TNFR2 Stimulation Promotes Mitochondrial Fusion via Stat3- and NF-kB-Dependent Activation of OPA1 Expression

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ABSTRACT

**Rationale:** Mitochondria are important cellular organelles and play essential roles in maintaining cell structure and function. Emerging evidence indicates that in addition to having pro-inflammatory and pro-apoptotic effects, tumor necrosis factor α (TNFα) can, under certain circumstances, promote improvements in mitochondrial integrity and function, phenomena that can be ascribed to the existence of TNFα receptor 2 (TNFR2).

**Objective:** The present study aimed to investigate whether and how TNFR2 activation mediates the effects of TNFα on mitochondria.

**Methods and Results:** Freshly isolated neonatal mouse cardiac myocytes (NMCMs) treated with shRNA targeting TNFR1 were used to study the effects of TNFR2 activation on mitochondrial function. NMCMs exhibited increases in mitochondrial fusion, a change that was associated with increases in mitochondrial membrane potential, intracellular ATP levels and oxygen consumption capacity. Importantly, TNFR2 activation-induced increases in OPA1 protein expression were responsible for the above enhancements, and these changes could be attenuated using siRNA targeting OPA1. Moreover, both Stat3 and RelA bound to the promoter region of OPA1 and their interactions synergistically upregulated OPA1 expression at the transcriptional level. Stat3 acetylation at lysine 370 or lysine 383 played a key role in the ability of Stat3 to form a supercomplex with RelA. Meanwhile, p300 modulated Stat3 acetylation in HEK293T cells, and p300-mediated Stat3/RelA interactions played an indispensable role in OPA1 upregulation. Finally, TNFR2 activation exerted beneficial effects on OPA1 expression in an in vivo transverse aortic constriction (TAC) model, whereby TNFR1-knockout mice exhibited better outcomes than in mice with both TNFR1 and TNFR2 knocked out.

**Conclusions:** TNFR2 activation protects cardiac myocytes against stress by upregulating OPA1 expression. This process was facilitated by p300-mediated Stat3 acetylation and Stat3/RelA interactions, leading to improvements in mitochondrial morphology and function.

**Keywords:** OPA1, acetylation, TNFR2, Stat3, RelA, molecular dynamics simulation, mitochondria, heart failure, cardioprotection.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Bis-I</td>
<td>Bisindolylmaleimide I</td>
</tr>
<tr>
<td>CCD</td>
<td>Coiled-coil domain</td>
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<tr>
<td>CHIP</td>
<td>Chromatin immunoprecipitation</td>
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<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
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<tr>
<td>Drp1</td>
<td>Dynamin-related protein 1</td>
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<tr>
<td>HATs</td>
<td>Histone acetyltransferases</td>
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<tr>
<td>HDACs</td>
<td>Histone deacetylases</td>
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<tr>
<td>HW/BW</td>
<td>Heart weight-to-body weight ratio</td>
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<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
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<tr>
<td>I/R</td>
<td>Ischemia-reperfusion</td>
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<td>KO</td>
<td>Knockout</td>
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<td>LK</td>
<td>Linker domain</td>
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<tr>
<td>L-OPA1</td>
<td>Long OPA1 form</td>
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<tr>
<td>LVEDD</td>
<td>Left ventricular end-diastolic diameter</td>
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<td>LVFS</td>
<td>Left ventricular fractional shortening</td>
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<tr>
<td>LW/BW</td>
<td>Lung weight-to-body weight ratio</td>
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<td>MDS</td>
<td>Molecular dynamic simulation</td>
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<tr>
<td>Mfn1</td>
<td>Mitofusin1</td>
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<td>Mfn2</td>
<td>Mitofusin2</td>
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<td>NMCMs</td>
<td>Neonatal mouse cardiac myocytes</td>
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<tr>
<td>MM/PBSA</td>
<td>Molecular mechanics/Poisson-Boltzmann surface area</td>
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<tr>
<td>NTD</td>
<td>N-terminal domain</td>
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<td>OMM</td>
<td>Outer mitochondrial membrane</td>
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<td>OPA1</td>
<td>Optic atrophy 1</td>
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<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
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<tr>
<td>PDTC</td>
<td>Pyrrolidine dithiocarbamate</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1-alpha</td>
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<tr>
<td>RCSs</td>
<td>Respiratory chain supercomplexes</td>
</tr>
<tr>
<td>Ruxo</td>
<td>Ruxolitinib</td>
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<tr>
<td>SAFE</td>
<td>Survival activating factor enhancement</td>
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INTRODUCTION

Tumor necrosis factor α (TNFα) is a cell signaling protein (cytokine) that participates in systemic inflammation, and increased serum TNFα levels are observed in patients with advanced heart failure. TNFα transmits its signals through two receptors, and a large body of evidence indicates that TNFα receptor1 (TNFR1)-dependent pathways mediate adverse cardiac remodeling and impairments in cardiac function, whereas TNFα receptor2 (TNFR2) signaling pathways counter cardiac injury and ameliorate heart failure progression. TNFα can protect the heart against apoptosis in the setting of ischemia-reperfusion (I/R) injury. Specifically, administering low concentrations of exogenous TNFα (0.5 ng/ml, in vitro) prior to I/R enhances cell survival in a dose-dependent manner, whereas administering higher concentrations (10-20 ng/ml, in vitro) leads to dose-dependent toxic effects on cells. Given that TNFR2 exhibits a higher affinity for TNFα than TNFR1, we propose that the cardioprotective effects of low concentrations of TNFα are attributable to TNFR2 activation and that the toxic effects of higher concentrations of TNFα are attributable to TNFR1 activation. In addition, the failure of previous clinical trials evaluating anti-TNFα therapies, such as RENAISSANCE, RECOVER, RENEWAL and ATTACH (where etanercept and infliximab were used to inhibit TNFα), may be attributable to the blockade of TNFR2 signaling.

Mitochondria serve as key regulators of cellular metabolic activity. In the heart, mitochondrial fitness is required for the maintenance of cardiac myocyte homeostasis. Mitochondrial fitness is facilitated largely by the following two distinct phenomena: mitochondrial biogenesis, a process that controls mitochondrial mass and copy number, and mitochondrial dynamics, which comprises mitochondrial fission and fusion. Mitochondrial biogenesis is modulated mainly by several key transcription factors, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which regulates the de novo generation of mitochondrial proteins, whereas mitochondrial dynamics is regulated mainly by a family of dynamin-related large GTPases. One of the members of this family, dynamin-related protein...
(Drp1), is largely localized in the cytoplasm and can translocate to the mitochondria to participate in fission of the outer mitochondrial membrane (OMM)\textsuperscript{18}. Two other members of this family, mitofusin (Mfn) 1 and 2, are located in the OMM, and another member of the family, optic atrophy 1 (OPA1), is located in the inner mitochondrial membrane (IMM); Mfn1 and Mfn2 control OMM, whereas OPA1 controls IMM fusion\textsuperscript{19, 20}. The boundary membrane and the cristae are two IMM sub-compartments connected via tubular structures known as cristae junctions, which are characterized by relatively small diameters\textsuperscript{21}. Cristae are studded with proteins, including respiratory chain supercomplexes (RCSs) and ATP synthase, and provide a sufficient surface area for chemical reactions to occur, a feature that enables them to serve as the major sites of aerobic cellular respiration and ATP synthesis\textsuperscript{22}. OPA1 comprises at least eight isoforms, which exist as a long OPA1 form (L-OPA1) and a short OPA1 form (S-OPA1). The oligomers formed by L-OPA1 and S-OPA1 tighten cristae junctions to stabilize RCSs, thereby increasing mitochondrial respiration efficiency\textsuperscript{23-25}.

\textbf{TNFα} can cause mitochondrial fragmentation\textsuperscript{26} and decrease both mitochondrial metabolism-related gene expression\textsuperscript{27} and RCS activity\textsuperscript{28, 29}; however, studies have also shown that TNFα can enhance mitochondrial integrity\textsuperscript{30} and stimulate mitochondrial metabolism\textsuperscript{31}. These seemingly contradictory data support the notion that TNFR2 can compromise the therapeutic effects of the anti-TNFα agents used to treat patients with heart failure. Moreover, TNFR2 activation has previously been demonstrated to protect the heart against ischemia\textsuperscript{5, 6}. Given that mitochondria play a significant role in maintaining normal cellular structure and function, investigating whether TNFR2 activation can protect cardiac myocytes by regulating mitochondrial morphology and function is worthwhile. In this study, we used cultured neonatal mouse cardiac myocytes (NMCMs) in which TNFR1 was knocked down using siRNA to study the effects of TNFR2 activation at the cellular level. We also subjected both TNFR1-knockout (KO) mice and TNFR1 and TNFR2-double (TNFR1/2)-KO mice to transverse aortic constriction (TAC)-induced stress to determine whether TNFR2 activation can protect against cardiac remodeling. Our data demonstrated that TNFR2 activation protected cardiac myocytes by modulating p300/Stat3/RelA/OPA1 signaling, resulting in cristae junction tightening and significant improvements in mitochondrial integrity, adaptive responses that enabled the indicated cells to withstand the indicated stressors.
METHODS

Generation of Stat3-knockdown and put-back cells.
Lentiviruses containing shRNA targeting mouse Stat3 (Hanheng Biotechnology, Shanghai, China) were transfected into NMCMs to knock down endogenous Stat3. To render the put-back different exogenous Stat3 resistant to siRNA, two silent nucleotide substitutions (the siRNA-resistant nucleotide sequence 5′-CTACATTGGTGTTTCATAA-3′ was used to replace the original nucleotide sequence 5′-CCACGTTGGTGTTTCATAA-3′) were introduced into Flag-tagged mouse wild-type (WT) Stat3 and two other Flag-tagged mutant forms of Stat3 for which we either generated a lysine (K)-to-glutamine (Q) mutation at residue 370 (K370Q) to mimic constitutive acetylation, or introduced lysine (K)-to-arginine (R) mutations at residues 370 and 383 (K370/383R) to mimic acetylation resistance. Adenoviruses containing the sequences of the Flag-tagged WT Stat3 and Stat3 mutants as described above were produced by Hanheng Biotechnology (Shanghai, China) for transfection into Stat3-knockdown NMCMs. Additional information regarding this procedure is available in the Supplemental Experimental Procedures.

Immunoprecipitation and western blotting.
Cultured cells were lysed in immunoprecipitation (IP) lysis buffer, after which the cell lysates were incubated in IP washing buffer with the appropriate antibodies overnight at 4 °C, with gentle agitation. The resultant protein-antibody immunocomplexes were precipitated following 1 hour of incubation with protein A/G plus agarose (Santa Cruz Biotechnology, Shanghai, China). Protein A/G plus agarose absorbed with protein-antibody immunocomplexes were washed five times in lysis buffer before SDS–PAGE and immunoblotting with the indicated antibodies. Additional information regarding this procedure is available in the Supplemental Experimental Procedures.

ATP measurements.
Cellular ATP content was measured using a luciferin/luciferase-based kit (Beyotime Biotechnology, Shanghai, China), according to the manufacturer’s instructions.

Measurement of cardiac myocyte oxygen consumption.
An Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria) was used to measure respiration in intact cells. Approximately 1×10^6 cells were suspended in MiR05 (respiration media containing 0.5 mM EGTA, 3 mM MgCl2, 6H2O, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, and 1 g/L fatty acid-free BSA, pH 7.1) and then added to the chamber with 2ml in volume (37°C). The basal oxygen consumption rate (OCR) of the above cells was measured in MiR05 containing no substrates, and the maximum OCR of the cells was measured in MiR05 when adding 1μM titration of carbonyl cyanide m-chlorophenylhydrazone (CCCP).

Transmission electron microscopy (TEM).
Murine heart specimens of the indicated genotypes were fixed in glutaraldehyde, post-fixed in osmium tetroxide, stained with ranyl acetate, dehydrated by ethanol solutions and embedded in epoxy resin.
Thereafter, the blocks were trimmed and cut into ultra-thin sections (120 nm) of the indicated thickness. The sections were subsequently observed under a transmission electron microscope. We randomly captured images to measure mitochondrial area, circularity and cristae width using the Multi-measure ROI tool developed by the manufacturers of ImageJ. Additional information regarding this procedure is available in the Supplemental Experimental Procedures.

**p300-induced Stat3 acetylation site prediction**

Protein-protein docking was performed using ZDOCK, and the indicated acetylation residues and their adjacent residues in Stat3 were set as the receptor binding sites. Any one of the following residues in p300 could serve as a donor binding site, as each residue in question was within the indicated distance (15 Å) of the receptor binding sites: ILE1456, ARG1461 and TRP1465. The binding status of each docking model was evaluated using ZRANK scores.

**Protein docking and molecular dynamics simulation (MDS).**

Mutated Stat3 (i.e., S1, S2, S3 and S4)-RelA protein docking was performed using ZDOCK. For this procedure, the angular step size was set at 15, the residues between LEU358 and ILE368 served as the receptor binding residues, and the docking models were ranked using ZRANK scores. For each of the docking complexes, i.e., S1-RelA, S2-RelA, S3-RelA and S4-RelA, the top ranked structure was selected for subsequent MDSs and binding free energy calculations. To conduct the MDS, we first optimized each docking complex. We then heated the entire system to 300 K in 50 ps. A weak restraint force constant of \( k = 10 \text{ kcal mol}^{-1} \text{ Å}^{-2} \) was applied to the protein atoms. The time for this step of the procedure was 1 fs. The complex was then simulated for 5 ns with a time step of 2 fs at 300 K. A quadratic constraint force constant of \( k = 1 \text{ kcal mol}^{-1} \text{ Å}^{-2} \) was applied to RelA during the simulation. The SHAKE algorithm was used to constrain all the chemical bonds containing hydrogen atoms, and the particle-mesh Ewald (PME) method was used to quantify long-range electrostatic interactions. The non-bonded cutoff was 8 Å.

**Binding free energy calculation.**

The binding free energy (\( \Delta G \)) of each complex was calculated using the molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) equation,

\[
\Delta G = \Delta E_{ele} + \Delta E_{vdw} + \Delta G_{PB} + \Delta G_{nondr} - T \Delta S
\]

where \( \Delta E_{ele} \) and \( \Delta E_{vdw} \) are the electrostatic and van der Waals interaction energies characterizing the interactions between the receptor and ligand; \( \Delta G_{PB} \) and \( \Delta G_{nondr} \) are the polar and nonpolar components of the desolvation energy based on the PB model, respectively; and \( T \Delta S \) is the change in conformational entropy induced by protein binding. For the binding free energy calculation, 20 snapshots of the 5-ns trajectory were taken for analysis. The value of the exterior dielectric constant was set to 80, and the value of the solute dielectric constant was set to 1. The non-polar solvation term was calculated.
from the solvent-accessible surface area (SASA\textsuperscript{39}) using the following equation:

\[ \Delta G_{\text{mispolar}} = \gamma \times \Delta \text{SASA} \]

[where \( \gamma = 0.0072 \text{ kcal/(mol} \cdot \text{Å}^2) \), and the unit of \( \Delta \text{SASA} \) is \( \text{Å}^2 \).]

Additional information regarding the antibodies, chemicals, vectors, viruses, cell culture and transfection methods, western blot analyses, real-time PCR analyses, chromatin immunoprecipitation (co-IP) assays, immunofluorescence assays, mitochondrial DNA quantification experiments, mitochondrial flow cytometry analyses, mitochondrial respiration measurements, in silico structure optimization for protein optimization experiments, echocardiography studies, animal models and histological analyses performed in the study is available in the Supplemental Experimental Procedures.

Statistical analysis.
All values are presented as the mean±SE. After confirming that all variables were normally distributed using the Kolmogorov-Smirnov test, we evaluated the significance of the differences between groups by Student’s t-test or two-way ANOVA followed by Bonferroni’s multiple comparison test. \( P<0.05 \) was considered statistically significant.

RESULTS

TNFR2 activation increased mitochondrial fusion and function in NMCMs.

To quantitatively assess the protective effects of TNFR2 activation on mitochondrial homeostasis, we transduced freshly isolated NMCMs and H9C2 cells with lentiviruses containing shRNA targeting mouse TNFR1 (TNFR1-shRNA\textsubscript{m}) and rat TNFR1 (TNFR1-shRNA\textsubscript{r}), respectively. TNFR1 silencing efficiency was pre-validated in experiments involving NMCMs and H9C2 cells transfected with negative-control shRNA (NC-shRNA) (Fig. S I A&B). Twelve hours after transduction, NMCMs with TNFR1 knocked down (NMCMs-TNFR1-KD) were treated with TNF\( \alpha \) (0.5 ng/ml) for different time periods and then harvested for TEM examination to analyze the mitochondrial morphology. Twelve hours after TNF\( \alpha \) treatment, NMCMs-TNFR1-KD featured mitochondria exhibiting a tubular morphology (Fig. 1A). We quantified the changes in mitochondria morphology induced by TNF\( \alpha \) treatment and found that TNFR2 activation in NMCMs-TNFR1-KD resulted in a 76% increase in mitochondrial size in the corresponding cells compared with placebo-treated cells, a change that was associated with a decrease in the circularity index (from 0.83±0.01 to 0.73±0.01) and a 45% increase in mitochondrial density (Fig. 1B). These data indicate that treatment with TNF\( \alpha \) enhanced mitochondrial fusion in NMCMs-TNFR1-KD.

Previous studies have shown that mitochondrial fusion is associated with increased mitochondrial oxidative phosphorylation (OXPHOS)\textsuperscript{25,40}. To confirm this finding, we studied the effects of treatment with TNF\( \alpha \) on ATP levels. Treatment with TNF\( \alpha \) resulted in a 35% increase in ATP levels in NMCMs-TNFR1-KD compared with placebo-treated cells (Fig. 1C). Moreover, treatment with TNF\( \alpha \)
resulted in a 35% increase in maximal OCRs (under CCCP treatment) but not basal OCRs in NMCMs-TNFR1-KD compared with placebo-treated cells (Fig. 1D). In addition, TNFR2 activation increased the $\Delta\psi_m$ (+18%) in TNFα-treated H9C2 cells with TNFR1-shRNAr compared with placebo-treated cells at 12 h post-treatment (Fig. 1E).

**TNFR2 activation did not promote mitochondrial biogenesis in NMCMs.**

We observed that TNFR2 activation induced significant changes in mitochondrial morphology and function. We subsequently aimed to determine whether these changes were attributable to enhanced mitochondrial biogenesis. We assessed cytoB expression levels (representing mtDNA levels), which were normalized to β-actin levels, by quantitative real-time PCR (qPCR) to measure the total mitochondrial mass in NMCMs-TNFR1-KD. Treatment with TNFα induced no changes in the above parameters in the corresponding cells compared with placebo-treated cells, indicating that TNFR2 activation does not stimulate enhancements of mitochondrial biogenesis (Fig. 1F). We also performed flow cytometry analysis, in which MitoTracker Deep Red was used to stain the mitochondria (Fig. 1G). The flow cytometry results also showed that treatment with TNFα elicited no significant changes in mitochondrial mass in the corresponding cells compared with placebo-treated cells. Moreover, we quantified the expression levels of the three core proteins involved in OXPHOS (i.e., ATP5A, MTCO1 and SDHB) by western blotting, with both HSP60 and β-actin serving as loading controls. Treatment with TNFα did not induce significant changes in the expression levels of the indicated proteins (Fig. 2A) in NMCMs-TNFR1-KD compared with placebo-treated cells. In addition, we noted no changes in the protein expression levels of PGC1α, a central regulator of mitochondrial biogenesis, in TNFα-treated cells compared with placebo-treated cells (Fig. 2A).

**TNFR2 activation modulated OPA1 expression in NMCMs.**

To further evaluate the changes in mitochondrial fusion induced by TNFR2 activation in NMCMs-TNFR1-KD, we measured the expression levels of the key proteins involved in mitochondrial dynamics$^{41-43}$ by western blot analysis. Mfn1, Mfn2 and Drp1 protein expression levels did not change significantly in TNFα-treated cells compared with placebo-treated cells (Fig. 2B); however, surprisingly, total OPA1 expression levels were significantly increased by TNFR2 activation (Fig. 2C). The results of the western blot analysis, which was performed using two different antibodies specific for OPA1, showed that the expression levels of five OPA1 isoforms (five bands) recognized by polyclonal antibodies increased by 1.52±0.1 fold in NMCMs in response to TNFR2 stimulation. Moreover, the expression levels of the L and S isoforms of OPA1 (two bands), which were detected using monoclonal antibodies, increased by 1.73 ± 0.2 fold (Fig. 2C). Thus, our data strongly suggested that the improvements in mitochondrial fusion and function induced by TNFR2 activation are due to enhancements of OPA1-dependent mitochondrial fusion.
TNFR2 activation-induced OPA1 upregulation was responsible for enhancing mitochondrial fusion and function in NMCMs.

We subsequently assessed whether enhancements of OPA1 expression were responsible for improving mitochondrial fusion and function after TNFR2 stimulation. We transfected NMCMs-TNFR1-KD with either NC-siRNA or siRNA targeting mouse OPA1 (OPA1-siRNA). The efficiency of the siRNAs was tested previously (Fig. S I C). Consistent with the results shown in Fig. 1, the results of this experiment showed that NMCMs-TNFR1-KD transfected with NC-siRNA exhibited a 56% increase in mitochondrial area, a 10% decrease in the circularity index, and a 42% decrease in mitochondrial density compared with untransfected cells following TNFR2 stimulation. All of these changes were attenuated in NMCMs-TNFR1-KD transfected with OPA1-siRNA (Fig. 2D&E). Similar results were observed in the experiments in which intracellular ATP levels (Fig. 2F) and maximum oxygen consumption capacity (Fig. 2G) were quantified, indicating that OPA1 upregulation contributed to enhancements of mitochondrial function. Interestingly, we also observed that Drp1 mitochondrial localization was slightly decreased in OPA1-siRNA-transfected cells compared with NC-siRNA-transfected cells (Fig. S II).

Both Stat3 and NF-kB signaling promoted OPA1 expression following TNFR2 activation.

To identify the specific signaling pathway that regulates OPA1 expression, we used specific inhibitors to block the signaling pathways downstream of TNFR2. Specifically, we pre-treated NMCMs-TNFR1-KD with the following agents: pyrrolidine dithiocarbamate (PDTC, at 50 µmol/L), which inhibits the NF-kB signaling pathway; bisindolylmaleimide I (Bis-I, at 50 µmol/L), which inhibits the PKC signaling pathway; wortmannin (Wort, 500 nM/L), which inhibits the PI3K signaling pathway; ruxolitinib (Ruxo, 250 nM/L), which inhibits the Stat3 signaling pathway; or KN-62 (3 µM/L), which inhibits the CaMKII signaling pathway. We found that the enhancements of OPA1 expression that were induced by TNFR2 activation were largely abrogated by pre-treatment with either PDTC or Ruxo however were unaffected by treatment with Bis-I, Wort or KN-62 (Fig. 3A), suggesting that Stat3 and NF-kB both participate in regulating OPA1 expression.

To confirm that Stat3 and NF-kB participate in regulating OPA1 expression in response to TNFR2 activation, we used siRNA specifically targeting either mouse Stat3 (Stat3-siRNA) or mouse RelA (RelA-siRNA). The silencing efficiency of the indicated siRNAs was tested previously (Fig. S I D&E). Treatment with either Stat3-siRNA or RelA-siRNA significantly attenuated the indicated enhancements of OPA1 expression in the corresponding cells compared with NC-siRNA-treated cells following TNFR2 stimulation, as shown by the results of experiments in which protein (by western blotting) and mRNA expression (by PCR) levels in the indicated cells were assessed (Fig. 3B-E). As Stat3 and RelA are both well-known important transcription factors, we aimed to determine whether Stat3 and RelA regulated OPA1 expression at the transcriptional level following TNFR2 activation. Analysis of the OPA1 promoter using Jaspar software (http://jaspar.genereg.net/) identified two putative binding sequences for Stat3 and RelA (as indicated in Fig. 3F) that were adjacent to each other. To directly determine how Stat3
and RelA mediated OPA1 expression at the transcriptional level in NMCMs-TNFR1-KD, we performed chromatin immunoprecipitation (CHIP) assays. We designed a pair of primers that targeted the R1 region covering the two putative binding sequences (Supplementary Table I). Primers targeting the R2 region were used as negative controls. The results of the CHIP analysis, in which antibodies targeting either mouse Stat3 or mouse RelA were used, showed that TNFR2 stimulation resulted in the binding of both Stat3 and RelA to the OPA1 promoter (Fig. 3F). These data implied that Stat3 and RelA can synergistically enhance OPA1 transcription by binding to adjacent regions of the OPA1 promoter.

**TNFR2 activation-mediated increases in Stat3 acetylation enhanced Stat3/RelA interactions and thus upregulated OPA1 expression.**

The roles of Stat3/RelA interactions in the nuclear retention of both proteins and the modulation of the expression of pro-survival genes have been studied previously. In the present study, we tested whether TNFR2 activation also facilitated Stat3/RelA interactions to increase OPA1 expression. We designed shRNAs targeting rat TNFR1 (TNFR1-shRNAr) and rat TNFR2 (TNFR2-shRNAr) and a negative control shRNA (NC-shRNA) and then transfected them into H9C2 cells. The gene silencing efficiency of each siRNA was tested before the experiment (Fig. S I B&F). We examined post-TNFα treatment Stat3/RelA co-localization in the nucleus by confocal immunofluorescence assay. Stat3/RelA clearly co-localized in the nuclei of H9C2 cells infected with NC-shRNA and TNFR1-shRNAr; however, only RelA localized in the nuclei of H9C2 cells infected with TNFR2-shRNAr (Fig. 4A). To confirm that TNFR2 activation modulates the interactions between Stat3 and RelA in mice, we performed an experiment involving NMCMs. We transfected shRNA targeting mouse TNFR2 (TNFR2-shRNAm) into NMCMs (NMCMs-TNFR2-KD). The silencing efficiency of the indicated siRNA was tested previously (Fig. S I G). We then performed a co-IP assay to test the interactions between endogenous Stat3 and RelA in NMCMs-TNFR2-KD and NRCMs-TNFR1-KD after the cells were stimulated by TNFα. As expected, Stat3 and RelA interacted in NRCMs-TNFR1-KD (Fig. 4B) but not in NRCMs-TNFR2-KD (Fig. 4C).

Protein acetylation has been demonstrated to play essential roles in protein-protein interactions, and the acetylation status of Stat3 has also been thoroughly investigated. Moreover, the crosstalk and interactions between Stat3 and NF-kB signaling have been described in previous studies. In the present study, we sought to determine whether Stat3 acetylation could also affect Stat3/RelA interactions following TNFR2 activation. We thus evaluated the acetylation status of Stat3 in TNFα-treated NMCMs-TNFR1-KD. We transfected shRNA targeting total endogenous Stat3 and then assayed the expression of the protein by western blotting using anti-acetyl-lysine antibodies. We observed that TNFR2 activation resulted in a dose-dependent increase in acetylated Stat3 levels (Fig. 4D). Additionally, p300-mediated modulation of Stat3 acetylation has also been described in previous studies. In the present study we determined whether p300 participated in the enhancements of Stat3 acetylation induced by TNFR2 activation. Indeed, TNFR2 activation in NMCMs-TNFR1-KD significantly increased the interactions between p300 and the Stat3/RelA complex in the nucleus but not in the cytosol (Fig. 4E). Therefore, we co-transfected myc-p300 and Flag-Stat3 into HEK293T cells to recapitulate the process through which p300 acetylates Stat3. We then immunoprecipitated the resulting Flag-Stat3 complexes using antibodies.
against Flag-Stat3 and found that p300 increased acetylated Flag-tagged Stat3 levels in a dose-dependent manner (Fig. 4F). To confirm the role of p300 in Stat3/RelA interactions, we co-transfected HEK293T cells with myc-p300, Flag-Stat3 and HA-RelA. Protein-protein interactions between Flag-Stat3 and HA-RelA were minimally detectable in HEK293T cells in which p300 was absent but were significantly enhanced in HEK293T cells in which exogenous myc-p300 was co-expressed (Fig. 4G). By contrast, when NMCMs-TNFR1-KD were transfected with p300-specific siRNA (p300-siRNA), whose knockdown efficiency was tested previously (Fig. S I H), endogenous p300 downregulation attenuated the above enhancements of the interactions between Stat3 and RelA that were induced by TNFR2 activation in NMCMs (Fig. 4H). Data collected by previous studies indicate that p300/CBP-associated factor (PCAF), another acetyltransferase, may also acetylate Stat3 61, 62. To test the hypothesis that PCAF regulates Stat3/RelA interactions in response to TNFR2 activation, we transfected NMCMs-TNFR1-KD with mouse PCAF-specific siRNA (PCAF-siRNA), whose knockdown efficiency was tested previously (Fig. S I I), and performed a co-IP assay using the methods described above. The results showed that Stat3 and RelA could still form a complex when PCAF was knocked down, suggesting that TNFR2 promotes Stat3/RelA interactions in a PCAF-independent manner (Fig. S III). The results also showed that transfecting NMCMs-TNFR1-KD with p300-siRNA eventually attenuated the enhancements of OPA1 expression induced by TNFR2 stimulation (Fig. 4I). Taken together, the results of these experiments strongly supported our hypothesis that p300 modulates Stat3 acetylation to enhance Stat3/RelA interactions, leading to enhanced OPA1 expression in TNFR2-stimulated NMCMs.

Stat3 DBD acetylation by p300 facilitated Stat3/RelA interactions.

As shown in Fig. 4F-H, p300-induced Stat3 acetylation is essential for Stat3/RelA interactions. We subsequently aimed to elucidate the molecular mechanism responsible for the interactions. Previous studies have demonstrated that Stat3 contains several functional domains, including an N-terminal domain (NTD), a coiled-coil domain (CCD), a DNA-binding domain (DBD), a linker domain (LK), an Src homology 2 domain (SH2) and a transactivation domain (TAD) 63. We first conducted ZDOCK analysis to identify the potential sites at which Stat3 is acetylated, as ZDOCK studies can simulate Stat3-p300 protein-protein docking and thus identify multiple acetylation sites on full-length (FL) Stat3. Forty-eight lysine sites in Stat3, as well as their neighboring residues, were set as receptor-binding site residues 34, and one of the three residues in p300 carrying an acetyl group to acetylate substrates (such as Stat3), namely, isoleucine 1456 (ILE1456), arginine 1461 (ARG1461) and tryptophan 1465 (TRP1465), was set as a donor binding site. The most likely potential acetylation sites were listed according to their ZRANK score. Of the 48 lysine sites in Stat3, twelve candidate residues (685, 531, 87, 409, 615, 383, 517, 49, 140, 631, 244, 370) were selected according to their ZRANK score (listed in Fig. S IV) and were assessed in subsequent experiments to quantify their ability to undergo acetylation 35. We also performed a parallel experiment, in which we generated a series of Flag-tagged truncated Stat3 mutants (Fig. 5A&B) and transfected them into HEK293T cells alone or with HA-tagged RelA and/or myc-tagged p300. We then immunoprecipitated the cell extracts with Flag-specific antibodies before immunoblotting them using antibodies targeting either HA or Myc. The FL Stat3 can bind to RelA in p300-over-expressing HEK293T cells. However, the truncated Stat3 mutants, which were devoid of their DBD (Fig. 5A), lost
the ability to interact with RelA, as demonstrated by the co-IP assay (Fig. 5B). This observation suggested that the DBD (from amino acids 330–465) of Stat3 (Fig. 5A) served as a RelA-affiliated binding domain. Importantly, this interaction was p300 dependent (lane 5 in Fig. 5B). Notably, we observed that the depletion of more Stat3 domains (Fig. 5A) gradually decreased the p300-binding capacity of Stat3 (lanes 9-11 on IB for myc-p300, Fig. 5B), indicating that multiple acetylation sites in Stat3 may participate in its interaction with p300. Thus, our data indicate that the Stat3 DBD was the domain responsible for mediating Stat3/RelA interactions. Interestingly, the ZDOCK study results pertaining to the predicted Stat3 acetylation sites (listed in Fig. SⅣ) showed that lysine (LYS)409, LYS383 and LYS370 were the most likely acetylation sites in the Stat3 DBD. Thus, we selected these three sites as receptor-binding residues; while residues of ILE1456, ARG1461 and TRP1465 within p300 that carry acetyl groups were set as donor-binding residues. We then analyzed the binding interface between Stat3 and p300 (Fig. 5C). The distance between LYS370 and its closest donor site, ILE1456, was approximately 3.457 Å, whereas the minimum distances between LYS383 and LYS409 (as the receptor residues) and their closest donor sites both exceeded 10 Å (Fig. 5C). We also evaluated the binding interfaces between the remaining nine putative acetylated lysine residues in Stat3 and p300 (Fig. SⅤ (a-i)).

*p300-mediated Stat3 acetylation at LYS370 and/or LYS383 facilitated its interaction with RelA in HEK293T cells.*

Based on the data obtained from the ZDOCK-based bioinformatic analysis, we determined the role of acetylation of the indicated lysine residues in Stat3/RelA interactions. We generated Stat3 mutants in which lysine (K) was replaced by arginine (R) to mimic the constitutively non-acetylated form of Stat3. We then constructed eight different plasmids containing Flag-tagged WT Stat3 DBDs or Flag-tagged mutant Stat3 DBDs, which featured mutations at LYS370, LYS383 or LYS409 or combinations of these mutations (Fig. 5D). We then co-transfected Flag-tagged WT Stat3 DBDs and different mutant Stat3 DBDs and myc-tagged p300 into HEK 293T cells. We first evaluated the roles of these three lysine residues in Stat3 DBD acetylation by co-IP assay. We observed WT Stat3 DBD acetylation in p300-over-expressing HEK 293T cells. Surprisingly, the K-to-R mutation on LYS409 (K409R) had no effect on Stat3 DBD acetylation, and the single K-to-R mutation on LYS370 (K370R) or LYS383 (K383R) and the combination of one of those mutations with the mutation on LYS409 (i.e., K409/370R or K409/383R) had limited effects on Stat3 DBD acetylation (Fig. 5D). Interestingly, K-to R mutations on both LYS370 and LYS383 (K370/380R) or all three lysine residues, i.e., LYS370, LYS383 and LYS409 (3K/R), abolished the capacity of Stat3 to undergo acetylation (Fig. 5D), suggesting that p300 was capable of acetylating the Stat3 DBD at LYS370 and LYS383.

We then evaluated how the acetylation status of the Stat3 DBD affects its interactions with RelA. We co-transfected HEK 293T cells with different Flag-tagged mutant Stat3 DBDs and myc-p300, as well as HA-tagged RelA. Consistent with our previous results (refer to Fig. 4G), the interaction between the WT Stat3 DBD and WT-RelA was significantly enhanced by p300 over-expression (Fig. 5E). Single K-to-R mutations (either K370R or K383R) partially reduced the ability of the Stat3 DBD to bind WT-RelA. By contrast, double K-to-R mutations (K370/383R) abolished the interaction between Stat3
MDS analysis of the contribution of Stat3 acetylation to Stat3/RelA interactions.

As co-IP assay enables only semi-quantitative assessments of protein-protein interactions, we conducted MDS to simulate the binding of RelA to an unmodified Stat3 (S1), a LYS(K)383-acetylated Stat3 (S2), a K370-acetylated Stat3 (S3) or both acetylated Stat3 species (S4; Fig. 6A). After establishing the four binding models, i.e., S1-RelA, S2-RelA, S3-RelA and S4-RelA, we calculated the binding free energy ($\Delta G_{\text{total}}$) value for each model using the MM/PBSA equation (Fig. 6B). S3-RelA had the lowest binding free energy of -77.37 kcal/mol and the binding free energy of the other models gradually increased from S4-RelA to S2-RelA and then to S1-RelA (Fig. 6B). In addition, the electrostatic interaction energy ($\Delta E_{\text{ele}}$) is a greater contributor to the above protein-protein interactions than van der Waals interaction energy ($\Delta E_{\text{vdw}}$). Moreover, in silico analysis showed that Stat3 acetylation at K370 alone led to the formation of the most stable Stat3-RelA binding interface.

We also investigated how the acetylation statuses of K370 and K383 affect the binding free energy that drives their interactions with their respective RelA binding residues using information provided by the docking model. We calculated the electrostatic and van der Waals interaction energies from twenty snapshots of the last 1 ns of the corresponding trajectories.

When K383 was acetylated, the repulsive interactions between this site and A385 and A389 of RelA decreased (Fig. 6C-“(a) and (b)”). As shown in Fig. 6D-(b) and E-(b), the distance between S2 and RelA was smaller than that between S1 and RelA. The shorter distance between S2 and RelA resulted in stronger electrostatic interactions between L388 and K383. These results indicated that acetylation at K383 may facilitate stronger binding between Stat3 and RelA.

When K370 was acetylated, the dynamics of the binding interface fluctuated, and the binding spectrum of the amino acids of RelA changed from A377-L401 (Fig. 6C-(b)) to S484-S549 (Fig. 6C-(c)). Careful quantification revealed that the energy levels of the interactions between K370 and the residues on RelA (namely, T515, L544, I547 and S548) were less than 1 kcal/mol (Fig. 6C-(c)), which indicated that the acetylation of K370 resulted in a weaker interaction between Stat3 and RelA and that the K370 residue on Stat3 was not spatially adjacent to the corresponding residues on RelA (Fig. 6F-(c)). Intriguingly, K370 acetylation contributes to enhanced electrostatic interactions between K383 and residues on RelA, enhancements that are partially attributable to strong electrostatic interactions between the positively charged lysine and the indicated negatively charged amino acids (aspartate and glutamate; Fig. 6C-“(c) and (d)”).

We also assessed the major interactions that occurred following the acetylation of both K370 and K383 and found that both residues were neutral (Fig. 6C-(d)). The interaction between S4 and RelA (Fig. 6G) was weaker than that between S3 and RelA (Fig. 6F), as the acetylated K383 was neutral, and the electrostatic interactions between K383 on Stat3 and the E489, D539 and S549 residues on RelA in the
indicated model were weaker than the corresponding interactions in the S3-RelA model. Acetylation at both K370 and K383 also enhanced the electrostatic interactions between R382, a residue in close proximity to K383 on Stat3, and S549 on RelA (Fig. 6C-(d)). These findings indicated that the variations in electrostatic energy that characterized the indicated binding interface arose from the acetylated residues and their adjacent residues. The backbone RMSD changes that occurred during 40 ns of MDS (overall, 5000 snapshots were taken during the 50-ns trajectory; the first 10 ns represented the equilibrium run, and the last snapshot was set as the RMSD reference point) are shown in Fig. S VI-(a-d). All RMSDs were converged during the simulation, and the last 1-ns trajectory was used to calculate the binding free energy.

*Acetylation of K370 and K383 was essential for OPA1 expression and, ultimately, mitochondrial fusion in NMCMs following TNFR2 activation.*

As stated above, we confirmed that p300-induced acetylation of the K370 and K383 residues of Stat3 was indispensable for the interactions between Stat3 and RelA in HEK293T cells. We also showed that knocking down p300 can abolish TNFR2-induced enhancements of OPA1 expression in NMCMs (Fig. 4I). Thus, we aimed to confirm that acetylation of Stat3 by p300 at residues K370 and K383 played key roles in its binding to RelA and the subsequent formation of a transcriptional complex (Stat3/RelA complex) that promoted OPA1 expression at the transcriptional level after TNFR2 activation.

We knocked down endogenous Stat3 in freshly isolated NMCMs and co-transfected the cells with various vectors, including WT Stat3, acetylation-mimetic Stat3 (K370Q-Stat3) and acetylation-resistant Stat3 (K370/383R-Stat3), to re-express exogenous shRNA-resistant Stat3. We then identified the resulting Stat3-knockdown and put-back cells (Fig. S VII). The co-IP assay showed that the interactions between Flag-tagged WT-Stat3 and endogenous RelA increased significantly following TNFR2 stimulation (Fig. 7A). Moreover, expressing acetylation-mimetic K370Q-Stat3 in endogenous Stat3-deprived NMCMs-TNFR1-KD substantially enhanced the interactions between Stat3 and endogenous RelA even in the absence of TNFR2 stimulation (Fig. 7A). By contrast, expressing deacetylation-resistant K370/383R-Stat3 in these cells resulted in a failure of Stat3 to bind endogenous RelA even after TNFα stimulation (Fig. 7A). Re-expressing WT Stat3 in the indicated cells resulted in increased OPA1 expression following TNFα therapy, whereas expressing K370Q-Stat3 increased OPA1 protein expression even in the absence of TNFR2 stimulation (Fig. 7B). However, as expected, no increase in OPA1 expression was observed in cells transfected with K370/383R-Stat3 (Fig. 7B). Thus, our data supported the idea that K370 and K383-dependent Stat3 acetylation plays an essential role in the Stat3/RelA interactions that lead to enhanced OPA1 expression.

When we assessed mitochondrial morphology (Fig. 7C&D) (mitochondrial area, the circularity index and mitochondrial density) and function (Fig. 7E&F) (ATP production and both basal and maximal oxygen consumption), we found that the patterns of the changes in mitochondrial morphology induced by the above treatments were consistent with the corresponding changes in Stat3/RelA interactions and OPA1 expression (as shown in Fig. 7A&B). We observed no changes in the above parameters in response to TNFR2 activation when NMCMs-TNFR1-KD were deprived of endogenous Stat3; however,
re-expressing WT Stat3 re-established the abovementioned response to TNFR2 activation. Interestingly, spontaneous increases (i.e., increases not induced by TNFα stimulation) in mitochondrial fusion (Fig. 7C&D) and function (Fig. 7E&F) were observed when K370Q-Stat3 was put back. By contrast no increases in mitochondrial fusion (Fig. 7C&D) and function (Fig. 7E&F) occurred when NMCMs-TNFR1-KD were transfected with K370/383R-Stat3.

Effects of TNFR2 on mitochondrial fusion in TAC-induced heart failure.

To determine whether TNFR2 activation-induced OPA1 expression protects the heart against stress, we generated TAC mice model to create pressure-overload induced heart failure. A total of sixty, eight-week-old mice, including thirty TNFR1-KO male mice and thirty TNFR1/TNFR2 double (TNFR1/2)-KO male mice, were evaluated in the in vivo study. Mice were randomly assigned to either sham (n=12 of each mouse genotype) or TAC surgery (n=18 of each mouse genotype) groups, and followed up for eight weeks after TAC surgery. All sham-operated TNFR1-KO and TNFR1/2-KO mice survived the eight-week postoperative period, whereas 7 TAC-treated TNFR1/2-KO mice died during the indicated period. By contrast, only 3 TAC-treated TNFR1-KO mice died; thus, survival in this group was superior to that in the double-KO group (Fig. SⅧ). Autopsy revealed that the mice in the two groups that underwent sham surgery did not differ with respect to their heart weight-to-body weight (HW/BW) or wet lung weight-to-body weight (LW/BW) ratios. However, TNFR1/2-KO mice subjected to TAC surgery had significantly increased HW/BW and LW/BW ratios compared with sham-operated mice. These changes were significantly attenuated in TNFR1-KO mice subjected to TAC surgery (Fig. 8A&B, Supplementary Table IV).

Echocardiography showed that sham-operated TNFR1-KO and TNFR1/2-KO mice did not differ with respect to their left ventricular end-diastolic diameters (LVEDDs) or left ventricular fractional shortening (LVFS) measurements at 8 weeks after the procedure (P>0.05, respectively. Fig. 8C, Supplementary Table IV). However, TNFR1/2 KO mice subjected to TAC surgery exhibited signs of severe cardiac remodeling, as well as significant increases in LVEDD and decreases in LVFS compared with sham-operated mice (P<0.05, respectively. Supplementary Table IV); however, these changes were significantly attenuated in TNFR1-KO mice subjected to TAC surgery (P<0.05, compared with TNFR1/2-KO TAC mice; Supplementary Table IV) (Fig. 8C).

We then quantified OPA1 protein expression levels in each group. Sham-operated TNFR1-KO and TNFR1/2-KO mice did not differ with respect to OPA1 expression at eight weeks post-surgery; by contrast, TNFR1/2 KO mice subjected to TAC displayed significantly decreased OPA1 expression levels compared with sham-operated mice. These changes were significantly attenuated in TNFR1-KO mice, whose OPA1 expression levels were similar to those of sham-operated mice (Fig. 8D).

To evaluate the beneficial effects of preserving of OPA1 expression, we analyzed mitochondrial morphology and function. TEM showed that sham-operated mice displayed round or rectangular mitochondria with normal morphological features (Fig. 8E&F). By contrast, TAC-treated TNFR1/2-KO
mice displayed fragmented mitochondria exhibiting significantly decreased areas and increased circularity ($P<0.05$). These changes were significantly attenuated in TAC-treated TNFR1-KO mice ($P<0.05$) (Fig. 8E&F). Flow cytometric assay of isolated mitochondria from freshly harvested hearts showed that mitochondrial size decreased in TNFR1/2-KO mice subjected to TAC surgery compared with sham-operated mice. This change was partially attenuated in TNFR1-KO mice subjected to TAC surgery (Fig. 8G).

OPA1 is critical for cristae junction tightening. As expected, cristae widths were similar between TNFR1-KO and TNFR1/2-KO mice subjected to sham surgery. By contrast, cristae were significantly wider (26%) in TAC-treated TNFR1/2-KO mice compared with sham-operated mice and were unaltered in TAC-treated TNFR1-KO mice compared with sham-operated mice (Fig. 8H). We also quantified mitochondrial respiratory capacity in each group mice. Specifically, we measured the substrate-driven OCRs for ETC complexes I, II, and IV and found that complex I and II activity levels were significantly decreased in TAC-treated TNFR1-KO mice compared with sham-operated mice ($P<0.01$) and were even more significantly decreased in TAC-treated TNFR1/2-KO mice compared with sham-operated mice (Fig. 8I).

Finally, histological examination demonstrated that TAC-treated TNFR1-KO mice displayed significantly less severe fibrosis than TAC-treated TNFR1/2-KO mice (Fig. 8A), indicating that the former group of mice experienced better outcomes than the latter group of mice following TAC (Fig. SⅧ).

DISCUSSION

The protective effects of TNFR2 against cardiac injury have been recognized$^{5, 6, 64}$; however, the mechanisms underlying these effects remain poorly understood. In this study, we showed for the first time that TNFR2 activation confers mitochondria-dependent cardioprotective effects. As a mitochondrial pro-fusion protein, OPA1 has received much attention because it plays significant roles in modulating mitochondrial morphology and function$^{65, 15, 23, 25}$. Our data demonstrated that TNFR2 activation mediates interactions between Stat3 and RelA to synergistically upregulate OPA1 expression, an effect that can be abolished by knocking down either Stat3 or RelA. Furthermore, using in vitro and in silico (MDS) studies, we verified that the acetylation statuses of the two lysine residues within the Stat3 DBD, i.e., K370 and K383, can be modulated by p300, which facilitates Stat3/RelA interactions, and that K370 acetylation resulted in a more stable Stat3/RelA complex than K383 acetylation. We confirmed that Stat3 acetylation plays an essential role in TNFR2 activation-induced increases in OPA1 expression via the following two approaches: first, we knocked down endogenous Stat3 in NMCMs and ectopically expressed the acetylation-mimetic K370Q mutant (a knockdown-put-back approach) to recapitulate its pro-OPA1 expression effects even in the absence of TNFα stimulation. Second, we transfected NMCMs deprived of endogenous Stat3 with an acetylation-resistant K370/383R mutant. We found that no changes in OPA1
expression occurred in these cells despite TNFR2 stimulation. Taken together, our data indicate that
TNFR2 can activate p300/Stat3/RelA/OPA1 signaling to improve mitochondrial fusion and function and
thus ameliorate heart failure.

In the setting of I/R injury, Stat3 signaling, which is also a key component of the survival activating
factor enhancement (SAFE) pathway, plays an essential role in the protection conferred by ischemic pre-
or post-conditioning5, 6. Given the short time frame associated with the indicated setting, it is difficult to
believe that Stat3 would exert its effects as a transcription factor and/or cofactor. It is more likely that
phosphorylation at serine 727 in Stat3 mediates its mitochondrial translocation66 and that this
non-canonical Stat3 signaling pathway may ameliorate I/R injury by reducing ROS production and
inhibiting mitochondrial permeability transition pore opening at the onset of reperfusion67. Whether and
how mitochondria are involved in the cardioprotective effects of Stat3 under chronic stress conditions has
seldom been reported. We showed for the first time that Stat3 signaling plays an essential protective role
in settings characterized by chronic stress, such as chronic pressure overload-induced cardiac remodeling,
during which Stat3 activation enhances mitochondrial integrity and function by regulating OPA1
transcriptional activity.

NF-kB has been shown to have multiple physiological effects in cardiac disease, effects that can be
either protective or detrimental68, 69. Intriguingly, both TNFR1 and TNFR2 have been shown to be able to
activate RelA, a central component of NF-kB signaling70. Therefore, it is reasonable to speculate that
TNFR1 and TNFR2 may recruit different cofactors for RelA, thereby enhancing either pro-survival or
pro-apoptotic gene expression. Based on data obtained from previous studies, as well as data obtained by
our group, we surmised that p300 served as a co-transcriptional factor to regulate RelA to produce
different gene repertoires. It is known that p300-dependent RelA acetylation increases RelA nuclear
retention and transcriptional activity49. Moreover, p300-mediated Stat3 acetylation can guide NF-kB
transcriptional activity to regulate gene expression and induce shifts in transcriptional patterns resulting in
the downregulation of pro-inflammatory or apoptotic genes and the upregulation of pro-survival genes.
These shifts in transcriptional patterns, which are driven by different upstream regulators, such as
TNFR1/RelA and TNFR2/p300/RelA, were validated in our study, which showed that TNFR1/RelA
activation had no effect on OPA1 expression (Fig. SIX)

Some studies have also demonstrated that p300-dependent Stat3 acetylation plays an essential role in
regulating mitochondrial function. It has been shown that p300-mediated Stat3 acetylation increases its
dimerization and nuclear retention and also enhances its transcriptional activity71. Heart failure is
characterized by and may be ascribed to increased glucose- and reduced fatty acid- metabolism in cardiac
myocytes72. p300-induced Stat3 acetylation inhibits gluconeogenesis52, which may counteract the
conversion of mitochondrial metabolic substrates from fatty acid to glucose to attenuate heart failure
progression. We showed that in addition to inhibiting gluconeogenesis, p300-induced Stat3 acetylation
also enhanced mitochondrial RCS assembly and stability to increase mitochondrial respiration efficiency.
These findings indicated that p300/Stat3 signaling is important for mitochondrial function in the heart.
Lysine acetylation is an evolutionarily conserved post-translational modification that regulates a variety of cellular processes, particularly nuclear transcription and cytoplasmic metabolism. Protein acetylation is regulated by enzymes with contrasting effects, histone acetyltransferases (HATs) and histone deacetylases (HDACs). A series of non-histone proteins, which serve as signal transducers and transcription factors, are also substrates of HATs and HDACs. Our results highlight that p300, a HAT family member, plays an important role in regulating mitochondrial dynamics by upregulating OPA1 expression. The data obtained in the present study support the notion that p300 plays a critical role in maintaining cardiac mitochondrial function and sustaining cell survival in postnatal hearts. Unsurprisingly, mitochondrial fragmentation was observed in p300C/H3 transgenic mice in which p300 lacked a functional domain. HDAC inhibitors have previously been reported to promote mitochondrial fusion, which can be abolished by either OPA1 or Mfn1 downregulation. Thus, we could not totally exclude the possibility that HDAC inhibitors played a role in modulating Stat3 acetylation levels in our study; nevertheless, our data strongly supported the idea that Stat3 acetylation and RelA play key roles in upregulating OPA1 expression and thus improving mitochondrial dynamics.

It should be noted that Stat3 phosphorylation at Y705 is a prerequisite for Stat3 translocation to the nucleus. The role of p300-mediated Stat3 acetylation in Stat3/RelA interactions and, consequently, OPA1 expression modulation has been thoroughly investigated; however, the p300-dependent Stat3 acetylation at K370 and K383 that occurred in the nucleus was originally facilitated by Stat3 phosphorylation. We observed that TNFR2 activation increased both Stat3 (at Tyrosine705 and Serine 727) and RelA phosphorylation levels (Fig. SXA). In addition, Ruxo, a Stat3 phosphorylation inhibitor, could prevent Stat3 accumulation in the nucleus and p300-mediated Stat3 acetylation at K370 and K383, which eventually blocked the interaction between Stat3 and RelA (Fig. SXB). Moreover, in silico analysis of the docking interface between Stat3 and RelA enabled us to determine whether Stat3-Y705 and Stat3-S727 phosphorylation was associated with Stat3/RelA interactions. As shown in Fig. SXI, the indicated phosphorylation sites, Y705 and S727, are located far away from the docking interface (the Stat3 DBD), a finding that supports the idea that Stat3 acetylation, but not Stat3 phosphorylation, contributes to Stat3/RelA interactions.

In summary, the present study has provided novel evidence indicating that TNFR2 activation can modulate mitochondrial dynamics by upregulating OPA1 expression, a process in which p300-mediated Stat3 acetylation at K370 and K383 played an essential role, as the indicated events enabled Stat3 to interact with RelA to activate OPA1 at the transcriptional level. Thus, TNFR2 activation, which facilitates enhancements of mitochondrial integrity and function, can serve as another novel therapeutic target for the treatment or prevention of cardiac dysfunction and remodeling (Fig. 8J).
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DISCLOSURES
The authors declare no conflicts of interest.

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FIGURE LEGENDS

**Figure 1. TNFR2 activation increased mitochondrial fusion and function in NMCMs.**
(A) Neonatal mouse cardiac myocytes (NMCMs) were transfected with shRNA targeting mouse TNFR1 (NMCMs-TNFR1-KD) and treated with either TNFα (0.5 ng/ml) or placebo for 12 h. Cells obtained before and after TNFα exposure were subjected to transmission electron microscopy (TEM) examination. Representative images are shown (bar=1 μm); (B) The average mitochondrial area (μm²), circularity index and number of mitochondria per μm² were quantified via TEM analysis (n=3 separate studies, 100 mitochondria/group); (C) Intracellular ATP levels were determined via luciferin/luciferase-based assays (n=3) involving NMCMs-TNFR1-KD; (D) OCRs in NMCMs-TNFR1-KD were assayed under both basal and maximal conditions (i.e., under CCCP treatment) (n=3); (E) Mitochondrial membrane potential (Δψm) was measured in H9C2 cells transfected with TNFR1-shRNA and treated with either CCCP (50 μmol/L) or oligomycin (10 μmol/L), which served as negative and positive controls, respectively (n=3). The results are shown in the bar graph; (F) Real-time PCR was performed to quantify mitochondrial cytochrome B (cytob as mtDNA) expression levels, which were normalized to β-actin expression levels in NMCMs-TNFR1-KD to assess the total mitochondrial mass; (G) MitoTracker Red staining was performed on H9C2 cells transfected with TNFR1-shRNA to stain the mitochondria for flow cytometric analysis (n=3). * denotes a P<0.05, ** denotes a P<0.01.

**Figure 2. TNFR2 activation enhanced mitochondrial fusion by upregulating OPA1 expression.**
(A) NMCMs-TNFR1-KD were treated with TNFα (0.5 ng/ml) for the indicated times and then harvested, after which the total protein was obtained. The protein expression levels of PGC1α, a transcriptional factor that regulates mitochondrial biogenesis, and selected proteins that participate in mitochondrial OXPHOS (including ATP5A, MTC01 and SDHB) were determined by western blotting (n=3) and did not change following TNFR2 activation. Both HSP60 (a mitochondrial marker protein) and β-actin (a cytosolic marker protein) served as loading controls; (B)&(C) The expression levels of mitochondrial dynamics-related proteins, including OPA1, Drp-1, Mfn1 and Mfn2, were determined by western blotting. Representative western blots are shown (n=3). Two antibodies against OPA1, including a polyclonal antibody (the first lane in panel C) and a monoclonal antibody (the fourth lane in panel C), were used to detect OPA1. The densitometry of each protein was quantified and normalized to that of either β-actin or GAPDH, and the differences in protein expression between the groups are shown in the relevant bar graphs; (D-G) To determine whether upregulated OPA1 was responsible for enhancements of mitochondrial fusion, we co-transfected NMCMs-TNFR1-KD with OPA1-siRNA before treating them with TNFα for 12 h. TEM examinations were performed, and representative images for each group are shown (D, bar=1 μm). The average mitochondrial area (μm²), circularity index and number of mitochondria per μm² were quantified and are shown in a bar graph (E, n=3, 100 mito/group). Intracellular ATP levels (F) and OCRs (G) were also determined (n=3, respectively). ** denotes P<0.01.
Figure 3. The Stat3 and NFκB signaling pathways participated in TNFR2 activation-induced OPA1 upregulation in NMCMs.

(A) Various pharmacological inhibitors of different signaling pathways, including bisindolylmaleimide I (PKC inhibitor), ruxolitinib (Stat3 inhibitor), PDTC (NF-κB inhibitor), KN-62 (CaMKII inhibitor) and wortmannin (PI3K inhibitor), were pre-administered to NMCMs-TNFR1-KD. TNFR2 activation-induced OPA1 expression levels were then quantified; (B-E) Stat3 (B&C) or RelA (D&E) was knocked down by siRNA against Stat3 or RelA, respectively, in NMCMs-TNFR1-KD, after which the cells were treated with TNFα. OPA1 protein (B&D) and mRNA expression levels (C&E) were quantified; (F) ChIP-PCR assay was performed to identify the binding sites for Stat3 and RelA in the promoter region of OPA1. ** denotes P<0.01.

Figure 4. p300 was essential for Stat3-NF-κB interactions and, consequently, OPA1 expression upon TNFR2 activation.

(A) H9C2 cells were transfected with shRNA targeting rat TNFR1 (TNFR1-shRNAr), shRNA targeting rat TNFR2 (TNFR2-shRNAr) or negative control (NC)-shRNA. These cells were then treated with TNFα (0.5 ng/ml) or placebo, which served as a control, for 3 h. Confocal microscopy examination was performed. Fluorescence staining was performed to detect RelA (in green) and Stat3 (in red), and DAPI used to identify the nucleus; (B)&(C) Co-IP experiments were performed to assess the interactions between Stat3 and RelA in NMCMs-TNFR1-KD and NMCMs-TNFR2-KD following TNFα treatment. IP and IB denote immunoprecipitation and immunoblotting, respectively; (D) Stat3 was immunoprecipitated in NMCMs-TNFR1-KD treated with incremental doses of TNFα and then immunoblotted using pan-anti-acetyl lysine antibodies (a-Ac). (E) Cytosolic (left panel) and nuclear (right panel) protein extracts were prepared from the same cells described in panel D. The proteins were then precipitated with antibodies against RelA followed by IB detecting RelA, Stat3 and p300; (F) Plasmids containing myc-p300 and Flag-Stat3 were co-transfected into HEK293T cells. Co-IP was performed again using antibodies against Flag to pull down Stat3, IB was then performed using a-Ac; (G) Plasmids containing myc-p300, Flag-Stat3 and HA-RelA were then co-transfected into HEK293T cells, as indicated, followed by co-IP to detect the interactions of Stat3 with myc-p300 and HA-RelA; (H)&(I) NMCMs-TNFR1-KD were transfected with p300-siRNA and then treated with TNFα. The interactions between endogenous Stat3 and RelA were subsequently assessed by co-IP (H), as described above. (I) OPA1, HSP60 and VDAC protein expression levels were determined by western blot analysis.

Figure 5. Acetylation of Stat3 at LYS(K)370, LYS(K)383, or both enhanced its interactions with RelA.

(A) Schematic diagram for various truncated mutant of Stat3 that span different domains of Stat3 as denoted by straight black line. FL indicates full-length Stat3. The truncated mutant Stat3 lacking NTD is denoted by ΔNTD. The same scheme was used for the other truncated mutants, which are noted by ΔCCD, ΔDBD, and ALK. TAD indicates that only the TAD domain was present in the corresponding truncated mutant. Different amino acid (aa) sites were also marked for each domain. All the lysine residues located within the DBD, which is labeled in gray, are outlined; (B) HEK293T cells were transfected with HA-RelA and/or myc-p300, as well as different vectors containing various Flag-tagged Stat3 mutants (as

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indicated in panel A). The Flag-tagged Stat3 mutants were immunoprecipitated and then immunoblotted using specific antibodies against Flag, myc and HA. (C) The stat3-p300 interface was simulated by an *in silico* analysis focusing on the lysine residues K370 (a) K383 (b) and K409 (c), which served as binding sites. The distance between the residues on stat3 and p300 is shown in the figure. (D) Various vectors containing a Stat3 DBD in which the putative lysine residues found in Flag-Stat3(DBD), namely, LYS(K)409, K383 and K370, were mutated to arginine (R) either alone or in combination, as indicated, were generated and transfected into HEK293T cells along with myc-p300. Whole-cell proteins were then harvested for immunoprecipitation, which was performed using antibodies against Flag-tag, followed by immunoblotting using antibodies against acetyl-lysine to assess the acetylation of the mutant Stat3 DBD. (E) The same HEK293T cells described in (D) were also transfected with HA-RelA before undergoing the same tests to determine whether p300-mediated Stat3 acetylation also affects Stat3/RelA interactions. NTD, N-terminal domain; CCD, coiled-coil domain; DBD, DNA-binding domain; LK, linker domain; SH2, Src homology 2 domain and TAD, transactivation domain. ARG denotes arginine; ASP, aspartic acid; CYS, cysteine; GLN, glutamine; GLU glutamic acid; HIS, histidine; ILE, isoleucine; SER, serine; TRP, tryptophan; TYR: tyrosine.

**Figure 6. MDS analysis of the contribution of Stat3 acetylation to Stat3/RelA interactions.**

(A) Four Stat3 mutants with different acetylated residues were constructed for MDS. S1, S2, S3 and S4 denote non-acetylated Stat3, K383-acetylated Stat3, K370-acetylated Stat3 and K370/K383-acetylated Stat3, respectively; (B) Free binding energy calculation for the Stat3-RelA binding complexes, namely, S1-RelA, S2-RelA, S3-RelA and S4-RelA. The energy for each interaction was expressed in kcal/mol. The definition of each equation term was provided in text; (C) The energies for the interactions between each Stat3 mutant (i.e., (a) S1; (b) S2; (c) S3 and (d) S4) and the residues located on RelA. The electrostatic and van der Waals contributions to the interactions are colored in blue and red, respectively; (D-G) The interfaces for the four binding complexes described in B and C are illustrated. (a) The RelA (with cyan surface)-Stat3 (in yellow) interactions involving residues K370 and K383 are represented by balls and sticks; (b) Close-up view of K383 in Stat3 and the residues on RelA. (c) Close-up view of K370 in Stat3 and the residues on RelA. K383(A) and K370(A) denote K383 acetylation and K370 acetylation, respectively. A, denotes alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; I, isoleucine; L, Leucine; P, proline; Q, glutamine; S, serine; T, threonine; V, valine.

**Figure 7. Acetylation of K370 and K383 was essential for OPA1 expression and, consequently, mitochondria fusion in TNFR2-treated cardiac myocytes.**

(A&B) NMCMs-TNFR1-KD were first transfected with Stat3-shRNA to knock down Stat3. Adenoviruses containing WT Stat3, Stat3 with a K370Q or K370/380R mutation, and empty vectors (which served as controls) were simultaneously transfected into the cells, which were then treated with either TNFα (0.5 ng/ml) or placebo for 3 h. Co-IP was performed using antibodies against RelA precipitation followed by IB using antibodies against Flag or RelA. OPA1 protein expression levels were detected by western blotting with HSP60, VDAC and β-actin as loading controls; (C&D) TEM images of the mitochondria were obtained using cells treated as described in panels A & B, and the average mitochondrial area (μm²), circularity index and number of mitochondria per μm² were quantified in a bar.
Figure 8. TNFR2 activation prevented pressure-overload-induced mitochondrial fragmentation and improved cardiac function in vivo.

(A) Cardiac autopsy was performed eight weeks after TAC induction in each group of mice, and hematoxylin and eosin (H&E), PSR and wheat germ agglutinin (WGA) staining were carried out; (B) The heart weight-to-body weight and lung weight-to-body weight ratios for each group of mice are also shown (n=7~8); (C) Echocardiographic examinations were performed, and representative M-mode images are shown. LVFS and LVEDD were measured to evaluate cardiac morphology and function (n=7~8/group); (D) OPA1 and HSP60 protein expression levels were detected by western blotting; (E) TEM examination was done to show the myofibrillary array and mitochondrial morphology; (F) The mitochondrial area, mitochondrial circularity index and number of mitochondria per μm² in the indicated groups were also quantified (n=3, 200 mitochondria/group); (G) Mitochondria were isolated from the heart and then stained with Mito-tracker Red before being subjected to flow cytometry analysis. Mitochondrial sizes were quantified using the FSC-A index (n=4-6); (H) Representative TEM images of the mitochondrial cristae (scale bar, 500 nm) are shown, and mitochondrial cristae widths were analyzed morphometrically (n=3, 80 mitochondria/group); (I) The specific substrate-driven OCR of each mitochondrial complex (complex I, complex II and complex IV) was determined using freshly isolated mitochondria from each group of mice. * denotes P<0.05; **, P<0.01; (J) Schematic figure demonstrating the role of p300-mediated acetylation in the Stat3-NFκB(RelA) cross-talk responsible for TNFR2-induced OPA1 upregulation and, consequentially, enhancements of mitochondrial fusion and function.
NOVELTY AND SIGNIFICANCE

What Is Known?

- Previous clinical trials evaluating anti-TNFα therapies failed to show beneficial effects against heart failure, possibly because of activation of the existence of TNFα receptor 2 (TNFR2).

- Mitochondrial fusion and fission process (mitochondrial dynamics) orchestrates the metabolic performance of the cardiac myocytes.

- Cardiac diseases are closely associated with dysregulation of mitochondrial dynamics.

What New Information Does This Article Contribute?

- We report that TNFR2 activation upregulates OPA1 expression, enhances mitochondrial fusion, promotes respiratory activity, and increases ATP content.

- Acetylation of STAT3 at lysine 370 and/or 383 by p300 is essential for the interaction of STAT3 and RelA and binding to the promoter region of OPA1 and enhancing transcription.

- We show in an in vivo transverse aortic constriction-induced heart failure mouse model that activation of TNFR2 in TNFR1 KO mice improved mitochondrial morphology and respiratory activity, leading to improved cardiac function and survival rate, as compared with TNFR1/2 double knockout (KO) mice.

Our data demonstrated that TNFR2 activation enhances mitochondria function via an OPA1-mediated mitochondrial fusion process. Thus, the TNFR2 signaling pathway might be a therapeutic target in heart failure.
**FIGURE 6**

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**A**

**B**

**C**

**D**

**E**

**F**

**G**

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Activation of OPA1 Expression

TNFR2 Stimulation Promotes Mitochondrial Fusion via Stat3- and NF-kB-Dependent

Jinliang Nan, Hengxun Hu, Yong Sun, Lianlian Zhu, Yingchao Wang, Zhiwei Zhong, Jing Zhao, Na Zhang, Ya Wang, Yaping Wang, Jian Ye, Ling Zhang, Xinyang Hu, Wei Zhu and Jian'an Wang

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SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Reagents
Tetramethylrhodamine methyl ester (TMRM; Sigma-Aldrich, Saint Louis, MO, USA) and MitoTracker Deep Red (Invitrogen, Carlsbad, CA, USA) were used to stain the mitochondria. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was obtained from Sigma-Aldrich (Saint Louis, MO, USA), and oligomycin was obtained from Santa Cruz Biotechnology (Shanghai, China). Recombinant murine TNF-α (PeproTech, Rocky Hill, USA) and recombinant rat TNF-α (PeproTech, Rocky Hill, USA) were obtained from PeproTech. Bisindolylmaleimide I, a PKC inhibitor (Selleck Chemicals, Houston, TX, USA); wortmannin, a PI3K inhibitor; pyrrolidinedithiocarbamic acid ammonium salt, an NFκB inhibitor (Santa Cruz Biotechnology, Shanghai, China); ruxolitinib, a JAK1/2 inhibitor (Selleck Chemicals); and KN-62, a CaMKII inhibitor (Selleck Chemicals, Houston, TX, USA), were administered to cardiomyocytes after TNFR2 activation. siRNAs targeting mouse RelA, p300 and PCAF, and a negative control siRNA were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA).

Cells and cell culture conditions
Neonatal mouse cardiomyocytes (NMCMs) were isolated from the hearts of neonatal C57BL6 mice via collagenase II (0.05% [w/v] (Invitrogen, Carlsbad, CA, USA) and trypsin (0.05% [w/v], Genom, China) digestion, as previously described. These cells, as well as H9C2 cells and HEK 293T cells, were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Corning, Manassas, VA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies, Paisley, UK) and 100 U/ml penicillin/streptomycin (v/v) at a density of 1×10^6 cells/ml. All cells were cultured in a 5% CO_2 atmosphere at 37°C.

Animal model and transverse aortic constriction surgery
TNFR1/2−/− and TNFR1−/− mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) (stock Nos. 003243 and 003242, respectively). The animals were fed a standard laboratory diet, allowed free access to food and water and maintained in a room with a controlled temperature (22°C±1°C) and humidity (65%–70%) under a 12:12-h light/dark cycle. All procedures were approved by the Animal Ethics Review Committee of Zhejiang University and were performed in accordance with the guidelines in NIH Publication No. 85-23 (revised 1996). Male mice (aged 6 to 8 weeks) were subjected to TAC surgery, which resulted in the development of pressure-overload conditions. The mice were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally), after which a left-sided thoracotomy was created at the second intercostal space. A 7-0 silk suture was placed around the transverse aorta and tied around a 26-gauge blunt needle, which was subsequently removed, resulting in the creation of small stenotic area. The mice in the sham-operation group underwent a similar surgical procedure but were not subjected to aortic constriction.

siRNA transfection
siRNAs targeting specific proteins and a control siRNA were transfected into NMCMs, according to the manufacturer's instructions. Briefly, the cells were transfected with 50 nM siRNA diluted in OPTIMEM (Gibco Life Technologies, NY, USA) using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). After 48 h of transfection, the cells were harvested to measure the expression levels of the indicated proteins. The sequences of the siRNA oligos for Stat3 and OPA1 are shown in online supplemental Table II.

**Plasmid vectors and transfection**
FL Flag-tagged mouse Stat3, various truncated flag-tagged mouse Stat3 mutants (see Fig. 5B for specific mutant information) and HA-tagged RelA were cloned into vectors containing the CMV promotor (Hanheng Biotechnology, Shanghai, China). Myc-tagged p300, which was obtained from Addgene (Cambridge, MA, USA), was cloned into a pCMVβ-vector. Transient transfections were performed with Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions.

**Recombinant virus vectors construction and infection**
Recombinant adenoviruses expressing FL mouse Stat3 (including the WT, K370Q mutant and K370/383R mutant Stat3) cDNA that was Flag tagged and lentiviruses carrying shRNAs against specific genes (the shRNA targeting sequences are provided in online supplemental Table III) were all provided by Hanheng Biotechnology (Shanghai, China). The viruses were amplified in HEK 293T cells and titrated according to the manufacturer's instruction. Adenoviruses containing empty plasmids (vectors) and lentiviruses containing non-specific shRNAs (NC shRNA, NC-shRNA) served as controls. Cardiomyocytes were infected with purified viruses at multiplicities of infection (MOI) of 50 (for adenoviruses) and 20 (for lentiviruses treated with polybrene at final concentration of 8 μg/ml, Sigma) overnight. Each viral suspension was replaced with fresh medium the day after infection, and the expression of the indicated proteins was determined by western blotting.

**qPCR for OPA1**
OPA1 expression levels were determined by qPCR using SYBR Green (Applied Biosystems), as previously described^3^. The expression levels of the indicated genes were normalized to those of β-actin, which was used as an internal control, and calculated using the standard \(2^{-\Delta\Delta Ct}\) method. The mRNA primers were selected using Primer3 input online software (available at: http://primer3.ut.ee/), as shown in the online supplemental Table I.

**Mitochondrial DNA quantification**
mtDNA content was determined by qPCR, as previously described^3^. β-actin and mitochondrial cytochrome-b were used as nuclear and mtDNA markers, respectively. The primers are shown in online supplemental Table I.

**Protein sample preparation and western blotting**
Western blot analysis was performed as described previously^1^· The proteins were isolated from snap-frozen hearts and cultured cells, which were extracted in RIPA solution (Beyotime, Shanghai, China) supplemented with a protease inhibitor cocktail (Roche, Base, Switzerland).
Nuclear and cytoplasmic protein fractions were obtained using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, Shanghai, China), according to the manufacturer’s instructions. Protein concentrations were determined by BCA assay (Pierce, Rockford, IL, USA). Equal amounts of protein were separated by SDS-PAGE and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The blots were subsequently incubated with primary antibodies to the following proteins: Stat3 (1:1,000, Cell Signaling Technology, Danvers, MA, USA), phospho-Stat3 (1:500, Cell Signaling Technology, Danvers, MA, USA), p300 (1:200, Abcam, Cambridge, MA, USA), TNFR1 (1:200, Santa Cruz Biotechnology, Shanghai, China), TNFR2 (1:500, Santa Cruz Biotechnology, Shanghai, China), NF-kB p65 (1:1,000, Cell Signaling Technology, Danvers, MA, USA), phospho-NF-kB p65 (1:500, Cell Signaling Technology, Danvers, MA, USA), Mfn1 (1:1,000, Cell Signaling Technology, Danvers, MA, USA), Mfn2 (1:1,000, Cell Signaling Technology, Danvers, MA, USA), Drp1 (1:1,000, Cell Signaling Technology, Danvers, MA, USA), Mfn1 (1:1,000, Abcam, Cambridge, MA, USA), PGC-1α (1:1,000, Abcam, Cambridge, MA, USA), ATP5A (1:1,000, Abcam, Cambridge, MA, USA), MTCO1 (1:1,000, Abcam, Cambridge, MA, USA), SDHB (1:1,000, Abcam, Cambridge, MA, USA), HSP60 (1:1,000, Cell Signaling Technology, Danvers, MA, USA), poly-OPA1 (1:1,000, Abcam, Cambridge, MA, USA), mono-OPA1 (1:1,000, Abcam, Cambridge, MA, USA), VDAC (1:1,000, Cell Signaling Technology, Danvers, MA, USA), Tom20 (1:1,000, Abcam, Cambridge, MA, USA), acetyl-lysine (1:1,000, Cell Signaling Technology, Danvers, MA, USA), IKB-α (1:1,000, Abcam, Cambridge, MA, USA), phospho-IKB (1:1,000, Abcam, Cambridge, MA, USA), β-actin (1:3,000, KANGCHEN, Shanghai, China), Tubulin (1:2,000, Santa Cruz Biotechnology, Shanghai, China), GAPDH (1:3,000, KANGCHEN, Shanghai, China), and Histone H3 (1:1,000, Cell Signaling Technology, Danvers, MA, USA). The blots were subsequently incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was visualized using a chemiluminescence ECL Western Blot System (Millipore, Boston, MA, USA).

Chromatin immunoprecipitation PCR
CHIP was performed as described in our previous report4. Briefly, NMCMs (5×10⁷ cells) were incubated with recombinant HPA (100 mg/ml) and cross-linked with 1% formaldehyde for 10 min at 37°C. Cross-linking was stopped by the addition of 0.125 M glycine. The cells were then washed three times with ice-cold PBS and kept on ice for 10 min in 25 mM HEPES (pH 7.8), 1.5 mM MgCl₂, 10 mM KCl, 0.1% Nonidet P 40 (NP-40), 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Roche, Base, Switzerland). The nuclei were subsequently collected and sonicated on ice to shear the DNA into fragments with an average length of 200 bp. After sonication, a chromatin solution (500 ug) was incubated with CHIP-grade antibodies against Stat3 (Cell Signaling Technology, Danvers, MA, USA), NF-kB (Cell Signaling Technology, Danvers, MA, USA), and rabbit IgG (Abcam, Cambridge, MA, USA) overnight at 4°C. The resulting antibody-bound complexes were precipitated, and the DNA fragments extricated from these complexes were purified using a QIAquick PCR Purification Kit (Qiagen, Dusseldorf, Germany). Pre-immunoprecipitated input DNA was used as a control in each reaction. The purified CHIPed DNA samples were analyzed by conventional PCR using forward and reverse primers specific for the mouse
OPA1 promoter (see online supplemental Table I).

**Immunoprecipitation and western blotting**
The cultured cells were lysed in ice-cold NP-40 buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40 and protease inhibitors. The samples were then incubated with the appropriate primary antibody overnight at 4°C, and the resulting immunocomplexes were precipitated and then incubated with protein A/G plus agarose for 1h (Santa Cruz Biotechnology, Shanghai, China). The agarose-antibody pellets were washed and collected before undergoing western blot analysis, as described above. Antibodies against the following proteins were used for western blotting: Stat3 (1:1,000, Cell Signaling Technology, Danvers, MA, USA), NF-κB (1:1,000, Cell Signaling Technology, Danvers, MA, USA), acetyl-lysine (1:300, Abcam, Cambridge, MA, USA), Flag-tag (1:1,000, Abcam, Cambridge, MA, USA), HA-tag (1:1,000, Abcam, Cambridge, MA, USA), and myc-tag (1:1,000, Abcam, Cambridge, MA, USA). For western blotting for acetylated proteins, 50 mM Tris (pH 7.5) supplemented with 10% (V/V) Tween-20 and 1% peptone (Sigma-Aldrich, Saint Louis, MO, USA) was used for blocking. The appropriate primary and secondary antibodies were prepared with 5% fat-free milk and 50 mM Tris (pH 7.5) with 0.1% peptone.

**Mitochondria isolation and respiration measurements**
Before measuring mitochondrial respiratory chain complex respiration, we isolated the mitochondria with a mitochondria isolation kit (Beyotime Biotechnology, Shanghai, China), according to the manufacturer’s instructions. Briefly, the mice were sacrificed by cervical dislocation, and their hearts were excised and weighed. Each heart was washed in ice-cold BIOPS and minced in 1 ml of BIOPS, after which the tissues and 2 ml of isolation buffer were transferred to a pre-cooled glass Potter homogenizer. The tissues were homogenized with 6-8 strokes at medium speed and then centrifuged at 800 g for 10 min 4°C. The supernatant was then transferred to a new tube and centrifuged at 10,000 g for 10 min at 4°C. After centrifugation, the supernatant was carefully discarded. The mitochondrial pellet was washed in 2 ml of isolation buffer and then resuspended in 100 µl of isolation buffer. Some isolated heart mitochondria were stored on ice until use, whereas other freshly isolated mitochondria were used immediately to measure oxygen consumption.

Mitochondrial respiration was measured in MiR05 containing 0.5 mM EGTA, 3 mM MgCl2·6H2O, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, and 1 g/L fatty acid-free BSA, pH 7.1, using an OROBOROS Oxygraph-2k at 30°C (Oroboros Instruments). We used the combination of 5 mM glutamate/2.5 mM malate as substrates to measure complex I-mediated respiration. Succinate (10 mM) plus rotenone (1.25 mM) was used to quantify complex I-dependent respiration after backflow into complex I was blocked. TMPD (0.5 mM), ascorbate (2 mM) and antimycin A (5 µM) were used to evaluate complex IV-mediated respiration. State 3 respiration was recorded after the addition of ADP at a final concentration of 0.6 mM.

**Echocardiography**
Transthoracic echocardiography was performed at day 56 after TAC surgery. The mice were
anesthetized by isoflurane inhalation. A comprehensive echocardiographic study, during which 2-dimensional and M-mode images were acquired and analyzed to assess cardiac morphology and function, was performed using a Vevo 2100 system (VisualSonics, Toronto, Canada).

**Immunofluorescence**
Cardiomyocytes were seeded on coverslips in 24-well cell culture plates. The cells were fixed with 2% paraformaldehyde for 20 min, permeabilized with PBS containing 0.1% Triton X-100 (PBS-T) for 5 min and then blocked with 3% BSA in PBS-T. Immunostaining was performed using the indicated primary antibodies (anti-Stat3 or RelA). After incubating with the appropriate secondary antibodies, the cells were washed with PBS, and the nuclei were stained with DAPI.

**Histological analysis**
Whole hearts were arrested in diastole, embedded in paraffin, and then sectioned at a thickness of 4 mm before being stained with fluorescein isothiocyanate (FITC)-labeled WGA, H&E, or Sirius red to visualize their histological changes.

**Flow cytometric analysis of mitochondrial membrane potential, mitochondrial mass and mitochondrial size**
Mitochondrial membrane potential (ψmt) and mitochondrial mass were measured after cardiomyocytes were treated with TMRM (200 nmol/L) or MitoTracker Deep Red (100 nmol/L) for 30 min. Thereafter, the cardiomyocytes underwent trypsinization, and then cell fluorescence was assessed by flow cytometry with a BD FACS Canto II Flow Cytometer (BD Biosciences, San Jose, CA, USA). The mitochondria were also incubated with CCCP (50 μM) and oligomycin (10 μM) for 30 min. These reagents were used as positive and negative controls, respectively, for the ψmt measurements.

To index mitochondrial size, we isolated mitochondria from the heart, as described above, before subjecting them to MitoTracker Deep Red staining. Mitochondrial size was examined using a forward scatter detector (FSC; 488 nm argon laser and diode detector). MitoTracker Deep Red was used to selectively stain intact mitochondria and exclude debris. The FSC data were displayed in histograms in which they were plotted against the number of gated events. Geometric means (arbitrary units) obtained via FSC (on a logarithmic scale) were used as indicators of mitochondrial size.

**Transmission electron microscopy (TEM)**
Murine heart specimens of the indicated genotype were fixed in 2% formaldehyde and 2.5% (V/V) glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) for 2 h at room temperature and then incubated overnight at 4°C. The sections were then washed three times in 0.1 M PBS for 15 min each before being post-fixed in 1% osmium tetroxide for 1 h. The sections were stained en bloc with uranyl acetate, dehydrated with a graded series of ethanol solutions, cleared in propylene oxide and then embedded in epoxy resin. Thereafter, the blocks were trimmed and cut into ultra-thin sections (120 nm), which were subsequently observed under a
transmission electron microscope (H7500 TEM, Hitachi, Tokyo, Japan, http://www.hitachi.com). Images were acquired, and measurements of mitochondrial area, circularity, and cristae width were performed with the Multi Measure ROI tool developed by the manufacturers of ImageJ software.

**Structure optimization**
The structures of p300, Stat3 and RelA were optimized via the addition of counter ions and an 8-Å TIP3P water box using the Sander module in the AMBER14 program. The optimization procedure comprised the following two steps: 1) the solvent and hydrogen atoms of the protein were optimized, and 2) all the atoms of the system were optimized. Five-hundred steps of the steepest descent method and 500 steps of the conjugate gradient algorithm were applied for each procedure. The Amber ff14SB Force Field was used for protein optimization. The force field parameters of the acetylated residues were obtained using the ANTECHAMBER module, according to the Generalized Amber Force Field (GAFF), during structure optimization and MDS. The charges of the phosphorylated and acetylated residues were fitted by the AM1-bcc method. The mutated Stat3 proteins, namely, S1-non-acetylation, S2-K383-acetylation, S3-K370-acetylation and S4-K370/383-acetylation, were also optimized as described above.
REFERENCES


FIGURES

(A) & (G) NMCMs were transduced with NC-shRNA, TNFR1-shRNA or TNFR2-shRNA for 48 h. Total extracts were obtained, and TNFR1 and TNFR2 protein expression levels were determined by western blotting; (C-E) & (H-I) NMCMs were transfected with OPA1-siRNA, Stat3-siRNA, RelA-siRNA, p300-siRNA or PCAF-siRNA for 36 h. The cell lysates were analyzed to determine the expression levels of the proteins targeted by the siRNAs; (B) & (F) The proteins were extracted from H9C2 cells transfected with NC-shRNA, TNFR1-shRNA or TNFR2-shRNA for 48 h, and TNFR1 and TNFR2 expression levels were analyzed by western blotting.

Figure I. Verification that siRNA transfection induced the downregulation of targeted proteins
Figure II. Subcellular localization of Drp1 in NMCMs-TNFR1-KD upon TNFα treatment
(A) Mitochondrial and cytosolic protein extracts were prepared from NMCMs-TNFR1-KD treated with TNFα (0.5 ng/ml) for 12 h. The protein expression levels of Drp1, Tubulin and Tom20 in each extract were determined by western blotting. Tom20 and Tubulin were mitochondrial and cytosolic markers, respectively.
Figure III. The interaction between Stat3 and RelA in PCAF-knockdown NMCMs-TNFR1-KD upon TNFα treatment
(A) NMCMs-TNFR1-KD were transfected with PCAF-siRNA and then treated with TNFα (0.5 ng/ml) for 3 h. The interaction between endogenous Stat3 and RelA was analyzed by western blotting.
Figure IV. Twelve candidate lysine residues were ranked and selected by ZDOCK analysis and ZRANK scoring to identify the sites at which Stat3 was acetylated. (A) Protein-protein docking studies were performed to simulate the acetylation of Stat3 by p300 to identify the potential sites at which Stat3 was acetylated. Forty-eight lysine sites in Stat3 were set as receptor binding residues. Any one of the three residues in p300 (isoleucine 1456-ILE1456, arginine 1461-ARG1461 and tryptophan 1465-TRP1465) carrying an acetyl group to acetylate p300 substrate(s) was set as a donor binding residue. ZDOCK analysis was performed, and ZRANK scoring was used to rank the 48 docking sites, after which the lysine residues on Stat3 that were most likely acetylated by p300 were determined based on the ZRANK scoring results. The top twelve sites are shown.

<table>
<thead>
<tr>
<th>Order</th>
<th>Residue</th>
<th>ZDock score</th>
<th>ZRank score</th>
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<td>2</td>
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<td>4</td>
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<td>6</td>
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<td>7</td>
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<td>8</td>
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<td>9</td>
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<td>12</td>
<td>370</td>
<td>15.34</td>
<td>-17.248</td>
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TNFR2 activation promotes mitochondrial fusion
Figure V. The Stat3-p300 docking structure interfaces

Each cartoon in (a–i) depicts a binding interface corresponding to one of the top twelve Stat3-p300 docked structures, as determined by ZRANK scoring (including the three structures shown in Fig. 5C), and the LYS49, LYS87, LYS140, LYS244, LYS370, LYS383, LYS409, LYS517, LYS531, LYS615, LYS631 and LYS685 residues in Stat3 were selected as receptor residues. ILE1456, ARG1461 and TRP1465 are the p300 residues that carry acetyl groups. Each of them was set as a donor-binding residue. The minimum distance for each docking between a receptor residue in Stat3 and the corresponding donor residue in p300 is shown in the cartoon figures.
Figure VI. The RMSD structures of the four docked complexes during the simulation
(A) Backbone RMSD changes during a 40-ns MDS (overall, 5000 snapshots were taken in 50-ns trajectories; the first 10 ns represented the equilibrium run, and the last snapshot was set as the RMSD reference). All RMSDs were converged during the simulation, and the last 1-ns trajectory was used to calculate the free binding energy. (a)-(d) indicate the S1-RelA-, S2-RelA-, S3-RelA-, and S4-RelA docking models, respectively.
Figure VII. Identification of Stat3-knockdown and put-back NMCMs

(A) Endogenous Stat3 was knocked down by Stat3-shRNA. Then, NMCMs-TNFR1-KD were transfected with exogenous Flag-tagged WT Stat3 and Flag-tagged Stat3 mutants, as indicated. Knockdown and put-back efficiency was determined by western blotting using antibodies against Flag-tag and Stat3, respectively.
Figure VIII. Kaplan-Meier survival curves for the TNFR1/2<sup>−/−</sup> and TNFR1<sup>−/−</sup> groups
(A) Kaplan-Meier survival curves were generated for TNFR1/2<sup>−/−</sup> and TNFR1<sup>−/−</sup> mice subjected to either TAC surgery or sham surgery (n=12 for sham surgery and n=18 for TAC surgery).
Figure IX. Determination of OPA1 protein levels in NMCMs following TNFR1 stimulation

(A) NMCMs knocked down with TNFR2 (NMCMs-TNFR2-KD) were treated with or without TNFα (0.5 ng/ml) for 24 h, after which OPA1 protein expression levels were determined by western blotting; (B) After the cells were treated with TNFα (0.5 ng/ml) for the indicated times, OPA1 protein levels were detected by western blotting. Representative western blots are shown.
Figure X. The effects of Stat3 phosphorylation and RelA signaling on Stat3/RelA interactions in NMCMs-TNFR1-KD upon TNFα treatment

(A) NMCMs-TNFR1-KD were treated with TNFα (0.5 ng/ml) for 3 h, and Stat3 phosphorylation (at different sites) levels and NF-kB signaling pathway (IkBα and RelA) activity levels were determined by western blot analysis; (B) NMCMs-TNFR1-KD were pretreated with Ruxo (250 nM/L) and then treated with TNFα (0.5 ng/ml) for 3 h. The cell lysates were subjected to IP to pull down Stat3, after which the resulting complexes were analyzed by western blotting to assess the interactions between Stat3 and RelA. Cellular extracts were also assessed by western blotting to assess Stat3 and RelA input.
Figure XI. The spatial relationship between Stat3 phosphorylation residues and Stat3/RelA docking interface

(A) The docking interface between Stat3 and RelA was assessed by in silico simulation. The white circle highlights the docking interface between the Stat3 DBD and RelA. The phosphorylation sites in Stat3, namely, Tyrosine 705 and Serine 727, are also marked.
# TABLES

**Table I.** Forward and reverse sequences of the mRNA and DNA primers

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<tr>
<th>mRNA primer</th>
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<tr>
<td>OPA1</td>
<td>TGACAAA ACTTAAGGAGGCTGTG</td>
<td>CATTGTGCTGAATAACCCCTCAA</td>
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<td>AGATCAAGATCATTGCTCCTCCT</td>
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<table>
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<tr>
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<tr>
<td>β-Actin</td>
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<td>Stat3 and RelA binding region</td>
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### Table II. Sense and antisense sequences of the siRNA specific for Stat3 and OPA1

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<th>Antisense</th>
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<td>Stat3</td>
<td>CCACGUUGGUGUUUCAUAA</td>
<td>UUAUGAAACACCAACGUGG</td>
</tr>
<tr>
<td>OPA1</td>
<td>GGAAGAUCUUGCAGCAUAAG</td>
<td>UAAUGCUGCAAGAUCUCCUC</td>
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### Table III. The sequences of the genes targeted by the shRNAs

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<tr>
<th>shRNA</th>
<th>Target sequences of the shRNAs</th>
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<td>Stat3 shRNA</td>
<td>5’-CCACGTTGGTGTTTCATAA-3’</td>
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<tr>
<td>Tnfrsf1a shRNAm</td>
<td>5’-GCTAGGTCTTTGCCTTTATC-3’</td>
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<td>Tnfrsf1b shRNAm</td>
<td>5’-CCAAGTAGACTCCAGGCTT-3’</td>
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<tr>
<td>Tnfrsf1a shRNAr</td>
<td>5’-GGTTATCTTCCAGGTCTTTG-3’</td>
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<tr>
<td>Tnfrsf1b shRNAr</td>
<td>5’-GTCAGATGTGCTGTGCTA-3’</td>
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**Table IV.** Evaluation of the cardiac function of mice of two different genotypes that underwent either TAC surgery or sham surgery

<table>
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<tr>
<th>Group</th>
<th>Sham</th>
<th>TAC</th>
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<tbody>
<tr>
<td></td>
<td>TNFR1/2−/−</td>
<td>TNFR1−/−</td>
</tr>
<tr>
<td>Heart wt (mg) / Body wt (g)</td>
<td>4.62±0.16</td>
<td>4.66±0.15</td>
</tr>
<tr>
<td>Lung wt (mg) / Body wt (g)</td>
<td>5.37±0.28</td>
<td>5.24±0.21</td>
</tr>
<tr>
<td>Left ventricular fractional shortening (LVFS)</td>
<td>52.57±2.85</td>
<td>54.13±2.06</td>
</tr>
<tr>
<td>Left ventricular internal diameter, diastole (LVIDd)</td>
<td>2.66±0.12</td>
<td>2.63±0.05</td>
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#TNFR1/2−/− (TAC) vs TNFR1/2 (sham)
§ TNFR1−/− (TAC) vs TNFR1/2 (TAC)