Caveolin-1 Modulates Cardiac Gap Junction Homeostasis and Arrhythmogenecity by Regulating cSrc Tyrosine Kinase

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Background—Genome-wide association studies have revealed significant association of caveolin-1 (Cav1) gene variants with increased risk of cardiac arrhythmias. Nevertheless, the mechanism for this linkage is unclear.

Methods and Results—Using adult Cav1−/− mice, we revealed a marked reduction in the left ventricular conduction velocity in the absence of myocardial Cav1, which is accompanied with increased inducibility of ventricular arrhythmias. Further studies demonstrated that loss of Cav1 leads to the activation of cSrc tyrosine kinase, resulting in the downregulation of connexin 43 and subsequent electric abnormalities. Pharmacological inhibition of cSrc mitigates connexin 43 downregulation, slowed conduction, and arrhythmia inducibility in Cav1−/− animals. Using a transgenic mouse model with cardiac-specific overexpression of angiotensin-converting enzyme (ACE8/8), we demonstrated that, on enhanced cardiac renin–angiotensin system activity, Cav1 dissociated from cSrc because of increased Cav1 S-nitrosation at Cys356, leading to cSrc activation, connexin 43 reduction, impaired gap junction function, and subsequent increase in the propensity for ventricular arrhythmias and sudden cardiac death. Renin–angiotensin system–induced Cav1 S-nitrosation was associated with increased Cav1–endothelial nitric oxide synthase binding in response to increased mitochondrial reactive oxidative species generation.

Conclusions—The present studies reveal the critical role of Cav1 in modulating cSrc activation, gap junction remodeling, and ventricular arrhythmias. These data provide a mechanistic explanation for the observed genetic link between Cav1 and cardiac arrhythmias in humans and suggest that targeted regulation of Cav1 may reduce arrhythmic risk in cardiac diseases associated with renin–angiotensin system activation. (Circ Arrhythm Electrophysiol. 2014;7:701-710.)

Key Words: arrhythmias, cardiac • caveolin 1 • connexin 43 • renin–angiotensin system

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Methods

Animals were handled in accordance with the National Institutes of Health 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.

In vivo electrophysiological studies, including ECG recordings, programmed stimulation, and ventricular conduction velocity, were performed on Cav1−/− and angiotensin-converting enzyme (ACE8/8) mice (all in C57/Bl6 background) that were derived and maintained as described previously.9–11 Left ventricular (LV) tissue and cardiomyocytes isolated from Cav1−/−, Cav3−/−, and ACE8/8 mice were used for Western blotting, immunoprecipitation, S-nitrosation assay, NO measurement, and transcript analyses.
All measurements were presented in dot plots with mean±SEM. The inducibility of ventricular tachycardia 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 the Mann–Whitney U test or Fisher exact test, followed by Holm test to correct for multiple comparisons; P values <0.05 are considered statistically significant. Detailed methods are available in the Data Supplement.

**Results**

**Loss of Cav1 Results in Slowed Cardiac Conduction and Increased Risk of Ventricular Arrhythmia**

To determine the potential impact of genetic deletion of Cav1 on cardiac electric functioning, adult (2–4 months) wild-type (WT) and Cav1−/− mice were first subjected to surface ECG recordings (Figure 1A). Cav1−/− mice were viable and fertile without evidence of cardiac structural abnormality ≤5 months of age.10 The ECG recordings revealed that the morphologies of the P, J, and T waves, as well as the durations of the PR, QRS, and corrected QT intervals (Figure 1B) measured in WT and Cav1−/− animals were indistinguishable, although the R wave amplitudes were trending lower in Cav1−/− compared with WT mice (Figure 1A and 1B). Using a 72-electrode Flex-Multi-electrode array, the LV epicardial conduction velocity was measured in WT and Cav1−/− mice. As shown in Figure 1C, the LV conduction velocity in Cav1−/− (n=6; 0.35±0.03 mm/ms; median, 0.32 mm/ms) was significantly (P=0.004) lower than that in WT (n=6; 0.50±0.09 mm/ms; median 0.44 mm/ms) mice. To test whether the reduced LV conduction velocity observed in Cav1−/− mice is associated with increased arrhythmia risk, epicardial programmed electric stimulation was conducted in WT and Cav1−/− mice. These experiments revealed that none of the WT mice (8 with double and 14 with triple extrastimuli) were inducible for ventricular tachycardia, whereas 70% (7 of 10; P<0.001 by Fisher exact test) and 79% (11 of 14; P<0.0001) of the Cav1−/− mice were inducible for ventricular tachycardia using double and triple extrastimuli, respectively (Figure 1D; Table in the Data Supplement). Single extrastimulus failed to induce arrhythmias in any of the animals studied. Taken together, initial electrophysiological studies demonstrated that loss of Cav1 resulted in slowed LV conduction velocity and increased ventricular arrhythmia inducibility.

**Electric Abnormalities Observed in Cav1−/− Mice Result From LV Connexin 43 Downregulation by Activated cSrc tyrosine Kinase**

Slow myocardial conduction velocity can result from reduced Na+ current (INa) or from increased cell–cell conduction resistance caused by increased fibrosis or decreased gap junction function.12 Whole-cell voltage clamp experiments in LV cardiomyocytes, as well as Mason-trichrome staining of the LV cross...

**Figure 1.** Knockout of caveolin-1 (Cav1) leads to reduced left ventricular (LV) conduction velocity and increased inducibility of ventricular arrhythmias, both of which can be prevented by cSrc inhibition. **A,** Representative ECG (lead II) waveforms from anesthetized adult (2–4 months) wild-type (WT) and Cav1−/− mice are illustrated; **B** means±SEM PR, QRS, and corrected QT intervals (QTc) measured in WT and Cav1−/− animals were indistinguishable, although the R wave amplitudes were trending lower in Cav1−/− compared with WT mice (Figure 1A and 1B). Using a 72-electrode Flex-Multi-electrode array, the LV epicardial conduction velocity was measured in WT and Cav1−/− mice. As shown in Figure 1C, the LV conduction velocity in Cav1−/− (n=6; 0.35±0.03 mm/ms; median, 0.32 mm/ms) was significantly (P=0.004) lower than that in WT (n=6; 0.50±0.09 mm/ms; median 0.44 mm/ms) mice. To test whether the reduced LV conduction velocity observed in Cav1−/− mice is associated with increased arrhythmia risk, epicardial programmed electric stimulation was conducted in WT and Cav1−/− mice. These experiments revealed that none of the WT mice (8 with double and 14 with triple extrastimuli) were inducible for ventricular tachycardia, whereas 70% (7 of 10; P<0.001 by Fisher exact test) and 79% (11 of 14; P<0.0001) of the Cav1−/− mice were inducible for ventricular tachycardia using double and triple extrastimuli, respectively (Figure 1D; Table in the Data Supplement). Single extrastimulus failed to induce arrhythmias in any of the animals studied. Taken together, initial electrophysiological studies demonstrated that loss of Cav1 resulted in slowed LV conduction velocity and increased ventricular arrhythmia inducibility.

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sections, were conducted in WT and Cav1−/− mice to determine whether changes in \(I_{\text{Na}}\) currents or the presence of cardiac fibrosis may contribute to the conduction abnormality and increased arrhythmia inducibility observed in Cav1−/− mice. As shown in Figure 1A and IB in the Data Supplement, the densities of \(I_{\text{Na}}\), as well as the steady-state inactivation properties of \(I_{\text{Na}}\), were similar in WT and Cav1−/− LV myocytes. Also similar to WT LV, there was no significant fibrosis detected in Cav1−/− LV (Figure IC and ID in the Data Supplement). In contrast, Western blot analyses revealed a 42% reduction of the connexin 43 (Cx43) expression in Cav1−/− compared with WT LV (Figure 2A and 2B). Quantification of Cx43 of different phosphorylation states, P0, P1, and P3, relative to total Cx43 levels, did not reveal significant difference between WT and Cav1−/− LV samples (Figure II in the Data Supplement), suggesting that genetic deletion of Cav1 does not affect the phosphorylation state of cardiac Cx43. In addition, immunofluorescent staining of Cx43 and N-cadherin, a protein marker of the intercalated discs (Figure IIIA and IIIB in the Data Supplement), revealed that the percentage of Cx43 colocalized with N-cadherin was similar in WT and Cav1−/− LV, suggesting that the proportion of cellular Cx43 incorporated into gap junctions was not affected in the absence of Cav1. Isolated myocytes from WT and Cav1−/− LV were used in additional Western blots to confirm that Cx43 expression levels were indeed markedly reduced in Cav1−/− compared with WT LV cardiomyocytes (52% reduction; \(P=0.004\); Figure 2C). Taken together, these data suggest that the conduction abnormality and increased inducibility for ventricular arrhythmias observed in Cav1−/− mice can be attributed largely to Cx43 downregulation.

It is known that Cav1 negatively regulates a redox-sensitive tyrosine kinase cSrc, the activation of which has been shown to cause the downregulation of cardiac Cx43.13 We hypothesized that the observed Cx43 downregulation, slow conduction, and increased arrhythmic inducibility in Cav1−/− mice resulted from loss of Cav1 inhibition of cSrc. To test this, we first examined the expression levels of phosphorylated cSrc at Tyr416 (p-cSrc, the active form of cSrc) in the ventricular myocardium and isolated LV cardiomyocytes from WT and Cav1−/− mice. As shown in Figure 2A and 2C, the protein expression level of p-cSrc was significantly upregulated in Cav1−/− LV (by 2.8-fold; \(P=0.002\)) and isolated LV cardiomyocytes (by 2.6-fold; \(P=0.002\)) compared with WT. In addition, pharmacological inhibition of cSrc activity with 4 weeks of the cSrc inhibitor PP1 (1.5 mg/kg per dose; 3× per week for 4 weeks IP) in Cav1−/− mice normalized LV p-cSrc and Cx43 expression to levels similar to that in WT (Figure 2A and 2B).

Consistent with the notion that cSrc regulates Cx43 post-translationally,13 quantitative reverse transcription polymerase chain reaction did not reveal a significant difference in the LV Cx43 mRNA expression levels in WT, Cav1−/−, and PP1-treated Cav1−/− mice (Figure IV in the Data Supplement; see Discussion section of this article).

In line with the reversal of Cx43 downregulation with cSrc inhibition, the slow LV conduction and increased ventricular arrhythmia inducibility observed in Cav1−/− mice could be mitigated by 4-week treatment with cSrc inhibitor PP1 (mean LV conduction velocity, 0.43±0.01 mm/ms; median, 0.45 mm/ms; 0% inducible for ventricular tachycardia with double extrastimuli; \(n=8\); Figure 1C and 1D; Table in the Data Supplement). In contrast to Cav1−/− mice, the LV p-cSrc and Cx43 expression in Cav3−/− LV were similar to that in WT (Figure 2D), suggesting no obvious role of Cav3 in cSrc/Cx43 regulation. Taken together, these results suggest that Cav1, but not Cav3, plays a critical role in maintaining cardiac Cx43 homeostasis through regulating cSrc activity. In the absence of Cav1, cSrc becomes activated, leading to Cx43 downregulation, subsequent conduction abnormality, and increased inducibility for arrhythmias.

**Reduced Binding Between Cav1 and cSrc Results in cSrc Activation and Subsequent Cx43 Downregulation on Enhanced Cardiac Renin–Angiotensin System Signaling**

The electrophysiological abnormalities linked to Cx43 dysregulation observed in Cav1−/− mice were reminiscent of the phenotype of the mouse models with increased cardiac renin–angiotensin system (RAS) activity.9,14 These animals have a

![Figure 2. Loss of caveolin-1 (Cav1) results in cardiac cSrc activation and connexin 43 (Cx43) downregulation, which can be reversed by cSrc inhibition. A, Representative Western blots of the left ventricular (LV) protein lysates from wild-type (WT), Cav1−/− mice, and Cav1−/− mice treated with 4 weeks of cSrc inhibitor PP1 (1.5 mg/kg per dose immunoprecipitation, 3× per week). B, cSrc phosphorylation was significantly (\(P=0.002\)) increased in Cav1−/− (\(n=6\)) compared with WT (\(n=6\), LV), whereas Cx43 was markedly reduced in Cav1−/− LV. Four weeks of PP1 treatments prevented cSrc phosphorylation/activation and Cx43 downregulation in Cav1−/− LV (\(n=6\)). C, Representative Western blots of the isolated LV cardiomyocytes from WT (\(n=4\)) and Cav1−/− (\(n=4\)) mice confirmed markedly reduced Cx43 (by 52%; \(P=0.004\)) and increased p-cSrc (by 2.6-fold; \(P=0.002\)) in cardiomyocytes with genetic deletion of Cav1. D, The p-cSrc and Cx43 protein levels were not different in WT and Cav3−/− LV.
high incidence of conduction block, ventricular arrhythmias, and sudden death resulting from reduced cardiac Cx43 and impaired gap junction function. Using a gene-targeted mouse model of cardiac-specific ACE overexpression (ACE8/8),9,15 we have previously demonstrated that enhanced cardiac RAS signaling can lead to cSrc activation, Cx43 degradation, reduce myocyte coupling, increased inducibility of ventricular arrhythmias, and sudden cardiac death, all of which can be reversed by pharmacological inhibition of cSrc.18-21 Given the similarity in the electrophysiological phenotypes of Cav1−/− and ACE8/8 mice, we hypothesized that Cav1 was likely involved in RAS-induced cardiac cSrc activation and Cx43 reduction.

Increased cardiac RAS activity in ACE8/8 mice was accompanied by a 3.4-fold increase (P<0.001) in cSrc activation/phosphorylation and 77% reduction in Cx43 (P=0.002) compared with WT LV (Figure 3A and 3B). The intrinsic kinase activity of cSrc is controlled by autophosphorylation of Tyr416 located within the kinase domain that results in cSrc activation and by phosphorylation at Tyr527 that results in cSrc inactivation.18 Phosphorylation of Tyr527 is mediated by the C-terminal Src kinase (CSK),17,19 whereas cSrc Tyr14 phosphorylation can be suppressed by the direct binding with the scaffolding proteins Cav1 and Cav3.20 Cav1 is also necessary for CSK recruitment to cSrc.5 We hypothesized that enhanced RAS signaling activated cSrc either through decreasing the availability of the negative regulator(s) or through abrogating the interaction between cSrc and its negative regulator(s).

To test this, the protein expression levels of CSK, Cav3, Cav1, as well as phosphorylated Cav1 (at Tyr14), the active form of Cav1 shown to inhibit cSrc activity,16 were examined and compared in WT and ACE8/8 LV samples. As shown in Figure 3A and 3B, the protein expression of cSrc negative regulators, CSK, Cav3, and Cav1/p-Cav1, was not significantly different in WT and ACE8/8 LV. Next, we assessed the interaction between cSrc and its negative regulators in the mouse LV. Interestingly, cSrc failed to coimmunoprecipitate with CSK (Figure V in the Data Supplement) or Cav3 (Figure 3C and 3D), whereas cSrc coimmunoprecipitated with Cav1 in mouse LV (Figure 3E).

In addition, the interaction between cSrc and Cav1 was markedly reduced (by 50%; P=0.003) in ACE8/8 compared with WT LV (Figure 3E and 3F). Taken together, these results suggest that reduced interaction between Cav1 and cSrc abrogates the inhibitory effects of Cav1 on cSrc, thereby contributing to cSrc activation on enhanced RAS signaling in mouse ventricular myocardium.

Enhanced RAS Signaling Increases S-Nitrosation of Cav1, Resulting in Reduced Cav1–cSrc Interaction in LV Cardiomyocytes

It is known that the interaction between Cav1 and cSrc at the cell membrane depends on the coupling between the N-terminal myristoyl moiety of cSrc and the palmitoylated Cys156 of Cav1.20 Protein palmitoylation can be disrupted by nitrosation of cysteine residues (S-nitrosation) by direct competition for cysteine or by the displacement of palmitate.21 S-Nitrosation of Cav1 to cSrc activation on enhanced RAS signaling in mouse ventricular myocardium.

To test this hypothesis directly, a biotin-switch assay to detect protein S-nitrosation was conducted using isolated cardiomyocytes from WT and ACE8/8 LV. As shown in Figure 4A, there was a 5.5-fold increase (P=0.03) of Cav1 S-nitrosation in isolated myocytes from ACE8/8 compared with WT LV. The increased Cav1 S-nitrosation with increased RAS activity was accompanied by a 50% reduction (P=0.03) in cSrc activation. Western blots revealed significantly increased cSrc activation (phosphorylation at pY416) and Cx43 downregulation in angiotensin-converting enzyme (ACE8/8; n=6) compared with wild-type (WT; n=6) left ventricle (LV; P<0.001). The protein expression levels of C-terminal Src kinase (CSK), Cav1, Cav3, and p-Cav1 (pY14) were not different in ACE8/8 and WT LV. Immunoprecipitation with either Cav3 (C) or cSrc (D) antibody did not show an interaction between Cav3 and cSrc in mouse LV. By contrast, cSrc coimmunoprecipitated with Cav1 in mouse LV (E and F), and the interaction between cSrc and Cav1 was significantly reduced (P=0.003, by 50%) in ACE8/8 (n=4) compared with WT (n=4) LV. IB indicates immunoblot; and IP, immunoprecipitation.

Figure 3. Cardiac renin–angiotensin system–induced cSrc activation and connexin 43 (Cx43) downregulation were accompanied by decreased caveolin-1 (Cav1)–cSrc binding. Western blots (A and B) revealed significantly increased cSrc activation (phosphorylation at pY416) and Cx43 downregulation in angiotensin-converting enzyme (ACE8/8; n=6) compared with wild-type (WT; n=6) left ventricle (LV; P<0.001). The protein expression levels of C-terminal Src kinase (CSK), Cav1, Cav3, and p-Cav1 (pY14) were not different in ACE8/8 and WT LV. Immunoprecipitation with either Cav3 (C) or cSrc (D) antibody did not show an interaction between Cav3 and cSrc in mouse LV. By contrast, cSrc coimmunoprecipitated with Cav1 in mouse LV (E and F), and the interaction between cSrc and Cav1 was significantly reduced (P=0.003, by 50%) in ACE8/8 (n=4) compared with WT (n=4) LV. IB indicates immunoblot; and IP, immunoprecipitation.
S-nitrosation directly disrupted the Cav1–cSrc interaction. cSrc binding (Figure 4C), suggesting that increased Cav1 S-nitrosation directly disrupted the Cav1–cSrc interaction.

Figure 4. Renin–angiotensin system activation induces caveolin-1 (Cav1) S-nitrosation, resulting in Cav1–cSrc dissociation. 

A. Cav1 S-nitrosation (SNO) was assessed using biotin-switch assay in the cardiomyocytes isolated from wild-type (WT; n=4) and angiotensin-converting enzyme (ACE8/8; n=4) left ventricle, which showed the level of Cav1 SNO was significantly (P=0.03) higher in ACE8/8 than in WT LV myocytes. B. Coimmunoprecipitation experiments revealed that the interaction between cSrc and Cav1 was reduced in ACE8/8 (n=4) compared with WT (n=4), LV myocytes. C. Human embryonic kidney (HEK) cells cotransfected with mouse cSrc and Cav1 cDNA were subjected to NO donor (S-nitroso-N-acetyl-DL-penicillamine [SNAP], 20 μmol/L; 10 minutes) treatment, where Cav1 SNO was increased, resulting in reduced interaction between cSrc and Cav1 (P=0.03; n=4 in each group). IB indicates immunoblot; and IP, immunoprecipitation.

Cardiac Cav1 S-nitrosation on Enhanced RAS Signaling Is Facilitated by Increased Endothelial Nitric Oxide Synthase–Cav1 Association

Physiologically, the chemical reaction of protein S-nitrosation is favored on increased availability of NO, either through increased NO production or by close proximity to the enzymes that synthesize NO, NO synthase (NOS). To test whether the increased Cav1 S-nitrosation on enhanced cardiac RAS signaling was the result of elevated NO production, we examined the protein expression levels of NOS in isolated LV cardiomyocytes from WT and ACE8/8 animals. As shown in Figure 5C, the protein expression levels of neuronal (nNOS) and endothelial NOS (eNOS), as well as phospho-eNOS, the active form of eNOS, were not significantly different in WT and ACE8/8 cardiomyocytes. In addition, a direct quantification of NO concentration did not reveal a measurable difference in NO production from isolated WT and ACE8/8 ventricular cardiomyocytes (Figure VI in the Data Supplement). To test whether enhanced RAS signaling makes NO available to Cav1 by bringing NOS in proximity to Cav1, we examined the amount of NOS that could be coimmunoprecipitated with Cav1 in WT and ACE8/8 LV myocytes. As shown in Figure 5D, Western blots of the Cav1-pull down lysates revealed a 2.2-fold increase (P=0.03) in the binding between eNOS and Cav1 in ACE8/8 compared with WT isolated LV myocytes. nNOS, however, did not coimmunoprecipitate with Cav1 in either WT or ACE8/8 LV myocytes (data not shown). Taken together, these data suggest that increased Cav1 S-nitrosation with enhanced cardiac RAS signaling is related to increased Cav1–eNOS binding.

Cardiac RAS-Induced eNOS–Cav1 Association Is Dependent on Increased Mitochondrial Reactive Oxidative Species

Using the same ACE8/8 mouse model, we have demonstrated recently that cardiac reactive oxidative species (ROS), specifically mitochondrial ROS (mitoROS), is markedly increased with enhanced RAS signaling. Treatment with mitochondria-targeted antioxidant MitoTEMPO, but not the other types of antioxidants, restores the Cx43 expression, normalizes gap junction conduction, as well as ameliorates ventricular arrhythmias and sudden cardiac death in ACE8/8 mice. We hypothesized that increased mitoROS on enhanced RAS signaling mediated Cx43 degradation.
through modulating the Cav1–cSrc interaction and cSrc activity. To test this, 4-week ACE8/8 animals were treated with (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride (MitoTEMPO) (0.7 mg/kg per day IP) for 2 weeks, a regimen that has been demonstrated to normalize elevated mitoROS in ACE8/8 hearts to the levels similar to WT controls. As shown in Figure 6A and consistent with previous results, MitoTEMPO treatment in ACE8/8 mice resulted in reduced cardiac cSrc phosphorylation (by 65%; \( P = 0.002 \)) and increased Cx43 expression (by 1.9-fold; \( P = 0.002 \)) compared with untreated ACE8/8 animals. Importantly, coimmunoprecipitation experiments revealed that the increased Cav1–eNOS binding and decreased Cav1–cSrc interaction observed in ACE8/8 LV were both reversed with the treatment of MitoTEMPO (Figure 6B), suggesting the increased Cav1–eNOS binding and subsequent Cav1–cSrc dissociation on enhanced RAS signaling were dependent on mitochondrial ROS.

Figure 5. Caveolin-1 (Cav1) is nitrosated at Cys156 and Cav1 S-nitrosation (SNO) on renin–angiotensin system activation is associated with increased endothelial NO synthase (eNOS)–Cav1 binding. \( A \), Schematic illustration of mouse Cav1, containing 3 cysteine residues (C133, C143, and C156) close to the C terminus, among which only C156 is predicted to be nitrosated. \( B \), Human embryonic kidney (HEK) cells transfected with mouse cSrc, as well as with either wild-type (WT) mouse Cav1 CDNA or Cav1 containing Cys133, Cys143, or Cys156 to Ser (nitrosation-resistant) single amino acid mutation, were subjected to S-nitroso-N-acetyl-DL-penicillamine (SNAP) treatment. SNAP treatment significantly increased SNO in WT, C133S, and C143S, but not in C156S, Cav1 molecule (\( P = 0.03; n = 4 \) in each pair), suggesting C156 is the only cysteine residue in Cav1 that can be nitrosated. \( C \), Western blot did not reveal significant differences in the protein expression levels of neuronal NOS (nNOS), eNOS or phosphorylated endothelial nitric oxide synthase (p-eNOS) in the isolated left ventricular (LV) myocytes from WT (\( n = 6 \)) and angiotensin-converting enzyme (ACE8/8; \( n = 6 \)) mice. \( D \), Coimmunoprecipitation experiments demonstrated significantly (\( P = 0.03 \)) increased eNOS–Cav1 binding in ACE8/8 (\( n = 4 \)) compared with WT (\( n = 4 \)), isolated LV cardiomyocytes. IB indicates immunoblot; and IP, immunoprecipitation.

Figure 6. Mitochondria-targeted antioxidant (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride (MitoTEMPO) ameliorates cardiac renin–angiotensin system activation–induced cSrc activation and connexin 43 (Cx43) downregulation through reducing caveolin-1 (Cav1)–endothelial NO synthase (eNOS) interaction and restoring Cav1–cSrc binding. \( A \), Two weeks of MitoTEMPO (0.7 mg/kg per day, IP) treatment significantly attenuated cSrc activation/phosphorylation (\( P = 0.002 \)) and Cx43 downregulation (\( P = 0.002 \)) in angiotensin-converting enzyme (ACE8/8) left ventricular (LV; \( n = 6 \) in each group). \( B \), MitoTEMPO treatment significantly reduced Cav1–eNOS interaction (\( P = 0.002 \)) and restored Cav1–cSrc binding (\( P = 0.002 \)) in ACE8/8 LV (\( n = 6 \) in each group). IB indicates immunoblot; and IP, immunoprecipitation.
Discussion

Accumulating evidence has suggested that Cav1 is involved in the regulation of cardiac electric functioning. For example, Cav1 binds to the human ether-a-go-go-related gene K^+ channel and regulates its function and degradation. L-type Ca^{2+} channels, as well as Cx43, have been shown to be targeted by lipid rafts/caveolae and directly interact with Cav1. Importantly, human genome-wide association studies have revealed significant association of Cav1 variants with increased risk of cardiac arrhythmias. Using 2 different mouse models (Cav1^-/- and ACE8/8) in the present study, we have demonstrated the essential role of Cav1 in maintaining the homeostasis of cardiac Cx43 by modulating cSrc activity. With the abrogation of Cav1-mediated cSrc inhibition, either through genetic deletion of Cav1 or via Cav1 S-nitrosation induced by enhanced RAS signaling, cSrc became activated, leading to downregulation of Cx43, reduced ventricular conduction velocity, and increased propensity for ventricular arrhythmias.

The RAS is a critical component of the physiological and pathological responses of the cardiovascular system. Angiotensin II, the central signaling effector of RAS, binds to angiotensin II type 1 receptor and activates NAD(P)H oxidases leading to increased production of cytosolic and mitochondrial ROS. It has been demonstrated that mitochondrial, but not cytosolic, ROS plays a critical role in RAS-mediated connexon remodeling and ventricular arrhythmias. It has been demonstrated that mitochondrial, but not cytosolic, ROS plays a critical role in RAS-mediated connexon remodeling and ventricular arrhythmias.

The present study provides a mechanistic link between RAS-induced oxidative stress and ventricular arrhythmias, where RAS-induced mitochondrial ROS triggers increased eNOS–Cav1 association and Cav1–S-nitrosation, resulting in cSrc activation, Cx43 downregulation, and subsequent electric abnormalities.

It has been demonstrated previously that increased cardiac p-cSrc can compete with Cx43 for the binding with ZO-1 protein at the intercalated disc, promoting Cx43 internalization and degradation. With the robust increases in p-cSrc, it is likely that Cx43 downregulation observed in Cav1^-/- LV can be attributed to p-cSrc–mediated Cx43 depletion. Indeed, Cx43 mRNA expression levels were not different in the LV from WT, Cav1^+/-, and Cav1^-/-+PP1 mice (Figure IV in the Data Supplement), suggesting that the production of Cx43, at least on the transcriptional level, is not affected in Cav1^-/- LV. Our experiments, however, could not exclude the possibility that the efficiency of ventricular Cx43 protein translation or trafficking could be impaired in the absence of Cav1.

Intriguingly, Cav3, the muscle-specific caveolin isoform that is essential for caveolae formation in cardiomyocytes, was not involved in the regulation of cSrc and Cx43 because Cav3 did not interact with cSrc (Figure 3C and 3D) and knockout of Cav3 did not alter cardiac cSrc activity or Cx43 expression levels (Figure 2D). The observation that cSrc is not activated in Cav3^-/- LV suggests that Cav1-mediated cSrc inhibition is unaffected in Cav3^-/- hearts. Because caveolae are completely absent in Cav3^-/- cardiomyocytes, the preserved Cav1–cSrc interaction in Cav3^-/- hearts suggests that Cav1 interacts with and regulates cSrc outside of caveolae in cardiomyocytes. Indeed, recent studies indicate that caveolin can regulate cellular functions in noncaveolar regions. Examples include cell adhesion, reactive neuronal plasticity, and oxidative stress–induced responses. Taken together, the data presented here provide evidence suggesting the noncaveolar role of Cav1-mediated cSrc and Cx43 regulation in cardiomyocytes.

Cav1 is known to negatively regulate eNOS activity in endothelial cells in a caveolae-dependent manner. In cells where Cav1 does not drive caveolae assembly, however, the ability of Cav1 to inhibit eNOS activity is diminished, albeit the Cav1–eNOS interaction remains. The observation that Cav1–eNOS binding increased without altering eNOS activity (levels of p-eNOS [phosphorylated endothelial nitric oxide synthase]) in ACE8/8 cardiomyocytes (Figure 5C and 5D) suggests that the Cav1–eNOS interaction in cardiomyocytes is noncaveolar. Therefore, on enhanced RAS activity and increased mitoROS, eNOS actively redistributes to noncaveolar compartments, allowing spatially confined NO release to targets such as Cav1. This observation highlights the importance of the spatial coupling and direct
interaction between eNOS and its targets in NO-mediated signaling pathways. In addition, the paradox that binding between eNOS and its negative regulator Cav1 in ACE8/8 mouse hearts allows nitrosation of Cav1 suggests that Cav1 may cease to inhibit eNOS if an appropriate signal is given. It is possible that on an enhanced RAS state, the noncaveolar interaction between eNOS and Cav1 is increased and this leads to potential increased local activity of eNOS to facilitate Cav1 S-nitrosation. The differential eNOS activities in caveolar and noncaveolar compartments also suggest that the lipid environment may contribute to the negative regulation of eNOS, where eNOS targeted to noncaveolar regions can be activated even in the presence of Cav1.

The present study also revealed that increased eNOS–Cav1 binding on RAS activation in cardiomyocytes was dependent on mitoROS. In line with the recent evidence showing that mitochondrial-targeted, but not general, antioxidants can ameliorate RAS activation–induced Cx43 downregulation and ventricular arrhythmias, these findings reflect the critical role of mitoROS in cardiac cSrc and Cx43 regulation. These data are also consistent with the emerging role of mitoROS as signaling molecules in regulating physiological functions. It is intriguing to understand how mitoROS signals the redistribution of eNOS to noncaveolar Cav1, causes Cav1 S-nitrosation, and contributes to subsequent cSrc and Cx43 dysregulation. It has been reported that a subpopulation of eNOS is docked to the mitochondrial outer membrane both in endothelial cells and neurons. It is possible that this subpopulation of eNOS senses the increased mitoROS on RAS activation, resulting in its displacement from the mitochondria outer membrane and redistribution to noncaveolar compartments where eNOS–Cav1–cSrc interaction occurs. Further experiments are required to test this hypothesis.

The observation that the LV conduction velocity is reduced by 30% in Cav1−/− mice, with a ≤50% reduction in Cx43 comparing with the WT, is intriguing. Based on the observation in connexin knockout mice, it is generally considered that there exists a significant redundancy of myocardial gap junctions, and significant myocardial conduction slowing occurs only with near-complete connexin depletion. Several studies in human and animal myocardium, however, reported significant changes in ventricular conduction velocity with relatively small changes in Cx43 levels. In a recent study by Dhillon et al., a continuous conduction velocity with relatively small changes in Cx43 comparing with the WT, is intriguing. Based on the observation in connexin knockout mice, it is generally considered that there exists a significant redundancy of myocardial gap junctions, and significant myocardial conduction slowing occurs only with near-complete connexin depletion. Several studies in human and animal myocardium, however, reported significant changes in ventricular conduction velocity with relatively small changes in Cx43 levels.

In summary, the present study, for the first time, demonstrates the critical role of Cav1 in maintaining the homeostasis of cardiac Cx43 by interacting with and inhibiting cSrc tyrosine kinase. The disrupted Cav1–cSrc interaction on pathological conditions, such as enhanced RAS signaling, resulted in the activation of cSrc, Cx43 reduction, slow conduction, and increased risk for ventricular arrhythmias. As summarized in the schematic illustration (Figure 7), our data suggest that mitoROS production increases on RAS activation, which triggers the redistribution of eNOS and increased Cav1–eNOS interaction, resulting in Cav1 S-nitrosation, Cav1–cSrc dissociation, cSrc activation, Cx43 downregulation, and subsequently, slow cardiac conduction and increased propensity for arrhythmias. Our findings provide a potential explanation for the genetic association of Cav1 and human arrhythmias, as well as the insights into the mechanistic link between RAS-induced mitochondrial ROS and Cx43 hemichannel regulation. These results suggest the potential therapeutic approach of targeting the regulation of Cav1 or mitochondrial ROS to ameliorate arrhythmic risk caused by RAS activation in various cardiac diseases.

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Disclosures
Dr Dudley is an inventor of 13/551 790, a method for ameliorating or preventing arrhythmic risk associated with cardiomyopathy by improving conduction velocities and 13/507 319, a method for modulating or controlling connexin 43 level of a cell and reducing arrhythmic risk. The other authors report no conflicts.

References
Yang et al  Caveolin-1 Modulates Gap Junction and Arrhythmias
CLINICAL PERSPECTIVE

Activation of the cardiac renin–angiotensin system (RAS) in heart failure is associated with increased risk of ventricular arrhythmia and sudden cardiac death. Inhibition of angiotensin II signaling reduces this risk. Increased cardiac RAS activity leads to slow conduction and spontaneous ventricular arrhythmias as a result of connexin 43 downregulation mediated by increased cardiac oxidative stress and activation of redox-sensitive tyrosine kinase cSrc. In this article, we demonstrate how oxidative stress activates cSrc to contribute to arrhythmic risk. We show a significant reduction in the binding of cSrc to caveolin-1 (Cav1) and subsequent cSrc activation as a result of S-nitrosation of Cav1 at Cys156 (Cav1–S-nitrosation) on enhanced RAS signaling. RAS-induced Cav1–S-nitrosation and cSrc activation was mediated by endothelial NO synthase–derived NO in response to increased mitochondrial oxidative stress. Knockout of Cav1 (but not caveolin-3) resulted in activation of cSrc, degradation of connexin 43, reduced cardiac conduction velocity, and increased arrhythmic risk. Arrhythmic risk in Cav1−/− mice was mitigated by pharmacological inhibition of cSrc. In summary, oxidative stress–induced Cav1 S-nitrosation in response to cardiac RAS signaling promotes Src-dependent disruption of gap junctions and ventricular arrhythmia. These findings may explain the genetic association of Cav1 with arrhythmias and suggest that targeted regulation of Cav1 or endothelial NO synthase may be a novel approach to reduce arrhythmic risk during heart failure.
Caveolin-1 Modulates Cardiac Gap Junction Homeostasis and Arrhythmogenicity by Regulating cSrc Tyrosine Kinase
Kai-Chien Yang, Cody A. Rutledge, Mao Mao, Farnaz R. Bakhshi, An Xie, Hong Liu, Marcelo G. Bonini, Hemal H. Patel, Richard D. Minshall and Samuel C. Dudley, Jr

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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/(√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 decreasing cycle lengths between 20-55 ms, and inducible ventricular tachycardia was defined as > 3 consecutive ventricular beats.\textsuperscript{6}

**Ventricular conduction velocity measurement**

LV conduction velocity was measured in anesthetized WT (n=6) and Cav1\textsuperscript{−/−} (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\textsuperscript{−/−} mice with and without 4 weeks treatment of cSrc inhibitor 1-(1,1-dimethylethyl)-1-(4-methylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amino (PP1, see below); in some cases, protein lysates were prepared from the LV cardiomyocytes isolated from ACE8/8 animals using described methods.\textsuperscript{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\textsuperscript{416}, Cx43, C-terminal Src kinase [CSK] and Tyr\textsuperscript{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-immunoprecipitated 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'-gggcggttgtacccgagcacataagacgctc-3'
    anti-sense 5'-gaagctcttgatgctcggtaaccagcc-3'

C143S: sense 5'-cctgattgagacgacgctcagccgctctca-3'
    anti-sense 5'-tagacgcggctgatgctctgaatctcaatcagg-3'

C156S: sense 5'-tttaacgcctccatatcctcgcctgatctgattc-3'
    anti-sense 5'-ttcaagaggtggagtctgataacagctgtag-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. 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 methylmethanethiosulphate (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'-O-
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 Cav1 was 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. Using equal amounts of RNA, transcript analyses of Cx43 and GAPDH were carried out using SYBR green RT-PCR in a two-step process. 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°C 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


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\(_{\text{Na}}\)) densities in WT and Cav1\(^{-/-}\) LV myocytes (n=20 in each group). There was no significant differences in I\(_{\text{Na}}\) 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\(_{\text{Na}}\) 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 \(\mu\)m
(B) Quantification of the proportion of Cx43 colocalized with N-cadherin did not reveal significant differences in WT, Cav1<sup>-/-</sup> and PP1-treated Cav1<sup>-/-</sup> LV.

Supplemental Figure 4. The transcript expression of Cx43 is not different in WT, Cav1<sup>-/-</sup> and PP1-treated Cav1<sup>-/-</sup> mouse LV

Quantitative RT-PCR revealed that the transcript expression levels of Cx43 in the LV from WT, Cav1<sup>-/-</sup> and Cav1<sup>-/-</sup>+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

<table>
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<th>WT</th>
<th>Cav1(^{-/-})</th>
<th>Cav1(^{-/-})+PP1</th>
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<td></td>
<td>(0%)</td>
<td>(79%)</td>
<td>(7%)</td>
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Supplemental Figure 1

A: Plot showing the relationship between test potential and INa density for WT and Cav1^-^- mice.

B: Graph depicting the normalized INa/INamax across different prepulse potentials for WT and Cav1^-^- mice.

C: Mason Trichrome staining of Cav1 WT tissue.

D: Mason Trichrome staining of Cav1^-^- tissue.
Supplemental Figure 2

Protein Expression Relative to total Cx43

WT Cav1−/− Cav1−/− + PP1

<table>
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P = 0.10

P = 0.07
Supplemental Figure 3

A

WT

Cav1-/-

Cav1-/-+PP1

Percentage of Cx43 colocalized with N-Cadherin

P = 0.53

P = 0.83

B

WT Cav1-/- Cav1-/-+PP1

Percentage of Cx43 colocalized with N-Cadherin
Supplemental Figure 4

WT
Cav1−/−
Cav1−/−+PP1

Relative Cx43 mRNA Expression

$P=0.59$

$P=0.82$
Supplemental Figure 5
Supplemental Figure 6

WT ACE 8/8

NO (nmol/mg protein)

P = 0.70