Novel Protective Role for Ubiquitin-Specific Protease 18 in Pathological Cardiac Remodeling

Xiaoying Ying,* Yichao Zhao,* Tianbao Yao, Ancai Yuan, Longwei Xu, Lingchen Gao, Song Ding, Hongyi Ding, Jun Pu, Ben He

Abstract—Ubiquitin-specific protease 18 (USP18), a USP family member, is involved in antiviral activity and cancer inhibition. Although USP18 is expressed in heart, the role of USP18 in the heart and in cardiac diseases remains unknown. Here, we show that USP18 expression is elevated in both human dilated hearts and hypertrophic murine models. Cardiomyocyte-specific overexpression of USP18 in mice significantly blunted cardiac remodeling as evidenced by mitigated myocardial hypertrophy, fibrosis, ventricular dilation, and preserved ejection function, whereas USP18-deficient mice displayed exacerbated cardiac remodeling under the same pathological stimuli. Similar results were observed for in vitro angiotensin II–induced neonatal rat cardiomyocyte hypertrophy. The antihypertrophic effects of USP18 under hypertrophic stimuli were associated with the blockage of the transforming growth factor-β–activated kinase 1-p38/c-Jun N-terminal kinase 1/2 signaling cascade. Blocking transforming growth factor-β–activated kinase 1-p38/c-Jun N-terminal kinase 1/2 signaling with a pharmacological inhibitor (5Z-7-oxooazaenol) greatly reversed the detrimental effects observed in USP18-knockout mice subjected to aortic banding. Our data indicate that USP18 inhibits cardiac hypertrophy and postpones cardiac dysfunction during the remodeling process, which is dependent on its modulation of the transforming growth factor-β–activated kinase 1-p38/c-Jun N-terminal kinase 1/2 signaling axis. Thus, USP18 is a potent therapeutic target for heart failure treatment. (Hypertension. 2016;68:1160-1170. DOI: 10.1161/HYPERTENSIONAHA.116.07562.) ● Online Data Supplement

Key Words: angiotensin II ■ cardiac remodeling ■ heart failure ■ hypertyrophy ■ signal transduction

Cardiovascular diseases such as hypertension and aortic stenosis impose a hemodynamic overload on ventricular walls, activating complicated signaling transduction pathways and biological responses, leading to cardiac remodeling.1,2 Cardiac remodeling starts as compensatory left ventricular hypertrophy but eventually evolves into maladaptive remodeling, triggering the transition to heart failure.3 Numerous intracellular signaling pathways are involved in this pathological process, resulting in fetal gene re-expression, cardiac myocyte enlargement, and fibrosis, which all facilitate the development of end-stage heart failure.4,14 Elucidating the mechanisms underlying this pathological process and discovering more selective targets is a future endeavor in the treatment of heart failure.

Ubiquitin-specific protease 18 (USP18) is a USP family member.5 As an interferon-stimulated gene, USP18 inhibits type I interferon signaling, thus forming a negative feedback loop.6 USP18 also functions as an isopeptidase responsible for cleaving the ubiquitin-like protein ISG15 and removing polyubiquitin chains from specific substrates.7,8 USP18 is highly expressed in hematopoietic organs such as the thymus and the fetal spleen.5,9 The thorough dissection of USP18 unraveled its role in antiviral activity and cancer inhibition.10–12 Intriguingly, the gene encoding human USP18 is located on chromosome 22q11.2 in a minimal region deleted in DiGeorge syndrome, which is characterized by immune deficiencies and congenital cardiac abnormalities.9 Emerging evidence has shed light on the relevance of USP18 to several well-known signaling pathways that participate in cardiac remodeling, including nuclear factor κB, calcineurin–nuclear factor of activated T cells, transforming growth factor-β–activated kinase 1 (TAK1) signaling.10,13 The functional impact on these pivotal signaling pathways in the heart suggest that USP18 may play a novel role in cardiac pathophysiology. Although USP18 expression is evident in the heart,9 its function remains unexplored. Thus, a detailed analysis of the consequences of USP18 ablation and overexpression in hearts and the mechanisms by which USP18 is involved in this remodeling process will provide insight.
USP18-deficient mice were first generated on a C57BL/6 and 129S mixed background. These mice displayed neurological disorders and died within 20 weeks.14 Whereas, USP18 null mice on a pure C57BL/6 background were embryonically lethal.15 USP18-knockout (KO) mice on an FVB (Friend Virus B) background spontaneously developed tumors between 6 and 12 months.16 In this study, we generated USP18-KO mice on a CD1 background; these mice were viable and did not exhibit apparent abnormalities at baseline. The USP18-KO CD1 mice together with the USP18 cardiac-specific transgenic (TG) mice on a C57BL/6 background were then used to determine the role of USP18 in pathological cardiac remodeling. USP18 overexpression mitigated cardiac hypertrophy, alleviated pathological fibrosis, and eventually postponed cardiac dysfunction. Furthermore, our results indicate that USP18 functions as an inhibitor of pathological cardiac remodeling by modulating TAK1-dependent p38 and c-Jun N-terminal kinase 1/2 (JNK1/2) signaling.

Materials and Methods
Detailed methods are available in the online-only Data Supplement.

Generation of Cardiac-Specific USP18-Transgenic (USP18-TG) Mice
To generate USP18-TG mice, cDNA containing full-length mouse USP18 was cloned, and the transgene vector pCAG-loxP-CAT-loxP-USP18 was constructed by replacing the lacZ gene in pCAG-loxP-CAT-loxP-lacZ with the cDNA of full-length mouse USP18. CAG-CAT-USP18 TG mice was produced by microinjecting this construct into fertilized C57BL/6J mouse embryos.17 To induce USP18 expression specifically in the heart, CAG-CAT-USP18 TG mice were bred with mice that carried the Cre gene under the control of the cardiac-specific α-myosin heavy chain (α-MHC) promoter. Cardiac-specific expression of the USP18 was induced in CAG-CAT-USP18/α-MHC-Cre TG mice by tamoxifen treatment, which allowed Cre-mediated CAT gene excision. Tail genomic DNA was amplified by polymerase chain reaction with the following primers: forward: 5′-CCCCCTGAACTGAAACATA-3′; reverse: 5′-TCCGAAGGCACGTGTATCCTC-3′. The expected size for the amplification product was 601 bp. Western blot analysis was used to confirm the protein expression of USP18 (Figure S1).

Statistical Analysis
All of the data are expressed as the mean±SD.18 We used SPSS 19.0 software to perform the statistical analysis in this study. Student unpaired t tests (between 2 groups) and 1-way ANOVA (for >2 groups) followed by Bonferroni (equal variances assumed) or Tamhane T2 (equal variances not assumed) were used where applicable. P<0.05 was considered statistically significant.

Results
USP18 Is Functionally Expressed in Human and Murine Hearts
We first investigated the cellular localization of USP18, which showed mainly cytoplasm distribution (Figure S6). We then evaluated whether the expression of USP18 is altered under pathological stimuli. Western blot analysis showed significant increase in the expression of hypertrophic markers (atrial natriuretic factor and β-MHC) in hearts of patients with dilated cardiomyopathy (Figure 1A). Moreover, we observed markedly increased USP18 protein levels in dilated cardiomyopathy hearts compared with normal donor hearts (Figure 1B). Furthermore, treatment with the angiotensin II (Ang II) agonist induced USP18 expression in cultured neonatal rat cardiomyocytes (NRCMs) (Figure 1C).
(Figure 1A). Next, we analyzed USP18 expression in pressure overload–induced hypertrophic murine models. The cardiac USP18 expression in the aortic banding (AB)–treated mice was ≈2-fold higher after AB surgery and remained higher even at 8 weeks compared with sham-operated mice (Figure 1B). Consistently, USP18 expression was tripled in neonatal rat cardiomyocytes (NRCMs) after stimulation with angiotensin II for 48 hours (Figure 1C). Collectively, we can conclude that cardiac USP18 expression is elevated during the hypertrophic process, and this alteration may imply functional relevance.

USP18 Inhibits Cardiomyocyte Hypertrophy In Vitro
To test the assumption that USP18 may participate in cardiac hypertrophy, NRCMs were infected with AdshUSP18 to silence USP18 expression or with AdUSP18 to induce USP18 overexpression (Figure 2A), followed by angiotensin II stimulation. NRCMs were assayed by immunofluorescence studies, and we observed that the overexpression of USP18 inhibited cardiomyocyte hypertrophy, whereas silencing USP18 exaggerated the hypertrophic response (Figure 2B through 2F). Meanwhile, USP18 overexpression inhibited atrial natriuretic factor and β-MHC expression, whereas USP18 deficiency augmented it (Figure 2E and 2F). Thus, the in vitro data suggest that USP18 exerted a protective role in the heart with respect to cardiac hypertrophy.

USP18 Ablation Deteriorates Cardiac Remodeling
To further confirm the regulatory effect of USP18 in vivo, we generated systemic USP18 gene KO (USP18-KO) mice (Figure 2). Ubiquitin-specific protease 18 (USP18) inhibits angiotensin II (Ang II)–induced hypertrophy. A, Representative images (left) and quantitative results (right) of USP18 expression in neonatal rat cardiomyocytes (NRCMs) transfected with AdshUSP18 or AdUSP18 (n=3 independent experiments). B, Representative images of NRCMs transfected with AdUSP18 or AdshUSP18 and stimulated with Ang II (1 μmol/L) for 48 h (scale bars=20 μm). Cell surface area of NRCMs transfected with AdshUSP18 or AdUSP18 (C) and AdUSP18 or AdGFP (D), then stimulated with Ang II (1 μmol/L) for 48 h (n=50 cells/group, *P<0.05 vs AdshRNA/PBS or AdGFP/PBS; #P<0.05 vs AdshRNA/Ang II or AdGFP/Ang II). Real-time polymerase chain reaction analysis of atrial natriuretic peptide (ANP) and β-myosin heavy chain (β-MHC) mRNA levels in NRCMs transfected with AdshUSP18 or AdshRNA (E) and AdUSP18 or AdGFP (F), then stimulated with Ang II (1 μmol/L) for 48 h (n=4 independent experiments, *P<0.05 vs AdshRNA/PBS or AdGFP/PBS; #P<0.05 vs AdshRNA/Ang II or AdGFP/Ang II).
Pressure overload by AB caused a marked increase in the heart weight (HW)/body weight ratio, the HW/tibia length ratio, and the HW/lung weight ratio in wild-type (WT) mice, whereas these alterations were augmented in USP18-KO mice subjected to AB (Figure 3A). Functionally, echocardiographic measurements showed enlarged left ventricular end-diastolic and end-systolic dimensions and reduced ejection fraction in USP18-KO mice compared with WT mice after AB surgery (Figure 3B). Morphologically, the cardiomyocytes cross-sectional area was much larger in the USP18-KO mice compared with the AB-operated WT mice (Figure 3C and 3D). In addition, the mRNA levels of hypertrophic markers including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC), collagen I, collagen III, and connective tissue growth factor (CTGF) were significantly increased in USP18-KO mice compared with WT mice after AB surgery (Figure 3F).
atrial natriuretic factor, brain natriuretic peptide, and β-MHC were significantly increased in the AB-operated USP18-KO mice compared with the WT mice (Figure 3F).

Analysis of the picrosirius red–stained heart sections showed marked perivascular and interstitial fibrosis in the USP18-KO AB-operated mice compared with the WT mice (Figure 3C and 3E). The subsequent assessment of collagen volume and fibrotic markers (collagen I, collagen III, and connective tissue growth factor) demonstrated that USP18 ablation worsened the pathological fibrosis in response to AB surgery (Figure 3F). Altogether, these results indicated that USP18 deficiency exaggerated pressure overload–induced remodeling in AB-treated mice.

**USP18 Overexpression Alleviates Cardiac Remodeling**

We next investigated whether the cardiac-specific overexpression of USP18 (USP18-TG) attenuated cardiac remodeling.

![Figure 4. Overexpression of ubiquitin-specific protease 18 (USP18) alleviates aortic banding (AB)–induced cardiac remodeling.](http://hyper.ahajournals.org/)

A, Heart weight (HW)/body weight (BW), HW/tibia length (TL), and lung weight (LW)/BW in the indicated groups after AB surgery (n=12/group). B, Echocardiographic parameters in the indicated groups after AB surgery (n=6–7/group). C, Hematoxylin and eosin (H&E) staining, fluorescein isothiocyanate–conjugated wheat germ agglutinin (WGA) staining, and picrosirius red–staining heart slices from the indicated groups (n=6–7/group, scale bars are as indicated). D, Cross-sectional area of cardiomyocytes in the indicated groups (n=5/group, 100 cells were calculated). E, Collagen volume of fibrotic areas in the indicated groups (n≥25 fields/group). F, Real-time polymerase chain reaction analyses of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC), collagen I, collagen III, and connective tissue growth factor (CTGF) in the indicated groups (n=4/group). *P<0.05 vs nontransgenic (NTG)/sham; #P<0.05 vs NTG/AB. The data are expressed as mean±SD. EF indicates ejection fraction; LVESd, LV end-systolic dimension; and LVEDd, LV end-diastolic dimension.
We generated and established 4 lines of USP18-TG mice and verified the protein expression of USP18 in these TG lines with Western blot assay (Figure S1). TG line 4, with the highest USP18 protein expression, was chosen for subsequent studies. As shown in Figure 4A, USP18-TG mice showed a blunted hypertrophic response under AB surgery, which was indicated by the decreased HW/body weight, lung weight/body weight, and HW/tibia length ratios in the USP18-TG mice compared with the nontransgenic (NTG) mice. Functionally, the echocardiography measurement showed postponed cardiac dilation and dysfunction in USP18-TG mice subjected to AB compared with NTG mice (Figure 4B). In addition, the cardiomyocyte cross-sectional area of the USP18-TG mice was much smaller than that of the NTG mice after AB (Figure 4C and 4D), accompanied by reduced mRNA expression of hypertrophic markers (atrial natriuretic factor, brain natriuretic peptide, and β-MHC) in the TG group compared with the NTG mice (Figure 4F).

Furthermore, the USP18-TG mice exhibited alleviated pathological fibrosis as demonstrated by reduced perivascular and interstitial collagen deposition, reduced collagen volume, and decreased mRNA expression of fibrotic indicators compared with the NTG AB-operated mice (Figure 4C, 4E, and 4F). Therefore, the cardiac-specific overexpression of USP18 protects against cardiac remodeling induced by pressure overload.

**USP18 Regulates TAK1-p38/JNK1/2 Signaling via Deubiquitination of TAK1**

The cardiac mitogen–activated protein kinase (MAPK) signaling pathways can be activated by various stimuli to facilitate maladaptive remodeling. At 4 weeks after AB, the levels of phosphorylated p38 and JNK1/2 were greatly increased in the WT hearts. In addition, the degree of p38 and JNK1/2 activation was markedly augmented in the USP18-KO hearts than in the WT hearts (Figure 5A). Conversely, the AB-induced activation of p38 and JNK1/2 was primarily inhibited in the USP18-TG mice (Figure 5B), which was consistent to our in vitro study (Figure 5C and 5D).

The activation of TAK1 is an upstream event of p38 and JNK1/2 phosphorylation. We, therefore, investigated whether TAK1 activation is regulated by USP18 in current model. Our experiments clearly revealed that the expression level of phosphorylated TAK1 was significantly higher in the USP18-KO mice than in the WT mice subjected to AB surgery (Figure 5A). By contrast, the elevated expression levels of phosphorylated TAK1 after AB were significantly reduced in USP18 TG hearts compared with NTG mice (Figure 5B), which was further confirmed by the in vitro experiment (Figure 5C and 5D). The above results indicated that the antihypertrophic effect of USP18 is associated with the inhibition of TAK1-p38/JNK1/2 activation.

To further investigate the mechanism underlying the effect of USP18 on TAK1 signaling, we first performed co-immunoprecipitation assay to determine whether endogenous USP18 physically interacted with TAK1. As shown in Figure 5E, we observed apparent binding of USP18 with TAK1 and vice versa. Considering that USP18 is a deubiquitinating enzyme, and ubiquitination of TAK1 is essential for its activation, we tested whether USP18 regulated TAK1 ubiquitination in hypertrophied cardiomyocytes. After angiotensin II stimulation for 24 hours, ubiquitination of TAK1 showed more robust augmentation in NRCMs transfected with AdshUSP18 compared with AdshRNA groups. Consistently, NRCMs transfected with AdUSP18 showed the diminished level of ubiquitinated TAK1 in Western blot assay compared with AdGFP group (Figure 5F), suggesting USP18 deubiquitinated TAK1 in hypertrophied cardiomyocytes. Taken together, USP18 may regulate TAK1-p38/JNK1/2 signaling via direct binding with and deubiquitination of TAK1 in current model.

**TAK1 Blockage Ameliorates Cardiac Remodeling in USP18-KO Mice**

These results indicated that the inhibitory effect of USP18 in cardiac hypertrophy was likely depending on the regulation of TAK1 signaling. To confirm these findings, we evaluated whether the abnormalities in USP18-KO mice could be reversed by blocking TAK1 signaling with a pharmacological TAK1 inhibitor (5Z-7-ox) in vivo after AB surgery. Western blot analysis showed that 5Z-7-ox treatment significantly reduced the expression levels of phosphorylated TAK1, p38, and JNK1/2 in hearts compared with dimethyl sulfoxide–treated controls 4 weeks after AB (Figure 6A). Remarkably, 5Z-7-ox treatment rescued the detrimental phenotype of USP18-KO mice in response to pressure overload as shown by the following observations: (1) reduced HW/lung weight, HW/tibia length, and lung weight/body weight ratios (Figure 6B); (2) smaller heart and myocyte size (Figure 6C); (3) decreased cross-sectional myocyte area (Figure 6D); and (4) improved myocardial function (Figure 6F) in 5Z-7-ox-treated USP18-KO mice compared with dimethyl sulfoxide–treated USP18-KO mice on AB surgery. In addition, AB-induced cardiac fibrosis was significantly limited in 5Z-7-ox-treated USP18-KO mice compared with dimethyl sulfoxide–treated USP18-KO mice (Figure 6C and 6E). Thus, the above findings demonstrate that TAK1 inhibition could offset the negative effect of USP18 ablation in response to pressure overload. Taken together, the cardiac protective role of USP18 is likely attributed to the blockage of TAK1 signaling.

**Discussion**

We identified USP18 as an essential component of cardiac remodeling in response to pressure overload. Its overexpression protected hearts against maladaptive hypertrophy, fibrosis, and dilatation, which might contribute to the preserved systolic function because left ventricle dilation and cardiac fibrosis lead to distorted and ineffective contractile performance. By contrast, its ablation hastened the transition toward decompensated heart failure. Mechanistically, we demonstrated that the antihypertrophic effect of USP18 was attributed to the repression of TAK1, thereby suppressing downstream p38 and JNK1/2 signaling, as the decrease in TAK1 activity induced by a TAK1 inhibitor (5Z-7-ox) rescued the exaggerated response of pathological cardiac remodeling in USP18-KO mice. Thus, for the first time, our work defines the functional role of USP18 in the heart and indicates that USP18 is a promising therapeutic target in treating pathological cardiac remodeling.
Figure 5. Ubiquitin-specific protease 18 (USP18) regulates transforming growth factor-β–activated kinase 1 (TAK1)–p38/c-Jun N-terminal kinase 1/2 (JNK1/2) signaling via deubiquitination of TAK1. A, Representative Western blots (top) and quantitative results (bottom) of the phosphorylated and total levels of p38, JNK1/2, and TAK1 in wild-type (WT) and USP18-knockout (USP18-KO) mice. (Continued)
In this study, the upregulation of USP18 expression was observed in both failing human hearts and hypertrophic murine hearts. There are several hypotheses for this increased USP18 protein expression. First, a compensatory response may occur in pathological remodeling. Similar to other negative cardiac regulators, USP18 may increase to play a role in the pathophysiology of cardiac remodeling. This hypothesis is supported by the findings that USP18 expression was higher in failing human hearts and hypertrophic murine hearts compared to normal controls.

Figure 5 Continued. (n=4/group, *P<0.05 vs WT/Sham; #P<0.05 vs WT/AB). B, Representative Western blots (top) and quantitative results (bottom) of the phosphorylated and total levels of p38, JNK1/2, and TAK1 in hearts from nontransgenic (NTG) and transgenic (TG) mice (n=4/group, *P<0.05 vs NTG/sham; #P<0.05 vs NTG/AB). C, Representative Western blots (top) and quantitative results (bottom) showing the phosphorylated and total levels of p38, JNK1/2, and TAK1 in neonatal rat cardiomyocytes (NRCMs) transfected with AdshUSP18 or AdshRNA on the addition of angiotensin II (Ang II; 1 μmol/L) for 60 min (n=3 independent experiments/group, *P<0.05 vs AdshRNA/PBS; #P<0.05 vs AdshRNA/Ang II). D, Representative Western blots (top) and quantitative results (bottom) of the phosphorylated and total levels of p38, JNK1/2, and TAK1 in NRCMs transfected with AdUSP18 or AdGFP on the addition of Ang II (1 μmol/L) for 60 min (n=3 independent experiments/group, *P<0.05 vs AdGFP/PBS; #P<0.05 vs AdGFP/Ang II). The data are expressed as the mean±SD. E, Co-immunoprecipitation analysis for detection of interaction between endogenous USP18 and TAK1 in NRCMs. F, USP18 was precipitated from NRCMs lysates using anti-USP18 antibody, followed by Western blotting analysis performed with antibodies against USP18 or TAK1.

Figure 6. Transforming growth factor-β-activated kinase 1 (TAK1) blockade reverses the cardiac abnormalities in ubiquitin-specific protease 18 knockout (USP18-KO) mice after aortic banding (AB). A, Representative Western blots (top) and quantitative results (bottom) of the phosphorylated and total levels of p38, JNK1/2, and TAK1 in hearts from nontransgenic (NTG) and transgenic (TG) mice (n=4/group, *P<0.05 vs NTG/Sham; #P<0.05 vs NTG/AB). B, Representative Western blots (top) and quantitative results (bottom) showing the phosphorylated and total levels of p38, JNK1/2, and TAK1 in NRCMs transfected with AdshUSP18 or AdshRNA on the addition of angiotensin II (Ang II; 1 μmol/L) for 60 min (n=3 independent experiments/group, *P<0.05 vs AdshRNA/PBS; #P<0.05 vs AdshRNA/Ang II). C, Representative Western blots (top) and quantitative results (bottom) showing the phosphorylated and total levels of p38, JNK1/2, and TAK1 in NRCMs transfected with AdUSP18 or AdGFP on the addition of Ang II (1 μmol/L) for 60 min (n=3 independent experiments/group, *P<0.05 vs AdGFP/PBS; #P<0.05 vs AdGFP/Ang II). The data are expressed as the mean±SD. E, Co-immunoprecipitation analysis for detection of interaction between endogenous USP18 and TAK1 in NRCMs. F, USP18 was precipitated from NRCMs lysates using anti-USP18 antibody, followed by Western blotting analysis performed with antibodies against USP18 or TAK1.
protective role under pathological stimuli. Second, USP18 is expressed at high levels in monocyte/macrophage cell lines. Local macrophage proliferation and monocyte recruitment are enhanced in mammalian hearts facing hemodynamic stress, the elevated USP18 level might be a result of macrophage expansion through monocyte recruitment. In this study, we cannot draw a conclusion on this issue, and further investigation is necessary.

MAPKs are a well-studied family of proteins that play an integral role in cardiac signaling events, which are all activated in end-stage heart failure and contribute to the deterioration of cardiac remodeling. To examine the underlying mechanisms by which USP18 retards cardiac remodeling, we investigated the protein phosphorylation levels of ERK1/2, p38, and JNK1/2 under hypertrophic stimuli and revealed that only p38 and JNK1/2 were further enhanced by the loss of USP18 in response to AB. In addition, cardiac-specific USP18 overexpression almost completely blocked p38 and JNK1/2 activation in response to AB surgery or angiotensin II treatment. P38 and JNK1/2 are collectively called stress-activated MAPKs. The overexpression of MKK6 (p38 activation) and MKK7 (JNK1/2 activation) exhibited the disease-causing effects of these kinases, inducing extreme cardiac dilation and contractile dysfunction. Inhibiting p38 and JNK1/2 signaling with an appropriate pharmacological agent could delay the transition toward heart failure. These results suggest that the heart-protective role of USP18 is likely attributed to its interference with p38 and JNK1/2 signaling.

We further investigated an upstream kinase of p38 and JNK1/2. TAK1 is a key kinase that activates downstream p38 and JNK1/2 kinase and plays a pivotal role during cardiac remodeling. Considering that TAK1 is an upstream regulator of p38 and JNK1/2 kinase, we predicted that USP18 may regulate TAK1 activity. Indeed, our results showed that USP18 deficiency enhanced TAK1 activity, whereas USP18 overexpression displayed the opposite response. TAK1-deficient mice fail to activate MAPKs. In our study, the inhibition of TAK1 activity with a specific TAK1 inhibitor (5Z-7-ox), which has been widely used to demonstrate the specific involvement of TAK1 in diverse conditions, not only reduced the elevated levels of phosphorylated TAK1 and its downstream targets p38 and JNK1/2 but also abrogated the prohypertrophic effect of USP18 deficiency in response to pressure overload. Thus, we conclude that the mechanisms of the USP18-elicited antihypertrophic effects may plausibly be attributed to the inhibition of the TAK1–p38/JNK1/2 axis.

In this study, we found that USP18-mediated cardiac protection was attributed to the inhibition of the TAK1–p38/JNK1/2 signaling axis, which was confirmed by utilizing a TAK1 inhibitor in USP18-KO mice and manifested a reversed effect during pathological remodeling. Modulation of signal transduction involves ubiquitination and deubiquitination. USP18 belongs to the USP family and was shown to have deubiquitinating enzyme activity early in 1999. TAK1 is a ubiquitin-dependent kinase, and the polyubiquitination of TAK1 results in its autoactivation, which is also important for the activation of its downstream MAPK effectors in response to different stimuli. Recently, a study by Liu et al confirmed that USP18 binds to and inhibits the ubiquitination of the TAK1–TAB1 (TAK1-binding protein 1) complex, thereby suppressing TAK1 activation. This was consistent with our findings that USP18 physically interacted with and deubiquitinated TAK1, as well as decreased phosphorylation of TAK1 in hypertrophied hearts. Chen et al discovered that the mutation of Lys562, a key Lys63-linked ubiquitination site of TAK1, leads to decreased TAK1 phosphorylation and the subsequent inhibition of MAPK signaling. Thus, USP18 may also restrain TAK1 activation via its deubiquitinating enzymatic activity in cardiac remodeling. Notably, inflammatory response also plays an indispensable pathological role in the pathogenesis of cardiac hypertrophy and heart failure. Considering that USP18 is an important negative regulator of inflammation via interferon signaling in various organs (eg, pancreas and liver), the regulatory effect of USP18 on inflammation may be another potential mechanism responsible for its protective role in this study, which warrants further study.

Together, our present work provides evidence of a previously unrecognized role for USP18 in protecting hearts against cardiac hypertrophy development in response to pressure overload. We reveal a novel interaction between USP18 and TAK1–p38/JNK1/2 signaling, providing new insights into the molecular basis of cardiac hypertrophy. Our data suggest that USP18 may hopefully delay the development of adverse remodeling after stable cardiac hypertrophy, considering its inhibitory effect against cardiac fibrosis and functional deterioration in current model. However, further study is still warranted to investigate whether USP18 can reverse stable cardiac hypertrophy.

Perspectives
This study provides in vivo and in vitro evidence that USP18 functions as a negative regulator of pathological cardiac remodeling by blocking the TAK1–p38/JNK1/2 signaling pathway. These findings uncover a novel USP18-TAK1–p38/JNK1/2 signaling axis in the hypertrophic hearts, which sheds light on a potential target for the treatment of heart failure.

Acknowledgments
We thank Hong-Liang Li and Ding-Sheng Jiang for providing experimental support with the generation of knockout (KO) and transgenic (TG) mice.

Sources of Funding
This study was supported by the National Natural Science Foundation of China (81500200, 81330006, 81170192, 81470389, and 81270282).

Disclosures
None.

References


40. Chen IT, Hsu PH, Hsu WC, Chen NJ, Tseng PH. Polyubiquitination of Transforming Growth Factor β-activated Kinase 1 (TAK1) at lysine 562
What Is New?

• The ubiquitin-specific protease 18 (USP18) protein level increases in failing human hearts and is upregulated both in vivo and in vitro in response to hypertrophic stimuli.

• USP18 exerts a protective role under remodeling, as evidenced by alleviated hypertrophy, relieved fibrosis, and blunted cardiac dysfunction.

• USP18 negatively regulates the transforming growth factor-β–activated kinase 1-p38/c-Jun N-terminal kinase 1/2 signaling pathway, and blocking transforming growth factor-β–activated kinase 1 offsets the beneficial effects of USP18 in cardiac remodeling.

What Is Relevant?

• Hypertrophic activators have been well investigated, but little is known about the negative modulators of cardiac hypertrophy.

• The role of USP18 in the heart is unexplored.

Novelty and Significance

This work advances our understanding of the important role of the unknown inhibitor that modulates cardiac remodeling by interacting with the well-defined signal transduction, thus providing a novel therapeutic target for heart failure.

Summary

This study clarifies that loss of USP18 exacerbates the hypertrophic response to aortic banding, whereas the overexpression of USP18 negatively regulates pressure overload–induced cardiac hypertrophy, fibrosis, and cardiac dysfunction. The USP18-transforming growth factor-β–activated kinase 1-p38/c-Jun N-terminal kinase 1/2 signaling axis is a key point for USP18 to participate in cardiac remodeling. These findings indicate that USP18 is a novel target for the treatment of heart failure.
Novel Protective Role for Ubiquitin-Specific Protease 18 in Pathological Cardiac Remodeling

Xiaoying Ying, Yichao Zhao, Tianbao Yao, Ancai Yuan, Longwei Xu, Lingchen Gao, Song Ding, Hongyi Ding, Jun Pu and Ben He

Hypertension. 2016;68:1160-1170; originally published online August 29, 2016;
doi: 10.1161/HYPERTENSIONAHA.116.07562

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/68/5/1160

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2016/08/29/HYPERTENSIONAHA.116.07562.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/
ONLINE SUPPLEMENT
Novel Protective Role for Ubiquitin-Specific Protease 18 in Pathological Cardiac Remodeling

Xiaoying Ying¹#, Yichao Zhao¹#, Tianbao Yao¹, Ancai Yuan¹, Longwei Xu¹, Lingchen Gao¹, Song Ding¹, Hongyi Ding¹, Jun Pu¹*, Ben He¹*

¹Department of Cardiology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China.
#These authors contributed equally to this work.

*Correspondence to: Ben He or Jun Pu
Department of Cardiology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University
No.160 Pujian Road, Shanghai 200127, China
Tel: +86-21-68383477
Fax: +86-21-68383609
E-mail: heben1025@hotmail.com or pujun310@hotmail.com
Supplementary Material and Methods

Generation of Cardiac-Specific USP18 Transgenic (USP18-TG) Mice

To generate USP18-TG mice, cDNA that contained full-length mouse USP18 was cloned, and the transgene vector pCAG-loxP-CAT-loxP-USP18 was constructed by replacing the lacZ gene in pCAG-loxP-CAT-loxP-lacZ with the cDNA of full-length mouse USP18. By microinjecting this construct into fertilized C57BL/6J mouse embryos, we produced CAG-CAT-USP18 TG mice. To induce USP18 expression specifically in the heart, CAG-CAT-USP18 TG mice were bred with TG mice that carried the Cre gene under the control of the cardiac-specific α-MHC promoter. Cre-mediated CAT gene excision was induced in 6-week-old CAG-CAT-USP18/α-MHC-Cre TG mice by intraperitoneal injection of tamoxifen (80 mg/kg per day, Sigma) for 5 days, allowing the cardiac-specific expression of the USP18 gene. Tail genomic DNA was amplified by PCR. Western blot analysis was used to confirm the protein expression of USP18. The PCR primers used were as follows: forward: 5’-CCCCCTGAACCTGAAACATA-3’; reverse: 5’-TCCGAGGCCACTGTATTCCTC-3’. The expected size for the amplification product was 601 bp. Four TG lines were generated (Supplemental Figure S1). TG line 4, which had the highest protein expression level, was used in the subsequent studies. In order to eliminate the potential bias caused by tamoxifen treatment associated cardiac toxicity, we set tamoxifen-treated α-MHC Cre mice as controls (Supplemental Figure S2).

Generation of USP18 Knockout (USP18-KO) Mice

The online CRISPR design tool (http://crispr.mit.edu) was used to predict the guide sequences of the target site for the USP18 gene in the mouse genome. A pair of oligomers (oligo1: TAGGGCGGCTCAGCCACAACTGAC; oligo2: AAACGTCAGTTTGCTGAGCCGC) was annealed to form dsDNA and cloned into the BsAI restriction site of the pUC57-sgRNA expression vector (Addgene 51132) to form the pUC57-USP18-sgRNA vector. A DNA fragment spanning the T7 promoter and sgRNA regions was amplified with primers (forward primer: 5’-GATCCCTAATACGACTCACTATAG-3’; reverse primer: 5’-AAAAAAAGCACCAGACTCGGT-3’). After purification, sgRNA was transcribed using the MEGAshortscript Kit (Ambion, AM1354) and purified with the miRNeasy Micro Kit (Qiagen, 217084). A Cas9 expression plasmid (Addgene 44758) was linearized with PmeI and used as the template for in vitro transcription. The FemtoJet 5247 microinjection system was used to inject one-cell embryos with Cas9 and sgRNA mRNA. Genomic DNA from mouse tails was extracted by phenol-chloroform and alcohol precipitation. For F0 offspring genotyping, a 366 bp DNA fragment overlapping the sgRNA target site was amplified with the following primers: USP18-F (5’-GGGTCTATTTGTCTCCGGCTT-3’) and USP18-R (5’-TAAGCACCGAAGTGCTGCT-3’). The purified PCR products were denatured and reannealed in NEB Buffer 2 (NEB) to form heteroduplex DNA which was digested with T7EN (NEB, M0302L) for 45 min and analyzed on a 3.0% agarose gel. The same primers were used to screen F1 and F2 offspring. The PCR products were analyzed by 3.0% agarose gel electrophoresis. The wildtype (WT) allele yielded an amplicon of 366
bp, whereas the mutant allele yielded an amplicon of 314 bp. The USP18 KO mice were obtained from heterozygotes bred for over 5 generations on a CD1 background. WT CD1 littermates were used as controls. Western blot analyses were used to confirm the absence of USP18 protein expression in the USP18-KO mouse hearts (Supplemental Figure S3).

**Reagents**
The following antibodies were obtained from Cell Signaling Technology: p-TAK1 (9339), TAK1 (4505), p-MEK1/2 (9154), MEK1/2 (9122), p-ERK1/2 (4370), ERK1/2 (4695), p-JNK (4668), JNK (9258), p-p38 (4511), p38 (9212), and GAPDH (2118). Antibodies against USP18 (sc98431), ANP (sc20158), and β-MHC (sc53090) were purchased from Santa Cruz Biotechnology. Peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. Cell culture reagents were obtained from Sigma. The BCA protein assay kit was purchased from Pierce.

**Human Heart Biopsy**
Heart samples of failing myocardium were obtained from patients with DCM undergoing heart transplantation. Control hearts that were unsuitable for transplantation were obtained from healthy donors who died in accidents. Informed consent was signed by the patients or the families of the donors. All of the procedures were conducted according to the Declaration of Helsinki and the ethical regulations of the Renji Hospital affiliated with the Shanghai Jiaotong University Review Board, Shanghai, China.

**Animals and Aortic Banding (AB) Surgery**
The protocol for animal experiments was approved by the Animal Care and Use Committee of Renji Hospital affiliated with Shanghai Jiaotong University and were conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The CD1 mice were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China), and the C57BL/6 mice were purchased from The Jackson Laboratory. We used 6 to 8-week-old male mice in our experiments. To establish a pressure overload-induced murine hypertrophic model, AB surgery was performed according to previously described methods. Briefly, the mice were anesthetized peritoneally with pentobarbital sodium (50 mg/kg), and the left chest was opened to dissect the thoracic aorta at the second intercostal space. After tying the thoracic aorta against a 27G needle with a 7-0 silk suture, the needle was promptly removed. We used Doppler analysis for later confirmation of the aortic constriction. The sham-operated group underwent the same procedure except for the aortic constriction.

**TAK1 Inhibitor (5Z-7-ox) Treatment**
The specific TAK1 inhibitor 5Z-7-oxozeaenol (5Z-7-ox; O9890-1 MG; Sigma, St. Louis, MO, USA) was administered intraperitoneally to USP18-KO mice (5 mg/kg) every three days to inhibit TAK1 activation. This dosage selected here was based on our preliminary study, which confirmed 5Z-7-oxozeaenol treatment at 5mg/kg significantly inhibited cardiac TAK1 signaling in hypertrophied heart (Supplemental Figure S4). The control group was administered a similar volume of DMSO.
**Echocardiography Measurements**
To evaluate the cardiac function of the mice, we performed echocardiography measurements at the indicated times as previously described.\(^3\) The mice were anesthetized with 1.5-2% isoflurane before echocardiography. We used an echocardiographic imaging system (Vevo 770; Visual Sonic) in the present study. All of the measurements were obtained from three beats and averaged. LV end-systolic dimension (LVESD) and LV end-diastolic dimension (LVEDD) were assessed at the smallest and largest LV areas, respectively.

**Histological Analysis**
The mice were euthanized at 4 weeks after AB or sham surgery to assess hypertrophic growth and cardiac fibrosis. Paraffin-embedded heart sections were stained with hematoxylin and eosin (H&E) for histopathology or with picrosirius red (PSR) to clarify collagen deposition. To calculate cardiomyocyte cross-sectional areas, the slides were stained with FITC-conjugated wheat germ agglutinin (WGA, Invitrogen). A fluorescence microscope was used for acquiring images, and a digital image analysis system (Image-Pro Plus 6.0) was used to measure individual cell sizes.

**Neonatal Rat Cardiomyocytes Culture and Treatment**
We isolated primary neonatal rat cardiomyocytes (NRCMs) from 24 to 48-hour-old Sprague-Dawley rats as previously described.\(^2\) To assess cardiomyocyte hypertrophy, NRCMs were plated in DMEM/F12 medium containing 20% FCS, BrdU (0.1 mM), and penicillin. The medium was exchanged for serum-free DMEM/F12 before treatment with Ang II (1 μM). The dosage of Ang II used here was based on our preliminary experiments (Supplemental Figure S5). For measurement of the cell surface area, α-actinin (Sigma) and DAPI staining was performed. Image-Pro Plus 6.0 software was used for quantitative analysis.

**Recombinant Adenoviral Vectors**
Recombinant AdUSP18 vectors were generated by introducing rat USP18 cDNA into a replication-defective adenoviral vector. Rat short hairpin USP18 (shUSP18) was obtained from SABiosciences (KR68253G), and AdshUSP18 adenovirus was subsequently generated. AdGFP and AdshRNA were used as controls, and the efficiency and specificity of these adenoviruses were validated. NRCMs were infected with AdUSP18, AdGFP, AdshUSP18, or AdshRNA at a multiplicity of infection (MOI) of 100 particles/cell for 24 hours.

**Immunofluorescence Analysis**
After infection and hypertrophic stimulation, the NRCMs were fixed in formaldehyde for 15 minutes at RT, permeabilized with 0.1% Triton X-100 for 40 minutes, and blocked in 10% BSA solution. Subsequently, the cells were incubated with primary antibodies against α-actin (1:100 dilution), followed by fluorescent secondary antibodies. The cell surface areas were calculated with Image-Pro Plus 6.0 software.

**Western Blot**
Proteins extracted from tissues or cells were homogenized and then prepared as previously described.\(^4\) After sonication, the extracts were incubated on ice for 15 minutes and then centrifuged at 4°C. Lysates (20 μg protein samples) were subjected to SDS-PAGE (Invitrogen) and transferred to a PVDF membrane (Millipore). Five
percent non-fat milk was used for blocking. The membrane was then incubated with the indicated primary antibodies at 4°C overnight, followed by incubation with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, 1:10,000 dilution). A Bio-Rad system was used to detect protein signals. The densitometric values of the bands were measured using densitometry software (Image Lab 5.1.0; Bio-Rad). The specific protein expression levels were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on the same PVDF membrane.

**RT-PCR**

TRIzol reagent (Invitrogen) was used to extract the total RNA from tissues or cells. Reverse transcription was performed with 2 µg of RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche). For quantitative assessment of the indicated genes, SYBR Green (Roche) and a Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA) were used for quantitative real-time PCR analysis. The data are expressed in arbitrary units and normalized to GAPDH mRNA in the same cDNA preparation.

**Immunoprecipitation**

The immunoprecipitation was performed as previously described. Briefly, cultured NRCMs cells were collected and sonicated in an IP buffer (20mM Tris-HCl (pH 8.0), 150mM NaCl, 1mM EDTA and 0.5% NP-40) with protease inhibitor cocktail (Roche). After incubating and centrifuging, the cell lysates were precleared with immunoglobulin G and protein A/G-agarose beads (11719394001, 11719386001, Roche), then incubated with antibody and protein A/G-agarose beads overnight. All the above procedure were performed under 4 °C. The immunocomplex was collected, washed five to six times before blotted with the indicated antibodies. Co-IP for endogenous proteins was performed in the cytoplasmic protein of NRCMs isolated using an NE-PER nuclear and cytoplasmic extraction kit (Pierce, Cat number: 78835).

**Ubiquitination assay**

The ubiquitination assay was performed as previously described. Briefly, cells were lysed with lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% SDS) containing Protease Inhibitor Cocktail Tablets (04693132001, Roche) following manufacturer’s instructions, and then denatured. The supernatant fluids diluted with the above prepared lysis buffer containing Protease Inhibitor Cocktail Tablet subsequently were used for immunoprecipitation.

**Statistical Analysis**

All of the data are expressed as the mean±SD. We used SPSS 19.0 software to perform the statistical analysis in the present study. Student’s unpaired t-tests (between two groups) and one-way ANOVA (for more than two groups) followed by Bonferroni (equal variances assumed) or Tamhane’s T2 (equal variances not assumed) were used where applicable. P<0.05 was considered statistically significant.
References:
**Supplementary Figure S1.** Generation of cardiac-specific USP18 transgenic (USP18-TG) mice. A, Schematic diagram for the construction of the cardiac-specific expression of the USP18-transgenic (USP18-TG) mice. B, Western blot analysis of USP18 levels in the USP18-TG mice and their NTG littermates (n=4/group).
Supplementary Figure S2. Effect of tamoxifen treatment on murine hearts. A, Hematoxylin and eosin (HE)-stained and picrosirius red (PSR)-stained heart slices of the indicated groups (n=5-7/group, scale bars=50 μm for HE, scale bars=100 μm for PSR). B, Heart weight (HW)/body weight (BW) ratio in the indicated groups after sham or AB surgery (n=12/group). C, Cross-sectional area of cardiomyocytes in the indicated groups (n=5/group, 100 cells were calculated). D, Echocardiographic parameters in the indicated groups after sham or AB surgery (n=6-7/group). LVEDd, LV end-diastolic dimension; LVESd, LV end-systolic dimension; FS, fractional shortening. The data are expressed as the mean±SD (N.S: not significant).
Supplementary Figure S3. Schematic diagram of the construction of ubiquitin-specific protease 18 knockout (USP18-KO) mice and identification of USP18 expression. A, A single guide RNA (sgRNA) that targeted a region downstream of the 5′ end of exon 2 in the USP18 mouse gene was constructed. B, The T7E1 assay indicated that five of eight pups contained cleavage products, suggesting a mixture of mutant and wild-type (WT) DNA templates. C, After subcloning the PCR products, eight subclones of each mouse were sequenced. All of the subclones carried a single mutant allele, whereas only two indels (#37-3-3 and #37-5) produced frameshift mutations. D, Founder #37-3 (allel3) was mated to CD1 mice to produce the F1 generation, which were then backcrossed over five generations for the USP18-KO mouse strain. E, Western blot analysis of USP18 expression in the hearts of WT and USP18-KO mice (n=4/group).
Supplemental Figure S4

Supplementary Figure S4. Inhibitory effect of 5Z-7-oxozeaenol (5Z-7-ox) on TAK1 activation at different dosages. Representative Western blots (A) and quantitative results (B) of the phosphorylated and total levels of TAK1 in WT murine hearts after 4 weeks aortic bind (AB) surgery with different dosage of 5Z-7-ox (* p<0.05 vs DMSO; N.S: not significant).
Supplementary Figure S5. Neonatal rat cardiomyocytes (NRCMs) treated with different dosages of Ang II. (TOP), Representative images of NRCMs treated with different dosages of AngII for 48 hours (scale bars=20μm). (Bottom), Cell surface area of NRCMs treated with different dosages of AngII. (n=50 cells/group, *P<0.05 vs PBS; N.S: not significant).
Supplementary Figure S6. The expression of USP18 in neonatal rat cardiomyocytes (NRCMs). Immunofluorescent staining showed that USP18 was mainly expressed in the cytoplasm of cardiocytes. Red: USP18; Blue: DAPI.