

## Article

# BACH1–CHAC1–Glutathione Axis Aggravates Myocardial Ischemia–Reperfusion Injury by Enhancing Ferroptosis and Oxidative Stress

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## Abstract

Myocardial ischemia–reperfusion injury (MIRI) is a pathological process in which reperfusion-induced oxidative stress and metabolic derangement further aggravate myocardial damage and blunt the benefit of reperfusion. Ferroptosis is increasingly implicated in MIRI, with the glutathione (GSH)–glutathione peroxidase 4 (GPX4) axis constituting a key antioxidant barrier. Although GSH depletion is recognized as a critical event, its upstream regulation in MIRI remains unclear. Against this background, we investigate the BACH1–CHAC1–GSH pathway as a putative upstream regulatory axis of ferroptosis in MIRI and a potential molecular target. Here, using an oxygen–glucose deprivation/reoxygenation (OGD/R) model in AC16 and the reversibility conferred by the ferrostatin-1, RNA sequencing identified the GSH-degrading enzyme CHAC1 as a modulator that is induced by stress and promotes ferroptosis. Experiments showed that CHAC1 overexpression aggravated OGD/R-induced injury, depleted GSH, suppressed GPX4 and enhanced lipid peroxidation, whereas CHAC1 knockdown was partially protective. N-acetylcysteine (NAC) replenished GSH, restored GPX4 activity and partially rescued CHAC1-driven injury. In a mouse myocardial I/R model, cardiotropic adeno-associated virus-mediated CHAC1 overexpression worsened cardiac dysfunction, enlarged infarct and fibrosis areas, and increased myocardial iron deposition. Dual-luciferase assays revealed that the transcription factor BACH1 activates the CHAC1 promoter, and BACH1 silencing attenuated ferroptosis by suppressing CHAC1 and restoring the GSH–GPX4 axis. Collectively, our data identify the BACH1–CHAC1–GSH axis as an upstream amplifier of ferroptosis in MIRI through glutathione depletion and impairment of GPX4-dependent antioxidant defense. These findings refine the mechanistic link between reperfusion-phase redox imbalance and ferroptosis and highlight BACH1/CHAC1 inhibition or augmentation of GSH precursors as potential cardioprotective strategies in ischemic heart disease.

**Keywords:** myocardial ischemia–reperfusion injury; ferroptosis; oxidative stress; glutathione (GSH); glutathione peroxidase 4 (GPX4); CHAC1; BACH1; lipid peroxidation



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## 1. Introduction

Ischemic heart disease (IHD) remains a leading global cause of mortality and disability [1]. While coronary reperfusion efforts, such as thrombolysis or primary percutaneous

coronary intervention (PPCI), are standard-of-care procedures to restore blood flow and preserve myocardial viability, these interventions can paradoxically induce myocardial ischemia–reperfusion injury (MIRI). However, the restoration of blood flow itself can induce MIRI, a phenomenon characterized by cellular and tissue damage triggered by the reestablishment of coronary perfusion following ischemia, which independently extends infarction and increases cardiomyocyte death [2]. Although MIRI involves multiple pathological processes such as oxidative stress, calcium overload, and inflammation [3], and can activate various modes of cell death including necroptosis, apoptosis [4], pyroptosis, autophagy [5], and ferroptosis [6], the precise mechanisms linking MIRI-mediated pathological events to cardiomyocyte death remain incompletely understood. Notably, these cell death programs are not mutually independent but may be concomitantly activated and intertwined during reperfusion [4], as accumulating evidence has reported crosstalk between autophagy-related processes and ferroptotic susceptibility [7]. Therefore, a deeper understanding of the triggers and amplification mechanisms of cardiomyocyte death during reperfusion, along with the identification of dominant pathological pathways that are amenable to intervention, is critical for developing novel cardioprotective strategies with clinical translatability.

In recent years, cell ferroptosis has become increasingly prominent as a novel form of regulated cell death, characterized by iron-dependent, intense lipid peroxidation ultimately leading to plasma membrane rupture. The key regulatory mechanism of ferroptosis depends on the delicate balance between cellular oxidative stress and the antioxidant defense system [8]. The System  $Xc^-$ –GSH–GPX4 axis is recognized as the central pathway suppressing ferroptosis [9]. Glutathione, a natural tripeptide, represents the most abundant small-molecular-weight thiol and a core antioxidant factor in living cell [10]. Glutathione peroxidase 4 (GPX4) is the key antioxidant enzyme, which utilizes GSH to reduce toxic lipid hydroperoxides into nontoxic lipid alcohols [11], thereby preventing the ferroptosis cascade driven by the accumulation of phospholipid peroxides in the plasma membrane. In parallel, ferroptotic susceptibility is also modulated by membrane lipid remodeling. Acyl-CoA synthetase long-chain family member 4 (ACSL4) facilitates the incorporation of polyunsaturated fatty acids into membrane phospholipids, thereby expanding the pool of peroxidation-prone lipid substrates and enhancing lipid peroxidation in a pro-ferroptotic pathophysiological context [12].

Previous studies have revealed significant correlations between cell ferroptosis and MIRI [9,13,14]. Metabolic disorders and the burst of oxidative stress constitute the key biochemical background for ferroptosis initiation during reperfusion. In early reperfusion, rapid oxidation of succinate accumulated triggers mitochondrial ROS surge and promotes polyunsaturated phospholipid peroxidation [15,16]. Simultaneously, the System  $Xc^-$ –GSH–GPX4 antioxidant pathway function is suppressed [9,17,18] and limited cysteine uptake and GSH synthesis lead to GSH depletion, while decreased GPX4 expression and activity result in insufficient phospholipid hydroperoxide clearance [14,17]. Intense oxidative stress coupled with the collapse of the core antioxidant defense system ultimately irreversibly initiates the ferroptosis program. In preclinical studies, ferroptosis inhibitors and GSH precursor supplements (such as NAC) can significantly reduce infarct size and improve cardiac function, demonstrating that targeting ferroptosis is a promising new therapeutic strategy for MIRI [13,19].

During early reperfusion, a surge in ROS and suppression of the System  $Xc^-$ –GSH–GPX4 pathway make GSH metabolism a major determinant of cardiomyocyte resistance to ferroptosis [20]. In 2012, CHAC1 was first identified as a highly efficient and specific cytosolic enzyme for GSH hydrolysis [21], exhibiting minimal catalytic activity toward other  $\gamma$ -glutamyl peptides or oxidized glutathione (GSSG). CHAC1 cleaves reduced GSH into 5-oxoproline

and Cys-Gly, thereby directly depleting the intracellular GSH pool [22]. In various stress and disease models—such as amino acid starvation, oxidative stress, and inflammatory stress—CHAC1 is often induced and accelerates GSH depletion, subsequently limiting GPX4 substrate availability and amplifying lipid peroxidation and ferroptosis [22–24]. Conversely, silencing CHAC1 can partially restore GSH levels and attenuated cell ferroptosis in multiple models [22].

In this study, we identified differentially expressed genes ( $|\text{FC}| \geq 2$ , adjusted  $p < 0.05$ ) induced by OGD/R in AC16 cells and focused on those genes whose expression was reversed by ferrostatin-1 (Fer-1), a canonical small-molecule inhibitor of ferroptosis that acts as a lipid radical-trapping antioxidant to reduce iron-dependent phospholipid peroxidation [25], as candidates. Integrated with enrichment analysis, the CHAC1 was selected as the key subject of this study. To further investigate the upstream transcriptional mechanisms regulating CHAC1 in MIRI, we extended our focus to transcription factors closely involved in oxidative stress responses. BACH1 is a CNC-bZIP transcription factor that functions as a key redox-responsive regulator, classically acting in opposition to NRF2 at ARE/MARE-related elements [26]. BACH1 has been reported to influence antioxidant defenses and iron homeostasis pathways [27], thereby shaping cellular susceptibility to lipid peroxidation and ferroptosis [28]. Collectively, these properties position BACH1 as a plausible upstream transcriptional regulator of stress-inducible genes involved in glutathione homeostasis. Subsequently, we performed a dual-luciferase reporter assay to evaluate the effect of BACH1 on CHAC1 promoter activity, to examine whether BACH1 regulates CHAC1 promoter-driven transcriptional activity. Based on these findings, we propose the hypothesis that during myocardial ischemia–reperfusion, activation of the BACH1–CHAC1–GSH axis contributes to glutathione depletion, impairs GPX4-dependent peroxidation-clearing capacity, and thereby amplifies lipid peroxidation, promotes ferroptosis, and exacerbates myocardial injury. This hypothesis may provide both a druggable molecular target and a theoretical foundation for elucidating the mechanisms of myocardial ischemia–reperfusion injury and informing future research on cardioprotective interventions.

## 2. Materials and Methods

Detailed methods are available in the Supplementary Methods.

### 2.1. Cell Culture and Establishment of the OGD/R Model

The human cardiomyocyte line AC16 (Fu Heng Biotechnology, Shanghai, China) was grown in DMEM/F12 (C11330500BT, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in a humidified incubator at 37 °C with 5% CO<sub>2</sub>, and was subcultured when reaching approximately 80–90% confluence.

To mimic myocardial ischemia–reperfusion in vitro, AC16 cells were subjected to oxygen–glucose deprivation followed by reoxygenation (OGD/R). For the deprivation phase, the culture medium was replaced with glucose- and serum-free DMEM/F12, and the plates were placed into an AnaeroPack<sup>®</sup> anaerobic system (GEN-90007, Mitsubishi Gas Chemical, Tokyo, Japan) equilibrated with 95% N<sub>2</sub>/5% CO<sub>2</sub> at 37 °C for 6 h. Reoxygenation was then achieved by returning cells to complete DMEM/F12 under normoxic conditions (95% air/5% CO<sub>2</sub>) for 12 h. Experimental groups included untreated control, OGD/R alone, OGD/R with ferrostatin-1 pretreatment (1 µM, 24 h before OGD), and OGD/R with N-acetylcysteine pretreatment (1 mM, 2 h before OGD).

### 2.2. Cell Viability and Cytotoxicity Assays

Cell viability was assessed using a CCK-8 assay in 96-well plates. Cytotoxicity and plasma membrane damage were evaluated by measuring lactate dehydrogenase

(LDH) activity in culture supernatants. Live and dead cells were visualized by Calcein-AM/propidium iodide double staining and fluorescence microscopy.

### 2.3. Assessment of Ferroptosis, Oxidative Stress, and Mitochondrial Function

Ferroptosis-related biochemical changes were profiled using multiple probes and colorimetric assays. Labile  $\text{Fe}^{2+}$  was detected with FerroOrange; total intracellular ROS with DCFH-DA; lipid ROS with BODIPY 581/591 C11; and mitochondrial membrane potential ( $\Delta\Psi\text{m}$ ) with JC-1. MDA levels and  $\text{Fe}^{2+}$  content were measured with commercial kits. Total, reduced, and oxidized glutathione (T-GSH, GSH, GSSG) were quantified and normalized to protein content.

### 2.4. Transmission Electron Microscopy

To examine mitochondrial ultrastructure, treated AC16 cells were fixed with glutaraldehyde, post-fixed with osmium tetroxide, embedded in resin, and sectioned. Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a Hitachi transmission electron microscope to assess ferroptosis-associated mitochondrial changes.

### 2.5. Immunofluorescence Staining and Confocal Imaging

Cells grown on coverslips were fixed with paraformaldehyde, permeabilized with Triton X-100, and blocked with bovine serum albumin. Primary antibodies against CHAC1 and BACH1 were applied overnight at 4 °C, followed by fluorophore-conjugated secondary antibodies. Nuclei were counterstained with DAPI. Images were acquired using a Leica TCS SP8 confocal microscope and analyzed with ImageJ (version 1.54p; National Institutes of Health, Bethesda, MD, USA).

### 2.6. Gene Silencing and Lentiviral Overexpression

Knockdown of CHAC1 and BACH1 was accomplished by transfecting specific siRNAs using Lipofectamine RNAiMAX, with a scrambled siRNA serving as negative control. For stable overexpression, AC16 cells were infected with lentiviral vectors encoding FLAG-tagged CHAC1 or the corresponding empty control vector and then subjected to puromycin selection to generate stable cell lines. The efficiency of gene knockdown and overexpression was validated by RT-qPCR and Western blotting.

### 2.7. RNA Sequencing and Bioinformatic Analysis

Total RNA was extracted from the indicated samples and subjected to high-throughput sequencing. Differentially expressed genes were identified using DESeq2 with  $|\text{FC}| \geq 2$ , adjust  $p < 0.05$  as thresholds. Gene Ontology and KEGG pathway enrichment, as well as gene set enrichment analysis (GSEA), were performed using R-based pipelines.

### 2.8. Animals and Ethical Approval

Male C57BL/6J mice (7–8 weeks, 20–25 g) were purchased from GemPharmatech (GemPharmatech, Nanjing, Jiangsu, China) and housed under controlled temperature and humidity with a 12 h light/dark cycle, with free access to standard chow and water. All procedures followed the institutional guidelines of Tongren Hospital, Shanghai Jiao Tong University School of Medicine and the NIH Guide for the Care and Use of Laboratory Animals, and complied with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. All animal protocols were approved by the Institutional Animal Care and Use Committee of Tongren Hospital (approval ID: A2025-053-01, approval date: 26 September 2025). Every effort was made to minimize animal suffering and reduce the number of animals used.

### 2.9. Murine Myocardial Ischemia–Reperfusion and AAV-Mediated Chac1 Overexpression

Myocardial ischemia–reperfusion (I/R) was induced by reversible ligation of the left anterior descending coronary artery. After acclimatization, mice were randomly assigned to the sham, I/R + AAV-Vector, or I/R + AAV-CHAC1 groups. For cardiomyocyte-targeted Chac1 overexpression, mice in the I/R + AAV-CHAC1 and I/R + AAV-Vector groups received systemic tail-vein injection of a myoAAV1A vector encoding Chac1 or the corresponding empty vector ( $1.5 \times 10^{11}$  vg per mouse), respectively, two weeks before I/R. I/R was then induced by reversible ligation of the left anterior descending coronary artery. Under isoflurane anesthesia, a left thoracotomy was performed, the LAD was occluded with a slipknot for 45 min, and then the ligature was released to allow reperfusion; sham mice underwent the same procedure without LAD ligation. Hearts were collected at 4 h, 1 day, and 7 days of reperfusion for downstream analyses.

### 2.10. Echocardiography and Histological Assessment

Cardiac function was evaluated by transthoracic echocardiography using a Vevo 2100 system with a high-frequency transducer under light isoflurane anesthesia. M-mode images obtained from the parasternal short-axis view were used to measure LVIDd and LVIDs and to calculate LVEF and LVFS. Myocardial infarct size was determined by TTC staining of transverse heart slices; infarct size and area at risk were quantified with ImageJ. Myocardial fibrosis was assessed by Masson's trichrome staining of paraffin sections. Myocardial iron deposition was detected by DAB-enhanced Perls' Prussian blue staining.

### 2.11. Western Blotting

Proteins from cells or heart tissues were extracted in RIPA buffer containing protease and phosphatase inhibitors, quantified by BCA assay, separated by SDS–PAGE, and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk, incubated with primary antibodies against ferroptosis-related and antioxidant proteins, and then with HRP-conjugated secondary antibodies. Bands were visualized by enhanced chemiluminescence and quantified by ImageJ;  $\beta$ -actin or  $\beta$ -tubulin served as loading controls.

### 2.12. Quantitative Real-Time PCR

Total RNA from cultured cells and myocardial tissue was reverse-transcribed to cDNA and analyzed by SYBR-based RT-qPCR. Relative mRNA expression was calculated using the  $2^{-\Delta\Delta C_t}$  method with ACTB as the internal reference. Primer sequences are listed in Supplementary Table S1, and representative amplification and melt curves for CHAC1 are shown in Supplementary Figure S3.

### 2.13. Statistical Analysis

Data are expressed as mean  $\pm$  SEM. Statistical analyses were performed using Graph-Pad Prism 9. Two-group comparisons used unpaired two-tailed Student's *t*-tests; comparisons among three or more groups used one-way ANOVA followed by Tukey's post hoc test. A *p* value  $< 0.05$  was considered statistically significant. Exact *n* values and statistical tests are specified in the figure legends.

## 3. Results

### 3.1. Fer-1 Alleviates OGD/R-Induced Cardiomyocyte Injury and Ferroptosis

To determine whether ferroptosis contributes to OGD/R-induced cardiomyocyte injury, we established an oxygen–glucose deprivation/reoxygenation (OGD/R) model in AC16 cells and pretreated cells with 1  $\mu$ M Ferrostatin-1 (Fer-1) for 24 h before OGD/R. Cell viability and membrane damage were assessed by CCK-8 and LDH release assays.

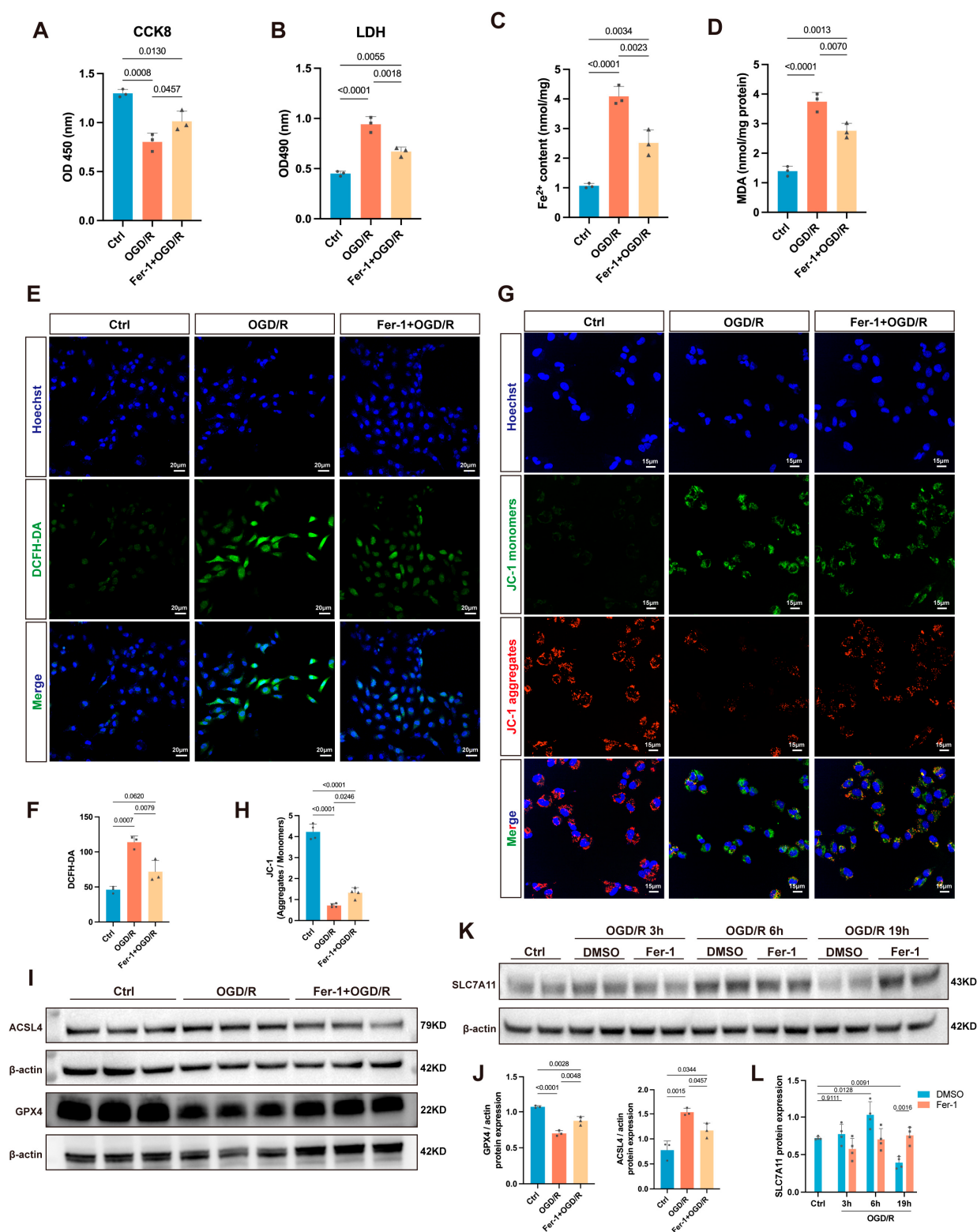
OGD/R markedly reduced cell viability and increased LDH release compared with the control group (Figure 1A,B). Fer-1 pretreatment significantly restored cell viability and attenuated LDH release, indicating that Fer-1 effectively protects AC16 cardiomyocytes from OGD/R-induced injury.

Ferroptosis is driven by iron-dependent lipid peroxidation, and ferrous iron levels and MDA, a key end product of lipid peroxidation, are commonly used as biomarkers. Intracellular  $\text{Fe}^{2+}$  content (Figure 1C) and MDA levels (Figure 1D) were significantly elevated after OGD/R treatment, whereas Fer-1 largely reversed these changes, reducing iron accumulation and lipid peroxidation. Mitochondrial dysfunction and morphological changes are also hallmarks of ferroptosis [29]. Changes in mitochondrial membrane potential ( $\Delta\Psi\text{m}$ ) reflect the degree of cellular injury and are commonly used to assess mitochondrial dysfunction during ferroptosis-associated injury [30]. JC-1 staining (Figure 1G) showed a significant decrease in  $\Delta\Psi\text{m}$  after OGD/R, which was partially reversed by Fer-1. In parallel, intracellular ROS levels increased after OGD/R and were reduced by Fer-1 (Figure 1E), supporting a key role of oxidative stress in OGD/R-induced ferroptotic injury.

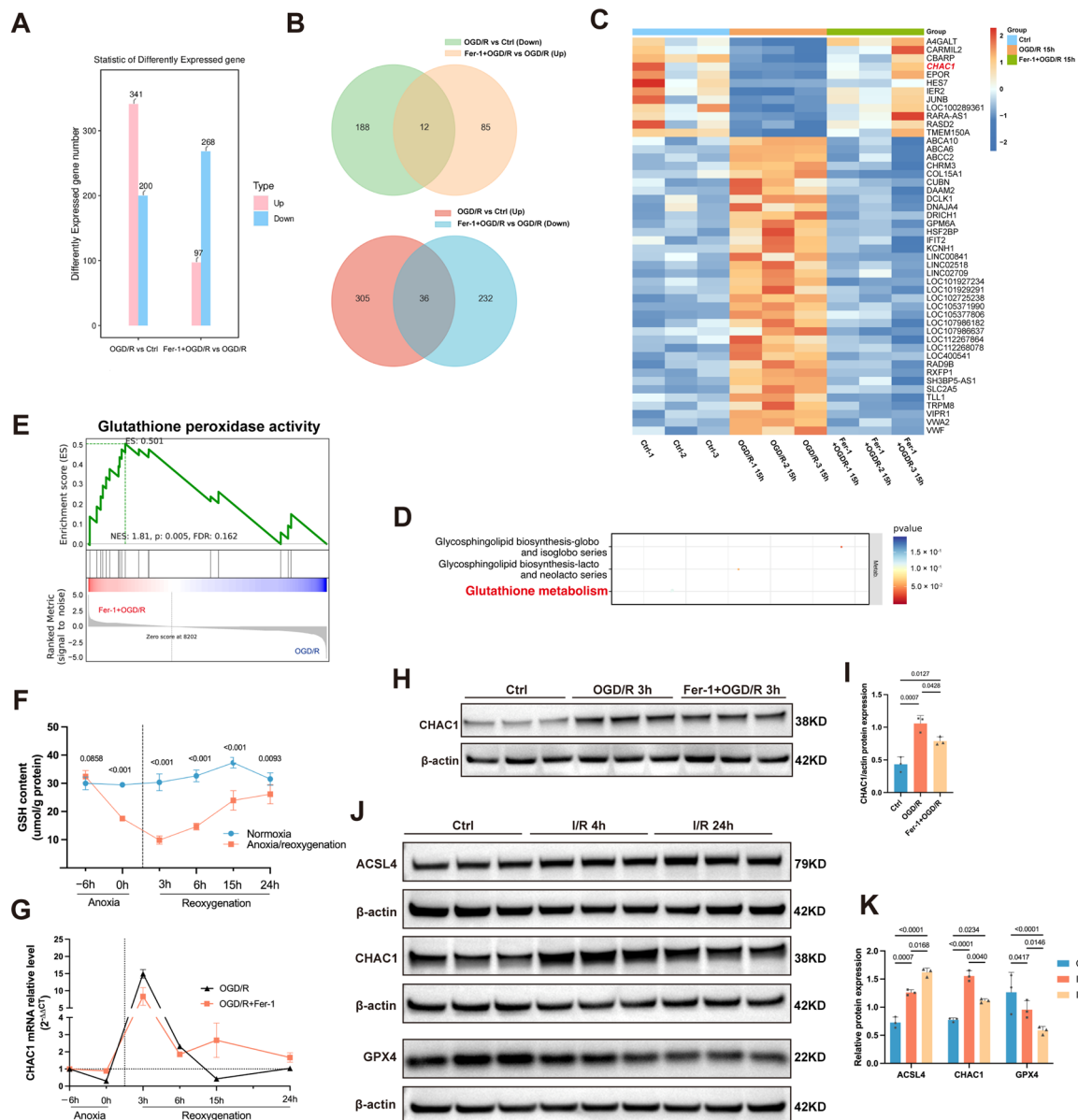
We further examined ferroptosis-related proteins. OGD/R increased ACSL4 expression and decreased GPX4 expression (Figure 1I), consistent with a shift toward a peroxidation-prone state and impaired GPX4-dependent detoxification of phospholipid hydroperoxides. Notably, ferroptosis was evaluated based on an integrated set of readouts, including key ferroptosis-related proteins (e.g., ACSL4/GPX4/SLC7A11), lipid peroxidation, iron accumulation, ROS burden, and mitochondrial dysfunction, rather than any single marker alone. Additionally, SLC7A11, a key subunit of system  $\text{Xc}^-$ , is often suppressed during ferroptosis [31]. Interestingly, in this study, its expression showed an initial increase followed by a decrease over the reperfusion period (Figure 1K). Fer-1 pretreatment attenuated ACSL4 upregulation and restored the OGD/R-induced decrease in GPX4 and SLC7A11 (Figure 1I–K). This suggests that Fer-1 exerts protection by stabilizing the  $\text{Xc}^-$ -GSH-GPX4 antioxidant barrier and inhibiting pro-peroxidation pathways. Together, these results support that OGD/R induces cardiomyocyte ferroptosis, and that Fer-1 effectively rescues this process, thereby stabilizing membrane lipids, alleviating iron-dependent oxidative damage, and ameliorating the ferroptotic phenotype.

### 3.2. CHAC1 Dynamically Changes After OGD/R Treatment

To identify key molecules related to cell ferroptosis after OGD/R treatment, we performed RNA-seq on AC16 cells from Control group, OGD/R group, and Fer-1+OGD/R groups. Differentially expressed genes (DEGs) were identified using  $|\text{Fold Change}| \geq 2$  and adjusted  $p < 0.05$  (Figure 2A), and 48 candidate genes were selected based on the intersection of genes “OGD/R group and Fer-1+OGD/R group” (Figure 2B). GO/KEGG enrichment analysis of these candidate genes revealed significant enrichment in the glutathione metabolic pathway (Figure 2D). Given the small number of candidate genes, to exclude the possibility of chance enrichment for glutathione metabolism, we relaxed the screening criteria and performed enrichment analysis on all DEGs with  $|\text{FC}| \geq 2$ , adjusted  $p < 0.05$ . GSEA also showed significant enrichment for glutathione peroxidase activity (Figure 2E), corroborating the GO/KEGG results and suggesting a key role for glutathione metabolism in OGD/R-associated ferroptosis. Based on this, we focused on CHAC1, which is both a member of the glutathione metabolic pathway (Supplementary Figure S2) and among the candidate genes (Figure 2C).



**Figure 1.** OGD/R modeling in AC16 cells and the effects of Fer-1 on injury and ferroptosis. (A,B) Cell viability and membrane integrity: CCK-8 and LDH release ( $n = 3$  independent experiments). (C,D) Iron and lipid peroxidation: intracellular Fe<sup>2+</sup> and MDA ( $n = 3$  independent experiments). (E,F) Intracellular ROS: DCFH-DA imaging and quantification ( $n = 3$  independent experiments). (G,H)  $\Delta\Psi_m$ : JC-1 imaging showing red J-aggregates (high  $\Delta\Psi_m$ ) vs. green J-monomers (low  $\Delta\Psi_m$ ); red/green ratio ( $n = 4$  independent experiments). (I–L) Immunoblots of ACSL4, GPX4, and SLC7A11 at indicated reperfusion times;  $\beta$ -actin loading control; densitometry ( $n = 3$  independent experiments). Data are presented as mean  $\pm$  SD. One-way ANOVA with Tukey's post hoc test; exact  $p$  values in the figure.



**Figure 2.** Transcriptomic nomination and time-course validation of CHAC1 and its link to glutathione metabolism. (A–E) Transcriptomic analyses based on RNA-seq ( $n = 3$  biological replicates per group). (A,B) Transcriptomic screening: DEG counts and Venn selection of candidates altered by OGD/R and reversed by Fer-1 ( $|\text{fold change}| \geq 2$ ; adjust  $p < 0.05$ ). (C) Candidate visualization: heatmap highlighting CHAC1 across groups. (D,E) Pathway enrichment: GO/KEGG analysis of candidate genes and GSEA for the “glutathione peroxidase activity” gene set (The red-to-blue color bar indicates the ranking metric (signal-to-noise) from Fer-1+OGD/R (red) to OGD/R (blue)). (F,G) Time course after OGD/R: reduced glutathione (GSH) content and CHAC1 mRNA levels at indicated hypoxia/reperfusion time points ( $n = 3$  independent experiments). (H,I) CHAC1 protein in AC16 cells: immunoblots with β-actin loading control and densitometry ( $n = 3$  independent experiments). (J,K) In vivo validation: cardiac tissue immunoblots and densitometry for CHAC1, GPX4, and ACSL4 in the mouse I/R model ( $n = 3$  independent experiments). Data are presented as mean  $\pm$  SD. One-way ANOVA with Tukey’s post hoc test for bar or line graphs; exact  $p$  values in the figure.

To investigate the relationship between glutathione metabolic and CHAC1 after OGD/R treatment, we measured GSH (reduced glutathione) content (Figure 2F) and CHAC1 mRNA levels (Figure 2G) at different reperfusion time points. Results showed that GSH was already reduced at the end of hypoxia, declined further in early reperfusion, and

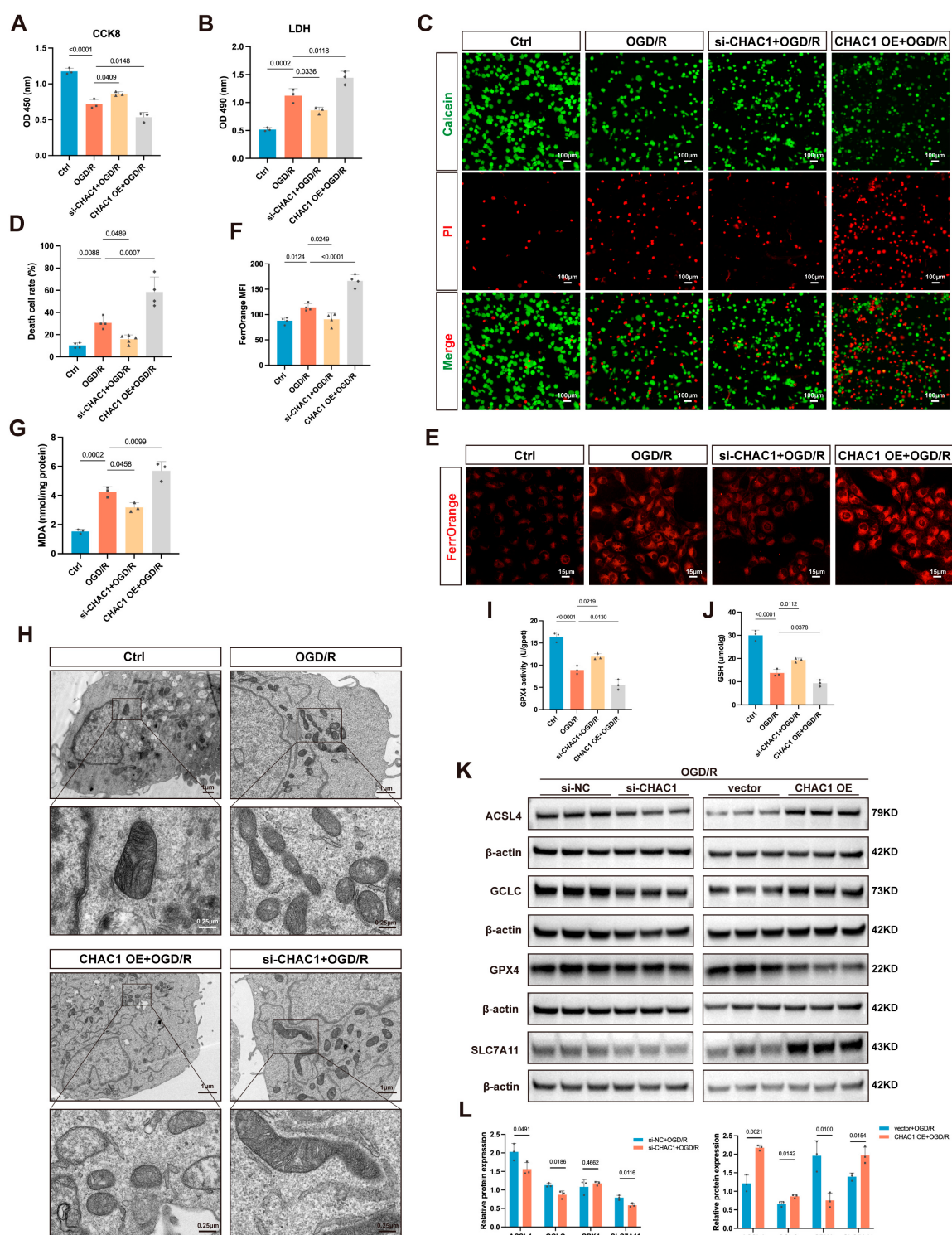
then gradually recovered toward baseline levels in the mid-to-late phase. Correspondingly, CHAC1 mRNA expression exhibited a dynamic pattern of “early increase—subsequent decrease—return to baseline”: it rose rapidly in early reperfusion, then decreased significantly, and finally returned to baseline. Fer-1 treatment did not change this trend but markedly attenuated the expression level. CHAC1 protein expression at 3 h of reperfusion followed the same trend as the PCR results (Figure 2H,I). Overall, during reperfusion, the rapid upregulation of CHAC1 coincides with the nadir of GSH, indicating a functional antagonism between the two and suggesting that the marked increase in CHAC1 in early reperfusion may aggravate oxidative stress by degrading GSH, thereby driving cells toward irreversible injury and initiating ferroptosis.

To validate these findings *in vivo*, we assessed CHAC1 expression in a C57 mouse myocardial I/R model. Results showed that GPX4 expression decreased and ACSL4 expression increased at 4 h of reperfusion, with more pronounced changes after 24 h (Figure 2J,K), indicating ferroptosis occurrence in the mouse I/R model that worsened with prolonged reperfusion [14,17]. CHAC1, the focus of our study, began to increase at 4 h of reperfusion, and the magnitude of increase subsided by 24 h (Figure 2J,K), consistent with the cell experiments. These data indicate that during reperfusion, I/R induces a rapid early increase in CHAC1 expression, followed by a subsequent decline. Based on these findings, we identified the CHAC1–GSH–GPX4 axis as a major candidate pathway for mechanistic dissection and therapeutic targeting in OGD/R-induced ferroptosis.

### 3.3. CHAC1 Modulate Cell Ferroptosis Under OGD/R Treatment

To validate the role of CHAC1 in ferroptosis under OGD/R conditions, CHAC1 was knocked down or overexpressed in AC16 cells. Compared with OGD/R alone, si-CHAC1 significantly increased cell viability and reduced LDH release and the proportion of dead cells, whereas CHAC1 overexpression (OE-CHAC1) decreased survival and exacerbated cell injury (Figure 3A–D). FerroOrange staining showed that intracellular  $\text{Fe}^{2+}$  levels were markedly elevated in the OGD/R group relative to control, attenuated by CHAC1 knockdown, and further enhanced by OE-CHAC1 (Figure 3E,F). Consistently, MDA assays revealed that si-CHAC1 reduced OGD/R-induced MDA accumulation, whereas OE-CHAC1 produced the opposite effect, indicating aggravated lipid peroxidation. Transmission electron microscopy (TEM) (Figure 3H) showed that OGD/R induced typical ferroptotic mitochondrial changes, including loss of cristae, vacuolization, and outer-membrane rupture; these abnormalities were partially corrected by si-CHAC1 (relatively intact membranes and attenuated cristae loss), whereas OE-CHAC1 induced nearly complete cristae loss and severe vacuolization. In summary, CHAC1 upregulation significantly increases susceptibility to OGD/R-induced ferroptosis—manifested by decreased viability, increased membrane damage,  $\text{Fe}^{2+}$ /MDA accumulation, and mitochondrial destruction—whereas CHAC1 inhibition exerts a protective effect.

To further investigate how CHAC1 exacerbates cardiomyocyte ferroptosis in the setting of myocardial ischemia–reperfusion injury (MIRI), and given its core biological function as a specific intracellular GSH hydrolase, we next measured GSH content (Figure 3I). OE-CHAC1 significantly decreased GSH levels, whereas si-CHAC1 partially restored GSH after OGD/R. GPX4 uses GSH as a substrate to detoxify phospholipid peroxides, and its activity is strongly dependent on GSH availability [32]; GSH depletion directly lowers GPX4 activity, triggering lipid peroxidation and ferroptosis [33]. Accordingly, GPX4 activity (Figure 3J) changed in parallel with GSH content, decreasing with OE-CHAC1 and increasing with CHAC1 knockdown.



**Figure 3.** Effects of CHAC1 manipulation on ferroptosis phenotypes under OGD/R in AC16 cells. (A,B) Cell injury: CCK-8 viability (OD450) and LDH release (OD490) ( $n = 3$  independent experiments). (C,D) Cell death: Calcein-AM/PI live/dead imaging and quantification ( $n = 4$  independent experiments). (E,F) Intracellular  $\text{Fe}^{2+}$ : FerroOrange fluorescence imaging and quantification ( $n = 4$  independent experiments). (G) Lipid peroxidation: MDA levels ( $n = 3$  independent experiments). (H) TEM images of mitochondrial ultrastructure. (I,J) GSH content and GPX4 enzymatic activity. (K,L) Immunoblots for the indicated proteins with  $\beta$ -actin as loading control and corresponding densitometry ( $n = 3$  independent experiments). Data are presented as mean  $\pm$  SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test; exact p values are indicated in the figures.

At the protein level (Figure 3K,L), OE-CHAC1 increased ACSL4 and decreased GPX4, whereas CHAC1 knockdown reduced ACSL4 but did not significantly rescue GPX4. Unexpectedly, SLC7A11 and GCLC were upregulated under CHAC1 overexpression and downregulated after CHAC1 knockdown. We speculate that these changes in SLC7A11 and GCLC represent a compensatory response to CHAC1-induced GSH depletion and oxidative stress [34,35]. Collectively, these findings support a “CHAC1–GSH depletion–GPX4 inhibition–amplified lipid peroxidation” axis.

### 3.4. Cardiomyocyte-Targeted CHAC1 Overexpression Exacerbates I/R Injury in Mice

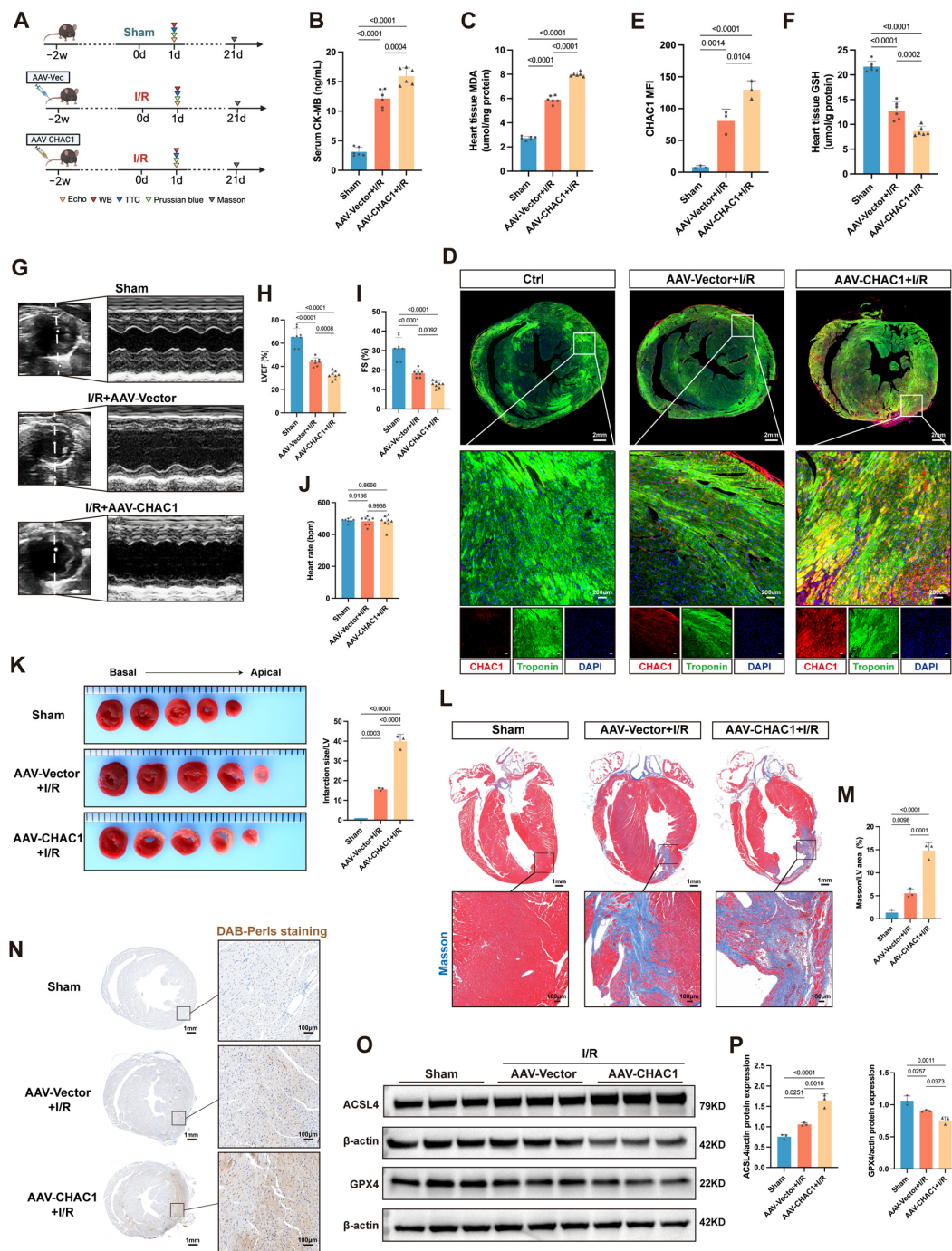
To assess the impact of CHAC1 on I/R injury in vivo, we intravenously injected C57BL/6J mice with cardiomyocyte-targeted AAV-CHAC1 or control AAV-Vector (Figure 4A). Two weeks later, myocardial CHAC1 expression was markedly increased in the AAV-CHAC1 group (Figure 4D,E), and a left anterior descending coronary artery ligation/reperfusion model was established. Compared to sham group, the AAV-Vector +I/R group showed significantly increased plasma creatine kinase-MB (CK-MB) and myocardial MDA, which were further exacerbated by AAV-CHAC1+I/R (Figure 4B,C). Echocardiography (Figure 4G–J) revealed that I/R-induced impairment of systolic function (EF, FS) was more pronounced in myocardial CHAC1-overexpressing mice.

Histologically, TTC staining demonstrated an expansion of infarct size in the AAV-CHAC1+I/R group compared with AAV-Vector +I/R group (Figure 4K). In parallel, compared with AAV-Vector +I/R, the AAV-CHAC1+I/R group exhibited more pronounced interstitial fibrosis on Masson staining at 7 days post-reperfusion, accompanied by ventricular dilation and myocardial wall thinning (Figure 4L,M), while DAB-Perls staining revealed more diffuse dark-brown iron deposits within the myocardium, indicating aggravated iron overload (Figure 4N). Moreover, I/R led to a significant decrease in myocardial GSH content, which was further exacerbated by CHAC1 overexpression. Concurrently, CHAC1 overexpression also enhanced the I/R-induced upregulation of ACSL4 level and downregulation of GPX4 level (Figure 4O,P).

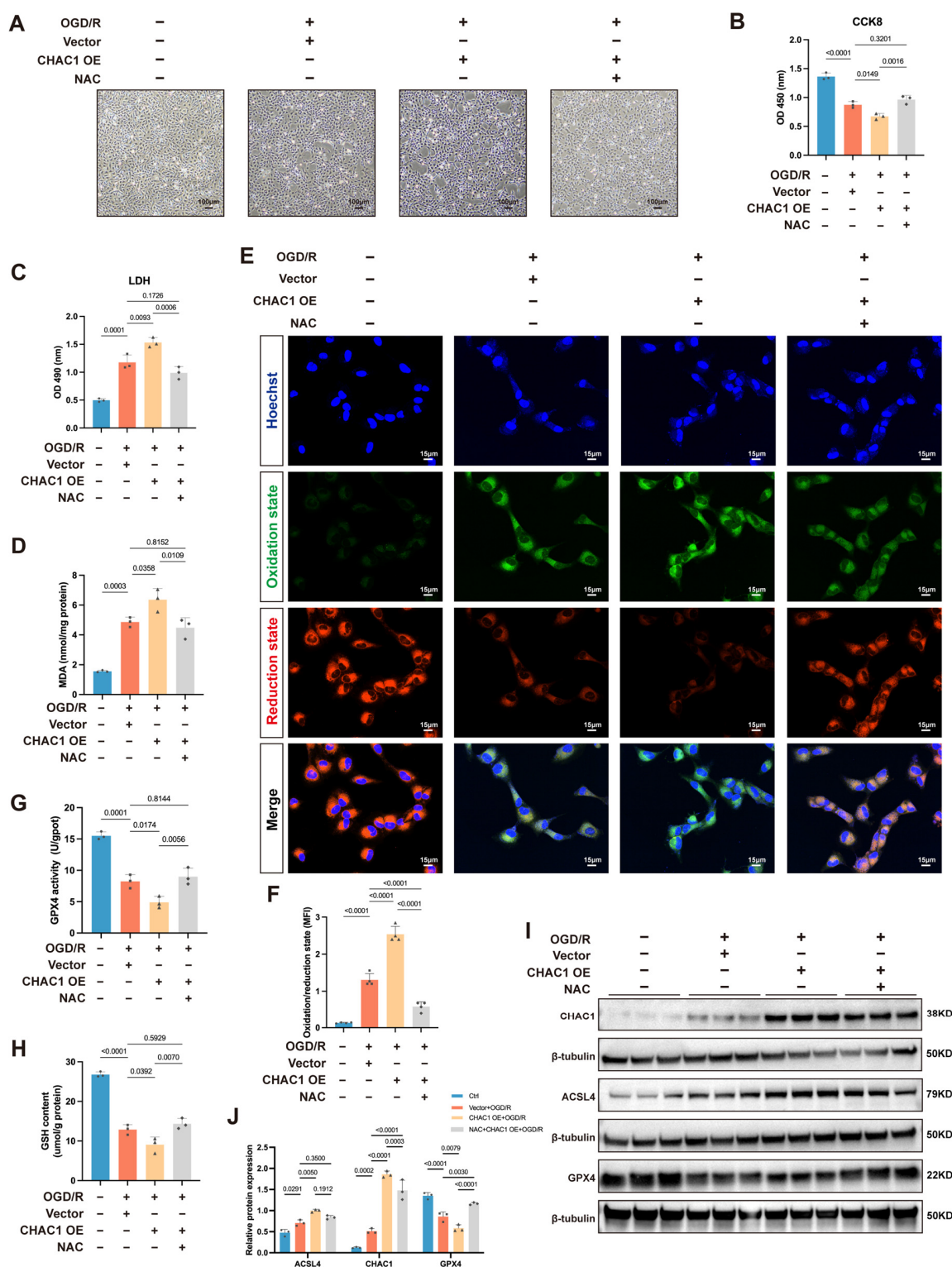
In summary, CHAC1 expression is upregulated in mouse myocardium subjected to I/R, and CHAC1 overexpression aggravates I/R injury, manifested as worsened cardiac function, enlarged infarct size, increased fibrosis and iron deposition, and promotion of GSH depletion and ferroptosis, suggesting that CHAC1 plays a key role in promoting I/R injury both in vitro and in vivo.

### 3.5. NAC Rescues CHAC1-Exacerbated OGD/R Injury

In order to detect the causality and reversibility of the CHAC1–GSH–GPX4 axis, we evaluated whether GSH supplementation could inhibit CHAC1-mediated OGD/R-induced cardiomyocyte ferroptosis. N-acetylcysteine (NAC), a precursor of GSH synthesis, can effectively increase the intracellular GSH reserve and supplement the cysteine pool [36]. Cells were pretreated with 1 mM NAC 2 h before OGD/R, and this concentration was maintained during reperfusion. Morphologically, OGD/R induced cell rounding, increased intercellular spaces, and accumulation of cellular debris; CHAC1 overexpression further led to extensive cell rounding, balloon-like swelling, and large-area detachment, whereas NAC partially reversed these changes (Figure 5A). CCK-8 and LDH analyses showed that NAC partially alleviated the CHAC1 overexpression-induced decrease in cell viability and membrane integrity (Figure 5B,C). In addition, MDA content and lipid ROS fluorescence intensity were significantly decreased after NAC treatment (Figure 5D–F).



**Figure 4.** Functional, histological, and molecular assessments in mice with cardiomyocyte-specific CHAC1 overexpression subjected to I/R. (A) Experimental timeline and AAV intervention. (B,C) Serum CK-MB and myocardial MDA levels ( $n = 6$  animals per group). (D,E) Cardiac CHAC1 expression: representative immunofluorescence staining and mean fluorescence intensity ( $n = 3$  animals per group). (F) Myocardial GSH content ( $n = 6$  animals per group). (G–J) Echocardiography: representative M-mode images (G) and quantification of LVEF (H), LVFS (I), and HR (J) ( $n = 8$  animals per group). (K) TTC-stained serial short-axis sections from base to apex and infarct size quantification ( $n = 3$  animals per group). (L,M) Masson staining at 7 days post-reperfusion and collagen fraction quantification ( $n = 3$  animals per group). (N) DAB-enhanced Perls staining showing myocardial iron deposition (brown) and nuclei (light blue). (O,P) Immunoblots of the indicated proteins, with  $\beta$ -actin serving as the loading control, and the corresponding densitometry ( $n = 3$  animals per group). Data are shown as mean  $\pm$  SD, and individual animals are displayed as dots. Statistical comparisons were made by one-way ANOVA with Tukey's post hoc test; exact  $p$  values are reported in the figures.



**Figure 5.** NAC reverses CHAC1-overexpression-induced ferroptosis phenotypes during OGD/R. (A) Bright-field images of cell morphology. (B,C) Cell injury: CCK-8 viability (OD450) and LDH release (OD490) ( $n = 3$  independent experiments). (D) MDA levels ( $n = 3$  independent experiments). (E,F) Lipid peroxidation: BODIPY 581/591 C11 fluorescence imaging (E) and green/red ratio quantification (F) ( $n = 3$  independent experiments). (G,H) GPX4 enzymatic activity and GSH content ( $n = 3$  independent experiments). (I,J) Immunoblots for the indicated proteins with  $\beta$ -actin as loading control and corresponding densitometry ( $n = 3$  independent experiments). Data are presented as mean  $\pm$  SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test; exact  $p$  values are indicated in the figures.

Mechanistically, in the context of CHAC1 overexpression, NAC effectively reversed OGD/R-induced GSH depletion and inhibition of GPX4 activity (Figure 5G,H). Consistently, NAC reduced GSSG levels and partially improved the GSH/GSSG ratio (Figure S5). Western blot results showed that NAC promoted the recovery of GPX4 expression and suppressed ACSL4 levels; notably, NAC also slightly reduced CHAC1 levels, suggesting that NAC might indirectly dampen CHAC1 expression by alleviating oxidative stress.

In summary, NAC attenuated the ferroptotic process exacerbated by CHAC1 overexpression. By replenishing GSH, NAC helps maintain the peroxide-scavenging capacity of GPX4 and thereby reduces ferroptosis; these findings further consolidate the link between the CHAC1–GSH–GPX4 axis and cardiomyocyte ferroptosis.

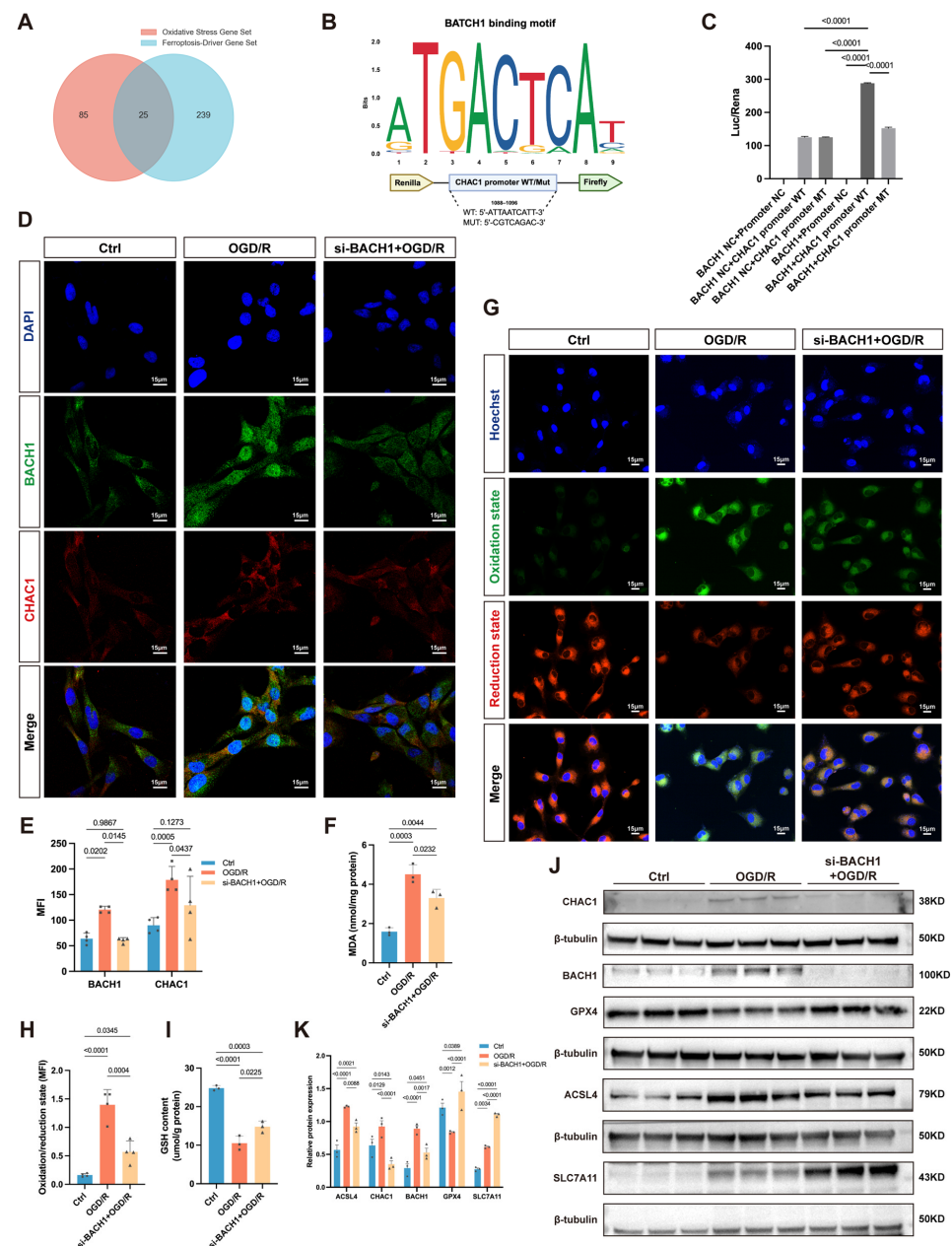
### 3.6. BACH1 Regulates CHAC1 Expression After OGD/R Treatment

To identify the upstream regulator of CHAC1 in cells, we established a three-step process of “candidate screening–prediction–validation” based on public databases. First, we constructed a human oxidative stress-related gene set by merging gene sets closely associated with oxidative stress from MSigDB ([https://www.gsea-msigdb.org/gsea/msigdb/human/geneset/GOBP\\_RESPONSE\\_TO\\_OXIDATIVE\\_STRESS](https://www.gsea-msigdb.org/gsea/msigdb/human/geneset/GOBP_RESPONSE_TO_OXIDATIVE_STRESS); accessed on 3 August 2025) (GO:0006979 Response to Oxidative Stress, HALLMARK Reactive Oxygen Species Pathway, Reactome Detoxification of ROS) (Table S3). Oxidative stress overwhelms the endogenous antioxidant defense, leading to lipid peroxidation and cell damage after myocardial ischemia–reperfusion, thus making oxidative stress a key link in the pathogenesis of I/R [15,37,38]. Similarly, we extracted ferroptosis drivers from FerrDb (<http://www.zhounan.org/ferrdb/>; accessed on 3 August 2025) to form a ferroptosis driver gene set (Table S2). Then, we took the intersection of these two gene sets (Figure 6A) and screened for transcription factor (TF) members within the intersection, thereby obtaining a set of upstream transcription factors connecting the oxidative stress–ferroptosis pathway. Finally, we performed JASPAR motif prediction for each candidate TF against the CHAC1 promoter sequence. Integrating multi-database consistency and site reliability, BACH1 was predicted to be an upstream transcription factor of CHAC1. Based on this clue, we further validated the BACH1–CHAC1 regulatory relationship through dual-luciferase reporter assays.

We constructed pGL3-CHAC1-WT vectors and the corresponding point-mutated pGL3-CHAC1-MT vectors, co-transfected them with a BACH1 overexpression vector into HEK293T cells, and performed dual-luciferase assays (The promoter construct map and raw dual-luciferase readouts are provided in Figure S1). Results showed that BACH1 significantly enhanced CHAC1-WT promoter activity, indicating that BACH1 can bind the CHAC1 promoter and enhance its transcriptional activity (Figure 6B,C).

Previous reports indicate that BACH1 is upregulated in ischemia/reperfusion models [39–41], consistent with our observation of increased BACH1 protein levels in mouse hearts after ischemia/reperfusion (I/R) (Figure S4), and that BACH1 knockdown alleviates cell ferroptosis and protects cardiomyocytes [40]. Immunofluorescence results showed that OGD/R induced BACH1 upregulation and nuclear translocation, with a concurrent increase in CHAC1 expression; knocking down BACH1 led to a decrease in both CHAC1 and BACH1 (Figure 6D,E). Furthermore, analysis of MDA content (Figure 6F) and lipid ROS fluorescence intensity (Figure 6G,H) indicated that BACH1 interference partially alleviated OGD/R-induced lipid peroxidation. Metabolically, si-BACH1 effectively mitigated OGD/R-induced GSH depletion. Western blot results (Figure 6J,K) showed that BACH1 knockdown suppressed the OGD/R-induced decrease in GPX4 expression and increase in ACSL4 expression, and promoted the recovery of SLC7A11 expression; CHAC1 levels also decreased with BACH1 knockdown, consistent with the immunofluorescence results. These findings are largely consistent with reports in *J. Biol. Chem.* [42] that BACH1, by

inhibiting antioxidant genes such as SLC7A11, depleting GSH reserves (Figure 6I), and impairing GPX4 function, predisposes cells to lipid peroxidation and induces ferroptosis.



**Figure 6.** BACH1 controls CHAC1 at the promoter level and modulates ferroptosis under OGD/R. (A) Venn diagram showing the overlap between oxidative stress-related and ferroptosis-driver gene sets. (B) Schematic of predicted BACH1 binding motifs within the human CHAC1 promoter. (C) Dual-luciferase reporter assays in HEK293T cells comparing pGL3-CHAC1-WT and motif-mutant pGL3-CHAC1-MT with or without BACH1 co-transfection; Firefly/Renilla ratios normalized to control ( $n = 3$  independent experiments). (D,E) Immunofluorescence staining of BACH1 (green) and CHAC1 (red) with DAPI nuclear counterstain, and quantification of fluorescence intensity ( $n = 4$  independent experiments). (F) MDA levels  $n = 3$  independent experiments). (G,H) Lipid peroxidation: BODIPY 581/591 C11 fluorescence imaging and green/red ratio ( $n = 4$  independent experiments). (I) Cellular GSH content ( $n = 3$  independent experiments). (J,K) Immunoblots for the indicated proteins with  $\beta$ -tubulin as loading control and corresponding densitometry ( $n = 3$  independent experiments), BACH1 and GPX4 were detected on the same membrane and share the same  $\beta$ -tubulin control. Data are presented as mean  $\pm$  SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test; Exact  $p$  values are shown in the figures.

In summary, through a systematic process of “database screening–site prediction–dual-luciferase reporter validation–functional knockdown experiments”, this study identifies BACH1 as an important upstream transcription regulator for CHAC1. BACH1 activates CHAC1 transcription, accelerating GSH depletion and disrupting the GSH–GPX4 antioxidant defense system, thereby amplifying lipid peroxidation and ultimately exacerbating ferroptosis after OGD/R injury in cardiomyocytes.

#### 4. Discussion

This study focuses on myocardial ferroptosis during the reperfusion phase and proposes and validates the BACH1–CHAC1–GSH axis as a novel upstream regulatory pathway that amplifies ferroptosis in this setting. Both in vitro and in vivo experiments demonstrated that CHAC1 overexpression aggravates ferroptosis, whereas silencing CHAC1 or supplementing with the GSH precursor N-acetylcysteine (NAC) substantially mitigates these phenotypes. Importantly, CHAC1 knockdown reduced but did not abolish ferroptotic injury, indicating that CHAC1 primarily acts as an upstream amplifier that modulates cardiomyocyte susceptibility to ferroptosis rather than an indispensable executor of the death program. Under myocardial ischemia–reperfusion (I/R) conditions, CHAC1-mediated cytosolic GSH cleavage limits the substrate supply for GPX4, leading to the accumulation of lipid peroxidation and exacerbating ferroptotic cell death. Notably, we reveal for the first time a mechanistic link in which BACH1 regulates CHAC1 in HEK293T cells, while also providing a new explanatory pathway for GSH depletion after myocardial I/R injury.

To focus on ferroptosis-related signaling after myocardial I/R injury, we used the effect of Ferrostatin-1 (Fer-1) as a functional reference to define ferroptosis candidate genes. Genes whose expression changes were reversed by Fer-1 treatment were considered more likely to be directly involved in the ferroptotic process. Although this strategy identified relatively few genes, it markedly increased the specificity and verifiability of the candidates, providing strong support for subsequent mechanistic and functional studies. Multiple models indicate that CHAC1, a  $\gamma$ -glutamylcyclotransferase (GGCT), degrades cytosolic glutathione (GSH) into 5-oxoproline and Cys-Gly, thereby limiting GPX4 substrate availability, weakening cellular defenses against lipid peroxidation, and lowering the threshold for ferroptosis [21,43,44], functionally linking CHAC1 to oxidative stress and ferroptosis [22,24].

RNA-seq analysis showed that CHAC1 was downregulated at 15 h of reperfusion. However, this finding differs from most oxidative stress and ferroptosis models, in which CHAC1 is generally reported to remain upregulated [22,23]. To clarify this discrepancy, we examined CHAC1 expression at multiple reperfusion time points. qPCR revealed that CHAC1 mRNA rose sharply at 3 h of reperfusion, then declined to below control levels by 15 h before gradually returning toward baseline at later time points. Western blotting showed a similar trend, with CHAC1 protein increasing in early reperfusion and then declining at later time points. Previous studies indicate that oxidative stress is most pronounced during early reperfusion [2,45]. Based on this, we propose that, in the MIRI model, the critical contribution of CHAC1 to ferroptosis occurs mainly in the early reperfusion phase: during this window, CHAC1 is rapidly upregulated and, against a background of already impaired antioxidant defenses, further depletes GSH, thereby lowering the threshold for ferroptosis and promoting its activation. By contrast, the decrease in CHAC1 expression during mid-to-late reperfusion is more likely to result from early death of CHAC1-high cells together with partial restoration of redox status in surviving cardiomyocytes, rather than indicating that CHAC1 is no longer involved in the regulation of ferroptosis or oxidative stress. Taken together, although CHAC1 appears reduced at 15 h, the qPCR and Western blot time-course data demonstrate a pronounced early induction,

supporting the view that the pro-ferroptotic activity of the BACH1–CHAC1–GSH axis in MIRI is concentrated in the early reperfusion phase and justifying our focus on this period in subsequent mechanistic experiments.

BACH1 belongs to the CNC-bZIP transcription factor family and functionally antagonizes NRF2 at ARE/MARE sites [26,46]. NRF2 suppresses ferroptosis by upregulating antioxidant and iron homeostasis genes such as SLC7A11, GCLM/GCLC, FTH1/FTL, and SLC40A1, thereby maintaining GSH homeostasis and limiting labile iron accumulation [47,48]. Conversely, BACH1 transcriptionally represses these genes, weakening antioxidant capacity and promoting an increase in the labile iron pool (LIP), thereby amplifying lipid peroxidation and increasing susceptibility to ferroptosis [42,49]. NRF2 and BACH1 thus act as opposing oxidative stress-responsive regulators of redox and iron homeostasis, jointly shaping cellular susceptibility to ferroptosis [42,49]. Under myocardial I/R conditions, we observed upregulated BACH1 expression; BACH1 knockdown subsequently upregulated SLC7A11 and GPX4, downregulated ACSL4, restored GSH levels, and alleviated ferroptosis. Furthermore, BACH1 upregulation has been reported in various organs, including liver, lung and brain, under I/R conditions [39,50,51], supporting a broad pathological role for BACH1 in I/R injury. In this study, dual-luciferase reporter assays further suggested that BACH1 can activate CHAC1 transcription. It is noteworthy that previous studies on BACH1 have mainly focused on its “transcriptional repression” function, such as promoting ferroptosis by inhibiting the antioxidant network. The “BACH1–CHAC1–GSH–ferroptosis” axis revealed here adds a positive transcriptional activation role for BACH1 on a downstream gene, expanding the understanding of its molecular functions and providing a new perspective for exploring BACH1’s role in related pathological processes.

This study has several limitations. First, the coordinated changes in SLC7A11 after CHAC1 intervention suggest crosstalk between the BACH1–CHAC1–GSH pathway and the canonical system  $Xc^-$ –GSH–GPX4 anti-ferroptosis axis; however, the specific molecular mechanisms involved remain unknown. Second, while this study primarily focuses on the increased susceptibility to ferroptosis that is mechanistically linked to CHAC1-dependent GSH degradation, some studies also indicate that CHAC1 products can replenish the mitochondrial cysteine pool, thereby supporting Fe–S cluster synthesis and respiratory chain function [52]. Therefore, CHAC1 may exert dual physiological and pathological roles in maintaining cellular metabolic balance.

Third, although this study highlights the BACH1–CHAC1–GSH axis as an important oxidative stress-responsive pathway, previous work has also demonstrated that, in integrated stress response and endoplasmic reticulum stress models, CHAC1 has been identified as a downstream transcriptional target of ATF4 and related stress-responsive transcription factors, including ATF3 and CHOP, which activate CHAC1 via ATF/CRE and related cis-regulatory elements within its promoter [24,43,53]. Thus, within the upstream regulatory network governing CHAC1 expression, BACH1 should be regarded as an important but not exclusive node, and under different stress contexts CHAC1 is likely to be co-regulated by multiple upstream factors acting in parallel or in a complementary manner. Fourth, Ferrostatin-1 (Fer-1) was used here as a widely adopted experimental ferroptosis inhibitor to support mechanistic interrogation; however, its clinical translation has been limited by poor aqueous solubility and unfavorable biodistribution [54]. Encouragingly, emerging evidence suggests that certain clinically approved cardioprotective agents, such as SGLT2 inhibitors and GLP-1 receptor agonists, may ameliorate myocardial ischemia–reperfusion injury by modulating ferroptosis-related redox and lipid peroxidation pathways [55,56]. Finally, we speculate that a positive-feedback regulatory loop might exist between BACH1 and CHAC1: GSH depletion and oxidative stress can

activate NRF2 and induce HMOX1/HO-1 expression [26]; because HO-1 degrades free heme [57] (whose binding promotes BACH1 nuclear export and degradation), this may in turn favour BACH1 stabilization [58], forming an NRF2–HMOX1–BACH1 axis that could further amplify CHAC1 expression and the ferroptosis process. Although this hypothesis requires validation, it provides a potential direction for future research.

## 5. Conclusions

In summary, this study proposes the “BACH1–CHAC1–GSH” axis as a novel ferroptosis upstream regulatory pathway in cardiomyocytes. Our findings not only expand the current understanding of the ferroptosis-related antioxidant regulatory network centered on the system  $Xc^-$ –GSH–GPX4 axis, but also provide a new theoretical basis for understanding the mechanisms of glutathione depletion during myocardial reperfusion. Developing specific inhibitors targeting CHAC1 enzymatic activity or BACH1 transcriptional activity could represent a promising strategy for inhibiting ferroptosis and alleviating reperfusion injury, thereby offering new intervention options for the clinical treatment of ischemic heart disease.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox15020215/s1>, Supplementary Materials and Methods: Detailed experimental procedures and reagent information are provided in the Supplementary Materials. Supplementary unedited Western blots: Full-length, unedited Western blots for all main-text Western blot panels in Figures 1–5. Figure S1: Map of the pGL3-basic-H\_CHAC1 promoter (–2000 to +50) mutant reporter construct, together with the raw Firefly and Renilla luciferase readouts from the dual-luciferase reporter assays. Figure S2: Glutathione metabolism pathway map from KEGG used for pathway annotation and illustration. Figure S3: Representative RT-qPCR quality control for CHAC1. Figure S4: Immunoblots and densitometry of BACH1 in sham and I/R mouse hearts. Figure S5: GSSG content and GSH/GSSG ratio corresponding to Figure 5. Table S1: Primer sequences and accession numbers of genes used for RT-qPCR. Table S2: Public ferroptosis driver gene set. Table S3: Public oxidative stress-related gene set.

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**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The raw sequencing data used and described in this study are openly available in NCBI BioProject at <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1418956> (accessed on 26 January 2026). The original contributions presented in this study are included in the article and Supplementary Materials. Further inquiries can be directed to the corresponding authors.

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