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Cyclin D1 Represses Gluconeogenesis via Inhibition of the Transcriptional Coactivator PGC1 α



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Hepatic gluconeogenesis is crucial to maintain normal blood glucose during periods of nutrient deprivation. Gluconeogenesis is controlled at multiple levels by a variety of signal transduction and transcriptional pathways. However, dysregulation of these pathways leads to hyperglycemia and type 2 diabetes. While the effects of various signaling pathways on gluconeogenesis are well established, the downstream signaling events repressing gluconeogenic gene expression are not as well understood. The cell-cycle regulator cyclin D1 is expressed in the liver, despite the liver being a quiescent tissue. The most well-studied function of cyclin D1 is activation of cyclin-dependent kinase 4 (CDK4), promoting progression of the cell cycle. We show here a novel role for cyclin D1 as a regulator of gluconeogenic and oxidative phosphorylation (OxPhos) gene expression. In mice, fasting decreases liver cyclin D1 expression, while refeeding induces cyclin D1 expression. Inhibition of CDK4 enhances the gluconeogenic gene expression, whereas cyclin D1-mediated activation of CDK4 represses the gluconeogenic gene-expression program *in vitro* and *in vivo*. Importantly, we show that cyclin D1 represses gluconeogenesis and OxPhos in part via inhibition of peroxisome proliferator-activated receptor γ coactivator-1 α (PGC1 α) activity in a CDK4-dependent manner. Indeed, we demonstrate that

PGC1 α is novel cyclin D1/CDK4 substrate. These studies reveal a novel role for cyclin D1 on metabolism via PGC1 α and reveal a potential link between cell-cycle regulation and metabolic control of glucose homeostasis.

Hepatic gluconeogenesis is crucial for the body to maintain normal blood glucose levels during fasting or extended periods of nutrient deprivation. Gluconeogenesis can also be maladaptive, contributing to the hyperglycemia observed in type 2 diabetes. Therefore, understanding how gluconeogenesis is controlled has been intensely studied because of its role in hyperglycemia and type 2 diabetes.

Peroxisome proliferator-activated receptor γ coactivator-1 (PGC1) is a family of multifunctional transcriptional coactivators that have emerged as playing a central role in cellular and systemic metabolism (1,2). PGC1 α is the founding member of this family and was initially shown to be a central regulator of brown fat thermogenesis and mitochondrial biogenesis (3,4). Subsequent studies showed that PGC1 α plays a critical role in the regulation of gene-expression programs, driving oxidative phosphorylation (OxPhos) and hepatic gluconeogenesis (5–9). Given the ability of PGC1 α to promote gluconeogenesis, identification of mechanisms that control PGC1 α activity has received significant attention.

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In the fed state, various growth factors and signal transduction pathways lead to repression of gluconeogenesis and increased glucose uptake into peripheral tissues (10,11). Although the initial signaling events of these growth factors in the liver have been well studied, many of the downstream events are not clear (12). Activation of signaling pathways by growth factors activate the D-type cyclins, of which cyclin D1 is the best studied. The classic function of cyclin D1 is regulation of the cell cycle (13,14). Cyclin D1 promotes the G1 to S phase transition of the cell cycle by binding and activating Cdk4 or Cdk6. However, cyclin D1 affects other cellular processes. Cyclin D1 plays a role in both activation and repression of gene expression (14–20). Indeed, a recent article described the presence of cyclin D1 on hundreds of promoters throughout the genome (21). Other recent studies have pointed to a role for cyclin D1 in metabolism (22–24). Early studies as well as microarray analysis show that cyclin D1 is expressed in human and murine liver (25–27). Most studies on cyclin D1 in the liver are in the context of pathogenic conditions such as regeneration, carcinogenesis, and liver damage. However, most hepatocytes are quiescent/in G_0 in the absence of chemical or physical damage. This raises the question as to the role of cyclin D1 in the liver under normal physiological conditions. Despite previous studies showing that cyclin D1 represses mitochondrial function and biogenesis in the livers of mice (23), to date, no studies have directly examined the effect of cyclin D1 on liver metabolism. We describe a new role for cyclin D1 as a repressor of PGC1 α and, in particular, the ability of cyclin D1 to inhibit metabolic gene-expression programs induced by PGC1 α .

RESULTS

Repression of Cyclin D1 Expression Correlates With Induction of a Program of Gluconeogenic Gene Expression

Previous studies and gene-expression analysis data show that cyclin D1 is expressed in a wide range of tissues, including the liver (26,27). We examined several tissues from mice for expression of cyclin D1 mRNA and protein. Cyclin D1 was expressed to varying degrees in a number of different tissues (Supplementary Fig. 1A and B). While spleen and intestine contained the highest levels of cyclin D1, almost no cyclin D1 was observed in pancreas or brain. The liver expressed abundant levels of cyclin D1 at both the protein and the RNA level. The presence of cyclin D1 in liver, despite the fact that hepatocytes, which comprise the bulk of the liver, are typically in G_0 , raises the question as to the normal physiological function of cyclin D1 in the liver.

Next we wanted to determine the role of cyclin D1 in a biologically relevant context. One of the functions of the liver is gluconeogenesis during fasting. Therefore, we examined a published microarray data set (National Center for Biotechnology Information Gene Expression Omnibus [NCBI GEO] accession GSE24504) for the

expression of cyclin D1 in the liver during fasting, in which the gluconeogenic gene-expression program was induced (28). As the length of the fast progressed, cyclin D1 mRNA expression decreased (Fig. 1A). This prompted us to confirm and directly examine cyclin D1 expression, along with gluconeogenic genes induced during 6 and 24 h of fasting. As expected, glucose levels were reduced in fasted mice at 6 and 24 h (Supplementary Fig. 2A and B). Fasting significantly reduced the expression of liver cyclin D1 mRNA at both time points (Fig. 1B and Supplementary Fig. 2C). PEPCK is the rate-limiting step in gluconeogenesis that converts oxaloacetate into phosphoenolpyruvate. G6Pase controls the last step in gluconeogenesis, which converts glucose-6-phosphate to glucose. PGC1 α induces the expression of PEPCK and G6Pase during fasting. The reduction in cyclin D1 mRNA during fasting correlated with a concomitant induction of mRNA for *Pepck* (and protein), *G6pase*, and *Pgc1 α* at both 6 and 24 h (Fig. 1C and Supplementary Fig. 2D and E).

Previous studies demonstrate that loss of cyclin D1 led to an increased mitochondrial biogenesis. Since genes involved in OxPhos are induced during fasting/gluconeogenesis, we examined the expression of OxPhos genes and *Pgc1 α* in liver RNA from cyclin D1 knockout mice (*Cyclin d1*^{-/-}). Cytochrome C (*Cyt-C*), *Cox4i*, and *Atpsynthase F1* (*AtpsynF1*) expressions were elevated in the livers of *Cyclin d1*^{-/-} mice, demonstrating an increase in OxPhos gene expression in the absence of cyclin D1 (Fig. 1D–F). In addition, the expression of the *Pgc1 α* was increased in livers from *Cyclin d1*^{-/-} mice (Fig. 1G). As a whole, these studies suggest that cyclin D1 may be regulating metabolic function in the liver. These data prompted us to examine a published data set of livers from obese patients with and without diabetes (NCBI GEO accession GSE15653). PEPCK and G6Pase were significantly elevated in livers from diabetic patients compared with nondiabetic patients (Fig. 1H). In contrast, cyclin D1 levels were reduced in the livers of diabetic patients (Fig. 1I). This demonstrates a possible link between cyclin D1 and glucose homeostasis.

The gluconeogenic gene-expression program is repressed following refeeding. Therefore, we examined the effect of refeeding on cyclin D1 expression. While fasting reduced cyclin D1 expression (Fig. 1B), cyclin D1 expression was significantly induced following refeeding (Fig. 2A). This induction of cyclin D1 correlated with reduced *Pepck*, *G6pase*, *Pgc1 α* , and OxPhos gene expression (Supplementary Fig. 3A–F).

The inverse correlation between gluconeogenic/OxPhos gene expression and cyclin D1 gene expression suggests a role for cyclin D1 in regulating the gluconeogenic and OxPhos gene-expression programs. Cyclin D1 functions primarily by activating the cyclin-dependent kinase 4 (CDK4). However, cyclin D1 also has CDK4-independent effects. Therefore, we used a pharmacological loss of function approach to determine the role of cyclin D1 on gluconeogenic and OxPhos gene expression and, in particular, whether these effects

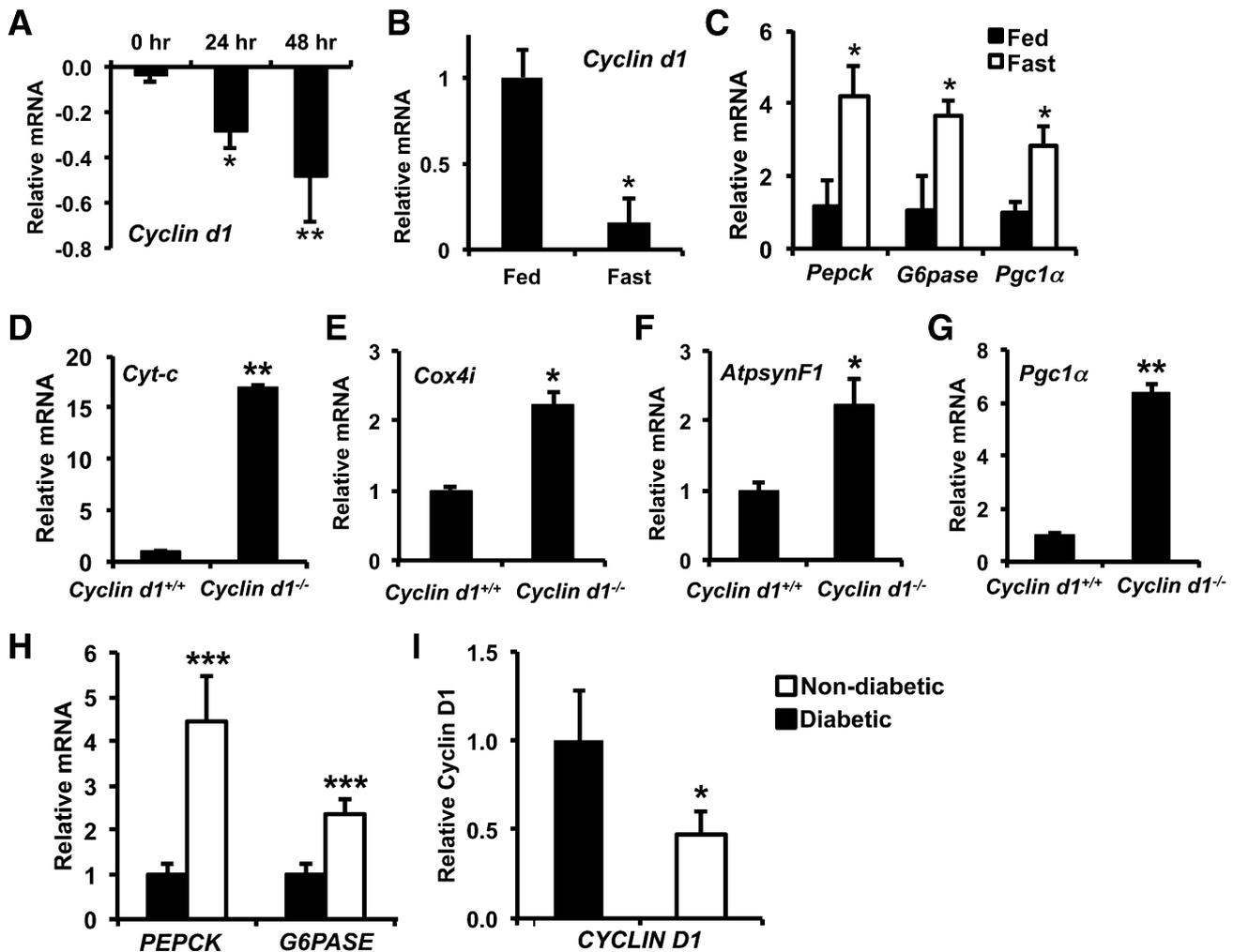


Figure 1—A reduction in cyclin D1 is associated with increased gluconeogenic and OxPhos gene expression. **A:** A published microarray data set of livers from fasted mice was reanalyzed for expression of cyclin D1 as described in RESEARCH DESIGN AND METHODS. Mice were fed or fasted overnight, livers were removed, and RT-PCR was performed for **(B)** *Cyclin d1* or **(C)** *G6pase*, *Pepck*, and *Pgc1α*; $n = 4-6 \pm$ SD. OxPhos and PGC1 α gene expression are increased in the absence of *cyclin d1*. RNA was isolated from the livers of *Cyclin d1*^{+/+} and *Cyclin d1*^{-/-} mice. cDNA was synthesized and RT-PCR was performed for **(D)** *Cyt-C*, **(E)** *Cox4i*, **(F)** *AtpsynF1*, or **(G)** *Pgc1α*; $n = 4 \pm$ SD. Analysis of a published microarray data set from livers of obese nondiabetic and obese diabetic patients for **(H)** PEPCK, G6Pase, and **(I)** cyclin D1. * $P < 0.05$; ** $P < 0.0001$; *** $P < 0.01$.

are CDK4 dependent. For these studies, we used a potent and specific inhibitor of cyclin D-CDK4/6 kinases, PD0332991 (29). C57Bl/6J mice were fasted overnight, administered PD0332991 or PBS control, and refed for 4 h. After 4 h, glucose levels in refed control mice increased to a normal glucose concentration (198 mg/dL) (Fig. 2B). In fed mice treated with PD0332991, plasma glucose levels increased to ~240 mg/dL, a level indicating hyperglycemia. While refeeding reduced the expression of gluconeogenic and OxPhos gene expression (Supplementary Fig. 3A–F), in the presence of PD0332991, *Pepck*, *G6pase*, *Pgc1α*, and OxPhos gene expression were significantly higher (Fig. 2C–H). These data suggest that inhibition of cyclin D1/CDK4 via administration of PD0332991 either promotes gluconeogenic gene expression or prevents the repression of gluconeogenic gene expression, resulting in increased plasma glucose.

Next we used a gain-of-function approach to directly determine whether cyclin D1 is involved in the gluconeogenesis and OxPhos gene-expression programs. We used a transgenic *Cyclin d1* mouse strain in which overexpression of cyclin D1 was targeted to the liver using the –596 to +21 fragment of the FABP1 promoter (*Cyclin d1*^{LITG}) (30). *Cyclin d1*^{LITG} mice were either fed or subjected to an overnight fast, and the expression of gluconeogenic genes *Pepck1*, *G6pase*, and *Pgc1α* examined. *Pepck* and *G6pase* were induced approximately fourfold and *Pgc1α* induced ~2.5-fold in the wild-type mice during fasting (Fig. 3A). However, in *Cyclin d1*^{LITG} mice, fasting-induced *Pepck*, *G6pase*, and *Pgc1α* gene expression was significantly reduced along with OxPhos gene expression (Supplementary Fig. 4A–C). Blood glucose levels were not altered in fed *Cyclin d1*^{LITG} mice. However, fasting glucose levels were reduced in *Cyclin d1*^{LITG} mice, underscoring a physiological

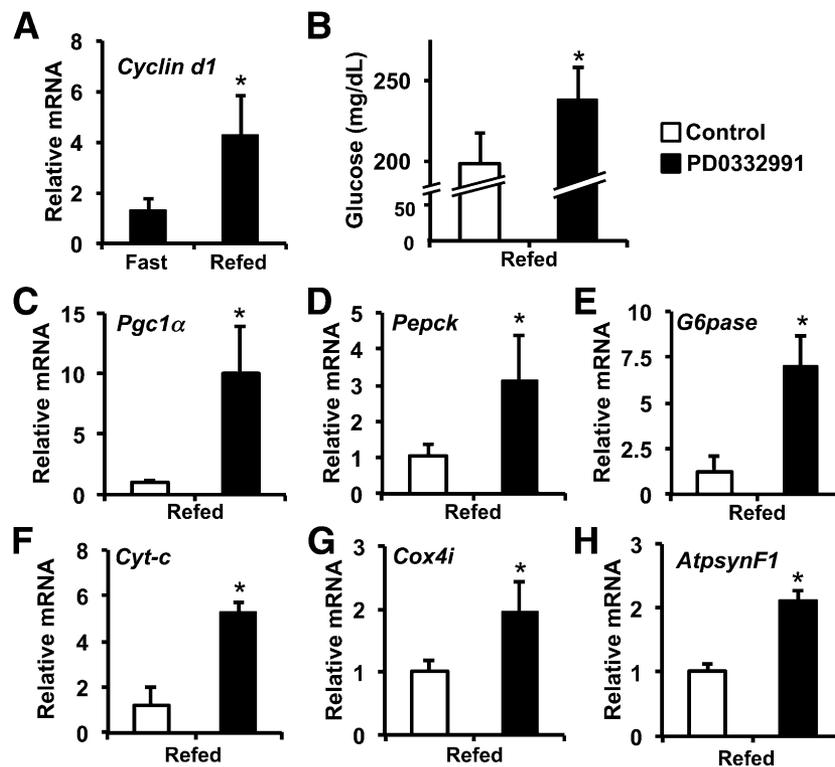


Figure 2—Inhibition of cyclin D1/CDK4 promotes gluconeogenesis. **A**: Cyclin D1 is increased following refeeding of mice subjected to an overnight fast. **B–H**: Wild-type C57Bl/6J mice were fasted overnight then treated with vehicle control or 75 mg/kg PD0332991 for 4 h. **B**: Blood glucose was measured after 4 h. **C–H**: Mice were killed, livers were removed, RNA was isolated, and RT-PCR was performed as indicated. $n = 4–6 \pm$ SD. * $P < 0.05$.

effect of the decrease in gluconeogenic gene expression (Fig. 3B). We also wanted to determine if acute expression of cyclin D1 affected the fasting-induced gluconeogenic response. Adenoviral-mediated expression of cyclin D1 in the liver of mice via tail vein injection significantly repressed fasting-induced *Pepck*, *G6pase*, and *Pgc1 α* gene expression (Fig. 3C). Similar to the cyclin D1 liver transgenic mice, adenoviral cyclin D1 expression blunted fasting glucose (Fig. 3D).

The pharmacological and genetic data show that cyclin D1 represses gluconeogenic and OxPhos gene expression. This raises the question as to how cyclin D1 represses these programs of gene expression. PGC1 α is a key regulator of gluconeogenesis and OxPhos. Therefore, we hypothesized that the reduction in gluconeogenic and OxPhos gene expression by cyclin D1 is a result of either cyclin D1-mediated reduction of PGC1 α expression or PGC1 α transcriptional activity. We examined whether cyclin D1 reduces the activity of PGC1 α by using a full-length PGC1 α cDNA fused to a heterologous Gal4 DNA-binding domain (Gal4-PGC1 α). Gal4-PGC1 α potently coactivated a UAS-TATA luciferase construct as expected (Fig. 4A). Increasing amounts of cyclin D1 repressed Gal4-PGC1 α activity in a dose-dependent manner, demonstrating that cyclin D1 reduces the activity of PGC1 α .

PGC1 α promotes gluconeogenic gene expression by coactivating PEPCK and G6Pase (7). Therefore, we investigated

whether cyclin D1 can repress PGC1 α -mediated coactivation of PEPCK and G6Pase using promoter luciferase reporter constructs. As expected, PGC1 α strongly coactivated PEPCK and G6Pase promoters (Fig. 4B and C). Cyclin D1 on its own had no effect activation of the PEPCK and G6Pase promoter activity in the absence of PGC1 α . However, cyclin D1 reduced PGC1 α -mediated activation of both promoters by at least 50%.

Although our experiments with the CDK4 inhibitor PD0332991 supports the role for CDK4 mediating the effects of cyclin D1, we wanted to further determine if CDK4 activity is responsible for the repression of PGC1 α by cyclin D1. We carried out promoter luciferase studies using a mutant of cyclin D1 with a lysine to glutamic acid mutation at amino acid 112 (cyclin D1^{K112E}) (31,32). This mutant can bind CDK4 and CDK6, but it is unable to activate their kinase activity. Wild-type cyclin D1 repressed PGC1 α activation of the PEPCK and G6Pase promoters (Supplementary Fig. 5A and B). However, not only did the mutant cyclin D1 not reduce PGC1 α -mediated activation of the PEPCK and G6Pase promoters, but it actually increased the activity of PGC1 α . This is in line with the ability of the cyclin D1^{K112E} mutant to reduce endogenous CDK4 activity and further suggests that CDK4 kinase activity is required for the activity of cyclin D1 to repress PGC1 α .

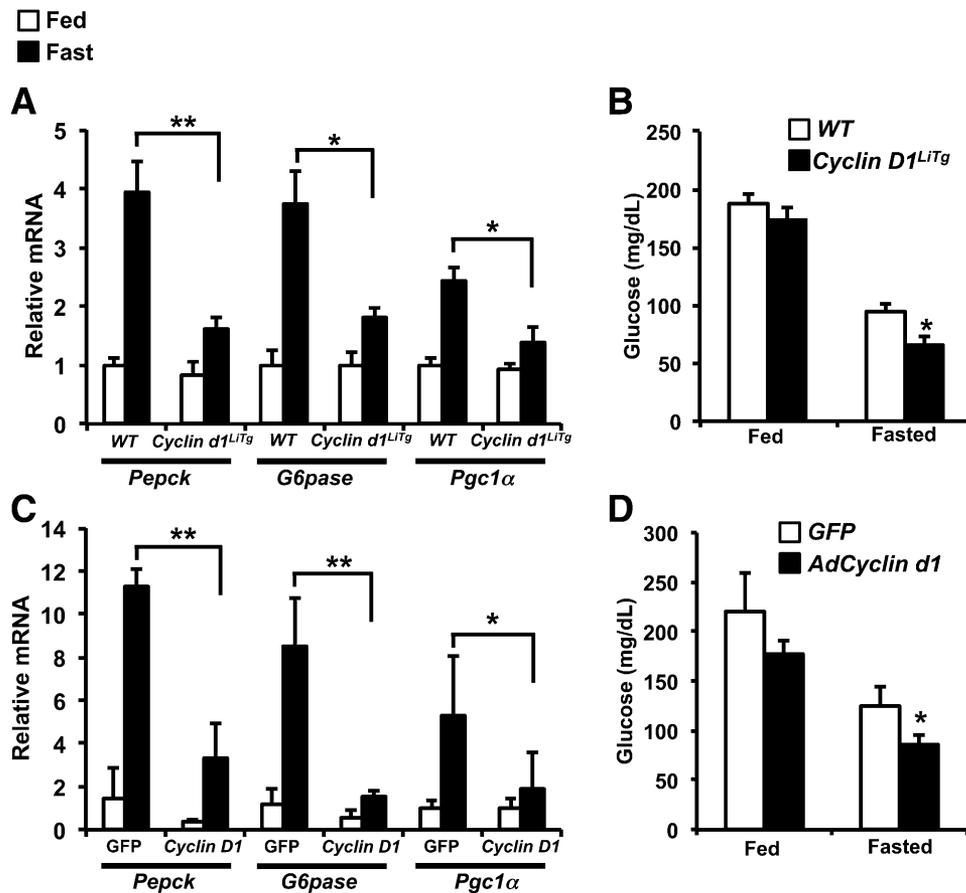


Figure 3—Liver-specific cyclin D1 expression reduces gluconeogenic gene expression during fasting. Wild-type mice and liver-specific *Cyclin d1^{LITg}* mice were either fed or fasted overnight. Glucose was measured, and then livers were harvested, RNA was isolated, cDNA was synthesized, and RT-PCR was performed. **A:** Expression of *Pepck*, *G6pase*, and *Pgc1α* RNA from livers of fed and fasted mice. **B:** Blood glucose; $n = 4 \pm$ SD. Adenovirus expressing GFP or cyclin D1 was injected via tail vein into mice. **C:** After 48 h, mice were fed or fasted for 6 h, blood glucose was taken, livers were removed, and RNA was isolated for *Pepck*, *G6pase*, or *Pgc1α* gene expression. **D:** Fed and fasting glucose levels. * $P < 0.05$; ** $P < 0.0001$. Not significant, $P > 0.05$. WT, wild type.

The data above also show that cyclin D1 suppresses OxPhos gene expression. The ability of PGC1 α to promote the expression of OxPhos genes is one of its most well-studied functions. Therefore, we wanted to determine whether cyclin D1 represses PGC1 α transcriptional activity on the OxPhos gene promoters that it controls. We used the 5' upstream promoter region of the cytochrome C (*Cyt-C*) gene linked to a luciferase reporter construct (33). PGC1 α significantly increased *Cyt-C* promoter activity, whereas cyclin D1 on its own had little effect on the *Cyt-C* promoter (Fig. 4D). However, the ability of PGC1 α to activate the *Cyt-C* promoter was reduced significantly in the presence of cyclin D1. Next we used a multimerized NRF1 response element (NRF1 \times 4) to determine whether cyclin D1 affected the ability of PGC1 α to coactivate endogenous NRF1 (34). NRF1 is coactivated by PGC1 α and is one of the main transcription factors driving OxPhos gene expression (35). PGC1 α alone increased NRF1 \times 4 activity approximately threefold (Fig. 4E). Cyclin D1 by itself had little effect on the activity of the NRF1 \times 4 luciferase construct, indicating that in this

system, cyclin D1 is not directly suppressing NRF1 activity. In contrast, PGC1 α -induced NRF1 \times 4 luciferase activity was reduced by cyclin D1.

Several different cyclins regulate the cell cycle, so we wanted to determine whether the effect of cyclin D1 was specific for D-type cyclins or also other cell cycle-related cyclins. Cyclin E controls the entry of cells from G₁ into S phase. Cells were transfected with the cytochrome C or NRF1 \times 4 luciferase constructs along with PGC1 α in the absence and presence of cyclin D1 or cyclin E. While cyclin D1 repressed PGC1 α activity, cyclin E had no effect on PGC1 α activity on either luciferase promoter (Supplementary Fig. 6A and B). These data demonstrate the specificity and ability of cyclin D1 to repress PGC1 α activity.

Results from the cyclin D1 knockout studies above show that loss of cyclin D1 is associated with increased *Pgc1α* expression. PGC1 α is part of an autoregulatory loop whereby PGC1 α induces its own expression (36). We used a 10-Kb fragment of the PGC1 α promoter linked to luciferase (PGC1 α -10Kb luciferase) to examine the role of cyclin D1 on induction of PGC1 α . Expression of PGC1 α

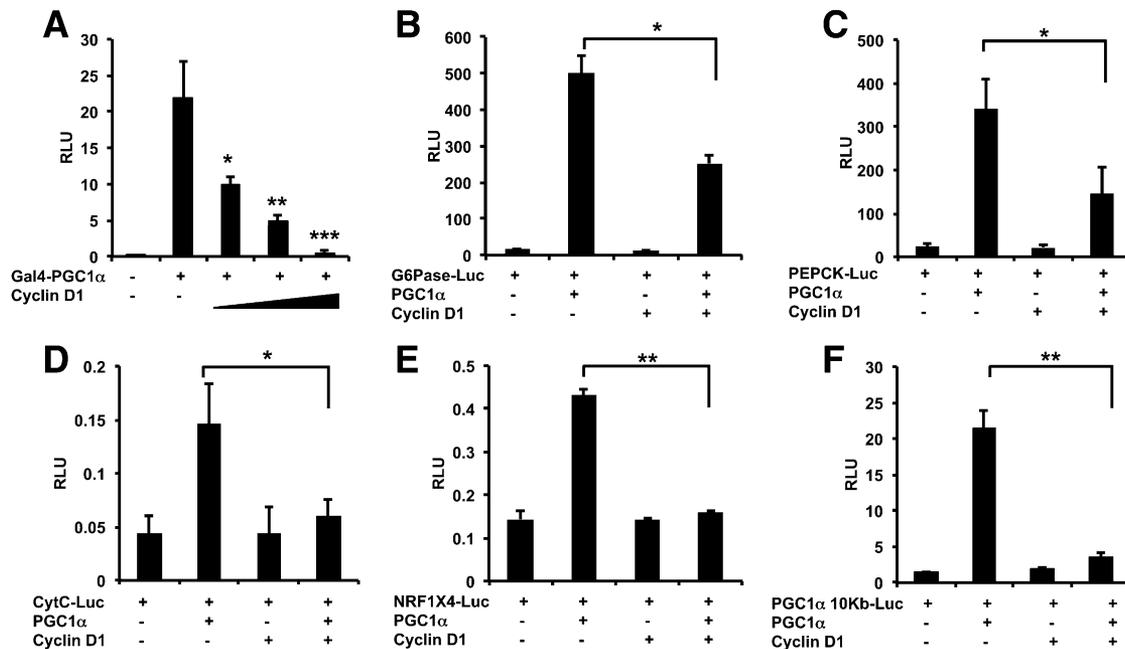


Figure 4—Cyclin D1 reduces the transcriptional activity of PGC1 α . **A:** Cos7 cells were transfected with 100 ng of UAS-TATA luciferase construct and 50 ng of Gal4-PGC1 α and increasing amounts of cyclin D1 expression construct (100–500 ng). Cells were harvested after 20 h, and luciferase activity was measured as described in RESEARCH DESIGN AND METHODS. Cos7 cells were transfected with (B) 100 ng HNF4 and 100 ng G6Pase promoter luciferase construct, (C) 100 ng HNF4 and 100 ng PEPCK promoter luciferase construct, (D) 50 ng of cytochrome C promoter luciferase construct, (E) 100 ng of multimerized NRF1 binding site (NRF1 \times 4) luciferase constructs, or (F) 100 ng of 5' 10-Kb promoter of PGC1 α . For all experiments, cells were also transfected with 250 ng of PGC1 α and 250 ng of cyclin D1 expression constructs as indicated and described in RESEARCH DESIGN AND METHODS. Cells were harvested after 20 h, and luciferase activity was measured. $n = 3 \pm$ SD. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$. Luc, luciferase; RLU, relative luciferase units.

cDNA increased the PGC1 α promoter activity several fold (Fig. 4F), supporting the autoregulatory role of PGC1 α . Cyclin D1 on its own had little effect on basal promoter activity. However, cyclin D1 significantly blocked the ability of PGC1 α to activate its own promoter in a dose-dependent manner, which shows that cyclin D1 decreases PGC1 α expression, in part, by inhibiting PGC1 α activity.

The promoter luciferase data strongly suggest that effects of cyclin D1 are mediated in part via effects on PGC1 α . Therefore, we wanted to determine whether cyclin D1 could directly affect PGC1 α activity on endogenous gluconeogenic and OxPhos gene expression. We ectopically expressed PGC1 α in Huh7 and HepG2 liver-derived cancer cell lines in the presence and absence of PD0332991 and examined the ability of PGC1 α to induce gluconeogenic gene expression. PGC1 α induced PEPCK in both cell lines (Fig. 5A and B). PD0332991 significantly enhanced the PGC1 α -mediated induction of PEPCK and G6Pase in both cell lines. G6Pase was induced robustly by PGC1 α in both cell lines. This induction was further potentiated by PD0332991, further suggesting that PGC1 α is the target of cyclin D1/CDK4. We also examined whether inhibition of CDK4 by PD0332991 could increase PGC1 α -mediated induction of OxPhos gene expression. PGC1 α induced Cyt-C and Atp5b1 in the liver-derived cancer cell lines (Supplementary Fig. 7A and B). In the presence of PD0332991, the induction by PGC1 α was significantly increased.

Next we examined the effect of cyclin D1 on PGC1 α activity in primary hepatocytes by ectopically expressing cyclin D1 and PGC1 α adenovirus. *Pepck* and *G6pase* expression were induced robustly by PGC1 α (Fig. 5C). The *Pepck* and *G6pase* induction were blunted 40 and 80% in the presence of cyclin D1, respectively. In order to confirm that the effect was mediated via CDK4, we examined the effect of cyclin D1 on PGC1 α in the presence of the CDK4 inhibitor PD0332991. PD0332991 increased PGC1 α -mediated induction of *Pepck* and *G6pase* \sim 50–60% (Fig. 5D). Moreover, repression of PGC1 α by cyclin D1 was significantly reduced by PD0332991.

We also used *Cdk4*^{+/+} and *Cdk4*^{-/-} mouse embryonic fibroblasts (MEFs) to further confirm the role of CDK4 since even specific inhibitors can have off-target effects. For these studies, we focused on OxPhos genes since MEFs express little to no PEPCK or G6Pase (Supplementary Fig. 8). Cells were infected with retrovirus expressing a control vector or PGC1 α , and gene expression was examined. PGC1 α alone induced *Cyt-c* and *Atp5b1* \sim 25 and 50% in *CDK4*^{+/+} MEFs. While there was a trend toward increased *Cox4i* and *Cox5b* induction, it was not significant. However, in *CDK4*^{-/-} MEFs, PGC1 α significantly induced all four OxPhos genes (Fig. 6A–D). This demonstrates genetically that CDK4 is regulating the activity of PGC1 α . Since MEFs do not express appreciable amounts of gluconeogenic genes, we also knocked

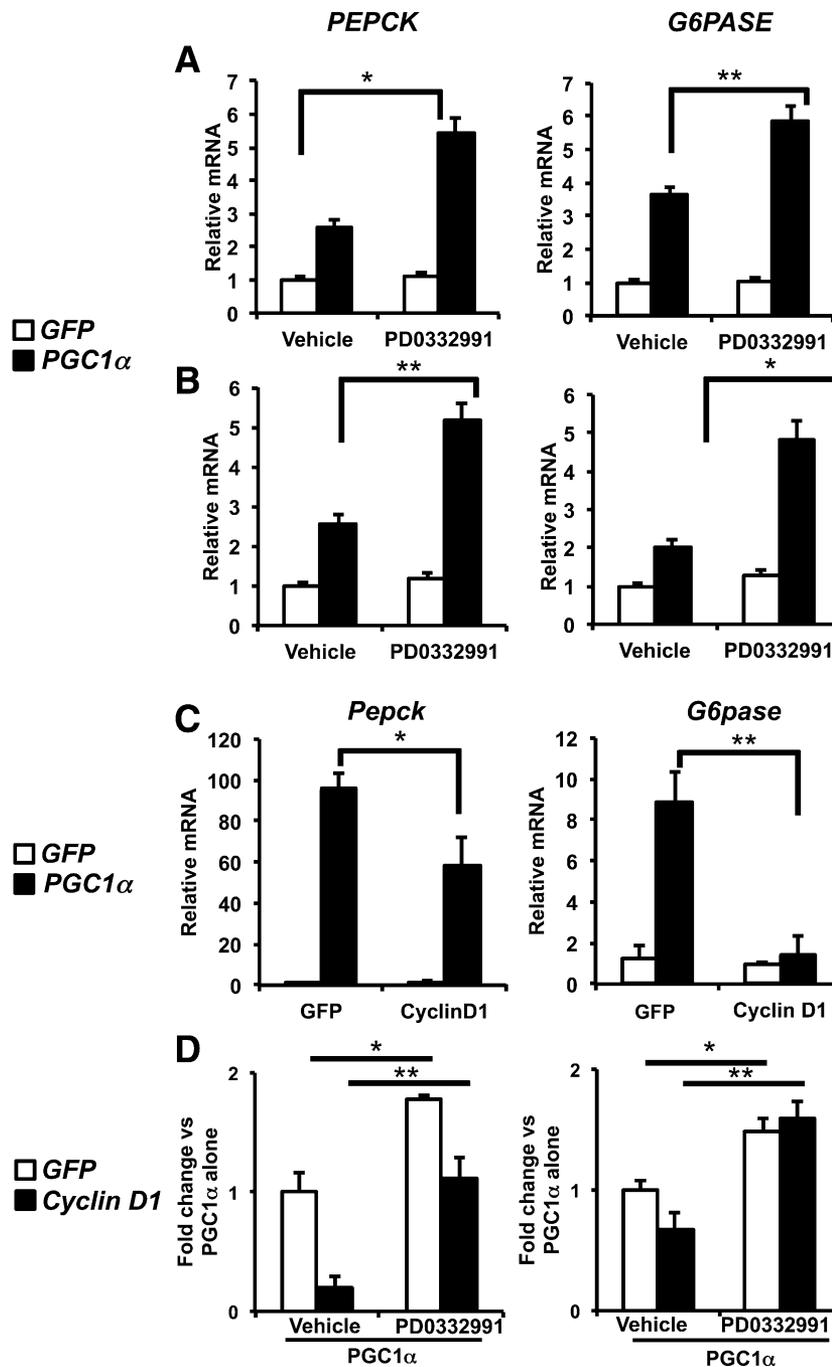


Figure 5—Pharmacological inhibition of CDK4 increases PGC1 α transcriptional activity. *A*: HepG2 or *B*: Huh7 cells were infected with GFP control or PGC1 α adenovirus and then treated with 1 μ M PD0332991 for 20 h. *C*: Primary hepatocytes were infected with GFP control, PGC1 α , cyclin D1, or cyclin D1 and PGC1 α adenovirus. *D*: Primary hepatocytes were infected with adenoviral PGC1 α in the absence and presence of cyclin D1 and treated with PD0332991 for 20 h. Following treatment, RNA was isolated, cDNA was synthesized, and RT-PCR was performed for *Pepck* or *G6pase*. $n = 3 \pm$ SD. * $P < 0.05$; ** $P < 0.005$.

down CDK4 in Huh7 cells (Supplementary Fig. 9). While PGC1 α induced PEPCK and G6Pase expression by itself, knockdown of CDK4 resulted in increased induction of PEPCK and G6Pase (Fig. 6E and F). These inhibitor and genetic loss of function data strongly suggest that CDK4 is, in part, responsible for the effects of cyclin D1 on PGC1 α .

Next we wanted to determine whether cyclin D1/CDK4 are regulating gene expression via PGC1 α in vivo. We again used the CDK4 inhibitor PD0332991 in PGC1 α liver-specific knockout (*Pgc1 α ^{LKO}*) mice. *Pgc1 α ^{LKO}* and wild-type (*Pgc1 α ^{WT}*) mice were fasted overnight and then administered vehicle control or PD0332991 by intraperitoneal injection and refed for 4 h. Previously (Fig.

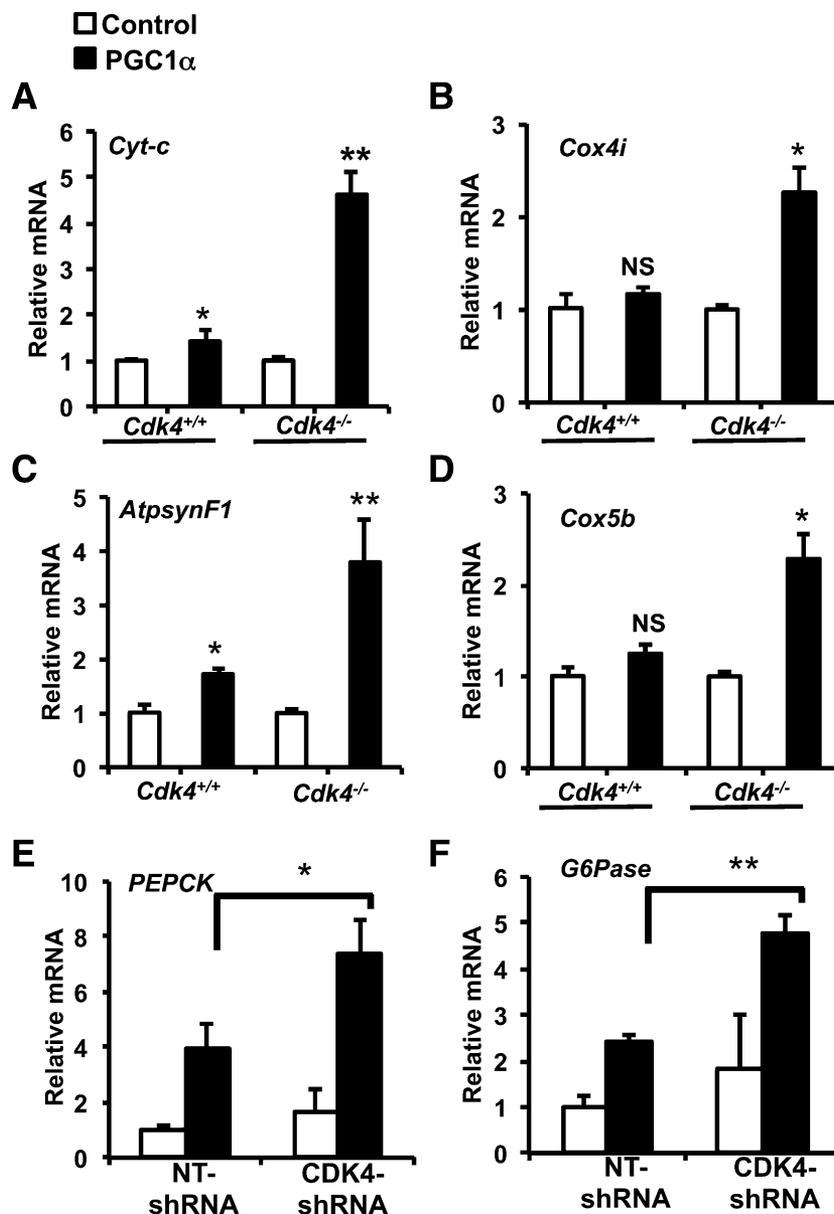


Figure 6—Genetic loss of CDK4 increases PGC1 α transcriptional activity. Loss of CDK4 increases PGC1 α activity. A–D: MEFs were isolated from *Cdk4*^{+/+} or *Cdk4*^{-/-} 13-day-old embryos as described in RESEARCH DESIGN AND METHODS. MEFs were infected with GFP- or PGC1 α -expressing adenovirus for 24 h, RNA was isolated, cDNA was synthesized, and RT-PCR was performed. E and F: Short hairpin RNA-mediated knockdown of CDK4 increases PGC1 α activity in Huh7 cells. Short hairpin RNA against CDK4 (validated/Addgene) was transfected into Huh7 cells for 48 h, and then cells were infected with PGC1 α adenovirus for 24 h. RNA was isolated, and RT-PCR was run for (E) PEPCK and (F) G6Pase. *n* = 3 \pm SD. **P* < 0.05; ***P* < 0.005. NT, nontarget; shRNA, short hairpin RNA.

2), we saw that inhibition of CDK4 in wild-type mice blocked the inhibitory effects of refeeding on gluconeogenic and OxPhos gene expression. In *Pgc1 α* ^{LKO} mice, we did not observe a significant increase in *Pepck* or *G6pase* expression (Fig. 7A and B) (*P* > 0.05). Similarly, we did not observe an increase in OxPhos gene expression (Fig. 7C–E) (*P* > 0.05). These data demonstrate that the effects of cyclin D1 on gluconeogenic and OxPhos gene expression are, in large part, mediated via PGC1 α .

Our in vitro and in vivo pharmacological and genetic loss of function data strongly suggest that cyclin

D1/CDK4 mediate their effects through PGC1 α . Although most kinases have multiple substrates, only a handful of substrates have been identified for CDK4 (37). Therefore, we carried out in CDK4/cyclin D1 in vitro kinase assays on purified full-length PGC1 α . PGC1 α was effectively phosphorylated by cyclin D1/CDK4 (Fig. 8A). We then performed in silico analysis to determine the potential phosphorylation sites on PGC1 α . Two highly conserved sites were identified in the transcriptional repression domain, threonine 298 and serine 312 (Fig. 8B). Next we mutated the potential sites to alanine (PGC1 α ^{AA}) and

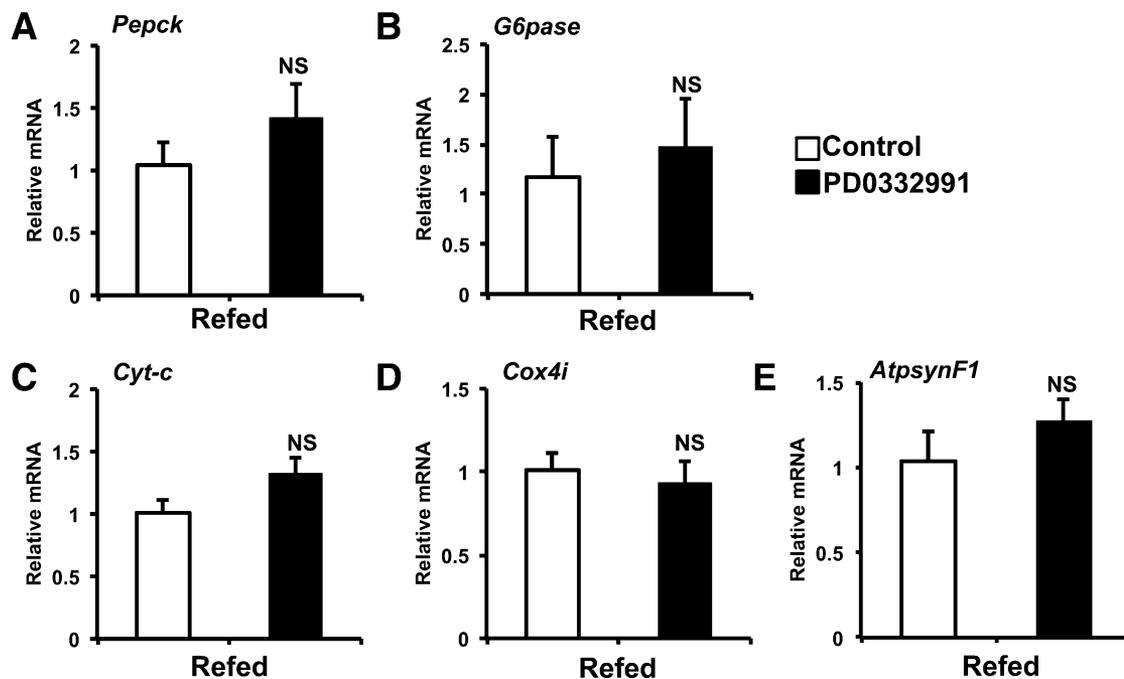


Figure 7—Cyclin D1 mediates its effects on gene expression in vivo via PGC1 α . *Pgc1 α* liver-specific knockout mice were fasted overnight and then treated with vehicle control or 75 mg/kg PD0332991 for 4 h. Livers were harvested, RNA was isolated, and RT-PCR was performed as indicated for *Pepck*, *G6pase*, *Cyt-c*, *Cox4i*, *AtpsynF1*. $n = 4\text{--}5 \pm$ SD. NS, not statistically significant.

conducted reporter luciferase experiments using the PEPCK, G6Pase, and Cyt-C luciferase promoters (Fig. 8C–E). As expected, cyclin D1 reduced the activity of PGC1 α^{WT} on all three promoters. However, cyclin D1 had little to no effect on the activity of the PGC1 α^{AA} mutant. These data identify PGC1 α as a novel CDK4 substrate and further confirm that cyclin D1/CDK4 repress PGC1 α .

DISCUSSION

Research on cyclin D1 has focused mainly on its ability to regulate the cell cycle and cancer. Cyclin D1 is expressed in the liver, a tissue that is relatively quiescent, raising the question as to the role of cyclin D1 in the liver. We describe here a novel metabolic role for cyclin D1, whereby cyclin D1 represses gluconeogenesis (Fig. 8F). Fasting reduces the expression of cyclin D1, leading to reduced CDK4 activity and hence increased PGC1 α activity. By contrast, feeding induces cyclin D1, causing the activation of CDK4, phosphorylation of PGC1 α , and repression of gluconeogenic gene expression.

PGC1 α plays a key role in gluconeogenesis by coactivating various transcription factors, stimulating the expression of genes involved in gluconeogenesis such as PEPCK and G6Pase. The ability of PGC1 α to induce gene-expression programs of OxPhos and gluconeogenesis is well established. It is metabolically advantageous for the liver to induce both of these programs of gene expression. The liver will use OxPhos to generate ATP so that it can generate glucose for the rest of the body via

gluconeogenesis and maintain its own bioenergetics. The repression of gluconeogenesis and OxPhos gene expression by cyclin D1 in our studies was lost in mice lacking liver PGC1 α , strongly suggesting that the repressive effect of cyclin D1 is via PGC1 α . Therefore, our data demonstrate another potential mechanism whereby feeding induces cyclin D1, which leads to inactivation of PGC1 α and its ability to promote gluconeogenesis and oxidative metabolism.

In addition to its effects on the cell cycle, cyclin D1 controls transcription by interacting with transcription factors, coactivators, and corepressors, promoting or repressing gene expression depending on the setting (14–20). The ability of cyclin D1 to regulate transcription was highlighted in a recent study that showed cyclin D1 bound to the promoters of hundreds of genes (21). A role for cyclin D1 in metabolism was suggested several years ago in studies showing that loss of cyclin D1 increased mitochondrial biogenesis (22,23). Furthermore a recent study showed that cyclin D1 inhibited liver lipogenesis, in part, by inhibiting HNF4 and ChREBP in a CDK4-dependent and CDK4-independent manner (24). Interestingly, in our studies, cyclin D1 repressed coactivation of HNF4 by PGC1 α but failed to repress HNF4 on its own (Fig. 4B and C, column 3). We also showed that cyclin D1 does not repress gene expression or promoter activity controlled by other transcription factors, such as NRF1, in the absence of PGC1 α . Therefore, PGC1 α may be required for the repression of HNF4 observed in previous studies, or alternatively, the target of cyclin

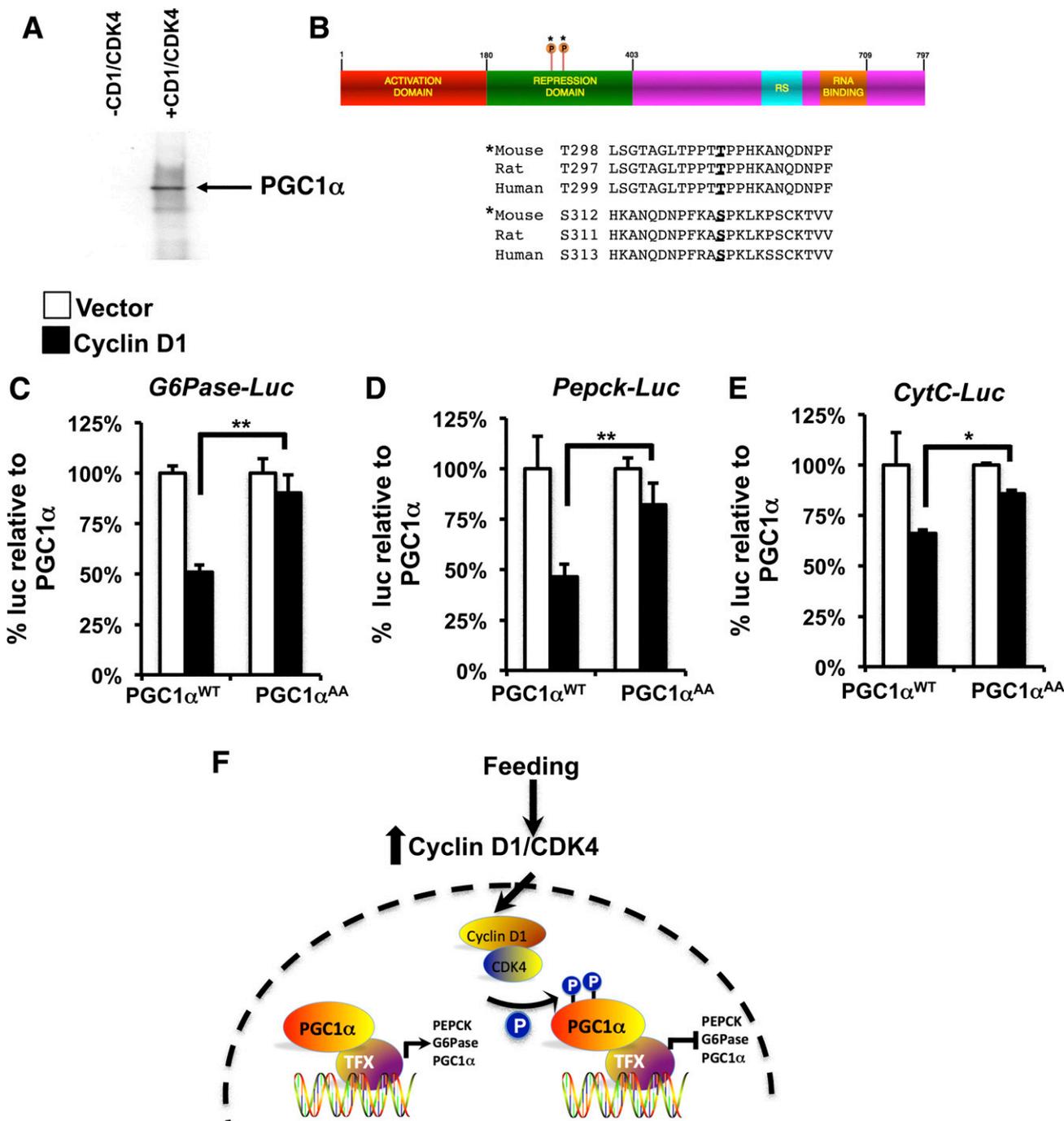


Figure 8—PGC1 α is a substrate for cyclin D1/CDK4. **A**: In vitro phosphorylation of PGC1 α by using GST-PGC1 α expressed in bacteria and purified cyclin D1/CDK4 and ³²P ATP. **B**: Schematic of cyclin D1/CDK4 sites in PGC1 α identified by using Scansite. Promoter luciferase activity for **(C)** G6Pase promoter reporter, **(D)** Pepck promoter reporter, and **(E)** CytC promoter reporter. Cells were transfected with luciferase reporter constructs along with wild-type PGC1 α or PGC1 α ^{T298A/S312A} (PGC1 α ^{AA}) in the presence and absence of cyclin D1. Renilla luciferase was used as a transfection control; *n* = 3 \pm SD. **F**: Schematic of cyclin D1-mediated effects on PGC1 α activity and expression. Fasting represses cyclin D1, leading to reduced CDK4 activity, enabling PGC1 α to exert full coactivation activity, inducing the gluconeogenic gene-expression program. Feeding induces cyclin D1, activating CDK4, leading to phosphorylation of PGC1 α and reduced transcriptional coactivation and the gluconeogenic gene-expression program. **P* < 0.05; ***P* < 0.001. Luc, luciferase; TFX, transcription factor; WT, wild type.

D1-mediated repression may be both gene specific and cell-type dependent.

Cell-cycle control by cyclin D1 is mostly dependent upon CDK4. However, its regulation of transcription has

been shown to be both CDK4 dependent and independent. Our studies strongly suggest a CDK4-dependent effect. A mutant of cyclin D1 that can bind but fails to activate CDK4 did not repress PGC1 α and actually

increased its activity. This is most likely due to the fact that the mutant of cyclin D1 interacts with but does not activate CDK4, thereby reducing the available active CDK4 (31,32). Finally, only a handful of CDK4 substrates have been identified to date (13,37,38). Our data strongly suggest that PGC1 α is a substrate for CDK4. In support of this analysis, recently, a screening assay suggested that PGC1 α is a potential CDK4 substrate (37). Although we cannot completely rule out CDK4-independent effects, our data strongly suggest that CDK4 mediates the effects of cyclin D1 on PGC1 α .

These studies have implications beyond the response to fasting and gluconeogenesis. The Warburg effect describes a metabolic alteration where cancer cells display increased glycolytic metabolism and reduced OxPhos with concomitant production of ATP and lactic acid even in the presence of oxygen. In recent years, this phenomenon has been shown to be a prominent feature in cancer. Several years ago, it was shown that PGC1 α expression is reduced in different cancers, including the liver, compared with normal adjacent tissues (39). Since cyclin D1 overexpression represents an early lesion in cancer (40), it is tempting to speculate that downregulation of PGC1 α activity by cyclin D1 is a potential mechanism responsible, in part, for the Warburg effect and the downregulation of OxPhos observed in cancer. Indeed, the cyclin D1 liver transgenic mice used in these studies develop hepatocellular carcinoma in ~15 months (30).

Cyclin D1 is most well-known for regulating the cell cycle, raising the question as to why cyclin D1 is expressed in a quiescent tissue like the liver. The ability of a cell-cycle regulator to regulate glucose homeostasis would be advantageous for a cell. Progression through the cell cycle and cell division require abundant amounts of nutrients. In contrast, gluconeogenesis is a state of nutrient deprivation. Therefore, it makes sense that a molecule that controls cell-cycle progression would also play a role in glucose homeostasis. Therefore, cyclin D1 activation represents a mechanism that links the cell cycle with nutrient homeostasis and sensing via its interaction with PGC1 α and explains the presence of a cell-cycle regulator in a quiescent tissue. Finally, our data show that livers from obese patients with type 2 diabetes express less cyclin D1 mRNA than their nondiabetic counterparts, although caution should be taken given the procancer role of cyclin hence as a therapeutic target.

RESEARCH DESIGN AND METHODS

Cell Culture and Treatments

HepG2 and Cos7 cells were obtained from ATCC. Huh7 cells originated from the Japanese Collection of Research Bioresources. All cells were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin. PD0332991 was obtained from Selleckchem. Cells were seeded into six-well plates and treated 24 h later as indicated in the figure legends.

Microarray Analysis of Published Data Set

Normalized data and probe annotations for GSE15653 and GSE24504 were downloaded from the NCBI GEO website. Data were used for reanalysis. Time points (0, 12, 24, and 48 h) were used as factor in one-way ANOVA analysis, and significant probes were selected by false discovery rate ≤ 0.1 .

Animal Experiments

For all experiments, male mice were used between 8–12 weeks of age. Wild-type C57Bl/6J, cyclin D1 liver-specific transgenic (*Cyclin d1*^{LiTG}), PGC1 α floxed, and albumin Cre mice were obtained from The Jackson Laboratory and previously described (30). *Cyclin d1*^{+/+} and *Cyclin d1*^{-/-} mice have been described previously (41,42). Mice flanked by LoxP sites at the PGC1 α allele (PGC1 α ^{fl/fl}) were bred to Alb-Cre mice to generate liver-specific PGC1 α knockout mice (*Pgc1 α* ^{LKO}). We did not observe a difference in weight of cyclin D1Tg or PGC1 liver knockout mice compared with control mice at these time points, similar to previous studies (30,43). For fasting experiments, mice were either fed or fasted for 6 h or overnight (16 h) as previously described (44). For refeeding experiments, mice were refed for 4 h. For experiments with PD0332991, mice were administered 75 mg/kg of PD0332991 or vehicle following overnight fasting and then refed for 4 h. Following the described time points, mice were killed, and the livers removed for RNA analysis. Blood glucose was measured following fasting, as well as following refeeding in control and PD0332991-treated mice as previously described (44). *Cdk4*^{+/+} and *Cdk4*^{-/-} MEFs were generated by the Sicinski Laboratory (45). In brief, *Cdk4*^{+/+} and *Cdk4*^{-/-} day 13.5 embryos were harvested, internal organs were removed, and remaining tissues were minced and digested. Tissue was resuspended by pipetting in DMEM supplemented with 10% FBS and plated out in T75 flasks. Once the cells reached confluence, they were trypsinized and passaged at 1:4. Cells were cultured in DMEM plus 10% FBS. For most of the experiments, cells from passages 1 to 2 were used.

RNA Isolation and Measurement of Gene Expression

RNA from cells and tissue was isolated by using TRIzol reagent (Invitrogen), and RNA was reverse transcribed as previously described (46). RT-PCR was performed using human- or mouse-specific primers (Supplementary Table 1).

Transfections

Gal4-PGC1 α , UAS-TATA luciferase, G6Pase luciferase, PEPCK luciferase, PGC1 α -10Kb promoter luciferase, Cyt-C luciferase, NRF1 \times 4 luciferase, PGC1 α , cyclin D1, and cyclin D1^{K112E} have been described previously and were obtained from Addgene (Cambridge, MA) (33,34,36,44). pRL-SV40 renilla luciferase was used as a transfection control (Promega). Cos7 cells were transfected with DNA as indicated in the figure legends by using SuperFect or Attractene for 3 h. Cell extracts were prepared and

luciferase activity was measured as previously described (47). Mutation of T298 and S312 of PGC1 α was accomplished using site-directed mutagenesis. The resulting product was sequenced for fidelity and subcloned into pCDNA3.1.

Adenovirus Experiments

GFP and PGC1 α adenovirus were prepared as previously described (47). HepG2, Huh7, *Cdk4*^{+/+} and *Cdk4*^{-/-} MEFs were infected with GFP- or PGC1 α -expressing adenovirus. Media were changed after 6 h. For MEFs, RNA was harvested as described above 24 h later, and RT-PCR was performed as indicated. HepG2 and Huh7 cells were treated with 1 μ mol/L PD0332991 overnight, RNA was isolated, and RT-PCR was performed.

Primary Hepatocyte Isolation

Mouse primary hepatocytes were prepared by perfusing C57Bl/6J mice with liver digest medium (Invitrogen) followed by filtration through a 70 μ mol/L mesh and Percoll gradient centrifugation. Cells were seeded at a density of 4×10^5 cells/well in six-well plates in DMEM containing 10% FBS, 2 mmol/L sodium pyruvate, 2% penicillin/streptomycin, 1 μ mol/L dexamethasone, and 0.1 μ mol/L insulin. After 4 h, cells were switched to 0.2% BSA, 2 mmol/L sodium pyruvate, 2% penicillin/streptomycin, 0.1 μ mol/L dexamethasone, and 1 nmol/L insulin (maintenance medium). Cells were infected with adenovirus as indicated and RNA harvested for gene expression.

In Vitro Kinase Reactions

Glutathione S-transferase (GST) substrate proteins were expressed in *Escherichia coli* and purified as previously described (37). Briefly, GST fusion proteins were eluted from the glutathione resin in 100 mmol/L HEPES, 100 mmol/L NaCl, 0.2% Tween 20, 20 mmol/L glutathione, and 2.5 mmol/L 1,4-dithiothreitol. Then 10 μ L of the eluate was mixed with 10 μ L of complementation buffer (100 mmol/L HEPES, 20 mmol/L MgCl₂, 2 mmol/L EGTA; pH 7.4). Samples were supplemented with 10 μ Ci γ -³²P-ATP (PerkinElmer, Waltham, MA) and 40 μ mol/L ATP followed by addition of 0.5 μ L recombinant cyclin D1-CDK4 (ProQinase, Freiburg, Germany). Reactions were run at 30°C for 20 min and terminated by adding 10 μ L of Laemmli buffer. Samples were loaded on SDS gels and transferred to nitrocellulose membranes, followed by staining with Amido black 10B protein stain. Stained membranes were dried and exposed to X-ray film.

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performed experiments and contributed research data. S.B. contributed research data. S.G.D. contributed research data, contributed to discussion, and reviewed the manuscript. Y.S. contributed research data and reviewed the manuscript. P.S. contributed reagents, contributed to discussion, and reviewed the manuscript. G.D.G. designed and performed experiments, contributed research data, contributed to discussion, and wrote the manuscript. G.D.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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