Tyr-301 phosphorylation inhibits pyruvate dehydrogenase by blocking substrate binding, and promotes the Warburg effect

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Background: Current understanding of mitochondrial PDH inhibition involves S293 phosphorylation that impedes active site accessibility.

Results: Y301 phosphorylation also inhibits PDHA1, likely by blocking pyruvate binding, which is important for the glycolytic switch and tumor growth.

Conclusion: Tyrosine phosphorylation may function to regulate PDH activity.

Significance: These data provide novel insights into the molecular mechanisms underlying PDC regulation and the Warburg effect.

ABSTRACT

The mitochondrial pyruvate dehydrogenase complex (PDC) plays a crucial role in regulation of glucose homoeostasis in mammalian cells. PDC flux depends on catalytic activity of the most important enzyme component pyruvate dehydrogenase (PDH). PDH kinase (PDK) inactivates PDC by phosphorylating PDH at specific serine residues including S293, while dephosphorylation of PDH by PDH phosphatase (PDP) restores PDC activity. The current understanding suggests that S293 phosphorylation of PDH impedes active site accessibility to its substrate pyruvate. Here we report that phosphorylation of a tyrosine residue Y301 also inhibits PDHA1 by blocking pyruvate binding through an novel mechanism in addition to S293 phosphorylation. In addition, we found that multiple oncogenic tyrosine kinases directly phosphorylate PDHA1 at Y301, and Y301 phosphorylation of PDHA1 is common in EGF-stimulated cells as well as diverse human cancer cells and primary leukemia cells from human patients. Moreover, expression of a phosphorylation-deficient PDHA1 Y301F mutant in cancer cells resulted in increased oxidative phosphorylation, decreased cell proliferation under hypoxia, and reduced tumor growth in mice. Together, our findings suggest that phosphorylation at distinct serine and tyrosine residues inhibits PDHA1 through distinct mechanisms to impact active site accessibility, which act in concert to regulate PDC activity and promote the Warburg effect.

In mammalian cells, mitochondrial PDC converts pyruvate to acetyl-CoA (pyruvate decarboxylation). Thus PDC is at the center of aerobic metabolism of carbohydrate, which regulates the flow of energy in mammalian cells by determining when pyruvate generated in glycolysis should be used for oxidative phosphorylation (OXPHOS) or, under hypoxic condition, converted to lactate to sustain
PDC is a large complex that is organized around a 60-meric dodecahedral core formed by acetyltransferase (E2p) and E3-binding protein (E3BP) (3), which binds pyruvate dehydrogenase (PDH, a.k.a. E1p), PDH upstream pyruvate dehydrogenase kinase (PDK) and phosphatase (PDP), and dihydrolipoamide dehydrogenase (E3) (4). PDH is the most important enzyme component of PDC and transforms pyruvate into acetyl-CoA, which, along with the acetyl-CoA from the fatty acid β-oxidation, enters the Krebs cycle to produce ATP and electron donors including NADH (5).

The activity of PDH is regulated by reversible phosphorylation of three serine residues on the E1α subunit. The phosphorylation of these sites is catalyzed by PDK, which is a Ser/Thr kinase that inactivates PDC by phosphorylating PDH at least at one of three specific serine residues (Sites 1, 2 and 3 are S293, S300, and S232, respectively), while dephosphorylation of PDH by PDH phosphatase (PDP) restores PDC activity (6). Among these three sites, phosphorylation of S293 was suggested to impede active site accessibility of PDH to its substrate pyruvate (7). However, it is not clear whether other types of post-translational modifications such as tyrosine phosphorylation and lysine acetylation are also involved in PDH regulation in mammalian cells.

Accumulating evidence suggests that aerobic glycolysis appears to be a key metabolic factor in human tumors (2) and leukemia (8,9), however, the detailed mechanisms underlying the glycolytic switch in tumor/leukemia cells remain unclear. It was suggested that the metabolic switch allowing cancer cells to rely more on glycolysis is, in part, due to functional attenuation of mitochondria (10). We recently reported that acetylation at K321 and K202 inhibits PDHA1 and PDP1, respectively, which is common in EGF-stimulated cells and diverse human cancer cells, and is important for tumor growth (11). Moreover, we found that oncogenic tyrosine kinases promote the Warburg effect in cancer cells by attenuating mitochondria function via tyrosine phosphorylation and activation of PDK1 (12) as well as tyrosine phosphorylation and inhibition of PDP1 (11,13).

Here we report that phosphorylation of PDHA1 at tyrosine residue Y301 is also common in EGF-stimulated cells as well as diverse human cancer cells, which inhibits PDHA1 by blocking pyruvate binding through a novel molecular mechanism in addition to S293 phosphorylation, and promotes the Warburg effect.

**EXPERIMENTAL PROCEDURES**

Reagents - PDHA1 cDNA image clone (Open Biosystems) was used to engineer several PDHA1 variants with a FLAG epitope tag, and were subsequently subcloned into pDEST27 for GST-tagged PDHA1 expression and purification in mammalian cells and pET53 vectors (Invitrogen) for His-tagged PDHA1 expression and purification in bacteria, respectively. Point mutations were introduced using QuikChange-XL site-directed mutagenesis kit (Stratagene). [5-3H]-glucose, [1-14C]-pyruvate and [2-14C]-pyruvate were purchased from Perkin Elmer. Stable knockdown of endogenous PDHA1 was achieved using a lentiviral vector harboring an shRNA construct (Open Biosystems; 5’-CGAATGGAGTTGAAAGCAGAT-3’). PDHA1 rescue H1299 cell lines were generated as previously described (11). Briefly, retroviral vector pLHCX (Clontech) containing shRNA-resistant, Flag-tagged human PDHA1 wild type (WT) or mutant forms harboring silent mutations in the shRNA targeted region were transfected into H1299 cells containing shRNA directed against endogenous PDHA1. Antibody against PDHA1 was purchased from Invitrogen. Phospho-PDHA1 (S293) antibody was purchased from Calbiochem. Phospho-Tyr antibody pY99 was purchased from Santa Cruz Biotechnology. Anti-FLAG, β-actin and GST antibodies were purchased from Sigma. Specific antibody against phospho-PDHA1 (pY301) and acetyl-PDHA1 (K321-Ac) was generated by Cell Signaling Technology (CST) specifically for the current project and is not currently commercially available.

Cell culture – Human lung cancer FGFR1-expressing H1299 (14) cells and A549 (15) cells, and leukemogenic tyrosine kinase expressing leukemia HEL (16), KG-1a (17), MO91 (18), EOL1 (19), Molm14 (20) and K562 (21) cells were cultured in RPMI 1640 medium with 10% bovine serum (FBS). 293T cells, human breast cancer MDA-MB231 (22) and MCF7 (23) cells, as well as normal control human proliferating cells including human foreskin fibroblasts (HFF) (24) and immortal human keratinocyte HaCaT cells (25) were cultured in Dulbecco Modified Eagle
Medium (DMEM) with 10% FBS. TKI258 (generously provided by Novartis Pharma) treatment was performed by incubating cells with 1 μM TKI258 for 4 hrs. For the cell proliferation assay, 125 nM oligomycin was added in the cell culture medium as previously described (26). Imatinib was purchased from Santa Cruz Biotechnology.

Purification of PDHA1 proteins - (His)₆-tagged PDHA1 protein were purified by sonication of high expressing BL21(DE3)pLysS cells obtained from a 250 ml culture subjected to IPTG-induction for 16 hrs. Cell lysates were resulted by centrifugations and loaded onto a Ni-NTA column in 20 mM imidazole. After washing twice, the centrifugations and loaded onto a Ni-NTA column in 20 mM imidazole. Proteins were desalted on a PD-10 column and the purification efficiency was examined by Coomassie staining and western blotting.

PDC activity assay - The PDC activity was measured by conversion rate of [1-¹⁴C]-pyruvate (Perkin Elmer) to ¹⁴CO₂ as described (11,27). In brief, ¹⁴CO₂ production through PDC was measured using isolated mitochondria (1 mg) in radioactive mitochondria resuspension buffer (1 ml) containing [1-¹⁴C]-pyruvate (0.1 μCi/ml), 200 mM sucrose, 10 mM Hepes-HCl (pH 7.4), 1 mM pyruvate, 1 mM malate, 2 mM sodium monophosphate, 1 mM EGTA. The incubation mixture was placed at the bottom of a vial with a rubber stopper and maintained in agitation. The ¹⁴CO₂ produced during incubation was trapped by hyamine hydroxide placed in an eppendorf tube in 0.5 ml of 50% TCA to the reaction after 1h. Twenty minutes after the TCA injection, all the samples in hyamine hydroxide were transferred to mini-vials together with 5 ml of scintillation fluid, and radioactivity was assayed on a scintillation counter. The results were normalized based on mitochondrial protein levels assayed by Bradford assay using BSA as a standard.

In vitro kinase assay - For FGFR1 kinase assay, 200 ng of purified recombinant PDHA1 WT, Y301F or Y289F proteins were incubated with 250 ng of recombinant active FGFR1 in FGFR1 kinase buffer [10 mM Hepes (pH 7.5), 150 mM NaCl, 10 mM MnCl₂, 0.01% Triton X-100, 5 mM DTT, 200 μM ATP] for 1 hr at 30°C. For ABL kinase assay, purified recombinant PDHA1 proteins were incubated with 175 ng of recombinant active ABL in ABL kinase buffer [50 mM Tris (pH 7.5), 10 mM MgCl₂, 0.01% NP-40, 1 mM DTT, 200 μM ATP] for 1 hr at 30°C. For JAK2 kinase assay, purified recombinant PDHA1 proteins were incubated with 200 ng of recombinant active JAK2 in JAK2 kinase buffer [25 mM Hepes (pH 7.5), 10 mM MgCl₂, 0.5 mM EGTA, 0.01% Triton X-100, 2.5 mM DTT, 0.5 mM Sodium orthovanadate, 5 mM -glycerophosphate, 200 μM ATP] for 1 hr at 30°C. The samples were electrophoresed on 10% SDS-polyacrylamide gel, transferred on a nitrocellulose membrane, and then detected with the phospho-PDHA1 (pY301) and anti-phophotyrosine (pY99) antibodies.

Pyruvate binding assay - Purified recombinant His-FLAG-PDHA1 proteins including WT, Y301F and control Y289F that were immobilized on anti-Flag beads were treated with or without rFGFR1 in an in vitro kinase assay as described above. The beads were incubated with 0.1 μM [2-¹⁴C]-pyruvate for 2 hrs at room temperature. The beads were then washed twice with TBS to remove the unbound ¹⁴C pyruvate. The PDHA1 proteins were eluted and the retained [2-¹⁴C]-pyruvate on PDHA1 was measured using a scintillation counter.

PDHA1 assay - PDHA1 activity was assayed by the formation of NADH after reconstitution of recombinant human protein PDHA1, E2-E3BP and E3 in the ratio 1:3:3. The mixture was incubated in 37°C for 5 min in PDHA1 buffer containing 50 mM potassium phosphate buffer, pH 7.5, containing 2 mM MgCl₂, 2 mM NAD⁺, 156 mM CoA, 4 mM cysteine, 0.2 mM TPP. The assay was then initiated by the addition of 2 mM pyruvate (Sigma) and the formation of NADH was monitored using a spectrofluorometer (ex.340 nm; em.460 nm).

Lactate production, oxygen consumption and intracellular ATP assays - Cellular lactate production under normoxia was measured using a fluorescence-based lactate assay kit (MBL). Phenol red-free RPMI medium without FBS was added to a 6 well-plate of subconfluent cells, and was incubated for 1 hr at 37 °C. After incubation, 1 µl of media from each well was assessed using the lactate assay kit. Cell numbers were determined by cell counting using a microscope (x40). Oxygen consumption rates were measured with a Clark type electrode equipped with 782 oxygen meter (Strathkelvin Instruments). 1 x 10⁷ cells were resuspended in RPMI 1640 medium
with 10% FBS and placed into a water-jacked chamber RC300 (Strathkelvin Instruments) and recording was started immediately. Intracellular ATP concentration was measured by an ATP bioluminescent somatic cell assay kit (Sigma). Briefly, 1 x 10^6 cells were trypsinized and resuspended in ultrapure water. Luminescence was measured with spectrofluorometer (SpectraMax Gemini; Molecular Devices) immediately after the addition of ATP enzyme mix to cell suspension.

**Glycolytic rate assay** Glycolytic rate was measured by monitoring the conversion of 5-^3^H-glucose to ^3^H_2>O. In brief, 0.5 x 10^6 cells were washed once in PBS prior to incubation in 1 ml of Krebs buffer without glucose for 30 min at 37°C. The Krebs buffer were replaced with Krebs buffer containing 10 mM glucose spiked with 10 μCi of ^3^H-glucose. After incubation for 1 hr at 37°C, triplicate 50 μl aliquots were transferred to uncapped PCR tubes containing 50 μl of 0.2 N HCl, and each tube was transferred into an eppendorf tube containing 0.5 ml of H_2>O for diffusion. The tubes were sealed, and diffusion were allowed to occur for a minimum of 24 hrs at 34°C. The amounts of diffused ^3^H_2>O were determined by scintillation counting.

**Cell proliferation assays** - Cell proliferation assays were performed by seeding 5 x 10^4 cells in a 6-well plate and culturing the cells at 37°C in normoxia (5% CO_2 and 95% air). Twenty-four hrs after seeding, cells that were used for further culture under hypoxia were cultured at 37°C in a sealed hypoxia chamber filled with 1% O_2, 5% CO_2, and 94% N_2. Cell proliferation was determined by cell numbers recorded by TC10 Automated Cell Counter (Bio-Rad) at indicated days.

**Xenograft studies** - Approval of use of mice and designed experiments was given by the Institutional Animal Care and Use Committee of Emory University. Nude mice (nu/nu, male 4–6-week-old, Harlan Laboratories) were subcutaneously injected with 20 x 10^6 rescue H1299 cells stably expressing hPDHA1 WT and hPDHA1 Y301F with stable knockdown of endogenous hPDHA1. Statistical analyses were performed using a two-tailed paired Student’s t test.

**RESULTS**

**FGFR1 inhibits PDHA1 via phosphorylation at Y301.** Our phospho-proteomics studies (11,12) and multiple proteomics-based studies performed by our collaborators at Cell Signaling Technology (CST) (http://www.phosphosite.org) revealed that, in addition to its upstream kinase PDK1 (12) and phosphatase PDP1 (11,13), PDHA1 is phosphorylated at a group of tyrosine residues in human cancer cells (Fig. 1A). Consistently, we found that co-expression of FGFR1 wild type (WT) but not a kinase dead form (KD) resulted in tyrosine phosphorylation of GST-tagged PDHA1, and such FGFR1-dependent tyrosine phosphorylation was attenuated upon treatment with FGFR1 small molecule inhibitor TK1258 (Fig. 1B). Moreover, treatment with active, recombinant FGFR1 (rFGFR1) resulted in tyrosine phosphorylation of purified His and FLAG-tagged PDHA1 in an in vitro kinase assay, leading to decreased PDHA1 catalytic activity (Fig. 1C). To examine the effect of tyrosine phosphorylation on PDHA1, we performed mutational analysis and generated diverse phospho-deficient Y→F mutants of PDHA1 to replace each of the tyrosine residues that were identified as phosphorylated. We found that treatment with FGFR1 significantly reduced PDHA1 enzyme activity (Figs.1C-1D), whereas only substitution of PDHA1 Y301 but not Y242, Y289, Y366 or Y369 abolished FGFR1-dependent inhibition of PDHA1 (Fig. 1D). These results together suggest that FGFR1 inhibits PDHA1 by phosphorylating Y301.

**Y301 phosphorylation of PDHA1 is common in EGF-stimulated human cancer cells.** We next generated a specific phospho-PDHA1 antibody that recognizes Y301-phosphorylated PDHA1. Using this antibody, we found that EGF treatment resulted in increased phosphorylation levels of PDHA1 at Y301 and S293 as well as previously reported K321 acetylation (11) in 3T3 cells (Fig. 2A). In addition, we found that inhibition of FGFR1 by TK1258, BCR-ABL by imatinib, JAK2...
by AG490, and FLT3 by TKI258 resulted in decreased Y301 phosphorylation of PDHA1 in the pertinent human leukemia cell lines (Figs. 2B-2E left panels, respectively). Consistently, purified rFGFR1, rABL, rJAK2 and rFLT3 (Figs. 2B-2E right panels, respectively) directly phosphorylated purified rPDHA1 WT and control Y289F mutant at Y301 in vitro, whereas substitution at Y301 of PDHA1 abolished such phosphorylation.

In addition, we found that Y301 phosphorylation of PDHA1 is common in diverse human tumor cells including MCF-7 and MDA-MB-231 breast cancer cells, A549 and H1299 lung cancer cells, as well as a group of leukemia cells associated with distinct leukemogenic tyrosine kinases including EOL1 (HIP1L1-PDGFRα), HEL (JAK2 V617F), K562 (BCR-ABL), KG-1a (FOP2-FGFR1), Molm14 (FLT3-ITD) and Mo91 (TEL-TrkC) cells (Fig. 3A), but not in normal proliferating human foreskin fibroblasts (HFF) and HaCaT keratinocyte cells (Fig. 3A). Furthermore, we observed that Y301 phosphorylation levels of PDHA1 were increased in primary leukemia cells from three acute myeloid leukemia (AML) and three chronic myeloid leukemia (CML) patients compared to peripheral blood cells from a representative healthy donor (Fig. 3B).

Expression of PDHA1 Y301F mutant in H1299 cells leads to decreased proliferation under hypoxia and increased oxidative phosphorylation. We next tested whether Y301 phosphorylation-dependent inhibition of PDHA1 is important for glycolysis and cancer cell proliferation. We generated “rescue” H1299 cells with stable knockdown of endogenous human PDHA1 (hPDHA1), followed by rescue expression of shRNA-resistant FLAG-PDHA1 WT and Y301F that harbor silent mutations (SM) in the target regions of shRNA (Fig. 4A). As shown in Fig. 4B; left, rescue expression of PDHA1 WT did not affect H1299 cell proliferation under normoxic or hypoxic conditions till day 5. In contrast, rescue expression of phospho-deficient PDHA1 Y301F significantly attenuated cell proliferation under hypoxia but not normoxia, while neither normoxia nor hypoxia affected Y301 phosphorylation of PDHA1 in H1299 cells (Fig. 4B; right). Consistent with these findings, PDHA1 Y301F expressing cells demonstrated increased PDC flux rate (Fig. 4C), along with decreased lactate production and glycolytic rate (Fig. 4D) under normoxia compared to WT cells. In addition, PDHA1 Y301F cells were more sensitive to treatment with ATP synthase inhibitor oligomycin in terms of inhibition of ATP production (Fig. 4E; left) and oxygen consumption rate (Fig. 4F), compared to control PDHA1 WT rescue cells, while oligomycin treatment did not affect Y301 phosphorylation of PDHA1 in H1299 cells (Fig. 4E; right). These data together suggest that abolishment of Y301 phosphorylation of PDHA1 leads to a metabolic change to allow cells to rely more on oxidative phosphorylation with decreased glycolysis, providing a metabolic disadvantage to cell proliferation under hypoxia.

It has been reported that long term (>72 hrs) culture under hypoxia results in increased PDK1 (28) and LDHA1 (29) expression, leading to decreased PDC activity and increased lactate production, respectively. We found that, even although short-term (2 hrs) hypoxic condition were insufficient to affect the increased PDC activity (Fig. 4G; left) or the decreased lactate production (Fig. 4G; middle) in PDHA1 Y301F expressing cells, such a low oxygen condition resulted in decreased ATP levels in these cells (Fig. 4G; right), compared to control PDHA1 WT rescue cells where short term hypoxic exposure had not affected PDC activity and lactate production yet. This is consistent with our hypothesis that cells expressing PDHA1 Y301F mutant rely more on OXPHOS for ATP production compared to control WT cells, so under hypoxic condition where oxygen is insufficient to sustain OXPHOS level, PDHA1 Y301F cells showed decreased ATP levels and subsequently reduced cell proliferation.

Y301 phosphorylation of PDHA1 is important for tumor growth. We next performed xenograft experiments and found that the growth rate (Fig. 5A) and masses of tumors (Fig. 5B) derived from PDHA1 Y301F rescue H1299 cells were significantly reduced with decreased Y301 phosphorylation of PDHA1 in H1299 cells (Fig. 5C) compared to those of tumors formed by the control PDHA1 WT rescue cells. Together these data demonstrate an important role for Y301 phosphorylation of PDHA1 in tumor growth.

Y301 phosphorylation inhibits PDHA1 by blocking substrate binding through a novel mechanism in addition to S293 phosphorylation. In order to
determine whether Y301 phosphorylation functions independent of S293 phosphorylation, we examined whether alteration of phosphorylation levels of these sites in PDHA1 would affect each other. We found that abolishment of Y301 phosphorylation did not alter S293 phosphorylation or K321 acetylation levels of PDHA1 Y301F mutant, while altered K321 acetylation attenuated phosphorylation levels of S293 as previously reported (11) but not Y301 in PDHA1 K321R mutant, compared to PDHA1 WT (Fig. 6A). Moreover, treatment with PDK1 inhibitor dichloroacetate (DCA) resulted in decreased S293 but not Y301 phosphorylation levels of PDHA1 in H1299 cells (Fig. 6B).

**Y301 phosphorylation inhibits PDHA1 by blocking substrate pyruvate binding.** Y301 of PDHA1 is evolutionarily conserved (Table 1). Structural analysis revealed that Y301 is close to the active site (less than 10Å away), a similar proximity to the active site as S293, whereas, for example, the nonfunctional phosphorylation site Y289 is more distal (Fig. 7A). Since phosphorylation of S293 impedes active site accessibility (7), we hypothesized that Y301 phosphorylation could also impact active site accessibility. We tested this hypothesis by incubating recombinant PDHA1 (rPDHA1) with 14C-labeled pyruvate (substrate) in the presence and absence of active, recombinant FGFR1 (rFGFR1). Phosphorylation of rPDHA1 by rFGFR1 resulted in decreased 14C-labeled pyruvate binding to PDHA1 WT and control Y289F mutant proteins, whereas substitution of Y301 abolishes FGFR1-dependent inhibition of substrate binding (Fig. 7B).

**DISCUSSION**

Our findings shed new insight into the molecular mechanisms by which growth factors and oncogenic tyrosine kinase signaling attenuate mitochondrial function by inhibiting PDHA1 through direct phosphorylation at Y301. The consequently decreased PDC activity in part contributes to the metabolic switch to allow proliferating and cancer cells to rely more on glycolysis instead of mitochondrial oxidative phosphorylation, providing a metabolic advantage to proliferating cells and cancer cell proliferation/tumor growth, respectively. Interestingly, Y301 phosphorylation also inhibits PDHA1 by impacting substrate (pyruvate) binding, similar as the well-known inhibitory S293 phosphorylation of PDHA1 regulated by its upstream kinase PDK1 and phosphatase PDP1. However, we demonstrated that Y301 phosphorylation represents a novel and distinct molecular mechanism underlying regulation of PDHA1 involving active site accessibility. These findings, in addition to our previous reports (11-13), together showcase the beauty of precise and organized signal transduction-based regulation of cellular processes, in which growth factors as well as oncogenic tyrosine kinases regulate PDHA1 activity directly through Y301 phosphorylation and indirectly through tyrosine phosphorylation of PDK1 (12) and PDP1 (11,13) to control S293 phosphorylation (Fig. 7C). Thus, these distinct mechanisms act in concert to provide parallel regulation of PDHA1 that ensures appropriate control of PDC in normal proliferating cells and cancer cells.

Our findings also suggest that tyrosine phosphorylation of PDHA1 is common in diverse human tumor and leukemia cells, as well as primary leukemia cells from human patients, which represents an acute mechanism to mediate upstream oncogenic tyrosine kinase signaling-dependent regulation to mitochondrial PDC. These findings add another example to the emerging concept that supports the importance of tyrosine phosphorylation of metabolic enzymes including PGAM1 (30), PKM2 (31), LDH-A (26), PDP1 (11,13) and PDK1 (12), in cancer cell metabolism and proliferation, as well as tumor growth. Future studies are warranted to explore how tyrosine kinase signaling coordinates phosphorylation and regulation of these enzymes to provide an ultimately optimized metabolic advantage to normal cell proliferation, which is, unfortunately, “hijacked” by the Warburg effect in cancer cells and tumor growth.

Lastly, our findings suggest that functional activation of PDHA1 may be explored as a therapeutic strategy in treatment of human cancers that heavily depend on glycolysis. Future studies are warranted to develop PDHA1 activators as novel anti-cancer agents.

**REFERENCES**


**FOOTNOTES**

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**FIGURE LEGENDS**

**FIGURE 1. FGFR1 inhibits PDHA1 via phosphorylation at Y301.** (A) Schematic representation of PDHA1. Five identified FGFR1-direct tyrosine phosphorylation sites are shown. (B) Western blot detecting tyrosine phosphorylation of purified GST-PDHA1 from cells co-expressing FGFR1 wild type (WT) or an FGFR1 kinase dead form (KD) using a pan phospho-Tyr antibody. Cells treated with FGFR1 inhibitor TKI258 were incubated for 4 hrs at indicated concentrations. (C) Active recombinant FGFR1 (rFGFR1) directly phosphorylates purified His-FLAG-PDHA1 at tyrosine residues in an in vitro kinase assay and attenuates PDHA1 activity. (D) Purified His-FLAG-PDHA1 variants were incubated with rFGFR1, followed by PDHA1 activity assay. The error bars represent mean values +/- SD from three replicates of each sample (*:0.01<p<0.05; **: 0.001<p<0.01; ns: not significant).

**FIGURE 2. EGF stimulation and diverse oncogenic tyrosine kinases lead to Y301 phosphorylation of PDHA1.** (A) 3T3 cells treated with EGF for increasing time were examined for phospho-PDHA1 (p-Y301) level using immunoblotting. (B-E) *Left panels:* Immunoblotting shows phosphorylation levels of PDHA1 Y301 in FGFR1-expressing human lung cancer H1299 cells treated with FGFR1 inhibitor TKI258 (B), BCR-ABL-expressing leukemia K562 cells treated with ABL inhibitor imatinib (C), JAK2 V617F-expressing leukemia HEL cells treated with JAK2 inhibitor AG490 (D), or FLT3-ITD-expressing leukemia Molm 14 cells treated with FLT3 inhibitor TKI258 (E). *Right panels:* Purified His-FLAG-PDHA1 WT, Y301F or Y289F were incubated with rFGFR1 (B), rABL (C), rJAK2 (D) or rFLT3 (E), followed by immunoblotting to detect Y301 phosphorylation of PDHA1.

**FIGURE 3. Y301 phosphorylation of PDHA1 is common in human cancer cells.** (A-B) Immunoblotting to detect phosphorylation levels of PDHA1 Y301 in diverse human tumor and leukemia cells (A) as well as human primary leukemia cells isolated from peripheral blood (PB) or bone marrow (BM) samples from representative AML and CML patients (B). Normal proliferating human foreskin fibroblasts (HFF), HaCaT keratinocyte cells and PB cells from healthy human donors were included as controls.
FIGURE 4. Expression of PDHA1 Y301F mutant in H1299 cells leads to decreased proliferation under hypoxia and increased oxidative phosphorylation. (A) Generation of H1299 cells with stable knockdown of endogenous human PDHA1 (hPDHA1), followed by stable “rescue” expression of FLAG-tagged hPDHA1 variants, which harbor silent mutations (SM) that confer PDHA1 shRNA-resistance. (B) Left: PDHA1 WT and Y301F rescue H1299 cells were tested for cell proliferation rate under normoxia (17% oxygen) or hypoxia (1% oxygen). Cell proliferation was determined based on cell numbers counted daily. Right: Phosphorylation level of PDHA1 Y301 was examined in H1299 cells under normoxic or hypoxic conditions using immunoblotting. (C-D) PDHA1 WT or Y301F rescue H1299 cells were tested for PDC flux rate (C) as well as lactate production and glycolytic rate (D; left and right, respectively) under normoxia. (E-F) PDHA1 rescue cells were tested for intracellular ATP level (E) and oxygen consumption (F) in the presence and absence of ATP synthase inhibitor oligomycin under normoxia. E; right: Phosphorylation level of PDHA1 Y301 was examined in H1299 cells in the presence or absence of oligomycin. (G) PDHA1 rescue cells were tested for PDC flux rate (left), lactate production (middle) and intracellular ATP level (right) under normoxic or hypoxic conditions for 2 hrs. The error bars represent mean values +/- SD from three replicates of each sample (*:0.01<p<0.05; **: 0.001<p<0.01; ns: not significant).

FIGURE 5. Y301 phosphorylation of PDHA1 is important for tumor growth. (A-B) Tumor growth (A) and (B) masses in xenograft nude mice injected with PDHA1 Y301F rescue cells compared to mice injected with control PDHA1 WT rescue cells are shown. p values were determined by a two-tailed paired Student's t test. (C) Top: Dissected tumors in a representative nude mouse injected with PDHA1 WT and Y301F rescue cells on the left and right flanks, respectively, are shown. Bottom panel shows detection of Y301 phosphorylation levels of PDHA1 in tumor lysates using specific phospho-PDHA1 (Y301) antibody.

FIGURE 6. Y301 phosphorylation provides a novel and distinct mechanism to inhibit PDHA1. (A) Distinct PDHA1 rescue cells were tested in immunoblotting to detect Y301 phosphorylation, K321 acetylation and S293 phosphorylation levels. (B) Immunoblotting results detecting phosphorylation levels of S293 and Y301 in H1299 cells treated with PDK inhibitor DCA.

FIGURE 7. Y301 phosphorylation inhibits PDHA1 by blocking substrate pyruvate binding. (A) Cartoon representation of PDHA1 structure (PDB ID: 1NI4; (32)). Y301 is proximal to the PDHA1 catalytic site where substrate (pyruvate) binds (less than 10Å), a distance similar to S293-pyruvate. (B) Purified His-FLAG-PDHA1 variants were incubated with rFGFR1, followed by incubation with [2-14C]-labeled pyruvate. PDHA1-bound [2-14C]-pyruvate was assessed by scintillation counting. The error bars represent mean values +/- SD from three replicates of each sample (*:0.01<p<0.05; **: 0.001<p<0.01; ns: not significant). (C) Proposed model shows that phosphorylation at Y301 inhibits PDHA1 and blocks substrate pyruvate binding through a novel and distinct mechanism in addition to S293 phosphorylation in EGF-stimulated cells and cancer cells where tyrosine kinase signaling is commonly upregulated.

TABLE 1. Phylogenetic analysis of PDHA1 in different species.

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<th>Species</th>
<th>PDHA1 sequence (Y301)</th>
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<td>Caenorhabditis elegans (C. elegans)</td>
<td>MSDPGRSYRTREEIQ</td>
</tr>
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<tr>
<td>Saccharomyces cerevisiae</td>
<td>MSDPGRTRYRDEIQ</td>
</tr>
</tbody>
</table>
Figure 2

A

EGF (100 ng/ml) 0 2 4 6 8 16 hrs
WB: PDHA1 Y301
WB: PDHA1 S293
WB: PDHA1 K321
WB: PDHA1

3T3 cells

B

TKI258 0 1 (μM)

His-FLAG-PDHA1
p-rPDHA1 (Y301)
p-PDHA1
p-HGF1
p-β-actin

H1299 cell (FGFR1)

C

Imatinib 0 10 (μM)

His-FLAG-PDHA1
p-rPDHA1 (Y301)
p-PDHA1
p-β-actin

H1299 cell (FGFR1)

D

AG490 0 50 (μM)

His-FLAG-PDHA1
p-rPDHA1 (Y301)
p-PDHA1
p-β-actin

HEL cell (JAK2 V617F)

E

TKI258 0 0.5 (μM)

His-FLAG-PDHA1
p-rPDHA1 (Y301)
p-PDHA1
p-β-actin

Molm14 cell (FLT3-ITD)

In vitro kinase assay
Figure 7

A

PDHA1
S293
Y301
Thiamine
Diphosphate

B

Relative [14C]-pyruvate
binding (%)

C

EGF stimulation
Oncogenic tyrosine kinases

Y301
p-PDHA1 (Y301)

PDHA1
p-FGFR1
p-PDHA1
PDHA1

Substrate (pyruvate) binding

PDC

rFGFR1
rPDHA1

WT
Y301F
Y289F
Tyr-301 phosphorylation inhibits pyruvate dehydrogenase by blocking substrate binding, and promotes the Warburg effect
Jun Fan, Hee-Bum Kang, Changliang Shan, Shannon Elf, Ruiting Lin, Jianxin Xie, Ting-Lei Gu, Mike Aguiar, Scott Lonning, Tae-Wook Chung, Martha Arellano, Hanna J. Khoury, Dong M. Shin, Fadlo R. Khuri, Titus J. Boggon, Sumin Kang and Jing Chen

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