RICH1 is a novel key suppressor of isoproterenol- or angiotensin II-induced cardiomyocyte hypertrophy

SIQI WANG[1,2*], XIN WANG[3*], LI LING[1], CAIRONG LI[4] and ZHANHONG REN[2]

[1]School of Pharmacy, Xianning Medical College, Hubei University of Science and Technology; [2]Hubei Key Laboratory of Diabetes and Angiopathy, Medicine Research Institute, Xianning Medical College, Hubei University of Science and Technology; [3]School of Mathematics and Statistics, Hubei University of Science and Technology; [4]School of Clinical Medicine, Xianning Medical College, Hubei University of Science and Technology, Xianning, Hubei 437100, P.R. China

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Abstract. Cardiac hypertrophy is one of the key processes in the development of heart failure. Notably, small GTPases and GTPase-activating proteins (GAPs) serve essential roles in cardiac hypertrophy. RhoGAP interacting with CIP4 homologs protein 1 (RICH1) is a RhoGAP that can regulate Cdc42/Rac1 and F-actin dynamics. RICH1 is involved in cell proliferation and adhesion; however, to the best of our knowledge, its role in cardiac hypertrophy remains unknown. In the present study, the role of RICH1 in cardiomyocyte hypertrophy was assessed. Cell viability was analyzed using the Cell Counting Kit-8 assay and cells surface area (CSA) was determined by cell fluorescence staining. Reverse transcription-quantitative PCR and western blotting were used to assess the mRNA expression levels of hypertrophic marker genes, such as *Nppa*, *Nppb* and *Myh7*, and the protein expression levels of RICH1, respectively. RICH1 was shown to be downregulated in isoproterenol (ISO)- or angiotensin II (Ang II)-treated H9c2 cells. Notably, overexpression of RICH1 attenuated the upregulation of hypertrophy-related markers, such as *Nppa*, *Nppb* and *Myh7*, and the enlargement of CSA induced by ISO and Ang II. By contrast, the knockdown of RICH1 exacerbated these effects. These findings suggested that RICH1 may be a novel suppressor of ISO- or Ang II-induced cardiomyocyte hypertrophy. The results of the present study will be beneficial to further studies assessing the role of RICH1 and its downstream molecules in inhibiting cardiac hypertrophy.

Correspondence to: Dr Zhanhong Ren, Hubei Key Laboratory of Diabetes and Angiopathy, Medicine Research Institute, Xianning Medical College, Hubei University of Science and Technology, 88 Xianning Avenue, Xianning, Hubei 437100, P.R. China
E-mail: renzhanhong@hbust.edu.cn

Dr Cairong Li, School of Clinical Medicine, Xianning Medical College, Hubei University of Science and Technology, 88, Xianning Avenue, Xianning, Hubei 437100, P.R. China
E-mail: xnlcr@163.com

*Contributed equally

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Introduction

Cardiac hypertrophy is an early pathological structural feature of heart failure, and is a risk factor for cardiac dysfunction, arrhythmia and myocardial infarction (1). It is characterized by an increase in heart mass and individual cardiomyocyte volume, which is accompanied by reactivated fetal genes, increased protein synthesis and altered metabolism (1,2). Research into the molecular mechanisms of cardiac hypertrophy serve a role in preventing and treating cardiomyopathy and heart failure (1,2). However, there are few feasible targets, such as Rho and Rho-associated kinase (ROCK), for preventing or reversing cardiac hypertrophy (3).

It has been reported that small GTPases are involved in the development of cardiac hypertrophy (3-6). Small GTPases are molecular switches that either exist in the active GTP-bound or inactive GDP-bound states (7-9). Small GTPases are divided into subfamilies, including Ras, Rho, Rab, Ran and Arf/Sar (10). Activated Ras and the upregulation of Rab1 are causes of cardiac hypertrophy (5,6). Conversely, inactivation of RhoA can suppress cardiac hypertrophy (11,12). GTPase-activating proteins (GAPs) can induce inactivation of small GTPases by inducing GTP hydrolysis (13,14). Notably, the inhibitory roles of RabGAPs and RasGAPs in cardiac hypertrophy have previously been reported (15-18). The ablation of the RabGAP TBC1 domain family member 1 results in significant cardiac hypertrophy in male rats and increased myocardial damage after ischemia/reperfusion (15,16). Similarly, depletion of Carabin, another RabGAP, can exacerbate cardiac hypertrophy and significantly reduce fractional shortening in mice (17,18). Furthermore, the RasGAP neurofibromatosis type 1 (NF1) is involved in cardiac hypertrophy. It has been reported that the cardiomyocyte-specific NF1 knockout promotes cardiac hypertrophy and dysfunction in mice (19). However, compared with RabGAPs and RasGAPs, the role of RhoGAPs in cardiac hypertrophy is unclear.

RhoGAP interacting with CIP4 homologs protein 1 (RICH1) is a RhoGAP that is also known as ARHGAP17. RICH1 contains three functional domains: An N-terminal BAR domain; a RhoGAP domain; and a C-terminal tail consisting of multiple proline-rich motifs (20,21). RICH1 is a selective GAP of Cdc42 and Rac1, and associates with tight junctions in epithelial cells (21,22). The GAP activity of RICH1 and regulation of Cdc42 by RICH1 is required for maintaining proper tight junctions in epithelial cells (21). RICH1 is widely distributed and highly expressed in human heart and placenta tissues (23), and it has been reported that RICH1 has multiple functions *in vivo* (24-26). RICH1 can inhibit the activity of Rac1 by catalyzing the cleavage of Rac1-GTP to Rac1-GDP, which then inhibits the MAPK signaling pathway, which in turn reduces cellular proliferation and tumorigenesis (27,28). It has been reported that the overexpression of RICH1 inhibits the invasion and metastasis of breast cancer through down-regulating Hippo signaling (29). However, the role of RICH1 in cardiac hypertrophy remains unknown. The present study aimed to clarify whether RICH1 is associated with myocardial cell hypertrophy and how it serves a role in myocardial cell hypertrophy.

Materials and methods

Cell culture and treatments. The H9c2 cardiomyocyte cell line was purchased from Procell Life Science & Technology Co., Ltd. Cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Procell Life Science & Technology Co., Ltd.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Biosharp Life Sciences) in a 5% CO_2 incubator at 37˚C. To induce the cardiomyocyte hypertrophy model, H9c2 cells were treated with either 60 μM ISO (MilliporeSigma) dissolved in phosphate-buffered saline (PBS) for 48 h, or 3 μM Ang II (Shanghai Yuanye Biotechnology Co., Ltd.) in PBS for 24 h in a 5% CO_2 incubator at 37˚C. The control group cells were treated with an equal volume of PBS in a 5% CO_2 incubator at 37˚C for 48 h (when compared with ISO) and 24 h (when compared with Ang II).

Small interfering (si)RNA transfection of H9c2 cells. H9c2 cells were transfected with 1 μg/ml siRICH1 using siRNA-Mate (Shanghai GenePharma Co., Ltd.) and incubated in 5% CO_2 at 37˚C for 6 h, according to the manufacturer's instructions. A total of 18 h after the 6-h transfection, cells were treated with 60 μM ISO and incubated in a 5% CO_2 incubator at 37˚C for 48 h. A total of 42 h after the 6-h transfection, 3 μM Ang II was added and incubated in a 5% CO_2 incubator at 37˚C for 24 h. The siRNA sequences were as follows: siRICH1 (Shanghai GenePharma Co., Ltd.) sense, 5'-GGUGAAGAAGGAGAG CUUUTT-3' and anti-sense, 5'-AAAGCUCUCCUUCUU CACCTT-3'; and negative control (siNC; Suzhou GenePharma Co., Ltd.) sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and anti-sense, 5'-ACGUGACACGUUCGGAGAATT-3'.

Plasmid transfection of H9c2 cells. H9c2 cells were transfected with 3 μg/ml pCDNA3.1-RICH1 plasmid (TsingKe Biological Technology) with siRNA-Mate and incubated in 5% CO_2 at 37˚C for 6 h to overexpress RICH1. Cells transfected with 3 μg/ml pCDNA3.1 plasmid (TsingKe Biological Technology) were used as a control. A total of 18 h after the 6-h transfection, cells were treated with 60 μM ISO and incubated in a 5% CO_2 incubator at 37˚C for 48 h. A total of 42 h after the 6-h transfection, 3 μM Ang II was added and incubated in a 5% CO_2 incubator at 37˚C for 24 h.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), after which, 1 μg RNA was reverse transcribed into cDNA using an ABScript III RT Master Mix (ABclonal Biotech Co., Ltd.) according to the manufacturer's protocol. After RT, qPCR was performed using a SYBR Green PCR kit (ABclonal Biotech Co., Ltd.) The amplification procedure was as follows: Pre-denaturation at 95˚C for 3 min, followed by 45 cycles at 95˚C for 5 sec and 60˚C for 30 sec. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (30). Data were normalized to *Gapdh*. Primer sequences are shown in Table I.

Western blotting. Total protein was extracted from H9c2 cells using RIPA lysis buffer containing phosphatase inhibitors and protease inhibitors (Wuhan Servicebio Technology Co., Ltd.). Protein concentration was determined using the BCA protein assay kit (Wuhan Servicebio Technology Co., Ltd.). Proteins (15 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels and were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk in 1X TBS-0.5% Tween 20 (Biosharp Life Sciences) for 1 h at room temperature. The blots were then incubated with primary antibodies against RICH1 (1:500; cat. no. sc-514438; Santa Cruz Biotechnology, Inc.) and α-tubulin (1:2,000; cat. no. GB15201; Wuhan Servicebio Technology Co., Ltd.) overnight at 4˚C, followed by anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:50,000; cat. no. BL001A; Biosharp Life Sciences) or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:50,000; cat. no. BL003A; Biosharp Life Sciences) for 1 h at room temperature. Protein bands were visualized using a Chemiluminescence Kit (Biosharp Life Sciences). Bio-ID software (Version 6.0; Bio-Rad Laboratories, Inc.) was used to semi-quantify protein expression.

Cell viability. Cell viability was assessed using Cell Counting Kit-8 (CCK-8; Biosharp Life Sciences). H9c2 cells were seeded in 96-well plates at $5x10^3$ cells/well. After culturing for 24 h, cells were treated with different concentrations of ISO (0, 20, 40, 60, 80 and 100 μM) for 48 h or Ang II (0, 1, 2, 3, 4 and 5 μM) for 24 h at 37˚C. The cells were then incubated with 10% CCK-8 solution in DMEM for 1 h and the absorbance was measured at 450 nm.

Cell fluorescence staining. H9c2 cells were seeded into 35-mm cell culture dishes at $4x10^4$ cells/dish. Cells were fixed with 4% paraformaldehyde at 37˚C for 20 min and then permeabilized with 0.1% Triton X-100 in PBS at 37˚C for 15 min. Cells were incubated with Acti-stain™ 488 phalloidin (Cytoskeleton, Inc.) in the dark for 30 min at 37˚C. ProLong™ Gold antifade reagent was mixed with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 37˚C and used

Table I. Primer sequences.

Gene	Species	Forward primer, 5'-3'	Reverse primer, 5'-3'
Nppa	Rat	GAAGATGCCGGTAGAAGATGAG	AGAGCCCTCAGTTTGCTTTTC
Nppb	Rat	CTGGAGACTGGCTAGGACTTC	GGTGCTGCCCCAGATGATT
Myh7	Rat	GCCCCAAATGCAGCCAT	CGCTCAGTCATGGCGGAT
Gapdh	Rat	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTACTCC

for nuclear staining. Images were captured using an Olympus FV3000 confocal microscope (Olympus Corporation). Cell surface area (CSA) was calculated using CellSens software (Version 4.2.1; Olympus Corporation).

Statistical analysis. Experiments were repeated at least three times and statistical analyses were performed using GraphPad Prism 9 (Dotmatics). Before analysis, data were tested for normality and homogeneity using Shapiro-Wilk and Levene's tests, respectively. Data were analyzed by unpaired Student's t-test to compare the differences between two groups and one-way ANOVA with Tukey's post hoc test to compare the means among >2 groups. $P<0.05$ was considered to indicate a statistically significant difference

Results

RICH1 is downregulated in ISO- or Ang II-treated cardiomyocytes. ISO or Ang II are two common inducers of myocardial cell hypertrophy. In this study, ISO or Ang II were used to induce cardiomyocyte hypertrophy and to verify whether the expression levels of RICH1 were changed under the induction of ISO or Ang II. Notably, 0, 20, 40, 60 and 80 μM ISO, or 0, 1, 2, 3, 4 and 5 μM Ang II were demonstrated to have no negative effect on cell viability at the concentrations tested (Fig. S1). According to these cell viability experiments results, the protein expression levels of RICH1 were assessed after treatment with ISO or Ang II at different concentrations, which have no negative effect on cell viability. In particular, the protein expression levels of RICH1 were significantly decreased in response to 60 μM ISO (Fig. S2A and C) and 3 μM Ang II (Fig. S2B and D). Therefore, these concentrations were selected for further experimentation, and it was verified that the protein expression levels of RICH1 were markedly decreased in 60 μM ISO-treated (Fig. 1A and C) and 3 μM Ang II-treated (Fig 1B and D) H9c2 cells. Therefore, it was suggested that RICH1 may serve a role in cardiomyocyte hypertrophy.

RICH1 can attenuate ISO or Ang II-induced cardiomyocyte hypertrophy. To assess the role of RICH1 in cardiomyocyte hypertrophy, RICH1 was overexpressed in H9c2 cells by transfecting cells with pCDNA3.1-RICH1 plasmids. Compared with in the control group, the protein expression levels of RICH1 were significantly increased in the RICH1 overexpression group (Fig. 2A and B).

The increase in CSA and upregulation of hypertrophy-related genes are two critical features of cardiomyocyte hypertrophy. Notably, it was demonstrated that CSA was significantly increased in ISO-treated (Fig. 2C and E) and Ang II-treated (Fig. 2D and F) cells compared with in the control group. There was no significant difference in CSA between the control group and the RICH1 overexpression group (Fig. 2C and E). Compared with in the ISO group, overexpression of RICH1 inhibited CSA. No difference in CSA was detected between the control group and the RICH1 overexpression group. However, the overexpression of RICH1 inhibited the CSA compared with that in the Ang II group (Fig. 2D and F).

Furthermore, the cardiac hypertrophy marker genes *Nppa*, *Nppb* and *Myh7* were assessed by RT-qPCR. It was demonstrated that the relative mRNA expression levels of these genes were significantly increased in ISO- or Ang II-treated cells compared with those in the control groups, whereas they were significantly decreased following overexpression of RICH1 in ISO- or Ang II-treated cells compared with those in the ISO or Ang II single treatment groups, respectively (Fig. 2G-L). These results suggested that RICH1 can alleviate cardiomyocyte hypertrophy induced by ISO or Ang II.

KD of RICH1 can promote ISO or Ang II-induced cardiomyocyte hypertrophy. To further assess the role of RICH1 in ISO- and Ang II-induced cardiomyocyte hypertrophy, siRNA was used to KD RICH1 in H9c2 cells. First, a siRNA fragment that can induce RICH1 KD in H9c2 cells was identified (Fig. 3A and B). It was then demonstrated that RICH1 KD in ISO- and Ang II-treated cells resulted in a significantly larger CSA compared with that in ISO- and Ang II-treated cells transfected with the siNC, respectively (Fig. 3C-F). Similarly, the relative mRNA expression levels of the cardiac hypertrophy-related genes *Nppa*, *Nppb* and *Myh7* were significantly higher in the siRICH1 + ISO group and siRICH1 + Ang II groups compared with those in the siNC + ISO and siNC + Ang II groups, respectively, as demonstrated by RT-qPCR (Fig. 3G-L). These results suggested that siRNA-mediated RICH1 KD can exacerbate cardiomyocyte hypertrophy induced by ISO or Ang II.

Discussion

Heart failure is the final stage of cardiovascular diseases, which results in high morbidity and mortality, and cardiac hypertrophy is an important stage in the occurrence of heart failure. Inhibiting cardiac hypertrophy is of great significance for preventing and alleviating heart failure. It has been reported that various signaling pathways, such as the MAPK/ERK, RhoA and PI3K/Akt/NF-κB pathways, are involved in cardiac hypertrophy (1,4-7). Previous studies have

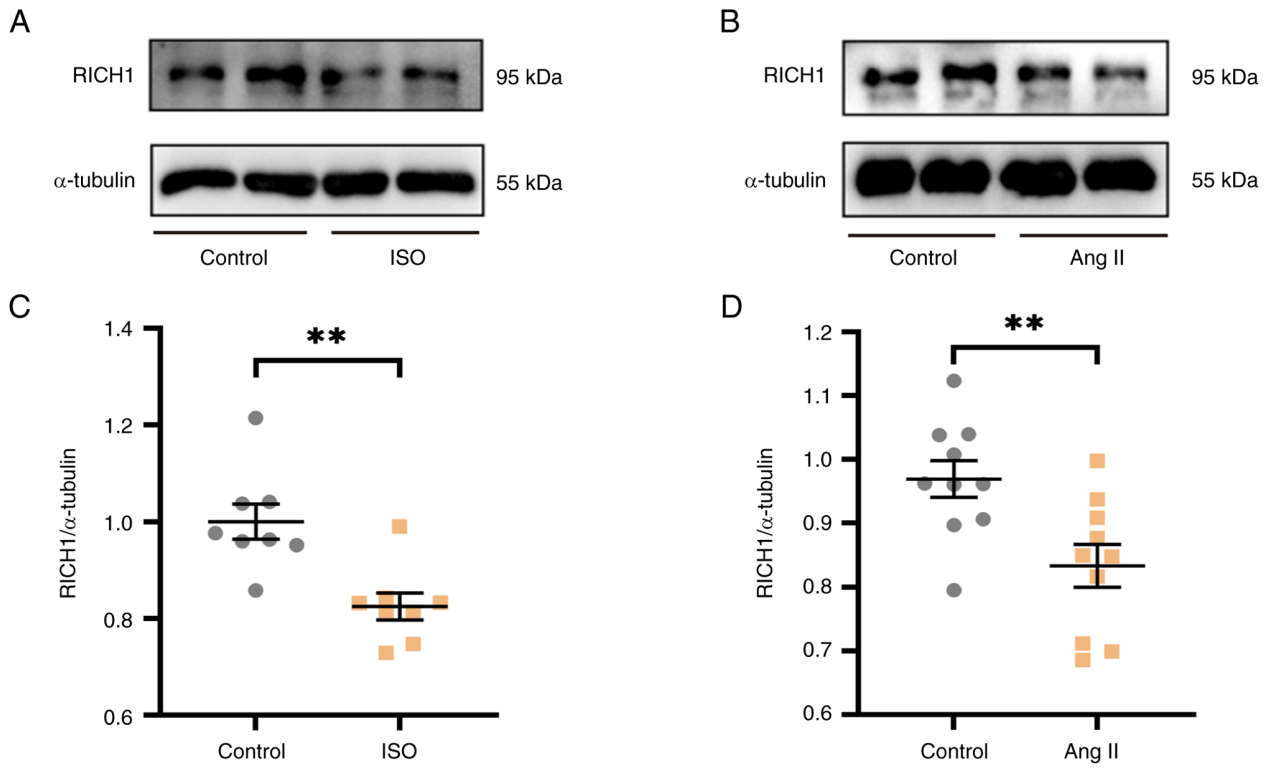

Figure 1. RICH1 is downregulated in ISO- or Ang II-treated H9c2 cells. Western blotting results of RICH1 and α-tubulin in (A) ISO- and (B) Ang II-treated cells. H9c2 cells were treated with phosphate-buffered saline in the control group, and 60 μM ISO or 3 μM Ang II in the experimental groups, and total protein was extracted for western blotting. Statistical evaluation of protein expression in cells treated with (C) 60 μM ISO (n=8 experimental repeats/group) and (D) 3 μM Ang II (n=10 experimental repeats/group) normalized to α-tubulin in each group. Data were analyzed using unpaired Student's t-test and presented as the mean ± SEM. **P<0.01. ISO, isoproterenol; Ang II, angiotensin II; RICH1, Rho GTPase-activating protein interacting with CIP4 homologs protein 1.

reported that RICH1 is a type of RhoGAP and a key regulatory factor in numerous signaling pathways, such as ERK and Rho signaling pathways, and diseases, including breast cancer (26,29,31). However, to the best of our knowledge, the relationship between RICH1 and cardiac hypertrophy has not been reported.

In the present study, it was demonstrated that RICH1 can act as a novel suppressor of ISO- or Ang II-induced cardiomyocyte hypertrophy. RICH1 was downregulated in ISO- or Ang II-induced cardiomyocyte hypertrophy, and the overexpression of RICH1 inhibited the increase in CSA and decreased the relative mRNA expression levels of cardiac hypertrophic markers, including *Nppa*, *Nppb* and *Myh7*, in ISO- or Ang II-treated H9c2 cells. By contrast, siRNA-mediated RICH1 KD exacerbated ISO- or Ang II-induced increases in CSA, as well as the expression of genes related to cardiomyocyte hypertrophy. The present study demonstrated that RICH1 may be a novel target in the development of treatments for cardiac hypertrophy. It has been reported that RICH1 can directly interact with small GTPases Cdc42 and Rac1, and serves a key role in Cdc42/Rac1/ERK1/2 signaling (32). Notably, RICH1 can inhibit the phosphorylation of ERK1/2 in epithelial cells through inactivating Cdc42 and Rac1 (30). It is thus implied that RICH1 may inhibit cardiac hypertrophy through regulating the Cdc42/Rac1/ERK1/2 signaling pathway.

Based on previous studies and the results of the present study, a prospective molecular mechanism by which RICH1 mediates cardiomyocyte hypertrophy has been proposed. It was demonstrated that RICH1 is downregulated in ISO- or Ang II-induced cardiomyocyte hypertrophy; therefore its downstream effectors, such as Cdc42 and Rac1, may remain active, thus leading to cardiomyocyte hypertrophy (Fig. 4). However, RICH1 overexpression may inactivate its downstream effectors and result in the attenuation of ISO or Ang II-induced cardiomyocyte hypertrophy (Fig. 4). To elucidate more detailed roles and mechanisms of RICH1 in cardiac hypertrophy, cardiac-specific overexpression or KD of RICH1 should be assessed in future studies.

RICH1 is highly expressed in cardiac tissue (23), which implies that the maintenance of high concentrations of RICH1 may be important for myocardial structure and function; therefore, downregulation of RICH1 may trigger myocardial remodeling. The use of RICH1 could thus be beneficial as a prospective diagnostic indicator of myocardial hypertrophy; however, further studies are required. For example, it is worth testing what changes occur in downstream proteins or signaling molecules after the dysregulation of RICH1.

Previous studies have reported that autophagy; actin-binding proteins, such as cofilin, Formin and CapZ; and the MAPK/ERK signaling pathway serve roles in cardiac hypertrophy (33-35). It has been reported that autophagosome formation depends on small GTPases (36). In addition, small GTPases, such as Cdc42 and Rac1, are the upstream regulators of actin-binding proteins and the ERK signaling pathway (37,38). Likewise, the abnormal upregulation of Rho GTPase functions can induce cardiac hypertrophy. Fasudil, an inhibitor of ROCK, which is a RhoA effector, can inhibit cardiac remodeling and hypertrophy (39). In addition, vitamin

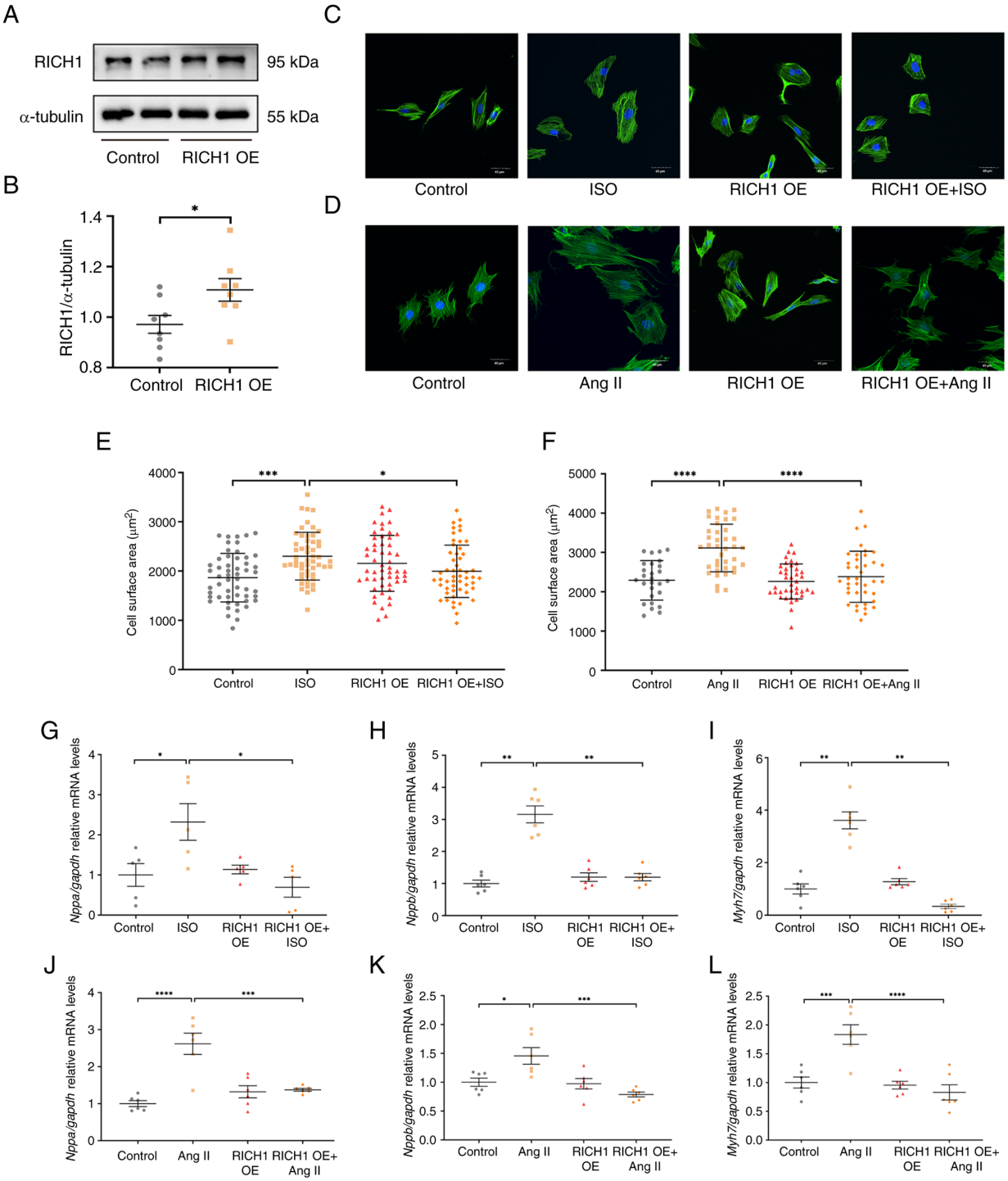

Figure 2. Overexpression of RICH1 attenuates ISO- or Ang II-induced cardiomyocyte hypertrophy. (A) Protein expression levels of RICH1 in H9c2 cells transfected with pCDNA3.1-RICH1 plasmids were assessed by western blotting and (B) RICH1 expression was normalized to α-tubulin (n=8 experimental repeats/group). Data were analyzed by unpaired Student's t-test and presented as the mean ± SEM. H9c2 cells were stained with Acti-stain™ 488 phalloidin and captured using an Olympus FV3000 confocal microscope. Overexpression of RICH1 can inhibit the enlargement of CSA induced by (C) ISO or (D) Ang II. Scale bar, 40 μm. CSA quantification in response to (E) ISO (n=53/group) and (F) Ang II (n=39 number of cells assessed/group). This experiment was repeated three times. Data were analyzed by one-way ANOVA with Tukey's post hoc test and are presented as the mean ± SD. Reverse transcription-quantitative PCR was used to assess the expression levels of the cardiac hypertrophy-related genes (G and L) *Nppa*, (H and K) *Nppb* and (I and L) *Myh7*, which were normalized to *Gapdh*. Overexpression of RICH1 can inhibit the upregulation of cardiac hypertrophy marker genes induced by (G-I) ISO or (J-L) Ang II (n=5-6 experimental repeats/group). Data were analyzed using one-way ANOVA with Tukey's post hoc test and are presented as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. CSA, cell surface area; ISO, isoproterenol; Ang II, angiotensin II; OE, overexpression; Rho GTPase-activating protein interacting with CIP4 homologs protein 1.

D can inhibit the expression of Rac1 and alleviate pressure overload-induced cardiac hypertrophy (40), and statins can relieve cardiac hypertrophy by inhibiting Rac1-mediated oxidative stress (41). Molecular switches act as a critical and specific 'brakes' of Rho GTPases. RICH1 is upstream of Rho GTPases; therefore, RICH1 may be considered a promising

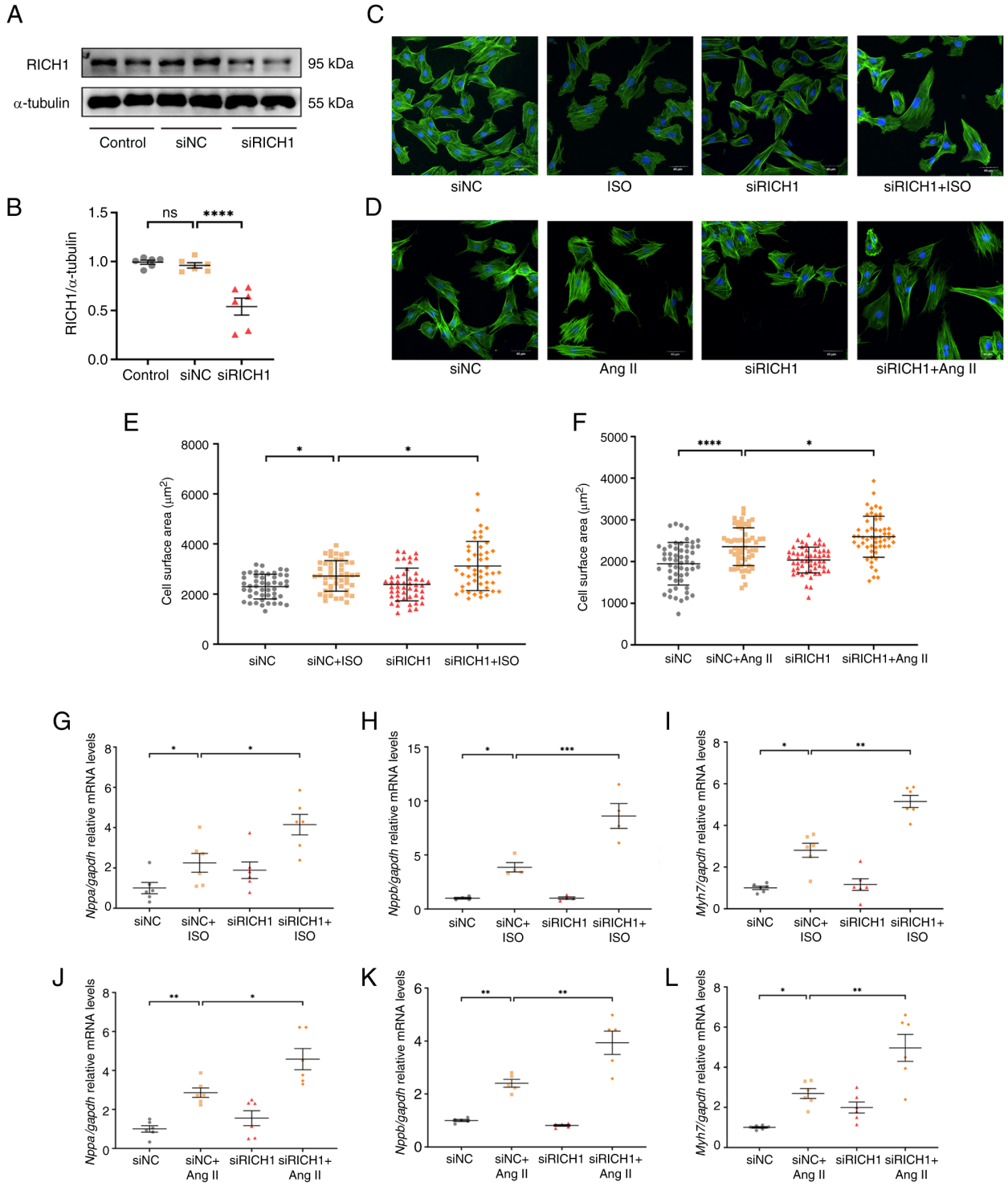

Figure 3. siRNA-directed RICH1 knockdown exacerbates ISO- or Ang II-induced cardiomyocyte hypertrophy. (A) Protein expression levels of RICH1 in H9c2 cells transfected with siRICH1 and siNC were assessed by western blotting, and (B) RICH1 expression was normalized to α-tubulin (n=6 experimental repeats/group). Data were analyzed using one-way ANOVA with Tukey's post hoc test and are presented as the mean ± SEM. H9c2 cells were stained with Acti-stain™ 488 phalloidin and captured under an Olympus FV3000 confocal microscope. Knockdown of RICH1 enhanced the enlargement of CSA induced by (C) ISO or (D) Ang II. Scale bar, 40 μm. CSA quantification in response to (E) ISO (n=48 number of cells assessed/group) and (F) Ang II (n=55 number of cells assessed/group). This experiment was repeated three times. Data were analyzed using one-way ANOVA with Tukey's post hoc test and are presented as the mean ± SD. Expression levels of the cardiac hypertrophy-related genes (G and J) *Nppa*, (H and K) *Nppb* and (I and L) *Myh7* treated with (G-I) ISO or (J-L) Ang II were assessed by reverse transcription-quantitative PCR and normalized to *Gapdh* (n=4-6 experimental repeats/group). Data were analyzed using one-way ANOVA with Tukey's post hoc test and are presented as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. CSA, cell surface area; ISO, isoproterenol; Ang II, angiotensin II; NC, negative control; si, small interfering; ns, not significant; RICH1, Rho GTPase-activating protein interacting with CIP4 homologs protein 1.

therapeutic target for the treatment of cardiac hypertrophy. The present study provides insights into the molecular mechanisms underlying cardiac hypertrophy, which should be further assessed in future studies.

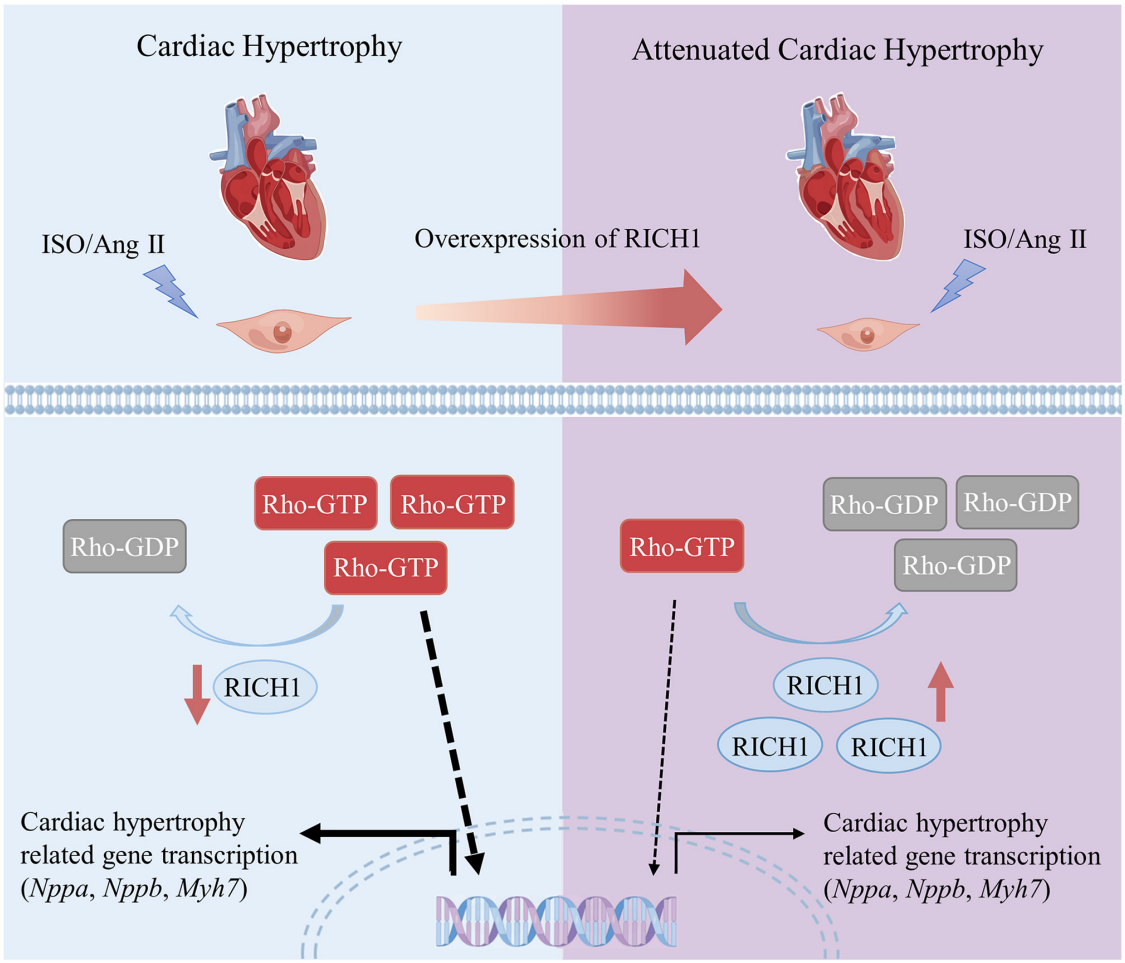

Figure 4. Schematic diagram of molecular mechanisms by which RICH1 mediates cardiomyocyte hypertrophy. Overexpression of RICH1 inhibited cardiomyocyte hypertrophy induced by ISO and Ang II. Active Rho GTPase proteins can positively promote the expression of cardiac hypertrophy-related genes, *Nppa*, *Nppb* and *Myh7*. RICH1 can induce the inactivation of Rho. Under the stimulation of ISO and Ang II, RICH1 is downregulated. Therefore, more Rho GTPase proteins remain active and enhance the expression of cardiomyocyte hypertrophy-related genes. When RICH1 is overexpressed, Rho GTPase proteins are inactivated and cardiac hypertrophy-related genes are downregulated. ISO, isoproterenol; Ang II, angiotensin II; RICH1, Rho GTPase-activating protein interacting with CIP4 homologs protein.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SW, LL and CL made substantial contributions to the conception or design of the work: acquisition, analysis and interpretation of data; drafting the work or reviewing it critically for important intellectual content; and finally approval of the version to be published; they also agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. XW completed and checked data statistics and analysis. ZR conceived, edited and finalized the manuscript. SW and ZR confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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