

Research Article

Rhoifolin Attenuates Ferroptosis in Renal Tubular Epithelial Cells in Diabetic Nephropathy by Targeting CDKN1A to Modulate the CDK2/E2F1 Pathway

Zhengyu Zhang¹, Jing Wu¹, Xinyi Chen¹, Peng Zheng¹, Jingjing Liu^{2,*} 

¹Department of Endocrinology, Lishui Hospital of Traditional Chinese Medicine, 323000 Lishui, Zhejiang, China

²Department of Endocrinology, The Third Affiliated Hospital of Zhejiang Chinese Medical University, 310005 Hangzhou, Zhejiang, China

*Correspondence: liujingjing34@163.com (Jingjing Liu)

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Abstract

Background: Current therapeutic strategies for Diabetic nephropathy (DN) remain limited, underscoring the urgent need to identify novel pharmacological agents and therapeutic targets. **Methods:** Network pharmacology analysis was conducted to identify key genes as principal target genes of Rhoifolin (ROF) in treating DN. A mouse model of DN was established using streptozotocin induction combined with a high-fat diet. Mice were randomly allocated into six groups: Control, DN, DN+ROF-low (L), DN+ROF-medium (M), DN+ROF-high (H), and DN+metformin (MET). *In vitro*, a high glucose (HG)-induced injury model was established in HK-2 cells. Cells were assigned to the normal control (NC), HG, HG+ROF-L, HG+ROF-M, HG+ROF-H, HG+ROF-H+overexpression negative control (oe-NC), HG+ROF-H+oe-cyclin dependent kinase inhibitor 1A (CDKN1A), HG+ROF-H+oe-CDKN1A+oe-NC, HG+ROF-H+oe-CDKN1A+oe-cyclin-dependent kinase 2 (CDK2), and HG+ROF-H+oe-CDKN1A+ferrostatin-1 (Fer-1) groups. Fasting blood glucose (FBG), body weight, and urinary albumin-to-creatinine ratio were measured; histopathological alterations were evaluated using periodic acid-schiff and hematoxylin-eosin staining; glutathione and malondialdehyde levels were quantified using corresponding detection kits; reactive oxygen species levels were assessed using dihydroethidium fluorescent probes; and the protein expression levels of CDKN1A, glutathione peroxidase 4 (GPX4), Acyl-CoA synthetase long chain family member 4 (ACSL4), CDK2, E2F transcription factor 1 (E2F1) were analyzed by western blot. Cell viability was determined using CCK-8; cell death was quantified by flow cytometry; levels of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6) were determined using enzyme-linked immunosorbent assay; and Fe²⁺ concentrations were measured with fluorescent probes. **Results:** ROF treatment significantly alleviated renal injury in mice with DN, enhanced the viability of HK-2 cells, activated the CDK2/E2F1 pathway, and inhibited ferroptosis. Overexpression of CDKN1A in the HG+ROF-H group resulted in decreased cell viability, increased cell death, elevated secretion of TNF- α , IL-1 β , IL-6, enhanced ferroptosis, and suppression of the CDK2/E2F1 pathway. Administration of the ferroptosis inhibitor Fer-1 in the HG+ROF-H+oe-CDKN1A group attenuated CDKN1A-induced cellular injury. Furthermore, overexpression of CDK2 in the HG+ROF-H+oe-CDKN1A group inhibited ferroptosis and mitigated the cellular injury caused by CDKN1A overexpression. **Conclusion:** ROF mitigates the progression of DN by targeting CDKN1A and modulating CDK2/E2F1 pathway-mediated ferroptosis in HK-2 cells.

Keywords: diabetic nephropathy; ferroptosis; network pharmacology

1. Introduction

Diabetic nephropathy (DN) is characterized by a progressive decline in glomerular filtration function and persistent proteinuria, with its pathogenesis closely linked to inflammatory responses, metabolic dysregulation, oxidative stress, and hemodynamic abnormalities [1]. Current therapeutic strategies for DN primarily focus on the management of major risk factors, including hyperglycemia, hypertension, and proteinuria, with the aim of alleviating symptoms and delaying disease progression [2]. In clinical practice, first-line treatment typically involves inhibitors of the renin-angiotensin system, including agents such as dapagliflozin [3]. These medications exert renoprotective effects largely by reducing sodium and glucose reabsorption in the proximal tubules, thereby promoting natriuresis, lowering intraglomerular pressure, attenuating glomerular

hyperfiltration, and ultimately slowing the decline in renal function [4]. Nonetheless, despite these therapeutic advances, the efficacy of current interventions remains limited, and their use may be accompanied by adverse effects. Consequently, there is an urgent need to further elucidate the pathophysiological mechanisms underlying DN and to identify novel therapeutic strategies.

Traditional Chinese medicine has recently shown promise for multi-target intervention in DN. Rhoifolin (ROF), a flavonoid glycoside abundantly found in citrus fruits [5], possesses notable pharmacological properties. Accumulating evidence has demonstrated its anti-tumor efficacy against multiple malignancies, such as pancreatic cancer [6], breast cancer [7], and hepatocellular carcinoma [8]. Moreover, recent studies indicate that ROF attenuates diabetes-induced hepatic injury through mechanisms



such as lowering blood sugar and lipid levels, as well as providing antioxidant and anti-inflammatory effects [9,10]. However, its potential role in DN management has not yet been explored. The present investigation aimed to identify ROF's putative pharmacological targets through network pharmacology and to validate its mechanisms of action in cellular models, with an attempt to provide a robust theoretical foundation for the potential clinical application of ROF in the treatment of DN.

2. Materials and Methods

2.1 Screening of Differentially Expressed Genes (DEGs)

The DN-related dataset GSE228960, comprising 11 db/m and 17 db/db mice, was obtained from the Gene Expression Omnibus database. Differential expression analysis was conducted using the limma package (v 3.60.3, <https://bioinf.wehi.edu.au/limma/>), with thresholds of false discovery rate <0.05 and $|\log_2\text{FoldChange}| >0.5$. Functional annotation and pathway enrichment analyses of the identified DEGs were subsequently performed using the ClusterProfiler package (v 4.12.0, <https://yulab-smu.top/biomedical-knowledge-mining-book/>), encompassing Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology-Biological Process (GO-BP) analyses. The terms were visualized with the use of ggplot2 (v 3.5.1, <https://ggplot2.tidyverse.org/>).

2.2 Prediction of Target Genes for ROF in Treating DN

Several online platforms were employed to predict the potential therapeutic targets of ROF and to identify genes associated with DN. The potential therapeutic targets of ROF were predicted via SwissTargetPrediction (<http://swisstargetprediction.ch/>), TargetNet (<http://targetnet.scbdd.com/home/download/>), the Comparative Toxicogenomics Database (<https://ctdbase.org/>), and PharmMapper (<https://www.lilab-ecust.cn/pharmmapper/>). Simultaneously, DN-related target genes were retrieved from MalaCards (<https://www.malacards.org/>), Disease Gene Network (DisGeNET, <https://disgenet.com/>), the Online Mendelian Inheritance in Man (OMIM, <https://www.omim.org/>) database. Finally, a Venn diagram was plotted to obtain overlapping genes among the DEGs, predicted target genes of ROF, and DN-related target genes, which were defined as candidate ROF-DN target genes.

2.3 Machine Learning (ML) Algorithms

To identify genes playing critical roles in DN progression from the ROF-DN target genes, three ML algorithms were applied to the GSE228960 dataset. Specifically, the Least Absolute Shrinkage and Selection Operator (LASSO) analysis was performed using the glmnet package (v 4.1-4), the random forest analysis was carried out with the randomForest package (v 4.7.1-1, <https://CRAN.R-project.org/package=randomForest>), the Support Vector Machine-Recursive Feature Elimination (SVM-RFE)

was implemented via the e1071 package (v 1.7-14, <https://CRAN.R-project.org/package=e1071>).

2.4 Protein-Protein Interaction (PPI) Network Analysis

The candidate ROF-DN target genes were imported into the STRING database (<https://string-db.org/>) to obtain protein-interaction data, which were subsequently visualized in Cytoscape (v 3.10.3, <https://cytoscape.org/>). The cytoHubba plugin (v 0.1, <https://apps.cytoscape.org/apps/cytohubba>) was used to identify the top 20 hub genes based on the data management and neural computing algorithm.

2.5 Molecular Docking

The three-dimensional structures of the hub gene-encoded protein receptors were downloaded from the Protein Data Bank database (<https://www.rcsb.org/>), while those of the ligand molecule ROF were retrieved from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). The ligand and receptor structures were imported into AutoDockTools (v 1.5.7, <https://ccsb.scripps.edu/autodock-suite/adt/>) for preprocessing. During this process, water molecules, metal ions, and endogenous ligands were removed from the receptor structures; hydrogen atoms were added to the ligand molecule; and charges were calculated. Semi-flexible docking mode was selected. The coordinates and dimensions of the docking grid box were determined using PyMOL (v 2.5.0, <https://pymol.org/>), and a genetic algorithm was applied to search for optimal binding conformations. The final protein-ligand binding conformations were visualized via PyMOL (v 2.5.0, <https://pymol.org/>) and Discovery Studio (v 4.5, Dassault Systèmes, MD, USA).

2.6 Animal Grouping and Treatment

36 male C57BL/6J mice (20 ± 2 g) were housed under controlled environmental conditions (relative humidity of $50 \pm 10\%$, a 12-h light/dark cycle, temperature of 25 ± 2 °C), and provided with food and water *ad libitum*. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang Center of Laboratory Animals (Approval No. ZJCLA-IACUC-20020245). A mouse model of DN was established using a high-fat diet (60% of calories from fat, D12492, Research Diets, NJ, USA) combined with streptozotocin (STZ, S1312, Selleck Chemicals, TX, USA). After a one-week acclimatization period, mice were randomly assigned to six groups using the random number table method: Control, DN, DN+ROF-low (L), DN+ROF-medium (M), DN+ROF-high (H), and DN+metformin (MET, S5958, Selleck Chemicals, TX, USA). Mice in the Control group were fed with standard chow, whereas those in the remaining groups were maintained on a high-fat diet for six weeks. Following a 12-h fast (with free access to water), mice in all groups except the Control group received intraperitoneal injections of 50 mg/kg STZ once daily for five consecutive

days. At 72 h and one week after the final STZ injection, mice were fasted for 12 h, and fasting blood glucose (FBG) levels were measured using tail vein blood samples. Mice with consecutive FBG levels ≥ 16.7 mmol/L were included for further study. ROF was dissolved in dimethyl sulfoxide (Beyotime, ST038, Shanghai, China) and prepared at the required concentrations for administration. Mice in the DN+ROF-L, DN+ROF-M, and DN+ROF-H groups received ROF daily via oral gavage at 10 mg/kg, 20 mg/kg, and 40 mg/kg, respectively [11]. Mice in the DN+MET group received MET (150 mg/kg) by oral gavage. Mice in the Control and DN groups were administered equal volumes of normal saline. All treatments were administered once daily for eight weeks. After the experiment, mice were euthanized using CO₂ and cervical dislocation. The specific operation is as follows: the mice were placed inside a transparent euthanasia chamber pre-filled with CO₂ at a concentration of 30%. CO₂ was then continuously infused at a flow rate of 10%–30% of the chamber volume per minute. After cessation of respiration and loss of consciousness, the CO₂ flow was maintained for an additional 1–2 min, and death was subsequently confirmed by cervical dislocation. XC divided the mice into groups, JW prepared the models and conducted drug intervention on each group of mice, ZZ conducted the detection of various experimental indicators, and ZZ and PZ processed and analyzed the experimental result data.

2.7 Cell Grouping

The human renal tubular epithelial cell line HK-2 (Procell, CL-0109, Wuhan, China) was cultured in MEM medium (Procell, PM150410, Wuhan, China) and maintained at 37 °C in an incubator containing 5% CO₂. HK-2 cells were assigned to ten groups: normal control (NC), high glucose (HG), HG+ROF-L, HG+ROF-M, HG+ROF-H, HG+ROF-H+overexpression negative control (oe-NC), HG+ROF-H+oe-cyclin dependent kinase inhibitor 1A (CDKN1A), HG+ROF-H+oe-CDKN1A+oe-NC, HG+ROF-H+oe-CDKN1A+oe-cyclin-dependent kinase 2 (CDK2), and HG+ROF-H+oe-CDKN1A+ferrostatin-1 (Fer-1, S7243, Selleck Chemicals, TX, USA). Cells in the NC group were cultured in medium containing glucose (5.5 mmol/L), whereas those in all other groups were cultured in medium containing glucose (30 mmol/L). ROF was administered to cells in the HG+ROF-L, HG+ROF-M, and HG+ROF-H groups at concentrations of 12.5 µg/mL, 25 µg/mL, and 50 µg/mL, respectively, followed by continued incubation for 48 h. Cells in the HG+ROF-H+oe-CDKN1A group and the HG+ROF-H+oe-NC group were transfected with the CDKN1A overexpression vector and the corresponding control vector, respectively, using Lipofectamine 3000 transfection reagent for 6 h. Cells in the HG+ROF-H+oe-CDKN1A+oe-CDK2 group and HG+ROF-H+oe-CDKN1A+oe-NC group were transfected with the CDK2 overexpression vector and the corre-

sponding control vector, respectively, using Lipofectamine 3000 transfection reagent for 6 h. Cells in the HG+ROF-H+oe-CDKN1A+Fer-1 group were treated with 100 µM Fer-1 for 24 h to inhibit ferroptosis. Both overexpression vectors were synthesized by Sangon Biotech (Shanghai, China), and transfection efficiency was verified by Western blot. All cell lines were validated by STR profiling and tested negative for mycoplasma.

2.8 Biochemical Testing of Urine Samples

After drug administration, 24-h urine samples were collected from each mouse. Creatinine and urine protein levels were measured using a Hitachi 3100 automated biochemical analyzer. The urine albumin-to-creatinine ratio (UACR) was subsequently calculated as the ratio of urine protein concentration (mg) to creatinine concentration (g).

2.9 Evaluation of the Pathological Changes in Mouse Renal Tissues

Following euthanasia of the mice, kidney tissues were quickly excised and fixed with 4% paraformaldehyde. The fixed tissues were dehydrated using an ethanol gradient, cleared with xylene, embedded in paraffin, and sectioned using a paraffin microtome. Then, hematoxylin-eosin (HE) staining (Beyotime, C0105S, Shanghai, China) and periodic acid-schiff (PAS) staining (Beyotime, C0142S, Shanghai, China) were performed to evaluate pathological changes in mouse renal tissues. For PAS staining, paraffin sections were deparaffinized, rehydrated, and incubated with periodic acid solution for 10 min, followed by washing for 5 min. Sections were subsequently stained with Schiff reagent at 37 °C for 30 min. Hematoxylin counterstaining was performed for 30 s, followed by two rinses with distilled water. Sections were subsequently dehydrated, mounted, and examined microscopically. For HE staining, paraffin sections were deparaffinized, rehydrated, stained with hematoxylin for 5 min, rinsed, and counterstained with eosin for 1 min. After dehydration and mounting, the sections were imaged to assess renal morphological changes.

2.10 Measurement of Glutathione (GSH) and Malondialdehyde (MDA) Levels in Mouse Renal Tissue

MDA levels in mouse renal tissues were measured using the MDA Assay Kit (Beyotime, S0131S, Shanghai, China). Briefly, the collected renal tissues were homogenized in lysis solution, and 0.1 mL of the homogenate was mixed with 0.2 mL of the MDA detection solution. The mixture was incubated in a water bath at 100 °C for 15 min, then centrifuged at 1000 ×g for 10 min. The supernatant was collected, and the absorbance was measured at 532 nm using a microplate reader. Similarly, GSH level in mouse renal tissues was measured using the GSH Assay Kit (Beyotime, S0053, Shanghai, China). Tissues were first pulverized in liquid nitrogen and thoroughly homogenized with 100 µL of protein removal reagent M solution. After in-

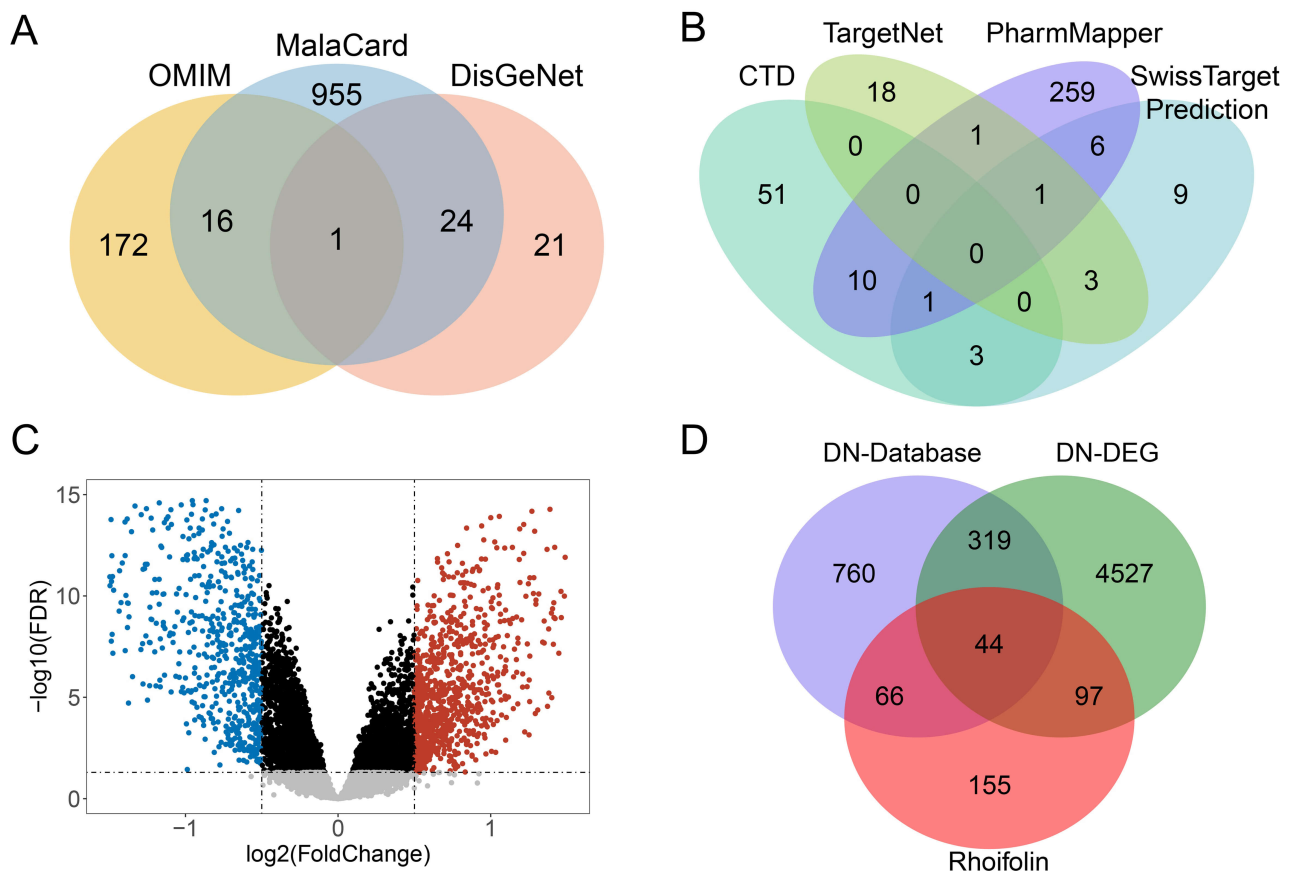


Fig. 1. Screening of ROF-DN target genes. (A) The overlap among DN-related genes obtained from MalaCards, DisGeNET, and OMIM, respectively. (B) Venn diagram illustrating the overlap among predicted ROF target genes obtained from SwissTargetPrediction, TargetNet, the Comparative Toxicogenomics Database, and PharmMapper, respectively. (C) Volcano plot illustrating the DEGs identified from the GSE228960 dataset. (D) Venn diagram illustrating the overlap among DN-related genes, predicted ROF target genes, and DEGs. ROF, Rhoifolin; DN, diabetic nephropathy; DisGeNET, Disease Gene Network; OMIM, Online Mendelian Inheritance in Man; DEGs, Differentially Expressed Genes; CTD, Comparative Toxicogenomics Database.

cubation at 4 °C for 10 min, the tissue samples were centrifuged at 10,000 ×g for 10 min, and the supernatant was subsequently collected. For each assay, 10 µL of the tissue supernatant was mixed with 150 µL of the GSH detection working solution and incubated at 25 °C for 5 min. Subsequently, 50 µL of 0.5 mg/mL NADPH solution was added, and the mixture was incubated for 25 min. The absorbance was then measured at 412 nm using a microplate reader.

2.11 Measurement of Intracellular Reactive Oxygen Species (ROS) Levels

Intracellular ROS levels were assessed using dihydroethidium (DHE) staining (Solarbio, ID3560, Beijing, China). DHE powder was dissolved in dimethyl sulfoxide, and paraffin-embedded tissue sections were incubated with the DHE solution in the dark for 30 min, followed by fluorescence detection.

2.12 Western Blot

Protein was extracted from cells or renal tissues using a lysis buffer supplemented with 1% protease inhibitor. Protein concentrations were quantified using the bicinchoninic acid (BCA) kit (Beyotime, P0012S, Shanghai, China). Proteins were separated on polyacrylamide gels and subsequently transferred onto PVDF membranes. The membranes were blocked with 5% skim milk for 70 min, and subsequently incubated with the corresponding primary antibodies [glutathione peroxidase 4 (GPX4, CST, 52455, MA, USA, 1:1000), Acyl-CoA synthetase long chain family member 4 (ACSL4, CST, 38493, MA, USA, 1:1000), CDKN1A (CST, 37543, MA, USA, 1:1000), CDK2 (CST, 2546, MA, USA, 1:1000), E2F transcription factor 1 (E2F1, Abcam, ab112580, MA, USA, 1:1000), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, CST, 2118, MA, USA, 1:1000)] overnight at 4 °C. After that, the proteins were incubated with secondary antibodies (CST, 7074, MA, USA, 1:1000) conjugated to horseradish

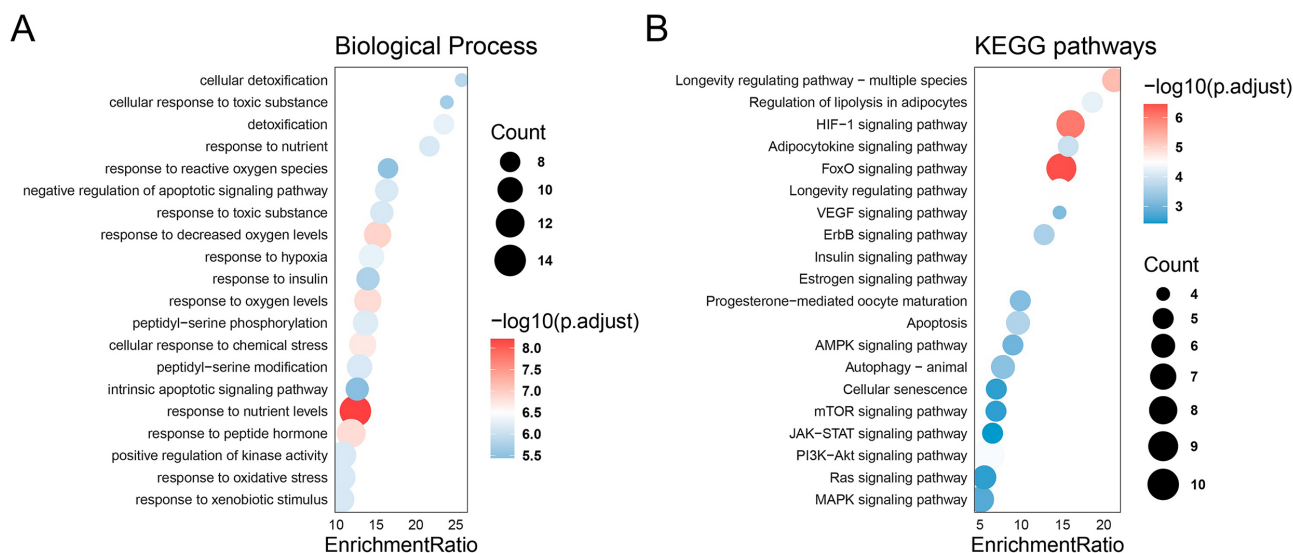


Fig. 2. Functional enrichment analysis of the ROF-DN target genes. (A) The top 20 enriched Gene Ontology-Biological Process (GO-BP) terms. (B) The top 20 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

peroxidase for 50 min. Finally, the target protein bands were visualized via development with enhanced chemiluminescence reagent.

2.13 Detection of Cell Viability

Cells were seeded into 24-well plates at a density of 2×10^6 cells per well. Subsequently, 10 μ L of CCK-8 solution was added to each well and incubated for 4 h.

2.14 Measurement of Cell Death

Cell death was assessed by flow cytometry using an Apoptosis Detection Kit (Yeasen Biotechnology, 40302ES20, Shanghai, China). Single-cell suspensions were prepared, followed by the addition of 5 μ L Annexin V-FITC and 10 μ L PI solution. After incubation in the dark for 10 min, 400 μ L of binding buffer was added, and the samples were gently mixed. The rates of cell death were subsequently determined using a flow cytometer. The operational steps are as follows: after collecting more than 10,000 cells, first set a gate in the FSC-A/SSC-A scatter plot to encompass all cellular events and exclude debris (P1 gate). Then, in the FSC-A/FSC-H scatter plot, gate single cells from within the P1 gate to exclude doublets (P2 gate). Finally, analyze the PI-positive cells within the P2 gate.

2.15 Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of inflammatory cytokines Interleukin-1 β (IL-1 β), Interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α) in cells were quantified using corresponding ELISA kits: IL-6 (Beyotime, PI325, Shanghai, China), IL-1 β (Beyotime, PI305, Shanghai, China), and TNF- α (Beyotime, PT518, Shanghai, China). Cell supernatant was collected by centrifugation at 500

$\times$ g for 5 min. 100 μ L of the supernatant was added to each well of a 96-well plate, and the plate was sealed and incubated for 2 h. Next, the plate was washed, and 100 μ L of the biotinylated antibody was added to each well of the plate, followed by sealing and incubation for 1 h. Subsequently, the plate was washed again, and 100 μ L of horseradish peroxidase labeled streptavidin was added to each well, followed by plate sealing and incubation in the dark for 20 min. After a final wash of the plate, 100 μ L of 3,3',5,5'-tetramethylbenzidine chromogenic solution was added to each well and the plate was sealed and incubated in the dark for 15 min. Finally, 50 μ L of termination solution was added to each well, and absorbance at 450 nm was measured immediately after mixing.

2.16 Detection of Intracellular Fe²⁺ Levels

Cells from each experimental group were seeded into 6-well plates at a density of 2×10^6 cells per well and treated accordingly. Intracellular Fe²⁺ levels were subsequently measured using a Fe²⁺ fluorescence assay kit (Elabscience, E-BC-F101, Wuhan, China) following the protocol. The specific experimental procedure was as follows: Cells were seeded into cell culture plates and cultured overnight at 37 $^{\circ}$ C in an incubator containing 5% CO₂. After cell attachment, the original culture medium was discarded, and the cells were gently washed with phosphate buffered saline. Subsequently, cells were subjected to respective treatments according to the experimental design. Following treatment, the medium was completely removed, and cells were washed with cell staining buffer. Freshly prepared staining working solution was added to each well to ensure complete coverage of the cell layer. The plates were placed in an incubator containing 5% CO₂ and incubated at 37 $^{\circ}$ C in

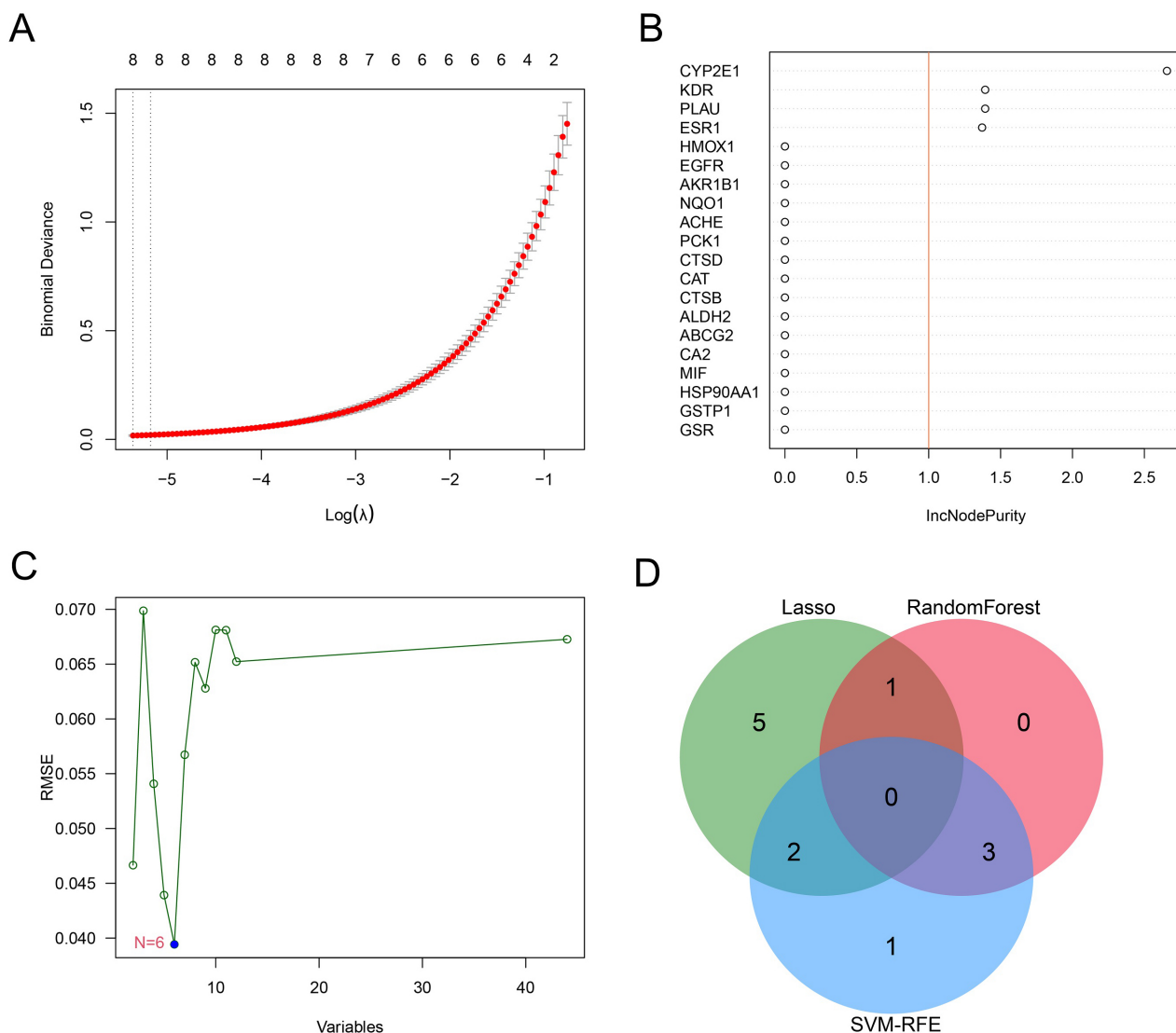


Fig. 3. ML-based screening of feature genes. (A) LASSO analysis shows that the difference between the predicted and actual values of the computational model is minimized when the number of feature genes is 8. (B) Random forest analysis shows the importance scores and ranking of each feature gene. (C) SVM-RFE analysis shows that the difference between the predicted and actual values of the computational model is minimized when the number of feature genes is 6. (D) The overlap among genes identified by LASSO, random forest, and SVM-RFE analyses. ML, machine learning; LASSO, Least Absolute Shrinkage and Selection Operator; SVM-RFE, Support Vector Machine–Recursive Feature Elimination.

the dark for 1 h. After incubation, fluorescence staining of cells in each group was observed and recorded under a fluorescence microscope.

2.17 Statistical Analysis

For bioinformatics analyses, differential expression analysis was conducted using the limma package (v 3.60.3). Statistical comparisons of the experimental data were performed using Student's *t*-test in GraphPad Prism (v 10.1.2, Dotmatics, MA, USA). $p < 0.05$ denotes statistical significance.

3. Results

3.1 Screening of ROF-DN Target Genes

996, 46, and 189 DN-related genes were retrieved from the MalaCards, DisGeNET, and OMIM databases, respectively. After deduplication, 1189 DN-associated genes were obtained (Fig. 1A). 23, 23, 65, and 278 putative targets of ROF were identified from SwissTargetPrediction, TargetNet, the Comparative Toxicogenomics Database, and PharmMapper, respectively, yielding 362 unique predicted targets (Fig. 1B). Differential expression analysis of the GSE228960 dataset identified 4987 DEGs (Fig. 1C). Integration of DN-related genes, predicted ROF targets, and

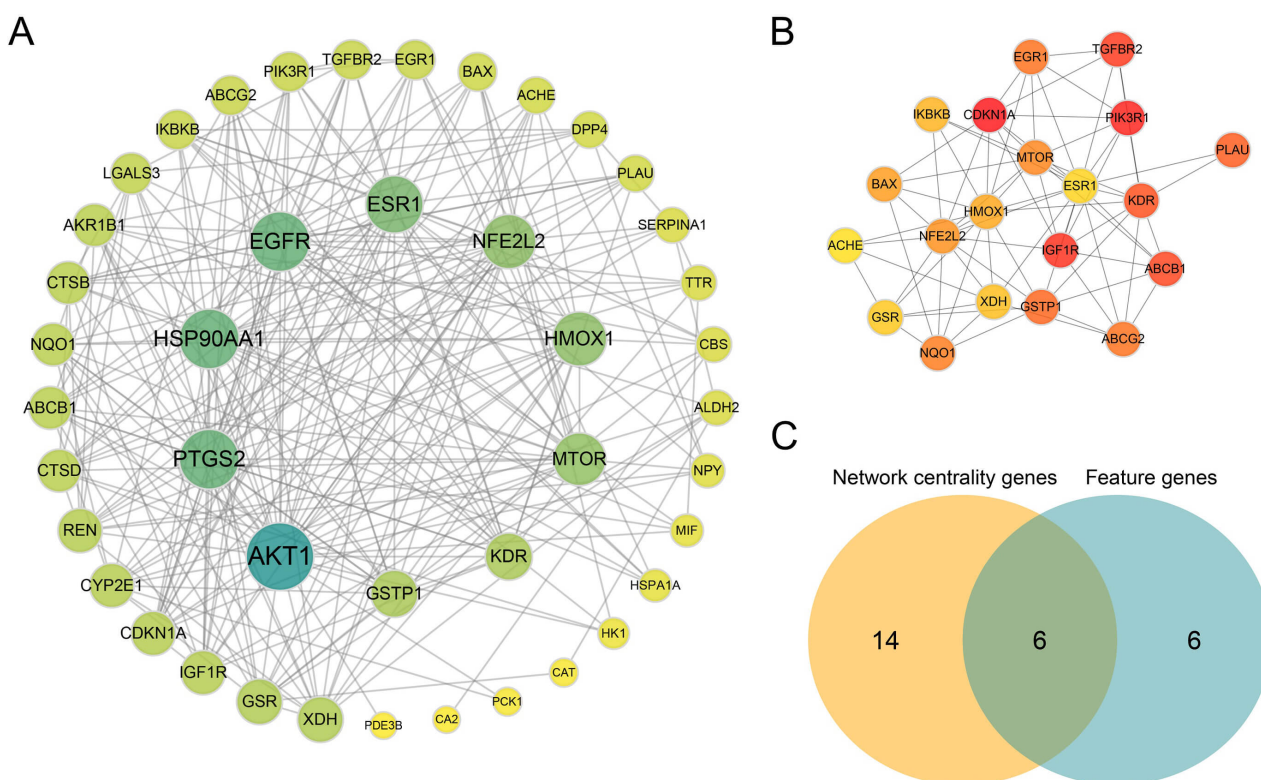


Fig. 4. Identification of hub genes. (A) Protein-Protein Interaction (PPI) network of the 44 ROF-DN target genes, in which each circle represents a gene and circle size corresponds to the number of interaction relationships. (B) Subnetwork displaying the 20 hub nodes identified through CytoHubba analysis within the PPI network. (C) Venn diagram illustrating the overlapping genes identified through PPI network analysis and ML-based screening.

DEGs using a venn diagram revealed 44 overlapping genes, which were designated as ROF-DN target genes for downstream analyses (Fig. 1D).

3.2 Functional Enrichment Analysis of the ROF-DN Target Genes

GO-BP and KEGG enrichment analyses revealed 803 biological processes (Fig. 2A) and 70 pathways (Fig. 2B). The top 20 enriched terms indicated that the ROF-DN target genes were involved in pathways and processes related to cellular detoxification, cellular response to toxic substances, response to ROS, positive regulation of kinase activity, and key pathways including hypoxia-inducible factor-1, Forkhead Box O, and Adenosine 5'-monophosphate-activated protein kinase.

3.3 ML-Based Screening of Feature Genes

To further identify the pivotal genes among the 44 ROF-DN targets, three ML algorithms—LASSO regression (Fig. 3A), random forest (Fig. 3B), and SVM-RFE (Fig. 3C)—were applied to the GSE228960 dataset. These approaches identified 8, 4, and 6 feature genes, respectively. After deduplication, 12 feature genes were ultimately retained: carbonic anhydrase 2, macrophage migration inhibitory factor, cystathionine beta-synthase, mech-

anistic target of rapamycin kinase (*MTOR*), NAD(P)H quinone dehydrogenase 1 (*NQO1*), galectin 3, *CDKN1A*, plasminogen activator, urokinase (*PLAU*), cytochrome P450 family 2 subfamily E member 1, kinase insert domain receptor (*KDR*), estrogen receptor 1 (*ESR1*), and aldo-keto reductase family 1 member B (Fig. 3D).

3.4 Identification of Hub Genes

A PPI network comprising 257 edges and 43 nodes (Fig. 4A) was constructed after uploading the 44 ROF-DN target genes to the STRING database. Identification using CytoHubba revealed the top 20 hub nodes within this network (Fig. 4B). Integration of these hub nodes with the 12 ML-derived feature genes via Venn analysis yielded six overlapping hub genes: *PLAU*, *KDR*, *ESR1*, *MTOR*, *CDKN1A*, and *NQO1* (Fig. 4C).

3.5 Molecular Docking Analysis of ROF with Hub Genes

The three-dimensional structures of the protein receptors corresponding to the six hub genes were obtained from the Protein Data Bank, while the three-dimensional structure of the ligand molecule ROF was retrieved from the PubChem database. Docking simulations were subsequently performed to predict the binding conformations be-

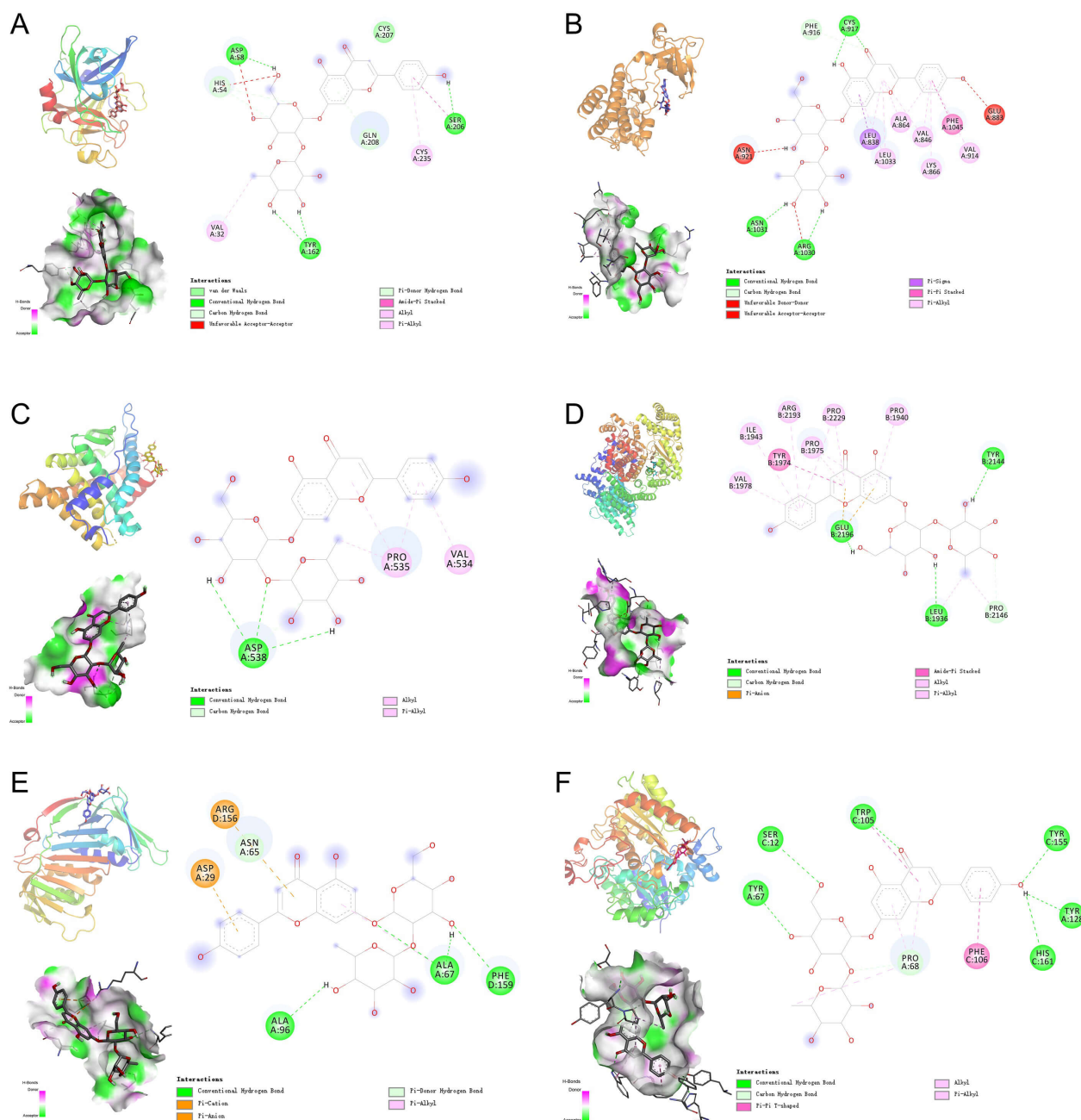


Fig. 5. Molecular docking analysis of ROF with hub genes. (A) PLAU-ROF binding conformation. (B) KDR-ROF binding conformation. (C) ESR1-ROF binding conformation. (D) MTOR-ROF binding conformation. (E) CDKN1A-ROF binding conformation. (F) NQO1-ROF binding conformation. PLAU, plasminogen activator, urokinase; KDR, kinase insert domain receptor; ESR1, estrogen receptor 1; MTOR, mechanistic target of rapamycin kinase; CDKN1A, cyclin dependent kinase inhibitor 1A; NQO1, NAD(P)H quinone dehydrogenase 1.

tween each protein and ROF. The analyses revealed robust binding affinities, with binding energies of -8.9 kcal/mol for PLAU, -8.6 kcal/mol for KDR, -6.4 kcal/mol for ESR1, -10.0 kcal/mol for MTOR, -8.3 kcal/mol for CDKN1A, and -10.4 kcal/mol for NQO1 (Fig. 5A–F). Further analysis of key interacting residues between the ligand and the target proteins (Supplementary Table 1) indicated that the non-

bonded interactions were primarily composed of hydrogen bonds and π -alkyl interactions. In hydrogen bonding, the ligand and the receptor protein residues could act as either hydrogen bond donors or acceptors. In π -alkyl interactions, the ligand typically provided the π -electron cloud, while the receptor protein residues contributed alkyl hydrophobic groups.

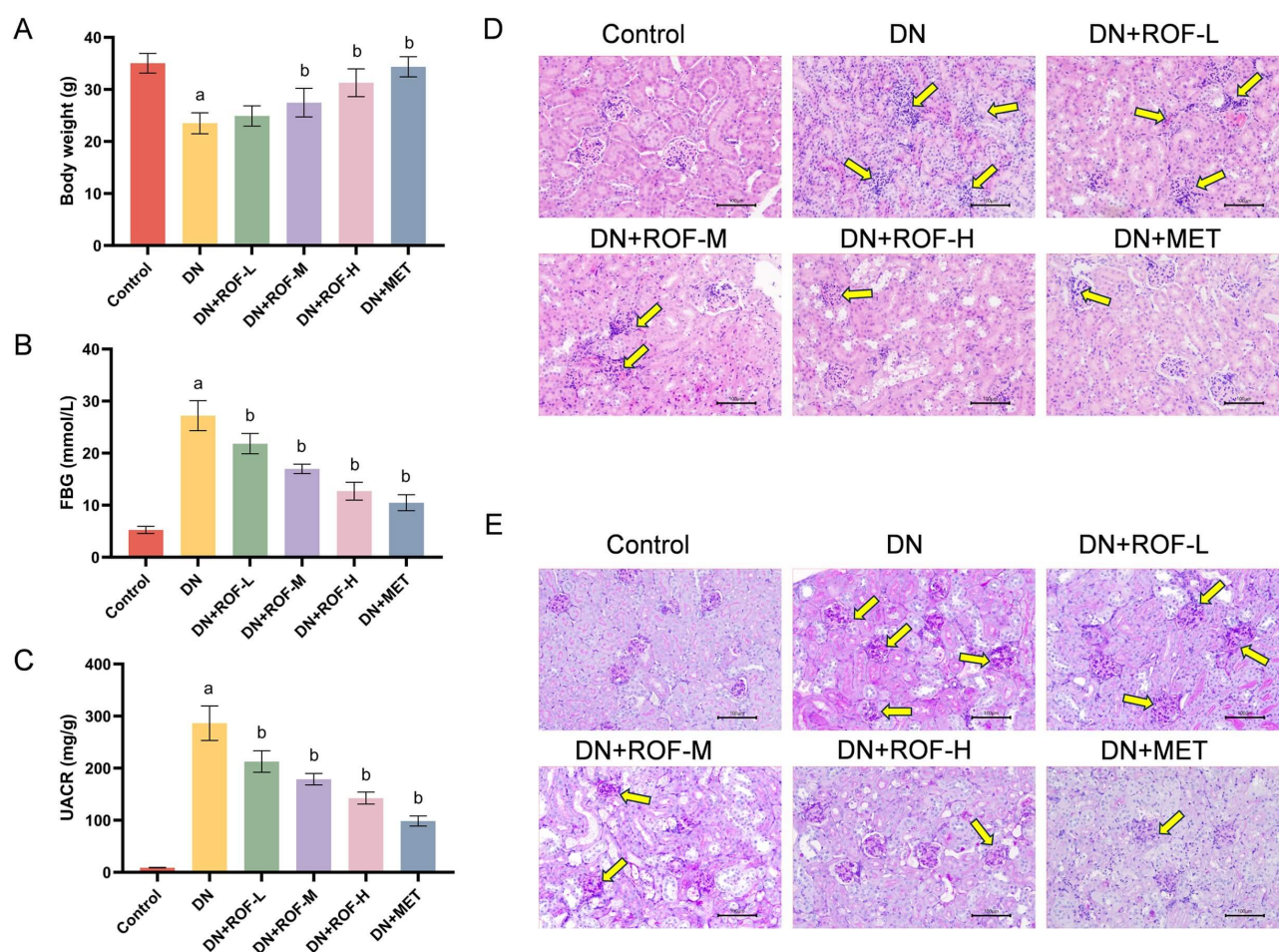


Fig. 6. ROF improves renal injury in mice with DN. (A) Body weight of mice ($n = 6$). (B) Fasting blood glucose (FBG) levels of mice ($n = 6$). (C) The UACR in each group ($n = 6$). (D) The pathological conditions of mouse renal tissues were assessed by hematoxylin-eosin (HE) staining ($n = 6$). Scale bar, 100 μm . The arrow indicates the area where inflammatory cells have infiltrated. (E) The pathological conditions of mouse renal tissues were assessed by periodic acid-schiff (PAS) staining ($n = 6$). Scale bar, 100 μm . The arrow indicates the area where glycogen is deposited. a, compared with the Control group, $p < 0.05$; b, compared with the DN group, $p < 0.05$.

3.6 ROF Improves Renal Injury in Mice With DN

Compared with the Control group, mice with DN exhibited a reduction in body weight (Fig. 6A), a marked elevation in FBG levels (Fig. 6B), and a substantial increase in the UACR (Fig. 6C). PAS and HE staining demonstrated pronounced renal histopathological abnormalities in mice in the DN group, including glomerular and tubular injury, mesangial expansion, tubular dilation and rupture, and interstitial inflammatory infiltration (Fig. 6D,E). By contrast, mice in the DN+ROF-L, DN+ROF-M, DN+ROF-H, and DN+MET groups showed increased body weight (Fig. 6A), significantly reduced FBG levels (Fig. 6B), and markedly decreased UACR (Fig. 6C) relative to those in the DN group. Histological analyses further indicated notable improvements in renal morphology across all treatment groups, with the DN+ROF-H and DN+MET groups displaying the most pronounced amelioration (Fig. 6D,E).

3.7 ROF Inhibits Ferroptosis and Downregulates *CDKN1A* Expression in the Renal Tissues of Mice With DN

Analysis of the six hub genes in the GSE228960 dataset revealed that, in comparison to the Control group, the DN group showed markedly increased expression of *CDKN1A* and *NQO1*, while exhibiting decreased expression of *KDR*, *PLAU*, *MTOR*, and *ESR1* (Fig. 7A). *CDKN1A* was initially selected for experimental validation. The results confirmed that *CDKN1A* expression was upregulated in the renal tissues from the DN group compared to those from the Control group (Fig. 7B). In contrast, the DN+ROF-L, DN+ROF-M, DN+ROF-H, and DN+MET groups demonstrated significantly reduced *CDKN1A* expression relative to the DN group (Fig. 7B). Simultaneously, it was detected that compared with the Control group, the DN group displayed decreased expression of the ferroptosis inhibitor GPX4, increased expression of ACSL4 (Fig. 7B), accompanied by elevated MDA levels (Fig. 7C),

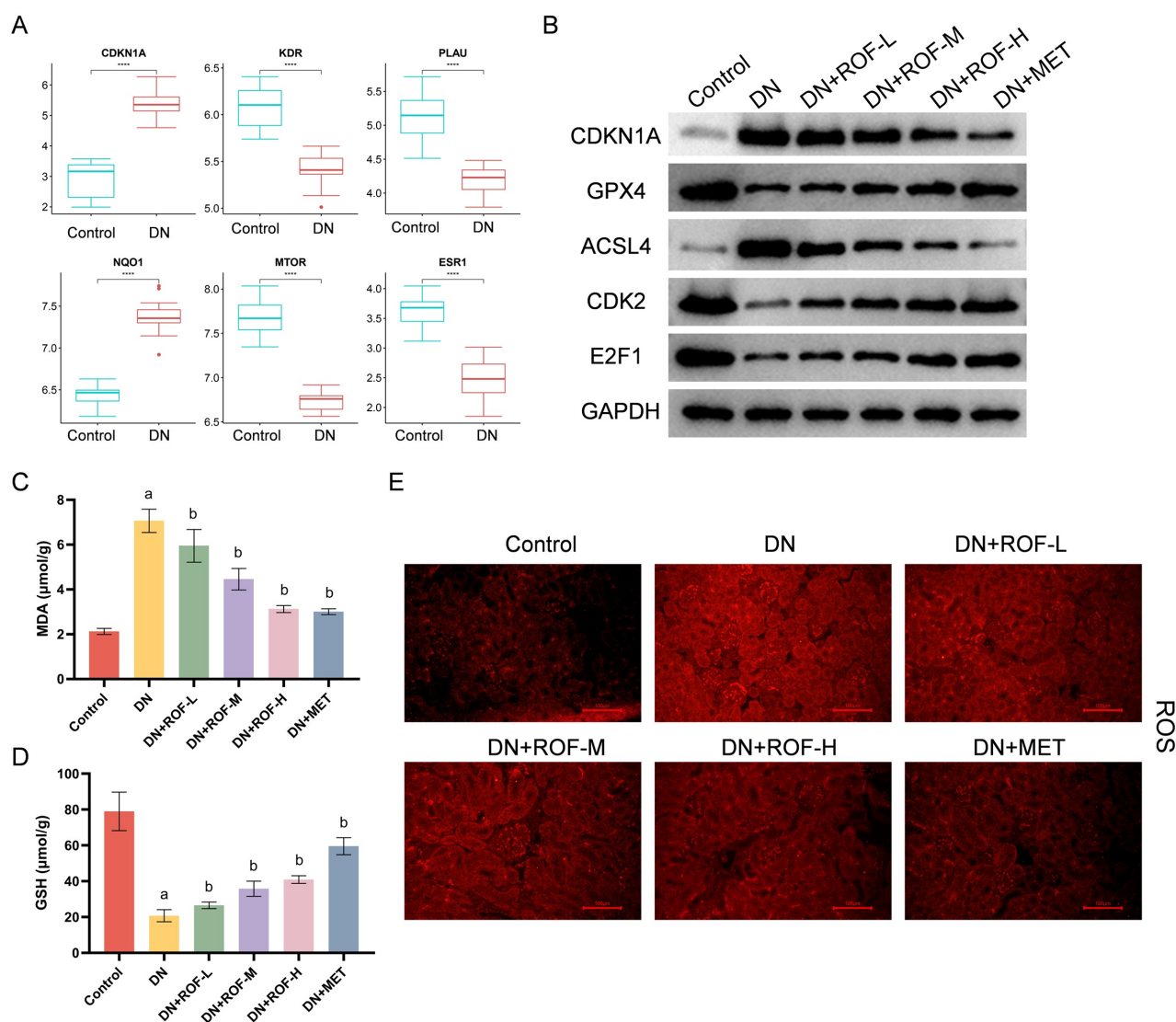


Fig. 7. ROF inhibits ferroptosis and downregulates CDKN1A expression in the renal tissues of mice with DN. (A) A box plot illustrating the expression levels of hub genes in the GSE228960 dataset. (B) The expression of CDKN1A, ferroptosis, and CDK2/E2F1 pathway-related proteins in renal tissues (n = 6). (C) The level of malondialdehyde (MDA) in renal tissue (n = 6). (D) The level of glutathione (GSH) in renal tissue (n = 6). (E) The reactive oxygen species (ROS) level in renal tissue (n = 6). Scale bar, 100 μm. a, compared with the Control group, $p < 0.05$; b, compared with the DN group, $p < 0.05$. ****, compared with DN group, $p < 0.0001$.

reduced GSH (Fig. 7D), heightened ROS (Fig. 7E), and significantly downregulated expression of CDK2 and E2F1 (Fig. 7B). In comparison to the DN group, the DN+ROF-L, DN+ROF-M, DN+ROF-H, and DN+MET groups exhibited increased GPX4 expression and decreased ACSL4 expression (Fig. 7B), reduced MDA levels (Fig. 7C), elevated GSH (Fig. 7D), suppressed ROS (Fig. 7E), and significantly upregulated CDK2 and E2F1 expression (Fig. 7B).

3.8 ROF Alleviates Renal Tubular Epithelial Cell Injury

Compared to the NC group, the HG group exhibited upregulated CDKN1A expression (Fig. 8A), reduced cell viability (Fig. 8B), increased cell death (Fig. 8C), elevated levels of inflammatory factors IL-1 β , IL-6, TNF-

α (Fig. 8D–F), heightened Fe²⁺ levels (Fig. 8G), decreased GPX4 expression, increased ACSL4 expression (Fig. 8A), along with significantly downregulated CDK2 and E2F1 expression (Fig. 8A). In contrast, relative to the HG group, the HG+ROF-L, HG+ROF-M, and HG+ROF-H groups showed markedly downregulated CDKN1A expression (Fig. 8A), enhanced cell viability (Fig. 8B), diminished cell death (Fig. 8C), reduced levels of IL-1 β , IL-6, TNF- α (Fig. 8D–F), decreased Fe²⁺ levels (Fig. 8G), elevated GPX4 expression, suppressed ACSL4 expression (Fig. 8A), and significantly upregulated CDK2 and E2F1 expression (Fig. 8A).

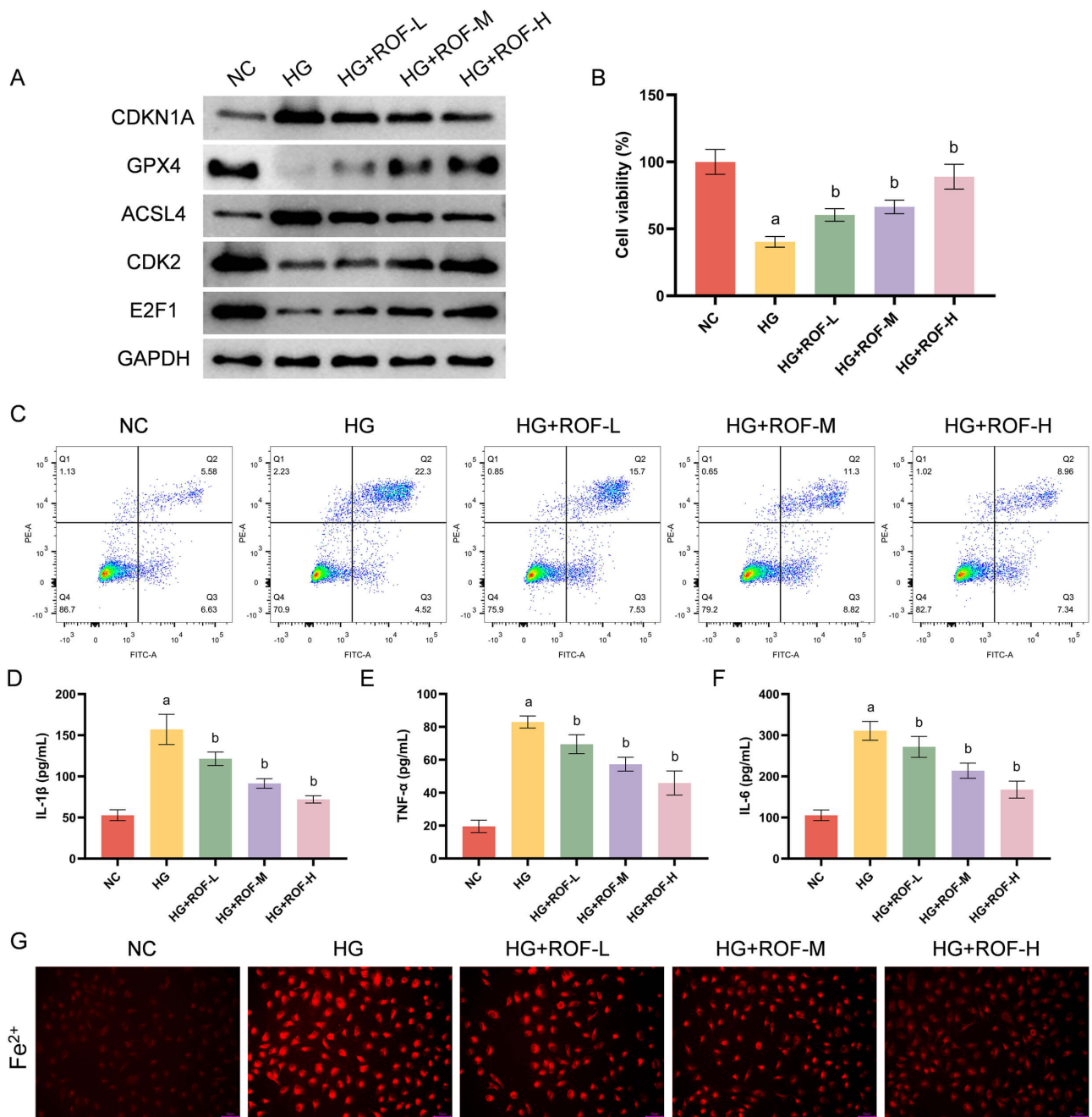


Fig. 8. ROF alleviates renal tubular epithelial cell injury. (A) The expression of CDKN1A, ferroptosis, and CDK2/E2F1 pathway-related proteins in cells (n = 3). (B) Cell viability (n = 3). (C) Cell death (n = 3). (D) Intracellular secretion levels of IL-1β (n = 3). (E) Intracellular secretion levels of TNF-α (n = 3). (F) Intracellular secretion levels of IL-6 (n = 3). (G) Intracellular levels of Fe²⁺ (n = 3). Scale bar, 50 μm. a, compared with the NC group, *p* < 0.05; b, compared with the HG group, *p* < 0.05.

3.9 ROF Alleviates Renal Tubular Epithelial Cell Injury by Targeting CDKN1A

To investigate the role of CDKN1A, CDKN1A was overexpressed in HG+ROF-H-treated cells. CDKN1A overexpression significantly increased CDKN1A levels (Fig. 9A), decreased cell viability (Fig. 9B), enhanced cell death (Fig. 9C), and led to elevated levels of IL-1β, IL-6, TNF-α (Fig. 9D–F), increased intracellular levels of Fe²⁺

(Fig. 9G), reduced GPX4 expression, increased ACSL4 expression, as well as decreased CDK2 and E2F1 expression (Fig. 9A).

3.10 ROF Regulates Ferroptosis by Targeting CDKN1A to Alleviate Renal Tubular Epithelial Cell Injury

To determine whether CDKN1A mediates ferroptosis, cells in the HG+ROF-H+oe-CDKN1A group were treated with Fer-1. Fer-1 treatment resulted in decreased Fe²⁺

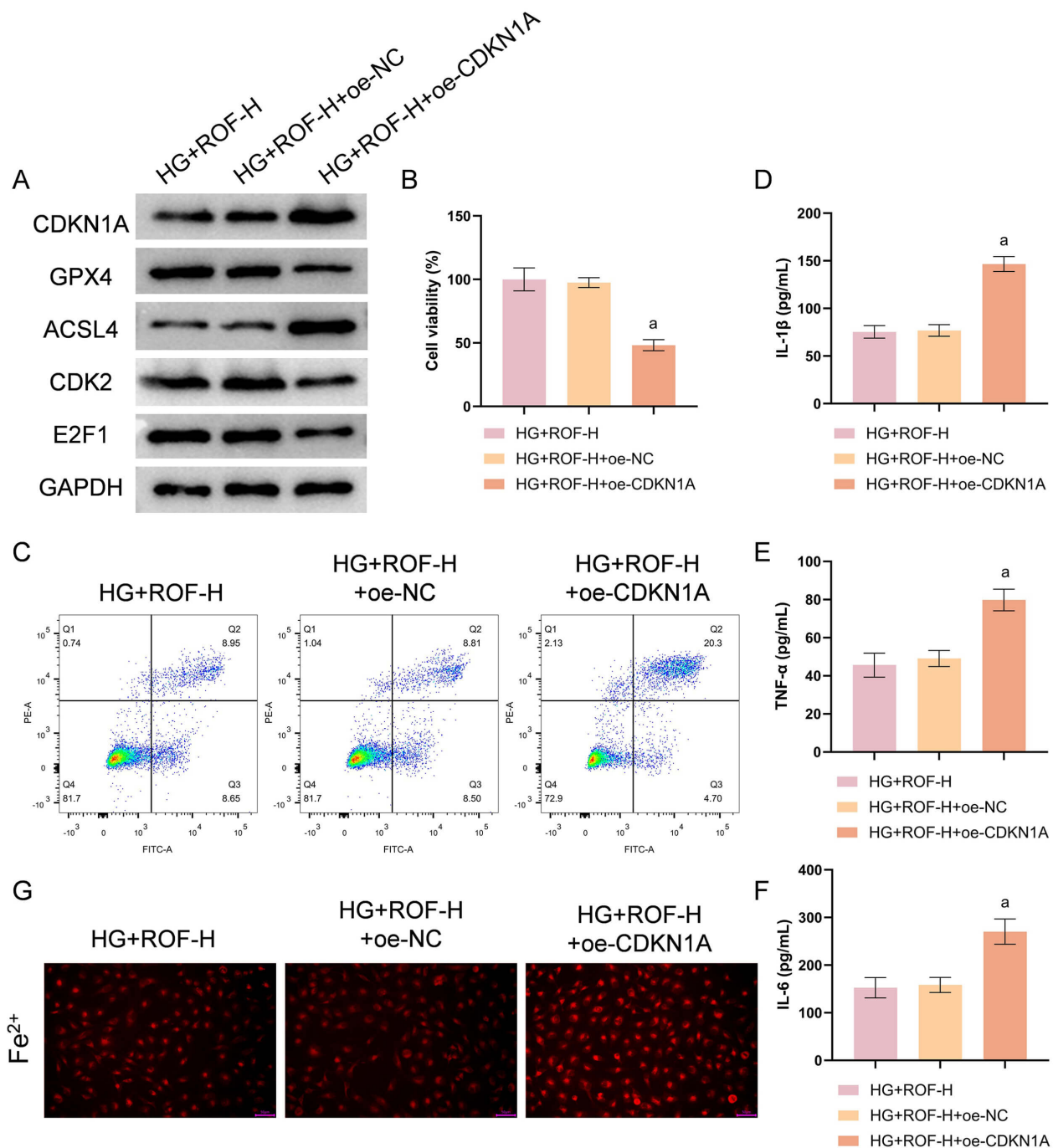


Fig. 9. ROF alleviates renal tubular epithelial cell injury by targeting CDKN1A. (A) The expression of CDKN1A, ferroptosis, and CDK2/E2F1 pathway-related proteins in cells (n = 3). (B) Cell viability (n = 3). (C) Cell death (n = 3). (D) Intracellular secretion levels of IL-1 β (n = 3). (E) Intracellular secretion levels of TNF- α (n = 3). (F) Intracellular secretion levels of IL-6 (n = 3). (G) Intracellular levels of Fe²⁺ (n = 3). Scale bar, 50 μ m. a, compared with the HG+ROF-H+oe-NC group, $p < 0.05$.

levels (Fig. 10A), increased GPX4 expression, decreased ACSL4 expression (Fig. 10B), unchanged CDKN1A (Fig. 10B), enhanced cell viability (Fig. 10C), reduced cell death (Fig. 10D), and decreased IL-6, IL-1 β , and TNF- α levels (Fig. 10E–G).

3.11 ROF Alleviates Ferroptosis by Targeting CDKN1A and Mediating the CDK2/E2F1 Pathway in Renal Tubular Epithelial Cells

To further evaluate whether CDKN1A regulates the CDK2/E2F1 pathway, CDK2 was overexpressed in cells in the HG+ROF-H+oe-CDKN1A group. CDK2 overexpression markedly increased CDK2 and E2F1 expression (Fig.

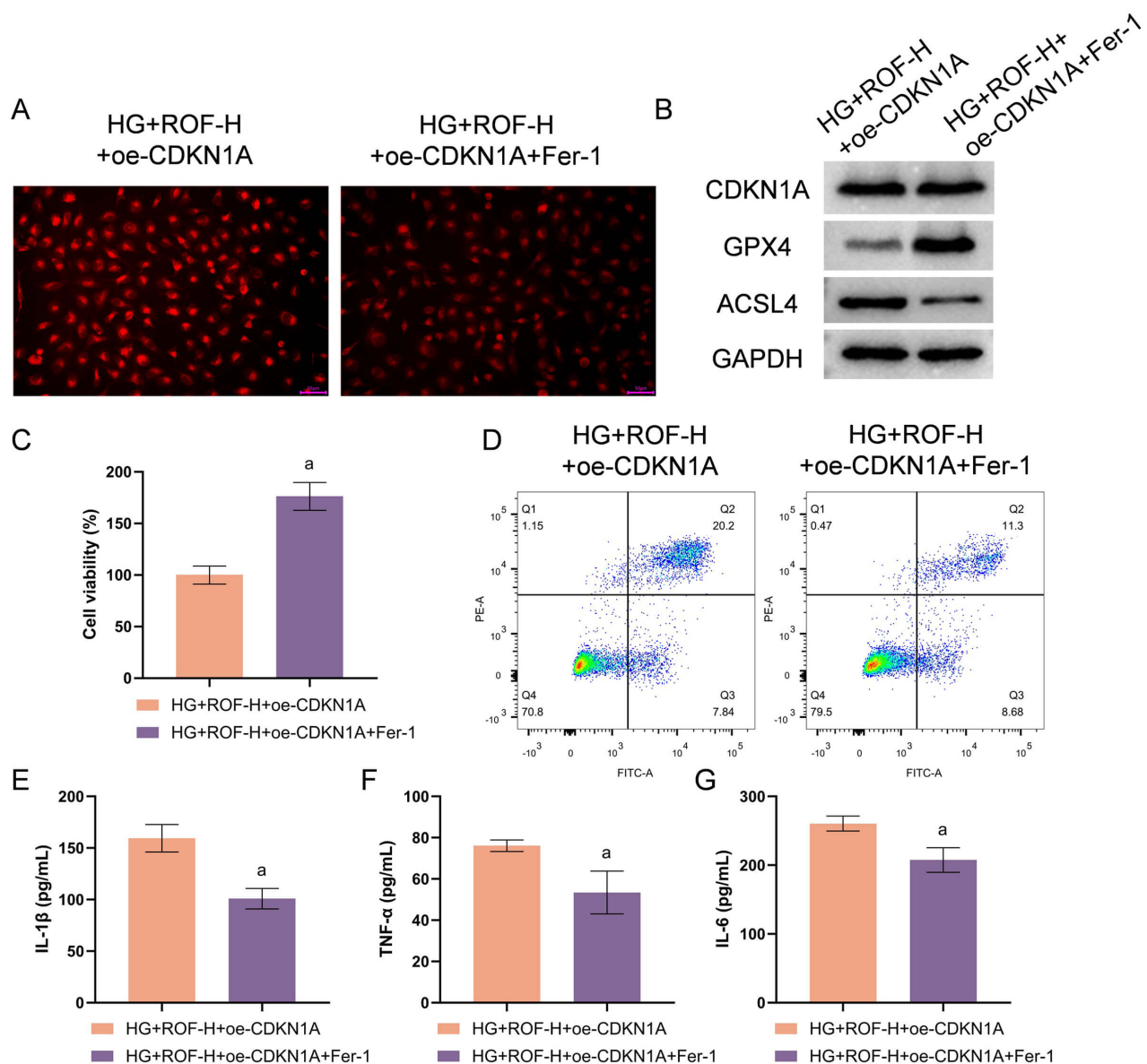


Fig. 10. ROF regulates ferroptosis by targeting CDKN1A to alleviate renal tubular epithelial cell injury. (A) Intracellular level of Fe^{2+} ($n = 3$). Scale bar, 50 μm . (B) The expression of CDKN1A, ferroptosis-related proteins in cells ($n = 3$). (C) Cell viability ($n = 3$). (D) Cell death ($n = 3$). (E) Intracellular secretion levels of IL-1 β ($n = 3$). (F) Intracellular secretion levels of TNF- α ($n = 3$). (G) Intracellular secretion levels of IL-6 ($n = 3$). a, compared with the HG+ROF-H+oe-CDKN1A group, $p < 0.05$.

11A), without affecting CDKN1A levels. CDK2 overexpression significantly enhanced viability (Fig. 11B), reduced cell death (Fig. 11C), decreased the intracellular secretion of IL-1 β , IL-6, TNF- α (Fig. 11D–F), reduced intracellular Fe^{2+} levels (Fig. 11G), increased GPX4 expression, and decreased ACSL4 expression (Fig. 11A).

4. Discussion

ROF, a flavonoid compound, has been documented to exert significant glucose-lowering effects [9,10]. However, its therapeutic potential in DN or other renal diseases has not yet been reported. In the present study, our prelimi-

nary experiments demonstrated that ROF improved renal function in mice with DN. Evidence from previous studies demonstrates that ROF exhibits considerable therapeutic promise across multiple diseases, including intestinal inflammation, arthritis, and cancer. For instance, ROF has been shown to mitigate colitis in mice by modulating the gut microbiota [12], ameliorate osteoarthritis through the regulation of autophagy [13], inhibit osteoclastogenesis via suppressing receptor activator of nuclear factor- κB ligand-induced mitogen-activated protein kinase 1 pathways [14], and suppress proliferation while inducing apoptosis in hepatocellular carcinoma cells [8].

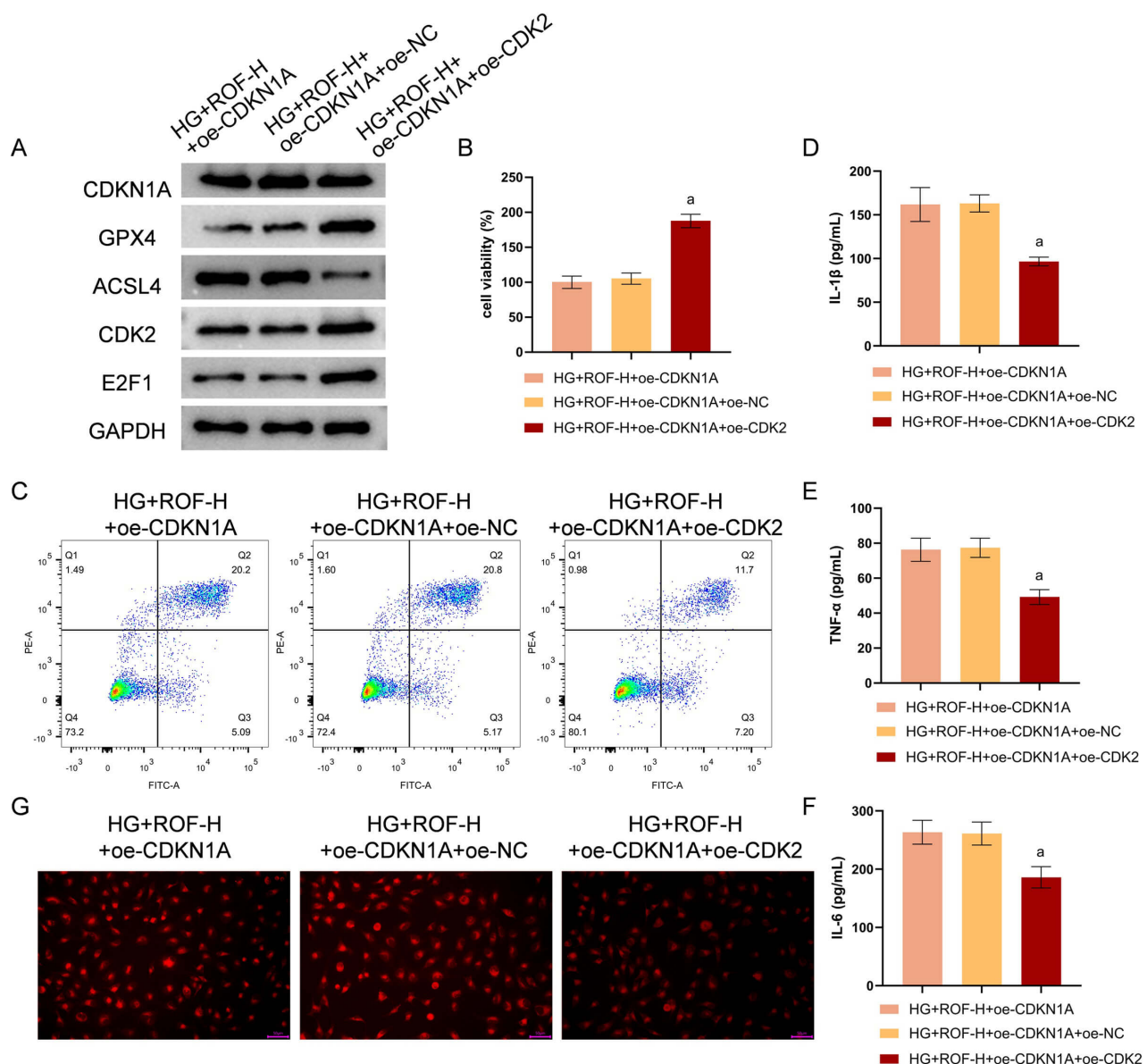


Fig. 11. ROF alleviates ferroptosis by targeting CDKN1A and mediating the CDK2/E2F1 pathway in renal tubular epithelial cells. (A) The expression of CDKN1A, ferroptosis, and CDK2/E2F1 pathway-related proteins in cells (n = 3). (B) Cell viability (n = 3). (C) Cell death (n = 3). (D) Intracellular secretion levels of IL-1 β (n = 3). (E) Intracellular secretion levels of TNF- α (n = 3). (F) Intracellular secretion levels of IL-6 (n = 3). (G) Intracellular levels of Fe²⁺ (n = 3). Scale bar, 50 μ m. a, compared with the HG+ROF-H+oe-CDKN1A+oe-NC group, $p < 0.05$.

In this study, low-, medium-, and high-dose ROF were administered by oral gavage to mice with DN induced by a high-fat diet combined with STZ, and the results demonstrated that ROF improved renal function in mice with DN. To elucidate the potential therapeutic targets of ROF, network pharmacology analysis was performed, leading to the identification of 44 candidate ROF-DN target genes. Subsequent PPI analysis and ML-based screening revealed six hub genes—*CDKN1A*, *NQO1*, *KDR*, *PLAU*, *MTOR*, and *ESR1*—as key regulators associated with DN progression. *CDKN1A* was selected for further expression validation and

mechanistic interrogation. Experimental results revealed that CDKN1A expression was elevated in renal tissues of mice with DN and in HG-stimulated renal tubular epithelial cells, whereas ROF treatment effectively suppressed CDKN1A expression. Consistent with our observations, evidence from previous studies supports the pivotal role of CDKN1A in DN pathogenesis. Jindou Yang et al. [15] demonstrated that X-inactive specific transcript promotes renal interstitial fibrosis in DN by binding to miR-93-5p and targeting CDKN1A, while Zhenjin Li et al. [16] reported that LINC00968 epigenetically regulates CDKN1A

expression via recruitment of enhancer of zeste homolog 2, thereby facilitating cellular proliferation and fibrosis in DN. To further determine whether ROF attenuates DN progression through CDKN1A, CDKN1A was overexpressed in HK-2 cells treated with both HG and ROF. The results demonstrated that CDKN1A overexpression markedly attenuated the protective effects of ROF, as reflected by reduced cell viability, enhanced apoptosis, and increased secretion of inflammatory mediators.

It was also observed that, beyond improving renal function, ROF modulated the CDK2/E2F1 signaling axis and ferroptosis in both renal tissues and renal tubular epithelial cells. Further mechanistic investigation revealed that CDKN1A overexpression promoted ferroptosis while concurrently suppressing the CDK2/E2F1 pathway. Ferroptosis, a distinct form of non-apoptotic cell death, has emerged as a crucial contributor to the progression of DN and other diabetic complications [17]. Existing evidence indicates that the vitamin D receptor agonist Paricalcitol alleviates DN-associated renal injury by suppressing ferroptosis in renal tubular epithelial cells [18]. Jia Hong et al. [19] demonstrated that protein arginine methyltransferase 6 interacts with signal transducer and activator of transcription 1 to co-regulate transcription of the ferroptosis-promoting gene acyl-CoA synthetase long chain family member 1, thereby exacerbating renal dysfunction in DN. Additionally, Zijun Liu et al. [20] reported that endoplasmic reticulum stress triggers ferroptosis-associated epithelial-mesenchymal transition, promoting renal tubulointerstitial fibrosis in DN. Collectively, the foregoing findings establish ferroptosis as an emerging therapeutic target for DN treatment and drug development. Current evidence confirms that CDKN1A mediates ferroptosis in renal tubular epithelial cells [21,22], while ROF has been reported to inhibit epithelial cell ferroptosis in colitis models [23]. However, whether ROF regulates ferroptosis by targeting CDKN1A in DN has remained unknown. To address this issue, cells in the HG+ROF-H+oe-CDKN1A group were treated with Fer-1. Notably, Fer-1 administration markedly attenuated the cellular injury induced by CDKN1A overexpression.

CDK2 plays critical roles in cell cycle regulation, particularly in DNA replication and mitosis [24]. The CDK2-encoded protein interacts with and is regulated by CDKN1A. Jianbo Liu et al. [25] demonstrated that knock-down of CDKN1A upregulates CDK2 expression, a regulatory mechanism functionally significant in cumulus cells [25], cervical cancer cells [26], and gastric cancer cells [27]. However, the potential effect of ROF on the CDK2/E2F1 pathway has not been previously investigated. To determine whether ROF modulates the CDK2/E2F1 pathway via CDKN1A, CDK2 was overexpressed in cells in the HG+ROF-H+oe-CDKN1A group. CDK2 overexpression significantly reversed the cellular injury triggered by CDKN1A overexpression.

5. Limitations

However, clinical evidence regarding ROF remains limited, and its potential adverse effects warrant further investigation. Future studies should therefore include larger cohorts of participants to systematically evaluate the therapeutic efficacy and clinical safety of ROF. Additionally, ferroptosis in the present study was primarily evaluated using Western blot and fluorescent probe-based assays, which have certain limitations. Notably, direct observation of mitochondrial morphology was not included. Future investigations will incorporate transmission electron microscopy and complementary approaches to enable comprehensive analysis of mitochondrial structure, thereby providing a more comprehensive elucidation of organelle changes during ferroptosis.

6. Conclusion

In summary, CDKN1A was identified in the present study as a critical therapeutic target of ROF in DN through integrated bioinformatic analyses. Comprehensive *in vivo* and *in vitro* evidence demonstrated that ROF attenuates ferroptosis in HK-2 cells and mitigates the progression of DN by targeting CDKN1A to regulate the CDK2/E2F1 pathway.

Availability of Data and Materials

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

Author Contributions

ZZ designed the research study and drafted the manuscript. JW, XC, and PZ performed experiments and prepared figures. JL designed the research study, provided methodological support, and critically reviewed the manuscript. All authors contributed to critical revisions in the manuscript, read and approved the final manuscript, and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Institutional Animal Care and Use Committee of Zhejiang Center of Laboratory Animals (Approval No. ZJCLA-IACUC-20020245), and conducted following the ARRIVE guidelines. The experiments were performed in line with the Laboratory animal—Guideline for ethical review of animal welfare (GB/T 35892-2018). Throughout the experiments, all animals received humane care, and every procedure was carried out under the oversight of this committee.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/IJP48847>.

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