SUPPLEMENTAL MATERIAL

Data S1:

RT PCR: Total RNA was isolated from PNCM on day 4 in culture and H9C2 cells at passages 18, 23, 24 (Ambion RNAqueous kit). To reduce DNA contamination in the preparations, the isolated total RNA was subject to precipitation with lithium chloride and DNase digestion (Ambion DNase free). cDNA was generated from mRNA using Takara Primescript cDNA synthesis kit with oligo dT primers. Previously described primers, forward: GGCCACAGAGGATCTGGAAAAG and reverse: CAACCCTGCTGGTTCCCTAAG for rat TRPV1[1], were used to amplify the full length coding sequence from the cDNA using Platinum Taq (Invitrogen). The amplified PCR product was subcloned into the pCR TOPO vector (Invitrogen) and sequenced.

Quantitative PCR: cDNA from mRNA was generated from H9C2, PNCM, and heart tissues (RA, LA, RV, LV) as described above. The qPCR reactions were performed using Fast SYBR Green Master Mix (Life Technology). Primers used were forward: CTGACGGCAAGGATGACTACC reverse: ACCTCAGGGAGAAGCTCAGG, previously described[2]. The reactions were done in a total of 20ul containing 10ng of cDNA and 200nM of primers per manufacturer's recommendation. The cycling protocol was as follows: initial denaturing template at 95^0C for 30 secs, followed by 40 cycles of 95^0C denaturing for 3 sec of and 30 secs of annealing/extension at 61^0C. After completion, melting curve analysis was performed by gradually increasing the temperature from 60^0C to 95^0C in a graded manner of 0.3^0C every 15 secs.

Immunofluorescence: PNCM were seeded onto poly-D-lysine coated glass coverslips at a density 150,000 cells and anti-TRPV1 (1:500; NeuroMab) and anti-TOM20 (1:250; Santa Cruz Biotechnology) were used. PNCM at day 4 in culture were fixed with 2% paraformaldehyde/2% sucrose solution for 10 minutes at room temperature (RT). The cell preparations were washed thrice with phosphate buffer saline (PBS) at five minutes intervals. A blocking solution (5% dry milk, 2% Goat Serum, 150mM sodium Chloride, 40 mM HEPES pH 7.4) with 0.2% Triton-X100 (Sigma-Aldrich) was applied for 30 minutes at RT to permeabilize cell preparations. The samples were washed twice with PBS and blocking solution without Triton-X100 was applied for 15 minutes. Antibody labeling of cell was performed by diluting antibodies in blocking solutions. The following antibodies were used: mouse monoclonal anti-TRPV1 (1:500; NeuroMab), and rabbit polyclonal anti-TOM20 (1:250; Santa Cruz Biotechnology). The antibodies in blocking solution were applied to cell preparation in various combinations for one hour at RT. The antibody labeled cell preparations were washed thrice with PBS at five minute intervals. Blocking solution without Triton-X100 was applied to cells for 30 at room temperature. Antibodies labeled cell preparations were stained with secondary antibodies, goat anti-mouse Alexa 488 and/or donkey anti-rabbit Alexa 594 (1:1000; Invitrogen), diluted in blocking solution. The cell preparations were washed thrice with PBS and mounted on glass slides using Prolong Gold antifade reagent.

The slide preparations were visualized by either epifluorescent or confocal microscopy. For the epifluorescent microscopy, the slide preparations were visualized using Ziess Axioplan 2 imaging system with a 63X 1.4 NA oil immersion apochromatic lens. The image acquisition and adjustment of brightness and contrast of cell preparations was done on the Leica SP8 AOBS Confocal system with a 63X HC PL APO, CS2, 1.4 NA lens. The software used for image acquisition and analysis was Leia's LASAF software v 3.3.10134.0

Western Blot: Heart tissue was homogenized and centrifuged at 800g to remove cellular debris. The supernatant was kept as the total fraction. PNCM, H9C2 and F11 cells (stably overexpressing TRPV1, a gift from Dr. Yoneda) were lysed in RIPA buffer (0.150M NaCl, 1% Triton X-100, 50mM HEPES, 1mM EDTA) containing a cocktail of protease and phosphatase inhibitors. 75µg of each homogenate was run on 7.5% SDS-PAGE gels and antibodies to TRPV1 (1:500, NeuroMab) and GAPDH (1:1000, Sigma) were used.

Transmission Electron Microscopy: Primary neonatal cardiomyocytes (PNCM) were prepared as previously described[3]. PNCM were washed in PBS (Ca^{2+}, Mg^{2+}) twice at room temperature, trypsinized and centrifuged at 1200 rpm for 3 minutes. Cell pellet was suspended and fixed in 1 ml of fixative containing paraformaldehyde (2% v/v), Glutaraldehyde (0.01% v/v) and Sucrose (2% w/v) in PBS (pH 7.4) for 20 minutes at room temperature. Cells were then washed in 20mM Glycine-PBS twice at room temperature followed by washing twice in PBS. Samples were dehydrated in a series of ethanol washes for 15 min at 4 °C beginning at 30%, 50%, 70%, and 95% ethanol in water. Samples were then infiltrated with Medium Grade LR White resin to 1 part 95% ethanol for 1 hour then changed 2 times in LR white resin. The samples were then placed into molds with labels and fresh resin, orientated and placed into 50^0 C heat block overnight. Sections were taken between 75 and 90 nm, picked on formvar/Carbon coated 100 mesh Ni grids. Sections were briefly washed three times in PBS-Tween 20 (PBST) for 5 minutes each, blocked with Standard block solution (0.5% BSA, 0.5% Ovalbumin in PBST) for 30 minutes followed by staining with Anti-TRPV1 antibody (1:25 in 1% BSA-Tris buffer, Santa Cruz). Sections were washed thrice with PBST and stained with secondary antibody for 30 minutes at room temperature. Sections were fixed in 8% Glut, washed with ultrapure water, stained with contrast stain (3.5% Uranyl acetate in 50% acetone) for 35 seconds, rinsed and then stained in 0.2% Lead citrate for 35 seconds. Images were acquired using a JEOL JEM-1400 transmission electron microscope at 80 kV.

Peptide Stability Assay:

The stability of the peptide designed against the calcineurin interaction site on TRPV1, which we named V1-cal, was determined by HPLC using modified trypsin, which cleaves peptide bonds after lysine and arginine. V1-cal (1 mg/mL, 400 µL) was dissolved in NH_4HCO_3 buffer (pH 8.0, 200 mM), and was mixed with a trypsin solution (1 µL, 1 mg/mL in NH_4HCO_3, pH 8.0) (Promega, WI, USA). The peptide was incubated at 37^OC, and samples (100 µL) were taken after 5, 10, 15 and 20 min. A solution of 2% trifluoroacetic acid and 5% acetonitrile in water (100 µL) was added, and the samples were analyzed by high performance liquid chromatography and mass spectrometry[4].

Live Cell Imaging:

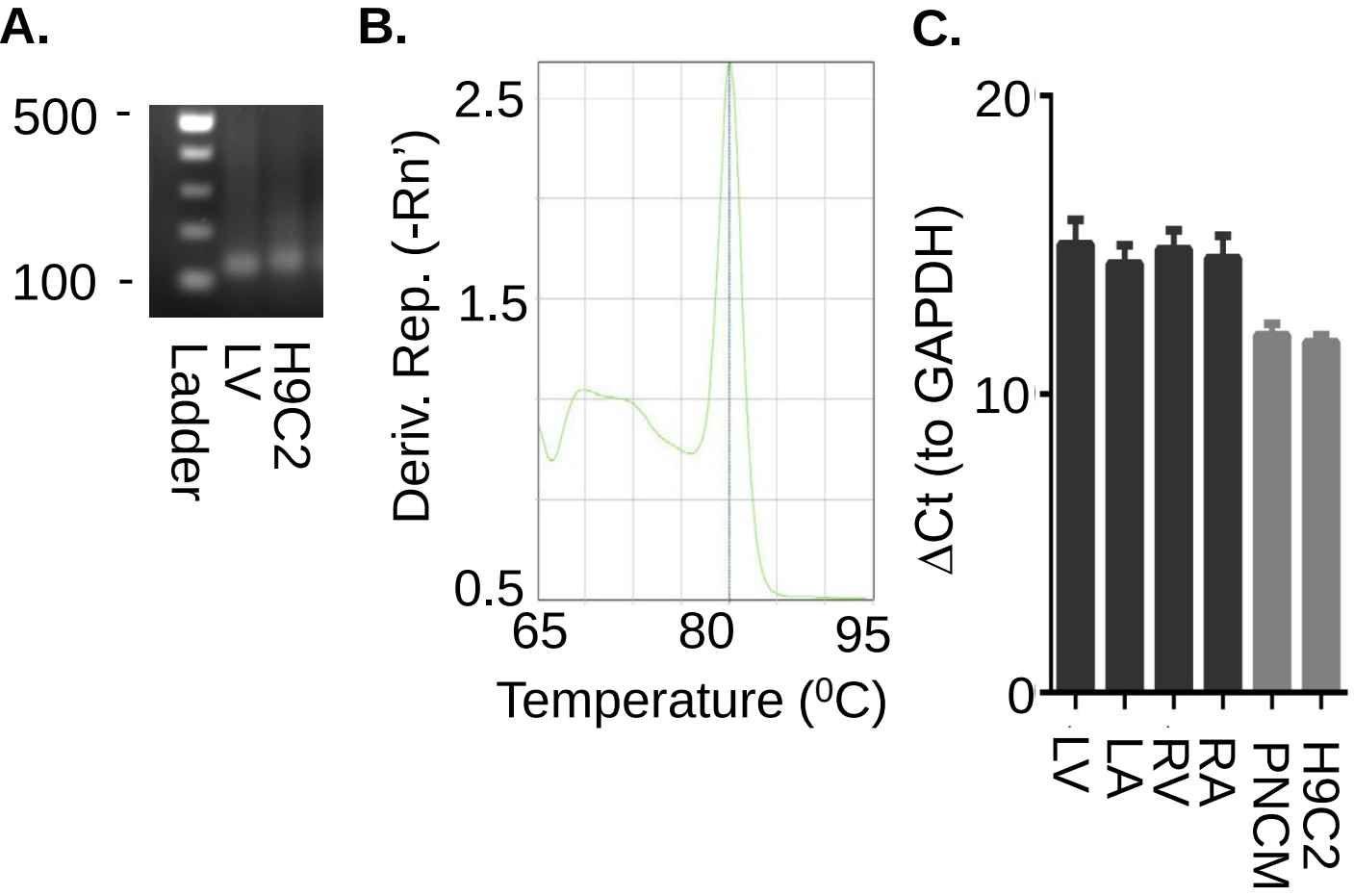
TRME and CMXRos fluorescence images were acquired using 592/24 nm excitation and

675/100 nm emission filter settings. For either protocol, images were taken using a Zeiss Axiovert 200M microscope with Hamamatsu CCD camera (C4742-95). The cellular fluorescent intensities of the dyes used were collected and quantified by fully automated software for all data (Axiovision software version 4.8.1). Additionally for these studies, the partial pressure of oxygen in media was continually assessed at 5 Hz throughout the experiment using a Neofox(TM) oxygen phase fluorimeter (Neofox-GT, SpectrEcology, Tampa Bay, FL) with a borosilicate fiber optic sensor atmospherically sealed within the imaging chamber. Each individual cell provided a baseline for subsequent analysis of the various treatments. This is in order to mitigate the effect of variations in dye loading due to baseline differences in mitochondria membrane potential between cells.

Sprague-Dawley Rats used for study:

Figure Number	Total Number of Animals Used	Number Excluded	Reason for Exclusion
Figure 2	33	3	Ventricuar fibrillation (1), preconditioned (1), suture not released at reperfusion (1); all 3 rats exluded were within the CAP (0.1mg/kg) group
Supplemental Figure 3	8	0	None Excluded
Figure 3	25	1	Remained in TTC overnight instead of formaldehyde (1); CPZ group
Figure 6	15	3	Cannulation unsuccessful (2), perfused with air (1); both prior to assigning to a group
Figure 7	25	1	Died 2 days prior to experiment in animal care facility
Figure 8	13	1	Small AAR/LV (1); TRPV1 knockout group

Table S1. Number of rodents used total for the study, including those included, excluded, and reasons for exclusion.



D.

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ATEDLERMEQRASLDSEESESPPQENSCLDPPDRDPNCKPPPVKPHIFTTRSRTRLFGKG   60
DSEEASPLDCPYEEGGLASCPIITVSSVLTIQRPGDGPASVRPSSQDSVSAGEKPPRLYD  120
RRSIFDAVAQSNCQELESLLPFLQRSKKRLTDSEFKDPETGKTCLLKAMLNLHNGQNDTI  180
ALLLDVARKTDSLKQFVNASYTDSYYKGQTALHIAIERRNMTLVTLLVENGADVQAAANG  240
DFFKKTKGRPGFYFGELPLSLAACTNQLAIVKFLLQNSWQPADISARDSVGNTVLHALVE  300
VADNTVDNTKFVTSMYNEILILGAKLHPTLKLEEITNRKGLTPLALAASSGKIGVLAYIL  360
QREIHEPECRHLSRKFTEWAYGPVHSSLYDLSCIDTCEKNSVLEVIAYSSSETPNRHDML  420
LVEPLNRLLQDKWDRFVKRIFYFNFFVYCLYMIIFTAAAYYRPVEGLPPYKLKNTVGDYF  480
RVTGEILSVSGGVYFFFRGIQYFLQRRPSLKSLFVDSYSEILFFVQSLFMLVSVVLYFSQ  540
RKEYVASMVFSLAMGWTNMLYYTRGFQQMGIYAVMIEKMILRDLCRFMFVYLVFLFGFST  600
AVVTLIEDGKNNSLPMESTPHKCRGSACKPGNSYNSLYSTCLELFKFTIGMGDLEFTENY  660
DFKAVFIILLLAYVILTYILLLNMLIALMGETVNKIAQESKNIWKLQRAITILDTEKSFL  720
KCMRKAFRSGKLLQVGFTPDGKDDYRWCFRVDEVNWTTWNTNVGIINEDPGNCEGVKRTL  780
SFSLRSGRVSGRNWKNFALVPLLRDASTRDRHATQQEEVQLKHYTGSLKPEDAEVFKDSM  840
VPGEK*WTLCRDQCGVFGWSA*GTSRV
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Figure S1. A. Representative TRPV1 DNA gel for qPCR experiments. **B.** Representative TRPV1 melt curve for qPCR experiments. **C.** Delta Ct relative to GAPDH for heart homogenate of different heart chambers and cells tested. **D.** Sequence of TRPV1 in rat neonatal primary cardiomyocytes. Underlined portions are regions sequenced prior to and after the TRPV1 start and end sequence.

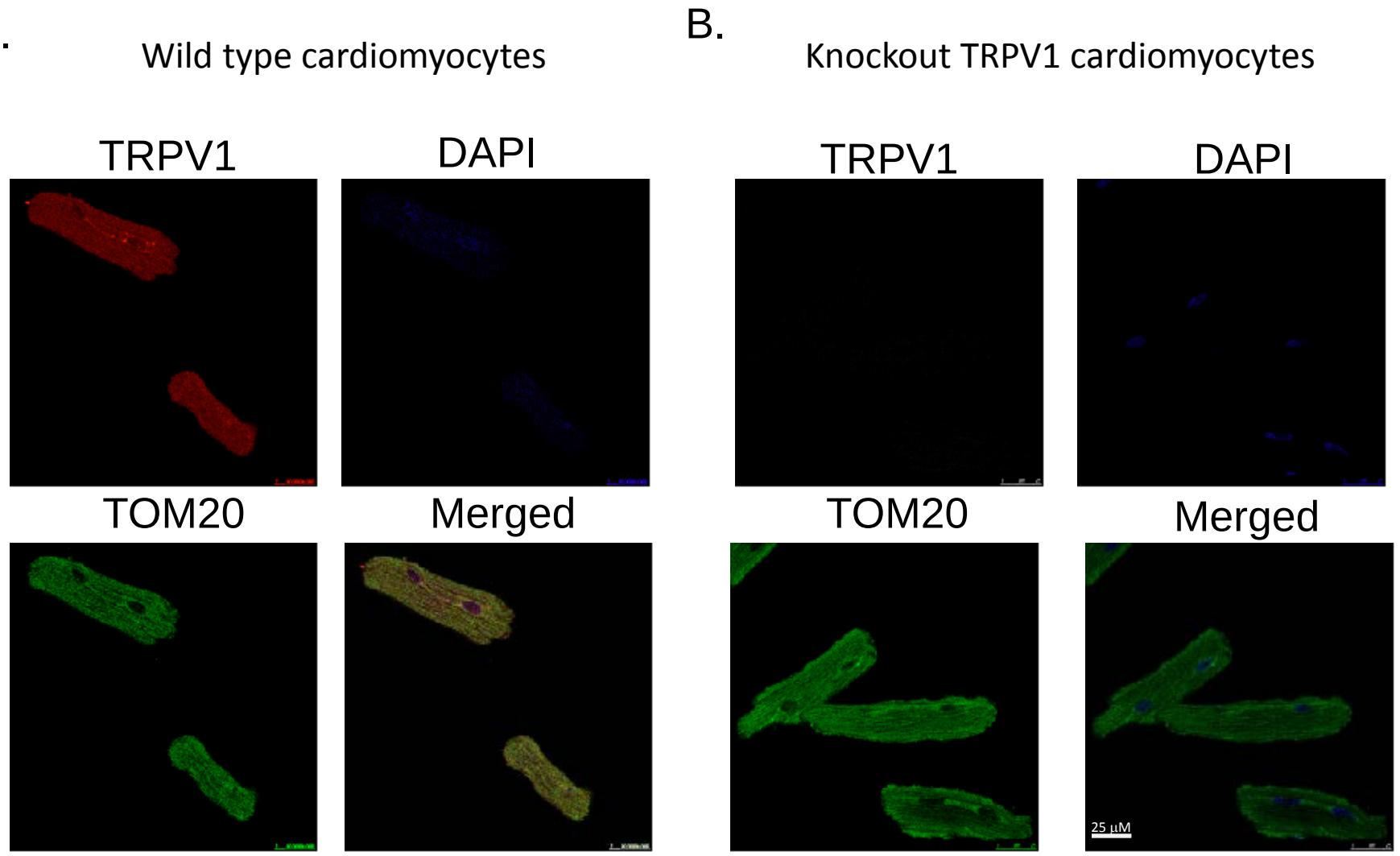

Figure S2. A. TRPV1 co-localizes with TOM20 in primary adult cardiomyocytes. **B.** Knockout verification of the antibody for selectivity using TRPV1 knockout rats.

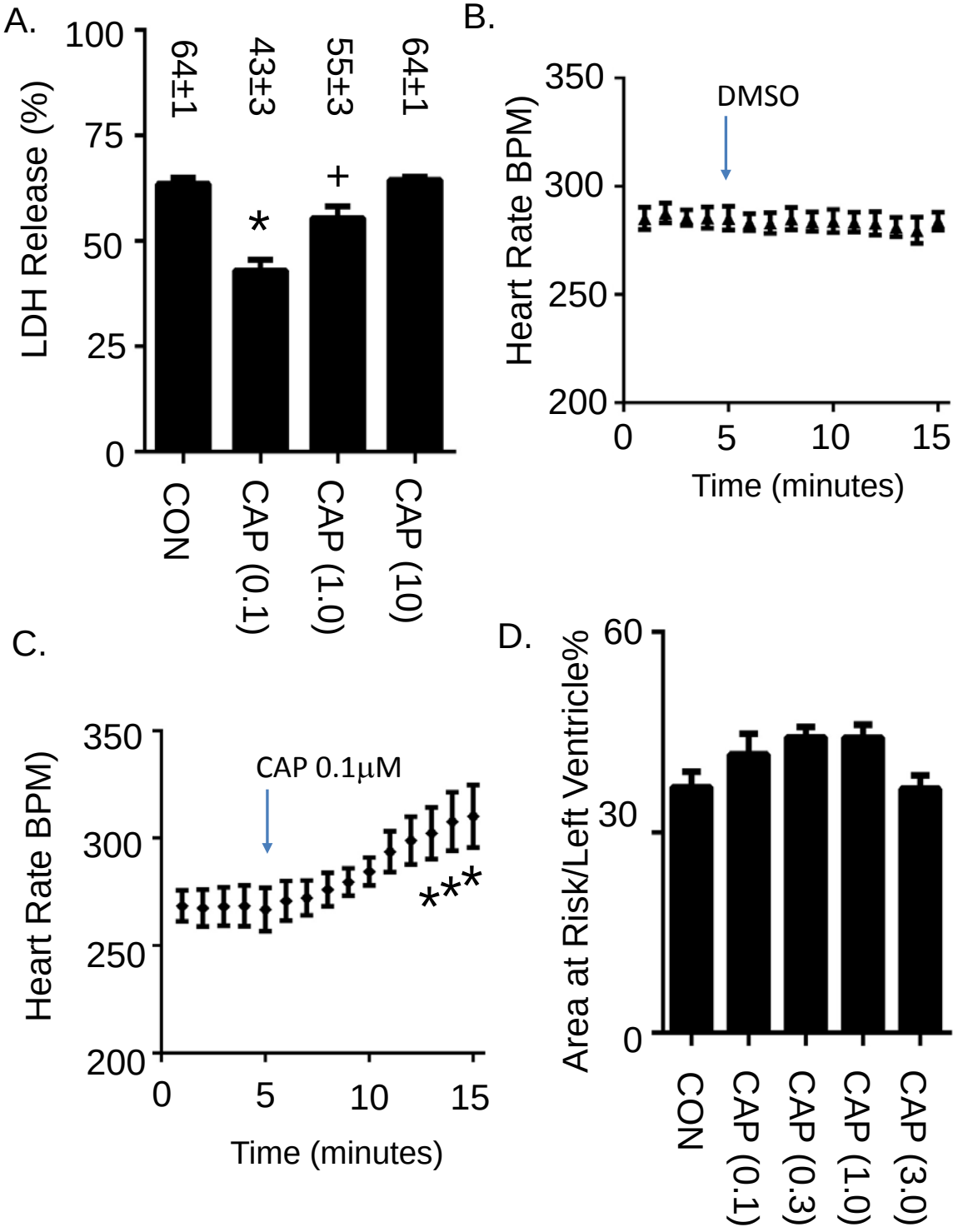

Figure S3. **A.** Cell ischemia-reoxygenation experiments with H9C2 cells. Capsaicin reduced LDH release only for a lower dose (0.1μM) when compared to higher doses of capsaicin (1 and 10μM), n=8/group, *P<0.01, +P<0.05 versus control. Percentage of LDH release noted as mean±SEM **B,C.** In isolated hearts after capsaicin administration, capsaicin (0.1μM) increases heart rate unlike vehicle (DMSO) $^{+}$P<0.05 versus prior to administration, n=4/group. **D.** Area at risk per left ventricle percentage for each group.

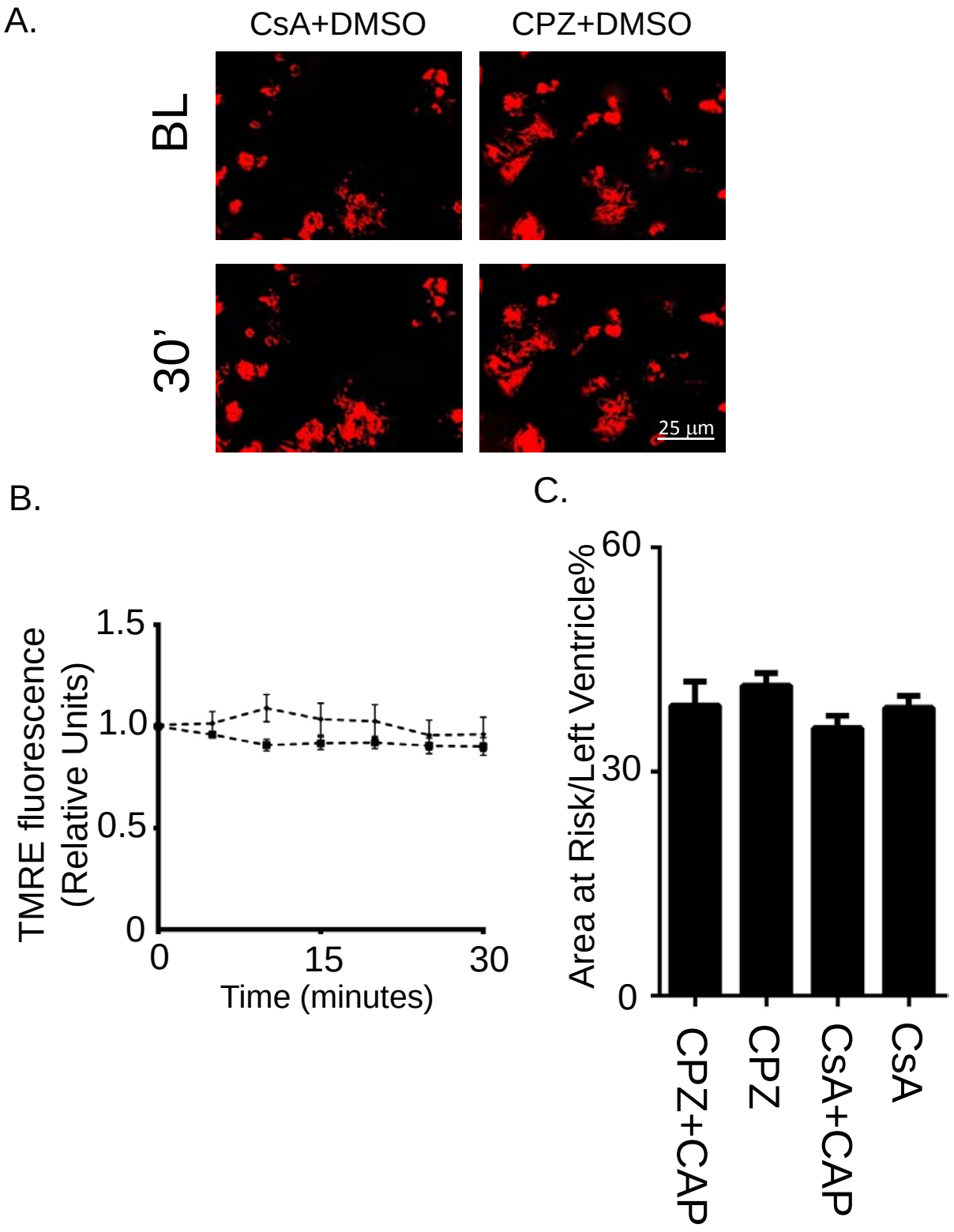

Figure S4. **A.** Representative images of PNCM at time 0 and after 30 minutes of treatment with DMSO alone, CPZ+DMSO and CsA+DMSO groups. **B.** Graph of data collected for groups including the DMSO alone (triangle), CPZ+DMSO (square) and CsA+DMSO (diamond) groups (n=3 biological replicates per group) **C.** Area at risk per left ventricle for each individual group.

A.

TRPV1 701-711	
RAITILDTEKS	Human (839 aa)
RAITILDTEKS	Cow (837 aa)
RAITILDTEKS	Rat (838 aa)
RAITILDTEKS	Mouse (839 aa)
RAITILDTEKS	Guinea Pig (839 aa)
RAITILDTEKG	Rabbit (842 aa)
RAITILDTEKS	Canine (840 aa)

B.

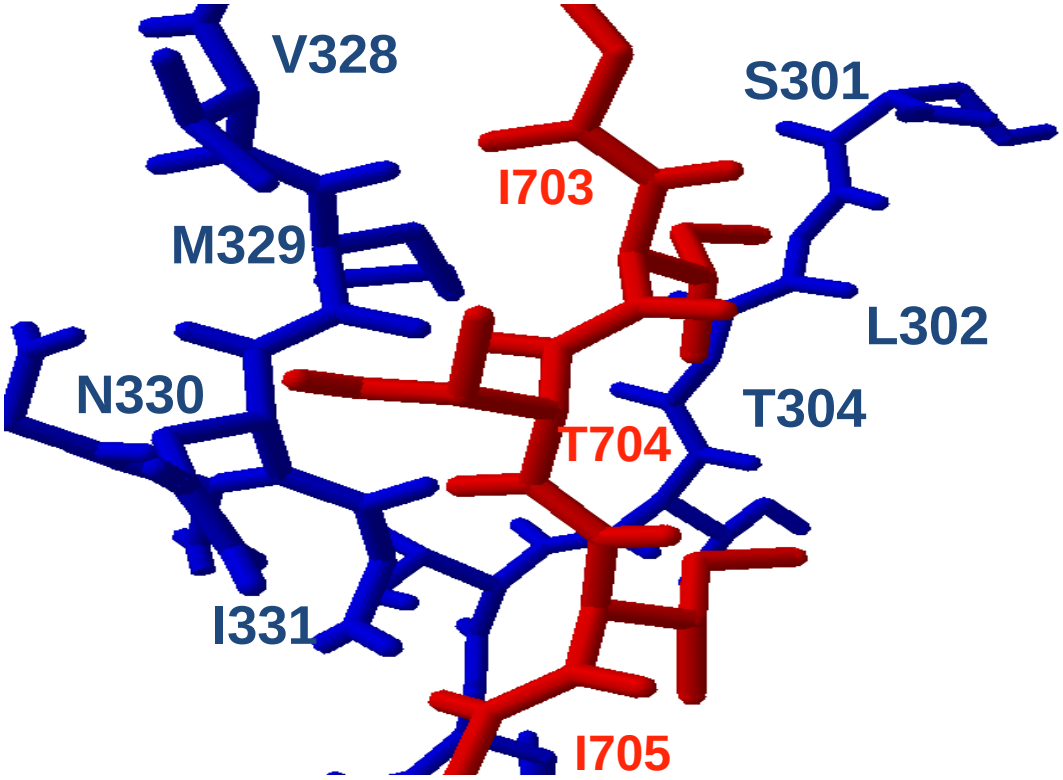

Figure S5. **A.** The TRPV1 region of where V1-cal was constructed is strongly conserved in mammals. **B.** Structure showing Thr704 of TRPV1 is near Asn330 site of calcineurin A.

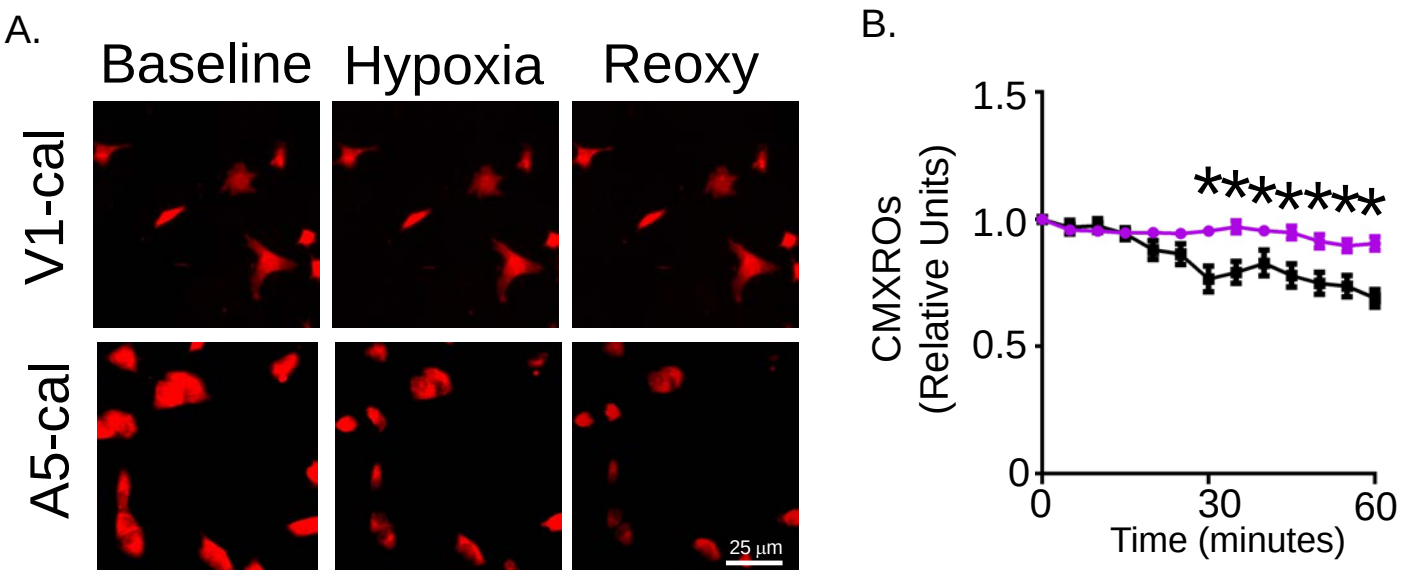

Figure S6. Single cell and isolated heart experiments A. Representative CMXRos images of PNCM at time 0 and after hypoxia-reoxygenation with A5-cal (black) or V1-cal (purple). **B.** Mitochondrial membrane potential assessed by CMXRos for PNCM treated with A5-cal (1mM) or V1-cal (1mM) (n=3 biological replicates/group, *P<0.01 versus A5-cal).

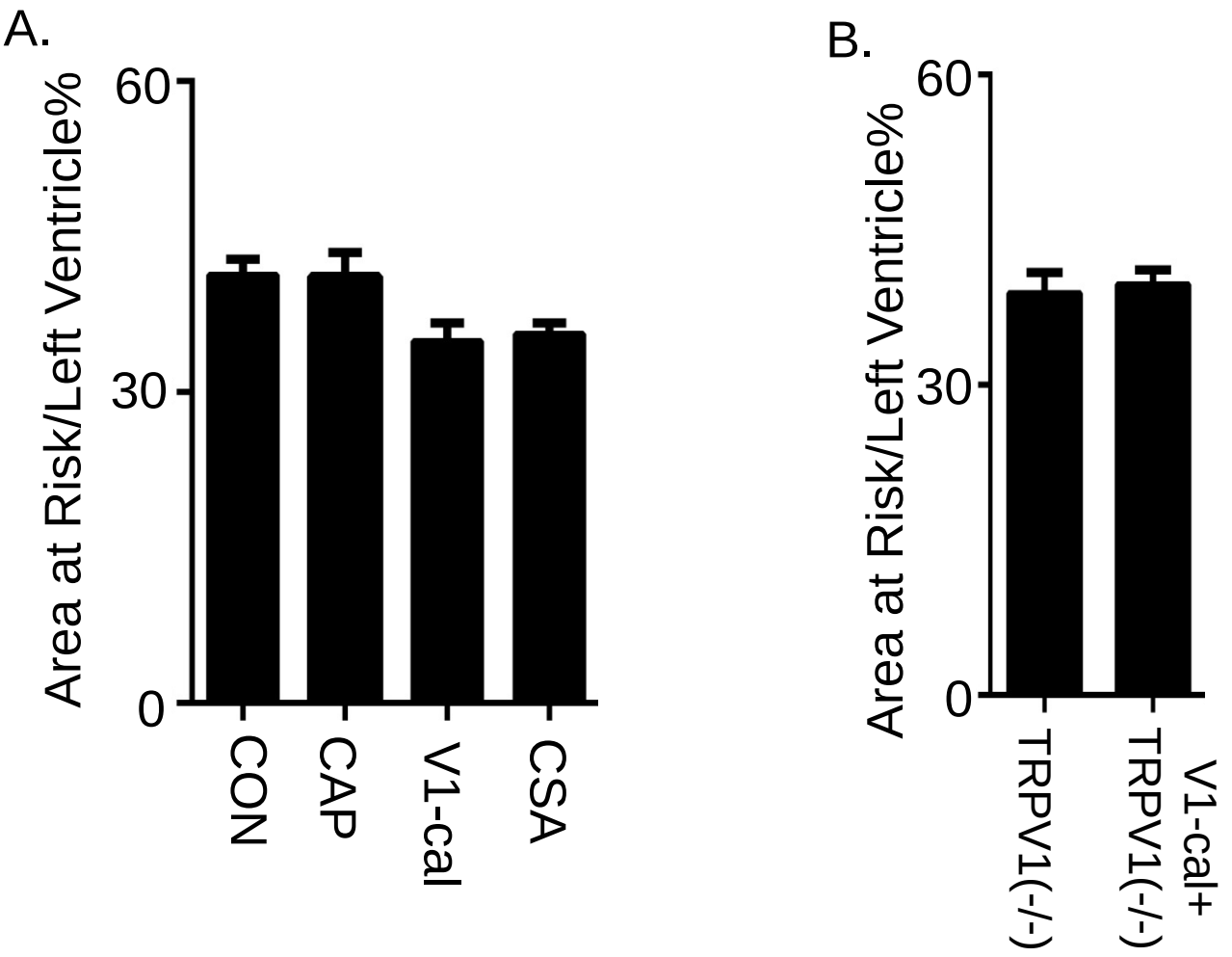

Figure S7. In vivo experiments. A. Area at risk per left ventricle for each group in Figure 9. **B.** Area at risk per left ventricle for each group in Figure 10.

References:

1. Tian W, Fu Y, Wang DH, Cohen DM. Regulation of trpv1 by a novel renally expressed rat trpv1 splice variant. *Am J Physiol Renal Physiol*. 2006;290:F117-126
2. Nakanishi M, Hata K, Nagayama T, Sakurai T, Nishisho T, Wakabayashi H, Hiraga T, Ebisu S, Yoneda T. Acid activation of trpv1 leads to an up-regulation of calcitonin gene-related peptide expression in dorsal root ganglion neurons via the camk-creb cascade: A potential mechanism of inflammatory pain. *Mol Biol Cell*. 2010;21:2568-2577
3. Yogalingam G, Hwang S, Ferreira JC, Mochly-Rosen D. Glyceraldehyde-3-phosphate dehydrogenase (gapdh) phosphorylation by protein kinase cdelta (pkcdelta) inhibits mitochondria elimination by lysosomal-like structures following ischemia and reoxygenation-induced injury. *J Biol Chem*. 2013;288:18947-18960
4. Pakkala M, Hekim C, Soininen P, Leinonen J, Koistinen H, Weisell J, Stenman UH, Vepsalainen J, Narvanen A. Activity and stability of human kallikrein-2-specific linear and cyclic peptide inhibitors. *Journal of peptide science : an official publication of the European Peptide Society*. 2007;13:348-353