ORIGINAL RESEARCH

The E3 Ligase TRIM16 Is a Key Suppressor of Pathological Cardiac Hypertrophy

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BACKGROUND: Pathological cardiac hypertrophy is one of the leading causes of heart failure with highly complicated pathogeneses. The E3 ligase TRIM16 (tripartite motif–containing protein 16) has been recognized as a pivotal regulator to control cell survival, immune response, and oxidativestress. However, the role of Trim16 in cardiac hypertrophy is unknown.

METHODS: We generated cardiac-specific knockout mice and adeno-associated virus serotype 9–Trim16 mice to evaluate the function of Trim16 in pathological myocardial hypertrophy. The direct effect of TRIM16 on cardiomyocyte enlargement was examined using an adenvirus system. Furthermore, we combined RNA-sequencing and interactome analysis that was followed by multiple molecular biological methodologies to identify the direct target and corresponding molecular events contributing to TRIM16 function.

RESULTS: We found an intimate correlation of Trim16 expression with hypertrophy-related heart failure in both human and mouse. Our functional investigations and unbiased transcriptomic analyses clearly demonstrated that Trim16 deficiency markedly exacerbated cardiomyocyte enlargement in vitro and in transverse aortic constriction–induced cardiac hypertrophy mouse model, whereas Trim16 overexpression attenuated cardiac hypertrophy and remodeling. Mechanistically, Prdx1 (peroxiredoxin 1) is an essential target of Trim16 in cardiac hypertrophy. We found that Trim16 interacts with Prdx1 and inhibits its phosphorylation, leading to a robust enhancement of its downstream Nrf2 (nuclear factor–erythroid 2–related factor 2) pathway to block cardiac hypertrophy. Trim16-blocked Prdx1 phosphorylation was largely dependent on a direct interaction between Trim16 and Src and the resultant Src ubiquitinational degradation. Notably, Prdx1 knockdown largely abolished the anti-hypertrophic effects of Trim16 overexpression.

CONCLUSIONS: Our findings provide the first evidence supporting Trim16 as a novel suppressor of pathological cardiac hypertrophy and indicate that targeting the Trim16-Prdx1 axis represents a promising therapeutic strategy for hypertrophy-related heart failure.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: heart failure ▪ hypertrophy ▪ mice ▪ oxidative stress ▪ peroxiredoxin

Cardiac hypertrophy is an adaptive response of the myocardium to mechanically increased workload caused by various diseases.1 Compensatory myocardial hypertrophy helps to increase cardiac work during the early stages of cardiovascular disease, whereas persistent pathological cardiac hypertrophy gradually develops into decompensation, resulting in irreversible heart failure and sudden death.2,3 The pathogenesis of myocardial

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hypertrophy is extremely complex and comprises extensive changes in protein synthesis, cell volume, contractile function, collagen synthesis, and fibrosis. Previous studies from our group and others demonstrated that mechanical stretching, oxidative stress, and inflammation are essential molecular mechanisms underlying those cellular dysfunctions. Despite numerous previous studies, no effective pharmacological interventions for cardiac hypertrophy have been approved. Therefore, the discovery of specific regulatory targets for myocardial hypertrophy will provide new therapeutic strategies for the treatment of this disease.

TRIM (tripartite motif–containing protein) family members are conserved RBCC (RING B-box coiled-coil) domain–containing factors and are characterized by a triple motif structure formed by three domains (tripartite motif)—the zinc finger domain (RING finger), one or two B-boxes and one crimp helix domain. TRIM family is related to a variety of cardiac and pathological processes, such as cardiac development, protein degradation, autophagy regulation, and cardiomyopathy. Notably, the TRIM family is related to a variety of cardiac and pathological processes, such as cardiac development, protein degradation, autophagy regulation, and cardiomyopathy.10–12 These cardio-regulatory functions of TRIMs are mainly dependent on their RING domain. Intriguingly, there is another subfamily of TRIMs without a RING domain, as represented by Trim16 (tripartite motif–containing protein 16). Trim16 was first identified as an EBBP (estrogen-responsive B-box protein) that responds to cardiac hypertrophy. Genetic overexpression Trim16 ameliorates myocardial hypertrophy and fibrosis. Mechanistically, Trim16 promotes Src K48-linked ubiquitination and degradation depending on its E3 ubiquitin ligase activity, leading to inhibited Prdx1 phosphorylation and regulated oxidative stress. Taken together, our study identified Trim16 as an essential suppressor of pathological cardiac hypertrophy.

Therapeutic targets for preventing or reversing the progression of cardiac hypertrophy are scarce. Here, we validated a previously unappreciated E3 ligase Trim16 that responds to cardiac hypertrophy. Genetic overexpression Trim16 ameliorates myocardial hypertrophy and fibrosis. Mechanistically, Trim16 promotes Src K48-linked ubiquitination and degradation depending on its E3 ubiquitin ligase activity, leading to inhibited Prdx1 phosphorylation and regulated oxidative stress. Taken together, our study identified Trim16 as an essential suppressor of pathological cardiac hypertrophy.
possesses transcriptional activity. Further reports identified its function in interleukin-1β secretion and transcriptional regulation of retinoic acid receptor β₃. In addition, Trim16 can interact with and regulate multiple substrates, including galectin-3 and other TRIM family members, through a coiled-coil domain. An increasing number of studies have confirmed the role of the above pathological process in modulating cardiac hypertrophy. We thus hypothesize that Trim16 might closely participate in cardiac hypertrophy, which, however, has not been illuminated.

Here, we provide the first evidence supporting the anti-hypertrophic activity of Trim16 in pathological cardiac hypertrophy. We found that Trim16 markedly restrained the development of cardiac hypertrophy by directly binding to Sra and promoting its K48-type ubiquitination degradation, leading to decreased tyrosine phosphorylation of Prdx1 (peroxiredoxin 1) and improved oxidative stress in cardiomyocytes.

METHODS

Data Availability

Accession Codes
Gene Expression Omnibus: Online data of RNA-sequencing are available in NCBI with BioProject ID: PRJNA763721 and PRJNA763720.

A Methods section and expanded Materials are available in the Supplemental Methods, which involves information on experiments of mouse models with transverse aortic constriction (TAC), adeno-associated virus (AAV) serotype 9-Trim16 mice, and generation of gene knockout mice, echocardiographic analyses, histological analyses, primary neonatal rat ventricular myocyte (NRVM) cell culture, immunofluorescence staining, chromatin immunoprecipitation, real-time polymerase chain reaction, dihydroethidium assay, immunoprecipitation assay, immunoblotting analysis, RNA-sequencing and analysis, immunoprecipitation assays, and in vivo ubiquitination assays, which are listed in the Supplemental Material.

RESULTS

Trim16 Protects Against Cardiac Hypertrophy

To investigate whether Trim16 expression is associated with cardiac hypertrophy, we first detected the expression of Trim16 in the hearts of patients diagnosed with heart failure. Immunoblot assays showed that Trim16 protein levels were dramatically increased in heart failure patients compared with healthy controls (Figure 1A). Consistent with changes in protein levels, the mRNA level of Trim16 was also upregulated in individuals with heart failure compared to the controls (Figure 1B). We further verified the results in a TAC surgery-induced hypertrophic mouse model. Compared with those in the sham groups, the protein and mRNA levels of Trim16 in the TAC surgery groups were dramatically elevated (Figure 1C and 1D), which was further confirmed by the immunohistochemistry results (Figure 1E). As shown in Figure S1A and S1B, Trim16 was significantly increased in NRVMs in response to phenylephrine stimulation.

We further explored the molecular mechanisms of Trim16 expression induced by hypertrophic stimulation. Find individual motif Occurrences was used to predict the transcription factors that could bind to Trim16 in humans and mice. We set a Q<0.05 as the screening standard, filtered
Figure 1. Trim16 (tripartite motif–containing protein 16) is upregulated in cardiac hypertrophy.

A, Immunoblotting and quantitation of TRIM16, MYH7/β-MHC (β-myosin heavy chain), and ANP (atrial natriuretic peptide) protein levels in human heart tissue samples from normal donors (n=3) and heart failure patients (n=6). B, Relative mRNA levels of TRIM16 in human heart tissue samples from normal donors (n=3) and heart failure patients (n=6). C, Immunoblotting and quantitation of Trim16, Myh7, and Anp protein levels in the hearts of mice subjected to sham or transverse aortic constriction (TAC) surgery at the indicated time points (n=3). D, Relative mRNA levels of Trim16 in heart tissues from mice at the indicated time points after sham operations or TAC surgery (n=5). E, Representative images and quantitation of immunohistochemical staining of Trim16 in the hearts of mice subjected to sham or TAC surgery (n=5). Scale bar, 25 μm. F, Transcription factors of EGR2 (early growth response), ESR1 (estrogen receptor protein 1), EWSR1 (EWS RNA binding protein 1), TFAP2A (transcription factor AP2A), TFAP2A (var.2), TFAP2C, RREB1 (ras responsive element binding protein 1), IRF1 (interferon regulatory factor 1), KLF1 (Kruppel-like factor 1), KLF9, ZNF263 (zinc finger protein 263), ZNF384 were predicted to bind to Trim16 by Find Individual Motif Occurrences. Predictive intersection of human (32) and mouse (59). Screening condition Q < 0.05. G, Gene expression of publicly available transcriptomic data obtained in heart tissues of heart failure patients or TAC mice in indicated Gene Expression Omnibus (GEO) database. Dot color indicates the log2-fold fold change in gene expression compared to the control group. H, Relative luciferase activity of the Trim16 promoter transfected with Flag, Flag-EGR2, Flag-ESR1 and Flag-KLF9 plasmids in HEK293T (human embryonic kidney cell) cells (n=4 independent experiments). I, Chromatin immunoprecipitation enrichment of the combination of Egr2 and different regions of the Trim16 promoter in neonatal rat ventricular myocytes (NRVMs) infected with the indicated Ad-Trim16 (adenovirus expressing Trim16) or the corresponding control (Ad-GFP [adenovirus expressing-green fluorescent protein]; n=4 independent experiments). J, Relative mRNA levels of Trim16 in NRVMs infected with Ad-GFP or Ad-Egr2 followed by stimulation with phenylephrine (20 μM) for 24 h (n=4 independent experiments). K, Protein levels of Trim16 in NRVMs infected with Ad-GFP or Ad-Egr2 followed by stimulation with (20 μM) for 24 h (n=3 independent experiments). Data in B are presented as the mean±SD; data in (D and E and H–J) are presented as the median±interquartile range. For statistical analysis, 2-tailed Student t test was used for (B); Mann-Whitney Test was used for E and I and J; and Kruskal-Wallis was used for D and H. PE indicates phenylephrine.
Trim16 Alleviates Phenylephrine-Induced Cardiac Hypertrophy In Vitro

To directly investigate the role of Trim16 in cardiomyocyte enlargement, we constructed an effective adenovirus expressing Trim16 (Ad-Tm16) and shRNA (short hairpin RNA)-targeting Trim16 (Ad-shTrim16) to infect NRVMs (Figure S2A). As shown in Figure 2A through 2C, compared with the corresponding controls, Ad-Tm16 notably inhibited enlargement of the cellular surface area of cardiomyocytes, whereas the cell surface area of cardiomyocytes was markedly increased in the Ad-shTrim16 cell group. To determine the impact of Trim16 at the molecular level, we performed RNA-sequencing analysis of NRVMs infected with Ad-Tm16 and Ad-GFP control virus. The transcriptomic profiles were clearly separated into two clusters via unsupervised hierarchical clustering analysis (Figure 2D). Gene set enrichment analysis revealed that Trim16-regulated genes were enriched in cardiac hypertrophy, protein synthesis, and oxidative response (Figure 2E). The Heat Map demonstrated that Trim16 overexpression significantly inhibited the expression of genes involved in the abovementioned pathways (Figure 2F). The key differentially expressed transcripts detected by RNA-Seq were verified by quantitative real-time polymerase chain reaction (Figure 2G through 2R). Correspondingly, RNA-sequencing analysis in Trim16 knockout cells revealed that pathways and genes related to cardiac hypertrophy, protein synthesis, and oxidative response were substantially enriched and upregulated in the Ad-shTrim16 group (Figure S2B through S2G). These results indicate that Trim16 plays a protective role against cardiomyocyte enlargement in vitro.

Trim16 Inhibits TAC-Induced Cardiac Hypertrophy in Mice

To evaluate the function of Trim16 in pathological myocardial hypertrophy in vivo, we generated a cardiac-specific Trim16 knockout mouse line (Trim16-CKO), and the efficiency of Trim16-CKO was verified by Western blotting (Figure S4A and S4B). Four weeks after TAC, the heart weight/body weight, lung weight/body weight, and heart/tibia length ratios of Trim16-CKO mice were substantially higher than those of Flox mice (Figure 3A). In addition, echocardiographic evaluation of left ventricular function and architecture, including left ventricular end-diastolic diameter, left ventricular end-systolic diameter, fraction shortening, and ejection fraction, further verified the worsened cardiomegaly and reduced myocardial function in Trim16-CKO mice compared with wild-type controls (Figure 3B). Trim16-CKO mice also showed a larger heart size and cross-sectional area of cardiomyocytes in the TAC-induced cardiac hypertrophy model (Figure 3C). Furthermore, Trim16-CKO mice showed obviously exacerbated TAC-induced cardiac fibrosis, as shown by Picosirius Red staining of heart sections (Figure 3D). The expression of genes related to cardiac hypertrophy (Anp [atrial natriuretic peptide], Bnp [β-type natriuretic peptide], Myh7/β-MHC [β-myosin heavy chain]) and fibrosis (Col1a1 [collagen type I alpha 1], Col3a1 [collagen type III alpha 1], Ctgf [connective tissue growth factor]) was also obviously upregulated in Trim16-CKO mice (Figure 3E through 3F). Overall, deletion of cardiomyocyte Trim16 aggravates pressure overload–induced cardiac hypertrophy. The exacerbat- ing effect of Trim16 knockout was consistently observed in global Trim16-knockout mice (Figure S3).

The functional role of Trim16 in pathological cardiac hypertrophy was further validated in mice with AAV serotype 9–delivered Trim16 specific overexpression in cardiomyocytes (AAV serotype 9–Trim16; Figure S4C–S4E). The cardiomyocyte specificity of AAV serotype 9–delivered Trim16 overexpression was enhanced by the cTnT promoter. In contrast to our observations in Trim16-CKO mice, Trim16 overexpression effectively inhibited TAC surgery-induced cardiac hypertrophy, heart dysfunction, and heart remodeling (Figure 4A through 4J).

Trim16 Directly Binds to Prdx1 and Inhibits Its Phosphorylation during Cardiac Hypertrophy

Given the essential role of Trim16 in pathological cardiac hypertrophy, we tried to uncover the molecular mechanism of Trim16 in the regulation of myocardial hypertrophy. We performed combined analysis of RNA-sequencing data and immunoprecipitation–mass spectrometry analysis to identify the potential targets of Trim16 (Figure 5A). First, volcano diagrams revealed the differentially expressed genes regulated by Trim16 with a reverse expression trend of 2 pairwise comparisons Ad-Tm16 versus Ad-GFP; Ad-shTrim16 versus Ad-vector [adenovirus expressing blank vector]; Figure 5B). Immunoprecipitation–mass spectrometry data suggested the interaction of Trim16 with Atp5a1 (ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1), Prdx1, desmin, Cs (citrate synthase), Hnrnpa2b1 (heterogeneous nuclear ribonucleoprotein...
Figure 2. Trim16 (tripartite motif–containing protein 16) overexpression alleviates phenylephrine-induced cardiac hypertrophy in neonatal rat ventricular myocytes (NRVMs).

A, Representative immunofluorescence images of α-actinin staining of NRVMs infected with Ad-GFP (adenovirus expressing-green fluorescent protein) or Ad-Trim16 (adenovirus expressing Trim16) and treated with phenylephrine (20 μM) or PBS for 24 h. Scale bar, 25 μm. PE, phenylephrine; DAPI, 4’,6-Diamidino-2-phenylindole.

B, Representative immunofluorescence images of α-actinin staining of NRVMs infected with Ad-vector (adenovirus expressing blank vector) or Ad-shTrim16 (adenovirus expressing short hairpin RNA targeting Trim16) and treated with phenylephrine (Continued)

C, Box plots showing the expression levels of selected genes in NRVMs infected with Ad-GFP or Ad-Trim16 and treated with phenylephrine or PBS. *p < 0.05.

D, GSEA analysis showing enrichment of muscle tissue development, oxidative response, and protein synthesis pathways in Ad-Trim16-treated NRVMs compared to Ad-GFP-treated NRVMs.

E, Gene expression heatmap showing the expression changes of various genes in Ad-Trim16-treated NRVMs compared to Ad-GFP-treated NRVMs.

F, Heatmap showing the expression changes of genes involved in protein synthesis and oxidative response pathways in Ad-Trim16-treated NRVMs compared to Ad-GFP-treated NRVMs.

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A2/B1), Idh2 (isocitrate dehydrogenase NADP+2) and Nnt (nicotinamide nucleotide transhydrogenase) (Figure 5C). To further identify the downstream targets of Trim16, we constructed a protein-protein interaction network-based connection between Trim16-binding proteins and cardiac hypertrophy-related genes affected by Trim16 from RNA-sequencing (Figure 5D). Among these candidates, Prdx1 belongs to the superfamily of antioxidant protein and has been reported closely related to pathological myocardial hypertrophy, we thus speculated that Prdx1 might be required for Trim16 function.

We then validated the interaction of Trim16 and Prdx1 by confocal imaging as well as exogenous immunoprecipitation experiments and endogenous immunoprecipitation assays (Figure 5E through 5G). In addition, a GST (glutathione S-transferase) pull-down assay confirmed the direct interaction between Trim16 and Prdx1 (Figure 5H). We further showed that the N-terminal B-box region (amino acids 1-165) of Trim16 was necessary for its interaction with Prdx1 (Figure 5I). Because the phosphorylation modification of Prdx1 predominantly and negatively mediates its activity,30,31 we explored whether Trim16 could affect Prdx1 phosphorylation. Notably, Trim16 significantly decreased the phosphorylation level of Prdx1 in a dose-dependent manner in NRVMs exposed to phenylephrine (Figure 5J).

**Trim16-Prdx1 Axis Effectively Regulates Oxidative Stress and Cardiomyocyte Enlargement**

To further clarify the role of Prdx1 in cardiac hypertrophy, we detected the expression of p-Prdx1 (phosphorylation-Prdx1) in human heart failure samples and mouse TAC samples. The results showed that p-Prdx1 was significantly activated in heart failure samples and mouse heart tissues after TAC operation (Figure S5A and S5B). In addition, we found that Prdx1 played a protective role against cardiomyocyte enlargement, as overexpression and knockdown of Prdx1 reduced and increased the cell surface area of NRVMs, respectively (Figure S5C and S5D). As previously reported, Prdx1 robustly inhibits oxidative stress that promotes pathological cardiac hypertrophy.32 As shown in Figure S5E and S5F, overexpression of Prdx1 reduced the level of oxidative stress, whereas deletion of Prdx1 made oxidative stress more severe, as visualized by dihydroethidium staining. We found that Prdx1 significantly upregulated the expression of Nrf2 (nuclear factor–erythroid 2–related factor 2) and HO-1 (heme oxygenase-1), two well-recognized antioxidative stress factors,32–34 in cardiomyocytes (Figure S5G and S5H).

Consistent with the function of Prdx1 in oxidative stress, **Trim16 overexpression significantly reduced dihydroethidium-positive signals, and Trim16 knockdown or Trim16 knockout produced the opposite result (Figure S5I and S5J, Figure S6A).** Furthermore, malondialdehyde content was reduced by **Trim16 overexpression** (Figure S6B and S6C) but SOD (superoxide dismutase) activity was enhanced. **Trim16 knockdown produced the opposite result**. In contrast, oxidative stress was markedly aggravated by Trim16 knockdown or knockout (Figure S6D and S6E). The results of endogenous experiments confirmed that Trim16 interacts with Prdx1 and inhibits its phosphorylation, leading to a robust enhancement of its downstream targets (Nrf2 and HO-1) in the mouse heart (Figure S6F and S6G).

Consistently, we observed that Trim16 increased Nrf2 and HO-1 expression both in vitro and in vivo (Figure S5K and S5L, Figure S6F through S6J). To further verify whether the function of Trim16 in cardiac hypertrophy is oxidative stress dependent, we pretreated cardiomyocytes with the reactive oxygen species scavenger N-acetylcysteine followed by adenovirus-mediated Trim16 knockdown and phenylephrine challenge. Notably, N-acetylcysteine administration significantly abolished the exacerbating effect of Trim16 knockdown on cardiomyocyte enlargement (Figure S6K). Furthermore, the role of Trim16 knockdown in enhancing Anp and Myh7 expression was also largely reversed by N-acetylcysteine treatment (Figure S6L through S6N). These data clarified that **TRIM16-Prdx1 axis-induced protection against pathological cardiac hypertrophy was largely dependent on the blockage of oxidative stress.**

**Trim16 Inhibits Prdx1 Phosphorylation by Promoting Src Degradation**

Considering the intrinsic mechanism of the Trim16-Prdx1 axis on cardiomyocyte enlargement, we next detected the influence of Trim16 on Prdx1 ubiquitination status. However, Trim16 did not show any significant regulation of Prdx1 ubiquitination (Figure 6A and S7A). Notably, when the ubiquitinating activity of Trim16 was removed (Trim16-DUB [Trim16-deubiquitinase fusion proteins]; Figure 6B),35,36 the effective regulatory effect of Trim16 on Prdx1 phosphorylation almost completely disappeared (Figure 6C). These

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**Figure 2 Continued.** (20 μM) or PBS for 24 h. Scale bar, 25 μm. C, Quantitative results of the cell surface area **(top)** of NRVMs infected with Ad-GFP/Ad-Trim16 or Ad-vector/Ad-sh Trim16 **(bottom)** followed by treatment with phenylephrine (20 μM) or PBS for 24 h (n ≥ 50 cells per group). D, Hierarchical clustering analysis showing the global sample distribution profiles between groups based on the RNA-seq (RNA-sequencing) data. E, GESA (gene set enrichment analysis) of molecular events involved in cardiac hypertrophy, protein synthesis, and oxidative response in RNA-seq data. F, Heatmaps showing the significantly altered genes related to cardiac hypertrophy, NES, normalized enrichment score. G–R, Relative mRNA levels of cardiac hypertrophy (G–J), protein synthesis (K–N) and oxidative response (O–R) marker genes in NRVMs infected with Ad-GFP and Ad-Trim16 followed by stimulation with phenylephrine (20 μM) for 24 h (n=6 independent experiments). Data in C, G, M, and O–Q are presented as the mean±SD, and data in (N and R) show the box median±interquartile range. For statistical analysis, 1-way ANOVA with Tamhane T2 post hoc analysis was used for (C), 2-tailed Student t test was used for (G–M and O–Q), and the Mann-Whitney test was used for (N and R). GFP indicates green fluorescent protein; and PC, principal component.
Figure 3. Trim16-CKO (cardiac myocyte-specific tripartite motif–containing protein 16 knockout) in cardiomyocytes exacerbates transverse aortic constriction (TAC)–induced cardiac hypertrophy in vivo.

A, Heart weight (HW), HW/body weight (BW), lung weight (LW)/BW, and HW/tibia length (TL) ratios in Flox and cardiac-specific Trim16 knockout (Trim16-CKO) mice at 4 wk after sham or TAC surgery (n=10). B, Assessments of echocardiographic parameters of left ventricular (LV) end-diastolic dimension (LVEDd), left ventricular end-systolic dimension (LVESd), ejection fraction (EF), and fraction shortening (FS) in Flox and Trim16-CKO mice at 4 wk after sham or TAC surgery (n=10). C, Representative images of hematoxylin-eosin (HE, left) staining of LV cross-sections in the hearts of Flox and Trim16-CKO mice at 4 wk after sham or TAC surgery (n=6). Scale bar, 1 mm for the top set and 25 μm for the bottom parts. Quantitative results of average cross-sectional areas (right) from the indicated groups. D, Representative images of picrosirius red (left) staining of LV cross-sections in the hearts of Flox and Trim16-CKO mice at 4 wk after sham or TAC surgery (n=6). Scale bar, 50 μm. Quantitative results of LV interstitial collagen volume (right) from the indicated groups. E and F, Relative mRNA levels of hypertrophy and fibrosis marker genes in heart tissues from the indicated mice (n=4). Data in (A–D) are presented as the mean±SD, and data in (E and F) show the group median±interquartile range. For statistical analysis, 1-way ANOVA with Bonferroni post hoc analysis was used for (A–D), and the Mann-Whitney test was used for (E and F).
**Figure 4.** Specific overexpression of \textit{Trim16} (tripartite motif–containing protein 16) gene inhibits transverse aortic constriction (TAC)–induced cardiac hypertrophy in mouse hearts. 

\textbf{A–D}, Heart weight (HW), HW/body weight (BW), lung weight (LW)/BW, and HW/tibia length (TL) ratios in adeno-associated virus serotype 9 (AAV9)-GFP (green fluorescent protein) and AAV9-\textit{Trim16} mice at 4 wk after sham or TAC surgery (n=10). \textbf{E–H}, Assessments of echocardiographic parameters of left ventricular (LV) end-diastolic dimension (LVEDd), left ventricular end-systolic dimension (LVESd), ejection fraction (EF), and fraction shortening (FS) in AAV9-GFP and AAV9-\textit{Trim16} mice at 4 wk after sham or TAC surgery (n=10). \textbf{I}, Representative images of hematoxylin-eosin (HE, \textit{left}) staining of LV cross-sections in the hearts of AAV9-GFP and AAV9-\textit{Trim16} mice at 4 wk after sham or TAC surgery (n=6). Scale bar, 1 mm for the \textit{top} set and 25 μm for the \textit{bottom} parts. Quantitative results of average cross-sectional areas (\textit{right}) from the indicated groups. \textbf{J}, Representative images of picrosirius red (\textit{left}) staining of LV cross-sections in the hearts of AAV9-GFP and AAV9-\textit{Trim16} mice at 4 wk after sham or TAC surgery (n=6). Scale bar, 50 μm. Quantitative results of LV interstitial collagen volume (\textit{right}) from the indicated groups. All data are presented as the mean±SD. For statistical analysis, 1-way ANOVA with Bonferroni post hoc analysis was used for (\textbf{A–C} and \textbf{E–I}), and Tamhane T2 post hoc analysis was used for (\textbf{D} and \textbf{J}).
**Figure 5.** Prdx1 (peroxiredoxin 1) was determined to be a potential downstream target of Trim16 (tripartite motif–containing protein 16).

**A**, Schematic diagram showing the conjoint analysis with RNA-seq (RNA-sequencing) (Ad-Trim16 [adenovirus expressing Trim16] group vs Ad-GFP [adenovirus expressing-green fluorescent protein] group and Ad-sh trim16 [adenovirus expressing short hairpin RNA targeting Trim16] group vs Ad-vector [adenovirus expressing blank vector] group) and MS (mass spectrometry) analysis to identify the specific target of Trim16. **B**, DEGs (Differentially expressed genes; adjusted P<0.05) with reverse expression trends of 2 pairwise comparisons (Ad-Trim16 group vs Ad-GFP group and Ad-sh Trim16 group vs Ad-vector group). **C**, Result of the screening of Trim16-binding protein (Atp5a1 [ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1], Atp5b, Prdx1, DES [desmin], Atp2a2 [ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2], mdh2 [malate dehydrogenase 2], Cs [citrate synthase], Hnrnpa2b1 [heterogeneous nuclear ribonucleoprotein A2/B1], Idh2 [isocitrate dehydrogenase NADP+2], Nnt [nicotinamide nucleotide transhydrogenase]) using co-IP (co-immunoprecipitation) assays in NRVMs (neonatal rat ventricular myocytes). (unique peptides >2 in MS analysis) **D**, Protein-protein interaction network relationship between Trim16-interacting proteins and phenotypic term genes enriched by gene ontology analysis. **E**, Representative confocal images of the colocalization of Trim16 and Prdx1 in NRVMs. Scale bar, 20 μm. **F**, Co-IP assays of the interaction between Trim16 and Prdx1 in HEK293T (human embryonic kidney) cells transfected with the indicated plasmids. **G**, Endogenous immunoprecipitation analysis of the interaction of Trim16 and Prdx1 in heart tissues of mice using anti-IgG or anti-Trim16 antibodies. **H**, GST (glutathione S-transferase)-pull-down assays for the direct binding between Trim16 and Prdx1. Purified GST was used as a control. **I**, Schematic diagram showing the domains of Trim16 and its related truncated mutants (top). B1 and B2, B-box domain-1 and B-box domain-2; B30.2, SPRY (B30.2) domain. Co-IP analysis of the binding regions of Trim16 and Prdx1 (bottom). **J**, Immunoblotting analysis of p-Prdx1 (phosphorylation-Prdx1), Prdx1, and Flag-Trim16 in NRVMs infected with the indicated adenovirus followed by stimulation with phenylephrine (20 μM) for 24 h. For (C, E–J), experiments were repeated independently 3×. aa indicates amino acids; FL, full length; and PE, phenylephrine.
Figure 6. Trim16 (tripartite motif–containing protein 16) promotes the degradation of Src proteasome and inhibits the phosphorylation of Prdx1 (peroxiredoxin 1).

A, Results of ubiquitination assays confirming the ubiquitination of Prdx1 after overexpression of Trim16 for 24 h in HEK293T (human embryonic kidney) cells. B, Top, schematic diagram of the TRIM16-DUB (Trim16-deubiquitinase fusion protein) protein structure. Bottom, the results of ubiquitination assays in HEK293T cells after transfection with the indicated plasmids for 24 h. C, Immunoblotting analysis of p-Prdx1 (phosphorylation-Prdx1), Prdx1, Flag-Trim16, and Flag-Trim16-DUB in neonatal rat ventricular myocytes (NRVMs) infected with the indicated adenovirus and stimulated with phenylephrine (20 μM) for 24 h. D, Possible pattern diagram of Trim16 regulation of phosphorylated Prdx1. E, Immunoblotting analysis of p-Prdx1, Prdx1, and Src in NRVMs infected with Ad-GFP (adenovirus expressing-Green fluorescent protein) or Ad-shSrc (adenovirus expressing short hairpin RNA targeting Src) and treated with phenylephrine (20 μM) for 24 h. F, co-IP (co-immunoprecipitation) assays of the interaction between Trim16 and Src in HEK293T cells transfected with the indicated plasmids. G, Endogenous immunoprecipitation analysis of the interaction of Trim16 and Src in adenosine expressing Trim16 (Ad-Trim16) and Src in adenosine expressing Trim16 and Src in HEK293T cells transfected with the indicated plasmids. H, Immunoblotting analysis of Src in NRVMs infected with Ad-GFP and Ad-Trim16 and treated with phenylephrine (20 μM) for 24 h and cycloheximide (CHX, 50 μM) for the indicated time points. I, Immunoblotting analysis of Src in NRVMs after Trim16 overexpression for 24 h and treatment with phenylephrine (20 μM) for 24 h, dimethyl sulfoxide (DMSO), MG132 (50 μM) or chloroquine (CQ, 50 μM) for 6 h. J, Results of ubiquitination assays confirming the ubiquitination of Src after overexpression of Trim16 and Src for 24 h and treated with MG132 (50 μM) for 6 h in HEK293T cells. K, Results of ubiquitination assays confirming the K48° ubiquitination of Src after overexpression of Trim16 and Src for 24 h and treatment with MG132 (50 μM) for 6 h in HEK293T cells. L, Immunoblotting analysis of p-Prdx1, Prdx1, Src, and Trim16 in NRVMs after infection with the indicated adenovirus (Ad-vector [adenovirus expressing blank vector], Ad-shSrc and Ad-ShTrim16 [adenovirus expressing short hairpin RNA targeting Trim16]) and treatment with phenylephrine (20 μM) for 24 h. All experiments were repeated independently 3×. PE indicates phenylephrine; and Ub, ubiquitination.
data clearly support that E3 ligase activity is required for the protective effect of Trim16 on regulating p-Prdx1.

The E3 ligase activity-dependent Trim16-mediated inhibition of Prdx1 phosphorylation suggested that there might be other key proteins, particularly kinases, mediating the Trim16-Prdx1 interaction (Figure 6D). A previous report indicated that Src is a tyrosine kinase that serves as a critical upstream regulator of Prdx1 phosphorylation, which was confirmed in our study (Figure 6E). In addition, the in vivo results showed that the expression of Src was upregulated after TAC (Figure S7B). Regarding the stress-dependent manner of Trim16-regulated Src expression, we speculate that there might be different interacting patterns of Trim16 and Src in the pathological setting compared to those at baseline. Indeed, we found that Trim16 could directly interact with Src and that this interaction was enhanced in cardiomyocytes after phenylephrine challenge (Figure 6F and 6G). Furthermore, overexpression of Trim16 aggravated the degradation of Src induced by cycloheximide (Figure 6H), which was reversed by MG132 (Figure 6I). We thus hypothesized that Trim16 might block Prdx1 phosphorylation by promoting Src ubiquitination and degradation. To evaluate this hypothesis, we further detected the regulatory effect of Trim16 on Src ubiquitination and found that Trim16 could significantly promote Src K48-type ubiquitination (Figure 6J and 6K, Figure S7C). When Src was knocked down, the regulatory effects of Trim16 on Prdx1 phosphorylation were largely abolished (Figure 6L). Notably, Trim16-DUB showed no effects on Src protein stability or K48-type ubiquitination (Figure S7D through S7E). Consistently, Trim16-DUB abolished the effect of Trim16 on cardiomyocyte enlargement in response to phenylephrine challenge (Figure S7F).

**Trim16 Inhibits Cardiac Hypertrophy and Oxidative Stress in A Prdx1-dependent Manner**

We next explored whether Prdx1 is required for the protective effect of Trim16 against oxidative stress and cardiac hypertrophy. As shown in Figure 7A, we used Ad-Trim16 and Ad-shPrdx1 adenoviruses to infect NRVMs. Notably, Prdx1 knockdown abolished the protective role of Trim16 against phenylephrine-induced cardiomyocyte hypertrophy and related cardiac marker expression (Figure 7B through 7F). Furthermore, Prdx1 depletion abrogated the effect of Trim16 on antioxidative stress and upregulating Nrf2 expression in hypertrophic cardiomyocytes (Figure 7G and 7H). Taken together, these data suggest that Prdx1 serves as an essential target of Trim16 in cardiac hypertrophy and that targeting the Trim16-Prdx1 axis may provide therapeutic strategies for pathological myocardial hypertrophy.

**DISCUSSION**

Pathological cardiac hypertrophy is one of the most important components of heart failure. In recent years, studies of cardiac hypertrophy have made significant progress in the identification of key molecular targets and signaling pathways. However, clinical pharmacological approaches for treating cardiac hypertrophy are far from satisfactory. In our study, we found that the expression of Trim16 was significantly upregulated in heart samples from patients diagnosed with heart failure. Consistently, we observed that Trim16 expression was dramatically upregulated in cardiac hypertrophy induced by pressure overload in vivo or phenylephrine in vitro. Our experimental results showed that the upregulation of Trim16 was caused by the transcriptional regulation of EGR2. Importantly, we demonstrated that Trim16 ameliorated cardiac hypertrophy by promoting the Src degradation-mediated decline in Prdx1 phosphorylation. These results suggest that Trim16 has cardioprotective effects in cardiac hypertrophy and may provide effective therapeutic strategies for pathological cardiac hypertrophy.

A large proportion of TRIM family proteins, such as Trim8, Trim24, Trim32, and Trim72, have been demonstrated to play prominent roles in cardiac hypertrophy and other cardiovascular diseases, implying a critical role of the TRIM family in heart disease. Our study for the first time demonstrates the important role of Trim16 in cardiac hypertrophy. To date, the majority of functions of Trim16 have focused on protein autophagy and ubiquitin modification. Previous reports suggested that Trim16 regulates oxidative stress and inflammation, which are critical events in cardiovascular lesions. In our research, based on global gene expression profiling through RNA-sequencing, we systematically revealed that Trim16 downregulated genes related to oxidative stress, cardiac hypertrophy, and protein synthesis.

It is well known that oxidative stress plays an important role in cardiac hypertrophy. PRDXs are peroxidases that belong to the superfamily of antioxidant proteins. Prdx1 is a member of the PRDX family and plays a protective antioxidant role by scavenging oxygen free radicals and regulating the oxidative stress signaling pathway in cells in the form of molecular chaperones. As one of the major downstream effectors of Prdx1, Nrf2 functions on multiple antioxidant stress-related molecular processes, including the inflammatory response, metabolic process, and cell proliferation. Prdx1-induced upregulation of Nrf2 protein expression in the nucleus was reported to be induced by enhanced nuclear distribution and reduced protein ubiquitination and degradation in the cytoplasm. Correspondingly, Nrf2 and its downstream target HO-1 hold a widely protective function on myocardial hypertrophy, myocardial ischemia-reperfusion, and other myocardial injuries. In line with our current study, Trim16 could effectively attenuate myocardial ischemia-reperfusion injury by affecting Nrf2. Here, we clearly illuminated the detailed molecular events underlying Trim16-regulated Prdx1–Nrf2–HO-1 signaling that critically control pathological cardiac hypertrophy and related heart diseases.

Most TRIM family members have E3 ubiquitin ligase activity, which plays key role in the posttranslational
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modification of certain proteins. Several TRIM superfamily members have been reported to degrade myosin (such as myosin and troponin I) and hypertrophy signaling factors (such as PKC (protein kinase C), SRF (serum response factor) and IGF-1 (insulin like growth factor 1) to regulate myocardial growth, development, and function via a ubiquitin–proteasome-dependent pathway. Although Trim16 does not have a RING domain, it possesses 2 B-box domains with similar functions related to E3 ubiquitin ligase activity. Intriguingly, Trim16 had no obvious effect on the ubiquitination of Prdx1, but promoting its phosphorylation and downstream Nr2–HO-1 axis activity was dependent on an E3 ligase. A previous report indicated that Src is a tyrosine kinase that serves as a critical upstream regulator of Prdx1 phosphorylation. The results of both exogenous and endogenous immunoprecipitation validated the binding of Src to Trim16. Nevertheless, Src was not identified in Flag-Trim16 pull-down mass spectrometry data, which might be due to its relatively low abundance in conventional coimmunoprecipitation/mass spectrometry techniques. Removal of nonspecific background proteins can also induce inevitable loss of interacting proteins, or the cellular copy numbers of Src are low. Nevertheless, our study provided the first evidence that Trim16 could significantly

Figure 7. Trim16 (tripartite motif–containing protein 16) inhibits cardiac hypertrophy through Prdx1 (peroxiredoxin 1).

A, Immunoblotting analysis of Prdx1 and Flag-Trim16 after infection with the indicated adenovirus (Ad-Trim16 [adenovirus expressing Trim16] group, Ad-GFP [adenovirus expressing-green fluorescent protein] group, Ad-shPrdx1 [adenovirus expressing short hairpin RNA targeting Prdx1] group and Ad-vector [adenovirus expressing blank vector] group) and phenylephrine (20 μM) for 24 h in neonatal rat ventricular myocytes (NRVMs). B, Representative immunofluorescence images (left) and quantitative results of the cell surface area (right) of NRVMs infected with the indicated adenovirus and treated with phenylephrine (20 μM) or PBS for 24 h (n=20 cells per group). Scale bar, 20 μm. C, Immunoblotting analysis of Anp (atrial natriuretic peptide) and Myh7/β-MHC (β-myosin heavy chain) in NRVMs infected with the indicated adenovirus and treated with phenylephrine (20 μM) for 24 h. D–F, Relative mRNA levels of cardiac hypertrophy marker genes (Anp, Bnp [b-type natriuretic peptide] and Myh7) in NRVMs infected with the indicated adenovirus and treated with phenylephrine (20 μM) for 24 h (n=6). G, Dihydroethidium (DHE) staining (left) and quantitative results of DHE relative fluorescence (right) in NRVMs infected with the indicated adenovirus and treated with phenylephrine (20 μM) for 24 h (n=20 fields). Scale bar, 100 μm. H, Immunoblotting analysis of Nrf2 (nuclear factor–erythroid 2–related factor 2) and HO-1 (heme oxygenase-1) in NRVMs after infection with the indicated adenovirus for 24 h and treatment with phenylephrine (20 μM) or PBS for 24 h. All experiments were repeated independently 3 times. Data in B and D–G are presented as the mean±SD. For statistical analysis, 1-way ANOVA with Bonferroni post hoc analysis was used for D, and 1-way ANOVA with Tamhane T2 post hoc analysis was used for (B and E–G). Ad-Trim16 indicates adenovirus expressing Trim16. PE indicates phenylephrine.
promote Src K48-linked ubiquitination and degradation, thereby relieving the phosphorylation of Prdx1. Therefore, targeting the Trim16-Src-Prdx1 axis might represent a promising strategy for treating pathological cardiac hypertrophy.

In summary, our results revealed for the first time that Trim16 is a critical suppressor of pathological myocardial hypertrophy. In the setting of this disease, Trim16 directly interacts with Src and Prdx1 to facilitate Src ubiquitinational degradation and the subsequently reduced Prdx1 phosphorylation. The E3 ligase activity of Trim16 is required for the hypertrophy. In the setting of this disease, Trim16 directly interacts with Src and Prdx1 to facilitate Src ubiquitinational degradation and the subsequently reduced Prdx1 phosphorylation. The E3 ligase activity of Trim16 is required for the hypertrophy. These findings provide new insights into the pathogenesis of pathological myocardial hypertrophy and offer new therapeutic strategies for cardiac hypertrophy.

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