SUPPLEMENTAL MATERIALS

Inhibition of PFKFB3 in HER2-positive gastric cancer improves sensitivity to trastuzumab by inducing tumor vessel normalization

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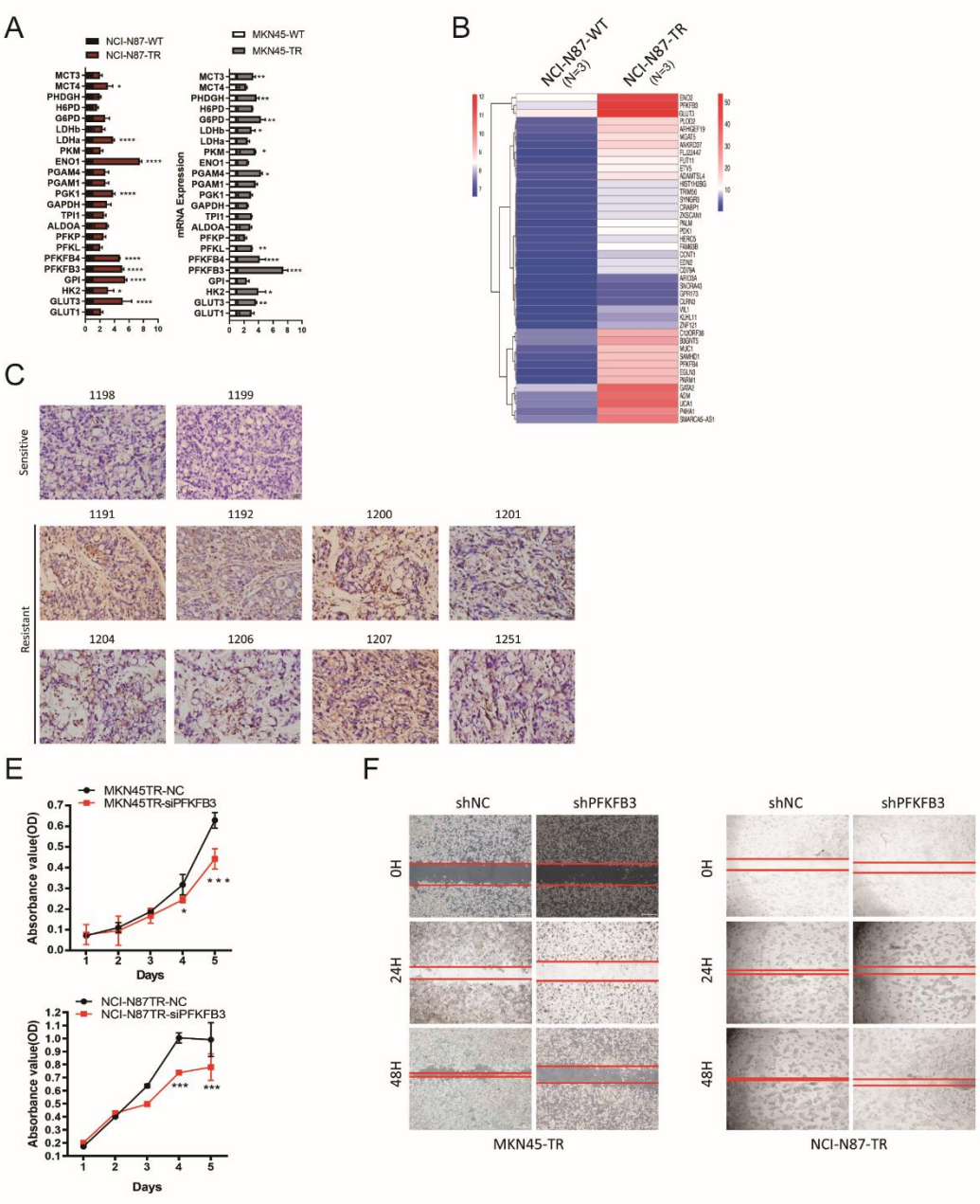
Xingxing Yao and Zhanke He contributed equally to this work.

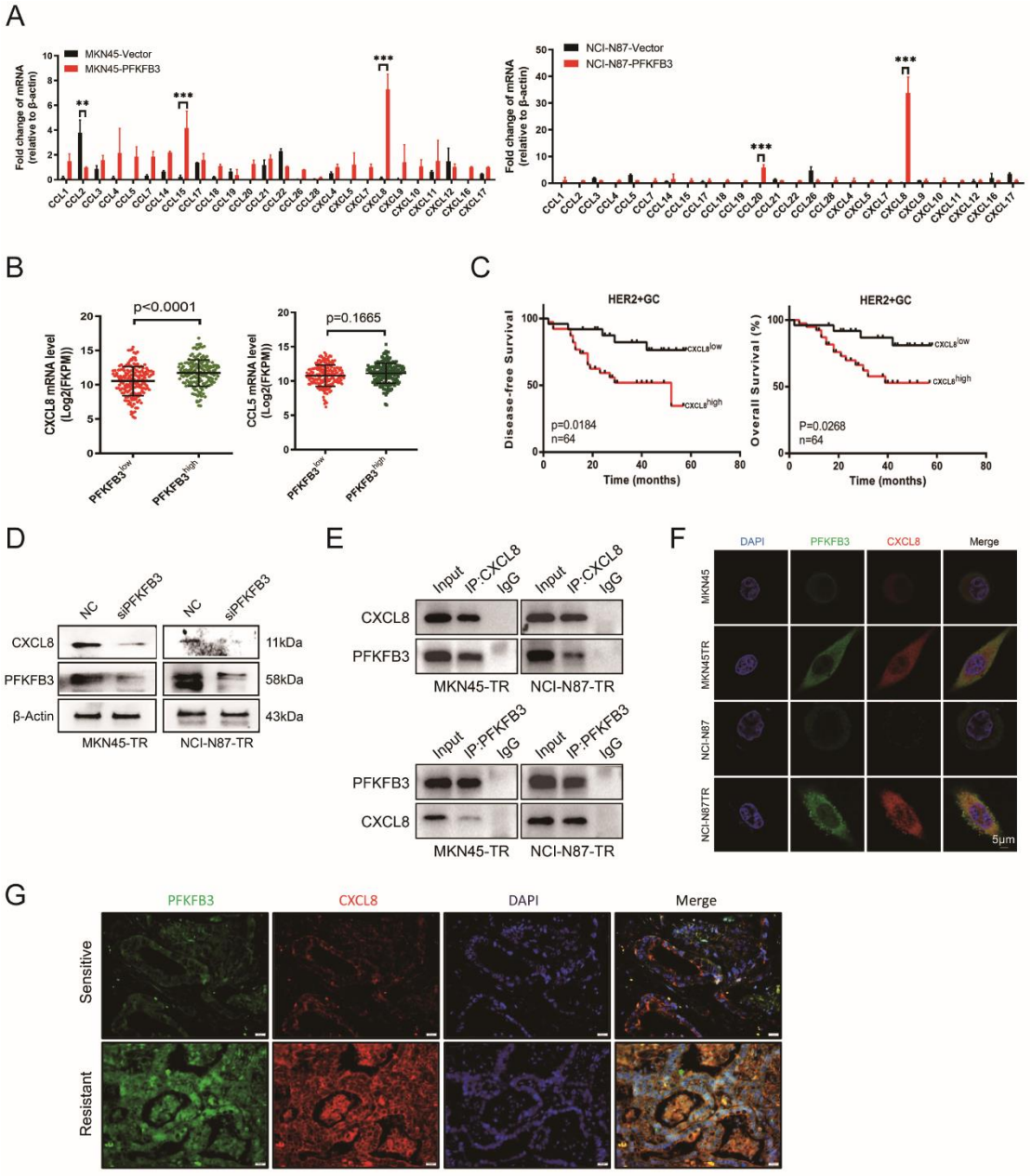
Supplementary Figure 1



Supplementary Figure 1. PFKFB3 increases glucose metabolism fuels contribute to the trastuzumab resistance in HER2+ gastric cancer. (A) RT-PCR was used to detect the expression levels of glycolysis enzymes in WTs and TRs. (B) Unsupervised hierarchical clustering of significantly deregulated metabolites between cells lines in NCI-N87-WT and NCI-N87-TR cells. (C) IHC

staining of PFKFB3 in selected species of trastuzumab-sensitive and -resistant PDX tumors, respectively. Scale bar, 50 μm (20×) (D) CCK8 assays were used to assess cell proliferation after knockdown PFKFB3 in MKN45-TR and NCI-N87-TR. (E) The wound healing assay was performed to assess the migration capacity of silencing PFKFB3 in MKN45TR cells and NCI-N87-TR cells for 48 h. Each experiment was conducted triple and results are presented as mean ± SEM. Student's t-test, one-way ANOVA or two-way ANOVA was used to analyze the data (*P < .05, **P < .01, ***P < .001)

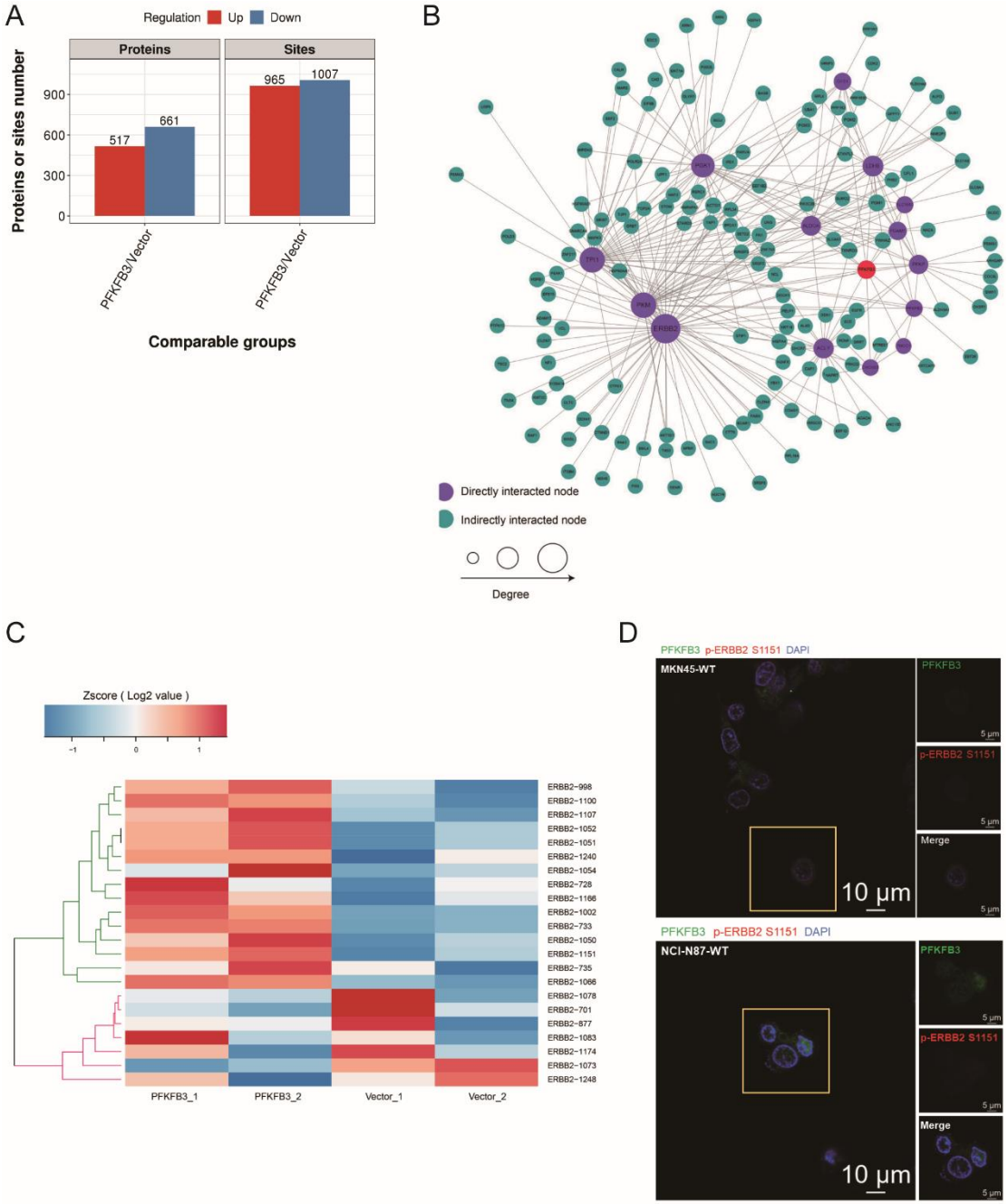
Supplementary Figure 2



Supplementary Figure 2. PFKFB3 promotes CXCL8 secretion in Her2 positive gastric cancer. (A) Fold changes of chemokine mRNA levels in PFKFB3 overexpressed gastric cancer cells compared with control cells, as analyzed by real-time PCR. Values are mean ± SD. *P < .05, **P < .01, ***P < .001, based on Student's t-test. (B) RNA-seq data from The Cancer Genome Atlas dataset showed expression levels of PFKFB3 correlated with CXCL8 (ρ

< 0.0001) or CCL5 (ρ = 0.166) expression. (C) Cumulative overall survival and progression-free survival curves of patients. The patients were divided into 2 groups according to the CXCL8 density. Paraffin-embedded gastric cancer samples (n=64) were stained with an anti-CXCL8 antibody. (D) Western blot assays showed the protein expression of CXCL8 and CCL5 induced by PFKFB3 in WTs. (E) Co-immunoprecipitation of PFKFB3 with p-ERBB2 S1151 in WT cells. (F) IF staining of PFKFB3 and CXCL8 in WTs and TRs. (G) IF staining of PFKFB3 and CXCL8 in tissues from trastuzumab-sensitive (Tra-S) and resistant (Tra-R) patients.

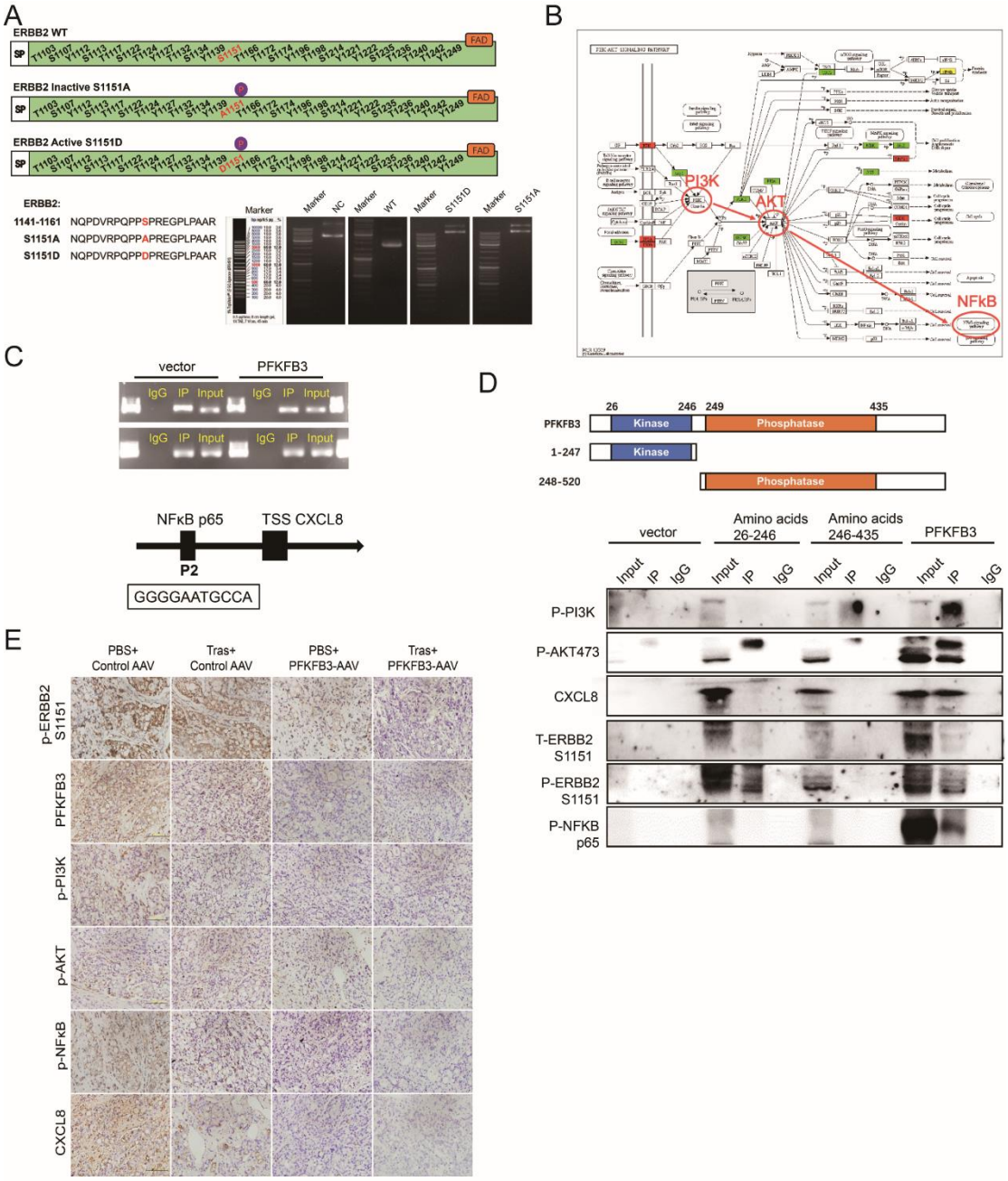
Supplementary Figure 3.



Supplementary Figure 3. (A) The number of differentially modified proteins and modified sites in Vector and PFKFB3 groups. (B) The differentially modified protein screened from different comparison groups were compared with the STRING (v.11.0) protein network interaction database, according to the score ≥ 0.7 (high confidence). (C) Heat map of motif enrichment of upstream and

downstream amino acids at all identified ERBB2 phosphorylation modification sites. Red represents the significant enrichment of this amino acid near the modification site, while blue represents the significant reduction of this amino acid near the modification site. (D) The location of PFKFB3 and p-ERBB2 S1151 in TRs was assessed by IF staining.

Supplementary Figure 4.



Supplementary Figure 4. (A) Schematic representation of ERBB2 WT, ERBB2 S1151A and ERBB2 S1151D (upper). The cysteines are shown as rectangle of green and the phosphorylation sites are shown circles of purple. Amino acid sequence alignments of ERBB2 are shown in black and ERBB2 Ser1151 shown

in red (lower). The DNA gel electrophoresis were used to verify the molecular weight of eukaryotic expression vectors. (B) The PI3K/AKT/NFkB signal pathway was enriched by KEGG pathway analysis. (C) ChIP-qPCR and PCR analysis of NFκB p65 with CXCL8 promoter regions. Data are shown as fold enrichment relative to input, *P < 0.5, mean ± SEM (n= 3), based on Student's t-test. (D) Construction of plasmid composed of kinase or phosphatase domains of PFKFB3. The 6-phosphofructo-2-kinase domain plasmid from 26 amino acids to 246 amino acids and the phosphatase domain plasmid from 249 amino acids to 435 amino acids was established. Co-immunoprecipitation of the kinase domain or the phosphatase domain with indicated proteins in 293T cells. (E) Representative PFKFB3 IHC staining images of PDX models. Scale bar, 100 μm (40×).

Supplementary Methods

MicroPET/CT

The xenograft bearing mice were fasted overnight and anesthetized with inhaled isoflurane. ^{18}F-FDG of about 200 μCi per mouse was injected into the tail vein. After 60 min of nonspecific clearance, the mouse was scanned in microPET/CT Inveon scanner (Siemens, Knoxville, TN, USA) and images were then reconstructed using a two-dimensional ordered subsets expectation maximization algorithm. PET and CT image fusion and image analysis were performed using software ASIPro 5.2.4.0 (Siemens).

siRNA-mediated gene silencing

Expression of human PFKFB3 was knocked down with siRNA duplexes targeting their sequences. The siRNAs were designed and chemically synthesized (Shanghai GenePharma Co., Shanghai, China) for targeting different coding regions of the genes. Negative control siRNA targeting an unknown mRNA sequence was used as a control. The sequences used are shown in Table S1. All siRNAs were synthesized from GenePharma (Shanghai, China). A BLAST search of the human genome verified that the selected sequences were specific for the target gene. Exponential growth phase cells were transfected with 20nM of siRNA in OPTI-MEM according to the manufacturer's protocol.

Supplementary Table 1. siRNA sequences used for transfection

Gene name	Sense 5’ to 3’	Antisense 5’ to 3’
PFKFB3-780	GGAGACACAUGAUCCUUCATT	UGAAGGAUCAUGUGUCUCCTT
PFKFB3-949	GAGGAUCAGUUGCUAUGAATT	UUCAUAGCAACUGAUCCUCTT
PFKFB3-1083	GCAUCGUGUACUACCUGAUTT	AUCAGGUAGUACACGAUGCTT
PFKFB3-1803	CUCGCAUCAACAGCUUUGATT	UCAAAGCUGUUGAUGCGAGTT
Negative control (NC)	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Elisa

Cells ($2x10^6$/100 mm dish) were cultured for 24 hours. Media were removed and replaced with 10 ml serum-free DMEM. Supernatants were collected 24 hours later with any floating cells removed by 0.45 μm filtration. The amount of CXCL8 protein in the supernatant was determined using a CXCL8 specific Human IL-8 High Sensitivity ELISA kit (Multisciences, EK108HS-24) following the manufacturer’s instructions.

Tumor vessel perfusion and leakage

At indicated days, tumor vessel leakage was analyzed after i.v. injection of 100ul of rhodamine-conjugated dextran (25mg/ml, 70kDa, Sigma-Aldrich) 30 min before sacrifice. For vascular perfusion studies, 100 ul of DyLight ® 594-

conjugated tomato lectin (1mg/ml, Vector laboratory) was i.v. injected 30 min before sacrifice. Mice were anesthetized and perfused by intracardiac injection of 1% PFA to remove circulating dextran or lectin.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) performed by the Molecular Signature Database (MsigDB) was used to identify the pathways that were significantly enriched in PFKFB3^{high} tumor samples. If a gene set had a positive enrichment score, most of its members had higher expression accompanied with higher risk score, and the set was termed “enriched”.

Extracellular acidification rate and oxygen consumption rate analysis

$3.5x10^4$ cells were seeded per well in 24-well cell culture plates (seahorse Biosciences, North Billerica, MA) in DMEM with 10% FBS and incubated at 37°C, overnight in 5% CO_2 incubator. Next day, growth medium was replaced with bi-carbonate free DMEM and cells were incubated at 37°C for 1 hour in CO_2 free incubator to equilibrate media temperature and pH. By utilizing a Seahorse XF24 analyzer, extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured in baseline conditions and under treatment with 2,4-dinitrophenol (2,4 DNP; 100μM), 2-deoxy glucose (2-DG; 100mM) and rotenone (1μM). Values are presented as mean ± standard error of mean.

Extracellular Lactate Analysis

Lactate levels secreted into the media were analyzed by colorimetric assays. The assay was performed as per the manufacturer's protocol utilizing Lactate Assay Kit II (Eton Bioscience Inc).

Glucose uptake assay and extracellular glucose analysis

To measure glucose uptake, $5x10^4$ cells were seeded per well in 24 well plate and incubated overnight at 37°C with 5% CO_2. Next day, cells were starved for glucose for 2 hour and then treated with 1µl 2-Deoxy-2- [(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG) for 20 min. After incubation, cells were washed twice with PBS and the fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. To measure the residual glucose in culture medium, the cells were incubated in FBS-free DMEM. After incubation for 24 h, the supernatant was collected to measure residual glucose. The reaction mixture was incubated for 30 min at room temperature in the dark. The glucose levels were measured at 490 nm in a microplate reader. The values were normalized with respective cell counts.

Mass Spectrometric Metabolomics Analysis

Cells were seeded in 6 cm plates and 2 hours before the collection of metabolites the culture medium was replaced with fresh medium. Polar

metabolites were extracted and then analyzed with LC-MS/MS using the selected reaction monitoring (SRM) method with positive/negative ion polarity switching on a Xevo TQ-S mass spectrometer (Gunda et al., 2016; Yuan et al., 2012). Peak areas integrated using MassLynx 4.1 (Waters Inc.) were normalized to the respective protein concentrations and the resultant peak areas were subjected to relative quantification analyses by utilizing Metaboanalyst 3.0 (www.metaboanalyst.ca) (Xia and Wishart, 2016).

Construction of Mutant

The GV141 plasmids encoding human ERBB2 or PFKFB3 were products by Shanghai Genechem Company. The mutations of PFKFB3 (1-247), PFKFB3 (248-520), ERBB2 S1151A and ERBB2 S1151D were created using the Fast Mutagenesis System (TransGen). All constructs were verified by DNA sequencing (Invitrogen). Detailed plasmid sequence was showed in supplemental figure 5.

AAV-GFP construction and intratumor injection

PFKFB3 knockdown and control recombinant Adeno-associated virus-green fluorescence protein vectors (AAV-GFP) were constructed (GENECHEM Biotech). The administration procedures were performed according to previous studies. Briefly, 1×10^9 physical particles of AAV in 100 μl of PBS were injected into the tumor of nude mice.

Mass Spectrometric Metabolomics Analysis

Cells were seeded in 6 cm plates, and 2 hours before the collection of metabolites the culture medium was replaced with fresh medium. Polar metabolites were extracted and then analyzed with LC-MS/MS using the selected reaction monitoring (SRM) method with positive/negative ion polarity switching on a Xevo TQ-S mass spectrometer. Peak areas integrated using MassLynx 4.1 (Waters Inc.) were normalized to the respective protein concentrations and the resultant peak areas were subjected to relative quantification analyses by utilizing Metaboanalyst 3.0 (www.metaboanalyst.ca).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed according to the protocol of Chromatin Immunoprecipitation kit (Cell Signal Tech). Immunoprecipitation reactions were performed with 5μg antibodies against p-NFkB p65 (1:50, Cell Signal Tech), PFKFB3 (1:20, Proteintec) or with IgG used as a negative control. Purified DNA was then suspended for following qRT-PCR analysis using primers of CXCL8 promoters. PCR products were then run on 1.2% agarose gels and visualized with ethidium bromide. Relative chromatin enrichment was calculated as the amount of amplified DNA normalized to input and relative to values obtained after normal IgG immunoprecipitation.

Phosphoproteomic profiling

Protein Extraction and Trypsin Digestion

NCI-N87 cells transfected with or without PFKFB3 were sonicated three times on ice using a high intensity ultrasonic processor (Scientz) in lysis buffer (8 M urea, 1% Protease Inhibitor Cocktail). (Note: For PTM experiments, inhibitors were also added to the lysis buffer, e.g., 3 μM TSA and 50 mM NAM for acetylation.) The remaining debris was removed by centrifugation at 12,000 g at 4 °C for 10 min. Finally, the supernatant was collected, and the protein concentration was determined with BCA kit according to the manufacturer's instructions.

For digestion, the protein solution was reduced with 5 mM dithiothreitol for 30 min at 56 °C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. The protein sample was then diluted by adding 100 mM TEAB to urea concentration less than 2M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h-digestion.

TMT/iTRAQ Labeling and HPLC Fractionation

After trypsin digestion, peptide was desalted by Strata X C18 SPE column (Phenomenex) and vacuum-dried. Peptide was reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for TMT kit/iTRAQ kit. Briefly, one unit of TMT/iTRAQ reagent were thawed and reconstituted in

acetonitrile. The peptide mixtures were then incubated for 2 h at room temperature and pooled, desalted and dried by vacuum centrifugation.

The tryptic peptides were fractionated into fractions by high pH reverse-phase HPLC using Thermo Betasil C18 column (5 μm particles, 10 mm ID, 250 mm length). Briefly, peptides were first separated with a gradient of 8% to 32% acetonitrile (pH 9.0) over 60 min into 60 fractions. Then, the peptides were combined into 6 fractions and dried by vacuum centrifuging.

Affinity Enrichment

Pan antibody-based PTM enrichment:

To enrich modified peptides, tryptic peptides dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0) were incubated with pre-washed antibody beads (Lot number 001, PTM Bio) at 4°C overnight with gentle shaking. Then the beads were washed four times with NETN buffer and twice with H2O. The bound peptides were eluted from the beads with 0.1% trifluoroacetic acid. Finally, the eluted fractions were combined and vacuum-dried. For LC-MS/MS analysis, the resulting peptides were desalted with C18 ZipTips (Millipore) according to the manufacturer's instructions. Bio-material-based PTM enrichment (for phosphorylation):

Peptide mixtures were first incubated with IMAC microspheres suspension with vibration in loading buffer (50% acetonitrile/6% trifluoroacetic acid). The IMAC microspheres with enriched phosphopeptides were collected by centrifugation,

and the supernatant was removed. To remove nonspecifically adsorbed peptides, the IMAC microspheres were washed with 50% acetonitrile/6% trifluoroacetic acid and 30% acetonitrile/0.1% trifluoroacetic acid, sequentially. To elute the enriched phosphopeptides from the IMAC microspheres, elution buffer containing 10% NH4OH was added and the enriched phosphopeptides were eluted with vibration. The supernatant containing phosphopeptides was collected and lyophilized for LC-MS/MS analysis.

LC-MS/MS Analysis

The tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a home-made reversed-phase analytical column (15-cm length, 75 μm i.d.). The gradient was comprised of an increase from 6% to 23% solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, 23% to 35% in 8 min and climbing to 80% in 3 min then holding at 80% for the last 3 min, all at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system.

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by

20 MS/MS scans with 15.0s dynamic exclusion. Automatic gain control (AGC) was set at 5E4. Fixed first mass was set as 100 m/z.

Database Search and Analysis

The resulting MS/MS data were processed using Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against human uniprot database concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to 4 missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in First search and 5 ppm in Main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification and Acetylation modification and oxidation on Met were specified as variable modifications. FDR was adjusted to < 1% and minimum score for modified peptides was set > 40.

Soft MoMo (motif-x algorithm) was used to analysis the model of sequences constituted with amino acids in specific positions of modify-21-mers (10 amino acids upstream and downstream of the site， but phosphorylation with modify-13-mers that 6 amino acids upstream and downstream of the site) in all protein sequences. And all the database protein sequences were used as background database parameter. Minimum number of occurrences was set to 20. Emulate original motif-x was ticked, and other parameters with default.

Proteins were classified by GO annotation into three categories: biological process, cellular compartment and molecular function. For each category, a

two-tailed Fisher's exact test was employed to test the enrichment of the differentially modified protein against all identified proteins. The GO with a corrected p-value < 0.05 is considered significant. For further hierarchical clustering based on differentially modified protein functional classification (such as: GO, Domain, Pathway, Complex). We first collated all the categories obtained after enrichment along with their P values, and then filtered for those categories which were at least enriched in one of the clusters with P value <0.05. This filtered P value matrix was transformed by the function x = −log10 (P value). Finally, these x values were z-transformed for each functional category. These z scores were then clustered by one-way hierarchical clustering (Euclidean distance, average linkage clustering) in Genesis. Cluster memberships were visualized by a heat map using the "heatmap.2" function from the "gplots" R-package.

All differentially expressed modified protein database accession or sequence were searched against the STRING database version 11.0 for protein-protein interactions. Only interactions between the proteins belonging to the searched data set were selected, thereby excluding external candidates. STRING defines a metric called "confidence score" to define interaction confidence; we fetched all interactions that had a confidence score ≥ 0.7 (high confidence). Interaction network form STRING was visualized in R package "networkD3".