N6-Methyladenosine-Mediated Phase Separation Suppresses Notch1 Boosts Mitochondrial Fission in Diabetic Cardiac Fibrosis

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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.

Animal experiments and models

All animal experimental protocols were conducted in accordance with the National Institutes of Health 'Guidelines for the Use of Laboratory Animals' and received approval from the Ethics Committee of Anhui Medical University and the Second Affiliated Hospital of Anhui Medical University.

In our study, we utilized the Leptin receptor-deficient (db/db) model, high fat diet (HFD)/streptozotocin (STZ) model, Notch1 conditional knockout (POSTN-Cre × Notch1$^{flox/flox}$, Notch1-cKO)-HFD/STZ model, Notch cKO-AAV9 model, and db/db-AAV9 model.

db/db model [1]

Leptin receptor-deficient (db/db, male, 6 weeks old) and lean control (db/+, male, 6 weeks old) mice were obtained from Hangzhou Ziyuan Laboratory Animal Technology Co., Ltd. (Zhejiang, China). The mice were housed in a specific pathogen-free facility with 4 animals per cage and a 12-hour light-dark cycle, with food and water provided ad libitum. Weekly measurements of blood glucose levels were taken, and food and water intake were recorded for all mice.

HFD/STZ model [1]

C57BL/6 mice were given a high-sugar, high-fat diet for a period of 3 months. When the mice were 8 weeks old, they were either injected with 100 mg/kg of STZ or an equal volume of normal saline on an empty stomach. Blood glucose was measured, and food and water intake recorded in all mice weekly.

Notch cKO model [2]

GemPharmatech generated the Notch1 loxP (Notch1$^{f/f}$) mouse, which was subsequently bred with the POSTN [3]-creER mouse to produce Notch1 pCKO mice.

AAV9 model

At 16 weeks old [4], db/db mice in the empty virus vector group received an injection of an empty virus vector via the tail vein. The other groups were administered AAV9-oeNotch1-NICD, AAV9-oeALKBH5, AAV9-shYTHDF2, and AAV9-shNotch1 (200μl/mouse) through tail vein injections, respectively.

Mice were anesthetized using a specialized animal anesthesia system for echocardiography and subsequent heart tissue collection. Anesthesia was induced with 2% isoflurane in oxygen for about 3-5 minutes and sustained through a nasal cone connected to an isoflurane anesthesia machine (E-Z Anesthesia, Euthanex Corp., PA, USA) to reach the desired level of anesthesia. Following the evaluation of cardiac function, the mice were humanely euthanized by cervical dislocation.

Echocardiography [5]

Transthoracic echocardiography was conducted post-modeling using a Vevo 2100 Imaging System (VisualSonics, Toronto, ON, Canada) on anesthetized mice with 2% isoflurane inhalation. Echocardiographic M-mode tracings were recorded in the long-axis view, with measurements taken for parameters including left ventricular ejection fraction (LVEF%), fractional shortening (FS%), left ventricular internal dimension in diastole (LVIDd), left ventricular internal dimension in systole (LVIDs), interventricular septal thickness in diastole (IVSd), and interventricular septal thickness in systole (IVSs), over a minimum of six cardiac cycles. All parameters were measured at least 6 times, and averages are presented.

Histopathology [6]

Histopathological analysis of mouse tissue was carried out at the Core Experimental Facility of The Second Hospital of Anhui Medical University. The tissue samples were processed, embedded in paraffin, and sectioned at a thickness of 6 μm. Collagen was visualized using picric acid Sirius red staining, while fibrosis was identified using Masson's trichrome staining. Six heart samples were examined for each staining technique. The staining intensity was analyzed with ImageJ software to calculate the volume fraction. At least 10 fields per animal were evaluated.

Immunofluorescence [7]

Fixed hearts were dehydrated in 30% sucrose at 4°C for 12 hours, then embedded in Tissue-Tek O.C.T. medium, and sectioned at 6 μm thickness. The sections were permeabilized with using 0.3% Triton X-100 and fixed with 4% paraformaldehyde. Subsequently, after blocking with fetal bovine serum, samples were incubated overnight underwent overnight incubation at 4°C with primary antibodies (refer to Table S2) diluted at a ratio of 1:300-1:500. Subsequently, the samples underwent PBST washes, followed by the application of secondary antibodies (Table S2) for 1.5 hours at room temperature in the dark. Nuclei were stained with DAPI and then imaged using confocal fluorescence microscopy. Six separate experiments were conducted. Immunostaining intensity was quantified from three distinct regions of each section using ImageJ software to evaluate the levels of immunostaining. The relative integral density represents the quantitative data derived from the color signal analyzed through the ImageJ IHC Toolbox plug-in (https://imagej##nih##gov/ij/plugins/ihc-toolbox/index##html).

The Coloc 2 plugin in ImageJ (Analyze-Colocalization-Coloc 2) generates Pearson correlation coefficients to calculate the colocalization rate.

TEM [1]

TEM samples were prepared as described previously and examined using a transmission electron microscope (TALOS L120C, Thermo Scientific). Six separate experiments were conducted on each sample to obtain images.

Mito Tracker red staining [8]

The sections were prepared as previously described and stained before sealing. Alternatively, cells were stained with Mito Tracker Red (Invitrogen, California) following the manufacturer's instructions during the final 30 minutes of culture. Nuclei were stained with DAPI for 20 minutes and visualized using confocal microscopy.

For tissue mitochondrial staining, frozen sections were utilized. Fresh tissue blocks, approximately 0.5 cm thick, were prepared, and embedding and sectioning were performed after removing excess moisture.

Subsequent procedures were conducted according to experimental requirements following the completion of mitochondrial staining.

Primary cardiac fibroblasts isolation and cell culture [9]

Cardiac fibroblasts (CF) were obtained from the ventricles of 1-3 day-old C57BL/6J mice. Neonatal mice were humanely euthanized by cervical dislocation, and their hearts were excised and rinsed with cold PBS.

The heart tissues were finely chopped and enzymatically digested using a combination of 0.16% trypsin (Beyotime, Shanghai, China) and 0.67% collagenase type II (Biofrox) at 37°C for 30-60 minutes.

Following digestion, the cells were cultured in DMEM medium supplemented with 15% FBS (5.5 mM glucose) and incubated at 37°C with 5% CO2 for 2 hours to allow primary cardiac fibroblasts to adhere based on their specific adhesion properties. The purity of the isolated fibroblasts was validated through Vimentin staining. Subsequently, the cells were split at a 1:1 ratio for culture expansion, with second- and third-generation cells utilized for subsequent genetic and functional analyses.

Cell culture and treatments

H9C2 (rat cardiomyocytes) and 3T3 (mouse embryonic fibroblasts) were procured from the Cell Bank of the Type Culture Collection, Chinese Academy of Sciences (Shanghai, China). They were maintained in DMEM (Hyclone) supplemented with 10% FBS (Gibco, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Beyotime) under a humidified atmosphere with 5% CO2 at 37°C. Moreover, primary cardiac fibroblasts (CFs), 3T3 cells, and cardiomyocytes were cultured in low glucose medium (5.5 mmol/L glucose and 27.5 mmol/L mannitol) as well as high glucose combined with palmitic acid and oleic acid medium (33 mmol/L glucose, 200 mmol/L palmitate, and 200 mmol/L oleate) for 24 hours to replicate type 2 diabetes conditions in vitro.

RNA interference and plasmid transfection [10]

The small interfering RNAs (siRNA) for Notch1, YTHDF1, YTHDF2, OE-NICD, OE-ALKBH5, and mutation plasmid were generated by Shanghai Genechem Co., Ltd. (Shanghai, China). Cardiac fibroblasts were seeded into six-well plates. After HG/HF stimulated 24h, the transfections for siRNA or plasmid were performed using the PolyPlus-Transfection (Illkirch, France) following the manufacturer's instructions.

Western blotting [11]

Cell lysates and cardiac tissue homogenates were prepared using RIPA lysis buffer containing protease and phosphatase inhibitors. Protein concentrations were determined with a BCA protein assay kit (Beyotime, China). Subsequently, protein samples were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Merck Millipore, Darmstadt, Germany). The membrane was then incubated overnight with the diluted primary antibody on a shaker at 4°C. Following three washes with TBST, the membrane underwent incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody. Enhanced chemiluminescence (ECL) substrates were utilized to visualize the proteins of interest, aiding in the detection of bound antibodies. Western blots are representative of six independent experiments. Data are presented as mean ± standard deviation. P-values were calculated using a two-tailed independent samples Student's t-test.

Quantitative real-time reverse transcription (qRT)-PCR [12]

Total RNA was isolated from cells and tissues using the Trizol method (Invitrogen) as per the manufacturer's instructions. Subsequently, RNA purity and concentration were assessed through spectrophotometry. The isolated RNA was then reverse transcribed into cDNA utilizing PrimeScript RT Master Mix (TaKaRa) following the provided guidelines.

Quantitative real-time PCR (qPCR) was conducted on all individual samples using SYBR Premix Ex Taq II (TaKaRa), with beta-actin serving as the internal control for normalization. The relative RNA expression levels were determined using the 2^-ΔΔCt method. Primer details for this analysis are listed in (Table S3). A minimum of six independent experiments were conducted, with three replicates performed for each group in each experiment.

EdU assay

Cell proliferation was evaluated by measuring the proportion of proliferating cells using the Cell-Light EdU DNA Cell Proliferation Kit (RiboBio Co., Ltd, Guangzhou, China). The EdU labeling reagent was introduced into the cell culture medium at a 1:1000 dilution during the final 3 hours of cultivation. Subsequently, the cells were fixed in 4% formaldehyde and subjected to a 30-minute incubation in the Apollo reaction buffer. Nuclei were stained with DAPI, and each sample was observed under confocal microscopy (Zeiss, Germany). Five distinct fields were imaged for each group across six independent experiments using a fluorescence microscope, and the number of EdU-positive cells was counted in approximately 1,000 DAPI-stained cells.

Transwell migration assay [13]

Cells were seeded in the upper chamber of a Matrigel-free 24-well plate, with the lower chamber filled with 20% FBS-DMEM and the upper chamber containing serum-free DMEM. After 24 hours of incubation, cells were fixed with paraformaldehyde and stained with 0.5% crystal violet. Migrated cells in the upper chamber were removed using a cotton swab. Five randomly selected fields of view (x200) were photographed with an inverted microscope. Six independent experiments were conducted, and cell counts were analyzed using ImageJ software.

Luciferase activity assay [14]

The wild-type Drp1 sequence was cloned into a dual luciferase reporter vector from Promega (USA). Following this, 3T3 fibroblasts were co-transfected with siRNA or plasmid along with the vector.

Luciferase activity was then measured after 48 hours using Promega's dual luciferase reporter system. Luciferase activity was assessed in six independent biological replicates using the Dual-Luciferase Reporter Assay Kit (Promega), following the manufacturer's instructions.

Co-immunoprecipitation [1]

Different groups of CFs were lysed using an IP/CO-IP kit (Absin, China) following the manufacturer's instructions. The extracted proteins were subsequently analyzed by Western blot. Co-immunoprecipitation experiments were conducted in three independent biological replicates.

CHIP assays and Chip-qPCR [15]

ChIP assays were conducted using the Simple ChIP Plus Enzymatic Chromatin IP Kit (CST) as per the provided protocol. Cells were exposed to 1% formaldehyde for cross-linking, and the chromatin was fragmented by sonication to break down the genomic DNA. The non-precipitated chromatin was utilized as the positive control (input). The remaining chromatin was incubated with relevant antibodies, and the DNA was purified for PCR with ChIP-specific primers (Table S4). The reaction products were then examined through 2% agarose gel electrophoresis and subjected to qPCR for relative quantification. Each ChIP sample was pooled from at least three independent ChIP experiments. ChIP-qPCR values represent six independent ChIP assays, each assessed in duplicate by qPCR.

m^{6}A prediction

The sequence-based RNA adenosine methylation site predictor (SRAMP) software (http://www.cuilab.cn/sramp) and RMBase (https://rna.sysu.edu.cn/rmbase/) were used for m^{6}A prediction and analysis.

RNA m^{6}A dot-blot assay [16]

Total RNA was extracted from CF cells or heart tissue using TRIzol. Following pretreatment, either 200 or 400 ng of RNA was applied to a nitrocellulose (NC) membrane (Biosharp, China) and cross-linked under ultraviolet light for 1 hour. Subsequently, the membranes underwent washing with PBST and were then incubated with 0.02% methylene blue (Beyotime, China) or 5% BSA for 1.5 hours. After several PBST washes to clear the background, ,the membrane was scanned to generate a methylene blue dot image. The fully blocked membrane was then washed with PBST and incubated overnight at 4°C with an m^{6}A antibody (Proteintech, 68055-1-Ig). The next day, the membrane was incubated with HRP-conjugated anti-rabbit IgG secondary antibody and detection was carried out using the ECL system. ImageJ software was utilized for quantifying the m^{6}A dot density, which was then normalized to the relative density of methylene blue staining. The dot blot shown is representative of at least six independent experiments.

RNA Decay Assay [8]

An RNA decay assay was carried out using CFs in 6-well plates. The cells were either overexpressed with ALKBH5, YTHDF2 knockout, or treated with 1,6 hexanediol after exposure to HG/HF. The effects of these interventions on RNA stability were evaluated. Transcription was halted by the addition of actinomycin D (Catalog No. GC16866, Glpbio) at a concentration of 1 μg/mL to each well. Cells were harvested at specific time points (0, 1, 2, 3, and 4 hours post-treatment), and total RNA was extracted. RT-qPCR analysis was performed to quantify the remaining levels of Notch1 mRNA, normalized to the initial time point (0 hours). The decay rates of Notch1 mRNA were determined using linear regression analysis. A minimum of three independent experiments were conducted, with three replicates performed for each group in each experiment.

MeRIP-RT-qPCR [17]

A fraction of the total mRNA was subjected to immunoprecipitation using magnetic beads conjugated with m^{6}A antibodies (Synaptic Systems, 202203) and IgG antibodies (Proteintech, 30,000-0-AP), which were pre-mixed and then combined with the sample, while an equal separate sample was retained as input. Following a brief centrifugation, the m^{6}A -bound magnetic beads were isolated using a magnetic rack. The mRNA-antibody complex was then treated with proteases to degrade the bound proteins, subsequently washed, air-dried, and re-suspended in DEPC-treated water. A specific primer targeting Notch1 was employed for qPCR amplification of both the initial sample and the m^{6}A-enriched sample, with the level of m^{6}A modification of Notch1 being determined based on the amplification results. Please refer to the Table S4 for details on the specific primers used. Each MeRIP sample was pooled from at least six independent MeRIP experiments. MeRIP-qPCR values represent six independent MeRIP assays, each assessed in duplicate by qPCR.

RIP-RT-qPCR [18]

Cells were lysed using Passive Lysis Buffer, and the extracted RNA was divided into three groups: IP group, IgG group, and input group. Specific antibodies or non-specific IgG (Proteintech, 30,000-0-AP) were incubated overnight with IP group or IgG group, respectively. The following day, Protein A/G beads were added to precipitate the resulting complexes, which were then collected by centrifugation and purified. mRNA was extracted using a phenol-chloroform-isoamyl alcohol mixture. The purified mRNA underwent reverse transcription, followed by RT-qPCR to quantitatively evaluate the relative binding affinity of the specific target gene to the target protein. Each RIP sample was pooled from at least six independent RIP experiments. RIP-qPCR values represent six independent RIP assays, each assessed in duplicate by qPCR.

Protein-protein interaction (PPI) networks

Mouse PPI network collected by GeneMANIA60 (http://genemania.org/).

RNA pull-down assay [19]

The RNA sequences were biotin-labeled using Roche's Biotin RNA Labeling Mix and purified with the GenElute™ mRNA Miniprep Kit (Sigma, St. Louis, MO). Following labeling, the RNA (1 mg) was heated in RNA structure buffer containing Tris (pH = 7) at 10 mmol/L, KCl at 0.1 mol/L, and MgCl2 at 10 mmol/L for 2 minutes at 95°C. It was then cooled on ice for 3 minutes and incubated at room temperature for 30 minutes to promote secondary structure formation. Cardiac fibroblasts were added to the cell lysis solution and left at 4°C for 30 minutes. The lysate was centrifuged at 12,000 g and 4°C for 15 minutes, and the supernatant was transferred to an RNase-free centrifuge tube. Subsequently, 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 portion of the cell lysate was saved as the input control. Streptavidin magnetic beads were introduced to each binding reaction and left at 4°C for 2 hours. The proteins bound to the beads were eluted using Biotin Elution Buffer and further analyzed by Western blotting. Pictures are representatives of three independent pull-down experiments.

Protein expression and purification [20]

N-terminal 6X-His tagged YTHDF2 and YTH domain constructs were created through a standard PCR-based cloning approach using HEK293T cDNA primed with oligo-d(T)25. These constructs were then expressed in E. coli strain Setta2 (DE3) using the pET-28(+) vector (Novagen.) from E. coli cells were cultured in 500 ml LB medium and induced overnight at 18°C with 0.1 mM IPTG. The cells were lysed in 30 ml buffer (50 mM Tris pH 7.5, 500 mM NaCl, 10 mM imidazole, 0.01% NP40, 1 mM β-mercaptoethanol) and homogenized at 4°C. Following centrifugation at 13,000 rpm for 20 minutes at 4°C, the supernatant was purified using Ni-NTA beads (Smart-Lifesciences) and eluted with buffer containing 50 mM Tris pH 7.5, 500 mM NaCl, 200 mM imidazole, and 1 mM β-mercaptoethanol. The eluted proteins were assessed through Coomassie staining and subsequently dialyzed in a storage buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM β-mercaptoethanol).

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

AAV9 vectors targeting periostin+ cells were engineered by incorporating the periostin promoter upstream of the sequences for Notch1, ALKBH5, and YTHDF2. Consequently, specific AAV9 constructs for Notch1, ALKBH5, and YTHDF2 in cardiac fibroblasts were generated：

ATTCTTTCAAGCTAACAATCTTTTTTTTTTTTTTAAAGTGGCCTCAGTCAAAGACACTAAAGATCAC

CGAGTCTTGCATAGAGTTTCCATTTACAGGACTAGAGAAAGCTAGTGGAGACACAGATCGGGTG

CGGAGGTAGTGAGAAGCACTTTTCCTAAGAAGGTGCAGGGTTGACTCCAAGGCTTGGCTGGGT

TATAAGAGTTACATGTATTATTTATTCTATATGTAAGCAACTTTTGAGCTCATGTGCCATGGCAACCT

ATGGACCGCATGTTAATATAGAAGCATTTTAAAATTAGTGATACAATCAAGACCAAGGGCATCCTG

CTTATGGTTTGTGTGCACAGGCTTACAGAGTGCAGAGTCCGCGAGGAGTCCCAGGGACTGCTG

GAGTTTGAGGTTGGTTTCACAGTGGTGAGTAAGCGTGGCAGTGTAATGACCTCATGGTCTCCCG

AGGCCAGATAACAGAGAACTGCCTATAAATCAGCATGCCGCGGCTAGAGAGAAACGGCCCTGTT

TCTCAGACACACTATCTCTCTTCAGCTACATAATGAACCATTTCTTTCTCAGTAATGACTTACATCT

CTGGGTCAGACTTTGCAGCCCTGGAAAGTCGGACTTCATTTTCATGATTTCCGTCATCTTCCCGA

CTGGTAGGAAAATTGCAGGGGTCAGTAGTGTCAGCATAGTTTCACAGAGCTGAAGAGAAAGGGC

CCTGTGTGGAGAGCGACTTTTGATGAGAGCCCCGGAAGAGAGTGTGCCCTTCCGGGGATTTTT

TTCCCAGTCTCTTCTACAACTTCAGACATCTATCCAGGATTTGGGTTAAATGCCCCTGTGATTTCT

CTTCTCCGTGTTCTGCTGTGGAGTGATTTAAGTGCAATCAGATCAAACCAGGAAAGTAACTGAGC

TCAGAGACACAGAGTGTGGTGGCAGAGACAGAAGGCAGAGAGATCCCTAAACTCAGAATCAGC

TCTTTTCGCAATGTAAACCTATAGAAGTGAAAAACGGGCTCACCATGATTGAAAACAAATAGGAGA

CAGAGTTCAGATTGCTCAGAACCCAGGAGATTTCCAGGGACAGCCCAGGGCTGCTGGTGCTTC

TGTAAGGCCATCGCAAGCTTCAGGTTGGCCCAGCGCCCCCTCCCACAGCCTTGCTCCCTCCCA

CAGCCCAGAGCTATATAAACTCAGCTCTCCAGAGCACAGGCCAGATCTCTTCCTGGACGGAGCT

CAGGGCTGAA

Fluorescence recovery after photobleaching (FRAP) [19]

FRAP was performed using a fluorescence microscope equipped with a 488 nm laser. Photobleaching was induced in an area of approximately 1 μm² using 100% laser power, and images were captured at intervals of 5 seconds. For reproducibility, three repetitions were performed and representative images were selected.

Human samples

Samples were collected from 30 DCM patients and 30 non-diabetic healthy controls at the Second Affiliated Hospital of Anhui Medical University. This study was approved by the Ethics Committee of Anhui Medical University and the Second Affiliated Hospital. Inclusion criteria for DCM patients included diagnosed diabetes mellitus, absence of other causes of decreased left ventricular function, no history of hypertension, myocardial infarction, or suspected coronary heart disease, normal liver and kidney function, and normal lipids. The control group met inclusion criteria of normal blood lipid, blood pressure, and blood sugar levels, normal cardiac function and pulmonary artery pressure, and age and sex matching with the DCM group. After obtaining informed consent for surgery and follow-up, heart tissue was collected during heart valve replacement or cardiac catheterization and stored at -80° C for further experiments.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 8. The data are presented as mean ± standard deviation. Student's t-test was applied to compare means, while one-way analysis of variance (ANOVA) and Tukey's post hoc test were used for comparing multiple groups. A significance level of $P < 0.05$ was considered statistically significant.

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Table S1. Baseline Characteristics of Healthy Control and Diabetic cardiomyopathy patients

Characteristics	Healthy Control	DCM Patients	P Value
Cases (*n*)	30	30	—
Age (years)	63.7931±14.32126	66.3793±12.43961	—
Male (%)	66.6666%（20/30）	66.6666%（20/30）	—
BMI(kg/m^2)	23.3080±3.65742	25.7955±3.86350	0.027985
Duration of diabetes illness(years)	-	10.6667±8.80569	-
Heart rates (/min)	81.6250±13.72656	83.4583±19.65790	0.761253
SBP (mm Hg)	132.6538±18.99883	140.9231±20.98747	0.179876
DBP (mm Hg)	76.1923±12.10956	79.0000±12.29634	0.412342
FBG(mmol /L)	5.4572±1.48885	8.2712±2.93339	0.000163
HbA1c（%）	5.7750±0.60886	7.7875±1.16550	0.003553
TC (mmol/L)	4.5915±1.43250	4.3230±1.65354	0.553734
Triglycerides(mmolL)	1.5604±1.15568	2.9558±4.87398	0.182228
HDL (mmol/L)	1.2613±0.51299	1.0943±0.32679	0.185933
LDL (mmo/L)	2.9126±0.86530	2.3517±1.05242	0.108354
Creatinine (umolL)	90.1250±64.70959	91.1667±36.77862	0.938961
BUN (umol/L)	6.8389±2.71380	8.3144±4.01232	0.077413
uric acid (umol/L)	364.8889±84.88967	356.5926±113.47391	0.743253
Cystatin c (mg/L)	1.9139±1.97085	1.4678±0.52778	0.378954
LVDd(mm)	44.6333±9.45947	49.1667±8.48562	0.038731
LA (mm)	37.7000±9.81607	38.8333±6.99795	0.616801
RV (mm)	21.2667±2.47656	22.2333±1.81342	0.127899
RA Major (mm)	43.5357±9.59738	44.9643±5.81494	0.530590
RA Minor (mm)	34.3571±6.23143	35.0357±4.86470	0.685132
IVS (mm)	10.4667±2.88556	11.6000±2.11073	0.112630
LVPW (mm)	9.6000±1.47625	10.6333±1.42595	0.008609
LVEF(%)	60.5067±5.40063	57.2000±9.30480	0.048542
LVFS(%)	32.9286±2.37157	30.10345±5.97223	0.0234

Table S2. Reagents and antibodies

Name	Manufacturers	Article Number
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
jetPRIME® Versatile DNA/siRNA transfection reagent	Polyplus	101000015
anti-Notch1 antibody	Proteintech	20687-1-AP
anti-Notch2 antibody	Proteintech	28580-1-AP
anti-Notch3 antibody	Proteintech	55114-1-AP
anti-Notch4 antibody	Abcam	Ab166605
anti-Drp1 antibody	Proteintech	12957-1-AP
anti-MID49 antibody	Proteintech	28718-1-AP
anti-MFN1 antibody	Proteintech	13798-1-AP
anti-MFN2 antibody	Proteintech	12186-1-AP
anti-NRF1 antibody	Proteintech	12482-1-AP
anti-TFAM antibody	Proteintech	22586-1-AP
anti-PINK1 antibody	Proteintech	23274-1-AP
anti-Parkin antibody	Proteintech	14060-1-AP
anti-Fibronectin antibody	Proteintech	15613-1-AP
anti-Collagen Type III antibody	Proteintech	22734-1-AP
anti-MFF antibody	Proteintech	66527-1-Ig
anti-MID51 antibody	Proteintech	20164-1-AP
anti-Fis1 antibody	Proteintech	66635-1-Ig
anti-YAP antibody	Proteintech	13584-1-AP
anti-YY2 antibody	Proteintech	66839-1-Ig
anti-ZEB1 antibody	Proteintech	21544-1-AP
anti-NR4A1 antibody	Proteintech	25851-1-AP
anti-m^6A antibody	Synaptic Systems	202003
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-PCNA antibody	Proteintech	81302-6-RR
anti-POSTN antibody	Proteintech	66491-1-Ig
anti-YTHDF1 antibody	Proteintech	66745-1-Ig
anti-YTHDF2 antibody	Proteintech	24744-1-AP
anti-YTHDF3 antibody	Proteintech	25537-1-AP
anti-IGF2BP1 antibody	Proteintech	22803-1-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
Mito Tracker Red	Beyotime	C1032

Table S3. Sequences of primers

Target genes		Forward primer	Reverse primer
Human			
	Notch1	5′-TGCGACACCAACCCTGTCAAT-3′	5′-CGTCGATCTCGCATCGGGG-3′
Mouse			
	Notch1	5′-ACACCGTGTAAGAATGCTGGA-3′	5′-GCCTGCTGACATGATTTTCCTG-3′
	Notch2	5′-GAGAAAAACCGCTGTCAGAATGG-3′	5′-GGTGGAGTATTGGCAGTCCTC-3′
	Notch3	5′-AGTGCCGATCTGGTACAACTT-3′	5′-CACTACGGGGTTCTCACACA-3′
	Notch4	5′-GAACGCGACATCAACGAGTG-3′	5′-GGAACCCAAGGTGTTATGGCA-3′
	Drp1	5′-CCTCAGATCGTCGTAGTGGGA-3′	5′-GTTCCTCTGGGAAGAAGGTCC-3
	MID49	5′-TCGATTGGTGCTAGGTGTG-3	5′-GTCATCCTCATCTGGAGGG-3′
	Mfn1	5′-GTAGACAGCCCAGGTACAG-3′	5′-AACAAAGACATCAGCATCAAGG-3′
	Mfn2	5′-GGTGGAAATACAGGGCTACAG-3	5′-ACACTCAGGAAGCAGTTGG-3′
	NRF1	5′-AACTGTGAAGCTGTCCAGGG-3′	5′-TGCTGCTGGGATCTTGCTTT-3′
	TFAM	5′-AACACCCAGATGCAAAACTTTCA-3′	5′-GACTTGGAGTTAGCTGCTCTTT-3′
	PINK1	5′-GGCTTCCGTCTGGAGGATTAT-3′	5′-AACCTGCCGAGATATTCCACA-3′
	Parkin	5′-AATGTGGAGCACACCCAACC-3′	5′-ACGACCTATTGCACAGTCCT-3′
	Beclin 1	5′-ATGGAGGGGTCTAAGGCGTC-3′	5′-TCCTCTCCTGAGTTAGCCTCT-3′
	P62(Sqstm1)	5′-GAACTCGCTATAAGTGCAGTGT-3′	5′-AGAGAAGCTATCAGAGAGGTGG-3′
	Bax	5′-AGGCCTCCTCTCCTACTTCG-3′	5′-CCTTTCCCCTTCCCCCATTC-3′
	BCL-2	5′-AACATCGCCCTGTGGATGAC-3′	5′-TATGCACCCAGAGTGATGCAG-3′
	NLRP3	5′-ATCAACAGGCGAGACCTCTG-3′	5′-GTCCTCCTGGCATACCATAGA-3′
	GSDMD	5′-CAGTTCCAGTGCCTCCATGA-3′	5′-AAACAGGTCATCCCCACGAC-3′
	Hes1	5′-CCAAGTGTGCTGGGGAAGTA-3′	5′-CACCTCGGTATTAACGCCCT-3′
	Hey1	5′-CTGAGCAAAGCGTTGACA-3′	5′-TCCACCAACACTCCAAA-3′
	MFF	5′-ATGTACTTGAGTGGCAGCCT-3	5′-TCACGACGAACCAGGAAGAA-3′
	MID51	5′-TCTATGATGATCTGCAGGTGG-3	5′-ATGACCACAGGTTCTGCTC-3’
	Fis1	5′-AGAGCACGCAATTTGAATATGCC-3	5′-ATAGTCCCGCTGTTCCTCTTT-3
	PCNA	5′-TGGAATCCCAGAACAGGAG-3′	5′-TCAGAGCAAACGTTAGGTG-3′
	POSTN	5′-GAAGTGATCCACGGAGAGCC-3′	5′-TGTTTCTCCACCTCCTGTGG-3′
	Collagen I	5′-CGATGGATTCCCGTTCGAGT-3′	5′-CGATCTCGTTGGATCCCTGG-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′

Table S4. Sequences of IP-specific primers and *Transfection*

	Target genes	site	Product	Forward primer	Reverse primer
Notch1-qPCR					
Mouse					
	Notch1-Site 1	568-672	105	5′-GTCAGATGATGTGGTTTGTACAC-3′	5′-GCAGACTCACGACTGGTACT-3′
	Notch1-Mut			5′-TTAGAAACACGCCCAGACCTT-3′	5′-CTCTGCACATGCTGAGGACAC-3′
	Notch1-Site 2	815-943	129	5′-TCTGTGCATCCCTCCAAACAG-3′	5′-TACTCTGGCGAGTCCACAATG-3′
	Notch1-Site 3	1188-1300	113	5′-GTGTCGTGTGTCAAGCTGATG-3′	5′-ATCCTGGGTTGTGCTCTTAGG-3′
Drp1-promoter region					
Mouse					
	Primer 1	16176882-16177182	301	5′-CACGCGCCACGACAGA-3′	5′-GGATTTCACTATGTAGTACAGACCT-3′
	Primer 2	16177159-16177489	331	5′-GGTCTGTACTACATAGTGAAATCCT-3′	5′-GGCTGGAGCGGTAGAAATGT-3′
	Primer 3	16177474-16177859	386	5′-TTCTACCGCTCCAGCCTACC-3′	5′-TAGGACTCCTTCTTCCCACCTA-3′
	Primer 4	16177853-16178148	296	5′-AGTCCTAGAAAGGAAGGGGGAA-3′	5′-AACCAGCCACCATCTGTGTT-3′
	Primer 5	16178129-16178447	319	5′-AACACAGATGGTGGCTGGTT-3′	5′-CCAAACTAGCTCTTAGCACACA-3′
	Primer 6	16178411-16178613	203	5′-TCCTTTGTCTTTTACTGTGTGCT-3′	5′-CTTAAGCACTGCCTGTGGGA-3′
	Primer 7	16178626-16179015	390	5′-TACATTACTGCCAGGATTGCCC-3′	5′-GAGCATTTTATCACCCCCACC-3′
Transfection					
Mouse					
	NC			5′-UUCUCCGAACGUGUCACGUTT-3′	5′-ACGUGACACGUUCGGAGAATT-3′
	siRNA-Notch1			5′-CGGUCAGGAUAACACAUGUTT-3′	5′-ACAUGUGUUAUCCUGACCGTT-3′
	siRNA-Drp1			5′-GAATGAGGCCTTAGAGTTA-3	
	siRNA-METTL3			5′-UACACCACCUCUCAGAUCUTT-3′	5′-AGAUCUGAGAGGUGGUGUAGC-3′
	siRNA-METTL14			5′-GACCTTGGAAGAGTGTGTTTACG-3′	5′-CTTTGATCCCCATGAGGCAGT-3′
	siRNA-WTAP			5′-GCAAGAGUGUACUACUCAATT-3	
	siRNA-YTHDF1			5′-GCGUAAGGAAAGCAAGAAUTT-3′	5′-AUUCUGUCUUUCCUUACGCTT-3′
	siRNA-YTHDF2			5′-GGACGUUCCCAAUAGCCAATT-3′	5′-UUGGCUAUUGGGAACGUCCUU-3′
	siRNA-ALKBH5			5′-CUGCGCAACAAGUACUUCUTT-3	

Table S5. Echo analysis data —— Figure S1K- db/+ vs db/db

Characteristics	db/+	db/db	P Value
Cases (*n*)	6	6	
LVIDd(mm)	3.72483±0.330849	4.23833±0.110815	0.014612
LVIDs(mm)	2.28567±0.316154	3.22700±0.134802	0.001897
LVEF(%)	72.8333±2.78687	52.5000±1.87083	0.000069
LVFS(%)	36.31317±2.955793	30.13967±1.591229	0.001852
E/A	1.5060±0.04355	1.9035±0.02115	0.000009

Table S6. Echo analysis data —— Figure S1L-STZ+HFD+WT vs STZ+HFD+cKO-Notch1

Characteristics	STZ+HFD+WT	STZ+HFD+cKO-Notch1	P Value
Cases (*n*)	6	6	
LVIDd(mm)	4.09000±0..112205	4.64883±0.103619	0.000599
LVIDs(mm)	3.26400±0.114261	3.97017±0.080024	0.000010
LVEF(%)	54.6667±2.65832	43.3333±2.16025	0.000882
LVFS(%)	29.51683±1.164923	24.08950±1.152029	0.000062
E/A	1.8552±0.05420	1.9472±0.03752	0.019511

Table S7. Echo analysis data —— Figure S2J- AAV9-POSTN-NC/Notch1 cKO vs AAV9-POSTN-oeNotch1-NICD/Notch1 cKO

Characteristics	AAV9-POSTN-NC/Notch1 Cko	AAV9-POSTN-oeNotch1-NICD/Notch1 cKO	P Value
Cases (*n*)	6	6	
LVIDd(mm)	4.01383±0.082754	3.76900±0.194200	0.033812
LVIDs(mm)	2.83217±0.032682	2.32400±0.156233	0.000204
LVEF(%)	44.0000±2.60768	69.1667±3.54495	0.000060
LVFS(%)	22.94717±3.222722	39.62883±3.604105	0.000411
E/A	1.9193±0.02945	1.5468±0.02691	0.000036

Table S8. Echo analysis data —— Figure S2J- AAV9-Postn-NC/ db/db vs AAV9-Postn-oeNotch1-NICD/ db/db

Characteristics	AAV9-Postn-NC/ db/db	AAV9-Postn-oeNotch1-NICD/ db/db	P Value
Cases (*n*)	6	6	
LVIDd(mm)	4.01717±0.156735	3.76867±0.163765	0.044360
LVIDs(mm)	2.75117±0.073969	2.39933±0.061383	0.000431
LVEF(%)	49.8333±3.31160	64.5000±3.93700	0.001582
LVFS(%)	27.14783±1.754194	34.98167±2.228482	0.000659
E/A	1.8735±0.02672	1.5305±0.08650	0.000068

Table S9. Echo analysis data —— Figure S6E 16-week-old AAV9-Postn-NC vs AAV9-Postn-oeALKBH5

Characteristics	AAV9-Postn-NC	AAV9-Postn-oeALKBH5	P Value
Cases (*n*)	6	6	
LVIDd(mm)	3.91033±0.061020	3.67867±0.092385	0.002028
LVIDs(mm)	2.567833±0.0682156	2.34083±0.082228	0.008720
LVEF(%)	54.0000±2.82843	65.8333±3.65605	0.003539
LVFS(%)	28.74133±0.873964	35.958167±2.3856754	0.002411
E/A	1.9168±0.03026	1.5642±0.03380	0.000010

Table S10. Echo analysis data —— Figure S6E 16-week-old AAV9-Postn-NC vs AAV9-Postn-shYTHDF2

Characteristics	AAV9-Postn-NC	AAV9-Postn-shYTHDF2	P Value
Cases (*n*)	6	6	
LVIDd(mm)	4.01967±0.164348	3.71900±0.167450	0.018528
LVIDs(mm)	2.69867±0.098413	2.29450±0.108896	0.000072
LVEF(%)	48.5000±3.39116	70.8333±3.43026	0.000026
LVFS(%)	27.30250±1.343866	37.69033±1.267243	0.000001
E/A	1.9037±0.03110	1.5690±0.04635	0.000069

Table S11. Echo analysis data —— Figure S6F 13-week-old AAV9-Postn-NC vs AAV9-Postn-oeALKBH5

Characteristics	AAV9-Postn-NC	AAV9-Postn-oeALKBH5	P Value
Cases (*n*)	6	6	
LVIDd(mm)	3.86817±0.078443	3.76900±0.095396	0.075721
LVIDs(mm)	2.34917±0.103037	2.33867±0.101202	0.867287
LVEF(%)	64.6667±4.22690	66.0000±8.27043	0.633598
LVFS(%)	37.28267±1.853383	37.54800±1.185610	0.683924
E/A	1.4020±0.10824	1.4798±0.03912	0.073516

Table S12. Echo analysis data —— Figure S6F 13-week-old AAV9-Postn-NC vs AAV9-Postn-shYTHDF2

Characteristics	AAV9-Postn-NC	AAV9-Postn-shYTHDF2	P Value
Cases (*n*)	6	6	
LVIDd(mm)	3.69933±0.149244	3.666833±0.1033391	0.554103
LVIDs(mm)	2.33600±0.060166	2.27950±0.079822	0.084973
LVEF(%)	66.33333±4.54606	69.00000±2.756810	0.186973
LVFS(%)	36.79450±1.739068	37.43883±1.953703	0.408957
E/A	1.4400±0.07898	1.457333±0.0483101	0.429309

Table S13. Echo analysis data —— Figure S6I AAV9-Postn-NC/oe ALKBH5 vs AAV9-Postn-shNotch1/oe ALKBH5

Characteristics	AAV9-Postn-NC/oe ALKBH5	AAV9-Postn-shNotch1/oe ALKBH5	P Value
Cases (*n*)	6	6	
LVIDd(mm)	3.56500±0.057699	3.97383±0.115761	0.000616
LVIDs(mm)	2.12183±0.107354	2.54083±.099839	0.002334
LVEF(%)	74.0000±2.36643	51.6667±2.16025	0.000004
LVFS(%)	40.65250±1.059901	28.75400±0.945580	0.000002
E/A	1.5122±0.03942	1.9132±0.01981	0.00809

Table S14. Echo analysis data —— Figure S6J AV9-Postn-NC/sh YTHDF2 vs AAV9-Postn-shNotch1/ sh YTHDF2

Characteristics	AV9-Postn-NC/oe ALKBH5	AAV9-Postn-shNotch1/ sh YTHDF2	P Value
Cases (*n*)	6	6	
LVIDd(mm)	3.58583±0.041441	3.96000±0.091564	0.000002
LVIDs(mm)	2.30033±.070165	2.66417±0.058150	0.000190
LVEF(%)	72.6667±2.16025	53.1667±2.48328	0.000015
LVFS(%)	40.676167±1.3927038	28.685833±.08951705	0.000014
E/A	1.5135±0.04374	1.9033±0.03401	0.000016