Supporting Online Material

Materials and Methods

Cell culture, construction of PKM1 and PKM2 expressing cells, and PKM2 shRNA knockdown. H1299, A549, MCF7, and HEK293 cells were obtained from ATCC and cultured in RPMI or DMEM based media according to ATCC protocols. FL5.12 cells were cultured in RPMI based media supplemented with interleukin 3. Flag-tagged mouse PKM1 or PKM2 cDNA was stably expressed in the indicated cells and an shRNA that caused efficient knockdown of endogenous human PKM2 was introduced by lentiviral infection as described previously (*S1*). After selection for cells containing both the PKM1 or PKM2 cDNA and the shRNA to knockdown endogenous PKM2, cells were lysed in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM DTT, and 4 μg/mL each of pepstatin, leupeptin and aprotinin prior to determination of protein concentration by Bradford assay (BioRad) and pyruvate kinase activity as described below. The same shRNAs were used for PKM2-knockdown experiments as described previously (*S1*).

Recombinant proteins and enzyme activity. Human PKM1, PKM2 and PGAM1 were cloned into pET28a, expressed as N terminal His_6 fusion proteins in *E.coli*, and purified using Ni-NTA resin as described previously (*S2, S3*). Pyruvate kinase activity was measured using an assay coupled to lactate dehydrogenate following NADH fluorescence in a Victor^3 plate reader (Wallac) as described previously (*S2, S3*). Reaction conditions used 10-40 ng of recombinant protein or 2 μg of cell lysates (or equivalent lysates fractions) with 0.6 mM ADP and 0.5 mM PEP. Where indicated FBP was added at a concentration of 200 μM. Enolase enzyme activity was determined by using 0.5 mM 2PG as the substrate, omitting PEP, and adding 5 units of rabbit muscle pyruvate kinase (Sigma) to the same reaction conditions described for pyruvate kinase.

Expression of epitope tagged PGAM1, immunoprecipitation, and limited proteolysis. Human PGAM1 cDNA was cloned into pcDNA3 (Invitrogen) with an in frame N-terminal Flag-tag or p3XFLAG-CMV-14 (Sigma) with an in frame C-terminal Flag-tag, and a mutation of histidine 11 to asparagines was introduced into the N-Flag-PGAM1 cDNA construct using standard molecular biology techniques. These constructs were transfected into HEK293 cells using polyethylenimine (PEI) (Polyscience) according to standard protocols. Lysates were prepared 2 days after transient transfection. Flag-tagged PGAM1 was immunoprecipitated using ANTI-FLAG M2 agarose (Sigma) according to standard protocols. For limited proteolysis experiments, endoproteinase Glu-C from *S. aureus* V8 was added at a concentration of 10 mg/mL and incubated for 30 minutes at 30^o C prior to SDS-PAGE or acetone precipitation for 2D gel analysis.

Preparation of ^{32}P-labeled PEP. 600 μCi of 10 mCi/mL of ^{32}P-γ-ATP (~2.2 μM final concentration) was incubated with 800 mM pyruvate, 50 mM Tris (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 5 units of rabbit muscle pyruvate kinase (Sigma) at 37^o C for 1 hour. ^{32}P-labeled PEP was immediately separated from ^{32}P-γ-ATP using anion exchange chromatography according to a modification of previously published method (*S4*) using a Vivapure Q column (Sartorius). Bound product was washed with reaction buffer, and the ^{32}P-labeled PEP eluted stepwise using 0.3 mM and 0.6 mM triethylammonium bicarbonate (TEAB) (pH 8.5). No difference in ^{32}P-labeled PEP activity was noted in either the 0.3 mM or 0.6 mM fraction and they were used interchangeably as a source of ^{32}P-labeled PEP. Determining ATP and PEP concentrations in each fraction confirmed the separation of PEP from ATP using this technique. A modification of the lactate dehydrogenase coupled assay for pyruvate kinase activity described above was used to determine PEP concentration, and a luciferase assay

described previously (Promega) (*S5*) was used to determine ATP concentration. Separation of ^{32}P-labeled PEP from ^{32}P-γ-ATP and $^{32}P_i$ was also confirmed by thin layer chromatography using PEI cellulose and 0.25 M ammonium bicarbonate as the mobile phase as described previously (*S4*).

Preparation of ^{18}O-labeled PEP. ^{18}O-labeled PEP was prepared as described previously (*S6*). Briefly, PEP was dissolved in $H_2{}^{18}O$ (80 mg/mL) with 1N HCL and heated to 98° C for 5 minutes and immediately transferred to ice. Concentrated KOH was titrated to bring the sample to pH 7.0, the ^{18}O-labeled PEP recovered using ion exchange chromatography, and the concentration determined enzymatically as described above for ^{32}P-labeled PEP. Using this method ^{18}O preferentially exchanges with the phosphate oxygens on PEP (*S6*). The efficiency of PEP ^{18}O-labeling was confirmed using ion trap mass spectrometry in MS-only negative ion mode.

ATP- and PEP-dependent phosphorylation assay. Intact cells were collected, washed in PBS, and pelleted prior to resuspension in a small volume of hypotonic lysis buffer (20 mM HEPES (pH 7.0), 5 mM KCl, 1 mM $MgCl_2$, 5 mM DTT, and 4 μg/mL each of pepstatin, leupeptin and aprotinin). After a 10 minute incubation on ice, the cells were passed 3 times through a 26g needle and centrifuged at 15,000 x g for 5 minutes. The resulting supernatant was used as a hypotonic lysate in phosphorylation reactions or processed further. Protein concentrations of the lysates or further processed fractions were determined by Bradford assay (BioRad). For phosphorylation reactions, 10-30 μg (or the fraction equivalent) was incubated with 50 mM Tris (pH 7.5), 50 mM KCl, 15 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT. ~2-10 μCi ^{32}P-γ-ATP or ^{32}P-PEP was included in the reaction, as was cold ATP or PEP at the concentration indicated for each experiment. Where indicated, non-radioactive competitor purine nucleotides were added to a final concentration of 1 mM, NaF was added to a final concentration of 1 mM, or 3 μL of rabbit muscle enolase (Sigma) was included in the reaction. Reactions were performed for 10 minutes at 30° C and stopped by the addition of SDS-loading dye. Reactions were analyzed using standard SDS-PAGE and autoradiography. Where indicated, HCl or NaOH was added to reactions (to achieve a pH of 1 or 13 respectively), heated to 65° C for two hours, and then neutralized prior to SDS-PAGE.

Phosphoaminoacid analysis. 'Standard' phosphoaminoacid analysis was performed as described previously (*S7, S8*). Briefly, ^{32}P-γ-ATP- or ^{32}P-PEP-phosphorylation reactions were separated by SDS-PAGE, transferred to PVDF membrane, the desired ^{32}P-labeled protein excised, added to 50 μL 6N HCl, and heated to 100° C for 1 hour. The PVDF was discarded and the sample dried completely prior to resuspension in 1:3:16 mixture of 88% formic acid : glacial acetic acid : water. The entire sample was spotted on a cellulose TLC plate along with phosphoserine, phosphothreonine and phosphotyrosine standards (Sigma), and separated by 2D thin layer electrophoresis. The migration of ^{32}P labeled species within the TLC plate was determined by autoradiography and phosphoaminoacid standards were visualized by ninhydrin staining. For reverse phase TLC phosphoaminoacid analysis (*S9*), samples were prepared as described above except the desired ^{32}P-labeled protein sample on PVDF was added to 50 μL 3M KOH, heated to 100° C for 3 hours, and the sample neutralized with perchloric acid. The sample was centrifuged to remove precipitated KCl and concentrated using a speed vac prior to spotting onto a $RP_{18}F_{254}$ TLC plate. Phosphoserine, phosphothreonine and phosphotyrosine standards (Sigma), along with phosphohistidine and phospholysine standards (see below) were included as indicated and the amino acids separated using a mobile phase of 65:10:16 Ethanol : NH_4OH : water. The locations of ^{32}P-labeled species were determined by autoradiography and phosphoaminoacid standards visualized by ninhydrin staining. Phosphohistidine and phospholysine standards were synthesized as described previously (*S10*). Briefly, triethylamine was added to an aqueous solution of poly-L-histidine or poly-L-lysine until saturated, and phosphoryl chloride added along with NaOH to keep the pH between 9.5 and

12.5. Following incubation on ice for 30 minutes, the reaction was dialyzed against 0.1 N NaOH prior to base hydrolysis using 3 N KOH as described above.

Preparation of C11NP resin. C11NP resin was prepared by washing EAH sepharose 4b (GE Lifesciences) sequentially in water (pH 4.5 with HCl), 0.5 M NaCl, and 0.1 M imidazole (pH 6.0). A 100 mM PEP solution was prepared in 10 mM MES (pH 6.0) and added to 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (Pierce) for a final EDC concentration of 50 mg/mL. The washed EAH sepharose 4b along with imidazole (pH 6.0, final concentration ~60 μM) was immediately added to the PEP/EDC solution and incubated with end-over-end mixing for 8 hours at room temperature. The sepharose beads were then washed 6 times alternating the wash solution between 90 mM MES (pH 5.5), 500 mM NaCl and 100 mM Tris (pH 8.0), 500 mM NaCl. The beads were then washed with hypotonic lysis buffer and stored in a buffer containing 20% ethanol until used.

Fractionation of cell lysates and isolation of PGAM1. Hypotonic cell lysates prepared as described above were centrifuged for 1 hour at 100,000 x g and the resulting supernatant used as an S100 fraction. Lysates were further fractionated by column chromatography using the following resins: C11NP (see above), sulphonic acid (S) (Sartorius), CHT ceramic hydroxyapatite (HAP) (BioRad), diethylamine (DEAE) (Sartorius), and quaternary ammonium (Q) (Sartorius). All resins were equilibrated with the input buffer prior to addition of the S100 or derivative fractions, and eluted stepwise as indicated for each column. The S100 was passed through C11NP resin and then S resin. Proteins in the resulting flow through fractions were labeled with ^{32}P-PEP as described above and applied to CHT ceramic HAP, washed with hypotonic lysis buffer and the 50 mM $NaHPO_4$ fraction that contained the 25kD target protein was collected. This fraction was then diluted 1:1 with hypotonic lysis buffer and applied to a DEAE column, washed with loading buffer and then buffer containing 100 mM NaCl prior to elution with buffer containing 200 mM NaCl. This fraction was diluted 1:4 and applied to a Q column, washed with loading buffer and buffer containing 100 mM NaCl prior to elution with buffer containing 350 mM NaCl. This final fraction was precipitated by addition of 1 volume of ice-cold acetone and the resulting protein pellet was analyzed by 2D gel electrophoresis followed by autoradiography and/or coomassie staining.

2D gel electrophoresis. Cell/tissue lysates or cell lysate fractions were mixed 1:1 with -20^{o} C acetone and centrifuged at 16,000 x g for 30 minutes to precipitate proteins. The pellet was resuspended in buffer containing 7 M urea, 2 M thiourea, 1.2% CHAPS, 0.2% bromophenol blue, 44 mM DTT and 0.25% ampholytes (Invitrogen). IEF was performed using ZOOM strips (pH range 3-10NL or 4-7) according to the manufacturer's instructions (Invitrogen). Prior to SDS-PAGE, IEF strips were equilibrated sequentially in buffer containing 50 mM Tris (pH 6.8), 6 M urea, 20% glycerol, 2% SDS, 0.1% bromophenol blue and 2% DTT or 2.5% iodoacetamide respectively.

LC/MS/MS identification. Gel separated protein bands were reduced with DTT, alkylated with iodoacetamide and digested at 37°C (pH 8.3) overnight with modified trypsin (Promega). Tryptic peptides were extracted from the gel bands and then analyzed by data-dependent reversed-phase microcapillary tandem mass spectrometry (LC/MS/MS) using a hybrid LTQ - Orbitrap XL-ETD mass spectrometer (Thermo Scientific) operated in positive ion mode. Peptides were injected using an EASY-nLC HPLC (Proxeon Biosystems) at a flow rate of 275 nL/min. A 75 μm (i.d.) x 15 cm (length) x 15μm (tip) PicoFrit microcapillary column (New Objective) was self-packed with Magic C_{18} resin (Michrom Bioresources). The column was equilibrated and peptides were loaded using buffer A (0.1% formic acid/0.9% acetonitrile/99% water) then eluted with a gradient from 5% buffer B (acetonitrile) to 38% B, followed by 95% B for washing. One FT-MS survey scan was followed by five MS/MS ion trap scans using collision induced dissociation (CID) with helium gas. The Sequest algorithm in Proteomics Browser

Software (PBS) (Thermo Scientific) was used for database searching of all MS/MS spectra against the reversed and concatenated Swiss-Prot protein database (UniProt, UK, v. 55.2) with the fixed modification carbamidomethyl (+57.0214) Cys and the differential modifications: oxidation (+15.9949 Da) of Met and deamidation (+0.9840 Da) of Gln and Asn. Peptide sequences were initially accepted if they matched the forward database and passed the following Sequest Browser scoring thresholds: 2+ ions, Xcorr ≥ 1.9, Sf ≥ 0.4, P ≥ 0; 3+ ions, Xcorr ≥ 2.50, Sf ≥ 0.45, P ≥ 0. Peptides with gas phase charges of 1+ and 4+ were generally not accepted as valid due to difficulty of interpretation of such ions. Proteins with consensus scores greater than 1.0 against the forward database with at least two peptides were accepted as valid. After passing the scoring thresholds, MS/MS were then manually inspected to confirm that all **b-** (fragment ions resulting from amide bond breaks from the peptide's N-terminus) and **y-** ions (fragment ions resulting from amide bond breaks from the peptide's C-terminus) aligned with the assigned protein database sequence. False discovery rates were estimated at ≤ 1.5% based on the number of reversed database hits using the above criteria.

Phosphopeptide analysis and identification of the histidine phosphorylated peptide. Phosphotryptic peptide mapping was performed using a modification of a method described previously (*S8*). Lysates prepared from HEK293 cells transiently transfected to express N-terminal Flag-tagged PGAM1 were incubated with ^{32}P-labeled PEP and PGAM1 collected by immunoprecipitation. The immunoprecipitated beads were washed 2x in hypotonic lysis buffer and 2x in 20 mM NH_4HCO_3 (pH 8.3) and the PGAM1 eluted into 20 mM NH_4HCO_3 (pH 8.3) using 3x FLAG peptide (Sigma). The beads were pelleted and the supernatant containing ^{32}P-labeled PGAM was digested with 1 μg trypsin (Promega) at 37° C for 4 hours. The peptide digest was dried completely, resuspended in 10 mg/mL $(NH_4)_2CO_3$ (pH 8.9), and spotted onto a cellulose TLC plate. Peptides were separated using thin layer electrophoresis (10 mg/mL $(NH_4)_2CO_3$ (pH 8.9)) at 1000 kV for 40 minutes followed by thin layer chromatography in the second dimension using a mobile phase of 37.5:25:7.5:30 n-Butanol : pyridine : acetic acid : water. The location of ^{32}P-labeled peptides was determined by autoradiography. To purify the ^{32}P-labeled PGAM1 peptide by HPLC, 100 μg of recombinant PGAM1 was incubated in a reaction with 30 μg of HEK293 hypotonic lysate to generate phosphorylated recombinant PGAM1. The reaction was diluted with NaCl (final concentration 100 mM NaCl) and Ni-NTA agarose used to collect the labeled His_6-tagged PGAM1. The Ni-NTA agarose beads were then washed 2x in 200 mM NaCl, 20 mM Tris (pH 7.5) and 2x in 20 mM NH_4HCO_3 (pH 8.3). The beads were resuspended in 20 mM NH_4HCO_3 (pH 8.3), 1 mM DTT and trypsin added at 0.2 mg/ml. The reaction was mixed with end-over-end shaking at 37° C for 4 hours, the Ni-NTA agarose beads pelleted and the resulting peptide mixture in the supernatant separated by HPLC. Peptides were separated using a 2.1 mm i.d x 15 cm Atlantis dC18 column (Waters) at 0.4 mL/min using 0.1% trifluoroacetic acid as the mobile phase with a linear gradient from 1% to 40% acetonitrile. Peptide peaks were identified by absorbance at 208 nm and the presence of ^{32}P determined by inline scintillation counting. Fractions were collected that corresponded to the peptide peak that was also labeled with ^{32}P, and these fractions were immediately brought to pH >7 through the addition of NH_4HCO_3.

Determination of Phosphohistidine using HCD-LC/MS/MS. The relevant fractions were combined, concentrated using a speed vac and subjected immediately to LC/MS/MS analysis using the LTQ Orbitrap XL-ETD in positive ion data dependent acquisition mode with the higher energy collision dissociation cell (HCD) on the Orbitrap as the detector and scanning down to 50 Da in MS/MS mode. Nitrogen was used as the collision gas at 35% normalized collision energy. HCD outperformed collision induced dissociation (CID) because it produced more useful **y-** series fragment ions and the low mass range in MS/MS was necessary to accurately determine the site of phosphorylation as His11. Electron transfer dissociation (ETD) is not efficient with tryptic peptides with 2+ charges and it does not produce low mass ion s as

fragmentation occurs in the ion trap. After determining the peptide sequence via Swiss-Prot database searching using Sequest and the putative modification site of phospho-His at His11, the MS/MS spectrum was interpreted both manually and with GraphMod software in PBS (see Fig. S6) to ensure that all **b-** and **y-** ions and immonium ions were consistent with His11 phosphorylation. The **b-** series ions are all shifted by 79.97 Da, indicative of phosphorylation. The $\mathbf{b_1}$-$\mathbf{b_3}$ ions contain a phosphate group in the peptide region where Ser, Thr, and Tyr residues are not present. In addition, the entire detected **y-** ion series does not contain a phosphate group, and the **b-** series ions $\mathbf{b_2}$-$\mathbf{b_5}$ show phosphate losses, consistent with the site of phosphorylation on His at position 1 of the peptide. Additionally, the $\mathbf{a_1}$ ion and/or pHis immonium ion is present at *m/z* 190.04. In addition, Mascot was used to statistically confirm the His11 phosphorylation and the hit with phosphorylation at His received a Mascot score of 44 and an Expectation value of 0.078 against the reversed IPI_HUMAN protein database compared to a score of 27.2 and an Expectation value of 3.8 for the unlikely Ser14 possibility. An analogous procedure using ^{18}O-labeled PEP (see below) in the presence of normal isotopic ATP was performed to label PGAM1 and purify the H11 containing peptide by HPLC. The efficiency of PEP-^{18}O-labeling was determined using a microcapillary liquid chromatography with the high resolution and high mass accuracy LTQ Orbitrap XL mass spectrometer in FT-MS mode at 30,000 resolution calibrated to obtain sub-2ppm mass accuracy with the aid of a lock mass using the m/z 445.12002 background ion.

Detection of enolase and PGAM activity using mass spectrometry. To measure enolase activity, interconversion of 2PG and PEP was determined using triple quadrupole mass spectrometry. Relative 2PG and PEP concentrations were measured following a 10 minute reaction at 30^{o} C in the presence or absence of exogenously added rabbit muscle enolase (Sigma) or 1 mM NaF. Reactions were stopped with the addition of cold methanol (final concentration 80%), dried completely, and then resuspended in 20 µL of LC/MS grade water (OmniSolv). The relative 2PG and PEP amounts in each sample were determined by a selected reaction monitoring (SRM) experiment. 7µL were analyzed using a 5500 QTRAP triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) coupled to a Prominence UFLC HPLC system (Shimadzu) via single reaction monitoring (SRM) (ESI voltage –4500V) in negative ion mode. The dwell time was 5 ms per SRM transition. The Q1/Q3 transition for 2PG was 185.0 Da/97.0 Da with collision energy of -17.0V and the Q1/Q3 transition for PEP was 167.0 Da/79.0 Da using collision energy of -22.0V. Scheduled SRMs were not utilized. Samples were delivered to the MS via normal phase chromatography using a 2.0 mm i.d x 10 cm Luna NH_2 HILIC column (Phenomenex) at 275 µL/min. Gradients were run starting from 85% buffer B (HPLC grade acetonitrile) to 42% B from 0-5 minutes; 42% B to 0% B from 5-16 minutes; 0% B was held from 16-24 minutes; 0% B to 85% B from 24-25 minutes; 85% B was held for 7 minutes to re-equilibrate the column. Buffer A was comprised of 20 mM ammonium hydroxide/20 mM ammonium acetate in 95:5 water : acetonitrile. Metabolomic measurements were performed in triplicate. Peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v1.1 software (Applied Biosystems). PGAM1 activity was measured using an analogous method where isolated PGAM1 was incubated with 3PG, reactions stopped with methanol, and 2,3-BPG levels determined by a selected reaction monitoring (SRM) experiment as above.

Measurement of 2,3-BPG and PEP in cells. Equal numbers of H1299 cells engineered to express PKM1 or PKM2 were placed in fresh media for 2 hours prior to a 10 minute treatment with pervanadate as described previously (*S2*). Media was removed from control and pervanadate stimulated cells and metabolome quenching and extraction were conducted as described previously (*S11*). Briefly, extraction solvent (80% methanol, 20% water) at −75 °C was added directly to the tissue culture dish to quench metabolic activity and extract metabolites. Cells were then scraped from the dish and the resulting cell

suspension was completely dried under nitrogen gas and re-dissolved in 50% methanol (50% water). Cell extracts were then analyzed by reversed-phase liquid chromatography coupled by negative mode electrospray ionization (ESI) to a Thermo TSQ Quantum triple quadrupole mass spectrometers operating in single reaction monitoring (SRM) mode as previously described (*S12*). These methods separate many but not all isomers. In the case of BPG, 1,3-BPG standard is not available, but the observed endogenous signal matches exactly the retention time of a 2,3-BPG standard.

Western blotting of cell lines and mouse tumors. Western blot analysis was performed using standard procedures as indicated. The following antibodies were used: isoform specific rabbit anti-PKM1 or anti-PKM2 antibodies (*S1*), goat anti-pyruvate kinase M (Abcam), goat anti-PGAM (Novus), H-300 N-terminus rabbit anti-enolase (Santa Cruz), C-19 C-terminus goat anti-enolase (Santa Cruz), rabbit anti-nm23 (Abcam), and rabbit anti-GAPDH (Cell Signaling). Western blots were developed using appropriate HRP-linked secondary antibodies and chemiluminescence. For 2D western blot analysis, cell or tissue lysates were prepared, protein concentration determined by Bradford assay (BioRad), and proteins immediately precipitated by the addition of 1:1 -20^{o} C acetone as described above. IEF followed by SDS-PAGE was then performed as described above prior to Western blot using standard protocols. Cell lysates were prepared using hypotonic lysis as described above, or RIPA buffer (10 mM Tris (7.5), 150 mM NaCl, 1% Nonidet P-40, 1% Deoxycholic acid, 0.1% SDS, and 4 μg/mL each of pepstatin, leupeptin and aprotinin). Tissue lysates were prepared using 25 mM Tris (7.4), 10 mM EDTA, 10 mM EGTA, 100 mM NaF, 50 mM NaPyrophosphate, 5 mM Na_3VO_4, 1 mM DTT, 2 mM PMSF, 1% Nonidet P-40, and 8 μg/mL each of pepstatin, leupeptin and aprotinin. Neoplastic and normal prostate tissue was harvested from 12 week old $Pten^{pc-/-}$ and $Pten^{pc+/+}$ mice respectively (*S13*). The presence of neoplastic and normal prostate in these samples was confirmed using histology (not shown). Breast tumor tissue was harvested from a 9 month old $Brca^{mmtv\text{-}cre-/-}$:$p53^{+/-}$ mouse (*S14*) and normal breast tissue harvested from an age matched mouse of the same strain which did not carry the mmtv-cre transgene ($Brca^{fl/fl}$:$p53^{+/+}$).

NMR detection of ^{13}C labeled PEP and pyruvate. The S100 cytosolic fraction of a HEK293 cell lysate was fractioned as described above. 2,3-^{13}C labeled PEP (Cambridge Isotope Laboratories) (final concentration 2 mM) was incubated in a reaction with 200 μg of S100 cell extract (or the fraction equivalent) for 10 minutes at 30^{o} C. Reactions were stopped by the addition of -80^{o} C methanol to a final methanol concentration of 80%. Samples were dried completely under nitrogen gas and analyzed by NMR. Samples were dissolved in 700 μL of NMR buffer containing 2 mM DSS as an internal standard and 50 mM $NaPO_4$ (pH 7.0). [^{1}H,^{13}C] HSQC spectra were collected on a Bruker Avance 750MHZ spectrometer equipped with a Bruker TXI cryogenic probehead. HSQC spectra with echo/antiecho sensitivity enhancement (*S15, S16*), and adiabatic refocussing and inversion pulses (*S17*) were acquired with spectral widths of 12000Hz in the direct dimension and 31850Hz in the indirect dimension. The total acquisition time for the indirect dimension was 25.6 milliseconds. 2048 points were collected in the direct dimension, with a recycle delay of 1.5 seconds. 4 dummy scans were collected prior to the first increment, and 64 scans were acquired per increment. Spectra were processed using NMRpipe and referenced to the location of the DSS peak. Peak integration was carried out using the Sparky software package (http://www.cgl.ucsf.edu/home/sparky/) using a fit to a Lorentzian linewidth. Errors were obtained by computing the RMS residuals from the curve fit to the Lorentzian function. Error bars in the ratios of the pyruvate peaks to the DSS peaks were obtained by standard propagation of error.

Measurement of phosphate release by thin layer chromatography. The S100 cytosolic fraction of a HEK293 cell lysate was fractioned as described above. ^{32}P-PEP was incubated in a reaction with the

fractional equivalent of 30 μg of S100 cell extract in the presence or absence of 1500 ng of added recombinant PGAM1 as indicated. Reactions were incubated at 30^{o} C and aliquots spotted onto a PEI cellulose thin layer chromatography plate at the indicated time points. $^{32}P_i$ was separated from ^{32}P-PEP using 0.25 M ammonium bicarbonate as the mobile phase as described previously (*S4*), and quantitated using a PhosphorImager.

Figures and Legends

Vander Heiden et al., Figure S1

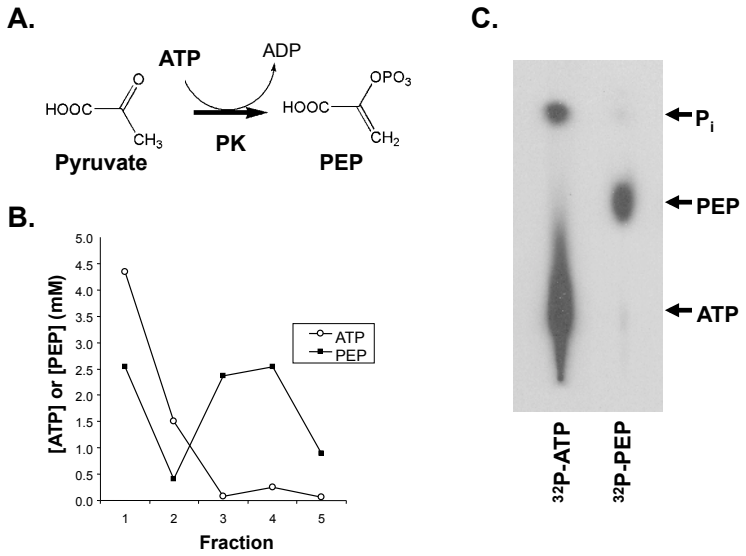


Fig. S1. Generation of ^{32}P-labeled PEP

A. Despite being thermodynamically unfavorable under physiological condition, pyruvate kinase can be used to transfer the γ-phosphate from ATP to pyruvate to generate PEP. In theory, approximately 50% of the ^{32}P-labeled phosphate from γ-^{32}P-labeled ATP will be transferred to pyruvate under the conditions described in the materials and methods.

B. PEP can be separated from ATP using ion exchange chromatography. A mixture of ATP and PEP was applied to a strong anion exchange (Q) column and eluted stepwise using TEAB buffer. Fraction 1 represents the flow through, while fractions 2-5 correspond to fractions eluted by increasing the molarity of TEAB. The concentration of ATP and PEP in each fraction was determined enzymatically and is plotted as shown. Fractions 3 and 4 correspond to 0.3 M and 0.6 M TEAB respectively, and these concentrations were used in subsequent separation of PEP from ATP.

C. A mixture of ^{32}P-labeled PEP and ^{32}P-labeled ATP was generated as described in (A) and the ^{32}P-labeled PEP separated by ion exchange chromatography as described in (B). The purity of the ^{32}P-labeled PEP was assessed by thin layer chromatography and autoradiography as shown. A ^{32}P-labeled ATP standard containing both ^{32}P-labeled ATP and ^{32}P-labeled inorganic phosphate (P_i) is shown for comparison.

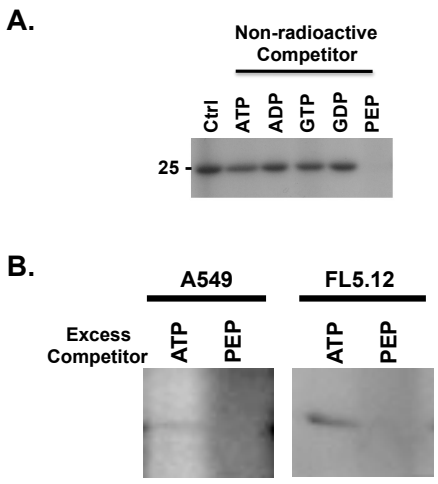


Fig. S2. Evidence of PEP-dependent phosphorylation of a 25-kD protein in multiple cell lines.
A. HEK293 cell extracts were incubated with ^{32}P-labeled PEP in the absence (ctrl) or presence of non-radioactive competitor ATP, ADP, GTP, GDP, or PEP as shown. The reactions were analyzed by SDS-PAGE and autoradiography as shown.
B. A549 cells or FL5.12 cells were hypotonically lysed and incubated with ^{32}P-labeled PEP in the presence of 1 mM non-radioactive competitor ATP or PEP. These reactions were analyzed by SDS-PAGE and autoradiography as shown.

Vander Heiden et al., Figure S3

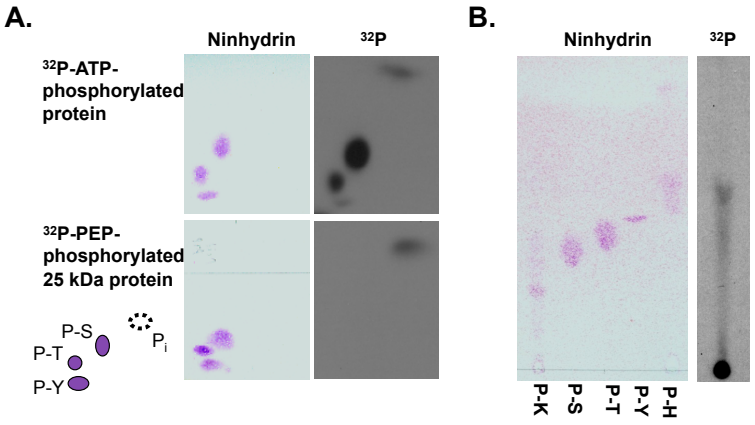


Fig. S3. Phosphoaminoacid analysis demonstrates that a phosphate is transferred from PEP to a histidine residue of the 25-kD protein.

A. Cell lysates were incubated with ^{32}P-labeled ATP or ^{32}P-labeled PEP and proteins separated by SDS-PAGE. The 25-kD protein phosphorylated in ^{32}P-labeled PEP reaction was identified by autoradiography and excised from the gel. A band was excised at random from a gel containing the products of a ^{32}P-labeled ATP reaction as a control. The gel fragments were boiled in HCl for one hour and the hydrolyzed amino acids were combined with phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) standards prior to separation by 2-dimensional thin layer electrophoresis. The location of the standards was determined by ninhydrin staining and the location of ^{32}P-labeled amino acids determined by autoradiography as shown. The expected location of inorganic phosphate (P_i) is also indicated (*S8*).
B. Cell lysates were incubated with ^{32}P-labeled PEP, proteins separated by SDS-PAGE, the 25-kD PEP-phosphorylated protein identified by autoradiography, excised, and boiled in KOH for three hours to hydrolyze the protein into amino acids. Phospholysine (P-K) and phosphohistidine (P-H) standards were synthesized as published previously (*S10*) and spotted onto a thin layer chromatography plate along with commercially available phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) standards adjacent to the base hydrolysate of the 25-kD protein from the ^{32}P-labeled PEP reaction. The amino acids were separated by reverse phase thin layer chromatography (*S9*). The location of the standards was determined by ninhydrin staining and the location of the ^{32}P-labeled amino acid was determined by autoradiography as shown.

Vander Heiden et al., Figure S4

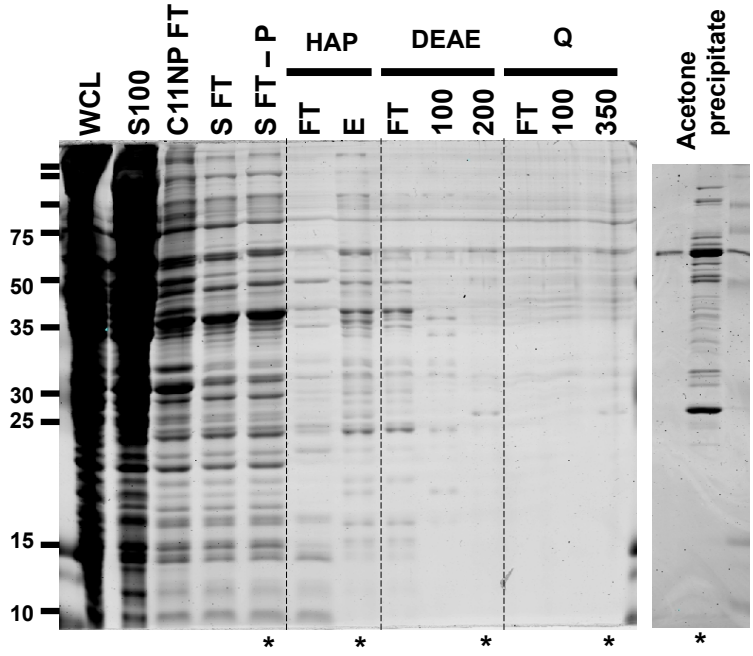


Fig. S4. Purification of the 25-kD protein phosphorylated by PEP.
HEK293 cell lysate (WCL) was centrifuged at 100,000 x g and the supernatant (S100) passed through a C11NP resin prepared as a possible PEP affinity column. The flow through from this column (C11NP FT) was passed through a strong cation exchange column and the flow through (S FT) incubated with PEP (including ^{32}P-labeled PEP) (S FT – P). This reaction was then applied to a hydroxyapatite (HAP) column and eluted with 50 mM $NaHPO_4$. The salt elution (E) containing the ^{32}P-labeled species was diluted to < 25 mM $NaHPO_4$ and applied to a weak anion exchange (DEAE) column and eluted sequentially with 100 mM and 200 mM salt as indicated. The 200 mM salt fraction containing the ^{32}P-labeled species was diluted to 50 mM NaCl and applied to a strong anion exchange (Q) column and eluted sequentially with 100 mM and 350 mM salt as indicated. The 350 mM salt fraction containing the ^{32}P-labeled species was acetone precipitated for analysis by 2D IEF and SDS-PAGE. A coomassie stained SDS-PAGE gel containing an aliquot of each step is shown. Flow through fractions are indicated as (FT). Fractions determined to contain the 25-kD ^{32}P-labeled protein are indicated with a (*) (see Fig. 2A).

Vander Heiden et al., Figure S5

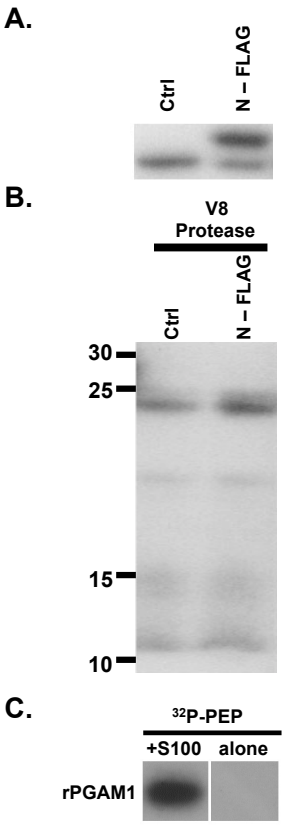


Fig. S5. PGAM1 is the target of PEP-dependent phosphorylation at 25-kD, but is not autophosphorylated by PEP.

A. HEK293 cells were transiently transfected with control plasmid (Ctrl) or N-terminally FLAG tagged PGAM1 (N-FLAG) and lysates incubated with ^{32}P-labeled PEP prior to analysis by SDS-PAGE and autoradiography.

B. The same reactions shown in (A) were subjected to limited proteolysis with endoproteinase Glu-C from *Staphylococcus aureus* type V8 (V8 Protease) and analyzed by SDS-PAGE and autoradiography as shown.

C. Recombinant 6xHis-tagged PGAM1 (rPGAM1) was incubated with ^{32}P-PEP in the presence (+S100) or absence (alone) of cell extract. The labeling of PGAM1 was determined by SDS-PAGE and autoradiography as shown.

Vander Heiden et al., Figure S6

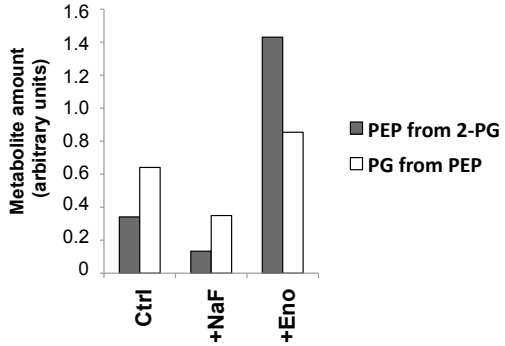


Fig. S6. Modulation of enolase activity in cell lysates by the addition of purified enzyme or NaF. The respective increase and decrease in the enolase activity present in cell lysate was confirmed by measuring the conversion of 2PG to PEP as well as the conversion of PEP to 2PG in lysates with or without the addition of exogenous enolase or NaF. 2PG or PEP was added to cell lysates at a concentration of 1 mM and incubated under the same conditions used to phosphorylate PGAM1 with PEP in the absence (Ctrl) or presence of NaF or exogenously added rabbit muscle enolase enzyme (Eno) as indicated. The reactions were stopped by the addition of cold methanol and the amount of PG generated from PEP, or PEP generated from 2PG was determined by mass spectrometry.

Vander Heiden et al., Figure S7

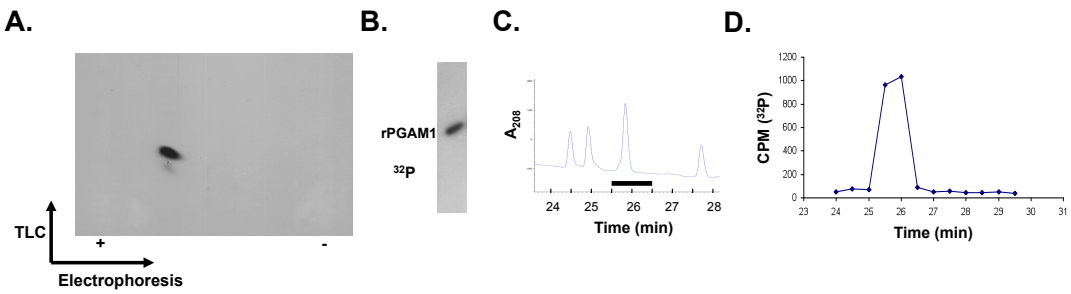


Fig. S7. The phosphate from PEP is transferred to a single peptide in PGAM1 that can be isolated by HPLC.

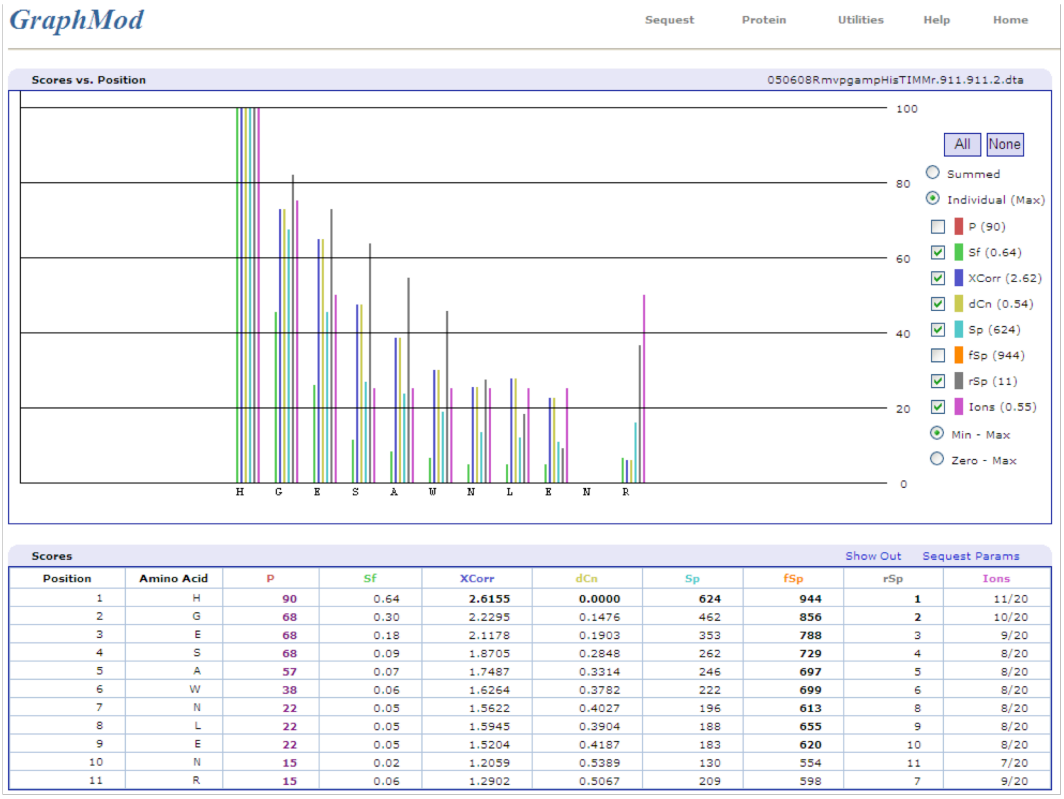
A. A cell lysate was incubated with ^{32}P-labeled PEP to label PGAM1, digested with trypsin, and the peptides spotted onto a TLC plate. Peptides were separated first by thin layer electrophoresis then the plate rotated 90 degrees and the peptides separated by thin layer chromatography. The location of ^{32}P-labeled peptide(s) was determined by autoradiography as shown.

B. Recombinant PGAM1 (rPGAM1) was phosphorylated in a reaction containing cell extract and ^{32}P-PEP. ^{32}P-labeled rPGAM1 was then recovered by binding to Ni-agarose beads with ^{32}P-labeling verified by SDS-PAGE and autoradiography as shown.

C. ^{32}P-labeled rPGAM isolated as described in (B) was digested with trypsin and the peptides separated using HPLC. A chromatograph identifying peptide peaks by absorbance at 208 nm that elute between 24 and 28 minutes is shown. The dark line corresponds to the fractions collected which contained ^{32}P (see panel D).

D. PGAM1 tryptic peptides were separated by HPLC and 0.5 mL fractions collected every 0.5 minutes from 24 to 28 minutes. The amount of ^{32}P in each fraction was determined by scintillation counting as shown. The fractions collected between 25.5 and 26.5 minutes were collected for peptide sequencing by mass spectrometry (see Fig. 3B).

Vander Heiden et al., Figure S8



Scores — Show Out — Sequest Params

Position	Amino Acid	P	Sf	XCorr	dCn	Sp	fSp	rSp	Ions
1	H	**90**	0.64	**2.6155**	**0.0000**	**624**	**944**	**1**	11/20
2	G	**68**	0.30	2.2295	0.1476	462	**856**	**2**	10/20
3	E	**68**	0.18	2.1178	0.1903	353	**788**	3	9/20
4	S	**68**	0.09	1.8705	0.2848	262	**729**	4	8/20
5	A	**57**	0.07	1.7487	0.3314	246	**697**	5	8/20
6	W	**38**	0.06	1.6264	0.3782	222	**699**	6	8/20
7	N	**22**	0.05	1.5622	0.4027	196	**613**	8	8/20
8	L	**22**	0.05	1.5945	0.3904	188	**655**	9	8/20
9	E	**22**	0.05	1.5204	0.4187	183	**620**	10	8/20
10	N	**15**	0.02	1.2059	0.5389	130	554	11	7/20
11	R	**15**	0.06	1.2902	0.5067	209	598	7	9/20

Fig. S8. Evidence for phosphorylation of PGAM1 H11.
The GraphMod algorithm in Proteomics Browser Software (PBS) (Thermo Scientific) tested the score of the phosphorylation modification at every amino acid residue in the peptide sequence HGESAWNLENR based on eight different scoring parameters from a Sequest database search. Note that phosphorylation at the first position, H, in the sequence outscores all other amino acid positions by a large margin.

Vander Heiden et al., Figure S9

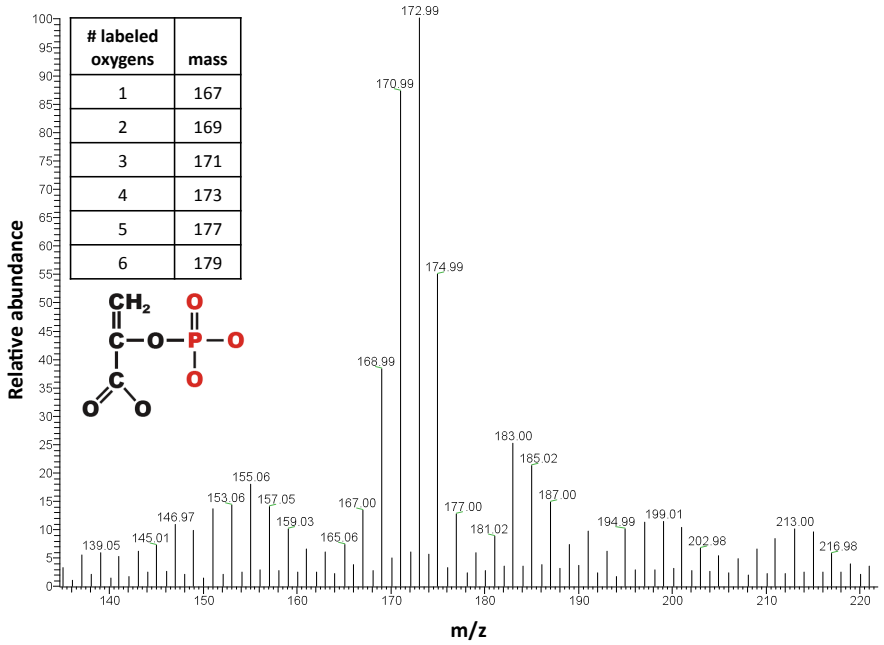


Fig. S9. Generation of PEP containing ^{18}O-labeled phosphate.
An 80 mg/mL solution of PEP was prepared in $H_2{}^{18}O$ at pH 0 and heated to 98° C for 5 minutes as described previously (*S6*). This results in preferential exchange of oxygens on the phosphate group (shown in red on the inset structure of PEP). Exchange with the carboxylic acid group oxygens also occurs with lower efficiency resulting in PEP with 4 and 5 labeled oxygens. The degree of isotopic labeling of PEP was determined by ion trap mass spectrometry as shown. The expected *m/z* of singly charged PEP (mass) corresponding to the number of labeled oxygens is shown for reference.

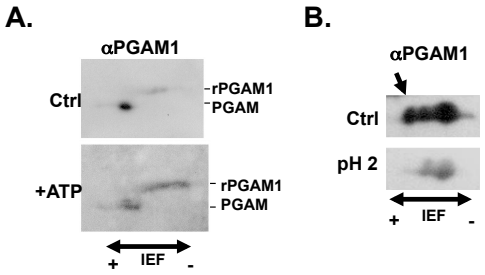


Fig. S10. Assessment of histidine phosphorylated PGAM1 by 2D Western blot.
A. Recombinant PGAM1 (rPGAM1) was added to HEK293 cell extract in the absence (Ctrl) or presence of ATP (+ATP). The reactions were analyzed by 2D IEF and SDS-PAGE followed by Western blot using an anti-PGAM1 antibody as shown.
B. HEK293 cell extract was incubated with PEP and then retained at neutral pH (Ctrl) or incubated at pH 2 as indicated. The reactions were then analyzed by 2D IEF and SDS-PAGE followed by Western blot using an anti-PGAM1 antibody as shown. The arrow points to the species which is lost under acidic conditions.

Vander Heiden et al., Figure S11

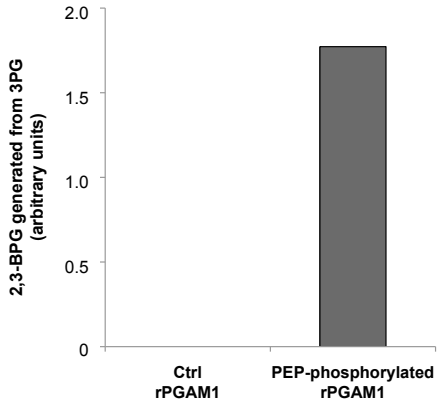


Fig. S11. PEP-phosphorylated PGAM1 is catalytically active.
Recombinant 6xHis-tagged PGAM1 (rPGAM1) was added to a cell extract in the absence (Ctrl) or presence (PEP-phosphorylated) of PEP and recovered by binding to Ni-agarose beads. 3PG was added to the isolated rPGAM and the reaction stopped by the addition of cold methanol. The amount of 2,3-BPG generated from 3PG was determined by mass spectrometry as shown.

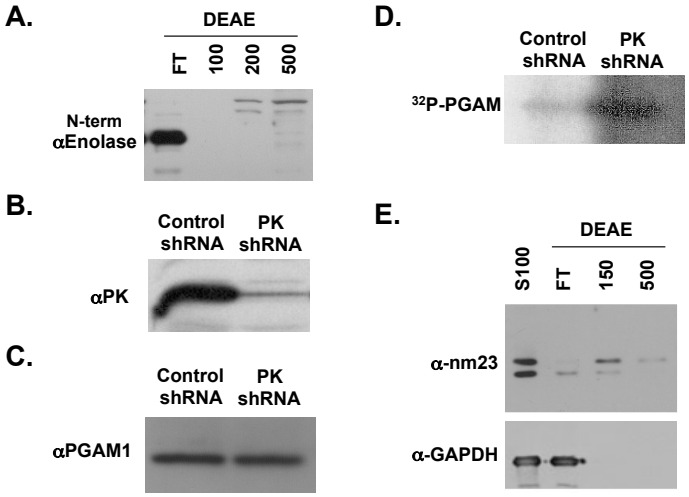


Fig. S12. PGAM1 phosphorylation occurs in the absence of enolase or pyruvate kinase.
A. HEK293 cell lysate was centrifuged at 100,000 x g and the supernatant (S100) fractionated over a weak anion exchange (DEAE) column. The flow through (FT) and fractions eluted sequentially with 100 mM, 200 mM and 500 mM NaCl were collected. The amount of enolase present in each fraction was determined by Western blot using an antibody raised against the N-terminus of enolase. The Western blot shown in Fig. 4B utilized a different antibody raised against the C-terminus of enolase.
B. A549 cells were infected with a virus expressing an shRNA directed against PKM2 (PK shRNA) or a virus expressing a control shRNA. The level of PKM2 expression was determined by Western blot using an antibody that recognizes both PKM1 and PKM2 as shown.
C. The amount of PGAM1 expressed in A549 cells following shRNA-mediated PKM2 knock-down (PK shRNA) or control shRNA expression (control) was determined by Western blot as shown.
D. Lysates prepared from the A549 cells described in (B) were incubated with ^{32}P-PEP and PGAM1 phosphorylation was determined by SDS-PAGE and autoradiography as shown.
E. HEK293 cell lysate was centrifuged at 100,000 x g and the supernatant (S100) fractionated over a weak anion exchange (DEAE) column. The presence of nm23 and GAPDH in the S100, the flow though (FT), 150 mM salt (D150) and 500 mM salt (D500) fractions from the DEAE column was determined by Western blot as shown.

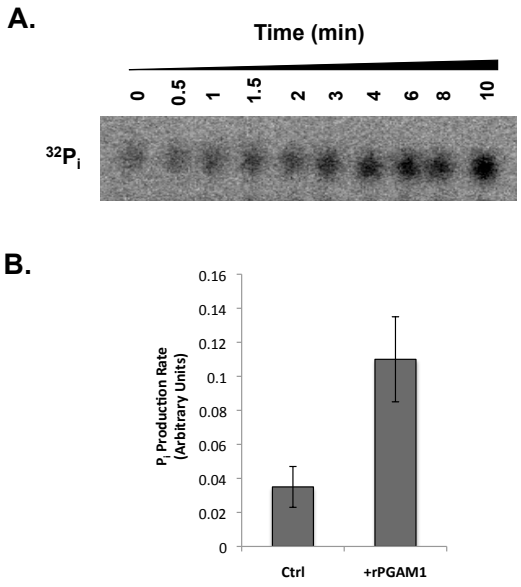


Fig. S13. Loss of P_i from PEP in the absence of pyruvate kinase is promoted by PGAM1.
A. HEK293 cell lysate was centrifuged at 100,000 x g and the S100 supernatant fractionated over a weak anion exchange (DEAE) column. The 500 mM NaCl (D500) fraction was collected and incubated with ^{32}P-PEP in the presence of exogenously added rPGAM1. Samples from the reaction were taken at the indicated time points and $^{32}P_i$ separated from ^{32}P-PEP using thin layer chromatography. The increase in $^{32}P_i$ over time is shown.
B. The D500 fraction prepared as described in (A) was incubated with ^{32}P-PEP in the absence (Ctrl) or presence (+rPGAM1) of added recombinant PGAM1. The D500 fraction contains a small amount of endogenous PGAM1. The P_i release was assessed over time by thin layer chromatography, and the relative rate of P_i release was obtained from linear regression of the time course with error bars obtained from RMS residuals and graphed as shown.

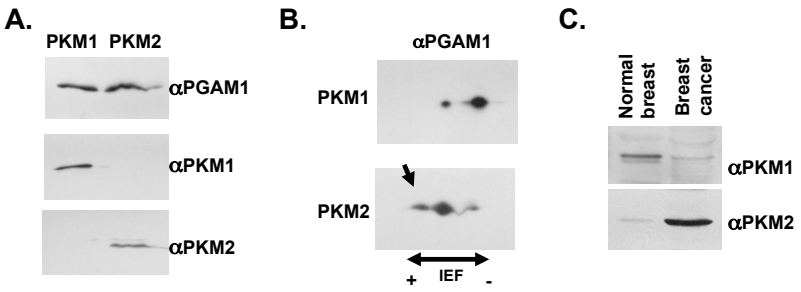


Fig. S14. PGAM1 is histidine phosphorylated in cells expressing PKM2.
A. H1299 lung cancer cells were engineered to exclusively express either PKM1 or PKM2 as described previously (*S1*). The expression of PKM1, PKM2 and PGAM1 was determined by Western blot as shown.
B. Lysates prepared from the same cells described in (A) were subjected to 2D IEF and SDS-PAGE and analyzed by Western blot using an anti-PGAM1 antibody as shown. The most acidic species corresponding to H11 phosphorylation is indicated with an arrow.
C. A breast tumor (cancer) was removed from 9 month old mouse harboring a conditional allele of the *Brca1* tumor suppressor gene and a transgene to express Cre recombinase in the breast to delete *Brca1*. Normal breast tissue was removed from a mouse not expressing Cre and hence where *Brca1* was not deleted in the breast. The expression of PKM1 or PKM2 in each tissue was determined by Western blot as shown.

Vander Heiden et al., Figure S15

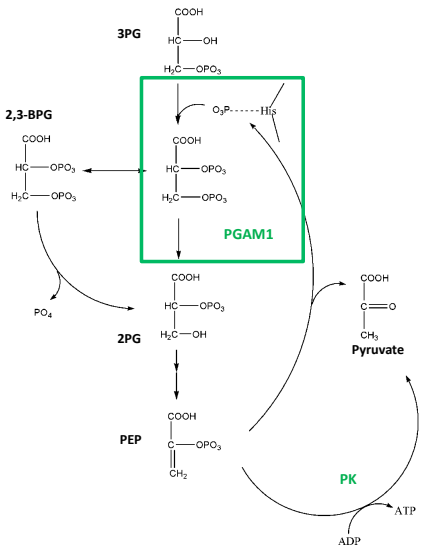


Fig. S15. Alternate regulation of glycolysis involving PEP-dependent PGAM1 phosphorylation.
A proposed model for the glycolytic pathway from 3PG to pyruvate that includes PEP-dependent phosphorylation of PGAM1 is shown. PEP donates its phosphate to PGAM1 resulting in pyruvate production and priming of H11 on PGAM1.

Vander Heiden et al., Table S1

Protein Group
Reference ID = sp|P18669|PGAM1_HUMAN
Protein Name = Phosphoglycerate mutase 1 [Homo sapiens]

Sequence	Accession	Scan	Charge	Ret. time	Sf	MH+	Xcorr	deltaCn	Sp	TIC
DAGYEFDICFTSVQK	P18669	5799-5909	2	45.58	0.97	1780.6	5.62	0.3	2094	322831
FSGWYDADLSPAGHEEAK	P18669	4677-4698	2	38.49	0.97	1980.8	5.48	0.31	2110	135673
HGESAWNLENR	P18669	3299-3300	2	30.14	0.95	1312.6	4.22	0.32	1590	147523
YADLTEDQLPSCESLKDTIAR	P18669	5117-5119	2	41.1	0.93	2424.8	5.53	0.28	834	45666
HGESAWNLENR	P18669	3641-3642	2	32.2	0.91	1312.4	3.67	0.24	1326	7802
PM*QFLGDEETVR	P18669	3749	2	32.85	0.89	1437.7	3.36	0.05	1784	7041
AM*EAVAAQGK	P18669	617-785	2	14.29	0.87	992.4	3.62	0.04	1342	33628
FSGWYDADLSPAGHEEAK	P18669	4703-4856	3	39.05	0.86	1980	4.37	0.26	1091	1051311
DAGYEFDICFTSVQK	P18669	6665	2	50.47	0.83	1779.1	3.45	0.35	919	2647
YADLTEDQLPSCESLKDTIAR	P18669	4915-4992	3	40.1	0.78	2425.9	4.19	0.29	785	27934
YADLTEDQLPSCESLK	P18669	4500-4523	2	37.43	0.78	1866.5	3.98	0.11	709	16653
AM*EAVAAQGK	P18669	1397	2	18.54	0.77	990.7	3.11	0.12	812	2308
KAM*EAVAAQGK	P18669	668-705	2	14.2	0.77	1121.5	3.19	0.01	954	54201
FSGWYDADLSPAGHEEAK	P18669	4370	3	36.58	0.65	1981.3	3.2	0.12	1065	22046
FSGWYDADLSPAGHEEAK	P18669	5175-5190	3	41.49	0.62	1982.5	3.7	0.18	648	23180
RYADLTEDQLPSCESLK	P18669	4329	2	36.33	0.61	2024.9	3.29	0.22	542	2829
FSGWYDADLSPAGHEEAK	P18669	5415	3	42.91	0.58	1978.4	3.14	0.17	784	8950
HGESAWNLENR	P18669	4637	2	38.19	0.54	1313.8	2.57	0.07	645	5944
HGESAWNLENR	P18669	4548	2	37.65	0.53	1313.8	2.43	0.25	472	5509
HYGGLTGLNK	P18669	2867-2876	2	27.55	0.53	1059.5	2.45	----	657	92241
FSGWYDADLSPAGHEEAK	P18669	5040	2	40.62	0.5	1979.7	2.53	0.32	454	2426
AM*EAVAAQGK	P18669	889	1	15.43	0.31	992.4	2.11	0.19	171	2810
FSGWYDADLSPAGHEEAK	P18669	6098	3	47.05	0.29	1980.7	2.66	0.08	569	3250
HGESAWNLENR	P18669	5082	2	40.88	0.28	1312.8	2.25	0.19	224	4781
FSGWYDADLSPAGHEEAK	P18669	5766	3	45.05	0.24	1980.8	2.65	0.06	522	4731

Table S1. List of phosphoglycerate mutase 1 (PGAM1) peptides identified by LC/MS/MS analysis of the ^{32}P-labeled species identified with an arrow in Fig. 2C.

References

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