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Cyclin D1 inhibits hepatic lipogenesis via repression of carbohydrate response element binding protein and hepatocyte nuclear factor 4α

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Abbreviations: ADV, adenovirus(es); AR, androgen receptor; CBP, CREB-binding protein; cdk, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; ChREBP, carbohydrate response element binding protein; HNF4α, hepatocyte nuclear factor 4 alpha; PH, partial hepatectomy; PPARγ, peroxisome proliferator-activated receptor γ; TRβ, thyroid hormone receptor β

Following acute hepatic injury, the metabolic capacity of the liver is altered during the process of compensatory hepatocyte proliferation by undefined mechanisms. In this study, we examined the regulation of de novo lipogenesis by cyclin D1, a key mediator of hepatocyte cell cycle progression. In primary hepatocytes, cyclin D1 significantly impaired lipogenesis in response to glucose stimulation. Cyclin D1 inhibited the glucose-mediated induction of key lipogenic genes, and similar effects were seen using a mutant (D1-KE) that does not activate cdk4 or induce cell cycle progression. Cyclin D1 (but not D1-KE) inhibited the activity of the carbohydrate response element-binding protein (ChREBP) by regulating the glucose-sensing motif of this transcription factor. Because changes in ChREBP activity could not fully explain the effect of cyclin D1, we examined hepatocyte nuclear factor 4α (HNF4α), which regulates numerous differentiated functions in the liver including lipid metabolism. We found that both cyclins D1 and D1-KE bound to HNF4α and significantly inhibited its recruitment to the promoter region of lipogenic genes in hepatocytes. Conversely, knockdown of cyclin D1 in the AML12 hepatocyte cell line promoted HNF4α activity and lipogenesis. In mouse liver, HNF4α bound to a central domain of cyclin D1 involved in transcriptional repression. Cyclin D1 inhibited lipogenic gene expression in the liver following carbohydrate feeding. Similar findings were observed in the setting of physiologic cyclin D1 expression in the regenerating liver. In conclusion, these studies demonstrate that cyclin D1 represses ChREBP and HNF4α function in hepatocytes via Cdk4-dependent and -independent mechanisms. These findings provide a direct link between the cell cycle machinery and the transcriptional control of metabolic function of the liver.

Introduction

A primary function of the liver is to maintain systemic energy homeostasis through the metabolism of glucose and lipids. For example, in fed animals, excess dietary carbohydrates are converted into triglycerides in the liver through de novo lipogenesis, a process that is controlled by enzymes including liver-type pyruvate kinase (Pklr) and fatty acid synthase (Fasn). Hepatic lipogenesis is regulated primarily at the level of transcription in response to signals generated by glucose, insulin and other stimuli. Although the transcriptional control of lipogenesis has been extensively characterized, the regulation of this process in physiologic and pathologic states is still incompletely understood.

The differentiated functions of hepatocytes are maintained by a complex network of transcription factors. The most abundant liver-enriched transcription factor is hepatocyte nuclear factor 4α (HNF4α), a member of the nuclear receptor family, which plays a critical role in the development of differentiated hepatic function.[1] HNF4α-knockout mice die during embryogenesis and fail to develop functional livers. Deletion or knockdown of HNF4α in the liver of postnatal mice leads to altered expression of a number of different metabolic genes, particularly those related to lipid homeostasis.[2-4]

Hepatocytes have the capacity to induce lipogenesis in response to high glucose levels. The principle mediator of this response is the carbohydrate response element binding protein (ChREBP, gene name Mlxipl), a basic helix-loop-helix/leucine zipper transcription factor that is activated by high glucose concentrations and that promotes transcription of key lipogenic genes.[5,6] Although the precise mechanisms of ChREBP activation

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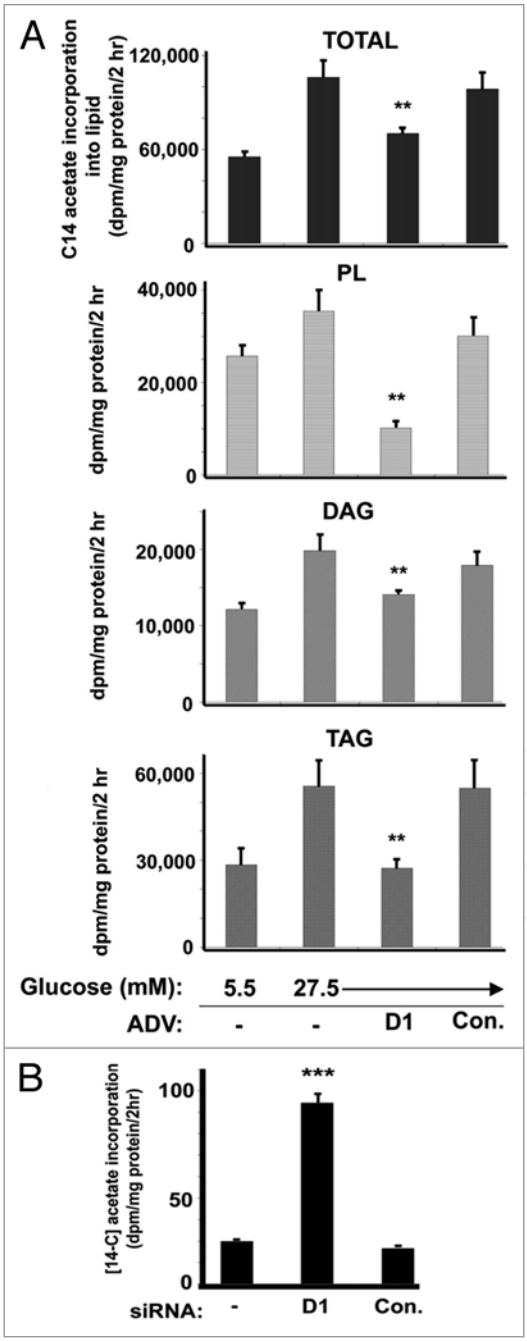

Figure 1. Cyclin D1 inhibits lipogenesis in hepatocytes. (A) Primary rat hepatocytes were cultured in low glucose (5.5 mM) conditions for 24 h, at which time medium was replaced with low or high (27.5 mM) glucose for an additional 24 h. Cells were transduced with adenoviruses (ADV) encoding cyclin D1 or GFP (control) as indicated. A low dose of insulin (1 nM) was used throughout the experiment. ^{14}C-acetate incorporation into total lipid, phospholipids, diacylglycerol or triacylglycerol was measured as described in the **Supplemental Materials and Methods**. (B) AML12 cells were cultured and treated with cyclin D1 or control siRNA as indicated. ^{14}C-acetate incorporation into total lipid was determined as above.

remain unsettled, glucose appears to regulate a motif in the N terminus of this protein.[7-9] ChREBP acts in concert with co-regulators such as the histone acetylases CREB binding protein (CBP) and p300 to induce the expression of genes involved in lipogenesis.[10,11]

Hepatocytes have a remarkable ability to undergo compensatory proliferation following acute or chronic liver injury, and this property is an important adaptive response in liver diseases.[12] In the standard model of liver regeneration, that of 70% partial hepatectomy (PH) in rodents, a large population of hepatocytes enter the cell cycle in a relatively synchronous manner in the first 1–2 d, and liver mass is restored within 1–2 weeks. The driving stimuli for hepatocyte replication are incompletely understood but include growth factors, hormones, nutrients and metabolic factors. The net effect of these pro-proliferative signals results in activation of the cell cycle machinery comprised of cyclin/cyclin-dependent kinase (cdk) complexes that regulate discrete phases of cell division. In many types of cells (including hepatocytes), induction of cyclin D1 in late G_1 phase appears to be a critical event in driving the cell cycle. Cyclin D1 complexes with its cdk partners (primarily cdk4) to phosphorylate the retinoblastoma protein (Rb) and drives cells through the G_1 restriction point, which, in general, commits the cell to proceed through cell division. Expression of cyclin D1 is sufficient to drive hepatocyte proliferation and liver growth, even under conditions that are normally inhibitory.[13] Importantly, deregulated expression of cyclin D1 contributes to autonomous cell cycle progression in many cancers, including hepatocellular carcinoma (HCC).[14,15]

In addition to its role in activating cdk4, cyclin D1 has been shown to control transcription in a cdk-independent manner.[16] For example, cyclin D1 binds and inhibits the transcriptional activity of several members of the nuclear receptor family, including the androgen receptor (AR), peroxisome proliferator-activated receptor γ (PPARγ) and thyroid hormone receptor β (TRβ). A central portion of cyclin D1 not involved in cdk activation, called the "repressor domain" (RD, amino acids 141–250), has been shown to be sufficient to inhibit AR and TRβ activity.[17] Furthermore, cyclin D1 has been shown to repress the p300 histone acetylase[18] and can either promote or inhibit CBP activity, depending on the target gene.[19,20] Additional evidence of a major transcriptional role of cyclin D1 has been provided by a recent study in developing mice, where this protein bound to the promoter of hundreds of genes.[19] Cyclin D1 may therefore participate in metabolic reprogramming to accommodate the energetic and synthetic demands of cell cycle progression and growth. However, the interaction of cyclin D1 with transcriptional regulatory proteins and the metabolic effects in specific tissues and conditions remains to be fully explored.[19,21]

In the current study, we examine the regulation of de novo lipogenesis in hepatocytes by cyclin D1. We find that cyclin D1 inhibits glucose-mediated lipogenesis, lipogenic gene expression and the activity of two key transcriptional factors involved in lipid metabolism, ChREBP and HNF4α. These data provide further insight into the complex interaction between the hepatocyte cell cycle and metabolic function of the liver. Furthermore, these studies offer additional evidence that cyclin D1 acts downstream of mitogenic signals to directly regulate both the cell cycle machinery and metabolism in the liver, a concept that may be highly relevant to its role in normal and malignant cells.

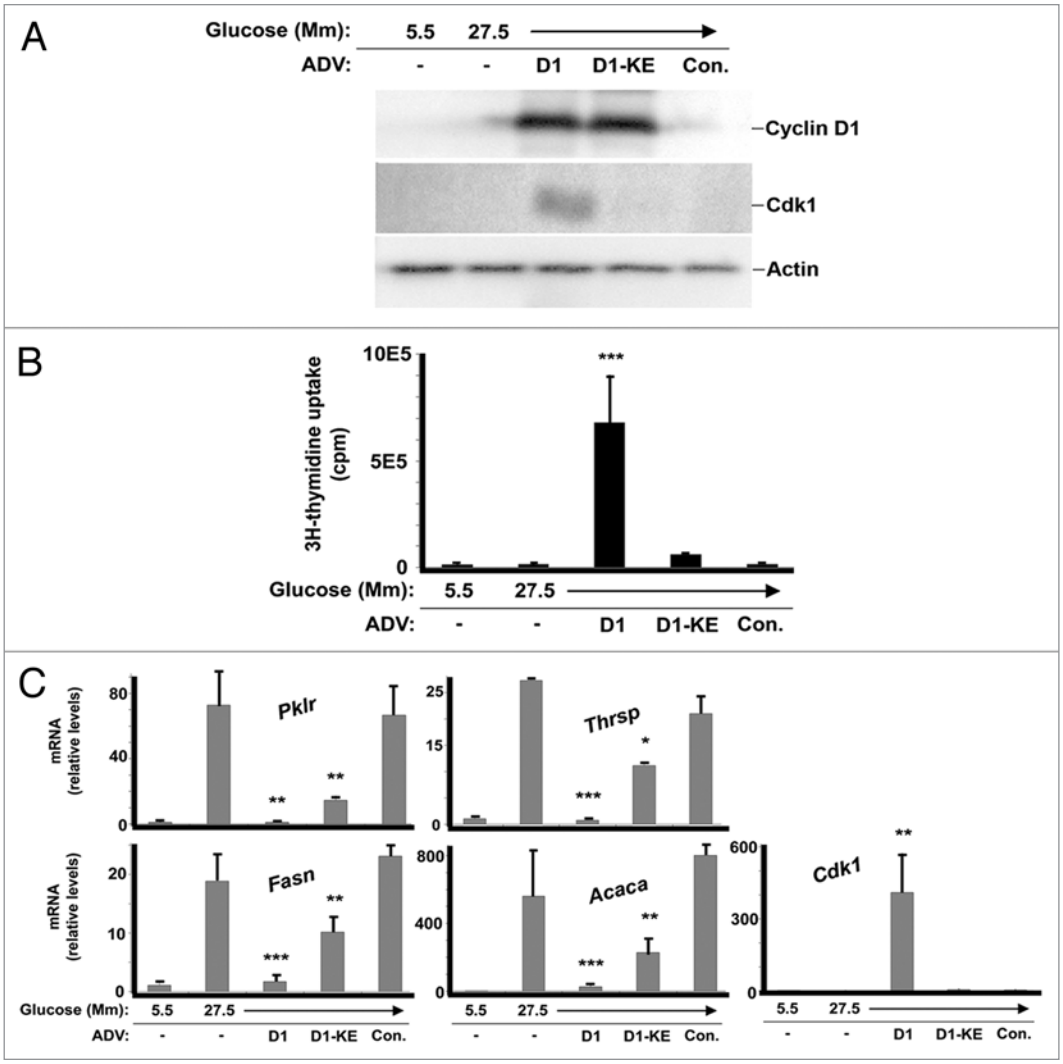

Figure 2. Cyclin D1 regulates lipogenic gene expression independently of cdk4 activation or proliferation. Hepatocytes were cultured for 48 h as in **Figure 1** and transduced with cyclin D1, cyclin D1-KE, or a control vector. (A) Western blot analysis of hepatocyte lysates for cyclin D1 and cdk1 (a marker of cell cycle progression). (B) DNA synthesis as measured by ^{3}H-thymidine incorporation. (C) Expression of the indicated lipogenic mRNA and cdk1 as measured by RT-PCR.

Results

In a prior study in mouse liver, we found that transduction with cyclin D1 using an adenoviral vector led to changes in gene expression that would predict altered lipogenesis.[22] To test this directly, we examined de novo lipogenesis in isolated primary rat hepatocytes (**Fig. 1A**). Cells were cultured in the presence of low concentrations of insulin (1 nM) and glucose (5.5 mM) for 24 h and then provided either low (5.5 mM) or high (27.5 mM) glucose media for the final 24 h. As expected, transition from a low to high concentration of glucose triggered increased de novo lipogenesis as measured by ^{14}C-acetate incorporation into total cellular lipids, phospholipids, diacylglycerol and triglycerides. Transduction of these cells with cyclin D1 substantially blunted the glucose-mediated induction of hepatocyte lipogenesis. Conversely, siRNA-mediated knockdown of cyclin D1 in the AML12 mouse hepatocyte cell line led to markedly increased lipogenesis (**Fig. 1B**). These studies demonstrate that cyclin D1 represses lipogenesis in hepatic cells.

We next investigated the effect of cyclin D1 on the expression of representative lipogenic genes that are upregulated by glucose in hepatocytes. In **Figure 2**, hepatocytes were transduced with either cyclin D1 or cyclin D1-KE, a point mutant that possesses a lysine-to-glutamine replacement in the cyclin box and does not activate cdk4 [23,24] (**Fig. S4**). As previously shown,[13] transduction of cyclin D1 induced proliferation (as evidenced by increased DNA synthesis and cdk1 expression), whereas cyclin D1-KE promoted minimal cell cycle progression (**Fig. 2A and B**). Prior studies have shown that expression of lipogenic genes including Pklr, Fasn, Acaca (acetyl-CoA carboxylase 1) and Thrsp (Spot 14) is significantly induced by a high concentration of glucose,[6] and similar results were noted in **Figure 2C**. Cyclin D1 substantially repressed the glucose-mediated induction of these transcripts. Cyclin D1-KE also significantly inhibited the expression of these

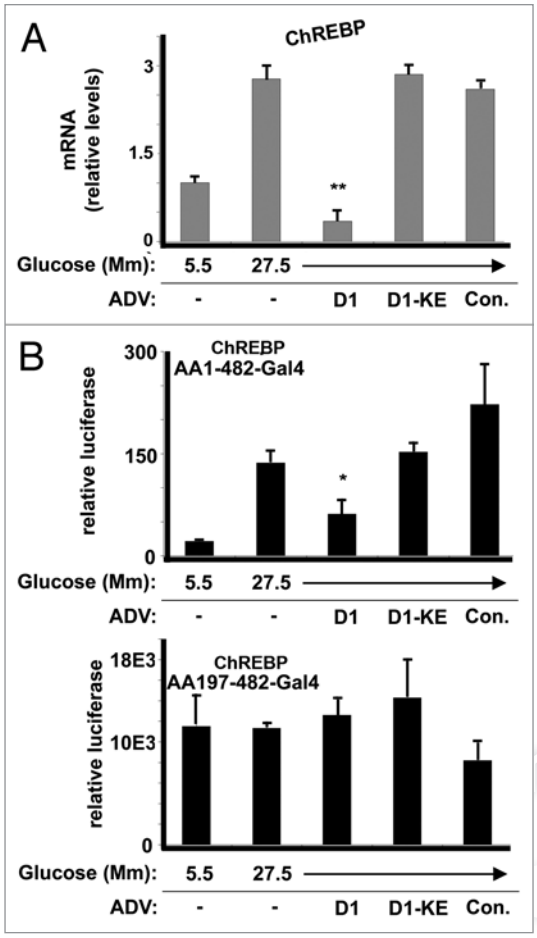

Figure 3. Cyclin D1 regulates ChREBP expression and activity in a cdk4-dependent fashion. (A) Hepatocytes were cultured as in **Figure 1**, and ChREBP mRNA expression was determined by RT-PCR. (B) Hepatocytes were co-transfected with expression plasmids containing Gal4-ChREBP amino acids 1–482 or 197–482, a reporter gene plasmid consisting of the firefly luciferase gene fused to a promoter region containing five copies of the Gal4 response element, and pRL-SV40. Firefly luciferase activity was normalized to Renilla activity.

genes, although the effect was diminished as compared with cyclin D1. In the presence of low glucose, cyclin D1 did not further reduce the low expression of Pklr and Fasn (**Fig. S2**). These data indicate that cyclin D1 inhibits the induction of lipogenic transcripts in response to glucose, and this effect is partly independent of cdk4 activation or cell cycle progression.

Since a principle mediator of glucose-mediated lipogenesis is ChREBP, we investigated whether this transcription factor was inhibited by cyclin D1. We were unable to detect ChREBP in hepatocytes using commercially available antibodies (data not shown), which limited our evaluation of the native protein. As previously shown,[6] transition from low- to high-glucose containing medium increased expression of the ChREBP mRNA (**Fig. 3A**). This induction was abrogated by cyclin D1 (**Fig. 3A**). This suggests that cyclin D1 inhibits the expression of ChREBP itself, which could impair its ability to activate genes in response to glucose. The downregulation of ChREBP mRNA levels by cyclin D1 was not recapitulated by cyclin D1-KE, suggesting that activation of cdk4 is required for this process. Hence, inhibition of ChREBP expression by cyclin D1 alone would not be sufficient to explain the effects of cyclin D1-KE on lipogenic gene expression seen in **Figure 2**.

We next inquired whether cyclin D1 affected the activity of ChREBP protein, which is modulated by glucose concentrations through incompletely identified mechanisms. To examine this, we employed an established assay using ChREBP constructs fused to the DNA-binding domain of Gal4 along with a plasmid containing a Gal4-responsive promoter linked to luciferase.[7,9] The use of constructs linked to Gal4 allowed us to study the effect of cyclin D1 on the transfected fusion proteins independently of endogenous ChREBP. Two ChREBP-Gal4 constructs were tested. The first coded for amino acids 1–482 of ChREBP and was highly responsive to glucose in pancreatic cells and hepatocytes. The second construct coded for amino acids 197–482 and showed very high constitutive activity regardless of glucose concentrations, because the glucose-sensing portion of ChREBP (amino acids 1–197) is removed. In **Figure 3B**, glucose induced the activity of the 1–482 ChREBP construct as previously shown, and this activity was diminished by cyclin D1 but not cyclin D1-KE. Cyclin D1 did not affect the activity of the constitutively active (197–482) ChREBP construct (**Fig. 3B**). These data suggest that cyclin D1 regulates a motif in the N-terminal portion (1–197) of ChREBP through a cdk4-dependent process. Importantly, both the changes in ChREBP mRNA levels and ChREBP activity in **Figure 3** were induced by cyclin D1 but not cyclin D1-KE, indicating that additional factors are responsible for the cdk4-independent effects.

To examine other mechanisms by which cyclin D1 may inhibit de novo lipid synthesis in hepatocytes, we examined HNF4α, because this transcription factor critically regulates many aspects of hepatic function, including lipogenesis and other facets of lipid metabolism.[2,3] Furthermore, HNF4α is a member of the nuclear receptor family, and cyclin D1 is known to regulate other nuclear receptors. HNF4α and its co-activator CBP were expressed in nuclear extracts of hepatocytes (**Fig. 4A**), and their expression was not altered by cyclin D1. Immunoprecipitation of HNF4α from hepatocyte lysates co-precipitated cyclins D1 and D1-KE (**Fig. 4B**), indicating that the two proteins associate. To determine whether cyclin D1 affected HNF4α binding to the promoters of target genes, we performed ChIP analysis (**Fig. 4C**). Binding of HNF4α to its binding region in the promoters of Pklr and Fasn was increased by glucose, and this was inhibited by both cyclins D1 and D1-KE. Binding of the co-activator protein CBP, which itself is regulated by cyclin D1,[19,20] was regulated in a similar manner. Furthermore, binding of RNA polymerase II to the proximal region of these promoters was inhibited by cyclins D1 and D1-KE, indicating decreased transcriptional activation. These results identify HNF4α as a target of cyclin D1 in hepatocytes. Cyclin D1 inhibited the recruitment of HNF4α to target promoters in a manner that did not require cdk4 activation or proliferation.

The studies in **Figure 4B and C** demonstrate that cyclin D1 prevented recruitment of HNF4α to lipogenic promoters. However, cyclin D1 regulates ChREBP and possibly other

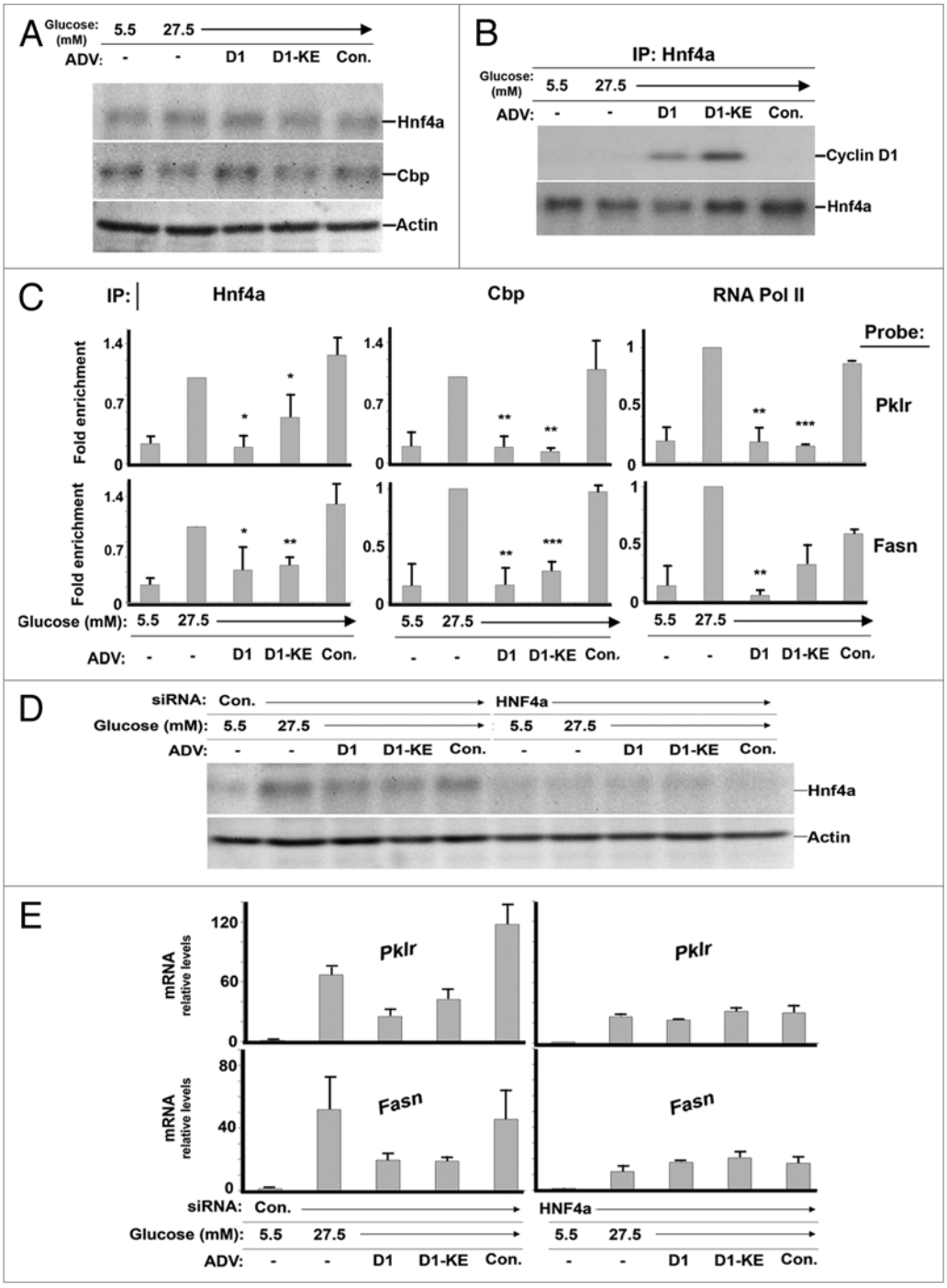

Figure 4. Cyclin D1 regulates binding of HNF4α to lipogenic genes. Hepatocytes were cultured as in **Figure 1**. (A) Western blot analysis of HNF4α and CBP in nuclear extracts. (B) Immunoprecipitation of HNF4α followed by western blot analysis of cyclin D1. (C) Chromatin immunoprecipitation analysis of HNF4α, CBP and PolII binding to the promoter regions of Pklr and Fasn. The combined results of three independent results are shown. (D) Hepatocytes were transfected with control siRNA or siRNA targeted to HNF4α, followed by western blot of HNF4α expression. (E) Expression of lipogenic transcripts in hepatocytes treated with control or HNF4α siRNA.

transcription factors involved in the induction of lipogenesis by glucose, and the relative contribution of decreased HNF4α activity is not known. To address the role of HNF4α in our system, we used a knockdown approach to repress the expression of this protein. Treatment of hepatocytes with siRNA to HNF4α markedly decreased the expression of this protein (**Fig. 4D**). Cells treated with control siRNA showed repression of Pklr and Fasn gene expression by cyclins D1 and KE (**Fig. 4E**), similar to what was found in the absence of siRNA (**Fig. 2C**). Inhibition of HNF4α expression blunted but did not eliminate the induction of these mRNA by high concentrations of glucose (**Fig. 4E**), which is consistent with prior studies suggesting that HNF4α plays a role

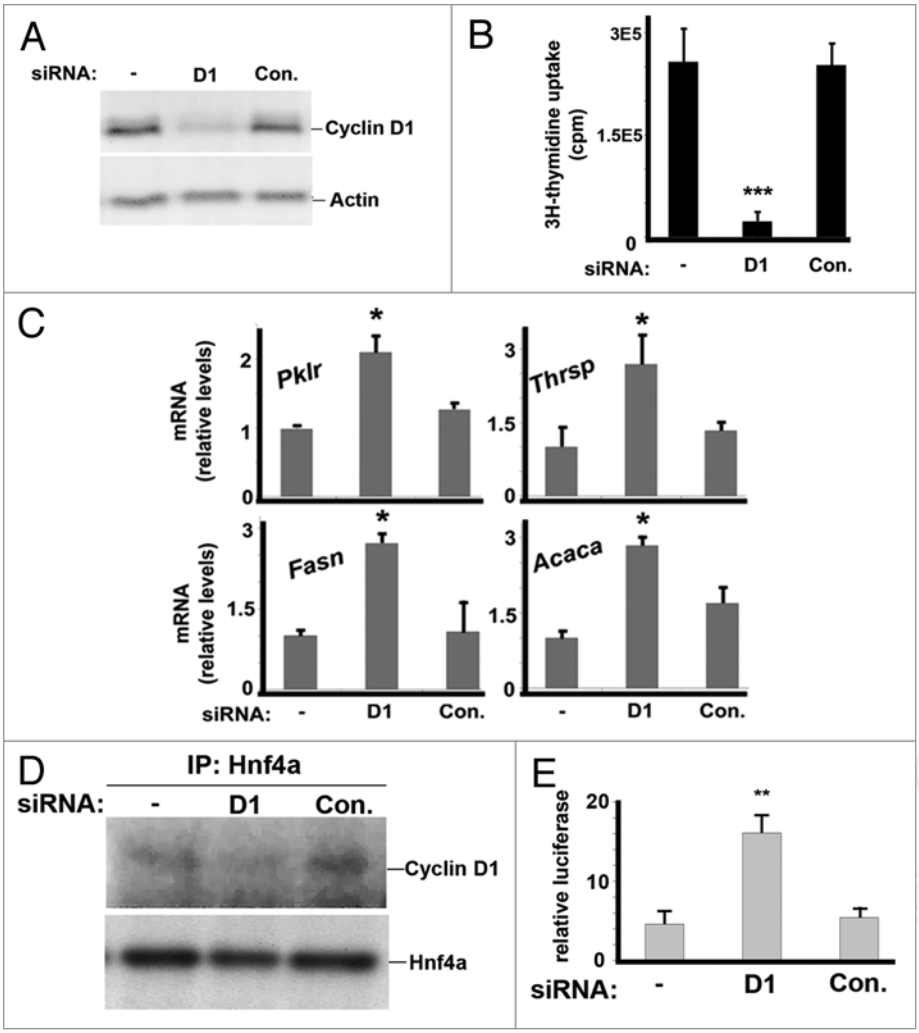

Figure 5. Knockdown of cyclin D1 promotes lipogenic gene expression and HNF4α activity in AML12 cells. AML12 cells were treated with siRNA as indicated. (A) Western blot analysis of cyclin D1. (B) DNA synthesis. (C) Expression of lipogenic mRNA. (D) Lysates were subjected to HNF4α immunoprecipitation followed by western blot for cyclin D1. (E) Cells were transfected with HNF4α and a HNF4α-responsive promoter linked to luciferase as in **Figure 3**. Firefly luciferase was normalized to Renilla activity.

in the induction of Pklr and Fasn by glucose.[25,26] Notably, in cells treated with HNF4α siRNA, cyclin D1 did not further affect the regulation of the transcripts by glucose. These results strongly suggest that HNF4α is a key target of cyclin D1 in regard to the regulation of these lipogenic genes by glucose.

To substantiate these findings, we examined whether endogenous cyclin D1 repressed HNF4α or lipogenic gene expression. Cyclin D1 is expressed at very low levels in quiescent liver or non-proliferating hepatocytes.[13] In the current studies, we cultured hepatocytes at a high density, which promotes differentiated function, including lipid synthesis, but represses mitogen-stimulated proliferation. We therefore used the well-differentiated mouse hepatocyte cell line AML12 to study the effect of cyclin D1 knockdown. In the presence of serum, these cells proliferate readily and express cyclin D1. Cyclin D1 siRNA markedly decreased expression of this protein and diminished proliferation as measured by DNA synthesis (**Fig. 5B**). Cyclin D1 knockdown resulted in increased expression of lipogenic transcripts (**Fig. 5C**), corresponding with the increased lipogenesis observed in **Figure 1B**. Immunoprecipitation studies demonstrated binding of endogenous cyclin D1 with HNF4α (**Fig. 5D**). Using an established reporter gene system, knockdown of cyclin D1 enhanced HNF4α transcriptional activity (**Fig. 5E**). These studies confirm that endogenously expressed cyclin D1 inhibits HNF4α activity in proliferating hepatic cells.

To further study the interaction of cyclin D1 and HNF4α, we used adenoviruses to transduce variants of cyclin D1 into the liver in vivo as shown in **Figure 6A**. In addition to cyclins D1 and D1-KE, a truncated version containing only the putative "repressor domain" (RD) (amino acids 141–250) was used, along with a mutant in which the RD had been excised (XMN).[17] Finally, a common polymorphism called cyclin D1b, which contains a distinct C terminus, was also examined. As is shown in **Figure 6B**, each of these vectors led to expression of the transduced protein after one day. Immunoprecipitation of HNF4α demonstrated that this protein associated with wild-type cyclin D1, cyclin D1-KE, the RD region and cyclin D1b (**Fig. 6C**). On the other hand, the XMN variant did not co-precipitate. These studies indicate that cyclin D1 binds HNF4α via a motif in the RD, which has been shown to inhibit the activity of other nuclear receptors.[17,18]

We next examined whether cyclin D1 regulated hepatic lipogenesis in vivo. Mice were fasted for 24 h and then fed a high-carbohydrate diet for 24 h, which induces a marked lipogenic response in the liver. Animals were transduced with cyclins D1, D1-KE and the RD variant one day prior to harvest (**Fig. 6D**). As expected, high carbohydrate feeding led to substantially increased expression of lipogenic genes Pklr, Fasn, Thrsp and Acaca (**Fig. 6E**). This response was markedly inhibited by cyclins D1 and D1-KE. The RD variant of cyclin D1 inhibited expression of Pklr, Fasn and Acaca. These data indicate that cyclin D1 inhibits the lipogenic response of hepatocytes in vivo to carbohydrate feeding in a cdk4-independent manner, and this effect involves a motif in the putative transcriptional repressor domain.

Finally, we examined whether cyclin D1 induction during liver regeneration was associated with a similar decrease in lipogenic gene expression in the liver. Mice were fasted and fed

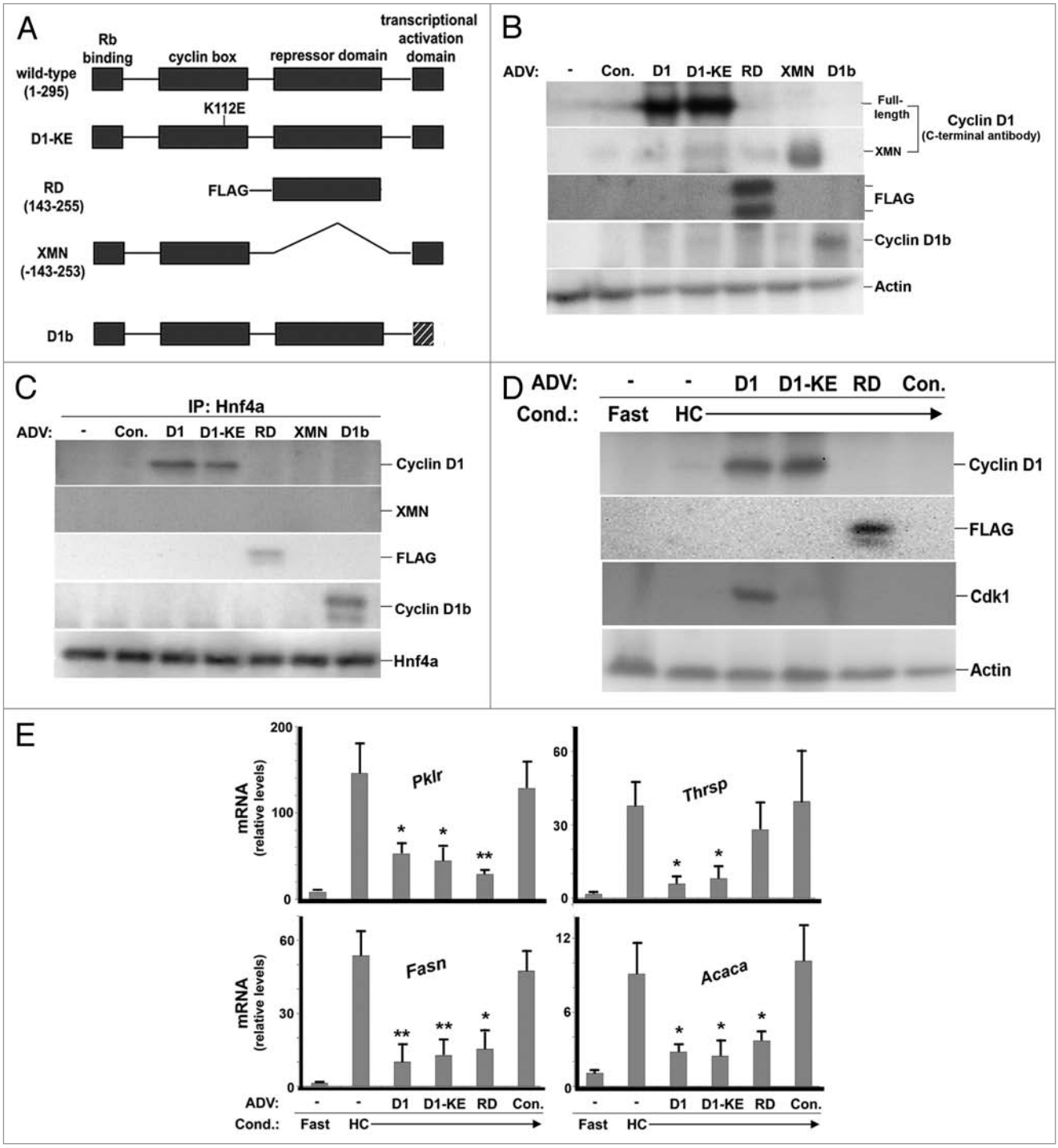

Figure 6. Cyclin D1 binds to HNF4α via a region in the repressor domain and represses the induction of lipogenic transcripts in the liver following high-carbohydrate feeding. Mice were transduced with the indicated adenoviral vectors and livers were harvested 24 h later. (A) Diagram of cyclin D1 mutants used. (B) Western blot analysis of liver lysates using antibodies directed against the C-terminal region of cyclin D1, the FLAG epitope (which is linked to the RD construct), and cyclin D1b as indicated. (C) Immunoprecipitation of HNF4α followed by western blot using the indicated antibodies. (D) Mice were fasted for one day or were fasted for one day followed by one day of high carbohydrate feeding (HC) ad libitum, followed by western blot of liver lysates as shown. (E) Mice underwent the fast-refeed protocol as above and livers were harvested for RNA, followed by RT-PCR for the lipogenic transcripts.

a high-carbohydrate diet as in **Figure 6D and E**. In addition, we performed 70% PH (or sham surgery) 42 and 72 h before harvest. As previously shown,[13] PH induces significant cyclin D1 expression at 42 h, which is accompanied by induction of hepatocyte DNA synthesis (**Fig. S5**) and cdk1 (**Fig. 7A**). Cyclin D1 expression persists at 72 h after PH, although hepatocyte

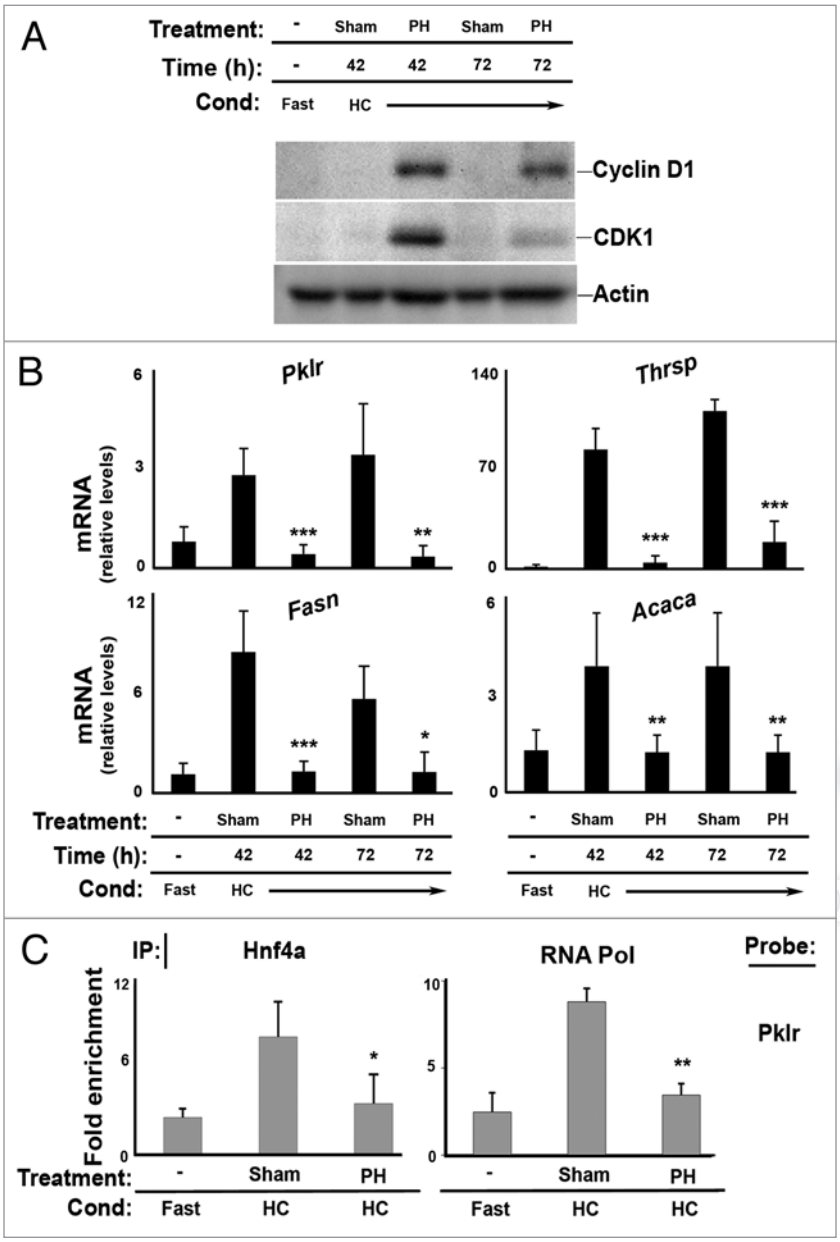

Figure 7. Repression of lipogenic gene expression and HNF4α in regenerating liver. Mice were subjected to 70% PH or sham surgery and livers were harvested at 42 or 72 h. In addition, mice were fasted for 24 h and refed for 24 h before harvest as in **Figure 6D and E**. (A) Western blot of cyclin D1 and cdk1 expression. (B) Expression of lipogenic mRNA. (C) ChIP analysis of HNF4α and PolII binding to the Pklr gene at 42 h after PH or sham surgery.

proliferation decreases at this time point.[13] As compared with refed sham-operated animals, PH caused a significant decrease in the expression of lipogenic genes Pklr, Fasn, Thrsp and Acaca at 42–72 h (**Fig. 7B**). Furthermore, PH reduced binding of HNF4α and PolII to Pklr promoter (**Fig. 7C**). Interpretation of these findings after PH is somewhat difficult given the potential for altered glucose metabolism, although we did not observe statistically significant changes in glucose levels in these mice (**Fig. S5**). In conjunction with our other findings, however, these data suggest that physiologic induction of cyclin D1 in the regenerating liver reduces lipogenic gene expression and HNF4α activity.

Discussion

In this study, cyclin D1 was found to inhibit de novo lipogenesis, an important component of liver metabolism. Our data indicate that cyclin D1 suppressed the activity of two key transcription factors involved in lipid metabolism, ChREBP and HNF4α. Cyclin D1 appears to inhibit ChREBP via a cdk-dependent mechanism, whereas it inhibits HNF4α in a cdk-independent manner. These findings suggest that the changes in metabolic function in the regenerating liver may not simply be due to decreased functional hepatic mass but may also be a result of the induction of cyclin D1. Our studies provide a novel link between the cell cycle machinery and the regulation of hepatic metabolism.

At first glance, the finding that cyclin D1 inhibited lipogenesis in hepatocytes may seem at odds with the observation that fatty acids and triglycerides typically accumulate in the liver after PH in rodents, although this is not required for liver regeneration.[27] Older studies have shown increased incorporation of ^3H_2O into hepatic fatty acids in rats following PH,[28,29] which led to the conclusion that lipogenesis rates increase in the regenerating liver. An alternative possibility is that systemically administered ^3H_2O may be converted to fatty acids at extrahepatic sites (i.e., adipose tissue) and then imported into the liver. Indeed, it is likely that fatty acids derived from adipose tissue are the primary source of fatty acids incorporated into liver lipids after PH.[30-32] To our knowledge, previous studies have not directly examined hepatic fatty acid synthesis in vivo in the regenerating liver. One study used perfused rat livers after PH and showed a trend toward decreased de novo lipogenesis as compared with controls.[28] Furthermore, mitogen stimulation of cultured primary rat hepatocytes leads to decreased lipogenesis.[33] Hepatic lipid metabolism is likely to be regulated by numerous factors in the regenerating liver,[27,31] and our data indicate that the effect of cyclin D1 is to diminish lipogenesis. Further studies will be required to determine how hepatic expression of cyclin D1 regulates other aspects of lipid metabolism in the liver and peripheral sites. However, the data presented here provide a mechanistic framework to examine these processes in greater detail.

Cyclin D1 has been shown to modulate the activity of numerous transcription factors and co-regulators including ERα, AR, TRβ, PPARγ, p300 and CBP. Moreover, a recent study using mice transgenic for affinity-tagged cyclin D1 showed that this

protein binds to the promoters of an unexpectedly large number of genes, suggesting that it may be involved in diverse cellular processes.[19] The ability of cyclin D1 to repress the nuclear receptors AR, TRβ and PPARγ does not require cdk4 activity and maps to motifs within the RD.[17,18] Our findings indicate that cyclin D1 binds to HNF4α, another member of the nuclear receptor family, via the RD (**Fig. 6**), and that it inhibits recruitment of HNF4α to target promoters in a manner that does not require cdk4 activity or cell cycle progression (**Fig. 4**). We further show that knockdown of HNF4α prevents cyclin D1 from inhibiting the expression of target lipogenic genes (**Fig. 4**), which strongly suggests that HNF4α is a relevant target of cyclin D1. Previous studies have shown that HNF4α regulates the lipogenic genes Pklr and Fasn,[25,26] and that knockdown of HNF4α inhibits hepatic lipogenesis;[3] thus, our findings that cyclin D1 appears to inhibit lipogenesis (at least in part) via repression of HNF4α (**Fig. 4**) are consistent with established models. Further studies will be required to identify the domain(s) of HNF4α required for its interaction with cyclin D1 and to determine whether these two proteins associate directly or through another protein that binds to both.

Our studies of ChREBP were limited by the inability of commercially available antibodies to detect this protein in our hands. However, the data in **Figure 3** show that cyclin D1 inhibits the glucose-mediated induction of ChREBP mRNA; this may be due to decreased activity of the ChREBP protein, since it can auto-regulate its own gene.[6] We used an established transfection system to show that cyclin D1 inhibits activity of ChREBP via a domain in the N terminus that is also responsible for the glucose-mediated regulation of this transcription factor.[7,9] Interestingly, this activity of cyclin D1 was not reproduced by cyclin D1-KE, suggesting a kinase-dependent mechanism in contrast to the regulation of HNF4α. Of note, the N-terminal region of ChREBP contains several potential phosphorylation sites[7] and thus could be directly or indirectly phosphorylated by cyclin D1/cdk4. As in the case of HNF4α, more investigation will be necessary to fully define the mechanism by which cyclin D1 inhibits ChREBP.

We cannot exclude the possibility that cyclin D1 may regulate other relevant pathways and transcriptional mediators involved in lipid metabolism aside from HNF4α and ChREBP. Indeed, this seems likely given prior literature demonstrating its regulation of numerous transcription factors and co-regulators, and the fact that it appears to bind the promoter region of a surprisingly large number of genes in vivo.[19] In the current report, we have focused on the induction of lipogenesis by glucose, and it is possible that cyclin D1 may also regulate components of the insulin-signaling pathway. In addition to its effect on lipid metabolism, we have recently found that cyclin D1 regulates estrogen and androgen metabolism in the liver,[34] and gene array data suggest that it may impact other aspects of hepatic function in vivo.[22] It is therefore plausible that cyclin D1 has diverse metabolic effects in the liver and other tissues.

Our finding that cyclin D1 inhibits HNF4α activity could have implications for normal and malignant cell cycle progression in the liver. It is tempting to speculate that in the setting of physiologic hepatocyte proliferation, transient repression of HNF4α by cyclin D1 may allow for a shift in cell metabolism away from activities that support systemic homeostasis (e.g., synthesis of lipid for energy storage). Indeed, the data in **Figure 7** show that the physiologic induction of cyclin D1 in vivo after 70% PH was associated with decreased lipogenic gene expression and diminished binding of HNF4α to a target promoter. This could allow more cellular resources to be directed toward cell growth and cell cycle progression, which are highly energy-intensive processes.[35] Furthermore, cyclin D1 is commonly overexpressed in HCC and other cancers.[14,15] By decreasing HNF4α activity, cyclin D1 may contribute to the de-differentiated phenotype observed in HCC. HNF4α is inhibited in HCC, and forced expression of this protein has been used as "differentiation therapy" in experimental models of this tumor, leading to decreased cell proliferation and tumor formation.[36-38] Repression of HNF4α function may therefore be a novel oncogenic effect of cyclin D1.

In summary, the data presented here demonstrate that cyclin D1 suppresses lipogenesis in hepatocytes and thus affects a major component of hepatic metabolism. Cyclin D1 inhibits two well-characterized transcription factors that modulate hepatic lipogenesis, ChREBP and HNF4α. The effect on HNF4α is of particular interest because this protein controls a number of key metabolic pathways in hepatocytes, and its interaction with cyclin D1 may affect other important aspects of liver function. Our findings provide a framework to better understand the mechanisms by which the cell cycle is coupled to hepatic metabolism, which is relevant to both physiologic cell proliferation in the regenerating liver and abnormal proliferation in HCC.

Materials and Methods

Please see **Supplemental Material** for details of the methods used.

Statistical analysis. Results were subjected to one-way ANOVA. Significance was determined against a p-value threshold of < 0.05. Data shown is statistically significant and denoted as such where necessary. Statistical significance is denoted in the figures if the indicated condition is different from the comparable control condition as follows: *< 0.05, **< 0.01 and ***< 0.001.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental materials may be found here:
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