

Hippo Deficiency Leads to Cardiac Dysfunction Accompanied by Cardiomyocyte Dedifferentiation During Pressure Overload

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Rationale: The Hippo pathway plays an important role in determining organ size through regulation of cell proliferation and apoptosis. Hippo inactivation and consequent activation of YAP (Yes-associated protein), a transcription cofactor, have been proposed as a strategy to promote myocardial regeneration after myocardial infarction. However, the long-term effects of Hippo deficiency on cardiac function under stress remain unknown.

Objective: We investigated the long-term effect of Hippo deficiency on cardiac function in the presence of pressure overload (PO).

Methods and Results: We used mice with cardiac-specific homozygous knockout of WW45 (WW45cKO), in which activation of Mst1 (Mammalian sterile 20-like 1) and Lats2 (large tumor suppressor kinase 2), the upstream kinases of the Hippo pathway, is effectively suppressed because of the absence of the scaffolding protein. We used male mice at 3 to 4 month of age in all animal experiments. We subjected WW45cKO mice to transverse aortic constriction for up to 12 weeks. WW45cKO mice exhibited higher levels of nuclear YAP in cardiomyocytes during PO. Unexpectedly, the progression of cardiac dysfunction induced by PO was exacerbated in WW45cKO mice, despite decreased apoptosis and activated cardiomyocyte cell cycle reentry. WW45cKO mice exhibited cardiomyocyte sarcomere disarray and upregulation of TEAD1 (transcriptional enhancer factor) target genes involved in cardiomyocyte dedifferentiation during PO. Genetic and pharmacological inactivation of the YAP-TEAD1 pathway reduced the PO-induced cardiac dysfunction in WW45cKO mice and attenuated cardiomyocyte dedifferentiation. Furthermore, the YAP-TEAD1 pathway upregulated OSM (oncostatin M) and OSM receptors, which played an essential role in mediating cardiomyocyte dedifferentiation. OSM also upregulated YAP and TEAD1 and promoted cardiomyocyte dedifferentiation, indicating the existence of a positive feedback mechanism consisting of YAP, TEAD1, and OSM.

Conclusions: Although activation of YAP promotes cardiomyocyte regeneration after cardiac injury, it induces cardiomyocyte dedifferentiation and heart failure in the long-term in the presence of PO through activation of the YAP-TEAD1-OSM positive feedback mechanism. (*Circ Res.* 2019;124:292-305. DOI: 10.1161/CIRCRESAHA.118.314048.)

Key Words: apoptosis ■ cell cycle ■ cell proliferation ■ heart failure ■ mice

Introduction

Heart failure is a major cause of mortality in developed countries. Despite remarkable advances in the development of medical interventions, the mortality rate of heart failure

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patients remains high.¹ Pressure overload (PO), imposed by high blood pressure or aortic valve stenosis, induces cardiac

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Novelty and Significance

What Is Known?

- The Hippo pathway is an evolutionarily conserved signaling pathway that controls organ size by promoting apoptosis and inhibiting cell proliferation.
- Mst1 (Mammalian sterile 20-like 1) and Lats2 (large tumor suppressor kinase 2), upstream kinases of the Hippo pathway, are activated by stress in cardiomyocytes and promote heart failure.
- Inhibition of the Hippo pathway and consequent activation of YAP (Yes-associated protein) promote myocardial regeneration after myocardial infarction.

What New Information Does This Article Contribute?

- Chronic suppression of the Hippo pathway and activation of YAP are detrimental in the presence of pressure overload, despite suppression of apoptosis and promotion of cell cycle reentry in cardiomyocytes.
- Detrimental effects of YAP during pressure overload are mediated through activation of a positive feedback loop consisting of YAP, TEAD (transcriptional enhancer factor), and OSM (oncostatin M).

The Hippo pathway is activated during heart failure and promotes apoptosis of cardiomyocytes and inhibits cardiomyocyte

proliferation or hypertrophy. Recent evidence suggests that downregulation of the Hippo pathway or activation of YAP, the terminal nuclear factor promoting cell survival and proliferation, is effective in inducing myocardial regeneration after myocardial infarction. This raises a possibility that either strong suppression of the Hippo pathway or stimulation of YAP may represent a promising therapeutic intervention to treat heart failure and induce cardiac regeneration. However, here we show that complete suppression of the Hippo pathway can be detrimental in the presence of pressure overload due to stimulation of a positive feedback mechanism consisting of YAP, TEAD1, and OSM, which leads to a reduction in the contractility of individual cardiomyocytes. Thus, caution should be exercised when Hippo suppression or stimulation of YAP is considered as a modality to prevent the progression of heart failure or to stimulate myocardial regeneration, especially in the presence of pressure overload. Although excessive activation of the Hippo pathway is detrimental for the heart, excessive inhibition can also be detrimental under some conditions. We propose that the level/activity of the Hippo pathway should be maintained at appropriate levels.

Nonstandard Abbreviations and Acronyms

ACTA2	smooth muscle actin alpha 2
cKO	cardiac-specific knockout
IL	interleukin
Lats2	Large tumor suppressor kinase 2
Mst1	Mammalian sterile 20-like 1
OSM	oncostatin M
pHH	phospho-histone H3
PO	pressure overload
RUNX1	Runt-related transcription factor 1
TAC	transverse aortic constriction
TEAD	transcriptional enhancer factor
TnT	troponin T
YAP	Yes-associated protein
WGA	wheat germ agglutinin

hypertrophy to maintain wall stress and cardiac output but eventually leads to cardiac failure with increases in the death of cardiomyocytes. Because the capacity of the adult heart to compensate for cardiomyocyte loss through regeneration is limited, death of cardiomyocytes triggers a progressive reduction in the total number of cardiomyocytes and induces heart failure.²

Among the signaling mechanisms involved in the death of cardiomyocytes, the Hippo signaling pathway may play a particularly important role in the progression of heart failure.³ The Hippo signaling pathway generally serves as a major regulator of organ size, through regulation of both apoptosis and cell proliferation.⁴ Activation of upstream components of the Hippo pathway, including Mst1 (mammalian sterile 20-like 1) and Lats2 (large tumor suppressor kinase 2), promotes phosphorylation and inactivation of YAP (Yes-associated protein),

a transcription factor cofactor involved in cell survival and proliferation, thereby promoting cell death.^{5–9} We have shown previously that activation of Mst1 by myocardial stress and genetic downregulation of YAP both promote the progression of heart failure, indicating that downregulation of YAP below physiological levels is detrimental for the heart.^{10–13} In contrast, inactivation of the Hippo pathway or YAP activation through genetic intervention suppresses cell apoptosis and enhances cell survival and proliferation.^{14,15} In addition, it is recently reported that both constitutively active YAP and inactivation of the Hippo pathway could stimulate myocardial regeneration after adult myocardial injury, and finally protects the cardiac function.^{15–18} However, the physiological function of the Hippo pathway in the heart remains unknown. Although recent studies support therapeutic inhibition of the Hippo pathway during heart failure, can this concept be applied to all types of heart failure caused by distinct mechanisms without harmful consequences?

To address this question, we examined how suppression of the endogenous Hippo pathway affects the phenotype of adult hearts in the presence of a pathologically relevant stress, namely PO, using cardiac-specific homozygous knockout of WW45 (WW45cKO), a scaffolding protein that induces activation of Lats by upstream kinases Mst1/2.^{3,4} Although the Hippo pathway promotes apoptosis and suppresses cardiomyocyte proliferation/cell cycle reentry, effects that usually negatively affect cardiac function, and despite recent reports showing the salutary effect of Hippo suppression in the heart after myocardial infarction (MI),^{14,15,18,19} our preliminary results suggest that chronic suppression of the Hippo pathway and persistently elevated YAP facilitate the progression of heart failure in response to PO in WW45cKO. Given that stimulation of YAP through suppression of the Hippo pathway is assumed to be one of the most significant modalities for

facilitating myocardial regeneration, it is important to further clarify potentially detrimental effects of YAP stimulation in the heart in the presence of PO and to elucidate the potential underlying mechanisms.

Thus, the goals of this study were to elucidate the molecular mechanism by which persistent suppression of the Hippo pathway and persistent activation of YAP in WW45cKO mice promote heart failure in response to PO and investigate whether suppression of YAP prevents progression of left ventricular (LV) dysfunction in mouse models of cardiovascular disease. Our result suggests that chronic suppression of the Hippo pathway in the presence of PO activates a gene expression profile promoting cardiomyocyte dedifferentiation through activation of a positive feedback mechanism consisting of YAP, TEAD1, and OSM (oncostatin M), thereby causing contractile dysfunction in individual cardiomyocytes.

Methods

To minimize the possibility of unintentionally sharing information that can be used to reidentify private information, a subset of the data generated for this study are available at NCBI GEO and can be accessed at GSE50637 and GSE56813 and GSE92970.

Detailed methods are provided in the [Online Data Supplement](#).

Mouse Models

Generation of mice harboring a floxed WW45/Salvador allele (C57BL/6 background)²⁰ and *Myh6*-Cre recombinase (C57BL/6 background) has been reported.²¹ We generated systemic TEAD1 (transcriptional enhancer factor)^{+/−} mice using homologous recombination strategies, and the mice were backcrossed into a C57BL/6 background.²² Briefly, an 8954 bp fragment containing exon 3 and the alternatively spliced exon 4 (the DNA binding TEA domain) was used to insert LoxP sites into the flanking introns.²³ The recombined allele in the embryonic stem cells used to generate mice inadvertently contained a duplicated exon 3. It was embryonic lethal in the homozygous mice. Downregulation of TEAD1 in the heterozygous knockout mice was confirmed at both protein and mRNA levels (Online Figure I). YAP floxed mice have been described.^{11,24} We used age-matched male mice in all animal experiments to compare our results with the past studies conducted with mice with the loss of function of the Hippo pathway.¹¹ Age- and sex-matched mice without *Myh6* promoter-driven Cre recombinase or targeting allele were used as littermate controls (controls). All experiments involving animals were approved by the Rutgers New Jersey Medical School's Institutional Animal Care and Use Committee.

Results

PO-Induced Activation of YAP Was Transient in Control Mice but Was Sustained in WW45cKO Mice

To evaluate the effects of PO, mice were subjected to either transverse aortic constriction (TAC) or sham operation. In the control hearts, total YAP protein increased within 1 day, reached a peak around 7 days, and gradually returned to the basal level by 4 weeks. The level of total YAP was lower than in sham-operated mice at 8 and 12 weeks of PO. Phosphorylation of YAP at Ser127, which is mediated by Lats2 and induces nuclear exit of YAP, normalized by the total amount of YAP was not significantly altered until 7 days but was significantly elevated by 8 weeks and further increased at 12 weeks (Figure 1A and 1B). Immunostaining of heart sections showed that nuclear staining of YAP was increased significantly 1 week but not 4 weeks after TAC compared

with sham-operated mice (Figure 1C). Thus, although PO induces transient upregulation and nuclear accumulation of YAP through a Ser127 phosphorylation-independent mechanism, activation of YAP gradually returns to baseline around 4 weeks and further decreases thereafter, and this gradual decline in activation is accompanied by increased phosphorylation of YAP at Ser127 normalized by total YAP.

Because cardioprotective and regenerative effects of YAP have been reported in the adult heart,^{15,17} we asked whether we can prevent or delay the development of heart failure by inducing sustained activation of YAP after TAC. In WW45cKO, a loss-of-function mouse model of the Hippo pathway, TAC significantly increased YAP, peaking at 7 days and remaining elevated thereafter. The level of YAP in WW45cKO mice was significantly higher than in control mice under basal conditions and 4, 8, and 12 weeks after TAC (Figure 1A and 1B). The Ser127 phosphorylated YAP/total YAP ratio was significantly smaller in WW45cKO mice than in control mice 8 and 12 weeks after TAC. Immunostaining of heart sections showed that nuclear staining of YAP in cardiomyocytes was significantly greater in WW45cKO than in control mice after sham operation and at 1 and 4 weeks of TAC (Figure 1C and Online Figure II).

Thus, WW45cKO mice can be used to elucidate whether sustained elevation of YAP is protective during PO.

In control mice, TAC activated Mst1, as evaluated with their phospho-MST1/2(Thr183/180)/total-MST1, and Lats2, as evaluated with their phospho-Lats2(Thr1041)/total-Lats2, with a similar time course as phosphorylation of YAP(Ser127) after PO. Activation of Mst1/2 and Lats2 and consequent phosphorylation of YAP, including at Ser127, were significantly attenuated in WW45cKO mice (Online Figure III). These results suggest that the nuclear accumulation of YAP observed after 1 week of PO cannot be sustained in control hearts because of activation of Mst1/2 and Lats2 and that endogenous WW45 plays an essential role in mediating activation of Mst1/2 and Lats2 and consequent phosphorylation of YAP at Ser127. Thus, WW45cKO mice can be used to elucidate the functional significance of chronic activation of Mst1/2 and Lats2, components of the canonical Hippo pathway, during PO.

WW45cKO Mice Exhibited Greater Cardiomyocyte Cell Cycle Reentry Without Organ Enlargement in Response to PO

Homozygous deletion of WW45 induced marked enlargement and tumor formation in the liver,²⁵ and homozygous knockout of WW45 at the fetal stage increased heart size.²⁶ Postnatal knockout of WW45 in the heart with *Myh6*-Cre, however, did not significantly affect LV size (Figure 2A) or LV weight/tibial length (Figure 2B) at baseline or in the presence of PO for 4 weeks. Cardiomyocyte cross-sectional area in WW45cKO mice, evaluated using WGA (wheat germ agglutinin) staining, was similar to that in control mice at baseline but was significantly smaller than in control mice after 4 weeks of PO (Figure 2C). Smaller cardiomyocyte size after stress often indicates the presence of newly generated cardiomyocytes because of regeneration or proliferation.^{27,28} Alternatively, activation of antihypertrophic signaling mechanisms may be responsible for

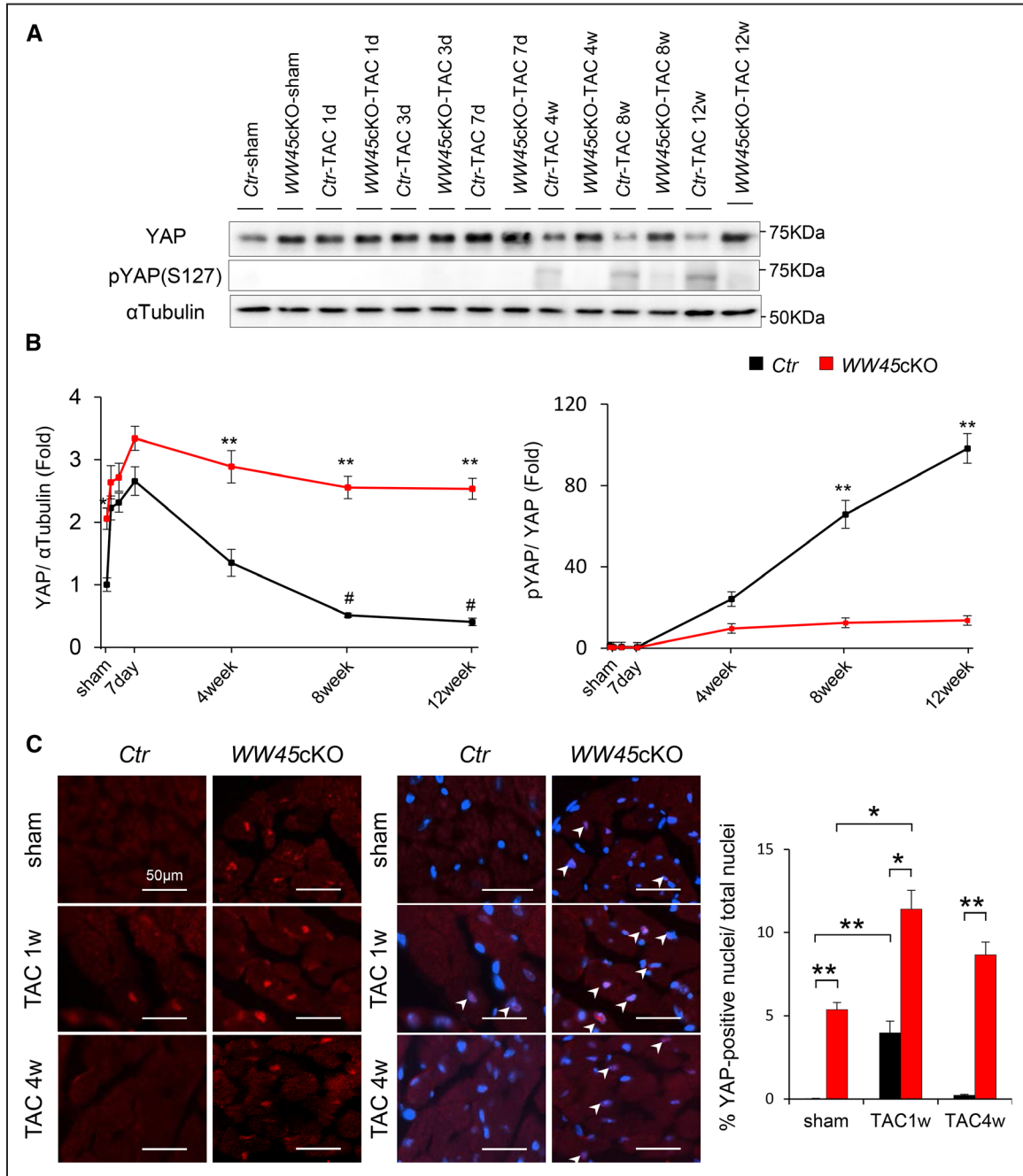


Figure 1. Pressure overload–induced YAP (Yes-associated protein) activation was transient in control (Ctr) mice but sustained in cardiac-specific homozygous knockout of WW45 (WW45cKO) mice. **A** and **B**, Representative gel pictures and quantitative analyses of immunoblot in hearts of Ctr and WW45cKO mice after operation, at the indicated time points ($n=4$, each). * $P<0.05$, ** $P<0.01$ by ANOVA, compared with Ctr–transverse aortic constriction (TAC). # $P<0.05$ compared with Ctr–sham. **C**, Representative immunostaining and quantitative analysis of YAP (YAP, red; DAPI [4',6'-diamidino-2-phenylindole], blue) in Ctr and WW45cKO 1 and 4 wk after TAC. Nuclear accumulation of YAP is indicated by arrowheads ($n=4$, each). p-YAP indicates phospho-YAP.

the smaller cardiomyocyte size. In tissue sections and isolated cardiomyocytes, the number of Ki-67-, pHH3 (phospho-histone H3)- or BrdU (bromodeoxyuridine)-positive nuclei in cardiac TnT (troponin T)-positive cardiomyocytes was similar for WW45cKO and control mice at baseline but was significantly greater in WW45cKO mice 4 weeks after TAC than in control mice (Figure 2D and Online Figure IVA and IVB). The number of pHH3 positive cardiomyocytes was also significantly greater in WW45cKO mice after TAC than in control mice when the analysis was conducted using freshly isolated cardiomyocytes

(Online Figure IVC). These results suggest that postnatal down-regulation of WW45 and consequent upregulation of endogenous YAP stimulates cardiomyocyte cell cycle reentry without significantly affecting organ size in the presence of PO.

WW45cKO Mice Exhibited More Severe Cardiac Dysfunction in Response to PO Despite Decreases in Apoptosis

Echocardiographic and hemodynamic measurements showed that WW45cKO mice have normal cardiac function at baseline.

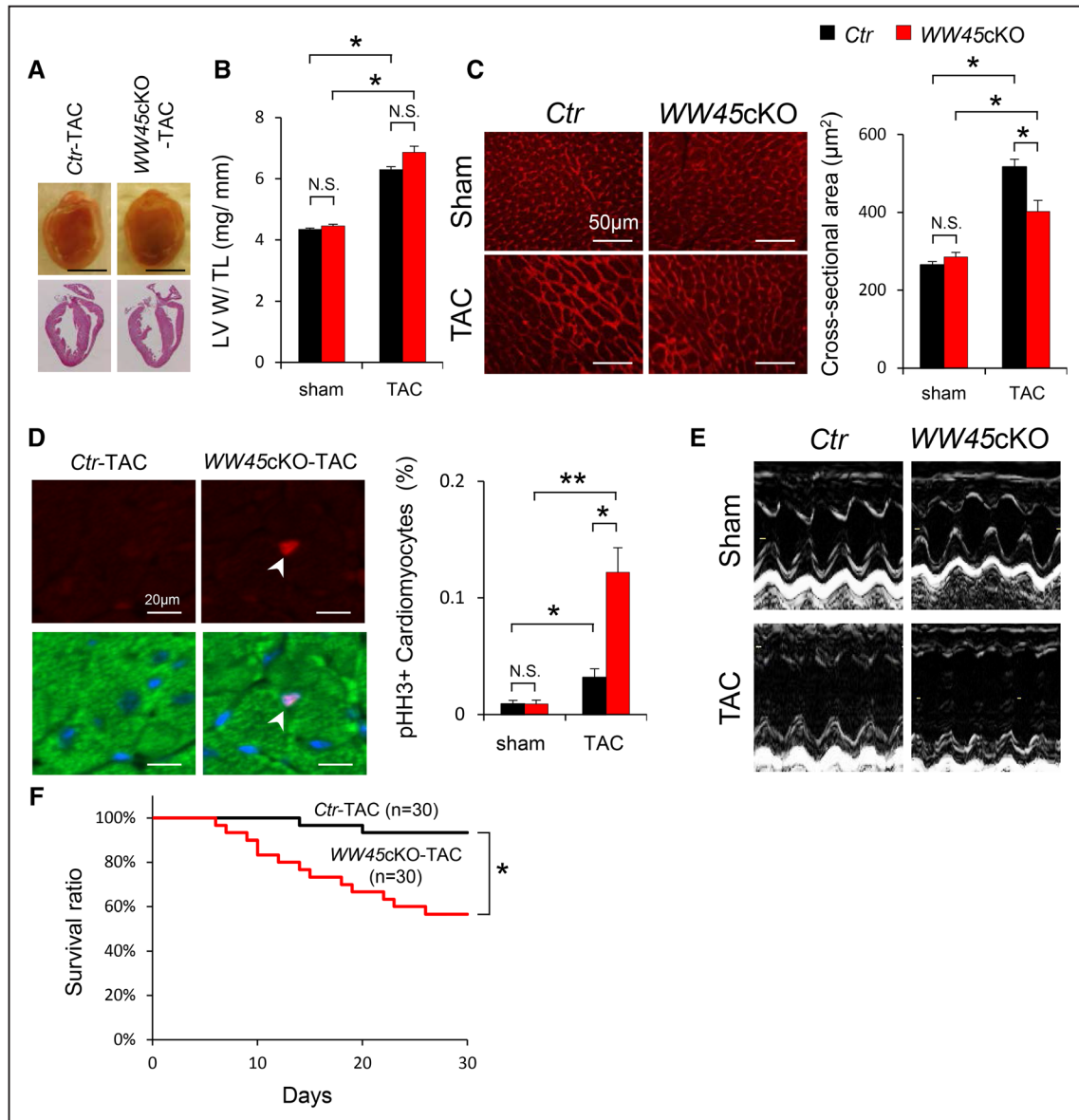


Figure 2. Cardiac-specific homozygous knockout of WW45 (WW45cKO) mice shows severe cardiac dysfunction in response to pressure overload, with higher level of markers of cardiomyocyte cycle reentry. **A**, Representative gross morphology and longitudinal heart sections from control (Ctrl) and WW45cKO mice, stained with hematoxylin-eosin 4 wk after transverse aortic constriction (TAC). Scale bars, 5.0 mm. **B**, Left ventricular weight to tibial length ratio ($n=7$, each). **C**, Representative WGA (wheat germ agglutinin) staining to assess cross-sectional area 4 wk after operation (sham: $n=6$, each; TAC: $n=8$, each). **D**, Representative immunostaining and quantitative analysis of pHH3 (phospho-histone H3) in the hearts of Ctrl and WW45cKO mice 1 wk after operation (pHH3, red; Sarc actinin, green; DAPI [4',6'-diamidino-2-phenylindole], blue; $n=6$, each). **E**, Representative echocardiographic tracings of the hearts of Ctrl and WW45cKO mice 4 wk after operation. **F**, Kaplan-Meier survival curves after TAC. All results are expressed as mean \pm SEM. * $P<0.05$, ** $P<0.01$ by ANOVA. NS indicates nonsignificant.

However, after 4 weeks of TAC, WW45cKO mice developed more severe heart failure. WW45cKO mice had more severe LV dysfunction, as indicated by decreases in LV fractional shortening (%), and a greater LV end-diastolic dimension than control mice (Figure 2E; Online Figure VA and Online Table I). Hemodynamic measurements showed prominent signs of heart failure in WW45cKO mice, including a significantly higher LV end-diastolic pressure and a lower pressure gradient than in control mice (Online Figure VB and Online Table II). WW45cKO mice also exhibited a significantly higher lung weight/tibial length, an index of lung congestion, than control mice (Online Figure VC). Consistently, WW45cKO mice exhibited significantly greater mortality in response to

TAC than control mice (Figure 2F). Histological analyses of the heart showed that there was significantly more interstitial fibrosis, but not perivascular fibrosis, in WW45cKO than in control mice after 4 weeks of TAC (Online Figure VD). The number of TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling)-positive cardiomyocytes and the myocardial level of cleaved caspase 3 after 4 weeks of TAC were significantly lower in WW45cKO mice than in control mice (Online Figure VE and VF), consistent with reduced activation of the upstream Hippo signaling pathway. Another important mechanism facilitating cardiac dysfunction during PO is inflammation. Myocardial infiltration of CD45-positive cells (leukocytes), CD68-positive cells (macrophages), and

Ly6G-positive cells (neutrophils) was not significantly different between control and WW45cKO mice 1 week after PO. Infiltration of inflammatory cells was significantly, but only slightly, enhanced 4 weeks after PO in WW45cKO mice than in control mice (Online Figure VI).

Contraction of Individual Cardiomyocytes Is Decreased in WW45cKO Mice in Response to PO

Because increased apoptosis was not observed in WW45cKO compared with control mouse hearts in the presence of PO, we hypothesized that heart failure occurs because of decreases in the contractility of individual cardiomyocytes. Contraction of freshly isolated cardiomyocytes, evaluated using the edge detection method,²⁹ was not significantly altered at baseline but was significantly decreased in cardiomyocytes isolated from WW45cKO mice compared to in those from control mice after PO (Online Figure VIIA). Although there was no effect on myofilament Ca²⁺ sensitivity or the Hill coefficient (data not shown), the maximum development of force in skinned cardiomyocytes was significantly reduced in WW45cKO compared with control mice ($P < 0.0002$) after PO (Online Figure VIIB). Consistently, electron microscopic analyses showed sarcomeric disarray, especially in M-line structures, and more irregularly shaped intercalated disks in WW45cKO than in control mouse hearts after PO (Online Figure VIIC).

YAP Mediates the Detrimental Phenotype in WW45cKO Mice in Response to PO

To investigate the role of YAP in the exacerbation of LV dysfunction in WW45cKO mice in response to PO, we generated WW45cKO-YAP heterozygous cKO (YAPhcKO) mice. Although both WW45cKO mice and YAPhcKO mice exhibited reduced cardiac function and LV dilation in the presence of PO, WW45cKO-YAPhcKO mice exhibited significantly better LV function and less LV dilation than either WW45cKO or YAPhcKO mice (Online Figure VIIIA and VIIB). WW45cKO-YAPhcKO mice exhibited significantly better survival than WW45cKO mice during PO (Online Figure VIIC). Similarly, treatment of WW45cKO mice with verteporfin, a small molecule that inhibits interaction between YAP and TEADs,³⁰ rescued LV dysfunction and dilation in response to PO (Online Figure IXA and IXB). These results suggest that upregulation of YAP mediates the detrimental phenotype in WW45cKO mice in the presence of PO. However, the reversal of the detrimental phenotypes in YAPhcKO mice and control mice treated with verteporfin when they were crossed with WW45cKO mice suggests that both upregulation and suppression of YAP are detrimental for the heart during PO and that maintaining YAP at appropriate levels is critical.

WW45cKO Induces Dedifferentiation of the Heart in Response to PO, Together With TEAD1 Activation

To elucidate the mechanism by which WW45cKO mice develop more severe cardiac dysfunction in response to PO, RNA sequencing analyses were conducted. We previously identified a set of genes whose expression changes during development are reversed in TAC.³¹ These genes are commonly regarded as differentiation/development markers (Gene Ontology:0055007). Thus, we first tested whether

WW45cKO affects changes in expression in this group of genes. As shown in Figure 3A, the difference in regulation between WW45cKO-TAC mice and control-TAC for genes that belong to Gene Ontology: 0055007 was much greater than for other genes ($P = 8 \times 10^{-5}$ and 3×10^{-7} for genes downregulated and upregulated in embryonic development, Kolmogorov-Smirnov test), indicating that, under TAC, WW45cKO mice launch a stronger dedifferentiation gene program than control mice. Online Table III and a heatmap (Figure 3B) of the genes that belong to Gene Ontology:0055007 indicate that genes promoting cardiomyocyte dedifferentiation, including *Bmp2*, *Cav3*, *Dll1*, *Fzd7*, *G6pdx*, *Pak1*, *Pi16*, *Rgs2*, and *Rgs4*, are upregulated, whereas those promoting cardiomyocyte differentiation (or inhibiting dedifferentiation), including *Arrb2*, *Efnb2*, *Ep300*, *Fdps*, *Gsk3b*, *Mef2c*, *Nr3c1*, and *Slc25a4*, are downregulated in WW45cKO mice than in control mice in the presence of TAC (Figure 3B, Online Table III, and Online Figure X). Gene Ontology analyses also indicated that genes involved in mitochondria are downregulated, those involved in apoptosis are upregulated, whereas those involved in contractile fibers are both up and downregulated in WW45cKO-TAC compared with wild-type-TAC (Online Table IV).

Transcription factor binding site analyses of regulated genes suggested that TEAD1, a TEAD family transcription factor and known target of YAP, may play a role in mediating the changes in gene expression profile observed in WW45cKO mice or in the presence of PO (Figure 3C). Importantly, TEAD1 was upregulated in cardiomyocyte nuclei after PO in the heart in WW45cKO mice (Figure 3D). mRNA expression of *Myh7*, *Acta2*, and *Acta1*, fetal-type contractile proteins possessing TEAD1 binding sites in their promoters, was upregulated in WW45cKO hearts (Online Figure XI A). However, mRNA expression of *Myh6*, an adult-type contractile protein that does not possess a TEAD1 binding site, was not affected. ChIP (chromatin immunoprecipitation) assays showed that YAP binding to TEAD1 binding motifs located in the promoters of *Acta2* and *Myh7* in cardiomyocytes was increased significantly in the presence of TEAD1 overexpression (Online Figure XI B), consistent with the notion that YAP binds to the TEAD1 sites in the *Acta2* and *Myh7* promoters through TEAD1.

Immunostaining of myocardial sections showed that cardiac TnT staining was significantly reduced, whereas the number of cardiomyocytes expressing MYH7 (myosin heavy chain 7), ACTA2 (smooth muscle actin alpha 2), or RUNX1 (Runt-related transcription factor 1), markers of cardiomyocyte dedifferentiation, was significantly higher after 4 weeks of TAC in WW45cKO than in control mouse hearts (Online Figure XI C–XI E). Immunoblot analyses confirmed that downregulation of WW45 enhances upregulation of MYH7, ACTA2, and RUNX1 in response to TAC (Online Figure XI F), an effect that was attenuated by heterozygous downregulation of YAP (Online Figure VIII D–VIII H). The level of ACTA2 was significantly greater in cardiomyocytes isolated from WW45cKO mice after TAC than in those from control mice after TAC (Online Figure XI G). These results are consistent with the notion that the heart is more dedifferentiated in WW45cKO mice in the presence of PO, which may be mediated by a YAP-TEAD1-dependent mechanism.

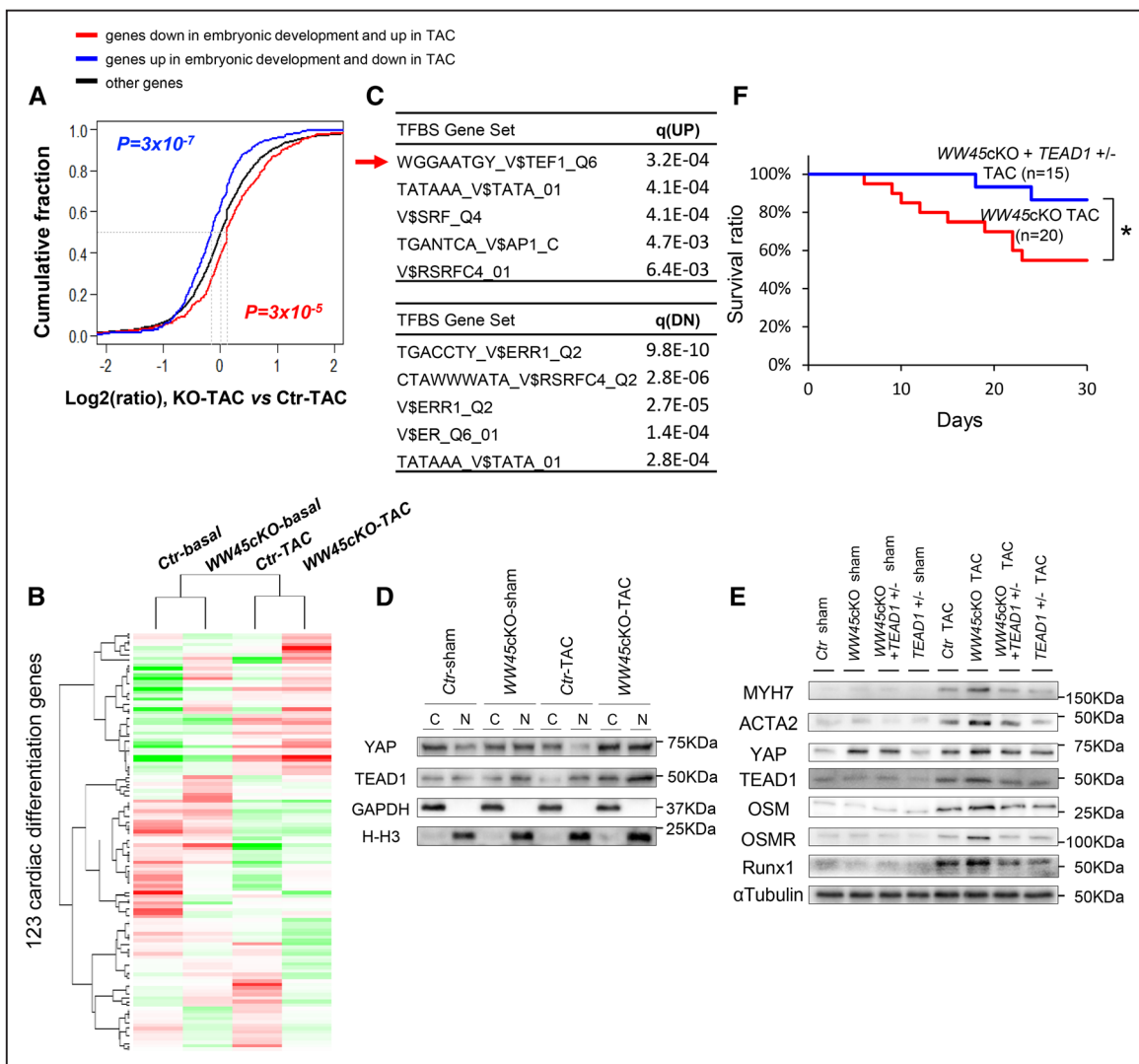


Figure 3. TEAD1 (transcriptional enhancer factor) plays an essential role in mediating the exacerbation of heart failure in cardiac-specific homozygous knockout (KO) of WW45 (WW45cKO) mice in response to pressure overload by facilitating cardiomyocyte dedifferentiation. **A**, Empirical cumulative distribution function showing the difference in regulation between WW45cKO-transverse aortic constriction (TAC) and control (Ctr)-TAC for genes oppositely regulated in cardiac development and disease. Red line: genes downregulated (DN) in embryonic development and upregulated (UP) in TAC (n=438); Blue line: genes UP in embryonic development and DN in TAC (n=365); black line: other genes. *P* are based on the Kolmogorov-Smirnov test comparing red or blue line genes with black line genes. **B**, Heatmap showing relative expression of genes regarding cardiac differentiation. Gene set derived from the association of Gene Ontology:0055007. The normalized read counts were subject to median centering before visualization. **C**, Transcription factor binding site (TFBS) analysis is based on UP or DN genes in WW45cKO vs Ctr (arrow: TEAD1). Top 5 TFBS gene sets are shown with the *Q* values for false discovery rate control. **D**, Representative gel pictures of immunoblot in the cytosolic and nuclear fractions of Ctr and WW45cKO mice 4 wk after operation (n=4, each). **E**, Representative gel pictures of immunoblot in the hearts of Ctr, WW45cKO, WW45cKO+TEAD1^{+/-} and TEAD1^{+/-} mice 4 wk after operation. **F**, Kaplan-Meier survival curves after TAC. Results are expressed as mean±SEM. **P*<0.05, ***P*<0.01 by ANOVA. ACTA2 indicates smooth muscle actin alpha 2; OSM, oncostatin M; OSMR, oncostatin M receptor; Runx1, Runt-related transcription factor 1; and YAP, Yes-associated protein.

TEAD1 Plays an Essential Role in the Exacerbation of Heart Failure in WW45cKO Mice in Response to PO by Facilitating Dedifferentiation of Cardiomyocytes

To further elucidate the critical involvement of TEAD1 in the exacerbation of cardiac dysfunction observed in WW45cKO mice during PO, WW45cKO mice were crossed with TEAD1^{+/-} mice (Online Figure IA). After confirmation that TEAD1 protein and mRNA expression in the heart are significantly reduced in TEAD1^{+/-} mice (Online Figure IB and IC), the mice were subjected to TAC for 4 weeks. As expected, heterozygous downregulation of TEAD1 inhibited the upregulation of TEAD1 during PO

in WW45cKO mice (Figure 3E and Online Figure XIID). Although heterozygous downregulation of TEAD1 did not affect the cardiac phenotype at baseline or in response to TAC in the absence of WW45cKO, it abrogated the exacerbation of PO-induced cardiac dysfunction observed in WW45cKO mice and significantly reduced the mortality of WW45cKO mice during PO (Figure 3F and Online Figure XIIB and XIIC). Heterozygous downregulation of TEAD1 normalized both decreases in cardiomyocyte cross-sectional area and increases in cardiac fibrosis in WW45cKO mice in response to PO (Online Figure XIIC and XIID). Importantly, heterozygous downregulation of TEAD1 also normalized the enhanced upregulation of

MYH7, ACTA2, ACTA1, and RUNX1 protein and mRNA in WW45cKO mice during PO (Figure 3E; Online Figures XII and XIII). Electron microscopic analyses showed that the sarcomere disarray and disturbances in the Z and M-line structures observed in WW45cKO mice in response to TAC were normalized when WW45cKO mice were crossed with TEAD1^{+/-} mice (Online Figure XIII). These results suggest that TEAD1 plays an essential role in the exacerbation of cardiac dysfunction and cardiomyocyte dedifferentiation in WW45cKO mice during PO.

OSM Is a Downstream Target of YAP/TEAD1

Because a previous study has suggested that OSM, a member of the IL (interleukin)-6 family of cytokines, is a major mediator of cardiomyocyte dedifferentiation,³² we hypothesized that OSM plays an important role in mediating cardiomyocyte dedifferentiation in WW45cKO mice during PO. Immunoblot analyses showed that both OSM and OSMR (oncostatin M receptor) are upregulated by PO and that their levels are further increased in the presence of WW45cKO (Figure 4A). Increases in OSM staining were observed both in the cytosol

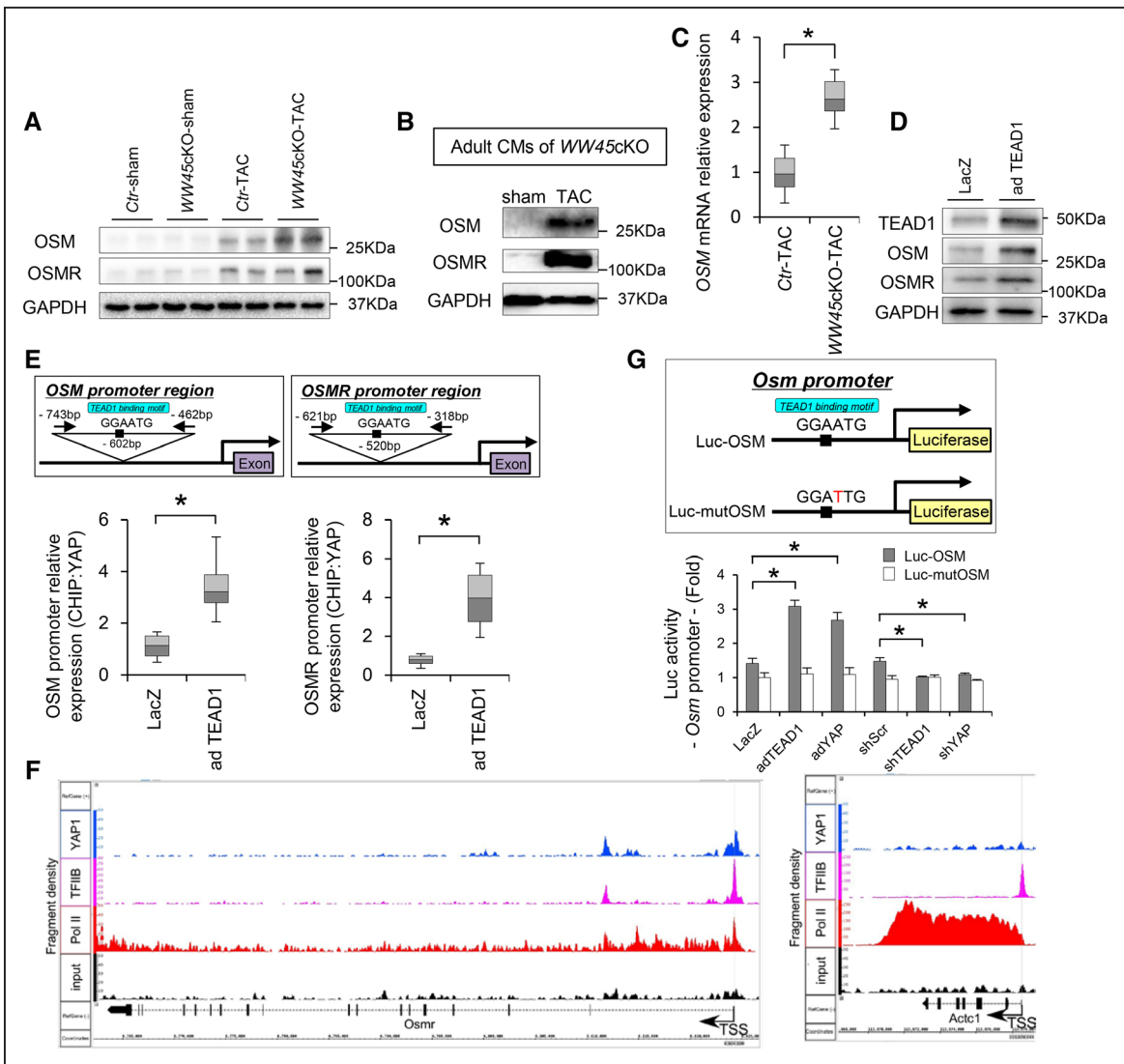


Figure 4. YAP (Yes-associated protein)-TEAD1 (transcriptional enhancer factor) activity regulates OSM (oncostatin M). **A**, Representative gel pictures of immunoblot in the hearts of control (Ctr) and cardiac-specific homozygous knockout of WW45 (WW45cKO) mice 4 wk after operation (n=6, each). **B**, Representative gel pictures of immunoblot in cardiomyocyte (CMs) isolated from adult WW45cKO mice 1 wk after operation. **C**, Relative mRNA expression in adult CMs isolated from the hearts 1 wk after transverse aortic constriction (TAC; n=3, each). **D**, Representative gel pictures of immunoblot in neonatal CMs transduced with adenovirus (Ad) harboring LacZ or TEAD1. **E**, ChIP (chromatin immunoprecipitation) assay of YAP binding to the *Osm* promoter (left) and the *Osmr* (oncostatin M receptor) promoter (right) in neonatal CMs transduced with adenovirus harboring LacZ (beta-galactosidase) or TEAD1. Polymerase chain reaction target regions and quantitative analyses are shown (n=4, each). **F**, Representative pictures of ChIP-sequencing in the *Osmr* promoter at the transcription start site (TSS) using YAP1, TFIIIB, Pol II, or control IgG antibodies. Mice were subjected to TAC for 4 days (n=3). The fragment densities (y axis) were aligned with the chromosomal coordinates (x axis) using the Integrated Genome Browser. Shown are the binding sites of YAP1, TFIIIB (transcription factor IIB), and Pol II (RNA polymerase II) along the *Osmr* (left) and *Actc1* (right) genes. The arrow defines the direction of the transcription start sites. **G**, Reporter genes assays to evaluate the role of the TEAD1 binding site in the *Osm* promoter in neonatal CMs transduced with adenovirus harboring LacZ, TEAD1, or YAP (n=6, each). Results are expressed as mean±SEM. *P<0.05, **P<0.01 by ANOVA. For box plots, whiskers show minima and maxima within 1.5 interquartile range. Ad-YAP indicates adenovirus harboring YAP; mutOSM, OSM promoter containing a mutated TEAD binding site; sh-TEAD1, short hairpin RNAs targeting TEAD1; sh-Scr, scrambled short hairpin RNA; and sh-YAP, short hairpin RNAs targeting YAP.

and in the perinuclear areas of cardiomyocytes in WW45cKO mice after TAC (Online Figure XIV). The staining was not observed when the secondary antibody was used alone or when the primary antibody was preadsorbed with blocking peptide. OSM and OSMR were also increased in cardiomyocytes isolated from WW45cKO mice after TAC (Figure 4B). OSM was upregulated at the level of mRNA (Figure 4C), suggesting that it is produced in the cardiomyocytes rather than it is solely taken up from the extracellular space. Similarly, OSM and OSMR protein levels were increased when TEAD1 was adenovirally overexpressed in cultured neonatal rat ventricular cardiomyocytes (Figure 4D). ChIP assays showed that YAP binds to a GGAATG element, the TEAD1 binding motif, in the promoter regions of the *Osm* and *Osmr* genes in cardiomyocytes (Figure 4E). ChIP-sequencing analyses with anti-YAP antibody showed that YAP binds to the promoter of *Osmr* near the transcription start site, where there is a TEAD1 binding sequence, but not to the promoter of *Actc1*, in which there is no TEAD1 binding sequence, in mouse hearts subjected to PO in vivo (Figure 4F). Overexpression of TEAD1 or YAP significantly increased the activity of a reporter gene driven by the *Osm* promoter in cardiomyocytes, but not when the TEAD1 site was mutated (Figure 4G). Conversely, short hairpin RNAs targeting YAP and TEAD1 significantly decreased the OSM reporter activity (Figure 4G). These results suggest that OSM and OSMR are downstream targets of the YAP-TEAD1 pathway in cardiomyocytes. Consistently, immunoblot analyses showed that the enhancement of OSM and OSMR upregulation observed during PO in WW45cKO mice was attenuated by heterozygous deletion of either YAP or TEAD1 (Figure 3E; Online Figures VIII, VIII, VIII, and XI).

OSM Induced Cardiomyocyte Dedifferentiation Through YAP/TEAD1

To investigate whether OSM is sufficient to induce cardiac dysfunction, we treated mice with OSM without stress. Treatment of mice with OSM (60 $\mu\text{g}/\text{kg}$ per day intraperitoneal, twice daily for 14 days; Online Figure XVA) increased MYH7 and ACTA2, together with YAP and TEAD1, in the heart at baseline, and the upregulation was further enhanced in the presence of TAC, consistent with cardiac dedifferentiation (Online Figure XVB). Interestingly, although OSM alone did not induce cardiac dysfunction without PO, OSM promoted the progression of LV dysfunction and LV dilation in response to TAC (Online Figure XVC). OSM treatment after TAC also exhibited similar results (Online Figure XVI). Taken together, these results suggest that OSM is sufficient to exacerbate PO-induced cardiac dysfunction and cardiomyocyte dedifferentiation, thereby mimicking the effect of WW45cKO during PO. Although YAP and TEAD1 upregulated OSM and OSMRs, OSM also upregulated YAP, TEAD1, OSMRs, and MYH7 in cardiomyocytes isolated from adult mouse hearts (Figure 5A) and induced nuclear accumulation of YAP (Figure 5B). OSM also upregulated the total amount of the YAP-TEAD1 complex in cultured cardiomyocytes in vitro (Online Figure XVII). Luciferase assays with a reporter gene driven by 8X TEAD binding sites showed that OSM stimulates the transcriptional activity of *Tead1* in a YAP-dependent manner in cardiomyocytes (Figure 5C). Eventually, OSM increases transcription of

the *Osm* gene itself through a YAP- and TEAD1-dependent mechanism in cardiomyocytes (Online Figure XVII B). Functionally, OSM increased the number of BrdU-positive cardiomyocytes, indicating activation of cardiomyocyte cell cycle reentry, as well as YAP staining in the nuclei of BrdU-positive cardiomyocytes (Online Figure XVIIC). OSM also increased the number of ACTA2-positive cardiomyocytes, and most ACTA2-positive cardiomyocytes exhibited nuclear accumulation of YAP (Figure 5D). These results suggest that the cardiomyocyte cell cycle reentry and dedifferentiation induced by OSM are accompanied by upregulation of TEAD1 and activation of YAP in cardiomyocytes. Indeed, in ChIP assays, OSM significantly increased binding of YAP to TEAD1 binding sequences in the *Acta2* and *Myh7* promoters in cardiomyocytes (Online Figure XVII D). Furthermore, immunoblot analyses showed that OSM upregulated YAP and TEAD1 and that OSM-induced upregulation of MYH7, ACTA2, and OSMR is suppressed in the presence of either short hairpin RNAs targeting YAP or short hairpin RNAs targeting TEAD1 in cardiomyocytes (Figure 5E and Online Figure XVIII). Likewise, immunostaining showed that OSM-induced upregulation of ACTA2 in cardiomyocytes is attenuated in the presence of short hairpin RNAs targeting TEAD1 or short hairpin RNAs targeting YAP (Figure 5F). OSM-induced cardiomyocyte cell cycle reentry was also attenuated in the presence of short hairpin RNAs targeting TEAD1 or short hairpin RNAs targeting YAP (Online Figure XVIIIE and XVIIIF). These results suggest that OSM induces cell cycle reentry and dedifferentiation of cardiomyocytes through YAP-TEAD1-dependent mechanisms. OSM inhibits p38 α and Thr1041 phosphorylation of LATS2 (Figure 5E and Figure XVIIIG), effects that have been implicated in the upregulation of TEAD1³³ and activation of YAP,³⁴ respectively, suggesting that OSM may stimulate YAP-TEAD1 through suppression of LATS2 and p38 α . Thus, YAP and TEAD1 upregulate OSM in cardiomyocytes, and OSM upregulates YAP and TEAD1, as well as OSMR through YAP and TEAD1, in cardiomyocytes, suggesting that YAP, TEAD1, and OSM/OSMR form an amplification loop to enhance OSM function and promote dedifferentiation of cardiomyocytes.

If the detrimental phenotype in WW45cKO mice during PO depends on this amplification loop, suppression of OSM would dramatically suppress the exacerbation of heart failure observed in WW45cKO mice. To address this hypothesis, we treated control and WW45cKO mice after PO with either control IgG or anti-OSM blocking antibody (Figure 6A). Anti-OSM blocking antibody treatment remarkably suppressed the exacerbation of LV dysfunction in response to TAC in WW45cKO mice, although the cardiac phenotype of control mice was not affected (Figure 6B–6D). Furthermore, expression of MYH7, ACTA2, and RUNX1 was substantially reduced and YAP and TEAD1 were partially reduced in the presence of anti-OSM blocking antibody in WW45cKO mice during PO (Figure 6E and Online Figure XIXA–XIXE). Anti-OSM antibody also inhibited upregulation of MYH7 and ACTA2 in cardiomyocytes cotransduced with Ad-YAP and Ad-TEAD1 (Online Figure XX). These results suggest that OSM is an essential component of the YAP-TEAD1-OSM amplification loop that

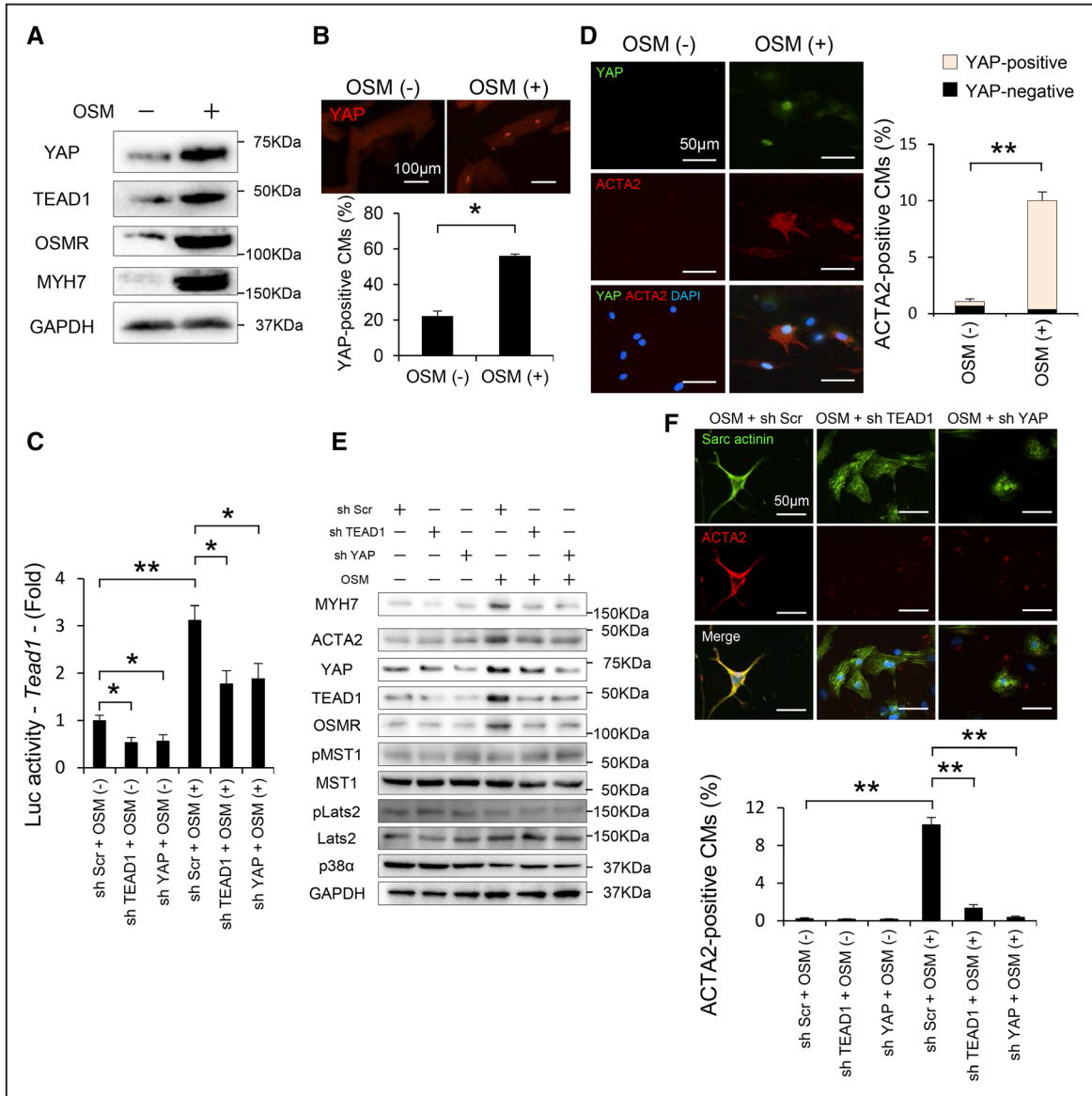


Figure 5. OSM (oncostatin M) induces cardiomyocyte (CM) dedifferentiation through YAP (Yes-associated protein)-TEAD1 (transcriptional enhancer factor). **A**, Representative gel pictures of immunoblot in CMs isolated from adult mice with or without OSM treatment. **B**, Representative immunostaining and quantitative analysis of YAP in CMs isolated from adult mice with or without OSM treatment ($n=3$, each). **C**, Reporter gene assays were conducted to evaluate the transcriptional activity of *Tead1* in response to OSM in neonatal CMs transduced with Ad-Scr, Ad-sh-TEAD1 or Ad-sh-YAP, and then transfected with 8xGTIIIC-luciferase plasmid in the presence or absence of OSM ($n=6$, each). **D**, Representative double-immunostaining and quantitative analysis of YAP and ACTA2 (smooth muscle actin alpha 2) in neonatal CMs with or without OSM treatment (YAP, green; ACTA2, red; DAPI [4',6-diamidino-2-phenylindole], blue; $n=4$, each). **E**, Representative gel pictures of immunoblot in neonatal CMs transduced with Ad-Scr, Ad-sh-TEAD1, or Ad-sh-YAP, in the presence or absence of OSM ($n=4$, each). **F**, Representative double-immunostaining and quantitative analysis of Sarcomeric actinin and ACTA2 in neonatal CMs transduced with adenovirus with or without OSM treatment (Sarc actinin, green; ACTA2, red; DAPI, blue; $n=4$, each). Results are expressed as mean \pm SEM. * $P<0.05$, ** $P<0.01$ by ANOVA. Ad-Scr indicates adenovirus-scrambled; Ad-sh-TEAD1, adenovirus- short hairpin RNA targeting TEAD1; Ad-sh-YAP, adenovirus- short hairpin RNA targeting YAP; Lats2, large tumor suppressor kinase 2; Mst1, Mammalian sterile 20-like 1; OSMR, oncostatin M receptor; pLATS, phospho-LATS; and pMST1, phospho-MST1.

facilitates the progression of heart failure in response to PO in the Hippo-deficient mice. YAP may induce cardiomyocyte cell cycle reentry through the Wnt and IGF (insulin-like growth factor) pathways. However, although expression of β -catenin was increased after 4 weeks of TAC, there was no significant difference between wild-type and WW45cKO mice. Moreover, there were no significant changes in protein expression of IGF-1R (IGF-1 receptor) in wild-type or WW45cKO mice in either the presence or absence of PO (Online Figure XIXF).

Discussion

One of the most surprising findings in this work is that chronic downregulation of Hippo and consequent activation of YAP promote cardiac dysfunction in response to PO, despite the fact that they decrease cardiomyocyte apoptosis and increase cardiomyocyte cell cycle reentry, both of which are generally thought to be cardioprotective under conditions of stress. Our study further suggests that YAP-TEAD1-mediated mechanisms, intensified by positive feedback between YAP-TEAD1 and OSM/OSMR, decreases the contraction of individual

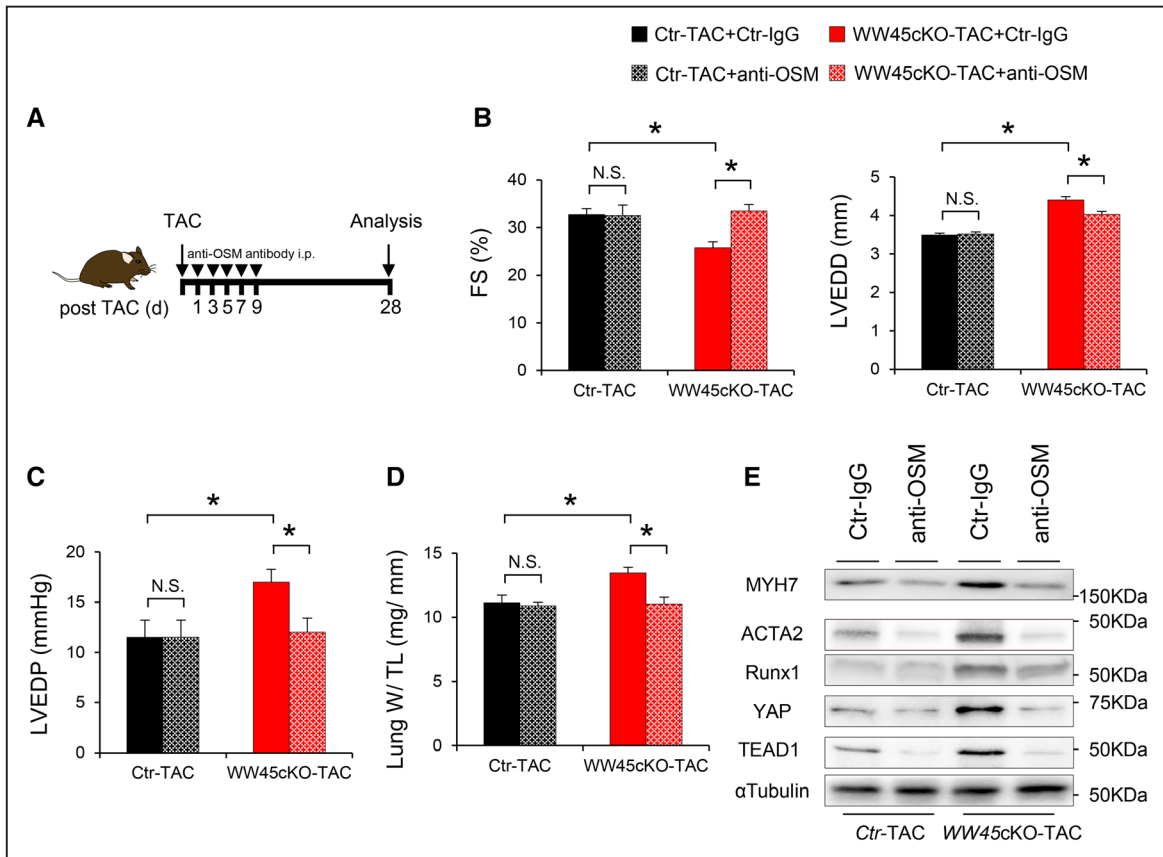


Figure 6. Anti-OSM (oncostatin M) blocking antibody improves cardiac dysfunction in cardiac-specific homozygous knockout of WW45 (WW45cKO) during pressure overload through suppression of cardiomyocyte dedifferentiation. WW45cKO mice were subjected to transverse aortic constriction (TAC) for 4 wk in the presence of either Ctr-IgG or anti-OSM blocking antibody (anti-OSM). **B**, Fractional shortening (%FS) and left ventricular end-diastolic dimension (n=4, each). **C**, Left ventricular end-diastolic dimension (LVEDD; n=4, each). **D**, Lung weight (W)/tibial length (TL; n=4, each). **E**, Representative gel pictures in the hearts after TAC in the presence of either Ctr-IgG or anti-OSM. Results are expressed as mean±SEM. **P*<0.05, ***P*<0.01 by ANOVA. ACTA2 indicates smooth muscle actin alpha 2; NS, nonsignificant; Runx1, Runt-related transcription factor 1; TEAD, transcriptional enhancer factor; and YAP, Yes-associated protein.

cardiomyocytes, thereby reducing LV function during PO. Previous studies have suggested that suppression of the Hippo pathway or exogenously supplied YAP effectively promotes myocardial regeneration and prevents the transition from compensation to decompensation in the post-MI heart primarily subjected to volume overload.^{14,19} Thus, our results suggest that the Hippo pathway and YAP have both detrimental and salutary roles in the heart depending on the nature of upstream stress.

OSM Is a Novel Target of YAP

Both RNA sequencing and CHIP-sequencing analyses suggest that YAP directly stimulates transcription of genes involved in cardiomyocyte dedifferentiation through TEAD1. This process is dramatically facilitated by a positive feedback loop consisting of OSM/OSMR, YAP, and TEAD1. In the presence of persistent upregulation of YAP- and PO-induced TEAD1 upregulation, OSM and OSMR are both upregulated in cardiomyocytes, which in turn upregulates YAP and TEAD1, most likely through suppression of Lats2 and p38 α , respectively.^{33,35} Alternatively, OSM may modulate VGLL4 (transcription cofactor vestigial-like protein 4), thereby stabilizing TEAD1 and enhancing YAP-TEAD1 interaction.³⁶ Upregulation of OSM/OSMR and activation of TEAD1 targets are dramatically stimulated in WW45cKO mice in the presence of PO, and this effect

is inhibited by attenuating any single component of the feedback loop, including YAP, TEAD1, and OSM. However, considering the fact that WW45 is a scaffolding protein and, thus, may possess known partners, we cannot formally exclude the possibility that WW45cKO may enhance TAC-induced upregulation of OSM in part through TEAD1-independent mechanisms as well.

Diverse Functions of YAP

We have shown previously that downregulation of YAP below physiological levels through either heterozygous or homozygous knockout exacerbates cardiac dysfunction at baseline and in response to chronic MI in mice because of increases in cardiomyocyte death and a lack of compensatory cardiac hypertrophy.¹¹ These results clearly suggest that a minimum level of YAP must be maintained and that the loss of function at sub-physiological levels is detrimental for the heart. The salutary effects of YAP are mediated in part through interaction with FoxO (Forkhead box-containing protein, O sub-family)¹⁶ and upregulation of either miR (microRNA)-206³⁷ or Akt.^{11,38} However, the current investigation suggests that persistent elevation of YAP above baseline and concomitant upregulation of TEAD1 induce dedifferentiation and cardiac dysfunction in the presence of PO. Thus, it is likely that YAP must be maintained at appropriate levels for the heart to maintain normal LV function. In addition, the in vivo

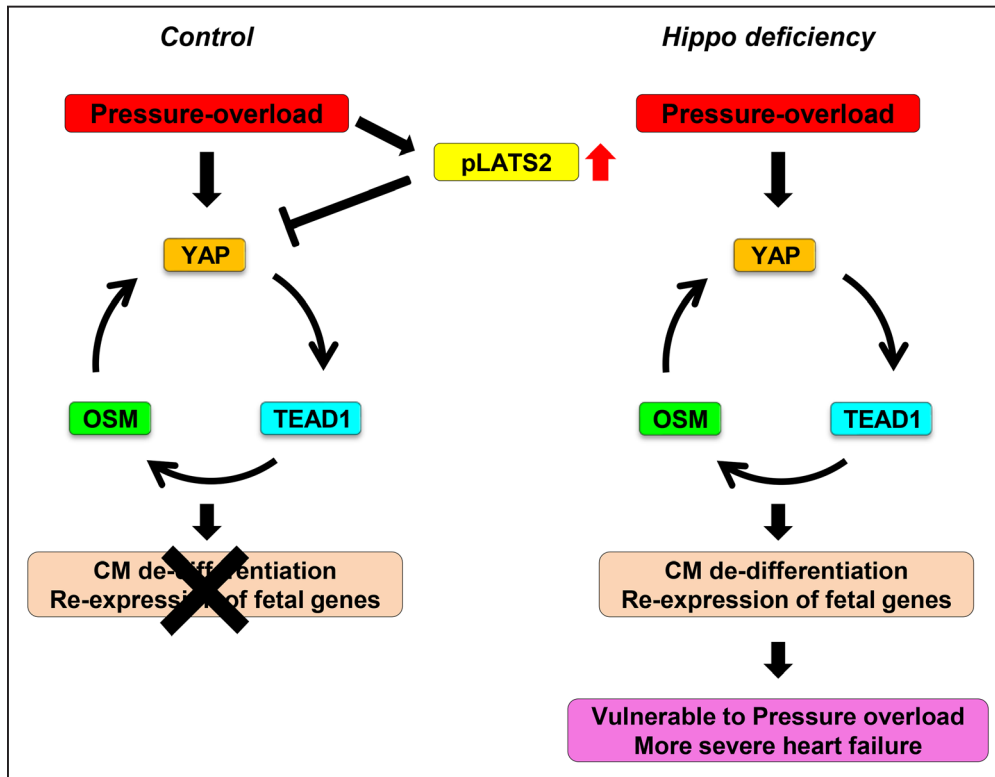


Figure 7. A schematic representation of the current hypothesis. Transverse aortic constriction (TAC) suppresses the feedback loop of YAP-TEAD1 (transcriptional enhancer factor 1)-OSM (oncostatin M) through activation of the Hippo pathway and consequent suppression of YAP. In the absence of Hippo activation, TAC dramatically stimulates the feedback loop of YAP-TEAD1-OSM, thereby inducing dedifferentiation of cardiomyocytes (CM) and preventing the heart from maintaining cardiac output in the presence of pressure overload. pLATS2 indicates phospho-LATS2.

effect of YAP is determined by the availability of downstream transcription factors in a given condition.

Underlying Mechanisms Exacerbating Heart Failure in WW45cKO Mice in Response to PO

WW45cKO mice exhibited more severe heart failure than control mice in the presence of PO. Neither apoptosis nor fibrosis seems to explain the exacerbation of heart failure in WW45cKO mice. Because decreases in maximum force generation and sarcomere disarray were observed in individual cardiomyocytes isolated from WW45cKO mouse heart subjected to TAC, it is likely that heart failure is caused by dysfunction of individual cardiomyocytes. RNA sequencing and bioinformatics analyses suggest that the chronic activation of YAP in the presence of PO facilitate gene expression pattern mimicking the fetal heart. In addition, OSM treatment, which is known to induce cardiomyocyte dedifferentiation, induces heart failure in control hearts similar to WW45cKO mouse hearts in the presence of PO. Thus, cardiomyocyte dedifferentiation may in part mediate cardiomyocyte dysfunction in the presence of PO. However, other targets of the YAP-TEAD1 pathway may also be involved in this process. For example, gene ontology analyses suggest that several groups of genes regulating cardiac function are affected in response to TAC in WW45cKO mice compared with in control hearts, although whether they are direct targets of the YAP-TEAD pathway remains to be elucidated. In addition, OSM is known to stimulate inflammation, which would, in turn, facilitate cardiac dysfunction in the presence of PO. Although modest increases in infiltration of inflammatory cells were observed in

WW45cKO mouse hearts in the presence of PO compared with control heart, the mechanistic involvement of inflammation in the exacerbation of heart failure remains to be elucidated.

Role of YAP During Distinct Forms of Hemodynamic Stress

It has been shown that upregulation of YAP promotes myocardial regeneration in the postnatal heart subjected to MI.^{14,15,17,19} In these experiments, YAP promoted proliferation of either cardiomyocytes or progenitor cells, maintained the wall thickness of the infarcted areas, and improved LV function after MI.^{14,15,17} These results seem to conflict with our finding that chronic elevation of YAP is detrimental for the heart during PO, but some differences in conditions may help explain the apparent discrepancy. Perhaps the most important difference in the conditions where upregulation of YAP is salutary or different is that whether YAP is upregulated post-MI or during pressure overload. Similar to the dichotomous roles of YAP, functional roles of OSM seem to be also context-dependent. OSM is cardioprotective during ischemia/reperfusion or in the post-MI heart,³⁸⁻⁴¹ whereas it is detrimental in some forms of cardiomyopathy, including diabetic cardiomyopathy and that induced by increased inflammation.^{31,42-44} The heart requires distinct signaling mechanisms to adapt to stress induced by volume overload, which is imposed by chronic MI, and that by pressure overload. Further investigation is required to elucidate the underlying mechanisms to explain the differential effects of YAP and downstream signaling mechanisms, including OSM, in these conditions.

YAP-TEAD1 Is a Therapeutic Target in Some Cardiovascular Conditions

YAP is generally excluded from cardiomyocyte nuclei in the adult heart at baseline, most likely because Mst1 and Mst2 have baseline activities. Although modest upregulation of YAP alone may not induce significant effects in the heart, simultaneous activation of TEAD1 or TEAD family transcription factors by a superimposed stress, such as high blood pressure, turns on the positive feedback loop of YAP-TEAD1-OSM, thereby inducing dedifferentiation and contractile dysfunction in cardiomyocytes. The consensus DNA binding sequence for TEAD family transcription factors is conserved in the *OSM*, *OSMR*, *ACTA2*, and *MYH7* genes in humans (Online Table I). Thus, suppression of the YAP-TEAD1-OSM-positive feedback loop may be effective in a subpopulation of cardiovascular patients. It is recently reported that some patients with hypertrophic cardiomyopathy exhibit high level of YAP in the heart,⁴⁵ whereas patients with heart failure exhibit elevated OSM levels in plasma.⁴⁶ In the future study, it is important to identify additional conditions in which YAP and TEAD1 are coactivated and test the efficacy of YAP inhibitors in these conditions. Moreover, the conditions of hemodynamic stress under which activation of the YAP pathway is detrimental should be specifically defined in humans because TAC may not faithfully mimic the conditions of PO in patients with chronic hypertension.

Experimental Limitations

Because of limited resources and time constraints, we conducted this investigation using only male mice, as in our previous investigations of the role of the Hippo signaling pathway in the heart.^{5,6,10–13,16,37} Whether the Hippo signaling pathway functions identically in the hearts of female mice is an important question that should be addressed in the future.

In summary, our study shows that an endogenous Hippo pathway functions to maintain cardiomyocyte differentiation so that the heart can cope with hemodynamic overload. In conditions where the YAP-OSM feedback loop is not opposed by the Hippo pathway, the heart undergoes failure because of dedifferentiation of individual cardiomyocytes.

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Disclosures

None.

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