Supplementary Information for
A specific JMJD6 inhibitor potently suppresses multiple types of cancers both in vitro and in vivo

Rong-quan Xiao, Ting Ran, Qi-xuan Huang, Guo-sheng Hu, Da-meng Fan, Jia Yi, Wen Liu

Wen Liu
Email: w2liu@xmu.edu.cn

This PDF file includes:

Supplementary Materials and Methods
Figures S1 to S14
Table S1
SI References
Supplementary Materials and Methods

Plasmids and cloning procedures

JMJD6 (136-310), KDM3A/JHDM2A (1053-1286), KDM4C/JMJD2C (161-337), KDM5C/SMCX (396-572), KDM6B/JMJD3 (1334-1507), KDM7B/PHF8 (190-356), JMJD8 (141-290), and JARID2 (879-1053) were PCR-amplified from cDNAs from HEK293T cells by using PrimeSTAR HS DNA Polymerase (Takara) and then cloned into pGEX-6P-1 (Promega) expression vector. c-Myc and CCND1 were PCR-amplified from cDNAs from HeLa cells and then cloned into pCDH-CMV-MCS-EF1-Puro (Youbio, VT1480) lentiviral vector. JMJD6 (W174A), JMJD6 (W206A), and JMJD6 (H187A) were generated by overlap extension PCR method using PrimeSTAR HS DNA Polymerase from p3×FLAG-CMV-10-JMJD6 we reported previously (1).

SiRNAs, antibodies, and compounds

SiRNAs specifically targeting JMJD6 (GCUAUGGUGAACACCCUAA and GGACCCGGCACAACUCUAA) have been described previously (1). The information on commercial antibodies used in this study are listed as following: His (Santa Cruz, sc-803), GST (Proteintech, 66001-1-Ig), Flag (Sigma, F3165), HSP70 (Santa Cruz, sc-24), H4R3me2(s) (Abcam, ab5823), H3R17me2(a) (Abcam, ab8284), JMJD6 (Abcam, ab10526), Actin (Proteintech, 66009-1-Ig), c-Myc (Santa Cruz, sc-40), N-myc (Santa Cruz, sc-53993), and CCND1 (Proteintech, 60186-1-Ig). Anti-HSP70 R469me1 antibody was reported previously (2). JQ1 was purchased from MedchemExpress (HY-13030). Fulvestrant (ICI) was purchased from Beyotime Biotechnology (SD0002).

In vitro demethylation assay and formaldehyde release assay

His- and GST- tagged proteins were expressed in BL21 (DE3) bacterial cells (Stratagene)
and purified by using Ni-NTA agarose (Thermo Fisher Scientific) and Glutathione agarose (Sigma), respectively, following the manufacturers’ protocols. The purified proteins were buffer exchanged to PBS by using PD-10 desalting column (GE Healthcare) after elution. The final concentration of the purified proteins was 5 µg/µL. Bulk histone substrates was dissolved in ddH₂O at the concentration of 5 µg/µL. The in vitro demethylation assay was performed by mixing JMJD6 (1 µL) with control or iJMJD6 in the demethylation buffer (50 mM HEPES-KOH, pH 8.0, 20 µM [NH₄]₂Fe[SO₄]₂, 1 mM α-ketoglutarate, 500 µM ascorbic acid) at room temperature (RT) for 10 minutes (min), followed by adding bulk histone substrates (1 µL) and incubating at 37 °C for 1 hour (h). The reaction was stopped and the released formaldehyde was measured by adding 60 mM ammonium acetate and acetoacetonilide followed by fluorescence measurement on an EnVision® Multi-mode plate reader (PerkinElmer, EnVision). The fluorescence was measured immediately after addition of ammonium acetate and acetoacetonilide (T₀) and 2 h after incubation at 25 °C (T₁) (excitation: 355 nm; emission: 460 nm). The activity of iJMJD6 was calculated based on the difference in fluorescence intensity between T₁ and T₀.

**In vitro hydroxylation assay followed by MALDI-TOF MS analysis and Succinate-Glo™ assay**

Histone H4, histone H3, and U2AF65 peptide were dissolved in ddH₂O at the concentration of 1 µg/µL. The in vitro hydroxylation assay was performed by mixing JMJD6 protein (1 µL) and iJMJD6 in the hydroxylation buffer (50 mM Tris-HCl, pH 7.5, 100 µM [NH₄]₂Fe[SO₄]₂, 500 µM α-ketoglutarate, 100 µM ascorbic acid) for 10 min, followed by adding substrate peptide (1 µL) and incubating at 37 °C for 2 h. For MALDI-TOF MS analysis, the samples were prepared by applying 1 µL mixture solution of sample and α-
cyano-4-hydroxycinnamic acid matrix (1:1, v/v) onto the stainless steel MALDI target plate, allowing the droplet to dry in the air at room temperature (RT) before transferring into mass spectrometer. Data was collected on a Bruker Autoflex II mass spectrometer (Bruker Daltonics) in reflection positive mode. For Succinate-Glo™ assay, the samples were transferred into white 96-well plates (Perkin-Elmer), and 25 µL of the Succinate-Glo reagent I (Promega) were added and incubated for 1 h at 25 °C, followed by adding 50 µL of the Succinate-Glo reagent II (Promega) and incubating for 10 min at 25 °C. The luminescence was recorded using a GloMax plate reader (Promega). The IC$_{50}$ of iJMJD6 was calculated using GraphPad Prism (GraphPad Software).

**Immunoblotting assay**

The immunoblotting assay were performed following the protocols as described previously (1). Briefly, the samples were boiled directly in SDS sample buffer at 100 °C for 10 min, resolved by SDS-PAGE gel in SDS running buffer and transferred to nitrocellulose membrane (Bio-Rad). The membrane was then blocked at RT in blocking buffer for 1 h, incubated with primary antibody diluted in blocking buffer, and washed five times with TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20), followed by incubation with HRP-conjugated secondary antibody. Membranes were then rinsed with TBST extensively before imaging.

**Isothermal titration calorimetry**

Experiments were carried out on a VP-ITC titration calorimeter (MicroCal) instrument at 25 °C while stirring at 1000 rpm in ITC buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5% DMSO). The sample cell was filled with His-JMJD6 (0.05 mM in ITC buffer), and the titration springe was loaded with iJMJD6 (1 mM in ITC buffer). The injection volume was
2 μL and the injection time was 4 seconds (s) with 2 min intervals. The data were analyzed using the origin 2016 software (OriginLab).

**Differential scanning calorimetry**

Differential scanning calorimetry (DSC) were carried out using the VP-DSC machine (Ge Healthcare). JMJD6 (20 μM) were mixed with control or iJMJD6 (50 μM) in a reaction buffer (50 mM NaCl, 25 mM Tris-HCl, pH 7.8) at RT before loaded into the sample cell. The measurements were carried out between 20 ℃ and 90 ℃ at a heating rate of 120 ℃/h. Reaction buffer alone was used as a reference. The DSC data were analyzed using the origin 2016 software (OriginLab).

**Thermal shift assay**

For in vitro thermal shift assay (TSA), JMJD6 protein or other JmjC demethylases (1 μM) were mixed with iJMJD6 in the reaction buffer (50 mM NaCl, 25 mM Tris-HCl, pH 7.8) at a final volume of 40 μL. Aliquots were then heated for 3 min at different temperature and centrifuged at 15,000 g for 15 min at 4 ℃. The supernatant was collected, lysed in 4× SDS loading buffer, resolved by SDS-PAGE, and subjected to western blotting analysis.

For cellular thermal shift assay (CETSA), cells were transfected with Flag-tagged, wild-type JMJD6 or JMJD6 mutants, H187A, W174A, or W206A, using Lipofectamine 2000 according to the manufacturer’s protocol, and treated with or without iJMJD6 overnight. Cells were then collected and equal volumes of cell suspensions were heated at different temperatures for 3 min followed by cooling to RT for additional 3 min. Cells were then lysed by three freeze-thaw cycles in liquid nitrogen. Lysates were centrifuged at 15,000 g for 15 min at 4 ℃ and the supernatant was kept for SDS-PAGE gel and western blotting analysis.
Measurement of the protein binding activity of iJMJD6 in cell culture media

Cell culture medium composed of 10% fetal calf serum (FBS) in DMEM were spiked with iJMJD6 dissolved in methanol to a final concentration of 0.1 μg/mL at 37 ℃. Medium were centrifuged (223,000 g) for 4.2 h at 37 ℃. The supernatant was analyzed by UPLC-HRMS to detect the unbound iJMJD6. The UPLC-HRMS method employed a Q Exactive Orbitrap LC-MS/MS equipped with electrospray ionization (Thermo Fisher Scientific) in positive and negative ion modes, which was controlled by Thermo Xcalibur 3.0.63 (Thermo Fisher Scientific). A Hypersil GOLD columns (Thermo Fisher Scientific; 2.1 mm × 150 mm, 3 μm) was used to separate the sample with the temperature set at 35 ℃. Mobile phase A was H2O with 10 mM ammonium acetate, and mobile phase D was 100% acetonitrile with a flow rate of 0.3 mL/min. The gradient was set as follows: 0-15 min (5% A, 95% D); 15-19 min (100% A); 19-25 min (5% A, 95% D). The injection volume was set as 3 μL, and the eluent was monitored at λ 205, 254, and 310 nm.

Measurement of the stability of iJMJD6 in cell culture medium

iJMJD6 dissolved in methanol were spiked into cell culture medium with 10% FBS to a concentration of 0.1 μg/mL at 37 ℃. Aliquots of spiked sample were immediately snap-frozen in dry ice, and the remaining samples were maintained in a humidified incubator (5% CO2) at 37 ℃ over 48 h. These samples were analyzed by UPLC-HRMS as described above.

Measurement of cell permeability of iJMJD6

Caco-2 cells (2 × 10⁴) were plated in MEM medium with 20% FBS in the Transwell® inserts (Corning). The medium was changed every 2-3 days. The transport experiment was conducted using confluent cell monolayers on day 21 post-seeding. The integrity of the
cell monolayers was determined on the day of the permeability experiment by measuring the transepithelial electrical resistance (TEER). Only monolayers with TEER value >300 Ω·cm² were kept. Permeability experiments were performed using Hanks balanced salt solution containing 20 mM HEPES buffer (pH 7.4) in both the apical and basolateral chambers. iJMJD6 was added to the apical compartment at a final concentration of 0.1 μg/mL at 37 °C. Samples were taken from the compartments 2 h before and after incubation, and analyzed by UPLC-HRMS as described above after precipitation with acetonitrile. The apparent permeability coefficient (Papp) was calculated from the apical to basolateral.

**Generation of JMJD6 knockout cell lines using CRISPR/Cas9 gene editing technology**

JMJD6 knock out (KO) HeLa cells were generated according to a previously published protocol (1). Briefly, sgRNA sequence (5’-GCTCTCGTAGTAGTTGTGCCGGG-3’) targeting JMJD6 was cloned into gRNA cloning vector (Addgene, 41824). The gRNA expression vector and pcDNA3.3-hCas9 (Addgene 41815) were mixed and transfected with Lipofectamine 2000. After G418 (1 mg/mL) selection, single colonies were subjected to immunoblotting to select knock-out clones, which were further validated by PCR using genomic DNA as template followed by Sanger sequencing.

**Cell proliferation assay**

Cell proliferation was measured by the CellTiter 96 AQueous one solution cell proliferation assay kit (Promega) according the protocols as described previously (1). Briefly, cells were seeded into a 96-well plate at 5,000 cells per well in triplicates and cultured overnight before adding iJMJD6 at different concentration for 72 h or for different duration at concentrations as indicated in the figure legend. To measure cell proliferation, 20 μL of MTS was added per 100 μL of medium, and then incubated for 1 h. The reaction
was stopped by adding 25 μL of 10% SDS. Data was recorded at the wavelength of 490 nm using a Multiskan MK3 Microplate Reader (Thermo Fisher Scientific). The data shown for cell proliferation assay are from three technical repeats, and the experiments were repeated at least for three times. The data were highly reproducible and representative data are shown. The EC\textsubscript{50} was calculated using GraphPad Prism (GraphPad Software).

**Colony formation assay**
2,000 cells were seeded in one well in a 6-well plate, and treated with or without iJMJD6 for 10 days. Colonies were examined after by fixation with 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet for 15 min. The data shown for colony formation assay are from three technical repeats, and the experiments were repeated at least for three times. The data were highly reproducible and representative data are shown.

**Anchorage-independent growth assay**
A VitroGel™ 3D (TheWell Bioscience) kit was used according to the manufacturer’s instructions. Briefly, the cell suspension (25,000 cells per well in DMEM with 10% FBS) was gently mixed with the diluted VitroGel solution (20% hydrogel solution in deionized water), and transferred to 24-well plate. After hydrogel stabilization, the cell culture media with control or appropriate concentration of iJMJD6 were added to cover hydrogel. The colonies were photographed and counted 14 days later. The data shown for anchorage-independent growth assay are from three technical repeats, and the experiments were repeated at least for three times and the data were highly reproducible.

**Wound healing assay**
Wound healing assay was performed following the protocol described previously (1). Briefly, cells were grown to confluence in 6-well plate, and wounds were performed with
a pipette tip. The cellular debris was then removed by washing cells with PBS, followed by adding iJMJD6. Media was replaced with fresh media containing iJMJD6 at the indicated concentration every day. Three images of each well were taken. The wounded area was measured by using image J and recorded as A0. The cells were allowed to migrate back into the wounded area, and the wounded area was measured again 24 h later and recorded as A1. Cell migration was presented as wound closure (%) = (wounded area (A0-A1)/wounded area A0) × 100%. The data shown for wound healing assay are from three technical repeats, and the experiments were repeated at least for three times. The data were highly reproducible and representative data are shown.

**Transwell assay**

A total of 20,000 cells were plated in serum-free medium in the apical chambers with membranes (Corning; 24-well insert; pore size, 8 μm), treated with or without iJMJD6, and then allowed to migrate to the basolateral chambers contained medium with 10% FBS in a humidified, 5% CO₂ atmosphere incubator at 37 ℃. After 24 h, cells were fixed with 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet for 15 min. Cells in the apical chambers were wiped away with a cotton swab. Migrated cells in the basolateral chambers were photographed. The data shown for transwell assay are from three technical repeats, and the experiments were repeated at least for three times. The data were highly reproducible, and representative data are shown.

**Fluorescence-activated cell sorting (FACS) analysis**

Cells were treated with or without iJMJD6 for 24 h before harvesting and fixing with 70% ethanol overnight at 4 ℃. After washed twice with PBS, cells were incubated with PI/Triton X-100 staining solution (0.1% (v/v) Triton X-100, 0.2 mg/mL DNase-free RNase
A (Sangon Biotech), 0.02 mg/mL propidium iodide (Roche)) at 37 ℃ for 15 min. DNA content was then measured by Attune NxT Flow Cytometer (Invitrogen) and about $10^5$ events were analyzed for each sample. The cell cycle distribution was analyzed with ModFit LT (Verity Software House).

**Apoptosis assay**

Cells were stained with propidium iodide and fluorescein isothiocyanate-conjugated annexin V (BD Pharmingen™) according to the manufacturer’s instructions. Briefly, cells were treated with iJMJD6 for 0, 12, 24, 36, 48, or 60 h, harvested by trypsin, washed twice with PBS, and resuspended in 500 μL binding buffer solution. Cells were then incubated with 5 μL Annexin V and 5 μL PI at RT for 15 min in the dark. Stained cells were subjected to flow cytometry analysis within 1 h. The data shown for apoptosis assay are from three technical repeats, and the experiments were repeated at least for three times. The data were highly reproducible and representative data are shown.

**RNA isolation and RT-qPCR**

Cells were seeded into 6-well plates and treated with or without iJMJD6 for 24 h followed by washing with PBS and RNA extraction using RNeasy Mini Kit (Qiagen) following the manufacturer’s protocol. First-strand cDNA synthesis from total RNA was carried out using GoScript Reverse Transcription System (Promega), followed by quantitative PCR (qPCR) using AriaMx Real-Time PCR machine (Agilent Technologies). All RT-qPCRs were repeated three times and representative data was shown. Significance test was performed using Student’s t-test. The sequences of primers for qPCR analysis were presented in Table S1.

**Animal experiments**
Pharmacokinetics

Male C57BL/6 mice were fasting for 12 h before iJMJD6 administration. Mice were randomized for treatment, either intravenously or orally. Blood samples were collected into labeled eppendorf tube containing K$_2$EDTA as an anticoagulant from mice under light isoflurane anesthesia from retro orbital plexus at different time points after administration. Plasma was immediately separated from blood by centrifugation at 1,500 g for 10 min at 4 °C and then stored at -80 °C. Concentration of iJMJD6 in mouse plasma was determined by UPLC-HRMS as described above. The plasma concentration-time curve was plotted, and the pharmacokinetic parameters, including the area under the concentration–time curve (AUC), maximum plasma concentration ($C_{\text{max}}$), half-life ($t_{1/2}$) and oral bioavailability (F), were estimated by means of a non-compartmental analysis using Drug and Statistics 3.0 (DAS 3.0).

Drug safety assessment

BALB/c nude mice (at least 6 weeks old) were randomized and treated with either vehicle control or iJMJD6 intraperitoneally every other day. Mice were euthanized 26 days after. Organs including heart, lung, liver, stomach, spleen, kidney, and gut were collected, photographed, and weighed, which were then fixed in 4% paraformaldehyde for 24 h, processed and embedded in paraffin wax, and serially sectioned at 5 μm thickness for hematoxylin and eosin (H&E) staining. The peripheral blood samples were collected and subjected to complete blood content analysis by using ProCyte Dx Hematology Analyzer (IDEXXX Laboratories). The serum samples were sent to plasma biochemical analysis by using BS-240 VET Clinical Chemistry Analyzer (Mindray).

Xenograft tumor assay
HeLa, SMCC7721, MCF7, MDA-MB-231, U87, and HCT116 cells \( (5 \times 10^6) \) were inoculated subcutaneously into the flank of female BALB/c nude mice (6 weeks-old). Tumor fragments from hepatoma, triple negative breast cancer, glioblastoma, and colon cancer PDX-bearing mice were subcutaneously implanted into female BALB/c nude mice that were anesthetized. Tumor size was measured using bilateral Vernier calipers and tumor volume was calculated based on the short and long measurement (tumor volume = (long measurement \( \times \) short measurement\(^2 \)) \times 0.5). Mice were randomized when tumor volume reached about 100 mm\(^3\). For monotherapy, mice were randomized into two groups, which were treated with vehicle or iJMJD6 intraperitoneally every other day. For combination therapy, mice were randomized into four groups, which were treated with vehicle, iJMJD6 alone, JQ1 or ICI alone, or in combination. IJMJD6 (12.5 mg/kg) and JQ1 (25 mg/kg) was injected intraperitoneally every other day, while ICI (5 mg) dissolved in peanut oil was injected subcutaneously every week. To supplement MCF7 cell proliferation, each nude mouse was brushed with estrogen (E\(_2\), \( 10^{-2} \) M) every 3 days for the duration of the experiments. Tumor size and body weight was recorded every other day.

All animals used in this study were maintained in animal room with 12 h light/12 h dark cycles at Animal Facility in Xiamen University under pathogen-free conditions. They were cared with free access to standard rodent chow and water in accordance with institutional guidelines. All of the animal experiments were approved by the Xiamen Animal Care and Use Committee.

**Chromatin immunoprecipitation coupled with high throughput sequencing (ChIP-seq) and data analysis**

HeLa cells were treated with or without iJMJD6 for 1 h before ChIP or ChIP-seq. ChIP
was performed following the protocol described previously (1). Briefly, Cells were fixed with 1% formaldehyde (Sigma) for 10 min at RT. Fixation was stopped by adding glycine (0.125 M) and incubated for 5 min at RT, followed by washing with PBS twice. Cells were lysed in lysis buffer (1% SDS, 50 mM Tris-HCl pH 7.8, 10 mM EDTA, and 1× complete protease inhibitor cocktail (Roche)) and chromatin DNA was sheared to 200-500 bp average in size through sonication. Resultant was diluted in dilution buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl pH 7.8, 2 mM EDTA, and 1× complete protease inhibitor cocktail) and immunoprecipitated with anti-H4R3me2(s) or H3R17me2(a) antibody overnight at 4 ℃, followed by incubation with protein G magnetic beads (Bio-Rad) for an additional 2 h. The bound fractions were sequentially washed for 15 min at 4 ℃ with TSE I buffer (0.1% SDS, 1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl pH 7.8, 2 mM EDTA, and 1× complete protease inhibitor cocktail), TSE II buffer (0.1% SDS, 1% Triton X-100, 400 mM NaCl, 20 mM Tris-HCl pH 7.8, 2 mM EDTA, and 1× complete protease inhibitor cocktail), and TE buffer (20 mM Tris-HCl pH 7.4, and 1mM EDTA). After washing and elution, the protein-DNA complex was reversed by heating at 65 ℃ overnight. Immunoprecipitated DNA was purified by using QIAquick spin columns (Qiagen) and subjected to high throughput sequencing or qPCR analysis. The sequences of primers for ChIP-qPCR analysis were presented in Table S1. ChIP-seq sample preparation and computational analysis of ChIP-seq data were performed as described previously with minor modifications (1, 3, 4). Library construction: the libraries were constructed following Illumina ChIP-seq Sample prep kit. Briefly, ChIP DNA was end-blunted and added with an ‘A’ base so the adaptors from Illumina with a ‘T’ can ligate on the ends. Then 200-400 bp fragments are gel-isolated and purified. The library
was amplified by 18 cycles of PCR. Primary analysis of ChIP-Seq datasets: the image analysis and base calling were performed by using Illumina’s Genome Analysis pipeline. The sequencing reads were aligned to hg19 Refseq database by using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) with default parameters. Both uniquely aligned reads and reads that align to repetitive regions were kept for downstream analysis (if a read aligned to multiple genomic locations, only one location with the best score was chosen). The identification of ChIP-seq peaks was performed using HOMER (http://biowhat.ucsd.edu/homer). The threshold for the number of tags that determined a valid peak was selected at a false discovery rate (FDR) of 0.001. Fourfold more tags relative to the local background region (10 kb) were also required to avoid identifying regions with genomic duplications or non-localized binding (poisson p-value < 0.0001). Genomic distribution was done by using the default parameters from HOMER with minor modifications, in which promoter peaks were defined as those with peak center falling between 1,000 bp downstream and 5,000 bp upstream of transcript start sites (TSS). Tag density for histograms (50 bp/bin) were generated by using HOMER.

All ChIP-seq data were deposited in the Gene Expression Omnibus database under accession GSE201988. JMJD6 ChIP-seq in HeLa was from GSE51633.

**Compound synthesis**

iJMJD6 was synthesized according to the procedure as described in Figure S1A.

Step1: synthesis of 6-methyl-2-oxo-2H-chromene-3-carboxylic acid (4). A mixture of 2-Hydroxy-5-methylbenzaldehyde (1) (27.2 g, 0.2 M), 2,2-dimethyl-1,3-dioxane-4,6-dione (2) (28.8 g, 0.2 M), piperidine (3) (340 mg, 4 mM), and AcOH (240 mg, 4 mM) in anhydrous EtOH (300 mL) were mixed and stirred at RT for 1 h. The reaction mixture was
heated to reflux for 3 h. TLC showed compound 2-Hydroxy-5-methyl benzaldehyde was consumed completely. The mixture was allowed to cool down to RT and then filtered. The solid was washed with ice EtOH and dried under vacuum to give a white solid (33.28 g, 81%). Its structure was confirmed by LC-MS and $^1$HNMR. LC-MS: [M+1]$^+$ = 204.85, $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.44 (s, 1H), 7.66 - 7.59 (m, 1H), 7.49 (dd, $J$ = 8.5, 2.1 Hz, 1H), 7.31 (d, $J$ = 8.4 Hz, 1H), 2.37 (s, 3H).

Step2: synthesis of 3-(1H-imidazo[4,5-c]pyridin-2-yl)-6-methyl-2H-chromen-2-one (6). PPA (polyphosphoric acid, 200 g) was heated to 140 °C and stirred under N$_2$. 6-methyl-2-oxo-2H-chromene-3-carboxylic acid (20.4 g, 0.1 M) and pyridine-3,4-diamine (5) (10.9 g, 0.1 M) was then added with some portions. The reaction mixture was continued at 140 °C for 3-4 h. TLC showed compound 6-methyl-2-oxo-2H-chromene-3-carboxylic acid was consumed completely. The mixture was allowed to cool down to 50 °C and poured into ice water while stirring. The pH was adjusted to 7-8 with 4N NaOH solution. The mixture was stirred at RT for 30 min and filtered. The resulting solid was washed with ice MeOH and dried under reduced pressure at 50 °C for 5 h (11.35 g, 41%). Its structure was confirmed by its LC-MS, $^{13}$C-NMR, and $^1$H-NMR. LC-MS: [M+1]$^+$=278; $^{13}$C-NMR (151 MHz, DMSO-d6) δ 159.54 (C8), 152.60 (C4), 136.02 (C10, C16), 135.26(C15, C18, C20), 134.09 (C1, C13,), 130.30 (C2), 118.85 (C6, C9), 116.67 (C5), 115.25(C3, C21), 20.76 (C11); $^1$H-NMR (400 MHz, DMSO-$d_6$) δ 13.03 (brs, 1H), 9.20 - 9.12 (m, 1H), 9.05 (s, 1H), 8.36 (d, $J$ = 5.7 Hz, 1H), 7.81 (d, $J$ = 2.1 Hz, 1H), 7.71 (dd, $J$ = 5.6, 1.0 Hz, 1H), 7.57 (dd, $J$ = 8.5, 2.1 Hz, 1H), 7.45 (d, $J$ = 8.4 Hz, 1H), 2.41 (s, 3H).
Figure S1. iJMJD6 synthesis and its binding with JMJD6 protein. Related to Figure 1.
(A) Schematic representation of chemical synthesis of iJMJD6. The detailed procedure is described in Materials and Methods.

(B) iJMJD6 was diluted with ddH2O to a final concentration of 50 µM from a DMSO stock solution, followed by absorption and fluorometric measurements. Absorbance spectrum was detected on a SHIMADZU UV-2600i UV-VIS-NIR spectrophotometer. Fluorescent emission spectrum was collected on a HITACHI F-7000 fluorescence spectrophotometer.

(C-E) The synthesized iJMJD6 as described in (A) was validated by LC-MS (C), 13C-NMR (D), and 1H-NMR (E). MW: molecular weight.

(F) JMJD6 protein incubated with DMSO or iJMJD6 were subjected to differential scanning calorimetry (DSC) analysis. Melting temperature (Tm) is shown as indicated.

(G-I) Computational analysis of the binding mode of iJMJD6 with JMJD6 (W174A) (G), JMJD6 (W206A) (H), and JMJD6 (H187A) (I) is shown (PDB ID: 3ldb). iJMJD6 is shown as magenta sticks. Amide acids are shown as green lines except that His 187, Trp 174, and Trp 206 are highlighted with sticks. The surfaces of Trp206 and Trp174 are indicated by dots. The iron (Fe2+) is shown as an orange sphere. The chelating bonds and the PI-Pl interactions are shown as red and blue dashes, respectively.

(J) The interaction between iJMJD6 and Fe2+ is shown.

(K, L) The distance between Fe2+ and O2 (K) or N2 (L) in iJMJD6 in the context of JMJD6 (WT), (W174A), (H187A), and (W206A) was calculated based on molecular dynamics stimulations (PDB ID: 3ldb).
Figure S2. iJMJD6 suppresses the growth of multiple types of cancer cells, but not normal cells. Related to Figure 2.

(A) Protein binding activity of iJMJD6 in cell culture medium is shown.
(B) The stability of iJMJD6 in cell culture medium is shown.

(C, D, E, F, G, H, K) HeLa (C), SMCC7721 (D), MCF7 (E), Ect1/E6E7 (F), LO2 (G),
MCF10A (H), and endometrial stem cells (K) were treated with iJMJD6 for 72 h at
concentrations as indicated followed by cell proliferation assay. EC$_{50}$ is shown.

(I, J) Ect1/E6E7, HeLa, LO2, SMCC7721, MCF10A, and MCF7 cells were subjected to
immunoblotting (I) and RT-qPCR (J) analysis to examine the protein and mRNA levels of
JMJD6, respectively ($\pm$ SEM; ***$p < 0.001$).
Figure S3. JMJD6 promotes the malignant behaviors of HeLa cervical cancer cells.

Related to Figure 2.
(A, B, C) HeLa cells were transfected with control siRNA (siCTL) or siRNA specifically targeting JMJD6 (siJMJD6) followed by cell proliferation assay (A), RT-qPCR analysis (B), and immunoblotting analysis (IB) (C) (± s.e.m., **P<0.01, ***P<0.001).

(E) Genomic DNA was extracted from JMJD6 knockout (KO) cells generated by CRISPR/Cas9 system, followed by PCR using specific primer sets surrounding gRNA targeting region (boxed in blue). The resultant PCR products were subjected to Sanger sequencing and one base pair, A, was inserted as shown in red.

(F) Wild type (WT) and JMJD6 KO HeLa cells were subjected to immunoblotting (IB) using the antibodies as indicated.

(D, G, H, J, L, N, P) Cell proliferation assay (D) (± SEM; ***p < 0.001), FACS analysis (G), colony formation assay (H), anchorage-independent growth assay (J), wound healing assay (L), and transwell (N), and apoptosis assay (P) were performed in Wild type (WT) and JMJD6 knockout (KO) HeLa cells.

(I, K, M, O, Q) Quantification of colony numbers (I, K), wound closure (M), migrated cells (O), and apoptotic cells (Q) as shown in (H, J), (L), (N), and (P), respectively (± SEM; **p < 0.01; ***p < 0.001).
Figure S4. iJMJD6 suppresses the malignant phenotypes in SMCC7721 liver cancer cells. Related to Figure 2.

(A, B, C, E, G, I, K) SMCC7721 cells were treated with DMSO or iJMJD6 (10 μM) followed by cell proliferation assay (A) (± SEM; **p < 0.01; ***p < 0.001), FACS analysis (B), colony formation assay (C), anchorage-independent growth assay (E), wound healing assay (G), and transwell (I), and apoptosis assay (K). Representative images are shown.

(D, F, H, J, L) Quantification of colony numbers (D, F), wound closure (H), migrated cells (J), and apoptotic cells (L) as shown in (C, E), (G), (I), and (K), respectively (± SEM; **p < 0.01; ***p < 0.001).
Figure S5. iJMJD6 suppresses the malignant phenotypes in MCF7 breast cancer cells.

Related to Figure 2.

(A, B, C, E, G, I, K) MCF7 cells were treated with DMSO or iJMJD6 (10 μM) followed by cell proliferation assay (A) (± SEM; **p < 0.01; ***p < 0.001), FACS analysis (B), colony formation assay (C), anchorage-independent growth assay (E), wound healing assay (G), and transwell (I), and apoptosis assay (K). Representative images are shown.

(D, F, H, J, L) Quantification of colony numbers (D, F), wound closure (H), migrated cells (J), and apoptotic cells (L) as shown in (C, E), (G), (I), and (K), respectively (± SEM; **p < 0.01; ***p < 0.001).
Figure S6. iJMJD6 inhibits JMJD6-mediated HSP70R469me1 demethylation. Related to Figure 4.

(A) HeLa Cells were treated with or without iJMJD6 for 1 h followed by immunoblotting (IB) analysis with antibodies as indicated.

(B, C) HeLa cells were treated with or without iJMJD6 (1 μM) for 1 h followed by H3R17me2(a) ChIP-seq, and the ChIP-seq tag distribution surrounding the center of JMJD6-bound (B) or random genomic sites (C) is shown.

(D, E, F) HeLa (D), SMCC7721 (E), and MCF7 (F) cells were treated with or without iJMJD6 (HeLa, 1 μM; SMCC7721, 10 μM; MCF7, 10 μM) for duration as indicated followed by immunoblotting analysis using anti-HSP70 R469me1 or HSP70 antibody. Intensity of HSP70 R469me1 was measured by image J and normalized to that of day 0.

(G) WT or JMJD6 (KO) HeLa cells were treated with or without iJMJD6 (1 μM) for duration as indicated followed by immunoblotting analysis using anti-HSP70 R469me1 or
HSP70 antibody. Intensity of HSP70 R469me1 was measured by image J and normalized to that of day 0.
Figure S7. iJMJD6 displays no significant impact on JMJD6-mediated hydroxylation of lysyl C-5. Related to Figure 4.
(A, C) *In vitro* hydroxylation assay was performed by mixing purified JMJD6 with various concentrations of iJMJD6 as indicated before adding synthetic short peptides from H4 (left), H3 (middle), or U2AF65 (right) proteins, the reactions were then subjected to MALDI-TOF MS analysis (A) or Succinate-Glo™ Assay (C). The reactions without JMJD6 were served as a control. Representative MALDI-TOF MS spectra are shown.

(B) The inhibition rate of JMJD6 hydroxylation activity, as measured by peak cluster areas using MALDI-TOF MS, was plotted versus log iJMJD6 concentration.
Figure S8. iJMJD6 inhibits JMJD6-mediated demethylation to inhibit the expression of Myc and CCND1 in cancer cells. Related to Figure 4.

(A, B) SMCC7721 cells were transfected with control siRNA (siCTL) or two independent siRNAs specifically targeting JMJD6 (siJMJD6 (1) and siJMJD6 (2)) followed by RT-qPCR (A) and immunoblotting (IB) (B) analysis to examine the mRNA and protein levels of c-Myc, N-Myc, and CCND1, respectively (± SEM; *p < 0.05; **p < 0.01).

(C, D) SMCC7721 cells were treated with or without iJMJD6 (10 μM) followed by RT-qPCR (C) and immunoblotting (IB) (D) analysis to examine the mRNA and protein levels of c-Myc, N-Myc, and CCND1, respectively (± SEM; **p < 0.01; ***p < 0.001).

(E, F) MCF7 cells were transfected with control siRNA (siCTL) or two independent siRNAs specifically targeting JMJD6 (siJMJD6 (1) and siJMJD6 (2)) followed by RT-
qPCR (E) and immunoblotting (IB) (F) analysis to examine the mRNA and protein levels of c-Myc, N-Myc, and CCND1, respectively (± SEM; **p < 0.01; ***p < 0.001).

(G, H) MCF7 cells were treated with or without iJMJD6 (10 μM) followed by RT-qPCR (G) and immunoblotting (IB) (H) analysis to examine the mRNA and protein levels of c-Myc, N-Myc, and CCND1, respectively (± SEM; **p < 0.01).

(I) HeLa cells were treated with or without iJMJD6 (1 μM) for 1 h followed by H4R3me2(s) ChIP, and the occupancy of H4R3me2(s) on the regions of 5 kb upstream from the transcription start site (TSS (-5kb)) and 5kb downstream from the transcription terminal site (TTS (+5kb)) of genes is shown. ChIP signals are presented as % of inputs (± SEM; N.S., not significant).
Figure S9. Myc and CCND1 are important target genes of iJMJD6. Related to Figure 4.

(A, B, C, E) HeLa cells were transfected with control vector or vectors expressing c-Myc or CCND1, and treated with or without iJMJD6, followed by cell proliferation assay (A), FACS analysis (B), wound healing assay (C), and immunoblotting analysis (E) (± SEM; ***p < 0.001).

(D) Quantification of wound closure as shown in (C) (± SEM; ***p < 0.001).
Figure S10. iJMJD6 has favorable pharmacokinetic properties and safety. Related to Figure 5.
(A) The plasma concentration of iJMJD6 versus time curve is shown when iJMJD6 was administrated orally (p.o., 100 mg/kg) or intravenously (i.v., 100 mg/kg).

(B) Pharmacokinetic parameters of iJMJD6 was calculated based on the curves as shown in (A). AUC\(_{(0-t)}\), area under the concentration-time curve from time zero to time t; AUC\(_{(0-\infty)}\), extrapolated area under the concentration-time curve from time zero to infinity; AUMC\(_{(0-t)}\), area under the first moment of the plasma concentration-time curve from time zero to time t; AUMC\(_{(0-\infty)}\), area under the first moment of the plasma concentration-time curve from time zero to infinity; MRT\(_{(0-t)}\), mean residence time from time zero to time t; MRT\(_{(0-\infty)}\), mean residence time from time zero to infinity; VRT\(_{(0-t)}\), variance of residence time from time zero to time t; VRT\(_{(0-\infty)}\), variance of residence time from time zero to infinity; t\(_{1/2}\), elimination half-life; T\(_{\text{max}}\), time until C\(_{\text{max}}\) is reached; V\(_{z}\), Apparent volume of distribution during terminal phase; CL, plasma clearance; C\(_{\text{max}}\), maximum plasma concentration; F, absolute bioavailability.

(C, D) Mice (n = 6/group) were treated with iJMJD6 at dosages as indicated for 28 days followed by complete blood count (C) and comprehensive metabolic panel analysis (D). RBC, red blood cells; HCT, hematocrit; HGB, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; RETIC, reticulocyte; WBC, white blood cells; NEU, neutrophil count; LYM, lymphocytes; MONO, monocytes; EOS, eosinophils; BASO, basophils; PLT, platelets; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit; ALT, alanine aminotrasferase; AST, aspartate aminotrasferase; ALP, alkaline phosphatase; TP, total protein; TC, total cholesterol; UA, uric acid; UREA, urea; Glu, glucose; CREA, creatinime; CK, creatine kinase; ALB, albumin.
(E) Organs from mice as described in (C) and (D) are shown.

(F) Sections from organs as shown in (E) were subjected to hematoxylin and eosin (H&E) staining, and representative images are shown.

(G) The weight of organs as shown in (E) is shown.
Figure S11. iJMJD6 exhibits potent anti-tumor activities. Related to Figure 5.

(A) Tumor sections from Fig. 5A were subjected to hematoxylin and eosin (H&E) staining.

(B) The body weight of mice as described in Fig. 5A is shown.

(C, E, G) Female BALB/c nude mice were inoculated with MDA-MB-231 (C), U87 (E), or HCT116 (G) cells, randomized, treated with DMSO or iJMJD6 at dosage as indicated
intraperitoneally every other day. Tumor growth curve is shown (± SEM; **p < 0.01, ***p < 0.001).

(D, F, H) The weight of tumors as described in (C), (E), and (G) is shown in (D) (F), and (H), respectively (± SEM; *p < 0.05; **p < 0.01).

(I, K, M) BALB/c nude mice were implanted with tumor fragments from triple-negative breast cancer (TNBC) patient- (I), glioblastoma (GBM) patient- (K), or colon cancer (CRC) patient- (M) derived xenografts, randomized, treated with DMSO or iJMJD6 at dosages as indicated intraperitoneally every other day. Tumor growth curve is shown (± SEM; **p < 0.01, ***p < 0.001).

(J, L, N) The weight of tumors as described in (I), (K), and (M) is shown in (J), (L), and (N), respectively (± SEM; *p < 0.05; ***p < 0.001).
Figure S12. Co-treatment with iJMJD6 and JQ1 exhibits synergistic effects on suppressing oncogene expression and cancer cell growth. Related to Figure 6.

(A, B) SMCC7721 (A) and MCF7 (B) cells were treated with iJMJD6 (5 μM) or JQ1 (2.5 μM) alone or in combination for 12 h, followed by immunoblotting (IB) with antibodies as indicated.

(C, D) SMCC7721 (C) and MCF7 (D) cells were treated with iJMJD6 (5 μM) or JQ1 (2.5 μM) alone or in combination followed by proliferation assay (± SEM; ***p < 0.001).
Figure S13. Co-treatment with iJMJD6 and ICI improves the efficacy ICI in suppressing oncogene expression and ERα-positive breast cancer cell growth. Related to Figure 7.

(A) T47D cells were treated with iJMJD6 (5 μM) or ICI (fulvestrant, 2 μM) alone or in combination for 12 h, followed by immunoblotting (IB) with antibodies as indicated.

(B) T47D cells were treated with iJMJD6 (5 μM) or ICI (2 μM) alone or in combination, followed by cell proliferation assay (± SEM; ***p < 0.001).
Figure S14. Repeats that are related to Figure 2D, Figure 3D, Figure 6D, Figure 7D, Figure S3H, Figure S4C, and Figure S5C.
(A, B) Repeats of colony formation assay in Fig. S3H. Colony formation assay were performed in Wild type (WT) and JMJD6 knockout (KO) HeLa cells (± SEM; ***p < 0.001).

(C-H) Repeats of colony formation assay in Fig. 2D, S4C and S5C. HeLa (C, D), SMCC7721 (E, F) and MCF7 (G, H) cells were treated with DMSO or iJMJD6 (HeLa, 1 μM; SMCC7721, 10 μM; MCF7, 10 μM) for 10 days followed by colony formation assay (± SEM; ***p < 0.001).

(I, J) Repeats of colony formation assay in Fig. 3D. WT and JMJD6 (KO) HeLa cells were treated with DMSO or iJMJD6 at the indicated concentration for 10 days, followed by colony formation assay (± SEM; **p < 0.01; ***p < 0.001; N.S., not significant).

(K, L) Repeats of colony formation assay in Fig. 6D. HeLa cells were treated with iJMJD6 (0.5 μM) or JQ1 (2.5 μM) alone or in combination for 10 days, followed by colony formation assay (± SEM; ***p < 0.001).

(M, N) Repeats of colony formation assay in Fig. 7D. MCF7 cells were treated with iJMJD6 (5 μM) or ICI (2 μM) alone or in combination for 10 days, followed by colony formation assay (± SEM; ***p < 0.001).
Table S1. Sequence information for all qPCR primers used in the current study.

Sequence information of qPCR primers designed to detect the expression of c-Myc, N-myc, and CCND1, and ChIP primers specifically targeting the enhancer regions, 5 kb upstream of translation start sites (TSS (-5kb)), or 5 kb downstream of translation terminal sites (TTS (+5kb)) of c-Myc, N-myc, and CCND1 are shown. F: forward; R: reverse.

| Name     | Application            | Sequence                                                |
|----------|------------------------|---------------------------------------------------------|
| c-Myc    | mRNA                   | F: 5'-GGATTTTTTTCGGGTAGGGAA-3'                         |
|          |                        | R: 5'-TTCTGTGTTGGAAGCTAAGTTT-3'                        |
| N-myc    | mRNA                   | F: 5'-CGACCACAGGGCCCTCAGTA-3'                          |
|          |                        | R: 5'-CAGCTTGGTGTTGGAGGAG-3'                           |
| CCND1    | mRNA                   | F: 5'-CCGAGAAGCTGTGCATCTAC-3'                          |
|          |                        | R: 5'-AGGTCCAAGCTGTGGAGGAG-3'                          |
| c-Myc    | ChIP (enhancer)        | F: 5'-CTCTGGAAACAGGCAGACACA-3'                         |
|          |                        | R: 5'-TGCAAGCTATCTGGATTGG-3'                           |
| c-Myc    | ChIP (TSS (-5kb))      | F: 5'-GTCTCACCCCCTAAGTTCGCT-3'                         |
|          |                        | R: 5'-CGACCCAACACCTTCTTGTG-3'                          |
| c-Myc    | ChIP (TTS (+5kb))      | F: 5'-TCCCAGTGATCCTCTCCAC-3'                          |
|          |                        | R: 5'-TGGAAGCTTGGAGGTAGG-3'                            |
| N-myc    | ChIP (enhancer)        | F: 5'-TTAGAAGCATCGGTCTCCCC-3'                          |
|          |                        | R: 5'-CAGAGAGGAGCTGCCAGAC-3'                           |
| N-myc    | ChIP (TSS (-5kb))      | F: 5'-CAAACCTCCTCCAGCTCA-3'                            |
|          |                        | R: 5'-AGTCAGGTAGGTTGGTC-3'                             |
| N-myc    | ChIP (TTS (+5kb))      | F: 5'-GCCTAGGAATTGGAGTCCG-3'                           |
|          |                        | R: 5'-CCTGGTCAGTGGCCTATGAA-3'                          |
| CCND1    | ChIP (enhancer)        | F: 5'-CGCCTTGCATATCTGCTG-3'                            |
|          |                        | R: 5'-AAAGACCTCCACCACCTG-3'                            |
| CCND1    | ChIP (TSS (-5kb))      | F: 5'-AGGATGGGTGAGTGAAG-3'                             |
|          |                        | R: 5'-GGTCCCCATGCTTTTAGACA-3'                          |
| CCND1    | ChIP (TTS (+5kb))      | F: 5'-ATCCACCCCCCCAGAAGAG-3'                           |
|          |                        | R: 5'-GGGGATGACTCAGGAGAGG-3'                           |
SI References

1. W.-w. Gao et al., JMJD6 licenses ERα-dependent enhancer and coding gene activation by modulating the recruitment of the CARM1/MED12 co-activator complex. Mol cell 70, 340-357. e348 (2018).

2. W.-w. Gao et al., Arginine methylation of HSP70 regulates retinoid acid-mediated RARβ2 gene activation. Proc Natl Acad Sci U S A 112, E3327-E3336 (2015).

3. H. f. Shen et al., The Dual Function of KDM5C in Both Gene Transcriptional Activation and Repression Promotes Breast Cancer Cell Growth and Tumorigenesis. Advanced Science 8, 2004635 (2021).

4. W. Liu et al., Brd4 and JMJD6-associated anti-pause enhancers in regulation of transcriptional pause release. Cell 155, 1581-1595 (2013).