



Apoptosis-inducing factor is a target gene of C/EBP α and participates in adipocyte differentiation

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ABSTRACT

Besides the contribution in cell death, apoptosis-inducing factor (AIF) also exerts roles in other cellular activities, which are largely unknown. The CCAAT-enhancer binding protein alpha (C/EBP α) is required for differentiation of adipocytes and granulocytes. Here we report that, during 3T3-L1 adipocyte differentiation, AIF expression is robustly upregulated via transcriptional regulation by C/EBP α . The upregulated effect is also confirmed by knockdown and ectopic expression of C/EBP α in U937 and MCF-7 cells respectively with and without endogenous expression of C/EBP α protein. We also reveal that AIF knockdown attenuates 3T3-L1 adipocyte differentiation, presumably due to the mitochondrial respiratory chain deficiency. These results provide evidence for the role of AIF in adipocyte differentiation.

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1. Introduction

Apoptosis-inducing factor (AIF), a mitochondrial flavoprotein, is well known as a caspases-independent cell death inducing factor [1]. During apoptotic signaling, AIF is released from the mitochondria with permeabilization of the mitochondrial membrane, and translocates to the nucleus where it induces chromatin condensation and large-scale DNA cleavage [2].

It is supposed that AIF also plays an important role in mitochondrial function. Under physiological conditions, AIF is confined to the inter-membrane space of mitochondria and possesses an NADH oxidase activity [3]. Notably, cells lacking AIF exhibit severe reduction of respiratory chain complex I activity [4]. Inactivating AIF in the early mouse embryo causes abnormal cell death presumably because of reduced mitochondrial complex I activity [5]. Targeted deletion of AIF protects mouse from obesity and diabetes, due to decreased mitochondrial oxidative phosphorylation [6]. Very recently, a disease-segregating mutation of AIFM1 gene, encoding AIF that deletes arginine 201 was reported in two male

infant patients who were given a diagnosis of progressive mitochondrial encephalomyopathy [7].

These evidences from cell lines, rodent model and clinical reports indicate that besides apoptosis induction, AIF may also play important roles in other cellular activities, which are largely unknown to date. Here we report that AIF gene is a transcriptional target of CCAAT/enhancer binding protein α (C/EBP α), which is required for differentiation of adipocytes [8] and neutrophil granulocytes [9], and controls cellular proliferation in vivo [10]. We also show that AIF expression is positively controlled by C/EBP α in leukemic U937 cells, mouse liver, as well as during the process of 3T3-L1 adipocyte differentiation. In turn, AIF participates in adipocyte differentiation, probably through maintaining the function of mitochondrial respiratory chain complex I.

2. Materials and methods

2.1. Cell culture and adipocyte differentiation

293T, MCF-7 and 3T3-L1 cells were cultured in high-glucose DMEM (Invitrogen, Carlsbad, CA) containing 10% FBS (Gibco BRL, Gaithersburg, MD). Leukemic cell lines U937, U937-NC, U937-siC/EBP α (generated as previously reported [11]) were cultured in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MI) supplemented with 10% FBS. 3T3-L1 differentiation was induced according to a previous procedure [8].

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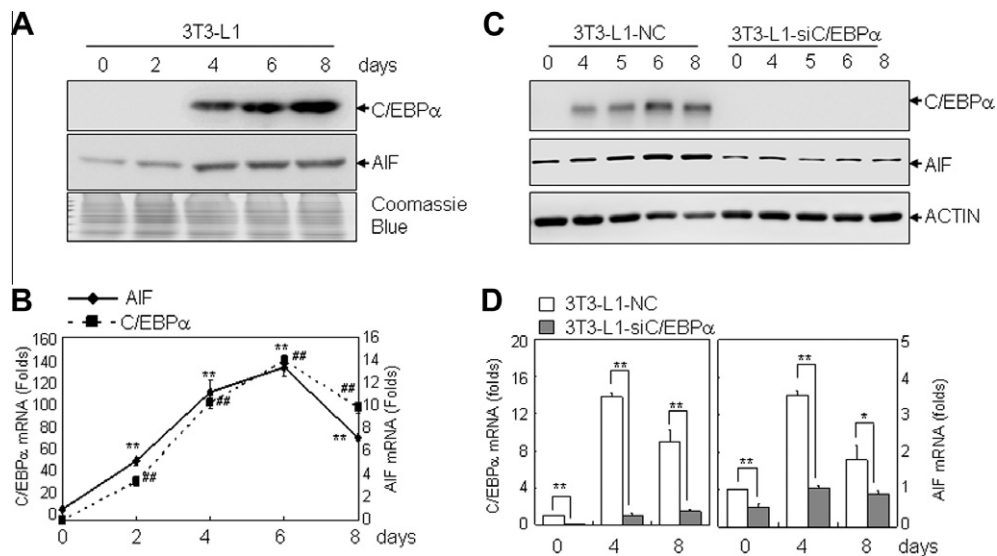


Fig. 1. AIF expression increases coincidentally with C/EBP α during 3T3-L1 adipocyte differentiation. 3T3-L1 adipocyte differentiation was induced by treatment with a hormone mixture. (A) Total protein at indicated time points were subjected to Western blot with indicated antibodies. Coomassie blue staining was used as loading control. (B) Total RNA at indicated time points were subjected to quantitative realtime PCR with β -actin as an internal control. Error bars represent S.D. ($n = 3$; **## $P < 0.005$ compared to the control; Student's t -test). (C and D) 3T3-L1-NC or 3T3-L1-siC/EBP α cells were induced to differentiate. Cells were collected at indicated time points to detect protein (C) and mRNA (D) levels with indicated antibodies and primers ($n = 3$; * $P < 0.05$, ** $P < 0.005$; Student's t -test).

2.2. Oil-Red-O staining

To visualize lipid accumulation, cells were stained with Oil-Red-O (Sigma) [8]. For quantification of triglycerides production, 1 ml of isopropyl alcohol was added to the stained culture dish. Absorbance of the extracted dye was monitored spectrophotometrically at 510 nm.

2.3. Animals

Liver-specific C/EBP α -null mice were generated by crossing C/EBP α -floxed mice (purchased from The Jackson Laboratory) with mice carrying the albumin-*Cre* (AlbCre) transgene (purchased from Model Animal Research Center of Nanjing University). Experiments were performed using liver and kidney cells from C/EBP α ^{fllox/fllox} × AlbCre⁺ (KO) and C/EBP α ^{fllox/fllox} × AlbCre⁻ (FLOX) mice. All procedures were approved by the local ethics committee of Shanghai Jiao-Tong University School of Medicine.

2.4. Plasmids and constructs

cDNAs of C/EBP α , PU.1, RUNX1, C/EBP β (kindly provided by Professor Qi-Qun Tang) and CHOP were cloned and inserted into pcDNA3 (Invitrogen). For siRNA in 3T3-L1 cells, the following oligonucleotides were inserted into RNAi-Ready pSIREN-RetroQ vector (Clontech, Palo Alto, CA): 5'-CCTTGTGCCTTGATACTC-3' for mouse C/EBP α , 5'-CTGGTATCCGTTCCGAGAG-3' for mouse AIF, and 5'-ACT-ACCGTTGTATAGGTG-3' for scrambled negative control.

2.5. Luciferase reporter assays

The human AIF promoter (−593 to +1337) or mouse AIF promoter (−551 to +1360) was subcloned into pGL3-Basic (Promega, Madison, WI), respectively. Luciferase assay was performed as previously described [12].

2.6. ChIP assay

U937 cells were subjected to ChIP assay as previously described [12] using the specific anti-human C/EBP α antibody. PCR was

performed with the following primers targeting different regions of human AIF promoter: 5'-CCACAGCAGGAGACTGTGTATC-3' (forward) and 5'-GATCCGGCGTGTAC TTCCATC-3' (reverse) for P1, 5'-CTAGAGCCAGCGTCTTTGCG-3' (forward) and 5'-ACTCGCAGCGGTAGCACTC-3' (reverse) for P2, 5'-GAATTCGGGTCTCTCCCACT-3' (forward) and 5'-TTGCCCATGAAGTAACG-3' (reverse) for P3, 5'-GT GCTGCCATGTTATCAAAGT-3' (forward) and 5'-TGGCAGTGTCCAGGAAGCA G-3' (reverse) for P_{>3000}.

2.7. Retroviral infection

Retroviruses were produced as previously described [13].

2.8. Quantitative realtime-PCR

Quantitative realtime PCR was performed as previously described [13]. Additional primers were used: 5'-CAGA-GAAGAGCCATTGCCTCC-3' (forward) and 5'-ATACAATCAGGACCTGGCCCC-3' (reverse) for mouse AIF, 5'-TTACAACAGGC-CAGGTTCC-3' (forward) and 5'-GGCTGGCGACATACAGT ACA-3' (reverse) for mouse C/EBP α , 5'-CCTTCTTCTTGGGT ATGGA-3' (forward) and 5'-CTTGCTGATCCACATCTGCT-3' (reverse) for mouse β -actin.

2.9. Western blots

Western blot was performed as described previously [13]. The proteins were probed by antibodies against human C/EBP α (sc-9314, Santa Cruz Biotech, Santa Cruz, CA), mouse C/EBP α (sc-61, Santa Cruz Biotech, Santa Cruz, CA), AIF (#4642, Cell Signaling, Beverly, MA), PPAR γ (#2443, Cell Signaling), NDUFS1, NDUFS3, NDUFB6 and NDUFA9 (12444-1-AP, 15066-1-AP, 16037-1-AP, 20312-1-AP, Proteintech Group, Chicago, IL), NDUFA10 (ab19131, abcam, San Francisco, CA) with mouse anti- β -actin mAb (JLA20, Merck, Darmstadt, Germany) to confirm equal loading.

2.10. Assessment of mitochondrial complex I activity

Mitochondria were isolated as previously described [14]. Complex I activity was assayed according to a previous report [15] by

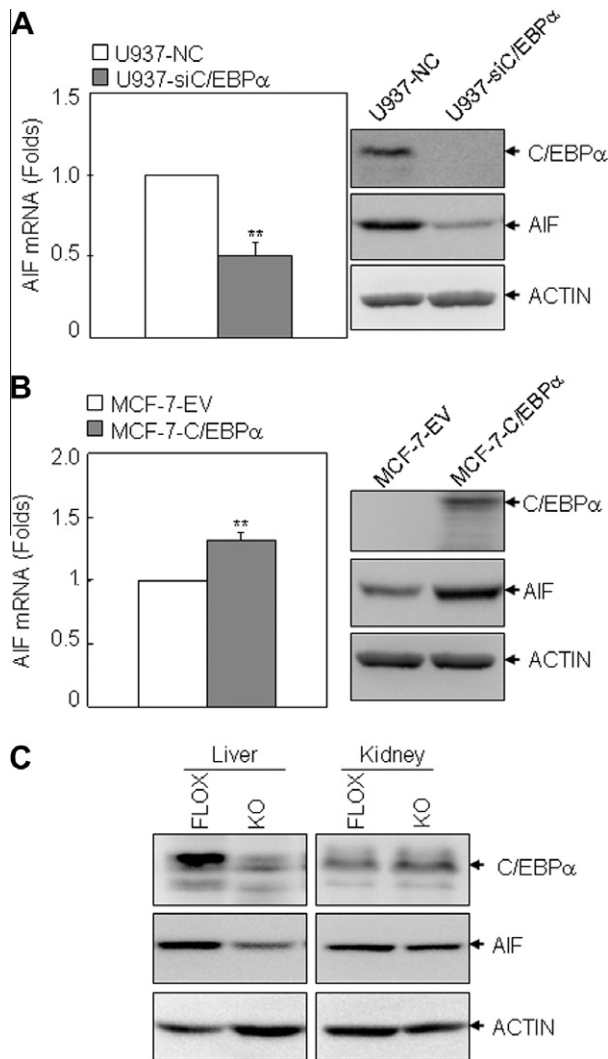


Fig. 2. AIF expression is positively regulated by C/EBP α . (A and B) mRNA and proteins from U937-NC or U937-siC/EBP α cells (A) and MCF-7-EV or MCF-7-C/EBP α cells (B) were analyzed respectively by quantitative realtime PCR and Western blot ($n = 3$; ** $P < 0.005$ compared to the control; Student's t -test). (C) Total proteins extracted from 4-week-old mouse were subjected to Western blot. The indicated proteins were assessed.

monitoring the reduction of DCPIP at 600 nm upon addition of assay buffer (10 \times buffer containing 500 mM Tris-HCl, pH 8.1, 1% BSA, 20 μ M antimycin A, 20 μ M NaN $_3$, 700 μ M decylubiquinone). Final concentration of mitochondrial protein was 20 μ g/ml with DCPIP concentration of 80 μ M. The reaction was started by adding 200 μ M NADH and scanned spectrophotometrically at 600 nm. Rotenone (3 μ M) was added into above system as the blank.

3. Results

3.1. AIF expression increases coincidentally with C/EBP α during 3T3-L1 adipocytic differentiation

When 3T3-L1 cells were induced to differentiate upon exposure to a mixture hormonal stimuli containing 100 nM insulin, 1 μ M dexamethasone and 0.5 mM 1-methyl-3-isobutyl-xanthine, a robust upregulation of AIF expression was observed by Western blot (Fig. 1A) and quantitative realtime PCR (Fig. 1B) analyzes. Significant upregulation of AIF protein could be observed from cells 4 days post-differentiation and reached maximal levels at day 8, while AIF mRNA started to increase as early as 2 days

and began to drop after day 6. To our interest, the expression of AIF paralleled to that of C/EBP α (Fig. 1A and B), which led us to hypothesize that C/EBP α modulates AIF expression during 3T3-L1 adipocyte differentiation. In order to verify this hypothesis, 3T3-L1 cells were transfected with retrovirus vector harboring shRNA against C/EBP α (siC/EBP α) or scrambled negative control (NC). C/EBP α was efficiently knocked down (Fig. 1C and D, upper panel), and as a result, 3T3-L1 differentiation was almost completely blocked (data not shown). Intriguingly, the up-regulation of AIF was greatly attenuated after loss of C/EBP α (Fig. 1C and D, lower panel). Furthermore, AIF was regulated by basal levels of C/EBP α in 0 day cells in which C/EBP α expression was extremely low, indicating a strong regulation of C/EBP α upon AIF.

3.2. AIF is positively regulated by C/EBP α

Besides C/EBP α , a batch of transcription factors is activated during the process of adipocyte differentiation. To reveal the relationship between C/EBP α and AIF more directly, we employed a leukemic cell line U937, which expresses endogenous C/EBP α under normal conditions. Knockdown of C/EBP α in U937 cells resulted in the reduced expression of both AIF protein and mRNA (Fig. 2A). On the contrary, when C/EBP α was ectopically expressed in breast cancer cell line MCF-7 which contains undetectable endogenous C/EBP α , AIF expression was upregulated (Fig. 2B).

To further verify the impact of C/EBP α on AIF expression, a liver-specific C/EBP α -null mouse line was generated. C/EBP α was lost in the liver, but not in the kidney of KO mouse. As a result, liver AIF of KO mouse was greatly reduced while AIF in kidney remained unchanged. Taken together, these results demonstrated that AIF expression is regulated by C/EBP α both in vitro and in vivo.

3.3. C/EBP α activates the AIF promoter

Because C/EBP α is a transcription factor, we conducted the following studies to determine whether C/EBP α regulates AIF at the transcriptional level. A 2 kb DNA fragment flanking human or mouse AIF promoter was inserted into the promoterless luciferase reporter plasmid pGL3-Basic, respectively. The reporter plasmids were transfected into 293T cells. Analysis of cell lysates 36 h later revealed that both promoters were activated by cotransfection of an expression vector encoding C/EBP α (Fig. 3A). Furthermore, C/EBP α activates AIF promoter in a dose-dependent manner (Fig. 3B).

C/EBP α is one of the hematopoiesis-related transcription factors including C/EBPs, PU.1/spi-1 and runt-related transcription factor 1 (RUNX1). Overlapping of target genes between these transcription factors is commonly seen. To find out the situation here, AIF promoter was cotransfected with C/EBP α , PU.1 and RUNX1 into 293T cells respectively. As shown in Fig. 3C, although statistically significant, the activation of AIF promoter by PU.1 and RUNX1 was very weak compared to that of C/EBP α . To further verify the specificity of C/EBP α upon AIF activation, two other C/EBP family members were also tested. C/EBP β activated AIF promoter only to a very low extent while CHOP showed no effect (Fig. 3D).

Next, we sought to verify C/EBP α binding to the AIF gene promoter by chromatin immunoprecipitation (ChIP) in U937 cells. The precipitated DNA was subjected to PCR amplification using three pairs of primers, P1, P2 and P3, specific to different regions of AIF gene promoter, as illustrated in Fig. 3E. It was astonishing to find that C/EBP α associated with all three regions. In addition, P $_{>3000}$, a pair of primers designed to 3 kb downstream of the transcription start site was used as a negative control. Taken together, these results clearly showed that C/EBP α binds to AIF promoter and activates its transcription.

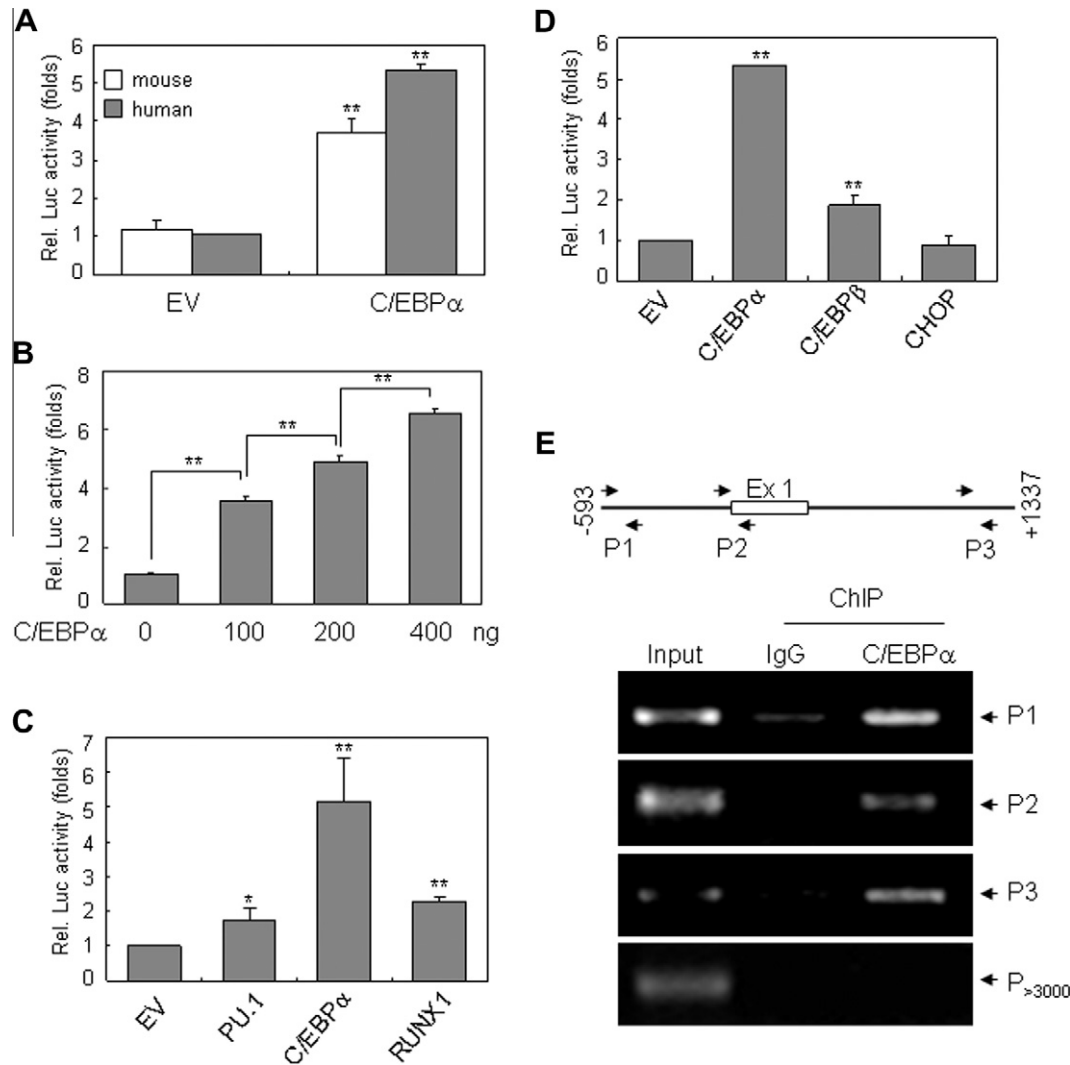


Fig. 3. C/EBP α activates and binds to multiple sites within AIF promoter. (A) Mouse or human AIF-luciferase reporters were cotransfected with EV or vector encoding C/EBP α into 293T cells. Mean luciferase activity is shown relative to the cells transfected with EV. Error bars represent S.D. ($n = 3$; $**P < 0.005$ compared to EV; Student's t -test). (B) 293T cells were transfected with human AIF-luciferase reporter and increasing amounts of vector encoding C/EBP α ($n = 3$; $**P < 0.005$; Student's t -test). (C) Human AIF-luciferase reporter was cotransfected with EV, vectors encoding C/EBP α , PU.1 or RUNX1 into 293T cells ($n = 3$; $*P < 0.05$, $**P < 0.005$ compared to EV; Student's t -test). (D) Human AIF-luciferase reporter was cotransfected with EV, vectors encoding C/EBP α , C/EBP β and CHOP into 293T cells ($n = 3$; $**P < 0.005$ compared to EV; Student's t -test). (E) C/EBP α binding to AIF promoter was analyzed in U937 cells. Chromatin was immunoprecipitated with IgG or anti-C/EBP α antibody and analyzed by PCR using primers indicated above.

3.4. AIF knockdown suppresses C/EBP α mediated 3T3-L1 adipocyte differentiation

C/EBP α is a master adipogenic transcription factor that modulates gene expression during adipocyte differentiation [8]. Thus, we speculated that AIF upregulation by C/EBP α may play a role in adipocyte differentiation. To address the function of AIF, we examined the effect of inhibiting it. When 3T3-L1 cells were infected with retrovirus vector harboring either NC or siAIF and then hormonally stimulated to differentiate, Oil-Red-O staining on day 6 after induction showed that knockdown of AIF reduced the accumulation of lipid droplets by approximately 50% (Fig. 4A and B). Furthermore, the expression levels of adipocyte markers C/EBP α and PPAR γ were also downregulated in AIF knockdown cells compared to those in NC cells at day 6 (Fig. 4C), indicating that AIF participated in 3T3-L1 adipocyte differentiation.

3.5. AIF knockdown compromised mitochondrial respiratory chain complex I activity

It has been reported that mitochondria inhibition resulted in notable suppression on adipocyte differentiation [16,17]. AIF is required for efficient oxidative phosphorylation through its role in ensuring proper assembly and functionality of mitochondrial respiratory chain complex I [4]. In view of these, we speculated whether AIF plays its role in adipocyte differentiation by maintaining the activity of complex I. The protein levels of several complex I subunits, including NADH dehydrogenase (ubiquinone) Fe-S protein 1 and 3 (NDUFS1 and NDUFS3), NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6 (NDUFB6), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9 and 10 (NDUFA9 and NDUFA10) were detected. As expected, 3T3-L1-siAIF cells displayed complex I deficiency manifested both on protein and functional

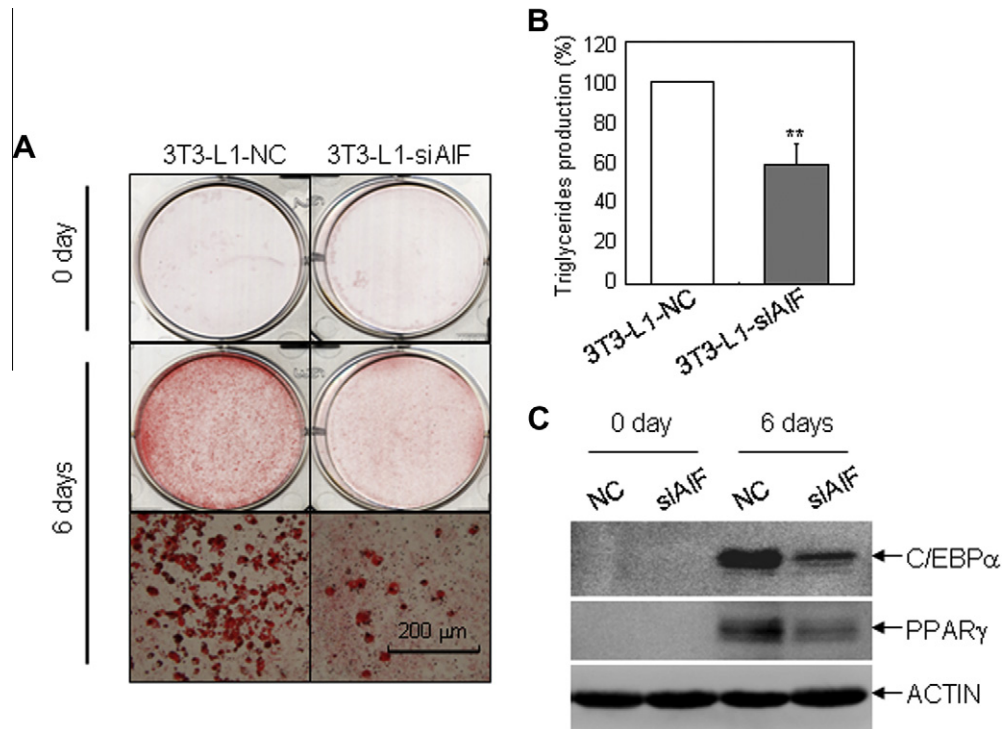


Fig. 4. AIF knockdown attenuated 3T3-L1 adipocyte differentiation. (A) On day 6 after induction of differentiation, 3T3-L1-NC or 3T3-L1-siAIF cells were stained with Oil-Red-O. (B) Quantification of triglycerides production after Oil-Red-O staining ($n = 3$; $**P < 0.005$ compared to the control; Student's t -test). (C) Total protein was collected and subjected to Western blot with indicated antibodies.

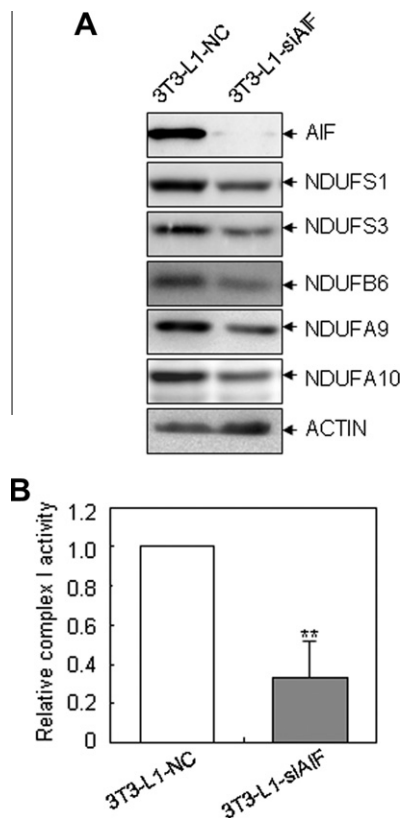


Fig. 5. AIF knockdown attenuated mitochondrial respiratory chain complex I activity. (A) Western blot determination of the subunit composition of complex I in 3T3-L1-NC or 3T3-L1-siAIF cells using indicated antibodies. (B) Mitochondrion-enriched fractions from 3T3-L1-NC or 3T3-L1-siAIF cells were monitored for the complex I activity ($n = 3$; $**P < 0.005$ compared to the control; Student's t -test).

levels revealed by Western blot (Fig. 5A) and spectrophotometry of isolated mitochondria (Fig. 5B). These results suggest that AIF participates in adipocyte differentiation, probably through maintaining mitochondrial function.

4. Discussion

In this study we showed for the first time that AIF is robustly upregulated during 3T3-L1 adipocyte differentiation, for which C/EBP α is responsible. Next, we identified C/EBP α as a transcription factor for AIF gene. C/EBP α activates AIF promoter through association with multiple sites. Multiple-site binding is possibly a common mechanism for C/EBP α as a strategy for strong activation. For example, C/EBP α binds to seven sites in the human AFP gene promoter and enhancer regions [18].

C/EBP α is a master regulator for adipocyte differentiation. We found that the ability of C/EBP α to induce adipocyte differentiation is partially mediated by AIF, as knockdown of AIF could attenuate 3T3-L1 differentiation to adipocytes. It is one of the few cellular activities that AIF was found to be involved irrespective of its well-defined apoptosis-induction role. One possible reason as we speculated is the decreased stability of mitochondrial complex I and the consequently impaired mitochondrial functionality caused by AIF loss. As reported, mitochondria underwent massive proliferation during adipocytes differentiation process [16,17]. Treatments with mitochondrial respiratory chain inhibitors resulted in notable suppression on adipocyte differentiation [17]. As a result of the accelerated mitochondrial biogenesis, the cellular abundance of complex I increased splendidly [16]. In order to maintain the high levels of complex I, more molecules that help with assembly are needed, which led us to infer that AIF expression is elevated partly to meet the demand of complex I stabilization.

In this work, we demonstrated that AIF is a target gene of C/EBP α and participates in C/EBP α mediated 3T3-L1 adipocyte differentiation. Considering the fact that the known functions of C/EBP α

and AIF are poorly related, in the future, it is of great interest for us to explore whether AIF is involved in other cellular activities driven by C/EBP α or whether AIF endows C/EBP α with new functions.

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