Phosphoglycerate Mutase Cooperates with Chk1 Kinase to Regulate Glycolysis

HIGHLIGHTS
PGAM cooperates with Chk1 kinase to boost glycolysis in a non-enzymatic manner
PGAM interacts with Chk1 in cancer with oncogenic Ras but not in standard cells
Chemical and genetical interference with PGAM-Chk1 axis abrogates the Warburg effect

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Phosphoglycerate Mutase Cooperates with Chk1 Kinase to Regulate Glycolysis

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SUMMARY
Dysregulated glycolysis, including the cancerous Warburg effect, is closely involved in pathological mechanisms of diseased states. Among glycolytic enzymes, phosphoglycerate mutase (PGAM) has been known to exert certain physiological impact in vitro, whereas its regulatory role on glycolysis remains unclear. Here, we identified that PGAM plays a key role in regulating glycolysis in cancer cells but not in standard cells. Cancer-prone phenotype by PGAM overexpression in vivo was associated with upregulated glycolytic features. PGAM interacts and cooperates with Chk1 to regulate the enhanced glycolysis in cancer cells, especially under oncogenic Ras expressing conditions. Genetic or chemical interference of the PGAM-Chk1 interaction, with intact PGAM activity, abrogated the maintenance of cancerous enhanced glycolysis. Thus, the nonenzymatic function of PGAM is essential for the Warburg effect that accompanies cancerous proliferation.

INTRODUCTION
Glycolysis constitutes an essential metabolism among various organisms, serving not only as an energy source but also for the synthesis of macromolecules (Vander Heiden et al., 2009). The regulation of glycolysis depends on several factors including growth conditions, differentiation status, and environmental stress. In mammalian cells, metabolic adaptation in glycolysis is mediated partly by the activation of specific signaling modules, followed by the transcriptional upregulation of glycolytic enzymes (Dang and Semenza, 1999). In addition to transcriptional factors, several other molecules including signals from nutritional stress, oncogenic stimuli, and posttranscriptional regulators are also deeply involved in glycolytic regulation (Mikawa et al., 2015).

Fine-tuning of glycolysis is required to maintain physiological homeostasis in normal cells and tissues, and dysregulated glycolysis is closely related to pathological features. Impaired glycolysis in vivo is associated with dysfunction in various tissues and degenerative disorders (Goyal et al., 2017; Taylor et al., 2001). Conversely, the pathological enhancement of glycolysis is also observed in several diseased states, such as inflammation (Chimenti et al., 2015), ischemia, and the cancerous Warburg effect in association with increased activity and protein levels for multiple glycolytic enzymes (Warburg, 1956). Although normal cells may adapt to hypoxic conditions by enhancing anaerobic glycolysis and limiting energy demands, cancer cells in vivo continue to grow even in hypoxia, which requires excess glycolysis as a maladaptive metabolism in the core of solid tumors. However, the Warburg effect cannot be explained simply as a consequence of cellular adaptation to hypoxia, as cancer cells maintain enhanced glycolysis even in standard tissue culture conditions (20% oxygen) or in circulating blood (Koppenol et al., 2011). Therefore, a rising question is how the Warburg effect is linked to the other cancerous properties besides adaptation to hypoxia.

Phosphoglycerate mutase (PGAM) is a glycolytic enzyme that converts 3-phosphoglycerate into 2-phosphoglycerate as an isomerase (Rodwell et al., 1957). PGAM consists of two isoforms, PGAM1 and PGAM2, termed also as brain- and muscle-forms, respectively, both of which display a significant similarity in their sequences (79% identity) and enzymatic activities (Kondoh et al., 2005; Mikawa et al., 2014; Zhang et al., 2001). Recent reports suggest that PGAM represents a key factor connecting glycolysis to physiological homeostasis. PGAM supports anti-oxidative defense not only by the reduction of mitochondrial reactive oxygen species (Kondoh et al., 2005, 2007) but also via activation of the pentose phosphate pathway...
(Hitosugi et al., 2012). Moreover, the p53/Mdm2 axis promotes proteolysis of PGAM during senescence-inducing stress (Mikawa et al., 2014), which is consistent with reports that p53 inactivation enhances glycolysis in cancer (Bensaad and Vousden, 2007). In addition to the ubiquitination, PGAM activity is post transcriptionally modulated by the phosphorylation or acetylation (Wang et al., 2017; Xu et al., 2014). Several studies also implicate the involvement of PGAM in human disease. Although PGAM protein and activity are upregulated in many cancerous tissues (Durany et al., 1997), patients with PGAM deficiencies are also reported (Naini et al., 2009). However, the precise regulatory role of PGAM in glycolysis remains unclear.

Here, we report a previously unappreciated role for PGAM in cancerous glycolytic regulation. We observed that PGAM significantly affected the global profiles of glycolysis in cancerous cells. PGAM cooperated with Chk1, previously known as a checkpoint kinase for p53, to boost glycolysis under oncogenic conditions, but not in standard cells. The significance of the PGAM-Chk1 interaction in cancerous glycolysis was validated by several lines of evidence with genetic or chemical ablation of PGAM-Chk1 binding, especially under oncogenic Ras expressing conditions. Thus, PGAM and Chk1 cooperated to regulate cancerous glycolysis.

RESULTS
PGAM Overexpression In Vivo Promotes Chemically Induced Tumorigenesis with Global Increase in Glycolytic mRNAs
We previously reported that heart-specific Pgam2 transgenic (Tg) mice displayed almost normal glycolytic features in the heart (Okuda et al., 2013). However, the impact of global PGAM overexpression in vivo remains unclear. As it has been demonstrated that the overexpression of either PGAM isoform confers similar physiological impact (Kondoh et al., 2005; Mikawa et al., 2014; Zhang et al., 2001), we utilized Pgam2-Tg mice, which exhibited a significant increase of PGAM protein in the whole body by the CAG-promoter driven Pgam2-FLAG transgene (Figure S1A) (Mikawa et al., 2014). Global overexpression of PGAM in vivo did not affect the profiles for glycolytic mRNAs among the various tissues (skin, liver, kidney, muscle, WAT, lung, or heart) under physiological conditions or in MEFs under standard culture conditions (Figures S1B and S1C). Although both isoforms of PGAM were expressed in skin of wild-type mice (Mikawa et al., 2014), however, we noticed that skin in Pgam2-Tg mice was more vulnerable to inflammation caused by a proinflammatory agent, 12-O-tetradecanoylphorbol-13-acetate (TPA) (Figures S2A and S2B). These findings prompted further investigation into the possibility that PGAM overexpression in vivo may affect glycolysis under TPA stress. For this purpose, we applied a protocol of chemically induced carcinogenesis utilizing 7,12-dimethylbenz[a]anthracene (DMBA) initiation followed by TPA promotion in mice skin from control and Pgam2-Tg mice (Figure S2C). We observed that the total number of tumors in Pgam2-Tg mice was slightly increased (Figure S2D), whereas the tumor sizes were strikingly larger than those of control mice throughout the protocol. At the early stage of the experimental process (8–12 weeks) small tumors in Pgam2-Tg mice were already observed (Figure 1A, top panel). Subsequently (12–24 weeks), some tumors in the Pgam2-Tg mice grew to over 6 mm in their diameters, which was not observed in control mice (Figure 1A, middle and bottom panels). Notably, some of the largest tumors in the Pgam2-Tg mice were accompanied by moderate-to-severe ulceration as shown in Figure 1B. At the end of the protocol (24 weeks), pathologic analysis was performed for all tumors in mice. Histologically, tumors in both groups showed acanthisis and hyperkeratosis. All tumors in the control mice were pathologically benign (squamous cell papillomas), whereas 3 of 25 tumors that developed in Pgam2-Tg mice showed marked dyskeratosis, numerous mitoses, and severe disturbances of stratification, which are characteristic of squamous cell carcinoma (SCC) (Figures 1C and 1D).

Notably, we identified clear differences in the mRNA profiles of glycolytic enzymes in the skins of this setting. The levels of glycolytic mRNAs were markedly increased both in papillomas and in skins without tumors from Pgam2-Tg mice compared with those in control mice (Figure 1E). In addition, the expression of these mRNAs was further enhanced in malignant tumors from Pgam2-Tg skin than in benign papillomas of the same genetic background (Figure 1E). Thus, the overexpression of PGAM promotes the expression of glycolytic mRNAs in vivo in chemically induced tumorigenesis.

Abrogation of PGAM Decreases Glycolytic mRNAs in Cancer Cells but Not in Standard Cells In Vitro
We next examined the impact of PGAM inactivation on glycolytic profiles in vitro. First, PGAM1 siRNA was transfected into cancer cell types in which PGAM1 is dominantly expressed: non-small cell lung carcinoma (H1299) and squamous cell carcinoma of human skin (HSC-1) cell lines (Figures S3A–S3C). We observed that
Figure 1. PGAM Overexpression In Vivo Promotes Chemically Induced Tumorigenesis with Significant Increment in Glycolytic mRNAs

Chemical-induced skin tumorigenesis was performed. Control (n = 7) and Pgam2-Tg mice (n = 7) were first treated with DMBA followed by TPA treatment twice a week.

(A) Comparison of tumor numbers between control (wild-type) and Pgam2-Tg mice. Tumors were classified into three subgroups according to their diameters (top panel for tumor size <3 mm; middle panel, 3–6 mm; and bottom panel, >6 mm). The average number of tumors per mouse was compared at the indicated time points.

(B) Representative tumors in control or Pgam2-Tg mice at 24 weeks are indicated.

(C and D) Histopathological analysis of skin tumors. (C) Skin papillomas in control (left panel) and SCC in Pgam2-Tg (right panel) with hematoxylin and eosin staining. Scale bar indicates 200 μm. (D) Summary of pathologic diagnoses of skin tumors in control and Pgam2-Tg mice.

(E) Comparison of mRNA levels for glycolytic enzymes between control and Pgam2-Tg mice. The results in non-tumor forming skin, skin papillomas, and skin SCCs are shown. Data for the indicated genes are shown as relative values against the counterparts in non-tumor-forming skin from control mice. Abbreviations for glycolytic enzymes were shown in Figure S1. *p < 0.05 and **p < 0.005, Dunnett’s multiple comparison test. Data represent the mean ± SEM.

See also Figures S1 and S2.
**Figure A**

H1299

Population doublings

![Graph showing population doublings vs passages](image)

**Figure E**

H1299

Relative value of mRNA

![Bar graph showing relative mRNA values](image)

**Figure B**

H1299

Relative glucose consumption

![Bar graph showing relative glucose consumption](image)

**Figure C**

H1299

Relative deteritted glucose

![Bar graph showing relative deteritted glucose](image)

**Figure D**

H1299

Relative lactate production

![Bar graph showing relative lactate production](image)

**Figure G**

H1299

Relative enzymatic activity

![Bar graph showing relative enzymatic activity](image)

**Figure H**

Pgam1 flox/flox MEFs

Cre-ER

Days

- 2

4

2

4

Control

4-OHT

![Western blot showing enzymatic activity](image)

**Figure I**

Pgam1 flox/flox MEFs

Relative value of mRNA

![Bar graph showing relative mRNA values](image)
PGAM1 knockdown in these cells significantly decreased their proliferative capacities (Figures 2A and S3D), accompanied by a marked reduction in glycolytic measurements such as glucose consumption, glycolytic flux, and lactate production (Figures 2B–2D and S3E–S3G). We observed that mRNA, protein levels, and enzymatic activities of other glycolytic enzymes were also reduced in both cell lines (Figures 2E–2G and S3H–S3J). Notably, the decline in overall glycolytic mRNAs after PGAM1 knockdown was restored by ectopic expression of either mouse Pgam1 or Pgam2 (Figures S4A and S4B), probably due to the high similarity in their sequences and enzymatic activities.

Next, Pgam1flox/lox MEFs expressing Cre-ER were generated by crossing CAG-Cre-ER-Tg mice and Pgam1flox/lox mice, which we established (Figure S4C). Pgam1 mRNA and protein were efficiently ablated after 4-hydroxytamoxifen (4-OHT) treatment of these MEFs (Figures 2H and 2I), followed by a premature senescent growth arrest (Figures S4D and S4E). However, glycolytic mRNA expression in these cells was similar to Pgam1-intact cells (Figure 2I). Similar results were obtained following transfection with Pgam1 siRNA in primary MEFs and human fibroblast WI-38 cells, which predominantly express PGAM1 (Figures S4F and S4G). Collectively, the abrogation of PGAM in standard cells did not affect the levels of other glycolytic mRNAs, in sharp contrast to our findings in cancer cells.

**PGAM Attenuates p53 Phosphorylation and Interacts with Chk1**

In addition to the susceptibility of globally PGAM-overexpressing mice to skin carcinogenesis (Figure 1), we previously reported that PGAM was ubiquitinated via the Mdm2/p53 axis under senescence-inducing stress (Mikawa et al., 2014). We also noticed that, in premature senescent MEFs by Pgam1 ablation, the levels of several p53 targets (p21CIP, Bax, and Fas) and p16Ink4a were significantly increased (Figure 3A). To gain insights into the molecular mechanism of glycolytic regulation by PGAM, we further investigated the possible link between PGAM and the tumor suppressor p53. As p53 activity and protein stability are regulated largely by post-transcriptional modification including its phosphorylation, we examined the p53 profile under oncogenic stress both in Pgam1-Tg and control MEFs expressing Ras-G12V. The protein levels of p21 and Mdm2, downstream effectors of p53, were upregulated in Ras-G12V-expressing control MEFs but were moderately impaired in Ras-G12V-expressing Pgam2-Tg MEFs (Figure 3B). Oncogene-induced stress also upregulated p53 protein levels in control MEFs, accompanied by the enhanced phosphorylation of serine residues 18 and 23 (Di Micco et al., 2006). In contrast, in Ras-G12V-expressing Pgam2-Tg MEFs, p53 serine 23 phosphorylation was largely impaired compared with control, whereas serine 18 phosphorylation was moderately downregulated (Figure 3B). We also observed that ectopic expression of PGAM1 also suppresses p53 serine 23 phosphorylation in MEFs under oncogene-induced stress (Figure S5A). We also examined the DNA damage response in H1299 cell line with transfected WT p53. Similarly, p53 phosphorylation at serine 20, corresponding to serine 23 in mouse p53, was attenuated by ectopic PGAM expression during etoposide-induced DNA damage in H1299 cells (Figure S5B). These data indicate that PGAM partially interferes with p53 function by impacting phosphorylation at serine 23.
p53 phosphorylation at serine 23 after DNA damage and oncogenic stress is mediated either by Chk1 or Chk2, checkpoint serine/threonine kinases, followed by p53 stabilization and activation (Shieh et al., 2000). In cells ectopically expressing PGAM, total protein levels and phosphorylation status of Chk1 and Chk2 (serine 345 and threonine 68, respectively) (Ahn et al., 2000; Zhao and Piwnica-Worms, 2001) were comparable with control cells (Figure 3B). However, we observed that both PGAM1 and PGAM2 physically
interacted with Chk1, but not with Chk2, in p53<sup>−/−</sup> MEFs, as shown with an immunoprecipitation assay (Figures 3C and S5C). To identify the Chk1-interacting domain in PGAM, a series of deletions in PGAM1- and PGAM2-FLAG were assessed for their ability to bind Chk1 (Figures S5D and 3D). Chk1-myc-his efficiently co-immunoprecipitated with full-length PGAM1- and 2-FLAG (T1) and the N-terminal one-third of PGAM (TN) but not with the other central (T4) or C-terminal fragments (TC). Consistently, endogenous PGAM protein co-immunoprecipitated with endogenous Chk1 protein in H1299 cancer cells but not in human standard cells WI-38 (Figure 3E).

Although PGAM has been shown to function as a phosphatase for metabolite (White and Fothergill-Gilmore, 1992), it is unclear whether PGAM possesses activity as a protein phosphatase. We assessed the potential of PGAM to display protein phosphatase activity. Recombinant PGAM1 or PGAM2 proteins with intact PGAM activity were prepared from Escherichia coli (Figure S5E). None of the recombinant PGAM proteins displayed any phosphatase activity against phospho-Ser/Thr peptides as a substrate, at any concentrations (125, 250, or 500 ng) or in any pH conditions tested (pH 6.2, 7.2, 8.2, and 9.2), whereas 3′PPase exhibited clear phosphatase activity (Figures S5F–S5H). In addition, recombinant PGAM proteins exhibited no phosphatase activity in vitro against phosphopeptides that mimicked phospho-p53 (Ser15, Ser20, or Ser15/Ser20; Figures 3F and S5F). Taken together, these data imply that PGAM interacts with and impairs p53 phosphorylation by Chk1, although PGAM is less likely to function as a protein phosphatase.

**PGAM and Chk1 Cooperate to Regulate Glycolytic mRNAs**

We noticed that the interaction of PGAM with Chk1 in p53<sup>−/−</sup> MEFs was enhanced under TPA stress (Figures 4A and S6A), which upregulates glycolytic mRNAs during in vivo chemical tumorigenesis protocol (Figure 1E). To investigate the physiological significance of the interaction between PGAM and Chk1, we evaluated the impact of Chk1 on glycolytic mRNA profiles in vitro. We observed that Chk1 knockdown in the p53-null cell line H1299 was followed by a significant reduction in glucose consumption, glycolytic flux, and lactate production (Figures 4B–4D). Significant reductions in glycolytic mRNAs and proteins were also observed in these cells (Figures 4E and 4F). Treatment with the Chk1 kinase inhibitor UCN01, but not that with the Chk2 kinase inhibitor (Chk2-inhibitor II), induced the downregulation of glycolytic flux and glycolytic mRNAs in H1299 cells (Figures S6B and S6C). Interestingly, although a low concentration of UCN01 (50 nM) did not affect glycolytic mRNA levels in wild-type MEFs, the downregulation of glycolytic mRNAs was observed in Pgam1<sup>−/−</sup> MEFs (Figure 4G), which displayed the same glycolytic profiles and proliferative capacity as those in wild-type MEFs under standard culture condition (Figures S6D–S6G). These data support the notion that PGAM and Chk1 cooperatively affect glycolytic mRNA profiles.

**Oncogenic Ras Pathway Is Required for the PGAM-Chk1 Interaction**

To evaluate the clinical relevance of PGAM-Chk1 cooperation, the impact of PGAM/Chk1 axis on prognosis of patients with cancer was evaluated. According to the database of non-small cell lung cancer (NSCLC; jacob-00182-MSK) (Director’s Challenge Consortium for the Molecular Classification of Lung Adenocarcinoma, et al., 2008) in ProgoScan (http://dna00.bio.kyutech.ac.jp/ProgoScan/) (Mizuno et al., 2009), we divided 104 patients with NSCLC into four groups in terms of the abundance of PGAM1 and Chk1 in cancers; Low-PGAM1 + Low-Chk1 (n = 23), Low-PGAM1 + High-Chk1 (n = 12), High-PGAM1 + Low-Chk1 (n = 29), and High-PGAM1 + High-Chk1 (n = 40) (Figure S7A). We noticed that the prognostic values were most significantly declined in High-PGAM1 + High-Chk1 group, compared with the others (Figure 5A).

Then, we addressed the question of whether the PGAM/Chk1 axis is involved in any cancer-related genetic events. In addition to H1299 and HSC-1 (Figures 2E and S3H), we evaluated the impact of PGAM1 siRNA among thirteen other cancer cells, including skin, breast, colon, bone, and liver cancer cell lines. Among these 15 in total, four cancer cell lines (H1299, HSC-1, SW480, and DLD-1) displayed the most prominent changes in glycolytic mRNAs (ALDO1, TPI, GAPDH, and ENO1) following PGAM1 knockdown (Figures S5B and S7B). For the clinical practice of precision medicine, subsets of oncogene and tumor suppressors are tested to select tailor-made therapy for individuals; for example, 50 cancer-related genes are listed according to Tsongalis et al. (2014) (Table S1). We surveyed 15 cancer cell lines we tested, regarding the mutations in these 50 cancer-related genes according to previous studies (Cancer Genome Atlas Research, 2008; Fujii et al., 1995; Hori et al., 2009; Klijn et al., 2015; Oliner et al., 1992). We noticed that aberrations both in the p53 and Ras pathways were the most common genetic features, shared in these four cell lines (H1299, HSC-1, SW480, and DLD-1) displaying significant glycolytic decline by PGAM1 knockdown (Table S1). As patients with NSCLC with High-PGAM1 + High-Chk1 were classified in low-prognosis subgroup...
(Figure 5A), we also analyzed the PGAM/Chk1 axis in 26 human lung adenocarcinoma cells. Based on the database of Kashiwa Encyclopedia for human genome mutations in Regulatory regions and their Omics contexts (DBKERO; http://kero.hgc.jp/) (Suzuki et al., 2015), these 26 cells are classified into two subgroups; cells with genetic aberrations either in the p53 or Ras pathway (n = 14) and those with aberrations in both (n = 12) (Table S2). Then, we analyzed Pearson correlation between the levels of \( \text{Chk1} \) and glycolytic enzymes. Correlation coefficient between \( \text{Chk1} \) and most glycolytic enzymes (\( \text{HK2}, \text{GPI}, \text{ALDOA}, \text{TPI}, \text{GAPDH}, \text{PGK1}, \text{PGAM1}, \text{ENO1}, \text{and PKM1} \)) is high (over 0.4) in the subgroup with aberrations in both, whereas only that between \( \text{Chk1} \) and \( \text{TPI} \) is high in the former subgroup (Figures 5C, S7C and S7D).

Consistent with these implications on the significance of Ras pathway against PGAM/Chk1 axis, we noticed that PGAM-Chk1 binding is enhanced by the expression of oncogenic Ras-G12V in \( \text{p53}^{-/-} \) MEFs (Figure 5D). To gain the molecular insight on the impact of Ras pathway against PGAM-Chk1 interaction, we applied several inhibitors for the downstream kinases of canonical Ras pathway in H1299; U126 (MEK inhibitor), BI-D1870 (inhibitor for ribosomal S6 kinase [RSK], a downstream kinase of MEK), and Triciribine (Akt kinase inhibitor). Although Akt inhibition unlikely affected the PGAM-Chk1 interaction (Figure S8A), the
Figure 5. Oncogenic Ras Enhances PGAM-Chk1 Interaction

(A) Association between the survival and PGAM1/Chk1 levels was analyzed in patients with non-small cell lung cancer (NSCLC) by PrognoScan database. The survivals of 104 patients with NSCLC (Dataset Jacob-00182-MSK) were plotted with Kaplan-Meier curve. Patients with NSCLC were divided into four groups: Low-PGAM1+Low-Chk1 (black; n = 23), Low-PGAM1+High-Chk1 (blue; n = 12), High-PGAM1+Low-Chk1 (green; n = 29), and High-PGAM1+High-Chk1 (orange; n = 40). *p < 0.05 and **p < 0.005, Log rank test.

(B) Summary of glycolytic mRNA downregulation by PGAM1-knockdown in 15 cancer cell lines tested. Original data of glycolytic mRNA expression were shown in Figure S7B. (−), <20% reduction; (+), reduction from 20 to 40%; (++), >40% reduction. *p < 0.05 and **p < 0.005, Student’s t test.

(C) Summary of correlation coefficients between Chk1 and glycolytic genes, which were evaluated in dataset of 26 NSCLC cell lines. All data were obtained from DBKERO. NSCLC cell lines were classified into two subgroups according to gene aberrations of p53 and Ras pathways as shown in Table S2.

(D) The impact of Ras-G12V expression on PGAM-Chk1 binding. His-tagged protein pull-down assay was performed in indicated cells. PGAM1- or PGAM2-FLAG proteins were detected in left or right panel, respectively.

(E–H) The impact of MEK and RSK inhibition against PGAM-Chk1 interaction. H1299 cells were treated with either U126 (MEK inhibitor) or BI-D1870 (RSK inhibitor). Immunoprecipitation assay was performed to evaluate the interaction.
inactivation of MEK/RSK interferes the binding between PGAM-Chk1 (Figures 5E and 5F), followed by significant reduction in glycolytic flux (Figures 5G and 5H). Thus, oncogenic Ras signals via MEK/RSK pathway are much involved in PGAM-Chk1 interaction.

Nutlin-3a Interferes with the PGAM-Chk1 Interaction

Next, we addressed the question of whether interference with the PGAM-Chk1 interaction affects glycolysis. Nutlin-3a is a compound originally designed to inhibit the physical binding between p53 and Mdm2 (Figure S8B) (Vassilev et al., 2004). We examined the effect of Nutlin-3a on PGAM protein, as PGAM also binds Mdm2 under senescence-inducing stress (Mikawa et al., 2014). Nutlin-3a did not affect the enzymatic activity of recombinant PGAM in vitro, even with the increased concentrations tested (Figure S8C). Although PGAM-Mdm2 binding was not affected by Nutlin-3a (Figures 6A and S8D), Nutlin-3a treatment strikingly abolished PGAM-Chk1 binding in p53/C0/C0 MEFs (Figures 6B and S8E). We also treated p53-null H1299 cells with Nutlin-3a to disrupt PGAM-Chk1 binding to evaluate the effect on glycolytic regulation (Figure S8F). Indeed, glycolytic flux and glycolytic mRNAs in H1299 cells were downregulated by Nutlin-3a in a dose-dependent manner (Figures 6C and 6D).

Recently, the consensus sequence ([L/I/V/M]−[W/Y/F]−x−x−[L/I/V/W]) for Nutlin-3a responsive proteins, including p53, was determined by proteomic analysis (Nicholson et al., 2014). We noticed that two repeats of this consensus motif are located and conserved in the N terminus of human and mouse PGAM, which overlap with the Chk1-interacting domains (Figures 3D and 6E). PGAM mutants (W68A and W78A) with amino acid substitutions in these two repeats of the consensus sequence were generated by PCR mutagenesis (Figure 6E). Although the enzymatic activity and protein levels were maintained in both W68A and W78A mutants (Figures 6F and S8G), PGAM-Chk1 binding was largely impaired (Figures 6Ga and S8H). Thus, Nutlin-3a interferes with the PGAM-Chk1 interaction and the maintenance of glycolytic features but not with the enzymatic activity of PGAM.

Physical Interaction between PGAM and Chk1 Is Essential for Both Maintenance of Glycolysis and Proliferative Potential in Cancerous Cells

As PGAM mutants for Nutlin consensus motif (W68A and W78A) attenuate the interaction with Chk1 in p53+/− MEFs with or without Ras-G12V expression (Figures S8I and S8J), we evaluated the physiological impact of these mutants in the conditions with or without Ras-G12V. For this purpose, Chk1-expressing p53+/− MEFs with or without Ras-G12V were prepared, followed by retroviral infection with various versions of PGAM. In the absence of Ras-G12V, co-expression of Chk1 with WT-, W68A-, or W78A-PGAM resulted in similar profiles for overall glycolytic mRNAs and proliferative capacity (Figures S8K, S9D, 7F, S8L, S9F, and S9G). In sharp contrast, in the presence of Ras-G12V, ectopic expression of PGAM-WT in Chk1-expressing p53+/− MEFs increased the glycolytic parameters (glucose consumption, glycolytic flux, and lactate production), compared with the vector control (Figures 7A–7C and S9A–S9C). Consistently, the expression of glycolytic mRNAs and proteins were upregulated in these cells (Figures 7D, 7E, S9D, and S9E). In the same genetic background, however, PGAM-W68A and W78A mutants displayed no such enhancement in glycolytic profiles (Figures 7A–7E and S9A–S9E).

Strikingly, in the presence of Ras-G12V, both the in vitro proliferative capacity and in vivo tumor growth of PGAM-WT overexpressing cells were much enhanced than control (Figures 7F–7H, S8L, and S9F–S9I). In the same genetic background, however, the cells with binding-deficient mutations (W68A and W78A) showed the similar proliferative potentials in vitro and in vivo to those in control (Figures 7F–7H, S8L, and S9F–S9I). Next, we addressed the question whether W68A or W78A mutation abolished PGAM-Chk1 binding through modulating PGAM dimerization. For this purpose, NanoLuc Binary Technology (NanoBiT) system (Promega) is applied, which enables us to quantify protein-protein interaction between the proteins with large and small NanoBiT tag (Figure S10A) (Dixon et al., 2016). We purified recombinant PGAM1, 2-WT, W68A, and W78A proteins with NanoBiT tags from the extract of bacteria (Figure S10B). We observed these recombinant PGAM proteins displayed similar intact enzymatic activity (Figure S10C). Consistently, the
Figure 6. Cooperation of PGAM and Chk1 for Glycolytic Regulation Is Abolished by Interfering with Their Binding

(A and B) Immunoprecipitation assay or His-tagged protein pull-down assay was performed to evaluate the effect of Nutlin-3a on the interaction between PGAM and Mdm2 (A) or PGAM-Chk1 binding (B). p53−/− MEFs expressing indicated vectors were prepared. Cells were exposed to 5 or 10 μM Nutlin-3a for 48 h. After treatment with 20 μM MG132 for 3 h, cell lysates were collected. PGAM2-FLAG proteins were immunoprecipitated by anti-FLAG antibody (A) or Chk1-myc-his proteins were precipitated by Ni-NTA beads (B).

(C and D) The effect of Nutlin-3a on glycolytic profile. Comparison of glycolytic flux (C) and glycolytic mRNAs (D) among H1299 cells treated with indicated concentration of Nutlin-3a for 48 h (n = 3). Data are relative to control cells. *p < 0.05 and **p < 0.005, Dunnett’s multiple comparison test.

(E) Diagram of the consensus motif of Nutlin-3-target proteins in the amino terminus of PGAM and p53. Consensus motifs, [L/I/V/M]-[W/Y/F]-x-x-[L/I/V/W], are indicated in blue and green. The alignments of the conserved consensus sequence in the amino terminus of PGAM1, 2, and p53 (human and mouse) are also presented. Orange bar, a site for Chk1 binding identified in Figure 3D. Purple bar, site for Mdm2 binding.

(F) PGAM enzymatic activity was measured in Chk1-expressing p53−/− MEFs retrovirally infected with the indicated vectors (empty vector, PGAM2-WT-FLAG, PGAM2-W68A-FLAG, and PGAM2-W78A-FLAG) (n = 3 each; upper panel). Protein levels for various versions of PGAM2-FLAG were shown by western blotting (lower panels). **p < 0.005, Dunnett’s multiple comparison test.

(G) The Chk1-binding activity of PGAM2 mutants (W68A and W78A) compared with that of PGAM2-WT was analyzed with a His-tagged protein pull-down assay. Data represent the mean ± SEM.

See also Figure S8.
Figure 7. W68A and W78A in PGAM, Binding-Deficient Mutations with Intact Enzyme Activity, Abolished the Enhancement of Glycolytic Profiles and Proliferative Capacity in the Presence of Ras-G12V.

(A–E) Comparison of glycolytic profiles among the cells expressing PGAM2 variants (WT, W68A, or W78A) in the presence of Ras-G12V. Glucose consumption (A), glucose flux (B), and lactate production (C) were analyzed among indicated cells on the common genetic background of p53−/− with Ras-G12V and Chk1 (n = 3). The mRNA and protein levels of glycolytic enzymes were also evaluated in indicated cells (D and E). Intensity of immunoblotting bands was normalized to that of actin. Data are relative to controls expressing the empty vector.

(F) The effect of indicated PGAM2 variants on cell growth was assessed by crystal-violet staining in Chk1-expressing p53−/− MEFs with or without Ras-G12V.

(G and H) In vivo tumor growth assay in nude mice. p53−/− MEFs, expressing Ras-G12V, Chk1, and the indicated versions of PGAM2, were injected subcutaneously into nude mice (n = 6). (G) Representative images of tumors. (H) Measurement of
dimerization of either PGAM versions displayed the similar values of $K_d$ (dissociation constant), shown by the saturation curves of NanoBiT detection; 62.5 to 67.1 nM for various versions of PGAM1 and 60.6 to 65.9 nM for those of PGAM2 (Figure S10D). Thus, PGAM-W68 and W78A did not affect dimerization and enzymatic activity of PGAM. However, we noticed that monomer forms of PGAM were much abundant in H1299 cancer cells, compared with those in standard WI-38 cells (Figure S10E). These findings support the possibility that nonenzymatic PGAM is more accumulated in cancer cells.

Finally, we evaluated the impact of enzymatic PGAM mutants in the same setting. As the patients with PGAM deficiency were previously reported (Naini et al., 2009), the point mutation $R90W$ in the patients abolished PGAM enzymatic activity (Tsujino et al., 1995). We generated cDNA clone of PGAM1 and 2 with $R90W$ mutation. We observed that such mutation in both PGAM isoforms abolished PGAM activity (Figures S11A and S11B) but still retains proliferative advantage compared with vector control, in Chk1 and Ras-G12V-expressing $p53^{-/-}$ MEFs (Figures S11C and S11D).

Thus, in accordance with the observation of chemically interfering by Nutlin, the genetical interference of PGAM-Chk1 binding with intact PGAM activity ($W68A$, $W78A$ mutations) abolished cancerous proliferation and enhanced glycolytic profiles, in the presence of oncogenic Ras (Figure 7I).

DISCUSSION

Here we demonstrated a previously unappreciated regulatory mechanism for cancerous glycolysis via the PGAM-Chk1 interaction. Genetic and chemical dissection for its interaction disclosed a “non-enzymatic” role for PGAM, especially under oncogenic Ras expressing conditions. Thus, we unveiled the noteworthy impact of interfering with the PGAM/Chk1 axis in the cancerous glycolysis.

First, our data indicate the evident genetic link between PGAM and the global enhancement of glycolytic profiles frequently observed in cancerous cells. We found a molecular interaction between PGAM and Chk1 as notable glycolytic regulator, which is operating in cancer cells, not in standard cells. Moreover, in addition to the data on the prognosis of patients with cancer, several close correlations between Chk1/PGAM and glycolytic enzymes identified oncogenic Ras mutations, which are highly prevalent both in human cancers and DMBA-treated tissues (Karnoub and Weinberg, 2008), as enhancer of PGAM-Chk1 interaction. Although the other research has also emphasized the distinct impact of PGAM in cancer metabolism (Evans et al., 2005; Vander Heiden et al., 2010), we identified the essential effect of PGAM on the boost of glycolysis in the cancerous conditions.

Second, our study also disclosed the key metabolic role of Chk1 for glycolytic regulation in cancer. Chk1, initially identified as a checkpoint kinase, was known to induce cell-cycle arrest after its activation during DNA damage or senescence-inducing stress (Sancar et al., 2004). Other studies, however, have suggested the opposing notions that Chk1 would be the critical driver of cellular proliferation in ES cells and cancer cells in vitro (Liu et al., 2000; Shimada et al., 2008) and in carcinogenic events in vivo (Tho et al., 2012). Interestingly, Chk1 was reported to be regulated by RSK, the downstream kinase of Ras pathway, during cell cycle progression (Li et al., 2012). RSK is upregulated in many types of cancer including lung cancer and promotes cancerous proliferation (Houles and Roux, 2018), whose impacts on the Warburg effect were scarcely reported. As our findings suggest that oncogenic Ras/RSK activation are closely involved in PGAM-Chk1 binding, the opposing roles of Chk1 with respect to cell proliferation would be cellular-context dependent, including the modulation of Ras/RSK pathway.

Third, our findings in the binding-deficient mutants of PGAM implicate that regardless of its enzymatic activity, PGAM plays a critical role in the regulation of glycolytic upregulation. Although neither the phosphatase nor enzymatic activity of PGAM was found to be required for the functional cooperation with Chk1, the physical interaction between PGAM and Chk1 is essential for glycolytic upregulation. These findings are
rather consistent with the previous notion that the multifaceted roles of some enzymes are independent of their catalytic activity (Mdm2 for Poyurovsky et al. [2003]; PP2A for Takemoto et al. [2009]). It is possible that in cancerous cells with accumulation of PGAM proteins, “nonenzymatic” PGAM cooperates with the Chk1 pathway to boost glycolysis, whose activation is required for the survival of cancers bearing oncogenic Ras mutation (Dietlein et al., 2015). A plausible explanation for the Warburg effect has been proposed as follows: besides hypoxic adaptation, it might not only enable cancer cells to meet demands both for energy and biosynthesis (Lunt and Vander Heiden, 2011) but also protect them from oxidative damages (Kondoh et al., 2007). Interestingly, the recent studies suggested that PGAM interacts with actin proteins as nonenzymatic role, to facilitate cancer motility (Huang et al., 2019; Zhang et al., 2017). In addition to the above, the identification of nonenzymatic role for PGAM suggested that enhanced glycolysis might support proliferative capacity of rapidly dividing cells through the cooperation with Chk1.

Finally, our analysis also supported the clinical significance of PGAM-Chk1 interaction. Nutlin-3a, an anti-cancer drug as inhibitor of p53-Mdm2 binding (Burgess et al., 2016), interfered with the PGAM-Chk1 interaction in a p53-independent manner but not with PGAM enzymatic activity. Thus, Nutlin-3a likely also functions as a glycolytic modulator through interfering with the PGAM-Chk1 interaction. Since the discovery of the Warburg effect, cancer metabolism has long been assumed to be a potential anticancer therapeutic target. However, enzymatic inhibition of glycolysis has failed in clinical trials as anticancer drugs owing to their profound adverse effects (Granchi and Minutolo, 2012). Therefore, targeting the binding between PGAM and Chk1 would represent a potential candidate strategy for anti-cancer therapy.

Limitation of the Study
Skin carcinogenesis protocol was performed in Pgam2-Tg mice, whereas in vivo tumor formation assay in nude mice was performed in PGAM1- or PGAM2-expressing cells. In the future, it would be worthy to explore the PGAM/Chk1 axis in human clinical samples.

Resource Availability
Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Hiroshi Kondoh, hkondoh@kuhp.kyoto-u.ac.jp.

Materials Availability
All unique reagents generated in this study are available on reasonable request with a completed Materials Transfer Agreement.

Data and Code Availability
All data are available in the main text or the Supplemental Information.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101306.

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AUTHOR CONTRIBUTIONS
T.M., E.S., M.S., K.L., J.T.I., H. Kanda, K.T., and M.E.L. researched and analyzed the data. N.I. and M.Y. provided technical support and contributed to discussions. H. Kondoh wrote the paper and supervised this study. All the authors read and approved the manuscript.

DECLARATION OF INTERESTS
T.M. and H. Kondoh have submitted relevant patent on the impact of PGAM-Chk1 for glycolysis.

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Supplemental Information

Phosphoglycerate Mutase Cooperates with Chk1 Kinase to Regulate Glycolysis

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Figure S1. Glycolytic profiles in Pgam2-Tg mice. Related to Figure 1

(A) Ectopic expression of PGAM2-FLAG was detected by immunoblotting in various tissues from control (wild-type) and Pgam2-Tg mice. Anti-FLAG, anti-PGAM, and anti-GAPDH antibodies were used. (B) Relative mRNA levels for glycolytic enzymes in control (WT; n = 4) and Pgam2-Tg (n = 4) mice. Total RNA was extracted from various tissues, including skin, liver, kidney, muscle, white adipose tissue (WAT), lung, and heart. (C) Relative mRNA levels for glycolytic enzymes in control (n = 3) and Pgam2-Tg MEFs (n = 3). Data are relative to those expressed in control. Hk1: hexokinase1; Hk2: hexokinase2; Hk3: hexokinase3; Gpi: glucose phosphate isomerase; Pfkl: phosphofructokinase L; Pfkm: phosphofructokinase M; Aldoa: aldolase A; Aldob: aldolase B; Tpi: triose phosphate isomerase; Gapdh: glyceraldehyde 3-phosphate dehydrogenase; Pgk1: phosphoglycerate kinase1; Pgam1: phosphoglycerate mutase1; Eno1: enolase1; Eno3: enolase3; Pkm1: pyruvate kinase M1.
Mikawa et al Figure S2

A

![Graph showing relative ear thickness over time for Control and Pgam2-Tg groups.](image)

B

![Comparative images of Control and Pgam2-Tg ears.](image)

C

![Diagram illustrating treatment schedule for Control and Pgam2-Tg groups.](image)

D

![Bar graph showing tumors per mouse over weeks for Control and Pgam2-Tg groups.](image)
Figure S2. TPA-induced skin inflammation and chemically induced tumorigenesis in *Pgam2-Tg* mice. Related to Figure 1

(A-B) Comparison of proinflammatory TPA-treated ears between control (wild-type) (n=3) and *Pgam2-Tg* mice (n=3). (A) Mice at 35 weeks were treated with 10 ng TPA on the ears whose thickness were monitored for 8 hours. (B) Representative pictures of TPA-treated ears in control and *Pgam2-Tg* mice. *P*<0.05, Student’s t-test. Data represent the mean ± SEM. (C) Schematic of the chemically-induced skin tumorigenesis protocol. Mice were treated with DMBA on Day 0, followed by treatment with TPA twice a week for 20 weeks. Histological examination of tumors was performed at 24 weeks. (D) Summary of total tumors during the protocol.
Figure S3. Abrogation of PGAM in HSC-1 cells. Related to Figure 2

(A-C) The profiles of PGAM isoforms in H1299, HSC-1, and WI-38 cells. (A) Validation of antibodies against PGAM1 and 2. Western blotting was performed using the extracts from the transfected cells with indicated plasmids. Antibodies against PGAM1 (Abcam, ab129191) or PGAM2 (Abcam, ab183027) were applied. (B) The mRNA profiles of PGAM isoforms in indicated cells were evaluated by RT-PCR. (C) The protein profiles of PGAM isoforms in indicated cells. Anti PGAM antibody detects both isoforms, while anti PGAM1 or PGAM2 antibody detects relevant isoforms, as shown in panel (A). (D-J) The impact of PGAM1 knockdown in HSC-1 cells in vitro. HSC-1 cells were transfected with siPGAM1 (n=3) or scrambled RNA (n=3). (D) The proliferation curves of PGAM1-knockdown HSC-1 cells. Cells were passaged according to 3T3 cell culture protocol. (E-I) The glycolytic profiles were evaluated in PGAM1-knockdown HSC-1 cells. Glucose consumption was evaluated by measuring glucose concentration in medium (E), whereas glycolytic flux was evaluated using 3H-labeled glucose (F). Lactate production was determined by the measurements of lactate concentration in medium (G). (H and I) The mRNA and protein levels for glycolytic enzymes were assessed. The protein levels of the indicated glycolytic enzymes were analyzed by immunoblot (I). (J) Enzymatic activity of aldolase, GAPDH, PGK, PGAM and enolase was measured by spectrometric assay. *P < 0.05 and **P < 0.005, Student’s t-test.
Mikawa et al. Figure S4

A. Relative value of mRNA

- HK1
- GPI
- PFKL
- ALDOA
- TPI
- GAPDH
- PGK1
- PGAM1
- ENO1
- PKM1

siPGAM1 scrambled MEFS

**

B. Mouse PGAM1-FLAG

- mouse PGAM2-FLAG
- siPGAM1

α-FLAG (PGAM) 36
α-PGAM 28
α-Actin 48 (kDa)

C. Mus musculus Pgam1 Locus

- 5'UTR
- Ex1
- Ex2
- Ex3
- Ex4
- 3'UTR

- Floxed Allele
- Deleted Allele

- FRT
- Lox-P

D. Cre (-) Cre (-) +4-OHT Cre (+) Cre (+) +4-OHT

- Pgam1 flox/flox MEFS

- Population doublings

- 4 5 6 7 8 9 10

E. Pgam1 flox/flox MEFS

- Cre (-) +4-OHT
- Cre (+) +4-OHT

- PDS 6

- PDS 10

F. MEFs

- scrambled siPgam1

- Relative value of mRNA

- HK1
- GPI
- PFKL
- ALDOA
- TPI
- GAPDH
- PGAM1
- ENO1
- PKM1

G. WI-38

- scrambled siPGAM1

- Relative value of mRNA

- HK1
- GPI
- PFKL
- ALDOA
- TPI
- GAPDH
- PGAM1
- ENO1
- PKM1
Figure S4. Abrogation of PGAM in H1299 and standard cells.

Related to Figure 2

(A-B) H1299 cells with retroviral expression of empty vector, mouse PGAM1-FLAG, or mouse PGAM2-FLAG were prepared. Indicated cells were transfected with siRNA against endogenous human PGAM1 or scrambled RNA. (A) The levels of glycolytic mRNAs were evaluated (n = 3). *P < 0.05 and **P < 0.005, Dunnett’s multiple comparison test. (B) Immunoblotting detected both ectopically-expressed mouse PGAM-FLAG and endogenous human PGAM proteins in the indicated cells. (C) Construction of Pgam1-conditional KO mice is illustrated by a schematic diagram of the wild-type (WT), floxed, and Pgam1 (exon 2–4) deleted alleles. (D and E) Pgam1flox/flox MEFs with or without Cre-ER were treated with 4-OHT, followed by serial passages according to the 3T3 cell culture protocol. Cre (−) and (+) indicate Pgam1flox/flox MEFs without or with Cre-ER, respectively. (D) Growth curves of indicated cells during 3T3 protocol. (E) SA-β-Gal staining was performed at passage 6 (PD6) and passage 10 (PD10) in Pgam1 ablated MEFs (Pgam1flox/flox + Cre-ER with 4OHT) or control MEFs (Pgam1flox/flox with 4OHT). Scale bar indicates 200 µm. (F and G) Levels of glycolytic mRNAs in siPgam1-transfected standard cells. Primary MEFs (F) or human WI-38 (G) cells were prepared. Data are relative to expression in cells transfected with scrambled RNA. *P < 0.05 and **P < 0.005, Student’s t-test. Data represent the mean ± SEM.
A. MEFs
- PGAM1 FLAG
- Ras-G12V
- α-pS18 (p53)
- α-pS23 (p53)
- α-p53
- α-pS345 (Chk1)
- α-Chk1
- α-FLAG (PGAM)
- α-Actin

(kDa)

B. H1299
- HA-p53
- PGAM1-FLAG
- PGAM2-FLAG
- α-pS15 (p53)
- α-pS20 (p53)
- α-p53
- α-FLAG (PGAM)
- α-Actin

(kDa)

C. p53−/− MEFs
- HA-Chk2
- PGAM1-FLAG
- PGAM2-FLAG
- α-HA (Chk2)
- α-FLAG (PGAM)

(kDa)

D. PGAM activity (mmol/mg/min)
- PGAM1
- PGAM2
- Recombinant protein

(kDa)

E. Phosphopeptide sequence
| Substrate | Phosphopeptide sequence |
|-----------|-------------------------|
| Phospho Ser/Thr | RRAT(p)VA |
| Phospho p53 (Ser15) | PPLS(p)QETFS |
| Phospho p53 (Ser20) | SQETFS(p)DLWKL |
| Phospho p53 (Ser15/Ser20) | PPLS(p)QETFS(p)DL |

G. Phospho Ser/Thr
- pmol phosphate / min
- PGAM1
- PGAM2
- λ-PPase

H. Phospho Ser/Thr
- pmol phosphate / min
- PGAM1
- PGAM2
- λ-PPase

- pH 6.2
- pH 7.2
- pH 8.2
- pH 9.2
Figure S5. The impact of PGAM on p53 phosphorylation and the assessment of protein phosphatase activity of PGAM. Related to Figure 3

(A-B) The impact of PGAM on the phosphorylation status of p53 was evaluated by western blotting. Primary MEFs were stressed by oncogenic Ras-G12V expression (A), while H1299 cells with indicated plasmids were treated with 100 µM Etoposide for 3 h (B). (C) The interaction between PGAM and Chk2 was examined in p53−/− MEFs transfected with HA-Chk2 and PGAM1- or 2-FLAG (upper and lower panels, respectively). (D) The evaluation of the interaction between Chk1 and various versions of PGAM1-FLAG. Left panel shows schematic diagram of various fragments of PGAM1. The binding of each fragment to Chk1 was assessed by co-immunoprecipitation (right panels). Extracts from p53−/− MEFs transfected with the indicated plasmids were immunoprecipitated with an anti-FLAG antibody. (E) Assessment of enzymatic activity for recombinant PGAM1 and PGAM2 protein. (F) The sequence of the phosphopeptides used for the in vitro phosphatase assay. RRAT(p)VA phosphopeptide (Phospho Ser/Thr) is compatible as a substrate for several serine/threonine phosphatases such as protein phosphatases 2A, 2B, and 2C. Phospho p53 (Ser15, Ser20, and Ser15/Ser20) phosphopeptides mimicked the p53 fragment phosphorylated at Ser15, Ser20, or Ser15/Ser20, respectively. (G and H) Phosphatase activity of recombinant PGAM1 and PGAM2 was evaluated. 5000 pmol phosphopeptides were incubated with recombinant PGAM1, PGAM2, or λPPase for 30 min, and the amount of released phosphate from phosphopeptides was monitored. (G) Phosphatase activity against phospho-Ser/Thr peptides was assessed with increasing amounts of recombinant PGAM (125, 250, and 500 ng) or λPPase (62.5, 125, and 250 ng). (H) Phosphatase activity against phospho-Ser/Thr peptides was assessed using 500 ng PGAM1 or PGAM2, or 250 ng λPPase in the indicated pH conditions. Data represent the mean ± SEM.
Figure S6. Impact of Chk1 on glycolytic mRNAs. Related to Figure 4

(A) The impact of TPA treatment on the interaction between PGAM and Chk1. p53−/− MEFs with expression of Chk1-myc-his and PGAM2-FLAG were treated with or without 0.1 µg/ml TPA for 48 hours, followed by treatment with MG132 for 3 h. The cell lysates were immunoprecipitated with an anti-FLAG antibody. (B and C) Effect of UCN01 (Chk1 inhibitor) or Chk2-inhibitor II on glycolytic profiles in H1299 cells (n = 3). H1299 were exposed to 500 nM UCN01 or 10 µM Chk2 inhibitor II for 12 h (upper and lower panel, respectively). (B) Glycolytic flux was evaluated in indicated cells (n=3). (C) The glycolytic mRNA expression was detected by qRT-PCR. Data are relative to expression in control cells. *P < 0.05 and **P < 0.005, Student’s t-test. (D-G) Cytological characterization of Pgam1 heterozygous KO MEFs. (D) Measurement of total PGAM enzymatic activity in WT (n = 3) and Pgam1+/− MEFs (n = 3). (E) Glucose consumption (left), glucose flux (middle), and lactate production (right) in WT (n = 3) or Pgam1+/− MEFs (n = 3). (F) Comparison of glycolytic mRNAs by real-time qRT-PCR between wild-type (WT) (n = 3) and Pgam1+/− MEFs (n = 3). Data are shown as relative values against those in WT. (G) Growth curves of WT and Pgam1+/− MEFs Three independent lines of each MEF were passaged according to 3T3 cell culture protocol. *P < 0.05 and **P < 0.005, Student’s t-test. Data represent the mean ± SEM.
**A**

Genetic aberrations:
- Either in p53 or Ras (14 cells)
- Both in p53 and Ras (12 cells)

**B**

Mikawa et al. Figure S7

**C**

Genetic aberrations: Either in p53 or Ras pathway (14 cells)
- RPKM of TUBA1A
- RPKM of Chk1

**D**

Genetic aberrations:
- Either in p53 or Ras (14 cells)
- Both in p53 and Ras (12 cells)
- $r = 0.09$
- $r = 0.19$

**E**

Genetic aberrations: Both in p53 and Ras pathways (12 cells)
- $r = 0.09$
- $r = 0.19$

Figure S7. Analysis of patient prognosis and cell lines for lung cancer. Related to Figure 5

(A) The comparison plot for PGAM1 and Chk1 levels in 104 NSCLC patients (Dataset Jacob-00182-MSK). According to the expression levels of PGAM1 or Chk1, NSCLC patients were categorized into four groups, as shown in Figure 5A. Cut-off points for PGAM1 and Chk1 were 7.546 and 4.509, respectively. (B) The levels of the glycolytic mRNAs, ALDO1, TPI, GAPDH and ENO1, were evaluated in PGAM1-knockdown cancer cell lines (n = 3 per cell line). Values for indicated targets are relative to those in cells transfected with scrambled siRNA. *P < 0.05 and **P < 0.005, Student’s t-test. (C and D) Association of expression levels among indicated genes was evaluated in dataset of 26 non-small cell lung adenocarcinoma cell lines as shown in Figure 5C. (C) Association between Chk1 and glycolytic enzymes in two indicated subgroups; 14 cells with genetic aberration either in p53 or Ras pathway, and 12 cells mutated in both. (D) Association between expressions of Chk1 and TUBA1A. Correlation-coefficient and significance-probability were presented as r-value and p-value, respectively.
Mikawa et al. Figure S8

**A**

| IP: IgG α-Chk1 | Tric | α-PGAM | α-Chk1 | α-PGAM | α-Chk1 |
|----------------|------|--------|--------|--------|--------|
| α-PGAM        | 28   | 48     |        |        |        |
| α-Chk1        | 28   | 48     |        |        |        |
| Total lysate  |      |        |        |        |        |
| α-Phospho Akt(Ser473) |       |        |        |        |        |
| α-Akt         |       |        |        |        |        |

(kDa)

**B**

| p53/- MEFs | HA-p53 | Nutlin-3a (µM) | Total lysate | IP: α-p53 |
|------------|--------|----------------|--------------|-----------|
| +          | +      | 0              | +            | +         |
| +          | +      | 5              | +            | +         |
| +          | +      | 10             | +            | +         |

**C**

| Nutlin-3a (µM) | 2.5 | 5  | 10 | 20 |
|----------------|-----|----|----|----|
| Relative PGAM activity |     |     |     |     |

**D**

| p53/- MEFs | myc-Mdm2 | PGAM1-FLAG | Total lysate | IP: α-FLAG |
|------------|----------|------------|--------------|------------|
| +          | +        | -          | +            | +          |
| +          | +        | +          | +            | +          |
| +          | +        | +          | +            | +          |

**E**

| p53/- MEFs | PGAM1-FLAG | Chk1-myc-his | Total lysate | Ni-NTA bound |
|------------|------------|--------------|--------------|--------------|
| +          | +          | +            | +            | +            |
| +          | +          | +            | +            | +            |
| +          | +          | +            | +            | +            |

**F**

| H1299 | Nutlin-3a (µM) | IP: IgG α-Chk1 |
|-------|----------------|----------------|
| +     | 0              | 28             |
| +     | 5              | 36             |
| +     | 10             | 63             |

(kDa)

**G**

| PGAM1-FLAG | Chk1-myc-his | α-Actin (kDa) |
|------------|--------------|--------------|
| +          | -            | 36           |
| +          | +            | 48           |

(kDa)

**H**

| p53/- MEFs | PGAM1-FLAG | Chk1-myc-his | Total lysate | Ni-NTA bound |
|------------|------------|--------------|--------------|--------------|
| +          | +          | +            | +            | +            |
| +          | +          | +            | +            | +            |
| +          | +          | +            | +            | +            |

**I**

| p53/- MEFs | PGAM1-FLAG | Ras-G12V | Chk1-myc-his | Total lysate | Ni-NTA bound |
|------------|------------|----------|--------------|--------------|--------------|
| +          | +          | -        | +            | +            | +            |
| +          | +          | +        | +            | +            | +            |
| +          | +          | +        | +            | +            | +            |

**J**

| p53/- MEFs | PGAM2-FLAG | Chk1-myc-his | Total lysate | Ni-NTA bound |
|------------|------------|--------------|--------------|--------------|
| +          | +          | -            | +            | +            |
| +          | +          | +            | +            | +            |
| +          | +          | +            | +            | +            |

**K**

| p53/- MEFs + Chk1 | Vector | WT | W68A | W78A |
|-------------------|--------|----|------|------|
| Relative value of mRNA |      |    |      |      |

**L**

| p53/- MEFs | Ras-G12V + Chk1 | Vector | WT | W68A | W78A |
|------------|-----------------|--------|----|------|------|
| Relative cell number |      |    |    |      |      |

**Figures**

A: Gel images showing protein bands after IP: IgG α-Chk1.
B: Bar graphs showing relative PGAM activity for p53/- MEFs.
C: Bar graphs showing relative PGAM activity for Nutlin-3a (µM).
D: Gel images showing protein bands after IP: α-FLAG.
E: Gel images showing protein bands for p53/- MEFs.
F: Gel images showing protein bands for H1299.
G: Gel images showing protein bands for p53/- MEFs.
H: Gel images showing protein bands for p53/- MEFs.
I: Gel images showing protein bands for p53/- MEFs.
J: Gel images showing protein bands for p53/- MEFs.
K: Bar graphs showing relative value of mRNA.
L: Bar graphs showing relative cell number.

**Notes**

1. Nutlin-3a (µM) concentration levels: 0, 2, 5, 10, 20.
2. PGAM activity levels: 20, 10, 5, 2.5, ng.
3. Protein bands sizes: 28, 48, 63, kDa.
Figure S8. Nutlin and binding-deficient mutations in PGAM interfere with the interaction between PGAM and Chk1, but not with PGAM enzymatic activity. Related to Figure 5, 6 and 7

(A) The impact of AKT inhibition against PGAM-Chk1 interaction. H1299 cells were treated with Triciribine. Immunoprecipitation assay were performed using anti-Chk1 antibody. Ser473 phosphorylation on AKT was evaluated. (B) The interaction between p53 and Mdm2 during Nutlin-3a treatment was evaluated by immunoprecipitation assay. p53−/− MEFS expressing the indicated vectors were exposed to 5 µM or 10 µM Nutlin-3a for 48 h, followed by treatment with 20 µM MG132 for 3 h. The lysates were immunoprecipitated with anti-p53 antibody. (C) Enzymatic activity of recombinant PGAM1 and 2 proteins was assessed in the presence of Nutlin-3a. Various amounts of recombinant PGAM1 and 2 proteins (2.5, 5, 10, and 20 ng) were incubated with indicated concentrations of Nutlin-3a (upper and lower panel, respectively). Data represent the mean ± SEM. (D) The effect of Nutlin-3a on PGAM1 and Mdm2 interaction was evaluated by the immunoprecipitation assay. (E) The evaluation of PGAM1 and Chk1 interaction under Nutlin-3a treatment by the immunoprecipitation assay. (F) The effect of Nutlin-3a on endogenous PGAM-Chk1 binding in H1299 cells. Cells were exposed to Nutlin-3a for 48 h. After treatment with 20 µM MG132 for 3 h, the immunoprecipitation assay was performed, using the indicated antibodies. (G) PGAM enzymatic activity in Chk1-expressing p53−/− MEFs retrovirally infected with the indicated vectors (empty vector, PGAM1-WT-FLAG, PGAM1-W68A-FLAG, and PGAM1-W78A-FLAG) (n = 3 each; right panel). Protein levels for various versions of PGAM1-FLAG were shown by western blotting (left panels). (H and I) His-tagged protein pulldown assay. The Chk1-binding activity of PGAM1 mutants (W68A and W78A) compared to that of PGAM1-WT was analyzed in the absence or presence of oncogenic Ras (H and I, respectively). (J) The interaction of PGAM2-W68A or -W78A mutant with Chk1. The Chk1-binding activity in various versions of PGAM2 (WT, W68A, or W78A) was assessed by His-tagged protein pulldown assay in the presence or absence of Ras-G12V. Chk1-expressing p53−/− MEFs with indicated genes were analyzed. (K) Comparison of mRNA levels for glycolytic enzymes in indicated Chk1-expressing p53−/− MEFs with PGAM2 variants (WT, W68A, or W78A) (n = 3). The data are relative to expression in control cells with the empty vector. (L) Comparison of cell proliferation in vitro. The effect of PGAM2 variants (WT, W68A, or W78A) on cell growth was assessed by crystal violet staining in Chk1-expressing
p53−/− MEFs with or without Ras-G12V as shown in Figure 7F. The staining intensities for three independent assays were shown as relative cell numbers against control. **P < 0.005, Dunnett’s multiple comparison test. Data represent the mean ± SEM.
Mikawa et al Figure S9

A. Relative glucose consumption

B. Relative dephosphorylation

C. Relative lactate production

D. Relative value of mRNA

E. Western Blot

F. Tumor volume (mm3)

G. Relative cell number

H. Matrix invasion assay

I. Tumor volume (mm3)
Figure S9. PGAM1 mutants with W68A or W78A abolished the enhancement of glycolysis and proliferation in oncogenic Ras expressing cells. Related to Figure 7

(A-E) Comparison of glycolytic profiles among the cells expressing PGAM1 variants (WT, W68A, or W78A) in the presence of Ras-G12V. Glucose consumption (A), glucose flux (B), and lactate production (C) were analyzed among indicated cells on the common genetic background of p53−/− with Ras-G12V and Chk1 (n = 3). The mRNA and protein levels of glycolytic enzymes were also evaluated in indicated cells (D and E). (F and G) The effect of indicated PGAM1 variants on cell growth in vitro. Crystal-violet staining was assessed in Chk1-expressing p53−/− MEFs with or without Ras-G12V (F). Intensities of crystal-violet staining were measured (G). (H and I) in vivo tumor growth assay in nude mice. p53−/− MEFs, expressing Ras-G12V, Chk1, and the indicated versions of PGAM1, were injected subcutaneously into nude mice (n=6). (H) Representative images of tumors. (I) Measurement of tumor volumes are shown. *P < 0.05 and **P < 0.005, Dunnett’s multiple comparison test. Data represent the mean ± SEM.
Mikawa et al Figure S10

A

PGAM dimerization

LgBit  SmBit

PGAM

Dimerization

PGAM dimer

Luminescence

B

PGAM1

PGAM2

C

Relative PGAM activity

WT  W68A  W78A

PGAM1

PGAM2

D

PGAM1 WT

PGAM2 WT

Kd=62.5 nM

Kd=65.9 nM

PGAM1 W68A

PGAM2 W68A

Kd=60.6 nM

Kd=65.9 nM

PGAM1 W78A

PGAM2 W78A

Kd=67.1 nM

Kd=62.1 nM

E

Total lysate  WI-38  H1299

Glutaraldehyde

α-PGAM

α-Actin

(kDa)

-  +  +  +

+  +  +  +

-  +  +  +

Dimer  Monomer

-  +  +  +

-  +  +  +

-  +  +  +

-  +  +  +

Total Binding  Specific Binding  Background

Concentration of LgBit protein (nM)
**Figure S10. The dimerization efficiency in various versions of PGAM1 and PGAM2. Related to Figure 7**

(A) Schematic diagrams of NanoBiT systems. Dimerization of PGAM proteins with large and small NanoBiT tag produces luminescence. (B) Coomassie Brilliant Blue staining for indicated recombinant proteins prepared from bacteria. (C) Measurement of PGAM enzymatic activity in indicated LgBiT-tagged PGAM recombinant proteins. (D) Saturated binding of SmBiT-PGAM to LgBiT-PGAM was assessed. 0.25 nM SmBiT-PGAM proteins were incubated with various concentration of indicated LgBiT-PGAM proteins. NanoBiT luminescence of dimerized PGAM proteins was measured. The left panels show dimerization of PGAM1 WT (top), PGAM1 W68A (middle), PGAM1 W78A (bottom), while the right panels indicate those of PGAM2 WT (top), PGAM2 W68A (middle) and PGAM2 W78A (bottom). Triangle; total binding curve of the complex between LgBiT and SmBit proteins. Square; background curve of LgBiT proteins only. Specific binding curve (circle) is obtained by subtracting background signals from total binding ones. Binding dissociation constant (Kd) was estimated from the curve of specific binding. (E) Evaluation of monomer and dimer forms of PGAM in H1299 and WI-38 cells. After 100kd cutoff filtration, the protein extracts from indicated cells were immunoprecipitated by anti-PGAM1 antibody. After the elution of PGAM protein from PGAM1 antibody-conjugated beads by specific peptides, eluted proteins were cross-linked by 0.5% glutaraldehyde for 5 min. Immunoblotting detected monomer or dimer of PGAM proteins (right panel). Left panels showed the immunoblotting in total cell lysates of indicated cells without cross-link.
A. p53-/- MEFs Ras-G12V + Chk1

PGAM1

\(\alpha\)-FLAG (PGAM)
\(\alpha\)-Actin

(kDa)

B. p53-/- MEFs Ras-G12V + Chk1

PGAM2

\(\alpha\)-FLAG (PGAM)
\(\alpha\)-Actin

(kDa)

C. Vector
PGAM1 WT
PGAM1 R90W

D. Vector
PGAM2 WT
PGAM2 R90W

Relative PGAM activity

Relative cell number

**
**
Figure S11. The impact of enzymatic mutant PGAM-R90W on cell growth. 
Related to Figure 7

(A and B) Enzymatic mutation R90W was introduced in PGAM1 (A) and PGAM2 (B). Western blotting was performed to detect FLAG tagged PGAM proteins (left panels). PGAM enzymatic activities were measured in indicated cells (right panels). (C and D) Crystal-violet (CV) staining was assessed in indicated cells to evaluate their proliferative capacity (left panels). The intensities of CV staining were evaluated (right panels).
| Table S1. The list of cancer-related genetic aberrations |
|-------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                             | H1299 | HSC-1 | HSC-5 | Hs578T | MDA-MB-231 | BT-474 | HCT116 | RKO | SW480 | DLD-1 | HT-29 | SJSA-1 | U2OS | Hek293 | HepG2 |
| ABL1                         |        |        |        |        | K7R        |        |        | Y257C |        |        |        |        |        |        |        |        |        | 1
| AKT1                         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1
| ALK                          |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1
| APC                          |        |        |        |        | K1454E    |        |        | Q1338* |        |        |        |        |        |        |        |        |        |        | *1 |
| ATM                          |        |        |        |        | N1005I    | E2469K | A1127V | R2461P |        |        |        |        |        |        |        |        |        |        | 24 |
| BRAF                         |        |        |        |        | G464V     |        |        | V600E |        |        |        |        |        |        |        |        |        |        | 1 |
| CDH1                         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| CDKN2A                       |        |        |        |        | c.1_471   | c.1_471 | R244** | 20    |        |        |        |        |        |        |        |        |        |        | 1 |
| CSF1R                        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| CTNNB1                       |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| EGFR                         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| EGFR                         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| ERBB2                        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| ERBB4                        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| EZH2                         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| FBXW7                        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| FGFR1                        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| FGFR2                        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| FGFR3                        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| FLT3                         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| GNA11                        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| GNAS                         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| GNAQ                         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| HNF1A                        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| HRAS                         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |
| IDH1                         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | 1 |

- * denotes activity change
- ** denotes frameshift mutation
- *** denotes nonsense mutation
Table S1. The list of cancer-related genetic aberrations (continued)

| Gene  | H1299 | HSC-1 | HSC-5 | Hs578T | MDA-MB-231 | BT-474 | HCT116 | RKO | SW480 | DLD-1 | HT-29 | SJSA-1 | U2OS | Hek293 | HepG2 |
|-------|-------|-------|-------|--------|------------|--------|--------|-----|--------|-------|-------|--------|------|--------|-------|
| IDH2  | "1"   | "1"   |       |        | G190D      |       |        |     |        |       |       |        |      |        |       |
| JAK2  | "1"   |       |       |        |            |       |        |     |        |       |       |        |      |        |       |
| JAK3  | "1"   |       |       |        |            |       |        |     |        |       |       |        |      |        |       |
| KIT   | "1"   |       |       |        |            |       |        |     |        |       |       |        |      |        |       |
| KRAS  | "1"   |       |       | G13D   | G13D       | G12V  | G13D   | Q61H|       |       |       |        |      |        |       |
| MET   | "1"   |       |       |        | L238fs*25 |       |        |     |        |       |       |        |      |        |       |
| MLH1  | "1"   |       |       |        | S252*     | L323M | A120S  |     |        |       |       |        |      |        |       |
| MPL   | "1"   |       |       |        |           |       |        |     |        |       |       |        |      |        |       |
| NOTCH1| "1"   |       |       | P915L  | G1195R    | Q2343R|       |     |        |       |       |        |      |        |       |
| NPM1  | "1"   |       |       |        |           |       |        |     |        |       |       |        |      |        |       |
| NRAS  | Q61K  | "1"   |       |        |           |       |        |     | Q61K  |       |       |        |      |        |       |
| PDGFRA| "1"   |       |       |        | Y172F     |       |        |     |        |       |       |        |      |        |       |
| PIK3CA| "1"   |       |       |        | K111N     | H1047R| H1047R |     | D549N | E545K | P449T |        |      |        |       |
| PTEN  | "1"   |       |       |        |           |       |        |     |        |       |       |        |      |        |       |
| PTPN11| "1"   |       |       |        |           |       |        |     |        |       |       |        |      |        |       |
| RB1   | "1"   |       |       |        |           |       |        |     |        |       |       |        |      |        |       |
| RET   | "1"   |       |       |        |           |       |        |     |        |       |       |        |      |        |       |
| SMAD4 | "1"   |       |       |        |           |       |        |     |        |       |       |        |      |        |       |
| SMARCB1| "1" |       |       |        |           |       |        |     |        |       |       |        |      |        |       |
| SMO   | "1"   |       |       |        | V404M     |       | T640A  |     |        |       |       |        |      |        |       |
| SRC   | "1"   |       |       |        |           |       |        |     |        |       |       |        |      |        |       |
| STK11 | "1"   |       |       |        |           |       |        |     |        |       |       |        |      |        |       |
| TP53  | del   | V173L | "1"  | V157F  | R280K     | E285K | P309S | R273H| S241F | R273H | Mdm2 amp |        |      |        |       |
| VHL   | "1"   |       |       |        |           |       |        |     |        |       |       |        |      |        |       |
**Table S1. List of cancer-related mutations in 15 cancer cell lines. Related to Figure 5**

Summary of identified mutations in 15 cancer cell lines. These cells were tested in Figure 5B and S7B. Fifty cancer-related genes were proposed by Tsongalis et al (Tsongalis et al., 2014).

Gene mutations in 15 cancer cells were described in the following literatures (Cancer Genome Atlas Research, 2008; Fujii et al., 1995; Hori et al., 2009; Klijn et al., 2015; Oliner et al., 1992).

*1 Not determined,
*2 amp stands for amplification,
Table S2 Genetic aberrations of lung adenocarcinoma cell lines

| Cells having genetic aberrations either in p53 or Ras pathway | Genetic aberrations |
|--------------------------------------------------------------|---------------------|
|                                                              | EGFR    | KRAS    | NRAS    | HRAS    | p53     |
| A427                                                          | G12D    |         |         |         |         |
| A549                                                          | G12S    |         |         |         |         |
| H1437                                                         | G12S    |         |         |         | R267P   |
| H1648                                                         |         |         |         | L35Fs*8 |         |
| H1703                                                         |         |         | c919+1G>T|         |         |
| H1819                                                         |         |         | c933+1G>T|         |         |
| H2126                                                         |         |         |         | E62*    |         |
| H2228                                                         |         |         |         | Q331*   |         |
| H2347                                                         | L19F    | R481H   | Q61R    |         |         |
| H322                                                          |         |         |         | Arg248Leu|         |
| PC-3                                                          |         |         |         | K139fs*31|         |
| RERF-LC-KJ                                                    |         |         |         | E224D   |         |
| VMRC-LCD                                                      |         |         |         | R175H   |         |
| LC2                                                          |         |         |         | S241C   |         |
Table S2. Genetic aberrations of lung adenocarcinoma cell lines (continued)

Cells having genetic aberrations both in p53 and Ras pathways

| Cells                | Genetic aberrations          |
|----------------------|------------------------------|
|                      | EGFR | KRAS | NRAS | HRAS | p53       |
| ABC-1                | L858R|      |      |      | P278S     |
| H1299                |      | Q61K | c1_954>AAG |      |           |
| H1650                | E746_A750del |      | c673-2A>G |      |           |
| H1975                | T790M, L858R |      | R273H |      |           |
| II-18                | L858R|      |      |      | K164*     |
| PC-14                | E746_A750del |      | Arg248Trp |      |           |
| PC-7                 | G719S| G12V |      |      | His214Arg |
| PC-9                 | E746_A750del |      | Arg248Gln |      |           |
|                      | L858R|      |      |      |           |
| RERF-LC-MS           |      | K12S | F134fs*14 |      |           |
| RERF-LC-OK           | L858R|      |      |      | F113C     |
| RERF-LC-ad1          |      | G12A | P278F |      |           |
| RERF-LC-ad2          | L747_A750>P |      | A159V |      |           |

Table S2. Classification of 26 non-small cell lung cancer (NSCLC) cell lines. Related to Figure 5

26 NSCLC cell lines were classified into two subgroups according to gene aberrations of p53 and Ras pathways.

Upper list shows 14 cells harboring genetic aberrations either in p53 or Ras pathway, while lower list indicates 12 cells with genetic aberrations in both pathways.

Gene mutations of NSCLC cells were described in the following literatures (Barretina et al., 2012; Chen et al., 1993; Fujita et al., 1999; Iwakawa et al., 2010; Kashii et al., 1994; Kaufman et al., 2017; Nagai et al., 2005; Notsuda et al., 2013; Wei et al., 2003)
Table S3. The primer sequences for real-time qRT-PCR

| Name   | Sequence                                    |
|--------|---------------------------------------------|
| mouse  |                                             |
| Rpl13a qRT Fw | 5'-TGC TGC TCT CAA GGT TGT TCG -3'        |
| Rpl13a qRT Re | 5'-GCC TTT TCC TTC CGT TTC TCC -3'        |
| Hk1 qRT Fw   | 5'-AAG AAT GGC CTC TCC CGG-3              |
| Hk1 qRT Re   | 5'-CGC CGA GAT CCA GTG CAA TG-3'          |
| Hk2 qRT Fw   | 5'-ATA TGG TTG CCT CAT CTT GG-3'          |
| Hk2 qRT Re   | 5'-CTC CCT CCC TCC CAA TG-3'              |
| Hk3 qRT Fw   | 5'-ATT CCT GGA TGC ATA CCC CGT -3'        |
| Hk3 qRT Re   | 5'-GCC GCT GCA CCT AAA ACC TTT -3'        |
| Gpi qRT Fw   | 5'-CCA ATG CAG AGA CAG CAA AGG-3'         |
| Gpi qRT Re   | 5'-CACCCTGGCGCTTGTCTAGA-3'                |
| Pfkm qRT Fw  | 5'-TGG AGC GAC TTG CTG AAT GAT -3'        |
| Pfkm qRT Re  | 5'-TCA TGG TCG ATT GAG CCA ACC -3'        |
| Pfkl qRT Fw  | 5'-GCT GCA ATG GAG TTG TG-3'              |
| Pfkl qRT Re  | 5'-GTA GCC AGG TAGC CAC AG-3'             |
| Tpi qRT Fw   | 5'-ATC AGA AGC ATG TGA CCG GTG-3'         |
| Tpi qRT Re   | 5'-TGC CAA ACA ATG AGC ACT GC-3'          |
| Aldoa qRT Fw | 5'-CTG GCC ATC ATG GAA AAT GC-3'          |
| Aldoa qRT Re | 5'-TCA AGT CAT GGT CCC CAT CAG-3'         |
| Aldob qRT Fw | 5'-ATC GGC GGA GTG ATC CTT TT-3'          |
| Aldob qRT Re | 5'-TCC AAC TTG ATG CCC ACC A -3'          |
| Gapdh qRT Fw | 5'-AGC CTC GTC CCG TAG ACA AAA-3'         |
| Gapdh qRT Re | 5'-TGG CAA CAA TCT CCA CTT TGC-3'         |
| Pgk1 qRT Fw  | 5'-TTT GGA CAA GCT GGA GTG GAA-3'         |
| Pgk1 qRT Re  | 5'-GCT TGG AAC AGC AGC CTT GAT-3'         |
| Pgam1 qRT Fw | 5'-GCT GCG AGA TGC TGG CTA TGA-3'         |
| Pgam1 qRT Re | 5'-CAC ATC TGG TCA ATG GCA TCC-3'         |
| Pgam2 qRT Fw | 5'-TGG AAT GAG GAG ATC GCA CCT -3'        |
| Pgam2 qRT Re | 5'-TCG GAC ATC CCT TCC AGA TGT -3'        |
| Eno1 qRT Fw  | 5'-TAT TGC GCC TGC TCT GTG TAG-3'         |
| Eno1 qRT Re  | 5'-GGA TGG CAT TTG CAC CAA AT-3'          |
| Eno3 qRT Fw  | 5'-GGA GAA GAA GGC CTG CAA TTG -3'        |
| Eno3 qRT Re  | 5'-CCC AGC CAT TAG ATT GTG CAA -3          |
| Pkm1 qRT Fw  | 5'-CTG TTT GAA GAG CTT GTG GCG -3         |
| Pkm1 qRT Re  | 5'-CTG CTA AAC ACT TAT AAG AGG CC -3      |
| p16INK4 qRT Fw | 5'-CCC AAC GCC CCG AAC T -3'             |
| p16INK4 qRT Re | 5'-GCA GAA GAG CTG CTA CGT GAA -3       |
| Name       | Sequence                                      |
|------------|-----------------------------------------------|
| mouse p21CIP1 qRT Fw | 5'-AGA CAT TCA GAG CCA CAG GCA-3'             |
| mouse p21CIP1 qRT Re  | 5'- ATG AGC GCA TCGCAA TCA C -3             |
| mouse Bax qRT Fw   | 5'-AGA AAA CTG GTG CTC AAG C -3'             |
| mouse Bax qRT Re   | 5'-AGA AAA CTG GTG CTC AAG C -3'             |
| mouse Fas qRT Fw   | 5'-CAG ACA TGC TGTGGA TCT GG -3'             |
| mouse Fas qRT Re   | 5'-CCT CAG CTT TAA ACT CTC GGA -3             |
| human Rpl13a qRT Fw| 5'-CTG GAC CGT CTC AAG GTG TT -3'             |
| human Rpl13a qRT Re| 5'-GCC CCA GAT AGG CAA ACT T -3'             |
| human Hk1 qRT Fw   | 5'-ACA TTG TCT CCT GCA TCT CTG -3'            |
| human Hk1 qRT Re   | 5'-GCC TTA AAA CCC TTT GTC CAC -3'            |
| human Gpi qRT Fw   | 5'-GCT TCT ACC AAT GGG CTC ATC -3'            |
| human Gpi qRT Re   | 5'-TGT CCA GGA ACA TGC AGT G -3'             |
| human Pfk1 qRT Fw  | 5'-AAC GAG AAG TGC CAT GAC TAC -3'            |
| human Pfk1 qRT Re  | 5'-GTC CCA TAG TTC CGG TCA AAG -3'            |
| human Tpi qRT Fw   | 5'-TCA TCG CAG ATA ACG TGA AGG -3'            |
| human Tpi qRT Re   | 5'-CAT CAG AGA CTG TGC ACT TCA G -3'          |
| human Aldoa qRT Fw | 5'- GGT GTC ATC CTC TTC CAT GAG -3'          |
| human Aldoa qRT Re | 5'-GTA GTC TCG CCA TTT GTC CC -3'            |
| human Gapdh qRT Fw | 5'-CTT TGTCAA GCT CAT TTD CGT G -3'          |
| human Gapdh qRT Re | 5'-TCT TCC TCG TCT GC GTC G -3'              |
| human Pkg1 qRT Fw  | 5'-GCT TCT GGG AAG AAG GTT AAA G-3'          |
| human Pkg1 qRT Re  | 5'-CTG TGG CAG ATT GAC TCC ATC -3'            |
| human Pgam1 qRT Fw | 5'-GGA GGC GCT CCT ATG ATG TC -3'            |
| human Pgam1 qRT Re | 5'-ATC TGG TAT GTG TGG ATG TCA C -3'          |
| human Enol qRT Fw  | 5'-TTT GAG CAG AGG TTT ACC AC -3'            |
| human Enol qRT Re  | 5'-TTC CCA ATA GCA TGC TTT GC GC -3'          |
| human Pkm1 qRT Fw  | 5'-CCA TAA TCG TCC TCA CCA AGT C-3'          |
| human Pkm1 qRT Re  | 5'-GGA AGA TGC CAC GGT ACA -3'               |
| human Chk1 qRT Fw  | 5'-GAG AAT CCA TCA GCA AGA ATT ACC -3'       |
| human Chk1 qRT Re  | 5'-GAA TGT GCT TAG AAA ATC CAC TGG-3'        |
| human Hif1 qRT Fw  | 5'-AAC ATA AAG TCT GCA ACA TGG AAG -3'       |
| human Hif1 qRT Re  | 5'-TTT GAG TGG TGA GGA ATG GG -3'            |

Table S3. Primers for real-time qRT-PCR. Related to Figure 1 to 7
Sequences of primer used for real-time qRT-PCR in this study.
Transparent Methods

Cell culture
The human cell lines H1299, RKO, SW480, DLD-1, HT-29, Hs578t, MDA-MB-231, BT-474, U2OS, and SJSA-1 were obtained from American Type Culture Collection, and HCT116, Hek293, HepG2, and WI-38 were from RIKEN Bioresources Center. HSC-1 and HSC-5 were obtained from the Japanese Collection of Research Bioresources. The PLAT-A packaging cell line was a kind gift from Dr. Toshio Kitamura (University of Tokyo). The human cell lines (H1299, Hs578t, MDA-MB-231, BT-474, HCT116, RKO, SW480, DLD-1, HT-29, HEK293, HepG2, SJSA-1, U2OS, and WI-38) were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). HSC-1 cells were cultured in DMEM with 20% FBS. HSC-5 cells were cultured in Iscove’s modified Dulbecco’s medium with 10% FBS. MEFs isolated from embryos (postcoital day 13.5) of C57BL/6 mice were grown in DMEM with 10% FBS and antibiotics (Carnero et al., 2000). Pgam1flox/flox ER-Cre MEFs were generated by crossing between CAG-ER-Cre mice (Hayashi and McMahon, 2002) and Pgam1flox/flox mice. At passage 4, Pgam1flox/flox ER-Cre MEFs or Pgam1flox/flox MEFs were treated with 0.5 µM 4-OHT for 4 days. Ablation of PGAM was detected by immunoblotting. All cell lines were tested and shown to be negative for mycoplasma contamination.

Cytochemical staining for SA-β-Gal was performed (Dimri et al., 1995). At passage 6 and 10, Pgam1flox/flox ER-Cre or Pgam1flox/flox MEFs were washed twice with ice-cold phosphate-buffered saline (PBS) buffer and were treated by fixation solution (2% formaldehyde and 0.2% glutaraldehyde in PBS buffer) for 5 min at room temperature. After removing the fixation solution, cells were washed twice with PBS buffer and were incubated with staining solution (40 mM citric acid/Na phosphate buffer, 5 mM K4[Fe(CN)6] 3H2O, 5 mM K3[Fe(CN)6], 150 mM NaCl, 2 mM MgCl2, 1 mg/ml X-gal) for overnight at 37°C. Images were recorded using a microscope (IX-73, Olympus, Tokyo, Japan).

Cells were subjected to retroviral infection or transfection of plasmids. For the proliferation assay, 5 × 10^3 cells were plated on 6-cm dishes. After 10 days of culture, cells were fixed with 2% glutaraldehyde, followed by crystal violet staining. Then the staining was resolved by 1% sodium dodecyl sulfate. The optical density was determined at 590 nm.

Plasmid DNA
Expression of full-length mouse Pgam1 and Pgam2 with a C-terminal 3X FLAG tag and deletion mutants of Pgam2 (TN, T4, and TC) with a C-terminal 3X FLAG tag driven by the CMV promoter of the p3xFLAG-CMV14 expression vector were constructed previously (Mikawa et al., 2014). The deletion mutants of Pgam1 (TN, T4, and TC) with a C-terminal 3X FLAG tag were generated by PCR. The relevant variants of Pgam1 and Pgam2 mutantos (W68A, W78A and R90W) were generated
by PCR-based mutagenesis. pHygro MarxIV retroviral vectors encoding mouse
Pgam1 and Pgam2 variants were also generated. The relevant variant Pgam1 and
Pgam2 cDNAs were ligated into the XhoI site of Hygro Marx IV vectors. The pBabe-
puro-Ras-G12V plasmid was a gift from Dr. Kayoko Maehara (Kio University, Nara,
Japan). The pcDNA3.1 Chk1-myc-his vector was previously described (Niida et al.,
2007). Chk1 cDNAs were sub-cloned into WZLneo retroviral vectors. Human p53
cDNA was sub-cloned into the pcDNA3.1 vector containing a 3X HA sequence
between the Nhel and XhoI sites. Expression of full-length p53 with an N-terminal
3X HA-tag was driven by the CMV promoter. Expression vector of human Mdm2
with an N-terminal HA-tag was constructed previously (Mikawa et al., 2014).
NanoBiT vectors were generated using Flexi Vector Systems (Promega). In brief,
wild type and relevant mutants of Pgam1 and Pgam2 (W68A, W78A) were
subcloned into pF4A plasmid. Subsequently, those relevant variants of Pgam were
subcloned into N-terminus LgBiT-tagging plasmid (pFN33K) or SmBiT-tagging
plasmid (pFN35K).

Reagents
Reagents were obtained as follows. UCN01, DMBA, TPA, and Nutlin-3a from
Sigma–Aldrich (St. Louis, MO). MG132 from Peptide Institute, Inc. (Osaka, Japan).
U126 and Triciribine from FUJIFILM Wako Pure Chemical Corporation (Osaka,
Japan). BI D-1870 from Selleck (Houston, TX).

Mouse models
All procedures for animal experiments were performed in accordance with the
principles and guidelines of the Animal Care and Use Committees of Kyoto
University Graduate School of Medicine.

Pgam2-Tg mice were generated previously (Mikawa et al., 2014). Pgam2-
Tg is a strain of transgenic C57BL/6 mice that overexpresses the Pgam2 with a
3xFLAG tag under the cytomegalovirus immediate-to-early enhancer element and
chicken β-actin promoter (CAG) (Niwa et al., 1991) For generation of the Pgam1
KO mouse, the targeting vector was constructed as follows. Exons 2–4 of mouse
Pgam1 were flanked by two loxP sequences, and a FLP recognition target (FRT)-
flanked neo-cassette was inserted upstream of exon 2. The targeting vector was
introduced into mouse embryonic stem cells (C57BL/6), which were injected into
blastocysts (BALB/c). Generated founder mice (Pgam1\textsuperscript{floxF/\textsuperscript{+}} [neo+]) were crossed
with FLP transgenic mice for removal of the neo-cassette from heterozygous flox
mice. Pgam1\textsuperscript{+/−} mice were generated by crossing Pgam1\textsuperscript{floxF/\textsuperscript{+}} mice and CAG-Cre
Tg mice, which ubiquitously express Cre recombinase under the CAG promoter.
Genotyping was performed using PCR.
Mouse experiments
For assessment of inflammatory response, control or \textit{Pgam2-Tg} mice (18 weeks old, male) were treated with 10 ng TPA on the ears, and the thickness of the ears was measured by constant pressure thickness gauge (PG-201, TECLOCK, Japan).

Chemically induced skin tumorigenesis was performed (Takeuchi et al., 2010). Briefly, seven age-matched (12–15 weeks old, male) pairs of control or \textit{Pgam2-Tg} mice were shaved and treated with 100 µg DMBA in 100 µl acetone. Mice were subsequently treated twice weekly with 12.5 µg TPA in 100 µl acetone for 20 weeks. The number and size of papillomas per mouse were recorded every 2 weeks. The nude mouse xenograft assay was performed (Mikawa et al., 2014). \textit{CAnN.Cg-Foxn\textsuperscript{nu}/CrlCrlj} mice (8 weeks old, male) were injected subcutaneously with $5 \times 10^6$ cells suspended in 100 µl phosphate-buffered saline. Tumor formation was assessed after 2 weeks.

Transfection and retroviral infection
Transfection of siRNA for mouse \textit{Pgam1}, human \textit{Pgam1} (Life Technologies, Carlsbad, CA), and human \textit{Chk1} (Sigma–Aldrich, St. Louis, MO) was performed using Lipofectamine siRNA MAX (Invitrogen, Carlsbad, CA). Expression vectors were transfected by polyethyleneimine MAX (Polysciences, Warrington, PA). The PLAT-A packaging cell line was used for retroviral production (Morita et al., 2000). Retrovirus-infected cells were treated with hygromycin (75 µg/mL), G418 (400 µg/mL), or puromycin (2 µg/mL) for positive selection of infected cells.

Immunoblotting and immunoprecipitation
For immunoblotting, cell lysates were prepared (Carnero et al., 2000). Cells were washed twice with ice-cold PBS and lysed in buffer containing 50 mM Tris–HCl (pH 7.5), 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 0.5% Triton-X100, 50 mM NaF, 1 mM dithiothreitol (DTT), 1 mM Na\textsubscript{3}VO\textsubscript{4}, 1 mM phenylmethanesulfonyl fluoride (PMSF), and protease inhibitor cocktail (Sigma–Aldrich). After 30 min on ice, lysates were cleared by centrifugation. Equivalent amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Anti-PGAM antibody (1:1000), which recognizes both PGAM1 and PGAM2 (Mikawa et al., 2014). Anti-FLAG (F1804, 1:1000), anti-human Chk1 (C9358, 1:3000), and anti-Actin (A4700, 1:1000) from Sigma–Aldrich. Anti-p21\textit{CIP1} (ab7960, 1:500), anti-phospho-p53 (Ser23; ab59206, 1:500), anti-Chk2 (ab8108, 1:1000), anti-Aldolase (ab169544, 1:1000), anti-PGK1 (ab38007, 1:1000), anti-ENO1 (ab155102, 1:1000), anti-PGAM1 (ab129191, 1:1000) and anti-PGAM2 (ab183027, 1:1000) from Abcam (Cambridge, UK). Anti-c-myc (9E10, 1:200), anti-
human p53 (DO-1, 1:1000), anti-mouse Chk1 (Sc-8404, 1:1000) and anti-Mdm2 (SMP-14, 1:200) from Santa Cruz Biotechnology (Dallas, TX). Anti-mouse p53 (1C12; #2524, 1:1000), anti-phospho-p53 (Ser20; #9287, 1:200), anti-phospho-p53 (Ser15; #9284, 1:1000), anti-phospho-Chk1 (Ser345; #2341, 1:1000), and anti-phospho-Chk2 (Thr68; #2661, 1:500), anti-ERK1/2 (#4370, 1:2000), anti-phospho-ERK1/2 (Thr202/Tyr204, #4370, 1:2000), anti-Bad (#9339, 1:1000), anti-phospho-Bad (Ser112, #9391, 1:500), anti-Akt(pan) (#4691, 1:1000) and anti-phospho-Akt (Ser473, #9271, 1:1000) from Cell Signaling Technology (Danvers, MA). Anti-GAPDH (MAB374, 1:3000) from Merck Milipore (Burlington, MA).

For immunoprecipitation assays, cell lysates were precipitated with the relevant antibody for 2 h. Immune complexes were recovered with protein G-agarose beads, washed four times with lysis buffer, and boiled in 2× Laemmli sample buffer for 5 min. Denatured immune complexes were resolved by SDS-PAGE. The quantification of the immunoblot bands were performed using ImageJ.

Dimer and monomer forms of proteins were detected by the modification of the previous protocol as follows (Jagemann et al., 2008). Cultured cells were lysed with lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton-X100, 50 mM NaF, 1 mM Na3VO4, 1 mM PMSF and protease inhibitor cocktail (Sigma–Aldrich). After the fractionation by centrifugal 100 kDa cutoff filter (Amicon Ultra-4, Merck Milipore Ltd) for 45 min, the resulting extracts were immunoprecipitated by anti-PGAM1 antibody (ab2220, Abcam) for 2 hours. Immunoprecipitants were incubated with 100 µg/ml synthetic peptide (KAMEAVAAQGKAKK), whose sequence matched with those in C-terminus of PGAM1 protein, the antigenic determinant of anti-PGAM1 antibody. Eluted PGAM proteins were cross-linked by 0.5% glutaraldehyde for 5 min at 37°C. Forms of PGAM proteins were evaluated by immunoblotting.

**Measurement of glycolytic enzyme activity**
Enzymatic activity of aldolase, GAPDH, PGK and enolase were measured spectrophotometrically using enzyme activity assay kit (BioVision, Milpitas, CA). PGAM enzymatic activity was measured (Kondoh et al., 2005). Briefly, cell lysates or recombinant proteins were incubated in reaction buffer (100 mM Tris–HCl [pH 8.0], 100 mM KCl, 0.5 mM EDTA, 2 mM MgCl₂, 0.2 mM NADH, 3 mM ADP, and 10 µM 2,3-diphosphoglycerate) with enzyme mixture (0.6 U lactate dehydrogenase, 0.5 U pyruvate kinase, and 0.1 U enolase). Next, 1mM 3-phosphoglyceric acid was added and incubated at 37°C. Activity was measured as NAD⁺ release.
Glycolytic flux measurement
Glycolytic flux was measured (Kondoh et al., 2005). After cells were cultured for 11 h in DMEM containing 25 mM glucose, medium was exchanged for DMEM containing 4.25 mM glucose. After 10 h, D-[3-3H] glucose was added to the medium. After 6 h, an aliquot of medium was precipitated with perchloric acid. The supernatant was applied to a column filled with DOWEX 1X8 200–400 MESH Cl resin (Sigma–Aldrich), and the amount of [3H] water in the flow through was normalized to protein content. The glucose and lactate concentrations in the culture medium were determined using a glucose or lactate assay kit (BioVision), respectively. The value of glucose consumption or lactate production was normalized to the protein content of the corresponding cell lysate.

RNA analysis
Total RNA was extracted with TRIzol (Invitrogen). cDNA pools were generated using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Real-time quantitative PCR was performed using the Thermal Cycler Dice Real-Time system (Takara Bio., Kusatsu, Japan) and Thunderbird SYBR qPCR mix (Toyobo). Gene expression levels were normalized to Rpl13a mRNA and presented as values relative to controls. The primers used are shown in Table S3.

In vitro phosphatase assay
Phosphatase activity was determined using a serine/threonine phosphatase assay system (Promega). Phosphopeptides were obtained from Scrum Inc. (Tokyo, Japan). Briefly, 5000 pmol phosphopeptide was preincubated in various pH conditions (pH 6.2, 7.2, 8.2, and 9.2) in phosphatase reaction buffer (50 mM imidazole, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 5 mM MgCl2, 0.02% beta-mercaptoethanol, and 0.1 mg/mL bovine serum albumin) for 3 min at 30°C (Dhananjaya and D'Souza, 2011). Recombinant PGAM protein or λPPase was added to the reaction and incubated for 30 min at 30°C. Free phosphate was detected as the absorbance by the complex of molybdate, malachite green, and phosphate, according to the manufacturer’s protocol. RRA(pT)VA phosphopeptide is compatible as a substrate for several serine/threonine phosphatases such as protein phosphatases 2A, 2B, and 2C (Donella Deana et al., 1990). For assessment of phosphatase activity against phospho-p53 (Ser15), phospho-p53 (Ser20), and phospho-p53 (Ser15 and Ser20) peptides, 5000 pmol phosphopeptides were incubated with 500 ng recombinant PGAM1 or PGAM2 protein or 250 ng λPPase in phosphatase reaction buffer (pH 7.2). The phospho-p53 peptide sequences were described previously (Shreeram
NanoBiT assay
NanoLuc Binary Technology (NanoBiT) assay (Promega) is utilized to quantify protein-protein interaction between the proteins with large and small NanoBiT tag (Dixon et al., 2016). NanoLuc is newly invented smaller luciferase than standard luciferase, but much more stable with production of much brighter luminescence. NanoBiT comprises two complementary small and large subunits (SmBiT and LgBiT, respectively), whose assembly generates luminescence. This technology was applied for accurate measurement of protein interactions (Song et al., 2020). We generated recombinant PGAM1, 2-WT, W68A, and W78A proteins with NanoBiT tags from the extract of bacteria. A saturation assay was performed using low levels (0.25 nM) of SmBiT fusion proteins for the binding with LgBiT partners at increasing concentrations (0.98 to 500 nM). LgBiT fusion proteins were incubated with or without SmBiT fusion protein in binding buffer (1% BSA in PBS) for 60 min. At the end of incubation, binding intensity was measured as luminescent emission by GloMAX navigator (Promega). Specific binding intensity was obtained by subtraction of background signal (luminescence of LgBiT protein only) from total binding signal (luminescence of LgBiT protein with SmBiT protein). For estimation of binding dissociation constant (Kd), we used 1:1 binding model and the curve of specific binding was fitted using GraphPad prism 8 (GraphPad software, CA).

Prognosis analysis
The correlation between the expression level of PGAM1 or Chk1 and the survival rates of NSCLC patients was assessed by the minimum P-value approach using the PrognoScan database (Mizuno et al., 2009). Dataset of Jacob-00182-MSK was evaluated (Director's Challenge Consortium for the Molecular Classification of Lung et al., 2008). NSCLC patients were classified into two groups according to PGAM1 or Chk1 expression levels in their tumors at all possible cutoff points. The P-value of risk differences between any two groups were analyzed using the log-rank test. The cutoff was selected at the point giving the most significant P-value.
**Statistical analysis**

All data are expressed as the mean ± standard error of the mean (SEM) from at least three independent experiments. Comparisons between two independent groups were analyzed using an unpaired Student’s two-tailed t-test. Comparisons between multiple groups were analyzed using one-way analysis of variance and Dunnett’s multiple comparison test.
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