Supplementary information

Contents：

Materials and Methods

Cell Lines

U251 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MCF7, ZR-751, 22RV1, and LNCaP cell lines were a gift from the Xiaobing Wang Laboratory. The hTERT RPE-1 cell line was obtained from ATCC. U251 and MCF7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin (100 U/mL)/streptomycin (0.1 mg/mL). ZR-751, 22RV1, and LNCaP cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. hTERT RPE-1 cells were cultured in DMEM: F-12 medium with 10% fetal bovine serum.

Animals

The animal studies were approved by the animal ethical committee at the Cancer Hospital, Chinese Academy of Medical Sciences (Beijing, China). All animal studies were conducted according to protocols approved by the Animal Ethics Committee of the Cancer Hospital, Chinese Academy of Sciences.

CRISPR-Cas9 synthetic lethal screens

U251-MEN1$^{+/+}$ (MEN1-WT) and U251-MEN1$^{-/-}$ (MEN1-KO) cells were transfected with the Brunello library at an MOI of ~0.3 and an average coverage of 500-fold. Cells were transfected for 24 h and then selected in puromycin for 48 h. 3.822×10^{8} transduced cells were harvested for T0, and the remaining cells were passaged every 72 h for 14

population doublings. After 21 days in culture, cells were harvested for T21. Genomic DNAs from MEN1-WT and MEN1-KO at T0 and T21 were isolated, using the Blood and Cell Culture DNA Maxi Kit (QIAGEN; Germantown, MD, USA), according to the manufacturer's protocol. To prepare the sgRNA sequencing library, the sgRNA integrated region was amplified through two rounds of PCR. In order to achieve a coverage of 250 × for 76,441 sgRNAs, 126 μg input gDNA (6.6 pg × 76,441 × 250 = 126 μg) was used in the first PCR. A total of 42 PCR reactions were performed using Q5 High-Fidelity DNA Polymerase (New England Biolabs; Rowley, MA, USA). 2 μL of the first PCR product was used for the second PCR, and the final PCR products were purified using AMPure XP Beads (Beckman Coulter; Brea, CA, USA). NGS reactions were performed on a HiSeq X10 platform (Illumina; San Diego, CA, USA). The sequences of the primers used for PCR are shown in Table S3.

Lentivirus production

The lentiCRISPR v2 system was used to produce the lentiviruses containing individual sgRNAs for validation. The day before transfection, HEK293T cells (4×10^7 cells/well) were seeded in 6-well plates and transfections were performed when cells were ~ 70% confluent. HEK293T cells were transfected with lentiCRISPR v2, psPAX2 (Addgene plasmid #12260) and pMD2.G (Addgene plasmid #12259) plasmids using Neofect transfection reagent (Neo Biotech; Beijing, China) according to the manufacturer's instructions. Supernatants were collected after 60 h, clarified by centrifugation, and filtered through a PVDF filter membrane (0.45 μm, Millipore SteriCup 250 mL,

Millipore; Shanghai, China). The virus was aliquoted and frozen at -80°C.

SiRNA transfection

SiRNAs were designed by GenePharma. Cells were plated in 6-well plates, and transfections were performed with siRNAs at a final concentration of 20 μM using RNAimax (Life Technologies, Thermo Fisher Scientific Distributer; Brendale QLD, Australia) according to the manufacturer's instructions. Cells were collected 24 h after transfection. The sequences of the siRNAs used are shown in Table S4.

Quantitative Real-time PCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific), and cDNA was prepared from total RNA (500 ng) using the PrimeScript RT Reagent Kit (TaKaRa; Tokyo, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR® Premix Ex Taq™ (TaKaRa; Tokyo, Japan) on the ABI V7 (ABI; Indianapolis, IN, USA). The sequences of the primers used are shown in Table S5.

Cell viability assay

Cell viability was measured with the Cell Counting Kit-8 (CCK-8) (DOJINDO; Kumamoto, Japan). After siRNA transfection, cells were plated in 96-well plates and cultured overnight. The absorbance was measured at 450 nm daily for 6 days and plotted to generate growth curves to evaluate the cell viability. One representative

experiment of three is shown with n = 3 ± SD for each data point. *P < 0.05, **P < 0.01, ***P < 0.001 (student t test, three technical replicates averaged in each).

Metabolomics profiling

Targeted metabolomics of MEN1-WT and MEN1-KO cells were conducted as previously described[1]. The data are presented as the mean ± SEM. *P < 0.05, **P < 0.01, (student t test, three biological replicates averaged in each).

Western blot analysis

Cells were lysed in buffer containing 1M Tris-HCl (PH6.8), 80% glycerin, and 10% SDS, and the lysates were heated at 100°C for 10 min and centrifuged at 15,000 rpm at 4°C for 5 min. Proteins were quantified using the BCA kit (Beyotime Biotech; Nantong, China) according to the manufacturer's instructions. Equal amounts of protein were resolved on an SDS-PAGE gel and transferred to a PVDF membrane (Millipore; Shanghai, China). Membranes were blocked with 5% milk in TBST, and incubated with primary antibodies overnight at 4°C and subsequently HRP-conjugated secondary antibodies to detect proteins with chemiluminescence. Antibodies used to determine protein expression were the following: menin (#sc-374372; RRID: AB_10988942; Santa Cruz Biotechnology), DHODH (#14877-1-AP; RRID: AB_2091723; Proteintech), GAPDH (# 60004-1-Ig; RRID: AB_2107436; Proteintech).

Clonogenic survival assay

1×10^4 cells were plated in 6-well plates and cultured for 10 days. Surviving colonies were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet.

Immunohistochemistry

Tumors were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned for immunohistochemistry. Antigen retrieval was performed on sections with citrate buffer. Sections were then blocked with sheep serum working solution for 30 min at room temperature, incubated with primary antibody overnight at 4°C, rinsed three times with TBST, and incubated with biotinylated antibody. Coloration was performed with the DAB Horseradish Peroxidase Color Development Kit (ZSGB-Bio; Beijing, China), and counterstaining was performed with hematoxylin.

RNA-seq data analysis

Total RNA of MEN1-WT and MEN1-KO cells was isolated using TRIzol. RNA-seq was performed by HaploX company (Beijing, China). Sequencing reads were aligned to human genome (hg38) using Hisat2[2] with default parameters. Counts for genes were counted using summarizeOverlaps function in Bioconductor[3]. Then, we used DESeq2[4] to identify differentially expressed genes (FDR < 0.05, fold change > 2). Genes that were not expressed (mean FPKM of three replicates < 1) in both MEN1-WT and MEN1-KO were removed during differential expression analysis. Gene Ontology (GO) and KEGG pathway enrichment were performed using Enrichr[5] and Gene Set Enrichment Analysis (GSEA)[6] was performed using GSEA desktop application on

normalized counts from DESeq2.

ATAC-seq library preparation

ATAC-seq was performed as previously described[7]. Briefly, MEN1-WT and MEN1-KO cells were harvest and count separately, 50,000 cells were collected and centrifuge for 5 min at 500g at 4°C. Cell pellets were washed with PBS and lysed by buffer containing 10 mM Tris-HCl (pH 7.4),10 mM NaCl, 3 mM MgCl2, 0.1% (v/v) Igepal CA-630. Transposition reaction and purification was used TruePrepTM DNA Library Prep Kit (Vazyme biotech co., ltd). The transposed DNA fragments were amplified using for library preparation. Fragment sizes of ATAC-seq libraries were determined by gel electrophoresis and purified library were sequenced on a HiSeq X10 platform (Illumina; San Diego, CA, USA).

ATAC-seq data analysis

Sequencing quality was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters and low quality reads were removed using TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Then, reads were aligned to human genome (hg38) using Bowtie2[8] with default parameters and -X 1000. Reads with low mapping quality and those mapped to mitochondrion DNA were removed and MarkDuplicates algorithm in Picard software (https://broadinstitute.github.io/picard/) was used to remove duplicates. MACS2[9] was

used for peak calling (parameters: --keep-dup all --nomodel --shift -100 --extsize 200). Differentially ATAC-seq peaks were identified using DiffBind (https://bioconductor.org/packages/release/bioc/html/DiffBind.html) and the cutoff was set to FDR $\leqslant$0.05. Peak annotation was performed using ChIPseeker[10] and regions within 3 kb upstream or downstream of gene transcription start site were regarded as promoters. IGV[11] and Circos[12] were used to visualize genomic data.

Animals

Mice were raised in a sterile environment and supplied with food and water. *Men1*$^{-/+}$ mice were created by Beijing Biocytogen (Beijing, China). Briefly, two sgRNAs were designed to target distinct positions in introns 3 and 6 of *Men1* in mice. The C57BL/6J mouse strain was used as the embryo donor, and correctly targeted ES clones were injected into C57BL/6J blastocysts to generate *Men1*$^{+/-}$ mice. Treatments with leflunomide were the following: continuous treatment, 100 mg/kg orally for 6 months started at the age of 6 months; and intermittent treatment, 100 mg/kg orally for 28 consecutive days every two months for 5 times started at the age of 3 months. All mice were killed at the age of about 19 months, and the pancreases were collected for further analysis. In addition, spontaneous tumors formed in *Men1*$^{-/+}$ mice were inoculated into 40 normal mice on average and divided into two subgroups: 20 mg/kg leflunomide or vehicle administered by gavage, once every 3 days. After 7 doses, tumor morbidity (if the tumor volume was less than 10 mm^3 or no tumor grows, it was recorded as no tumor, otherwise it was recorded as tumor) and tumor volume were evaluated. Mice were

weighed every three days during the experiment.

Xenograft model

MEN1-WT-U251 and MEN1-KO-U251 cells were injected subcutaneously into the female BALB/C nude mice (Beijing HFK Bioscience CO. Ltd.; Beijing, China). When tumor volume reached 100 mm^3, mice from each group were randomly divided into two subgroups: 20 mg/kg leflunomide or vehicle (corn oil) administered by gavage, once every 3 days. Tumor volume was measured manually every 3 days, and the total volume was calculated as $(a \times b^2)/2$ (a = longest length of diameter, b = shortest length in diameter). Mice were sacrificed when the tumor grew to 1000 mm^3.

Clinical Trial of Leflunomide

Study design

This single arm, single center, phase II clinical study was designed to determine the safety, efficacy of leflunomide in MEN1-mutant neuroendocrine tumor patients. The clinical trial was approved by Medical Ethics Committee of Cancer Hospital, Chinese Academy of Medical Sciences (approval ID: 20/158-2354). Informed consents were obtained from all patients.

Inclusion criteria

1) 18-75 years old

2) pathologically confirmed neuroendocrine tumors

3) NGS validation of *MEN1* germline mutation or tumor with somatic *MEN1* mutation

4) Unresectable neuroendocrine tumors

5) Standard first-line treatment system failed

6) At least one evaluable lesion according to the RECIST criteria

7) ECOG physical status score 0-1

Exclusion criteria

1) Receiving any anti-tumor treatment within 4 weeks

2) Participated in clinical trials of other drugs/therapies within 4 weeks before the first use of leflunomide

3) Undergoing major surgery within 4 weeks

4) With other malignant tumors

5) Other severe, acute or chronic medical diseases or laboratory abnormalities

6) Poor compliance

7) Poor conditions that made patients unsuitable to participate in this trial, including but not limited to: poorly controlled high blood pressure (blood pressure consistently greater than 150/90 mmHg); poorly controlled diabetes; heart disease (Class III/IV congestive heart failure or heart block as defined by the New York Heart Association); deep vein thrombosis or pulmonary embolism et al.

Treatment and Assessments

Patients received oral administration of leflunomide tablets (oral administration before bedtime, once daily, 20 mg each). The test would be terminated if progress disease or

untolerant adverse reactions. Patients were reviewed every 2 cycles (1 cycle per 28 days), including CT, MRI examination, blood routine examination, coagulation examination, biochemical examination and tumor markers (CEA, CA199 and NSE). The clinical response to leflunomide was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) by three experienced imaging doctors.

Statistical analysis

Analysis was performed using GraphPad Prism6 software. The two-tailed unpaired student's t test was used to assess statistical significance where $p < 0.05$ was considered to be significant. *P < 0.05, **P < 0.01, ***P < 0.001.

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Fig. S1 Construction of *MEN1* knockout cell line.

(a) Validation of MEN1-KO in U251 cells by Sanger sequencing. (b) The mutation patterns on the two alleles were marked in U251 cells. (c) Validation of MEN1-KO in U251 cells by western blot. (d) Validation of MEN1-KO in hTERT RPE-1 cells by Sanger sequencing. (e) The mutation patterns on the two alleles were marked in hTERT RPE-1 cells. (f) Validation of MEN1-KO in hTERT RPE-1 cells by western blot.

Fig. S2 The analysis of the genome-wide CRISPR-Cas9 screen in U251 cells.

(a) MAGeCK mle beta score for each gene in MEN1-WT and MEN1-KO. The blue dots represent the genes that do not affect the growth of MEN1-WT cells, while the orange dots represent genes that are synthetic lethal with MEN1 deficiency. (b) MAGeCK mle beta score for each gene in MEN1-WT cells. The blue dots represent the genes that do not affect the proliferation of MEN1-WT cells. (c) MAGeCK mle beta score for each gene in MEN1-KO cells. The blue dots represent the genes that do not affect the proliferation of MEN1-KO cells and the orange dots represent the genes essential for the growth of MEN1-KO cells. (d) Differences in beta score of CAD and DHODH between MEN1-WT and MEN1-KO cells after 14 doublings in the CRISPR-Cas9 screen. (e) The ratio of KO beta score/WT beta score in the genes that were detected from the screen.

Fig. S3 The validation of the synthetic lethality between DHODH and MEN1.

(a) and (b) The knockdown of DHODH or CAD in U251 cell line were verified by qPCR. (c) The morphology and (d) the colony formation staining of WT (MEN1-WT) and KO (MEN1-KO) in U251 cell line with or without DHODH knockdown. (e) and

(f) The knockdown of DHODH or CAD in hTERT RPE-1 cell line were verified by qPCR. (g) Cell viability was measured using CCK8 assay of knockdown of DHODH in WT (MEN1-WT) and KO (MEN1-KO) in hTERT RPE-1 cell line. (h) Cell viability was measured using CCK8 assay of knockdown of CAD in WT (MEN1-WT) and KO (MEN1-KO) in hTERT RPE-1 cell line. (i) The morphology and (j) the colony formation staining of WT (MEN1-WT) and KO (MEN1-KO) in hTERT RPE-1 cell line with or without DHODH knockdown.

Fig. S4 The DHODH inhibitor was synthetic lethal with MEN1 deficiency.

(a) The IC50 of leflunomide in MEN1-WT, MEN1-KO and MEN1-reconstituted cells detected with CCK8. (b) The MEN1 expression was detected by western blot in LNCaP, 22RV1, MCF7 and ZR-751 cells. (c) and (d) The IC50 of leflunomide or A771726 in MEN1-WT and MEN1-mutated (MEN1-MUT) cell lines (LNCaP vs 22RV1; MCF7 vs ZR-751).

Fig. S5 The synthetic lethal interaction is due to the insufficient metabolites of pyrimidine biosynthesis.

(a) Western blots to detect expression levels of MEN1 and DHODH using protein lysates prepared from MEN1-WT, MEN1-KO and KO-reconstituted cells in U251 cell line. (b) IGV tracks showing ATAC-seq signals and gene expression levels of *DHODH* in WT and KO cells. (c) and (d) The relative cell viability of MEN1-mutant cells (22RV1 and ZR751) treated with 100 μM leflunomide in combination with different concentrations of orotate 48 h assessed with CCK8 assay.

Fig. S6 The validation of MEN1-deficient tumors.

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Fig. S7 Leflunomide prevents the development of pancreatic neuroendocrine tumors in *Men1*$^{-/+}$ mice.

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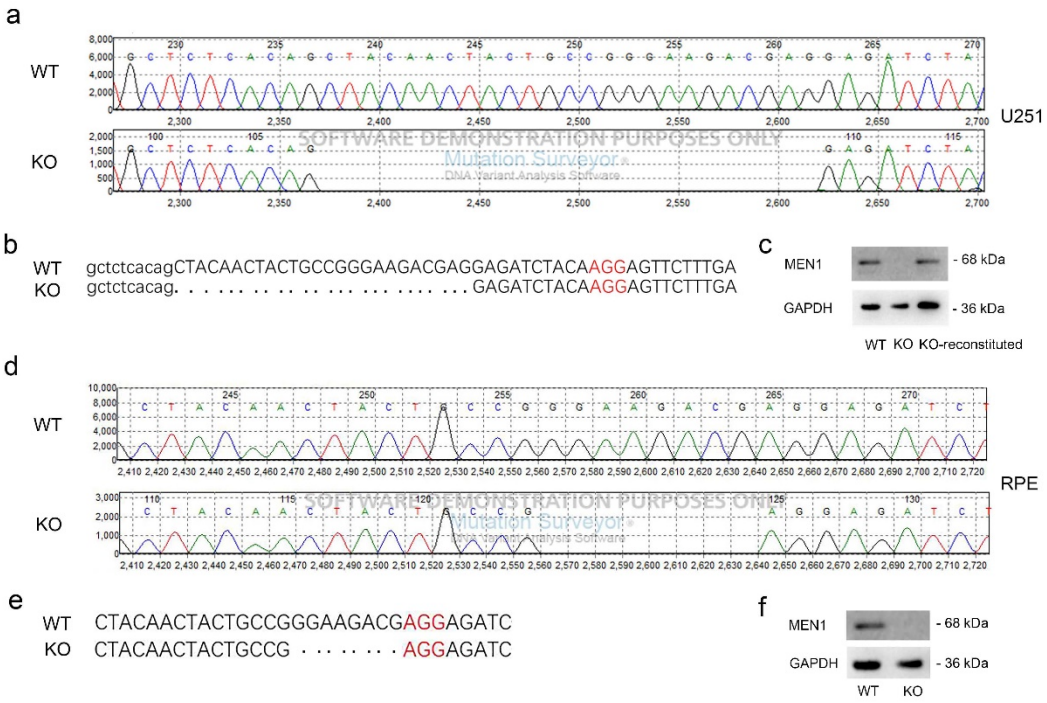

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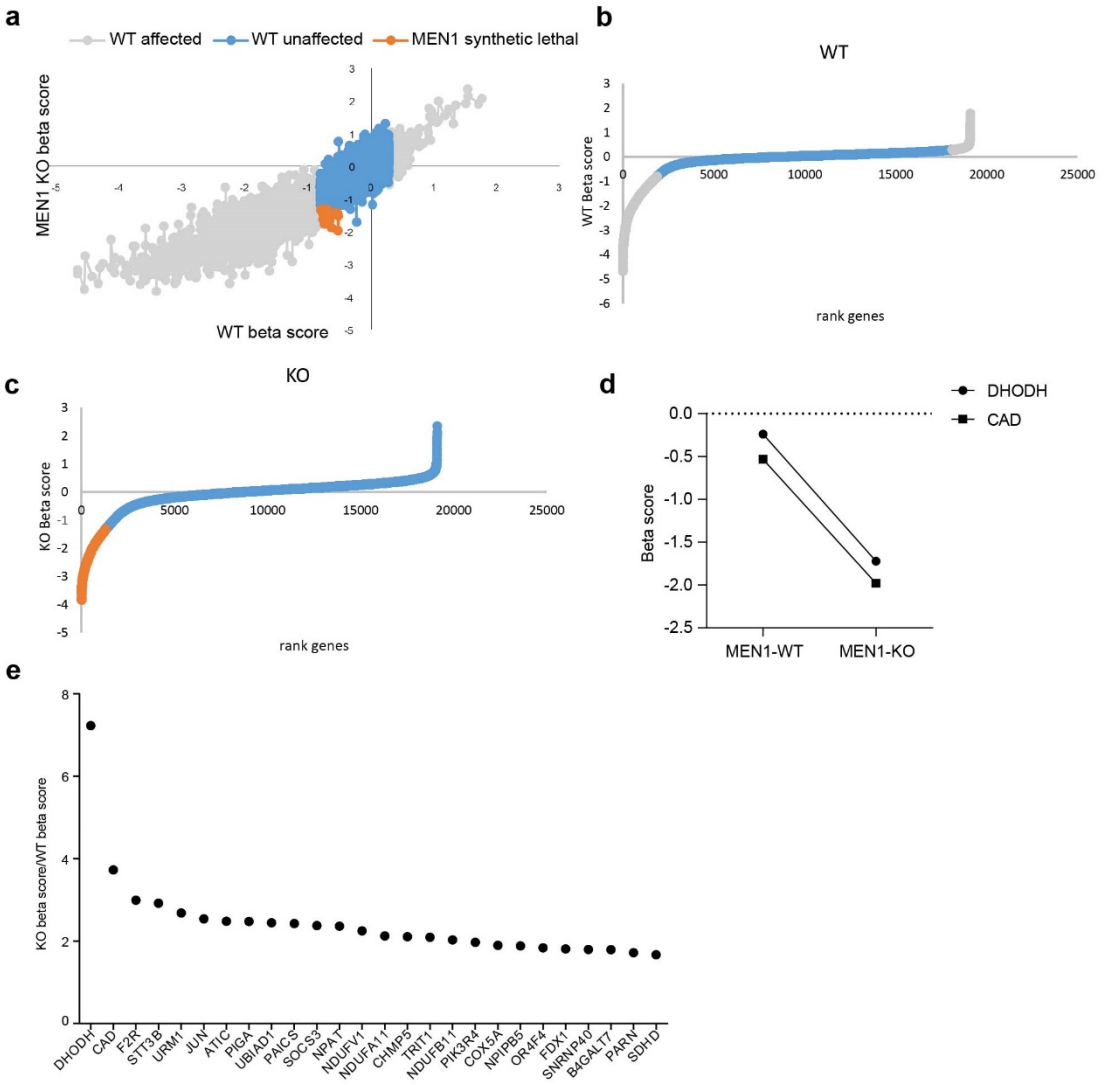

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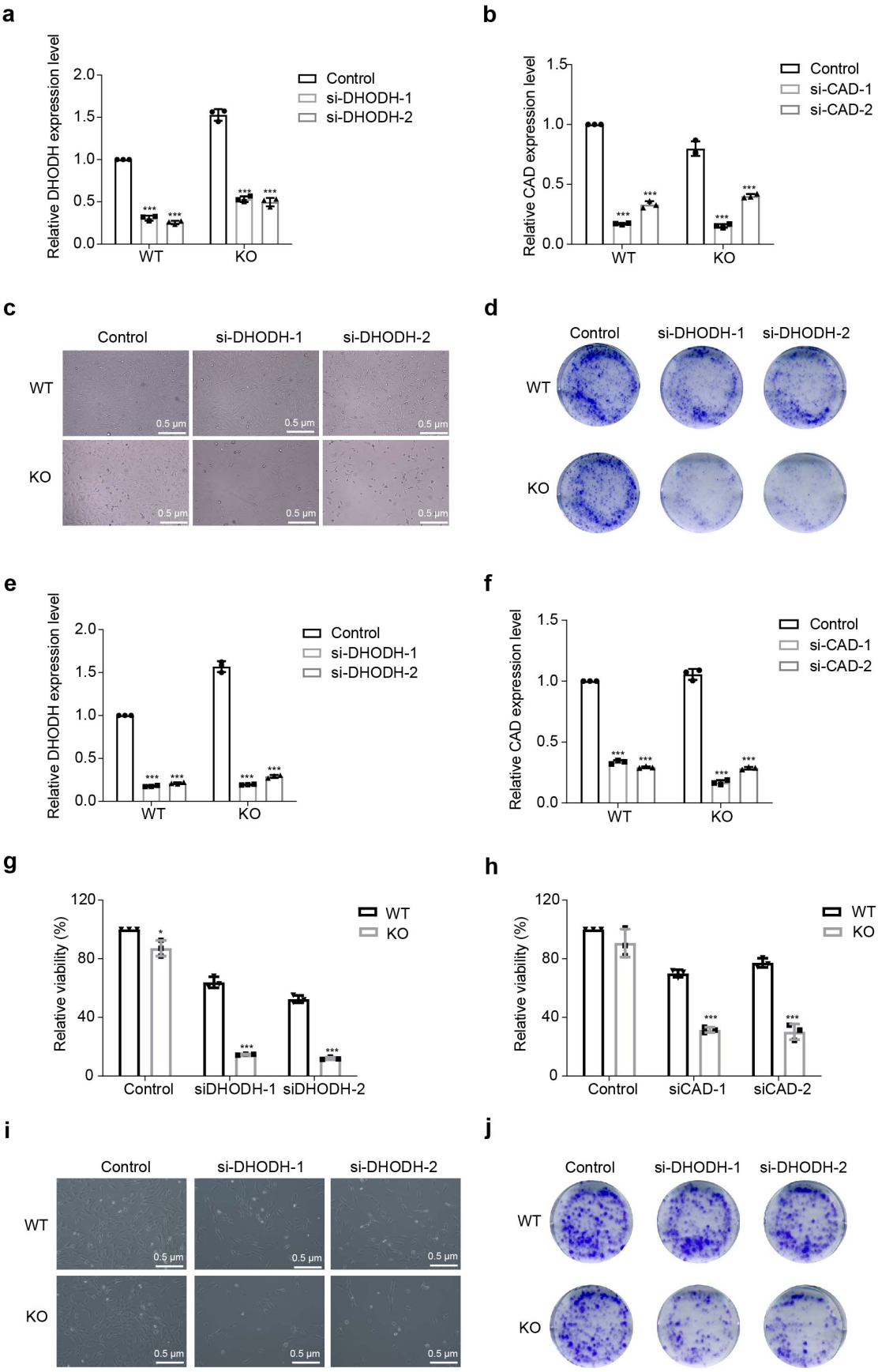

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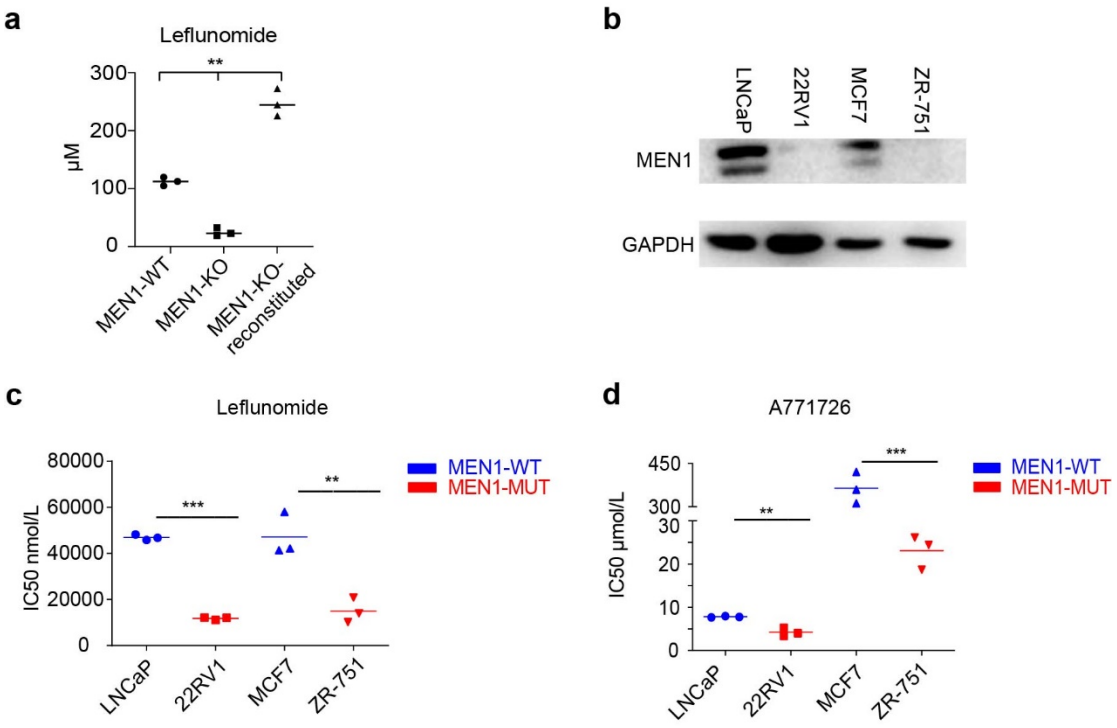

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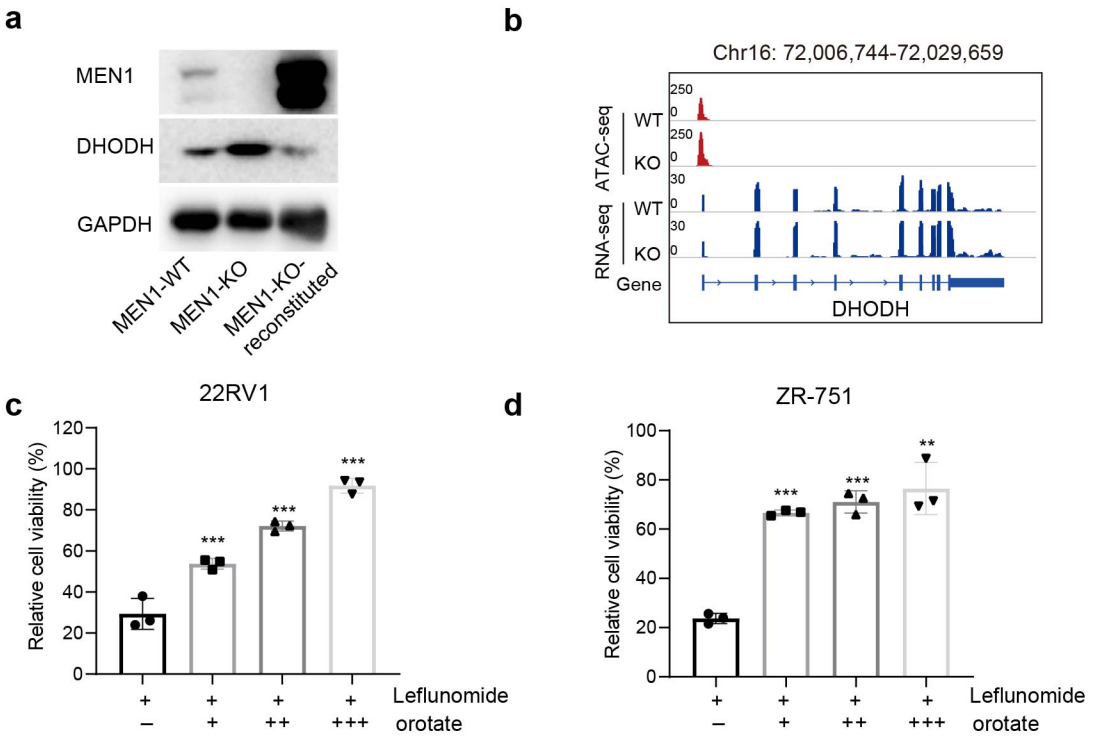

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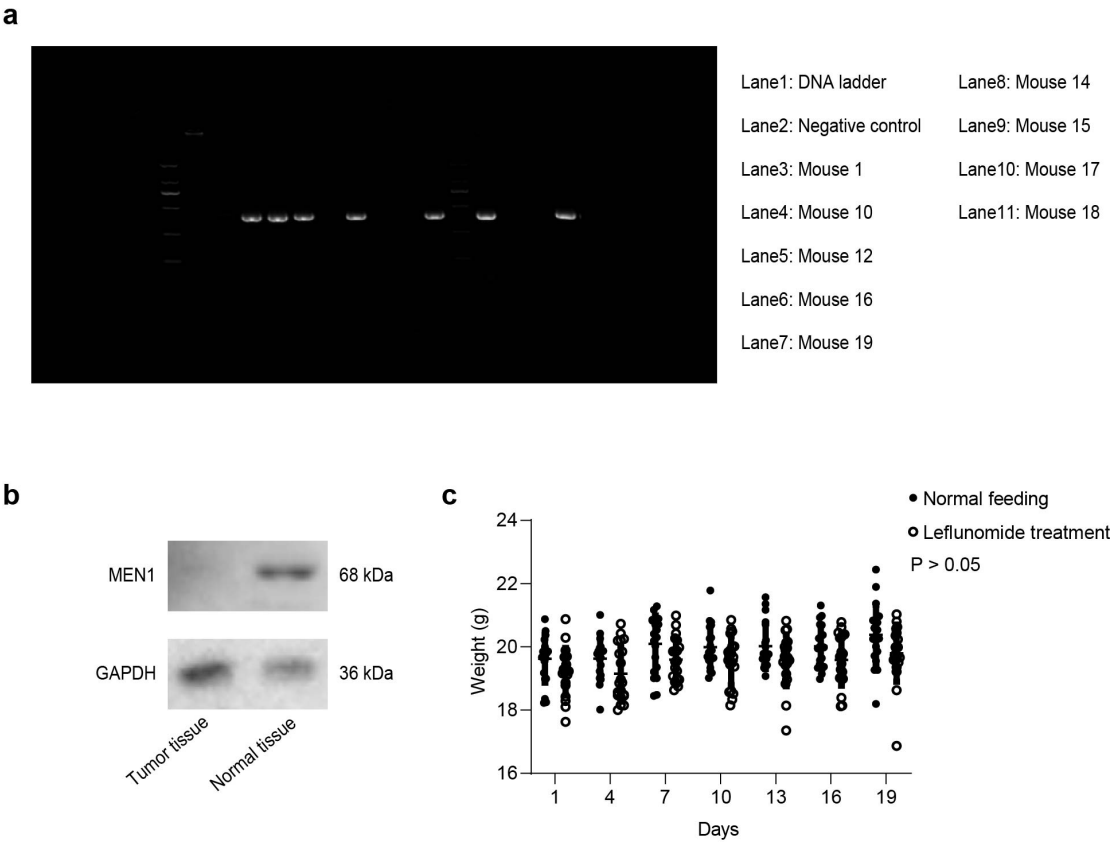

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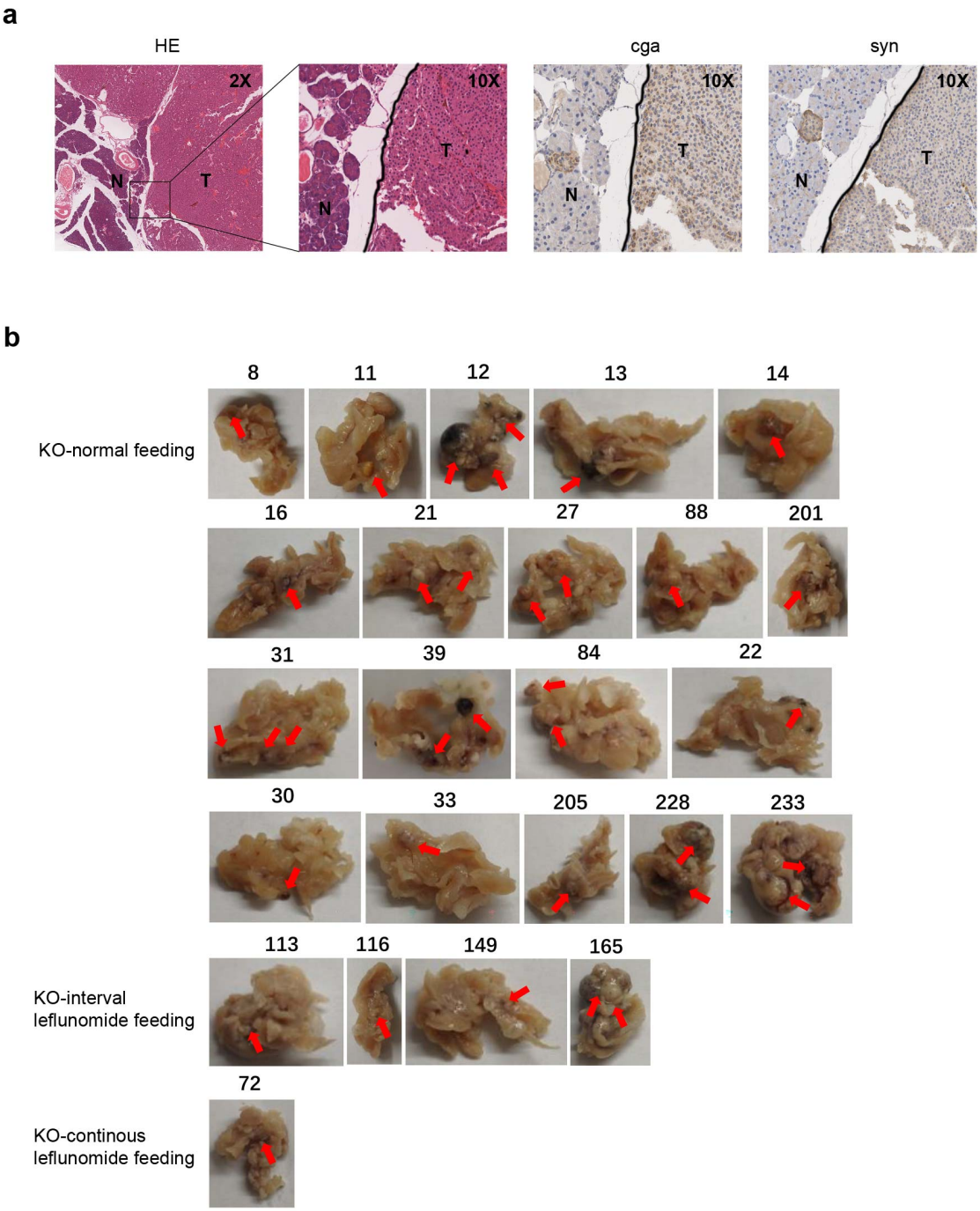

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