FRIGID ZONE **MEDICINE**
Original Article • DOI: 10.1515/fzm-2026-0001

Cold exposure aggravates myocardial ischemia-reperfusion injury *via* m6A-mediated circRNA-mRNA regulatory networks

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Abstract

Objective: Myocardial ischemia-reperfusion (I/R) injury remains a major contributor to cardiac morbidity and mortality, and accumulating evidence suggests that epitranscriptomic regulation may critically influence cardiac stress responses. N6-methyladenosine (m6A) modification and circular RNAs (circRNAs) have emerged as important regulators of cardiovascular pathology; however, their integrated roles in myocardial I/R injury, particularly under chronic cold stress, remain poorly defined. **Methods:** A mouse model of myocardial I/R injury was established under room-temperature or chronic cold exposure conditions. Cardiac function, infarct size, histopathology, and serum injury markers were assessed. Global m6A levels were quantified, and m6A-modified circRNA profiles were analyzed using epitranscriptomic microarrays and bioinformatics approaches. Differentially expressed circRNAs were validated *in vivo* and in hypoxia-reoxygenation-treated neonatal cardiomyocytes. Circular structures and stability were confirmed by Sanger sequencing, divergent/convergent PCR, and actinomycin D assays. Competing endogenous RNA (ceRNA) networks were constructed to identify downstream regulatory pathways. **Results:** Myocardial I/R injury resulted in significant cardiac dysfunction, increased infarct size, histological damage, and elevated serum CK-MB and LDH levels, accompanied by a marked increase in global m6A methylation. Epitranscriptomic profiling identified 391 circRNAs with altered m6A modification following I/R injury, involving pathways related to molecular binding, cellular processes, and kinase signaling. Multiple circRNAs exhibited consistent dysregulation in both *in vivo* and *in vitro* I/R models and displayed high structural stability. Importantly, chronic cold exposure significantly exacerbated I/R-induced cardiac dysfunction and infarct severity while further modulating the expression of specific m6A-modified circRNAs. ceRNA network analysis revealed that cold-responsive circRNAs potentially regulate myocardial injury through miRNA-mediated signaling pathways. **Conclusion:** This study identifies m6A-modified circRNAs as key epitranscriptomic regulators of myocardial I/R injury and demonstrates that chronic cold stress amplifies circRNA-mediated regulatory networks. These findings provide novel mechanistic insight into temperature-dependent epigenetic regulation in ischemic heart disease and highlight m6A-circRNAs as potential therapeutic targets.

Keywords

circRNAs; m6A; myocardial I/R injury; cold stress

Received 21 March 2025, accepted 25 November 2025

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1 Introduction

Myocardial ischemia-reperfusion (I/R) injury refers to the tissue damage that occurs following the restoration of blood flow to ischemic myocardium[1]. This phenomenon is particularly prevalent in ischemic heart diseases and involves a complex cascade of pathophysiological processes, including calcium overload[2], oxidative stress[3], and inflammatory responses[4]. Although multiple preventive and therapeutic strategies have been developed, the clinical management of myocardial I/R

injury remains challenging. Therefore, further investigation is required to improve existing treatment strategies and to identify novel therapeutic targets aimed at mitigating I/R-associated myocardial damage.

N6-methyladenine (m6A) methylation is the most abundant internal RNA modification in eukaryotic cells and represents a critical epigenetic regulatory mechanism. m6A modification occurs in diverse RNA species, including messenger RNA (mRNA) and non-coding RNAs[5], and plays essential roles in regulating RNA metabolism, such as RNA stability, splicing, nuclear export, and translation[6]. The dynamic and reversible nature of m6A modification is mediated by a group of specialized proteins known as “writers,” “erasers,” and “readers,” which coordinate the deposition, removal, and recognition of m6A marks in response to intracellular and environmental cues[7]. Importantly, dysregulation of m6A methylation has been implicated in the development and progression of various cardiovascular diseases, including myocardial I/R injury[8]. Consequently, elucidating the molecular mechanisms underlying m6A-mediated regulation may provide new insights into the pathogenesis of myocardial I/R injury.

Circular RNAs (circRNAs) are a class of endogenous non-coding RNAs characterized by a covalently closed loop structure that lacks canonical 5′ caps and 3′ poly(A) tails, conferring high stability compared with linear RNAs[9]. CircRNAs exert diverse biological functions through multiple mechanisms. One well-recognized function is their ability to act as competing endogenous RNAs or molecular sponges for microRNAs (miRNAs), thereby regulating miRNA availability and downstream target gene expression[10]. In addition, circRNAs can interact with RNA-binding proteins (RBPs) to modulate transcriptional or post-transcriptional processes[11], serve as scaffolds for protein complexes, or, in some cases, be translated into functional peptides[12]. Increasing evidence highlights the critical involvement of circRNAs in myocardial I/R injury. CircRNAs can influence cardiomyocyte survival during I/R by regulating apoptosis-, inflammation-, and stress-related signaling pathways. For example, CircHIPK3 has been shown to protect against myocardial I/R injury by sponging miR-124-3p, thereby reducing cardiomyocyte apoptosis[13]. Similarly, Circ_0050908 regulates inflammatory and oxidative stress responses by sponging miR-324-5p and upregulating TRAF3, ultimately modulating I/R-induced myocardialinjury[14]. These findings underscore the multifaceted regulatory roles of circRNAs and highlight their potential as novel therapeutic targets in myocardial I/R injury.

In recent years, increasing attention has been directed toward the impact of cold environments on cardiovascular health. Epidemiological studies have demonstrated a strong association between low ambient temperatures and increased cardiovascular morbidity and mortality[15]. Exposure to cold induces vasoconstriction, elevates blood pressure, and triggers a series of physiological stress responses that collectively increase cardiovascular risk[16]. Cold exposure activates the sympathetic nervous system and promotes inflammatory responses, further contributing to the progression of cardiovascular diseases[17]. Notably, low temperature has been shown to be significantly associated with a higher incidence of cardiovascular events, including hypertension[18] and coronary heart disease[19]. Despite these observations, the molecular mechanisms by which cold exposure exacerbates cardiovascular injury, particularly in the context of myocardial I/R, remain poorly understood and warrant further investigation.

2 Materials and methods

2.1 Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Harbin Medical University (approval number: IRB3106724) and were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2 Mouse model of I/R injury

Eight-week-old male C57BL/6J mice were anesthetized by intraperitoneal injection of avertin (200 mg/kg) and intubated for mechanical ventilation. Following left thoracotomy, myocardial ischemia was induced by ligation of the left anterior descending coronary artery (LAD) using a 7-0 nylon suture for 45 mins. Reperfusion was initiated by releasing the ligature, allowing restoration of coronary blood flow for 24 h to establish the I/R model. Sham-operated control mice underwent the same surgical procedures without LAD ligation. Cardiac tissue were subsequently dissected for downstream analyses.

2.3 Cold exposure and experimental groups

Eight-week-old male C57BL/6J mice were assigned to experimental groups based on baseline cardiac function and body weight. Mice in the cold exposure group were housed at 4 °C for 4 weeks, whereas mice in the room-temperature group were maintained at 20-26 °C under a 12-h light/12-h dark cycle. After the environmental conditioning period, mice underwent I/R surgery or sham surgery, followed by ultrasound imaging and other experimental procedures. After the environmental conditioning period, mice were subjected to either myocardial I/R surgery or sham surgery, followed by echocardiographic assessment and subsequent experimental analyses.

2.4 Echocardiography

Mice were anesthetized *via* intraperitoneal injection of 2% avertin prior to echocardiographic examination. Cardiac function was assessed using a Vevo 2100 echocardiography system (VisualSonics, Toronto, Ontario, Canada) equipped with a 10.0 MHz phased-array transducer. M-mode images of the left ventricular long axis were acquired to evaluate left ventricular systolic function. Measurements were averaged from three consecutive cardiac cycles for each animal.

2.5 Evans blue-2,3,5-triphenyl tetrazolium chloride (TTC) double staining

Following anesthesia, 2% Evans blue solution (Solarbio, Beijing, China) was injected into the abdominal aorta to delineate the area at risk. Hearts were rapidly excised, frozen at −80 °C, and sectioned transversely into 1-3 mm thick slices. The slices were incubated in 2% TTC solution (Solarbio, Beijing, China) at 37 °C for 30 min to distinguish viable and infarcted myocardium. Images were captured under a microscope for infarct size analysis.

2.6 H&E staining

Heart tissue sections were dewaxed in xylene and rehydrated through a graded ethanol series. The sections were stained with hematoxylin and eosin (H&E) (Solarbio, Beijing, China) according to standard protocols. After dehydration and mounting with neutral balsam, histopathological changes were examined under a light microscope.

2.7 Assay kits

Blood samples were collected from the retro-orbital venous plexus and allowed to clot at room temperature for 2 h. Serum was obtained by centrifugation at 4000 rpm for 5 min. Serum lactate dehydrogenase (LDH) activity was measured using a commercial LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Creatine kinase-MB (CK-MB) levels were quantified using ELISA kits (Elabscience, Wuhan, China) according to the manufacturer's instructions.

2.8 RNA immunoprecipitation (RIP) assay

RIP was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, MA, USA) following the manufacturer's instructions. Briefly, tissue lysates were incubated with 50 μl of Protein A/G magnetic beads (Roche, USA) conjugated with an m6A antibody (#202003, Synaptic Systems, Goettingen, Germany) at 4 °C. After immunoprecipitation, m6A-modified RNA was extracted and subjected to quantitative real-time PCR (qRT-PCR) analysis.

2.9 m6A-circRNA epitranscriptomic microarray and bioinformatics analysis

Total RNA was extracted from myocardial tissues for m6A-circRNA profiling. m6A-modified RNA was enriched using by immunoprecipitation using an m6A-specific antibody (Synaptic Systems, Goettingen, Germany) for immunoprecipitation. Both immunoprecipitated and supernatant RNA fractions were collected, and linear RNAs were removed by RNase R digestion. The purified RNA was labeled using the Super RNA Labeling Kit (Arraystar, USA) and further purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany). The labeled RNA was hybridized to the Arraystar circRNA epitranscriptome microarray (8×15k, Arraystar), and arrays were scanned using an Agilent Scanner G2505C (Agilent, Beijing, China). Differentially m6A-modified circRNAs were identified through microarray analysis based on normalized signal intensities.

2.10 QRT-PCR

Total RNA was extracted from cardiomyocytes and cardiac tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were assessed using a Nanodrop ND-8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized using a reverse transcription kit (Toyobo, Japan). Quantitative PCR was performed using SYBR Green Master Mix (Toyobo, Japan) on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA. The expression levels of selected circRNAs (mmu-circRNA-43003, mmu-circRNA-35933, mmu-circRNA-40722, mmu-circRNA-21003, mmu-circRNA-017142, mmu-circRNA-29852, mmu-circRNA-19351, mmu-circRNA-006547, mmu-circRNA-015196, mmu-circRNA-40368, mmu-circRNA-26402, mmu-circRNA-37908, mmu-circRNA-29837, mmu-circRNA-33764, mmu-circRNA-29391, mmu-circRNA-34515, mmu-circRNA-41542, mmu-circRNA-26189, mmu-circRNA-42133, and mmu-circRNA-42424) were normalized to 18S rRNA or GAPDH, and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in Supplementary Table S1.

2.11 Neonatal mouse ventricular cardiomyocytes (NMVCs) isolation and culture

Neonatal mouse ventricular cardiomyocytes (NMVCs) were isolated from hearts of 1- 3-day-old neonatal mice. Hearts were excised and rinsed in phosphate-buffered saline containing penicillin-streptomycin to remove blood and debris. After trimming excess tissue, hearts were digested with trypsin (Solarbio, Beijing, China) at 4 °C for 8-10 h, followed by digestion with Type II collagenase (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C with gentle agitation.

Supernatants were collected every 10 min and replaced with fresh collagenase until complete tissue dissociation. Cells were pooled, centrifuged, resuspended, and pre-plated for 2 h to allow fibroblast adhesion. Non-adherent cardiomyocytes were collected and seeded for subsequent experiments.

NMVCs were exposed to hypoxic conditions (5% CO_2 and 95% N_2) for 12 h, followed by reoxygenation for 24 h to establish a hypoxia-reoxygenation model[20].

2.12 Actinomycin D (ActD) treatment

To assess circRNA stability, NMVCs were treated with actinomycin D (5 μg/mL; MedChemExpress, New Jersey, USA) for 0 or 24 h. CircRNA expression levels were subsequently measured by qRT-PCR.

2.13 Data analysis

All data are expressed as the mean ± standard error of the mean (SEM) from at least three independent experiments. Statistical analyses were performed using GraphPad Prism 7.0. (GraphPad Software, San Diego, CA, USA). Comparisons between two groups were conducted using two-tailed Student's t-tests. For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was applied. A P-value < 0.05 was considered statistically significant.

3 Results

3.1 Upregulation of m6A methylation modification I/R mice

An *in vivo* myocardial I/R model was established in eight-week-old C57BL/6 mice and successfully validated. Echocardiographic assessments revealed a significant reduction in both ejection fraction (EF%) and fractional shortening (FS%) in the I/R group compared to the Sham group (Fig. 1A-C). Consistently, TTC staining demonstrated a markedly increased infarct size in I/R mice (Fig. 1D, E). Histological examination using H&E staining further revealed disorganized cardiomyocyte architecture in the I/R group (Fig. 1F). In addition, serum levels of LDH and CK-MB were significantly elevated in I/R mice compared to Sham controls (Fig. 1G, H). Importantly, global m6A methylation levels were markedly increased in myocardial tissues from I/R mice (Fig. 1I), suggesting that m6A modification is involved in myocardial I/R injury

3.2 Profiling of differentially m6A-modified circRNAs and Gene Ontology analysis

To investigate the epigenetic mechanisms underlying myocardial I/R injury, we conducted m6A-circRNA epitranscriptomic microarray analysis combined with bioinformatics profiling on ischemic myocardial tissues obtained from three Sham-operated mice and three I/R mice. This analysis identified circRNAs exhibiting altered m6A methylation levels following I/R injury. In total, 391 circRNAs displayed significant differences in m6A modification in the I/R group compared with the Sham group (Fig. 2A). Scatter plot analysis demonstrated increased m6A methylation in 37 circRNAs (red) and decreased methylation in 354 circRNAs (blue) (Fig. 2B). Volcano plot analysis further identified 38 circRNAs with significantly increased expression and 231 circRNAs with markedly decreased expression (Fig. 2C).

To explore potential regulatory mechanisms, particularly miRNA sponging activity, we constructed a Venn diagram based on predicted target miRNAs, including mmu-let-7j and mmu-miR-27b-3p, identifying 875 circRNAs with shared miRNA-binding potential (Fig. 2D). Given that circRNAs originate from exonic or intronic regions, we further analyzed the genomic distribution of differentially expressed circRNAs (Fig. 2E, F). Gene Ontology (GO) enrichment analysis revealed that these circRNAs were primarily associated with biological processes related to molecular binding, cellular components, cellular processes, and protein tyrosine kinase activity (Fig. 3A-C). GO pathway enrichment further highlighted their involvement in multiple signaling pathways relevant to myocardial injury (Fig. 3D).

3.3 Validation of circRNAs expression levels *in vivo* and *in vitro*

Although microarray analysis identified 391 circRNAs with altered m6A modification, the expression levels of the corresponding circRNAs themselves required further validation. Therefore, we selected 10 circRNAs with increased m6A modification and 10 circRNAs with decreased m6A modification for quantitative analysis. qRT-PCR was performed using myocardial tissues from I/R mice *in vivo* and hypoxia-reoxygenation (HR)-treated neonatal mouse ventricular cardiomyocytes (NMVCs) *in vitro*.

In vivo, compared with Sham mice, the expression levels of mmu-circRNA-35933, mmu-circRNA-40722, mmu-circRNA-017142, mmu-circRNA-29852, mmu-circRNA-19351, mmu-circRNA-006547, mmu-circRNA-015196, mmu-circRNA-40368, mmu-circRNA-29837, mmu-circRNA-29391, and mmu-circRNA-41542 were significantly decreased in I/R mice. In contrast, mmu-circRNA-26402, mmu-circRNA-37908, mmu-circRNA-33764, mmu-circRNA-26189, mmu-circRNA-42133, and mmu-circRNA-42424 were significantly upregulated (Fig. 4A, B).

In vitro, HR-treated NMVCs exhibited significantly reduced expression of mmu-circRNA-35933, mmu-circRNA-40722,

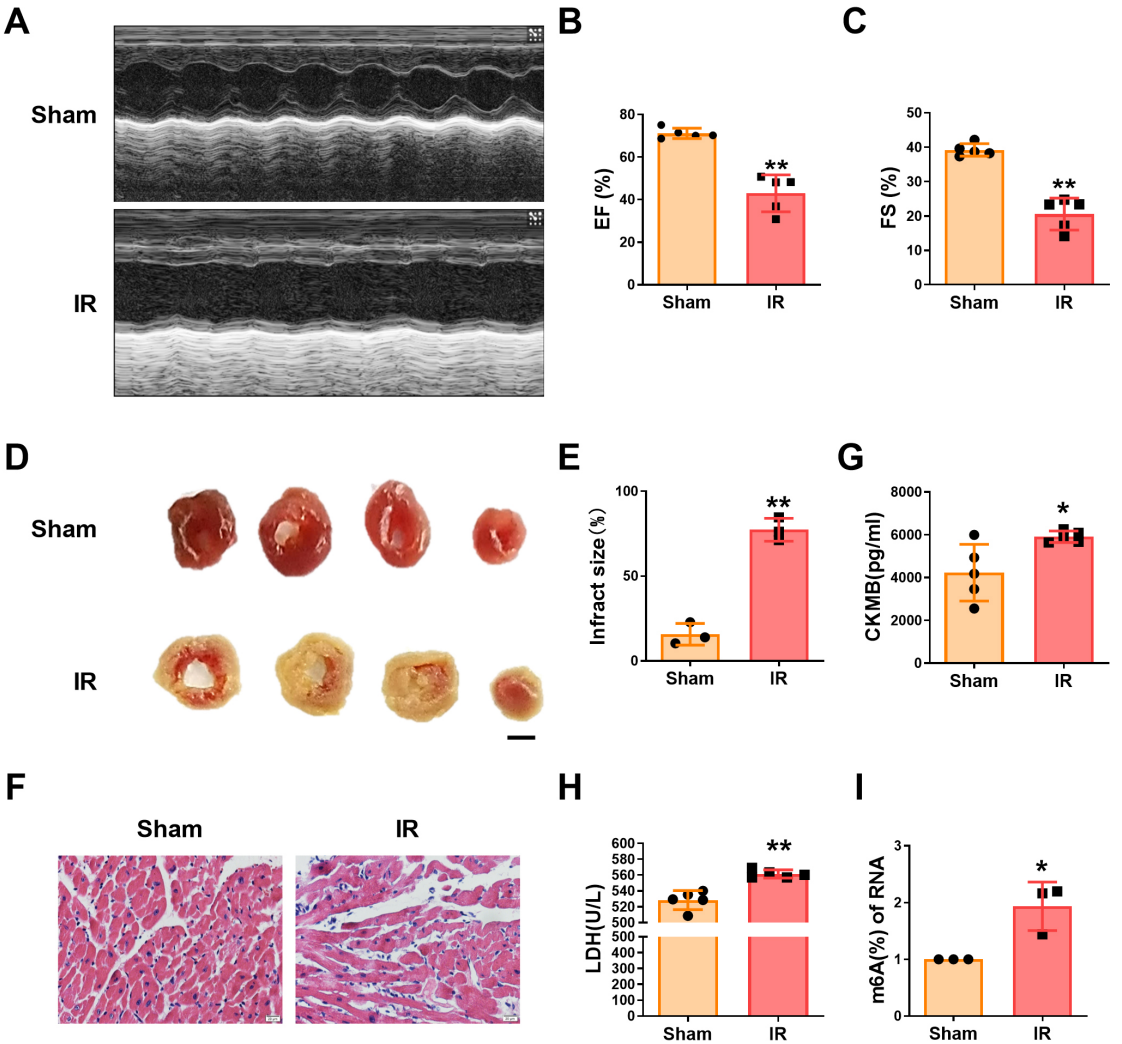

Fig. 1 Establishment of a mouse myocardial ischemia-reperfusion (I/R) model

(A) Representative echocardiographic images showing cardiac function. Ejection fraction (EF%). (C) Fractional shortening (FS%). $^{**}P < 0.01$ versus Sham, $N = 5$. (D-E) Representative images and quantitative analysis of infarct size assessed by blue-2,3,5-triphenyl tetrazolium chloride (TTC) staining. $^{**}P < 0.01$ versus Sham, $N = 3$. (F) Representative H&E-stained sections showing myocardial histopathological changes. (G-H) Serum levels of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH). $^{*}P < 0.05$, $^{**}P < 0.01$ versus Sham, $N = 5$. (I) Global N6-methyladenosine (m6A) modification levels in cardiac tissue, $^{*}P < 0.05$ versus Sham, $N = 3$.

mmu-circRNA-017142, mmu-circRNA-29852, mmu-circRNA-006547, mmu-circRNA-015196, and mmu-circRNA-29391 compared with control cells. Conversely, the expression levels of mmu-circRNA-26402, mmu-circRNA-37908, mmu-circRNA-33764, mmu-circRNA-34515, mmu-circRNA-26189, and mmu-circRNA-42424 were significantly increased following HR treatment (Fig. 4C, D).

3.4 Validation of the circular structure of circRNAs

CircRNAs are generated through back-splicing events that join the 3′ end of an upstream exon or intron to the 5′ end of a downstream exon or intron, forming a covalently closed loop structure. To confirm the circular nature of selected circRNAs, two candidates were randomly chosen for validation. Sanger sequencing confirmed the presence of back-splice junctions characteristic of these circRNAs (Fig. 5A, D).

Using divergent and convergent primers, qRT-PCR analysis demonstrated that mmu-circRNA-35933 and mmu-circRNA-37908 could be amplified from cDNA but not from genomic DNA when

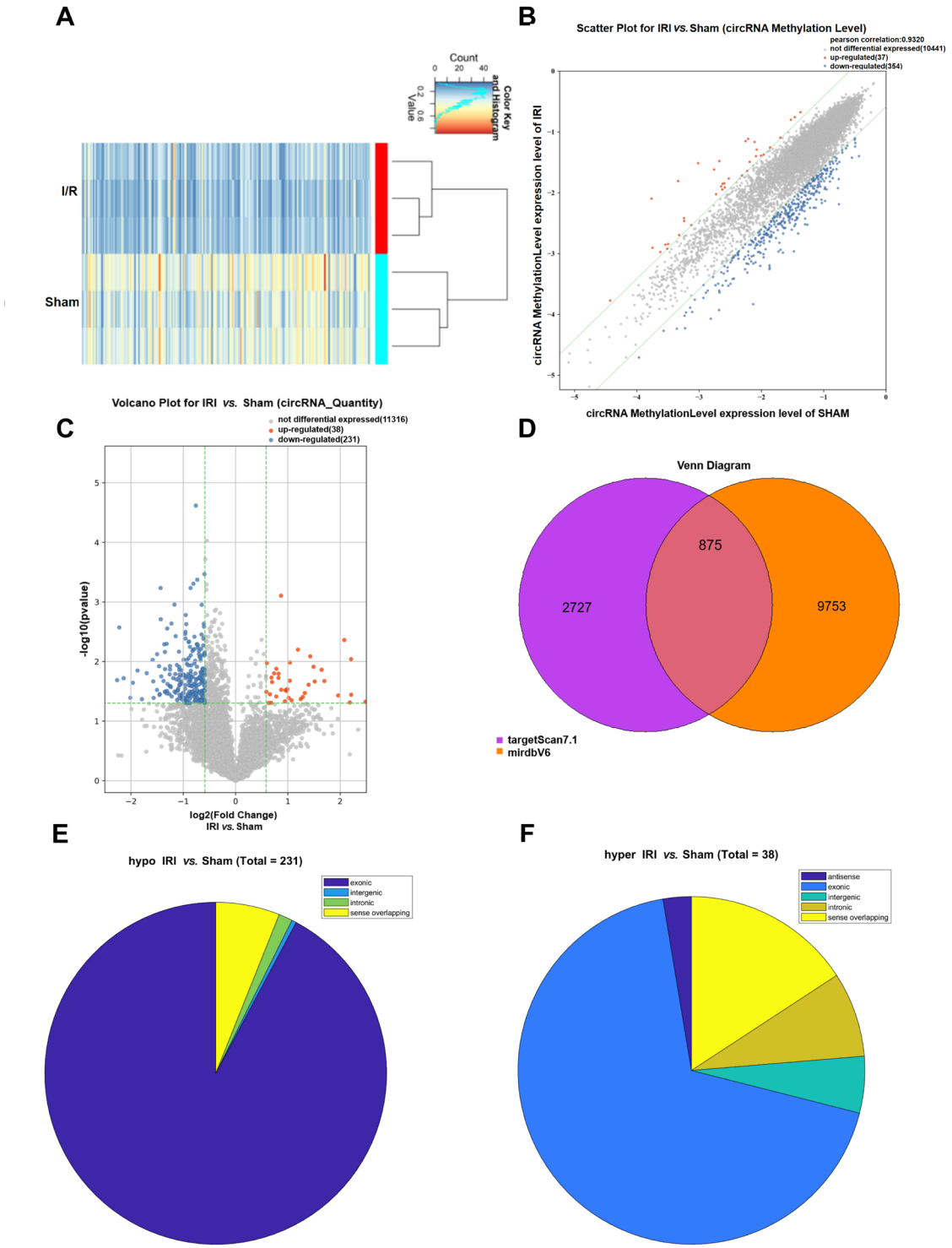

Fig. 2 Differential N6-methyladenosine (m6A)-methylated circRNAs and Gene Ontology (GO) pathway analysis

(A) m6A-circRNA epitranscriptomic microarray and bioinformatics analysis showing differential m6A modification levels. *N* = 3. (B) Scatter plot analysis of differentially expressed circRNAs. (C) Volcano plot analysis of differentially expressed circRNAs. (D) Venn diagram showing circRNAs targeting common miRNAs. (E, F) Distribution of hypermethylated and hypomethylated circRNAs in myocardial I/R injury.

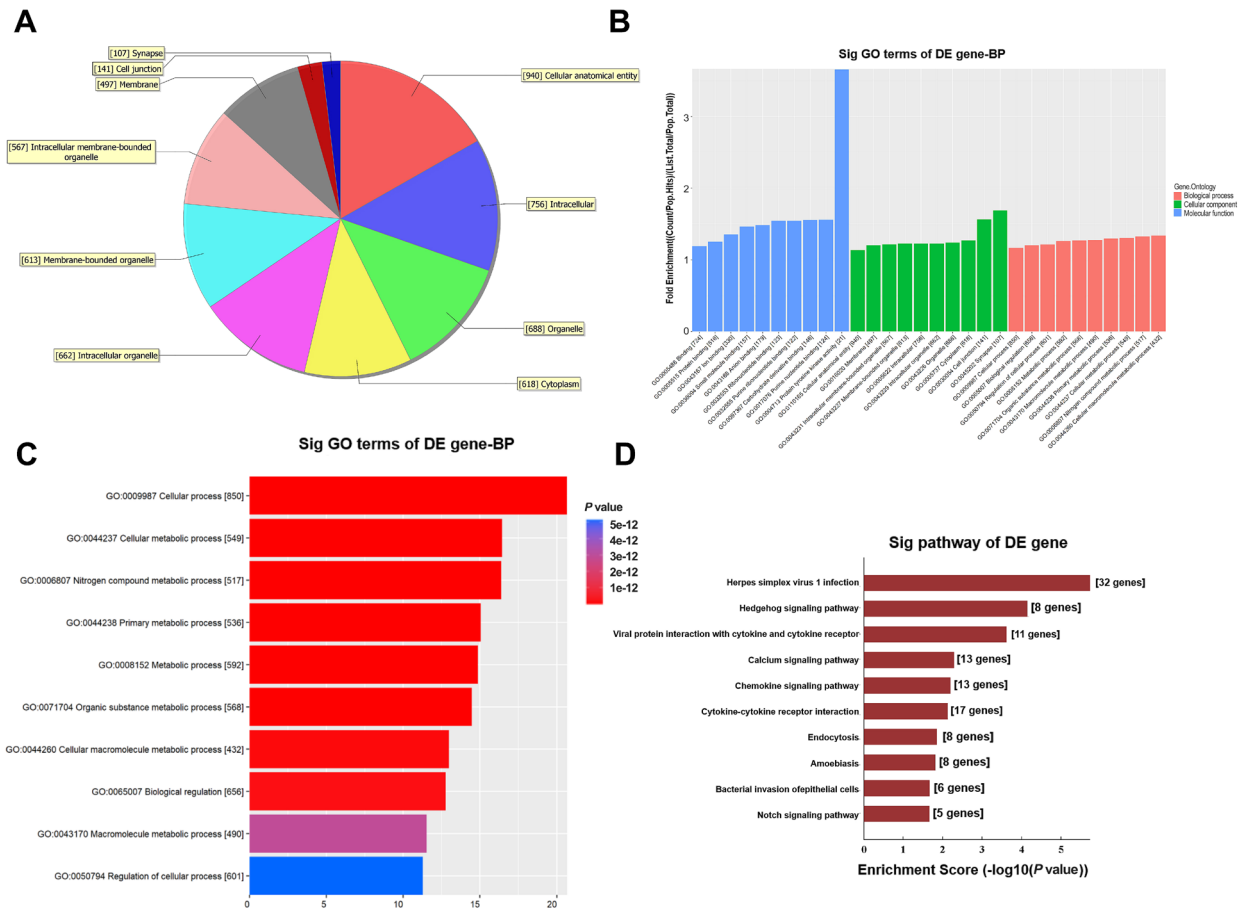

Fig. 3 Gene Ontology and pathway enrichment analysis of differentially expressed circRNAs in biological processes, cellular components, and molecular functions (A-C) Gene Ontology (GO) enrichment analysis of differentially expressed circRNAs in biological processes, cellular components, and molecular functions.(D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially expressed genes.

divergent primers were used, whereas convergent primers amplified both cDNA and genomic DNA templates, confirming their circular configuration (Fig. 5B, E). In addition, treatment with actinomycin D revealed that these circRNAs exhibited greater stability than linear RNAs, further supporting their circular nature (Fig. 5C, F).

3.5 Expression of m6A-modified circRNAs in chronic cold stress-induced I/R mice

Accumulating evidence indicates that adverse cardiovascular events occur with increased morbidity and mortality during cold seasons. To investigate the effects of chronic cold exposure on myocardial I/R injury, eight-week-old C57BL/6 mice were randomly assigned to four experimental groups based on baseline cardiac function (Fig. 6A-C). The mice were housed at 4 °C for four weeks prior to I/R surgery to establish a cold--exposed I/R mice, as evidenced by reduced EF % and FS % (Fig. 6D-F), which was further confirmed by Evans blue-TTC staining (Fig. 6G-I).

Cold-exposed mice also exhibited a significantly higher body weight compared with room-temperature controls Supplementary Fig. S1. To determine whether m6A-modified circRNAs identified previously were involved in cold stress-associated myocardial I/R injury, we assessed their expression levels under cold conditions. Several circRNAs, including mmu-circRNA-35933, mmu-circRNA-40722, mmu-circRNA-017142, mmu-circRNA-29852, mmu-circRNA-19351, mmu-circRNA-006547, mmu-circRNA-015196, mmu-circRNA-29837, mmu-circRNA-29391, mmu-circRNA-26402, mmu-circRNA-37908, and mmu-circRNA-33764, showed significantly altered expression compared with room temperature Sham mice (Fig. 7A-B).

Notably, mmu-circRNA-40722, mmu-circRNA-017142, mmu-circRNA-19351, and mmu-circRNA-015196 were further

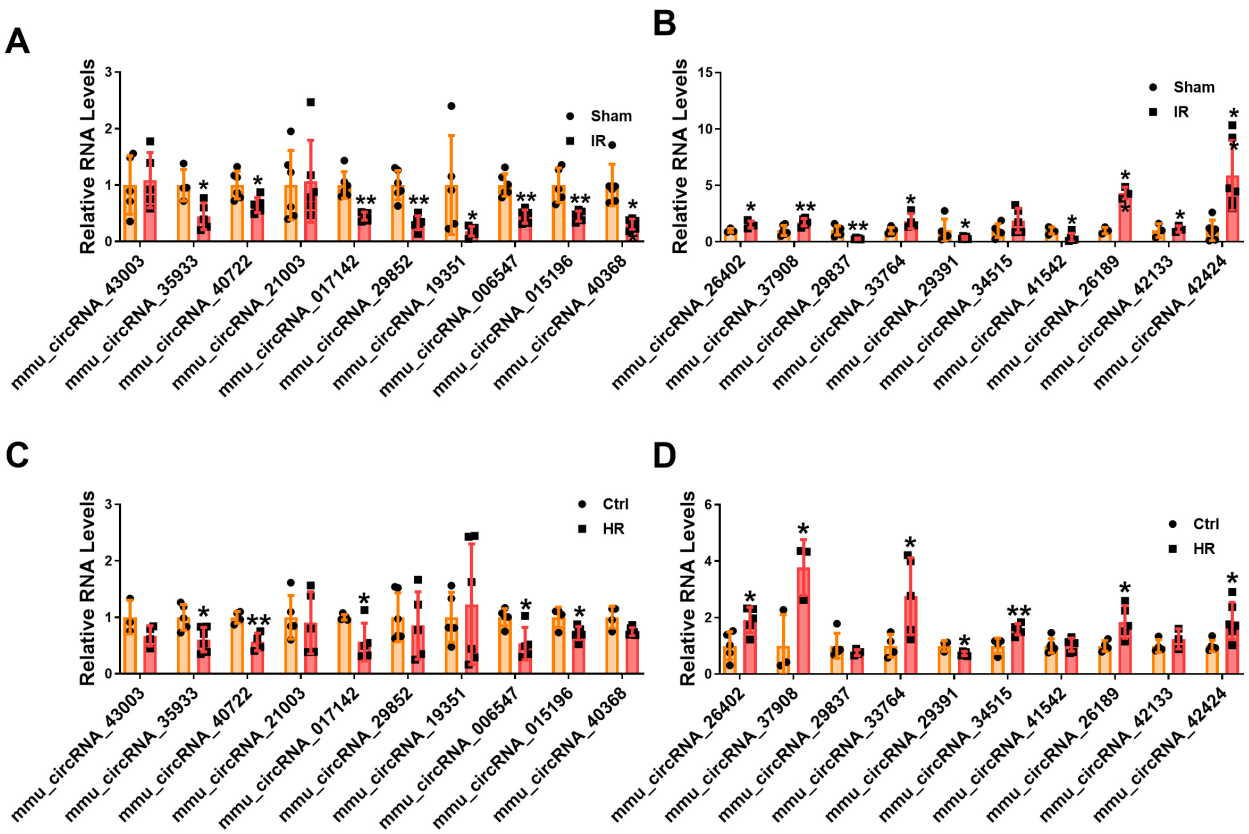

Fig. 4 Assessment of circRNA expression levels *in vivo* and *in vitro*

(A, B) Relative expression levels of circRNAs in cardiac tissues. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Sham, $N = 3$-4. (C, D) Relative expression levels of circRNAs in cardiomyocytes. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Sham, $N = 3$-4.

downregulated in cold-exposed I/R mice compared to I/R mice maintained at room temperature, whereas mmu-circRNA-26402 was significantly upregulated under cold conditions (Fig. 7A-B). These findings indicate that m6A-modified circRNAs may play critical regulatory roles in myocardial I/R injury under cold stress.

3.6 Identification o downstream targets of cold-responsive circRNAs

To further elucidate the functional significance of circRNAs responsive to cold stress, we selected 10 circRNAs that exhibited marked differential expression under cold exposure across both physiological and pathological conditions. Bioinformatics analysis was performed to construct a competing endogenous RNA (ceRNA) network, illustrating the interactions among circRNAs, their sponge miRNAs, and downstream target genes (Supplementary Data Fig. S2).

Among these, five circRNAs demonstrated consistent expression changes under both normal and cold conditions. Further analyses of their associated miRNAs and target genes were conducted, followed by functional enrichment analysis to identify related biological pathways. These results revealed that cold-responsive circRNAs are involved in multiple pathways relevant to myocardial injury and stress responses (Fig. 8A, B; Supplementary Fig. S3).

4 Discussion

Cardiovascular diseases (CVDs) remain the leading cause of mortality and morbidity worldwide[21-22]. Although timely restoration of blood flow is essential for salvaging ischemic myocardium, reperfusion itself often induces additional myocardial injury and cardiac dysfunction, collectively referred to as myocardial ischemia-reperfusion (I/R) injury[23]. Despite extensive basic and clinical investigations, myocardial I/R injury remains a major unresolved clinical challenge. In the present study, we demonstrated that myocardial I/R injury in mice resulted in significant cardiac dysfunction, increased infarct size, histopathological damage, and elevated serum levels of CK-MB and LDH, confirming the successful establishment of the I/R injury model

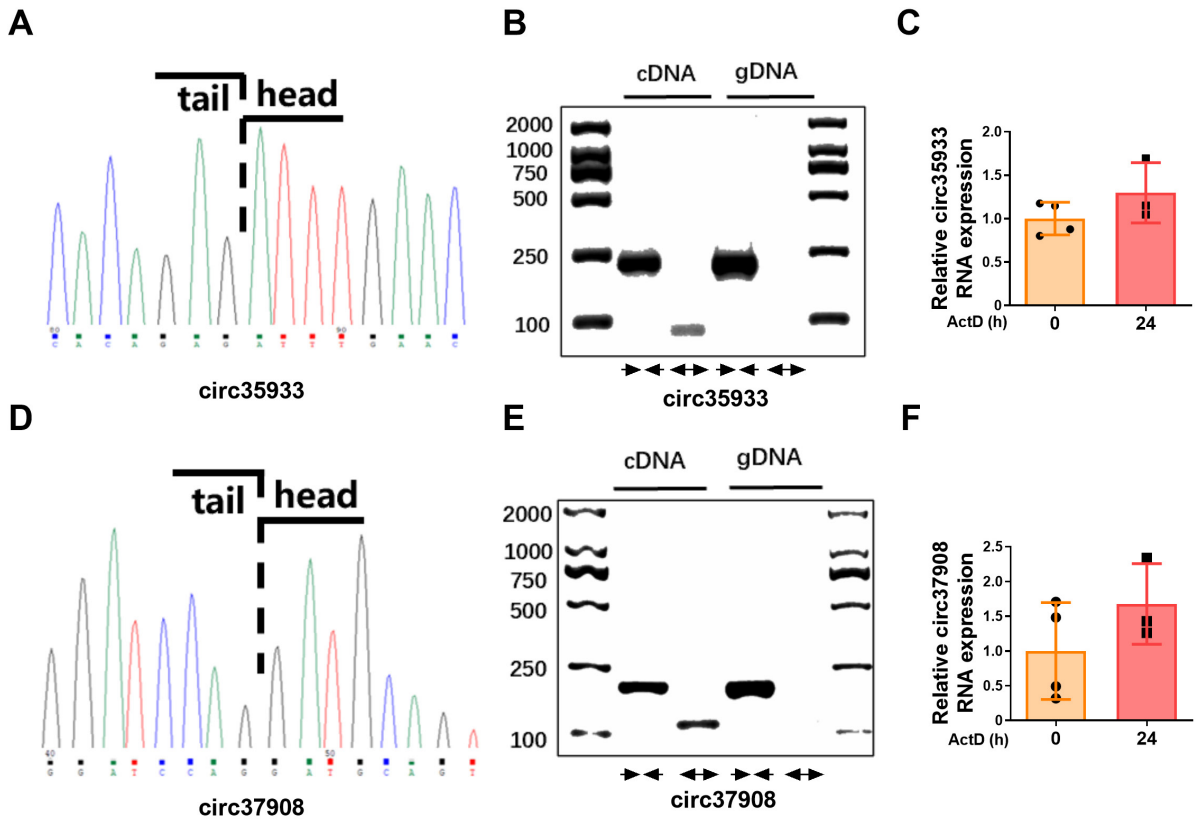

Fig. 5 Validation of the circular structure of circRNAs

(A, D) Identification of circRNA back-splice junctions by Sanger sequencing. (B, E) Validation of circular structures using divergent and convergent primers with cDNA and genomic DNA templates. (C, F) CircRNA stability assessed by actinomycin D treatment. *N* = 3-4.

and the severity of myocardial damage.

N6-methyladenosine (m6A) methylation, which occurs at the N6 position of adenine residues in RNA molecules, has emerged as an important epigenetic modification involved in the regulation of cardiovascular diseases[8,22,24]. In this study, we observed a marked increase in global m6A methylation levels in myocardial tissues following I/R injury, as detected by RNA immunoprecipitation assays, suggesting that RNA methylation is dynamically regulated during myocardial injury. m6A methylation is a reversible and tightly regulated process that influences RNA stability, translation efficiency, nuclear export, and degradation, thereby participating in both physiological and pathological processes in the cardiovascular system[25-26]. Importantly, m6A modification is not restricted to messenger RNAs; a growing body of evidence indicates that non-coding RNAs, including circular RNAs (circRNAs), long non-coding RNAs, and transfer RNAs, also undergo m6A modification and contribute to I/R injury[27-29]. However, studies focusing specifically on m6A-modified circRNAs in myocardial I/R injury remain limited. To address this gap, we performed m6A-circRNA epitranscriptomic microarray analysis combined with bioinformatics profiling and identified 37 circRNAs with increased m6A methylation and 354 circRNAs with decreased m6A methylation following I/R injury. Functional enrichment analyses suggested that these circRNAs are involved in cellular processes, metabolic regulation, and other biological pathways relevant to cardiac physiology and pathology.

CircRNAs are widely expressed in mammalian tissues and represent a class of non-coding RNAs characterized by covalently closed loop structures and high stability[30-31]. Due to their evolutionary conservation tissue specificity, and resistance to exonuclease-mediated degradation, circRNAs have attracted increasing attention as potential biomarkers and therapeutic targets in various diseases. Numerous studies have reported the important regulatory roles of circRNAs in myocardial I/R injury. For example, circUtrn has been shown to reduce oxygen-glucose deprivation/reoxygenation-induced apoptosis, attenuate acute myocardial I/R injury, and inhibit post-I/R remodeling by interacting with protein phosphatase 5[31]. Similarly, circCHSY1 enhances heme

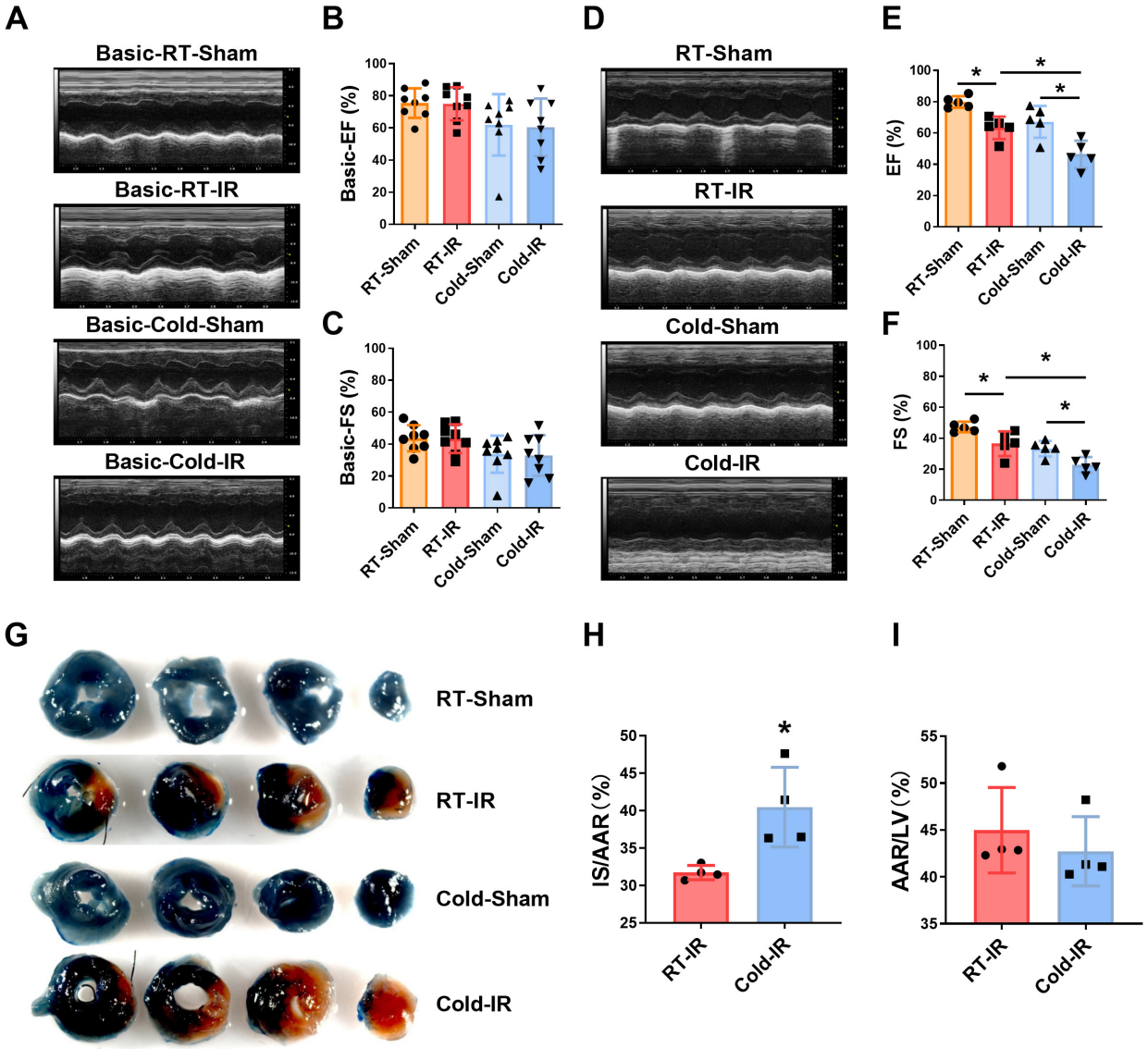

Fig. 6 Dynamic changes in N6-methyladenosine (m6A)-circRNA expression in chronic cold stress-induced I/R mice

(A) Baseline echocardiographic assessment of cardiac function. (B) Ejection fraction (EF%). (C) FS%. $N = 8$. (D) Echocardiographic assessment after four weeks of cold exposure. (E) EF%. (F) FS%. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Sham, $N = 5$. (G-I) Representative images and quantitative analysis of myocardial injury assessed by Evans blue-TTC double staining. $^{*}P < 0.05$ versus RT-I/R, $N = 4$.

oxygenase 1 expression by sponging miR-24-3p, thereby maintaining mitochondrial homeostasis and protecting against myocardial I/R injury[32]. Consistent with these findings, our study identified multiple circRNAs with altered m6A modification and expression levels in both *in vivo* and *in vitro* I/R models. Among the circRNAs examined, several exhibited consistent expression changes across experimental systems. Furthermore, due to their lack of free 5′ and 3′ ends, circRNAs possess longer half-lives and exhibit resistance to transcriptional inhibition by actinomycin D[33-34]. We selected two representative circRNAs, circ35933 and circ37908, and confirmed their circular structures using Sanger sequencing and actinomycin D treatment, further supporting their stability and potential functional relevance in myocardial I/R injury.

Exposure to long-term cold environments has been increasingly recognized as a significant risk factor for cardiovascular dysfunction and mortality. Epidemiological evidence indicates that adverse cardiovascular events occur with higher morbidity and

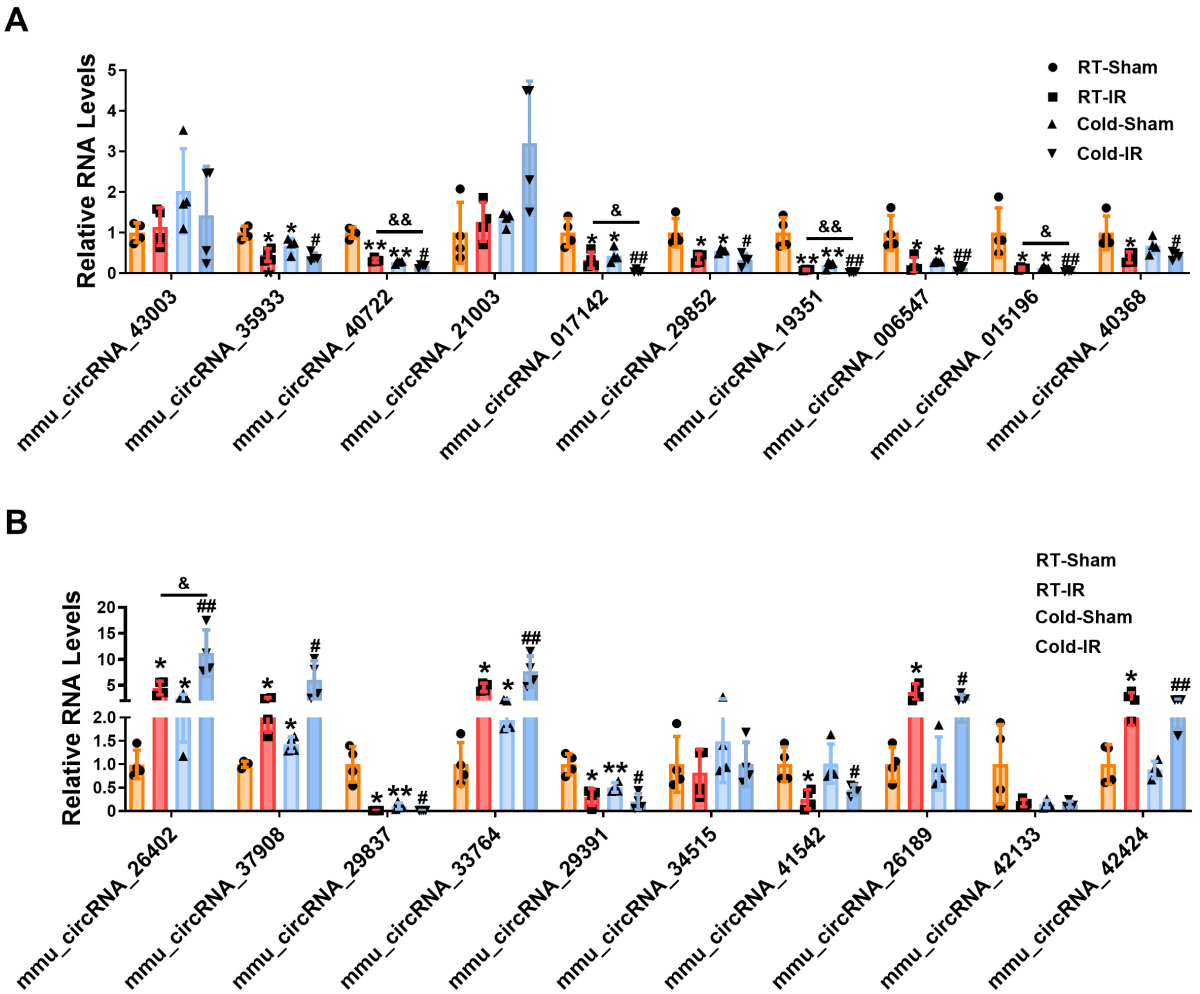

Fig. 7 Expression of N6-methyladenosine (m6A)-methylated circRNAs in chronic cold stress-induced myocardial I/R injury

(A) Relative expression levels of circRNAs in cardiac tissue. $^{*}P < 0.05$, $^{**}P < 0.01$ versus RT-Sham, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Cold-Sham, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ versus Cold-I/R $N = 4$.

mortality during winter months[35-39]. Cold exposure can trigger a series of detrimental cardiovascular responses, including increased blood viscosity, impaired hemodynamics, heightened sympathetic activation, and elevated arrhythmia risk, ultimately contributing to the development and progression of myocardial infarction and heart failure[36,39-43]. Previous studies have identified multiple molecular mediators involved in cold-induced cardiovascular dysfunction, such as TRPM8[44], β-adrenergic receptors[45], uncoupling protein 1 (UCP1)[46] and inflammatory signaling pathways[16]. In line with these observations, our results demonstrated that chronic cold exposure significantly exacerbated cardiac dysfunction and increased infarct size following myocardial I/R injury in mice. Interestingly, mice exposed to cold conditions exhibited increased body weight compared with those maintained at room temperature, which may be attributed to reduced physical activity and altered energy metabolism under cold stress, as previously reported[47-48].

To date, most studies examining the relationship between cold exposure and m6A modification have focused on plant biology. For instance, m6A methylation has been shown to be essential for cold tolerance in Arabidopsis, where disruption of m6A methyltransferase activity impairs cold-responsive gene translation and adaptation. These findings suggest that m6A modification plays a conserved role in regulating organismal responses to cold stress. In mammals, emerging evidence indicates that m6A methylation may influence cold and ischemia tolerance by regulating metabolic and stress-response pathways, including those involved in energy homeostasis and antioxidant defense. However, the role of m6A-modified circRNAs in mediating cardiovascular responses

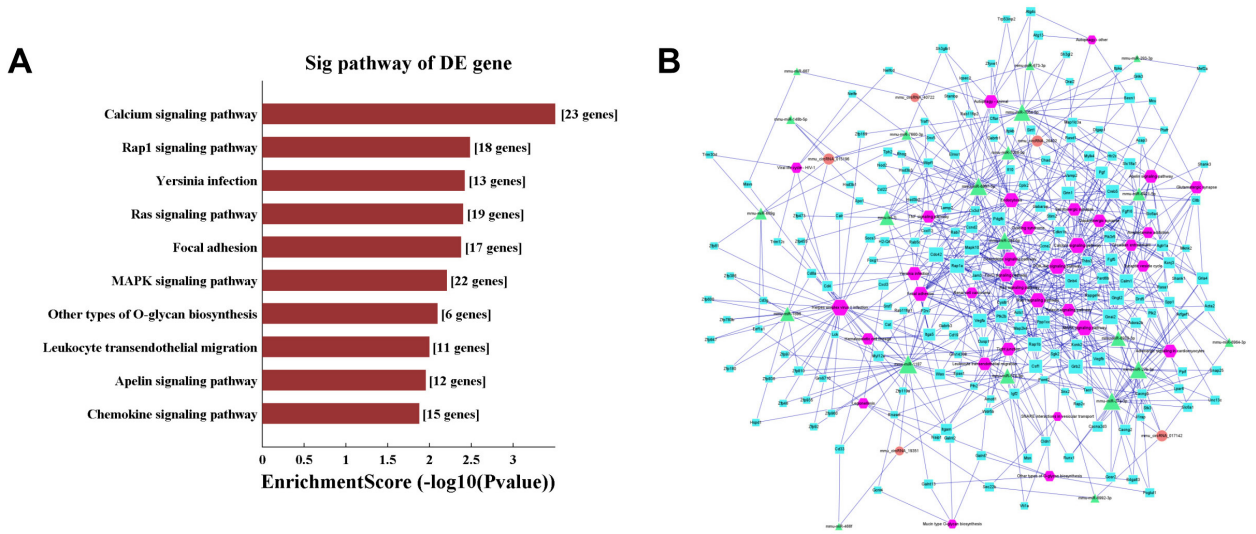

Fig. 8 Downstream target analysis of cold-responsive circRNAs

(A) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of cold-responsive circRNAs. (B) ceRNA network of five cold-responsive circRNAs and their sponge miRNAs under cold stress conditions. CircRNAs are shown as orange nodes, miRNAs as green nodes, and downstream target genes as blue nodes.

to cold stress remains largely unexplored.

Despite the novel findings presented in this study, several limitations should be acknowledged. Further investigations are required to elucidate the precise molecular mechanisms by which m6A-modified circRNAs regulate myocardial I/R injury under cold stress, including their interactions with specific miRNAs, RNA-binding proteins, and downstream signaling pathways. In addition, functional gain- and loss-of-function studies will be necessary to establish causal relationships between candidate circRNAs and cardioprotection or injury.

In conclusion, our study demonstrates that global m6A methylation levels are significantly increased following myocardial I/R injury and identifies a subset of circRNAs with altered m6A modification and expression profiles. Bioinformatics analyses suggest that these m6A-modified circRNAs are involved in key biological pathways relevant to myocardial injury. Importantly, chronic cold stress further modulates the expression of specific m6A-modified circRNAs, indicating that environmental temperature may influence epigenetic regulation during myocardial I/R injury. These findings provide new insights into the interplay between m6A modification, circRNAs, and cold stress in cardiovascular disease and highlight potential targets for future therapeutic intervention.

Acknowledgements

Not applicable.

Research ethics

All animal experiments were conducted in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care International and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Harbin Medical University (ethical approval number: IRB3017723).

Informed consent

Not applicable.

Author contributions

Yang B F and Bian Y designed the experiments and supervised the project. Wu H and Jiang WT performed the molecular biology experiments and drafted the manuscript. Zhang X Y, Yao F T, and Pang P conducted the design and implementation of the animal experiments. Pan T F and Lutokhina Y critically revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Use of large language models, AI and machine learning tools

No large language models, AI or machine learning tool was used for any part of the present study.

Conflict of interests

Yang B F is the Editor-in-Chief of Frigid Zone Medicine. The manuscript was handled according to the journal's standard editorial procedures, with peer review conducted independently of the Editor-in-Chief and his research groups.

Research funding

This work was supported by the National Natural Science Foundation of China (Nos. 82330011); Key Research and Development Program of Heilongjiang Province (Grant No. 2025ZX05A01); the Basic Research Support Program for Excellent Young Teachers in Heilongjiang Province (YQJH2025126).

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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