Cold-Inducible RNA-Binding Protein Regulates Cardiac Repolarization by Targeting Transient Outward Potassium Channels

Jun Li,* Duanyang Xie,* Jian Huang,* Fei Lv,* Dan Shi, Yi Liu, Li Lin, Li Geng, Yufei Wu, Dandan Liang, Yi-Han Chen

Rationale: Cold-inducible RNA-binding protein (CIRP) is constitutively expressed at low levels across various tissues. It is rapidly upregulated by multiple stresses, underlying a general role for CIRP in organic adaptations to pathophysiological conditions. However, the role of CIRP in the heart remains unclear.

Objective: To examine the biofunctions of CIRP in the mammalian heart.

Methods and Results: Rats with targeted disruption of Cirp were generated using the TALEN (transcription activator-like effector nucleases)-based genome editing technique. The Cirp-knockout rats had structurally and functionally normal hearts. Resting ECG recordings revealed a short rate-corrected QT (QTc) interval in Cirp-null rats without any abnormalities in PR interval, RR interval or QRS waves as compared to wild-type animals. The shortened QTc interval from Cirp ablation was tightly linked to an abbreviated action potential duration in cardiac myocytes, which was attributable to increased transient outward potassium current (I_{to}). Furthermore, our findings uncovered that CIRP protein selectively bonded to KCND2 and KCND3 mRNAs encoding the functional α-subunits of I_{to} channel proteins. Cirp deficiency did not change the transcriptional activity of KCND2 or KCND3, but it facilitated their translation. Cirp knockout had no effect on the functional expression of ion channels other than I_{to} channels.

Conclusions: CIRP modulates cardiac repolarization by negatively adjusting the expression and function of I_{to} channels. Our study may open a window to decipher the potential function of RNA-binding proteins in bioelectrical activity. (Circ Res. 2015;116:1655-1659. DOI: 10.1161/CIRCRESAHA.116.306287.)

Key Words: action potential ■ cold shock proteins ■ ion channels ■ myocytes, cardiac ■ repolarization

RNA-binding proteins are key regulators of gene expression.1 Cold-inducible RNA-binding protein (CIRP) is an 18-kDa protein consisting of an amino-terminal RNA-binding domain and a carboxyl-terminal glycine-rich domain. The expression of Cirp was first identified in murine germ cells, a cell type naturally exposed to temperatures lower than body temperature.2 Subsequent studies reveal that Cirp is expressed in a large variety of tissues, including the testis, brain, lung, kidney, liver, stomach, bone marrow, and heart.3,4 CIRP has been characterized as a cold-inducible protein that plays an essential role in cold-induced cell growth suppression.5 The induction of CIRP exerts antiapoptotic and cell-protective effects under cold temperatures.6-8 In addition to cold stress, CIRP is also inducible by other stimuli such as ultraviolet radiation and hypoxia.9,10 Recent reports have demonstrated that CIRP can modulate circadian gene expression11 and functions as a damage-associated molecular pattern molecule promoting inflammatory responses in hemorrhagic shock and sepsis.4 These findings suggest that CIRP may be extensively involved in physiological and pathological processes. However, the biofunction of CIRP in the heart has remained to be elucidated.

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Methods
An expanded Methods section is available in the Online Data Supplement and includes detailed information on the generation of Cirp

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1655
knockout rats, ECG monitoring, cardiomyocyte action potential and ion channel currents recording, quantitative immunoblotting, RNA immunoprecipitations, luciferase reporter assay, and statistical analyses.

**Results**

**Cirp Knockout Shortens QTc Interval in Rats**

To explore the physiology of CIRP in the mammalian heart, we generated genetically engineered rat lines using TALEN (transcription activator-like effector nucleases)-based genome editing to target exon 3 of *Cirp* to ablate its expression in intact rats (Figure 1A). Heterozygous rats were interbred to produce homozygous-deficient rats. The offspring exhibited the expected Mendelian ratios. Sequencing analysis of the targeted *Cirp* locus

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Ablation of cold-inducible RNA-binding protein (CIRP) shortens QTc interval in rats. **A**, A schematic overview of the TALEN (transcription activator-like effector nucleases)-based genome-editing strategy used to generate the Cirp knockout (KO). **Bottom**, Representative chromatogram showing a microdeletion and representative sequences of mutated alleles identified from clonal amplicons. Red dashes indicate the deleted bases. **B**, Western blot analysis of CIRP in rat hearts. **Upper**, Representative blots; **lower**, pooled data. The presented blots are representative of 3 separate experiments. **C**, Representative electrocardiograms from wild-type (WT) vs Cirp-null rats. **D**–**G**, Analysis of ECGs parameters. WT: n=8; Cirp-KO: n=8.
revealed a mutation comprising a 7-bp deletion (Figure 1A). Western blotting analysis confirmed the gene-targeting efficiency of Cirp, demonstrating a significant reduction in CIRP protein expression (Figure 1B). The homozygous Cirp-knockout (Cirp-KO) rats were viable, fertile, and showed no apparent health defects.

Next, we analyzed the cardiac phenotype of Cirp-KO rats. At 2 months of age, the echocardiographic and histologic analysis revealed no significant differences in cardiac mechanical function, left ventricular posterior wall thickness, ventricular wall diameter, and interstitial fibrosis between wild-type and Cirp-KO rat hearts (Online Figure I). We then evaluated the effects of Cirp on cardiac electrophysiological homeostasis. Telemetric ECG recording of Cirp-KO rats demonstrated normal PR interval, RR interval, and QRS duration, but there was a prominent shortening of QTc interval (Figure 1C–1G).

**Cirp Ablation-Mediated Increase of Transient Outward Potassium Current Contributes to Cardiac Repolarization Acceleration**

To investigate the cellular mechanisms underlying the phenotype of QTc interval shortening on ECG from Cirp ablation, we examined whether Cirp null affects action potential in ventricular myocytes. As shown in Figure 2A to 2D, there was a dramatic abbreviation of action potential duration in isolated Cirp-null cardiac myocytes, indicating an acceleration of repolarization. Next, using the whole-cell patch-clamping technique, we analyzed the ion-channel currents responsible for the alterations in action potential duration and the ECG changes mentioned above in Cirp-null rats. Notably, in Cirp-deficient cardiac myocytes, the current densities of $I_{\text{Ko}}$, $I_{\text{Na}}$, and $I_{\text{Ca.L}}$, which, respectively, contribute to different phases of action potential in rat ventricular myocytes, were comparable with those in wild-type myocytes (Online Figure II). In contrast, we observed a marked increase in transient outward potassium current ($I_o$) current density without alterations in the activation, inactivation, or reactivation parameters (Figure 2E–2H).

**Cirp Modulates the Functional Expression of Transient Outward Potassium Channel α-Subunits Post-Transcriptionally**

To gain insight into the increase in $I_o$ current in Cirp-null cardiac myocytes, the protein levels of $I_o$ channels were measured. The current consensus is that Kv4.2 and Kv4.3 are the primary α-subunits in rat ventricular myocytes. We found that Cirp ablation upregulated the protein levels of subunits Kv4.2 and Kv4.3, and there was no significant alteration in the auxiliary subunit Kv channel–interacting protein 2 (KChIP2) in Cirp-null cardiac myocytes (Figure 3A). Considering the inherent capacity of CIRP to target RNAs, we further analyzed the potential binding of CIRP to KCND2, KCND3, and KChIP2 mRNAs using a CIRP antibody-based RNA-immunoprecipitation technique. The polymerase chain reaction assay identified the expression of KCND2 and KCND3 mRNAs in CIRP-bound transcripts (Figure 3B). We then investigated the function of CIRP in KCND2 and KCND3 mRNAs metabolism. As shown in Figure 3C and 3D, whole-cell and cytoplasmic KCND2 and KCND3 mRNA levels were comparable in all the conditions tested. The luciferase reporter–based assay indicated that CIRP did not affect the transcriptional activity of KCND2, KCND3, and KChIP2 mRNAs (Figure 3E). Collectively, CIRP post-transcriptionally modulates expression of the α-subunits of $I_o$ channels. A deficiency of CIRP facilitates the protein expression of $I_o$ channels.

**Discussion**

In this study, we identified a novel physiological role of CIRP in cardiac repolarization. First, ablation of Cirp by in vivo gene targeting demonstrated a shortened QTc interval ECG phenotype with structurally and functionally normal hearts.

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**Figure 2.** Cirp deficiency abbreviates action potential duration (APD) and amplifies transient outward potassium current in rat ventricular myocytes. A to D, Representative ventricular action potentials from all study groups with summary data (n=11 for wild-type [WT], n=14 for Cirp-knockout [KO]). E to H, Representative outward current recordings from all study groups and summary data (n=10 for WT, n=12 for Cirp-KO). *P<0.05 compared with WT. APA indicates action potential amplitude; APD_{50} and APD_{90}, action potential duration at 50% and 90% repolarization, respectively.
Second, a abbreviated action potential duration was observed in Cirp-null ventricular myocytes. Third, reduction in CIRP amplified $I_{\text{Na}}$ channel current without any effect on other sarcocennell ion channels. Finally, CIRP deficiency facilitated the protein expression of $I_{\text{Na}}$ channel α-subunits. Therefore, it is shown here that CIRP regulates cardiac repolarization by controlling the expression and function of $I_{\text{Na}}$ channels in cardiac myocytes.

RNA-binding proteins are essential components in the post-transcriptional regulation of gene expression, and they represent a set of homeostatic regulators for protein expression.1 Once RNAs are transcribed, they are cotranscriptionally associated with RNA-binding proteins to form ribonucleoprotein complexes, which regulate every aspect of RNA metabolism including RNA splicing, nuclear export, localization, and translation.1 As an RNA-binding protein, CIRP has been described to modulate gene expression at the post-transcriptional level.6,11 In the cardiomyocyte of CIRP-KO rats, there were no changes in whole cell and cytosolic KCND2/3 mRNAs. Luciferase reporter–based experiments revealed that CIRP did not affect KCND2/3 gene transcription activity (Figure 3). In contrast to the constancy of KCND2/3 mRNAs, the amount of KCND2/3 protein changed in the direction opposite to that of CIRP, suggesting a negative regulation of KCND2/3 translation by CIRP.

Considering that abnormal ventricular repolarization is tightly linked to a variety of arrhythmic manifestations,13 the constitutive expression of CIRP in the heart and the CIRP-dependency of cardiac repolarization may imply the critical involvement of CIRP in cardiac physiology and pathophysiology. Importantly, RNA-binding proteins are important molecules with universal biological activities. The present findings may provide insight into the pivotal roles of these molecules in cardiac electrophysiology. Uncovering the potential roles of these RNA-binding proteins in cardiac electrophysiology will be one of the important research directions in the field of cardiology.

Sources of Funding

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Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- RNA-binding proteins are essential components in the post-transcriptional regulation of gene expression and represent a set of homeostatic regulators for protein expression.
- Cold-inducible RNA-binding protein (CIRP) is rapidly upregulated by multiple stresses, underlying a general role for CIRP in organic adaptations to pathophysiological conditions.
- Cardiac repolarization plays an important role in the clinical development of a variety of arrhythmic manifestations and its prolongation has been identified as a risk factor for sudden cardiac death.

**What New Information Does This Article Contribute?**

- Ablation of *Cirp* promoted prominent shortening of QT interval, which resulted from elevated transient outward potassium current ($I_{to}$)-induced abbreviation of the action potential duration in ventricular cardiac myocytes.
- CIRP negatively modulates translation of the $\alpha$-subunits of $I_{to}$ channels without any effect on other sarcolemmal ion channels.

RNA-binding proteins are key regulators of gene expression. However, their roles in the heart remain unclear. We reveal that ablation of *Cirp* induced prominent shortening of the QT interval. Abbreviation of the action potential duration was observed in *Cirp*-null ventricular myocytes, which was tightly linked to the increased protein expression of $I_{to}$ channel $\alpha$-subunits and the consequent amplification of $I_{to}$ currents. This finding brings new insight into the mechanisms underlying cardiac repolarization abnormalities that might be important in the pathophysiology of the long and short QT syndromes and opens a window to decipher the potential function of RNA-binding proteins in regulating bioelectric activity.
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SUPPLEMENTARY MATERIAL

Methods and Materials

Generation of Cirp-Knockout Rats

All animal protocols were approved by the Animal Care and Use Committee at the Tongji University School of Medicine and conducted according to the NIH Guidelines for Care and Use of Animals in Research (NIH Publication No. 85-23, revised 1996).

A TALEN-based genome editing technique was adopted to produce knockout rats by targeting exon 3 of Cirp. To confirm the efficiency of Cirp deletion, quantitative real-time polymerase chain reaction (PCR) using TaqMan primer sets spanning the constitutively expressed exon 3 were used to measure the total transcription of the Cirp gene using cDNA from wild-type (WT) and Cirp-knockout rat cardiomyocytes. Quantitative real-time PCR reactions were performed in a 384-well format using an ABI 7900HT instrument (Applied Biosystems). Sequencing analysis and western blotting were performed to detect base pair deletions and frameshift mutations of Cirp and CIRP protein levels, respectively. Experiments were performed on adult (2-month-old) rats.

Echocardiographic Analysis and ECG Monitoring

To evaluate left ventricular function and dimension, transthoracic two-dimensional echocardiography was performed on rats sedated with 5% isoflurane using a Visual Sonics Vevo 770 ultrasound (Visual Sonics) equipped with a 30-MHz linear array transducer. M-mode tracings in the parasternal short axis view were used to measure left ventricular anterior and posterior wall thicknesses and left ventricular internal diameter at end-systole and end-diastole, which were used to calculate left ventricular fractional shortening and the ejection fraction. Telemetric ambulatory long-term ECG recordings were obtained with implantable transmitters. Rats were anesthetized with ketamine hydrochloride (150 mg/kg, IP) and pentobarbital (54 mg/kg, IP), and a midline incision was made along the spine. An implantable 3.5-g wireless radiofrequency transmitter (DataSciences International) was aseptically inserted into a subcutaneous tissue pocket in the peritoneum. The rats were placed in cages overlying a receiver for transmission of ECG signals to a computer for display and analysis, including assessment of heart rate variability parameters with spectral analysis of time and frequency domains.

Cardiomyocyte Isolation and Electrophysiology

Ventricular myocytes were isolated from 2-month-old WT rats and Cirp-knockout littersmates using a previously described method.1 Each cellular experiment employed cardiomyocytes isolated from three sex-matched rats per genotype. Whole-cell patch clamping was applied for
ionic current and AP recording at room temperature. Ionic currents were recorded with a tight-seal patch clamp in voltage clamp mode, and APs were recorded in current clamp mode. Borosilicate-glass electrodes had tip resistance between 2 and 5 MΩ. Current is expressed as current density (normalized to cell capacitance).

Standard Tyrode’s solution contained (mmol/L) NaCl 136, KCl 5.4, MgCl₂ 1, CaCl₂ 1, NaH₂PO₄ 0.33, HEPES 5, and dextrose 10 (pH 7.35 with NaOH). Standard pipette solution contained (mmol/L) K-aspartate 110, KCl 20, MgCl₂ 1, MgATP 5, GTP 0.1, HEPES 10, Na-phosphocreatine 5, and EGTA 5 (for current recording) or 0.025 (for AP recording) (pH 7.3, KOH).

For AP recording, nystatin (60 μg/mL) was back-filled into the pipette tip, and external solutions contained 2 mmol/L CaCl₂. For K⁺ currents other than transient-outward current (Iₜₒ), 1 mmol/L 4-aminopyridine was added. Inward-rectifier current (Iᵣᵣ) was studied as 1 mmol/L Ba²⁺-sensitive current.

For L-type calcium current (I_{cal}) studies, external solutions contained (mmol/L) tetraethylammonium chloride 136, CsCl 5.4, CaCl₂ 2, MgCl₂ 0.8, HEPES 10, and dextrose 10 (pH 7.4, CsOH). Niflumic acid (50 μmol/L) was added to inhibit I_{Cl,Cs}. The pipette solution contained (mmol/L) CsCl 20, Cs-aspartate 110, MgCl₂ 1, MgATP 5, GTP 0.1, Na₂-phosphocreatine 5, EGTA 10, and HEPES 10 (pH 7.2, CsOH).

To record Nav1.5 currents, we used an external K⁺-free solution that contained (in mmol/L): 135 NaCl, 1.8 CaCl₂, MgCl₂ 1.0, TEA-Cl, 10 HEPES, and 10 glucose with a pH of 7.4 adjusted with NaOH. Glass electrodes were filled with an internal solution that contained (in mmol/L): CsF 120, NaCl 10, CaCl₂ 1, MgCl₂ 1, EGTA 10, TEA-Cl 10 and HEPES 10 with a pH of 7.3 adjusted with CsOH. To better control I_{Na} recording, the internal and external Na⁺ concentrations were lowered to 20 mmol/L.

For Iₚ₆ recordings, atropine (1 μmol/L) and CdCl₂ (200 μmol/L) were added in external solutions to eliminate muscarinic K⁺-currents and to block Ca²⁺ currents. Na⁺ current contamination was avoided by using a holding potential (HP) of -50 mV or by substitution of equimolar Tris HCl for external NaCl. The pipette solution contained (mmol/L) CsCl 20, Cs-aspartate 110, MgCl₂ 1, MgATP 5, GTP 0.1, Na₂Phosphocreatine 5, EGTA 10 and HEPES 10 (pH 7.2 with CsOH).

**RNA Immunoprecipitation (RIP)**

RNA-IP was performed using the kit protocol (Magna RIP™ RNA-binding Protein Immunoprecipitation Kit, Millipore, Billerica, MA, USA).

**Real-time PCR**
Total RNA was extracted from cell samples with homogenization in TRIzol Reagent (Invitrogen), chloroform extraction and isopropanol precipitation. Genomic DNA was eliminated by incubation in DNase I (0.1-U/µL, 37°C) for 30 minutes, followed by phenol-chloroform acid extraction and gel verification. RNA was quantified spectrophotometrically at 260-nm wavelength and integrity was confirmed on a denaturing agarose gel. RNA samples were stored in DEPC H₂O at -80°C. First-strand cDNA was synthesized by RT with 2 µg of RNA sample, random primers and MMLV reverse transcriptase (High Capacity cDNA Archive Kit, Applied Biosystems). Real-time PCR was conducted with a Stratagene Mx3000P QPCR detection system and was performed with the Taqman quantitative assay. Commercially purchased 18s rRNA (Applied Biosystems) was used as the internal control. Primers and probes for the real-time PCR reactions are listed in on-line Table 1. Each sample was run in duplicate, and PCR products were verified with gel electrophoresis.

**DNA Constructs**

The bidirectional Renilla/Firefly luciferase constructs containing zero or eight MS2-CP-binding sites were described elsewhere. Plasmids encoding CIRP-MS2-CP were obtained by inserting the full-length sequences flanked by the Flag epitope between the BamHI and XbaI sites of pCMS2. All the constructs were subsequently sequenced.

**Luciferase Assay**

Cell lysis and luciferase assays were performed using the Promega Dual-luciferase system and a TD-20/20 luminometer (Turner Designs). Each assay was conducted using three batches of independently transfected cells. The statistical analyses were performed using Graphpad Prism 5 software.

**Immunoblotting**

Protein concentration was determined with the Bradford method. From 20-40 µg of membrane protein sample, total cell lysate, total cell extract or nuclear extract from cells was denatured with Laemmli buffer and fractionated on 8% or 12% SDS-polyacrylamide gels, then proteins were transferred electrophoretically to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore) in 25-mmol/L Tris-base, 192-mmol/L glycine and 20%-ethanol at 0.3 A for 1 hour. Membranes were blocked in 1 X TBS-T with 5% non-fat dry milk for 1 hour and incubated with primary antibodies (goat anti-Kv4.3/Kv4.2, 1:500, Santa Cruz; mouse anti-KChIP2, 1:5000, Badrilla; mouse anti-phospholamban [total], 1 µg/mL, Affinity Bioreagent (ABR); mouse anti-Cav1.2, 1:5000, BD Transduction; mouse anti-Nav1.5, 1:2500, ABR; mouse anti-KCNJ2, 1:2500, ABR; mouse anti-CIRP, 1:1000, Santa Cruz) overnight at 4°C. After washing and re-blocking, the membranes were incubated with
horseradish peroxidase-conjugated goat anti-rabbit, donkey anti-goat or goat anti-mouse IgG secondary antibody (1:10,000, Jackson Immunolabs or Santa Cruz). Antibodies were detected with Western-Lightening Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences). Later, the same membranes were also probed with anti-GAPDH at room temperature for 2 hours to control for protein loading. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000, Santa Cruz).

Statistics

All data are expressed as means ± SEM. GraphPad Prism 5.0 was used for data analysis; curve fitting was performed with nonlinear least-square algorithms. Group comparisons were performed with unpaired Student's t tests. Differences were considered to be significant at P<0.05.

References


Online Figure Legends

Online Figure I. Baseline functional and structural characteristics of Cirp-knockout rat hearts. A, Echocardiographic evaluation of heart function. Top, Representative echocardiographic images. Bottom, quantitative statistics of ejection fraction (EF), fractional shortening (FS) and left ventricular wall thickness (LVPWd) in WT and Cirp-KO rats. WT: n=8; Cirp-KO: n=8. P < 0.05 compared with WT, by one-tailed unpaired Student’s t-test with Welch’s correction. B, Representative histological sections of 2-month-old WT and Cirp-knockout rat hearts stained with hematoxylin and eosin (H&E). C, Masson’s trichrome staining for fibrosis of histological sections of 2-month-old rat hearts. Three to five animals were employed at the indicated time point in different groups.

Online Figure II. Cirp knockout did not alter protein expression and function of ion channels other than I\textsubscript{Na} channel. A, Western blotting examination of HERG, KCNQ1, Kir2.1, Nav1.5, Cav1.2 and KIf15 proteins in the Cirp-null cardiomyocytes. Left, representative blots. Right, pooled data. B-D, Representative I\textsubscript{K1}, I\textsubscript{Na} and I\textsubscript{Ca,L} currents from all study groups with summary data in bar graphs (n = 13 for wild-type, n = 11 for Cirp-KO).

Online Table I: Collection of primer sequences for PCR experiments.

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