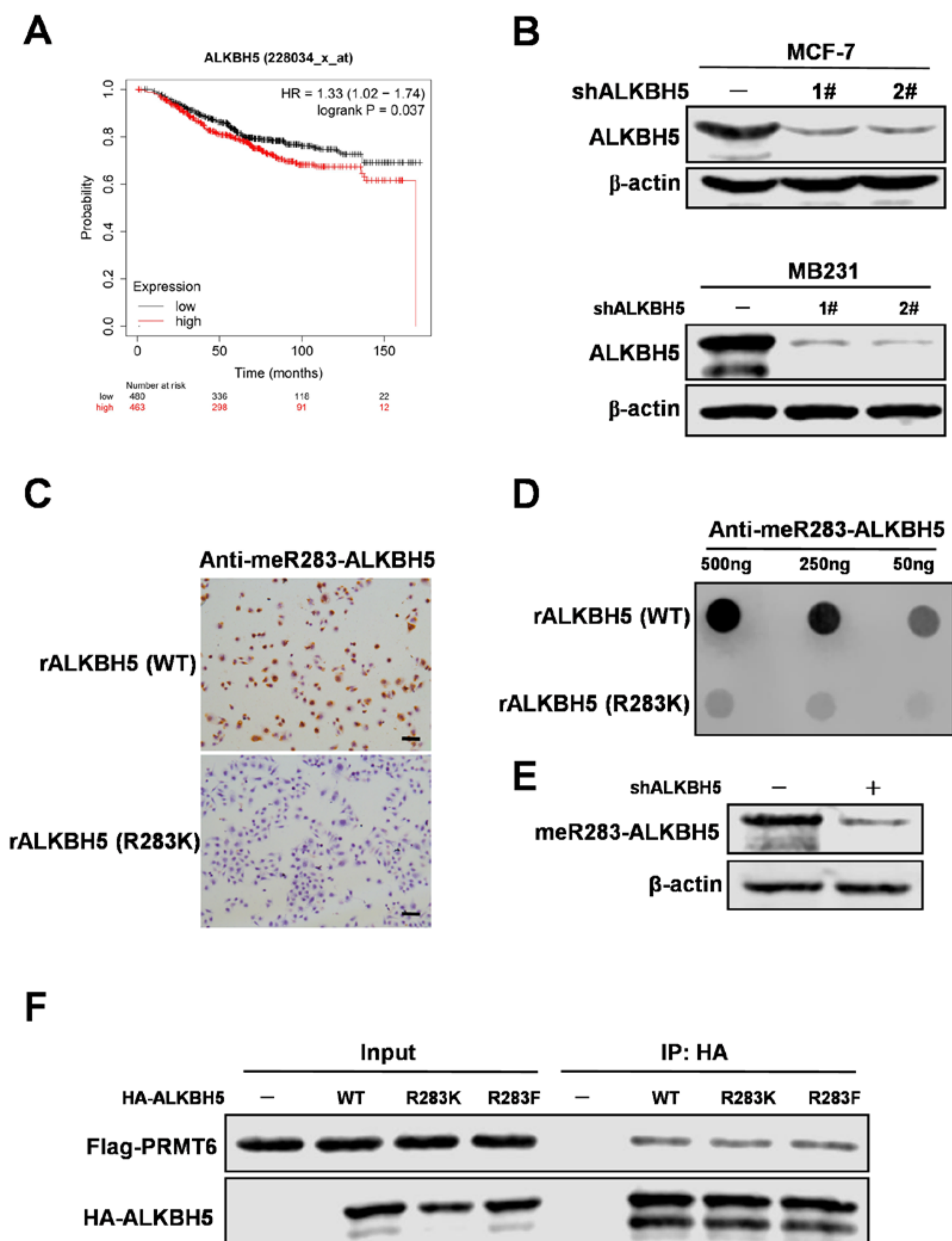
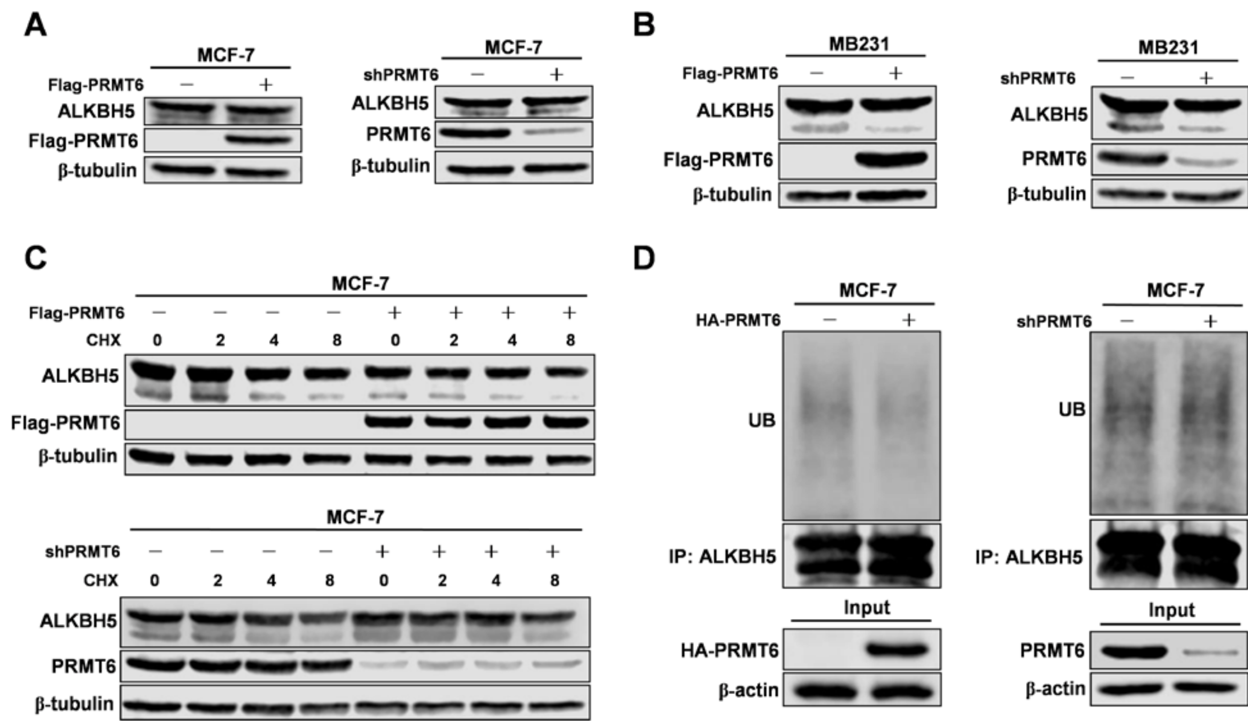
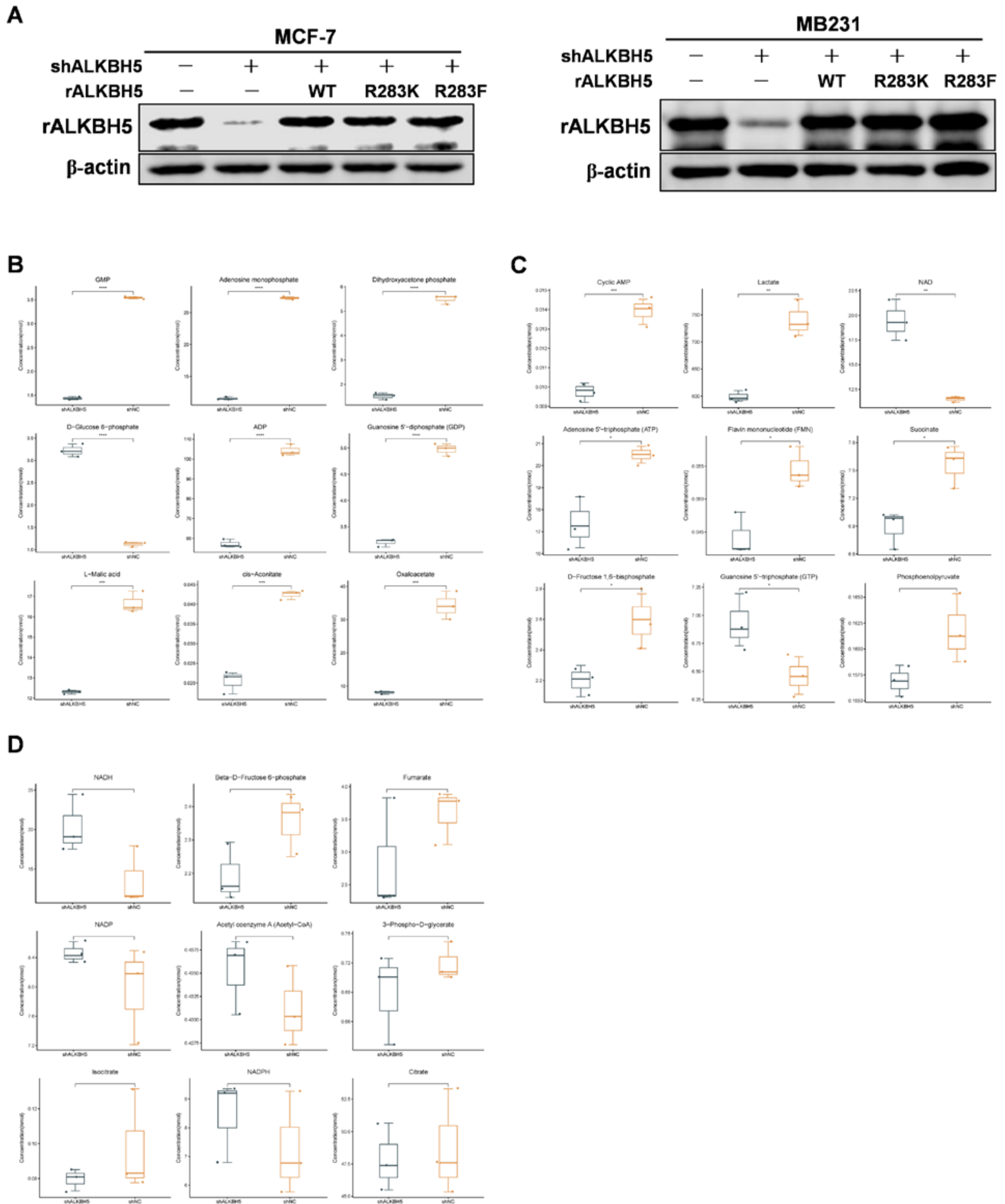
Supplementary Data



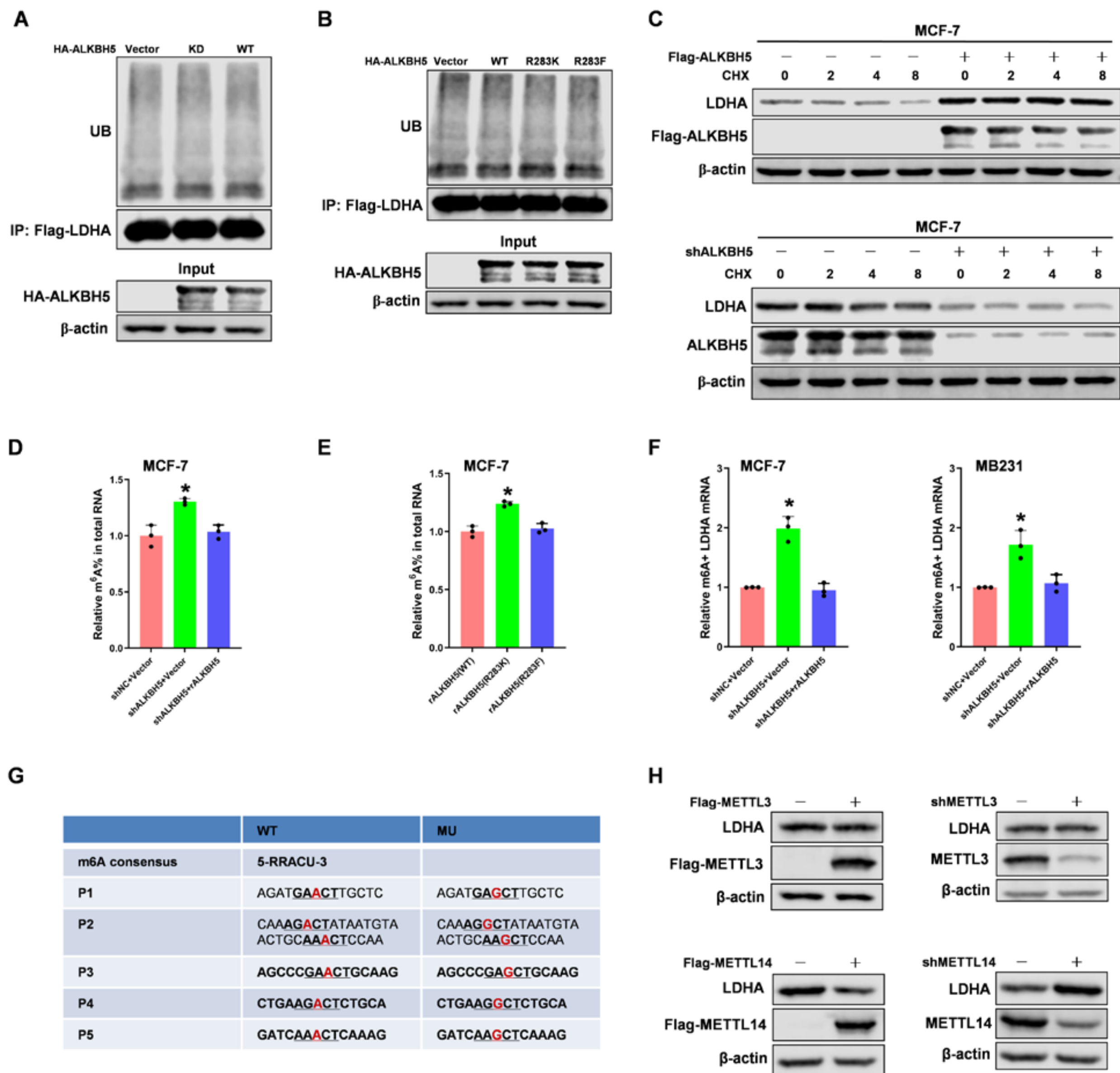
Supplementary Fig. 1 (A) Kaplan-Meier plotter Overall Survival curve for breast cancer patient groups with high and low ALKBH5 mRNA expression using Kaplan-Meier plotter. *p<0.05. (B) Breast cells were stably knocked down ALKBH5. Immunoblotting analyses were performed with the indicated antibodies. (C) Validation of the anti-meR283-ALKBH5 antibody by immunohistochemistry analyses of ALKBH5-depleted MCF-7 cells reconstituted with ALKBH5 (WT or R283K) (scale bar, 20 μm). (D) Validation of the anti-meR283-ALKBH5 antibody by dot-blot analyses of ALKBH5-depleted MCF-7 cells reconstituted with ALKBH5 (WT or R283K). (E) Validation of the anti-meR283-ALKBH5 antibody by immunoblotting analyses of ALKBH5-depleted MCF-7 cells. (F) HEK293T cells were co-transfected with HA-tagged ALKBH5 (WT, R283K or R283F) and Flag-tagged PRMT6 plasmids, Immunoprecipitation assays were performed with anti-HA antibody followed by immunoblotting analyse.



Supplementary Fig. 2 (A, B) Breast cancer cells were overexpressed Flag-tagged PRMT6 or stably knocked down PRMT6. Immunoblotting analyses were performed with the indicated antibodies. (C) MCF-7 cells with overexpression of Flag-tagged PRMT6 or stable knockdown of PRMT6 were treated with CHX for indicated time. Immunoblotting analyse was performed. (D) MCF-7 cells were overexpressed Flag-tagged PRMT6 or stably knocked down PRMT6. After 48hr transfection, cells were treated with MG132 for 8hr followed by IP and immunoblotting analyse.

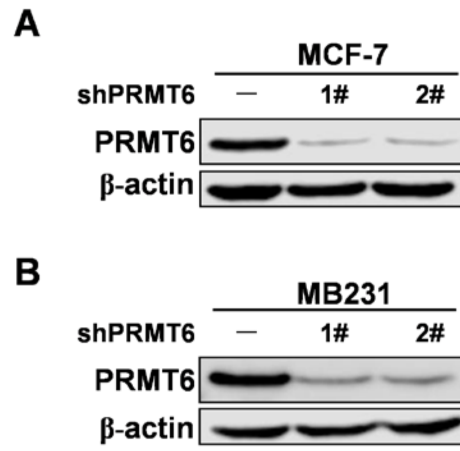


Supplementary Fig. 3 (A) ALKBH5-depleted breast cancer cells were reconstituted with the indicated protein expression. Immunoblotting analyses were performed with the indicated antibodies. (B-D) MCF-7 cells were stably knocked down ALKBH5. The levels of 27 metabolites related to energy metabolism were shown. (All data represent mean ± SEM n = 3), *p<0.05.



	WT	MU
m6A consensus	5-RRACU-3	
P1	AGATGAACTTGCTC	AGATGAGCTTGCTC
P2	CAAAGACTATAATGTA ACTGCAAACTCCAA	CAAAGGCTATAATGTA ACTGCAAGCTCCAA
P3	AGCCCGAACTGCAAG	AGCCCGAGCTGCAAG
P4	CTGAAGACTCTGCA	CTGAAGGCTCTGCA
P5	GATCAAACTCAAAG	GATCAAGCTCAAAG

Supplementary Fig. 4 (A) HEK293T cells were co-transfected with Flag-tagged LDHA and HA-tagged ALKBH5 (WT or KD) proteins. After 48hr transfection, cells were treated with MG132 for 8hr followed by IP and immunoblotting analyse. (B) HEK293T cells were co-transfected with Flag-tagged LDHA and HA-tagged ALKBH5 (WT, R283K or R283F) proteins. After 48hr transfection, cells were treated with MG132 for 8hr followed by IP and immunoblotting analyse. (C) MCF-7 cells with overexpression of Flag-tagged ALKBH5 or stable knockdown of ALKBH5 were treated with CHX for indicated time. Immunoblotting analyse was performed. (D) ALKBH5-depleted MCF-7 cells were reconstituted expression of rALKBH5. The m^{6}A level in total RNA was determined. (E) ALKBH5-depleted MCF-7 cells were reconstituted expression of rALKBH5 (WT, R283K or R283F). The m^{6}A level in total RNA was determined. (F) ALKBH5-depleted breast cancer cells were reconstituted expression of rALKBH5. The m^{6}A immunoprecipitation and RT-PCR were performed to determine the percentage of LDHA mRNA with methylation. (G) The m^{6}A sequence motif identified from sequencing profile. (H) MCF-7 cells were overexpressed Flag-tagged METTL3/METTL14 or stably knocked down METTL3/METTL14. Immunoblotting analyses were performed with the indicated antibodies. (All data represent mean ± SEM n = 3), *p<0.05.



Supplementary Fig. 5 Breast cells were stably knocked down PRMT6. Immunoblotting analyses were performed with the indicated antibodies.

MATERIALS AND METHODS

Immunofluorescent analyse

The indicated cells were fixed with 4% formaldehyde for 10min, and permeabilized with PBS containing 0.1% Triton X-100. Then, it was blocked with 1% normal bovine serum in PBS for 1hr at room temperature. Immunostaining was performed following the appropriate primary and secondary antibodies. At last, the cells were counterstained with DAPI-containing mounting solution (Abcam). Images were pictured with a ZEISS LSM 900 Scope.

Lentiviral production and infection

Lentiviral constructs expressing shRNA plasmids, or other overexpressing lentiviral plasmids were transfected into HEK293T using Lipofectamine 3000 according to the manufacturer's instructions. For stable knockdown experiments, the lentivirus was collected at 48hr after transfection and infected target cells with polybrene. After 48hr of transduction, the cells were subjected to 1μg/mL puromycin selection for one week. For stable overexpression experiments, the cells were selected using 1mg/mL G418 for one week. For CRISPR-mediated PRMT6 knockout, the cells were subjected to 1μg/mL puromycin selection for one week.

Luciferase assay

Luciferase activity was performed by Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions. Briefly, the E box in ALKBH5 promoter was constructed into pGL3-promoter. For the m^6A modification, the indicated fragments were constructed into pMIR-Report Luciferase. After transfection for 48hr, the cells were detected with the kit.

Antibody production

Rabbit anti-ALKBH5 R283me2 antibody was commercially produced at Dia-An, Inc, in Wuhan, in China by immunizing animals with a synthetic asymmetrically dimethylated-peptide corresponding to residues surrounding R283 of human ALKBH5. This antibody was then affinity purified and characterized for their specificity using IB, IP and IHC with or without corresponding modified and unmodified peptides.

Immunohistochemistry

The experiments using human tissues were approved by Human Assurance Committee of Weifang Medical University. Breast tissue sections were from surgical resection at the affiliated hospital of Weifang Medical University. All patients signed an informed consent, and the investigators got the informed consent before the researches. This study was conducted according to the Declaration of Helsinki of the World Medical Association.

The breast xenografts or specimens tissue slides were deparaffinized, rehydrated using different concentrations of

ethanol followed by antigen retrieval with sodium citrate buffer. Endogenous peroxidase was blocked with 3% H_2O_2. Then, the slides were blocked with 5% normal bovine serum in PBS for at least1hr. Slides were incubated with the indicated primary antibody diluted with PBS overnight at 4℃. Slides were incubated secondary antibody for 1hr. At last, slides were performed with DAB reagent and counterstained with hematoxylin. Observation and statistical analysis were performed. The images were pictured using Leica microscope.

PRMT6, meR283-ALKBH5 and LDHA expressions in breast tissues were scored blindly and independently by two scientists. The scores for staining frequency (0 = 0–5%, 1 = 6–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%) and intensity (0 = negative, 1 = weak, 2 = moderate and 3 = strong staining) were added to obtain an overall staining score.

Cell proliferation assay

The indicated cells were plated in triplicates onto 96-well plates. After 24, 48 or 72hr, the cells were incubated 100μL cultured medium containing CCK-8 working solution (10μL/well), which were sequentially cultured at 37℃ for 1hr. The OD value was detected at 450nm.

Plate colony formation, wound healing and cell invasion assays

For the plate colony formation assay, the indicated cells were plated into 6-well plates with 200-600 cells per well. After the cells adhered to the well, they continued to culture for two weeks. The cells were fixed with 4% paraformaldehyde and stained by 0.1% crystal violet [1].

For the scratch assay, the indicated cells were plated into 6-well plates with 90% confluence. Then, the cells were scratched with a micropipette tip. The wound healing area was measured at 24hr using a microscope [2].

For cell invasion assay, transwell chamber filters (8μm) were coated with 100μL Matrigel (1:8 dilutions in DMEM without FBS). The indicated cells were suspended in 200mL serum-free DMEM medium, and were placed in the upper chambers of the transwell. The chambers were then put back to wells containing 500μL DMEM medium containing 10% FBS. After culturing for 24hr, the cells in the upper chambers were cleaned by wiping the top of the membrane with cotton swabs. The invasion cells were then stained and counted.

Glucose consumption and lactate production

Extracellular levels of glucose and lactate were tested using lactate assay kit (BioVision) and glucose assay kit (Sigma) according to the manufacturer's instructions. Briefly, the indicated cells were plated onto 6-well plates. After 24hr, cells were washed and cultured in serum-free DMEM for 16-24hr.

Liquid chromatography-massspectrometry/mass spectrometry analysis

For identification of interacting proteins, ALKBH5 was immunoprecipitated from the indicated cells and the SDS-PAGE was performed. The SDS-PAGE gel was stained with Coomassie brilliant blue using Protein Stains H. The

In-gel ALKBH5 proteins were digested overnight in 12.5ng/μL trypsin in 25mmol/L NH_4HCO_3. The peptides were extracted three times with 60% acetonitrile (ACN)/0.1% trifluoroacetic acid and dried completely in a vacuum centrifuge. LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Fisher Scientific) that was coupled to an Easy nLC instrument (ThermoFisher Scientific) for 1hr.

For detection of arginine methylation sites, in vitro arginine methylation of ALKBH5 by PRMT6 was performed according to the in vitro arginine methylation assay protocol described above. Then, the arginine methylation protein samples were digested using trypsin and analysed using Bruker timsTOF Pro mass spectrometer. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethyl on Cys were specified as fixed modification and asymmetric methylation on Arg was specified. Peptide confidence was set at high, and peptide ion score was set > 20.

Glycolytic rate assay

Glycolytic rate was determined with Seahorse XFe24 Analyzer (Angilent). Breast cancer cells were seeded at 2×10^4 cells per well and cultured in indicated medium. After 24hr, the tests were performed using the Glycolytic Rate Assay Kit (Angilent) according to manufacturer's instructions.

Generation of PRMT6 knockout mice and mouse embryonic fibroblasts (MEFs) cells

PRMT6 knockout (PRMT6−/−) mice were generated using CRISPR/Cas9-mediated genome editing in C57BL/6J embryonic stem cell (gRNA1: CGGTGTGCCAAGATTGACTCTGG; gRNA2: CACTCCGTGAATTAAGCGTAAGG). The mice were bred to get homozygous PRMT6−/− mice. MEFs cells (PRMT6−/−) were obtained from embryonic day 13.5 embryos of these PRMT6−/− mice. All mice experimental procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethical Committee for Animal Experimentation of Weifang medical University.

Table S1. Primary antibodies and reagents used in this study.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-PRMT6	Santa Cruz	Cat#sc-271744
Rabbit anti-PRMT6	Abcam	Cat#ab271091
Mouse anti-ALKBH5	Proteintech	Cat#67811-1-Ig
Rabbit anti-ALKBH5	Abcam	Cat#ab195377
Rabbit anti-ALKBH5 R283me2	Dia-An, Inc, in Wuhan, in China	N/A
ADME Rabbit mAb	Cell Signaling Technology	Cat #13522S
Mouse anti-LDHA	Santa Cruz	Cat#sc-137243
Rabbit anti-LDHA	Abcam	Cat#ab52488

Mouse anti-HA	Sigma-Aldrich	Cat#H3663
Rabbit anti-N6-methyladenosine (m^{6}A)	Abcam	Cat#ab208577
Mouse anti-Flag	Sigma-Aldrich	Cat#F1804
Mouse anti-GFP	Sigma-Aldrich	Cat#G6539
Mouse anti-GST	Sigma-Aldrich	Cat#SAB5300159
Mouse anti-His	Sigma-Aldrich	Cat#SAB2702220
Mouse anti-β-actin	Abcam	Cat#ab6276
Mouse anti-β-tubulin	Sigma-Aldrich	Cat#T8328
Rabbit anti-HA	Proteintech	Cat#51064-2-AP
Rabbit anti-Flag	Proteintech	Cat#20543-1-AP
Rabbit anti-GFP	Proteintech	Cat#50430-2-AP
Rabbit anti-GST	Proteintech	Cat#10000-0-AP
Rabbit anti-His	Proteintech	Cat#10001-0-AP
Rabbit anti-β-actin	Abcam	Cat#ab8227
Rabbit anti-Ubiquitin	Abcam	Cat#ab134953
Rabbit anti-Ki67	Abcam	Cat#ab92742
Mouse anti-METTL3	Proteintech	Cat#67733-1-Ig
Rabbit anti-METTL14	Proteintech	Cat#26158-1-AP
IRDye 800CW goat anti-rabbit	LI-COR	Cat#925-32210
IRDye 800CW goat anti-mouse	LI-COR	Cat#925-32211
IRDye 680LT goat anti-mouse	LI-COR	Cat#925-68020
IRDye 680LT goat anti-rabbit	LI-COR	Cat#925-68021
Anti-HA Affinity Gel	Sigma-Aldrich	Cat#E6779
ANTI-FLAG Affinity Gel	Sigma-Aldrich	Cat#F2426
Ni-NTA Agarose	Thermo Fisher	Cat#R90101
MagnaBind™ Protein A Beads	Thermo Fisher	Cat#21348
Pierce™ Glutathione A garose	Thermo Fisher	Cat#16100
Dual-Luciferase® Reporter Assay	Promega	Cat#E1960
Cell Counting Kit-8	MedChemExpress	Cat#HY-K0301
m^{6}A RNA Methylation Quantification Kit	Epigentek	Cat#P-9005
Lactate Colorimetric Assay Kit	Biovision	Cat#K627
Glucose Assay Kit	Sigma-Aldrich	Cat#GAGO20
Glycolytic Rate Assay Kit	Agilent Technologies	Cat#103344-100
Bacterial Strain		
E. coli DH5α	Thermo Fisher	Cat#18258012
E. coli Stable 3	Thermo Fisher	Cat#C737303
E. coli BL21	Thermo Fisher	Cat#C600003
Mouse: BALB/c nude	Nanjing model animal center	N/A
Mouse: C57BL/6-PRMT6$^{-/-}$	Cyagen Biosciences, Inc.	N/A
Experimental Models: Cell Lines		
Human: 293T cells	Cell Bank of the Chinese Academy of Sciences	Cat#GNHu17

Human: MCF-7 cells	Cell Bank of the Chinese Academy of Sciences	Cat#TCHu 74
Human: MDA-MB231 cells	Cell Bank of the Chinese Academy of Sciences	Cat#TCHu227
Recombinant DNA		
pCDNA3.0/neo-HA-PRMT6	This paper	N/A
pCDNA3.0/neo-Flag-PRMT6	This paper	N/A
pCDNA3.1/neo-Flag-PRMT6 (mutants)	This paper	N/A
pCDNA3.0/neo-HA-ALKBH5	This paper	N/A
pCDNA3.1/neo-Flag-ALKBH5	This paper	N/A
pCDNA3.1/neo-Flag-ALKBH5 (mutants)	This paper	N/A
pCDNA3.1/neo-HA-ALKBH5 (mutants)	This paper	N/A
pEGFP-C1-PRMT6 (P1, P2 or P3)	This paper	N/A
pEGFP-C1-ALKBH5 (A1, A2 or A3)	This paper	N/A
pCDNA3.1/neo-Flag-PRMT1	This paper	N/A
pCDNA3.1/neo-Flag-PRMT2	This paper	N/A
pCDNA3.1/neo-Flag-PRMT3	This paper	N/A
pCDNA3.1/neo-Flag-PRMT4	This paper	N/A
pCDNA3.1/neo-Flag-PRMT8	This paper	N/A
pCDNA3.1/neo-Flag-METTL3	This paper	N/A
pCDNA3.1/neo-Flag-METTL14	This paper	N/A
pGEX-4T-1-ALKBH5	This paper	N/A
PET28a-His-PRMT6 (WT and mutants)	This paper	N/A
PET28a-His-ALKBH5	This paper	N/A
pMIR-Report Luciferase-LDHA (WT or MU)	This paper	N/A
pLVX-shRNA1-PRMT6	This paper	N/A
pGE-2(pU6gRNACas9puro)-sgRNA PRMT6	This paper	N/A
pLVX-shRNA1-ALKBH5	This paper	N/A
pLVX-shRNA1-METTL3	This paper	N/A
pLVX-IRES-neo-rALKBH5 (WT or MU)	This paper	N/A

Table S2. shRNA and sgRNA sequences used in our research.

shRNA	Sense (5'-3')	Anti-sense (5'-3')
shRNA-Control	TTCTCCGAACGGTCACGT	ACGTGACCGTTCGGAGAA
shRNA-PRMT6-1#	CACGGACGTTTCAGGAGAGAT	ATCTCTCCTGAAACGTCCGTG
shRNA-PRMT6-2#	CACCGGCATTCTGAGCATCTT	AAGATGCTCAGAATGCCGGTG
sgRNA-PRMT6	CTCGGACGTTTCGGTCCACG	CGTGGACCGAAACGTCCGAG
shRNA-ALKBH5-1#	GAAAGGCTGTTGGCATCAATA	TATTGATGCCAACAGCCTTTC
shRNA-ALKBH5-2#	CCTCAGGAAGACAAGATTAGA	TCTAATCTTGTCTTCCTGAGG
shRNA-METTL3	GCCAAGGAACAATCCATTGTT	AACAATGGATTGTTCCTTGGC
shRNA-METTL14	CCATGTACTTACAAGCCGATA	TATCGGCTTGTAAGTACATGG

Table S3. The primers used for real-time PCR in our research.

Real-time PCR		
Gene	**Sense (5'-3')**	**Anti-sense (5'-3')**
PKM1	GGGGTTCGGAGGTTTGATGAA	AGGTCTGTGGAGTGACTTGAG
PKM2	GGGGTTCGGAGGTTTGATGAA	TTGCAAGTGGTAGATGGCAGC
HK1	CGAGAGTGACCGATTAGCACT	AGACAGGAGGAAGGACACGTT
HK2	ACGGAGCTCAACCATGACCAA	AAGATCCAGAGCCAGGAACTC
PGK1	GGGTCGTTATGAGAGTCGACT	AGGTGGCTCATAAGGACTACC
PGK2	AAGTCAGCCATGTCAGCACTG	GCCTGCTGCTTGTCCATTACA
PFKFB1	CTCCATCTACCTTTGCCGACA	GCCCTGGGACTGAATGAAGTT
PFKFB2	CACCAATACAACCCGGGAGA	GCAGCAATGACATCAGGATCA
PFKFB3	CTCGCATCAACAGCTTTGAGG	TCAGTGTTTCCTGGAGGAGTC
PFKFB4	CCAACTGCCCAACTCTCATTG	GCGATACTGGCCAACATTGAA
ENO1	GCCCTGGTTAGCAAGAAACTG	TTCTCAACGGCACCAGCTTTG
ENO2	AACAGTGAAGCCTTGGAGCTG	TCCTCAATGGAGACCACAGGA
PGM1	TCCAGAGTATCATCTCCACCG	ATGATGCAGGATACAGCAGGG
PGM2	AGCAGAAGGTTTGCCCGACTT	TTCACGCTCCTGTGGAAACAG
PGM3	CTCCTGGTGGAGATTGGAGAA	CTAAACAGTGCAGTGCCATGC
PDK1	GCTAGGCGTCTGTGTGATTTG	AACACCTCTGTTGGCATGGTG
PDK2	CAACCAGCACACCCTCATCTT	GCCCTCATGGCATTCTTGAAG
PDK3	TCGCCGCTCTCCATCAAACAA	CTGAACCAATCCCACTGAAGG
LDHA	GATTCCAGTGTGCCTGTATGG	CTACAGAGAGTCCAATAGCCC
LDHB	GCGTGTGCTATCAGCATTCTG	TTCTCTGCACCAGATTGAGCC
GLUT1	GCAGTTTGGCTACAACACTGG	TTTCGAGAAGCCCATGAGCAC
GLUT3	TGGCTACAACACTGGGGTCAT	TTGAATTGCGCCTGCCAAAGC
ALDOA	AGATGAGTCCACTGGGAGCAT	CACGCCCTTGTCTACCTTGAT
ALDOB	AGACCCTCTACCAGAAGGAC	CTTGTACTGAGCACAGCGCT
ALDOC	GGATGAGTCTGTAGGCAGCAT	GAGTGGTGGTTTCTCCATCAG
TIGAR	GGACAAAGCAGACCATGCATG	ACCCCGTATTTCCTTTCCCGA
GPI	ATCAACTACACCGAGGGTCGA	CCAATGTTGATGACGTCCGTG
TPI	GTCAGATGAGCTGATTGGGCA	GGTGTTGCAGTCTTGCCAGTA
18S	GTTGAACCCCATTCGTGATG	GCCTCACTAAACCATCCAA

Reference:

1. Yang, T., Ren, C., Qiao, P., Han, X., Wang, L., Lv, S., Sun, Y., Liu, Z., Du, Y. & Yu, Z. (2018) PIM2-mediated phosphorylation of hexokinase 2 is critical for tumor growth and paclitaxel resistance in breast cancer, *Oncogene.* **37**, 5997-6009.
2. Han, X., Ren, C., Yang, T., Qiao, P., Wang, L., Jiang, A., Meng, Y., Liu, Z., Du, Y. & Yu, Z. (2019) Negative regulation of AMPKalpha1 by PIM2 promotes aerobic glycolysis and tumorigenesis in endometrial cancer, *Oncogene.* **38**, 6537-6549.