Cardiac Hypertrophy

Cardiac-Specific EPI64C Blunts Pressure Overload–Induced Cardiac Hypertrophy

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Abstract—The calcium-responsive molecule, calcineurin, has been well characterized to play a causal role in pathological cardiac hypertrophy over the past decade. However, the intrinsic negative regulation of calcineurin signaling during the progression of cardiomyocyte hypertrophy remains enigmatic. Herein, we explored the role of EPI64C, a dual inhibitor of both Ras and calcineurin signaling during T-cell activation, in pressure overload–induced cardiac hypertrophy. We generated a cardiac-specific Epil64c conditional knockout mouse strain and showed that loss of Epi64c remarkably exacerbates pressure overload–induced cardiac hypertrophy. In contrast, EPI64C gain-of-function in cardiomyocyte-specific Epil64c transgenic mice exerts potent protective effects against cardiac hypertrophy. Mechanistically, the cardioprotective effects of EPI64C are largely attributed to the disrupted calcineurin signaling but are independent of its Ras suppressive capability. Molecular studies have indicated that the 406 to 446 C-terminal amino acids in EPI64C directly bind to the 287 to 337 amino acids in the catalytic domain of calcineurin, which is responsible for the EPI64C-mediated suppressive effects. We further extrapolated our studies to cynomolgus monkeys and showed that gene therapy based on lentivirus-mediated EPI64C overexpression in the monkey hearts blunted pressure overload–induced cardiac hypertrophy. Our study thus identified EPI64C as a novel negative regulator in cardiac hypertrophy by targeting calcineurin signaling and demonstrated the potential of gene therapy and drug development for treating cardiac hypertrophy.

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As the heart undergoes unremitting overload stress (eg, pressure or volume overload), it initially exhibits adaptive hypertrophic growth to augment contractile performance.1,2 However, emerging evidence suggests that ventricular hypertrophy is unnecessary for maintaining circulatory sufficiency under stress but renders the heart vulnerable to morbidity and mortality.3,5 At the molecular level, increased cardiac contractility is a result of incremental degrees of binding between myosin and actin filaments, which is secondary to elevated intracellular calcium concentration during contraction.6 Apart from regulating excitation-contraction coupling, calcium also acts as an important second messenger in cardiomyocytes and regulates calcium-dependent signaling cascades, known as, excitation-contraction coupling. Persistent pro-hypertrophic stimulation is associated with profound alterations in calcium handling and sustained rises in cytosolic calcium, which, in turn, promotes the nonmitotic growth of cardiomyocytes and enhances sarcomere reorganization.7 Thus, understanding the regulatory molecular mechanisms of calcium-associated signaling and developing methods of uncoupling hypertrophic growth and hemodynamic compensation will provide therapeutic opportunities in the treatment of heart failure. Among the focal regulators of cardiac hypertrophy that respond to elevated calcium, calmodulin-activated serine/threonine phosphatase calcineurin has been extensively characterized for its sufficiency in mediating pathological hypertrophy but not physiological hypertrophy.8

Mammalian calcineurin consists of a catalytic calcineurin A (CnA) subunit, a regulatory calcineurin B subunit, and
calmodulin. Calcineurin B is loaded with Ca\(^{2+}\) once the cytosol calcium concentration increases. The Ca\(^{2+}\)-saturated calcineurin B then facilitates the binding of calmodulin to CnA, which causes conformational changes in CnA to expose the active site. Activated calcineurin directly dephosphorylates the N-terminal serine residues of cytoplasmic nuclear factor of activated T cells (NFAT), which then translocates into the nucleus and acts as a transcriptional effector to activate downstream pro-hypertrophic gene (eg, GATA-4, MEF-2, and AP-1) transcription, eventually leading to pathological cardiac hypertrophy. Despite the description of several endogenous inhibitors (eg, Cabin1/Cain, MCIP, and AKAP79) of calcineurin, the intrinsic negative regulation of calcineurin in the context of cardiac hypertrophy remains to be investigated.

EPI64C, also known as Carabin or TBC1D10C, belongs to the EPI64/TBC1D10 GTPase-activating protein (GAP) family based on its conserved catalytic Trc2/Sub2/Cdc16 (TBC) domain at the N-terminal. EPI64C was first described as a negative regulator of T-cell activation via dual inhibition of both the Ras and calcineurin signaling pathways. Herein, our study was designated to elucidate the role of EPI64C in cardiac hypertrophy.

Using a cardiomyocyte-specific Epi64c knockout mouse strain and a transgenic mouse strain that overexpresses EPI64C specifically in the myocardium, we demonstrated that EPI64C is a negative regulator in pressure overload–induced cardiac hypertrophy and associated pathology. Unlike the molecular coupling that occurs in T cells, EPI64C have no Ras inhibitory activity in cardiomyocytes. In particular, we obtained both in vitro and in vivo evidence showing that EPI64C directly interacts with calcineurin and acts as a bona fide repressor of calcineurin-NFAT signaling, which is responsible for the cardioprotective effect of EPI64C. Finally, by means of lentivirus transfection-mediated EPI64C overexpression in the hearts of Cynomolgus monkeys, we showed that EPI64C gain-of-function remarkably retarded the progression of hypertrophy triggered by aortic constriction. Therefore, our results identify EPI64C as an endogenous inhibitor of calcineurin in the heart and indicate that it holds great potential as a therapeutic target in gene therapy and pharmacological intervention in cardiac hypertrophy.

**Materials and Methods**

Human heart samples were collected as we previously described. All of the procedures involving human samples conformed to the principles outlined in the Declaration of Helsinki and were approved by the Human Research Ethics Committees of Renmin Hospital of Wuhan University and Tongji Hospital of Huazhong University of Science and Technology. All of the animal experimental procedures complied with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committees of Renmin Hospital of Wuhan University and Tongji Hospital of Huazhong University of Science and Technology. A detailed description about the generation of cardi-specific Epi64c conditional knockout (Epi64c\(^{-}\)) mice, full-length Epi64c (Epi64c-TG), deletion mutant Epi64c (ΔEpi64c-TG), deplet of the A406–426 amino acid and site mutant Epi64c (Epi64c\(^{-}\))/Epi64c double knockout (Pp33r\(^{-}\)/Epi64c\(^{-}\), DKO) mouse is provided in the online-only Data Supplement. The methodology for aortic banding (AB) surgery and echocardiographic measurements in mice, morphometric analysis, cell culture experiments of primary neonatal rat cardiomyocyte (NRCM), quantitative real-time polymerase chain reaction, Western blot assay, coimmunoprecipitation, glutathione-S-transferase (GST) pull-down assay, and luciferase assay was performed as we previously described and is illustrated in the online-only Data Supplement. The cynomolgus monkeys (Macaca fascicularis) were purchased from Hainan Primate Laboratory Animal Developing Company Co. Ltd. (Hainan, China). The research protocols about monkeys adhered to the legal and regulatory requirements of the People’s Republic of China and were approved by the Department of Forestry of Hubei Province in China. The details about the husbandry of, lentivirus transfection in, and supravalvular AB surgery in cynomolgus monkeys are provided in the online-only Data Supplement. Experiments of lentivirus construction, active Ras detection, and calcineurin activity assay were performed using established protocols and described in the online-only Data Supplement.

The data are represented as means±SD. Two-tailed Student’s t-tests were used for comparisons between 2 groups. Differences between 3 or more groups were compared using ANOVA followed by a Bonferroni posttest. Differences of echocardiographic measurements in monkeys were analyzed by 2-way ANOVA with repeated measurements followed by a Bonferroni posttest. All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) software, version 13.0. A P value <0.05 was considered significant.

**Results**

**EPI64C is Downregulated in Failing Hearts and Acts as a Negative Regulator in the Cardiomyocyte Hypertrophic Response In Vitro**

Abundant expression of EPI64C has been described in T and B cells. We initially sought to determine the expression profile of EPI64C in ventricular samples procured from normal and failing human hearts. Left ventricle tissues procured from failing dilated cardiomyopathy (Figure 1A) and hypertrophic cardiomyopathy (Figure 1B) hearts showed increased expression of the prototypical hypertrophic markers atrial natriuretic peptide (ANP) and β-myosin heavy chain (β-MHC). However, compared with the robust expression in normal human hearts, EPI64C expression was dramatically reduced in hypertrophic cardiomyopathy and dilated cardiomyopathy hearts (Figure 1A and 1B). A similar expression profile was observed in murine hearts when hypertrophy was induced in wild-type C57BL/6J mice by AB for 4 or 8 weeks. As shown in Figure 1C, EPI64C expression levels were inversely correlated with hypertrophic markers ANP and β-MHC after AB stress.

The greatly decreased expression of EPI64C suggests a possible role of EPI64C in cardiac hypertrophy. To confirm this hypothesis, an in vitro experiment was performed on NRCMs. As displayed in Figure 1D, when hypertrophy was induced in isolated NRCMs by angiotensin II (Ang II) stimulation, EPI64C expression was greatly downregulated, reminiscent of that observed in human and mouse hearts. We thus conducted EPI64C gain- and loss-of-function assays in these cells. Increased or decreased EPI64C expression was approached by adenoviral EpI64c (AdEpI64c) and lentiviral short hairpin EpI64c (Lenti-shEpI64c) transfection, respectively (Figure S1 in the online-only Data Supplement). Intriguingly, EpI64c knockdown remarkably promoted Ang II-provoked cardiomyocyte hypertrophy, as measured by individual cell surface area (Figure 1E and 1F) and mRNA levels of hypertrophic markers (ANP, β-MHC, Figure 1H). In contrast, forced EPI64C overexpression almost abolished the cardiomyocyte hypertrophic response (Figure 1E, 1G, and 1I).
These results suggest that EPI64C negatively regulates cardiomyocyte hypertrophy in vitro.

**Induced Cardiac-Specific Epi64c Knockout Exacerbates Pressure Overload–Induced Cardiac Hypertrophy**

The above findings prompted us to pursue whether EPI64C was functionally significant in the cardiac hypertrophic response in vivo. For this purpose, we generated a conditional knock-out mouse strain by flanking the Epi64c exon 10 with 2 loxP sites (Epi64cfl/fl) mouse was bred with α-MHC-MCM, expressing a tamoxifen-sensitive Cre recombinase under the control of the cardiomyocyte-specific α-MHC-MCM promoter) mice to generate α-MHC-MCM; Epi64cΔ/Δ mutant mice, in which cardiomyocyte Epi64c inactivation (Epi64cΔ/Δ) could be induced in adult mice on tamoxifen injection (Figure 2A). Indeed, Western blot analysis revealed that the loss of EPI64C expression after induction was restricted to the heart (Figure 2B).

Then, the hypertrophic response in the Epi64cΔ/Δ mice was tested by pressure overload (AB) stress for 4 weeks. Age-, weight-, and sex-matched α-MHC-MCM and Epi64cΔ/Δ mice were used as controls. Remarkably, the loss of Epi64cΔ/Δ substantially enhanced the hypertrophic response compared with the control counterparts, as manifested by the increased heart weight (heart weight/body weight, HW/BW; HW/tibia length) increment (Figure 2C and 2D). Lung weight increment (lung weight/body weight, LW/BW), reflecting pulmonary congestion after left ventricle failure, was also enhanced in the Epi64cΔ/Δ mice (Figure 2E). The echocardiography results revealed augmented ventricle chamber enlargement in the Epi64cΔ/Δ mice (Figure 2F and 2G). Specifically, aggravated hypertrophy was accompanied by deteriorated cardiac function, as exhibited by a decrease in percent fractional shortening assessed by echocardiography (Figure 2H). Likewise, morphometric studies demonstrated that both the visible heart enlargement (Figure 2I, first panel) and individual cardiomyocyte hypertrophy (analyzed by cardiomyocyte cross-sectional area, Figure 2I and 2J) were exaggerated by Epi64cΔ/Δ mice. In particular, the analysis of picrosirius red-stained cardiac sections revealed that the massive hypertrophy that occurred in the Epi64cΔ/Δ mice developed rampant cardiac fibrosis (Figure 2I and 2J).

EPI64C Overexpression in Cardiomyocytes Attenuates Cardiac Hypertrophy in Response to Pressure Overload

The massive hypertrophy that occurred in the Epi64cΔ/Δ mice suggests that EPI64C is a brake against hypertrophic...
growth. However, naturally occurring EPI64C downregulation in this process, in turn, promotes cardiac hypertrophy. We then investigated whether restoring EPI64C expression would rescue this maladaptive response. For this goal, we generated several Epi64c transgenic (Epi64c–TG) mouse strains, in which EPI64C was overexpressed in cardiomyocytes driven by the αMHC promoter (Figure 3A). Four lines of transgenic mice were produced and exhibited no overt abnormalities. The transgenic line with the 4.6-fold transgene expression (TG 4, Figure S2) was subjected to experimental use.

The Epi64c–TG and control nontransgenic (NTG) mice were challenged with pressure overload for 8 weeks, a longer period and a more stringent model to test the cardioprotective effect of EPI64C. Surprisingly, EPI64C overexpression exerted a potent suppressive effect against the pressure overload–induced heart mass increase (Figure 3B and 3C). The absence of pulmonary congestion in Epi64c–TG mice suggests preserved ventricle function (Figure 3D). Accordingly, echocardiographic analysis showed that the ventricular dimensions (Figure 3E and 3F) and cardiac contractile function (Figure 3G) were largely preserved in Epi64c–TG mice after AB.

Figure 2. Cardiomyocyte-specific Epi64c knockout aggravates pressure overload–induced cardiac hypertrophy. A, A schematic diagram showing the generation of cardiomyocyte-specific Epi64c knockout mice. The mouse Epi64c gene is shown with the targeting vector and the targeted allele. Homologous recombination resulted in a floxed Epi64c exon 10 (Epi64cfl). On αMHC-MerCreMer (αMHC-MCM)-mediated recombination, the floxed Epi64c allele became an Epi64c null allele (Epi64cΔ). B, Homozygous Epi64c–floxed mice (Epi64cfl/fl) and Epi64c null mice (Epi64cΔ/Δ) were analyzed for the protein levels of EPI64C in different organs. Representative results of Western blotting are shown. Mice with the indicated genotypes were subjected to aortic banding (AB) or sham surgery. Then, 4 weeks later, the ratios of (C) heart weight (HW)/body weight (BW), (D) HW/tibia length (TL), and (E) lung weight (LW)/BW in indicated groups were calculated and compared (n=10–15). F–H, Statistical results of echocardiographic parameters in the indicated groups (n=5–8). I. The representative images of gross hearts (the first panel), hematoxylin and eosin-stained (the second panel), and picrosirius red-stained (third and fourth panels) heart sections from the indicated groups (scale bar: 50 μm). J. Morphometric results of cardiomyocyte cross-sectional area in the indicated groups. K. Statistical results of left ventricle collagen volume determined by picrosirius red staining. L. The relative mRNA levels of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), β-myosin heavy chain (β-MHC), collagen I, collagen III, and connective tissue growth factor (CTGF) in the left ventricle of mice from the indicated groups (n=4). *P<0.05 vs αMHC-MCM sham; §P<0.05 vs Epi64cfl/fl sham; #P<0.05 vs αMHC-MCM AB. FS indicates fractional shortening; LVEF, left ventricular ejection fraction; LVEDd, left ventricular end-diastolic diameter; and LVESd, left ventricular end-systolic diameter.
Consistently, both macroscopic and microscopic examinations of cardiac histology revealed compromised cardiomyocyte hypertrophy in Epi64c-TG mice (Figure 3H and 3I). The Epi64c-TG cardiac sections also displayed less collagen deposition as visualized by picrosirius red staining (Figure 3H and 3J). Correspondingly, mRNA levels of hypertrophic (ANP, brain natriuretic peptide, and \(\beta\)-MHC) and fibrotic (collagen I, collagen III, and connective tissue growth factor) markers were all decreased on EPI64C overexpression (Figure 3K).

Notably, EPI64C overexpression had no impact on baseline cardiac performance.

Calcineurin Suppressive Capability but not the Ras GAP Activity of EPI64C Is Responsible for the EPI64C-Mediated Anti-Hypertrophic Effect

A study by Pan et al\(^\text{12}\) uncovered a role of EPI64C as a Ras GAP, which inactivates Ras activity and, in turn, impairs downstream Raf-mitogen-activated protein kinases (MAPK) kinase (MEK)-extracellular signal-regulated kinase (ERK) signaling. It has been well established that various stimuli transmit pro-hypertrophic signals through the Ras-ERK signaling cascade in cardiomyocytes.\(^\text{17}\) We, therefore, sought to determine whether the Ras GAP activity of EPI64C interfered with its anti-hypertrophic effects. Heart lysates from the above described groups of mice were analyzed to determine the activation state of MEK1/2 and ERK1/2 by Western blotting. Unexpectedly, we failed to observe differences in MEK1/2-ERK1/2 activation in Epi64c-depleted and Epi64c-overexpressed hearts under pressure overload (Figure S3A and S3B). Furthermore, the activation of the other 2 branches of MAPK, the c-Jun N-terminal kinase 1/2 (JNK1/2) and the p38 pathway, was also not affected by altered EPI64C expression (Figure S3A and S3B). We then directly examined the levels of activated Ras in ventricle lysates. Ras becomes bio-logically active when bound to guanosine triphosphate (GTP). In an active Ras-GTP pull-down assay, we found that AB
elevated the levels of Ras-GTP in control mice (Figure 4A and 4B). Nevertheless, neither Epi64c knockout nor overexpression affected the activation of Ras in response to pressure overload (Figure 4A and 4B).

Given the inhibitory role of EPI64C in calcineurin-NFAT signaling in T-cell activation,12 we assessed whether the calcineurin-NFAT cascade could be the downstream effector of EPI64C. We first examined the calcineurin phosphatase activity in ventricle lysates from different groups of mice. The results revealed that AB greatly enhanced calcineurin activity in ventricular tissues of the control mice (Figure 4C and 4D), which is consistent with previous studies. The increased calcineurin activity was further enhanced in the in Epi64c transgenic mice (Figure 4C), whereas the Epi64c transgene led to a reduction in the increases in calcineurin activity (Figure 4D). To measure the impact of EPI64C on NFAT transcription activity, NRCMs were coinfected with lentiviral NFAT-luciferase reporter together with either AdEpi64c or Lenti-shEpi64c. The results demonstrated that Ang II-evoked NFAT-luciferase activity was dramatically enhanced by EPI64C knockdown (Figure 4E) but restrained by EPI64C overexpression (Figure 4F). To further validate these results, we examined the dephosphorylation state and nuclear translocation of NFATc3 (also known as NFAT4, NFATx) in ventricular lysates. In agreement with previous studies,18 phospho-NFATc3 in the cytoplasm was reduced in response to pressure overload in control mice (Figure 4G and 4H). However, loss of EPI64C greatly potentiated the dephosphorylation (Figure 4G), whereas EPI64C overexpression largely preserved the phosphorylation state of NFATc3 (Figure 4H). Accordingly, the degree of NFATc3 nuclear translocation was elevated, which resulted in increased accumulation of NFATc3 in the nucleus of the Epi64c transgenic mice (Figure 4I). Conversely, EPI64C overexpression prevented the nuclear translocation of NFATc3 (Figure 4J). Further gain- and loss-of-function experiments performed on NRCMs yielded similar results (Figure S4A through S4D).

EPI64C Directly Interacts With Calcineurin
To dissect the molecular mechanisms by which EPI64C regulates calcineurin-NFAT signaling, we first examined whether EPI64C and calcineurin interact directly. Cotranslation of Myc-tagged CnA and Flag-tagged EPI64C in HEK293T cells, followed by immunoprecipitation with anti-Myc antibody and immunoblotting with anti-Flag antibody, revealed that EPI64C coimmunoprecipitated with CnA (Figure 5A). Conversely, CnA could also well precipitated with EPI64C (Figure 5B). To exclude the potential that the 2 proteins might bind indirectly, we purified GST fusion proteins in bacteria and tested the formation of EPI64C-CnA complexes in an in vitro GST pull-down assay. As shown in Figure 5C, Flag-tagged EPI64C in cell extracts could bind to and elute with immobilized GST-CnA but not GST alone. Consistently and inversely, Myc-tagged CnA could well elute with GST-EPI64C (Figure 5D).

To map the determinants of this interaction more precisely, we tested a series of CnA deletion mutants for the ability to coimmunoprecipitated with full-length EPI64C. Using extracts of HEK293T cells coexpressing tag-flagged full-length EPI64C and Myc-tagged CnA deletion mutants, we found that amino acid residue 287 to 337 constituted the bona fide module in CnA that binds to EPI64C (Figure 5E).

Figure 4. The calcineurin suppressive capability but not Ras GTPase-activating protein activity of EPI64C is responsible for the suppressive effect of EPI64C on cardiac hypertrophy. The Epi64c null (Epi64c<sup>−/−</sup>), Epi64c transgenic (Epi64c<sup>+/+</sup>-TG), and corresponding control mice were stressed with aortic banding (AB) or sham surgery for 4 or 8 weeks as indicated. A and B, Protein levels of total Ras (Ras) and GTP-bound Ras (GTP-Ras) in the left ventricle (LV) lysates of mice from the indicated groups. Shown are representative results of 2 independent active Ras pull-down assays. C and D, Calcineurin phosphatase activity in the lysates of the LV from the indicated experimental groups (n=4). NFAT-luciferase activity in neonatal rat cardiomyocytes (NRCMs) after transfection with E and F lentiviral short hairpin Epi64c (Lenti-shEpi64c) or (F) adenoviral Epi64c (AdEpi64c) followed by Ang II or PBS stimulation for 24 hours (n=4). G and H, Protein levels of phosphorylated nuclear factor of activated T-cells c3 (p-NFATc3) and total NFATc3 in the cytoplasm of the LV from mice in the indicated groups (n=4). I and J, Protein levels of NFATc3 in the nucleus of the LV from mice in the indicated groups (n=4). *P<0.05 vs αMHC-MCM sham or NTG sham; #P<0.05 vs αMHC-MCM AB or NTG AB.
The results revealed the smallest interaction region mapped in our assay spanned the C-terminal amino acids 406 to 446 of EPI64C (Figure 5F). To substantiate these results, we produced the same series of GST-fused CnA deletion mutants in bacteria and tested their interaction with full-length Flag-EPI64C in a GST pull-down assay, which corroborated these results (Figure 5I). The GST-fused truncated EPI64C also displayed a consistent pattern of binding with Myc-CnA (Figure 5J).

**Calcineurin Binding-Defective EPI64C Mutant Loses Anti-Hypertrophic Capability In Vivo**

To further corroborate these observations, we constructed a transgenic mouse line, in which a Ras GAP-defective truncated EPI64C also displayed a consistent pattern of binding with Myc-CnA (Figure 5J).
EPI64C mutant (Epi64c<sup>R141A</sup>)<sup>12</sup> was overexpressed specifically in the cardiomyocytes (Epi64c<sup>R141A-TG</sup>, Figure 6A and Figure S5). The substitution of the critical catalytic finger, the 141 amino acid arginine in the TBC domain, to alanine has been shown to eliminate the GAP activity of EPI64C. To better characterize the functional significance of the interaction between EPI64C and calcineurin, we also generated a deletion mutant Epi64c transgenic mouse strain (∆Epi64c-TG, Figure 6A and Figure S6). This truncated form of EPI64C was depleted of amino acids 406 to 426 and was unable to bind to calcineurin, as demonstrated by the above coimmunoprecipitation and GST pull-down assays. Then, these mice were challenged with AB for 8 weeks. Intriguingly, the Epi64c<sup>R141A-TG</sup> mice retained the same degree of anti-hypertrophic capacity under pressure overload as that in Epi64c-TG mice. In contrast, the loss of calcineurin binding capability almost completely abrogated the cardioprotective effects of Epi64c in ∆Epi64c-TG mice. The Epi64c<sup>R141A-TG</sup> and Epi64c-TG mice showed comparable reductions in increased levels of the phenotypic measurements. On the contrary, these measurements in ∆Epi64c-TG mice were indistinguishable from those in NTG mice and were much higher than those in the Epi64c-TG and Epi64c<sup>R141A-TG</sup> mice. These measurements are ratios of HW/BW (Figure 6B), HW/tibia length (TL), and LW/BW (Figure 6D); echocardiographic examinations of ventricle geometry (Figure 6E and 6F); morphometric examinations of cardiomyocyte hypertrophy (Figure 6H and 6I) and extracellular fibrosis (Figure 6H and 6J); and mRNA levels of hypertrophic markers ANP and β-MHC (Figure 6K). Collectively, these results demonstrated that calcineurin interaction is essential and solely responsible for EPI64C-mediated inhibition of cardiac hypertrophy.
Blocking Calcineurin Signaling Overrides the Effects of Epi64c Knockout on Hypertrophy

If calcineurin signaling but without the involvement of Ras acts downstream of EPI64C, calcineurin interruption should reverse the deleterious effects of Epi64c knockout on cardiac hypertrophy. To consolidate this theory, the calcineurin subunit B type 1 encoding gene floxed mice (Ppp3r1fl/fl) were mated with αMHC-MCM and αMHC-MCM;Epi64cfl/fl mice to generate the αMHC-MCM;Ppp3r1fl/fl and αMHC-MCM;Ppp3r1fl/fl;Epi64cfl/fl mice, respectively (Figure S7A). On tamoxifen injection, Ppp3r1 was solely (Ppp3r11/1) knocked out, or both Ppp3r1 and Epi64c were doubly (double knockout) knocked out specifically in cardiomyocytes (Figure S7B). All mouse strains exhibited no abnormalities in baseline cardiac functions. Because Ppp3r1 encodes the only isoform of the essential calcineurin regulatory subunit B in the heart, Ppp3r1 knockout results in complete interruption of calcineurin signaling.26 The indicated mice were subjected to AB for 4 weeks to induce cardiac hypertrophy. In accordance with the established role of calcineurin in hypertrophy, targeted Ppp3r1 knockout in cardiomyocytes attenuated the expected hypertrophy, compared with that in the αMHC-MCM control mice. More importantly, Ppp3r1 deficiency completely reversed the deleterious effects of Epi64c depletion on cardiac hypertrophy in double knockout mice. These conclusions were based on the following phenotypic examinations: ratios of HW/BW (Figure S7C), HW/ibfia length (Figure S7D), and LW/BW (Figure S7E); ventricle chamber dimensions (Figure S7F and S7G) and cardiac function (Figure S7H) analyzed by echocardiography; heart size and cross-sectional area of cardiomyocytes (Figure S7I and S7J); fibrosis (Figure S7I and S7K); and mRNA levels of hypertrophic markers (ANP and β-MHC, Figure S7L).

Inhibitory Role of EPI64C in Pathological Cardiac Hypertrophy Is Conserved in Cynomolgus Monkeys

The impressive cardioprotective effect of EPI64C overexpression against pathological cardiac hypertrophy motivated us to test the preclinical application in a nonhuman primate model. EPI64C was overexpressed in the heart of cynomolgus monkey by lentiviral Epi64c (Lenti-Epi64c) mediated antegrade intracoronary transfection, and cardiac hypertrophy was subsequently induced by ascending aorta constriction (Figure 7A; Figure S8A through S8C). As shown in Figure 7B, lentivirus transfection using our technique successfully generated a 1.7-fold increase in EPI64C protein expression 70 days after surgery. The hypertrophic phenotype was determined by echocardiographic examinations before, postoperative day 35, and postoperative day 70 (Figure 7A). As shown in Figure 7C and Table S1, echocardiographic measurements of ventricle wall thickness were all gradually increased after aortic constriction in control Lenti-Vector–infected monkeys, indicating progressive development of cardiac hypertrophy. Importantly, EPI64C overexpression delayed the progress of pressure overload–triggered ventricle wall hypertrophy compared with the controls (Figure 7C; Tables S1 and S2). However, our technique failed to provoke ventricle chamber enlargement (Figure S9A; Tables S1 and S2) and cardiac function deterioration (Figure S9B and S9C; Tables S1 and S2) compared with preoperative measurements during the 70-day observation period. Measurements of HW/BW at postoperative day 70 confirmed the alleviated hypertrophy in EPI64C–overexpressed hearts (Figure 7D). However, no difference of LW/BW was obtained (Figure S10). Consistently, cardiac histology (Figure 7E) and cardiomyocyte size (Figure 7F) were largely preserved by EPI64C gain-of-function.

Discussion

EPI64C was first discovered in a yeast 2-hybrid assay designed to screen the calcineurin interaction proteins.13 However, there have been few studies on the in vivo functions of EPI64C since this study. In this study, EPI64C gain- and loss-of-function studies performed in both Ang II–induced cardiomyocyte hypertrophy in vitro and pressure overload–induced cardiac hypertrophy in vivo revealed that cardiomyocyte-specific EPI64C was a critical negative regulator of cardiomyocyte hypertrophy. Moreover, our molecular studies showed that the direct interaction with EPI64C inhibited calcineurin phosphatase activity and blocked the subsequent dephosphorylation and nuclear translocation of NFAT both in vivo and in vitro. Importantly, the repressed calcineurin–NFAT signaling was solely responsible for the EPI64C–mediated suppressive effect on cardiac hypertrophy. This result is supported by the observation that minimally truncated EPI64C that was incapable of binding to calcineurin completely lost its protective effect against hypertrophy in transgenic mice. Furthermore, simultaneously interrupting calcineurin–NFAT signaling by depleting the calcineurin B gene fully rescued the devastating effect of Epi64c knockout on cardiac hypertrophy and associated pathology.

It has been established that calcineurin interacts with substrates or regulatory proteins that possess 2 distinct consensus motifs: the PxIxIT motif or the LxVP (the x could be any aa) motif.9 Of special interest is the PxIxIT motif, which is harnessed by several endogenous inhibitors of calcineurin to interact with the C-terminus of the catalytic domain of calcineurin, eg, MCIP1,21 DSCR1,22 and AKAP79.23 By competitively binding to calcineurin, these inhibitors blocked the docking domain for NFAT on calcineurin, eg, MCIP1,21 DSCR1,22 and AKAP79.23 By competitively binding to calcineurin, these inhibitors blocked the docking domain for NFAT on calcineurin, thus prevents the dephosphorylation of NFAT.9 Unexpectedly, we failed to locate either the PxIxIT motif or the LxVP motif in the C-terminal 406–446aa, or even the full-length EPI64C. Hence, we mapped the precise interactive domains in EPI64C in CnA, respectively. In EPI64C, the C-terminal 406–446aa was shown to be the minimum domain that was required for calcineurin interaction. We further identified the module (287–337aa) lying in the catalytic domain but in proximity to the active site of calcineurin A was the docking site for EPI64C, which is similar to those PxIxIT-bearing inhibitors.24 This phenomenon raised the possibility that EPI64C may also tune the calcineurin signaling via competitive binding. However, further biochemical investigations are needed to address the structural basis for EPI64C–mediated calcineurin inhibition. Moreover, this increased understanding will be instructive for designing novel synthetic mimetics as a tool to block calcineurin signaling and protect the heart, therefore, providing an alternative of PxIxIT motif based peptide VIVIT.25

The conserved TBC domain present at the N-terminal (residues 89–294) of EPI64C has been shown to possess Ras
GAP activities to negatively regulate IL-2 production and cellular proliferation during T-cell activation in cultured T cells. In this scenario, EPI64C facilitates the switch of Ras from the GTP-bound on state to the guanosine diphosphate (GDP)-bound off state through accelerating the intrinsic rate of GTP hydrolysis, thus terminating the Ras-MEK-ERK signaling (GDP)-bound off state through accelerating the intrinsic rate of GTP hydrolysis, thus terminating the Ras-MEK-ERK signaling. However, we failed to observe differences in the activation state of Ras-MEK-ERK signaling in either the EPI64C-depleted or the EPI64C-overexpressed hearts in response to pressure overload. This phenomenon suggests that the Ras GAP activity of EPI64C does not take effect in cardiac muscle cells, eg, endothelial cells or fibroblasts, caused by the off-target effects of the genetic techniques used in Bisserier’s reports. Then, they demonstrated that the Ras GAP-inactivated mutant EPI64CΔR141 was less potent for inhibiting hypertrophy in cultured cardiomyocytes. Nevertheless, in our study, cardiac-specific EPI64C harboring the same mutation transgene remains potent in suppressing pressure overload–induced hypertrophy, and calcineurin interruption fully reversed the exaggerated phenotype in EpiΔE mice. However, we obtained negative results in our cardiomyocyte-specific EPI64C gain- and lose-of-function mice. We speculate the reason for this disparity might be caused by that the Ras GAP activity of EPI64C does not take effect in cardiac muscle cells, eg, endothelial cells or fibroblasts, caused by the off-target effects of the genetic techniques used in Bisserier’s reports. Then, they demonstrated that the Ras GAP-inactivated mutant EPI64CΔR141 was less potent for inhibiting hypertrophy in cultured cardiomyocytes. Nevertheless, in our study, cardiac-specific EPI64C harboring the same mutation transgene remains potent in suppressing pressure overload–induced hypertrophy, and calcineurin interruption fully reversed the exaggerated phenotype in EpiΔE mice. However, we obtained negative results in our cardiomyocyte-specific EPI64C gain- and lose-of-function mice.

At this writing, Bisserier et al published their elegant research on the same topics as those in our study. Although different techniques of genetic engineering were used, we reached the same conclusion regarding the phenotype. With respect to the associated molecular mechanisms, consensus was reached in the conclusion that calcineurin is the downstream effector of EPI64C. However, divergence appeared when Bisserier et al demonstrated that the Ras GAP activity of EPI64C is also account for repressing pro-hypertrophic signaling. In pressure overload-stressed hearts, they detected differential ERK activation in global Epi64c knockout mice, and adeno-associated virus mediated EPI64C overexpressed mice. However, we obtained negative results in our cardiomyocyte-specific EPI64C gain- and lose-of-function mice. This divergence may reflect changes that happened in nonmuscle cells, eg, endothelial cells or fibroblasts, caused by the off-target effects of the genetic techniques used in Bisserier’s reports. Then, they demonstrated that the Ras GAP-inactivated mutant EPI64CΔR141 was less potent for inhibiting hypertrophy in cultured cardiomyocytes. Nevertheless, in our study, cardiac-specific EPI64C harboring the same mutation transgene remains potent in suppressing pressure overload–induced hypertrophy, and calcineurin interruption fully reversed the exaggerated phenotype in EpiΔE mice. However, we obtained negative results in our cardiomyocyte-specific EPI64C gain- and lose-of-function mice.

By introducing genetic materials into cells or tissues to repair causative genes involved in diseases or to add novel functions to the target cells, the so-called gene therapy has showed great promise in the war against heart failure over the past decade. Abnormal calcium handling in cardiomyocytes, as mentioned above, was prevalent in heart failure because of various etiologies. In an attempt to repair calcium handling in the context of gene therapy, restoring decreased sarcoplasmic/endoplasmic reticulum Ca2+ATPase2a (SERCA2a) expression has attracted the most attention and was shown to be efficacious in both
animal studies and clinical trials. In this study, we found that EPI64C expression is downregulated in hypertrophied human myocardium, which, in turn, relieved the inhibition of calcineurin-NFAT signaling and the subsequent progression of hypertrophy. We then showed that lentivirus-mediated increased EPI64C expression in monkey hearts exerted a strong anti-hypertrophic effect in response to pressure overload. Thus, we have provided an alternative target for gene therapy in calcium-associated signaling and demonstrated the preclinical feasibility of replenishing EPI64C to treat hypertrophy in nonhuman primates. However, with respect to the large-scale clinical application of gene therapy, the following problems regarding the gene vector and delivery route are still the topics of ongoing debate: (1) gene vectors with high transfection efficiency, long-term cardiorestrictive expression, yet minimal immunogenicity, and biosafety concerns still need to be developed; (2) Improved techniques to produce high-quality and large-quantity vectors that meet the clinical need are required; and (3) minimally invasive delivery systems are needed. However, we should expect that the continued technological evolution of gene therapy will aid in translating advances in molecular cardiology from bench top to bedside to treat heart failure.

In conclusion, our present study demonstrated that EPI64C negatively regulates cardiac hypertrophic response and associated pathology via direct inhibition of calcineurin signaling. Moreover, gene therapy by replenishing EPI64C expression showed preclinical efficacy in large animals. These observations suggest that EPI64C could be a therapeutic target for the treatment of cardiac hypertrophy.

**Perspectives**

Central to the regulation of excitation-contraction coupling, calcium also plays a vital role in regulating cardiac hypertrophy and associated molecular cardiology. A deeper insight into the calcium-associated regulatory network will provide clues for the treatment and prevention of cardiac hypertrophy and heart failure.

**Acknowledgments**

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**Disclosures**

All authors have read and agreed to the content within the article. All authors declare no financial or other conflict of interest relevant to the subject of this article.

**References**


What Is New?
- The protein levels of EPI64C are decreased in failing human and mouse hearts.
- EPI64C negatively regulates pressure overload–induced cardiac hypertrophy.
- The cardioprotective effect of EPI64C is largely mediated by inhibiting calcineurin activation but is independent of its Ras GAP activity.
- Lentivirus-mediated EPI64C overexpression in the hearts of cynomolgus monkeys dampened cardiac hypertrophy in response to pressure overload.

What Is Relevant?
- Knowledge regarding the regulatory network of calcineurin signaling during cardiac hypertrophy is poor.
- The function and underlying mechanism of EPI64C with respect to cardiac hypertrophy have not been investigated.
- Explorations of gene therapy for treating cardiac hypertrophy are rare.

Summary
Restoring or increasing EPI64C expression during cardiac hypertrophy could be a novel therapeutic concept for the prevention and treatment of heart failure.
Cardiac-Specific EPI64C Blunts Pressure Overload–Induced Cardiac Hypertrophy
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Cardiac-specific EPI64C Blunts Pressure Overload-Induced Cardiac Hypertrophy

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Supplementary Materials and Methods

Human Heart Samples

Left ventricle tissues were collected as we previously described. The failing heart tissues were procured from patients with dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM) who were undergoing heart transplantation. Normal ventricle samples were obtained from heart donors who died in accidents, but whose hearts were unsuitable for transplantation for non-cardiac reasons. Written informed consent was obtained from the families of the prospective heart donors. All of the procedures involving human samples conformed to the principles outlined in the Declaration of Helsinki and were approved by the Human Research Ethics Committees of Renmin Hospital of Wuhan University and Tongji Hospital of Huazhong University of Science and Technology.

Genetically Engineered Mice

All of the animal experimental procedures complied with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committees of Renmin Hospital of Wuhan University and Huazhong University of Science and Technology.

Epi64c-floxed mice (Epi64c^{flox/flox}) were generated as follows. The targeting construct for the generation of conditional Epi64c^{flox} mice was prepared using 129/Sv genomic BAC clone (clone No. 192F10) harboring the complete Epi64c locus (2005,86:753-758) and subcloned into the PL451 vector (2003,13:476-484). The construct consisted of a 2.6-kb 5’ homology arm, a PGK-EM7-Neo selection cassette for positive selection by G418, and a 1.3-kb 3’ homology arm as depicted in Fig. 2A. The sequence containing Epi64c exon 10 and the PGK-EM7-Neo cassette was flanked by two loxP sites, whereas the PGK-EM7-Neo cassette alone was additionally flanked by 2 flippase recognition target (FRT) sites. Linearized targeting vectors were electroporated into the V6.5 embryonic stem cell line, which was derived from C57BL/6 and 129S6SvEv F1 hybrid mice. Colonies resistant to G418 were picked and expanded for screening. Then, the FRT flanked Neo cassette were removed through Flp-mediated recombination. Next, 2 heterozygous embryonic stem clones that had undergone homologous recombination were injected into C57BL/6 and 129S6SvEv F1 hybrid mice. Colonies resistant to G418 were picked and expanded for screening. Then, the FRT flanked Neo cassette were removed through Flp-mediated recombination. Next, 2 heterozygous embryonic stem clones that had undergone homologous recombination were injected into C57BL/6J blastocysts, and the resulting chimeric male mice were mated with C57BL/6J female mice to obtain homozygous Epi64c^{flox/flox} (Epi64c^{fl/fl}) mice. None of the viable mice showed any baseline abnormalities. The primer pairs, P1 and P2 and P5 and P6 were used for the polymerase chain reaction (PCR)-based screening of ES cells, and the primers P3 (5’-TTGCCAGCATCAAAGCCAGC3’) and P4 (5’-TGAAGTGCCTCTCTGCGGACTCA3’) were used for genotyping of the mice. The wild type (WT) band was 381 bp, and the floxNeo allele band was 2 kb and did not produce a band.

The transgenic mice expressing a tamoxifen-inducible Cre-mediated recombinase (MerCreMer, MCM) driven by the cardiomyocyte-specific alpha-myosin heavy chain promoter (αMHC-MCM) were obtained from The Jackson Laboratory (stock No. 005650). Then, the αMHC-MCM mice were mated with Epi64c^{flox} to generate the αMHC-MCM;Epi64c^{flox} mutant mice. To induce the Epi64c knockout (Epi64c^{Δ/Δ}) in adult mouse cardiomyocytes, tamoxifen (Sigma-Aldrich, T-5648) dissolved in cornoil was intraperitoneally injected into αMHC-MCM;Epi64c^{flox} mice (25 mg/kg) for 5 consecutive days as previously described. Two types of littermate controls were used in our study: αMHC-MCM transgenic mice with tamoxifen injection; and αMHC-MCM non-transgenic Epi64c^{flox} mice with cornoil injection. No mice died or showed any morbidity after tamoxifen injection. Mice with the indicated genotype were given tamoxifen at the age of 6-7 weeks. Then, at 9-10 weeks of age, these mice were sacrificed for protein assessment or subjected to surgery.

The plasmid constructs encoding full-length Epi64c, deletion mutant Epi64c (AEpi64c, depleted of the 406-426 amino acid) and site mutant Epi64c (Epi64c^{R141A}) were kindly provided by Jun O. Liu at Johns
Hopkins University School of Medicine (Baltimore, Maryland, USA). Full-length Epi64c, ΔEpi64c, or Epi64cR141A were separately cloned into the downstream region of the αMHC promoter. The linearized αMHC-Epi64c plasmid, αMHC-ΔEpi64c plasmid and αMHC-Epi64cR141A plasmid were microinjected into fertilized mouse embryos to produce cardiac-specific Epi64c-transgenic (Epi64c-TG) mice, ΔEpi64c-transgenic mice (ΔEpi64c-TG), and Epi64cR141A–transgenic mice (Epi64cR141A-TG), respectively. Successful production of the transgene was identified based on PCR analysis of tail genomic DNA using the following primers: αMHC-Forward (5'-ATCTCCCCCATAAGAGTTTGAGTC-3') and Epi64c-Reverse (5'-CGGTCGATACAGGGTGTAGG'). A successful transgene generated a 550 bp band. The transgenic line with the highest transgene expression in the heart, as verified by Western blot analysis, was subjected to experimental use. All transgenic mice were fertile, viable and indistinguishable in appearance from normal mice.

The calcineurin subunit B type 1 floxed mice (Ppp3r1fl/fl) were obtained from The Jackson Laboratory (stock No. 006581). The Ppp3r1fl/fl mice were crossed with the αMHC-MCM mice and the Epi64cfl/fl mice produce the αMHC-MCM;Epi64cfl/fl mice and αMHC-MCM;Ppp3r1fl/fl;Epi64cfl/fl mice, respectively. Then, these mice were intraperitoneally injected with tamoxifen (Sigma-Aldrich, T-5648, 25 mg/kg) for 5 consecutive days to generate cardiomyocyte specific Ppp3r1 knockout (Ppp3r1Δ/Δ) mice and Ppp3r1 and Epi64c double knockout (Ppp3r1Δ/Δ;Epi64cΔ/Δ, DKO) mice, respectively.

**Mouse Aortic Banding (AB) Surgery**

Male mice aged 8 to 10 weeks and weighing 24-27 g were used in this study. Pentobarbital (80 mg/kg, Sigma-Aldrich, P3761, delivered intraperitoneally) was used to anesthetize the mice during surgery. AB surgery was performed as we previously described.1-3 Briefly, the thoracic aorta was accessed through the second intercostal space. The descending aorta distal and adjacent to the left common carotid artery was banded against a 26/27-gauge needle using 7-0 silk suture. The needle was quickly removed upon knotting and the thoracic cavity was closed. Doppler analysis was performed to ensure that appropriate constriction of the aorta was produced. Control animals underwent the same procedures without AB (sham groups).

**Husbandry of, Lentivirus Transfection in, and Supravavular AB Surgery in Cynomolgus Monkeys**

The cynomolgus monkeys (Macaca fascicularis) were purchased from Hainan Primate Laboratory Animal Developing Company Co. Ltd. (Hainan, China). The research protocols adhered to the legal and regulatory requirements of the People’s Republic of China and were approved by the Department of Forestry of Hubei Province in China. All monkeys were male, weighed 3~5 kg, and were 4-5 years old. The healthy status of each monkey was determined by a local Veterinary Department. After transport to the animal facility at Tongji Medical College, the animals were individually housed in stainless steel wire-bottomed cages with sufficient space for three months to acclimate to the environment. The monkeys were feed a special chow for non-human primates and seasonal fruits with water available ad libitum. The room lighting was on a 12 h dark/12 h light automatically cycle and the room temperature was maintained at 22~26°C. The monkeys were randomly assigned into the experimental group (n=10) and the control group (n=10).

Prior to surgery, the monkeys were fasted and deprived of water for 12 and 4 h, respectively. Anesthesia was induced with an intramuscular injection of 50 mg of ketamine hydrochloride. Then, the monkey was fixed on an operating table in the supine position. Atropine (0.05 mg/kg) was injected intramuscularly to prevent excessive airway secretions. Blood pressure and electrocardiogram results were monitored using a Dash 2000 Patient Monitor (GE Healthcare). General anesthesia suitable for a median thoracotomy was achieved using a bolus intravenous injection of ketamine hydrochloride (15 mg/kg) and midazolam (1.5 mg/kg). Then, the
monkey was intubated and ventilated with a tidal volume 10 ml/kg at 40 breaths per minute. All traumatic operations were performed using sterile techniques. To expose the heart, a median thoracotomy was performed, and the epicardium was suspended. Then, the ascending aorta was bluntly mobilized from the surrounding connective tissues.

To conduct lentivirus intracoronary transfection, the inferior vena cava was cross-clamped to reduce the venous drainage and intracardiac pressure. Then, the ascending aorta was cross-clamped. Lentivirus (10⁹ transfection units) in 1 ml of medium was injected into the aortic root proximal to the aortic clamp through a 25G needle. The aorta was clamped for 30 more seconds to allow virus transfection through the coronary circulation; then, all clamps were released. Prior to chest closure, another 1 ml of lentivirus was sprayed pericardially. Monkeys in the experimental group were transfected with lentivirus carrying the Epi64c-expressing vector, whereas the control group received only the lentiviral vector. Rational of optimal lentivirus dose and transfection route for in vivo cardiac transfection were obtained from preliminary experiments performed in miniature pig.

When hemodynamic stability was achieved after virus transfection, aortic constriction was performed. A 2-0 silk suture was used to ligate the ascending aorta against a bent stainless steel stick (3.2 mm in diameter for 3~4kg monkey, 4 mm for 4~5kg monkey). After firmly knotting, the stick was promptly removed, and proper constriction was evaluated by Doppler analysis. Aortic constriction performed by our method produced approximately 80% narrowing of the ascending aorta. Then, the sternum, muscle and skin were closed in three layers, with evacuation of any pneumothorax. The total operative time from intubation to skin closure was less than 40 min. The monkeys were extubated when able to breathe spontaneously, intramuscularly injected with sufentanil (5 ug/kg) to alleviate operation-associated pain, and returned to their cages until full resuscitation. To prevent infection, 1.2 million IU benzathine benzylpenicillin was injected intramuscularly. The operative mortality associated with the invasive procedures was 10% and occurred the day after operation. At 70 days after the operation, the monkeys were deeply sedated as described above and euthanized using an intravenous injection of 10% potassium chloride. The heart tissues were removed after brain death.

**Lentivirus Construction**

A lentivirus carrying the Epi64c coding gene (Lenti-Epi64c) and Gfp-coding vector (Lenti-Vector) was constructed. Briefly, full length Epi64c cDNA was inserted into the lentiviral vector construct and driven by the cytomegalovirus (CMV) promoter. Lentiviral particles were produced by co-transfection of the lentiviral vector construct, packaging construct and envelope plasmid into 293T cells. At 48 h after transfection, supernatants were harvested and viral particles were concentrated by ultracentrifugation. Virus stocks were resuspended in serum free culture medium and stored at -80°C until use. Virus titers of transfection units were assayed by transfecting 293T cells for 48 h with serial dilutions of concentrated lentivirus and counting GFP-positive cells under fluorescence microscopy.

**Echocardiographic Measurements**

Echocardiographic measurements in mice were performed using the Mylab30CV (Biosound Esaote Inc.) machine equipped with a 15-MHz probe as we previously described. M-mode tracings derived from the short axis of the left ventricle (LV) at the level of the papillary muscles were recorded to obtain measurements of LV end-diastolic diameter (LVEDd), LV end-systolic diameter (LVESd) and LV fractional shortening [FS(%)=(LVEDd-LVESd)/LVEDd ×100%]. All of the parameters were obtained from at least three beats and then averaged.

Echocardiography measurements in monkeys were performed on a Vivi7 Ultrasonic Doppler System (GE
Healthcare) equipped with an 8-MHz 10S probe (GE Healthcare) by the same sonographer. The animals were chemically restrained using ketamine hydrochloride (10 mg/kg, intramuscularly). M-mode images captured from the left parasternal long axis views were recorded to measure the following parameters: interventricular septal thickness at end-diastole (IVSd), interventricular septal thickness at end-systole (IVSs), left ventricular posterior wall thickness at end-diastole (LVPWd), left ventricular posterior wall thickness at end-systole (LVPWs), left ventricular internal diameter at end-diastole (LVIDd), left ventricular internal diameter at end-systole (LVIDs), ejection fraction (EF%), and fractional shortening (FS%).

**Morphometric Analysis**

Mouse and monkey cardiac arrest was induced with 10% potassium chloride intravenous injection. Then, the hearts were removed, fixed in 10% formalin, and embedded in paraffin after dehydration. Subsequently, heart paraffin blocks were transversely sectioned at 5 µm. Heart sections were subjected to hematoxylin and eosin (H&E) staining for histology, or picrosirius red (PSR) staining for fibrosis assessments. FITC-conjugated wheat germ agglutinin (WGA, Invitrogen) staining was performed to determine the myocyte cross-sectional area. Myocyte cross-sectional area and collagen volume were measured using a quantitative digital image analysis system (Image-Pro Plus 6.0). More than 100 myocytes from 5 mice or 7-8 monkeys per experimental group were traced to determine the myocyte cross-sectional area. At least 50 high-power fields of PSR-stained cardiac slides per experimental group were measured to determine the left ventricle collagen volume.

**Primary Neonatal Rat Cardiomyocyte (NRCM) Culture, Virus Transfection and Immunofluorescence**

NRCMs were prepared and cultured as previously described. Briefly, NRCMs were obtained from the 1- to 2-day-old Sprague-Dawley rat hearts and seeded at a density of 1×10^6 cells/well into gelatin-coated six-well culture plates. Replication-defective adenoviral vectors were used to overexpress EPI64C (referred to as AdEpi64c) under the control of the CMV promoter, and a similar adenoviral vector expressing GFP was used as a control. To knockdown EPI64C expression, four lines of rat EPI64C short hairpin RNA lentiviral particles (TL704019V) were purchased from Origene Technologies, and one line that produced the most significant reduction in EPI64C levels was selected for in vitro experiments (designated as Lenti-shEpi64c). Scramble Lenti-shRNA was used as a non-targeting control. NRCMs were infected with adenovirus at a multiplicity of infection (MOI) of 100 or lentivirus at a MOI of 10 for 24 hours. Then, DMEM/F12 medium supplemented with 15% fetal calf serum (FCS, Hyclone), 0.1 mM BrdU (to inhibit the proliferation of fibroblasts), and 1% penicillin/streptomycin was used to culture the NRCMs for 48 h. Afterward, the culture medium was replaced with DMEM/F12 medium containing 1% FCS for 12 hours prior to stimulation with Ang II (1 µmol/L) for 48 h.

The cells cultured on cover slips were subjected to immunofluorescence staining after Ang II stimulation. To quench the GFP signal, the cardiomyocytes were fixed in prewarmed (37°C) 100% methanol for 20 min at room temperature. After three washes, the cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 40 min and stained with α-actinin antibody (Abcam, ab9465, 1:100 dilution) using standard immunofluorescence staining techniques.

**Quantitative Real-Time PCR**

Total mRNA were extracted from cultured cells or left ventricles using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). The relative mRNA levels of the indicated genes were determined by quantitative real-time PCR using SYBR green (Roche), and the results were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The
primers used were as follows:

rat ANP forward: 5'-AAAGCAAACTGAGGCTCTGCTCG-3';
rat ANP reverse: 5'-TTCCGGTACCAGGCTCTGGCA-3';
rat β-MHC forward: 5'-TCTGGGACAGCTCCCATTCT-3';
rat β-MHC reverse: 5'-CAAGGCTAACCTGGAGAGATG-3';
mouse ANP forward: 5'-ACCACCTGGAGGAGAAGA-3';
mouse ANP reverse: 5'-TTCAGAGGGCAGATCTATC-3';
mouse BNP forward: 5'-GAGGTCACTCCTATCCTCG-3';
mouse BNP reverse: 5'-GCCATTTCCCTCCAGCTTTTC-3';
mouse β-MHC forward: 5'-CCGAGTCCCAGGTCAACAA-3';
mouse β-MHC reverse: 5'-CTTCACGGGCACCCTGGGA-3';
mouse collagen I forward: 5'-TGGTACATCAGCCCGAAC-3';
mouse collagen I reverse: 5'-GTCAGCTGGATAGCGACA-3';
mouse collagen III forward: 5'-CCCAACCCAGAGATCCCATT-3';
mouse collagen III reverse: 5'-GAAGCACAGGAGCAGGTGTAGA-3';
mouse CTGF forward: 5'-TGACCCCTGCGACCCACA-3';
mouse CTGF reverse: 5'-TACACCGACCACCGAAGACACAG-3'

Western Blot Assay

Total protein from the left ventricles or cultured cells was extracted using RIPA lysis buffer. Enriched fractions of cytoplasmic and nuclear proteins were extracted using differential centrifugation as previously described. Protein was loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Invitrogen), transferred to polyvinylidene fluoride membranes (Millipore) and subsequently probed with the indicated primary antibodies at 4°C overnight. The membranes were then incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature. Prior to visualization using the ChemiDoc™ XRS+ system (Bio-Rad), the membranes were incubated in ECL reagents (Bio-Rad). Specific protein expression levels were normalized to GAPDH for total cell and cytoplasmic lysates or to Lamin B for the nuclear proteins.

The following primary antibodies were used in this study: antibodies against EPI64C (ab77625) and PPP3R1 (ab154650) were obtained from Abcam; antibodies against ANP (sc-20158), β-MHC (sc-53090), NFATc3 (sc-8405) and Lamin B (sc6217) were purchased from Santa Cruz Biotechnology; antibody against p-NFATc3 (GTK52339) were provided by GeneTex; antibodies against GAPDH (MB001) were ordered from Bioworld Technology; antibodies against Flag (F3165) were purchased from Sigma-Aldrich; antibodies against Myc (11814150001) were ordered from Roche; antibodies to detect ERK1/2 (#4695), phospho-ERK1/2 (#4370), MEK1/2 (#9122), phosphor-MEK1/2 (#9154), JNK1/2 (#9258), phosphor-JNK1/2 (#4668), p38 (#9212), and phospho-p38 (#4511) were purchased from Cell Signaling Technology.

Co-Immunoprecipitation (Co-IP)

HEK293T cells were co-transfected with plasmids encoding the indicated proteins and then collected and sonicated in an immunoprecipitation (IP) buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA and 0.5% NP-40) supplemented with a protease inhibitor cocktail (Roche). After incubation for 20 min at 4°C, the homogenized cell suspension was centrifuged at 13,000 g for 15 min. To avoid non-specific binding of irrelevant proteins, the supernatant was pre-cleared with normal mouse or rabbit immunoglobulin G and protein A/G-agarose beads (11719394001, 11719386001, Roche) for 3 h at 4°C. After centrifugation, the pre-cleared lysates (500 μl) were incubated with 1 μg of indicated antibody and 10 μl of protein A/G-agarose beads on a
rocking platform at 4°C overnight. Then, the immunocomplexes were collected and washed six times using cold IP buffer and eluted from agarose beads. Finally, eluted proteins were immunoblotted using the indicated primary antibodies.

**Glutathione-S-transferase (GST) pull-down assay**
The GST-fused full-length and truncated forms of EPI64C and calcineurin were expressed in Rosetta (DE3) *Escherichia coli* and purified after being immobilized on Glutathione-Sepharose 4B beads (GE Healthcare). Then, protein-bound beads were incubated with Myc-Calcineurin- or Flag-EPI64C- expressed HEK293T cell lysates in IP buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA and 0.5% NP-40 supplemented with protease inhibitor cocktail) for 4 h at 4°C. The beads were then washed four times with IP lysis buffer in the absence of protease inhibitor cocktail. Finally, the bound proteins on the beads were eluted, resolved by SDS-PAGE and analyzed by Western blotting.

**Active Ras Detection**
The GTP-bound active form of Ras (Ras-GTP) in left ventricle lysates was determined using the Active Ras Pull-Down and Detection Kit (ThermoFisher) according to the manufacturer’s instructions. Briefly, ventricle lysates were incubated with GST-Raf1-RBD and immobilized glutathione resin for 1 h at 4°C. Then, the protein-bound resin was washed three times and eluted with SDS loading buffer. The eluted protein was further separated by SDS-PAGE and analyzed for the protein level of Ras using Western blotting.

**Calcineurin Activity Assay**
Calcineurin phosphatase activity was measured using the Cellular Calcineurin Phosphatase Activity Assay Kit (Abcam) according to the manufacturer’s instruction. Briefly, left ventricular tissues were lysed and desalted to prepare the test sample. Then, the total phosphatase activity was measured at OD620 nm and calculated against a phosphate (PO₄) standard curve. Calcineurin activity was determined by subtracting the “EGTA buffer”-sensitive phosphatase activity from the total phosphatase activity.

**Luciferase Assay**
The luciferase reporter assay was performed as we previously described. Briefly, the lentivirus encoding a NFAT-responsive sequence followed by a firefly luciferase reporter gene was ordered from QIAGEN (Lenti-NFAT-luc, CLS-015L). The Lenti-NFAT-luc was used to transfect NRCMs in combination with AdEpi64c or Lenti-shEpi64c and their corresponding control virus for 24 h as described above. Cells were then stimulated with Ang II for 24h, harvested, washed for three times and lysed in passive lysis buffer (Promega). After centrifugation, the supernatant was used for the luciferase assay with a GloMax® 20/20 Luminometer (Promega).

**References:**


### Supplementary Tables

**Table S1.** Echocardiographic measurements of each lentiviral vector (Lenti-Vector) transfected Cynomolgus monkey before and after aortic banding surgery.

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Pre-AB, pre-aortic banding; POD, postoperative day; IVSd, interventricular septal thickness at end-diastole; IVSs, interventricular septal thickness at end-systole; LVPWd; left ventricular posterior wall thickness at end-diastole; LVPWs; left ventricular posterior wall thickness at end-systole; LVIDd, left ventricular internal diameter at end-diastole; LVIDs, left ventricular internal diameter at end-systole; EF, ejection fraction; FS, fractional shortening.
### Table S2. Echocardiographic measurements of each lentiviral Epi64c (Lenti-Epi64c) transfected Cynomolgus monkey before and after aortic banding surgery.

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Pre-AB, pre-aortic banding; POD, postoperative day; IVSd, interventricular septal thickness at end-diastole; IVSs, interventricular septal thickness at end-systole, LVPWd; left ventricular posterior wall thickness at end-diastole, LVPWs; left ventricular posterior wall thickness at end-systole; LVIDd, left ventricular internal diameter at end-diastole; LVIDs, left ventricular internal diameter at end-systole; EF, ejection fraction; FS, fractional shortening.
Supplementary Figures

**Supplemental Figure 1**

**Figure S1.** EPI64C expression levels in Neonatal rat cardiomyocytes (NRCMs) were knocked down or overexpressed by lentiviral short hairpin EPI64C (Lenti-shEpi64c) and adenoviral *Epi64c* (AdEpi64c) transfection, respectively. Protein levels were verified by Western blot analysis after transfection (representative of two independent experiments) and normalized against GAPDH (n=2; *P*<0.05 vs. Lenti-shRNA, #P<0.05 vs. AdGfp).
Figure S2. EPI64C protein levels in the heart lysates derived from the non-transgenic mice (NTG) and four independent Epi64c transgenic (Epi64c-TG) lines were determined by Western blot analysis (representative of two independent experiments). Relatively expression levels were normalized against GAPDH (n=2-4).
**Supplemental Figure 3**

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<td>NTG Epi64c-TG</td>
</tr>
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</tr>
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<td>GAPDH</td>
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**Figure S3.** Altered EPI64C expression have no impact on the activation of mitogen-activated protein kinases (MAPKs) under pressure overload. A and B. The Epi64cΔ/Δ, Epi64c-TG and corresponding control mice were stressed with aortic banding (AB) or sham surgery. Protein levels of phosphorylated mitogen-activated protein kinase kinase 1/2 (p-MEK1/2), total MEK1/2 (t-MEK1/2), phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2), total ERK1/2 (t-ERK1/2), phosphorylated c-Jun N-terminal kinase 1/2 (p-JNK1/2), total JNK1/2 (t-JNK1/2), phosphorylated p38 (p-p38), and total p38 (t-p38) in the left ventricle (LV) lysates of mice from the indicated groups. Shown are representative results of two independent experiments.
Supplemental Figure 4

**Figure S4.** EPI64C inhibits nuclear factor of activated T-cells c3 (NFATc3) nuclear translocation. A and B, Neonatal rat cardiomyocytes (NRCMs) were transfected with the indicated virus and stimulated with Ang II for 2 h. Protein levels of phosphorylated nuclear factor of activated T-cells c3 (p-NFATc3) and total NFATc3 in the cytoplasm of NRCMs were determined by Western blot analysis and normalized against GAPDH (n=4). C and D, Protein levels of NFATc3 in the nucleus of the indicated NRCMs were assayed by Western blotting and normalized against Lamin B (n=4). *P<0.05 vs. Lenti-shRNA or AdGfp PBS; #P<0.05 vs. Lenti-shRNA or AdGfp Ang II.
**Figure S5.** Protein levels of EPI64C<sup>R141A</sup> in the heart lysates of non-transgenic mice (NTG) and two independent Epi64c<sup>R141A</sup>-TG lines were determined by Western blot analysis (n=2-4) and normalized against GAPDH. Shown are representative of two independent experiments.
**Figure S6.** The $\Delta$EPI64C-Flag expression levels in the hearts of different transgenic lines and non-transgenic (NTG) mice were measured by Western blot analysis ($n=2-4$) and normalized against GAPDH. Shown are representative of two independent experiments.
**Figure S7.** Ppp3r1 knockout reverses the detrimental effects of Epi64c deficiency in pressure overload-induced cardiac hypertrophy. **A.** The breeding strategy for producing cardiac-specific Ppp3r1 knockout (Ppp3r1Δ/Δ) and Epi64c;Ppp3r1 double knockout (DKO) mice. The structures of the floxed genes and their recombined alleles are shown. **B.** Representative Western blot of EPI64C and PPP3R1 in the hearts of αMHC-MCM, Epi64cΔ/Δ, Ppp3r1Δ/Δ, and DKO mice. The results are representative of two independent experiments. **C-E.** Statistical results for the ratios of (C) heart weight (HW)/body weight (BW), (D) HW/tibia length (TL), and (E) lung weight (LW)/BW in the indicated groups at 8 weeks after aortic banding (AB) or sham surgery (n=10-13). **F-H.** Statistical results of echocardiographic parameters in the indicated groups (n=5-6). LVEDd: left ventricular end-diastolic diameter; LVESd: left ventricular end-systolic diameter; FS: fractional shortening. **I.** The gross hearts (first panel) and histological analysis of the hematoxylin and eosin (H&E)-stained (second panel) and the picrosirius red-stained (third and fourth panels) cardiac sections (scale bar: 50 μm). **J-K.** The statistical results for (J) cardiomyocyte cross-sectional area and (K) the left ventricle (LV) collagen volume measured in the indicated groups. **L.** The relative mRNA levels of atrial natriuretic peptide (ANP) and β-myosin heavy chain (β-MHC) in the LV of mice from the indicated groups (n=4). *P<0.05 vs. Epi64cΔ/Δ AB; n.s. indicates not statistically significant.
Supplemental Figure 8

Figure S8. A-C, Surgical illustrations of (A) lentivirus transfection and (B) supravalvar AB surgery performed in monkeys during thoracic open surgery. Image (C) shows the heart and aorta after the operation and before sternal closure. ① The needle used for lentivirus transfusion; ② the clamp used for cross-clamping the supravalvar aorta; ③ the right ventricle; ④ the clamp used for cross-clamping the inferior vena cava; ⑤ the left ventricle; ⑥ the aortic band; ⑦ the bent stainless steel stick used for aortic banding; and ⑧ The supravalvar aorta.
Figure S9. Echocardiographic measurements in lentiviral vector (Lenti-Vector) and lentiviral Epi64c (Lenti-Epi64c) transfected Cynomolgus monkeys before and after aortic banding surgery. A, Echocardiographic measurements of left ventricular chamber dimensions. LVIDd, left ventricular internal diameter at end-diastole; LVIDs, left ventricular internal diameter at end-systole. B and C, Echocardiographic measurements of cardiac contractile functions. (B) Ejection fraction (EF%), and (C) fractional shortening (FS%). Pre-AB, pre-aortic banding; POD, postoperative day; n.s. indicates not statistically significant.
Figure S10. Statistical results for the ratios of lung weight (LW)/ body weight (BW) in the indicated groups of Cynomolgus monkeys at 70 days after lentiviral Epi64c (Lenti-Epi64c) or lentiviral vector (Lenti-Vector) transfection and aortic banding surgery (n=9). n.s. indicates not statistically significant.