Supplementary Materials for

Targeting Pyruvate Kinase M2 phosphorylation reverses aggressive cancer phenotypes

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This PDF file includes:

Supplementary Materials and Methods

Plasmid constructs

pWZ-Neo-Myr-Flag-PKM2 plasmid (Addgene #20585) was used for amplification and cloning of the human PKM2 wild type (WT) sequence into the pFRT-TO-DEST-Flag-HA plasmid using gateway technology (Invitrogen). NLS coding sequence (5'-cctgctgccaagagggtcaagttggac-3') were inserted c-terminally to PKM2 WT sequence. PKM2 S37 mutagenesis was carried out using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) according to manufacturer's guidelines. Final PKM2 constructs (FH-PKM2-WT, FH-PKM2-S37A, FH-PKM2-S37E, FH-PKM2-NLS) were PCR amplified, using primers FW: 5'-ACTGGATCCGCCACCATGGACTAC-3', RV: 5'-GTCGAATTCTTACGGCACAGGAA-3' or 5'-GTCGAATTCTTAGTCCAACTTGAC-3' and cloned into pFUGW expression plasmid (Addgene #63592) at BamHI/EcoRI cloning sites under EF-1a promoter after deletion of the Cas9 gene.

PKM2-S37A mutagenesis oligomers

5'-TGTGCCGCCTGGACATTGATGCACCACCCATC-3'

5'-GATGGGTGGTGCATCAAATGTCCAGGCGGCACA-3'

PKM2-S37E mutagenesis oligomers

5'-CATGTGCCGCCTGGACATTGATGAGCCACCCATCACAGCC-3'

5'-GGCTGTGATGGGTGGCTCATCAATGTCCAGGCGGCACATG-3'

pLKO.1 plasmid (Addgene #84530) was used to express shRNA against 3'UTR of PKM2. DNA oligomers for shPKM2 and control scramble shRNA were annealed and cloned into pLKO.1 plasmid at AgeI/EcoRI cloning sites.

shPKM2 DNA oligomers

5'-CCGGCAACGCTTGTAGAACTCACTCCTCGAGGAGTGAGTTCTACAAGCGTTGTTTTTG-3'

5'-AATTCAAAAACAACGCTTGTAGAACTCACTCCTCGAGGAGTGAGTTCTACAAGCGTTG-3'

Scramble-shRNA DNA oligomers

5'-CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTG-3'

5’-AATTCAAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG-3’

Custom antibody production and purification

A synthetic peptide mimicking the PKM2pS37 epitope (Ac-CRLDID(pS)PPIT-amide) was conjugated and injected into 2 animals (Specific Pathogen Free (SPF) rabbits). After 2 rounds of immunization secreted antibodies were collected into 20-23 ml of blood serum. All work was performed with Covance Research Products (Denver, PA).

Total IgG antibody title was first purified using a chromatography column packed with immobilized Protein A (Pierce). Purified IgG antibodies were tested against the physiological PKM2pS37 epitope and positive fractions were further purified using a chromatography column for peptide conjugation (Pierce) packed with immobilized immunogen (PKM2pS37 synthetic peptide). Specific antibodies were eluted in 0.1-0.2 M Glycine-HCl buffer pH=2.5-3.0, neutralized in 1M Tris-HCl pH=8.0, buffer exchanged into 50 mM Tris-HCl pH=7.4, 150 mM NaCl, 20% glycerol, and concentrated to 1.4 mg/ml for storage at -20 ^{o}C.

Immunoblotting

Additional antibodies used in the analysis include p44/42 MAPK (Erk1/2) (Cell Signaling Technology, Cat# 9102, RRID: AB_330744), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling Technology, Cat# 9101), Phospho-Akt (Ser473) (Cell Signaling Technology, Cat# 9271, RRID: AB_329825), Phospho-cdc2 (Tyr15) (Cell Signaling Technology, Cat# 9111, RRID: AB_331460).

Immunohistochemistry analysis

Tissue sections were subjected to PKM2 staining on Leica Bond Rx using Leica Refine Polymer Detection Kit as per manufacturer’s instructions. Briefly, sections were baked online at 60 ^{o}C for 30 minutes followed by standard dewax and rehydrate program using Bond Dewax and 100% ethanol. Post rehydration HIER (heat induced antigen retrieval) was performed using ER2 buffer (EDTA pH=9) for 20 minutes at 100 ^{o}C and slides were cooled to ambient temperatures. Peroxide block was added for 5 minutes at room temperature (RT) followed by primary anti-PKM2 (RRID: AB_1904096) and anti-PKM2pS37 (rabbit polyclonal, custom-made), 1:2000 dilution, for 1 hour at RT. Post

Primary reagent was added for 8 minutes at RT followed by Polymer for 8 minutes at RT. Mixed DAB Refine was added to slides (2x) at ambient temperatures and finally Hematoxylin was added for 5 minutes at RT. Between each step after peroxide addition, standard Bond Rx washing protocol was applied (3x Bond wash at ambient temperatures). Slides were removed from Leica Bond Rx and subject to manual dehydration. Slides were submerged in ascending alcohol concentrations [70, 80, 90 and 100% Ethanol (3x)] for 3 minutes each, rinsed in Xylene and submerged in Xylene for 15 minutes (2x). Slides were mounted using Cytoseal mountant media and left to dry overnight.

Quantitative immunofluorescence by AQUA Method

Tissue microarrays (TMAs) were processed according to published methods (1,2). Briefly, sections were deparaffinized (baked at 60 ºC and undergo two incubations in xylenes, rehydrated in two 100% ethanol washes followed by one wash in 70% ethanol and rinsed in streaming tap water) and subjected to antigen retrieval in EDTA buffer (pH 8) at 97 ºC for 20 minutes in a pressure heating container (PT module, Lab Vision). Subsequently, a sequential multiplexed immunofluorescence staining was performed with primary antibodies to detect epithelial tumor cells (pan-cytokeratin, polyclonal, Agilent, 1:100 dilution) and PKM2 (RRID: AB_1904096, 1:2000 dilution) or PKM2pS37 (rabbit polyclonal, custom-made, 1:2000 dilution) on serial TMA sections, overnight at 4 ºC. Detection of proteins was performed using isotype-specific horseradish peroxidase (HRP)-conjugated secondary antibodies (Life Technologies) diluted 1:100 in EnVision reagent (Dako). Cy5/Cy3-Tyramide-based systems (Perkin Elmer) were used for signal amplification. Nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI). Two TBS/Tween and one TBS wash were performed between each step. Slides were mounted with ProLong™ Gold Antifade Mountant (Invitrogen). Control slides from index arrays created for this specific purpose were included in each staining experiment to ensure reproducibility.

AQUA Analysis. Immunofluorescence was quantified using automated quantitative analysis (AQUA) (1). Fluorescent images of DAPI, Cy3, Cy5 for each TMA spot were collected. Image analysis was carried out using the AQUA analysis software (Navigate

Biopharma Inc.), which generates an AQUA score for each compartment (tumor/stroma) by dividing the sum of target pixel intensities by the area of the compartment in which the target is measured. AQUA scores were normalized to the exposure time and bit depth at which the images were captured, allowing scores collected at different times to be directly comparable. Specimens with less than 5% tumor area per region of interest are not included in AQUA analysis for not being representative of the corresponding tumor specimen.

Proteomics analysis

Phosphoproteomics workflow was carried out as described previously with modifications (3).

Lysis and protein extraction. 2-3 x 10^7 cells from each cell line (INS-1, Jurkat, MCF-7, OVCAR-3) was lysed using 1 ml 1x Cytobuster reagent (Sigma) supplemented with protease inhibitor (cOmplete, Roche), phosphatase inhibitor (Phosphatase Inhibitor Cocktail 1, Sigma), 14.3 mM β-mercaptoethanol, 50 mM NaF, and 1 mM NaVO4, incubated for 45 minutes on ice, and sonicated (30s ON/ 30s OFF for 4 cycles at 55% amplitude). Next, the proteins were precipitated using methanol/chloroform extraction and vacuum dried.

Digestion. 5 mg of dried protein were solubilized by sonication with a 2.5% solution of ALS-110 (Protea) in 50 mM Tris-HCl pH=8.5 with 5 mM EDTA, and 50 mM DTT. Samples were diluted (1:3) with 0.1 M Tris-HCl pH=8.5, incubated at 55 °C for 35 minutes to reduce cysteines, cooled down to RT and alkylated with iodoacetamide for 30 minutes in the dark. Samples were then quenched with the addition of excess DTT and digested with LysC endoproteinase (Wako) at 1:100 enzyme to protein ratio for 4 hours at 37 °C. Reactions were next diluted under 0.025% ALS-110 (1:100) and Tris-HCl pH=8.5 and $CaCl_2$ were added to 0.1 M and 5 mM respectively. Trypsin (Promega) was then added at 1:50 enzyme to protein ratio and proteins left to digest for 16 hours at 37 °C under rotation. ALS-110 was finally cleaved with the addition of 20% trifluoroacetic acid (TFA) until pH< 3 and samples were incubated at 25 °C for 15 minutes. Peptides were desalted using Sep-Pak SPE cartridges (Waters). Column elution was performed in 80% acetonitrile (ACN) / 0.1% TFA and peptides were dried by centrifugal vacuum.

Titanium dioxide (TiO_2) Enrichment. 1 mg peptides from each sample resuspended in 50% ACN / 2 M lactic acid and incubated with TiO_2 beads (GL Sciences) with a 4:1 TiO_2 to peptide ratio for 2 hours at 25 °C under rotation. Samples were then spun down to separate phosphopeptides bound to beads and flow through was collected as the unenriched peptide fraction. Beads were washed 3 times with 50 % ACN / 0.1 % TFA and wash fluid was added to flow through fractions. Enriched phosphopeptides were then sequentially eluted with 0.2 M Sodium Phosphate pH=7.8 (2x), 5% NH_4OH (1x) and 5% pyrrolidine (1x). All elutions were combined and 20% TFA was added until pH< 3. Phosphopeptides were then desalted with C18 Microspin Columns (The Nest Group). Unenriched peptide fractions were desalted using Sep-Pak C18 Light cartridges (Waters). All peptides were eluted in 80% ACN / 0.1% TFA, dried by centrifugal vacuum, and reconstituted for LC-MS.

Mass spectrometry. LC-MS/MS was performed using an ACQUITY UPLC M-Class (Waters) paired with a Q Exactive Plus (Thermo). Approximately 3-4 ug of enriched phosphopeptides and 2 ug of unenriched peptides were run for each of the samples with at least two blank runs in between to avoid peptide carryover. Peptides were separated on a 65-cm-long, 75-µm-internal-diameter PicoFrit column (New Objective) packed in-house to a length of 50 cm with 1.9 µm ReproSil-Pur 120 Å C18-AQ (Dr. Maisch) using methanol as the packing solvent, on a non-linear 120 min gradient from 1% ACN / 0.1% formic acid to 90% ACN / 0.1% formic acid with a flowrate of 250 nl/min.

Bioinformatics. Mass spectra were searched using Maxquant version 1.6.2.6 (4) with Acetyl (N-Term), Oxidation (M) and Phosphorylation (S/T/Y) as variable modifications, with up to three missed trypsin cleavages, a 7 amino acid minimum length, and 1% false discovery rate (FDR) against a custom database containing all annotated pyruvate kinase proteins (Uniprot).

Protein synthesis and purification

Plasmids and strains. pET28a-LIC bacterial expression plasmid containing the N-term 6xHis tagged WT Human PKM2 sequence (Addgene #25360) was a gift from Dr. Sriram Subramaniam, NCI, NIH. DNA sequences encoding either the full-length N-term 6xHis tagged Human PKM2 or the truncated S37 PKM2 epitope (28-46) including a S37TAG

mutation were assembled into pCRT7/NT-TOPO bacterial expression plasmid (Addgene #73446) at NdeI/SacI and KpnI/HindIII cloning sites respectively. pET28a-LIC-PKM2 plasmid was transformed into *E. coli* BL21(DE3) cells (Invitrogen). pCRT7/NT-TOPO transformed into genomically recoded *E. coli* (C321.ΔA) (Addgene #68306) cells along with SepOTSλ plasmid for phosphoprotein production (Addgene #68292) (5,6).

Expression and purification. For the PKM2 WT protein, expression was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.7-0.8 OD600 for 18 hours at 20 ^{o}C. Bacterial cells were lysed in 50 mM Tris-HCl pH=7.4, 300 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA buffer supplemented with 1 mg/ml lysozyme (Sigma), and protease inhibitor (cOmplete, Roche). Recombinant protein was purified using a NiNTA column (Qiagen) (elution with 250 mM imidazole), followed by size exclusion chromatography (Superdex 200 10/300 GL, GE Healthcare) with 50 mM Tris-HCl pH=7.4, 150 mM NaCl buffer containing 1mM DTT and 5% glycerol. Expression and purification of full-length PKM2pS37 protein and GST-fused truncated PKM2pS37 peptide were performed as described before with modifications (7). In brief, expression was induced with 0.2% arabinose at 0.8-0.9 OD600 for 20 hours at 30 ^{o}C. For the full-length PKM2pS37 protein, bacterial cells were lysed in 20 mM Tris-HCl pH=7.2, 500 mM NaCl, 1 mM Tris (2-carboxyethyl) phosphine (TCEP), 50 mM NaF, 1 mM $NaVO_4$ buffer supplemented with 1 mg/ml lysozyme (Sigma), and protease inhibitor (cOmplete, Roche). Recombinant protein was purified on a HisTrap HP column (GE Healthcare) using continuous imidazole gradient (10-500 mM), followed by size exclusion chromatography on a Superdex 200 10/300 GL (GE Healthcare) with 20 mM Tris-HCl pH=7.2, 100 mM NaCl buffer containing 0.5 mM DTT and 5% glycerol. For the GST-fused PKM2pS37 truncated peptide bacterial cells were lysed in 50 mM Tris-HCl pH=7.4, 500 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 50 mM NaF, 1 mM $NaVO_4$, 10% glycerol buffer supplemented with 1 mg/ml lysozyme (Sigma), and protease inhibitor (cOmplete, Roche). Recombinant protein was purified using a NiNTA column (Qiagen) (elution with 250 mM imidazole), followed by HiCap GST resin (Qiagen) (elution with 50 mM reduced glutathione pH=8.0). When needed GST was cleaved by PreScission protease (GE Healthcare) at 4 °C under rotation for 16 hours and peptides were separated using a 30 kDa Amicon filter unit (Sigma) according to

manufacturer's protocol. All proteins and peptides were buffer exchanged into 50 mM Tris-HCl pH=7.4, 150 mM NaCl, and 20% glycerol for storage at -20 °C.

For peptide competition assay purified IgG against PKM2pS37 were incubated with a 5-fold excess of GST-fused PKM2pS37 truncated peptide (EHMCRLDIDpSPPITARNTG) mimicking the PKM2pS37 epitope.

In vitro CDK Kinase assay

Kinase reactions and LC-MS/MS analysis were performed as described previously (8). In brief, activated CDKs 1, 2, 5, and 6 (Signal Chem) were added in 50 ul of reaction mixture, containing 1x Kinase Assay Buffer I (Signal Chem), 10 ug of purified human PKM2 protein and 50 uM ATP to the following final concentrations, 1.6 ng/ul CDK1, 1 ng/ul CDK2, 2 ng/ul CDK5, 4 ng/ul CDK6. Reactions were incubated for 30 minutes at 30 °C, desalted using C18 Ultramicrospin columns (The Nest Group) and vacuum dried. Subsequently, samples were digested with trypsin (Promega) and desalted. 1/5th of the sample was directly reconstituted for mass spectrometry (unenriched fraction) and the remaining sample was enriched for phosphopeptides using TiO_2 (enriched fraction). Samples were then separated using a NanoAquity HPLC (waters) on a non-linear 90 min gradient from 5% ACN 0.1% formic acid to 95% ACN 0.1% formic acid and analyzed with a LTQ Orbitrap Velos (Thermo).

Cell viability assays

Cell viability assays were performed using an alamarBlue assay (Bio-Rad) according to manufacturer's instructions. In brief, 0.5 x 10^4 cells were seeded in 80 ul complete medium per well (96-well solid black plates, Corning). 20 ul of each treatment mixture [TEPP-46 (10 uM) or TEPP-46 (10 uM) / Dinaciclib (100 nM) combination] was added after 4 hours and 10 ul of alamarBlue reagent after additional 4 or 28 hours. Measurements were taken after 24 and 48 hours of treatment on a VICTOR Multilabel Plate Reader (PerkinElmer) using an exposure time of 0.1s and 535 nm excitation / 590 nm emission filters for fluorescence.

RealTime-Glo Annexin V Apoptosis and Necrosis assay

Cell apoptosis and necrosis was quantified using RealTime-Glo Annexin V Apoptosis and Necrosis detection assays (Promega). 10^4 cells were seeded in 50 ul complete medium per well (96-well solid white plates, Corning) in presence or absence of drugs or drug combinations mixed after 4 hours with equal volume of detection mixture and recorded for luminescence and fluorescence for additional 40 hours. Measurements were performed on a VICTOR Multilabel Plate Reader (PerkinElmer) using an exposure time of 1s for both luminescence and fluorescence and 485 nm excitation / 535 nm emission filters for fluorescence.

Clonogenic assay

0.5×10^4 cells were seeded in 2 ml of the appropriate complete medium in 6-well plates. Dinaciclib (100 nM) and TEPP-46 (10 uM) were added after 24 hours alone or in combination. Cells subjected to continuous treatment for 2 weeks. Subsequently, stained with 40% Methanol, 10% glacial acetic acid, 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad) and washed with 1x DPBS until complete reduction of background staining.

Supplementary Figures

Figure S1

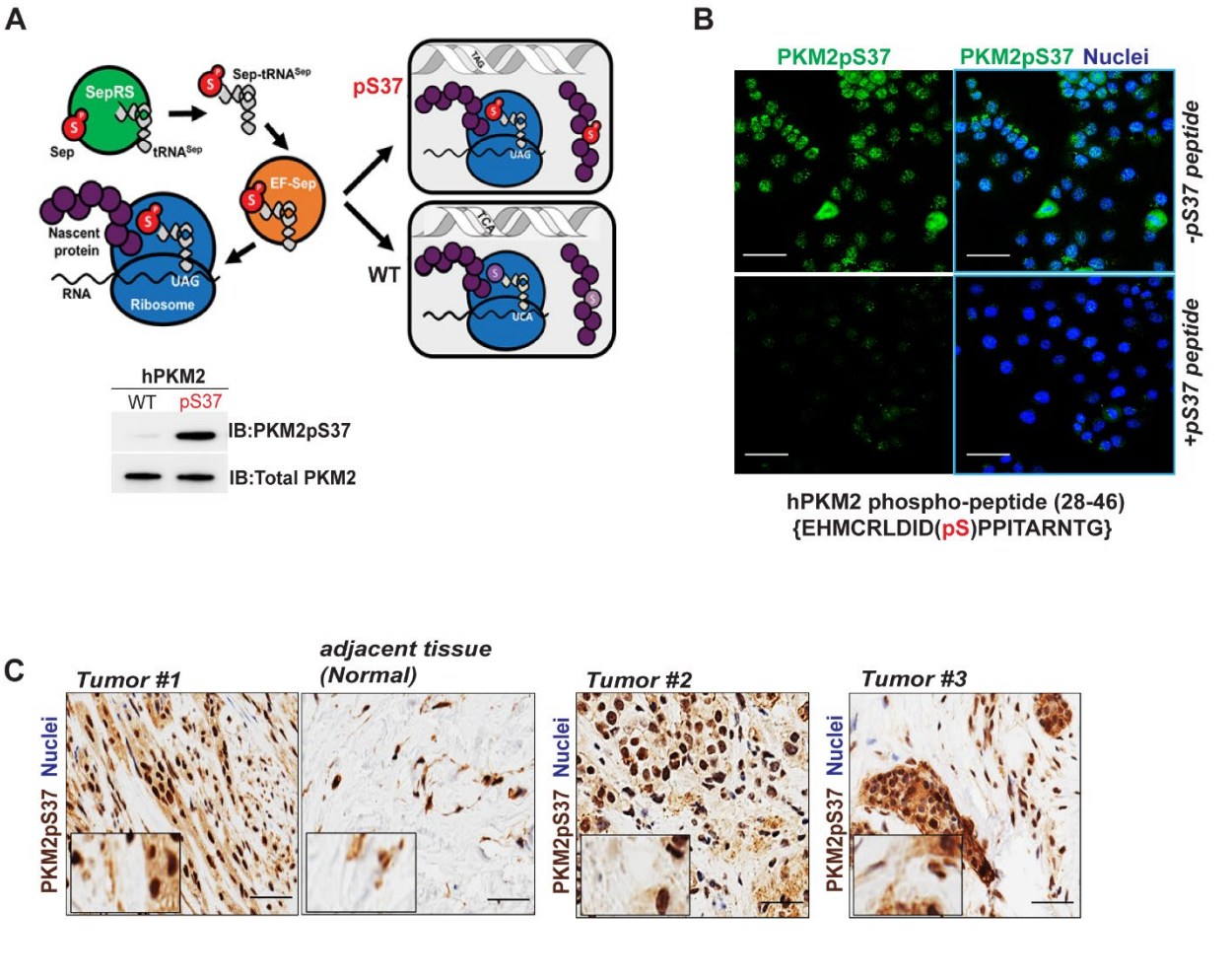

Figure S1: Validation of the custom-made PKM2pS37 phospho-specific antibody. A) (Upper panel) Expression of PKM2 proteins authentically phosphorylated at serine 37 (pS37) or non-phosphorylated (WT) using our novel biotechnology platform to produce full length human proteins and peptides in an engineered bacterial system as described before (5,6,8). (Lower panel) The custom-made phospho-specific antibody recognizes only the pS37 epitope of the authentically phosphorylated human PKM2 protein. B) Peptide competition experiment for further validation of the phospho-specific antibody. The purified IgG were preincubated or not with the PKM2pS37 phospho-peptide prior to immunofluorescence analysis. PKMpS37 staining is dramatically reduced after the addition of the phospho-peptide mimicking the authentic PKM2 phosphorylated epitope

in OVCAR-3 ovarian cancer cells. Nuclear staining, Hoechst 33342 (blue); Scale bar, 60 μm. C) Immunohistochemical analysis of PKM2pS37 expression *in vivo*. TNBC patient tumors and normal tissue adjacent to the tumor were co-stained with hematoxylin (blue) and PKM2pS37 (brown); Scale bar, 100 μm.

Figure S2

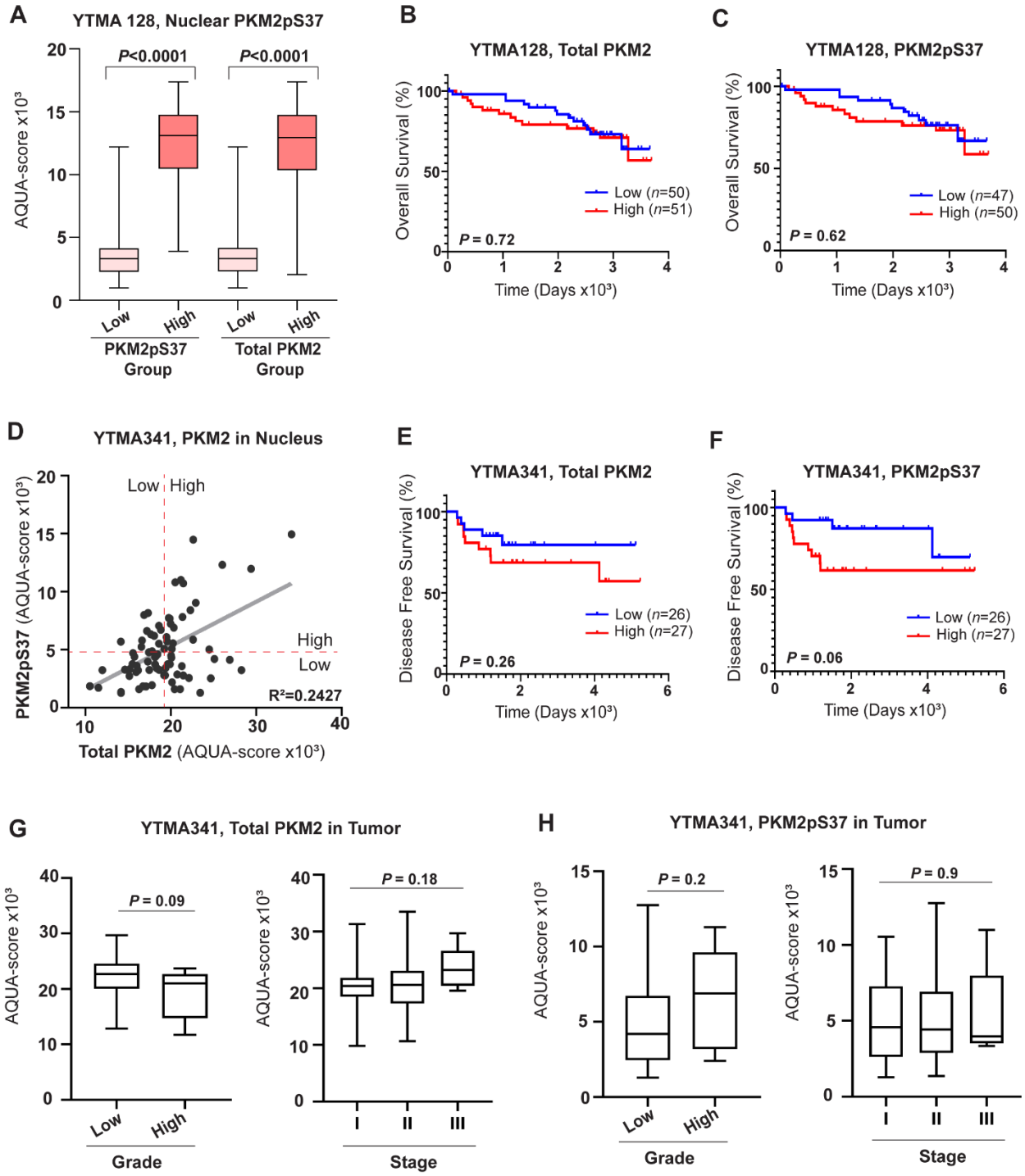

Figure S2: AQUA analysis of PKM2pS37 and total PKM2 staining of YTMA128 and YTMA341 patient cohorts. A) Comparison of nuclear PKM2pS37 expression in tumor

between Low- (*n* = 45) and High- (*n* = 48) expressing PKM2pS37 or total PKM2 patient groups using AQUA scores obtained from YTMA128 analysis. Significance, one-way ANOVA (Tukey's HSD). B), C) Kaplan-Meier curves for overall survival with respect to total PKM2 and PKM2pS37 expression. Significance, Log-rank (Mantel-Cox) test. D) Correlation between AQUA (QIF) scores for nuclear PKM2pS37 and total PKM2 quantified in tumor mask on each YTMA341 spots. Dashed red lines correspond to median cut-points used for patient Low/High grouping. E), F) Kaplan-Meier curves for disease free survival with respect to total PKM2 and PKM2pS37 expression in TNBC. Significance, Log-rank (Mantel-Cox) test. G), H) Association of PKM2pS37 and total PKM2 expression with patient stage and grade in TNBC. Significance, unpaired *t* test.

Figure S3

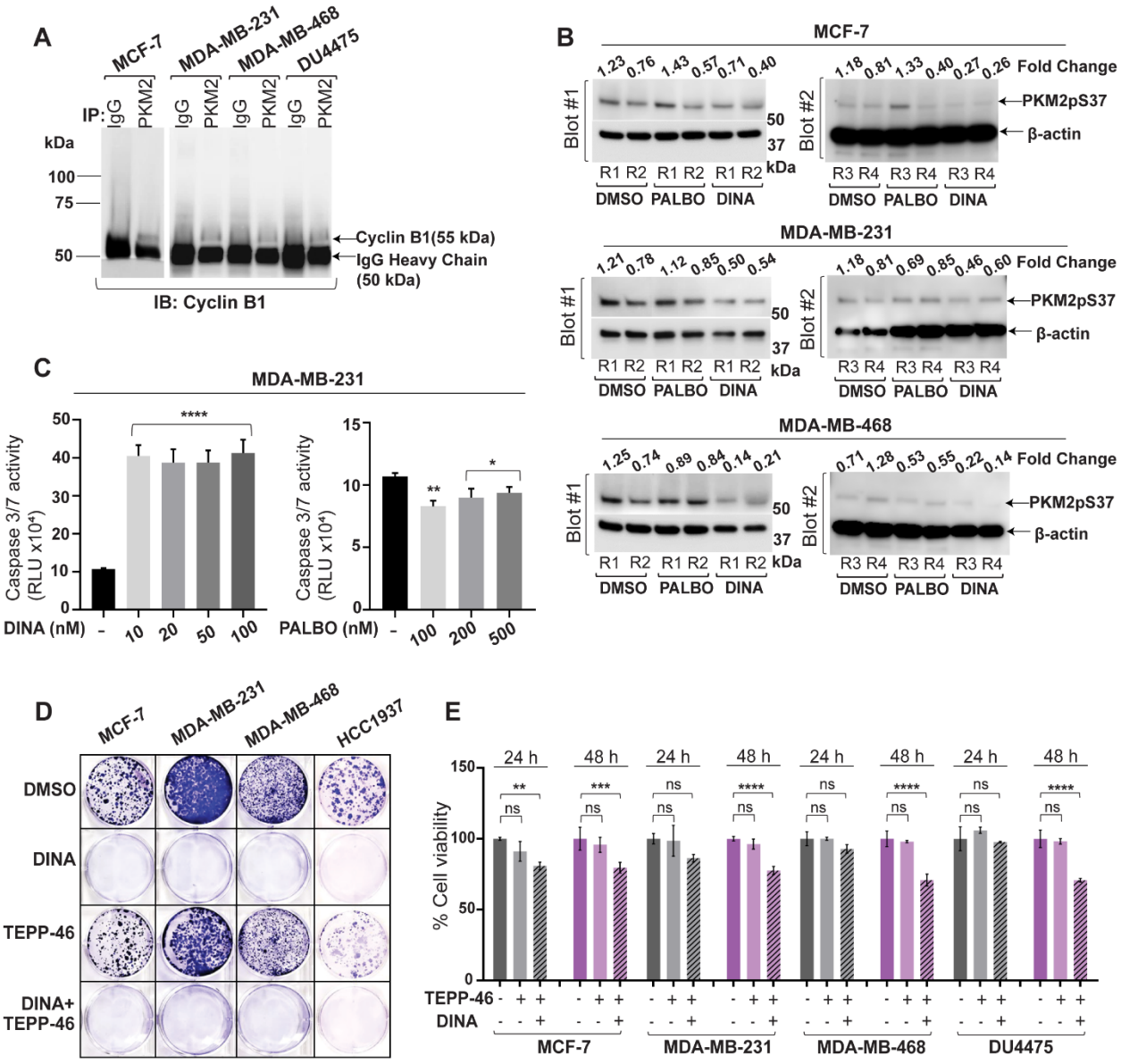

Figure S3: Dinaciclib presents a strong effect on cell growth and clonogenicity by reducing PKM2pS37 in breast cancer cells. A) Immunoprecipitation of PKM2 and CDK1/cyclinB1 complexes in whole cell lysates with anti–PKM2 or normal IgG antibodies. Original blot of cyclin B1 detection presented in Figure 3B. B) Immunoblot analysis of PKM2pS37 in whole cell lysates after treatment with Dinaciclib (1 uM) or Palbociclib (1 uM) for 20 hours. Quantification of original blots presented in Figure 3C. C) Apoptosis in MDA-MB-231 cells treated with Dinaciclib or Palbociclib for 20 hours. Bars, mean values, *n* = 3; error bars, SD; one-way ANOVA (Tukey's HSD), **P* < 0.05, ***P* = 0.002, *****P* < 0.0001; ns,

nonsignificant. D) Long-term clonogenic assay of breast cancer cells treated with Dinaciclib (100 nM) or TEPP-46 (5 uM) or the combination. Formed colonies were stained after 2 weeks of continuous treatment. E) AlamarBlue assay for cell viability assessment of breast cancer cells treated with TEPP-46 (10 uM) and Dinaciclib (100 nM) for 24 or 48 hours. Bars, mean values of cell growth proportions, n = 3; error bars, SD. One-way ANOVA (Tukey's HSD), $^{**}P$ = 0.002, $^{***}P$ = 0.0002, $^{****}P$ < 0.0001; ns, nonsignificant.

Figure S4

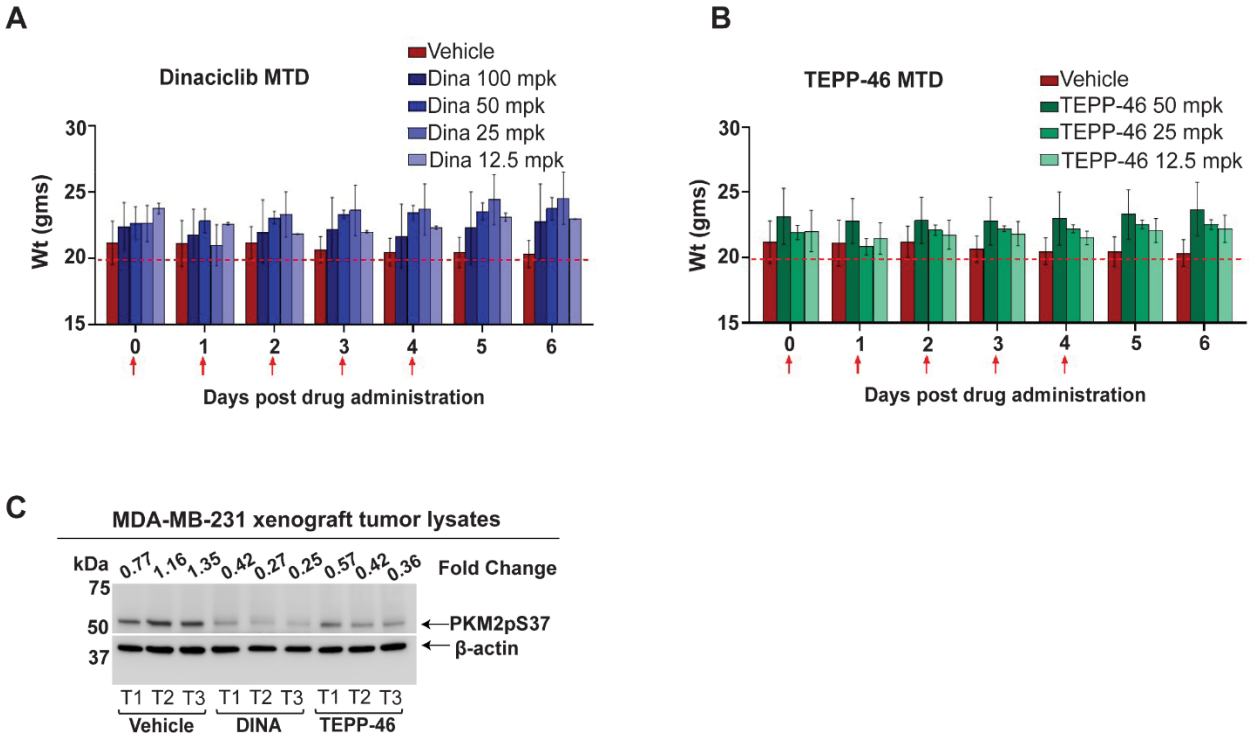

Figure S4: Both Dinaciclib and TEPP-46 are tolerated in R2G2 mice. Maximum tolerated dose (MTD) of either Dinaciclib or TEPP-46 was determined by intraperitoneal administration once daily at different doses and body weights of mice were measured. Red arrows indicate the days of drug administration. Bars, mean values, *n* = 2; error bars, SD. C) Immunoblot analysis of dissected tumors at treatment end point. Quantification of original blots presented in Figure 6C.

Figure S5

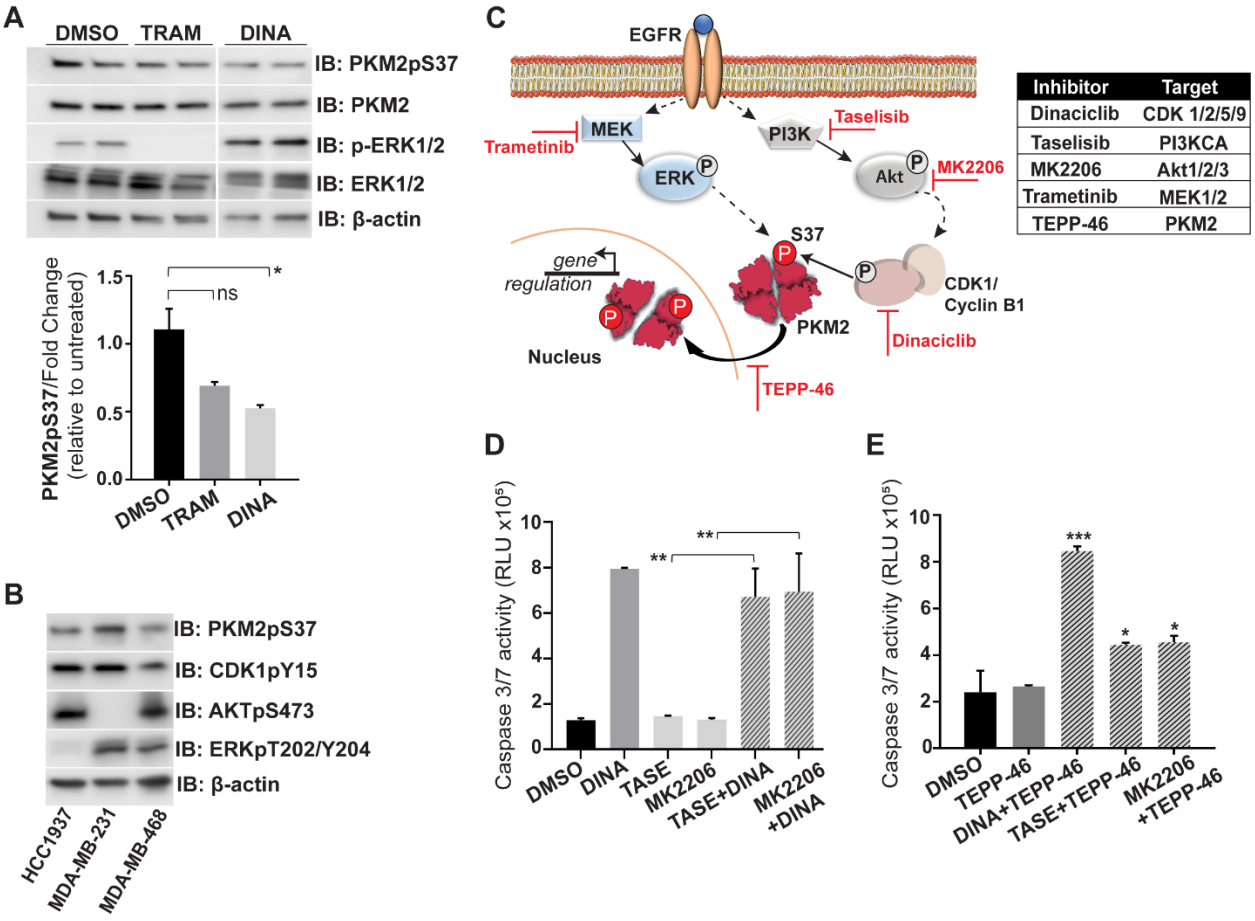

Figure S5: Dinaciclib and TEPP-46 combination treatment against EGFR/CDK/PKM2 axis. A) Immunoblot analysis of MDA-MB-231 cells upon Trametinib (0.1 uM) or Dinaciclib (1 uM) treatment. Bars, mean values, *n* = 2; error bars, SD; One-way ANOVA (Tukey's HSD), **P* = 0.03; ns, nonsignificant. B) Immunoblot analysis showing the phosphorylation status of CDK1, AKT and ERK kinases in TNBC cells. C) Schematic illustration of the hypothesized EGFR/CDK/PKM2 axis which is responsible for PKM2 phosphorylation and nuclear transportation in TNBC tumor model (left panel). The list of compounds that tested and target central components of EGFR downstream signaling including the newly identified CDK/PKM2 node (right panel). D) Apoptosis detection in MDA-MB-231 cells treated with Dinaciclib or PI3K/Akt inhibitors for 24 hours. Bars, mean values *n* = 2; error bars, SD; one-way ANOVA (Tukey's HSD), ***P* = 0.002. E) Apoptosis detection in MDA-MB-231 cells treated with TEPP-46/Dinaciclib or TEPP-46/PI3K/Akt small molecule

combinations for 24 hours. Bars, mean values $n = 2$; error bars, SD; one-way ANOVA (Tukey's HSD), *$P < 0.05$, ***$P = 0.0002$.

Figure S6

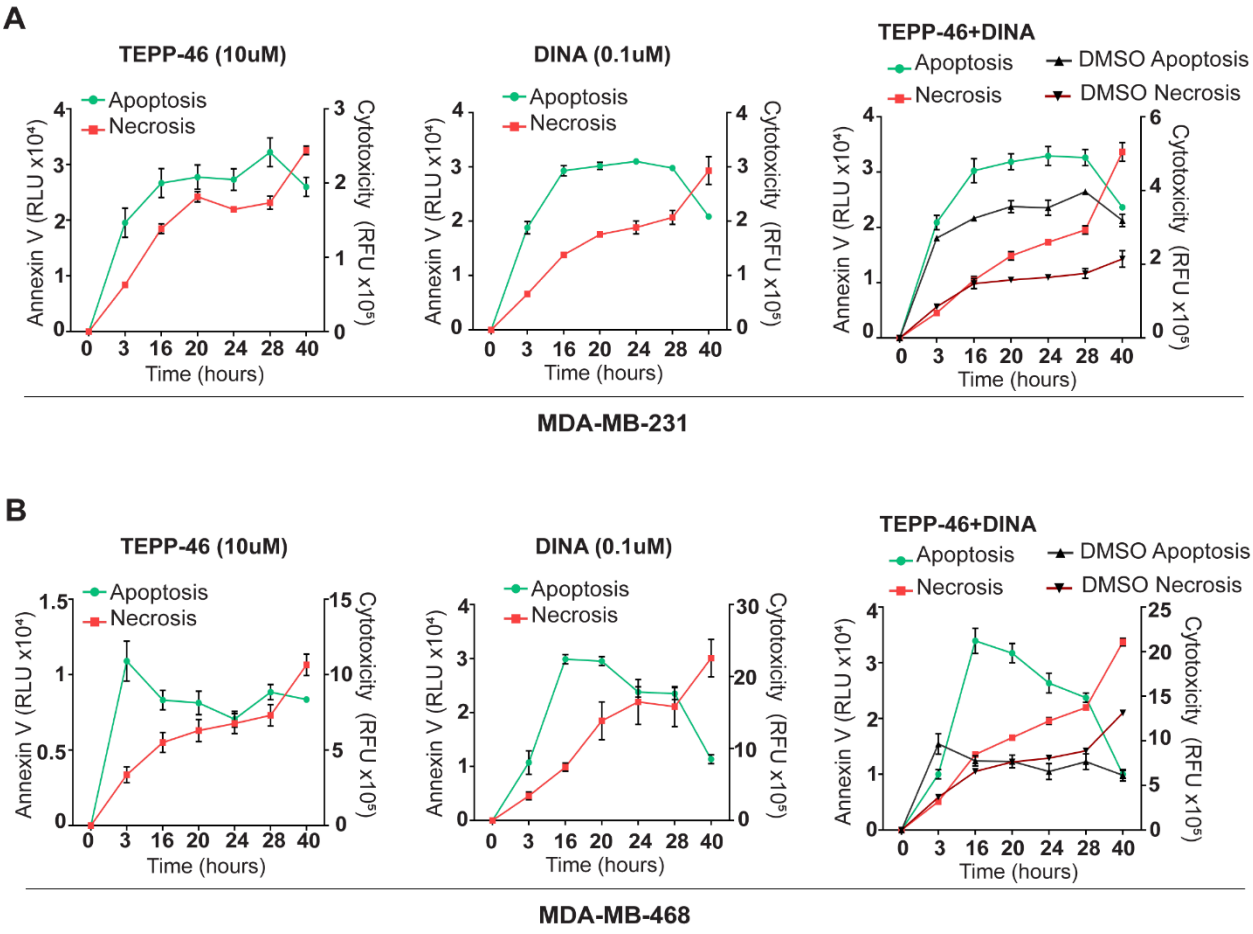

Figure S6: Analysis of the synergistic apoptotic effect of TEPP-46/Dinaciclib combination in TNBC cell lines. Annexin V real-time assay for apoptosis detection using luminescence in A) MDA-MB-231 or B) MDA-MB-468 cells treated with Dinaciclib or TEPP-46 or the combination for 40 hours. Cytotoxicity was measured by fluorescence for same 40 hours in real time. Bars, mean values, *n* = 3.

Supplementary Tables

Table S1: (Microsoft Excel format). PKM2 gene expression analysis in TCGA Pan-Cancer database.

Table S2: (Microsoft Excel format). MaxQuant analysis of the PKM2 phosphopeptides detected in cell lines.

Table S3

YTMA	128		341	
Characteristic	N	%	N	%
Age				
Median	57	-	51	-
Range	30-89	-	29-81	-
Gender				
Male	0	0	0	0
Female	101	100	94	100
Histotype				
ER positive	78	77.2	0	0
PR positive	61	60.4	0	0
HER2 positive	59	58.4	0	0
Triple negative	8	7.9	94	100
Stage (at diagnosis)				
I	52	51.5	24	25.5
II	35	34.6	32	34.0
III	10	9.9	11	11.7
IV	4	3.9	2	2.1
Surgery				
Yes	101	100	92	97.9
	0	0	2	2.1
Adjuvant radiotherapy				
Yes	62	61.4	34	36.2
No	32	31.7	22	23.4
Adjuvant chemotherapy				
Yes	86	85.1	46	48.9
No	10	9.9	7	7.4
Disease recurrence				
Yes	12	11.9	18	19.1
No	83	82.2	45	47.9
Dead of disease				
Yes	27	26.7	15	16.0
No	74	73.3	48	51.1

Table S3: Tumor microarray patient characteristics.

Table S4: (Microsoft Excel format). AQUA analysis of YTMA128 and YTMA341 breast cancer patient cohorts.

Table S5: (Microsoft Excel format). CDK/cyclin gene expression analysis in TCGA BRCA database.

Table S6: (Microsoft Excel format). MaxQuant analysis of the PKM2 phosphopeptides detected after *in vitro* treatment with activated CDKs.

Supplementary References

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