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Original article

Zishen Huoxue decoction (ZSHX) alleviates ischemic myocardial injury (MI) *via* Sirt5-*β*-tubulin mediated synergistic mechanism of "mitophagy-unfolded protein response" and mitophagy

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ABSTRACT

Zishen Huoxue decoction (ZSHX) enhances cardiomyocyte viability following hypoxic stress; however, its upstream therapeutic targets remain unclear. Network pharmacology and RNA sequencing analyses revealed that ZSHX target genes were closely associated with mitophagy and apoptosis in the mitochondrial pathway. *In vitro*, ZSHX inhibited pathological mitochondrial fission following hypoxic stress, regulated FUN14 domain-containing protein 1 (FUNDC1)-related mitophagy, and increased the levels of mitophagy lysosomes and microtubule-associated protein 1 light chain 3 beta II (LC3II)/translocase of outer mitochondrial membrane 20 (TOM20) expression while inhibiting the over-activated mitochondrial unfolded protein response. Additionally, ZSHX regulated the stability of beta-tubulin through Sirtuin 5 (SIRT5) and could modulate FUNDC1-related synergistic mechanisms of mitophagy and unfolded protein response in the mitochondria (UPR^{mt}) *via* the SIRT5 and -*β*-tubulin axis. This targeting pathway may be crucial for cardiomyocytes to resist hypoxia. Collectively, these findings suggest that ZSHX can protect against cardiomyocyte injury *via* the SIRT5-*β*-tubulin axis, which may be associated with the synergistic protective mechanism of SIRT5-*β*-tubulin axis-related mitophagy and UPR^{mt} on cardiomyocytes.

1. Introduction

Ischemic cardiomyopathy (ICM) is a cardiovascular disorder characterized by myocardial damage or necrosis resulting from hypoxia and ischemia [1, 2]. The maintenance of mitochondrial morphology, structure, and functional integrity is crucial for normal cardiomyocyte physiological function [3]. Mitochondria fulfill various physiological requirements for cardiomyocyte changes and provide energy for cardiomyocyte activity by generating ATP and participating in the tricarboxylic acid cycle [4, 5]. Alterations in mitochondrial morphology primarily involve fission and fusion processes [6]. Previous studies have demonstrated that under myocardial ischemia/hypoxia conditions, the impairment of mitophagy and the unfolded protein response in the mitochondria (UPR^{mt}) leads to an inability to eliminate damaged mitochondria and nonfunctional mitochondrial organelles in cardiomyocytes [7, 8]. Zishen Huoxue decoction (ZSHX) is composed of Astragali Radix (20 g), Codonopsis (6 g), Mulberry (9 g), Notoginseng Radix et Rhizoma (3 g), Trichosanthes Fructus (9 g), Allium macrostemonis bulbus (9 g), and Glycyrrhizae Radix et Rhizoma (6 g). ZSHX is a traditional formulation used in the treatment of cardiovascular diseases, known for its ability to supplement qi and blood, promote blood circulation, and protect the myocardium. Moreover, ZSHX has been shown to effectively enhance the energy metabolism of mitochondria in cardiomyocytes and sinus node cells under hypoxic conditions, activate mitophagy, and inhibit apoptosis [9, 10].

Previous research has confirmed the protective effects of quercetin, the active component of ZSHX, on mitochondria damaged by hypoxia, inflammation, and high glucose levels in cardiomyocytes and vascular endothelial cells [9, 11]. Quercetin has been shown to improve heart failure following transverse aortic constriction (TAC), increase the ejection fraction, and suppress myocardial remodeling and cardiomyocyte hypertrophy [12]. Additionally, a prior study demonstrated that quercetin could effectively inhibit apoptosis and mitochondrial oxidative stress following myocardial infarction (MI) *via* the janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathway [13]. However, the mitochondrial protective function of ZSHX remains to be fully elucidated.

Mitophagy denotes the selective elimination of damaged mitochondria. Along with UPR^{mt}, mitophagy serves as a crucial regulatory mechanism for mitochondrial quality control. Proper protein folding and maturation are essential for mitochondrial function; any deviation, such as irreversible oxidation or misfolding, can lead to proteomic instability and loss of function, thereby activating cellular sensors through unfolded protein responses (UPRs) at the endoplasmic reticulum (UPRer) and UPR^{mt} sites [14, 15]. Previous research has demonstrated that the balanced

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regulation of mitophagy and UPR^{mt} is fundamental for maintaining mitochondrial quality and quantity, as well as the primary regulatory mechanism for cardiomyocyte function [14, 16]. Further investigations into the dysregulated collaboration between mitophagy and UPR^{mt} in ischemic MI, and the identification of relevant target drugs, are critical for mitigating injury [17]. Inhibition of FUNDC1-associated mitophagy in AC16 cardiomyocytes results in a significant loss of mitochondrial membrane potential (MMP). Mitophagy inhibition triggers UPR^{mt}, subsequently affecting mitochondrial self-renewal. Additional research has shown that FUNDC1-mediated mitophagy remains unaffected and synchronously activated when UPR^{mt} is blocked [knockdown of activating transcription factor 6 (ATF6)]. The collaborative action of these two factors maintains mitochondrial homeostasis in cardiomyocytes. However, the upstream regulatory mechanisms during ischemic myocardial injury (MI) remain to be elucidated.

Sirtuin 5 (SIRT5) is a member of the silent information regulator protein family. In certain instances, multiple pathways influenced by SIRT5 have consistently yielded similar outcomes [18]. Previous animal studies have shown that the active ingredients of ZSHX enhance IDH2 by increasing SIRT5 expression, thereby inhibiting pathological mitochondrial fission and inflammatory injury in cardiomyocytes, while promoting mitochondrial fusion and biosynthesis [12]. Our previous research revealed that SIRT5 is predominantly localized in mitochondria, but also present in peroxisomes through its *N*-terminal sequence, further regulating hydrogen peroxide (H_2O_2) production. SIRT5 directly modulates the activity of antioxidant enzymes and the "inflammation-oxidative stress" mediated pathway, influencing the redox homeostasis of cardiomyocytes. Furthermore, ZSHX regulates the structural integrity of *β*-tubulin *via* sirtuin 1 (SIRT1), activates mitophagy, and maintains mitochondrial homeostasis. However, the precise regulatory mechanisms of mitophagy and UPR^{mt} remain to be fully elucidated. Consequently, identifying upstream proteins or drugs that effectively modulate SIRT5 is crucial for understanding the regulatory roles of mitophagy and the UPR^{mt} in ICM. Empagliflozin (EMPA), a sodium-glucose cotransporter2 (SGLT2) inhibitor, demonstrates cardioprotective effects, including reduced cardiovascular mortality and improved cardiac function after MI in clinical settings [19]. Given that EMPA's myocardial protection is associated with regulating mitochondrial homeostasis [20], and our previous study confirmed its role in ischemic MI and coronary microvascular injury [21], we utilized EMPA as a positive control drug in this study.

This study illuminates the mechanisms by which ZSHX mitigates ICM through its protective effects against ischemia- and hypoxia-induced cardiomyocyte damage. These effects are mediated *via* the SIRT5-*β*-tubulin axis, which is associated with mitophagy and the UPR^{mt}.

2. Materials and Methods

2.1. Drug preparation

The composition of ZSHX is detailed in the introduction. All herbs are sourced and authenticated by Guang'anmen Hospital (Beijing, China). The herbs underwent decoction for 45 min, after which the solution was filtered. Subsequently, the two filtered solutions were combined in a 75 °C water bath to produce a 1 g·mL^{-1} ZSHX stock solution. The HPLC analysis of the decoction is presented in Supplementary Material Figs. j1–j2.

2.2. Experimental animals

We developed SIRT5 heart-specific knockout mice (Cyagen Biosciences). SIRT5$^{f/f}$ and SIRT5^{CKO} mice were generated following previously established protocols [22, 23]. To mitigate potential sex-related variations, male mice were exclusively used in this study. In accordance with the ARRIVE guidelines for animal studies, and with approval from the Ethics Committee of Guang'anmen Hospital (Approval number: IACUC-GAMH-2023-054-SQ), adult male C57BL/6 mice (7–8 weeks old) were procured from the laboratory animal center at Guang'anmen Hospital. All mice were maintained under controlled laboratory conditions at 23–25 °C, with a 12-h light/dark cycle, and 55% ± 5% humidity. The animals were provided with standard approved food and unrestricted access to water for a minimum of 7 days prior to experimentation.

2.3. Myocardial ischemic injury model and intervention measures

This study utilized a previously established MI model [24]. Mice were anesthetized with pentobarbital (2 mg·kg^{-1}) and artificially ventilated using a ventilator with a tidal volume of 0.2 mL·min^{-1} and a rate of 120 breaths/min. The left anterior descending branch (LAD) of the coronary artery was ligated using a 7–0 surgical silk thread. After 30 min of ligation, the suture was untied to initiate reperfusion. The mice were euthanized 120 min post-reperfusion for serum and heart collection and staining [14]. Mice in the low-, medium-, and high-dose groups were pretreated with ZSHX at concentrations of 2.015, 4.03, and 8.06 g·kg^{-1}·d^{-1}, respectively. For all treatment groups, ZSHX administration commenced on the second day of modeling and concluded the day before sampling. In accordance with a previous study [21], mice were treated with EMPA (10 mg·kg^{-1}·d^{-1}) seven days prior to MI. EMPA was kindly provided by Boehringer Ingelheim Pharma GmbH & Co., KG, Germany, and was ainistered *via* oral gavage with 0.5% hydroxyethylcellulose as the vehicle.

2.4. Echocardiography

Transthoracic echocardiography was conducted on multiple groups of anesthetized mice. Cardiac ejection function parameters (LVEF, fractional shortening (FS), LV Vol-D, LV Vol-S, and E/A ratio) were evaluated using a diagnostic ultrasound system (SSA-700A; Toshiba) [23].

2.5. Cardiomyocytes culture

In vitro modeling was conducted following the isolation of primary cardiomyocytes from WT (wild-type) and SIRT5 heart-specific knockout mice. The cardiomyocyte hypoxia-reoxygenation model was implemented, as previously described [11]. To examine the role of ZSHX in regulating *β*-tubulin stability through SIRT5, *β*-tubulin was subjected to knockdown and overexpression. Cardiomyocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM) with glutamine, supplemented with 10% fetal bovine serum (FBS) and 100 μg·mL^{-1} penicillin/streptomycin. Cardiomyocytes from the SIRT5/*β*-tubulin overexpression and knockdown groups were generated *via* adenovirus transfection. The cardiomyocyte incubator was maintained at 37 °C and 5% CO_2 [21, 25].

2.6. Preparation of drug-containing serum, drug, and dose screening

Prior to conducting cell experiments, we prepared ZSHX drug-containing serum. Sprague-Dawley (SD) rats, obtained from the laboratory animal center at Guang'anmen Hospital and raised in standard conditions, were divided into low-, medium-, and high-dose groups based on human-to-animal body surface area ratios. ZSHX was administered at 1, 2, and 4 times the equivalent dose (5.58, 11.16, and 22.32 g·kg^{-1}·d^{-1}, respectively). The control group received an equal volume of saline *via* oral gavage. After

7 days of continuous gastric lavage, anesthesia was administered one hour following the final treatment, and blood was collected. Rat serum was isolated *via* centrifugation, and inactivated by heating at 56 °C for 30 min, and filtered using a 0.22 μmol·L^{-1} microporous filter. The resulting serum containing the nourishing kidney and activating blood formula was stored at −20 °C. Following our previous study [10], cardiomyocytes were treated with ZSHX at concentrations of 50, 100, and 150 mg·L^{-1}. The viability of cardiomyocytes after hypoxic stress was evaluated using the cell counting kit-8 (CCK-8) assay. All three concentrations of ZSHX drug-containing serum (50, 100, and 150 mg·L^{-1}) enhanced cardiomyocyte viability following hypoxia and mitigated hypoxic injury. The high-concentration group (150 mg·L^{-1}) demonstrated the most significant improvement in cardiomyocyte function post-hypoxia and was consequently selected for subsequent experiments.

2.7. Cardiomyocytes grouping and drug intervention

The hypoxia/reoxygenation (H/R) injury model of cardiomyocytes has been previously described [9, 11]. Cardiomyocytes were categorized into control and model groups (H/R), as well as ZSHX low-concentration (50 mg·L^{-1}), medium-concentration (100 mg·L^{-1}), and high-concentration (150 mg·L^{-1}) groups. EMPA at low-concentration (5 μmol·L^{-1}), medium-concentration (10 μmol·L^{-1}), and high-concentration (15 μmol·L^{-1}) was administered to the cardiomyocytes 12 h prior to model injury [26]. To induce mitophagy, FCCP (5 μmol·L^{-1}) was applied for 120 min, as previously described. Conversely, to inhibit mitophagy, cells underwent pretreatment with 3-MA (10 mmol·L^{-1}) for 2 h [27].

2.8. Real time quantitative polymerase chain reaction (PCR) and Western Blotting

Total RNA was extracted from cardiomyocytes using TRIzol reagent, purified with chloroform, and precipitated with isopropanol. The PrimeScriptTM RT kit was employed for RNA reverse transcription using a fast real-time PCR instrument. TB Green Premium Ex Taq II (RR820A, Takara, Japan) was utilized to amplify cDNA. The experimental results were analyzed using the $2^{-\triangle\triangle CT}$ method. The primers used are listed in Supplementary Material Table 1.

The samples underwent washing with PBS, and protein concentrations were determined using a PBCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Cells were incubated with 5% skim milk in Tris Buffered Saline with Tween-20 (TBS-T) buffer for 90 min at 4 °C, followed by overnight incubation with primary antibodies. Immunoblots were visualized using an enhanced chemiluminescence (ECL) reagent. The primary antibodies utilized in this study included Anti-*β*-tubulin (Abcam, #ab18207), Anti-*β*-actin (Abcam, #ab200658), Anti-FUNDC1 (Abcam, #ab224722), Anti-Mitofusin 1 (Abcam, #ab198410), Anti-Mitofusin 2 (Abcam, #ab124773), Anti-GAPDH (Abcam, #ab8245), and Anti-Drp1 (Abcam, #ab184247).

2.9. Laser-confocal and immunofluorescence

Primary cardiomyocyte samples underwent washing with PBS and incubation with the primary antibody overnight at 4 °C. Following this, the cells were subjected to staining with a fluorescent secondary antibody for 30 min and DAPI for nuclear visualization. Image acquisition was performed using confocal laser microscopy.

2.10. Immunofluorescence and TUNEL staining

For immunofluorescence analysis of myocardial tissue, frozen heart sections and cardiomyocyte samples underwent overnight incubation, followed by incubation with secondary antibodies. To evaluate cardiomyocyte apoptosis, a TdT-mediated dUTP Nick-End Labeling (TUNEL) assay was performed, with nuclei counterstained using DAPI. Subsequent quantitative data analysis was conducted.

2.11. Analysis of MMP and mito-ROS\mPTP detection

Cardiomyocytes subjected to hypoxia-reoxygenation were incubated in darkness for 15 minutes with the copolymerized sensing agent JC-1 (10 nmol·L^{-1}; T3168; Invitrogen). The production of mitochondrial reactive oxygen species (ROS) was evaluated using MitoSOX (5 mmol·L^{-1}, M36008, Invitrogen), which was incubated with the cardiomyocyte cores. The opening of the mitochondrial permeability transition pore (mPTP) was observed as a rapid dissipation of tetramethylrhodamine ethyl fluorescence. A confocal microscope was utilized to capture the images [28].

2.12. Analysis of mitochondrial energy, metabolism level, and respiratory chain function

Cardiomyocyte mitochondrial energy metabolism was assessed using an XF-24 Extracellular Flux Analyzer (Agilent 103592-100; Hippocampal Biological Sciences, California, USA). Cardiomyocytes were seeded in XF24 cell plates (catalog No. 100777-004; Agilent, Santa Clara, USA) to achieve 95% confluence (3–4 × 10^4 cells/well). XF unbuffered medium (XF DMEM, pH 7.4, Catalog number 103757-100; Agilent, Santa Clara, USA) was utilized. The cartridges were then loaded with XF real-time ATP rate assay compounds. Data analysis was performed using the Agilent ATP Assay Report Generator, and statistical analysis was conducted using Prism 9.0 software (GraphPad).

2.13. Enzyme-linked immunosorbent assay (ELISA)

The antioxidant enzyme activity was determined using commercially available ELISA kits (SOD, cat. no. EIASODC, Invitrogen; Malondialdehyde (MDA) assay kit (TBA method) (A003-1-2), Caspase-9 activity assay kit (G018-1-1), Caspase-3 activity assay kit (G015-1-1); BCL-2 associated X protein Assay Kit (H379-1), and BCL-W (BCL2L2) Rat ELISA Kit (H073) were purchased from Nanjing Jiancheng Biotech. Co., Ltd.). Samples were thawed on ice from −80 °C storage. The kits were removed from 4 °C storage and allowed to reach room temperature. Sample processing was conducted according to the manufacturer's instructions.

3. Statistical analysis

Data analysis and organization were performed using GraphPad Prism 9.0, with results presented as means ± standard error of the mean (SEM). For comparisons between two groups, either the parametric Student's *t*-test or the nonparametric Mann-Whitney *U* test was employed. One-way ANOVA with Bonferroni post-hoc tests were utilized for comparisons involving two or more groups. Statistical significance was defined as $P < 0.05$.

4. Results

4.1. Effective target and pathway prediction of ZSHX targeting mitochondrial injury in cardiomyocytes

The active ingredients of ZSHX were identified using the PubChem database, and their 2D structures in SDF format were downloaded and imported into the PharmMapper database for target prediction. Targets with Fit Scores exceeding 3 were selected. Additionally, ZSHX targets were predicted using the Swiss Target Prediction database, with targets having probability val-

ues greater than 0.1 chosen as predicted targets. Using "Ischemic cardiomyopathy" as a keyword, disease targets related to ICM were searched and screened in three disease gene databases, including GeneCards. To elucidate the relationships between these targets, common targets of ZSHX and ICM were analyzed using the STRING11.5 database (https://string-db.org/), and a protein-protein interaction (PPI) network was constructed. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed using the DAVID database, with a significance threshold set at $P < 0.01$. The results suggested that the therapeutic mechanism of ZSHX in ICM primarily targets the mitochondria, both inner and outer mitochondrial membranes, intracellular molecular complexes, hypoxia/inflammatory damage, skeletal protein damage, dysfunction in protein phosphorylation, and apoptosis (Figs. 1A–1F).

4.2. ZSHX inhibits ischemic MI and inflammatory response in a dose-dependent manner

We further investigated the optimal dose-response relationship and the therapeutic mechanism of ZSHX. Various doses were administered for the intervention. Following the induction of ischemic MI, the mice exhibited different degrees of myocardial damage and elevated TNT levels. These changes were accompanied by alterations in myocardial mitochondrial morphology and the activation of apoptosis (Figs. 1G–1O). Notably, myocardial ischemic injury in mice was associated with increased LDH and IL-17 levels, as well as activation of the caspase-3 pathway (Figs. 1P–1R). These findings suggest that myocardial ischemic injury in mice is linked to cardiomyocyte apoptosis through the mitochondrial pathway mediated by inflammation, aligning with the results of network pharmacology.

Intervention with varying doses of ZSHX and EMPA demonstrated inhibitory effects on myocardial remodeling and fibrotic changes following ischemic injury. This intervention reduced TNT expression, suppressed apoptosis, maintained normal mitochondrial structure (Figs. 1G–1O), and inhibited the increase in LDH and IL-17 levels, as well as the activation of the caspase-3 pathway (Figs. 1P–1R). Notably, the high dose of ZSHX exhibited comparable efficacy to EMPA. These findings suggest that a high dose

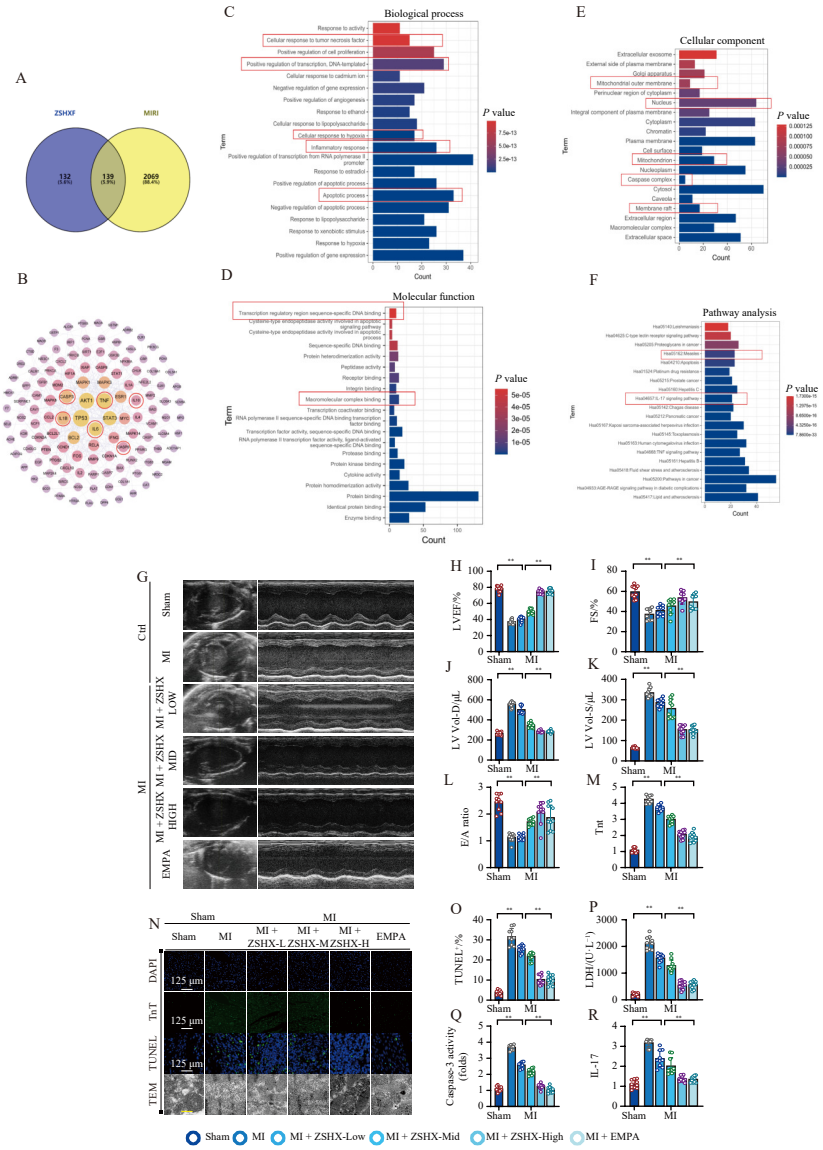

Fig. 1 Network pharmacology analysis of ZSHX and its dose-dependent effect on ischemic MI and inflammatory response (A) Disease and drug VENN diagram. (B) MCODE analysis diagram of intersection targets. (C–F) KEGG bubble diagram; GO-BP bubble diagram; GO-CC bubble diagram; GO-MF bubble diagram. (G–L) Echocardiographic analysis of cardiac function following intervention with different doses of ZSHX: left ventricular ejection fraction (LVEF), left ventricular FS, diastolic left ventricular volume (LV Vold), systolic left ventricular volume (LV Vols), and E/A (mitral flow velocity ratio). (M–O) Trinitrotoluene (TNT) fluorescent staining and TUNEL cell apoptosis detection. (K) Myocardial mitochondria observed *via* transmission electron microscopy. (P–R) Lactate dehydrogenase (LDH), Caspase-3, and IL-17 expression levels; Data are presented as mean ± SEM (n = ten independent cell isolations per group). $^{**}P < 0.01$.

of ZSHX may ameliorate ischemic MI through mitochondrial pathways. However, the specific regulatory mechanisms underlying mitochondrial injury require further investigation.

4.3. ZSHX attenuates mitochondrial damage following hypoxic stress through regulation of mitochondrial quality control.

Network pharmacology demonstrated that ZSHX could ameliorate ischemic MI *via* the mitochondrial pathway. The specific mechanism by which ZSHX improves hypoxia-induced mitochondrial damage was validated through *in vitro* experiments. These experiments revealed that treatment with varying concentrations of ZSHX enhanced cardiomyocyte viability following hypoxia and reduced hypoxic cardiomyocyte injury, with the high-concentration group exhibiting the most significant improvement (Fig. 2A). A similar dose-dependent effect was observed with EMPA intervention (Fig. 2B). Furthermore, ZSHX increased TOM20 expression, elevated ATP production, and mitigated excessive mitochondrial fission and abnormal mPTP opening in cardiomyocytes following hypoxic injury, demonstrating efficacy comparable to EMPA (Figs. 2C–2J). Additionally, ZSHX inhibited the overproduction of ROS and MDA, restored antioxidant enzyme activity and MMP, and suppressed Caspase-3/-9 activation (Figs. S1A–S1L). These findings suggest that hypoxic stress significantly impairs cardiomyocyte activity, and that disruption of mitochondrial homeostasis likely contributes to cardiomyocyte apoptosis.

4.4. Mitochondrial membrane injury and homeostasis mechanism in cardiomyocytes after hypoxia stress analyzed through RNA sequencing enrichment

The mechanism through which ZSHX enhances cardiomyocyte mitochondrial function involves mitophagy and mitochondrial fusion/fission; however, the specific regulatory mechanism responsible for mitochondrial damage remains unclear. To further elucidate the correlation between the pathological mechanism of ICM and mitochondrial injury, genetic alterations and injury mechanisms in cardiomyocytes before and after modeling were analyzed using RNA sequencing. The results demonstrated that during H/R, cardiomyocytes were primarily enriched in processes related to MMP damage, mitochondrial matrix, mitophagy, and membrane damage (Figs. S2A–S2J). KEGG/GO enrichment analysis and Gene Set Enrichment Analysis (GSEA) strongly suggested that hypoxia-induced cardiomyocyte injury was predominantly associated with mitochondrial energy metabolism, mitophagy, and the integrity of the mitochondrial membrane (Figs. S2A–S2J).

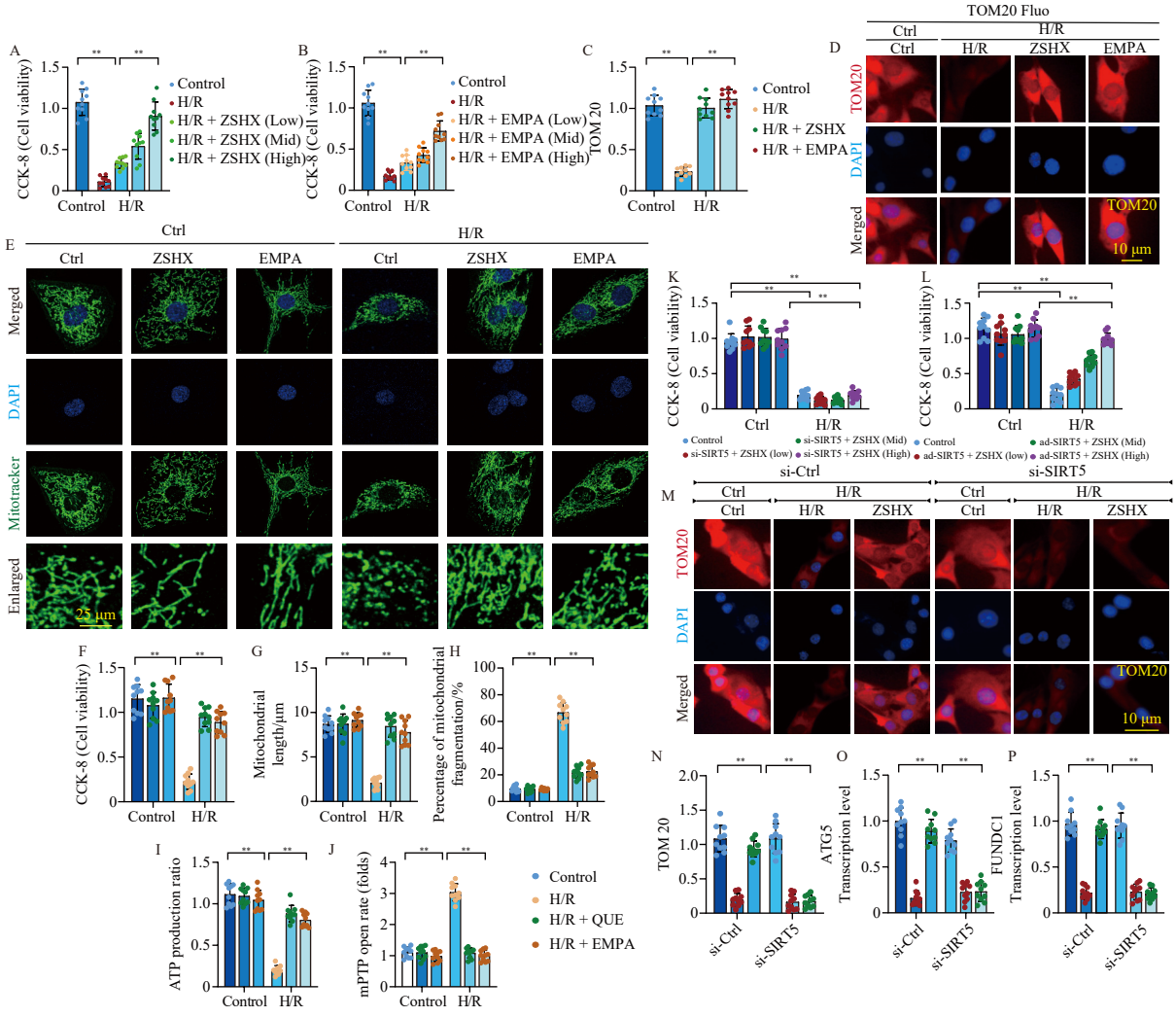

Fig. 2 ZSHX alleviates mitochondrial damage following hypoxic stress by modulating mitochondrial quality control through SIRT5. (A–B) Cell viability was assessed using CCK-8 assay. (C–D) Fluorescence expression level of TOM20 was analyzed by laser confocal microscopy. Scale bar, 25 μm. (E) Mitochondrial length measurement across different groups. (F) Cell viability was evaluated using CCK-8 assay. (G–H) Mitochondrial fission visualized with mito-tracker, Scale bar, 25 μm. (I) ATP production measurement. (J) mPTP opening level assessment. (K–L) Cell viability analysis using varying doses of ZSHX after SIRT5 knockdown and overexpression. (M–N) Fluorescent expression analysis of mitochondrial TOM20 in cardiomyocytes, Scale bar, 25 μm. (O) ATG5 transcription level quantification. (P) FUNDC1 transcription level quantification. Data are presented as mean ± SEM (n = ten independent cell isolations per group). $^{**}P < 0.01$.

Subsequent sequencing analyses revealed that genes enriched in myocardial ischemic injury were closely associated with mitochondrial nucleoids, the mitochondrial matrix, and various mitochondrial components (Figs. S3A–S3J). These genetic alterations directly influenced mitochondrial respiratory chain function (complex-I/V), the tricarboxylic acid cycle, and oxidative phosphorylation within the mitochondria (Figs. S3A–S3J). These findings further corroborate the hypothesis that myocardial ischemic injury is intricately linked to mitochondrial homeostasis and quality control, potentially affecting the structural integrity of the mitochondrial membrane. This mechanism may represent the primary pathway through which ZSHX enhances cardiomyocyte viability.

4.5. ZSHX enhances mitochondrial homeostasis via SIRT5-mediated mitophagy

To validate mitophagy involvement in myocardial cell injury following hypoxia, we investigated the role of SIRT5-mediated mitophagy in the ZSHX therapeutic pathway. *In vivo*, ZSHX increased cardiomyocyte viability in a concentration-dependent manner following H/R treatment, an effect negated by SIRT5 knockdown (Figs. 2K and 2L). The regulatory effects of ZSHX on TOM20 were also inhibited by si-SIRT5, as was the activation of ATG and FUNDC1 by ZSHX (Figs. 2M–2P). These findings indicate that ZSHX enhances mitochondrial homeostasis *via* SIRT5-mediated mitophagy. ZSHX also inhibited mitochondrial oxidative stress injury and prevented the abnormal opening of mPTP. However, SIRT5 knockdown disrupted the regulatory effects of ZSHX on mitochondrial function (Figs. 3A–3I). Further evaluation revealed that ZSHX restored the MMP, inhibited proton leakage, and restored respiratory chain function after hypoxia-induced injury, effects abrogated by SIRT5 knockdown (Figs. 3J–3T). These observations collectively suggest that SIRT5 plays a crucial role in the ZSHX decoction-induced protection against mitochondrial injury following hypoxia.

4.6. Interactions between SIRT5 and β-tubulin regulate mitophagy induced by ZSXH

Hypoxic stress diminished the expression of *β*-tubulin cytoskeletal protein, while ZSHX partially restored *β*-tubulin expression in a dose-dependent manner (Figs. 4A and 4B). However, specific knockdown of SIRT5 hindered the regulatory effect of ZSHX on *β*-tubulin. Protein docking data further corroborated the hypothesis that SIRT5 may interact with *β*-tubulin, influencing mitochondrial homeostasis (Figs. 4N–4P), although the exact mechanism necessitates additional investigation. These findings suggest that ZSHX could inhibit apoptosis *via* the mitochondrial pathway. However, treatment with ad-SIRT5/si-*β*-tubulin or 3-methyladenine (3MA) impeded the regulatory effects of ZSHX on apoptosis through the mitochondrial pathway. Notably, ad-SIRT5/ad-*β*-tubulin did not affect ZSHX's targeted regulation (Figs. 4E and 4F).

These findings indicate that restoring SIRT5 expression alone does not ameliorate *β*-tubulin damage and cytoskeletal abnormalities. However, the normalization of *β*-tubulin through SIRT5 modulation by ZSHX may offer an effective approach to mitigate mitochondrial damage. Furthermore, ZSHX demonstrated the ability to suppress Drp1 expression, reinstate TOM20/Mfn1/Mfn2 expression, phosphorylate FUNDC1 at Tyrosine 18 in the LC3 interaction region (LIR), and regulate mPTP release (Figs. 4C–4M). These observations suggest that hypoxia-induced imbalance in mitochondrial fission/fusion may result in aberrant mitophagy, leading to abnormal mPTP opening and subsequent activation of the mitochondrial apoptotic pathway.

However, si-SIRT5/3MA or ad-SIRT5/si-*β*-tubulin thwarted the inhibitory effect of ZSHX on pathological mitochondrial fis-

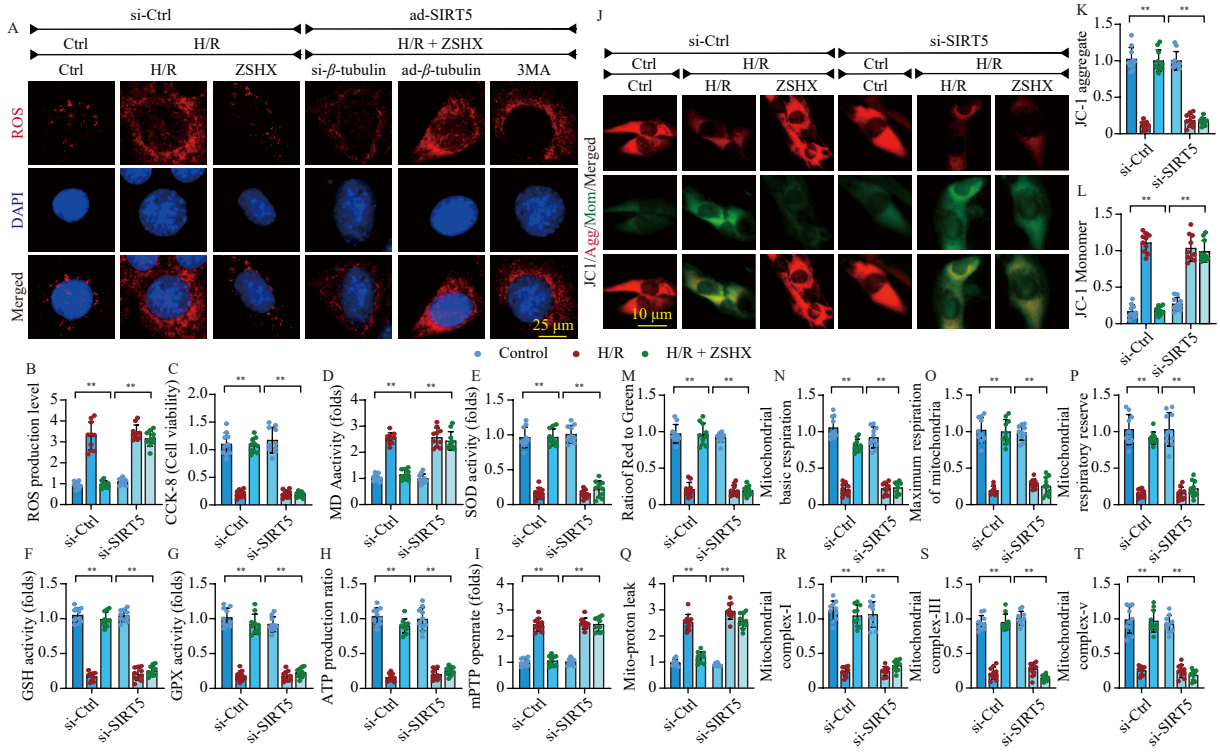

Fig. 3 ZSHX regulates mitochondrial homeostasis and mitochondrial energy metabolism through SIRT5. (A–B) Mito-ROS fluorescence expression. Scale bar, 25 μm. (C) CCK-8 detection. (D) Detection of MDA activity by ELISA. (E–G) Detection of SOD/GPX/GSH activity by ELISA. (H) ATP production. (I) MDA, an indicator of oxidative stress, was measured using ELISA. (E–G) ELISA was employed to assess the activity of antioxidant enzymes SOD (Superoxide dismutase)/GPX (glutathione peroxidase) and GSH (Glutathione). (J–M) JC-1 immunofluorescence detection of MMP level. Scale bar, 10 μm. (N) Mitochondrial basal respiration. (O) Mitochondrial maximal respiration. (P) Mitochondrial respiratory reserve. (Q) Mitochondrial proton leakage. (R–T) Mitochondrial respiratory chain function. Data are presented as mean ± SEM (n = ten independent cell isolations per group). $^{**}P < 0.01$.

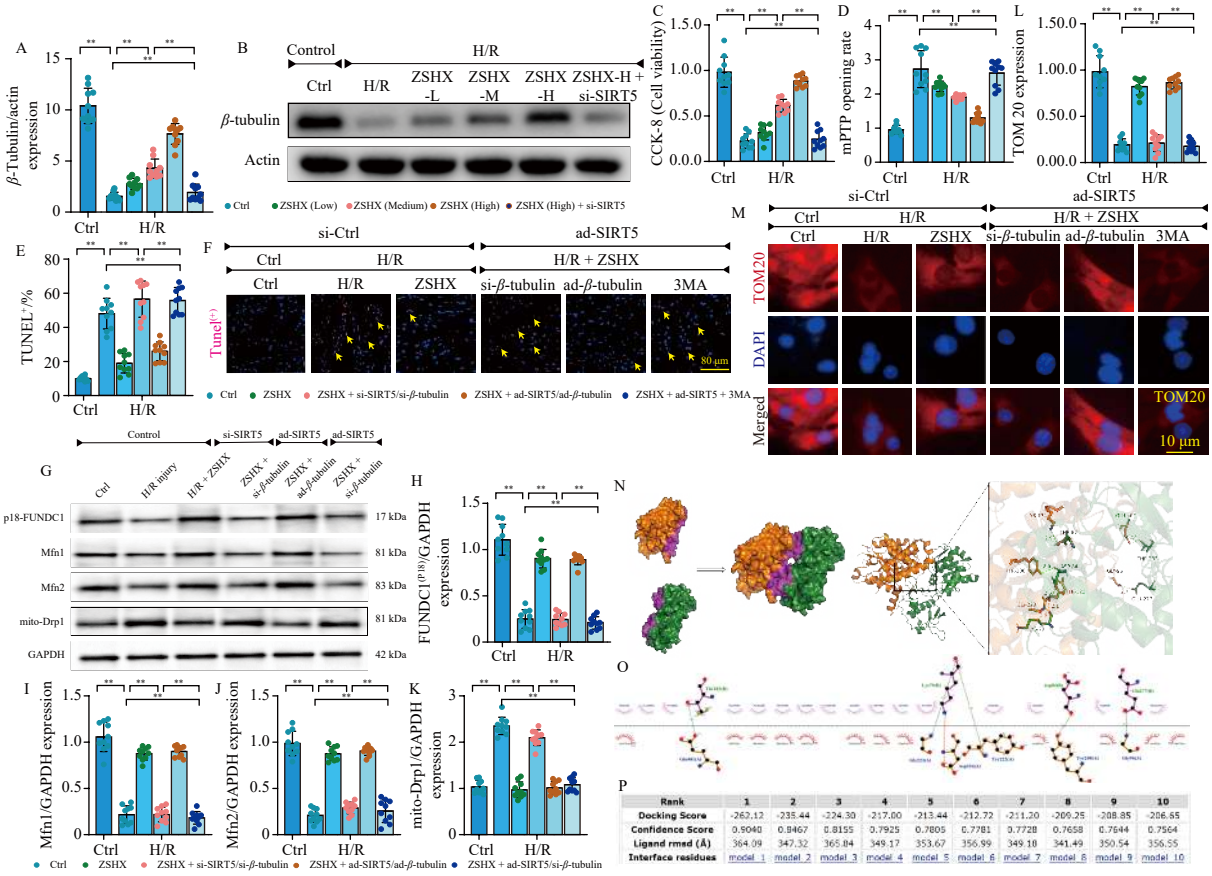

Fig. 4 ZSHX maintains β-tubulin stability and regulates mitochondrial quality control in cardiomyocytes through SIRT5 (n = 3 three independent cell isolations per group). (A–B) Protein expression of β-tubulin. (C) CCK-8 (Cell viability). (D) mPTP (Mitochondrial permeability transition pore) opening rate. (E–F) TUNEL assay. (G–K) Protein expression of mitochondrial dynamics and mitophagy-related proteins (phosphorylation). (L–M) Fluorescent expression of TOM20. (N–P) Protein-protein docking prediction of the interaction mechanism between SIRT5 and β-tubulin. $^{+}P < 0.01$.

sion and reversed the regulatory effect of ZSHX on FUNDC1-mediated mitophagy (Figs. 4C–4M). In conclusion, the interaction between SIRT5 and β-tubulin may be the primary mechanism by which ZSHX improves mitochondrial homeostasis.

4.7. ZSHX regulates mitophagy and UPRmt synergistically to maintain mitochondrial homeostasis via the SIRT5-β-tubulin axis

Our findings demonstrate that the hypoxia-induced injury model resulted in excessive activation of the UPR^{mt}, while ZSHX inhibited the transcription of UPR^{mt}-related genes (Figs. 5A–5L). However, specific knockdown of SIRT5 and β-tubulin counteracted ZSHX's inhibitory effect on UPR^{mt} overactivity (Figs. 5A–5L). Treatment with Si-SIRT5/ad-β-tubulin or ad-SIRT5/si-β-tubulin enhanced or inhibited UPR^{mt} activation to varying degrees (Figs. 5M–5R). These results suggest that hypoxic stress leads to cytoskeletal protein damage and SIRT5-mediated UPR^{mt} dysfunction. ZSHX appears to regulate the UPR^{mt} through the SIRT5-β-tubulin axis. Additionally, further experiments involving MMP and transmission electron microscopy revealed that si-SIRT5/ad-β-tubulin or ad-SIRT5/si-β-tubulin treatment could block or enhance ZSHX's regulatory effect on MMP and mitochondrial homeostasis. This also affected the transcription levels of FUNDC1 and Drp1 (Fig. 5S–5W). These observations suggest that ZSHX exerts a bidirectional influence on UPR^{mt}-mitophagy through SIRT5-β-tubulin and indirectly impacts the mitochondrial homeostasis mechanism.

The findings also substantiated the crosstalk mechanism between SIRT5 and β-tubulin mediated by ZSHX. Further investigations demonstrated that ZSHX enhanced the expression of LC3-II and TOM20 following hypoxic stress while suppressing the excessive activation of Bax and caspases-3 and -9 and the aberrant opening of the mPTP. Additionally, it augmented mitochondrial biosynthesis (Figs. 6A–6N). Furthermore, ZSHX increased the expression level of LC3II and TOM20 (Figs. 6O-6Q). However, SIRT5^{CKO} blocked the regulatory effects of ZSHX on these mechanisms (Figs. 6O-6Q). However, the administration of si-SIRT5/3MA and ad-SIRT5/si-β-tubulin counteracted the effects of ZSHX on the mitochondrial pathways to apoptosis and dysregulation of mitochondrial quality control (Figs. 6A–6N). These observations indicate that ZSHX can modulate cardiomyocyte homeostasis following hypoxic stress through SIRT5, preserving the integrity and stability of the β-tubulin structure, and regulating the synergistic protective mechanism of mitophagy and UPR^{mt}.

5. Discussion

This study provides experimental evidence for ZSHX's role in ICM and demonstrates the following key findings: 1) ZSHX protects cardiomyocytes from IR injury by regulating mitochondrial quality control, with efficacy comparable to EMPA, which has been shown to reduce major adverse cardiovascular events, cardiovascular death, and myocardial IR injury in numerous clinical and pre-clinical studies [29]. 2) SIRT5-mediated mitochondrial damage plays a critical role in ischemic MI and cardiomyocyte dysfunction, contributing to myocardial fibrosis and cardiac ejection impairment following ischemia. 3) ZSHX regulates β-tubulin stability through SIRT5, thereby promoting the synergistic effects of mitophagy and UPR^{mt}, which inhibit cardiomyocyte apoptosis *via* the mitochondrial pathway. 4) ZSHX modulates mitochondrial quality control *via* interaction between SIRT5 and β-tubulin.

These findings corroborate previous research demonstrating

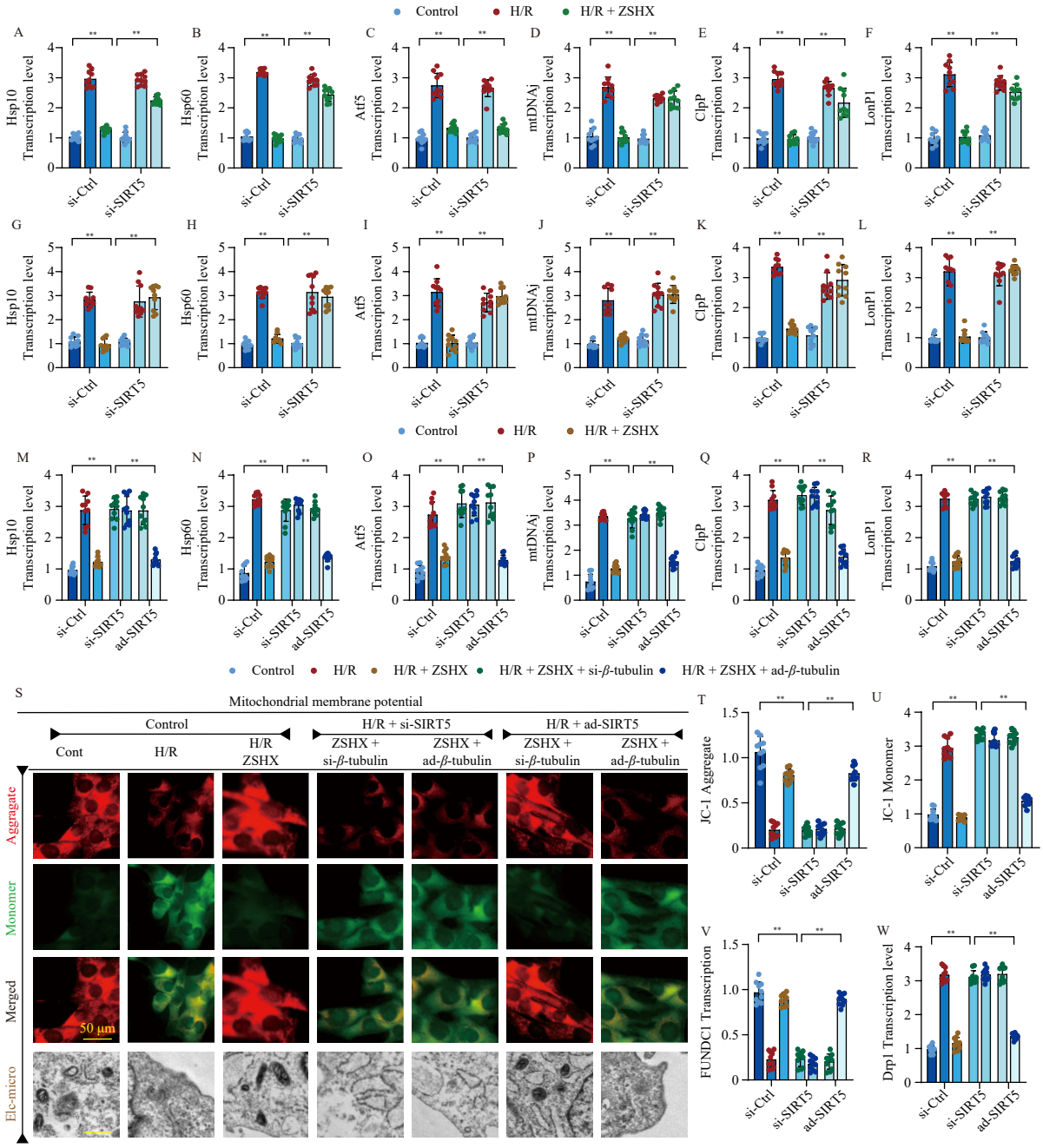

Fig. 5 ZSHX activates FUNDC1 dependency-mitophagy through SIRT5 and inhibits excessive mitochondrial unfolded protein reaction. (A) HSP10 transcriptional level (si-SIRT5). (B) HSP60 transcriptional level (si-SIRT5). (C) ATF5 transcriptional level (si-SIRT5). (D) mtDNAj transcriptional level (si-SIRT5). (E) ClpP transcriptional level (si-SIRT5). (F) Lonp1 transcriptional level (si-SIRT5). (G) HSP10 transcriptional level (si-*β*-tubulin). (H) HSP60 transcriptional level (si-*β*-tubulin). (I) ATF5 transcriptional level (si-*β*-tubulin). (J) mtDNAj transcriptional level (si-*β*-tubulin). (K) ClpP transcriptional level (si-*β*-tubulin). (L) Lonp1 transcriptional level (si-*β*-tubulin). (M) HSP10 transcriptional level (ad/si-SIRT5/ad/si-*β*-tubulin). (N) HSP60 transcriptional level (ad/si-SIRT5 and ad/si-*β*-tubulin). (O) ATF5 transcriptional level (ad/si-SIRT5 and ad/si-*β*-tubulin). (P) mtDNAj transcriptional level (ad/si-SIRT5 and ad/si-*β*-tubulin). (Q) ClpP transcriptional level (ad/si-SIRT5 and ad/si-*β*-tubulin). (R) Lonp1 transcriptional level (ad/si-SIRT5 and ad/si-*β*-tubulin). (S–U) Levels of MMP. (V–W) Transcript levels of FUNDC1/Drp1. Data are presented as mean ± SEM (n = ten independent cell isolations per group). $^{**}P < 0.01$.

the antioxidant, anti-inflammatory, and vasodilatory properties of ZSHX, supporting its potential application as a targeted therapy for ICM. Prior studies have established that ZSHX effectively mitigates ROS and restores the function of the intrinsic antioxidant system, thereby preventing oxidative stress-induced cardiomyocyte injury. Ischemic myocardium initiates a pro-inflammatory response, typically occurring between 6 and 24 h post-ischemia [30], and disrupts overall mitochondrial integrity in cardiomyocytes [31]. To the best of our knowledge, this study represents the first investigation into the mechanisms by which ZSHX regulates mitophagy and the UPR^{mt} to alleviate cardiac ischemic injury.

Mitophagy and the UPR^{mt} collaboratively facilitate the degradation and renewal of mitochondria and proteins, thereby dual-regulating mitochondrial proteins in cardiomyocytes [32]. Diminished mitophagy exacerbates oxidative injury, resulting in ROS accumulation and subsequent cardiac dysfunction. In a previous study, we demonstrated that the active ingredient in ZSHX could enhance cardiomyocyte viability under hypoxic conditions by modulating mitophagy through SIRT1/TMBIM6, thereby ameliorating mitochondrial oxidative stress [11]. In the current study, we further established that ZSHX could activate FUNDC1-related mitophagy through SIRT5-*β*-tubulin crosstalk, thus improving mitochondrial function and suppressing mitochondrial unfolded overreaction, consistent with our previous findings. Based on these research results, we posit that mitophagy exhib-

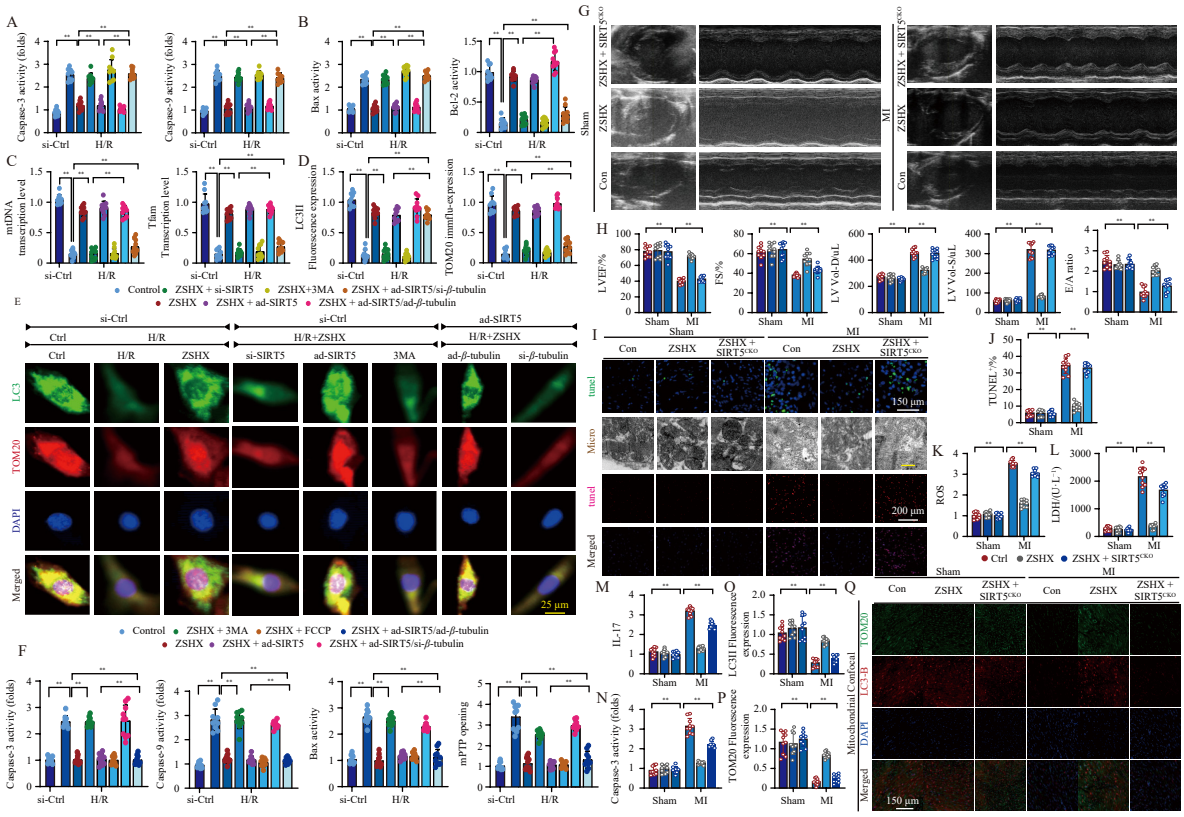

Fig. 6 ZSHX protects mitochondria from damage by regulating mitophagy, mitochondrial biosynthesis and inflammatory response through SIRT5. (A) Caspase-3 and Caspase-9 activities. (B) Bax activity and BcL-2 (B-cell lymphoma-2) activity. (C) mtDNA and Tfam transcription levels. (D) LC3II and TOM20 fluorescence expression levels. (E) LC3II and TOM20 fluorescence. (F) Caspase-3/-9 and Bax activities and mPTP opening levels. (G–H) Echocardiographic analysis of cardiac function: left ventricular ejection fraction (LVEF), left ventricular FS, diastolic left ventricular volume (LV Vold), systolic left ventricular volume (LV Vols) and E/A (mitral flow velocity ratio). (I–J) TUNEL cell apoptosis and myocardial mitochondria transmission electron detection. (K) ROS production level. (L–N) LDH, Caspase-3, and IL-17 expression levels. (O–Q) Laser confocal detection of TOM20 and LC3II fluorescence expression levels. Scale bar, 25 μm. Data are presented as mean ± SEM (n = ten independent cell isolations per group). $^{**}P < 0.01$.

its "double-edged-sword effects" and plays a crucial role in mitigating mitochondrial stress [33]. Both insufficient and excessive mitophagy can disrupt the UPR^{mt}, leading to the perturbation of mitochondrial protein homeostasis [34]. However, mitophagy and protein homeostasis may directly influence the mitochondrial biosynthesis. When mitophagy is over-activated or down-regulated, it is accompanied by dysregulation of mitochondrial protein homeostasis, which in turn leads to insufficient mitochondrial DNA synthesis and impaired ATP supply capacity, subsequently mediating the onset of apoptosis in the mitochondrial pathway [35]. The results suggest that under hypoxic stress conditions, cardiomyocyte mitochondrial content volume cannot be renewed promptly due to dysfunctional mitophagy, necessitating subsequent activation of mitochondrial biosynthesis to promote mitochondrial network regeneration. An overstressed state leading to the suppression of regulatory gene expression levels would further impede mitochondrial regenerative recycling pathways and mediate mitochondrial energy metabolism dysfunction [36]. Conversely, ZSHX can normalize mitochondrial biosynthesis by regulating β-tubulin *via* SIRT5 and maintaining the stability of mitophagy and mitochondrial proteins.

β-Tubulin, a crucial tubulin protein in cardiomyocytes, maintains the dynamic equilibrium of cytoskeletal microtubule distribution and dynamics, directly influencing mitochondrial quality. Previous studies have shown that β-tubulin is regulated by SIRT family proteins and is directly involved in the mechanism of myocardial I/R injury, with increased β-tubulin density enhancing mitochondrial structure and function stability. This aligns with our findings demonstrating that SIRT5 directly affects β-tubulin stability and regulates mitophagy. In hypoxia-induced cardiomyocytes, both SIRT5 and β-tubulin are downregulated, and ZSHX can enhance the protective effects of mitophagy and UPR^{mt} by regulating the interaction between SIRT5 and β-tubulin. These data further revealed that hypoxia could excessively activate the UPR^{mt} and mitophagy, ultimately leading to pathological disturbances in mitochondrial quality control. This includes the disruption of mitochondrial biosynthesis and the mitochondrial respiratory chain. Given that the UPR^{mt} enhances mitochondrial function through a series of stress-protective mechanisms and that mitophagy primarily eliminates severely damaged mitochondria [37], we hypothesize that the UPR^{mt} may precede mitophagy activation. Severely impaired mitochondria are eliminated through mitophagy, and continued mitochondrial damage ultimately leads to programmed cell apoptosis.

Mitochondria are dynamic organelles that undergo continuous fusion and fission in response to various environmental stimuli. This process is essential for mitochondrial biosynthesis and plays a crucial role in regulating mitochondrial energy metabolism and the function of intra- and extracellular ion channels [38]. In the heart, fusion of Mfn1 and Mfn2 or deletion of fission-associated proteins may result in cardiomyocyte damage [39]. Excessive mitochondrial fission can lead to mitochondrial damage, further contributing to mitochondrial energy metabolism dysfunction and increased oxidative stress. Under hypoxic conditions, pathological mitochondrial fission typically increases, while mitochondrial fusion is suppressed [40]. Notably, the normalization of SIRT5-mediated mitophagy and UPR^{mt} inhibited Drp1-mediated excessive mitochondrial fission, enhanced mitochondrial fusion, and restored MMP. These observations align with our previous findings demonstrating that mitophagy activation can protect against mitochondrial stress damage resulting from excessive fission [16]. This suggests that SIRT5-mediated mitophagy and UPR^{mt} can mitigate excessive mitochondrial fission and restore mitochondrial quality control, a process closely associated

with β-tubulin-mediated myocardial cytoskeleton protein homeostasis. Our experimental results further confirmed that ZSHX could regulate the disruption of mitochondrial homeostasis following excessive mitochondrial fission. β-Tubulin facilitates the migration of mitochondrial Drp1 and enhances the expression of mitochondrial fusion proteins, potentially constituting a pivotal mechanism in the regulation of mitochondrial quality. However, when the stability of the SIRT5-β-tubulin interaction is disrupted, the coordinated regulation of ZSHX on UPR^{mt} and mitophagy is impeded. This necessitates further investigation into the SIRT5-β-tubulin interaction mechanism in the context of UPR^{mt}.

SIRT5, a prominent member of the silent information regulator family, regulates various cellular metabolic processes. It also plays diverse biological roles, including countering oxidative stress, regulating cellular homeostasis, and mitigating apoptosis through the mitochondrial pathway [41]. The mechanisms underlying mitochondrial damage and homeostasis are intricately linked with SIRT5. Specific SIRT5 knockout in mouse hearts elevated the levels of succinylated, glutarylated, and malonylated cell membranes and nucleoproteins, while acetylation levels remained unaffected. Further research revealed that SIRT5 catalyzes the deacetylation of K271 and K290 sites on the transcription factor forkhead box O3 (FOXO3), promoting its nuclear translocation and inhibiting cell apoptosis [42]. *In vivo* studies have also demonstrated that SIRT5 deficiency in mice is associated with increased mortality following acute myocardial ischemic injury, a significant decline in cardiac function compared to normal mice, and exacerbated MI.

In contrast, mice overexpressing SIRT5 (SIRT5^{OE}) exhibited reduced ischemic injury, decreased left ventricular dilation, and preserved ejection fraction [43]. These findings indicate that the specific knockdown of SIRT5 disrupts ZSHX's regulation of β-tubulin stability and the synergy between mitophagy and UPR^{mt}. Under hypoxia-induced stress, mitochondrial activation in cardiomyocytes initiates a network-like stress response involving excessive mitochondrial fission [44] and the regulation of deficient mitophagy and mitochondrial biosynthesis. This process ultimately leads to a detrimental cycle of mitophagy and UPR^{mt} reactions [45]. ZSHX targets SIRT5 and β-tubulin to regulate mitophagy and protein homeostasis, thereby maintaining the stability of mitochondrial quality control. These findings enhance our understanding of the molecular basis of myocardial cell mitochondrial damage and identify the SIRT5-β-tubulin axis-regulated mitochondrial quality control as the primary intervention target against myocardial damage.

This study presents several limitations. Firstly, while we elucidated the targeting and modulating effects of ZSHX on the synergistic action of mitophagy and the UPR^{mt}, further research is necessary to investigate its impact on mitochondrial biosynthesis at the molecular level. Secondly, although we observed pathological mitochondrial fission activation during hypoxic stress, the underlying mechanism, including Drp1 migration to mitochondria and its relationship with the mitochondrial fusion protein Mfn1/Mfn2, requires additional examination. Despite SIRT5 and β-tubulin emerging as promising pharmacological targets for ZSHX in ameliorating ischemic MI, the interaction mechanism of pathological mitochondrial fission with mitophagy and UPR^{mt} necessitates further validation. Moreover, animal studies should be conducted to verify the mechanisms of action and regulatory targets associated with SIRT5 and mitochondria using immunohistochemistry and other molecular biology techniques. Finally, a dose-dependent verification for ZSHX remains necessary.

6. Conclusion

In conclusion, our findings demonstrate that ZSHX has the capacity to restore integrity and normalize β-tubulin through SIRT5, thereby alleviating myocardial damage through the synergistic effects of mitophagy and UPR^{mt}. As a targeted therapeutic agent, ZSHX shows promise in advancing the traditional treatment of cardiovascular diseases by modulating mitochondrial homeostasis.

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Supporting Information

Supporting data can be requested by sending E-mail to the corresponding authors.

Declaration of competing interest

These authors have no conflict of interest to declare.

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