

REEP5 (Receptor Accessory Protein 5) Acts as a Sarcoplasmic Reticulum Membrane Sculptor to Modulate Cardiac Function

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Background—Heart failure is a complex syndrome characterized by cardiac contractile impairment with high mortality. Defective intracellular Ca^{2+} homeostasis is the central cause under this scenario and tightly links to ultrastructural rearrangements of sarcolemmal transverse tubules and the sarcoplasmic reticulum (SR); however, the modulators of the SR architecture remain unknown. The SR has been thought to be a specialized endoplasmic reticulum membrane system. Receptor accessory proteins (REEPs)/DP1/Yop1p are responsible for shaping high-curvature endoplasmic reticulum tubules. This study aimed to determine the role of REEPs in SR membrane shaping and thus cardiac function.

Methods and Results—We identified REEP5 (receptor accessory protein 5) as more highly expressed than other REEP members in adult rat ventricular myocardium, and it was downregulated in the failing hearts. Targeted inactivation of *REEP5* in rats specially deformed the cardiac SR membrane without affecting transverse tubules, and this was visualized by focused ion beam scanning electron microscopy-based 3-dimensional reconstruction. Accordingly, simultaneous recordings of depolarization-induced Ca^{2+} currents and Ca^{2+} transients in *REEP5*-null cardiomyocytes revealed normal L-type Ca^{2+} channel currents but a depressed SR Ca^{2+} release. Consequently, the excitation–contraction coupling gain of cardiomyocytes and consequent cardiac contractility were compromised. REEP5 deficiency did not alter the expression of major proteins involved in Ca^{2+} handling in the heart.

Conclusions—REEP5 modulates cardiac function by shaping the SR. REEP5 defect deforms the SR architecture to depress cardiac contractility. REEP5-dependent SR shaping might have potential as a therapeutic target for heart failure. (*J Am Heart Assoc.* 2018;7:e007205. DOI: 10.1161/JAHA.117.007205.)

Key Words: cardiac myocyte • excitation–contraction coupling • electron microscopy • heart failure • sarcoplasmic reticulum

The prominent hallmark of heart failure is contractile dysfunction. Intracellular Ca^{2+} mishandling critically underlies the mechanical defects in failing myocardium.¹ A tight junction between specialized domains of the sarcoplasmic reticulum (SR) and sarcolemmal transverse tubules (t-tubules), called *calcium release units*, determines intracellular Ca^{2+} homeostasis and consequent cardiomyocyte contractility.^{2–4} Ultrastructural rearrangements to SR and t-tubule networks represent a major feature in cases of

heart failure.^{5–7} Several regulators for t-tubule integrity have been identified, for example, BIN-1 (bridging integrator 1) and JPH2 (junctophilin 2)^{8,9}; however, understanding of molecular entities underlying the structure-based function of SR is still very limited.

The SR has been considered merely a specialized endoplasmic reticulum (ER) network.¹⁰ High-curvature ER tubules are generated and maintained by an integral membrane protein superfamily consisting of DP1 in mammals.^{11,12}

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Accompanying Figures S1 through S3 are available at <http://jaha.ahajournals.org/content/7/3/e007205/DC1/embed/inline-supplementary-material-1.pdf>

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Clinical Perspective

What Is New?

- REEP5 (receptor accessory protein 5) is an important molecule for normal cardiac sarcoplasmic reticulum shaping and function.
- REEP5 ablation results in abnormal Ca^{2+} metabolism in the heart by disrupting the spatial contiguity between the sarcoplasmic reticulum and transverse tubules, leading to depressed cardiac contractility.

What Are the Clinical Implications?

- This discovery helps us further understand the molecular mechanisms for intracellular Ca^{2+} activity and heart failure and implicates a new therapeutic target for cardiac dysfunction.

Receptor accessory proteins (REEPs) are members of the DP1 family.¹² Six REEPs (REEP1 to REEP6) have been identified in humans and other mammals.¹³ They are classified into 2 subfamilies: (1) REEP1 to REEP4 and (2) REEP5 and REEP6. A direct role for REEP5/DP1 in shaping ER tubules has been established.¹¹ Whether REEPs contribute to SR membrane shaping and the associated SR Ca^{2+} handling and cardiac function remains to be characterized.

In this study, using focused ion beam scanning electron microscopy-based 3-dimensional (3D) reconstruction, we provided compelling visual evidence that knockout of *REEP5*, which was highly expressed in intact cardiomyocytes and downregulated in the failing hearts, deformed the SR membrane structure and disrupted the physical coupling of the SR to t-tubules. Consequently, the uncoupling of the SR from t-tubules induced the depression of SR Ca^{2+} release and cardiac contraction.

Methods

The data, analytic methods, and study materials have been made available to other researchers for purposes of reproducing the results or replicating the procedure. The authors declare that all supporting data are available within the article and its online supplementary files.

Generation of *REEP5* Knockout Rats and Cardiomyocyte Isolation

All animal protocols were approved by the animal care and use committee at the Tongji University School of Medicine and were conducted according to the *Guidelines for the Care and Use of Animals in Research* (US National Institutes of Health Publication No. 85-23, revised 1996).

A CRISPR/Cas9-based genome-editing technique was adopted to produce knockout rats by targeting exon 2 of *REEP5*. We designed single guide RNAs for SpCas9 target selection using the CRISPR design tool (<http://crispr.mit.edu/>), which directed Cas9 DNA endonuclease to cleave the *Reep5* gene and to create a double-strand break, disrupting the expression of REEP5 by frameshift mutation. To confirm the efficiency of *REEP5* deletion, quantitative real-time polymerase chain reaction (PCR) using TaqMan primer sets spanning the constitutively expressed exon 2 were used to measure total transcription of the *REEP5* gene using cDNA from wild-type (WT) and *REEP5*-knockout (*REEP5*-KO) rat cardiomyocytes. Quantitative real-time PCR reactions were performed in a 384-well format using an ABI 7900HT instrument (Applied Biosystems). Sequencing analysis was performed to detect base pair deletions and frameshift mutations.

Ventricular myocytes were isolated from 8-week-old WT and *REEP5*-KO littermates using a previously described method.¹⁴ Each cellular experiment used cardiomyocytes isolated from 5 sex-matched rats per genotype.

Immunostaining

Dissociated cardiomyocytes were allowed to attach to glass coverslips precoated with laminin before fixation. For all immunocytochemistry experiments, the cells were fixed in methanol at room temperature for 5 minutes. After fixation, the cardiomyocytes were permeabilized with 0.1% Triton X-100 for 15 minutes and successively blocked with 10% normal goat serum in PBS for 1 hour at room temperature. Next, the cells were incubated in antibodies (1:50 for REEP5 and RyR2 [ryanodine receptor 2]) overnight at 4°C. After several washes with PBS after primary antibody incubation, the cells were then incubated in goat antimouse and antirabbit IgG conjugated to AlexaFluor 488 and 555, respectively. All cardiomyocytes were imaged by confocal microscopy (Leica TCS SP8) and analyzed using LAS AF and IPP 6.0 software.

Transmission Electron Microscopy

Transmission electron microscopy analysis of the rat ventricular tissue ultrastructure was performed as described previously.¹⁵ Tissue sections were imaged at $\times 20\,000$ and $\times 80\,000$ magnification using a transmission electron microscope (JEOL TEM1230 from the Electron Microscopy Core of the Shanghai Institute of Biological Science at the Chinese Academy of Sciences). For immunogold localization, the sections were incubated with anti-REEP5 for 3 days and then with antirabbit IgG conjugated to 4-nm gold particles for 3 hours at 21°C. Finally, the sections were post-fixed in 1% osmium tetroxide and 2% uranyl acetate, dehydrated, and flat-embedded in Durcupan ACM (Fluka) for light and electron microscopic examinations.

Focused Ion Beam Scanning Electron Microscopy Imaging and 3D Reconstruction

The hearts of WT and *REEP5* KO rats were harvested rapidly after anesthesia and fixed in a 0.1 mol/L phosphate buffer solution containing 4% paraformaldehyde. Focused ion beam scanning electron microscopy (from the Institute of Biophysics at the Chinese Academy of Sciences) was used to obtain continuous sectional slices at a spacing of 20 nm, according to a previous study.¹⁶ The 3D reconstruction of SR and t-tubules

was performed using ImageJ 1.5.1 (US National Institutes of Health) and the Imaris 7.2 software package (Bitplane).

Simultaneous Recording of Depolarization-Induced Ca^{2+} Currents and Ca^{2+} Transients

L-type Ca^{2+} channel (LTCC) currents and SR Ca^{2+} transients were simultaneously measured in acute isolated adult rat ventricular cardiomyocytes loaded with Fluo-4 pentapotassium salt (10 $\mu\text{mol/L}$; Thermo Fisher) via a patch-clamp

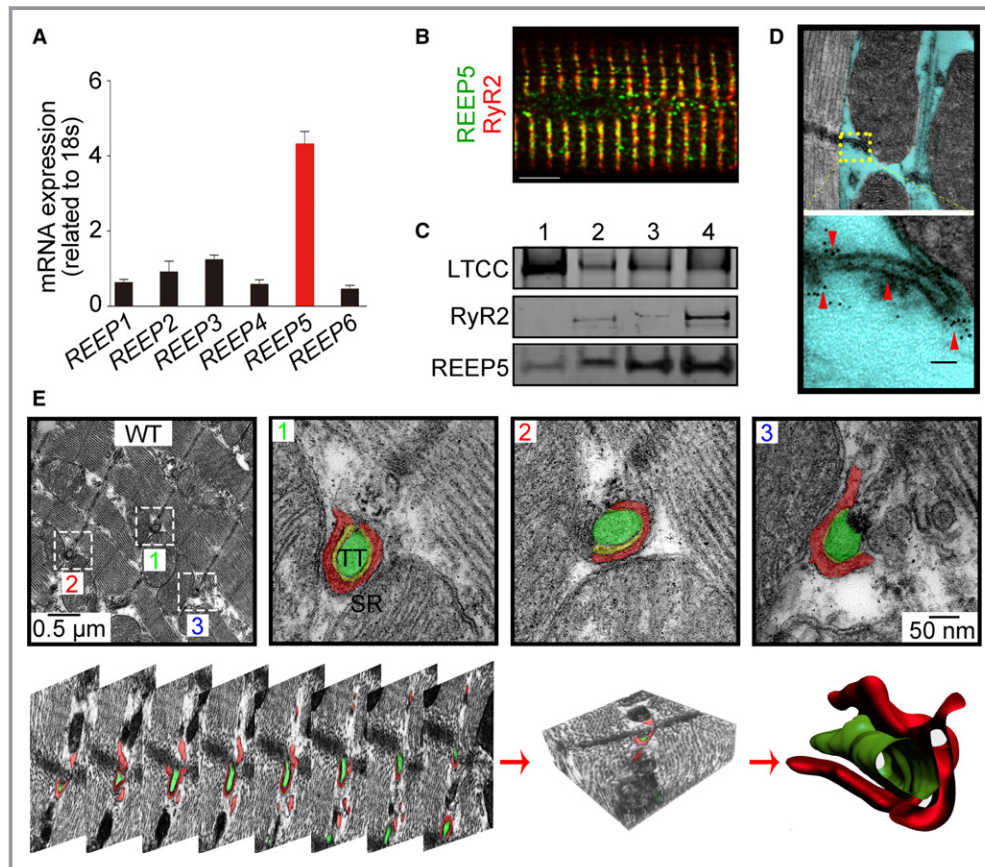


Figure 1. REEP5 mediates membrane shaping of the cardiac SR. **A**, Quantitative polymerase chain reaction measurements of *REEP1* through *REEP6* levels in adult rat ventricular myocardium. **B**, Immunofluorescence imaging of REEP5 in isolated intact adult rat ventricular myocytes. Scale bar: 5 μm . **C**, Identification of REEP5 proteins in sucrose gradient centrifugation–based membrane fractions. Numbers 1 to 4 indicate <23%, 23% to 29%, 29% to 32%, and 32% to 35% sucrose contents, respectively. **D**, Two-dimensional transmission EM images of normal heart sections immunolabeled for REEP5. The yellow box identifies the SR structure, and the red arrowheads denote the REEP5 proteins situated on the SR. **E** and **F**, Three-dimensional reconstruction of SR from serial EM images of WT and *REEP5*-KO heart sections. The top panels in (**E**) and (**F**) show EM images of axial cross-sections of heart tissue, and the bottom panels present serial aligned images from the same section. Bottom right shows digitized 3D reconstruction of the SR with an associated t-tubule from the serial images. The green regions indicate t-tubules, and the red regions indicate the SR. **G** and **H**, The apparent length of SR/t-tubule junction (**G**) and its distribution (**H**). Data are from 90 (WT) and 108 (*REEP5*-KO) SR/t-tubule junction images in 3 independent experiments per group. * $P < 0.05$ compared with WT controls. The blots and images shown are representative of 3 to 5 independent experiments. EM indicates electron microscopy; KO, knockout; LTCC, L-type Ca^{2+} channel; REEP, receptor accessory protein; RyR2, ryanodine receptor 2; SR, sarcoplasmic reticulum; t-tubule, transverse tubule; WT, wild type.

recording amplifier (EPC-10 USB; HEKA Elektronik) coupled to a confocal laser scanning microscope (Leica TCS SP8).

Measurement of Cardiac Function

In vivo pressure–volume analysis was performed in 8-week-old WT and *REEP5*-KO rats, as described previously.¹⁷ Briefly, steady-state data were recorded after 10 to 15 minutes of stabilization. Load-independent measurements were recorded during transient intrathoracic inferior vena cava occlusion. Data were recorded using LABCHART 7 software (AD Instruments) and analyzed offline using PVAN 3.6 software (Millar Instruments).

Real-Time PCR

Messenger RNA was extracted from rat heart tissue using TRIzol reagent. The reverse transcription reaction was performed using PrimeScript RT reagent (Takara), and real-time PCR was performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems).

Preparation of Subcellular Membrane Fractions From Cardiomyocytes

Membrane fractions of rat cardiomyocytes were prepared according to a sucrose gradient centrifugation–based approach, as described previously.^{18,19}

Western Blot Analyses and Antibodies

Western blot was performed according to standard protocols. The following antibodies were used: anti-REEP5, anti-Cav1.2, anti-RyR2, anti-SERCA2 α , anti-IP₃R, anti-PLB, anti-CaMKII, anti-CASQ, and anti- β -tubulin.

Statistical Analyses

The data are presented as the mean \pm SEM. Prism 6 software (GraphPad) was used for statistical analyses. For comparison between 2 groups, a paired or unpaired 2-tailed Student *t* test was performed. $P < 0.05$ was considered statistically significant.

Results

REEP5 Is Predominantly Concentrated at the Cardiac SR

To ascertain whether REEPs contribute to membrane shaping of the SR in the heart, we first examined the transcriptional profiles of REEPs in cardiomyocytes. REEP5 was more highly expressed than the other members screened in adult rat ventricular myocytes (Figure 1A). Next, we analyzed the subcellular localization of REEP5 in intact cardiomyocytes. Immunocytochemical imaging showed that

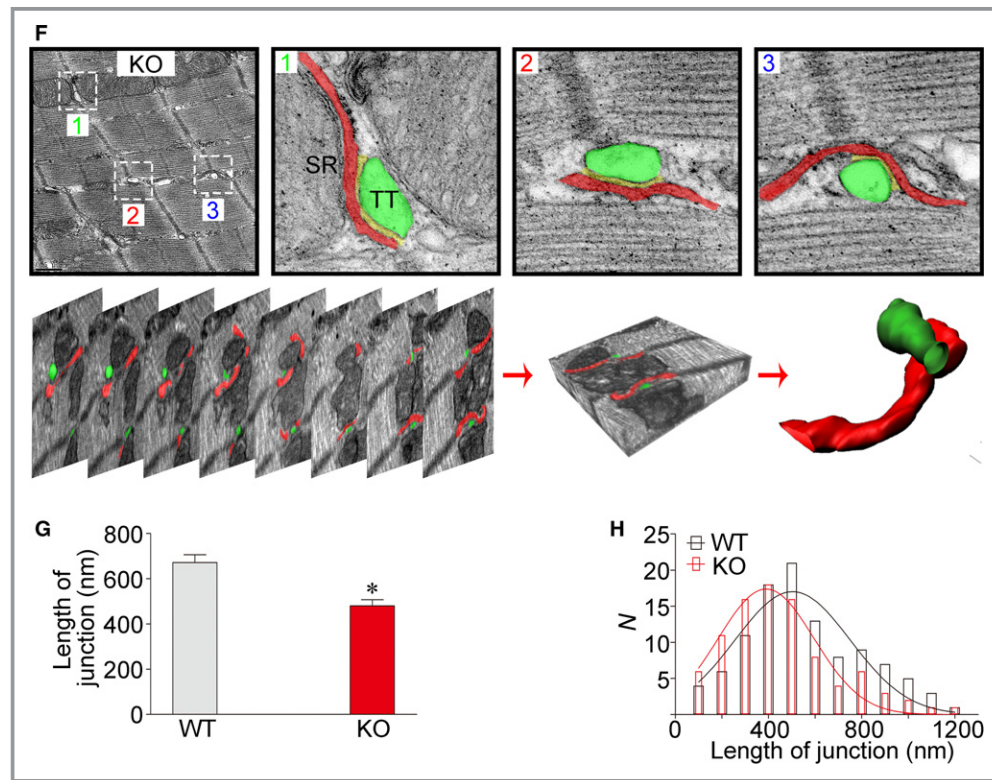


Figure 1. Continued

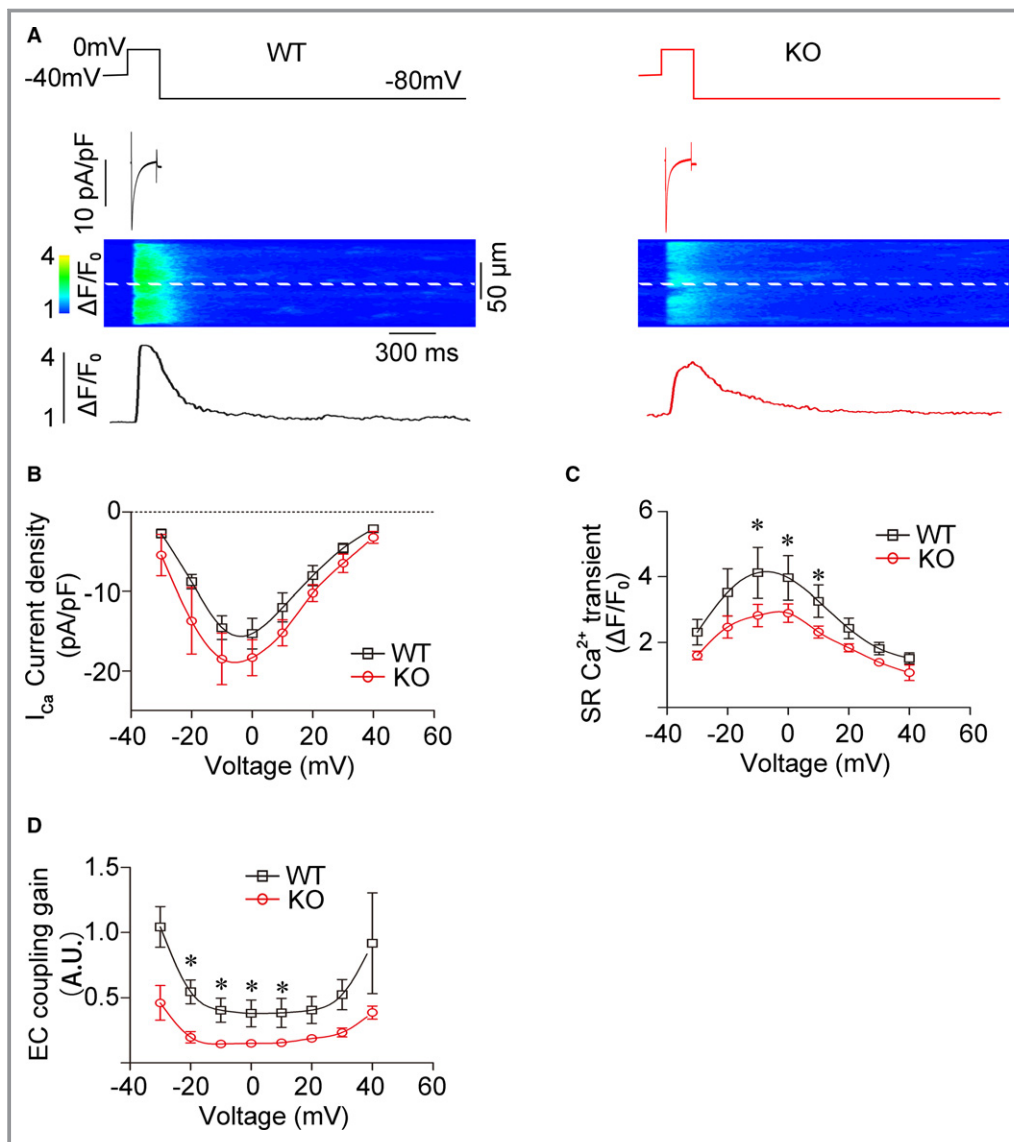


Figure 2. *REEP5* ablation depresses depolarization-induced SR Ca^{2+} release. Simultaneous recordings of LTCC currents and Ca^{2+} transients in freshly isolated rat ventricular myocytes. A, A typical LTCC current and a synchronously evoked Ca^{2+} transient. B through D, Pooled data. $n=9$ for WT, $n=13$ for *REEP5*-KO. * $P<0.05$ compared with WT controls. EC indicates excitation–contraction; KO, knockout; LTCC, L-type Ca^{2+} channel; REEP, receptor accessory protein; SR, sarcoplasmic reticulum; WT, wild type.

REEP5 extensively overlapped with RyR2, a representative SR membrane protein (Figure 1B). A sucrose gradient centrifugation–based approach also detected abundant *REEP5* in the subcellular fractionations in which RyR2 was enriched (Figure 1C), suggesting the predominant localization of *REEP5* at the SR membrane. This finding was further validated by immunogold labeling and transmission electron microscopy (Figure 1D).

REEP5 Knockout Deforms SR Membranes

We next generated genetically engineered rats using CRISPR/Cas9-based genome editing to target exon 2 of *REEP5* to

ablate its expression (Figure S1A). Heterozygous rats were interbred to produce homozygous animals. Offspring exhibited the expected mendelian ratios. Sequencing analyses of the targeted *REEP5* locus revealed that 1 founder possessed a mutation comprising an 8-bp deletion. Western blot analysis confirmed the gene-targeting efficiency of *REEP5*, revealing a significant reduction in *REEP5* protein expression (Figure S1B and S1C). Homozygous *REEP5*-KO rats were viable and fertile and appeared healthy.

To directly visualize the impact of *REEP5* deletion on the SR network in situ, we utilized focused ion beam scanning electron microscopy, which allows automatic connectome tracing for serial ultrathick sections of heart tissues to

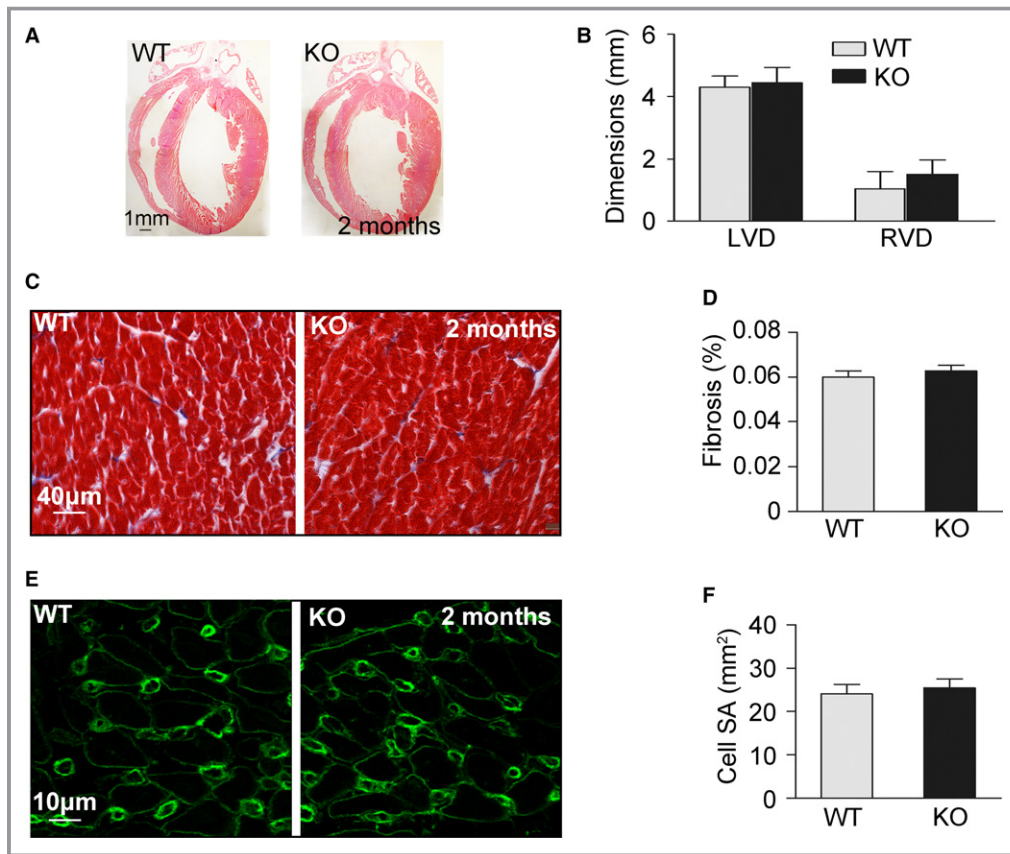


Figure 3. *REEP5*-KO compromises cardiac contractility in vivo. A, Representative histological sections of 2-month-old rat hearts stained with HE. B, Cardiac wall dimensions were assessed from HE-stained histological sections. C, Masson's trichrome staining for fibrosis in histological sections of rat hearts. D, Percentage of fibrosis quantified from a Masson trichrome stain. E, Representative WGA fluorescence stains of rat cardiomyocytes. F, Cardiomyocyte surface area calculated from WGA stains. WT, n=5; *REEP5*-KO, n=5 (A through F). G and H, Representative in vivo PV loops during transient inferior vena caval occlusion in a *REEP5*-KO rat and a WT control. ESPVR relationships are presented. I and J, Steady-state PV data demonstrating decreased PRSW and increased peak velocities of pressure change (peak+dP/dt) during isovolumic contraction in *REEP5*-KO rats. WT, n=8; *REEP5*-KO, n=8. **P*<0.05 compared with WT controls. ESPVR indicates end-systolic pressure–volume relationship; HE indicates hematoxylin and eosin; KO, knockout; LV, left ventricular; LVD, left ventricular wall dimension; PRSW, preload recruitable stroke work; PV, pressure–volume; REEP, receptor accessory protein; RVD, right ventricular wall dimension; SR, sarcoplasmic reticulum; WGA, wheat germ agglutinin; WT, wild type.

produce data sets optimized for final 3D reconstruction of the SR membrane structures. We found that the gross morphology of SR network is preserved in *REEP5*-KO cardiomyocytes. In high-magnification axial cross-sectional images of the SR, we observed that, relative to WT SR, *REEP5*-KO SR consistently exhibited fewer membrane contortions surrounding t-tubules. As revealed by 3D tomography (Figure 1E and 1F), the junctional SR membrane folds, which envelop t-tubules, were significantly outstretched and dissociated from t-tubules in *REEP5*-KO cardiomyocytes.

To quantitatively analyze the connection between SR and t-tubule structures, we measured the length of the SR/t-tubules junction from multiple samples (Figure 1G and 1H).

We found that the SR/t-tubule junction length in *REEP5*-KO cardiomyocytes was dramatically reduced (Figure 1G), suggesting that REEP5 controls SR membrane shaping and the spatial connection of the SR with t-tubules in cardiomyocytes.

REEP5-Defective SR Membrane Remodeling Depresses Depolarization-Induced SR Ca²⁺ Release

The physical contiguity between the SR and t-tubules determines the excitation–contraction (EC) coupling efficiency in ventricular cardiomyocytes.⁴ This permits the influx of Ca²⁺

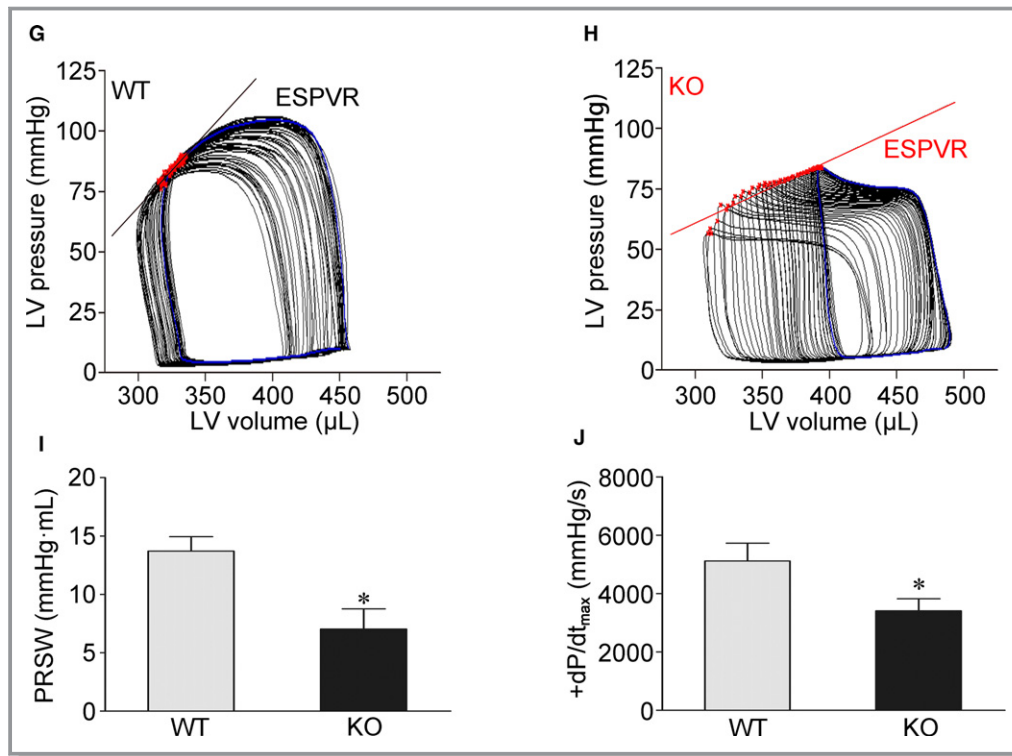


Figure 3. Continued

through LTCCs, which rapidly diffuses across the dyadic cleft to activate RyR2, and causes SR Ca^{2+} release and synchronous myocyte contraction. Simultaneous recording of LTCC current and Ca^{2+} transients showed that LTCC current in *REEP5*-KO myocytes was comparable to that in WT cells (Figure 2A and 2B), but the peak amplitude of the Ca^{2+} transient was significantly decreased in *REEP5*-KO myocytes (Figure 2C), leading to a reduced EC coupling gain (Figure 2D). Notably, the *REEP5*-defective SR Ca^{2+} release seemed not to be correlated with the levels of SR Ca^{2+} -handling proteins such as RyR2, SERCA2a (sarco/endoplasmic reticulum Ca^{2+} ATPase cardiac isoform 2a), and CASQ2 (calsequestrin 2), in that *REEP5* deficiency did not significantly alter their expression (Figure S2). These results indicate that structural deformation of the SR membrane induced by *REEP5* deficiency depresses depolarization-induced SR Ca^{2+} release by decreasing the efficiency of the EC coupling in cardiomyocytes.

Depressed SR Ca^{2+} Release Compromises Cardiac Contractility in *REEP5*-KO Rats

In the heart, cardiomyocyte SR Ca^{2+} release is coupled to cardiac contraction.⁴ In view of the effects of *REEP5* reduction on SR Ca^{2+} release, we further studied *in vivo* cardiac contractile function in *REEP5*-KO rats. We found that the hearts of *REEP5*-KO rats were structurally normal (Figure 3A

through 3F). However, dynamic measures of intact heart function using a rigorous ventricular pressure–volume catheter technique demonstrated that contractile function (end-systolic pressure–volume relationship, preload recruitable stroke work, and time-varying maximal elastance) was significantly compromised in *REEP5*-KO animals (Figure 3G through 3J). Collectively, *REEP5*-mediated membrane shaping of the SR modulates cardiac contraction.

Discussion

Our studies in both cells and genetically engineered animals have uncovered a novel role of *REEP5* in the structure-based function of the cardiac SR. First, we identified the predominant expression of *REEP5* in cardiomyocytes and its spatial localization at the SR. Second, we found that *REEP5* ablation induced a reduction of SR membrane folds and disrupted the spatial contiguity between the SR and t-tubules. Third, the reduction in *REEP5* depressed SR Ca^{2+} release without any effect on the expression of critical SR Ca^{2+} -handling proteins and sarcolemmal LTCC currents. Finally, knockout of *REEP5* compromised cardiac contractility. Therefore, we demonstrated that *REEP5* serves as an SR membrane sculptor to control cardiac function.

REEP family members were originally identified by their ability to enhance the membrane expression of G protein–

coupled receptors.²⁰ REEP members such as REEP5, a member of the DP1 family, regulate intracellular trafficking by affecting ER membrane structure and cargo capacity and by acting as adapter proteins.¹² Contrasting findings regarding the intracellular localization and mechanisms of action of the REEP proteins have been described.^{20,21} The present findings expand our understanding of REEP5 as a molecule that shapes the ER. Furthermore, our discovery of the restricted distribution of REEP5 at the SR and of the effects of REEP5 on SR membrane curvature and related SR Ca²⁺ release reveals an important modulatory mechanism for cardiac EC coupling.

The SR is an extremely motile organelle that is capable of molecular motor protein–driven structural changes.²² Under physiological conditions, SR vesicles move dynamically throughout the cell, approaching or moving away from the myocyte sarcolemma. SR dysfunction is a major feature of cardiac diseases, and its underlying mechanisms have been intensively studied.^{23–25} Abnormal SR Ca²⁺ release occurs in human cardiomyocytes isolated from patients with heart failure, regardless of etiology.²⁶ This mirrors findings concerning SR/t-tubule coupling disruption in animal models of heart failure that resulted from ischemic, hypertensive, and tachycardic phenotypes and suggests that the disruption of the SR network and its connection to t-tubules is a common pathway in heart failure. In addition, we found that REEP5 proteins were significantly reduced in the failing hearts (Figure S3). Therefore, our identification of depressed SR Ca²⁺ release and cardiac contractility in REEP5-defective rats may yield novel perspectives on the mechanisms underlying heart failure.

In sum, REEP5 serves as an SR membrane sculptor to modulate cardiac function. Its deficiency deforms the SR membranes, with the result that EC coupling of cardiomyocytes and cardiac contractility are compromised. As such, REEP5-dependent SR shaping could be of pathophysiological significance, and targeting REEP5 appears to be a novel therapeutic approach for heart failure.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Supplemental Figure Legends:

Figure S1. Validation of *REEP5* knockout in rats. **A**, A schematic overview of the CRISPR/Cas9-based genome-editing strategy used to generate the *REEP5* knockout (KO). Bottom, Example chromatogram showing a microdeletion and representative sequences of mutated alleles identified from clonal amplicons. Yellow dashes indicate the deleted bases. **B**, Western blot analysis of *REEP5* expression in the hearts of rats carrying homozygous 8-bp deletion mutations. *Left*, representative blots; *right*, pooled data. WT: n=6; *REEP5* KO: n=6. $P < 0.05$ compared with WT. **C**, Analysis of the effects of CRISPR/Cas9-mediated *REEP5* gene editing on the mRNA expression of *REEPs* other than *REEP5*. n=3 for each group. $P < 0.05$

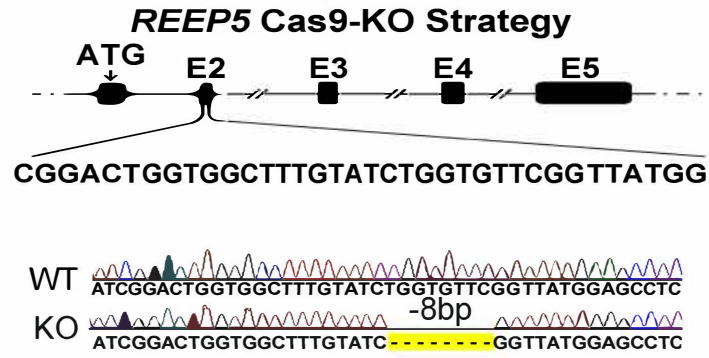
Figure S2. *REEP5* deficiency does not affect the expression of SR Ca^{2+} -handling proteins. Analysis of critical Ca^{2+} -handling proteins on the SR. *Left*, typical blots; *Right*, pooled data from three separate experiments.

Figure S3. Measurement of *REEP5* protein during the development of heart failure. **A**, Echocardiographic analysis of cardiac function during transverse aortic constriction (TAC)-induced heart failure, as demonstrated by ejection fraction. **B**, Western blotting examination of *REEP5* protein in the heart tissues. *Top*, typical blots; *bottom*, pooled data. Three to five

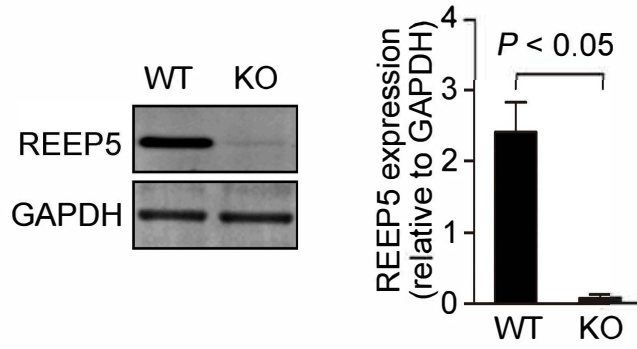
animals were analyzed at indicated time points in WT and REEP5-KO rats.

Figure S1.

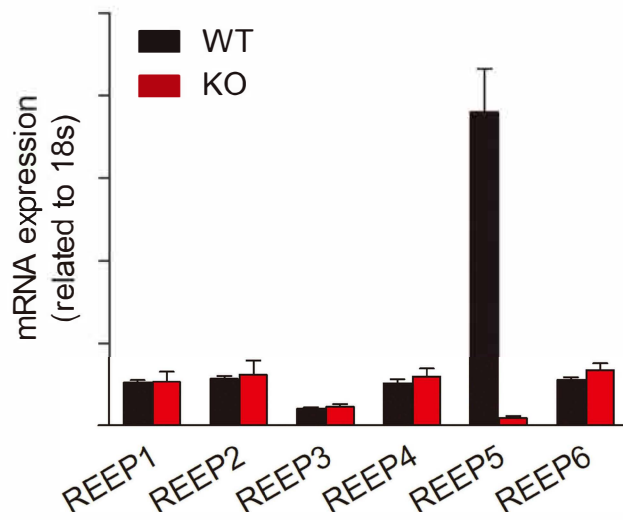
A



B



C



A

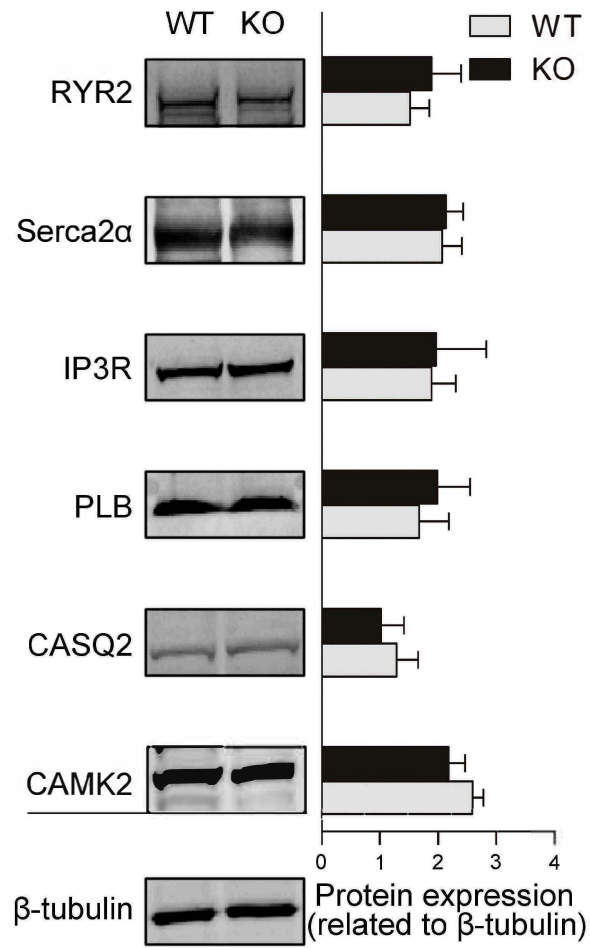
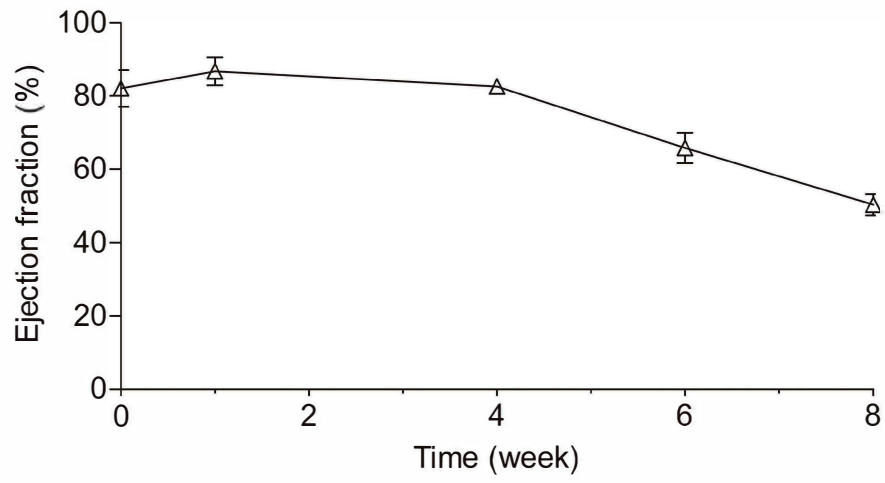


Figure S3

A



B

