Cardiac Hypertrophy

Ubiquitin-Specific Protease 4 Is an Endogenous Negative Regulator of Pathological Cardiac Hypertrophy

Ben He, Yi-Chao Zhao, Ling-Chen Gao, Xiao-Ying Ying, Long-Wei Xu, Yuan-Yuan Su, Qing-Qi Ji, Nan Lin, Jun Pu

Abstract—Dysregulation of the ubiquitin proteasome system components ubiquitin ligases and proteasome plays an important role in the pathogenesis of cardiac hypertrophy. However, little is known about the role of another ubiquitin proteasome system component, the deubiquitinating enzymes, in cardiac hypertrophy. Here, we revealed a crucial role of ubiquitin specific protease 4 (USP4), a deubiquitinating enzyme prominently expressed in the heart, in attenuating pathological cardiac hypertrophy and dysfunction. USP4 levels were consistently decreased in human failing hearts and in murine hypertrophied hearts. Adenovirus-mediated gain- and loss-of-function approaches indicated that deficiency of endogenous USP4 promoted myocyte hypertrophy induced by angiotensin II in vitro, whereas restoration of USP4 significantly attenuated the prohypertrophic effect of angiotensin II. To corroborate the role of USP4 in vivo, we generated USP4 global knockout mice and mice with cardiac-specific overexpression of USP4. Consistent with the in vitro study, USP4 depletion exacerbated the hypertrophic phenotype and cardiac dysfunction in mice subjected to pressure overload, whereas USP4 transgenic mice presented ameliorated pathological cardiac hypertrophy compared with their control littermates.

Molecular analysis revealed that USP4 deficiency augmented the activation of the transforming growth factor β–activated kinase 1 (TAK1)-(JNK1/2)/P38 signaling in response to hypertrophic stress, and blockage of TAK1 activation abolished the pathological effects of USP4 deficiency in vivo. These findings provide the first evidence for the involvement of USP4 in cardiac hypertrophy, and shed light on the therapeutic potential of targeting USP4 in the treatment of cardiac hypertrophy.

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Key Words: heart failure □ ligases □ phenotype □ signal transduction □ ubiquitin-specific protease

Cardiac hypertrophy is an initially adaptive reaction to maintain cardiac output in response to various stresses (eg, pressure/volume overload and ischemic injury). Despite its initial compensatory nature, pathological cardiac hypertrophy results in ultimate maladaptation and heart failure, and represents a potent independent risk factor for cardiac morbidity and mortality. The development of cardiac hypertrophy involves a complex process of myocyte molecular modifications, secondary to the reactivation of the fetal gene expression program after excessive activation of multiple signaling cascades. Existing antihypertrophic therapies generally targeting membrane receptors seem to have limited efficacy and lead to systemic and off target effects, possibly because of nonspecific suppression of downstream signaling. Identification of novel molecular regulators with pathway specificity within the intracellular hypertrophic signaling network may allow to characterize novel intervention targets for the treatment of cardiac hypertrophy and heart failure.

Deubiquitinating enzymes (DUBs), consisting of diverse enzymes targeting specific substrates in a highly regulated manner, have drawn enhanced interest as drug targets. In particular, the ubiquitin-specific protease (USP) family is the largest subclass among the 5 DUB subclasses, with the highest substrate specificity, and exhibit diverse tissue-specific expression patterns, representing an intriguing pool for drug discovery. USPs are pivotally involved in DNA repair, endocytosis, immune response, and carcinogenesis; however, the role of the USPs in cardiac pathology remains almost unknown. Interestingly, USP4 is prominently expressed in the heart and skeletal muscle at the protein level, indicating a potential role of USP4 in cardiac biology. USP4 knockout mice are viable and developmentally normal, suggesting that USP4 may be preferentially involved in pathological processes rather than in physiological functions. Indeed, USP4 plays pivotal roles in noncardiac pathologies by regulating specific intracellular pathways in a stimulus-dependent manner. In response to proinflammatory stimuli, USP4 quenches excessive inflammatory response by suppressing nuclear factor κB signaling, whereas in response to viral infection, USP4 promotes antiviral responses by augmenting retinoic acid–inducible gene 1-β interferon signaling. In addition, USP4...
promotes ionizing radiation–induced cell apoptosis by specifically reducing p53 expression. Of note, USP4 is a putative proto-oncogene, which promotes epithelial to mesenchymal transition and breast cancer cell migration. Interestingly, mounting evidence supports pivotal roles of proto-oncogenes in modulating cardiac hypertrophy, indicating a potential involvement of USP4 in cardiac hypertrophy. We therefore posited that USP4, a DUB prominently expressed in the heart, might be a potential signaling regulator implicated in cardiac hypertrophy.

In this study, we observed reduced levels of USP4 in hearts from patients with dilated cardiomyopathy and in animal models of cardiac hypertrophy induced by pressure overload. By subjecting USP4 knockout mice and transgenic mice with cardiac-specific USP4 overexpression to aortic banding (AB), we observed that USP4 deficiency aggravated hypertrophic growth and cardiac dysfunction. Conversely, restoration of USP4 level remarkably protected the heart against pathological hypertrophy. Mechanistically, the beneficial effect of USP4 was largely dependent on the blockade of the transforming growth factor β–activated kinase 1 (TAK1)-(JNK1/2)/P38 signaling.

Materials and Methods

The animal protocol was approved by the Institute’s Animal Ethics Committee of Shanghai Jiao Tong University, and the investigation complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male USP4-knockout mice and their wild-type (WT) littermates as well as cardiac-specific USP4 transgenic mice and their nontransgenic littermates (aged 8–10 weeks) were used in the present experiments. Human failing heart samples were obtained from patients with dilated cardiomyopathy who underwent heart transplant surgery. Nonfailing heart samples were collected from healthy donor hearts, which were not suitable for transplantation. Informed written consent was obtained from the families of the patients and prospective heart donors. All the studies involving human samples were approved by the Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiao tong University, and conformed to the principles outlined in the Declaration of Helsinki. An expanded Materials and Methods section is available in the online-only Data Supplement, which includes detailed information on the following aspects: generation of global USP4 knockout and USP4 transgenic mice, surgical procedure of AB, histological analysis, echocardiographic measurement, culture of neonatal rat ventricular myocytes and adenovirus transfection, immunofluorescence analysis, ubiquitination assay, immunoprecipitation, Western blot and quantitative real-time polymerase chain reaction, and human heart samples.

Figure 1. Ubiquitin-specific protease 4 (USP4) expression is reduced in human failing hearts and murine hypertrophied hearts.

A. Transcriptional levels of USP4 were determined by real-time quantitative polymerase chain reaction in heart samples from patients with dilated cardiomyopathy (DCM) and normal heart tissues (n=4 samples per experimental group, *P<0.05 vs donor hearts).

B. mRNA levels of USP4 in heart samples from sham-operated mice or mice subjected to pressure overload (n=4 mice per experimental group, *P<0.05 or **P<0.01 vs sham).

C. Left, Western blot bands of USP4, atrial natriuretic peptide (ANP), and β-myosin heavy chain (β-MHC) in heart samples from normal donors and patients with DCM. Right, Protein expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and compared between indicated groups (n=4, *P<0.01 vs donor hearts).

D. Left, USP4, ANP, and β-MHC levels in heart tissues from mice subjected to sham or aortic banding (AB) surgery were detected by Western blot at indicated time points. Right, Quantitative representation of protein expression levels in indicated groups (n=6, *P<0.05 or **P<0.01 vs sham).

E. Left, Western blot analysis of USP4, ANP, and β-MHC levels in heart tissues from mice subjected to sham or angiotensin II (1 μmol/L) for the indicated time.

The data are presented as the mean±SD.
Statistical Analysis
Data are presented as the mean±SD. Comparisons between 2 groups were performed using a 2-tailed Student t test. One-way ANOVA with the Bonferroni post hoc test (equal variances assumed) test or the Tamhane post hoc test (equal variances not assumed) was used to determine differences among multiple groups. P value <0.05 (2-tailed) were considered significant.

Results
USP4 Expression Is Decreased in Human Failing Hearts and Murine Hypertrophic Hearts
To investigate the potential involvement of USP4 in cardiac hypertrophy, we first determined if the development of pathological cardiac hypertrophy was associated with altered USP4 expression. USP4 transcription levels determined using real-time polymerase chain reaction were significantly reduced in heart tissues of patients with dilated cardiomyopathy when compared with normal donor hearts (Figure 1A); similar findings were apparent in a murine model of cardiac hypertrophy induced by pressure overload (Figure 1B). Consistent with the polymerase chain reaction results, USP4 protein levels were significantly reduced in human failing hearts compared with those of normal donor hearts, which was accompanied by increased levels of fetal genes atrial natriuretic peptide (ANP) and β-myosin heavy chain (β-MHC; Figure 1C). Consistently, USP4 protein expression was progressively downregulated in the murine model by 30% and 75% at 4 and 8 weeks after AB surgery, respectively, along with increased levels of ANP and β-MHC (Figure 1D). Moreover, endogenous expression of USP4 in cardiomyocytes progressively decreased in response to angiotensin II (Ang II) exposure (Figure 1E). These data indicate that endogenous USP4 is consistently downregulated in human failing hearts and hypertrophied murine hearts.

USP4 Suppresses Ang II–Induced Cardiomyocyte Hypertrophy In Vitro
To characterize the pathological consequence of decreased USP4 expression in response to hypertrophic stimulation, we performed loss-of-function studies in isolated neonatal rat cardiomyocytes (NRVMs) using an adenovirus harboring
USP4 short hairpin RNA to artificially downregulate USP4 expression (Figure 2A), followed by treatment with Ang II (1 μmol/L) for 48 hours to elicit cellular hypertrophy. USP4 silencing dramatically augmented Ang II–induced cardiomyocyte hypertrophy (Figure 2B and 2C) and significantly increased expression of fetal genes ANP, brain natriuretic peptide, and β-MHC compared with transfection with control short hairpin RNA (Figure 2D). To further determine if restoration of USP4 levels by expression of exogenous USP4 attenuated myocyte hypertrophy, we transfected isolated NRVMs with an adenovirus harboring USP4 cDNA (AdUSP4) to upregulate USP4 expression. Conversely, USP4 overexpression dramatically blunted the prohypertrophic effect of Ang II (Figure 2E) and suppressed expression of ANP, brain natriuretic peptide, and β-MHC compared with AdGFP transfection (Figure 2F). Thus, decreased USP4 levels seem to be essential for the development of myocyte hypertrophy induced by Ang II, and restoration of USP4 levels potently alleviates myocyte enlargement.

**USP4 Deficiency Aggravates AB-Induced Cardiac Hypertrophy and Dysfunction In Vivo**

Development of pressure overload–induced cardiac hypertrophy in vivo involves more complex cellular modifications than Ang II–induced myocyte hypertrophy in vitro. To determine whether USP4 deficiency promotes cardiac hypertrophy in vivo, we subjected USP4 global knockout mice and their WT littermates to pressure overload. Details on the design and testing of the knockout animals are included in the online-only Data Supplement (Figure S1–S4). Of note, knockout
mice were viable and developmentally normal compared with WT mice. Four weeks after AB surgery, knockout mice exhibited remarkably increased heart weight index and lung weight index (Figure 3A), indicating that USP4 deficiency exacerbated pressure overload–induced heart enlargement and cardiac dysfunction. Consistently, echocardiographic measurements confirmed that USP4 depletion dramatically promoted ventricular dilation and contractile dysfunction (Figure 3B). Furthermore, more severe cardiomyocyte hypertrophy and interstitial and perivascular fibrosis were observed in knockout mice compared with WT controls in response to pressure overload (Figure 3C–3E), accompanied by significantly decreased transcription levels of sarcoplasmic reticulum Ca\(^{2+}\) ATPase (Serca2a) and elevated levels of hypertrophic and fibrotic markers, including ANP, brain natriuretic peptide, β-MHC, collagen I, collagen III, and connective tissue growth factor (Figure 3F). Collectively, these loss-of-function studies suggest that USP4 deficiency contributes to...
the development of pathological cardiac hypertrophy induced by chronic pressure overload.

**Cardiac-Specific USP4 Overexpression Attenuates Cardiac Hypertrophy and Dysfunction In Vivo**

On the basis of the pathological effects of reduced USP4 levels on cardiac hypertrophy, we further determined if restoring cardiac USP4 levels exerted a beneficial effect, and for this purpose generated mice with cardiac-specific overexpression of USP4 (Figure 4A, left). Overexpression of cardiac USP4 was validated in 4 transgenic lines by Western blot (Figure 4A, right). Line transgenic 4 was randomly selected for further experiments. At baseline, transgenic mice were viable and showed no obvious cardiac morphological or functional abnormalities. In response to pressure overload, USP4 overexpression dramatically reduced heart weight index and lung weight index (Figure 4B), and improved cardiac function after AB surgery compared with nontransgenic mice (Figure 4C). In parallel, the USP4 transgene inhibited development of cardiomyocyte hypertrophy and cardiac fibrosis (Figure 4D–4F) and significantly normalized expression of hypertrophic and fibrotic markers (Figure 4G). Taken together, these data indicate that maintaining cardiac USP4 expression protects against pressure overload–induced cardiac hypertrophy and cardiac dysfunction.

**USP4 Regulates TAK1-(JNK1/2)/P38 Signaling Via Its Deubiquitinating Activity**

The aforementioned results provided robust evidence supporting the detrimental effect of USP4 deficiency in cardiac hypertrophy development. To further gain insight into the downstream molecular events mediating the pathological effects of reduced USP4 levels on cardiac hypertrophy, we investigated the potential involvement of the TAK1-JNK1/2 and P38 mitogen-activated protein kinase (MAPK) signaling pathways.

**Figure 5.** Ubiquitin-specific protease 4 (USP4) reduces JNK1/2 and P38 phosphorylation levels in hypertrophied hearts and myocytes. **A,** The effect of endogenous USP4 on phosphorylation levels of prohypertrophic kinases in vivo. Top, Western blots results showing the phosphorylation and total protein levels of MEK1/2, ERK1/2, JNK1/2, and P38 in heart tissues from USP4 deleted mice and wild-type (WT) mice 4 weeks after aortic banding (AB) surgery. Bottom, Graphic representation of the phosphorylation levels of JNK1/2 and p-P38 in knockout (KO) and WT mice 4 weeks after AB (n=6, *P<0.01 vs WT/sham, #P<0.05 or ##P<0.01 vs WT/AB). **B,** The effect of exogenous USP4 overexpression on phosphorylation levels of prohypertrophic kinases in vivo. Top, Western blot results showing the phosphorylation and total protein levels of MEK1/2, ERK1/2, JNK1/2, and P38 in heart tissues from USP4 transgenic mice and nontransgenic mice 4 weeks after sham or AB surgery. Bottom, Statistical analysis of the phosphorylation levels of JNK1/2 and p-P38 in knockout (KO) and WT mice 4 weeks after AB (n=6, *P<0.05 or **P<0.01 vs NTG/sham, #P<0.05 or ##P<0.01 vs NTG/AB). **C,** Effect of endogenous USP4 on phosphorylation levels of prohypertrophic kinases in vitro. Top, Representative Western blots showing the phosphorylation and total protein levels of MEK1/2, ERK1/2, JNK1/2, and P38 in NRVMs infected with shUSP4 or shRNA followed by treatment with phosphate buffered saline (PBS) or angiotensin II (Ang II). Bottom, densitometry analyses of JNK1/2 and P38 phosphorylation levels in indicated groups (n=3 independent experiments, *P<0.05 or **P<0.01 vs shRNA+PBS, #P<0.05 or ##P<0.01 vs NTG/Ang II). **D,** Effect of exogenous USP4 overexpression on phosphorylation levels of prohypertrophic kinases in vitro (n=3 independent experiments, *P<0.01 vs AdGFP+PBS, #P<0.05 or ##P<0.01 vs AdGFP+Ang II). Protein expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are expressed as mean±SD.
consequences of USP4 downregulation, we tested the effect of USP4 on kinases involved in promoting cardiac hypertrophy. Our results suggested that the phosphorylation levels of kinases MEK1/2, ERK1/2, c-Jun N-terminal kinase 1/2 (JNK1/2), and P38 were significantly increased after AB surgery (Figure 5A), and USP4 depletion significantly augmented phosphorylation levels of JNK1/2 and P38 compared with WT controls (Figure 5A), which in turn suggested that USP4 acts as a negative regulator of JNK1/2 and P38 signaling in hypertrophied hearts. The latter finding was further supported by the observation that cardiac-specific USP4 overexpression significantly suppressed phosphorylation levels of JNK1/2 and P38 in hypertrophied heart (Figure 5B). Consistently, USP4 knockdown in primary NRVMs significantly increased the phosphorylation levels of JNK1/2 and P38 after Ang II stimulation (Figure 5C), whereas USP4 overexpression remarkably suppressed JNK1/2 and P38 phosphorylation levels (Figure 5D). Collectively, these findings suggest that the antihypertrophic effect of USP4 is associated with suppression of JNK1/2 and P38 phosphorylation levels.

JNK1/2 and P38 are mitogen-activated kinases activated by upstream kinases such as TAK1 in response to various stresses.29,30 To investigate the underlying mechanism responsible for effect of USP4 on JNK1/2 and P38 phosphorylation in hypertrophied hearts, we examined the potential effects of USP4 on TAK1. We observed that USP4 depletion significantly elevated the phosphorylation level of TAK1 in hypertrophied heart compared with WT control (Figure 6A), whereas USP4 overexpression dramatically suppressed TAK1 phosphorylation (Figure 6B). Consistent with in vivo observations, USP4 silencing significantly increased the phosphorylation level of TAK1 in Ang II–treated NRVMs compared with control shRNA transfection (Figure 6C), whereas AdUSP4 transfection dramatically suppressed TAK1 phosphorylation in Ang II–treated NRVMs compared with AdGFP transfection (Figure 6D). Together, these data indicate TAK1 activation

Figure 6. Ubiquitin-specific protease 4 (USP4) inhibits the transforming growth factor β activated kinase 1 (TAK1) phosphorylation in hypertrophied hearts and myocytes. A. Effect of endogenous USP4 on phosphorylation levels of TAK1 in vivo. Top, Western blots results showing the phosphorylation and total protein levels of TAK1 in heart tissues from USP4 knockout mice and wild-type (WT) mice 4 weeks after aortic banding (AB) surgery. Bottom, Graphic representation of the phosphorylation levels of TAK1 in knockout (KO) and WT mice 4 weeks after AB (n=6, *P<0.01 vs WT/sham, #P<0.05 vs WT/AB). B. Effect of exogenous USP4 overexpression on phosphorylation levels of TAK1 in vivo. Top, Western blots results showing the phosphorylation and total protein levels of TAK1 in heart tissues from USP4 transgenic mice and nontransgenic mice 4 weeks after sham or AB surgery. Bottom, Statistical analysis of the phosphorylation levels of TAK1 in transgenic (TG) and nontransgenic (NTG) mice (n=6, *P<0.01 vs NTG/sham, #P<0.01 vs NTG/AB). C. Effect of endogenous USP4 on phosphorylation levels of TAK1 in vitro. Top, Representative Western blots showing the phosphorylation and total protein levels of TAK1 in neonatal rat cardiomyocytes infected with shUSP4 or shRNA followed by treatment with phosphate buffered saline (PBS) or angiotensin II (Ang II). Bottom, densitometry analyses of TAK1 phosphorylation levels in indicated groups (n=3 independent experiments, *P<0.05 vs shRNA+PBS, #P<0.05 vs shRNA+Ang II). D. Effect of exogenous USP4 overexpression on phosphorylation levels of TAK1 in vitro (n=3 independent experiments, *P<0.01 vs AdGFP+PBS, #P<0.01 vs AdGFP+Ang II). Protein expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are expressed as mean±SD.
was negatively regulated by USP4 in response to prohypertrophic stresses.

To further determine if USP4 regulates TAK1 activation in a direct manner, we first examined if USP4 physically interacts with TAK1. To this end, we transfected Flag-tagged TAK1 and hemaglutinin-tagged USP4 into HEK293T cells; coimmunoprecipitation of cell lysates demonstrated that the ectopically expressed USP4 interacted with TAK1 and vice versa (Figure 7A). Because USP4, a DUB, is primarily involved in regulating substrate ubiquitination, we further investigated if USP4 deubiquitinated TAK1 in hypertrophied myocytes. As shown in Figure 7B, Ang II stimulation augmented polyubiquitination levels of TAK1 in NRVMs, and USP4 deficiency markedly increased TAK1 polyubiquitination, suggesting that endogenous USP4 was responsible for deubiquitinating TAK1 in hypertrophied myocytes. Because it has been reported that TAK1 polyubiquitination is responsible for autophosphorylation of TAK1 and subsequent phosphorylation of JNK and p38 mitogen-activated kinase, we further tested if USP4 regulates TAK1, JNK1/2, and p38 phosphorylation levels via deubiquitination of TAK1 in hypertrophied myocytes. To this end, WT USP4 or a USP4 mutant (C311A) without deubiquitinating activity were overexpressed in NRVMs, followed by treatment with Ang II. We observed that WT USP4 overexpression markedly suppressed polyubiquitination levels of TAK1 (Figure 7C), and reduced subsequent phosphorylation of TAK1, JNK1/2, and p38 in Ang II–treated myocytes (Figure 7D). By contrast, USP4 (C311A) that failed to suppress TAK1 polyubiquitination was unable to inhibit phosphorylation of TAK1, JNK1/2, and p38 in Ang II–treated myocytes. Collectively, these data indicate that the inhibitory effect of USP4 on TAK1-(JNK1/2)/P38 signaling in hypertrophied myocytes was dependent on its deubiquitinating activity.

**USP4 Deficiency Promotes Cardiac Hypertrophy Depending on TAK1-(JNK1/2)/P38 Signaling Activation**

The aforementioned results indicated that USP4 is a negative regulator of TAK1-(JNK1/2)/P38 signaling in response to hypertrophic stress. To further identify if the excessive activation of TAK1-(JNK1/2)/P38 signaling is essential for USP4
deficiency–mediated cardiac hypertrophy progression, USP4 knockout mice and WT control were intraperitoneally injected with 5z-7-oxozeaenol (an inhibitor of TAK1) for 4 weeks after AB surgery. As shown in Figure 8A, 5z-7-oxozeaenol effectively inhibited phosphorylation of TAK1-(JNK1/2)/P38 signaling in hypertrophied hearts. Notably, blockade of TAK1-(JNK1/2)/P38 signaling attenuated hypertrophic growth, cardiac fibrosis, and cardiac dysfunction to similar extents in knockout and WT mice subjected to AB (Figure 8B–8F), implying that the pathological effect of USP4 deficiency is largely dependent on excessive activation of TAK1-(JNK1/2)/P38 signaling.

Discussion
Cardiac hypertrophy is a common response to a variety of pathological stimuli and eventually leads to heart failure, making it essential to discover impactful therapeutic targets to suppress its progression. This study identified that expression of USP4 was downregulated in human failing heart samples and in a murine model of cardiac hypertrophy induced by pressure overload. Adenovirus-mediated gain-and loss-of-function approaches indicated that decreased expression of endogenous USP4 promoted myocyte hypertrophy induced by Ang II in vitro, whereas restoration of USP4 significantly attenuated the prohypertrophic effect of Ang II. To further confirm the cardiac roles of USP4 in an in vivo model, we generated mice with cardiac-specific overexpression of USP4 or global knockout of USP4. Consistent with in vitro data, USP4 deficiency aggravated cardiac hypertrophy and fibrosis induced by pressure overload, whereas overexpression of cardiac USP4 protected against these pathological consequences. Mechanistically, the beneficial effect of USP4 on cardiac hypertrophy was largely dependent on the blockade of TAK1-(JNK1/2)/P38 signaling based on its deubiquitinating activity. Our results provide novel insights...
into the pathogenesis of cardiac hypertrophy, and for the first time suggest that USP4 may be an indispensable endogenous negative regulator of pressure overload–induced cardiac remodeling and dysfunction.

The ubiquitin proteasome system, counterbalanced by 2 of its important components, ubiquitin ligases (E3s) and DUBs, controls many fundamental biological processes by targeting specific substrates. Emerging data indicate the ubiquitin proteasome system is dysregulated and plays a role in cardiac pathologies such as cardiac hypertrophy, ischemic heart injury, and heart failure. In this regard, there has been more focus on investigating the cardiac roles of E3s, and many E3s such as MuRF1/2/3, Atrogin-1, CHIP, and MDM2 have been implicated in the pathogenesis of cardiac hypertrophy. By contrast, data on the roles of DUBs in cardiac hypertrophy are relatively lacking. In particular, although the largest DUB subclass USPs, consisting of ≈60 members, have stimulated the development of various inhibitors for their potential therapeutic value in cancer, only 1 USP member (CYLD) was demonstrated to be involved in the pathogenesis of cardiac hypertrophy. Here, by utilizing global knockout and cardiac-specific transgenic manipulation, we provided the first evidence that USP4, unlike CYLD, which is a mediator of cardiac hypertrophy, represents a cardiomyocyte-specific negative regulator of pathological cardiac hypertrophy. The different roles of USP4 and CYLD in the pathogenesis of cardiac hypertrophy by regulating distinct signaling pathways imply that the USPs family may represent a novel reservoir of signaling molecules involved in maintaining cardiac homeostasis.

Mechanistic studies demonstrated that USP4 exerted antihypertrophic effects by suppressing activation of TAK1 in response to pressure overload. TAK1 is a member of the MEKK family central to many critical physiological processes and is designated as a pivotal strategic point involved in the development of cardiac hypertrophy. Activation of TAK1 was well-documented to pivotally induce cardiac hypertrophic growth at baseline or in response to pressure overload, whereas a recent study reported that cardiac-specific depletion of TAK1 induced cell death and cardiac dysfunction. Such discrepancy may be reconciled by the dual role of TAK1 in the regulation of myocyte survival and hypertrophy. Under physiological circumstances, maintaining adequate levels of TAK1 is indispensable for myocyte survival, whereas hyperactivation of TAK1 stimulates cell enlargement and chamber remodeling, suggesting that TAK1 represents a molecular node in the heart, which requires delicate regulation to maintain cardiac homeostasis. Our results indicated that USP4 depletion dramatically augmented TAK1 activation in response to hypertrophic stimuli, whereas overexpression of USP4 suppressed TAK1 phosphorylation to levels similar to those in sham-operated mice, indicating that USP4 is a potent molecular switch, which dampens the hyperactivation of TAK1 in the setting of cardiac hypertrophy. Furthermore, the administration of a TAK1 inhibitor (5z-7-oxozeaenol), which has been used to demonstrate the specific involvement of TAK1 in diverse conditions, significantly suppressed phosphorylation levels of TAK1 and reversed cardiac hypertrophy in USP4 knockout mice, suggesting that USP4 regulates hypertrophic phenotype in a TAK1-dependent manner. 5z-7-oxozeaenol treatment also significantly suppressed phosphorylation levels of JNK1/2 and p38 in hypertrophied hearts. The inhibitory effect of 5z-7-oxozeaenol on JNK1/2 and p38 might be an indirect effect mediated by TAK1 inhibition because TAK1 is an upstream kinase responsible for the phosphorylation of JNK and p38. Collectively, these findings suggest that cardiac USP4 represents a promising antihypertrophic target, which serves as a potent negative regulator of a central hypertrophic mediator TAK1.

Cardiac fibrosis is another important hallmark of pressure overload–induced pathological cardiac remodeling, which exacerbates cardiac performance by disarranging cardiac muscle fibers. Results of this study surprisingly indicated that cardiomyocyte-specific overexpression of USP4 dramatically reduced interstitial and perivascular fibrosis. This is plausible when considering that TAK1-(JNK1/2)/P38 serves as an important pathway in the fibrotic response by promoting production of profibrotic factors, such as connective tissue growth factor. Here, we observed that cardiomyocyte-specific USP4 overexpression drastically inhibited TAK1-(JNK1/2)/P38 signaling activation and connective tissue growth factor production in hypertrophic myocardium. Connective tissue growth factor is one of the most important profibrotic growth factors that can be transcriptionally induced by hypertrophic stimuli in myocytes and can propagate the fibrotic response as a paracrine factor. Therefore, these lines of evidence imply that cardiomyocyte-specific overexpression of USP4 may decrease cardiac fibrosis in a paracrine manner.

To conclude, we unfolded a novel role of USP4 in regulating pathological cardiac hypertrophy via inhibition of the TAK1-(JNK1/2)/P38 signaling pathway. These findings provide the first evidence for the involvement of USP4 in cardiac hypertrophy, and imply the USPs family may represent a novel reservoir of signaling molecules involved in maintaining cardiac homeostasis. Our results also shed light on the therapeutic potential of USP4 manipulation in the treatment of cardiac hypertrophy. Because therapeutic pharmacological activation of an enzyme is difficult and not an optimal strategy in a disease state where the expression level of the target is low, genetically restoring USP4 levels might be a more reasonable therapeutic strategy for cardiac hypertrophy and heart failure.

**Perspectives**

USPs are DUBs as important as E3 ubiquitin ligases; however, their roles in the pathogenesis of cardiac hypertrophy are less well understood. Our study provides the first direct evidence that USP4, a DUB, is a critical negative regulator of pathological cardiac hypertrophy via inhibition of the TAK1-(JNK1/2)/P38 signaling pathway. These findings expand our understanding of the cardiac roles of USPs, and indicate that maintaining cardiac USP4 levels may be a promising therapeutic strategy for reversing cardiac hypertrophy.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is New?**

- Ubiquitin-specific protease 4 (USP4) expression is downregulated in human failing hearts and pressure overload–induced cardiac hypertrophy in mouse.

- USP4 deficiency exacerbates pressure overload–induced cardiac hypertrophy and cardiac dysfunction, whereas restoration of cardiac USP4 levels attenuates these cardiac structural and functional impairments.

- Administration of 5z-7-oxozeaenol, an inhibitor of the transforming growth factor β–activated kinase 1, reverses pressure overload–induced cardiac hypertrophy and cardiac dysfunction in USP4 knockout mice.

**What Is Relevant?**

- Effective therapeutic strategies to reverse pathological cardiac hypertrophy are limited, and the molecular mechanisms underlying the pathogenesis of cardiac hypertrophy are not fully elucidated.

- Pressure overload–induced animal models of cardiac hypertrophy closely mimic the hypertension-induced cardiac impairments observed in patients.

**This study provides the first evidence for the involvement of USP4 in cardiac hypertrophy, highlighting the USPs family as a novel reservoir of signaling molecules involved in maintaining cardiac homeostasis.**

**Summary**

Pressure overload reduces cardiac USP4 levels, which exacerbates cardiac hypertrophy and dysfunction via excessive activation of the transforming growth factor β–activated kinase 1 (TAK1/2)/p38 signaling pathway, whereas restoration of USP4 levels alleviates these pathological consequences. These observations provide clues for the involvement of USPs in maintaining cardiac homeostasis, and imply that restoring USP4 levels may represent a strategy for the treatment of cardiac hypertrophy and heart failure.
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Ubiquitin Specific Protease 4 Is an Endogenous Negative Regulator of Pathological Cardiac Hypertrophy

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Running Title: USP4 alleviates cardiac hypertrophy

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Reagents

Anti-phospho-MEK1/2 (#9154), anti-MEK1/2 (#9122), anti-phospho-ERK1/2 (#4370), anti-ERK1/2 (#4695), anti-phospho-JNK1/2 (#4668), anti-JNK1/2 (#9258), anti-phospho-P38 (#4511), anti-P38 (#9212), anti-TAK1 (#4505), and anti-ubiquitin (#3933) antibodies were obtained from Cell Signaling Technology (Beverly, MA); anti-ANP (#ab91250), anti-β-MHC (#ab50967), and anti-phospho-TAK1 (#ab192443) antibodies were purchased from Abcam (Cambridge, MA); anti-USP4 (#SC292321) and anti-GAPDH (#SC25778) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-HA (H6908) and anti-Flag (F3165) antibodies were obtained from Sigma (St. Louis, MO, USA). Fetal calf serum (FCS) was obtained from HyClone (Logan, UT). The bicinchoninic acid (BCA) protein assay kit was from Pierce (Rockford, IL).

Generation of global USP4 knockout mice

The animal protocol was approved by the Institute’s Animal Ethics Committee of Shanghai Jiao Tong University, and the investigation complied with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Global USP4 knockout mice were generated using CRISPR/Cas9 methods (Supplemental Figure S1). Briefly, guide sequences for the USP4 gene target site in the mouse genome were predicted using an online CRISPR design tool (http://crispr.mit.edu). A pair of oligomers (oligo1: TAGGGGTATCTTATTGACAGCCGG and oligo2: AAACCCGGCTGTCAATAAGATACC) were annealed and cloned into the BsaI restriction site of the Puc57-sgRNA expression vector (Addgene, 51132). DNA was amplified by PCR with primers spanning the T7 promoter and sgRNA regions (F primer: GATCCCTAATACGACTCACTATAG; R primer: AAAAAAGCACCAGCTCGGT). After purification, sgRNA was transcribed using the MEGShorntscript Kit (Ambion, AM1354) and purified using the miRNeasy Micro Kit (QiaGen, 217084). The Cas9 expression plasmid (Addgene 44758) was linearized with PmeI and used as the template for in vitro transcription using the T7 Ultra Kit (Ambion, AM1345). Cas9 and sgRNA mRNA injections of one-cell embryos were performed using the FemtoJet 5247 microinjection system (Eppendorf, Hamburg, Germany) under standard conditions. Genomic DNA from mouse tail was extracted by phenol-chloroform and alcohol precipitation. A 372-bp DNA fragment that overlaps the sgRNA target site was amplified with PCR using the following primers: Forward (5'-CCCCCAAACGTGCAAAACATAATGC-3') and Reverse (5'-GTTAGAGATACACGCAGCCCC-3'). The purified PCR product was denatured and re-annealed in NEB Buffer 2 (NEB) to form heteroduplex DNA, which was subsequently digested with T7EN (NEB, M0302L) for 45 minutes and analyzed on a 3.0% agarose gel. These mice were sequenced to select for frameshift mutations. The primers Forward (5'-GGAGAGCTGCAAAACATAATGC-3') and Reverse (5'-CAGCTGTCAAAGCCACATA-3') were used to screen F1 and F2 offspring.
The PCR products were analyzed using 3.0% agarose gel electrophoresis. The wild-type (WT) allele yielded an amplicon of 149 bp, whereas the mutant allele yielded an amplicon of 126 bp. USP4 knockout did not significantly alter mRNA levels of other USP members (Supplemental Figure S2) and proteasomal activity (Supplemental Figure S3) in heart compared with wild type mouse heart. In addition, the levels of total protein ubiquitination were slightly higher in USP4 knockout hearts compared with wild type hearts (Supplemental Figure S4).

**Generation of cardiac-specific USP4 transgenic (USP4-TG) mice**
Cardiac-specific overexpression of USP4 was achieved by mating mice carrying a pCAG-loxP-CAT-loxP-USP4 transgene with α-MHC-MerCreMer transgenic mice (Jackson Laboratory, 005650). Briefly, to generate the pCAG-loxP-CAT-loxP-USP4 transgenic mice, vector of pCAG-loxP-CAT-loxP-USP4 was constructed by replacing the lacZ gene in pCAG-loxP-CAT-loxP-lacZ with the cDNA of full-length mouse USP4. The construct was linearized and purified using the QIAquick Gel Extraction Kit (Qiagen, 28704), and was then used for pronuclear microinjection. Founder transgenic mice were identified by tail DNA amplification and then bred with C57BL/6J mice. Tail genomic DNA was identified using polymerase chain reaction (PCR). Primers for PCR include CAG gene promoter-forward (F) (5'-CCCCCTGAACCTGAAACATA-3') and USP4-reverse (R) (5'-ACCCCGGCTGTCAATAAGA-3'). The expected size for the amplification product was 426 bp. After mating these two transgenic mice, four independent double transgenic lines (CAG-CAT-USP4/MEM-Cre) were established. To commence USP4 overexpression, these double transgenic mice (6-week) were injected intraperitoneally with tamoxifen (80 mg/kg per day, Sigma, T-5648) for five consecutive days to induce Cre-mediated CAT gene excision. The double transgenic mice without tamoxifen administration (NTG) served as control.

**Surgical procedure of aortic banding (AB) and TAK1 inhibitor treatment**
Cardiac hypertrophy was induced by aortic banding (AB) according to previously described methods (1). In brief, the left chest of each mouse was opened at the second intercostal space to expose the thoracic aorta under adequate anesthesia (pentobarbital sodium, i.p., 50 mg/kg, Sigma) confirmed by the absence of a toe pinch reflex. Subsequently, the exposed aorta was tied against a 27G or 26G needle with a 7-0 silk suture depending on the body weight. After ligation, the needle was removed gently. Sham surgery followed a similar procedure without ligation. A technician blinded to the mice strain performed all surgeries and subsequent analyses. When needed, a TAK1 inhibitor, 5z-7-ox dissolved in dimethyl sulfoxide (DMSO) or DMSO alone was administered by intraperitoneal injection (5mg/kg) every 3 days for 4 weeks following AB surgery.

**Histological analysis**
Four weeks after AB surgery, mice were sacrificed under adequate anesthesia, and the heart, lung and tibia were collected and weighed for further analysis. Hearts were
fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE), fluorescein isothiocyanate-conjugated wheat germ agglutinin (WGA), or picrosirius red (PSR) to assess myocyte hypertrophy and cardiac fibrosis, respectively. Following photography, the cross-sectional area of myocytes and fibrotic ratio were measured using a digital image analysis system (Image-Pro Plus, version 6.0).

**Echocardiographic measurements**
Echocardiography was performed to evaluate cardiac performance. In brief, a Mylab30CV (ESAOTE) ultrasound system with a 15-MHz probe was utilized for echocardiographic assessment. M-mode tracings derived from the short axis of the left ventricle were recorded at the level of the papillary muscles. LV end-diastolic dimension (LVEDd) and LV end-systolic dimension (LVESd) were measured, and the LV fractional shortening (LVFS) was calculated as previously described (2).

**Culture of neonatal rat ventricular myocytes and adenovirus transfection**
Neonatal rat ventricular myocytes (NRVMs) were isolated from 1-day-old Sprague-Dawley rats as previously described (2, 3). To silence USP4 expression, we constructed adenovirus harboring USP4 short hairpin RNA (shUSP4). The non-targeting shRNA was used as a control. To overexpress USP4 expression, we constructed adenoviruses carrying sequences encoding rat full-length USP4 (AdUSP4) and inactive USP4 mutant AdUSP4 (C311A), and an adenoviral vector encoding the green fluorescent protein (GFP) gene served as a control. NRVMs were infected with corresponding adenoviruses at a multiplicity of infection (MOI) of 100 particles/cell for 24 hours, followed by subsequent experiments.

**Immunofluorescence analysis**
To assess the cell surface area of NRVMs, immunofluorescence staining was performed. Following induction of cellular hypertrophy, NRVMs infected with corresponding adenoviruses were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 30 min and stained with α-actinin (1:100 dilution), followed by a fluorescent secondary antibody. The cellular size was measured using Image-Pro Plus 6.0 software.

**Immunoprecipitation**
For immunoprecipitation, pcDNA5-Flag-TAK1 was generated by cloning the mouse TAK1 gene into pcDNA5-Flag-C1, and pcDNA5-HA-USP4 was generated by cloning the mouse USP4 gene into pcDNA5-HA-C1. HEK293T cells were co-transfected with the indicated plasmids. At 48 h after transfection, cells were lysed with lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, and 1 mM EDTA) in combination with Protease Inhibitor Cocktail Tablets (04693132001, Roche). After being pre-cleared with immunoglobulin G and protein A/G-agarose beads (11719386001 and 11719394001, Roche), lysates were incubated with the indicated primary antibodies and Protein G-agarose (11243233001, Roche) at 4 °C overnight.
The immunoprecipitated proteins were washed five or six times with lysis buffer, boiled with SDS loading buffer, and subjected to Western blot analysis with the indicated primary and secondary antibodies.

**Ubiquitination assay**

The ubiquitination assay was performed as previously described (4). Briefly, cells were lysed with lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% SDS) in combination with Protease Inhibitor Cocktail Tablets (04693132001, Roche) and subsequently denatured. The supernatant fluids were diluted with lysis buffer containing Protease Inhibitor Cocktail Tablets. The supernatant fluids were subjected to immunoprecipitation after centrifugation at 20,000 rpm for 25 min at 4 °C.

**Western blot and quantitative real-time PCR**

Following extraction and determination of concentration of total protein from heart tissues and primary cells in lysis buffer (5, 6), 30-40 micrograms of protein were subjected to SDS-PAGE (Invitrogen), transferred to a polyvinylidene fluoride membrane (Millipore), and incubated with corresponding primary antibodies overnight at 4 °C. After incubation with Peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, at 1:10000 dilution), bands were visualized with The Bio-Rad ChemiDoc™ XRS+ (Bio-Rad). Background-subtracted densities of bands of each protein were normalized to corresponding GAPDH bands, and expressed as fold change over sham group. Total mRNA was extracted using TRIzol reagent (Invitrogen), and converted to cDNA using oligo (dT) primers with the Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative real-time PCR amplification was performed using SYBR Green (Roche). Gene expression was normalized against corresponding GAPDH gene levels. The primers used for real-time PCR are presented in Supplemental Table S1.

**Human heart samples**

Human failing hearts samples were obtained from the patients with dilated cardiomyopathy (DCM) who underwent heart transplant surgery. Non-failing heart samples were collected from healthy donor hearts, which were not suitable for transplantation. Information of normal donor hearts and human DCM hearts is presented in Supplemental Table S2 in the online-only Data Supplement. Informed written consent was obtained from the families of the patients and prospective heart donors. All the studies involving human samples were approved by the Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiaotong University, and conformed to the principles outlined in the Declaration of Helsinki.

**Statistical analysis**

Data are presented as the mean ± standard deviation (SD). Comparisons between two groups were performed using a two-tailed Student’s t-test. One-way ANOVA with the Bonferroni post hoc test (equal variances assumed) test or the Tamhane post hoc test.
(equal variances not assumed) was used to determine differences among multiple groups. P values < 0.05 (2-tailed) were considered significant.

References
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### Supplemental Table S2

**Characteristics of normal heart donors and DCM patients**

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<th>Digoxin</th>
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DCM: Dilated cardiomyopathy; LVEF: Left ventricular ejection fraction; LVEDD: Left ventricular end-diastolic diameter. F: Female; M: Male. N/A: not available. Y: Yes; N: No.
Supplemental Figure S1. Construction of ubiquitin specific protease 4 (USP4) global knockout mice using the CRISPR-Cas9 method. (A) One single guide RNA (sgRNA) that targeted a region downstream of the 5′ end of exon 2 in the USP4 mouse gene was constructed. (B) Evaluation of
successful deletions in founder mice. Genomic DNA was extracted from tails of founder mice, and a DNA fragment that overlaps the sgRNA target site was amplified and digested with T7EN. Results of genotyping suggested non-homologous deletions in the Usp4 locus in several lines (existence of uncut bands). (C) Mice with non-homologous deletions in the Usp4 locus were sequenced to confirm frameshift mutations. (D) Termination codon sites in the USP4 mouse gene in founder line #14-8 (allel2) and wild type (WT) C57BL/6J mouse were predicted using SnapGene software (GSL Biotech, available at snapgene.com). The termination codon appeared at 962aa in WT mice (top panel), while a termination codon at 44aa was observed in the tested founder line (bottom panel). (E) Founder #14-8 (allel2) was mated to C57BL/6J mice to obtain the F1 offspring. Heterozygous F1 offspring were interbred to generate the USP4-depleted (KO) mice strain. (F) Representative western blot bands for USP4 expression in hearts from WT and KO mice.

**Supplemental Figure S2**

![Graph](image)

**Supplemental Figure S2.** Transcription levels of USP members in wild type and USP4 knockout mice heart. Transcription levels of USP members were measured by real-time quantitative PCR, and the results indicated that several USPs were not detectable in wild type mice heart (USP17, USP26, USP29, USP34, USP44, USP50 and USP51), and that USP4 knockout did not significantly alter mRNA levels of other USP members.

**Supplemental Figure S3**

![Bar graph](image)

**Supplemental Figure S3.** The effect of USP4 knockout on proteasomal activities in heart.
Proteasomal activity was determined by measuring both chymotryptic (A) and tryptin-like activity (B) in heart from KO and WT mice. WT, wild type; KO, USP4 knockout; sham, sham-operated.

**Supplemental Figure S4**

*Supplemental Figure S4.* Levels of total protein ubiquitination analyzed by Western blot in indicated groups. Levels of total protein ubiquitination were slightly higher in USP4 knockout hearts compared with wild type hearts at baseline (sham) or after aortic banding (AB) surgery. WT, wild type; KO, USP4 knockout; sham, sham-operated; AB, aortic banding surgery.