TRIM38 protects H9c2 cells from hypoxia/reoxygenation injury via the TRAF6/TAK1/NF-κB signalling pathway

Zhengri Lu[1,*], Mengen Deng[1,*], Genshan Ma[1] and Lijuan Chen[1,2]

[1] Department of Cardiology, Zhongda Hospital, Southeast University, Nanjing, Jiangsu, China
[2] Department of Cardiology, Nanjing Lishui People's Hospital, Zhongda Hospital Lishui Branch, Nanjing, Jiangsu, China
[*] These authors contributed equally to this work.

Corresponding author
Lijuan Chen, chenlijuan@seu.edu.cn

ABSTRACT

Tripartite motif (TRIM) 38 is a ubiquitin E3 protein ligase that is involved in various intracellular physiological processes. However, the role of TRIM38 in myocardial ischaemia/reperfusion (I/R) injury remains to be elucidated. We aimed to establish an *in vitro* cellular hypoxia/reperfusion (H/R) model to explore the role and potential mechanisms of TRIM38 in H9c2, a rat cardiomyoblast cell line. Recombinant adenoviruses for silencing or overexpressing TRIM38 were constructed and transfected into H9c2 cells. Western blotanalysisshowed that TRIM38 expression was significantly decreased after H/R injury. Functionally, TRIM38 expression relieved inflammatory responses and oxidative stress, and inhibited H/R-induced apoptosis in H9c2 cells. Mechanistically, TRIM38 overexpression inhibited H/R-induced transforming growth factor beta-activated kinase 1 (TAK1)/nuclear factor-kappa B (NF-κB) pathway activity in H9c2 cells. The opposite results were observed after TRIM38 knockdown. Furthermore, H/R-induced injury aggravated by TRIM38 deficiency in H9c2 cells was reversed upon treatment with 5Z-7-oxozeaenol, a TAK1 inhibitor. Therefore, TRIM38 reduction attenuated the anti-apoptotic capacity and anti-inflammatory potential of H/R-stimulated H9c2 cells by activating the TAK1/NF-κB signalling pathway. Specifically, TRIM38 alleviated H/R-induced H9c2 cell injury by promoting TNF receptor-associated factor 6 degradation, which led to the inactivation of the TAK1/NF-κB signalling pathway. Thus, our study provides new insights into the molecular mechanisms underlying H/R-induced myocardial injuries.

INTRODUCTION

Myocardial infarction (MI) is a significant cause of disability and mortality globally (*Thygesen et al., 2012*). Reperfusion therapy after MI is primarily used to reduce MI size and improve its prognosis. However, restoring blood flow to the ischaemic myocardium may cause myocardial ischemia/reperfusion (I/R) injury (*Yellon & Hausenloy, 2007*). The pathogenesis of myocardial I/R injury is an intricate pathophysiological process involving numerous mechanisms, including oxidative stress, endoplasmic reticulum stress, inflammation, and autophagy (*Kalogeris et al., 2012*). Thus, in-depth studies to clarify

the critical cellular and molecular mechanisms of I/R injury and develop new preventive prophylactic and therapeutic strategies are vital.

Ubiquitin ligase contributes to myocardial I/R injury by regulating various pathological processes (*Wang et al., 2020*; *Tang et al., 2021*). Our group previously reported that tripartite motif (TRIM) 8 and TRIM32, which are E3 ligase family members, participated in the regulation of pathological cardiac remodelling (*Chen et al., 2016*; *Chen et al., 2017*). TRIM38, an E3 ubiquitin ligase that contains three domains (RING, B-box/coiled coil, and SPRY), mediates inflammatory responses by targeting lysosome-dependent degradation of transforming growth factor (TGF)-beta-activated kinase 1 (TAK1) and MAP3K7-binding protein 2 (TAB2/3) and negatively regulates TLR3/4-mediated inflammatory responses *via* the proteasomal degradation system (*Hu et al., 2014*; *Hu et al., 2015*). Furthermore, TRIM38 ameliorates IL-1β-mediated induction of chondrocyte apoptosis by regulating NF-κB signals (*Hu et al., 2021*). However, a possible mechanism by which TRIM38 protects cardiac cells against myocardial I/R injury has not yet been reported.

Nuclear factor-κB (NF-κB) is an essential transcription factor that regulates various biological processes, including immunity, inflammation, and apoptosis (*Karin & Greten, 2005*; *Courtois & Gilmore, 2006*). In unstimulated cells, NF-κB is sequestered in the cytoplasm as a heterodimeric protein complexed with NF-κB-alpha (IκBα) inhibitors. When cells are stimulated, the NF-κB kinase inhibitor IκB kinase (IKK) is activated, resulting in the phosphorylation and degradation of IκBα. Subsequently, the nuclear localisation signal on NF-κB is exposed, leading to the nuclear translocation of NF-κB and activation of transcription (*Takaesu et al., 2003*). Ubiquitination is a fundamental post-translational modification that plays an essential role in the NF-κB pathway. Numerous TRIM members participate in the regulation of NF-κB signals, indicating the importance of ubiquitination in maintaining fine regulation.

Several critical upstream signalling molecules are involved in IKK activation, including TNF receptor-associated factor 6 (TRAF6) and TAK1. TRAF6 is an E3 ubiquitin ligase containing a RING domain that recruits the TAK1-TAB complex by catalysing Lys63-linked polyubiquitin chain synthesis, which activates the endogenous TAK1 complex and downstream NF-κB signalling pathways (*Wang et al., 2001*). TAK1 is an intracellular mitogen-activated protein kinase (MAP3K) necessary for activating the NF-κB signalling pathway, as it is an upstream kinase of IKK (*Hayden & Ghosh, 2008*; *Mihaly, Ninomiya-Tsuji & Morioka, 2014*). TAK1/NF-κB pathway regulates numerous physiological processes, including inflammation and apoptosis (*Mihaly, Ninomiya-Tsuji & Morioka, 2014*). Ubiquitin modifications play a crucial role in regulating TAK1-mediated NF-κB activation. For instance, the ubiquitin E3 ligase, TRIM8, positively modulates TNF-α-and IL-1β-triggered NF-κB activation by catalysing K63-linked polyubiquitination of TAK1 (*Li et al., 2011*). TRIM27 and TRIM29 negatively regulate NF-κB activation by targeting TAB2 degradation (*Dou et al., 2019*; *Chen et al., 2021*). Additionally, TRIM38 negatively regulates NF-κB and MAPK activation by controlling K48-linked ubiquitination of TRAF6 (*Zhao et al., 2012*). These findings suggest that TRIM proteins are essential regulators of TAK1/NF-κB signals. Therefore, the functional identification of cellular TRIM38 might help in understanding the pathological mechanism of hypoxia/reperfusion

(H/R) and exploring the relevance of TRIM38 as a therapeutic target in myocardial H/R injury.

Here, we identified TRIM38 as a novel repressor of TAK1 activation. TRIM38-mediated inactivation of NF-κB may be associated with the TRAF6/TAK1 pathway. TRIM38 interacts with TRAF6 and specifically catalyses the polyubiquitination and degradation of TRAF6, resulting in suppressed TAK1 activation and downstream signal transduction. Based on these results, we elucidated a new molecular mechanism for H/R-mediated myocardial injury and identified novel therapeutic targets for MI.

MATERIAL AND METHODS

Cell culture

The H9c2 rat cardiomyoblast cell line, which is originally derived from embryonic rat heart tissues, was purchased from Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Shanghai, China) containing 10% foetal bovine serum (Gibco, Shanghai, China), at 37 °C and 5% CO_2.

In vitro establishment of H/R model

To simulate hypoxia/reoxygenation conditions, H9c2 cells were cultured in serum/glucose-free Dulbecco's modified Eagle's medium (Gibco, Shanghai, China) under hypoxia (94% N_2, 5% CO_2 and 1% O_2) for 6 h and subsequently cultured in normal medium under reoxygenation (95% air and 5% CO_2) for another 6 h. Control groups were cultured in normoxic conditions for 12 h.

Adenovirus infection

Recombinant adenoviruses for silencing or overexpressing TRIM38 were purchased from GenePharma (Shanghai, China). Three shTRIM38 constructs were also obtained from GenePharma to generate three lines of AdshTRIM38 adenoviruses. The following sequences were used: shTRIM38-1: 5′-GCACAGTGAAGTCAAGAAAGG-3′, shTRIM38-2: 5′-GGTGGAAGACTAGTGACTTAC-3′, and shTRIM38-3: 5′-GCTGTTGTTGGGAAGATCAGC-3′. H9c2 cells were infected with different adenoviruses in diluted medium at a multiplicity of infection of 100 for 24 h.

Western blot analysis

Total protein was extracted from H9c2 cells using RIPA lysis buffer (Solarbio, Beijing, China) containing protease and phosphatase inhibitors. A BCA kit (Biosharp, Hefei, China) was used to measure the protein concentration. Total protein samples (50 μg) were separated by SDS–PAGE and transferred to methanol-preactivated PVDF membranes and then blocked overnight with 10% non-fat milk in tris-buffered saline at 4 °C for 2 h. The membranes were probed with primary antibodies including TRIM38 (dilution: 1:200; MA5-26235; Invitrogen), Bax (dilution: 1:5,000; 60267-1; Proteintech), Bcl-2 (dilution: 1:5,000; 26593-1-AP; Proteintech), caspase 3 (dilution: 1:1,000; #14220; CST), C-caspase3 (dilution: 1:1,000; #9661, CST), p-Ikkα (dilution: 1:1000; PA5-121282; Invitrogen), IKKα (dilution:

1:1,000; #2682; CST) p-p65 (dilution: 1:1,000; #3033; CST), p65 (dilution: 1:1,000; #8242; CST), IkBα (dilution: 1:500; AF5002; Affinity Biosciences), p-IkBα (dilution: 1:1,000, # 2859; CST), TAK1 (dilution: 1:1,000; #5206; CST), p-TAK1 (dilution: 1:1,000; #9339; CST) overnight at 4 °C with gentle shaking. After washing three times with 1× tris-buffered saline tween (TBST) buffer, the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibodies (dilution: 1:5,000; SA00001-2; Proteintech) and HRP-conjugated goat anti-mouse secondary antibodies (dilution: 1:5,000; SA00001-1; Proteintech) for 2 h at room temperature.

Lactate dehydrogenase (LDH) measurement

The LDH leak rate was detected using an LDH Cytotoxicity Assay Kit (Beyotime, Shanghai, China), following standard procedures according to the manufacturer's instructions. The absorbance intensity at OD490 nm was measured using a microplate reader (Bio-Rad Laboratories Ltd., Shanghai, China).

Malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) measurement

The levels of intracellular MDA, SOD, and GSH-Px were measured using corresponding assay kits and detected using a microplate reader.

ELISA assay

Culture supernatants were collected and the concentrations of TNF-α, IL-1β, and IL-6 in cell supernatants were detected by enzyme-linked immunosorbent assay (ELISA) (Proteintech, Wuhan, China) according to the manufacturer's instructions.

Immunoprecipitation (IP) assay

The IP assay was performed as previously described (*Chen et al., 2017*). Briefly, cultured H9c2 cells were collected and lysed in IP buffer containing 20 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP-40, one mmol/L EDTA, and protease inhibitors. Cell lysates were incubated with protein A/G agarose beads for 3 h at 4 °C before blotting with primary antibodies.

Ubiquitination assay

The ubiquitination assay was performed as previously described (*Chen et al., 2017*). Briefly, H9c2 cells were lysed in SDS lysis buffer containing 20 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, one mmol/L EDTA, 1% SDS, and protease inhibitors. Then, the lysate was heated for 5 min to denature. The supernatant was diluted 10-fold with lysis buffer containing 20 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, one mmol/L EDTA, 1% Triton X-100, and protease inhibitor cocktail. After centrifugation at 4 °C for 30 min, the supernatants were collected and subjected to IP with the indicated antibodies. The supernatants were then incubated with anti-ubiquitin antibodies to determine ubiquitination.

Statistical analysis

Continuous variables are presented as the mean ± standard deviation (SD). The unpaired Student's t-test was used for comparisons between two groups. One-way ANOVA followed

by Bonferroni's *post-hoc* test or Tamhane's T2 post hoc test was used for comparisons among multiple groups. A two-tailed *P*-value of <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism Version 8.0 (GraphPad Prism Inc., San Diego, CA, USA) and IBM SPSS Version 25.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

H/R increased H9c2 apoptosis and reduced TRIM38 expression

To investigate the role of TRIM38 expression in myocardial H/R injury, a cellular model of H/R injury was established. H9c2 cells were cultured under hypoxic conditions for 6 h and then under normoxic conditions for 6 h. We analysed the expression of apoptosis-related proteins in the control and H/R groups using western blotting. Compared with the control group, H/R increased the levels of pro-apoptotic proteins Bcl-2-associated X protein (Bax) and cleaved caspase-3 and decreased the levels of anti-apoptotic protein Bcl-2, leading to a higher bax/Bcl-2 ratio, which showed that H/R induced H9c2 cell apoptosis (Figs. 1A and 1B). Next, we examined the effects of H/R on TRIM38 expression in H9c2 cells. TRIM38 protein expression was remarkably downregulated by H/R compared to that in the control group (Fig. 1A). These results suggest that TRIM38 may be associated with H/R injury-induced H9c2 cell apoptosis.

TRIM38 overexpression alleviated H/R-induced myocardial apoptosis

To assess the effect of TRIM38 on H/R-induced apoptosis, we constructed a TRIM38 overexpressing adenovirus and transfected it into H9c2 cells, the AdGFP-infected H9c2 cells were used as controls (Fig. 2A). TRIM38 overexpression had little effect on the expression of the apoptosis markers under normoxic conditions. However, TRIM38 overexpression substantially decreased bax and cleaved caspase-3 expression, while increasing Bcl-2 expression, and thus reducing the bax/Bcl-2 ratio following H/R (Figs. 2B and 2C). The membrane integrity of damaged cells is disrupted, and the rate of LDH leakage can reflect the degree of cardiomyocyte damage. Subsequently, H/R markedly increased LDH release in H9c2 cells, whereas TRIM38 overexpression inhibited LDH release (Fig. 2D). Upon H/R, the excess production of ROS leads to oxidative stress and cellular damage. ROS lead to increased levels of MDA, which is the product of membrane lipid peroxidation. SOD and GSH-PX are the main enzymes for scavenging free radicals and preventing ROS accumulation. To analyse the impact of TRIM38 on cellular oxidative stress following H/R, we examined SOD and GSH-Px activity as well as MDA content. Overexpression of TRIM38 reduced the MDA content (Fig. 2E) and improved SOD and GSH-Px activity (Figs. 2F and 2G). These results demonstrate that TRIM38 protects against H/R injury.

TRIM38 knockdown inhibits apoptosis in H9c2 cells after H/R

To confirm the effect of TRIM38 on H/R-induced H9c2 cell injury, we transfected H9c2 cells with a TRIM38 interference adenovirus, the AdshRNA-infected H9c2 cells were used as controls. As shown in Fig. 3A, compared with AdshRNA group, TRIM38 protein expression in H9c2 cells was downregulated by AdshTRIM38-1, AdshTRIM38-2, and
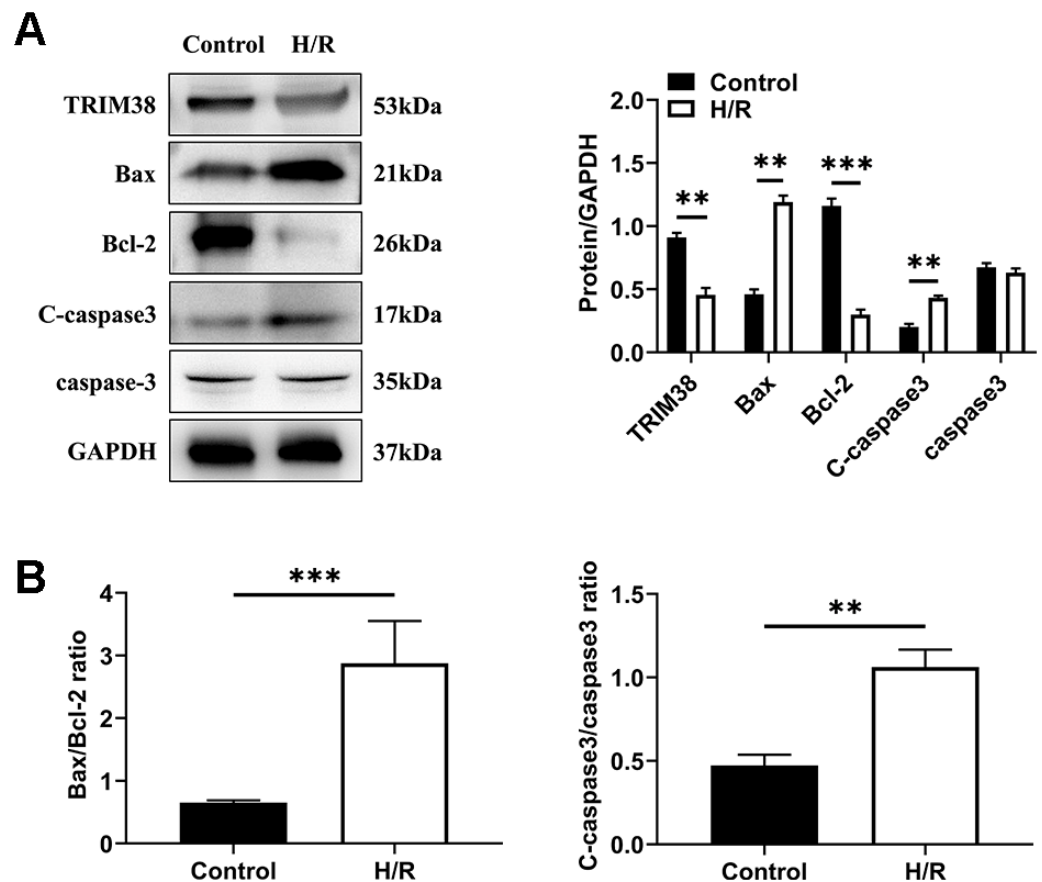
Figure 1 **TRIM38 expression and its roles in H9c2 cells exposed to H/R. H9c2 cells were exposed to 6 h of hypoxia, followed by 6 h of reoxygenation.** (A) Representative immunoblotting analysis and quantification of TRIM38, Bax, Bcl-2, C-caspase3, and caspase3 protein levels in H9c2 cells insulted by H/R or not. (B) Calculation of the ratio of Bax/Bcl2 and C-caspase3/caspase3. $n = 3$ for each group. $**p < 0.01$ and $***p < 0.001$ *versus* control. Unpaired two-tailed student t test for A and B.

AdshTRIM38-3. Compared with AdshTRIM38-1 and AdshTRIM38-3, AdshTRIM38-2 had a higher knockdown efficiency (Fig. 3A) and was therefore used in subsequent experiments. Conversely, TRIM38 knockdown promoted H/R-induced apoptosis by upregulating Bax and cleaved caspase-3 and downregulating Bcl2 expression; the Bax/Bcl2 ratio also increased (Figs. 3B and 3C).

TRIM38 inhibits the NF-κB signalling pathway to mediate inflammation during H/R injury

An excessive inflammatory response is a major cause of H/R injury (*Hu et al., 2016*). The H/R induced the secretion of TNF-α, IL-1β, and IL-6, which was markedly downregulated by the overexpression of TRIM38 (Figs. 4A and 4C). In addition, the H/R-induced expression of TNF-α, IL-1β, and IL-6 was further promoted by TRIM38 knockdown (Figs. 4B and 4D). NF-κB is a transcription factor and plays an important role in inflammation and apoptosis. To further explore the mechanisms of TRIM38 in myocardial H/R injury, we
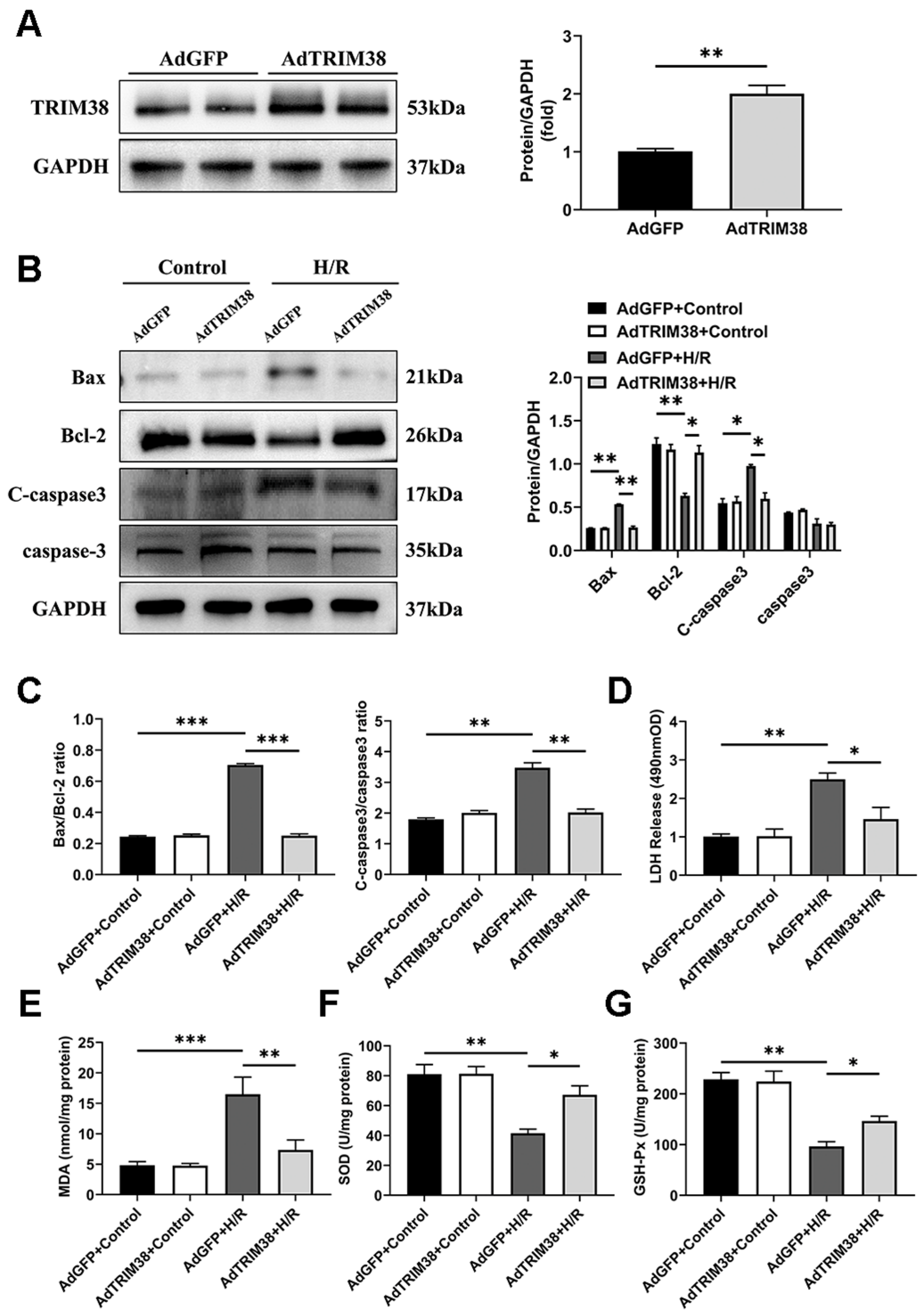
Figure 2 **TRIM38 attenuates apoptosis and oxidative stress in H/R model.** (A) Representative immunoblotting analysis (Left) and quantification (right) of TRIM38 expression in cultured H9c2 cells transfected with AdGFP or AdTRIM38. $n = 4$ for each group. (B) Representative immunoblotting analysis (Left) and quantification (right) of Bax, Bcl-2, C-caspase3, and caspase3 protein levels in H9c2 cells transfected with AdGFP or AdTRIM38 after H/R or not.

Figure 2 (…continued)
(C) Calculation of the ratio of Bax/Bcl2 and C-caspase3/caspase3 in Control or H/R-treated H9c2 cells.
(D) Effects of TRIM38 overexpression on LDH release in H/Rmodel. (E) Cellular MDA content in H/R
model. (F) Cellular SOD activities in H/R model. (G) Cellular GSH-Px activities in H/R model. $n = 3$ for
each group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Unpaired two-tailed student t test for A. One-way
ANOVA followed by *post-hoc* tests for B–G.

examined the activation of NF-κB signalling in H9c2 cells after H/R. Western blot analysis
showed that TRIM38 overexpression blocked the increase in p-IKKα, p-p65, and p-IκBα
levels that was induced by H/R (Fig. 4E). Furthermore, TRIM38 deficiency promoted
NF-κB signalling, as indicated by an increase in p-IKKα, p-p65, and p-IκBα levels (Fig.
4F). These results show that TRIM38 inhibits the activation of NF-κB signalling during
H/R injury.

TRIM38 triggers NF-κB signalling by activating TAK1

TAK1 is a critical upstream molecule for IKK activation and is activated by a broad range of
stimuli (*Mihaly, Ninomiya-Tsuji & Morioka, 2014*; *Mukhopadhyay & Lee, 2020*). To assess
whether TRIM38 inhibits TAK1 activation in H9c2 cells following H/R stimulation, TAK1
and phosphorylated TAK1 levels in H9c2 cells were determined using western blotting.
The results showed that TRIM38 overexpression significantly attenuated H/R-enhanced
phosphorylation of TAK1, whereas TRIM38 knockdown had the opposite effect (Figs. 5A
and 5B). We used the TAK1 inhibitor 5Z-7-oxozeaenol to further explore its effect on the
H/R-induced phosphorylation of TAK1. As shown in Figs. 5C and 5D, TRIM38 knockdown
resulted in the H/R-induced upregulation of TAK1 and NF-κB phosphorylation. The
increase in TAK1 and NF-κB phosphorylation following TRIM38 knockdown was
markedly alleviated by 5Z-7-oxozeaenol (Figs. 5C and 5D). Furthermore, the inhibition
of TAK1/NF-κB signalling blocked the pro-apoptotic effects of TRIM38 knockdown in
H/R-stimulated H9c2 cells. This was confirmed by the increased expression of Bcl2 and
decreased expression of bax and caspase-3 proteins (Figs. 5C and 5E). Collectively, these
results demonstrate that TRIM38 protects against myocardial I/R injury by suppressing
NF-κB activity *via* the regulation of the TAK1 signalling pathway.

TRIM38 degraded TRAF6 *via* ubiquitination

We explored the mechanism by which TRIM38 regulates the TAK1/NF-κB pathway.
TRAF6 interacts with downstream TAK1 and IκB kinases to activate specific signalling
pathways (*Dong et al., 2006*). As TRIM38 can modulate TRAF6 ubiquitination and
degradation in response to LPS administration in macrophages (*Zhao et al., 2012*), we
verified the interaction between endogenous TRIM38 and TRAF6 in H9c2 cells. As shown
in Fig. 6A, TRAF6 co-precipitated with TRIM38 following H/R. Normal IgG, which was
used as a control, did not co-precipitate with TRIM38. Next, we examined the effect of
TRIM38 on the polyubiquitination and degradation of TRAF6. Western blot analysis
showed that TRIM38 knockdown and overexpression markedly increased and decreased
the baseline and post-H/R expression of TRAF6, respectively, compared with that in the
control group (Figs. 6B and 6C). In response to H/R induction, ubiquitinated TRAF6
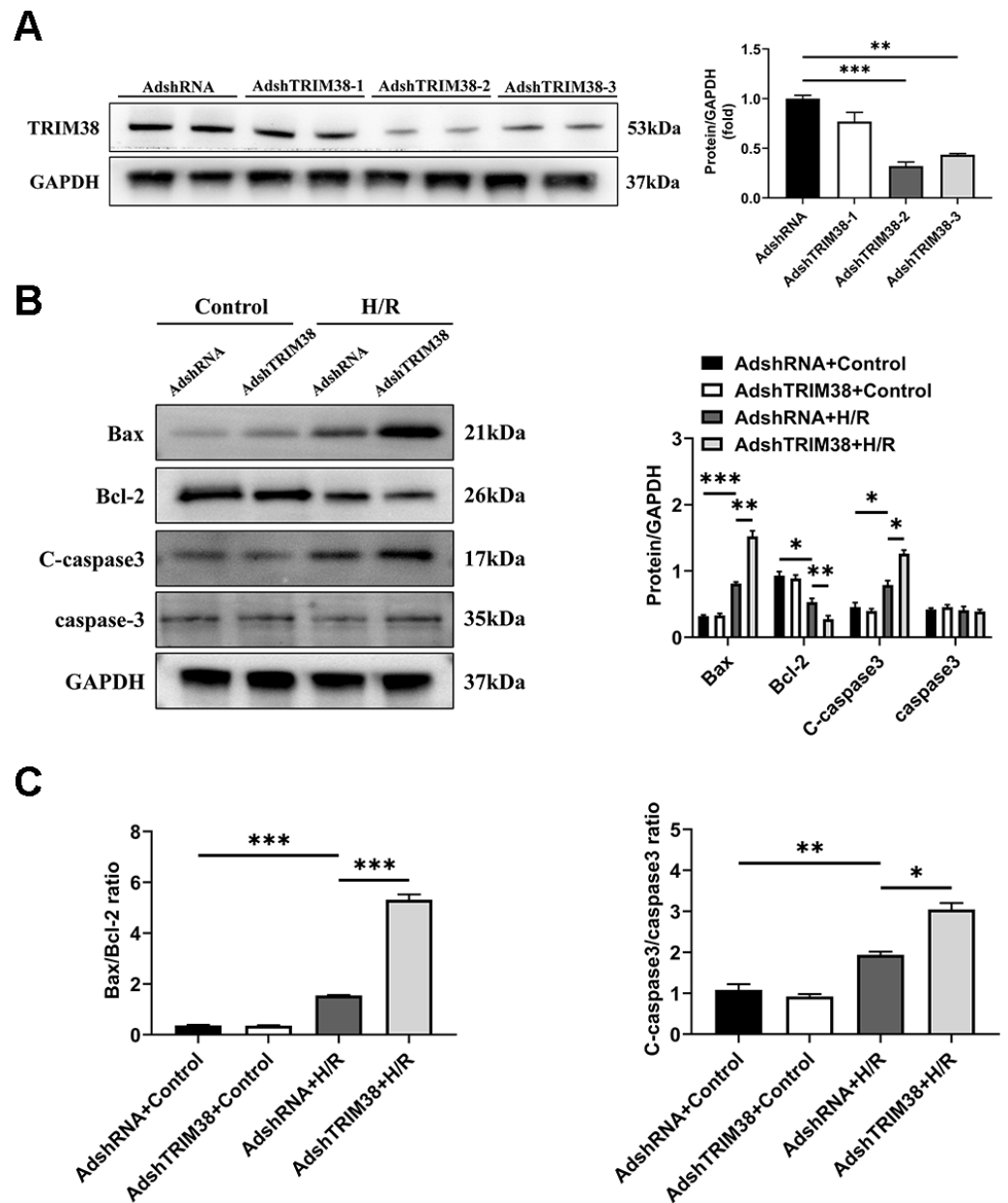
Figure 3 **TRIM38 knockdown enhanced apoptosis in H9c2 Cells After H/R.** (A) Representative immunoblotting analysis (Left) and quantification (right) of TRIM38 expression in cultured H9c2 cells transfected with AdshRNA or AdshTRIM38. (B) Representative immunoblotting analysis (Left) and quantification (right) of Bax, Bcl-2, C-caspase3, and caspase3 protein levels in H9c2 cells transfected with AdshRNA or AdshTRIM38 after H/R or not. (C) Calculation of the ratio of Bax/Bcl2 and C-caspase3/caspase3 in Control or H/R-treated H9c2 cells. $n = 3$ for each group. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. One-way ANOVA followed by *post-hoc* tests for A–C.
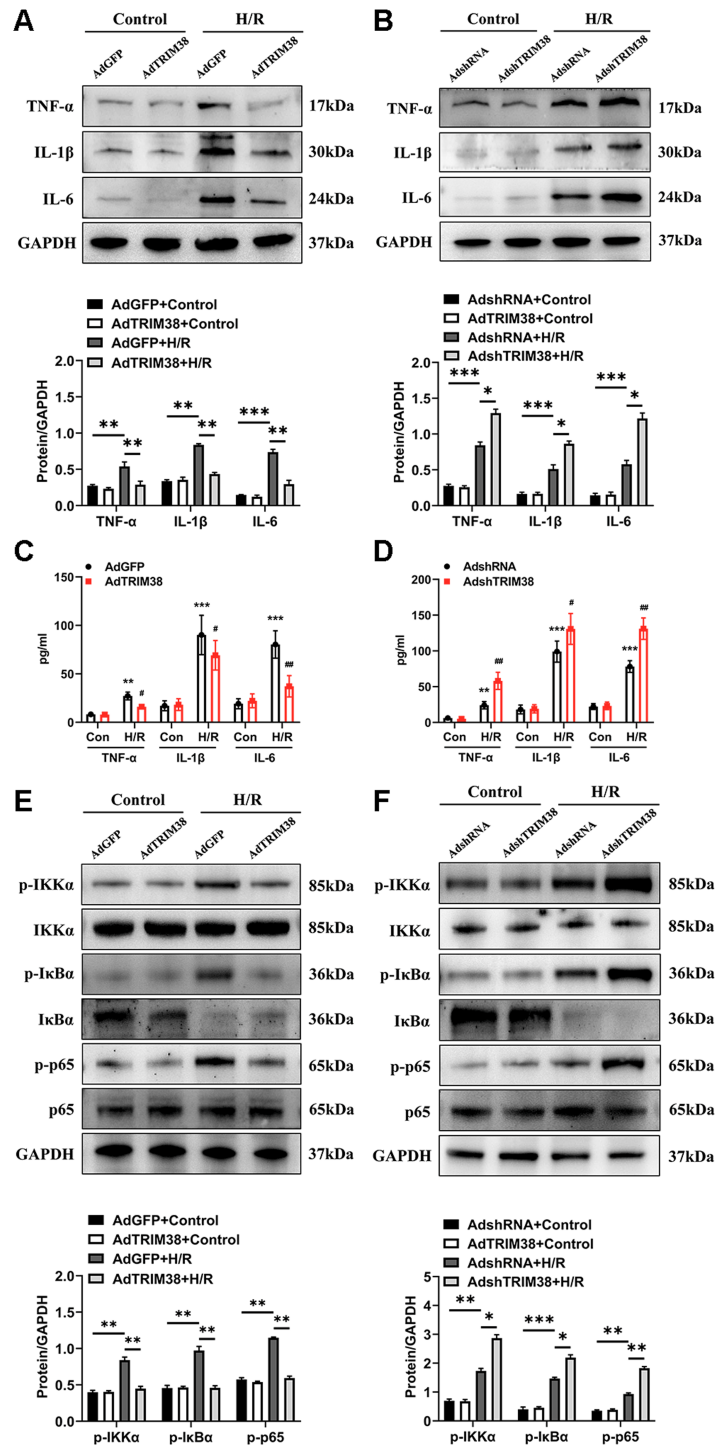
Figure 4 **TRIM38 ameliorates inflammation in H/R-treated H9c2 cells *via* the NF-*κ* B pathways.** (A) Representative immunoblotting analysis and quantification of proinflammatory cytokines in H9c2 cells transfected with AdGFP or AdTRIM38 after H/R stimuli or not. (B) Representative immunoblotting analysis and quantification of proinflammatory cytokines in H9c2 cells transfected with AdshRNA or AdshTRIM38 after H/R stimuli or not. (C–D) Evaluation of TNF-α, IL-1β, and IL-6 in the supernatants of cells using ELISA analysis . $^{**}p < 0.01$ and $^{***}p < 0.001$

levels increased, but TRIM38 deficiency and overexpression decreased and enhanced polyubiquitinated TRAF6 levels, respectively (Figs. 6B and 6C).

DISCUSSION

The molecular mechanisms of myocardial I/R injury involve apoptosis, oxidative stress, imbalance in calcium homeostasis, and inflammation (*Sun et al., 2018*). Unfolded or misfolded proteins resulting from oxidative stress, inflammatory responses, and cytotoxic events are ubiquitinated and transferred to the ubiquitin-proteasome system for degradation (*Davies, 2001*). Ubiquitination is an enzymatic post-translational modification, in which ubiquitin is attached to a substrate protein. Studies have demonstrated that ubiquitin E3 ligase is involved in inflammation and apoptosis during myocardial I/R injury (*Patterson et al., 2007*; *Sun et al., 2019*). In this study, we show, for the first time, that TRIM38 expression is significantly decreased in H9c2 cells following H/R injury. Functionally, TRIM38 relieved inflammatory responses and oxidative stress and inhibited apoptosis triggered by H/R. Mechanistically, TRIM38 protected H9c2 cells against H/R injury by regulating the TAK1/NF-κB pathway. In particular, TRIM38 alleviated H/R-induced H9c2 cell injury by promoting TRAF6 degradation, which led to the inactivation of TAK1—an upstream NF-κB signalling molecule (Fig. 6D).

Apoptosis is the principal factor associated with cardiomyocyte loss in I/R pathogenesis. Emerging evidence suggests that ubiquitination plays a pivotal role in myocardial I/R injury by regulating apoptosis (*Li et al., 2020*; *Zhang et al., 2021*). TRIM38 attenuates IL-1β-stimulated chondrocyte apoptosis (*Hu et al., 2021*). In the present study, western blotting analysis suggested that TRIM38 expression was downregulated in cultured H9c2 cells subjected to H/R. Subsequently, the anti-apoptotic effect of TRIM38 on H9c2 cells during H/R was confirmed by functional experiments. Western blotting analysis indicated that TRIM38 overexpression notably protected H9c2 cells from H/R-induced apoptosis. In contrast, reduction of TRIM38 expression promotes apoptosis by regulating the Bax/Bcl-2 ratio and caspase-3 activity. These results preliminarily demonstrated that TRIM38 protected H9c2 cells from H/R-induced apoptosis.

Accumulating evidence suggests that oxidative stress and inflammatory response are critical factors associated with pathological process of myocardial I/R injury (*Liu et al., 2021*). Cellular reactive oxygen species (ROS) are major stressors of oxidative stress during myocardial ischaemia. Overproduction of oxygen free radicals exacerbates lipid peroxidation and inflammation, ultimately contributing to myocardial injury, particularly during reperfusion (*Wu et al., 2020*). Therefore, antioxidant and anti-inflammatory therapies may alleviate myocardial I/R injury to some degree (*Hu et al., 2016*; *Liu et al., 2021*). TRIM38 plays an important role in the regulation of inflammatory responses (*Hu et*
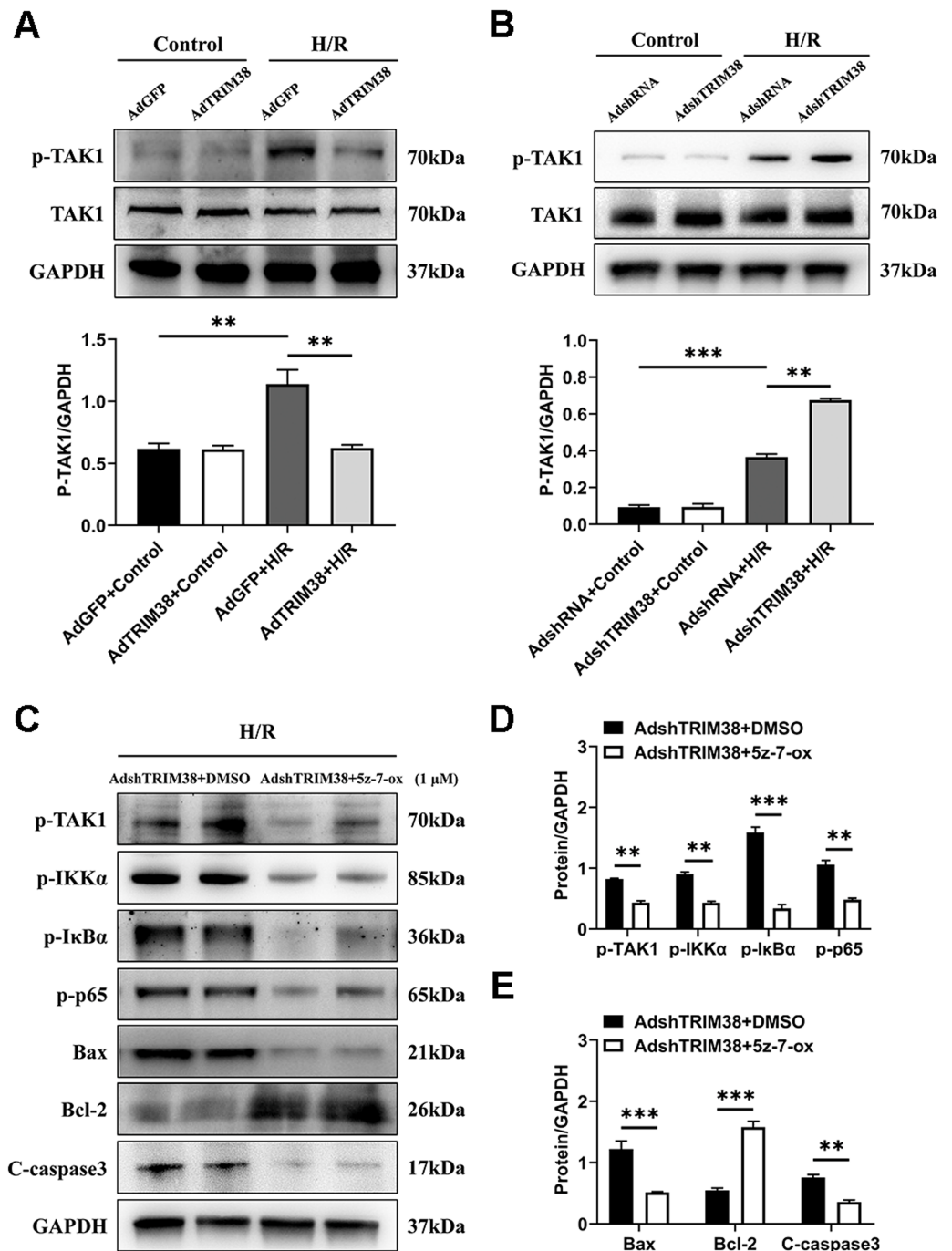
Figure 5 TRIM38 inhibited TAK1-NF-κB signaling axis in H9c2 cells. (A–B) The protein expression of total and phosphorylated TAK1 in control or H/R-treated H9c2 cells from indicated groups. $n = 3$ for each group. (C–E) Representative immunoblottinganalysis and quantification of p-TAK1, p-IKKα, p-IκBα, and p-p65 after pretreated with 5z-7-ox (1 μM) inH9c2 cells, and subjected to H/R for 12 h. $n = 6$ for each group. **$p < 0.01$ and ***$p < 0.001$. Unpaired two-tailed student t test for C–E. One-way ANOVA followed by *post-hoc* tests for A–B.
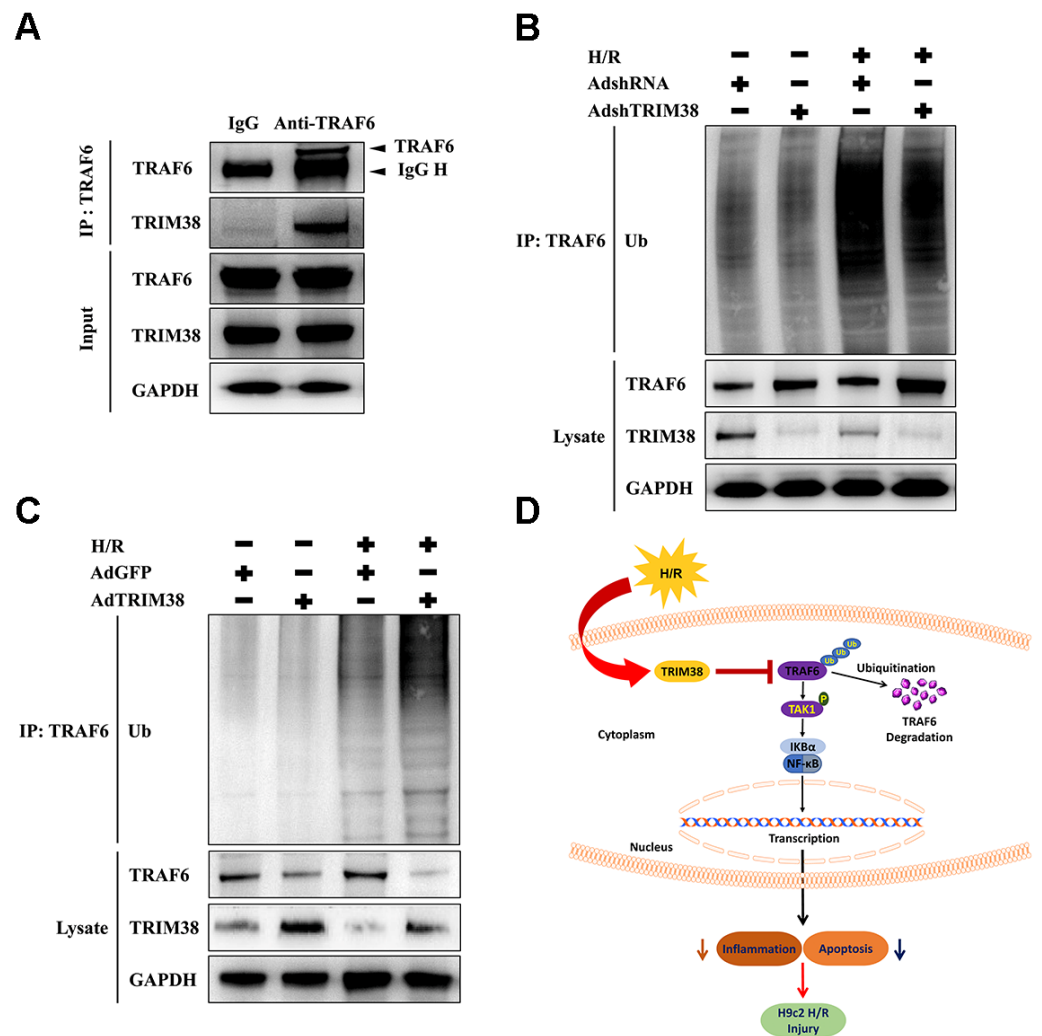
Figure 6 Under H/R conditions, TRIM38 degraded TRAF6 viaubiquitination. (A) H9c2 cells were subjected to H/R stimulation. Cell lysate and normal IgG antibody or TRAF6 antibody were subjected to Co-IP, and the indicated antibodies were used for immunoblotting analysis. (B–C) TRIM38 knockdown or overexpression cells were exposed with H/R. Immunoprecipitation assay was performed using lysates with anti-TRAF6 antibody. IB assay was performed using anti-TRAF6, anti-TRIM38, and anti-Ub antibodies. (D) Working model of TRIM38 protecting H9c2 cells from H/R injury by inhibiting TRAF6/TAK1/NF-κB signaling pathway. TRIM38 alleviated H/R-induced H9c2 injury by promoting TRAF6 degradation, which led to the inactivation of TAK1, an upstream NF-κB signalling molecule.

al., 2014; *Hu et al., 2015*). Therefore, we explored whether TRIM38 overexpression could inhibit the production of inflammatory cytokines and oxidative stress substances. The levels of MDA and SOD reflect the degree of oxidative stress (*Zhou et al., 2019*). Our study revealed that TRIM38 overexpression significantly elevated the SOD levels, decreased the MDA levels, and inhibited the release of proinflammatory cytokines in H9c2 cells under conditions of H/R stimulation. These results indicate that TRIM38 attenuates H/R-induced H9c2 cell injury, partly by reducing the levels of inflammatory factors and

inhibiting oxidative stress. In the pathological mechanism underlying myocardial I/R injury, inflammatory cells are the main cell type mediating inflammatory responses (*Frangogiannis, 2012*). Among them, monocytes and their progeny macrophages dominate cell infiltration and release inflammatory mediators and reactive oxygen species, thereby promoting inflammation and phagocytosis (*Yellon & Hausenloy, 2007*; *Nahrendorf, Pittet & Swirski, 2010*). Previous studies have demonstrated that TRIM38 inhibits TNF-α/IL-1 β signalling in mouse bone marrow-derived macrophages (*Hu et al., 2015*). However, it is unclear whether TRIM38 can inhibit NF-κB-mediated pro-inflammatory responses in cardiomyocytes. Our findings suggest that TRIM38 can inhibit NF-κB-mediated pro-inflammatory responses in cardiomyocytes. During myocardial I/R injury, cardiac-resident cells (*e.g.*, cardiomyocytes) can produce pro-inflammatory cytokines in response to various stimuli, thereby favouring a pro-inflammatory environment. However, given the dominant role of monocytes and macrophages in mediating inflammatory responses, exploring the link between TRIM38 and inflammatory cells and cardiomyocytes in myocardial tissues is worthy of further research (*Fan et al., 2019*; *Algoet et al., 2022*).

The NF-κB family plays a critical role in numerous biological processes, including apoptosis and inflammatory responses (*Karin & Greten, 2005*; *Courtois & Gilmore, 2006*). When cardiomyocytes are stimulated by various chemical and mechanical signals, such as I/R, NF-κB is activated (*Xiong et al., 2021*; *Zhang et al., 2022*). Therefore, we investigated the effect of TRIM38 on NF-κB signalling. TRIM38 overexpression blocked the H/R-induced increase in the levels of p-IKKα, p-p65, and p-IκBα. In contrast, TRIM38 knockdown displayed the opposite effects. These findings suggest that TRIM38 exerts protective effects on H/R-induced apoptosis and inflammation in H9c2 cells, at least in part, by inhibiting the NF-κB signalling pathway. TAK1 linked cytokine stimulation with the activation of inflammatory signals. When stimulated by TGF-β, TNF-α, and IL-1, TAK1 phosphorylates and binds to the IKK complex, resulting in IκB phosphorylation (*Fan et al., 2010*). This was further corroborated by the results of our *in vitro* rescue experiments. Treatment with a specific TAK1 inhibitor markedly attenuated the phosphorylation of TAK1 and the downstream signalling pathways in H9c2 cells subjected to H/R. Interestingly, we found that TRIM38 knockdown promoted H/R-induced apoptosis; however, the inhibitor 5Z-7-oxozeaenol blocked the effects caused by TRIM38 knockdown. In TRAF6-TAK1 pathway, TRAF6 ubiquitination promotes TAK1 activation. Therefore, we examined whether TRIM38 modulated the ubiquitination of TRAF6 in H9c2 cells. The results obtained from co-immunoprecipitation experiments showed that endogenous TRIM38 interacted with TRAF6, mediating its ubiquitination. Furthermore, we verified the negative correlation between TRIM38 and TRAF6 expression. An *in vivo* ubiquitination assay confirmed that TRIM38 deficiency and overexpression reduced and enhanced TRAF6 ubiquitination, respectively. Increased TRAF6 levels are involved in the activation of the TAK1/NF-κB signalling pathway to regulate H9c2 cell injury.

Nevertheless, this study has a few limitations. First, we chose H/R-treated H9c2 cells rather than primary cardiomyocytes to explore the mechanism underlying the protective action of TRIM38. Although the H9c2 rat cardiomyoblast cell line demonstrates a reliable availability and consistency of responses, these cells may not fully reflect the interactions

among primary cardiomyocytes. Moreover, the pathophysiological mechanisms underlying myocardial I/R injury are multifactorial and may involve factors other than inflammation and apoptosis. Furthermore, an *in vitro* model was constructed using standardised H/R conditions. Thus, additional experiments are needed to ascertain the protective effects of TRIM38 on cardiomyocytes under various hypoxic conditions.

In conclusion, we confirmed the novel cardioprotective effects of TRIM38 *in vitro*; these effects occur *via* the TRIM38-mediated blockage of the TRAF6/TAK1/NF-κB signalling pathway, which suppresses apoptosis and inflammation in H/R-induced H9c2 cells. Furthermore, this study elucidated the molecular mechanism underlying the activity of TRIM38 in H/R-treated H9c2 cells and underscored a novel target for the alleviation of myocardial ischaemic injury.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Zhengri Lu conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Mengen Deng performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Genshan Ma conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Lijuan Chen conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.

Data Availability

The raw data is available in the Supplemental Files.

Supplemental Information

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REFERENCES

Algoet M, Janssens S, Himmelreich U, Gsell W, Pusovnik M, Van den Eynde J, Oosterlinck W. 2022. Myocardial ischemia-reperfusion injury and the influence of inflammation. *Trends in Cardiovascular Medicine* Epub ahead of print Feb 16 2022 DOI 10.1016/j.tcm.2022.02.005.

Chen L, Huang J, Ji YX, Mei F, Wang PX, Deng KQ, Jiang X, Ma G, Li H. 2017. Tripartite motif 8 contributes to pathological cardiac hypertrophy through enhancing transforming growth factor beta-activated kinase 1-dependent signaling pathways. *Hypertension* **69(2)**:249–258 DOI 10.1161/HYPERTENSIONAHA.116.07741.

Chen L, Huang J, Ji Y, Zhang X, Wang P, Deng K, Jiang X, Ma G, Li H. 2016. Tripartite motif 32 prevents pathological cardiac hypertrophy. *Clinical Science* **130(10)**:813–828 DOI 10.1042/CS20150619.

Chen SY, Zhang HP, Li J, Shi JH, Tang HW, Zhang Y, Zhang J-K, Wen P-H, Wang Z-H, Shi X-Y, He Y-T, Hu B-W, Yang H, Guo W-Z, Zhang S-J. 2021. Tripartite motif-containing 27 attenuates liver ischemia/reperfusion injury by suppressing transforming growth factor beta-activated kinase 1 (TAK1) by TAK1 binding protein 2/3 degradation. *Hepatology* **73(2)**:738–758 DOI 10.1002/hep.31295.

Courtois G, Gilmore TD. 2006. Mutations in the NF-kappaB signaling pathway: implications for human disease. *Oncogene* **25(51)**:6831–6843 DOI 10.1038/sj.onc.1209939.

Davies KJ. 2001. Degradation of oxidized proteins by the 20S proteasome. *Biochimie* **83(3–4)**:301–310 DOI 10.1016/S0300-9084(01)01250-0.

Dong W, Liu Y, Peng J, Chen L, Zou T, Xiao H, Liu Z, Li W, Bu Y, Qi Y. 2006. The IRAK-1-BCL10-MALT1-TRAF6-TAK1 cascade mediates signaling to NF-kappaB from Toll-like receptor 4. *Journal of Biological Chemistry* **281(36)**:26029–26040 DOI 10.1074/jbc.M513057200.

Dou Y, Xing J, Kong G, Wang G, Lou X, Xiao X, Vivier E, Li XC, Zhang Z. 2019. Identification of the E3 ligase TRIM29 as a critical checkpoint regulator of NK cell functions. *Journal of Immunology* **203(4)**:873–880 DOI 10.4049/jimmunol.1900171.

Fan Q, Tao R, Zhang H, Xie H, Lu L, Wang T, Su M, Hu J, Zhang Q, Chen Q, Iwakura Y, Shen W, Zhang R, Yan X. 2019. Dectin-1 Contributes to myocardial ischemia/reperfusion injury by regulating macrophage polarization and neutrophil infiltration. *Circulation* **139(5)**:663–678 DOI 10.1161/CIRCULATIONAHA.118.036044.

Fan Y, Yu Y, Shi Y, Sun W, Xie M, Ge N, Mao R, Chang A, Xu G, Schneider MD, Zhang H, Fu S, Qin J, Yang J. 2010. Lysine 63-linked polyubiquitination of TAK1

at lysine 158 is required for tumor necrosis factor alpha- and interleukin-1beta-induced IKK/NF-kappaB and JNK/AP-1 activation. *Journal of Biological Chemistry* **285(8)**:5347–5360 DOI 10.1074/jbc.M109.076976.

Frangogiannis NG. 2012. Regulation of the inflammatory response in cardiac repair. *Circulation Research* **110(1)**:159–173 DOI 10.1161/CIRCRESAHA.111.243162.

Hayden MS, Ghosh S. 2008. Shared principles in NF-kappaB signaling. *Cell* **132(3)**:344–362 DOI 10.1016/j.cell.2008.01.020.

Hu S, Li Y, Wang B, Peng K. 2021. TRIM38 protects chondrocytes from IL-1beta-induced apoptosis and degeneration via negatively modulating nuclear factor (NF)-kappaB signaling. *International Immunopharmacology* **99**:108048 DOI 10.1016/j.intimp.2021.108048.

Hu X, Ma R, Lu J, Zhang K, Xu W, Jiang H, Da Y. 2016. IL-23 promotes myocardial I/R injury by increasing the inflammatory responses and oxidative stress reactions. *Cellular Physiology and Biochemistry* **38(6)**:2163–2172 DOI 10.1159/000445572.

Hu MM, Xie XQ, Yang Q, Liao CY, Ye W, Lin H, Shu H-B. 2015. TRIM38 negatively regulates TLR3/4-mediated innate immune and inflammatory responses by two sequential and distinct mechanisms. *Journal of Immunology* **195(9)**:4415–4425 DOI 10.4049/jimmunol.1500859.

Hu MM, Yang Q, Zhang J, Liu SM, Zhang Y, Lin H, Huang Z-F, Wang Y-Y, Zhang X-D, Zhong B, Shu H-B. 2014. TRIM38 inhibits TNFalpha- and IL-1beta-triggered NF-kappaB activation by mediating lysosome-dependent degradation of TAB2/3. *Proceedings of the National Academy of Sciences of the United States of America* **111(4)**:1509–1514 DOI 10.1073/pnas.1318227111.

Kalogeris T, Baines CP, Krenz M, Korthuis RJ. 2012. Cell biology of ischemia/reperfusion injury. *International Review of Cell and Molecular Biology* **298**:229–317 DOI 10.1016/B978-0-12-394309-5.00006-7.

Karin M, Greten FR. 2005. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nature Reviews Immunology* **5(10)**:749–759 DOI 10.1038/nri1703.

Li X, Wang X, Liu YS, Wang XD, Zhou J, Zhou H. 2020. Downregulation of miR-3568 protects against ischemia/reperfusion-induced cardiac dysfunction in rats and apoptosis in H9C2 cardiomyocytes through targeting TRIM62. *Frontiers in Pharmacology* **11**:17 DOI 10.3389/fphar.2020.00017.

Li Q, Yan J, Mao AP, Li C, Ran Y, Shu HB, Wang Y-Y. 2011. Tripartite motif 8 (TRIM8) modulates TNFalpha- and IL-1beta-triggered NF-kappaB activation by targeting TAK1 for K63-linked polyubiquitination. *Proceedings of the National Academy of Sciences of the United States of America* **108(48)**:19341–19346 DOI 10.1073/pnas.1110946108.

Liu S, Liao Q, Xu W, Zhang Z, Yin M, Cao X. 2021. MiR-129-5p protects H9c2 cardiac myoblasts from hypoxia/reoxygenation injury by targeting TRPM7 and inhibiting NLRP3 inflammasome activation. *Journal of Cardiovascular Pharmacology* **77(5)**:586–593.

Mihaly SR, Ninomiya-Tsuji J, Morioka S. 2014. TAK1 control of cell death. *Cell Death & Differentiation* **21**(11):1667–1676 DOI 10.1038/cdd.2014.123.

Mukhopadhyay H, Lee NY. 2020. Multifaceted roles of TAK1 signaling in cancer. *Oncogene* **39**(7):1402–1413 DOI 10.1038/s41388-019-1088-8.

Nahrendorf M, Pittet MJ, Swirski FK. 2010. Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. *Circulation* **121**(22):2437–2445 DOI 10.1161/CIRCULATIONAHA.109.916346.

Patterson C, Ike C, PWt Willis, Stouffer GA, Willis MS. 2007. The bitter end: the ubiquitin-proteasome system and cardiac dysfunction. *Circulation* **115**(11):1456–1463 DOI 10.1161/CIRCULATIONAHA.106.649863.

Sun T, Ding W, Xu T, Ao X, Yu T, Li M, Liu Y, Zhang X, Hou L, Wang J. 2019. Parkin regulates programmed necrosis and myocardial ischemia/reperfusion injury by targeting cyclophilin-D. *Antioxidants & Redox Signaling* **31**(16):1177–1193 DOI 10.1089/ars.2019.7734.

Sun T, Zhang Y, Zhong S, Gao F, Chen Y, Wang B, Cai W, Zhang Z, Li W, Lu S, Zheng F, Shi G. 2018. N-n-butyl haloperidol iodide, a derivative of the antipsychotic haloperidol, antagonizes hypoxia/reoxygenation injury by inhibiting an Egr-1/ROS positive feedback loop in H9c2 cells. *Frontiers in Pharmacology* **9**:19 DOI 10.3389/fphar.2018.00019.

Takaesu G, Surabhi RM, Park KJ, Ninomiya-Tsuji J, Matsumoto K, Gaynor RB. 2003. TAK1 is critical for IkappaB kinase-mediated activation of the NF-kappaB pathway. *Journal of Molecular Biology* **326**(1):105–115 DOI 10.1016/S0022-2836(02)01404-3.

Tang LJ, Zhou YJ, Xiong XM, Li NS, Zhang JJ, Luo XJ, Peng J. 2021. Ubiquitin-specific protease 7 promotes ferroptosis via activation of the p53/TfR1 pathway in the rat hearts after ischemia/reperfusion. *Free Radical Biology and Medicine* **162**:339–352 DOI 10.1016/j.freeradbiomed.2020.10.307.

Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD, Katus HA, Lindahl B, Morrow DA, Clemmensen PM, Johanson P, Hod H, Underwood R, Bax JJ, Bonow RO, Pinto F, Gibbons RJ, Fox KA, Atar D, Newby LK, Galvani M, Hamm CW, Uretsky BF, Steg PG, Wijns W, Bassand J-P, Menasché P, Ravkilde J, Ohman EM, Antman EM, Wallentin LC, Armstrong PW, Simoons ML, Januzzi JL, Nieminen MS, Gheorghiade M, Filippatos G, Luepker RV, Fortmann SP, Rosamond WD, Levy D, Wood D, Smith SC, Hu D, Lopez-Sendon J-L, Robertson RM, Weaver D, Tendera M, Bove AA, Parkhomenko AN, Vasilieva EJ, Mendis S. Joint ESC/ACCF/AHA/WHF Task Force for the Universal Definition of Myocardial Infarction. 2012. Third universal definition of myocardial infarction. *Circulation* **126**(16):2020–2035 DOI 10.1161/CIR.0b013e31826e1058.

Wang SJ, Chen H, Tang LJ, Tu H, Liu B, Li NS, Luo X-J, Peng J. 2020. Upregulation of mitochondrial E3 ubiquitin ligase 1 in rat heart contributes to ischemia/reperfusion injury. *Canadian Journal of Physiology and Pharmacology* **98**(5):259–266 DOI 10.1139/cjpp-2019-0285.

Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412(6844)**:346–351 DOI 10.1038/35085597.

Wu L, Xiong X, Wu X, Ye Y, Jian Z, Zhi Z, Gu L. 2020. Targeting oxidative stress and inflammation to prevent ischemia-reperfusion injury. *Frontiers in Molecular Neuroscience* **13**:28 DOI 10.3389/fnmol.2020.00028.

Xiong W, Zhou R, Qu Y, Yang Y, Wang Z, Song N, Liang R, Qian J. 2021. Dexmedetomidine preconditioning mitigates myocardial ischemia/reperfusion injury via inhibition of mast cell degranulation. *Biomedicine & Pharmacotherapy* **141**:111853 DOI 10.1016/j.biopha.2021.111853.

Yellon DM, Hausenloy DJ. 2007. Myocardial reperfusion injury. *The New England Journal of Medicine* **357(11)**:1121–1135 DOI 10.1056/NEJMra071667.

Zhang J, Jiang S, Lu C, Pang J, Xu H, Yang F, Zhuang S. 2021. SYVN1/GPX5 axis affects ischemia/reperfusion induced apoptosis of AC16 cells by regulating ROS generation. *American Journal of Translational Research* **13(5)**:4055–4067.

Zhang D, Wang Q, Qiu X, Chen Y, Yang X, Guan Y. 2022. Remifentanil protects heart from myocardial ischaemia/reperfusion (I/R) injury via miR-206-3p/TLR4/NF-kappaB signalling axis. *Journal of Pharmacy and Pharmacology* **74(2)**:282–291 DOI 10.1093/jpp/rgab151.

Zhao W, Wang L, Zhang M, Yuan C, Gao C. 2012. E3 ubiquitin ligase tripartite motif 38 negatively regulates TLR-mediated immune responses by proteasomal degradation of TNF receptor-associated factor 6 in macrophages. *Journal of Immunology* **188(6)**:2567–2574 DOI 10.4049/jimmunol.1103255.

Zhou K, Chen H, Lin J, Xu H, Wu H, Bao G, Li J, Deng X, Shui X, Gao W, Ding J, Xiao J, Xu H. 2019. FGF21 augments autophagy in random-pattern skin flaps via AMPK signaling pathways and improves tissue survival. *Cell Death and Disease* **10(12)**:872 DOI 10.1038/s41419-019-2105-0.