ORIGINAL RESEARCH

Regulator of G Protein Signaling 6 Facilities Cardiac Hypertrophy by Activating Apoptosis Signal–Regulating Kinase 1–P38/c-JUN N-Terminal Kinase 1/2 Signaling

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***Background*—**Regulator of G protein signaling 6 (RGS6) is an important member of the RGS family and produces pleiotropic regulatory effects on cardiac pathophysiology. However, the role of RGS6 protein in cardiomyocytes during angiotensin II– and pressure overload–induced cardiac hypertrophy remain unknown.

***Methods and Results*—**Here, we used a genetic approach to study the regulatory role of RGS6 in cardiomyocytes during pathological cardiac hypertrophy. RGS6 expression was significantly increased in failing human hearts and in hypertrophic murine hearts. The extent of aortic banding–induced cardiac hypertrophy, dysfunction, and fibrosis in cardiac-specific RGS6 knockout mice was alleviated, whereas the hearts of transgenic mice with cardiac-specific RGS6 overexpression exhibited exacerbated responses to pressure overload. Consistent with these findings, RGS6 also facilitated an angiotensin II–induced hypertrophic response in isolated cardiomyocytes. According to the mechanistic studies, RGS6 mediated cardiac hypertrophy by directly interacting with apoptosis signal–regulating kinase 1, which further activates the P38-c-JUN N-terminal kinase 1/2 signaling pathway.

***Conclusions*—**Based on our findings, RGS6 aggravates cardiac hypertrophy, and the RGS6-apoptosis signal–regulating kinase 1 pathway represents a potential therapeutic target to attenuate pressure overload–driven cardiac remodeling. **(*J Am Heart Assoc*. 2018;7:e009179. DOI: 10.1161/JAHA.118.009179.)**

Key Words: angiotensin II • apoptosis signal–regulating kinase 1 • cardiac hypertrophy • hypertension • regulator of G protein signaling protein 6

Cardiac hypertrophy is an early hallmark of the clinical course of heart failure and is an independent risk factor for cardiac and noncardiac mortality.[1] Cardiac hypertrophy results from an adaptive response to persistent stresses by increasing muscle mass and volume.[2] Cardiac hypertrophy and failure are highly complex disorders that arise from a combination of genetic, physiological, and environmental factors. Multiple molecular mechanisms have been proposed and proven to be involved in the pathogenesis of this cardiac abnormality. Pathological cardiac hypertrophy is largely associated with G protein–coupled receptor (GPCR)–mediated signaling.[3,4] Among the many essential regulators of GPCR signaling, regulator of G protein signaling proteins (RGSs) were first identified as a group of GTPase-accelerating proteins that promote GTP hydrolysis by the alpha subunit of heterotrimeric G proteins, thereby inactivating the G protein and rapidly switching off signaling pathways.[4,5] More recently, however, studies by our group and others have provided evidence that RGSs, including RGS3, 10, 12, and 14, regulate cardiac hypertrophy by inhibiting mitogen-activated protein kinase kinase 1/2 (MEK1/2)–extracellular signal–regulated kinase 1/2 (ERK1/2) signaling through a mechanism independent of GTPase-activating protein activity.[6–9]

RGS6, a member of the R7 subfamily of RGSs, is robustly expressed in the heart and is reported to be a key regulator of cardiac rhythm and ischemic injury through its canonical G

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Accompanying Data S1, Tables S1 through S4, and Figures S1, S2 are available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.118.009179

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Clinical Perspective

What Is New?

- This study demonstrates that regulator of G protein signaling 6 (RGS6) in cardiomyocytes aggravates cardiac remodeling and fibrosis independent of its role in regulation of G protein–coupled receptor–mediated signaling.
- Mechanistic study reveals that RGS6 mediated cardiac hypertrophy by directly interacting with apoptosis signal–regulating kinase 1 and further activates the apoptosis signal–regulating kinase 1–P38-JUN N-terminal kinase 1/2 signaling pathway.

What Are the Clinical Implications?

- These findings suggest the potential for RGS6 to be an alternative therapeutic target to prevent the progression of cardiac remodeling and fibrosis with response to hypertrophic stimulus.
- Cardiac hypertrophy is a preclinical phase of heart failure and is an independent risk factor for cardiac and noncardiac mortality. The potential to prevent cardiac hypertrophy to improve functional outcomes and decrease mortality is highly clinically relevant.
- Pleiotropic RGS6 and other RGS proteins are able to elicit tissue-specific pathways in response to various stimuli. This feature of the RGS protein is important yet poorly characterized in multicellular organisms.

protein actions.[10,11] RGS6 is also unique because it remains the only member of the R7 protein family that has been shown to regulate G protein–independent pathways, as evidenced by its function as an activator in reactive oxygen species–dependent apoptosis in both doxorubicin-induced and alcohol-induced cardiomyopathy.[12,13] Because of its potential in regulation of cardiac pathophysiology and disease, in the current study, we sought to clarify whether RGS6 in cardiomyocytes is involved in cardiomyocyte hypertrophy and therefore modulates cardiac remodeling. Here, we provide new evidence that the pleiotropic RGS6 protein is a critical upstream mediator of angiotensin II (Ang II)– and pressure overload–induced cardiac hypertrophy. RGS6 expression is markedly increased in the heart of individuals with heart failure, and mouse cardiomyocytes lacking RGS6 exhibit impaired development of cardiomegaly. According to mechanistic studies, RGS6 directly interacts with and phosphorylates apoptosis signal–regulating kinase 1 (ASK1), facilitating the activation of P38 and c-JUN N-terminal kinase 1/2 (JNK) signaling in response to pressure overload. These actions of RGS6 are entirely novel as they are independent of its canonical function as a GTPase-activating protein for heterotrimeric G proteins.

Methods

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure on request from the Department of Cardiology of the Third Xiangya Hospital, Central South University, Hunan, China.

Animal Models

The animal protocol was approved by the Animal Care and Use Committee of the Third Xiangya Hospital of Central South University. The investigation complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Generation of Cardiac-Specific RGS6 Transgenic Mice

Cardiac-specific transgenic mice were generated as previously described.[7] Briefly, the pCAG-loxP-CAT-loxP-RGS6 transgene vector was constructed by replacing the lacZ gene in pCAG-loxP-CAT-loxP-lacZ with the full-length mouse RGS6 cDNA. The construct was linearized and purified using the QIAquick Gel Extraction Kit (Qiagen, 28704) and then used for pronuclear microinjections. Founder transgenic mice were identified by tail DNA amplification and then bred with C57BL/6J mice. Tail genomic DNA was amplified using polymerase chain reaction (PCR). Primers for PCR include the CAG gene promoter: forward primer 5′-CATGTCTGGATCGATCCCCG-3′ and reverse primer TCCGGTCAATCTTCACTTGT-3′. The expected size of the amplification product was 686 bp. Cardiac-specific RGS6 transgenic mice were generated by mating the founders with α-myosin heavy chain–MerCreMer transgenic mice (Jackson Laboratory, 005650). These double-transgenic mice (6 weeks of age) were intraperitoneally injected with tamoxifen (80 mg/kg per day; Sigma, T-5648) for 5 consecutive days to specifically induce Cre-mediated CAT gene excision and RGS6 expression in the heart. The CAG-CAT-RGS6/α-myosin heavy chain–MerCreMer mice that were not administered tamoxifen served as the control group.

Generation of Cardiac-Specific RGS6 Knockout Mice

The fifth exon was flanked by loxP sites, and thus, 2 single guide RNAs (sgRNA1 and sgRNA2) targeting RGS6 introns 4 and 5 were designed to obtain mice carrying a conditional knockout RGS6 allele. Targeting constructs were generated by introducing the loxP-flanked RGS6 exon 5 into the left and right short homology arms (40 bp) of microhomology-mediated end-joining, which were flanked by the 2 gRNA

target sequences (Tsg). The targeting construct, gRNA1 and gRNA2, Tsg and Cas9 mRNAs were coinjected into 1-cell-stage embryos using the FemtoJet 5247 microinjection system under standard conditions (Figure S1). DNA fragments containing the entire left and right homology arms and the floxed exon 5 were amplified using primers P1 and P2. All PCR products were subcloned and sequenced. Founder mice contained loxP-flanked (floxed) exon 5 on the same allele. We used genomic DNA for in vitro Cre–loxP-mediated recombination to confirm that the floxed allele was functional. Primer pairs RGS6-P1 and RGS6-P2 as well as RGS6-P3 and RGS6-P4 were used to detect the deletion product and the circle product, respectively. All products were confirmed by sequencing. We also used primers RGS6-P5 and RGS6-P6 to avoid random integration. The founder mouse was mated with a C57BL/6J female mouse to obtain homozygous RGS6-floxed mice. PCR primers RGS6-P1 and RGS6-P3 were used to screen the progeny. Cardiac-specific RGS6 knockout (RGS6 CKO) mice were generated by mating RGS6 flox/flox (RGS6-Flox) mice with α-myosin heavy chain–MerCreMer (The Jackson Laboratory, 005650). All primer sequences (RGS6-P1 to RGS6-P6) are listed in Table S1.

Echocardiography Evaluation

Echocardiography measurements were performed before tissue harvest to evaluate the cardiac function of the mice using previously described methods (detailed in Data S1).[6,8]

Histological Analysis and Immunofluorescence Staining

The histological staining of hematoxylin and eosin, Picrosirius red, and immunofluorescence staining of wheat germ agglutinin using heart sections were performed as previously described (detailed in Data S1).[6,8] Cardiomyocyte cross-sectional area, interstitial collagen deposition, and perivascular collagen deposition were measured using Image-Pro Plus 6.0 software (Media Cybernetics, Inc).

Quantitative Real-Time PCR and Western Blotting

Quantitative real-time PCR (qRT-PCR) and Western blotting of left ventricle cardiac tissue and cultured cardiomyocytes were performed using previously described methods.[6,8] The primers are listed in Table S2.

Cardiomyocyte Culture and Infection With Recombinant Adenoviral Vectors

Primary neonatal rat cardiomyocytes (NRCMs) and cardiac fibroblasts were isolated ventricles and cultured as previously described (detailed in Data S1).[6,8] We infected cardiac myocytes with adenoviruses expressing RGS6 (AdRGS6), green fluorescent protein, a short hairpin RNA targeting RGS6 (AdshRGS6), or a short hairpin RNA for 12 hours. The culture medium was then replaced with serum-free medium overnight, and then medium containing 1 μmol/L Ang II was incubated with the cells for 48 hours. The sequence of RGS6 short hairpin RNA are listed in Table S3.

Human Heart Samples

All procedures involving human samples complied with the principles outlined in the Declaration of Helsinki and were approved by the ethics committee at the Third Xiangya Hospital of Central South University. Written informed consent was obtained from the family of prospective heart donors. Samples were collected from the left ventricle of patients with dilated cardiomyopathy who were undergoing heart transplant surgery. Control samples were collected from normal heart donors who had died of accidental injuries and whose hearts were not suitable for transplantation. Informed consent was obtained from the families of the prospective heart donors.

Plasmid Constructs

Plasmids containing the full-length human HA-ASK1 and Flag-RGS6 cDNAs were obtained by cloning the appropriate cDNA into pcDNA5-HA and psi-Flag vectors, respectively. The sequences of ASK1 and RGS6 were amplified by PCR using human or mouse cDNAs as a template. The PCR-amplified fragments were inserted into the pcDNA5 expression vector. GST-ASK1 and GST-RGS6 were obtained by cloning the ASK1 or RGS6 cDNA into the pGEX-4T-1 vector. Expression plasmids encoding truncated ASK1 or RGS6 were constructed using standard molecular biology techniques. The primers used in this study are listed in Table S4.

Immunoprecipitation Assay

Human embryonic kidney 293T cells were transfected with ASK1 and RGS6 expression plasmids for 24 hours to examine the interaction between these 2 proteins. Cells were then collected and lysed in an ice-cold immunoprecipitation buffer containing 20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP-40, 1 mmol/L EDTA, and a protease inhibitor cocktail (Roche). The obtained lysates were incubated with beads conjugated with the anti-FLAG (#F3165, Sigma) or anti-HA (#H6908, Sigma) antibody for 3 hours at 4°C. Interactions between the proteins of interest were measured via immunoblotting with the indicated primary and secondary antibodies.

Statistical Analysis

Data are presented as means±SDs. Normality and variance equality analysis was performed before any test using Shapiro–Wilk and Levene tests. When normally distributed and equal variances assumed, comparisons between 2 groups were performed using an independent 2-tailed Student *t* test. One-way ANOVA with the least significant difference (equal variances assumed) test or Tamhane T2 (equal variances not assumed) test was used to determine the significance of differences between >2 groups. $P<0.05$ was considered statistically significant. Statistical analyses were performed with SPSS version 22 software (IBM).

Results

RGS6 Expression is Increased in the Myocardium of Patients With Heart Failure and in Hypertrophic Mouse Hearts

We tested the hypothesis that RGS6 expression was dysregulated in the hypertrophic myocardium. Human heart tissues were collected from patients receiving a heart transplant. As shown in Figure 1A, the expression of the RGS6 protein was upregulated 6.5-fold in failing hearts of patients with dilated cardiomyopathy compared with levels in normal donors (6.48±1.62 versus 1.00±0.73, $P<0.01$). Levels of atrial natriuretic peptide (ANP) and β-myosin heavy chain (MYH7) proteins, which have been reported to be involved in the pathological process of myocardial hypertrophy,[6,8] were increased, similar to RGS6 levels (ANP: 4.81±1.62 versus 1.00±0.17 [$P<0.01$]; MYH7: 3.51±0.50 versus 1.00±0.37 [$P<0.01$]). These results were consistent with the observations in aortic banding (AB)–induced cardiac hypertrophy in murine hearts and in Ang II–treated NRCMs. Levels of the RGS6 protein increased over time, by 2.3- and 6.3-fold at 4 and 8 weeks after AB, respectively (2.33±0.18 versus 1.00±0.19 [$P<0.01$]; 6.29±0.40 versus 1.00±0.19 [$P<0.01$]). Levels of the ANP and MYH7 proteins also significantly increased (Figure 1B). In vitro, exposure to 1 μmol/L Ang II for 24 and 48 hours induced time-dependent increases in the levels of the RGS6, ANP, and MYH7 proteins in primary cultured NRCMs (Figure 1C). Thus, the upregulation of RGS6 expression in the hypertrophic myocardium indicates that an RGS6-mediated pathway may be involved in cardiac remodeling and hypertrophy.

RGS6 Mediates Ang II–Induced Cardiomyocyte Hypertrophy In Vitro

We manipulated the RGS6 gene expression in primary cultured NRCMs by transfecting them with AdshRGS6, AdRGS6, or the respective adenoviral controls to determine whether RGS6 expressed in cardiomyocytes plays an essential role in hypertrophy in vitro. As shown in Figure 2A, RGS6 expression was sufficiently inhibited by AdshRGS6 and upregulated by AdRGS6. NRCMs exposed to 1 μmol/L Ang II for 48 hours exhibited a significant increase in cell size. However, AdshRGS6 transfection blocked the Ang II–induced increase in cell size by ≈38.3% (1223.22±160.72 versus 1691.53±174.44, $P<0.01$), and AdRGS6 transfection further augmented the Ang II–driven expansion in cell size by 52% compared with controls (2455.56±245.61 versus 1615.43±135.80, $P<0.01$). Adenoviral manipulation of RGS6 expression did not change the cell size at baseline in NRCMs (Figure 2B). Levels of mRNAs encoding the myocardial hypertrophy markers ANP and MYH7 were upregulated in NRCMs 48 hours after Ang II treatment compared with the control groups, as evidenced by the qRT-PCR results. AdshRGS6 transfection significantly inhibited the Ang II–induced increase in ANP and MYH7 mRNA expression (ANP: 2.63±0.18 versus 9.14±0.87 [$P<0.01$]; MYH7: 2.03±0.13 versus 7.29±0.05 [$P<0.01$]), and AdRGS6 infection further enhanced the Ang II–induced increase in ANP and MYH7 mRNA expression (ANP: 13.60±0.14 versus 8.84±0.78 [$P<0.01$]; MYH7: 10.76±1.03 versus 6.78±0.24 [$P<0.05$]) (Figure 2C).

Cardiac-Specific Deletion of RGS6 Attenuates AB-Induced Cardiac Hypertrophy

We selectively deleted the RGS6 gene from the cardiomyocytes by crossing RGS6-floxed mice with mice carrying an α-myosin heavy chain–MerCreMer transgenic (MCM-Cre) to produce mice carrying MCM-Cre and floxed RGS6 genes (refer to the "Methods" section) and further determine whether RGS6 expression in cardiomyocytes is required for the development of cardiac hypertrophy in vivo. Results from representative Western blots showed that RGS6 expression was sufficiently inhibited in heart tissues from RGS6 cardiomyocyte-specific knockout (RGS6 CKO) mice compared with RGS6-floxed mice and MCM-Cre mice (Figure 3A and 3B). The baseline cardiac phenotype of RGS6 CKO mice was not altered. RGS6 CKO mice exhibited a significant reduction in the AB-induced increase in cardiac weight compared with RGS6-floxed mice and MCM-Cre mice, as evidenced by the heart weight/body weight ratio (HW/BW), lung weight/BW ratio, and HW/tibia length ratio. RGS6 CKO mice also displayed an amelioration of AB-induced cardiomegaly (Figure 3C through 3E) and cardiomyocyte hypertrophy (Figure 3F), as well as levels of mRNAs encoding hypertrophy markers (such as ANP, brain natriuretic peptide, and MYH7) in vivo (Figure 3K). We also determined whether cardiac RGS6 expression mediates AB-induced cardiac dysfunction. Left ventricular end-diastolic dimension, left ventricular

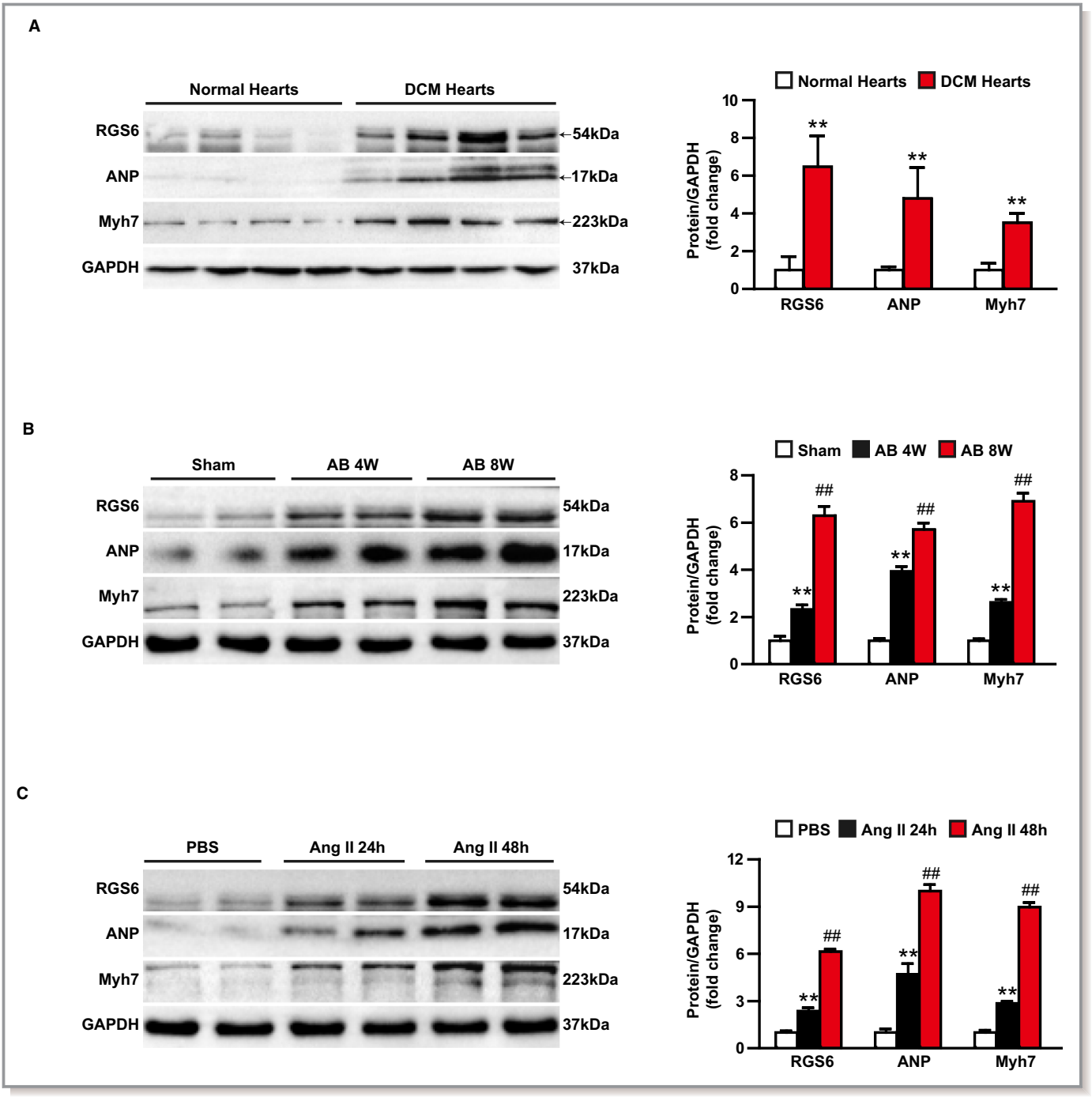

Figure 1. Regulator of G protein signaling 6 (RGS6) is expressed in heart and is upregulated in response to hypertrophic stimuli. A, Tissue lysates were prepared from the normal donor hearts (n=8) and failing hearts of patients with dilated cardiomyopathy (DCM) (n=8) and were subjected to Western blotting analysis with anti–atrial natriuretic peptide (ANP), β-myosin heavy chain (MYH7), RGS6, and GAPDH antibodies. B, Aortic banding (AB) procedures were performed on wild-type mice, and the heart tissue was harvested 4 and 8 weeks after surgery (n=4 per group). ANP, MYH7, RGS6, and GAPDH were detected by Western blotting. C, Neonatal rat cardiomyocytes were treated with 1 μmol/L angiotensin II (Ang II) for 24 to 48 hours. ANP, MYH7, RGS6, and GAPDH were detected by Western blotting. Representative Western blots from the indicated groups and quantification of the results are shown. Protein levels were normalized to GAPDH levels. The graph shows the quantitative data from 4 to 5 independent experiments. Data are presented as means±SDs. $^{**}P<0.01$ compared with the controls. $^{\#\#}P<0.01$ compared with the group 4 weeks after AB or 24 hours after Ang II treatment.

end-systolic dimension, and fractional shortening percentage were captured by echocardiography. AB successfully increased the size of the hearts and impaired ejection functions in RGS6-floxed mice and MCM-Cre mice. RGS6 CKO mice were significantly protected from the AB-induced increase in heart size and impaired function (Figure 3G

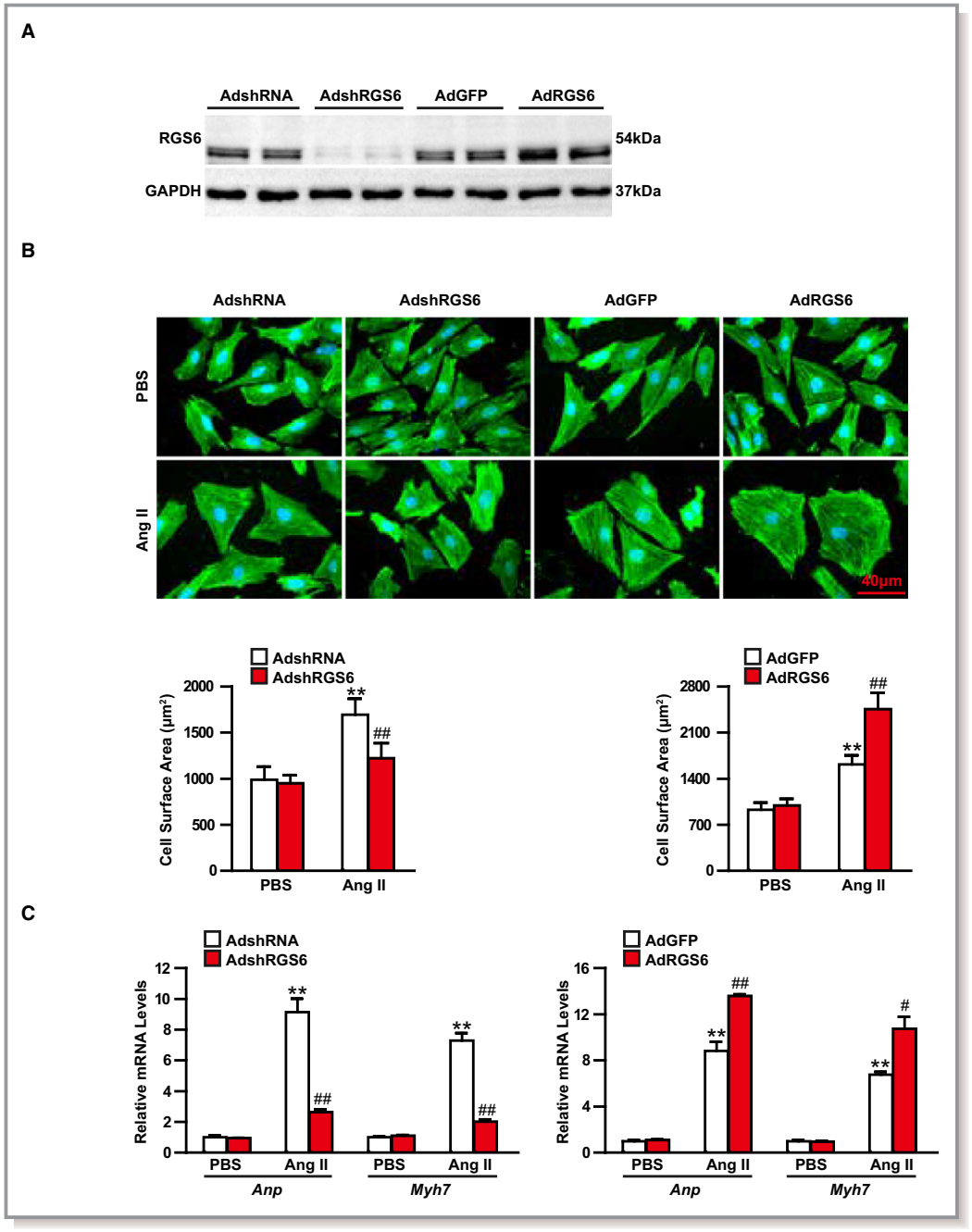

Figure 2. Regulator of G protein signaling 6 (RGS6) regulates angiotensin II (Ang II)–induced cardiomyocyte hypertrophy in vitro. A, Neonatal rat cardiomyocyte (NRCM) lysates were prepared from cells infected with adenoviruses expressing short hairpin RNA (AdshRNA), a short hairpin RNA targeting RGS6 (AdshRGS6), green fluorescent protein (AdGFP), or RGS6 (AdRGS6) and subjected to Western blotting with anti-RGS6 and GAPDH antibodies. Representative Western blots are shown. B, Representative fluorescent images of NRCMs infected with the indicated adenoviruses and treated with 1 μmol/L Ang II for 48 hours are shown. NRCMs were stained with an α-actinin antibody (green). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Cell surface areas were quantified by planimetry after treatments. Fifty cells were measured for each group. C, Atrial natriuretic peptide (ANP) and β-myosin heavy chain (MYH7) mRNA levels were measured in NRCMs 48 hours after Ang II exposure using quantitative real-time polymerase chain reaction and normalized to levels of the GAPDH transcript. The graph shows the quantitative data from 4 to 5 independent experiments. Data are presented as means±SDs. $^{**}P<0.01$ compared with the respective adenovirus control groups. $^{\#}P<0.05$; $^{\#\#}P<0.01$ compared with the respective adenovirus control group treated with Ang II.

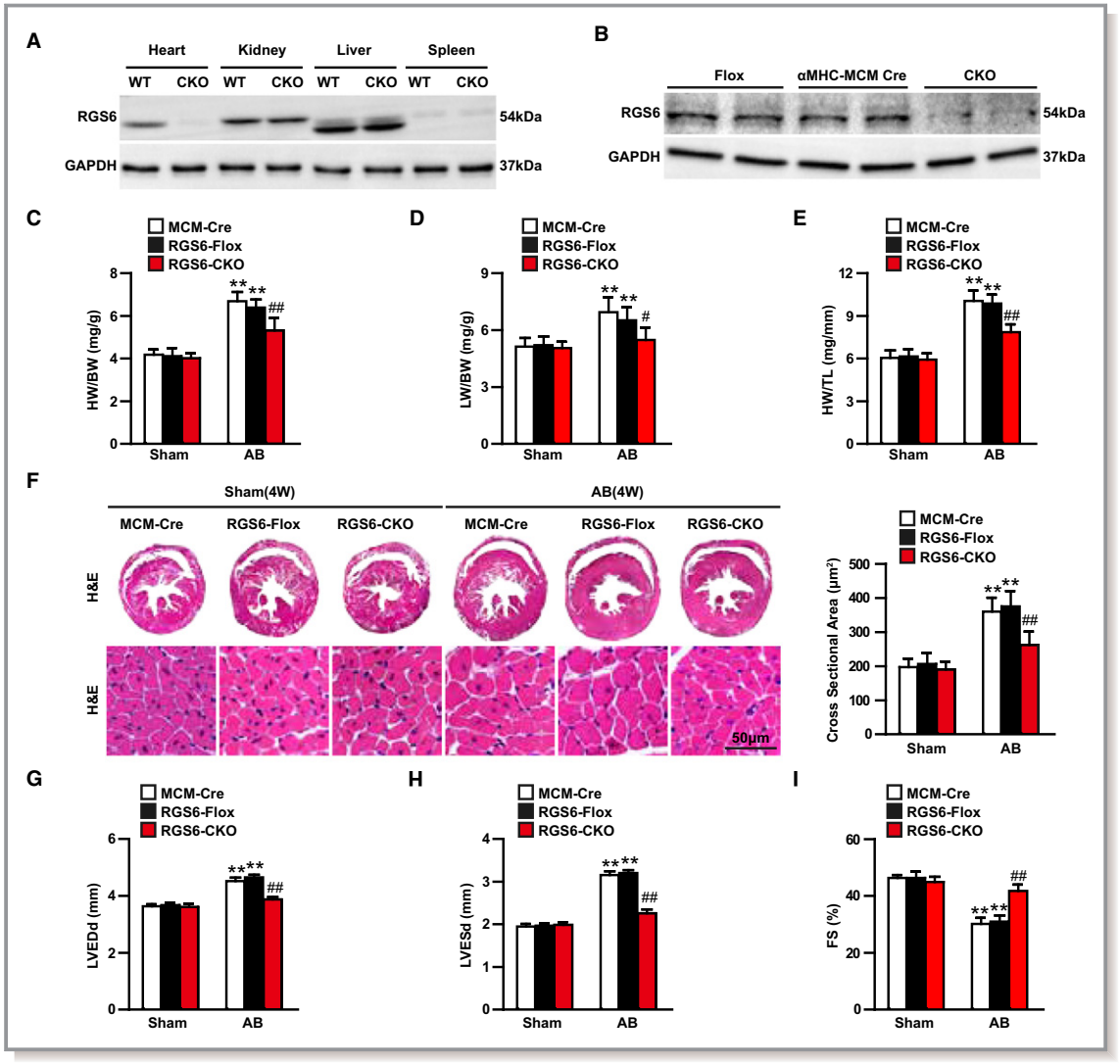

Figure 3. Cardiac-specific deletion of regulator of G protein signaling 6 (RGS6) attenuates aortic banding (AB)–induced cardiac hypertrophy. A, We established the cardiac-specific RGS6 knockout (RGS6 CKO) strain. Tissue lysates from the heart, kidney, liver, and spleen were prepared from wild-type (WT) mice (n=4) and RGS6 CKO mice (n=4) and subjected to Western blotting analysis. Representative Western blots showing RGS6 expression levels in the 2 strains. B, Tissue lysates from the heart in the RGS6-floxed mice (n=4), α-myosin heavy chain (αMHC)–MerCreMer transgenic (MCM-Cre) mice (n=4), and RGS6 CKO mice (n=4) and subjected to Western blotting analysis. Representative Western blots showing RGS6 expression levels in the 3 strains. C through E, Pressure overload–induced cardiac hypertrophy was alleviated in RGS6 CKO mice. The heart weight/body weight (HW/BW), lung weight/body weight (LW/BW), and heart weight/tibia length (HW/TL) ratios were calculated in RGS6-floxed mice, MCM-Cre mice, and RGS6 CKO mice 4 weeks after the AB procedure (n=12 mice per group). F, Representative images of hematoxylin and eosin (H&E)–stained heart sections from RGS6-floxed mice, MCM-Cre mice, and RGS6 CKO mice are shown (n=12 mice per group). The cross-sectional areas of the indicated groups were quantified using planimetry. G through I, left ventricular (LV) end-diastolic dimension (LVEDd), LV end-systolic dimension (LVESd), and fractional shortening (FS) percentage of the indicated groups were measured using echocardiography 4 weeks after AB (n=4 mice per group). J, Representative images of Picrosirius red–stained sections of perivascular and interstitial areas in heart tissues from RGS6-floxed mice, MCM-Cre mice, and RGS6 CKO mice. The fibrotic areas of the indicated groups were quantified by planimetry (n=5 mice per group). K and L, Levels of the atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (MYH7), connective tissue growth factor (CTGF), and collagen I and collagen III mRNAs in the indicated groups were measured 4 weeks after AB using quantitative real-time polymerase chain reaction and were normalized to levels of the GAPDH transcript (n=4 mice per group). Data are presented as means±SDs. **P*<0.05; ***P*<0.01 compared with the RGS6-floxed groups that underwent the sham procedure; $^{\#}P$<0.05, $^{\#\#}P$<0.01 compared with the RGS6-floxed groups subjected to the AB procedure.

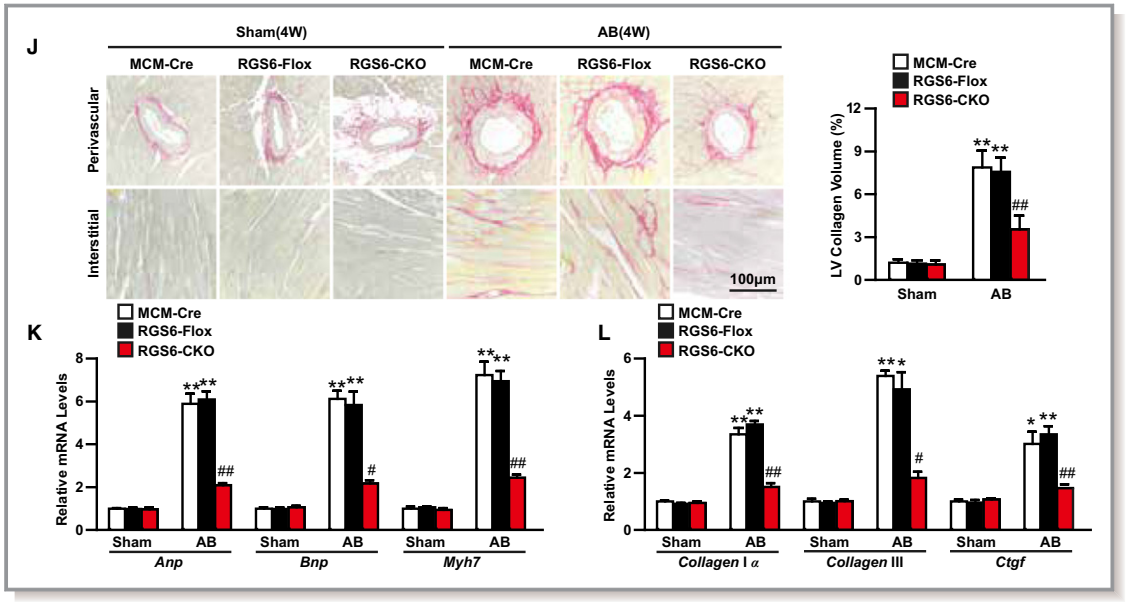

Figure 3. Continued.

through 3I). We next investigated whether RGS6 expression in cardiomyocytes contributed to AB-induced cardiac fibrosis. According to the Picrosirius red staining, the volume of deposited collagen increased in the perivascular and interstitial areas at 4 weeks. The increases in the volume of deposited collagen were attenuated in heart tissues from RGS6 CKO mice (Figure 3J). Based on the qRT-PCR data, levels of the connective tissue growth factor and collagen I and III mRNAs, which are required for cardiac fibrosis, were increased at 4 weeks after AB, whereas the increases in levels of the connective tissue growth factor and collagen I and III mRNAs were suppressed in cardiac tissue from RGS6 CKO mice (Figure 3L). Thus, the deletion of RGS6 in cardiomyocytes mitigates cardiac hypertrophy and fibrosis and improves ejection function in vivo.

RGS6 Overexpression Exacerbates AB-Induced Cardiac Hypertrophy

We further explored whether RGS6 overexpression exacerbates AB-induced cardiac hypertrophy. We established cardiac-specific RGS6 transgenic mice for these experiments. The baseline cardiac phenotype was not altered in cardiac-specific RGS6 transgenic mice (Figure 4A and 4B). RGS6 transgenic mice exhibited a significant increase in the AB-induced increase in cardiac weight compared with the nontransgenic control (NTG) mice, as determined by the HW/BW, lung weight/BW, and HW/tibia length. RGS6 transgenic mice also displayed increased AB-induced cardiomegaly, cardiomyocyte hypertrophy (Figure 4C through 4E), and levels of mRNAs encoding hypertrophy markers (such as brain natriuretic peptide, ANP, and MYH7) in vivo (Figure 4K). Echocardiography revealed a significantly enlarged cardiac chamber and impaired ejection function in RGS6 transgenic mice after AB challenge compared with the NTG group (Figure 4G through 4I). We next investigated whether RGS6 overexpression in cardiomyocytes exacerbates AB-induced cardiac fibrosis. Results from Picrosirius red staining revealed a substantial increase in the volume of deposited collagen in the perivascular and interstitial areas of heart tissues from RGS6 transgenic mice compared with NTG mice (Figure 4J). According to the qRT-PCR data, AB-induced increases in levels of the connective tissue growth factor and collagen I and III mRNAs were further exacerbated by RGS6 overexpression (Figure 4L). Based on these data, RGS6 overexpression in cardiomyocytes exacerbates cardiac hypertrophy and fibrosis and further impairs ejection function in vivo.

RGS6 Promotes Cardiac Hypertrophy Via the ASK1-P38/JNK1/2 Signaling Pathway

Mitogen-activated protein (MAP) kinase signaling plays a crucial role in the pathophysiological mechanisms leading to cardiac hypertrophy.[6,8,14] The expression and activity of MAP kinase signaling molecules were detected in various mouse groups to determine whether RGS6 induced cardiac hypertrophy by activating MAP kinase signaling. As shown in Figure 5A, levels of phosphorylated MEK1/2, ERK1/2, JNK1/2, and P38 were significantly elevated 4 weeks after the AB procedure, whereas levels of the total proteins remained unchanged. Deletion of RGS6 in cardiomyocytes reduced the phosphorylation of JNK1/2 and P38 to basal

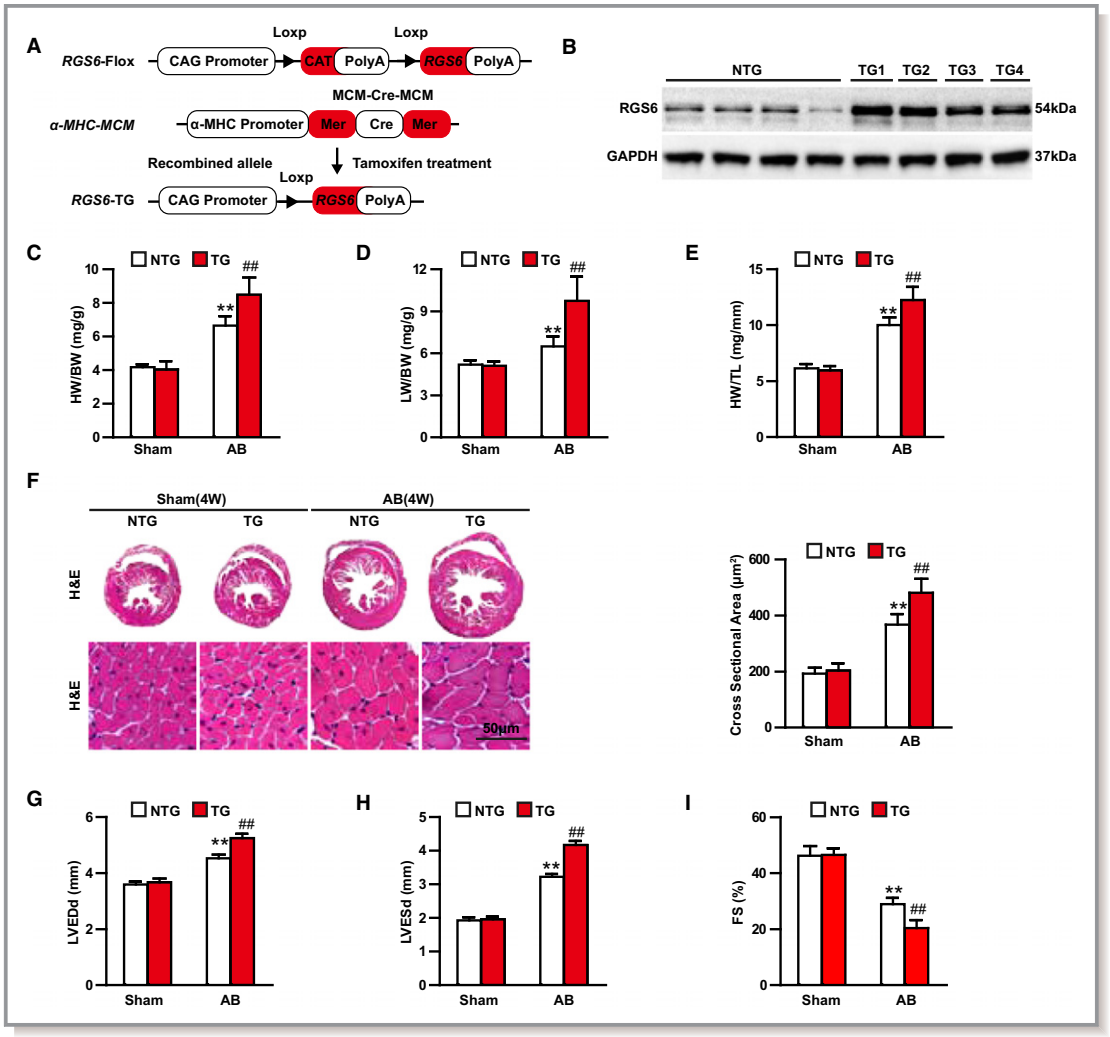

Figure 4. Cardiac-specific regulator of G protein signaling 6 (RGS6) overexpression exacerbates aortic banding (AB)–induced cardiac hypertrophy. A and B, We established RGS6 transgenic (TG) mice. Tissue lysates were prepared from the hearts of non-TG control (NTG) mice (CAG-CAT-RGS6/myosin heavy chain–Cre littermates without tamoxifen administration) and from the RGS6 TG mice 4 weeks after the AB procedure and were subjected to Western blotting analysis. Representative Western blots showing RGS6 expression levels in the 2 strains are depicted. C through E, Pressure overload–induced cardiac hypertrophy was exacerbated in RGS6 TG mice. The heart weight/body weight (HW/BW), lung weight/body weight (LW/BW), and heart weight/tibia length (HW/TL) ratios in NTG mice and RGS6 TG mice were calculated 4 weeks after the AB procedure (n=12 mice per group). F, Representative images of hematoxylin and eosin (H&E)–stained heart sections from NTG mice and RGS6 TG mice are shown. The cross-sectional areas of the indicated groups were quantified by planimetry (n=5 mice per group). G through I, left ventricular (LV) end-diastolic dimension (LVEDd), LV end-systolic dimension (LVESd), and fractional shortening (FS) percentage of the indicated groups were measured using echocardiography 4 weeks after AB (n=4 mice per group). J, Representative images of Picrosirius red–stained sections of perivascular and interstitial areas in heart tissues from NTG mice and RGS6 TG mice are shown. The fibrotic areas of the indicated groups were quantified by planimetry (n=5 mice per group). K and L, Levels of the atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (MYH7), connective tissue growth factor (CTGF), and collagen I and collagen III mRNAs in the indicated groups were measured by quantitative real-time polymerase chain reaction 4 weeks after AB and normalized to levels of the GAPDH transcript (n=4 mice per group). Data are presented as means±SDs. **P*<0.05; ***P*<0.01 compared with the NTG groups that underwent the sham procedure; #*P*<0.05; ##*P*<0.01 compared with the NTG groups that underwent the AB procedure.

levels (p-JNK1/2: 0.28±0.04 versus 1.00±0.43 [*P*<0.01]; p-P38: 0.15±0.03 versus 1.00±0.04 [*P*<0.01]) (Figure 5A), whereas RGS6 overexpression further increased the levels of phosphorylated JNK1/2 and P38 by 2.9- and 2.5-fold (p-JNK1/2: 2.93±0.13 versus 1.00±0.04 [*P*<0.01]; p-P38: 2.54±0.10 versus 1.00±0.09 [*P*<0.01]), respectively,

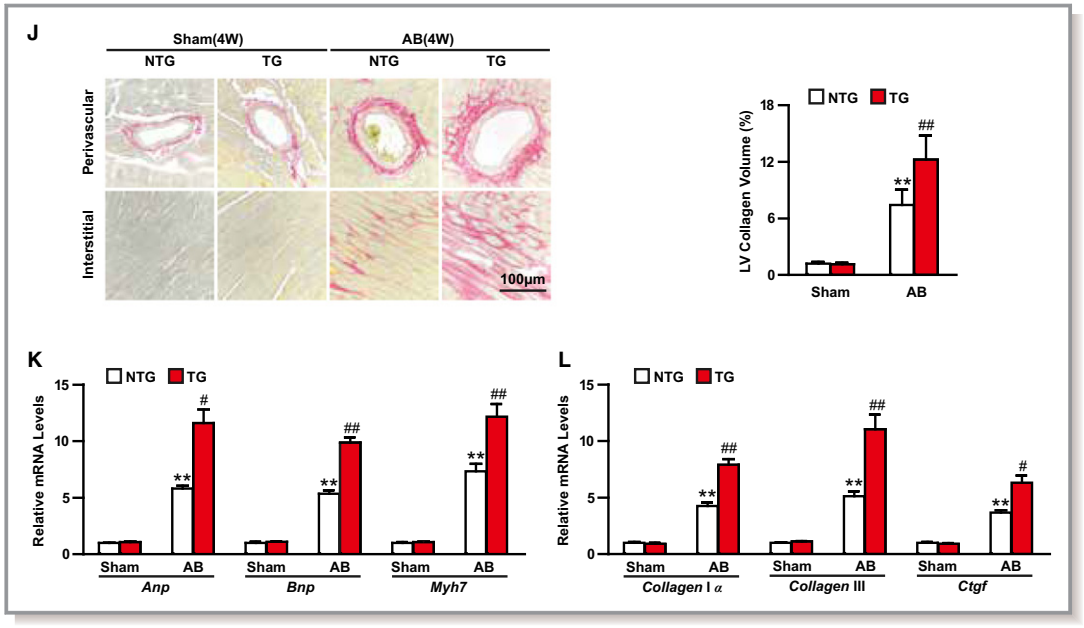

Figure 4. Continued.

compared with NTG mice subjected to AB (Figure 5B). The AB-induced increase in MEK1/2 and ERK1/2 phosphorylation was not altered in the hearts from RGS6 CKO or RGS6 transgenic mice.

NRCMs transfected with either AdshRGS6 or AdRGS6 were treated with Ang II for 48 hours to further confirm that RGS6 induces myocardial hypertrophy by inducing JNK1/2 and P38 phosphorylation in vitro. Ang II increased MEK1/2, ERK1/2, JNK1/2, and P38 phosphorylation. The increased levels of phosphorylated JNK1/2 and P38 were almost completely abolished by RGS6 deletion, whereas MEK1/2 and ERK1/2 activation did not depend on RGS6 (Figure 5C). Meanwhile, RGS6 overexpression further facilitated Ang II–induced JNK1/2 and P38 phosphorylation without interfering with MEK1/2 and ERK1/2 activation (Figure 5D).

ASK1, a member of the MAP kinase kinase kinase family, activates JNK1/2 and P38 in a Raf-independent manner in response to stress.[15] We next determined whether RGS6 participates in the activation of ASK1 to exacerbate cardiac hypertrophy. As shown in Figure 5E, levels of phosphorylated ASK1 were significantly elevated after the AB procedure, whereas total ASK1 expression remained unchanged. Deletion of RGS6 in cardiomyocytes restored ASK1 phosphorylation to the basal level. However, RGS6 overexpression further increased the level of phosphorylated ASK1 by 2.9-fold compared with the NTG mice subjected to AB (2.94±0.12 versus 1.00±0.14, $P<0.01$) (Figure 5F). NRCMs transfected with either AdshRGS6 or AdRGS6 were treated with Ang II for 48 hours in vitro. The Ang II treatment increased ASK1 phosphorylation. The increase in levels of phosphorylated ASK1 was completely abolished by RGS6 deletion, and RGS6 overexpression further enhanced the Ang II–induced increase in ASK1 phosphorylation (Figure 5G and 5H). These data indicate that the recruitment of RGS6 is involved in AB and Ang II–induced ASK1 signaling activation.

RGS6 Mediates Ang II–Induced Cardiac Hypertrophy by Directly Binding to ASK1

Based on the results presented above, RGS6 coactivates ASK1 phosphorylation and the activation of its downstream kinases JNK1/2 and P38 during cardiac hypertrophy. We further investigated how RGS6 regulated ASK1 activation. Immunoprecipitation assays revealed the interaction of RGS6 with ASK1 in the human embryonic kidney 293T cell line (Figure 6A and 6B). We tested the effect of mutated Asn^{401}→Val in RGS6 protein ($RGS6^{N401V}$),[16] which is required for RGS protein interaction with and GTPase-activating protein activity toward Gα subunits, on the ASK1 interaction and binding. We found that $RGS6^{N401V}$ was as effective as RGS6 in binding with ASK1. These results demonstrate that the ability of RGS6 to induce ASK1 interaction does not require its GTPase-activating protein activity (Figure 6C). The RGS6 (1–120) fragment interacted with ASK1, whereas the ASK1 (1–678) fragment was responsible for the binding of ASK1 to RGS6 (Figure 6D and 6E). RGS6 overexpression increased the size of NRCMs by 46.6% after Ang II exposure for 48 hours (2513.44±280.88 versus 1714.36±206.27, $P<0.01$). Inactivation of ASK1 by the transfection of AddnASK1 ameliorated the RGS6-mediated increase in cardiomyocyte size

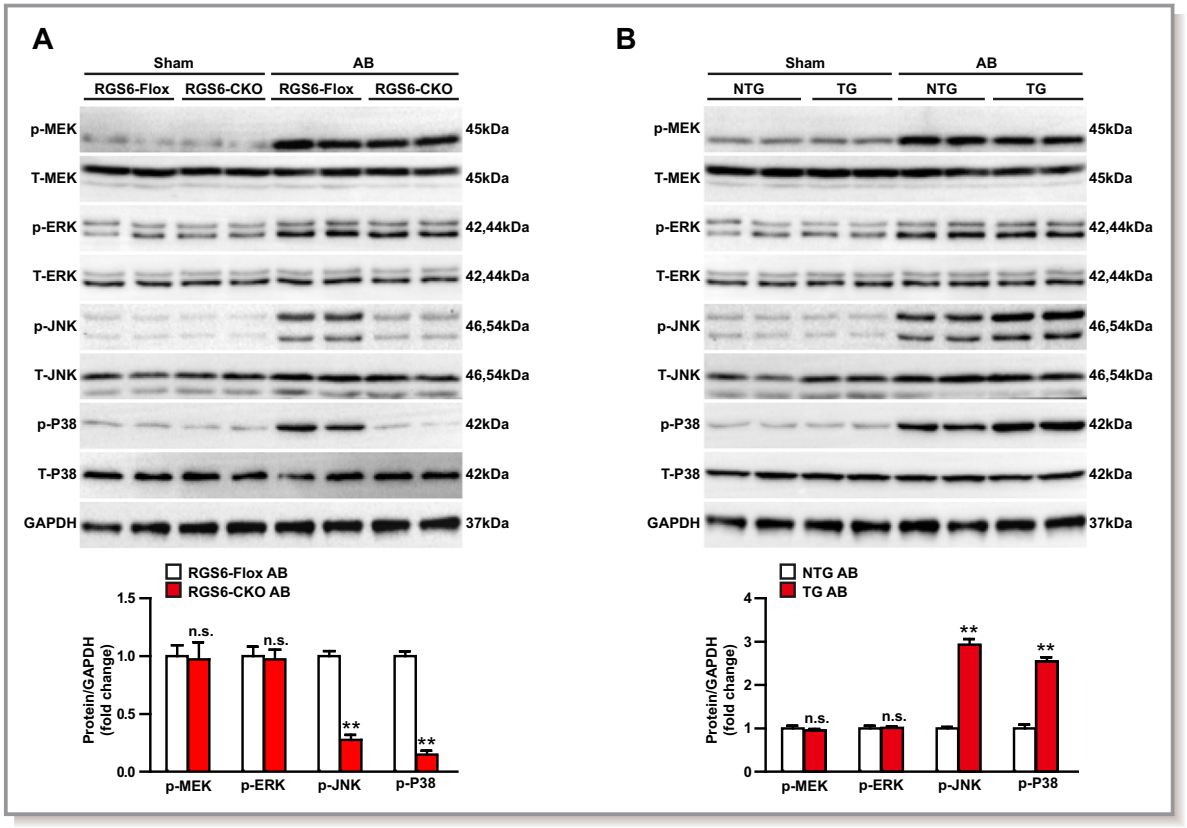

Figure 5. Regulator of G protein signaling 6 (RGS6) modulates apoptosis signal–regulating kinase 1 (ASK1)-P38-c-JUN N-terminal kinase 1/2 (JNK1/2) signaling in hypertrophic cardiomyocytes and hearts. A and B, Tissue lysates were prepared from the hearts of RGS6-floxed mice (n=4), cardiac-specific RGS6 knockout (RGS6 CKO) mice (n=4), nontransgenic control (NTG) mice (CAG-CAT-RGS6/myosin heavy chain–Cre littermates without tamoxifen administration) (n=4), and RGS6 transgenic (TG) mice (n=4) 4 weeks after the aortic banding (AB) procedure and subjected to Western blotting analysis. Representative Western blots showing the levels of the phosphorylated and total mitogen-activated protein kinase kinase 1/2 (MEK1/2), extracellular signal-regulated kinase 1/2 (ERK1/2), JNK1/2, and P38 proteins in the indicated strains are depicted. Bar graphs present the quantitative data for the levels of the phosphorylated and total JNK1/2 and P38 proteins in the indicated strains. $^{**}P<0.01$ compared with RGS6-floxed mice subjected to the AB procedure or NTG mice subjected to the AB procedure. C and D, Neonatal rat cardiomyocyte (NRCM) lysates were prepared from cells infected with adenoviruses expressing short hairpin RNA (AdshRNA), a short hairpin RNA targeting RGS6 (AdshRGS6), green fluorescent protein (AdGFP), or RGS6 (AdRGS6) and treated with angiotensin II (Ang II) for 48 hours, which were then subjected to Western blot analysis. Representative Western blots showing the levels of the phosphorylated and total MEK1/2, ERK1/2, JNK1/2, and P38 proteins in the indicated groups are presented. Bar graphs present the quantitative results for the levels of the phosphorylated and total JNK1/2 and P38 proteins in the indicated groups. $^{**}P<0.01$ compared AdshRNA-infected cells treated with Ang II or AdGFP-infected cells treated with Ang II. E and F, Representative Western blots and qualitative results for levels of the phosphorylated and total ASK1 proteins in hearts from RGS6-floxed mice (n=4), RGS6 CKO mice (n=4), NTG mice (n=4), and RGS6 TG mice (n=4) 4 weeks after the AB procedure are shown. $^{**}P<0.01$ compared with RGS6-floxed or NTG mice subjected to the AB procedure. G and H, Representative Western blots and qualitative results for levels of the phosphorylated and total ASK1 proteins in NRCMs infected with AdshRNA, AdshRGS6, AdGFP, or AdRGS6 and subsequently treated with Ang II treatment for 48 hours are shown. Data are presented as means±SDs. $^{**}P<0.01$ vs AdshRNA or AdGFP groups with Ang II treatment. Protein expression was quantified and normalized to GAPDH. Graphic data show quantification of 4 to 5 independent experiments. n.s. indicates not significant.

(Figure 6F) and the increase in levels of mRNAs encoding hypertrophy markers (such as ANP and MYH7) in vitro (Figure 6G). Based on these data, RGS6 binds to ASK1 and exacerbates cardiomyocyte hypertrophy in response to Ang II.

Discussion

In the present study, we identified RGS6 as a critical regulator of cardiac remodeling. Levels of the RGS6 protein were significantly increased in hypertrophic hearts.

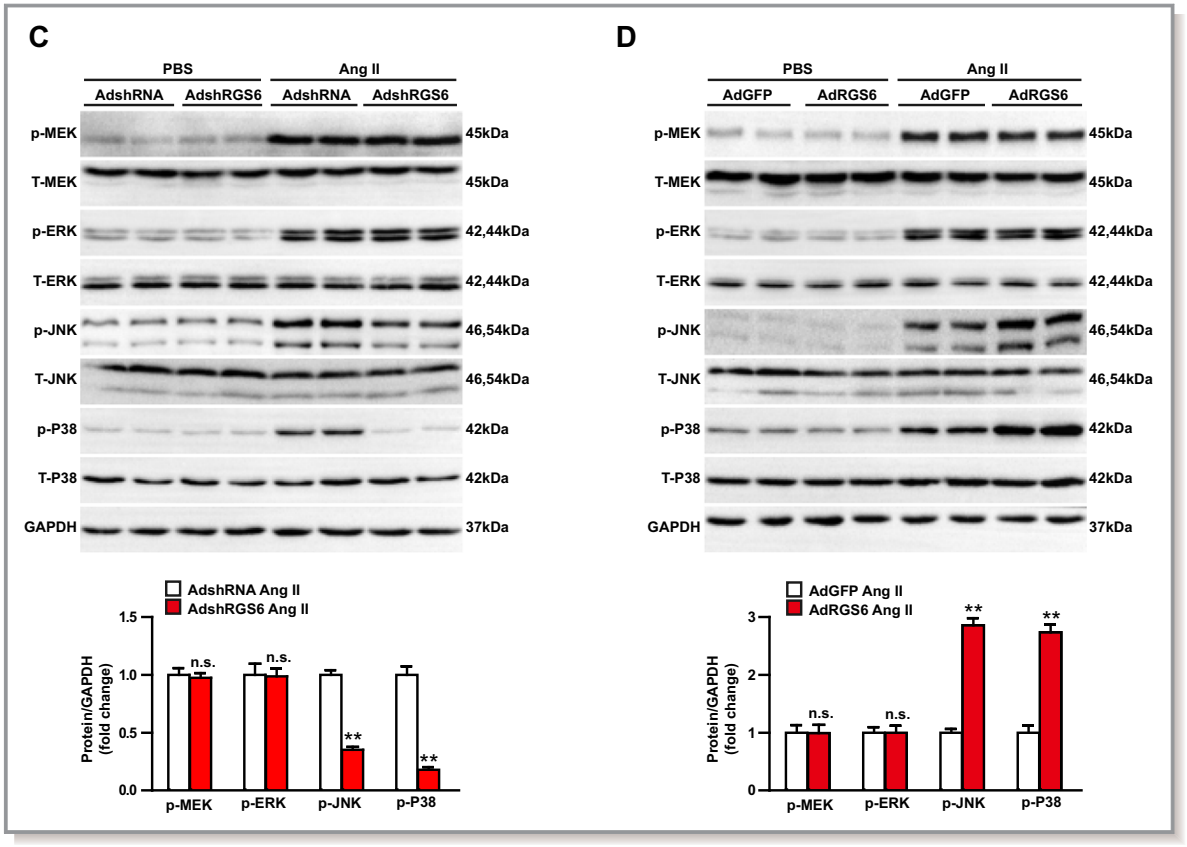

Figure 5. Continued.

Furthermore, RGS6 deletion in cardiomyocytes attenuated the hypertrophic response to AB in mouse hearts, whereas the overexpression of RGS6 in cardiomyocytes exacerbated AB-induced cardiac hypertrophy. Consistent with these findings, in vitro experiments indicated a role for RGS6 in inhibiting NRCM hypertrophy. Mechanistic studies delineated that RGS6 act as a coactivator for ASK1 phosphorylation and activation of the P38-JNK signaling axis in response to pressure overload. Thus, this study provides evidence of an essential function for RGS6 in cardiac remodeling induced by Ang II or pressure overload (Figure 6H).

RGS proteins were discovered as a set of proteins that lead to the pronounced acceleration of Gα GTPase activity, which attenuates the amplitude and duration of both Gα- and Gβγ-mediated signaling. RGS proteins have been categorized into 4 subfamilies: RZ/A, R4B, R7/C, and R12/D. In mammals, the R7/C subfamily consists of 4 highly homologous proteins: RGS6, RGS7, RGS9, and RGS11.[17,18] Among these proteins, RGS6 was first discovered in the nervous system and is best known for its role in regulating neuronal processes.[19–21] Recently, RGS6 was discovered to function in the heart as well. RGS6 has been reported as a key regulator of parasympathetic signaling and bradycardia in the heart through its interactions with components of GPCR complexes.[11,22] RGS6 exhibits a unique robust expression pattern in the heart, particularly in the sinoatrial and atrioventricular nodal regions.[23] Deletion of RGS6 exacerbates bradycardia and inhibits sinoatrial and atrioventricular nodal action potential firing and G protein–coupled inwardly rectifying K(+) current in response to acetylcholine.[23] Additionally, RGS6 also exerts a cardioprotective effect on ischemic heart disease by suppressing β2-AR–induced $G\alpha_{i3}$ signaling.[10] However, in the current study, specifically deleted RGS6 from cardiomyocytes do not interfere with the heart rhythm (Figure S2). The specific cell type involved in the RGS6-mediated parasympathetic activity requires further investigation.

RGS6 also interacts with signaling proteins that are not involved in classical GPCR pathways. As shown in the present study, RGS6 mediates cardiac hypertrophy by directly interacting with ASK1 and participates in activation of the ASK1-P38-JNK signaling axis. RGS6 is also a critical mediator of chronic alcohol-induced cardiac hypertrophy. RGS6 deficiency protects against alcohol-induced cardiac hypertrophy and fibrosis. Alcohol-mediated toxicity in the heart partially mediates the canonical function of RGS6 as a G protein regulator but also involves the ability of RGS6 to promote reactive oxygen species–dependent apoptosis, an

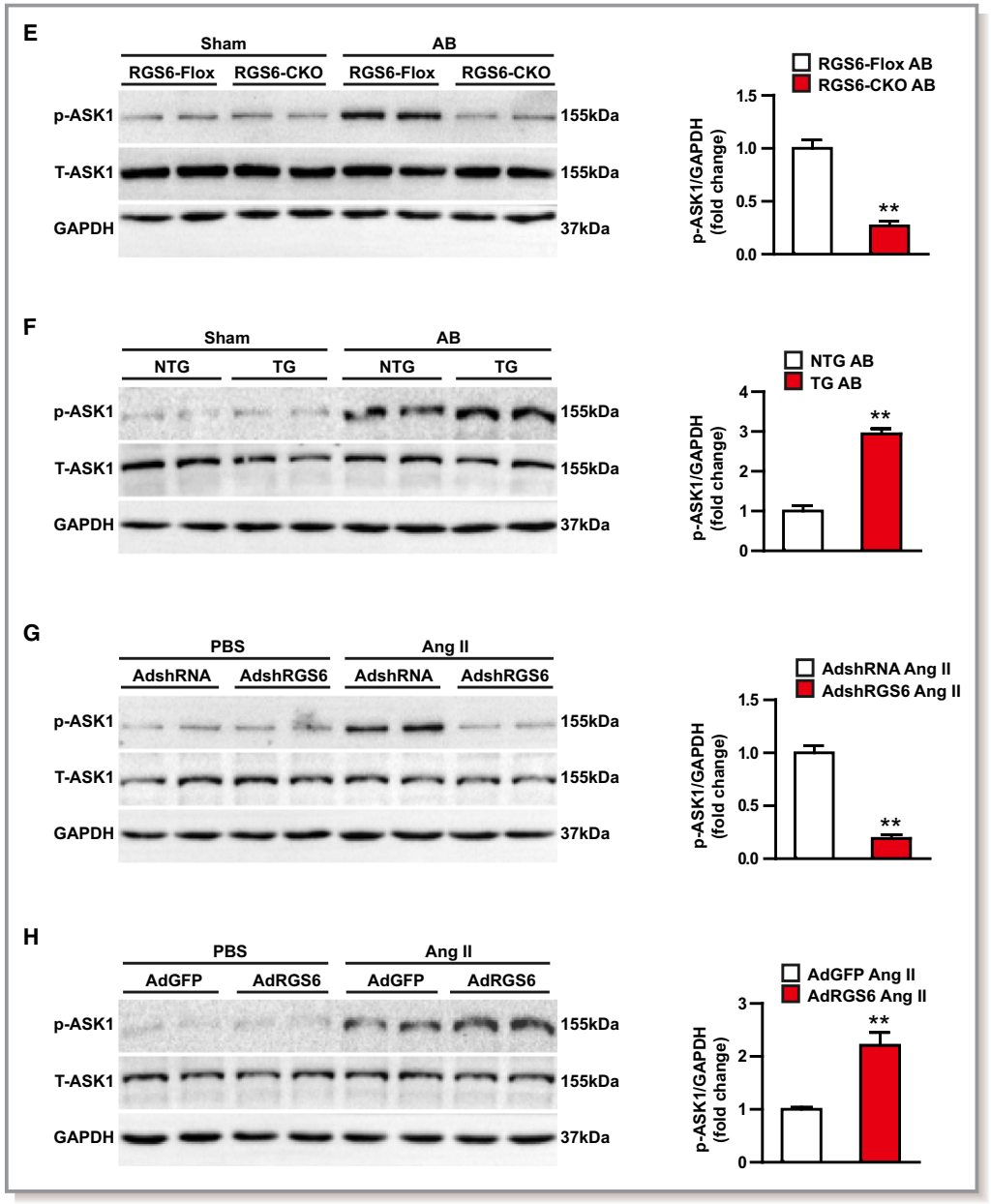

Figure 5. Continued.

action independent of its G protein regulatory capacity.[13] According to Huang et al,[24] RGS6 promotes doxorubicin-induced p53 and ataxia telangiectasia mutated activation in chemotherapy-induced cardiomyopathy through a mechanism independent of its canonical interactions with G proteins. Thus, RGS6 participates in a broad range of pathways in the pathogenesis of various cardiomyopathies, which depend on the cell types, stimulus, and stresses.

ASK1 is a member of the MAP kinase kinase kinase family that has been shown to be involved in infectious diseases, cancer, and metabolic, cardiovascular, and neurodegenerative diseases. ASK1 mediates cardiac remodeling and hypertrophy derived from a variety of etiologies, such as myocardial infarction, pressure overload, and endocrine disorders. The ASK1-mediated JNK signaling pathway and cardiac apoptosis seem to be mainly involved in post–myocardial infarction, AB-, and Ang II infusion–related cardiac hypertrophy.[25–28] Cardiac hypertrophy induced by certain GPCR agonists (eg, Ang II, endothelin-1, and phenylephrine) is mediated by ASK1-NF-κB activation and reactive oxygen species production.[26] ASK1 enhances cardiac nicotinamide adenine dinucleotide phosphate oxidase–mediated oxidative stress and the activation of the cardiac renin-angiotensin system in aldosterone-induced

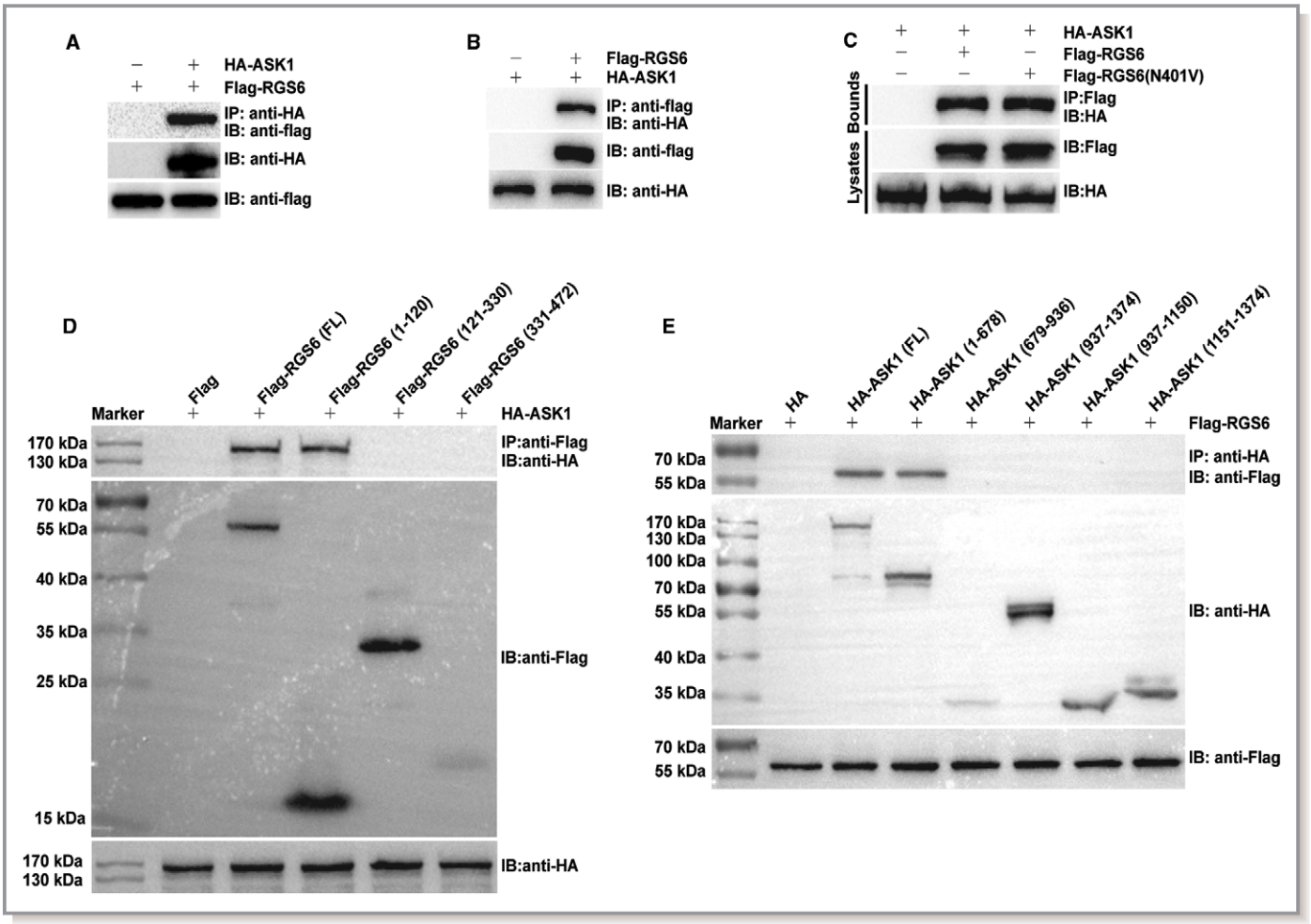

Figure 6. RGS6 mediates Ang II-induced cardiac hypertrophy by directly binding to ASK1. A-B. The interaction between RGS6 and ASK1 in HEK293T cells transfected with FLAG-RGS6 and HA-ASK1 (or FLAG-ASK1 and HA-RGS6) was examined by co-IP and Western blotting analysis. C. The interaction between mutant RGS6N401V and ASK1 in HEK293T cells transfected with FLAG- RGS6N401V (or FLAG- RGS6) and HA-ASK1 was examined by co-IP and Western blotting analysis. D-E. Representative Western blots of the mapping analyses showing the binding domains responsible for the RGS6-ASK1 interaction. F. Representative fluorescent images of NRCMs infected with the indicated adenoviruses and treated with 1 μg/mL Ang II for 48 h are shown (n=50 cells per group). NRCMs were stained with an α-actinin antibody (green). Nuclei were stained with DAPI (blue). Cell surface areas were quantified by planimetry after treatments. G. Levels of the ANP and MYH7 mRNAs in NRCMs treated with Ang II for 48 h were measured by qRT-PCR and normalized to levels of the GAPDH transcript. The graph shows quantitative data from four-five independent experiments. H. An illustration of potential mechanisms in cardiac hypertrophy is depicted. The data are presented as the means ± SD. $^{**}P<0.01$ compared with AdGFP-infected cells treated with Ang II. $^{\#\#}P<0.01$ compared with AdRGS6-infected cells treated with Ang II. n.s. compared with AddnASK1-infected cells treated with Ang II.

cardiac remodeling and fibrosis.[29] Moreover, ASK1/P38 signaling pathway, activated by pathological stimuli, leads to maladaptive cardiac remodeling and at the same time attenuates the physiological adaptive pathway via crosstalk with insulin-like growth factor 1/Akt signaling cascade.[30] These results indicate that ASK1 has a dual adaptive function: (1) to suppress physiological hypertrophy, and (2) to promote pathological hypertrophy. Based on studies from our laboratory and other groups, ASK1, a key intermediate, lies at the intersection of proinflammatory, profibrotic, and proapoptotic pathways during the progression of cardiac hypertrophy and heart failure.[29,31–34] Thus, ASK1 might be a potential target for ameliorating cardiac remodeling.

The most common mechanisms regulating ASK1 are mediated by protein-protein interactions due to the ability of ASK1 to form a scaffold structure and recruit interacting proteins. Numerous proteins, not only kinases, have been shown to bind ASK1 to facilitate its oligomerization, phosphorylation, nitrosylation, methylation, or ubiquitination.[35–37] The present study is the first to show that RGS6 is a crucial coregulator for ASK1 phosphorylation during pressure overload– and Ang II–induced cardiac remodeling. Blockade of basal RGS6 expression did not further diminish this phosphorylation. Thus, the strategy of preventing ASK1 phosphorylation by inhibiting RGS6 expression or recruitment may represent a better approach than complete inhibition of ASK1 activation.

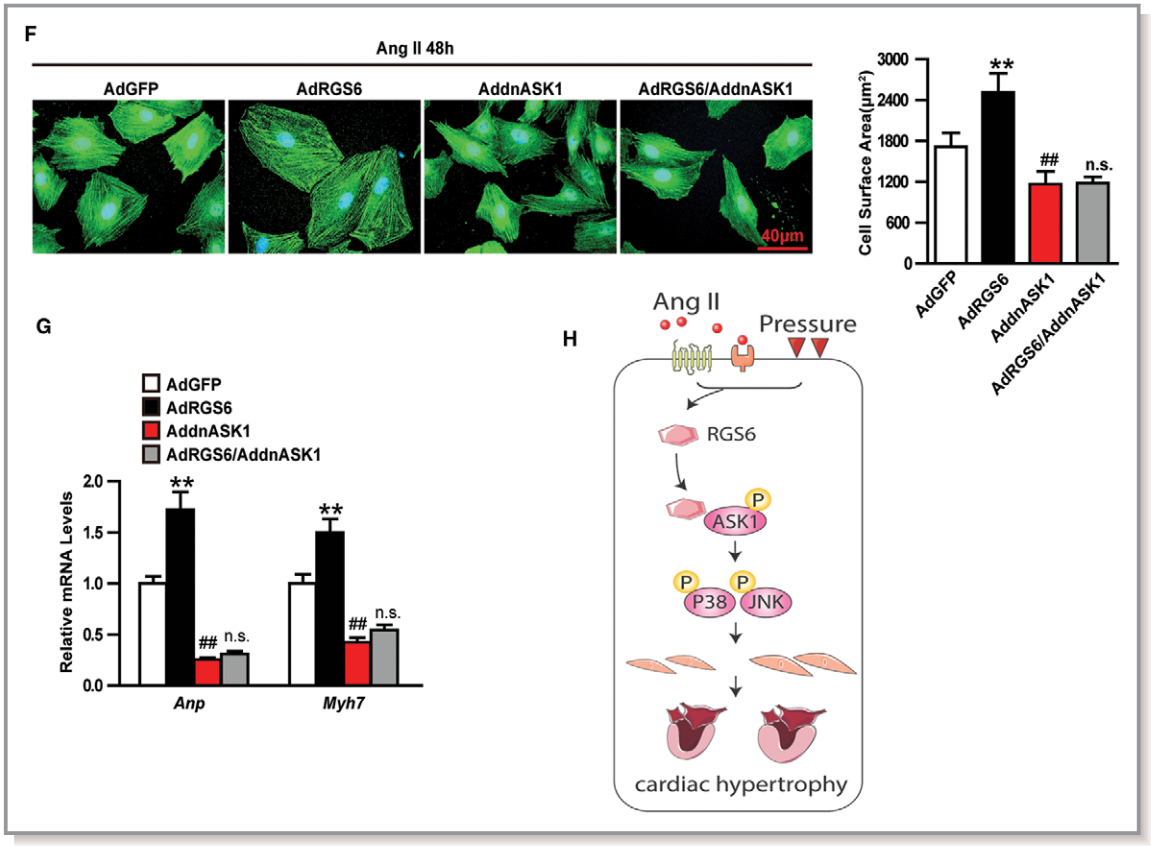

Figure 6. Continued.

Conclusions

Our present study provides in vitro and in vivo evidence supporting the hypothesis that RGS6 is a critical mediator of cardiac hypertrophy and contributes to the pathogenesis of cardiac remodeling by facilitating ASK1 phosphorylation–mediated P38-JNK1/2 signaling. These observations implicate the RGS6-ASK1 pathway as a novel therapeutic target for the treatment of pathological cardiac hypertrophy.

Perspectives

Most RGS proteins were reported as a negative regulator of GPCR-mediated signaling activation in response to cardiac hypertrophy stimulus. Unique among the members of the RGS family, this work identifies RGS6 in cardiomyocytes as a positive modulator of pressure overload– and Ang II–induced cardiac hypertrophy via regulation of G protein–independent signaling cascades. These findings extend our understanding of pleiotropic effects mediated by RGS proteins underlying cardiac hypertrophy, establishing RGS6 as a potential therapeutic target for such conditions.

Sources of Funding

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Reagents

The following antibodies from the respective commercial sources were used in the present study: Cell Signaling Technology: anti-phospho-MEK1/2 (#9154), anti-MEK1/2 (#9122), anti-phospho-ERK1/2 (#4370), anti-ERK1/2 (#4695), anti-phospho-JNK1/2 (#4668), anti-JNK1/2 (#9258), anti-phospho-P38 (#4511), anti-P38 (#9212), and anti-phospho-ASK1 (#3765); Santa Cruz Biotechnology: anti-ANP (#sc20158) and anti-MYH7 (#sc53090); **GeneTex, Inc:** ASK1 (#GTX107921); Bioworld Technology: anti-GAPDH (#MB001); Abcam: anti-GATA4 (ab84593 and ab5245) and anti-RGS6 (ab128943). The bicinchoninic acid (BCA) Protein Assay Kit was obtained from Pierce (Rockford, IL, USA). Fetal calf serum (FCS) was obtained from HyClone. All other reagents, including cell culture reagents, were purchased from Sigma.

Animal surgery

Mice were anesthetized via an intraperitoneal injection of sodium pentobarbital (50 mg/kg, Sigma). After blunting dissection at the second intercostal space, the left chest was opened, and the thoracic aorta was identified. We tied the thoracic aorta to a 27-G (for animals with body weights of 24-25 g) or 26-G (for animals with body weights of 26-27 g) needle with a 7-0 silk suture. Immediately after ligation, the needle was removed quickly, and the thoracic cavity was closed. Finally, the adequacy of aortic constriction was determined using a Doppler analysis. Sham-treated groups received an untied suture in the same aortic banding (AB) location.

Echocardiography evaluation

A Mylab30CV (ESAOTE) ultrasound system switched to M-mode with a 15-MHz probe was used to determine echocardiographic parameters. Left ventricles (LV) end-diastolic

dimension (LVEDd), LV end-systolic dimension (LVESd) and LV fractional shortening (FS, FS(%)=(LVEDd-LVESd)/LVEDd×100%) were measured from the short axis of the LV at the level of the papillary muscles. Measurements were obtained from three beats and averaged.

Histological analysis and immunofluorescence staining

Hearts were harvested, arrested in diastole with 10% potassium chloride solution, fixed with 10% formalin, dehydrated and embedded in paraffin. Next, the hearts were transversely sectioned at 5-μm intervals. Sections at the mid-papillary muscle level were stained with hematoxylin and eosin (H&E) and picrosirius red (PSR) to calculate the cardiomyocyte cross-sectional area (CSA) and collagen deposition volume, respectively. Fluorescein isothiocyanate-conjugated wheat germ agglutinin (WGA, Invitrogen) was also used to visualize the size of the cardiomyocytes. The immunofluorescence analysis was performed using the standard immunocytochemical techniques. Cardiomyocyte CSA, interstitial collagen deposition and perivascular collagen deposition were measured using the Image-Pro Plus 6.0 software.

Quantitative real-time PCR and Western blotting

Total mRNA was extracted from primary cardiac cells and ventricles with TRIzol reagent (Invitrogen). The cDNA templates were synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative real-time PCR was performed using SYBR Green (Roche) to determine the expression levels of the genes of interest, and the results were normalized to GAPDH expression. Cardiac ventricles and cultured cardiac myocytes were lysed in RIPA lysis buffer, and the protein concentration was determined with the BCA Protein Assay Kit. 50 μg of protein were loaded and separated on SDS-PAGE gels (Invitrogen); gels were then transferred to polyvinylidene fluoride membranes (Millipore) that were subsequently blocked and incubated with primary antibodies. Membranes were washed twice or three times and then incubated with a secondary antibody for one hour at room temperature. Finally, enhanced chemiluminescence-treated membranes were visualized

using a Bio-Rad ChemiDoc^{TM} XRS^{+}(Bio-Rad). The results were normalized to the GAPDH signal.

Cardiomyocyte culture and infection with recombinant adenoviral vectors

Primary neonatal rat cardiomyocytes (NRCMs) were prepared from the ventricles of one- to two-day-old Sprague-Dawley rats. First, neonatal hearts were excised from the thoracic cavities, and the ventricles tissue was finely minced and digested in a phosphate-buffered saline (PBS) solution containing 0.03% trypsin and 0.04% collagenase type II. After removing fibroblasts using a differential attachment technique, NRCMs were planted in six-well plates and cultivated in DMEM/F12 containing 20% FCS, bromodeoxyuridine (0.1 mM, to inhibit fibroblast proliferation), and penicillin/streptomycin. Neonatal rat hearts were digested as described above for cardiomyocytes. The adherent fibroblast fractions obtained during pre-plating were grown in DMEM containing 10% FCS until they reached confluence and were then passaged. Cells in the first three passages were used in subsequent experiments. The prepared cells were incubated with serum-free medium overnight, followed by medium containing 1 μM Ang II for 24 or 48 h.

Table S1. The primers used to genotype the Cardiac-specific *RGS6* knockout mice.

Primer name	Sequence 5'---3'
RGS6-P1	CCATGAGCAGAGGGAGTCAT
RGS6-P2	GTGTGAAGAGGGTCCCAGAG
RGS6-P3	CAGGGTCACCTAAGACCATCA
RGS6-P4	TAGCGTTTGGATGACCAACA
RGS6-P5	AGCTTGGCGTAATCATGGTC
RGS6-P6	CAATACGCAAACCGCCTCTC

RGS6-P1+ *RGS6*-P2 to detect the deletion product

RGS6-P3+ *RGS6*-P4 to detect the circle product

RGS6-P5+ *RGS6*-P6 to avoid random integration

RGS6-P1+ *RGS6*-P3 to detect the existence of left Loxp site：

The wild-type (WT) allele yields an amplicon of 222 bp, whereas the floxed allele yields an amplicon of 274 bp.

Table S2. PCR primers.

Gene	Forward Primer	Reverse Primer
ANP Rat	AAAGCAAACTGAGGGCTCTGCTCG	TTCGGTACCGGAAGCTGTTGCA
Myh7 Rat	TCTGGACAGCTCCCCATTCT	CAAGGCTAACCTGGAGAAGATG
GAPDH Rat	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT
ANP Mouse	ACCACCTGGAGGAGAAGA	TTCAAGAGGGCAGATCTATC
BNP Mouse	GAGGTCACTCCTATCCTCTGG	GCCATTTCCTCCGACTTTTCTC
Myh7 Mouse	CCGAGTCCCAGGTCAACAA	CTTCACGGGCACCCTTGGA
Collagen I Mouse	TGGTACATCAGCCCGAAC	GTCAGCTGGATAGCGACA
Collagen III Mouse	CCCAACCCAGAGATCCCATT	GAAGCACAGGAGCAGGTGTAGA
Ctgf Mouse	TGACCCCTGCGACCCACA	TACACCGACCCACCGAAGACACAG
GAPDH Mouse	ACTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA

Table S3. The sequence of RGS6 shRNA.

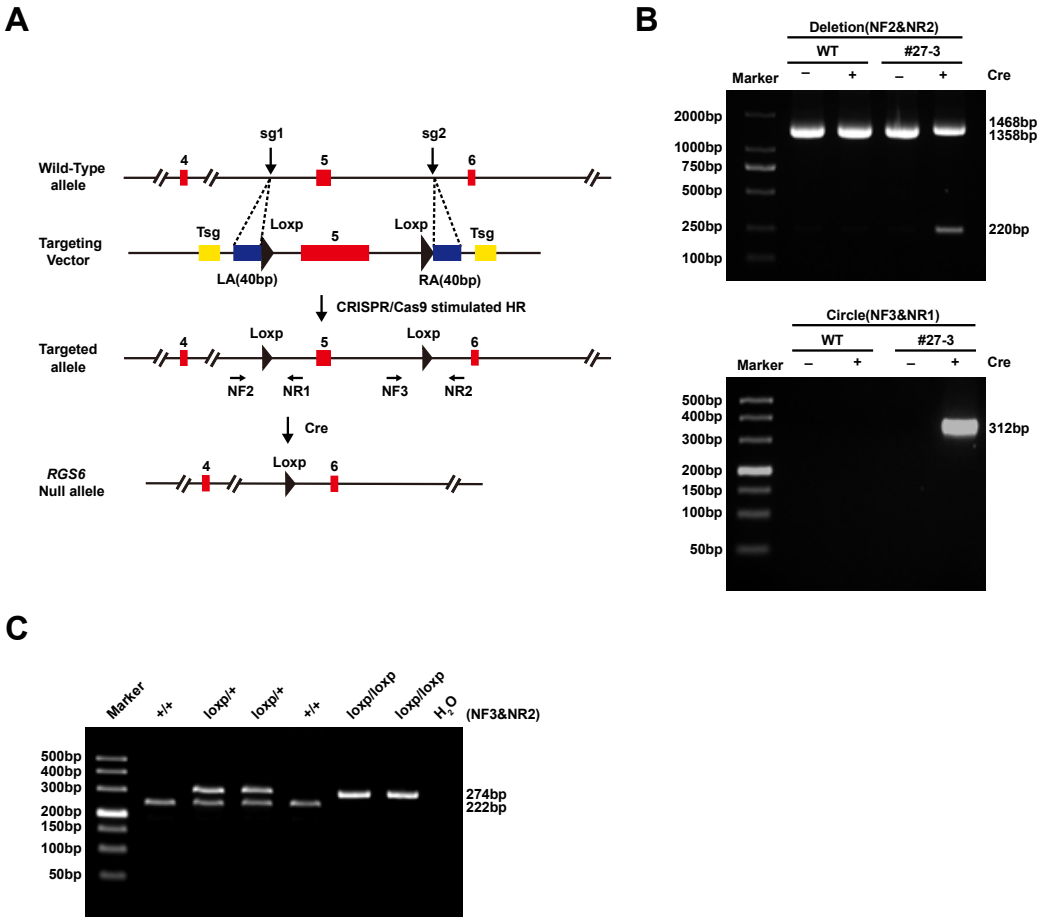
	Sequence
rat-RGS6-shRNA-F	CCGGCTTACACGGAGCAGTATGTTTCTCGAGAAACATACTGC TCCGTGTAAGTTTTTG
rat-RGS6-shRNA-R	AATTCAAAAACTTACACGGAGCAGTATGTTTCTCGAGAAAC ATACTGCTCCGTGTAAG

Table S4. PCR primers.

Plasmid		Primers
pcDNA5-Flag-RGS6 (FL)	pc5-flag-RGS6-S	TAAGAGCCCGGGCGGATCCATGGCTCAAGGATCCGGGGA
	pc5-flag-RGS6-AS	CGGGCCCTCTAGACTCGAGTCAGGAGGACTGCATCAGGC
pcDNA5-Flag-RGS6 (1-120)	pc5-flag-RGS6-S	TAAGAGCCCGGGCGGATCCATGGCTCAAGGATCCGGGGA
	pc5-flag-RGS6-AS120	CGGGCCCTCTAGACTCGAGTCAAGGCCAGAAGTACGGAGCCT
pcDNA5-Flag-RGS6 (121-330)	pc5-flag-RGS6-S121	TAAGAGCCCGGGCGGATCCTCGAACTGCTGGGAACCTGA
	pc5-flag-RGS6-AS330	CGGGCCCTCTAGACTCGAGTCATACTCGCTGTTGGCTGGGCT
pcDNA5-Flag-RGS6 (331-472)	pc5-flag-RGS6-S331	TAAGAGCCCGGGCGGATCCAAAAGATGGGGCTTCTCTTTCG
	pc5-flag-RGS6-AS	CGGGCCCTCTAGACTCGAGTCAGGAGGACTGCATCAGGC
pcDNA5-HA-ASK1 (FL)	pc5-HA-h ASK1-S	CCAGATTACGCTGGATCCATGAGCACGGAGGCGGACGA
	pc5-HA-h ASK1-AS	TGCGGCCGCATTCTCGAGTCAAGTCTGTTTGTTTCGAAAG
pcDNA5-HA-ASK1 (1-678)	pc5-HA-h ASK1-S	CCAGATTACGCTGGATCCATGAGCACGGAGGCGGACGA
	pc5-HA-h ASK1-AS678	TGCGGCCGCATTCTCGAGTCAATCATATTCATAGTCATACTCCAG
pcDNA5-HA-ASK1 (679-936)	pc5-HA-h ASK1-S679	CCAGATTACGCTGGATCCATGGAAAATGGTGACAGAGTCGT
	pc5-HA-h ASK1-AS936	TGCGGCCGCATTCTCGAGTCACTCATCAACAAGCAAGTCGT
	pc5-HA-h ASK1-S937	CCAGATTACGCTGGATCCATGTTTTTAAAAGTTTCAAGCAA

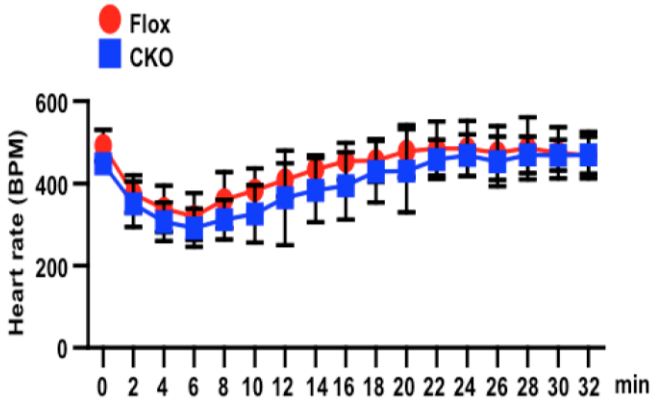
pcDNA5-HA-ASK1 (937-1374)	pc5-HA-h ASK1-AS	TGCGGCCGCATTCTCGAGTCAAGTCTGTTTGTTTCGAAAG
pcDNA5-HA-ASK1 (937-1150)	pc5-HA-h ASK1-S937	CCAGATTACGCTGGATCCATGTTTTTAAAAGTTTCAAGCAA
	pc5-HA-h ASK1-AS1150	TGCGGCCGCATTCTCGAGTCAGTGCGGCTTGATGTTATGAT
pcDNA5-HA-ASK1 (1151-1374)	pc5-HA-h ASK1-S1151	CCAGATTACGCTGGATCCATGTGGATGTTTGCCTTAGACAG
	pc5-HA-h ASK1-AS	TGCGGCCGCATTCTCGAGTCAAGTCTGTTTGTTTCGAAAG

Figure S1. Construction and phenotypes of Cardiac-specific RGS6 knockout (RGS6-CKO) mouse strain.



A. Schematic workflow showing the establishment of RGS6 CKO mouse strain. Two single guide RNAs, sgRNA1 and sgRNA2, targeting RGS6 introns 4 and 5 were designed to obtain mice carrying a conditional knockout RGS6 allele. Targeting constructs were generated by introducing the loxP-flanked RGS6 exon 5 into the left and right short homology arms (40 bp) of microhomology-mediated end-joining (MMEJ), which were flanked by the two gRNA target sequences (Tsg). B. Mouse genotyping was confirmed by PCR. *RGS6*-P1+ *RGS6*-P2 to detect the deletion product, *RGS6*-P3+ *RGS6*-P4 to detect the circle product. C. Representative genotyping PCR-based amplification of wildtype (RGS6+/+), RGS6loxP/loxP, and RGS6loxP/+ alleles. PCR primers RGS6-P1 and RGS6-P3 were used. Allele containing loxP sites: 274 bp, wild-type allele: 222 bp

Figure S2. Effects of loss of RGS6 on CCh-induced bradycardia.



Heart rates were recorded continuously by cardiac echo in RGS6-floxed (n=4) and RGS6-CKO mice (n=5) at rest and following CCh (0.1 mg/kg i.p.). p>0.05 vs RGS6-CKO mice.