SUPPLEMENTARY INFORMATION

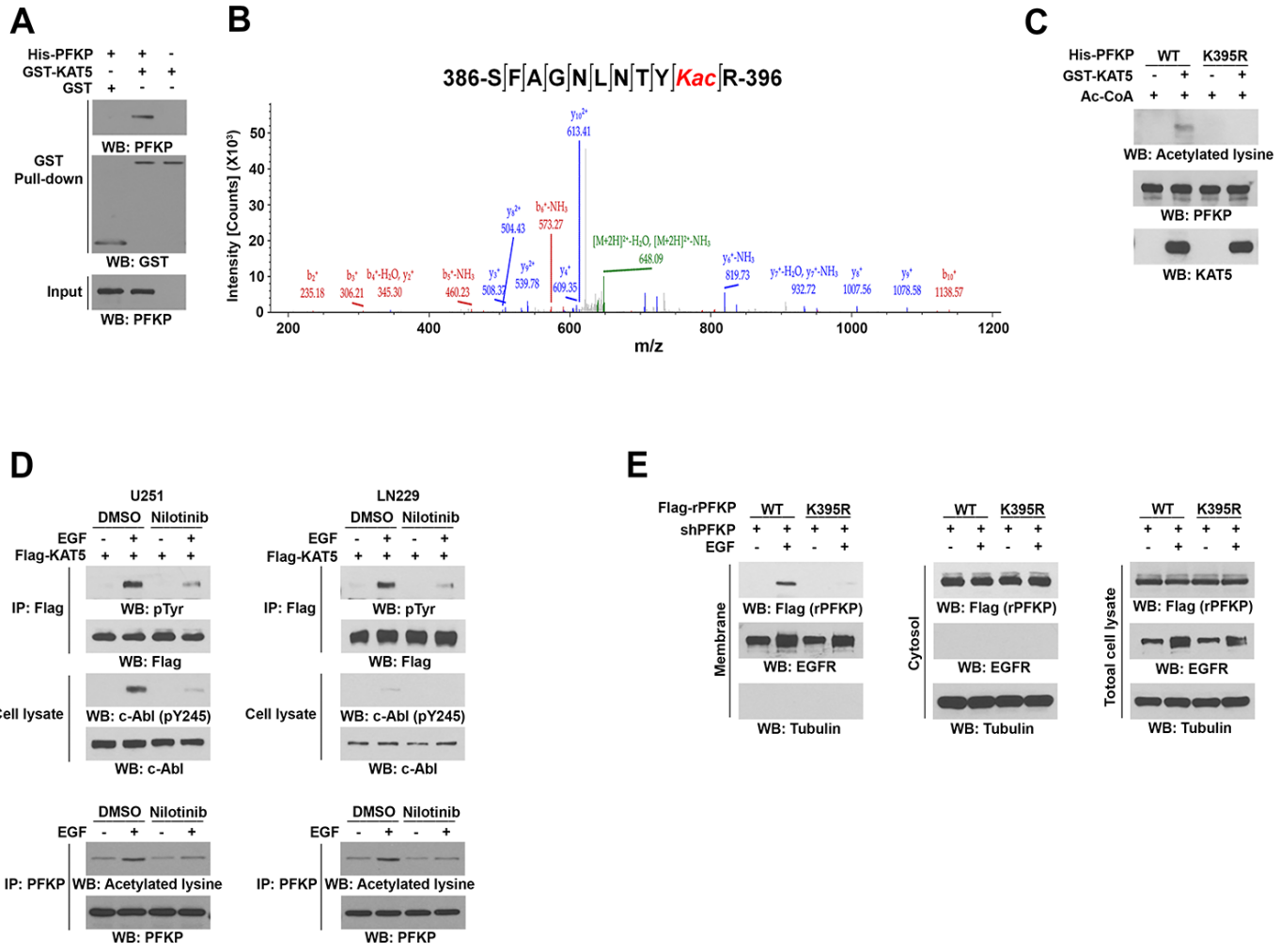


Figure S1 related to Figure 1. KAT5-Mediated PFKP K395 Acetylation Promotes Binding of PFKP to EGFR.

(**A**) A GST pull-down assay was performed by mixing bacterially purified His-PFKP with GST or GST-KAT5. Immunoblotting analyses were performed with the indicated antibodies.

(**B**) An *in vitro* acetylation assay was performed by mixing purified KAT5 and purified PFKP in the presence of acetyl-CoA. Mass spectrometric analysis of a tryptic fragment of PFKP at mass-

to-charge ratio (m/z) 656.83704 (mass error, 1.85 ppm) matched the 2+ charged peptide 386-SFAGNLNTYKR-396 suggesting that K395 was acetylated. The Mascot score was 27, and the expectation value was 0.021.

(**C**) An *in vitro* acetylation assay was performed by mixing purified KAT5 and purified WT PFKP or PFKP K395R mutant in the presence of acetyl-CoA. Immunoblotting analyses were performed with the indicated antibodies.

(**D**) U251 and LN229 cells expressing Flag-KAT5 were pretreated with DMSO or Nilotinib (5 μM) for 60 min before stimulation with or without EGF (100 ng/ml) for 15 min. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies.

(**E**) Serum starved PFKP-depleted U87/EGFR cells with reconstituted expression of WT Flag-rPFKP or Flag-rPFKP K395R mutant were treated with or without EGF (100 ng/ml) for 5 min. Cell fractionation assay was carried out. Immunoblotting analyses were performed with the indicated antibodies.

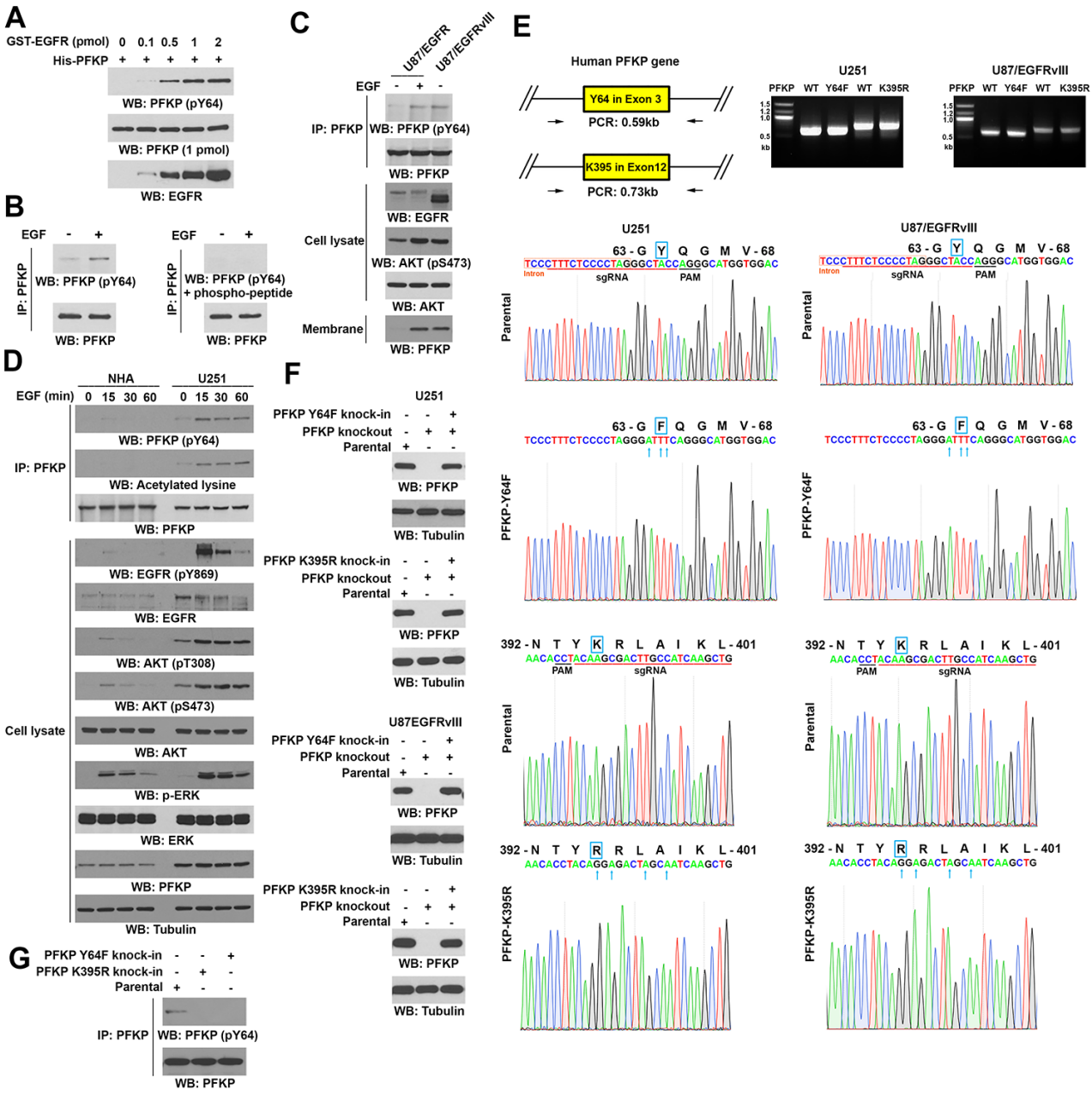


Figure S2 related to Figure 2. EGFR Phosphorylates PFKP at Y64.

(**A**) The stoichiometry of PFKP phosphorylation by EGFR was examined using *in vitro* phosphorylation analyses by mixing the purified His-PFKP with the indicated amounts of bacterial purified GST-EGFR in the presence of ATP. Immunoblotting analyses were performed with the indicated antibodies.

(**B**) U251 cells were treated with or without EGF (100 ng/ml) for 15 min. Immunoprecipitation with an anti-PFKP antibody was followed by immunoblotting analyses with an anti-phospho-PFKP Y64 antibody in the presence or absence of specific competing phosphopeptides.

(**C**) U87 cells stably transfected with the plasmids expressing EGFR or EGFRvIII were stimulated with or without EGF (100 ng/ml) for 15 min. Cell fractionation and immunoprecipitation analyses were carried out. Immunoblotting analyses were performed with the indicated antibodies.

(**D**) Serum-starved normal human astrocytes (NHA) and U251 cells were treated with or without EGF (100 ng/ml) for the indicated time periods. Endogenous PFKP was immunoprecipitated. Immunoblotting analyses were performed with the indicated antibodies.

(**E, F**) CRISPR/cas9-mediated knock-in of PFKP Y64F and K395R mutants in U251 and U87/EGFRvIII cells. Genomic DNA was extracted from the indicated cells. PCR products for the indicated DNA fragments were separated on an agarose gel and sequenced. The red lines indicate the sgRNA-targeting sequences. The black lines indicate the protospacer adjacent motifs (PAMs). The mutated nucleotides are highlighted by cyan arrows. The mutated amino acids and their WT counterparts are highlighted by the solid blue box. Silent mutations of the indicated nucleotides were introduced into the sgRNA-targeting sequences to avoid repeat cutting by hSpCas9 (**E**). Immunoblotting analyses were performed with the indicated antibodies. MG132 (10 μM) was added to the cells 6 h before harvesting to eliminate the potential effect of proteasomal degradation on PFKP proteins (**F**).

(**G**) Endogenous PFKP in parental U87/EGFRvIII cells and the indicated U87/EGFRvIII cells with knockin expression of PFKP Y64F or K395R was immunoprecipitated. MG132 (10 μM) was added to the cells 6 h before harvesting to eliminate the potential effect of proteasomal degradation on PFKP proteins. Immunoblotting analyses were performed with the indicated antibodies.

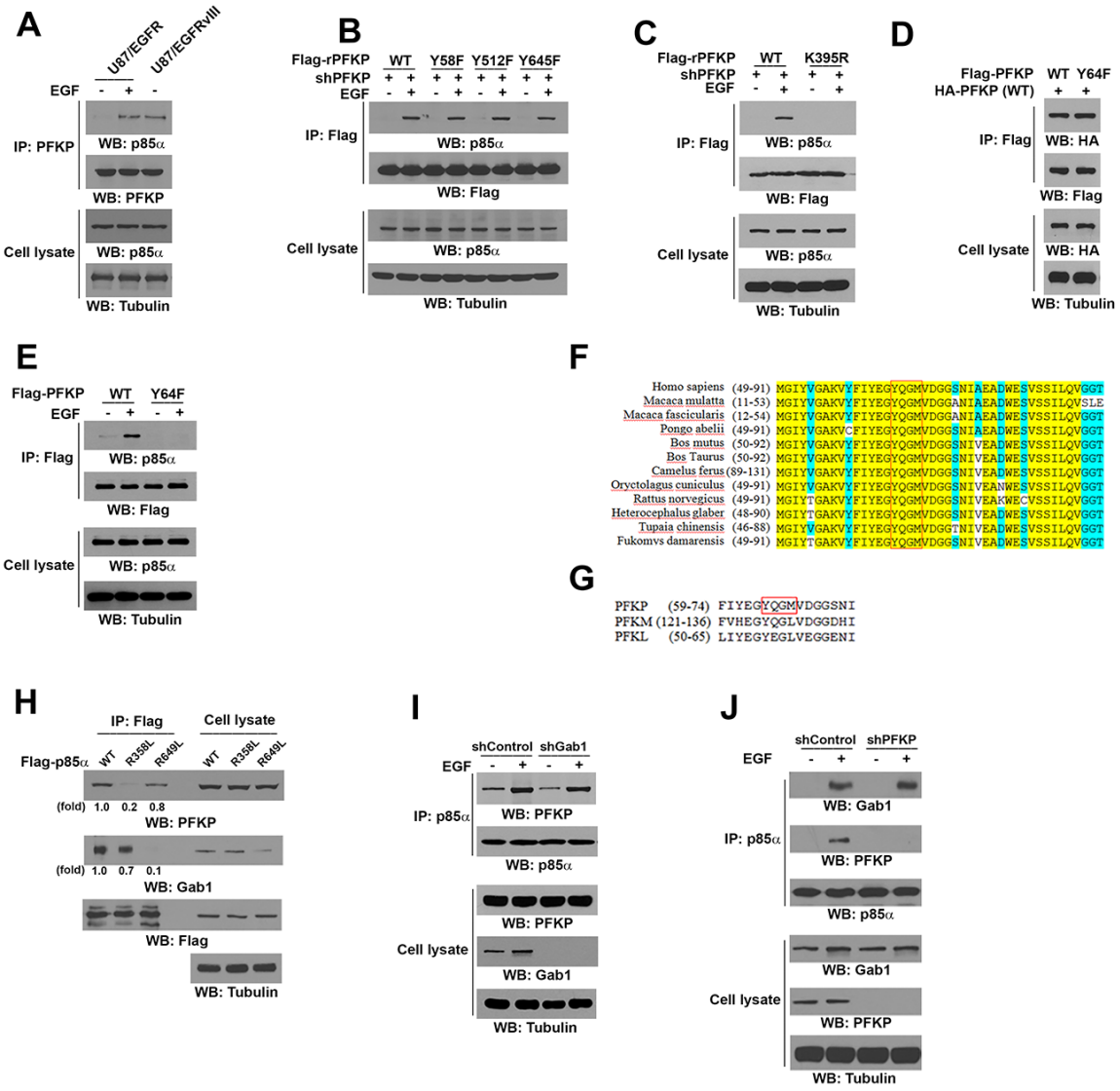


Figure S3 related to Figure 3. PFKP Binds to N-Terminal SH2 Domain and Gab1 Binds to C-Terminal SH2 Domain of p85α.

(**A-E, H-J**) Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies.

(**A**) U87 cells stably transfected with the plasmids expressing EGFR or EGFRvIII were stimulated with or without EGF (100 ng/ml) for 15 min.

(B) Serum starved PFKP-depleted U251 cells with reconstituted expression of the indicated

plasmids were treated with or without EGF (100 ng/ml) for 15 min.

(**C**) Serum starved PFKP-depleted U251 cells with reconstituted expression of WT Flag-rPFKP or Flag-rPFKP K395R mutant were treated with or without EGF (100 ng/ml) for 15 min.

(**D**) WT HA-PFKP, WT Flag-PFKP, and Flag-PFKP Y64F were expressed in U251 cells.

(**E**) Serum starved U251 cells expressing WT Flag-PFKP or Flag-PFKP Y64F mutant were treated with or without EGF (100 ng/ml) for 15 min.

(**F**) Sequence alignment of N-terminal sequence of PFKP from the indicated species shows amino acid conservation. Invariant residues are highlighted in yellow, and variant residues are shown in blue. The YQGM sequence shown in the box is conserved.

(**G**) Sequence alignment of the indicated PFK1 isoforms shows that the Y-X-X-M motif (in the red box) appears only in PFKP.

(**H**) U87/EGFRvIII cells expressed the indicated Flag-tagged p85α WT, R358L mutant, or R649L mutant proteins.

(**I**, **J**) Serum-starved U251 cells with or without expressing Gab1 shRNA (**I**) or PFKP shRNA (**J**) were treated with or without EGF (100 ng/ml) for 15 min.

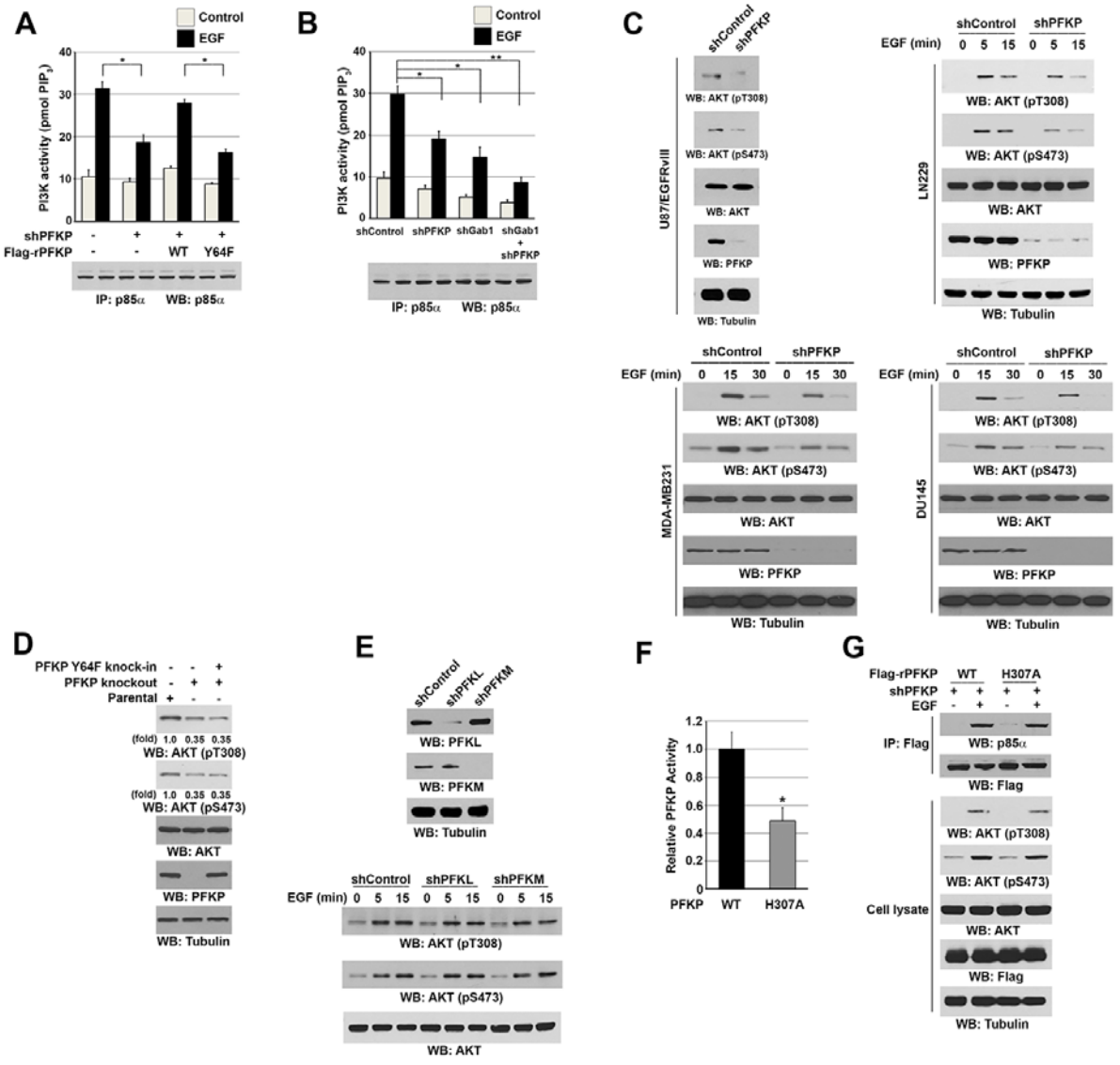


Figure S4 related to Figure 4. Phosphorylation of PFKP at Y64 Enhances EGF-Induced Activation of PI3K and AKT.

(**A**) Serum-starved U251 cells with or without expressing PFKP shRNA and with or without reconstituted expression of WT Flag-rPFKP or Flag-rPFKP Y64F mutant were treated with or without EGF (100 ng/ml) for 15 min. The PI3K activity of the p85α immunoprecipitates was determined. Data represent the means ± SD of three independent experiments. *$p < 0.01$, based on the Student's *t* test.

(**B**) Serum-starved U251 cells with or without expressing PFKP shRNA, Gab1 shRNA, or combined PFKP shRNA and Gab1 shRNA were treated with or without EGF (100 ng/mL) for 15 min. The PI3K activities of the p85α immunoprecipitates were determined. Data represent the means ± SD of three independent experiments. *$p < 0.01$; **$p < 0.001$, based on the Student's *t* test.

(**C**) A control shRNA or PFKP shRNA was expressed in U87/EGFRvIII cells. Serum-starved LN229, MDA-MB231, and DU145 cells with or without expressing PFKP shRNA were treated with EGF (100 ng/ml) for the indicated periods of time. Immunoblotting analyses were performed with the indicated antibodies.

(**D**) U87/EGFRvIII cells with or without knockout of PFKP or knock-in of PFKP Y64F mutant were subjected to immunoblotting analyses with the indicated antibodies. MG132 (10 μM) was added to the cells 6 h before harvesting to eliminate the potential effect of proteasomal degradation on PFKP proteins.

(**E**) Serum-starved U251 cells with or without expressing PFKL shRNA or PFKM shRNA (top panel) were treated with or without EGF (100 ng/ml) for the indicated time periods (bottom panel). Immunoblotting analyses were performed with the indicated antibodies.

(**F**) The PFKP enzymatic activity of bacterially purified WT PFKP (0.1 μg) and PFKP H307A (0.1 μg) was measured. Data represent the means ± SD of three independent experiments. *$p < 0.001$, based on the Student's *t* test.

(**G**) Serum starved PFKP-depleted U251 cells with reconstituted expression of WT Flag-rPFKP or Flag-rPFKP H307A mutant were treated with or without EGF (100 ng/ml) for 15 min. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies.

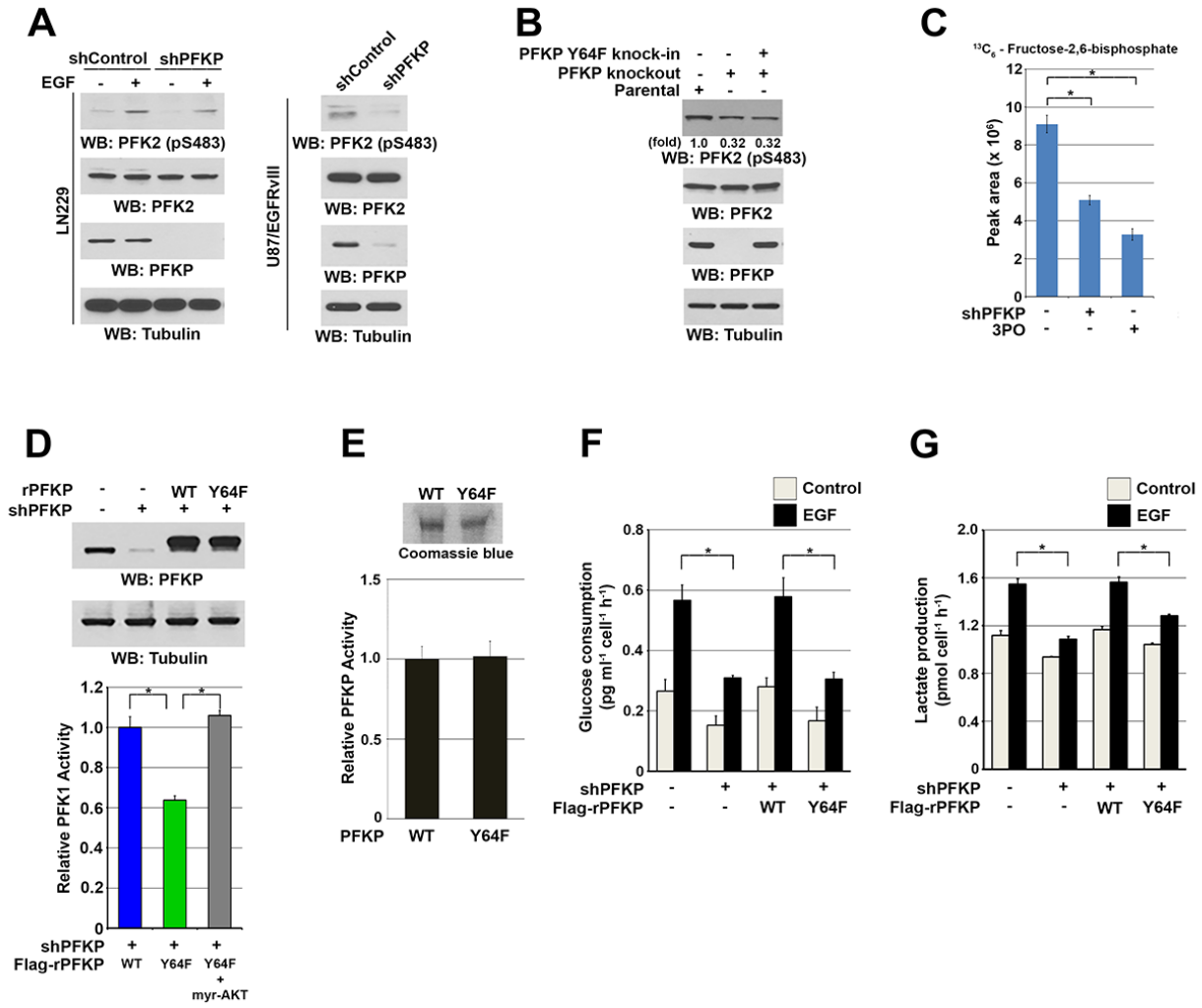


Figure S5 related to Figure 5. PFKP Y64 Phosphorylation Enhances PFK1 Activity and GLUT1 Expression via AKT Activation.

(**A**) Serum-starved LN229 cells with or without expressing PFKP shRNA were treated with or without EGF (100 ng/ml) for 15 min. A control shRNA or PFKP shRNA was expressed in U87/EGFRvIII cells. Immunoblotting analyses were performed with the indicated antibodies.

(**B**) U87/EGFRvIII cells with or without knockout of PFKP or knock-in of PFKP Y64F mutant were subjected to immunoblotting analyses with the indicated antibodies. MG132 (10 μM) was added to the cells 6 h before harvesting to eliminate the potential effect of proteasomal degradation on PFKP proteins.

(**C**) U87/EGFRvIII cells with or without expressing PFKP shRNA were pretreated with or without 3PO (20 μM) for 6 h, followed by metabolically labeling with $^{13}C_6$-glucose (10 mM) for 30 min. The indicated $^{13}C_6$-labeled fructose-2,6-bisphosphate was measured by LC-MS/MS. All results were normalized to the final cell number. Data represent the means ± SD of three independent experiments. *p < 0.001, based on the Student's *t* test.

(**D**) U87/EGFRvIII cells with or without PFKP depletion and reconstituted expression of WT Flag-rPFKP or Flag-rPFKP Y64F mutant were subjected to immunoblotting analyses with the indicated antibodies. (top panel). The indicated serum-starved U87/EGFRvIII cells were transfected with a control vector or a vector expressing HA-tagged myr-AKT. PFK1 enzymatic activity was checked. Data represent the means ± SD of three independent experiments. *p < 0.01, based on the one-way ANOVA (bottom panel).

(**E**) The PFKP enzymatic activity of bacterially purified WT PFKP (0.1 μg) and PFKP Y64F (0.1 μg) was measured. Data represent the means ± SD of three independent experiments.

(**F, G**) U251 cells with or without expressing PFKP shRNA and with or without reconstituted expression of WT Flag-rPFKP or Flag-rPFKP Y64F mutant were cultured in no-serum DMEM with or without EGF (100 ng/ml) for 24 h. The media were collected for analysis of glucose consumption (**F**) or lactate production (**G**). All results were normalized to the final cell number. Data represent the means ± SD of three independent experiments. *p < 0.001, based on the Student's *t* test.

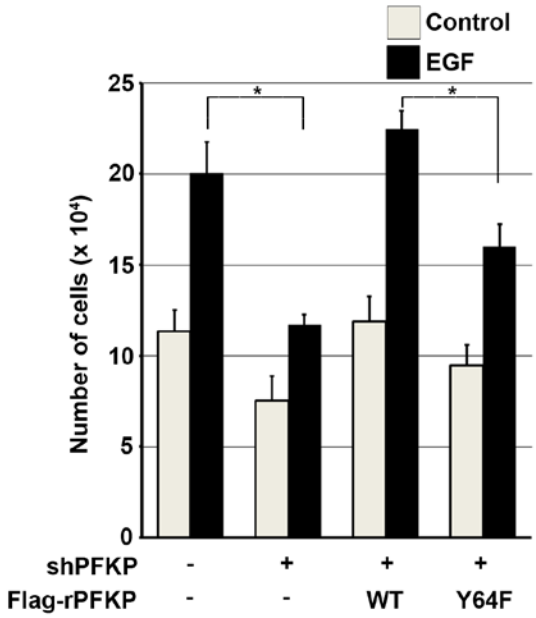


Figure S6 related to Figure 6. PFKP Y64 Phosphorylation Promotes Tumor Cell Proliferation.

U251 cells with or without expressing PFKP shRNA and with or without reconstituted expression of WT Flag-rPFKP or Flag-rPFKP Y64F mutant were cultured in 1% serum medium with or without EGF (100 ng/ml) for 4 days. The cells were collected and counted. Data represent the mean ± SD of three independent experiments. *$p < 0.001$, based on the Student's *t* test.

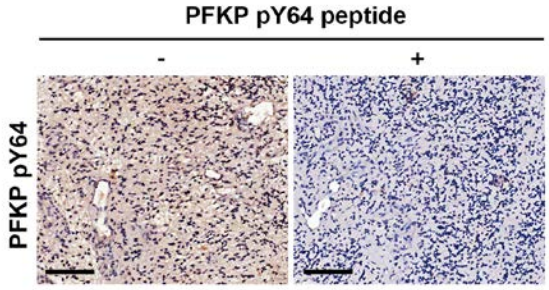


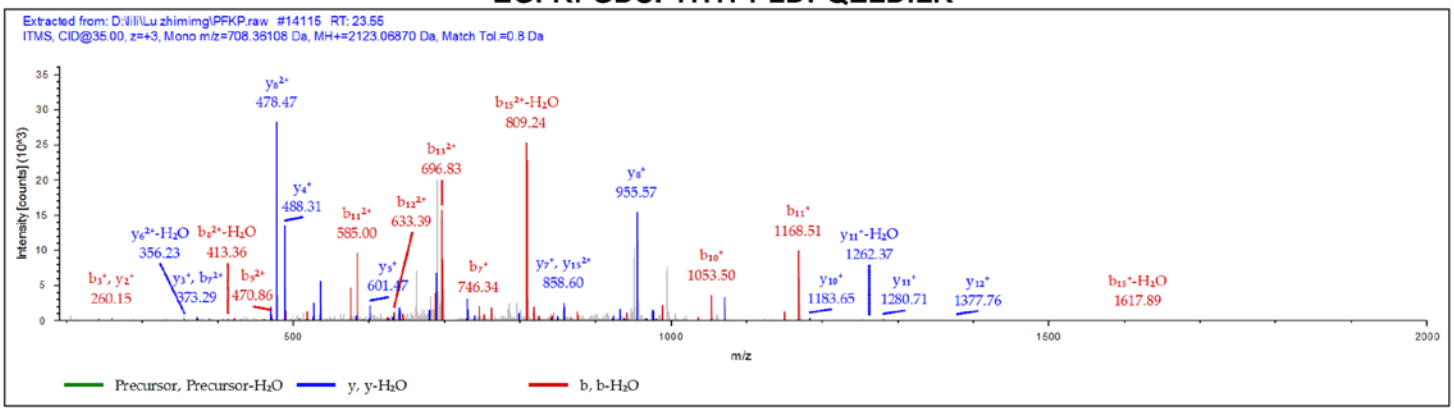
Figure S7 related to Figure 7. PFKP Y64 Phosphorylation Is Positively Correlated with EGFR Y1068 Phosphorylation and PFK2 S483 Phosphorylation and with Poor Patient Prognosis in Human GBM Specimens. Antibody specificity was validated in human GBM specimens, in the presence or absence of a blocking peptide that was specific for phosphorylated PFKP Y64. Scale bar, 100 μm.

Supplementary Table S1 related to Figure 1. LC-MS/MS analysis of PFKP-interacting proteins.

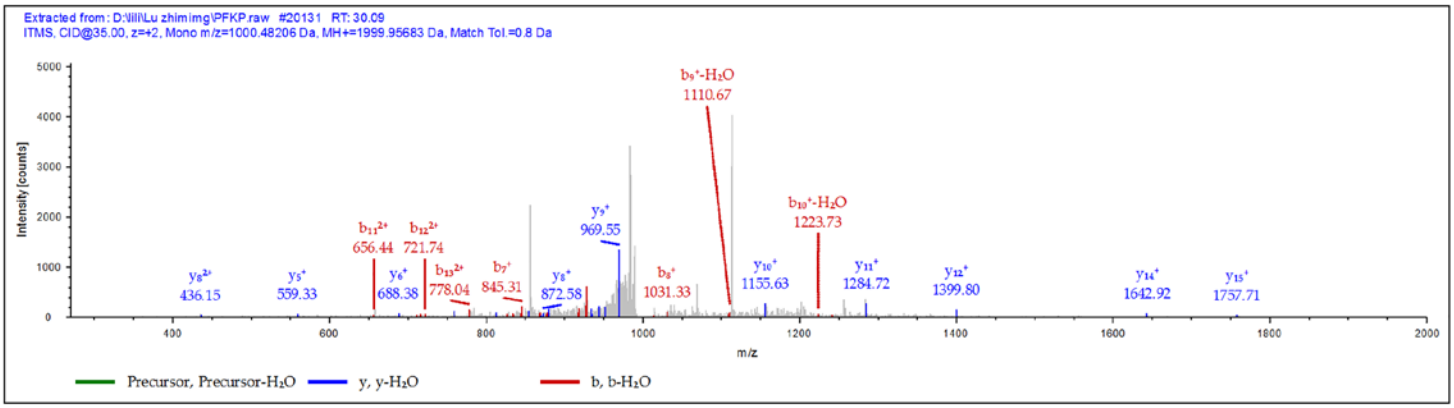
Accession number and peptide sequences of PFKP-associated polypeptides by LC-MS/MS analysis. These PFKP-associated proteins were not detected in IgG control pull-down samples. EGFR and KAT5 were identified by mass spectrometry as indicated.

Protein name	Accession number	Peptide sequences
EGFR	ADZ75461	YLVIQGDER
		NLQEILHGAVR
		SLKEISDGDVIISGNK
		LTQLGTFEDHFLSLQR
		ELVEPLTPSGEAPNQALLR
		GDSFTHTPPLDPQELDILK
		GSHQISLDNPDYQQDFFPK
		YSSDPTGALTEDSIDDTFLPVPEYINQSVPK
KAT5	NP_874369	RLDEWVTHER
		LLIEFSYELSK
		NQDNEDEWPLAEILSVK
		NGLPGSRPGSPEREVK
PFKL	AAH08964	MGIYVGAK
		TFVLEVMGR
		SEWGSLLEELVAEGK
		AIGVLTSGGDAQGMNAAVR
		IMEVIDAITTTAQSHQR
		SNFSLAILNVGAPAAGMNAAVR
		GSRLNIIIIAEGAIDRNGKPISSSYVK
		LRASGAGKAIGVLTSGGDAQGMNAAVR
		QANWLSVSNIIQLGGTIIGSAR
		VFLIYEGYEGLVEGGENIK
		MPREQWWLSLRLMLK
		ISMAAYVSGELEHVTR
PFKM	NP_001341664	IPKEQWWLK
		VLVVHDGFEGLAK
		DLQANVEHLVQK
		SEWSDLLSDLQK
		IMEIVDAITTTAQSHQR
		SSYLNIVGLVGSIDNDFCGTDMTIGTDSALHR
		MGVEAVMALLEGTPDTPACVVSLSGNQAVR
		TPQRTAGEASTSSMLIPKPPPKTDILK
		FNIQGLVIIGGFEAYTGGLELMEGRK
		ESYRNGRIFANTPDSGCVLGMR
Actin	P60709	EITALAPSTMK
		LDLAGRDLTDYLMK
		SYELPDGQVITIGNER
Tubulin α-1A	NP_001290043	DVNAAIATIK
		AVFVDLEPTVIDEVR
		VGINYQPPTVVPGGDLAK
		TIGGGDDSFNTFFSETGAGK
Tubulin β-2A	NP_001297244.1	LAVNMVPFPR
		AILVDLEPGTMDSVR
Tubulin β-4A	AAP35617	LAVNMVPFPR
		AVLVDLEPGTMDSVR
HSP60	ABB01006	ALMLQGVDLLADAVAVTMGPK

EGFR: GDSFTHTPPLDPQELDILK



KAT5: NQDNEDEWPLAEILSVK



Supplementary Table S2 related to Figure 2. Oligos used in CRISPR/cas9-mediated knock-in study.

Name	Sequence (5'-3')	Note
PFKP-Y64-sgRNA -F	caccgCTTTCTCCCCTAGGGCTACC	For constructing sgRNA of PFKP Y64F knock-in mutation
PFKP-Y64-sgRNA -R	aaacGGTAGCCCTAGGGGAGAAAGc	
PFKP-Y64F-Template	TTTTCCCGTTTTATATAATTTAAGGATCACTTCCC ATTTAGTGAAGTTTATCTCATTTTTAAAAGATTCT CCCTTTCTCCCCTAGGGaTttCAGGGCATGGTGGAC GGAGGCTCAAACATCGCAGAGGCCGACTGGGAG AGTGTCTCCAGCATCCTGCAAGTGGTAGGTACTG GGCTGCG	Template for PFKP Y64F knock-in mutation
PFKP Y64F DNA – F	GTTGGGTGAAAGTGACCCCT	For identification of PFKP Y64F knock-in mutation
PFKP Y64F DNA – R	AGTGACTCACAGATTAACACATGC	
PFKP-K395-sgRNA-F	caccgCTTGATGGCAAGTCGCTTGT	For constructing sgRNA of PFKP K395R knock-in mutation
PFKP-K395-sgRNA-R	aaacACAAGCGACTTGCCATCAAGc	
PFKP-K395R-Template	TGAGCCCTGAGTTGTGTCCGGTATTCTCGACTCC GGTCCAACGACACCCTTTTCCTTTAGGAGCTTTGC GGGCAACCTGAACACCTACAgGaGACTaGCaATCA AGCTGCCGGATGATCAGATCCCAAAGGTAGGTG GCCGGCCTCCCGCGATGCCCCGACCTCTCCTGCG GGCCTCCCC	Template for PFKP-K395R knock-in mutation
PFKP K395R DNA – F	CCATTTCCACCAGACACGGA	For identification of PFKP K395R knock-in mutation
PFKP K395R DNA – R	ATCACAGGATCCCGGACTGA	

Table S3. Information of GBM patients, Related to STAR Methods.

Order	Gender	Age	Histology	Grade
1	M	49	GBM	4
2	M	21	GBM	4
3	M	17	GBM	4
4	M	52	GBM	4
5	M	40	GBM	4
6	M	43	GBM	4
7	M	59	GBM	4
8	M	51	GBM	4
9	F	50	GBM	4
10	F	45	GBM	4
11	M	57	GBM	4
12	M	62	GBM	4
13	F	70	GBM	4
14	M	62	GBM	4
15	M	38	GBM	4
16	M	33	GBM	4
17	F	42	GBM	4
18	M	30	GBM	4
19	F	35	GBM	4
20	M	13	GBM	4
21	M	59	GBM	4
22	M	62	GBM	4
23	F	45	GBM	4
24	M	55	GBM	4
25	F	59	GBM	4
26	M	60	GBM	4
27	M	53	GBM	4
28	F	35	GBM	4
29	M	42	GBM	4
30	F	63	GBM	4
31	M	51	GBM	4
32	F	58	GBM	4
33	M	50	GBM	4
34	M	61	GBM	4
35	M	51	GBM	4
36	M	48	GBM	4
37	F	54	GBM	4
38	F	55	GBM	4
39	F	37	GBM	4
40	M	55	GBM	4
41	F	59	GBM	4
42	F	56	GBM	4

43	F	48	GBM	4
44	M	62	GBM	4
45	F	58	GBM	4
46	M	59	GBM	4
47	M	38	GBM	4
48	F	42	GBM	4
49	M	50	GBM	4
50	M	60	GBM	4
51	F	65	GBM	4
52	M	29	GBM	4
53	F	35	GBM	4
54	M	53	GBM	4
55	M	49	GBM	4
56	F	58	GBM	4
57	M	56	GBM	4