Supplemental Materials for:

Akihiro Hino, et al. Enhanced binding of calmodulin to the ryanodine receptor corrects contractile dysfunction in failing hearts

EXPANDED MATERIALS AND METHODS

1. Expression and Purification of CaM and Gly-Ser-His-GSH (GSH-CaM)

The expression vector of human calmodulin (CaM), kindly provided by Dr. Z. Grabarek (Boston Biomedical Institute, Boston, MA, USA) was transformed into BL21-CodonPlus (DE3)-RP (Stratagene). Purification of CaM was carried out following the method of Tan et al.[1] The bacteria were cultured in LB-ampicillin medium for 16hrs at 37°C after overnight preculture at 37°C. For protein expression, 1mM isopropyl-ß-D-thiogalactopyranoside (IPTG) (Wako Purechemicals) was added to the medium and incubated 5 hrs at 20°C. After centrifugation, the bacteria pellets were washed with Dulbecco's PBS (D-PBS) (Sigma-Aldrich) and sonicated with 50mM Tris-HCl buffer (pH7.5) containing 2mM EDTA, 1mM dithiothreitol (DTT) and Protease Inhibitor Cocktail for general use (Sigma-Aldrich). The supernatant of ultracentrifugation was applied to Phenyl-Cellulofine (Seikagaku Corporation) column at room temperature, equilibrated with 50mM Tris-HCl buffer (pH7.5) containing 5mM CaCl2, 0.1M NaCl and 1mM DTT. The resin was washed with 50mM Tris-HCl buffer (pH7.5) containing 0.1mM CaCl2 and 1mM DTT and the same buffer containing 0.5M NaCl. The recombinant CaM was eluted with 50mM Tris-HCl buffer (pH7.5) containing 1mM EGTA and 1mM DTT.

For construction of the GSH-CaM expression vector, the human CaM cDNA was PCR amplified with oligonucleotide primers designated to contain two restriction enzyme sites (the forward primer 5'- ACACAGGGGATCCCATATGGCTGAC - 3' and the reverse primer 5'- CAAGCTTGGCTCGAGTCACTTTGC - 3') and inserted into a pGEX4T-1 vector (GE Healthcare). The expression vector was transformed into DH5α E. coli (Nippongene). The strain was preincubated with LB-ampicillin for 16 hrs at 30°C followed by 2 hrs incubation with 10 times volumes of LB- ampicillin at 37°C. For protein expression, 1mM IPTG was added to the medium and incubated 16 hrs at 20°C. After centrifugation, the bacteria pellets were washed with D-PBS and sonicated with 20mM Tris-HCl buffer (pH7.5) containing 1mM EDTA, 1mM DTT and 10% sucrose. After ultracentrifugation at 100,000xg for 30 min, the supernatant fractions were applied to Glutathion Sepharose-4B Fast Flow column (GE Healthcare). The resin was washed with 20mM Tris-HCl buffer (pH7.5) containing 1mM EDTA, 1mM DTT, 1M NaCl and 1% CHAPS , and then with D-PBS. The washed resin absorbing the GST-fused recombinant protein was suspended in D-PBS into 50% slurry and incubated with 1U/mL thrombin (GE Healthcare) for 16hrs at 4°C. The digested fragment (GSH-CaM) was eluted from the resin with D-PBS and applied to HiTrap Benzamidine FF (GE Healthcare) equibrilated with D-PBS. GSH-CaM bound to the resin under these conditions and eluted with D-PBS containing 1M NaCl.

Both purified proteins were applied to Hi Trap Desalting column (GE Healthcare) to exchange the buffer solutions to 20mM HEPES-NaOH buffer (pH7.9) and stored at -80 °C until use.

2. Preparation of SR vesicles [2]

Left ventricles were homogenized in a solution containing 30 mmol/L Tris–malate, 0.3 mol/L sucrose, protease inhibitor cocktail (complete EDTA-free, Roche), pH 7.0 (Solution I). The homogenate was centrifuged at 5,500 g for 15 minutes and the resultant supernatant fraction was filtered through four layers of cheesecloth before centrifugation at 12, 000 g for 25 minutes. The supernatant was again filtered through cheesecloth and centrifuged at 143,000 g for 35 minutes. The pellet was resuspended in a solution containing 0.6 mol/L KCl, 30 mmol/L Tris–malate, 0.3 mol/L sucrose, protease inhibitor cocktail (complete EDTA-free, Roche), pH 7.0. This suspension was centrifuged at 143,000 g for 50 minutes. The pellet was suspended in Solution I and centrifuged at 143,000 g. The resultant pellet represents the microsomal fraction that is enriched in SR vesicles, and it was homogenized in a solution containing 0.1 mol/L KCl, 20 mmol/L Tris–malate, 0.3 mol/L sucrose, protease inhibitor cocktail (complete EDTA-free, Roche), pH 7.0, to a final concentration of about 10–20 mg protein/mL. This fraction was rapidly frozen with liquid nitrogen and stored at –80°C.

3. Site-directed fluorescent labeling of DPc10 binding site in the RyR2 [2, 3]

Peptide-SAED conjugate was formed by incubating 0.2 mmol/L peptide with 0.2 mmol/L SAED in a 20 mmol/L HEPES (pH 7.5) solution for 60 min at 22 °C in the dark. The reaction was quenched by 20 mmol/L Tris-HCl, pH 7.5. Unreacted SAED was removed using ion exchange column (GE HiTrap Q XL). The peptide-SAED conjugate (5 μmol/L in a final concentration) was mixed with 1 mg/mL SR protein in the sample solution in the dark and photolyzed with UV light in a Pyrex tube at 4 °C for 2 minutes. Beta-mercaptoethanol was added (100 mmol/L in a final concentration) to cleave the disulfide bond of SAED and to remove the carrier (DPc10). After incubation on ice for

1 hour, the mixture was centrifuged at 100,000g for 15 minutes, and the sedimented vesicles were resuspended in the sample solution to a final protein concentration of 5-10 mg/mL.

4. Preparation of isolated cardiomyocytes [2]

A wedge of LV free wall, perfused by a branch of the left circumflex coronary artery, was dissected free of the heart and perfused with a collagenase-containing buffer solution. LV myocardium was minced with scissors in the fresh collagenase-containing buffer solution. Then, rod-shaped adult canine cardiomyocytes were prepared by retrograde perfusion of quickly excised hearts with 95%O_2/5%CO_2 -bubbled Minimal Essential Medium (Sigma) supplemented with 50 μmol/L Ca^{2+}, 0.5 mg/mL collagenase B, 0.5 mg/mL collagenase D and 0.02 mg/mL protease type XIV. The concentration of Ca^{2+} was then gradually increased to a final concentration of 100 μmol//L by changing the incubation medium (50 μmol/L and 100 μmol/L). The isolated canine cardiomyocytes were transferred to laminin-coated glass culture dishes, and incubated for 12 hours at 37°C in 5%CO_2/95%O_2 atmosphere. Only quiescent, rod-shaped, and pacing-responsible cells with clear cross striations were used for the study.

5. Analysis of Ca^{2+} sparks in saponin-permeabilized cardiomyocytes with laser scanning confocal microscopy [4]

Ventricular myocytes were superfused with relaxing solution containing EGTA 0.1 mmol/L, ATP 5 mmol/L, HEPES 10 mmol/L, K- aspartate 150 mmol/L, $MgCl_2$ 0.25 mmol/L, and reduce-glutathione 10 mmol/L, at 23°C. The sarcolemma was permeabilized with saponin (100 μg/mL) for 30-40 seconds. After permeabilization,

myocytes were placed in internal solution composed of: EGTA 1 mmol/L; HEPES 10 mmol/L; K-aspartate 120 mmol/L; ATP 3 mmol/L; free [Mg^{2+}] 1 mmol/L; reduced glutathione 10 mmol/L; free [Ca^{2+}] 75 nmol/L (calculated using MaxChelator (http://www.stanford.edu/~cpatton/webmaxcS.htm)); creatine phosphokinase 5 U/ml; phosphocreatine 10 mmol/L; dextran (Mr: 40,000) 4%; Fluo-3 20 μmol/L; pH 7.2. Fluo-3 was excited by 488 nm laser lines, and fluorescence emission was acquired at wavelengths of 505-530 nm.

Ca^{2+} sparks were measured in saponin-permeabilized cardiomyocytes with a laser scanning confocal microscope (LSM-510, Carl Zeiss). equipped with an argon ion laser coupled to an inverted microscope (Axiovert 100, Carl Zeiss) with a Zeiss x40 oil-immersion Plan-Neofluor objective (numerical aperture, 1.3; excitation at 488 nm; emission > 505 nm). [4] The images of Ca^{2+} sparks were acquired in the linescan mode of the confocal microscope at a rate of 1.92 ms per scan, with the 3000 scan lines (5.76sec) oriented along the longitudinal axis of the cell. We sampled and analyzed Ca^{2+} sparks from 20-40 cells. Data were analyzed with SparkMaster, an automated analysis program which allows rapid and reliable spark analysis. [5] The analysis involved general image parameters (number of detected sparks, spark frequency) as well as individual spark parameters (Amplitude, FWHM: full width at half maximum, FDHM: full duration at half maximum).

6. Cell shortening and Ca^{2+} transient measurement [2, 6]

Measurements of myocyte sarcomere shortening and intracellular Ca^{2+} were performed using fura-2 AM. Cells were stimulated by a field electric stimulator (IonOptix, MA) by 0.5 Hz. After stimulation of the cells, the sarcomere shortening and

peak Ca^{2+} transient gradually increased and reached a steady state within 2 minutes. At the steady state (2 minutes after initiation of pacing), the intracellular calcium concentration was monitored by a dual-excitation spectrofluorometer as the ratio of the fluorescence emission intensities (at 505 nm) of fura-2 AM elicited by excitation at 340 and 380 nm. Mean sarcomere length was calculated using a fast Fourier Transform of a video image (IonOptix) recorded simultaneously. The domain peptide (DPc10 or DP4090-4123) was introduced using a protein delivery reagent (Bioporter, Gene Therapy Systems, Inc, CA) into the cells. The successful introduction of the peptide into the cell was confirmed by detecting the intracellular fluorescence signal of the peptide prelabeled with Alexa Fluor 350 (Molecular Probe). Briefly, peptide–Alexa Fluor conjugate was formed by incubating 1 mmol/L peptide with 1 mmol/L Alexa Fluor carboxylic acid and succinimidyl ester in a 20 mmol/L HEPES buffer (pH 7.6) for 60 minutes at 22°C in the dark. Free Alexa Fluor was removed by Sephadex G15 gel filtration. The final concentration of peptide conjugated with the Alexa Fluor was estimated by measuring the absorbance at 280 nm. After the introduction of peptide-Alexa Fluor conjugate into the cell, the fluorescence intensity of Alexa Fluor was measured with confocal microscopy (LSM 510, Carl Zeiss). The intracellular peptide concentration was determined from the concentration-fluorescence intensity plot.

The successful incorporation of the domain peptide into the cell was confirmed by detecting the fluorescence signal which was pre-labeled with Alexa Fluor® 350 (Molecular Probe, OR).

7. Cross-linking of CaM-SANPAH to the RyR2 [4]

For CaM binding on the RyR2, we first tried to perform the conventional pull-down assay using the anti-RyR2 antibody, followed by detection of the bound CaM using the anti-CaM antibody. However, there was marked non-specific CaM binding on Sepharose A or G even without the anti-RyR2 antibody, which precluded the reliable assessment of the specific CaM binding on the RyR2. Therefore, we assessed the CaM binding to the RyR2 using a photoreactive crosslinker, sulfosuccinimidyl-6-[4'-azido-2'-nitrophenylamino]hexanoate(Sulfo-SANPAH, PIERCE). First, CaM-SANPAH conjugate was formed by incubating 0.2 mmol/L CaM with 0.8 mmol/L SANPAH in a 20 mmol/L HEPES (pH 7.5) solution for 60 minutes at 22 °C in the dark. The reaction was quenched by 20 mmol/L Tris-HCl, pH 7.5. Free Sulfo-SANPAH was removed using Sephadex G25 column. The CaM-SANPAH conjugate (16 nmol/L-1μmol/L in a final concentration) was mixed with 0.5mg/mL SR protein in the sample solution containing 150 mmol/L NaCl, 50 mmol/L MOPS, 1 mmol/L EGTA/calcium buffer (0.3 μmol/L free [Ca^{2+}]) pH6.8, in the dark and photolyzed with UV light in a Pyrex tube at 4 °C for 30 seconds. Then, the mixture was immunoblotted with a monoclonal anti-CaM antibody (MILLIPORE, CA) to detect the RyR2-bound CaM.

8. Direct binding of exogenous CaM to the RyR2 in saponin-permeabilized cardiomyocytes [4]

Recombinant calmodulin was labeled with Alexa Flour 633 carboxylic acid succinimidyl ester as follows. Since calmodulin has eight Lysine residues, there should be nine succinimidyl ester targets including amino terminal. Therefore, we mixed recombinant calmodulin(100 μmol/L) and Alexa Flour 633 succinimidyl ester (300

μmol/L) with one to three molar ratio in reaction buffer (150mmol/L NaCl, 20 mmol/L HEPES, pH7.4). After 30min conjugation reaction at room temperature, the reaction was quenched by addition of 30mmol/L Lysine. The hydrolyzed or quenched Alexa Flour 633 was removed using Sephadex™ G25 size exclusion column. With this method, we could obtain fluorescent labeled calmodulin.

The exogenous CaM, fluorescently labeled with Alexa Fluor 633 (Molecular Probes, OR), was added to the saponin-permeabilized normal and failing cardiomyocytes under the same condition as the above-mentioned Ca^{2+} spark measurements. Then, quantification of the distribution of localized CaM was achieved through densitometric measurement of CaM-Alexa fluorescence. Briefly, the fluorescently labeled cardiomyocytes were laser-scanned with the confocal microscope system (LSM-510, Carl Zeiss). (numerical aperture, 1.3; excitation at 632 nm; emission 647 nm). The sarcomere-related periodical increase in the Alexa633 fluorescence intensity from baseline was integrated with respect to the longitudinally selected distance (~25 μm) and then divided the value by the distance. Thus obtained signal-averaged Alexa633 fluorescence intensity was expressed as the ratio to the maximum value obtained in the presence of CaM-Alexa (1 μmol/L). The CaM-Alexa labeled cardiomyocytes were also applied to the immunofluorescent staining by a monoclonal anti-RyR antibody (C-teminal Ab4963, Sigma; cf. ref 10), as follows. The cardiomyocytes were fixed with 4% paraformaldehyde in PBS for 10 minutes, washed three times with PBS, and permeabilized in 0.5% Triton X-100 and 1% BSA for 15 minutes. Then, the cardiomyocytes were incubated overnight at 4°C with the anti-RyR antibody (C-teminal Ab4963, Sigma) in 1% BSA and 0.5 % Triton X-100, followed by labeling with an Alexa488-conjugated goat anti-rabbit secondary antibody (Molecular

Probes, OR). The cardiomyocytes were washed three times with PBS. Images for co-localization of CaM-Alexa and RyR were acquired at 488nm excitation wavelength using a BP 505-530 nm band-pass detection filter for green channel (anti-RyR antibody) and 633 nm excitation wavelength in conjunction with a LP 650 nm long pass filter for red channel (CaM-Alexa). Secondary antibody labeling alone showed no detectable fluorescence pattern.

9. Determination of the binding of endogenous CaM to the RyR2 in intact cardiomyocytes [4]

The isolated cardiomyocytes were plated on glass-based dishes, fixed with 4% paraformaldehyde in PBS for 10 min, washed three times with PBS, and permeabilized in 0.5% Triton X-100 and 1% BSA for 15 min. These cardiomyocytes were incubated overnight at 4°C with a monoclonal mouse anti-CaM antibody (MILLIPORE, CA) and polyclonal rabbit anti-RyR antibody (C-teminal Ab4963, Sigma) in 1% BSA and 0.5 % Triton X-100, followed by each labeling with an Alexa488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, OR) and Alexa633-conjugated rabbit anti-mouse secondary antibody (Molecular Probes, OR). Cardiomyocytes were then washed three times with PBS. For co-localization of anti-CaM antibody (Alexa633; red) and anti-RyR antibody (Alexa488; green), images were acquired at 633 nm and 488 nm excitation in conjunction with LP 650 filter and BP505-530 nm, respectively. Both secondary antibody labeling showed no detectable fluorescence pattern and did not cross-react with each primary antibody. For co-localization analysis, the fluorescent images were acquired sequentially in order to prevent spectral cross-talk.

References

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Supplemental figure legends.

Supplementary Figure 1.

Concentration-dependent effect of wild-type calmodulin (WT-CaM) or Gly-Ser-His-calmodulin (GSH-CaM) on Ca^{2+}/CaM-dependent protein kinase-II (CaMKII) activity. CaMKII activity was measured using the CycLex® CaM kinase II Assay Kit (Medical & Biological Laboratories). Data represent means ± standard deviations (SD) of 2 experiments.

Supplementary Figure 2

The localization of exogenously introduced CaM in saponin-permeabilized cardiomyocytes. The CaM was fluorescently labeled with Alexa Fluor 633 (Molecular Probes, OR) and delivered into the cardiomyocytes. The fluorescently labeled cardiomyocytes were laser-scanned using a confocal microscope system (LSM-510, Carl Zeiss). (Left panel) Representative images of exogenous WT-CaM or GSH-CaM co-localized with RyR2 in normal cardiomyocytes. Left: GSH-CaM-Alexa (red), middle: RyR2 (green), right: merged image. (Right panel) Periodic increases in the Alexa fluorescence signals of either RyR2 (green) or WT-CaM or GSH-CaM (red).

Supplementary Figure 3

Representative images of CaM (detected by the anti-CaM antibody) co-localized with RyR2 in normal cardiomyocytes after incorporation of exogenous WT-CaM or GSH-CaM into intact normal and failing cardiomyocytes using a protein delivery kit (Bioporter, Gene Therapy Systems, Inc., CA). Left: CaM (red), middle: RyR2 (green),

right: merged.

Supplementary Figure 4

Effect of WT-CAM (or GSH-CaM) (200 nmol/L) on Ca^{2+} spark frequency and SR Ca^{2+} content in (non-permeabilized) normal cardiomyocytes and failing cardiomyocytes. SR Ca^{2+} content was measured by adding 10 mmol/L caffeine. **A.** Representative images of Ca^{2+} sparks. **B.** Summarized data of the full duration at half-maximum (FDHM), full width at half-maximum (FWHM), Ca^{2+} spark amplitude, Ca^{2+} spark frequency, and SR Ca^{2+} content. Data represent the means ± SD.

Supplementary Figure 5

Effect of KN93 on Ca^{2+} spark frequency and SR Ca^{2+} content in (non-permeabilized) normal cardiomyocytes and failing cardiomyocytes. SR Ca^{2+} content was measured by adding 10 mmol/L caffeine. **A.** Representative images of Ca^{2+} sparks. **B.** Summarized data of the full duration at half-maximum (FDHM), full width at half-maximum (FWHM), Ca^{2+} spark amplitude, Ca^{2+} spark frequency, and SR Ca^{2+} content. Data represent the means ± SD.

Supplementary Figure 6

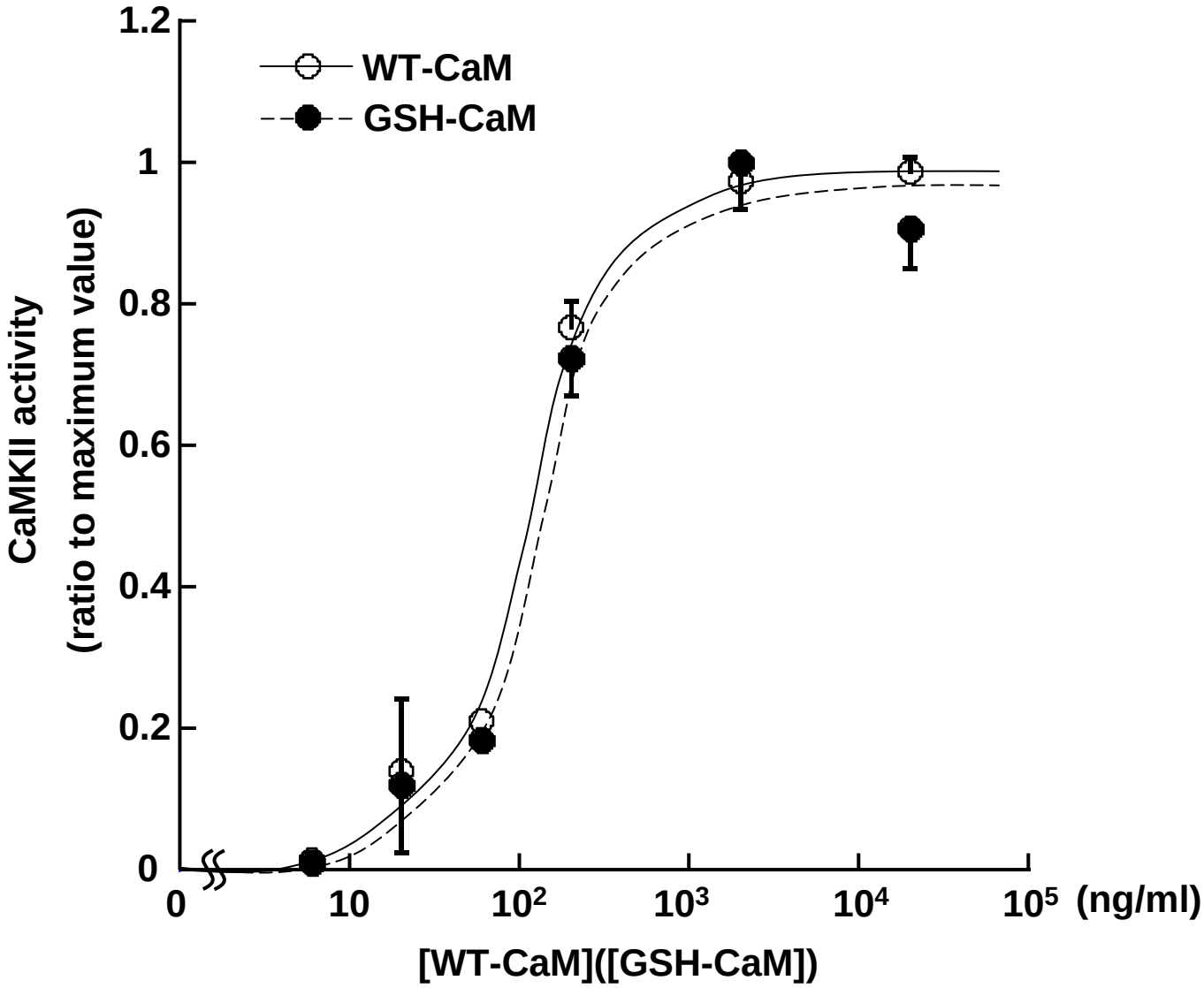
Relationship between % cell shortening and the magnitude of Ca^{2+} transient in normal and failing cardiomyocytes.

Supplementary Figure 8

A schematic diagram which illustrates the interaction between domain unzipping of

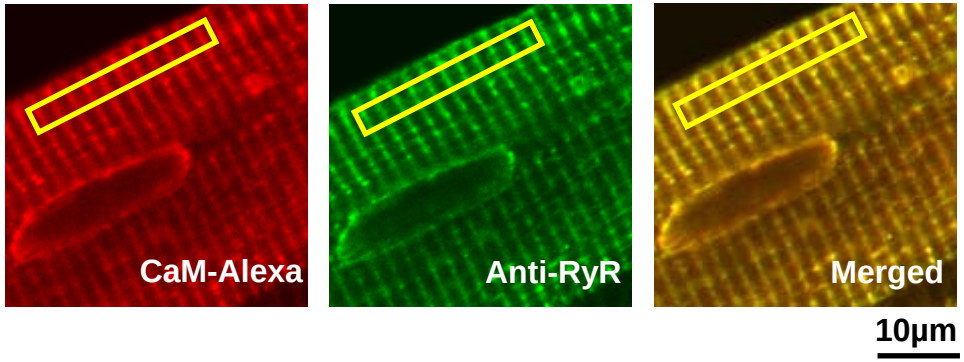
N-terminal and central inter-domain interaction, and CaM binding. Defective N-terminal domain/central domain interaction reduces the binding affinity for CaM, destabilizing the closed state of the channel, leading to heart failure. In contrast, enhancing CaM binding affinity stabilizes the closed state of the channel and inhibits Ca^{2+} leak.

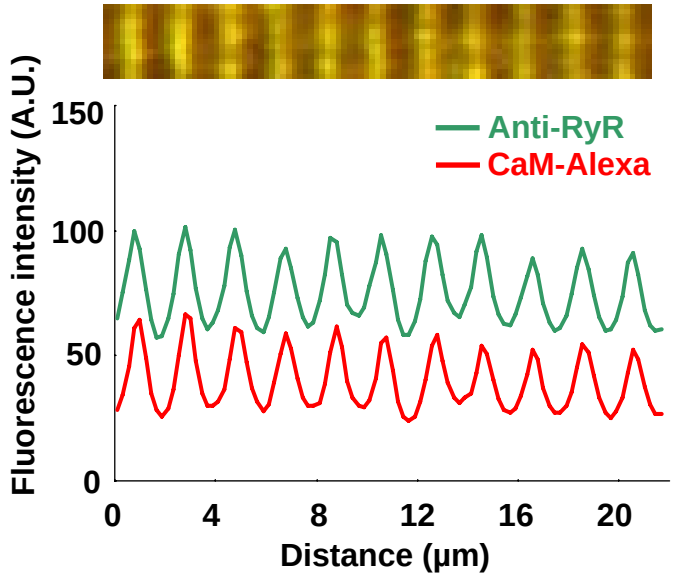
Supplementary Figure 1



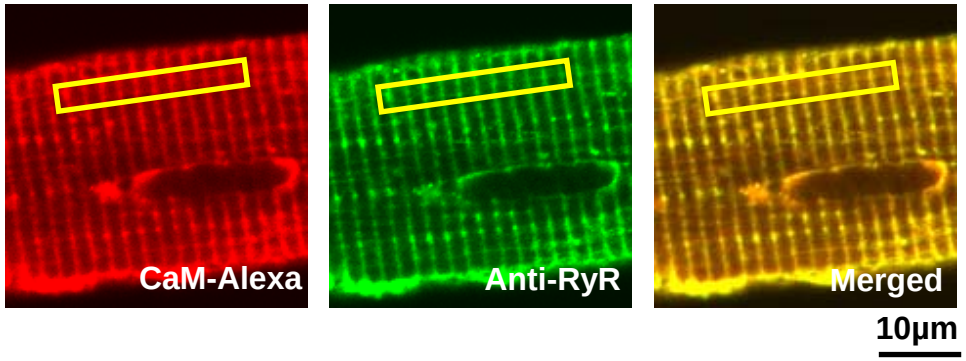
Supplementary Figure 2

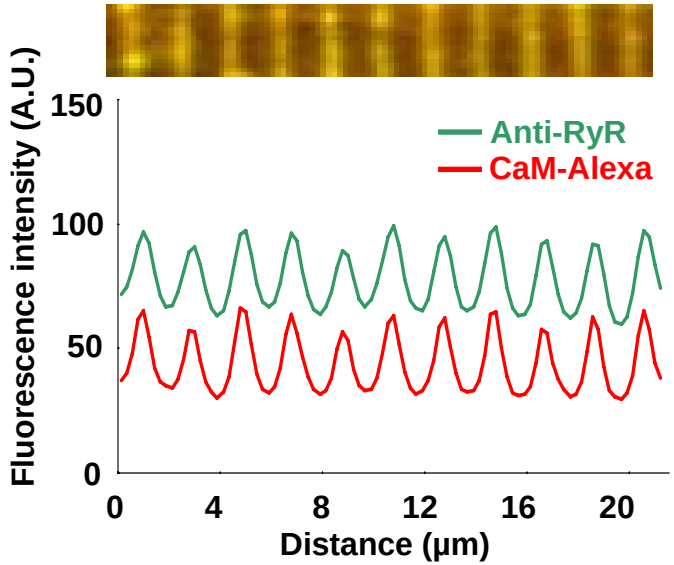
WT-CaM





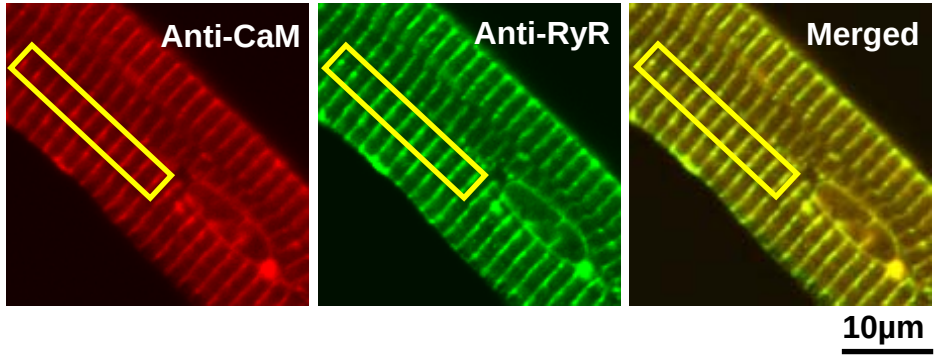
GSH-CaM

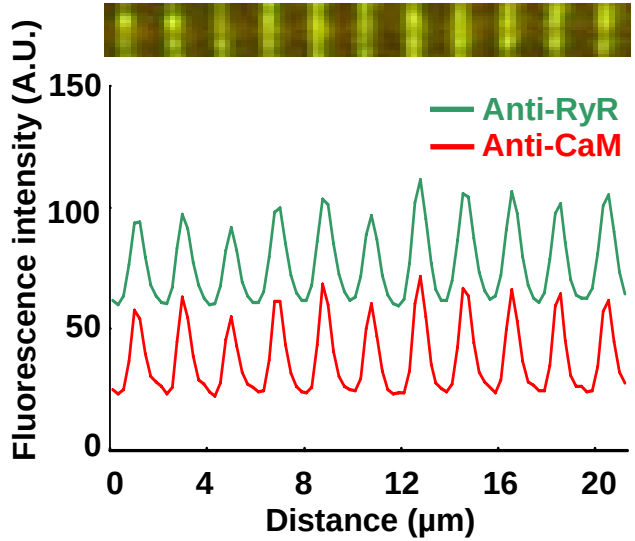




Supplementary Figure 3

WT-CaM





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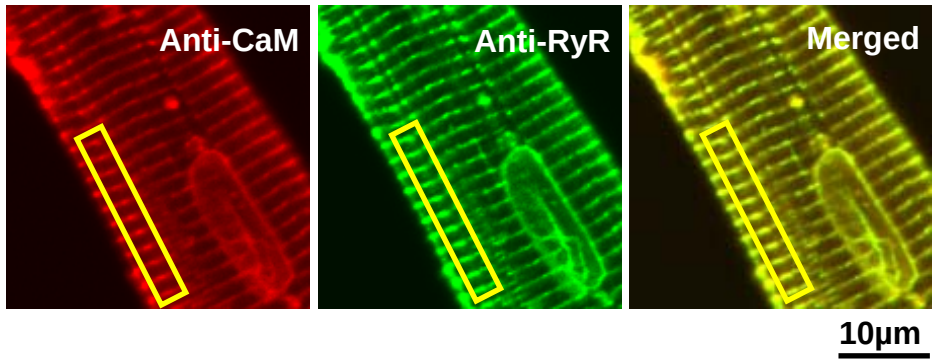
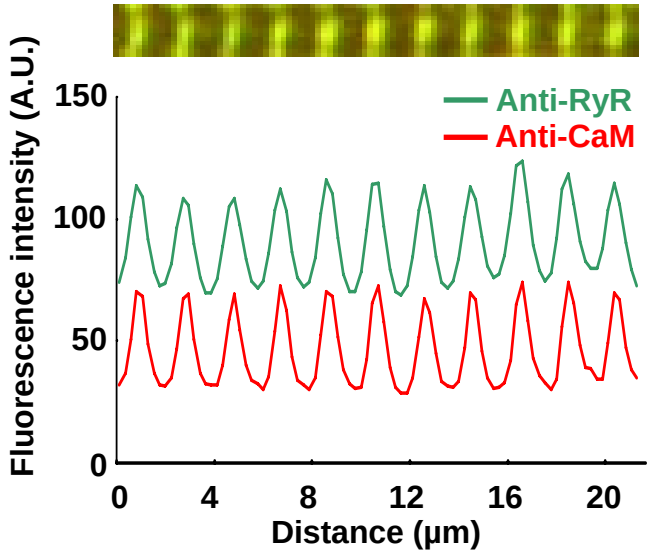
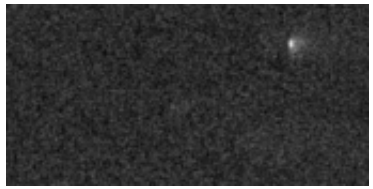




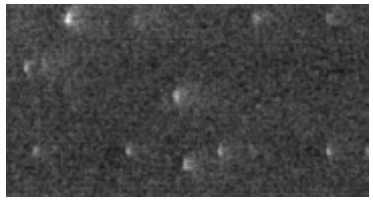
Supplementary Figure 4

Normal cardiomyocyte

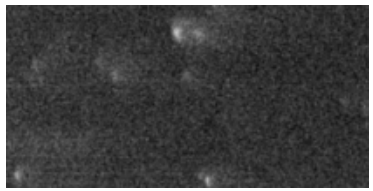


Failing cardiomyocyte

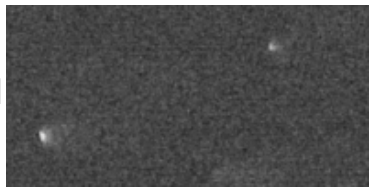
control

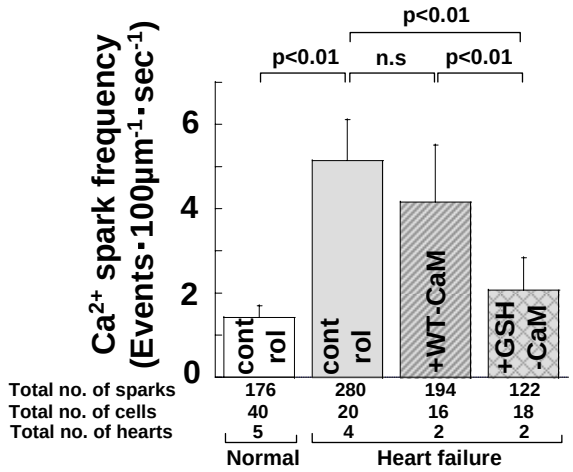


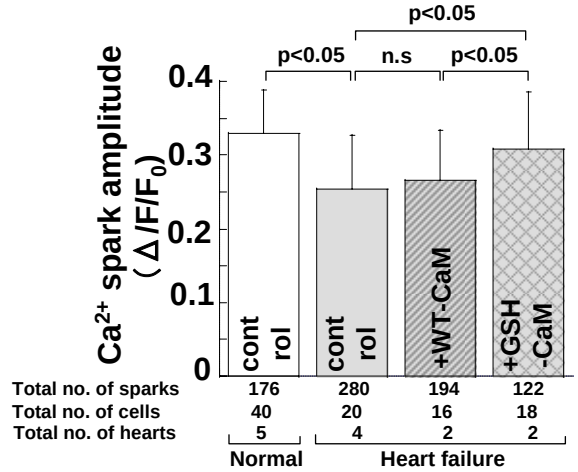
+WT-CaM

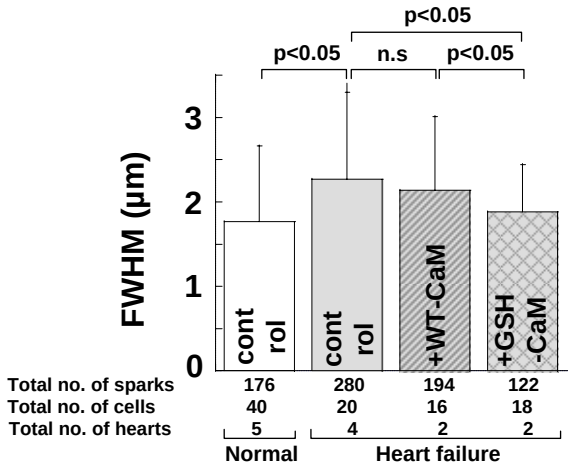


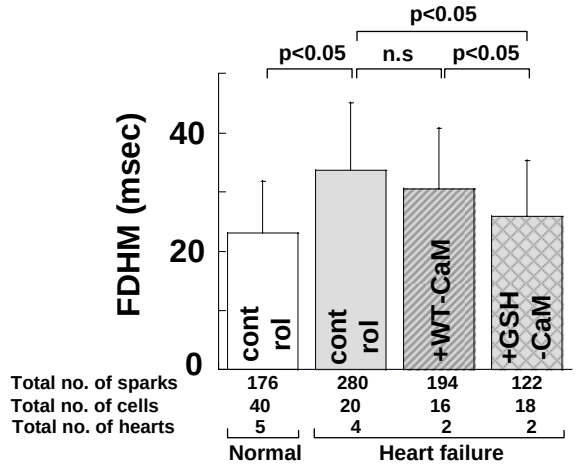
+GSH-CaM

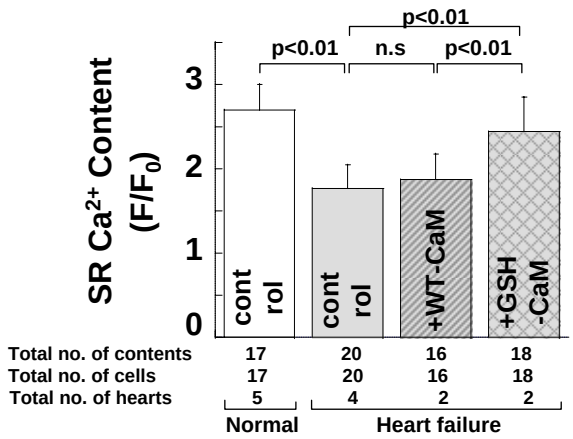






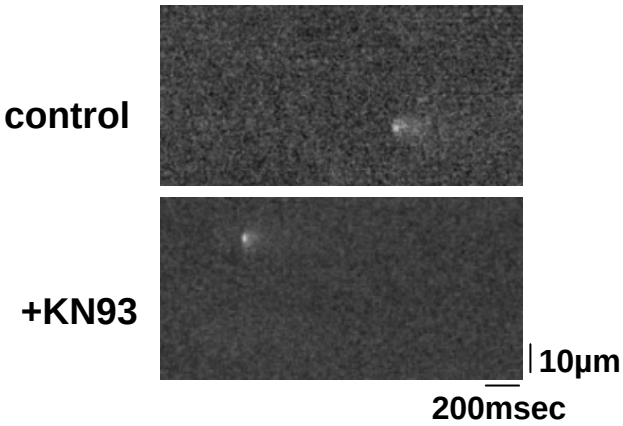




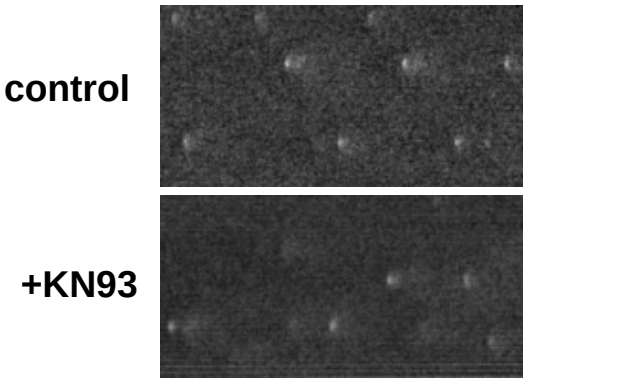


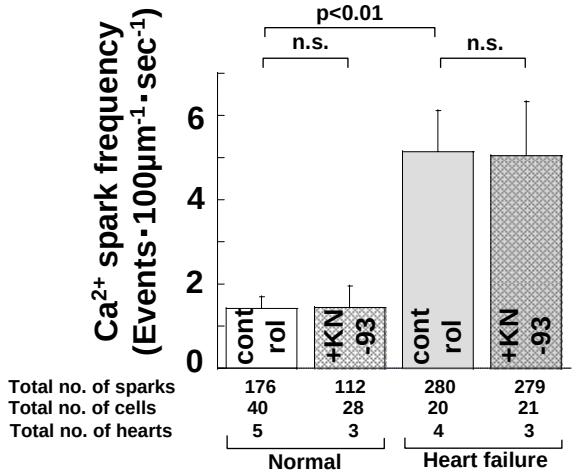
Supplementary Figure 5

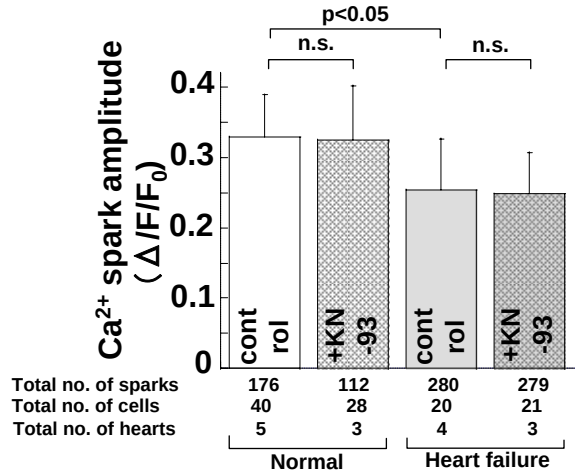
Normal cardiomyocyte

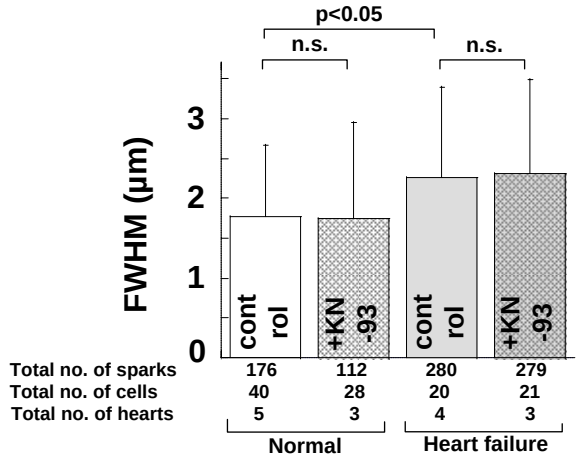


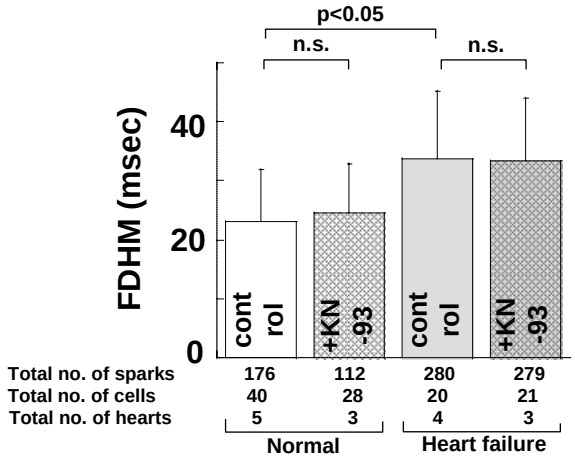
Failing cardiomyocyte

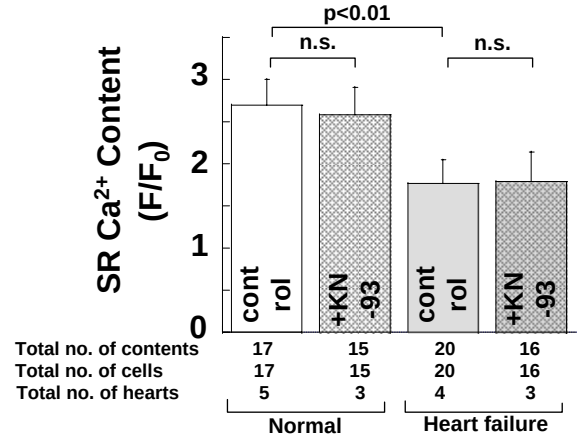




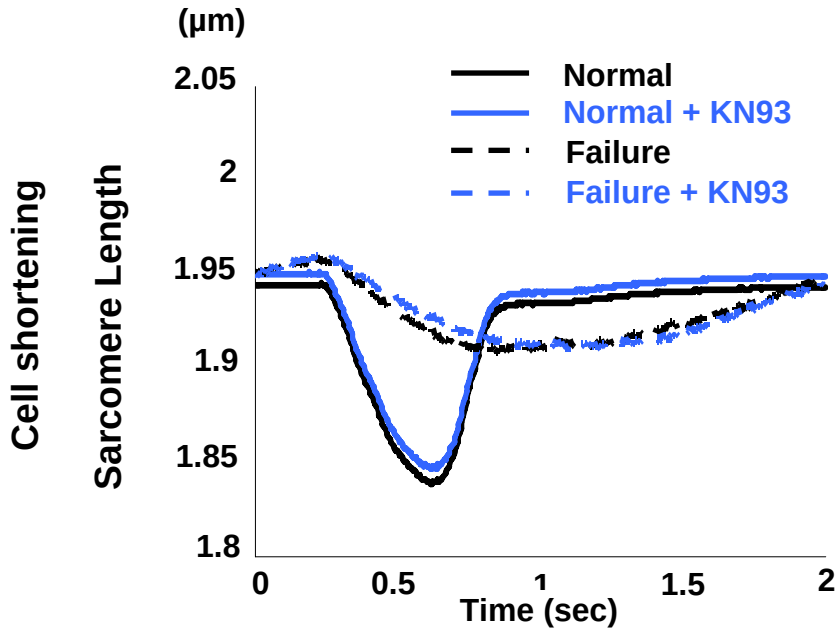


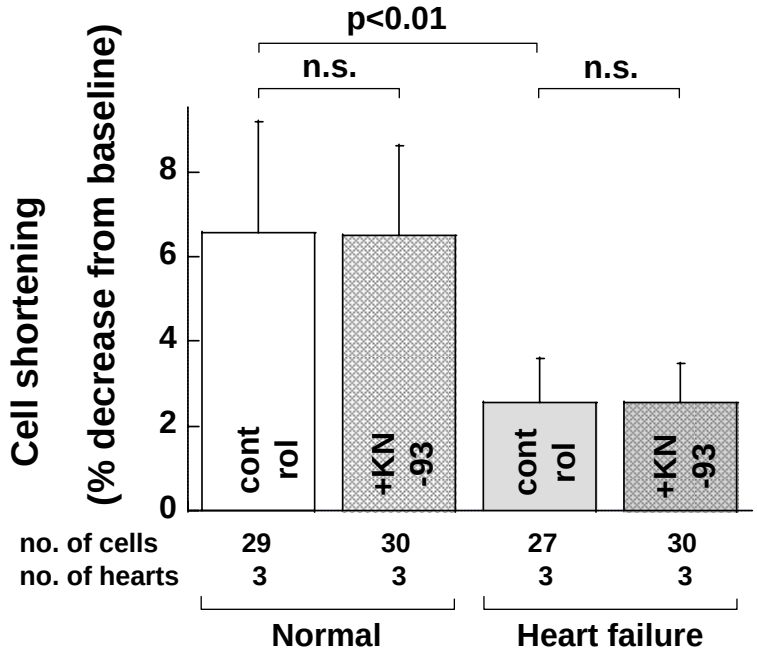


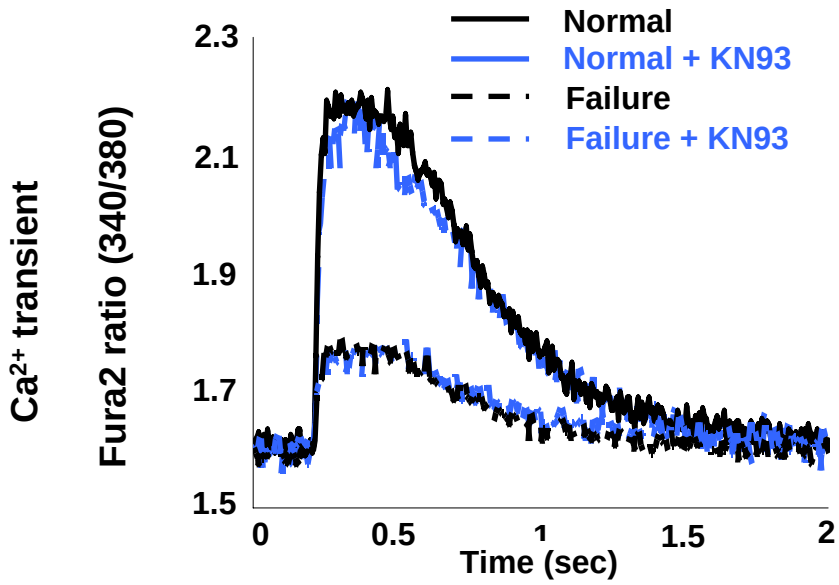


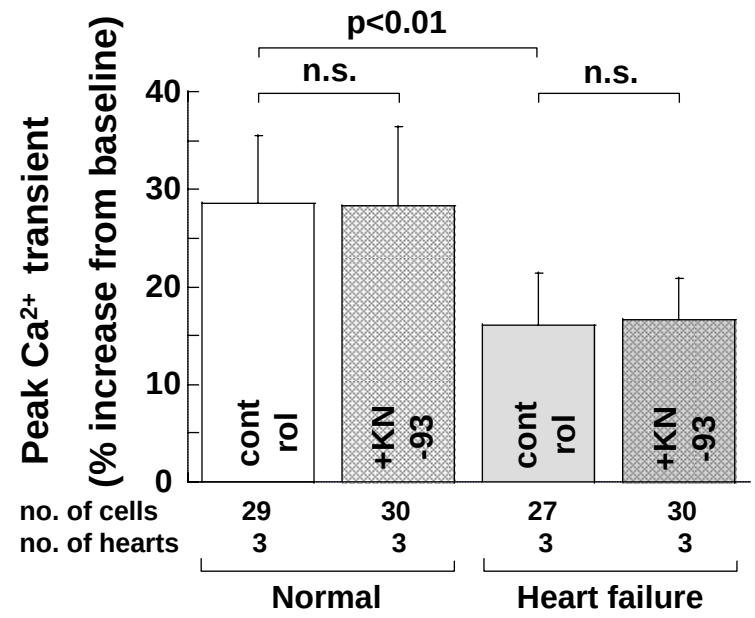


Supplementary Figure 6

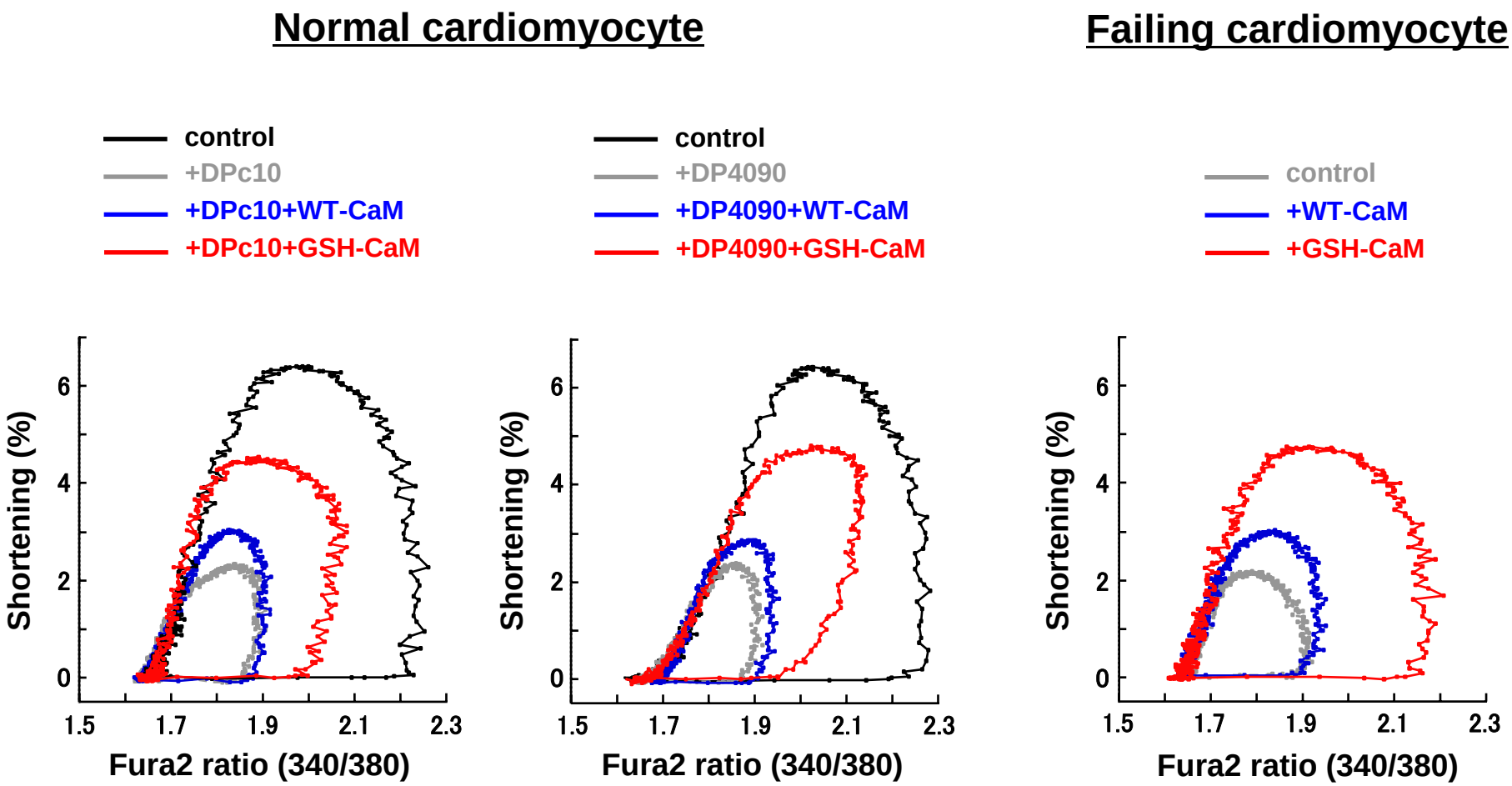




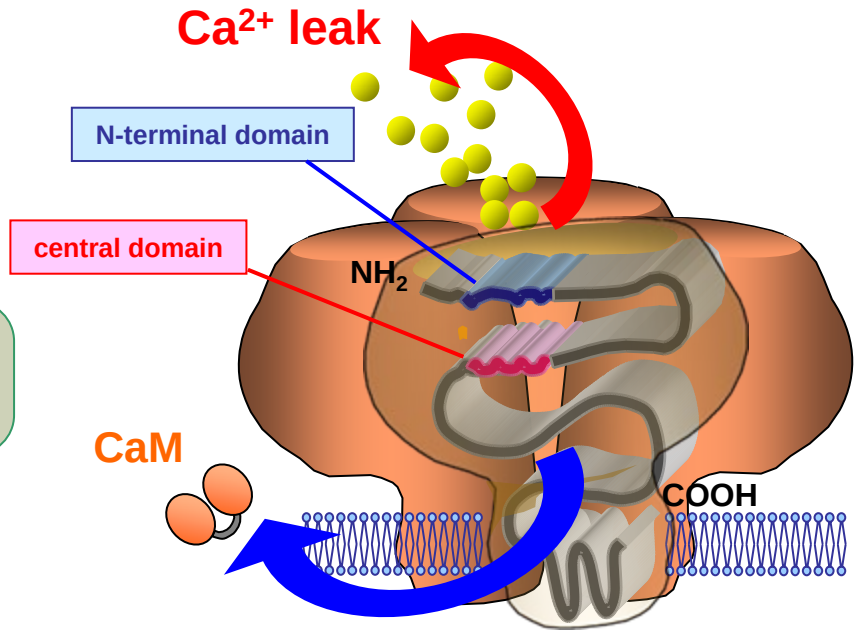




Supplementary Figure 7



Supplementary Figure 8



de-stabilized channel

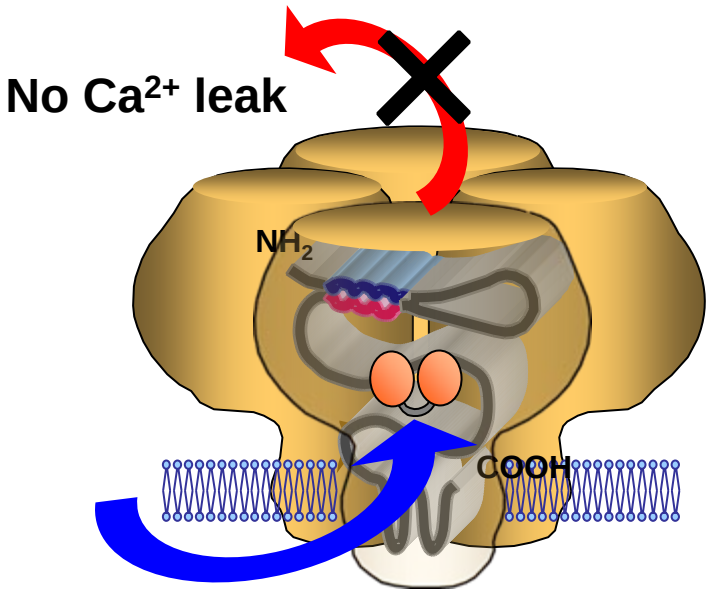
defective inter-domain interaction

↓

reduced CaM-binding

↓

Ca^{2+} leak



stabilized channel

correction of the reduced CaM-binding

↓

Restored CaM binding

↓

No Ca^{2+} leak

Supplementary Table 1

Characteristics of hearts

	LVDd (mm)	LVDs (mm)	LVFS (%)
Normal (n=6)	31.8 ± 0.8	20.3 ± 1.2	35.2 ± 2.6
Heart failure (n=6)	39.7 ± 0.5 †	34.0 ± 0.9 †	13.3 ± 2.4 †

Each data point represents the mean ± SD. †P＜0.01: Normal vs Heart failure.

N: the number of hearts. LVDd = left ventricular end-diastolic diameter; LVDs = left ventricular end-systolic diameter; LVFS = left ventricular fractional shortening

Supplementary Table 2

Characteristics of isolated cardiomyocytes

	Cell length (μm)	Cell width (μm)
Normal (n=6, 120 cells)	119.3 ± 7.8	15.5 ± 0.9
Heart failure (n=6, 120 cells)	137.9 ± 7.3 †	16.4 ± 1.3 †

Each data point represents the mean ± SD. †P＜0.01: Normal vs Heart failure.

N: the number of hearts.