

Original Article

Phillyrin protects against myocardial ischemia/reperfusion injury by promoting KNL1 K605 acetylation to inhibit the p53/p21 pathway

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Abstract

Reperfusion therapy is critical for acute myocardial infarction but is often accompanied by myocardial ischemia/reperfusion injury (MIRI). Phillyrin, a natural lignan from *Forsythia suspensa*, exerts anti-inflammatory and antioxidant effects; however, its role and mechanism in MIRI remain unclear. In this study, HL-1 cardiomyocytes are subjected to oxygen-glucose deprivation/reperfusion (OGD/R). Cell viability, apoptosis, oxidative stress, and inflammation are measured after phillyrin treatment. Multiomics (mRNA-seq, proteomics, and acetylproteomics) is used to identify key targets and pathways. Molecular docking, co-immunoprecipitation, site-directed mutagenesis, and western blot analysis are used to validate posttranslational regulation. A mouse MIRI model is established to confirm the *in vivo* cardioprotective effects of phillyrin. Phillyrin preserves cell viability and reduces apoptosis, oxidative stress, and inflammation in OGD/R-injured HL-1 cells. Multiomics integration reveals that phillyrin acts primarily through posttranslational regulation and highlights kinetochore scaffold 1 (KNL1) as the only protein that is both upregulated and hyperacetylated at lysine 605 (K605). Mechanistically, phillyrin may bind to the KNL1 C-terminus and enhance the interaction between KNL1 and acetyltransferase p300/CBP. KNL1 K605R mutation and *Kn1* knockdown reduce KNL1 protein expression and reverse the inhibitory effects of phillyrin on p53 pathway-mediated apoptosis, oxidative stress, and inflammation. In mouse MIRI models, phillyrin reduces infarct size, myocardial damage, and cardiomyocyte apoptosis; these effects are abolished by knockdown of *Kn1*. Therefore, phillyrin promotes KNL1 acetylation at K605 to increase KNL1 protein expression, thereby inhibiting p53 signaling and alleviating apoptosis, oxidative stress, and inflammation in MIRI. This study identifies KNL1 acetylation at K605 as a novel posttranslational modification target for cardioprotection.

Key words myocardial ischemia/reperfusion injury, phillyrin, acetylation, KNL1, p53 signaling pathway

Introduction

Acute myocardial infarction (AMI) is a severe cardiovascular disease that significantly threatens human health. Reperfusion therapy, which rapidly restores coronary blood flow, facilitates the metabolic and functional recovery of cardiomyocytes, and ameliorates the pathophysiological alterations induced by myocardial ischemia, represents the most effective strategy for reducing

ischemic damage and limiting infarct size [1]. However, myocardial ischemia/reperfusion may lead to a range of adverse effects, including myocardial injury, arrhythmias, and inflammatory responses [2–4], which can further exacerbate ischemic damage [5]. To date, although several therapeutic strategies (e.g., beta-blockers and remote ischemic conditioning) have been applied to alleviate myocardial ischemia/reperfusion injury (MIRI) in clinical

practice, their efficacy remains limited by factors such as narrow application scope, potential side effects, or incomplete inhibition of MIRI-related pathological processes [6,7]. Therefore, more effective and safe therapeutic approaches to improve MIRI outcomes are urgently needed.

The pathophysiology and mechanisms underlying MIRI are complex and multifactorial and involve reactive oxygen species (ROS) generation, apoptosis, autophagy, inflammatory responses, mitochondrial dysfunction, and immune reactions [2,8,9]. Although these pathological processes have long been recognized as MIRI drivers, recent advances have highlighted that epigenetic regulation acts as a “master switch” to orchestrate their progression: it not only modulates the development of cardiovascular diseases such as cardiac hypertrophy, hypertension, and heart failure but also plays an indispensable role in MIRI by fine-tuning the expression and function of key regulatory proteins [4,10–12]. This has made epigenetic targeting a highly promising therapeutic strategy for MIRI.

Among diverse epigenetic and posttranslational modifications (PTMs), protein acetylation stands out as a central, dynamically regulated mechanism in cardioprotection against MIRI. Acetylation is a rapid, energy-efficient PTM that governs protein stability, enzymatic activity, and protein–protein interactions and plays critical roles in cell cycle progression, apoptosis, oxidative stress, and inflammation. In MIRI, ischemia/reperfusion disrupts acetyl-CoA homeostasis and acetyltransferase/deacetylase activity, leading to the aberrant acetylation of mitochondrial, cytoskeletal, and signaling proteins. This dysregulation directly promotes cardiomyocyte apoptosis, cell cycle arrest, oxidative stress, and inflammation. For instance, the acetylation of mitochondrial enzymes enhances their antioxidant capacity and metabolic function, thereby mitigating ROS overproduction and mitochondrial dysfunction [13,14]. The acetylation of transcription factors or chromatin regulators can repress the expression of proapoptotic or proinflammatory genes, further alleviating cardiomyocyte death and inflammatory infiltration [11,15]. Despite these insights, the precise molecular targets of acetylation and the mechanisms by which acetylation dynamically coordinates MIRI-related pathways remain incompletely understood, creating a critical gap in the translation of acetylation-based strategies into effective MIRI therapies.

Phillyrin, a natural compound extracted from the medicinal plant *Forsythia suspensa*, is widely used in traditional Chinese medicine for the treatment of cold, fever, and cough [16]. It exhibits a range of pharmacological properties, including anti-inflammatory, antibacterial, antipyretic, and analgesic effects, contributing to its efficacy in alleviating pain and reducing fever [16–18]. Moreover, phillyrin has been applied in the management of various inflammatory conditions, including rheumatoid arthritis, gout, inflammatory bowel disease, and skin inflammation [16,19]. Studies on the role of phillyrin in epigenetic modification are relatively limited, but existing studies have provided some preliminary insights. It has been reported that phillyrin activates adenosine monophosphate-activated protein kinase (AMPK) signaling, which promotes autophagy and inhibits apoptosis [20]. AMPK activation can regulate the activity of various histone-modifying enzymes through phosphorylation, thereby affecting histone acetylation levels [21]. These findings suggest a potential link between phillyrin and the regulation of acetylation.

Given the anti-inflammatory and antioxidant properties of

phillyrin, we hypothesized that phillyrin exerts protective effects against MIRI by modulating epigenetic mechanisms, particularly acetylation. Herein, we aimed to verify the cardioprotective efficacy of phillyrin against MIRI and further elucidate its acetylation-dependent molecular mechanisms. Findings from this work will not only facilitate the development of epigenetic-targeted therapeutics for ischemic myocardial injury but also expand our mechanistic insights into natural product-driven epigenetic modulation in cardiovascular disorders.

Materials and Methods

Cell culture

HL-1 cardiac muscle cell line was purchased from Bohui Biotechnology (Guangzhou, China). Cells were cultured in Claycomb medium (51800C; Sigma-Aldrich, St Louis, USA) supplemented with 10% fetal bovine serum (KGA6008-50; KeyGEN BioTECH, Nanjing, China), 100 μ M norepinephrine (HY-13715; MedChemExpress, Monmouth Junction, USA), 2 mM L-glutamine (HY-N0390; MedChemExpress), and 1% penicillin-streptomycin (HY-K1006; MedChemExpress). Cells were maintained in a humidified incubator at 37°C with 5% CO₂. The culture medium was changed every 24–48 h. Upon reaching 80%–90% confluence, the cells were passaged.

Oxygen-glucose deprivation/reperfusion (OGD/R) model

To establish the OGD/R model in HL-1 cells, the following protocol was used. When the cells reached approximately 70% confluence, the culture medium was replaced with glucose-free and serum-free medium (PM150455; Procell, Wuhan, China). The cells were then transferred to a hypoxic chamber and exposed to a gas mixture of 5% CO₂ and 95% N₂ at 37°C for 4 h to simulate ischemic conditions. After 4 h of hypoxia, the glucose-free and serum-free medium was replaced with complete medium. The cells were returned to normoxic conditions (5% CO₂, 37°C) for a reperfusion period of 12 h.

HL-1 cells were treated with various concentrations (0, 1.562, 3.125, 6.25, 12.5, 25, 50, 100, and 200 μ g/mL) of phillyrin (B20725; Yuanye Bio-Technology, Shanghai, China) for 24 h to assess its safety profile using CCK-8 assay. On the basis of the determined safe concentration range, cells in the logarithmic growth phase were divided into four experimental groups: control, OGD/R, OGD/R + 5 μ g/mL phillyrin, and OGD/R + 50 μ g/mL phillyrin. In accordance with the grouping design, the cells were pretreated with phillyrin for 8 h before being subjected to OGD/R for an additional 16 h.

CCK-8 assay

HL-1 cells were digested, counted, and resuspended at a concentration of 1×10^5 cells/mL. Subsequently, 100 μ L of the cell suspension was added to each well of a 96-well plate, which was then incubated at 37°C in a 5% CO₂ incubator overnight to allow cell adherence. The cells were subsequently treated with phillyrin (5 μ g/mL or 50 μ g/mL) or diltiazem (1 μ M) for 8 h, followed by OGD for 4 h and reoxygenation with glucose for 12 h. A negative control group was also established. Subsequently, 10 μ L of CCK-8 solution (KGA9306-500; KeyGEN BioTECH) was added to each well and incubated at 37°C for 2 h. Finally, the optical density at 450 nm was measured using a microplate reader (SPARK; Tecan, Männedorf, Switzerland), and the cell viability was calculated.

Flow cytometry

For cell cycle analysis, cells were treated according to their respective groups and then collected by digestion with 0.25% trypsin (KGL2102-100; KeyGEN BioTECH). The collected cells were then fixed in 70% ethanol for 2 h. Before staining, the fixed cells were washed with phosphate-buffered saline (PBS; KGL2206-500; KeyGEN BioTECH) to remove the ethanol. A propidium iodide (PI) working solution was prepared by mixing RNase A and PI staining solution at a ratio of 1:9 (KGA9101-50; KeyGEN BioTECH). The cells were stained with this PI working solution for 30 min at 4°C in the dark. The cell cycle was analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Indianapolis, USA) by detecting fluorescence at an excitation (Ex) of 488 nm and an emission (Em) of 620 nm. The cell cycle distribution was determined by analyzing the DNA content of the cells. The percentage of cells in each phase of the cell cycle (G1, S, and G2) was calculated using ModFit LT software (Verity Software House, Topsham, USA).

For cell apoptosis analysis, cells were collected, washed with cold PBS, and resuspended in 1× binding buffer. Annexin V-APC (5 µL) and 7-AAD (5 µL) (KGA1106-100; KeyGEN BioTECH) were added to the cell suspension, followed by incubation in the dark for 15 min at room temperature. Apoptosis was quantified within 1 h using the CytoFLEX flow cytometer. Cells were classified as viable (Annexin V-PI⁻), early apoptotic (Annexin V+PI⁻), late apoptotic (Annexin V+PI⁺), or necrotic (Annexin V-PI⁺). Data were analyzed using FlowJo software.

ROS levels in the cells were detected using a DCFH-DA fluorescent probe (KGA7308-100; KeyGEN BioTECH). The cell suspensions were incubated with 10 µM DCFH-DA at 37°C in the dark for 20 min. The intracellular ROS levels were subsequently measured using the CytoFLEX flow cytometer at an Ex of 488 nm and an Em of 525 nm.

mRNA-seq

mRNA-seq was conducted at KeyGEN BioTECH. mRNA in the cells in the OGD/R and OGD/R + phillyrin groups ($n = 3$) was enriched via RNA extraction and poly-A selection, fragmented, and converted into cDNA. After end repair, 3' end adenylation, and adapter ligation, the cDNA was amplified by PCR. The library quality was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) and RNA 6000 Pico LabChip Kits (Agilent Technologies) and sequenced on the Illumina HiSeq 2500 platform, yielding 100 bp paired-end reads. The sequencing data were quality controlled using FastQC and Trimmomatic to remove low-quality reads and adapter sequences. High-quality reads were aligned with the mouse reference genome, and gene expression levels were quantified using HTSeq. Principal component analysis (PCA) was performed using R (<https://www.r-project.org/>). Differential expression analysis was conducted using DESeq2 (<https://bioconductor.org/packages/release/bioc/html/DESeq2.html>) to identify significantly differentially expressed genes (DEGs). Volcano plots were generated using the R package ggplot2 (<https://ggplot2.tidyverse.org/>). Heatmaps were generated using the R package pheatmap (<https://cran.r-project.org/web/packages/pheatmap/>). Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.bioinformatics.com.cn/>) enrichment analyses were performed to explore the biological functions and pathways associated with these genes. Raw sequence data were deposited in the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>).

[ncbi.nlm.nih.gov/geo/](https://www.ncbi.nlm.nih.gov/geo/)).

Proteomics

Proteomic analysis was performed by PTM BioLab (Hangzhou, China). Briefly, proteins were extracted from the cells in the OGD/R and OGD/R + phillyrin groups ($n = 3$), reduced, alkylated, and trypsin digested. The resulting peptides were separated using liquid chromatography and analyzed by tandem mass spectrometry (LC-MS/MS) on a timsTOF Pro instrument. Raw data were processed using MaxQuant software (version 2.0.3.0) for protein identification and quantification against a relevant UniProt database. Significantly differentially expressed proteins (DEPs) were selected on the basis of a threshold of $|\log_2(\text{fold change})| > 1$ and an adjusted P value < 0.05 . PCA, volcano plots, and heatmaps were generated using R packages. A functional enrichment analysis of the DEPs was conducted using clusterProfiler (version 4.0) for the KEGG pathway analysis. Raw proteomics data were deposited in the iProX database (<https://www.iprox.cn/>).

Integrated transcriptomic and proteomic analysis

All genes and proteins detected separately from mRNA-seq and proteomics data were annotated with unified mouse gene symbols for subsequent cross-omics comparison. Pearson correlation analysis was performed in R to assess the global correlation of expression fold changes for commonly identified molecules between two omics datasets. Venn diagrams were drawn using the R package VennDiagram (<https://cran.r-project.org/web/packages/VennDiagram/>) to visualize the overlapping molecules of the DEGs and DEPs, which were further screened and grouped into different regulatory patterns on the basis of their consistent or inconsistent expression characteristics. Subsequent KEGG pathway enrichment analysis was performed on these coreregulated molecules to characterize key functional pathways. The remaining multiomics diagrams were visualized using R.

Acetylproteomics

Acetylproteomic analysis was conducted in collaboration with Jingjie Biotechnology (Hangzhou, China). Briefly, proteins were extracted from the cells in the OGD/R and OGD/R + phillyrin groups ($n = 3$) and digested with trypsin. Enrichment of the acetylated peptides was performed using an antibody-based affinity purification approach. The enriched peptides were analyzed by LC-MS/MS on a timsTOF Pro instrument. Raw data were processed using MaxQuant software (<https://www.maxquant.org/>) with integrated acetylation settings for identification and site localization. Differentially acetylated proteins/sites were filtered with thresholds of $|\log_2(\text{fold change})| > 1$ and adjusted P value < 0.05 . Functional enrichment analysis of the differentially acetylated proteins was performed for the KEGG pathways using clusterProfiler. Venn diagram analysis was performed to identify overlapping proteins among DEPs and differentially acetylated proteins (acetylation-up and acetylation-down) using the R package VennDiagram. Raw acetylproteomics data were deposited in the iProX database (<https://www.iprox.cn/>).

Sequence conservation analysis

Multi-species sequence alignment was performed using the NCBI COBALT tool (<https://www.ncbi.nlm.nih.gov/tools/cobalt/>) to evaluate the evolutionary conservation of lysine 605 (K605) in KNL1.

Molecular docking

Molecular docking analysis was performed by TargetMol Chemicals (Wellesley Hills, USA) using the Molecular Operating Environment (MOE) software package (<https://www.chemcomp.com>). The three-dimensional structure of the mouse kinetochore scaffold 1 (KNL1) protein (UniProt: Q66JQ7, residues 1915–2119) was obtained from the AlphaFold2 database (<https://alphafold.ebi.ac.uk>). The protein structure was prepared using the QuickPrep module in MOE, including hydrogenation, energy minimization, and removal of water molecules. Potential ligand-binding pockets were predicted using the Site Finder module. The 3D structure of phillyrin (PubChem CID: 135829, <https://pubchem.ncbi.nlm.nih.gov>) was optimized, and a conformational search was conducted using the LowModeMD algorithm. Docking was performed with the Triangle Matcher algorithm, followed by rescoring using the GBVI/WSA ΔG function. Protein-ligand interaction fingerprints (PLIFs) were analyzed to identify key residues involved in the interaction. The optimal binding pose and interaction patterns were visualized using PyMOL software (<https://pymol.org>).

Plasmid construction

The full-length coding sequence of wild-type *Kn1l* was cloned and inserted into the pcDNA3.1-flag vector to generate a *Kn1l*-flag plasmid. For the acetylation mutation assay, the lysine at position 605 was mutated to arginine (K605R), and the mutant sequence was constructed into a pcDNA3.1-flag to generate a pcDNA3.1-*Kn1l*-R605-flag plasmid. All recombinant plasmids were synthesized and verified by KeyGEN BioTECH.

Cell transfection and experimental design

HL-1 cells were transfected with KeygenMAX 3000 transfection reagent (KGA9705; KeyGEN BioTECH) according to the manufacturer's instructions. Briefly, cells were seeded in 6-well plates at a density of 1×10^5 cells per well and cultured until they reached 70% confluence. Next, 7.5 μ L of KeygenMAX 3000 reagent was diluted in 125 μ L of Opti-MEM® I reduced-serum medium and gently mixed at room temperature. For siRNA transfection, 75 pmol of siRNA (three sequences targeting *Kn1l* or negative control, synthesized by KeyGEN BioTECH; sequences listed in [Supplementary Table S1](#)) was diluted in 125 μ L of Opti-MEM® I medium. For pcDNA3.1 plasmid transfection, 2.5 μ g of plasmid was diluted in 125 μ L of Opti-MEM® I, followed by the addition of 5 μ L of Enhancer. The respective nucleic acid mixture was then combined with the transfection reagent mixture (total volume $\sim$ 250 μ L), gently pipetted, and incubated at room temperature for 10–15 min to form transfection complexes. The resulting mixture was added directly into the culture medium of each well in a dropwise manner. After 4–6 h, the transfection medium was replaced with fresh complete MEM. The cells were further incubated at 37°C in a humidified incubator with 5% CO₂ for 48 h before subsequent analyses.

For co-immunoprecipitation (Co-IP) assays, cells were transfected with a *Kn1l*-flag plasmid for 24 h and then treated with phillyrin for another 24 h. The two groups were as follows: (1) *Kn1l*-flag transfection alone and (2) *Kn1l*-flag transfection plus high-concentration phillyrin.

For KNL1 K605 acetylation mutation assays, cells were transfected with wild-type pcDNA3.1-*Kn1l*-flag or mutant pcDNA3.1-*Kn1l*-R605-flag plasmids. After 24 h, the cells were pretreated with 50 μ g/mL phillyrin or 200 μ M kevetrin hydrochloride (HY-16271;

MedChemExpress) for 8 h, followed by OGD/R.

For the phillyrin treatment and knockdown experiments, HL-1 cells in the logarithmic growth phase were transfected with knockdown siRNA (siKn1l) or negative control siRNA (siNC) 24 h before drug pretreatment. Additionally, the cells were pretreated with phillyrin or diltiazem for 8 h, followed by OGD/R exposure for 16 h. HL-1 cells were transfected with siKn1l or siNC. After 32 h of transfection, the cells were subjected to OGD/R injury (4 h OGD + 12 h reperfusion). The p53 inhibitor pifithrin- α (PFT- α) hydrobromide (10 μ M; HY-15484; MedChemExpress) was administered during OGD/R modeling.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted from the cells using TRIzol reagent (KGF5101; KeyGEN BioTECH) following the manufacturer's instructions. The RNA concentration and purity were measured using a UV spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan). cDNA was subsequently synthesized from the extracted RNA using a cDNA first-strand synthesis kit (RR036B; TaKaRa, Kusatsu, Japan). qPCR was performed in a total reaction volume of 20 μ L containing 10 μ L of 2 $\times$ TB Green® Premix Ex Taq™ II (RR820A; TaKaRa), 0.4 μ L of forward primer (10 μ M), 0.4 μ L of reverse primer (10 μ M), 2 μ L of cDNA template, and 7.2 μ L of sterile water. The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s. qPCR was conducted using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, USA), and the fluorescence signals were monitored in real time. The primers used for qPCR were as follows: for *Kn1l*, forward: 5'-AGCCTACA GAAGCCGAAAGT-3' and reverse: 5'-CCTCTCCAAGCCGCTAT TA-3'; for *Gapdh*, forward: 5'-AAGGTCGGTGTGAACGGATT-3' and reverse: 5'-TGAGTGGAGTCATACTGGAACAT-3'. The relative expression levels of the *Kn1l* mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) serving as the reference gene for normalization.

Co-IP

Co-IP was performed to determine KNL1 acetylation and the interaction between KNL1 and p300/CBP. Cells were lysed in IP lysis buffer (KGB5202-50; KeyGEN BioTECH) supplemented with PMSF (KGB5105-10; KeyGEN BioTECH). Equal amounts of protein were incubated with anti-FLAG antibody (details are provided in [Supplementary Table S2](#)) overnight at 4°C, followed by incubation with Protein A/G agarose beads (P2055; Beyotime, Shanghai, China) for 3 h at 4°C. After being washed, the immunocomplexes were eluted with 1 $\times$ SDS loading buffer and boiled for 5 min.

Western blot analysis

Total protein was extracted using a whole-cell lysis assay kit (KGB5303; KeyGEN BioTECH). The protein concentration was determined using a BCA protein assay kit (KGB2101; KeyGEN BioTECH). Equal amounts of protein (20 μ g) were separated by 8%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to PVDF membranes. The membranes were blocked and incubated with primary and secondary antibodies as detailed in [Supplementary Table S2](#). The protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (KGC4601; KeyGEN

BioTECH), and images were captured with a chemiluminescence imaging system (Bio-Rad, Hercules, USA). Grayscale analysis was performed using Gel-Pro32 software.

Immunofluorescence staining

HL-1 cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.2% Triton X-100 for 15 min. The cells were inactivated with 3% H₂O₂-methanol, blocked with 1% BSA, and then incubated with anti-nuclear factor erythroid 2-related factor 2 (Nrf2) primary antibody (1:100; AF0639; Affinity Biosciences, Liyang, China) at 37°C for 2 h, followed by TRITC-conjugated goat anti-rabbit IgG (1:100; KGC6216-0.1; KeyGEN BioTECH) for 2 h in the dark. Nuclei were counterstained with 1 µg/mL DAPI (KGA1807-100; KeyGEN BioTECH) for 5 min. Images were captured using a laser scanning confocal microscope (FV3000; Olympus, Tokyo, Japan) at 600× magnification.

MIRI model

Female C57BL/6 mice (eight-week-old) were obtained from SiPeiFu Biotechnology (Suzhou, China). The mice were kept in the Laboratory Animal Center and housed in CP-4 cages (L × W × H: 290 mm × 178 mm × 160 mm). The number of mice per cage did not exceed 6. The housing environment was maintained at a temperature of 24 ± 2°C, a relative humidity of 40%–60%, and a light-dark cycle for 12 h. All animals had free access to Co60-irradiated maintenance diet (Beijing Keao Xieli Feed, Beijing, China) and autoclaved sterile water. After one week of acclimatization, the mice were randomly divided into five groups: the sham, model, model + diltiazem, model + phillyrin, and model + phillyrin + shKnl1 groups. Animals in the model group were anesthetized by intraperitoneal injection of 100 mg/kg Zoletil 50 (Virbac S.A., Carros, France), fixed in the supine position, and connected to a ventilator for mechanical respiration at a frequency of 60–80 breaths/min, a tidal volume of 10–12 mL/kg, and an inspiration/expiration ratio of 1:1. The left anterior chest hair was removed, and the area was disinfected and draped. A skin incision approximately 1 cm in length was made along the intercostal space between the third and fourth ribs on the left side of the sternum. The subcutaneous tissue and muscle were dissected layer by layer, and the thoracic cavity was entered by spreading the intercostal space. The thymus was gently pushed upward to expose the heart and great vessel roots. The pericardium was torn open, and the heart was carefully extruded by applying slight pressure to the thorax. The left anterior descending coronary artery was visualized near the origin between the left auricle and the pulmonary conus. An atraumatic suture needle was passed underneath the artery at a depth of 0.1 cm and a width of 0.1–0.2 cm, followed by rapid ligation. The heart was then returned to the thoracic cavity. After 40 min of ischemia, the ligature was released to allow reperfusion, thereby establishing MIRI. The mice were euthanized 24 h after reperfusion, after which the myocardial tissues were collected. The sham group animals underwent identical procedures, without coronary ligation. Animals in the model + diltiazem group received daily oral gavage of diltiazem (10 mg/kg; HY-B0632; MedChemExpress) starting one week before modeling until sacrifice at 24 h post-reperfusion. The model + phillyrin group was administered phillyrin (50 mg/kg) via daily gavage one week before ischemia/reperfusion (I/R) induction and continued until sacrifice. The model + phillyrin + shKnl1 group was intraperitoneally injected

with adeno-associated virus serotype 9 (AAV9) carrying shKnl1 (5 × 10¹¹ viral particles per mouse) one week before modeling. Moreover, this group received daily oral phillyrin (50 mg/kg) starting one week before I/R surgery until sacrifice at 24 h post-reperfusion. The shKnl1 virus was constructed by KeyGEN BioTECH.

The animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Ramda Pharmaceutical Co., Ltd (Nanjing, China) (approval number: IACUC-2025030511) and were performed in accordance with the guidelines of the Animal Care Committee.

Triphenyltetrazolium chloride (TTC) staining

After the mice were anesthetized, the hearts were perfused with precooled PBS (KGL2206-500; KeyGEN BioTECH) to remove blood. Once the effluent was cleared, the mice were euthanized, and the hearts were excised. The hearts were then frozen at –20°C for 30–60 min. Each heart was sectioned into 2–3 mm thick slices using a blade. The sections were immersed in 1% TTC solution (KGH4702-100; KeyGEN BioTECH), ensuring complete coverage of the tissue, and incubated at 37°C in the dark for 15–30 min until viable myocardium-stained brick red and infarcted areas appeared pale. After staining, the slices were gently rinsed with PBS to stop the reaction. Images were captured for subsequent analysis.

Hematoxylin and eosin (HE) staining

Myocardial tissues were fixed in 4% paraformaldehyde (KGC3121-100; KeyGEN BioTECH) at 4°C overnight, dehydrated, and embedded in paraffin. Paraffin sections (4 µm thick) were prepared and mounted on glass slides. The sections were deparaffinized in xylene and rehydrated through a graded ethanol series. The sections were then stained with hematoxylin (KGE1201-100; KeyGEN BioTECH) for 10 min, followed by eosin (KGE1205-100; KeyGEN BioTECH) for 30 s, and finally washed in distilled water. Stained sections were dehydrated, cleared in xylene, and mounted with neutral resin. The stained sections were observed under a BX43 microscope (Olympus).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Apoptosis was detected using a TUNEL assay kit (KGA1400-100; KeyGEN BioTECH). Paraffin-embedded myocardial sections were deparaffinized in xylene, rehydrated through a graded ethanol series, and then washed with PBS. The sections were treated with Proteinase K working solution at 37°C for 15–20 min, rinsed with PBS, and incubated with TdT enzyme reaction solution in a humidified dark chamber at 37°C for 1 h. After being washed with PBS, the sections were incubated with streptavidin-HRP at 37°C for 30 min in the dark. Following additional PBS washes, color development was performed using freshly prepared DAB chromogen solution (KGB4101-100; KeyGEN BioTECH). The reaction was stopped under microscopic guidance by rinsing with tap water and distilled water. Counterstaining was carried out with hematoxylin for 10 min, and the sections were then dehydrated through an ethanol series, cleared in xylene, and mounted with neutral balsam. Images were acquired using a light microscope (BX63; Olympus).

Oxidative stress detection

The levels of oxidative stress markers, including ROS, malondial-

dehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD), in the cell and serum samples were measured using the corresponding assay kits (KGA7308, KGA7101, KGA7302, and KGA7305) respectively from KeyGEN BioTECH following the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

To evaluate the changes in the levels of proinflammatory cytokines, tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and the anti-inflammatory cytokine IL-10, ELISAs were performed on both cellular and serum samples. The concentrations of TNF- α , IL-1 β , and IL-10 were measured using the corresponding ELISA kits (KGC1211, KGC1201, and KGC1205, respectively) respectively from KeyGEN BioTECH following the manufacturer's instructions.

Statistical analysis

The data are presented as the mean $\pm$ standard deviation (SD). All experiments were performed with at least three independent biological replicates ($n \geq 3$) to ensure reproducibility. Differences between multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test to correct for multiple testing. A P value < 0.05 was considered to indicate statistical significance.

Results

Phillyrin attenuates OGD/R-induced injury in HL-1 cells

To investigate whether phillyrin protects HL-1 cells against OGD/R-induced injury, we first determined its safe concentration using a CCK-8 assay. Phillyrin at concentrations ≤ 100 $\mu\text{g/mL}$ had no cytotoxic effect on HL-1 cells, with 50 $\mu\text{g/mL}$ having the most favorable effect on cell proliferation (Figure 1A). Therefore, 50 $\mu\text{g/mL}$ was selected as the high concentration, and 5 $\mu\text{g/mL}$ (one-tenth of 50 $\mu\text{g/mL}$) was selected as the low concentration for subsequent experiments. After OGD/R exposure, HL-1 cells exhibited significantly decreased viability and increased apoptosis rates ($P < 0.001$), whereas phillyrin treatment notably increased cell viability and reduced apoptosis in a concentration-dependent manner ($P < 0.05$) (Figure 1B,C). Furthermore, phillyrin suppressed the OGD/R-induced increase in ROS and MDA levels in HL-1 cells in a concentration-dependent manner ($P < 0.001$) (Figure 1D,E). These findings suggest that phillyrin protects HL-1 cells from OGD/R-induced injury by inhibiting apoptosis and oxidative stress while promoting cell proliferation.

Phillyrin mainly improves OGD/R-induced cell damage in HL-1 cells through protein-level regulation

To explore the potential mechanisms underlying the protective

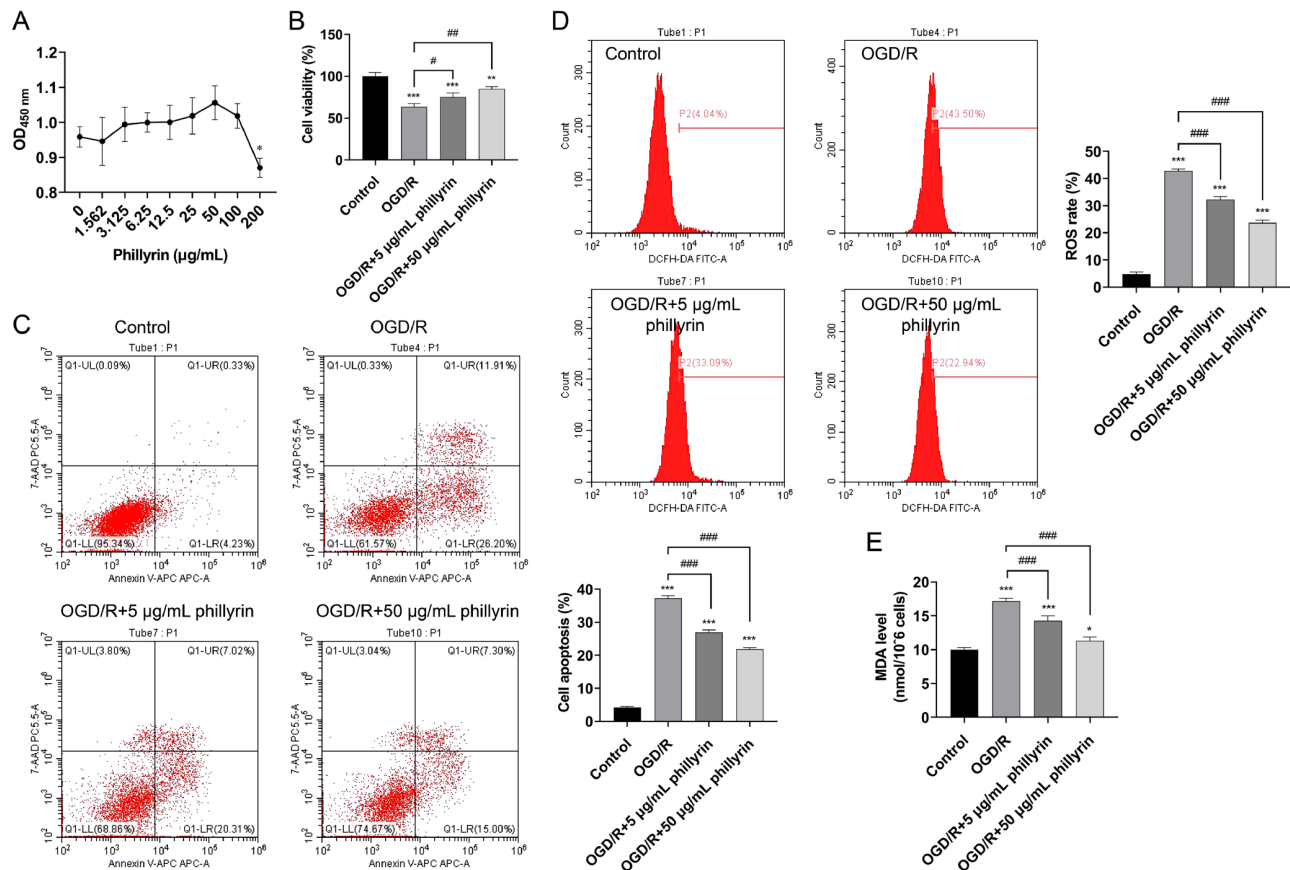


Figure 1. Phillyrin alleviates OGD/R-induced injury in HL-1 cardiomyocytes (A) The viability of HL-1 cells treated with gradient concentrations of phillyrin was determined by CCK-8 assay. (B) Cell viability in the control, OGD/R, OGD/R + 5 $\mu\text{g/mL}$ phillyrin, and OGD/R + 50 $\mu\text{g/mL}$ phillyrin groups was measured by CCK-8 assay. (C) The apoptosis rate was assessed using Annexin V-APC/7-AAD double staining and flow cytometry. (D) Intracellular ROS levels were detected using a DCFH-DA fluorescent probe. (E) Intracellular MDA content was quantified by the corresponding assay kit. The data are presented as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs the control group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$.

effects of phillyrin against OGD/R-induced damage in HL-1 cardiomyocytes, mRNA sequencing and proteomic profiling were performed to analyze the transcriptomic and proteomic expression signatures, as well as the signaling pathways modulated by phillyrin. PCA revealed that phillyrin treatment reshaped the global gene expression profile in OGD/R-challenged HL-1 cells (Figure 2A). Volcano plot and heatmap analyses revealed 73 significantly upregulated and 105 significantly downregulated genes in the phillyrin-treated group compared with those in the OGD/R model group (Figure 2B,C and Supplementary Table S3). KEGG enrichment analysis revealed that these DEGs were predominantly enriched in pathways such as cytokine-cytokine receptor interactions, the cAMP signaling pathway, and the calcium signaling pathway (Figure 2D). Proteomic analysis further revealed 63 significantly upregulated and 94 significantly downregulated proteins in the phillyrin intervention group compared with those in the OGD/R group (Figure 2E–G and Supplementary Table S4). These DEPs were enriched mainly in biological processes and pathways related to the cell cycle and motor proteins (Figure 2H).

Integrated transcriptomic and proteomic analysis revealed a total of 15,522 transcripts and 7430 proteins. Matching of protein IDs and transcript IDs yielded 6806 paired protein-transcript candidates that were coquantified in both the OGD/R group and the phillyrin-treated group (Figure 3A). Pearson correlation analysis revealed an extremely weak correlation between protein expression levels and their corresponding transcript levels (Pearson $R = 0.04$) (Figure 3B). Only two genes, *Ii33* and *Ngfr*, overlapped between the DEGs and DEPs in the phillyrin intervention group (Figure 3C). Collectively, these findings suggest that the cytoprotective effects of phillyrin against OGD/R injury are not dominated by transcriptional changes but are more likely to be controlled by protein-level regulation rather than transcriptional changes.

To dissect the functional characteristics of distinct regulatory patterns, KEGG enrichment analysis was conducted on nine classified protein-transcript expression clusters (Figure 3D). The cytokine-cytokine receptor interaction pathway was enriched specifically in the “transcription-downregulated-protein-downregulated” cluster. The “transcription unchanged-protein downregulated” cluster was significantly enriched in metabolic pathways, autophagy, and O-glycan and N-glycan biosynthesis. No significantly enriched pathways were observed in the “transcription downregulated-protein unchanged” or “transcription upregulated-protein unchanged” clusters. Notably, the “transcription unchanged-protein upregulated” cluster exhibited the most abundant enrichment, including cellular senescence, p53 signaling, cell cycle, and motor protein-related pathways (Figure 3D). These results imply that phillyrin may exert its cardioprotective effects by modulating the translational efficiency, protein stability, or PTMs of key proteins in these pathways.

Phillyrin may promote KNL1 K605 acetylation and protein expression in OGD/R-injured HL-1 cardiomyocytes by enhancing the p300/CBP interaction with KNL1

PTMs are pivotal regulators of acute MIRI because of their rapid response kinetics and low energy consumption. Among these, protein acetylation has emerged as a particularly promising therapeutic target owing to its high dynamicity, reversibility, and druggability. Here, we investigated whether phillyrin ameliorates OGD/R-induced cardiomyocyte injury by modulating protein

acetylation. Quantitative acetylproteomic analysis was performed to profile global protein acetylation changes in OGD/R-injured HL-1 cells with or without phillyrin treatment. A total of 232 differentially acetylated proteins (185 hyperacetylated and 47 hypoacetylated) at 243 distinct lysine residues were identified (Figure 4A,B and Supplementary Table S5), indicating that phillyrin predominantly drives protein hyperacetylation under ischemic stress. KEGG enrichment analysis revealed that these differentially acetylated proteins were significantly enriched in pathways associated with purine metabolism, antigen processing and presentation, and basal transcription factors (Figure 4C).

To determine the key effector protein that mediates the protective effects of phillyrin, the DEPs were compared with the differentially acetylated proteins by Venn diagram analysis. KNL1 was the only protein present in both the upregulated protein set and the hyperacetylated protein set (Figure 4D). Further site localization revealed that phillyrin specifically increased KNL1 acetylation at K605, a residue that is evolutionarily conserved across multiple mammalian species (Figure 4E,F). Western blot analysis confirmed that OGD/R moderately increased KNL1 expression and its panacetylation, effects that were amplified by phillyrin (Figure 4G).

The molecular mechanism underlying phillyrin-induced KNL1 K605 acetylation remains unclear. Two potential scenarios were considered: direct binding of phillyrin to KNL1 to induce conformational changes and subsequent site-specific acetylation or indirect regulation via the modulation of upstream acetyltransferases/deacetylases. Molecular docking analysis was conducted to explore potential direct physical interactions between phillyrin and KNL1. The AlphaFold-predicted structure of the KNL1 C-terminal domain (residues 1915–2119) was processed for hydrogenation and energy minimization, and one major potential small-molecule binding pocket (site 1) was identified on the protein surface (Figure 4H). PLIF analysis of 92 valid docking conformations revealed that phillyrin formed stable interactions with residues Trp1922, Asp1924, Lys1954, and Arg1957, with an optimal docking score of -7.18 (Figure 4I,J). However, the acetylated K605 residue is not located within or adjacent to this predicted C-terminal binding pocket, excluding the possibility that phillyrin directly induces K605 acetylation in KNL1 by binding to this specific site. Given that p300/CBP is the primary acetyltransferase responsible for the lysine acetylation of most cellular proteins, the physical interaction between p300/CBP and KNL1 was examined. Co-IP assays revealed that phillyrin enhanced the interaction between KNL1 and the major acetyltransferase p300/CBP in HL-1 cells (Figure 4K). Collectively, these data demonstrate that phillyrin treatment enhances the interaction between p300/CBP and KNL1, which coincides with elevated KNL1 acetylation at K605 and increased KNL1 protein expression.

Phillyrin ameliorates OGD/R-induced injury in HL-1 cells by regulating KNL1 acetylation at K605 to suppress p53 pathway activation

To investigate whether phillyrin upregulates KNL1 expression through K605 acetylation, a K605R mutation was subsequently introduced to induce KNL1 deacetylation. As shown in Figure 5A, OGD/R exposure increased KNL1 protein expression and acetylation in HL-1 cells, and these effects were further enhanced by phillyrin treatment. Strikingly, the K605R mutation abrogated this phillyrin-induced acetylation, confirming that K605 is the primary acetylation

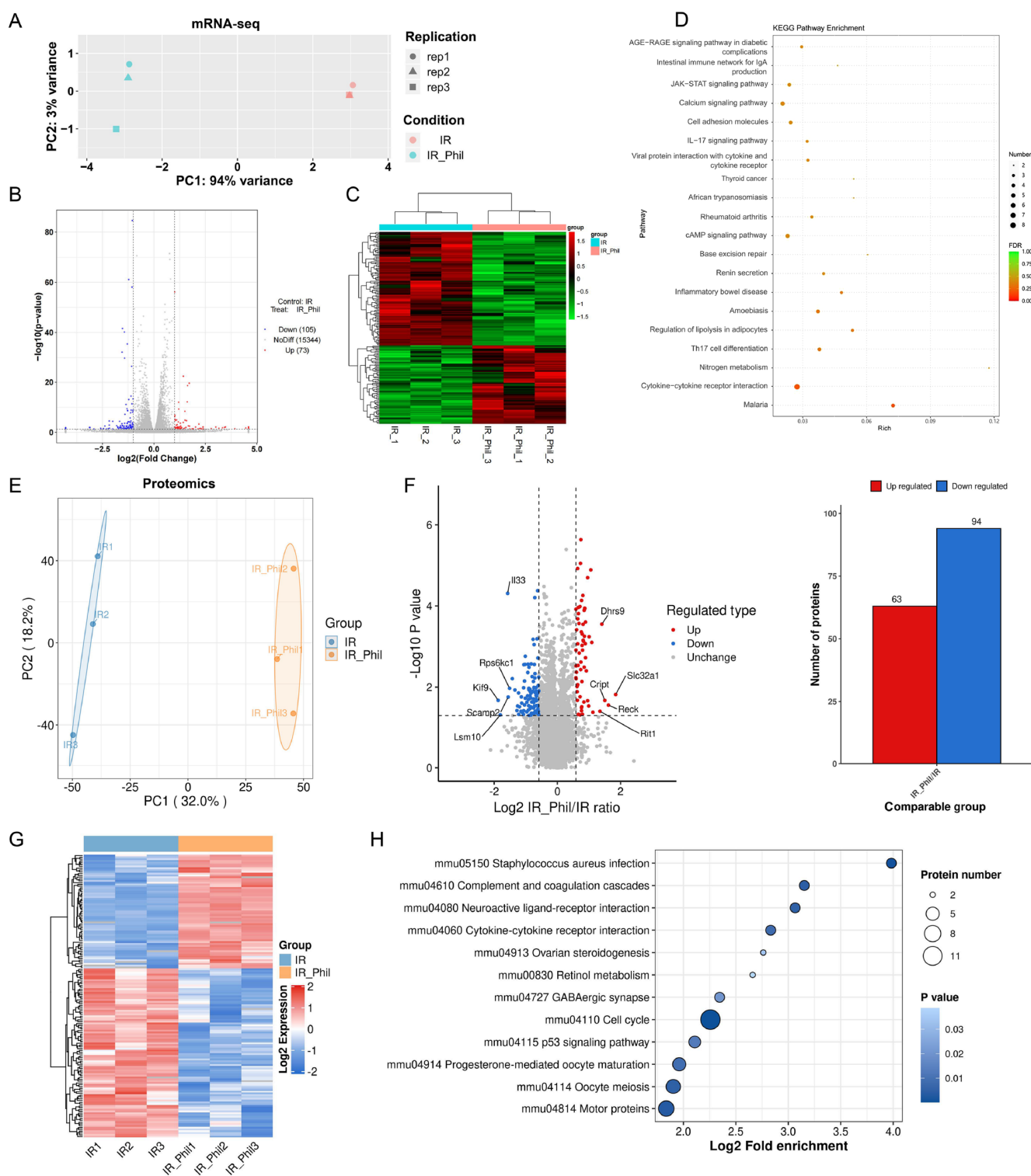


Figure 2. Phillyrin reshapes the transcriptomic and proteomic profiles of OGD/R-injured HL-1 cells (A) Principal component analysis (PCA) of mRNA-seq data between the OGD/R (IR) and OGD/R + phillyrin (IR_Phil) groups ($n = 3$). (B,C) Volcano plot and hierarchical clustering heatmap of differentially expressed genes (DEGs). (D) KEGG pathway enrichment analysis of DEGs. (E) PCA of proteomic data ($n = 3$). (F,G) Volcano plot and hierarchical clustering heatmap of differentially expressed proteins (DEPs). (H) KEGG pathway enrichment analysis of the DEPs.

site targeted by phillyrin. After the KNL1 K605R mutation, the effects of phillyrin on promoting proliferation, cell cycle progression, and antiapoptotic activity in OGD/R-induced HL-1 cells were attenuated (Figure 5B–D). Given that the p53 signaling pathway is a major posttranslational target of phillyrin, we also examined the effects of the p53

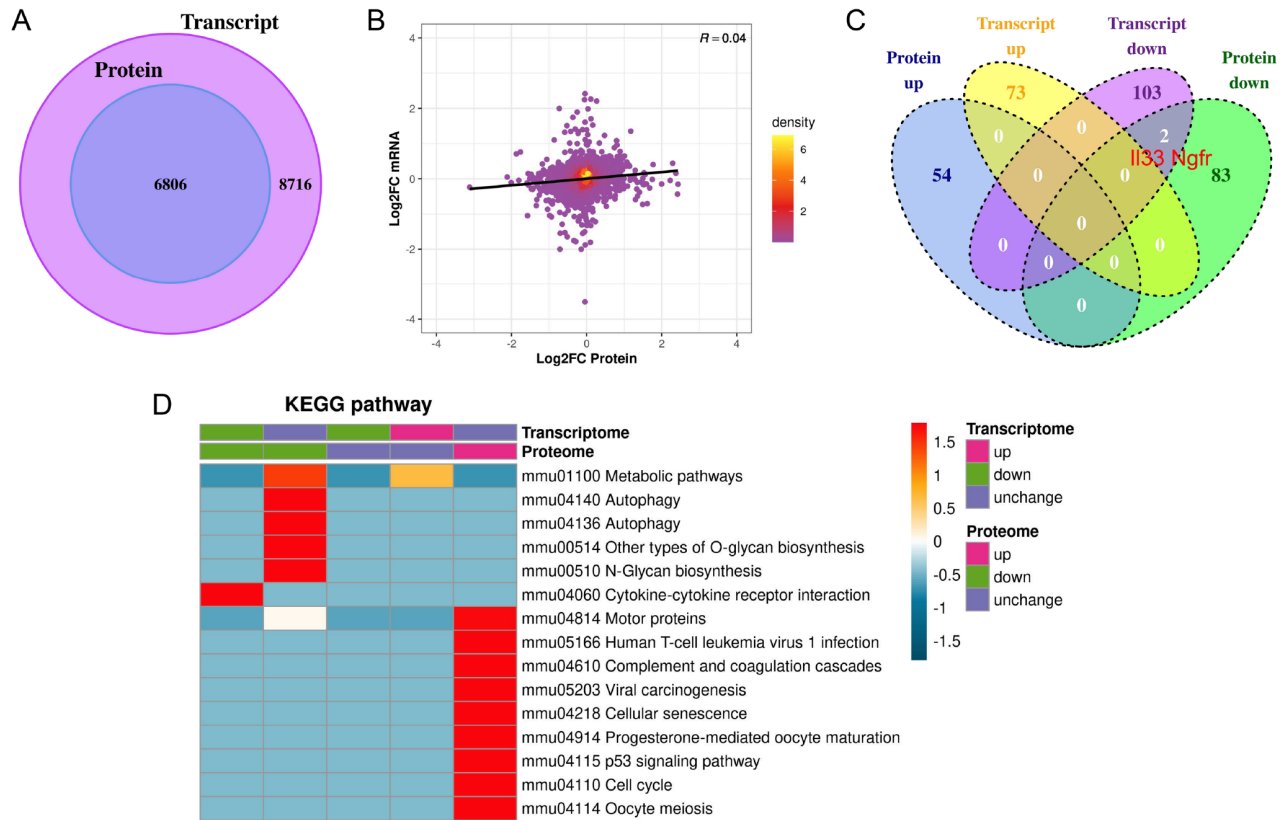


Figure 3. Integrated transcriptomic and proteomic analyses reveal predominant protein-level regulation by phillyrin (A) Venn diagram showing the overlap between quantified transcripts and proteins. (B) Correlation analysis between transcript and protein expression levels (Pearson $R = 0.04$). (C) Venn diagram of overlapping DEGs and DEPs. (D) KEGG pathway enrichment heatmap of proteins classified by transcript-protein expression patterns.

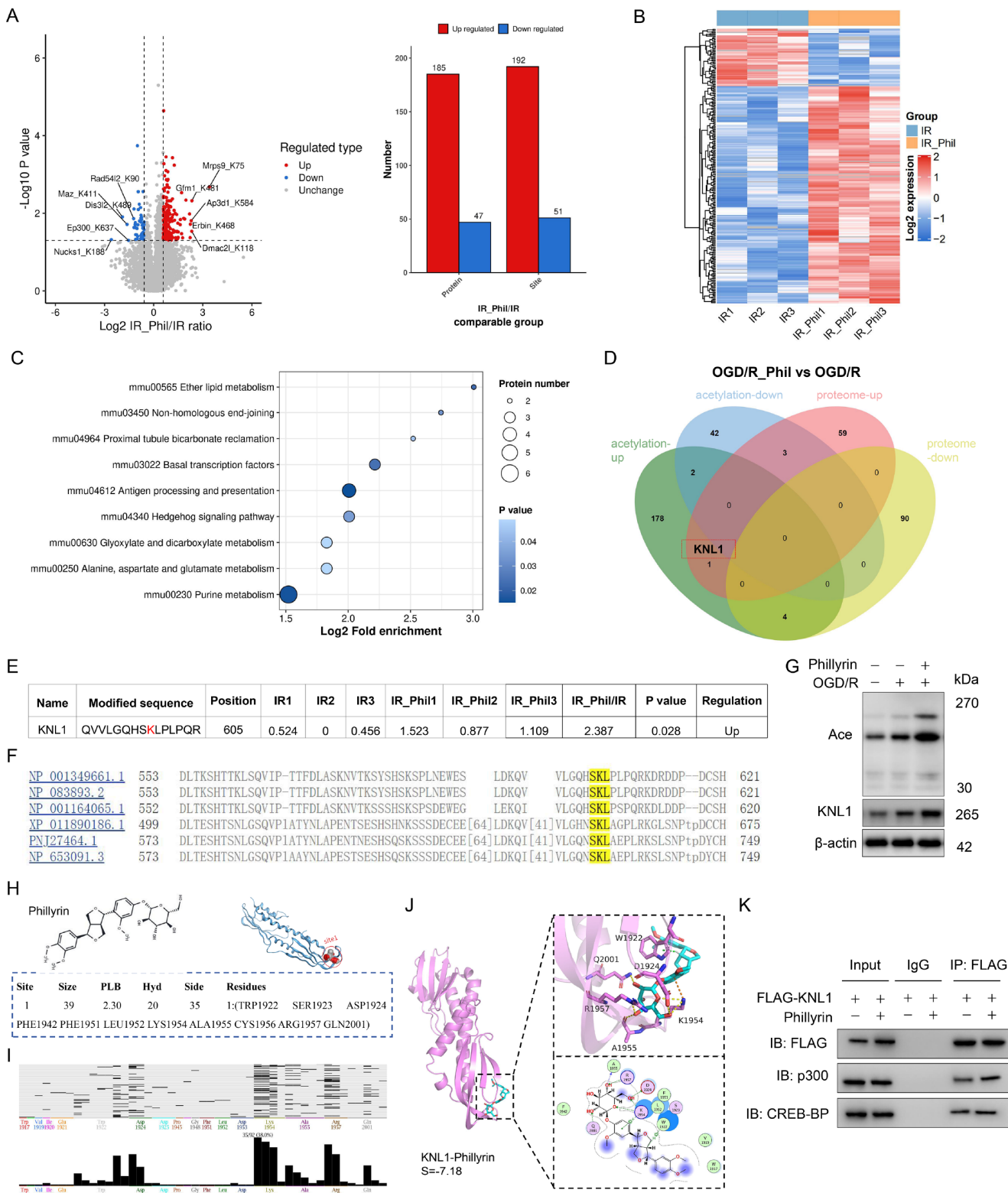
agonist Kevetrin. Notably, compared with K605 (wild-type), the K605R mutation increased the anti-proliferative, cell cycle arrest, and pro-apoptotic effects of the p53 agonist Kevetrin (Figure 5B–D). Furthermore, the K605R mutation reversed the inhibitory effects of phillyrin on oxidative stress, inflammation, and p53/p21 pathway activation in OGD/R-induced HL-1 cells, whereas it increased the pro-oxidative, pro-inflammatory, and p53/p21-activating effects of Kevetrin (Figure 5E,F). Importantly, the K605R mutation abolished the phillyrin-induced increase in global acetylation levels and reduced KNL1 protein expression (Figure 5G and Supplementary Figure S1). These results indicate that phillyrin promotes KNL1 protein expression and inhibits p53 pathway activation in a manner dependent on KNL1 K605 acetylation, thereby alleviating OGD/R-induced injury, oxidative stress, and inflammation in HL-1 cells.

Phillyrin ameliorates OGD/R-induced HL-1 cardiomyocyte injury via the KNL1/p53 axis

To investigate whether phillyrin ameliorates OGD/R-induced injury in HL-1 cardiomyocytes by inhibiting p53/p21 pathway activation through the upregulation of KNL1 expression, HL-1 cells were subjected to OGD/R followed by incubation with phillyrin alone or in combination with *Kn1* siRNA. First, the knockdown efficiency of the three pairs of *Kn1* siRNAs was verified, and the results revealed that compared with the siNC, siKn1#3 had the most potent knockdown effect on *Kn1* gene expression (Supplementary Figure S2). Consequently, siKn1#3 was selected for subsequent combined

treatment with phillyrin in HL-1 cells under OGD/R conditions. The results of functional experiments demonstrated that the protective effects of phillyrin against OGD/R-induced HL-1 cell injury were significantly attenuated by knockdown of *Kn1*. Specifically, the inhibitory effects of phillyrin on OGD/R-induced apoptosis and cell cycle arrest and its ability to promote cell proliferation were reversed by knockdown of *Kn1* (Figure 6A–C). Furthermore, knockdown of *Kn1* abolished the suppressive effects of phillyrin on OGD/R-induced oxidative stress and inflammatory responses in HL-1 cells. Compared with the phillyrin single-treatment group, the *Kn1*-silenced group presented significant increases in the levels of ROS, MDA, TNF- α , and IL-1 β but markedly decreased the levels of SOD, GSH, and IL-10 (Figure 6D,E). Mechanistically, knockdown of *KNL1* significantly reduced the protein expression level of KNL1 and the overall acetylation level in HL-1 cells (Figure 6F and Supplementary Figure S3). Importantly, phillyrin treatment significantly inhibited OGD/R-induced activation of the p53/p21 signaling pathway; however, this inhibitory effect was completely reversed by knockdown of *Kn1* (Figure 6F and Supplementary Figure S3). Notably, the protective effect of phillyrin was comparable to that of diltiazem, a well-recognized positive control drug for MIRI.

To further confirm that the protective effect of KNL1 is mediated by the p53 pathway, we subsequently performed experiments in which *Kn1* was knocked down alone or in combination with PFT- α , a specific p53 inhibitor. *Kn1* knockdown significantly promoted



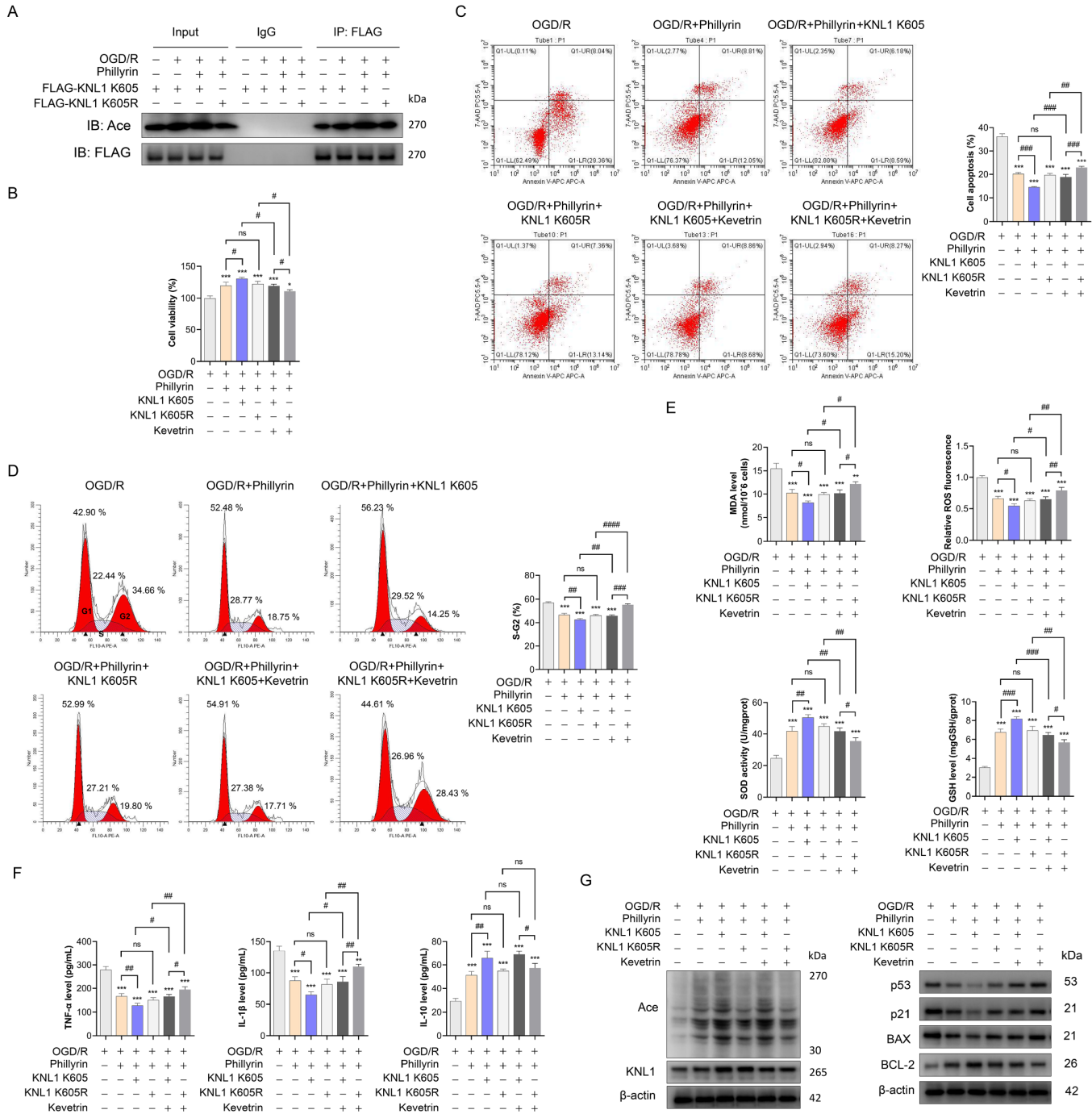


Figure 5. KNL1 K605 acetylation is required for phillyrin-mediated protection against OGD/R injury (A) Co-IP verification of phillyrin-induced KNL1 acetylation at K605. (B) Cell viability detected by CCK-8 assay in the KNL1 wild-type (K605) and mutant (K605R) groups with or without phillyrin/kevetrin. (C) Apoptotic rate measured by Annexin V-APC/7-AAD staining. (D) Cell cycle distribution analyzed by PI staining. (E) Intracellular levels of MDA, ROS, SOD, and GSH. (F) Secretion of inflammatory cytokines (TNF- α , IL-1 β , and IL-10) determined using ELISA. (G) Western blot analysis of p53, p21, BAX, BCL-2, KNL1, and global acetylation. The data are presented as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs the OGD/R group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$. ns, not significant.

OGD/R-induced apoptosis, cell cycle arrest, oxidative stress, and inflammatory responses but inhibited cell proliferation; in contrast, PFT- α treatment had the opposite effects (Figure 7A–J). In addition, OGD/R promoted Nrf2 nuclear translocation, induced heme oxygenase-1 (HO-1) expression, activated the nuclear factor- κ B (NF- κ B) p65 inflammatory pathway, upregulated BAX expression,

and downregulated BCL-2 expression. *Kn1* knockdown further exacerbated OGD/R-induced injury responses while suppressing the compensatory activation of the Nrf2/HO-1 pathway; in contrast, PFT- α , a specific p53 inhibitor, partially reversed the regulatory effect of *Kn1* knockdown on these proteins and pathways (Figure 7K,L). These findings demonstrate that KNL1 is a critical mediator

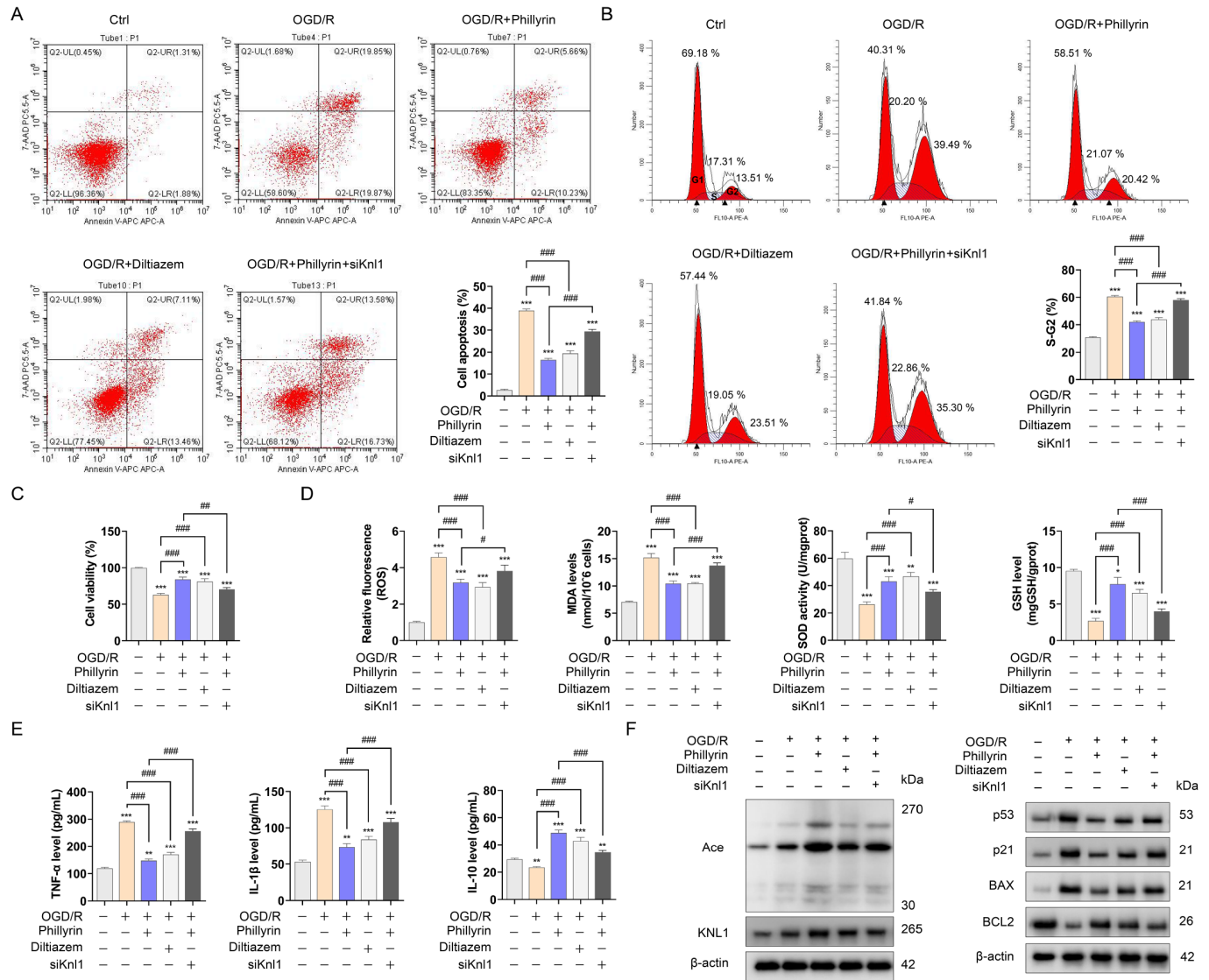


Figure 6. KNL1 knockdown abolishes the cardioprotective effects of phillyrin in OGD/R-injured HL-1 cells (A) Apoptotic rate assessed by Annexin V-APC/7-AAD staining. (B) Cell cycle distribution analyzed by PI staining. (C) Cell viability was measured by CCK-8 assay. (D) Intracellular levels of MDA, ROS, SOD, and GSH. (E) Inflammatory cytokine levels (TNF- α , IL-1 β , and IL-10) were quantified by ELISA. (F) Western blot analysis of p53, p21, BAX, BCL-2, KNL1, and global acetylation. The data are presented as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs the control group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$.

of the protective effects of phillyrin against OGD/R-induced HL-1-induced cardiomyocyte injury and that the KNL1/p53 axis regulates cardiomyocyte survival by modulating the Nrf2/HO-1 oxidative stress axis, NF- κ B p65 inflammatory pathway, p21-dependent cell cycle regulation, and the BAX/BCL-2 apoptotic axis.

Phillyrin ameliorates myocardial injury in a MIRI mouse model via KNL1-mediated suppression of the p53/p21 pathway

As presented in Figure 8A, a mouse MIRI model was established by transthoracic ligation. TTC staining revealed significant myocardial infarction in the model group, whereas diltiazem and phillyrin treatment reduced the infarct size. Notably, knockdown of *Kn1* reversed the protective effect of phillyrin on the infarct area (Figure 8B). HE staining revealed normal myocardial cell structure with clear striations, minimal interstitial hyperemia or edema, and

occasional inflammatory cell infiltration in the Sham group. Conversely, the model group exhibited a loss of striations, significant myocardial degeneration, transparent cytoplasmic changes, and extensive inflammatory infiltration. Both the diltiazem and phillyrin groups exhibited visible striations, attenuated myocardial degeneration, and reduced inflammatory cell infiltration. However, knockdown of *Kn1* resulted in the loss of striations, pronounced degeneration, and marked inflammatory cell infiltration (Figure 8C). Histopathological scoring confirmed that compared with the model group, the diltiazem and phillyrin groups had lower pathological scores, whereas the *Kn1* knockdown group had higher scores (Figure 8C). Compared with the sham group, the model group had more apoptotic cells. Diltiazem and phillyrin treatment reduced I/R-induced cardiomyocyte apoptosis, whereas knockdown of *Kn1* abolished the protective effects of phillyrin (Figure 8D).

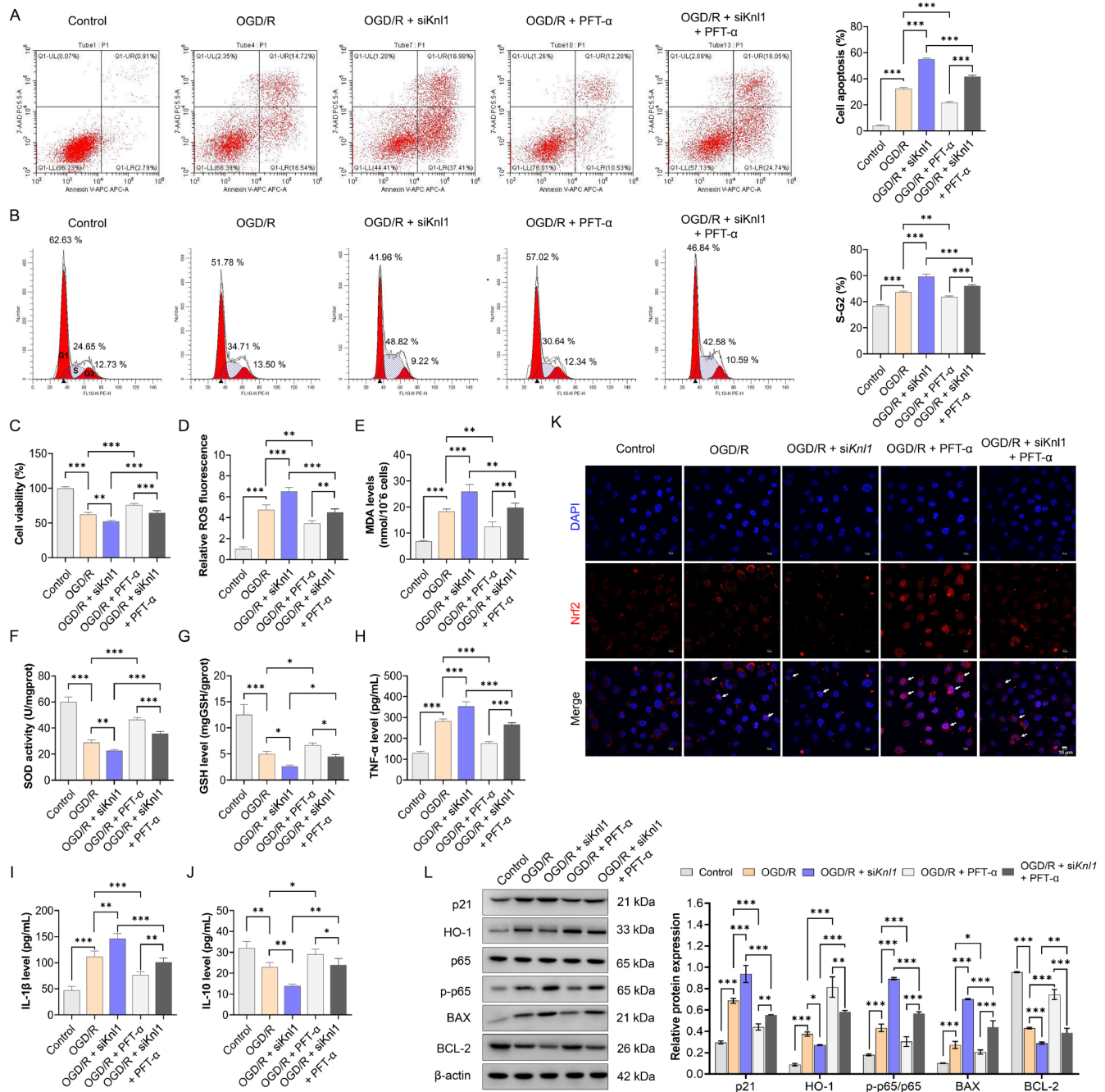


Figure 7. The KNL1/p53 axis regulates OGD/R-induced injury via oxidative stress, inflammation, and apoptosis pathways (A) Apoptotic rate assessed by Annexin V-APC/7-AAD staining. (B) Cell cycle distribution analyzed by PI staining. (C) Cell viability was measured by CCK-8 assay. (D–G) Intracellular levels of MDA, ROS, SOD, and GSH. (H–J) Inflammatory cytokine levels (TNF- α , IL-1 β , and IL-10) were quantified by ELISA. (K) Immunofluorescence staining showing the nuclear translocation of Nrf2 (600 $\times$). (L) Western blot analysis of p21, HO-1, p-p65, p65, BAX, and BCL-2 expression. The data are presented as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs the control group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$.

Further studies revealed that the inhibitory effects of phillyrin on oxidative stress and inflammatory responses, including decreases in the levels of ROS, MDA, TNF- α , and IL-1 β and increases in the levels of SOD, GSH, and IL-10, were reversed by knockdown of *Kn1* (Figure 8E,F). *Kn1* knockdown significantly reduced KNL1 expression and acetylation levels (Figure 8G and Supplementary Figure S4). Phillyrin treatment suppressed the activation of the p53/p21

pathway in the myocardial tissues of model mice, an effect that was reversed by knockdown of *Kn1* (Figure 8G and Supplementary Figure S4).

Discussion

Reperfusion therapy is the first-line clinical strategy for treating AMI; however, its therapeutic benefit is severely compromised by

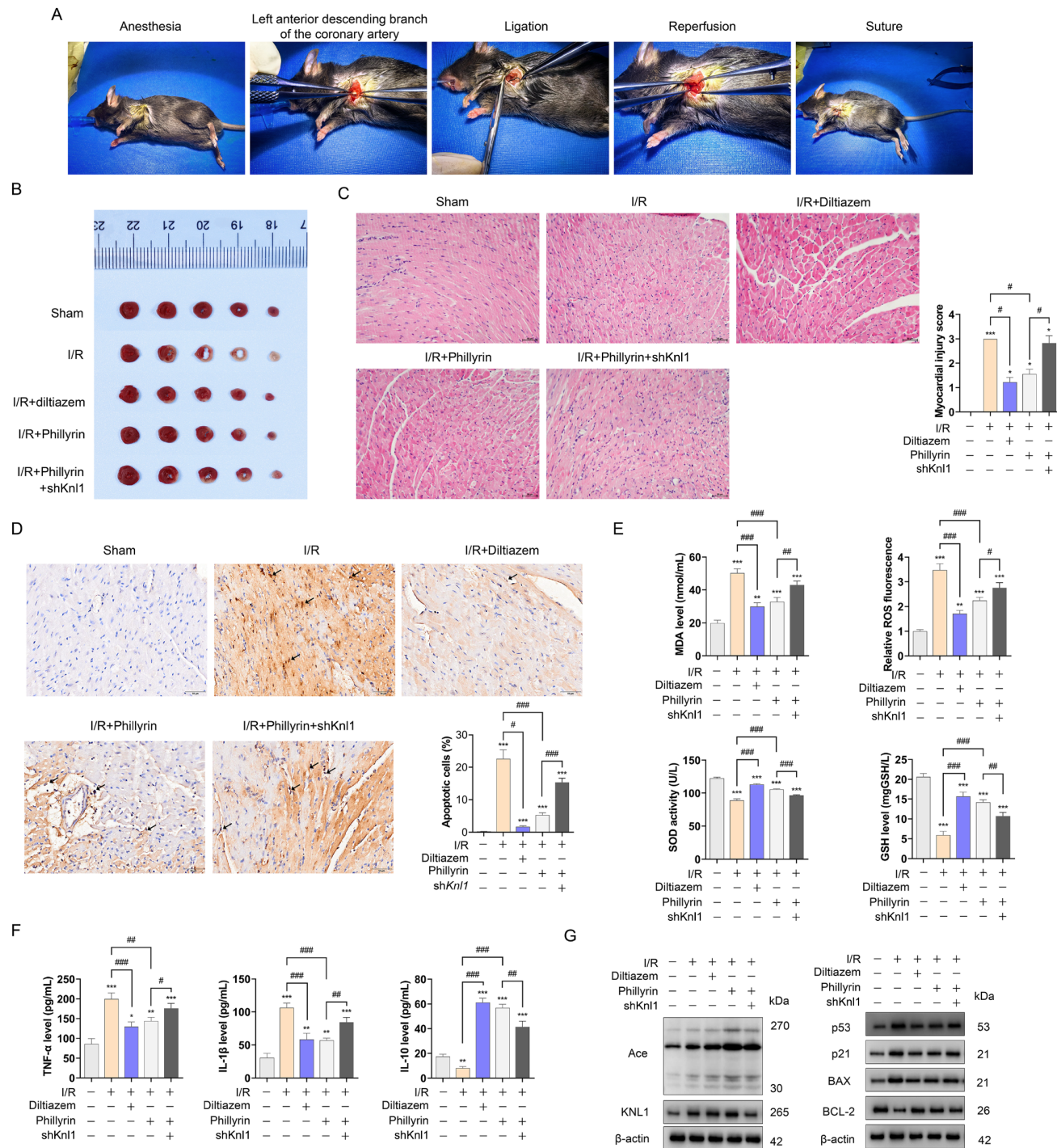


Figure 8. Phillyrin mitigates myocardial ischemia/reperfusion injury in mice via a KNL1-dependent mechanism (A) Schematic illustration of mouse model establishment. (B) Myocardial infarct size evaluated by TTC staining. (C) Myocardial pathological changes were assessed by HE staining. (D) Cardiomyocyte apoptosis detected by TUNEL staining. (E) Serum levels of MDA, ROS, SOD, and GSH. (F) Serum concentrations of TNF- α , IL-1 β , and IL-10 were measured by ELISA. (G) Western blot analysis of p53, p21, BAX, BCL-2, and KNL1 expression in myocardial tissues. Scale bar: 50 μ m. The data are presented as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs the sham group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$.

MIRI, which involves excessive oxidative stress, inflammatory responses, and cardiomyocyte apoptosis. Effective and safe interventions to alleviate MIRI are urgently needed. Phillyrin is a natural lignan extracted from *Forsythia suspensa* with recognized anti-inflammatory and antioxidant properties. In this study, phillyrin significantly improved viability, suppressed apoptosis, and reduced oxidative stress and inflammation in OGD/R-injured HL-1 cardiomyocytes. Integrated transcriptomic, proteomic, and acetylproteomic analyses further revealed that phillyrin predominantly acts through post-translational regulation and revealed KNL1 as the only

protein upregulated and hyperacetylated at K605. Mechanistically, phillyrin may increase KNL1 acetylation at Lys605 via p300/CBP, thereby promoting KNL1 protein expression. This in turn suppresses p53-mediated p21 and NF- κ B p65 signaling while activating the Nrf2/HO-1 pathway, ultimately conferring protection against MIRI. In a mouse model of MIRI, phillyrin notably reduced myocardial infarct size, pathological injury, and cardiomyocyte apoptosis, and these cardioprotective effects were abolished by knockdown of *Kn1* *in vivo*.

Acetylation is a PTM that is vital for regulating protein function, stability, and localization [22,23]. As a rapid and energy-efficient modification, acetylation is suited for the acute stress response during MIRI. It critically governs two core pathological processes in MIRI: cell cycle regulation and apoptosis. Acetylation stabilizes mitotic scaffold proteins to maintain proper chromosome segregation and prevent abnormal cell cycle arrest while also modifying apoptotic regulators, including p53, to suppress excessive cardiomyocyte death. In the context of MIRI, acetylation has been shown to modulate the activity of various proteins involved in oxidative stress, apoptosis, and inflammation [24–26]. For instance, mitochondrial protein acetylation can influence mitochondrial function and ROS production, which are critical factors in MIRI [13]. Our study contributes to the growing body of evidence supporting the importance of acetylation in MIRI by demonstrating that phillyrin enhances the acetylation of KNL1, a protein essential for cell cycle progression and cell division.

KNL1 is a key protein involved in mitosis and plays an essential role in cell division. Previous studies have indicated that KNL1 positively regulates cell cycle progression and cellular proliferation [27]. In a mouse model of myocardial infarction, high expression of KNL1 was closely associated with cardiomyocyte proliferation, cardiac regeneration, and attenuation of myocardial injury [28]. Currently, studies on KNL1 are focused mainly on tumors, mitosis, and other related fields [23,27,29]. Our findings suggest that phillyrin can promote KNL1 protein expression by facilitating its acetylation at K605. This promotes cell cycle progression and proliferation while inhibiting apoptosis and reducing oxidative stress in HL-1 cells. Consistent with these *in vitro* observations, phillyrin exerted comparable cardioprotective effects in a mouse model of MIRI through the upregulation of KNL1. This KNL1-dependent mechanism underlies the cardioprotective action of phillyrin against MIRI in both cellular and animal models.

However, a closer look at the molecular details reveals an interesting puzzle. Although molecular docking indicated that phillyrin can bind to the C-terminal region of KNL1, the K605 acetylation site lies outside this predicted binding pocket. Therefore, phillyrin is unlikely to directly induce K605 acetylation through steric occlusion or catalytic activation. Whether phillyrin binding instead induces conformational changes in KNL1 that facilitate p300/CBP engagement remains to be tested by future structural studies, such as cryo-EM or hydrogen-deuterium exchange mass spectrometry. Currently, our data support a model in which phillyrin indirectly regulates KNL1 K605 acetylation by enhancing the protein-protein interaction between KNL1 and p300/CBP rather than by directly targeting the acetylation site or acting as a conventional acetyltransferase activator.

The p53 signaling pathway is a master regulator of cell cycle arrest and apoptosis [30–32]. Sustained p53/p21 signaling pathway activation promotes cardiomyocyte cycle arrest and apoptosis,

thereby exacerbating MIRI [33,34]. Moreover, p53 signaling also interacts with oxidative stress and inflammatory pathways, including Nrf2/HO-1 and NF- κ B p65, to collectively modulate cardiomyocyte survival. Our study demonstrated that phillyrin markedly suppressed the activation of the p53/p21 pathway in OGD/R-injured HL-1 cells, and these effects were significantly reversed by knockdown of this gene. Further mechanistic experiments revealed that knockdown of *Kn1* promoted apoptosis, oxidative stress, and inflammation, whereas these detrimental effects were partially abrogated by the p53 inhibitor PFT- α , which restored Nrf2/HO-1 antioxidant signaling and suppressed NF- κ B p65 inflammatory activity. These results indicate that the cardioprotective action of phillyrin is mediated through a KNL1-dependent mechanism that modulates p53 signaling to coordinately inhibit apoptosis, oxidative stress, and inflammatory responses during MIRI.

Although our study reveals a novel epigenetic mechanism underlying the cardioprotective effects of phillyrin against MIRI, several limitations must be addressed. First, although KNL1 was identified as a key effector, the specific intermediate molecules that connect KNL1 to the p53/p21 signaling pathway remain to be identified, and the complete regulatory cascade requires further validation. Second, although AAV9 exhibited favorable cardiac tropism and cardiac *Kn1* knockdown was confirmed by western blot analysis both *in vitro* and *in vivo*, the strict cardiomyocyte specificity of gene intervention has not been fully verified. Third, the long-term safety, optimal dose, and pharmacodynamic properties of phillyrin for myocardial protection require further evaluation. Future studies will focus on elucidating the detailed KNL1-p53 network, determining the long-term safety and appropriate dosage of phillyrin, extending our findings to large animal models, and using cardiomyocyte-specific conditional knockout or cTnT-driven AAV9 systems to confirm the cardiac-specific role of KNL1.

In conclusion, our study demonstrated that phillyrin exerts potent cardioprotective effects against MIRI by increasing KNL1 acetylation at K605 to increase KNL1 protein expression, thereby suppressing p53 signaling pathway-mediated apoptosis, oxidative stress, and inflammation. These findings identify phillyrin as a promising candidate for the prevention and treatment of MIRI and highlight KNL1 acetylation at K605 as a novel PTM target for cardioprotection.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

Data Availability

The raw RNA sequencing data generated in this study have been deposited in the GEO database under accession number GSE333299. The raw proteomics and acetylproteomics data have been deposited in the iProX database under project ID IPX0017418000. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

Dianyuan Li conceived and designed the overall research framework, supervised the entire study, and revised the manuscript critically. Dongsheng He, Zhipeng Ren, and Shangxuan Li performed the majority of the experiments, collected and analyzed

the experimental data, and drafted the initial manuscript. Ziqiang Dai, Gen Zhang, Huan Wang, and Guanzheng Cui assisted with cell culture, animal modeling, molecular biology experiments, and data processing. All the authors contributed to the interpretation of the data, revised the manuscript for important intellectual content, and approved the final submitted version.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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