CKIP-1 Inhibits Cardiac Hypertrophy by Regulating Class II Histone Deacetylase Phosphorylation Through Recruiting PP2A

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despite recent treatment advances, heart failure continues to impose a substantial healthcare burden. One of the major risk factors for developing heart failure is preexisting cardiac hypertrophy with aging and hypersensitivity to pressure overload–induced pathological cardiac hypertrophy, as well. Transgenic mice with cardiac-specific overexpression of CKIP-1 showed resistance to cardiac hypertrophy in response to pressure overload. The results of GST pull-down and coimmunoprecipitation assays showed the interaction between CKIP-1 and histone deacetylase 4 (HDAC4), through which they synergistically inhibited transcriptional activity of myocyte-specific enhancer factor 2C. By directly interacting with the catalytic subunit of phosphatase 2A, CKIP-1 overexpression enhanced the binding of catalytic subunit of phosphatase-2A to HDAC4 and promoted HDAC4 dephosphorylation.

Conclusions—CKIP-1 was found to be an inhibitor of cardiac hypertrophy by upregulating the dephosphorylation of HDAC4 through the recruitment of protein phosphatase 2A. These results demonstrated a unique function of CKIP-1, by which it suppresses cardiac hypertrophy through its capacity to regulate HDAC4 dephosphorylation and fetal cardiac genes expression. (Circulation. 2012;126:3028-3040.)

Key Words: hypertrophy • molecular biology • cardiomyopathy • heart failure

Received February 29, 2012; accepted November 1, 2012.
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The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.112.102780/-DC1.

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Circulation is available at http://circ.ahajournals.org DOI: 10.1161/CIRCULATIONAHA.112.102780

Clinical Perspective on p 3040

CKIP-1 was first identified as an interactive protein of casein kinase 2a and was implicated in tumor cell proliferation, muscle cell differentiation, cell apoptosis, the regula-

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tion of cell morphology, and the actin cytoskeleton.\textsuperscript{14–21} We previously showed that CKIP-1–deficient mice displayed increased osteoblast activity and accelerated bone formation.\textsuperscript{22} However, the physiological role of CKIP-1 in the adult heart is unknown. In this study, we identified CKIP-1 as a novel regulator of class II HDAC-MEF2 pathway and cardiac hypertrophy. CKIP-1–deficient mice exhibited spontaneous cardiac hypertrophy with aging and sensitivity to pressure overload–induced cardiac hypertrophy. Hearts from CKIP-1 transgenic mice were protected from pressure overload–induced hypertrophy. CKIP-1 repressed the activity of MEF2 via the regulation of HDAC4 phosphorylation and localization. Overexpression of CKIP-1 could enhance the interaction between HDAC4 and PP2A, inhibit HDAC4 phosphorylation levels, and promote HDAC4 translocation from cytoplasm to nucleus. These results indicate that CKIP-1 would be a potential target for treating cardiac hypertrophy and failure.

Methods

Animals

The experimental procedures in mice have been approved by the Animal Care and Use Committee of China Astronaut Research and Training Center. For this study, we used male CKIP-1–deficient, CKIP-1 transgenic mice, and age-matched wild-type (WT) controls. CKIP-1–deficient mice have been reported previously.\textsuperscript{22}

Generation of Myocardial-Specific CKIP-1 Transgenic Mice

A cDNA encoding mouse Flag-tagged CKIP-1 was cloned from the mouse cDNA. The pGALPHA MHC plasmid (gift of Dr J. Robbins) containing the 5.5-kbp mouse \(\alpha\)-myosin heavy chain (\(\alpha\)-MHC) promoter was cut with HindIII. CKIP-1, including its Flag tag, was cut with the same restriction enzymes (HindIII) and cloned within the HindIII ends of the pGALPHA MHC plasmid that contained the 5.5-kbp mouse \(\alpha\)-MHC promoter. For details, see online-only Data Supplement Text.

Transverse Aortic Constriction

See online-only Data Supplement Text.

Histological Analysis

Sections for Masson trichrome staining were generated from paraffin-embedded hearts. Frozen sections were used to visualize cardiomyocyte cell membranes by staining with FITC-conjugated wheat-germ agglutinin (Sigma-Aldrich).

RNA Extraction and Real-Time Polymerase Chain Reaction

Total RNA was extracted from cultured cells or heart tissues by the use of Trizol reagent according to the manufacturer’s protocol. For the details about the Primers (synthesized by Invitrogen, China) for CKIP-1, atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), \(\beta\)-MHC, and GAPDH, please refer to online-only Data Supplement Text.

Plasmids

CKIP-1 plasmids were described previously.\textsuperscript{22} Full-length and truncated of HDACs and 3XMEF2C-luc plasmids were provided by Dr Eric Olson. The pcDNA3.1-MEF-2C plasmid was a gift of Dr Xiaohong Peng.

Immunoprecipitation and Immunoblotting

See online-only Data Supplement Text.

GST Pull-Down and Mass Spectrometry

See online-only Data Supplement Text.

Small Interfering RNA

Small interfering RNAs (siRNAs) targeted to human CKIP-1, HDAC4, PP1A, and PP2AC genes were designed and synthesized by Shanghai GenePharma Co, Ltd.

CKIP-1 siRNA: GUGUGAAGAGCUCGGGAAGT

PP1A siRNA: GAGCGUACAAACAUCAACTUT

PP2AC siRNA: GAGUGAUUCCGUUT

HDAC4 siRNA: GUCCAGGCUAAAGCAGAATT

Reporter Gene Assays

For details, see online-only Data Supplement Text.

Statistical Analysis

Data are presented as mean \(\pm\) SEM per experimental condition unless noted otherwise. Considering the presence of unequal variance for the data, we first test the equality of variances across groups. If it shows that the variances are unequal, we then use mixed model with heterogeneous variances for 2-way analysis or the Welch \(t\) test for 1-way analysis. Otherwise, we use the regular linear model or the Student \(t\) test. For the comparison of the time-series data, we used the mixed model for repeated-measures analysis of variance. A probability value \(<0.05\) was considered statistically significant. A probability value \(<0.01\) was considered very significant. Bonferroni adjustment was used for multiple comparisons. All the statistical tests are analyzed by Prism software (Graphpad prism for windows, version 5.01) and SPSS (Version 14.0). For details, see online-only Data Supplement Text.

Results

Characterization of Myocardial CKIP-1 Expression

CKIP-1 was highly expressed in the heart under physiological conditions (Figure 1A, online-only Data Supplement Figure IA). CKIP-1 mRNA and protein levels were significantly increased in the hearts of mice during the first 2 weeks after transverse aortic constriction (TAC) (Figure 1B and 1C). However, in comparison with the sham control, CKIP-1 levels were much lower in the late phase of cardiac hypertrophy. In contrast, real-time polymerase chain reaction analysis showed that transcripts for the hypertrophic marker genes ANF, brain natriuretic peptide BNP, and \(\beta\)-MHC were constantly increased in the hearts of mice after transverse aortic constriction (TAC) (Figure 1B and 1C). However, in comparison with the sham control, CKIP-1 levels were much lower in the late phase of cardiac hypertrophy. In contrast, real-time polymerase chain reaction analysis showed that transcripts for the hypertrophic marker genes ANF, brain natriuretic peptide BNP, and \(\beta\)-MHC were constantly increased in the development of pressure overload–induced cardiac hypertrophy (Figure 1B and 1C, online-only Data Supplement Figure IB). An analysis of CKIP-1 myocardial expression in vivo by immunohistochemistry also revealed high levels in the early phase and low levels in the late phase of pressure overload–induced cardiac hypertrophy (Figure 1D, online-only Data Supplement Figure IC). CKIP-1 reduction was also observed in the histological sections of hypertrophied failing human hearts, which is consistent with our observations in the late phase of hypertrophied mouse hearts (Figure 1E). CKIP-1 protein levels were also significantly reduced in the hypertrophied failing human hearts (online-only Data Supplement Figure ID). These results suggested that CKIP-1 played an important role in the regulation of cardiac function.

CKIP-1–Deficient Mice Develop Age-Dependent Cardiac Hypertrophy

To address the potential role of CKIP-1 in cardiac hypertrophy, hearts from WT and CKIP-1–deficient (knockout [KO]) mice at different ages were assessed for alterations in morphol-
ogy and function. Histological analysis demonstrated increases in the size of whole hearts and individual cardiomyocytes at both 2 and 8 months of age. Masson trichrome staining revealed the formation of cardiac fibrosis in the hearts of CKIP-1–deficient mice by 8 months of age (Figure 2A).

In comparison with WT mice, the ratios of heart weight to body weight and left ventricular (LV weight to body weight were all significantly increased in CKIP-1–deficient mice at both 2 and 8 months of age (Figure 2B and 2C). At 2 months of age, the results of echocardiography demonstrated that the fractional shortening and ejection fraction of CKIP-1–deficient mice were higher than that of WT mice. However, by 8 months of age, the levels of fractional shortening and ejection fraction were decreased and lower than those of WT mice (Figure 2D and 2E). The basal echocardiographic parameters demonstrated thickening of the LV walls. At 2 months of age, in comparison with WT mice, the left ventricular posterior wall dimensions in diastole (LVPWd) (“0.05) and systole
Figure 2. CKIP-1 deficiency causes cardiac hypertrophy, fetal gene activation, and fibrosis in the heart with age. A, H&E-stained sections of hearts from wild type (WT) and CKIP-1 knockout (KO) mice at 2 and 3 months of age show gross changes of cardiac hypertrophy (2 upper lanes). Sections of hearts are stained with Masson trichrome (MTT) to detect fibrosis (blue). Wheat germ agglutinin (WGA) staining is used to demarcate cell boundaries. Scale bars, 1 mm for the upper lane and 50 μm for the others. Heart weight to body weight ratios (B) and left ventricular (LV) mass to body weight ratios (C) of CKIP-1 KO mice and WT littermates were analyzed at 2 and 8 months of age. Echocardiographic assessment of fractional shortening (FS) (D) and ejection factor (EF) (E) at 2 and 8 months in CKIP-1 KO mice and WT littermates. F, CKIP-1 deficiency leads to fetal cardiac gene induction in vivo. mRNA levels of ANF, BNP, and β-MHC were analyzed by Q-PCR. The samples are from CKIP-1 KO mice and WT littermates at 2 and 8 months. The relative abundances of transcripts were quantified and normalized to GAPDH. The number of mice each group is indicated within the bar. *P<0.05, **P<0.01. Data represent the means±SEM. CKIP-1 indicates casein kinase-2 interacting protein-1; H&E, hematoxylin and eosin; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; β-MHC, β-myosin heavy chain; and Q-PCR, real-time polymerase chain reaction.
(LVPWs) \((P<0.01)\) were increased significantly in CKIP-1–deficient mice, the interventricular septal thicknesses in diastole \((\text{IVSd})\) \((P<0.05)\) and systole \((\text{IVSs})\) \((P<0.05)\) displayed the same trend. At 8 months of age, the LV internal diameter in diastole \((P<0.01)\) and systole \((P<0.01)\) were increased significantly in the hearts of CKIP-1–deficient mice (online-only Data Supplement Table I). At 2 months of age, the expression levels of the heart hypertrophy marker genes ANF, BNP, and \(\beta\)-MHC were much higher in the hearts of CKIP-1–deficient mice than those of WT mice. These differences were further increased by 8 months of age (Figure 2F). All of these results indicated that CKIP-1 deficiency induced age-dependent cardiac hypertrophy.

**CKIP-1–Deficient Mice Are Hypersensitive to Pressure Overload–Induced Cardiac Hypertrophy**

To further investigate the potential role of CKIP-1 as a suppressor of hypertrophy, we compared the responses of WT and CKIP-1–deficient mice to TAC. Hearts from CKIP-1–deficient mice showed an exaggerated response to TAC (Figure 3A through 3C). Notably, the amount of cardiac hypertrophy in CKIP-1–deficient mice after TAC was significantly greater than that of WT mice (heart weight/body weight: \(P<0.01\), LV weight/body weight: \(P<0.01\)) (Figure 3B and 3C). The histological analysis with Masson trichrome staining revealed increased fibrosis in the hearts of CKIP-1–deficient mice in comparison with the fibrosis of WT mice after TAC (Figure 3A). Morphometric quantification of cardiomyocyte cross-sectional area from mouse LV also demonstrated a significant increase in the size of cardiomyocyte in KO mice (online-only Data Supplement Figure II). Real-time polymerase chain reaction analysis showed that transcripts for ANF, BNP, and \(\beta\)-MHC were significantly increased in CKIP-1–deficient mice after TAC (Figure 3D). Echocardiographic analysis revealed the occurrence of hypertrophy in CKIP-1–deficient and WT mice after TAC, as indicated by the increase in the interventricular septal thickness (IVS), LV posterior wall thickness (LVPW), and LV internal diameter, at both 2 and 4 weeks after TAC (Figure 3E). Although significant differences in LVPWd \((P<0.05)\) and IVS \((P<0.05)\) between WT and KO mice exist, we did not observe any obvious difference at 2 weeks after TAC. However, the values of LVPW and IVS in KO mice were significantly higher than these values in WT mice at 4 weeks after TAC (Figure 3E). Although the ejection fraction and fractional shortening values are higher in KO mice than in WT mice, they are significantly lower in KO mice than in WT mice after 4 weeks of TAC \((P<0.05)\). Collectively, these results indicate that CKIP-1–deficient mice were hypersensitive to pressure overload–induced cardiac hypertrophy.

**Myocardial CKIP-1 Overexpression Protects From Pressure Overload–Induced Cardiac Hypertrophy**

To determine whether overexpression of CKIP-1 could protect mice from developing cardiac hypertrophy, we generated transgenic (TG) mice with CKIP-1–specific overexpression in cardiomyocytes by using the \(\alpha\)-MHC promoter (Figure 4A). Two independent lines of transgenic mice (TG1 and TG2) were established. Western blotting results revealed high levels of CKIP-1 expression in these 2 lines (Figure 4B). Real-time polymerase chain reaction analysis showed that CKIP-1 mRNA expression level in the hearts from TG2 mice was 15 times that of WT mice (Figure 4C). Here, offspring of TG2 were used for heart phenotype examination. CKIP-1 transgenic mice developed normally with no obvious phenotype under basal conditions. WT and CKIP-1 TG littermates at 2 months of age underwent TAC surgery for 4 weeks. As predicted, WT mice exhibited marked cardiac hypertrophy, as revealed by size enlargements, large areas of fibrosis, increased heart weight/body weight and LV weight/body weight ratios (Figure 4D through 4F, online-only Data Supplement Figure III). In comparison with WT mice, CKIP-1 TG mice exhibited a decreased response to pressure overload–induced cardiac hypertrophy. The heart weight/body weight ratios of TG mice were significantly decreased after TAC (Figure 4E). Masson trichrome staining revealed less fibrosis in the heart of TG mice (Figure 4D).

Echocardiographic analysis showed significant decreases in ejection fraction and fractional shortening in WT mice after 4 weeks of TAC, whereas the changes in TG mice are not apparent (Figure 4G and 4H). In WT mice, 4 weeks of TAC caused increases in IVSd \((P<0.01)\), IVSs \((P<0.05)\), and LVPWd \((P<0.05)\). This response was markedly reduced in TG mice (online-only Data Supplement Table II). The reactivations of the fetal genes ANF, BNP, and \(\beta\)-MHC were also not as evident in the TG hearts as in the WT hearts (Figure 4I). These results support a critical role for CKIP-1 in the repression of the cardiac hypertrophy response.

**HDAC4 Interacts With CKIP-1 and Mediates Its Repression of MEF2C Transcription Activity**

CKIP-1 deficiency promoted pathological cardiac hypertrophy, and myocardial CKIP-1 overexpression protected from pressure overload–induced cardiac hypertrophy. In an effort to identify the proteins that interact with CKIP-1, we used affinity purification and mass spectrometry to identify the proteins that were associated with CKIP-1. The binding proteins were eluted and resolved by sodium dodecyl sulfate-polyacrylamide electrophoresis. The different protein bands were retrieved and analyzed by mass spectrometry (Figure 5A, online-only Data Supplement Table III). Among the copurified proteins, histone deacetylase 4 (HDAC4) is one of the most important repressors of cardiac hypertrophy.9,23,24 HDAC4 is a member of class IIa histone deacetylases (HDACs).25 Class IIa HDACs can suppress hypertrophic growth of cardiomyocytes via their N-terminal peptide sequence by binding MEF2 when mediating transcriptional repression of hypertrophic genes.4,5,26 The interaction between CKIP-1 and HDAC4 was confirmed by a GST pull-down assay (Figure 5B). Coimmunoprecipitation experiments demonstrated an association between CKIP-1 and HDAC4 in heart tissue and overexpression systems (Figure 5C). A deletion analysis implicated the N-terminal domain region (residues 210–660) of HDAC4 as the interacting domain (online-only Data Supplement Figure IVA). Coimmunoprecipitation experiments also revealed an association between CKIP-1 and class IIa HDACs-HDAC5/9 (online-only Data
**Figure 3.** CKIP-1–deficient mice are hypersensitive to pressure overload–induced cardiac hypertrophy. **A,** CKIP-1–deficient mice and WT littermates at 2 months of age were subjected to transverse aortic banding (TAC) or sham surgery. Histological sections from hearts taken 28 days after TAC or sham were stained with H&E (scale bars, 1 mm) and MTT to detect fibrosis (scale bars, 50 μm). The ratios of heart weight to body weight (B) and left ventricular (LV) mass to body weight (C) in WT and CKIP-1 KO mice subjected to TAC for 4 weeks were analyzed. **D,** mRNA levels of ANF, BNP, and β-MHC were analyzed by Q-PCR from CKIP-1 KO and WT mice at 4 weeks after TAC or sham surgery. The relative abundance of transcripts were quantified and normalized to GAPDH. **E,** Quantitative analysis of the diastolic and systolic left ventricular internal diameter (LVIDd and LVIDs), interventricular septal thickness (IVSd and IVSs), and LV posterior wall thickness (LVPWd and LVPWs) from 6 CKIP-1 KO and 6 WT mice by echocardiography at 2 and 4 weeks after TAC surgery. **F,** Echocardiographic assessment of FS and EF in CKIP-1 KO mice and WT littermates at 2 and 4 weeks after TAC surgery. *P*<0.05; **P**<0.01, ns, no significance (E and F, versus WT). Data represent the means±SEM. CKIP-1 indicates casein kinase-2 interacting protein-1; H&E, hematoxylin and eosin; WT, wild type; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; β-MHC, β-myosin heavy chain; MTT, Masson trichrome staining; Q-PCR, real-time polymerase chain reaction; FS, fractional shortening; and EF, ejection fraction.
Supplement Figure IVB). These results identified CKIP-1 as a class IIa HDAC-interacting partner.

HDAC4 is involved in the regulation of cardiac hypertrophy through the repression of MEF2 transcriptional activity. To measure the impact of CKIP-1 on the HDAC4/MEF2 signaling pathway, HEK293T cells were transfected with a 3XMEF2-luciferase reporter and MEF2C expression plasmids with or without CKIP-1. MEF2-luciferase reporter activity was inhibited by CKIP-1 in a dose-dependent manner (Figure 5D). The inhibitory effect of CKIP-1 on MEF2C activity was significantly decreased by HDAC4 siRNA (Figure 5E). However, CKIP-1 could enhance the repression effect of HDAC4 on MEF2 activity (Figure 5F). CKIP-1 also could regulate the repression effect of HDAC5 on MEF2 activity (online-only Data Supplement Figure IVC). These results demonstrated that CKIP-1 could repress MEF2 activity through its interaction with class IIa HDACs.

CKIP-1 Regulates HDAC4 Localization via Dephosphorylation Through PP2A

It has been shown that HDAC phosphorylation plays an important role in mediating extracellular signals to gene regulation. Serine/threonine phosphorylation controls the function of HDACs directly or indirectly by the achievement of subcellular relocalization of the HDACs. Thus, we investigated whether CKIP-1 could regulate the localization of HDAC4. C2C12 cells were transfected with expression plasmids encoding green fluorescent protein-HDAC4 and red fluorescent protein-CKIP-1 either separately or together.
Figure 4. Myocardial CKIP-1 overexpression protects from pressure overload-induced cardiac hypertrophy. A, Schematic representation of the transgenic construct used to generate CKIP-1 transgenic mouse lines. B, CKIP-1 protein levels in WT and 2 CKIP-1 transgenic mouse lines at 8 weeks were assessed with both anti-Flag and anti-CKIP antibodies. C, The expression levels of CKIP-1 and GAPDH (control) mRNA in hearts of 8-week-old WT and CKIP-1 transgenic mice were analyzed by Q-PCR. D, CKIP-1 transgenic mice and WT littermates at 2 months of age were subjected to TAC for 28 days. Gross changes in the hearts are shown in the upper 2 lanes (scale bars, 1 mm). Representative heart sections were stained with H&E and MTT at 4 weeks after TAC surgery (scale bars, 50 μm). The ratios of heart weight to body weight (E) and left ventricular (LV) mass to body weight (F) were analyzed 4 weeks after TAC in 2-month-old WT and CKIP-1 transgenic mice. Echocardiographic assessment of EF (G) and FS (H) 4 weeks after TAC in 2-month-old WT and CKIP-1 transgenic mice. I, CKIP-1 overexpression in the heart inhibits fetal cardiac gene expression in vivo after TAC surgery. mRNA levels of ANF, BNP, and β-MHC from CKIP-1 TG mice and WT littermates were analyzed by Q-PCR. The relative abundances of transcripts were quantified and normalized to GAPDH. *P<0.05, **P<0.01. Data represent the means±SEM. CKIP-1 indicates casein kinase-2 interacting protein-1; H&E, hematoxylin and eosin; TAC, transverse aortic constriction; WT, wild type; TG, transgenic; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; β-MHC, β-myosin heavy chain; MTT, Masson trichrome staining; Q-PCR, real-time polymerase chain reaction; FS, fractional shortening; and EF, ejection fraction.
the cytoplasm, whereas simultaneous overexpression of CKIP-1 with HDAC4 increased HDAC4 nuclear accumulation (Figure 6A). These results indicated that CKIP-1 overexpression in C2C12 cells promoted the translocation of HDAC4 from the cytoplasm to the nucleus. This effect was further confirmed in vivo by immunohistochemical analysis of HDAC4 localization in heart cryosections from CKIP-1–deficient and CKIP-1 TG mice. Consistent with the cellular

Figure 5. HDAC4 interacts with CKIP-1 and mediates its repression on MEF2C transcriptional activity. A, Identification of HDAC4 as a CKIP-1 interacting protein by mass spectrometry. GST and a GST-CKIP fusion protein were conjugated to glutathione agarose beads. Cellular extracts from HEK293T cells were incubated with the beads, and the binding proteins were eluted and resolved by sodium dodecyl sulfate-polyacrylamide electrophoresis. The different protein bands were retrieved and analyzed by mass spectrometry. B, CKIP-1 interacts with HDAC4 directly. Cellular extracts from HEK293T cells were pulled down with GST or GST-CKIP-1 conjugated to glutathione agarose beads (top, Coomassie blue staining) and then analyzed by Western blot with anti-HDAC4 antibody. C, The interaction between HDAC4 and CKIP-1. HDAC4 was coimmunoprecipitated with CKIP-1 in heart tissues. Heart lysate was immunoprecipitated by HDAC4 antibody, anti-HA antibody was used as negative control. CKIP-1 precipitates were analyzed by Western blot with CKIP-1 antibody (left). 293T cells transfected with Myc-CKIP-1 and Flag-HDAC4. CKIP-1 proteins were immunoprecipitated with anti-Myc antibody, anti-HA antibody was used as negative control. Whole cell lysates and anti-Myc precipitates were analyzed by Western blot with anti-Myc or anti-HDAC4 antibody (middle). HDAC4 proteins were immunoprecipitated with anti-HDAC4 antibody, followed by Western blot analysis (right). D, MEF2C-dependent luciferase assay in 293T cells transfected with CKIP-1, MEF2C expression plasmid, and the 3XMEF2-luciferase reporters. Values are expressed as relative luciferase activity normalized to cotransfected renilla luciferase activity and represent the means ± SD of 3 separate experiments. E, HDAC4 knockdown decreased the inhibition effect of CKIP-1 on MEF2C activity. 293T cells were transfected with HDAC4 siRNA or a negative control and were then transfected with CKIP-1 or a control vector along with 3XMEF2-luciferase reporters. F, The effect of HDAC4 on MEF2C activity was promoted by CKIP-1. 293T cells were transfected with CKIP-1, HDAC4, or both. MEF2C activity was assayed as previously described. **P < 0.01. Data represent the means ± SEM. CKIP-1 indicates casein kinase-2 interacting protein-1; HDAC4, histone deacetylase 4; MEF2C, myocyte-specific enhancer factor 2C; GST, Glutathione S-Transferase; HA, hemaglutinin; and siRNA, small interfering RNA.
Figure 6. CKIP-1 regulates HDAC4 localization via dephosphorylation through PP2A. A, CKIP-1 promotes HDAC4 nuclear translocation in vitro. C2C12 cells were transfected with expression plasmids encoding green fluorescent protein-HDAC4 or red fluorescent protein-CKIP-1 (100 ng) either separately or together. After 36 hours, nuclei were stained with DAPI (blue) followed by confocal fluorescent microscopy (scale bars, 10 μm). B, Immunohistochemical analysis of HDAC4 (green) localization in heart cryosections from CKIP-1 KO and TG mice and WT littermates. CKIP-1 was red. DAPI (blue) was used to mark nuclei (scale bars, 10 μm). C, Western blot analysis of the HDAC4 protein and its phosphorylation levels in C2C12 cells after transfection with Flag-CKIP-1 or its control. D, Western blot analysis of HDAC4 protein expression and phosphorylation in the hearts of WT, KO, and TG mice. E, Western blot analysis of HDAC4 phosphorylation in 293T cells overexpressed with CKIP-1 with or without the treatment of the protein serine/threonine phosphatase inhibitor-okadaic acid. F, The effect of CKIP-1 overexpression on HDAC4 phosphorylation under PP1A knockdown. G, The effect of CKIP-1 overexpression on HDAC4 phosphorylation under PP2AC knockdown. H, GST pull-down assay of the interaction between CKIP-1 and PP2AC. Cellular extracts from 293T cells were pulled down by GST or GST-CKIP-1 conjugated to glutathione agarose beads followed by Western blotting with anti-PP2AC antibody. PP2AC was coimmunoprecipitated with CKIP-1 in 293T cells. 293T cells were transfected with Flag–CKIP-1 or empty vector. CKIP-1 was immunoprecipitated with Flag antibody, followed by PP2AC detection with anti-PP2AC antibody. I, The effect of CKIP-1 on the interaction between HDAC4 and PP2AC. Heart lysate from WT, KO, and TG mice was immunoprecipitated by HDAC4 antibody, followed by PP2AC detection with anti-PP2AC antibody. J, Model of CKIP-1.
experiment, increases in HDAC4 cytoplasmic-to-nuclear ratios were detected in the TG hearts, whereas CKIP-1 deficiency caused cytoplasmic accumulation of HDAC4 in cardiomyocytes (Figure 6B).

A set of conserved serine residues (S246, S467, and S632) is the critical determinant of HDAC localization. These serines, once phosphorylated, become attachment sites for 14-3-3 chaperone proteins, which escort HDACs into the cytoplasm and relieve transcriptional repression. Next, we tested whether CKIP-1 could affect the phosphorylation of HDAC4. In C2C12 cells, CKIP-1 overexpression decreased the phosphorylation of HDAC4 at S246 and S632 sites (Figure 6C). In vivo, CKIP-1 deficiency increased the phosphorylation of HDAC4 at the S246 site in the heart of KO mice, whereas CKIP-1 TG mice demonstrated reduced HDAC4 phosphorylation, and the changes of phospho-S632 were not apparent (Figure 6D). HDAC4 phospho-S246 has previously been shown to be the most important of the 3 conserved phosphorylation sites necessary to regulate HDAC4 localization. These results suggest that CKIP-1 could promote dephosphorylation of HDAC4 at S246. The phosphomimetic mutant of HDAC4 at 246 localized in the cytoplasm and failed to function downstream of CKIP-1. When they were coexpressed in C2C12 cells, CKIP-1 could not promote its nuclear localization online-only Data Supplement Figure VA). In HL-1 cardiomyocytes, CKIP-1 siRNA could increase the expression of ANP and BNP. However, HDAC4, which is a conserved serine residues (S246, S467, and S632) could restore their expression to the normal levels (online-only Data Supplement Figure V8).

HDAC4 could be dephosphorylated by phosphatase 2A (PP2A). To determine whether PP2A is also involved in this modulation, we investigated the effect of CKIP-1 on HDAC4 phosphorylation under the treatment of a PP2A inhibitor, okadaic acid. The results revealed that HDAC4 phospho-S246 was decreased by CKIP-1 in a dose-dependent manner, and okadaic acid antagonizes the action of CKIP-1 (Figure 6E). Inhibition of PP2A by7 using okadaic acid could affect CKIP-1–dependent HDAC4 nuclear localization (online-only Data Supplement Figure VC) and transcription function by MEF2C luciferase reporter assay (online-only Data Supplement Figure VE). Thus, CKIP-1, HDAC4, and PP2AC may have formed a protein complex in the cells. The interaction of HDAC4 and PP2AC is important for the dephosphorylation of HDAC4. To explore whether the influence of CKIP-1 on HDAC4 phosphorylation is through the regulation of the interaction between HDAC4 and PP2AC, we performed coimmunoprecipitation experiments in WT, KO, and TG mice. The results demonstrated that CKIP-1 expression could promote the interaction between HDAC4 and PP2AC (Figure 6f). In vitro, the results of immunoprecipitation under CKIP-1 overexpression and CKIP-1 knockdown also indicated the effect of CKIP-1 on the interaction between PP2AC and HDAC4 (online-only Data Supplement Figure VF). Together, these data demonstrated that PP2A mediated the influence of CKIP-1 on HDAC4 phosphorylation.

In summary, CKIP-1 could interact with both HDAC4 and PP2AC to increase the interaction between HDAC4 and PP2AC, thus promoting the dephosphorylation of HDAC4 by PP2AC and its accumulation in the nucleus. On the contrary, CKIP-1 deficiency in vivo enhances the export of HDAC4 from nucleus to the cytoplasm. By which, MEF2C activity is upregulated, and the expression of its target genes is increased. All of these events resulted in the occurrence of cardiac hypertrophy (Figure 6K).

**Discussion**

Here, we identified CKIP-1 as a novel regulator of pathologic cardiac hypertrophy and class II HDACs/MEF2 signaling in cardiomyocytes. Pressure overload–induced heart hypertrophy was exaggerated in CKIP-1–deficient mice, as evidenced by gravimetric, echocardiographic, and cell size analysis. CKIP-1 transgenic mice exhibited a strong reduction of the hypertrophy-induced fetal gene expression by 50% to 70%. The expression of CKIP-1 was sharply reduced in the late phases of cardiac hypertrophy in mouse and human hearts. CKIP-1 levels in the heart were closely related to the development of cardiac hypertrophy and failure in the HDAC4-dependent pathway. So, CKIP-1 manifests more important functional significance during the cardiac stress response resulting from hypertrophic overload.

The class IIa HDACs (HDACs 4, 5, 7, and 9) share a common structure, including a C-terminal catalytic domain and an N-terminal regulatory domain, which mediates interactions with transcription factors, coactivators, and corepressors. The transcriptional activity of MEF2 is tightly governed by its interaction with HDACs. Stress-inducible kinases, such as calcium/calmodulin-dependent protein kinase D, protein kinase C, and protein kinase C have been.
identified as critical class II HDAC kinases that are activated by prohypertrophic signals, resulting in the phosphorylation and subsequent nuclear export of class II HDACs, with consequent activation of MEF2 and its downstream target genes that are involved in cardiac remodeling.\textsuperscript{4,40–43} The dephosphorylation of N-terminal regulatory domain of class IIa HDACs has been shown to be regulated by PP2A in chordocytes, endothelial cells, and T cells.\textsuperscript{12,44,45} The balance between phosphatases and kinases sets the level of HDAC4 phosphorylation.\textsuperscript{46,47} The phosphorylation of HDAC4 by calcium/calmodulin-dependent protein kinase usually occurs in the cell nucleus. However, CKIP-1 is mainly located in the cytoplasm and its membrane. Therefore, it seems impossible for CKIP-1 to be involved in the regulation of HDAC4 phosphorylation by calcium/calmodulin-dependent protein kinase because of their different localization.

HDAC4 forms a complex with the PP2A holoenzyme C\textsubscript{\alpha}, A\textsubscript{\alpha}, and B\textsubscript{\alpha}/PR55\textsubscript{\alpha}.\textsuperscript{11} HDAC4 is dephosphorylated by PP2A. However, the regulation of class IIa HDACs dephosphorylation in cardiac hypertrophy was unknown. Our results demonstrated that CKIP-1 could regulate the repression of HDAC4 on MEF2 transcription activity via direct interaction with HDAC4. CKIP-1 deficiency caused cytoplasmic accumulation of HDAC4 in cardiomyocytes, whereas CKIP-1 myocardial overexpression inhibited the cytoplasmic accumulation of HDAC4. The experiments using okadaic acid or RNA interference have revealed that PP2A mediated the regulation of CKIP-1 on HDAC4 dephosphorylation. CKIP-1, HDAC4, and PP2A may form a protein complex in vivo. The interaction of CKIP-1 with HDAC4 promotes its interaction with PP2A, thus enhancing the dephosphorylation of HDAC4 by PP2A.

In recent years, much progress has been made in the identification of hypertrophic negative regulators. However, relatively little is known about how to use them either independently or in combination to treat cardiac hypertrophy and heart failure. CKIP-1 is clearly required for inhibiting HDAC4 export during maladaptive cardiac hypertrophy, suggesting a novel strategy for positively affecting hypertrophic heart disease through the upregulation of CKIP-1 expression and its interaction with HDAC4.

Acknowledgments

We thank Dr Yumei Ye (Biochemistry & Molecular Biology, University of Texas Medical Branch) for constructive suggestions to this manuscript. We thank Dr Eric N. Olson (Department of Molecular Biology, University of Texas Southwestern Medical Center) for kindly providing the full-length and truncated of HDACs and 3MEF2C-luc plasmids. We thank Dr Jeffrey Robbins (Division of Molecular Cardiovascular Biology, The Children’s Hospital Research Foundation, Cincinnati, OH) for providing us with the pG/ALPHA MHC plasmid. We thank Dr Xiaozhong Peng (The Peking Union Medical College, China) for kindly providing the pcDNA3.1-MEF-2C plasmid. We thank Dr Lianfeng Zhang (Key Laboratory of Human Disease Comparative Medicine, Ministry of Health, Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences and Comparative Medical Center) for kindly providing us with the human heart samples. We thank Dr William C. Claycomb (Department of Biochemistry & Molecular Biology, New Orleans, LA) for providing the HL-1 cardiomyocytes. We thank Dr Yaqiang Zhao (China Agricultural University) for the help of statistical analysis.

Sources of Funding

This work was supported by the National Basic Research Program of China (973 programs, 2011CB711003, 2011CB707704, and 2011CB910802), National Natural Science Foundation Projects (31271225, 31125010, and 30830029), State Key Lab of Space Medicine Fundamentals and Application grants (SMFA09A05), and Advanced Space Medico-engineering Research Project of China (SJ200810, SJ200909).

Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Among the intracellular signaling pathways involved in the regulation of cardiac hypertrophy, class II histone deacetylases (HDACs) have been identified as important signal-responsive repressors for cardiac hypertrophy. Little is known about the regulation of HDAC4 in response toextracellular stimuli leading to cardiac hypertrophy. Here we identified casein kinase-2interacting protein-1 (CKIP-1) as a novel regulator of pathological cardiac hypertrophy and class II HDACs/myocyte enhancer factor-2 signaling in cardiac myocytes. In this research, our data showed that CKIP-1 is induced early after pressure overload, but sharply reduced in the late phases of cardiac hypertrophy in mouse and human hearts. CKIP-1 systemically deficient mice exhibit spontaneous pathological cardiac hypertrophy by 8 months and display exaggerated responses to pressure overload. Conversely, cardiac specific overexpression of CKIP-1 attenuates pathological remodeling during chronic pressure overload. HDAC4 was identified to be a CKIP-1 interaction partner through unbiased interaction screening. HDAC4 is involved in the regulation of myocyte enhancer factor-2 transcription activity via decreasing the phosphorylation of HDAC4. CKIP-1 could increase the association of HDAC4 with protein phosphatase 2A and promote the dephosphorylation of HDAC4. These results demonstrated that CKIP-1 was required to inhibit the occurrence of cardiac hypertrophy and represented a promising candidate target for preventing hypertrophic cardiomyopathy.
CKIP-1 Inhibits Cardiac Hypertrophy by Regulating Class II Histone Deacetylase Phosphorylation Through Recruiting PP2A
Shukuan Ling, Qiao Sun, Yuheng Li, Luo Zhang, Pengfei Zhang, Xiaogang Wang, Chunyan Tian, Qi Li, Jinping Song, Hongju Liu, Guanghan Kan, Hongqing Cao, Zengming Huang, Jielin Nie, Yanqiang Bai, Shanguang Chen, Yinghui Li, Fuchu He, Lingqiang Zhang and Yingxian Li

Circulation. 2012;126:3028-3040; originally published online November 14, 2012; doi: 10.1161/CIRCULATIONAHA.112.102780
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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Supplemental Methods

Animals

Mice were bred and maintained at the Animal Research Center of Astronaut Center of China (ACC). The experimental procedures in mice and protocol used in this study were approved by the Animal Care and Use Committee of China Astronaut Research and Training Center. For this study, we used male CKIP-1 deficient and CKIP-1 transgenic mice and age-matched WT controls. All the experiments were repeated three times and were performed with homozygous CKIP-1 deficient mice (2 and 8 months old) and CKIP-1 TG mice (2 months) with age-matched WT littermates. CKIP-1 deficient mice have been reported previously.

Generation and identification of myocardial-specific CKIP-1 transgenic mice

A cDNA encoding mouse Flag-tagged CKIP-1 was cloned from the mouse cDNA. The forward primer containing a flag peptide sequence: 5'-CCCAAGCTTATGGACTACAAAGACGATGACGACAAGATGAAGAAGAGCGGCTCCGGCAAG-3', and the reverse primer was: 5'-CCCAAGCTTTTCACATCAGGCTCTCCCGGAAG-3'. The pJG/ALPHA MHC plasmid (gift of Dr. J. Robbins, Cincinnati Children's Hospital, USA) containing the 5.5-kbp mouse α-MHC promoter was cut with Hind III. CKIP-1, including its Flag tag, was cut with the same restriction enzymes (Hind III) and cloned within the Hind III ends of the pJG/ALPHA MHC plasmid that contained the 5.5-kbp mouse α-MHC promoter. The fragment containing the transgene (Flag-CKIP-1 cDNA under the control of the α-MHC promoter) was isolated and microinjected.
into C57BL/6 mouse oocytes, then the oocytes were surgically transferred to pseudopregnant C57BL/6 dams at Xiehe Medical University Animal Model Center. For genotype identification, genomic DNA was isolated from mouse tail biopsies and analyzed by PCR using specific primers:

5′–GACTAACTAGAAGCTTATGGACTA-3’ and 5′-CCAGGGTGAACTTGCTGTGAT-3’. We screened all the transgenic founders we obtained, and the ones that had the highest expression of CKIP-1 were selected. The transgenic mouse was screened at the weaning time, and the transgenic negative littermates were served as wild-type (WT) controls.

**Transverse Aortic Constriction (TAC)**

TAC was performed on 8 to 12-week-old male CKIP-1 deficient, CKIP-1 transgenic and wild type mice. Briefly, mice (8–10 weeks old, 21–26 g body weight) were anesthetized by intraperitoneal injection of 2,2,2-tribromoethanol (0.2 ml/10 g body weight of a 1.2% solution). The animals were then placed in a supine position, an endotracheal tube was inserted, and the animals were ventilated using a volume-cycled rodent ventilator with a tidal volume of 0.4 ml room air and a respiratory rate of 110 breaths/minute. The chest cavity was exposed by cutting open the proximal portion of the sternum. After the aortic arch between the innominate and left common carotid arteries was isolated, it was constricted with a 7-0 nylon suture tied firmly 3 times against a 25-gauge blunted needle for TAC. The needle was immediately withdrawn after the ligation. Sham-operated mice were subjected to identical interventions except for the constriction of the aorta. After echocardiographic analysis at different time points, mice were sacrificed by cervical dislocation, and hearts were removed and weighed promptly.
**Echocardiography**

Animals were lightly anesthetized with 2,2,2-tribromoethanol (0.2 ml/10 g body weight of a 1.2% solution) and set in a supine position. Two dimensional (2D) guided M-mode echocardiography was performed using a high resolution imaging system (Vevo 770, Visual-Sonics Inc., Toronto, ON, Canada). Two-dimensional images are recorded in parasternal long- and short-axis projections with guided M-mode recordings at the midventricular level in both views. Left ventricular (LV) cavity size and wall thickness are measured in at least three beats from each projection. Averaged LV wall thickness [interventricular septum (IVS) and posterior wall (PW) thickness] and internal dimensions at diastole and systole (LVIDd and LVIDs, respectively) are measured. LV fractional shortening ((LVIDd – LVIDs)/LVIDd), relative wall thickness ((IVS thickness + PW thickness)/LVIDd), and LV mass (LV Mass = 1.053×[(LVID;d+LVPW;d+IVS;d)³−LVID;d³]) are calculated from the M-mode measurements. LV ejection fraction (EF) was calculated from the LV cross-sectional area (2-D short-axis view) using the equation \( \text{LV %EF} = \frac{(\text{LV Vol;d}−\text{LV Vol;s})}{\text{LV Vol;d}} \times 100\% \). The studies and analysis were performed blinded as to experimental groups.

**Histological Analysis**

Sections for Masson’s Trichrome staining were generated from paraffin embedded hearts. Frozen sections were used to visualize cardiomyocyte cell membranes by staining with TRITC-conjugated wheat-germ agglutinin (Sigma-Aldrich). Immunostaining for CKIP-1 and HDAC4 was performed on cryosections of human and mouse myocardium. After fixation, the primary antibody for CKIP-1 (Santa Cruz Biotechnologies) or HDAC4 (Cell Signaling
Technology) was incubated overnight before it was visualized with ALEXA 488 or ALEXA 568 linked secondary antibodies (Invitrogen). As a negative control, the primary antibody incubation was omitted in the procedure. Paraffin-embedded sections of normal human heart tissue and hypertrophic heart tissue were provided by Dr. Lianfeng Zhang (Institute of Laboratory Animal Science CAMS & PUMC, Beijing, China).

**RNA extraction and Real-time PCR**

Total RNA was extracted from cultured cells or heart tissues using Trizol reagent according to the manufacturer’s protocol. First-strand cDNA synthesis was performed with 1 μg of total RNA, random hexamers, and SuperScript III Reverse Transcriptase (Invitrogen). Real-time PCR was performed using a SYBR Green PCR kit (Takara) in a Light Cycler (Eppendorf). The expression level of each gene was normalized to that of GAPDH, which served as an endogenous internal control. Primers (synthesized by Invitrogen, China) for CKIP-1, ANF, BNP, β-MHC, GAPDH were as follows:

- **CKIP-1 sense primer:** 5’-GCCGTGAGTCTCTGAAGAGAAG-3’,
- **CKIP-1 anti-sense primer:** 5’-CGAGTAGGGTGGGCAAGATAG-3’;
- **ANF sense primer:** 5’-TTCGGGGGTAGGATTGACAG-3’,
- **ANF anti-sense primer:** 5’-CACACCACAAGGGCTTAGGA-3’;
- **BNP sense primer:** 5’-TGTTTCTGCTTTTCTTTTATCTG-3’,
- **BNP anti-sense primer:** 5’-TCTTTTTGCGCTGTCTTTTGTGA-3’;
- **β-MHC sense primer:** 5’-TCCCACAACCGCATCTCTAT-3’,
- **β-MHC anti-sense primer:** 5’-CAGTTTCTCAGCCCTTCTC-3’;
GAPDH sense primer: 5′-ACTCCACTCACGGCAAATTCA-3′,

GAPDH anti-sense primer: 5′-GGCCTCACCCCATTTGATG-3′.

**Plasmids**

CKIP-1 plasmids were described previously. Full-length and truncated of HDACs and 3MEF2C-luc plasmids were provided by Dr. Eric Olson (UT Southwestern, USA). The pcDNA3.1-MEF-2C plasmid was a gift of Dr. Xiaozhong Peng (The Peking Union Medical College, China).

**Immunoprecipitation and immunoblotting**

Human embryonic kidney HEK293T cells were cultured in DMEM medium (Hyclone, USA) containing 10% fetal bovine serum (FBS) (Hyclone, USA). Transfection was performed with Lipofectamine 2000 (Invitrogen, USA). At 24–48 h after transfection, cells were harvested and lysed in HEPES lysis buffer (20 mM HEPES pH 7.2, 50 mM NaCl, 0.5% Triton X-100, 1 mM NaF, 1 mM dithiothreitol) supplemented with protease inhibitor cocktail (Roche, Indianapolis, Indiana, USA). Immunoprecipitation and immunoblotting were performed as described previously. Anti-Myc and anti-Flag monoclonal antibodies were from Cell Signaling (Danvers, Massachusetts, USA) and Sigma (St Louis, Missouri, USA), respectively.

To detect endogenous interaction between CKIP-1 and HDAC4, immunoprecipitation was performed with 2 µg of agarose-conjugated rabbit anti-HDAC4 antibody (Cell Signaling). Both total cell lysates and immunoprecipitates were detected by immunoblotting with HDAC4 (dilution 1:1000) and CKIP-1 (1:500) antibodies, Other antibodies used in analysis of HDAC4
phosphorylation were all from Cell Signaling Technology.

**GST pull-down and Mass Spectrometry.**

GST or GST-CKIP-1 were expressed and extracted from *Escherichia coli* strain BL21 (DE3) cells. Purified GST fusion proteins were immobilized on Glutathione-Sepharose 4B beads (GE Healthcare, USA) and washed with PBS (150 mM NaCl, 16 mM Na2HPO4, 4 mM NaH2PO4, pH 7.3), then beads were incubated with whole HEK293T cells extract for 3 h at 4 °C. Beads were washed with GST binding buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40 and protease inhibitor cocktail) and proteins were eluted with PBS buffer containing 10 mM reduced glutathione, followed by western blotting and LC-MS/MS sequencing.

**siRNA**

siRNAs targeted to human CKIP-1, HDAC4 PP1A and PP2AC gene were designed and synthesized by Shanghai GenePharma Co., Ltd.

CKIP-1 siRNA: GUGUGAAGAGCUCCGGAAGTT,
PP1AC siRNA: GACGCUACAACAUCAAACUTT,
PP2AC siRNA: GAGUUGAUUCCGUU TT,
HDAC4 siRNA: GUCCAGGCUAAGCAGAAAT T.

**Reporter gene assays**

HEK293T cells were seeded on 24-well plates (5 × 10^4 cells per well) and transfected with pRL-TK, p3MEF2-luc reporter gene plasmids with or without pcDNA3.1-CKIP-1 or pFlag
CMV-HDAC4 plasmid as described. Luciferase activity was measured as described. Renilla luciferase was co-transfected for the purposes of normalization. The activity of luciferase were detected by Dual-Luciferase® Reporter Assay System (Promega). Error bars are derived from the standard deviations of multiple experiments.

**Statistical Analysis**

To detect the CKIP-1 expression cryosections obtained from mouse hearts at 2 weeks and 6 weeks after TAC surgery, we firstly did homogeneity of variance test using Levene's method, and the test indicates the variances across groups are equal, then we used one-way ANOVA to test the differences the data of the immunohistochemistry of CKIP-1, followed by the Bonferroni post hoc test for multiple comparisons.

For quantitative analysis the difference of heart function between KO and WT mice at different time points, data are expressed as mean ± s.e.m. of the values obtained for each time point from WT and KO mice. The values of EF and FS are from echocardiography. The values of relative fetal cardiac gene expression are from Q-PCR. They are normalized to the internal control (GAPDH). We use the two-way analysis of variance with unequal variances to account for 2 factors and their interactions. First, we test the equality of variances using a factorial effects analysis of variance on the absolute values of the residuals. If the variances are unequal, we then fit a mixed model with heterogeneous variances. Otherwise, we use the regular linear model. We also used one-way ANOVA to compare the differences for multiple groups. Considering the presence of unequal variance for the data in different groups, we firstly did homogeneity of variance test using Levene’s method. If the test indicates the variances across groups are unequal, we then reported statistics and corresponding p value adjusted by the Welch's method. Otherwise,
For quantitative analysis the changes of heart function under the condition of transverse aortic constriction, we analyzed the data of heart weight index and cardiac gene expression as above. To compare the changes of heart function after TAC, we collected the data at 2 and 4 weeks from the same mouse and use repeated measures data analysis to examine and compare response trends over time between WT and KO. The data were collected serially using the same experimental animals, we used the mixed model for repeated measures analysis of variance. We did separate tests for the differences between WT and KO at 4 weeks after TAC. We also addressed the unequal variance problem for these comparisons. In case of unequal variances, we used the Welch's t test; otherwise we use the regular Student's t-test.

In Figure 4, we are comparing the difference between WT and TG mice in response to TAC surgery. The data were collected as before mentioned. We firstly test the equality of variances across groups. If it shows the variances are unequal, we then use mixed model with heterogeneous variances for 2-way analysis or Welch’s t test for 1-way analysis. Otherwise we use the regular linear model or Student's t-test. In Figure 5, the effect of CKIP-1 on MEF2C activity were analyzed through luciferase repoter assy. MEF2C luciferase activity were used to reflect MEF2C activity. The values were normalized by internal control (Renilla luciferase activity). We firstly did homogeneity of variance test using Levene's method. If the test indicates the variances across groups are unequal, we then reported statistics and corresponding p value adjusted by the Welch's method. Otherwise, we reported unadjusted ANOVA statistics. We also did the Bonferroni adjustments for multiple comparisons.
Table 1 Basal echocardiographic parameters in mice with CKIP-1 deletion and their WT littermates at 2 and 8 months of age.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2 months</th>
<th></th>
<th>8 months</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT(n=11)</td>
<td>KO(n=13)</td>
<td>WT(n=10)</td>
<td>KO(n=13)</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.78 ± 0.14</td>
<td>0.91 ± 0.14</td>
<td>0.97 ± 0.065</td>
<td>0.99 ± 0.17</td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>1.07 ± 0.24</td>
<td>1.30 ± 0.24&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.27 ± 0.14</td>
<td>1.22 ± 0.25</td>
</tr>
<tr>
<td>LVPWd(mm)</td>
<td>0.63 ± 0.13</td>
<td>0.89 ± 0.28&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.96 ± 0.27</td>
<td>0.92 ± 0.18</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>0.95 ± 0.15</td>
<td>1.20 ± 0.14&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.27 ± 0.17</td>
<td>1.17 ± 0.15</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.81 ± 0.15</td>
<td>3.90 ± 0.36</td>
<td>3.44 ± 0.30</td>
<td>3.75 ± 0.28&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.76 ± 0.31</td>
<td>2.54 ± 0.24</td>
<td>2.27 ± 0.32</td>
<td>2.73 ± 0.30&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as mean ±SD of values. <sup>A</sup><sub>P<0.05</sub>, <sup>B</sup><sub>P<0.01</sub> versus WT.
### Supplementary Table 2

**Table 2** LV dimension, wall thickness, and function in mice with myocardial CKIP-1 overexpression and their WT littermates in response to 4-week TAC.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT 4-wk TAC</th>
<th>TG 4-wk TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham(n=8)</td>
<td>TAC(n=6)</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.85±0.080</td>
<td>1.08±0.11(^B)</td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>1.09±0.091</td>
<td>1.37±0.19(^A)</td>
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<tr>
<td>LVPWd(mm)</td>
<td>0.84±0.096</td>
<td>1.00±0.16(^A)</td>
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<td>LVPWs (mm)</td>
<td>1.18±0.10</td>
<td>1.19±0.087</td>
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<tr>
<td>LVIDd (mm)</td>
<td>3.64±0.22</td>
<td>3.77±0.41</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.44±0.19</td>
<td>2.75±0.40</td>
</tr>
</tbody>
</table>

Data are expressed as mean ±SD of values. \(^{A}P<0.05, \(^{B}P<0.01\) versus Sham. \(^{C}P<0.05\) versus WT.
### Table 3 Mass Spectrometry Analysis of CKIP-1 Interacting Protein

<table>
<thead>
<tr>
<th>Band</th>
<th>Identified Protein</th>
<th>Peptides</th>
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<td>140 kDa</td>
<td>ATP-dependent RNA helicase A</td>
<td>K.LPIEPR.F</td>
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<td>K.YPSPPFVFGEK.I</td>
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<tr>
<td></td>
<td></td>
<td>R.DINTDFLLYVLR.D</td>
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<tr>
<td></td>
<td></td>
<td>K.AIEPPPLDAVIEAEHTLR.E</td>
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<tr>
<td>130 kDa</td>
<td>histone deacetylase 4</td>
<td>R.LAVGCVVVELVK.V</td>
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<tr>
<td>109 kDa</td>
<td>Elongation factor Tu GTP binding domain containing 2</td>
<td>R.SFVEFILEPLYK.I</td>
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<td></td>
<td>K.AFIPAI DFSGFTDLR.T</td>
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<td></td>
<td></td>
<td>K.NGQDLGVAFLK.I</td>
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<td></td>
<td></td>
<td>R.NFILDQTNVSAAAAQR.R</td>
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<tr>
<td>76 kDa</td>
<td>nucleolin</td>
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<tr>
<td></td>
<td></td>
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<td></td>
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<td>K.VTQDELKEVFEDAAEI.R</td>
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<td>58 kDa</td>
<td>keratin</td>
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<tr>
<td></td>
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<td>K.IEGYEDQVLITEHGDGLGNSR.F</td>
</tr>
</tbody>
</table>
Supplementary Figure

Sup Fig 1a

Sup Fig 1b

Sup Fig 1c

Sup Fig 1d

Supplementary Figure 1 (a) Relative CKIP-1 expression in adult tissues. Graph showing densitometric analysis of immunoblot from 3 independent experiments. (b) Relative CKIP-1 expression from heart tissue after TAC or Sham at 2 days, 2w, 4w. (c) To quantify the Immunohistochemistry of CKIP-1 in cryosections obtained from mouse hearts at 2 weeks and 6 weeks after TAC surgery, the 10 to 20 areas at 400 × magnification of each section, and at least 3 sections of each heart were captured with a digital camera and analyzed using Image Pro Plus software (version 6.0, Media Cybernetics, USA). (d) The expression of CKIP-1 in heart tissue from advanced heart failure specimens and normal control was analyzed by western blot. **P<0.01. Data represent the means ±s.e.m.
Supplementary Figure 2 The cardiomyocyte crosssectional area was measured from 8-μm-thick heart sections that had been stained with HE by using ImageJ software (NIH). Only myocytes that were round were included in the analysis. The studies and analysis were performed blinded as to experimental. *P<0.05, **P<0.01. Data represent the means ± s.e.m.
**Supplementary Figure 3** The cardiomyocyte cross-sectional area was measured from 8-μm-thick heart sections that had been stained with HE by using ImageJ software (NIH). Only myocytes that were round were included in the analysis. The studies and analysis were performed blinded as to experimental. *P<0.05, **P<0.01. Data represent the means ± s.e.m.
Sup Fig 4a

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Co-IP with CKIP-1

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**Supplementary Figure 4** (a) Mapping of HDAC4 and CKIP-1 binding regions. The indicated HDAC4 deletion mutations and full-length CKIP-1 were analyzed for their binding capacities by a co-immunoprecipitation assay in 293T cells. (b) The interaction between CKIP-1 and HDAC4, 5 and 9. 293T cells were transfected with Flag-HDAC4 and Myc-CKIP-1. HDAC4 proteins were immunoprecipitated with anti-Flag antibody, followed by anti-Myc antibody detection. (c) The effect of HDAC5 on MEF2C activity was promoted by CKIP-1. 293T cells were transfected with CKIP-1, HDAC5 or both. MEF2C activity was assayed as previously described. **P<0.01. Data represent the means ±s.e.m.
Supplementary Figure 5 (a) The effect of CKIP-1 on the localization of HDAC4 S246D. C2C12 cells were transfected with expression plasmids encoding GFP-HDAC4 S246D (upper) or with RFP-CKIP-1 (100 ng) (lower). After 36 h, nuclei were stained with DAPI (blue) followed by confocal fluorescent microscopy investigation (scale bars, 10 μm). (b) CKIP-1 deficiency on the expression of ANP and BNP and the regulation of nuclear localized HDAC4 S3A on it. HL-1 cardiomyocytes were transfected with CKIP-1 siRNA or CKIP-1 siRNA with HDAC4 S3A. The expression of ANP and BNP were quantified by Q-PCR. (c) The regulation of OA on the effect of CKIP-1 on the localization of HDAC4. C2C12 cells were transfected with expression plasmids encoding GFP-HDAC4 and RFP-CKIP-1 (100 ng) (lower) under the treatment of OA or not. After 36 h, nuclei were stained with DAPI (blue) followed by confocal fluorescent microscopy investigation (scale bars, 10 μm). (d) The regulation of OA on the effect of CKIP-1 on MEF2C activity. 293T cells were transfected with CKIP-1, HDAC4 or both under the treatment of OA or not. MEF2C activity was assayed as previously described. (e) The effect of CKIP-1, CaMK II inhibitor or both on the phosphorylation of HDAC4. 293T cells were treated with CaMKII inhibitor (KN-93, Upstate) with CKIP-1 over-expression or not, then the levels of HDAC4 phosphorylation was analyzed by western blotting. (f) The effect of CKIP-1 on the interaction between HDAC4 and PP2AC. 293T cells were transfected with CKIP-1 (left) or CKIP-1 siRNA (right). The interaction between HDAC4 and PP2AC was analyzed by co-immunoprecipitation.