Ligand-dependent corepressor (LCoR) represses the transcription factor C/EBPβ during early adipocyte differentiation

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Nuclear receptors (NRs) regulate gene transcription by recruiting coregulators, involved in chromatin remodeling and assembly of the basal transcription machinery. The NR-associated protein ligand-dependent corepressor (LCoR) has previously been shown to suppress hepatic lipogenesis by decreasing the binding of steroid receptor coactivators to thyroid hormone receptor. However, the role of LCoR in adipogenesis has not been established. Here, we show that LCoR expression is reduced in the early stage of adipogenesis *in vitro*. LCoR overexpression inhibited 3T3-L1 adipocyte differentiation, whereas LCoR knockdown promoted it. Using an unbiased affinity purification approach, we identified CCAAT/enhancer-binding protein β (C/EBPβ), a key transcriptional regulator in early adipogenesis, and corepressor C-terminal binding proteins as potential components of an LCoR-containing complex in 3T3-L1 adipocytes. We found that LCoR directly interacts with C/EBPβ through its C-terminal helix-turn-helix domain, required for LCoR's inhibitory effects on adipogenesis. LCoR overexpression also inhibited C/EBPβ transcriptional activity, leading to inhibition of mitotic clonal expansion and transcriptional repression of *C/EBPα* and peroxisome proliferator-activated receptor γ2 (*PPARγ2*). However, LCoR overexpression did not affect the recruitment of C/EBPβ to the promoters of *C/EBPα* and *PPARγ2* in 3T3-L1 adipocytes. Of note, restoration of PPARγ2 or C/EBPα expression attenuated the inhibitory effect of LCoR on adipogenesis. Mechanistically, LCoR suppressed C/EBPβ-mediated transcription by recruiting C-terminal binding proteins to the *C/EBPα* and *PPARγ2* promoters and by modulating histone modifications. Taken together, our results indicate that LCoR negatively regulates early adipogenesis by repressing C/EBPβ transcriptional activity and add LCoR to the growing list of transcriptional corepressors of adipogenesis.

Nuclear receptors (NRs)[3] regulate target gene transcription by dynamically recruiting a variety of coregulators, which play various roles in chromatin remodeling and assembly of the basal transcription machinery (1–3). Ligand-dependent corepressor (LCoR) was originally reported as a corepressor that interacts with estrogen receptor α and some other NRs only in the presence of ligands through an L*XX*LL motif, also called the NR box (4). It has been shown that LCoR is able to recruit transcriptional suppressors such as histone deacetylase and C-terminal binding protein (CtBP) to repress target gene transcription (4–6). LCoR can also act as a transcriptional corepressor in the absence of NR ligands. We previously reported that LCoR can physically interact with thyroid hormone receptor (TR) and repress the transcriptional activity of TR in a hormone-independent manner (7). Additionally, LCoR has

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This article contains supplemental Fig. S1 and Tables S1–S4.

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[3] The abbreviations used are: NR, nuclear receptor; LCoR, ligand-dependent corepressor; C/EBP, CCAAT/enhancer-binding protein; CtBP, C-terminal binding protein; HTH, helix-turn-helix; PPARγ, peroxisome proliferator-activated receptor γ; TR, thyroid hormone receptor; KLF, Krüppel-like factor; ap2, adipocyte fatty-acid binding protein 4; SVF, stromal vascular fraction; F-LCoR, FLAG-tagged LCoR; shLCoR, short hairpin RNA targeting LCoR; IP, immunoprecipitation; Gins1, GINS complex subunit 1; Mcm3, minichromosome maintenance complex component 3; shC/EBPβ, short hairpin RNA targeting C/EBPβ; Rosi, rosiglitazone; plin2, perilipin 2; shCtBP, short hairpin RNA targeting CtBP; H3K9acK14ac, acetylation of histone 3 lysines 9 and 14; H3K9me2, dimethylation of histone 3 lysine 9; CCN, cyclin; NCoR, nuclear corepressor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptors; SRC, steroid receptor coactivator; qPCR, quantitative PCR; shLacZ, shRNA targeting LacZ.

been shown to interact with transcriptional factor Krüppel-like factor 6 (KLF6) and repress KLF6 target gene transcription (8). These findings suggest that different mechanisms are involved in the regulation of transcription by LCoR.

NR coregulators have been implicated in not only developmental but also metabolic processes and diseases (9). It is now well-accepted that NR coregulators are important metabolic switches and regulate metabolic pathways through their interactions with not only NRs but also other transcription factors. The potential pathological role of LCoR has been studied extensively in breast and prostate cancers using various cell lines (5, 8, 10). However, the physiological and pathological functions of LCoR in metabolic regulation are largely unknown. In a previous study, we demonstrated that LCoR is able to repress TR-mediated expression of lipogenic genes and acts as a negative regulator of hepatic lipogenesis. Down-regulation of hepatic LCoR might contribute to the dysregulation of lipogenesis and the development of fatty livers in obese mouse models and patients with non-alcoholic fatty liver disease. More importantly, hepatic LCoR could serve as a potential target for treating hepatic steatosis (7). Besides liver, adipose tissue is also a key metabolic organ and plays a vital role in the regulation of energy homeostasis. We showed that LCoR is highly expressed in white adipose tissue; however, its role in adipose tissue has never been studied (7).

Understanding the molecular mechanism underlying adipogenesis will provide insights into adipose tissue plasticity and remodeling in adaptive energy homeostasis as well as in metabolic disease. It has been proposed that the development of adipose depots can be divided into two stages, commitment and terminal differentiation, which gives rise to adipocytes from preadipocytes. As a result of intensive studies using cellular models including 3T3-L1 cells and mouse embryonic fibroblasts, it is known that adipogenesis is orchestrated by a complex network of transcriptional cascades (11–13). As the master transcriptional regulators, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα) control almost all of the genes involved in the morphological changes and lipid accumulation during adipocyte differentiation and lipid droplet formation. C/EBPβ and C/EBPδ, two transcription factors induced in the early stage of adipogenesis, play crucial roles in the initiation of the differentiation program by activating the expression of PPARγ and C/EBPα, which ultimately leads to terminal adipogenic differentiation.

In this study, we showed that LCoR is a negative regulator of adipogenesis. Moreover, our data suggested that LCoR can interact with C/EBPβ and repress the transcriptional activity of C/EBPβ by recruiting CtBP in the early stage of adipogenesis. Our results provided novel insights into the molecular mechanism involved in the regulation of early adipogenesis.

Results

The expression of LCoR is dynamically regulated during adipogenesis

LCoR is expressed in a wide variety of fetal and adult human tissues (4), but its expression profile during adipogenesis is unclear. To explore the potential roles of LCoR in adipogenesis, we initiated our study by examining the expression levels of LCoR during 3T3-L1 adipocyte differentiation. The cells were induced to differentiate into mature adipocytes using a standard induction hormone mixture. We observed that the mRNA levels of LCoR were decreased gradually within the first 8 h after induction of differentiation, then increased gradually, and reached a peak at the end of the differentiation (Fig. 1*A*). Similarly, the protein levels of LCoR declined from the initial stage of differentiation, were maintained at a very low level until day 1, and then increased considerably from day 2 to the end of the differentiation (Fig. 1*B*). The mRNA expression of C/EBPβ, C/EBPα, PPARγ, and adipocyte fatty-acid binding protein 4 (ap2), which are the key players and/or adipogenic markers during adipogenesis, were also examined (supplemental Fig. S1, A–D). We also assessed the expression of LCoR in primary adipocytes using stromal vascular fraction (SVF) cells as described under "Experimental procedures." Similar to the LCoR expression pattern that was observed during 3T3-L1 adipocyte differentiation, the mRNA levels of LCoR were reduced in the early stage and increased gradually afterward during the adipogenesis of SVF cells (Fig. 1*C*). The dynamic expression of LCoR indicated that LCoR might have a potential role in adipogenesis.

Overexpression of LCoR inhibits 3T3-L1 adipocyte differentiation

To investigate the effect of LCoR on adipocyte differentiation, we stably overexpressed FLAG-tagged LCoR (F-LCoR) in 3T3-L1 preadipocytes using retroviral vectors. Overexpression of LCoR was first confirmed at both mRNA and protein levels (Fig. 1, *D* and *E*). Analysis using the Agilent mouse gene expression microarrays revealed that the overexpression of LCoR down-regulated the expression of those genes implicated in multiple metabolic pathways in differentiated 3T3-L1 adipocytes, including the PPAR signaling pathway, fatty acid metabolism, and fatty acid elongation (supplemental Table S1). Because the PPAR signaling pathway, fatty acid metabolism, and fatty acid elongation are essential for mature adipocyte formation, we hypothesized that LCoR is able to regulate adipogenesis.

To test our hypothesis, the adipogenic abilities of 3T3-L1 preadipocytes overexpressing F-LCoR were accessed by Oil Red O staining after differentiation induction. Compared with control cells, the cells overexpressing F-LCoR showed a severely impaired ability to accumulate lipids (Fig. 1*F*). We then examined several adipogenic markers to confirm the inhibitory effect of F-LCoR on adipocyte differentiation. The mRNA and protein levels of early adipogenic marker C/EBPβ were not altered, whereas the mRNA and protein expression levels of late adipogenic markers C/EBPα, PPARγ, and their downstream target gene, ap2, were significantly reduced in cells overexpressing F-LCoR (Fig. 1, *G–K*). These results indicated that overexpression of LCoR is able to suppress adipogenesis.

Knockdown of LCoR facilitates 3T3-L1 adipocyte differentiation

To verify the inhibitory effect of LCoR on adipocyte differentiation, we established a stable LCoR knockdown 3T3-L1 cell line by using retrovirus-mediated short hairpin RNA targeting LCoR (shLCoR). Significant reduction of endogenous LCoR

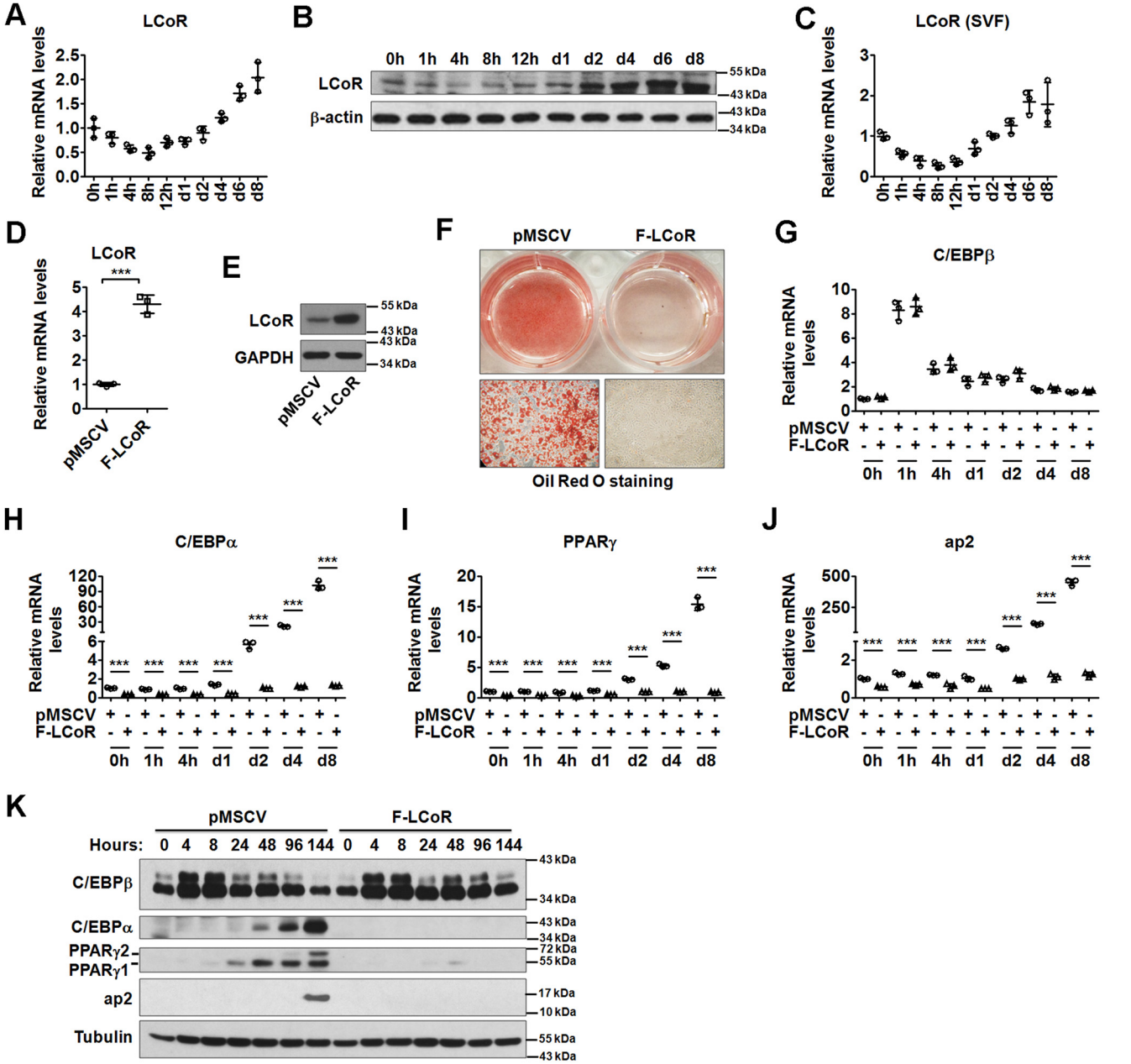

Figure 1. Overexpression of LCoR inhibits adipogenesis. *A–C*, the expression levels of LCoR during adipogenesis. 3T3-L1 (*A* and *B*) or SVF (*C*) cells were harvested at the indicated times after adipogenic induction. The mRNA and protein levels of LCoR were measured by qPCR (*A* and *C*) or Western blotting (*B*), respectively. *D* and *E*, the overexpression of LCoR in 3T3-L1 preadipocytes was confirmed by qPCR (*D*) and Western blotting (*E*). 3T3-L1 preadipocytes were infected with a retroviral vector containing F-LCoR or an empty vector (pMSCV) as a control. *F*, Oil Red O staining of 3T3-L1 cells stably overexpressing F-LCoR or pMSCV was performed on day (*d*) 8 of differentiation. *G–K*, the expression levels of adipogenic genes in 3T3-L1 cells overexpressing LCoR during adipogenesis. 3T3-L1 cells stably overexpressing F-LCoR or pMSCV were harvested at the indicated times after adipogenic induction. The mRNA and protein levels of C/EBPβ, C/EBPα, PPARγ, and ap2 were examined by qPCR (*G–J*) or Western blotting (*K*), respectively. Immunoblots and Oil Red O staining shown here are representative of at least three independent experiments, which yielded similar results. Data are represented as mean ± S.D. ($n = 3$). *Error bars* represent S.D. ***, $p < 0.001$.

expression by shLCoR was confirmed in 3T3-L1 preadipocytes at both mRNA and protein levels (Fig. 2, *A* and *B*). Then these LCoR knockdown 3T3-L1 preadipocytes were subjected to a differentiation assay. Compared with control cells, LCoR knockdown cells accumulated greater amounts of lipids (Fig. 2*C*). To confirm the effect of LCoR knockdown on adipogenesis, the expression of adipogenic markers was examined (Fig. 2, *D–H*). Consistent with our findings in 3T3-L1 cells overexpressing LCoR, we found that the knockdown of LCoR did not lead to changes in C/EBPβ mRNA and protein expression (Fig. 2, *D* and *H*). In contrast, the mRNA and protein levels of adipogenic factors C/EBPα, PPARγ, and ap2 were relatively higher in LCoR knockdown cells than those in control cells (Fig. 2, *E–H*). These results further suggested that inhibition of LCoR expression is able to promote adipogenesis.

LCoR inhibits adipogenesis through directly interacting with C/EBPβ

To reveal the mechanisms underlying the antiadipogenic effect of LCoR, we used an unbiased affinity purification

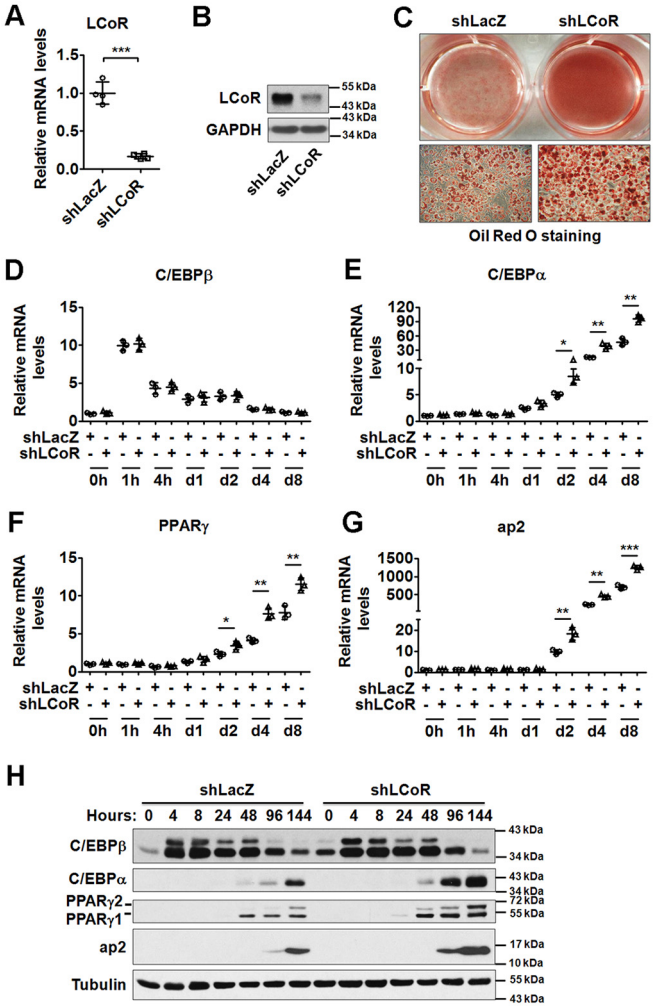

Figure 2. Knockdown of LCoR enhances adipogenesis. *A* and *B*, the efficiency of the knockdown of LCoR in 3T3-L1 preadipocytes was determined by qPCR (*A*) and Western blotting (*B*). 3T3-L1 preadipocytes were infected with a retroviral vector containing shLCoR or shRNA targeting LacZ (shLacZ) as a control. *C*, Oil Red O staining of 3T3-L1 cells stably overexpressing shLCoR or shLacZ was performed on day (*d*) 8 of differentiation. *D–H*, 3T3-L1 cells stably overexpressing shLCoR or shLacZ were harvested at the indicated times after adipogenic induction. The mRNA and protein levels of C/EBPβ, C/EBPα, PPARγ, and ap2 were investigated by qPCR (*D–G*) or Western blotting (*H*), respectively. The results of Western blotting and Oil Red O staining are typical of at least three independent experiments. Data are represented as mean ± S.D. ($n = 3–4$). *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

approach to identify the binding proteins of LCoR in 3T3-L1 cells overexpressing F-LCoR as described under "Experimental procedures" (Fig. 3*A*). The mass spectrometric analysis revealed that C/EBPβ, a key regulator of early adipogenesis, was a potential component of LCoR-containing complex (Fig. 3*A* and supplemental Table S2). Given that C/EBPβ controls the transcription of *C/EBPα* and *PPARγ* during adipogenesis and LCoR regulates the expression of C/EBPα and PPARγ without affecting C/EBPβ, we speculated that LCoR may inhibit adipogenesis through suppressing the transcriptional activity of C/EBPβ via direct binding. To test this hypothesis, we first confirmed the interaction of LCoR with C/EBPβ by coimmunoprecipitation (co-IP) assay in HEK 293T cells transfected with F-LCoR and C/EBPβ constructs (Fig. 3*B*). Importantly, the interaction of endogenous LCoR with endogenous C/EBPβ was also observed in 3T3-L1 cells by co-IP assay (Fig. 3*C*). To identify which region of LCoR is responsible for the interaction with C/EBPβ, we constructed FLAG-tagged N- or C-terminally truncated mutants of LCoR, F-LCoRΔN (LCoR lacking the N-terminal NR box), and F-LCoRΔC (LCoR lacking the C-terminal HTH domain) as indicated (Fig. 3*D*). We found that C/EBPβ interacted with F-LCoRΔN but not F-LCoRΔC, indicating that the HTH domain of LCoR is required for the interaction of LCoR with C/EBPβ (Fig. 3*E*). A similar result was obtained from a GST pulldown assay (Fig. 3*F*). We found that the GST-LCoR fusion protein bound C/EBPβ *in vitro*, suggesting that the interaction between C/EBPβ and LCoR is direct (Fig. 3*F*). Moreover, GST-LCoR fusion protein lacking HTH domain lost the ability to bind to C/EBPβ, further supporting the notion that the interaction between C/EBPβ and LCoR requires the C-terminal HTH domain of LCoR (Fig. 3, *D* and *F*).

To determine whether the interaction between C/EBPβ and LCoR is required for the antiadipogenic effect of LCoR, 3T3-L1 preadipocytes stably overexpressing F-LCoRΔN or F-LCoRΔC were used for a differentiation assay. We found that overexpression of F-LCoRΔN inhibited adipogenesis and lipid accumulation to the same extent as overexpression of F-LCoR did. In contrast, overexpression of F-LCoRΔC, which lost the ability to bind to C/EBPβ, did not affect 3T3-L1 adipocyte differentiation and lipid accumulation (Fig. 3*G*). Together, these results indicated that LCoR plays an inhibitory role in adipogenesis by physically interacting with C/EBPβ, and the HTH domain of LCoR is required for the interaction with C/EBPβ and the antiadipogenic effect of LCoR.

LCoR inhibits the transcriptional activity of C/EBPβ

Because either overexpression or knockdown of LCoR altered the expression of C/EBPα and PPARγ (Figs. 1, *H*, *I*, and *K*, and 2, *D* and *E*), two downstream target genes of C/EBPβ, we speculated that LCoR may inhibit the transcriptional activity of C/EBPβ through direct binding. To test this hypothesis, we performed luciferase assays using a reporter containing *PPARγ2* promoter region with C/EBPβ-responsive element in HEK 293T cells. We found that overexpression of LCoR repressed the promoter activity of *PPARγ2* in a dose-dependent manner, whereas knockdown of LCoR by specific siRNA enhanced the promoter activity of *PPARγ2* (Fig. 4, *A* and *B*). In addition, we found that overexpression of F-LCoRΔN but not F-LCoRΔC inhibited the transcriptional activity of C/EBPβ (Fig. 4*C*). We also examined the effects of LCoR overexpression and knockdown as well as the effects of its mutants on the promoter activity of *PPARγ2* in 3T3-L1 cells when C/EBPβ levels were greatly elevated after differentiation induction (Fig. 4, *D–F*). In agreement with the findings obtained in HEK 293T cells, we found that overexpression of LCoR repressed the promoter activity of *PPARγ2* in 3T3-L1 cells after adipogenic induction, whereas knockdown of LCoR by shLCoR enhanced the promoter activity of *PPARγ2* in 3T3-L1 cells after adipogenic induction (Fig. 4, *D* and *E*). Accordingly, we found that LCoRΔN but not LCoRΔC had an inhibitory effect on the promoter activity of *PPARγ2* in 3T3-L1 cells after adipogenic induction (Fig. 4*F*). Notably, the suppressive potencies of these

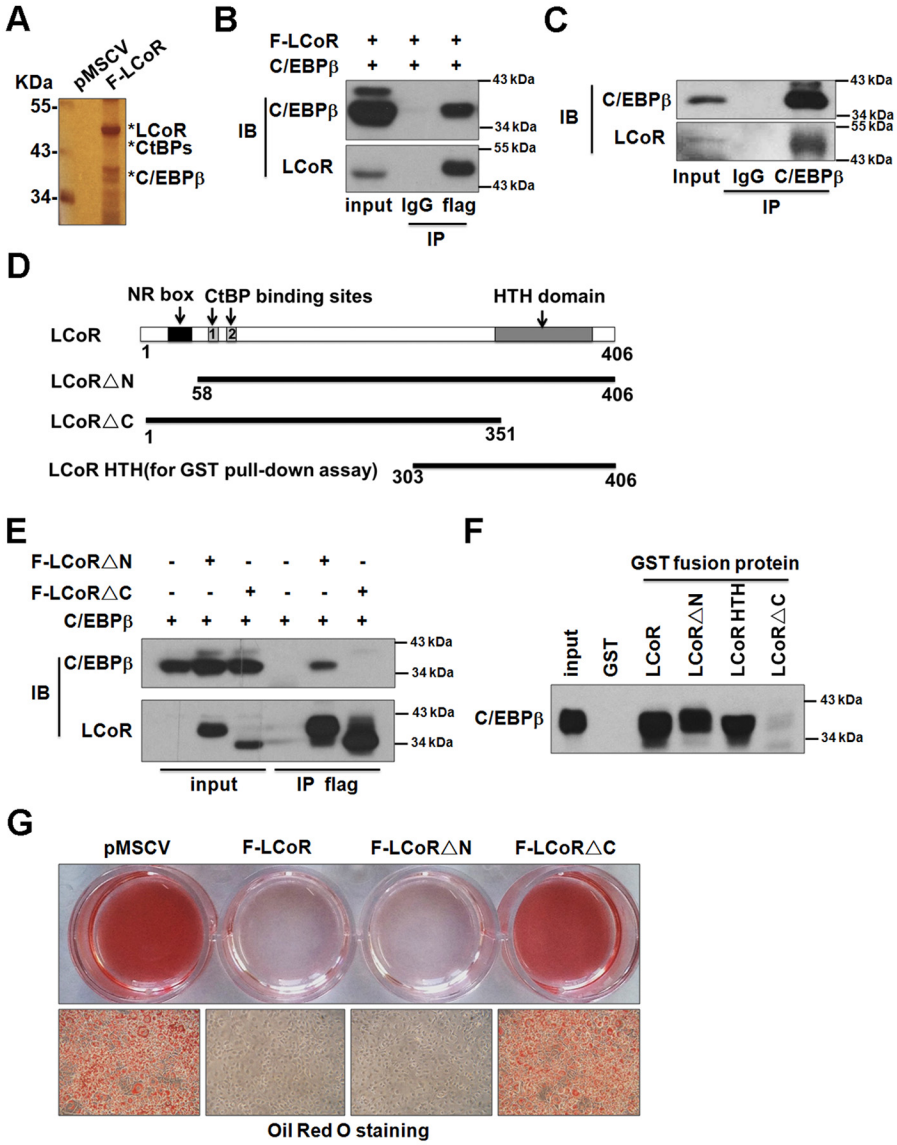

Figure 3. LCoR inhibits adipogenesis through directly interacting with C/EBPβ. *A*, 3T3-L1 preadipocytes stably overexpressing F-LCoR or an empty vector (pMSCV) were induced to differentiate for 2 days. LCoR-associated proteins purified from nuclear extracts of indicated cells were separated by SDS-PAGE and visualized by silver staining. The positions of identified proteins are indicated on the *right*, and molecular mass markers (kDa) are shown on the *left*. *B*, HEK 293T cells were transiently transfected with F-LCoR and C/EBPβ constructs. After 36 h, the cell lysates were immunoprecipitated with antibodies against IgG or FLAG and immunoblotted (*IB*) with antibodies against C/EBPβ or LCoR as indicated. *C*, the lysates of 3T3-L1 cells after differentiation for 48 h were immunoprecipitated with antibodies against IgG or C/EBPβ and then immunoblotted with antibodies against C/EBPβ or LCoR as indicated. *D*, schematic representation of full-length and truncated LCoR proteins with main functional motifs and boundaries indicated. *E*, HEK 293T cells were transiently transfected with the indicated constructs. After 36 h, the cell lysates were immunoprecipitated with antibodies against FLAG and immunoblotted with antibodies against C/EBPβ or LCoR. *F*, GST pulldown analysis was performed using *in vitro* translated C/EBPβ with GST control or with full-length or truncated LCoR GST fusion proteins as indicated. *G*, Oil Red O staining of 3T3-L1 cells stably overexpressing F-LCoR or truncated LCoR was performed on day 8 of differentiation. Blots and Oil Red O staining shown here are representative of at least three independent experiments, which yielded similar results.

LCoR mutants were correlated with their abilities to bind to C/EBPβ and inhibit adipogenesis (Fig. 3, *E*–*G*). These results suggested that the inhibitory effect of LCoR on the transcriptional activity of C/EBPβ requires direct binding.

The transcriptional activity of C/EBPβ is important not only for the expression of C/EBPα and PPARγ but also for the mitotic clonal expansion during 3T3-L1 adipocyte differentiation (14, 15). To evaluate the effect of LCoR on mitotic clonal expansion, 3T3-L1 cells stably overexpressing F-LCoR were induced to differentiate for 24 h followed by a BrdU incorporation assay. A significant decrease in the number of BrdU-positive cells was observed in cells overexpressing F-LCoR (Fig. 4, *G* and *H*). To further investigate the role of LCoR during mitotic clonal expansion, we measured the expression of cyclin D2 (CCND2) and cyclin E1 (CCNE1), two cyclin genes that are important for cell cycle progression. We found that overexpression of LCoR led to a significant decrease in CCND2 and CCNE1 mRNA levels at both 24 and 48 h after adipogenic induction (Fig. 4, *I* and *J*). In addition, the mRNA levels of GINS complex subunit 1 (Gins1) and minichromosome maintenance complex component 3 (Mcm3), two target genes of C/EBPβ that are required for mitotic clonal expansion (16), were also significantly down-regulated (Fig. 4, *K* and *L*). Similar results were obtained when C/EBPβ expression was knocked down

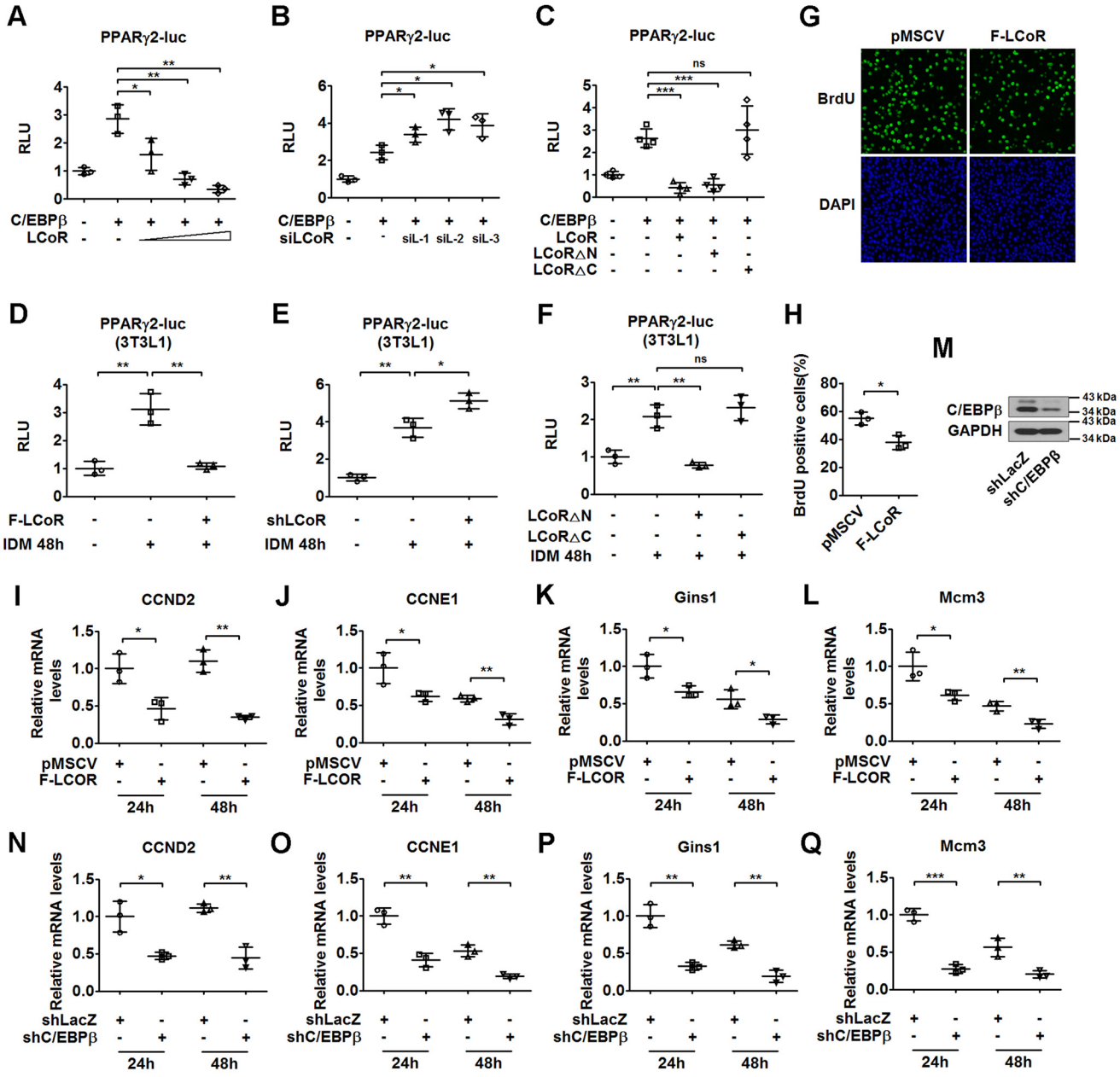

Figure 4. LCoR inhibits the transcriptional activity of C/EBPβ and mitotic clonal expansion. *A–C*, HEK 293T cells were transiently transfected with a luciferase reporter plasmid containing *PPARγ2* promoter (−602 to +52) (*PPARγ2-luc*) with or without a C/EBPβ construct in the absence or presence of increasing amounts of LCoR (*A*), siRNAs targeting LCoR (*B*), or full-length or truncated LCoR as indicated (*C*). The luciferase activity was measured and is shown as relative luciferase units (*RLU*). *D–F*, 3T3-L1 cells were infected with retrovirus expressing a *PPARγ2* promoter (−602 to +52)-driven luciferase reporter with or without retrovirus expressing F-LCoR (*D*), shLCoR (*E*), or truncated LCoR (*F*) as indicated. The luciferase activities were analyzed before or 48 h after differentiation induction using insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (*IDM*) and shown as relative luciferase units (*RLU*). *G* and *H*, representative image (*G*) and percentages of BrdU-positive cells (*H*) are shown. 18 h after adipogenic induction, 3T3-L1 cells stably overexpressing F-LCoR or an empty vector (pMSCV) were labeled with BrdU for 2 h and then stained with DAPI. The fluorescence of BrdU (*green*) and DAPI (*blue*) was detected with a fluorescence microscope. *I–L*, 3T3-L1 cells stably overexpressing F-LCoR or pMSCV were harvested at the indicated times after adipogenic induction. The mRNA levels of CCND2 (*I*), CCNE1 (*J*), Gins1 (*K*), and Mcm3 (*L*) were measured by qPCR analysis. *M*, the efficiency of the knockdown of C/EBPβ in 3T3-L1 cells was determined by Western blotting. 3T3-L1 preadipocytes were infected with a retroviral vector containing shC/EBPβ or shLacZ as a control. *N–Q*, 3T3-L1 cells infected with shC/EBPβ or shLacZ were harvested at the indicated times after adipogenic induction. The mRNA levels of CCND2 (*N*), CCNE1 (*O*), Gins1 (*P*), and Mcm3 (*Q*) were measured by qPCR analysis. Blots shown here are representative of at least three independent experiments. Data are represented as mean ± S.D. ($n = 3–4$). *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *ns*, not significant.

using retrovirus-mediated short hairpin RNA targeting C/EBPβ (shC/EBPβ) in 3T3-L1 cells after adipogenic induction (Fig. 4, *M–Q*). Together, these data suggested that overexpression of LCoR is able to inhibit mitotic clonal expansion by suppressing the transcriptional activity of C/EBPβ, which also contributes to the repression of adipogenesis.

Because C/EBPβ is a transcriptional factor and stimulates the expression of *C/EBPα* and *PPARγ* by directly binding to their promoters, we examined whether LCoR inhibits the transcriptional activity of C/EBPβ by affecting its DNA binding ability. 3T3-L1 cells stably overexpressing LCoR or its mutants were induced to differentiate for 24 h. Afterward, a chromatin

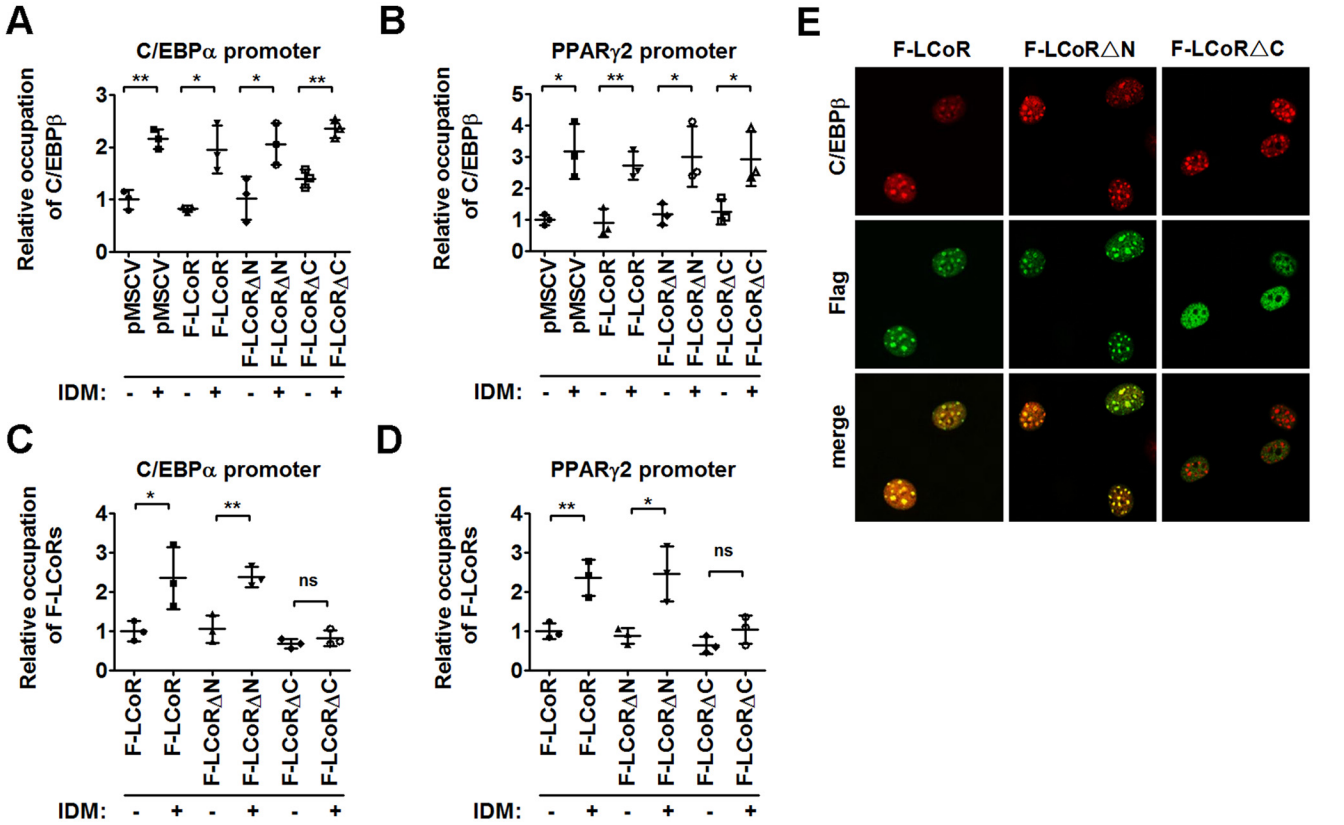

Figure 5. The interaction of LCoR with C/EBPβ does not affect the binding of C/EBPβ to DNA. *A–D*, ChIP analysis of C/EBPβ (*A* and *B*) or F-LCoRs (wild-type or truncated LCoR) (*C* and *D*) enrichment on the *C/EBPα* (*A* and *C*) or *PPARγ2* (*B* and *D*) promoter. At 0 and 24 h after adipogenic induction, ChIP experiments were performed using antibodies against C/EBPβ or FLAG as indicated. The precipitated DNA was analyzed by analysis with primers targeting the *C/EBPα* or *PPARγ2* promoter region. *E*, confocal microscope images of C/EBPβ (*red*) and F-LCoRs (*green*) in 3T3-L1 cells stably overexpressing FLAG-tagged wild-type or truncated LCoR. Cells were induced to differentiate for 24 h before they were fixed. C/EBPβ and LCoR proteins were detected using antibodies against C/EBPβ and FLAG, respectively. The confocal microscope images shown here are representative of at least three independent experiments. Data are represented as mean ± S.D. ($n = 3$). *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$; *ns*, not significant. *IDM*, insulin, dexamethasone, and 3-isobutyl-1-methylxanthine.

immunoprecipitation (ChIP) assay was performed to test whether overexpression of LCoR or its mutants could affect the recruitment of C/EBPβ to the promoters of its target genes. In agreement with the current notion that C/EBPβ is recruited to the promoters of *C/EBPα* and *PPARγ2* after adipogenic induction, we found that C/EBPβ was enriched in the promoter region of *C/EBPα* and *PPARγ2* at 24 h after adipogenic induction (Fig. 5, *A* and *B*). In addition, we found that overexpression of LCoR as well as of its mutants did not affect the recruitment of C/EBPβ to the promoters of *C/EBPα* and *PPARγ2* in 3T3-L1 cells either before or after adipogenic induction (Fig. 5, *A* and *B*). Because LCoR is able to interact with C/EBPβ, as expected, we observed an increased recruitment of LCoR to the promoters of *C/EBPα* and *PPARγ2* after the induction of adipogenesis (Fig. 5, *C* and *D*). Consistent with our finding that F-LCoRΔN but not F-LCoRΔC is able to interact with C/EBPβ, we only found the enrichment of LCoR and F-LCoRΔN but not F-LCoRΔC on the promoters of *C/EBPα* and *PPARγ2* after adipogenic induction (Fig. 5, *C* and *D*). These results suggested that LCoR is recruited to the promoters of *C/EBPα* and *PPARγ2* together with C/EBPβ after adipogenic induction through direct binding.

It is known that once C/EBPβ acquires DNA binding ability it is localized to centromeres and forms a punctate pattern in the nuclei (17). To further confirm that the DNA binding ability of C/EBPβ is not affected by LCoR overexpression, we examined the centromeric localization of C/EBPβ during adipogenesis. The localization of C/EBPβ and LCoR was analyzed by immunofluorescence in 3T3-L1 cells stably overexpressing LCoR or it mutants after adipogenic induction for 24 h. We found that C/EBPβ formed a punctate pattern in all groups, indicating that the overexpression of LCoR or its mutants is not able to affect the centromeric localization of C/EBPβ (Fig. 5*E*). As expected, we found that either F-LCoR or F-LCoRΔN formed a punctate pattern and was colocalized with C/EBPβ (Fig. 5*E*). In contrast, because F-LCoRΔC could not bind to C/EBPβ, F-LCoRΔC was distributed diffusely within whole nuclei and was not colocalized with C/EBPβ (Fig. 5*E*). These results further suggested that the interaction of LCoR with C/EBPβ during early adipogenesis is required for the inhibitory effect of LCoR.

Overexpression of PPARγ2 or C/EBPα attenuates the inhibitory effect of LCoR on adipogenesis

Given that LCoR is able to repress the transcriptional activity of C/EBPβ, we examined whether reintroducing a target gene of C/EBPβ (C/EBPα or PPARγ) could attenuate the inhibitory effect of LCoR on adipogenesis. The expression of PPARγ2 or C/EBPα in 3T3-L1 preadipocytes overexpressing LCoR was measured at both mRNA and protein levels (Fig. 6, *A–D*). Cells were

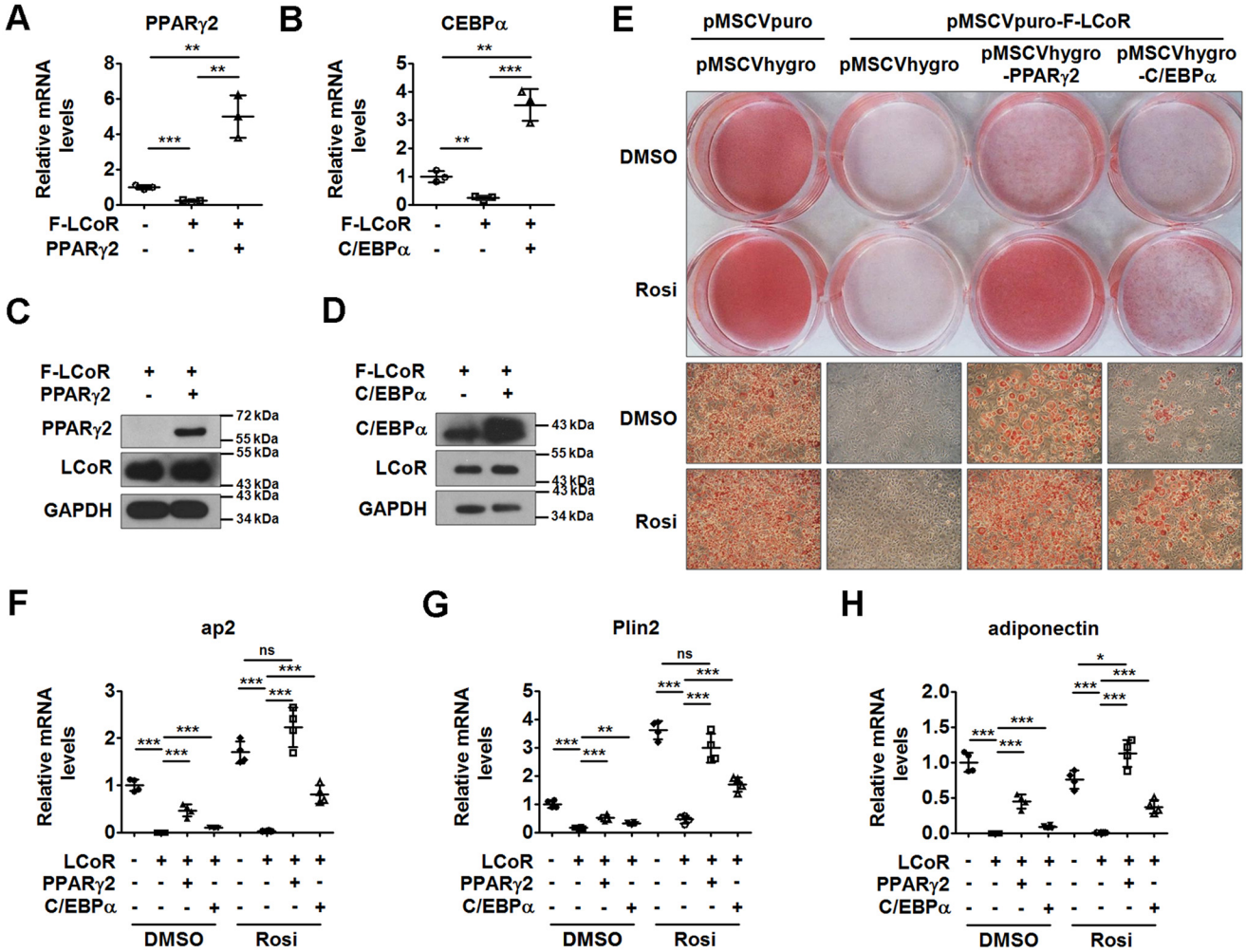

Figure 6. Overexpression of PPARγ2 or C/EBPα attenuates the inhibitory effect of LCoR on adipogenesis. *A–D*, the overexpression of PPARγ2 or C/EBPα in 3T3-L1 preadipocytes was confirmed by qPCR (*A* and *B*) and Western blotting (*C* and *D*). 3T3-L1 preadipocytes infected with retroviruses (pMSCVpuro) expressing F-LCoR were then infected with retroviruses (pMSCVhygro) expressing PPARγ2 or C/EBPα. *E*, Oil Red O staining was performed on day 8 of differentiation. Modified cells were induced to undergo adipogenesis in the presence or absence of 0.5 μM synthetic PPARγ ligand Rosi or vehicle (DMSO) alone. *F–H*, the expression levels of ap2, plin2, and adiponectin were examined by qPCR analysis on day 8 of differentiation. Immunoblots and Oil Red O staining shown here are representative of at least three independent experiments. Data are represented as mean ± S.D. ($n = 3–4$). *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *ns*, not significant.

induced to undergo adipogenesis in the presence of the synthetic PPARγ ligand rosiglitazone (Rosi) or vehicle (DMSO) alone. The adipogenic abilities were accessed by Oil Red O staining (Fig. 6*E*). In the absence of Rosi, C/EBPα induced about 5–10% and PPARγ2 induced about 40–50% of cells overexpressing LCoR to resume adipogenesis (Fig. 6*E*). Treatment with Rosi further enhanced the C/EBPα-induced differentiation, whereas PPARγ2 totally restored the adipogenic abilities of the cells overexpressing LCoR in the presence of Rosi (Fig. 6*E*). The effect of PPARγ2 or C/EBPα overexpression on the expression of mature adipocytes makers, including ap2, perilipin 2 (plin2), and adiponectin, in the cells overexpressing LCoR was also analyzed (Fig. 6, *F–H*), and similar results were obtained. Of note, overexpression of PPARγ2 totally restored the expression of ap2, plin2, and adiponectin in those cells overexpressing LCoR in the presence of Rosi. These results suggested that LCoR might suppress adipogenesis via C/EBPβ-mediated transcription of *C/EBPα* and *PPARγ2*.

LCoR inhibits C/EBPβ-mediated transcription and adipogenesis by recruiting CtBP

It has been shown that CtBPs play a regulatory role in adipogenesis (18–20). We noticed that both CtBP1 and CtBP2 were also identified as potential components of LCoR-containing complex in differentiated 3T3-L1 adipocytes (Fig. 3*A*). Based on these findings, we hypothesized that CtBPs may mediate the suppressive effect of LCoR on the transcriptional activity of C/EBPβ and early adipogenesis. Given the fact that CtBP1 and CtBP2 are highly related and functionally redundant (20–22), we made expression constructs carrying *CtBP1* gene for further studies. The interaction between CtBP1 and LCoR was first analyzed by using a GST pulldown assay. We found that LCoR interacted with CtBP1 directly (Fig. 7*A*). However, we could not detect the binding of CtBP1 to C/EBPβ in a GST pulldown assay, suggesting that CtBP1 does not interact with C/EBPβ directly (Fig. 7*A*). We also examined the localization of CtBP1 in 3T3-L1 cells at 24 h after differentiation induction. Immu-

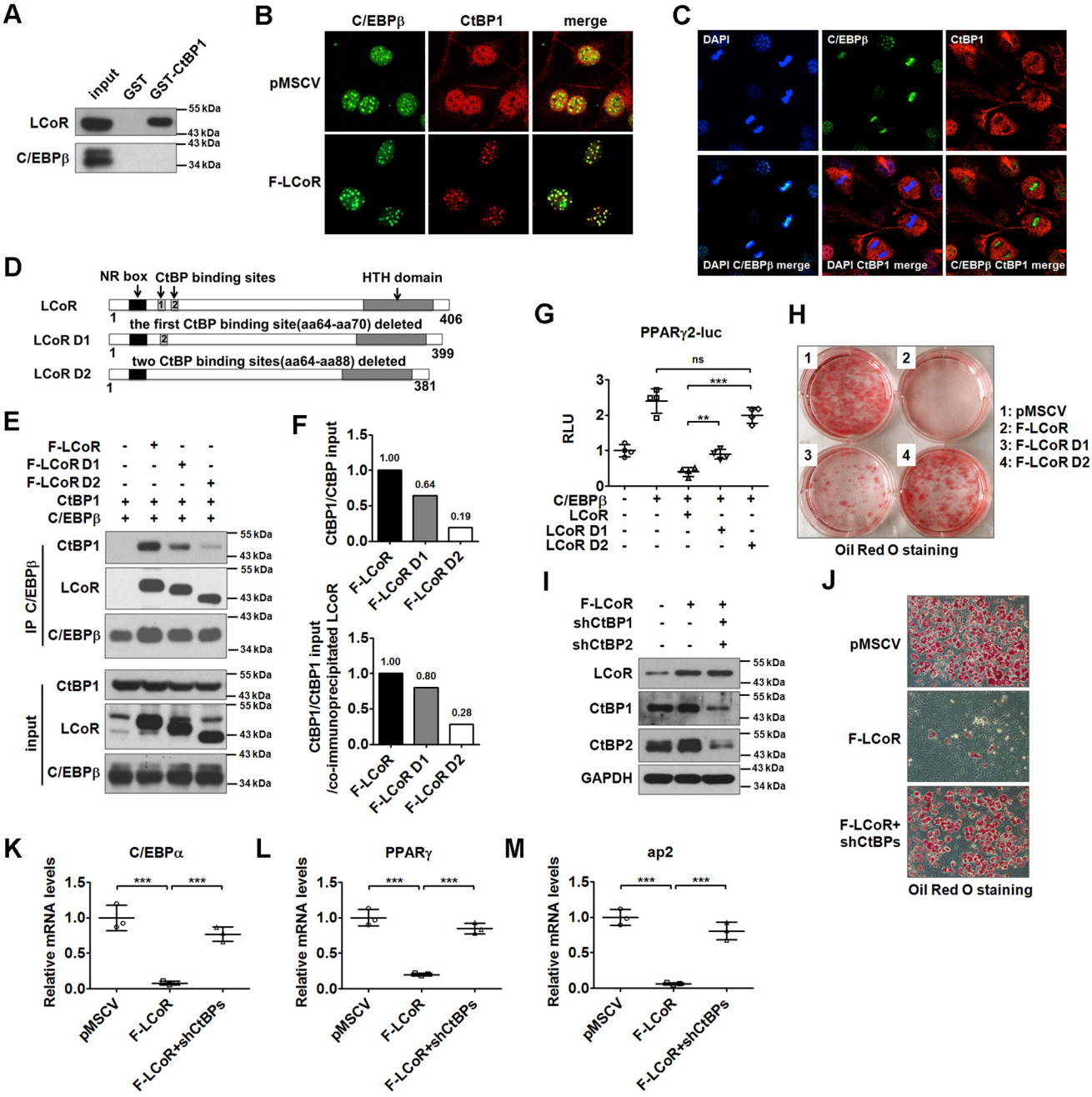

Figure 7. LCoR inhibits adipogenesis through recruiting CtBP. *A*, GST pulldown analysis was performed using *in vitro* translated LCoR or C/EBPβ with GST or GST-CtBP1 fusion proteins as indicated. *B*, confocal microscope images of endogenous C/EBPβ (*green*) and CtBP1 (*red*) in 3T3-L1 cells stably expressing F-LCoR or an empty vector (pMSCV). Cells were induced to differentiate for 24 h before they were fixed. *C*, confocal microscope images of C/EBPβ (*green*) and CtBP1 (*red*) in 3T3-L1 cells. Cells were induced to differentiate for 24 h before they were fixed. The different localization of CtBP1 and C/EBPβ was much more obvious in 3T3-L1 cells undergoing mitosis. *D*, schematic representation of mutant LCoRs (LCoR D1 and LCoR D2) with CtBP-binding sites deleted. *E*, HEK 293T cells were transiently transfected with the indicated constructs. After 36 h, the cell lysates were immunoprecipitated with antibodies against C/EBPβ and immunoblotted with antibodies against CtBP1, C/EBPβ, or FLAG. *F*, the amount of CtBP1 that was coimmunoprecipitated with C/EBPβ was normalized to the amount of CtBP1 input (*top panel*) or further normalized by the amount of coimmunoprecipitated LCoR or LCoR mutants (*bottom panel*). *G*, HEK 293T cells were transiently transfected with a luciferase reporter plasmid containing *PPARγ2* promoter (*PPARγ2-luc*) with or without a C/EBPβ construct in the absence or presence of LCoR, LCoR D1, or LCoR D2 as indicated. The luciferase activity was measured and is shown as relative luciferase units (*RLU*). *H*, Oil Red O staining of 3T3-L1 cells stably overexpressing F-LCoR, F-LCoR D1, or F-LCoR D2 was performed on day 8 of differentiation. *I*, the efficiency of the knockdown of CtBP1 and CtBP2 by shCtBP1 and shCtBP2, respectively, in 3T3-L1 preadipocytes was determined by Western blotting. *J*, Oil Red O staining of 3T3-L1 cells infected with F-LCoR or both F-LCoR and shCtBPs (shCtBP1 + shCtBP2) was performed on day 8 of differentiation. *K–M*, the mRNA levels of C/EBPα, PPARγ, and ap2 were examined by qPCR analysis in these differentiated 3T3-L1 cells infected with F-LCoR or both F-LCoR and shCtBPs. Immunoblots, confocal microscope images, and Oil Red O staining shown here are representative of at least three independent experiments. Luciferase assay and qPCR data are represented as mean ± S.D. ($n = 3–4$). *Error bars* represent S.D. **, $p < 0.01$; ***, $p < 0.001$; *ns*, not significant. *aa*, amino acids.

nofluorescence analysis revealed that CtBP1 was distributed diffusely in the nuclei, whereas C/EBPβ formed a punctate pattern after differentiation induction (Fig. 7*B*). The different localization of CtBP1 and C/EBPβ was much more obvious in 3T3-L1 cells undergoing mitosis (Fig. 7*C*). These results indicated that CtBP1 normally does not colocalize with C/EBPβ in

3T3-L1 cells after adipogenic induction, especially when the cells are dividing. In contrast, we observed that CtBP1 colocalized with C/EBPβ and formed a punctate pattern in 3T3-L1 cells overexpressing LCoR after induction of adipogenesis, suggesting that LCoR serves as a bridge to tether CtBP1 to C/EBPβ, and overexpression of LCoR could lead to the colocalization of CtBPs with C/EBPβ (Fig. 7*B*). These results also indicated that the down-regulation of LCoR during early adipogenesis could keep most of the CtBPs away from C/EBPβ, thereby facilitating mitotic clonal expansion and promoting adipogenesis.

It has been shown that LCoR can interact with CtBPs through two CtBP-binding sites, PLDLPVR (site 1) and VLDLSTK (site 2), within its N-terminal region (4). To determine whether the recruitment of CtBPs by LCoR is required for the inhibitory effect of LCoR on the transcriptional activity of C/EBPβ and adipogenesis, we constructed FLAG-tagged LCoR mutants, F-LCoR D1 (lacking CtBP-binding site 1) and F-LCoR D2 (lacking two CtBP-binding sites) (Fig. 7*D*). Then we examined whether these LCoR mutants could affect the recruitment of CtBP1 to LCoR-C/EBPβ complex by performing a co-IP assay in HEK 293T cells. We found that both F-LCoR D1 and F-LCoR D2 were coprecipitated with C/EBPβ, suggesting that these two mutations did not affect the binding of LCoR to C/EBPβ (Fig. 7*E*). As expected, we observed that, in the presence of wild-type LCoR, CtBP1 could be coprecipitated with C/EBPβ (Fig. 7*E*). However, in the presence of LCoR mutants that lost one or both CtBP-binding sites, the coprecipitation of CtBP1 with the LCoR-C/EBPβ complex was inhibited accordingly (Fig. 7, *E* and *F*). Consistently, we found that LCoR D1 had less inhibitory effect on *PPARγ2* promoter activity compared with wild-type LCoR, whereas LCoR D2 has almost no inhibitory effect as revealed by luciferase assay (Fig. 7*G*). Moreover, Oil Red O staining results showed that F-LCoR D1 had less antiadipogenic ability, whereas F-LCoR D2 had nearly lost the antiadipogenic effect on 3T3-L1 adipocyte differentiation (Fig. 7*H*). We also tested whether knockdown of CtBPs could attenuate the effect of LCoR on adipogenesis. In agreement with our previous findings, we found that knockdown of CtBPs using retrovirus-mediated short hairpin RNA targeting CtBP1 and CtBP2 (shCtBPs) restored the adipogenic capacity of LCoR-overexpressing 3T3-L1 cells (Fig. 7, *I* and *J*). Consistently, the mRNA expression of C/EBPα, PPARγ, and ap2 was derepressed in LCoR-overexpressing 3T3-L1 cells after knockdown of CtBPs by shCtBPs (Fig. 7, *K*–*M*). Together, these results indicated that CtBPs mediate the inhibitory effect of LCoR on C/EBPβ-mediated transcription and adipogenesis.

LCoR alters histone modifications on adipogenic promoters

Histone modification plays an important role in regulating gene transcription. Generally, histone H3 acetylation, for example acetylation of histone 3 lysines 9 and 14 (H3K9acK14ac), is correlated with transcriptional activation, whereas dimethylation of histone 3 lysine 9 (H3K9me2) is correlated with repression (23, 24). It has been reported that CtBPs can form a functional complex with histone deacetylases 1/2 and histone methyltransferase G9a and result in transcriptional repression by converting active Lys-9-acetylated histone H3 to repressive Lys-9-methylated H3 (25). Based on our findings, we hypothesized that LCoR may alter the histone modifications on the promoters of *C/EBPα* and *PPARγ* through the recruitment of CtBPs, thereby repressing transcription. To test our hypothesis, we first examined whether CtBP1 could be recruited to the promoters of *C/EBPα* and *PPARγ2* by performing a ChIP assay. As expected, we found that overexpression of LCoR significantly increased the occupancy of CtBP1 on the promoter of *PPARγ2* (Fig. 8*A*). Overexpression of LCoR also significantly reduced the levels of H3K9acK14ac and increased the levels of H3K9me2 on the promoter of *PPARγ2* (Fig. 8, *B* and *C*). We also tested whether knockdown of CtBPs could attenuate the effect of LCoR on the histone modification on the *PPARγ2* promoter. As expected, we found that knockdown of CtBPs by shCtBPs not only reduced the occupancy of CtBP1 on the promoter of *PPARγ2* in LCoR-overexpressing 3T3-L1 cells but also abolished the effect of LCoR overexpression on the enrichment of H3K9acK14ac and H3K9me2 on the promoter of *PPARγ2* in 3T3-L1 cells (Fig. 8, *D*–*F*). These results suggested that CtBPs might mediate the suppressive effect of LCoR on C/EBPβ-controlled transcription through modulating histone modifications on adipogenic promoters.

Taken together, our study here demonstrated that LCoR is a negative regulator of adipogenesis. LCoR is able to interact with C/EBPβ and inhibit its activity by recruiting CtBP corepressor complex during early adipogenesis, which might result in chromatin remodeling and transcription repression (Fig. 8*G*).

Discussion

Uncovering the molecular basis of adipogenesis is crucial for a better understanding of adipose tissue plasticity and remodeling in metabolic homeostasis and disease (26). It is known that the regulation of adipogenesis is orchestrated by regulatory circuits controlled to a great extent by transcriptional mechanisms (11, 12). A growing body of evidence suggests that transcriptional coregulators play pivotal roles in transcriptional regulation during adipogenesis (11, 27, 28). For example, NCoR, SMRT, and SRC-3 were found to be key regulators of adipocyte differentiation (29–32). However, the role and importance of LCoR in adipogenesis have not been reported before. Here, we demonstrated that LCOR is a negative regulator of adipogenesis. By repressing the transcriptional activity of C/EBPβ, LCoR not only suppresses the C/EBPβ-mediated expression of C/EBPα and PPARγ but also inhibits mitotic clonal expansion. Taken together, our study reveals a physiological role of LCoR in the control of mitotic clonal expansion and adipocyte differentiation, adding LCoR to the growing list of key transcriptional coregulators of energy homeostasis.

Growing evidence has demonstrated that nuclear receptor coregulators are able to modulate transcription controlled by other types of transcriptional factors. For example, SMRT is able to control adipogenesis through C/EBPβ and KAISO (31), whereas SRC-3 is able to regulate PPARγ expression by targeting C/EBPα and C/EBPδ (33). In our study, we showed that LCoR is able to interact with both C/EBPβ and CtBP1, whereas C/EBPβ and CtBP1 did not interact with each other directly. Given that CtBP was colocalized with C/EBPβ and enriched on the promoter regions of C/EBPβ target genes upon LCoR overexpression, we speculated that LCoR serves as a bridge to tether

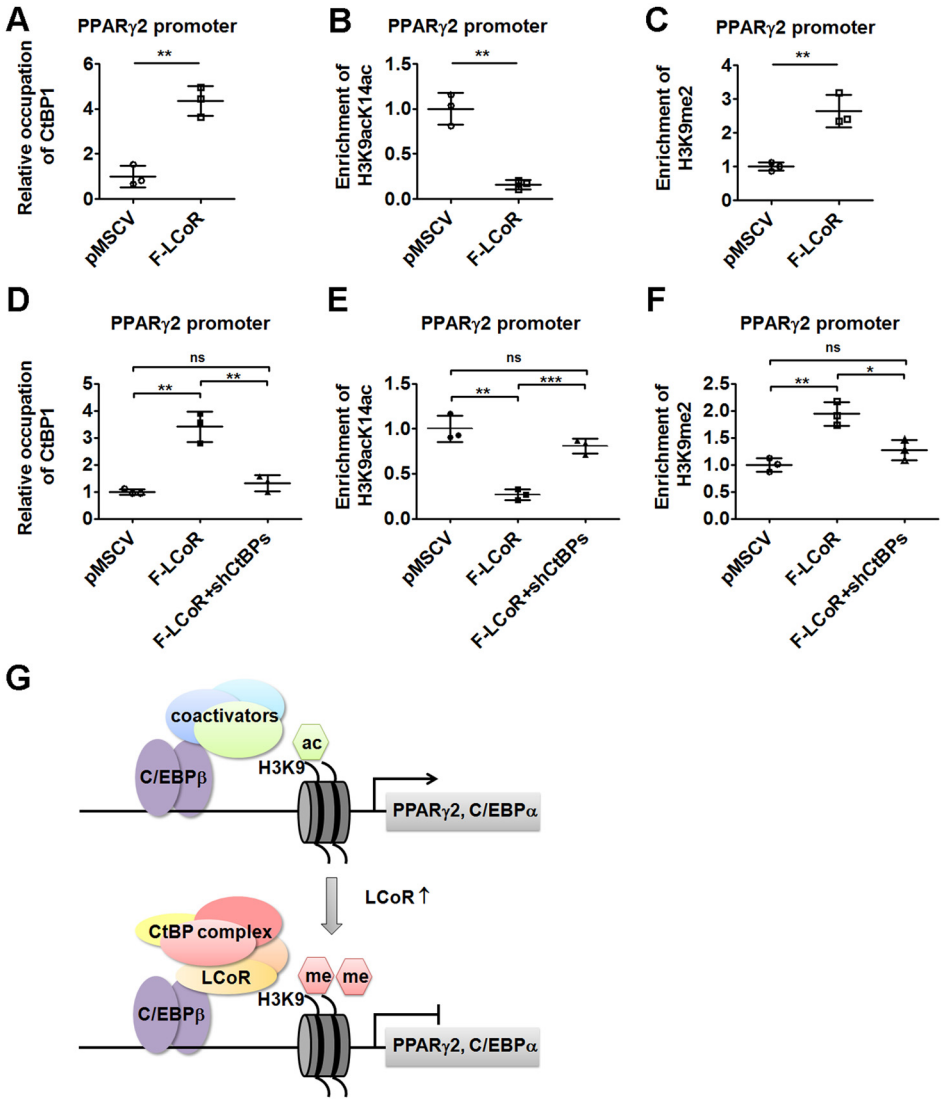

Figure 8. LCoR alters histone modifications on adipogenic promoters. *A*, ChIP analysis of the recruitment of CtBP1 on the *PPARγ2* promoter in 3T3-L1 adipocytes overexpressing F-LCoR. *B* and *C*, ChIP analysis of histone modification on the *PPARγ2* promoter in 3T3-L1 adipocytes overexpressing F-LCoR. ChIP experiments were performed using CtBP1 antibody (*A*), H3K9acK14ac antibody (*B*), and H3K9me2 antibody (*C*), respectively. *D*, ChIP analysis of the recruitment of CtBP1 on the *PPARγ2* promoter in 3T3-L1 adipocytes infected with F-LCoR or both F-LCoR and shCtBPs. *E* and *F*, ChIP analysis of histone modification on the *PPARγ2* promoter in 3T3-L1 adipocytes infected with F-LCoR or both F-LCoR and shCtBPs. ChIP experiments were performed using CtBP1 antibody (*A*), H3K9acK14ac antibody (*E*), and H3K9me2 antibody (*F*), respectively. *G*, schematic diagram of the working model of LCoR during early adipogenesis. Data are represented as mean ± S.D. of independent experiments ($n = 3$). *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *ns*, not significant.

CtBP1 to C/EBPβ, which results in transcription repression and adipogenesis inhibition. It has been reported that CtBP can regulate adipogenesis through multiple transcription factors, including KLF3 and GATA (18, 19). Therefore, our result revealed a new regulatory mechanism for CtBP in the regulation of adipogenesis.

LCoR, originally identified as a corepressor of estrogen receptor α, was shown to interact with estrogen receptor α and some other nuclear receptors only in the presence of ligands (4). Previously, we demonstrated that LCoR interacts with TR and represses the transcriptional activity of TR in a hormone-independent manner. Furthermore, we found that LCoR acts as a competitor for coactivators SRC-1/3 in binding to TR in liver (7). Because both TR and SRCs are involved in the regulation of adipogenesis (28, 32, 34), whether LCoR also could modulate adipogenesis through TR and/or SRC transcription requires further study. In contrast, our study suggested that the down-regulation of LCoR expression in the early stage might facilitate mitotic clonal expansion and promote C/EBPβ-mediated C/EBPα and PPARγ expression. However, it is still not clear why the expression of LCoR increases gradually at the end of adipogenesis. SMRT has been shown to control adipogenesis through either C/EBPβ or PPARγ, suggesting that this corepressor may play distinct roles at different stages. Therefore, further studies using an inducible system or adenoviral system to study the effect of LCoR in the late stage are required to address these questions.

In summary, our study indicates that LCoR acts as a negative regulator of early adipogenesis. LCoR suppresses adipogenesis by targeting C/EBPβ-mediated gene expression and mitotic clonal expansion. Further mechanistic studies revealed that LCoR is able to interact with C/EBPβ and recruit another core-

pressor, CtBP, to LCoR-C/EBPβ complex, which led to chromatin remodeling and transcription repression (Fig. 8*G*). Our studies and others suggest the existence of a transcription coregulator network that modulates adipogenesis. Further study of this network will help us understand adipose biology and identify potential drug targets for metabolic disease.

Experimental procedures

RNA extraction and quantitative PCR

Total RNA of cells was extracted using TRIzol reagent (Invitrogen 15596018) according to the manufacturer's instructions. Reverse transcription was performed using a PrimeScript RT reagent kit (TaKaRa). cDNAs were quantified in an ABI Real-Time System (Applied Biosystems). Relative gene expression levels were calculated using comparative C_T (TATA-binding protein was used as the reference gene) and normalized as indicated. Primer sequences used for qPCR are provided in supplemental Table S3.

Western immunoblotting

Cells were solubilized in radioimmune precipitation assay lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, and a protease and phosphatase inhibitor mixture (Roche Applied Science). The protein concentration was measured using the Coomassie protein assay kit (Pierce) and diluted to 5 μg/μl in 6× SDS loading buffer. Protein samples (50 μg of each sample in a total volume of 10 μl) were subjected to SDS-PAGE followed by transfer to PVDF membranes (Millipore). The membranes were blocked with 5% nonfat dry milk in TBS with Tween 20 followed by incubation with the indicated primary antibodies and corresponding secondary antibodies. Detection was performed using a SuperSignal West Pico Chemiluminescent Substrate kit (Pierce 34080). Antibodies used for Western blotting are as follows: anti-LCoR (Santa Cruz Biotechnology sc-134674), anti-FLAG M2 (rabbit polyclonal; Sigma F7425), anti-C/EBPβ (rabbit polyclonal; Santa Cruz Biotechnology sc-150), anti-C/EBPα (Santa Cruz Biotechnology sc-61), anti-PPARγ (Santa Cruz Biotechnology sc-7273), anti-ap2 (Cell Signaling Technology 3544), anti-CtBP1 (Santa Cruz Biotechnology sc-11390), anti-CtBP2 (Cell Signaling Technology 13256S), anti-GAPDH (Santa Cruz Biotechnology sc-25778), anti-β-actin (Sigma A5316), and anti-tubulin (Sigma T6199).

Plasmid constructs

pGEX-6P-1-LCoR and pcDNA6-LCoR have been described previously (7). FLAG-tagged LCoR and truncated mutants, F-LCoR (full length; amino acids 1–407), F-LCoRΔN (LCoR lacking the N-terminal NR box; amino acids 57–407), F-LCoRΔC (LCoR lacking the C-terminal HTH domain; amino acids 1–350), F-LCoR D1 (LCoR lacking one CtBP-binding site; amino acids 64–70 were deleted), and F-LCoR D2 (LCoR lacking two CtBP-binding sites; amino acids 64–88 were deleted), were subcloned into pMSCVpuro vector to generate retroviral plasmids. LCoR D1 and LCoR D2 were constructed using a KOD-Plus-Mutagenesis kit (Toyobo) according to the manufacturer's instructions. LCoRΔN, LCoRΔC, and LCoR HTH (amino acids 303–407) were subcloned into pGEX-6P-1 to generate GST fusion proteins. Oligonucleotides targeting mouse LCoR, C/EBPβ, CtBP1, or CtBP2, respectively, were annealed and cloned into the pSIREN-RetroQ vector (Clontech). Oligonucleotides targeting LacZ were used as a control. pGL3-PPARγ2 promoter-reporter construct (−602 to +52) and pMSCVpuro-C/EBPβ were generously provided by Kai Ge. To generate a retroviral *PPARγ2* promoter-reporter construct, *PPARγ2* promoter and the promoter-driven firefly luciferase sequence were subcloned from pGL3-PPARγ2 promoter reporter construct to pSIREN-RetroQ vector using BglII and BamHI restriction enzymes. C/EBPβ was subcloned into pcDNA6. Full-length cDNA encoding mouse *CtBP1* was generated from 3T3-L1 preadipocyte RNA by RT-PCR and cloned into pcDNA6. Primer sequences used for cloning are provided in supplemental Table S3.

Isolation of SVF cells

Inguinal fat pads from C57BL/6J mice were minced and digested in Dulbecco's modified Eagle's medium (DMEM) containing 2 mg/ml type I collagenase (Invitrogen 17100017) and 10 mM HEPES (Invitrogen 15630080) for 40 min at 37 °C with shaking, and an equal volume of DMEM containing 10% fetal bovine serum (FBS) was added to terminate the digestion. The digested fat was filtered through a sterile 100-μm cell strainer (BD Falcon) to remove undigested fragments. The filtrate was centrifuged at 250 × *g* for 10 min. The supernatant containing mature adipocytes was collected and washed twice with PBS before harvesting. The cell pellet was resuspended in PBS and further filtered through a sterile 40-μm cell strainer (BD Falcon) to obtain SVF cells. The SVF cells were either harvested for RNA and protein analyses or cultured for adipogenic differentiation.

Cell culture

3T3-L1 preadipocytes were cultured in high-glucose DMEM supplemented with 10% newborn calf serum. SVF and HEK 293T cells were cultured in DMEM supplemented with 10% FBS. For adipogenic differentiation, 2-day postconfluent 3T3-L1 preadipocytes were induced by adipogenic differentiation medium containing high-glucose DMEM, 10% FBS, 1 μg/ml insulin (Sigma I1882), 1 μM dexamethasone (Sigma D1756), and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma I5879) for 2 days. Cells were then cultured in maintenance medium containing high-glucose DMEM, 10% FBS, and 1 μg/ml insulin. For adipogenesis of SVF cells, 0.5 μM rosiglitazone (Cayman Chemical 71740) was supplemented in 3T3-L1 differentiation medium and maintenance medium. Cells were either harvested for RNA and protein analyses or stained with Oil Red O at the indicated time.

Retroviral infection

Retroviral packaging was achieved by cotransfection of pMSCV constructs or pSIREN-RetroQ with pCL-Eco vectors at a ratio of 1:1 into HEK 293T cells using Lipofectamine® 2000 transfection reagent (Invitrogen 11668019). 36 and 48 h after transfection, the viral supernatants were collected, supplemented with 8 μg/ml Polybrene, filtered through 0.45-μm filter

(Millipore SLHV033RB), and added to cells. Stable cell lines were selected in the presence of 2.5 μg/ml puromycin (Sigma P8833).

Oil Red O staining

Cells were rinsed with PBS and fixed with 10% formalin in PBS for 15 min at room temperature. After rinsing three times with PBS, cells were stained with freshly prepared Oil Red O working solution for 1 h at 37 °C followed by three rinses with double distilled H_2O and photographed. Oil Red O working solution was prepared by mixing six parts Oil Red O stock solution (0.5% Oil Red O in isopropanol) with four parts double distilled H_2O and filtered through a 0.45-μm filter.

Coimmunoprecipitation

HEK 293T cells were transfected using Lipofectamine® 2000 transfection reagent with pMSCVpuro-FLAG-LCoR (F-LCoR), pMSCVpuro-FLAG-LCoRΔN (F-LCoRΔN), pMSCVpuro-FLAG-LCoRΔC (F-LCoRΔC), pMSCVpuro-FLAG-LCoR D1 (F-LCoR D1), pMSCVpuro-FLAG-LCoR D2 (F-LCoR D2), pMSCVpuro-C/EBPβ, or pCDNA6-CtBP1 as indicated. After transfected for 36 h, cells were lysed in IP lysis buffer (pH 7.4; 50 mM NaCl, 20 mM Tris-Cl, 2 mM EDTA, 1% Nonidet P-40) supplemented with phosphatase inhibitor for 30 min on ice. Subsequently, the cell lysate was centrifuged, and the supernatant was incubated with the indicated antibodies and Protein A/G PLUS-agarose (Santa Cruz Biotechnology sc-2003) at 4 °C overnight. The immunoprecipitates were separated by SDS-PAGE and detected by Western blotting. Antibodies used for immunoprecipitation are as follows: anti-normal mouse IgG (Santa Cruz Biotechnology sc-2025), anti-FLAG M2 (mouse monoclonal; Sigma F3165), and anti-C/EBPβ (mouse monoclonal; Santa Cruz Biotechnology sc-7962).

GST pulldown

LCoR- or CtBP1-GST fusion proteins were expressed and purified using glutathione-agarose (Santa Cruz Biotechnology sc-2009). *In vitro* transcription/translation was performed using the TnT® T7 Quick Coupled Transcription/Translation System (Promega L1170) according to the manufacturer's instructions. Purified GST fusion proteins and *in vitro* translated proteins were mixed and incubated in BII buffer (20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 2 mM EDTA, 0.1% Nonidet P-40, 2 mM DTT, 0.05% BSA, 5% glycerol) supplemented with phosphatase inhibitor at 4 °C overnight. After washing five times with PBS, GST-precipitated complexes were separated by SDS-PAGE and detected by Western blotting.

LCoR protein complex purification

3T3-L1 preadipocytes stably expressing either pMSCVpuro-F-LCoR or pMSCVpuro empty vector were induced to differentiate as described under "Cell culture." After 24-h induction, the cells were harvested, and the nuclear proteins were extracted as described under "Cytoplasmic and nuclear protein extraction." The nuclear proteins were further diluted in IP lysis buffer and incubated with anti-FLAG M2 affinity gel (mouse; Sigma A2220) at 4 °C overnight. The purified nuclear extracts were eluted by 0.1 M glycine HCl (pH 3.5). The eluted proteins were separated by SDS-PAGE and visualized by silver staining. Several prominent bands between 55 and 34 kDa were excised and analyzed by mass spectrometry at the research center for proteome analysis in Shanghai Applied Protein Technology Co., Ltd. Briefly, the immunoprecipitated proteins were digested by trypsin and subjected to reverse-phase liquid chromatography with tandem mass spectrometry (LC-MS/MS) using a high-resolution hybrid mass spectrometer (LTQ-Orbitrap, Thermo Scientific). MS/MS spectra were automatically searched against ipi.HUMAN.v3.53 using Bioworks-Browser rev. 3.1 (Thermo Electron, San Jose, CA). Protein identification results were extracted from SEQUEST.out files with BuildSummary.

Chromatin immunoprecipitation

ChIP was performed using a Magna ChIP G kit (Millipore 17-611) according to the manufacturer's instructions. Briefly, 3T3-L1 preadipocytes stably expressing pMSCVpuro-F-LCoR, pMSCVpuro-F-LCoRΔN, pMSCVpuro-F-LCoRΔC, or pMSCVpuro empty vector were grown in 10-cm dishes and induced to differentiate as described under "Cell Culture." The cells were cross-linked at the indicated time with 1% formaldehyde at room temperature for 10 min. Unreacted formaldehyde was quenched by adding glycine to a final concentration of 0.125 M at room temperature for 5 min. After washing twice with ice-cold PBS, cells were harvested by scraping and pelleted. Then the cells were resuspended in 0.5 ml of cell lysis buffer containing protease inhibitor and incubated on ice for 15 min. The cell suspension was centrifuged at 800 $\times$ *g* at 4 °C for 5 min. The pellet was resuspended in 0.5 ml of nuclear lysis buffer and sonicated to shear cross-linked DNA to 200–1000 base pairs. After centrifugation at 12,000 $\times$ *g* at 4 °C for 10 min, protein-DNA complexes were diluted at a ratio of 1:10 with ChIP dilution buffer, and 1% of the mixture was removed as "input." The chromatin fractions were incubated at 4 °C overnight with 20 μl of Protein G magnetic beads and 2 μg of one of the following antibodies: anti-FLAG M2 (mouse monoclonal), anti-C/EBPβ (mouse monoclonal), anti-CtBP1, anti-H3K9acK14ac (Millipore 06-599), anti-H3K9me2 (Millipore 17-648). After washing in low-salt buffer, high-salt buffer, LiCl buffer, and Tris-EDTA buffer, proteins were eliminated using Proteinase K at 62 °C for 2 h with shaking. DNA was purified using the kit column and analyzed by qPCR. Primer sequences used in the ChIP assays are provided in supplemental Table S3.

Cytoplasmic and nuclear protein extraction

Cytoplasmic and nuclear proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce 78833) according to the manufacturer's instructions. Briefly, 3T3-L1 preadipocytes were induced to differentiate for 2 days as described under Cell culture." The cells were harvested with trypsin-EDTA at the indicated time and centrifuged at 500 $\times$ *g* for 5 min. After washing twice with PBS, cells were resuspended in Cytoplasmic Extraction Reagent (CER) I and incubated on ice for 10 min. CER II was then added and vortexed for 5 s. The cells were centrifuged at 16,000 $\times$ *g* for 5 min, and supernatants were collected as the cytoplasmic extract. The pellets were

resuspended in ice-cold Nuclear Extraction Reagent (NER) and incubated on ice for 40 min; during this incubation, lysates were vortexed for 15 s every 10 min. Finally, cells were centrifuged at 16,000 × *g* for 10 min to obtain the nuclear extract.

BrdU incorporation assay

3T3-L1 cells were induced to differentiate as described under "Cell culture." 18 h after induction, cells were labeled for 2 h with 10 μM BrdU and washed three times with PBS before fixation with 4% formaldehyde. After denaturation (2 N HCl for 20 min) and neutralization (0.1 M sodium borate, pH 8.5), cells were incubated with anti-BrdU monoclonal antibody (Santa Cruz Biotechnology sc-32323) at 4 °C overnight and then incubated with Alexa Fluor 488-conjugated secondary antibody for 1 h at room temperature. Cells were also stained with DAPI for 15 min at room temperature to enable nuclear detection. Staining was evaluated and photographed by high-content screening instruments (Thermo Fisher Scientific Cellomics).

Immunofluorescence

3T3-L1 cells cultured on coverslips were induced to differentiate for 24 h before fixation in a solution containing 4% formaldehyde (Polysciences Inc.) and 4% sucrose (Sigma-Aldrich) in PBS for 30 min. After washing three times with PBS, the cells were blocked with blocking buffer containing 0.5% Triton X-100 and 5% serum in PBS for 30 min and then coincubated overnight at 4 °C with primary antibodies against FLAG (mouse monoclonal) and C/EBPβ (rabbit polyclonal) or coincubated with primary antibodies against C/EBPβ (mouse monoclonal) and CtBP1 as indicated. Primary antibodies were diluted with blocking buffer. After washed three times again with PBS, the cells were incubated with Alexa Fluor 488- and 555-conjugated secondary antibodies (Invitrogen A21202 and A31572) for 1 h. Secondary antibodies were diluted with 0.5% Triton X-100 and 2% serum in PBS. Nuclei were stained with DAPI in PBS for 5 min. Confocal images were obtained with a confocal microscope (Olympus FW1000).

Luciferase reporter assay

Transient transfection assay was performed in 293T cells to analyze C/EBPβ activity. 293T cells were seeded in 24-well plates and grown to 80–90% confluence, and then cells in each well were transfected with 125 ng of pGL3-PPARγ2 promoter-reporter construct (−602 to +52) alone or in combination with 25 ng of pMSCVpuro-C/EBPβ in the absence or presence of increasing quantities of pMSCVpuro-LCoR (5, 10, and 20 ng), 25 nM siRNAs targeting human LCoR, or 20 ng of full-length or truncated LCoR as indicated in the legends with Lipofectamine 2000 transfection reagent. The quantity of transfected DNA was kept constant in all conditions by adding a corresponding amount of empty vector or scrambled siRNAs. Sequences of siRNAs targeting human LCoR are provided in supplemental Table S3. 5 ng of pRL-TK (Promega) carrying *Renilla* luciferase was cotransfected as an internal control. 36 h after transfection, 293T cells were harvested, and the luciferase activities were analyzed using the Dual-Luciferase assay kit (Promega) according to the manufacturer's instructions. To further analyze the effect of LCoR on C/EBPβ activity in 3T3-L1 cells, 3T3-L1 cells were infected with retrovirus expressing a *PPARγ2* promoter (−602 to +52)-driven luciferase reporter with or without retrovirus expressing LCoR, shLCoR, LCoRΔN, or LCoRΔC as indicated in the legends. The relative luciferase activity was normalized against the protein concentration of each cell lysate sample. 48 h after induction, the 3T3-L1 cells were harvested, and the luciferase activities were analyzed using a luciferase assay kit (Promega).

Statistical analysis

All experiments were performed at least three times. Data are presented as means ± S.D. Error bars represent S.D. Student's *t* test was performed to assess whether the means of two groups are statistically significant from each other ($p < 0.05$). GraphPad Prism 5.0 software was applied to all statistical analyses. The density of specific bands from immunoblots shown here was measured by ImageJ (1.29v, National Institutes of Health) and normalized to either protein input in GST pull-down and immunoprecipitation assays as indicated or protein levels of GAPDH, tubulin, or β-actin because we did not see any evidence showing that our experimental manipulations would affect their expression (supplemental Table S4).

Author contributions—H. C., W. L., and H. Y. designed the experiments. H. C. carried out most of the experiments and analyzed the data. S. Z., S. S., C. S., Y. L., H. W., and S. Y. provided technical assistance. Y. L., F. G., Q. Z., Y. W., J. J., H. W., and J. Y. contributed to the discussion and supervised the project. H. C., W. L., and H. Y. wrote the manuscript.

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