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Proviral Insertion in Murine Lymphomas 2 (PIM2) Oncogene Phosphorylates Pyruvate Kinase M2 (PKM2) and Promotes Glycolysis in Cancer Cells*[S]

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Zhenhai Yu[‡], Xiaoping Zhao[§], Liangqian Huang[¶], Teng Zhang[§], Fajun Yang[‖], Lei Xie[§], Shaoli Song[§], Ping Miao[§], Li Zhao[§], Xiaoguang Sun[§], Jianjun Liu[§1], and Gang Huang[‡§¶2]

From the [‡]School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai 200030, China, the [§]Department of Nuclear Medicine, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China, the [¶]Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China, and the [‖]Department of Medicine and Developmental and Molecular Biology, Albert Einstein College of Medicine, New York, New York 10461

Background: The protein-serine/threonine kinase PIM2 regulates glycolysis, but the mechanism is not fully elucidated.
Results: PIM2 interacts with PKM2 and phosphorylates PKM2 on the Thr-454 residue.
Conclusion: This phosphorylation of PKM2 increases glycolysis and proliferation in cancer cells.
Significance: PIM2-dependent phosphorylation of PKM2 is critical for regulating the Warburg effect in cancer, highlighting PIM2 as a potential therapeutic target.

Pyruvate kinase M2 (PKM2) is a key player in the Warburg effect of cancer cells. However, the mechanisms of regulating PKM2 are not fully elucidated. Here, we identified the protein-serine/threonine kinase PIM2, a known oncogene, as a novel binding partner of PKM2. The interaction between PIM2 and PKM2 was confirmed by multiple biochemical approaches *in vitro* and in cultured cells. Importantly, we found that PIM2 could directly phosphorylate PKM2 on the Thr-454 residue, resulting in an increase of PKM2 protein levels. Compared with wild type, PKM2 with the phosphorylation-defective mutation displayed a reduced effect on glycolysis, co-activating HIF-1α and β-catenin, and cell proliferation, while enhancing mitochondrial respiration of cancer cells. These findings demonstrate that PIM2-dependent phosphorylation of PKM2 is critical for regulating the Warburg effect in cancer, highlighting PIM2 as a potential therapeutic target.

Tumor cell proliferation proceeds only when sufficient energy and building blocks are available. Compared with normal tissues, most tumors exhibit a significant increase of glucose utilization, namely the Warburg effect (1). Such a characteristic of increased glucose uptake, which accompanies the aerobic glycolysis, has been exploited for diagnosis of cancer using ^{18}F-deoxyglucose position emission tomography (2). Due to the changes of glycolytic enzymes, tumor cells shift glucose metabolism from oxidative phosphorylation to glycolysis even in the presence of oxygen. To date, pyruvate kinase (PK)[3] is considered a key regulator of the Warburg effect (3). PK catalyzes the dephosphorylation of phosphoenolpyruvate to pyruvate with concomitant formation of ATP, which is a rate-limiting step in glycolysis. There are four isoenzymes of PK, L, R, M1, and M2, which are encoded by two separate genes. The L and R isoforms of PK, which are expressed exclusively in the liver and red blood cells, respectively, are originated from the *PKL* gene by alternative splicing (4). PKM1 and PKM2 isoforms are alternative-splicing products of the *PKM* gene (exon 9 for PKM1 and exon 10 for PKM2) (5). During embryogenesis, PKM2 is progressively replaced by PKM1. Conversely, during tumorigenesis, the L-PK or PKM1 isoenzymes are down-regulated and PKM2 is reexpressed, suggesting unique roles of PKM2 in cancer cells. Because PKM2 has a lower enzymatic activity compared with PKM1, it will channel more glycolytic intermediates into building blocks, such as nucleic acids, amino acids, and lipids, to support cancer cell proliferation. The enzymatic activity of PKM2 is under the control of metabolic intermediates, oncogenes, and growth factors (6). Growing evidence indicates that oncogenes reprogram glycolysis, impacting the tumor aggressive phenotype via regulating PKM2 (7). In addition to its direct roles in glycolysis, recent studies have also demonstrated that PKM2 can function as a transcriptional co-activator or a protein kinase to promote gene transcription and tumorigenesis (8–11).

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[1] To whom correspondence may be addressed: Dept. of Nuclear Medicine, Renji Hospital Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China. Tel.: 86-21-68383962; Fax: 86-21-68383962; E-mail: ljjsh@133sh.com.
[2] To whom correspondence may be addressed: Dept. of Nuclear Medicine, Renji Hospital Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China. Tel.: 86-21-63867812 or 63853125; Fax: 86-21-63842916; E-mail: huang2802@163.com.

[3] The abbreviations used are: PK, pyruvate kinase; Co-IP, co-immunoprecipitation; KD, kinase-dead; PIM, proviral insertion in murine lymphomas; PMK2, pyruvate kinase M2; TRITC, tetramethylrhodamine isothiocyanate; HIF, hypoxia-inducible factor.

Transcription regulation appears not to be the primary mechanism of regulating PKM2. Throughout mitosis, PKM2 mRNA and activity decline whereas the protein levels continue to increase (12). The decrease of PKM2 activity is due to post-translational modifications (13). It has been shown that acetylation of PKM2 at Lys-305 promotes its degradation via chaperone-mediated autophagy (14). Interestingly, phosphorylation at tyrosine or serine residues has been implicated in regulating PKM2. In pp60v-src kinase-transformed cells, increased tyrosine phosphorylation of PKM2 correlates with its inactivation (15, 16). In addition, fibroblast growth factor receptor 1 phosphorylates PKM2 on Tyr-105, which inhibits the formation of active, tetrameric PKM2 by disrupting binding of PKM2 cofactor fructose-1,6-biophosphate (17). Protein-tyrosine phosphatase 1B reverses this phosphorylation (18). A-Raf can bind to and phosphorylate PKM2 on serine residues, inducing a transition of dimeric to tetrameric active form of PKM2 (19). Although it is not fully clear, PKCδ is believed to regulate PKM2 protein stability via phosphorylation (20). Moreover, ERK1/2 has been shown to phosphorylate PKM2 on Ser-37 and promote its nuclear translocation, which is important to tumor growth (12).

Proviral insertion in murine lymphomas (PIM) protein kinases are highly conserved oncogenic serine/threonine kinases and have three isoforms: PIM1, PIM2, and PIM3 (21). It has been reported that PIM kinases are aberrantly expressed in multiple types of cancer (22). PIM kinases are responsible for cell cycle regulation, antiapoptotic activity, and other malignant phenotypes of cancer (23). PIM kinases mediate their oncogenic activity through phosphorylating a wide range of cellular proteins (23). All three PIM kinases can phosphorylate Thr-157 and Thr-198 of $p27^{Kip1}$, promoting its binding to the 14-3-3 proteins, resulting in nuclear exclusion and degradation (24). PIM1 can phosphorylate the intracellular domain of CXCR4 at Ser-339, a site critical for CXCR4 recycling (25). PIM2 has been reported to phosphorylate the ribosomal protein 4E-BP1, causing its dissociation from Eif-4e, which impacts protein synthesis (26). Therefore, inhibiting PIM kinases may lead to apoptosis, cell cycle arrest, and senescence. For that reason, PIM kinase inhibitors have been actively developed for cancer treatment (27).

Here, we identify PIM2 as a novel binding partner of PKM2 from a yeast two-hybrid screen. We show that PIM2 critically regulates multiple aspects of PKM2 functions through direct phosphorylation. Thus, our results provide a new insight into the regulation of PKM2 and its contribution to the Warburg effect in cancer cells.

EXPERIMENTAL PROCEDURES

Materials—Rabbit anti-PIM2 antibody was purchased from GeneTex; rabbit anti-PKM2 antibody from Abcam; rabbit anti-phosphoserine antibody from Invitrogen; rabbit anti-phosphothreonine antibody from Cell Signaling; mouse anti-HA, -FLAG, or β-actin antibody from Sigma; and rabbit or mouse IgG from Santa Cruz Biotechnology. Goat anti-mouse or rabbit second antibodies were purchased from LI-COR Biosciences. The plasmids used in this study were generated by subcloning the indicated human cDNA fragments into expression vectors. All plasmids were verified by DNA sequencing. The sequences for siRNA and PCR primers are listed in supplemental Table S2.

Yeast Two-hybrid Screen—The C-terminal portion (amino acids 354–531) of human PKM2 was subcloned into the yeast expression vector pGBKT7 (Clontech) in-frame with the Gal4DNA binding domain. This bait was used to screen human cDNA libraries (Human Kidney Matchmaker® cDNA Library pACT2 638816, Human Liver Matchmaker® cDNA Library pACT2 638802, and Mate & Plate™ Library-Human Brain (Normalized) 630486) (Clontech). The yeast two-hybrid screens were performed according to the manufacturer's instructions (Clontech).

Cell Culture and Transient Transfection—All cell lines were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 100 mg/ml penicillin, and 100 mg/ml streptomycin sulfate (GIBCO) at 37 °C and 5% CO_2. Hypoxic treatment was performed in a specially designed hypoxia incubator (Thermo Electron, Forma, MA) with 1% O_2, 5% CO_2, and 94% N_2. Lipofectamine 2000 (Invitrogen) was used in transient transfection according to the manufacturer's protocol.

Co-immunoprecipitation (Co-IP) and GST Pulldown Assay—Cell extracts were incubated with antibodies and protein A-agarose (Pierce) overnight at 4 °C. Normal mouse or rabbit IgG (Santa Cruz Biotechnology) was used as negative control. After being washed five times with lysed buffer (RIPA; Beyotime, P0013), the resulting beads were eluted with 2× SDS sample buffer by boiling for 8 min at 100 °C, and the samples were analyzed by Western blotting. GST alone and GST-tagged and His-tagged proteins were expressed in *Escherichia coli* BL21. GST-tagged proteins were purified by glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer's instructions. His-tagged proteins were prepared and purified using nickel affinity resins (Merck). His-PIM2 protein was mixed with GST or GST-PKM2 fusion protein in PBS-binding buffer (Takara's PBS, pH 7.4) at 4 °C for 2 h, followed by the addition of 20 μl of glutathione-Sepharose 4B beads. After 1 h of incubation with nutation, the beads were washed five times with PBS. The resulting beads were eluted with 2× SDS sample buffer and analyzed by Western blotting (28).

Confocal Immunofluorescence Microscopy—Cells were plated into 6-well plates with a density of 8×10^4 cells/well. Approximately 36 h after transfection, cells were fixed with 4% paraformaldehyde for 20 min, washed three times with PBS, and treated with 0.2% Triton X-100 for 20 min followed by another three washes with PBS. The cells were blocked with 3% BSA/PBS for 1 h followed by incubation with primary antibody (1:100 anti-HA; 1:100 anti-FLAG) for 2 h at room temperature. After extensive washing, cells were incubated with a secondary antibody conjugated with goat anti-mouse IgG/TRITC antibody for 1 h and counterstained with DAPI for 10 min. The resulting signals were visualized by a confocal laser-scanning microscope (Olympus BX61).

In Vitro Kinase Assay—For *in vitro* kinase assays, PKM2-WT or PKM2-T454A recombinant proteins were incubated with PIM2 in kinase buffer (20 mM MOPS, pH 7.4, 150 mM NaCl, 12.5 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EGTA, 1 mM DTT, 10 μM ATP) (Sigma). The reaction mixtures were incubated at

37 °C for 30 min (29). Aliquots of reaction mixtures were analyzed by Western blotting using rabbit anti-phosphothreonine antibody.

Luciferase Reporter Assays—Cells were seeded onto 6-well plates, transfected with PKM2-WT or PKM2-T454A together with the reporter plasmid p2.1 (30) and control pSV40-*Renilla*, and exposed to either 20% or 1% O_2 for 24 h. Cell lysates were analyzed using the Dual-Luciferase Assay system (Promega) according to the manufacturer's instructions (31, 32).

Glucose Consumption and Lactate Production—Cells were seeded onto 6-well plates and transfected with plasmids or siRNAs. Approximately 48 h after transfection, cells were washed and cultured in serum-free DMEM for approximately 16 h (12). Glucose levels in medium were measured using a glucose assay kit (Sigma), and lactate levels in medium were measured using a lactate assay kit (CMA; Microdialysis). These readouts were normalized to corresponding protein amounts (Beyotime).

Cellular Oxygen Consumption Rate—Oxygen consumption rate was analyzed using a Seahorse XF24 Extracellular Flux Analyzer by real-time monitoring mitochondria respiration. Cells were plated onto XF24 cell culture plates (Seahorse Bioscience) at a density of 2×10^4 cells/well and incubated for 24 h in a normal incubator. Then, cells were equilibrated with bicarbonate-free buffered DMEM in a 37 °C incubator for 60 min without CO_2 immediately before XF assay. Substrates or perturbation compounds were prepared in an identical assay medium as in the corresponding well and were injected from the reagent ports automatically to the wells at the designated time points. The first compound injected was oligomycin, which inhibits complex V in the electron transport chain and causes a decrease in respiration. The second was carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which drives mitochondrial respiration to its maximal capacity, referred to as the total reserve capacity. Finally, antimycin/rotenone was injected to inhibit oxidative phosphorylation at complex I to block all mitochondrial oxygen consumption (33).

Cell Proliferation Analysis—Cells were seeded onto 6-well plates, transfected with PKM2-WT or PKM2-T454A. After 24 h 1×10^4 cells were harvested and seeded in triplicates onto 24-well plates, and cell numbers were counted every 24 h over a 4-day period (14).

$^{14}CO_2$ Release Assay—To determine $^{14}CO_2$ release, cells were incubated in glucose-free medium containing 1 μCi/ml either [6-^{14}C]glucose or [1-^{14}C]glucose for 30 min at 37 °C. Phenylethylamine was used to absorb the released CO_2. The radioactivity of collected $^{14}CO_2$ was quantified by scintillation counting of phenylethylamine for three times with 30-min intervals. This method is designed according to the principle and protocol reported previously (34). The radioactivity was normalized to cell numbers.

Quantitative Real-time PCR—Total RNA was isolated using a TRIzol kit (Omega), and cDNA was synthesized using a cDNA synthesis kit (Takara). Quantitative real-time PCR was performed using the SYBR Green PCR Master Mix (Takara) on the Roche 480 system (Roche Applied Bioscience). The primers used in this study are listed in supplemental Table S2.

Statistical Analysis—We determined the significance of differences using Pearson's correlation test and Student's *t* test (two-tailed). $p < 0.05$ was considered to be significant.

RESULTS

PIM2 Is a Novel Binding Partner of PKM2—To better understand the regulation of PKM2, we screened cDNA libraries of human brain, liver, and kidney by yeast two-hybrid using a C-terminal portion (amino acids 354–531) of PKM2 as the bait because the N terminus of PKM2 tends to form tetramers (35). Fifty-eight positive clones were identified from the screens (supplemental Table S1). Among them, five clones encoding the serine/threonine kinase PIM2 were identified from the human kidney cDNA library. As shown in Fig. 1*a*, the interaction between PKM2 and PIM2 was then validated by independent yeast two-hybrid. To further analyze their interaction, we transiently overexpressed HA-tagged PKM2 and FLAG-tagged PIM2 in HEK293T cells. By Co-IP analysis, we showed that PKM2 could pull down PIM2 (Fig. 1*b*) and vice versa (Fig. 1*c*), although the latter is weak. These results demonstrate that PIM2 and PKM2 interact with each other.

PKM2 consists of four domains: N, A1, B, A2, and C (Fig. 1*d*). To determine which domain(s) of PKM2 bind to PIM2, we generated a series of HA-tagged truncation mutants of PKM2 (Fig. 1*d*) and tested their binding affinity with FLAG-tagged PIM2 in HEK293T cells by Co-IP. As shown in Fig. 1*e*, full-length PIM2 could interact with all the fragments of PKM2 with various affinities, suggesting the presence of multiple interaction surfaces. However, its interaction with the amino acid sequence 219–531 of PKM2 was much stronger than any other fragments, consistent with our yeast two-hybrid results (Fig. 1*e*). Furthermore, to determine whether PIM2 can interact directly with PKM2, we performed GST pulldown assays using purified recombinant His-tagged PIM2 and GST-tagged PKM2. As shown in Fig. 1*f*, GST-tagged PKM2 could efficiently and specifically pull down His-tagged PIM2, suggesting that PIM2 can interact directly with PKM2.

Because PIM2 is highly homologous to PIM1 and PIM3, we examined whether PKM2 could also interact with PIM1 and PIM3. Interestingly, overexpressed PKM2 interacted with PIM3 even stronger than with PIM2, but failed to interact with PIM1 (Fig. 2*a*). In addition, because PKM1 is an alternative splice product of the *PKM* gene (36), we asked whether PKM1 could also interact with PIM2. As shown in Fig. 2*b*, overexpressed PKM1 bound to PIM2 in HEK293T cells as measured by Co-IP. Moreover, to determine whether endogenous PIM2 interacts with PKM2, we performed Co-IP experiments with cell lysates from HeLa or A549 cells. Using an anti-PKM2 antibody, we show that endogenous PKM2 could Co-IP endogenous PIM2 in A549 cells (Fig. 2*c*) or HeLa cells (Fig. 2*d*). Conversely, endogenous PIM2 could Co-IP endogenous PKM2 in A549 cells (Fig. 2*e*). Furthermore, immunofluorescence confocal microscopy analyses showed that PIM2 partially overlapped with PKM2 in the nuclei of HeLa cells (Fig. 2*f*). Taken together, our results demonstrate that PIM2 is a novel binding partner of PKM2.

PIM2 Phosphorylates PKM2 on Thr-454—PIM2 has been shown to regulate the functions of proteins, such as BAD (37),

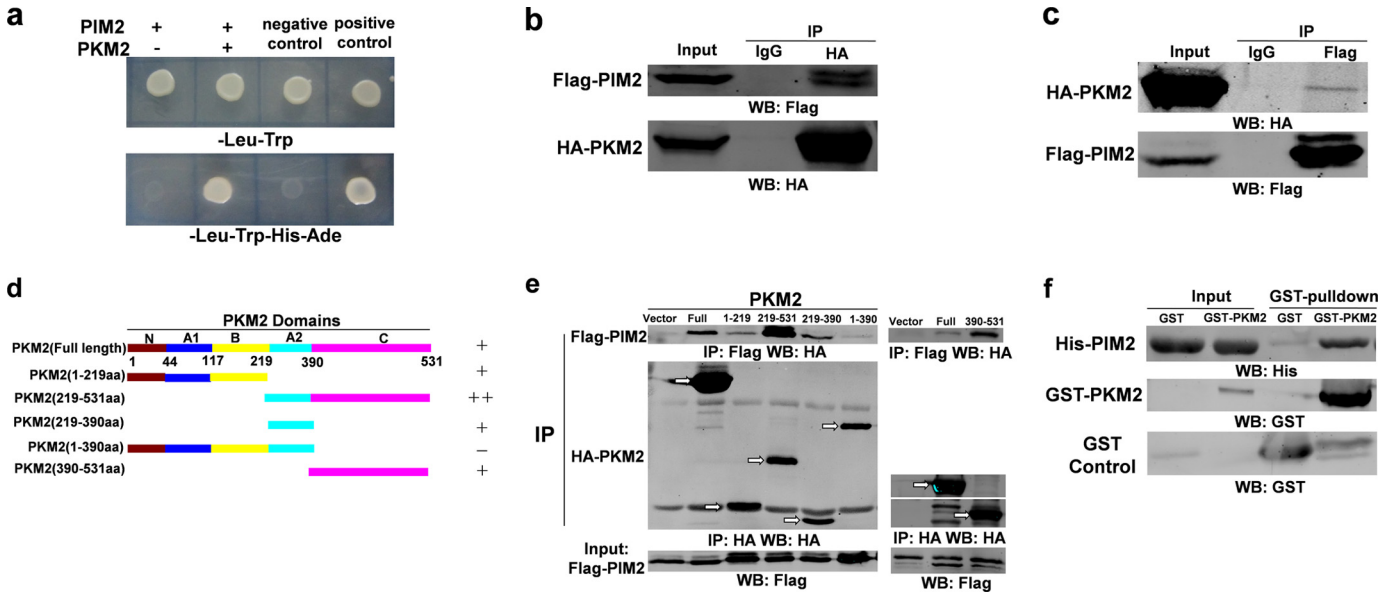

FIGURE 1. **PIM2 is a novel binding partner of PKM2.** *a*, interaction between PKM2 (amino acids 354–531) and PIM2 (amino acids 182–311) was reexamined by yeast two-hybrid using SD-Leu-Trp or SD-Leu-Trp-His-Ade culture medium. Vector pGADT7-T with pGBKT7-p53 or pGBKT7-Lam was used as positive control and negative control, respectively. *b* and *c*, interaction between FLAG-tagged PIM2 and HA-tagged PKM2 full-length proteins was examined by Co-IP followed by Western blotting using anti-HA antibody or anti-FLAG antibody. Tagged proteins were overexpressed in HEK293T cells by transient transfection. *d*, schematic represents PKM2 protein fragments used in the mapping analysis. *e*, FLAG-tagged PIM2 was co-expressed in HEK293T cells with HA-tagged PKM2 fragments as indicated. Co-IP followed by Western blotting was performed to determine their interaction. *f*, GST pulldown assays were performed to examine the direct interaction between PKM2 and PIM2 using recombinant His-tagged PIM2 and GST-tagged PKM2.

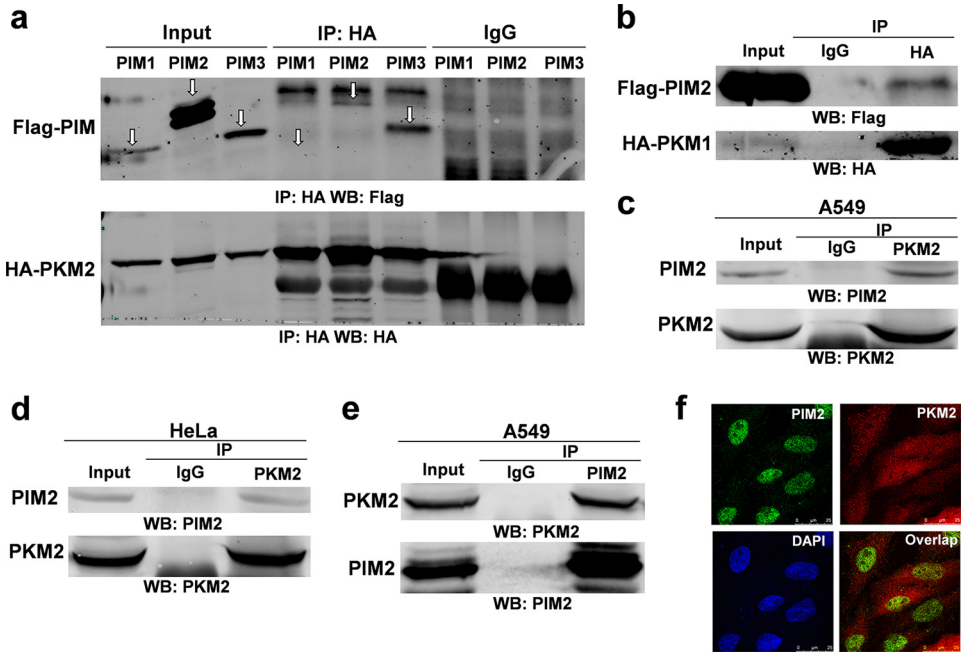

FIGURE 2. **PIM2 interacts with PKM2.** *a*, HA-tagged PKM2 was co-expressed with FLAG-tagged PIM1, PIM2, or PIM3 in HEK293T cells. Co-IP followed by Western blotting was performed to determine their interaction. *b*, interactions between HA-tagged PKM1 and FLAG-tagged PIM2 were examined by Co-IP followed by Western blotting. *c*–*e*, the association of endogenous PIM2 and PKM2 in A549 (*c* and *d*) or HeLa (*e*) cells was analyzed by Co-IP followed by Western blotting using anti-PIM2 antibody and anti-PKM2 antibody. *f*, confocal immunofluorescence microscopy was performed to analyze localization of PKM2 and PIM2 in HeLa cells.

p21 (29), p27 (24), and 4E-BP1 (38) via direct phosphorylation. We then examined whether PIM2 could also phosphorylate PKM2. For that purpose, we co-transfected FLAG-tagged PIM2 (vector, wild type, or kinase-dead (KD) PIM2) with HA-tagged PKM2 into HEK293T cells. Compared with the kinase-dead PIM2 or vector control, transfection of wild type PIM2 caused an increase in PKM2 phosphorylation on threonine residues, as detected by immunoblotting with a phospho-Thr-specific antibody (Fig. 3*a*). PIM2 did not affect the phosphorylation levels of PKM2 on serine residues (Fig. 3*b*), whereas as shown in Fig. 3*c*, it increased the serine phosphorylation of the known PIM2 kinase substrate, BAD (37), under the same condition.

To determine whether PIM2 could phosphorylate PKM2 directly, we performed *in silico* analyses for potential PIM sub-

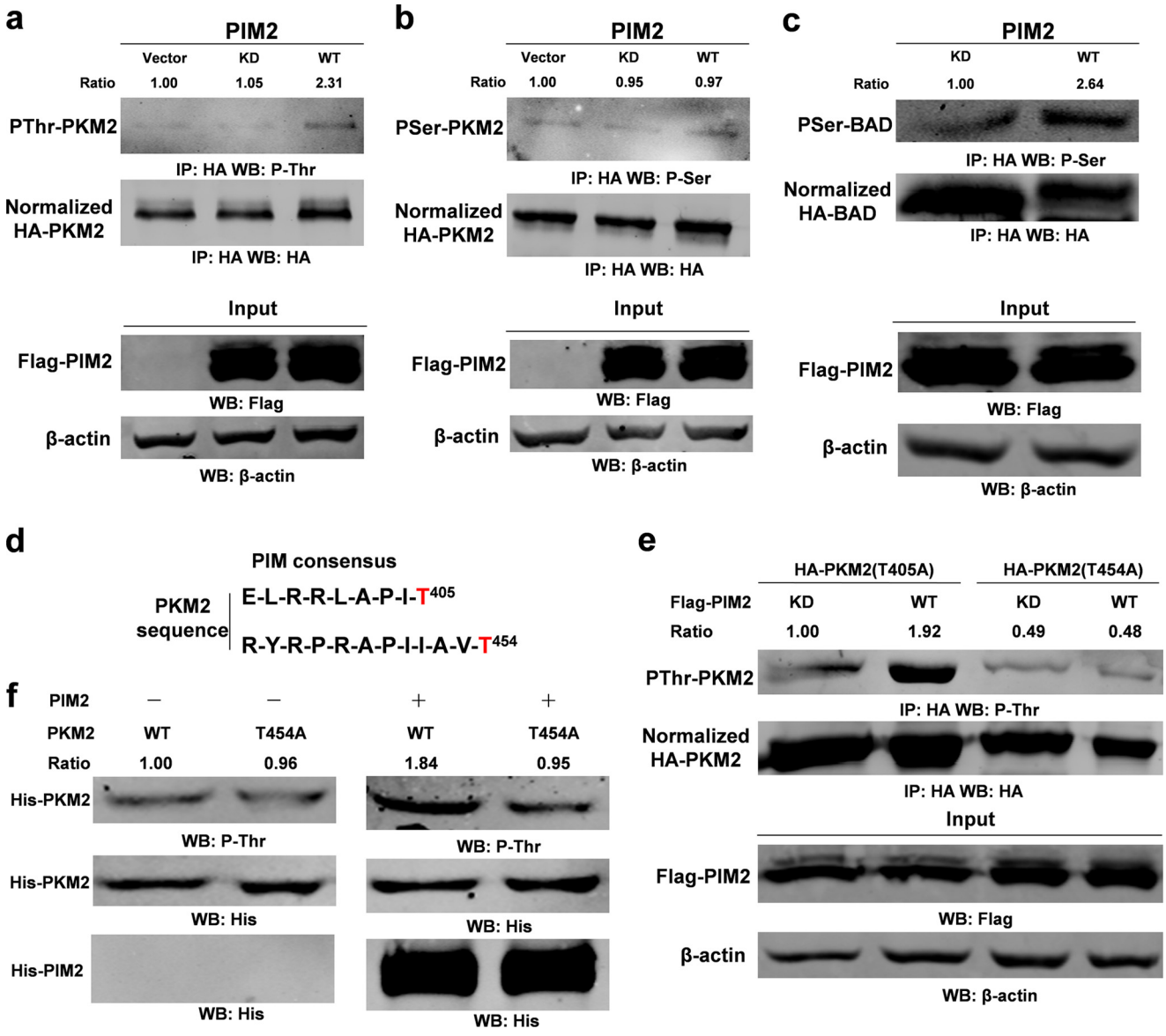

FIGURE 3. **PIM2 phosphorylates PKM2 on Thr-454.** *a* and *b*, HA-tagged PKM2 was co-transfected with empty vector, FLAG-tagged PIM2-KD, or PIM2-WT (wild type) in HEK293T cells. Two days after transfection, PKM2 proteins were immunoprecipitated (*IP*) using anti-HA antibody, and HA-tagged PKM2 protein levels were normalized before phosphothreonine (*a*) or phosphoserine (*b*) levels were detected by Western blotting using the indicated antibodies. *c*, PIM2 affects phosphoserine levels of HA-tagged BAD in HEK293T cells. *d*, two putative PIM2 substrate motifs are identified in PKM2. Thr-405 and Thr-454 residues are highlighted in *red*. *e*, effects of FLAG-tagged PIM2 (KD or WT) on threonine phosphorylation of HA-tagged PKM2-T405A or T454A in HEK293T cells are shown. *f*, *in vitro* kinase assay was used to determine the effects of recombinant PIM2 on threonine phosphorylation of His-tagged PKM2-WT or T454A.

strate motifs in PKM2 (38). As shown in Fig. 3*d*, we identified two putative PIM phosphorylation sites (Thr-405 and Thr-454) in PKM2. To determine which site(s) could be phosphorylated by PIM2, we generated phosphorylation-defective mutants by mutating the threonine residue to alanine (T405A or T454A). Interestingly, in HEK293T cells mutation of Thr-405 had no effect on threonine phosphorylation of PKM2, whereas mutation of Thr-454 significantly reduced PIM2-induced PKM2 phosphorylation (Fig. 3*e*), suggesting that the Thr-454 residue of PKM2 was targeted by PIM2 for phosphorylation. *In vitro* kinase assays further confirmed that PIM2 could directly phosphorylate PKM2 on the Thr-454 residue. As shown in Fig. 3*f*, recombinant wild type PIM2 increased the threonine phosphorylation of wild type PKM2 by approximately 5-fold, whereas it had no effect on the PKM2-T454A mutant. These results strongly demonstrate that PIM2 directly phosphorylates PKM2 on the Thr-454 residue.

PIM2 Positively Regulates PKM2 Protein Levels—Posttranslational regulation of PKM2, such as phosphorylation or acetylation, has been reported to regulate PKM2 function by affecting its protein stability, translocation, and homodimerization (14). Thus, we hypothesized that T454 phosphorylation of PKM2 by PIM2 regulates PKM2 protein stability. To this end, PKM2 protein levels were analyzed when HEK293T cells were co-transfected with FLAG-tagged PIM2 and HA-tagged PKM2. Compared with the vector control, overexpression of PIM2 caused an increase in both overexpressed and endogenous PKM2 proteins in HEK293T cells (Fig. 4*a*). Similarly, overexpression of PIM2 also increased endogenous PKM2 protein levels in A549 cells (Fig. 4*b*). Conversely, when PIM2 was knocked

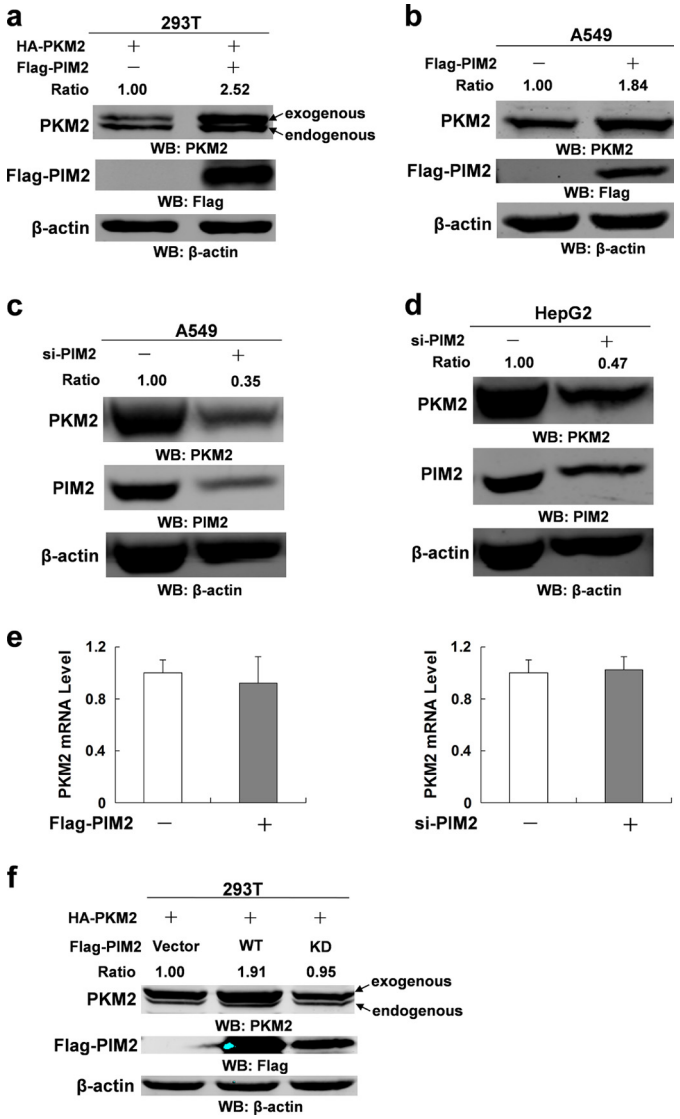

FIGURE 4. **PIM2 regulates PKM2 protein levels.** *a*, HEK293T cells co-transfected with FLAG-tagged PIM2-WT (or empty vector as control) with HA-tagged PKM2. Two days after transfection, the protein levels were analyzed by Western blotting using the indicated antibody. *b*, effects of overexpressing PIM2 on PKM2 protein levels in A549 cells. A549 cells were transfected with FLAG-tagged PIM2. Two days after transfection, the protein levels were analyzed by Western blotting using the indicated antibody. *c* and *d*, effects of PIM2 knockdown by specific siRNA on PKM2 protein levels in A549 (*c*) and HepG2 (*d*) cells. *e*, effects of overexpressing or knocking down PIM2 on PKM2 mRNA levels in 293T cells. HEK293T cells were transfected with FLAG-tagged PIM2 or siRNA-PIM2. Two days after transfection, the mRNA levels of PKM2 were analyzed by quantitative RT-PCR. Data represent mean ± S.E. (*error bars*; $n = 3$); *, $p < 0.05$. *f*, requirement of kinase activity of PIM2 in regulating PKM2 protein levels in HEK293T cells. HEK293T cells were transfected with FLAG-tagged PIM2 (WT or KD) or empty vector as control. Two days after transfection, the protein levels were analyzed by Western blotting using the indicated antibody.

down by specific siRNA in A549 (Fig. 4*c*) or HepG2 (Fig. 4*d*) cells, PKM2 protein levels were significantly reduced. However, the mRNA levels of PKM2 were not significantly changed when PIM2 was overexpressed or knocked down (Fig. 4*e*), suggesting that PIM2 regulates PKM2 protein stability. To determine whether PIM2 stabilizes PKM2 by Thr-454 phosphorylation, the KD form of PIM2, K61A (37), was used. Compared with the vector control, transfection of wild type PIM2 led to an increased levels of overexpressed PKM2, whereas KD PIM2 had little effect on or slightly decreased PKM2 levels in HEK293T cells (Fig. 4*f*). Together, these data suggest that PIM2-mediated phosphorylation of PKM2 on the Thr-454 residue controls the abundance of PKM2 proteins.

PIM2 Promotes PKM2-dependent Glycolysis and Reduces Mitochondrial Respiration—A recent study has shown that PIM1 and PIM3 regulate energy metabolism and cell growth (39). Because PIM2 interacts with PKM2, which regulates glycolysis in cancer cells, we hypothesize that PIM2 regulation of glycolysis depends on PKM2. Indeed, overexpression of PIM2 increased glucose consumption in both HEK293T (Fig. 5*a*) and HepG2 (Fig. 5*b*) cells. Moreover, overexpression of PIM2 also increased lactate production in those cells (Fig. 5, *c* and *d*). These data suggest that PIM2 regulates glycolysis in these cells. To determine the role of endogenous PIM2 in glycolysis, we knocked down PIM2 using specific siRNA. As shown in Fig. 5, *a–d*, PIM2 knockdown decreased both glucose consumption and lactate production in HEK293T and HepG2 cells, further confirming the role of PIM2 on glycolysis. Next, we asked whether PKM2 was required for PIM2 regulation of glycolysis. For that purpose, we first knocked down PKM2 in HEK293T or HepG2 cells using siRNA specifically targeting PKM2 and then overexpressed FLAG-tagged PIM2. As shown in Fig. 5, *a–d*, both glucose consumption and lactate production were no longer increased when PKM2 was knocked down, suggesting that PKM2 is indeed required for PIM2-induced glycolysis in cancer cells.

To determine whether PIM2 stimulation of glycolysis depends on PKM2 phosphorylation at the Thr-454 residue, we transfected HEK293T cells with HA-tagged wild type or T454A mutant PKM2. As shown in Fig. 5*e*, overexpression of the T454A mutant PKM2 decreased both glucose consumption and lactate production compared with overexpression of wild type PKM2. Interestingly, overexpression of the T454A mutant PKM2 also resulted in a higher PK enzyme activity (Fig. 5*f*). These data suggest that PKM2 Thr-454 phosphorylation is involved in regulating glycolysis and controlling its enzymatic activity.

Changes in glycolysis may affect mitochondrial functions. To determine whether PIM2 regulation of PKM2 influences mitochondrial functions, we examined the metabolic flux by Seahorse analysis and using ^{14}C (1-^{14}C and 6-^{14}C)-labeled glucose. As shown in Fig. 6, *a* and *b*, the mitochondrial respiration, as indicated by oxygen consumption, was increased in cells expressing the T454A mutant PKM2. In contrast to wild type, T454A mutant PKM2 significantly increased the release of [6-^{14}]CO_2 from [6-^{14}C]glucose (Fig. 6*c*) but had no effect on the release of [1-^{14}]CO_2 from [1-^{14}C]glucose (Fig. 6*d*). Thus, the ratio of [1-^{14}]CO_2 to [6-^{14}]CO_2 was decreased (Fig. 6*e*). Together, our results suggest that PKM2 phosphorylation at Thr-454 by PIM2 reduces mitochondrial respiration.

Thr-454 Phosphorylation of PKM2 Promotes Its Cofactor Functions—Previous studies have reported that PKM2 can function as a transcriptional cofactor to stimulate HIF-1α and β-catenin-mediated gene transcription (9, 10). To determine whether PIM2-dependent phosphorylation of PKM2 on Thr-454 affects its transcriptional cofactor functions, we first examined the effects of wild type and T454A mutant PKM2 on

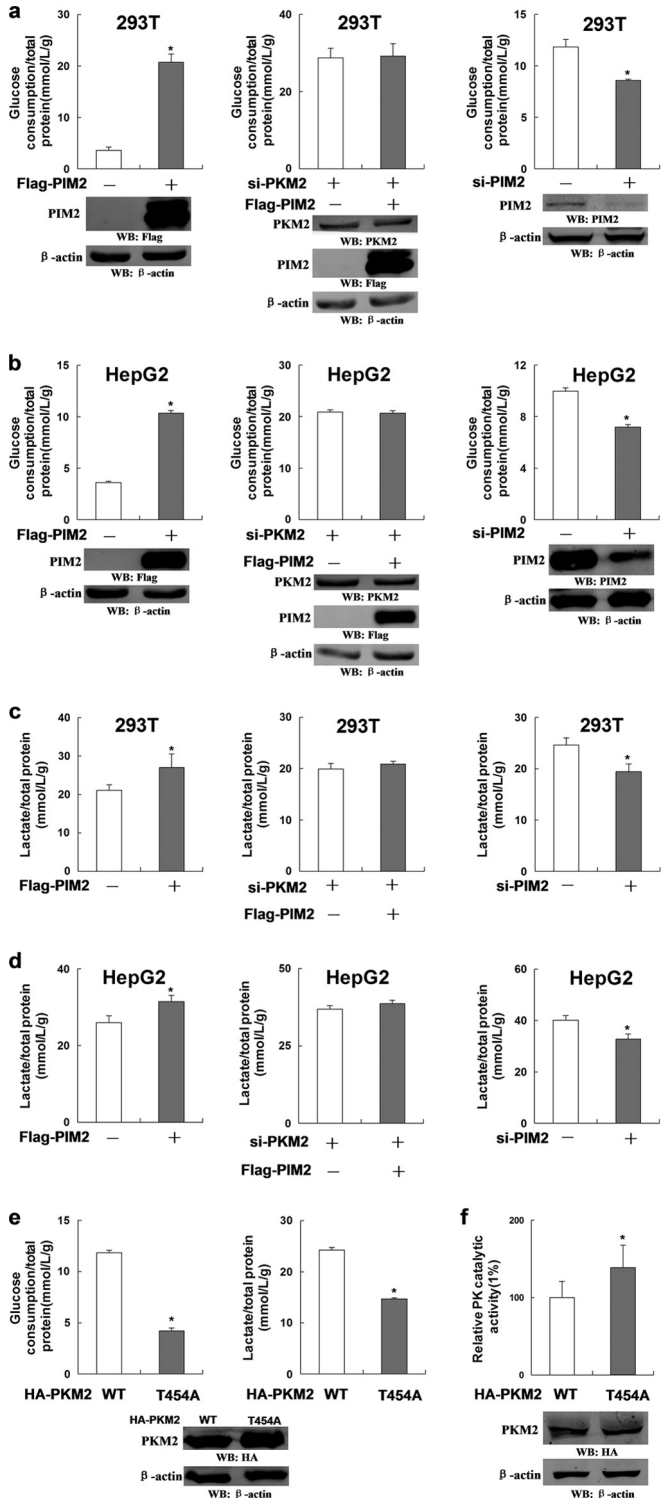

FIGURE 5. **PIM2 promotes PKM2-dependent glycolysis.** *a* and *b*, HEK293T (*a*) or HepG2 (*b*) cells were transfected with an empty vector or FLAG-tagged PIM2-WT, PKM2 or control siRNA followed by FLAG-tagged PIM2-WT, or PIM2 or control siRNA. Two days after transfection, the medium was replaced by serum-free medium for another 12–16-h culture. Glucose levels in medium were examined. *c* and *d*, same treatments as in *a* and *b* were done. Lactate levels in medium were examined. *e*, A549 cells were transfected with either HA-tagged PKM2-WT or T454A. Two days after transfection, the medium was replaced by serum-free medium for another 12–16-h culture. Glucose and lactate levels in medium were examined. *f*, A549 cells were transfected with either HA-tagged PKM2-WT or T454A. Two days after transfection, PK activities in cell lysates were examined. All data represent the means ± S.E. (*error bars*) of three independent experiments; *, $p < 0.05$.

hypoxia-induced gene transcription. For that purpose, we co-transfected HEK293T cells with HA-tagged PKM2 (wild type or T454A mutant) with the p2.1 plasmid, a luciferase reporter construct containing hypoxic responsive elements of the HIF-1α target gene *ENO1* (30) and pSV40-*Renilla* as the control for transfection. After 24 h of culture, cells were incubated in either 20% or 1% O_2 for another 24 h. As shown in Fig. 7*a*, wild type PKM2 could strongly activate the promoter under the hypoxic condition, whereas T454A mutant PKM2 was only approximately half as active as wild type PKM2 on HIF-1α-mediated transcription. Moreover, T454A mutant PKM2 was significantly less potent to activate endogenous HIF-1α target genes, such as *LDHA*, *PDK1*, *ENO1*, *VEGF*, and *GLUT1*, in HEK293T, HepG2, or HeLa cells (Fig. 7, *b–d*). Similarly, T454A mutant PKM2 was also significantly less potent to activate endogenous β-catenin target genes, such as *Myc* and *CCND1*, in HEK293T, HepG2, or HeLa cells (Fig. 7, *e–g*). These results demonstrated that phosphorylation of PKM2 on Thr-454 is important for its co-activator functions on HIF-1α and β-catenin.

Thr-454 Phosphorylation of PKM2 Increases Cancer Cell Proliferation—To determine whether phosphorylation of PKM2 on Thr-454 regulates proliferation of cancer cells, we transfected A549 cells with HA-tagged PKM2 (wild type or T454A mutant). As shown in Fig. 8*a*, cells transfected with T545A mutant PKM2 grew significantly slower than those transfected with wild type PKM2, suggesting that phosphorylation of PKM2 on Thr-454 is required to promote cancer cell proliferation. Previous studies have shown that the ratio of 1-$^{14}CO_2$ to 6-$^{14}CO_2$ reflects the malignant degree of tumors (40). In our study, the ratio of 1-$^{14}CO_2$ to 6-$^{14}CO_2$ was lower in the presence of T454A mutant than wild type PKM2 (Fig. 6*e*), suggesting that Thr-454 phosphorylation of PKM2 is required for malignant phenotype of cancer cells.

DISCUSSION

Reprogrammed energy metabolism is a hallmark of cancer cells. The Warburg effect, which is characterized by an increased glycolysis even in the presence of oxygen, is currently the best known metabolic abnormality in cancer cells (1). Numerous studies in recent years have demonstrated that the PKM2 isoform of pyruvate kinase is a key regulator of the Warburg effect (41). Supporting this notion, during tumorigenesis PKM1, L and R isoforms of pyruvate kinase are gradually diminished and replaced by PKM2 (35). Moreover, it has been shown that knockdown of PKM2 in cancer cells could decrease the rate of glycolysis in addition to inhibiting cell proliferation (36). Importantly, introduction of PKM2, but not PKM1, could stimulate glycolysis and promote tumorigenesis (36). Despite such extensive studies on the oncogenic role of PKM2, the underlying mechanisms of PKM2 regulation are still not fully understood.

In this study, using a C-terminal portion (amino acids 354–531) of PKM2 as the bait in yeast two-hybrid, we identified multiple clones encoding PIM2 from the human kidney cDNA library. The interaction between PIM2 and PKM2 was confirmed through independent yeast two-hybrid and Co-IP of overexpressed or endogenous proteins. GST pulldown data

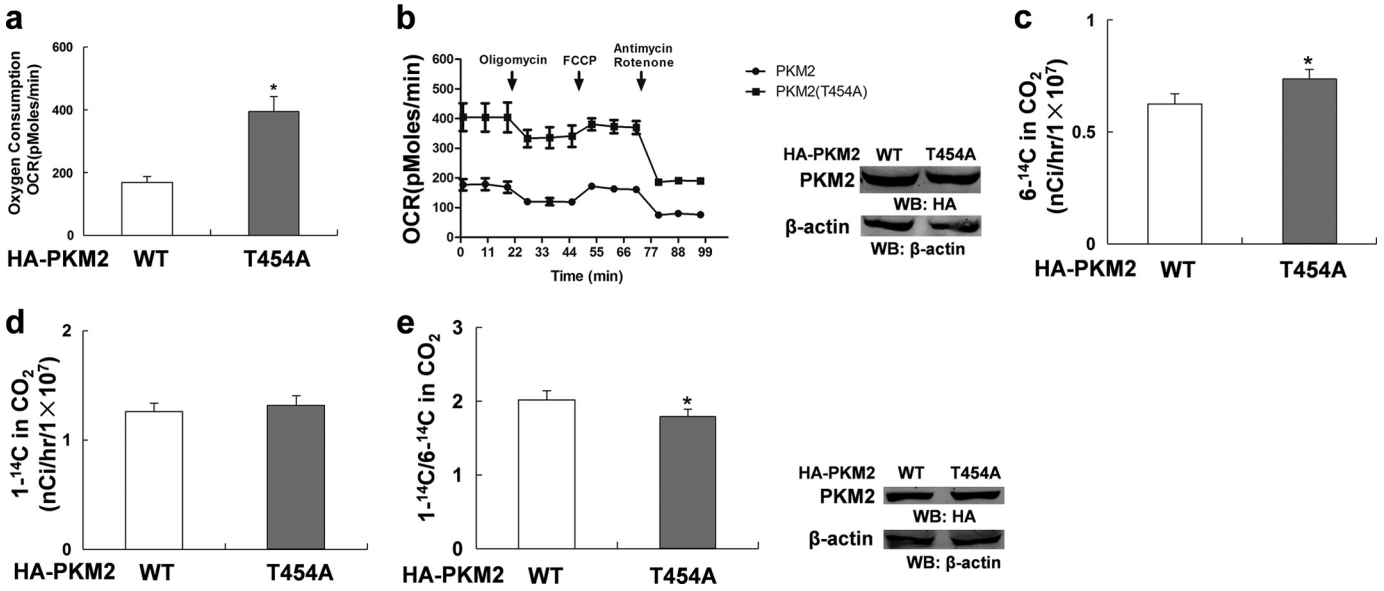

FIGURE 6. **PIM2 phosphorylation of PKM2 regulates mitochondrial respiration.** *a–d*, A549 cells were transfected with HA-tagged PKM2-WT or T454A. One day after transfection, cells were replated into appropriate plates for analysis of O_2 consumption (*a*) and OCR (*b*) by a Seahorse XF24 extracellular flux analyzer, [6-^{14}]CO_2 (*c*) or [1-^{14}]CO_2 (*d*) release from glucose. *e*, ratio of [1-^{14}]CO_2 to [6-^{14}]CO_2 from *c* and *d* is shown. All data represent the means ± S.E. (*error bars*) of three independent experiments; *, $p < 0.05$. *OCR*, oxygen consumption rate.

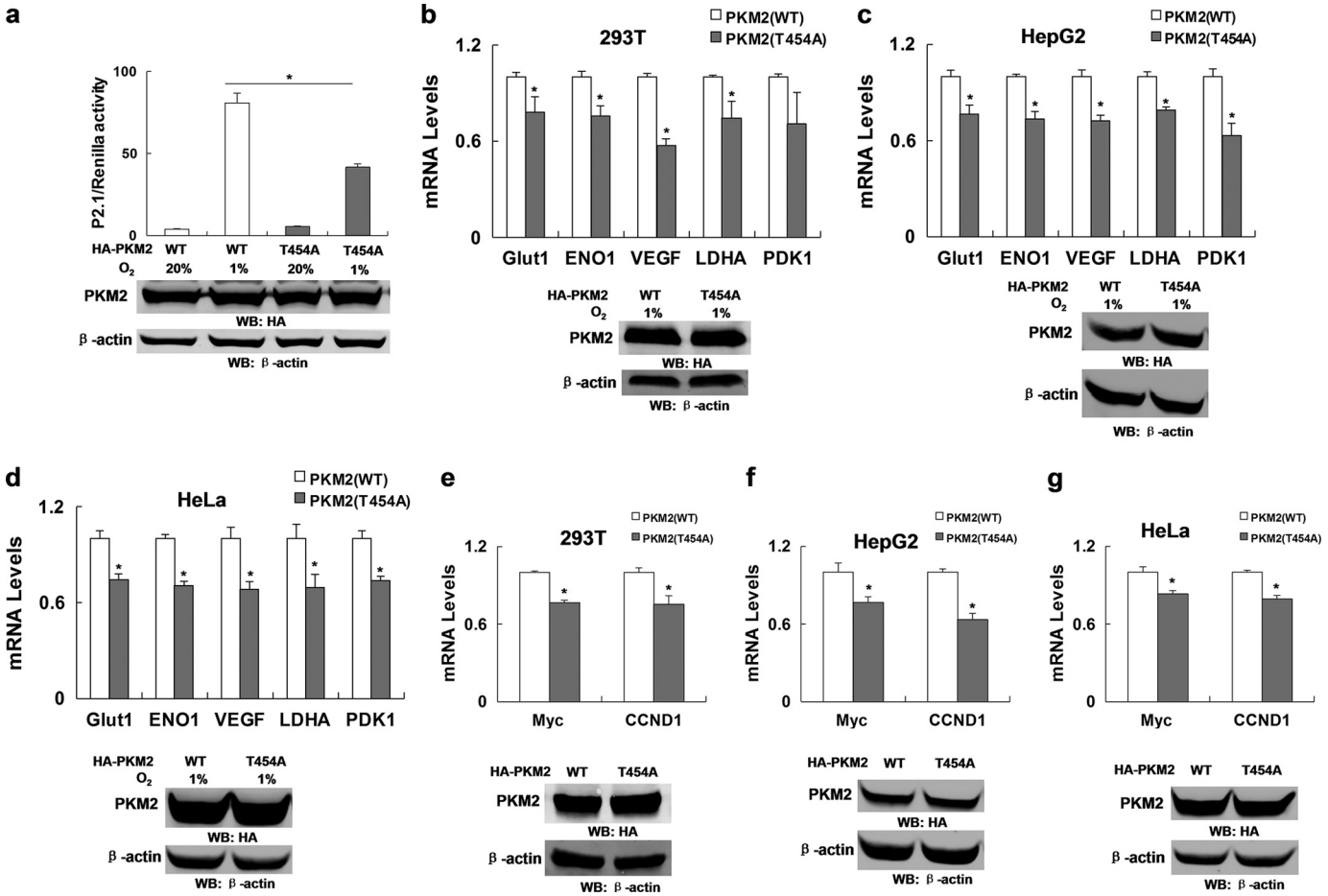

FIGURE 7. **Thr-454 phosphorylation of PKM2 promotes its cofactor functions.** *a*, HEK293T cells were co-transfected with HA-tagged PKM2-WT or T454A, p2.1, and pSV40-*Renilla*. Transfected cells were exposed to 20% O_2 or 1% O_2 for 24 h. The ratio of firefly to *Renilla* luciferase activity was determined. *b–d*, HEK293T (*b*), HepG2 (*c*), or HeLa (*d*) cells were transfected with HA-tagged PKM2-WT or T454A, Two days after transfection, the cells were exposed to 1% O_2 or 20% O_2 for another 24 h. The mRNA levels of the indicated genes were examined by quantitative RT-PCR. *e–g*, HEK293T (*e*), HepG2 (*f*), or HeLa (*g*) cells were transfected with HA-tagged PKM2-WT or T454A. Two days after transfection, the mRNA levels of the indicated genes were examined by quantitative RT-PCR. All data represent the means ± S.E. (*error bars*) of three independent experiments; *, $p < 0.05$.

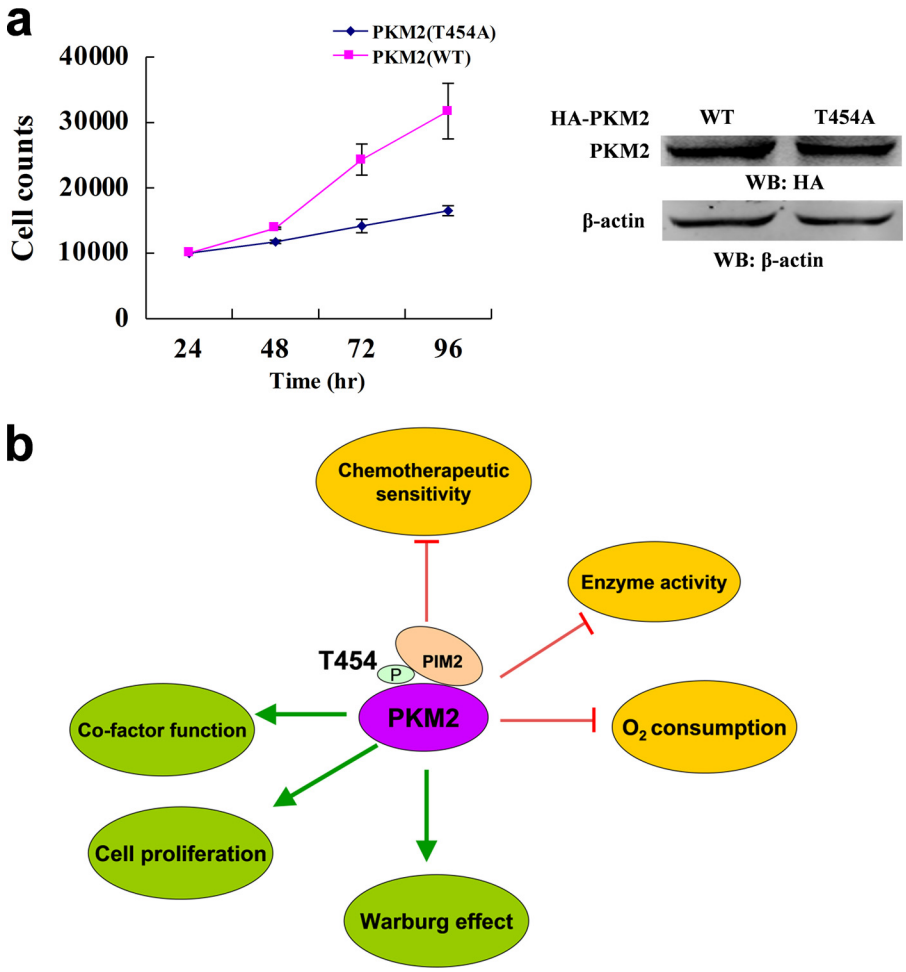

FIGURE 8. **Thr-454 phosphorylation of PKM2 increases cancer cell proliferation.** *a*, A549 cells were transfected with HA-tagged PKM2-WT or T454A. One day after transfection, cells were replated and analyzed for cell growth by counting cell number at the indicated time points. *b*, PIM2 regulation of PKM2 functions in cancer development is summarized. All data represent the means ± S.E. (*error bars*) of three independent experiments; *, $p < 0.05$.

show that recombinant PIM2 and PKM2 bind to each other, suggesting that PIM2 interacts directly with PKM2. Interestingly, by Co-IP we found that PIM2 could interact with all fragments of PKM2, which we tested, with various affinities, suggesting a complicated interaction between PIM2 and PKM2. However, it is also likely that some of these interactions were indirect and were mediated by other cellular proteins. Nevertheless, consistent with our yeast two-hybrid data, a fragment covering amino acids 219–531 of PKM2 displayed the strongest binding with PIM2.

On one hand, PKM2 is the alternative splicing form of PKM1, and they differ by only 23 amino acids. Thus, we predicted that PKM1 could also interact with PIM2. Indeed, by Co-IP we show that overexpressed PIM2 binds to PKM1. Thus, it would not be surprising if PIM2 also regulates PKM1 functions. However, because PKM1 is often down-regulated and replaced by PKM2 in cancer cells, the relevance of PKM1 regulation by PIM2 is probably not significant at least in cancer development. On the other hand, PIM2 belongs to a family of protein-serine/threonine kinases. The other members of this family include PIM1 and PIM3. Thus, we also examined the affinities of PKM2 binding to all three PIMs. Interestingly, our Co-IP data show that PKM2 could interact with PIM2 and PIM3, but not PIM1. In addition, it appears that PIM3 has the strongest interaction with PKM2. However, since PIM1, 2, and 3 are highly similar in structure (21), it is possible that all three PIMs may phosphorylate PKM2 and regulate its functions. Consistent with the data in this study, PIMs are known proto-oncogenes (21). It would be interesting to test whether the oncogenic functions of PIM1 and PIM3 in cancer cells also require PKM2 in future studies.

Because PIM2 is a protein-serine/threonine kinase and PKM2 is a known phosphorylated protein, we tested the hypothesis that PKM2 is a novel substrate of PIM2. Previous studies have identified several substrates of PIM2, including BAD (37), p21 (29), p27 (24), and 4E-BP1 (38). From these studies, a consensus substrate motif for PIM2 has been concluded (21). Interestingly, through *in silico* analysis, we have identified two PIM2 substrate motifs containing potential phosphorylation at Thr-405 and Thr-454. However, mutation analyses and *in vitro* kinase assays demonstrated that only the Thr-454 residue of PKM2 could be targeted by PIM2. Thus, PKM2 is a new substrate of PIM2.

Posttranslational modifications of PKM2 and protein-protein interaction have been reported to regulate its enzymatic activity or protein stability (41). For example, glucose-stimulated acetylation decreases PKM2 enzymatic activity and pro-

motes CMA-mediated degradation (14). In this study, we found that cells transfected with T454A mutant PKM2 displayed higher pyruvate kinase activity than those with wild type PKM2, suggesting that Thr-454 phosphorylation of PKM2 inhibits its enzymatic activity. In addition, our data suggest that PIM2 positively regulates the abundance of PKM2, and such regulation is most likely posttranslational because PIM2 does not affect PKM2 mRNA expression. Thus, PIM2 functionally regulates PKM2.

A previous study has reported a stimulatory role of PIM2 on glycolysis in cancer cells (42). However, the underlying mechanism of PIM2 in regulating glucose metabolism was not clear. Through loss-of-function and gain-of-function analyses we show that PIM2-dependent stimulation of glycolysis requires PKM2, further suggesting the significance of their interaction. Consistent with these data, we show that T454A mutant PKM2 is less potent to promote glycolysis than wild type, suggesting that PIM2-mediated phosphorylation on Thr-454 residue is critical for PIM2/PKM2-induced glycolysis in cancer cells. In addition to increased glycolysis, our data also show that PIM2-mediated phosphorylation of PKM2 inhibits mitochondrial oxidative phosphorylation, suggesting that Thr-454 phosphorylation of PKM2 is essential for the switch of glucose metabolism. Previous study indicates that high pyruvate kinase activity suppressed glycolysis and cell proliferation (35). In our study we found that T454A mutant PKM2 also had higher PK catalytic activity than wild type. In the meantime, glycolysis and cell proliferation were down-regulated. T454A mutant probably promotes tetramer formation of PKM2 and suppresses tumorigenesis, which is consistent with a previous study (35).

Besides its role in glycolysis, PKM2 has recently been reported as a transcriptional co-activator for oncogenic transcription factors, such as HIF-1α (9) and β-catenin (10). Thus, we tested whether Thr-454 phosphorylation of PKM2 affects the cofactor functions of this protein. Indeed, our results demonstrated that T454 mutant PKM2 is less potent to activate both HIF-1α and β-catenin target gene expression than wild type, suggesting that PIM2-mediated phosphorylation of PKM2 affects PKM2 functions broadly.

In summary, we have identified PIM2 as a novel regulator of PKM2. Multiple approaches show that PIM2 and PKM2 interact with each other. Functionally, through phosphorylating the Thr-454 residue, PIM2 regulates several aspects of PKM2 functions in metabolic reprogramming of cancer cells (Fig. 8*b*). Our results demonstrate that PIM2-dependent phosphorylation of PKM2 is critically involved in the Warburg effect in cancer cells. So far, PIM2 has been extensively targeted for cancer therapy. Several inhibitors of PIM2 are in clinical trials. Our results will provide a novel mechanistic basis of targeting PIM2 in treating cancer.

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