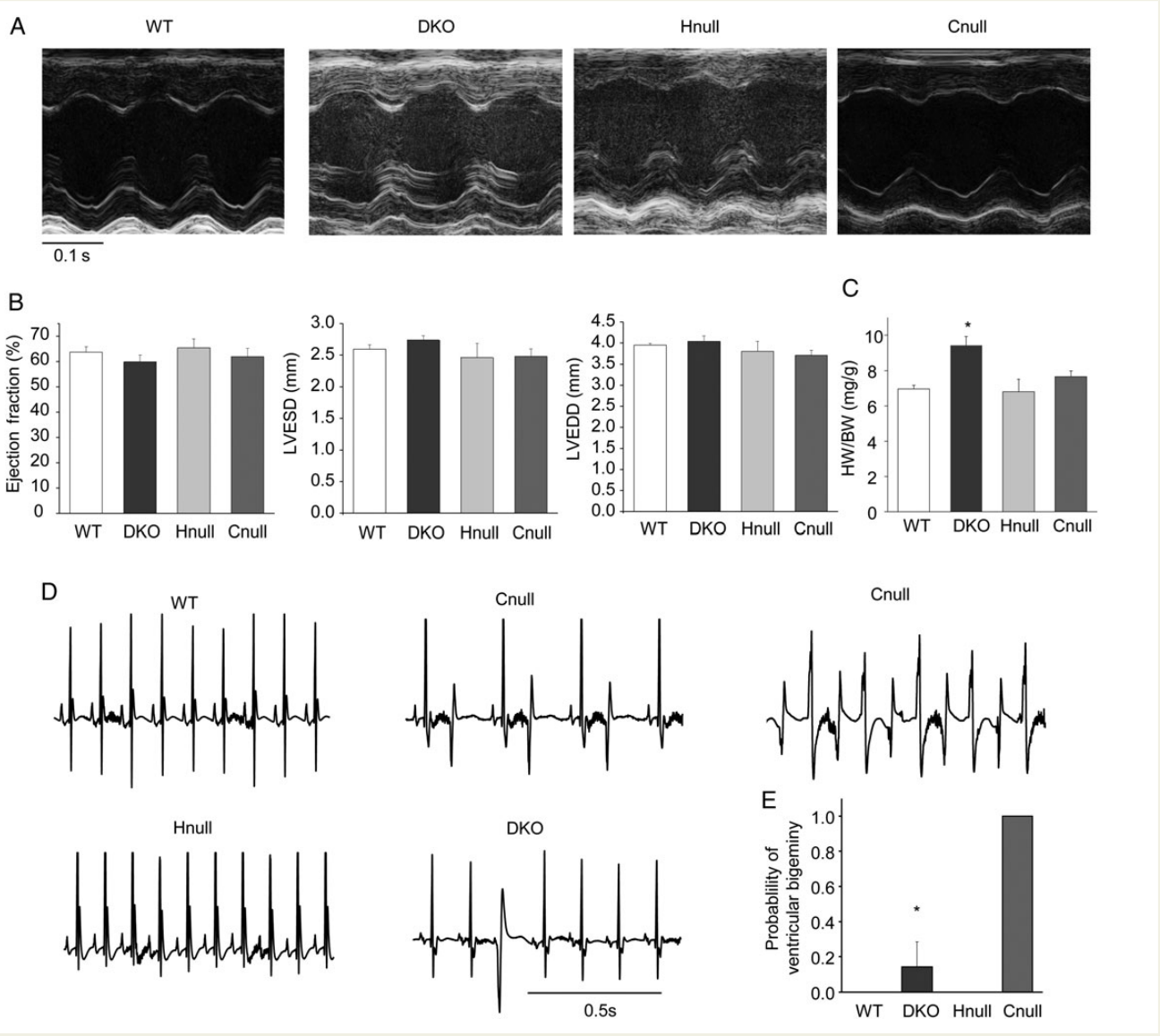

Figure 1 Deletion of HRC in Cnull did not affect *in vivo* cardiac function, but alleviated arrhythmia vulnerability. (*A*) Representative images of M-mode echocardiography of left ventricle of WT, DKO, Hnull, and Cnull mice, respectively. (*B*) Mean ± SEM of LVEF, LVESD, and LVEDD in 2-month-old mice of WT ($n = 4$), DKO ($n = 8$), Hnull ($n = 5$), and Cnull ($n = 7$). (*C*) Mean ± SEM of heart weight/body weight (HW/BW) ratio in WT ($n = 4$), DKO ($n = 4$), Hnull ($n = 4$), and Cnull ($n = 5$) mice. *$P < 0.05$ vs. WT. (*D*) Representative surface ECG traces of WT ($n = 4$), DKO ($n = 7$), Hnull ($n = 6$), and Cnull ($n = 4$) mice after catecholamine challenge (1.5 mg/kg ISO and 120 mg/kg caffeine, IP). (*E*) Probability of ventricular bigeminy following catecholaminergic stimulation. *$P < 0.05$ vs. Cnull.

sparks was similar in DKO, Hnull, and WT cells, which was lower than in Cnull cells (*Figure 5C*).

Together, these results suggest that whereas the deletion of CASQ2 increases arrhythmic potential of cardiac cells, the additional deletion of HRC alleviates this effect.

3.5 DKO myocytes had longer Ca release refractoriness compared with Cnull myocytes

It has been demonstrated previously that the functional recovery, i.e. refractoriness, of SR Ca release is abnormally abbreviated in Cnull myocytes, thus facilitating the generation of pro-arrhythmic SCWs.[18] We hypothesized that the reduced arrhythmic potential of DKO myocytes compared with Cnull cells is at least partly due to slowing of the rate of Ca release refractoriness. A standard two-pulse protocol was utilized to measure Ca transient restitution time as an estimate of Ca release refractoriness in DKO vs. Cnull myocytes.[19] During this protocol, the time interval between two pulses was gradually shortened, and the extent of Ca transient recovery at each time point was quantified to determine Ca transient restitution rate. As shown in *Figure 5D* and *E*, the restitution of the Ca transient was significantly slower in DKO myocytes than in Cnull myocytes. Thus, longer release refractoriness could indeed contribute to the reduced arrhythmic potential of DKO myocytes compared with Cnull myocytes.

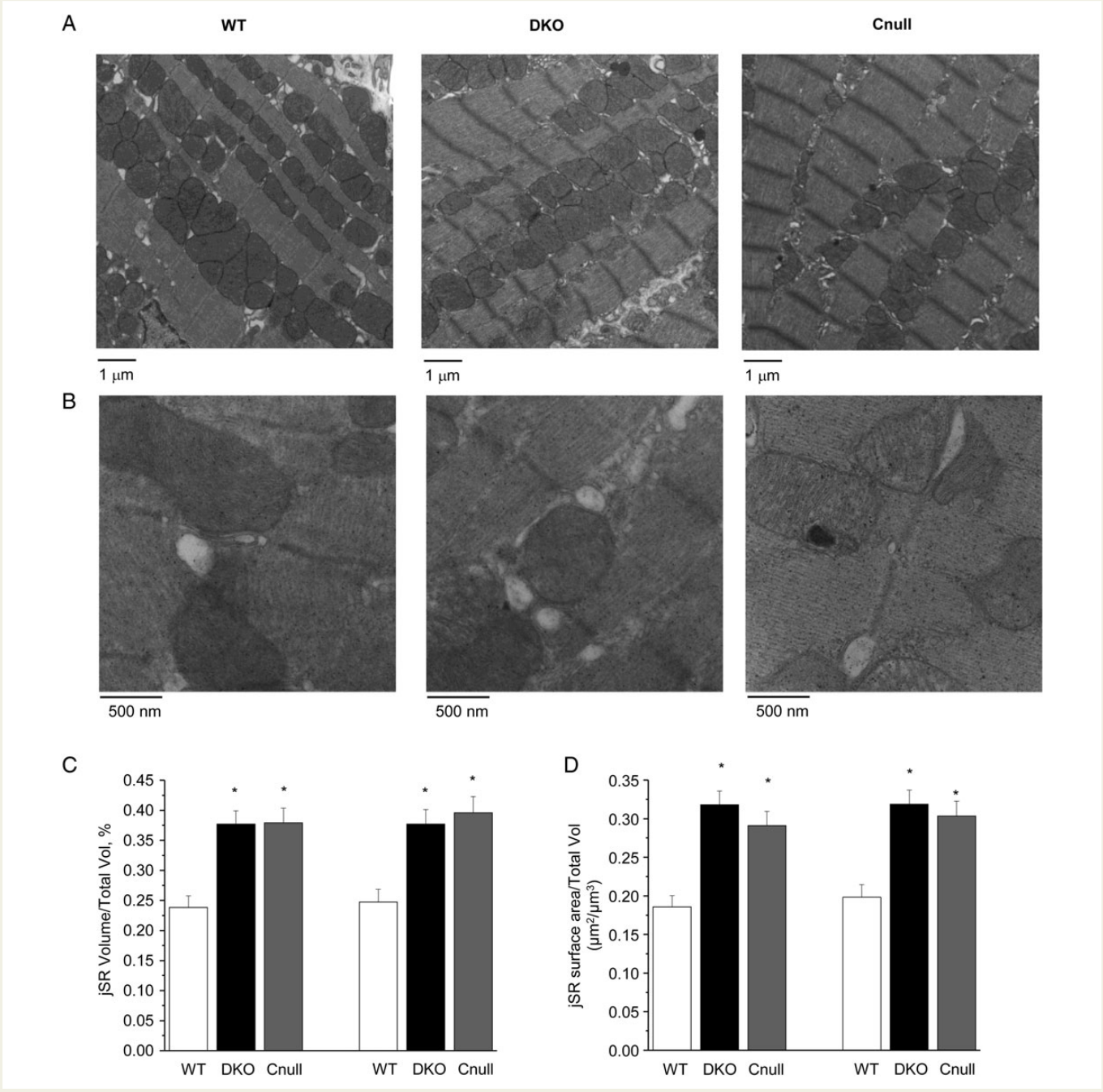

Figure 2 Myocardium ultrastructure was preserved in DKO. (*A*) Overview of myofibrillar organization of WT, DKO, and Cnull hearts. (*B*) jSR ultrastructure. (*C*) Quantification of jSR volume, calculated by counting points (first set of bars from the left) or pixels (second set of bars). (*D*) Quantification of jSR surface area, calculated by perimeter (first set of bars from the left) or intersection (second set of bars) measurements. Both approaches give almost identical statistics results. $n = 39–42$ micrographs, from three mice per group.

3.6 Deletion of CASQ2 and HRC impairs frequency-dependent contractile performance of intact cardiac muscle

To further examine whether frequency-dependent alterations in Ca handling in DKO myocytes translate into myocardial functional deficiency, we performed force measurements in intact cardiac muscle preparations paced at 4–12 Hz at 37°C. WT muscles exhibited a characteristic force–frequency relationship (FFR), maintaining a stable developed tension at 4–6 Hz, which then gradually declined to ~60% of its initial value at 12 Hz. Notably, both Cnull and Hnull muscles also preserved a fairly typical FFR. However, in DKO muscles as the frequency increased, the developed tension quickly declined (*Figure 6A*); this result was consistent with our myocyte Ca transient measurements. Moreover, at frequencies above 8 Hz, all the DKO muscles displayed contraction alternans, which were rarely observed in either Cnull or Hnull preparations (*Figure 6C*). When alternans were present, the alternans magnitude (the difference in force between

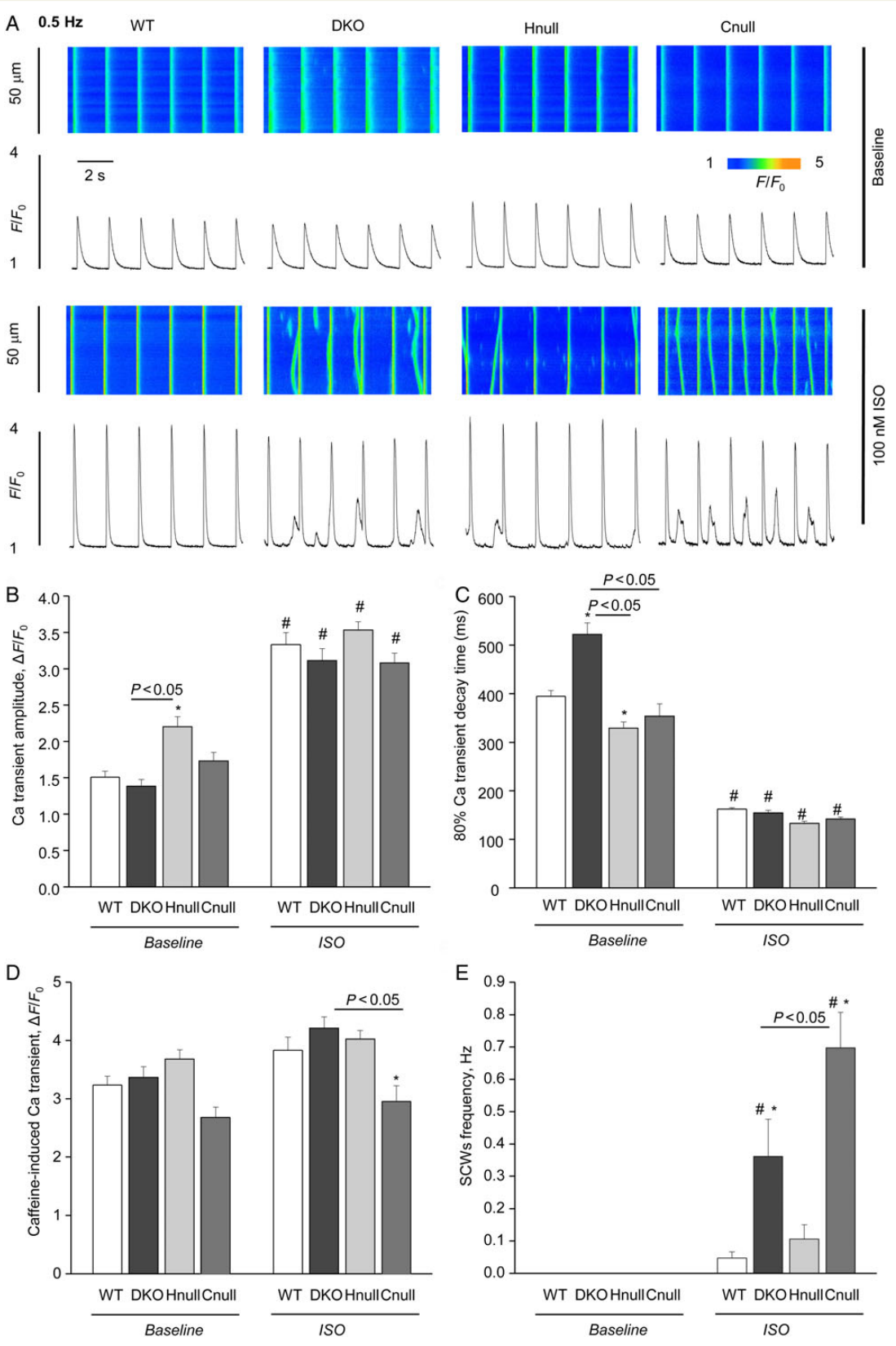

Figure 3 Deletion of HRC in Cnull alleviated pro-arrhythmic Ca waves in isolated myocytes. (*A*) Representative line-scan images and time-dependent fluorescence profiles of SCWs under control conditions (baseline) and in the presence of 100 nM ISO in myocytes isolated from WT, DKO, Hnull, and Cnull mice. (*B*) Average Ca transient amplitude, $n = 9–26$ cells; (*C*) Average 80% Ca transient decay time, $n = 11–32$ cells; (*D*) Average caffeine-induced Ca transient amplitude, $n = 10–25$ cells; (*E*) Average SCWs frequency, $n = 13–34$ cells. $^{*}P < 0.05$ vs. WT, $^{\#}P < 0.05$ vs. baseline, other significant differences vs. DKO were labelled as $P < 0.05$; two-way ANOVA. Data were obtained from three mice hearts per group.

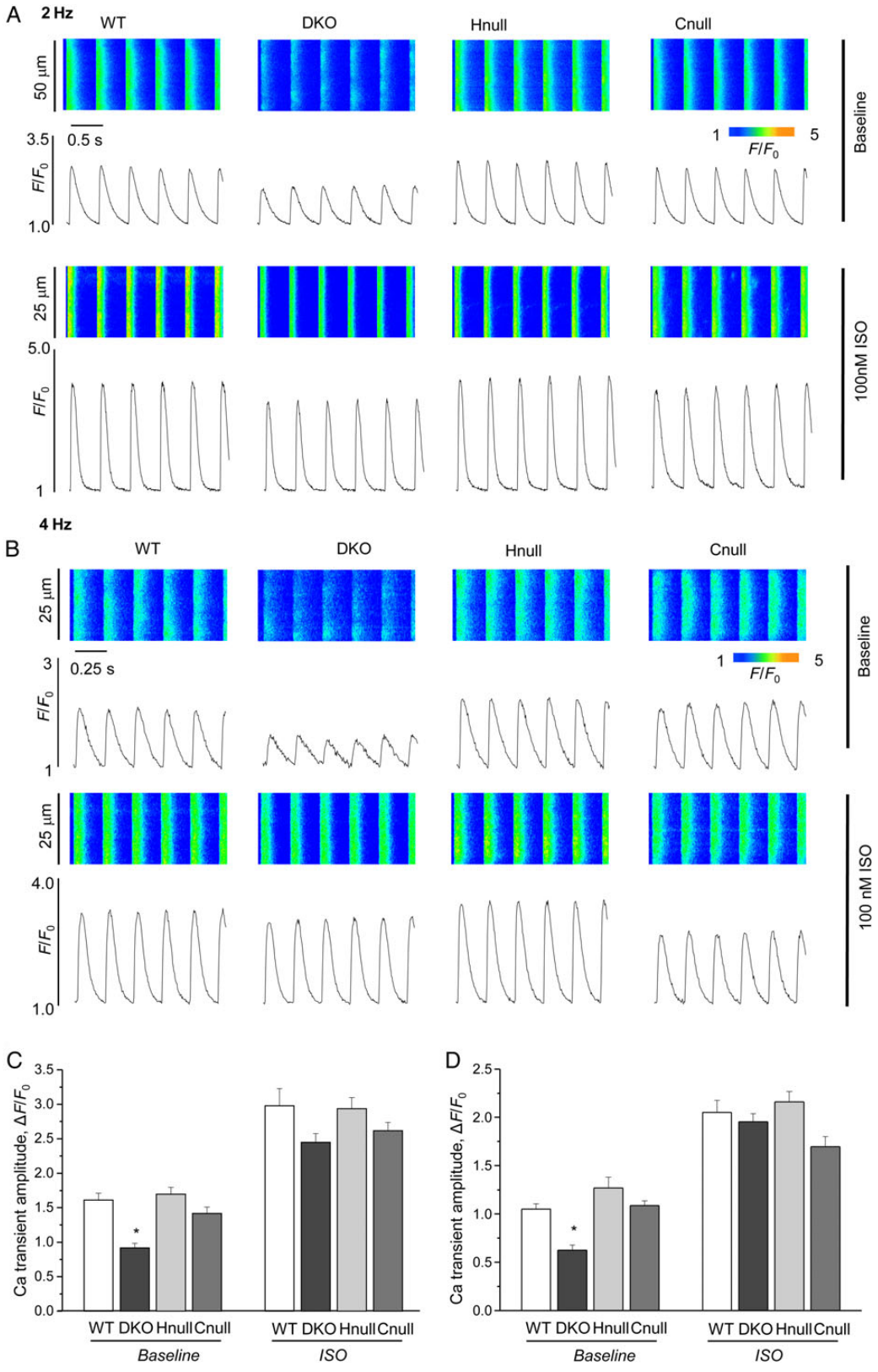

Figure 4 Deletion of HRC in Cnull depressed Ca transient amplitude in isolated myocytes at high pacing frequencies. Representative line-scan images (top) and time-dependent fluorescence profiles (bottom) of Ca transients under control conditions (baseline) and in the presence of 100 nM ISO at 2 Hz (*A*) and 4 Hz (*B*) stimulation frequencies. Average Ca transient amplitude in myocytes stimulated at 2 Hz (*C*) and 4 Hz (*D*). $n = 8–24$ cells. *$P < 0.05$ vs. WT; two-way ANOVA. Data were obtained from three mice hearts per group.

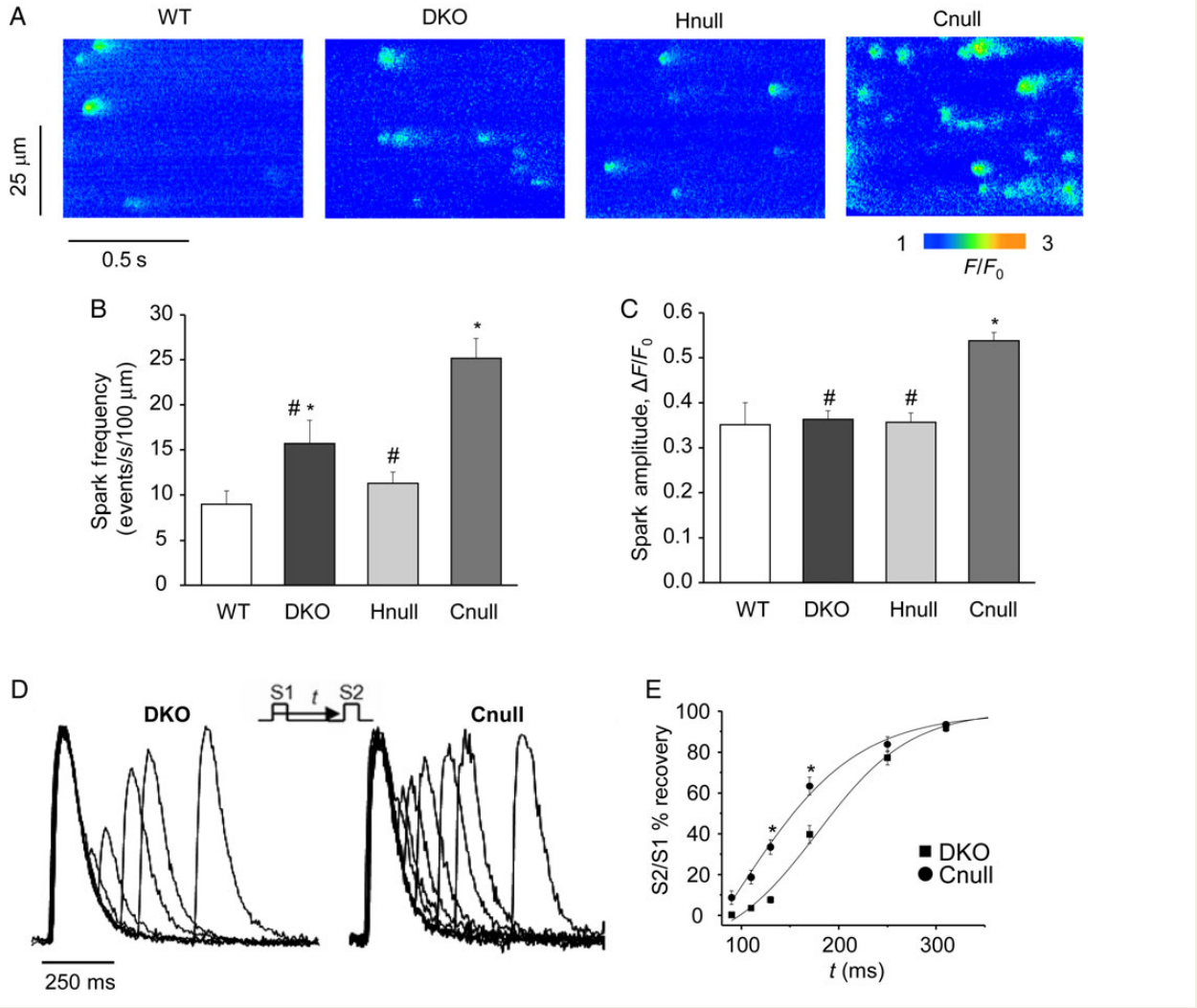

Figure 5 Deletion of HRC in Cnull decreased Ca sparks frequency and increased RyR2 refractoriness in isolated myocytes. (*A*) Representative line-scan images of Ca sparks in the presence of 100 nM ISO. (*B*) Average Ca spark frequency, $n = 13–21$ cells; (*C*) Average Ca spark amplitude, $n = 13–21$ cells. $^{*}P < 0.05$ vs. WT, $^{\#}P < 0.05$ vs. Cnull. (*D*) Representative traces of SR Ca release attained during an S1–S2 restitution protocol. Experiments were carried out in the presence of 100 nM ISO. (*E*) Average time course of Ca transient restitution shows faster recovery of SR Ca release in Cnull myocytes compared with DKO cells (time to half-maximal recovery was 146 ± 8 vs. 187 ± 7 ms, respectively, $P < 0.05$). Each data point was recorded in 6–14 DKO and 5–16 Cnull myocytes, respectively. Restitution of Ca transients in each group was fitted to logistic functions. Data were obtained from three mice hearts per group.

large and small contractions) in DKO muscles was significantly higher than in the other groups at all frequencies tested (*Figure 6C* and *E*).

Exposure to ISO alleviated the differences between FFR curves obtained in DKO, Hnull, and WT muscles observed under baseline conditions (*Figure 6B*). At the same time, the FFR curve for Cnull muscles shifted upward at frequencies above 6 Hz compared with the other muscle groups (Hnull, DKO, and WT) (*Figure 6B*). Notably Cnull muscles showed regular extra systolic contractions (ESCs) at 4–6 Hz that were absent in all the other groups (see Supplementary material online, *Figure S3A* and *B*). These ESCs only occurred at 4–6 Hz, not at 8–12 Hz (see Supplementary material online, *Figure S3B*). Aberrant Ca release associated with these ESCs could contribute to the upward tilt of the normalized FFR curve for Cnull muscles by decreasing the amplitude of the following stimulated contractions (via either lowering the SR Ca content and/or causing inactivation of RyR2s) (see Supplementary material online, *Figure S3C*). Interestingly, ISO failed to inhibit contraction alternans in DKO muscles observed under baseline conditions (*Figure 6D*). These results suggest that CASQ2 and HRC are required for maintaining stable myocardium contractile performance at a broad range of pacing frequencies.

3.7 jSR volume and surface area were similarly increased in DKO and Cnull hearts compared with WT

To test the possibility of ultrastructural remodelling, we quantified the jSR ultrastructure using TEM imaging. We used two methods for the quantification (described in the Methods section). As shown in *Figure 2C* and *D*, both methods gave similar results. Both jSR volume and surface area increased significantly in the DKO hearts compared

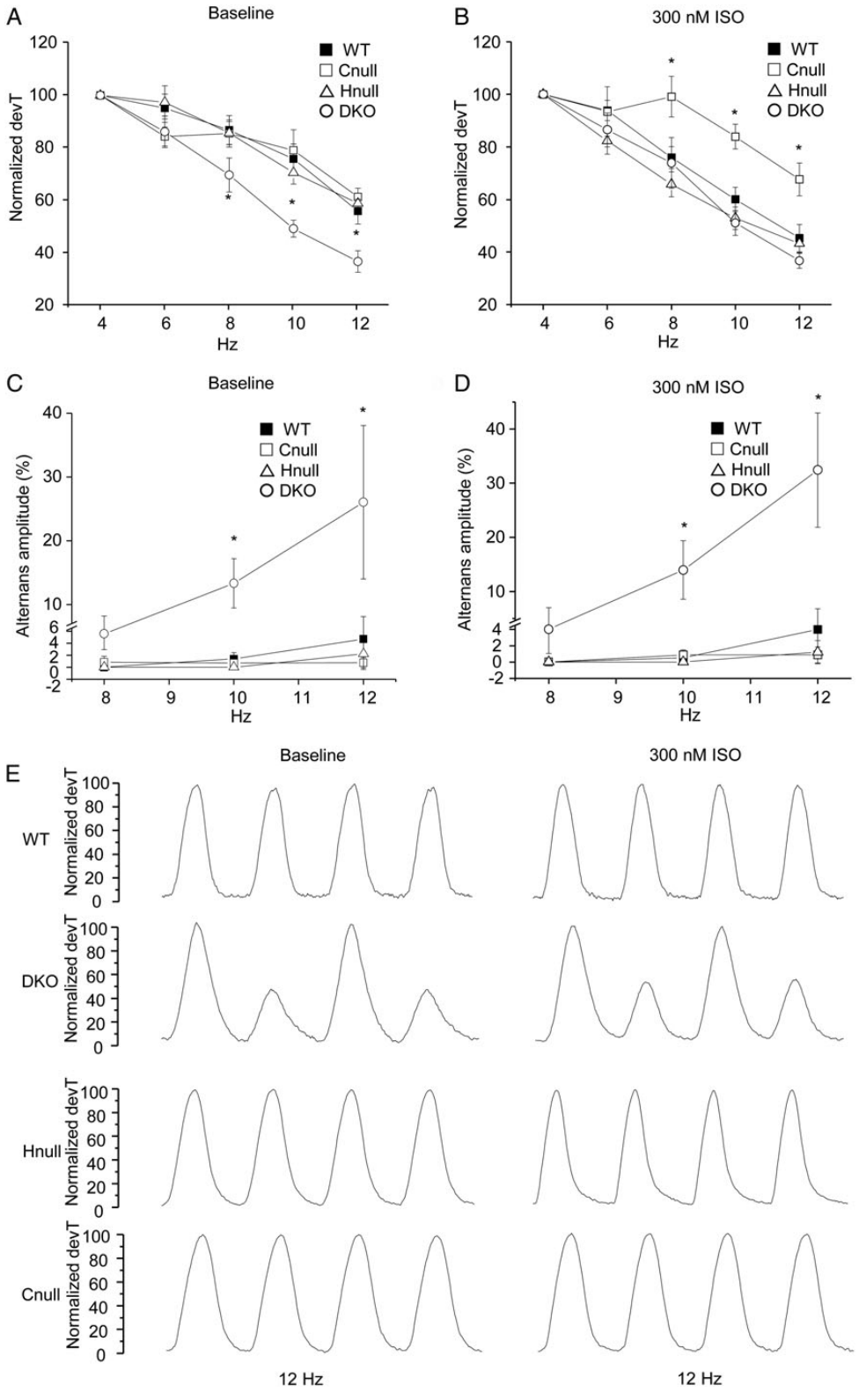

Figure 6 Cardiac contractile performance was impaired at high pacing frequencies in DKO cardiac muscles. FFR plots for WT, Hnull, Cnull, and DKO under baseline conditions ($n = 7, 4, 5,$ and 4, respectively) (*A*) and after exposure to 300 nM ISO ($n = 6, 4, 5,$ and 4, respectively) (*B*). For each muscle, the force amplitude (developed tension, devT) at all pacing frequencies was normalized to the corresponding value recorded at a stimulation frequency of 4 Hz. Contraction alternans magnitudes at different pacing frequencies (8–12 Hz) in WT, Hnull, Cnull, and DKO muscles under baseline conditions ($n = 7, 4, 5,$ and 4, respectively) (*C*) and in the presence of 300 nM ISO ($n = 6, 4, 5,$ and 4, respectively) (*D*). (*E*) Representative traces of contraction alternans of WT, DKO, Hnull, and Cnull muscles recorded at a pacing frequency of 12 Hz. $^*P < 0.05$ vs. WT; two-way ANOVA.

with WT. Consistent with previous studies, jSR volume and surface area also increased in the Cnull hearts.[6] Of note, the extent of increase relative to WT was similar in the DKO and Cnull hearts. Thus, the deletion of HRC on the background of Cnull did not seem to cause further ultrastructural remodelling.

3.8 Expression levels of key Ca handling proteins were preserved in DKO mice

To investigate the potential adaptive changes in protein expression that could account for the phenotype of the DKO mice, we performed western blot analyses of key Ca handling proteins. As shown in *Figure 7*, we did not detect significant differences in the expression levels of SERCA2a, RyR2, and PLB between WT and DKO hearts. Consistent with previous studies, the expression of TRD was down-regulated in Cnull hearts.[6] The additional deletion of HRC in the background of CASQ2 KO did not lead to further changes in the expression of TRD, which was down-regulated to a similar level in the DKO as in the Cnull hearts.

4. Discussion

In this study, we investigated the consequences of combined deletion of the two SR Ca-binding proteins, CASQ2 and HRC, in the heart. Lack of either functional CASQ2 or HRC has been reported to result in increased vulnerability towards cardiac arrhythmias but otherwise mild cardiac phenotypes in human and mice.[2,3,6,8,20–22] We hypothesized that simultaneous ablation of both proteins would exacerbate the cardiac phenotype compared with the single-protein KOs. Surprisingly, the DKO mouse missing both proteins presented only mild hypertrophy and nearly normal *in vivo* and *in situ* contractility. Furthermore, combined deletion of CASQ2 and HRC ameliorated the predisposition to ventricular arrhythmia and arrhythmogenic Ca waves characteristic of the Cnull mouse. These results provide new unexpected insights into the regulation of RyR2-mediated Ca release by these SR proteins in the heart.

4.1 Modulation of SR Ca release by CASQ2 and HRC

As low-affinity, Ca-binding proteins, CASQ2 and HRC contribute to the SR Ca-binding capacity of cardiac myocytes.[2,6] Moreover, both proteins have been reported to regulate RyR2 activity via binding to TRD.[2,3] Whereas CASQ2 has been shown to inhibit RyR2,[6,23] HRC's role in regulating RyR2 and SR Ca release is less clear. Additionally, HRC (but not CASQ2) also modulates SERCA by inhibiting its Ca transport function.[2] Given the apparent overlapping roles of CASQ2 and HRC as both luminal Ca buffers and modulators of SR Ca release, one would expect Ca handling to become substantially deteriorated in DKO mice compared with the KO mice missing either one of these proteins. In contrast to this expectation, combined ablation of CASQ2 and HRC affected Ca handling only mildly, predominantly at high pacing frequencies. Moreover, the predisposition to arrhythmias was ameliorated, and SCWs and spark-mediated SR Ca leak were reduced in DKO mice compared with Cnull mice (albeit more pronounced than in Hnull) (*Figures 3E*, *5A* and *B*). Two possibilities can be considered to rationalize these unexpected findings.

One possibility is that compensatory mechanisms induced by the deletion of both proteins prevented further deterioration of Ca handling and counteracted increased arrhythmia vulnerability in DKO mice. However, the expression of relevant Ca handling proteins, including SERCA, PLB, and RyR2, remained stable in these mice (*Figure 7*). Furthermore, expression of TRD, which was down-regulated in Cnull, was not further altered in DKO (*Figure 7B*). Moreover, DKO did not exhibit further ultrastructural changes in SR organization, including SR volume and surface area, compared with the alterations previously reported for the Cnull mouse (*Figure 2C* and *D*).[6] Thus, no obvious molecular or ultrastructural adaptations were detected that could account for the relatively mild phenotype of the DKO mouse. As with any genetic KO model, however, it cannot be ruled out that the DKO phenotype is confounded by undetected compensatory changes.

Alternatively, the regulation of RyR2 by CASQ2 and HRC could be more complex than previously thought. An interesting possibility supported by our results is that RyR2 is subject to dual regulation by CASQ2 and HRC via inhibition and activation of the channels, respectively (*Figure 8*). Thus, the propensity for SCWs and spark-mediated Ca leak being the highest in Cnull myocytes among all groups (*Figures 3E* and *5A*) is consistent with the loss of an inhibitory influence of CASQ2 while retaining the stimulatory action of HRC. The lowest arrhythmogenic propensity and SR Ca leak observed in Hnull cells could be attributed to the loss of stimulatory influence of HRC coupled with the inhibitory action of CASQ2. Finally, the intermediate phenotype of the DKO myocytes could be a consequence of the simultaneous loss of both the inhibitory and stimulatory influences of CASQ2 and HRC, respectively. The inhibitory effects of CASQ2 on RyR2 proposed here are consistent with previous findings.[6,23] While previously HRC has been suggested to inhibit SR Ca release,[2,24] those results could be ascribed to the inhibitory effects of HRC on SERCA and corresponding alterations in the SR Ca content when HRC was deleted.[8]

Of note, both CASQ2 and HRC are known to interact with the RyR2 complex via TRD at overlapping sequences (amino acids 210–224 and 204–221, for CASQ2 and HRC, respectively).[2,3,25] Therefore, CASQ2 and HRC could regulate RyR2 by competing for the same binding site on TRD (*Figure 8*). Interestingly, whereas CASQ2 binding to TRD weakens at high luminal Ca^{3}, HRC binding to TRD is stabilized at high luminal Ca^{25}. This raises the possibility of a dynamic process in which interactions of CASQ2 and HRC with the RyR2 complex driven by changes in luminal Ca influence the functional state (i.e. refractory status) of RyR2 on a beat-to-beat basis. In particular, it could be speculated that CASQ2 contributes to RyR2 deactivation and refractoriness early after release, whereas HRC facilitates functional recovery of RyR2s later in diastole when the SR stores are filled with Ca. Consistent with this possibility, functional restitution of SR Ca release was slower in DKO myocytes than in Cnull myocytes (*Figure 5D* and *E*).

4.2 The role of CASQ2 and HRC in maintaining Ca cycling at high frequencies

A notable feature of DKO mice was the exaggerated suppression of the Ca transient and force amplitudes at high stimulation frequencies (>8 Hz) compared with the other groups (*Figures 4* and *6A*). This altered frequency response was associated with slowed SR Ca reuptake evidenced by prolonged Ca transient decay in DKO myocytes (*Figure 3C*). Slowed SR Ca reaccumulation could account for the altered frequency dependency of SR Ca release by resulting in a progressive decrease in the SR Ca content at high pacing rates.

In contrast to DKO cells, Ca transient decay was accelerated in Hnull myocytes. This effect was also demonstrated in previous reports[8] and can be attributed to the loss of inhibition of SERCA by HRC in Hnull cells. Why this effect was reversed by the additional deletion of

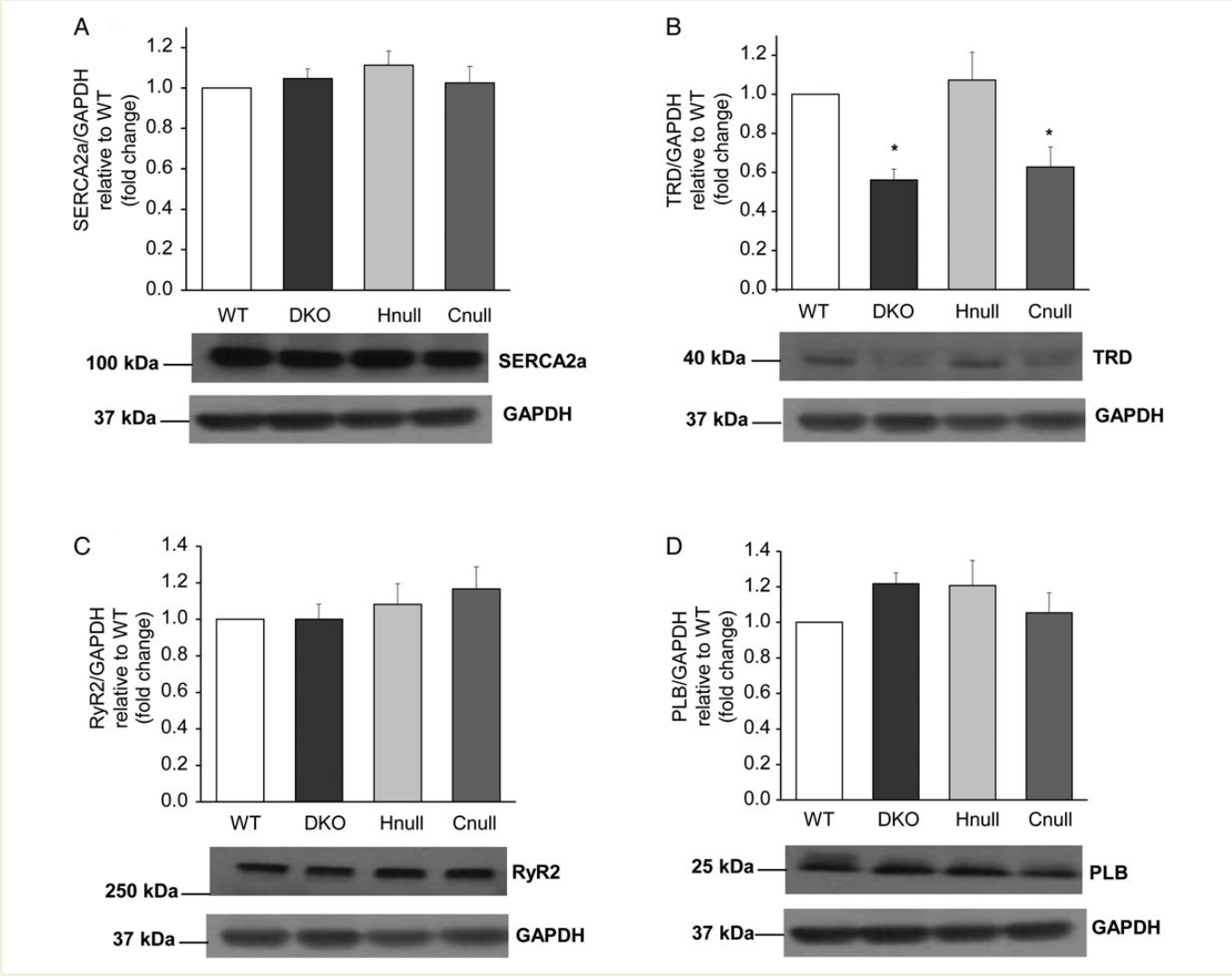

Figure 7 Expression levels of key Ca handling proteins was preserved in DKO. Representative western blots and the quantifications for SERCA2a (*A*), TRD (*B*), RyR2 (*C*), and PLB (*D*). The expression levels of Ca handling proteins (normalized to loading control GAPDH) from three mutant groups (DKO, Hnull, and Cnull) were expressed as arbitrary units relative to WT. Data are mean ± SEM, $^*P < 0.05$ vs. WT, $n = 4$ hearts for each group.

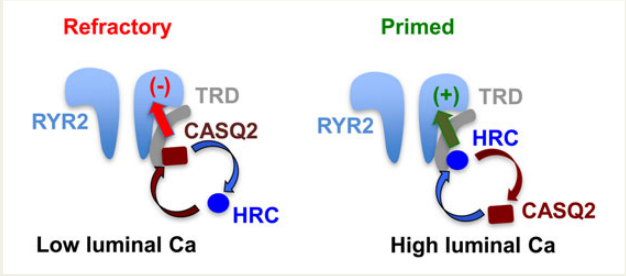

Figure 8 Dual regulation of RyR2 by CASQ2 and HRC. Both CASQ2 and HRC interact with the RyR2 complex via TRD at overlapping sequences. It is hypothesized that whereas CASQ2 inhibits, HRC stimulates (primes for activation) RyR2 through TRD binding, in an alternating, SR luminal-Ca dependent manner during a single Ca release-uptake cycle.

CASQ2 in DKO myocytes is unclear. One possibility is that it is a consequence of reduced luminal Ca buffering in the DKO myocytes lacking two intra-SR Ca binding proteins. Indeed, luminal Ca buffers have been suggested to facilitate SR Ca refilling by absorbing Ca and thus preventing the buildup of free Ca at the luminal side of SERCA, which would inhibit the transport activity of the pump.[26,27] Consistent with this possibility, stimulation of SERCA activity by ISO both accelerated SR Ca reuptake and blunted the differences in Ca transient amplitude between DKO cells and the other groups at higher stimulation frequencies (*Figure 4*). Regardless of the precise underlying mechanisms, these results show that the presence of both proteins is essential for maintaining robust Ca release in the high frequency regime of Ca cycling (>8 Hz). Of note, the resting HR of ~7 Hz is at the edge of the decline of the force–frequency curve (*Figure 6A*), consistent with the unaltered cardiac contractile function in the DKO mouse at the resting heart rate. Additionally, the HR was reduced to a similar extent in both Cnull and

DKO mice compared with the WT control (see Supplementary material online, *Table S1*). The reduced HR in mice lacking CASQ2 could be attributed to abnormal SR Ca release and selective interstitial fibrosis in the sino-atrial (SA) pacemaker complex, which disrupt SA node pacemaking.[28] Thus, the phenotype differences between the Cnull and DKO mice could not be due to a difference in HR.

4.3 Mechanism of arrhythmogenesis in CPVT

Consistent with the two demonstrated functional roles of CASQ2 in the SR, i.e. a RyR2 luminal Ca sensor and a Ca buffer,[3] two mechanisms have been proposed as to how loss/mutations of CASQ2 can result in CPVT: (i) impaired regulation prevents RyR2s from becoming properly deactivated, or refractory, at reduced SR Ca levels following systolic release, thus permitting the spontaneous activation of Ca-induced Ca release[3]; or (ii) decreased SR Ca buffering allows Ca level to rise too quickly near the luminal face of the RyR2, thus activating it.[29] The present study showed that the ablation of another SR Ca buffer (i.e. HRC) besides CASQ2, instead of exacerbating, alleviated the propensity to SCWs and CPVT in DKO mice. Thus, Ca-dependent arrhythmogenesis in CPVT seems to be due to deficiency in CASQ2 regulatory function rather than reduced SR Ca buffering by this protein. Moreover, we demonstrated that ablation of HRC on the background of Cnull prolonged restitution of SR Ca release compared with Ca release restitution in Cnull myocytes (*Figure 5D* and *E*). These results suggest that in addition to being regulated by CASQ2, RyR2 functional activity/readiness is also influenced by HRC. However, in contrast to the stabilizing action of CASQ2, HRC appears to facilitate SR Ca release and its restitution during systole, thereby resulting in slowed restitution, inhibition of arrhythmogenic Ca waves, and decreased arrhythmia burden in the DKO mouse.

4.4 Summary

In summary, we found that combined deletion of the SR Ca-binding proteins CASQ2 and HRC failed to exacerbate the cardiac phenotype compared with the respective single-protein knockouts (Hnull and Cnull). Moreover, it alleviated the Ca-dependent arrhythmias characteristic of the Cnull mouse. Rather than being a result of compensatory changes in myocyte Ca handling, this phenotypic stability in the face of losing two SR Ca-binding proteins seems to be attributable to the opposing roles these proteins play in the regulation of RyR2-mediated SR Ca release. While CASQ2 stabilizes RyR2s and renders them refractory, HRC enhances RyR2 activity and facilitates their functional recovery in the diastolic period. Although alleviating arrhythmias, deletion of both proteins reduced SR Ca release and contractile strength at high pacing frequencies, thus suggesting that the simultaneous presence of these proteins is required for maintaining robust Ca cycling at high pacing rates.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

We thank Dr Przemyslaw Radwanski for critical reading of the manuscript. We thank Dr Igor Kubasov (Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences) for his valuable suggestions on the manuscript and help with the morphometric analysis of cardiac ultrastructure.

Conflict of interest: none declared.

Funding

This work was supported by the National Institutes of Health (RO1 HL088635 to B.C.K., RO1 HL074045 and HL063043 to S.G.) and American Heart Association (postdoctoral fellowship 15POST25460007 to B.L.). Additionally funding was provided by the Russian Science Foundation (N15-15-20008), which was used to support the cardiac ultrastructure morphometry studies.

References

1. Gyorke S, Carnes C. Dysregulated sarcoplasmic reticulum calcium release: potential pharmacological target in cardiac disease. *Pharmacol Ther* 2008;**119**:340–354.
2. Arvanitis DA, Vafiadaki E, Sanoudou D, Kranias EG. Histidine-rich calcium binding protein: the new regulator of sarcoplasmic reticulum calcium cycling. *J Mol Cell Cardiol* 2011;**50**:43–49.
3. Gyorke S, Terentyev D. Modulation of ryanodine receptor by luminal calcium and accessory proteins in health and cardiac disease. *Cardiovasc Res* 2008;**77**:245–255.
4. Terentyev D, Viatchenko-Karpinski S, Gyorke I, Volpe P, Williams SC, Gyorke S. Calsequestrin determines the functional size and stability of cardiac intracellular calcium stores: mechanism for hereditary arrhythmia. *Proc Natl Acad Sci USA* 2003;**100**:11759–11764.
5. Radwanski PB, Belevych AE, Brunello L, Carnes CA, Gyorke S. Store-dependent deactivation: cooling the chain-reaction of myocardial calcium signaling. *J Mol Cell Cardiol* 2013;**58**:77–83.
6. Knollmann BC, Chopra N, Hlaing T, Akin B, Yang T, Ettensohn K, Knollmann BE, Horton KD, Weissman NJ, Holinstat I, Zhang W, Roden DM, Jones LR, Franzini-Armstrong C, Pfeifer K. Casq2 deletion causes sarcoplasmic reticulum volume increase, premature Ca2+ release, and catecholaminergic polymorphic ventricular tachycardia. *J Clin Invest* 2006;**116**:2510–2520.
7. Brunello L, Slabaugh JL, Radwanski PB, Ho HT, Belevych AE, Lou Q, Chen H, Napolitano C, Lodola F, Priori SG, Fedorov VV, Volpe P, Fill M, Janssen PM, Györke S. Decreased RyR2 refractoriness determines myocardial synchronization of aberrant Ca2+ release in a genetic model of arrhythmia. *Proc Natl Acad Sci USA* 2013;**110**:10312–10317.
8. Park CS, Chen S, Lee H, Cha H, Oh JG, Hong S, Han P, Ginsburg KS, Jin S, Park I, Singh VP, Wang HS, Franzini-Armstrong C, Park WJ, Bers DM, Kranias EG, Cho C, Kim do H. Targeted ablation of the histidine-rich Ca(2+)-binding protein (HRC) gene is associated with abnormal SR Ca(2+)-cycling and severe pathology under pressure-overload stress. *Basic Res Cardiol* 2013;**108**:344.
9. Jaehnig EJ, Heidt AB, Greene SB, Cornelissen I, Black BL. Increased susceptibility to isoproterenol-induced cardiac hypertrophy and impaired weight gain in mice lacking the histidine-rich calcium-binding protein. *Mol Cell Biol* 2006;**26**:9315–9326.
10. Singh VP, Rubinstein J, Arvanitis DA, Ren X, Gao X, Haghighi K, Gilbert M, Iyer VR, Kim do H, Cho C, Jones K, Lorenz JN, Armstrong CF, Wang HS, Gyorke S, Kranias EG. Abnormal calcium cycling and cardiac arrhythmias associated with the human Ser96Ala genetic variant of histidine-rich calcium-binding protein. *J Am Heart Assoc* 2013;**2**:e000460.
11. Mohler PJ, Schott JJ, Gramolini AO, Dilly KW, Guatimosim S, duBell WH, Song LS, Haurogne K, Kyndt F, Ali ME, Rogers TB, Lederer WJ, Escande D, Le Marec H, Bennett V. Ankyrin-B mutation causes type 4 long-QT cardiac arrhythmia and sudden cardiac death. *Nature* 2003;**421**:634–639.
12. Gyorke S, Lukyanenko V, Gyorke I. Dual effects of tetracaine on spontaneous calcium release in rat ventricular myocytes. *J Physiol* 1997;**500** (Pt 2):297–309.
13. Liu B, Ho HT, Velez-Cortes F, Lou Q, Valdivia CR, Knollmann BC, Valdivia HH, Gyorke S. Genetic ablation of ryanodine receptor 2 phosphorylation at Ser-2808 aggravates Ca(2+)-dependent cardiomyopathy by exacerbating diastolic Ca2+ release. *J Physiol* 2014;**592**:1957–1973.
14. Mobley BA, Eisenberg BR. Sizes of components in frog skeletal muscle measured by methods of stereology. *J Gen Physiol* 1975;**66**:31–45.
15. Mulieri LA, Hasenfuss G, Ittleman F, Blanchard EM, Alpert NR. Protection of human left ventricular myocardium from cutting injury with 2,3-butanedione monoxime. *Circ Res* 1989;**65**:1441–1449.
16. Varian KD, Raman S, Janssen PM. Measurement of myofilament calcium sensitivity at physiological temperature in intact cardiac trabeculae. *Am J Physiol Heart Circ Physiol* 2006;**290**:H2092–H2097.
17. Raman S, Kelley MA, Janssen PM. Effect of muscle dimensions on trabecular contractile performance under physiological conditions. *Pflugers Arch* 2006;**451**:625–630.
18. Kornyeyev D, Petrosky AD, Zepeda B, Ferreiro M, Knollmann B, Escobar AL. Calsequestrin 2 deletion shortens the refractoriness of Ca(2)(+) release and reduces rate-dependent Ca(2)(+)-alternans in intact mouse hearts. *J Mol Cell Cardiol* 2012;**52**:21–31.

19. Belevych AE, Terentyev D, Terentyeva R, Ho HT, Gyorke I, Bonilla IM, Carnes CA, Billman GE, Gyorke S. Shortened Ca2+ signaling refractoriness underlies cellular arrhythmogenesis in a postinfarction model of sudden cardiac death. *Circ Res* 2012;**110**:569–577.
20. Arvanitis DA, Sanoudou D, Kolokathis F, Vafiadaki E, Papalouka V, Kontrogianni-Konstantopoulos A, Theodorakis GN, Paraskevaidis IA, Adamopoulos S, Dorn GW II, Kremastinos DT, Kranias EG. The Ser96Ala variant in histidine-rich calcium-binding protein is associated with life-threatening ventricular arrhythmias in idiopathic dilated cardiomyopathy. *Eur Heart J* 2008;**29**:2514–2525.
21. Postma AV, Denjoy I, Hoorntje TM, Lupoglazoff JM, Da Costa A, Sebillon P, Mannens MM, Wilde AA, Guicheney P. Absence of calsequestrin 2 causes severe forms of catecholaminergic polymorphic ventricular tachycardia. *Circ Res* 2002;**91**:e21–e26.
22. Pritchard TJ, Kranias EG. Junctin and the histidine-rich Ca2+ binding protein: potential roles in heart failure and arrhythmogenesis. *J Physiol* 2009;**587**:3125–3133.
23. Gyorke I, Hester N, Jones LR, Gyorke S. The role of calsequestrin, triadin, and junctin in conferring cardiac ryanodine receptor responsiveness to luminal calcium. *Biophys J* 2004;**86**:2121–2128.
24. Fan GC, Gregory KN, Zhao W, Park WJ, Kranias EG. Regulation of myocardial function by histidine-rich, calcium-binding protein. *Am J Physiol Heart Circ Physiol* 2004;**287**:H1705–H1711.
25. Arvanitis DA, Vafiadaki E, Fan GC, Mitton BA, Gregory KN, Del Monte F, Kontrogianni-Konstantopoulos A, Sanoudou D, Kranias EG. Histidine-rich Ca-binding protein interacts with sarcoplasmic reticulum Ca-ATPase. *Am J Physiol Heart Circ Physiol* 2007;**293**:H1581–H1589.
26. Inesi G, Lewis D, Toyoshima C, Hirata A, de Meis L. Conformational fluctuations of the Ca2+-ATPase in the native membrane environment. Effects of pH, temperature, catalytic substrates, and thapsigargin. *J Biol Chem* 2008;**283**:1189–1196.
27. Inesi G, Tadini-Buoninsegni F. Ca(2+)/H (+) exchange, lumenal Ca(2+) release and Ca (2+)/ATP coupling ratios in the sarcoplasmic reticulum ATPase. *J Cell Commun Signal* 2014;**8**:5–11.
28. Glukhov AV, Kalyanasundaram A, Lou Q, Hage LT, Hansen BJ, Belevych AE, Mohler PJ, Knollmann BC, Periasamy M, Gyorke S, Fedorov VV. Calsequestrin 2 deletion causes sinoatrial node dysfunction and atrial arrhythmias associated with altered sarcoplasmic reticulum calcium cycling and degenerative fibrosis within the mouse atrial pacemaker complex1. *Eur Heart J* 2015;**36**:686–697.
29. Jiang D, Wang R, Xiao B, Kong H, Hunt DJ, Choi P, Zhang L, Chen SR. Enhanced store overload-induced Ca2+ release and channel sensitivity to luminal Ca2+ activation are common defects of RyR2 mutations linked to ventricular tachycardia and sudden death. *Circ Res* 2005;**97**:1173–1181.