Genetic Deletion of Rnd3/RhoE Results in Mouse Heart Calcium Leakage Through Upregulation of Protein Kinase A Signaling

Xiangsheng Yang,* Tiannan Wang,* Xi Lin, Xiaojing Yue, Qiongling Wang, Guoliang Wang, Qin Fu, Xun Ai, David Y. Chiang, Christina Y. Miyake, Xander H.T. Wehrens, Jiang Chang

Rationale: Rnd3, a small Rho GTPase, is involved in the regulation of cell actin cytoskeleton dynamics, cell migration, and proliferation. The biological function of Rnd3 in the heart remains unexplored.

Objective: To define the functional role of the Rnd3 gene in the animal heart and investigate the associated molecular mechanism.

Methods and Results: By loss-of-function approaches, we discovered that Rnd3 is involved in calcium regulation in cardiomyocytes. Rnd3-null mice died at the embryonic stage with fetal arrhythmias. The deletion of Rnd3 resulted in severe Ca\(^{2+}\) leakage through destabilized ryanodine receptor type 2 Ca\(^{2+}\) release channels. We further found that downregulation of Rnd3 attenuated β\(_{-}\)adrenergic receptor lysosomal targeting and ubiquitination, which in turn resulted in the elevation of β\(_{-}\)-adrenergic receptor protein levels leading to the hyperactivation of protein kinase A (PKA) signaling. The PKA activation destabilized ryanodine receptor type 2 channels. This irregular spontaneous Ca\(^{2+}\) release can be curtailed by PKA inhibitor treatment. Increases in the PKA activity along with elevated cAMP levels were detected in Rnd3-null embryos, in neonatal rat cardiomyocytes, and noncardiac cell lines with Rnd3 knockdown, suggesting a general mechanism for Rnd3-mediated PKA signaling activation. β\(_{-}\)-Adrenergic receptor blocker treatment reduced arrhythmia and improved cardiac function.

Conclusions: Rnd3 is a novel factor involved in intracellular Ca\(^{2+}\) homeostasis regulation in the heart. Deficiency of the protein induces ryanodine receptor type 2 dysfunction by a mechanism that attenuates Rnd3-mediated β\(_{-}\)-adrenergic receptor ubiquitination, which leads to the activation of PKA signaling. Increased PKA signaling in turn promotes ryanodine receptor type 2 hyperphosphorylation, which contributes to arrhythmogenesis and heart failure. (Circ Res. 2015;116:e1-e10. DOI: 10.1161/CIRCRESAHA.116.304940.)

Key Words: adrenergic receptor, beta-2 • arrhythmias, cardiac • calcium • rho GTP-binding proteins • ubiquitination

R

nd3, also known as RhoE, is one of the Rnd subsets of the Rho family GTPases. It is generally thought that it functions as a repressor of Rho-associated coiled-coil protein kinase 1 (ROCK1).\(^{1,2}\) In contrast to the canonical mode of Rho family proteins, members of the Rnd subfamily, including Rnd1, Rnd2, and Rnd3, bind but do not hydrolyze GTP. They are defective in GTPase activity, even in the presence of RhoGAPs.\(^{3,4}\) The study of the regulatory activation of ROCK1 demonstrated that Rnd3 inhibits RhoA-mediated ROCK1 signaling by specifically binding to ROCK1, but not ROCK2.\(^{1,2}\) Rnd3 is the only endogenous ROCK1 antagonist discovered to date.\(^{5}\) The current knowledge about the biological functions of Rnd3 has been achieved mainly by in vitro cell culture studies and tissue screening assays including gain-of-function and loss-of-function approaches. Overexpression of Rnd3 inhibited ROCK1-mediated biological effects including stress fiber formation, phosphorylation of myosin light chain phosphatase, and apoptosis. Reduced expression of Rnd3 potentiated ROCK1 activity.\(^{1,2}\) Rnd3 is also a substrate of ROCK1. The ROCK1–Rnd3 interaction mediates Rnd3 phosphorylation by ROCK1 at...
Excess β2-adrenergic receptor protein kinase A (β2AR-PKA) signaling pathway contributes to the dysfunction of RyR2 calcium release channels. The pathological consequence of Rnd3 downregulation in human heart failure and arrhythmogenesis remains obscure. Clearly, our Rnd3-null mouse study provides a mechanistic base for the future investigation in humans.

Methods

Methods and any associated references are provided in detail in the Online Data Supplement.

Results

Genetic Deletion of the Rnd3 Gene in Mice

Results in Embryonic Lethality With Fetal Heart Arrythmias

Rnd3 knockout mice were generated from a gene trap embryonic stem cell line. The targeting vector was inserted at Rnd3 intron 2 (Figure 1A). The deletion of the Rnd3 gene was verified by polymerase chain reaction genotyping (Figure 1B) and Southern blotting (Figure 1C). Quantitative polymerase chain reaction (Figure 1D) and Western blot (Figure 1E) assessments of the Rnd3 transcript and protein levels confirmed the Rnd3 knockout. No obvious morphological changes were observed in the mutant embryonic hearts (Online Figure I). Genotyping results revealed that the majority of Rnd3-null mice died between E10.5 and E12.5 (Online Table I). To evaluate embryonic mouse cardiac functions in vivo, we performed echocardiographic analyses in female mouse wombs. Rnd3-null mice showed severe decreases in the cardiac ejection fraction and fractional shortening compared with wild-type (WT) control mice (Online Table II). Because of the technological challenges, we were not able to measure an individual mouse embryo electrocardiography (ECG);
therefore, we assessed heart beats using echocardiography and detected cardiac arrhythmias in E11 Rnd3-null mice (Online Movies I and II).

**Rnd3**−/− Cardiomyocytes Display an Abnormal Intracellular Ca2+ Release

To explore a possible mechanism contributing to the arrhythmogenicity, we analyzed cardiomyocytes isolated from E10.5 embryonic hearts. We found that Rnd3−/− cardiomyocytes exhibited a significant increase in spontaneous rhythm (Figure 2A) as shown by the increased frequency of Ca2+ oscillations (1.62±0.16/Hz) compared with the WT (1.04±0.12/Hz; Figure 2B). The amplitude of the spontaneous Ca2+ transient, however, was reduced by 27% in Rnd3−/− cardiomyocytes (1.72±0.09 F1/F0) compared with WT myocytes (2.35±0.12 F1/F0; Figure 2C, upper and lower). Further experiments revealed obvious Ca2+ leakage occurring in Rnd3−/− cardiomyocytes under sodium- and calcium-free perfusate conditions (without the influences of Ca2+ pump and Na+/Ca2+ exchanger (NCX; Figure 2D). The cardiomyocyte sarcoplasmic reticulum (SR) Ca2+ load showed no significant differences between the 2 groups (Figure 2E), suggesting normal or compensated sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) and NCX functions. Measurements of the fluorescence decay times (Tau) for Ca2+ transient decline and Ca2+ decline in caffeine showed no statistically significant differences between the WT and the Rnd3 mutant hearts, indicating no impairment of the SERCA and NCX functions in the mutant heart (Figure 2F and 2G). However, we observed a higher Tau value in SERCA function analysis (Figure 2F), which could be because of the downregulation of the SERCA expression levels and a lower SERCA activity. To rule out these possibilities, NCX1 and SERCA2α protein levels and phospholamban phosphorylation at serine 16 in the animal hearts were assessed by immunoblot analyses. No changes in the NCX1 and SERCA2α protein levels were observed in the Rnd3-null heart compared with the WT heart (Figure 2H). Hyperphosphorylation of phospholamban at serine 16 was detected in the Rnd3 mutant heart (Figure 2I). This indicates that the mutant hearts compensate for the Ca2+ leakage by enhancing SERCA function, although this counter effect may not be sufficient to overcome the overall Ca2+ leakage shown by a prolonged Tau of Ca2+ transient decline (Figure 2F). Together, the genetic deletion of Rnd3 resulted in an obvious Ca2+ leakage phenotype in mouse cardiomyocytes. This leak did not lead to a lower SR Ca2+ load because of the compensations by SERCA and NCX functions, therefore suggesting a RyR2 Ca2+ release channel dysfunction.

**Figure 2. Abnormal intracellular Ca2+ release in Rho family GTPase 3 (Rnd3)**−/− cardiomyocytes. 

A. Representative tracings of Ca2+ transients in wild-type (WT) and Rnd3−/− embryonic cardiomyocytes. B. Rnd3−/− myocytes exhibited increased spontaneous Ca2+ transient activity. C. Upper, Representative single tracing of Ca2+ transient in WT and Rnd3−/− cardiomyocytes. Lower, Rnd3−/− cardiomyocytes exhibited decreased transient amplitudes. D. Representative tracings of Ca2+ transients showed a noticeable Ca2+ leakage in Rnd3−/− cardiomyocytes under sodium- and calcium-free perfusate conditions followed by caffeine treatment. E. No change of sarcoplasmic reticulum (SR) Ca2+ load in Rnd3−/− cardiomyocytes compared with the WT cells. F. No compromised sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) function was found in Rnd3−/− cardiomyocytes assessed by fluorescence decay time (tau). G. The Na+/Ca2+ exchanger (NCX) function remained intact in Rnd3-null cardiomyocytes. H. Immunoblotting analysis showed no difference in NCX1 and SERCA2α protein levels between the WT and Rnd3−/− hearts. I. The hyperphosphorylation of phospholamban (PLN) at serine 16 was observed in Rnd3−/− hearts. The number in each column represents the number of cardiomyocytes analyzed from 10 embryonic hearts in each group. At least 40 embryos were pooled and used for the immunoblotting analysis in each group. Statistical significance was determined by an unpaired, 2-tailed Student t test. Data are mean±SD. The number at the top of each immunoblot band (H and I) represents the average of densitometries from 3 experiments, normalized by GAPDH.
RyR2 Ca\textsuperscript{2+} Release Channels Are Destabilized in Rnd3\textsuperscript{−/−} Cardiomyocytes

We then assessed RyR2 single channel function in planar lipid bilayers.\textsuperscript{20} Although the average RyR2 basal opening probability was not statistically different between Rnd3\textsuperscript{−/−} mice (0.41±0.06) and WT mice (0.49±0.07; Figure 3A and 3B), the frequency of the RyR2 opening was 3× higher in Rnd3\textsuperscript{−/−} mouse hearts (44.6±5.5 s\textsuperscript{−1}) compared with WT hearts (14.0±3.1 s\textsuperscript{−1}; Figure 3A and 3C). Both RyR2 channel open and close times were significantly shortened in Rnd3\textsuperscript{−/−} mouse hearts (mean open time, \(T_o=67.1±30.7\) ms; mean close time, \(T_c=149.4±43.1\) ms; Figure 3D and 3E, filled black bar, respectively) compared with WT (mean open time, \(T_o=207.3±66.0\) ms; mean close time, \(T_c=34.9±9.7\) ms; Figure 3D and 3E, empty bar, respectively). These data suggest that RyR2 channels are destabilized in Rnd3-null cardiomyocytes, contributing to abnormal intracellular Ca\textsuperscript{2+} release and an arrhythmogenic phenotype.

Rnd3 Knockdown Leads to Elevated PKA Activity and Hyperphosphorylation of RyR2

Next, we explored the molecular mechanism of Rnd3 deficiency-mediated RyR2 destabilization. RyR2 Ca\textsuperscript{2+} release channels, tetramer proteins, are critical for calcium handling and are tightly regulated. We, as well as others, have demonstrated that RyR2 channel hyperphosphorylation by PKA is closely associated with Ca\textsuperscript{2+} dysregulation including SR Ca\textsuperscript{2+} leakage, which contributes to cardiac arrhythmias and heart failure.\textsuperscript{21–24} We therefore assessed the protein levels of the active PKA isoform and the phosphorylation status of cAMP-responsive element-binding protein, a known PKA substrate. The immunoblot data displayed remarkable increases in the PKA isoform protein levels and the hyperphosphorylation of cAMP-responsive element-binding protein in the Rnd3 mutant heart (Figure 4A). The elevated PKA activation in the Rnd3 mutant heart was also confirmed by the observation of an increase in phospholamban phosphorylation at serine 16, a major target of PKA in the early immunoblot of this study (Figure 2I). In parallel with the assessment of PKA activity, the cAMP levels were measured in mouse embryos. More than a 2-fold increase in the cAMP concentration was detected in Rnd3\textsuperscript{−/−} E10.5 embryos compared with WT embryos (Figure 4B). Meanwhile, obvious increases in RyR2 phosphorylation levels at serines 2080 and 2030 were observed in Rnd3-null hearts with no changes in the RyR2 total protein expression levels (Figure 4C). Thus, several lines of evidence in vivo indicate that Rnd3 deficiency results in activation of PKA signaling, which leads to RyR2 channel hyperphosphorylation.

**Figure 3.** Single channel tracings revealed aberrant ryanodine receptor type 2 (RyR2) openings in Rho family GTPase 3 (Rnd3)\textsuperscript{−/−} cardiomyocytes. **A,** Representative tracings of RyR2 channels from wild-type (WT) and Rnd3\textsuperscript{−/−} mice, showing similar probability (Po) in Rnd3\textsuperscript{−/−} mice (B). **C,** Increased RyR2 open frequency (open event/s) along with decreased mean open time (\(T_o\); D) and closed time (\(T_c\); E) was observed in Rnd3\textsuperscript{−/−} cardiomyocytes compared with WT. The numbers in each column indicate the total number of RyR2 channels recorded over a total number of mouse embryos. Statistical significance was determined by unpaired, 2-tailed Student t test. Data are mean±SD. c indicates channel closed state; and o, channel open state.

**Figure 4.** Rho family GTPase 3 (Rnd3) knockdown led to elevated protein kinase A (PKA) activity and hyperphosphorylation of ryanodine receptor type 2 (RyR2) in vivo and in vitro. **A,** The active protein PKA isoform (PKAc) was detected by an immunoblot and an obvious increase in PKAc was found along with hyperphosphorylated cAMP-responsive element-binding protein (pCREB) in Rnd3 mutant hearts. **B,** More than a 2-fold increase in the cAMP level assessed by the ELISA assay was observed in E10.5 mutant embryos compared with wild-type (WT) embryos. The data were pooled from 8 embryos of 6 female mice (8/6) for the WT group and 11 embryos of 6 female mice (11/6) for the Rnd3\textsuperscript{−/−} group. Western blot analysis displayed significant increases in RyR2 phosphorylation at serine 2080 and serine 2030 in Rnd3 mutant hearts. The same results were achieved in the neonatal rat cardiomyocytes that showed small interfering RNA specific for Rnd3 (D–F). The cAMP data from rat cardiomyocytes were pooled from 3 repeated experiments in each group with triplicate analyses for each time. At least 40 embryos were pooled and used for the immunoblotting analysis in the Rnd3-null heart experiment. Statistical significance was determined by an unpaired, 2-tailed Student t test. Data are mean±SD. The number at the top of each immunoblot band represents the average of densitometries from 3 experiments, normalized by CREB for pCREB and GAPDH for PKAc in **A** and **D** and by RyR2 in **C** and **F**.
To demonstrate that the Rnd3-mediated change in the PKA activity was not because of any secondary or compensated effects by the animal in vivo, we knocked down Rnd3 in neonatal rat cardiomyocytes in vitro using the specific siRNA for Rnd3. Increases in the levels of PKA isoform protein, cAMP-responsive element-binding protein hyperphosphorylation, and intracellular cAMP concentrations paralleled the knockdown of Rnd3 expression in the same cohort of rat cardiomyocytes (Figure 4D and 4E). Noticeable increases in RyR2 phosphorylation at serines 2808 and 2030 were detected in siRNA-treated rat cardiomyocytes as well (Figure 4F). These data are consistent with the in vivo results from the Rnd3 mutant mice.

Because PKA signaling is essential for a broad array of biological activities, we examined the Rnd3 deficiency–mediated PKA activation in noncardiac cells as well. In accordance with our cardiomyocyte results, significant increases in the PKA activity and cAMP-responsive element-binding protein hyperphosphorylation were observed in mouse embryonic fibroblasts isolated from Rnd3+/− mice (Online Figure IIA), and HEK293T (human embryonic kidney) epithelial cells (Online Figure IIB), and C2C12 myoblasts (Online Figure IIC) treated with the siRNA for Rnd3 knockdown. The higher cAMP levels were also found in the Rnd3 downregulated groups compared with the controls (Online Figure IID), suggesting a general mechanism of PKA activation regulated by Rnd3. We demonstrated the increased cAMP levels along with the elevated PKA activities both in vivo in the Rnd3-null mouse hearts and embryos and in vitro in the Rnd3 knockdown cardiac and noncardiac cells. These data suggest that the downregulation of Rnd3 is sufficient to initiate PKA signaling activation that leads to RyR2 channel hyperphosphorylation.

PKA Activation Is Involved in the Rnd3 Deficiency–Mediated Ca2+ Leakage

We have demonstrated the activation of PKA induced by Rnd3 downregulation. Now we would like to know whether Rnd3 deficiency–mediated Ca2+ leakage can be blocked by the inhibition of PKA activity. We treated cardiomyocytes with a PKA inhibitor, H89. Consistent with the previous data in this study, we observed 4× higher spontaneous Ca2+ release events in Rnd3+/− cardiomyocytes compared with WT cells (Figure 5A). However, the Ca2+ leakage was blocked by the H89 treatment (Figure 5A). The percentage of the mutant cardiomyocyte cells with spontaneous Ca2+ release was double that of the WT cardiomyocytes (Figure 5B). After H89 treatment, this high percentage returned to the level of the WT cardiomyocytes (Figure 5B). This result suggests that PKA activation is involved in the Rnd3 deficiency–mediated Ca2+ leakage.

In the assessment of PKA activation, we also evaluated RyR2 phosphorylation at serine 2814, a Ca2+/calmodulin-dependent protein phosphorylation site. The enhancement of the phosphorylation was detected in the Rnd3+/− heart (Online Figure IIIA). Treatment of the mutant cardiomyocytes with Ca2+/calmodulin-dependent protein inhibitor KN93 partially attenuated the Ca2+ leakage (Online Figure IIIIB). However, the inhibitory effect on the Ca2+ leakage by KN93 was moderate compared with the blockage by H89 treatment (Figure 5). Therefore, we focused on PKA activation in this study.

Adult Cardiomyocytes With Rnd3 Haploinsufficiency (Rnd3+/−) Also Exhibit an Abnormal Intracellular Ca2+ Release and the Ca2+ Leakage Is Attenuated by PKA Inhibitor Treatment

To investigate whether the calcium leakage occurred in the adult cardiomyocytes as well, we conducted the same cohort of experiments in the cardiomyocytes isolated from adult Rnd3+/− mouse hearts. The spontaneous calcium transient incidence in Rnd3+/− cells was 5× higher than that in the WT cells (Online Figure IVA), and significantly higher calcium transient frequencies were also detected in the mutant cells compared with the controls (Online Figure IVB). These abnormal intracellular calcium releases were attenuated by H89 treatment (Online Figure IVA and IVB). The calcium leakage in Rnd3 haploinsufficient cardiomyocytes was further augmented by isoproterenol treatment (Online Figure IVC and IVD). Consistent with the embryonic cardiomyocytes, the calcium leakage did not lead to a lower SR Ca2+ load (Online Figure IVF). The representative tracings of Ca2+ transients were presented in Online Figure IIIG. Overall, the results indicate that Rnd3 downregulation leads to calcium leakage in both embryonic and adult mouse cardiomyocytes, and the leakage phenotype is attenuated by PKA inhibitor treatment.

Adult Rnd3 Haploinsufficient Mice Displayed Ventricular Arrhythmias

Because Rnd3-null mice are embryonically lethal, we evaluated the electrophysiology of adult Rnd3 heterozygous mice. Nine mice (5 Rnd3+/− and 4 WT) underwent the electrophysiology study protocol. All mice exhibited sinus rhythm before pacing studies started.
Rnd3 Deficiency Results in the Elevation of β2-AR Protein Levels Through the Ubiquitination Mechanism

Finally, we explored the molecular mechanism of the Rnd3 deficiency–mediated PKA activation and found an increase in the β2-AR, but not β1-AR, protein levels in the Rnd3-null mouse heart (Figure 6A). Because the assessment of β2-AR protein expression levels is critical, we validated the specificity of the β2-AR antibody by probing for β2-AR expression in the β2AR/β2AR double-knockout and the β2-AR single knockout mouse hearts, respectively (Online Figure VI). To further verify and quantify the β2-AR protein increase detected by the immunoblot analysis, the 125I-iodocyanopindolol binding assay, a more stringent radioligand method, was used. We observed that the membrane-associated β2-AR was 10x higher in Rnd3−/− hearts (227.4 fmol/mg) compared with the WT control hearts (21.36 fmol/mg; Figure 6B). Interestingly, we did not detect a correlated increase in the β2-AR mRNA levels in the same cohort of the mutant hearts (Figure 6C). To rule out any possible secondary or compensated effects leading to this increase in β2-AR protein levels by the animal in vivo, we conducted the same set of experiments in vitro in the neonatal rat cardiomyocytes treated with the siRNA specific for Rnd3. Again, upregulated β2-AR protein levels were detected by both immunoblot analyses (Figure 6D) and 125I-iodocyanopindolol binding assay (Figure 6E), but the β1-AR protein levels remained the same. The disconnection of the increase in β2-AR protein levels without an increase in its transcript levels was also observed (Figure 6F). Finally, we forced expression of Rnd3 and found a downregulation of β2-AR protein levels (Online Figure VII).

In the previous experiments, we demonstrated that Rnd3 deficiency–mediated PKA activation was a general mechanism that happened in both cardiac and noncardiac cells. Therefore, we wanted to know whether the β2-AR protein upregulation occurs without a change in its transcript levels in noncardiac cells with Rnd3 downregulation. The associated assessments of the β2-AR protein and mRNA levels in the noncardiac mouse embryonic fibroblast cells isolated from Rnd3−/− mice (Online Figure VIII A and VIII B, respectively) and the HEK293T cells (Online Figures VIIIIC and VIID, respectively), and myoblast C2C12 cells (Online Figure VIIIIE and VIIF, respectively) with Rnd3 knocked down by siRNA were performed. Again, noticeable increase in the β2-AR protein, but not mRNA, levels was found. These data strongly suggest that β2-AR is under post-translational regulation through Rnd3.

Because ubiquitination is a critical post-translational regulatory mechanism for β2-AR, we investigated whether Rnd3 plays a role in β2-AR ubiquitination. First, we determined the interaction of Rnd3 and β2-AR ex vivo by cotransfection of Rnd3 and β2-AR in HEK293T cells followed by mutual communoprecipitations as shown in Figure 7A and 7B. Then, the levels of β2-AR ubiquitination were assessed by immunoprecipitation of β2-AR followed by antiubiquitin immunoblotting analysis under both baseline and isoproterenol treatment conditions. As shown in Figure 7C (in the untreated group), the amount of ubiquinated species of β2-AR was significantly reduced by the knockdown of Rnd3 with about a 22% decline in the normalized amount of ubiquitinated β2-AR compared with the control without isoproterenol treatment (Figure 7D in the untreated group). Consistent with the baseline result, a 41% reduction in the normalized amount of ubiquitinated β2-AR in the Rnd3 knockdown group was observed relative to the control (Figure 7C in isoproterenol group and Figure 7D in isoproterenol group), whereas the total amount of β2-AR ubiquitination was increased by the isoproterenol treatment as expected. In parallel with the reductions in β2-AR ubiquitination, higher expression levels of β2-AR protein were once again detected in the Rnd3 knockdown groups under both baseline (untreated) and isoproterenol-treated conditions by cycloheximide chase analysis (Figure 7E and 7F).

β2-AR lysosomal targeting has been shown as an important mechanism for ubiquinated β2-AR degradation.25 We conducted
isoproterenol-stimulated β2AR lysosomal trafficking experiments to monitor β2AR receptor dynamic subcellular localization in a flag-β2AR stable expression cell line (293TΔβ2AR cells). Immunostaining for lysosome-associated membrane protein 2, a lysosome marker, and β2AR was performed followed by fluorescent microscopy analysis. The receptor internalization into lysosomes and endocytic vesicles was visualized (Figure 8A) and quantified by the colocalization of β2AR with lysosome-associated membrane protein 2 (Figure 8B). In the control group without isoproterenol stimulation, β2AR was evenly distributed across the cell membrane with a basal level of β2AR within lysosomes. However,Rnd3 deficiency significantly reduced β2AR receptor internalization into lysosomes (10.7%) compared with the control group (21.3%), and this attenuated lysosomal trafficking of β2AR was still detected 10, 20, and 60 minutes after isoproterenol stimulation. These data indicate that Rnd3 is a critical factor for the integrity of lysosome-mediated β2AR regulation. The result is consistent with the regulatory role of Rnd3 in β2AR degradation observed earlier in this study.

Regulation of post-translational modification–mediated β2AR degradation is complicated and involves multiple biological processes and factors. Although it is not the focus of the study, we still examined the expression levels of β2-arrestin, an adaptor protein of E3 ligase for β2AR ubiquitination, and G-protein–coupled receptor kinase 2 (Grk2) in the mutant heart because both factors are involved in β2AR ubiquitination. No significant differences of the 2 protein expression levels were detected between the Rnd3-null heart and the WT heart (Online Figure IX).

We then assessed hydroxylation regulation of β2AR receptor and sumoylation modification of arrestin 3 and caveolin-3 in Rnd3-deficient mouse hearts because hydroxylation and sumoylation are involved in β2AR receptor post-translational modification degradation.26–28 The former is through Egf-9 family hypoxia-inducible factor 3 (EGLN3), a member of the EGLN family of prolyl hydroxylases, also called prolyl hydroxylase domain-containing protein 3, which catalyzes hydroxylation of β2AR and facilitates the receptor ubiquitination by von Hippel-Lindau E3 ubiquitin ligase complex (pVHL).29 In the sumoylation modification mechanism, arrestin 3 and caveolin-3 sumoylation enhanced β2AR internalization and endocytosis.27,28 We evaluated the amount of EGLN3 and pVHL binding to β2AR by immunoprecipitation followed by immunoblot specific for EGLN3 and pVHL in mouse hearts. As displayed in Online Figure XA, we did not find significant differences in EGLN3 and pVHL proteins bound to β2AR among WT, Rnd3−/−, and Rnd3−/− animal hearts. Meanwhile, the immunoprecipitation from the same cohort of mouse hearts showed no obvious changes of arrestin 3 and caveolin-3 sumoylation modifications (Online Figure XB), indicating a minimal role of hydroxylation and sumoylation in Rnd3-mediated β2AR regulation.

Finally, we conducted a mouse rescue experiment using IC1118551, a β2AR antagonist. The drug significantly improved Rnd3−/− embryo mouse cardiac contractility (Online Table II) and reduced arrhythmia. We only detected arrhythmia in 1 of 16 mutant embryos.

Together with the previous results, we provide the evidence that Rnd3 is a factor involved in β2AR ubiquitination and RyR2 stabilization regulation. Rnd3 deficiency attenuates β2AR lysosomal trafficking and ubiquitination, resulting in the prolonged activation of PKA signaling. The latter targets RyR2 channels and leads to channel dysfunction, which eventually contributes to arrhythmias and heart failure in Rnd3-null embryonic hearts. The β2AR antagonist treatment reduced arrhythmia and improved cardiac function.

### Discussion

The destabilization of RyR2 calcium release channels is responsible for many heart diseases, including cardiac arrhythmias and heart failure. Phosphorylation of RyR2 has been demonstrated as an important modification for its functional integrity. In patients with RyR2 channel mutations, the RyR2 channels exhibit leaky defects under PKA phosphorylation conditions, a situation that mimics sympathetic activation during exercise.29,30 The PKA-mediated destabilization of the RyR2 channels was demonstrated in several mouse models as well.21–23,31–33 Overexpression of PKA catalytic domain in mouse heart tissues was sufficient to lead to dilated cardiomyopathy and sudden cardiac death.22 Genetic inhibition of PKA-mediated RyR2 phosphorylation attenuated RyR2 leakage23 and prevented heart failure induced by chronic stimulation of β-adrenergic signaling.34 We would like to indicate that whether PKA-mediated RyR2 phosphorylation at serine 2808 (S2808) directly leads to RyR2 dysfunction remains controversial.35 Several studies found that genetic alteration of RyR2 channels at S2808, a situation that was suggested to mimic the PKA hyperphosphorylation, showed no different phenotypes in
More recent studies demonstrated that the adult Rnd3 haploinsufficient mice were hypersensitive to arrhythmogenic stimuli and displayed severe ventricular arrhythmias after cardiac pacing, particularly with the β1-adrenergic receptor (β1-AR) blocker. Despite the β1-AR receptor degradation, we assessed the expression levels of the 2 proteins and did not detect the changes of Grk2 and arrestin expression levels in the Rnd3-null heart. Emerging evidence shows that the process of β1-AR receptor trafficking and degradation are dynamic and complex. Additional new factors have been continually identified in the signalosome complex. We report that Rnd3 is another regulator in the β1-AR ubiquitination regulatory complex. The lack of Rnd3 prevents the ubiquitination of β1-AR, despite the normal expression levels of β-arrestin1/2 and Grk2, resulting in the accumulation of β1-AR protein, which promotes the activation of PKA signaling. Finally, we demonstrated that application of β1-AR blocker can rescue the mouse phenotype by preserving cardiac contractility and reducing cardiac arrhythmia.

It is important to realize that there is a limitation in this study. It is unclear whether Rnd3 is a constitutively associated factor in the multimeric complex or just a hit and run mediator of β1-AR function.
regulatory protein during β2-AR receptor trafficking and degradation. Further sets of experiments are absolutely necessary. The answer will provide a further mechanism for our understanding of β2-AR dynamic regulation and calcium release regulation.

Rnd3 has also been shown to play an essential role in the Notch signaling pathway by facilitating NICD protein degradation through ubiquitin proteasome system in mouse brains in our recent study. Given the fact that there are >600 putative E3 ligases in humans, our future study will investigate whether Rnd3 directly binds to an E3 ligase functioning as an ancillary protein or interacts with arrestin family members.

In summary, there are 5 findings in this study. First, the Rnd3 gene is indispensable for mouse development. Rnd3-null mice are embroyonically lethal with cardiac arrhythmias. Second, our data reveal that Rnd3 is essential for the normal function of RyR2 Ca2+ release channels in both the embryonic and adult mouse heart. Genetic deletion of Rnd3 results in Ca2+ leakage through RyR2 channel destabilization that in turn prompts arrhythmias. Third, we validate that hyperphosphorylation of RyR2 by PKA contributes to Rnd3 deficiency–mediated RyR2 dysfunction in mouse hearts. Fourth, we demonstrate that the downregulation of Rnd3 is sufficient to initiate activation of PKA signaling in vivo in animal hearts and in vitro in both cardiac and noncardiac cells, suggesting a general mechanism of Rnd3-mediated PKA activation. Finally, we discover the molecular mechanism of Rnd3-mediated PKA activation and determine that Rnd3 is a regulator in the β2-AR ubiquitination regulatory complex. The lack of Rnd3 prevents the ubiquitination of β2-AR, resulting in the accumulation of β2-AR protein, which promotes the activation of PKA signaling (Online Figure XI). Given the fundamental roles of the RyR2 channels in cell signaling, arrhythmogenicity, heart failure, and the β2-AR-PKA signaling pathway, the present findings have both basic and clinical significances and provide a potential new target for pharmacological manipulations.

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Disclosures

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27. Fuhs SR, Insel PA, Cavenoli-3 undergoes SUMOylation by the SUMO E3 ligase PIAS4: sumoylation affects G-protein-coupled receptor desensitz-
Rnd3 deficiency results in Ca^2+ leakage through ryanodine receptor

• Rnd3 participates in intracellular Ca^2+ homeostasis regulation in the central nerve system.

→ It plays an essential role in central nerve system development.

→ It modulates SR luminal Ca^2+ sensitivity.

→ Melanin distribution pattern is associated with polymorphic ventricular tachycardia associated with calcium release channel (ryanodine receptor) leak.


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SUPPLEMENTAL MATERIAL

Materials and Methods

Generation and Verification of Rnd3 Knockout Mouse Lines and Mouse Studies
We generated the Rnd3 knockout mice derived from a TIGM (Texas Institute for Genomic Medicine) gene trap ES cell line. The targeting vector was inserted into the Rnd3 intron 2. The chimeras were bred with wild-type C57 mice for 10 generations. Mutant Rnd3 alleles were verified by PCR genotyping and Southern blot. The PCR genotyping primers were (5' to 3'): TCCATAGAGGGTAAAGGCCATCC/AAAGGTACTCCCAGAGGCTAAGG; TCCATAGAGGGTAAAGGCCATCC/ATAAACCCTTTCAGTTGCA. The oligo probe for Southern blot was synthesized by PCR with primers: TGTAAGGGGATGGAGTGA/TCTCCAGCCTCCTTCCTGTA. For Southern blot, 10 µg of genomic DNA was extracted from mouse tails followed by HindIII digestion overnight. The digested DNA was separated by gel electrophoresis, transferred onto Zeta membranes, and blotted using an α-32P dCTP-labeled oligo probe. The β1AR and β2AR double-knockout mouse heart tissue was purchased from the Jackson Laboratory (stock number: 003810; Bar Harbor, Maine). The β2AR knockout mouse heart tissue was kindly provided by Yang K. Xiang (Department of Pharmacology, University of California at Davis). For the rescue experiment, the pregnant mice were allowed access to drinking water containing ICI118,551 (1µg/ml, Sigma-Aldrich, St. Louis, MO), a β2AR antagonist, from embryonic days E3.5 to E11. All experiments with animals were approved by the Institutional Animal Care and Use Committee of the Texas A&M University Health Science Center-Houston.

Cardiomyocyte and Mouse Embryonic Fibroblast Preparation and Cell Cultures
Mouse and rat cardiomyocytes (CMs) were isolated as described previously. Briefly, hearts from E10.5 mouse embryos or 10-12 week-old male mice were dissected followed by collagenase type II ( Worthington, 75 U/ml) digestion for 30 min. The digestion was terminated with KB buffer (85 mM KCl, 30 mM K2HPO4, 5 mM MgSO4, 1 mM EGTA, 2 mM Na2ATP, 5 mM pyruvate, 5 mM creatin, 20 mM taurine, 20 mM glucose). The isolated cells were plated with DF-10 culture medium (DMEM/F12, adjusted to 17 mM sodium bicarbonate, 2 mM L-glutamine, 10% FBS). The beating CMs were chosen for the cell calcium assessments. For neonatal rat cardiomyocytes, ventricles from two day-old rats were used. Mouse embryonic fibroblasts (MEF) were isolated from E10.5 embryos by 0.05% trypsin digestion for 30 min. HEK293T cells (human embryonic kidney A293T) and C2C12 myoblasts were cultured in DMEM medium with 10% FBS. Isoproterenol was purchased from Sigma (I5627, St. Louis, IL). The siRNA specific for Rnd3 was purchased from Thermo Scientific Dharnacon RNAi Technologies (L-007794-00-0005, Lafayette, CO), and their sequences were CUACAGUGUUGAGAAUUA; UAGUAGACUCUCGAUAUCA; CAGCAAAUCUAUCAUGGAU; GGGAGACAGUUGUAGUAC. Cycloheximide was used at 10 μg/ml in the cycloheximide chase analysis.

Immunoblotting, Immunofluorescent Staining, Immunoprecipitation Analyses, and Plasmids
The immunoblots, immunofluorescent staining, and immunoprecipitation analyses were performed using the following antibodies: anti-Rnd3 (Cocalico Biologicals, Inc., Pennsylvania), anti-sarco/endoplasmic reticulum Ca2+-ATPase 2 (SERCA2) (Badrilla, A010-20), anti-PKAc (Abcam, ab82545,MA), anti-Na+/Ca2+ exchanger 1 (NCX1) (Abcam, ab6495, MA ), anti-RyR2-p2808 (Badrilla, A010-30, UK), anti-RyR2-p2030 (Badrilla, A010-32, UK), anti-RyR2 (Pierce Antibodies, MA3-916), anti-PKAc (Abcam, ab82545, Cambridge, MA), anti-pCREB (Santa Cruz, sc-7978, CA), anti-CREB (Santa Cruz, sc-186), anti-PLB (Badrilla, A010-14), anti-PLN-pSer16 (Badrilla, A010-12), anti-β1AR (Abcam, ab3442, MA), anti-β2AR (Thermo Scientific, PA5-19649,
IL), anti-β2AR (Santa Cruz, sc-569, for lysosome trafficking experiment), LAMP2 (Santa Cruz, sc-18822), anti-Ub (Santa Cruz, sc-8017), anti-caveolin-3 (Abcam, ab2912) and anti-arrestin2/3 (Cell signaling, 4674S), anti-SUMO1 (Cell signaling, 4930S) and anti-SUMO2/3 (Thermo Scientific, PA1-41153). Even protein loading was verified by the intensity of the GAPDH blot (Santa Cruz, sc-20357). Expression plasmid for human Rnd3 was modified from Origene (sc322482, Rockville, MD). Expression plasmid for human β2-adrenergic receptor was received from Addgene (14697, Cambridge, MA) \(^3\). For β2AR Ubiquitination assessment, HEK293T cells were treated by 1.0 µM isoproterenol for 10 min followed by immunoprecipitation with anti-β2AR antibody conjugated to protein G beads. For lysosome trafficking experiments, 293T-FLAG-β2AR cells, a stable β2AR expression cell line, were transfected with the siRNA specific for Rnd3. Cells were harvested and fixed with 4% PFA (Paraformaldehyde) after post-isoproterenol treatments (1.0 µM) for 10, 20, and 60 minutes, respectively. Images were acquired by fluorescence microscopy (Leica, DM2000). The immunoblotting densitometry was quantified by Gel Logic 6000 PRO Imaging System (Carestream Health, Inc.), and the immunofluorescent image quantifications were conducted by Leica Application Suite Imaging Software (Version 4.0, Germany).

### q-PCR (Quantitative Polymerase Chain Reaction) Analysis and cAMP Measurement
Transcripts were quantified by q-PCR analysis (Applied Biosystems StepOnePlus) using the SYBR green method with the MasterMix buffer system containing Taq polymerase as described previously\(^2\). Total RNA was prepared by TRIZOL extraction (GIBCO BRL). The forward/reverse PCR primers were as follows (5’ to 3’): Rnd3: GACAGCCAGTGGAGAAG/AGAGAGTGGACGACGTTGT; β2AR (human): GGACCCACGCAGTCACGCGAG/TGATGAACTGTTGGTGACC; β2AR (mouse): ATACACACCATTGCAAGTT/TAAGCGATCAGCTGAAATC; GAPDH: GGTGAAGTTCGTGTGACCGATT/CGAGAAAGGGCCGGAGATGTA. Expression levels were determined by the 2^ΔΔCT method. The q-PCR product sizes are 183bp for Rnd3, 158bp for human β2AR, and 233bp for mouse β2AR. cAMP levels were measured by the cyclin AMP assay kit from R&D System (Cat# KGE002, Minneapolis, MN). E10.5 embryos and cells were homogenized and lysed, respectively. The sample supernatants were achieved by centrifugation and were immediately used for cAMP measurement by the enzyme-linked immunosorbent assay (ELISA) method using a microplate reader at 450 nm (MRX revelation microplate reader, Dynex Technologies, Inc., Chantilly, VA).

### Mouse Embryonic Cardiac Function Assessment by Echocardiography
A Vevo770 High-Resolution Micro-Imaging System (VisualSonics Inc., Toronto, Canada) with a 30 MHz probe (RMV-707B) was used for cardiac function analysis in mouse embryos. The pregnant mice at stage E11.0 were anesthetized by 3-4% isoflurane and then switched to 1-1.5% isoflurane mixed with 100% oxygen. The uterus carrying the embryos was taken out of the lower abdomen, and the M-mode echocardiography was immediately obtained from the embryos within the uterus as described previously\(^4\).

### Adult Mouse Cardiac Electrophysiology Assessment
Mice were anesthetized with isoflurane (1.75%) in 100% oxygen, enough to maintain adequate levels of anesthesia. Intracardiac bipolar atrial and ventricular electrograms were obtained using a 1.1F octapolar catheter (EPR-800; Millar Instruments), as described previously\(^5\). Baseline recordings were made after a 10 minute stabilization period following catheterization. Pacing protocols included ventricular pacing with single and double extrastimuli, at a basic cycle length (BCL) of 90 and 70 ms. Ventricular burst pacing was performed at a drive train of 60 and 40 ms. After a full series of programmed electrical stimulation protocols were complete, mice were injected with isoproterenol (3mg/kg i.p.) and caffeine (120 mg/kg i.p.). Ventricular extrastimulus
pacing protocols were repeated at a BCL of 70 ms, and burst pacing at both 60 and 40 ms. An arrhythmia was counted as ventricular tachycardia if the same stimulus protocol reproducibly induced 4 or more consecutive ventricular beats.  

Confocal Calcium Imaging  
After being cultured in the DF-10 medium at 37°C overnight, the E10.5 and adult mouse cardiomyocytes were exposed to 5 and 2 μM Fluo-4/AM (Invitrogen), respectively, in KB solution (90 mM KCl, 30 mM KH2PO4, 5 mM MgSO4, 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) for at least 30 min, and then were switched into the Tyrode solution containing 1.8 mM Ca2+ (1.8Ca Ty’s) with a final 1.0 μM Ca2+ concentration for another 30 min. The [Ca2+]i was measured by a laser scanning confocal microscope (Zeiss LSM 510, Thornwood, NY) at the excitation wavelength of 488 nm and the emission wavelength of 505 nm. The fluorescence images were recorded in line-scan mode with 512 pixels per line. For the rescue experiment, 1.0 μM H89 (Sigma, MO), a PKA inhibitor, was added when the spontaneous Ca2+ transient baseline signal was stabilized. The cardiomyocyte basal spontaneous [Ca2+]i transient was recorded for 20 sec. The cells were then switched to sodium and calcium-free perfusate for an additional 40 sec recording, which blocked the Ca2+ exchange via the NCX and the L-type Ca2+ channel activity. The spontaneous Ca2+ leakage events were calculated during this period. The steady-state SR Ca2+ load was assessed by F1/F0 by a caffeine-induced (10 μM) peak. In the assessment of NCX functions, we measured the time constant (tau) of Ca2+ decline during caffeine-induced Ca2+ transients under 1.8 mM Ca2+ conditions. SERCA functions were evaluated by the decline (tau) of spontaneous Ca2+ transients.  
For adult mouse cardiomyocytes, once steady-state Ca2+ transient induced by 1Hz-pacing (5 ms, 10 V) was observed, pacing was halted for 20-40 sec, and the spontaneous Ca2+ transients were recorded. The steady-state SR Ca2+ content was estimated by rapid application of caffeine (10 mM) at the end of the non-pacing period.  

Single-Channel Recordings  
Single-channel recordings were obtained under voltage-clamp conditions at 0 mV as previously described. Cardiac SR (sarcoplasmic reticulum) membrane preparations were incorporated into lipid bilayer membranes comprised of a mixture of phosphatidylethanolamine and phosphatidylserine at a ratio of 3:1 (Avanti Polar Lipids, Alabaster, AL) dissolved in n-decane (25 mg/ml). Bilayers were formed across a 150 μm aperture of a polystyrene cuvette. The cis and trans chambers corresponded to the cytosolic and the luminal side of the SR, respectively. The solution in the trans chamber included 250 mM HEPES, 50mM KCl, and 53 mM Ca(OH)2, and the solution in the cis chamber contained 250 mM HEPES, 125 mM Tris-base, 50 mM KCl, 1 mM EGTA, and 0.5 mM CaCl2 with pH 7.35. Ryanodine (5 μM) was added in the cis chamber to verify RyR2 channels at the end of the experiments. Data were collected using Digidata 1322A (Axon instruments, Foster City, CA) and Warner Bilayer Clamp Amplifier BC-535 under voltage-clamp conditions. Data were analyzed from digitized current recordings using pCLAMP 9.2 software (Axon Instruments, Foster City, CA).  

Radioligand Binding Assays  
The radioligand binding assay was modified from previous studies. Briefly, E10.5 mouse hearts or Rat cardiomyocytes were homogenized by sonication in a cold lysis buffer (5 mM Tris-HCl, pH7.4, 5 mM EDTA with proteinase inhibitors). The homogenized samples were centrifuged at 25,000 g to pellet the membrane proteins. The pellets were resuspended in the binding buffer (75 mM Tris-HCl, pH7.4, 12.5 mM MgCl2, 2 mM EDTA with proteinase inhibitors), and 10 μg of membrane proteins from each sample were incubated with 10 nM 125I-iodocyanopindolol, a βAR agonist, at room temperature for 2 hours followed by cold PBS wash and vacuum filtration over fiber filters to remove non-binding free probes. The radioactive binding activities were assessed.
by a gamma counter. The nonspecific radioactive binding activity was determined by co-incubation of membrane proteins with 10 µM propranolol, a βAR blocker. A series of competition binding assays were performed using ICI118551 with dosages from 1.0 pM to 1.0 µM in the presence of 20 pM of 125I-iodocyanopindolol. All of the measurements were triplicated. The receptor density was normalized by the amount of membrane proteins used for the assay.

**Statistical Analysis**
Chi-square analysis was performed for mouse genotyping data (Table S1) based on Mendelian ratios. In two independent group comparisons, the unpaired, two-tailed t test was conducted. For multiple comparisons, one-way ANOVA followed by Students-Newman-Keuls analysis was performed (SigmaPlot, version 11.0, Systat, Inc., San Jose, CA, USA). The data were expressed as mean ± s.d.
REFERENCES

Online Figure I. E10.5 Embryos with H&E Staining Showed no Obvious Morphological Defects in Rnd3-Null Heart. EC: endocardial cushion; RV: right ventricular; LV: left ventricular; OFT: outflow tract; IVS: interventricular septum; VT: ventricular; AT: atrium; AVC: AV canal. Objective lens: 10x.O
**Online Figure II. Rnd3 Knockdown Activated PKA Signaling in Non-Cardiac Cells.** The levels of active protein PKA isoform (PKAc) and CREB phosphorylation (pCREB) were elevated in mouse embryonic fibroblasts (MEF) isolated from Rnd3−/− mice (A), as well as HEK293T epithelial cells (B), and C2C12 myoblasts (C) treated by siRnd3. Significant increases in the cAMP levels were observed in HEK293T and C2C12 cells transfected with siRnd3 and MEF cells isolated from Rnd3−/− mice (D). The cAMP data were pooled from 3 repeated experiments in each group with triplicate analyses each time. cAMP: cyclic adenosine monophosphate. WT: wild-type. Ctrl: control. Statistical significance was determined by unpaired, two-tailed student’s t test. Data are means ± s.d.
Online Figure III. Western blot analysis displayed an increase in RyR2 phosphorylation at serine 2814 in the Rnd3⁻/⁻ heart. The Ca²⁺ leakage was moderately attenuated by KN93 treatment.
Online Figure IV. Abnormal intracellular Ca^{2+} release in adult Rnd3^{+/−} cardiomyocytes; Ca^{2+} leakage was attenuated by PKA inhibitor treatment. Rnd3^{+/−} myocytes exhibited increased spontaneous Ca^{2+} transient incidence and frequency, which were attenuated by H89 treatment (A and B). The Ca^{2+} leakage was further augmented by isoproterenol (ISO) treatment (C and D). E. No significant changes of SR load in Rnd3^{+/−} cardiomyocytes compared to the WT cells (E and F). Representative tracings of Ca^{2+} transients showed a noticeable Ca^{2+} leakage in Rnd3^{+/−} cardiomyocytes under sodium- and calcium-free perfusate conditions followed by caffeine treatment along with H89 or ISO treatment (G). The number in each column represents the number of cardiomyocytes analyzed. Statistical significance was determined by an unpaired, two-tailed student’s t test. Data are means ± s.d.
Online Figure V. Ventricular Arrhythmia was Detected in Adult Rnd3 Haploinsufficient Mice.  A. Baseline sinus rhythm in WT and Rnd3+/− mice under baseline, non-paced conditions. B. Example of ventricular tachycardia evoked by ventricular extrastimulus pacing in the Rnd3+/− mouse. C. Baseline sinus rhythm in WT and Rnd3+/− mice after isoproterenol (iso) and caffeine injection, before pacing. D. Example of ventricular tachycardia evoked by ventricular burst pacing after iso/caffeine injection. E. Summary of the results showed the total incidence of ventricular tachycardia after ventricular overdrive or burst pacing protocols. * P<0.05. Statistical significance was determined by an unpaired, two-tailed student’s t test. Data are means ± s.d.
**Online Figure VI. Validation of Specificity of the Antibody for β₂AR.**

Immunoblotting analysis for the verification of antibody for β₂AR protein expression in wild-type (WT), β₁AR/β₂AR double-knockout (DKO), and β₂AR knockout mouse heart tissues.
Online Figure VII. Western blot showed that overexpression of Rnd3 downregulated β₂AR protein expression level.
Online Figure VIII. Rnd3 Knockdown Resulted in the Elevation of $\beta_2$AR Protein Levels But Not in Its mRNA Levels in Non-Cardiac Cells. An immunoblotting analysis showed a remarkable increase in $\beta_2$AR protein levels (A) but not in $\beta_2$AR mRNA levels (B) in MEF cells isolated from Rnd3$^{-/-}$ mice. The same observations were also detected in HEK293T (C and D) and C2C12 cells (E and F) transfected with siRnd3. Statistical significance was determined by unpaired, two-tailed student’s t test. Data are means ± s.d.
Online Figure IX. Immunoblotting analyses showed that the expression levels of β-arrestin1/2 and G-protein coupled receptor kinase 2 (Grk2) protein remained constant in Rnd3−/− mouse heart tissues compared to wild-type (WT) heart tissues.
Online Figure X. Rnd3 Downregulation Did Not Alter the Hydroxylation- and Sumoylation-Mediated β2AR Protein Degradation Mechanism in Mouse Hearts. (A) Immunoprecipitation of β2AR followed by immunoblotting for EGLN3 and pVHL. No obvious differences of EGLN3 and pVHL bound to β2AR were observed among WT, Rnd3+/−, and Rnd3−/− mouse hearts. (B) Immunoprecipitation of CaV3 and Arrestin2/3 from the same cohort of animal hearts showed no status changes of CaV3 and Arrestin2/3 sumoylation induced by Rnd3 deficiency. EGLN3: Egl-9 family hypoxia-inducible factor 3; pVHL: von Hippel-Lindau E3 ubiquitin ligase complex; CaV3: caveolin-3; WT: wild-type.
Online Figure XI. Proposed Model Outlining the Molecular Mechanism of the Rnd3-deficiency-Mediated Calcium Dysregulation. The downregulation of Rnd3 attenuated β2-adrenergic receptor (β2AR) lysosomal targeting and ubiquitination, which in turn resulted in the elevation of β2AR protein levels leading to the hyperactivation of protein kinase A (PKA) signaling. The PKA activation destabilized ryanodine receptor type 2 (RyR2) Ca²⁺ release channels, contributing to calcium leakage.
<table>
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<th>Development stage</th>
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<th>Heterozygote</th>
<th>Homozygote</th>
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<td>64 (50.0%)</td>
<td>32 (25.0%)</td>
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<td>169</td>
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<td>75 (44.4%)</td>
<td>49 (29.0%)</td>
<td>0.313</td>
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<td>138 (58.0%)</td>
<td>47 (19.7%)</td>
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<td>42 (26.3%)</td>
<td>98 (61.2%)</td>
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<tr>
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<td>94 (61.0%)</td>
<td>12 (7.8%)</td>
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<td>postnatal</td>
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<td>282 (31.3%)</td>
<td>610 (67.8%)</td>
<td>8 (0.9%)</td>
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Chi-square analysis was performed for mouse genotyping data based on Mendelian ratios.
Online Table II. Cardiac Dysfunction was Detected in E11.0 Rnd3\(^{-/-}\) Mice and was Improved by \(\beta_2\)AR Antagonist Treatment

<table>
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<tr>
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<th>WT (n=9)</th>
<th>Rnd3(^{-/-}) (n=5)</th>
<th>Rnd3(^{-/-}) + ICI118,551 (n=16)</th>
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<td>56.34±3.70</td>
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<td>FS (%)</td>
<td>26.18±2.16</td>
<td>15.36±1.49*</td>
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</table>

The cardiac function was assessed by echocardiography EF: ejection fraction. FS: fractional shortening. *P<0.001 compared to wild-type (WT). Statistical significance was determined by one-way ANOVA followed by Students-Newman-Keuls analysis. Data are means ± s.d.