

Original Article

Targeting the Zbp1-Ripk3 axis attenuates ischemic neuronal injury by suppressing PANoptosis

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Abstract

Cerebral ischemia-reperfusion injury (CIRI) significantly exacerbates neuronal damage following ischemic stroke. Emerging evidence implicates PANoptosis, a novel inflammatory form of programmed cell death, in CIRI pathogenesis. However, the role of Z-DNA-binding protein 1 (Zbp1), a key regulator of PANoptosis and innate immune responses, remains poorly understood in this context. Here, we establish a middle cerebral artery occlusion/reperfusion (MCAO/R) mouse model and an oxygen-glucose deprivation/re-oxygenation (OGD/R) model in which HT-22 cells are used to simulate CIRI and investigate the effects of Zbp1. Western blot analysis is performed to assess Zbp1 and PANoptosis-related protein expressions. Cell viability and death are evaluated using Hoechst 33342 staining and Calcein AM/PI assays, and the cerebral infarct volume is quantified using 2,3,5-triphenyltetrazolium chloride staining. Immunoprecipitation and mass spectrometry identify Ripk3 as a potential downstream effector of Zbp1. Furthermore, Zbp1 and PANoptosis-related proteins are significantly upregulated following OGD/R treatment. Zbp1 knockdown markedly reduces PANoptosis and cell injury in both models, decreases infarct volume and improves neurological outcomes in MCAO/R model mice. Conversely, Zbp1 overexpression exacerbates OGD/R-induced neuronal injury and PANoptosis in HT-22 cells. This effect is partially reversed by Ripk3 knockdown, indicating that Ripk3 mediates Zbp1-induced neuronal damage. These findings highlight Zbp1 as a promising therapeutic target in ischemic stroke and underscore the need for further research into Zbp1-mediated PANoptotic pathways to aid in the development of novel neuroprotective strategies.

Key words cerebral ischemia/reperfusion injury, Zbp1, PANoptosis, ischemic stroke, Ripk3

Introduction

Cerebral ischemia-reperfusion injury (CIRI) significantly exacerbates brain injury during the acute phase of ischemic stroke and is closely associated with poor clinical outcomes in stroke patients [1]. Multiple cell death mechanisms, including necroptosis, apoptosis, necrotic apoptosis, and autophagic cell death, contribute to CIRI [2]. Furthermore, these pathways interact in a highly intricate manner, and inhibiting a single pathway is insufficient to prevent cell death or adequately mitigate ischemia-reperfusion-induced brain injury [3]. Thus, identifying key molecules that can simultaneously modulate multiple cell death pathways to identify more effective therapeutic targets for neuroprotection is critical.

PANoptosis, a recently identified inflammatory form of programmed cell death, is associated with numerous conditions, including inflammation, infection, and cancer [4]. PANoptosis

shares key features with pyroptosis, apoptosis, and necroptosis but cannot be fully characterized by any of these distinct forms of programmed cell death. Recent research has indicated that PANoptosis plays a role in ischemia-reperfusion injury [5]. Xie *et al.* [6] demonstrated that neuronal PANoptosis is activated during spinal cord ischemia-reperfusion in rats; furthermore, a slow-releasing H₂S donor provided enhanced spinal neuroprotection by inhibiting neuronal PANoptosis. Despite these advances in understanding, the molecular mechanisms involved in PANoptosis in CIRI remain incompletely understood. Several critical aspects require further elucidation: (i) the molecular sensors that trigger PANoptosis in sterile neuronal injury, (ii) the regulatory networks orchestrating the convergence of multiple cell death pathways, and (iii) the identification of druggable targets within the PANoptotic machinery for therapeutic intervention. Addressing these knowl-

edge gaps is essential for developing effective neuroprotective strategies targeting PANoptosis in CIRI.

Z-DNA-binding protein 1 (Zbp1) is a recently identified nucleic acid recognition receptor implicated in various pathological processes, including inflammation, necroptosis, and PANoptosis [7]. However, studies have suggested that the role of Zbp1 may differ significantly under various stress conditions. Saada *et al.* [8] demonstrated that damaged mitochondrial DNA (mtDNA) binds to Zbp1 in retinal pigment epithelial cells, activating it and leading to the upregulation of pro-inflammatory markers. These findings underscore the crucial role of Zbp1 in retinal inflammation. Another study recently demonstrated that elevated concentrations of mtDNA can induce the upregulation of Zbp1 and inflammatory mediators in cardiomyocytes; however, Zbp1 silencing further exacerbates myocardial inflammation, suggesting that Zbp1 serves as an endogenous inhibitor of myocardial inflammation and plays a protective role in cardiac remodeling [9]. In our previous study, bioinformatics analyses suggested that Zbp1 may play a role in regulating PANoptosis during CIRI [10]; however, the precise mechanism involved requires further investigation.

Receptor-interacting protein kinase 3 (Ripk3) is a key member of the receptor-interacting protein kinase (RIP) family and plays a pivotal role in several signaling pathways that regulate cell death, inflammation, and immune responses [11]. Studies have shown that Zbp1/Ripk3 signaling is crucial for the activation of PANoptosis under specific stress conditions, such as influenza A virus infection and heat stress [12,13]. However, it remains unclear whether the Zbp1/Ripk3 signaling pathway plays a role in regulating PANoptosis following cerebral ischemia-reperfusion.

Here, we established a middle cerebral artery occlusion/reperfusion (MCAO/R) mouse model and an oxygen-glucose deprivation/re-oxygenation (OGD/R) model in HT-22 cells to simulate ischemia-reperfusion. Building upon our previous bioinformatics analyses [10], in the present study we aimed to investigate the role of Zbp1 as a potential regulator of PANoptosis in CIRI and examine its downstream signaling relationship with Ripk3. Although a comprehensive understanding of PANoptotic mechanisms remains elusive, this work provides preliminary evidence supporting the involvement of the Zbp1-Ripk3 axis in ischemic neuronal injury, identifying a potential therapeutic target that warrants further investigation.

Materials and Methods

Cells

Mouse hippocampal neuronal HT-22 cells (Fuheng Biology, Shanghai, China) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Cytiva, Hangzhou, China) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (Gibco, Waltham, USA) under humidified conditions with 5% CO₂ at 37°C. The medium was refreshed every 2–3 days.

OGD/R model

HT-22 cells were subjected to OGD/R as previously described [14]. Briefly, after two washes with PBS (Beyotime, Shanghai, China), the cells were cultured in DMEM without glucose or serum under hypoxic conditions (5% CO₂, 94% N₂, and 1% O₂) for 6 h. The cells were subsequently returned to normoxic conditions, and the medium was subsequently replaced by

complete DMEM for 24 h.

Animals

Male C57BL/6 mice aged 10–12 weeks (25–30 g) were obtained from Shanghai Zhan Yuan Biotechnology (Shanghai, China). The mice were housed in standard cages at 22–25°C with 55%–60% humidity, with *ad libitum* access to food and water and a 12/12-h light/dark cycle. This study was conducted according to the Animal Research: Reporting of *In Vivo* Animal Experiments guidelines. The research and protocols were approved by the Institutional Animal Care and Use Committee of Fudan University (Shanghai, China) (approval No. 2022JS Huadong Hospital-087) and adhered to Chinese government guidelines for animal experimentation. All the animal experiments were conducted according to the relevant guidelines, standard protocols, and animal welfare regulations.

MCAO model and adeno-related virus (AAV) transfection

MCAO/R was performed as previously described [15]. Specifically, under anesthesia with 2% isoflurane in O₂ (RWD Life Science, Shanghai, China), the right external carotid artery (ECA) of each mouse was ligated, followed by the insertion of a monofilament with a silicone coating (diameter of 0.22 ± 0.01 mm; Beijing Cinontech Co., Ltd., Beijing, China) into the external carotid artery (ECA), which advanced into the internal carotid artery (ICA) until mild resistance was felt. After 60 min, the filament was removed to allow reperfusion. The mice were maintained at 37 ± 0.5°C throughout the procedure. The sham operation followed the same procedure, with MCA occlusion omitted. Following recovery, the animals were assessed by blinded investigators via a five-point neurological deficit score.

AAV-shRNA-Zbp1 and control AAV-shRNA-NC (5'-CGCTGAG TACTTCGAAATGTC-3') were designed and synthesized by Gene-Chem (Shanghai, China). The AAV-shRNA-Zbp1 sequences used were as follows: (F) 5'-CCCUCAAUCAAGUCCUUUATT-3' and (R) 5'-UAAAGGACUUGAUUGAGGGTT-3'. The mice were immobilized using a stereotaxic apparatus (Chengdu Techman, Chengdu, China), and a 10-μL syringe was inserted into the right lateral ventricle through a burr hole. AAV-shRNA-Zbp1 or the negative control was injected at a rate of 0.5 μL/min, delivering a total of 4.51 × 10¹² viral genomes, as previously described [16]. The microinjector was held in place for 5 min before removal. MCAO/R was performed 3 weeks after AAV microinjection. The animals were subsequently divided into the following four groups (*n* = 10 per group): Sham + shRNA-NC, Sham + shRNA-Zbp1, MCAO/R + shRNA-NC, and MCAO/R + shRNA-Zbp1.

Cell transfection

Small interfering RNAs (siRNAs) targeting Zbp1, Ripk3, and their corresponding negative controls were obtained from GenePharma (Shanghai, China). The sequences were as follows: Zbp1 (sense) 5'-CCCUCAAUCAAGUCCUUUATT-3', (antisense) 5'-UAAAGGACUUGAUUGAGGGTT-3'; Ripk3 (sense) 5'-GCAGUCAAGAUCGUGA CUTT-3', (antisense) 5'-AGUUCACGAUCUUGACUGCTT-3'. HT-22 cells were seeded in 6-well plates with complete medium lacking antibiotics, followed by transient transfection with siRNA or a negative control (NC) (sense: 5'-UUCUCCGACGUGUACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3') using Rfect Transfection Reagent (BIOG, Changzhou, China) and OGD/R treatment. For Zbp1 knockdown, the cells were divided into the

si-NC, siZbp1, si-NC + OGD/R, and si-Zbp1 + OGD/R groups. For *Ripk3* knockdown, the cells were divided into the si-NC, si-Ripk3, si-NC + OGD/R, and si-Ripk3 + OGD/R groups. In the rescue experiments, following *Ripk3* siRNA transfection, the cells were subjected to OGD/R and divided into the LV-NC + si-NC + OGD/R, LV-NC + si-Ripk3 + OGD/R, LV-Zbp1 + si-NC + OGD/R, and LV-Zbp1 + si-Ripk3 + OGD/R groups. All the transfections were performed for 48 h, and the efficiency was assessed via western blot analysis and PCR analysis.

Lentiviral overexpression of Zbp1

We overexpressed Zbp1 in HT-22 cells to further investigate the role of Zbp1. The recombinant lentiviral vectors for Zbp1 (LV-Zbp1) and the empty vector (LV-NC) were provided by Gene-Pharma. Before transfection, the cells were cultured in 6-well plates with complete medium until they reached 50%–60% confluence. Puromycin (2 µg/mL; Santa Cruz Biotech, Santa Cruz, USA) was added 72 h post-transfection for the selection of transfected cells.

Cell viability and cytotoxicity assays

A calcein AM/PI kit (Beyotime) and a CCK-8 assay kit (Epizyme, Shanghai, China) were used to assess cell viability and cytotoxicity, respectively. HT-22 cells in 96-well plates were cultured overnight and subjected to different treatments. After treatment, the HT-22 cells were incubated for 2 h with 10 µL of CCK-8 reagent at 37°C. The absorbance at 450 nm was recorded with a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, USA). For the calcein AM/PI assay, the cells were incubated for 30 min with calcein AM/PI buffer after overnight treatment in 6-well plates. Dead (red, PI⁺) and live (green, calcein-AM⁺) cells were examined via fluorescence microscopy. Cytotoxicity was calculated via the following formula: cell cytotoxicity (%) = (PI⁺ cells)/(PI⁺ cells + Calcein-AM⁺ cells) × 100%. Viability was calculated as follows: cell viability = (calcein-AM⁺ cells)/(PI⁺ cells + calcein-AM⁺ cells). All the experiments were performed in triplicate.

Hoechst 33342 staining

After treatment, the cells on coverslips in 24-well plates were fixed for 30 min at room temperature (RT) with 4% paraformaldehyde (Servicebio, Wuhan, China), washed three times with PBS, stained with Hoechst 33342 (Thermo Fisher Scientific) for 30 min, and evaluated under a fluorescence microscope (Nikon, Tokyo, Japan).

Transmission electron microscopy (TEM)

HT-22 cells were harvested via trypsin digestion and centrifuged at low speed (1000 rpm) for 5 min to form visible pellets. The cell pellets were fixed in electron microscopy fixative at room temperature for 30 min in the dark and then stored at 4°C until use. The samples were washed three times with 0.1 M phosphate buffer (PB, pH 7.4) for 15 min each and subsequently post-fixed with 1% osmium tetroxide in 0.1 M PB at room temperature for 1–2 h in the dark. Following three additional washes with 0.1 M PB (15 min each), the cells were dehydrated using a graded ethanol series (15 min per step) followed by isoamyl acetate for 15 min. Ultrathin sections were prepared and examined using a Hitachi TEM system (Tokyo, Japan). Images were captured at 80.0 kV with appropriate magnification.

Tissue preparation

The animals were deeply anesthetized 24 h after sham or MCAO surgery. Mouse brains were harvested for histological analysis after cardiac perfusion with PBS and paraformaldehyde. The tissues were then immersed in paraformaldehyde overnight, dehydrated in 30% sucrose, and sectioned (20 µm). Tissues for western blot analysis were collected after cardiac perfusion with PBS without fixation. The cortex tissues of the ipsilateral infarct hemisphere were collected, snap-frozen in liquid nitrogen, and stored at –80°C until use.

Assessment of cerebral infarction volume

Cerebral infarct volumes were measured by staining with 2,3,5-triphenyltetrazolium chloride (TTC) solution (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The mice were sacrificed 24 h after reperfusion following MCAO surgery. Four 2-mm coronal brain sections were cut and incubated in 2% TTC solution for 15 min at 37°C in the dark. The sections were fixed overnight in paraformaldehyde, subsequently imaged with a digital camera, and analyzed using ImageJ software (NIH, Bethesda, USA). To correct for hemispheric edema, infarct volumes were calculated using an indirect method, as previously described [17], with the following formula: corrected infarct volume = contralateral hemisphere volume – (ipsilateral hemisphere volume – direct infarct volume). The infarct volume percentage was calculated as follows: (corrected infarct volume/contralateral hemisphere volume) × 100%.

Hematoxylin-eosin (HE) and Nissl staining

HE and Nissl staining were used to assess hippocampal and cortical histology in the ipsilateral infarct hemisphere. The sections were dewaxed, stained with HE, dehydrated, cleared with xylene, and coverslipped. The sections were subsequently treated with toluidine blue for 1 min at RT for Nissl staining, dehydrated, cleared with xylene, and coverslipped.

Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) staining

TUNEL (Servicebio) staining was used to assess apoptotic cell death in cortical tissues. DAPI was used for nuclear staining, and fluorescence was visualized via fluorescence microscopy. Images were analyzed using ImageJ.

Neurological scoring

Neurological deficits in the mice were examined with the Longa test. The scores were as follows: 4 points, unable to walk, with loss of consciousness; 3 points, collapsing on the paralyzed side when walking; 2 points, turning to the paralyzed side when walking; 1 point, unable to extend the front paw on the paralyzed side maximally; and 0 points, no deficits. Higher scores indicate worse injury. In addition to the Longa test, the modified neurological severity score (mNSS) was performed 24 h after reperfusion to assess neurological function comprehensively. The mNSS is a composite scoring system ranging from 0 (no neurological deficit) to 14 (maximal neurological deficit) that evaluates four functional domains: motor performance, sensory responses, reflex integrity, and balance capability. According to the total score, neurological impairment was classified as mild (1–4 points), moderate (5–9 points), or severe (10–14 points). Blinded investigators conducted all the assessments.

Western blot analysis

Total protein was extracted by lysing samples in ice-cold RIPA buffer (Beyotime) containing phosphatase and protease inhibitors, clarified by centrifugation, and quantified using a BCA kit (Beyotime). Proteins (20 µg/sample) were separated on 12.5% SDS-PAGE gels (Epizyme) and electroblotted onto PVDF membranes (Millipore, Billerica, USA). After being blocked, the membranes were incubated overnight with primary antibodies and then with HRP-conjugated secondary antibodies for 1 h at RT. ECL (Tanon, Beijing, China) was used for visualization, and band densities were semi-quantified using ImageJ, with β -actin/GAPDH as a reference. The primary antibodies used were as follows: 1:1000 anti-Zbp1 (AG-20B-0010; AdipoGen, Beijing, China), anti-caspase-3 (C9662; CST, Danvers, USA), anti-caspase-7 (9492T; CST), anti-BAX (2772T; CST), anti-MLKL (AP14272b; Abgent, St Louis, USA), anti-pMLKL (ab196436; Abcam, Cambridge, UK), anti-RIPK3 (17563-1-AP; Proteintech, Wuhan, China), anti-pRIPK3 (ab195117; Abcam), anti-GSDMD (ab209845; Abcam), anti- β -actin (AF5003; Beyotime), and 1:2000 anti-GAPDH (SB-AB0037; Share-Bio.) antibodies. The secondary antibody used was 1:1000 HRP-AffiniPure goat anti-rabbit IgG (A0208; Beyotime).

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected via cardiac puncture into serum collection tubes and left undisturbed for 30 min at RT. The samples were subsequently centrifuged at 1000 *g* for 10 min at 4°C, after which the serum was transferred to fresh tubes. Serum IL-1 β and IL-18 concentrations were measured using commercial ELISA kits (IL-1 β : Cat. #RK04599; IL-18: Cat. #RK00104; ABclonal, Wuhan, China) according to the manufacturer's directions, with duplicate analysis for reproducibility.

Immunoprecipitation and mass spectrometry analysis (IP-MS)

The cells were lysed in IP lysis buffer (Beyotime). The cell lysates were centrifuged at 16,000 *g* for 20 min, and protein A/G agarose was added to the supernatant. After overnight incubation at 4°C, the protein A/G beads were washed, and the immune complexes were eluted. SDS-PAGE and western blot analysis were used to separate and identify the eluted proteins. For immunoprecipitation followed by mass spectrometry (IP-MS), proteins were visualized by silver staining of the SDS-PAGE gel.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using the EZ-press RNA Purification Kit (EZBioscience, Roseville, USA). An aliquot of 1 µg of RNA from each sample was reverse transcribed to cDNA via 4 $\times$ Reverse Transcription Master Mix with gDNA remover (EZBioscience) and amplified using Color SYBR Green qPCR Mix (EZBioscience) on a StepOne Plus Real-Time PCR instrument (Applied Biosystems, Foster City, USA). All steps were performed according to the manufacturer's directions. Relative mRNA expression compared with that of *GAPDH* was determined using the $2^{-\Delta\Delta C_t}$ method. The sequences of primers used for qRT-PCR were as follows: *Zbp1* (F), 5'-CACATCTCCATTGCCAATTCAA-3' and (R), 5'-TGTCCATGTA GATGTACTGAGC-3'; *Ripk3* (F), 5'-GAAGACACGGCACTCCTTGG TATC-3' and (R), 5'-CTTGAGGCAGTAGTTCTTGGTGGTG-3'; and *GAPDH* (F) 5'-AGGTCGGTGTGAACGGATTG-3' and (R), 5'-TG TAGACCATGTAGTTGAGGTCA-3'.

Statistical analysis

Data were analyzed using GraphPad Prism. Data are presented as the mean $\pm$ standard deviation (SD) obtained from at least three replicates. Student's *t* tests were used for two-group comparisons. For the Longa test and mNSSs, the Kruskal-Wallis test was employed for multiple-group comparisons, followed by Dunn's post hoc test. Other multiple-group comparisons of continuous data were analyzed using one-way ANOVA with Tukey's post hoc test. *P* < 0.05 was considered to indicate statistical significance.

Results

OGD/R induces Zbp1 upregulation and PANoptosis in HT-22 cells

The cells were subjected to OGD/R (OGD, 6 h; R, 24 h). *Zbp1* mRNA and protein levels were elevated after OGD/R compared with those in the controls (Figure 1A). Western blot analysis revealed increased Bax, cleaved caspase-3, and cleaved caspase-7 levels, with no changes in total caspase-3 or caspase-7 levels, indicating the induction of apoptosis (Figure 1B,C). The upregulation of phosphorylated Ripk3 (p-Ripk3), phosphorylated Mlkl (p-Mlkl), Ripk3, and Mlkl expressions indicated necroptosis (Figure 1B,C). The expression of Gsdmd, a pyroptosis driver, was also upregulated (Figure 1B,C), suggesting that PANoptosis was induced. Hoechst 33342 staining revealed significant cell death (Figure 1D), and the CCK-8 and calcein AM/PI assays revealed increased cytotoxicity and reduced viability (Figure 1E). Therefore, OGD/R caused significant cell injury.

Zbp1 knockdown in HT-22 cells mitigates damage and PANoptosis following OGD/R

Zbp1 was knocked down in HT-22 cells using si-*Zbp1* to examine its role in OGD/R-induced damage and cell death. qRT-PCR and western blot analysis confirmed that *Zbp1* expression was reduced after transfection (Figure 2A). Western blot analysis revealed lower expression levels of apoptosis-related proteins (Bax, cleaved caspase-3, and cleaved caspase-7), necroptosis-related proteins (p-Mlkl, p-Ripk3, Mlkl, and Ripk3), and the pyroptosis marker Gsdmd in si-*Zbp1* + OGD/R-treated cells (Figure 2B,C), indicating reduced PANoptosis. Hoechst 33342 staining and CCK-8 assays revealed reduced cell death and increased viability in si-*Zbp1* + OGD/R-treated cells (Figure 2D). Calcein AM/PI assays further confirmed fewer PI⁺ cells and a greater number of Calcein⁺ cells in the knockdown group (Figure 2E). We performed TEM analysis of HT-22 cells under different treatment conditions to obtain ultrastructural data (Figure 2F). The control cells (si-NC and si-*Zbp1*) presented a normal cellular morphology with intact nuclear membranes and a well-organized cytoplasm. In contrast, si-NC + OGD/R-treated cells presented severe ultrastructural damage, including marked chromatin condensation and margination, cytoplasmic vacuolization, and organelle swelling. Compared with si-NC + OGD/R, *Zbp1* knockdown (si-*Zbp1* + OGD/R) partially preserved the cellular architecture, resulting in less severe chromatin changes and reduced cytoplasmic damage. Thus, *Zbp1* knockdown reduced OGD/R-induced cell damage.

Zbp1 overexpression augments HT-22 cell damage and PANoptosis

Zbp1 was overexpressed by transfecting HT-22 cells with recombinant lentiviral vectors (LV-*Zbp1*). *Zbp1* levels were significantly

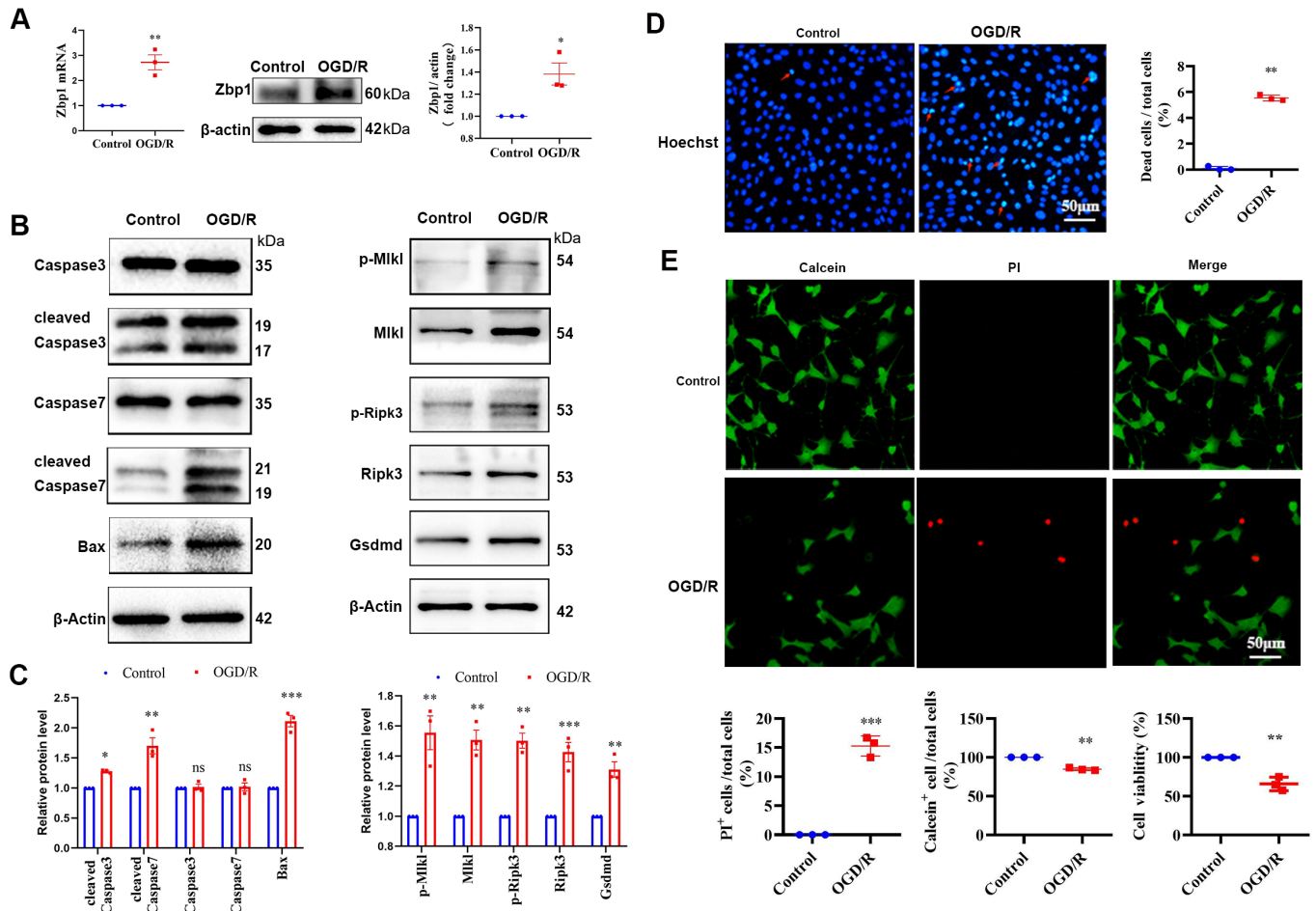


Figure 1. Zbp1 expression is upregulated and PANoptosis is induced in HT-22 cells following OGD/R (A) Zbp1 mRNA and protein levels after OGD/R were analyzed using qRT-PCR and western blot analysis. (B,C) Western blot analysis of key apoptotic proteins (cleaved caspase-3, caspase-7, and Bax), necroptotic proteins (p-MiK1, p-Ripk3, total MiK1, and total Ripk3), and pyroptotic protein (Gsdmd). (D) Hoechst 33342 staining was used to assess nuclear damage, with condensed/fragmented nuclei indicating cell death. (E) Calcein AM/PI staining and CCK-8 assays were used to evaluate cell viability and cytotoxicity. Calcein⁺ (green) indicates live cells; PI⁺ (red) indicates dead cells. Scale bar: 50 μ m. Data are presented as the mean $\pm$ SD; * P < 0.05, ** P < 0.01, *** P < 0.001, and ns, not significant (vs control). n = 3 per group.

elevated in the LV-Zbp1 group (Figure 3A). Western blot analysis revealed increased expressions of apoptosis markers, including Bax, cleaved caspase-3, and cleaved caspase-7, in the LV-Zbp1 + OGD/R group compared with the control group (Figure 3B,C). The expression levels of necroptosis markers (p-MiK1, p-Ripk3, total MiK1, and total Ripk3) and the pyroptosis protein Gsdmd were also increased in the LV-Zbp1 + OGD/R group (Figure 3B,C). These results indicate that Zbp1 overexpression exacerbates PANoptosis after OGD/R. Hoechst 33342 staining revealed increased cell death in the LV-Zbp1 + OGD/R group (Figure 3D). CCK-8 and calcein AM/PI assays confirmed the reduced cell viability and increased cytotoxicity in the LV-Zbp1 + OGD/R group (Figure 3E). Thus, Zbp1 overexpression worsened OGD/R-induced cell damage.

Zbp1 knockdown reduces brain damage following MCAO/R in mice

To investigate whether Zbp1 knockdown had similar effects *in vivo*, we injected AAV-shRNA-Zbp1 and AAV-shRNA-NC into the right lateral ventricles 3 weeks before MCAO surgery. Western blot analysis confirmed that Zbp1 expression was lower in the ipsilateral

cortex of AAV-shRNA-Zbp1 mice than in that of AAV-shRNA-NC mice (Figure 4A). TTC staining revealed reduced infarct volumes in the AAV-shRNA-Zbp1 + MCAO/R group relative to those in the AAV-shRNA-NC + MCAO/R group (Figure 4B). To comprehensively evaluate neurological function, we performed a Longa test and mNSS assessment 24 h after reperfusion. Compared with those of the control mice, the Longa test scores of the AAV-shRNA-Zbp1 mice were lower 24 h after reperfusion. Consistent with these findings, the mNSS evaluation revealed significantly improved neurological outcomes in the AAV-shRNA-Zbp1 + MCAO/R group compared with the AAV-shRNA-NC + MCAO/R group (Figure 4C). These behavioral assessments suggest that Zbp1 knockdown may contribute to neuroprotection and functional recovery following ischemic stroke. We subsequently measured serum cytokine levels to assess systemic inflammatory responses. ELISAs revealed that the IL-1 β and IL-18 concentrations were significantly greater in the AAV-shRNA-NC + MCAO/R group than in the sham control group. Notably, Zbp1 knockdown substantially reduced cytokine levels in AAV-shRNA-Zbp1 + MCAO/R mice (Figure 4D). HE staining revealed well-organized and normal cortical and hippocampal cells in

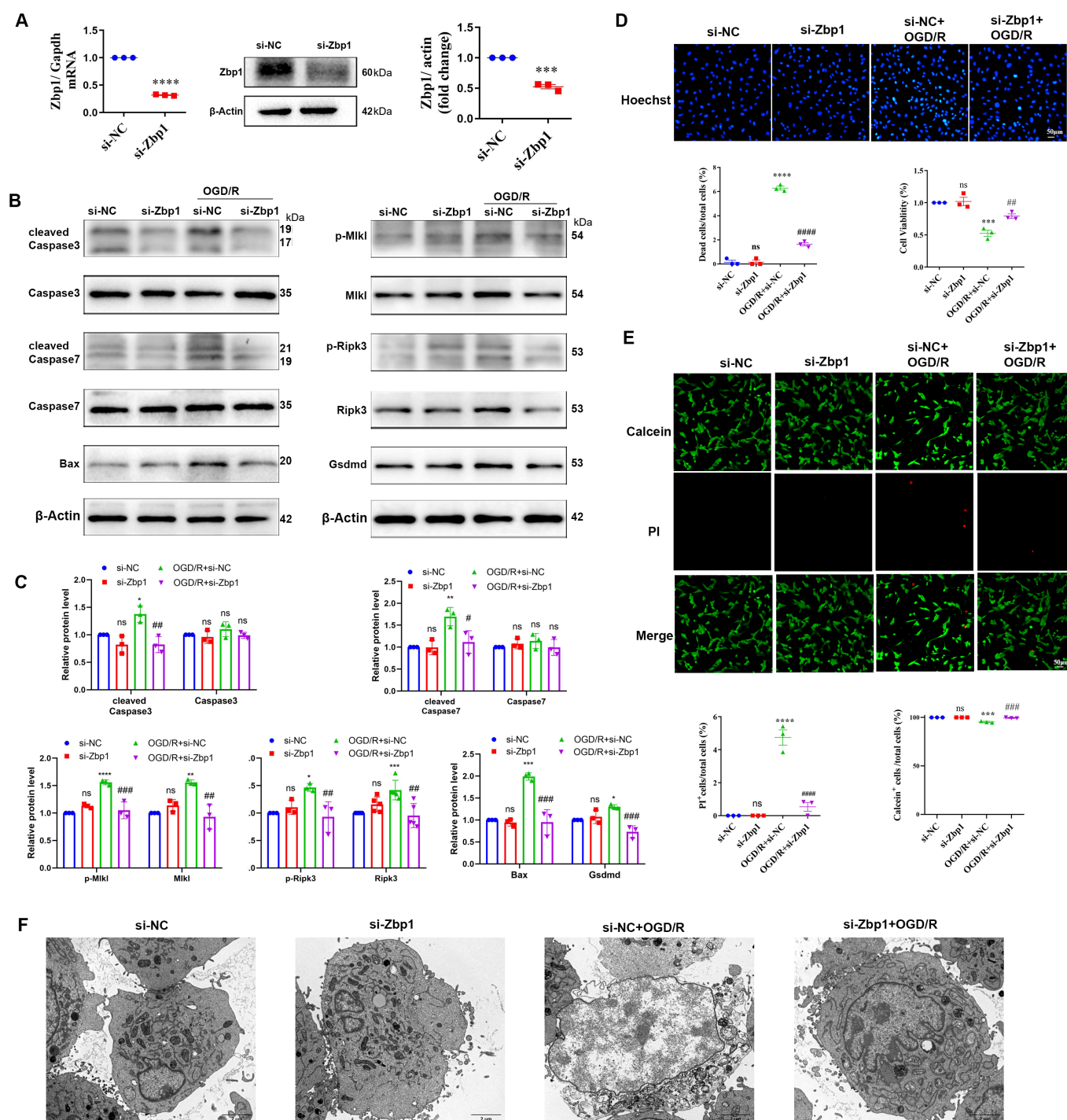


Figure 2. Zbp1 knockdown reduces PANoptosis and cell damage in HT-22 cells after OGD/R (A) qRT-PCR and western blot analysis of Zbp1 expression 48 h after transfection with si-Zbp1 or si-NC. (B,C) Western blot analysis of apoptotic proteins (cleaved caspase-3, caspase-7, and Bax), necroptotic (p-MiK1, p-Ripk3, total MiK1, and total Ripk3), and pyroptotic (Gsdmd) proteins. (D) Hoechst 33342 staining and CCK-8 assays were used to measure cell damage and viability. (E) Calcein AM/PI staining was used to assess cell viability and cytotoxicity [calcein⁺ (green) and PI⁺ (red)]. Scale bar: 50 μm. (F) Representative TEM images of HT-22 cells. Scale bar: 2 μm. $n \geq 3$ per group; data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ vs si-NC; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, and #### $P < 0.0001$ vs OGD/R + si-NC; and ns, not significant.

the sham group, whereas the AAV-shRNA-NC group exhibited loose cell arrangement and nuclear shrinkage after MCAO. These changes were less pronounced in the AAV-shRNA-Zbp1 mice (Figure 4E).

Similar improvements were observed through Nissl staining (Figure 4F), indicating that Zbp1 knockdown mitigated cerebral damage after MCAO.

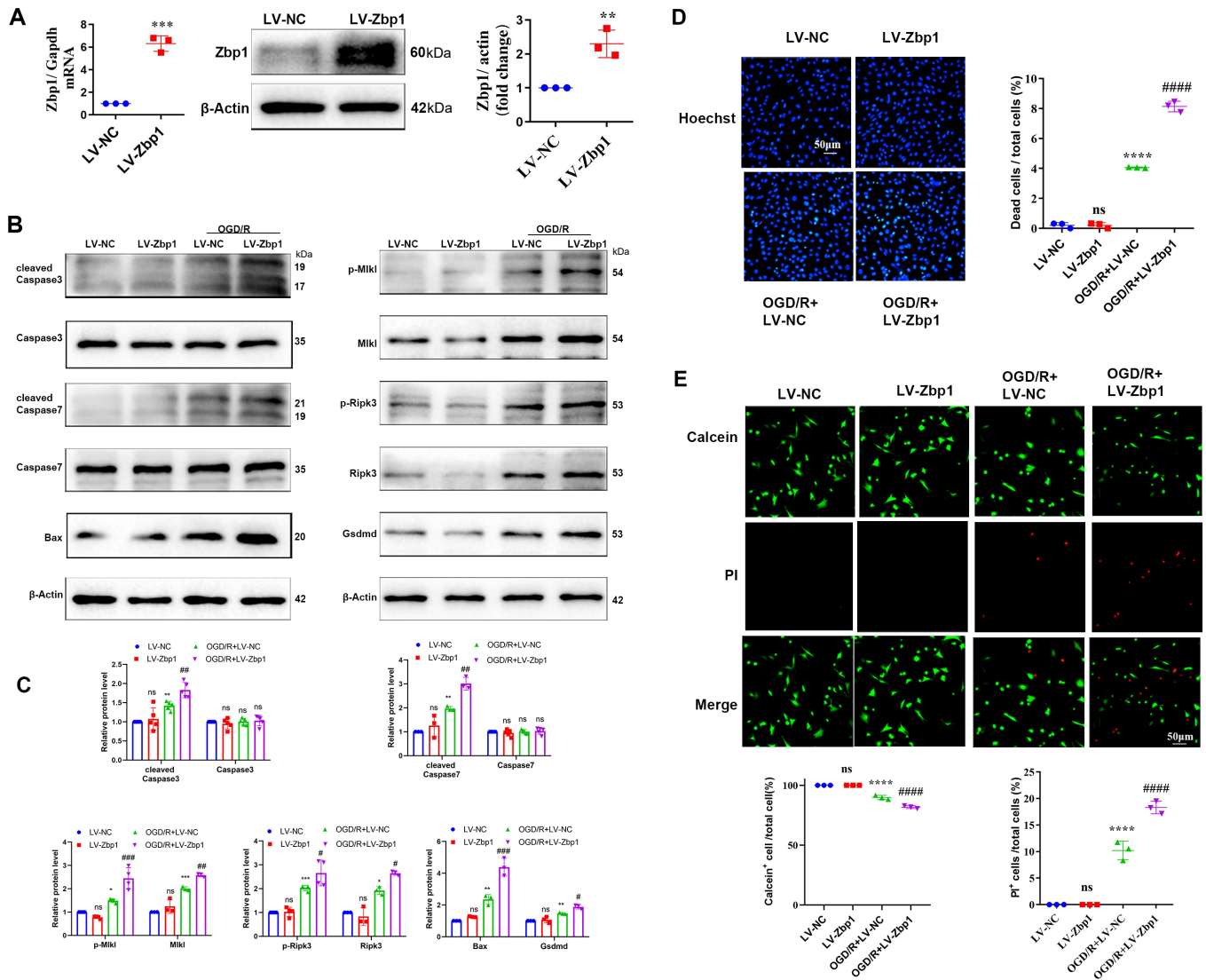


Figure 3. Zbp1 overexpression exacerbates HT-22 cell damage and PANoptosis following OGD/R (A) qRT-PCR and Western blot analysis of Zbp1 expression after transfection with LV-Zbp1 or LV-NC. (B,C) Western blot analysis of apoptotic proteins (cleaved caspase-3, caspase-7, and Bax), necroptotic proteins (p-Mkl1, p-Ripk3, total Mkl1, and total Ripk3), and pyroptotic protein (Gsdmd). (D) Hoechst 33342 staining was used to assess cell damage. (E) Calcein AM/PI staining was used to evaluate cell viability and cytotoxicity [calcein⁺ (green) and PI⁺ (red)]. Scale bar: 50 μ m. $n \geq 3$ per group; data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ vs LV-NC; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, and #### $P < 0.0001$ vs OGD/R + LV-NC; and ns, not significant.

Zbp1 knockdown reduces cell death and attenuates PANoptosis following MCAO/R in mice

Fewer TUNEL⁺ cells were observed in the cortices of the AAV-shRNA-Zbp1 mice than in those of the control mice after MCAO surgery (Figure 5A,B). Western blot analysis revealed increased levels of apoptosis markers (Bax, cleaved caspase-3, and cleaved caspase-7) in the ipsilateral cortices of AAV-shRNA-NC + MCAO/R mice, which were significantly reduced by Zbp1 knockdown (Figure 5C). Similarly, p-Ripk3, p-Mkl1, total Ripk3, total Mkl1, and Gsdmd levels followed a comparable trend. These results suggest that Zbp1 knockdown reduced PANoptosis following MCAO/R in mice.

Ripk3 knockdown alleviates HT-22 cell damage and PANoptosis after OGD/R

The above findings suggest that Zbp1 may regulate PANoptosis in

CIRI; however, the exact mechanism remains unclear. We employed IP-MS and analyzed the STRING database to identify potential Zbp1-associated proteins. Ripk3 was found to be a potential Zbp1-associated protein (Figure 6A–C). Consistent with previous results, OGD/R upregulated Ripk3 expression, which was decreased upon Zbp1 knockdown but increased with Zbp1 overexpression. These findings suggest that Zbp1 may regulate PANoptosis through Ripk3.

Ripk3 siRNA or a negative control was transfected into HT-22 cells to further investigate the role of Ripk3 in PANoptosis under OGD/R. Ripk3 expression (at both the mRNA and protein levels) was significantly reduced in Ripk3-silenced cells (Figure 6D). Western blot analysis revealed lower levels of cleaved caspase-3, cleaved caspase-7, and Bax in si-Ripk3 + OGD/R-treated cells than in control cells (Figure 6E), with similar trends observed for p-Mkl1,

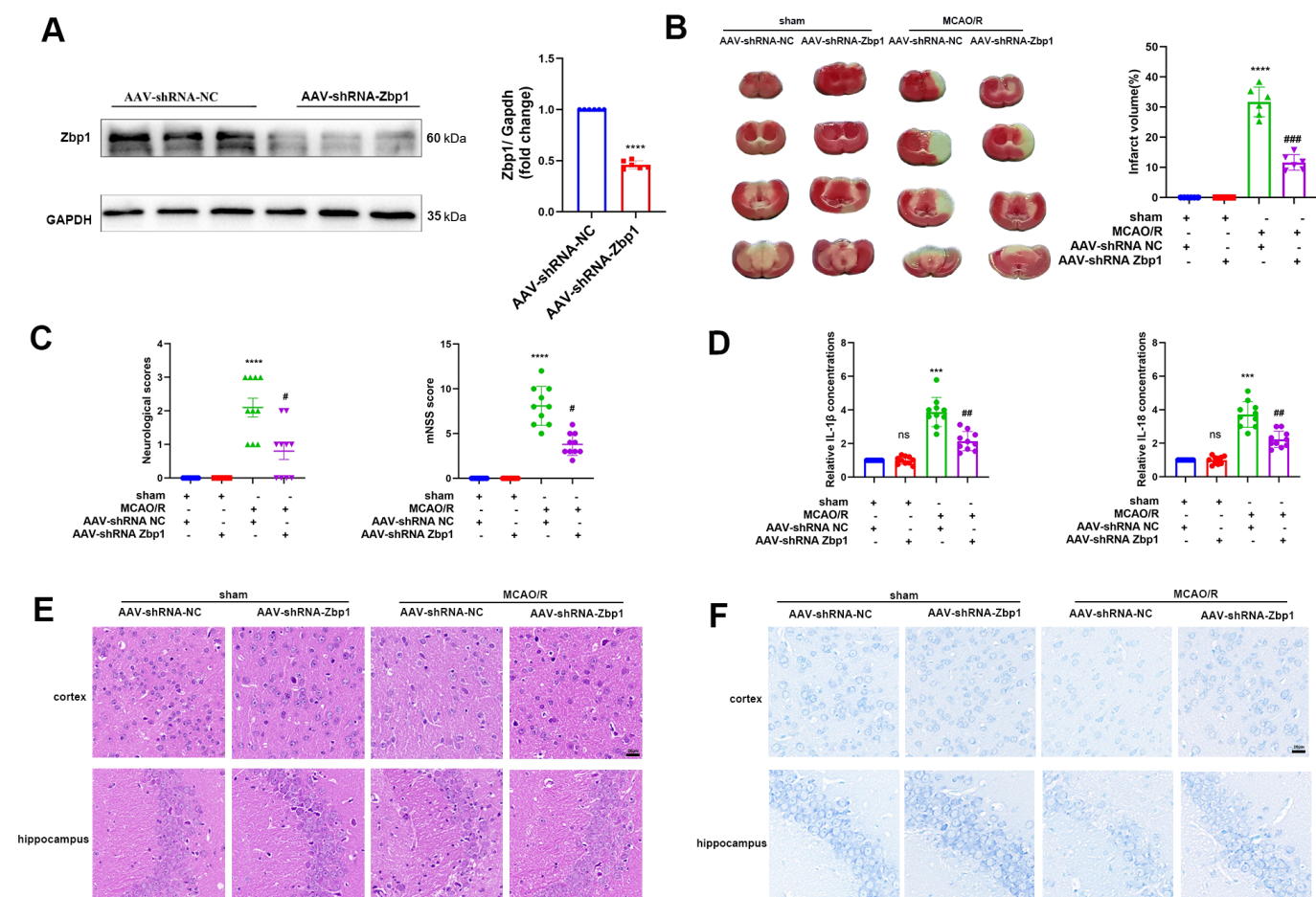


Figure 4. Zbp1 knockdown alleviates brain injury following MCAO/R in mice (A) Western blot analysis of Zbp1 protein expression ($n = 6$). (B) TTC staining of cerebral infarct volumes and quantification of infarct volumes ($n = 6$). (C) Neurological scores were assessed via the Longa test and the mNSS in mice ($n = 10$). (D) ELISA results showing the serum IL-1 β and IL-18 concentrations in the different treatment groups ($n = 10$). (E,F) HE and Nissl staining of ipsilateral cortical and hippocampal sections ($n = 6$). Scale bar: 20 μ m. Data are presented as the mean $\pm$ SD. *** $P < 0.001$, **** $P < 0.0001$ vs AAV-shRNA-NC; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ vs MCAO/R + AAV-shRNA-NC; and ns, not significant.

total Mlkl, and Gsdmd (Figure 6E). These results suggest that *Ripk3* knockdown reduced PANoptosis following OGD/R.

Hoechst 33342 staining revealed significantly lower cell death rates in si-*Ripk3* + OGD/R cells than in control cells (Figure 6F), whereas calcein AM/PI assays revealed reduced cytotoxicity and increased viability in si-*Ripk3* + OGD/R cells (Figure 6G). These findings demonstrate that *Ripk3* knockdown mitigated OGD/R-induced cell damage.

Ripk3 knockdown partially reverses the cell damage and PANoptosis induced by Zbp1 overexpression and OGD/R in HT-22 cells

To determine whether Zbp1 regulates OGD/R-induced cell injury and PANoptosis through *Ripk3*, we transfected *Ripk3* siRNA into HT-22 cells overexpressing Zbp1. Western blot analysis revealed that Zbp1 overexpression increased the levels of cleaved caspase-3, cleaved caspase-7, and Bax 24 h after reperfusion; this effect was partially reversed by *Ripk3* knockdown (Figure 7A). Similarly, the p-Mlkl, total Mlkl, and Gsdmd levels followed the same trend (Figure 7A). Zbp1 overexpression increased cell death and the percentage of PI⁺ cells, which were partially reversed by *Ripk3*

knockdown (Figure 7B,C). These results indicate that *Ripk3* knockdown partially reversed the cell damage and PANoptosis induced by Zbp1 overexpression in HT-22 cells.

Discussion

CIRI is a complex pathological condition characterized by extensive neuronal loss, largely driven by dysregulated programmed cell death pathways [2]. Recent studies suggested that PANoptosis, a unique inflammatory form of regulated cell death, plays a pivotal role in ischemia-reperfusion injury [5]. Appropriate molecular markers are essential for distinguishing PANoptosis from individual death pathways in CIRI. In the present study, we evaluated representative markers from each of the three constituent pathways, which is consistent with established approaches in recent PANoptosis investigations [6,12,13]: apoptosis (Bax and cleaved caspase-3/7), necroptosis (*Ripk3*, Mlkl, and their phosphorylated forms), and pyroptosis (Gsdmd). The concurrent upregulation of markers from all three pathways serves as a hallmark of PANoptosis, distinguishing this mode of cell death from the isolated activation of individual cell death mechanisms.

We identified the Zbp1-*Ripk3* axis as a central mediator of

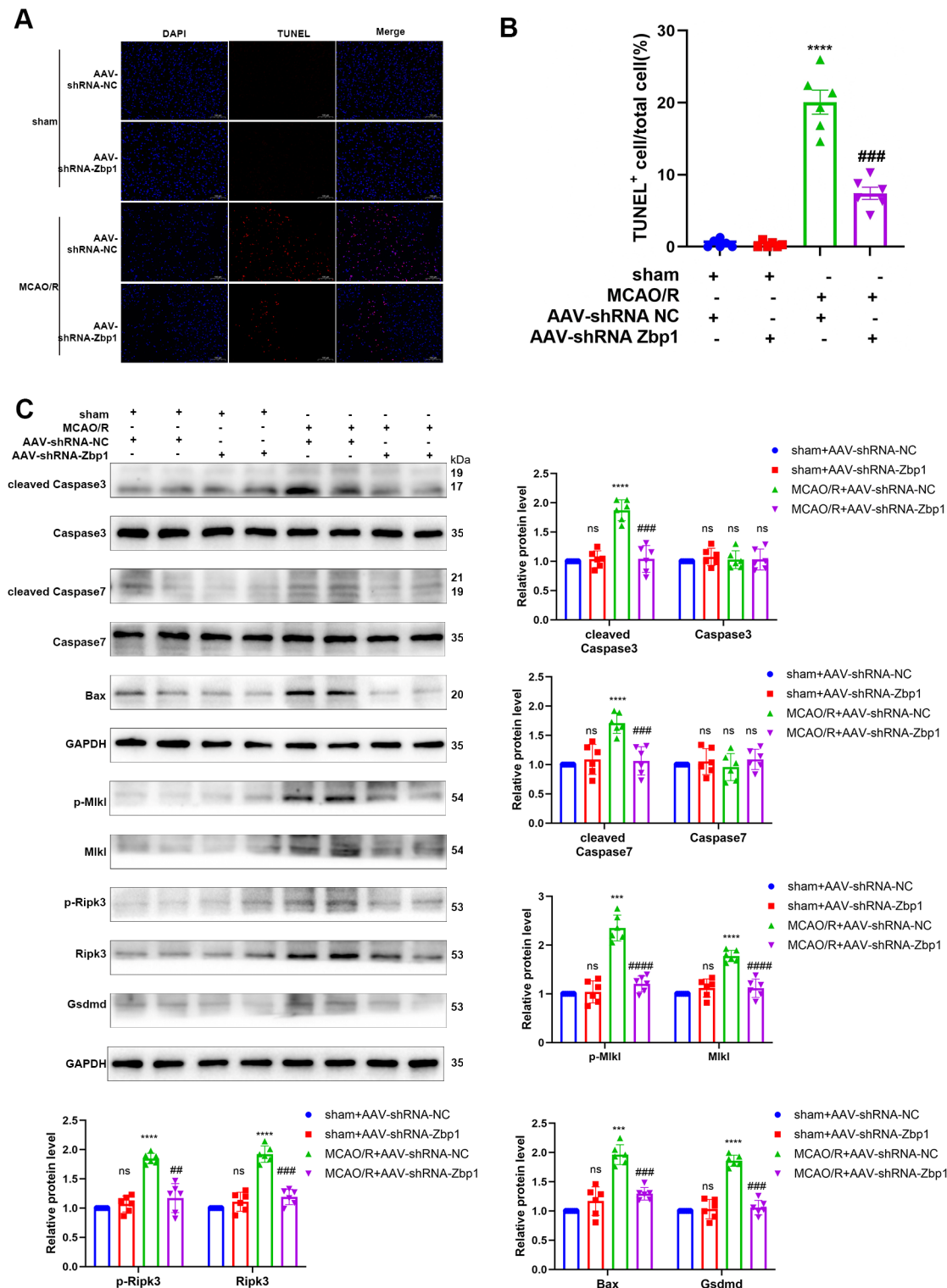


Figure 5. Zbp1 knockdown reduces cell death and attenuates PANoptosis following MCAO/R in mice (A) TUNEL staining of cortical sections from mice subjected to MCAO/R or sham surgery. Scale bar: 100 μ m. (B) Quantification of TUNEL⁺ cell ratios ($n = 6$). (C) Western blot analysis of PANoptosis-related proteins, including apoptotic proteins (cleaved caspase-3, cleaved caspase-7, and Bax), necroptotic proteins (p-MiIκ, total MiIκ, p-Ripk3, and total Ripk3), and pyroptotic protein (Gsdmd) ($n = 6$). Data are presented as the mean $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ vs Sham + AAV-shRNA-NC; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ vs MCAO/R + AAV-shRNA-NC; and ns, not significant.

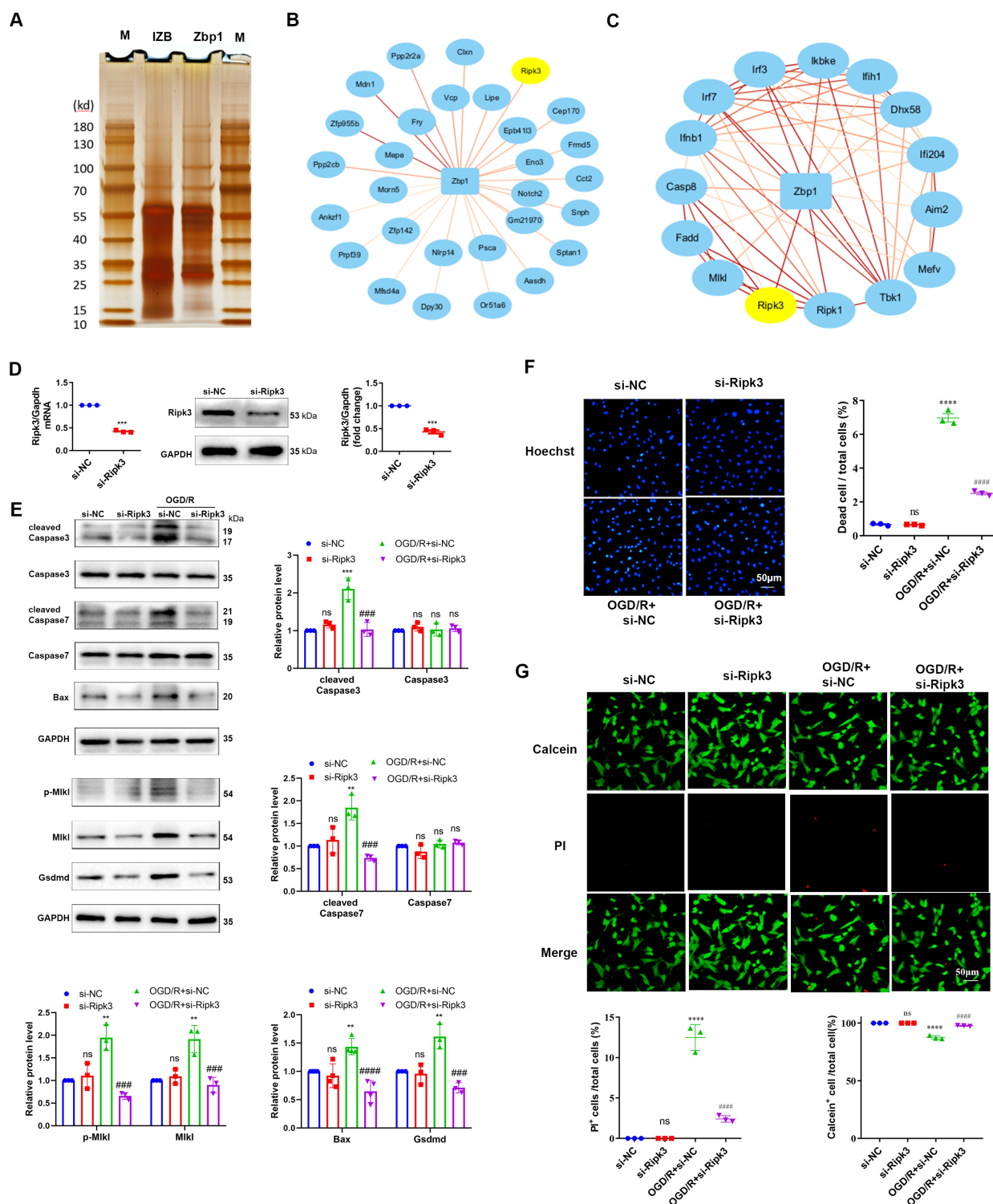


Figure 6. *Ripk3* knockdown mitigates HT-22 cell damage and PANoptosis following OGD/R (A) Immunoprecipitation with a Zbp1 antibody, followed by silver staining and mass spectrometry-based protein identification. (B) MS analysis of Zbp1-interacting proteins, with the line color intensity indicating LFQ intensity values. (C) STING database prediction of Zbp1-interacting proteins, with the line color intensity reflecting combined score values. (D) qRT-PCR and western blot analysis of *Ripk3* expression 48 h after transfection with si-*Ripk3* or si-NC. (E) Western blot analysis of apoptotic proteins (cleaved caspase-3, caspase-7, and Bax), necroptotic proteins (p-MiKl and total MiKl), and pyroptotic protein (Gsdmd). (F) Hoechst 33342 staining was used to assess cell damage. Scale bar: 50 μ m. (G) Calcein AM/PI staining was used to evaluate cell viability and cytotoxicity [calcein⁺ (green) and PI⁺ (red)]. Scale bar: 50 μ m. Data are presented as the mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001 vs si-NC; # P < 0.05, ## P < 0.01, ### P < 0.001, and **** P < 0.0001 vs OGD/R + si-NC; and ns, not significant.

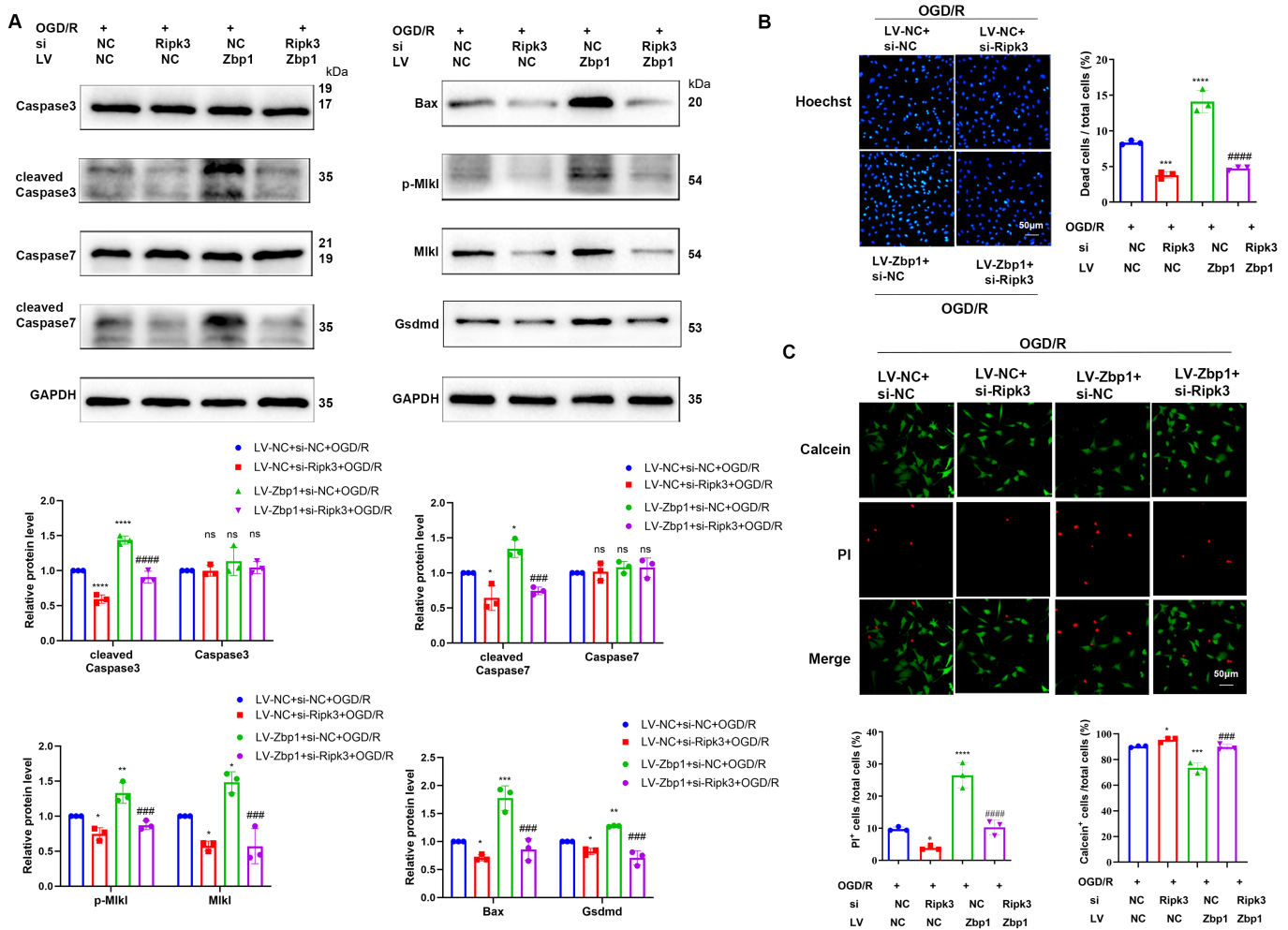


Figure 7. *Ripk3* knockdown partially reverses *Zbp1* overexpression-induced cell damage and PANoptosis in HT-22 cells (A) Western blot analysis of apoptotic proteins (cleaved caspase-3, caspase-7, and Bax), necroptotic proteins (p-MiKl and total MiKl), and pyroptotic protein (Gsdmd). (B) Hoechst 33342 staining was used to assess cell damage. Scale bar: 50 μ m. (C) Calcein AM/PI staining was used to evaluate cell viability and cytotoxicity [calcein⁺ (green) and PI⁺ (red)]. Scale bar: 50 μ m. $n = 3$ per group; data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ vs si-NC + LV-NC + OGD/R; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, and #### $P < 0.0001$ vs si-NC + LV-Zbp1 + OGD/R; and ns, not significant.

PANoptosis in CIRI. Our findings demonstrate that Zbp1 expression is significantly upregulated following ischemia-reperfusion, promoting neuronal injury through PANoptotic mechanisms. Notably, *Zbp1* knockdown effectively reduced the infarct volume, mitigated neuronal damage, and enhanced neurological recovery. Conversely, its overexpression exacerbated ischemic injury. Furthermore, we established Ripk3 as a downstream effector of Zbp1; *Ripk3* knockdown attenuated PANoptosis and partially reversed Zbp1-induced neuronal injury. Collectively, these findings define the Zbp1-Ripk3 axis as a key driver of PANoptosis in CIRI, highlighting its potential as a therapeutic target for ischemic stroke.

Emerging evidence suggests that PANoptosis is a common pathological mechanism across various organ ischemia-reperfusion injuries. Zhang *et al.* [18] identified IRF1 and IL-1A as PANoptosis-associated biomarkers of lung ischemia-reperfusion injury, and their elevated expression correlated with primary graft dysfunction. Similarly, in hepatic ischemia-reperfusion injury, Wu *et al.* [19] revealed that STING-mediated mtDNA release triggers hepatocyte PANoptosis, with STING deficiency significantly improving liver

function. The retina also exhibits PANoptosis following ischemia-reperfusion injury. Wan *et al.* [20] demonstrated that VDAC1 oligomerization drives PANoptosis through mitochondria-derived reactive oxygen species. Building upon our previous bioinformatics analysis [10], the present study provides direct experimental evidence that PANoptosis occurs in ischemic neurons, with concurrent upregulation of apoptotic, necroptotic, and pyroptotic markers following OGD/R and MCAO/R. Notably, while the specific molecular triggers may vary across tissues, ranging from STING activation in the liver to VDAC1 oligomerization in the retina, the convergence of PANoptotic cell death appears to be a unifying feature of ischemia-reperfusion pathophysiology. Furthermore, Qiu *et al.* [21] demonstrated that the m⁶A demethylase ALKBH5 protects against CIRI by suppressing SNHG3-mediated PANoptosis in astrocytes, suggesting that PANoptosis occurs in multiple cell types within the ischemic brain and that epigenetic regulation plays a crucial role in its activation. Importantly, therapeutic interventions targeting PANoptosis have shown promise across different organ systems. Collectively, these findings support the hypothesis that

PANoptosis contributes to ischemia-reperfusion pathophysiology and warrants investigation as a potential therapeutic target.

The role of Zbp1 in regulating cell death and inflammation demonstrates remarkable heterogeneity across different tissues and disease contexts. While Zbp1 has been characterized as a pro-inflammatory mediator that promotes PANoptosis in immune cells and various pathological conditions [22], emerging evidence has revealed that it has paradoxical functions. Zbp1 promotes retinal ganglion cell death through PANoptosis activation in acute glaucoma models [23] and acts as a pathogenic factor in hepatic and endothelial ischemia-reperfusion injury [24,25]. However, Enzan *et al.* [9] reported an unexpected protective role of Zbp1 in heart failure, where it suppresses myocardial inflammation. Our current study demonstrated that Zbp1 overexpression promotes neuronal injury in CIRI, serving as a key driver of PANoptosis.

The contrasting roles of Zbp1 in cardiac tissue warrant careful examination. While Enzan *et al.* [9] reported that Zbp1 protects against myocardial inflammation in heart failure, other studies, including that of Cui *et al.*, demonstrated that Zbp1 promotes cardiac injury during myocardial ischemia-reperfusion [24]. These seemingly contradictory findings might be reconciled by considering the following potential factors: (1) Disease severity: chronic heart failure represents a gradual pathological process with moderate inflammation, whereas ischemia-reperfusion injury involves acute, severe oxidative stress and massive cell death, which may differentially activate Zbp1 signaling pathways. A recent study suggested that the intensity of cellular stress determines cell fate decisions between survival and death pathways [26]. (2) Activation threshold: it is possible that under mild stress conditions, Zbp1 functions as a homeostatic regulator, while severe injury triggers pro-death signaling. This severity-dependent switch in protein function has been observed in other cardiac stress response pathways [27]. (3) Metabolic status: the metabolic environment in chronic heart failure patients differs substantially from that in acute ischemic injury patients, potentially affecting Zbp1 downstream signaling [28]. (4) Compensatory mechanisms: under chronic conditions, cells may develop adaptive responses that modify Zbp1 function. Such protective adaptations in chronic cardiac stress have been documented [29].

In our neuronal CIRI model, acute injury severity combined with neuronal vulnerability to ischemic stress [30] likely explains why Zbp1 acts predominantly as a PANoptosis driver. However, we acknowledge that these explanations remain speculative and require experimental validation. This context-dependent functionality suggests that the role of Zbp1 is determined by the specific cellular environment and pathological context; however, further mechanistic studies are needed.

Zbp1 is a well-established nucleic acid sensor that detects Z-DNA, Z-RNA, and mtDNA, triggering downstream PANoptotic pathways [31]. The role of Zbp1 in infection-induced inflammation and PANoptosis has been extensively characterized; however, its involvement in sterile tissue injury, particularly CIRI, remains largely unexplored. Our study provides the first direct evidence that Zbp1 expression is markedly upregulated in CIRI. Given that ischemia-reperfusion induces mitochondrial dysfunction and subsequent mtDNA release [32], it is possible that Zbp1 recognizes cytoplasmic mtDNA as a key upstream activator of PANoptotic signaling, thereby contributing to neuronal cell death. However, whether Zbp1 activation in ischemic neurons is directly mediated

by mtDNA binding or through alternative stress pathways warrants further investigation.

As a key downstream effector of Zbp1, Ripk3 has been widely recognized for its role in necroptosis through M1k phosphorylation. However, recent evidence suggested that the role of Ripk3 extends beyond necroptosis, mediating apoptotic signaling within the PANoptotic framework [33]. Our IP-MS screening experiments identified Ripk3 as a potential Zbp1-associated protein, and functional studies demonstrated that *Ripk3* knockdown partially rescued Zbp1-induced neuronal injury. These findings strongly suggest a functional relationship between Zbp1 and Ripk3 in PANoptosis; however, direct co-immunoprecipitation experiments are needed to confirm whether these proteins physically interact with or function through intermediate proteins. The partial rescue effect of *Ripk3* knockdown could indicate either the involvement of additional downstream effectors or an indirect regulatory mechanism.

Although this study provides novel insights, several limitations must be acknowledged. First, while our IP-MS screening and functional studies suggest a regulatory relationship between Zbp1 and Ripk3, the absence of reciprocal co-immunoprecipitation experiments prevents us from confirming direct physical interactions. Second, our research focused primarily on the Zbp1-Ripk3 axis, leaving the roles of other PANoptotic regulators (e.g., Ripk1 and Tak1) unexplored. The precise mechanisms underlying Zbp1 activation in CIRI remain unclear, particularly whether mtDNA or other DAMPs serve as key triggers. Additionally, the absence of *in vivo* Zbp1 overexpression experiments represents an asymmetry in our experimental design that should be addressed in future studies. Finally, the use of HT-22 cells and MCAO/R mice may not fully represent the complexity of PANoptotic regulation across diverse neuronal populations.

Future research should focus on elucidating the precise mechanisms of Zbp1 activation in ischemic neurons, particularly its potential interactions with mtDNA and other DAMPs. Additionally, identifying downstream effectors beyond Ripk3 will be crucial for fully understanding the role of Zbp1 in PANoptosis. Exploring Zbp1-targeted therapeutic strategies may offer novel translational opportunities for ischemic stroke treatment.

In summary, our findings establish Zbp1 as a central regulator of PANoptotic cell death in CIRI. Furthermore, the Zbp1-Ripk3 axis has emerged as a key driver of ischemic neuronal injury, indicating that Zbp1 is a promising therapeutic target. Future research should prioritize the development of pharmacological strategies to inhibit Zbp1-mediated PANoptosis, potentially offering novel neuroprotective approaches for ischemic stroke.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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