

Regulation of the PDK4 Isozyme by the Rb-E2F1 Complex^{*[S]}

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Loss of the transcription factor E2F1 elicits a complex metabolic phenotype in mice underscored by reduced adiposity and protection from high fat diet-induced diabetes. Here, we demonstrate that E2F1 directly regulates the gene encoding *PDK4* (pyruvate dehydrogenase kinase 4), a key nutrient sensor and modulator of glucose homeostasis that is chronically elevated in obesity and diabetes and acutely induced under the metabolic stress of starvation or fasting. We show that loss of *E2F1* *in vivo* blunts *PDK4* expression and improves myocardial glucose oxidation. The absence of *E2F1* also corresponds to lower blood glucose levels, improved plasma lipid profile, and increased sensitivity to insulin stimulation. Consistently, enforced E2F1 expression up-regulates PDK4 levels and suppresses glucose oxidation in C₂C₁₂ myoblasts. Furthermore, inactivation of Rb, the repressor of E2F-dependent transcription, markedly induces *PDK4* and triggers the enrichment of E2F1 occupancy onto the *PDK4* promoter as detected by chromatin immunoprecipitation analysis. Two overlapping E2F binding sites were identified on this promoter. Transactivation assays later verified E2F1 responsiveness of this promoter element in C₂C₁₂ myoblasts and IMR90 fibroblasts, an effect that was completely abrogated following mutation of the E2F sites. Taken together, our data illustrate how the E2F1 mitogen directly regulates *PDK4* levels and influences cellular bioenergetics, namely mitochondrial glucose oxidation. These results are relevant to the pathophysiology of chronic diseases like obesity and diabetes, where *PDK4* is dysregulated and could have implications pertinent to the etiology of tumor metabolism, especially in cancers with Rb pathway defects.

Insulin responsiveness and the efficient utilization of glucose are greatly influenced by the activity of the mitochondrial pyruvate dehydrogenase kinase (PDK)³ family (PDK1 to -4). As part of a large multimeric complex known as the pyruvate dehydro-

genase complex, these kinases phosphorylate and inactivate the pyruvate dehydrogenase enzyme, another pyruvate dehydrogenase complex component, to inhibit the transition (decarboxylation) of glycolysis end product pyruvate to mitochondrial acetyl-CoA, with the net result of curtailing mitochondrial glucose oxidation (1–4). As such, PDKs are widely viewed as critical nutrient sensors and modulators of cellular metabolic patterns in response to nutrient shortage like starvation or fasting and important regulators of cell bioenergetics during postprandial states as well.

Experiments to date suggest that PDK4 is a major isoenzyme responsible for changes in pyruvate dehydrogenase complex activity (1, 2). PDK4 in turn is regulated by multiple factors. For instance, metabolic stress conditions, such as starvation, fasting, or glucose deprivation markedly induce muscle PDK4 expression (5–7), presumably to prevent hypoglycemia and spare glucose for glucose-dependent tissues like the brain. Refeeding restores PDK4 levels to fed states. Increased fatty acid (FA) flux also up-regulates PDK4 expression in muscle, and this effect is reversed by insulin treatment (7). In addition, hypolipidemic drugs like PPAR α agonists, which promote FA uptake and utilization, induce PDK4 expression (7). PDK4 is also induced by steroid hormones, such as retinoic acids (8), and glucocorticoids like dexamethasone (4) and is co-activated by the global metabolic regulator PGC-1 α (9). Importantly, constitutively high PDK4 levels are documented in chronic diseases, such as diabetes (10), and are believed to promote insulin resistance at least through the inhibition of efficient muscle glucose utilization. Recently, we showed that increased FA flux, via the membrane translocase CD36, regulates PDK4 expression in a PPAR δ / β -dependent manner. This was reported as a potential mechanism for transcriptional control of glucose homeostasis in muscle under the metabolic stress of fasting (11).

Here, we investigate the interplay between the transcription factor E2F1 and the PDK4 isoenzyme. E2F1 is a critical regulator of survival and proliferation. It controls timely and accurate cell cycle progression by activating S-phase entry genes (12–15) and regulating components of the G₂/M checkpoint (16, 17). Consistent with such a role, E2F1 is a key component of the Rb (retinoblastoma)-E2F tumor suppressor complex, and its activity is largely controlled by the Rb tumor suppressor, a protein that is highly inactivated in cancer and that typically binds E2F1 and represses its transcription activities and functions. Using *in vitro* and *in vivo* systems where expression and function of E2F1

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. 1.

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³ The abbreviations used are: PDK, pyruvate dehydrogenase kinase; FA, fatty acid; ER, estrogen receptor; OHT, 4-hydroxytamoxifen; WT, wild type; MRI, magnetic resonance imaging; IWHP, isolated working heart perfusion;

ChIP, chromatin immunoprecipitation; ER-E2F1, E2F1-estrogen receptor fusion construct.

and Rb were manipulated, we characterize a novel mechanism through which Rb inactivation and unrestrained E2F1 activity can chronically alter muscle bioenergetics, namely glucose oxidation, and transcriptionally regulate *PDK4* expression. The direct interaction between E2F1, a potent mitogen-stimulated cell cycle regulator on the one hand, and PDK4, a critical nutrient sensor and modulator of glucose homeostasis on the other hand, provides a framework for a dynamic regulation of cellular fuel preference by Rb-E2F1 and underscores the intimate coupling between cell cycle events and the regulation of mitochondrial glucose oxidation. We suggest that the findings of this study are relevant to the pathophysiology of chronic metabolic diseases where PDK4 is dysregulated. The results could also have implications related to the etiology of cancer-associated metabolic patterns by means of characterizing a transcriptional and functional link coupling the Rb-E2F1 tumor suppressor complex to a key component of the mitochondrial metabolic machinery, the pyruvate dehydrogenase kinase.

EXPERIMENTAL PROCEDURES

Cells, Gene Transfer, and Drug Treatment—IMR90, U2OS, and C_2C_{12} cell lines were obtained from the American Type Culture Collection. Primary mouse embryo fibroblasts from wild type and $Rb^{-/-}$ mice were isolated and cultured as described (16, 18). C_2C_{12} myoblasts were maintained in low glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 200 units/ml penicillin, and 50 μ g/ml streptomycin. For gene transfer, cells were infected as described (18) with high titer recombinant retroviruses expressing *E1A* (LPC-12S), *E2F1* (LPC-E2F1), inducible *E2F1* constructs ER-E2F1, and a transactivation-defective mutant of *E2F1* (pBabeHAERE2F-1-(1–374)), all expressing drug-selectable markers and selected with puromycin (2 μ g/ml). For ER constructs, *E2F1* activity was induced by the addition of 250 nM 4-hydroxytamoxifen (OHT; Sigma). Cell viability was determined using a trypan blue exclusion assay. For experiments involving cycloheximide, cells were incubated with cycloheximide (10 μ g/ml) for the indicated times.

Protein and Gene Expression—Hearts were excised immediately following animal sacrifice and then wetted with 0.5 ml of buffer (100 mM KCl, 50 mM Tris, 5 mM NaN_3 , 100 μ M phenylmethylsulfonyl fluoride, pH 7.4) and blotted on Whatman papers to remove fluids, finely minced on ice with a razor blade, and homogenized on ice in a hand-held electric homogenizer (PowerGen 125; Fisher) in SDS sample buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol), followed by boiling for 5 min. For experiments involving cell cultures, cells were collected according to common procedures. Protein concentration was quantified using the Bio-Rad protein assay, and 30 μ g of total lysate were resolved by SDS-PAGE and transferred to Immobilon-P (Millipore, Billerica, MA) membranes, which were blotted with the appropriate antibodies: *E2F1* (1:1000; sc-251, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), *E1A* (1:1000; sc-430; Santa Cruz Biotechnology), caspase 7 (1:1000; 9492, Cell Signaling Technologies, Danvers, MA), *PDK4* (1:1000 (AP7041b (Abgent, San Diego, CA)) or 1:500 (sc14495 (Santa Cruz Biotechnology))), *Ran* (1:2000; sc-1156; Santa Cruz Biotechnology), and α -tubulin (1:2000; B-5-1-2;

Sigma). Anti-mouse horseradish peroxidase (1858413; Amersham Biosciences) and anti-rabbit horseradish peroxidase (1858415; Amersham Biosciences) were used as secondary antibodies. Proteins were visualized using ECL detection (RPN 2132; Amersham Biosciences).

Mouse Models—*E2F1*^{-/-} mice (B6;129S4-E2F1tm1Meg/J) and wild type (WT) controls (B6;129SF2/J) were obtained from Jackson Laboratory. *E2F1*^{-/-} (FVB) and controls were provided by Dr. G. Leone (Ohio State University). All animal procedures were approved by the Washington University Animal Studies Committee.

Serum Analysis—Whole blood was collected in EDTA by the cheek pouch method of fasted (overnight) animals. Plasma was collected by centrifugation (2940 \times g for 5 min). Total cholesterol (Sigma), triglycerides (Sigma), glucose (Sigma), and non-esterified fatty acids (Wako Chemicals U.S. Inc., Richmond, VA) were analyzed using commercial kits according to the manufacturers' specifications.

Insulin Tolerance Test—Adult male mice (11–12 weeks old) fasted for 6 h received an intraperitoneal injection of human insulin at a dose of 0.75 units/kg of body weight. Glucose was measured via the tail vein before the insulin injection and afterward at 30 and 60 min.

Echo MRI—Measurements of body fat, lean mass, body fluids, and total body water in WT and *E2F1*^{-/-} mice without the need for anesthesia or sedation were obtained using the EchoMRI 3-in-1TM, a Quantitative Nuclear Magnetic Resonance (QNMR) system. Adult male mice (12 weeks old) were used.

Isolated Working Heart Perfusion (IWHF) and in Vivo Metabolic Measurement—Adult male mice (11–12 weeks old with 25–30-g body weight) fasted for 16 h were heparinized 10 min before anesthesia. Animals were then deeply anesthetized, and hearts were excised and placed in ice-cold Krebs-Henseleit bicarbonate solution. Hearts were cannulated first via the aorta with an 18-gauge plastic cannula and perfused retrogradely by the Langendorff method with Krebs-Henseleit bicarbonate solution. During this time, the left atrium was cannulated through a pulmonary opening with a 16-gauge steel cannula. Once the cannulation for the working heart mode was complete, the Langendorff line was closed, and the left atrial and aortic lines were opened and perfused with Krebs-Henseleit bicarbonate solution containing 5.0 mM glucose, 0.4 mM palmitate bound to 3% fatty acid-free bovine serum albumin and 600 pM insulin with a preload pressure of 11.5 mm Hg and an after load pressure of 50 mm Hg. Spontaneously beating hearts were subjected to a 60-min aerobic perfusion with oxygenated buffer solution. To determine glucose oxidation, trace amounts of [U -¹⁴C]glucose (0.1 μ Ci/ml) was used. Functional measurements were acquired for 10 s every 10 min with the MP100 system from AcqKnowledge (BioPac Systems, Santa Barbara, CA).

Real Time PCR Analysis—Total RNA was isolated from tissues and cells using Trizol (Invitrogen) according to the manufacturer's directions, and reverse transcription-PCR was performed as described (11). Primer sequences used were as follows: *18 S*, AGTCCCTGCCCTTTGTACACA and GATCGAGGGCCTCACTAAAC; *PDK4*, TTTCTCGTCTCTACGCCAAG and GATACACCAGTCATCAGCTTCG.

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Immunofluorescence Analysis—Cells were grown on coverslips and fixed with 2% paraformaldehyde for 30 min. Following permeabilization with Triton X-100 and blocking with 3% bovine serum albumin, the cells were incubated with primary antibodies and then with FITC-conjugated secondary antibodies with 3 washes after each incubation. Nuclear localization was analyzed by fluorescence microscopy.

Glucose Oxidation—For glucose oxidation in cell culture, [U - ^{14}C] labeled glucose was used and the amount oxidized was evaluated by measuring the $^{14}CO_2$ produced as previously described (11, 19). Cells were washed three times with Krebs-Ringer Hepes containing 40 μM fatty acid-free bovine serum albumin and incubated for 1–2 h in the same buffer containing U - ^{14}C -labeled glucose (1 $\mu Ci/80 \mu M$). $^{14}CO_2$ trapping was performed (using a flask with a well containing a filter soaked with benzethonium hydroxide) overnight at 30 °C with gentle shaking. Glucose oxidation in the heart is described above.

Bioinformatic Analysis of the PDK4 Promoter—mRNA sequences of human PDK4 (NM_002612) were retrieved from the NCBI nucleotide data base, and its proximal promoter sequence (from –700 to +300 around the transcription start site) was obtained from the CSHLmpd mammalian promoter data base (20). The MATCH program associated with the TRANSFAC data base (21) was run to minimize the false negative rate in order to identify the potential E2F1 binding sites.

Chromatin Immunoprecipitation (CHIP) Analysis of PDK4 Promoter—CHIP was performed as previously described (16, 18), using an E2F1-specific antibody (E2F1 sc-193; Santa Cruz Biotechnology). DNA released from precipitated complexes was amplified using sequence-specific primers to detect PDK4 promoter elements (Fig. 4E, oligonucleotides 1 and 2). As controls, oligonucleotides corresponding to the indicated positions (Fig. 4E, oligonucleotides 3 and 4) were used to amplify sequences within the cyclin A gene.

Luciferase Assays—Genomic fragments corresponding to the human PDK4 promoter (described above) were amplified by PCR from human muscle genomic DNA using the sequence-specific primers (available upon request) to introduce KpnI/XhoI restriction enzyme sites. Following purification and digestion cycles, amplicons were cloned into the pGL3-Basic luciferase reporter vector (Promega, Madison, WI) at KpnI/XhoI sites. IMR90 and C₂C₁₂ cells were transfected using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions with 1 μg of the reporter construct, 200 ng of the E2F1 expression plasmid or a vector control, and 1.2 μg of the pRL- β -globin control plasmid (Promega). Cells were harvested 36 h after transfection (or as indicated otherwise). Luciferase activity was assayed using a luminometer (Promega 20/20), and data were normalized to the transfection control pRL- β -globin. The human Caspase 7 promoter was used as a positive control. Sequences of oligonucleotides are available on request.

Site-directed Mutagenesis—The E2F binding site in the human PDK promoter was mutated using site-directed mutagenesis performed using the QuikChange kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. PDK4 primer sequences are follows (with mutated nucleotides in lowercase type): CTTTCTCTGATCTGATTaaCGtGACCTGGA-GTTCAGGACGCG and CGCGTCCTGAACTCCAGGTCa-

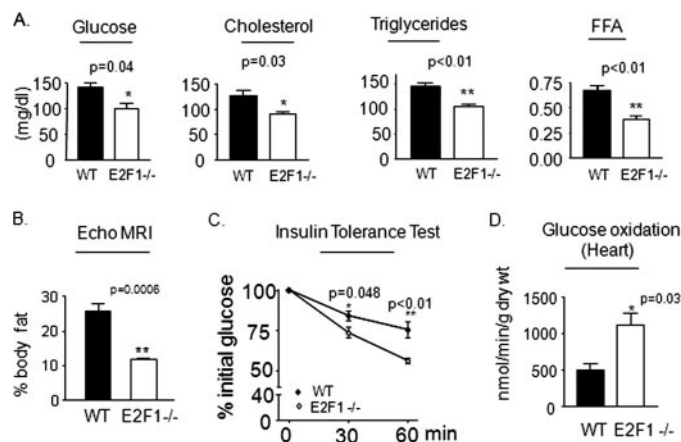


FIGURE 1. Loss of E2F1 promotes insulin sensitivity and improves myocardial glucose oxidation *in vivo*. A, plasma serum levels of glucose, cholesterol, triglycerides, and free fatty acids (FFA) in E2F1^{-/-} mice and WT counterparts (mice fasted for 16 h prior to blood collection). B, total fat composition measured by echo MRI and plotted as percentage body fat. C, intraperitoneal insulin tolerance test in fasted E2F1^{-/-} mice and WT counterparts. Mice ($n = 5$) received an intraperitoneal injection of human regular insulin (Lilly) at a dose of 0.75 units/kg of body weight. Tail blood glucose was determined at 0, 30, and 60 min after challenge using a B-GLUCOSE analyzer (Hemacue). Values are plotted relative to initial glucose levels. D, glucose oxidation measured by IWHP in E2F1-deficient mice and WT controls. IWHP is described in detail under "Experimental Procedures."

CGttAATCAGATCAGAGAAAG. Mutations were verified by ABI automated sequencing through the Protein and Nucleic Acid Chemistry Laboratory facility at Washington University, and clones were analyzed using Vector NTI, version 10.

Statistical Analysis—Values are expressed as means \pm S.E. Student's t test (two-tailed) was performed to determine the statistical difference between groups. p values of <0.05 were considered significantly different (*), and p of <0.01 was considered highly significant (**). All experiments were repeated at least three times, and each represents a minimum of three biological replicas.

RESULTS

E2F1 Loss Promotes Insulin Sensitivity and Improves Myocardial Glucose Oxidation—Homozygous loss of E2F1 influences basic metabolic patterns *in vivo*, as detected by the change in a number of metabolic markers. As shown in Fig. 1A, sera obtained from E2F1^{-/-} animals and their WT counterparts following an overnight fast shows lower blood glucose levels as well as features of improved plasma lipid profiles underscored by marked reduction in cholesterol, triglycerides, and nonesterified free fatty acids. Echo MRI was performed to determine body fat composition, and as shown in Fig. 1B, E2F1^{-/-} mice are significantly leaner, with 73% less body fat compared with WT. These results suggest an improved insulin response in E2F1^{-/-} mice, which would be consistent with earlier findings (22). An intraperitoneal insulin tolerance test was conducted to evaluate glucose disposal in response to insulin in our E2F1^{-/-} mice. Following insulin injections (0.75 units/kg of body weight), blood glucose was assayed as a function of time. Glucose clearance was significantly improved in E2F1^{-/-} compared with age- and gender-matched WT mice, as depicted in Fig. 1C. The function of the heart upon E2F1 loss was also examined by (i) echocardiography to assess the mechanical

aspect of the heart as a pump and (ii) IWHP to determine glucose oxidation efficiency. M-mode echocardiography did not reveal a detectable difference between the two genotypes, $E2F1^{-/-}$ and WT controls (data not shown). However, analysis of myocardial glucose oxidation by IWHP in the beating heart revealed significant improvement of glucose utilization in $E2F1^{-/-}$ mice. As shown in Fig. 1D, hearts of $E2F1$ -null mice are markedly better in oxidizing $[U-^{14}C]$ glucose compared with WT controls. Together, these data demonstrate that the loss of $E2F1$ enhances the overall metabolic profile, promotes insulin sensitivity, and stimulates myocardial glucose utilization *in vivo*.

E2F1 Expression Induces PDK4 and Blunts Glucose Oxidation—We determined whether factors known to regulate myocardial glucose oxidation, such as the PDK4 enzyme, are altered in the $E2F1$ -deficient hearts. As described earlier, PDK4 is a key inhibitor of glucose oxidation and a regulator of myocardial fuel utilization patterns. Moreover, recent data from a heart-specific $PDK4$ transgenic mouse model demonstrates that PDK4 overexpression in the heart is sufficient to reduce myocardial glucose oxidation (23). A reduction in PDK4 expression with $E2F1$ loss would be consistent with the aforementioned improved myocardial glucose oxidation in the $E2F1^{-/-}$ mice (Fig. 1D). Protein expression of PDK4 was analyzed in total heart lysates of $E2F1$ -deficient and -proficient mice. As shown by Western blot analysis in Fig. 2A, an expression pattern reflecting blunted PDK4 expression in the $E2F1^{-/-}$ heart was readily detected.

Since various hormonal and nutritional inputs contribute to PDK4 regulation, we asked if PDK4 expression is directly linked to E2F1 expression. An approach using an inducible E2F1-estrogen receptor fusion construct (ER-E2F1) (14, 18), where nuclear translocation of E2F1 can be induced by OHT, was applied. This circumvents the cytotoxic effects of prolonged ectopic E2F1 expression and enables us to determine the kinetics of PDK4 expression as a time course following E2F1 activation. C_2C_{12} myoblasts cells were stably infected with high titer retroviruses harboring ER-E2F1 and a gene encoding the antibiotic resistance marker. Following selection of transduced populations as described (18), immunofluorescence analysis was performed to confirm nuclear translocation of exogenous E2F1 upon OHT treatment. As shown in Fig. 2B, immunofluorescence analysis performed with E2F1-specific antibodies shows E2F1 nuclear localization following OHT treatment. To further validate the function of the ER-E2F1 system, we performed a viability assay following a DNA damage challenge. As shown in Fig. 2C, E2F1 sensitizes cells to the DNA-damaging agent adriamycin as expected. E2F1 also induces the known E2F1 target caspase 7 proenzyme (18), as shown in Fig. 2D. Importantly, PDK4 protein expression, determined in these same lysates, is rapidly induced following E2F1 activation as well (Fig. 2D). Consistently, glucose oxidation was markedly reduced in these cells with activated E2F1. As shown in Fig. 2E, glucose oxidation determined from $^{14}CO_2$ production in cells incubated with $D-[U-^{14}C]$ glucose as described (19) was significantly blunted upon E2F1 induction. Such effect of enforced E2F1 expression on PDK4 and glucose oxidation *ex vivo* is in line with the effects we detect in $E2F1^{-/-}$ hearts and collec-

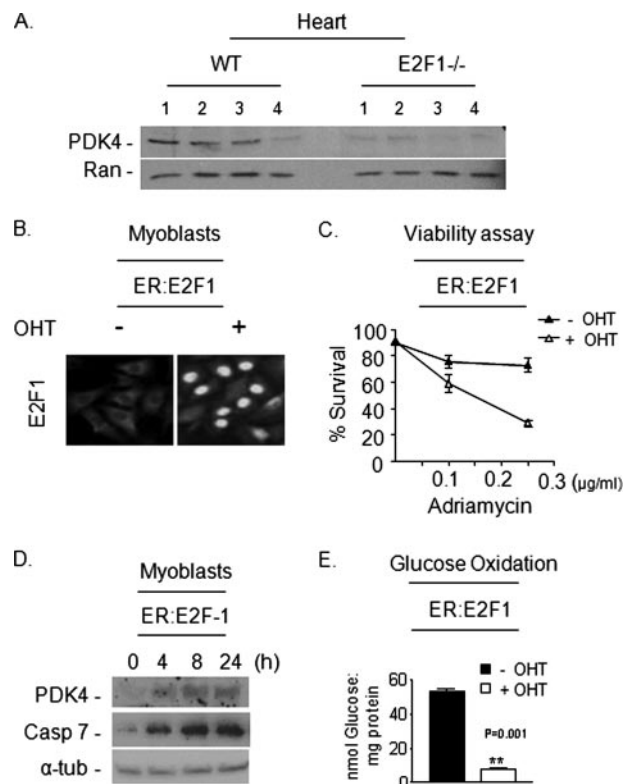


FIGURE 2. E2F1 expression induces PDK4 and blunts glucose oxidation in C_2C_{12} muscle cells. A, PDK4 protein levels as determined by Western blot analysis in total heart lysates of WT and $E2F1^{-/-}$ mice shown. Four mice per group are shown, and Ran was used as loading control. B, immunofluorescence analysis (IF) of E2F1 protein expression in myoblasts expressing ER-E2F1. Photomicrographs represent cells treated for 4 h with OHT or a solvent control. C, viability assay using a trypan blue exclusion assay in C_2C_{12} cells expressing ER-E2F1 in the presence or absence of OHT (which induces exogenous E2F1 translocation and activation) and incubated with increasing concentration of adriamycin (ADR). D, Western blot time course analysis of total PDK4 protein levels and procaspase 7 in ER-E2F1-expressing C_2C_{12} myoblasts following OHT treatment. The time indicates duration of OHT incubation, and α -tubulin is used as a loading control. E, $D-[U-^{14}C]$ glucose oxidation in C_2C_{12} myoblasts expressing ER-E2F1 and treated with OHT (20 h). Values are expressed as means \pm S.E. Statistical significance was determined by Student's *t* test, and *p* values of <0.05 are considered significant.

tively supports the interpretation that PDK4 expression and its effect on glucose oxidation is regulated by E2F1. Of note, we focus in this investigation on the PDK4 isoenzyme, because it is distinctive among the PDK family in its response to starvation and diabetes in the heart (10). This was also stimulated by the phenotype of the aforementioned heart-specific $PDK4$ transgenic mouse. Nevertheless, other PDKs may be the target of similar regulation by E2F1. In the same analysis, other E2F family members, in particular E2F2, which shares great sequence homology with E2F1, can be regulating the PDKs and influencing muscle glucose oxidation as well. To that extent, loss of $E2F1$ alone was sufficient to induce robust effects on PDK4 expression, underscoring the importance of this E2F family member in regulating this PDK isoenzyme.

Rb Inactivation and Oncogenic E1A Induce PDK4—Analysis of $PDK4$ transcript levels following induction by E2F1 shows up-regulation of $PDK4$ mRNA consistent with PDK4 protein expression in ER-E2F1-expressing C_2C_{12} myoblasts upon induction with OHT (Fig. 3A). Importantly, such induction is observed despite the presence of cycloheximide, an inhibitor of

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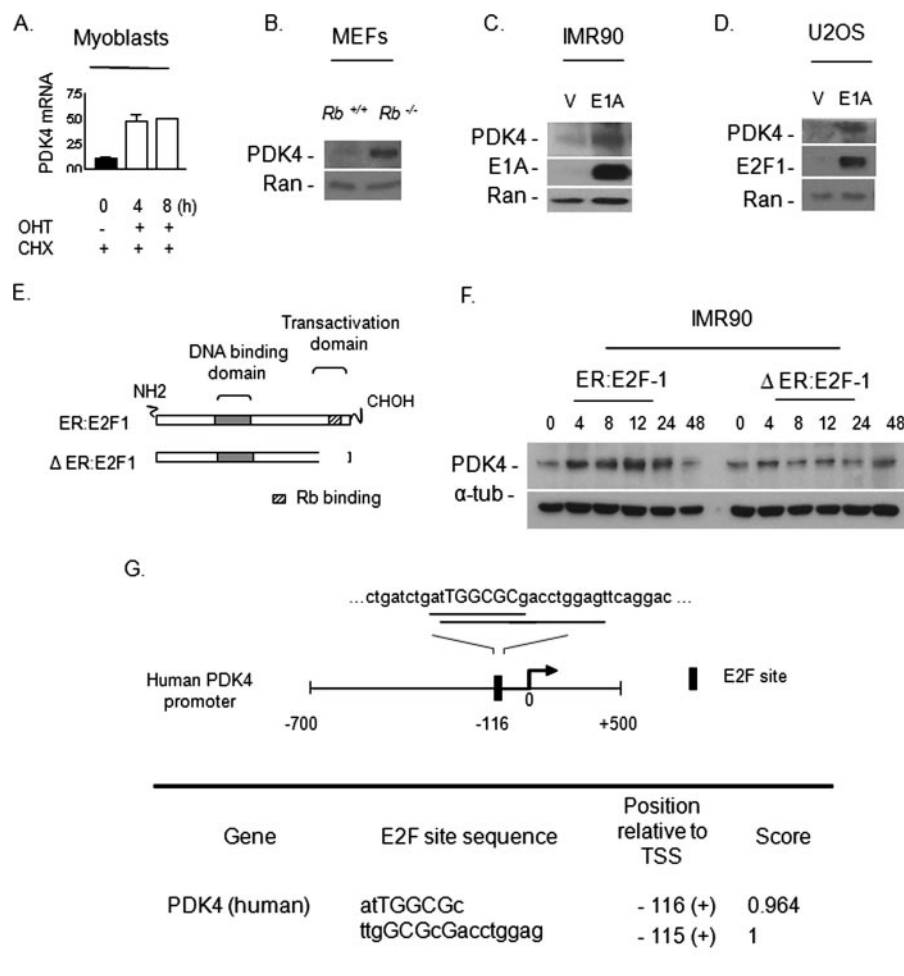


FIGURE 3. Rb inactivation and oncogenic E1A induce PDK4. A, mRNA expression of PDK4 in the presence or absence of OHT, as determined by quantitative PCR in C₂C₁₂ myoblasts stably expressing the inducible E2F1 construct and induced for the indicated time; all samples were incubated with cycloheximide (CHX; 10 μ g/ml). PDK4 values are expressed as means of -fold induction \pm S.E. relative to controls. Shown is Western blot analysis of PDK4 expression in *Rb*^{-/-} compared with *Rb*^{+/+} mouse embryo fibroblasts (MEFs) (B), IMR90 fibroblasts stably expressing E1A compared with cells expressing a vector control (C), and U2OS osteosarcoma cells stably expressing E1A compared with a vector control as in C (D). E, schematic representation of the ER-E2F1 mutant (Δ ER:E2F1) and the WT E2F1 construct (ER:E2F1). The blank area represents the approximate location of the deleted fragment. F, Western blot time course analysis (in hours) of total PDK4 protein levels in IMR90 cells expressing ER-E2F1 mutant following OHT treatment. G, schematic diagram and tabulation depicting genomic regions of the human PDK4 promoter. A black rectangle represents the putative E2F1 binding sites. The corresponding nucleotide sequence representing the sites is also shown (underlined). (+), sites are on the leading strand.

de novo protein synthesis. As such, transcriptional activation of E2F1 appears to be required for its ability to induce PDK4, implicating it as a direct E2F1 transcriptional target. The rapid induction of PDK4 is also characteristic of the induction kinetics of *bona fide* early response E2F1 targets like procaspases, HURP, and CDC16 determined using the same E2F1-inducible system (17, 18). To further characterize the mechanism of PDK4 induction by E2F1, we determined whether deregulation of endogenous E2F1 can induce PDK4 and recapitulate the effects of enforced expression of exogenous E2F1. Many of the endogenous E2F1 functions in proliferation and survival are regulated by the tumor suppressor Rb, which represses E2F-dependent transcription (13, 24). In quiescent cells, Rb physically associates with E2F1, resulting in its inhibition (13, 25). In cycling cells, cyclin-dependent kinases, in response to growth factors and mitogenic signals, periodically phosphorylate (inac-

tivate) Rb, freeing E2Fs and allowing unabated E2F1 transcription of target genes (12, 13, 18, 26, 27). As such, mouse embryo fibroblasts deficient for *Rb* were compared with *Rb*-proficient counterparts for PDK4 protein levels. As shown in Fig. 3B by Western blot analysis, mere loss of *Rb* markedly induces PDK4. Next, we asked whether inactivation of Rb in *Rb*-proficient cells using the E1A oncogene can also induce PDK4 expression. We have reported previously that E1A-mediated inactivation of Rb deregulates and induces E2F1 and consequently promotes the transcription of its direct targets like caspase 7 and cyclin A in human IMR90 fibroblasts (18). E1A physically binds and inactivates Rb (16, 28) and unrestrains E2F1 activity. Additionally, E1A and the transactivation domain of E2F1 were recently shown to compete for the same binding site on Rb (29). Therefore, E1A or a vector control were stably introduced into IMR90s as described (18). As shown by Western blot analysis in Fig. 3C, E1A was sufficient to markedly induce PDK4 protein expression. The same results were obtained in the *Rb*-proficient osteosarcoma U2OS cell line following introduction of E1A and induction of E2F1 (Fig. 3D). Inactivation of Rb by stable expression of a short hairpin *Rb* construct also induces PDK4 (data not shown). To this end, the aforementioned results suggest a transcriptional requirement for PDK4 induction by E2F1. We analyzed this further using an E2F1-inducible system identical to the one described in Fig. 2B but lacking the transactivation domain of E2F1 (Δ ER-E2F1; Fig. 3E). We determined whether PDK4 can still be induced in response to this E2F1 mutant. As shown in Fig. 3F, in IMR90 cells and upon activation with OHT, Δ ER-E2F1 fails to induce PDK4 expression compared with full-length ER-E2F1, indicating as such that the transactivation domain of E2F1 is required for the E2F1-mediated PDK4 induction. Taken together, data in Fig. 3 support the interpretation that Rb inactivation and E2F1-dependent transcription induce PDK4.

PDK4 Promoter Contains E2F Binding Sites—Above, we indicate the importance of E2F1 transcriptional activity for the induction of PDK4. Therefore, we undertook a nonbiased bioinformatics approach to locate and potentially characterize E2F1 binding sites on the *PDK4* promoter. This method iden-

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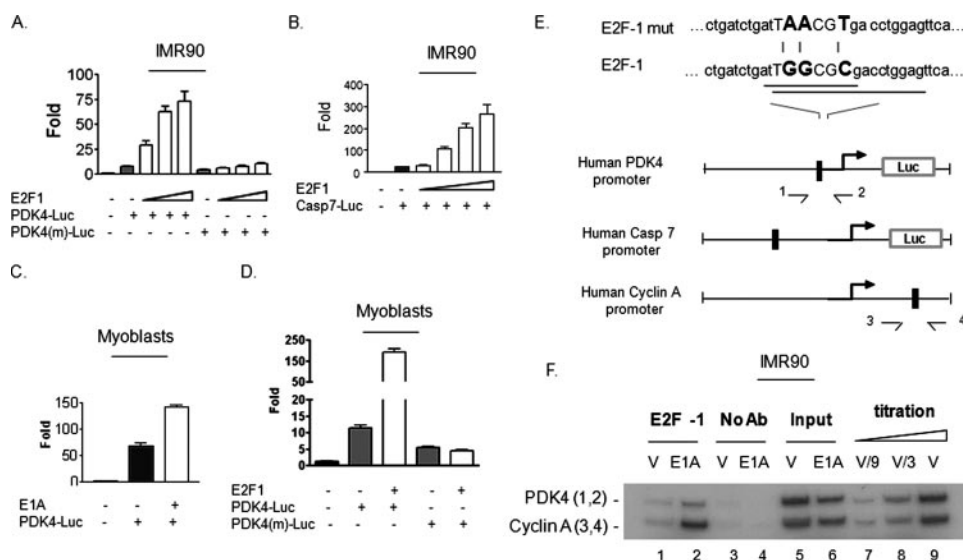


FIGURE 4. PDK4 is a direct transcriptional E2F1 target. A, transactivation assay using a *PDK4*-luciferase reporter system (*PDK4*-Luc) in IMR90 fibroblasts co-transfected with E2F1 plasmid in addition to the *PDK4*-Luc or the mutant *PDK4*-Luc (*PDK4*(m)-Luc) with mutated E2F binding sites. The presence (+) or absence (-) of particular plasmids in the transfection is indicated, and the triangle represents increasing amounts of E2F1 plasmid (20, 40, 80, 250, and 500 ng, respectively). B, luciferase experiments were performed as in A, using the Casp 7-Luc reporter instead of *PDK4*-Luc. Casp 7-Luc is known to be transactivated by E2F1 and is used here as a positive control. E2F1 concentrations used were 40, 80, 250, and 500 ng, respectively. C, transactivation assay in *C2C12* myoblasts co-transfected with E1A plasmid (200 ng) and *PDK4*-Luc (C) or E2F1 (500 ng) and *PDK4*-Luc and mutant *PDK4*-Luc (*PDK4*(m)-Luc) (D), as indicated. E, schematic representation of the human *PDK4* promoter (as in Fig. 3G), with a black rectangle representing the putative E2F1 binding sites location. Corresponding nucleotide site sequences are also shown, and mutated nucleotides are highlighted (boldface type). Also shown is a representation of the human caspase 7 and cyclin A promoters. Actual sequences of E2F1 sites in the *caspase 7* promoter and the cyclin A promoters are shown in supplemental Fig. 1. Primer sets (primers 1 and 2 and primers 3 and 4) represent flanking primers used to amplify genomic fragments for ChIP analysis (F). F, *in vivo* detection of promoter occupancy by E2F1 using ChIP. Chromatin was prepared from IMR90 cells (Fig. 3C) and immunoprecipitated with antibodies (Ab) specific to E2F1. Duplex PCR amplification was performed on corresponding templates using the indicated primer sets. Input corresponds to PCRs containing 0.5% of total chromatin used in immunoprecipitation reactions. Parallel immunoprecipitation without antibody failed to yield detectable signals after equivalent autoradiographic exposure. Loading of templates in E1A and vector (V) samples is shown in the input lanes, and a titration of DNA template was performed demonstrating the linearity of PCR amplification. Titration was performed on input genomic DNA of templates from cells infected with the vector control (V) following a serial dilution (by a factor of 3, hence V/3 and V/9).

transcription factor binding sites on the basis of position weight matrices constructed from known verified binding sites by assigning scores (0–1, 1 being highest match) for similarity between a candidate site and the position weight matrices. This approach has been previously used successfully to characterize several E2F1 targets (16–18). Briefly, we analyzed the first exons and their flanking regions, a 700-bp upstream promoter region, and a 300-bp downstream intron region of genomic sequences of the human *PDK4* promoter using the MATCH program associated with the TRANSFAC data base of cis-regulatory motifs. The *PDK4* promoter exhibit features characteristic of E2F1 response elements (Fig. 3G), including at least one predicted E2F1 binding site in very close proximity to the transcription start site.

PDK4 Is a Transcriptional E2F1 Target—Following identification of E2F binding sites within the *PDK4* promoter, we sought to determine whether the *PDK4* promoter containing the E2F sites is physiologically responsive to E2F1. If *PDK4* is a functional E2F1 transcriptional target, then *PDK4* promoter sequences containing E2F sites would confer responsiveness to a *PDK4* heterologous promoter. To address this experimentally, we cloned the genomic fragment containing the predicted E2F sites (residues -700 to +300 relative to the transcription

start site) into a promoterless luciferase reporter plasmid (PGL3; Promega). The construct *PDK4*-Luc was then co-transfected into IMR90 cells with either an empty vector control or increasing concentrations of an E2F1 expression plasmid and a normalization control. As shown in Fig. 4A, E2F1 induces significant transactivation of the *PDK4*-Luc reporter in a dose-dependent manner (0, 20, 40, 80, 250, and 500 ng of E2F1, respectively). E2F1 activity was confirmed by transactivation of the *Caspase 7* promoter luciferase construct (Casp7-Luc), a well characterized E2F1-responsive construct (16, 18), and used here as a control (Fig. 4B). *C2C12* myoblasts were also co-transfected with *PDK4*-Luc in the presence or absence of E1A, and as shown in Fig. 4C, E1A transactivates *PDK4* in these cells. Similar results were obtained when determining *PDK4* transactivation by E2F1 *C2C12* myoblasts (Fig. 4D) or E1A (data not shown) in IMR90 cells.

To demonstrate the specific involvement of the E2F1 binding site in the transcriptional regulation of the *PDK4* promoter element, the overlapping E2F sites were mutated, and the mutant promoter was cloned and tested for E2F1 responsiveness. Mutation was achieved using site-directed mutagenesis (Fig. 4E). Following sequence verification of successful mutations, the m*PDK4*-Luc was introduced into IMR90 fibroblasts (Fig. 4A) or *C2C12* myoblasts (Fig. 4D), and transcriptional activities were determined. As shown, the mutant *PDK4* promoter fails to drive transactivation of the luciferase gene in response to E2F1, unlike the wild type *PDK4* promoter. Mutation of the E2F1 binding site results in a complete inactivation of the *PDK4* promoter in response to E2F1, indicating the biological relevance of this site in regulating *PDK4* transactivation. Finally, we verified binding of E2F1 to the *PDK4* promoter *in vivo* using ChIP, which allows the detection of proteins bound to specific regions of DNA. Our analysis was performed after stably introducing E1A (same as in Fig. 3C). As such, we determined whether endogenous levels of E2F1 occupy the *PDK4* promoter. E1A- or vector-expressing human IMR90 fibroblasts were fixed in formaldehyde to cross-link proteins to the DNA. After sonication to generate ~500-base pair fragments of genomic DNA, E2F1-DNA complexes were immunoprecipitated using an antibody against E2F1. The DNA was released from immunoprecipitates and analyzed by radiolabeled PCR amplification using *PDK4*-specific primers (1 and 2) flanking the predicted E2F-binding sites (Fig. 4E). *PDK4* promoter

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sequences were readily amplified in chromatin immunoprecipitates from vector control cells (V) (Fig. 4F, lane 1), indicating E2F1 binding to this promoter element. Further enrichment in E1A-expressing cells (E1A) (Fig. 4F, lane 2) is also detected and is consistent with the effect of E1A on inducing PDK4 expression. Similar patterns were observed using primers flanking the known binding site in the cyclin A promoter shown here as a control. Taken together, our data demonstrate that PDK4 is a functional *bona fide* E2F1 transcriptional target.

DISCUSSION

In this study, we describe a new mechanism through which the mitogenic factor E2F1 physically interacts with the PDK4 promoter and is sufficient to regulate its expression and activity. Given the critical function of PDK4 in nutrient sensing and the integration of dietary signals, our findings highlight the intimate coupling between cell cycle regulators and mitochondria-mediated fuel processing and core fluxes. Since the Rb-E2F complex is the target of growth factors and external trophic signals, it is conceivable that the (Rb + E2F1 → PDK4) axis is part of a nutrient sensing network coupling external dietary conditions to the metabolic adaptation of the cellular milieu, in particular the regulation mitochondrial glucose oxidation.

Such E2F1-dependent transcriptional regulation of a key nutrient sensor like PDK4 is probably reminiscent of E2F1 function in promoting cellular growth during S phase, likely to spare the glycolysis end product pyruvate for the synthesis of lipid and protein intermediates needed for cell doubling. Precedents of other cell proliferators in promoting biosynthesis processes are credibly documented. For instance, oncogenes like *c-MYC* or *her2/neu* target glycolytic enzymes as well as the lactate dehydrogenase A that facilitates lactate production from pyruvate (30–32). E2F1 itself induces dihydrofolate reductase (33), a main regulator of *de novo* synthesis of purines, thymidylc acid, and certain amino acids, as well as stimulates glycolytic flux regulators like the PF2K enzymes (34, 35). E1A- or E2F1-dependent induction of PDK4, the inhibitor of pyruvate oxidation (3, 36), would be consistent with the overall effects of proliferator mitogens in supporting biosynthesis platforms. Unrestrained E2F1 activities, as we demonstrate by enforcing E2F1 expression or inactivation of Rb, would maintain elevated PDK4 expression and constitutively suppress glucose oxidation (Fig. 2), conceivably mimicking a prolonged synthesis phase. Similarly, a permanent loss of *E2F1* in an active metabolic organ like the heart blunts PDK4 expression (Fig. 2A) and improves glucose oxidation (Fig. 1D). Given the dysregulation of PDK4 activities in metabolic pathologies like obesity and diabetes (10, 36) and the postulation that counteracting high PDK4 expression can be of therapeutic use in the prevention of hyperglycemia (36), suppression of E2F1 could benefit, in principle, anomalies associated with chronic inhibition of muscle glucose oxidation. This could also be relevant for other diseases of metabolic inflexibility (37) and would require further investigation. Nonetheless, evidence from *E2F1*-null mice, where the loss of *E2F1* reduces glucose levels, promotes insulin sensitivity, and markedly improves plasma lipid profile (Fig. 1), elucidates perhaps a potential metabolic benefit of regulating E2F1 expression.

As we learn more about the interplay between cancer genes and metabolic regulation, it is becoming more and more conspicuous that the influence of the Rb-E2F1 tumor suppressor complex in cellular metabolism is profound. Several studies, including this one, support such an interpretation. For instance, inactivation of Rb in adipocytes was shown to protect mice from diabetes by promoting energy expenditure (38). In addition, Rb intrinsically drives erythropoiesis by coupling cell cycle exit with mitochondrial biogenesis, as recently reported (39). Compelling evidence for an E2F1-specific effect in metabolism is also underscored by the striking metabolic pathologies of *E2F1*-null mice, which exhibit reduced adiposity and resistance to high fat diet-induced obesity and diabetes (22). Furthermore, deficient *E2F1* expression has been shown to impair adipogenesis (40), myogenesis (41), and pancreatic β -cell growth and function (22) secondary to cell cycle defects. As such, abrogated *E2F1* inadvertently compromises critical functioning of these tissues in calibrating basal energy homeostasis and resting metabolic rates. Furthermore, E2F1 is directly linked to the regulation of a few metabolic genes, as previously described. As such, it is important to emphasize that the resulting complex metabolic phenotype of the *E2F1*^{-/-} mouse is probably the culmination of altered expression in a number of E2F1 targets and not one singular gene in particular. We believe that the direct regulation of PDK4 is nonetheless consistent with such a phenotype.

Finally, it is notable that cancer cells, the bulk of which have defects in the Rb-E2F pathway, are largely impaired in their mitochondrial glucose oxidation diverting glycolysis end products, even under normoxic conditions, to cytosolic events, such as lactate production, a tumor-associated phenotype better known as “aerobic glycolysis” and originally described under the Warburg hypothesis (42, 43). Considering the central role of the Rb-E2F complex in survival and proliferation and the widespread Rb pathway mutations found in human cancers, our findings could assist in better defining the etiology of tumor metabolism, in particular the perplexing phenotype of aerobic glycolysis and the lack of efficient mitochondrial oxidation of glucose despite the ostensible need for ATP in hyperproliferative cells. We show that oncogenic stimulation and inactivation of Rb by E1A transcriptionally activates *PDK4* and triggers a functional residency of E2F1 on the *PDK4* promoter. The direct induction by E2F1, E1A, or Rb loss of the PDK4 isoenzyme, an inhibitor of mitochondrial glucose oxidation whose expression is directly linked to metabolic inflexibility and suppression of glucose oxidation in chronic metabolic anomalies, could in fact be a major contributor to aerobic glycolysis. To the extent that it was not tested directly in tumor models in this study, our transcriptional model implies that aerobic glycolysis may not necessarily be a selected trait of cancer cells or an adaptation to low oxygen environments within tumors but could be, at least in part, rather simply an inadvertent consequence of coupling PDK4 expression to unrestrained E2F1 activity. It is conceivable that this coupling would lock pyruvate dehydrogenase complex into an inactive form, creating a semblance of a starvation-like phenotype typically associated with elevated PDK4 expression. Such a byproduct of a direct transcriptional link between Rb-E2F1 and PDK4 might be exploited therapeuti-

cally, especially in tumors with Rb pathway defects, possibly by ways of altering glucose oxidation. Nevertheless, transformed cells probably compensate by promoting glycolytic events or survival genes like *AKT* to ensure viability; ironically, this could happen through mechanisms that might involve transcriptional activities by E2F1 itself (34, 44, 45).

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