Caspase Recruitment Domain 6 Protects Against Cardiac Hypertrophy in Response to Pressure Overload

Liangpeng Li, Wen Chen, Yifan Zhu, Xiaodi Wang, Ding-Sheng Jiang, Fuhua Huang, Liming Wang, Fei Xiang, Wei Qin, Qiang Wang, Rui Zhang, Xueyong Zhu, Hongliang Li, Xin Chen

Abstract—Caspase recruitment domain 6 (CARD6), a crucial member of the CARD family, was initially shown to be involved in the immune system and oncogenesis. However, the role of CARD6 in chronic pressure overload–induced cardiac hypertrophy remains unexplored. To evaluate the impact of CARD6 on pathological cardiac hypertrophy, cardiac-specific CARD6 knockout mice and transgenic mice with cardiac-specific CARD6 overexpression were generated and subjected to aortic banding for 4 weeks. Our results demonstrated that CARD6-deficient mice aggravated aortic banding–triggered cardiac hypertrophy, ventricular dilation, fibrosis, and dysfunction, as measured by echocardiography, immunostaining, and molecular/biochemical analyses. Conversely, CARD6-overexpressing mice exhibited an attenuated hypertrophic response to chronic pressure overload. Similarly, using cultured neonatal rat cardiomyocytes, we found that adenovirus vector–driven overexpression of CARD6 dramatically limited angiotensin II–induced myocyte hypertrophy, whereas knockdown of CARD6 by AdshCARD6 (adenoviral short hairpin CARD6) exhibited the opposite phenotypes. Furthermore, analysis of the signaling events in vitro and in vivo revealed that CARD6-mediated protection against cardiac hypertrophy was attributed to the interruption of mitogen-activated protein kinase kinase (MEK) kinase–dependent MEK-extracellular signal-regulated protein kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase 1/2 (JNK1/2) activation. Altogether, these data indicated that CARD6 serves as a novel cardioprotective factor via negative regulation of MEK kinase–dependent MEK-ERK1/2 and JNK1/2 signaling. Thus, our study suggests that CARD6 may be a novel target for the treatment of pathological cardiac hypertrophy and failure.

Key Words: CARD6 protein, human ▪ cardiomegaly ▪ MEKK1 protein kinase ▪ signal transduction

Cardiac hypertrophy, an early milestone during the clinical course of heart failure, increases the risk of cardiac morbidity and mortality.1,2 In fact, clinical and epidemiological studies have identified that cardiac hypertrophy is an important independent risk factor for the development of heart failure and malignant arrhythmia.3 Initially, cardiac hypertrophy arises as an adaptive response to various common disease stimuli, for example, longstanding hypertension, valvular insufficiency, valvular stenosis, myocardial infarction, and coronary artery disease. However, sustained or excessive cardiac hypertrophy can progress to contractile dysfunction and cardiac decompensation, eventually leading to heart failure, arrhythmia, and sudden death.3 Recently, the multiple signaling mechanisms that control cardiomyocyte growth have been studied extensively, but the molecular mechanisms that mediate the development of cardiac hypertrophy and the transition to heart failure remain incompletely understood.

Caspase recruitment domain 6 (CARD6), also known as a microtubule-interacting protein, belongs to the CARD family.5 CARD6 expression can be induced by interferon in a variety of cell types, including T lymphocytes, B lymphocytes, monocytes, and splenocytes.8 Once expressed, CARD6 either transmits or amplifies the intracellular signals to downstream effector molecules, which activate or suppress intracellular signal pathways to regulate biological processes.9,10 Previous studies have implicated CARD6 as a tumor activator gene in gastric, colorectal, and esophageal cancers.8 In addition, CARD6 regulates the innate and adaptive immune responses, and it plays critical roles in infectious diseases.8 Interestingly, an increasing number of studies has supported the involvement of immune and tumor modulatory factors in modulating cardiac hypertrophy, such as signal regulatory protein-α, interferon regulatory factor 7, Ras-association domain family 1 isoform A, and A20.9,11 These functional impacts of modulatory factors on cardiac hypertrophy and pathological remodeling suggest that CARD6 may exert prominent regulatory effects on cardiac pathoogy. Notably, CARD6 is expressed ubiquitously in various tissues including the heart. However, to the best of our knowledge, the role of CARD6 in cardiac hypertrophy has not been studied yet. Therefore, it will be interesting to determine...
the consequences of ablation and overexpression of CARD6 in the heart after chronic aortic banding (AB).

In the present study, our findings indicated that CARD6 was strikingly altered in human dilated cardiomyopathic hearts and in pressure overload–induced hypertrophic mouse hearts. Moreover, we found that disruption of CARD6 accelerated pressure overload–triggered cardiac hypertrophy, fibrosis, and dysfunction. In contrast, cardiac-specific overexpression of CARD6 significantly mitigated hypertrophic responses. Importantly, inactivation of MEK kinase-1 (MEKK1) was required for CARD6-mediated cardioprotection through suppressing its downstream signaling, including MEK-ERK1/2 and JNK1/2. Our results indicate that CARD6 functions as a novel negative modulator of pathological cardiac hypertrophy via regulation of MEKK1-dependent MEK-ERK1/2 and JNK1/2 signaling.

Materials and Methods
The animal protocol was approved by the Animal Care and Use Committee of Nanjing Medical University. A detailed Methods section is available in the online-only Data Supplement, which includes detailed methods on the following: Reagents, Human Heart Samples, Animal Models and Procedures, Echocardiography Evaluation, Histological Analysis, Quantitative Real-Time Polymerase Chain Reaction (PCR) and Western Blotting, Cultured Neonatal Rat Cardiomyocytes (NRCMs) and Recombinant Adenoviral Vectors, and Statistical Analysis.

Results
CARD6 Expression in Failing Human Hearts and Hypertrophic Mouse Hearts
To investigate the potential role of CARD6 in the development of heart failure, we first analyzed whether expression levels of CARD6 were altered in pathological hypertrophic hearts. Both real-time PCR and Western blot assays revealed that the mRNA and protein expression levels of CARD6 were significantly increased by ≈110% in failing dilated cardiomyopathic hearts (n=7) compared with normal donor hearts (n=5; Figure S1A in the online-only Data Supplement; Figure 1A). In addition, the upregulation of CARD6 was correlated with the reinduction of a fetal gene profile in the ventricular myocardium, including atrial natriuretic peptide (ANP), brain natriuretic peptide, and β-myosin heavy chain (β-MHC; Figure S1B). Similarly, the expression levels of cardiac hypertrophy markers (ie, ANP, brain natriuretic peptide, and β-MHC) were dramatically elevated at week 2 and more pronounced from 4 to 8 weeks after AB (Figure S1D). Compared with the sham-operated hearts, the expression levels of CARD6 were significantly increased in the experimental mouse hearts subjected to AB from weeks 2 to 4 and then markedly reduced at week 8 (Figure 1B; Figure S1C; P<0.05 versus sham). Furthermore, after treating cultured NRCMs with angiotensin II (Ang II; 1 μmol/L) for 24, 48, and 72 hours to induce hypertrophy in vitro, we found that the protein expression levels of CARD6 were significantly decreased at 72 hours, but its expression was gradually increased in cardiomyocytes at 24 and 48 hours after Ang II stimulation (Figure 1C; P<0.05 versus PBS). Taken together, the altered pattern of CARD6 expression levels in human failing hearts, hypertrophic mouse hearts, and cardiomyocytes treated with Ang II in vitro suggested that CARD6 may be involved in cardiac hypertrophy and heart failure.

Figure 1. Caspase recruitment domain 6 (CARD6) expression in failing human hearts and hypertrophic mouse hearts. A, Representative Western blot analysis of CARD6 protein expression in normal donor hearts (n=5) and hearts collected from human patients with dilated cardiomyopathy (DCM; n=7, n indicates the number of samples per experimental group; *P<0.05 vs donor hearts). B, Western blot analysis of CARD6 in an experimental mouse model after aortic banding at the indicated time points (n=4 mice per experimental group; *P<0.05 vs sham). C, Western blot analysis of CARD6 in cultured neonatal rat cardiomyocytes treated with angiotensin II (Ang II; 1 μmol/L) for 24, 48, and 72 hours (n=4 independent experiments; *P<0.05 vs PBS). Left, Representative blots. Right, Bar graphs of the quantitative results. The data are presented as the mean±SEM and are representative of ≥3 independent experiments.
CARD6 Negatively Regulates Ang II–Induced Cardiomyocyte Hypertrophy In vitro

Considering the alterations of CARD6 expression in response to hypertrophic stimuli, we sought to determine whether CARD6 could regulate the progression of cardiac hypertrophy. To test this hypothesis, we performed gain-of-function and loss-of-function studies in cultured NRCMs. The levels of CARD6 were either reduced by infection of cardiomyocytes with AdshCARD6 or elevated by infection with adenoviral CARD6 (AdCARD6) (Figure S2). The cells were subsequently treated with either Ang II (1 μmol/L) or PBS (control) for 48 hours, followed by immunostaining with α-actinin to measure the size of the cells. Notably, under basal conditions (PBS), knockdown (AdshCARD6) and overexpression (AdCARD6) of CARD6 did not alter cardiomyocyte morphology or cell size compared with AdshRNA or AdGFP (adenoviral green fluorescent protein) transfection. However, in response to Ang II–triggered cell hypertrophy, CARD6-deficient cells showed remarkably increased cell surface area (3314±82 versus 4993±76 μm²; P<0.05; Figure 2A and 2B) compared with AdshRNA-infected controls. Conversely, Ang II–induced cell hypertrophy was reduced significantly in CARD6-overexpressing cardiomyocytes (3187±54 versus 2492±60 μm²; P<0.05; Figure 2A and 2C). Accordingly, the mRNA expression of hypertrophy markers (ANP and β-MHC) was enhanced dramatically in AdshCARD6-injected cardiomyocytes (Figure 2D), but the expression of these markers was suppressed remarkably in AdCARD6-infected cells (Figure 2E) compared with controls. These in vitro data confirmed that upregulation of CARD6 alleviates cardiac hypertrophy, whereas downregulation of CARD6 promotes pathological cardiac hypertrophy.

Cardiac-Specific Knockout of CARD6 Aggravates Cardiac Hypertrophy and Failure Induced by Pressure Overload

To examine the cardiac-specific effects of CARD6 during hypertrophy in vivo, we used a tamoxifen-inducible α-MHC-Cre system to delete CARD6 (knockout) in the hearts of adult mice...
(Figure S3A and S3B). It is important to note that at baseline, the cardiac-specific CARD6 knockout (cCARD6-KO) mice were viable, fertile, and had no pathological alterations in their heart morphology or contractile function (data not shown). However, 4 weeks after AB, CARD6-deficient hearts exhibited significant hypertrophic deterioration, as indicated by a greater gross heart size (Figure 3A), increased cardiomyocyte cross-sectional area (wheat germ agglutinin staining; Figure 3A and 3B), and higher ratios of heart weight (HW)/body weight (BW), lung weight/BW, and HW/tibia length (Figure 3C) compared with AB-treated CARD6-Flox hearts. Accordingly, the mRNA expression levels of hypertrophic markers, including ANP, brain natriuretic peptide, and β-MHC in hearts from cCARD6-KO mice were higher than those in CARD6-Flox mice after a 4-week course of AB (Figure S3C). Similar increases were also observed in the left ventricular (LV) chamber dimension and wall thickness, as assessed by echocardiographic parameters (LV end-diastolic dimension: CARD6-Flox/4.43±0.06 versus cCARD6-KO/5.32±0.21; P<0.05 and LV end-systolic dimension: CARD6-Flox/2.93±0.04 versus cCARD6-KO/4.07±0.21; P<0.05; Figure 3D; Table S1). Moreover, LV contraction was significantly aggravated in cCARD6-KO hearts (fractional shortening: cCARD6-KO/23%) compared with that in CARD6-FloX hearts (CARD6-Flox/34%; P<0.05; Figure 3D).

To further define the effects of CARD6 deficiency on maladaptive cardiac remodeling, we evaluated myocardial fibrosis, another hallmark of pathological cardiac hypertrophy. The extent of fibrosis was quantified by collagen volume through the visualization of the total amount of collagen present in the interstitial and perivascular spaces of the heart. Our results revealed that both interstitial and perivascular fibrosis, as measured by picrosirius red staining, were dramatically enhanced in cCARD6-KO hearts subjected to chronic AB, but they were markedly limited in CARD6-Flox hearts (Figure 3E). In addition, quantitative analysis showed a significantly increased collagen volume in cCARD6-KO hearts compared with CARD6-Flox hearts (Figure 3F). Finally, we examined the synthesis of collagen by analyzing the expression of mRNAs encoding collagen I, collagen III, and connective tissue growth factor (Figure S3D), which consistently indicated an increased fibrotic response in knockout mice. Overall, these in vivo findings indicated that absence of CARD6 in the heart aggravates cardiac hypertrophy and sensitizes mice to hypertrophic stimuli and associated derangements, such as fibrosis.

**Overexpression of CARD6 Attenuates AB-Induced Cardiac Remodeling**

Subsequently, we sought to determine whether CARD6 overexpression in the heart could prevent the progression of cardiac hypertrophy in vivo. To address this concern, we generated a transgenic mouse line carrying the full-length murine CARD6 cDNA under the control of the α-MHC promoter (Figure S4A). In total, 4 independent lines of transgenic mice (transgenic-2, transgenic-5, transgenic-6, and transgenic-8) were confirmed by Western blot analysis (Figure S4B). Under basal conditions, all the lines were healthy and exhibited no apparent morphological or pathological cardiac abnormalities (data not shown). Western blotting showed a spectrum of CARD6 overexpression ranging from moderate overexpression in the heart could prevent the progression of cardiac hypertrophy in vivo. To address this concern, we generated a transgenic mouse line carrying the full-length murine CARD6 cDNA under the control of the α-MHC promoter (Figure S4A). In total, 4 independent lines of transgenic mice (transgenic-2, transgenic-5, transgenic-6, and transgenic-8) were confirmed by Western blot analysis (Figure S4B). Under basal conditions, all the lines were healthy and exhibited no apparent morphological or pathological cardiac abnormalities (data not shown). Western blotting showed a spectrum of CARD6 overexpression ranging from moderate overexpression in the heart could prevent the progression of cardiac hypertrophy.
expression in the heart (transgenic-8) was selected for the subsequent experiments (Figure S4C). To determine the effect of increased CARD6 expression in hearts on chronic pressure overload, transgenic mice and their nontransgenic littermates were subjected to AB for 4 weeks. In contrast to the results observed in cCARD6-KO mice, overexpression of CARD6 significantly mitigated the pathological cardiac hypertrophy induced by chronic AB, as evidenced by a reduction in the cross-sectional area of cardiomyocytes (Figure 4A and 4B) and hypertrophy markers (ANP, brain natriuretic peptide, and β-MHC; Figure S4D) in transgenic mice compared with nontransgenic mice. In parallel, after 4 weeks of AB, the HW/BW, lung weight/BW, and HW/tibia length ratios were significantly decreased in transgenic mice compared with nontransgenic mice (Figure 4C). In accordance with these results, myocardial contractile function, as measured by echocardiography analyses, were greatly improved in transgenic mice compared with nontransgenic mice (Figure S4E; Table S2). Picrosirius red staining revealed that the fibrosis present in both interstitial and perivascular spaces was significantly reduced in transgenic mice compared with nontransgenic mice in response to AB (Figure 4D and 4E). Subsequent analysis demonstrated that the expression levels of fibrosis markers (collagen I, collagen III, and connective tissue growth factor) were consistently decreased in transgenic mice compared with those in nontransgenic mice (Figure S4F).

Considering that diverse hypertrophic stimuli can act on different receptors and induce different molecular responses,1,2 we then performed in vivo studies using Ang II infusion, a classic method for inducing hypertrophy. Consistent with the results observed during AB, the ratios of HW/BW, lung weight/BW, and HW/tibia length were dramatically lower in transgenic mice compared with nontransgenic mice after 4 weeks of Ang II infusion (2.0 mg/kg per day; Figure S5A).
In addition, compared with nontransgenic mice, transgenic mice exhibited more blunted hypertrophic responses to Ang II, as indicated by a smaller ventricular cross-sectional area and less cardiac fibrosis (Figure S5B–S5D). Accordingly, we assessed the cardiac structure and function (LV end-diastolic dimension, LV end-systolic dimension, and percentage of fractional shortening) of nontransgenic and transgenic mice. As expected, after 4 weeks of Ang II infusion, transgenic mice exhibited a mild ventricular dilatation and cardiac dysfunction compared with nontransgenic mice (Figure S5E). Together, these in vivo gain-of-function data supported the notion that CARD6 may mediate stress-dependent pathological cardiac hypertrophy and is generally required to protect the heart from cardiac remodeling.

CARD6 Inhibits Pressure Overload–Mediated MEK-ERK1/2 and JNK1/2 Signaling Pathway

The present results demonstrated that CARD6 plays a protective role in cardiac hypertrophy. However, the molecular mechanisms through which the upregulation and downregulation of CARD6 in hearts affect the hypertrophic responses on stress stimuli remain unknown. To elucidate these possible mechanisms, we next investigated the expression and activity of mitogen-activated protein kinase (MAPK) signaling molecules (ie, MEK1/2, ERK1/2, JNK1/2, and P38), as the MAPK pathway is known to play a crucial role in pathological cardiac hypertrophy.\(^{17}\) The expression levels of phosphorylated MEK1/2, ERK1/2, and JNK1/2 were greatly increased in cCARD6-KO hearts compared with CARD6-Flox hearts after 4 weeks of AB (Figure 5A). In contrast, CARD6 overexpression reduced AB-triggered activation of MEK1/2, ERK1/2, and JNK1/2, as evidenced by remarkably decreased phosphorylation of MEK1/2, ERK1/2, and JNK1/2 in transgenic hearts relative to control hearts (Figure 5B). Unexpectedly, under pressure overload, neither the overexpression nor the ablation of CARD6 in mouse hearts altered the expression level of phosphorylated P38 (Figure 5A and 5B). To further validate these results, we increased or decreased CARD6 expression in vitro by infecting cultured NRCMs with either AdshCARD6 or AdCARD6, followed by treatment with 1 \(\mu\)mol/L Ang II or PBS control for 48 hours. Western blotting revealed that in response to Ang II, knockdown of CARD6 in NRCMs markedly enhanced MEK1/2, ERK1/2, and JNK1/2 phosphorylation compared with control AdshRNA cells (Figure S6A). Conversely, the phosphorylation of MEK1/2, ERK1/2, and JNK1/2 was suppressed in the CARD6 overexpression compared with that observed in the control cells (Figure S6B). These results suggested that CARD6 may exert cardioprotective effects via negative regulation of MEK-ERK1/2 and JNK1/2 signaling.

CARD6-Mediated Antihypertrophic Effects Mainly Impair MEKK1 Signaling Pathway

Considering that activation of MEKK1, an upstream regulator of ERK1/2 and JNK1/2, has been shown to participate in cardiac

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**Figure 5.** Caspase recruitment domain 6 (CARD6) suppresses mitogen-activated protein kinase (MEK)-extracellular signal-regulated protein kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase 1/2 (JNK1/2) signaling pathway in vivo. **A**, Representative Western blots and quantitative results showing the phosphorylation and total protein levels of MEK1/2, ERK1/2, JNK1/2, and P38 in CARD6-Flox and cardiac-specific CARD6 knockout (cCARD6-KO) mice subjected to aortic banding (AB) or sham surgery for 4 weeks (n=5 mice per experimental group; \(^*P<0.05\) vs CARD6-Flox/Sham; \(^{#}P<0.05\) vs CARD6-Flox/AB). **B**, Representative Western blots and quantitative results of MEK1/2, ERK1/2, and P38 phosphorylation as well as total protein levels in hearts from nontransgenic (NTG) and transgenic (TG) mice at 4 weeks after AB surgery (n=5 mice per experimental group; \(^*P<0.05\) vs NTG/sham; \(^{#}P<0.05\) vs NTG/AB). GAPDH was used as a loading control. **Top**, Representative blots. **Bottom**, Bar graphs of the quantitative results.
hypertrophy, we investigated the activation of MEKK1 in our hypertrophic models. Our experiments clearly revealed that the expression level of phosphorylated MEKK1 was significantly increased in the AB-induced hypertrophic hearts of cCARD6-KO mice (Figure 6A). In contrast, phosphorylated MEKK1 was remarkably reduced in transgenic hearts in response to AB (Figure 6B). However, the total MEKK1 protein level did not differ among the tested groups (Figure 6A and 6B). Thus, we deduced that CARD6 may modulate cardiac hypertrophy through the activation of MEKK1. To further confirm our hypothesis, the in vitro data also demonstrated that CARD6 upregulation by infection with AdCARD6 remarkably limited Ang II–induced MEKK1 activation (Figure S7B); in sharp contrast, MEKK1 activation was aggravated largely by the expression of AdshCARD6 (Figure S7A). Collectively, both in vivo and in vitro data suggested that the protective role of CARD6 in pathological cardiac hypertrophy is dependent, at least partially, on the inactivation of MEKK1.

**Discussion**

In the present study, we elucidated the role of CARD6 in pressure overload–induced cardiac remodeling using loss-of-function and gain-of-function approaches. Our data revealed that cardiac-specific overexpression of CARD6 protected hearts against maladaptive hypertrophy, dilation, and fibrosis in response to chronic pressure overloading, whereas loss of CARD6 exhibited an exaggerated response of pathological cardiac remodeling. Mechanistically, we demonstrated that after AB, CARD6 exerted an antihypertrophic effect through repression of its target MEKK1, thereby suppressing downstream MEK-ERK1/2 and JNK 1/2 signaling, as summarized in Figure S8. Thus, we provide the first evidence indicating that CARD6 plays a critical role in attenuating pressure overload–induced cardiac remodeling.

CARD6, a microtubule-interacting protein, is structurally and functionally related to the superfamily of interferon-inducible GTPases, a group that comprises host defense proteins, which mediate cell-autonomous immunity. CARD6 itself is rapidly induced by interferon-β and interferon-γ in bone marrow–derived macrophages. Notably, CARD6 is expressed ubiquitously in various tissues including the heart, but it is unclear whether its expression levels are altered under pathological stimulus. In the present study, we found that in response to hypertrophic stress, CARD6 expression was increased progressively in adaptive hypertrophy (the first 2 and 4 weeks after hypertrophy stimuli) and then markedly decreased in maladaptive cardiac remodeling (the past 4 weeks). However, CARD6 exhibited a decrease during decompensated heart failure. The limitation of this study was that the mechanisms regulating CARD6 expression pattern were not clarified. However, a mechanism can be hypothesized based on the studies of other negative regulators of cardiac hypertrophy. Several endogenous factors, for example, inducible cAMP early repressor, interferon regulatory factor 3, calcineurin inhibitor 1, and suppressor of cytokine signaling 3, display increased expression in hypertrophic hearts.

The results of these studies were similar to our CARD6 finding, and this increased expression may be a compensatory response to cope with the detrimental effects of hypertrophy. These negative regulators of hypertrophy are relatively low under basal conditions but upregulated in response to hypertrophic stimuli, and they counteract the adverse effect on cardiac hypertrophy. Another mechanism may also contribute to the increased CARD6 level, which may include an endogenous feedback loop related to CARD6. Consistent with our results, the increased level of CARD6 induced by hypertrophic stress may in turn inhibit the activation of upstream pathways, which may eventually induce the downregulation of CARD6 expression during maladaptive cardiac remodeling. More importantly, this conclusion was supported by our data revealing that CARD6 deficiency accelerated the hypertrophic response of mouse heart to AB but that the overexpression of CARD6 displayed the opposite response. However, further studies are required to validate this hypothesis.

![Figure 6](image-url) Vue 6. Caspase recruitment domain 6 (CARD6) impairs mitogen-activated protein kinase kinase (MEK) kinase-1 (MEKK1) signaling involved in hypertrophy. **A**. Representative Western blots and quantitative results showing the phosphorylation and total protein levels of MEKK1 in CARD6-Flox and cardiac-specific CARD6 knockout (cCARD6-KO) mice at 4 weeks after aortic banding (AB) or sham operation (n=5 mice per experimental group; *P<0.05 vs CARD6-Flox/sham; #P<0.05 vs CARD6-Flox/AB). **B**. Representative Western blots and quantitative results of MEKK1 phosphorylation as well as total protein levels in nontransgenic (NTG) and transgenic (TG) mice at 4 weeks after AB surgery (n=5 mice per experimental group; *P<0.05 vs NTG/sham; #P<0.05 vs NTG/AB). GAPDH was used as a loading control. **Top**, Representative blots. **Bottom**, Bar graphs of the quantitative results.
The mechanism underlying the antihypertrophic effect of CARD6 remains largely unclear. Considerable evidence exists to indicate that the activation of the MAPK signaling pathway contributes to the pathogenesis of cardiac hypertrophy. This pathway is composed of a sequence of branches of serine/threonine kinases, including P38, JNKs, and ERks, along with the upstream activating kinases MAP2K and MAP3K, which collectively mediate signaling for diverse stress stimuli in cardiomyocytes. To clarify the molecular mechanisms involved in the suppressive role of CARD6 in cardiac hypertrophy, we evaluated the activation status of the MAPK pathway in our hypertrophic models. We found that MEK1/2, ERK1/2, and JNK1/2 activation was greatly enhanced by CARD6 deficiency, but almost completely blocked by the cardiac-specific overexpression of CARD6 in response to chronic pressure overload or Ang II stimulation. However, genetic manipulation of CARD6 did not have any impact on P38 protein expression in our hypertrophic models. Therefore, it is conceivable that CARD6 exerts its antihypertrophic effects through inhibition of MEK-ERK1/2 and JNK1/2 signaling.

Given that various molecules are components of \( \geq 2 \) pathways within the MAPK cascades, there is potential for synergistic or antagonistic cross-talk. For example, ERK1/2 and JNK1/2 cooperate in the differentiation of PC12 cells. Waetzig et al. reported that ERK1/2 and JNK1/2 cascades signal in parallel to induce and control neurite formation and that their actions converge only at the level of upstream target proteins. In this study, we tested whether CARD6 functioned through similar mechanisms to regulate cardioprotective effects. Importanty, previous studies have reported that expression of the catalytic domain of MEKK1 preferentially activates JNK1/2, more subtly influences ERK1/2, and has little or no effect on P38. When overexpressed, MEKK1 activates the ERK1/2 and JNK1/2 pathways. Indeed, the results presented in this study revealed that MEKK1 activity, a major upstream regulator of ERK1/2 and JNK1/2, was significantly enhanced in AB-treated CARD6 hearts, which was in agreement with previous reports, thus favoring a role for MEKK1 in the development of cardiac hypertrophy. Thus, we think that CARD6 may negatively regulate cardiac hypertrophy by inhibiting MEKK1-dependent MEK-ERK1/2 and JNK1/2 activation. Based on our research, we cannot explicitly state that MEKK1 directly or indirectly regulated by CARD6. Further studies are needed to precisely determine this interaction in heart.

Cardiomyocyte enlargement is a feature of both physiological and pathological hypertrophy, but fibrosis is a classical feature of pathological hypertrophy. Under pressure overload, cardiac fibrosis is associated with increased collagen accumulation within the adventitia of the coronary arteries (perivascular fibrosis), which progressively extends into the neighboring interstitial spaces (interstitial fibrosis). In the present study, we demonstrated that CARD6 blocked cardiac fibrosis and reduced the expression of several fibrotic mediators induced by chronic pressure overload. However, the mechanism by which CARD6 restrains cardiac fibrosis remains elusive. Previous studies have indicated that activation of the ERK1/2 and JNK1/2 signaling pathways by various stimuli is correlated with several cellular processes, such as cell proliferation and remodeling of the extracellular matrix.

Moreover, pharmacological inhibition of the MEK-ERK1/2 and JNK1/2 signaling pathways could have a beneficial effect on cardiac function by acting directly to decrease the proliferation of myocardial fibroblasts. Thus, in the current study, the blockade of MEK-ERK1/2 and JNK1/2 signaling as a consequence of CARD6 overexpression likely contributed, at least in part, to reduce the degree of cardiac fibrosis observed in response to pressure overload.

In conclusion, our present work provides in vivo and in vitro evidence to support the concept that CARD6 protects against pressure overload–induced cardiac hypertrophy and failure through negative regulation of MEKK1-dependent MEK-ERK1/2 and JNK1/2 signaling pathway. These findings suggest that CARD6 may be a novel therapeutic target for the prevention of pathological cardiac hypertrophy.

**Perspectives**

The current study demonstrates that the essential role of CARD6 under stressful conditions is to negatively regulate the hypertrophic response in the heart. The underlying mechanisms of the protective role of CARD6 in the development of cardiac hypertrophy seem to involve the inhibition of MEKK1-dependent MEK-ERK1/2 and JNK1/2 signaling pathways. These findings will provide potential therapeutic targets for cardiac hypertrophy and heart failure.

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**Disclosures**

None.

**References**


CARD6 Negatively Regulates Cardiac Hypertrophy

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Novelty and Significance

What Is New?

- Caspase recruitment domain 6 (CARD6) is significantly altered in both failing human hearts and hypertrophic mouse hearts.
- CARD6 is a cardioprotective factor that attenuates aortic banding-induced cardiac hypertrophy, fibrosis, and dysfunction in vivo, as well as angiotensin II–induced cardiomyocyte hypertrophy in vitro.
- CARD6 negatively regulates MEK kinase-1–dependent MEK-ERK1/2 and JNK1/2 signaling activation in the development of cardiac hypertrophy and heart failure.

What Is Relevant?

- Many hypertrophic activators have been investigated, but relatively little is known about the negative modulators of cardiac hypertrophy.
- CARD6 is structurally and functionally related to the superfamily of interferon-inducible GTPases, a group that is composed of host defense proteins, which mediate cell-autonomous immunity. However, it is unclear whether CARD6 induces defensive responses to hypertrophic stresses such as biomechanical stress and neurohumoral factors.
- This study reveals a previously unrecognized role for CARD6 in the hypertrophic response and also suggests a novel interaction between MEK kinase-1 signaling and CARD6, thereby providing new insights into the molecular basis of cardiac remodeling.

Summary

The salient finding of the current study is that the absence of CARD6 in the heart profoundly aggravates aortic banding or angiotensin II–induced hypertrophy, chamber dilatation, and fibrosis via enhancement of MEK kinase-1–dependent MEK-ERK1/2 and JNK1/2 signaling pathways. Conversely, overexpression of CARD6 in transgenic mice blunts pathological hypertrophic response. These observations suggest that CARD6 may be a novel therapeutic target for cardiac hypertrophy and heart failure.
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Caspase Recruitment Domain 6 Protects Against Cardiac Hypertrophy In Response to Pressure Overload

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Running title: CARD6 negatively regulates cardiac hypertrophy

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Materials and Methods

Reagents
Antibodies against the following proteins were purchased from Cell Signaling Technology: phospho-MEK1/2 (#9154), total-MEK1/2 (#9122), phospho-ERK1/2 (#4370), total-ERK1/2 (#4695), phospho-JNK1/2 (#4668), total-JNK1/2 (#9258), phospho-P38 (#4511), and total-P38 (#9212). The antibodies against CARD6 (#sc49403), phospho-MEKK1 (#sc13202), and total-MEKK1 (#sc252) were purchased from Santa Cruz Inc. The GAPDH (#MB001) antibody was purchased from Bioworld Technology. The BCA protein assay kit was purchased from Pierce. We used a FluorChem E imager (ProteinSimple, FluorChem E) for visualization. Fetal calf serum (FCS) was purchased from Hyclone. Cell culture reagents and all other reagents were purchased from Sigma.

Human ventricular samples
All the procedures involving human samples conformed to the principles outlined in the Declaration of Helsinki and were approved by the Ethics Committee at Nanjing Medical University. The samples of failing human hearts were collected from the left ventricles (LV) of dilated cardiomyopathy (DCM) patients undergoing heart transplants. The control samples were obtained from normal heart donors who had died from head trauma or intracranial bleeds and who had hearts that were unsuitable for transplantation for technical reasons. Written informed consent was obtained from each DCM patient undergoing transplantation and from the families of the prospective heart donors.

Experimental animal models
All the experiments involving animals were approved by the Animal Care and Use Committee of Nanjing Medical University. The conditional CARD6β/β mice (C57BL/6 background) were generously provided by Dr. Tak W. Mak.1 Cardiac-specific CARD6-knockout mice were generated by mating α-MHC-MerCreMer transgenic mice (MEM-Cre-Tg (Myh6-cre/Esr1, Jackson Laboratory, 005650)) with CARD6β/β mice. Cre-mediated recombination of floxed alleles were induced by intraperitoneal injection of 80 mg/kg/day of an emulsion containing 200 mg of tamoxifen (Sigma, T-5648) dissolved in 1 ml of ethanol and
added to 9 ml of corn oil (Sigma, C-8267) to attain a final tamoxifen concentration of 20 mg/ml for 5 consecutive days (Supplement Figure S3A).

To generate CARD6 transgenic mice, a full-length mouse CARD6 cDNA was cloned downstream of the cardiac α-myosin heavy chain (α-MHC) promoter (Supplement Figure S4A). This α-MHC-CARD6 construct was microinjected into fertilized murine embryos (C57BL/6J background), and the transgenic mice were genotyped by PCR using tail genomic DNA and the primers 5’-ATCTCCCCATAAGAGTTTGAGTC-3’ (forward) and 5’-GGCCACTTTCCCTTG TAGGTGA-3’ (reverse). Four independent transgenic lines were established and studied.

**Animal surgery**

Male mice ages 8-10 weeks (with body weights of 24-27 g) were used in aortic banding (AB) surgeries, and all experiments and subsequent analyses were conducted in a blinded fashion. In preparation for aortic banding, the animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p., Sigma). When the toe-pin reflex was absent, the left chest of each animal was opened to identify the thoracic aorta by blunt dissection at the second intercostal space. Partial aortic constriction with 7-0 silk sutures was performed by ligating the descending aorta (thoracic aorta) with a 26/27-gauge needle. The needle was removed before closing the thoracic cavity. Adequate constriction of the aorta was induced and confirmed by Doppler echocardiogram. Sham-operated animals underwent the same surgical procedure without AB. The internal diameter and wall thickness of the left ventricle (LV) were assessed by echocardiography at the indicated times after surgery. The hearts, lungs, and tibiae of the sacrificed mice were collected, and the ratios of heart weight (HW)/body weight (BW) (mg/g), HW/tibial length (TL) (mg/mm), and lung weight (LW)/BW (mg/g) were measured. For Ang II infusion model, mice were anaesthetized with 1.5-2% isoflurane to implant osmotic minipumps (Alzet model 2004; Alza Corp.). After loading with Ang II (Sigma), osmotic minipumps were primed at 37°C in normal saline overnight, and then implanted subcutaneously in the dorsal region of mice to obtain a delivery rate of 2.0 mg/kg/day over the course of 4 weeks. Osmotic minipumps containing saline solution were implanted in the control mice.

**Echocardiography measurements**
Echocardiographic measurements were performed on anesthetized (1.5-2% isoflurane) mice using a MyLab 30CV ultrasound (Biosound Esaote Inc.) with a 15-MHz probe. The LV was assessed in both parasternal long-axis and short-axis views at a frame rate of 120 Hz. End-systole and end-diastole were defined as the phases in which the areas of the LV were the smallest and largest, respectively. The LV end-systolic diameter (LVESD), LV end-diastolic diameter (LVEDD), and posterior wall end-diastolic thickness (PWT) were measured from the LV M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level. The percentage of fractional shortening (FS) was calculated as (LVEDD-LVESD)/LVEDD×100. Echocardiographic measurements were taken in M-mode in triplicate from more than 4 individual mice per group.

**Quantitative real-time PCR and western blotting**

For real-time PCR, total RNA was extracted from frozen cardiac tissue and cultured cardiomyocytes using TRIzol reagent (Invitrogen) and reverse-transcribed into cDNA using oligo (dT) primers with the Transcriptor First Strand cDNA Synthesis Kit (Roche). PCR amplifications were quantified using the SYBR Green PCR Master Mix (Applied Biosystems), and the results were normalized against GAPDH gene expression. The primers for real-time PCR are shown in Supplement Table S3.

Total proteins extracted from left ventricle tissue and cultured cardiomyocytes were first lysed in RIPA lysis buffer, and the protein concentrations were measured using the Pierce® BCA Protein Assay Kit (Pierce). The protein extract (50μg) was run on 8%–12% SDS-PAGE gels (Invitrogen) and transferred to PVDF membranes (Millipore). The membranes were blocked in TBST containing 5% skim milk powder for 1 h at room temperature and incubated with various primary antibodies overnight at 4°C. After incubation with secondary antibodies for 1 h at room temperature, the membranes were treated with ECL reagents (170-5061, Bio-Rad) prior to visualization using a FluorChem E imager (ProteinSimple, FluorChem E) according to the manufacturer’s instructions. The specific protein expression levels were normalized to GAPDH present on the same nitrocellulose membrane.

**Histological analysis**

Hearts were fixed in 4% paraformaldehyde, and embedded in paraffin using standard histological procedures. Subsequently, 5-μm-thick sections obtained at the
mid-papillary muscle level of each heart were stained with hematoxylin-eosin (H&E) for histopathology or with picrosirius red (PSR) to examine collagen deposition. To determine the cross sectional area (CSA) of the myocytes, sections were stained with FITC-conjugated wheat germ agglutinin (WGA, Invitrogen Corp) to identify membranes and with DAPI to identify nuclei. Single myocyte and fibrillar collagen were visualized by microscopy and measured with a quantitative digital analysis imaging system (Image-Pro Plus 6.0). More than 100 myocytes in the sections were outlined in each group.

**Cultured neonatal rat cardiomyocytes (NRCMs) and recombinant adenoviral vectors**

To overexpress CARD6, the entire coding region of the rat CARD6 gene under the control of the cytomegalovirus promoter was inserted in replication-defective adenoviral vectors. A similar adenoviral vector encoding the GFP gene was used as a control. To knock down CARD6 expression, three rat shCARD6 constructs were obtained from SABiosciences (KR55501G). Subsequently, we generated three AdshCARD6 adenoviruses, and the construct that decreased CARD6 levels to the greatest extent was selected for further experiments. AdshRNA was used as the non-targeting control. NCRMs were infected with AdshRNA, AdshCARD6, AdGFP, and AdCARD6 in diluted media at a multiplicity of infection (MOI) of 10 for 24 hours.

Primary cultures of neonatal rat cardiomyocytes (NRCMs) were performed according to a previously described protocol with minor revisions. Briefly, 1- to 2-day-old Sprague-Dawley rats were sacrificed by swift decapitation according to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. Cardiomyocytes were then isolated from the hearts of these animals, and seeded into six-well culture plates at a density of $3 \times 10^5$ cells/well in DMEM/F12 medium supplemented with 10% FCS, BrdU (0.1 mM, inhibits the proliferation of fibroblasts), and penicillin/streptomycin. After 48 hours, the culture medium was replaced with serum-free DMEM/F12 for 12 hours prior to stimulation with angiotensin II (Ang II, 1 µmol/L).

**Statistical analysis**

The data are presented as the mean ± SEM. Differences among groups were
determined by a two-way ANOVA followed by Tukey’s post hoc test. Comparisons between two groups were performed using an unpaired Student’s $t$ test. The significance level was set at $P<0.05$. 
Reference


## Supplemental Tables

**Table S1.** Echocardiographic and anatomic evaluation in control (CARD6-Flox) and cardiac-specific CARD6 knockout (cCARD6-KO) mice at 4 weeks after sham or aortic banding (AB) operation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>AB</th>
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</thead>
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<tr>
<td></td>
<td>CARD6-Flox</td>
<td>CARD6-Flox</td>
</tr>
<tr>
<td>Number</td>
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<td>11</td>
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<tr>
<td>BW (g)</td>
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<td>HW/BW (mg/g)</td>
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<td>4.99±0.17</td>
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<td>HR (beats/min)</td>
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<td>IVSD (mm)</td>
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<td>LVEDD (mm)</td>
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<td>LVPWD (mm)</td>
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<td>0.66±0.02</td>
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<td>IVSS (mm)</td>
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<td>1.02±0.02</td>
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<td>LVESD (mm)</td>
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<td>LVPWS (mm)</td>
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<td>FS (%)</td>
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<tr>
<td>LVEF (%)</td>
<td>83.1±0.7</td>
<td>80.8±0.8</td>
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BW=body weight; HW=heart weight; LW=lung weight; TL=tibia length; HR=heart rate; IVSD=left ventricular wall thickness at end diastole; LVEDD=left ventricular end-diastolic dimension; LVPWD=left ventricular posterior wall thickness at end diastole; IVSS=ventricular septum wall thickness at end systole; LVESD=left ventricular end-systolic dimension; LVPWS=left ventricular posterior wall thickness at end systole; FS=fractional shortening; LVEF=left ventricular ejection fraction.

*P<0.05 versus CARD6-Flox sham operation; †P<0.05 versus CARD6-Flox AB after 4 weeks.

All values are presented as means ± SEM.
Table S2. Echocardiographic and anatomic evaluation in transgenic (TG) mice and their non-transgenic (NTG) littermates at 4 weeks after sham or AB operation.

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<tr>
<th>Parameter</th>
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<th>NTG AB</th>
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<td>10</td>
<td>12</td>
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<td>BW(g)</td>
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<td>HW/BW(mg/g)</td>
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<td>7.12±0.21*</td>
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<td>LW/BW(mg/g)</td>
<td>4.76±0.18</td>
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<td>HW/TL(mg/mm)</td>
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<td>HR(beats/min)</td>
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<td>IVSD(mm)</td>
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<td>LVPWD(mm)</td>
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<td>FS(%)</td>
<td>43.3±0.6</td>
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<td>29.2±0.9*</td>
<td>36.9±0.4*†</td>
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<td>LVEF(%)</td>
<td>80.5±0.7</td>
<td>82.2±0.3</td>
<td>63.0±1.3*</td>
<td>73.7±0.5†*</td>
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*P<0.05 versus NTG sham operation; †P<0.05 versus NTG AB after 4 weeks.

All values are means ± SEM.
Table S3. The primers for RT-PCR.

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<th>Primer name</th>
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<td>BNP-Mouse</td>
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<td>III-Mouse</td>
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Supplementary Figures

Figure S1 CARD6 expression in failing human hearts and hypertrophic mouse hearts. A and B, Real-time PCR analyses of CARD6, ANP, BNP, and β-MHC mRNA levels in the left ventricles of normal donor hearts (n=4) and human dilated cardiomyopathic (DCM) patients (n=5). n indicates the number of samples per experimental group, *P<0.05 vs. normal donors. C and D, Real-time PCR analyses of CARD6, ANP, BNP, and β-MHC mRNA levels in an experimental mouse model with aortic banding (AB)-induced cardiac hypertrophy at the indicated time points (n=4 mice per experimental group, *P<0.05 vs. Sham).
Figure S2 CARD6 negatively regulates angiotensin II-induced cardiomyocyte hypertrophy *in vitro*. The protein expression level of CARD6 after infection with AdshCARD6, AdCARD6, or their respective controls (AdshRNA and AdGFP) was analyzed by western blotting (n=4 independent experiments). **Left**, Representative blots. **Right**, Quantitative results.
**Figure S3** Absence of CARD6 aggravates pressure overload-induced cardiac hypertrophy and failure. A, Schematic representation of the generation of cardiac-specific CARD6-knockout (cCARD6-KO) mice. B, Representative western blots of CARD6 expression in heart tissues from cardiac-specific CARD6 knockout (cCARD6-KO) and littermate control (CARD6-Flox) mice (n=4 mice per experimental group). C, Real-time polymerase chain reaction (RT-PCR) analysis of hypertrophic markers (atrial natriuretic peptide [ANP], brain natriuretic peptide [BNP], and β-myosin heavy chain [MHC]) induced by AB at 4 weeks in the indicated groups (n=4 mice per experimental group). D, Real-time PCR analyses of the fibrotic markers (collagen I, collagen III, and connective tissue growth factor [CTGF]) in the indicated groups (n=4 mice per experimental group). *P<0.05 vs. CARD6-Flox/Sham; #P<0.05 vs. CARD6-Flox/AB.
Figure S4 Overexpression of CARD6 blunts the development of cardiac hypertrophy and failure induced by pressure overload. A, Schematic diagram of the construction of transgenic (TG) mice with a full-length murine CARD6 cDNA under the control of the α-myosin heavy chain (MHC) promoter. B, Representative western blots from 4 lines of both TG and their non-transgenic (NTG) littermate mice for determination of CARD6 expression in heart tissues (n=3 independent experiments). C, Quantitation of the CARD6 protein level expressed in heart tissue from 4 TG and NTG mouse lines. D, Expression of the hypertrophic markers ANP, BNP, and β-MHC was examined by real-time PCR in NTG and TG mice (n=4 mice per experimental group). E, Echocardiographic results (LVEDD, LVESD, and FS%) for NTG and TG mice (n=8 to 12 mice per experimental group). F, Real-time PCR analyses of fibrotic markers (collagen I, collagen III, and CTGF) in the indicated groups (n=4 mice per experimental group). *P<0.05 vs. NTG/Sham; #P<0.05 vs. NTG/AB.
Figure S5 Overexpression of CARD6 attenuates Ang II-induced cardiac hypertrophy. **A**, The heart weight (HW)/body weight (BW), lung weight (LW)/body weight (BW), and HW/tibia length (TL) ratios in NTG and TG mice after saline or Ang II infusion (2 mg/kg/day for 4 weeks, n=12 mice per experimental group). **B**, Histological analysis of the hearts sections from NTG and TG mice treated with saline or Ang II (n=4 to 6 mice per experimental group). H&E staining (top row: scale bars represent 500 μm; second row: scale bars represent 50 μm); PSR staining (third and fourth rows: scale bars represent 50 μm). **C**, Quantification of cardiomyocyte cross-sectional area (CSA) in saline or Ang II-induced NTG and TG mice hearts (n=100+ cells per experimental group). **D**, Quantification of the total collagen volume in the indicated groups (n=30+ fields per experimental group). **E**, Measurements of echocardiographic parameters (LVEDD, LVESD, and FS%) in the indicated groups (n=6 to 10 mice per experimental group). *P<0.05 vs. NTG/Saline; #P<0.05 vs. NTG/Ang II.
Figure S6 CARD6 suppresses MEK-ERK1/2 and JNK1/2 signaling pathway in vitro. A, Representative western blots and quantitative results showing the phosphorylation and total protein levels of MEK1/2, ERK1/2, and JNK1/2 after infection of cardiomyocytes with AdshCARD6 or AdshRNA treated with PBS or angiotensin II (Ang II; 1 μmol/L) for 48 hours (n=3 independent experiments; *P<0.05 vs. AdshRNA/PBS, #P<0.05 vs. AdshRNA/Ang II). B, Representative blots and quantitative results of the MEK1/2, ERK1/2, and JNK1/2 phosphorylation as well as total protein levels in AdCARD6 or AdGFP adenoviral vector-infected cardiomyocytes after treatment with PBS or Ang II (1 μmol/L) for 48 hours (n=3 independent experiments; *P<0.05 vs. AdGFP/PBS, #P<0.05 vs. AdGFP/Ang II). GAPDH was used as a loading control. Top, Representative blots. Bottom, Bar graphs are quantitative results.
Figure S7 CARD6 impairs MEKK1 signaling involved in hypertrophy. A, Representative western blots and quantitative results showing the phosphorylation and total protein levels of MEKK1 after infection of cardiomyocytes with AdshCARD6 or AdshRNA treated with PBS or Ang II (1 μmol/L) for 48 hours (n=3 independent experiments; *P<0.05 vs. AdshRNA/PBS, #P<0.05 vs. AdshRNA/Ang II). B, Representative blots and quantitative results of MEKK1 phosphorylation as well as total protein levels in AdCARD6 or AdGFP adenoviral vector-infected cardiomyocytes after treatment with PBS or Ang II (1 μmol/L) for 48 hours (n=3 independent experiments; *P<0.05 vs. AdGFP/PBS, #P<0.05 vs. AdGFP/Ang II). GAPDH was used as a loading control. Top, Representative blots. Bottom, Bar graphs are quantitative results.
Figure S8 Proposed model for the anti-hypertrophic process involving CARD6 in the heart. Based on the present study, we propose that CARD6 expression is induced in cardiomyocytes upon stress stimulus. CARD6 functions as a negative regulator of the hypertrophic responses in the heart through suppressing MEKK1-dependent MEK-ERK1/2 and JNK1/2 pathways, thereby reduced expression of a series of cardiac fetal genes, and consequently mitigated cardiac hypertrophy and fibrosis in response to pressure overload.