

Cardiomyocyte-specific long noncoding RNA *Trdn-as* induces mitochondrial calcium overload by promoting the m⁶A modification of calsequestrin 2 in diabetic cardiomyopathy

Xiaohan Li^{1,2,3,*}, Ling Liu^{1,2,3,*}, Han Lou^{1,2,3,*}, Xinxin Dong^{1,2,3}, Shengxin Hao^{1,2,3}, Zeqi Sun^{1,2,3}, Zijia Dou^{1,2,3}, Huimin Li^{1,2,3}, Wenjie Zhao^{1,2,3}, Xiuxiu Sun^{1,2,3}, Xin Liu^{1,2,3}, Yong Zhang (✉)^{1,2,3}, Baofeng Yang (✉)^{2,3}

¹State Key Laboratory of Frigid Zone Cardiovascular Diseases (SKLFZCD), College of Pharmacy, and Department of Cardiology, the Second Affiliated Hospital, Harbin Medical University, Harbin 150081, China; ²Department of Pharmacology (SKLFZCD, State Key Laboratory–Province Key Laboratories of Biomedicine-Pharmaceutics of China, Key Laboratory of Cardiovascular Research, Ministry of Education), College of Pharmacy, Harbin 150081, China; ³Research Unit of Noninfectious Chronic Diseases in Frigid Zone (2019RU070), Chinese Academy of Medical Sciences, Harbin 150081, China

© Higher Education Press 2024

Abstract Diabetic cardiomyopathy (DCM) is a medical condition characterized by cardiac remodeling and dysfunction in individuals with diabetes mellitus. Sarcoplasmic reticulum (SR) and mitochondrial Ca²⁺ overload in cardiomyocytes have been recognized as biological hallmarks in DCM; however, the specific factors underlying these abnormalities remain largely unknown. In this study, we aimed to investigate the role of a cardiac-specific long noncoding RNA, *D830005E20Rik* (*Trdn-as*), in DCM. Our results revealed the remarkably upregulation of *Trdn-as* in the hearts of the DCM mice and cardiomyocytes treated with high glucose (HG). Knocking down *Trdn-as* in cardiac tissues significantly improved cardiac dysfunction and remodeling in the DCM mice. Conversely, *Trdn-as* overexpression resulted in cardiac damage resembling that observed in the DCM mice. At the cellular level, *Trdn-as* induced Ca²⁺ overload in the SR and mitochondria, leading to mitochondrial dysfunction. RNA-seq and bioinformatics analyses identified calsequestrin 2 (*Casq2*), a primary calcium-binding protein in the junctional SR, as a potential target of *Trdn-as*. Further investigations revealed that *Trdn-as* facilitated the recruitment of METTL14 to the *Casq2* mRNA, thereby enhancing the m⁶A modification of *Casq2*. This modification increased the stability of *Casq2* mRNA and subsequently led to increased protein expression. When *Casq2* was knocked down, the promoting effects of *Trdn-as* on Ca²⁺ overload and mitochondrial damage were mitigated. These findings provide valuable insights into the pathogenesis of DCM and suggest *Trdn-as* as a potential therapeutic target for this condition.

Keywords *Trdn-as*; calsequestrin 2; METTL14; mitochondrial dysfunction; calcium overload

Introduction

Diabetes mellitus is a metabolic condition distinguished by elevated blood glucose levels, and its prevalence has increased in recent years. Research on the global prevalence of prediabetes suggests that the global diabetic population will reach 638 million by 2045 [1,2]. Diabetic cardiomyopathy (DCM) is the most common complication of diabetes mellitus and is characterized by

myocardial hypertrophy, cardiac fibrosis, and diastolic dysfunction in the early stages, followed by progression to systolic dysfunction and ultimately resulting in heart failure [3]. Various factors, including Ca²⁺ homeostasis disruptions, mitochondrial dysfunction, and oxidative stress, contribute to DCM development [4–6]. However, the exact underlying mechanism is unclear. Thus, identifying key factors responsible for DCM progression is crucial to uncover potential therapeutic targets for its treatment.

Ca²⁺ is a crucial and second messenger within eukaryotic cells. Its elevation in the cytoplasm elicits various cellular responses, including cell proliferation, contraction, secretion, and cell death [7–9]. The

Received February 25, 2024; accepted July 26, 2024

Correspondence: Yong Zhang, zy@ems.hrbmu.edu.cn;

Baofeng Yang, yangbf@ems.hrbmu.edu.cn

*Xiaohan Li, Ling Liu, and Han Lou contributed equally to this work.

sarcoplasmic reticulum (SR) is the central intracellular Ca^{2+} storehouse that regulates intracellular Ca^{2+} levels and dynamics [10,11]. The mitochondria, comprising 30% of the overall volume of mature cardiomyocytes, determine cell destiny mainly through their involvement in the dynamic control of cellular Ca^{2+} [12]. These two organelles intimately communicate as the SR transmits proper Ca^{2+} signals to the mitochondria on a dynamic membrane platform. Elevated Ca^{2+} translocation from the SR to the mitochondria leads to excessive Ca^{2+} accumulation within the mitochondria, causing disruptions in mitochondrial processes such as reduced adenosine triphosphate (ATP) synthesis, heightened reactive oxygen species (ROS) production, and apoptosis. SR and mitochondrial Ca^{2+} overload in cardiomyocytes have been recognized as biological hallmarks in experimental animals and individuals with DCM [13]. However, the specific factors contributing to these abnormalities remain largely unknown.

The calcium binding protein calsequestrin 2 (Casq2), a constituent of the ryanodine receptor 2 (RyR2) channel located within junctional SR (jSR), is essential for modulating the Ca^{2+} storage capability of the SR, enabling the accumulation of approximately 20 mmol/L Ca^{2+} within the SR. Alterations and mutations in *Casq2* expression can disrupt intracellular Ca^{2+} homeostasis, leading to the onset of various diseases [14,15]. Mutations in the *Casq2* gene is associated with catecholaminergic polymorphic ventricular tachycardia [16]. An elevated Ca^{2+} content in the SR was observed in transgenic mice with *Casq2* overexpression, leading to the development of substantial cardiac hypertrophy [17]. *Casq2* overexpression led to increases in SR Ca^{2+} , RyRs' sensitivity to Ca^{2+} , and Ca^{2+} release [18]. The close location between junctional SR (jSR) and mitochondria in cardiomyocytes facilitates the transfer of Ca^{2+} from SR to mitochondria [19]. However, the precise role of Casq2 in regulating mitochondrial Ca^{2+} balance and the factors controlling Casq2 levels in DCM remain to be fully understood.

Human genome sequencing has revealed that a significant portion of the transcriptome consists of noncoding RNAs (ncRNAs) [20]. Among these ncRNAs, long noncoding RNAs (lncRNAs) exhibit diverse functional mechanisms, including the regulation of transcription, epigenetics, and posttranscriptional processes [21–24]. Our previous research uncovered the involvement of lncRNAs in the development of myocardial infarction, arrhythmia, and heart aging [25–28]. The potential role of lncRNAs in modulating cardiac function and Ca^{2+} homeostasis in DCM is a significant area of inquiry.

To identify cardiac-specific lncRNAs implicated in cardiac function and DCM development, we analyzed

dysregulated lncRNAs in heart tissues from DCM mice through transcriptomic sequencing. A cardiomyocyte-specific lncRNA named *Trdn-as* was identified and exhibited significant upregulation in the hearts of the DCM mice. Using loss- and gain-of-function methods *in vitro* and *in vivo*, we demonstrated that *Trdn-as* plays a detrimental role in DCM progression by promoting the m⁶A modification of *Casq2* mRNA. This modification leads to SR and mitochondrial Ca^{2+} overload and subsequent mitochondrial dysfunction. Therefore, *Trdn-as* represents a promising novel target for therapeutic intervention in DCM.

Materials and methods

Animal care

Db/m mice (8-week-old, 20–25 g) and db/db mice (8-week-old, 42–47 g) were provided by the Nanjing Military Laboratory Animal Science and Technology Co., Ltd., China, and reared in a controlled animal facility with optimal conditions of 22 ± 1 °C temperature and $55\% \pm 5\%$ humidity.

Construction of viral vectors for *Trdn-as* knockdown and overexpression

Adeno-associated viruses (AAVs) have become widespread due to their characteristics such as highly efficient infection, low immunogenic response, nonpathogenicity, sustained gene expression over an extended period, and extensive tissue transduction. Among them, AAV9 has the highest infectivity for myocardial transduction. *Trdn-as* overexpression was completed by injecting an AAV9 vector carrying *Trdn-as* sequence (AAV-*Trdn-as*) with a conjugated GMV bGlobin promoter (Genechem Co., Ltd., Shanghai, China). An AAV9 vector with a short RNA fragment for knocking down *Trdn-as* and a conjugated U6 promoter were also designed (sh-*Trdn-as*) (Genechem Co., Ltd., Shanghai, China). Saline was injected into the control group of mice through the tail vein.

In vivo gene delivery

Viral solutions were injected into the mice via the tail vein using random delivery (AAV-*Trdn-as*, AAV-NC, sh-*Trdn-as*, and sh-NC constructs diluted in 100 μL of saline solution). For data collection on single-gene infection, experimental measurements were conducted 3 months after the animals were injected with either AAV-*Trdn-as* to overexpress *Trdn-as* or sh-*Trdn-as* to silence *Trdn-as*.

Establishment of DCM model and study design

The animals were randomly allocated into the following groups: db/m, db/db (21-week-old db/db mice), + sh-*Trdn-as* (9-week-old db/db mice infected with AAV9-sh-*Trdn-as* once a month for 3 months), + sh-NC (9-week-old db/db mice infected with AAV9-sh-NC once a month for 3 months), AAV-*Trdn-as* (9-week-old db/m mice infected with AAV9-*Trdn-as* once a month for 3 months), and NC (9-week-old db/m mice infected with AAV9-NC once a month for 3 months).

Echocardiographic assessment of cardiac function

The mice were anesthetized through intraperitoneal injection of 0.2 g/kg avertin (Sigma, St. Louis, Missouri, USA). Cardiac function was evaluated using the Vevo2100 high-resolution imaging system (Visualsonics, Toronto, ON, Canada). Cardiac systolic function was assessed using left ventricular (LV) ejection fraction (EF) and short-axis fractional shortening (FS), both calculated from M-mode recordings. Cardiac diastolic function was detected using the ratio of the E peak (LV diastolic maximum blood flow) to the A peak (atrial systolic maximum blood flow through the mitral valve). LV posterior wall diastole (LVPWD) and LV mass were also measured.

Histopathological and morphometric observations

Hematoxylin and eosin (HE) and Masson's trichrome staining were applied to evaluate cardiac histopathological changes and collagen accumulation following the procedures described in our previous study [29]. Heart tissues were thoroughly fixed in 4% paraformaldehyde, embedded in paraffin, and transversely sliced into 5 μ m-thick sections for HE staining using HE reagents (Solarbio, Beijing, China) or Masson's trichrome staining with Masson's trichrome kit (Solarbio, Beijing, China). Each section was imaged using a light microscope (Carl Zeiss Microscopy, Jena, Germany). Collagen volume fraction was measured with Image J software.

Immunohistochemistry assessment

Prepared paraffin-embedded slices of mouse heart were heated at 65 °C for 20 min, deparaffinized through xylene, and rehydrated using different alcohol concentrations (100%, 95%, 80%, and 70%). The sections were soaked in citric acid buffer at 100 °C for 10 min without boiling for antigen retrieval and treated with 3% H₂O₂ (Aladdin, Shanghai, China) at room temperature for 10 min to quench endogenous peroxidase. Goat serum (Beyotime, Shanghai, China) was used to block the slices for 1 h, followed by staining with anti-Casq2 (1:500,

#ab3516; Abcam, Cambridge, UK) overnight at 4 °C, incubation with horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h, and diaminobenzidine color rendering (ZSGB-BIO, Beijing, China). Furthermore, the slices were counterstained with HE, treated with ethanol and xylene, and sealed using resin. Immunohistochemistry images were obtained using a panoramic scanning microscope (Leica, Wetzlar, Germany), and Casq2 expression was analyzed from the immunohistochemistry images with Image J software.

Immunofluorescence assessment

Frozen heart sections were prepared for immunofluorescence assessment to determine Casq2 expression. The sections were washed with PBS for 10 min and then fixed with 4% paraformaldehyde. After three subsequent PBS washes, the slices were treated with goat serum containing 0.3% Triton X-100 for 1 h and incubated with anti-Casq2 (1:100) at 4 °C overnight. On the second day, a fluorescence-conjugated secondary antibody was applied to the sections at room temperature for 1 h. Finally, DPAI staining was performed for 15 min. Immunofluorescence images of the heart were captured by a confocal fluorescence microscope (Olympus, Fluoview1000, Tokyo, Japan), and the fluorescence density of Casq2 was analyzed from the immunofluorescence images with Image J software.

Transmission electron microscopy

Heart samples were fixed with 2.5% glutaraldehyde at 4 °C overnight. The slices were dehydrated and stained with uranyl acetate and lead citrate. The ultrastructural changes of the heart tissues were observed under a JEOL-1200 electron microscope produced by JEOL Company in Tokyo, Japan.

Primary neonatal mouse cardiomyocytes (NMCMs)

Cardiomyocytes were obtained from neonatal Kunming mice aged 1–3 days procured from the Experimental Animal Center of the Second Affiliated Hospital of Harbin Medical University in Harbin, China. After heart dissection, the specimens were placed in a mixed solution of D-hanks and trypsin on a shaker at 4 °C overnight. Preheated type II collagenase (#17101015; Gibco, NY, USA) was used to digest the hearts on the second day. The obtained solution containing mixed cells was collected and centrifuged (1500 rpm/min, 5 min). The supernatant was discarded, and the cells were resuspended on DMEM (#BL301A; Biosharp, China) containing 10% fetal bovine serum (#C04001; Vivacell, Shanghai, China) and 1% penicillin–streptomycin solution ($\times 100$) (#C0222; Beyotime, Shanghai, China).

The cells were cultured in a CO₂ (5%) incubator with constant temperature (37 °C) and humidity. After 1.5 h, the supernatant suspension was collected, diluted, and dispersed with fresh culture solution and spread evenly in culture plates.

Transfection of *Trdn-as* siRNA and plasmids

For transfection, *Trdn-as*-overexpressing pcDNA3.1-plasmids or negative controls (IBSBIO, Shanghai, China) were transiently transfected into NCMs using Lipofectamine 2000 (Invitrogen, USA). *Trdn-as* siRNA and siNC (RiboBio Co., China) were also transfected using the X-treme GENE Transfection Reagent (Roche, USA). The transfection agents were combined with Opti-MEM (Gibco, NY, USA) and DMEM without serum and subsequently added to the NCMs. After 6 h, the medium was replaced, and the cells were treated with 33 mM glucose for 48 h and utilized for subsequent experiments.

RNA-interacting protein immunoprecipitation (RIP)

The RIP RNA binding protein immunoprecipitation kit (#Bes5101; Beisbio, Guangzhou, China) was utilized for RIP analysis. The ReverTraAce qPCR RT Kit (#FSQ - 101; Toyobo, Osaka, Japan) was employed to reverse-transcribe RNA into cDNA. The SYBR Green PCR Master Mix kit (#4309155; Invitrogen, Carlsbad, USA) and LightCycler 96 real-time polymerase chain reaction system (Roche, Basel, Switzerland) were utilized for qRT-PCR on the specific gene of interest.

JC-1 staining for mitochondrial membrane potential (MMP)

JC-1 is a fluorescent indicator used to detect MMP and emits red or green fluorescence in cells with high or low potential. Cardiomyocytes were treated with specific interventions and exposed to a 5 μM JC-1 dye solution (#C2006; Beyotime, Shanghai, China) at 37 °C for 20 min. The resulting images were then acquired using a confocal fluorescence microscope. The fluorescence intensity of the red and green signals was quantified with Image J software.

Immuno-FISH

A RiboTM fluorescence *in situ* hybridization kit (RiboBio, Guangzhou, China) was acquired to perform the FISH fluorescence probe staining of *Trdn-as* in accordance with the regulations and suggestions in the kit manual. The sample was sealed in a prehybridization buffer (37 °C, 30 min), followed by a 12 h hybridization in a buffer containing 20 μM *Trdn-as* FISH Probe Mix at room temperature. The sample was then washed with 0.1%

Tween-20 in 4 × SSC, 2 × SSC, and PBS for 10 min each. The nucleus was labeled with DAPI. Finally, the sample was mounted on a confocal fluorescence microscope for imaging.

Oxygen consumption rate (OCR)

OCR was measured using the Seahorse XF Cell Mito Stress Test Kit (#103015-100; Seahorse Bioscience, USA) and Seahorse XFe24 Extracellular Flux Analyzer following the manufacturer's instructions. NCMs were cultured in XFe24 plates and subjected to respective treatments. Before measurement, the cells were rinsed with an XF assay medium and incubated at 37°C without CO₂ for 1 h. Compounds oligomycin, FCCP, rotenone, and antimycin A were sequentially added to the XF probe card, and the plates were loaded onto the XFe24 analyzer for OCR detection.

Methylated RNA immunoprecipitation (MeRIP)-qPCR assays

MeRIP assay was conducted following the protocol outlined in the riboMeRIPTM m⁶A Transcriptome Profiling Kit (#C11051-1; RiboBio Co., China). RNAs were fragmented by RNA fragmentation buffer and heating and then prepared for MeRIP reaction. The m⁶A-specific antibody was incubated with magnetic beads A/G in an IP buffer at room temperature. The MeRIP reaction mixture, consisting of fragmented RNAs, was introduced to anti-m⁶A beads and incubated at 4 °C for 2 h on a rotator. Finally, the immunoprecipitated mixtures were treated with elution buffers at 4 °C for 1 h to extract the bound RNAs. The enriched RNA (*Casq2*) was examined by qPCR.

SR Ca²⁺ detection

SR Ca²⁺ level was detected using a specific fluorescent probe for SR Ca²⁺, Mag-Fluo-AM (GMS10267.1; GENMED SCIENTIFIC INC. USA). NCMs were applied to cell slides, subjected to specific treatments, and exposed to a dyeing solution at room temperature for 1 h after being washed with cleaning fluid. Images were capture by a confocal microscope, and fluorescence density (green) was measured with Image J software [30].

Mitochondrial Ca²⁺ detection

A specific fluorescent probe for mitochondrial Ca²⁺, Rhod-2-AM (GMS10153.1; GENMED SCIENTIFIC INC. USA), was utilized to explore mitochondrial Ca²⁺ levels [31]. NCMs were placed in a black 96-well plate, transfected, and treated with glucose. Cleaning fluid was added to the plate and mixed with the dyeing working

fluid. The cells were placed in a 37 °C cell incubator for 30 min away from the light. The solution was substituted with a cleaning fluid, and the cells were incubated at 37 °C for an additional 45 min. Mitochondrial Ca^{2+} concentration was determined by measuring relative fluorescence units using a fluorescence microplate reader.

ROS detection

ROS level was measured using a ROS detection kit (#S0033S; Beyotime Biotechnology, China). NCMs were rinsed with PBS three times and then exposed to a 10 μM DCFH-DA fluorescence probe at 37 °C for 20 min. Images were captured by a confocal microscope, and the fluorescence density of DCFH-DA was measured with Image J software.

Malonaldehyde (MDA) detection

MDA concentration was assessed using the CheKine™ Lipid Peroxidation (MDA) Assay Kit (#KTB1050; Abbkine, China) following the manufacturer's guidelines. Treated NCMs were collected and lysed with lysis buffer. After the solution was centrifuged at 13 000 g for 10 min at 4 °C, the supernatant was employed to assess the MDA level. The reaction mixture was added to the supernatant and incubated at 95 °C for 30 min. The mixed solution underwent centrifugation at 10 000 g for 10 min. The supernatant was collected and added to a 96-well plate for MDA detection. Absorbance was measured at 532 and 600 nm using a microplate reader.

Superoxide dismutase (SOD) detection

SOD level was measured by CheKine™ Superoxide Dismutases (SOD) Activity Assay Kit (#KTB1030; Abbkine, China) following the guidelines provided by the manufacturer. NCMs were lysed and then centrifuged at 12 000 g for 5 min. The supernatant was then added to the 96-well plate at 20 μL per well and mixed with 80 μL of working solution, which consisted of 5 μL of xanthine, 5 μL of WST-8, 1 μL of enhancer, and 74 μL of assay buffer. Finally, the mixture was incubated with 20 μL of xanthine Oxidase for 1 h at room temperature. The absorbance, indicative of the SOD level, was then measured at 450 nm using a microplate reader.

ATP content detection

The ATP levels in cardiomyocytes were determined using the ATP Assay Kit (#S0026; Beyotime, Shanghai, China). The heart tissue was lysed and centrifuged at 12 000 g for 5 min at 4 °C. ATP detection working fluid and the supernatant were sequentially added into the wells of the 96-well plate.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from heart tissues and cardiomyocytes was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA). Reverse transcription was carried out using the ReverTraAce qPCR RT Kit. qRT-PCR was performed using the SYBR Green PCR Master Mix kit and the LightCycler 96 real-time polymerase chain reaction system. β -actin served as the internal control. The sequences of primers used were listed below:

Trdn-as: F: 5'-GGGATGCAGAAGCAACAGAG-3'

R: 5'-GCAGCAAAGGTACACAGAGG-3'

Casq2: F: 5'-GACCGAGTGGTCAGCCTTTC-3'

R: 5'-ACAGGTTCGTGGTAATAGAGACA-3'

β -actin: F: 5'-CGTTGACATCCGTAAAGACC-3'

R: 5'-AACAGTCCGCCTAGAAGCAC-3'

Western blot analysis

For protein blot detection, the total protein samples from heart tissues and cardiomyocytes were subjected to electrophoresis (60–100 μg /well) and subsequently transferred onto a nitrocellulose filter membrane (PALL, New York, USA). The membrane was blocked with 5% nonfat milk dissolved in PBS for 2 h. Primary antibodies against Casq2 and β -actin (Proteintech, Chicago, USA), diluted at 1:1000 in PBS buffer, were utilized to probe the membrane at 4 °C overnight. The membrane was incubated with a fluorescence-conjugated anti-rabbit or anti-mouse IgG secondary antibody at room temperature for 1 h after being washed in PBS with 0.05% Tween-20 three times. β -actin served as the internal control. Western blot results were quantified with Odyssey v1.2 software (LICOR Biosciences, Lincoln, NE, USA).

Statistical analysis

The data are presented as mean $\pm$ SEM. Statistical comparison between the two experimental groups was performed using a two-tailed unpaired Student's *t*-test. The difference between groups was assessed using one-way ANOVA and multiple comparison tests with Tukey's post hoc analysis. Statistical analyses were conducted using Prism 8.0 (GraphPad, La Jolla, CA) and SPSS (Version 25). A significance level of $P < 0.05$ was considered statistically significant.

Results

Trdn-as is upregulated in the cardiomyocytes of DCM mice

To investigate the regulatory role of lncRNAs in DCM, we screened the differentially expressed lncRNAs using

the GEO data set (GSE161931) as illustrated in Fig. 1A. Heatmap analysis identified the top 30 dysregulated genes. Among them, the following lncRNAs with heart-specific expression patterns were identified: GM30556, GM35228, GNM31013, GM15543, GM26795, C130080G10Rik, and D830005E20Rik (*Trdn-as*) (Fig. 1B). *Trdn-as* exhibited the highest upregulation in the hearts of the DCM mice, indicating its potential

significance. Furthermore, its expression pattern was assessed in various organs of wild-type (WT) mice, including the heart, liver, kidney, spleen, pancreas, lung, skeletal muscle, small intestine, skin, and fat. The results revealed that *Trdn-as* was prominently enriched in the heart (Fig. S1A). To elucidate the specific role of *Trdn-as*, we also analyzed its expression patterns across various cardiac cell types using the Tabula Muris database. The

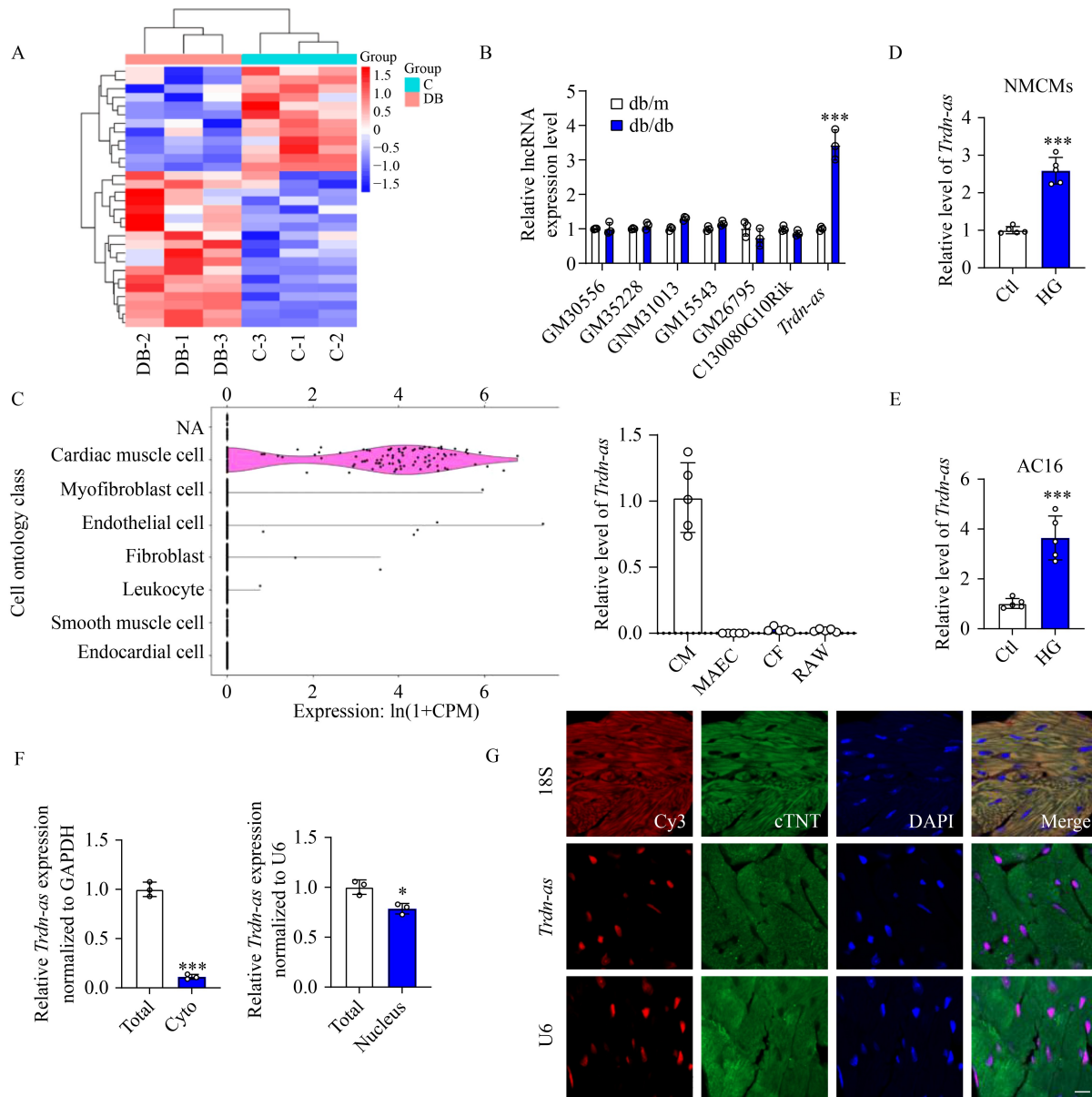


Fig. 1 *Trdn-as* is upregulated in diabetic cardiomyopathy (DCM) heart. (A) Heatmap of differential genes: red indicates the upregulation of differential genes, and blue indicates the downregulation of differential genes, $n = 3$. (B) *Trdn-as* expression in the heart tissue of DCM mice detected by qRT-PCR. (C) Cell distribution of *Trdn-as* in various cardiac cell types. $n = 5$. (D) *Trdn-as* expression in NCMs treated with high glucose for 48 h was significantly higher than that in the control group. $n = 5$. (E) *Trdn-as* expression was significantly increased in the AC16 cell line treated with high glucose for 48 h. $n = 5$. *** $P < 0.001$ vs. Ctl. (F) *Trdn-as* expression in the nucleus and cytoplasm of NCMs tested by qRT-PCR. $n = 3$. * $P < 0.05$, *** $P < 0.001$ vs. total. (G) Subcellular localization of *Trdn-as* in NCMs assessed using fluorescence *in situ* hybridization (FISH), with 18S and U6 serving as indicators for the cytoplasm and nucleus, respectively. Scale bar: 20 μ m. $n = 5$. The data were expressed as the mean $\pm$ SEM.

results revealed that *Trdn-as* was exclusively expressed in cardiomyocytes, indicating its potential involvement in modulating cardiomyocyte function. We also verified this conclusion in mouse aortic endothelial cells (MAEC), mouse RAW, cardiomyocytes (CM), and cardiac fibroblasts (CF) (Fig. 1C). qRT-PCR experiments demonstrated similar *Trdn-as* upregulation in NMCs and AC16 cells exposed to high glucose, a condition that mimics cardiomyocyte damage associated with DCM (Fig. 1D and 1E). To uncover its potential regulatory mechanism, we investigated the cellular localization of *Trdn-as*. qRT-PCR analysis of separated nuclear and cytoplasmic fractions and FISH staining revealed that *Trdn-as* was primarily sustained in the nucleus (Fig. 1F and 1G). These findings indicated the potential regulatory role of *Trdn-as* within cardiomyocytes in DCM.

***Trdn-as* induces cardiac dysfunction and heart injury in mice**

We then questioned whether the upregulation of *Trdn-as* in cardiomyocytes directly contributes to cardiac dysfunction. We developed an AAV9 plasmid to overexpress *Trdn-as* in WT C57BL/6J mice. The successful overexpression of *Trdn-as* in the hearts of the mice was confirmed 3 months after injection of the AAV9-*Trdn-as* plasmid (Figs. 2A and S1B). Echocardiography was utilized to assess the cardiac function. The results suggested that *Trdn-as* overexpression impaired the diastolic function of mice as evidenced by the reduced E/A ratio and increased LV mass and LVPWD. A significant decrease was observed in EF and FS, suggesting a compromised systolic function. Left ventricular end diastolic (LVEDD) and systolic (LVESD) dimensions were also increased after overexpressing *Trdn-as* (Figs. 2B and S1C). To examine the influence of *Trdn-as* on cardiac remodeling, we utilized HE and Masson staining to assess myocardial organization and interstitial fibrosis. The results revealed notable differences between the control mice and the mice overexpressing *Trdn-as* (Fig. 2C). In particular, the myocardial sarcomeres in the *Trdn-as*-overexpressing mice displayed disarray, enlarged myocardial cells and increased collagen production, indicating the presence of cardiac remodeling and interstitial fibrosis (Fig. 2C). To investigate the potential of *Trdn-as* as a therapeutic target for DCM, we performed a knockdown experiment by transfecting *Trdn-as* shRNA carried by an AAV plasmid in db/db mice. The knockdown efficiency of *Trdn-as* was confirmed using qRT-PCR and immunofluorescence staining (Figs. 2D and S1D). *Trdn-as* knockdown significantly improved the cardiac systolic and diastolic function of db/db mice (Figs. 2E and S1E). It also ameliorated the disorganized arrangement of cardiac sarcomeres, improved myocardial hypertrophy, and

decreased the production of cardiac collagen (Fig. 2C). These results strongly indicated that *Trdn-as* modulates cardiac function in DCM.

***Trdn-as* regulates *Casq2* expression in DCM**

To investigate the molecular mechanism underlying the role of *Trdn-as* in DCM, we performed RNA-seq analysis to identify potential genes regulated by *Trdn-as* following its overexpression in NMCs (Fig. 3A). Functional analysis of Gene Ontology and pathway enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes indicated the notable enrichment of pathways related to Ca^{2+} , including Ca^{2+} ion homeostasis, cardiac muscle contraction, SR, and regulation of cation transmembrane transport (Fig. 3B and 3C). Further analysis using a heatmap highlighted the enriched genes within these pathways (Fig. 3D), and subsequent verification identified *Casq2* as a common gene (Fig. 3E and 3F). *CASQ2* overexpression (GSE62203) was observed in DCM model (Fig. S2A–S2C). Furthermore, the observed upregulation of *Trdn-as* and *Casq2* in our sequencing data suggested that the model was effectively established and the analytical findings are reliable (Fig. S2D). Our data demonstrated the significant upregulation of *Casq2* at the mRNA and protein levels in the hearts of db/db mice. By contrast, *Trdn-as* knockdown resulted in decreased *Casq2* mRNA and protein levels. *Trdn-as* overexpression in the control mice increased *Casq2* expression (Fig. 3G and 3H). Furthermore, the expression and distribution of *Casq2* in cardiac tissues was confirmed by immunofluorescence staining (Fig. 3I and 3J). In NMCs, *Trdn-as* knockdown reversed the upregulation of *Casq2* induced by high glucose conditions, while overexpression of *Trdn-as* resulted in increased *Casq2* mRNA and protein expression (Fig. 3K and 3L).

***Trdn-as* induces SR and mitochondrial Ca^{2+} overload and mitochondrial damage**

Considering the role of *Casq2* in modulating Ca^{2+} storage within the SR, we evaluated the impact of *Trdn-as* on SR Ca^{2+} levels. Our findings demonstrated a substantial augmentation in SR Ca^{2+} levels upon *Trdn-as* overexpression (Fig. S3A). The transport of Ca^{2+} from the SR to the mitochondria is critical in regulating mitochondrial function, including mitochondrial biogenesis, respiration, and oxidative stress. *Trdn-as* upregulation led to a significant elevation in Ca^{2+} levels within the mitochondria (Fig. S3B). Excessive Ca^{2+} accumulation induced by *Trdn-as* in the mitochondria impaired mitochondrial function. JC-1 staining results indicated that *Trdn-as* overexpression led to a reduction in MMP (Fig. S3C). TEM results suggested that *Trdn-as*

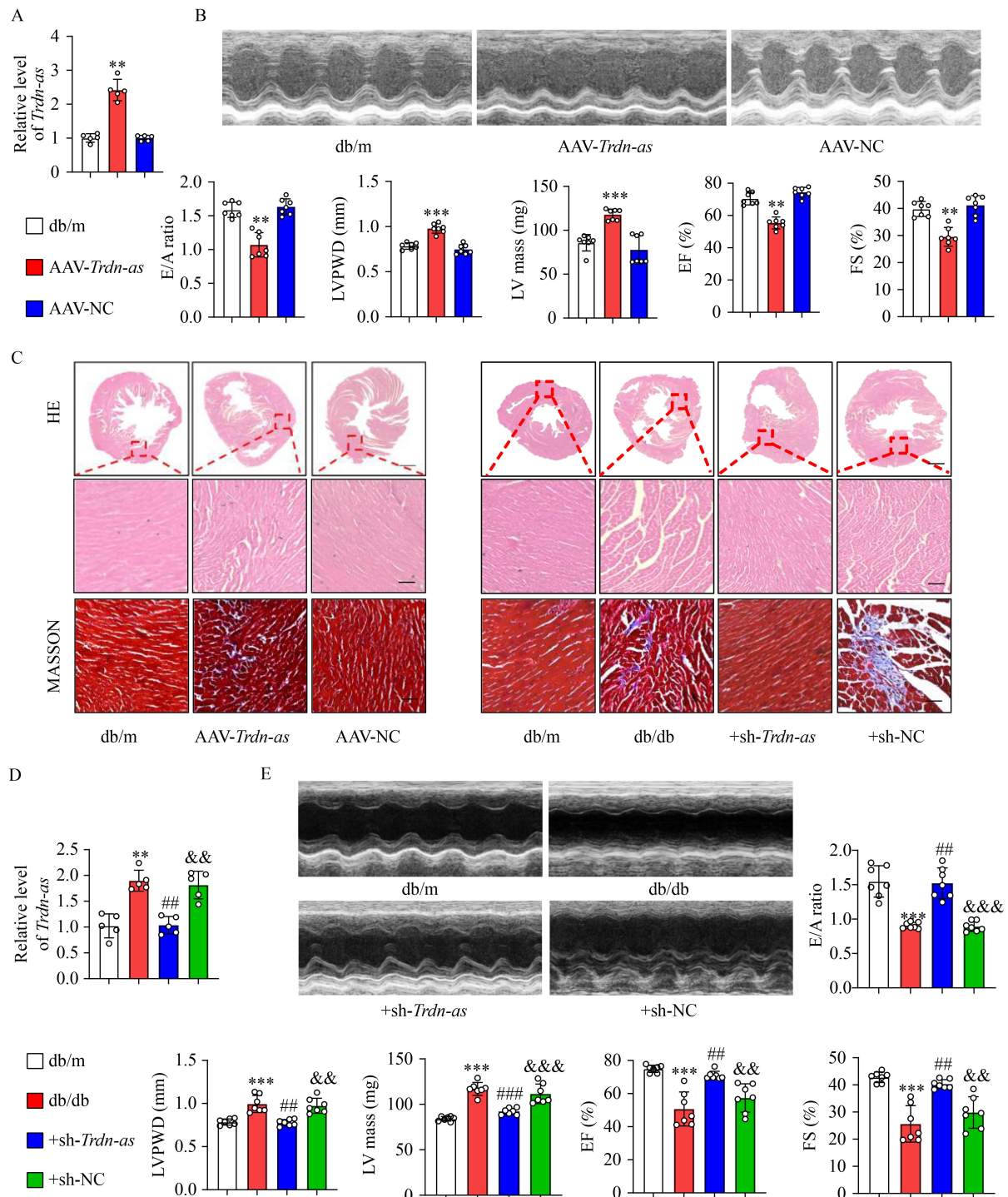


Fig. 2 *Trdn-as* induced cardiac dysfunction and tissue injury. (A) Expression efficiency of AAV-*Trdn-as* verified by qRT-PCR. $n = 5$. (B) Identification of alterations in heart function using echocardiography. $n = 7$. (C) Histological heart tissue sections from DCM mice were stained with hematoxylin and eosin (HE) and Masson's trichrome stain. Scale bar: 500 and 20 μm . $n = 5$. (D) qRT-PCR was used to verify the knockdown efficiency of sh-*Trdn-as*. $n = 5$. (E) Cardiac function of DCM mice was recovered by echocardiography after *Trdn-as* knockdown. $n = 7$. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. db/m, $^{##}P < 0.01$, $^{###}P < 0.001$ vs. db/db. $^{&&}P < 0.01$, $^{&&&}P < 0.001$ vs. sh-*Trdn-as*. Data were expressed by mean $\pm$ SEM.

overexpression markedly diminished normal mitochondrial content, leading to mitochondrial damage characterized by swelling and loss of ridge structure (Fig. S3D).

Subsequent analysis showed increased oxidative stress (as evidenced by ROS overload), decreased SOD levels, and increased MDA levels (Fig. S3E–S3G).

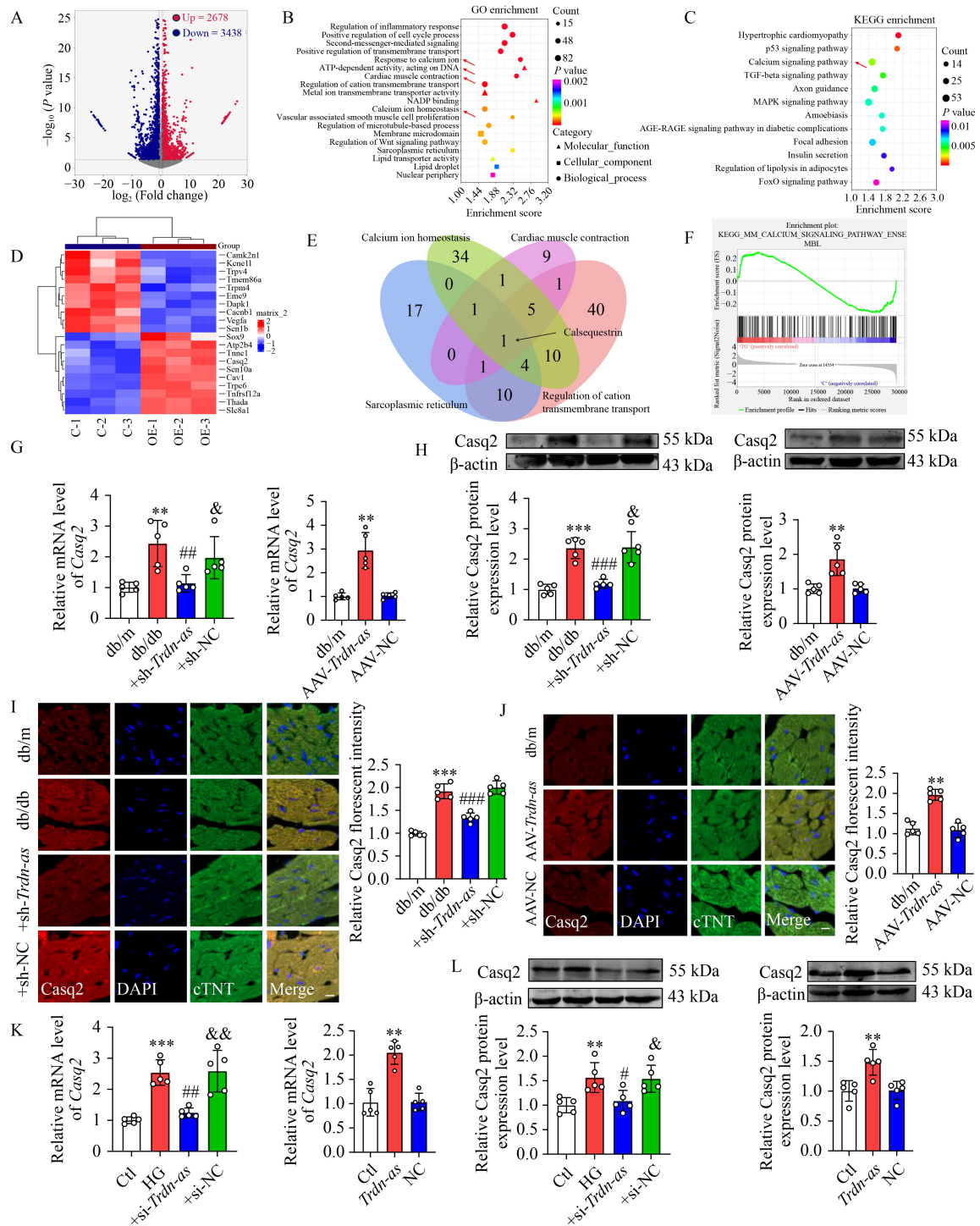


Fig. 3 RNA sequencing and identification and validation of target genes. (A) Volcano plot generated to visualize the differentially expressed mRNAs responding to *Trdn-as* overexpression. (B) A Gene Ontology functional analysis of differentially expressed genes. (C) Enrichment of KEGG pathways with differentially expressed genes. (D) Heat map of mRNA involved in Ca^{2+} homeostasis, cardiac muscle contraction, sarcoplasmic reticulum, and regulation of cation transmembrane transport. (E) Venn diagram takes the intersection of genes of four pathways to find the target. (F) Enrichment plot of Ca^{2+} signaling pathway. (G) *Casq2* mRNA levels tested by qRT-PCR in db/db mice. $n = 5$. (H) *Casq2* protein levels in hearts of mice tested by Western blot. $n = 5$. $**P < 0.01$, $***P < 0.001$ vs. db/m, $\#P < 0.01$, $\#\#P < 0.001$ vs. db/db, $\&P < 0.05$ vs. sh-*Trdn-as*. (I, J) Levels of *Casq2* (red) verified by immunofluorescence staining, nuclei (blue), cTNT (green). Scale bar: 20 μm . $n = 5$. (K) mRNA levels of *Casq2* assessed using qRT-PCR in NMCs. $n = 5$. (L) Protein levels of *Casq2* in NMCs assessed by Western blot. $n = 5$. $**P < 0.01$, $***P < 0.001$ vs. Ctl, $\#P < 0.05$, $\#P < 0.01$ vs. HG, $\&P < 0.05$, $\&P < 0.01$ vs. sh-*Trdn-as*. Data were expressed by mean $\pm$ SEM.

***Trdn-as* knockdown inhibits mitochondrial Ca^{2+} overload and mitochondrial damage caused by high glucose**

We then investigated the potential of *Trdn-as* knockdown to mitigate mitochondrial Ca^{2+} overload and damage. High glucose treatment induced significant SR and mitochondrial Ca^{2+} overload, which were attenuated upon *Trdn-as* knockdown (Fig. 4A and 4B). In addition, *Trdn-as* knockdown restored MMP and mitochondrial respiration (Fig. 4C and 4D). In the hearts of db/db mice, a notable reduction in normal mitochondrial content and many damaged mitochondria were observed. However, *Trdn-as* knockdown attenuated mitochondrial damage in db/db mice (Fig. 4E). In the presence of elevated glucose levels, the ROS production and MDA content increased and the SOD levels decreased. When *Trdn-as* was genetically disrupted, the ROS and MDA production were inhibited and the SOD levels increased (Fig. 4F–4H). ATP synthesis was observed in the myocardium of mice. The ATP levels in the cardiac muscle of DCM mice were significantly reduced, and those in the myocardium of *Trdn-as* knockout mice were restored. Conversely, *Trdn-as* overexpression led to a decrease in ATP content in the myocardium of mice (Fig. S4A). We also explored the mechanism of Ca^{2+} overload in mitochondria. VDAC1 and MCU were identified as mediators of Ca^{2+} entry into the mitochondria, and knocking them down successfully mitigated the mitochondrial Ca^{2+} overload induced by *Trdn-as*, indicating the contribution of these two calcium channels to this process (Fig. S4B). These findings demonstrated that *Trdn-as* knockdown can alleviate mitochondrial Ca^{2+} overload and dysfunction, thereby protect cardiomyocytes against high glucose-induced damage.

***Casq2* knockdown restores Ca^{2+} homeostasis and ameliorates mitochondrial dysfunction**

We subsequently confirmed the involvement of *Casq2* in the functional deterioration of cardiomyocytes caused by *Trdn-as*. When the calcium-sensing protein *Casq2* was disrupted under typical physiologic circumstances, the Ca^{2+} within the SR and mitochondria in cardiomyocytes were reduced (Fig. 5A and 5B). Our findings also suggested that the decrease in *Casq2* can ameliorate the mitochondrial dysfunction induced by *Trdn-as* overexpression, and this ability involved the restoration of MMP, enhancement of mitochondrial respiration, mitigation of ROS and MDA generation, and an increase in SOD activity (Fig. 5C–5F). However, knocking down *Casq2* leads to a reduction in Ca^{2+} in the SR and mitochondria, resulting in mitochondrial damage and dysfunction. The changes in SR Ca^{2+} are related to

contractile function, and we also detected the changes of SERCA2a. Western blot results showed no evident change in the SERCA2a expression of db/db mice. *Trdn-as* knockdown did not affect SERCA2a expression, indicating that *Trdn-as* did not influence the function of cardiomyocytes by affecting SERCA2a (Fig. S5A). These findings highlighted that *Trdn-as* disrupts Ca^{2+} homeostasis in cardiomyocytes mediated by *Casq2*, ultimately inducing mitochondrial dysfunction.

***Trdn-as* enhances *Casq2* mRNA stability by promoting METTL14-mediated m⁶A modification**

The above findings suggested that *Trdn-as* could regulate the mRNA expression of *Casq2*. Hence, molecular mechanism was further investigated. *Trdn-as* downregulation led to a significant decrease in the stability of *Casq2* mRNA (Fig. 6A). m⁶A modification promotes epigenetic reprogramming in mRNA, often leading to changes in mRNA stability. To examine the potential involvement of m⁶A modification in the modulation of *Casq2* mRNA stability by *Trdn-as*, we analyzed the total m⁶A levels following the overexpression or knockdown of *Trdn-as*. Our results demonstrated that *Trdn-as* overexpression increased the m⁶A level in normal cardiomyocytes (Fig. 6B). Conversely, *Trdn-as* knockdown inhibited the high glucose-induced elevation of m⁶A levels (Fig. 6C). The regulatory potential of *Trdn-as* on m⁶A modification encouraged us to investigate the m⁶A modification sites on *Casq2* mRNA.

The online tool SRAMP suggested a high confidence of m⁶A modification position in the *Casq2*, including 748, 1681, and 1788 locus (Fig. 6D and 6E). MeRIP results revealed that relying on the 748 locus, *Trdn-as* contributed to a significant increase in the m⁶A modification of *Casq2* mRNA (Fig. 6F). Furthermore, high glucose treatment could elevate the mRNA methylation level of *Casq2*, which was effectively inhibited by *Trdn-as* knockdown (Fig. 6F). To identify potential methylated proteins involved in regulating *Casq2* methylation, we employed the RPIseq web-based tool to assess the interaction potential of established methyltransferases and readers such as METTL3, METTL14, WTAP, and YTHDF2 with *Trdn-as* or *Casq2* mRNA. METTL14 emerged as the most prominent candidate, displaying significantly higher RF and SVM scores than other writer proteins (Fig. 6G). Subsequent RIP analysis revealed that METTL14 exhibited significant binding with *Trdn-as* (Fig. 6H). METTL14 expression remained unaltered, which was also confirmed through protein-level analysis (Fig. S5B and S5C). METTL14 demonstrated binding capabilities with *Casq2* mRNA. Under high glucose conditions, an increased interaction between METTL14 and *Casq2* mRNA was

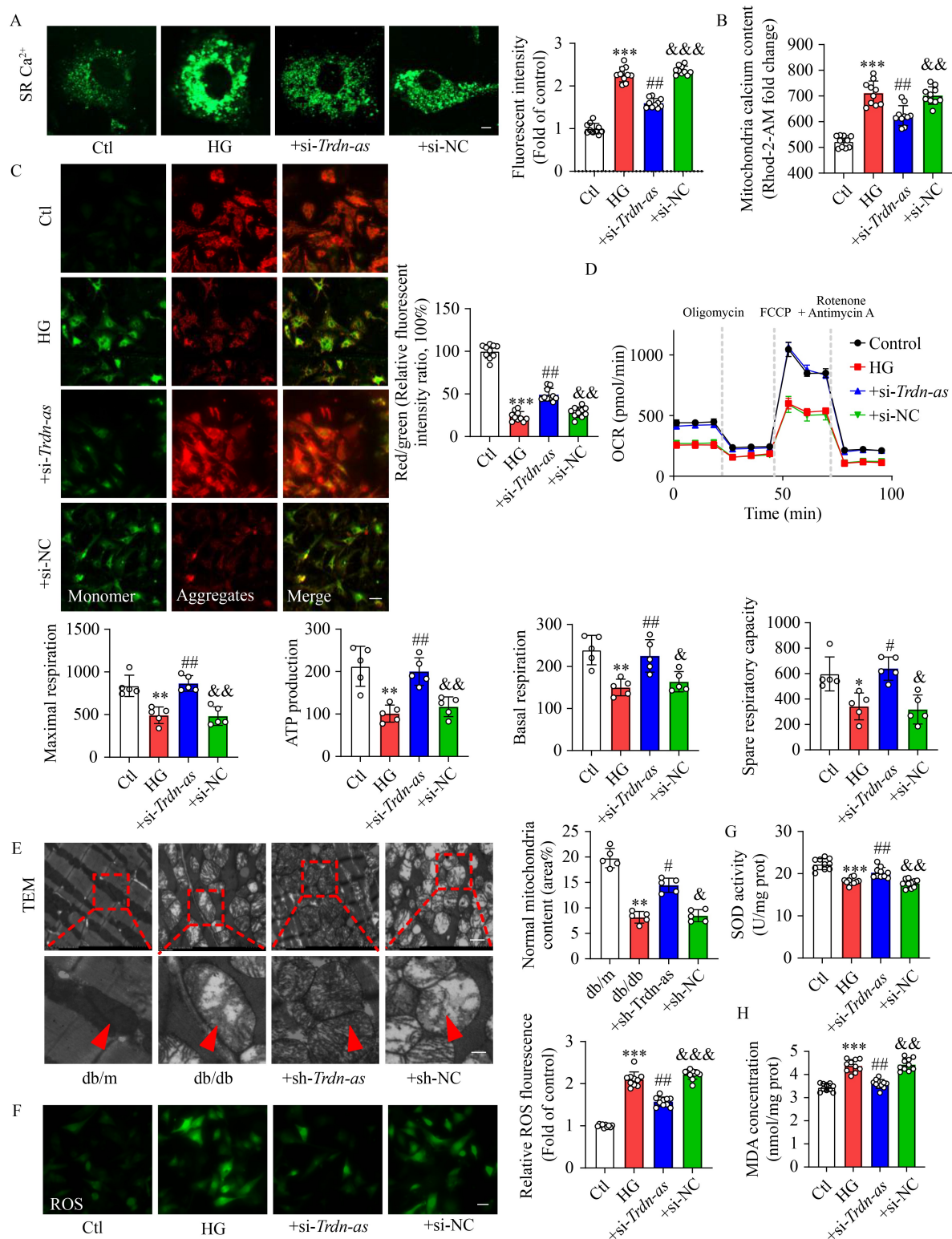


Fig. 4 *Trdn-as* induces mitochondria dysfunction in primary myocardial cells. (A) Image of SR Ca^{2+} fluorescence in NCMs. Scale bar: 10 μm . $n = 10$. (B) Relative mitochondria Ca^{2+} level in NCMs. $n = 10$. (C) Relative fluorescent intensity ratio of red/green for the JC-1 staining of NCMs. Scale bar: 20 μm . $n = 10$. (D) Oxygen consumption rate of myocardial cells under HG condition and si-*Trdn-as* treatment. $n = 5$. (E) Mitochondria detected by transmission electron microscopy. The red arrow indicates mitochondria. Scale bar: 2 and 1 μm . $n = 5$. ** P < 0.01 vs. db/m, # P < 0.05 vs. db/db, & P < 0.05 vs. sh-*Trdn-as*. (F) Reactive oxygen species (ROS) production in NCMs. Scale bar: 20 μm . $n = 10$. (G, H) Relative superoxide dismutase (SOD) activity and malonaldehyde (MDA) concentration in NCMs. $n = 10$. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. Ctl, # P < 0.05, ## P < 0.01 vs. HG, & P < 0.05, && P < 0.01, &&& P < 0.001 vs. si-*Trdn-as*. Data were expressed by mean $\pm$ SEM.

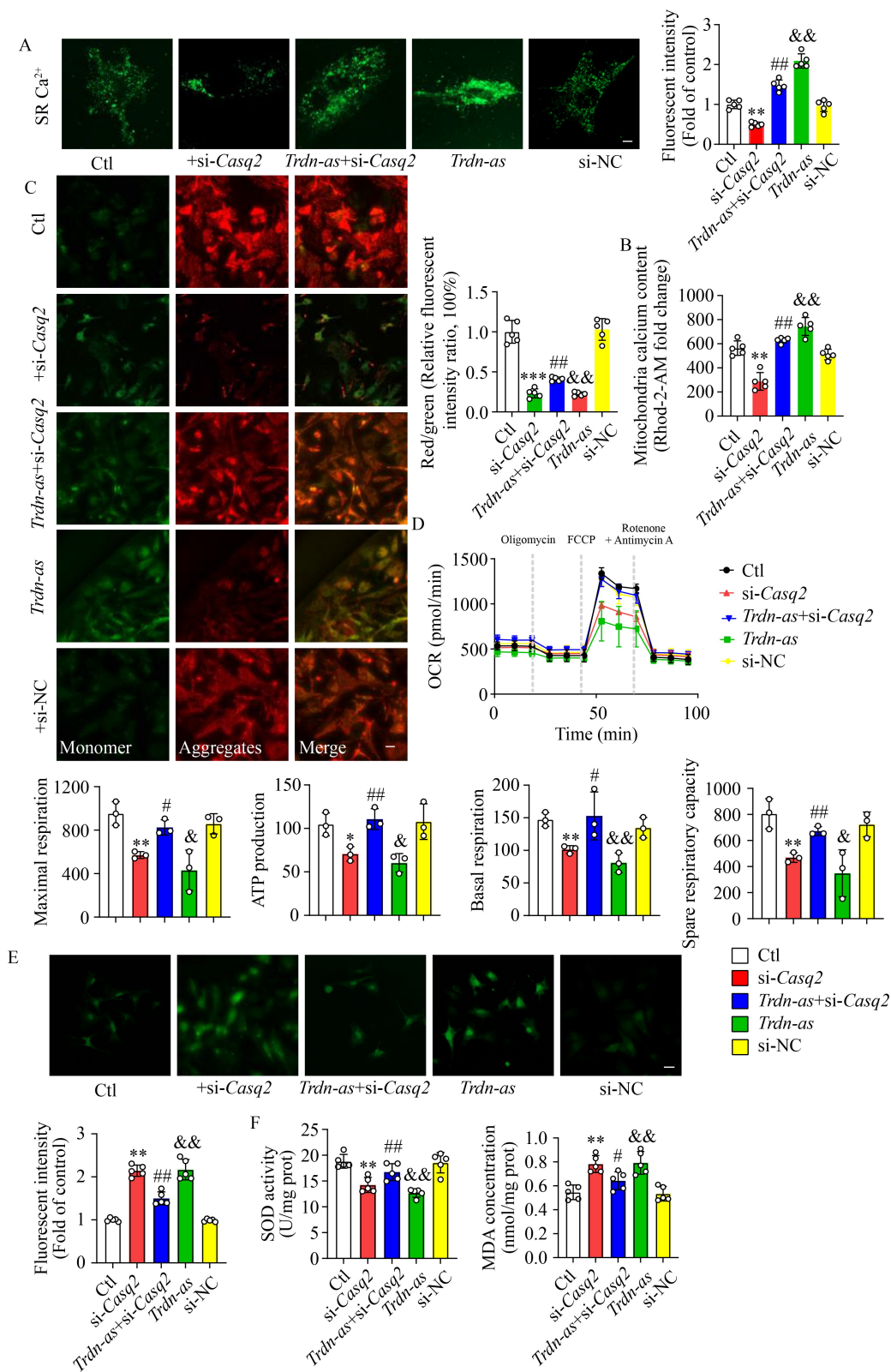


Fig. 5 *Casq2* knockdown improves mitochondrial dysfunction induced by *Trdn-as* overexpression. (A) Image of SR Ca^{2+} fluorescence in NCMs. Scale bar: 10 μm . $n = 5$. (B) Relative mitochondria Ca^{2+} level in NCMs. $n = 5$. (C) Fluorescence of JC-1 staining. Scale bar: 20 μm . $n = 5$. (D) Oxygen consumption rates of myocardial cells. $n = 3$. (E) Representative images of ROS staining. Scale bar: 20 μm . $n = 5$. (F) Relative SOD activity and MDA concentration in NCMs. $n = 5$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Ctl, # $P < 0.05$, ## $P < 0.01$ vs. *Trdn-as*, & $P < 0.05$, && $P < 0.01$ vs. Ctl. Data were expressed by mean $\pm$ SEM.

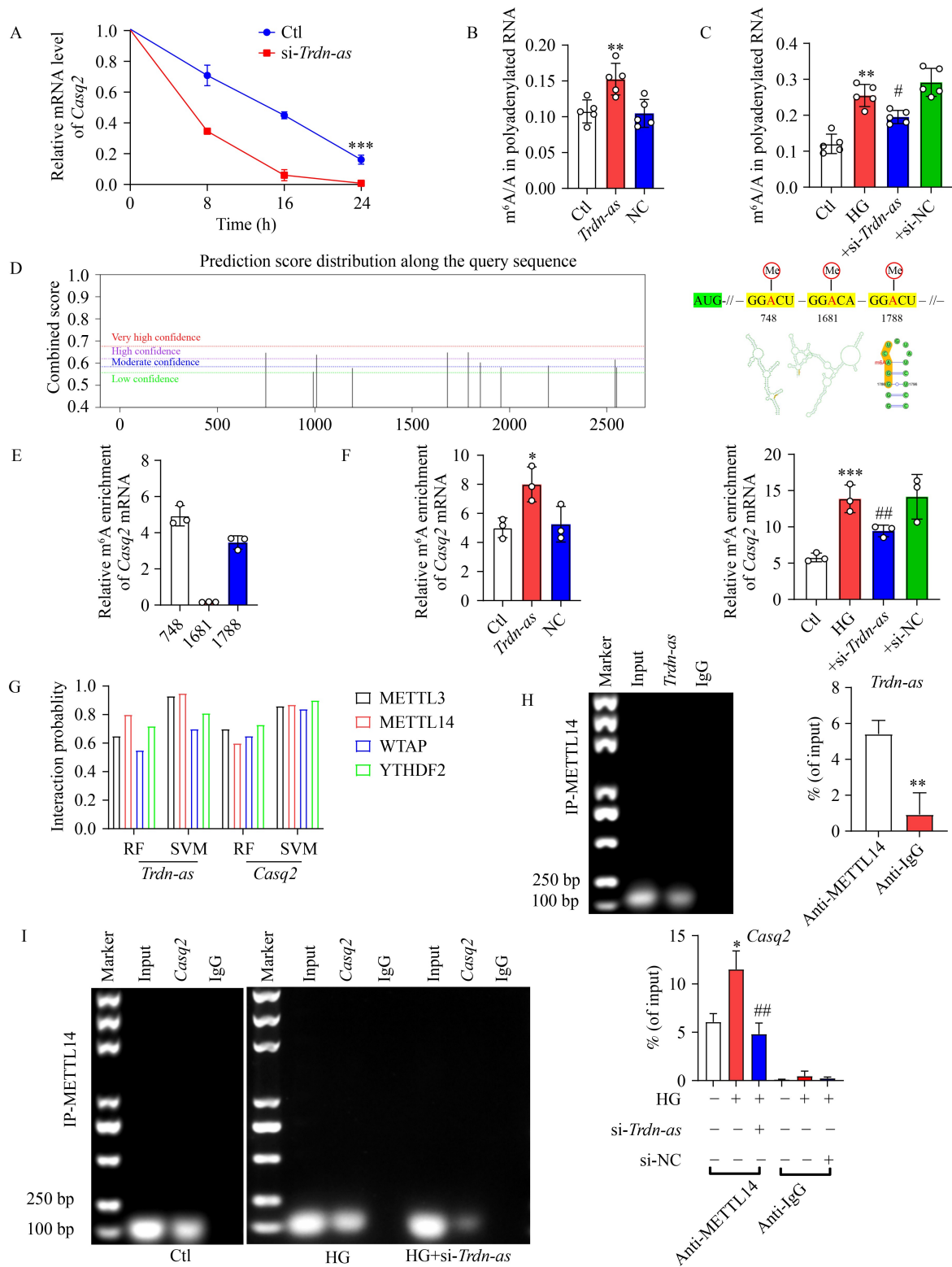


Fig. 6 *Trdn-as* elevated *Casq2* mRNA expression levels dependent on METTL14-mediated m^6A modification. (A) RNA stability analysis of *Casq2* after treatment with si-*Trdn-as* for 0, 8, 16, and 24 h. *n* = 5. (B, C) Methylation level of primary myocardial cells. *n* = 5. (D) SRAMP was utilized to predict results along the *Casq2* mRNA sequence. (E) MeRIP detection of effective methylation sites of *Casq2*. (F) MeRIP experiment was used to detect the modification level of the *Casq2* methylation site. (G) RPIseq results predicted the interaction probability. RF, random forest; SVM, support vector machine. (H) RNA binding protein immunoprecipitation (RIP) analysis for *Trdn-as*-METTL14 interaction. ** $P < 0.01$ vs. anti-METTL14. (I) Under high glucose condition, lncRNA-*Trdn-as* knockdown weakens the binding between *Casq2* mRNA and METTL14. *n* = 3. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Ctl, # $P < 0.05$, ## $P < 0.01$ vs. HG. Data were expressed by mean $\pm$ SEM.

observed, and *Trdn-as* knockdown attenuated this interaction (Fig. 6I). These findings suggested that *Trdn-as* can regulate the m⁶A modification of *Casq2* mediated by METTL14, thereby influencing the mRNA stability of *Casq2* in a glucose-dependent manner.

Discussion

This investigation elucidates the pathophysiological role of *Trdn-as* and its potential mechanism in DCM. Our findings indicate a significant increase in *Trdn-as* levels in the myocardium of db/db mice and cardiomyocytes treated with high glucose. *Trdn-as* knockdown prevents cardiac dysfunction and mitochondrial damage in db/db mice, and its overexpression has the opposite effects. From a mechanistic perspective, *Trdn-as* influences the mRNA methylation of *Casq2* mediated by METTL14, resulting in increased *Casq2* protein expression in myocardial cells and an imbalance in Ca²⁺ homeostasis in the SR and mitochondria. These findings suggest the crucial role for *Trdn-as* in developing cardiac dysfunction.

Similar to most lncRNAs, *Trdn-as* exhibits limited conservation across species, with a homology of approximately 45% between humans and mice. Nevertheless, the expression patterns of *Trdn-as* in cardiomyocytes exhibit remarkable consistency across various species. Aligning with our results, Zhao *et al.* [32] suggested that *Trdn-as* is specifically expressed in the cardiomyocytes of human and mouse hearts. *Trdn-as* downregulation has been observed in individuals with arrhythmia and heart failure, suggesting its diverse regulatory effects in the progression of cardiovascular diseases [33]. Our results revealed that *Trdn-as* overexpression resulted in cardiac dysfunction similar to that observed in db/db mice, and its knockdown improved cardiac dysfunction in db/db mice. Meanwhile, modifications in *Trdn-as* expression had no impact on the blood glucose levels in mice (the data were not shown), suggesting that the beneficial effects of *Trdn-as* arise from its direct regulation of cardiomyocytes, rather than its influence on blood glucose. We also observed the detrimental effect of *Trdn-as* on the myocardial structure in db/db mice. Cardiac remodeling of db/db mice was also influenced by *Trdn-as*. Given the relevance of fibroblasts and endothelial cells in cardiac remodeling, the potential impact of AAV-*Trdn-as* or AAV-*Trdn-as* shRNA on these cellular components must be examined in future studies.

SR Ca²⁺ in cardiomyocytes is responsible for regulating protein synthesis, cell contraction, and relaxation and participating in cell signaling. The Ca²⁺ level must be carefully controlled to ensure normal cell function, which is achieved through a delicate balance of introducing and releasing Ca²⁺ across the SR membrane [34]. SERCA

pumps Ca²⁺ from cytoplasm ([Ca²⁺] 100 nM) into SR ([Ca²⁺] 100 μM to 1 mM) to counteract Ca²⁺ concentration [35]. At the same time, SR Ca²⁺ is passively released into the cytoplasm to maintain a stable state and prevent an overload of SR Ca²⁺. Excessive Ca²⁺ accumulation in the SR triggers SR stress and results in a subsequent increase in Ca²⁺ levels in the mitochondria, a known factor in causing cell death [36–39]. In this study, RNA-seq and bioinformatics analyses revealed the impact of *Trdn-as* on intracellular Ca²⁺ signaling. Meanwhile, the Ca²⁺ levels within the SR and mitochondria were elevated following *Trdn-as* overexpression.

Ca²⁺ is transferred to the mitochondria through the IP3Rs-VDAC1 pathway, but other factors that may influence Ca²⁺ transfer must also be considered [40,41]. As the primary Ca²⁺ binding protein within the jSR of skeletal and cardiac muscles, *Casq2* assumes a pivotal role in the storage and regulation of intracellular Ca²⁺ within the Ca²⁺ release unit, which is composed of the ryanodine receptor, histidine-rich Ca²⁺ binding protein, triadin, and junction [18]. Approximately 75% of the releasable Ca²⁺ within the SR is bound to *Casq2* in muscle cells [42]. IP3Rs are predominantly localized at the intercalated discs of the myocardium, and RYR2 and *Casq2* are abundantly found in the jSR, which is close to the mitochondria and has been implicated in the transport of Ca²⁺ to the mitochondria [43]. However, the factors mediating SR-mitochondrial Ca²⁺ transport within jSR are poorly understood. In this study, we observed the significant upregulation of *Casq2* mRNA and protein levels in the heart tissues of *TRDN-as*-overexpressing mice. Knocking down *Casq2* markedly attenuated the mitochondrial Ca²⁺ overload and dysfunction induced by *Trdn-as* overexpression in cardiomyocytes. These findings suggested that *Casq2* plays a pivotal role in the regulation of mitochondrial Ca²⁺ overload by *Trdn-as*. Moreover, we found that knocking down *Casq2* in cardiomyocytes reduced SR Ca²⁺ levels, consistent with the findings reported by Knollmann *et al.* [44]. Our study also demonstrated the potential influence of *Casq2* on mitochondrial Ca²⁺ levels, leading to a notable decrease in MMP, impairments in mitochondrial respiratory function and energy production, and increased ROS production. Overall, this work provided additional compelling evidence to support the role of *Casq2* as the paramount Ca²⁺ storage protein in cardiac SR. We further elucidated its crucial involvement in regulating mitochondrial Ca²⁺ levels.

TRDN, the gene responsible for encoding *triadin*, is a downstream target of *Trdn-as*. However, RNA-seq analysis did not reveal any significant changes in *TRDN* expression following *Trdn-as* overexpression. In addition, the GEO database GSE62203 supported the lack of significant alterations in *TRDN* levels with DCM (Fig. S6A). These pieces of evidence suggested that

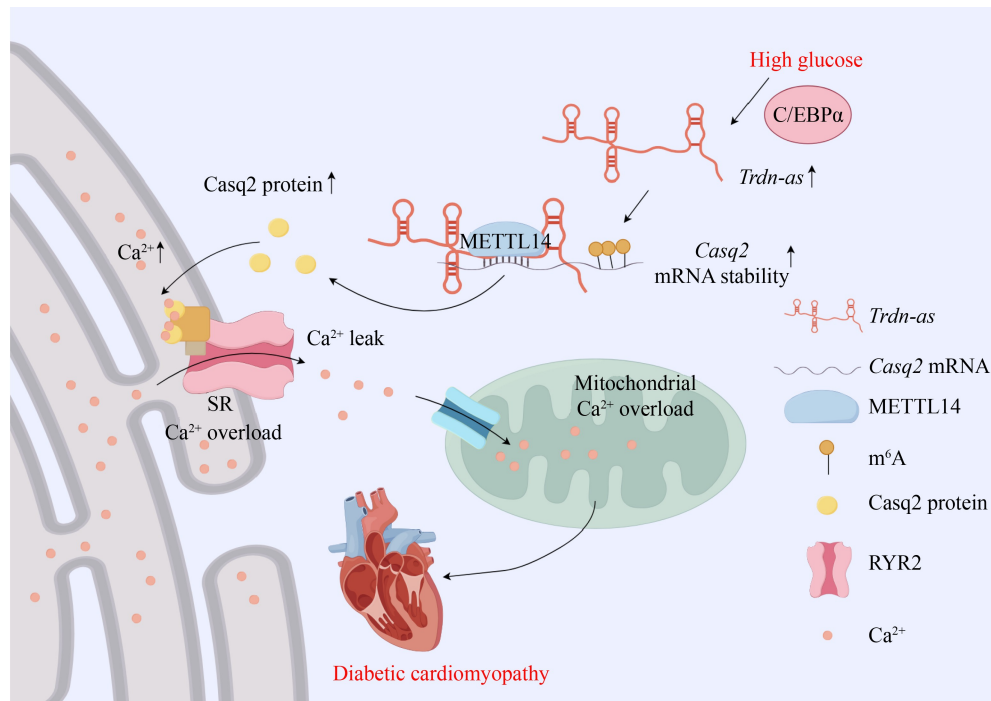


Fig. 7 At the onset of diabetic cardiomyopathy, *Trdn-as* expression increases, which regulates the methylation of *Casq2* mRNA mediated by METTL14, thus increasing the stability and expression of *Casq2* mRNA. This phenomenon leads to Ca²⁺ overload in the sarcoplasmic reticulum of myocardial cells, induce mitochondrial dysfunction and myocardial cell damage, and lead to diabetic cardiomyopathy. Figure 7 was created using Figdraw (figdraw.com).

TRDN may not be a potential therapeutic target of *Trdn-as* in DCM. Finally, we further explored the regulatory mechanisms of *Trdn-as*. Transcription factors that potentially bind to the promoter region of *Trdn-as* were predicted by PROMO. We selected some transcription factors related to metabolic diseases. The results showed that the expression of transcription factor C/EBPα increased under high glucose condition (Fig. S6B), leading to an elevated *Trdn-as* expression upon C/EBPα overexpression (Fig. S6C). Therefore, the upregulation of C/EBPα could potentially contribute to *Trdn-as* upregulation in DCM.

We also discovered that *Trdn-as* influenced the *Casq2* mRNA level, leading us to investigate its direct regulatory mechanism on *Casq2* mRNA. m⁶A is the most prevalent RNA modification and can regulate mRNA stability [45]. Our results indicated that the knocking down *Trdn-as* significantly reduced the stability of *Casq2* mRNA and the level of m⁶A, and overexpressing *Trdn-as* had the opposite effect. *Trdn-as* overexpression increased the m⁶A level of *Casq2* mRNA. Other studies also discovered the regulatory effects of lncRNAs on mRNA m⁶A modification. Zhou *et al.* [46,47] revealed that lncRNA STEAP3-AS1 competed with YTHDF2, leading to the upregulation of mRNA stability in STEAP3 and subsequently an increase in STEAP3 protein expression. A separate investigation highlighted the role of lncRNA

LINC02253 in promoting the m⁶A modification of KRT18 mRNA. This process stabilizes KRT18 mRNA by recruiting the m⁶A writer METTL3, ultimately exerting a favorable regulatory influence on the proliferation and spread of gastric cancer [48]. In our study, predictions from bioinformatics-related websites and experimental validation suggested that METTL14 may be partially involved in the regulation of *Casq2* methylation. RIP experiments confirmed that METTL14 binds to *Casq2* mRNA and *Trdn-as*. However, its expression level was not affected by *Trdn-as*, indicating that *Trdn-as* may function as a “molecular adhesive tape” to stabilize the METTL14–*Casq2* mRNA complex. Moreover, METTL14 is prone to interact with lncRNA in specific circumstances. Wang *et al.* [49] identified a series of RGG/RG motifs in the C-terminal intrinsically disordered region (IDR) of METTL14, which are recognized as the second most common RNA binding domains in the human genome. The arginine methylation in this C-terminal IDR enhanced METTL14–RNA interaction and amplified the methyltransferase activity of METTL3/METTL14 [49]. The lncRNA UCA1 has been identified as a positive regulator of keratinocyte-driven inflammation and psoriasis. UCA1 exerts its regulatory function by binding to METTL14 and activating the HIF-1α and NF-κB signaling pathways [50]. Furthermore, the LNC942 molecule directly recruits the METTL14

protein, leading to the stabilization of downstream targets such as CXCR4 and CYP1B1 through m⁶A methylation modification *in vitro* and *in vivo*, thereby facilitating the proliferation of breast cancer cells [51]. Whether other m⁶A-related proteins mediate the regulatory effects of *Trdn-as* on *Casq2* mRNA must be further investigated. Our study into *Casq2* is currently limited to cell experiments. Further research is necessary to validate the role of *Casq2* in DCM through animal experiments to provide valuable insights.

Previous studies have highlighted the RNA regulatory functions of this lncRNA. Zhang *et al.* [33] proposed a theoretical model suggesting that *Trdn-as* hinders the transcription of the skeletal muscle triadin by causing RNA polymerase II collision. Zhao *et al.* [32] indicated that *Trdn-as* is involved in mediating the alternative splicing of the triadin gene, thereby specifically controlling the levels of cardiac isoforms of triadin in cases of cardiac arrhythmias and heart failure. Although our data strongly suggested that *Trdn-as* regulates the m⁶A level of *Casq2* mRNA, we cannot completely dismiss the possibility that it also regulates *Casq2* expression in the heart through the previously proposed mechanism. Therefore, the role of *Trdn-as* in the regulation of transcription and alternative splicing of *Casq2* must be explored in future studies. Our findings proposed that *Trdn-as* regulates the m⁶A modification of *Casq2* mRNA to maintain cardiac function, contributing to the understanding of the RNA modulation effects of *Trdn-as*.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Nos. 82273919, 82270396, and U21A20339), the HMU Marshal Initiative Funding (No. HMUMIF-21022), and the Science Foundation for the Excellent Youth Scholars of Heilongjiang Province (No. JJ2023YX0509).

Compliance with ethics guidelines

Conflicts of interest Xiaohan Li, Ling Liu, Han Lou, Xinxin Dong, Shengxin Hao, Zeqi Sun, Zijia Dou, Huimin Li, Wenjie Zhao, Xiuxiu Sun, and Xin Liu declare no competing interests. Baofeng Yang is one of Honorary Editors-in-Chief of *Frontiers of Medicine*. Yong Zhang is a member of the Editorial Board of *Frontiers of Medicine*. Baofeng Yang and Yong Zhang were excluded from the peer-review process and all editorial decisions related to the acceptance and publication of this article. Peer-review was handled independently by the other editors to minimise bias.

The use of animals has been approved by the Ethics Committee of Harbin Medical University (No. IRB3052724) and the protocols conformed to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH

Publication No.85-23, revised in 1996).

Electronic Supplementary Material Supplementary material is available in the online version of this article at <https://doi.org/10.1007/s11684-024-1102-6> and is accessible to authorized users.

References

- Magliano DJ, Chen L, Islam RM, Carstensen B, Gregg EW, Pavkov ME, Andes LJ, Balicer R, Baviera M, Boersma-van Dam E, Booth GL, Chan JCN, Chua YX, Fosse-Edorh S, Fuentes S, Gulseth HL, Gurevicius R, Ha KH, Hird TR, Jermendy G, Khalangot MD, Kim DJ, Kiss Z, Kravchenko VI, Leventer-Roberts M, Lin CY, Luk AOY, Mata-Cases M, Mauricio D, Nichols GA, Nielen MM, Pang D, Paul SK, Pelletier C, Pildava S, Porath A, Read SH, Roncaglioni MC, Lopez-Doriga Ruiz P, Shestakova M, Vikulova O, Wang KL, Wild SH, Yekutieli N, Shaw JE. Trends in the incidence of diagnosed diabetes: a multicountry analysis of aggregate data from 22 million diagnoses in high-income and middle-income settings. *Lancet Diabetes Endocrinol* 2021; 9(4): 203–211
- Rooney MR, Fang M, Ogurtsova K, Ozkan B, Echouffo-Tcheugui JB, Boyko EJ, Magliano DJ, Selvin E. Global prevalence of prediabetes. *Diabetes Care* 2023; 46(7): 1388–1394
- Jin L, Geng L, Ying L, Shu L, Ye K, Yang R, Liu Y, Wang Y, Cai Y, Jiang X, Wang Q, Yan X, Liao B, Liu J, Duan F, Sweeney G, Woo CWH, Wang Y, Xia Z, Lian Q, Xu A. FGF21-Sirtuin 3 axis confers the protective effects of exercise against diabetic cardiomyopathy by governing mitochondrial integrity. *Circulation* 2022; 146(20): 1537–1557
- Lee CS, Zhai Y, Rodrigues B. Changes in lipoprotein lipase in the heart following diabetes onset. *Engineering (Beijing)* 2023; 20: 19–25
- Dia M, Gomez L, Thibault H, Tessier N, Leon C, Chouabe C, Ducreux S, Gallo-Bona N, Tubbs E, Bendridi N, Chanon S, Leray A, Belmudes L, Couté Y, Kurdi M, Ovize M, Rieusset J, Paillard M. Reduced reticulum-mitochondria Ca²⁺ transfer is an early and reversible trigger of mitochondrial dysfunctions in diabetic cardiomyopathy. *Basic Res Cardiol* 2020; 115(6): 74
- Yuan M, Gong M, He J, Xie B, Zhang Z, Meng L, Tse G, Zhao Y, Bao Q, Zhang Y, Yuan M, Liu X, Luo C, Wang F, Li G, Liu T. IP3R1/GRP75/VDAC1 complex mediates endoplasmic reticulum stress-mitochondrial oxidative stress in diabetic atrial remodeling. *Redox Biol* 2022; 52: 102289
- Zhang X, Chen X, Qian F, Zhu Y, He G, Yang J, Wu X, Zhang H, Yu X, Liu X. Deubiquitinase USP19 modulates apoptotic calcium release and endoplasmic reticulum stress by deubiquitinating BAG6 in triple negative breast cancer. *Clin Transl Med* 2023; 13(9): e1398
- Bootman MD, Bultynck G. Fundamentals of cellular calcium signaling: a primer. *Cold Spring Harb Perspect Biol* 2020; 12(1): a038802
- Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 2000; 1(1): 11–21
- Li J, Liu C, Li Y, Zheng Q, Xu Y, Liu B, Sun W, Li Y, Ji S, Liu

- M, Zhang J, Zhao D, Du R, Liu Z, Zhong G, Sun C, Wang Y, Song J, Zhang S, Qin J, Ling S, Wang X, Li Y. TMCO1-mediated Ca^{2+} leak underlies osteoblast functions via CaMKII signaling. *Nat Commun* 2019; 10(1): 1589
11. Stutzmann GE, Mattson MP. Endoplasmic reticulum Ca^{2+} handling in excitable cells in health and disease. *Pharmacol Rev* 2011; 63(3): 700–727
12. Hamilton S, Terentyeva R, Martin B, Perger F, Li J, Stepanov A, Bonilla IM, Knollmann BC, Radwański PB, Györke S, Belevych AE, Terentev D. Increased RyR2 activity is exacerbated by calcium leak-induced mitochondrial ROS. *Basic Res Cardiol* 2020; 115(4): 38
13. Jaquenod De Giusti C, Palomeque J, Mattiazzi A. Ca^{2+} mishandling and mitochondrial dysfunction: a converging road to prediabetic and diabetic cardiomyopathy. *Pflugers Arch* 2022; 474(1): 33–61
14. Rossi D, Gamberucci A, Pierantozzi E, Amato C, Migliore L, Sorrentino V. Calsequestrin, a key protein in striated muscle health and disease. *J Muscle Res Cell Motil* 2021; 42(2): 267–279
15. Viatchenko-Karpinski S, Terentev D, Györke I, Terentyeva R, Volpe P, Priori SG, Napolitano C, Nori A, Williams SC, Györke S. Abnormal calcium signaling and sudden cardiac death associated with mutation of calsequestrin. *Circ Res* 2004; 94(4): 471–477
16. Postma AV, Denjoy I, Hoorntje TM, Lupoglazoff JM, Da Costa A, Sebillon P, Mannens MM, Wilde AA, Guicheney P. Absence of calsequestrin 2 causes severe forms of catecholaminergic polymorphic ventricular tachycardia. *Circ Res* 2002; 91(8): e21–e26
17. Jones LR, Suzuki YJ, Wang W, Kobayashi YM, Ramesh V, Franzini-Armstrong C, Cleemann L, Morad M. Regulation of Ca^{2+} signaling in transgenic mouse cardiac myocytes overexpressing calsequestrin. *J Clin Invest* 1998; 101(7): 1385–1393
18. Shannon TR. Linking calsequestrin to lumenal control of SR Ca^{2+} release. *Circ Res* 2007; 101(6): 539–541
19. Kohlhaas M, Maack C. Calcium release microdomains and mitochondria. *Cardiovasc Res* 2013; 98(2): 259–268
20. Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, Tanzer A, Lagarde J, Lin W, Schlesinger F, Xue C, Marinov GK, Khatun J, Williams BA, Zaleski C, Rozowsky J, Röder M, Kokocinski F, Abdelhamid RF, Alioto T, Antoshechkin I, Baer MT, Bar NS, Batut P, Bell K, Bell I, Chakraborty S, Chen X, Chrast J, Curado J, Derrien T, Drenkow J, Dumais E, Dumais J, Duttagupta R, Falconnet E, Fastuca M, Fejes-Toth K, Ferreira P, Foissac S, Fullwood MJ, Gao H, Gonzalez D, Gordon A, Gunawardena H, Howald C, Jha S, Johnson R, Kapranov P, King B, Kingswood C, Luo OJ, Park E, Persaud K, Preall JB, Ribeca P, Risk B, Robyr D, Sammeth M, Schaffer L, See LH, Shahab A, Skancke J, Suzuki AM, Takahashi H, Tilgner H, Trout D, Walters N, Wang H, Wrobel J, Yu Y, Ruan X, Hayashizaki Y, Harrow J, Gerstein M, Hubbard T, Reymond A, Antonarakis SE, Hannon G, Giddings MC, Ruan Y, Wold B, Carninci P, Guigó R, Gingeras TR. Landscape of transcription in human cells. *Nature* 2012; 489(7414): 101–108
21. Bridges MC, Daulagala AC, Kourtidis A. LNCcation: lncRNA localization and function. *J Cell Biol* 2021; 220(2): e202009045
22. Zhang Z, Salisbury D, Sallam T. Long noncoding RNAs in atherosclerosis: JACC review topic of the week. *J Am Coll Cardiol* 2018; 72(19): 2380–2390
23. Li QW, Lei W, Chen C, Guo W. Recent advances of long noncoding RNAs involved in the development of multiple sclerosis. *Chin J Nat Med* 2020; 18(1): 36–46
24. Liu L, Hu L, Long H, Zheng M, Hu Z, He Y, Gao X, Du P, Zhao H, Yu D, Lu Q, Zhao M. lncRNA IL21-AS1 interacts with hnRNPU protein to promote IL21 overexpression and aberrant differentiation of Tfh cells in systemic lupus erythematosus. *Clin Transl Med* 2022; 12(12): e1117
25. Zhang Y, Sun L, Xuan L, Pan Z, Hu X, Liu H, Bai Y, Jiao L, Li Z, Cui L, Wang X, Wang S, Yu T, Feng B, Guo Y, Liu Z, Meng W, Ren H, Zhu J, Zhao X, Yang C, Zhang Y, Xu C, Wang Z, Lu Y, Shan H, Yang B. Long non-coding RNA CCRR controls cardiac conduction via regulating intercellular coupling. *Nat Commun* 2018; 9(1): 4176
26. Zhang Y, Sun L, Xuan L, Pan Z, Li K, Liu S, Huang Y, Zhao X, Huang L, Wang Z, Hou Y, Li J, Tian Y, Yu J, Han H, Liu Y, Gao F, Zhang Y, Wang S, Du Z, Lu Y, Yang B. Reciprocal changes of circulating long non-coding RNAs ZFAS1 and CDR1AS predict acute myocardial infarction. *Sci Rep* 2016; 6(1): 22384
27. Liu X, Bai X, Liu H, Hong Y, Cui H, Wang L, Xu W, Zhao L, Li X, Li H, Li X, Chen H, Meng Z, Lou H, Xu H, Lin Y, Du Z, Kopylov P, Yang B, Zhang Y. lncRNA LOC105378097 inhibits cardiac mitophagy in natural ageing mice. *Clin Transl Med* 2022; 12(6): e908
28. Zhang Y, Jiao L, Sun L, Li Y, Gao Y, Xu C, Shao Y, Li M, Li C, Lu Y, Pan Z, Xuan L, Zhang Y, Li Q, Yang R, Zhuang Y, Zhang Y, Yang B. lncRNA ZFAS1 as a SERCA2a inhibitor to cause intracellular Ca^{2+} overload and contractile dysfunction in a mouse model of myocardial infarction. *Circ Res* 2018; 122(10): 1354–1368
29. Li HM, Liu X, Meng ZY, Wang L, Zhao LM, Chen H, Wang ZX, Cui H, Tang XQ, Li XH, Han WN, Bai X, Lin Y, Liu H, Zhang Y, Yang BF. Kanglexin delays heart aging by promoting mitophagy. *Acta Pharmacol Sin* 2022; 43(3): 613–623
30. Fan G, Zhou F, Feng C, Wu F, Ye W, Wang C, Lin F, Yan J, Li Y, Chen Y, Bi Y. Lead-induced ER calcium release and inhibitory effects of methionine choline in cultured rat hippocampal neurons. *Toxicol In Vitro* 2013; 27(1): 387–395
31. Smolina N, Bruton J, Sjöberg G, Kostareva A, Sejersen T. Aggregate-prone desmin mutations impair mitochondrial calcium uptake in primary myotubes. *Cell Calcium* 2014; 56(4): 269–275
32. Zhao Y, Riching AS, Knight WE, Chi C, Broadwell LJ, Du Y, Abdel-Hafiz M, Ambardekar AV, Irwin DC, Proenza C, Xu H, Leinwand LA, Walker LA, Woulfe KC, Bristow MR, Buttrick PM, Song K. Cardiomyocyte-specific long noncoding RNA regulates alternative splicing of the triadin gene in the heart. *Circulation* 2022; 146(9): 699–714
33. Zhang L, Salgado-Somoza A, Vausort M, Leszek P, Devaux Y. A heart-enriched antisense long non-coding RNA regulates the balance between cardiac and skeletal muscle triadin. *Biochim Biophys Acta Mol Cell Res* 2018; 1865(2): 247–258
34. Lomax RB, Camello C, Van Coppenolle F, Petersen OH, Tepikin AV. Basal and physiological Ca^{2+} leak from the endoplasmic reticulum of pancreatic acinar cells. Second messenger-activated channels and translocons. *J Biol Chem* 2002; 277(29): 26479–26485
35. Wuytack F, Raeymaekers L, Missiaen L. Molecular physiology of the SERCA and SPCA pumps. *Cell Calcium* 2002; 32(5–6):

- 279–305
36. Skobeleva K, Shalygin A, Mikhaylova E, Guzova I, Ryazantseva M, Kaznacheyeva E. The STIM1/2-regulated calcium homeostasis is impaired in hippocampal neurons of the 5xFAD mouse model of Alzheimer's disease. *Int J Mol Sci* 2022; 23(23): 14810
 37. Patil CS, Li H, Lavine NE, Shi R, Bodalia A, Siddiqui TJ, Jackson MF. ER-resident STIM1/2 couples Ca^{2+} entry by NMDA receptors to pannexin-1 activation. *Proc Natl Acad Sci USA* 2022; 119(36): e2112870119
 38. Giorgi C, Baldassari F, Bononi A, Bonora M, De Marchi E, Marchi S, Missiroli S, Patergnani S, Rimessi A, Suski JM, Wieckowski MR, Pinton P. Mitochondrial Ca^{2+} and apoptosis. *Cell Calcium* 2012; 52(1): 36–43
 39. Liu Y, Ma Y, Xu J, Zhang G, Zhao X, He Z, Wang L, Yin N, Peng M. VMP1 prevents Ca^{2+} overload in endoplasmic reticulum and maintains naive T cell survival. *J Exp Med* 2023; 220(6): e20221068
 40. Rieusset J, Fauconnier J, Paillard M, Belaidi E, Tubbs E, Chauvin MA, Durand A, Bravard A, Teixeira G, Bartosch B, Michelet M, Theurey P, Vial G, Demion M, Blond E, Zoulif F, Gomez L, Vidal H, Lacampagne A, Ovize M. Disruption of calcium transfer from ER to mitochondria links alterations of mitochondria-associated ER membrane integrity to hepatic insulin resistance. *Diabetologia* 2016; 59(3): 614–623
 41. Wu S, Lu Q, Ding Y, Wu Y, Qiu Y, Wang P, Mao X, Huang K, Xie Z, Zou MH. Hyperglycemia-driven inhibition of AMP-activated protein kinase $\alpha 2$ induces diabetic cardiomyopathy by promoting mitochondria-associated endoplasmic reticulum membranes *in vivo*. *Circulation* 2019; 139(16): 1913–1936
 42. Xu H, Zhao M, Liang S, Huang Q, Xiao Y, Ye L, Wang Q, He L, Ma L, Zhang H, Zhang L, Jiang H, Ke X, Gu Y. The effects of Puerarin on rat ventricular myocytes and the potential mechanism. *Sci Rep* 2016; 6(1): 35475
 43. Rossini M, Filadi R. Sarcoplasmic reticulum-mitochondria kissing in cardiomyocytes: Ca^{2+} , ATP, and undisclosed secrets. *Front Cell Dev Biol* 2020; 8: 532
 44. Knollmann BC, Chopra N, Hlaing T, Akin B, Yang T, Ettensohn K, Knollmann BE, Horton KD, Weissman NJ, Holinstat I, Zhang W, Roden DM, Jones LR, Franzini-Armstrong C, Pfeifer K. *Casq2* deletion causes sarcoplasmic reticulum volume increase, premature Ca^{2+} release, and catecholaminergic polymorphic ventricular tachycardia. *J Clin Invest* 2006; 116(9): 2510–2520
 45. Wang J, Zha L. RNA modification by m^6A methylation in cardiovascular diseases: current trends and future directions. *Frigid Zone Med* 2022; 2(3): 158
 46. Wu G, Su J, Zeng L, Deng S, Huang X, Ye Y, Li R, Bai R, Zhuang L, Li M, Zhou Q, Zheng Y, Deng J, Zhang S, Chen R, Lin D, Zhang J, Zheng J. LncRNA BCAN-AS1 stabilizes c-Myc via N^6 -methyladenosine-mediated binding with SNIP1 to promote pancreatic cancer. *Cell Death Differ* 2023; 30(10): 2213–2230
 47. Deng P, Zhang H, Wang L, Jie S, Zhao Q, Chen F, Yue Y, Wang H, Tian L, Xie J, Chen M, Luo Y, Yu Z, Pi H, Zhou Z. Long-term cadmium exposure impairs cognitive function by activating lnc-Gm10532/ m^6A /FIS1 axis-mediated mitochondrial fission and dysfunction. *Sci Total Environ* 2023; 858(Pt 3): 159950
 48. Gao Z, Long Y, Wu Y, Pu Y, Xue F. LncRNA LINC02253 activates KRT18/MAPK/ERK pathway by mediating N^6 -methyladenosine modification of KRT18 mRNA in gastric cancer. *Carcinogenesis* 2022; 43(5): 419–429
 49. Wang Z, Pan Z, Adhikari S, Harada BT, Shen L, Yuan W, Abeywardana T, Al-Hadid Q, Stark JM, He C, Lin L, Yang Y. m^6A deposition is regulated by PRMT1-mediated arginine methylation of METTL14 in its disordered C-terminal region. *EMBO J* 2021; 40(5): e106309
 50. Hu Y, Lei L, Jiang L, Zeng H, Zhang Y, Fu C, Guo H, Dong Y, Ouyang Y, Zhang X, Huang J, Zeng Q, Chen J. LncRNA UCA1 promotes keratinocyte-driven inflammation via suppressing METTL14 and activating the HIF-1 α /NF- κB axis in psoriasis. *Cell Death Dis* 2023; 14(4): 279
 51. Sun T, Wu Z, Wang X, Wang Y, Hu X, Qin W, Lu S, Xu D, Wu Y, Chen Q, Ding X, Guo H, Li Y, Wang Y, Fu B, Yao W, Wei M, Wu H. LNC942 promoting METTL14-mediated m^6A methylation in breast cancer cell proliferation and progression. *Oncogene* 2020; 39(31): 5358–5372