

p53 Negatively Regulates Transcription of the Pyruvate Dehydrogenase Kinase Pdk2

Tanupriya Contractor¹ and Chris R. Harris^{1,2,3}

Abstract

In cancer cells, the aberrant conversion of pyruvate into lactate instead of acetyl-CoA in the presence of oxygen is known as the Warburg effect. The consequences and mechanisms of this metabolic peculiarity are incompletely understood. Here we report that p53 status is a key determinant of the Warburg effect. Wild-type p53 expression decreased levels of pyruvate dehydrogenase kinase-2 (Pdk2) and the product of its activity, the inactive form of the pyruvate dehydrogenase complex (P-Pdc), both of which are key regulators of pyruvate metabolism. Decreased levels of Pdk2 and P-Pdc in turn promoted conversion of pyruvate into acetyl-CoA instead of lactate. Thus, wild-type p53 limited lactate production in cancer cells unless Pdk2 could be elevated. Together, our results established that wild-type p53 prevents manifestation of the Warburg effect by controlling Pdk2. These findings elucidate a new mechanism by which p53 suppresses tumorigenesis acting at the level of cancer cell metabolism. *Cancer Res*; 72(2); 560-7. ©2011 AACR.

Introduction

Cancer and noncancer cells can differ in the metabolism of glucose. In cancer cell lines, as much as 90 percent of glucose is converted into lactate (1), whereas nontumor cells tend to fully oxidize glucose into carbon dioxide. High production of lactate by tumor cells occurs even in the presence of oxygen and is known as aerobic glycolysis or the "Warburg effect." Although first noted by Otto Warburg (2, 3), aerobic glycolysis has drawn more research interest lately, and it is hoped that drugs that attack the Warburg effect will prove to be effective in fighting tumor formation. It is not completely clear why the Warburg effect is favored by tumor cells, but presumably, it is beneficial for cell proliferation. Glycolysis produces ATP more quickly than oxidative phosphorylation, which may favor rapid growth if glucose is plentiful. Also, interruption of glycolysis can divert glycolytic intermediates toward synthesis of important cell-cycle molecules such as deoxyribonucleotides and phospholipids, which would be favorable for DNA replication and membrane synthesis (4). Another consideration is that complete oxidation of glucose produces reactive oxygen species (ROS; ref. 5), leading to apoptosis (6), so tumor cells may restrict oxidative phosphorylation as a survival mechanism.

In this study, we tested whether the tumor suppressor protein p53 helps to prevent the Warburg effect. Approximately half of all tumors harbor a mutation in the *p53* gene (7), and

the remaining tumors likely have mutations in one or more of the many pathways impacted by p53. p53 plays several key roles in the prevention of tumors, such as inducing cell-cycle arrest, senescence, or apoptosis in response to DNA damage (8). More recently, p53 has been shown to affect cellular metabolism, such as the mTOR pathway (9).

Clinically, *p53* mutations correlate with increased uptake of the glucose analog ¹⁸FDG (10), which is a marker for aerobic glycolysis. These data suggest that p53 could lower glucose uptake and/or glucose catabolism by tumors. Indeed, as detailed in the discussion section, p53 is known to increase the expression of genes that should decrease glycolysis (11, 12). However, p53 is also known to increase the expression of genes that should increase glucose metabolism (13, 14). Thus, the precise mechanism by which p53 alters glucose metabolism has not been completely clear.

We decided to test the effect of p53 on a key decision point in glucose metabolism: the conversion of pyruvate, the end product of glycolysis, into acetyl-CoA, which is one of the entry points into the citric acid cycle. This decision point is controlled in large part by the enzyme pyruvate dehydrogenase kinase isoenzyme-2 (Pdk2), which inactivates acetyl-CoA production by phosphorylating the Pdc. We show that transcription of Pdk2 is negatively regulated by p53. Thus p53 can affect the most singular feature of the Warburg effect, the production of lactate.

Materials and Methods

Plasmids

A plasmid expressing Pdk2 under *CMV* promoter control was purchased from Origene. pCMV-wtp53 was a gift from Arnold Levine, and the pCMV-fsp53 control plasmid was prepared by inserting 2 frame-shift mutations into the *p53* gene. pHA-E2F1, pHA-E2F2, and pCMV-neo plasmids were

Authors' Affiliations: ¹Raymond and Beverly Sackler Foundation; ²Cancer Institute of New Jersey; and ³Department of Pediatrics, University of Medicine and Dentistry of New Jersey, New Brunswick, New Jersey

Corresponding Author: Chris R. Harris, Cancer Institute of New Jersey, Room 3529, 195 Little Albany Street, New Brunswick, NJ 08901. Phone: 732-235-5590; Fax: 732-235-6618; E-mail: harrisch@umdnj.edu

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gifts from Alain Nepveu. Human Pdk2/luciferase was prepared by PCR of the *Pdk2* promoter from human genomic DNA, using primers 5'-AAAAGGTACCCTATTGGTTCGCCCGGAGTT-3' and 5'-TTTCTCGAGCGGCCGCGACTTTGGTTGTGG-3'. The PCR product was inserted into the *KpnI* and *XhoI* sites of pGL3-Basic (Promega). Murine Pdk2/luciferase was prepared by PCR of the *Pdk2* promoter from mouse genomic DNA, using primers 5'-AAAAGGTACCAGCACAGCCAGGAA-3' and 5'-AAAAGGATCCGGCTCGGGCTTGGATCTCGCT-3'. The PCR product was digested with *KpnI* and *BamHI*, then inserted into the *KpnI* and *BglII* sites of pGL3-Basic.

Cell lines

MCF7 was obtained from American Type Culture Collection. MCF7 CL.17 was constructed by transfection of a plasmid expressing Pdk2 under control of the *CMV* promoter. G418-resistant colonies were screened for Pdk2 expression in presence and absence of nutlin-3a. MCF7shp53 was kindly provided by Xinbin Chen. The cell lines EB and EB1 were gifts from Arnold Levine, and the HCT116, HCT116 (*p53*^{-/-}), and HCT116(*p21*^{-/-}) were gifts from Bert Vogelstein. Cell lines were not authenticated by the authors after receipt. Cells were grown in Dulbecco's modified Eagle's medium (DMEM)-10% FBS (MCF7, EB, and EB1) or in McCoy's-10% FBS (HCT116 and its derivatives). MCF7 CL.17 and MCF7shp53 were grown in DMEM-10% FBS supplement with 1 mg/mL G418 or 1 µg/mL puromycin, respectively. Media were purchased from Invitrogen and FBS was purchased from Sigma-Aldrich. Cells were maintained at 37°C in the presence of 5% carbon dioxide.

Western blots and RT-PCR

For protein analysis, lysates were isolated by radioimmunoprecipitation assay extraction in the presence of protease and phosphatase inhibitors (Sigma-Aldrich). Twenty micrograms of protein extracts were separated using NuPage 4% to 12% BisTris gels (Invitrogen). Pdk2 and PDH E1α antisera were purchased from Novus Biologicals. P-PDH E1α antisera were purchased from EMD Biosciences. Pdk1 antisera were purchased from Assay Designs. β-Actin and caspase 7 antisera were purchased from Sigma-Aldrich. Caspase 9 antisera were from Cell Signaling Technologies. For RNA analysis, RNA was extracted using RNeasy columns (Qiagen), then converted into cDNA using reverse transcription reagents (Applied Biosystems). All TaqMan assays were purchased from Applied Biosystems. Assays were read in real time using an Applied Biosystems 7500 and normalized to β-actin mRNA.

Chromatin immunoprecipitation assays

Two million EB1 cells were treated with 200 µmol/L zinc chloride, or mock treated, for 7 hours. Cells were then treated for 10 minutes with 0.01% formaldehyde at 37°C. Cells were washed and lysed, then chromatin was fragmented using a Sonic Dismembrator 50 (Fisher Scientific) at 30% to 40% output in 3 sets of 15 seconds. A kit for chromatin immunoprecipitation (ChIP) was purchased from Millipore. Antisera against E2F1 (Novus Biologicals) and acetyl-histone H4 (Millipore) were used for immunoprecipitation experiments. Immu-

noprecipitation was carried out as recommended by the manufacturer. Relative amounts of *Pdk2* promoter in the precipitated samples were assayed using 70 nmol/L concentrations of 2 oligonucleotides, 5'-CCGGAGTTGTTGTGAGTGG-3' and 5'-GCCTCCTCCCTACCCTTG-3'. Relative amounts of *Pdk2* promoter in the input (nonimmunoprecipitated) preparations were also determined and used for normalization. SYBR green was used to monitor PCR amplifications in real time.

Lactate assays

Cells were plated in 6-well plastic tissue culture dishes at a density of 500,000 cells. After 24 hours, fresh DMEM-10% FBS was added along with 10 µmol/L nutlin-3a (Sigma-Aldrich) or dimethyl sulfoxide (DMSO) alone. After 4 hours, media were removed and replaced with fresh media containing DMSO or nutlin-3a. After 12 additional hours, media were removed and centrifuged through 10K spin columns (BioVision) to remove cell debris and contaminating enzymes. The low molecular weight flow through was assayed for lactate using lactate assay kit II (BioVision). A no-cell control was used to get a basal level of lactate in the media. Each test condition was sampled in quadruplicate wells. The quantity of lactate was normalized to the number of viable cells in the same wells, which was determined by Guava ViaCount assay (Millipore).

Luciferase and other transient transfection experiments

HCT116(*p53*^{-/-}) cells were plated in 6-well plastic tissue culture dishes at a density of 500,000 cells. An amount of 0.8 µg of pPdk2-luciferase (either human or murine *Pdk2* promoter) was transfected along with 0.8 µg of purified native *Renilla* luciferase (Prolume Ltd). Native *Renilla* was used because *Renilla* reporters were sensitive to added E2F1, E2F2, and p53. Also added was 0.8 µg of pCMV-p53wt, pCMV-p53fs, pHA-E2F1, pHA-E2F2, or pCMV-neo. Lipofectamine 2000 (Invitrogen) was used for transfection as recommended by the manufacturer. After 24 hours, transfected cells were lysed and assayed for luciferase using a dual-glo luciferase kit (Promega). All siRNAs were purchased from Ambion and were transfected with siPORT, as recommended by the manufacturer. For p21 knockdown, HCT116(*p53*^{-/-}) cells were transfected and 24 hours later, cells were trypsinized and replated for luciferase experiments. For p53 knockdown, MCF7 were transfected and 24 hours later, cells were trypsinized and replated for nutlin-3a treatment.

Mouse experiments

Wild-type or isogenic *p53*^{-/-} male mice were irradiated with 10 Gray gamma from a Cesium source. Mice had no visible health problems at the time of assay. Three mice from both backgrounds were used, and 3 untreated mice from both backgrounds were also studied. Six hours after irradiation, mice were euthanized with carbon dioxide, in accordance with the Institutional Animal Care and Use Committee of Rutgers University. Spleen or epithelial lining of colon were isolated and RNA was extracted, converted into cDNA, and quantitated by real-time reverse transcriptase PCR (RT-PCR) as described above.

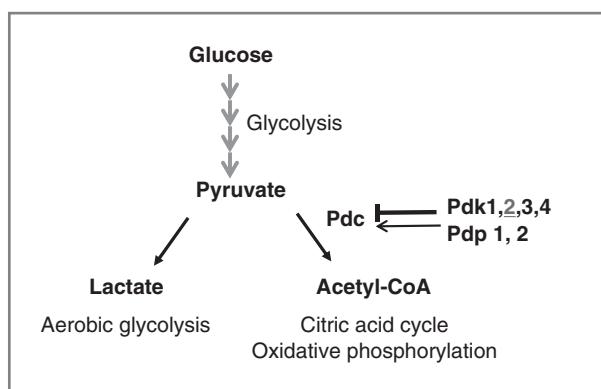


Figure 1. A decision point in pyruvate metabolism. In the left fork, pyruvate is converted into lactate, which regenerates NAD^+ , which can be used to drive more glycolysis and production of more pyruvate. In the right fork, pyruvate is converted into acetyl-CoA, which can be converted into carbon dioxide by the citric acid cycle. The right fork is catalyzed by Pdc, which is negatively regulated by phosphorylation. Pdk2 is the major inactivator of Pdc, but Pdk1, Pdk3, and Pdk4 are employed by certain cell types. Phosphorylation of Pdc can be reversed by either of 2 phosphatases, Pdp1 or Pdp2.

Results

Lactate is produced from pyruvate, the end product of glycolysis. As shown in Fig. 1, pyruvate can also be converted into acetyl-CoA, which can enter the citric acid cycle. Production of acetyl-CoA is catalyzed by the Pdc, whose activity is controlled by the action of several kinases and phosphatases. Pdc can be inactivated by phosphorylation, which should cause more lactate production. Because lactate production is a key component of the Warburg effect in cancer cells, we decided to test whether p53 can influence the phosphorylation of Pdc.

We ran our experiments on the MCF7 human breast carcinoma cell line, which encodes wild-type p53 from its natural promoter. To test the effects of p53 on lactate production, we challenged the cell line by addition of nutlin-3a, a compound that increases p53 activity by interfering with the interaction between p53 and its negative regulator, Mdm2 (15). As shown in Fig. 2A, the addition of nutlin-3a decreased the amount of phosphorylated Pdc, without altering the amount of unphosphorylated Pdc.

Phosphorylation of Pdc can be controlled by 4 different Pdk, that is, Pdk1 to 4 and by 2 different pyruvate dehydrogenase phosphatases (Fig. 1). Pdk1, 3, and 4 are expressed only in certain cell types, or under times of cellular stress (16). Pdk2 is more prevalent, with expression in all tissues. Indeed, under normal conditions, Pdk2 is seen as the key control point in conversion of pyruvate to acetyl-CoA reaction because it responds allosterically to rapid local changes in substrate and product: Pdk2-specific activity increases if acetyl-CoA and NADH levels increase, but decreases if pyruvate levels increase (16). We decided to test whether p53 affects Pdk2 expression.

As shown in Fig. 2A, Pdk2 levels dropped upon nutlin-3a treatment. Because nutlin-3a can produce off-target effects, we also looked for effects when p53 is knocked down using siRNA. Upon reduction of p53, the effect of nutlin-3a upon Pdk2 protein decreased (Fig. 2B). We also examined Pdk1 expression

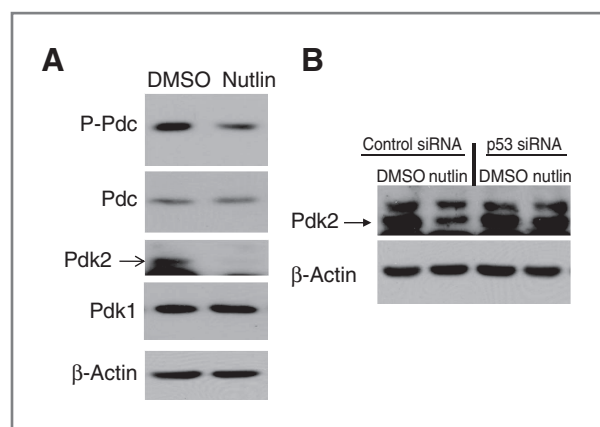


Figure 2. Western blots of proteins involved in pyruvate metabolism. A, MCF7 cells were treated with 10 $\mu\text{mol/L}$ nutlin-3a or mock-treated with DMSO for 24 hours. Protein was extracted then Western blotted using antisera against phospho-Pdc (P-Pdc), Pdc, Pdk2 or Pdk1, or β -actin. B, MCF7 cells transfected with negative control or p53 siRNA were treated with 10 $\mu\text{mol/L}$ nutlin or DMSO for 24 hours, then lysed and Western blotted for Pdk2.

and saw no effect of nutlin-3a by Western blot (Fig. 2A) or by RNA analysis (Fig. 3A). Transcript levels of *pdk3*, *pdp1*, and *pdp2* also did not change (Fig. 3A), although we were not able to analyze these proteins by Western blot, either due to poor antisera or low expression (not shown). MCF7 did not seem to express mRNA for *pdk4*.

Unlike the other mediators of Pdc phosphorylation, Pdk2 transcription decreased upon addition of nutlin-3a (Fig. 3A). This effect on Pdk2 mRNA levels is p53 dependent, because reduction of p53 by use of siRNA prevented transcriptional regulation by nutlin-3a (Fig. 3A). We also tested for effects of p53 on Pdk2 protein stability, but nutlin-3a treatment of MCF7 did not alter Pdk2 protein half-life (data not shown). Pdk2 transcription is downregulated by p53 in other cell lines, as shown in Fig. 3B. The EB1 human colon cell line expresses p53 from a zinc-inducible promoter, and zinc decreased transcription of Pdk2. In the EB cell line, which encodes no p53 but is otherwise isogenic, zinc did not affect the transcription of Pdk2.

We also fused a small segment of the human Pdk2 promoter to the firefly luciferase gene (Fig. 3C). This promoter fusion showed promoter activity in the absence of p53 but was strongly repressed in the presence of p53. A mouse Pdk2/luciferase promoter fusion was also downregulated by p53 (Fig. 3C).

Next, we tested whether p53 can control the expression of Pdk2 *in vivo*. Isogenic wild-type and p53 null mice were irradiated to activate p53. Six hours after irradiation, the mice were sacrificed, and colon and spleen were isolated from 3 treated or from 3 untreated animals. The p53 response to irradiation is very rapid in these tissues (17). As shown in Fig. 4A and B, the amount of Pdk2 mRNA in both colon and spleen decreased upon irradiation of wild-type mice but not upon irradiation of p53 knockout mice. Thus p53 decreases Pdk2 transcription *in vivo*. Interestingly, for both tissues the

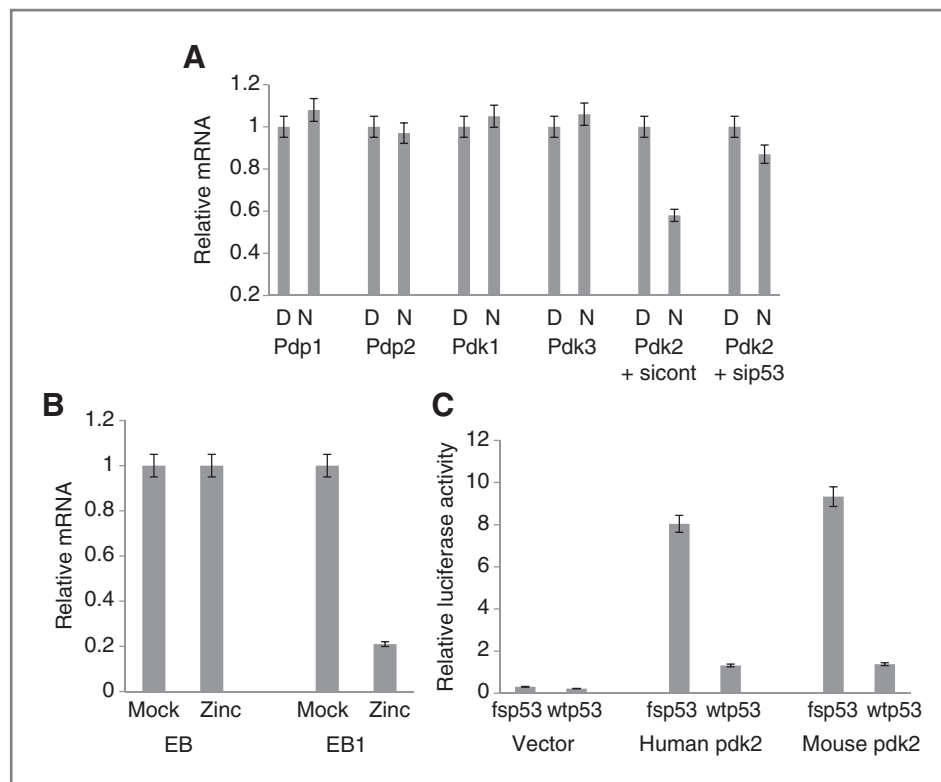


Figure 3. Transcriptional analysis of genes involved in the metabolism of pyruvate. A, mRNA concentrations were measured and normalized to β -actin mRNA using real-time RT-PCR following 24 hours of treatment with either DMSO (D) or 10 μ mol/L nutlin-3a (N). TaqMan assays were used for the following genes: Pdc phosphatases 1 or 2 (Pdp1, Pdp2); Pdk1, Pdk3, or Pdk2. For Pdk2 measurements, cells were first treated with either control siRNA (sicont) or p53 siRNA (sip53) prior to DMSO or nutlin-3a treatment. B, EB (*p53* null) or EB1 (zinc-inducible *p53*) cells were treated with 200 μ mol/L zinc for 8 hours, or else mock treated. mRNA for Pdk2 was measured by real-time RT-PCR and normalized to β -actin mRNA. C, promoter fusions were generated between a firefly luciferase reporter and either human Pdk2 or mouse Pdk2 sequences. These fusions, or empty vector pGL3-basic, were cotransfected into HCT116 (*p53*^{-/-}) cells with a plasmid expressing either wild-type p53 (wtp53) or a control plasmid in which the *p53* gene was inactivated with 2 frame-shift mutations (fsp53). After 24 hours, luciferase activity was assayed and normalized to a transfection control.

level of Pdk2 mRNA was higher in the untreated wild-type mice than in the untreated *p53* null mice.

We returned to cultured cell systems to investigate the mechanism of repression of the *Pdk2* promoter by p53. p53 can repress transcription of certain promoters by binding to DNA directly, but the *Pdk2* promoter has no p53 binding motifs nor did we observe p53 binding to the *Pdk2* promoter by ChIP (data not shown). Another common mechanism by which p53 represses promoters is shown in Fig. 5A, in which p53 inhibits the activity of E2F transcription factors (18, 19), which activate by promoting histone acetylation (20, 21). This type of regulation depends upon p53-dependent transcriptional activation of the cyclin-dependent kinase inhibitor p21, whose expression prevents phosphorylation of proteins of the retinoblastoma family (Rb, p107, and p130), which repress E2F factors (Fig. 5A). Analysis of the *Pdk2* promoter revealed many GC-rich sequences that might serve as E2F binding sites (data not shown).

Using a Pdk2/luciferase transcriptional fusion reporter construct, we tested 3 parts of the model in Fig. 5A: the influence of p21 expression on *Pdk2* promoter repression, the effect of E2F factors E2F1 and E2F2 on *Pdk2* promoter activation, and the effect of histone deacetylation on repression. First, we knocked

down p21 expression using siRNA. Reduction of p21 decreased repression of Pdk2/luciferase by p53 (Fig. 5B). Next, cotransfection with plasmids expressing either E2F1 or E2F2 activated Pdk2/luciferase transcription (Fig. 5C). Finally, a histone deacetylase inhibitor, trichostatin A, partially prevented repression of Pdk2 transcription by p53 (Fig. 5D).

Data from transcriptional fusions must be viewed with caution because these constructs lack more distal sequences that might also influence transcription. Therefore, we also looked for effects of p21, E2F1, and histone deacetylation on genomic Pdk2. To test for p21 effects, we used 3 versions of the human colon cell line HCT116. The parental line has wild-type *p53* and *p21*, whereas isogenic versions are knocked out for both copies of *p53* or for both copies of *p21*. We compared the amount of Pdk2 mRNA in each cell line with and without treatment with nutlin-3a. The wild-type cell line showed 60% repression of Pdk2 transcription after nutlin-3a treatment, but Pdk2 transcription was not repressed in either the *p53* or *p21* knockout lines (Fig. 6A). These data showed that both p53 and p21 are required for repression of Pdk2 transcription by nutlin-3a.

To test for the importance of E2F1 and histone deacetylation on genomic Pdk2, we carried out ChIP experiments on EB1

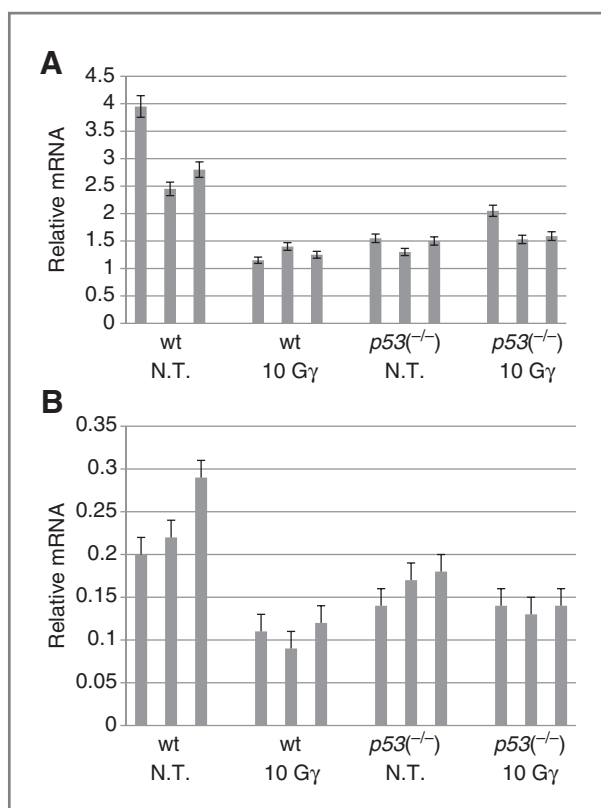


Figure 4. Analysis of *Pdk2* transcription in mice. The amount of *Pdk2* mRNA in wild-type or *p53* knockout mice colon (A) or spleen (B) was measured 6 hours after γ irradiation and normalized to β -actin mRNA. Data from 3 mice are shown. Nonirradiated (N.T.) mice were also examined.

cells, which repress *Pdk2* transcription in a *p53*-dependent fashion (Fig. 3B). E2F1 bound less well to the *Pdk2* promoter when *p53* is induced with zinc (Fig. 6B). This suggests that E2F1 activation of *Pdk2* transcription is blocked by *p53*. Moreover, when EB1 is treated with zinc, acetylation of histone H4 decreased, which is consistent with more compact chromatin and decreased transcription (Fig. 6C). Chromatin compaction may release E2F1 from the *Pdk2* promoter.

Finally, to determine the functional consequences of downregulation of *Pdk2* by *p53*, we engineered a cell line in which *Pdk2* expression is under control of a promoter, *CMV*, which is less repressed by *p53*. The new cell line, MCF7 CL.17, expresses near-physiologic levels of *Pdk2* in the presence of DMSO, but retains its *Pdk2* expression even in the presence of nutlin-3a (Fig. 7A). We then assayed lactate production. As shown in Fig. 7B, lactate production by MCF7 decreased more than 2-fold upon nutlin-3a treatment, but lactate production was mostly restored in MCF7 CL.17. Lactate production did not decrease at all in another cell line, MCF7shp53, in which *p53* expression is knocked down by short hairpin RNA. These data showed that *p53* decreases lactate production by MCF7 by decreasing the transcription of *Pdk2*.

A potential consequence of decreased *Pdk2* expression is increased oxidative phosphorylation, which can produce ROS

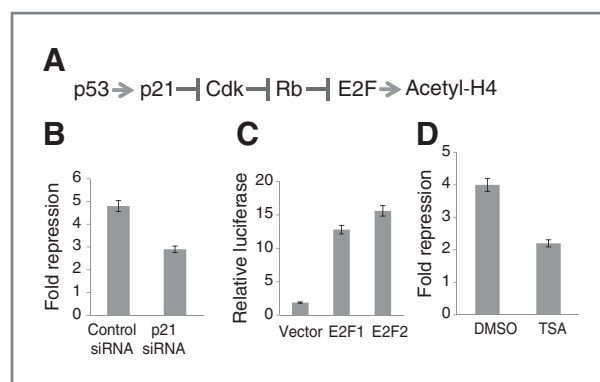


Figure 5. Testing a model of *p53*-dependent repression using *Pdk2* transcriptional fusions. A, repression model: *P53* induces *p21*, which inhibits cyclin-dependent kinases that phosphorylate proteins of the retinoblastoma family. Hypophosphorylated Rb proteins inhibit transcriptional activators of the E2F family, resulting in deacetylation of histones such as histone H4 and decreased promoter activity. B, the HCT116(*p53*^{-/-}) cell line was first treated with siRNA to knockdown *p21* expression, or else treated with a control siRNA. Then cells were transfected with a human *Pdk2*/luciferase reporter along with either a *p53*-expressing plasmid or frame-shifted *p53*. Fold-repression is the ratio of transcriptional activity in the absence versus presence of *p53*. C, plasmids that express the E2F1 or E2F2 transcription factors or empty vector pCMV-neo were cotransfected with the human *Pdk2*/luciferase transcriptional fusion. The amount of luciferase was measured after 24 hours. D, the human *Pdk2*/luciferase promoter fusion was cotransfected with either wild-type *p53* or frame-shifted *p53*. After 17 hours, trichostatin A (TSA) was added to inhibit histone deacetylation, or else the cultures were mock treated with DMSO. Luciferase was assayed 7 hours after TSA addition. Fold repression is the ratio of transcription in the absence versus presence of *p53*.

through the electron transport chain (5, 22). ROS can damage DNA, and a major mechanism of tumor suppression by *p53* is induction of apoptosis in the presence of high ROS. Indeed, *Pdk2* has previously been shown to lower ROS and to decrease apoptosis (23). We decided to test whether *p53*-dependent apoptosis requires downregulation of *Pdk2*. Because MCF7 does not express caspase 3 (24, 25), we were unable to view late apoptotic events in this cell line. However, a key early step in apoptosis is proteolytic processing of procaspases 7 and 9, which are upstream of caspase 3 (26). When we treated MCF7 with nutlin-3a, processing of caspases 7 and 9 occurred (Fig. 7C). Conversely, caspases 7 and 9 were not processed when MCF7 CL.17 was treated with nutlin-3a. These data clearly indicated that early apoptotic events do not occur unless *p53* can downregulate the transcription of *Pdk2*.

Discussion

A role for *p53* in downregulating the Warburg effect was previously surmised, mostly due to the effect of *p53* on the transcription of genes involved in glycolysis and oxidative phosphorylation. For instance, *p53* activates transcription of TIGAR, whose gene product can lower the quantity of fructose 2/6 biphosphate (11), which is a positive regulator of glycolytic enzymes phosphofructokinase and pyruvate kinase. *P53* also decreases the transcription of glucose transporter isoenzymes 1 and 4 (12). But the precise effect of *p53* on aerobic glycolysis

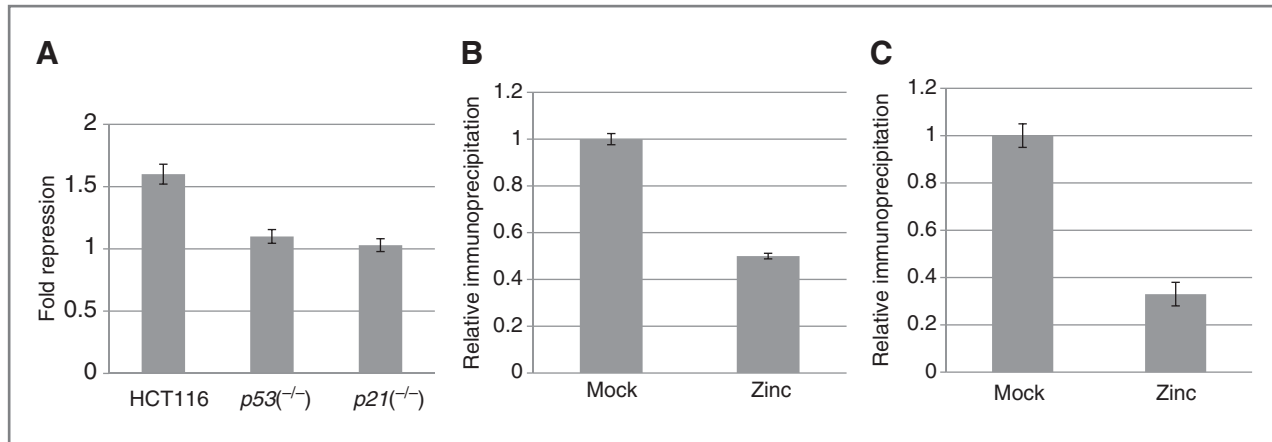
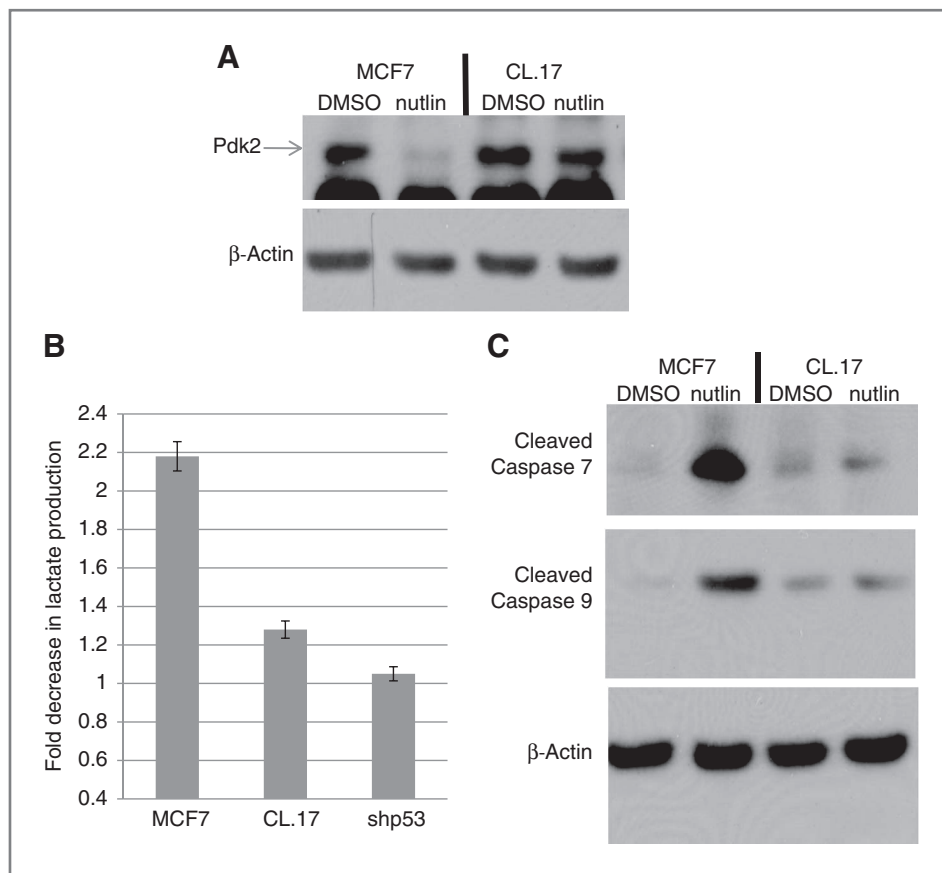


Figure 6. Tests of mechanism of p53-dependent repression of genomic Pdk2. A, the amount of Pdk2 mRNA relative to β -actin was determined following 24-hour nutlin-3a treatment (or mock DMSO treatment) of 3 isogenic cell lines: HCT116, HCT116(*p53*^{-/-}), or HCT116(*p21*^{-/-}). Fold repression was determined by comparing the amount of normalized mRNA in the absence to the amount of mRNA in the presence of nutlin-3a. B and C, the EB1 human colon cell line has zinc-inducible p53 and was treated with zinc for 7 hours or mock treated. Protein/chromatin cross-links were formed by formaldehyde treatment. The presence of *Pdk2* promoter in complex with either E2F1 (B) or acetyl-histone H4 (C) was determined by immunoprecipitation of the protein/chromatin complexes, followed by quantitation of *Pdk2* promoter sequences in real time by PCR.

was not certain because some activities of p53 would likely increase glycolysis. A key activity required for glucose uptake, hexokinase-2, is actually upregulated by p53 (13). The glycolytic enzyme phosphoglycerate mutase is also upregulated by p53 (14).

We show that activation of p53 causes a decrease in the production of lactate by the human breast carcinoma cell line MCF7. Because lactate production in the presence of oxygen is the key step in aerobic glycolysis, this study provides a critical link between p53 and downregulation of the Warburg effect.

Figure 7. Functional consequences of downregulation of Pdk2 by p53. A, MCF7 CL.17 was engineered to stably express Pdk2 under control of the *CMV* promoter as described in Materials and Methods. MCF7 and MCF7 CL.17 were treated with DMSO or 10 μ M nutlin-3a for 16 hours and lysed. The lysate was used to generate a Western blot against Pdk2 and β -actin, as shown. B, MCF7, MCF7 CL.17, and MCF7shp53 were treated with DMSO or 10 μ M nutlin-3a for 16 hours, followed by assay of lactate in the tissue culture supernatants. Fold decrease in lactate production upon nutlin-3a treatment is shown. The differences in fold decrease between MCF7shp53 and MCF7, and between MCF7 CL.17 and MCF7, were determined to be statistically significant (*P* value less than 0.01) by two-tailed Student's *t* test. C, MCF7 or MCF7 CL.17 were treated with DMSO or with 10 μ M nutlin-3a for 72 hours then lysed. Western blots against processed Caspases 7 or 9, or β -actin loading control, are shown.



We also show that activation of p53 results in higher amounts of the active, unphosphorylated form of Pdc and decreased amounts of Pdk2, a major kinase of Pdc. Finally, we show that adding back *Pdk2* under control of a heterologous promoter prevents p53 from downregulating lactate production. Pdk2 has long been seen as the primary gatekeeper over pyruvate metabolism. Thus p53, which has been called the cellular gatekeeper for growth and division (27), seems to downregulate the Warburg effect by controlling the expression of the gatekeeper of pyruvate metabolism.

Previously, Pdk2 activity was thought to be modulated in a strictly posttranslational fashion, through inhibition by pyruvate or activation by acetyl-CoA (16). Ours is the first study to suggest that control of Pdk2 transcription may also be important in pyruvate metabolism. P53 decreases the amount of Pdk2 transcription. In separate experiments, we show that p21 is required for p53-dependent repression of both a Pdk2 transcriptional fusion (Fig. 5B) and genomic Pdk2 (Fig. 6A). E2F1 can activate transcription of Pdk2/luciferase (Fig. 5C) and also binds to the active but not to the less active form of the genomic *Pdk2* promoter (Fig. 6B). Finally, a histone deacetylase inhibitor decreases repression of the Pdk2/luciferase fusion (Fig. 5D), whereas acetylation of histone H4 at the *Pdk2* promoter decreases upon p53 activation (Fig. 6C). These data support the model in Fig. 5A, in which p53 prevents activation of Pdk2 transcription by inhibiting an E2F activator. Although the model in Fig. 5A suggests that another key tumor suppressor protein, Rb, may also be involved in the Warburg effect through control over Pdk2 transcription, we did not directly show a role for Rb in Pdk2 transcription. It remains possible that one of the other members of the retinoblastoma protein family member, p107 or p130, is more important than Rb in Pdk2 transcription; alternatively, a mechanism independent of the Rb protein family cannot be ruled out at this time. However, it is noteworthy that Rb/E2F control over another *Pdk* gene, *Pdk4*, has been shown (28).

In addition to lowering Pdk2 transcription, p53 may also lower Pdk2 activity indirectly and posttranslationally. For instance, in mice p53 promotes the expression of the thiamine transporter (29). Greater uptake of thiamine increases cellular thiamine pyrophosphate, which is a cofactor of the Pdc and which is also an inhibitor of phosphorylation of Pdc by Pdk2 (30). Also, acetyl-CoA activates Pdk2, so more rapid conversion of acetyl-CoA into citrate during the citric acid cycle might serve the same role as downregulating Pdk2 transcription. This could be achieved by accelerating steps downstream of pyruvate oxidation. Indeed, in muscle p53 seems to control aerobic glycolysis through upregulation of SCO2 (31), which encodes a protein that aids in assembly of the cytochrome C oxidase component of the oxidative phosphorylation pathway. Higher

SCO2 activity may decrease acetyl-CoA and inactivate Pdk2. In liver, p53 can also upregulate glutaminase-2, which may accelerate the citric acid cycle by converting glutamine into the citric acid cycle intermediate α -ketoglutarate (32). Thus the mechanism by which p53 downregulates Pdk2 activity may differ depending on the cell and tissue type, as well as the available carbon sources.

The laboratory of Michelakis has previously shown that Pdk2 is important for the growth of some human cell lines, and that an inhibitor of Pdk2, dichloroacetic acid (DCA), can block growth of tumors in xenograft models (23). Moreover, DCA may have decreased the progression of glioblastomas in a very small human trial of 5 patients (33). Our work, which links Pdk2 to key cancer factors such as p53, p21, E2F1, and possibly Rb, reinforces the idea that Pdk2 plays a role in tumor biology. Other Pdk isoenzymes have been linked to cancer genes. As mentioned above, pdk4 can be activated by the Rb/E2F1 pathway (28). Pdk1 and 3 are activated by Hif-1 (34–36), which may allow tumors to control glucose metabolism under oxygen-limiting conditions. For these reasons, it seems likely that phosphorylation of the Pdc will turn out to be important in tumor formation or progression.

Control over Pdk2 expression may be central to some aspects of p53 biology. First, induction of apoptosis did not occur when p53 could not downregulate Pdk2 (Fig. 7C). DNA damage is thought to trigger p53 to activate transcription of genes that produce ROS, which cause more DNA damage that triggers even more p53 activity, thus creating a positive feedback loop that eventually leads to apoptosis (37, 38). Downregulation of Pdk2 by p53 may be sufficient to produce ROS at the levels required for apoptosis. Second, another role of p53 is to decrease tumor metastasis by preventing invasion (reviewed in Muller and colleagues; ref. 39). Conversely, lactate secretion, which acidifies the tumor microenvironment, is thought to increase invasion (40, 41). Therefore, the ability to decrease lactate production through repression of Pdk2 may help to explain the effect of p53 on metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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