Structures of human phosphofructokinase-1 and atomic basis of cancer-associated mutations

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Phosphofructokinase-1 (PFK1), the ‘gatekeeper’ of glycolysis, catalyses the committed step of the glycolytic pathway by converting fructose-6-phosphate to fructose-1,6-bisphosphate. AllostERIC activation and inhibition of PFK1 by over ten metabolites and in response to hormonal signalling fine-tune glycolytic flux to meet energy requirements1. Mutations inhibiting PFK1 activity cause glycogen storage disease type VII, also known as Tarui disease2, and mice deficient in muscle PFK1 have decreased fat stores3. Additionally, PFK1 is proposed to have important roles in metabolic reprogramming in cancer4–6. Despite its critical role in glucose flux, the biologically relevant crystal structure of the mammalian PFK1 tetramer has not been determined. Here we report the first structures of the mammalian PFK1 tetramer, for the human platelet isoform (PFKP), in complex with ATP–Mg2+ and ADP at 3.1 and 3.4 Å, respectively. The structures reveal substantial conformational changes in the enzyme upon nucleotide hydrolysis as well as a unique tetramer interface. Mutations of residues in this interface can affect tetramer formation, enzyme catalysis and regulation, indicating the functional importance of the tetramer. With altered glycolytic flux being a hallmark of cancers, these new structures allow a molecular understanding of the functional consequences of somatic PFK1 mutations identified in human cancers. We characterise three of these mutations and show they have distinct effects on allosteric regulation of PFKF activity and lactate production. The PFKF structural blueprint for somatic mutations as well as the catalytic site can guide therapeutic targeting of PFK1 activity to control dysregulated glycolysis in disease.

Previous attempts to obtain the structure of mammalian tetrameric PFK1 used native protein or recombinant protein generated in yeast or bacteria. A limitation of using native PFK1 is that most mammalian tissues express all three isoforms: muscle (PFKM), liver (PFKL) and platelet (PFKP)2. Although there are structures of PFK from prokaryotes8–11 and eukaryotes12–14, including dimeric rabbit PFKM expressed in Escherichia coli12, can be readily used for a detailed structural comparison with that of the dimeric structures (Fig. 1). The F6P substrate, as observed in the Saccharomyces cerevisiae PFK (ScPFK) structure12, is observed between the subdomains of the active site (Extended Data Fig. 1b, c). An effect of this conformational change is to open the catalytic interface or the conformational changes with regulation of the tetrameric mammalian enzyme. To overcome current limitations with structural studies of human PFK1, we produced recombinant PFKF by using a baculovirus expression system. The recombinant enzyme, purified to homogeneity (Extended Data Fig. 1a), is tetrameric as shown by transmission electron microscopy (TEM; Fig. 1a). The activity and regulation of recombinant PFKF, including high cooperativity for fructose-6-phosphate (F6P), a high affinity for ATP–Mg2+, and high sensitivity to ATP inhibition (Extended Data Fig. 1b, c), was similar to previously reported mouse PFKP expressed in yeast15. We determined the crystal structure of the PFKF tetramer in complex with ATP–Mg2+ at 3.1 Å resolution (Fig. 1b–d and Extended Data Fig. 2). The atomic model has good agreement with the crystallographic data and the expected geometric parameters (Extended Data Table 1). The asymmetric unit contains two tetramers, and the eight protomers have essentially the same conformation (with r.m.s.d. of ~0.3 Å between any pair of them, Extended Data Fig. 3). The overall organizations of the two tetramers are slightly different, reflected in part by changes in the relative orientations of the two dimers (Extended Data Fig. 3).

Each PFKP tetramer measures 13.8 nm by 10.3 nm, similar in size and shape to what we calculated from TEM images (Fig. 1a, b). The tetramer is composed of a dimer of dimers, and the interface between the two dimers is relatively small, with a buried surface area of 700 Å² for each subunit (arrow labelled ‘t’ in Fig. 1b, c). The two subunits of the dimer are arranged in an antiparallel orientation, confirming previous predictions17, with a buried surface area of 1,800 Å² for each subunit. The active site is located at the interface between the two subunits (arrow labelled ‘c’ in Fig. 1b, e).

The structure of PFKP is likely to represent the active conformation of the enzyme. The crystal was prepared at pH 7, near physiological pH, and residues in the active site that are important for substrate binding and/or catalysis have similar conformations in PFKF as in other PFK structures (Fig. 1e). The F6P substrate, as observed in the Saccharomyces cerevisiae PFK (ScPFK) structure12, can be readily accommodated in the PFKF active site for catalysis. The invariant substrate binding residues His208 and Arg210 from the second protomer of the dimer are located ~6 Å away from F6P, suggesting that a closure of this region of the active site may occur upon F6P binding and catalysis. PFKF contains only one ATP in each subunit bound to the active site, despite the presence of 10 mM ATP during crystallization and even though the allosteric adenine nucleotide-binding sites are functional (Extended Data Fig. 1b, c). We also observed the binding of two phosphate groups in each protomer at positions corresponding to the prokaryotic PFK effector sites (Extended Data Fig. 2c, d). The enzyme activity, regulation and stability of PFKF are controlled by binding phosphate or sulphate ions18. PFKF displayed a loss of ATP inhibition in the presence of 10 mM sodium sulphate (Extended Data Fig. 2e), suggesting that phosphate-binding and inhibitory site ATP-binding are mutually exclusive in the tetrameric structure.

We also determined the crystal structure of PFKF in complex with ADP at 3.4 Å resolution, at pH 7.5. The relatively low resolution of this structure precludes a detailed structural comparison with that of the ATP–Mg2+ complex. However, it is clear there is a dramatic change in the relative positions of the two domains in each protomer (Fig. 2a), and especially the overall structures of the dimer and tetramer (Fig. 2b). A rotation of ~12° is observed between the subdomains of the ADP complex protomer relatively to the ATP complex, leading to an 8 Å shift in the substrate binding domain relative to the nucleotide binding domain. An effect of this conformational change is to open the catalytic site (Fig. 2c, d), which may play a role in the release of products. The conformational changes observed between the ATP and ADP complexes of PFKF are different from those seen for the R- and T-states of bacterial PFK (Extended Data Fig. 4)19,20.

We tested the importance of hydrophobic and electrostatic interactions at the tetramer interface for enzyme activity (Fig. 3a). Most
residues at the interface are hydrophobic. Tyr645 and Phe649 from the two subunits form a π-stack of four aromatic side chains in the interface, with Phe649 in the middle (Fig. 3a). Despite the overall similarities in organization between the PFK tetramer to that of ScPFK, there are significant differences in the tetramer interface between the two enzymes (Extended Data Fig. 5). Phe649, which is evolutionarily conserved in metazoans but not in yeasts, is a leucine residue in ScPFK. However, this Leu residue has a completely different local environment in ScPFK compared with Phe649 in PFKP. We generated recombinant PFKP with Phe649 mutated to Leu (Extended Data Fig. 6a) to test whether Phe649 is required for tetramer formation. Previous studies showed that PFK1 assembles into tetramers in a concentration- and ligand-dependent manner, with allosteric activators favouring the formation of tetramers and allosteric inhibitors favouring the formation of dimers. ATP and F6P, TEM showed that wild-type (WT) PFKP particles had the dimensions and appearance of tetramers (Fig. 3b, c). In contrast, PFKP-F649L particles were the same width but half the length of WT, consistent with dimer formation along the catalytic interface (Fig. 3b, c and Extended Data Fig. 6b). We compared the PFKP-F649L particles with those induced by the inhibitor citrate, which cause PFKM to form dimers. In a buffer containing 1 mM citrate, we saw two sizes of particles with WT PFKP: one with dimensions of tetramers and the other with dimensions of dimers along the catalytic interface (Fig. 3b, c and Extended Data Fig. 6c), further confirming dimer formation by PFKP-F649L. The catalytic activity of PFKP-F649L was reduced 98% (Extended Data Fig. 6d) compared with WT enzyme, indicating that tetramer formation is necessary for PFK1 activity.

The structures suggest that an electrostatic interaction at the tetramer interface between Arg613 of one subunit and Glu657 of the adjacent subunit (Fig. 3a) may be important for enzyme function. This salt bridge was only observed in the ATP-bound structure but not in the ADP-bound PFKP structure or dimeric rabbit PFKM structures, suggesting that it may contribute to maintaining an active form of the mammalian tetramer. PFKP-E657A had reduced affinity of ATP and F6P, compared with 4.5 mM for F6P, compared with 0.8 mM in WT, and an approximately twofold decrease in maximum activity (Fig. 3d, Extended Data Fig. 6a and Extended Data Table 2). Our data indicate that hydrophobic interactions are essential for the formation of tetramers while electrostatic interactions are required for optimal enzyme activity.

The structure of PFKP provides a foundation for understanding the functional effects of somatic PFK1 mutations identified in cancers. Cancer cells rely on aerobic glycolysis to provide energy and cellular building blocks required to support rapid proliferation. PFK1 activity is increased in cancer cell lines and primary tumour tissues and expression of PFKP is upregulated in breast and liver cancers. The effect of somatic mutations in PFK1 on metabolic adaptation has not been reported. We mapped the 44 reported somatic mutations
in cancers\textsuperscript{27} that were not associated with single nucleotide polymorphisms\textsuperscript{28} onto the structure of PFKP (Fig. 4a and Extended Data Table 3). Analysis by Mutation Assessor\textsuperscript{29} predicted that 28 of these mutations would alter enzyme activity.

We selected three identified somatic mutations for biochemical analysis (Extended Data Fig.7). Arg48 interacts with a bound phosphate ion in the structure (Fig. 4b), and the R48C mutant had reduced citrate inhibition, shifting $E_{50}$ for WT to greater than 0.4 mM for WT to greater than 4 mM (Fig. 4d) but did not markedly change effects of ATP and F6P (Fig. 4e, f and Extended Data Table 2). Analogous mutations in PFKM have been described in Tarui disease\textsuperscript{20,30}. These data indicate that Arg48 is located in the citrate-binding site, which is occupied by phosphate ion in the structure\textsuperscript{28}. A serine substitution for Asn426, located close to the catalytic interface, is predicted to disrupt interactions with the backbone carbonyls of Gln472, Gly473 and Gly474 and the main-chain amide of Ile476, which are involved in positioning a loop at the catalytic interface (Fig. 4c). The N426S mutant partly relieves ATP inhibition, shifting EC\textsubscript{50} for ATP from $\sim$1 mM to greater than 3 mM (Fig. 4e). Located across the catalytic interface from Asn426, Asp564 forms an electrostatic interaction with Arg319 (Fig. 4b). The D564N mutant had decreased maximum velocity and affinity for F6P (Fig. 4f and Extended Data Table 2). We also stably expressed PFKP WT and mutants tagged with green fluorescent protein (GFP) in MTLn3 rat mammary adenocarcinoma cells expressing WT and mutant PFKP–GFP. Data are means $\pm$ s.e.m. of at least five determinations from two independent protein preparations.
production in the glucose-free cell-culture conditions we used. This finding could reflect the ability of PFKP to dynamically alter metabolic states by integrating multiple signals. Additionally, the functional significance of selective PFKP mutations will depend on the mutational signature of the respective cancer in which they occur as well as the relative expression of other PFK isoforms.

In addition to cancer, aberrant glycolytic flux is increasingly recognized as contributing to several other diseases such as obesity, diabetes, and cardiac disease. The biologically relevant tetrameric structures of the PFKP provide information on the catalytic interface and conformational changes upon ATP hydrolysis that contributes to a mechanistic understanding of the functional impact of disease-associated mutations. Additionally, these new structural insights will enable rational drug design for therapeutic development.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** B.A.W. and D.L.B. conceived initial studies with recombinant PFKP. B.A.W. expressed and purified recombinant PFKP, and performed thermobility screens to identify buffer conditions for protein stability. C.A.W. performed X-ray diffraction data collection and processing. F.F. determined and refined the structures. B.A.W. generated and biochemically characterized recombinant WT and mutant PFKP, and generated and analysed cells with heterologous PFKP expression. B.A.W., F.F., L.T., and D.L.B. contributed to writing the manuscript.

**Author Information** Structures of ATP-Mg$^{2+}$- and ADP-bound PFKP have been deposited in the Protein Data Bank (PDB) under accession numbers 4XYJ and 4XYK, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to D.L.B. (diane.barber@ucsf.edu) or L.T. (florent@columbia.edu).
METHODS

No statistical methods were used to predetermine sample size. Cloning, expression and purification of recombinant human PFKP. Homo sapiens PFKP complementary DNA (cDNA) (NM_002627.4) encoding the 794-amino-acid isoform 1 was cloned into the pFastBac HTA vector and baculovirus was generated using the Bac-to-Bac Expression system (Invitrogen) according to the manufacturer’s protocols. Two billion sf21 or Hi5 cells were used to express PFKP at a multiplicity of infection of 1 for 48 h. Cell pellets were resuspended in lysis buffer (20 mM tris(hydroxymethyl)aminomethane (Tris-HCl pH 7.5); 50 mM potassium phosphate; 1 mM 2-mercaptoethanol; 10% glycerol; 10 mM imidazole; cComplete Protease Inhibitor Cocktail tablet (Roche)) and lysed with 15 passes of a dounce homogenizer. Cell debris was removed by centrifugation and the pellet discarded. The supernatant was incubated with Talon resin (Clontech), washed with 20 bed volumes of lysis buffer and eluted with a minimal volume of elution buffer (lysis buffer with 100 mM imidazole). Protein was concentrated using an Amicon Ultracel-30K Centrifugal Filter Unit (Millipore) and buffer exchanged into EPLC buffer (20 mM HEPES, pH 7.5, 100 mM KC1, 1 mM TCEP, 1 mM ATP, 1 mM MgCl₂, and 5% glycerol). PFKP was passed over a Superose 6 10/300 GL column (GE Healthcare) and the peak corresponding to the tetrameric fraction collected. Buffer was exchanged to crystallization buffer (20 mM HEPES, pH 7.5, 100 mM KC1, 1 mM TCEP, 10 mM MgCl₂, and 5% glycerol) containing either 10 mM ADP or 10 mM ATP using an Amicon Ultracel-30K Centrifugal Filter Unit and recombinant PFKP concentrated to 5 mg ml⁻¹. Protein was stored at 4°C. Recombinant PFKP was tested for activity and allosteric regulation before crystallization.

PFKP activity assays. Activity assays for PFK1 were preformed using an auxiliary enzyme assay33. Kinetic studies were performed in 200 μl reaction containing 50 mM HEPES pH 7.4, 100 mM KC1, 10 mM MgCl₂, 0.15 mM NADH, 0.675 units ml⁻¹ aldolase, 5 units ml⁻¹ triosephosphate isomerase and 2 units ml⁻¹ glycerol phosphate dehydrogenase. ATP and F6P were used as indicated. Auxiliary enzymes were de-salted using an Amicon Ultracel-10K Centrifugal Filter Unit before use. The concentration of PFKP was normalized and samples diluted as a 10X stock in 10% glycerol, 20 mM Tris-HCl (pH7.5) and 1 mM DTT immediately before the assay. The temperature was equilibrated to 25°C for 10 min before initiating the reaction with the addition of PFKF. The absorbance at 340 nm was measured using a SpectraMax M5 microplate reader (Molecular Devices). Kinetic studies were performed by linear regression analysis of the Hill equation using Prism (GraphPad Software) and are the average of a minimum of three measurements from two independent preparations of protein (R² > 0.95 for all analyses). An unpaired t-test with equal variance was used to compare the activity of WT and F649L PFKP. One unit (U) of activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of fructose-1,6-bisphosphate per minute at 25°C. Data on the effect of phosphate on PFKP activity were obtained in the presence of 10 mM sodium phosphate or 10 mM sodium chloride as a control.

Transmission electron microscopy. Twenty microlitres of 25 μg ml⁻¹ PFKP was applied to glow-discharged carbon-coated grids and stained with 2% (w/v) uranyl acetate. Grids were examined and photographed with a JESOL 100CX II. For estimation of size of PFKP dimers and tetramers, the length and width of individual particles from TEM images were measured using FIJI ImageJ software32. The average length and width ± s.d. are reported. For experiments analysing the shape and size of PFKP for crystallography studies, the protein was diluted in TEM buffer (20 mM HEPES, pH 7.5, 100 mM KC1, 1 mM DTT, 1 mM ATP, 1 mM MgCl₂ and 5% glycerol). For experiments analysing the oligomeric state of the enzyme in the presence of activators, WT and F649L PFKP were diluted in TEM buffer containing 3 mM ADP, 3 mM ATP and 8 mM F6P. For experiments analysing the oligomeric state of the enzyme in the presence of inhibitors, PFKP was diluted in TEM buffer containing 1 mM citrate.

Crystallization and structure determination. PFKP was crystallized in two different complexed conditions of ADP and ATP-Mg⁺ by a microbatch method at 18°C. For the ADP complex, 2 μl of protein solution containing PFKP (6.35 mg ml⁻¹) was mixed with 1 μl of the precipitant solution consisting of 200 mM potassium sodium tartrate trihydrate, pH 7.4, and 20% (w/v) PEG 3350. The same protein concentration. PFK1 activity assays were performed on the lysate as prev-

Data Table 1. The Ramachandran plots suggest that 88.1% and 81.9% of residues are in the most favoured regions, and there is no residue in disallowed regions, respectively. The trajectory contains two tetramers. A single-wavelength native data set to resolution 3.1 Å was collected at the X4C beamline of the National Synchrotron Light Source. The diffraction images were processed with the HKL package35. The structure of PFK from rabbit skeletal muscle (PDB accession number 3O8L)12 was used to determine the ATP–Mg²⁺ structure of PFKP using the molecular replacement method, with the program MolRep36. Only a monomeric model of PFK from rabbit skeletal muscle resulted in a solution, which led to structure determination of the ATP-bound PFKP structure. The remainder of the PFKP model was built manually with the program Cerius². The structure refinement was performed with CNS35. A similar methodology was used for data collection and processing of the ADP-bound structure of PFKP, the crystal of which diffracted to 3.4 Å at the X4C beamline of the National Synchrotron Light Source. The ADP-bound structure was subsequently determined using a monomeric model of the ATP-bound complex of PFKP, with the program MolRep36 followed by structure refinement by CNS35. The data processing and refinement statistics are summarized in Extended Data Table 1. The Ramachandran plots suggest that 88.1% and 81.9% of residues in ATP-bound complex and ADP-bound complex of PFKP are in most favoured regions, and there is no residue in disallowed regions, respectively. The trajectory between the ATP–Mg²⁺–bound and the ADP-bound structures was generated using UCSF Chimera37. The structures were aligned with Matchmaker tool and the trajectory calculated with the Morph Conformation tool.

Selection of cancer mutations and generation of point mutants. Somatic mutations identified in human cancers were selected from the COSMIC database38 and known single nucleotide polymorphisms were disregarded39. The mutations were modelled onto the structure of PFKP and selected for further analysis. Point mutants at the tetramer interface, F649L and E657A, and cancer mutants, R48C N426S, and D564N, were generated by using a commercially available site-directed mutagenesis kit (QuikChange Lightning, Agilent). DNA primers were designed using the online primer design tool (http://www.genomics.agilent.com/primerDesignProgram.jsp) and purchased from Elix Biopharmaceuticals.

Analysis of cells expressing PFKP. A mammalian PFKP expression construct was generated by PCR amplification and the cDNA inserted into the multiple cloning site of pEGFP-N1 using the restriction enzymes Xhol and BamHI. Cancer mutations were generated by site-directed mutagenesis as described above. Constructs were expressed by transfecting MTLn3 rat mammary adenocarcinoma cells19 using FugenEH (Promega) transfection reagent. One-day post-transfection cells were re-plated into 100 mm dishes and 800 μg ml⁻¹ G418 was added to select for transfected cells. After 1 week of selection, fluorescence-activated cell sorting was used to sort cells expressing GFP. For metabolic assays, cells were seeded onto a six-well plate at a density of 3 × 10⁵ cells per well. One day after re-plating, cells were washed twice in serum- and glutamine-free media, and cells were incubated for 2 h in 1 ml of the same media. Fifty microlitres of the media were collected in triplicate and the amount of lactate in the media measured using an enzyme-linked assay30. One hundred microlitres of reagent A (300 mM hydrazine; 200 mM glycine, pH 9.5, 20 mM β-nicotinamide adenine dinucleotide) and 50 μl of reagent B (200 μM 1-L-lactate dehydrogenase from rabbit muscle (Sigma Aldrich)) were added to each well and incubated for 1 h at 22°C. The absorbance at 340 nm was measured and the amount of lactate was determined from a standard curve. Cells were lysed in buffer (10 mM potassium phosphate, pH 7.5; 0.5% Triton X-100; protease inhibitors; cComplete Protease Inhibitor Cocktail tablet (Roche)), and cellular debris removed by centrifugation and the protein concentration determined by the Bradford method. Lactic-acid levels in the media were normalized to protein concentration. PFK1 activity assays were performed on the lysate as previously described40. Enzyme-linked PFK1 activity assays were performed on 10 μg of total cell lysate as described above with the exception that 10 mM ammonium sulphamate was added to the assay mixture and the auxiliary enzymes were not de-

Levels of PFKP expression were determined by immunoblotting using rabbit anti-GFP (Invitrogen A-11122, 1DB-001-0000868907) and mouse anti-

actin clone C4 (ED Millipore MAB1501, 1DB-001-0000850281) antibodies. Two-sided paired t-tests were used to determine statistical significance.

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Extended Data Figure 1 | Activity of purified recombinant PFKP.

**a**, Coomassie-stained SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of purified PFKP. The molecular mass (MM) of protein standards is shown in kilodaltons (kDa). **b**, Allosteric regulation of PFKP by ATP and ADP; F6P saturation curve PFKP in the presence of 0.25 mM ATP (filled black squares), 3 mM ATP (filled grey circles), 0.25 mM ATP and 0.25 mM ADP (open black squares), and 3 mM ATP and 3 mM ADP (open grey circles). **c**, Effect of ADP on kinetic behaviour of PFKP in the presence of 0.25 mM (black squares) or 3 mM (grey circles) ATP. Data in **b** and **c** are means \( \pm \) s.e.m. of ten (b) or five (c) determinations from two separate protein preparations.
Extended Data Figure 2 | Structure, nucleotide binding and phosphate ion binding of PFKP.  

a, The structure of PFKP protomer can be divided into two halves: the amino (N)-terminal (cyan) and the carboxy (C)-terminal (yellow) subdomains. The N terminus of each subdomain begins with a nucleotide-binding domain (NBD) followed by a smaller substrate-binding domain (SBD). Each NBD closely resembles a canonical Rossmann fold composed of a seven-stranded β-sheet surrounded by six α-helices. Each SBD consists of a four-stranded β-sheet surrounded by five α-helices. Two phosphate ions (stick drawings) are bound in pockets equivalent to the effector binding sites of the E. coli PFK.  

b, Final 2Fo−Fc electron density at 3.1 Å resolution for ATP–Mg2⁺, contoured at 1σ. A strong electron density is observed near β- and γ-phosphate of the nucleotide, which was unambiguously modelled as Mg2⁺ ion. An extended but weaker electron density is also observed near the γ-phosphate of the nucleotide, which is surrounded by three backbone carbonyls of strictly conserved Ser32, Gly34 and Gly172. This electron density was modelled as a second metal ion, although it may belong to a water molecule.  

c, d, Structure of the two inorganic phosphate-binding sites in PFKP.  

e, Plot of concentration of ATP versus relative enzymatic activity of PFKP. Activity is expressed relative to maximal activity at this pH and F6P concentration. Data are means ± s.e.m. of three determinations.
Extended Data Figure 3 | Structural comparison of the two PFKP tetramers in the ATP–Mg²⁺ complex. 
a, Overlay of the structures of the eight PFKP subunits. Only two loops show substantial differences, indicated with the red arrows. 
b, Overlay of the two PFKP tetramers. A noticeable difference is the twisting of the second dimer in the two tetramers, indicated with the red arrow.
Extended Data Figure 4 | Structural comparison of ATP- and ADP-bound PFKP with R- and T-state of *E. coli* PFK. **a**, Structural overlay of ATP-bound (coloured) and ADP-bound (grey) PFKP. For comparison with structures of *E. coli* PFK, the N- and C-terminal subdomains of PFKP are coloured cyan and blue for subunit A, and yellow and orange for subunit B. **b**, The view in **a** is slabbed to highlight the difference between the two structures. **c**, Structural overlay of R-state (coloured; PDB accession number 4PFK) and T-state (grey; PDB accession number 6PFK) of *E. coli* PFK. **d**, The view in **c** is slabbed.
Extended Data Figure 5 | A unique tetramer interface in PFKP.

a, Alignment of residues from PFKP surrounding Phe649 (arrow) with human PFKM and PFKL and S. cerevisiae α- and β-subunits. b, Structure of PFKP tetramer. c, Structure of ScPFK tetramer, viewed roughly in the same orientation as PFKP. The tetramer interface is highlighted in the red box. d, Structure of rabbit PFKM. e, Stereo drawing of the overlay of the tetramer interface of PFKP (in colour) and ScPFK.
Extended Data Figure 6 | Purification and TEM analysis of PFKP tetramer mutants. a, Coomassie-stained SDS–PAGE of PFKP F649L and E657A. b, c, TEM images of PFKP F649L (b) in buffer with activator and substrates (3 mM ADP, 3 mM ATP and 8 mM F6P) and WT PFKP (c) in buffer containing inhibitor (1 mM citrate). Red arrows indicate dimers. Scale bar, 50 nm. d, Activity of WT PFKP and PFKP-F649L in buffer containing 3 mM ADP, 3 mM ATP and 8 mM F6P. Data are means ± s.e.m. of six (WT) and nine (F649L) determinations from two independent protein preparations ($P < 0.001$).
Extended Data Figure 7 | Purification of PFKP cancer mutants and their activity in cells. a, Coomassie-stained SDS–PAGE of purified recombinant PFKP mutants R48C, N426S and D564N. b, Immunoblot of GFP and actin from total cell lysates of MTLn3 rat mammary adenocarcinoma cells expressing PFKP–GFP. Blots are representative of three experiments from individual preparations of cells. c, PFK1 activity (micromoles of F1,6bP produced per minute per nanogram of total cell lysate) was measured in five independent preparations of cells. A two-sided paired t-test was used to determine significance. **P < 0.01; ***P < 0.001.
Extended Data Table 1 | Data collection and refinement statistics

| Data collection | PFKP (ATP-Mg\(^2+\) complex) | PFKP (ADP complex) |
|-----------------|---------------------------------|--------------------|
| Space group     | \( P2_1 \)                       | \( P2_1 \)          |
| Cell dimensions \( a, b, c \) (Å) | 137.2, 159.3, 170.5 | 79.3, 168.4, 133.3 |
| \( \alpha, \beta, \gamma \) (°) | 90, 104.2, 90 | 90, 103.8, 90 |
| Resolution (Å) | 50-3.1 (3.2-3.1) * | 45.4-3.4 (3.5-3.4) * |
| \( R_{merge} \) | 11.7 (68.4) | 15.6 (53.4) |
| \( I/\sigma I \) | 13.9 (1.8) | 4.3 (1.2) |
| Completeness (%) | 93.0 (84.1) | 84.8 (74.3) |
| Redundancy | 6.4 (5.3) | 2.1 (1.9) |

| Refinement | | |
| Resolution (Å) | 50-3.1 (3.3-3.1) | 45.4-3.4 (3.6-3.4) |
| No. reflections | 109,577 (10,980) | 34,657 (3,486) |
| \( R_{work} / R_{free} \) | 22.8/25.8 | 24.2/29.4 |
| No. atoms | 47,148 | 23,644 |
| Protein | 46,792 | 23,500 |
| Ligand/ion | 316 | 144 |
| Water | 40 | 0 |
| B-factors | | |
| Protein | 62.8 | 74.5 |
| Ligand/ion | 45.6 | 66.1 |
| Water | 22.3 | |
| R.m.s deviations | | |
| Bond lengths (Å) | 0.009 | 0.011 |
| Bond angles (°) | 1.3 | 1.2 |

One crystal was used for data collection for each structure.

*Highest resolution shell is shown in parenthesis.
Extended Data Table 2 | Saturation kinetics on WT and mutant PFKP

| Parameter          | Wild type | E657A | R48C | N426S | D564N |
|--------------------|-----------|-------|------|-------|-------|
| Maximum Velocity   | 59.27     | 32.29 | 58.19| 67.41 | 30.60 |
| $S_{50}^{F6P}$ (mM) | 0.83      | 4.51  | 0.84 | 0.82  | 2.04  |
| $n_{H}^{F6P}$ (mM)  | 3.41      | 4.13  | 2.94 | 3.64  | 3.18  |
| EC$_{50}^{ATP}$ (mM)| 0.96      | ND    | 1.19 | >3    | 0.68  |
| EC$_{50}^{Citrate}$ (mM)| 0.40      | ND    | >4   | 0.31  | 1.40  |

Kinetic properties of WT and mutant protein were determined by modelling the sigmoidal part of the curve (V$_{min}$ to V$_{max}$) to the Hill equations. Assays were performed at pH 7.4 with 0.25 mM ATP. For F6P affinity, assays were performed at pH 7.4 with 0.25 mM ATP. ATP and citrate inhibition assays were performed at pH 7.4 with 2 mM F6P (WT, R48C and N426S) or 4 mM F6P (D564N). Citrate inhibition assays were performed with 0.25 mM ATP. ND, not determined.
**Extended Data Table 3 | Somatic mutations of PFKP in cancer**

| Missense Mutation* | Mutation ID* | Ligand Interactions** | Predicted Impact on Activity** | Tissue/Cancer Type* |
|--------------------|--------------|------------------------|---------------------------------|---------------------|
| S32R               | 1603385      | ADP ATP                | High                            | Liver Carcinoma     |
| R48C               | 1347553      | PGA ADP                | High                            | Large Intestine Carcinoma |
| R48H               | 917671       | PGA ADP                | Medium                          | Endometrium Carcinoma |
| M49L               | 1675020      |                         | Medium                          | Lung Carcinoma       |
| S1V                | 538534       |                         | Medium                          | Lung Carcinoma       |
| D120G              | 1492251      | F1,6bP ADP ATP         | High                            | Kidney Carcinoma     |
| G129W              | 1107879      | ADP ATP                | High                            | Lung Carcinoma       |
| L131H              | 330744       | ATP                    | High                            | Lung Carcinoma       |
| Q153K              | 1236264      |                         | Neutral                         | Autonomic ganglia Neuroblastoma |
| A158V              | 917677       |                         | Low                             | Endometrium Carcinoma |
| D175Y              | 1347563      | ATP F6P F1,6bP         | High                            | Large Intestine Carcinoma |
| R219Q              | 1603390      | ATP ADP F6P F1,6bP     | High                            | Liver Carcinoma      |
| E245Q              | 684504       | ATP ADP                | Low                             | Lung Carcinoma       |
| R262Q              | 1347567      | PGA ADP                | Low                             | Large Intestine Carcinoma |
| E286K              | 1474583      |                         | Medium                          | Breast Carcinoma     |
| V293I              | 255484       |                         | Medium                          | Primitive neuroectodermal tumour - medulloblastoma |
| R301H              | 1347571      | F6P                    | High                            | Large Intestine Carcinoma |
| V308M              | 917679       |                         | Medium                          | Endometrium Carcinoma |
| E328Y              | 1603392      |                         | Medium                          | Liver Carcinoma      |
| A332T              | 291604       |                         | Medium                          | Large Intestine Carcinoma |
| P407S              | 233090       |                         | Neutral                         | Skin Malignant melanoma |
| A414D              | 1347575      |                         | High                            | Large Intestine Carcinoma |
| N426S              | 917681       |                         | High                            | Endometrium Carcinoma |
| A445T              | 1220252      |                         | Medium                          | Large Intestine Carcinoma |
| W463C              | 370998       | PGA ADP                | High                            | Lung Carcinoma       |
| G467A              | 332540       | PGA ADP                | Neutral                         | Lung Carcinoma       |
| T470I              | 1702000      |                         | Neutral                         | Skin Malignant melanoma |
| A492T              | 1675022      | F1,6bP                 | High                            | Lymphoid neoplasm    |
| A537S              | 26929        |                         | Low                             | Lung Carcinoma       |
| D564N              | 241318       |                         | Medium                          | Large Intestine Carcinoma |
| R575Q              | 1220254      |                         | Low                             | Large Intestine Carcinoma |
| A603T              | 1492249      |                         | Medium                          | Kidney Carcinoma     |
| K627E              | 1347584      | PGA ADP                | Low                             | Large Intestine Carcinoma |
| K627N              | 72151        | PGA ADP                | Medium                          | Ovary Carcinoma      |
| D648N              | 1560809      |                         | Low                             | Large Intestine Carcinoma |
| N667Y              | 1347586      |                         | Medium                          | Large Intestine Carcinoma |
| P680A              | 917689       |                         | Low                             | Endometrium Carcinoma |
| I689M              | 1128065      |                         | Neutral                         | Prostate Carcinoma   |
| E703D              | 684499       |                         | Medium                          | Lung Carcinoma       |
| K709I              | 255302       |                         | Low                             | Primitive neuroectodermal tumour - medulloblastoma |
| T713A              | 1239809      |                         | Low                             | Oesophagus Carcinoma |
| E734G              | 98079        |                         | Low                             | Upper aerodigestive tract Carcinoma |
| M758I              | 117577       |                         | Medium                          | Ovary Carcinoma       |
| L761P              | 538526       |                         | Medium                          | Lung Carcinoma       |

Residues highlighted in grey were chosen for further characterization.

* Missense mutations identified, mutation identification number and the tissue/cancer type each mutation was identified from COSMIC database31.

** Ligand interactions and predicted impact on activity obtained from Mutation Assessor33. Mutations with ‘High’ or ‘Medium’ impact are predicted to alter enzyme activity.