Heart Failure

Disrupted Junctional Membrane Complexes and Hyperactive Ryanodine Receptors After Acute Junctophilin Knockdown in Mice

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Background—Excitation-contraction coupling in striated muscle requires proper communication of plasmalemmal voltage-activated Ca\(^{2+}\) channels and Ca\(^{2+}\) release channels on sarcoplasmic reticulum within junctional membrane complexes. Although previous studies revealed a loss of junctional membrane complexes and embryonic lethality in germ-line junctophilin-2 (JPH2) knockout mice, it has remained unclear whether JPH2 plays an essential role in junctional membrane complex formation and the Ca\(^{2+}\)-induced Ca\(^{2+}\) release process in the heart. Our recent work demonstrated loss-of-function mutations in JPH2 in patients with hypertrophic cardiomyopathy.

Methods and Results—To elucidate the role of JPH2 in the heart, we developed a novel approach to conditionally reduce JPH2 protein levels using RNA interference. Cardiac-specific JPH2 knockdown resulted in impaired cardiac contractility, which caused heart failure and increased mortality. JPH2 deficiency resulted in loss of excitation-contraction coupling gain, precipitated by a reduction in the number of junctional membrane complexes and increased variability in the plasmalemma–sarcoplasmic reticulum distance.

Conclusions—Loss of JPH2 had profound effects on Ca\(^{2+}\) release channel inactivation, suggesting a novel functional role for JPH2 in regulating intracellular Ca\(^{2+}\) release channels in cardiac myocytes. Thus, our novel approach of cardiac-specific short hairpin RNA–mediated knockdown of junctophilin-2 has uncovered a critical role for junctophilin in intracellular Ca\(^{2+}\) release in the heart. (Circulation. 2011;123:979-988.)

Key Words: calcium ■ excitation ■ heart failure ■ junctophilin ■ sarcoplasmic reticulum

Excitation-contraction (EC) coupling is the fundamental mechanism by which depolarization of the voltage-gated Ca\(^{2+}\) channels (VGCCs) in the plasmalemma triggers a much greater release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) via type 2 ryanodine receptors (RyR2), a process known as Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR). This Ca\(^{2+}\) release amplification depends on the organization of VGCC and RyR2 within junctional membrane complexes (JMCs), also known as calcium release units. Disruption of JMC structure, as seen in heart failure, profoundly affects CICR and thus cardiac muscle contractility.

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The molecular mechanisms involved in organizing Ca\(^{2+}\) channels within the JMC remain poorly understood. One family of proteins, known as junctophilins (JPHs), has been proposed to provide a structural bridge between the plasmalemma and SR, thereby potentially ensuring approximation of VGCC and RyR2.* Junctophilin-2 (JPH2) is the major cardiac isoform among the 4 JPH isoforms, which are expressed within JMCs of all excitable cell types. JPH proteins comprise 8 N-terminal “membrane occupation and recognition nexus” domains, a space-spanning α-helix, and a C-terminal transmembrane domain. The membrane occupation and recognition nexus domains mediate binding to the plasmalemma, and the hydrophobic transmembrane domain is anchored into the SR membrane.

The role of junctophilin in myocytes has remained unclear because of the lack of adequate animal models. Mice deficient in JPH1, the major skeletal muscle isoform, suffer from

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Detailed methods are provided in the online-only Data Supplement.

Electrophysiology and Ca²⁺ Imaging

Western Blotting and Coimmunoprecipitation

Immunohistochemistry and Histology

Transthoracic Echocardiography

Electron Microscopy

Computational Model

Methods

Please refer to the online-only Data Supplement for a more detailed Methods section.

Generation of Transgenic Mice

The generation of mice with cardiac-specific inducible short hairpin RNA (shRNA)–mediated knockdown of JPH2 is described in detail in the online-only Data Supplement.

Northern Blot and Real-Time Polymerase Chain Reaction

Detailed methods are provided in the expanded Methods section in the online-only Data Supplement.

Western Blotting and Coimmunoprecipitation

Western blotting and coimmunoprecipitation were performed as described in detail previously.¹²

Immunohistochemistry and Histology

Detailed methods are provided in the online-only Data Supplement.

Transthoracic Echocardiography

Mice were anesthetized with the use of 1% to 2.0% isoflurane in 95% O₂. Cardiac function was assessed with M-mode echocardiograms acquired with a VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Ontario, Canada), as described.¹³

Electrophysiology and Ca²⁺ Imaging

Detailed methods are provided in the online-only Data Supplement.

Electron Microscopy

Detailed methods are provided in the online-only Data Supplement.

Computational Model

Detailed methods are provided in the online-only Data Supplement.

Statistical Analysis

Data are expressed as mean±SEM. Statistical significance of differences between experimental groups was compared with Student t test and 1-way ANOVA with pairwise multiple comparisons performed with the Student-Newman-Keuls method as appropriate. If data were not normal according to the Shapiro-Wilk test, the Wilcoxon rank sum or Kruskal-Wallis test was used, as appropriate. A value of P<0.05 was considered statistically significant.

Results

Cardiac-Specific, Inducible JPH2 Knockdown With RNA Interference

To circumvent embryonic lethality associated with germ-line JPH2 knockout, we developed a novel approach for inducible cardiac-specific JPH2 knockdown. We first cloned 4 candidate shRNA oligonucleotides targeting different regions in mouse JPH2 and tested the knockdown efficiency in vitro (Figure 1a in the online-only Data Supplement). Western blot analysis demonstrated efficient knockdown for 3 JPH2-shRNA constructs (Figure 1b in the online-only Data Supplement). The most efficient oligonucleotide (No. 2) was used to generate a conditional JPH2 knockdown (shJPH2) mouse. shJPH2 mice harbor a transgene composed of the JPH2 shRNA sequence downstream of a U6 promoter, which is inactive because of the insertion of a loxp flanked neo cassette.¹⁴ To ensure cardiac-specific shRNA expression, we crossed shJPH2 mice with α-myosin heavy chain (αMHC)-MerCreMer (MCM) mice, which express a tamoxifen-inducible mutant Cre recombinase under control of the cardiac-specific αMHC promoter.¹⁵ Tamoxifen administration in double transgenic MCM-shJPH2 mice results in cardiac-specific excision of the neo cassette, which activates the U6 promoter and induces expression of JPH2 shRNA (Figure 1A).

Northern blot analysis confirmed expression of JPH2 shRNA exclusively in the hearts of tamoxifen-injected MCM-shJPH2 mice. Moreover, indicative of the cardiac specificity, there was no JPH2 shRNA expression in skeletal muscle of tamoxifen-injected MCM-shJPH2 mice. Real-time polymerase chain reaction analysis revealed that JPH2 mRNA levels were significantly lower in hearts of tamoxifen-treated MCM-shJPH2 mice, whereas JPH1 mRNA levels were unaltered (Figure 1C). Western blot analysis of cardiac protein lysates demonstrated that knockdown of JPH2 mRNA resulted in a 60% reduction in JPH2 protein (Figure 1B). Because expression of double-stranded RNA might trigger a cellular interferon response with the potential to affect gene expression,¹⁶ we measured mRNA levels of oligoadenylate synthase-1 (OAS1) and signal transducer and activator of transcription 1 (STAT1). Real-time polymerase chain reaction analysis demonstrated unaltered levels of OAS1 and STAT1 in tamoxifen-treated MCM-shJPH2 mice (Figure 1C in the online-only Data Supplement).

Whereas all provided data were obtained in mice from 1 transgenic line (line 38), we confirmed similar findings in a second independent shJPH2 transgenic mouse line (line 21) in which JPH2 was reduced by 30% after tamoxifen administration (data not shown). These data show that we successfully created a novel shRNA-mediated approach to selectively reduce JPH2 protein levels in the adult murine heart.
Downregulation of JPH2 Leads to Acute Heart Failure

JPH2 knockdown in MCM-shJPH2 mice caused a significantly reduced survival rate compared with MCM mice (Figure 1D). Within 1 week after tamoxifen injections, mortality was 40% among MCM-shJPH2 mice compared with 0% among MCM mice ($P$=0.004). Histological analysis of surviving mice revealed cardiac dilatation in MCM-shJPH2 mice (Figure 1E). Transthoracic echocardiography revealed significantly decreased ejection fractions in MCM-shJPH2 mice (36.72±4.25%) compared with MCM controls (51.97±2.15%; $P$<0.01) (Figure 1F; Table in the online-only Data Supplement). Furthermore, end-systolic diameter and end-diastolic diameter were both significantly larger in MCM-shJPH2 (3.89±0.16 and 4.80±0.07 mm, respectively) compared with MCM control mice (3.19±0.07 and 4.35±0.06 mm; $P$<0.001) (Figure 1G and 1H). Similar findings were obtained in MCM-shJPH2 mice from line 21 (Table in the online-only Data Supplement). Left ventricular posterior wall thickness did not change between MCM-shJPH2 (0.80±0.03 mm) and MCM controls (0.79±0.02 mm; $P$=NS; Figure 1d in the online-only Data Supplement), suggesting that the observed changes were not due to compensatory cardiac remodeling. Consistently, there was no difference in cardiomyocyte cross-sectional areas between MCM-shJPH2 mice and MCM controls (Figure 1e in the online-only Data Supplement). On the other hand, the ratio of lung weight to tibia length was increased in MCM-shJPH2 mice, consistent with pulmonary congestion in MCM-shJPH2 mice (Figure 1f in the online-only Data Supplement). However, mRNA levels of cardiac stress markers skeletal muscle–1 actin (Acta1), atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), $\beta$-myosin heavy chain ($\beta$-MHC), and the exon 4 splice isoform of regulator of calcineurin 1 (RCAN 1-4), were not altered after JPH2 knockdown (Figure 1g in the online-only Data Supplement). Thus, acute loss of JPH2 in the heart results in sudden cardiac death caused by acute heart failure in the absence of hypertrophic remodeling.

Loss of EC Coupling Gain in JPH2 Knockdown Mice

We next determined the effects of JPH2 knockdown on EC coupling by simultaneously measuring VGCC $\mathrm{Ca^{2+}}$ influx and...
RyR2 Ca\textsuperscript{2+} release. Whereas Ca\textsuperscript{2+} influx through VGCC was unaffected by loss of JPH2, Ca\textsuperscript{2+} release from the SR (ie, Ca\textsuperscript{2+} transients) was significantly smaller in tamoxifen-treated MCM-shJPH2 mice (Figure 2A). As a result, the gain of EC coupling was reduced by >50% after JPH2 knockdown (MCM-shJPH2: 0.11 ± 0.02 versus MCM: 0.29 ± 0.06; P<0.05; Figure 2B). Further analysis revealed that neither inactivation kinetics (MCM: 35.79 ± 2.53 ms versus MCM-shJPH2: 31.37 ± 2.18 ms; P=0.19) nor current-voltage relationship of VGCC was affected by loss of JPH2 (Figure 2C and 2D). The total amount of Ca\textsuperscript{2+} entering the cell via VGCC was also comparable between MCM-shJPH2 (4.35 ± 0.21 pA/pF) and MCM mice (4.25 ± 0.27 pA/pF; P=NS; Figure 2E).

In addition to the Ca\textsuperscript{2+} influx trigger, the amount of SR Ca\textsuperscript{2+} release also depends on SR Ca\textsuperscript{2+} load and activity of RyR2 channels. Therefore, we assessed the amount of Ca\textsuperscript{2+} stored in the SR after steady state pacing at 1 Hz (Figure 2F). Consistent with EC coupling experiments (Figure 2A), the amplitude of pacing-evoked Ca\textsuperscript{2+} transients was decreased in MCM-shJPH2 mice (1.91 ± 0.06 F/F0) compared with MCM controls (2.30 ± 0.10; P<0.01; Figure 2G). SR Ca\textsuperscript{2+} content, measured by a caffeine dump protocol, was significantly decreased in MCM-shJPH2 mice (2.92 ± 0.25 F/F0 versus 4.10 ± 0.33 F/F0; P<0.01; Figure 2H). Surprisingly, however, we observed that the relative amount of SR Ca\textsuperscript{2+} released via RyR2 normalized to SR Ca\textsuperscript{2+} content (ie, fractional release) was increased when JPH2 was downregulated (Figure 2I). Because the Ca\textsuperscript{2+} decay curves in the presence of caffeine appeared shallower in MCM-shJPH2 mice compared with MCM controls, we further analyzed Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity. We found that τ values were significantly decreased after JPH2 knockdown (τ=2.70 ± 0.23 for MCM versus 5.11 ± 0.61 for MCM-shJPH2 mice, respectively; P<0.01). Combined, these data suggest that 1 consequence of JPH2 deficiency is reduced VGCC-RyR2 coupling leading to reduced cardiomyocyte contractility due to a novel role of JPH2 in directly regulating RyR2 activity.

JPH2 Knockdown Disrupts JMC Organization

To ascertain the effects of JPH2 deficiency on the structure of JMCs, we first examined colocalization of VGCC and RyR2 in ventricular myocytes from MCM-shJPH2 mice. Immunofluorescent imaging indicated that both types of Ca\textsuperscript{2+} channels were distributed in similar striated patterns (Figure 3A through 3D). However, colocalization between the 2 channels was significantly decreased after JPH2 knockdown, and this
was not due to a difference in protein levels (Figure 3E and 3F). Thus, loss of JPH2 results in defective colocalization of VGCC and RyR2 Ca\textsuperscript{2+} channels within the JMC.

We therefore examined the structure of transverse (T)-tubules using di-8-ANEPPS staining. A significant disruption of normal T-tubular structure was apparent in cardiomyocytes from tamoxifen-treated MCM-shJPH2 mice (Figure 4A and Figure II in the online-only Data Supplement). These alterations in T-tubular structure did not affect membrane capacitance (average membrane capacitance = 184.6 ± 22.6 pF for MCM versus 165.8 ± 22.6 pF for MCM-shJPH2 mice, respectively; *P = 0.27). Transmission electron microscopy was used to investigate ultrastructural changes in the JMCs (Figure 4B). Cardiomyocytes from MCM-shJPH2 mice had 40% fewer JMCs (0.36 ± 0.04 JMCs per sarcomere) than MCM control mice (0.59 ± 0.01; Figure 4C). Furthermore, the remaining JMCs were irregular in terms of their structure. Whereas the average distance between the T-tubular membrane and SR membrane was similar (12.45 ± 0.52 nm in MCM-shJPH2 versus 12.30 ± 0.26 nm in MCM), the gap between the 2 membranes within a single JMC (intradyad width) was more variable in MCM-shJPH2 mice (variance 1.96 nm\textsuperscript{2}) than in MCM controls (variance 1.21 nm\textsuperscript{2}; *P < 0.01; Figure 4D). In addition, dyad width among different JMCs (interdyad width) was more variable in MCM-shJPH2 mice (variance 0.26 nm\textsuperscript{2}) compared with MCM controls (variance 1.07 nm\textsuperscript{2}; Figure 4E). Therefore, acute loss of JPH2 leads to altered morphology and loss of JMCs.

**JPH2 Stabilizes the Ryanodine Receptor Ca\textsuperscript{2+} Release Channel**

The reduction in JMC number and disruption of VGCC/RyR2 colocalization could account for the attenuated EC coupling gain (Figure 2). However, structural uncoupling of VGCC and RyR2 would theoretically cause reduced RyR2 triggering, leading to increased SR Ca\textsuperscript{2+}. Because we observed the exact opposite, a reduction in SR Ca\textsuperscript{2+} content (Figure 2F and 2H), we decided to assess RyR2 channel behavior during cellular diastole in cardiomyocytes from MCM-shJPH2 mice (Figure 5A and 5B). Ventricular myocytes of MCM-shJPH2 mice exhibited an increase in cell-wide spontaneous Ca\textsuperscript{2+} release events after a 1-Hz pacing train compared with MCM mice (Figure 5A and 5C). Next, we used confocal line scan imaging to visualize Ca\textsuperscript{2+} sparks in isolated cardiomyocytes (Figure 5B). Knockdown of JPH2 in MCM-shJPH2 mice led to a significantly higher Ca\textsuperscript{2+} spark frequency (Figure 5B and 5D). Taken together, our data indicate that loss of JPH2 leads to increased diastolic Ca\textsuperscript{2+} leak via RyR2 channels. Moreover, this suggests that JPH2 is an important RyR2 regulator besides its structural role in the JMC. Immunoprecipitation of RyR2 from mouse heart tissue revealed that JPH2 binds to RyR2, further supporting a novel role of JPH2 in directly regulating RyR2 (Figure 5F). Thus, our data suggest that JPH2 is required for proper inactivation of RyR2 and that loss of JPH2 can cause diastolic Ca\textsuperscript{2+} leaks.

**Effect of JMC Alterations on EC Coupling Gain in a Computational Model**

Our experimental data in MCM-shJPH2 mice revealed that acute loss of JPH2 leads to a reduced number of JMCs and an increased variability in the distance between the plasmalemmal and SR membranes. In our in vivo model, however, it is not possible to distinguish the individual contributions of these alterations to EC coupling gain, which was severely impaired after JPH2 knockdown. Therefore, we used a mathematical model to quantitatively probe the effects of
JPH2 in the heart. Although shRNA-mediated knockdown of JPH2 expression was due to nonspecific side effects of transgene integration or off-target effects of shRNA. Although this seemed less likely to be the cause, it is theoretically possible that the observed phenotype represents a powerful tool to study the role of JPH2 in the heart.

Discussion

Proper conversion of plasmalemmal depolarization into release of intracellular Ca\(^{2+}\) from the SR is a prerequisite for myocyte contractility. This fundamental process known as CICR takes place within specialized subcellular domains known as JMCs. JMCs are found in all excitable cell types and are believed to be necessary to provide a structural and functional coupling between the plasma membrane and intracellular Ca\(^{2+}\) storage organelles such as the SR. In heart failure, the reduced CICR and disorganization of JMC are believed to contribute to contractile dysfunction, although the molecular mechanisms remain poorly understood.

After the initial discovery of the JPH family, it was postulated that this class of proteins provides a structural role in myocyte contractility. This fundamental process known as CICR takes place within specialized subcellular domains known as JMCs. JMCs are found in all excitable cell types and are believed to be necessary to provide a structural and functional coupling between the plasma membrane and intracellular Ca\(^{2+}\) storage organelles such as the SR. In heart failure, the reduced CICR and disorganization of JMC are believed to contribute to contractile dysfunction, although the molecular mechanisms remain poorly understood.

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bridge that anchors the SR membrane to the plasmalemma within JMCs. Previous studies in germ-line JPH2 knockout mice revealed embryonic lethality at E10.5 due to weakened contractility and heart failure. Although embryonic JPH2-deficient mice exhibited defective calcium release units, whether this was directly caused by the absence of JPH2 has remained controversial because other knockout mouse models, including triadin-deficient mice, display similar subcellular defects. To overcome these limitations, we developed a novel shRNA-based interference approach to induce acute knockdown of JPH2 expression levels exclusively in adult mice cardiac myocytes (MCM-shJPH2 mice). Our findings revealed reproducible JPH2 knockdown after tamoxifen administration in 2 independent transgenic MCM-shJPH2 lines. It is important to highlight that conventional tissue-specific gene knockout only produces heterozygous (50% knockout) and homozygous (100% knockout) genotypes. In contrast, our shRNA-based model allowed us to study a wide range of knockdown percentages in MCM-shJPH2 mice. Thus, our new approach to inducible and cardiac-specific gene knockdown enabled us to determine the specific role of JPH2 in the maintenance of cardiac structure and function. Specificity of the model was confirmed by demonstrating that transgenic overexpression of JPH2 in MCM-shJPH2 knockdown mice prevented loss of JPH2 and negated the development of cardiac and cellular phenotypes linked to JPH2 deficiency.

Our findings suggest that loss of JPH2 is directly responsible for impaired cardiac contractility, one of the hallmarks of congestive heart failure. Loss of JPH2 expression in MCM-shJPH2 mice caused development of acute contractile failure in the absence of myocardial remodeling or alterations in expression levels of other Ca2+ handling proteins, suggesting that JPH2 deficiency impaired EC coupling within the JMC. Furthermore, our data indicate that loss of cardiac JPH2, as observed in animal models for heart failure, is not a compensatory response during heart disease development but actually plays a causal role in the disease progression. The finding that genetic mutations in JPH2 cause hypertrophic cardiomyopathy and heart failure also implies a causal link between JPH2 defects and heart disease.

Previous studies in animals with congestive heart failure revealed reduced EC coupling due to an inability of VGCC to trigger sufficient SR Ca2+ release. Our findings in cardiomyocytes isolated from MCM-shJPH2 mice suggest that loss of JPH2 expression is directly responsible for defective CICR. Furthermore, JPH2 knockdown in adult cardiomyocytes increased variability in the distance between the SR and plasma membrane within a single JMC and among populations of JMCs. These data suggest that the remaining JPH2 proteins are sufficient to keep both membranes together as JMCs but that the fidelity of JMC morphology is significantly affected by the loss of JPH2.
The T-tubular system permits the formation of larger numbers of JMCs within cardiomyocytes. T-tubule remodeling has been demonstrated in rats with pressure overload–induced heart failure and patients with congestive heart failure. In a recent study, heart failure development was correlated with progressive defects in T-tubule organization and a decline in JPH2 expression levels. Our findings provide, for the first time, a causal link between acute loss of JPH2 in MCM-shJPH2 mice and a reduction in the total number of JMCs and loss of T-tubules in ventricular myocytes. Normalization of the number of JMCs per sarcomere in MCM-shJPH2 mice intercrossed with JPH2 OE mice further underscores the specific role of JPH2 in maintaining JMC structure. Thus, our data demonstrate that JPH2 is an essential component of the JMC and that loss of JPH2 leads to disappearance of JMCs within affected cardiomyocytes.

Our findings are consistent with previous reports that JPH2 regulates the frequency of spontaneous Ca\(^{2+}\) releases from the SR in frog and mammalian ventricular myocytes. However, the physiological roles of JPH2 in the regulation of Ca\(^{2+}\) handling in cardiomyocytes have not been fully elucidated. Our findings provide new insights into the role of JPH2 in the regulation of Ca\(^{2+}\) handling in cardiomyocytes and suggest that JPH2 downregulation can directly impair RyR2 Ca\(^{2+}\) release in failing hearts. Moreover, our findings suggest that JPH2 downregulation can directly impair RyR2 Ca\(^{2+}\) release in failing hearts.

In conclusion, we have developed a novel approach to conditionally reduce JPH2 protein levels using RNA interference. Our studies uncovered the physiological roles of JPH2 in cardiac myocytes and implicated JPH2 as an important factor involved in the loss of cardiac contractility in heart failure, a key feature of this prevalent disease. In addition to determining the spacing between the plasmalemma and SR...
membrane, JPH2 is also required for the structural integrity of JMCs in myocytes. Computational analysis provided further quantitative insights into the relative importance of each of these subcellular defects related to impaired EC coupling. Moreover, we found that JPH2 associates with and facilitates RyR2 inactivation, thereby preventing diastolic SR Ca\(^{2+}\) leak. This dual functional role for JPH2 in cardiomyocytes may have implications for the development of new therapeutic strategies for heart failure.

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**Disclosures**

None.

**References**

Impaired cardiac muscle contraction is a hallmark of congestive heart failure. At the cellular level, excitation-contraction coupling requires proper communication to contractile failure. Junctophilin-2 (JPH2) has recently been proposed to provide a structural connection between the plasma membrane and sarcoplasmic reticulum within the JMC, although its function has remained unclear because of the lack of adequate animal models. We have developed a novel approach to conditionally reduce JPH2 protein levels using RNA interference. Our studies revealed that acute knockdown of JPH2 leads to loss of cardiomyocyte contractility and heart failure in mice. JPH2 was found to determine the spacing between the plasmalemma and sarcoplasmic reticulum membrane, in addition to being required for the structural integrity of JMCs within myocytes. Computational analysis provided further quantitative insights into the relative importance of each of these subcellular defects related to impaired excitation-contraction coupling. Finally, our data showed that JPH2 associates with and facilitates type 2 ryanodine receptor inactivation, thereby preventing diastolic sarcoplasmic reticulum Ca\(^{2+}\) leak that could promote heart failure. Taken together, our studies suggest that downregulation of JPH2 could play an important role in the development of contractile dysfunction in heart failure.
Disrupted Junctional Membrane Complexes and Hyperactive Ryanodine Receptors After Acute Junctophilin Knockdown in Mice

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SUPPLEMENTAL INFORMATION

Supplemental Methods

Recombinant Plasmids, and Transfection
Full-length mouse JPH2 cDNA was amplified from pCMS-RFP-JPH2 \(^1\) and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). For JPH2 RNAi transfections, 4 sets of short hairpin RNA (shRNA) oligonucleotides specific to mouse JPH2 were cloned into pBS/U6-LoxP vector \(^2\) (a kind gift from Dr. Chu-Xia Deng).

Generation of Transgenic Mice
JPH2 shRNA oligonucleotides (Forward: 5'-gcacctgggaataacgcattcagataggcgcgtatatccagtgcttttgc-3', Reverse: 5'-aattcaaaaaggcacttggaataacgcctatctcttgaatatgcgcgcttattccaagtgc-3') were ligated and cloned into pBS/U6-ploxPneo transgenic vector \(^2\) (also a kind gift from Dr. Chu-Xia Deng) according to a previously described protocol \(^3\). Cardiac specific JPH2 overexpression (OE) mice were generated by subcloning mouse JPH2 cDNA into an αMHC transgenic vector (kindly provided by Dr. Thomas Cooper \(^4\)). The transgenic vectors were injected into the pronuclei of fertilized C57/BL6 oocytes, which were transferred to pseudopregnant recipients. Transgenic offspring (shJPH2) was crossed with MHC-MerCreMer (MCM) mice (Jackson laboratory, Bar Harbor, ME) \(^5\) to generate double transgenic mice (MCM-shJPH2). MCM-shJPH2 mice were crossed with OE mice to obtain triple transgenic MCM-shJPH2-OE mice. Three to five month old male MCM, MCM-shJPH2 and MCM-shJPH2-OE mice were treated with daily 100 µl tamoxifen (Sigma-Aldrich Co., St. Louis, MO) intraperitoneal injections (~30 mg/kg) for 5 consecutive days. All mice were treated in accordance with Baylor College of Medicine Animal Care and Use Committee.
**Northern Blot**

Total RNA was extracted from left ventricular tissue using TRIzol reagent (Invitrogen, Carlsbad, CA). Ten µg of RNA was run in a 15% denaturing urea gel, and transferred to a Hybond N+ membrane (GE Healthcare, UK). A JPH2 probe was prepared using the mirVana probe preparation kit (Ambion, Austin, TX), following the kit procedures.

**RT-PCR**

One µg of RNA was reverse transcribed using Superscript II and oligo(dT) primer (Invitrogen, Carlsbad, CA). RTPCR was performed in duplicate in 96-well plates using SYBR Green and a Mastercycler ep realplex (Eppendorf, Hamburg, Germany), as described 6, 7. Expression levels were compared using the relative Ct method.

**Western Blotting**

Heart lysates were prepared from flash-frozen mouse hearts as described previously 8. Heart lysate aliquots were size-fractionated on 6% (for RyR2), 7.5% (for JPH2), 12% (for Cav1.2, NCX1, SERCA2a, CaMKII, and GAPDH), or 15% (for PLN) SDS-polyacrylamide gels. Only for PLN monomer blots, heart lysates were heated at 70°C for 10 min in 1x sample loading buffer containing 5% β-mercaptoethanol before loading on gels. The resolved gels were electro-transferred on PVDF membranes. The membranes were probed with anti-pSer2808-RyR2 (1:1,000; 8), anti-pSer2814-RyR2 (1:500; 8), anti-pSer16-PLN (1:5,000), or anti-pThr17-PLN (1:2,500) (both from Badrilla Ltd., Leeds, United Kingdom) phosphoepitope-specific antibody, and/or anti-Cav1.2 (1:200; Alomone Labs, Jerusalem), or anti-NCX1 (1:500; Swant, Bellinzona, Switzerland), or anti-SERCA2a (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal antibody, or an anti-JPH2 antibody raised against a synthetic peptide consisting of the amino acid
sequence 458-CRPRESQHERETPQPEG-475 and/or anti-RyR2 (1:5,000), or anti-PLN (1:1,000) (both Thermo Fisher Scientific (Pierce), Rockford, IL), or anti-GAPDH (1:5,000; Millipore, Temecula, CA) monoclonal antibody at 4°C overnight or at room temperature for 4 h. Blots were developed using Alexa-Fluor680-conjugated anti-mouse (Invitrogen Molecular Probes, Carlsbad, CA) and/or IR800Dye-conjugated anti-rabbit fluorescent secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA), and scanned on an Odyssey infrared scanner (Li-Cor, Lincoln, NE). Integrated densities of protein bands were measured using ImageJ Data Acquisition Software (National Institute of Health, Bethesda, MD). Protein-signal densities were normalized to the corresponding GAPDH-signal densities, while phosphorylation signal densities were normalized to the corresponding total protein-signal densities, and used for plotting data.

**Co-Immunoprecipitation Assay**

In order to immunoprecipitate RyR2 from lysates, an anti-RyR2 antibody (Thermo Scientific, Rockland, IL) was incubated with Protein A-Sepharose beads (Rockland, Gilbertsville, PA) at RT for 1 h. Following which, this antibody attached beads were incubated with heart-lysate aliquots, containing 500 µg total protein, in a reaction volume of 300 µL at 4°C overnight. The reaction volume was made up with the homogenization buffer. At the end of the incubation, beads were washed with the homogenization buffer and then resuspended in 2x LDS buffer (Invitrogen, Carlsbad, CA) containing β-mercaptoethanol. The samples were heated at 50°C for 10 min, and were subjected to Western Blotting as described above.

**Immunohistochemistry**

Isolated cardiomyocytes were fixed in 2% paraformaldehyde for 10 min followed by 100 mM glycine in PBS (pH 7.4) for 10 min. Cells were washed twice for 10 min in PBS and permeabilized in PBS with 0.1% Triton X-100 for 10 min. Following two 10 min washes in PBS, the cells were incubated overnight at 4°C in antibody buffer (75 mM NaCl, 18 mM Na₃ citrate, 2% goat serum, 1%
BSA, 0.05% Triton X-100, 0.02% NaN₃, pH7.4) with mouse monoclonal RyR2 antibody (Thermo Scientific-Affinity BioReagents, Rockford, IL) and rabbit polyclonal Caᵥ1.2 antibody (Alomone Labs, Jerusalem, Israel). The cells were washed twice for 10 min in wash buffer (75 mM NaCl, 18 mM Na₃ citrate, 0.05% Triton X-100, pH7.4) and incubated in antibody buffer with anti-mouse Alexa 568 and anti-rabbit Alexa 488 secondary antibodies (Invitrogen, Carlsbad, CA) for 2 hrs. Cells were resuspended in 50 µl mounting buffer (90% glycerol, 10% 10x PBS, 2.5% triethylenediamine, 0.02% NaN₃) and transferred to frosted slides. Isolated cardiomyocytes were resuspended in HEPES buffer at room temperature, then plated on laminin-coated dishes in 1mL of HEPES buffer and incubated at 37°C for 15 min. Cells were then washed with HEPES buffer and incubated for 1hr at RT. Imaging was performed using a laser scanning confocal microscope (LSM 510, Carl Zeiss, Thornwood, NY) with a 63X oil immersion objective. Pearson’s correlation coefficient for colocalization was assessed using Zen 2008 software (Carl Zeiss, Thornwood, NY).

**Histology**

Hearts were fixed in 4% buffered formaldehyde, dehydrated with ethanol and histoclear, and embedded in paraffin. Tissues were sectioned longitudinally (5 µm), and stained with hematoxylin and eosin using standard protocols. Cardiomyocyte cross-sectional areas were measured in sections stained with wheat germ agglutinin as described before.

**Transthoracic Echocardiography**

Mice were anesthetized using 1-2.0% isoflurane in 95%. Cardiac function was assessed using M-mode echocardiograms acquired with a VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada), as described.

**Cardiomyocyte Isolation and Ca²⁺ Imaging**
Mouse ventricular myocytes were isolated as described \(^6\). Briefly, the heart was removed following isoflurane anesthesia and rinsed in 0 Ca\(^{2+}\) Tyrode solution (137 mM NaCl, 5.4 mM KCl, 1 mM MgCl\(_2\), 5 mM HEPES, 10 mM glucose, 3 mM NaOH, pH 7.4). The heart was cannulated through the aorta and perfused on a Langendorf apparatus with 0 Ca\(^{2+}\) Tyrode (3 ~ 5 minutes, 37 °C), then 0 Ca\(^{2+}\) Tyrode containing 20 \(\mu\)g/ml (0.104 a.u./ml) Liberase TH Research Grade (Roche Applied Science) for 10 ~ 15 minutes at 37 °C. After digestion, the heart was perfused with 5 ml KB solution (90 mM KCl, 30 mM K\(_2\)HPO\(_4\), 5 mM MgSO\(_4\), 5 mM pyruvic acid, 5 mM β-hydroxybutyric acid, 5 mM creatine, 20 mM taurine, 10 mM glucose, 0.5 mM EGTA, 5 mM HEPES, pH 7.2) to wash out collagenase. The left ventricle of the heart was minced in KB solution and gently agitated, then filtered through 210 \(\mu\)m polyethylene mesh. After settling, ventricular myocytes were washed once with KB solution, and stored in KB solution at room temperature before use. Ventricular myocytes were incubated with 2 \(\mu\)M Fluo-4-acetoxymethyl ester (Fluo-4 AM, Invitrogen, Carlsbad, CA) in normal Tyrode solution containing 1.8 mM Ca\(^{2+}\) for 30 minutes at RT. Cells were then washed with dye-free normal Tyrode solution for 15 minutes for de-esterification and transferred to a chamber with a pair of parallel electrodes on a laser scanning confocal microscope (LSM 510, Carl Zeiss, Thornwood, NY) After being paced at 1 Hz for at least 2 minutes and steady state Ca\(^{2+}\) transients were observed, pacing was stopped for 45 seconds and spontaneous Ca\(^{2+}\) release events and Ca\(^{2+}\) sparks were counted. Steady state SR Ca\(^{2+}\) content was estimated by rapid application of 10 mM caffeine after pacing.

**T-Tubule Imaging and Analysis.**

T-Tubules of ventricular myocytes was visualized by Di-8-ANEPPS staining (10 \(\mu\)M for 10 min) in normal Tyrode solution with 1.8 mM Ca\(^{2+}\). Quantitative analysis of spatial integrity of T-Tubules was modified from previous studies \(^{11,\ 12}\) and was performed with ImageJ software (http://rsb.info.nih.gov/ij/). Region of interest was selected within a cell and outside of nucleus.
Power spectrum was computed using Fast Fourier Transform (FFT). Normalized power at spatial frequency of ~0.55 μm⁻¹ (peak power at ~0.55 μm⁻¹ normalized to average power at spatial frequency of 0.2 to 0.4 μm⁻¹) was used as an index of the spatial integrity of T-Tubules (TT-Power).

**Electrophysiology**

Membrane currents were measured using whole cell patch clamp techniques with an Axopatch 200B amplifier, DigiData 1440a digitizer and pCLAMP v.10 software (Molecular Devices, Sunnyvale, CA). Ventricular myocytes were perfused with normal Tyrode solution with 1.8 mM Ca²⁺. Pipette resistances were 1.5 - 3 mΩ prior to sealing. Electrode solution contained 110 mM CsCl, 20 mM tetraethylammonium chloride, 10 mM glucose, 10 mm HEPES, and 5 mM Mg-ATP (pH 7.2 adjusted with CsOH). Cells were voltage clamped at -80 mV in whole cell configuration. Prior to recording the holding potential was changed from -80 mV to -40 mV to inactivate Na⁺ current (I_{Na}). I-V curves were generated using 500 ms depolarizing voltage steps from -40 to +40 mV in 10 mV increments. All currents were normalized to cell capacitances before comparison.

**Excitation-Contraction Coupling Gain**

To investigate EC coupling gain, confocal Ca²⁺ imaging and whole cell patch clamp techniques were employed simultaneously. Ten pre-pulses at 0.2 Hz were applied to equalize SR Ca²⁺ and the testing pulse was delivered 30 seconds later. The EC coupling gain is expressed as the ratio of the amplitude of Ca²⁺ transient (ΔF/F₀) and the amplitude of I_{Ca,L} (pA/pF). Data was analyzed offline using Clampfit (Axon Instruments, CA) and Zen 2008 (Carl Zeiss, Thornwood, NY).

**Electron Microscopy**
Small sections of left ventricular apex were fixed in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde, post-fixed in 1% tannic acid and transferred to 1% osmium tetroxide. Specimens were dehydrated through an acetone series and embedded in resin. Thin plastic sections (80 to 100 nm) were cut, stained with uranyl acetate and lead citrate, and imaged on a Tecnai G² Spirit BioTWIN (FEI Company, Hillsboro, OR) electron microscope.

Co-immunoprecipitation

Anti-RyR2 antibody (Thermo Scientific, Rockland, IL) was incubated with Protein A-Sepharose beads (Rockland, Gilbertsville, PA) at RT for 1 h and incubated with heart lysates containing 500 µg total protein. Beads were washed and resuspended in 2x LDS buffer (Invitrogen, Carlsbad, CA) containing β-mercaptoethanol. The samples were heated at 50°C for 10 min, and were resolved on SDS-PAGE gels.

Computational Model

The modifications of the Luo-Rudy myocyte simulation model \(^{14}\) will be described in detail elsewhere. The discrete Ca\(^{2+}\) release myocyte model was paced to a steady state at a basic cycle length (BCL) of 1000 ms. The myocyte was then held at a membrane potential of -88 mV for 100 ms before being clamped at 0 mV for a duration of 100 ms. The depth of the dyad is defined as the distance between the voltage-gated Ca\(^{2+}\) channels (VGCC) and type 2 ryanodine receptors (RyR2).

Statistical Analysis

Data are expressed as mean ± SEM. Statistical significance of differences between experimental groups was compared using Student’s t-test, one-way ANOVA with pairwise multiple comparisons performed using the Student-Newman-Keuls method as appropriate. If results failed a normality
test, the Wilcoxon rank sum or Kruskal-Wallis tests were used, as appropriate. A value of $P<0.05$ was considered statistically significant.
Supplemental References


Supplemental Figure Legends.

Legend Supplemental Figure 1. Inducible cardiac-specific JPH2 knockdown mice causes mortality and acute heart failure

a. Schematic representation of vectors used to express JPH2 and different shRNAs targeting JPH2 in HEK cells. b. Western blot analysis of HEK cell lysates after co-transfection with JPH2 and 4 different shRNAs or empty vector (e.v.), respectively. Mouse heart lysate (H) was used as positive control for JPH2 detection. c. Quantitative PCR analysis of OAS1 and STAT1 mRNA levels in cardiac tissue from MCM (n=7) and MCM-shJPH2 (n=7) mice, indicating the absence of interferon response activation. d. Left ventricular posterior wall thickness in diastole (LVPWd) of MCM (17) and MCM-shJPH2 (14) mice at 1 week after completion of tamoxifen administration. e. Bar graph showing normalized cardiomyocyte area calculated from wheat germ agglutinin (WGA) stained sections. Numbers indicate number of cells (number of mice). f. Bar graph showing increased lung weight to tibia length in MCM-shJPH2 mice compared to MCM. Numbers in bar indicate number of mouse. g. Quantitative PCR analysis of cardiac stress markers Acta1, ANF, BNP, βMHC, and RCAN1-4 in hearts from MCM and MCM-shJPH2 mice (n=7 each group). Data are represented as mean ± SEM; * P<0.05 versus MCM control.

Legend Supplemental Figure 2. Altered morphology of T-Tubules following knockdown of JPH2.

a-b. Bright field (a) and confocal fluorescence images (b) of representative di-8-ANEPPS-stained single ventricular myocytes isolated from tamoxifen-treated MCM (left) and MCM-shJPH2 (right) mice. c. Respective high resolution views and binary imaged (threshold at mean pixel intensity) of areas within the red rectangles in panel b. e. Corresponding Fast Fourier transformed images of T-
Tubule images shown in panel c. f. Representative power vs. spatial frequency along the x axis computed using Fourier analysis. g. Bar graph showing normalized power at spatial frequency of \~0.55 \mu m^{-1} (as indicated by the arrow in panel e). Scale bar = 10 \mu m. *** P<0.001 versus MCM control. n = 21 cells from 5 tamoxifen-treated MCM and 6 MCM-shJPH2 mice.

**Legend Supplemental Figure 3.** Inducible cardiac-specific JPH2 knockdown does not alter expression and phosphorylation levels of other calcium-handling proteins.

a. Representative Western blots of cardiac tissue lysates from MCM (n=6) and MCM-shJPH2 (n=6) mice showing expression levels of Na\(^+\)/Ca\(^{2+}\)-exchanger (NCX1), sarcoplasmic reticulum Ca\(^{2+}\) ATPase 2 (SERCA2) and calsequestrin 2 (Csq2). b. Bar graphs summarizing quantitative analyses of the Western Blots shown in a. Protein expression levels were normalized first to corresponding GAPDH levels and then to average MCM value. c, e. Representative Western blot analyses of cardiac tissue lysates from MCM (n=6) and MCM-shJPH2 (n=6) mice showing expression and phosphorylation levels of ryanodine receptor 2 (RyR2; panel c) and phospholamban (PLN; panel e) at protein kinase A site (pS2808) and calcium calmodulin kinase II site (pS2814). d, f. Bar graphs summarizing quantitative analyses of the Western Blots shown in c and e respectively. RyR2 and PLN phosphorylation levels were normalized first to respective total protein expression levels and then to average MCM value. Data are represented as mean ± SEM.

**Legend Supplemental Figure 4.** Inducible cardiac-specific JPH2 knockdown decreases expression level of JPH2 and its interaction with RyR2 to similar extents.

a. Representative Western blots of cardiac tissue lysates and immunoprecipitates (IP) from MCM (n=4) and MCM-shJPH2 (n=5) mice showing expression levels of ryanodine receptor 2 (RyR2) and junctophilin 2 (JPH2). RyR2 was immunoprecipitated using anti-RyR2 antibody from cardiac lysates (right). b. Bar graphs summarizing quantitative analyses of the Western Blots shown in a.
Protein expression levels were normalized to average MCM value. Data are represented as mean ± SEM; * P<0.05 and **P<0.005, both versus MCM control.
**Supplemental Table.** Left ventricular echocardiographic parameters of MCM, MCM-shJPH2 line 21, MCM-shJPH2 line 38 and MCM-shJPH2 line38-OE mice at one week after finalizing tamoxifen treatment.

<table>
<thead>
<tr>
<th></th>
<th>MCM (n=17)</th>
<th>MCM-shJPH2 line 21 (n=11)</th>
<th>MCM-shJPH2 line 38 (n=14)</th>
<th>MCM-shJPH2-OE (n=8)</th>
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<tr>
<td>BW (g)</td>
<td>37.0 ± 0.8</td>
<td>35.2 ± 1.0</td>
<td>37.5 ± 1.7</td>
<td>35.0 ± 2.1</td>
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<tr>
<td>HR (b.p.m.)</td>
<td>450 ± 7#</td>
<td>468 ± 10**,#</td>
<td>485 ± 11*,###</td>
<td>420 ± 4***</td>
</tr>
<tr>
<td>ESD (mm)</td>
<td>3.19 ± 0.07</td>
<td>4.11 ± 0.13***,#</td>
<td>3.89 ± 0.16***,#</td>
<td>3.16 ± 0.20</td>
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<tr>
<td>EDD (mm)</td>
<td>4.35 ± 0.06</td>
<td>4.80 ± 0.07**,#</td>
<td>4.73 ± 0.08**,#</td>
<td>4.37 ± 0.18</td>
</tr>
<tr>
<td>EF (%)</td>
<td>52.0 ± 2.2</td>
<td>30.3 ± 3.7***,#</td>
<td>36.7 ± 4.3**,#</td>
<td>54.2 ± 3.0</td>
</tr>
<tr>
<td>FS (%)</td>
<td>26.7 ± 1.3</td>
<td>14.5 ± 1.9***,#</td>
<td>18.2 ± 2.3**,#</td>
<td>28.1 ± 1.8</td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>0.86 ± 0.01</td>
<td>0.76 ± 0.02****,#</td>
<td>0.82 ± 0.02</td>
<td>0.83 ± 0.01</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.76 ± 0.01</td>
<td>0.71 ± 0.02</td>
<td>0.76 ± 0.02</td>
<td>0.74 ± 0.01</td>
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<tr>
<td>LVPWs (mm)</td>
<td>1.13 ± 0.02</td>
<td>1.02 ± 0.06</td>
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</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.79 ± 0.02</td>
<td>0.83 ± 0.04</td>
<td>0.80 ± 0.03</td>
<td>0.72 ± 0.03</td>
</tr>
</tbody>
</table>

BW = body weight; HR = heart rate; b.p.m. = beats per minutes; ESD = end-systolic diameter; EDD = end-diastolic diameter; EF = ejection fraction; FS = left ventricular fractional shortening; IVS = intraventricular septal wall thickness (s, systole d, diastole); LVPW = left ventricular posterior wall thickness (s, systole d, diastole). Data are expressed as mean ± SEM. * P<0.05, ** P<0.01, *** P<0.001 versus MCM. # P<0.05, ## P<0.01, ### P<0.001 versus MCM-shJPH2-OE.
Supplemental Figure 1.

(a) Schematic diagram of the expression plasmid. JPH2 is driven by CMV promoter and tagged with cDNA and polyadenylation signal (pA). shRNA is driven by U6 promoter and targets JPH2.

(b) Western blot analysis showing JPH2 expression levels. JPH2 is detected at ~88 kDa, while GAPDH is detected at ~35 kDa. JPH2 expression is shown with + and shRNA concentration levels are indicated by numbers 1, 2, 3, 4, and e.v. indicates empty vector.

(c) Bar graph showing fold expression levels of MCM and MCM-shJPH2. The black bars represent MCM and the white bars represent MCM-shJPH2. The numbers on the bars indicate the sample size.

(d) Bar graph showing left ventricular posterior wall thickness (LVPWd) in mm. The black bars represent MCM and the white bars represent MCM-shJPH2. The numbers on the bars indicate the sample size.

(e) Bar graph showing myocardial area (a.u.). The black bars represent MCM and the white bars represent MCM-shJPH2. The numbers on the bars indicate the sample size. The numbers in parentheses are the mean (SD).

(f) Bar graph showing lung weight/tibia length ratio (mg/mm). The black bars represent MCM and the white bars represent MCM-shJPH2. The numbers on the bars indicate the sample size.

(g) Bar graph showing fold expression of Acta1, ANF, BNP, βMHC, and RCAN1-4. The black bars represent MCM and the white bars represent MCM-shJPH2. The numbers on the bars indicate the sample size.
Supplemental Figure 2.
Supplemental Figure 3

(a) Western blot analysis of NCX1, SERCA2a, Casq2, and GAPDH with their molecular weights indicated. (b) Bar graph showing protein expression levels of NCX, SERCA2a, and Casq2. (c) Western blot analysis of RyR2, pS2808, and pS2814 with their molecular weights indicated. (d) Bar graph showing protein phosphorylation levels of pS2808/RYR2 and pS2814/RYR2. (e) Western blot analysis of PLN, pS16, and pT17 with their molecular weights indicated. (f) Bar graph showing protein phosphorylation levels of pS16/PLN and pT17/PLN.
Supplemental Figure 4.