

Adipogenesis licensing and execution are disparately linked to cell proliferation

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Coordination of cell differentiation and proliferation is a key issue in the development process of multi-cellular organisms and stem cells. Here we provide evidence that the establishment of adipocyte differentiation of 3T3-L1 cells requires two processes: the licensing of an adipogenesis gene-expression program within a particular growth-arrest stage, i.e., the contact-inhibition stage, and then the execution of this program in a cell-cycle-independent manner, by which the licensed progenitors are differentiated into adipocytes in the presence of inducing factors. Our results showed that differentiation licensing of 3T3-L1 cells during the contact-inhibition stage involved epigenetic modifications such as DNA methylation and histone modifications, whereas disturbing these epigenetic modifications by DNA methylation inhibitors or RNAi during the contact-inhibition stage significantly reduced adipogenesis efficiency. More importantly, when these licensed 3T3-L1 cells were re-cultured under non-differentiating conditions or treated only with insulin, this adipogenesis commitment could be maintained from one cell generation to the next, whereby the licensed program could be activated in a cell-cycle-independent manner once these cells were subjected to adipogenesis-inducing conditions. This result suggests that differentiation licensing and differentiation execution can be uncoupled and disparately linked to cell proliferation. Our findings deliver a new concept that cell-fate decision can be subdivided into at least two stages, licensing and execution, which might have different regulatory relationships with cell proliferation. In addition, this new concept may provide a clue for developing new strategies against obesity.

Keywords: adipogenesis, proliferation, contact inhibition, DNA methylation, *C/EBP α*

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Introduction

Cell proliferation and differentiation are two basic behaviors in the development process of multi-cellular organisms or stem cells, and the inter-regulation between these two behaviors is an important issue for understanding the mechanisms of development. It is commonly assumed that differentiation is tightly coordinated with

the cell cycle [1-4], and many experiments have shown that growth arrest resulting in an exit from the cell cycle is necessary for differentiation ([5-7]; reviewed in ref [4]). However, some studies have argued that differentiation and proliferation might be regulated independently ([8-10], reviewed in ref [11]).

Cell proliferation is typically regulated during the G1 phase of the cell cycle, and reversible growth arrest during the G1 phase is a basic way to exit from the cell cycle [12]. The state of growth arrest during the G1 phase, defined as cellular quiescence, can be derived from three different procedures. The first route for cell quiescence is contact inhibition at a high cell density of growing mammalian cells [12, 13]. Treatments such as mitogen deprivation or loss of cell adhesion could also induce

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a cellular quiescent state [12]. A recent study based on genome-wide transcription profiling showed that cells could enter different quiescent states that are determined by the growth-arrest-inducing signals [14].

The 3T3-L1 cell line is widely used as an *in vitro* model for studying adipocyte differentiation [15]. The standard differentiation process of 3T3-L1 preadipocytes can be subdivided into three distinct stages: cell-contact inhibition, mitotic clonal expansion and terminal differentiation (Figure 1A; see refs [15, 16]). Proliferating 3T3-L1 cells usually undergo 48-h contact inhibition, and these post-confluent cells are then induced with a cocktail of hormones, including isobutylmethylxanthine, dexamethasone and insulin (MDI) for adipogenesis. After the hormone induction, these quiescent cells re-enter the cell cycle for a period called mitotic clonal expansion, and then subsequently differentiate [15, 16].

In this study, we used the 3T3-L1 preadipocyte model to address the question of the inter-regulation between cell proliferation and differentiation. We showed that the differentiation process of adipogenesis could be subdivided into licensing and execution phases. The former depends on an exit from the cell cycle through the contact-inhibition process, whereas the latter is cell-cycle independent. Furthermore, the licensing phase could be uncoupled from the execution phase.

Results and Discussion

Requirement of contact inhibition for differentiation

Although the first stage, “contact inhibition”, is commonly assumed to be a prerequisite for adipocyte differentiation [16], the functional role of this stage remains unknown. Some reports have argued that adipogenesis can be uncoupled from contact inhibition [17]. To determine the role of contact inhibition, we induced the differentiation of 3T3-L1 cells at different cellular states (cycling cells and the contact-inhibited 24 h (CI-24h) or 48 h (CI-48h) cells). The results showed that the longer they stayed in the contact-inhibition stage, the more the 3T3-L1 cells were differentiated into adipocytes, whereas adipocytes were barely detected from the population of cycling 3T3-L1 cells without contact inhibition under the same inducing conditions (Figure 1B).

To understand whether general cellular quiescence is required for adipocyte differentiation, we analyzed the differentiation of 3T3-L1 cells cultured in serum-free medium for 60 h before hormone induction. The results showed that adipocytes were not detected among the cells previously treated by mitogen deprivation (Figure 1B). As Collier *et al.* [14] recently showed that gene-expression changes caused by contact inhibition in the initiation stage of quiescence were considerably different

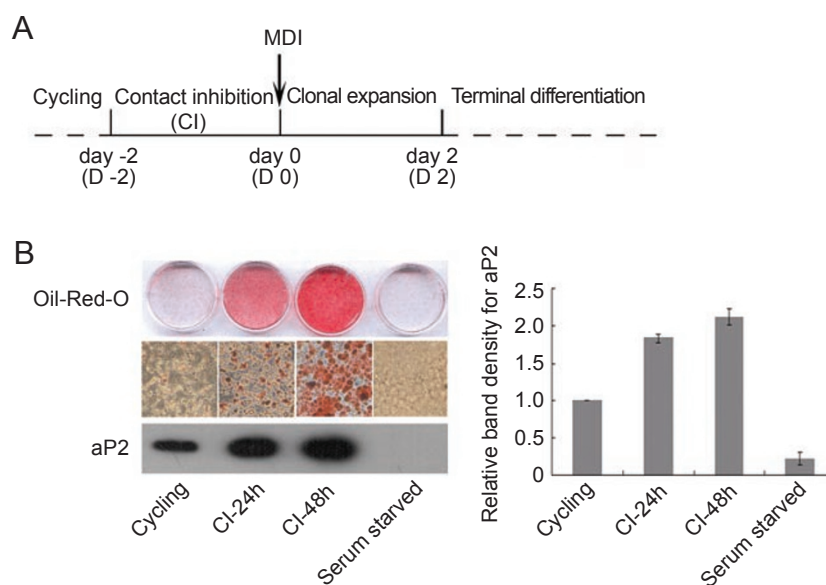


Figure 1 Contact inhibition is a prerequisite for the differentiation of preadipocytes. **(A)** Schematic for the process of 3T3-L1 cell differentiation. **(B)** Oil-Red-O staining with microscope images of the cells and western blot analysis of aP2. Cycling, CI-24h, CI-48h preadipocytes and cycling preadipocytes treated in serum-free medium for 60 h were subjected to adipogenesis induction. On day 8 of the induction, differentiated cells were stained with Oil Red O to measure the levels of triglyceride droplets and analyzed by western blot with an antibody against aP2, a marker protein for adipogenesis. Relative quantification analysis of band densities for aP2 is shown on the right. Data are presented as mean \pm s.e. from five independent experiments.

from those caused by mitogen-deprivation, we conclude that adipocyte differentiation of 3T3-L1 cells requires a unique contact-inhibition state rather than a simple state of cell-growth arrest.

As contact inhibition usually relies on high cell density, and the different cell density might result in differences in cellular communication [18], the effect of cell density on 3T3-L1 cell differentiation was also evaluated. 3T3-L1 cells were maintained in the contact-inhibition stage for 48 h and then reseeded at different cell densities (100%, 50% and 25%) under the differentiation-inducing conditions. A similar degree of adipocyte differentiation was observed among the populations of post-confluent 3T3-L1 cells plated at different cell densities (Supplementary information, Figure S1). On the other hand, cycling 3T3-L1 cells reseeded at the highest cell density (100%) failed to differentiate into adipocytes (Supplementary information, Figure S1). These results indicate that the process of contact inhibition itself is required for adipogenesis licensing of 3T3-L1 cells.

Involvement of epigenetic programming in adipogenesis licensing during contact inhibition

Recent findings indicate that the differentiation program involves epigenetic modifications of the cellular genome, including DNA methylation and histone modi-

fications [19, 20]. We reasoned that the stage of contact inhibition might provide a special window for 3T3-L1 cells to establish an adipogenesis-competency program through epigenetic modifications. To test this hypothesis, we investigated the change of 5-methylcytosine (5-MeC) distribution before and after the stage of contact inhibition through immunofluorescence using an antibody against 5-MeC. As shown in Figure 2A, punctated 5-MeC foci clearly formed and total nuclear 5-methylcytosine intensities increased in the 48-h contact-inhibited cells compared with cycling cells. In addition, the level of lysine 9 trimethylated histone H3 (H3K9) also increased, whereas H3K9 acetylation correspondingly decreased in the population of 48-h contact-inhibited cells (Figure 2B). These results indicate that epigenetic modifications occurred extensively in 3T3-L1 preadipocytes during contact inhibition.

To evaluate whether these epigenetic modifications that occurred during the contact-inhibition stage are important for adipogenesis licensing, we treated 3T3-L1 cells with two inhibitors of DNA methylation at different stages of adipogenesis. The results showed that 3T3-L1 cells in the contact-inhibition stage were highly sensitive to DNA-methylation inhibitors, whereas the cells after the stage of contact inhibition were less sensitive to the drugs (Figure 2C). The cytotoxicity of the inhibi-

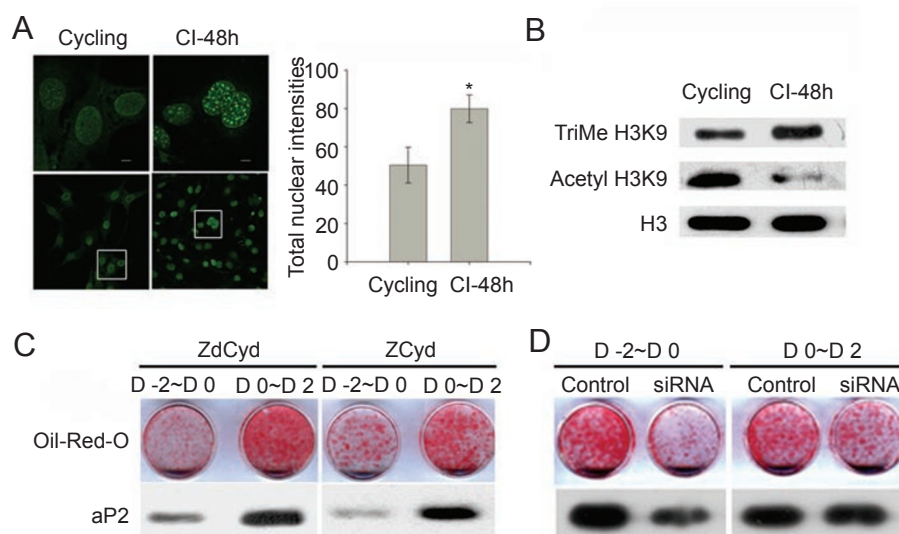


Figure 2 Licensing adipogenesis program through epigenetic modifications during contact inhibition. **(A)** 5-Methylcytosine immunofluorescence analysis. Cycling and CI-48h preadipocytes were probed with an antibody against 5-methylcytosine and an FITC-conjugated secondary antibody, respectively. Scale bars: 5 μ m. Quantification analyses of total nuclear 5-methylcytosine densities in cycling ($n = 121$) and CI-48h ($n = 151$) preadipocytes are shown on the left. Data were collected at the same voltage and are presented as mean \pm s.e. from three independent experiments ($*P < 0.01$). **(B)** Western blot analysis of modifications of histone H3 at lysine 9 in cycling and CI-48h preadipocytes. **(C)** Oil-Red-O staining and western blot analysis of aP2. Preadipocytes were treated with DNA methylation inhibitors ZnDcyd (0.2 μ M) and ZCyd (0.8 μ M) at the indicated time periods and then subjected to adipogenesis induction. **(D)** Oil-Red-O staining and western blot analysis of aP2. Preadipocytes were transfected with siRNA against *Dnmt3a* for the indicated time periods and then subjected to adipogenesis induction.

tors was evaluated by the MTT assay (Supplementary information, Figure S2A). Immunofluorescence analysis confirmed that these inhibitors led to the reduction of 5-MeC intensities (Supplementary information, Figure S2B). Furthermore, transfection of a siRNA against *Dnmt3a* during the stage of contact inhibition led to a stronger reduction in adipogenesis than did transfection after the stage of contact inhibition (Figure 2D). Silencing of *Dnmt3a* by the siRNA was confirmed by Western blot (Supplementary information, Figure S2C). Taking these results together, we conclude that adipogenesis competency is licensed, at least in part, through epigenetic modifications of the 3T3-L1 preadipocyte genome during the stage of contact inhibition.

Maintenance of adipogenesis licensing during subsequent cell cycles

It is known that epigenetic modifications can be maintained during the cell cycle [21]. Accordingly, we postulated that the licensed adipogenesis-competent state should be functionally maintained from one cell generation to the next. To test this hypothesis, we analyzed the differentiation efficiency of the pre-licensed 3T3-L1 cells that were re-cultured for different generations. 3T3-L1 cells after the 48-h contact-inhibition stage were collected as CI-48h cells and were then re-plated in a dish of the same size at different cell densities (40% as generation 1 (G1), 20% as G2, 10% as G3 and 5% as G4). Once these CI-48h cells that were re-cultured at different densities grew to the same cell density of 80%, they were subjected to adipogenesis induction with MDI, and the efficiency of adipogenesis was then quantitatively evaluated by aP2 western blot and Oil-Red-O staining. The results showed that, even after four generations of proliferation, the adipogenesis efficiency of the re-cultured CI-48h cells was still considerably higher than that of the cycling cells that had not previously experienced the stage of contact inhibition (Figure 3A; compare “negative” with “G4”). These findings suggest that the cell-fate commitment of the licensed progenitors can be maintained during subsequent cell cycles.

Treatment with the MDI cocktail results in a special proliferation process in the licensed 3T3-L1 cells after the 48-h contact-inhibition stage, referred to as mitotic clonal expansion, during which the number of 3T3-L1 cells would double 1 or 1.5 times within 48 h (refs [22, 23]; also see Supplementary information, Figure S3A). Although some studies suggested that mitotic clonal expansion might be necessary for adipogenesis of 3T3-L1 cells [23, 24], our previous work showed that this special proliferation is separable from adipocyte differentiation [22, 25]. In this study, we further showed that insulin

alone was sufficient to induce mitotic clonal expansion of 3T3-L1 cells, of which the cell numbers and DNA profiles were indistinguishable from those induced by the standard MDI cocktail (Supplementary information, Figure S3). Importantly, adipocyte differentiation could be induced by MDI after the CI-48h cells were first treated with insulin for 12, 24, 36 and 48 h, and the efficiency of adipocyte differentiation in these insulin-MDI experiments was quite similar to that of the standard sample that was treated with only MDI (Figure 3B). These data are in agreement with the result of the above cell-passage experiment showing that the licensed adipogenesis-competent state could be maintained after subsequent cell cycles. Our results clearly show that the adipogenesis competency of 3T3-L1 cells established in the contact-inhibition stage can be propagated during the subsequent process of mitotic clonal expansion (e.g., when cells were treated with insulin only), whereas the licensed program can be executed at any point of the mitotic-clonal-expansion stage once the induction condition is satisfied (by MDI treatment). In addition, this result also supports our above conclusion that the adipogenesis competency must have been completely established during the contact-inhibition stage. Otherwise, the phenomenon of propagating the adipogenesis-competent state would not have been observed in the insulin-MDI experiments.

Gene expression profiling at the stages of competency propagation and differentiation execution

We used microarray to analyze the global gene-expression profiles of post-confluent 3T3-L1 cells at the competency propagation and execution stages of adipocyte differentiation. The microarray result was confirmed by real-time quantitative PCR analyses (Supplementary information, Figure S4). Our microarray profiling detected more than 1 000 differentially expressed genes for each experimental condition (microarray data can be accessed from <http://202.127.18.238/webdownload>). Principal component analysis (PCA) showed that the overall gene-expression profile of MDI-treated cells that were pretreated with insulin for 12 h overlapped with that of the MDI-treated cells that were pretreated with insulin for 24 h, and that these profiles were quite close to those of cells treated with only MDI, whereas the overall gene-expression profile of cells treated with only insulin for 12 h was significantly different from that of cells treated with only insulin for 24 h (Figure 3C). In addition, cluster analysis of 11 selected adipogenesis-related genes showed that the expression patterns of these genes were clustered into one group for both the insulin-MDI-treated cells and the cells treated with only MDI, whereas the cells treated with only insulin were clustered into another

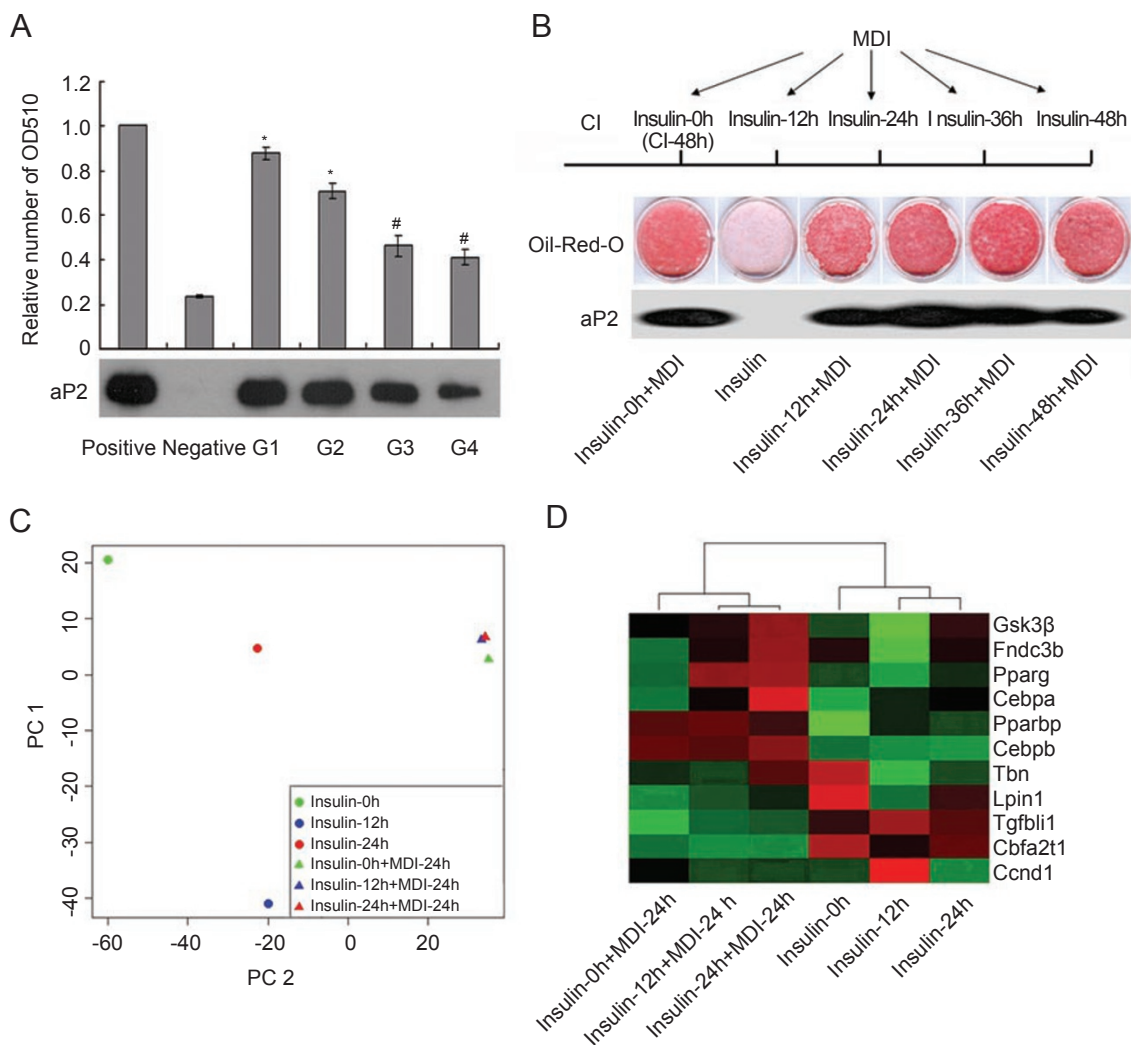


Figure 3 Licensed adipogenesis program is maintained through cell division. **(A)** Oil-Red-O staining as OD510 and western blot analysis of aP2. CI-48h preadipocytes were re-cultured at 40%, 20%, 10% or 5% cell densities (defined as G1, G2, G3 and G4, respectively). Once these cells grew to the same cell density of 80%, they were subjected to adipogenesis-inducing conditions. CI-48h preadipocytes were induced with MDI as a positive control (Positive). Cycling preadipocytes (not undergoing contact inhibition) at 80% cell density were induced with MDI as a negative control (Negative). The results displayed in the bar graphs are quantitative analytic data from three independent experiments and are presented as mean \pm s.e. Significance was determined by Student's *t*-test ($*P < 0.01$, $\#P < 0.05$ versus Negative). **(B)** Oil-Red-O staining and Western blot analysis of aP2. CI-48h preadipocytes were first treated with insulin and then induced with MDI at the indicated time periods (right panel). **(C)** Principal component analysis (PCA) result from the Affymetrix microarray approach of the overall gene-expression profiles of CI-48h preadipocytes treated first with insulin and then with MDI at the indicated time periods. **(D)** Gene-cluster analysis of the six samples over the expression profiles of 11 adipocyte-specific genes. The columns indicate the six indicated samples and the rows are the names of 11 genes. The shade changes from green to red as the expression value varies from low to high.

group (Figure 3D). Real-time quantitative PCR analyses showed that the expression levels of *PPAR γ* and *C/EBP β* were in agreement with the microarray results (Supplementary information, Figure S4). Taken together, these results indicate that the regulation of adipogenesis gene expression is separable from the regulation of gene ex-

pression for cell proliferation, and that the former must be actively maintained, because DNA replication and other cellular activities during the process of mitotic clonal expansion did not disrupt this differentiation competency-related regulation pattern.

As adipogenesis licensing during contact inhibition

was based, at least in part, on epigenetic modifications (Figure 2), the execution of adipogenesis gene expression must involve these particular epigenetic changes, whereas the regulation of gene expression for cell proliferation alone should not be related to these epigenetic changes. To test this hypothesis, we measured the epigenetic status of the CCAAT-enhancer-binding protein α (*C/EBP α*) gene, which is an essential gene in the early stage of 3T3-L1 cell differentiation [26]. As the promoter of the *C/EBP α* gene fulfills the definition of CpG islands [27], we selected the region of the promoter from -340 to -116 bp for analysis of DNA methylation status (Figure 4A). Bisulfite sequencing results showed that the CpG methylation level of the *C/EBP α* promoter region was up-regulated after contact inhibition (Figure 4B; compare the CI-48h panel with the cycling panel). Interestingly, when the post-confluent cells were induced by MDI for 24 h, the CpG methylation level of this region was down-regulated, although the region was able to maintain its hypermethylated status after treatment with insulin alone for 24 h (Figure 4B; compare the CI-48h + MDI-24h panel with the CI-48h + insulin-24h panel). We further confirmed this observation by a real-time quantitative PCR assay. The results showed that up-regulation of *C/EBP α* transcription was detected only in MDI-treated cells and could not be detected in insulin-treated cells (Figure 4C). These results support our earlier observation that the licensed adipogenesis-competent state involving epigenetic modifications can be propagated through the insulin-induced cell cycle, until these licensed cells are treated by MDI. Our suggestion is consistent with a recent study, showing that epigenetic memory of an ac-

tive gene state can persist through cell passages in the absence of transcription [28].

In conclusion, this study clearly indicates that licensing of adipogenesis relies on a particular stage of cell-growth arrest, which provides a special microenvironment for generating the programmed epigenetic modifications. Moreover, we show that the established adipogenesis-competent state can be propagated during subsequent cell cycles, whereas the execution of the differentiation program can be regulated independently from the cell cycle. Our results suggest that the cell differentiation process consists of at least two stages, a licensing stage and an execution stage, and that these two stages can be uncoupled in terms of their respective regulation.

Materials and Methods

Cell culture and differentiation induction of 3T3-L1 preadipocytes

3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% newborn calf serum (GIBCO). After 48 h contact inhibition (CI-48h), the preadipocytes (designated as day 0) were induced with 1 μ g/ml insulin, 1 μ M DEX and 0.5 mM MIX (MDI) in DMEM supplemented with 10% fetal bovine serum (GIBCO). After 48 h (day 2), the culture medium was replaced with DMEM supplemented with 10% fetal bovine serum and 1 μ g/ml insulin, and the cells were then fed every other day with DMEM containing 10% fetal bovine serum. Cytoplasmic triglyceride droplets were visible under the microscope by day 4.

For adipogenesis induction at different cell densities, the cycling preadipocytes were trypsinized from culture dishes and replaced at 100% cell density, whereas the CI-48h preadipocytes were trypsinized from the culture dishes and replaced at 100%, 50% or 25% cell densities. All the samples were subjected to the

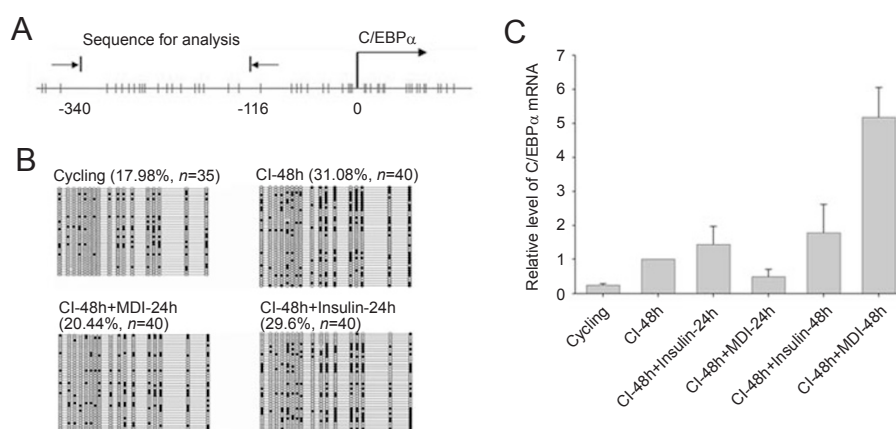


Figure 4 DNA methylation analysis of the *C/EBP α* promoter region. **(A)** CpG island analysis of the 5' flanking and 5' untranslated sequences of murine *C/EBP α* . **(B)** Bisulfite genomic sequencing results of the *C/EBP α* promoter (position -340 to -116 bp) from cells under the indicated conditions (*n* indicates the number of sequenced clones for each condition). The methylation status of each CpG dinucleotide is represented as a shaded circle if the position is methylated and as a white circle if it is not. **(C)** Real-time quantitative PCR analysis of the relative mRNA level of *C/EBP α* . Data are presented as mean \pm s.e. from three independent experiments.

differentiation induction conditions.

For adipogenesis induction at different cell generations, the CI-48h cells were trypsinized from the culture dishes and replaced at 40%, 20%, 10% or 5% cell densities. Once these CI-48h cells that were re-cultured at different cell densities grew to the same cell density of 80%, they were subjected to adipogenesis-inducing conditions. The CI-48h cells were directly induced with MDI as a positive control and the cells (not undergoing contact inhibition) at the 80% cell density were induced as a negative control.

Oil-Red-O staining, Oil-Red-O staining of OD510 and western blot analysis of aP2

3T3-L1 adipocyte monolayers were washed three times with phosphate-buffered saline (PBS) and then fixed for 2 min with 3.7% formaldehyde in PBS. Oil Red O (0.5%) in isopropanol was diluted with 2/3 volumes of water, filtered and added to the fixed cell monolayers for 1 h at room temperature. The cell monolayers were then washed with water, and the stained triglyceride droplets in the cells were visualized. Oil-Red-O staining of OD510 was carried out by extracting Oil-Red-O-stained triglyceride droplets with 100% isopropanol and then quantitating the extracts at OD510. Protein aP2 was used as an adipocyte-specific marker. Proteins of differentiated cells were collected and subjected to western blot analysis with aP2 antibody (R&D Systems). All protein samples from differentiated cells were quantified by a Lowry assay and the total protein loading was used as an input control for the aP2 western blot (data not shown).

5-Methylcytosine immunofluorescence

5-Methylcytosine (5-MeC) immunofluorescence staining was performed essentially as described in ref [29]. Briefly, the cells grown on coverslips were denatured in 70% formamide, $2 \times$ SSC for 3 min at 80 °C and then dehydrated in ice-cold ethanol. After brief air drying, the coverslips were blocked for 1 h in PBS containing 2% BSA (PBS-BSA 2%) and incubated with the anti-MeC polyclonal antibody (1:20 dilution in PBS-BSA 2%, Abcam) at 37 °C for 4 h, followed by washing with PBST (0.05% Tween-20) three times and incubation with FITC-conjugated anti-sheep secondary antibody (1:800, Abcam) in PBST (0.05% Tween-20) at room temperature for 1 h. Observations were performed with a Leica TCS SP2+ABOS confocal fluorescence microscope system using an HC x PL Apo \times 63 oil immersion objective (NA=1.4) and an excitation wavelength of 488 nm. For each indicated experiment, the same gain, black-level and aperture parameters were used. Nuclear densities were measured by manual outlining with Leica confocal analysis software. Student's *t*-test was used to compare the values from different conditions.

Western blot analysis of Dnmt3a and modified H3K9

Proteins from 3T3-L1 cell-lysate were separated by SDS-PAGE (15%) and transferred to PVDF membranes. The PVDF membranes were blocked with 5% BSA for 1 h and probed with H3K9^{3mcc} (Abcam) and H3K9^{9acc} (Cell Signaling) antibodies. H3 (Cell Signaling) was loaded as a control. For western blot analysis of Dnmt3a, proteins were separated by SDS-PAGE (6%) and transferred to PVDF membranes. The PVDF membranes were blocked with 5% BSA for 1 h and probed with Dnmt3a (Cell signaling) antibody.

siRNA transfection for Dnmt3a

The day -3 and day 0 cells were transfected with chemically synthesized siRNA (forward sequence 5'-GUG CAG AAC AAG CAG AUG A-3' and reverse sequence 5'-UCA UCU GCU UGU UCU GCA C-3') using Lipofectamine^{RT} 2000 (Invitrogen). The cells transfected on day 3 were grown for 3 days to reach 48-h post-confluence and then subjected to adipogenesis induction. The cells transfected on day 0 were induced 6 h after transfection. Scramble siRNA was used as a control.

Bisulfite genomic sequencing

Genomic DNA was extracted with a Wizard Genomic DNA Purification Kit (Promega) and treated with 3.9 M sodium bisulfite as described in www.protocol-online.org/prot/Protocols/Bisulfite-Modification--Conversion--of-DNA-3160.html. The DNA was then amplified by PCR and purified. The purified PCR products were ligated into a pGEM-T-vector (Promega) and transformed. For each indicated condition, about 40 separate clones were sequenced. The primers and PCR conditions are summarized in the Supplementary information (Supplementary information, Table S1).

DNA microarray and PCA analysis

Total RNA of 3T3-L1 cells was isolated with an RNeasy Total RNA Isolation kit (QIAGEN). The overall gene-expression profiles were detected by Affymetrix Mouse 430 2.0 according to Affymetrix GeneChip expression-assay protocols. Three independent experiments were done for each indicated condition. The chips were scanned with a Gene Array Scanner3000 and data were obtained by GCOS1.2. All data were analyzed with the standard method of PCA, and the expression data of 11 adipocyte-specific genes were selected for gene-cluster analysis.

Real-time quantitative PCR

Total RNA of 3T3-L1 cells was isolated with an RNeasy Total RNA Isolation kit (QIAGEN) and reverse transcribed (Takara). The SYBR Green quantitative PCR analysis reactions were then performed using the ABI Prism 7500 sequence detection system. The following primers were used for PPAR γ : forward 5'-TGT CGG TTT CAG AAG TGC CTT G-3' and reverse 5'-TTC AGC TGG TCG ATA TCA CTG GAG-3'. The following primers were used for *C/EBP β* : forward 5'-ACC GGG TTT CGG GAC TTG A-3' and reverse 5'-GTT GCG TAG TCC CGT GTC CA-3'. The following primers were used for *C/EBP α* : forward 5'-CGC AAG AGC CGA GAT AAA GC-3' and reverse 5'-CAG TTC ACG GCT CAG CTG TTC-3'. The gene GAPDH was detected simultaneously as a control.

MTT assay

After the cells were treated with ZcCyd (0.2 μ M) or ZCyd (0.8 μ M) for the indicated time, the cell culture medium was replaced with 0.5 mg/ml MTT solution, and the cells were incubated at 37 °C and 10% CO₂ for 3 h. The MTT formazan crystals were then dissolved with 1 ml of isopropanol and detected at OD570 and OD690 in a spectrophotometer.

Cell counting and FACS analysis

The CI-48h preadipocytes were induced by MDI and insulin at the indicated times. The cells were then trypsinized and the

number of cells was determined using a hemocytometer. For FACS analysis, the cells were fixed and permeabilized in 70% ethanol. The cells were then treated with 0.5 mg/ml RNase A at 37 °C for 1 h. After staining with 50 µg/ml propidium iodide, the DNA content in the cells was detected with a FACScan flow cytometer (Becton Dickinson).

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