Supplemental Figures

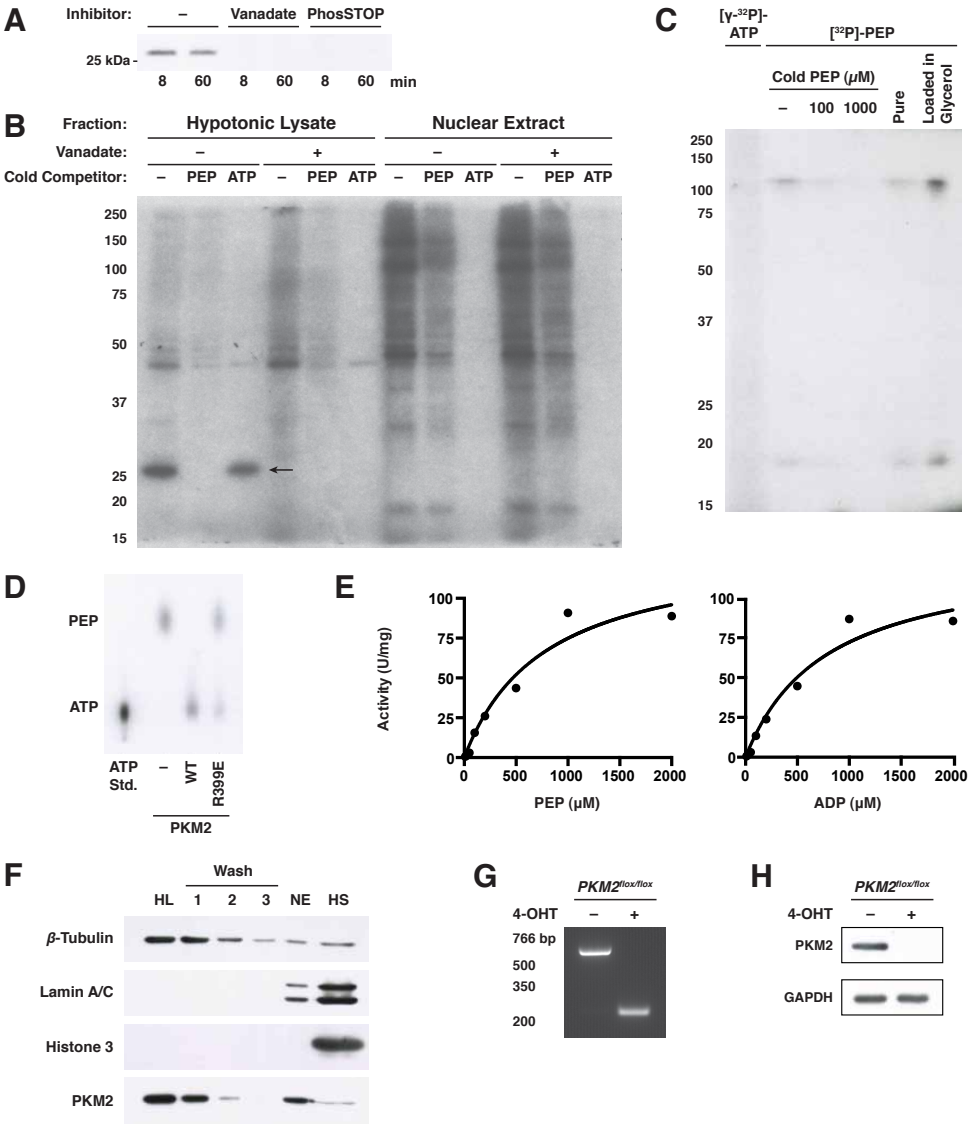

Figure S1, related to Figure 1. (*A*) Phosphoglycerate mutase 1 (PGAM1) labeling from [^{32}P]-PEP is inhibited by orthovanadate. H1299 hypotonic lysates were incubated for 8 min or 1 h with [^{32}P]-PEP and 1 mM cold competitor ATP. The reaction was carried out without added phosphatase inhibitors or with the addition of 1 mM sodium orthovanadate (vanadate) or PhosSTOP (Roche), and proteins were resolved by SDS-PAGE and visualized by autoradiography. (*B*) Orthovanadate (vanadate, 1 mM) does not inhibit PEP-dependent phosphorylation. H1299 cell hypotonic lysate and nuclear extract were incubated with [^{32}P]-PEP and cold competitor PEP or ATP with or without the addition of 1 mM sodium orthovanadate.

PGAM1 is indicated with an arrow. (*C*) [^{32}P]-PEP can label a polyacrylamide gel in the absence of added protein. [γ-^{32}P]-ATP (lane 1) and [^{32}P]-PEP (lanes 2-6) were analyzed by SDS-PAGE and autoradiography. These molecules migrate with the salt front of the gel as assessed by Geiger counting. This salt front is cut off before autoradiography, but [^{32}P]-PEP also labels what appears as two bands by autoradiography. Impure [^{32}P]-PEP was electrophoresed in the presence of no (lane 2), 100 μM (lane 3), or 1000 μM (lane 4) cold competitor PEP. HPLC-purified [^{32}P]-PEP (lane 5) and [^{32}P]-PEP loaded in ~7% (v/v) glycerol (as opposed to Lamelli sample buffer) (lane 6) produced a similar pattern. (*D*) Recombinant wild type and R399E mutant PKM2 display glycolytic pyruvate kinase activity. Lane 1: [γ-^{32}P]-ATP standard. Lanes 2-4: [^{32}P]-PEP and 0.5 mM ADP were incubated for 45 min with no enzyme or 10 μg/mL recombinant wild type or mutant (R399E) PKM2. PEP and ATP were resolved by TLC and visualized by autoradiography. (*E*) Specific activity of wild type rPKM2 with respect to substrate concentration determined by a linked assay to lactate dehydrogenase (Vander Heiden et al., 2010). In each case the substrate whose concentration was not varied was kept fixed at 5 mM. Data are the mean of N=3 ± S.D. (*F*) Protein levels of β-tubulin, lamin A/C, histone H3, and PKM2 were determined in cell extract fractions by western blotting. H1299 hypotonic lysate (HL) was prepared in buffer containing 10 mM KCl. The remaining material was washed three times with this buffer, and a nuclear extract (NE) was prepared from the resulting material using 500 mM NaCl. A high-salt (HS) extraction, containing histones, was then obtained by extraction with 2.5 M NaCl. (*G*) PKM2 can be deleted from MEFs. Cre-ER expressing MEFs homozygous for a *PKM* allele where exon 10 (specific to PKM2) is flanked by two loxP sites (*PKM2*$^{flox/flox}$) were treated with .1% (v/v) ethanol or 500 nM 4-hydroxytamoxifen (4-OHT) and .1% ethanol. Deletion of *PKM* exon 10 was confirmed by PCR using primers described previously (Israelsen et al., 2013). (*H*) PKM2 expression was analyzed by western blot analysis of cell lysates.

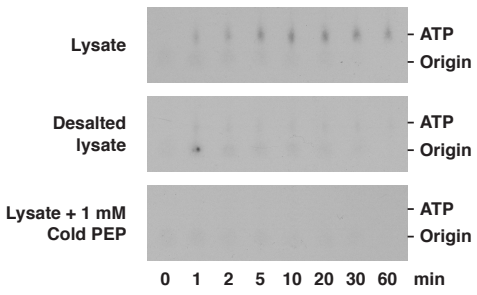

Figure S2, related to Figure 2. A hypotonic lysate contains enough ADP to enable synthesis of [γ-^{32}P]-ATP. H1299 hypotonic lysates were incubated for the indicated times with rPKM2 and [^{32}P]-PEP. Labeled ATP produced by this reaction was measured by TLC and autoradiography.

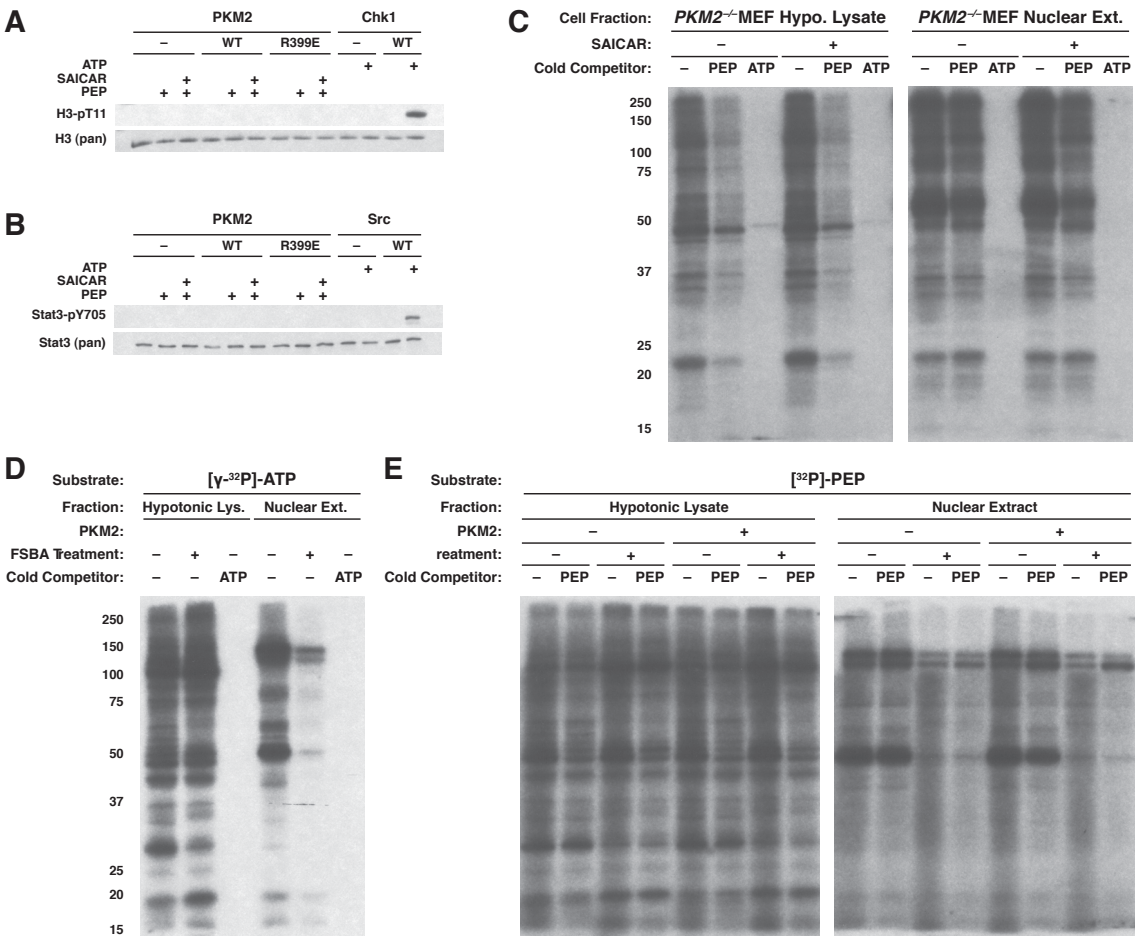

Figure S3, related to Figure 3. Recombinant PKM2 and (*A*) Stat3 or (*B*) histone H3 were incubated for 1 hr at 30 ºC with or without PEP and SAICAR as indicated. As a control, the same assay was repeated with recombinant protein kinases known to phosphorylate these proteins, (*A*) Chk1 and (*B*) Src, with the addition of cold ATP. (*C*) *PKM2*$^{-/-}$ MEF cell hypotonic lysate and nuclear extract were incubated for 1 h with [^{32}P]-PEP and rPKM2 with or without 0.5 mM SAICAR, and in the absence of competitor or in the presence of cold competitor PEP (1 mM) or ATP (1 mM) as indicated. (*D*) H1299 cell hypotonic lysate and nuclear extract were incubated with DMSO or 1 mM FSBA (5'-(4-fluorosulfonobenzoyl)adenosine) to inhibit ATP-dependent kinases. Protein phosphorylation events observed in lysates containing [γ-^{32}P]-ATP with or without FSBA are shown. (*E*) DMSO- or FSBA-treated cell extracts were incubated with [^{32}P]-PEP with or without the addition of PKM2 and cold competitor PEP to monitor PKM2 and PEP-dependent phosphorylation.

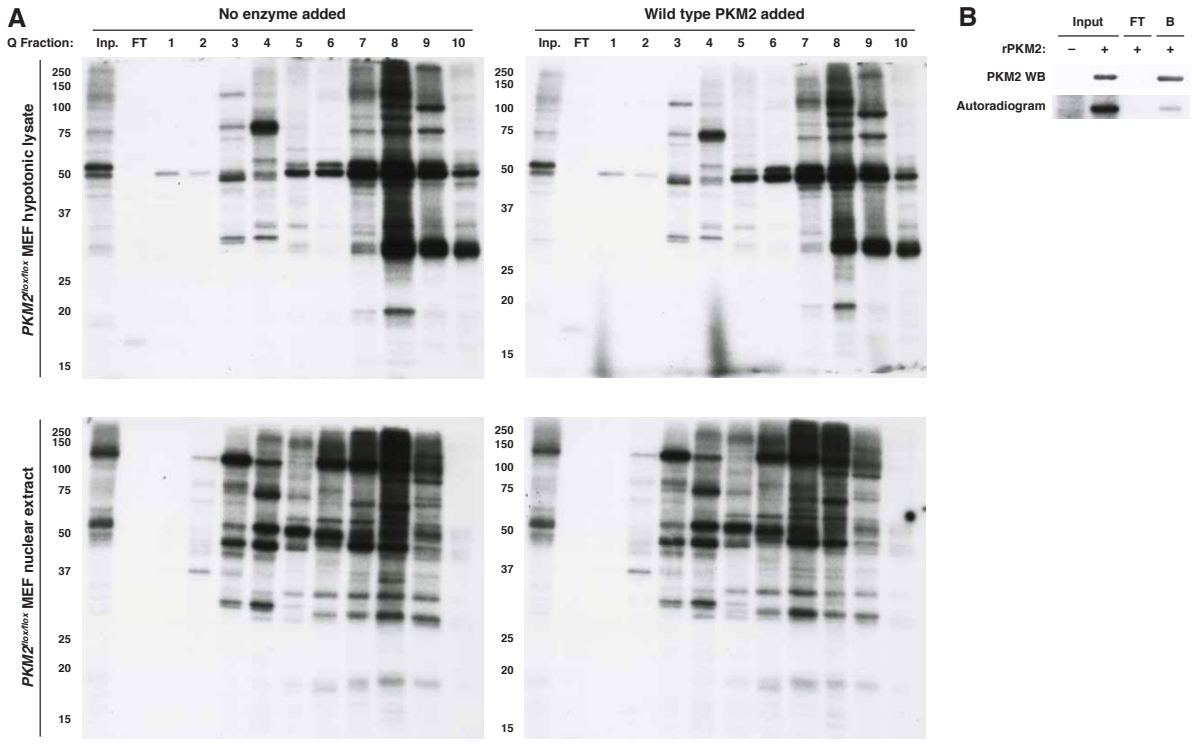

Figure S4, related to Figure 4. (*A*) rPKM2 was added to a fractionated hypotonic lysate and nuclear extract prepared from PKM2-expressing ($PKM2^{flox/flox}$) MEFs to identify potential PKM2 protein kinase activity using ATP as a substrate. (*B*) In Figure 4A, one protein (predominantly contained within H1299-hypotonic-lysate fractions 1 and 2) exhibited phosphorylation dependent on PKM2 addition. This phosphorylated protein co-purifies with PKM2 by nickel-affinity chromatography (Inp., Q column input; FT, nickel-affinity column flow through).

Supplemental Experimental Procedures

Protein Extraction and Purification. To prepare hypotonic lysates and nuclear extracts, cells were washed with ice cold PBS, collected by centrifugation at 650 × g, and incubated in three volumes of hypotonic lysis buffer (10 mM HEPES/KOH pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, .5 mM DTT, 1x cOmplete mini protease inhibitor (Roche)) for 10 min on ice. Lysis was accomplished by passing cells through a 22.5-gauge needle fifteen times. Nuclei were pelleted for 10 min at 855 × g, and washed five times with four volumes of hypotonic lysis buffer. They were next resuspended in one volume of nuclear extraction buffer (20 mM HEPES/KOH pH 7.6, 500 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, .5 mM DTT, 25% (v/v) glycerol, 1x cOmplete mini protease inhibitor (Roche)) and incubated for 30 min on ice with occasional mixing. Lysates were clarified for 10 min at 20 000 × g, and a high-salt extraction was accomplished by incubating the resulting pellet in high-salt solubilization buffer (Shechter et al., 2007) for 30 min. Proteins were desalted into hypotonic lysis buffer by passing samples through a Sephadex G-25 Fine (GE Healthcare) gravity column. For Q-column fractionation, samples were first concentrated to 1 mL on Pierce Concentrators with a 9 kDa molecular weight cutoff (Thermo Scientific). Samples were then desalted into buffer A (20 mM bis-tris-propane-HCl pH 8.7, 5 mM KCl, 1 mM $MgCl_2$) on a 5 mL HiTrap Desalting column (GE Healthcare) and then loaded onto a 1 mL HiTrap Q HP column (GE Healthcare). Proteins were eluted in a linear gradient from buffer A to buffer A with 1 M KCl, and 1 mL fractions were collected. Protein concentration was determined by standard Bradford assay.

Site-Directed Mutagenesis. The R399E mutation was introduced using the QuikChange II XL Site Directed Mutagenesis Kit (Agilent Technologies) and the following primers:
5’-CAATTATTTGAGGAACTCGAGCGCCTGGCGCCCATTACC-3’ and
5’-GGTAATGGGCGCCAGGCGCTCGAGTTCCTCAAATAATTG-3’.

Crude [^{32}P]-PEP Synthesis and Purification. [^{32}P]-PEP was synthesized as described previously (Mattoo and Waygood, 1983; Vander Heiden et al., 2010). Briefly, commercial rabbit muscle pyruvate

kinase (Sigma) catalyzed synthesis from pyruvate and [γ-^{32}P]-ATP (Perkin Elmer) at 37 ºC for 45-60 min, followed by crude separation of PEP from ATP using a Vivapure Q Mini H columns (Satorius) with sequential elution in 0.3 and 0.6 M triethylammonium bicarbonate pH 8.5 (Sigma).

***In Vitro* Phosphorylation Reactions.** Unless stated otherwise, reactions were carried out at 30 ºC for 1 h in previously published buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 50 mM $MgCl_2$, 1 mM DTT) with 1x PhosSTOP (Roche) (Gao et al., 2012; Yang et al., 2012). Protein extracts prepared as described above or calf thymus histones (Worthington Biochemical) were included at 100 µg/mL. Recombinant PKM2 was included in the reaction at 10 µg/mL. Where indicated, 1 mM Na_3VO_4, 0.5 mM SAICAR, or 1 mM cold competitor ATP or PEP were included in the reaction. Radioactive phosphate group donor substrates were ~6 nM (~20 µCi/mL) [γ-^{32}P]-ATP (Perkin Elmer) or ~4.5 nM (~13 µCi/mL) impure [^{32}P]-PEP, or ~3.5 nM (~10 µCi/mL) pure [^{32}P]-PEP or ~.31 nM (~.9 µCi/mL) pure [γ-^{32}P]-ATP. Reactions were quenched with addition of Lamelli sample buffer, and phosphorylated proteins were analyzed by 10% SDS-PAGE followed by autoradiography. Nickel-affinity purification was accomplished by incubating phosphorylation reactions with Ni-NTA Beads (Qiagen) for 30 min. Beads were then washed with reaction buffer supplemented with 30 mM imidazole, and proteins were eluted by boiling in Lamelli sample buffer. Where indicated, lysates were incubated with 1% (v/v) DMSO or 1 mM (final) 5'-(4-fluorosulfonobenzoyl)-adenosine (FSBA, Sigma) with 1% (v/v) DMSO at 30 ºC to inhibit endogenous ATP-dependent kinases. FSBA was removed by desalting in an Amicon Ultra centrifugal filter prior to phosphorylation assay and addition of PKM2. For *in vitro* phosphorylation detected by western blot, recombinant PKM2, Src, or Chk1 was present at 1 ng/µL and histone H3 or Stat3 was present at 3 ng/µL.

SAICAR Synthesis and Purification. SAICAR was synthesized enzymatically using adenosuccinate lyase (ADSL) purified by a previously described approach (Lee and Colman, 2007). ADSL (50 µg/mL) catalyzed the reaction between 13.5 mg/mL AICAR (Toronto Research Chemicals) and 50 mM fumarate at 37ºC overnight in a buffer containing 20 mM HEPES pH 7.4, 50 mM ammonium acetate, 50 mM KCl, 2 mM EDTA, and 1 mM DTT (Keller et al., 2012). The reaction was loaded on to a 5 mL HiTrap Q HP column and eluted in a linear gradient from water to 3 M ammonium acetate. Peak fractions were pooled

and lyophilized. SAICAR was resuspended in 10 mM HEPES-KOH pH 7.5 prior to use in *in vitro* reactions.

Enzyme-Linked Pyruvate Kinase Assay. Pyruvate kinase activity was measured as previously described (Anastasiou et al., 2012). Briefly, rPKM2 (0.5 µg/mL) was mixed with various concentrations of PEP and ADP in a reaction buffer containing 50 mM HEPES-KOH (pH 7.5), 20 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 180 µM NADH, and 11 U lactate dehydrogenase (LDH, Sigma Aldrich). LDH activity was verified to be in excess of pyruvate kinase activity in these conditions, enabling disappearance of NADH (absorbance at 340 nm) to be used as a measure of pyruvate kinase activity.

Western Blotting. Total protein extracts were prepared by incubating cells for 15 min in ice-cold RIPA buffer followed by clarification for 10 min at 20 000 × g. To confirm absence of PKM2 in MEFs, 15 µg of protein were resolved by 8% SDS-PAGE. To determine contents of cell extracts, volumes proportionate to the extraction buffer volumes were resolved by 8 or 10% SDS-PAGE. Proteins were transferred to a PVDF membrane and, following immunoblotting, were visualized by ECL assay. Antibodies used: PKM2, histone 3, lamin A/C, histone H3, H3 phospho-T11, Stat3, Stat3 phospho-Y705 (Cell Signaling Technologies: 4053, 4499, 4777, 4499, 9764, 9132, 9145, respectively), *β*-tubulin (Abcam: 21058).

DNA Extraction and Genotyping. Genomic DNA was extracted using the PureLink Genomic DNA Mini Kit (Life Technologies). *PKM* exon 10 was amplified by polymerase chain reaction as described previously (Israelsen et al., 2013), and reaction products were resolved on a 2% agarose gel.

Supplementary References

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