J Physiol 592.9 (2014) pp 1957–1973

The Journal of Physiology

Genetic ablation of ryanodine receptor 2 phosphorylation at Ser-2808 aggravates Ca^{2+}-dependent cardiomyopathy by exacerbating diastolic Ca^{2+} release

Bin Liu[1], Hsiang-Ting Ho[1], Florencia Velez-Cortes[1], Qing Lou[1], Carmen R. Valdivia[3], Bjorn C. Knollmann[2], Hector H. Valdivia[3] and Sandor Gyorke[1]

[1] *Department of Physiology and Cell Biology, College of Medicine, and Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH 43210, USA*

[2] *Division of Clinical Pharmacology, Vanderbilt University Medical School, Nashville, TN 37232, USA*

[3] *Center for Arrhythmia Research, Cardiovascular Division of the Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109, USA*

Key points

- Phosphorylation at Ser-2808 is suggested to result in RyR2 hyperactivity, i.e. 'leakiness', thus contributing to the pathology of cardiac diseases.
- We studied the effect of disabling phosphorylation at Ser-2808 of RyR2 in a genetic model of Ca^{2+}-dependent cardiomyopathy, which was caused by leaky RyR2.
- RyR2 phosphorylation was high at Ser-2808 in myocytes expressing wild-type (WT) RyR2; protein phosphatase increased RyR2 leakiness in cells expressing WT, but not in mutant RyR2s with disabled Ser-2808 phosphorylation sites.
- Rather than alleviating cardiac disease, ablation of the Ser-2808 exacerbated the disease phenotype by reducing survival, impairing *in vivo* cardiac function and enhancing RyR2 Ca^{2+} leak and mitochondrial damage.
- These results suggest a novel mode of RyR2 regulation via dephosphorylation at Ser-2808 in normal and diseased hearts.

Abstract Phosphorylation of the cardiac ryanodine receptor (RyR2) by protein kinase A (PKA) at Ser-2808 is suggested to mediate the physiological 'fight or flight' response and contribute to heart failure by rendering the sarcoplasmic reticulum (SR) leaky for Ca^{2+}. In the present study, we examined the potential role of RyR2 phosphorylation at Ser-2808 in the progression of Ca^{2+}-dependent cardiomyopathy (CCM) by using mice genetically modified to feature elevated SR Ca^{2+} leak while expressing RyR2s that cannot be phosphorylated at this site (S2808A). Surprisingly, rather than alleviating the disease phenotype, constitutive dephosphorylation of Ser-2808 aggravated CCM as manifested by shortened survival, deteriorated *in vivo* cardiac function, exacerbated SR Ca^{2+} leak and mitochondrial injury. Notably, the deteriorations of cardiac function, myocyte Ca^{2+} handling, and mitochondria integrity were consistently worse in mice with heterozygous ablation of Ser-2808 than in mice with complete ablation. Wild-type (WT) and CCM myocytes expressing unmutated RyR2s exhibited a high level of baseline phosphorylation at Ser-2808. Exposure of these CCM cells to protein phosphatase 1 caused a transitory increase in Ca^{2+} leak attributable to partial dephosphorylation of RyR2 tetramers at Ser-2808 from more fully phosphorylated state. Thus, exacerbated Ca^{2+} leak through partially dephosphorylated RyR2s accounts for the prevalence of the disease phenotype in the heterozygous S2808A CCM mice. These results do not support the importance of RyR2 hyperphosphorylation in Ca^{2+}-dependent heart disease, and rather suggest roles for the opposite process, the RyR2 dephosphorylation at this residue in physiological and pathophysiological Ca^{2+} signalling.

DOI: 10.1113/jphysiol.2013.264689

(Received 4 September 2013; accepted after revision 18 January 2014; first published online 20 January 2014)
Corresponding author S. Gyorke: Department of Physiology and Cell Biology, 507 Davis Heart & Lung Research Institute (office), 473 W. 12th Avenue, Columbus, OH 43210, USA. Email: sandor.gyorke@osumc.edu

Abbreviations CASQ2, calsequestrin 2; CCM, Ca^{2+}-dependent cardiomyopathy; DM, double mutant; ECG, electrocardiography; EF, ejection fraction; EM, electron microscopy; HF, heart failure; HW/BW, heart weight/body weight ratio; ISO, isoproterenol (isoprenaline); LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; ox, overexpression; PKA, protein kinase A; PLB, phospholamban; PP1, protein phosphatase 1; RyR2, cardiac ryanodine receptor; SCWs, spontaneous Ca^{2+} waves; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum; TEM, transmission electron microscopy; TM, triple mutant; TMRE, tetramethylrhodamine ethyl ester; TM-S2808A+/–, triple mutant with heterozygous RyR2 S2808A mutation (genotype: $CASQ2^{+/-}$-$SERCA1a^{ox}$-$S2808A^{+/-}$); TM-S2808A+/+, triple mutant with homozygous RyR2 S2808A mutation (genotype: $CASQ2^{+/-}$-$SERCA1a^{ox}$-$S2808A^{+/+}$); VT, ventricular tachycardia; WT, wild type.

Introduction

Abnormal calcium (Ca^{2+}) release from the sarcoplasmic reticulum (SR) is recognized to play a key role in pathological events in the heart. Evidence from human and animal models of disease suggests that the cardiac SR Ca^{2+} release channel (RyR2) becomes abnormally active, i.e. 'leaky', in the failing heart (Marx *et al.* 2000; Shannon *et al.* 2003; Eisner *et al.* 2004; Kubalova *et al.* 2005). Accelerated diastolic release is thought to contribute to heart failure by compromising systolic Ca^{2+} release and contractility, impairing diastolic relaxation, as well as by activating pathological hypertrophic pathways and inducing cell death (Molkentin, 2000; Backs & Olson, 2006; Bers *et al.* 2006; Heineke & Molkentin, 2006; Anderson *et al.* 2011).

Although the pathological role of increased diastolic release via leaky RyR2s in heart failure has been well established, the mechanisms underlying RyR2 dysfunction remain controversial. Marks and colleagues have put forth evidence that increased phosphorylation of the RyR2 homotetramer by protein kinase A (PKA) on Ser-2808 results in dissociation of FKBP12.6 from the RyR2 complex, causing RyR2s to become hyperactive (Marx *et al.* 2000; Wehrens *et al.* 2006; Shan *et al.* 2010). However, key facets of this hypothesis have been challenged by other groups (Xiao *et al.* 2004; Carter *et al.* 2006; Benkusky *et al.* 2007; Bers, 2012; Zhang *et al.* 2012; Fischer *et al.* 2013). A growing body of evidence suggests that rather than PKA phosphorylation at Ser-2808, phosphorylation by CaMKII at Ser-2814 contributes to HF by enhancing RyR2 leak (Belevych *et al.* 2011; Respress *et al.* 2012; Fischer *et al.* 2013). Further adding to the complexity of the issue, RyR2 phosphorylation at these different sites have been suggested to play different roles in ischaemic *vs.* non-ischaemic cardiac disease (e.g. Ser-2808 *vs.* Ser-2814, respectively; Wehrens *et al.* 2006; Respress *et al.* 2012).

Unlike other PKA phosphorylation target proteins thought to be involved in the β-adrenergic modulation of cardiac Ca^{2+} signalling, such as Ser-2030 of RyR2 and Ser-16 of phospholamban (PLB; Xiao *et al.* 2005; Benkusky *et al.* 2007), Ser-2808 is highly phosphorylated (>60–80%) even at baseline (i.e. absence of adrenergic stimulation), according to most reports (Carter *et al.* 2006; Xiao *et al.* 2006; Benkusky *et al.* 2007; Fischer *et al.* 2013). This would substantially limit the functional responsiveness of this site to PKA phosphorylation. Indeed, a number of studies reported no discernible effects of PKA stimulation on RyR2 Ca^{2+} release (Li *et al.* 2002; Stange *et al.* 2003; Benkusky *et al.* 2007). Interestingly, previous work by this group and others has shown that decreasing (rather than increasing) RyR2 phosphorylation at this site by exogenous phosphatases results in increased RyR2 activity and enhanced diastolic SR Ca^{2+} release (Lokuta *et al.* 1995; Valdivia *et al.* 1995; Terentyev *et al.* 2003). These controversial results further suggest that the role of RyR2 Ser-2808 phosphorylation is more complex than currently recognized. Thus, whether and how PKA phosphorylation status of RyR2 affects RyR2 function and contributes to degenerative cardiac disease requires further investigation.

Heart failure is associated with elevated plasma catecholamines and increased β-adrenergic tone as part of the compensatory response to decreased contractility (Packer, 1988). Given the complex interplay between Ca^{2+} homeostasis, β-adrenergic signalling, and heart failure, it is difficult to dissect the specific contribution of either pathway to the pathophysiology of this disease. To examine the relationship between Ca^{2+} leakiness and cardiac disease, we recently generated a mouse model that reproduces constitutive elevated diastolic Ca^{2+} release by combining deficiency in the expression of the RyR2 regulatory protein calsequestrin 2 (CASQ2) with over-expression of SERCA1a. The double mutant (DM) mice develop dilated cardiomyopathy and die prematurely of contractile failure due to mitochondrial Ca^{2+} damage and apoptosis, with severity of the phenotype dependent on the extent of leakiness (Kalyanasundaram *et al.* 2012).

In the present study, we sought to examine the potential role of RyR2 phosphorylation at Ser-2808 in the development of Ca^{2+}-dependent cardiomyopathy (CCM) by using triple mutant (TM) mice featuring elevated diastolic release while expressing RyR2s that cannot be phosphorylated at this site (TM-S2808A).

If phosphorylation of Ser-2808 indeed contributes to disease by exacerbating SR Ca^{2+} leak, ablation of this site would alleviate CCM. Rather than alleviating the disease phenotype, constitutive dephosphorylation of Ser-2808 markedly aggravated CCM. These results suggest a novel mode of regulation of RyR2, in which dephosphorylation from a highly phosphorylated state (rather than further phosphorylation) increases RyR2 activity.

Methods

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 1996). The authors have read, and the experiments comply with, the policies and regulations of *The Journal of Physiology* given by Drummond (1996).

Generation of triple mutant mouse models

Previously, a double mutant mouse model of Ca^{2+}-dependent cardiomyopathy was generated by cross-breeding the CASQ2 knockout (KO) mice with mice overexpressing SERCA1a (Kalyanasundaram *et al.* 2012). The double mutant mice ($CASQ2^{+/-}$-$SERCA1a^{ox}$) of mixed strain on a predominantly FVB/N background, were further crossbred with RyR2 S2808A mice (Sv129) (Benkusky *et al.* 2007; Ullrich *et al.* 2012) to obtain triple mutant mice. The genotypes of the crossbred mice were confirmed by polymerase chain reactions (PCR; for CASQ2 KO, SERCA1a overexpression, RyR2 S2808A mutation) using tail DNA. The detailed breeding scheme is shown in Fig. 1. Age- and litter- controlled mice were utilized for experiments. Since survival of DM mice was similar between males and females, animals of both genders were used in the study. Of note, the sex and strain backgrounds of the animals differ from our previous work (Kalyanasundaram *et al.* 2012), where only males were used (DM females exhibited longer survival, unpublished observation). To simplify the nomenclature of the genetically engineered mice involved in this study, the term mutant (as in double mutant, DM, or triple mutant, TM) is used as a generic term to include both transgenic and targeted mutant (knock-in and knock-out) mice.

Transthoracic echocardiography

Mice were lightly anaesthetized using 1% isoflurane in 95% oxygen. The body temperature of the mice was maintained at ∼37°C by a heating pad. *In vivo* cardiac function was assessed at different time points using a Visualsonic Vevo 2100 imaging system, with a 30 MHz probe (VisualSonics, Toronto, Canada). Data was analysed using VisualSonic Software.

Electrocardiographic Recordings

Mice were lightly anaesthetized using 1.5% isoflurane in 95% oxygen. Baseline ECG was recorded for 5 min, followed by an additional 25 min after administration of β-agonist isoproterenol (ISO, 1.5 mg kg^{-1}, I.P. injection). Response to toe-pinch reflex was examined to ensure the proper level of anaesthesia.

Cardiomyocyte isolation and confocal Ca^{2+} imaging

Myocyte isolation. Mouse ventricular myocytes were isolated as previously described (Gyorke *et al.* 1997). Briefly, hearts were quickly excised and perfused on a Langendorff's apparatus at 37°C. After ∼5 min perfusion with nominally Ca^{2+}-free Tyrode solution (containing, in mM: 140 NaCl, 5.4 KCl 0.5 $MgCl_2$, 10 Hepes and 5.6 glucose; pH 7.3), the perfusate was then switched to Tyrode solution containing Liberase Blendzymes (Roche, Applied Science, IN, USA) for digestion of the connective tissue. After ∼20 min digestion, single ventricular myocytes were isolated from dissected and triturated ventricles and stabilized in BSA containing Tyrode solution.

Ca^{2+} imaging. Ventricular myocytes were loaded with ∼8 μM Fluo 3 AM (Invitrogen, Carlsbad, CA, USA) for 25 min at room temperature (RT), followed by 25 min incubation at RT to allow the fluorescent dye to wash out, and deesterification. 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 (Olympus Fluoview 1000). Myocytes were paced at 0.5 Hz using extracellular platinum electrodes. To assess the SR Ca^{2+} load, 20 mM caffeine was applied at the end of the experiments. Fluo 4 pento potassium salt (Invitrogen) was used to record Ca^{2+} sparks. The myocytes were permeabilized with saponin (0.01% for ∼30 s) dissolved in the internal solution, which contained (mM): 120 potassium aspartate, 20 KCl, 0.81 $MgCl_2$, 1 KH_2PO_4, 0.5 EGTA, (free [Ca^{2+}] 50 nM), 3 MgATP, 10 phosphocreatine, 20 Hepes (pH 7.2) and 5 U ml^{-1} creatine phosphokinase.

Mitochondrial membrane potential measurement

A voltage-sensitive fluorescent indicator, tetramethylrhodamine ethyl ester (TMRE) was utilized to measure mitochondria membrane potential. Freshly isolated mouse ventricular myocytes were loaded with TMRE (75 nM, 20 min, 37°C). TMRE was excited at 543 nm with a helium–neon laser, and the emission signals were collected at 570–650 nm. TMRE fluorescence was measured as a series of *X*–*Y* confocal images.

To quantify the level of regional mitochondria damage, the areas devoid of mitochondrial staining were automatically labelled using a custom-written MATLAB program (See Fig. 10*A*). The program first rotated the confocal image so that the longitudinal direction of the cell was oriented vertically. Then the horizontal edges between the stained and non-stained areas were identified using image convolution with a Sobel operator. The algorithm then paired two edges if there was no staining between them. Finally, the area between the paired edges was measured and was considered as damaged region if its area was larger than the size of 15 pixels. The individual size and number of such regions were quantified, summarized, and compared among groups.

Western blots

Mouse ventricular homogenates were prepared as previously described (Jaehnig *et al.* 2006). Proteins were separated by SDS–PAGE (5%–15% gradient gel, Bio-rad) and transferred onto nitrocellulose membrane. Membranes were probed by primary antibodies against RyR2 (Thermo Scientific) and Ser-2808 phosphorylated RyR2 (Badrilla Ltd), followed by secondary anti-mouse or anti-rabbit antibodies. SuperSignal chemiluminescence (Pierce Biotechnology Inc, Rockford, IL, USA) was utilized to detect the horseradish peroxidase conjugated protein bands. ImageJ was utilized to quantify the protein expression levels.

[^{3}H]Ryanodine binding assay

[^{3}H]Ryanodine binding assay was performed as previously described with modifications (Li & Chen, 2001). The Ca^{2+}-dependent [^{3}H]ryanodine binding was determined with cardiac homogenates plus a standard mixture of 200 mM KCl, 25 mM Tris–50 mM Hepes (pH 7.4), 3 mM MgATP, 1 mM EGTA, 5 nM [^{3}H]ryanodine (68.4 Ci mmol^{-1}, Dupont NEN, Wilmington, DE, USA) and $CaCl_2$. Free Ca^{2+} concentration was set from pCa 8 to pCa 3 by using MaxChelator (http://www.stanford.edu/~cpatton/maxc.html). [^{3}H]ryanodine binding was normalized to RyR2 expression level which was determined by Western blots.

Transmission electron microscopy (TEM)

Mouse hearts were excised from the chest and dissected into thin slices. Left ventricular tissue was cut into 1 mm cube and immediately put it into a puddle of fixative on a wax plate. The fixative consists of 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The tissue was fixed overnight at 4°C. The tissue was further processed as previously described (Fraysse *et al.* 2010). Ultra-thin sections (70 nm) were produced on a Leica EM UC6 ultramicrostome and stained with 2% uranyl acetate and Reynold's lead citrate. TEM was performed on a FEI Tecnai G2 Spirit TEM at 80 kV. Images were captured using an AMT 2 ×2 digital camera.

Materials

Protein phosphatase 1 (PP1) was purchased from Sigma (St Louis, MO, USA). Fluorescent dyes were purchased from Invitrogen (Carlsbad, CA, USA).

Statistical analysis

Results are expressed as means ± SEM. Statistical significance of differences between experimental groups was determined using unpaired Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

Results

Ablation of RyR2 phosphorylation at Ser-2808 increased mortality in CCM

To investigate the role of PKA phosphorylation of RyR2 in Ca^{2+}-dependent cardiomyopathy (CCM), we cross-bred double mutant (DM) mice deficient in CASQ2 and expressing SERCA1a with mice in which Ser-2808 of RyR2 was replaced with Ala to prevent phosphorylation at this site. The crossing gave rise to triple mutant (TM) mice that were deficient in expression of CASQ2, overexpressed SERCA1a, and expressed RyR2s that had the Ser-2808 site either partially or completely disabled (TM-S2808A+/− and TM-S2808A+/+, respectively; Fig. 1). In line with our previous observations (Kalyanasundaram *et al.* 2012), DM mice ($CASQ2^{+/-}$-$SERCA1a^{ox}$) exhibited shorter survival than WT controls (albeit to a lesser extent than in the previous study). Disabling the Ser-2808 site increased the death rate in the TM mice. Surprisingly, early death was more pronounced in mice with heterozygous (TM-S2808A+/−), than with homozygous ablation of the Ser-2808 phosphorylation site (TM-S2808A+/+) (Fig. 2*A*). Thus, the survival studies showed that genetic disabling of PKA phosphorylation at Ser-2808 did not improve survival of CCM mice. Instead, ablation of the Ser-2808 site exacerbated mortality, most notably in TM mice heterozygous for S2808A.

Of note, the crossbreeding between DM and S2808A+/+ mice gave rise to a spectrum of mice with various genotypes. All the relevant control groups for this study, including DM controls, were littermates obtained from the crossbreeding to ensure validity of comparison.

Ablation of RyR2 phosphorylation at Ser-2808 exacerbated *in vivo* cardiac dysfunction in CCM but did not exacerbate arrhythmia vulnerability

Consistent with previous demonstration of structural remodelling in Ca^{2+}-dependent cardiomyopathy (Kalya-

nasundaram *et al.* 2012), postmortem examination showed elevated heart weight/body weight ratio (HW/BW) in DM mice. Ablation of Ser-2808 rather than preventing hypertrophy led to a further increase of HW/BW, especially in the TM-S2808A+/− mice (Fig. 2*B*). We next examined *in vivo* cardiac function and chamber dimensions by echocardiography in the four different mouse groups (Fig. 2*C* and *D*). At the age of 2 months, when DM mice only began to show signs of depressed ejection fraction (EF), EF was already markedly reduced in TM-S2808A+/− compared with wild type (WT) mice (39.8 ± 4.0 *vs.* 61.6 ± 1.4, $P < 0.05$, Fig. 2*C*), although not as much in TM-S2808A+/+ mice. Additionally, left ventricular end-systolic dimension (LVESD) was significantly increased in TM-S2808A+/− mice, but only tended to increase in TM-S2808A+/+ mice (Fig. 2*C*).

Surface electrocardiography (ECG) was recorded from lightly anaesthetized mice before and after catecholaminergic challenge with isoproterenol (ISO). At baseline, all groups showed a nearly normal sinus rhythm. With a catecholamine challenge (1.5 mg kg^{-1}, ISO), ventricular tachycardia was observed in one-third of mice from all three CCM groups, but not in the WT group (Fig. 3). Taken together, these results suggest that heterozygous ablation of Ser-2808 phosphorylation exacerbated contractile dysfunction, but did not seem to exacerbate arrhythmogenic propensity in our model.

Ablation of RyR2 phosphorylation at Ser-2808 exacerbated abnormal Ca^{2+} handling in CCM

Confocal Ca^{2+} imaging was performed in myocytes isolated from the same four mouse groups (WT, DM, TM-S2808A+/− and TM-S2808A+/+). At baseline, consistent with our previous studies (Kalyanasundaram *et al.* 2012), the DM myocytes displayed increased Ca^{2+} transient amplitude and increased frequency of diastolic Ca^{2+} waves compared to WT myocytes (Fig. 4). The frequency of Ca^{2+} waves was further increased in both TM groups, with the change being more pronounced in TM-S2808A+/− myocytes (Fig. 4*B*). The SR Ca^{2+} content was markedly increased in all three CCM groups (DM, TM-S2808A+/− and TM-S2808A+/+), with no significant differences between the groups (Fig. 4*D*). ISO further increased the frequency of Ca^{2+} waves in each group, with both TM-S2808A+/− and TM-S2808A+/+ myocytes displaying higher wave frequencies than DM myocytes (Fig. 4*B*). Of note, ISO did not further increase the SR Ca^{2+} content in the three CCM groups, as evidently maximum attainable SR Ca^{2+} content in these cells was already reached due to the activity of overexpressed SERCA1a. The ability of ISO to increase Ca^{2+} wave frequency in the TM cells expressing RyR2 that cannot be phosphorylated at Ser-2808 suggests that ISO-dependent Ca^{2+} waves are mediated by phosphorylation of sites distinct from Ser-2808, most likely the CaMKII site, Ser-2814, as suggested by several previous studies (Ferrero *et al.* 2007; Curran *et al.* 2010; Pereira *et al.* 2013), and possibly RyR2 oxidation, which has also been shown to increase upon stimulation with ISO (Bovo *et al.* 2012). Additionally Chen *et al* (Xiao *et al.* 2005, 2007) reported that PKA-dependent phosphorylation of RyR2 at Ser-2030 can increase RyR2 functional activity by modulating RyR2's sensitivity to luminal Ca^{2+} (Xiao *et al.* 2007). Thus the mechanisms of Ser-2808-independent effects of adrenergic stimulation remain to be clarified.

Rather than manifesting a direct modulatory influence, the observed effects of ablation of the Ser-2808 site on Ca^{2+} handling in CCM mice could result from RyR2 dysregulation due to lack of CASQ2. To examine this possibility, we performed experiments in SERCA1a

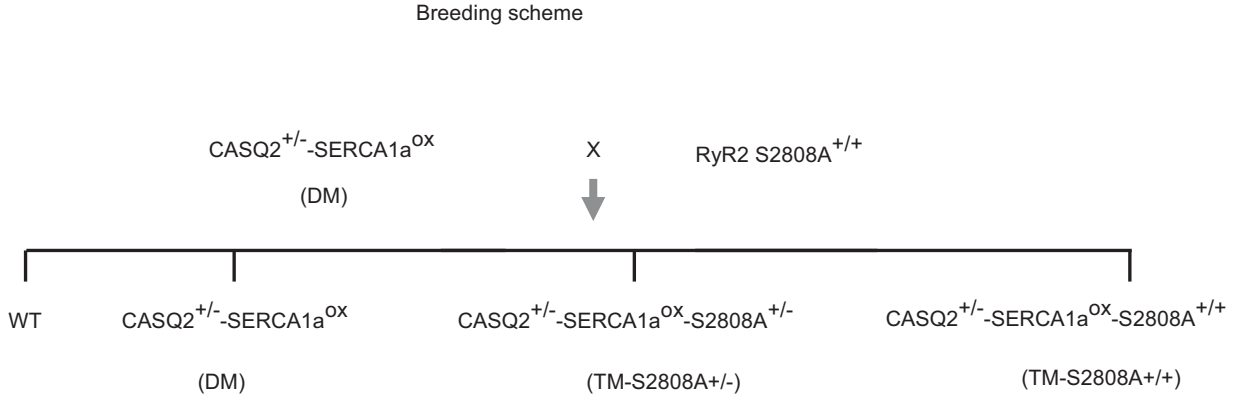

Figure 1. Breeding scheme and nomenclature for the mice involved in current study
This figure lists the simplified breeding process for the triple mutant mice, which were deficient in expression of CASQ2, overexpressed SERCA1a, and expressed RyR2s that had the Ser-2808 site either partially or completely disabled. Additionally, the abbreviations for the different mutant mice and its associated genotype are listed. Of note, the crossbreeding between the DM and RyR2 S2808A mice also gave rise to mice with other genotypes which were not listed here. This breeding scheme is a simplified version listing only the relevant study groups.

expressing mice carrying either WT or mutant RyR2 (S2808A), but normal for CASQ2. SERCA1a over-expression results in healthy mice with enhanced cardiac contractility, robust myocyte SR Ca^{2+} uptake and stable SR Ca^{2+} load (Loukianov *et al.* 1998). Similar to our results in the TM myocytes, ablation of Ser-2808 phosphorylation resulted in increased frequency of Ca^{2+} sparks in SERCA1a expressing myocytes (Fig. 5). Moreover, spark frequency tended to be higher in S2808A+/– than S2808A+/+ group. Thus, ablation of Ser-2808 is capable of increasing RyR2 activity independently of CASQ2 regulation of the channel.

Ablation of Ser-2808 phosphorylation blunted the increase in Ca^{2+} spark frequency by exogenous phosphatases

Consistent with previous studies (Carter *et al.* 2006; Xiao *et al.* 2006; Benkusky *et al.* 2007), RyR2 was highly phosphorylated (~75%) at Ser-2808 in WT and DM hearts expressing unmutated RyR2s, while substitution of Ser for Ala at 2808 rendered the sites either partially dephosphorylated or non-phosphorylatable in the TM-S2808A+/– or TM-S2808A+/+ group, respectively (Fig. 6*A* and *B*). Given the high phosphorylation status

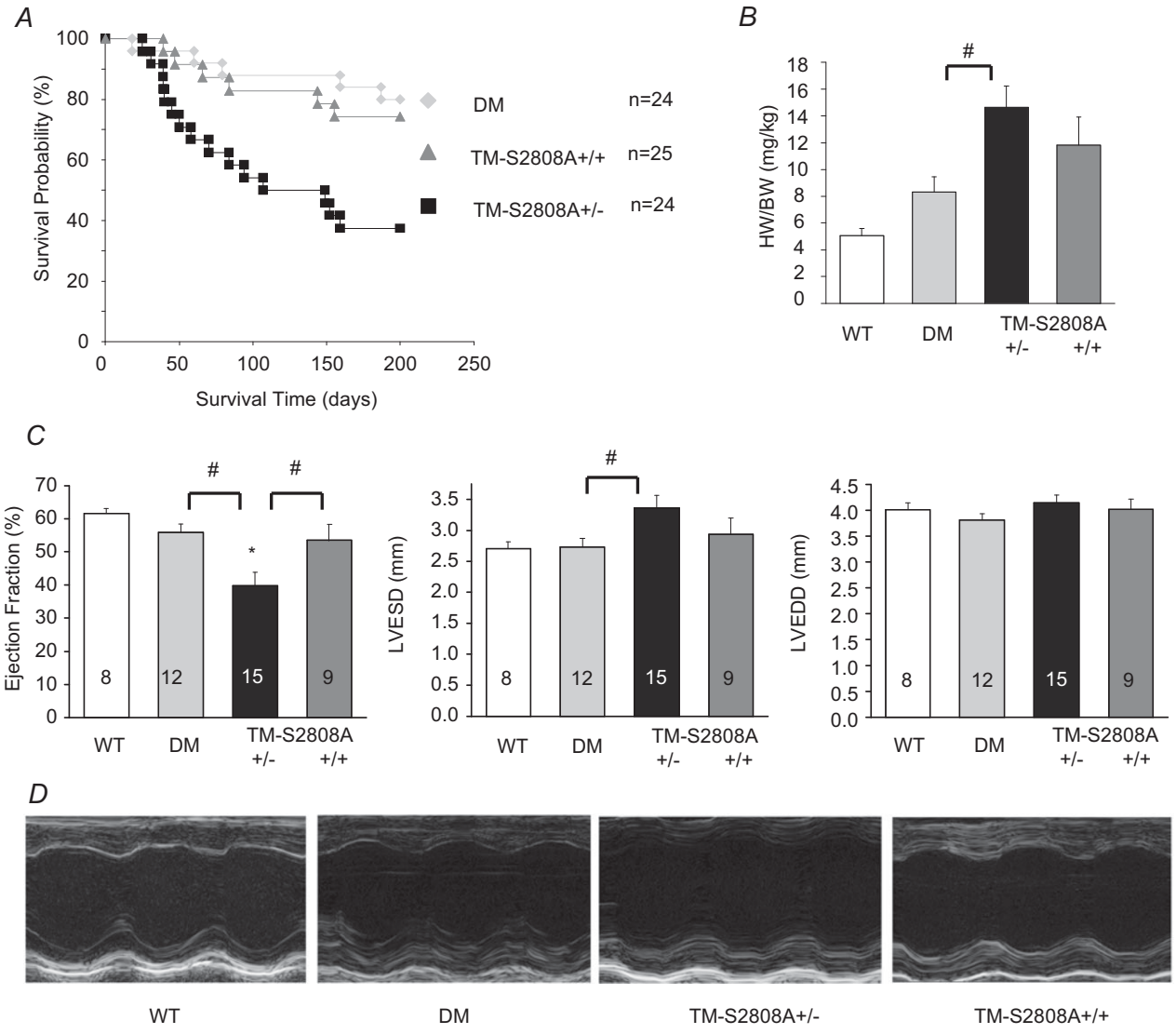

Figure 2. Ablation of RyR2 phosphorylation at Ser-2808 exacerbated survival and *in vivo* cardiac function in the CCM model
A, survival studies on DM mice and TM mice with Ser-2808 phosphorylation ablated. Sample sizes, indicated next to the curves, included 9 males and 15 females for DM; 11 males and 14 females for TM-S2808A+/+ and 11 males and 13 females for TM-S2808A+/–. *B*, mean ± SEM of HW/BW. *C*, means ± SEM of LV ejection fraction (EF%), left ventricular end-systolic dimension (LVESD) and left ventricular end-diastolic dimension (LVEDD) at the age of 2 months. $^*P < 0.05$ *vs.* WT control, $\#P < 0.05$ *vs.* TM-S2808A+/–. Sample sizes are indicated within the respective columns. *D*, representative images of M-mode echocardiography.

of RyR2 at baseline, it is possible that dephosphorylation (rather than further phosphorylation) of this site leads to exacerbated CCM and abnormal Ca^{2+} cycling by enhancing RyR2 leakiness in TM mice. To directly examine the functional consequences of RyR2 dephosphorylation at Ser-2808, we performed experiments with application of the protein phosphatase PP1 to permeabilized DM, TM-S2808A+/– and TM-S2808A+/+ myocytes. Consistent with the notion that dephosphorylation causes increased RyR2 leakiness, baseline spark frequency was significantly higher in TM myocytes than in DM myocytes. Interestingly, baseline frequency was highest in TM-S2808A+/– myocytes with incomplete dephosphorylation (Fig. 6*C* and *D*). Addition of PP1 (10 U ml^{-1}) significantly increased spark frequency measured within 30 s after PP1 application in DM cells (Fig. 6*C* and *D*). Notably, the increase in spark frequency in these cells was transient, despite overexpression of SERCA1a which should preclude significant SR Ca^{2+} depletion in these myocytes. Since on addition of PP1, phosphorylated RyR2s must initially pass through sets of partially dephosphorylated states before becoming completely dephosphorylated at all sites, these results reaffirm that partial dephosphorylation of RyR2 at Ser-2808 exacerbates RyR2 leakiness to a greater extent than complete dephosphorylation. In further support of this notion, addition of PP1 failed to produce a transient increase of spark frequency in TM-S2808A+/− cells from

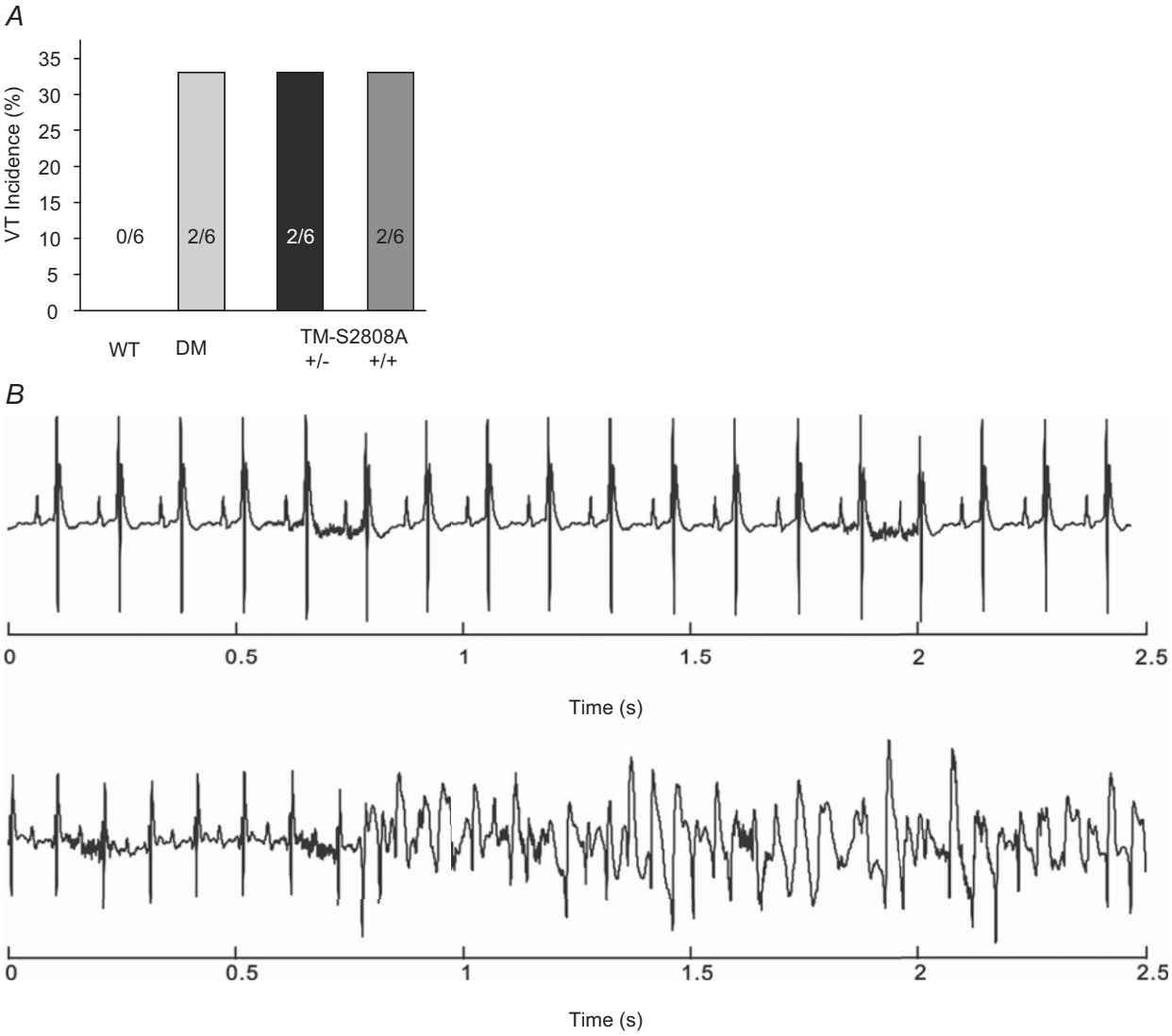

Figure 3. Ablation of RyR2 phosphorylation at Ser-2808 did not further increase the arrhythmogenic propensity in the Ca^{2+}-dependent cardiomyopathy models
A, the number of mice displaying ventricular tachycardia (VT) was similar among all three cardiomyopathy groups after catecholamine challenge (ISO, 1.5 mg kg^{-1} I.P.). *B*, representative traces of normal ECG (upper trace) and ventricular tachycardia (lower trace).

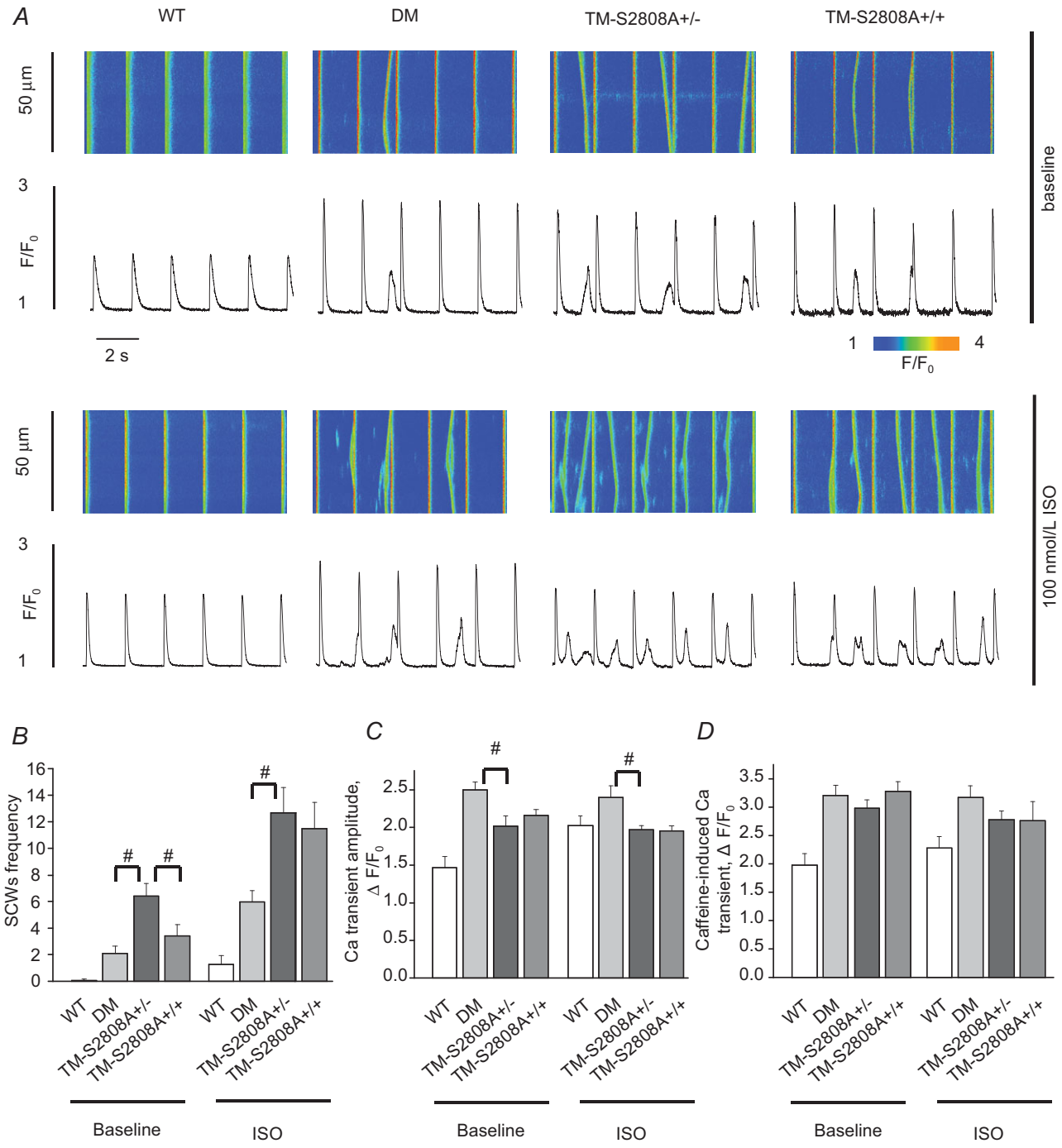

Figure 4. Ablation of RyR2 phosphorylation at Ser-2808 in the CCM model led to increased propensity for Ca^{2+} waves in isolated myocytes
A, representative line-scan images (top) and time-dependent profiles (bottom) of spontaneous Ca^{2+} waves (SCWs) under baseline condition and in the presence of 100 nmol ISO. *B*, pooled data for frequency of SCWs. *C*, pooled data for amplitude of Ca^{2+} transients, baseline and in 100 nmol ISO. #$P < 0.05$ *vs*. TM-S2808A+/–. *D*, pooled data for amplitude of caffeine induced Ca^{2+} transients, baseline and 100 nmol ISO.

its elevated baseline level, but instead led to significant decrease in steady-state Ca^{2+} spark frequency. PP1 failed to increase Ca^{2+} spark frequency in TM-S2808A+/+ myocytes, in which all 2808 sites are constitutively dephosphorylated (Fig. 6*C* and *D*). Taken together these results suggest that (1) RyR2 dephosphorylation at Ser-2808 enhances RyR2 leakiness and (2) the effects of partial dephosphorylation on leak are greater than those of complete dephosphorylation.

To further examine the effects of ablation of Ser-2808 phosphorylation on RyR2 activity in our CCM mouse models, we performed measurements of [^{3}H]ryanodine binding. Consistent with spark measurements, [^{3}H]ryanodine binding was significantly increased in both TM groups, and more substantially in TM-S2808A+/−, as compared with DM (Fig. 7).

Heterozygous ablation of RyR2 phosphorylation at Ser-2808 increased Ca^{2+} signalling heterogeneity

Apart from increasing leakiness of individual RyR2s, it is also possible that partial disruption of RyR2 phosphorylation by PKA plays a detrimental role by increasing spatial heterogeneity of Ca^{2+} signalling in cardiac myocytes. Assuming that WT and mutant (S2808A) RyR2 monomers randomly mix to assemble into a tetrameric channel, one would expect a substantial heterogeneity in the composition of individual RyR2 tetramers, as well as in the composition of clusters of RyR2 channels forming release sites. To look for such possible spatial heterogeneities of Ca^{2+} signalling, we examined Ca^{2+} spark amplitude characteristics in permeabilized myocytes from the two triple transgenic groups. Notably, spark amplitude was significantly increased in TM-S2808A+/− myocytes compared to TM-S2808A+/+ (Fig. 8*A* and *C*). The difference in spark amplitudes is further emphasized in Figs 8*A* and *B* which show that the increase in Ca^{2+} spark amplitude was due to the redistribution of Ca^{2+} sparks to a larger amplitude population. Additionally, the mean standard deviation (SD) of Ca^{2+} spark amplitude in the S2808A+/− group was significantly larger than that of the S2808A+/+ group (Fig. 8*C*), further suggestive of greater spark amplitude heterogeneity in S2808A+/− myocytes.

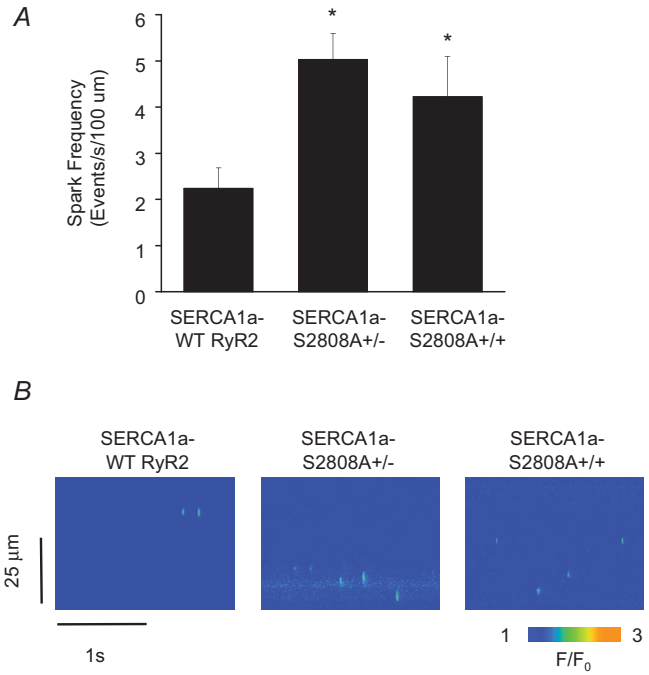

Figure 5. Ablation of Ser-2808 phosphorylation also increased SR Ca^{2+} leak in mice overexpressing SERCA1a
A, averaged spark frequency for mice with different genotypes for the RyR2s. Data are means ± SEM from at least 15 cells from each group. *$P < 0.05$ *vs.* SERCA1a-WT RyR2. *B*, representative line-scan images of Ca^{2+} sparks in intact myocytes.

Ablation of RyR2 phosphorylation at Ser-2808 exacerbated ultrastructural and regional mitochondrial damage

Elevated diastolic SR Ca^{2+} release has been shown to result in mitochondrial Ca^{2+} overload and injury, in turn leading to myocyte degeneration and death (Dorn, 2013). To examine whether deteriorated Ca^{2+} cycling in the TM myocytes is associated with increased ultrastructural and regional mitochondrial damage, we utilized electron microscopy (EM) and performed mitochondrial membrane potential measurements using tetramethylrhodamine ethyl ester (TMRE). As illustrated by the EM micrographs in Fig. 9, compared with the intact mitochondria structure of WT mice, DM did not show pronounced mitochondria damage, but both TM groups had severe mitochondria damage marked by partial or complete disruption of the mitochondria matrix. The extent of such mitochondria damage was more severe in the TM-S2808A+/− group than the TM-S2808A+/+ group (Fig. 9*A* and *B*).

Mitochondrial membrane potential measurements revealed further pathological changes in mitochondria associated with deteriorated Ca^{2+} handling in the TM myocytes. As shown in Fig. 10, WT myocytes displayed a relatively homogenous labelling, with organized arrangement of mitochondria bands along the myofibrils. However, such homogenous labelling was disrupted to different degrees in the three CCM groups, which is illustrated more clearly in the enlarged images shown in Fig. 10*A*. Myocytes from the three CCM groups displayed round areas of varying sizes devoid of mitochondrial potential staining indicative of regional damage (Fig. 10*A*). In TM-S2808A+/− myocytes, this 'Swiss cheese' pattern was most pronounced. When quantified, the average size

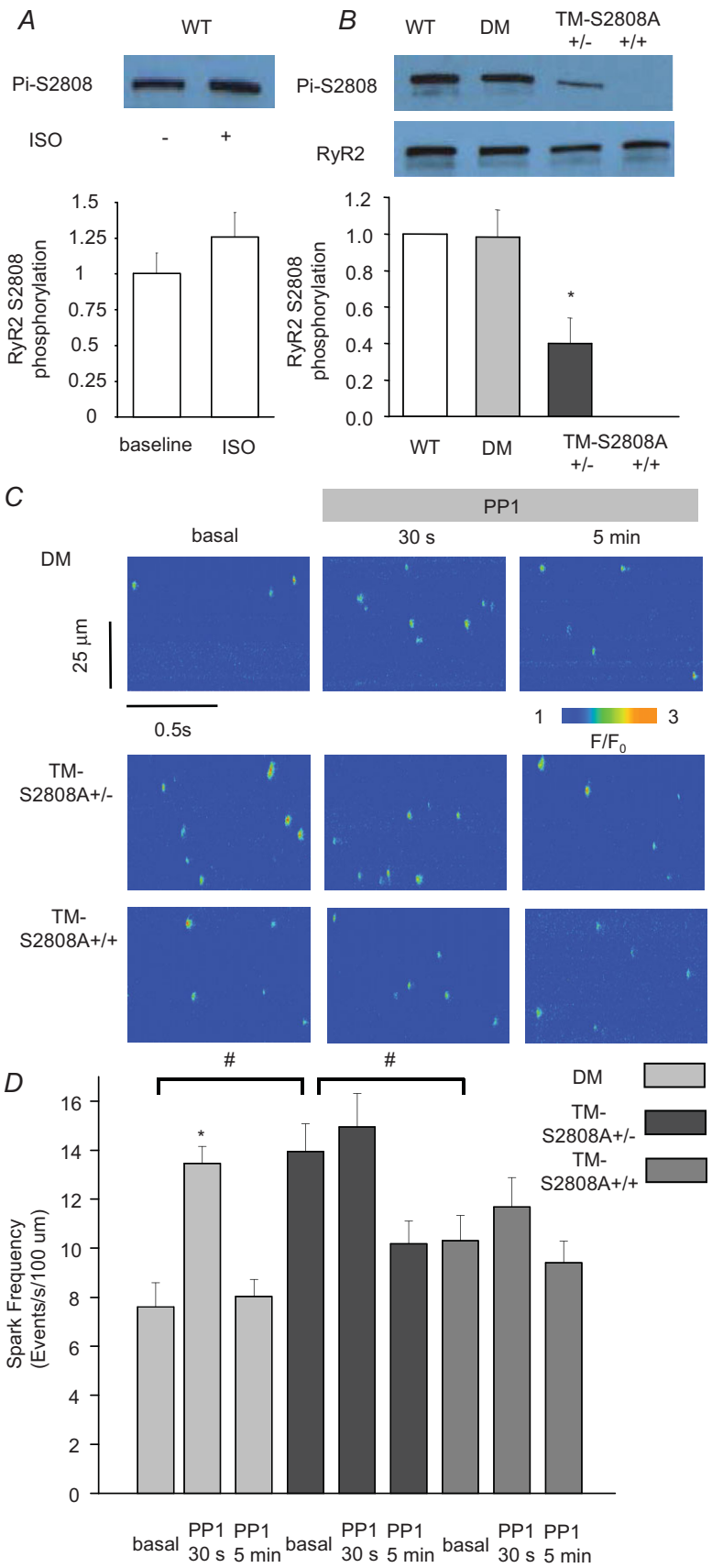

Figure 6. Ablation of Ser-2808 phosphorylation blunted the modulatory effect of phosphatase to increase RyR2 activity
A, Western blots of WT hearts perfused with or without ISO (100 nmol; $n = 3$). *B*, Western blots of cardiac tissue homogenates in WT and the three CCM groups, $^*P < 0.05$ *vs.* WT ($n = 4$). *C*, representative line-scan images of Ca^{2+} sparks in permeabilized myocytes before and after exposure of cells to 10 U ml^{-1} PP1. *D*, averaged spark frequency under different experimental conditions. Data are means ± SEM from at least 8 cells from each group. $^*P < 0.05$ *vs.* basal. #$P < 0.05$ *vs.* TM-S2808A+/−.

of regional damage (shown as holes in the TMRE staining of myocytes, highlighted in green in the lower panels of Fig. 10*A*) was significantly larger in the TM-S2808A+/− group than in the other two groups. When the distribution of hole size was plotted, the TM-S2808A+/− group also outnumbered the other two groups at the large-size scale (inset of Fig. 10*B*). Additionally, the size of mitochondria regional damage is also more heterogeneous in the TM-S2808A+/− group (Fig. 10*C*), supported by the increased SD of mitochondria damage size.

Discussion

Despite significant effort, the role of RyR2 phosphorylation by PKA in the 'fight or flight' response and pathophysiology of heart failure remains one of the most noted controversies in the field. Marks and coworkers have provided evidence in support of such a role via phosphorylation on Ser-2808 increasing RyR2 activity (Marx *et al.* 2000; Wehrens *et al.* 2006; Shan *et al.* 2010). Yet others found no indications that RyR2 PKA phosphorylation perceptibly affected Ca^{2+} handling and contractility in normal and diseased hearts (Li *et al.* 2002; Xiao *et al.* 2005; Benkusky *et al.* 2007; Respress *et al.* 2012; Zhang *et al.* 2012); some studies even reported that RyR2 dephosphorylation (rather than phosphorylation) at this site increased RyR2 activity (Valdivia *et al.* 1995; Terentyev *et al.* 2003). This long lasting controversy is a likely indication that RyR2 regulation is more complex than currently realized, thus resulting in a range of apparently inconsistent results. In the present study, we used a mouse model of Ca^{2+}-dependent cardiomyopathy to examine the consequences of preventing RyR2 phosphorylation at Ser-2808 on progression of Ca^{2+}-dependent disease. In this model, reduced expression of the RyR2 stabilizing protein, CASQ2, is combined with overexpression of SERCA1a to generate sustained diastolic Ca^{2+} release. These mice died prematurely, and displayed cardiac dysfunction and dilated hypertrophy associated with enhanced diastolic Ca^{2+} release, Ca^{2+}-dependent mitochondrial damage, and myocyte death in a manner proportional to the extent of diastolic release (Kalyanasundaram *et al.* 2012). In the present study, using this model we found that inhibition of RyR2 phosphorylation at Ser-2808, instead of alleviating, exacerbated Ca^{2+} mishandling and cardiac dysfunction. Notably, ablation of this site acted in a heterozygous disadvantage manner, that is, the consequences of partial, heterozygous ablation were more severe than those of complete, homozygous ablation. These results do not support the importance of RyR2 hyperphosphorylation in Ca^{2+}-dependent heart disease, and rather suggest roles for the opposite process, the RyR2 dephosphorylation at

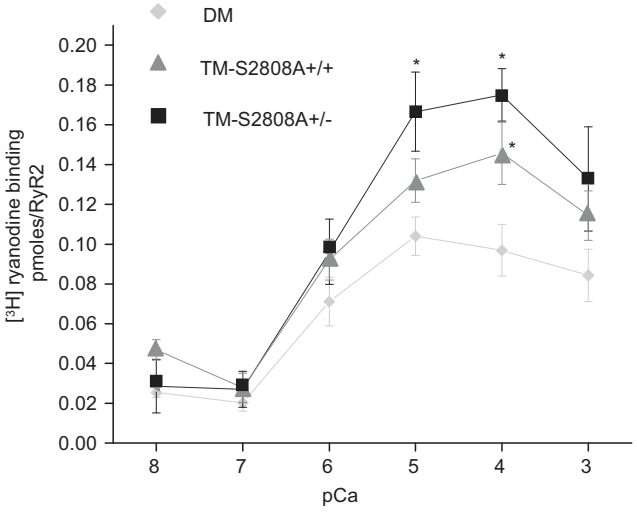

Figure 7. Ablation of RyR2 phosphorylation at Ser-2808 increased Ca^{2+} dependence of [^{3}H]ryanodine binding
[^{3}H]ryanodine binding in DM, TM-S2808A+/– and TM-S2808A+/+ at different Ca^{2+} concentrations normalized to total RyR2. *$P < 0.05$ *vs.* DM.

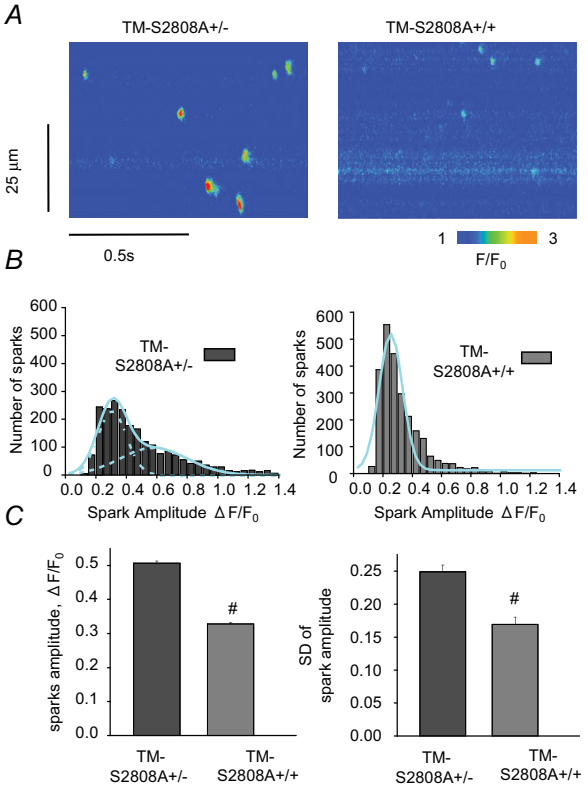

Figure 8. Heterozygous ablation of RyR2 phosphorylation at Ser-2808 in the CCM model increased Ca^{2+} signalling heterogeneity
A, representative line-scan images of Ca^{2+} sparks in saponin-permeabilized myocytes. *B*, the amplitude distribution of Ca^{2+} sparks. *C*, the average amplitude and standard deviation (SD) of Ca^{2+} sparks, #$P < 0.05$ *vs.* TM-S2808A+/−.

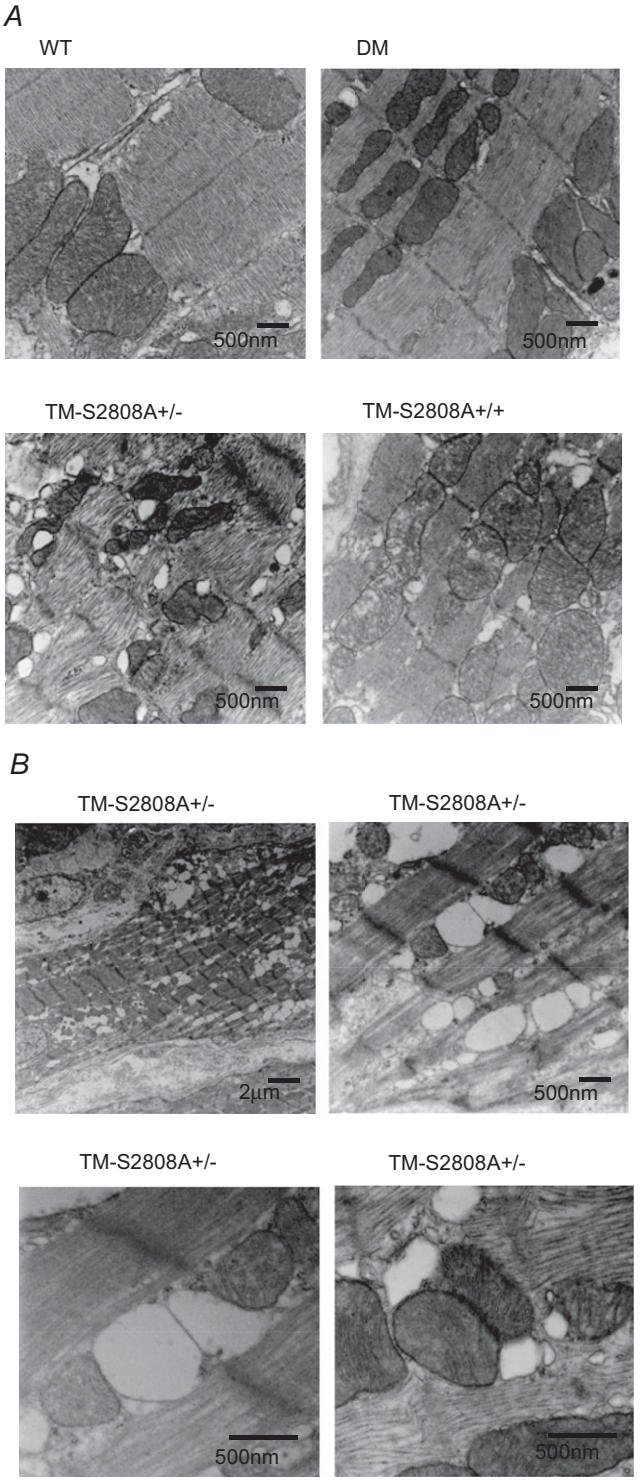

Figure 9. Heterozygous ablation of RyR2 phosphorylation at Ser-2808 in the CCM model led to more severe ultrastructural remodelling than homozygous ablation and unmodified Ser-2808
A, overview of myofibrillar organization in WT, DM, TM-S2808A+/– and TM-S2808A+/+. *B*, abnormal mitochondria morphology in TM-S2808A+/– at different resolutions.

this residue in physiological and pathophysiological Ca^{2+} signalling.

Mechanisms of action of constitutive dephosphorylation of Ser-2808 in CCM

Consistent with previous studies (Carter *et al.* 2006; Xiao *et al.* 2006; Benkusky *et al.* 2007), Ser-2808 phosphorylation was relatively high (75%) at baseline condition in both WT and DM mice, even in the absence of β-adrenergic stimulation (Fig. 6*A*). This is in sharp contrast with the phosphorylation status of other sites (e.g. Ser-2030 of RyR2, Ser-16 of PLB) that are low at baseline and increase upon β-adrenergic stimulation (Xiao *et al.* 2005; Benkusky *et al.* 2007). Therefore, to account for the deterioration of the CCM phenotype in TM mice, and in particular of those heterozygous for S2808A, we hypothesized that dephosphorylation from the more fully phosphorylated states at Ser-2808 makes the RyR2 channels leakier, thereby exacerbating CCM. In support of this hypothesis, application of PP1 produced a surge in spark frequency in DM myocytes expressing WT RyR2 highly phosphorylated at baseline, which was absent in TM myocytes expressing RyR2s with fully or partially disabled Ser-2808 phosphorylation sites (Fig. 6). Interestingly, the increase in sparks in DM myocytes was transient, consistent with a transition of RyR2s from highly phosphorylated states to only partially dephosphorylated, but functionally more active states, and then to completely dephosphorylated states with less pronounced leakiness (Fig. 6). In further support of this explanation, the baseline spark frequency for TM myocytes was significantly higher than that of DM myocytes, with the difference being most pronounced in the TM-S2808A+/– myocytes that mimic partially dephosphorylated states (Fig. 6). Moreover, [^{3}H]ryanodine binding was significantly increased in TM hearts, especially in the TM-S2808A+/– group, which is indicative of increased RyR2 activity in these groups (Fig. 7). These results are also supported by previous reports showing that RyR2 dephosphorylation can increase RyR2 activity in various experimental settings (Lokuta *et al.* 1995; Valdivia *et al.* 1995; Terentyev *et al.* 2003). The heterozygous-disadvantaged manner in which genetic modification affected the RyR2 tetramer is not unique to this channel. Similar effects have been reported for a mutation in the HCN4 channel (Duhme *et al.* 2014), which is also a tetramer. Apparently, partial modification of tetramers alters monomer interaction, thus affecting channel stability and functional activity (Duhme *et al.* 2014).

Notably, in addition to increased overall RyR2 leakiness and mitochondrial damage, TM myocytes, particularly those heterozygous for the S2808A modification, exhibited profound heterogeneities in Ca^{2+} signalling and regional mitochondrial degradation ('Swiss cheese'

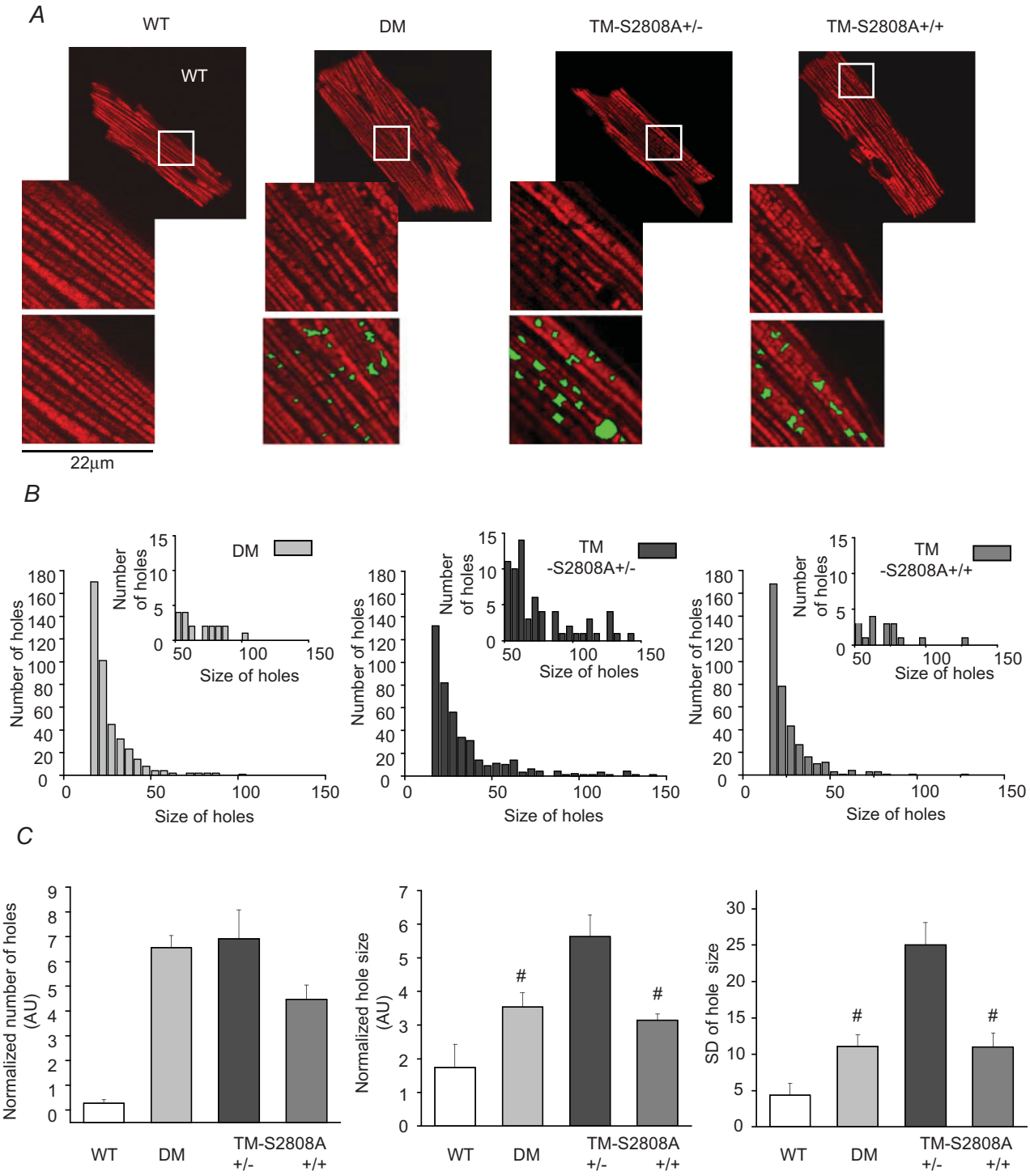

Figure 10. Mitochondrial membrane potential measurements revealed severe regional damage in TM-S2808A+/−

A, representative images of mitochondrial membrane potential measurements, the top panel shows the overview of TMRE stained cardiac myocytes, enlarged regions of the boxed areas are shown in the lower left corner, with regional damage or holes highlighted by a MATLAB program in green. *B*, the histograms of distribution of mitochondria regional damage size. *C*, quantification of regional damage or holes: the average number of holes (normalized to the size of myocyte), the average size of holes (normalized to the size of myocyte) and the average standard deviation (SD) of hole size. #$P < 0.05$ *vs*. TM-S2808A+/−.

pattern). These results suggest that the impact of increased leakiness of dephosphorylated RyR2s in TM-S2808A+/− myocytes is further amplified by heterogeneity of Ca^{2+} handling expected as a result of combinatorial variations in the monomer composition of individual RyR2 tetramers. Indeed, the RyR2 channel is a protein tetramer assembled from the available monomer variants (Nakai *et al.* 1990; Fill & Copello, 2002); thus, in myocytes expressing the two monomer variants, most channels will be composed of a mixture of the phosphorylated WT and non-phosphorylatable mutant monomers, with a smaller fraction of channels composed of the WT or mutant monomers only (i.e. 87.5%, 6.25% and 6.25%, respectively; Xu *et al.* 2008; Loaiza *et al.* 2012). Our results are consistent with other examples of degenerative processes and localized Ca^{2+}-dependent mitochondrial damage caused by disruption in homogeneity of RyR2 function in both cardiac and skeletal muscle (Litwin *et al.* 2000; Diaz *et al.* 2002; Aistrup *et al.* 2006; Zhou *et al.* 2010).

When recently examining Ca^{2+} handling and RyR2 function in WT *vs.* S2808A mice, Ullrich *et al.* (2012) found only subtle differences. However, these studies were performed in mice homozygous for S2808A; furthermore, most experiments were conducted in the presence of ISO, which is expected to lead to RyR2 modifications through other mechanisms, including CaMKII phosphorylation at Ser-2814 and oxidation (Ferrero *et al.* 2007; Curran *et al.* 2010; Bovo *et al.* 2012), thus masking potential effects specifically related to the phosphorylation status of Ser-2808. Therefore, future experiments are needed to determine as to what extent this phenomenon applies to other disease conditions and physiological settings.

Lastly, it is to be noted that survival of DM mice in this study is substantially longer than what we have previously reported in mice bearing the same genetically-engineered modifications (Kalyanasundaram *et al.* 2012). This difference could be ascribed to the differences in the genetic background and gender of the animals used as specified in the Methods. Genetic background and gender differences may also contribute, in part, to the multiplicity of findings in the literature and their role requires further investigation.

Implications for normal physiology and disease

Our study demonstrates an unexpected functional complexity of RyR2 modification through phosphorylation/dephosphorylation. Cardiac performance and Ca^{2+} handling are under balanced control of sympathetic and parasympathetic systems. Upon sympathetic stimulation, phosphorylation of RyR2 at Ser-2814 and other target proteins including L-type Ca^{2+} channel and PLB contributes to the 'fight or flight' response by facilitating Ca^{2+} cycling (De Jongh *et al.* 1996; Chu *et al.* 2000; Pereira *et al.* 2013). In contrast, Ser-2808 phosphorylation does not seem to play a major role in this process. This is supported by our results showing that ISO can facilitate Ca^{2+} cycling independent of the S2808A mutation (Fig. 4). Our investigation shed light on a novel mode of RyR2 operation, possibly evolved to mediate SR Ca^{2+} release at low levels of cardiac energy consumption upon shifting the regulatory balance to the parasympathetic side. The dependence of Ca^{2+} release on SR Ca^{2+} content is highly non-linear such that lowering SR Ca^{2+} content below a level as high as 40% of normal can abolish release (Bassani *et al.* 1995). Thus, increasing RyR2 activity through dephosphorylation at Ser-2808, as shown here, coupled with dephosphorylation of PLB and L-type Ca^{2+} channel aimed at reducing the SR Ca^{2+} load could maximize energy efficiency of Ca^{2+} cycling. In support of this notion, ablation of the Ser-2808 phosphorylation sites increased Ca^{2+} spark frequency not only in CCM myocytes but also in myocytes from healthy SERCA1a overexpression mice (Figs 5 and 6). Previously, it has been suggested that reducing RyR2 phosphorylation at Ser-2808 alleviates heart failure by decreasing SR Ca^{2+} leak. Our results obtained in a genetic model of Ca^{2+}-dependent cardiomyopathy suggest that the relationship between RyR2 phosphorylation/dephosphorylation at this site and cardiac disease is much more complex and warrants further investigation in clinically more relevant models.

References

Aistrup GL, Kelly JE, Kapur S, Kowalczyk M, Sysman-Wolpin I, Kadish AH & Wasserstrom JA (2006). Pacing-induced heterogeneities in intracellular Ca^{2+} signalling, cardiac alternans, and ventricular arrhythmias in intact rat heart. *Circ Res* **99**, e65–73.

Anderson ME, Brown JH & Bers DM (2011). CaMKII in myocardial hypertrophy and heart failure. *J Mol Cell Cardiol* **51**, 468–473.

Backs J & Olson EN (2006). Control of cardiac growth by histone acetylation/deacetylation. *Circ Res* **98**, 15–24.

Bassani JW, Yuan W & Bers DM (1995). Fractional SR Ca release is regulated by trigger Ca and SR Ca content in cardiac myocytes. *Am J Physiol Cell Physiol* **268**, C1313–C1319.

Belevych AE, Terentyev D, Terentyeva R, Nishijima Y, Sridhar A, Hamlin RL, Carnes CA & Gyorke S (2011). The relationship between arrhythmogenesis and impaired contractility in heart failure: role of altered ryanodine receptor function. *Cardiovasc Res* **90**, 493–502.

Benkusky NA, Weber CS, Scherman JA, Farrell EF, Hacker TA, John MC, Powers PA & Valdivia HH (2007). Intact β-adrenergic response and unmodified progression toward heart failure in mice with genetic ablation of a major protein kinase A phosphorylation site in the cardiac ryanodine receptor. *Circ Res* **101**, 819–829.

Bers DM (2012). Ryanodine receptor S2808 phosphorylation in heart failure: smoking gun or red herring. *Circ Res* **110**, 796–799.

Bers DM, Despa S & Bossuyt J (2006). Regulation of Ca^{2+} and Na^{+} in normal and failing cardiac myocytes. *Ann N Y Acad Sci* **1080**, 165–177.

Bovo E, Lipsius SL & Zima AV (2012). Reactive oxygen species contribute to the development of arrhythmogenic Ca^{2+} waves during β-adrenergic receptor stimulation in rabbit cardiomyocytes. *J Physiol* **590**, 3291–3304.

Carter S, Colyer J & Sitsapesan R (2006). Maximum phosphorylation of the cardiac ryanodine receptor at serine-2809 by protein kinase a produces unique modifications to channel gating and conductance not observed at lower levels of phosphorylation. *Circ Res* **98**, 1506–1513.

Chu G, Lester JW, Young KB, Luo W, Zhai J & Kranias EG (2000). A single site (Ser16) phosphorylation in phospholamban is sufficient in mediating its maximal cardiac responses to beta -agonists. *J Biol Chem* **275**, 38938–38943.

Curran J, Brown KH, Santiago DJ, Pogwizd S, Bers DM & Shannon TR (2010). Spontaneous Ca waves in ventricular myocytes from failing hearts depend on Ca^{2+}-calmodulin-dependent protein kinase II. *J Mol Cell Cardiol* **49**, 25–32.

De Jongh KS, Murphy BJ, Colvin AA, Hell JW, Takahashi M & Catterall WA (1996). Specific phosphorylation of a site in the full-length form of the alpha 1 subunit of the cardiac L-type calcium channel by adenosine 3′,5′-cyclic monophosphate-dependent protein kinase. *Biochemistry* **35**, 10392–10402.

Diaz ME, Eisner DA & O'Neill SC (2002). Depressed ryanodine receptor activity increases variability and duration of the systolic Ca^{2+} transient in rat ventricular myocytes. *Circ Res* **91**, 585–593.

Dorn GW 2nd (2013). Mitochondrial dynamics in heart disease. *Biochim Biophys Acta* **1833**, 233–241.

Duhme N, Schweizer PA, Thomas D, Becker R, Schroter J, Barends TR, Schlichting I, Draguhn A, Bruehl C, Katus HA & Koenen M (2014). Altered HCN4 channel C-linker interaction is associated with familial tachycardia-bradycardia syndrome and atrial fibrillation. *Eur Heart J* **34**, 2768–2775.

Eisner DA, Diaz ME, O'Neill SC & Trafford AW (2004). Physiological and pathological modulation of ryanodine receptor function in cardiac muscle. *Cell Calcium* **35**, 583–589.

Ferrero P, Said M, Sanchez G, Vittone L, Valverde C, Donoso P, Mattiazzi A & Mundina-Weilenmann C (2007). Ca^{2+}/calmodulin kinase II increases ryanodine binding and Ca^{2+}-induced sarcoplasmic reticulum Ca^{2+} release kinetics during β-adrenergic stimulation. *J Mol Cell Cardiol* **43**, 281–291.

Fill M & Copello JA (2002). Ryanodine receptor calcium release channels. *Physiol Rev* **82**, 893–922.

Fischer T, Herting J, Tirilomis T, Renner A, Neef S, Toischer K, Ellenberger D, Forster A, Schmitto J, Gummert J, Schondube F, Hasenfuss G, Maier L & Sossalla S (2013). CaMKII and PKA differentially regulate SR Ca^{2+}-leak in human cardiac pathology. *Circulation* **128**, 970–981.

Fraysse B, Nagi SM, Boher B, Ragot H, Laine J, Salmon A, Fiszman MY, Toussaint M & Fromes Y (2010). Ca^{2+} overload and mitochondrial permeability transition pore activation in living δ-sarcoglycan-deficient cardiomyocytes. *Am J Physiol Cell Physiol* **299**, C706–C713.

Gyorke S, Lukyanenko V & Gyorke I (1997). Dual effects of tetracaine on spontaneous calcium release in rat ventricular myocytes. *J Physiol* **500**, 297–309.

Heineke J & Molkentin JD (2006). Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol* **7**, 589–600.

Jaehnig EJ, Heidt AB, Greene SB, Cornelissen I & Black BL (2006). Increased susceptibility to isoproterenol-induced cardiac hypertrophy and impaired weight gain in mice lacking the histidine-rich calcium-binding protein. *Mol Cell Biol* **26**, 9315–9326.

Kalyanasundaram A, Lacombe VA, Belevych AE, Brunello L, Carnes CA, Janssen PM, Knollmann BC, Periasamy M & Gyorke S (2012). Up-regulation of sarcoplasmic reticulum Ca^{2+} uptake leads to cardiac hypertrophy, contractile dysfunction and early mortality in mice deficient in CASQ2. *Cardiovasc Res* **98**, 297–306.

Kubalova Z, Terentyev D, Viatchenko-Karpinski S, Nishijima Y, Gyorke I, Terentyeva R, da Cunha DN, Sridhar A, Feldman DS, Hamlin RL, Carnes CA & Gyorke S (2005). Abnormal intrastore calcium signalling in chronic heart failure. *Proc Natl Acad Sci U S A* **102**, 14104–14109.

Li P & Chen SR (2001). Molecular basis of Ca^{2+} activation of the mouse cardiac Ca^{2+} release channel (ryanodine receptor). *J Gen Physiol* **118**, 33–44.

Li Y, Kranias EG, Mignery GA & Bers DM (2002). Protein kinase A phosphorylation of the ryanodine receptor does not affect calcium sparks in mouse ventricular myocytes. *Circ Res* **90**, 309–316.

Litwin SE, Zhang D & Bridge JH (2000). Dyssynchronous Ca^{2+} sparks in myocytes from infarcted hearts. *Circ Res* **87**, 1040–1047.

Loaiza R, Benkusky NA, Powers PP, Hacker T, Noujaim S, Ackerman MJ, Jalife J & Valdivia HH (2012). Heterogeneity of ryanodine receptor dysfunction in a mouse model of catecholaminergic polymorphic ventricular tachycardia. *Circ Res* **112**, 298–308.

Lokuta AJ, Rogers TB, Lederer WJ & Valdivia HH (1995). Modulation of cardiac ryanodine receptors of swine and rabbit by a phosphorylation-dephosphorylation mechanism. *J Physiol* **487**, 609–622.

Loukianov E, Ji Y, Grupp IL, Kirkpatrick DL, Baker DL, Loukianova T, Grupp G, Lytton J, Walsh RA & Periasamy M (1998). Enhanced myocardial contractility and increased Ca^{2+} transport function in transgenic hearts expressing the fast-twitch skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase. *Circ Res* **83**, 889–897.

Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, Rosemblit N & Marks AR (2000). PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* **101**, 365–376.

Molkentin JD (2000). Calcineurin and beyond: cardiac hypertrophic signalling. *Circ Res* **87**, 731–738.

Nakai J, Imagawa T, Hakamat Y, Shigekawa M, Takeshima H & Numa S (1990). Primary structure and functional expression from cDNA of the cardiac ryanodine receptor/calcium release channel. *FEBS Lett* **271**, 169–177.

Packer M (1988). Neurohormonal interactions and adaptations in congestive heart failure. *Circulation* **77**, 721–730.

Pereira L, Cheng H, Lao DH, Na L, van Oort RJ, Brown JH, Wehrens XH, Chen J & Bers DM (2013). Epac2 mediates cardiac β1-adrenergic-dependent sarcoplasmic reticulum Ca^{2+} leak and arrhythmia. *Circulation* **127**, 913–922.

Respress JL, van Oort RJ, Li N, Rolim N, Dixit SS, Dealmeida A, Voigt N, Lawrence WS, Skapura DG, Skardal K, Wisloff U, Wieland T, Ai X, Pogwizd SM, Dobrev D & Wehrens XH (2012). Role of RyR2 phosphorylation at S2814 during heart failure progression. *Circ Res* **110**, 1474–1483.

Shan J, Betzenhauser MJ, Kushnir A, Reiken S, Meli AC, Wronska A, Dura M, Chen BX & Marks AR (2010). Role of chronic ryanodine receptor phosphorylation in heart failure and β-adrenergic receptor blockade in mice. *J Clin Invest* **120**, 4375–4387.

Shannon TR, Pogwizd SM & Bers DM (2003). Elevated sarcoplasmic reticulum Ca^{2+} leak in intact ventricular myocytes from rabbits in heart failure. *Circ Res* **93**, 592–594.

Stange M, Xu L, Balshaw D, Yamaguchi N & Meissner G (2003). Characterization of recombinant skeletal muscle (Ser-2843) and cardiac muscle (Ser-2809) ryanodine receptor phosphorylation mutants. *J Biol Chem* **278**, 51693–51702.

Terentyev D, Viatchenko-Karpinski S, Gyorke I, Terentyeva R & Gyorke S (2003). Protein phosphatases decrease sarcoplasmic reticulum calcium content by stimulating calcium release in cardiac myocytes. *J Physiol* **552**, 109–118.

Ullrich ND, Valdivia HH & Niggli E (2012). PKA phosphorylation of cardiac ryanodine receptor modulates SR luminal Ca^{2+} sensitivity. *J Mol Cell Cardiol* **53**, 33–42.

Valdivia HH, Kaplan JH, Ellis-Davies GC & Lederer WJ (1995). Rapid adaptation of cardiac ryanodine receptors: modulation by Mg^{2+} and phosphorylation. *Science* **267**, 1997–2000.

Wehrens XH, Lehnart SE, Reiken S, Vest JA, Wronska A & Marks AR (2006). Ryanodine receptor/calcium release channel PKA phosphorylation: a critical mediator of heart failure progression. *Proc Natl Acad Sci U S A* **103**, 511–518.

Xiao B, Jiang MT, Zhao M, Yang D, Sutherland C, Lai FA, Walsh MP, Warltier DC, Cheng H & Chen SR (2005). Characterization of a novel PKA phosphorylation site, serine-2030, reveals no PKA hyperphosphorylation of the cardiac ryanodine receptor in canine heart failure. *Circ Res* **96**, 847–855.

Xiao B, Sutherland C, Walsh MP & Chen SR (2004). Protein kinase A phosphorylation at serine-2808 of the cardiac Ca^{2+}-release channel (ryanodine receptor) does not dissociate 12.6-kDa FK506-binding protein (FKBP12.6). *Circ Res* **94**, 487–495.

Xiao B, Tian X, Xie W, Jones PP, Cai S, Wang X, Jiang D, Kong H, Zhang L, Chen K, Walsh MP, Cheng H & Chen SR (2007). Functional consequence of protein kinase A-dependent phosphorylation of the cardiac ryanodine receptor: sensitization of store overload-induced Ca^{2+} release. *J Biol Chem* **282**, 30256–30264.

Xiao B, Zhong G, Obayashi M, Yang D, Chen K, Walsh MP, Shimoni Y, Cheng H, Ter Keurs H & Chen SR (2006). Ser-2030, but not Ser-2808, is the major phosphorylation site in cardiac ryanodine receptors responding to protein kinase A activation upon β-adrenergic stimulation in normal and failing hearts. *Biochem J* **396**, 7–16.

Xu L, Wang Y, Yamaguchi N, Pasek DA & Meissner G (2008). Single channel properties of heterotetrameric mutant RyR1 ion channels linked to core myopathies. *J Biol Chem* **283**, 6321–6329.

Zhang H, Makarewich CA, Kubo H, Wang W, Duran JM, Li Y, Berretta RM, Koch WJ, Chen X, Gao E, Valdivia HH & Houser SR (2012). Hyperphosphorylation of the cardiac ryanodine receptor at serine 2808 is not involved in cardiac dysfunction after myocardial infarction. *Circ Res* **110**, 831–840.

Zhou J, Yi J, Fu R, Liu E, Siddique T, Rios E & Deng HX (2010). Hyperactive intracellular calcium signalling associated with localized mitochondrial defects in skeletal muscle of an animal model of amyotrophic lateral sclerosis. *J Biol Chem* **285**, 705–712.

Additional information

Competing interests

There are no competing interests.

Author contributions

B.L.and S.G. contributed to the conception, design, data interpretation and manuscript preparation. B.L., H.-T.H, F.V. and C.V. were responsible for experimental work. B.L. and Q.L. contributed to data collection and analysis. All authors approved the final version of the manuscript.

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 13POST16910102 to B.L.).

Acknowledgements

We thank Drs Jonathan Davis and Xander Wehrens for critical reading of the manuscript.

Translational perspective

The cardiac ryanodine receptor (RyR2) is the Ca^{2+} release channel of sarcoplasmic reticulum that provides the Ca^{2+} necessary to induce cardiac contraction. Excessive RyR2 activity during diastole (i.e. RyR2 Ca^{2+} leak) is thought to lead to a spectrum of cardiac pathologies (i.e. 'ryanopathies') including heart failure (HF) by compromising systolic Ca^{2+} release and contractility, impairing diastolic relaxation, and activating pathological hypertrophic pathways that trigger cell death. Post-translational modifications of RyR2, especially phosphorylation, are considered among the most important factors contributing to pathological RyR2 leakiness. However, the role of RyR2 phosphorylation in cardiac pathology remains incompletely understood and highly controversial. Previous work by some investigators has suggested that in HF, 'hyperphosphorylation' of RyR2 by PKA at Ser-2808 renders RyR2 leaky, and that genetic ablation of Ser-2808 prevents RyR2 leakiness and HF. However, these findings have not been reproduced by several other studies conducted in similar disease settings. In our current study, we utilized a genetic model of cardiomyopathy induced by excessive RyR2 Ca^{2+} leak. Remarkably, disabling Ser-2808 phosphorylation exacerbated rather than alleviated Ca^{2+}-dependent cardiomyopathy. Additionally, our results demonstrated that *de*phosphorylation, rather than phosphorylation of Ser-2808 – a site that is already highly phosphorylated at baseline – made RyR2 leakier and exacerbated the cardiomyopathy. Our results suggest that RyR2 modulation by Ser-2808 phosphorylation is more complex than previously thought, thereby suggesting the need for more tailored approaches in the design of HF therapies.