Cyclin C regulates adipogenesis by stimulating transcriptional activity of CCAAT/enhancer-binding protein α

Received for publication, January 10, 2017, and in revised form, March 27, 2017 Published, Papers in Press, March 28, 2017, DOI 10.1074/jbc.M117.776229

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Edited by George M. Carman

Brown adipose tissue is important for maintaining energy homeostasis and adaptive thermogenesis in rodents and humans. As disorders arising from dysregulated energy metabolism, such as obesity and metabolic diseases, have increased, so has interest in the molecular mechanisms of adipocyte biology. Using a functional screen, we identified cyclin C (CycC), a conserved subunit of the Mediator complex, as a novel regulator for brown adipocyte formation. siRNA-mediated CycC knockdown (KD) in brown preadipocytes impaired the early transcriptional program of differentiation, and genetic KO of CycC completely blocked the differentiation process. RNA sequencing analyses of CycC-KD revealed a critical role of CycC in activating genes co-regulated by peroxisome proliferator activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα). Overexpression of PPARγ2 or addition of the PPARγ ligand rosiglitazone rescued the defects in CycC-KO brown preadipocytes and efficiently activated the PPARγ-responsive promoters in both WT and CycC-KO cells, suggesting that CycC is not essential for PPARγ transcriptional activity. In contrast, CycC-KO significantly reduced C/EBPα-dependent gene expression. Unlike for PPARγ, overexpression of C/EBPα could not induce C/EBPα target gene expression in CycC-KO cells or rescue the CycC-KO defects in brown adipogenesis, suggesting that CycC is essential for C/EBPα-mediated gene activation. CycC physically interacted with C/EBPα, and this interaction was required for C/EBPα transactivation domain activity. Consistent with the role of C/EBPα in white adipogenesis, CycC-KD also inhibited differentiation of 3T3-L1 cells into white adipocytes. Together, these data indicate that CycC activates adipogenesis in part by stimulating the transcriptional activity of C/EBPα.

Adipose tissues play a pivotal role in energy homeostasis (1, 2). Because of the increased epidemic of obesity and metabolic diseases, a better understanding of the molecular events in adipocyte biology is important for treating this global health problem. As the major cell types in adipose tissues, white adipocytes are best known for energy storage, whereas uncoupling protein 1 (UCP1)-expressing[4] brown/beige adipocytes dissipate energy in the form of heat via dissociation of cellular respiration from ATP synthesis (3, 4). It has been shown that increased amounts and/or activity of brown/beige adipose tissues reduce metabolic defects in mice (5–8) and correlate with leanness in humans (9–11). Despite extensive studies of adipogenesis in the past, our understanding of the development of brown/beige adipocytes remains incomplete.

Transcriptional regulation is critical in adipogenesis; extracellular adipogenic stimuli activate a cascade of core transcription factors that control the differentiation of all types of adipocytes (2, 12, 13). Within this core transcriptional hierarchy, PPARγ is the master regulator, as it is both necessary and sufficient for adipogenesis (14–17). The induced early transcription factors include C/EBPβ, C/EBPδ, the glucocorticoid receptor (GR), STAT5A, and cAMP response element-binding protein (CREB) (2, 12, 13). These transcription factors then activate downstream transcription factors, including PPARγ and C/EBPα, which positively activate each other and coordinately regulate the expression of a large group of genes that define adipocyte phenotypes (2, 12, 13). Other transcription factors that modulate the expression or functions of the core adipogenic factors often affect adipogenesis. For example, EBF1 and EBF2 promote adipogenesis by activating the transcription of *Pparg* and *Cebpa* (18). ZFP423 determines white adipocyte cell fate by activating *Pparg* gene expression and suppressing EBF2 and PRDM16 functions (19, 20). EBF2 determines and

This work was supported in part by National Science and Technology Major Project of China Grant 2016ZX08006003 (to G. Y.) and the National Institutes of Health Grants P30 DK020541, R01 DK093623, DK098439, and DK110063. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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[4] The abbreviations used are: UCP, uncoupling protein; C/EBP, CCAAT/enhancer-binding protein; CycC, cyclin C; PPAR, peroxisome proliferator-activated receptor; qRT-PCR, quantitative RT-PCR; KD, knockdown; BAT, brown adipose tissue; NS, nonspecific; RNA-seq, RNA sequencing; IP, immunoprecipitation; TAD, transactivation domain; aa, amino acids; CDK, cyclin-dependent kinase; FAS, fatty acid synthase; ATGL, adipose triglyceride lipase; PPRE, peroxisome proliferator activated receptor responsive element.

maintains the identity of brown adipocytes by facilitating PPARγ binding to brown fat-specific binding sites during differentiation (21). The activities of these adipogenic transcription factors can be further regulated by various transcription cofactors or non-coding RNAs. For example, the PPARγ cofactor PGC1α controls mitochondrial biogenesis and respiration through induction of *Ucp1* and nuclear respiratory factors (22), and another PPARγ cofactor, PRDM16, has emerged as a brown and beige adipocyte determination factor (23, 24). Moreover, the microRNAs miR-133 and miR-155 regulate brown and beige adipocyte differentiation by targeting PRDM16 and C/EBPβ, respectively (25, 26). The long noncoding RNA Blnc1 in complex with EBF2 activates the thermogenic genes (27). Thus, overlapping and distinct transcriptional networks are involved in controlling white, brown, and beige adipogenesis.

The multisubunit protein complex Mediator is a transcriptional cofactor that was originally discovered in yeast (28–30) and is conserved in eukaryotes (31–33). The Mediator complex can be recruited by numerous transcription factors, including PPARγ (34–37) and C/EBPβ (38–40), and integrates cellular signals with the basal transcription machinery to activate or repress gene transcription (41–44). The Mediator complex as a whole may regulate a large number of genes, but each subunit displays remarkable context-dependent or pathway-specific functions (41–44). It has been shown that the Mediator subunits MED1 and MED14 directly bind to PPARγ and activate PPARγ-dependent transcription and adipogenesis (36, 37, 45). More recent studies show that MED1 activates the *Ucp1* gene in brown adipocytes by interacting with PRDM16 (46, 47). The Mediator subunit MED23 promotes white adipogenesis through ELK1 (40, 48). These studies suggest the involvement of the Mediator complex in adipogenesis, but the underlying mechanisms are distinct for different subunits. It is currently unclear whether other Mediator subunits also regulate adipogenesis.

In this study, we identify cyclin C (CycC) as a novel regulator of adipogenesis. Unlike classical cyclins, CycC does not directly regulate the cell cycle but acts as a conserved subunit of the Mediator complex. Here we show that CycC is essential for adipogenesis. In contrast to MED1, MED14, and MED23, CycC functions primarily to support C/EBPα-mediated gene expression in adipogenesis.

Results

Identification of novel regulators of brown adipogenesis

To study brown adipocyte biology, we established mouse brown preadipocyte cell lines according to protocols published previously (49, 50). Selection of single clones was based on the efficiency of lipid accumulation as assayed by oil red O staining (Fig. 1*A*) as well as the induction of marker genes (*i.e.* the common adipocyte marker *Fabp4*, Fig. 1*B*, and the brown adipocyte-specific marker *Ucp1*, Fig. 1*C*) as detected by qRT-PCR in the presence of differentiation mixture (49, 50). To identify novel regulators that control the formation of brown adipocytes, we transduced immortalized brown preadipocytes with a lentiviral library of shRNA, which included a total of 4551 protein-coding genes (Fig. 1*D*), and six or more independent sequences of shRNA were designed to target each gene. Before induction of differentiation, a small portion of those transduced cells was collected to determine the initial abundance of each shRNA in the library (*i.e.* control), and the remaining cells were cultured to confluence, followed by induction for 8 days (Fig. 1*D*). Taking advantage of the floating nature of lipid-filled adipocytes, we were able to isolate undifferentiated cells, which had little lipid droplets and were adherent, by two rounds of trypsin digestion and replating (Fig. 1*D*). We then extracted the genomic DNA from undifferentiated cells that were collected before and after induction. The gene identities were determined according to shRNA barcode sequences that were amplified by PCR and analyzed by Illumina next-generation sequencing (Fig. 1*D*).

Because shRNA-mediated knockdown (KD) may have off targets, we used the following cutoff to define a positive hit from this screen. Compared with the control, at least two different shRNA clones were enriched more than 1.5-fold, but none of the shRNA clones for that gene had less than a 0.3-fold reduction. Using these criteria, we identified a total of 543 genes (or 11.9%) as positive hits (data not shown). Functionally, those genes are involved in several biological processes, including cellular metabolism, macromolecule biosynthesis, regulation of gene expression, and protein modifications (Fig. 1*E*). Several genes that have been reported previously as regulators of adipogenesis, including *Cebpa* (17), *Cebpb* (51, 52), *Ebf1* (18), *Rbl1* (53), and *Stat5a* (54, 55), were among the positive hits (data not shown), indicating the validity of this screen.

CycC is down-regulated during brown adipogenesis and aging

Consistent with the critical role of gene transcription in adipogenesis, DNA-binding proteins or chromatin modifiers were particularly enriched among positive hits in this screen (a total of 156 genes or 28.7%, data not shown). Among them, 14 genes are transcription cofactors (Fig. 1*F*), including *Ccnc*, which encodes CycC. Although previous studies have reported that the Mediator subunits MED1 (36, 45), MED14 (37), and MED23 (40, 48) regulate either white adipogenesis or brown adipocyte-specific genes, the role of CycC in adipogenesis has not been investigated.

First, we examined the protein levels of CycC and its known partners CDK8 and CDK19, which are mutually exclusive subunits of the Mediator complex (56), during the differentiation of brown preadipocytes. As shown in Fig. 2*A*, during differentiation, CycC and CDK19 proteins were down-regulated, but CDK8 proteins had a trend of increase. Because aging affects brown fat, we compared the protein abundance in BAT between young (3 months old) and aged (24 months old) C57Bl/6J mice. As shown in Fig. 2, *B* and *C*, the protein levels of CycC and CDK19, but not CDK8, were significantly lower in BAT from aged mice. As expected, aging also reduced the UCP1 proteins (Fig. 2, *B* and *C*). Cold exposure is known to enhance BAT activity. Thus, we also examined CycC levels upon cold exposure. As shown in Fig. 2*D*, cold exposure at 4 °C for 6 h induced the expression of *Ucp1* and *Pgc1a* in BAT, as expected, but did not affect *Ccnc*, *Cdk8*, and *Cdk19* compared with room temperature (22 °C). At the protein level, cold expo-

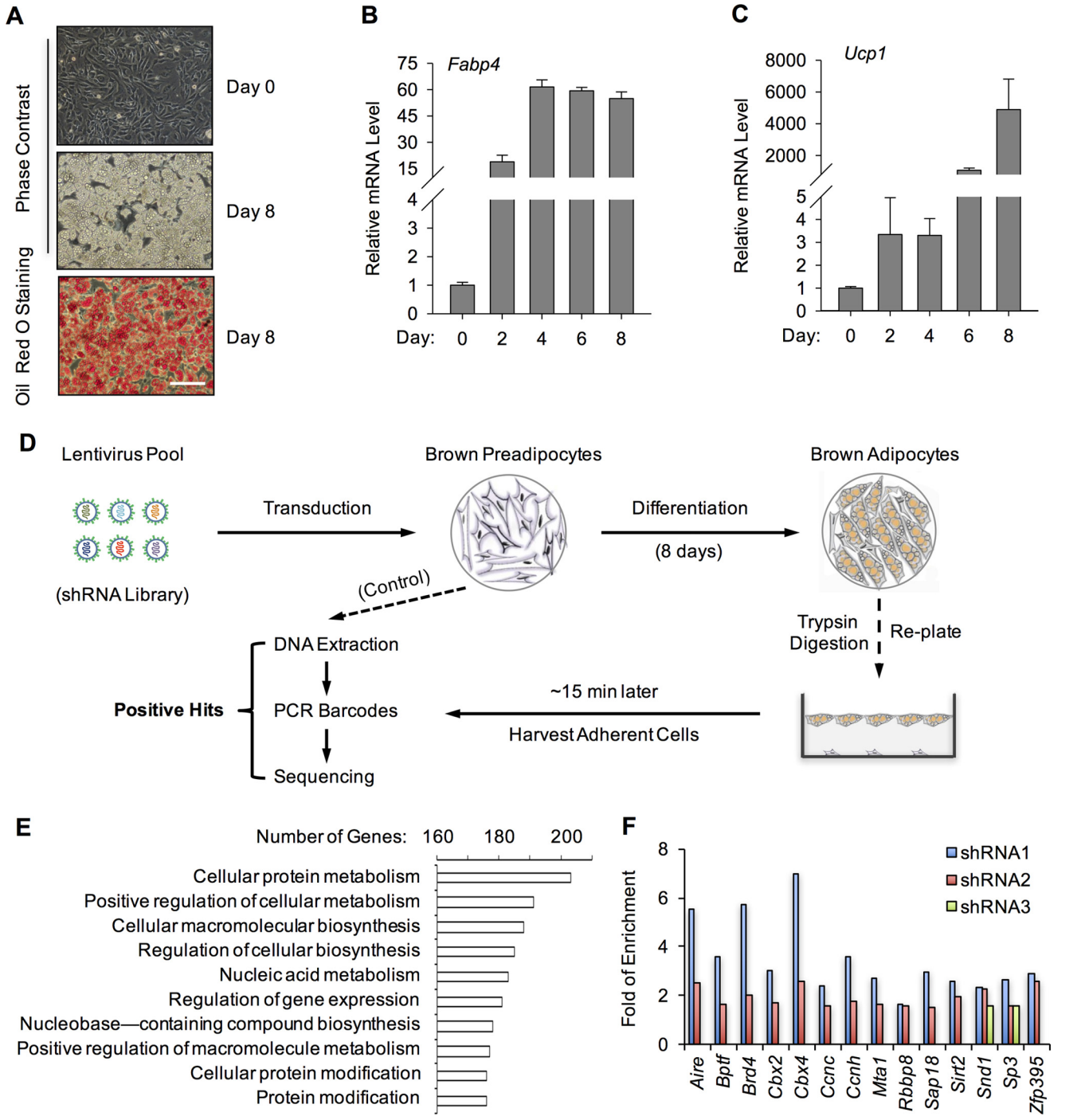


Figure 1. Screen for novel regulators of brown adipogenesis. *A–C*, establishment of mouse brown preadipocyte lines (*A*) and qRT-PCR analyses of *Fabp4* (*B*) and *Ucp1* (*C*) mRNA levels during differentiation. *D*, the shRNA screen scheme. *E*, biological process analyses of positive hits. *F*, enriched transcriptional cofactors.

sure slightly down-regulated CycC and CDK19 but increased CDK8 protein levels (Fig. 2*E*). Thus, different from what we observed in the liver (57), in BAT, CycC and CDK19, but not CDK8, appear to be coordinately regulated.

Although the best-known function of CycC is to be a subunit of the Mediator complex, recent studies have shown that CycC can also function in the cytoplasm to regulate mitochondrial fission (58, 59). Therefore, we examined the subcellular location of CycC in brown adipocytes by immunostaining. As shown in Fig. 2*F*, CycC proteins were exclusively located in the nucleus in brown preadipocytes. Although a small portion of CycC proteins became diffused in the cytoplasm after differentiation, the majority of CycC proteins were still in the nucleus (Fig. 2*F*). These data suggest that, in brown adiopocytes, CycC acts predominantly in the nucleus.

CycC is essential for brown preadipocyte differentiation

To verify the shRNA screen results, we transfected brown preadipocytes with a pool of siRNAs that were designed to target four different sequences of *Ccnc* mRNA for reducing off

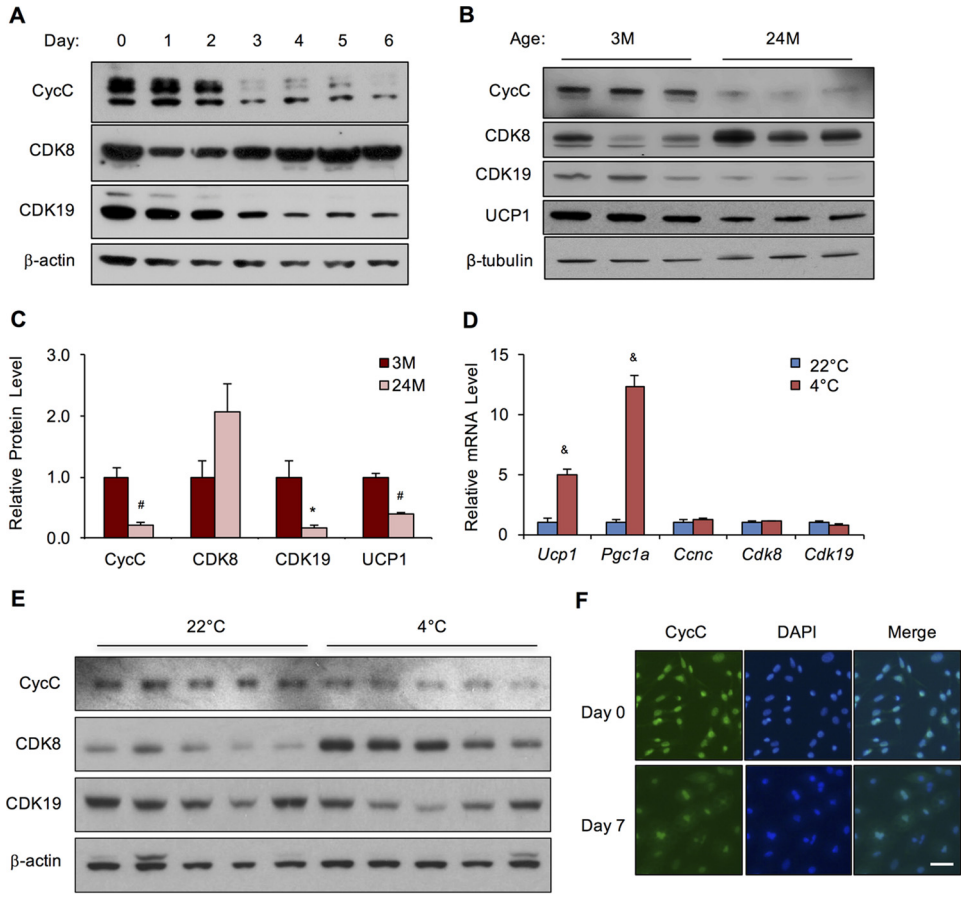


Figure 2. CycC in brown fat. *A* and *B*, immunoblotting of the indicated proteins during brown preadipocyte differentiation (*A*) and in BAT from 3- or 24-month-old (*M*) old male C57Bl/6J mice (*B*). *C*, densitometry data of *B* (*, $p < 0.05$; #, $p < 0.01$ *versus* 3 M; $n = 3$). *D* and *E*, real-time RT-PCR (*D*) and immunoblotting (*E*) analyses of the indicated genes at 22 °C and 4 °C (6-h exposure; &, $p < 0.001$ *versus* 22 °C; $n = 5$). *F*, CycC immunostaining of brown preadipocytes before (*Day 0*) and after (*Day 7*) differentiation. *Scale bar* = 50 μm.

targets. Nonspecific (NS) siRNA was used as a control. The CycC-KD efficiency was determined by qRT-PCR (Fig. 3*A*) and immunoblotting (Fig. 3*B*). The siRNA-treated cells were cultured until confluent and induced with differentiation mixture. After 2 days of induction, CycC-KD significantly inhibited the expression of adipocyte-enriched aP2, FAS, and ATGL proteins, whereas other markers, such as PPARγ1, C/EBPα, and Caveolin, were less affected (Fig. 3*C*). As shown in Fig. 3*D*, CycC-KD significantly inhibited cellular lipid accumulation, as examined by oil red O staining. Interestingly, CycC-KD resulted in a mixed pattern of gene expression. For common adipocyte markers, initially CycC-KD cells expressed significantly lower levels of *Pparg*, *Fabp4* (encoding aP2), *Cebpa*, *Adipoq* (encoding Adiponectin), and *Cd36* during 2 days of induction (Fig. 3, *E* and *F*), but after 5 days of induction, these differences vanished (Fig. 3, *E* and *F*). These data suggest that CycC may act primarily at the early stage(s) of adipogenesis. For lipogenic genes, such as *Srebf1c* and *Fasn*, and other genes, including *Acc1*, *Atgl*, *Hsl*, and *Slc2a4* (encoding GLUT4), CycC-KD reduced their expression throughout the differentiation process (Fig. 3*F*). Moreover, CycC-KD also significantly reduced the expression of most brown adipocyte-specific marker genes, including *Ucp1*, *Cidea*, *Otop1*, and *Fgf21* after 5 days of differentiation (Fig. 3*G*), suggesting that CycC is required to activate the brown adipocyte transcription program. Interestingly, *Prdm16*, the key activator of the brown adipocyte transcription program, was not affected by CycC-KD (Fig. 3*G*).

Because of the transient nature of siRNA-mediated KD and partial reduction of CycC proteins, we generated CycC-KO brown preadipocytes by treating brown preadipocytes isolated from $Ccnc^{flox/flox}$ mice with lentiviruses expressing Cre proteins. CycC-KO was verified by qRT-PCR using a pair of exon-specific primers (Fig. 4*A*). As shown in Fig. 4*B*, CycC-KO cells completely lost their adipogenic capability, as examined by oil red O staining for lipid accumulation. Consistently, gene expression analyses by qRT-PCR revealed that, in CycC-KO cells, the differentiation mixture was unable to induce general adipocyte makers, including *Pparg*, *Cebpa*, *Fabp4*, and *Adipoq*, brown adipocyte-specific markers, and mitochondrial genes, including *Ucp1*, *Cidea*, *Otop1*, *Fgf21*, *Cox5b*, and *Cox8b*, and lipogenic genes, including *Srebf1c* and *Fasn* (Fig. 4, *C* and *D*). At the protein level, CycC-KO almost completely abolished the induction of key brown and general adipocyte markers, including PPARγ, C/EBPα, UCP1, aP2, FAS, ATGL, GLUT4, and Caveolin (Fig. 4*E*). These results indicate that CycC is essential for brown preadipocyte differentiation *in vitro*.

To determine whether CycC overexpression in CycC-KO cells could rescue the adipogenic defects, we treated cells with retroviruses expressing CycC. The levels of CycC were exam-

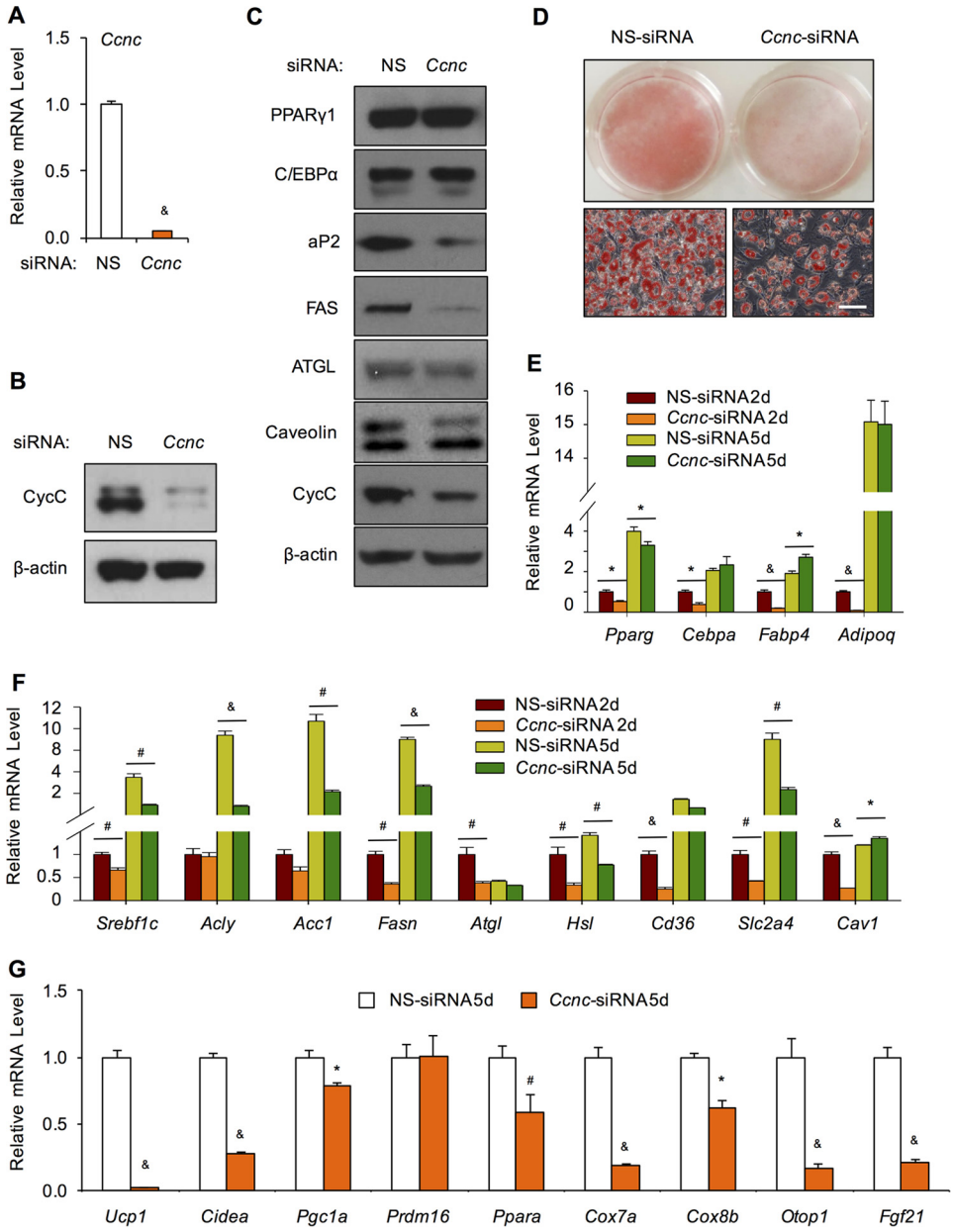


Figure 3. CycC-KD impairs brown adipogenesis. *A*, qRT-PCR analyses of CycC-KD. *B* and *C*, immunoblotting of the indicated proteins in siRNA-treated cells before (*B*) or after 2 days of differentiation (*C*). *D*, oil red O staining of siRNA-treated cells after day 5 of differentiation. *Scale bar* = 100 μm). *E*—*G*, qRT-PCR analyses of CycC-KD effects at day 2 and/or day 5 of brown preadipocyte differentiation. *, $p < 0.05$; #, $p < 0.01$; &, $p < 0.001$ *versus* NS-siRNA ($n = 3$).

ined by qRT-PCR (Fig. 4*F*) and immunoblotting (Fig. 4*G*). As shown in Fig. 4, *H* and *I*, CycC overexpression partially rescued the gene expression and triglyceride accumulation defects in CycC-KO cells, supporting a specific role of CycC in brown adipogenesis.

To determine whether CycC overexpression in WT brown preadipocytes could conversely enhance adipogenesis, we treated cells with CycC-expressing retroviruses. Surprisingly, although the mRNA levels of *Ccnc* were increased by 2.5-fold, adipogenesis was not significantly affected (Fig. 4, *K*–*M*). Immunoblotting revealed that CycC overexpression reduced endogenous CycC and, therefore, restricted the increase in the total amount of CycC proteins (Fig. 4*N*).

CycC activates the core adipogenic program

To understand the molecular mechanism(s) underlying CycC regulation of brown adipogenesis, we performed RNA-seq analyses to determine the effects of CycC-KD on genome-wide gene expression at day 2 of differentiation. As shown in Fig. 5*A*, of a total of 10,852 transcripts that were detected, CycC-KD resulted in up-regulation (>1.5-fold as cutoff) of 789 genes (or 7.3%) and down-regulation (<0.5-fold as cutoff) of 633 genes (or 5.8%). These data indicate that, although the Mediator complex as a whole may control the expression of many genes, the CycC subunit only regulates a small subset of genes in brown adipocytes. Biological pathway analyses revealed that the most significantly up-regulated pathways

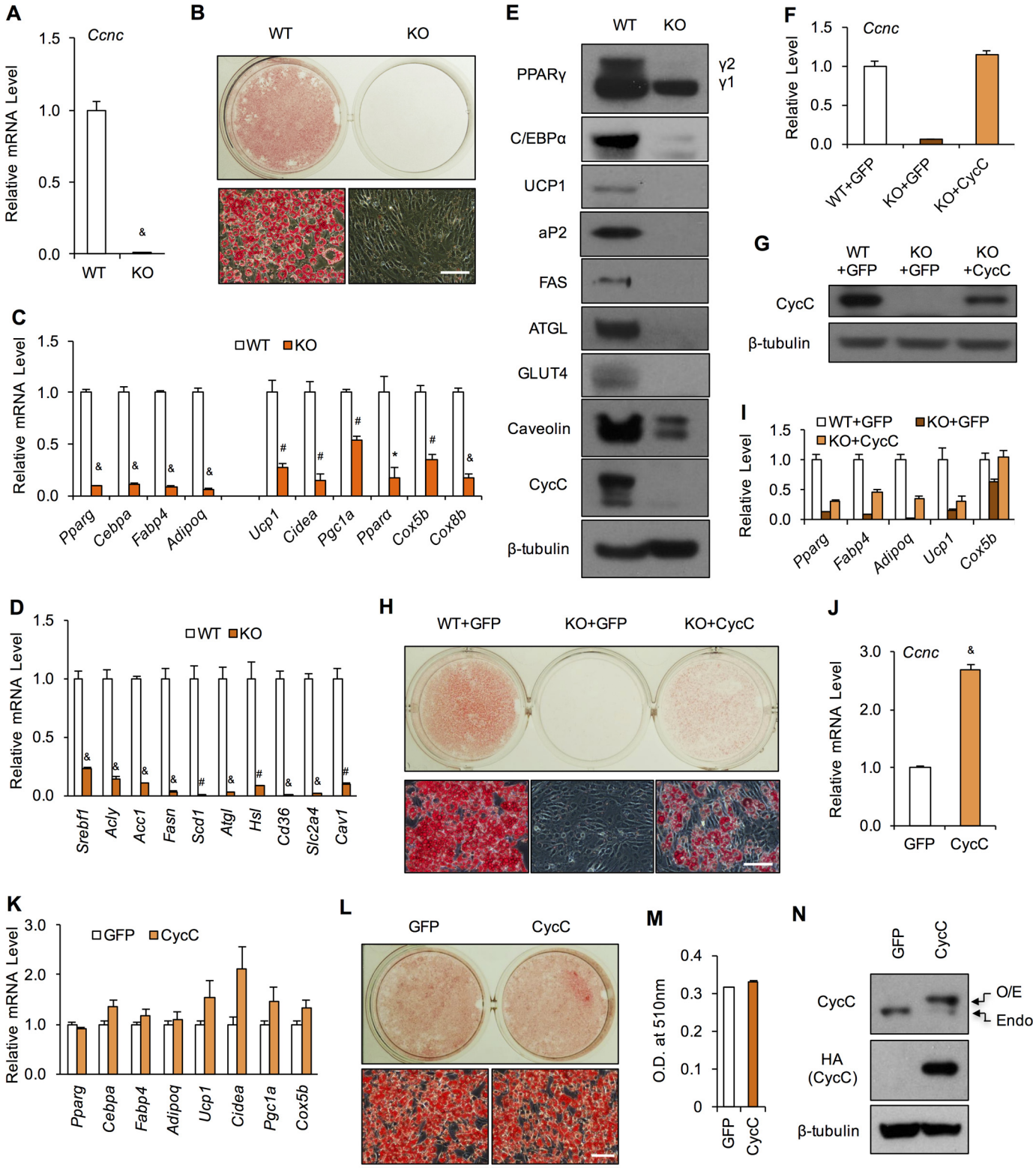


Figure 4. CycC-KO blocks brown adipogenesis. *A*, qRT-PCR analyses of *Ccnc* in brown preadipocytes. *B*—*E*, oil red O staining (*B*), qRT-PCR (*C* and *D*) and immunoblotting (*E*) analyses of CycC-KO on brown preadipocyte differentiation at day 8. *F* and *G*, CycC overexpression in CycC-KO brown preadipocytes analyzed by qRT-PCR (*F*) and immunoblotting (*G*). *H* and *I*, effects of CycC overexpression on differentiation in CycC-KO brown preadipocytes at day 10 as analyzed by oil red O staining (*H*) and qRT-PCR (*I*). *J*—*M*, effects of CycC overexpression in WT brown preadipocytes (*J*) on adipogenesis at day 7 as analyzed by qRT-PCR (*K*), oil red O staining (*L*), and semiquantitative measurement of lipids (*M*). *N*, immunoblotting analyses of CycC overexpression in WT brown preadipocytes. *Scale bars* = 100 μm. *, $p < 0.05$; #, $p < 0.01$; &, $p < 0.001$ *versus* control ($n = 3$).

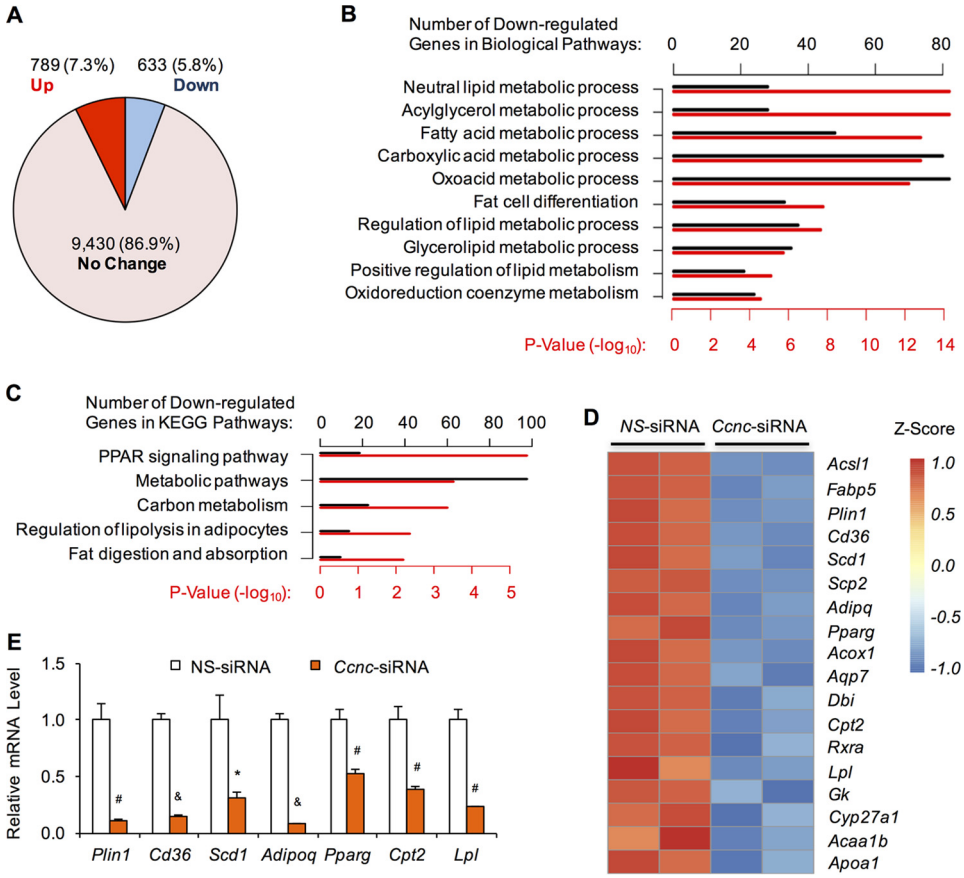


Figure 5. CycC is required to activate the PPAR pathway in brown adipogenesis. *A*, number of genes affected by CycC-KD at day 2 of differentiation. *B*, biological process analyses of CycC-KD down-regulated genes (*black bars*, numbers of genes; *red bars*, *p* values). *C*, KEGG pathway analyses of CycC-KD down-regulated genes. *D*, heat map of down-regulated genes in the PPAR pathway. *E*, qRT-PCR analyses of indicated genes. #, $p < 0.01$; &, $p < 0.001$ *versus* NS-siRNA ($n = 3$).

included regulation of cell migration, cell surface receptor signaling, cell motility, and cell development (data not shown), and the most significantly down-regulated pathways were involved in lipid metabolism and fat cell differentiation (Fig. 5*B*), indicating that gene down-regulation is the primary cause of the phenotype observed after CycC-KD. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses revealed that the most significantly down-regulated pathway was the PPAR signaling pathway (Fig. 5*C*). In fact, CycC-KD reduced the expression of many known PPARγ-target genes, including *Pparg*, *Plin1*, *Cd36*, *Adipoq*, and *Lpl* (Fig. 5*D*). Down-regulation of PPARγ target genes was also verified by qRT-PCR analyses (Fig. 5*E*).

CycC regulation of brown adipogenesis is not dependent on PPARγ transcriptional activity

Previous studies have demonstrated that the Mediator subunits MED1 (36, 45) and MED14 (37) are required to activate PPARγ-mediated gene transcription. To determine whether CycC is also required for PPARγ-dependent gene expression in brown adipocytes, we overexpressed PPARγ2 in WT and CycC-KO brown preadipocytes by retroviral transduction (Fig. 6*A*). As shown in Fig. 6*B*, overexpression of PPARγ2 was able to efficiently rescue CycC-KO defects of adipogenesis, as examined by oil red O staining. Consistent with the rescue of lipid accumulation, overexpression of PPARγ2 also rescued key marker gene expression (Fig. 6*C*). Similarly, addition of the PPARγ ligand rosiglitazone to the culture medium also rescued the CycC-KO-caused defects in adipogenesis (Fig. 6*D*) and key marker gene expression (Fig. 6*E*). These results suggest that CycC is not required to maintain PPARγ transcriptional activity. Indeed, PPARγ2 overexpression could similarly induce endogenous PPARγ target genes, such as *Cd36* and *Adipoq*, in the presence or absence of CycC (Fig. 6*F*). Furthermore, PPARγ2 overexpression by transient transfection could activate a synthetic PPAR-responsive (PPRE) promoter in both WT and CycC-KO brown preadipocytes (Fig. 6*G*). Interestingly, overexpressed PPARγ2 was even more active in KO cells (Fig. 6*G*). Together, these data indicate that CycC is not an activator of PPARγ-dependent transcription.

CycC is required for C/EBPα-dependent transcription in brown preadipocytes

A previous study has shown that PPARγ and C/EBPα co-regulate a series of genes, including adipocyte markers, in adipogenesis (60). We compared CycC-regulated genes with those defined PPARγ and/or C/EBPα target genes (60). As shown in Fig. 7*A*, nearly half of PPARγ-C/EBPα co-regulated genes were down-regulated upon CycC-KD. Thus, during brown adipogenesis, CycC is critically required to activate a subset of genes that are co-regulated by PPARγ and C/EBPα. Because CycC-KO did not attenuate PPARγ transcriptional activity, we

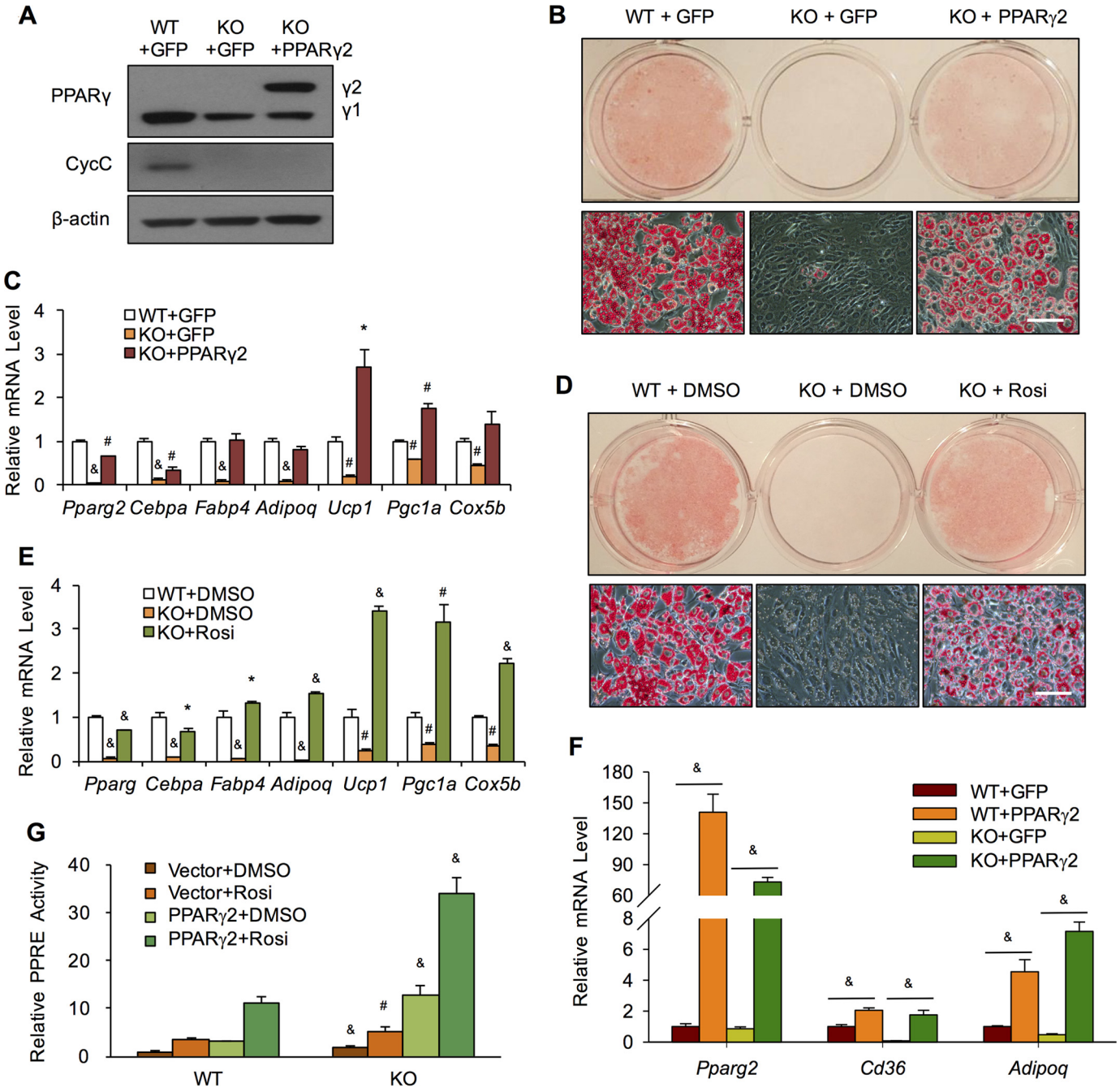


Figure 6. CycC is not required for PPARγ to activate targets in brown adipocytes. *A*—*E*, effects of PPARγ2 overexpression (*A*) or treatment with rosiglitazone (*Rosi*, 0.5 μM) in CycC-KO brown preadipocytes on differentiation (day 8) as analyzed by oil red O staining (*B* and *D*) (*scale bars* = 100 μm) and qRT-PCR (*C* and *E*). *F* and *G*, effects of PPARγ2 overexpression on the indicated genes (*F*) and the activity of a synthetic PPARγ-responsive promoter (PPRE) (*G*) in WT and KO cells. *, $p < 0.05$; #, $p < 0.01$, and &, $p < 0.001$ *versus* WT ($n = 4$).

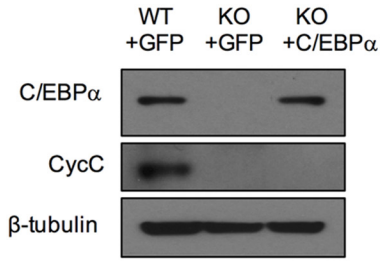
asked whether CycC regulates the transcriptional activity of C/EBPα. To this end, we examined the ability of C/EBPα to activate its target genes by overexpression (Fig. 7*B*). As shown in Fig. 7*C*, C/EBPα overexpression by retroviral transduction robustly induced endogenous C/EBPα target genes such as *Pparg2* in WT, but not CycC-KO, brown preadipocytes, although C/EBPα was overexpressed similarly in terms of -fold increase. These data suggest that CycC may be important for the transcriptional activity of C/EBPα. Consistent with gene expression, C/EBPα overexpression could not rescue the differentiation defects in CycC-KO cells (Fig. 7, *D* and *E*). Thus, our results indicate that CycC stimulates brown adipogenesis at least in part through activating C/EBPα-mediated gene expression.

Similar to CycC regulation of endogenous genes, overexpression of C/EBPα in CycC-KO cells was significantly less efficient to activate a synthetic C/EBP-responsive promoter (3×CBE-Luc) in luciferase reporter assays (Fig. 7*F*). By co-immunoprecipitation (co-IP) analyses, we found that CycC physically interacted with C/EBPα (Fig. 7*G*). Consistent with the transcriptional cofactor role of CycC, the transactivation domain (TAD) of C/EBPα is about 50% as active in CycC-KO cells as in

A

Binding Sites for	# of Genes			Total # Of Genes
	Up	Down	N.C.	
PPARγ	0	4	13	17
C/EBPα	8	44	132	184
Both	**11**	**204**	**208**	**423**

B



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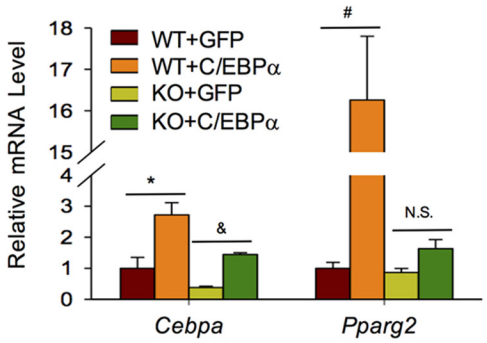

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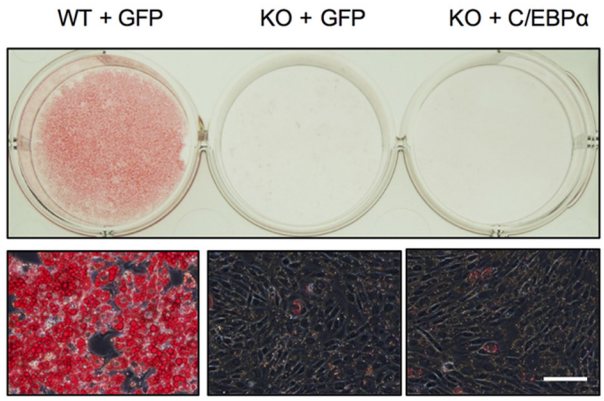

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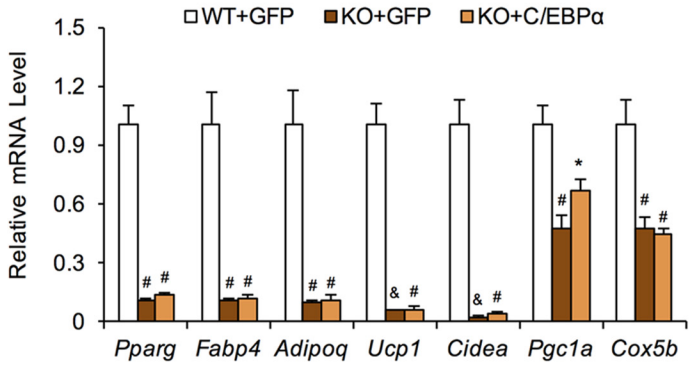

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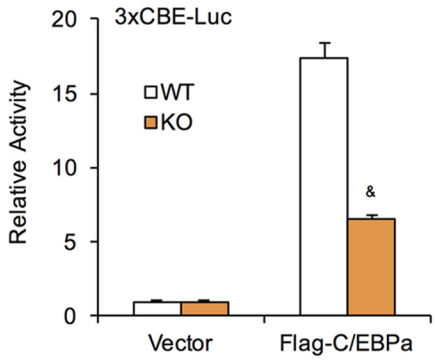

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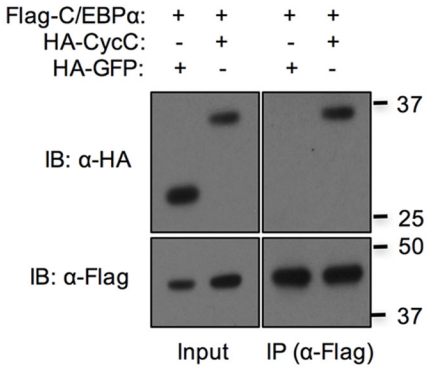

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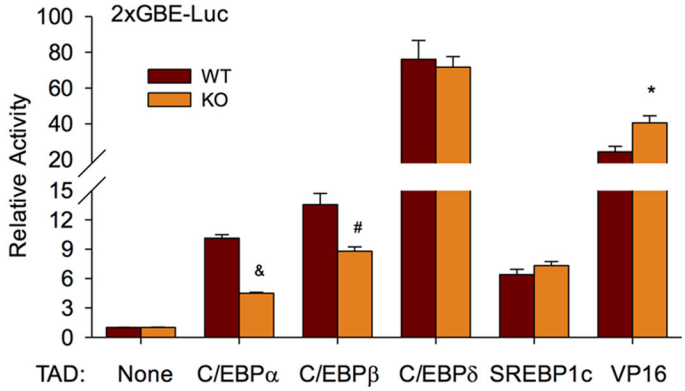

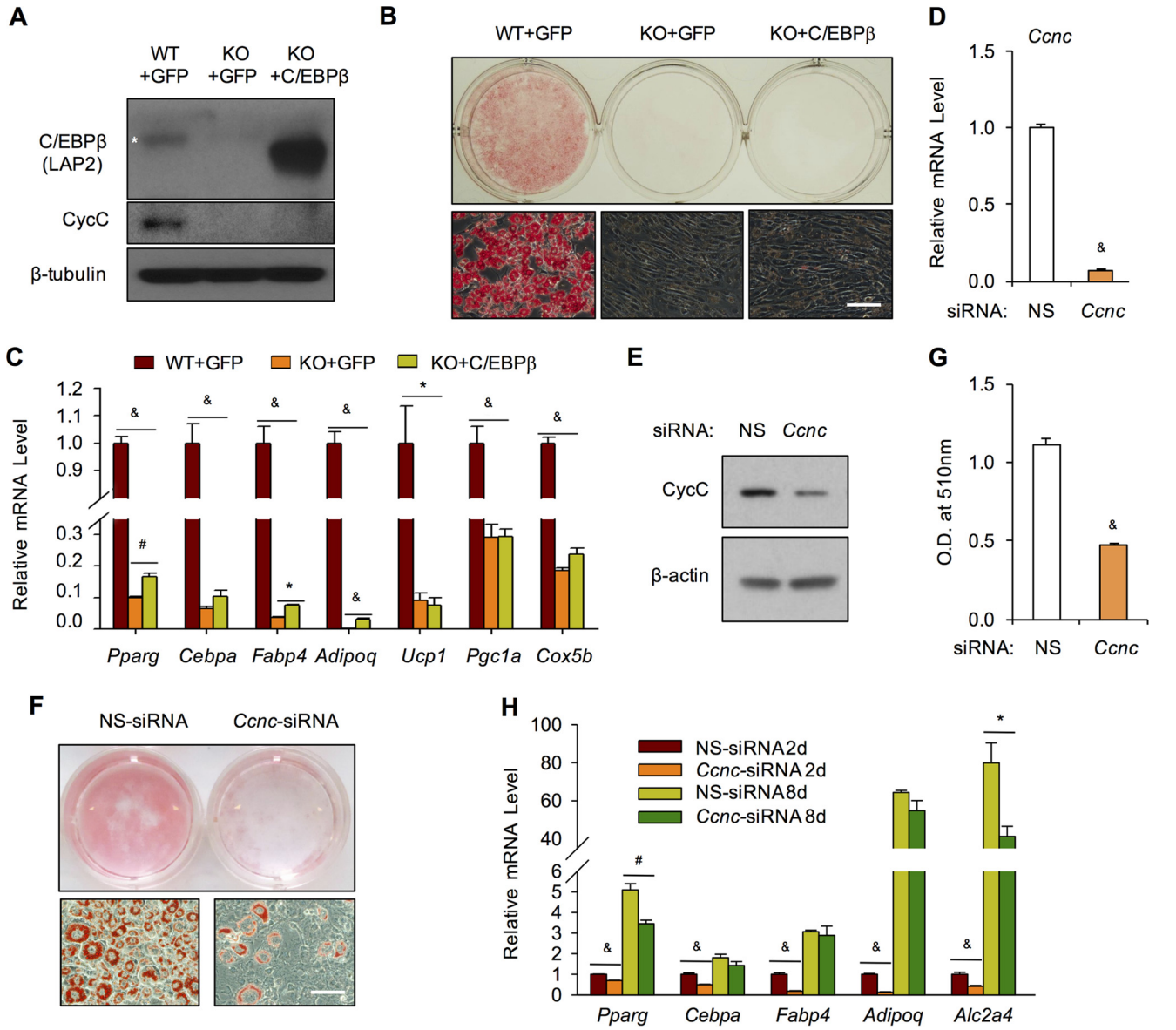

Figure 8. CycC-KD impairs white adipogenesis. *A*—*C*, effects of C/EBPβ overexpression (*A*; *asterisk*, nonspecific signal) in CycC-KO brown preadipocytes on differentiation (day 8) as assayed by oil red O staining (*B*) and qRT-PCR (*C*). *D*—*H*, effects of CycC-KD (*D* and *E*) on 3T3-L1 cell differentiation (day 8) as analyzed by oil red O staining (*F*), semiquantitative measurement of lipids (*G*), and qRT-PCR (*H*). *Scale bars* = 100 μm. *, $p < 0.05$; #, $p < 0.01$; &, $p < 0.001$ *versus* control ($n = 3$).

WT cells in luciferase reporter assays (Fig. 7*H*). CycC-KO in brown preadipocytes did not affect the TAD activities of C/EBPδ and SREBP1c but slightly reduced C/EBPβ-TAD activity and increased VP16-TAD activity (Fig. 7*H*). Together, our data suggest that CycC is required for C/EBPα-TAD activity.

C/EBPβ is an important transcription factor upstream of C/EBPα in adipogenesis. We wondered whether overexpression of C/EBPβ could rescue the defects in CycC-KO cells. To this end, we transduced brown preadipocytes with retroviruses expressing C/EBPβ (LAP2) (Fig. 8*A*). As shown in Fig. 8, *B* and *C*, C/EBPβ overexpression was unable to rescue the CycC-KO-caused defects in brown adipogenesis.

CycC regulates white adipogenesis

Because C/EBPα is an important activator for white adipocyte formation, we speculate that CycC may be also required for white adipogenesis. To test this hypothesis, we transfected 3T3-L1 cells with the *Ccnc* or NS siRNA pool, and CycC-KD efficiency in 3T3-L1 cells was analyzed by qRT-PCR (Fig. 8*D*) and immunoblotting (Fig. 8*E*). The siRNA-treated cells were

Figure 7. CycC is required for C/EBPα transcriptional activity. *A*, number of defined PPARγ and/or C/EBPα target genes affected by CycC-KD. *B*—*F*, effects of C/EBPα overexpression (*B*) in CycC-KO brown preadipocytes on gene expression (*C*), differentiation (day 8) as analyzed by oil red O staining (*D*) (*scale bars* = 100 μm) and qRT-PCR (*E*), and the activity of a synthetic C/EBP-responsive promoter (*3×CBE*) (*F*). *G*, co-IP analyses of HA-tagged CycC binding to FLAG-tagged C/EBPα in HEK293T cells. *H*, effects of CycC knockout on the activities of indicated TADs by luciferase reporter assays. *, $p < 0.05$; #, $p < 0.01$; &, $p < 0.001$ *versus* WT ($n = 4$).

cultured until confluent and then treated with differentiation mixture. As shown in Fig. 8*F*, CycC-KD significantly inhibited the formation of adipocytes, as examined by oil red O staining. Quantitative measurements further confirmed that CycC-KD reduced lipid accumulation during 3T3-L1 cell differentiation (Fig. 8*G*). Similar to what we observed in brown adipocytes, CycC-KD in 3T3-L1 cells also reduced the expression of key adipocyte markers, such as *Pparg*, *Fabp4*, *Cebpa*, and *Adipoq*, at the early stages (day 2) of adipogenesis (Fig. 8*H*). Thus, our data indicate that CycC is also required for white adipogenesis.

Discussion

In this study, we performed a loss-of-function screen to search for novel regulators of brown adipogenesis. Because this screen was based on the floating nature of lipid-rich adipocytes (Fig. 1*D*), the resulting positive hits may represent various mechanisms of action that could disturb the formation of mature brown adipocytes, including regulation of cell differentiation, fatty acid biosynthesis, and lipid droplet formation. The validity of this screen is evidenced by the presence of numerous reported regulators of adipogenesis among positive hits (data not shown) as well as the relatively low enrichment rate of positive hits (~12%). From the screen, we identified a number of genes that are potential regulators for BAT development. Particularly enriched are transcription factors, cofactors, and regulators of protein modifications, such as ubiquitination (data not shown). Thus, future studies are necessary to determine whether those genes play a role in the development of BAT.

One positive hit of the screen was the CycC subunit of the Mediator complex. Independent experiments confirmed that CycC-KD in brown preadipocytes impairs brown adipogenesis (Fig. 3). More strikingly, CycC-KO in brown preadipocytes completely abolished the ability to differentiate into mature brown adipocytes (Fig. 4), demonstrating the essential role of CycC for brown adipocyte formation *in vitro*.

The Mediator complex functions primarily as a transcriptional cofactor in eukaryotic cells (41, 44). Recent studies show that CycC also has functions outside of the Mediator complex (58, 59). However, our data suggest that CycC in brown preadipocytes functions most likely in the context of the Mediator complex. First, CycC proteins are predominantly located in the nuclei in brown fat cells (Fig. 2*F*). Second, it is difficult to increase CycC protein levels in brown preadiocytes (Fig. 4*N*), as CycC overexpression results in a reduction of endogenous CycC, likely because of the degradation of both endogenous and overexpressed CycC proteins. These data suggest that only CycC proteins in the Mediator complex are relatively stable, like in cancer cells (61).

The best known binding partners of CycC are CDK8 and CDK19 (56). Interestingly, our data suggest that CycC regulation of brown adipogenesis may be through the CDK19-Mediator complex, as CycC and CDK19, but not CDK8, in brown adipocytes are changed similarly at the protein level during *in vitro* differentiation and aging or cold exposure in mice. However, whether CDK19 and/or CDK8 are also involved in adipogenesis needs to be investigated in the future.

To elucidate the molecular mechanism(s) underlying CycC regulation of brown adipogenesis, we analyzed gene expression by RNA-seq. Our RNA-seq data show that CycC-KD affects ~13% of the expressed transcripts in brown adipocytes during differentiation (Fig. 5*A*). These data indicate that CycC regulation of gene transcription is relatively specific. Further supporting this conclusion, CycC depletion did not significantly affect brown preadipocyte growth (data not shown). Although previous studies have implicated the Mediator subunits MED1 (36, 45), MED14 (37), and MED23 (40, 48) in the adipogenic transcriptional program, the regulatory mechanisms are clearly distinct for different subunits. Therefore, the Mediator complex likely plays roles at multiple stages of adipogenesis through different transcription factors.

Among CycC-regulated transcripts, the PPAR pathway was the top down-regulated pathway. Nearly half of the known PPARγ target genes were significantly down-regulated upon CycC depletion. One assumption is that, like MED1 and MED14, CycC is required for the transcriptional activity of PPARγ. However, overexpressed PPARγ2 was able to efficiently activate both endogenous and synthetic PPARγ-responsive promoters in CycC-KO cells, indicating a dispensable nature of CycC for PPARγ-dependent gene expression. In fact, CycC-KO resulted in an increase of overall PPARγ transcriptional output (Fig. 6*G*). Although the mechanism(s) underlying such an increase are currently unclear and need to be further investigated, CycC regulation of PPARγ is not likely to be the cause of defects in adipogenesis that results from CycC-KO. Supporting this conclusion, overexpression of PPARγ2 or addition of the PPARγ ligand rosiglitazone could efficiently rescue the differentiation defects in CycC-KO cells. Interestingly, CycC-KD did not affect the expression of those same genes at a later stage of differentiation (Fig. 3*E*). One possibility is that self-activation of PPARγ and/or increased overall PPARγ transcriptional activity in CycC-KO cells resulted in this "catch-up expression" of PPARγ target genes. Alternatively, it is also possible that, in mature brown adipocytes, CycC may regulate both positive and negative events of gene transcription, neutralizing the overall outcome. Considering the fact that CycC proteins are down-regulated during brown preadipocyte differentiation (Fig. 2*A*), CycC may be important primarily at the early stages of differentiation. Nevertheless, different from MED1 or MED14 regulation of adipogenesis, multiple lines of evidence argue against a primary role of PPARγ in CycC regulation of brown adipogenesis.

Because CycC regulation of PPARγ could not explain CycC-KO phenotypes in brown adipogenesis, an alternative mechanism is that CycC regulates PPARγ target genes through other transcription factors, as any given promoter is often controlled by multiple transcription factors. It has been shown that most PPARγ target genes in adipogenesis are also co-regulated by C/EBPα (60). We examined whether CycC regulates C/EBPα. Indeed, CycC-KO significantly reduced C/EBPα transcriptional activity (Fig. 7, *C* and *F*). Mechanistically, CycC (likely in the context of the Mediator complex) physically binds to C/EBPα (Fig. 7*G*) and supports its TAD activity, although CycC may also regulate other transcription factors, such as C/EBPβ (Fig. 7*H*). Because C/EBPα can activate itself, its mRNA levels were also decreased in CycC-KO cells. Moreover, overexpression of C/EBPα could induce known PPARγ-C/

EBPα target gene expression only in WT cells but not in CycC-KO cells. Different from the results of increasing PPARγ level/activity, overexpression of C/EBPα could not rescue CycC-KO-caused differentiation defects in brown preadipocytes, suggesting that CycC regulation of C/EBPα-mediated gene expression is required for brown adipocyte formation. In agreement with the key role of C/EBPα in activating the adipogenic gene program, overexpression of an upstream transcription factor, C/EBPβ, could not rescue CycC-KO-caused defects either. Furthermore, consistent with the critical role of C/EBPα in adipogenesis and the role of CycC in regulating C/EBPα, CycC-KD also impairs white adipogenesis, as demonstrated using 3T3-L1 cells (Fig. 8*F*).

In summary, this study demonstrates that CycC is required for adipogenesis. Mechanistically distinct from MED1 and MED14, CycC regulation of adipogenesis is not through PPARγ. Although additional mechanisms cannot be excluded, our data indicate that CycC promotes adipogenesis by activating C/EBPα-dependent transcription.

Experimental procedures

Generation of CycC-KO brown preadipocytes

Ccnc-flox mice were generated and backcrossed with the C57Bl/6J strain for 10 generations. All mice were fed *ad libitum* with a standard chow diet (5053, LabDiet). The Albert Einstein College of Medicine Institutional Animal Care and Use Committee approved all animal experiments. Mouse brown preadipocyte cell lines were generated according to published protocols (49, 50). For generation of CycC-KO brown preadipocytes, immortalized $Ccnc^{flox/flox}$ cells were transduced with the CMV-Cre-GFP lentivirus and selected with puromycin (2.0 μg/ml). Single clones were selected.

Screen with the shRNA library

The Mouse Module I lentiviral shRNA library (Cellecta, Inc.) was packaged into lentiviruses in HEK293T cells using the calcium phosphate precipitation method. The viral supernatant was collected 48 h and 72 h after transfection, filtered, pelleted by ultracentrifugation, resuspended in 1× PBS, and then frozen at −80 °C. The final titer was 8×10^6 transduction units per ml. In total, 2.2×10^7 brown preadipocytes were plated at 50% confluence on 44 10-cm dishes and transduced with lentivirus stocks containing 4 μg/ml Polybrene. The medium was replaced 1 day after transduction. Upon confluence, two dishes of cells were collected as controls, and other cells were induced for differentiation for 7 days. The cells were collected after trypsin digestion, dispersed by pipetting several times, and then replated in 10-cm dishes. In ~15 min, the undifferentiated preadipocytes and poorly differentiated cells attached to the dish bottom, whereas well differentiated adipocytes remained floating in the medium. The adherent cells were washed gently with 1× PBS, followed by trypsin digestion and replating, and then collected. Genomic DNA was isolated using the Wizard genomic DNA purification kit and amplified by 10 rounds of PCR. The high-throughput sequencing was performed using a HiSeq 2500 instrument (Illumina) at the Einstein Genomics Core Facility. The enriched pathways and biological process Gene Ontology (GO) terms were obtained using the DAVID database (version 6.8).

Transfection of siRNA

ON-TARGETplus siRNA pools for *Ccnc* (047925) were purchased from GE Dharmacon. Non-targeting siRNA (001810) was used as a control. Preadipocytes were transfected with siRNA (25 pmol/2×10^5 cells) using DharmaFECT reagent by following the protocol of the manufacturer. The next day, fresh medium was added to allow cells to grow until confluent in 35-mm dishes or 12-well plates in DMEM with 20% FBS.

RNA-seq analysis

Total RNA was extracted from brown adipocytes transfected with NS-siRNA or *Ccnc*-siRNA and induced for 2 days (two biological replicates for each condition) using TRIzol reagent according to the instructions of the manufacturer. After removal of contaminating genomic DNA with RNase-free DNase I and quality analyses by Bioanalyzer (Agilent Technologies), RNA samples were processed at the Einstein Genomics Core Facility for library construction and sequencing using a HiSeq 2500 instrument (Illumina). Raw reads for each library were mapped using TopHat version 2.0.8 against the indexed mouse (mm9) genome, and transcripts were assembled using Cufflinks. Genes not expressed under both conditions (fragments per kilobase million (FPKM) < 1) were filtered out. Differentially expressed genes were analyzed using an RNA-seq processing tool, DESeq2, and selected by adjusted $p < 0.05$ and -fold change >1.5. The enriched pathways and biological process GO terms were obtained using the DAVID database (version 6.8), and the expressed genes were used as background. The heat map (18 down-regulated genes in the PPAR pathway) was generated based on the read count after variance-stabilizing transformation by DESeq2. The genes were ranked according to their p values.

Real-time PCR analysis

One μg of total RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed using FastStart Universal SYBR Green Master (Roche). Sequence information for specific primers used in this study is available upon request. All reactions were normalized to the *Tbp* level and performed in triplicate.

Immunoblotting

Protein extraction and immunoblotting were performed as described previously (62). Proteins in samples were resolved in 12% SDS-PAGE gels. The following antibodies and dilutions were used: anti-CDK8 (ab54561, 1:500) and anti-GLUT4 (ab654, 1:500) from Abcam; anti-CycC (558903, 1:500) from BD Pharmingen); anti-Caveolin (610406, 1:1000) from BD Transduction Laboratories; anti-C/EBPα (8178, 1:250), anti-aP2 (2120, 1:1000), anti-ATGL (2138, 1:500), and anti-FAS (3180, 1:500) from Cell Signaling Technology; anti-HA (MMS-101P, 1:1000) from Covance; anti-β-tubulin (1235662A, 1:500) from Invitrogen; anti-UCP1 (PA1-24894, 1:1000) from Pierce; anti-PPARγ (sc-7273, 1:100) from Santa Cruz Biotechnology;

and anti-CDK19 (HPA007053, 1:500), anti-β-actin (A5060, 1:200), and anti-FLAG (F1804, 1:1000) from Sigma-Aldrich. HRP-conjugated secondary antibody (anti-rabbit IgG, 111-035-003 or anti-mouse IgG, 115-035-003, Jackson ImmunoResearch Laboratories) was used (1:5000 dilution). The signals were detected using the Super Signal West Pico kit (Pierce) and exposure to X-ray films.

Co-IP

FLAG-C/EBPα (Addgene, 66978) was co-transfected with HA-GFP or HA-CycC. One day after transfection, cell lysates were prepared in the IP buffer as described previously (62). FLAG-tagged proteins were immunoprecipitated from the lysates using anti-FLAG antibody. After five washes with IP buffer, FLAG-tagged proteins were eluted with 3× FLAG peptides. Proteins in elutes were detected by immunoblotting using anti-FLAG or anti-HA antibody.

Immunostaining

Immortalized brown preadipocytes or differentiated brown adipocytes were fixed with 4% formaldehyde (Fisher Scientific) in 1× PBS for 10 min at room temperature, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 15 min, and blocked with 10% normal blocking serum for 30 min. The samples were then incubated with polyclonal anti-CycC antibody (AHF0072, Invitrogen, 1:200) in 1.5% normal blocking serum (ab166643, Abcam) for 2 h at room temperature, washed with 1× PBS three times, incubated with Alexa Flour 488-labeled secondary antibody (A-21206, Invitrogen, 1:500) in 1.5% normal blocking serum for 45 min at room temperature in the dark, and washed with 1× PBS three times. Nuclei were stained with Prolong Antifade reagent with DAPI (Invitrogen). Images were captured using a fluorescence microscope.

Oil red O staining

After various days of differentiation, cells were washed once with 1× PBS and fixed with formaldehyde (Fisher Scientific) for 1 h. The staining stock was prepared by dissolving 0.35 g oil red O (Sigma) in 100 ml of isopropanol, stirred overnight, filtered through a 0.22-μm filter, and stored at room temperature. For preparing the oil red O working solution, 6 ml of the stock was mixed with 4 ml of distilled water. After sitting at room temperature for 20 min, the staining solution was filtered again and then added to cells for 30 min. The staining solution was then removed, and cells were washed four times with distilled water. Images were captured using a digital camera and an inverted microscope.

Retroviral transduction

HEK293T cells were grown in DMEM containing 10% FBS. Retroviral vector, pMSCV-PIG (21654), pBABE-puro-PPARγ2 (8859), pMSCV-PIG-C/EBPα (66985), and pBABE-puro-LAP2 (15712) were obtained from Addgene. To make the pMSCV-PIG-CycC (with or without an HA tag) viral vector, the full-length coding sequence of mouse *Ccnc* was amplified from brown preadipocyte cDNA by PCR (one HA tag sequence was added to the 3′ end of *Ccnc* by PCR) and cloned into the EcoRI site of the pMSCV-PIG retroviral vector using In-Fusion cloning kits (Clontech), verified by DNA sequencing. To generate pseudotyped virus, pBABE-puro-PPARγ2 (pMSCV-PIG-C/EBPα, pMSCV-PIG-HA/CycC, pBABE-puro-LAP2, or the empty retroviral vector pMSCV-PIG) and retroviral packaging plasmids (pKat and pCMV-VSV-G) were co-transfected into subconfluent HEK293T cells using Lipofectamine 3000 (Invitrogen) according to the instructions of the manufacturer. The virus stocks were collected 2 and 3 days after transfection, filtered through a 0.45-μm filter, and then frozen at −80 °C. Brown preadipocytes with ~30% confluence were treated with virus stock containing 8 μg/ml Polybrene for 4 h, washed, and cultured in fresh medium. The transduction was repeated the next day.

Luciferase reporter assays

3×PPRE-Luc (1015) was obtained from Addgene. To make a luciferase reporter construct containing three C/EBP consensus binding sequences (3×CBE-Luc), the 3×PPRE-Luc construct was cut by HindIII and BamH1 to remove the 3×PPRE fragment, and then oligos containing 3×CBE (5′-AGATCTGTTGCGCAAGTGGAGGTTGCGCAAGTGGCAGGTTGCGCAAGCTCGAG-3′) were annealed and subcloned into HindIII/BamH1 sites and verified by DNA sequencing. GAL4 fusion constructs were made by linking the C/EBPα (aa 2–327), C/EBPβ (aa 2–83), or C/EBPδ (aa 2–102) transactivation domain to the Gal4 DNA-binding domain in pcDNA3-HA-Gal4 using In-Fusion cloning kits (Clontech). The procedures for transfection and luciferase reporter assays were described previously (63). Briefly, brown preadipocytes were seeded at a density of 1×10^5/well in 24-well plates. The next day, cells were co-transfected with 250 ng of pBABE-puro-PPARγ2, pcDNA3-FLAG-C/EBPα, or pMSCV-PIG (or pcDNA-HA-Gal4, pcDNA-HA-Gal4-C/EBP-TADs, pcDNA-HA-Gal4-SREBP1c-TAD, or pcDNA-HA-Gal4-VP16-TAD), 250 ng of 3×PPRE-Luc, 3×CBE-Luc, or 2×GBE-Luc and 50 ng of *Renilla* luciferase control plasmids using Lipofectamine 3000 transfection reagent. For 3×PPRE-Luc assays, rosiglitazone (1 μM) or DMSO (control) was added immediately following transfection. After incubation for 1 to 2 days, cells were lysed and analyzed using the Dual-Luciferase system (Promega) according to the instructions of the manufacturer. The activity of firefly luciferase was normalized by the corresponding activity of *Renilla* luciferase.

Statistical analyses

The results are expressed as mean ± S.E. Significance was determined by one-way analysis of variance. $p < 0.05$ was considered to be statistically significant. The statistical analyses were performed using SPSS15.0.

Author contributions—F. Y. conceived, designed, and supervised the study. Z. S. performed most of the experiments and data analyses. F. Y., Z. S., and J. E. P. wrote the manuscript. R. S. generated the Ccnc-flox mice, and A. M. X. performed background backcrossing of the mice. A. M. X., Y. Z., and E. S. T. Y. participated in the experiments. Q. Z., S. W., R. C., and Z. D. Z. performed data analyses for the shRNA screen and RNA-seq. G. Y. and J. E. P. provided scientific advice and discussions.

Acknowledgments—We thank Dr. John Reidhaar-Olson and Dr. Deborah Smith (Einstein shRNA Core Facility) for assistance with the shRNA screen and Dr. Shahina B. Maqbool (Einstein Epigenomics Core Facility) for assistance with RNA-seq.

Note added in proof—In the version of this article that was published as a Paper in Press on March 28, 2017, the construct used for the C/EBPβ-TAD sequence in Fig. 7*H* was missing a few amino acids because of a PCR cloning error. The C/EBPβ-TAD clone has been corrected and the experiments were repeated. This error does not affect the results or conclusions of this work.

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