Supplemental Material

Supplemental Methods

Experimental animals

Animals were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All protocols involving animals were approved by the Animal Studies Committee at the University of Illinois at Chicago, Lifespan, or the Veterans Administration San Diego Healthcare System. Experiments were performed on $Cav1^{-/-}$, $Cav3^{-/-}$ and ACE8/8 mice (all in C57/Bl6 background) that were derived and maintained as described previously.[9-11]

Surface Electrocardiogram Recording and Programmed Ventricular Stimulation

Surface electrocardiograms (ECG) were recorded and ventricular arrhythmia inducibility was determined in WT and $Cav1^{-/-}$ with and without 4 weeks of PP1 treatment (n=4-6 in each group) using described methods under general anesthesia with isofurane.[4] Surface electrocardiograms (ECG) were monitored and recorded with needle electrodes connected to a dual bioamplifier (PowerLab 26T, AD Instruments, Dunedin, New Zealand) as described previously.[1] Baseline ECG was acquired for 2 minutes; the data were stored and subsequently analyzed offline using the LabChart 7.1 (AD Instrument) software. Lead II recordings were chosen for analyses. The measurement is illustrated in Figures 1A. QT intervals were corrected for heart rate using the formula $QTc=QT/(\sqrt{RR}/100)$.[5]

Programmed ventricular stimulation was performed with a RV epicardial electrode connected to STG1008 stimulator (Multichannel systems, Reutlingen, Germany), where eight consecutive beats were paced at 60 ms basic cycle length, followed by single, double and triple

extrastimuli with incrementally deceasing cycle lengths between 20-55 ms, and inducible ventricular tachycardia was defined as > 3 consecutive ventricular beats.[6]

Ventricular conduction velocity measurement

LV conduction velocity was measured in anesthetized WT (n=6) and Cav1$^{-/-}$ (with and without 4 week PP1 treatment, n=6 in each group) mice using a flexible multielectrode array (Flex-MEA, 72 electrodes) system (Multichannel systems, Reutlingen, Germany) according to manufacturer's instructions. Mid-anterior LV epicardial electrical propagation was recorded under right-ventricular pacing (750 bpm); the color mapping of LV conduction propagation, as well as the calculation of LV conduction velocity, were carried out using Cardio 2D software (Multichannel systems, Reutlingen, Germany).

Western blotting

For Western blots, total protein lysates were prepared from the LV of 6 week-old WT control, ACE8/8 with and without 2 week treatment of mitochondria-targeted antioxidant (2-(2,2,6,6-Tetra-methylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)-triphenylphosphonium chloride (MitoTEMPO, see below), as well as from adult (2-4 month) Cav1$^{-/-}$ mice with and without 4 weeks treatment of cSrc inhibitor 1-(1,1-dimethylethyl)-1-(4-methylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP1, see below); in some cases, protein lysates were prepared from the LV cardiomyocytes isolated from ACE8/8 animals using described methods.[1] Total protein lysates were fractionated on 8-15% SDS-PAGE and transferred to PVDF membranes, incubated in 5% skim milk in PBS containing 0.1% Tween 20 (blocking buffer) for 1 h at room temperature, followed by overnight incubation at 4 °C with primary antibodies (rabbit monoclonal anti-cSrc, p-cSrc at Tyr^{416}, Cx43, C-terminal Src kinase [CSK] and Tyr^{14} p-Cav1 antibodies from Cell

Signaling, mouse monoclonal anti-Cav1 and Cav3 antibodies from BD Biosciences, rabbit monoclonal anti-eNOS, p-eNOS and nNOS antibodies from Santa Cruz). For a loading control, the membranes were blotted with primary antibodies against glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotech, Santa Cruz, California). After washing, the membranes were incubated for 1 h at room temperature with alkaline phosphatase-conjugated secondary antibody diluted in blocking buffer, and bound antibodies were detected using a chemiluminescent alkaline phosphate substrate. Protein band intensities were quantified by densitometry (Quantity One Basic, Bio-Rad Laboratory, Hercules, CA) and the band densities of each protein in individual samples were normalized to that of GAPDH (except for p-cSrc, which is normalized to total cSrc) in the same sample.

Immunoprecipitation of Cav1, Cav3 and cSrc

Immunoprecipitation (IP) of Cav1 was conducted using a magnetic IP kit from Thermo Scientific (Waltham, MA). In short, protein lysates from total LV or isolated LV myocytes (with 1000 μg total protein) from control and ACE8/8 mice were incubated with 10 μg of mouse anti-Cav1 monoclonal antibodies overnight at 4°C. The immune complex was bound to protein A/G magnetic beads and collected with a magnetic stand. Proteins co-immunoprecipitated with Cav1 were eluted and subjected to gel electrophoresis and Western blotting using the antibodies described above where appropriate. The amount of proteins co-immunoprecipitated with Cav1 was normalized to total Cav1 co-immunoprecipitated in each sample. Similar methods were used to analyze the proteins that co-immunopricipitated with Cav3 and cSrc using antibodies against Cav3 (mouse monoclonal, BD Biosciences, San Jose, CA) or cSrc (rabbit monoclonal, Cell Signaling Technology, Danvers, MA).

Generation of Cav1 Cysteine-to-Serine Mutants and Transfection

A full-length mouse Cav1 cDNA clone in pCMV-SPORT6 vector was acquired from Thermo Scientific (MGC mouse Cav1 cDNA, clone ID 4484857). The cysteine-to-serine Cav1 mutants (C133S, C143S and C156S) were generated from this WT mouse Cav1 clone using the QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions. The primer sequences used for generation of these Cav1 mutant clones are:

C133S: sense 5'-gggcggttgtaccgagcatcaagagcttc-3'

anti-sense 5'-gaagctcttgatgctcggtacaaccgccc-3'

C143S: sense 5'-cctgattgagattcagagcatcagccgcgtcta-3'

anti-sense 5'-tagacgcggctgatgctctgaatctcaatcagg-3'

C156S: sense 5'-tctacgtccataccttcagcgatccactctttgaa-3'

anti-sense 5'-ttcaaagagtggatcgctgaaggtatggacgtaga-3'

Transfection of HEK cells with designated plasmids was conducted using Lipofectamine 2000 according to manufacturer's protocol.

Detection of Cav1 S-Nitrosation

We detected S-nitrosated Cav1 in cells (isolated ventricular cardiomyocytes from control and ACE8/8 mice or HEK cells transfected with WT Cav1 or Cav1 mutants [C156S, C143S or C133S], with or without NO donor, SNAP [20 μM, 10 min], treatment) using described methods.[2,3] In brief, cells were lysed with HENS buffer (25 mM HEPES, pH 7.7, 0.1 mM EDTA, 0.01 mM neocuproine and 1% SDS) and centrifuged at 20,000 g for 15 min. The total cell protein was incubated in 20 mM methylmethanthiosulphate (MMTS) for 20 min at 50°C and vortexed for 5 s every 2 min. Cellular protein was precipitated with acetone. After removing acetone, protein pellet was resuspended in HENS buffer. *N*-[6-(biotinamido)hexyl]-3′-(2′-

pyridyldithio)propionamide (biotin–HPDP, 400μM) and sodium ascorbate (1mM) was added for 1 h at 25 °C in the dark. Cav1 was then immunoprecipitated from each sample using monoclonal Cav1 antibody (BD Biosciences, San Jose, CA), where S-nitrosated Cav1 was detected by HRP-conjugated streptavidin following gel electrophoresis and Western blotting. The amount of S-nitrosated Cav1was quantified and normalized to total Cav1 in each sample.

Measurement of nitric oxide (NO) production by chemiluminescence

Isolated LV cardiomyocytes from WT and ACE8/8 mice were plated in 6-well plates. After adherence, myocytes were washed twice with HBSS and incubated with serum free DMEM or HBSS at 37°C for one hour. After incubation, medium was collected and centrifuged shortly to remove floating cells. NO concentration in the culture media was assessed by measuring NO_2^- accumulation using a Sievers 280i Nitric Oxide Analyzer (Sievers Instruments, Boulder, CO). NO production was assessed from accumulated NO_2^- level in the media and reported as nmol NO per mg protein. A standard curve was generated using authentic sodium nitrite ($NaNO_2$) for calibration.

Transcript analyses

Total RNA from the LV of individual animals was isolated and treated with DNase using described methods.[7,8] Using equal amounts of RNA, transcript analyses of Cx43 and GAPDH were carried out using SYBR green RT-PCR in a two-step process.[7,8] Data were analyzed using the threshold cycle (C_T) relative quantification method and normalized to GAPDH. The normalized transcript expression values were then expressed relative to the mean of the WT LV samples.

Immunofluorescent staining and confocal imaging

Frozen cardiac samples from WT, Cav1$^{-/-}$ and PP1-treated Cav1$^{-/-}$ animals (n=4 in each group) were sectioned (short axis, 10μm), fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 30 min, permeabilized with 0.1% Triton X-100 for 30 min, and blocked with 1% bovine serum albumin for 1 h. Fixed cardiac sections were then incubated with the primary antibody, rabbit anti-Cx43 (1:200, Cell Signaling Technology, Danvers, MA) and mouse anti-N-cadherin (1:200, Life Technologies, Grand Island, NY) at 4℃ overnight. After washing with PBS (3 X 10 min), the sections were incubated with Alexa Fluor 594-labeled anti-rabbit and Alexa Fluor 488-labeled anti-mouse secondary antibodies (1:200, room temperature 1 h). The sections were then washed with PBS (3 X 10 min) and mounted with ProLong Gold (Life Technologies, Grand Island, NY). Fluorescent imaging was acquired using a Nikon C1si confocal (Nikon Inc. Mellville NY.) microscope. Serial optical sections were performed with EZ-C1 computer software (Nikon Inc. Mellville, NY). Deconvolution and projections were performed in Elements (Nikon Inc. Mellville, NY) computer software. Image analysis was performed using iVision image analysis software (BioVisions Technologies, Exton, PA.) Positive staining was defined through intensity thresholding. Area measurements of total Cx43 and area percentage of Cx43 colocalized with N-cadherin were determined.

Statistical analyses

All averaged WB densitometry, transcript analyses and LV conduction velocity measurements were presented in dot plots with means ± SEM. The inducibility of VT was presented as percentage of all tested animals in the same group. The statistical significance of differences between experimental groups was evaluated by the exact version of Mann-Whitney *U* test or Fisher's exact test with Holm test to correct for multiple comparisons; *P* values <0.05 are considered statistically significant.

Supplemental References

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Supplemental Figure Legends

Supplemental Figure 1. No evidence of sodium current change or increased fibrosis in Cav1$^{-/-}$ LV

(A) Current-voltage curves of Na^+ current (I_{Na}) densities in WT and Cav1$^{-/-}$ LV myocytes (n=20 in each group). There was no significant differences in I_{Na} densities between WT and Cav1$^{-/-}$ LV myocytes across the ranges of test potentials (-80 to 60 mV). (B) The steady state inactivation curves of I_{Na} of WT and Cav1$^{-/-}$ LV myocytes were indistinguishable. Mason trichrome staining of the LV cross-sections from WT (C) and Cav1$^{-/-}$ (D) mice did not reveal evidence of increased fibrosis in Cav1$^{-/-}$ LV.

Supplemental Figure 2. The phosphorylation state of Cx43 is not different in WT, Cav1$^{-/-}$ and PP1-treated Cav1$^{-/-}$ mouse LV

Representative Western blots of the phosphorylated (P1 and P2) and non-phosphorylated (P0) Cx43 in the LV samples from WT, Cav1$^{-/-}$ and PP1-treated Cav1$^{-/-}$ mice. The ratios of P0, P1 and P3 relative to total Cx43 levels were not significantly different among these three groups of samples, suggesting that the phosphorylation state of Cx43 is not different in WT, Cav1$^{-/-}$ and PP1-treated Cav1$^{-/-}$ mouse LV

Supplemental Figure 3. The proportion of Cx43 colocalized with N-cadherin was not different in WT, Cav1$^{-/-}$ and PP1-treated Cav1$^{-/-}$ mouse LV

(A)Representative immunofluorescent staining of LV sections from WT, Cav1$^{-/-}$ and PP1-treated Cav1$^{-/-}$ mice (n=5 in each group). Red: Cx43, Green: N-cadherin, Blue: DAPI. Scale bar: 10 μm

(B) Quantification of the proportion of Cx43 colocalized with N-cadherin did not reveal significant differences in WT, Cav1$^{-/-}$ and PP1-treated Cav1$^{-/-}$ LV.

Supplemental Figure 4. The transcript expression of Cx43 is not different in WT, Cav1$^{-/-}$ and PP1-treated Cav1$^{-/-}$ mouse LV

Quantitative RT-PCR revealed that the transcript expression levels of Cx43 in the LV from WT, Cav1$^{-/-}$ and Cav1$^{-/-}$+PP1 mice were not significantly different.

Supplemental Figure 5. C-terminal Src kinase (CSK) does not co-immunoprecipitate with cSrc in mouse left ventricle (LV)

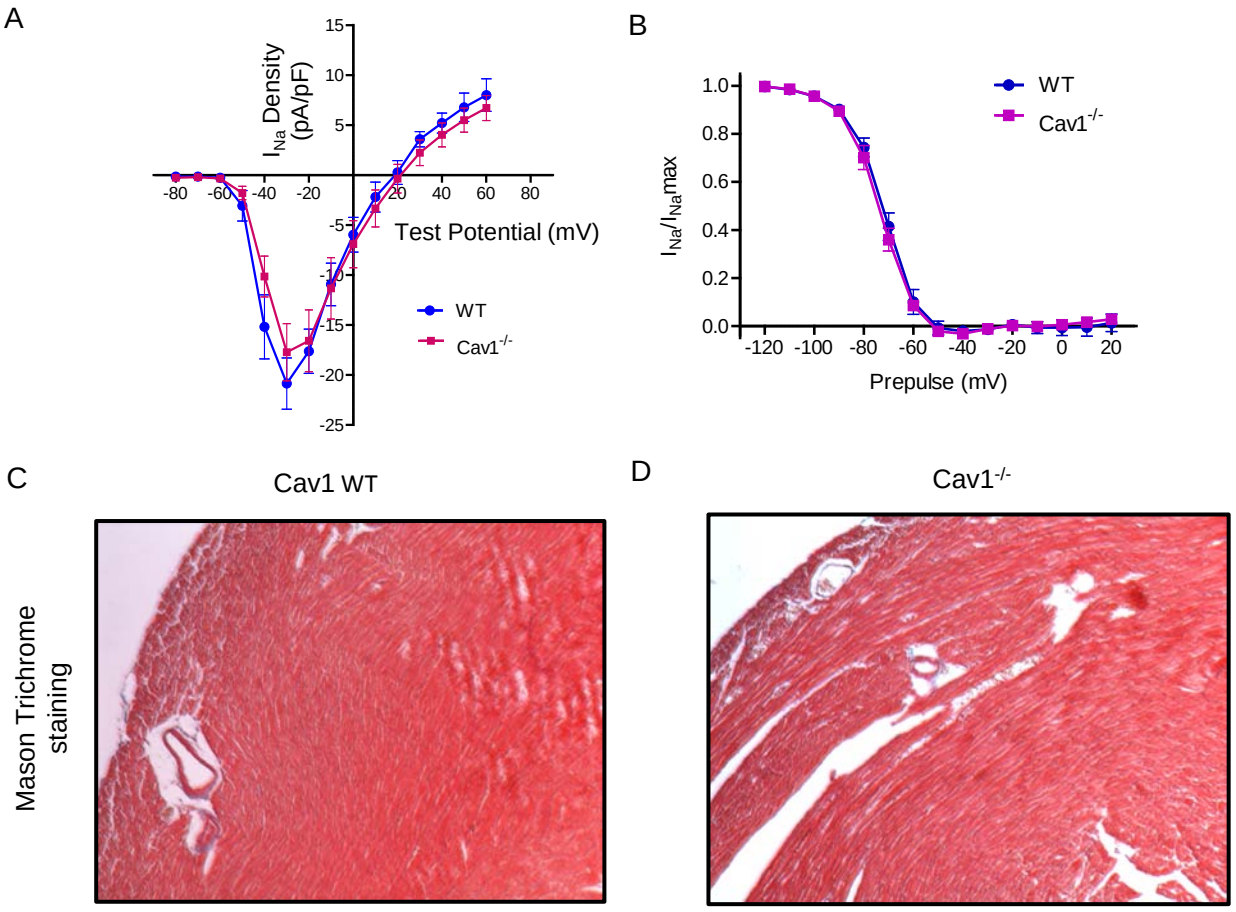
Immunopercipitation with cSrc antibody using the protein lysates from WT and ACE8/8 LV did not show evidence of interaction between CSK and cSrc.

Supplemental Figure 6. Nitric oxide (NO) production does not differ in WT and ACE8/8 ventricular cardiomyocytes

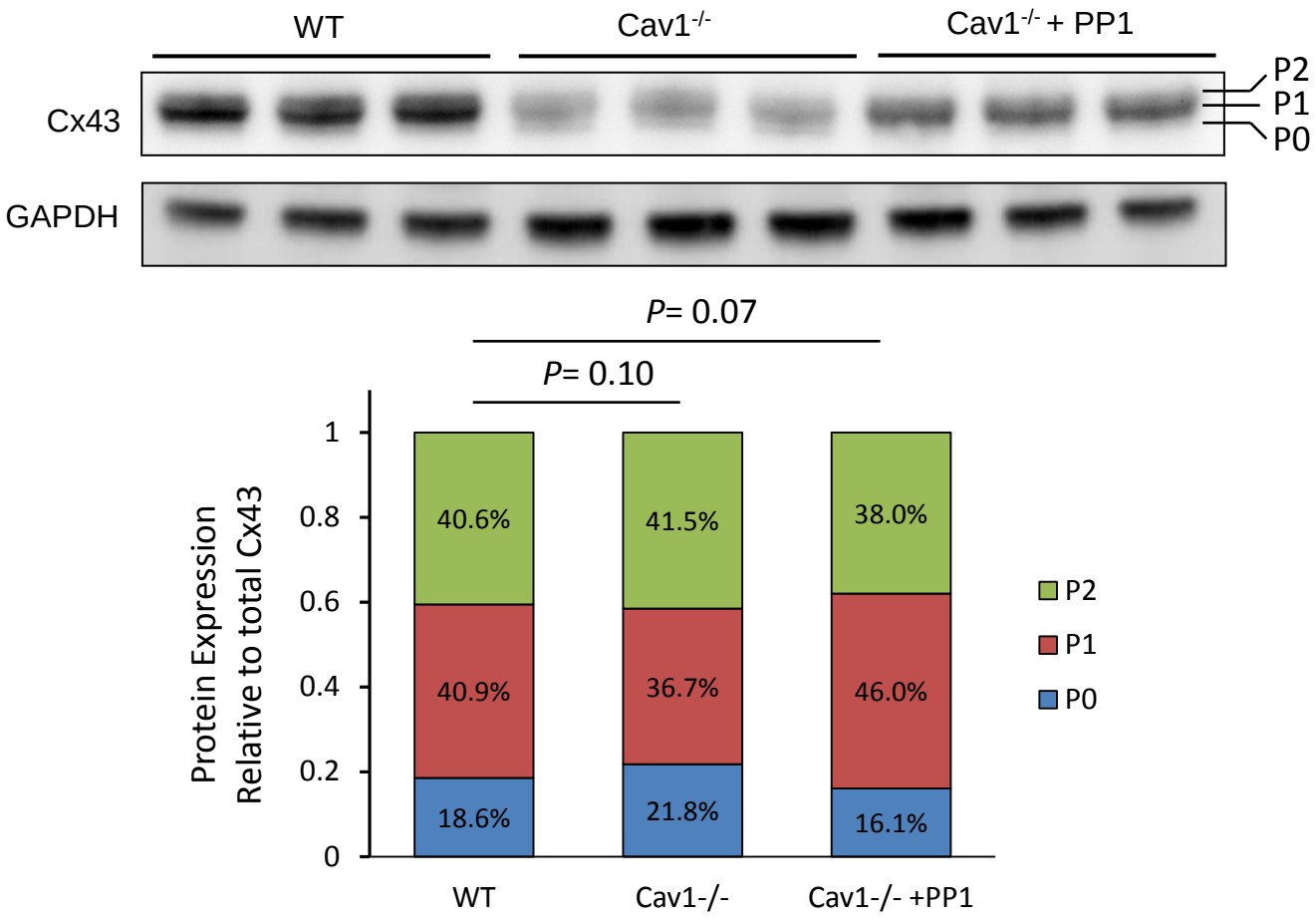
Chemiluminescence NO measurements did not reveal significant differences in NO production from WT and ACE8/8 ventricular cardiomyocytes.

Supplemental Table. Results of programmed electrical stimulation in WT, Cav1$^{-/-}$ and Cav1$^{-/-}$+PP1 mice

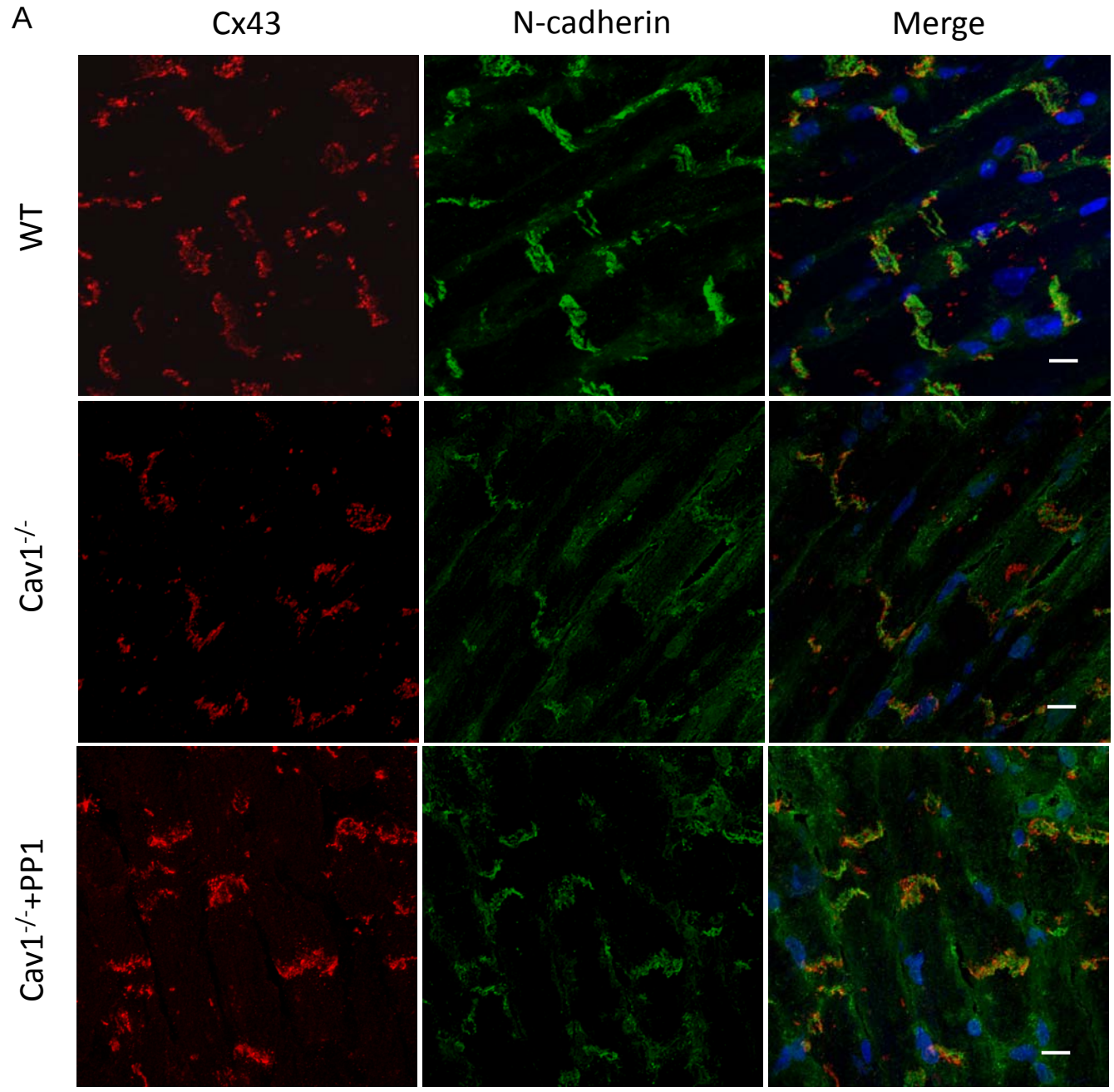
		WT	Cav1$^{-/-}$	Cav1$^{-/-}$+PP1	P value
Double extra-stimuli	n	8	10	8	
	VT	0 (0%)	7 (70%)	0 (0%)	0.007
Triple extra-stimuli	n	14	14	14	
	VT	0 (0%)	11 (79%)	1 (7%)	<0.0001

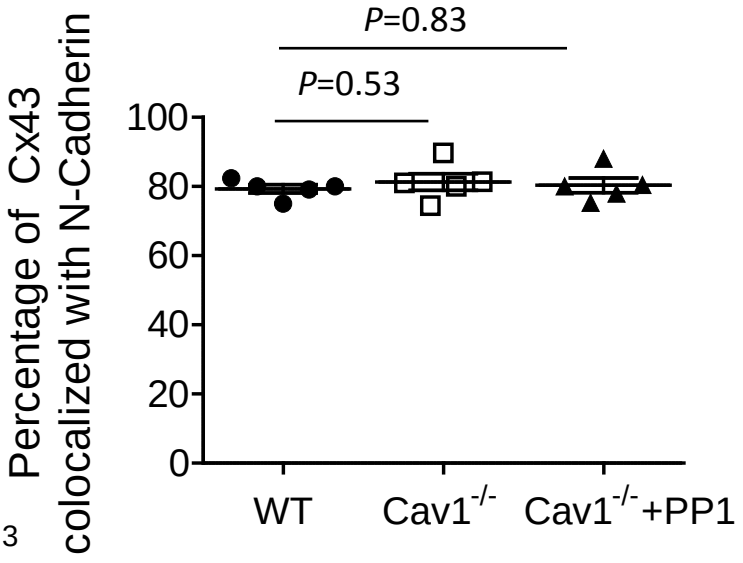


Supplemental Figure 1

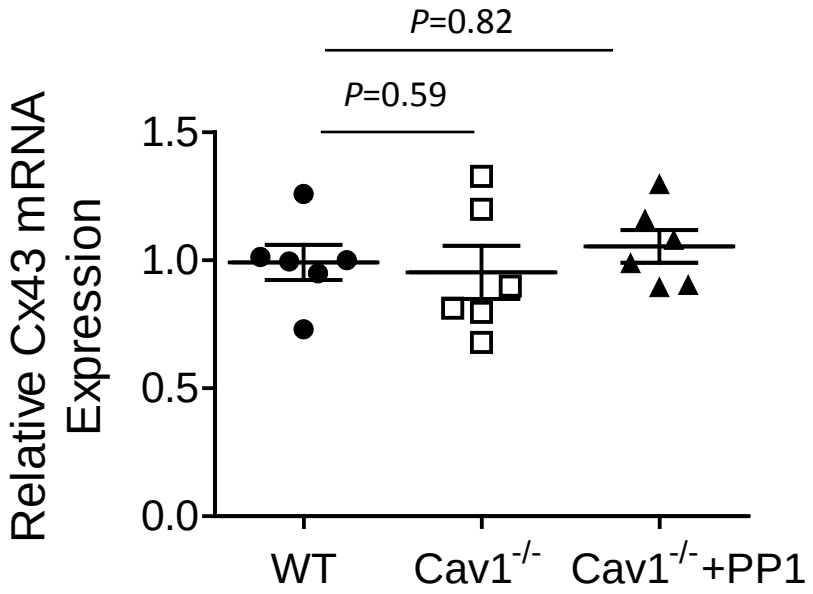


Supplemental Figure 2





Supplemental Figure 3



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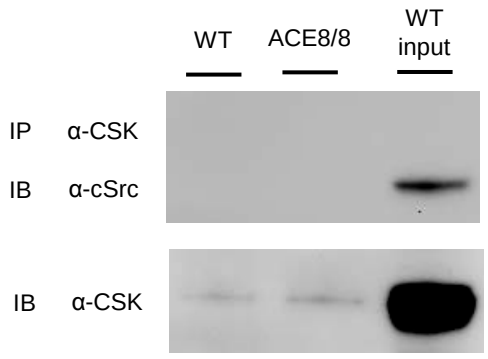
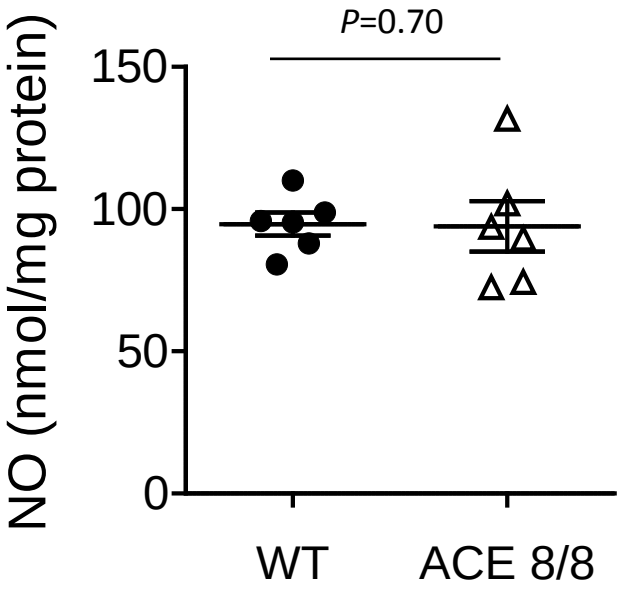


Supplemental Figure 5



Supplemental Figure 6