Expanded Materials and Methods

Mouse models

Experiments were performed using 12 - 16-week old male and female mice with four genotypes: C57BL/6J wild-type (WT), knockout mice with deletion of CaMKIIδ (CaMKIIδ-KO), and novel knock-in mouse models with a single mutation of the Cys-273 or Cys-290 *S*-nitrosylation sites on CaMKIIδ (CaMKIIδ-C273S and CaMKIIδ-C290A). The CaMKIIδ-C290A knock-in mice were generated by the UC Davis Mouse Biology Core and have been described previously [20]. CaMKIIδ-C273S animals were generated using CRISPR/Cas9 genome editing at the Australian Phenomics Facility (Australian National University, Australia). The cysteine codon "TGT" at position 273 was replaced with a serine codon "TCT" using two single guide RNAs: AGTGACTTAC**A**C**A**GATCCAT**GGG** and **CC**CATGGATC**T**G**T**GTAAGTCA CT and an oligonucleotide repair template: TTCTGTAAGATTATTTTAAACTTATGAAAAGTGAC TAGGGGTTTATCCTTCAGTTTGGCTCCTGGGGTGCATGCGAAAGTGACTTAC**A**G**A**GATCC AT**GGG**TGTTTCAGGGCCTCAGAGGCTGTGATACGTTTGGCAGGGTTGATGGTCAGCATTT TGTTGATGAGGTCTTTGG. All genetically modified animals were crossed with C57BL/6J mice to share common genetic background. CaMKIIδ-C273S mice were genotyped via PCR amplification of the oligonucleotide repair template with the following primers ATCTCATGTATACCAGGTTTCCC and CCTTCTGGGGTGCAGTAAGT and then ABI BigDye Terminator sequencing of that product. Ear notches from mice were incubated in 100µl of lysis buffer (100 mM Tris-HCL, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, pH 8.5 with Proteinase K at a final concentration of 100 ug/mL) for 3 hrs at 55°C, 15 min at 95°C and then stored overnight at 4°C. 1 µl of the lysate was used for template in 20 µl reactions. PCR was carried out using Phusion High-Fidelity DNA Polymerase (Thermo Fisher) with primers at 0.5 µM and initial denaturation of 1 min at 98°C; 35 cycles of 98°C for 10 sec, 62°C for 15 seconds (s), 72°C for 15 s; and the final extension at 72°C for 10 min. Products were run on 1% agarose gels and then gel-purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) according to manufacturer's instructions. This was then submitted for sequencing to Genetic Analysis Services at University of Otago with either of the primers listed above. Results were analyzed with SnapGene Viewer (Dotmatics).

Mice were fed Teklad Global 18% Protein Rodent diet (Envigo) *ad libitum* and housed at 20-22ºC under 12-hour light-dark cycles. The Animal Ethics Committee at both the University of Otago (AUP-18-42) and University of California, Davis (21572) approved use of animals in this study. For all mouse resources described here and materials listed below, please see the Major Resources Table in the Supplemental Materials.

Protein expression

The ventricles were dissected and snap frozen in liquid nitrogen for protein analysis as previously described [21]. Briefly, approximately 10 – 20 mg of ventricle tissue was homogenized in 20 volumes of buffer containing (in mM) 50 Tris-HCl pH 7.4 150 NaCl, 1 EDTA, 1 phenylmethylsulfonyl fluoride, 0.1% SDS, 1% triton X-100 (CaMKIIδ -KO) or 50 Tris-HCl pH 7.5, 1 phenylmethylsulfonyl fluoride, 3% SDS (RyR, SERCA, CaMKIIδ-C273S) and supplemented with cOmplete protease inhibitor (Roche). Lysates were then incubated on ice for 15 min and centrifuged for 15 min at 15000 rcf at 4°C. Lysates were stored at -80°C until use.

For CaMKIIδ expression in the CaMKIIδ-KO mice, 25µg of protein homogenate was separated on 10 % SDS polyacrylamide gel and transferred for 3 hrs on ice at 100 V to PVDF membrane, and then blocked for 1 hr at room temperature (RT) in 5% non-fat milk powder in Tris-bufferedsaline, 0.05% Twee-20 (TBST). CaMKIIδ was probed for using a primary antibody against CaMKIIδ (1:5000, ThermoFisher PA5-22168) and GAPDH (1:10000, GeneTex GTX627408) for a loading control. Blots were then incubated with secondary mouse or rabbit antibodies conjugated with horse-radish peroxidase

(1:10000; Thermo Fisher 31430, 31460), visualized by chemiluminescence detection with Super-signal West Pico (Thermo Fisher), and imaged using a Syngene gel doc system.

For the CaMKIIδ-C273S mice, 5 μg of protein homogenate was separated on Criterion™ XT Bis-Tris (RyR, SERCA, Actin) or 4–15% Mini-PROTEAN® TGX™ Precast stain-free (CaMKIIδ) gels (Bio-rad) and transferred at 4°C for 16 hrs at 30 V onto 0.45 μm supported nitrocellulose membrane (Bio-rad). Membranes were blocked in EveryBlot Blocking Buffer (Bio-rad) for 20 min at RT. Primary antibodies for RyR [34C] (1:500, Abcam ab2868), SERCA2a (1:5000, Badrilla A010-20), and CaMKIIδ (1:2000, Thermo Fisher PA5-22168) were incubated in EveryBlot Blocking Buffer (RyR) or TBST for 2 hrs at RT followed by mouse (1:20000, Thermo Fisher 31430) or rabbit (1:10000, Thermo Fisher 31460) secondary antibodies conjugated with horse-radish peroxidase in TBST for 1 hr at RT. Anti-beta Actin [AC-15] antibody HRP (1;20000, Abcam ab49900) was incubated in TBST for 1 hr at RT. Blots were visualized by chemiluminescence detection with Clarity Western ECL Substrate (Bio-rad) and imaged using a ChemiDoc MP (Bio-rad).

Heart Fibrosis

When ventricles were dissected as noted above the apex of the heart was fixed in 4% formalin (Sigma) for 24 hrs. The fixed tissue was then transferred to Phosphate-buffered saline (PBS) for 24 hrs then 30% sucrose in PBS for 48 hrs. The samples were then rinsed in PBS and cryopreserved in FSC 22 Frozen Section Media (Leica) and stored at -80°C until use. Sections were cut (8 μm) in a Leica CM 1950 Cryostat and 4 sections/sample mounted onto Superfrost Plus microscope slides (LabServ). Slides were stored at -20°C for up to one month before staining. Masson's trichrome staining of sections was performed by the Histology Unit at University of Otago. Slides were then scanned on an Aperio Slide Scanner (Leica) under 40X magnification.

Using Leica's ImageScope software, randomly selected regions of each section from blinded files were extracted and saved as tiff files. Files were imported into Adobe Photoshop and the background removed. After this, total pixels of the region were determined. Blue target pixels (representing collagen) were then identified. Percent collagen is the sum of all regions from sample sections of target pixels divided by total pixels.

Measurement of NO concentration

The amount of NO released following addition of NO donor S-nitrosoglutathione (GSNO) to our experimental buffer (KRH; see below) was measured using an Apollo 1000 Free Radical analyser with an ISO-NOPF100 NO microsensor (1 mm) (World Precision Instruments, USA). The sensor was calibrated with S-nitroso-N-Acetyl-D,L-Penicillamine (Toronto Research) in 0.1 M $CuCl_2$ solution [22]. Data were acquired using a Powerlab 2/25 and recorded in LabChart 8.1 (ADInstruments, New Zealand).

Isolation of cardiomyocytes

Ventricular cardiomyocytes were isolated from mouse hearts using a Langendorff-perfusion method [23]. Mice were anesthetized with isoflurane until loss of toe pinch reflex. Hearts were excised, aorta cannulated and subjected to retrograde perfusion with either Ca^{2+}-free Krebs-Ringer HEPES (KRH) Buffer containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgCl_2$, 25 HEPES, 6 glucose, pH 7.4 at 37ºC or MEM solution (for CaMKIIδ-C290A mice at UC Davis the KRH for myocyte isolation containing (in mM): 135 NaCl, 4.7 KCl, 1.2 $MgSO_4$•$7H_2O$, 0.6 KH_2PO_4, 0.6 Na_2HPO_4•$7H_2O$, 10 glucose, 20 HEPES, pH 7.4) gassed with 100% O_2. Hearts were then perfused for 5 - 10 min with 0.125 mM $CaCl_2$ KRH buffer supplemented with 1mg/mL of type II collagenase (Worthington Biochemical). Ventricles were cut into 0.1 mM $CaCl_2$ solution, and the tissue was minced into small pieces and further dissociated with gentle pipetting. Cardiomyocytes were collected by filtering solution though a 200 μm mesh and $CaCl_2$ concentration gradually increased.

Calcium imaging

Freshly isolated cardiomyocytes were loaded with 2 μM Fluo-4-AM (Thermo Fisher) for 20 min at room temperature, followed by wash and de-esterification for 30 min. Cells were added to a stimulation

bath mounted on either a Nikon A1+ confocal laser scanning microscope (excited at 488 nM and emission as collected through a 525/50 nm filter using a 63X oil immersion objective) or Bio-Rad Radiance 2100 (40X oil immersion objective lens) for CaMKIIδ-C290A myocytes only. Cardiomyocytes were field stimulated at 0.5 Hz for 30 s to establish a steady-state prior to recording Ca^{2+} transients in line-scan mode (2 ms/line, 0.15 x 0.15 μm pixel size). Ca^{2+} sparks and the occurrence of waves were measured under quiescent conditions (30 s after termination of 0.5 Hz pacing). Total SR Ca^{2+} content was determined at the end of each experiment with a rapid 20 mM caffeine exposure in Ca^{2+}-free KRH following a 30 s train of 0.5 Hz stimulations. Steady-state Ca^{2+} transients were averaged, and parameters analysed in custom written MATLAB software (version R2018a, MathWorks) and custom code written in Python. Ca^{2+} sparks were detected using the SparkMaster plugin in Fiji using a detection criteria of 3.8 [24]. All experiments were performed at room temperature and cardiomyocytes were constantly perfused with KRH buffer containing 1.5 mM $CaCl_2$ unless stated otherwise. Cardiomyocyte dimensions were measured in Fiji from confocal images acquired using frame-scan mode. Representative images have been chosen as illustrative examples of the results.

Animal Echocardiography

Echocardiography was carried out on WT and CaMKIIδ-C273S at 12 weeks of age using a Vivid E9 Imaging System (General Electric Vingmed Ultrasound, Norway) equipped with an ultrasound probe of 11 MHz frequency. Animals were maintained under anaesthesia with 0.5 L/min of 100% oxygen, 1-2% isoflurane. All captures were obtained and analysed as described previously [21].

Isolated Heart Function

Under anaesthesia (3% isoflurane, 0.5 L/min of 100% O_2), mice were injected intraperitoneally with heparin (10,000 U/kg). After 5 min, isoflurane inhalation was further increased to 5% to induce deep anaesthesia. The hearts were excised and arrested in Ca^{2+}- free Krebs-Henseleit buffer (KHB) on ice before being cannulated via the aorta and Langendorff-perfused with modified KHB. The modified KHB solution contained (in mM): 2.0 $CaCl_2.2H_2O$, 118.5 NaCl, 4.7 KCl, 1.2 $MgO_4S.7H_2O$, 1.2 $KH_2PO_4.H_2O$, 25 $NaHCO_3$, 11 glucose and 2.0 pyruvate. The modified KHB was aerated continuously with 95% oxygen and 5% carbon dioxide to maintain pH of 7.4. The coronary pressure was maintained at 75 mmHg and coronary flow rate range was between 1.5 - 4 mL/min. Each heart was allowed to stabilize in the organ bath while continuously perfused with KHB at 37ºC for 5 min. Afterwards, the left atrium was removed and a balloon connected to a pressure transducer (ADInstruments) was inserted into the left ventricle (LV) via the atrial appendage. The balloon was inflated with distilled water to achieve a Left Ventricular End Diastolic Pressure (LVEDP) of 5 - 10 mmHg. This was followed by a stabilization period of 30 min, during which the LV pressure trace was monitored for regular beats before obtaining basal heart function parameters.

Baseline data were recorded for 10 min, followed by drug infusion for 10 min. To determine the effect of NO and β-AR stimulation on heart function, the mouse hearts were randomly assigned to three groups depending on the order of drug infusion. The control group received isoproterenol (ISO, 100 nM) only followed by wash out with control KHB buffer. The treatment groups received either ISO (100 nM) followed by S-nitrosoglutathione (GSNO, 150 μM) or GSNO (150 μM) before ISO (100 nM). LV pressure, coronary flow, and temperature were recorded using a data acquisition system (LabChart 8.1, ADInstruments). The trace obtained was saved for offline analysis to measure the incidence of arrhythmias. Ventricular arrhythmic events from the LV pressure trace were evaluated and classified as outlined in Figure S1 and Table S1. The different types of arrhythmias observed at least once in the isolated hearts included; ventricular premature beats (VPBs), bigeminy, trigemini, potentiated contraction and ventricular tachycardia (VT) to form a five-point arrhythmia score classification which indicated severity of the arrhythmias (Table S2). Three minutes of the trace recording for each drug treatment were analysed for abnormal ventricular contractions. Isolated hearts were also Langendorff-

perfused without insertion of the balloon and treated with the same drug infusions or only KHB and immediately snap frozen in liquid nitrogen for analysis of protein *S*-nitrosylation.

Experimental solutions

All drugs were prepared fresh daily or from frozen aliquots. ISO (100 nM, Merck, Germany) was used to initiate the β-AR signalling pathway. The NO donor GSNO (150 μM, Cayman Chemical, USA) was used to induce *S*-nitrosylation in cardiomyocytes [19]. Sodium nitroprusside (SNP; 200 μM, Sigma, USA) was also tested as a clinical NO donor. The soluble guanylyl cyclase (sGC) inhibitor ODQ (10μM, Tocris, UK) was used to prevent activation of the NO-sGC-CGMP pathway.

Modified Biotin switch for *S*-nitrosylated protein detection

Frozen whole heart samples were homogenized at 4°C with a Precellys Evolution homogenizer (Bertin) in homogenization buffer containing (in mM); 300 sucrose, 250 HEPES-NaOH, pH 7.7, 1 EDTA, 100 neocuproine, 0.5% Triton-X 100 and 25 N-ethylmaleimide (NEM, Sigma). One tablet of EDTA-free protease inhibitor (Roche) was added just before use. All buffers were freshly prepared before use and subsequent procedures were carried out in the dark to prevent degradation of light-sensitive *S*-nitrosylated proteins. Homogenates were incubated on ice for 15 min and centrifuged at 14000 rcf for 10 min at 4°C. The supernatant was recovered as total crude homogenate, aliquoted and stored at -80°C. Total protein concentration was determined using the Bradford protein assay.

Homogenates (100 μg protein) were diluted in HEN buffer containing (in mM): 250 HEPES-NaOH, pH 7.7, 1 EDTA, 0.1 neocouproine, 2% SDS, 20 NEM and incubated and oscillated at 80 rpm, 50°C for 40 min. Excess NEM was removed via cold acetone precipitation (-20°C for 20 min) and the pellet was collected via centrifugation at 10,000 rcf for 10 min at 4°C. Between each precipitation, the sample pellet was air-dried to ensure total removal of acetone. Samples were then resuspended in HEN with 1% SDS (wt./vol) and incubated with ascorbate (Sigma) and 4 μM DyLight Maleimide 800 (Thermo Fisher) to reduce *S*-nitrosylated cysteine residues and label with the maleimide group. Excess dye was removed via cold acetone precipitation (-20°C for 20 min) and samples were resuspended in 50μL of 1X reducing buffer (Invitrogen loading dye and 5% β-mercaptoethanol), heated for 5 mins (95°C) and separated on 4-12% SDS PAGE gel at 75 V for 20 min and 150 V for 80 min. The gel was then rinsed with deionized water and fluorescence was visualized at 800 nm using an iBright Imaging System (Thermo Fisher).

Electrocardiogram recordings

Electrocardiograms (ECGs) were recorded from mice under 1.5-2 % isoflurane delivered via a nose cone with 100% O_2 at 0.5 L/min. Negative, positive and earth electrodes were inserted subcutaneously with acupuncture needles into the right front limb, left back limb and right back limb respectively. ECG measurements were acquired from lead II connected to a Powerlab and recorded in LabChart 8.1(ADInstruments). Recordings were made for 10 minutes with the last 5 minutes used for analysis performed in LabChart.

Data Analysis

All individual data points are shown for cardiomyocyte Ca^{2+} and isolated heart parameters along with the mean ± SEM. For results reported in the text, the data are mean ± SD. Isolated heart perfusion data were analysed on Lab Chart 8.1 (ADInstruments). Statistical analysis was performed using Prism 10 (GraphPad) and RStudio (Posit; McNemar's test only). Numeric data (n>10) were analysed for normality using Shapiro-Wilk test. Paired data (drug responses) with a normal distribution were analysed using paired 2-tailed student *t* test. Non-normally distributed data or n<10 were analysed using a non-parametric alternative test (Wilcoxon matched-pairs signed rank test or a Friedman test). For group comparisons (and SR content) an unpaired *t* test or an ordinary one-way ANOVA (Tukey's multiply comparisons test) was used for normally distributed data, while a Mann Whitney test or Kruskal-Wallis, was used for non-parametric data sets. Differences in the fraction of cardiomyocytes

displaying Ca^{2+} waves were determined by a Chi-squared test (between groups) or a McNemar's test (paired data). Values where $p \leq 0.05$ were considered statistically significant.

Data Supplement Tables S1-S3

Table S1. Arrhythmic events.

Name (Abbreviation)	Description
Ventricular premature beat (VPB)	an early contraction prior to relaxation and is often succeeded by a larger subsequent beat
Bigeminy	paired beats occurring in repetition
Trigeminy	a triplet beat occurring in repetition with every third beat as a premature beat
Potentiated contraction (PC)	normal sinus rhythm then a slight delay before an increased single contraction and followed by resumption of sinus rhythm
Ventricular tachycardia (VT)	four or more ventricular premature beats in quick succession

The different types of arrhythmic events identified from pressure trace and their descriptions.

Table S2. Arrhythmia Score Classification.

Arrhythmia Score	Characteristics
0	0 – 5 VPBs (no arrhythmias or isolated VPBs)
1	6 – 20 VPBs (very occasional bigeminy and trigeminy)
2	21 – 100 VPBs (frequent bigeminy, trigemini and PCs)
3	> 100 VPBs or 1 – 2 episodes of VT
4	> 3 VT
5	Ventricular fibrillation or death

A 5-point arrhythmia score classification to determine severity of arrhythmias observed in the isolated perfused mouse hearts. VPB = ventricular premature beats, PC = potentiated contraction, VT = ventricular tachycardia.

Table S3. Echocardiography parameters in WT and CaMKIIδ-C273S mice at 12 weeks

Parameter	WT	CAMKIIδ-C273S	Genotype effect (P value)
Animal weight (g)	28.18 ± 0.34	28.12 ± 0.67	9.3×10^{-1}
HR (beats/min)	419.9 ± 6.66	416.9 ± 11.34	8.1×10^{-1}
IVSd (mm)	0.99 ± 0.02	1.00 ± 0.02	7.5×10^{-1}
IVSs (mm)	1.29 ± 0.04	1.30 ± 0.03	9.0×10^{-1}
EF (%)	79.48 ± 1.20	69.54 ± 2.65	1.0×10^{-3}**
LVEDV (ml)	0.07 ± 0.02	0.11 ± 0.01	$<1.0 \times 10^{-4}$****
LVESV (ml)	0.01 ± 0.00	0.03 ± 0.00	$<1.0 \times 10^{-4}$****
R-R Interval (ms)	144.2 ± 2.33	146.1 ± 4.05	6.7×10^{-1}
E/A Ratio	2.31 ± 0.06	1.93 ± 0.07	5.0×10^{-4}***

HR, heart rate; IVSD, interventricular septum diastolic thickness; IVSS, interventricular septum systolic thickness; FS, Fractional shortening; EF, Ejection Fraction; LVEDV, left ventricular end diastolic volume; LVESV, left ventricular end systolic volume. Data are expressed as mean ± SEM. Independent t-test was used to compare between WT (n=20) and C273S (n=17) values, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, WT vs C273S.

Data Supplement Figures S1-S3

Figure S1. Arrhythmic events obtained from pressure trace

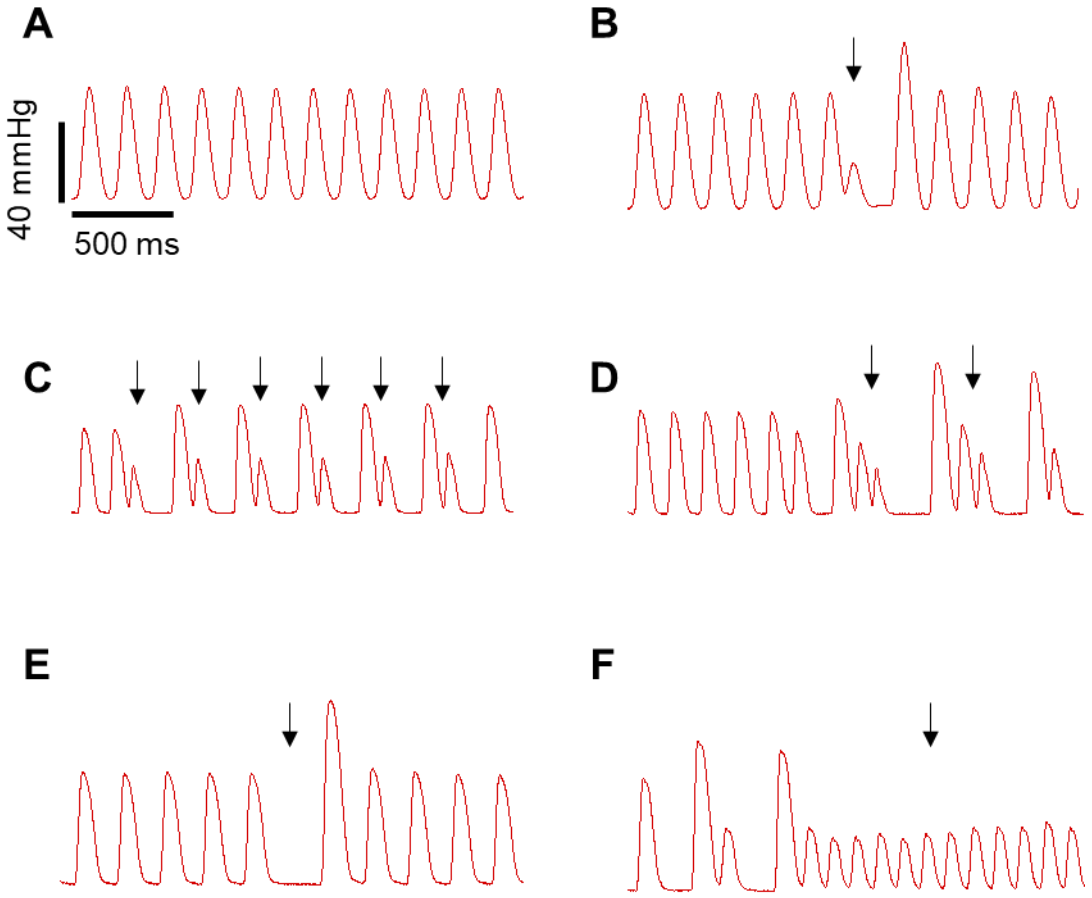

Figure S2. SR content for myocytes isolated from CaMKIIδ KO or C273S mice

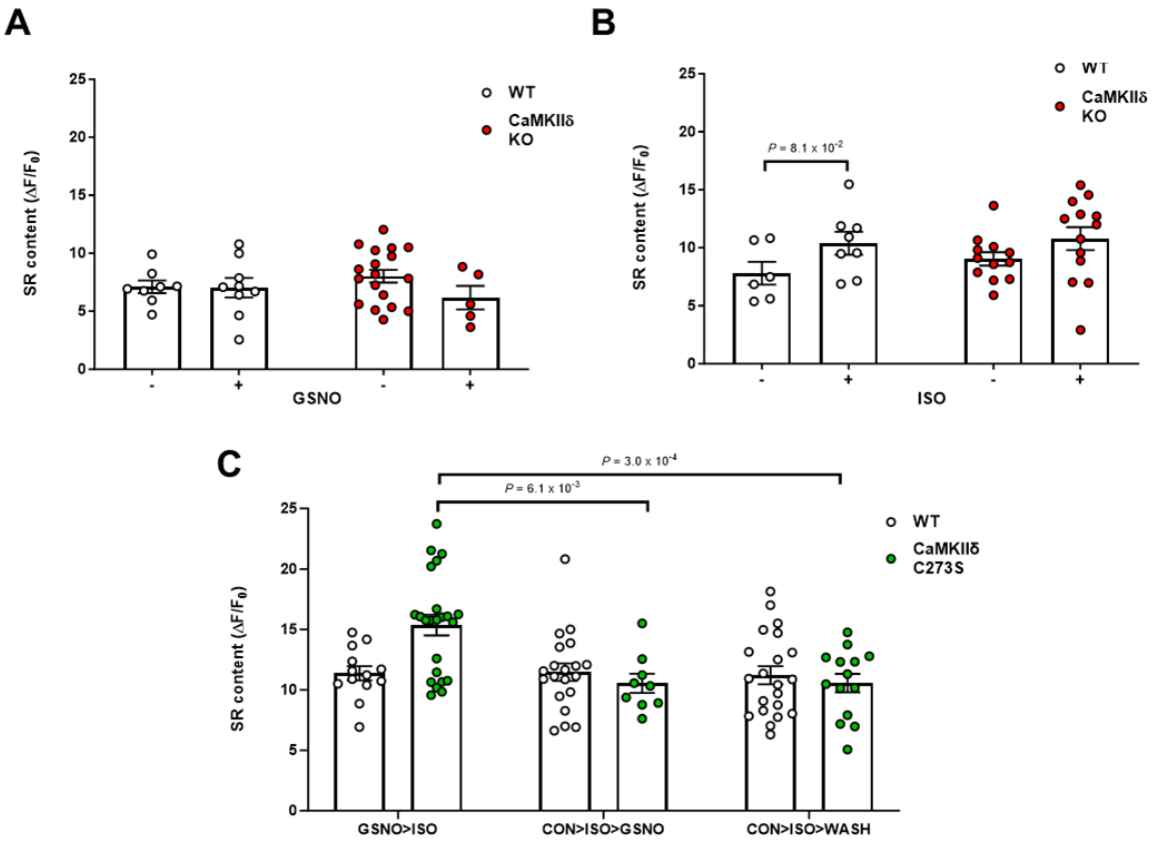

Figure S3. Whole mouse hearts exposed to ISO and GSNO have increased *S*-nitrosylation of cardiac proteins.

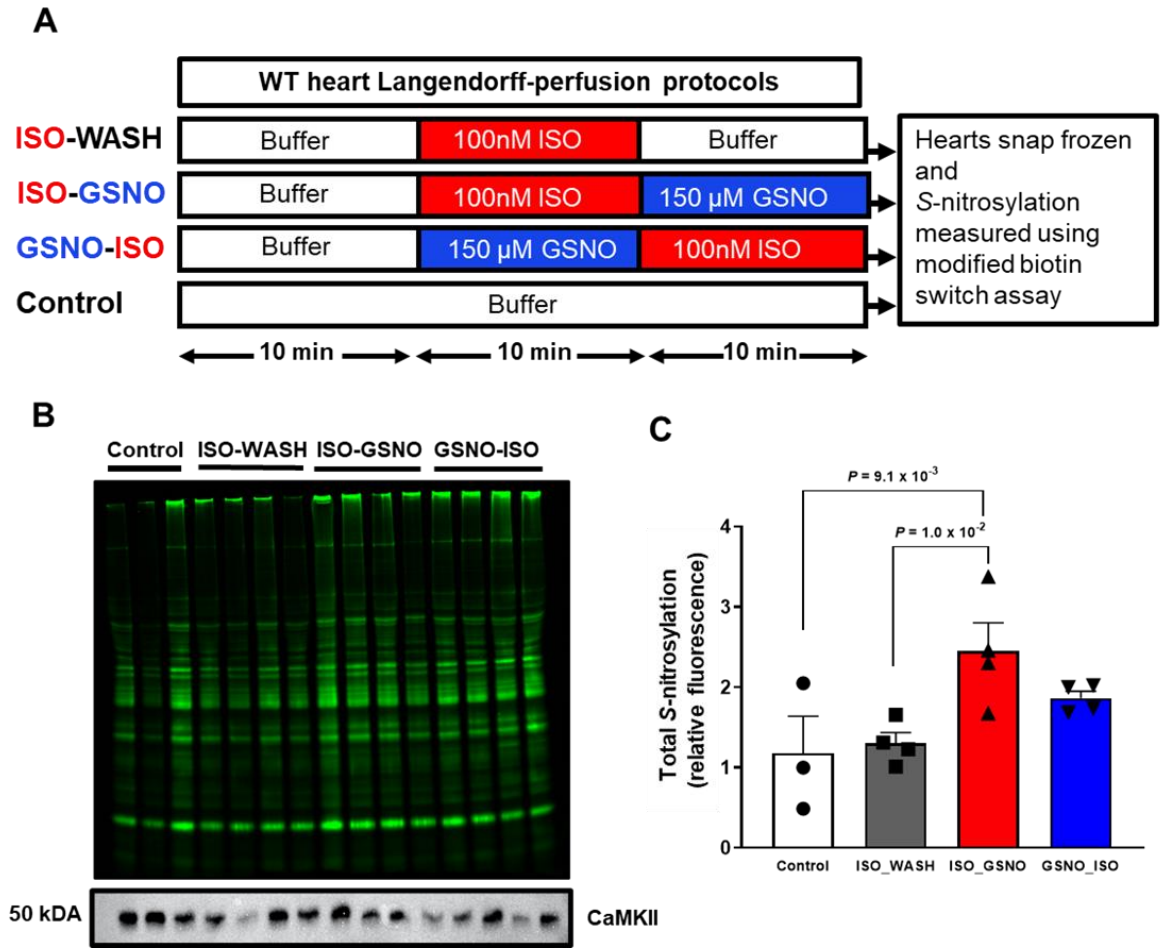

Figure S1. Arrhythmic events obtained from pressure trace. (**A**) sinus rhythm (**B**) ventricular premature beats (**C**) bigeminy (**D**) trigemini (**E**) potentiated contraction, and (**F**) ventricular tachycardia.

Figure S2. SR content for myocytes isolated from CaMKIIδ KO or C273S mice. At the end of each experimental protocol, a rapid application of caffeine was used to measure SR Ca^{2+} content. Ca^{2+} SR content for WT and CaMKIIδ-KO exposed to 150 μM GSNO (**A**) or 100 nM ISO (**B**). Time controls were also performed for the WT and CaMKIIδ-KO cardiomyocytes and were not exposed to iso or GSNO. (**C**) SR Ca^{2+} content for WT and CaMKIIδ-C273S cardiomyocytes exposed to ISO after GSNO pre-treatment (GSNO>ISO; WT: n=13 cells, N=6 hearts; CaMKIIδ C273S: n=23 cells, N=8 hearts), or GSNO following ISO (CON>ISO>GSNO; WT: n=21 cells, N=9 hearts; CaMKIIδ C273S: n=9 cells, N=5 hearts) or following washout of ISO with control buffer (CON>ISO>WASH; WT: n=21 cells, N=10 hearts; CaMKIIδ C273S: n=14 cells, N=8 hearts).

Figure S3. Whole mouse hearts exposed to ISO and GSNO have increased *S*-nitrosylation of cardiac proteins. (**A**) WT mouse hearts undergoing Langendorff-perfusion were treated with ISO and GSNO in varying order, snap-frozen and lysed, followed by a modified biotin switch assay to measure total protein *S*-nitrosylation. (**B**) Total cardiac protein *S*-nitrosylation in each lysate. (**C**) Treatment with ISO followed by GSNO (red bar) resulted in a significant increase in total cardiac protein *S*-nitrosylation compared to control (white bar, n=3-4 hearts per group).