ORIGINAL RESEARCH ARTICLE

Jmjd4 Facilitates Pkm2 Degradation in Cardiomyocytes and Is Protective Against Dilated Cardiomyopathy

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BACKGROUND: A large portion of idiopathic and familial dilated cardiomyopathy (DCM) cases have no obvious causal genetic variant. Although altered response to metabolic stress has been implicated, the molecular mechanisms underlying the pathogenesis of DCM remain elusive. The JMJD family proteins, initially identified as histone deacetylases, have been shown to be involved in many cardiovascular diseases. Despite their increasingly diverse functions, whether JMJD family members play a role in DCM remains unclear.

METHODS: We examined Jmjd4 expression in patients with DCM, and conditionally deleted and overexpressed Jmjd4 in cardiomyocytes in vivo to investigate its role in DCM. RNA sequencing, metabolites profiling, and mass spectrometry were used to dissect the molecular mechanism of Jmjd4-regulating cardiac metabolism and hypertrophy.

RESULTS: We found that expression of Jmjd4 is significantly decreased in hearts of patients with DCM. Induced cardiomyocyte-specific deletion of Jmjd4 led to spontaneous DCM with severely impaired mitochondrial respiration. Pkm2, the less active pyruvate kinase compared with Pkm1, which is normally absent in healthy adult cardiomyocytes but elevated in cardiomyopathy, was found to be drastically accumulated in hearts with Jmjd4 deleted. Jmjd4 was found mechanistically to interact with Hsp70 to mediate degradation of Pkm2 through chaperone-mediated autophagy, which is dependent on hydroxylation of K66 of Pkm2 by Jmjd4. By enhancing the enzymatic activity of the abundant but less active Pkm2, TEPP-46, a Pkm2 agonist, showed a significant therapeutic effect on DCM induced by Jmjd4 deficiency, and heart failure induced by pressure overload, as well.

CONCLUSION: Our results identified a novel role of Jmjd4 in maintaining metabolic homeostasis in adult cardiomyocytes by degrading Pkm2 and suggest that Jmjd4 and Pkm2 may be therapeutically targeted to treat DCM, and other cardiac diseases with metabolic dysfunction, as well.

Key Words: cardiomyopathy, dilated, chaperone-mediated autophagy, HSP70 heat-shock proteins, hydroxylation, JMJD4 protein, human, Pkm protein, mouse

Dilated cardiomyopathy (DCM) is the most common subtype of cardiomyopathy, which is estimated to affect 1 in 250 to 500 individuals. It is characterized by ventricular enlargement and impaired contractile function that led to increased morbidity despite treatment, making it a leading cause for heart transplantation. DCM represents a heterogeneous group of patients, because it results from a variety of genetic and acquired triggers.
Among the idiopathic DCM cases, 20% to 35% are familial, with autosomal dominant inheritance in most cases.6 More than 400 potentially causative mutations in 60 genes both in familial and sporadic DCM cases have been identified,6 and genes in mitochondrial metabolism are a major subgroup of these DCM-associated genes,5–8 indicating that defects in metabolism and mitochondrial function may contribute to DCM. However, little is known about upstream control points contributing to dysfunctional energy metabolism, especially in idiopathic DCM.

One of the nodal points of energy metabolism is pyruvate kinase that catalyzes the conversion of phosphoenolpyruvate to pyruvate, the last step of glycolysis. In mammals, there are 4 pyruvate kinase isoforms (PKM1, PKM2, PKL, and PKR). PKM1 and PKM2 are alternative splice isoforms of the PKM gene and differ only by the inclusion of 1 mutually exclusive exon.9 PKM1 is present in cardiomyocytes and other terminally differentiated tissues with high energy demand, and PKM2 is present in tissues with anabolic functions, proliferating cells, and cancer cells.10,11 PKM1 has constitutively high catalytic activity, whereas PKM2 enzyme activity is subject to complex allosteric regulation,12 which allows cells to switch between glycolysis and biosynthesis.11 Pkm2 was recently found to promote proliferation of cardiomyocytes,13 but it is still unknown whether it plays a role in DCM and what regulates Pkm2 expression and activity in cardiomyocytes.

Epigenetics has recently emerged as a mechanism important in the development of various cardiac diseases, including ischemia, DCM, and heart failure. Drugs targeting epigenetic modifiers, such as histone deacetylase inhibitors, have been shown to be effective in the treatment of cardiomyopathy in mouse and human studies.14,15 With the increasing number of epigenetic modifications and regulators identified, elucidating the function of such regulators in the heart will be crucial to understanding and treating heart diseases. JMJD (Jumonji C domain-containing) proteins have been characterized as epigenetic regulators that demethylate histones,16 and recent studies have implicated the roles of JMJD proteins in cardiac hypertrophy and fibrosis.17,18 Although the large JMJD (molecular weight>100 kDa) proteins are mostly histone demethylases, thus renamed as histone lysine demethylase family members, the small JMJD proteins mainly hydroxylate a broad range of proteins, and evidence for their histone demethylase activity is still scarce.16 Proteolytic (Jmjd5 and Jmjd7) and tyrosine kinase (Jmjd6) activities have been reported, setting the small JMJD proteins further apart from the other JMJD family members.19,20 Jmjd4, one of the small JMJD family proteins, was shown to hydroxylate a lysine residue of eukaryotic release factor 1 (eRF1) to facilitate translational termination efficiency12 without known function of histone modifications. Whether Jmjd4 plays a role in energy metabolism in cardiomyocytes and DCM has not been investigated.

Here, we generated mice with an inducible cardiomyocyte-specific deletion of Jmjd4 using Myh6-CreERT222 and found that mice with Jmjd4 ablated in adult cardiomyocytes rapidly developed DCM, and energy metabolism was severely disrupted. Exploration of how Jmjd4 regulates metabolism in cardiomyocyte uncovered novel
molecular mechanisms and possible therapeutic targets for treating DCM and other cardiac diseases with metabolic defects.

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

Data and Materials Availability

All echocardiography data associated with this study are present in this article or the Supplemental Material. RNA sequencing data generated in this study have been deposited into the Gene Expression Omnibus database under the accession number GSE211955. Original FASTQ data have been deposited into the Sequence Read Archive database under the accession number PRJNA873050.

Human Samples

All human studies were conducted in accordance with the official ethical guidelines and protocols approved by the Ethics Committee of Soochow University, and informed written consent was obtained from all subjects. The investigation further conformed to the principles outlined in the Declaration of Helsinki.

Compounds

CHIR-99021 (S1263), IWP-2 (S7085), TEPP-46 (S7032), QX-77 (S6797), CMC-Na (S6703), MG-182 (S2619), leupeptin (S7380), Bat-A1 (S1413), E64d (S7393), cycloheximide (NSC-185) were purchased from Selleck. Phenylephrine hydrochloride (R815791) was purchased from MACKLIN. Phenylephrine hydrochloride (R815791) was purchased from MACKLIN.

Antibodies

Antibodies used in this study were: GAPDH (CST, 5174S), Histone 3 (Abcam, ab1791), β-Actin (CST, 4970T), Jmjd4 (Sigma, HPA027260), Jmjd4 (Thermo, PA6-96889), Jmjd4 (Santa Cruz, sc-514881), Jmjd4 (Proteintech, 21572-1-AP), Pkm2 (CST, 4053s), Pkm1 (CST, 7067T), atrial natriuretic peptide (Proteintech, 19856-1-AP), hemagglutinin (HA; Thermo, 26183), FLAG (Sigma, F7425), Hsp70 (Abcam, ab51052).

Plasmids and Virus

PCR-amplified mouse Jmjd4 was cloned into pcDNA3.1-FLAG as previously described. Full-length construct of pcDNA-HA-Pkm2, pcDNA-HA-GFP, pcDNA-FLAG-Jmjd4, pcDNA-FLAG-GFP, N- and C-terminal–truncated constructs of Pkm2 and Jmjd4 were generated from the full-length constructs through polymerase chain reaction (PCR) with specific primers and inserted into pcDNA3.1-HA and pcDNA3.1-FLAG, respectively, yielding HA-Pkm2 and FLAG-Jmjd4 truncation mutants. Serotype 9 adeno-associated virus (AAV9)-Jmjd4 and AAV9-Pkm1 were generated through cloning full-length mouse Jmjd4 with PCR with specific primers into AAV9 vector from AAV9-cTNT-hYAP construct (Addgene, No. 86558). AAV9 virus was packaged in Shanghai Taitool Bioscience Co. Ltd.

Animals and Experimental Models

All animal experiments in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences and the National Institutes of Health. Animal protocols and performances were approved by the Animal Care and Use Committee of Tongji University and Use Committee at Center for Animal Experiment, medical school of Tongji University. Animals received humane care, and the abuse of animals was avoided.

Jmjd41/1 mice were constructed by Beijing ViewSoid Biotech Co., Ltd. Jmjd4OE-Flox mice (OE) were constructed by Shanghai Model Organisms Center, Inc. Tg(Myh6-cre/Esr1)1Jmk/J (MCM) were purchased from Jax(005657). Four-week-old mice were injected intraperitoneally once per day with vehicle (oil) or tamoxifen (75 mg/kg) for 5 consecutive days to induce CreERT2 activity. Mice (8–10 weeks old, 21–25 g body weight) were subjected to transverse aortic constriction (TAC) under pentobarbital anesthesia (1%) as previously described. The chest cavity was exposed by cutting open the proximal portion of the sternum. After the aortic arch between the innominate and left common carotid arteries was isolated, it was constricted with a 7-0 nylon suture tied firmly 3 times against a 25-gauge blunted needle for TAC. The needle was immediately withdrawn after the ligation. Sham-operated mice were subjected to identical interventions, with the exception of the constriction of the aorta. After echocardiographic analysis at different time points, mice were euthanized by cervical dislocation, and hearts were removed and weighed promptly.

We used the PASS software (V15.0.5) to determine the group size for animal experiments. For 2-group analysis, we chose the 2-sample t tests assuming equal variance method and assumed 20% minimum detectable difference, 5% SD, 80% power, and 5% type I error rate. The calculated result indicates that n=3 is the minimal number of animals for each group. For multiple group analysis, we chose the multiple comparisons method and assumed 4 groups, 20% minimum detectable difference, 5% SD, 80% power, and 5% type I error rate. The calculated result indicates that n=5 is the minimal number of animals for each group, and most experiments in animals were performed with >8 animals to ensure statistical power.

The MCM; Jmjd41/1 mice were given TEPP-46 (10 mg/kg) by oral gavage once a day for 2 weeks after tamoxifen inducement. The wild-type mice were given TEPP-46 (10 mg/kg) by oral gavage once a day for 2 weeks starting from 2 weeks after the TAC operation.

All animals used for experiments were selected by their cardiac function baseline, male mice weighing >25g, and ejection fraction >50%, and they were randomly assigned to different experimental groups by using a random number generator. Animals succumbing during experiments were excluded from analysis, except for the survival analysis. For echocardiography and TAC model surgery, the operator and data analyzer were both blinded to the allocation and group information. The data analyzer was blinded to the group information. In all blinded experiments, a supervisor was assigned to manage the whole process and uncover the final results.

Sequences of genotyping primers are listed in Table S1.
Cell Culture and Transfection

Human pluripotent stem cell–derived cardiomyocytes (hPSC-CMs) were produced from human induced pluripotent stem cells ZSSY001 (NuVaccell) by regulating the Wnt signaling pathway as previously described. In brief, hPSCs were grown on Matrigel (Coming, 354277) in Essential 8 Medium (Gibco, A15169-01, A15171-01). Cardiac differentiation was performed using dense monolayers of hPSCs treated with 6 μmol/L CHIR-99021 (Selleck, S1263) for 48 hours and 5 μmol/L IWP-2 (Sellect, S7065) for 2 days in RPMI 1640 (Gibco, 11875-093)/B-27 minus insulin Medium (Gibco, A18965-01) according to previously described protocol.

Spontaneously beating hPSC-CMs appeared at day 7. Then, the containing medium was replaced with RPMI 1640/B-27 Medium (Gibco, A17504-044). Metabolism selection was performed using Cardiomyocyte Selection Medium (ScienCell, 5911, 5962) for high-purity cardiomyocytes. Neonatal rat ventricular cardiomyocytes (NRVCs) were isolated from 1-day-old neonatal Sprague-Dawley rats (Shanghai Laboratory Animal Center, CAS) as previously described. In brief, neonatal rat's hearts were separated and dissociated in 0.25% trypsin (Gibco, 15090046) at 4°C overnight. The next day, rat tissue was digested in 0.1% collagenase type I (Worthington, LS004176) at 37°C. NRVCs were collected and seeded on collagen in DMEM medium containing 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. Twenty-four hours later, the medium was replaced with cardiomyocyte medium: DMEM:F12 (1:1) medium (Gibco, 11330032) supplemented with 0.25% fetal bovine serum, 3 mmol/L sodium pyruvate, 0.1 mmol/L Vitamin C, and 2 mmol/L L-glutamine (Gibco, 25030081).

The 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum. Transfection of cells was performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were harvested 24 hours posttransfection and washed in phosphate-buffered saline for further RNA extraction and 48 hours for whole-cell protein extraction. The sequences of the small interfering RNA used are listed in Table S2.

AAV9 viral infection of NRVCs was completed in suspension immediately after cell isolation as described previously. NRVCs were infected with the AAV9 virus at a multiplicity of infection of 100 000 for 40 hours. NRVCs were fixed for staining or lysed for protein extraction at 48 hours after removing virus from cardiomyocytes.

Proteomic and Metabolomics Analysis

Protein samples were electroplated with SDS-PAGE gels, and gel pieces were cut, destained with 30% acetonitrile/100 mmol/L NH₄HCO₃ until the gels were clear. The gels were dried in a vacuum centrifuge. The in-gel proteins were reduced with dithiothreitol (10 mmol/L dithiothreitol/100 mmol/L NH₄HCO₃) for 30 minutes at 56°C, then alkylated with iodoacetamide (200 mmol/L iodoacetamide/100 mmol/L NH₄HCO₃) in the dark at room temperature for 30 minutes. Gel pieces were briefly rinsed with 100 mmol/L NH₄HCO₃ and acetonitrile and digested overnight in 12.5 ng/μL trypsin in 25 mmol/L NH₄HCO₃. The peptides were extracted 3 times with 60% acetonitrile/0.1% trifluoroacetic acid. The extracts were pooled and dried completely by a vacuum centrifuge. Liquid chromatography with tandem mass spectrometry analysis was performed on a Q Exactive Exact mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific) for 60 minutes. The mass spectrometer was operated in positive ion mode. Mass spectrometry data were acquired using a data-dependent top 10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for higher-energy collisional dissociation fragmentation. Automatic gain control target was set to 3e6, and maximum inject time to 10 ms. Dynamic exclusion duration was 400 s. Survey scans were acquired at a resolution of 70 000 at m/z 200 and resolution for higher-energy collisional dissociation spectra was set to 17 500 at m/z 200.

For hydroxylation site MS detection, to avoid nonspecific hydroxylation site detection, JMJD4 enzymatic mutation H194A was used. Empty vector, wild-type JMJD4, JMJD4-H194A, and HA-PKM2 plasmids were transfected to 293T cells, HA magnetic beads were used to purify proteins. Protein samples were digested with Glu-C before tandem mass spectrometry; the remaining steps are as described above and analyzed at Shanghai iProteome Biotechnology.

For targeted metabolomics analysis, apex tissue from mice were analyzed at Applied Protein Technology (Shanghai).

Histological and Immunofluorescence Staining

Histological and immunofluorescence staining were performed on heart tissues of mice that were fixed in 4% paraformaldehyde at room temperature, embedded in paraffin, and serially sectioned at 5-µm thickness. Standard hematoxylin and eosin and Masson staining were performed on these sections to evaluate histopathology. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using the TUNEL Apoptosis Detection Kit (Alexa Fluor 488; YEASEN, 40307ES20) according to the manufacturer's instructions. Histopathological images were acquired with Nikon ECLIPSE E100. For immunofluorescence analyses, heart sections and fixed cells were incubated with primary antibodies overnight followed by incubation with corresponding secondary antibody. The dilutions were 1:50 for primary antibody and 1:500 for secondary antibody. NRVCs or hPSC-CMs seeded in 96-well plates (ThermoFisher, 167008) or 8 Chambered Coverglass System (Cellvis, C8-1.5H-N) were fixed with 4% paraformaldehyde at
4 °C overnight. Punctured with 0.5% Triton-X and 2% bovine serum albumin, cells were incubated with primary antibodies at room temperature or overnight at 4 °C. Primary antibodies were detected with appropriate secondary antibodies (ThermoFisher) and 4’,6-diamidino-2-phenylindole (Mbpio, 157574). Imaging was conducted using fluorescence microscopy (Zeiss, Axio Vert A1 or Leica TCS-SP8 SR) and analyzed using CellProfiler software or PerkinElmer Opera Phenix.

**Echocardiography**

Two-dimensional guided M-mode echocardiography was performed using a high-resolution imaging system (Vevo 2100, Visual-Sonics Inc., Toronto, Canada). Mice were anesthetized with the isoflurane, keeping the body temperature at 37 °C during the study. Parasternal long-axis view was acquired in heart rate monitored from 470 to 510 bpm. Left ventricular (LV) cavity size and wall thickness are measured in at least 3 beats from each projection. Averaged LV diastolic and systolic anterior wall thickness (LVAWd, LVAWs), LV diastolic and systolic posterior wall thickness (LVPWd, LVPWs), and LV diastolic and systolic internal dimensions (LVIDd, LVIDs) are measured. LV fractional shortening (FS) \((\text{LVIDd} - \text{LVIDs})/\text{LVIDd}\) and LV mass \((\text{LVIDd} + \text{LVPWd} + \text{LVAWd})/3 - \text{LVIDd}/3\) are calculated from the M-mode measurements. LV ejection fraction (EF) was calculated from the LV long-sectional area. The studies and analysis were performed blinded as to experimental groups. Raw data of all echocardiography were provided in Tables S3 through S11.

**Immunoprecipitation and Western Blot Assays**

Cell lysates used for immunoprecipitation assay were extracted in radioimmunoprecipitation assay (Epizyme, PC103) with cocktail (Epizyme, GRF 101) mixed buffer at 4 °C. Supernatants were clarified by centrifugation at top speed (16,000 g) for 30 minutes at 4 °C. The supernatants (1 mL) were incubated with 20 µL anti-FLAG magnetic beads (Bimake, B26101) or anti-HA magnetic beads (Bimake, B26201) at 4 °C overnight. The beads were washed 3 times with Tris-buffered saline supplemented with 0.1% Tween 20. Elution was performed by incubating the washed beads in 30 µL of 7.5 mmol/L Tris·HCl buffer (pH 8.0), 50 mmol/L dithiothreitol, and 1× SDS sample buffer (Epizyme, LT101) at 100 °C for 10 minutes. The input lysates and immunoprecipitates were separated by 5% to 15% SDS-PAGE and analyzed by light chromatography–tandem MS or Western blotting assay. Protein samples from total cell extracts, PAGE and analyzed by light chromatography–tandem MS or 1% agarose gels. RNA sequencing work was completed by Novogene.

**Mitochondrial Respiration Assay**

NRVCs or hPSC-CMs were seeded onto a Seahorse XF96 Cell Culture Microplate (Agilent) at a density of 4.0 (NRVCs) or 2.0 (hPSC-CMs) x 10^4 cells/well and allowed to adhere for 24 hours in 0.25% fetal bovine serum DMEM/F12 (NRVCs) or B27 medium (hPSC-CMs) at 37 °C and 5% CO₂. Mitochondrial oxygen consumption rates (OCRs) were determined using the Seahorse XF96 Analyzer (Agilent). The following drugs were injected at the final concentrations given: oligomycin (1 mmol/L), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (2 mmol/L), and rotenone (1 mmol/L) and antimycin A (1 mmol/L). Basal OCR was determined by subtracting the OCR measured after the addition of rotenone/antimycin A from the value before the addition of oligomycin. Maximal OCR was calculated by subtracting the OCR measured after the addition of rotenone/antimycin A from the value in the presence of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone.

The mitochondria from heart tissue were extracted using mitochondria extraction kit (Abcam 110168). Mitochondrial respiration was monitored using a Clark-type oxygen electrode coupled in a sealed glass chamber connected to a 782 Oxygen Meter (Strathkelvin Instruments), and respiratory parameters were determined according to Chance and Williams. One milligram of mitochondrial proteins was added to 1 mL of respiration buffer containing 125 mmol/L sucrose, 55 mmol/L KCl and 10 mmol/L HEPES-KOH (pH 7.4) plus 0.5 mmol/L/EGTA, and 10 mmol/L KH₂PO₄ at 308C. To measure OCRs, 50 µg of mitochondrial protein was added to each chamber. To assess respiration on substrates, different substrates combination, 50 µmol/L palmityl-β-carnitine + 2 mmol/L malate ± 2.5 mmol/L ADP + Mg²⁺ or 10 mmol/L glutamate + 2 mmol/L malate ± 2.5 mmol/L ADP + Mg²⁺ or 5 mol/L succinate + 2.5 mmol/L ADP + Mg²⁺ or 5 mol/L pyruvate + 2 mmol/L malate + 2.5 mmol/L ADP were used. OCRs were measured as picomoles O₂ s⁻¹ mg⁻¹ mitochondrial protein. Oxygen consumption rates were calculated using computer software (Strathkelvin Oxygen 782 System, Ver 3.0).

**Quantitative Real-Time Polymerase Chain Reaction**

RNA was extracted using TRIzol extraction reagent (Invitrogen, 15596018). Complementary DNA libraries were synthesized using the PrimeScript RT Reagent Kit (Takara, RR047A). We used 200 ng of cDNA for quantitative real-time polymerase chain reaction (qRT-PCR) performed using CFX384 Real-Time Systems (Bio-Rad, C1000, Touch), and the reactions were performed using SYBR Green Premix Ex Taq (Takara, AK8806). Sequences of qRT-PCR primers are listed in Table S1.

**RNA Sequencing**

Cardiomyocytes were collected and RNA was purified using TRIzol extraction reagent (Invitrogen, 15596018). The sequencing work was completed by Novogene. For RNA quantification and qualification, RNA degradation and contamination were monitored on 1% agarose gels. RNA
Figure 1. Deleting Jmjd4 in adult cardiomyocytes causes progressive dilated cardiomyopathy and heart failure.

A, Western blot and quantification of JMJD4 protein levels in the extract of heart tissues from human patients with DCM and healthy donors (n=8 healthy donors for donor group, n=8 patients for DCM group).

B, Strategy of generating a floxed allele of Jmjd4 in mouse. Maps of the WT Jmjd4 locus, the floxed allele, and the excised allele are shown. Exons are shown in bars.

C, Schematic of tamoxifen-induced Jmjd4 knockout in the adult MCM+; Jmjd4f/f mouse hearts.

D, Quantitative real-time polymerase chain reaction analysis of Jmjd4 mRNA levels in the heart tissue of MCM+, MCM+, and MCM+, Jmjd4f/f mice (n=7 animals per group). Data from MCM+; Jmjd4f/f mice in D through (Continued)
purity was checked using the NanoPhotometer spectrophotometer (IMPLEN). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies).

For library preparation for transcriptome sequencing, a total amount of 1 µg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext UltraTM DNA Library Prep Kit for Illumina (New England BioLabs) following manufacturer’s recommendations, and index codes were added to attribute sequences to each sample. In brief, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was performed using divalent cations under elevated temperature in NEBNext First Stranded Synthesis Reaction Buffer (5X). First-strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second-strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends through exo-nuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure was ligated to prepare for hybridization. To select cDNA fragments of preferentially 250 to 300 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter). Then 3 µL USER Enzyme (New England BioLabs) was used with size-selected, adapter-ligated cDNA at 37°C for 15 minutes followed by 5 minutes at 95°C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

For clustering and sequencing, the clustering of index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot HS (Illumia) according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated.

On the basis of Illumina Novaseq platform in paired-end mode, raw fastq data were received followed by removing low-quality reads and adapters. For quality control, raw data (raw reads) of fastq format were first processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N, and low-quality reads from raw data. At the same time, Q20, Q30, and GC content the clean data were calculated. All the downstream analyses were based on the clean data with high quality.

Clean data were mapped by hisat2 and transcripts were assembled by stringtie. In brief, reference genome and gene model annotation files were downloaded from the genome website directly. Index of the reference genome was built using hisat2 v2.0.5, and paired-end clean reads were aligned to the reference genome using hisat2 v2.0.5. We selected hisat2 as the mapping tool so that hisat2 can generate a database of splice junctions on the basis of the gene model annotation file and thus produce a better mapping result than other non-splice-mapping tools.

Differential expression analysis of the genes expression matrix was performed using the edgeR R package (version 3.4.4). In brief, before differential gene expression analysis, for each sequenced library, the read counts were adjusted by edgeR program package through one scaling normalized factor. Differential expression analysis of 2 conditions was performed using the edgeR package (3.18.1). The P values were adjusted using the Benjamini and Hochberg method. Corrected P value of 0.05 and absolute foldchange of 2 were set as the threshold for significantly differential expression.

RNA sequencing clean data are publicly accessible at Gene Expression Omnibus (GSE211955). The information on raw data obtained is also provided in Supplemental Material (Table S12).

Gene Ontology and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package, in which gene length bias was corrected. Gene Ontology terms with corrected P value <0.05 were considered significantly enriched by differential expressed genes. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism, and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome
sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). We used clusterProfiler R package to test the statistical enrichment of differential expression genes in KEGG pathways. Enrichment analysis results were also provided in Supplemental Material Excel Files (Files S1–S3).

Statistics
All experiments had at least 3 replicates. Results are expressed as mean±SEM. Unpaired t test, 1-way ANOVA, 2-way ANOVA, and log-rank (Mantel-Cox) test were used as appropriate using the Prism software (version 9.4.1). Differences at the level of P<0.05 were considered statistically significant.

RESULTS
Deleting Jmjd4 in Adult Cardiomyocytes Causes Progressive DCM and Heart Failure
To investigate whether JMJD4 plays a role in cardiac diseases like DCM, we examined the expression of JMJD4 protein in the ventricles of human patients with DCM, and healthy donors, as well (Table S13). JMJD4 protein level is significantly decreased in DCM hearts (Figure 1A), suggesting that it may be involved in heart disease progression. We then examined Jmjd4 protein level in adult mouse hearts after TAC and myocardial infarction and found that it is also decreased on these cardiac injuries (Figure S1A and S1B).

To examine the role of Jmjd4 in cardiac pathology, cardiomyocyte-specific inducible Jmjd4 knockout mice were created by generating a floxed allele of Jmjd4 (Figure S1A and S1B). 22 Myh6-CreERT2 crossing it with mice carrying Jmjd4+/f/f controls (Figure 1F, Figure S1F). 23 Jmjd4 loss-of-function, additional indicators of mitochondrial metabolism were enriched in commonly mitochondria, the intensity of which correlates to mitochondrial membrane potential, 36 we found that the mito-Jmjd4 deletion on the cardiac transcriptome. Gene Ontology term enrichment analyses of the differentially expressed genes downregulated in MCM+/f/f hearts (Figure 1H). Activation of hypertrophy response genes such as Nppa and Nppb, 23 together with reduced expression of Myh6 and increased expression of Myh7, and elevation of protein level of atrial natriuretic peptide, encoded by Nppa, were evident in the MCM+/f/f hearts (Figure 1I and 1J). MCM+/f/f hearts also have enlarged cardiomyocyte cross-sectional area measured by wheat germ agglutinin staining, and elevated cardiomyocyte death detected by TUNEL staining (Figure 1K and 1L), indicating a severe hypertrophic phenotype. 24 To corroborate with our findings in vivo, we knocked down Jmjd4 with small interfering RNA in NRVCs (Figure S2A and S2B) and hPSC-CMs (Figure S2C and S2D). Cardiomyocyte size was increased and cell death rate was elevated on small interfering (si-)Jmjd4 treatment versus si-negative control (NC) treatment (Figure 1M) in both rat and human cardiomyocytes (Figure 1M, Figure S2E), consistent with the phenotypes in MCM+/f/f mouse hearts. Taken together, these data demonstrate that the cardiomyocyte-specific deletion of Jmjd4 leads to hypertrophic response, cardiac dysfunction, and DCM.

Jmjd4 Is Required for Maintaining Oxidative Metabolism in Cardiomyocytes
RNA sequencing was performed on apical tissues of MCM+/f/f and control Jmjd4+/f/f hearts to assess the effect of Jmjd4 deletion on the cardiac transcriptome. We also analyzed the transcriptomic changes on Jmjd4 knockdown in NRVCs by RNA sequencing (File S2). Genes in mitochondrial metabolism were consistently enriched in downregulated differentially expressed genes on si-Jmjd4 treatment (Figure 2B, File S3), and similar mitochondrial metabolism pathways were enriched in commonly downregulated genes between the heart tissue and NRVCs (Figure 2C and 2D). Downregulation of genes in glycolysis, lipid metabolism, and mitochondrial biogenesis were confirmed by qRT-PCR (Figure S3A–S3C).

To confirm the effect on mitochondrial function with Jmjd4 loss-of-function, additional indicators of mitochondrial integrity, morphology, and function were analyzed. Mitochondrial membrane potential generated by respiratory chain complexes (I, III, and IV) is essential for mitochondrial ATP synthesis. 30 Using tetra-methylrhodamine ester, a cell-permeant dye that accumulates in polarized mitochondria, the intensity of which correlates to mitochondrial membrane potential, 36 we found that the mitochondria in si-Jmjd4–treated NRVCs were significantly depolarized (Figure 2E), and the ratio between mitochondrial area, measured by MitoTraker, 27 and cardiomyocytes area is significantly reduced by si-Jmjd4 treatment.
Jmjd4 Regulates Metabolism in Cardiomyocytes

Figure 2. Jmjd4 is required for maintaining oxidative metabolism in cardiomyocytes.

A, Gene ontology (GO) analysis of the differentially expressed genes (DEGs) that were downregulated in heart tissue of MCM++; Jmjd4ff mice 14 days after tamoxifen induction compared with Jmjd4ff controls (n=3 animals per group). B, GO analysis of DEGs that were downregulated in NRVCs transfected with si-Jmjd4 compared with si-NC, 48 hours after transfection (n=3 wells per group). C, Venn diagram showing overlap of genes downregulated in NRVCs with Jmjd4 knockdown and in MCM++; Jmjd4ff mice. D, GO analysis of the DEGs that were commonly downregulated in both NRVCs with Jmjd4 knockdown and MCM++; Jmjd4ff mice.

E, Baseline OCR (left) and OCR stimulated by pyruvate/malate (P/M), palmitoyl carnitine/malate (PC/M), or glutamate/malate (G/M) before or after the addition of ADP measured from isolated cardiac mitochondria from Jmjd4ff and MCM++; Jmjd4ff mice 14 days after tamoxifen induction (n=10 animals per group). F, Analysis and quantification of mitochondrial OCR in NRVCs 48 hours after transfection with si-NC and si-Jmjd4. The arrows indicate the time of adding oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and rotenone and antimycin A (Anti-A; n=12 wells/si-NC, 11 wells/si-Jmjd4).

G, Changes in metabolites in targeted metabolomics of left ventricles of MCM++; Jmjd4ff mice 14 days after tamoxifen induction compared with Jmjd4ff controls (n=3 animals per group).

H, Heatmap of significantly changed metabolites in targeted metabolomics dataset.

K, Schematic diagram of the glucose metabolic flux highlighting the changes of metabolites on Jmjd4 deletion. Two-tailed unpaired Student t test was performed (E–I). All in vitro experiments were assessed 3 times independently. All quantitative data are expressed as mean±SEM, *P<0.05, **P<0.01, ***P<0.001. CM indicates cardiomyocyte; DAPI, 4',6-diamidino-2-phenylindole; KD, knockdown; Mito, mitochondrial; NRVC, neonatal rat ventricular cardiomyocytes; OCR, oxygen consumption rate; si-NC, small interfering negative control; and TMRE, tetra-methyl-rhodamine ester.
Figure 3. Jmjd4 interacts with Pkm2 and regulates its stability.

A. Top proteins interacting with Jmjd4 from mass spectrometry analysis of peptides pulled down using anti-FLAG antibody in NRVCs 48 hours after infected with AAV9-FLAG-Jmjd4. B. Coimmunoprecipitation (coIP) of overexpressed FLAG-Jmjd4 and HA-Pkm2 in 293T cells. Arrow: HA-Pkm2. C. CoIP of overexpressed FLAG-Jmjd4 and endogenous Pkm2 in 293T cells. D. CoIP of overexpressed HA-Pkm2 and endogenous Jmjd4 in NRVCs. E. CoIP of Pkm2 and Jmjd4 in NRVCs. F. CoIP of Pkm2 and Jmjd4 in heart tissues of wild-type mouse with sham or TAC operation. G. Representative coIP images and quantification of overexpressed FLAG-Jmjd4 (WT), FLAG-Jmjd4-H194A and endogenous PKM2 in 293T cells (n=3 wells per group). H. Western blots and quantification of protein levels of Pkm1 and Pkm2 in heart apex of Jmjd4f/f (Continued).
(Figure 2F). Baseline OCR and OCR on different metabolic substrates of mitochondria isolated from MCM; Jmjd4+/− hearts were also significantly reduced compared with mitochondria from Jmjd4−/− hearts (Figure 2G), measured by the Strathkelvin Oxygen System. In addition, Seahorse experiments were performed to monitor the dynamics of OCRs in NRVCs on Jmjd4 knockdown. Significantly reduced basal and maximal OCRs and spare respiratory capacity and ATP production were observed in NRVCs treated with si-Jmjd4 compared with si-NC (Figure 2H). These mitochondrial dysfunctions were also observed in hPSC-CMs on Jmjd4 knockdown (Figure S4A–S4C). Taken together, these results indicate that Jmjd4 plays a critical role in maintaining mitochondrial respiration.

We then performed targeted metabolomics analyses to examine the detailed molecular metabolic phenotypes induced by loss of Jmjd4 (Figure 2I–2K). We found that acetyl-CoA production was reduced significantly (Figure 2I and 2J), consistent with insufficient respiration in mitochondria in vitro, and phosphoenolpyruvate (PEP) that are directly upstream of pyruvate in glycolysis were accumulated on Jmjd4 deletion (Figure 2I and 2J), consistent with insufficient respira-

Jmjd4 Interacts with Pkm2 and Regulates Its Stability

To investigate the molecular mechanism of Jmjd4 regulating metabolism in cardiomyocytes, we performed immunoprecipitation-mass spectrometry with AAV9 mediated overexpression of FLAG-tagged Jmjd4 in NRVCs. eRF1 has been reported as a Jmjd4 substrate on protein translation termination. However, eRF1 was not identified in our FLAG-Jmjd4 immuno-

precipitation-mass spectrometry result (Figure S6A), and Jmjd4 knockdown did not affect the total protein levels in NRVCs (Figure S6B). We identified Pkm2 among the interacting proteins of Jmjd4, which is a pyruvate kinase normally absent or expressed at low levels in adult cardiomyocytes. The enzymatic activity of Pkm2 is much lower than Pkm1, which is abundantly expressed in adult cardiomyocytes. Given the accumulation of metabolites upstream of pyruvate upon Jmjd4 loss-of-function, this interaction is particularly interesting and is consistent with our metabolic dysfunctional phenotype (Figure 3A). The interaction between Jmjd4 and Pkm2 was then validated by coimmunoprecipitation of overexpressed FLAG-Jmjd4 and HA-Pkm2 in 293T cells (Figure 3B), FLAG-Jmjd4 and endogenous Pkm2 in 293T cells (Figure 3C), HA-Pkm2 and endogenous Jmjd4 in NRVCs (Figure 3D). We also validated endogenous Jmjd4-Pkm2 interaction in NRVCs (Figure 3E) and heart tissues from mice with TAC operation with coimmunoprecipitation using Pkm2 antibody (Figure 3F).

To determine which regions in Jmjd4 are important for Jmjd4-Pkm2 interaction, we performed serial deletion of 80 residues from each end of Jmjd4 until they met in the middle (ΔN80, ΔN160, ΔN240, ΔC80, ΔC160, and ΔC240), and tagged these truncated mutants of Jmjd4 with a FLAG tag. 293T cells were cotransfected with each of the truncated FLAG-Jmjd4 and HA-Pkm2, followed by immunoprecipitation and Western blotting analysis (Figure S7A). A significant decrease of interaction with Pkm2 was found for the ΔN240 and ΔC160 mutants of Jmjd4, whereas little signal was detected for the ΔC240 mutant (Figure S7A). These results suggest that the most critical region in Jmjd4 for Pkm2 interaction may reside in the Jmjd4 catalytic domain where the deletion of ΔC240, ΔN240, and ΔC160 overlap (Figure S7A).

Previous studies showed that Jmjd4 possesses hydroxylase activity, so we next tested whether the Jmjd4-Pkm2 interaction is dependent on their catalytic activity by performing coimmunoprecipitation using catalytically inactive mutants (Jmjd4-H194A with Fe2+ binding sites required for hydroxylation activity mutated, and Jmjd4-K367M, a previously reported kinase-dead mutant) (Figure S7B). These results suggest that the most critical region in Jmjd4 for Pkm2 interaction may reside in the Jmjd4 catalytic domain where the deletion of ΔC240, ΔN240, and ΔC160 overlap (Figure S7A).
Figure 4. Jmjd4 regulates cardiac metabolism through Hsp70-mediated Pkm2 degradation.
A. Western blots and quantification of protein levels of Pkm2 in NRVCs treated with E64d for 8 hours (n=3 wells per group).
B. Western blot of Pkm2 in JMJD4 overexpressed NRVCs with or without E64d for 8 hours. C. Representative Western blot of Pkm2 and Jmjd4 in NRVCs transfected with si-NC or si-Jmjd4, with or without CMA agonist QX-77 (5 µmol/L) for 48 hours. D. Venn diagram showing the intersection between proteins interacting with Jmjd4 in NRVCs and CMA pathway–associated proteins from GSEA. E. Identities of the 5 CMA pathway–associated proteins interacting with Jmjd4. F. Western blots and quantification of protein levels of Pkm2 in NRVCs transfected with si-NC or si-Hsp70. G. Western blot of Pkm2 in JMJD4 overexpressed NRVCs transfected with si-Jmjd4. H. Coimmunoprecipitation of overexpressed HA-Pkm2 and endogenous Hsp70 in 293T cells transfected with si-NC or si-JMJD4. I. Hydroxylation of K66 of Pkm2 detected by mass spectrometry. Purified Pkm2 from 293T cells was digested with Glu-C before tandem mass spectrometry. Spectra show mass shift in all detected y ion fragments (y1–y17; blue) when comparing the upper (hydroxylated) and lower (unhydroxylated) panels. In contrast, the masses of detected b ion fragments (b2–b14) (red) are consistent with their predicted values, indicating K66 hydroxylation.
requires hydrolysis activity of Jmjd4, but not kinase activity of Pkm2.

Given that Jmjd4 is critical for cardiomyocyte metabolism and interacts with Pkm2, we next sought to determine whether Pkm2 level in cardiomyocytes changes on Jmjd4 loss-of-function. Pkm2 expression was significantly upregulated in MCMs; Jmjd4 knockout hearts compared with Jmjd4+/- hearts on tamoxifen induction (Figure 3H), and was upregulated by Jmjd4 knockdown in NRVCs (Figure 3I), whereas Jmjd4 overexpression in NRVCs suppressed Pkm2 expression (Figure 3J). The elevation of Pkm2 protein level on Jmjd4 knockdown was independent from Pkm1 and Pkm2 transcript levels (Figure S7C), suggesting that Jmjd4 regulates Pkm2 protein level posttranslationally. Accumulation of enzymatically ineffective Pkm2 may compete for PEP with Pkm1, thus reducing the overall metabolic efficiency, which could result in the accumulation of metabolites upstream of pyruvate and reduced acetyl-CoA on Jmjd4 knockout (Figure 2I–2K).

**Jmjd4 Hydroxylates K66 of Pkm2 to Promote Its Degradation Through the Chaperone-Mediated Autophagy Pathway**

Jmjd4 loss-of-function did not affect Pkm2 transcript level (Figure S7C), suggesting that Jmjd4 regulates Pkm2 protein level through translational or posttranslational mechanisms. To investigate whether Pkm2 stability is altered by Jmjd4, we used cycloheximide, a protein synthesis inhibitor, to block Pkm2 translation and assess the effects of Jmjd4 on its stability. Pkm2 protein level decreased significantly in NRVCs treated with cycloheximide (Figure S8A), and knocking down Jmjd4 in NRVCs blunted this effect (Figure S8B). Time course analysis revealed that knockdown of Jmjd4 in NRVCs substantially extended the half-life of Pkm2 protein in the presence of cycloheximide (Figure S8C). In contrast, overexpression of Jmjd4 remarkably decreased the half-life of Pkm2 protein (Figure S8D). These data indicate that Jmjd4 posttranslationally plays a role in the regulation of Pkm2 protein stability.

We next explored the specific pathway by which Jmjd4 regulates the stability of the Pkm2 protein. In NRVCs, Pkm2 protein level was not affected by pro teaseosome inhibitor MG-132 (Figure S8E), but it was increased by lysosome inhibitor E64d (Figure 4A), Baf-A1, and leupeptin (Figure S8F), which did not affect its transcript level (Figure S8G). Furthermore, E64d, but not MG-132, also rescued the decrease of Pkm2 induced by Jmjd4 overexpression (Figure 4B), suggesting that Jmjd4 may regulate Pkm2 stability through lysosome-related protein degradation pathway. Chaperone-mediated autophagy (CMA) is a major pathway for the degradation of cytosolic proteins in lysosomes, and its specific agonist QX-77 can reduce Pkm2 protein level in NRVCs (Figure S8H), suggesting that Pkm2 stability is regulated by the CMA pathway. Knocking down Jmjd4 in NRVCs blunted the effect of QX-77 (Figure 4C), suggesting that Jmjd4 is required for CMA degradation of Pkm2.

To investigate how Jmjd4 regulates Pkm2 degradation through CMA, we reexamined the proteins pulled down by Jmjd4 in our immunoprecipitation-mass spectrometry experiment (Figure 3A, Figure S6B), and identified 5 proteins in the CMA pathway (GO:0061684) present in the proteins interacting with Jmjd4 (Figure 4D), among which the most abundant protein was Hsp70 (Figure 4E), an essential chaperone in substrate degradation through autophagy pathways. Knocking down Hsp70 (Figure S8I and S8J) led to significantly elevated protein level of Pkm2 in NRVCs (Figure 4F), and rescued the decrease in Pkm2 protein level induced by Jmjd4 overexpression (Figure 4G), indicating a CMA-dependent degradation of Pkm2. These data indicate that Jmjd4 attenuates the protein stability of Pkm2 in cardiomyocytes by promoting CMA-induced protein degradation of Pkm2.

To examine if Jmjd4 and Hsp70 form a complex to regulate Pkm2 stability, we performed a coimmunoprecipitation experiment with HA-tagged Pkm2 and found that endogenous Hsp70 can be pulled down by Pkm2 in 293T cells (Figure 4H). In addition, this interaction was diminished by Jmjd4 knockdown (Figure 4I), suggesting that Jmjd4 is required for Pkm2-Hsp70 interaction.

We next explore how Jmjd4 regulates Pkm2 degradation through the CMA pathway. Jmjd family proteins are known as lysine hydroxylases. Previous studies have shown that posttranslational modifications, including phosphorylation and acetylation, are critical for the protein stability and functional activity of Pkm2. Therefore, we explored whether Jmjd4 decreases Pkm2 protein stability and abundance by modulating its posttranslational modifications. For this purpose, we overexpressed Jmjd4 and HA-Pkm2 in 293T cells, then we performed HA tag immunoprecipitation to purify Pkm2, followed by proteolysis and mass spectroscopy analysis to identify

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Figure 5. Activating Pkm2 rescues the phenotypes induced by Jmjd4 knockout in vitro and in vivo.

A. Representative images of TMRE staining (scale bars, 100 µm) and quantification of mitochondrial membrane potential in NRVCs transfected with si-NC or si-Jmjd4 in the presence of DMSO or TEPP-46 (20 µmol/L) for 48 hours (n=3 wells per group). B. Representative images of MitoTracker staining (scale bars, 100 µm) and quantification of mitochondrial distribution in NRVCs transfected with si-NC or si-Jmjd4 in the presence of DMSO or TEPP-46 (20 µmol/L) for 48 hours (n=3 wells per group). C. Analysis and quantification of mitochondrial OCR in NRVCs transfected with si-NC or si-Jmjd4 in the presence of DMSO or TEPP-46 (20 µmol/L) for 48 hours. The arrows indicate the time of adding oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and rotenone and antimycin A (Anti-A) (n=12 wells/si-NC+DMSO, 6 wells/si-NC+TEPP-46, 7 wells/si-Jmjd4+DMSO, 17 wells/si-Jmjd4+TEPP-46). D. Schematic of TEPP-46 administration and echocardiography (Echo) in Jmjd4°/° and MCM°+; Jmjd4°/° mice. E. Representative M-mode echocardiographic images and measurements of ejection fraction (EF), fractional shortening (FS), LVIDs, and LVIDd in Jmjd4°/° and MCM°; Jmjd4°/° mice 2 and 4 weeks after tamoxifen induction, and administered TEPP-46 (10 mg/kg) with oral gavage once a day for 2 weeks (2 weeks, n=6 animals/vehicle, 6 animals/TEPP-46; 4 weeks, n=5 animals/vehicle, 6 animals/TEPP-46). F. Representative Masson trichrome staining and quantification of fibrotic areas in the hearts of (Continued)
the exact amino acid residue of Pkm2 hydroxylated by Jmjd4. We identified a single hydroxylation site of Pkm2 at K66 (Figure 4J), with <5% hydroxylation in control cells without Jmjd4 overexpression and >70% hydroxylation in cells overexpressing FLAG-Jmjd4 (Figure 4K).

In addition, the inactive H194A mutant of Jmjd4 abolished Pkm2 K66 hydroxylation (Figure 4K), confirming that the hydroxylase activity of Jmjd4 is required for K66 hydroxylation, which is localized on the surface of Pkm2 (Figure 4L). To examine if Pkm2 K66 hydroxylation is necessary for its degradation through CMA, we performed coimmunoprecipitation to test the interaction between Pkm2 K66 mutant (Pkm2-K66R) and endogenous Hsp70 in 293T cells. It is not surprising that, compared with wild-type Pkm2, the K66R mutation significantly reduced its binding to Hsp70 (Figure 4M). Our data collectively demonstrate that Jmjd4 hydroxylates K66 of Pkm2 to promote its degradation through the CMA pathway, and the molecular mechanism of Jmjd4 regulating cardiomyocyte metabolism through Hsp70-mediated Pkm2 degradation is summarized in Figure 4N.

**Activating Pkm2 Rescues the Phenotypes Induced by Jmjd4 Knockout in Vitro and in Vivo**

To examine if Pkm2 accumulation is responsible for metabolic dysfunction and cardiomyopathy induced by Jmjd4 deletion, we used Pkm2 inhibitor shikonin to examine if it can rescue the metabolic defect induced by Jmjd4 knockout in NRVCs. Seahorse assay revealed that shikonin partially rescued metabolic defects in NRVCs treated with si-Jmjd4 (Figure S9A and S9B), suggesting that accumulated Pkm2 mediates the metabolic defects induced by knocking out Jmjd4.

Pkm2 has been found to primarily exist in 2 multimeric forms in tumor cells: an enzymatically active tetramer and a nearly inactive dimer at physiological concentrations of its substrate PEP. Because Pkm2 accumulates in cardiomyocytes without Jmjd4, we next investigated the Pkm2 forms in cardiomyocytes with Jmjd4 deleted. Native protein electroporation revealed that Pkm2 mainly existed in dimer form in hearts and tetrameric Pkm2 is barely detectable (Figure S9C), and Jmjd4 knockout induced accumulation of the inactive dimer form of Pkm2, with minimal other forms detected (Figure S9C and S9D), which is also the case in NRVCs with Jmjd4 knockout (Figure S9C and S9E). This is consistent with our hypothesis that the inactive Pkm2 accumulated on Jmjd4 deletion competes with enzymatically active Pkm1 for PEP, thus reducing the overall efficiency of pyruvate metabolism.

We then sought to find a small molecule that can alleviate the clinical phenotypes due to elevated Pkm2 caused by Jmjd4 deletion. Shikonin has been reported to induce apoptosis and other cytotoxicity, and we found that Shikonin led to significantly increased cell death in cardiomyocytes (Figure S9F). Although it rescued the metabolic defect induced by Jmjd4 knockout despite its toxicity, we decided not to use it for in vivo experiments. If the competition for substrate from the enzymatically inactive Pkm2 is responsible for the impaired metabolism, pharmacological activation of Pkm2, which makes the accumulated Pkm2 more efficient in producing pyruvate, should also rescue the metabolic phenotypes induced by Jmjd4 knockout. To test this hypothesis, we used TEPP-46, a Pkm2-specific activator that can transform Pkm2 dimers to tetramers. Despite the negative effect of TEPP-46 itself on mitochondrial membrane potential (Figure 5A), it rescued the suppression of membrane potential by si-Jmjd4 in NRVCs (Figure 5A), and TEPP-46 also rescued the decreased mitochondria size and cardiomyocyte hypertrophy (Figure 5B), and metabolic dysfunctions induced by si-Jmjd4 measured by Seahorse assay, as well (Figure 5C). To test whether TEPP-46 can also rescue the metabolic and DCM phenotypes in MCM−/−; Jmjd4−/− hearts, we treated MCM−/−; Jmjd4−/− mice with TEPP-46 through oral gavage for 14 days after tamoxifen induction (Figure 5D). Echo-cardiography showed significant improvement of EF and FS, and reduced LVIDd and LVIDs 2 weeks after TEPP-46 treatment (4 weeks after tamoxifen induction), as well (Figure 5E). Fibrosis induced by Jmjd4 knockout was significantly reduced by TEPP-46 treatment (Figure 5F), and cardiac hypertrophy in MCM−/−; Jmjd4−/− mice was also suppressed by TEPP-46 (Figure 5G). Native Western blot showed that TEPP-46 upregulated the protein level of Pkm2 tetramer significantly (Figure 5H), and qRT-PCR showed that downregulation of some genes in glycolysis, lipid metabolism, and mitochondrial biogenesis by Jmjd4 knockout were rescued in part by TEPP-46 (Figure S10A–S10C). Overexpression of the active pyruvate kinase Pkm1 can also consistently alleviate, in part, the metabolic abnormalities caused by Jmjd4 knockout (Figure S11). Together, these results showed that activating Pkm2 that is accumulated on Jmjd4 loss-of-function can rescue the metabolic and hypertrophic...
Figure 6. Overexpression of Jmjd4 in adult cardiomyocytes protects the heart from TAC-induced heart failure.

A. Representative images of TMRE staining (scale bars, 100 µm) and quantification of mitochondrial membrane potential in NRVCs treated with phenylephrine (PE, 100 nmol/L) and ddH2O with and without overexpression of Jmjd4 for 48 hours (n=3 wells per group). B. Representative images of MitoTracker staining (scale bars, 100 µm) and quantification of mitochondrial distribution in NRVCs treated with 100 nmol/L PE and ddH2O with and without overexpression of Jmjd4 for 48 hours (n=3 wells per group).

C. Analysis and quantification of mitochondrial OCR in NRVCs treated with 100 nmol/L PE and ddH2O with and without overexpression of Jmjd4 for 48 hours. The arrows indicate the time of adding oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and rotenone and antimycin A (Anti-A; n=11 wells/AAV.GFP+ddH2O, 10 wells/AAV.Jmjd4+ddH2O, 4 wells/AAV.GFP+PE, 10 wells/AAV.Jmjd4+PE).

D. Quantitative real-time polymerase chain reaction analysis of mRNA levels of Pkm1 and Pkm2 in heart tissue of wild-type mice at 2, 4, and 6 weeks after TAC operation (n=8 animals/sham, 4 animals for 2 weeks, 8 animals for 4 weeks, and 3 animals for 6 weeks).

E. Representative Western blot images and quantification of Pkm2 protein levels in heart tissue from wild-type mice at 2, 4, 6, and 8 weeks after TAC operation (n=3 animals/sham, 3 animals for 2 weeks, 3 animals for 4 weeks, 3 animals (Continued)
Overexpression of Jmjd4 in Adult Cardiomyocytes Protects the Heart From TAC-Induced Heart Failure

As we have shown, Jmjd4 is required for cardiomyocyte metabolism through regulating Pkm2 stability, we then sought to test whether overexpressing Jmjd4 could rescue metabolic dysfunction of cardiomyocyte induced by stress or pressure overload. First, we tested whether overexpression of Jmjd4 could offer protection against phenylephrine-induced mitochondrial dysfunction in NRVCs. With the use of AAV9 vector, Jmjd4 was overexpressed in NRVCs, and the mitochondrial membrane potential, area, and metabolic activity, which were all negatively affected by phenylephrine treatment, were rescued by Jmjd4 overexpression (Figure 6A–6C).

To investigate if overexpressing Jmjd4 could rescue metabolic dysfunction induced by pressure overload, we first examined Pkm2 expression after TAC operation. TAC operation led to suppressed EF, FS, and elevated LVIDd and LVIDs (Figure S12A–S12D), promoted moderate elevation of Pkm2 transcript level without affecting Pkm1 expression (Figure 6D), and induced a sustained elevation of Pkm2 protein level (Figure 6E), suggesting that promoting Pkm2 degradation may be beneficial for maintaining metabolic function after TAC. We then generated a mouse line with conditional overexpression of Jmjd4 by knocking in Jmjd4 OE-Flox into Jmjd4 OE-Flox mice (Figure S13A). Cardiomyocyte-specific expression is mediated by Myh6-CreERT2 (MCM) when induced with tamoxifen (Figure 6F, Figure S13B–S13D), and there is no difference of baseline heart function between MCM−; Jmjd4 OE-Flox mice without tamoxifen induction and control Jmjd4 OE-Flox mice (Figure S13E).

Four weeks after TAC operation, we found that the Pkm2 level was suppressed by Jmjd4 overexpression (Figure 6G), and cardiac function was protected in MCM−; Jmjd4 OE-Flox mice versus Jmjd4 OE-Flox control mice, with elevated EF and FS and decreased LVIDd and LVIDs (Figure 6H), consistent with our data in vitro. These data suggest that Jmjd4-dependent Pkm2 degradation not only is required for maintaining metabolic homeostasis of adult cardiomyocytes, but also can be protective against metabolic dysfunction induced by various pathological stimuli.

Activating Pkm2 Protects the Heart From TAC-Induced Heart Failure

Because Pkm2 is elevated after TAC, and activating Pkm2 using TEPP-46 can rescue metabolic dysfunction and hypertrophy induced by deleting Jmjd4, we then examined if TEPP-46 can be used to treat TAC-induced cardiac dysfunction. TEPP-46 was orally administered to TAC-operated mice from 2 to 4 weeks after TAC (Figure 7A). Mice treated with TEPP-46 showed a sustained cardiac function versus deteriorating cardiac function of TAC-operated mice from 2 to 4 weeks after TAC (Figure 7A). Mice treated with TEPP-46 showed a sustained cardiac function versus deteriorating cardiac function of TAC-operated mice from 2 to 4 weeks after TAC (Figure 7A).

These results suggest a broad therapeutic effect of TEPP-46 in the treatment of pathological cardiac conditions with elevated Pkm2 level, and TEPP-46 could potentially be a powerful drug candidate for treating heart failure. The molecular mechanism of modulating metabolism in cardiomyocytes through regulating Pkm2 abundance and activity by pressure overload, Jmjd4, and TEPP-46 is summarized in Figure 7F.

DISCUSSION

Previous studies indicated that Jmjd family proteins may have functions in heart diseases. However, whether Jmjd4, a small molecular weight member of this family with few known functions, plays a role in pathological cardiac remodeling remained elusive. Here we have shown, for the first time, that JMJD4 expression is decreased in DCM hearts, and it is a key endogenous regulator of energy metabolism in the mammalian heart. When Jmjd4 is conditionally deleted in adult cardiomyocytes, it led to lowered ΔΨm and dampened oxygen consumption, and a systemic downregulation of genes coding for
Figure 7. Activating Pkm2 protects the heart from TAC-induced heart failure.
A, Schematic of TAC operation, TEPP-46 oral gavage (10 mg/kg, once a day), and echocardiography (Echo) in WT mice. B, Representative M-mode echocardiographic images and corresponding measurements of ejection fraction (EF), fractional shortening (FS), LVIDd, and LVIDs at baseline, 2, 4, and 6 weeks after the TAC operation in TEPP-46 (10 mg/kg) group versus vehicle group for 2 weeks (n=7, 17, 16, and 19 animals in the vehicle group, and n=7, 7, 10, and 9 animals in TEPP-46 group for the 4 time points, respectively). C, Representative Masson trichrome staining and quantification of fibrotic areas in the WT mouse hearts 6 weeks after sham and TAC operation, treated with TEPP-46 (10 mg/kg) or vehicle for 2 weeks (n=6 animals/sham+vehicle, 10 animals/TAC+vehicle, 6 animals/TAC+TEPP-46). D, Ratios of heart weight to body weight (HW/BW) of the hearts of WT mice 6 weeks after sham and TAC operation, treated with TEPP-46 (10 mg/kg) or vehicle for 2 weeks (n=5 animals/sham+vehicle, 13 animals/TAC+vehicle, and 12 animals/TAC+TEPP-46). E, Western blot and quantification of dimer and tetramer of Pkm2 from native protein extraction from heart tissues of WT hearts 6 weeks after TAC operation treated with TEPP-46 (10 mg/kg) or vehicle for 2 weeks (n=3 animals/vehicle, 5 animals/TEPP-46). F, Graphical summary of Jmjd4 regulating metabolism by promoting Pkm2 degradation. One-way ANOVA with Bonferroni correction was performed (B–D). Two-tailed unpaired Student t test was performed (E). All quantitative data are expressed as mean±SEM, *P<0.05, **P<0.01, ***P<0.001. CMA indicates chaperone-mediated autophagy; LVIDd, left ventricular internal dimension at end-diastole; LVIDs, left ventricular internal dimension at end-systole; PEP, phosphoenolpyruvate; TAC, transverse aortic constriction; and WT, wild type.
mitochondrial respiratory chain components, as well. Cardiac performance deteriorates with cardiomyocyte hypertrophy, cell death, myocardial fibrosis, and dilation of the ventricles with thinning of the ventricular walls, all classic phenotypes of DCM. Conversely, increasing Jmjd4 abundance protects cardiomyocytes from metabolic dysfunction induced by phenylephrine and TAC operation, suggesting that Jmjd4 is required overall for metabolic homeostasis of cardiomyocytes and is protective against stresses altering cardiomyocyte metabolism.

Jmjd4 is dominantly present in the cytoplasm, and its only known hydroxylation substrate is eRF1 in transcriptional regulation in the cytoplasm. In our MS results, Jmjd4 consistently has no interactions with transcription factors (Figure S6A), suggesting that it is not functioning as a histone demethylase like the large Jmjd family members. However, we also did not find eRF1 in proteins interacting with Jmjd4 in cardiomyocytes (Figure S6A), suggesting that this interaction might be cell type specific. We found that Jmjd4 interacts with Pkm2 and Hsp70, both in the cytoplasm, and is required for degradation of Pkm2 through Hsp70-mediated CMA by hydroxylating K66 on Pkm2. This new role of a small Jmjd protein adds to the expanding functions of this family of proteins, highlighting the diversity of the understudied members of the Jmjd family.

Impaired homeostasis of proteins in the heart regulated by autophagy has emerged as a major mechanism of various cardiomyopathies, whereas Pkm2 degradation regulated by CMA has been reported in cell lines. Impaired homeostasis of proteins in the heart regulated by autophagy has emerged as a major mechanism of various cardiomyopathies, whereas Pkm2 degradation regulated by CMA has been reported in cell lines. Pkm2 is located at a gate position in the glycolytic flux to respond to various stimuli, and is best known as its crucial role in Warburg effect. Due to its low expression in the adult heart, its function in cardiomyocytes had not been thoroughly analyzed. We showed that Pkm2 accumulation caused by impaired CMA through Jmjd4 deletion or TAC is at least partially responsible for metabolic dysfunction, pathological hypertrophy, and fibrosis, all characteristics of DCM, emphasizing an active role of elevated Pkm2 in the pathology of DCM.

The striking metabolic dysfunction caused by Jmjd4 loss-of-function is accompanied by hypertrophic growth of cardiomyocyte, which is a signature of the decompensation process in DCM. But as we delineated the molecular mechanism of Jmjd4 regulating metabolism through Pkm2 degradation, the hypertrophic growth on insufficient energy production became puzzling, which may suggest that cardiomyocytes are wired to prioritize contractile functions at all costs, even with metabolic crisis. Mitophagy, autophagy, and other recycling strategies may be used by cardiomyocytes to grow in size to maintain mechanical output despite worsening metabolic conditions.

Metabolic interventions for cardiac diseases have been increasingly drawing attention in recent years. One of the most striking findings in our work is that the Pkm2 agonist TEPP-46 significantly rescued the DCM phenotypes not only in the MCM-; Jmjd4+/− mouse, but also in wild-type mice undergoing TAC operation. Given the nature of TEPP-46 as a small molecule with plenty of in vivo applications in kidney, immune system, and cancer, as well it may be a promising candidate for pharmacological intervention to treat a broad range of cardiac diseases with metabolic dysfunction and elevated Pkm2.

In summary, we demonstrated that Jmjd4 plays an essential role in maintaining metabolic homeostasis in cardiomyocyte and cardiac function through coordinating the degradation of Pkm2 through CMA, which provided novel targets for intervention for DCM.

ARTICLE INFORMATION
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Acknowledgments
Drs Wei, Xu, and Tang conceived the study. Drs Xu and Wei supervised the experiments. Dr Tang performed experiments on mice. Drs Tang, Feng, Shi, and Zhang performed experiments on neonatal rat ventricular cardiomyocytes. Drs Tang and Feng performed human pluripotent stem cell–derived cardiomyocyte experiments. Drs Hu and MW performed experiments on human samples. Dr Zhang performed bioinformatics analysis. Drs Su, Ma, Wang, Feng, and Tang bred and maintained mouse lines and prepared mouse samples. Drs Wei and Tang wrote the manuscript with significant input from Drs D. Xu, Feng, and Y. Xu and feedback from all authors. The authors thank the Peak Disciplines (Type IV) of Institutions of Higher Learning in Shanghai, and the Frontier Science Research Center for Stem Cells, Ministry of Education for their support.

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Disclosures
None.

Supplemental Material
Figures S1–S14
Tables S1–S13
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REFERENCES
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