

YB1 protects cardiac myocytes against H₂O₂-induced injury via suppression of PIAS3 mRNA and phosphorylation of STAT3

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Abstract. Oxidative stress serves important roles in cardiac injury during the process of ischemia/reperfusion (I/R). Y-box protein 1 (YB1), a member of the highly conserved Y-box protein family, is closely associated with inflammation and stress responses by regulating gene transcription, RNA splicing and mRNA translation. However, the roles of YB1 in oxidative stress-induced myocardial-I/R (M-I/R) injury are unknown. The aim of the present study was to examine the effects of YB1 on H₂O₂-induced cardiomyocyte injury and its underlying mechanism. The results demonstrated that YB1 expression was upregulated during H₂O₂-induced myocardial injury. YB1 knockdown through transfection of small interfering RNA significantly aggravated cardiac cell apoptosis. Furthermore, YB1 knockdown significantly reversed the H₂O₂-mediated increase in phosphorylated signal transducer and activator of transcription (STAT)3, but did not affect the phosphorylation of P38, extracellular signal-regulated kinases 1/2, c-Jun N-terminal kinases, P65, Janus kinase 1 and 2 or STAT1. Moreover, protein co-immunoprecipitation and RNA-binding protein immunoprecipitation assays revealed that YB1 interacted with protein inhibitor of activated STAT

3 (PIAS3) mRNA but not its translated protein. YB1 overexpression may have promoted PIAS3 mRNA decay, decreasing PIAS3 protein levels, and therefore increased the levels of phosphorylated STAT3. Finally, YB1 knockdown, mediated by a lentivirus carrying YB1 targeted short hairpin RNA, significantly decreased left ventricle percentage fractional shortening and ejection fraction values, while increasing the infarct sizes in a rat model of M-I/R injury. These results demonstrated for the first time (to the best of our knowledge) that YB1 may protect cardiac myocytes against H₂O₂ or M-I/R-induced injury by binding to PIAS3 mRNA and resulting in the phosphorylation of STAT3.

Introduction

Ischemic heart disease remains one of the leading causes of morbidity and mortality across the world (1). Currently, the primary treatment for ischemic heart disease is reperfusion of the blocked artery. However, this abrupt reperfusion frequently results in deleterious secondary damage termed ischemia-reperfusion (I/R) injury (2). I/R triggers a cascade of complex intracellular events, including the generation of intracellular reactive oxygen species (ROS), loss of intracellular and mitochondrial calcium homeostasis and dysfunction of microcirculation, which subsequently activate multiple pathways leading to myocardial stunning, cardiomyocyte death, microvascular obstruction and arrhythmias, all eventually leading to increased infarct sizes (3,4). Therefore, understanding the underlying mechanisms of cardiac I/R injury is necessary for the development of novel therapeutic strategies for the prevention and treatment of this pathology.

Numerous studies have reported that ROS, including the superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂) and the hydroxyl radical (•OH), are critically involved in the pathophysiology of myocardial I/R (M-I/R) injuries. Induction of oxidative modification of intracellular molecules by ROS further activates stress signaling pathways, which leads to cardiomyocyte apoptosis (5,6). An overload of ROS may trigger the activation of mitogen-activated protein kinase (MAPK) signaling and aggravate lethal cellular processes, particularly during I/R injury. Inhibition of p38, c-Jun NH₂-terminal kinases (JNK) and/or extracellular signal-regulated kinases (ERK) 1 and 2 (all three of which are MAPKs, namely MAPK 8, 3 and 1, respectively), may protect the heart from

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Abbreviations: YB1, Y-box protein 1; I/R, ischemia/reperfusion; M-I/R, myocardial ischemia-reperfusion; ROS, reactive oxygen species; JAK, Janus kinase; STAT, signal transducer and activator of transcription; COIP, co-immunoprecipitation; RIP, RNA-binding protein immunoprecipitation; LDH, Lactate dehydrogenase; LVIDd, LV internal diastolic diameter; LVIDs, LV internal systolic diameter; LVFS, LV percentage fractional shortening; LVEF, LV ejection fraction; SHP1, Src homology region 2 domain-containing phosphatase 1; PIAS3, protein inhibitor of activated STAT 3; ActD, actinomycin D; MAPKs, mitogen-activated protein kinases; ERK1/2, extracellular signal-regulated kinases; JNK, c-Jun NH₂-terminal kinases; NF-κB, nuclear factor κB

Key words: oxidative stress, M-I/R, YB1, PIAS3, STAT3

I/R injury by reducing cardiomyocyte death and infarct size, in addition to attenuating left ventricular function impairment, as demonstrated in M-I/R models (7-10). Moreover, ROS may also activate signal transducer and activator of transcription (STAT) 3 during I/R, which has been demonstrated to protect the myocardium from ischemia and oxidative stress through the upregulation of cardioprotective genes and the modulation of mitochondrial respiration (11,12). In fact, a number of compounds, including sevoflurane, cilostazol and selenium, have been demonstrated to have cardioprotective effects and to alleviate M-I/R injury by activating STAT3 signaling (13-15).

Y-box protein 1 (YB1), a member of the highly conserved Y-box protein family, is a multifunctional DNA/RNA-binding protein, which serves important roles during the process of inflammation, wound healing and the stress response by regulating gene transcription, RNA splicing and mRNA translation (16-18). In previous studies, YB1 was also reported to be an important regulator of metabolic stress by sequestering mRNA molecules necessary for cell survival during temporary periods of hypoxia, adenosine triphosphate depletion or oxidative stress (19-21). However, whether YB1 serves a role in M-I/R injury remains unknown.

In the present study, the role of YB1 in cardiomyocyte apoptosis was evaluated using H₂O₂-treated H9c2 cells *in vitro* and M-I/R injury models *in vivo*. The results, to the best of our knowledge, demonstrated for the first time that YB1 expression was upregulated during H₂O₂ stimulation, and that YB1 may protect cardiac myocytes against H₂O₂ or M-I/R-induced injury by binding to protein inhibitor of activated STAT 3 (PIAS3) mRNA, resulting in the activation of STAT3.

Materials and methods

Cell culture and treatment. Rat embryonic cardiomyoblast-derived H9c2 cells (CLR-1446; American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin at 37°C with 5% CO₂. H9c2 cells were seeded in 6-well plates (1×10⁶ cells/well), treated at 24 h post-seeding with 50 mM H₂O₂ for 0, 3 or 6 h at 37°C, and harvested for the subsequent experiments.

In vitro cell viability assay. H9c2 cells cultured in 96-well plates (4×10⁴ cells/well) were treated with 50 mM H₂O₂ for 0, 3 or 6 h. Cell viability was measured using the Cell Counting Kit-8 (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm.

Lactate dehydrogenase (LDH) release assay. H9c2 cells cultured in 96-well plates (4×10⁴ cells/well) were treated with 50 mM H₂O₂ for 0, 3 or 6 h. The activity of LDH was measured in cell culture supernatants or serum using an LDH Release Assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions.

Analysis of cell apoptosis by flow cytometry. H9c2 cells were cultured and treated with 50 mM H₂O₂ for different times as

described. Following treatment, cells were digested with 0.25% trypsin at 37°C for 5 min and collected by centrifugation at 1,000 × g for 5 min at 4°C. Following two washes with ice-cold PBS, the cells were fixed in ice-cold 70% ethanol overnight at -20°C and stained with Annexin V-fluorescein isothiocyanate and propidium iodide (Hangzhou MultiSciences Biotech Co., Ltd., Hangzhou, China) together for 15 min at room temperature. The apoptotic cells were identified by EPICS XL flow cytometry (Beckman Coulter, Inc., Brea, CA, USA) and analyzed by Cell Quest software version FCS2.0 (BD Biosciences, San Jose, CA, USA). PI and Annexin V-FITC-positive cells were considered apoptotic cells.

Western blotting. Following treatment, H9c2 cell samples were collected and lysed with NP40 lysis buffer (Beyotime Institute of Biotechnology). Following centrifugation at 12,000 × g for 10 min at 4°C, the supernatant was collected and quantified using a bicinchoninic acid kit (Beyotime Institute of Biotechnology). The proteins (15 µg/lane) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Following blocking with 5% non-fat milk for 60 min at 37°C, the membranes were incubated with primary antibodies for 60 min at 37°C. The primary antibodies used included rabbit-anti-YB1 (cat. no. ab76149; Abcam, Cambridge, UK), rabbit-anti-phosphorylated P38 (p-P38; cat. no. ab4822; Abcam), rabbit-anti-P38 (cat. no. ab170099; Abcam), rabbit-anti-phosphorylated JNK (p-JNK; cat. no. ab4821; Abcam), rabbit-anti-JNK (cat. no. ab112501; Abcam), rabbit-anti-phosphorylated ERK1/2 (p-ERK1/2; cat. no. ab215362; Abcam), rabbit-anti-ERK1/2 (cat. no. ab17942; Abcam), rabbit-anti-nuclear factor κ B (NF-κB) p65 (cat. no. ab16502; Abcam), rabbit-anti-phosphorylated NF-κB p65 (p-p65; cat. no. ab86299; Abcam), rabbit-anti-Janus kinase (JAK)1 (cat. no. ab133666; Abcam), rabbit-anti-PIAS3 (cat. no. ab22856; Abcam), mouse-anti-Src homology region 2 domain-containing phosphatase (SHP)1 (cat. no. ab76202; Abcam), mouse-anti-SHP2 (cat. no. ab76285; Abcam), mouse-anti-suppressor of cytokine signaling (SOCS) 1 (cat. no. ab211288; Abcam), mouse-anti-SOCS3 (cat. no. ab14939; Abcam), rabbit-anti-insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1; cat. no. ab82968; Abcam), rabbit-anti-GAPDH (cat. no. ab181602; Abcam), rabbit-anti-phosphorylated JAK1 (p-JAK1; cat. no. 74129; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit-anti-JAK2 (cat. no. 3230; Cell Signaling Technology, Inc.), rabbit-anti-phosphorylated JAK2 (p-JAK2; cat. no. 3771; Cell Signaling Technology, Inc.), rabbit-anti-STAT1 (cat. no. 9172; Cell Signaling Technology, Inc.), rabbit-anti-phosphorylated STAT1 (p-STAT1; cat. no. 9167; Cell Signaling Technology, Inc.), rabbit-anti-STAT3 (cat. no. 12640; Cell Signaling Technology, Inc.), rabbit-anti-phosphorylated STAT3 (p-STAT3; cat. no. 9145; Cell Signaling Technology, Inc.) and rabbit-anti-SHP2 (cat. no. 3397; Cell Signaling Technology, Inc.). The primary antibodies were all used at a dilution of 1:1,000 in 5% bovine serum albumin (BSA; Beyotime Institute of Biotechnology). Horseradish peroxidase-conjugated secondary antibodies against rabbit (cat. no. ab6721; Abcam) or mouse (cat. no. ab6789; Abcam) were used at a dilution of 1:5,000 in 5% BSA. The membranes were incubated with secondary antibodies for 60 min at 37°C. The protein levels were first normalized to GAPDH

and subsequently normalized to the experimental controls. Blots were visualized with an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology) and quantified with ImageJ version 1.42 software (National Institutes of Health, Bethesda, MD, USA).

Small interfering RNA (siRNA), plasmids and transfection. siRNA against YB1 (siYB1) consisted of 5'-UCAUCGCAA CGAAGGUUUUTT-3' and 5'-AAAACCUUCGUUGCG AUGATT-3'. The scrambled siRNA (siNC) oligonucleotide duplex used as the control had the following sequences: 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGA CACGUUCGGAGAATT-3'. siRNAs were synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). Total RNA was extracted from H9c2 cells using RNA Isolater Total RNA Extraction Reagent (Vazyme, Piscataway, NJ, USA) and then reverse-transcribed into cDNA using a HiScript 1st Strand cDNA Synthesis Kit (Vazyme), according to the manufacturer's instructions. The YB1 gene was amplified from the above cDNA by polymerase chain reaction (PCR) using Phanta Super-Fidelity DNA Polymerase (Vazyme), using forward primer, 5'-CCCAAGCTTATGAGCAGCGAGGCCGAG-3' and reverse primer, 5'-CCGCTCGAGCTCAGCTGGTG GATC-3', and then cloned into the pCMV-flag-N-expression vector (Clontech Laboratories, Inc., Mountainview, CA, USA) and sequenced. The PCR thermocycling conditions were as follows: 95°C for 3 min; 30 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 90 sec; and 72°C for 10 min. The PIAS3 gene was amplified from cDNA from H9c2 cells by PCR, using forward primer, 5'-GGAATTCGGATGGCGGAGCTG GCGAA-3' and reverse primer, 5'-GGGGTACCTCAGTCC AAGGAAATGC-3', cloned into the pCMV-Myc-N-expression vector (Clontech Laboratories, Inc.) and sequenced. siRNAs (100 nM) or plasmids (2 µg) were transfected into H9c2 cells, using Exfect Transfection Reagent (Vazyme), according to the manufacturer's instructions. At 24 h post-transfection, cells were processed for further analysis.

Protein co-immunoprecipitation (COIP). H9c2 cells were treated with 50 mM H₂O₂. After 6 h, the cells were lysed with NP40 lysis buffer (Beyotime Institute of Biotechnology). Following centrifugation at 12,000 x g for 10 min at 4°C, the cell lysates were precleared with protein A/G agarose beads (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and an unrelated antibody from the same species of origin. Subsequently, the samples were incubated with the anti-YB1 antibody (1:30; cat. no. ab76149; Abcam) and protein A/G agarose beads overnight at 4°C with continuous rotation. The beads were washed five times in lysis buffer, and the immunoprecipitates were eluted from protein A/G agarose beads by heating at 100°C for 5 min. Following centrifugation at 12,000 x g for 10 min at 4°C, the samples were analyzed by western blotting.

RNA preparation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using RNA Isolater Total RNA Extraction Reagent (R401-01; Vazyme). RNA (500 ng) from each sample was reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). RT-qPCR was performed using a 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific,

Inc.) with AceQ qPCR SYBR® Green Master Mix (Q111-02; Vazyme), under the following conditions: An initial denaturation at 95°C for 10 min, and 40 cycles of 95°C for 10 sec, 60°C for 30 sec and 72°C for 10 sec. The uniqueness and sizes of PCR products were confirmed by generating melting curves. Each sample was evaluated in triplicate and used for the analysis of the relative transcription data using the 2^{-ΔΔC_q} method (22). RT-qPCR primers were as follows: YB1, forward 5'-CACCTT ACTACATGCGGAGACCT-3', reverse, 5'-TTGTCAGCA CCGTCCATCACT-3'; SHP1, forward 5'-CAGGTCTGTCGCA CTATTCTGT-3', reverse, 5'-AGGCTACTGTCTTGGCTA GGA-3'; SHP2, forward 5'-AGAGGGAAGAGCAAATGT GTCA-3', reverse, 5'-CTGTGTTTCCTTGTCCGACCT-3'; SOCS1, forward 5'-CTGCGGCTTCTATTGGGGAC-3', reverse, 5'-AAAAGGCAGTCGAAGGTCTCG-3'; SOCS3, forward 5'-TGCGCCTCAAGACCTTCAG-3', reverse, 5'-GCT CCAGTAGAATCCGCTCTC-3'; PIAS3, forward 5'-TTCGCT GGCAGGAACAAGAG-3', reverse, 5'-GGGCGCAGCTAG ACTTGAG-3'; and GAPDH, forward 5'-TCAACAGCAACTC CCACTCTTCCA-3' and reverse, 5'-ACCCTGTTGCTGTAG CCGTATTCA-3'. The obtained data were normalized to the GAPDH expression levels in each sample.

Actinomycin D (ActD) treatment. H9c2 cells cultured in 6-well plates (1x10⁶ cells/well) were transfected with p-CMV-flag-vector or p-CMV-flag-YB1. At 24 h post-transfection, cells were treated with ActD (10 µg/ml) for 0, 1, 2, 4, 6 or 8 h at 37°C. Cellular RNA was extracted and PIAS3 mRNA expression levels were analyzed via RT-qPCR analysis, as described above.

RNA-binding protein immunoprecipitation (RIP). RIP was performed using a Magna RIP kit (Merck KGaA, Darmstadt, Germany). Briefly, H₂O₂-treated H9c2 cells were harvested following two washes with PBS and lysed with RIP lysis buffer. A total of one-tenth of the supernatant was retained for the RT-qPCR analysis of the input, and the rest was incubated with antibodies against YB1 or immunoglobulin G for 24 h. The 50 µl A/G magnetic beads were added to the supernatant. Subsequent to immobilizing magnetic bead-bound complexes with a magnetic separator (Merck KGaA), the supernatant was used to extract RNAs with phenol:chloroform:isoamyl alcohol reagent at a ratio of 125:24:1 (all chemicals were purchased from Aladdin Industrial Corporation, Ontario, CA, USA). A cDNA synthesis kit (Takara Bio, Inc.) was used to synthesize the first-strand cDNA. Finally, qPCR was performed for analysis using AceQ qPCR SYBR Green Master Mix, as described above.

Animal model of M-I/R injury. Adult male Sprague-Dawley (SD) rats (15 rats, 8-9 weeks old, 250-300 g) were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee of the Medical School of Ningbo University (Ningbo, China). The animals were housed at 22-24°C under a 12-h light/dark cycle, with 40-60% humidity and free access to food and water.

For the M-I/R model (23,24), rats were anesthetized using thiopental (60 mg/kg, intraperitoneal). The trachea was cannulated and ventilated using a rodent ventilator (tidal volume, 2-3 ml; respiratory rate, 65-70 breaths/min; rodent ventilator

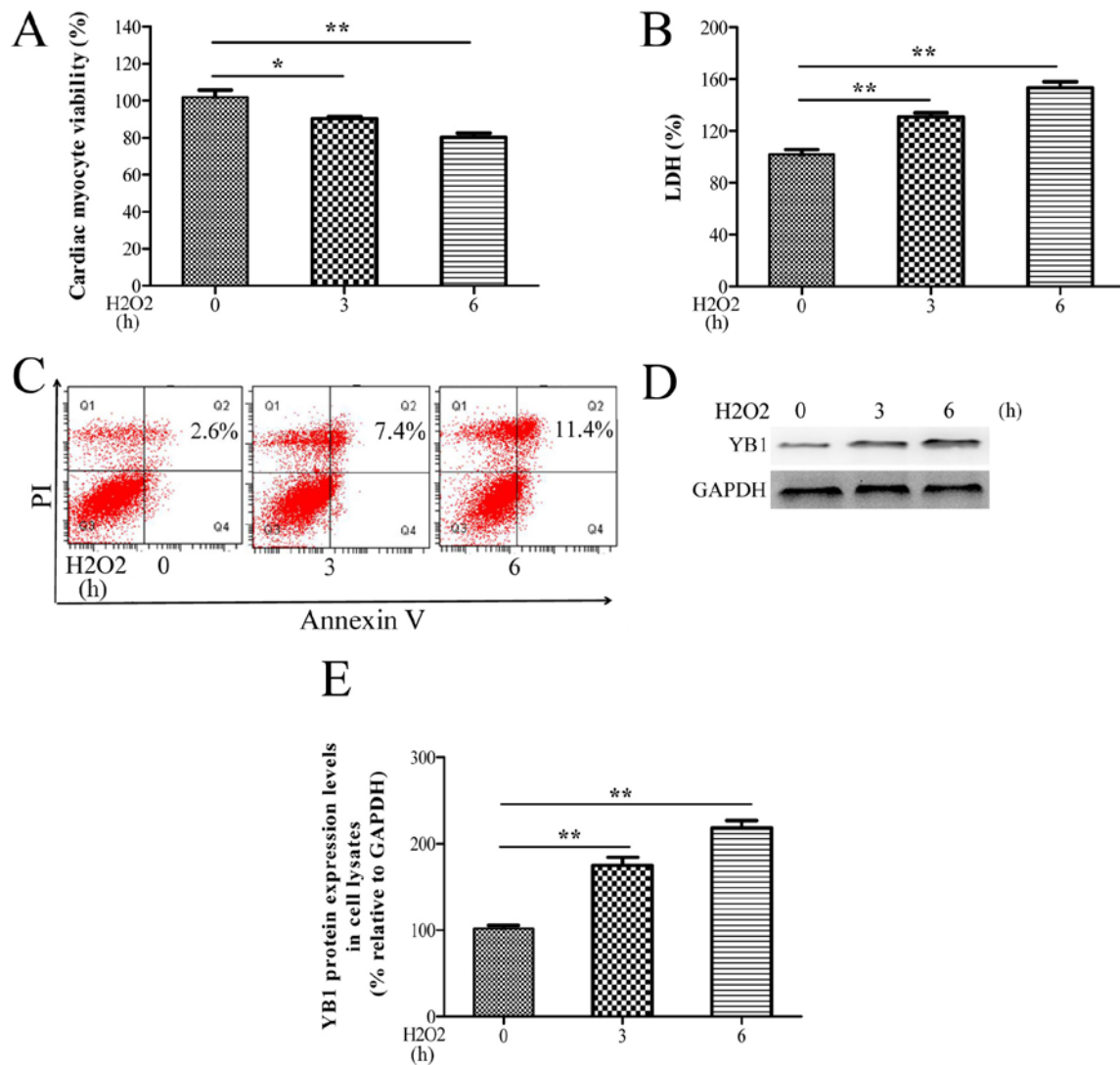


Figure 1. YB1 expression is upregulated following H₂O₂-induced myocardial injury. (A) H9c2 cells were treated with H₂O₂ for the indicated times, and cell viability was assessed using a Cell Counting Kit-8. (B) LDH levels were measured using a specific kit and (C) quadrant percentages of apoptotic cells were assessed using flow cytometry. (D) YB1 protein levels were detected by western blotting. (E) Relative expression levels of YB1 were calculated by normalizing to those of GAPDH. Data are presented as the mean \pm standard error of the mean (n=3). *P<0.05 and **P<0.01. YB1, Y-box protein 1; LDH, lactate dehydrogenase; PI, propidium iodide.

model 683; Harvard Apparatus, Holliston, MA, USA). The heart was exposed through left intercostal thoracotomy (between the fourth and fifth costal spaces) and the pericardium was cut. Subsequently, ischemia was induced for 30 min with a 6-0 silk suture, ~1-2 mm distal to the origin of the left anterior descending coronary artery (LAD), tightening over a pipette tip to ligate. Successful LAD ligation was characterized by elevation of the ST segment of the echocardiogram. Reperfusion was performed for 24 h by removing the tubes and loosening the suture.

A recombinant lentivirus carrying short hairpin RNA (shRNA) against YB1 (shYB1) or containing non-specific shRNA (shNC) as a control were provided by Shanghai GenePharma Co., Ltd. Healthy adult male SD rats were randomly divided into groups: i) The Sham group (n=5), sham operation without coronary artery ligation and received normal saline injection via the tail vein; ii) the I/R+shNC group (n=5), ischemia induction for 30 min followed by 6 h reperfusion, and injected with shNC via the tail vein; and iii) the I/R+shYB1

group (n=5), ischemia induction for 30 min followed by 6 h reperfusion, injected with shYB1 via the tail vein. After 6 h of reperfusion, echocardiography and cardiac hemodynamic measurements were performed to assess the heart function. Following the measurements, the animals were sacrificed and heart tissues and serum were collected.

Echocardiography analysis. Rats of the Sham group, I/R+shNC group, or I/R+shYB1 group were kept on a heating pad in a left lateral decubitus or supine position under isoflurane (2%) anesthesia, and two-dimensional images were recorded using a Vivid 7 echocardiography machine (GE Healthcare, Chicago, IL, USA) equipped with a 10 MHz phased array transducer. Left ventricle parameters including interventricular septum thickness, posterior wall thickness, left ventricle internal diastolic diameter (LVIDd) and left ventricle internal systolic diameter (LVIDs) were obtained from M-mode interrogation in a long-axis view. Left ventricle percentage fractional shortening (LVFS) and ejection fraction (LVEF) were calculated

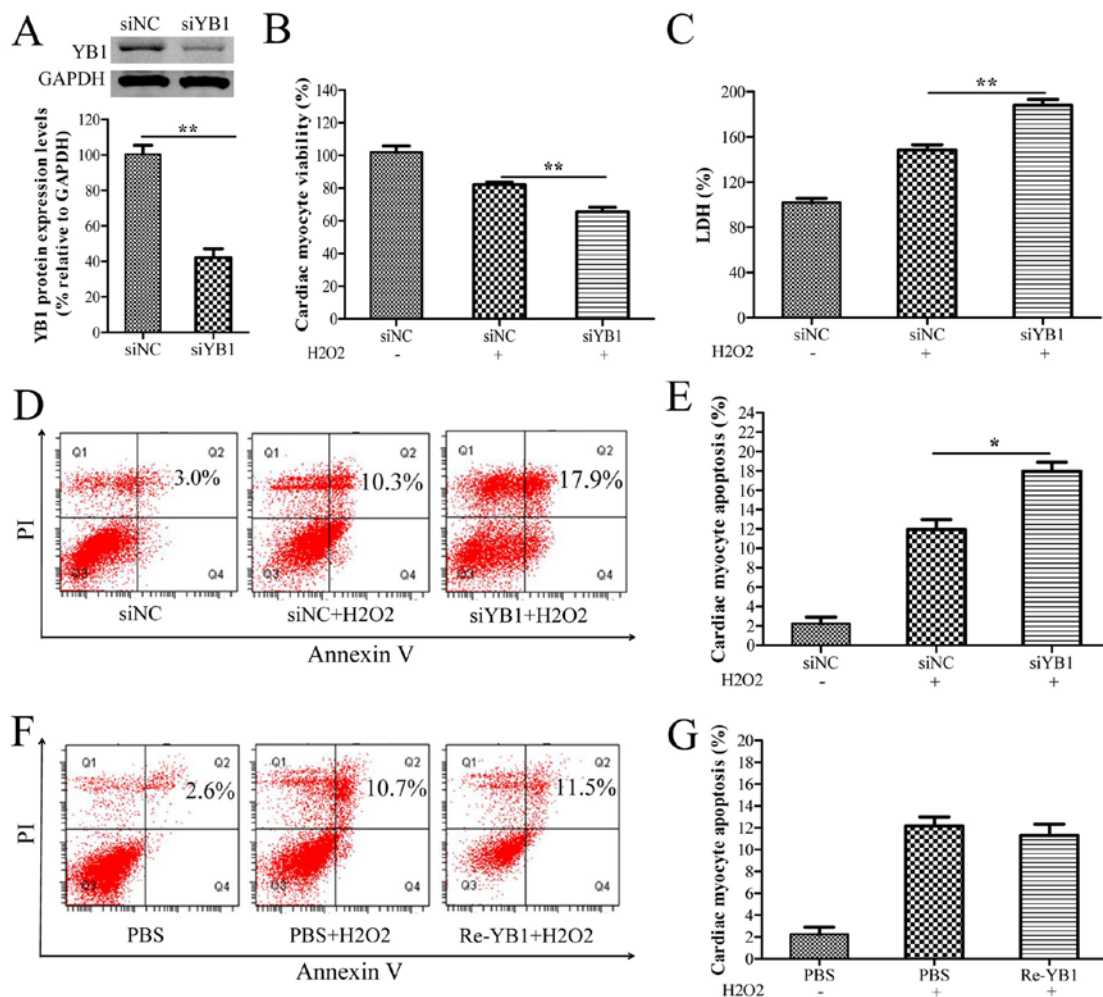


Figure 2. YB1 knockdown enhances H₂O₂-induced myocardial injury. (A) H9c2 cells were transfected with siYB1 or siNC (as a control) for 24 h, and YB1 protein was detected using western blotting with GAPDH as an endogenous control. (B) Viability of H9c2 cells (assessed using a Cell Counting Kit-8) transfected with siYB1 or siNC (as a control) for 24 h and treated with H₂O₂ for 6 h. (C) LDH levels were measured using a specific kit. (D) Quadrant percentages and (E) the proportion of apoptotic cells were assessed using flow cytometry. Prior to H₂O₂ treatment, H9c2 cells were pretreated with Re-YB1 or PBS (as a control) for 24 h. (F) Quadrant percentages and (G) the proportion of apoptotic cells were assessed using flow cytometry. Data are presented as the mean \pm standard error of the mean (n=3). *P<0.05 and **P<0.01. YB1, Y-box protein 1; siYB1, small interfering RNA against YB1; siNC, scrambled small interfering RNA; LDH, lactate dehydrogenase; PI, propidium iodide; Re-YB1, recombinant YB1.

as follows: LVFS%=(LVIDd - LVIDs)/ LVIDd x100; LVEF%=[(LVIDd)³-(LVIDs)³]/(LVIDd)³ x100. All echocardiographic measurements were averaged from at least three separate cardiac cycles.

Measurement of myocardial infarct size. Following I/R, the hearts were removed from the animals (n=5), and snap frozen at -20°C for 24 h. The hearts were sliced into 2 mm transverse sections, along the ape-base axis, using a stainless steel slicer matrix. The slices were incubated in 2% triphenyltetrazolium chloride (TTC in 0.1 M phosphate buffer, pH 7.4) for 20 min at 37°C. The reaction of TTC with viable parts of tissue produced a red region in the ventricle, which is distinct from the pale necrotic tissue observed following fixation in 10% formalin for 24 h at room temperature. The size of the total left ventricle area and the infarcted area were measured by planimetry of scanned slices using ImageJ version 1.42.

Statistical analysis. The data are presented as the mean \pm standard error of the mean of at least three independent

experiments. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The differences between two groups were analyzed using a Student's t-test, while the differences among three or more groups were analyzed using one-way analysis of variance, followed by Student-Newman-Keuls post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

YB1 expression is upregulated during H₂O₂-induced myocardial injury. To investigate whether YB1 is involved in cardiomyocyte injury, H9c2 cells grown *in vitro* were treated with H₂O₂. As observed in Fig. 1A-C, H₂O₂ treatment significantly decreased the cell viability, and increased the LDH release and apoptosis rates of H9c2 cells in a time dependent-manner. Moreover, YB1 protein levels progressively increased during treatment with H₂O₂ (Fig. 1D and E). These *in vitro* data suggested that H₂O₂-mediated upregulation

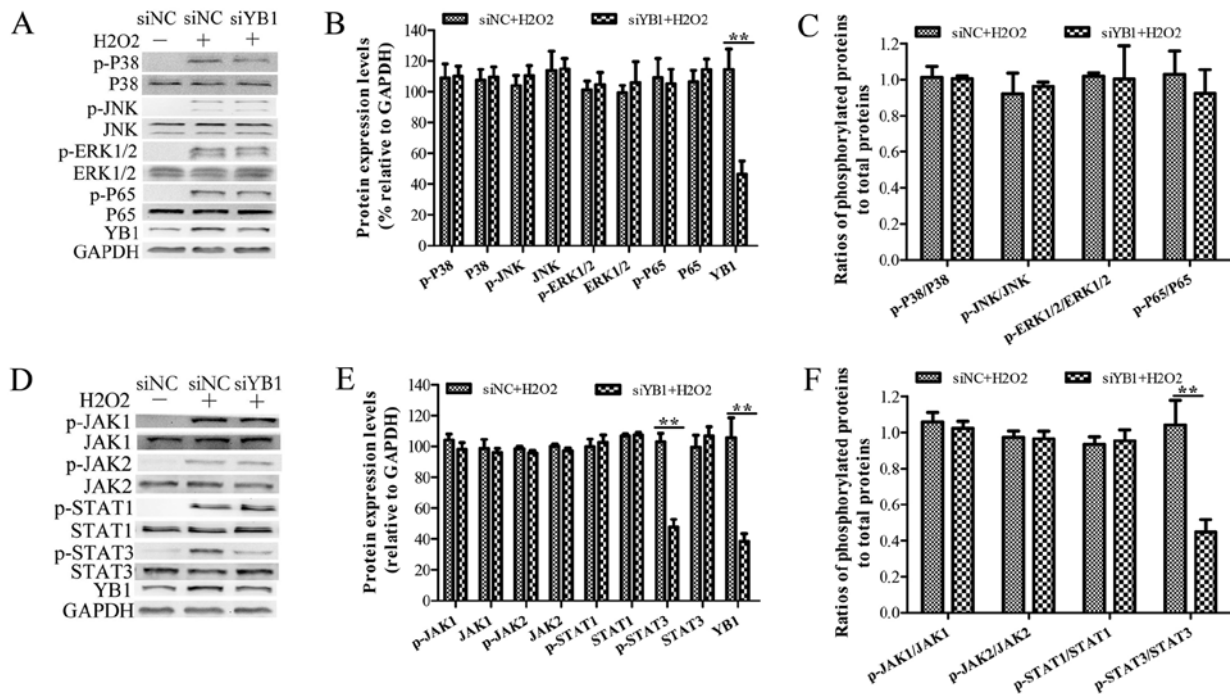


Figure 3. YB1 knockdown inhibits H_2O_2 -induced phosphorylation of STAT3. H9c2 cells were transfected with siYB1 or siNC (as a control) for 24 h, and treated with H_2O_2 for 6 h. (A) YB1, p-P38, p-JNK, p-ERK1/2, p-P65, P38, JNK, ERK1/2 and P65 protein expression levels were detected using western blotting. (B) Relative expression levels of these proteins were calculated by normalizing to those of GAPDH. (C) Ratios of phosphorylated protein to total proteins after normalization to GAPDH further demonstrates the observation these proteins are not recruited following treatment (D) YB1, p-JAK1, p-JAK2, p-STAT1, p-STAT3, JAK1, JAK2, STAT1 and STAT3 protein expression levels were detected using western blotting. (E) Relative expression levels of these proteins were calculated by normalizing to those of GAPDH. (F) Ratios of phosphorylated protein to total proteins after normalization to GAPDH demonstrates that STAT3 phosphorylation levels decrease in the absence of YB1 following treatment. Data are presented as the mean \pm standard error of the mean ($n=3$). ** $P<0.01$. YB1, Y-box protein 1; siYB1, small interfering RNA against YB1; siNC, scrambled small interfering RNA; p-, phosphorylated; JNK, c-Jun NH_2 -terminal kinase; ERK, extracellular signal-regulated kinase; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

of YB1 may be associated with H_2O_2 -induced cardiomyocyte injury.

YB1 knockdown enhances H_2O_2 -induced myocardial injury. To further investigate the roles of YB1 in H_2O_2 -induced cardiomyocyte injury, YB1 was knocked-down via siYB1 transfection in H9c2 cells. Western blot analysis demonstrated that YB1 expression was significantly decreased in siYB1-transfected cells compared with siNC-transfected cells (Fig. 2A). Furthermore, YB1 knockdown notably decreased cell viability, while further increasing the LDH release and apoptosis rates of H9c2 cells following treatment with H_2O_2 (Fig. 2B-E), suggesting that YB1 may have cardioprotective effects in H_2O_2 -induced cardiomyocyte injury. To further these observations, the effect of extracellular YB1 treatment on H_2O_2 -induced cardiomyocyte injury was evaluated. As presented in Fig. 2F and G, the flow cytometry results demonstrated that stimulation with recombinant YB1 protein did not affect the apoptosis rates of H_2O_2 -treated H9c2 cells.

YB1 knockdown inhibits H_2O_2 -induced STAT3 phosphorylation. Since H_2O_2 treatment is known to activate the p38, JNK, ERK and NF- κ B signaling pathways, which induce cell apoptosis (25,26), it was investigated whether YB1 knockdown enhanced H_2O_2 -induced myocardial injury. Western blot analysis demonstrated that the expression levels of phosphorylated P38 (p-P38), p-JNK, p-ERK1/2 and p-P65 were increased in H_2O_2 -treated H9c2 cells, compared with those in nontreated

H9c2 cells (Fig. 3A). Moreover, the expression levels of p-P38, p-JNK, p-ERK1/2 and p-P65 in siYB1-transfected H9c2 cells were similar to those in siNC-transfected cells, suggesting that YB1 knockdown did not affect the activation of p38, JNK, ERK and NF- κ B signaling pathways following treatment with H_2O_2 (Fig. 3B and C).

The JAK-STAT signaling pathway has also been reported to be involved in H_2O_2 -induced cardiomyocyte injury. Therefore, to investigate whether YB1 knockdown affected the JAK-STAT pathway and promoted H_2O_2 -induced myocardial injury, western blotting was performed. Just as observed in the aforementioned paragraph, treatment with H_2O_2 led to an increase in the phosphorylation levels of JAK1, JAK2, STAT1 and STAT3 (Fig. 3D). Furthermore, this assay demonstrated that YB1 knockdown did not significantly affect the levels of p-JAK1, p-JAK2 and p-STAT1 following H_2O_2 treatment (Fig. 3E and F). However, the protein levels of p-STAT3 in siYB1-transfected cells were decreased compared with those observed in siNC-transfected cells, suggesting that YB1 knockdown may inhibit H_2O_2 -induced STAT3 phosphorylation.

YB1 interacts with and promotes PIAS3 mRNA decay to facilitate STAT3 phosphorylation. Further to being activated by JAK, STAT3 is also negatively regulated by phosphatases, including SHP1, SHP2, SOCS1, SOCS3 and PIAS3. To further investigate how YB1 knockdown inhibited the H_2O_2 -induced phosphorylation of STAT3, the interactive association between YB1 and SHP1, SHP2, SOCS1, SOCS3 and PIAS3 was evaluated using

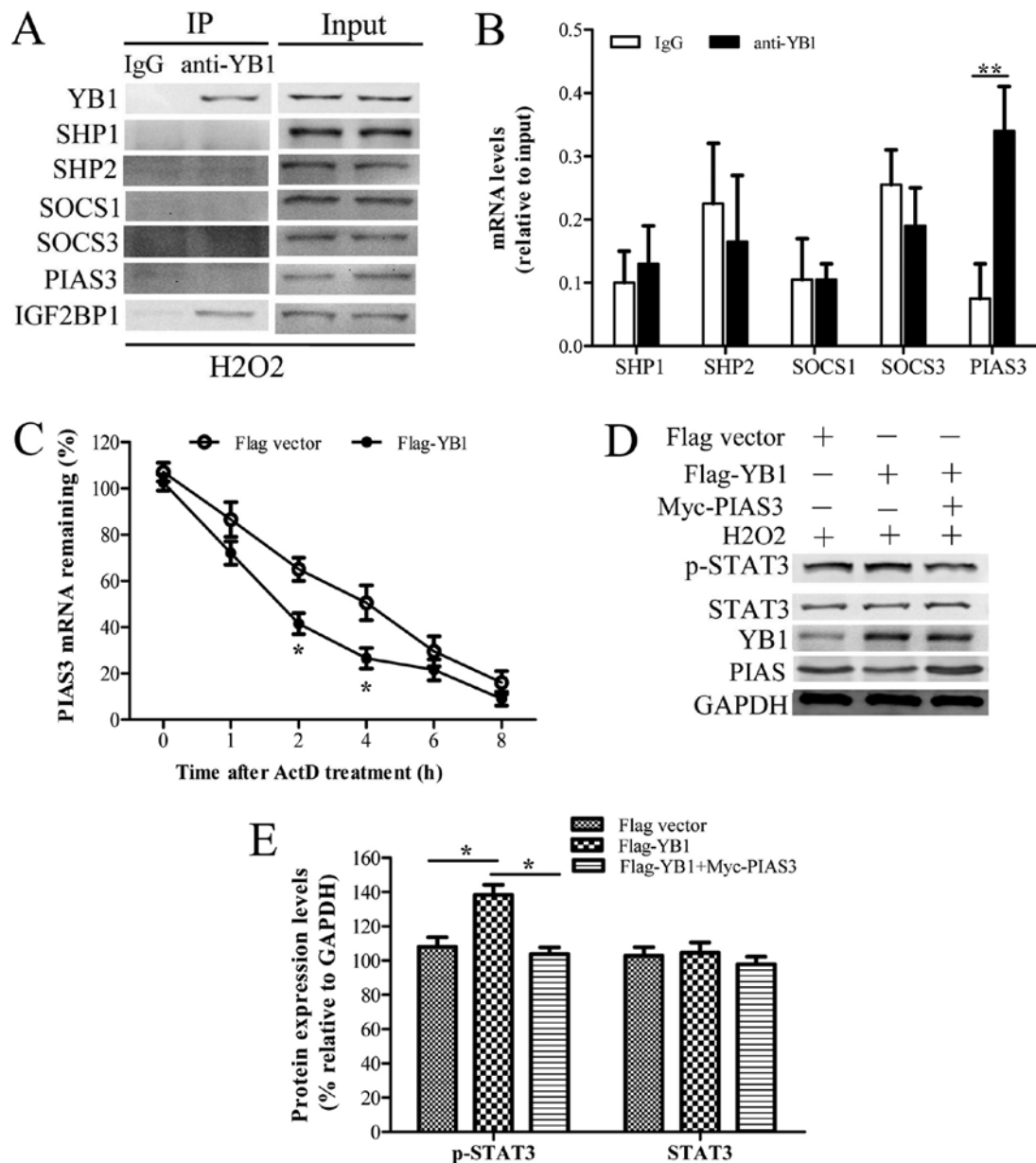


Figure 4. YB1 interacts with PIAS3 and promotes its mRNA decay, facilitating STAT3 phosphorylation. (A) H9c2 cells were treated with H₂O₂ for 6 h and immunoprecipitated with anti-YB1 antibody, with IgG as a control. Following cross-linking with protein A/G agarose beads, the immunoprecipitated complex was analyzed using western blotting. IGF2BP1 was used as a positive control. (B) mRNAs of indicated genes endogenously associated with YB1 in H9c2 cells were detected by RNA-binding Protein Immunoprecipitation using IgG as a control. (C) H9c2 cells were transfected with p-CMV-flag-vector (as the control) or p-CMV-flag-YB1 for 24 h, and treated with ActD (10 μ g/ml) for the indicated times. PIAS3 mRNA expression levels were analyzed with reverse transcription-quantitative polymerase chain reaction. (D) H9c2 cells were transfected with p-CMV-flag-vector or p-CMV-flag-YB1 and p-CMV-Myc-PIAS3 for 24 h, and treated with H₂O₂ for 6 h. YB1, p-STAT3, STAT3, PIAS3 and GAPDH protein expression levels were detected using western blotting. (E) Relative expression levels of these proteins were calculated by normalizing to those of GAPDH. Data are presented as the mean \pm standard error of the mean (n=3). *P<0.05 and **P<0.01. YB1, Y-box protein 1; STAT, signal transducer and activator of transcription; PIAS3, protein inhibitor of activated STAT 3; IgG, immunoglobulin G; IGF2BP1, insulin-like growth factor 2 mRNA-binding protein 1; ActD, actinomycin D; p-, phosphorylated; SHP, Src homology region 2 domain-containing phosphatase; SOCS, suppressor of cytokine signaling.

COIP and RIP assays (Fig. 4A and B). The results suggested that YB1 chiefly interacted with PIAS3 mRNA. Little interaction was observed between YB1 and SHP1 (protein and mRNA), SHP2 (protein and mRNA), SOCS1 (protein and mRNA), SOCS3 (protein and mRNA) and PIAS3 protein.

Subsequently, the effects of YB1 on PIAS3 mRNA stability were evaluated by RT-qPCR in YB1-overexpressing H9c2 cells treated with the transcriptional inhibitor ActD. The results (Fig. 4C) demonstrated that the amount of

PIAS3 mRNA in YB1-overexpressing H9c2 cells was lower compared with control cells during ActD exposure. Moreover, the half-life of PIAS3 mRNA in YB1-overexpressing H9c2 cells was also shorter compared with that of control cells. Therefore, YB1 overexpression may have decreased PIAS3 mRNA stability.

To further these observations, the effects of YB1 overexpression on the protein levels of PIAS3 and STAT3 in H9c2 cells treated with H₂O₂ were investigated. As exhibited in

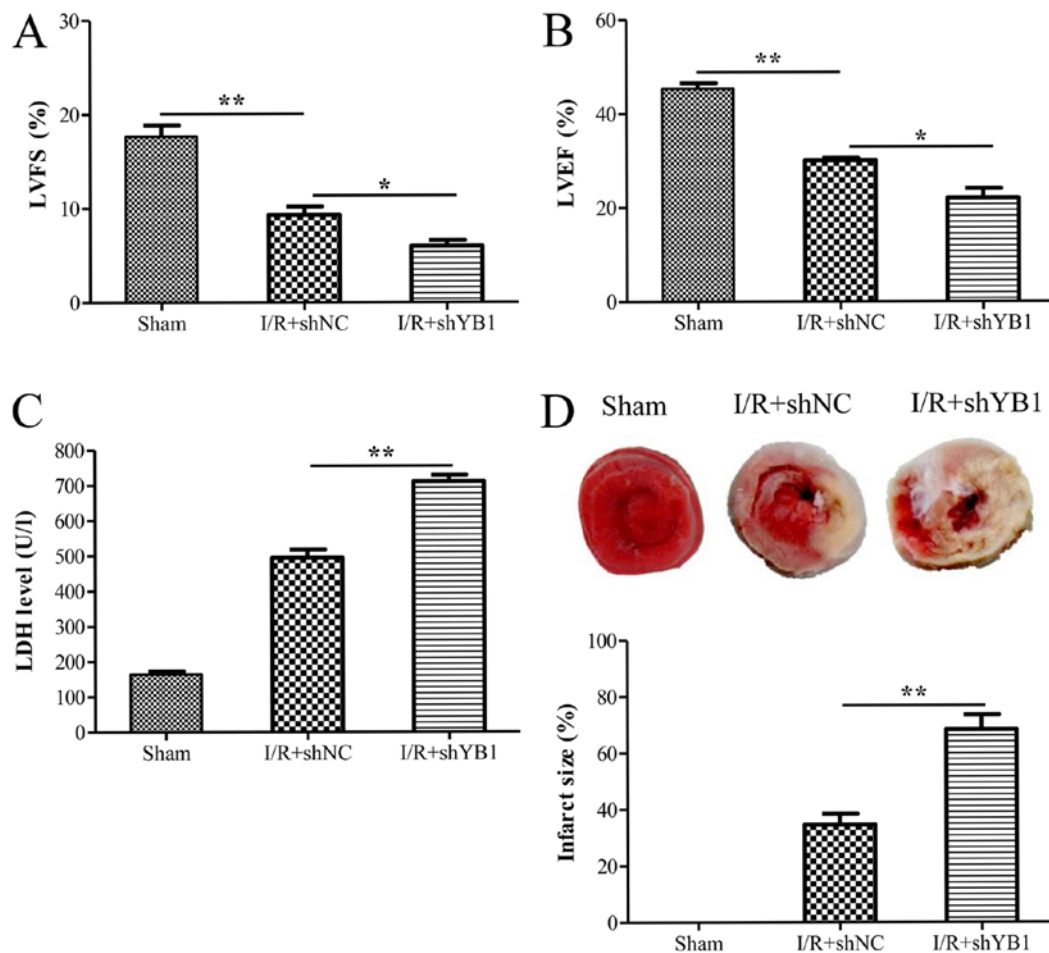


Figure 5. YB1 knockdown aggravates myocardial I/R injury *in vivo*. Rats were intravenously injected with lentivirus carrying shYB1 or shNC for 3 days and the myocardial I/R injury was performed. (A) LVFS% and (B) LVEF% were detected by echocardiography. (C) LDH release in blood plasma in rats *in vivo* was detected. (D) Infarct sizes were measured by triphenyltetrazolium chloride staining. Data are presented as the mean \pm standard error of the mean (n=5); *P<0.05 and **P<0.01. YB1, Y-box protein 1; I/R, ischemia/reperfusion; shYB1, short hairpin RNA against YB1; shNC, non-specific short hairpin RNA; LVFS, left ventricle percentage fractional shortening; LVEF, left ventricle ejection fraction; LDH, lactate dehydrogenase.

Fig. 4D and E, YB1 overexpression in H9c2 cells treated with H₂O₂ decreased PIAS3 and increased p-STAT3 protein levels without altering STAT3 total protein levels. However, PIAS3 overexpression reversed the YB1 overexpression phenotype by decreasing the phosphorylation levels of STAT3. Taken together, these results indicated that YB1 may interact with and promote PIAS3 mRNA decay, facilitating STAT3 phosphorylation in H9c2 cells.

YB1 knockdown aggravates M-I/R injury in vivo. An *in vivo* rat M-I/R model was employed to investigate the effects of YB1 on M-I/R injury. Transthoracic echocardiography and M-mode tracings were used to evaluate LVFS% and LVEF% via echocardiographic measurements. As expected, I/R injury significantly decreased LVFS% and LVEF% (Fig. 5A and B, respectively), compared with those of the sham group. Moreover, animals injected with shYB1 displayed a significantly decreased LVFS% and LVEF% compared with I/R-injured rats injected with shNC. Consistently, the LDH levels in the I/R+shYB1 group were also significantly higher compared with those of the I/R+shNC group (Fig. 5C). Furthermore, the infarct sizes in I/R+shYB1 group were significantly increased compared with those in the I/R+shNC

group (Fig. 5D). These results indicated that YB1 knockdown aggravated myocardial I/R injury *in vivo*.

Discussion

Cardiomyocyte apoptosis during the I/R process is responsible for multiple sequelae of myocardial infarction, including congestive heart failure, cardiac rupture and ventricular arrhythmia (27,28). In the present study, the cardioprotective effect of YB1 in the prevention and control of I/R-derived cardiac damage was evaluated *in vivo* and *in vitro*. With this approach, it was demonstrated that YB1 inhibited cardiomyocyte apoptosis by promoting STAT3 phosphorylation.

Previous studies reported that YB1 expression is increased in the regenerating heart following I/R injury, and the increased expression levels of YB1 have been attributed to myofibroblast infiltration, which is thought to take place 4-7 days following I/R (29,30). The present study demonstrated low expression of YB1 in cardiomyocytes, in contrast with the previously reported data from myofibroblasts (27). However, in a rat M-I/R model, pretreatment with lentivirus-shYB1, which caused YB1 knockdown in heart cells, including cardiomyocytes and myofibroblasts, aggravated

myocardial infarction sizes, which seems inconsistent with the work of Kamalov *et al* (29). Due to the positive role of YB1 on the proliferation and migration of myofibroblasts in the infarct regions (29), YB1 knockdown in myofibroblasts may hinder the local scar formation, which contributes to heart deterioration and heart failure. On the other hand, the low expression of YB1 in cardiomyocytes may partly sustain a higher tolerance to I/R induced apoptosis, since YB1 suppression in cardiomyocytes increased apoptosis following I/R.

To dissect the mechanism of YB1-mediated STAT3 phosphorylation, certain proteins involved in regulating STAT3 phosphorylation were detected, and PIAS3 mRNA was determined to be regulated by YB1. However, the present study did not evaluate in detail the YB1 binding region on PIAS3 mRNA, or whether YB1 functions through its cold shock domain or other domains (31,32). Future experiments should aim to investigate the role of YB1 in cardiomyocyte proliferation, and whether ectopic expression of YB1 in cardiomyocytes, but not in myofibroblasts, may alleviate I/R-induced cardiomyocyte apoptosis.

In conclusion, the present study demonstrated that YB1 expression levels increased in H9c2 cardiomyocytes following exposure to H₂O₂. Upregulation of YB1 may have increased STAT3 phosphorylation by promoting PIAS3 mRNA degradation. Moreover, lentivirus-mediated YB1 knockdown in a rat I/R model aggravated infarct size and may have exacerbated the possibility of heart failure. These results may have implications in the diagnosis and treatment of a variety of heart diseases associated with ROS damage, including cardiac hypertrophy, heart failure, myocardial infarction and M-I/R injury. The potential physiological roles of YB1 in other cardiac myocyte disease models should be evaluated in future studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SW, FH, ZL, YH and NH performed the experiments. SW, FH, ZL and XC analyzed the data. SW, FH, ZL, YH, NH and XC designed the study and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee of the Medical School of Ningbo University (Ningbo, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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