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Supplemental Information

Mitogenic and Oncogenic Stimulation of K433 Acetylation Promotes PKM2 Protein Kinase Activity and Nuclear Localization

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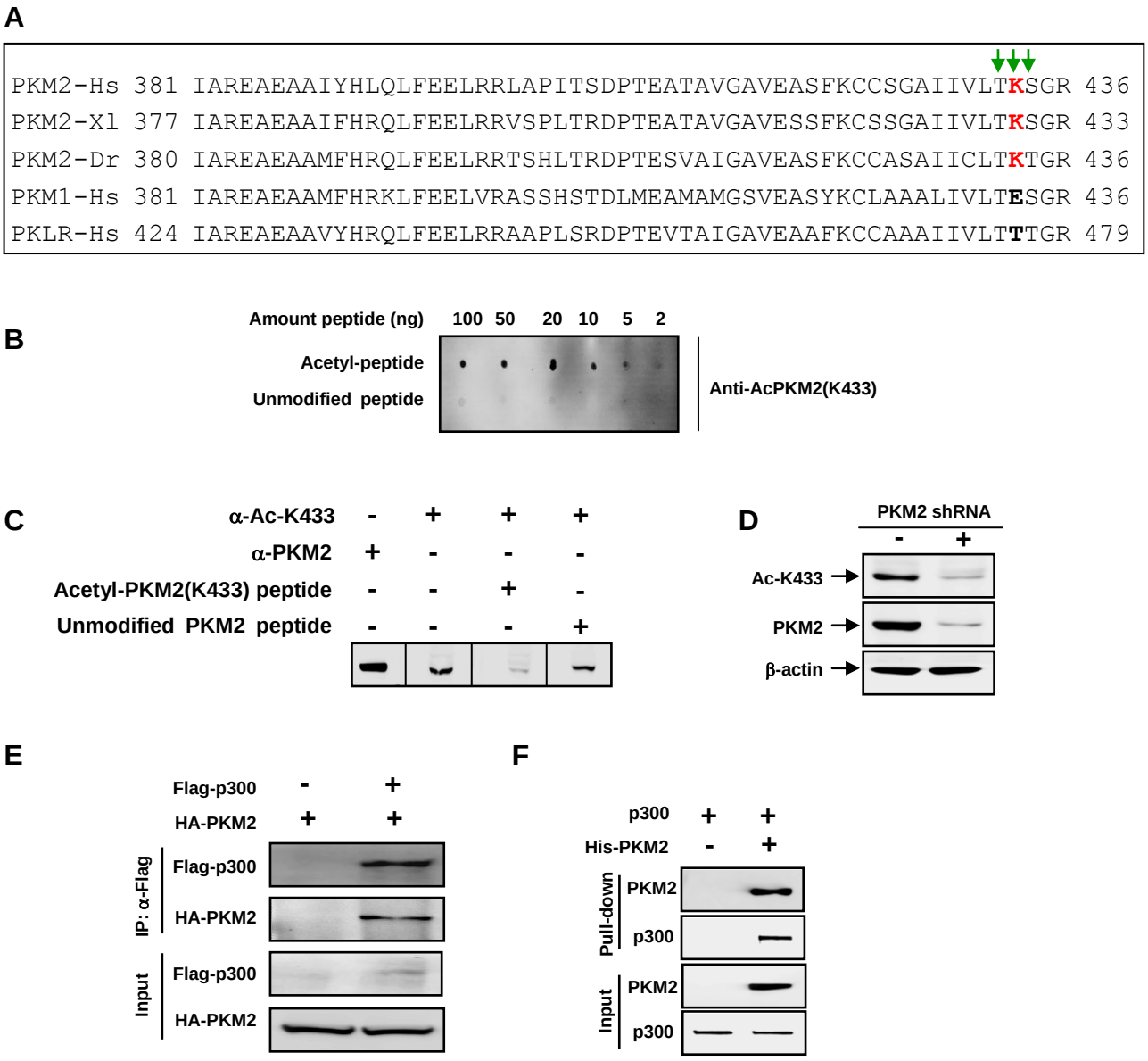

Figure S1: Linked to Figure 1

(**A**) Amino acid sequence comparison of PKM1-specific exon 9, PKM2-specific exon 10 and corresponding sequences in PKL and PKR. 56 amino acids encoded by the exon 10 of PKM2 from human (Hs; *Homo sapiens*), two representative vertebrates, frog (Xl; *Xenopus laevis*) and zebrafish (Dr; *Danio rerio*), exon 9 of human PKM1 and corresponding sequence in human PKL and PKR are aligned. The number in PKL/R refers to PKR. Of 11 residues involved in FBP binding, three are encoded by this sequence and indicated by a green arrow above. K433 in PKM2 and corresponding E433 in PKM1 and T476 in PKL/R are bolded.

(**B**) Specificity of antibody against acetyl-K433 of PKM2 was determined by dot blot assay. Nitrocellulose membrane was spotted with different amounts of acetyl-K433 peptide (CIVLTK(Ac)SGRSAHQ) or unmodified peptide (CIVLTKSGRSAHQ) and probed with the anti-AcPKM2(K433) antibody.

(**C**) Acetyl-K433, but not unmodified peptide can block the anti-AcPKM2(K433) antibody. Total cell lysates were prepared from HEK293T cells treated with deacetylase inhibitors (NAM and TSA), separated by SDS-PAGE and blotted separately with the anti-PKM2 antibody (lane 1), anti-AcPKM2(K433) antibody (lane 2), anti-AcPKM2(K433) antibody in the presence of molar excess of acetyl-PKM2(K433) peptide (lane 3) or unmodified PKM2 peptide (lane 4). The image was composited from the four separate western blots.
(**D**) Total cell extracts were prepared from untransduced cells or cells transduced with retrovirus expressing shRNA targeting PKM2, followed by western blotting using the indicated antibodies.
(**E**) p300 binds with PKM2. HEK293T cells were transfected with indicated plasmids and p300-PKM2 association was examined by IP-western analysis.
(**F**) p300 binds with PKM2 *in vitro*. The recombinant PKM2 was incubated with purified p300(sigma) *in vitro* and pull down was performed, direct interaction of the two proteins was determined by western blotting.

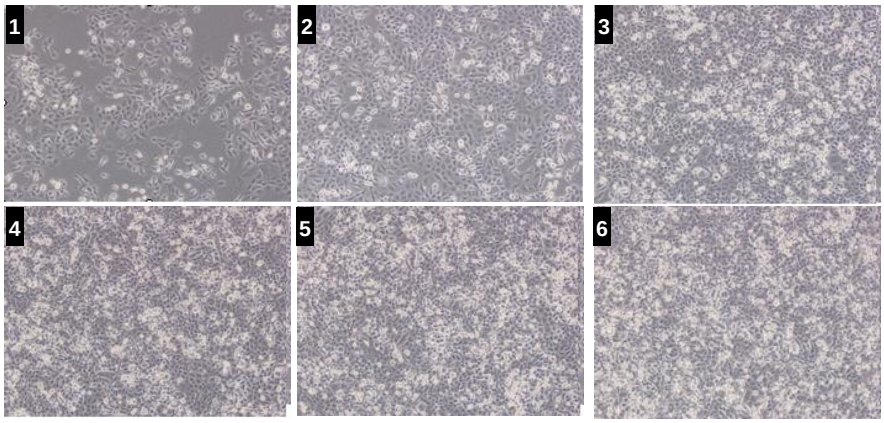

Figure S2: Linked to Figure 3

Cell-cell contact decreases acetylation of K433 of PKM2. H1299 cells were seeded at different densities as indicated in main Fig. 3E, cultured for 60 hours and photographed. The levels of both PKM2 and K433-acetylated PKM2 were determined by western blotting (main Fig. 3E).

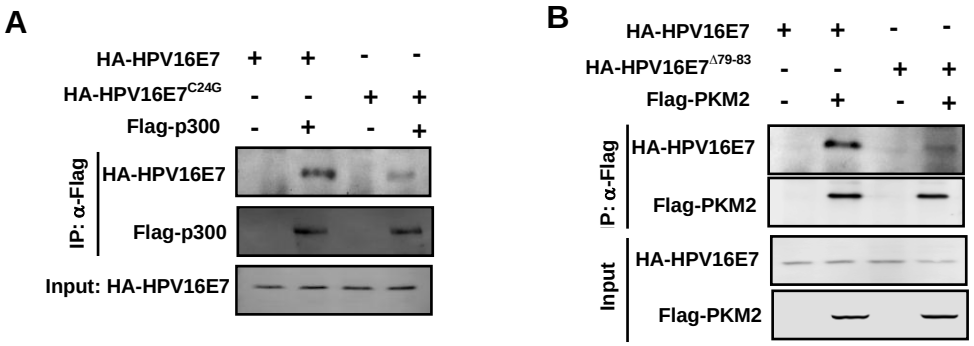

Figure S3: Linked to Figure 4

Mutations impairing the transforming activity of oncoprotein HPV16-E7 reduce its interaction with PKM2 and p300.

Transformation-deficient HPV16-E7^{C24G} and HPV16-E7$^{\Delta 79\text{-}83}$ mutants are defective in binding with p300 and PKM2, respectively. 3T3 cells were co-transfected with indicated plasmids. HPV16E7-p300 **(A)** and HPV16E7-PKM2 **(B)** associations were examined by IP-western analysis.

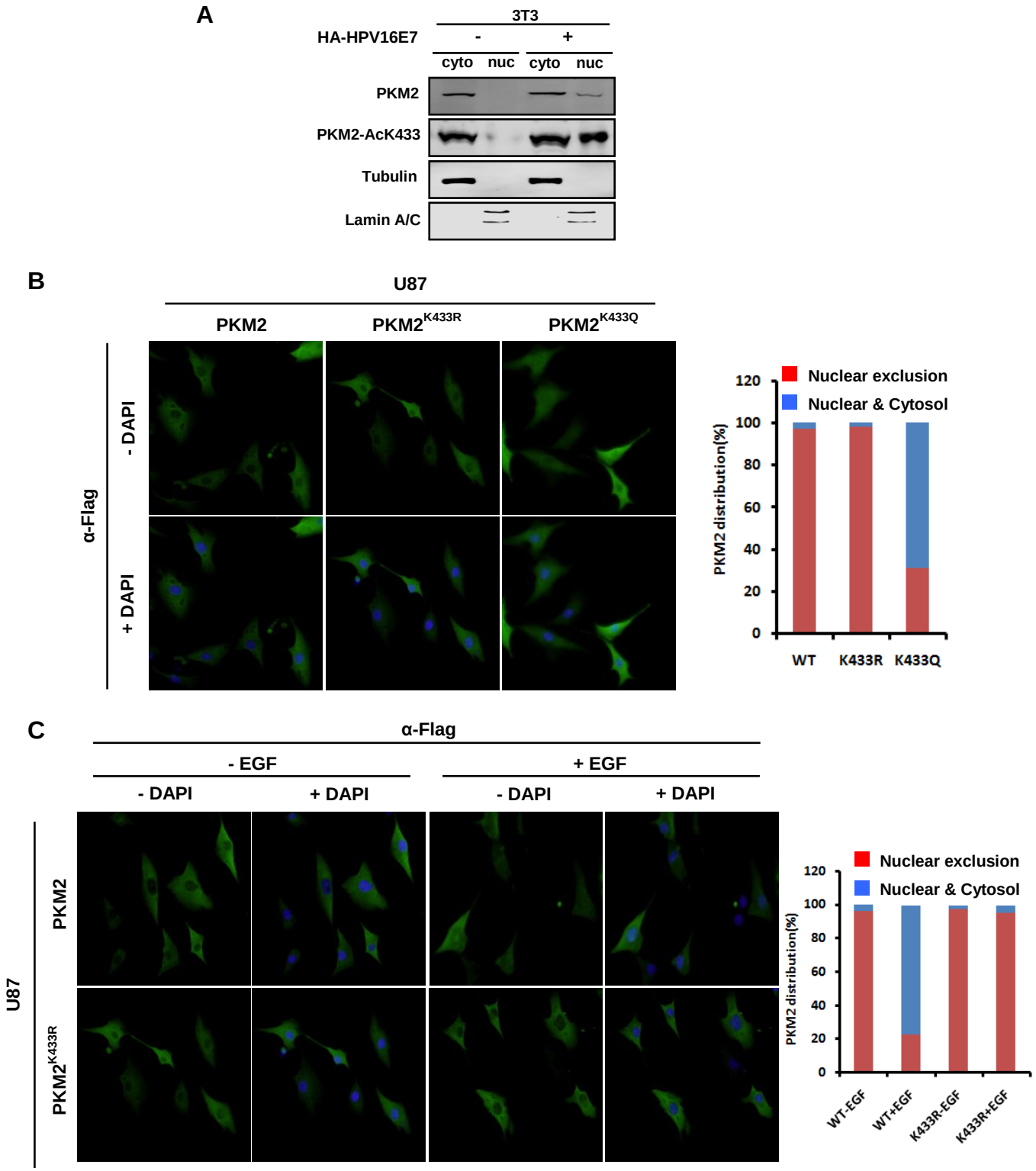

Figure S4, Linked to Figure 5.

(A) Oncoprotein E7 promotes nuclear translocation of PKM2.Cytosolic and nuclear extracts were prepared from 3T3 cells transfected with a plasmid expressing HPV16-E7, followed by western blotting analysis to determine the total and K433-acetylated PKM2.

(B) K433Q mutation promotes the nuclear localization of PKM2. Stable cell lines were established in U87 cells after knocking down endogenous PKM2 and ectopically expressing Flag-tagged, wild-type or K433R or K433Q mutant PKM2 (U87/Flag-PKM2). The knocking-down and putback cells were seeded for 24h. Subcellular localizations of Flag-PKM2 were examined by α-Flag immunofluorescence. 100 cells were microscopically examined for each line and scored for the nuclear accumulation or exclusion of Flag-PKM2.
(C) K433R mutation blocks EGF-induced PKM2 nuclear translocation. U87/Flag-PKM2 stable cell lines described above were seeded for 24h, starved for another 24h with 0.5% serum + DMEM, followed by treatment with EGF (100 ng/ml) for 6h. Subcellular localizations of Flag-PKM2 were examined by α-Flag immunofluorescence. 100 cells were microscopically examined for each line and scored for the nuclear accumulation or exclusion of Flag-PKM2.

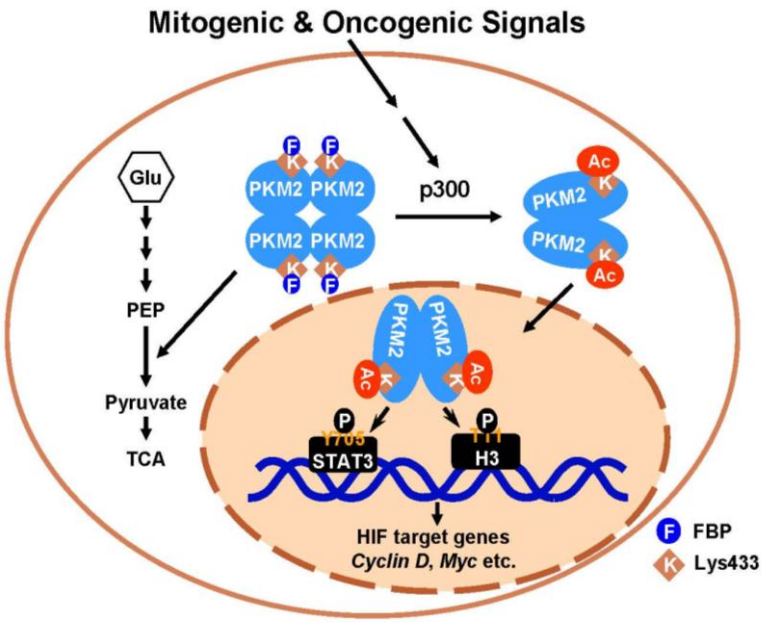

Figure S5: Linked to Figure 7

A schematic illustration on the control of nuclear translocation and protein kinase activity of PKM2 by Lys433 acetylation.

Supplemental Experimental Procedures

Cell lysis, antibodies and immunological procedures

Cells were lysed in an NP40 buffer containing 50mM Tris pH 7.5, 150mM NaCl, 0.3% Nonidet P-40, 1μg/ml aprotinin, 1μg/ml leupeptin, 1μg/ml pepstatin, 1mM Na_3VO_4 and 1mM PMSF. Western blot analysis was carried out according to standard methods. Antibodies specific to Flag (Sigma, F7425), HA (Santa Cruz, sc-7392), STAT3 (Cell Signaling Technology, CST#9132), pY705 STAT3 (CST #9145), His (CST #2366), histone H3 (CST #9715), pT11 H3 (Abcam, ab5168), p300 (Millipore #05-257), tubulin (NeoMarkers #581P), Lamin A/C (GenScript, A01455) and actin (GenScript, A00702) were purchased from commercial sources. Antibodies to pyruvate kinase (antigen peptide sequence: LRRLAPITSDPTEATAVGAV) and to acetyl-PKM2 (K433) (antigen peptide sequence: CIVLTK(Ac)SGRSAHQ) were generated by immunizing rabbits with indicated antigen peptides at Shanghai Genomic Inc.

RNA interference

p300 knockdown was carried out by using siRNA oligonucleotides synthesized from Genepharma, Shanghai. siRNA oligonucleotides were diluted to 20 μM using RNase free water. 12.5μl of siRNA were transfevted by using Lipofectamine 2000 (Invitrogen) and Opti-MEM (Invitrogen). Cells were harvested 72h after transfection. The knockdown efficiency was verified by q-PCR. The following sequence was used. P300 siRNA: 5'-AACAGAGCAGUCCUGGAUUAG. q-PCR primer: 5'-GCGGCCTAAACTCTCATCTC, 5'-TCTGGTAAGTCGTGCTCCAA.

PKM2-FBP binding assay

Recombinant His-tagged wild-type, K433Q and K433R mutant PKM2 were expressed in bacteria and purified using Ni-NTA beads (GE Healthcare). 5μg of each purified protein and control GST protein was added to a 25 μl of reaction mixture containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 5% glycerol and different amounts of mixture of cold (5 μM) and ^3H radiolabeled (0.1 μCi) FBP. The binding reactions were incubated at room temperature for 30 min. and then 4°C overnight. The next morning, samples were spotted on nitrocellulose membrane and washed with 50 mM Tris-HCl (pH 7.5). Radioactivity retained on the membrane was quantified on a scintillation counter.

Cell proliferation and contact inhibition

For serum starvation, H1299 cells were serum starved in RPMI 1640 (Gibco) without supplement of FBS for the length of time as indicated in the figures. For serum stimulation, H1299 cells were first serum starved for 2 days in RPMI 1640 (Gibco) without supplement of FBS and then serum stimulated by adding 10% FBS for the indicated length of time. For cell-cell contact inhibition, H1299 cells were seeded in 6-well plates at different densities as indicated, cultured in RPMI1640 for 60 hours, harvested and cell density was recorded by photograph and counting.

***In vitro* deacetylation and acetylation assay**

Ecoli His-CobB (10 μg/ml) was purified from *E.coli* and incubated with Flag-PKM2 (10 μg/ml) in a HEPES buffer (40 mM HEPES; 1mM $MgCl_2$, 1mM DTT, 5mM NAD^+) at 37°C for 1 h. The effect of CobB deacetylation of PKM2 on its protein kinase activity toward Y705 of STAT3 was assayed in the presence or absence of FBP and analyzed by western blotting.

For *in vitro* acetylation, Flag-PKM2 (10 μg/ml) was purified from HEK293T cells and incubated with recombinant p300 (Sigma) in HAT buffer (Millipore) in a 30°C shaking incubator for 1h. The effect of K433 acetylation is determined by western blotting.

Preparation and fractionation of human tumor extracts

Breast cancer samples were acquired from Affiliated Zhongshan Hospital of Fudan University. A physician obtained informed consent from the patients. The procedures related to human subjects were approved by Ethic Committee of the Institutes of Biomedical Sciences (IBS), Fudan University.

Fresh breast cancer samples and matched surrounding normal tissues from the same patients were homogenated, cytosolic and nuclear fractions were separated followed the protocol provided by the manufacturer (NE-PER Nuclear and Cytoplasmic Extraction Kit, Pierce), and total and K433-acetylated PKM2 were determined by western analysis.

Cell Proliferation and xenograft studies

A shRNA retrovirus targeting pyruvate kinase was constructed, the sequences of PKM2 shRNA and control shRNA were previously described (Christofk et al., 2008a). The sequence of PKM2 shRNA (kd):

5'-CCGGGCTGTGGCTCTAGACACTAAACTCGAGTTTAGTGTCTAGAGCCACAGCTTTTTG-3',

and sequence of control shRNA (cl):

5'-CCGGGAGGCTTCTTATAAGTGTTTACTCGAGTAAACACTTATAAGAAGCCTCTTTTTG-3'.

Retrovirus was made using a two-plasmid packaging system. Briefly, shRNAs in the pMKO-puro vector were co-transfected into HEK293T cells along with vectors expressing the *gag* and *vsvg* genes. Retrovirus was harvested 48 h after transfection and used to transducer H1299 and U87 cells in the presence of polybrene (8μg/ ml). Transduced cells were selected in 2 μg /ml puromycin for 1 week. Flag-tagged human PKM2, PKM2 K433Q and PKM2 K433R containing two silent nucleotide substitutions in the sequence corresponding to the shRNA-targeted region were cloned into the retroviral vector (pQCXIH) and were cotransfected into HEK293T cells together with vectors expressing the *gag* and *vsvg* genes. Retroviral supernatants were harvested 36 hr after initial plasmid transfection, mixed with polybrene (8 μg/ml) and transduce both H1299 and U87 cells were infected with retrovirus and selected in hygromycin (350 mg/ml) for 2 weeks.

For cell proliferation assay, $5x10^4$ H1299 cells were seeded in triplicate in 6-well plates, and cell numbers were counted every 24 hr over a 5 day period. For xenograft assay, nude mice (nu/nu, male 6 to 8-week-old) were injected subcutaneously with

$4x10^6$ U87 cells. Around 9 weeks after injection, the tumors were dissected, and the volume and weight of tumors were measured. All animal related procedures were performed under Division of Laboratory Animal Medicine regulations of Fudan University.