Supporting Information

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SI Methods

Animal Model. The use of animals in this study was in accordance with National Institutes of Health guidelines and approved by the Vanderbilt University Laboratory Animal Care and Use Committee. Sex-matched *Trdn*$^{+/+}$ and *Trdn*$^{-/-}$ mice, C57/black6 strain, of 4–7 months of age were used for all of the experiments.

Protein Analysis. Mouse ventricular homogenates and microsomes were prepared as previously described (1). Immunoblotting against RyR2, Serca2a, Casq2, triadin, and junctin was conducted with antibodies as described previously (1). Antibody-binding protein bands were visualized with ^{125}I-protein A and then quantified using a Bio-Rad Personal Fx phosphorimager. Quantitative immunoblotting for junctophilin 1 and 2 was performed using the Odyssey Infrared Imaging System (Li-COR Biosciences). The custom-made antibodies were kindly provided by Dr. H. Takeshima (2).

Electron Microscopy. Hearts from 6–7-month-old *Trdn*$^{+/+}$ and *Trdn*$^{-/-}$ mice were harvested, the aorta cannulated, and hearts fixed by retrograde reperfusion with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Hearts were processed, imaged by electron microscopy, and estimates of relative surface areas and volumes of the total obtained as previously described (3), with a factor of 2 correction. Lengths and widths of the jSR cisternae profiles were measured with Photoshop (Adobe Systems). All quantitative data were obtained from 3 hearts each for *Trdn*$^{+/+}$ and *Trdn*$^{-/-}$ mice.

Heart Histology. Hearts were harvested from 6 age- and sex-matched mice per genotype, fixed by perfusion with 10% buffered formaldehyde, and embedded in paraffin. A total of 24 transverse sections (4-μm thickness) were cut from each heart, with the first section at the level of the papillary muscle $\approx$2 mm below the mitral valve. Twelve sections were stained with hematoxylin and eosin and examined for the amount of inflammation present, individual cell hypertrophy, and myofiber disarray. The other 12 were stained with Masson's trichrome and evaluated for fibrosis. For each category, the histology was graded on a scale from 0 to 3 by an experienced pathologist blinded to the genotype.

Immunolabeling and Colocalization Experiments. Isolated ventricular myocytes from *Trdn*$^{+/+}$ and *Trdn*$^{-/-}$ mice were used. The techniques for fixation, permeabilization, and immunolabeling, as well as processing, deconvolving, and analyzing images have been previously described (4, 5). We used an anti-RyR2 monoclonal antibody and a polyclonal anti-CSQ from Affinity Bioreagents, as well as an affinity-purified rabbit polyclonal antibody against the pore-forming subunit of the voltage-gated calcium channel, Cav1.2 (6). The secondary antibodies were affinity purified and highly cross-adsorbed goat antimouse conjugated to Alexa 594 and goat antirabbit conjugated to Alexa 488 (Invitrogen). Images were acquired using an inverted Zeiss Axio Observer microscope equipped with a Plan Apo 63/1.4 objective and EXFO Xcite illumination. All filters were from Semrock.

Myocyte Isolation and Ca^{2+} Indicator Loading. Single ventricular myocytes were isolated by a modified collagenase/protease method as previously described (7). All chemicals, unless otherwise specified, were obtained from Sigma. Ventricular myocytes were incubated with 2 μM fura-2 acetoxymethyl ester (Fura-2 AM; Molecular Probes) for 8 min at room temperature to load the indicator in the cytosol. Myocytes were then washed twice for 10 min with 1.2 mM Ca^{2+} Tyrode's solution (TS) containing 250 μM probenecid to retain the indicator in the cytosol. A minimum of 30 min were allowed for de-esterification of the indicator before imaging the cells. All of the experiments were then conducted in 2 mM Ca^{2+} TS. The composition of TS was (in mM): NaCl, 134; KCl, 5.4; $MgCl_2$, 1; glucose, 10; and Hepes, 10, pH adjusted to 7.4 with NaOH.

Ca^{2+} Fluorescence Measurements. Fura-2–loaded healthy rod-shaped isolated ventricular myocytes were loaded in the experimental chamber, field-stimulated, and superfused with TS containing 2 mM Ca^{2+}. Intracellular Ca^{2+} transients were measured using a dual-beam excitation fluorescence photometry setup (IonOptix). After 5–10 min of steady-state pacing at 1 Hz, 4 10-s Ca^{2+} fluorescence records were obtained from each myocyte. After that, myocytes were exposed for 4 s to TS containing 10 mM caffeine using a rapid concentration clamp system. The amplitude of the caffeine-induced Ca^{2+} transient was used as estimates of total SR Ca^{2+} content (8). All experiments were conducted at room temperature ($\approx$23 °C). Where indicated, ISO was used at a concentration of 1 μM. Ca^{2+} transients were analyzed using commercial software (IonWizard; IonOptix). Excitation wavelengths of 360 and 380 nm were used to monitor the fluorescence signals of Ca^{2+}-bound and Ca^{2+}-free Fura-2. After subtracting background and cellular autofluorescence, intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ is proportional to the fluorescence ratio at 360 nm and 380 nm excitation (9). Because Fura-2 AM compartmentalizes into intracellular organelles (10), calculating $[Ca^{2+}]_i$ from Fura-2 fluorescence ratios may not be accurate in intact cells. Thus, $[Ca^{2+}]_i$ measurements are reported as fluorescence ratios (F_{ratio}).

Analysis of SCRs. An SCR was defined as any spontaneous increase of 0.1 ratiometric units (3 times the average background noise) or more from the diastolic F_{ratio} other than when triggered by field stimulation or caffeine (3). For each myocyte, SCRs were counted over a 20-s period. In a separate set of experiments, SCRs and SR Ca^{2+} content were analyzed in myocytes incubated with the Cav1.2 channel blocker nifedipine 20 μM for at least 20 min.

Voltage-Clamp Studies. Cav1.2 Ca^{2+} currents were measured as previously described (11). Briefly, mouse ventricular myocytes were whole-cell patched in K^+-free solution containing (in mM): NaCl, 137; MgCl2, 1; CaCl, 2; Hepes, 10; glucose, 10; pH 7.4. The pipette solution contained (in mM): CsCl, 110; TEA-Cl, 20; EGTA, 14; Hepes, 10; MgATP, 5; glucose, 10; pH 7.2. Na^+ currents were eliminated by adding tetrodotoxin (30 μM) to external solutions and a holding potential of $-$50 mV. I_{Ca}-L was elicited with 200-ms depolarization pulses ranging from $-$50 to 40 mV in 10-mV steps. Where indicated, ISO was used at a concentration of 1 μM and ryanodine at 10 μM.

ECG Recordings and Echocardiography. For the surface ECG and echocardiography, recordings were done as previously described (3, 11, 12). Isoproterenol challenge was performed on unrestrained telemetry implanted mice as described previously (3).

Measurement of [^{3}H]PN200–110 and [^{3}H]ryanodine Binding. Cardiac ventricles were dissected from age-matched *Trdn*$^{+/+}$ and *Trdn*$^{-/-}$ mice (117–192 days old, 6 hearts per group), snap

frozen, and stored at −80 °C. Ventricles were homogenized and membrane vesicles prepared with minor modifications as described by Lachnit et al. (13) Briefly, frozen ventricles were pulverized with a dry ice–cooled mortar and pestle. Equal masses of tissue were suspended in 40 volumes of a cold buffer consisting of 300 mM sucrose, 40 mM histidine, and 40 mM Tris, pH 7.0, supplemented by 20 μg/mL PMSF and 10 μg/mL leupeptin. Tissue suspensions were placed in an ice bath and homogenized with a PowerGen 700D (Fisher Scientific) set at 18,000 rpm, using 3 30-s cycles with 30 s between each cycle. Tissue homogenate was then centrifuged at 45,000 × g for 30 min. Pellets were resuspended with a Dounce homogenizer in cold buffer consisting of 300 mM sucrose and 5 mM imidazole, pH 7.0. Aliquots were snap-frozen in liquid nitrogen. Protein concentrations were determined with the BCA kit (Pierce). For [^{3}H]PN200–110 binding analysis, membranes (40–65 mg protein) were incubated in the dark at 25 °C with 0.01–3 nM [^{3}H]PN200–110 for 1 h in a buffer consisting of 140 mM NaCl, 15 mM KCl, 20 mM Hepes, and 50 mM CaCl2, pH 7.0. Nonspecific binding was determined by the fraction of [^{3}H]PN200–110 binding in the presence of 1 μM nifedipine. [^{3}H]ryanodine binding was measured by incubating 40–65 mg protein with 0.5–1 nM [^{3}H]ryanodine and 0–250 nM unlabeled ryanodine (cold titration) for 3 h at 37 °C in a buffer consisting of 250 mM KCl, 15 mM KCl, 20 mM Hepes, and 50 μM CaCl2, pH 7.1. Binding was quenched by filtration of membranes onto GF/B filters using a cell harvester and washed 3 times with 4 mL of ice-cold wash buffer (250 mM KCl, 10 mM Tris-HCl, and 50 μM CaCl2, pH 7.1). Radioactivity was quantified with a Beckman LS 6500 scintillation counter. For data analysis, binding isotherms were fit to a single site model and analyzed with Prism V5.00 software (GraphPad).

Statistical Analysis. All experiments were done in random sequence with respect to the genotype, and measurements were taken by a single observer who was blinded to the genotype. Differences between groups were assessed using a one-way analysis of variance (for normally distributed parameters) or by Kruskal-Wallis test (for parameters that are not normally distributed). If statistically significant differences were found, individual groups were compared with Student's t test or by nonparametric tests as indicated in the text. Results were considered statistically significant if the P value was <0.05. Unless otherwise indicated, results are expressed as arithmetic means ± SEM.

1. Kirchhefer U, et al. (2004) Transgenic triadin 1 overexpression alters SR Ca2+ handling and leads to a blunted contractile response to beta-adrenergic agonists. *Cardiovasc Res* 62:122–134.
2. Takeshima H, Komazaki S, Nishi M, Iino M, Kangawa K (2000) Junctophilins: A novel family of junctional membrane complex proteins. *Mol Cell* 6:11–22.
3. Knollmann BC, et al. (2006) Casq2 deletion causes sarcoplasmic reticulum volume increase, premature Ca2+ release, and catecholaminergic polymorphic ventricular tachycardia. *J Clin Invest* 116:2510–2520.
4. Scriven DR, Klimek A, Asghari P, Bellve K, Moore ED (2005) Caveolin-3 is adjacent to a group of extradyadic ryanodine receptors. *Biophys J* 89:1893–1901.
5. Scriven DR, Dan P, Moore ED (2000) Distribution of proteins implicated in excitation-contraction coupling in rat ventricular myocytes. *Biophys J* 79:2682–2691.
6. Hell JW, et al. (1993) Differential phosphorylation of two size forms of the neuronal class C L-type calcium channel alpha 1 subunit. *J Biol Chem* 268:19451–19457.
7. Knollmann BC, et al. (2003) Familial hypertrophic cardiomyopathy-linked mutant troponin T causes stress-induced ventricular tachycardia and Ca2+-dependent action potential remodeling. *Circ Res* 92:428–436.
8. Bers DM (2001) Sarcoplasmic reticulum. *Excitation and Contraction Coupling and Cardiac Contractile Force* (Kluwer Academic Publishers, Dordrecht, The Netherlands), pp 161–202.
9. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450.
10. Williford DJ, Sharma VK, Korth M, Sheu SS (1990) Spatial heterogeneity of intracellular Ca2+ concentration in nonbeating guinea pig ventricular myocytes. *Circ Res* 66:241–248.
11. Knollmann BC, Knollmann-Ritschel BE, Weissman NJ, Jones LR, Morad M (2000) Remodelling of ionic currents in hypertrophied and failing hearts of transgenic mice overexpressing calsequestrin. *J Physiol* 525(Pt 2):483–498.
12. Knollmann BC, et al. (2001) Inotropic stimulation induces cardiac dysfunction in transgenic mice expressing a troponin T (I79N) mutation linked to familial hypertrophic cardiomyopathy. *J Biol Chem* 276:10039–10048.
13. Lachnit WG, Phillips M, Gayman KJ, Pessah IN (1994) Ryanodine and dihydropyridine binding patterns and ryanodine receptor mRNA levels in myopathic hamster heart. *Am J Physiol* 267(3 Pt 2):H1205–H1213.

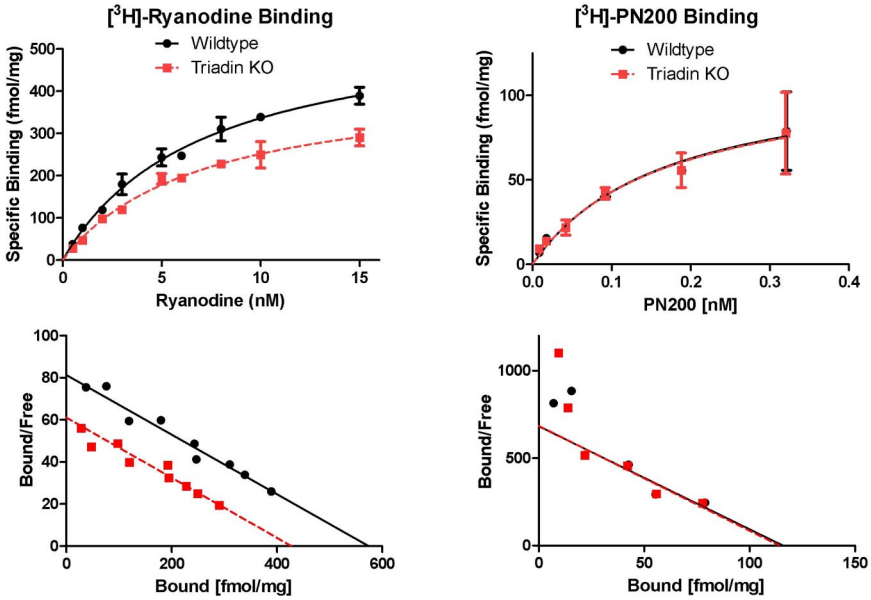

Fig. S1. [^{3}H]Ryanodine and [^{3}H]PN200–110 binding curves and their respective Scatchard plots demonstrate reduction in RyR2 without any change in L-type Ca^{2+} channels in *Trdn*$^{-/-}$ hearts.

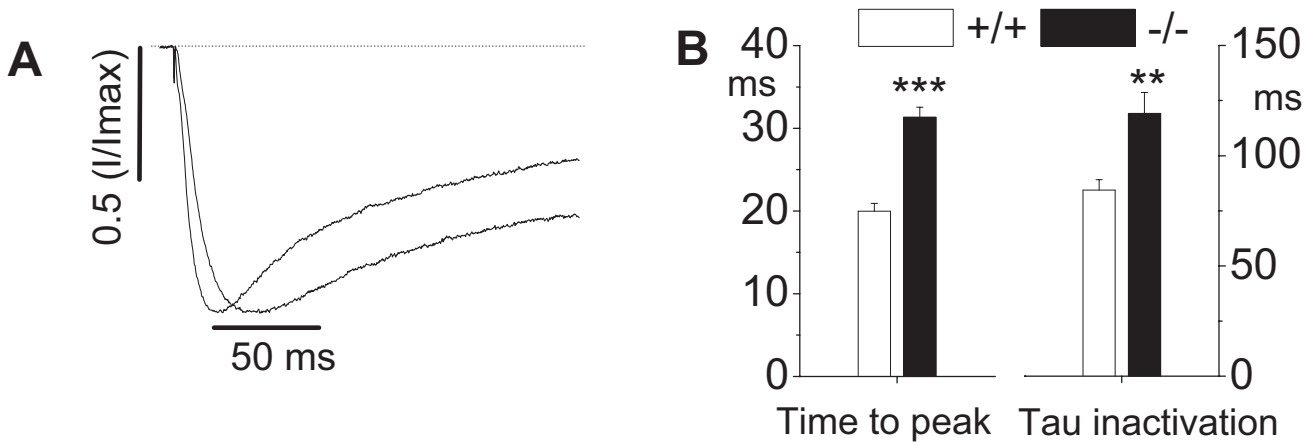

Fig. S2. I_{Ba} in voltage-clamped ventricular myocytes. Note the significantly slower activation and inactivation kinetics of I_{Ba} currents recorded from *Trdn*$^{-/-}$ compared with *Trdn*$^{+/+}$ myocytes. Myocytes were incubated in 10 μM ryanodine for 60 min and 1 μM ISO for 5 min. After whole-cell patch was obtained, extracellular Ca^{2+} was rapidly replaced with 2 mM Ba^{2+}. Example traces recorded at 0 mV (*A*) and average inactivation time constant (*B*). $n = 7$ myocytes per group. **, $P < 0.01$; ***, $P < 0.001$. Average cell capacitance, access resistance, and peak I_{Ba} were not statistically different between the 2 groups.

Table S1. Echocardiographic and ECG parameters of anesthetized mice

Parameter	*Trdn*$^{+/+}$ ($n = 16$)	*Trdn*$^{-/-}$ ($n = 16$)
HR (bpm)	475 ± 10.25	391 ± 6.48*
IVSD (mm)	0.71 ± 0.033	0.87 ± 0.031*
LVPWD (mm)	0.76 ± 0.035	0.88 ± 0.04*
LVIDd (mm)	3.9 ± 0.087	3.7 ± 0.077
LVIDs (mm)	2.6 ± 0.088	2.3 ± 0.085*
%FS	32 ± 1.14	38 ± 1.63*
PR (ms)	40.7 ± 1.1	38.5 ± 0.99
QRS (ms)	10.35 ± 0.21	10.8 ± 0.26
QT (ms)	53.5 ± 0.79	58.3 ± 1.32*
QTc (ms)	46.4 ± 0.61	46 ± 0.75

HR, heart rate; IVSD, interventricular septum diameter; LVPWD, left ventricular posterior wall diameter; LVIDd, LV internal dimension in diastole; LVIDs, LV internal dimension in systole; %FS, percentage fractional shortening (LVIDs/LVIDd × 100); QTc, heart rate–corrected QT interval. *, $P < 0.05$, Student's *t* test.