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 NFkB 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[1, 2]. 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]. ß-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 report[4]. Briefly, NMCMs (5×10^7 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-kB (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 $MgCl_2.6H_2O$, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 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 Ⅱ-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 FACSCanto 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[5] using the Sander module in the AMBER14 program[6]. 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[7] was used for protein optimization. The force field parameters of the acetylated residues were obtained using the ANTECHAMBER[8] module, according to the Generalized Amber Force Field[9] (GAFF), during structure optimization and MDS. The charges of the phosphorylated and acetylated residues were fitted by the AM1-bcc method[10]. 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

1. Zhang N, Ye F, Zhu W, Hu D, Xiao C, Nan J, Su S, Wang Y, Liu M, Gao K, Hu X, Chen J, Yu H, Xie X, Wang J. Cardiac ankyrin repeat protein attenuates cardiomyocyte apoptosis by upregulation of bcl-2 expression. *Biochimica et biophysica acta*. 2016;1863:3040-3049

2. Jiang Z, Hu X, Yu H, Xu Y, Wang L, Chen H, Chen H, Wu R, Zhang Z, Xiang C, Webster KA, Wang JA. Human endometrial stem cells confer enhanced myocardial salvage and regeneration by paracrine mechanisms. *Journal of cellular and molecular medicine*. 2013;17:1247-1260

3. Wang K, Jiang Z, Webster KA, Chen J, Hu H, Zhou Y, Zhao J, Wang L, Wang Y, Zhong Z, Ni C, Li Q, Xiang C, Zhang L, Wu R, Zhu W, Yu H, Hu X, Wang J. Enhanced cardioprotection by human endometrium mesenchymal stem cells driven by exosomal microrna-21. *Stem cells translational medicine*. 2017;6:209-222

4. Hu X, Chen P, Wu Y, Wang K, Xu Y, Chen H, Zhang L, Wu R, Webster KA, Yu H, Zhu W, Wang J. Mir-211/stat5a signaling modulates migration of mesenchymal stem cells to improve its therapeutic efficacy. *Stem cells*. 2016;34:1846-1858

5. Jorgensen WL, Jenson C. Temperature dependence of tip3p, spc, and tip4p water from npt monte carlo simulations: Seeking temperatures of maximum density. *Journal of Computational Chemistry*. 1998;19:1179-1186

6. Case D, Babin V, Berryman J, Betz R, Cai Q, Cerutti D, Cheatham Iii T, Darden T, Duke R, Gohlke H. Amber 14. 2014

7. Case D, VB JTB BR, Cai Q, Cerutti D, Cheatham III T, Darden T, Duke R, Gohlke H, Goetz A, Gusarov S. The ff14sb force field. *AMBER*. 2014;14:29-31

8. Wang J, Wang W, Kollman PA, Case DA. Antechamber: An accessory software package for molecular mechanical calculations. *J. Am. Chem. Soc*. 2001;222:U403

9. Wang J, Wolf RM, Caldwell JW, Kollman PA, Case DA. Development and testing of a general amber force field. *Journal of computational chemistry*. 2004;25:1157-1174

10. Jakalian A, Bush BL, Jack DB, Bayly CI. Fast, efficient generation of high‐quality atomic charges. Am1‐bcc model: I. Method. Journal of Computational Chemistry. 2000;21:132-146

FIGURES

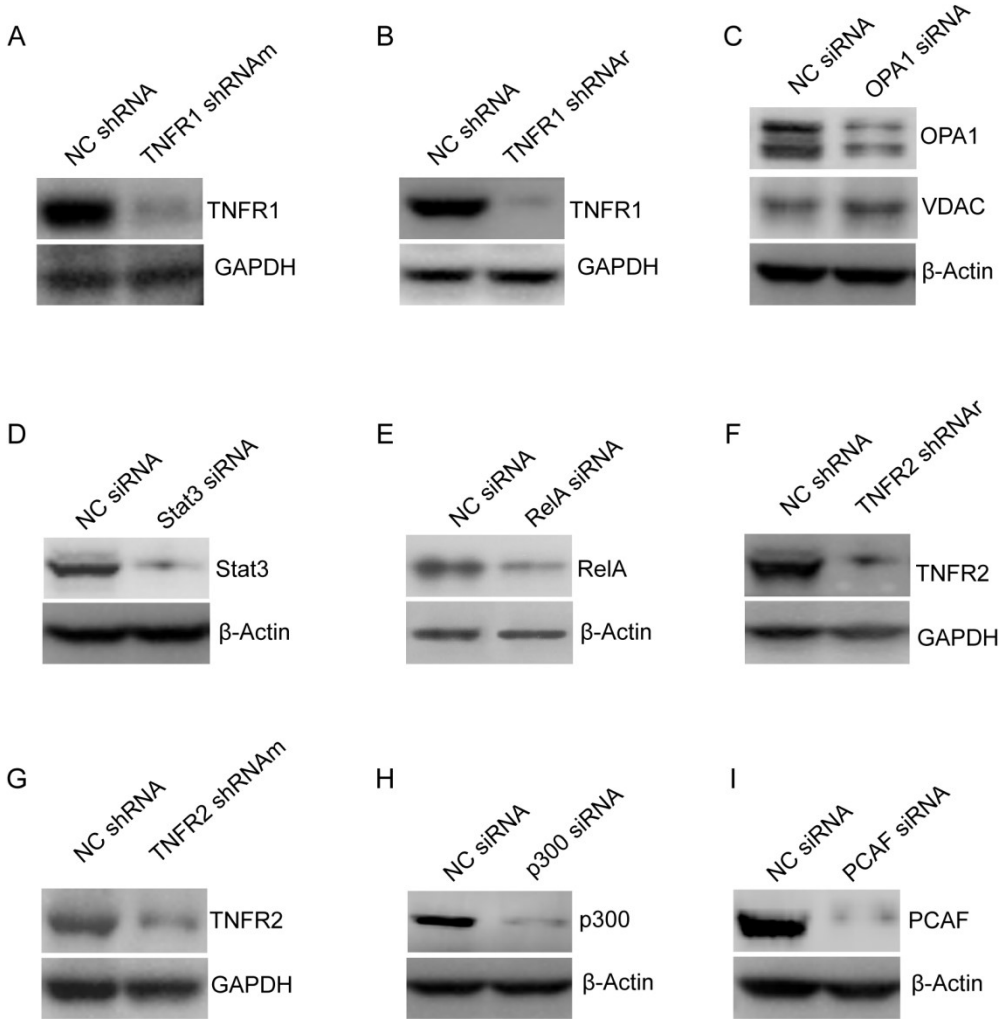


Figure Ⅰ. Verification that siRNA transfection induced the downregulation of targeted proteins

(A)&(G) NMCMs were transduced with NC-shRNA, TNFR1-shRNAm or TNFR2-shRNAm 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-shRNAr or TNFR2-shRNAr for 48 h, and TNFR1 and TNFR2 expression levels were analyzed by western blotting.

A

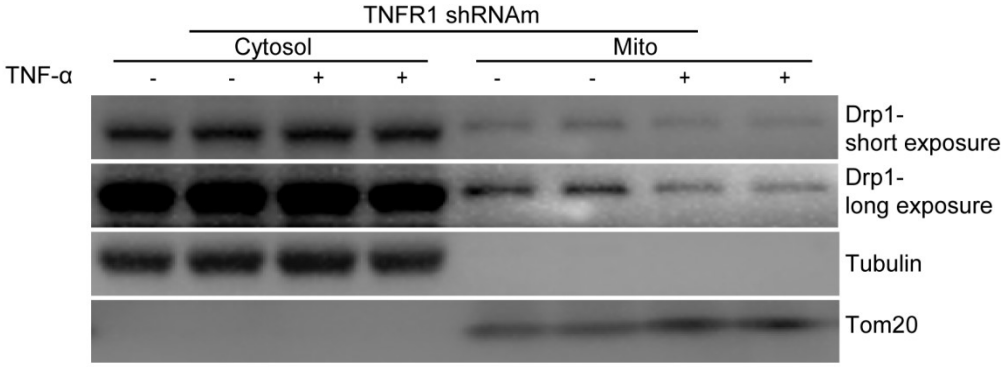


Figure Ⅱ. Subcellular localization of Drp1 in NMCMs-TNFR1-KD upon TNFα treatment
(A) Mitochondrial and cytosolic protein extracts were prepared from NMCMs-TNFR1-KD treated with TNFα (0.5 ng/ml) for 12 h. The protein expression levels of Drp1, Tubulin and Tom20 in each extract were determined by western blotting. Tom20 and Tubulin were mitochondrial and cytosolic markers, respectively.

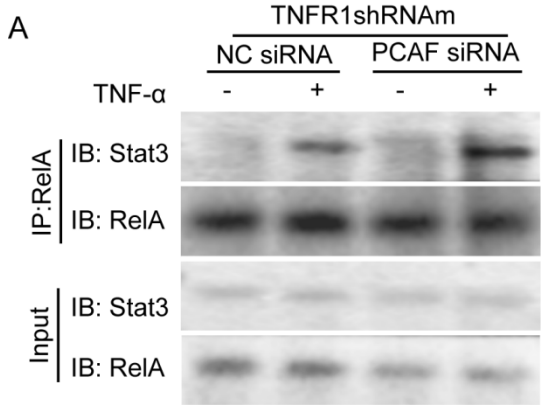


Figure Ⅲ. The interaction between Stat3 and RelA in PCAF-knockdown NMCMs-TNFR1-KD upon TNFα treatment

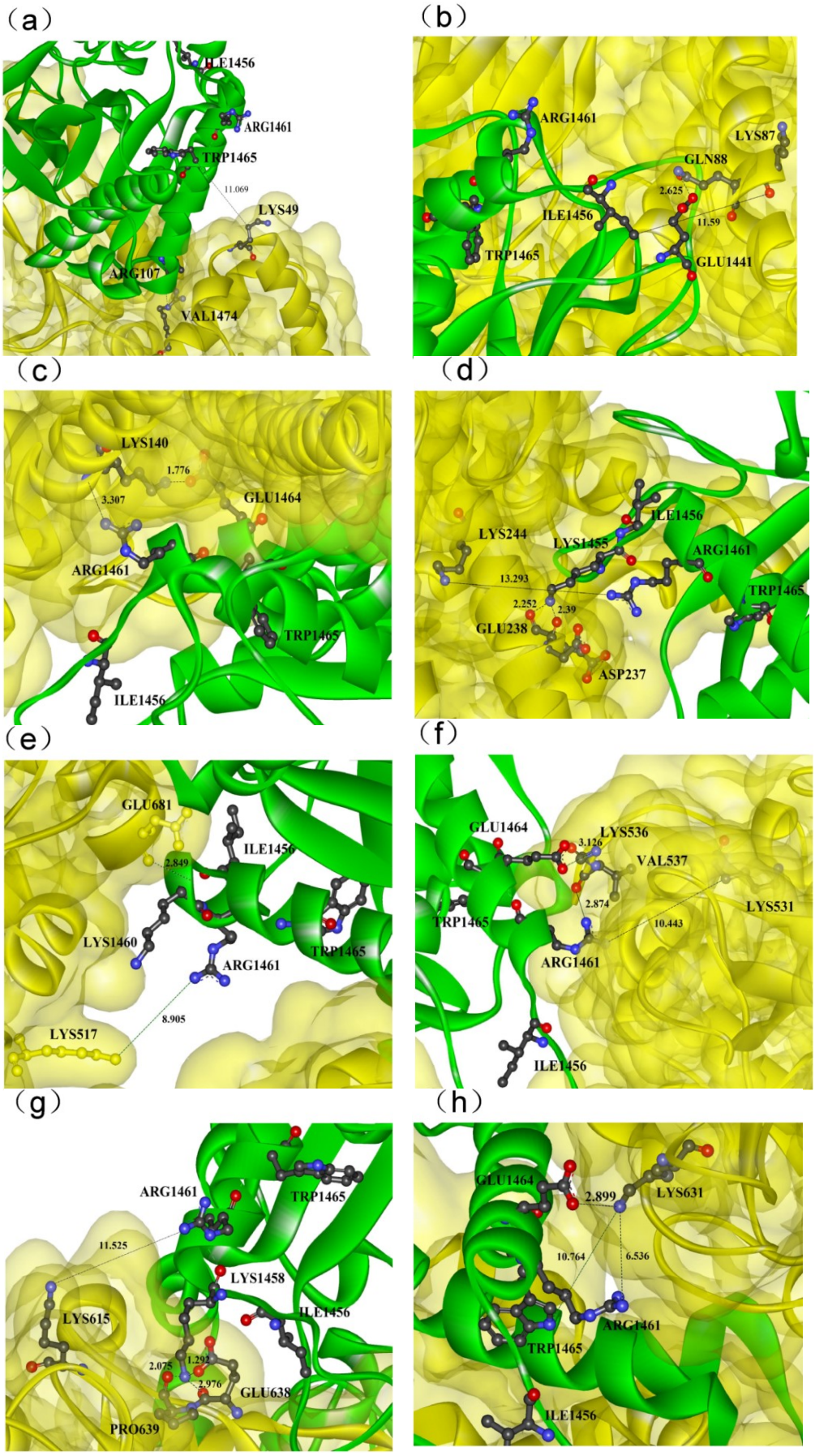
(A) NMCMs-TNFR1-KD were transfected with PCAF-siRNA and then treated with TNFα (0 .5 ng/ml) for 3 h. The interaction between endogenous Stat3 and RelA was analyzed by western blotting.

A

Order	Rsidue	ZDock score	ZRank score
1	685	14.98	-69.556
2	531	14.86	-59.459
3	87	17.08	-50.652
4	409	16.16	-49.637
5	615	14.48	-45.891
6	383	15.96	-44.366
7	517	14.84	-42.791
8	49	15.8	-40.226
9	140	14.64	-37.495
10	631	18.7	-35.735
11	244	13.56	-26.336
12	370	15.34	-17.248

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.


(a)
ILE1456
ARG1461
TRP1465
11.069
LYS49
ARG107
VAL1474
(b)
ARG1461
LYS87
GLN88
2.625
ILE1456
11.59
TRP1465
GLU1441
(c)
LYS140
1.776
3.307
GLU1464
ARG1461
TRP1465
ILE1456
(d)
ILE1456
LYS244
LYS1455
ARG1461
13.293
TRP1465
2.252
2.39
GLU238
ASP237
(e)
GLU681
ILE1456
2.849
LYS1460
TRP1465
ARG1461
8.905
LYS517
(f)
GLU1464
LYS536
3.126
VAL537
2.874
LYS531
TRP1465
10.443
ARG1461
ILE1456
(g)
ARG1461
TRP1465
11.525
LYS1458
ILE1456
LYS615
2.075
1.292
2.976
GLU638
PRO639
(h)
GLU1464
2.899
LYS631
10.764
6.536
ARG1461
TRP1465
ILE1456

(I)

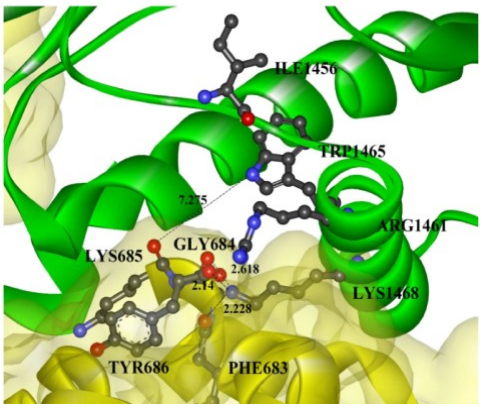


Figure Ⅴ. The Stat3-p300 docking structure interfaces

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.

A

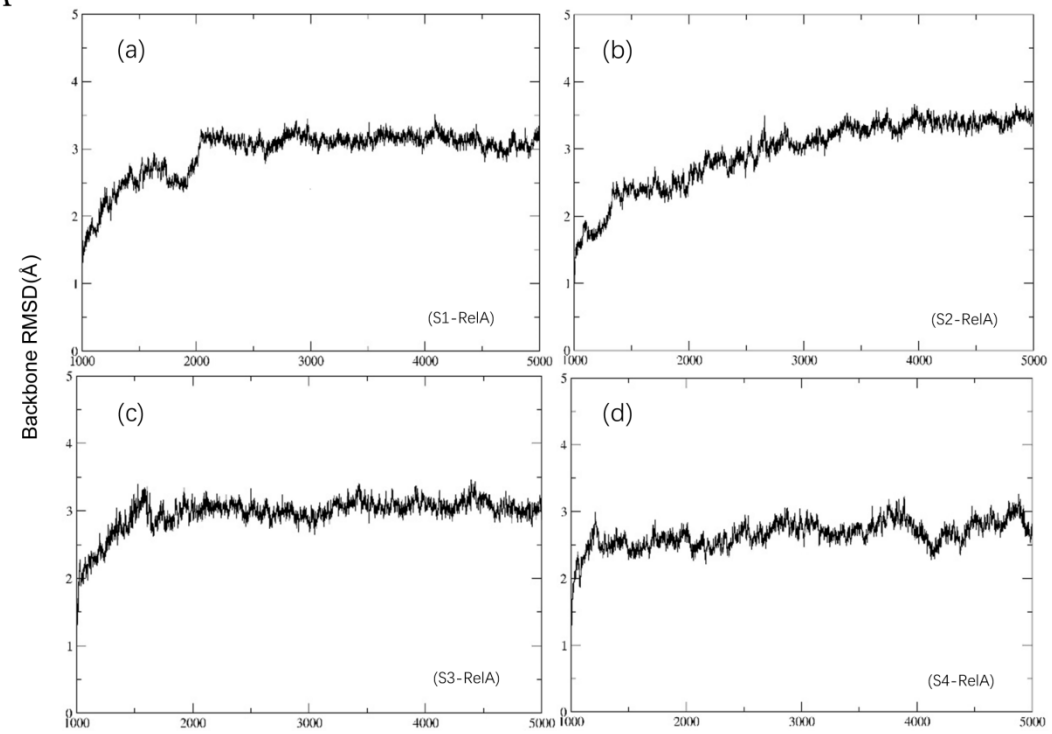


Figure Ⅵ. The RMSD structures of the four docked complexes during the simulation

(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.

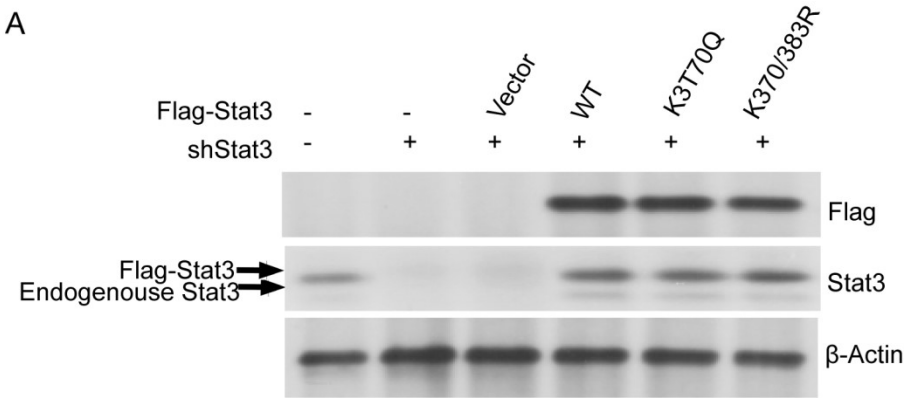


Figure Ⅶ. Identification of Stat3-knockdown and put-back NMCMs

(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.

A

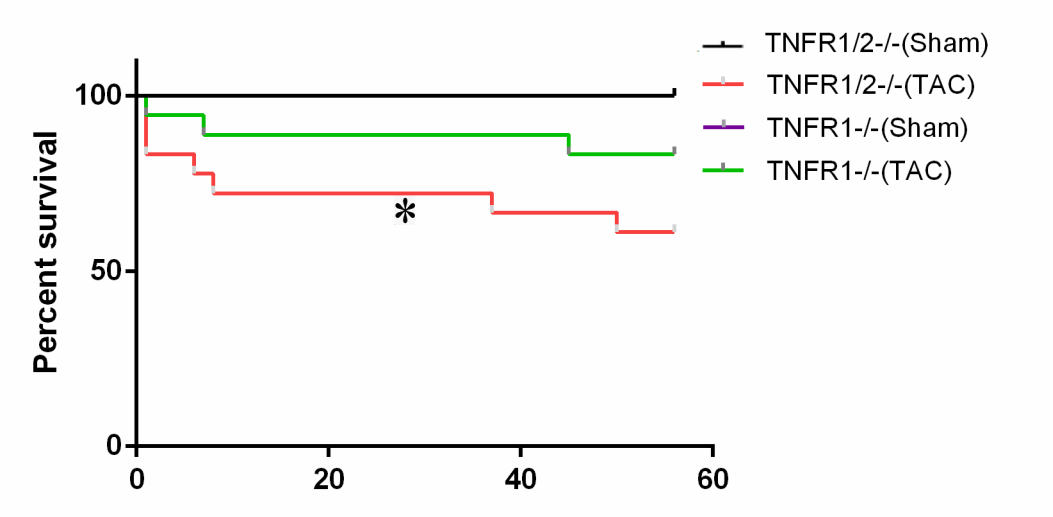


Figure Ⅷ. Kaplan-Meier survival curves for the TNFR1/2$^{-/-}$ and TNFR1$^{-/-}$ groups

(A) Kaplan-Meier survival curves were generated for TNFR1/2$^{-/-}$ and TNFR1$^{-/-}$ mice subjected to either TAC surgery or sham surgery (n=12 for sham surgery and n=18 for TAC surgery).

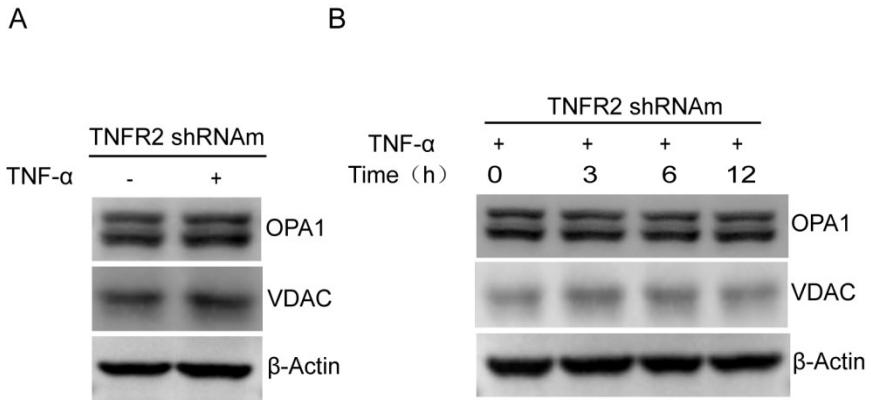


Figure Ⅸ . Determination of OPA1 protein levels in NMCMs following TNFR1 stimulation

(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.

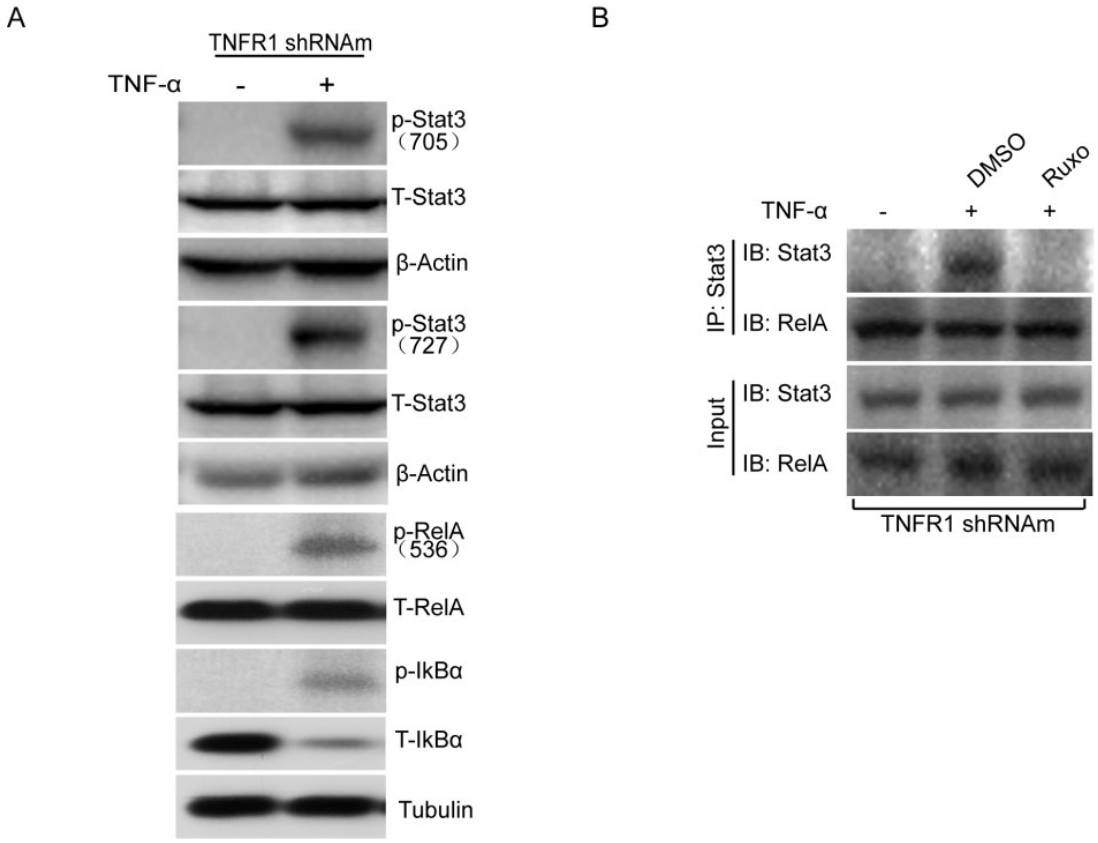


Figure X. The effects of Stat3 phosphorylation and RelA signaling on Stat3/RelA interactions in NMCMs-TNFR1-KD upon TNFα treatment

(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.

A

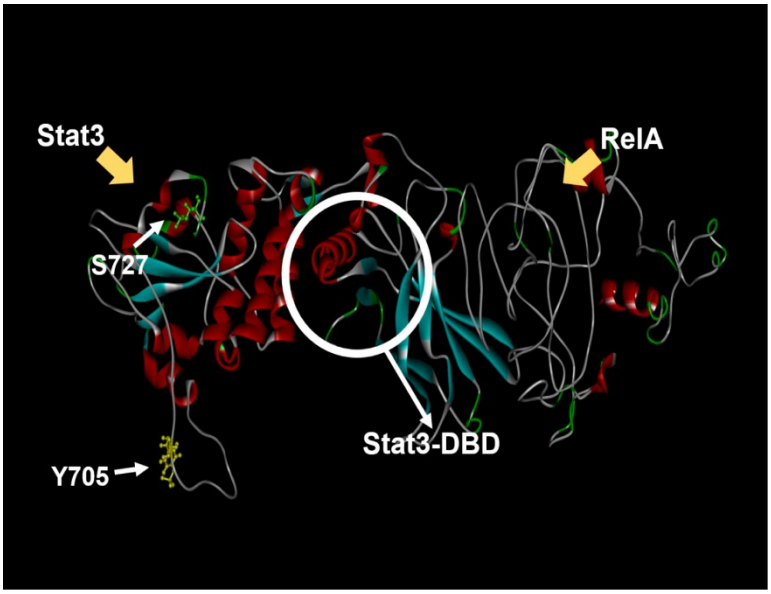


Figure XI. The spatial relationship between Stat3 phosphorylation residues and Stat3/ RelA docking interface

(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

mRNA primer		
OPA1	Forward	TGACAAACTTAAGGAGGCTGTG
	Reverse	CATTGTGCTGAATAACCCTCAA
β-Actin	Forward	AGATCAAGATCATTGCTCCTCCT
	Reverse	ACGCAGCTCAGTAACAGTCC
DNA primer		
cytochrome b (cytoB)	Forward	AACATACGAAAAACACACCCATT
	Reverse	AGTGTATGGCTAAGAAAAGACCTG
β-Actin	Forward	TGTTACCAACTGGGACGACA
	Reverse	CTATGGGAGAACGGCAGAAG
DNA primer for CHIP		
Stat3 and RelA binding region	Forward	GGTGTCTCGCATACTCGAT
	Reverse	ACAGGAACGCTAGGCAGGCT

Table II. Sense and antisense sequences of the siRNA specific for Stat3 and OPA1

Stat3	Sense	CCACGUUGGUGUUUCAUAA
	Antisense	UUAUGAAACACCAACGUGG
OPA1	Sense	GGAAGAUCUUGCAGCAUUAAG
	Antisense	UAAUGCUGCAAGAUCUUCCUC

Table III. The sequences of the genes targeted by the shRNAs

	Target sequences of the shRNAs
Stat3 shRNA	5′-CCACGTTGGTGTTTCATAA-3′
Tnfrsf1a shRNAm	5′-GCTAGGTCTTTGCCTTCTATC-3′
Tnfrsf1b shRNAm	5′-CCAAGTAGACTCCAGGCTT-3′
Tnfrsf1a shRNAr	5′-GGTTATCTTCCTAGGTCTTTG-3′
Tnfrsf1b shRNAr	5′-GCTCAGATGTGCTGTGCTA-3′

Table IV. Evaluation of the cardiac function of mice of two different genotypes that underwent either TAC surgery or sham surgery

Group	Sham		TAC	
	TNFR1/2$^{-/-}$	TNFR1$^{-/-}$	TNFR1/2$^{-/-}$	TNFR1$^{-/-}$
Heart wt (mg) / Body wt (g)	4.62±0.16	4.66±0.15	6.44±0.21$^{\#}$	5.56±0.20§
Lung wt (mg) / Body wt (g)	5.37±0.28	5.24±0.21	7.94±0.25$^{\#}$	6.27±0.23§
Left ventricular fractional shortening (LVFS)	52.57±2.85	54.13±2.06	18.95±3.18$^{\#}$	37.61±1.73§
Left ventricular internal diameter, diastole (LVIDd)	2.66±0.12	2.63±0.05	3.93±0.25$^{\#}$	2.94±0.07§

#TNFR1/2-/- (TAC) vs TNFR1/2 (sham)
§ TNFR1-/- (TAC) vs TNFR1/2 (TAC)