HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia

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Summary

Activation of glycolytic genes by HIF-1 is considered critical for metabolic adaptation to hypoxia through increased conversion of glucose to pyruvate and subsequently to lactate. We found that HIF-1 also actively suppresses metabolism through the tricarboxylic acid cycle (TCA) by directly *trans*-activating the gene encoding pyruvate dehydrogenase kinase 1 (PDK1). PDK1 inactivates the TCA cycle enzyme, pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl-CoA. Forced PDK1 expression in hypoxic HIF-1α null cells increases ATP levels, attenuates hypoxic ROS generation, and rescues these cells from hypoxia-induced apoptosis. These studies reveal a hypoxia-induced metabolic switch that shunts glucose metabolites from the mitochondria to glycolysis to maintain ATP production and to prevent toxic ROS production.

Introduction

The Pasteur effect, which describes the increased conversion of glucose to lactate in hypoxic cells, has been considered a critical cellular metabolic adaptation to hypoxia for over a century. The molecular basis for the Pasteur effect is thought to result solely from the activation of glycolysis by hypoxia-inducible transcription factors, with mitochondrial respiration passively decreasing due to the paucity of oxygen. The studies we report here along with studies by Papandreou et al. (2006), however, indicate that cellular adaptation to hypoxia also requires an active suppression of mitochondrial pyruvate catabolism and respiration.

Under normal oxygen tensions, cells catabolize glucose to pyruvate via glycolytic enzymes. Pyruvate is then taken up by the mitochondria for further catabolism through the tricarboxylic acid (TCA) or Krebs cycle, which transfers electrons to the respiratory chain. Electron transport through this chain results in ATP production and terminates in the donation of electrons to oxygen. In low oxygen tension, in which there is a paucity of oxygen as an electron acceptor, hypoxic cells are surmised to undergo anaerobic glycolysis as a default mode. Over the past decade, however, the adaptation of cells to hypoxia has emerged as an active process. Increased glycolytic flux requires transcriptional activation of genes encoding glucose transporters and glycolytic enzymes that is mediated by hypoxia-inducible factor 1 (HIF-1) and/or HIF-2 (Iyer et al., 1998; Seagroves et al., 2001). HIF-1 is a heterodimeric transcription factor, consisting of HIF-1α and HIF-1β subunits, which functions as a master regulator of oxygen homeostasis in all metazoan species (Schofield and Ratcliffe, 2004; Semenza, 2004). Hypoxic activation of HIF-1 promotes ATP production through increased anaerobic glycolysis, which partially compensates for hypoxic cellular energy demands. Increased ATP production, however, may not be sufficient for hypoxic adaptation since hypoxia paradoxically causes oxidative stress from uncontrolled mitochondrial generation of reactive oxygen species (ROS) that may pose a barrier for cell survival . ROS, a byproduct of mitochondrial respiration due to electron transfer to O_2, is neutralized by catalase, peroxiredoxins, and superoxide dismutase. Under hypoxic conditions, however, perturbation in electron transport is associated with leakage of electrons from the respiratory chain, resulting in increased ROS that could be toxic to cells if ROS levels are not attenuated. Here, we report that the hypoxia inducible pyruvate dehydrogenase kinase 1 (PDK1) is critical for the attenuation of mitochondrial ROS production, maintenance of ATP levels, and adaptation to hypoxia.

We initially sought to determine by microarray analysis of gene expression the overlap of genes responsive to both MYC and hypoxia using the human B lymphocyte cell line, P493-6, which contains a tetracycline-repressible MYC allele (Kim et al., 2004; Schuhmacher et al., 1999). Among the genes highly induced by hypoxia, PDK1 is also a potential MYC target that was identified by chromatin immunoprecipitation experiments (Li et al., 2003). PDK1 was selected for further study because of its involvement in the regulation of glucose metabolism by the TCA cycle. We found that the gene encoding pyruvate dehydrogenase kinase 1 (PDK1) is a direct target of HIF-1. PDK1 phosphorylates the pyruvate dehydrogenase (PDH) E1α subunit and inactivates the PDH enzyme complex that converts pyruvate to acetyl-coenzyme A, thereby inhibiting pyruvate metabolism via the tricarboxylic acid (TCA) cycle (Holness and Sugden, 2003). Because the TCA cycle is coupled to electron transport, regulation of the PDH complex by PDK is critical for the attenuation of mitochondrial respiration and ROS production.

We observed that under hypoxic conditions, HIF-1α null mouse embryo fibroblasts (MEFs) fail to activate PDK1 and undergo apoptosis that is associated with a dramatic rise in the level of reactive oxygen species (ROS). Forced expression of PDK1 increases the levels of ATP and prevents hypoxia-induced ROS generation and apoptosis. Our studies suggest a failure of the electron transport chain under hypoxic conditions, which necessitates the shunting of glucose metabolites away from

the mitochondria by HIF-1-mediated PDK1 expression. This results in the attenuation of mitochondrial respiration, prevention of toxic ROS production, and the maintenance of ATP levels.

Results

PDK1 is a direct HIF-1 target gene

Our microarray gene expression analysis of hypoxic P493 cells revealed the induction of many known hypoxia-responsive genes including those encoding glucose transporter, glycolytic enzymes, and VEGF, as well as previously unreported ones (deposited in Gene Expression Omnibus [GEO] database, GSE4186). PDK1, which had not been previously reported, is among 25 genes that were induced at least 4-fold by hypoxia in P493-6 cells (Table S1 in the Supplemental Data available with this article online). The hypoxic induction of *PDK1* gene expression was accompanied by a remarkable induction of PDK1 protein as demonstrated by immunoblot assay (Figure 1A). PDK1 levels were also increased in P493-6 cells exposed to $CoCl_2$ (Figure 1B), which induces HIF-1 activity by inhibiting O_2-dependent degradation of the HIF-1α subunit (Maxwell et al., 1999; Wang and Semenza, 1993). To determine whether HIF-1 is necessary for PDK1 induction, we analyzed HIF-1α null (*Hif1a*$^{-/-}$) MEFs (Feldser et al., 1999; Iyer et al., 1998) that also do not express HIF-2α (data not shown). The dramatic increase in PDK1 levels in isogenic wild-type MEFs exposed to hypoxia did not occur in *Hif1a*$^{-/-}$ MEFs (Figure 1C). Similar results were obtained by immunoblot assay of hexokinase 2 (HK2), which is the product of a known HIF-1 target gene (Iyer et al., 1998).

To determine whether *PDK1* is a direct target of HIF-1, chromatin immunoprecipitation (ChIP) was performed with an anti-HIF1α antibody, as described for anti-Myc antibody (Kim et al., 2004), using hypoxic P493-6 cells. For a positive control, we demonstrated binding of HIF-1 to a known HIF-1 target gene, *VEGF*, in chromatin from hypoxic, but not from nonhypoxic, cells (Figure S1). Since HIF-1 is absent in nonhypoxic cells, it is expected that HIF-1 bound to target genes would not be detected. In hypoxic cells, HIF-1α was crosslinked to the *PDK1* gene in DNA sequences flanking exon 1 that are enriched with consensus HIF-1 binding sites (Figure 1D). These results indicate that *PDK1* is a direct HIF-1 target gene.

PDK1 rescues hypoxic *Hif1a*$^{-/-}$ MEFs

The proliferation of *Hif1a*$^{-/-}$ embryonic stem cells may be impaired when cultured under hypoxic conditions for 24–48 hr (Carmeliet et al., 1998; Iyer et al., 1998). The proliferation of *Hif1a*$^{-/-}$ MEFs was also impaired after 48 hr of hypoxia (Figure 1E) as was previously observed (Seagroves et al., 2001). However, a more striking defect was observed after 72 hr, with a reduction in cell number indicating cell death, which was confirmed by demonstration of a dramatic increase in annexin V staining, an indicator of apoptosis (Figure 2C). In contrast, the immortalized wild-type MEFs are able to proliferate in hypoxia, presumably because SV40 T-antigen expression in these cells (Feldser et al., 1999) overcomes the RB-mediated G1 checkpoint that is observed in moderately hypoxic cells (Gardner et al., 2001).

We hypothesized that active suppression of the TCA cycle and shunting of pyruvate to lactate via inactivation of PDH by PDK1 is required for cell survival under prolonged hypoxic conditions. To test this hypothesis, we generated *Hif1a*$^{-/-}$ cell pools with forced overexpression of PDK1 by independent retroviral infections (Figure 2A). PDK1 overexpression resulted in increased PDH E1α subunit phosphorylation, which was also observed in hypoxic wild-type MEFs (Figure 3). Intriguingly, forced PDK1 expression was sufficient to permit the proliferation of hypoxic *Hif1a*$^{-/-}$ MEFs (Figure 2B) and protect them from hypoxia-induced apoptosis (Figure 2C). By contrast, forced expression of the murine glycolytic enzyme glucose phosphate isomerase (mGPI) could not rescue hypoxic *Hif1a*$^{-/-}$ MEFs (Figure S2).

Forced expression of PDK1 inhibits hypoxia-induced apoptosis in the absence of HIF1

Our observation that PDK1 rescued hypoxic HIF-1α null MEFs suggested that PDK1-mediated inactivation of the PDH complex and shunting of pyruvate away from the TCA cycle toward glycolysis was sufficient for the survival of hypoxic cells. We hypothesized that limited O_2 availability may lead to increased ROS production due to ineffective electron transfer in the mitochondria if flux through the TCA cycle is not attenuated (Balaban et al., 2005; Yankovskaya et al., 2003). Increased ROS levels could, in turn, trigger apoptosis (Balaban et al., 2005). As shown in Figure 4A, hypoxia caused an increase in intracellular H_2O_2 in *Hif1a*$^{-/-}$ MEFs in sharp contrast to the reduction in H_2O_2 levels that was observed when wild-type MEFs were exposed to hypoxia. These data taken together with the demonstration that forced PDK1 expression prevented hypoxia-induced apoptosis of *Hif1a*$^{-/-}$ MEFs raised the possibility that HIF-1-induced PDK1 activity may reduce ROS production. As shown in Figure 4B, production of H_2O_2 in hypoxic *Hif1a*$^{-/-}$ MEFs was significantly decreased by forced PDK1 expression. To further confirm that PDK1 reduces ROS production, we examined intracellular oxidants by staining cells with H_2DCFDA, which is oxidized by ROS to the highly fluorescent DCF. DCF fluorescence was markedly diminished by forced PDK1 expression in *Hif1a*$^{-/-}$ MEFs (Figure 4C). Furthermore, we hypothesized that inhibition of mitochondrial electron transport would also rescue the *Hif1a*$^{-/-}$ MEFs. While complex 3 inhibitors, myxothiazol and antimycin A, were toxic to both normoxic and hypoxic *Hif1a*$^{-/-}$ MEFs, the mitochondrial complex 1 inhibitor rotenone was able to rescue hypoxic *Hif1a*$^{-/-}$ MEFs, whereas it inhibited proliferation of normoxic *Hif1a*$^{-/-}$ MEFs (Figure 4D). These results support a novel regulatory mechanism for hypoxic adaptation in which PDK1 inactivates the PDH complex and inhibits the TCA cycle, thereby attenuating ROS production.

We hypothesized that PDK1-mediated inhibition of the TCA cycle would result in increased glycolysis and ATP levels by shunting pyruvate toward lactate production. ATP content in *Hif1a*$^{-/-}$ MEFs was reduced in hypoxia as compared with wild-type MEFs (Figure 4E). The decrease in ATP level could result from decreased ATP production as well as increased mitochondrial ATP consumption to maintain mitochondrial membrane potential. In contrast, hypoxic *Hif1a*$^{-/-}$ MEFs with forced PDK1 expression had elevated ATP levels as compared with hypoxic wild-type MEFs (Figure 4E). The increased ATP levels, hence, could result from increase ATP production by glycolysis and/or from decreased ATP consumption to maintain mitochondrial membrane potential in hypoxia.

Reduced PDK1 levels impair the growth of hypoxic P493-6 cells

To determine whether PDK1 is necessary for hypoxic adaptation of P493-6 cells, which express predominantly PDK1 (as

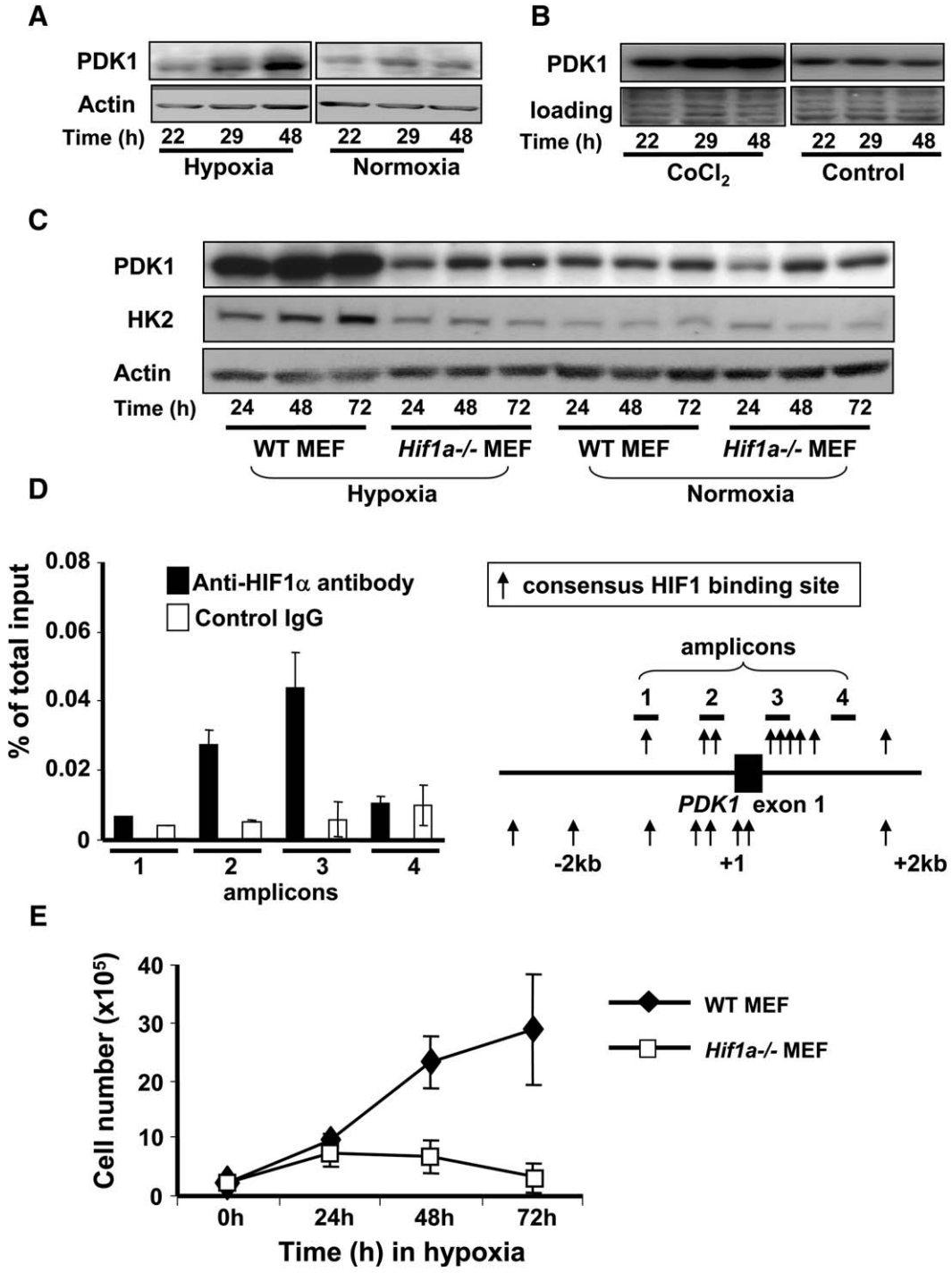

Figure 1. HIF-1-dependent induction of PDK1 expression in hypoxic cells

A) Immunoblot assay of PDK1 protein levels in hypoxic or normoxic P493-6 cells that were exposed to 0.1% or 20% O_2, respectively. β-actin is shown as a loading control.
B) PDK1 induction in P493-6 cells exposed to 100 μM $CoCl_2$ under normoxic (20% O_2) conditions. Total protein staining is shown as a loading control.
C) Immunoblot assay of PDK1 and hexokinase 2 expression in wild-type and *Hif1a*$^{-/-}$ MEFs under hypoxic (0.5% O_2) or normoxic (20% O_2) conditions. β-actin is shown as a loading control.
D) Chromatin immunoprecipitation assay of the human *PDK1* gene. Real-time PCR quantification of HIF1α binding to regions 1–4 (amplicons) is indicated as the percentage of total input chromatin DNA. Arrows indicate consensus HIF-1 binding site.
E) Growth curves of the wild-type and *Hif1a*$^{-/-}$ MEFs in hypoxic conditions (0.5% O_2). Cell numbers (mean ± SD) from three independent experiments are shown.

compared to the three other PDK isoforms), we reduced the expression of PDK1 by RNA interference (Figure 5A). The growth of P493-6 cells in hypoxia was impaired by small interfering RNA (siRNA) directed against PDK1 as compared to cells treated with a scrambled control siRNA that did not reduce PDK1 expression (Figure 5B). These results are consistent with the hypothesis that PDK1 is necessary for the proliferation of P493-6 cells under hypoxic conditions.

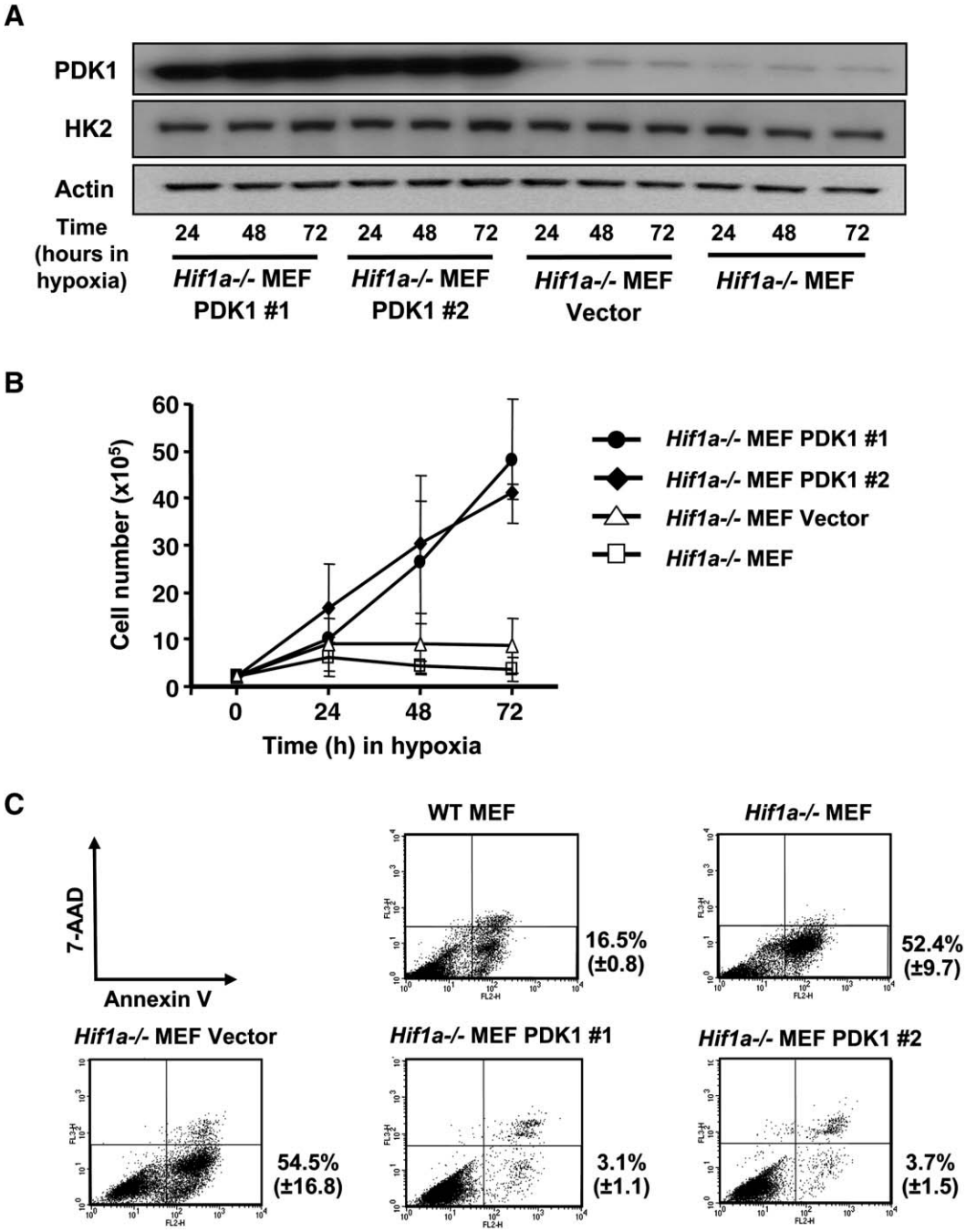

Figure 2. Effect of forced PDK1 expression on the survival of hypoxic *Hif1a*$^{-/-}$ MEFs

A) Immunoblot assays of PDK1 and HK2 expression in *Hif1a*$^{-/-}$ MEFs ectopically expressing PDK1 by pMSCVpuro-PDK1 retroviral transduction after 24–72 hr of hypoxia (0.5% O_2). Two independently transduced cell pools with pMSCVpuro-PDK1 retrovirus (#1 and #2) were used. *Hif1a*$^{-/-}$ MEFs and those transduced with empty pMSCVpuro vector were used as controls. β-actin is shown as a loading control.

B) Growth curves of retrovirally transduced *Hif1a*$^{-/-}$ MEFs under hypoxia (0.5% O_2). Cell numbers (mean ± SD) from four independent experiments are shown.

C) Measurement of apoptosis by Annexin V staining after 72 hr of hypoxia (0.5% O_2). Percentage of apoptotic cells (Annexin V positive, 7-AAD negative, right lower quadrant; mean ± S.D.) from three independent experiments are shown.

Discussion

Pasteur described over a century ago that hypoxic cells increase the conversion of glucose to lactate, an effect that to date had been primarily attributed to the activation of glycolysis by hypoxia-inducible transcription factors HIF1α and/or HIF2α. The accompanying decrease in cellular respiration in hypoxia was thought to result passively from the paucity of oxygen, which is required for accepting electrons from the mitochondrial respiratory chain. Our observations along with Papandreou et al. (2006) reveal that adaptation to hypoxia critically depends on the active inhibition of mitochondrial pyruvate metabolism and respiration.

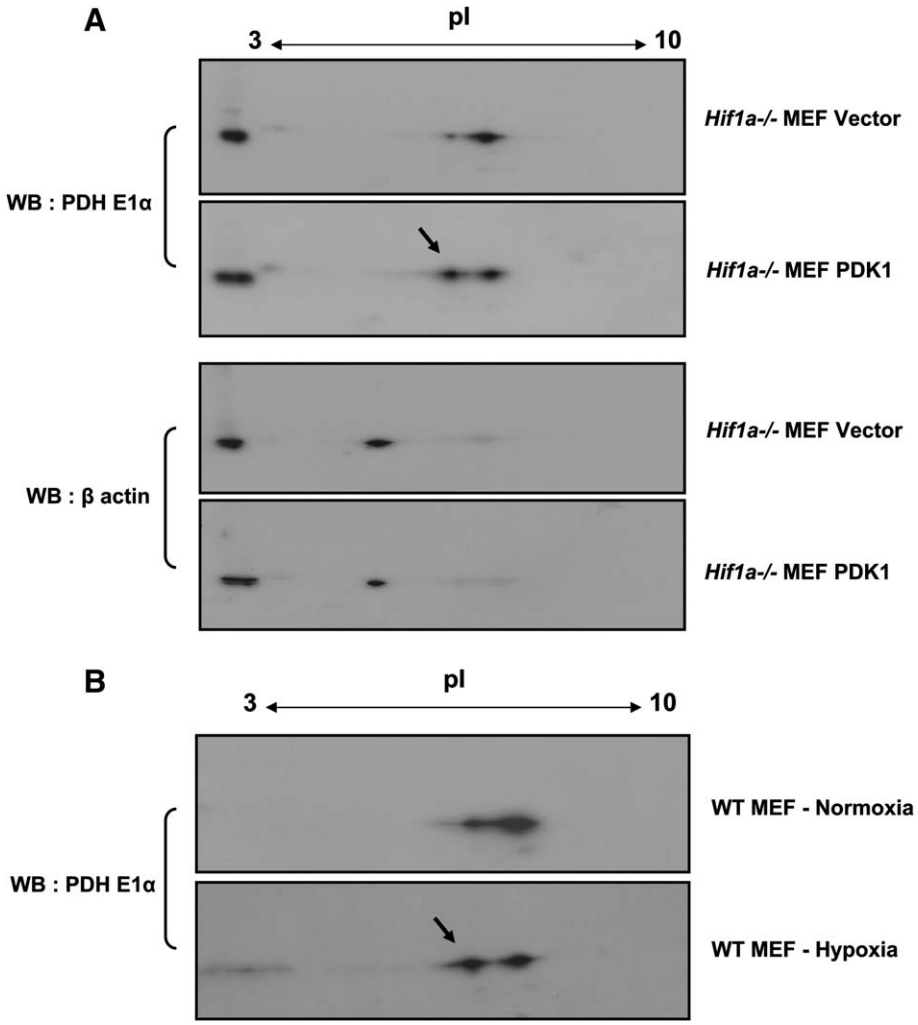

Figure 3. Phosphorylation of PDH E1α subunit by PDK1 expression

A) Immunoblot (IB) analysis of PDH E1α subunit (41 kDa) after two dimensional gel electrophoresis of lysates from the *Hif1a*$^{-/-}$ MEFs expressing PDK1 or those transduced with empty vector. Filters were stripped and re-probed for β-actin, which is shown as an intergel reference point for the immunoblot alignment. The very far left lane of each panel represents one-dimensional electrophoresis of the lysates.

B) Phosphorylation of PDHE1α in hypoxic (0.5% O_2) wild-type MEFs compared to normoxic (20% O_2) cells. Arrows indicate a phosphorylated form of PDH E1α subunit. pI = isoelectric points.

In addition to our finding that *PDK1* is hypoxia inducible from microarray gene expression analysis, we found that *PDK1* is a direct target of HIF-1 by chromatin immunoprecipitation, which demonstrates binding of HIF-1 to the promoter region of *PDK1*. The accompanying paper by Papandreou et al. (2006) further confirms that *PDK1* is a direct HIF-1 target through *PDK1* promoter-reporter assays using a constitutively active HIF-1 expression vector.

Having established that *PDK1* is a direct HIF-1 target, we were intrigued and surprised by our finding that PDK1 is sufficient to rescue hypoxic cells that lack the expression of HIF-1α or HIF-2α. Our further studies support a novel regulatory mechanism for hypoxic adaptation whereby hypoxia-induced PDK activity inhibits PDH, causing the shunting of pyruvate from the mitochondria and subsequently attenuating mitochondrial respiration and ROS production. Although we cannot rule out the possibility that PDK1 may have phosphorylation targets other than PDH that may promote survival in hypoxia, our study along with the study by Papandreou et al. (2006) strongly suggests that PDK1 suppression of the TCA cycle, mitochondrial respiration and ROS production, and augmentation of ATP levels are crucial for the survival of hypoxic cells. Thus, HIF-1 plays three critical roles in the hypoxia-induced metabolic switch from oxidative to glycolytic metabolism. HIF-1 induces expression of: (1) upstream glucose transporters and glycolytic enzymes to increase flux from glucose to pyruvate; (2) PDK1 to block the conversion of pyruvate to acetyl CoA; and (3) lactate dehydrogenase A to convert pyruvate to lactate (Figure 6). In this context, we propose that the induction of PDK1 is necessary to prevent excessive mitochondrial ROS production and to shunt pyruvate toward lactate and regenerate NAD^+, which permits continued glycolysis and ATP production under hypoxia (Figure 6). This model is compatible with findings that hypoxia-induced mitochondrial ROS is necessary and sufficient for the stabilization of HIF-1 (Brunelle et al., 2005; Guzy et al., 2005; Kaelin, 2005), which would in turn activate PDK1 and thereby attenuate the persistence of potentially lethal ROS.

It is notable that enforced PDK1 expression increased lactate production by *Hif1a*$^{-/-}$ MEFs under nonhypoxic conditions, but this increase was not readily apparent in hypoxia (data not shown), suggesting that increased ATP levels with enforced PDK1 expression in hypoxia may not be due only to glycolysis. Along with the findings by Papandreou et al. (2006) that PDK1 decreases mitochondrial oxygen consumption and function, our observations suggest that enforced PDK1 expression rescued *Hif1a*$^{-/-}$ MEFs by inactivating PDH, decreasing mitochondrial oxygen consumption and function, thereby reducing hypoxic mitochondrial ATP consumption for maintenance of mitochondrial membrane potential.

Further investigation of this fundamental mechanism for regulating cellular metabolism will allow us to better understand the

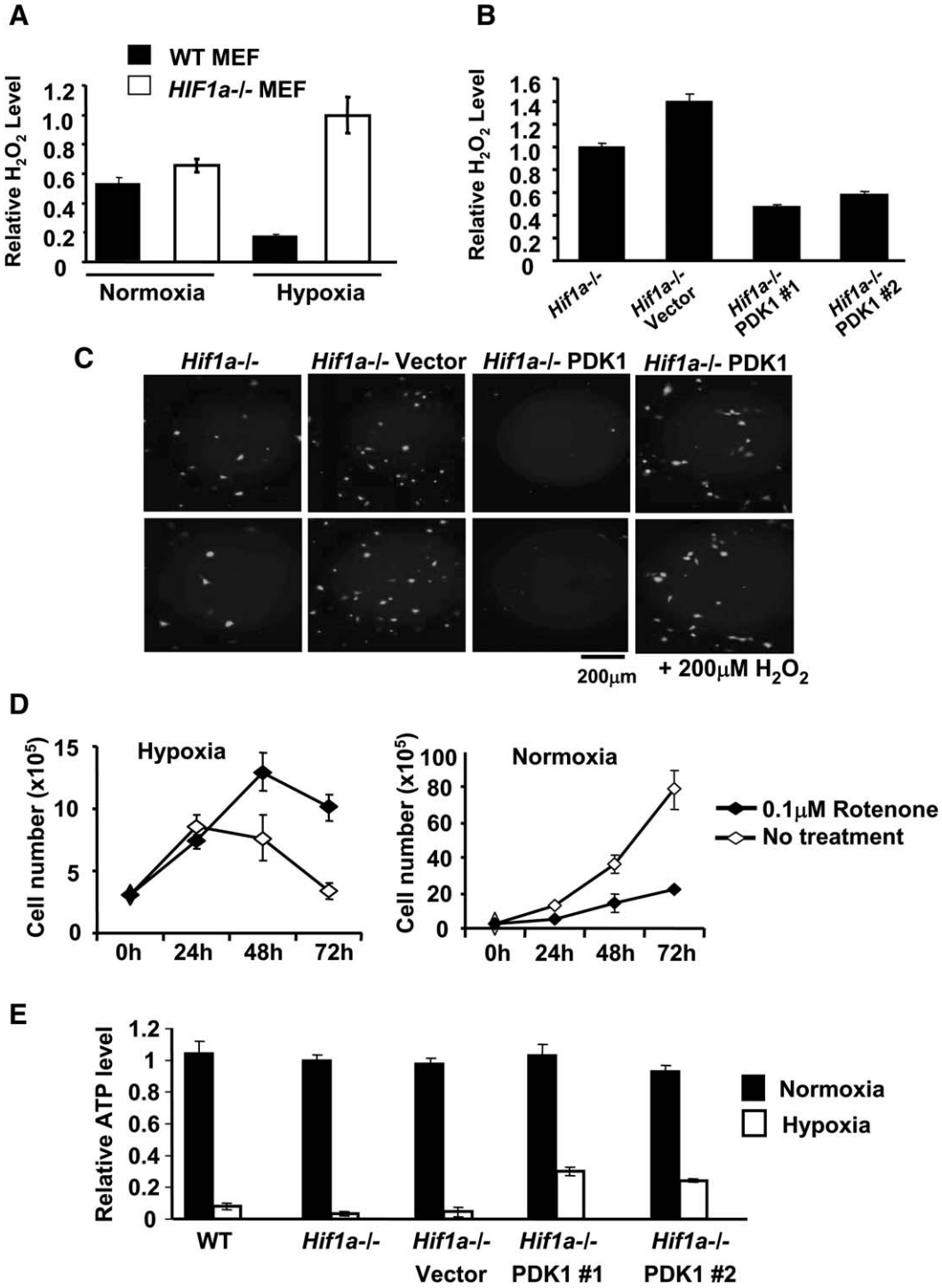

Figure 4. Effect of PDK1 on hypoxia-induced ROS production

A) Intracellular hydrogen peroxide levels in wild-type and *Hif1a*$^{-/-}$ MEFs after 72 hr at 0.5% or 20% O_2. The data are expressed as the mean fluorescence levels from two independent experiments normalized by protein concentration and shown as normalized (to *Hif1a*$^{-/-}$ MEF) values. Error bars represents SEM.

B) Intracellular hydrogen peroxide level in *Hif1a*$^{-/-}$ MEFs ectopically expressing PDK1 or transduced with empty vector after 72 hr of hypoxia (0.5% O_2). Four independent experiments were performed and error bars represent SEM.

C) DCF fluorescence staining of hypoxic (0.5% O_2) *Hif1a*$^{-/-}$ MEFs transduced with relevant retroviruses. Images were captured with identical photographic exposure times from two randomly selected fields. As a positive control, *Hif1a*$^{-/-}$ MEFs ectopically expressing PDK1 were incubated with 200 μM hydrogen peroxide for 4 hr in hypoxia before staining (right panels).

D) Growth curves of hypoxic (0.5% O_2, left panel) or normoxic (20% O_2, right panel) *Hif1a*$^{-/-}$ MEFs incubated with 0.1 μM rotenone. Cell numbers (mean ± S.D) from two independent experiments, each measured in duplicate are shown.

E) Intracellular ATP levels of wild-type MEFs, *Hif1a*$^{-/-}$ MEFs ectopically expressing PDK1 or transduced with empty vector after 72 hr of hypoxia (0.5% O_2) or normoxia (20% O_2). Values are normalized to those of normoxic *Hif1a*$^{-/-}$ MEFs.

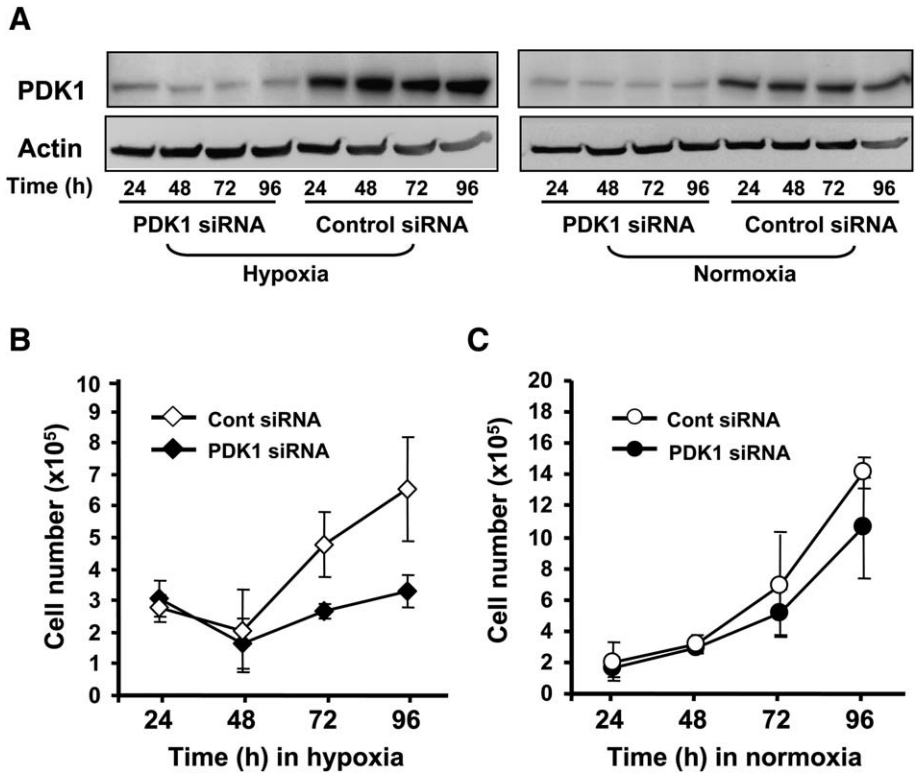

Figure 5. Effect of decreased PDK1 expression on P493-6 cells exposed to hypoxia

A) Immunoblot assays of PDK1 expression in cells following electroporation with PDK1 siRNA or control scrambled siRNA in hypoxic (0.1% O_2) or normoxic (20% O_2) conditions. β-actin is shown as a loading control.

B–C) Growth curves of cells electroporated with PDK1 siRNA or control scrambled siRNA in hypoxia (**B**) and normoxia (**C**). Cell numbers (mean ± SD) from two independent experiments, each measured in duplicate, are shown.

physiologic adaptation of normal cells to hypoxia and may also provide new insights into the Warburg effect or the reliance of cancer cells on glycolytic metabolism even under aerobic conditions (Dang and Semenza, 1999; Elstrom et al., 2004). In particular, the activation of PDK under nonhypoxic conditions would favor the conversion of glucose to lactate, which is a key feature of the Warburg effect. Both Myc and HIF-1 activate overlapping sets of glycolytic genes including lactate dehydrogenase (Kim et al., 2004), and hence Myc is hypothesized to play a role in the Warburg effect. While nonhypoxic activation of HIF-1 through specific signal transduction pathways or loss of VHL could increase PDK1 and trigger the Warburg effect, the role of Myc in activating PDK1 is currently unclear. Although Myc was observed to bind the promoters of PDK1, PDK2, and PDK3 in the Burkitt's lymphoma cell line, Daudi (Li et al., 2003), PDK1 expression was not further increased by enforced Myc expression in the P493-6 cells. Notwithstanding the as yet unclear role of Myc in regulating PDK1, which requires further study, our novel

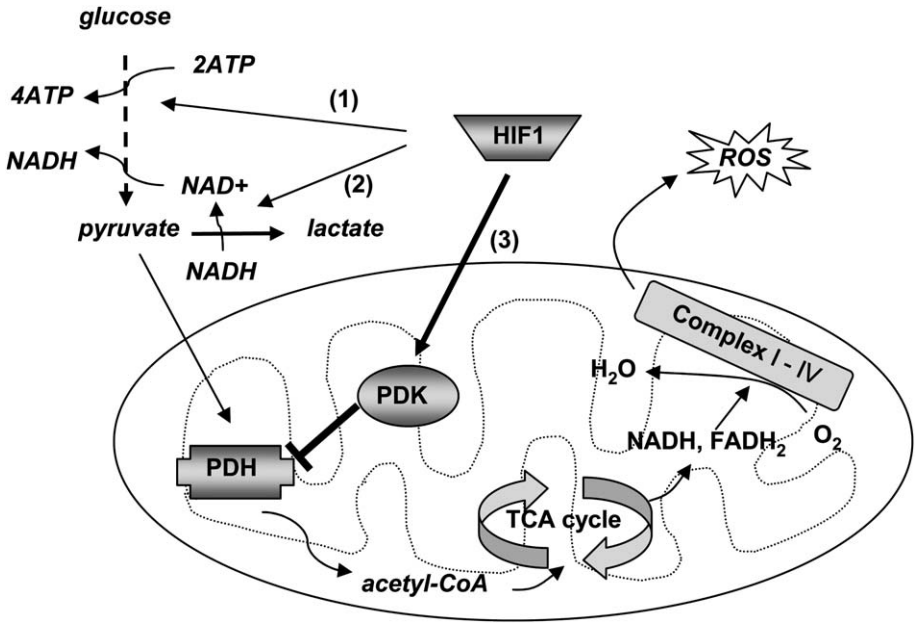

Figure 6. Coordinate regulation of hypoxia-induced metabolic switches by HIF-1

In hypoxic cells, HIF-1 stimulates increased glycolytic flux to pyruvate (1) and its reduction to lactate (2). In addition, HIF-1-induced PDK1 activity inhibits PDH (3), resulting in decreased flux through the TCA cycle. The resulting attenuation of oxidative phosphorylation is essential to prevent the generation of reactive oxygen species (ROS) resulting from ineffective electron transport under hypoxic conditions. HIF-1 mediates these effects through transcriptional activation of genes encoding glucose transporters and glycolytic enzymes (1), lactate dehydrogenase A (2), and PDK1 (3).

finding that HIF-1 dependent activation of PDK1 is essential for cellular adaptation to hypoxia suggests a possible target for cancer therapeutic intervention. In particular, the decreased viability of P493-6 cells with reduced PDK1 level in hypoxia suggests the possibility of novel approaches that induce apoptosis in hypoxic cancer cells by PDK inhibition.

Experimental Procedures

Cell culture and hypoxic exposures

Wild-type and *Hif1a*$^{-/-}$ MEFs were immortalized by SV-40 large T antigen and maintained in high glucose (4.5 mg/ml) DMEM (GIBCO/BRL) with 15% fetal bovine serum (FBS) (GIBCO/BRL), 2 mM sodium pyruvate (Sigma), nonessential amino acids (Sigma), and 1% penicillin-streptomycin (GIBCO/BRL)(Feldser et al., 1999). The human Burkitt's lymphoma cell line P493-6 was generated and maintained as described (Kim et al., 2004; Schuhmacher et al., 1999). Normoxic cells (20% O_2) were maintained at 37°C in a 5% CO_2/95% air incubator. Hypoxic cells (0.1%–0.5% O_2) were maintained in a control atmosphere chamber (Plas-Labs) at 37°C. Oxygen tension was monitored by a calibrated Series 200 Percent oxygen analyzer (Alpha Omega Instruments).

Vectors and retrovirus infection

Verified full-length cDNA clones for human PDK1 were purchased from Open biosystems. Full-length human PDK1 cDNA was ligated into the pMSCVpuro (Clontech) retroviral vector (pMSCVpuro-PDK1). Retroviruses were produced by transfecting the pMSCVpuro-PDK1 or empty pMSCVpuro vector into the ecotropic Phoenix packaging cell line. *Hif1a*$^{-/-}$ MEFs were infected with retroviruses in the presence of 8 μg/ml polybrene (Sigma). Infected cells were selected with 2 μg/ml puromycin (Sigma).

ChIP assay

Chromatin immunoprecipitation and real-time PCR quantification were performed as described (Kim et al., 2004). The rabbit polyclonal HIF1α antibody (Novus Biologicals) and rabbit IgG1 antibody (Zymed) were used for immunoprecipitation (see Table S2 for the primer sequences for the ChIP assay).

Western blot analysis

Proteins extracted from MEFs or P493-6 cells were fractionated by 10% SDS-PAGE. Polyclonal anti-PDK1 antibody (Stressgen Bioreagents), polyclonal anti-HK2 antibody (Santa Cruz) and monoclonal anti-β actin antibody (Sigma) were used for immunoblot assays.

Cell proliferation and apoptosis

For the cell proliferation assay, 2 × 10^5 MEFs were plated in a 10 cm dish 1 day before hypoxic exposure (0.5% O_2). At indicated times, cells were trypsinized and viable cells were counted. Apoptotic rate was measured by Annexin V-PE Apoptosis Detection kit (BD Biosciences) according to the manufacturer's instructions.

ROS measurements

Intracellular hydrogen peroxide levels were measured using an Amplex Red Hydrogen Peroxide Assay kit (Molecular Probes) according to the manufacturer's instructions. In brief, total cell lysates were harvested at 72 hr of hypoxic incubation (0.5% O_2) inside the hypoxic chamber and the reactions were initiated immediately by adding Amplex Red reaction mixture. Fluorescence was measured on a Cytofluor 2300 (Millipore). Fluorescence levels were normalized to the protein concentration. Intracellular ROS production was also measured by staining with dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes). After 72 hr of hypoxic incubation (0.5% O_2), cells were loaded with 5 μM H_2DCFDA for 1 hr, washed in PBS, and incubated with fresh media without H_2DCFDA for 30 min. DCF fluorescence was visualized using an Axiovert 200 inverted fluorescence microscope (Zeiss).

Intracellular ATP level measurements

Intracellular ATP levels were measured using an ATPLite assay kit (Perkin Elmer) according to the manufacturer's instructions. In brief, cell lysates were collected from hypoxic (0.5% O_2) or normoxic (20% O_2) MEFs. Luminescence was measured using an LMax microplate luminescence reader (Molecular Device), and normalized to the protein concentration.

siRNA experiments

siRNA targeting human PDK1 was designed and purchased from Dharmacon Research Inc. 3 × 10^6 P493-6 cells were electroporated (1500 μF and 240 volts) with 100 nM PDK1 siRNA (5′-CUACAUGAGUCGCAUUUCAdTdT-3′) or scrambled control siRNA (5′-CACGCUCGGUCAAAAGGUUdTdT-3′) in a 4 mm cuvette (BTX) using a Gene Pulser Xcell (Bio-Rad). On the following day, 5 × 10^5 viable cells subjected to the hypoxic exposure (0.1% O_2). At the indicated times, viable cells were counted and protein lysates were prepared for immunoblot assays.

2-D electrophoresis

After washes with low-salt wash buffer, cells were extracted in lysis buffer (8 M urea, 4% CHAPS, 1.5% 3-10 IPG buffer, protease and phosphatase inhibitor cocktail). The crude cell homogenate was sonicated on ice, and the first-dimension isoelectric focusing and second-dimension electrophoresis were performed as described with modifications(Gorg et al., 1988). After second-dimension electrophoresis, proteins were transferred to nitrocellulose membrane and immunoblotted with monoclonal anti-PDH E1α antibody (Molecular Probes) or monoclonal anti-β-actin antibody (Sigma).

Microarray analysis

P493-6 cells were incubated in normoxic (20% O_2) or hypoxic condition (0.1% O_2) for 29 hr. Duplicate mRNA samples were prepared from cells from biologically independent experiments and subjected to Affymetrix oligonucleotide microarray analysis by using an HG_U133A chip as described (Chou et al., 2004). To estimate the gene expression signals, data analysis was conducted on the microarrays' CEL file probe signal values at the Affymetrix probe pair (perfect match [PM] probe and mismatch [MM] probe) level, using the statistical algorithm Robust Multiarray Analysis (RMA) (Irizarry et al., 2003) with the bioconductor package *Affy*. This probe level data processing includes a normalization procedure utilizing quantile normalization (Bolstad et al., 2003) to reduce the obscuring variation between microarrays, which might be introduced during the processes of sample preparation, manufacture, fluorescence labeling, hybridization and/or scanning. Fold changes were computed based on the signal intensities estimated above. All computation was done in *R* environment.

Supplemental data

Supplemental data include two figures and three tables and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/3/3/177/DC1/.

Acknowledgments

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