Original Article

Smad3 Couples Pak1 With the Antihypertrophic Pathway Through the E3 Ubiquitin Ligase, FBXO32

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Abstract—Pathological cardiac hypertrophy is regarded as a critical intermediate step toward the development of heart failure. Many signal transduction cascades are demonstrated to dictate the induction and progression of pathological hypertrophy; however, our understanding in regulatory mechanisms responsible for the suppression of hypertrophy remains limited. In this study, we showed that exacerbated hypertrophy induced by pressure overload in cardiac-deleted Pak1 mice was attributable to a failure to upregulate the antihypertrophic E3 ligase, Fbxo32, responsible for targeting proteins for the ubiquitin-degradation pathway. Under pressure overload, cardiac overexpression of constitutively active Pak1 mice manifested strong resilience against pathological hypertrophic remodeling. Mechanistic studies demonstrated that subsequent to Pak1 activation, the binding of Smad3 on a critical singular AGAC²⁸⁶-binding site on the *FBXO32* promoter was crucial for its transcriptional regulation. Pharmacological upregulation of Fbxo32 by Berberine ameliorated hypertrophic remodeling and improved cardiac performance in cardiac-deficient Pak1 mice under pressure overload. Our findings discover Smad3 and Fbxo32 as novel downstream components of the Pak1-dependent signaling pathway for the suppression of hypertrophy. This discovery opens a new venue for opportunities to identify novel targets for the management of cardiac hypertrophy. (*Hypertension*. 2015;66:00-00. DOI: 10.1161/HYPERTENSIONAHA.115.06068.)

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• **Online Data Supplement**

Key Words: cardiomegaly ■ Fbxo32 protein, mouse ■ p21-activated kinases ■ Smad3 protein ■ ubiquitin

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Pathological cardiac hypertrophic growth is associated with many forms of heart disease, including ischemic disease, hypertension, myocardial infarction, and valvular disease, leading to eventual heart failure; 23 million people are affected worldwide.¹ The duration for progression from pathological hypertrophy to heart failure provides an opportunity for intervention; thus, the continuous identification of novel antihypertrophic mechanisms is necessary for the discovery of new therapeutic options targeting this time window. Pathological hypertrophy can be divided into the initial adaptive compensatory stage and the maladaptive decompensatory stage.^{2,3} The maladaptive signaling pathway leads to an enlargement of individual cardiomyocytes, reactivation of fetal genes, increased fibrosis, increased protein synthesis, and protein degradation.⁴

During pathological hypertrophy, increased protein synthesis causes an accumulation of misfolded proteins ultimately resulting in protein aggregates.⁵ To prevent protein aggregate accumulation, the rate of protein degradation is increased through autophagy and the ubiquitin-degradation system; suppression of autophagy has been shown to exacerbate pathological hypertrophy.^{6,7} The ubiquitin-degradation system pathway requires the substrate-specific ubiquitin

ligase (E3 ligase) that polyubiquitinates the target protein on lysine residue 48 resulting in degradation by the 26S proteasome.⁸ The antihypertrophic E3 ligases, mouse double minute 2 and muscle-specific ring finger, have been demonstrated to act through targeting telethonin and myosin-binding protein C, respectively.^{9,10} Another antihypertrophic E3 ligase is the muscle-specific F-box protein, Fbxo32, which is a component of the SCF ubiquitin ligase complex.¹¹ Fbxo32 was primarily identified as a vital effector involved in skeletal muscle atrophy, as mice lacking Fbxo32 were resistant to atrophy, and conversely, its overexpression led to atrophy.^{12,13} In cardiomyocytes, Fbxo32 was shown to colocalize with calcineurin (PP2B); furthermore, overexpression of Fbxo32 reduced PP2B levels resulting in cytoplasmic retention of the transcription factor, nuclear factor of activated T cells (NFAT), which in turn prevents its prohypertrophic effects.^{11,14–16}

P21-activated kinase 1 (Pak1) has been discovered as an important serine/threonine kinase with crucial functions in the heart.^{17–19} It belongs to the group I Pak family and is activated by GTP-bound Cdc42/Rac1 GTPases.²⁰ Studies by Liu et al^{17,21} reported on the antihypertrophic property of Pak1 through phosphorylation of NFAT by the c-jun N-terminal

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kinase 1/2 (JNK1/2) pathway. In this study, we have identified an AGAC⁻²⁸⁶-binding site within the *FBXO32* promoter region required by small mother against decapentaplegic 3 (Smad3), which is responsible for Fbxo32 transcriptional regulation. We have shown that Smad3 regulation of Fbxo32 is downstream of the Pak1-dependent antihypertrophic pathway. Furthermore, pharmacological upregulation of Fbxo32 was able to ameliorate exacerbated hypertrophy induced by the loss of Pak1 under pressure overload stress.

Methods

Detailed Methods section is available in the online-only Data Supplement.

Animal Models

Cardiomyocyte-specific male mouse models include Pak1 knockout (Pak1^{cko}) mice and its control counterpart homozygous Pak1 floxed (f/f) mice¹⁷; overexpression of constitutively active Pak1 (Pak1^{ctg}) mice and its control counterpart nontransgenic mice. Both mouse models and their control counterparts were subjected to transverse aortic constriction (TAC). Berberine (3 mg/kg per day) treatment was administered for the duration of TAC in f/f and Pak1^{cko}-mice. Detailed procedures are available in the online-only Data Supplement.

Chromatin Immunoprecipitation Assay

Neonatal rat cardiomyocytes (NRCMs) were cross-linked in 1% v/v formaldehyde and sonicated to ≈500 bps. Immunoprecipitation reactions with Smad3 antibody (Cell Signalling) or rabbit-IgG was performed according to manufacturers' protocol for SimpleChIPPlus Enzymatic ChromatinIP Agarose-Beads Kit (Cell Signalling). Eluted DNA was subjected to quantitative polymerase chain reaction, and CT values were normalized against rabbit-IgG; data are presented as fold change.

Results

Cardioprotective Effects of Pak1 Under Pressure Overload Stress

Our recent study showed exacerbated hypertrophy in cardiac-specific Pak1 knockout mice (Pak1^{cko}) under pressure overload stress.¹⁷ To directly examine Pak1 activation in the heart, mice with cardiac-specific overexpression of constitutively active Pak1 driven by an α -myosin heavy chain promoter (Pak1^{ctg}) were generated (Figure S1 in the online-only Data Supplement). Pak1^{ctg} mice were viable, fertile, and developed to adulthood. At baseline, echocardiographic analyses revealed normal cardiac function in Pak1^{ctg} and nontransgenic mice (Table S1).

We used pressure overload stress to ascertain the role of Pak1 activation in hypertrophic suppression. TAC-induced increases in left ventricular mass and fibrosis in nontransgenic/TAC mice were not apparent in Pak1^{ctg}/TAC mice (Figure S2A and S2B). Echocardiographic analyses (Figure S2C–S2G) revealed that increased left ventricular end-diastolic diameter, increased left ventricular end-systolic diameter, and decreased fraction shortening were only evident in nontransgenic/TAC mice. These results show that Pak1 activation suppresses pressure overload-induced hypertrophy.

Pak1 Negatively Regulates PP2B Through Fbxo32 in Response to Hypertrophic Stress

Led by the above results, we tested whether the central PP2B-NFAT prohypertrophic pathway is involved in our

experimental model. Post examination, we revealed exacerbated PP2B protein level in Pak1^{cko}/TAC mice, although its mRNA levels in Pak1^{cko}/TAC and f/f/TAC mice were equivalent (Figure 1A and 1B). These results indicate possible involvement of protein degradation mechanisms. To test this, we examined the protein levels of Fbxo32.^{12,13} Fbxo32 protein and mRNA levels in f/f/TAC mice almost doubled; this increase was abrogated in Pak1^{cko}/TAC mice (Figure 1A and 1B). Our data also showed comparable levels of Fbxo32 expression in Pak1^{ctg}/TAC and nontransgenic/TAC mice (Figure 1C). In addition, we examined desmin and troponin I (TnI), targets of Fbxo32.^{22,23} Desmin but not TnI expression increased in Pak1^{cko}/TAC mice (Figure S3).

To elucidate the link between Pak1-mediated Fbxo32 upregulation and resultant PP2B downregulation, a NRCM cellular model was used. Phenylephrine induced Fbxo32 mRNA and protein upregulation in Ad-shC2 (control)–infected NRCMs; this effect was abolished in Pak1 knockdown (Ad-shPak1) NRCMs, resulting in PP2B accumulation (Figure 2A and 2B). We also demonstrated that PP2B expression increased (Figure 2C) under phenylephrine stress in Fbxo32 knockdown NRCMs (Figure S4). Furthermore, upregulation of Fbxo32 protein level was recorded in NRCMs expressing constitutively active Pak1 (Ad-CaPak1); this was consistent with PP2B downregulation; these effects were not evident in the control counterpart (Ad-LacZ). To further establish possible ubiquitin-degradation system degradation of PP2B, Ad-CaPak1-NRCMs were treated with MG132, which inhibited PP2B degradation despite Fbxo32 upregulation (Figure 2D). Above results show that Pak1 signaling is vital for downstream Fbxo32-dependent PP2B degradation.

Smad3 Is a Potent Transcription Factor for Fbxo32 Upregulation

We sought to discover transcription factors responsible for Fbxo32 transcriptional upregulation. Thus, we identified the transcriptional start site of the *FBXO32* gene using in silico analysis (University of California Santa Cruz and National Center for Biotechnology Information). Forkhead box O 1/3 (Foxo1/3) and Smad2/3 binding sites were present in the *FBXO32* promoter region. As phenylephrine induced comparable Foxo1/3 phosphorylation levels in Ad-shC2 and Ad-shPak1-NRCMs, (Figure 3A), we focused on Smad3, which possesses DNA-binding ability.^{24,25} Interestingly, Smad3 phosphorylation increased in Ad-shC2-phenylephrine-NRCMs, also demonstrated in Ad-CaPak1-NRCMs (Figure 3A and 3B). Smad3 inhibitor, SIS3 (5 μ mol/L), abolished phenylephrine-induced Fbxo32 upregulation (Figure 3C). In vivo studies displayed augmented Smad3 phosphorylation in f/f/TAC mice, which was not evident in Pak1^{cko}/TAC mice (Figure 3D); comparable increases in Smad3 phosphorylation levels were observed in nontransgenic/TAC and Pak1^{ctg}/TAC mice (Figure 3E). These results indicate Smad3 as a potential transcription factor responsible for Fbxo32 transcriptional activation. In addition, increased transforming growth factor- β expression was found in Pak1^{cko}/TAC mice (Figure S5).

Next, we examined Smad3 nuclear translocation and discovered that phenylephrine-induced Smad3 nuclear translocation only occurred in Ad-shC2-NRCMs (Figure 4A). We

Figure 1. Absence of Pak1 causes aggravated calcineurin (PP2B) accumulation because of a failure to upregulate Fbxo32 under transverse aortic constriction (TAC). **A**, Immunoblots demonstrate exacerbated PP2B expression resulting from abrogation of Fbxo32 upregulation in Pak1^{cKO}(cKO)/TAC mice. **B**, Quantitative polymerase chain reaction analysis of *PPP3CA* (encoding PP2B) and *FBXO32*, gene expression normalized to GAPDH; n=6. **C**, Western blot analyses show comparable levels of PP2B and Fbxo32 in nontransgenic (ntg)/TAC and Pak1^{tg}(TG)/TAC mice. **A** and **C**, GAPDH represents protein-loading control; bar graphs represent Fbxo32/GAPDH and PP2B/GAPDH; n=6. **P*<0.05; ***P*<0.01; ****P*<0.001; and *****P*<0.0001. A.U indicates arbitrary units; and ns, not statistically significant.

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identified 2 Smad3-binding sites, a GTCT site at -92 bp and an AGAC site at -286 bp, relative to transcriptional start site. To study their respective transcriptional activity, we generated a construct containing both binding sites (COMB-construct), a GTCT-binding site construct (GTCT⁻⁹²-construct), and an AGAC-binding site construct (AGAC⁻²⁸⁶-construct).

Cotransfection of a plasmid expressing wild-type Smad3 with either COMB-construct or AGAC⁻²⁸⁶-construct induced an approximate 10-fold induction of reporter gene expression (Figure 4B). Further chromatin Immunoprecipitation analyses on phenylephrine-NRCMs revealed that Smad3 binds specifically to the AGAC⁻²⁸⁶ site (Figure 4C). Together, these results

Figure 2. Importance of Pak1 in the regulation of calcineurin (PP2B) through Fbxo32 in neonatal rat cardiomyocytes (NRCMs). **A**, Immunoblots demonstrated that phenylephrine (PE) augmented Fbxo32 expression and prevented the accumulation of PP2B in the presence of Pak1. **B**, Quantitative polymerase chain reaction analysis of *FBXO32*, data normalized to GAPDH. **C**, Immunoblot analyses showing PE-induced PP2B accumulation in Fbxo32 knockdown conditions. **D**, Expression of Fbxo32 increased in Ad-CaPak1-NRCMs; MG132 prevented the decline in PP2B expression. **A**, **C**, and **D**, GAPDH represents protein-loading control; bar graphs represent Fbxo32/GAPDH and PP2B/GAPDH; n=5 to 8 independent experiments. **P*<0.05; ***P*<0.01; ****P*<0.001; and *****P*<0.0001. A.U indicates arbitrary units; and ns, not statistically significant.

Figure 3. Pak1 is necessary for Smad3 phosphorylation under stressed conditions. **A**, Immunoblot analyses of neonatal rat cardiomyocytes (NRCMs) show that phenylephrine (PE)-induced Smad3 phosphorylation is Pak1 dependent. **B**, Expression of Smad3 phosphorylation increased in Ad-CaPak1-NRCMs. **C**, Immunoblots exhibit Smad3 inhibition (SIS3) prevented PE-induced Fbxo32 upregulation. Bar graphs represent Fbxo32/GAPDH; n=4 independent experiments; GAPDH represents protein-loading control. **D**, Immunoblotting shows that Smad3 phosphorylation is abrogated in Pak1^{CKO}(cKO)/transverse aortic constriction (TAC) mice. **E**, Western blot analysis of Smad3 phosphorylation in nontransgenic (ntg)/TAC and Pak1^{CKO}(TG)/TAC mice. **A** and **B**, GAPDH represents protein-loading control; bar graphs represent phosphorylation/total (P/T) ratios of Foxo1/3 and Smad3; n=4 to 5 independent experiments. **D** and **E**, GAPDH represents protein-loading control; bar graphs represent phosphorylation/total (P/T) Smad3; n=4 to 6. **P*<0.05; ***P*<0.01; and ****P*<0.001. A.U. indicates arbitrary units; and ns, not statistically significant.

demonstrate that the binding of Smad3 onto the AGAC⁻²⁸⁶ site transcriptionally upregulates the *FBXO32* gene.

Smad3 Acts Downstream of Pak1

Subsequently, we sought to obtain evidence that the Pak1 signaling cascade is responsible for Smad3 phosphorylation. Pak1 has previously been demonstrated to act via the MKK7-JNK pathway.¹⁷ We demonstrated that knockdown of MKK7 abrogated phenylephrine-induced Smad3 phosphorylation (Figure 5A). We further show that inhibition of JNK in NRCMs using JIP-1 also abolished phenylephrine-induced Smad3 phosphorylation (Figure 5B). Together, we have shown that Pak1 activates Smad3 through the MKK7-JNK signaling cascade.

Treatment Potential of Fbxo32 Induction in Ameliorating Pak1 Deficiency-Induced Cardiac Hypertrophy

Because Fbxo32 is an antihypertrophic proximal effector, using our hypertrophic model of Pak1 deficiency, we investigated whether Fbxo32 possessed therapeutic value. We pharmacologically upregulated Fbxo32 using Berberine, which is derived from the plant *Hydrastis canadensis* L.^{26,27} Application of Berberine to Ad-shPak1-phenylephrine-NRCMs upregulated Fbxo32 expression to similar levels when compared with Ad-shC2-phenylephrine-NRCMs; this resulted in PP2B downregulation (Figure 6A). These results indicate that Berberine does not act through Pak1. Instead, Berberine

induced phosphorylation of adenosine monophosphate kinase (Figure S6), presenting its potential mechanistic action.^{10,28}

Next, we investigated whether upregulation of Fbxo32 by Berberine would improve cardiac performance in f/f/TAC and Pak1^{CKO}/TAC mice. The level of Fbxo32 upregulation and subsequent PP2B downregulation was comparable between Berberine-treated Pak1^{CKO}/TAC and f/f/TAC mice (Figure 6B). Furthermore, Berberine significantly blunted hypertrophy and reduced interstitial fibrosis (Figure 7A–7C). Berberine also improved contractility, reduced end-diastolic left ventricular posterior wall thickness, left ventricular end-diastolic diameter and left ventricular end-systolic diameter in both TAC groups (Figure 7D). Collectively, these data suggest that Fbxo32 induction antagonizes pathological hypertrophic remodeling caused by Pak1 deficiency under TAC.

Discussion

In this study, we identified novel Pak1 antihypertrophic signaling components in the prevention of pathological hypertrophic remodeling in our pressure overload experimental model. The major findings of this study include the identification of a unique singular Smad3 AGAC⁻²⁸⁶-binding site on the *FBXO32* promoter region responsible for *FBXO32* transcriptional expression. We further discovered that pharmacological upregulation of Fbxo32 is able to rescue antihypertrophic effects in cardiac-deficient Pak1 mice under pressure overload.

Previous studies suggest that Foxo1/3 is able to regulate Fbxo32 through the phosphoinositide-3-kinase and protein

Figure 4. Smad3 transcriptional activation of *FBXO32* under stress is Pak1 dependent. **A**, Representative fluorescent images of neonatal rat cardiomyocytes (NRCMs) demonstrate that Pak1 is required for phenylephrine (PE)-induced Smad3 nuclear translocation (scale bar, 20 μ m; red, Smad3; green, α -actinin; blue, DAPI). Bar graph shows quantification from 300 NRCMs per group, represented as nuclear/cytoplasmic Smad3 ratio. **B**, Increased luciferase activity in COMB-construct (COMB) or AGAC⁻²⁸⁶-construct (AGAC) when cotransfected with plasmid expressing wild-type Smad3 (wtSmad3); n=8 independent experiments. **C**, Schematic diagram of the AGAC⁻²⁸⁶- and GTCT⁻⁹²-binding sites on the *FBXO32* promoter region. Chromatin immunoprecipitation analysis show increased binding of Smad3 to the AGAC⁻²⁸⁶-binding site. n=4 independent experiments. **P*<0.05; ***P*<0.01; and *****P*<0.0001. A.U arbitrary units; cDNA3, control plasmid; GTCT, GTCT⁻⁹²-construct; and ns, not statistically significant.

kinase B pathway.^{12,13,29,30} However, in our experimental model, Foxo1/3 is unlikely to participate in Pak1 regulation of Fbxo32 because its phosphorylation induced by phenylephrine was not affected by Pak1 knockdown in NRCMs. The antihypertrophic role of Smad3 has previously been reported. In skeletal muscle, overexpression of constitutively active Smad3 efficiently attenuated hypertrophy, whereas Smad3-null mice expressed increased susceptibility to TAC-induced hypertrophy in the heart.^{31,32} Consistent with these studies, we have identified a Pak1-dependent Smad3 antihypertrophic pathway through the transcriptional regulation of Fbxo32. Furthermore, our study has identified a specific potent singular AGAC⁻²⁸⁶-binding site responsible for Fbxo32 expression. This finding uncovers a novel ability of Smad3, which allows transcriptional activation of target genes after its binding onto a unique singular binding site. This is in contrast to the previously reported requirement of at least 2 core Smad3-binding sites, AGAC and GTCT, to allow transcriptional activation.^{25,33} In addition, this binding site is also conserved in the human *FBXO32* promoter region (-266 bps), indicating potential translational value (Figure S7).

Our study demonstrated that Pak1^{CTG}/TAC mice displayed attenuated pathological hypertrophy compared with nontransgenic/TAC mice, whereas Fbxo32 levels were comparable in both TAC groups. This phenomenon might be because of TAC-induced Smad3 phosphorylation reaching a saturated level in both Pak1^{CTG} and nontransgenic mice, considering that total Smad3 expression remains unchanged. In addition, the presence of a singular preferable Smad3-binding site could also provide controllable transcriptional regulation of Fbxo32 expression. It is likely that the observed beneficial effects in Pak1^{CTG}/TAC mice are a collective result from activation of a parallel Pak1 antihypertrophic pathway through JNK phosphorylation of NFAT.¹⁷

Previous studies have demonstrated that Smad3 can be phosphorylated by transforming growth factor- β -independent pathways, such as JNK in lung epithelial cells, p38 in breast carcinoma cells, or directly by Pak1 in mesangial cells.^{34–36} This is consistent with our data showing Pak1-dependent Smad3 phosphorylation through the MKK7-JNK signaling cascade. In addition, previous studies have reported that hypertrophy-induced microtubule network expansion can sequester Smad3, preventing it from being accessed and phosphorylated by the transforming growth factor- β receptor.^{37,38} This might explain although transforming growth factor- β expression was increased

Figure 5. Upstream regulatory pathway of Smad3 phosphorylation under phenylephrine (PE) in neonatal rat cardiomyocytes. **A**, Immunoblot analyses show that PE-induced Smad3 phosphorylation is MKK7 dependent. **B**, Western blots demonstrate inhibition of JNK1/2 (JIP-1)-abolished Smad3 phosphorylation under PE. **A** and **B**, GAPDH represents protein-loading control; bar graphs represent phosphorylation/total (P/T) Smad3; n=4 independent experiments. **P*<0.05; ***P*<0.01; and ****P*<0.001. A.U indicates arbitrary units.

Figure 6. Fbxo32 is crucial for calcineurin (PP2B) downregulation. **A**, In neonatal rat cardiomyocytes, immunoblots show that Berberine (Ber)-induced Fbxo32 upregulation is Pak1 independent; this correlated with reduced PP2B expression; $n=4$ independent experiments. **B**, Immunoblot analyses from left ventricular lysates demonstrate Berberine-restored Fbxo32 upregulation in Pak1^{CKO}(cKO)/transverse aortic constriction (TAC) mice; $n=6$. **A** and **B**, GAPDH represents protein-loading control; bar graphs represent Fbxo32/GAPDH and PP2B/GAPDH. * $P<0.05$; ** $P<0.01$; and *** $P<0.001$. A.U. indicates arbitrary units; and ns, not statistically significant.

in Pak1-deficient hypertrophied hearts, yet it still failed to phosphorylate Smad3.

Previous studies have identified desmin, TnI, and I-kB as targets of Fbxo32.^{22,23,39,40} Our results showed unaltered TnI expression in our Pak1^{CKO}/TAC hypertrophic model, whereas

other studies have reported a reduction in TnI expression in chronic heart failure or ischemia/reperfusion models.^{23,39} The difference observed is likely due to the dissimilar stress challenges. Usui et al⁴⁰ observed exacerbated I-kB expression in the absence of Fbxo32; however, I-kB expression was unchanged in our Pak1^{CKO}/TAC-model (Figure S8). Different from the Fbxo32 total knockout model used in their study, fluctuations in Fbxo32 expression in our Pak1^{CKO}/TAC model were restricted to cardiomyocytes. Thus, the discrepancy could be attributable to different models as the total absence of Fbxo32 in multiple cell types versus Fbxo32 downregulation in cardiomyocytes could yield distinctive targeting profiles. Elucidation of different Fbxo32 targeting profiles will require future investigation.

Our current study has identified an antihypertrophic mechanism through the Pak1-dependent signaling pathway for downstream Smad3-mediated Fbxo32 upregulation. We have also demonstrated the treatment potential of Fbxo32 through Berberine, which is consistent with a study using a cardiomyocyte-specific Fbxo32-overexpression mouse model to show resistance against TAC-induced hypertrophy through PP2B downregulation.¹¹ However, we do not exclude the possibility that Berberine activates other antihypertrophic pathways as previous studies have indicated upregulation of autophagy.^{10,41} Together, this study highlights the novel components, Smad3 and Fbxo32, as well as Pak1 itself as attractive drug targets for future studies on therapeutic treatment in halting or reversing the progression of hypertrophy.

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Perspectives

This study has discovered important components within the Pak1 signaling pathway for the identification of new

Figure 7. Berberine prevents transverse aortic constriction (TAC)-induced hypertrophic remodeling. **A**, Berberine (Ber) prevented hypertrophy in f/f/TAC and Pak1^{CKO}(cKO)/TAC mice as demonstrated by heart weight/tibia length (HW/TL) ratio. **B**, Mean cardiomyocyte cross-sectional measurements and representative hematoxylin and eosin-stained images of heart cross-sections (scale bar, 20 μm). **C**, Masson trichrome images (scale bar, 20 μm) demonstrate interstitial fibrosis; bar graph represents quantification of fibrotic area against total tissue area. **D**, Echocardiography analyses reveal Berberine-improved fractional shortening, decreased end-diastolic left ventricular posterior wall thickness (dPW), left ventricular end-diastolic diameter (LVEdD), and left ventricular end-systolic diameter (LVEsD). $n=6$ to 8. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; and **** $P<0.0001$. A.U. indicates arbitrary units; and ns, not statistically significant.

therapeutic targets against hypertrophy. Combined, our studies propose a dual mechanism for the antihypertrophic function of Pak1. One arm of the cascade involves inhibition of NFAT by the JNK pathway,^{17,18} and another arm of the cascade as discovered in this study is through Smad3 regulation of Fbxo32 for the degradation of PP2B (Figure S9).

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Disclosures

None.

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Novelty and Significance

What Is New?

- We have identified a unique singular Smad3-binding site on the *FBXO32* promoter region responsible for its transcriptional activation.
- Pak1 regulation of Fbxo32 is through Smad3 activation.
- We have elucidated the novel Pak1-Smad3-Fbxo32 cascade, which holds potential translational value.

What Is Relevant?

- Persistent hypertension commonly results in pathological hypertrophic remodeling, and our study has identified an important and novel antihypertrophic mechanism.
- We have discovered the potent Pak1-Smad3-Fbxo32 cascade, and furthermore, we shown that targeting the downstream component, Fbxo32,

can functionally and phenotypically antagonize pressure overload-induced pathological hypertrophy.

Summary

We have demonstrated that Pak1 is able to antagonize pressure overload-induced pathological hypertrophic remodeling through Smad3 regulation of Fbxo32. The prominence of downstream Fbxo32 is emphasized through pharmacological induction, and our findings support the elucidation of the Pak1 antihypertrophic pathway in the discovery of novel components as potential targets for the management of hypertrophy.

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