SI Materials and methods

Cell culture and transfection. Primary cultures of neonatal cardiac myocytes were prepared as described previously [1]. Hearts were removed from rat pups at postnatal day 1 (P1). Atria were discarded, and ventricular tissues were digested (51 U/ml collagenase, 0.29 mg/ml pancreatin, 116 mM NaCl, 20 mM HEPES, 1 mM NaH_2PO_4, 5.5 mM glucose, 5.4 mM KCl, and 0.8 mM $MgSO_4$, pH 7.4). Cells were counted with a hemocytometer and plated onto laminin-coated 6-well plates at a density of 2×10^6 cells/well. Cultures were incubated in DMEM/F12 medium (American Type Culture Collection, Manassas, VA, USA) supplemented with 5% horse serum, 1 μg/ml insulin, 10 ng/ml sodium selenite, 1 μg/ml transferrin (Tri-Mix, Invitrogen, Carlsbad, CA, USA), and 0.2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA). Transfections of cardiomyocytes with STIM1-GFP plasmids (3 μg/ml) were performed by electroporation in a Lonza Nucleofector with the kit for rat neonatal cardiac myocytes (Lonza Inc., Basel, Switzerland). Transfections were performed on 2.5×10^6 cells in a volume of 100 μl of Nucleofector solution (Lonza) immediately after isolation and prior to plating[1]. After 3 days of transfection, over 95% of the cells were infected when checked using confocal fluorescence imaging. Freshly isolated rat and mouse ventricular myocytes were cultured in M-199 (MEM for mouse) culture medium supplemented with 0.1% ITS and 10 μM blebbistatin (20 μM for mouse myocytes). Experiments were performed 48 h after transfection with an MOI of 50 for human Orai1-mCherry (Vector BioLabs, PA) and 100 MOI for STIM1-mCherry, STIM1-eYFP (Vector BioLabs, PA) or same amount of mCherry alone. STIM1-mCherry and Orai1-mCherry fluorescence was imaged with an excitation of 543 nm and an emission of >580 nm. STIM1-eYFP fluorescence was imaged with an excitation of 514 nm and an emission of >530 nm. When STIM1-eYFP and STIM1-mCherry were co-transfected, multitrack configuration was used for image. The correlation coefficient (i.e. Pearson's Coefficient) was estimated using ImageJ Plugin software JACoP at the identical parameters for control and thapsigargin treated cells.

Generation of transgenic animal models. Cardiac myocytes-specific STIM1 knockout mice were generated at Duke University. Briefly, STIM1flox/flox mice (C57BL/6) were generated and provided by Anjana Rao's lab. STIM1 flox/flox mice were crossbred with alpha-MCH-Cre mice (Center for Cardiovascular Development, Baylor college of Medicine) to generate STIM1flox$^{+/-}$ MHC-Cre$^{+/-}$ mice. The MHC-Cre STIM1flox+/- were bred with STIM1flox/flox mice to produce MHC-Cre+/- STIM1 flox/flox (cardiac specific STIM1KO). Wild-type (WT) animals consisted of STIM1flox/flox mice. Phospholamban knockout (PLN$^{-/-}$) mice (in the background of RyR2-R2474S heterozygote in C57BL/6N) were kindly provided by Prof. Stephan E. Lehnart, University Medical Center Goettingen.

Ca^{2+} imaging. Primary cultures of adult rat ventricular myocytes were loaded with fluo 4 (using 5 μM fluo 4-AM for 20 min at room temperature) and placed in a normal Tyrode's solution containing (in mM): 140 NaCl, 10 HEPES, 0.5 $MgCl_2$, 0.33 $NaHPO_4$, 5.5 Glucose, 1.8 $CaCl_2$ and 5 KCl (pH 7.4 with NaOH). Confocal imaging (Zeiss LSM-510 or Zeiss LSM-5 Live, Germany) was used to monitor Ca^{2+} signals using either a line scan method (at 1.92 ms per line) or an XY image time series (at 780 ms per image for Ca^{2+} waves and 100 ms per image for caffeine-induced Ca^{2+} transient measurement). Data were analyzed offline using ImageJ 1.44p (NIH).

Patch clamp electrophysiology. Ca^{2+} current ($I_{Ca\text{-}L}$) from wild type (WT) or cardiac specific STIM1 knock out (KO) ventricular myocytes was recorded using conventional whole-cell patch-clamp configuration (Axopatch 200A, Clampex 8.2). Specifically, cells were held at -80mV. A ramp from -80 mV to -45 mV was run before pre-pulse to inactivate Na^+ channel. $I_{Ca\text{-}L}$ was elicited by 50 ms pre-pulses from holding potential of -45 mV to test potentials from -40 mV to +60 mV with 10 mV step.

The glass pipette was pulled and fire polished. The resistance of the pipette is 2-3 MΩ when filled with pipette solution containing (in mM): 130 CsCl, 1 $MgCl_2$, 10 TEACl, 10 HEPES, 5 Mg-ATP, and 10 EGTA (pH 7.2 with CsOH). The bath solution contained (in mM): 140 NaCl, 10 HEPES, 0.5 $MgCl_2$, 0.33 $NaHPO_4$, 5.5 Glucose, 1.8 $CaCl_2$ and 5 CsCl (pH 7.4 with NaOH). Data was analyzed offline using Clampfit 10.2.

Western blot. Proteins were extracted from cells or tissues using SDS sample buffer for Western blot experiments. After protein quantification, 50 µg of total proteins were loaded per lane and separated by 4-20 % SDS-polyacrylamide gels (Life Biotech., NY) and transferred onto PVDF membranes. The membrane was blocked in Tris-buffered solution (TBS) containing 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk for 1 hour, followed by an overnight incubation with primary antibody anti-Orai1 (Alomone Labs, Israel) at a dilution of 1:500 in TBS-T containing 5% nonfat milk at 4°C. After three washes (10 min each) with TBS-T, the membrane was then incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody. After another 3 washes (15 min each) using TBS-T, the membrane was developed using enhanced chemiluminescence (Amersham, Arlington Heights, IL) for Orai1. Negative control was prepared as the same except that the primary antibody was pre-incubated with the epitope peptide (1:1) for 1 hour before incubation with the membrane.

ELISA. Costar 96 well plates were coated with neutravidin in $NaHCO_3$ (100 mmol, pH 8.5) overnight at 4°C. The wells were then blocked by 2% milk in $NaHCO_3$ at room temperature for 1 h followed by three washes using PBS-T. Then 4 pM purified biotin-STIM1c was added and incubated overnight at 4°C. Wells coated with 2% milk in PBS were set as negative control. Hearts isolated from 8 week old C57BL/6 mice were Langendorff-perfused with saline control or isoproterenol (10^{-8} M) for 20 min. The ventricles were then harvested and homogenized in RIPA buffer and incubated with the STIM1c or milk coated wells for 2 h at room temperature followed by 5 washes using PBS-T. Anti- PLN or anti-phospho-PLN (Cell Signaling Technology) was then incubated with the wells at a dilution of 1:1000 at room temperature for 2 h. After 5 washes using PBS-T, HRP conjugated secondary antibody (1:3000) was added and incubated for 1 h at room temperature followed by 5 washes with PBS-T. 100 microliters per well of ABTS with 0.05% H_2O_2 was then added and incubated for 30 min at room temperature. The plate was read at 405 nm with a plate reader. The data presented were background subtracted (milk) and normalized by input PLN level. Signal of PLN binding to STIM1c in control heart lysate was set as 100%. See Fig. S9.

Immunochemistry and STIM1 immunofluorescence analysis. Freshly isolated rat or mouse ventricular myocytes were fixed using 3.7% paraformaldehyde in phosphate-buffered saline solution (PBS) for 15 min. After three washes with PBS, cells were permeabilized and blocked in PBS containing 5% goat serum and 0.3% triton X-100 for 1 h at room temperature. Cells were then incubated overnight at 4ºC with polyclonal anti-STIM1 at a dilution of 1:100 in PBS containing 5% goat serum. After 3 washes, cells were incubated for 1 h at room temperature with Alexa Fluor 633-conjugated goat anti-rabbit. Following 6 washes with PBS, fluorescence images were acquired using a Zeiss LSM-510 laser scanning confocal microscopy at excitation of 633 nm and emission of >650 nm. STIM1 immunofluorescence was quantified using ImageJ 1.44 p (NIH). Specifically, the STIM1 immunofluorescence images were adjusted with threshold (the pixel value used to find the edge of STIM1 immunofluorescence closed regions) at an intensity of 2 x mean intensity + 3 x SD. The threshold area was outlined under particle analysis (size 0-infinity; circularity 0-1).The fraction of the outlined STIM1 immunofluorescence was measured and calculated as the following:

$$Fraction\ (\%) = 100\ \times \frac{pixel\ of\ outlined\ area}{\text{pixel of whole cell}}$$

Fluorescence recovery after photobleaching (FRAP). A 3 x 3 μm area (avoiding nucleus) was used for the photobleaching experiments using high illumination intensity (100% 488 nm laser) in STIM1-GFP transfected rat postnatal cardiac myocytes. The fluorescence recovery in the bleached area was monitored at 2 s per image. Data were analyzed offline using ImageJ (1.44p).

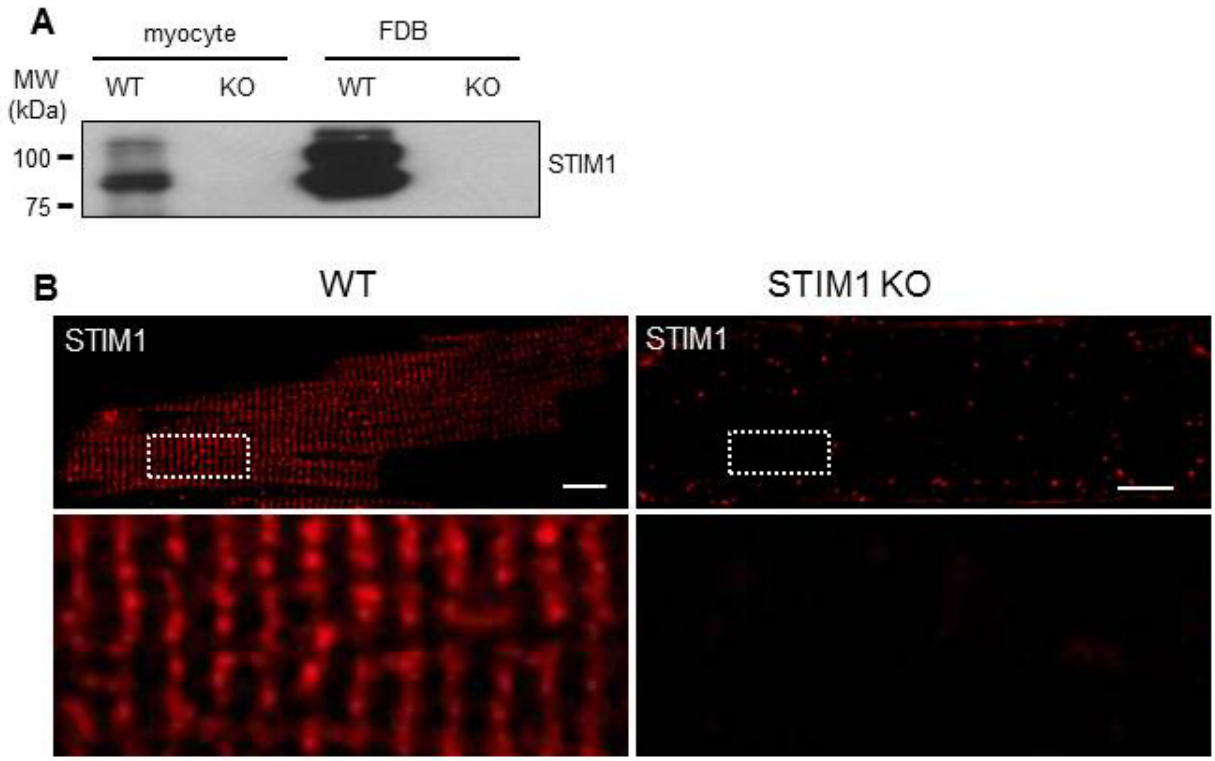

Figure S1. The specificity of the anti-STIM1. A. Western blot shows anti-STIM1 antibody specifically recognizes STIM1, with STIM1 null mouse cardiac myocytes and flexor digitorum brevis (FDB) skeletal muscle as negative control. **B.** Immunochemistry shows anti-STIM1 antibody specifically recognizes STIM1 in mouse ventricular myocyte. Lower panel, amplification of the corresponding boxed areas. Scale bar, 10 μm.

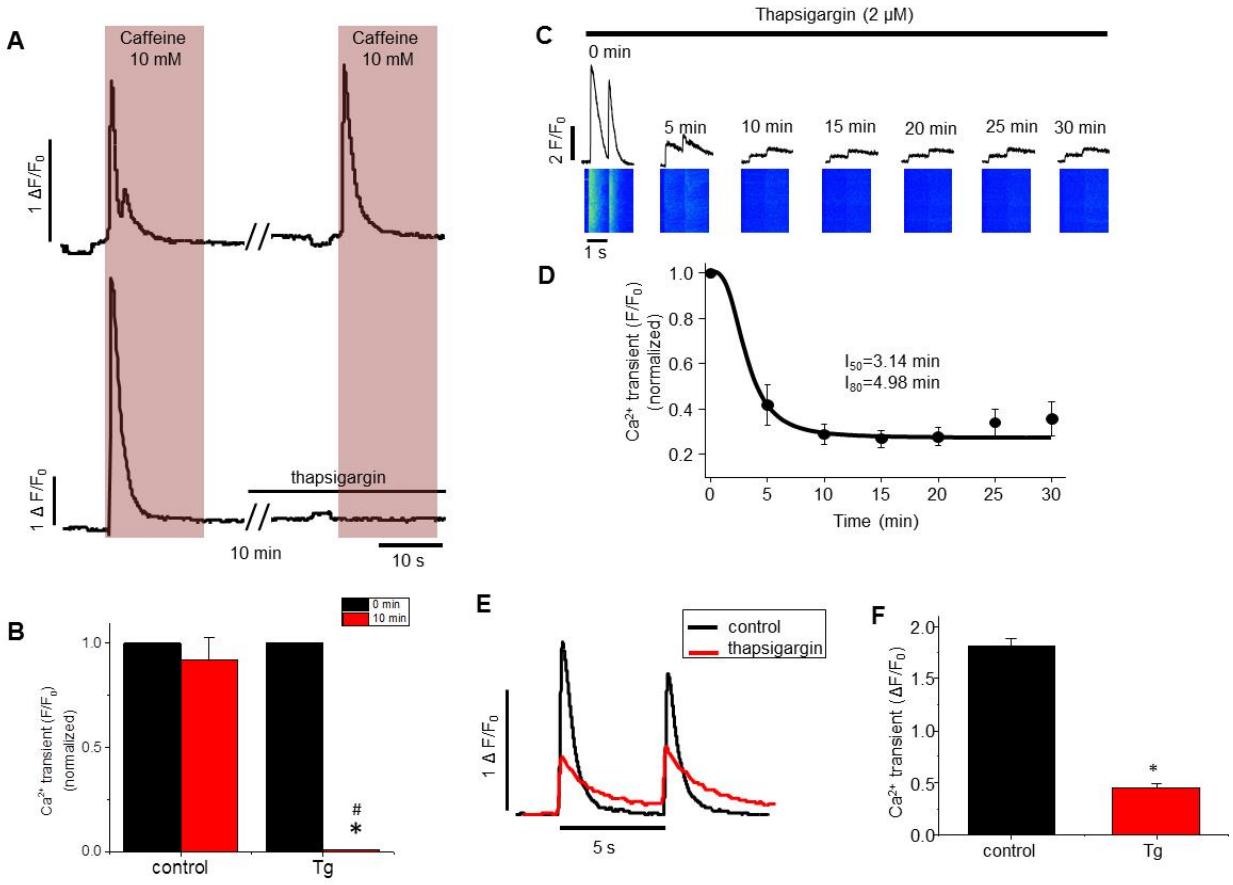

Figure S2. Effect of thapsigargin (10 μM) on SR Ca^{2+} depletion. **A.** An XY time series image (100 ms per image) profile show Ca^{2+} transients induced by caffeine (10 mM) at 0 and 10 min of perfusion with normal Tyrode's solution in the absence (upper panel) and presence of thapsigargin (10 μM) in adult rat ventricular myocytes . **B.** Summary data show the peak of the caffeine-stimulated $[Ca^{2+}]_i$ level in control and thapsigargin treated rat ventricular myocytes (n=10 and n=12 cells in control and thapsigargin groups, respectively). **C.** Line scan images of fluo-4 fluorescence show the time-course of the fall in SR Ca^{2+} release in rat ventricular myocytes due to SERCA2a inhibition by thapsigargin (2 μM). The residual $[Ca^{2+}]_i$ transients produced by field stimulations (FS) are due to Ca^{2+} entry through the L-type Ca^{2+} channels. Field stimulations (FS) imposed at 1 Hz. **D.** Summary data show the effect of thapsigargin on SERCA2a (n=5 cells). **E.** $[Ca^{2+}]_i$ transients induced by field stimulation (FS, 0.2 Hz) before and after 10 min application of thapsigargin (10 μM). **F.** Summary data show the decrease of $[Ca^{2+}]_i$ transient (FS induced) amplitudes in the presence of thapsigargin (n=5 cells). * $p<0.05$ vs control; # $p<0.05$ vs 0 min.

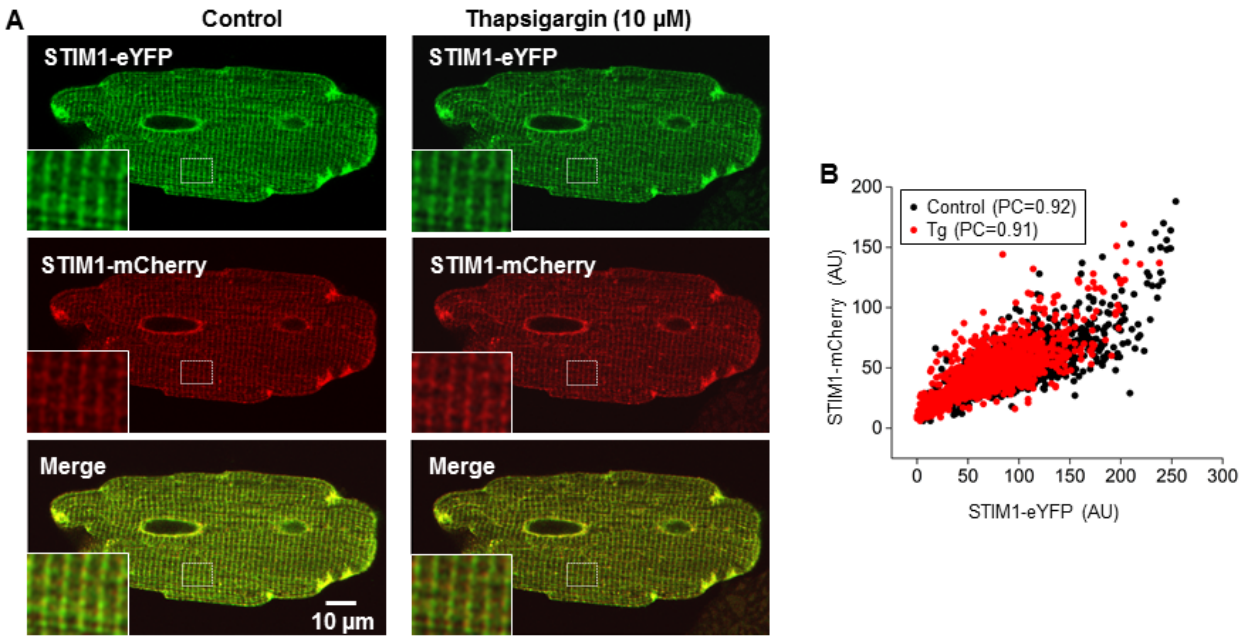

Figure S3. The co-localization of STIM1-mCherry and STIM1-eYFP. **A.** The fluorescence of STIM1-mCherry and STIM1-eYFP in short-term cultured rat ventricular myocytes in the absence and presence of thapsigargin (10 μM). Inset, zoom in of the boxed area. **B.** Correlation of STIM1-mCherry and STIM1-eYFP in the absence and presence of thapsigargin. The Pearson's Coefficients (PC) are 0.92 and 0.91 before and after treatment with thapsigargin for 30 min.

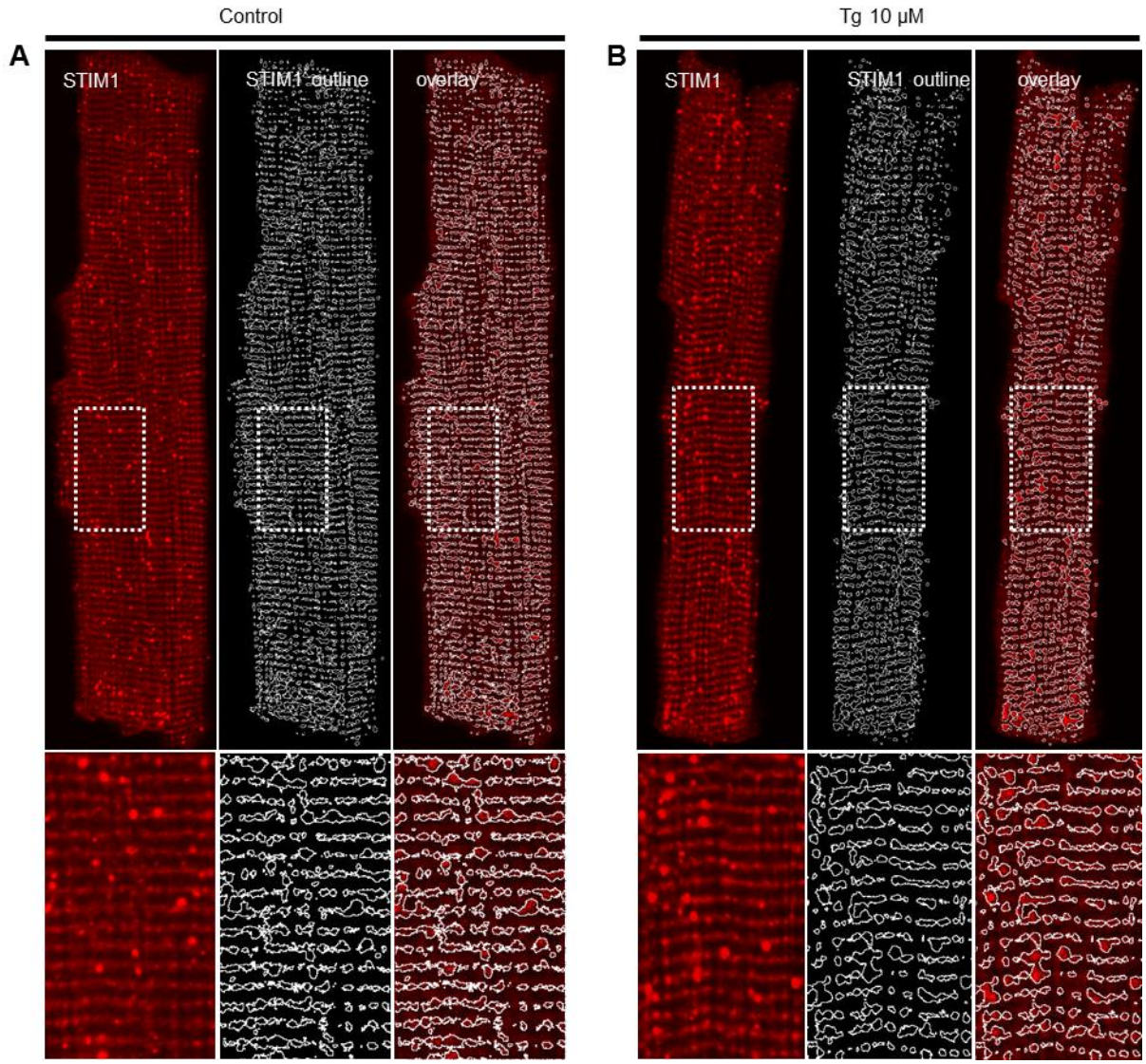

Figure S4. Subcellular distribution of STIM1 protein in control and SR Ca^{2+} depleted ventricular myocytes. A. Confocal immunofluorescence image (red) of the distribution of STIM1 in a control rat ventricular myocyte (left panel). The dashed whited rectangular region is enlarged in the panel (bottom left). Threshold-outline of the fluorescence intensity (see Methods) from the same cell is shown in the middle panel. The outline image is merged with the immunofluorescence image (right panel). **B.** Same as A. 20 minutes after treatment with 10 µM thapsigargin. Puncta and linear structures appear at z-disk in both A and B. The fractions of STIM1 immunofluorescence at or above the threshold are 20 ± 0.6, 21 ± 0.7 and 21 ± 0.4 % in control (n=9 cells), Tg (2 µM, n=5 cells) and Tg (10 µM, n=9 cells), respectively. No significant difference was detected between control and SR depleted rat ventricular myocytes at either 2 or 10 µM thapsigargin.

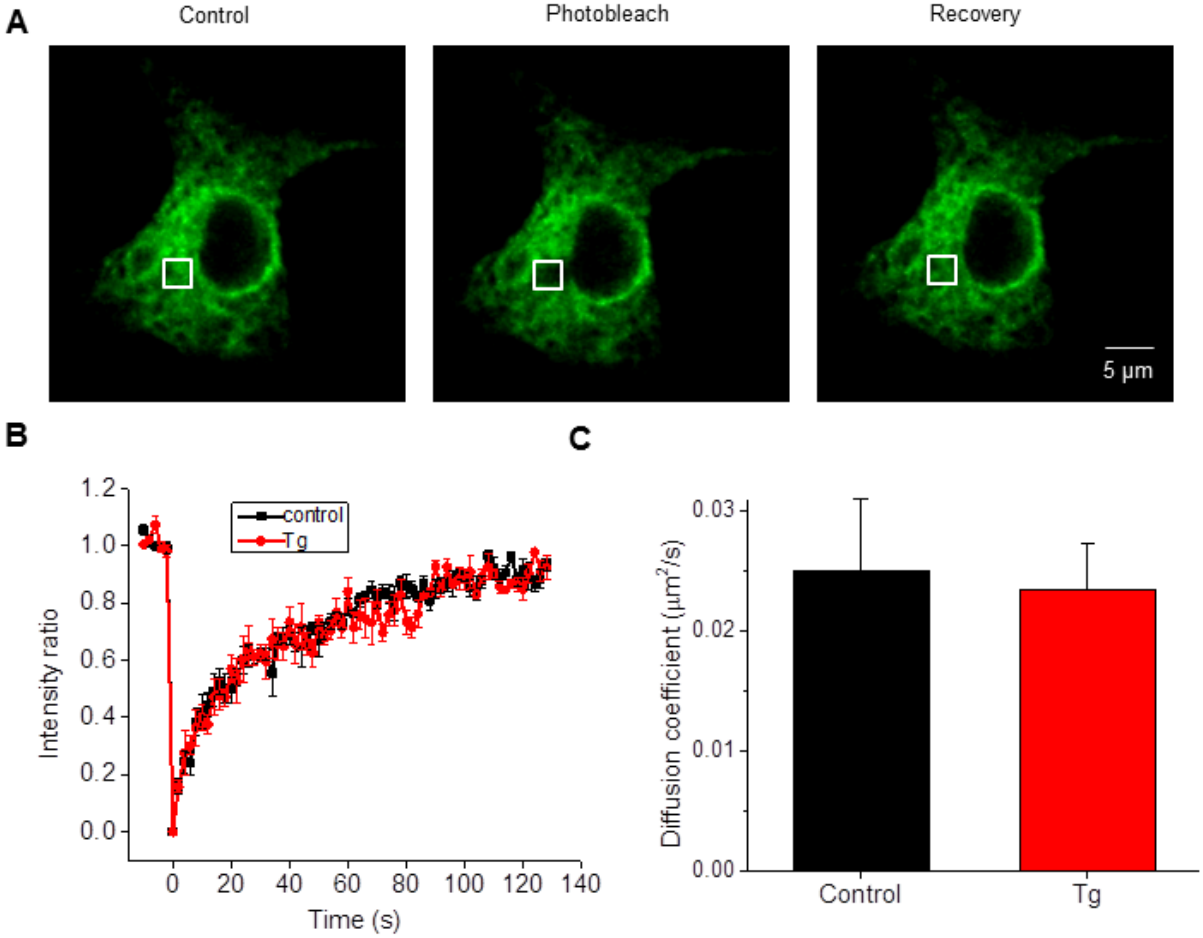

Figure S5. STIM1 mobility in neonatal rat cardiac myocytes. **A.** The fluorescence image shows the successful transfection of STIM1-GFP in neonatal rat cardiac myocytes in short-term primary culture. White boxes show before photo bleaching, right after photo bleaching, and after fluorescence recovery in a control cell. **B.** STIM1-GFP fluorescence intensity ratio change in fluorescence recovery after photobleaching (FRAP) in the absence and presence of thapsigargin (2 μM). **C.** Summary data show the diffusion coefficient of STIM1-GFP in the absence and presence of thapsigargin. n=5 cells for control and thapsigargin, respectively.

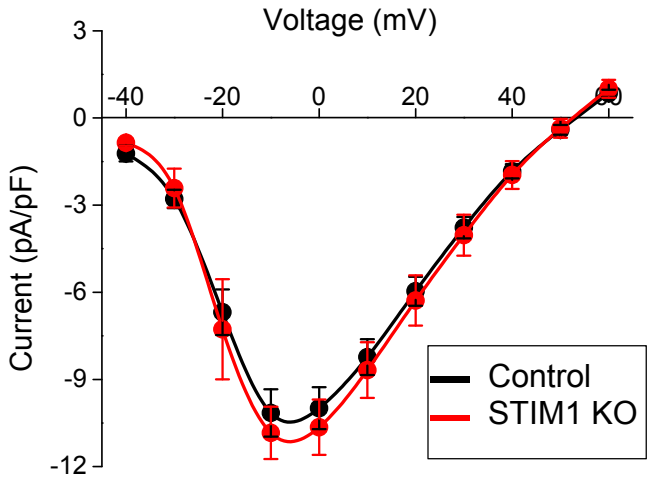

Figure S6. The L-type Ca^{2+} current in WT and cardiac specific STIM1 knockout ventricular myocytes. n=14 and 5 cells in WT and STIM1 KO group, respectively.

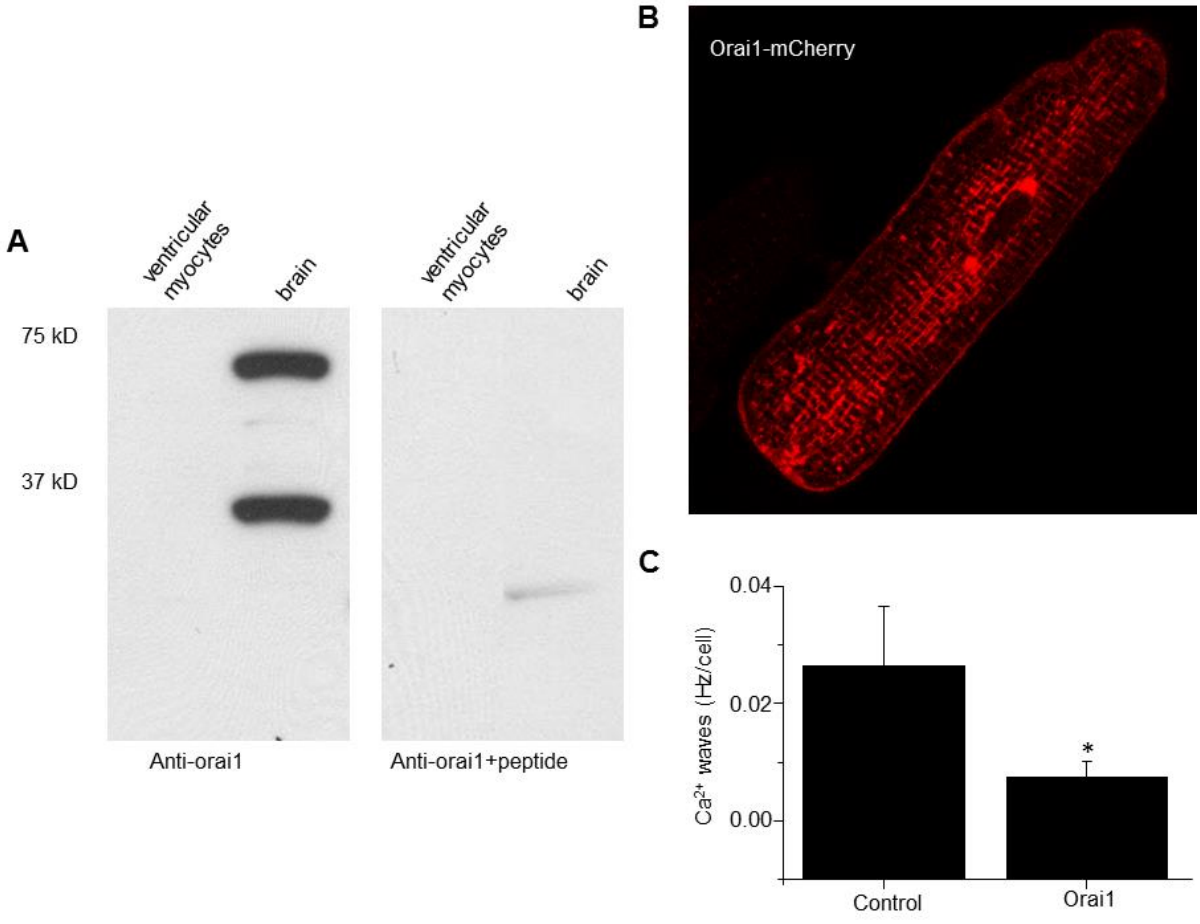

Figure S7. The effect of Orai1 overexpression on spontaneous Ca^{2+} wave frequency in ventricular myocytes. A. Western blot shows Orai1 is expressed in rat brain tissue but not in rat ventricular myocytes (Left panel). Western blot shows that incubation of anti-Orai1 with antigen peptide fails to detect Orai1 in rat brain tissue and ventricular myocytes, suggesting that

the bands seen in left panel are Orai1 protein (right panel). **B.** Fluorescence image from a rat ventricular myocyte expressing Orai1-mCherry. **C.** Summary data showing the spontaneous Ca^{2+} wave frequency in control (mCherry) and Orai1-mCherry groups (n=40 and 20 cells, respectively). Orai1-mCherry transfected cells show decreased frequency (0.007 Hz) of spontaneous Ca^{2+} waves comparing to that in control cells (0.026 Hz),*p<0.05 vs control.

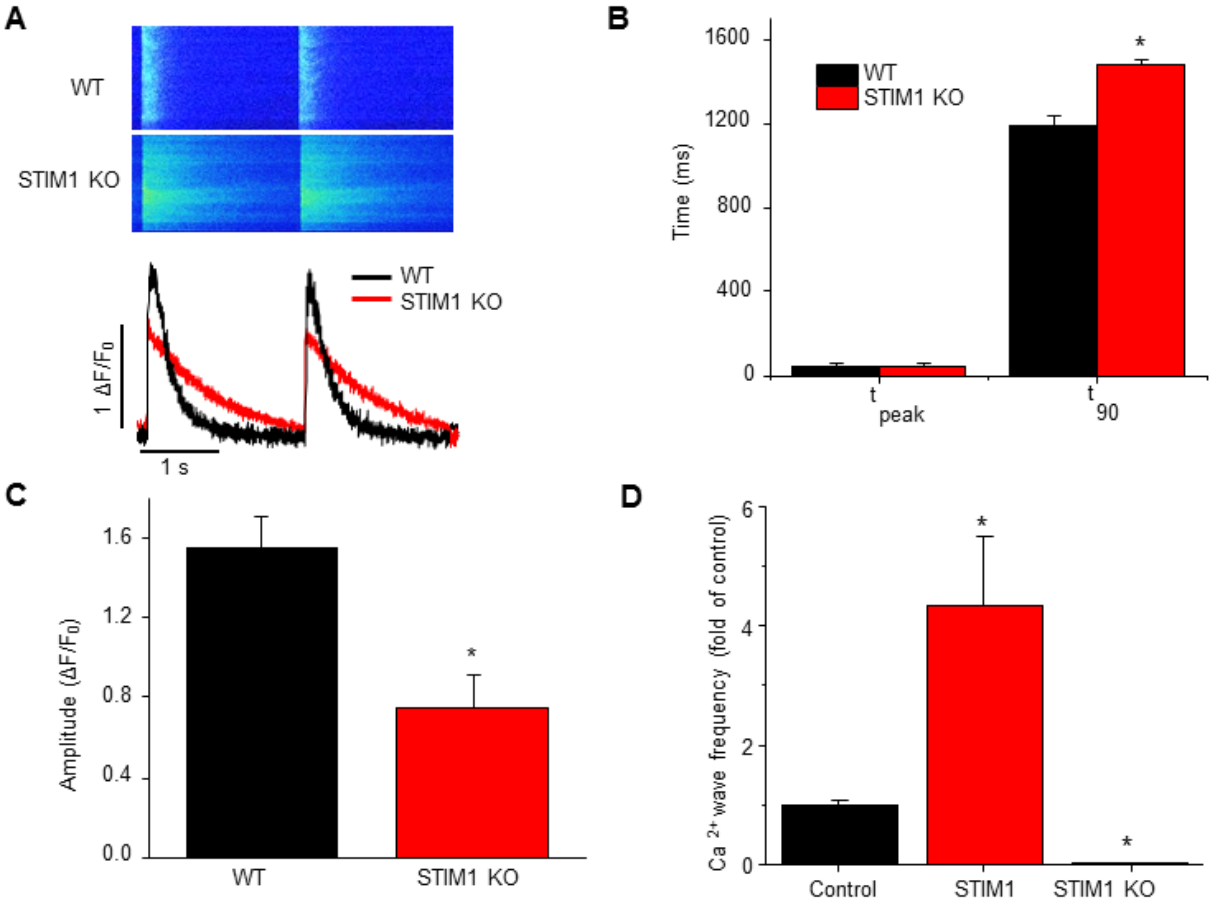

Figure S8. Ca^{2+} handling changes in STIM1 knockout (KO) mouse ventricular myocytes. **A.** Line scan images of fluo 4 fluorescence and the profiles show $[Ca^{2+}]_i$ transient induced by field stimulation (FS, 0.5 Hz) in wild type (WT) and STIM1 KO mouse ventricular myocytes. **B.** Summary data show the time to peak and 90% decay of $[Ca^{2+}]_i$ transient in WT and STIM1 KO myocytes. **C**. Summary data show the change of $[Ca^{2+}]_i$ transient amplitude in WT and STIM1 KO myocytes. * p<0.05 vs WT. n= 18 and 6 cells in WT and STIM1 KO groups, respectively. **D.** Summary data of Ca^{2+} wave frequency change in STIM1 KO and STIM1 overexpressing mouse ventricular myocytes. Data were normalized to control (STIM1 KO to WT control, STIM1-mCherry overexpressed to mCherry control); n=27, 16, 46, and 48 cells in WT, STIM1 KO, mCherry, and STIM1-mCherry overexpressing myocytes, respectively.

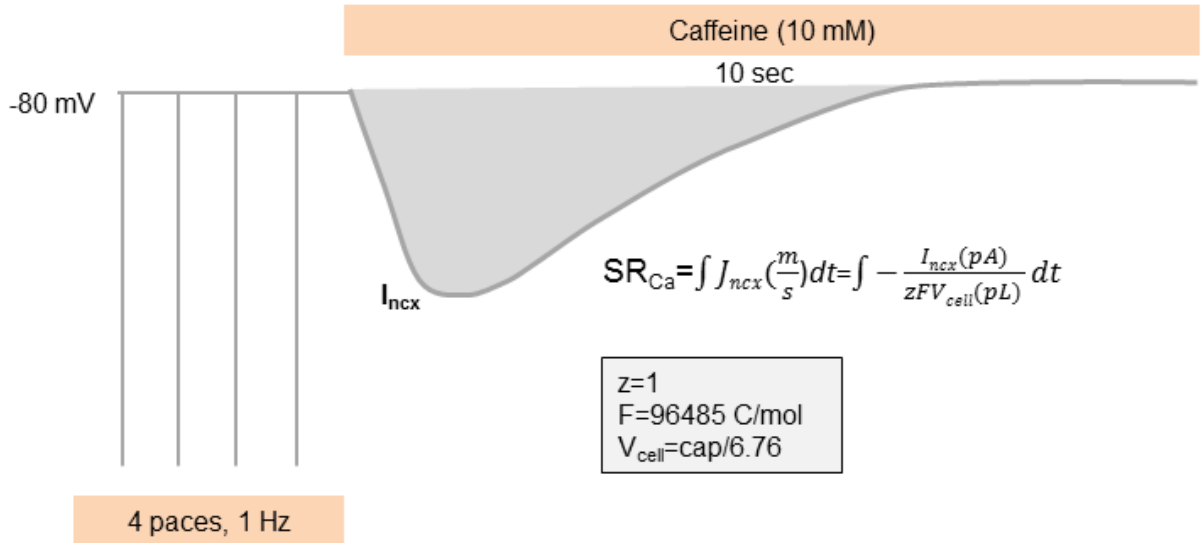

Figure S9. Illustration of the protocol and formula used to measure SR Ca^{2+} content. Four repetitive depolarizations from -80 mV to 0 mV were imposed before caffeine (10 mM) was applied. The 1.5 times integral of Na/Ca^{2+} exchanger current (NCX current, I_{ncx}) upon caffeine stimulation was regarded as SR Ca^{2+} content [2]. The following formula was used to integrate the I_{ncx}.

$$\int J_{ncx}(\frac{m}{s})dt=\int -\frac{I_{ncx}(pA)}{zFV_{cell}(pL)}dt$$

Where, z is the number of net charge movement (which is 1 for I_{ncx}); F, the Faraday constant; V_{cell}, the volume of the cell. The ventricular myocyte volume was calculated based on cell capacitance as previously described [2]: volume (pL)=capacitance (pF)/6.76.

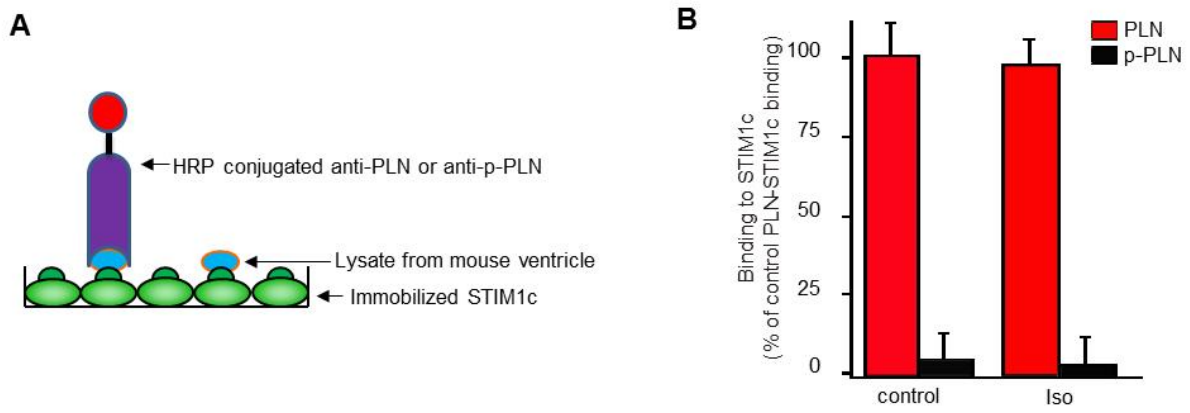

Figure S10. ELISA shows that non-phosphorylated phospholamban binds to STIM1 c-terminus in heart. A. Diagram of ELISA to characterize the interaction of STIM1c and endogenous PLN and p-PLN. Purified STIM1c protein was coated to a 96 well plate and incubated with lysates from mouse heart. Anti-PLN and anti-p-PLN antibodies were used to detect the amounts of PLN and p-PLN that bind to STIM1c. **B.** Interaction of PLN and p-PLN with STIM1c in mouse heart. Two control hearts and two isoproterenol-treated hearts were used. Each binding condition was performed in triplicate.

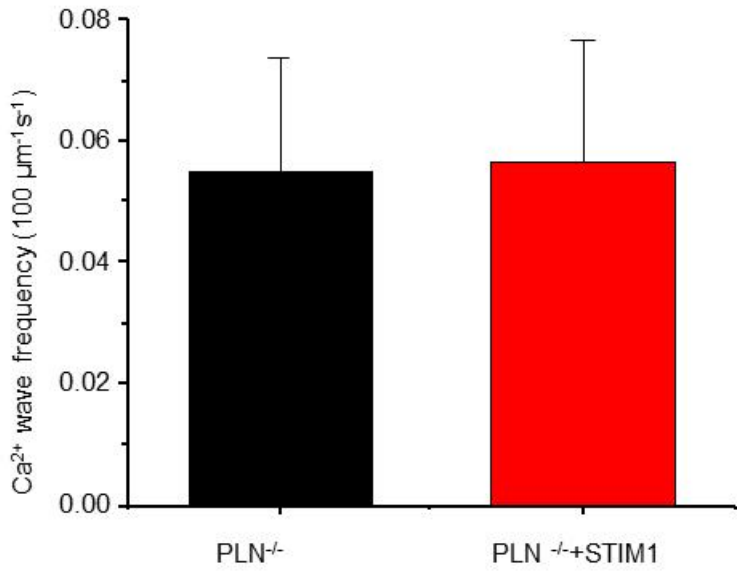

Figure S11. STIM1 action in phospholamban knockout ($PLN^{-/-}$) cardiac ventricular myocytes. The frequency of Ca^{2+} waves was measured in $PLN^{-/-}$ mouse ventricular myocytes (in the background of C57BL/6N crossed with RyR2-R2474S heterozygote). When STIM1 was over-expressed in these cells there was no change in the frequency of Ca^{2+} waves. (This mouse model was kindly provided by Prof. Stephan E. Lehnart, University Medical Center Goettingen). N=24 and 19 cells in $PLN^{-/-}$ control myocytes and STIM1 overexpressing myocytes, respectively.

References

1. Lund LM, Kerr JP, Lupinetti J, Zhang Y, Russell MA, Bloch RJ, Bond M. Synemin isoforms differentially organize cell junctions and desmin filaments in neonatal cardiomyocytes. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2012;26:137-148
2. Walden AP, Dibb KM, Trafford AW. Differences in intracellular calcium homeostasis between atrial and ventricular myocytes. *J.Mol.Cell Cardiol.* 2009;46:463-473