

ELAVL1 Stabilizes HMOX1 mRNA to Drive Ferroptosis in Diabetic Retinopathy

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Objective: This study aims to investigate the role of heme oxygenase 1 (HMOX1) in the pathogenesis of diabetic retinopathy (DR) and identify the RNA-binding protein embryonic lethal abnormal vision-like 1 (ELAVL1) as a novel regulator of HMOX1 mRNA stability driving ferroptosis, proposing ELAVL1 as a potential therapeutic target for DR.

Methods: DR was modeled in vitro using high glucose (HG)-treated ARPE19 cells and in vivo via streptozotocin (STZ)-induced diabetic rats. Cell damage was assessed through measuring cell viability, oxidative stress markers, iron content, and ferroptosis-related protein expression. HMOX1 expression and its regulatory interaction with ELAVL1 were analyzed using RT-qPCR, immunohistochemistry, Western blotting, RNA-fluorescence in situ hybridization, and RNA immunoprecipitation. Functional roles were validated by gene knockdown or overexpression experiments.

Results: HG stimulation induced ferroptosis-related damage and oxidative stress in ARPE19 cells and the retinas of DR rats, accompanied by a significant upregulation of HMOX1 expression (approximately 165% in vivo and 189% in vitro). Knockdown of either HMOX1 or ELAVL1 effectively suppressed ferroptosis and mitigated retinal degeneration.

Conclusion: HMOX1 is critical for ferroptosis-mediated retinal damage in DR, and ELAVL1 promotes ferroptosis predominantly by stabilizing HMOX1 mRNA.

Keywords: ELAVL1, HMOX1, diabetic retinopathy, streptozotocin, ferroptosis

Introduction

The escalating global prevalence of diabetes has precipitated a parallel rise in diabetic retinopathy (DR), imposing substantial socioeconomic burdens on healthcare systems.^{1,2} Recent epidemiological data indicate that more than 103 million individuals currently live with DR worldwide, with projections suggesting a rise to 161 million by 2045.³ Oxidative stress is recognized as a fundamental pathological mechanism in DR, where reactive oxygen species (ROS) overproduction triggers lipid peroxidation cascades that compromise retinal structure and function.⁴ Current standard-of-care interventions for DR, including retinal laser photocoagulation, intravitreal anti-vascular endothelial growth factor (anti-VEGF) agents, and vitrectomy, primarily target late-stage vascular complications.⁵ Critically, these approaches fail to halt early neural degeneration or reverse established vascular damage.⁶ Additionally, suboptimal patient responses to anti-VEGF therapies and injection-related complications further limit clinical efficacy. These therapeutic constraints underscore the urgent need to identify novel molecular targets that address fundamental disease mechanisms.

Ferroptosis, an iron-dependent regulated cell death pathway characterized by glutathione peroxidase 4 (GPX4) suppression and lethal lipid peroxidation,⁷ has recently emerged as a pivotal contributor to DR pathogenesis.⁸ Unlike apoptosis or necrosis, ferroptosis directly links iron dyshomeostasis with oxidative membrane damage, a process particularly detrimental to metabolically active retinal neurons, pigment epithelium, and vascular endothelium.^{9,10} This mechanistic distinction positions ferroptosis inhibition as a promising therapeutic strategy capable of protecting both neural and vascular compartments during early DR progression, where current interventions remain ineffective.

Heme oxygenase 1 (HMOX1), a stress-responsive enzyme regulating iron recycling and cellular antioxidant defense, exhibits a key role in redox homeostasis.¹¹ Growing evidence indicates that elevated HMOX1 activity releases free iron that catalyzes Fenton reactions, thereby promoting ferroptotic cascades through amplified lipid peroxidation.¹² In DR contexts, retinal HMOX1 upregulation exacerbates iron overload and oxidative stress, accelerating the damage and death of retinal cells.¹³ This nature establishes HMOX1 as a compelling therapeutic node for modulating ferroptosis in DR.

RNA-binding proteins (RBPs) represent master regulators of post-transcriptional gene expression through modulating mRNA stability, transport, and translation, allowing for precise control of cellular stress responses.¹⁴ Previous studies have revealed significant RBP dysregulation, including Fus, Hnrnpa2b1, Canx, and Calr, in diabetic retinas and hyperglycemia-stressed mouse retinal microvascular endothelial cells.¹⁵ Embryonic lethal abnormal vision-like 1 (ELAVL1/HuR), an RBP implicated in diabetes and related complications, stabilizes numerous stress-response transcripts.¹⁶ Bresciani et al have proven that ELAVL1 is an essential factor for retinal pigment epithelium homeostasis, providing mechanistic insights into age-related macular degeneration pathophysiology.¹⁷ Crucially, it has been demonstrated that ELAVL1 can binds AU-rich elements in the 3'-untranslated region of HMOX1 mRNA, enhancing its stability and transcriptional levels.¹⁸ While direct evidence of ELAVL1 involvement in DR remains limited, its established role in neuronal oxidative stress responses and recent detection in retinal diseases proteomic profiles suggest conserved functions in retinal pathophysiology.

Herein, the present study investigates the previously unexplored mechanistic axis wherein ELAVL1-mediated stabilization of HMOX1 mRNA drives ferroptosis in DR. We hypothesize that ELAVL1 upregulates HMOX1 expression by enhancing its mRNA stability, thereby triggering ferroptosis in retinal cells and accelerating DR progression. To validate this hypothesis, we will mechanistically dissect the ELAVL1-HMOX1-ferroptosis pathway using streptozotocin (STZ)-induced DR rat models and high-glucose-stressed ARPE-19 cells. Our integrated approach aims to assess: (1) spatiotemporal coordination between ELAVL1/HMOX1 expression and ferroptotic markers; (2) functional consequences of ELAVL1 perturbation on retinal ferroptosis; and (3) therapeutic efficacy of targeting this regulatory axis. Through these investigations, we hope to identify a novel RBP-regulated mechanism underlying DR pathogenesis and provide actionable molecular targets for early intervention strategies that directly address ferroptosis.

Methods

Cell Culture and Treatment

The adult retinal pigment epithelial cell line ARPE19 (SNL-227, Wuhan Shangen Bio-Tech Co., Ltd.) was commercially acquired and maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum and 100 U/mL streptomycin-penicillin. To establish hyperglycemic conditions, basal glucose concentrations were modified to 5.5 mM (Control group) and 25 mM high glucose (HG) group for 48-hour exposure periods.¹⁹ At 70% confluence, cells underwent transfection using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Transfection constructs included knockdown vectors: short hairpin RNA (shRNA) targeting HMOX1 (sh-HMOX1), ELAVL1 (sh-ELAVL1), and non-targeting negative control (sh-NC), as well as overexpression vectors: ELAVL1 overexpression vector (oe-ELAVL1) and empty vector negative (oe-NC).²⁰

Cell Counting Kit-8 (CCK-8) Assay

ARPE19 cells were seeded at a density of 5,000 cells per well in 96-well plates and subjected to experimental treatments. Following interventions, 10% CCK-8 reagent was added followed by 3-hour incubation. Optical density (OD) was then measured at 450 nm using a microplate reader (Thermo Fisher Scientific).

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Assay

Cell apoptosis was assessed using a TUNEL assay kit (C1089, Beyotime, Shanghai, China). ARPE19 cells were washed once with phosphate-buffer saline (PBS) and fixed with 4% paraformaldehyde for 30 minutes. Following an additional wash, the cells were exposed to a permeabilization solution and incubated at room temperature for 5 minutes. Next, 50 µL of TUNEL reaction mixture was introduced, and the cells were kept in the dark for incubation at 37°C for

60 minutes. After nuclear counterstaining with 4',6-diamidino-2-phenylindole (DAPI), the samples were mounted with an anti-fade mounting medium. Finally, the samples were observed and imaged under a fluorescence microscope, and TUNEL-positive cells were quantified using ImageJ software (V1.8.0.112, NIH, Madison, WI, USA).

Fe²⁺ Content Measurement

The intracellular Fe²⁺ levels were measured using an iron assay kit (EEA009, Thermo Fisher Scientific). Briefly, cell samples were processed and incubated with the provided colorimetric reagent. Subsequently, the OD values were determined using a microplate reader to obtain the final concentration of Fe²⁺. For retinal tissue analysis, the Fe²⁺ levels were quantified using a kit (E-BC-K304-S, Elabscience, Wuhan, China) according to the manufacturer's instructions.

Measurement of Intracellular ROS Levels

Intracellular ROS generation was quantified by incubating ARPE19 cells with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; S0033, Beyotime). After washing three times with DMEM, images of the cells were captured immediately using a fluorescence microscope. The analysis of relative fluorescence intensity was conducted using ImageJ software.

Biochemical Analysis

Superoxide dismutase (SOD), malondialdehyde (MDA), and ROS levels in tissues or cells were assessed using the SOD assay kit (D799594, Sangon, Shanghai, China), MDA assay kit (S0131S, Beyotime), and ROS detection kit (JL-T3037-96, Jianglai Biotechnology, Shanghai, China), respectively, according to manufacturers' protocols.

Western Blotting (WB)

Total protein from cells was acquired using radioimmunoprecipitation assay lysis buffer (ab170197, Abcam, Cambridge, UK). The protein concentration was measured using a bicinchoninic acid protein assay kit (P0010, Beyotime). Equal amounts of protein samples were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene fluoride membranes (ab133411, Abcam) using a wet transfer method. The membranes were blocked with 5% non-fat milk for 1 hour and then incubated overnight at 4°C with the following primary antibodies targeting ELAVL1 (1:500, A1608, Sigma-Aldrich, St. Louis, MO), HMOX1 (1:2,000, ab189491, Abcam), GPX4 (1:1,000, ab125066, Abcam), solute carrier family 7 member 11 (SCL7A11; 1:1,000, A13685, Abclonal, Wuhan, China), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:2,000, ab181602, Abcam). After washing the membranes with Tris-buffered saline with Tween 20 (ZS405-3, Zomanbio, Beijing, China), cells were cultured for 2 hours with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:2,000, ab205718, Abcam). Protein bands were visualized using enhanced chemiluminescence substrate (A38554, Thermo Fisher Scientific) and quantified with ImageJ software. GAPDH was used as an internal control to evaluate the relative levels of target proteins.

Animal Treatment and Grouping

Sprague-Dawley rats (aged 8 weeks old, 230–250 g) were acquired from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed under controlled conditions: temperature maintained at 25 ± 5°C, relative humidity at 60 ± 5%, 12-hour light/dark cycles, with free access to food and drinking water. After 7-day acclimatization, rats were assigned randomly to the following experimental groups: Control group, STZ group, sh-NC group, sh-HMOX1 group, sh-ELAVL1 group, sh-ELAVL1 + oe-NC group, and sh-ELAVL1 + oe-HMOX1 group, with 6 rats in each group. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition, 2011) and were approved by the Ethics Committee of Hunan Evidence-based Biotechnology Co., Ltd. (Approval Number: AB24072204).

Animal Modeling

Insulin-deficient diabetes was induced in rats by intraperitoneal injections of 60 mg/kg STZ (S0130, Sigma-Aldrich) for 5 consecutive days.²¹ To prevent acute weight loss and maintain chronic hyperglycemia, insulin (0.2 units, 12585014, Invitrogen, Carlsbad, CA, USA) was administered every 2–3 days. Rats in the Control group received intraperitoneal injections of citrate buffer as a control, for a duration of two months. Diabetes onset was confirmed when blood glucose readings sustained above 275 mg/dL for three consecutive days after STZ regimen. Post-diabetes confirmation, rats received intravitreal injections of recombinant adeno-associated virus (AAV) serotype 2 (selected for retinal tropism) carrying expression cassettes under the cytomegalovirus promoter. Constructs included scrambled shRNA control (sh-NC), HMOX1-targeting shRNA (sh-HMOX1), ELAVL1-targeting shRNA (sh-ELAVL1), empty vector control (oe-NC), and HMOX1 overexpression plasmid (oe-HMOX1). Each eye was injected with 2 μ L of viral solution at a concentration of 1.0×10^{10} viral genomes. Injections were administered on the second day following the initiation of diabetes induction and repeated once every month to maintain transgene expression. Retinal transduction efficiency was verified by *in vivo* fundus imaging of green fluorescent protein (GFP) reporters and *in vitro* reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Following two months of hyperglycemia, rats were euthanized via intraperitoneal injection of 150 mg/kg pentobarbital sodium, with retinal tissues harvested for further experiments.²²

Hematoxylin-Eosin (HE) Staining

The collected retinal tissue samples were fixed, embedded in paraffin, and sectioned at 4 μ m thickness. The sections were then deparaffinized in xylene I and xylene II (Sigma-Aldrich) for 20 minutes, followed by dehydration in gradient ethanol for 5 minutes each. After rinsing with distilled water, the sections were stained with hematoxylin, washed with running water, and then counterstained with eosin. After rinsing the excessive eosin with distilled water, the sections were dehydrated in gradient ethanol, cleared with xylene, and mounted with neutral resin. Cellular morphology was observed under an optical microscope (CX43, Olympus, Tokyo, Japan) to assess the pathological changes in rat retinal tissues.

Immunohistochemistry (IHC)

The tissue sections were placed in a 40°C water bath to avoid air bubbles from affecting the tissues. After deparaffinization, the sections were washed in distilled water and then immersed in 3% hydrogen peroxide to block endogenous peroxidase activity. Antigen retrieval was performed by incubating the sections in citrate buffer, followed by microwave heating for 3 minutes, cooling to room temperature, and reheating to ensure adequate exposure of antigen sites. After antigen retrieval, blocking of non-specific binding was performed. The sections were then incubated overnight at 4°C with primary antibodies against HMOX1 (1:20,000, ab189491, Abcam) and ELAVL1 (1:100, A1608, Sigma-Aldrich). After washing, the sections were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:2,000, ab205718) for 1 hour. Subsequently, color development was performed using 3,3'-diaminobenzidine working solution (P0203, Beyotime), followed by hematoxylin counterstaining. The staining results were visualized under an optical microscope (CX43). Cells were quantified using ImageJ software (positive area/total area $\times$ 100% = relative positive area).

RT-qPCR Assay

Total RNA was isolated from the tissues and cells using a Fast RNA extraction kit (DP419, Jiachu Biotechnology Co., Ltd, Shanghai, China). Subsequently, RT-qPCR was conducted using a fluorescence quantitative PCR detection kit (QR0100, Sigma-Aldrich). GAPDH was used as an internal reference gene, and the relative mRNA levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences are listed in Table 1.

Actinomycin D Transcription Inhibition Assay

To assess HMOX1 mRNA stability dynamics, ARPE19 cells were exposed to HG conditions or subjected to ELAVL1 knockdown, followed by treatment with 100 ng/mL actinomycin D (50–76-0, MedChemExpress, New Jersey, USA). Cells were collected at 0, 6, 12, and 24 hours post-treatment for total RNA extraction. RT-qPCR was performed to analyze the impact of HG or ELAVL1 knockdown on the stability of HMOX1 mRNA.

Table 1 Primer Sequences for RT-qPCR

Gene	Species	Direction	Sequence (5'-3')
HMOX1	Rattus norvegicus	Forward	CTCGCATGAACACTCTGGAGAT
		Reverse	AGCTCCTCAAACAGCTCAATGT
	Homo sapiens	Forward	CTGCTCAACATCCAGCTCTTTG
		Reverse	GTGTAAGGACCCATCGGAGAAG
ELAVL1	Rattus norvegicus	Forward	CCCGCCCGTGTTCAGATTTTTA
		Reverse	GAACAGACTCCGTAGTTCCTCC
	Homo sapiens	Forward	TGTTCTCTCGGTTTGGGCGGAT
		Reverse	TCTTCTGCCTCCGACCGTTTGT
GAPDH	Rattus norvegicus	Forward	CACCATCTCCAGGAGCGAG
		Reverse	CTCGTGGTTCACACCCATCA
	Homo sapiens	Forward	GTCTCCTCTGACTTCAACAGCG
		Reverse	ACCACCCTGTTGCTGTAGCCAA

Notes: Primers labeled with *Rattus norvegicus* are used for rat models, while those labeled with *Homo sapiens* are used for human models.

Abbreviations: HMOX1, Heme oxygenase 1; ELAVL1, embryonic lethal abnormal vision-like 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

RNA Immunoprecipitation (RIP)

ARPE19 cells under experimental conditions were lysed using a lysis buffer, and the supernatant was collected for further use. RIP was conducted following the instructions of the immunoprecipitation kit with protein A + G magnetic beads. Briefly, 500 μ L of anti-ELAVL1 antibody (1:30, ab200342, Abcam) or immunoglobulin G (IgG) working solution was incubated with 20 μ L of protein A + G magnetic beads at room temperature for 1 hour to obtain the beads coated with either anti-ELAVL1 antibody or IgG. The collected supernatant was then mixed with the antibody-coated magnetic beads and incubated overnight at 4°C. Afterward, the mixture was placed on a magnetic rack for 10 seconds to separate the beads, and the supernatant was subsequently removed. Bound mRNAs were eluted and cross-links were reversed, followed by collection of mRNAs for RT-qPCR analysis.

RNA Fluorescence in situ Hybridization (RNA-FISH) Combined with Immunofluorescence (IF)

Cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (9036–19-5, Sigma-Aldrich). RNA-FISH was performed using a fluorescently labeled probe specific for HMOX1 mRNA. Following pre-hybridization, the probe mixture was added and incubated at an appropriate temperature for sufficient time to allow binding to HMOX1 mRNA. After RNA-FISH, cells were washed with PBS and subjected to IF staining. A primary antibody against ELAVL1 (SAB5700123, Sigma-Aldrich) was applied to the cells at room temperature for 1 hour, followed by incubation with an Alexa Fluor-conjugated secondary antibody (ab150079, Abcam) at room temperature for 1 hour. DAPI was used for nuclear counterstaining. After the staining procedure, the samples were mounted with a mounting medium. Fluorescence microscopy was employed to observe the RNA-FISH signals (fluorescent signals of HMOX1 mRNA) and the IF signals (fluorescent signals of ELAVL1 protein). ImageJ software was utilized to analyze the co-localization of ELAVL1 protein and HMOX1 mRNA within the cells.

Statistical methods

Statistical analyses were conducted using GraphPad Prism 9 (Dotmatics, Boston, MA, USA). The normality of data distribution was assessed using the Shapiro–Wilk test. Comparisons between two groups were executed using the Student's *t*-test, whereas one-way analysis of variance (ANOVA) was utilized for assessing significance among three or more groups, followed by Tukey's post hoc test.

Results

Ferroptosis Mediates DR Pathogenesis

To model diabetic retinal pathology, ARPE19 cells were exposed to HG conditions. The results showed that HG treatment significantly decreased cell viability, an effect reversed by co-treatment with the ferroptosis inhibitor ferrostatin-1 (Fer-1) (Figure 1A). Further TUNEL assays confirmed HG-induced apoptosis in ARPE19 cells, which was similarly attenuated by Fer-1 (Figure 1B). Biochemical profiling revealed that HG increased intracellular Fe^{2+} levels, elevated ROS production, augmented lipid peroxidation marker MDA content, and reduced antioxidant enzyme SOD activity in ARPE19 cells, indicating ferroptotic dysregulation and oxidative stress. However, Fer-1 treatment normalized these perturbations, reducing Fe^{2+} and ROS accumulation while attenuating MDA elevation and restoring SOD activity (Figures 1C–E). WB analysis further demonstrated that ferroptosis-related proteins GPX4 and SLC7A11 were down-regulated after HG treatment, with Fer-1 restoring expression of these ferroptosis regulators (Figure 1F). These findings collectively suggest ferroptosis as a critical mechanism in hyperglycemia-induced retinal cell damage.

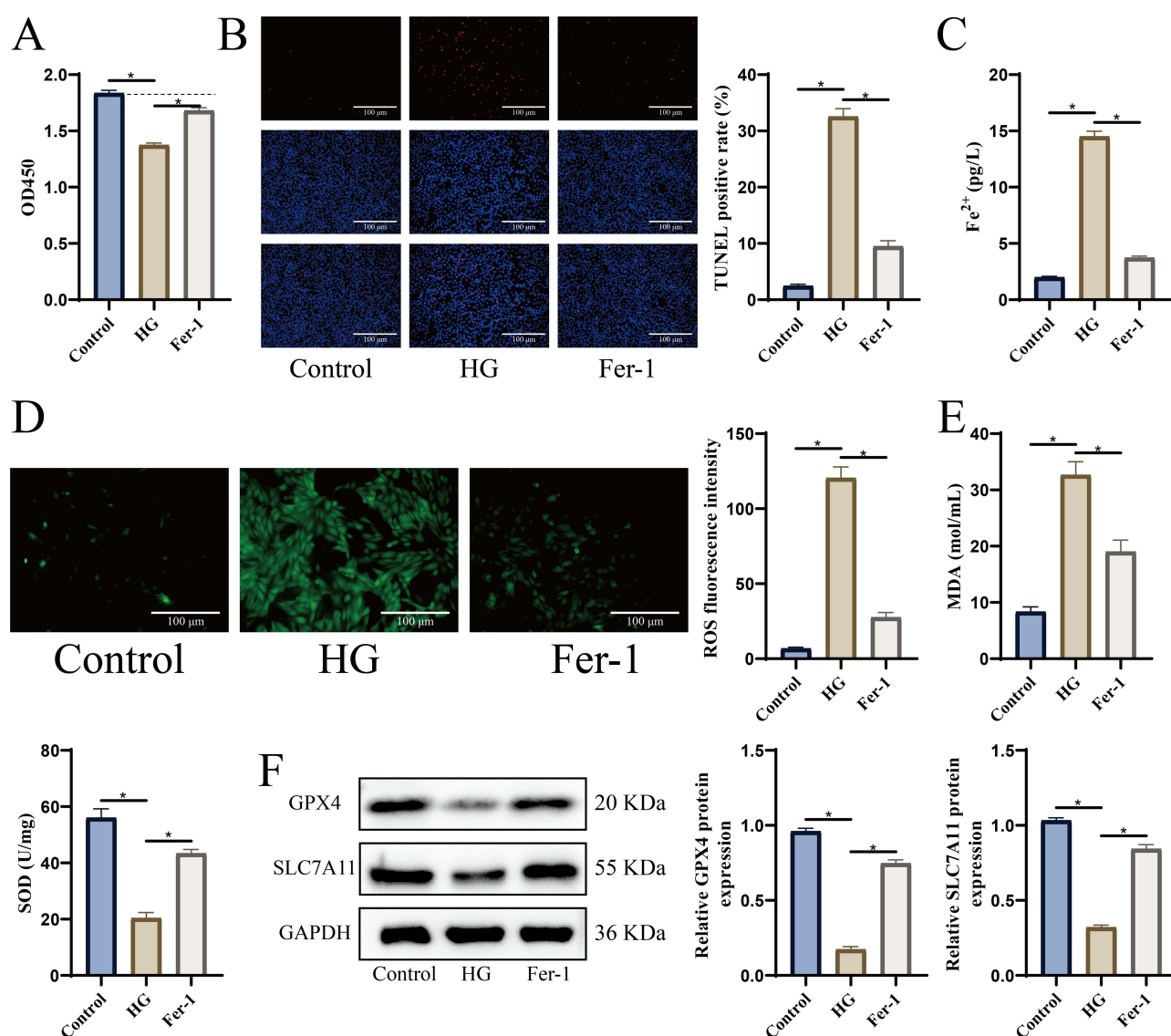


Figure 1 Ferroptosis contributes to HG-induced ARPE19 cell damage. (A) Cell viability assessed by CCK-8 assay. (B) Apoptosis detected by TUNEL staining (scale bar: 100 μm). (C) Measurement of intracellular Fe^{2+} levels. (D) ROS fluorescence intensity (scale bar: 100 μm). (E) Measurement of MDA content and SOD activity. (F) GPX4 and SLC7A11 protein expression analyzed by WB. N = 3; data were analyzed by one-way ANOVA, * $p < 0.05$.

HMOX1 is Highly Expressed in DR Rats

To identify molecular drivers of DR, we screened differentially expressed genes (DEGs) in DR from three Gene Expression Omnibus (GEO) datasets GSE102485, GSE94019, and GSE60436, with stringent thresholds of p -value < 0.01 and log fold-change > 2 (Figure 2A). Given established ferroptosis involvement in DR pathogenesis,⁸ we intersected these DEGs with ferroptosis-related factors from the FerrDb database (<http://www.zhounan.org/ferrdb/current/>), identifying HMOX1 as the sole overlapping gene (Figure 2B). This iron-regulatory enzyme modulates ferroptosis by affecting iron metabolism and oxidative stress levels.²³ In STZ-induced DR rat model, HE staining showed profound retinal disorganization compared to the Control group (Figure 2C). Additionally, IHC analysis confirmed a significant increase in the positive expression rate of HMOX1 in diabetic retinas versus controls (Figure 2D). These findings implicate that HMOX1 may play an important role in DR pathology, positioning its inhibition as a promising therapeutic strategy.

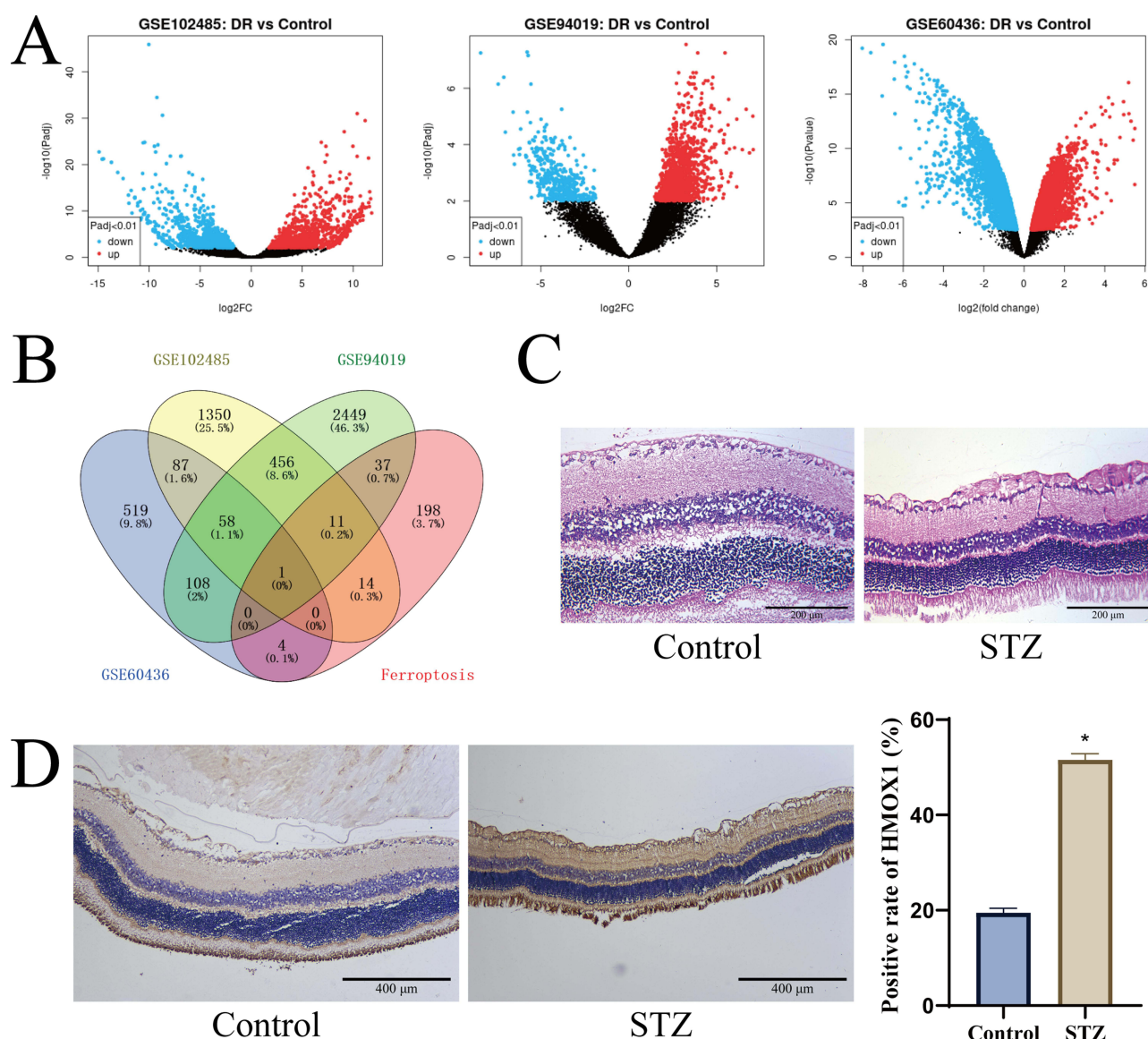


Figure 2 HMOX1 is upregulated in DR rats. **(A)** Volcano plots of DR-associated transcriptomes from GEO datasets GSE102485, GSE94019, and GSE60436. **(B)** Venn diagram of ferroptosis-related gene and DR-DEG intersection. **(C)** Morphological changes in rat retinal tissues by HE staining (scale bar: 200 μ m). **(D)** HMOX1 expression in rat retinal tissues measured via IHC (scale bar: 400 μ m). N = 6 (**C–D**); data were analyzed by one-way ANOVA, *p < 0.05.

HMOX1 Exacerbates HG-Induced ARPE19 Cell Damage by Driving Ferroptosis

HG exposure significantly upregulated HMOX1 expression in ARPE19 cells at both mRNA and protein levels (Figure 3A and B). To further investigate the functional role of HMOX1, we performed HMOX1 knockdown using three independent sh-HMOX1. RT-qPCR and WB results showed that sh-HMOX1-3 exhibited optimal knockdown efficiency (Figure 3C and D) and was chosen for subsequent experiments. CCK-8 assay demonstrated that HMOX1 knockdown partially restored hyperglycemia-induced cell viability compared to the sh-NC group (Figure 3E). TUNEL staining confirmed a significant decrease in apoptosis following HMOX1 knockdown (Figure 3F). Crucially, ferroptosis biomarkers were profoundly modulated: intracellular Fe^{2+} levels, ROS accumulation, and MDA contents were significantly reduced, while SOD activity was significantly increased after HMOX1 knockdown (Figure 3G–I). WB results further revealed that, compared to the sh-NC group, GPX4 and SLC7A11 were significantly upregulated in the sh-HMOX1 group (Figure 3J). These data demonstrate that inhibiting HMOX1 reduces retinal cell damage through ferroptosis potentiation, positioning it as a therapeutic target for DR.

HMOX1 Drives Ferroptosis-Mediated DR Pathology in vivo

To establish the therapeutic potential of HMOX1 inhibition in DR in vivo, we administered intravitreal sh-HMOX1 in STZ-induced DR rats. RT-qPCR and IHC confirmed robust HMOX1 knockdown in retinal tissues in the sh-HMOX1 group (Figure 4A and B). HE staining showed significant histological preservation in sh-HMOX1-treated retinas compared to the sh-NC group (Figure 4C). Biochemical analyses revealed that HMOX1 suppression substantially attenuated ferroptosis biomarkers, reducing retinal Fe^{2+} , MDA, and ROS levels, while increasing SOD activity (Figure 4D). At the molecular level, WB analysis demonstrated increased GPX4 and SLC7A11 expression in the sh-HMOX1 group compared to the sh-NC group (Figure 4E), indicating inhibited ferroptosis following HMOX1 knockdown. These results establish that HMOX1 promotes DR progression through ferroptosis activation and validate its targeting as a disease-modifying strategy.

ELAVL1 Enhances the mRNA Stability of HMOX1

Given the established role of RBPs in DR pathogenesis, we queried the ENCORI database (<https://rnasysu.com/encori/>) for RBPs regulating HMOX1. Intersection with ferroptosis-related proteins identified two candidates: ELAVL1/HuR and SRY-box transcription factor 2 (SOX2) (Figure 5A). ELAVL1, an N⁶-methyladenosine (m^6A) reader protein known to enhance mRNA stability, was prioritized due to its undefined role in HMOX1 regulation during DR. In STZ-induced DR rats, retinal ELAVL1 expression increased at both mRNA and protein levels versus controls (Figures 5B and C). Similarly, HG elevated ELAVL1 expression in ARPE19 cells (Figure 5D and E), implicating ELAVL1 in hyperglycemic stress responses. Knockdown screening identified sh-ELAVL1-1 as optimal (Figure 5F), concurrently reducing HMOX1 mRNA and protein expression (Figure 5G). Actinomycin D chase assays revealed that the half-life of HMOX1 mRNA in the sh-ELAVL1 group was significantly shortened, indicating that ELAVL1 enhances HMOX1 mRNA stability (Figure 5H). Additionally, RNA-FISH combined with IF confirmed significant co-localization of ELAVL1 protein and HMOX1 mRNA (Figure 5I), supporting the interaction between ELAVL1 and HMOX1. RIP assays validated that ELAVL1 directly bound to HMOX1 mRNA (Figure 5J), establishing ELAVL1 as a direct post-transcriptional regulator of HMOX1 in DR.

ELAVL1 Drives Ferroptosis Through HMOX1 mRNA Stabilization

To establish functional causality, we performed rescue experiments in ELAVL1-depleted ARPE-19 cells under HG. While sh-ELAVL1 reduced HMOX1 expression, concomitant HMOX1 overexpression (oe-HMOX1) restored its expression without altering ELAVL1 expression (Figure 6A and B). This molecular rescue abrogated the cytoprotective effects of ELAVL1 knockdown, decreasing cell viability (Figure 6C) and increasing cell apoptosis versus sh-ELAVL1 controls (Figure 6D). Ferroptosis biomarkers mirrored this reversal: intracellular Fe^{2+} contents, ROS accumulation, and MDA levels significantly increased, while SOD activity decreased after HMOX1 overexpression, indicating enhanced ferroptosis and oxidative stress following HMOX1 restoration (Figure 6E–G). Moreover, GPX4 and SLC7A11 expression

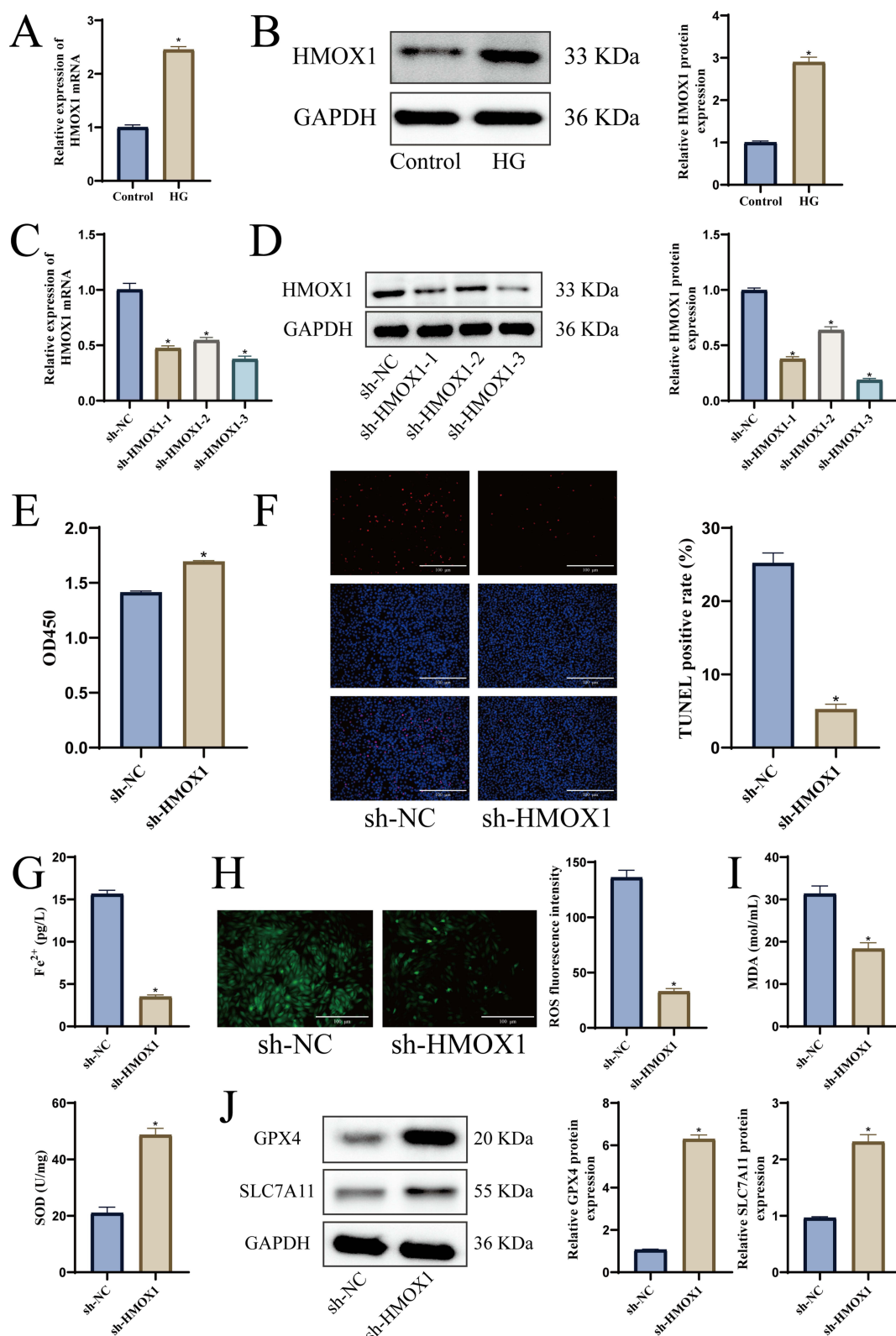


Figure 3 HMOX1 exacerbates HG-induced ARPE19 cell damage by promoting ferroptosis. **(A)** HMOX1 mRNA expression after HG treatment by RT-qPCR. **(B)** HMOX1 protein expression after HG treatment by WB. **(C)** Efficiency of HMOX1 knockdown monitored by RT-qPCR. **(D)** Efficiency of HMOX1 knockdown measured by WB. **(E)** Cell viability evaluated by CCK-8 assay. **(F)** Cell apoptosis assessed by TUNEL (scale bar: 100 μ m). **(G)** Intracellular Fe^{2+} quantification. **(H)** ROS fluorescence (scale bar: 100 μ m). **(I)** MDA content and SOD activity. **(J)** Levels of ferroptosis-related proteins GPX4 and SLC7A11 by WB. N = 3; data were analyzed by one-way ANOVA, * p < 0.05.

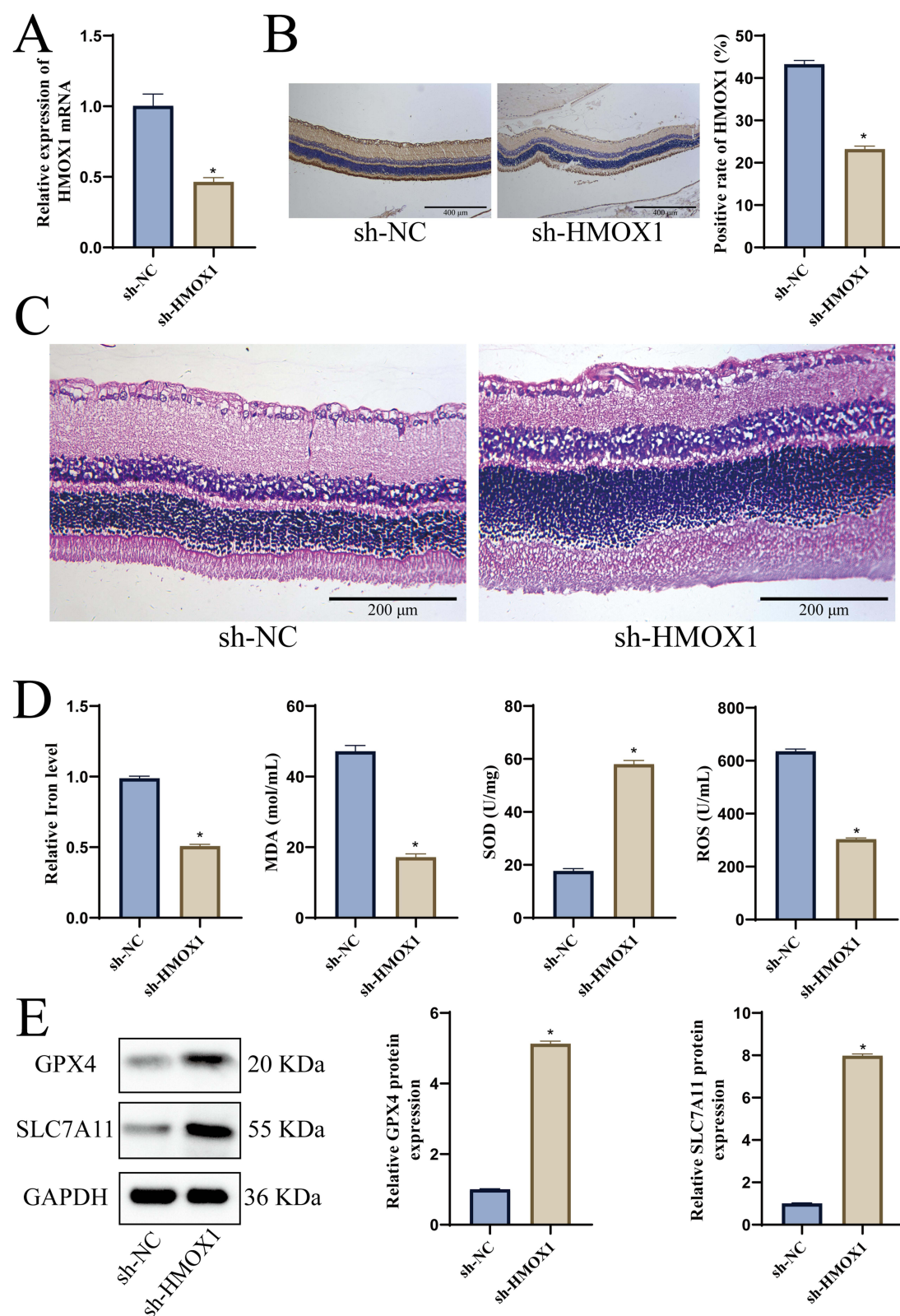


Figure 4 HMOX1 exacerbates DR in rats by promoting ferroptosis. **(A)** Retinal HMOX1 mRNA expression by RT-qPCR. **(B)** Retinal HMOX1 immunoreactivity by IHC (scale bar: 400 μ m). **(C)** Retinal histopathology via HE staining (scale bar: 200 μ m). **(D)** Measurement of Fe²⁺, MDA, ROS, and SOD levels. **(E)** Ferroptosis-related proteins GPX4 and SLC7A11 by WB. N = 6; data were analyzed by one-way ANOVA, * $p < 0.05$.

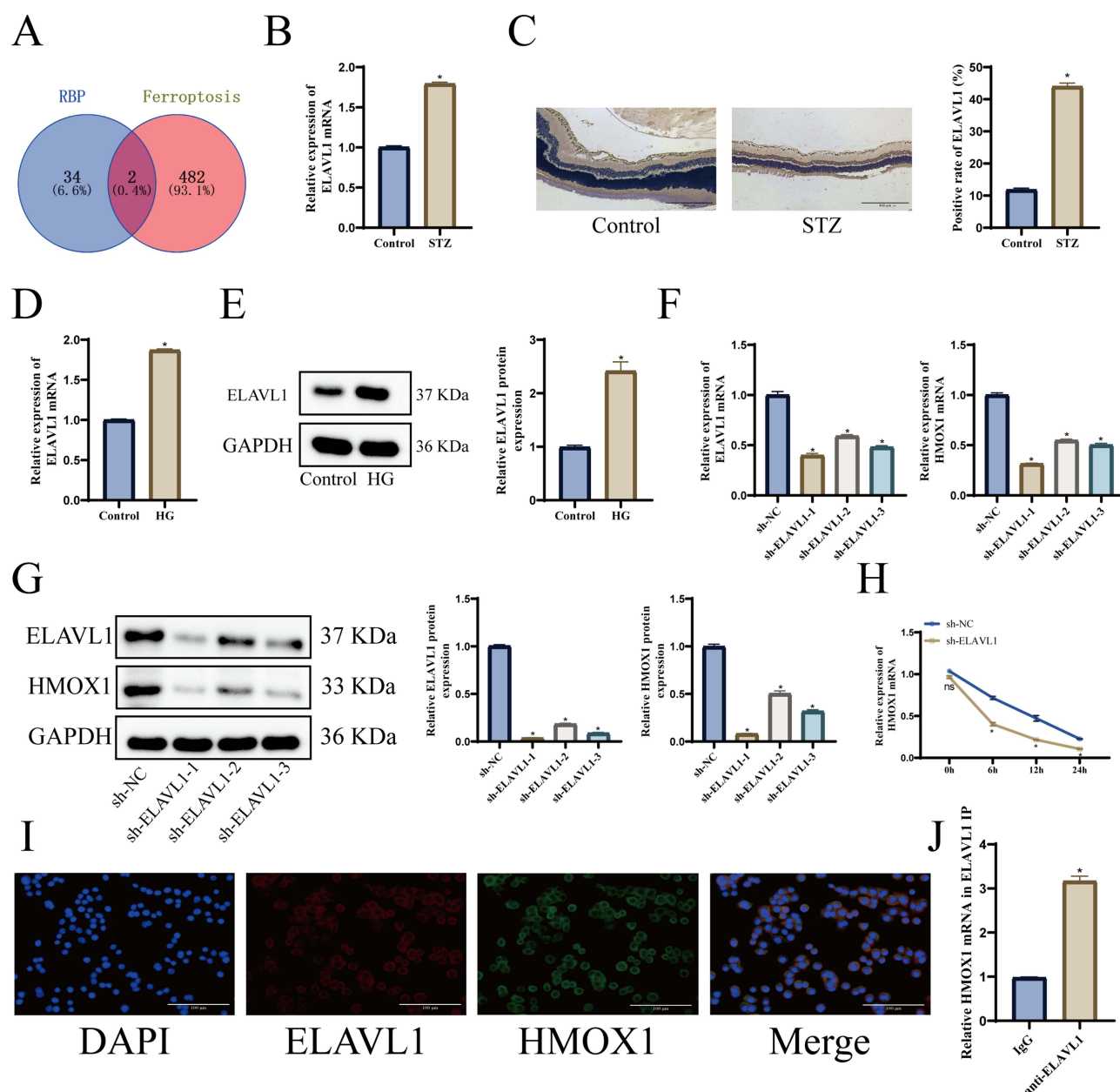


Figure 5 ELAVL1 enhances the stability of HMOX1 mRNA. **(A)** Venn diagram showing the intersection of HMOX1-interacting RBPs and ferroptosis-related proteins. **(B)** Retinal ELAVL1 mRNA expression by RT-qPCR. **(C)** Retinal ELAVL1 expression by IHC (scale bar: 400 μ m). **(D)** ELAVL1 mRNA expression in ARPE19 cells by RT-qPCR. **(E)** ELAVL1 expression in ARPE19 cells by WB. **(F)** ELAVL1 and HMOX1 mRNA expression post-ELAVL1 knockdown validated by RT-qPCR. **(G)** ELAVL1 and HMOX1 protein expression post-ELAVL1 knockdown verified by WB. **(H)** Half-life of HMOX1 mRNA after actinomycin D treatment. **(I)** Co-localization of ELAVL1 and HMOX1 mRNA by RNA-FISH/IF (scale bar: 100 μ m). The signals shown in the images were derived from the same cells. Co-localization analysis yielded a Pearson's correlation coefficient of $r = 0.909$, indicating a strong spatial correlation. **(J)** ELAVL1-HMOX1 binding validated by RIP. $N = 6$ (**B–C**) or $N = 3$ (**D–J**); data were analyzed using one-way ANOVA (**B–G** and **J**) or two-way ANOVA (**H**), $*p < 0.05$.

declined after overexpression of HMOX1 (Figure 6H). These results suggest that HMOX1 restoration reverses ELAVL1 knockdown-mediated ferroptosis protection, establishing ELAVL1-HMOX1 as the definitive regulatory axis driving retinal cell ferroptosis in diabetes.

In vivo Validation of the ELAVL1-HMOX1-Ferroptosis Axis

To further validate translational relevance, we conducted rescue experiments in DR rats via combinatorial intravitreal delivery of sh-ELAVL1 and oe-HMOX1. sh-ELAVL1 significantly reduced both ELAVL1 and HMOX1 expression compared to the sh-NC group. Crucially, oe-HMOX1 restored HMOX1 expression without rescuing ELAVL1 suppression (Figure 7A and B).

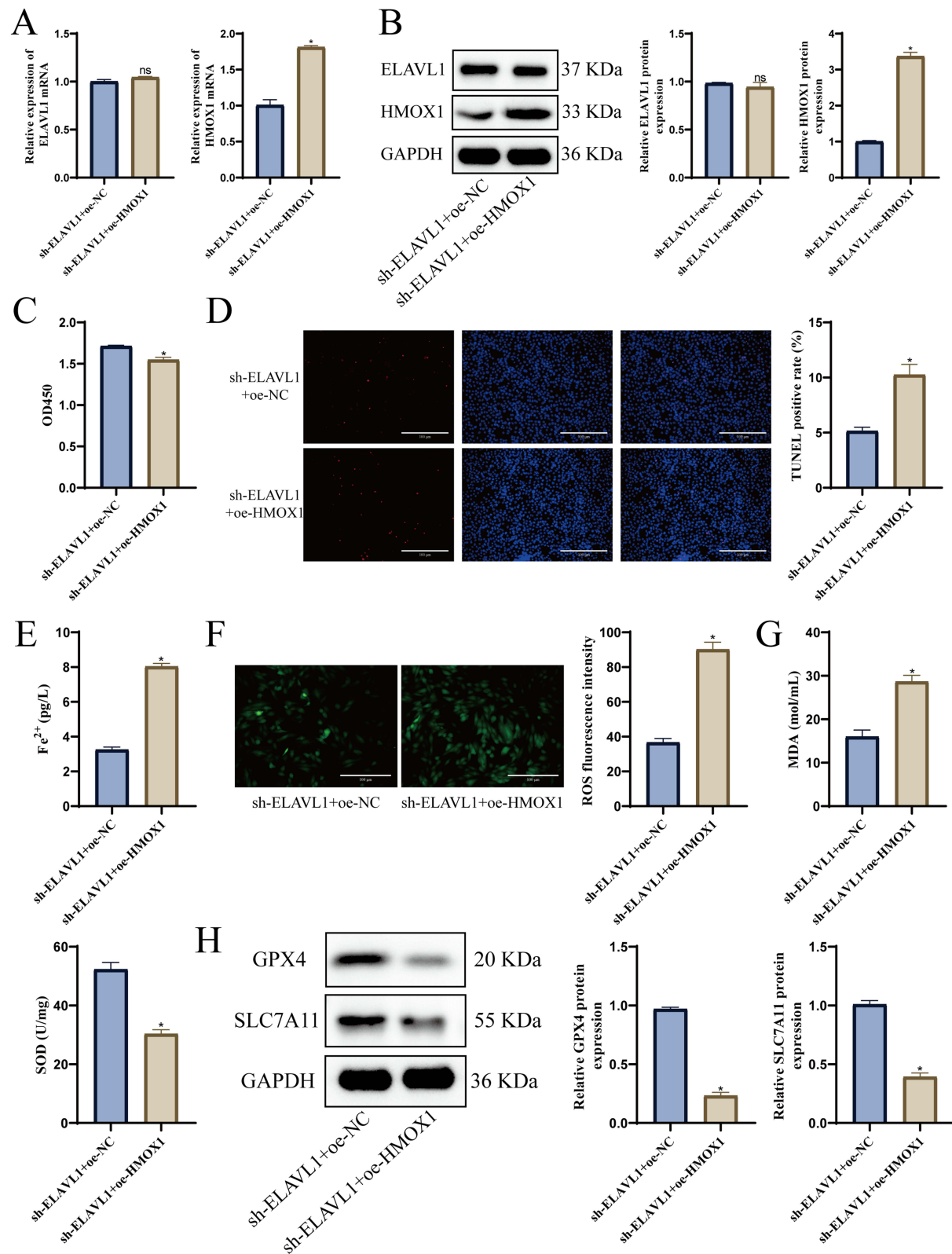


Figure 6 HMOX1 restoration reverses ELAVL1 knockdown-mediated ferroptosis protection in vitro. **(A)** ELAVL1 and HMOX1 mRNA expression post-HMOX1 overexpression by RT-qPCR. **(B)** ELAVL1 and HMOX1 expression post-HMOX1 overexpression by WB. **(C)** Changes in cell viability via CCK-8 assay. **(D)** Apoptosis assessed by TUNEL staining (scale bar: 100 μ m). **(E)** Intracellular Fe²⁺ content measurement. **(F)** ROS fluorescence (scale bar: 100 μ m). **(G)** Biochemical analysis of MDA levels and SOD activity. **(H)** Protein levels of GPX4 and SLC7A11 by WB. N = 3; data were analyzed using one-way ANOVA, **p* < 0.05.

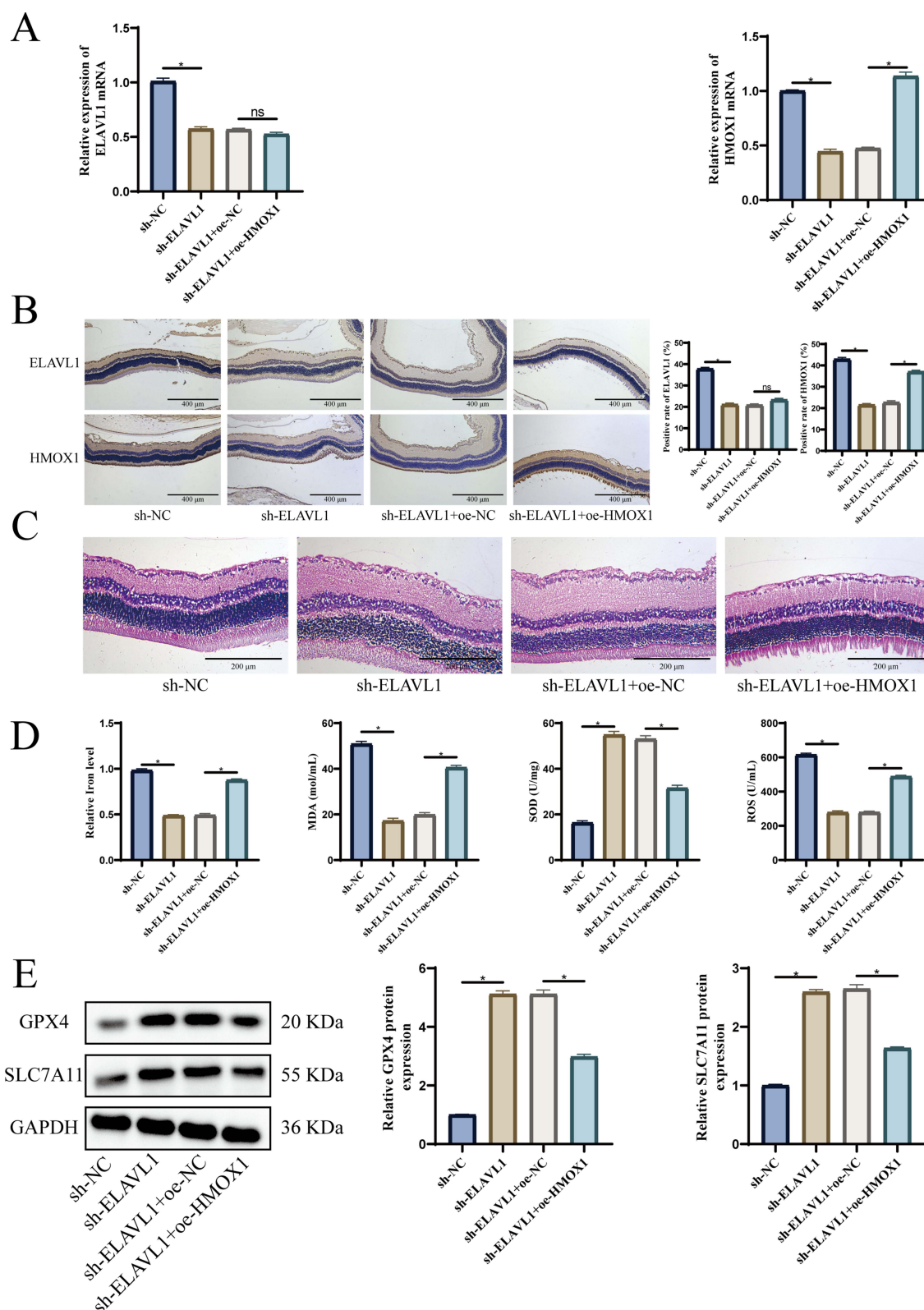


Figure 7 ELAVL1 promotes ferroptosis in DR rats by stabilizing HMOX1 mRNA. **(A)** Retinal ELAVL1 and HMOX1 mRNA expression by RT-qPCR. **(B)** Retinal ELAVL1 and HMOX1 protein expression by IHC (scale bar: 400 μ m). **(C)** HE-stained retinal tissue sections (scale bar: 200 μ m). **(D)** Fe^{2+} quantification and oxidative stress markers (ROS, MDA, SOD). **(E)** Levels of ferroptosis-related proteins GPX4 and SLC7A11 by WB. N = 6; data were analyzed using one-way ANOVA, * $p < 0.05$.

HE staining showed that sh-ELAVL1 preserved retinal structure, exhibiting more regular cell arrangement and reduced tissue damage, compared to the sh-NC group. This protection was abrogated by HMOX1 reconstitution, with oe-HMOX1 inducing greater photoreceptor disorganization than the sh-ELAVL1 + oe-NC group (Figure 7C). Ferroptosis biomarkers mirrored this reversal, with reduced retinal Fe^{2+} levels, while oe-HMOX1 increased Fe^{2+} levels versus the sh-ELAVL1 + oe-NC group (Figure 7D). Oxidative stress parameters further confirmed pathway dependency. sh-ELAVL1 decreased MDA and ROS while elevating SOD activity, indicating that ELAVL1 knockdown alleviated oxidative stress. HMOX1 restoration reversed these gains, increasing MDA and ROS levels while suppressing SOD activity (Figure 7D). WB analysis of ferroptosis-related proteins showed that sh-ELAVL1 upregulated GPX4 and SLC7A11, effects nullified by oe-HMOX1 co-expression (Figure 7E). These data conclusively demonstrate that ELAVL1 drives ferroptosis in DR through HMOX1 stabilization, with HMOX1 reconstitution abolishing ELAVL1-targeted protection.

Discussion

Accumulating evidence suggests that HMOX1 is closely related to ferroptosis due to its role in iron release and oxidative stress regulation.²⁴ Elevated HMOX1 expression may increase intracellular free iron, thereby triggering lipid peroxidation, which is a hallmark of ferroptosis.²⁵ Given the established significance of ferroptosis in DR, where it heightens cellular vulnerability and accelerates disease progression,²⁶ we hypothesized that inhibiting HMOX1 could offer protective effects against DR. This premise prompted further investigation into the relationship between upstream regulators of HMOX1 and its functional impact.

To elucidate HMOX1's role in DR, we successfully established a STZ-induced rat model of DR and a HG-treated retinal pigment epithelial (ARPE19) cell injury model. Our experimental results demonstrated that ferroptosis was abnormally activated in the retinal tissues of the DR rats and in HG-exposed ARPE19 cells, concurrent with significant upregulation of both HMOX1 mRNA and protein. Notably, HMOX1 knockdown substantially suppressed ferroptosis, highlighting its critical involvement in this cell death pathway. These findings suggest that HMOX1 overexpression drives ferroptosis by augmenting iron release and lipid peroxidation. This aligns with previous research linking ferroptosis in DR to dysregulated iron metabolism and enhanced oxidative stress commonly seen in diabetic patients.²⁷ Mechanistically, HMOX1 promotes ferroptosis primarily through heme degradation, releasing free iron that catalyzes ROS accumulation and lipid peroxidation, ultimately leading to cell death.²⁸ Supporting our findings, literature reports that destabilizing HMOX1 mRNA can significantly alleviate DR progression.²⁹ Therefore, HMOX1 emerges not only as a key regulatory factor in ferroptosis but also as a promising therapeutic target for DR. Our work demonstrated that targeting HMOX1 could effectively mitigate the ferroptotic process in DR. Integrating such molecular insights with risk prediction tools, exemplified by the use of continuous glucose monitoring (CGM) and machine learning for identifying individuals at high risk of incident DR³⁰ could facilitate early targeted interventions.

Given the central role of HMOX1 in DR, further exploring its upstream regulatory factors is essential for a comprehensive understanding of its regulatory mechanisms. Existing research indicates that the transcription and mRNA stability of HMOX1 are modulated by various upstream elements.³¹ Through analysis of the ENCORI database, we identified ELAVL1, an RBP, as a key candidate potentially regulating HMOX1 mRNA stability and participating in DR-associated ferroptosis. ELAVL1 is known to modulate mRNA stability under diverse stress conditions and plays significant regulatory roles in diabetes and its complications.³² To validate this hypothesis, we conducted relevant experiments and confirmed that ELAVL1 could enhance the stability of HMOX1 mRNA. Moreover, we suppressed ELAVL1 expression through sh-ELAVL1 transfection in HG-treated ARPE19 cells. The results indicated that ELAVL1 knockdown significantly enhanced the viability of damaged ARPE19 cells, reduced the apoptosis rate, and notably decreased oxidative stress levels and ferroptosis markers, with a concomitant downregulation of ROS generation. Importantly, co-transfection with an HMOX1 overexpression plasmid abrogated this protective effect, suggesting that ELAVL1 critically regulates DR pathogenesis primarily through its modulation of HMOX1. Subsequent *in vivo* experiments corroborated this conclusion, yielding results consistent with the *in vitro* data. Furthermore, existing studies have shown that overexpression of ELAVL1 exacerbates apoptosis and damage in ARPE19 cells,¹⁷ lending additional support to our findings. Collectively, these pieces of evidence establish that ELAVL1, by stabilizing HMOX1 mRNA, plays a key role in the regulatory mechanism of ferroptosis associated with DR. Targeting ELAVL1 thus represents a novel

therapeutic strategy to counteract HMOX1-driven retinal damage, offering new avenues for DR treatment. While other RBPs may also regulate HMOX1 expression, which will be an important focus of our future research, this study did not assess the pharmacological feasibility, ocular delivery methods, or potential toxicity of targeting ELAVL1, representing another critical direction for our future investigations. Subsequent studies should prioritize elucidating additional upstream regulators of HMOX1 and their interplay with ELAVL1, developing targeted inhibitors or modulators of ELAVL1 and HMOX1, and employing patient-derived retinal tissues and advanced *in vivo* models to validate the clinical relevance and therapeutic potential of this pathway. This research roadmap will address existing gaps and accelerate the development of innovative treatments for DR.

Conclusion

This study demonstrates that HMOX1 plays a critical role in DR pathogenesis, with its regulatory mechanism intimately linked to the RBP ELAVL1. ELAVL1 stabilizes HMOX1 mRNA, thereby facilitating ferroptosis and exacerbating DR pathology. Inhibition of either HMOX1 or ELAVL1 significantly attenuated ferroptosis markers and retinal damage in our models, highlighting the strength and therapeutic relevance of targeting this regulatory axis. Consequently, strategies aimed at inhibiting the HMOX1-ELAVL1 pathway represent a promising novel approach for DR treatment.

Future Perspectives

Despite these findings, several questions remain persist. Future research should focus on elucidating additional upstream regulators and downstream effectors involved in the ELAVL1-HMOX1 axis to fully delineate its role in DR and other diabetic complications such as nephropathy and neuropathy. Investigations into the functional impact of modulating this pathway on retinal cells under sustained hyperglycemic conditions will be essential. Critically, advancing the development of specific inhibitors (eg, small molecules, oligonucleotides) targeting ELAVL1 or HMOX1 necessitates parallel exploration of ocular delivery strategies. This includes evaluating the feasibility and safety of localized administration routes (eg, intravitreal injection) using viral vectors such as AAV or non-viral systems like lipid nanoparticles, given the current lack of studies on pharmacological ELAVL1 inhibition in ocular contexts. Integrating multi-omics approaches, such as transcriptomics and proteomics, with patient-derived models will facilitate a more comprehensive understanding of the molecular networks involved. Translational efforts will necessitate rigorous preclinical validation and well-designed clinical trials to realize the therapeutic potential of these findings. Future studies should incorporate effect size metrics (eg, Cohen's d , η^2 , or odds ratios) and confidence intervals in addition to p -values to enhance the statistical rigor and interpretability of the results.

Data Sharing Statement

The data used and/or analyzed during the current study are available from the corresponding author.

Ethics Statement

This study was approved by the ethics committee of Hunan Evidence-based Biotechnology Co., Ltd. (Approval Number: AB24072204).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflict of interest, financial or otherwise.

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