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Regulation of lipogenesis by cyclin-dependent kinase 8–mediated control of SREBP-1

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Altered lipid metabolism underlies several major human diseases, including obesity and type 2 diabetes. However, lipid metabolism pathobiology remains poorly understood at the molecular level. Insulin is the primary stimulator of hepatic lipogenesis through activation of the SREBP-1c transcription factor. Here we identified cyclin-dependent kinase 8 (CDK8) and its regulatory partner cyclin C (CycC) as negative regulators of the lipogenic pathway in Drosophila, mammalian hepatocytes, and mouse liver. The inhibitory effect of CDK8 and CycC on de novo lipogenesis was mediated through CDK8 phosphorylation of nuclear SREBP-1c at a conserved threonine residue. Phosphorylation by CDK8 enhanced SREBP-1c ubiquitination and protein degradation. Importantly, consistent with the physiologic regulation of lipid biosynthesis, CDK8 and CycC proteins were rapidly downregulated by feeding and insulin, resulting in decreased SREBP-1c phosphorylation. Moreover, overexpression of CycC efficiently suppressed insulin and feeding–induced lipogenic gene expression. Taken together, these results demonstrate that CDK8 and CycC function as evolutionarily conserved components of the insulin signaling pathway in regulating lipid homeostasis.

Introduction
Dysregulation of lipid metabolism is closely associated with major human diseases such as obesity, type 2 diabetes, and cardiovascular disease (1–4). However, the physiologic and pathophysiologic regulation of lipid metabolism is a highly complex and integrative process that remains poorly understood at the molecular level. Among the known lipogenic regulators, the SREBP transcription factors are pivotal activators of key enzymes responsible for hepatic biosynthesis of fatty acids and cholesterol (5–8) and play an important role in the development of fatty liver and dyslipidemia (9).

The three mammalian SREBP transcription factors, SREBP-1a, -1c, and -2, are synthesized as inactive precursors that are tethered to the ER membrane (10). Reduction of intracellular sterols results in the transportation of SREBP-2 to the Golgi, where it undergoes proteolytic maturation. Then, the N-terminal fragment of SREBP-2 translocates into the nucleus and activates transcription of target genes (11). Similarly, the two SREBP-1 isoforms, SREBP-1a and -1c, are also processed in the Golgi to generate the mature SREBP-1 protein (12, 13). Unlike SREBP-2, SREBP-1c is primarily activated by insulin (14). Both SREBP-1a and SREBP-1c proteins are produced by the same gene, SREBF1, by two distinct promoters and alternative splicing (15). Their amino acid sequences differ only at the very N-terminal end. The unique region of SREBP-1a is part of its transactivation domain (16). SREBP-1a and SREBP-1c have different expression profiles: SREBP-1a is highly expressed in proliferating cells, such as cancer cells, while SREBP-1c is the predominant form in normal cells, particularly hepatocytes (17).

CDK8 and its regulatory partner CycC have been reported as being subunits of the Mediator complexes in mammalian cells (18, 19). The mammalian Mediators are large protein complexes containing up to 30 distinct subunits, depending on starting materials and biochemical purification protocols, and play a critical role in bridging the signals from transcription factors to the basal transcription apparatus (20–22). Biochemical purifications have identified at least two groups of Mediator complexes, the small Mediator and the large Mediator, with the latter containing an extra submodule of CDK8, CycC, MED12, and MED13 (19). In chromatin-based in vitro transcription assays, the small Mediator can activate gene transcription, while the large Mediator is inactive (19). Interestingly, recent studies have established a role of CDK8–CycC in tumorigenesis (20). However, the in vivo functions and regulation of CDK8 and CycC are still poorly understood.

In this study, we identify CDK8 and CycC as key repressors of lipogenic gene expression, de novo lipogenesis, and lipid accumulation in Drosophila and mammals. This function of CDK8–CycC occurs through site-specific phosphorylation on nuclear SREBP-1c protein, resulting in rapid degradation of this central regulator of lipid metabolism. Since CDK8 and CycC are reduced upon feeding or by insulin, concomitant with increased levels of nuclear SREBP-1c protein, the CDK8–CycC complex appears to function downstream of the insulin signaling as a key regulator of de novo lipogenesis.
Figure 1
Role of CDK8 and CycC in lipid accumulation in fat bodies of Drosophila larvae. Representative light micrographic images for larvae (scale bars: 1.0 mm) of (A) wild-type (w1118), (B) Cdk8-null (k185), and (C) CycC-null (y5) Drosophila. (D) Quantitative measurement of oil red O staining in Drosophila larvae of the indicated genotypes. (E) Triglyceride levels in Drosophila larvae of the indicated genotypes. (F) Quantitative measurement of oil red O staining in Drosophila larvae with fat body–specific expression of the indicated RNAi; the background control was FB-Gal4. Data represent mean ± SD of 15 larvae per genotypes for oil red O staining or 3 random pools of the same genotype for triglyceride measurement. *P < 0.01, #P < 0.001 versus control. Data are representative of independent experiments repeated at least 3 times.

Results

CDK8 and CycC regulate lipid levels and lipogenic gene expression in Drosophila. CDK8 and CycC are highly conserved in eukaryotes; thus, we first used Drosophila as a model organism to study function and regulation of CDK8 and CycC in vivo. Compared with wild-type (Figure 1A), Cdk8-null (Figure 1B) and CycC-null (Figure 1C) larvae are less transparent, suggesting that the mutant larvae possess more fat tissue. Intrigued by this observation, we stained fat body of larvae, which is the predominant tissue for regulating lipid homeostasis in Drosophila, with oil red O for neutral lipids (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI61462DS1) and extracted the dye with isopropanol. As shown in Figure 1D, we detected significantly more oil red O staining in Cdk8- and CycC-null larvae. Quantitative measurements of triglycerides in Cdk8- and CycC-null larvae further confirmed the significant increase in neutral lipids as compared with control (Figure 1E). To avoid potential nonspecific effects of Cdk8 or CycC mutations on lipid metabolism during development, we generated transgenic RNAi lines that allow tissue-specific knockdown of CDK8 or CycC. Similarly, we observed a significant increase in lipid levels in larvae with either Cdk8 or CycC knockdown by RNAi whose expression is driven by fat body–specific FB-Gal4 (Figure 1F) or Adh-Gal4 (data not shown). Taken together, these results suggest that CDK8 and CycC are required for inhibition of lipid accumulation in Drosophila larvae.

To investigate the underlying mechanisms of increased lipid levels in Cdk8- and CycC-null larvae, we examined the global gene expression profiles in homozygous null mutant larvae of Cdk8 and CycC at the late third instar stage by cDNA microarray analysis. Both Cdk8<sup>K185</sup> and CycC<sup>y5</sup> alleles, and homozygous mutants are lethal as pupae (23). Compared with the control, a number of genes in Cdk8-null mutants were significantly altered, with a cutoff at 1.5-fold and a false discovery rate of less than 0.05 (Figure 2A). More than 80% of these genes (662 of upregulated and 807 of downregulated genes) were also altered in CycC-null mutants in the same fashion (Figure 2A). The high percentage of overlapping genes supports a model whereby CycC is required for activation of CDK8 (20, 23–26). In addition, the known dE2F1 target genes were also found to be upregulated in this microarray analysis (data not shown), confirming our previous finding that CDK8 inhibits E2F1-dependent transcription (27).

Consistent with our observation of the phenotypic changes in Cdk8- or CycC-null larvae, many genes involved in lipid metabolism were significantly upregulated in those mutants (Supplemental Table 1). These genes include fatty acid synthase (dFAS), acetyl-CoA carboxylase (dACC), and acetyl-CoA synthetase (dACS), which are important enzymes for fatty acid biosynthesis. As confirmed by reverse transcription and quantitative PCR (qRT-PCR), we observed a significant increase in transcripts of dACC, dACS, dFAS, and other genes in Cdk8- and CycC-null mutants compared with the control (Figure 2, B and C), suggesting that CDK8 and CycC are required for inhibition of the transcription of these genes.

Interestingly, the mammalian homologs of several genes in Supplemental Table 1 are known transcriptional targets of SREBP transcription factors (28, 29), which are the master regulators of lipid homeostasis in diverse organisms (10, 30, 31). Consistent with the lipogenic function of SREBP, knocking down Drosophila SREBP (dsREBP) in fat body significantly reduced lipid levels (Figure 1F). Thus, we postulate that CDK8 and CycC may regulate the expres-
sion of lipogenic genes through repression of SREBP-mediated transcription. To test this hypothesis, we knocked down both CDK8 (or CycC) and dSREBP in fat body and observed a significant reduction in neutral lipid levels compared with knockdown of CDK8 (or CycC) alone (Figure 1F). These results suggest that the effect of CDK8 or CycC knockdown on lipid accumulation is likely dependent on SREBP. However, the mRNA levels of dSREBP and dSCAP were not significantly changed in Cdk8- and CycC-null mutants (Supplemental Table 1), suggesting that the effect of CDK8 and CycC on SREBP target gene expression in Drosophila was less likely mediated through regulation of the transcription or maturation of dSREBP itself.

**CDK8 and CycC repress the lipogenic program in mammalian hepatocytes.** To determine whether CDK8 regulation of lipid accumulation and lipogenic gene expression is conserved in mammalian cells, we knocked down CDK8 or CycC in primary culture of rat hepatocytes using lentiviral shRNA (Figure 3A). Knockdown of CDK8 or CycC resulted in a significant increase in triglycerides (Figure 3B), suggesting that as in Drosophila larvae, CDK8 and CycC are required for inhibition of intracellular triglyceride accumulation in primary rat hepatocytes. To test whether CDK8 and CycC also regulate the transcription of FAS, we knocked down CDK8 or CycC in HepG2 cells that were cotransfected with the FAS luciferase reporter (32). As shown in Figure 3C, CDK8 or CycC knockdown with two independent shRNAs, as we used previously (27), resulted in significant elevation of FAS promoter activity, indicating that CDK8 and CycC negatively regulate the FAS promoter. Consistent with the concept that a functional interaction of CDK8 and CycC is necessary for stability of the CDK8-CycC complex, CDK8 knockdown in mammalian cells resulted in a concomitant reduction in CycC, while CycC knockdown caused a parallel reduction in CDK8 in cultured cells (Figure 3, A and C). In addition, CDK8 or CycC knockdown (Figure 3D) significantly increased the mRNA levels of endogenous SREBP target genes, such as FAS, ACC, ATP-citrate lyase (ACL), and stearoyl CoA desaturase (SCD1), in three hepatocyte lines: primary rat hepatocytes (Figure 3E), FAO cells (data not shown), and HepG2 cells (Supplemental Figure 2A). The effect of CDK8 or CycC knockdown was compromised when SREBP-1 was co-depleted in those cells by shRNA (Figure 3E and Supplemental Figure 2A), similar to what we observed in Drosophila (Figure 1F). These results support the hypothesis that regulation of lipogenic gene expression by CDK8 is dependent on SREBP-1. Furthermore, CDK8 knockdown significantly stimulated the activity of a promoter with wild-type SREBP-1 binding sites (SRE) in HEK293 cells, but had no effects when the SRE site was inactivated by point mutations (Supplemental Figure 2B), further suggesting that CDK8 represses SREBP-1–dependent gene expression. Consistent with the mRNA level and promoter activity of FAS gene, FAS protein levels in primary rat hepatocytes (data not shown) and HepG2 cells (Figure 4A) were also significantly elevated when CDK8 or CycC was knocked down. Taken together, our data suggest that CDK8 and CycC regulate SREBP-dependent gene expression in mammalian hepatocytes.

*Cdk8 and CycC are involved in regulating nuclear SREBP-1 protein stability.** To elucidate the molecular mechanisms of CDK8 inhibition of SREBP-1–mediated lipogenic gene expression, we first asked whether CDK8 affects the abundance of nuclear SREBP-1 in mammalian cells. CDK8 or CycC knockdown resulted in a significant increase in endogenous nuclear SREBP-1 protein in primary rat hepatocytes (Figure 3A and Figure 4A), with no significant change in the protein levels of the SREBP-1 precursor (Figure 3A and Figure 4A), suggesting that CDK8 regulates the abundance of the nuclear form of this transcription factor.

The nuclear/active form of SREBP-1 is processed from its precursor initially located in ER membrane (13). Since CDK8 is predominantly a nuclear protein (33), it is less likely that CDK8 can directly regulate the proteolytic maturation process of SREBP-1 in ER or Golgi apparatus, which is also supported by our data in primary rat hepatocytes (Figure 3A and Figure 4A). Thus, to further analyze the effect of CDK8 on nuclear SREBP-1 protein levels, we initially overexpressed a Flag-tagged nuclear form of SREBP-1a in HEK293 cells by transient transfection. As a control, an HA-tagged fusion protein of the Gal4 DNA-binding domain and Myb transactivation domain, which is neither physically nor functionally associated with CDK8 or CycC (32, 34), was coexpressed. As shown in Figure 4B, depletion of CDK8 by shRNAs caused accumulation of the nuclear form of SREBP-1a. Similar results were obtained when CycC was knocked down (data not shown). The increased accumulation of the nuclear form of SREBP-1a in HEK293 cells caused by CDK8 or CycC knockdown was due to decreased protein degradation as assayed by Western blots in the presence of cycloheximide, an inhibitor of protein synthesis (Figure 4, C and D). These data support a model in which CDK8-CycC regulates SREBP target gene expression at least in part by controlling the stability of nuclear SREBP-1 proteins.
CDK8 directly phosphorylates SREBP-1. Since ubiquitination is required for SREBP-1 degradation (35), we determined whether CDK8 and CycC affected SREBP-1a ubiquitination by coexpressing HA-tagged ubiquitin with Flag-tagged nuclear SREBP-1a in HEK293 cells. As shown in Figure 4E, the amount of ubiquitinated SREBP-1a was significantly decreased in cells with CDK8 or CycC knockdown by shRNAs. Moreover, CDK8 knockdown also decreased the ubiquitination levels of endogenous SREBP-1 (Figure 4F).

Phosphorylation of SREBPs has been shown to facilitate their binding to the E3 ligase SCFFbw7b, thus controlling their ubiquitination and subsequent degradation (35). Because CDK8 can directly phosphorylate several proteins in multicellular organisms (27, 36–38), we tested the possibility that CDK8 alters SREBP-1a stability through direct phosphorylation. Since the consensus sequence motifs for cyclin-dependent kinases are generally threonine (T) or serine (S) followed by a proline residue (TP or SP), we used a commercially available antibody that specifically recognizes phosphorylated TP (p-TP) as well as an antibody that can recognize phosphorylated serine (p-S) to detect the phosphorylation status of SREBP-1a. By Western blot analysis of immunoprecipitated SREBP-1a, we found that CDK8 or CycC knockdown in HEK293 cells dramatically decreased the phosphorylation levels of SREBP-1a at TP motifs, but had no significant effects on phosphorylation at serine residues (Figure 4F), suggesting that CDK8 or CycC only regulates threonine phosphorylation of SREBP-1a. To determine whether CDK8 can also directly phosphorylate SREBP-1c, we performed in vitro kinase assays in which endogenous CDK8 proteins were immunopurified from nuclear extracts of HEK293 cells, and recombinant GST–SREBP-1c (amino acids 1–426) protein was used as the potential substrate. As shown in Figure 5B, native CDK8, but not heat-inactivated CDK8, could phosphorylate SREBP-1c in vitro at TP motifs, but not serine residues. To eliminate the possibility of contamination by other cellular kinases, we immunopurified Flag-tagged CDK8 proteins from nuclear extracts of HEK293 cells that were transfected with either wild-type or a kinase-defective mutant of CDK8. As shown in Figure 5C, wild-type CDK8, but not the kinase-defective mutant, could phosphorylate SREBP-1c in vitro, confirming that SREBP-1c is a direct substrate of CDK8. Moreover, only CDK8, but not the closely related nuclear CDK7 and CDK9, was able to phosphorylate SREBP-1c at TP motifs, and none of them was able to phosphorylate SREBP-1c on serine residues in vitro (data not shown). Consistent with a previous report (39), all of these kinases could phosphorylate the CTD tail of RNA polymerase II (RNAPII) in vitro (data not shown).

There are 5 TP motifs within the nuclear form of human SREBP-1c (Figure 5D). To determine which TP motifs are targeted by CDK8, we performed in vitro kinase assays with several truncated forms of SREBP-1c (Figure 5D). To determine which TP motifs are targeted by CDK8, we performed in vitro kinase assays with several truncated forms of SREBP-1c (Figure 5D). Only the fragments containing the T402

Figure 3
CDK8 and CycC regulate SREBP-1–dependent lipogenic gene expression in mammalian hepatocytes. (A and D) Immunoblots for the indicated proteins in primary rat hepatocytes after treatment with lentivirus expressing the indicated shRNA. Pre-SREBP-1, precursor SREBP-1; nSREBP-1, nuclear SREBP-1. (B) Effects of CDK8 or CycC knockdown by lentiviral shRNA on triglyceride levels in isolated primary rat hepatocytes. (C) Effect of CDK8 or CycC knockdown by shRNA on FAS promoter–driven firefly luciferase mRNA levels in HepG2 cells as measured by qRT-PCR. The β-actin–driven Renilla luciferase served as control. (E) mRNA levels of endogenous SREBP target genes in primary rat hepatocytes were detected by qRT-PCR after treatments of the indicated lentiviral shRNA. Cyclophilin B served as the invariant control, and the mRNA levels of each gene were normalized to those in the non-silencing (NS) shRNA–treated sample. Data represent mean ± SD of 3 independent treatments. *P < 0.01 versus control.
residue (Figure 5E), which is located in a highly conserved region (Figure 5F), can be phosphorylated by CDK8 in vitro, suggesting that the T402 residue of human SREBP-1c is likely the direct target site of CDK8. To determine whether CDK8 affects phosphorylation of SREBP-1c T402 in cultured cells, we mutated this residue to alanine (T402A mutation) and transfected the Flag-tagged mutant with the wild-type SREBP-1c– but not T402A SREBP-1c–mediated activation of the transactivation domain of Myb and Gal4 DNA-binding domain served as control. (E) Effects of CDK8 or CycC knockdown on SREBP-1a ubiquitination. Flag-tagged SREBP-1a was cotransfected with HA-tagged ubiquitin (HA-Ub) and the indicated shRNA plasmids in HEK293 cells. At approximately 40 hours of culture followed by 3 hours of treatment with 0.1 mM MG132, cell lysates were prepared. The amount of SREBP-1a proteins was normalized after immunoprecipitation from cell lysate, and the presence of HA-ubiquitin modifications was detected by immunoblotting using anti-HA antibody. (F) Endogenous nuclear SREBP-1c proteins were immunoprecipitated from HEK293 cells after lentiviral CDK8 (or NS) shRNA treatment for 48 hours, followed by 3 hours of treatment with 0.1 mM MG132. The presence of ubiquitinated proteins was detected by anti-ubiquitin antibody.

**Figure 4**

CDK8 and CycC regulate SREBP-1 protein stability. (A) Effects of CDK8 and CycC knockdown on protein levels of FAS and SREBP-1 in HepG2 cells. (B) Effects of CDK8 knockdown on protein levels of overexpressed Flag-tagged nuclear SREBP-1a in HEK293 cells. The HA-tagged fusion protein of the transactivation domain of Myb and Gal4 DNA-binding domain served as control. (C) Representative immunobLOTS and (D) relative levels of overexpressed Flag-tagged nuclear SREBP1a in HEK293 cells in the presence of cycloheximide (CHX) for the indicated amount of time after CDK8 or CycC knockdown. (E) Effects of CDK8 or CycC knockdown on SREBP-1a ubiquitination. Flag-tagged nuclear SREBP-1a was cotransfected with HA-tagged ubiquitin (HA-Ub) and the indicated shRNA plasmids in HEK293 cells. At approximately 40 hours of culture followed by 3 hours of treatment with 0.1 mM MG132, cell lysates were prepared. The amount of SREBP-1a proteins was normalized after immunoprecipitation from cell lysate, and the presence of HA-ubiquitin modifications was detected by immunoblotting using anti-HA antibody. (F) Endogenous nuclear SREBP-1c proteins were immunoprecipitated from HEK293 cells after lentiviral CDK8 (or NS) shRNA treatment for 48 hours, followed by 3 hours of treatment with 0.1 mM MG132. The presence of ubiquitinated proteins was detected by anti-ubiquitin antibody.

Knockdown of CDK8 causes lipid accumulation in mice. The regulation of lipogenesis in cultured hepatocytes, particularly cancer cell lines, may not necessarily reflect the physiologic regulation in vivo. However, conventional CDK8 knockouts are embryonic lethal (40), and conditional CDK8-knockout mice are not yet available. Thus, to avoid the developmental defects of Cdks8 gene ablation, we transiently knocked down CDK8 in mouse livers by tail vein injection of adenoaviruses expressing specific shRNA against CDK8 (Figure 6A). Similar to our data from cell culture, CDK8 knockdown in mouse liver caused a significant increase in protein levels of FAS (Figure 6, A and B) and the nuclear, but not precursor, form of SREBP-1c (Figure 6A). In addition, we found that the SREBP-1c target genes FAS, ACS, and SCD1 were all significantly upregulated in CDK8-knockdown mouse livers compared with non-silencing shRNA–adenovirus infected samples (Figure 6C). In addition, CDK8 knockdown in liver had no significant effect on Srebp1 or Srebp2 mRNA expression (Supplemental Figure 4A) or L-pyruvate kinase (Figure 6C). The latter is not regulated by SREBP-1c (41), but it is a target of another important lipogenic transcription factor, carbohydrate regulatory element binding protein (ChREBP) (42, 43). These results suggest an inhibitory role of CDK8 on lipogenic gene expression in vivo through the regulation of nuclear SREBP-1c. Consistent with the increased lipogenic gene expression, the hepatic de novo synthesis rate of palmitate was significantly elevated by injection of adenoaviral CDK8 shRNA (Figure 6D). As a result, hepatic triglyceride levels were also significantly increased, by about 2-fold (Figure 6E), with accumulation of neutral lipids in mouse livers as detected by oil red O staining (Figure 6, G versus F), revealing a phenotype of early-stage fatty liver in CDK8-knockdown
mice. Strikingly, CDK8 knockdown also resulted in a concomitant increase in plasma triglycerides (~5-fold) (Supplemental Figure 4B), but no change in circulating insulin (Supplemental Figure 4C), free fatty acids (Supplemental Figure 4D), or glycerol (Supplemental Figure 4E). These results suggest that hypertriglyceridemia in CDK8-knockdown mice was not due to changes in adipocyte lipolysis. Thus, our data from mouse livers support the hypothesis that CDK8 plays a critical role in regulating de novo lipogenesis and triglyceride levels in mammals.

Regulation of CDK8 and CycC by nutrients. Since food intake generally stimulates lipogenesis in liver, we examined the protein levels of nuclear SREBP-1c, CycC, and CDK8 in mouse liver in fasted and re-fed states. Consistent with a previous report (44), compared with mice fasted for 12 hours, those re-fed for 5 hours had higher levels of nuclear SREBP-1c (Figure 7A). Interestingly, re-feeding resulted in a significant decrease in nuclear levels of both CDK8 and CycC proteins in mouse liver (Figure 7A), suggesting that CDK8 and CycC are regulated during fasting and re-feeding. Although a complicated set of changes takes place from the fasting to fed state, insulin is a primary lipogenic factor upon food intake, and it stimulates de novo lipogenesis primarily by stimulating SREBP-1 at the levels of transcription, processing, and nuclear protein stability (14). Thus, we tested whether insulin has any effects on CDK8 or CycC levels in hepatocytes. As shown in Figure 7B, insulin treatment caused downregulation of both CDK8 and CycC proteins in primary rat hepatocytes. We also observed a similar effect of insulin in FAO and HEK293 cells (data not shown). Moreover, insulin treatment also markedly decreased the phosphorylation of nuclear form of endogenous SREBP-1 at TP sites in primary rat hepatocytes (Figure 7C).

After establishing the role of feeding and insulin in downregulating CDK8-CycC, we examined whether overexpression of CycC and/or CDK8 can antagonize the effects of insulin. As expected, insulin treatment caused accumulation of overexpressed Flag-tagged nuclear form of SREBP-1c in HEK293 cells (Figure 7D, lane 3 vs. lane 1). Overexpression of CycC decreased the protein levels of SREBP-1c in the absence of insulin and blocked insulin-induced
The accumulation of SREBP-1a (Figure 7D). Furthermore, overexpression of CycC completely inhibited insulin-induced activation of the FAS promoter (Figure 7E). However, overexpression of wild-type CDK8 had little effect on the FAS promoter (data not shown). This is probably because increasing CDK8 alone is not sufficient for CDK8 kinase activity, substrate recognition, and/or localization in the absence of CycC. Finally, CycC overexpression in fat body of Drosophila larvae also inhibited re-feeding-induced upregulation of expression of lipogenic genes, such as dFAS (Figure 7F), and triglyceride accumulation (data not shown). Together, our data strongly support a functional role of CDK8-CycC in feeding/insulin-induced activation of nuclear SREBP-1c and de novo lipogenesis.

Discussion

In contrast to the sterol regulation of SREBP-2, insulin functions as the major activator of SREBP1c gene expression and inducer of SREBP-1c precursor processing to the mature nuclear form (5, 45–47). Numerous studies have established a key role of nuclear SREBP-1c on lipogenic gene expression and de novo lipogenesis (6, 16, 28, 44, 48). In this regard, SREBP-1c levels are elevated in mice and humans under a variety of pathophysiologic states, including obesity, insulin resistance associated with hyperinsulinemia, high carbohydrate diet, excessive alcohol consumption, and non-alcoholic fatty liver disease (NAFLD) — in the case of the latter being directly shown to increase the proportion of liver VLDL triglyceride production by de novo lipogenesis from 2%–5% to 20%–30% (41, 49–52). In those disease states, it is generally thought that the increased de novo lipogenesis results from high levels of insulin driving SREBP-1c transcription and precursor processing through PI3K- and mTORC1-dependent pathways, despite the marked insulin resistance to gluconeogenesis (14, 53, 54). However, these studies have only focused on the mechanisms of transcriptional activation of SREBP-1c expression and have not considered changes in the transcriptional activity or protein stability of nuclear SREBP-1c protein.

In this study, we identified a highly conserved role for CDK8 in the control of fatty acid and triglyceride metabolism. In Drosophila larvae, loss of CDK8 or CycC increased SREBP-dependent neutral lipid accumulation in fat body and lipogenic gene expression. Similarly, RNAi knockdown in hepatocytes in vitro or mouse liver in vivo also increased triglyceride levels, along with increasing SREBP target gene expression and de novo lipogenesis. Surprisingly, CycC and CDK8 levels were negatively regulated by food intake in mouse liver and by insulin in cultured cells. Thus, we propose a model whereby insulin stimulates de novo lipogenesis by downregulating the CDK8-CycC complex, which normally inhibits de novo lipogenesis through promoting nuclear SREBP-1c degradation. This new mechanism of insulin-induced lipogenesis is in addition to the well-documented function of insulin in stimulating SREBP-1c at the transcriptional level. Consistent with our model, overexpression of CycC blocks insulin-induced stabilization of nuclear SREBP-1 and lipogenic gene expression in vitro and in vivo. These observations establish an important and physiological role of CDK8-CycC in regulating fatty acid biosynthesis.

CDK8 and CycC are best known for their functions as the subunits of the Mediator complex, which serves as a transcription cofactor complex (26). Along with MED12 and MED13, they form the
CDK8 submodule of the Mediator complex. However, unlike that of CDK8 and CycC, fat body–specific knockdown of MED12 and MED13 did not cause statistically significant changes in lipid levels in Drosophila larvae, although there was a trend toward a decrease in lipids in MED13-knockdown larvae (Supplemental Figure 5). Similarly, MED12 or MED13 knockdown had no effect on SREBP-1 in mammalian cells (data not shown). These results support a specific role of CDK8-CycC in lipid metabolism and are consistent with a previous report demonstrating functions for the CDK8-CycC complex distinct from those of the MED12-MED13 complex in regulating developmental patterns in Drosophila (23). Our data also support a model whereby specific subunits of the Mediator complex are involved in only specific biological pathways, although the Mediator complex collectively may regulate most of the RNAPII-controlled gene expression. Alternatively, CDK8-CycC may function independent of the Mediator complex. Mechanistically, CDK8-CycC inhibits lipid accumulation largely by repressing SREBP-mediated gene expression. Our biochemical analyses have revealed that SREBP-1c can be directly phosphorylated by CDK8 at a conserved T402 residue (for human SREBP-1c) in vitro and in cultured mammalian cells. Furthermore, we observed that phosphorylation of SREBP-1 at T402 promotes its degradation. Thus, the phosphorylation event provides a critical control mechanism to regulate the level of nuclear SREBP-1c protein by increasing ubiquitination and subsequent rate of degradation without affecting the levels of the precursor SREBP-1c protein (Figure 3A, Figure 4A, and Figure 6A). These results suggest a simple explanation for the increased fat accumulation on CDK8 or CycC mutants or in cells with reduced levels of CDK8 and CycC. That is, reduction of CDK8 or CycC will result in hypophosphorylation of SREBP-1, which increases the stability of the nuclear SREBP proteins, thereby allowing increased expression of SREBP target genes involved in de novo lipogenesis.

It has been known that phosphorylation of nuclear SREBP proteins controls their stability (35, 55). GSK-3β, which functions downstream of insulin signaling, was previously reported to be involved in phosphorylation of SREBP proteins at several conserved sites, including T426 of SREBP-1a (the site corresponding to T402 of SREBP-1c) (35, 55). In this study, we identified that CDK8 can directly phosphorylate the T402 site of SREBP-1c. It is not uncommon that the same threonine or serine residue of a protein can be phosphorylated by multiple kinases. The fact that the T402 site of SREBP-1c can be targeted by both GSK-3β and CDK8 is consistent with the key role of phosphorylation on this site for the binding of the E3 ligase SCFFbw7b (35, 55).

The importance of these findings is underscored by the following two points. First, the observed high rates of lipogenesis and enhanced SREBP-target gene expression in dyslipidemic states in CDK8 and CycC mutants cannot be solely accounted for by changes in SREBP-1c transcription or maturation. It will be important to determine whether the control over nuclear SREBP-1c protein levels through the CDK8-CycC complex can provide a mechanistic basis for the enigmatic enhancement of lipogenesis that occurs during insulin resistance and diabetes. Second, CDK8 has been recently identified as an oncoprotein in melanoma and colorectal cancers.
(56, 57). Aberrant lipid and carbohydrate metabolism is a universal feature of human cancer cells; however, the mechanisms linking such aberrant metabolism and tumorigenesis remain poorly understood. Our results suggest that dysregulation of CDK8 may not only promote tumorigenesis, but also disrupt the cellular lipid homeostasis by lifting its repressive effect on SREBP-dependent transcription. It will be important to investigate in the future whether such a mechanism is functional in different types of human cancer cells.

**Methods**

*Drosophila* stocks and genetics. The w1118 and Oregon R *Drosophila* strains were used as controls. The null alleles of Cdk8 (w1122; †; FRT80B Cdk88165/TM3 Sb) and CycC (w1212; †; FRT2B CycC7/TM3 Sb) were provided by Henri-Marc Bourbon and Muriel Bouche (Centre de Biologie du Développement, Université Paul Sabatier, Toulouse, France), and we replaced thebalanced chromosome TM3 Sb with TM6B to facilitate the identification of homoyzygous mutant larvae. The fat body–specific Adh4-Gal4 and FB-Gal4 lines were obtained from Thomas Neufeld (Department of Genetics, University of Minnesota, Minneapolis, Minnesota, USA). The transgenic RNAi lines for Cdk8 and CycC were generated using the pVAlIUM10 vector (SB). Briefly, an 845-bp fragment of the dCdk8 gene and an 838-bp fragment of the dCycC gene were chosen using the online SnapDragon program developed by the Norbert Perrimon laboratory (http://www.flyrnai.org/cgi-bin/RNAi_find_primers.xml). The PCR primers were: dCdk8 forward, 5’TACCCAGGGTGATGATGGTGA-3’; dCdk8 reverse, 5’AGGAGACGGTTGAGTTAGTG-3’; dCycC forward, 5’ACCCGGGAATTTTGCGAGATTC-3’; and dCycC reverse, 5’TTGGCCATACCGACTGAGA-3’. The PCR fragments were cloned into entry vectors using a PENTR-D-TOPO cloning kit (Invitrogen), and the product was verified by diagnostic digestion. The fragments were then recombined into the pVAlIUM10 vector, and the final constructs were verified by DNA sequencing. To knock down dSREBP (HLH106), we generated a miRNA transgenic line using the pVAlIUM20 vector, which was shown to efficiently knock down target genes in both soma and germ line (59). The primers were: dSREBP-1F, 5’CTAGCATGCGGTTGATCT-3’; dSREBP-1R, 5’ATTCGACCATGGTGGATTT-3’; dSREBP-2F, 5’TACCCAGGGTGATGATGGTGA-3’; and dSREBP-2R, 5’AGGAGACGGTTGAGTTAGTG-3’. The final constructs were verified by DNA sequencing. UAS-CycC was constructed by inserting wild-type CycC cDNA (from Patrick O’Farrell, UCSF, San Francisco, California, USA) into the pTHFW vector (T. Murphy, Drosophila Genomic Resource Center, Bloomington, Indiana, USA), and the final construct was verified by sequencing. Transgenic flies were generated using antibody amplification with goat IgG (Sigma-Aldrich) and anti-HA (Covance), anti–p-TP (Cell Signaling Technology), anti–p-S (Invitrogen), anti–β-tubulin (Invitrogen), and anti-TBP (Fisher Scientific) antibodies were used in this study.

**Tissue culture.** HEK293, HepG2, and FAO cells were purchased from ATCC and cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 2 mM l-glutamine (Gibco), 100 μ/l penicillin (Gibco), and 100 μg/ml streptomycin (Gibco) at 37°C under humidified air containing 5% CO2. Primary rat hepatocytes were isolated by perfusion of rat livers by David Neufeld (Marion Bessin Liver Research Center, Albert Einstein College of Medicine). For all perfusions, C6e medium (pH 7.2) was supplemented with 10 mM HEPES. Washout medium was further supplemented with heparin (2.0 U/ml) and EGTA (0.5 mM), and digestion medium was supplemented with 500 μg/l collagenase. Viable primary rat hepatocytes were enriched by low-speed centrifugation (500 g) for 3 minutes. Typically, the yield of isolated hepatocytes was approximately 3 x 105 cells with a viability of greater than 80% as determined by trypan blue dye exclusion. The primary rat hepatocytes were further selected for experiments by seeding 1.5 x 105 cells on a 6-cm BD BioCoat dish (BD Biosciences) supplemented with low-glucose DMEM (Gibco), 5% FBS, 100 μ/l penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. After approximately 4 hours of incubation, non-adherent cells were washed twice with 1x PBS, and the resulting hepatocytes were cultured in the above DMEM medium for further treatment. For insulin treatment, cells were fasted in serum-free medium for 4 hours.

**Preparation of lentivirus.** pGIPZ-shRNA plasmids were purchased from Thermo Scientific. Lentivirus was packaged by the TransLenti Viral GIPZ Packaging System (Thermo Scientific). The day before transfection of pGIPZ plasmids, HEK293T cells were plated at a density of 5.5 x 106 cells per 100-mm plate. For each plate, 9 μg plasmids and 28.5 μg viral packaging mix were transfected into HEK293T cells by Arrest-In transfection reagent. Four hours after transfection, culture medium was replaced with regular DMEM. Transfection efficiency was then examined two days after transfection by the percentage of GFP-positive cells. Culture medium was collected at day 3 and centrifuged at 2,000 g for 20 minutes at 4°C. The virus-containing supernatant was kept at –80°C in aliquots.

**Protein extraction and immunoblotting.** For whole cell extracts, cells or homogenized mouse tissues were lysed in a buffer containing 50 mM Tris-
HCl (pH 8.0), 0.1 mM EDTA, 420 mM NaCl, 0.5% NP-40, 0.05% SDS, 10% glycerol, 1 mM dithiothreitol, 2.5 mM PMSF, 1 mM benzamidine, 1 mg/l aprotinin, and 0.1 mM of the calpain inhibitor ALLN (Santa Cruz Biotechnology Inc.). Supernatants were collected after centrifugation at 20,000 g for 20 minutes at 4°C. For nuclear extracts, cells or mouse tissues were first suspended in 5 volumes of buffer A (10 mM Tris-HCl pH 8.0, 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol, 2.5 mM PMSF, 1 mM benzamidine, 1 mg/l aprotinin), homogenized in a glass homogenizer until approxi-mately 90% cells were broken, and centrifuged at 2,000 g for 5 minutes at 4°C. The pellet was washed once with buffer A and resuspended in buffer C (20 mM Tris-HCl pH 8.0, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20% glycerol, 12% sucrose, 1 mM dithiothreitol, 2.5 mM PMSF, 1 mM benzamidine, and 1 mg/l aprotinin). This suspension of nuclei was rotated at 4°C for 40 minutes and centrifuged 20,000 g for 20 minutes at 4°C. Supernatant was dialyzed against 500 volumes of buffer D (20 mM Tris-HCl pH 8.0, 100 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 2.5 mM PMSF, and 1 mM benzamidine). After centrifugation at 20,000 g for 20 minutes at 4°C, the resulting supernatant was designated as nuclear extract. Protein concentrations were measured using a BCA kit (Pierce). A given amount of whole cell extract or nuclear extract was mixed with 5x SDS loading buffer (0.25 M Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 0.05% bromphenol blue, 500 mM dithiothreitol). After boiling for 3 minutes, the proteins were resolved by NuPage 4–12% Bis-Tris gel (Invitrogen) and transferred to nitrocellulose or PVDF membrane by iBlot Gel Transfer Kit (Invitrogen). After blocking in 5% nonfat milk in 1× TBST, the membrane was incubated with specific primary antibodies with appropriate dilution for 2 hours at room temperature and washed 3 times with 1× TBST (10 minutes each). Then, the membrane was incubated with the HRP-conjugated secondary antibodies (1:10,000 dilution) for 1 hour at room temperature. After washing 3 times with 1× TBST (10 minutes each), the HRP signals were visualized by the SuperSignal West Pico kit (Pierce) according to the manufacturer’s instructions.

*In vitro kinase assay.* Endogenous CDK8 proteins were immunoprecipitated from HEK293 cell nuclear extracts with anti-CDK8 antibody on protein A/G sepharose beads. Flag-tagged CDK8 proteins expressed in HEK293 cells by transient transfection were immunoprecipitated using anti-Flag M2 affinity gel (Sigma–Aldrich). After incubation for 2 hours at 4°C, protein A/G sepharose or anti-Flag beads were washed 3 times with IP buffer and mixed with GST proteins. The mixture was washed once with the kinase buffer (50 mM Tris–HCl pH 7.4, 50 mM NaCl, 10 mM MgCl₂, 1 mM MnCl₂, and 10% glycerol) and incubated in 50 μl of kinase buffer containing 50 μM ATP for 1 hour at 30°C. The reaction was terminated by boiling in 5x SDS loading buffer. The phosphorylation signals were detected using anti-specific p–p-TP and anti–p-S antibodies.

RNA preparation and quantitative PCR analysis. Total RNA was isolated from cells and mouse livers using the TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RNA concentration was measured by a NanoDrop spectrophotometer (Thermo). After removing genomic DNA with a QiA RNase-free DNase I (Promega), cDNA was synthesized by a First-Strand cDNA Synthesis Kit (GE Healthcare). Synthesized cDNA was diluted for real-time PCR. Each real-time PCR reaction mixture contained 10 μl FastStart universal SYBR Green Master mix (Roche), 1 μl primer (250 nM each), and 9 μl diluted cDNA. Real-time PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems). The cycling parameters consisted of 95°C incubation for 10 minutes for enzyme activation and DNA denaturation, followed by 40 PCR amplification cycles consisting of 95°C for 15 seconds and 60°C for 1 minute. The thermocycling program was followed by a melting program of 95°C for 15 seconds (denaturation), 60°C for 1 minute (annealing), and then 60–95°C at a transition rate of 0.3°C/s with continual monitoring of fluorescence.

Data analysis was performed by software provided with the StepOnePlus Real-Time PCR System. PCR primers are listed in Supplemental Table 2.

Mice treatment. Male C57BL/6J mice purchased from The Jackson Labora-tory were maintained under a 12-hour dark cycle with free access to water and standard mouse diet (10% calories from fat). For transient deletion of hepat-ic CDK8, 8- to 10-week-old mice were injected with adenovirus (~1.5 x 10⁸ infectious particles per mouse) expressing nonspecific shRNA (control) or CDK8 shRNA (Welgen Inc.) through tail veins. For de novo lipogenesis experiments, 9 days after viral injection, food was removed in the morning and mice received an i.p. injection of deuterated water (H₂O₂) containing 0.9% sodium chloride at a concentration of approximately 4% lean body mass. Mice were provided drinking water containing 4% H₂O₂. Five hours later, mice were sacrificed, and pieces of liver tissues and plasma were sent to the Metabolic Center at Albert Einstein College of Medicine to determine the palmitate levels by gas chromatography and mass spectrometry.

Metabolites. Plasma triglyceride, glycerol, and non-esterified fatty acid (NEFA) levels were measured using Infinity Triglycerides Reagent (Thermo Scientific), free glycerol reagent (Sigma–Aldrich), and HR Series NEFA-HR (Wako). Liver triglyceride levels were analyzed by an Adipogenesis Assay Kit (Biovision) and normalized by total protein levels.

Statistics. For quantitative measurement of mRNA, protein, triglycerides, oil red O staining, NEFA, glycerol, and insulin, the comparison of 2 differ-ent groups was carried out using 2-tailed unpaired Student’s t-test. Results are presented as mean ± SD. Differences was considered statistically sig-nificant when 𝑃 was less than 0.05.

Study approval. All experiments using mice and rats conformed to proto-cols approved by the Animal Care and Use Committee of Albert Einstein College of Medicine.

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