N6-methyladenosine Modification of CRLF1 Boosts Cardiac Fibroblasts Pyroptosis and Induces Fibrosis

Jian-Yuan Zhao et al

Data Supplementary

Methods

Ethics statement

The in vivo assay was approved by the Animal Care and Use Committee of Anhui Medical University. And all experimental procedures and animal care were in accordance with the institutional ethics guidelines for animal experiments.

Animals

GemPharmatech generated the CRLF1 loxP ($CRLF1^{flox/flox}$) and YTHDF1 loxP ($YTHDF1^{flox/flox}$) mouse. This mouse was crossed with the POSTN-creER mouse to create CRLF1 and YTHDF1 pCKO mice. Use a syringe to draw the required dose of the Tamoxifen solution (Beyotime, Shanghai). Administer 100 mg/kg of body weight per mouse. Inject the Tamoxifen solution intraperitoneally for 5 consecutive days. On the fifth day after the final injection, verify CreER activation using PCR to ensure its efficacy.

Establishment of Mouse model[2]

The C57BL/6J male and female mice, aged 8-10 weeks, were housed in a specific and opportunistic pathogen-free facility with a 12-hour light-dark cycle and ad libitum access to food and water. All mice, exception of the control group, were administered a 4-week subcutaneous injection of ISO at a dosage of 5 mg/kg/day. After 1 week, they were administered AAV9-NC, AAV9-POSTN-OECRLF1 (200μl/mouse) via tail vein injection. Following the 4-week treatment period, the mice were anesthetized with isoflurane and their hearts were dissected. Heart tissue samples were either frozen in liquid nitrogen, fixed in formalin, or frozen in OCT compound for histological analysis. Blood samples were collected in EDTA tubes for plasma isolation and stored at -80°C.

LPS/Nig induced sepsis model in mice[3]

In vivo experiments were conducted using mice aged 8 weeks. The mice were injected with either 25 mg/kg of Lipopolysaccharide (LPS) along with 5 mg/kg of the Nigericin (Nig) or PBS. After 4 hours, the mice were euthanized, and their hearts were collected and stored at -80°C. Blood samples were obtained to measure the levels of serum inflammatory factors using ELISA.

Echocardiography[4]

Before conducting a heart dissection, cardiac hemodynamics were assessed using an ultrasound machine known as VeVo2100. The mouse was anesthetized with 2% isoflurane and underwent transthoracic echocardiography to stabilize the heart rate at 450-500 beats per minute. Echocardiographic M-mode tracings were then recorded in the long-axis view, with measurements taken for parameters such as LVEF, LVFS, LVESD, and LVEDD using at least six cardiac cycles.

Histopathology[5]

Histopathology of mouse tissue was conducted at the Core Experimental Facility at The Second Hospital of Anhui Medical University. The tissue samples were processed, embedded in paraffin, and cut into 6 um sections. To identify collagen, picric acid Sirius red staining was performed, while fibrosis was detected using Masson's trichrome staining. Six heart samples were analyzed for each staining method.

Immunofluorescence[6]

Fixed hearts were dehydrated in 30% sucrose at 4°C for 12 hours before being embedded in Tissue-Tek O.C.T. medium and sliced into 6μm sections. The sections were treated with 0.3% Triton X-100 for permeabilization and 4% paraformaldehyde for fixation. Following blocking with fetal bovine serum, the samples were incubated with primary antibodies (Table S2) at a dilution of 1:300-1:500 overnight at 4°C. After washing with PBST, secondary antibodies (Table S2) were applied for 1.5 hours at room temperature in the dark. Nuclei were stained with DAPI, and confocal fluorescence microscopy was used for imaging. Image analysis was performed using the ImageJ software.

Data source and processing[7]

The RNA sequencing datasets GSE128188, GSE97358, GSE82294, GSE151466 were retrieved from the Gene Expression Omnibus (GEO) database at http://www.ncbi.nlm.nih.gov/geo/. The microarray dataset EMTAB-8810 was obtained from the Array Express database at http://www.ebi.ac.uk/arrayexpress.

Differential gene expression analysis was conducted using the Bioconductor package edgeR (Robinson et al., 2009) on primary read counts data via the Sangerboxonline tool (http://sangerbox.com). Genes were deemed significantly differentially expressed if the log2 fold-change was greater than 0.5 or less than -0.5 and the *p*-value was less than 0.05. Additionally, genes with a log2CPM<1 were excluded as low-expressed genes in the differential gene screening process.

Gene set enrichment analysis (GSEA)[8]

We utilized the GSEA software provided by the Broad Institute (version number, http://software.broadinstitute.org/gsea/). Firstly, we sorted genes based on their expression levels in order to identify genes that were significantly differentially expressed between different conditions or treatment groups. These genes were then used for enrichment analysis in the KEGG database to determine if there was a significant enrichment trend in biological pathways. Specifically, we focused on the extracellular matrix (ECM)-receptor interaction pathway. Using the GSEA software, we evaluated the enrichment of differentially expressed genes in this pathway. To determine if the enrichment was statistically significant, we set the threshold for normalized enrichment score (NES) > 1.2, false discovery rate (FDR) < 0.25, and nominal p-value < 0.05 as the significance criteria.

Single-cell RNA sequencing (scRNA-seq) analysis[9]

The single-cell sequencing data EMTAB-8810 was processed in R using the Seurat package (v4.1.1). Cells were filtered based on the criteria of having a number of RNA features between 1000 and 5000. Clusters were identified using the FindClusters and FindNeighbors functions with a resolution parameter of 0.8. Fibroblast cells were distinguished by the expression of fibroblast markers such as CRLF1, COLA1, and POSTN. The Nebulosa package (v1.6.0) was then used to analyze gene expression through weighted kernel density estimation with default settings using the plot_density function.

Primary cardiac fibroblasts isolation and culture

Primary cardiac fibroblasts were isolated from the hearts of 1-3 day old neonatal C57BL/6 mice using standard techniques. The hearts were carefully removed and washed with chilled PBS. The tissues were finely chopped and subjected to a thorough enzymatic digestion at 37° C for 30 to 60 minutes using 0.16% trypsin (Beyotime, Shanghai, China) and 0.67% type II collagenase (Biofrox). After digestion, the tissue mix was centrifuged at 1200 rpm for 5 minutes using a high-speed centrifuge. The collected pellet was then resuspended in Ham's F12k medium enriched with 5% FBS. The suspension was placed in a 37° C incubator with 5% CO2 for 2 hours, allowing primary cardiac fibroblasts to adhere, based on their selective adhesion properties. Cells that adhered within the initial 2 hours were identified as primary cardiac fibroblasts. The purity of the isolated fibroblasts was confirmed via Vimentin staining. These cells were then split at a 1:1 ratio for culture expansion, and cells from the second and third passages were used for further genetic and functional studies.

Plasmid construction, knockdown and transfection[10]

Plasmid-mediated overexpression models for CRLF1, DHHC7, GBP9, and ALKBH5 were obtained from GenePharma (Shanghai, China). Additionally, siRNAs targeting NC, CRLF1, DHHC7, GBP9, ALKBH5, and YTHDF1 were generated by GenePharma and used for individual cell transfections. Transfections were carried out using the jetPRIME® Versatile DNA/siRNA transfection reagent (Polyplus, France) following the manufacturer's instructions. Transfection efficiency was assessed by RT-qPCR and Western blot analysis. The sequences of the plasmids and siRNAs are listed in (Table S3).

Western blot analysis[11]

Protein extraction was performed using SDS lysis buffer (Beyotime, Shanghai) following the manufacturer's guidelines. Proteins were then quantified and equalized in concentration, followed by separation through SDS-PAGE (Beyotime, Shanghai). After separation, proteins were electro-transferred to PVDF membranes (Merck Millipore, Darmstadt, Germany). These membranes were blocked with 5% (w/v) non-fat milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 2 hours at room temperature. This step was followed by an overnight incubation at 4° C with primary antibodies diluted in the same blocking solution. The membranes were washed thrice with TBST and subsequently incubated with horseradish peroxidase (HRP)-linked secondary antibodies. The detection of bound antibodies was facilitated using enhanced chemiluminescence (ECL) substrate, visualizing the proteins of interest.

Quantitative real-time reverse transcription (RT-qPCR)[12]

Total RNA was extracted from both cells and tissues using the Trizol method (Invitrogen), in line with the manufacturer's protocol. The extracted RNA was then converted into cDNA using PrimeScript RT Master Mix (TaKaRa), according to the instructions provided. Quantitative real-time PCR (qPCR) was performed for all independent samples using SYBR Premix Ex Taq II (TaKaRa), with β-actin serving as the internal control for normalization. The relative expression levels of RNA were quantified using the 2^-ΔΔCt method. Primer details for this analysis are listed in (Table S3).

Cell pyroptosis[13]

The induction of pyroptosis in primary cardiac fibroblasts involves the following steps: Lipopolysaccharide (LPS) and Nigericin (Nig) are transfected into the cells, setting the final concentration of LPS at 5 μg/mL and Nig at 0.5 μg/mL. The cells are then incubated at 37° C in a 5% CO2 atmosphere for 24 hours. After this incubation period, the medium is changed to remove any residual LPS and Nig, and the cells are prepared for subsequent treatments.

Scanning Electron Microscopy (SEM)

Cells were carefully seeded onto 9mm glass slides and allowed to attach and grow. The slides were then fixed in a 2.5% glutaraldehyde solution at 4° C for 8 hours to preserve the cellular structure. Subsequently, the fixed cells on the glass slides were dehydrated using an ethanol gradient to remove excess water without compromising the sample integrity. Finally, the prepared samples were examined under a scanning electron microscope (GeminiSEM 300, Zeiss) to visualize the cellular morphology at high resolution. Images of the cells were captured to analyze any changes or effects induced by the treatments applied.

Caspase-1/PI activity assay[14]

After staining with the FAM-FLICA Caspase-1 Assay Kit, cells were incubated for 1 hour at 37° C in the dark. Following the incubation, cells were washed and resuspended in PBS. Flow cytometry analysis was then performed using the CytoFLEX Flow cytometer, with a minimum of 10,000 events collected for each sample. Data analysis was conducted using FlowJo software to quantify the percentage of cells undergoing pyroptosis in each experimental group. The results were expressed as the mean percentage of pyroptotic cells ± standard deviation. Statistical analysis was performed using a one-way ANOVA followed by post-hoc Tukey's test to compare differences between groups.

Co-immunoprecipitation assays[15]

CFs from different groups were lysed using an IP/CO-IP kit (Absin, China). The lysates were precleared and then immunoprecipitated with 3μg of specific antibodies overnight at 4 ℃, with IgG as a control. The immunocomplexes were then conjugated with protein A/G agarose for an additional 4h at 4 ℃. After washing the immunocomplexes three times with a washing buffer, the bound proteins were eluted by boiling. Finally, the proteins in each group were analyzed by immunoblotting.

m6A dot blot assay[16]

Polyadenylated mRNA was extracted from total RNA using the GenEluteTM mRNA Miniprep Kit. The isolated poly(A)+ RNA was then cross-linked to a Hybond-N+ membrane using UV light. The membrane was blocked with 5% nonfat milk, incubated with an m6A antibody overnight at 4° C, and detected using the ECL system after incubation with a horseradish peroxidase-conjugated anti-mouse IgG. Equal amounts of poly(A)+ RNA were also spotted on the membrane and stained with 0.02% methylene blue in 0.3M sodium acetate (pH=5.2). The density of m6A dots was quantified using Image J software and presented as the relative density normalized to the methylene blue staining.

mRNA stability (Actinomycin D treatment)[17]

Cells were exposed to Actinomycin D at a concentration of 1μg/mL in each well. Various time points (0, 2, 4, 6, 8, and 10 h) were assigned to different experimental groups, with corresponding negative controls established for each time point. Upon reaching the designated time points, cells were collected, and total RNA was extracted. Following the removal of genomic DNA, cDNA was generated through reverse transcription. Quantitative PCR (qPCR) was utilized to determine the levels of the target RNA and reference RNA. Subsequent data analysis was performed to ascertain the decay rate of the target RNA, which was then normalized to the 0 h time point.

MeRIP and MeRIP-qPCR[18]

RNA isolation was performed using Trizol reagent (Takara, Japan), followed by mRNA purification with the GenEluteTM mRNA Miniprep Kit (Sigma, Louis, MO). The RNA was randomly fragmented using RNA fragmentation reagents (NEB, Hertfordshire, UK). The m6A immunoprecipitation was carried out with the specific anti-m6A antibody (NEB, Hertfordshire, UK) pre-bound to Protein G magnetic beads. The fragmented mRNA was incubated with the antibody-bound beads at 4℃ for 1 hour, followed by washing with low salt and high salt buffers. The m6A-antibody-bound RNA was extracted from the Dynabeads using Buffer RLT (Qiagen, Hilden, German) and further processed with Dynabeads MyOne Silane (Life Technologies, West Palm Beach, FL). After precipitation with ethanol and resuspension in nuclease-free water, real-time PCR was conducted to analyze changes in m6A methylation of a specific target gene.

RNA-binding protein immunoprecipitation (RIP) assay[19]

The RIP assay was conducted using the EZ-Magna RIP RNA-binding protein immunoprecipitation kit (Merck Millipore, Germany) as per the manufacturer's instructions. Cells were lysed in RIP lysis buffer with protease and RNase inhibitors. Magnetic beads coated with Ago2 antibodies or IgG (Millipore) were added to the lysates and incubated overnight at 4 °C. After washing, proteinase K treatment was performed to remove proteins. RNA was extracted, followed by RT-qPCR analysis and agarose gel electrophoresis.

RNA pull-down assay[20]

The RNA sequences were biotin-labeled using the Biotin RNA Labeling Mix from Roche and purified with the GenEluteTM mRNA Miniprep Kit from Sigma. The labeled RNA (1 mg) was heated in RNA structure buffer containing Tris (pH = 7) at 10 mmol/L, KCl at 0.1 mol/L, and $MgCl_2$ at 10 mmol/L, reaching a temperature of 95℃. After 2 minutes, the RNA was cooled on ice for 3 minutes and left at room temperature for 30 minutes to form an appropriate secondary structure. Cardiac fibroblasts were then added to the cell lysis solution and incubated at 4℃ for 30 minutes. The lysate was centrifuged at 12,000 g and 4℃ for 15 minutes, and the supernatant was collected and transferred to an RNase-free centrifuge tube. 400 ng of biotinylated RNA was mixed with 500 mL of RIP buffer and incubated with the cell lysate at room temperature for 1 hour, while a part of the cell lysate was saved as the input control. Streptavidin magnetic beads were added to each binding reaction and incubated at 4℃ for 2 hours. The bound protein was collected using Biotin Elution Buffer and analyzed further by Western blot.

Periostin(POSTN)-promoter, AAV9 adeno-associated virus construction

AAV9 vectors targeting periostin+ cells were created by incorporating the periostin promoter before the sequences of CRLF1, resulting in CRLF1 cardiac fibroblast-specific AAV9 constructs:

ATTCTTTCAAGCTAACAATCTTTTTTTTTTTTTTAAAGTGGCCTCAGTCAAAGACACTAAAGATCAC
CGAGTCTTGCATAGAGTTTCCATTTACAGGACTAGAGAAAGCTAGTGGAGACACAGATCGGGTG
CGGAGGTAGTGAGAAGCACTTTTCCTAAGAAGGTGCAGGGTTGACTCCAAGGCTTGGCTGGGT
TATAAGAGTTACATGTATTATTTATTCTATATGTAAGCAACTTTTGAGCTCATGTGCCATGGCAACCT
ATGGACCGCATGTTAATATAGAAGCATTTTAAAATTAGTGATACAATCAAGACCAAGGGCATCCTG
CTTATGGTTTGTGTGCACAGGCTTACAGAGTGCAGAGTCCGCGAGGAGTCCCAGGGACTGCTG
GAGTTTGAGGTTGGTTTCACAGTGGTGAGTAAGCGTGGCAGTGTAATGACCTCATGGTCTCCCG
AGGCCAGATAACAGAGAACTGCCTATAAATCAGCATGCCGCGGCTAGAGAGAAACGGCCCTGTT
TCTCAGACACACTATCTCTCTTCAGCTACATAATGAACCATTTCTTTCTCAGTAATGACTTACATCT
CTGGGTCAGACTTTGCAGCCCTGGAAAGTCGGACTTCATTTTCATGATTTCCGTCATCTTCCCGA
CTGGTAGGAAAATTGCAGGGGTCAGTAGTGTCAGCATAGTTTCACAGAGCTGAAGAGAAAGGGC
CCTGTGTGGAGAGCGACTTTTGATGAGAGCCCCGGAAGAGAGTGTGCCCTTCCGGGGATTTTT
TTCCCAGTCTCTTCTACAACTTCAGACATCTATCCAGGATTTGGGTTAAATGCCCCTGTGATTTCT
CTTCTCCGTGTTCTGCTGTGGAGTGATTTAAGTGCAATCAGATCAAACCAGGAAAGTAACTGAGC
TCAGAGACACAGAGTGTGGTGGCAGAGACAGAAGGCAGAGAGATCCCTAAACTCAGAATCAGC
TCTTTTCGCAATGTAAACCTATAGAAGTGAAAAACGGGCTCACCATGATTGAAAACAAATAGGAGA
CAGAGTTCAGATTGCTCAGAACCCAGGAGATTTCCAGGGACAGCCCAGGGCTGCTGGTGCTTC
TGTAAGGCCATCGCAAGCTTCAGGTTGGCCCAGCGCCCCCTCCCACAGCCTTGCTCCCTCCCA
CAGCCCAGAGCTATATAAACTCAGCTCTCCAGAGCACAGGCCAGATCTCTTCCTGGACGGAGCT
CAGGGCTGAA

Human samples

Tissue samples were collected from 300 patients, of which 150 had Atrial Fibrillation (AF) and 150 were in Sinus Rhythm (SR). These samples were taken from the left atrial appendage (LAA) during heart valve replacement surgery at the Second Hospital of Anhui Medical University with patient consent. Gender and age were matched between the two groups. The samples were stored at -80° C. Ethical approval was obtained from the Anhui Medical University ethical committee. Inclusion criteria for the AF group included confirmation of AF through ECG and Holter recordings, recovery of cardiac function to NYHA class I or II after heart failure treatment, and signed informed consent for surgery and follow-up. The control group was chosen based on normal blood lipid levels, blood pressure, blood glucose, heart function, and pulmonary artery pressures, with age and sex matching the AF group.

Statistical Analysis

Perform data analysis and statistics using GraphPad Prism 9.0. Compare the means of two independent samples using t-test analysis (Student's t test); if the data does not follow a normal distribution or the variances are not equal, use Welch's t-test or Mann-Whitney U test. For comparisons among more than two independent samples, use one-way ANOVA (analysis of variance); if the data does not follow a normal distribution or the variances are not equal, use Welch's ANOVA or Kruskal-Wallis H test. A difference is considered statistically significant when $P < 0.05$. The number of biological replicates is indicated by the “n” in the figure legends.

Reference:

1. Schwanekamp JA, Lorts A, Vagnozzi RJ, Vanhoutte D, Molkentin JD. Deletion of Periostin Protects Against Atherosclerosis in Mice by Altering Inflammation and Extracellular Matrix Remodeling. Arterioscler Thromb Vasc Biol. 2016 Jan;36(1):60-8. doi: 10.1161/ATVBAHA.115.306397.
2. Tu B, Song K, Zhou Y, Sun H, Liu ZY, Lin LC, Ding JF, Sha JM, Shi Y, Yang JJ, Li R, Zhang Y, Zhao JY, Tao H. METTL3 boosts mitochondrial fission and induces cardiac fibrosis by enhancing LncRNA GAS5 methylation. Pharmacol Res. 2023 Aug;194:106840.doi: 10.1016/j.phrs.2023.106840.
3. Qin Y, Meng X, Wang M, Liang W, Xu R, Chen J, Song H, Fu Y, Li J, Gao C, Jia M, Zhao C, Zhao W. Posttranslational ISGylation of NLRP3 by HERC enzymes facilitates inflammasome activation in models of inflammation. J Clin Invest. 2023 Oct 16;133(20):e161935.doi: 10.1172/JCI161935.
4. Liu C, Spinozzi S, Chen JY, Fang X, Feng W, Perkins G, Cattaneo P, Guimarães-Camboa N, Dalton ND, Peterson KL, Wu T, Ouyang K, Fu XD, Evans SM, Chen J. Nexilin Is a New Component of Junctional Membrane Complexes Required for Cardiac T-Tubule Formation. Circulation. 2019 Jul 2;140(1):55-66. doi: 10.1161/CIRCULATIONAHA.119.039751.
5. Zhou Y, Song K, Tu B, Sun H, Ding JF, Luo Y, Sha JM, Li R, Zhang Y, Zhao JY, Tao H. METTL3 boosts glycolysis and cardiac fibroblast proliferation by increasing AR methylation. Int J Biol Macromol. 2022 Dec 31;223(Pt A):899-915. doi: 10.1016/j.ijbiomac.2022.11.042.
6. Cao P, Chen C, Liu A, Shan Q, Zhu X, Jia C, Peng X, Zhang M, Farzinpour Z, Zhou W, Wang H, Zhou JN, Song X, Wang L, Tao W, Zheng C, Zhang Y, Ding YQ, Jin Y, Xu L, Zhang Z. Early-life inflammation promotes depressive symptoms in adolescence via microglial engulfment of dendritic spines. Neuron. 2021 Aug 18;109(16):2573-2589.e9. doi:10.1016/j.neuron.2021.06.012.
7. Ding JF, Sun H, Song K, Zhou Y, Tu B, Shi KH, Lu D, Xu SS, Tao H. IGFBP3 epigenetic promotion induced by METTL3 boosts cardiac fibroblast activation and fibrosis. Eur J Pharmacol. 2023 Mar 5;942:175494. doi: 10.1016/j.ejphar.2023.175494.
8. Ganji E, Lamia SN, Stepanovich M, Whyte N, Goulet RW, Abraham AC, Killian ML. Optogenetic-induced muscle loading leads to mechanical adaptation of the Achilles tendon enthesis in mice. Sci Adv. 2023 Jun 23;9(25):eadf4683. doi: 10.1126/sciadv.adf4683.
9. Yerly L, Pich-Bavastro C, Di Domizio J, Wyss T, Tissot-Renaud S, Cangkrama M, Gilliet M, Werner S, Kuonen F. Integrated multi-omics reveals cellular and molecular interactions governing the invasive niche of basal cell carcinoma. Nat Commun. 2022 Aug 20;13(1):4897. doi: 10.1038/s41467-022-32670-w.
10. Han Z, Wang X, Xu Z, Cao Y, Gong R, Yu Y, Yu Y, Guo X, Liu S, Yu M, Ma W, Zhao Y, Xu J, Li X, Li S, Xu Y, Song R, Xu B, Yang F, Bamba D, Sukhareva N, Lei H, Gao M, Zhang W, Zagidullin N, Zhang Y, Yang B, Pan Z, Cai B. ALKBH5 regulates cardiomyocyte proliferation and heart regeneration by demethylating the mRNA of YTHDF1. Theranostics. 2021 Jan 1;11(6):3000-3016. doi: 10.7150/thno.47354.

Reference:

11. Song K, Sun H, Tu B, Zhou Y, Lin LC, Liu ZY, Li R, Yang JJ, Zhang Y, Zhao JY, Tao H. WTAP boosts lipid oxidation and induces diabetic cardiac fibrosis by enhancing AR methylation. iScience. 2023 Sep 15;26(10):107931. doi: 10.1016/j.isci.2023.107931.
12. Wang J, Yang Y, Sun F, Luo Y, Yang Y, Li J, Hu W, Tao H, Lu C, Yang JJ. ALKBH5 attenuates mitochondrial fission and ameliorates liver fibrosis by reducing Drp1 methylation. Pharmacol Res. 2023 Jan;187:106608. doi: 10.1016/j.phrs.2022.106608.
13. Qin Y, Meng X, Wang M, Liang W, Xu R, Chen J, Song H, Fu Y, Li J, Gao C, Jia M, Zhao C, Zhao W. Posttranslational ISGylation of NLRP3 by HERC enzymes facilitates inflammasome activation in models of inflammation. J Clin Invest. 2023 Oct 16;133(20):e161935. doi: 10.1172/JCI161935.
14. Sun H, Song K, Zhou Y, Ding JF, Tu B, Yang JJ, Sha JM, Zhao JY, Zhang Y, Tao H. MTHFR epigenetic derepression protects against diabetes cardiac fibrosis. Free Radic Biol Med. 2022 Nov 20;193(Pt 1):330-341. doi: 10.1016/j.freeradbiomed.2022.10.304.
15. Chen Y, Wang J, Xu D, Xiang Z, Ding J, Yang X, Li D, Han X. m6A mRNA methylation regulates testosterone synthesis through modulating autophagy in Leydig cells. Autophagy. 2021 Feb;17(2):457-475. doi: 10.1080/15548627.2020.1720431.
16. Wan W, Ao X, Chen Q, Yu Y, Ao L, Xing W, Guo W, Wu X, Pu C, Hu X, Li Z, Yao M, Luo D, Xu X. METTL3/IGF2BP3 axis inhibits tumor immune surveillance by upregulating N6-methyladenosine modification of PD-L1 mRNA in breast cancer. Mol Cancer. 2022 Feb 23;21(1):60.doi: 10.1186/s12943-021-01447-y.
17. Li B, Hu Y, Li X, Jin G, Chen X, Chen G, Chen Y, Huang S, Liao W, Liao Y, Teng Z, Bin J. Sirt1 Antisense Long Noncoding RNA Promotes Cardiomyocyte Proliferation by Enhancing the Stability of Sirt1. J Am Heart Assoc. 2018 Nov 6;7(21):e009700. doi: 10.1161/JAHA.118.009700.
18. Chen C, Yuan W, Zhou Q, Shao B, Guo Y, Wang W, Yang S, Guo Y, Zhao L, Dang Q, Yang X, Wang G, Kang Q, Ji Z, Liu J, Sun Z. N6-methyladenosine-induced circ1662 promotes metastasis of colorectal cancer by accelerating YAP1 nuclear localization. Theranostics. 2021 Feb 25;11(9):4298-4315. doi: 10.7150/thno.51342.
19. Song H, Feng X, Zhang H, Luo Y, Huang J, Lin M, Jin J, Ding X, Wu S, Huang H, Yu T, Zhang M, Hong H, Yao S, Zhao Y, Zhang Z. METTL3 and ALKBH5 oppositely regulate m6A modification of TFEB mRNA, which dictates the fate of hypoxia/reoxygenation-treated cardiomyocytes. Autophagy. 2019 Aug;15(8):1419-1437. doi: 10.1080/15548627.2019.1586246.
20. He T, Zhang Q, Xu P, Tao W, Lin F, Liu R, Li M, Duan X, Cai C, Gu D, Zeng G, Liu Y. Extracellular vesicle-circEHD2 promotes the progression of renal cell carcinoma by activating cancer-associated fibroblasts. Mol Cancer. 2023 Jul 22;22(1):117. doi: 10.1186/s12943-023-01824-9.

Table S1. Baseline Characteristics of Healthy Control and Atrial fibrillation patients

Characteristics	Healthy Control	AF Patients	P Value
Cases (*n*)	150	150	—
Age (years)	64	73	—
Male (%)	81/(54%)	85/(57%)	—
LV (mm)	45.00±4.46	49.50±7.67	<0.001 ***
LA (mm)	34.15±6.29	44.76±9.28	<0.001 ***
RV (mm)	21.00±2.47	22.50±3.32	<0.001 ***
RA Major (mm)	42.81±6.10	51.49±10.54	<0.001 ***
RA Minor (mm)	33.40±5.43	40.33±9.60	<0.001 ***
IVS (mm)	9.67±1.61	10.17±1.77	0.0111 *
LVPW (mm)	9.15±1.35	9.50±1.27	0.0593 **ns**
LVEF(%)	62.94±4.96	55.79±10.26	<0.001 ***
LVFS(%)	34.41±2.20	31.02±4.70	<0.001 ***

Note: P values were calculated using a 2-tailed Student's t-test values; $\overline{X}\pm$ s represents the mean ± standard deviation.

Table S2. Reagents and antibodies

Name	Manufacturers	Article Number
LPS	Merck	297-473-0
Nigericin	KKL.Med	28380-24-7
BCA kit	Beyotime	P0012
CO-IP kit	absin	abs9649
RNA Pull-Down kit	Bersinbio	Bes5102
RIP kit	Bersinbio	Bes5101
MeRIP kit	Bersinbio	Bes5203-1
Actinomycin D	GlpBio	GC16866
TRIzol	Invitrogen	15596018
Tamoxifen Solution	Beyotime	MFCD00010454
jetPRIME® Versatile DNA/siRNA transfection reagent	Polyplus	101000015
FAM FLICA Caspase 1 Assay Kit	ImmunoChemistry Technologies	ICT-97
anti-m6A antibody	Synaptic Systems	202003
anti-CRLF1 antibody	Affinity	DF8930
anti-COMP antibody	Affinity	DF13438
anti-LRRN3 Antibody	Affinity	DF8295
anti-ANGPTL2 Antibody	Affinity	DF12557
anti-CSRP3 Antibody	Affinity	DF12922
anti-DHHC7 Antibody	Affinity	DF4276
anti-GBP1 Antibody	Affinity	DF3598
anti-NLRP3 Antibody	Affinity	BF8029
anti-Caspase1 Antibody	Affinity	AF5418
anti-Cleaved-Caspase1 Antibody	Affinity	AF4005
anti-GSDMD-N Antibody	Affinity	DF13758
anti-ALKBH5 Antibody	Proteintech	16837-1-AP
anti-WTAP Antibody	Proteintech	60188-1-Ig
anti-FTO Antibody	Proteintech	27226-1-AP
anti-METTL3 Antibody	Proteintech	15073-1-AP
anti-METTL14 Antibody	Proteintech	26158-1-AP
anti-Collagen Type I antibody	Proteintech	14695-1-AP
anti-POSTN antibody	Proteintech	66491-1-Ig
anti-YTHDF1 antibody	Proteintech	66745-1-Ig
anti-YTHDF2 antibody	Proteintech	24744-1-AP
anti-Mouse IgG	Proteintech	B900620
anti-Rabbit IgG	Proteintech	30000-0-AP
anti-β-actin antibody	Proteintech	81115-1-RR
anti-GAPDH antibody	Proteintech	60004-1-Ig
Goat anti-Mouse IgG (H+L) HRP	Bioworld	BS12478
Goat anti-Rabbit IgG (H+L) HRP	Bioworld	BS13278
DAPI	Beyotime	P0131

Table S3. Sequences of primers

Target genes		Forward primer	Reverse primer
Human			
	POSTN	5′-CTGCTTCAGGGAGACACACC-3′	5′-CACTGAGAACGACCTTCCCT-3′
	Collagen I	5′- TAACATCGCTGACGGGAAGTG -3′	5′-CCGTGATTCCATTGGTATCAACA-3′
	CRLF1	5′-ATGAAGGACTTGACCTGCCG-3′	5′-GTCCTGGCCATACCACCTAAG-3′
	COMP	5′-CAAGGCCAACAAGCAGGTTT-3′	5′-GTTGATGCACACGGAGTTGG-3′
	LRRN3	5′-CCCGGACCCTGGCATCAT-3′	5′-TTGATGTACTTTTTGAAGGTGGAGT-3′
	TLL2	5′-AAAAGCCGACTGTGCTGGTA-3′	5′-GCCACAAAACCTGCCCAAAA-3′
	CSRP3	5′-GTTTCCACTGCAGTCCCCAA-3′	5′-TCGGACTCTCCAAACTTCGC-3′
	ANGPTL2	5′-GAGAACACCAACCGCCTCAT-3′	5′-CCCAAACCCTTGCTTGTACG-3′
	β-actin	5′-ACAGAGCCTCGCCTTTGC-3′	5′-GATATCATCATCCATGGTGAGCTGG-3′
	GAPDH	5′-GAAAGCCTGCCGGTGACTAA-3′	5′-GCCCAATACGACCAAATCAGAG-3′
Mouse			
	POSTN	5′-GAAGTGATCCACGGAGAGCC-3′	5′-TGTTTCTCCACCTCCTGTGG-3′
	Collagen I	5′-CGATGGATTCCCGTTCGAGT-3′	5′-CGATCTCGTTGGATCCCTGG-3′
	CRLF1	5′-GCTGTGGTCGCCTCTGTT-3′	5′-ATTACAGCTGTGTGGGCTCC-3′
	COMP	5′-AGTTGGCTACATCAGGGTGC-3′	5′-CGCAGGTTAGCCCAGATGAT-3′
	IL-1β	5′-TGCCACCTTTTGACAGTGATG-3′	5′-TGATGTGCTGCTGCGAGATT-3′
	IL-18	5′-TCCAACTGCAGACTGGCAC-3′	5′-GGCAGGAGTCCAGAAAGCAT-3′
	YTHDF1	5′-GGACAGTCCAATCCGAGTAACA-3′	5′-CCTCGCTGAGGGAGTAAGGA-3′
	YTHDF2	5′-CCTCTTGGAGCAGAGACCAAA-3′	5′-TTATTCGGCCTTGCCTGTGG-3′
	YTHDF3	5′-ATCAGAGACCTAAAGGGCAAGG-3′	5′-TTGGTGGATAGCTGTTATTCTGA-3′
	YTHDC1	5′-GCCGGGAGGAGAAAGATGG-3′	5′-TCATCCTGTTCTGGTACTTCAGTC-3′
	YTHDC2	5′-ACAAAACATGCTGTTAGGAGCC-3′	5′-TGCTCATTTCTCGGTTTTCAGC-3′
	IGF2BP1	5′-TCAAATCCGGCTACGCCTTC-3′	5′-TGCAGTTCTACTTTCCCCGAG-3′
	IGF2BP2	5′-CTGGCCGTTAACCAACAAGC-3′	5′-GCACAGACAGTCCAGTCGAA-3′
	IGF2BP3	5′-GAAGACGGGCTACGCGTTC-3′	5′-GAAGTTTACGAATCCTCTGCCG-3′
	ALKBH5	5′-GTGACTGTGCTCAGTGGGTA-3′	5′-TTCCAATCGCGGTGCATCTA-3′
	WTAP	5′-TGCAAGAGTGCACCACTCAA-3′	5′-GTTGATCTCAGTTGGGCCAC-3′
	FTO	5′-CACCAGGGAGACTGCTATTTCA-3′	5′-AGGTGCCTGTTGAGCACTCT-3′
	METTL3	5′-CTTGCCATCTCTACGCCAGA-3′	5′-TCTTGGAGGAGACCTCGCTT-3′
	METTL14	5′-TATGCTTGCGAAAGTGGGGT-3′	5′-CCATCAGGCAATGCTCCTTTG-3′
	β-actin	5′-CACTGTCGAGTCGCGTCC-3′	5′-TCATCCATGGCGAACTGGTG-3′

Target genes		Site	Product	Forward primer	Reverse primer
CRLF1-qPCR					
	Mouse				
	CRLF1-Site 1	2251-2350	100	5′-ACCAGTCGCTCGAAGGAGAA-3′	5′-GGGGAGTAATGCCCTTTTACCA-3′
	CRLF1-Site 2	5351-5450	100	5′-AGCTTCAATGAGTGCTTGGGT-3′	5′-CCTGCCTGGATGTTTCAAGAG-3′
	CRLF1-Site 3	6601-6700	100	5′-TAGAGGTCTTAGCCACAGCCA-3′	5′-TCTTGACTGTCTTGGGGGAA-3′
	CRLF1-Mut			5′-TGACTCTTGAAACATCCAGGCT-3′	5′-TCTGGGGTCAAAGACGACAC-3′
Transfection					
	Mouse				
	NC			5′-UUCUCCGAACGUGUCACGUTT-3′	5′-ACGUGACACGUUCGGAGAATT-3′
	siRNA-CRLF1			5′-CGGUCAGGAUAACACAUGUTT-3′	5′-ACAUGUGUUAUCCUGACCGTT-3′
	siRNA-DHHC7			5′-GCAUUCGAAAGAUGGACCATT-3′	5′-UGGUCCAUCUUUCGAAUGCTT-3′
	siRNA-GBP9			5′-UAUGACAAGUGUGGCCCGGTT-3′	5′-CCGGGCCACACUUGUCAUATT-3′
	siRNA-METTL3			5′-UACACCACCUCUCAGAUCUTT-3′	5′-AGAUCUGAGAGGUGGUGUAGC-3′
	siRNA-METTL14			5′-GACCTTGGAAGAGTGTGTTTACG-3′	5′-CTTTGATCCCCATGAGGCAGT-3′
	siRNA-YTHDF1			5′-GCGUAAGGAAAGCAAGAAUTT-3′	5′-AUUCUGUCUUUCCUUACGCTT-3′
	siRNA-YTHDF2			5′-GGACGUUCCCAAUAGCCAATT-3′	5′-UUGGCUAUUGGGAACGUCCUU-3′

Supplementary Figure 1

A. The expression of CRLF1, COMP, POSTN in TGF-β1-treated mouse cardiac fibroblasts were analyzed by RT-qPCR (n=6).

B. SR and AF heart tissues were embedded in paraffin, and tissue sections were evaluated by Masson's trichrome staining, Sirius red staining.

C. Confocal microscopy images showing the results of double staining with the CRLF1 and POSTN in SR and AF heart tissues.

D. Confocal microscopy images showing the results of double staining with the COMP and POSTN in SR and AF heart tissues.

"ns" was considered significant at $P>0.05$, Differences were considered significant if the $P<0.05$ the "n" in figure legends indicates biological replicates. The empty circles in the bar chart represent the mean values.

Supplementary Figure 2

A. Confocal microscopy images showing the results of double staining with the CRLF1 and POSTN in ISO-induced experimental cardiac fibrosis tissues.

B. ISO-induced experimental cardiac fibrosis tissues sections were evaluated Masson's trichrome staining, Sirius red staining (n=6).

C and D. Transthoracic echocardiography was performed on mice with ISO-induced experimental cardiac fibrosis. Cardiac function was assessed using transthoracic echocardiography, which measured HR(heart rate), EF (Ejection fraction), FS (Fraction shortening), LVIDd (Left ventricular internal dimension-diastole), LVIDs (Left ventricular internal dimension in systole) (n=6).

E. The expression levels of CRLF1, POSTN, and Collagen I were analyzed by Western blotting in AAV9-POSTN-oeCRLF1-treated mice and compared with vector-treated controls**F.** AAV9-POSTN-oeCRLF1-treated and vector-treated mice heart tissues sections were evaluated Masson's trichrome staining, Sirius red staining(n=6).

G. Transthoracic echocardiography was performed to assess cardiac function in AAV9-POSTN-oeCRLF1-treated mice (n=6) compared with vector-treated controls. Measurements included EF, FS, LVIDd, and LVIDs (n=6).

"ns" was considered significant at $P>0.05$, Differences were considered significant if the $P<0.05$, the "n" in figure legends indicates biological replicates. The empty circles in the bar chart represent the mean values.

Supplementary Figure 3

A. Cardiac fibroblasts were transfected with a CRLF1 overexpression plasmid, and the proteomics data were subsequently analyzed.

B. The expression of CRLF1, DHHC7 and GBP9 in CRLF1 knockdown cardiac fibroblasts were analyzed by western blotting.

C. The expression of PLAM-GSDMD in C192 cardiac fibroblasts treated with LPS/Nig or without LPS/Nig was analyzed by western blotting (n=3).

D. The expression of PLAM-GSDMD in DHHC7 knockdown cardiac fibroblasts treated with LPS/Nig or without LPS/Nig was analyzed by western blotting (n=3).

E. Disruption of the palmitoylation process or interference with depalmitoylation pathways reveals changes in pyroptosis in cardiac fibroblasts (n=3).

"ns" was considered significant at $P>0.05$, Differences were considered significant if the $P<0.05$, the “n” in figure legends indicates biological replicates. The empty circles in the bar chart represent the mean values.

Supplementary Figure 4

A. m6A motifs within CRLF1, using sramp software (http://www.cuilab.cn/sramp) in mice are shown.

B and C. Global m6A level of RNA extracted from ISO-induced experimental cardiac fibrosis tissues and TGF-β1-induced mouse cardiac fibroblasts were measured via m6A dot blot assays (2-tailed Student t test; n=6).

D. Cardiac fibroblasts treated with m6A inhibitor DAA show decrease stability of CRLF1, as demonstrated by RNA decay assay analysis (n = 3).

E. ALKBH5 expression in cardiac fibroblasts treated with ALKBH5 overexpression was analyzed using RT-qPCR and western blotting (n=6).

F. METTL3 expression in cardiac fibroblasts treated with METTL3 knockdown was analyzed using RT-qPCR and western blotting (n=6).

G. METTL14 expression in cardiac fibroblasts treated with METTL14 knockdown was analyzed using RT-qPCR and western blotting (n=6).

H. RT-qPCR analysis of CRLF1 mRNA levels in OE-ALKBH5 or siRNA-METTL13 or siRNA-METTL14 induced cardiac fibroblasts (n = 6).

I. The m6A enrichment of selected mRNAs and CRLF1 was detected by the MeRIP-qPCR method (n = 6); The m6A modification site of CRLF1 and the mutation site are predicted by this sequence.

J. Relative luciferase activity of CRLF1 with wild-type or mutated m6A sites after co-transfection with Vector, or OE-ALKBH5 in cardiac fibroblasts (n = 6).

K. Interaction between ALKBH5 and CRLF1 verified by RNA pull-down assay.

L. RIP experiments were conducted using the ALKBH5 antibody for immunoprecipitation (IP) and a primer of CRLF1 for detection. RIP enrichment was quantified as the ratio of RNA associated with ALKBH5 IP to the input control (n = 6).

M. MeRIP-qPCR analysis of fragmented CRLF1-Mut RNA with ALKBH5 overexpression (n = 6)**.**

N. Global m6A level of RNA extracted from TGF-β1-induced mouse cardiac fibroblasts treated with OE-ALKBH5 were measured via m6A dot blot assays (n=6).

"ns" was considered significant at $P>0.05$, Differences were considered significant if the $P<0.05$, the “n” in figure legends indicates biological replicates. The empty circles in the bar chart represent the mean values.

Supplementary Figure 5

A. A protein-protein interaction network revealed that ALKBH5 could interact with eight common m6A readers.

B. Bioinformatic analysis with SRAMP software revealed that the most frequent m6A motif of CRLF1 was a consistent RRACH (where R = G or A, H = A, C or U).

C. RIP experiments were conducted using the YTHDF1 or YTHDF2 antibody for immunoprecipitation (IP) and a primer of CRLF1 for detection. RIP enrichment was quantified as the ratio of RNA associated with YTHDF1 or YTHDF2 IP to the input control (n=3).

D. The m6A enrichment of selected mRNAs and CRLF1 was detected by the MeRIP-qPCR method (n = 6).

E. The m6A modification site of CRLF1 and the mutation site are predicted by this sequence.

F. Interaction between YTHDF1 and CRLF1 verified by RNA pull-down assay.

G. RIP experiments were conducted using the YTHDF1 antibody for immunoprecipitation (IP) and a primer of CRLF1 for detection. RIP enrichment was quantified as the ratio of RNA associated with YTHDF1 IP to the input control (n = 6).

H. MeRIP-qPCR analysis of fragmented CRLF1-Mut RNA with YTHDF1 knockdown (n = 6).

I. Cardiac fibroblasts treated with siRNA-ALKBH5 show decrease stability of CRLF1, as demonstrated by RNA decay assay analysis (n = 3).

J. Molecular docking experiments showed that YTDHF1 can bind to ALKBH5.

K and L. ALKBH5 and YTHDF1 expression in cardiac fibroblasts treated with ALKBH5 overexpression was analyzed using RT-qPCR and western blotting (n=6).

M and N. RIP experiments were observed that the binding ability of YTHDF1 with CRLF1 was altered by the knockdown or overexpression of ALKBH5 (n=6).

"ns" was considered significant at $P>0.05$, Differences were considered significant if the $P<0.05$, the “n” in figure legends indicates biological replicates. The empty circles in the bar chart represent the mean values.

Supplementary Figure 6

A. Confocal microscopy images of double staining for CRLF1 and POSTN in ISO-induced cardiac fibrosis tissues treated with the m6A inhibitor DAA compared to negative control (n=3).

B. EF and FS of cardiac function were assessed using transthoracic echocardiography in mice subjected to ISO treatment, the m6A inhibitor DAA, cardiac fibroblast-specific YTHDF1 deficiency, and treatment with LPS/Nig (n=3).

C. Heart tissue from mice subjected to ISO treatment, the m6A inhibitor DAA, cardiac fibroblast-specific YTHDF1 deficiency, and LPS/Nig treatment was embedded in paraffin for subsequent analysis and evaluated using Masson's trichrome and Sirius red staining (n=3).

D. The expression levels of IL-1β and IL-18 were analyzed by RT-qPCR in mice subjected to ISO treatment, the m6A inhibitor DAA, cardiac fibroblast-specific YTHDF1 deficiency, and LPS/Nig treatment (n=3).

"ns" was considered significant at $P>0.05$, Differences were considered significant if the $P<0.05$, the “n” in figure legends indicates biological replicates. The empty circles in the bar chart represent the mean values.