Published in final edited form as:

Exp Cell Res. 2008 April 15; 314(7): 1626–1639. doi:10.1016/j.yexcr.2008.01.028.

Regulation of apoptotic and growth inhibitory activities of C/EBP α in different cell lines

Guo-Li Wang¹, Xiurong Shi, Elizabeth Salisbury, and Nikolai A. Timchenko^{1,*}

¹Huffington Center on Aging and Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030

Abstract

C/EBP α is expressed in many tissues and inhibits cell growth. In this paper, we have examined mechanisms which regulate activities of C/EBP α in cell lines derived from different tissues. We found that C/EBP α possesses strong pro-apoptotic activity in NIH3T3 cells, while this activity is not detected in 3T3-L1, Hep3B2 and HEK293 cells. Micro array data show that C/EBP α activates many genes of apoptosis signaling in NIH 3T3 cells. One of these genes, ARL6IP5, is a direct target of C/EBP α and is a key mediator of the apoptosis. Using C/EBP α mutants which do not cause cell death; we have found that C/EBP α does not arrest proliferation of NIH3T3 cells. The lack of growth arrest in NIH3T3 cells correlates with the inhibition of p16INK4 and with low levels of cyclin D3. The limited growth inhibitory activity of C/EBP α is also observed in Hep3B2 cells which express low levels of cyclin D3. Elevation of cyclin D3 restores growth inhibitory activity of C/EBP α in NIH3T3 and in Hep3B2 cells. These data show that apoptotic and growth inhibitory activities of C/EBP α are differentially regulated in different cells and that cooperation of cyclin D3 and C/EBP α is required for the inhibition of proliferation.

INTRODUCTION

A member of C/EBP family, C/EBP α , is expressed in many tissues and is involved in the regulation of differentiation and in growth arrest [1–3]. C/EBP α regulates gene expression via direct interactions with the promoters, while C/EBP α -mediated growth arrest requires direct interactions of C/EBP α with cell cycle proteins. The growth inhibitory activity of C/EBP α in the liver, adipose tissue and in myeloid cells is displayed through different mechanisms [3]. Molecular mechanisms regulating growth inhibitory activity of C/EBP α in the liver present an example of a complicated network of several pathways operating upstream and downstream of C/EBP α . Expression of C/EBP α is first detected in livers at days 13–14 of gestation [4, 5, 6]. At that time, C/EBP α plays a critical role in transcriptional activation of the genes required for assembly of the liver architecture. Since liver needs to grow before birth, the growth inhibitory activity of C/EBP α is significantly reduced in prenatal livers and is displayed in restricted cell types of the liver. Sato and co-workers have shown that, in prenatal livers, a transient sumoylation of C/EBP α on 159-lysine blocks the interaction of C/EBP α with Brg1/Brm and blocks its inhibitory effect on liver proliferation [6]. After the birth, C/EBP α is not sumoylated and inhibits liver proliferation [6, 7, 8]. In

© 2008 Elsevier Inc. All rights reserved.

*Correspondent author: Nikolai A. Timchenko, Department of Pathology and Huffington Center on Aging, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, Tel: 713-798-1567, Fax: 713-798-4161, nikolait@bcm.tmc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

addition, recent publications show that the biological functions of C/EBP α during prenatal development of the liver depend on the cell type in which C/EBP α is expressed. At day 17 of gestation, the deletion of C/EBP α causes a 3.5-fold increase of proliferation in hepatocytes; while biliary epithelial cells do not show significant change in the rate of proliferation [9]. In young adult animals, C/EBP α is associated with cdk2 and p21 and inhibits liver proliferation by inhibiting cdk2 [2, 7, 10]. With age, C/EBP α changes the pathway of growth arrest and inhibits E2F-dependent promoters as the component of a high MW C/EBP α -Brm complex [11]. In the liver, cyclin D3 is a key positive regulator of C/EBP α inhibitory activity and the expression of cyclin D3 is increased with age leading to enhancement of growth inhibitory activity of C/EBP α [12]. Cyclin D3 belongs to a family of D-type cyclins which consists of three members: cyclins D1, D2 and D3. On the contrary to cyclins D1 and D2, cyclin D3 is expressed in a number of quiescent tissues and displays functions that support differentiation status of the tissues [13–15]. Protein levels of cyclin D3 are increased during differentiation of myocytes and are involved in the regulation of the MyoD-mediated program of gene expression [16]. Cyclin D3 is also increased during differentiation of 3T3-L1 cells [17] and contributes to differentiation of adipocytes by activation of PPAR γ [18]. High levels of Cyclin D3 are observed in the quiescent liver [19]. These studies showed that cyclin D3 supports growth arrest and differentiation of several tissues through activation of certain signal transduction pathways.

The initial goal of the work presented in this paper was to determine the mechanisms which regulate growth inhibitory activities of C/EBP α in different cell lines. In the course of these studies, we have found that C/EBP α also possesses pro-apoptotic activity which is very strong in NIH3T3 cells and which prevents investigations of C/EBP α growth arrest in these cells. Searching for the molecular mechanisms by which C/EBP α causes death of NIH3T3 cells, we have performed micro array studies and found that C/EBP α increases expression of several apoptotic proteins in NIH3T3 cells, while in 3T3-L1 cells these proteins are not activated. Among activated proteins, ARL6IP5 was increased up to 10–15 folds. We have found that the ARL6IP5 is a key regulator of apoptotic activities of C/EBP α in NIH 3T3 cells and that C/EBP α activates this protein through direct binding to its promoter. We have generated C/EBP α mutant which is not able to cause cell death and used this mutant for examination of the inhibitory activities of C/EBP α in NIH3T3 cells. Our further data demonstrated a critical role of the cooperation of cyclin D3 and C/EBP α in the inhibition of cell proliferation.

Materials and Methods

Growth arrest studies in cultured cells

In these studies, we have used NIH3T3, 3T3-L1, Hep3B2 and HEK293 cells. Examination of growth arrest in these cells was performed as described in our previous papers [11, 20]. Briefly, WT C/EBP α and C/EBP α mutants (shown in Fig 1A) and mouse cyclin D3 were cloned into pAdTrack plasmid under CMV promoter. This plasmid also expresses green fluorescent protein (GFP) which allows a visualization of transfected cells. The pAdTrack-cyclin D3 or pAdTrack-C/EBP α plasmids were transfected into cells and the levels of corresponding protein were examined in transfected cells by Western blotting and calculated as ratios to β -actin. Number of single green colonies (growth arrest) and number of colonies containing two, three and more cells per colony was counted. The efficiency of transfections did not differ significantly between Hep3B2, NIH3T3 and 3T3-L1 cell lines and was in the range of 15–25%. For HEK293 cells, we have higher efficiency which reached up to 40–60%. Since C/EBP α is expressed from CMV promoter, the levels of C/EBP α as ratios to β -actin. were approximately identical in HEK293, Hep3B2 and 3T3-L1 cells. In NIH3T3 cells; however, C/EBP α / β -actin ratio was lower due to apoptotic-mediated selection of un-transfected cells. Since the WT C/EBP α induces apoptosis in cultured cells at days 4–5 after

transfections (see Fig 1), the C/EBP α growth arrest was examined at days 1, 2 and 3 after transfections. Data in the manuscript present summaries with at least three independent experiments. 200–300 colonies were calculated in each experiment.

Examination of cell death and apoptosis

Survival studies—pAdTrack -C/EBP α plasmids and an empty vector were transfected in cultured cells. Both empty vector and pAdTrack-C/EBP α plasmids express GFP protein; and the transfected cells are visualized by green fluorescence. We have also examined expression of GFP protein from the control vector and C/EBP α plasmid by Western blotting (Fig 1C). At day one, the majority of cells in all transfections were single. At later time points, proliferating cells formed colonies containing 2, 3 and more cells, while cells inhibited by C/EBP α remain as single cell colony. Therefore, we have calculated number of green colonies (transfected cells) at days 1, 2, 3, 4 and 5 after transfections independently on number of cells in the colonies. The percent of survived colonies was calculated as ratio to a number of colonies observed at day one after transfections. **Apoptosis** was examined by measuring DNA laddering.

Inhibition of ARL6IP5 and C/EBP α by siRNAs

To inhibit expression of ARL6IP5 or C/EBP α , siRNAs to mRNAs of these proteins were transfected into cells and mRNA and protein levels of ARL6IP5 and C/EBP α were examined by RT-PCR and by Western blotting. Nucleotide sequence of siRNA to ARL6IP5 is as follows. 5'-GGGACATTTCCAAATGGAACAACCG-3'. The sequence of siRNA to C/EBP α and conditions for the inhibition are described in our previous papers [1, 20].

C/EBP α constructs and antibodies

Generation of C/EBP α mutant constructs was described in our previous papers [1, 20]. Antibodies to cyclin D3, C/EBP α (14AA and N19), cdk4 (C-22), cdk2 (M2), Brm and Rb (C-15), p21 (H164), p16 and HDAC1 were purchased from Santa Cruz Biotechnology. Antibodies to ARL6IP5 (JWA) and to β -actin are from Abcam and Sigma. The generation and characterization of S193-ph-C/EBP α antibodies was described in our previous paper [12].

Examination of C/EBP α -mediated regulation of ARL6IP5 promoter, mRNA and protein

Gelshift assay was performed with DNA probes which cover C/EBP α consensus within the ARL6IP5 promoter (see Fig 4A). The conditions for EMSA were described in our previous papers [1, 11]. To examine activation of the ARL6IP5 promoter by C/EBP α , DNA fragment containing 216 nucleotide of the promoter was linked to luciferase. The ARL6IP5-luc construct was co-transfected with WT C/EBP α or with C/EBP α -R290A mutant into cells. The luciferase activity was determined in 16 hours after transfections. To examine effects of C/EBP α on the expression of ARL6IP5 in NIH3T3 cells, C/EBP α was delivered into cells by adenovirus based vectors, mRNA and proteins were isolated and examined by semi quantitative RT-PCR and by Western blotting correspondingly. Note that examination of the ARL6IP5 promoter, levels of mRNA and protein in NIH3T3 cells were performed within 16 hours after transfections since cells die in two-three days after transfections.

Protein isolation, Western blotting and Co-Immunoprecipitation

Nuclear extracts were isolated as described in previous papers [1, 7]. Proteins (50–100ug) were loaded on gradient (4–20% or 8–16%) poly acrylamide gel, transferred on the membrane and probed with antibodies to C/EBP α , cdk2, cyclin D3, E2F4 or Brm. To verify protein loading, each filter was re-probed with β -actin and then stained with coomassie. C/EBP α was immunoprecipitated from nuclear extracts with polyclonal antibodies (14AA,

Santa Cruz), and the presence of Brm, E2F4, cdk2 in C/EBP α IPs was examined by Western blotting with monoclonal antibodies to mentioned proteins. Different conditions were used for detection of endogenous C/EBP α in Hep3B2 cells and in cells transfected with C/EBP α . For detection of endogenous C/EBP α , the filter was incubated with antibodies for C/EBP α for overnight and then for 2 hours with secondary antibodies. For examination of C/EBP α after transfection in cultured cells, the membranes were incubated for 30 min with primary antibodies and then for 1 hour with secondary antibodies. Under these conditions, the signals of endogenous C/EBP α in Hep3B2 cells are weak or not detectable (see Fig 1C).

Kinase assay

To determine activities of kinases associated with cyclin D3, we have precipitated cyclin D3 from nuclear extracts of 3T3-L1 and NIH3T3 cells and added to a kinase reaction with GST-Rb substrate. Mock agarose was used as a negative control. Baculovirus expressed purified cyclin D1-cdk4 complex was used as a positive control. The mixtures were separated by gradient PAGE and transferred on the membrane. The membrane was stained with coomassie blue to verify loading of GST-Rb and exposed with the X ray film.

Examination of gene expression by micro array

NIH 3T3 and 3T3-L1 cells were transfected with WT C/EBP α or with C/EBP α -R290A mutant by adenovirus delivery. The efficiency of transfections was 85–90%. Total RNA was isolated and used for micro array assay. Two hundred nanograms of total RNA were amplified and purified using Illumina TotalPrep RNA Amplification Kit (Ambion, Cat# IL1791) following kit instructions. Briefly, first strand cDNA was synthesized by incubating RNA with T7 oligo(dT) primer and reverse transcriptase mix at 42 °C for 2 hours. RNase H and DNA polymerase master mix were immediately added into the reaction mix following reverse transcription and were incubated for 2 hours at 16 °C to synthesize second strand cDNA. RNA, primers, enzymes and salts that would inhibit in vitro transcription were removed through cDNA filter cartridges (part of the amplification kit). In vitro transcription was performed and biotinylated cRNA was synthesized by 14-hour amplification with dNTP mix containing biotin-dUTP and T7 RNA polymerase. Amplified cRNA was subsequently purified and concentration was measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, DE). An aliquot of 1.5 micrograms of amplified products were loaded onto Illumina Sentrix Beadchip Array Mouse-6 arrays, hybridized at 55°C in an Illumina Hybridization Oven (Illumina, Cat# 198361) for 17 hours, washed and incubated with streptavidin-Cy3 to detect biotin-labeled cRNA on the arrays. Arrays were dried and scanned with BeadArray Reader (Illumina, CA). Data were analyzed using BeadStudio software (Illumina, CA). Clustering and pathway analysis were performed with BeadStudio and Ingenuity Pathway Analysis (Ingenuity Systems, Inc.) softwares respectively.

Chromatin-immunoprecipitation (chip) assay

The chromatin immunoprecipitation assay was performed using the ChIP-IT kit (Active Motif). The chromatin solutions were isolated from 3T3-L1 and NIH3T3 cells, and DNA was sheared by enzymatic digestion according to the instruction manual. The size of DNA fragments produced averaged between 500–1000 bp in length. Antibodies against C/EBP α were added to each aliquot of pre-cleared chromatin and incubated for overnight. Protein G beads were added and incubated for 1.5h at 4 °C. After reversing the crosslinking, DNA was isolated and used for PCR reactions with primers to the ARL6IP5 promoter. The sequences of the primers are as follows. F: 5'-GCACGGGTCGCTAGTTGCTTTCTGTCATTTC-3'; R: 5'-GAAGATCTTCTCAGCTTTGCGTTCTTCG-3'. PCR mixtures were amplified for 1 cycle of 95°C for 5 min, annealing temperature for primers (55°C) for 5 min, and 72°C for 2 min. Then PCR mixtures were amplified for 34 cycles of 95°C for 1 min, annealing temperature for 2 min, and 72°C for 1.5 min. PCR products were separated by 4% PAGE.

RESULTS

Ectopic expression of C/EBP α in NIH3T3 cells causes a massive death of the cells

Since biological activities of C/EBP α are regulated by cell-type specific mechanisms, we have performed studies of C/EBP α growth arrest in four cell lines, Hep3B2, HEK293, 3T3-L1 and NIH3T3. In the course of these studies, we have found that the response of NIH3T3 cells to C/EBP α expression differs dramatically from that observed in 3T3-L1, HEK293 and Hep3B2 cells. We have also realized that the investigation of NIH3T3 cells requires a number of C/EBP α mutants which are shown in Fig 1A. We have surprisingly observed that NIH3T3 cells die shortly after transfections with wild type C/EBP α , while C/EBP α does not cause death in Hep3B2, HEK293 and 3T3-L1 cells. To compare survived cells in these tissue culture cell lines, we have counted number of colonies which survived after several days after transfections. Figure 1B shows a typical result of these studies. As one can see, 50% of transfected NIH3T3 cells died at day 2 and less than 10% cells survived at day 3. Note that even at day 1 the number of green transfected with C/EBP α cells was 2–3-fold lower than that in cells transfected with an empty vector. WT and C/EBP α -S193A mutant possess identical capabilities to cause cell death in NIH3T3 cells. Under the same conditions, transfections of 3T3-L1, HEK293 and Hep3B2 cells with WT C/EBP α do not show reduction in the number of colonies within 3 days; a small reduction is observed at days 4–5. Examination of C/EBP α in protein extracts isolated from the experimental plates showed the lack of NIH3T3 cells expressing WT C/EBP α (Fig. 1C) confirming that C/EBP α causes cell death in NIH3T3 cells. Since NIH3T3 cells die shortly after transfections with WT C/EBP α , we have performed studies to determine regions of C/EBP α responsible for the death of NIH3T3 cells and to find conditions under which mechanisms of C/EBP α -mediated inhibition of NIH3T3 cells are possible to examine.

DNA binding activity of C/EBP α is required for cell death in NIH3T3 cells

We have first examined if C/EBP α initiates cell death in NIH3T3 cells using DNA laddering assay. Figure 2A shows that the apoptotic laddering is detectable in NIH3T3 cells at 8 hours after transfections with WT C/EBP α . Previous studies mapped several single amino acids of C/EBP α which are involved in the regulation of biological activities of C/EBP α . Mutation of one of these residues, S193 to Ala (which blocks growth inhibitory activity of C/EBP α in the liver and 3T3-L1 cells), does not block C/EBP α -mediated apoptosis (Fig 1). Therefore, we have tested if DNA binding activity of C/EBP α is required for the apoptosis. Since the mutation of R290 to Ala abolishes DNA binding activity of C/EBP α [1], we have transfected NIH3T3 cells with WT C/EBP α and with R290A mutant and counted survived cells. Parallel examinations of C/EBP α binding activity and protein levels by EMSA and Western blotting using nuclear extracts isolated at day 1 after transfections showed that the R290A mutant does not bind to DNA despite high levels of expression, while WT C/EBP α interacts with DNA (Fig 2B and C). We have found that C/EBP α -R290A mutant does not cause death in NIH3T3 cells. The survival curve for NIH3T3 cells transfected with the C/EBP α -R290A mutant is identical to that of cells transfected with an empty vector (Fig. 2D). Thus, these studies revealed that DNA binding activity of C/EBP α is required for the initiation of apoptosis in NIH3T3 cells.

C/EBP α activates apoptotic pathways and diminishes expression of p16 family in NIH3T3 cells

Given quite different effects of C/EBP α on cell fate in NIH3T3 cells, we have next examined pathways by which C/EBP α causes death of NIH3T3 cells. Since DNA binding activity of C/EBP α is required for the initiation of apoptosis, we have focused our search on the transcriptional targets of C/EBP α which might be involved in the initiation of apoptosis. For this goal, we have compared C/EBP α -mediated activation of genes in NIH3T3 cells and

in 3T3-L1 cells since both these cell lines are derived from disaggregated day 17 Swiss NIH mouse embryos. WT C/EBP α was delivered into these cells using adenovirus vectors; RNA was isolated and used for the global micro array analysis. Comparison of the up-regulated genes showed that C/EBP α increases mRNA levels of 71 and 122 genes in NIH3T3 and 3T3-L1 cells correspondingly. Only 14 identical genes (12–28%) are up-regulated by C/EBP α in these cells (Fig. 3A). Similar examination of the down-regulated genes also showed that only small portion (5–7%) of identical genes is down-regulated in the NIH3T3 and 3T3-L1 cells (Fig 4B). We have further compared differentially up and down-regulated genes in NIH3T3 and 3T3-L1 cells. Among several changes in alterations of signal transduction pathways, most significant differences were found for apoptotic signaling and for expression of cell cycle genes. The list of selected genes with highest differences is shown in the table (Fig. 3C). Four pro-apoptotic genes, ARL6IP, calpain 5, Ras and Fas, were increased in NIH-3T3 cells up to 10–26 fold compared to the levels of these proteins in 3T3-L1 cells (Fig 3C). We have also observed a down-regulation of certain apoptotic proteins; however, this down-regulation is within 1.7–2.3 folds. Analysis of the differences in the expression of cell cycle genes revealed dramatic differences in the expression of inhibitors of cyclin dependent kinases, p14INK4, p15INK4, p16INK4 and p19ARF. These inhibitors are required for cell cycle arrest. Despite C/EBP α is a strong inhibitor of cell proliferation, surprisingly micro array studies showed that C/EBP α inhibits expression of this family of proteins (Fig 3C). Interestingly, C/EBP α also up-regulates three members of HDAC family HDAC1, HDAC4 and HDAC5 in NIH3T3 cells, but not in 3T3-L1 cells. These findings suggested that WT C/EBP α might not inhibit proliferation of NIH3T3 cells due to these alterations in cell cycle proteins (see below). Taken together, global micro array search showed quite different patterns of the C/EBP α -mediated regulation of genes in NIH3T3 and 3T3-L1 cells and has identified a dramatic activation of pro-apoptotic proteins in NIH3T3 cells.

C/EBP α increases expression of a pro-apoptotic protein ARL6IP5 via direct activation of the ARL6IP5 promoter

Among several pro-apoptotic proteins activated in NIH3T3 cells, the high level of activation by C/EBP α was observed for ARL6IP5 protein which is also known as DER11, GTRP3-18, HSPC127, JWA and hp22. In addition to our analyses, a paper by Mao et al has shown that all-trans retinoic acid inhibits proliferation and causes apoptosis through the activation of ARL6IP5 and that this activation is mediated by C/EBP α through direct binding to the promoter of ARL6IP5 [21]. Therefore, we suggested that the ARL6IP5 gene might be a key protein which mediates pro-apoptotic activity of C/EBP α in NIH3T3 cells and have investigated the role of ARL6IP5 in pro-apoptotic activity of C/EBP α . We have cloned a short region (214 bp) of the mouse ARL6IP5 promoter and examined the interactions of C/EBP α with the promoter. The nucleotide sequence of the ARL6IP5 promoter is shown in Fig. 4A. In addition to previously described two C/EBP binding sites (sites 1 and 2, ref #22) we have found a new C/EBP α consensus (site 3). Therefore, we have examined interactions of C/EBP α with these three sites. Gelshift assay with corresponding oligomers revealed that C/EBP α binds to all three sites (Fig 4B). The strongest interaction is observed for site 3, while the interaction with sites 1 and 2 is weaker but well detectable. Thus these studies showed that C/EBP α interacts with the ARL6IP5 promoter and that this interaction involves at least three regions of the promoter.

We have next examined the effects of C/EBP α on the ARL6IP5 promoter. The ARL6IP5 promoter was linked to the luciferase reporter construct and co-transfected with WT C/EBP α and C/EBP α -R290A into different cultured cells. Figure 4C shows a typical result of these experiments. In 3T3-L1 cells, the ARL6IP5 promoter has a weak activity and C/EBP α does not activate this promoter in these cells. Although the ARL6IP5 promoter is active in both Hep3B2 and NIH3T3 cells, the effects of C/EBP α on the ARL6IP5 promoter are quite

different. In Hep3B2 cells, C/EBP α inhibits the activity of the ARL6IP5 promoter; while in NIH3T3 cells, the expression of C/EBP α leads to a 7–10 fold activation of the promoter. These C/EBP α effects are mediated through a direct binding of C/EBP α to the promoter since the R290A mutant does not affect activity of the ARL6IP5 promoter in the transfected cells. The activation of the ARL6IP5 promoter by WT C/EBP α , but not by R290A mutant, correlates with the ability of WT C/EBP α to cause apoptosis and with a lack of the apoptosis in cells transfected with the R290A mutant. We next examined if the transfections of C/EBP α into NIH3T3 cells activate expression of endogenous ARL6IP5 gene. WT and R290A mutant C/EBP α proteins were transfected into the cells using adenovirus-based delivery, mRNAs and proteins were isolated in 8 hours after transfections and ARL6IP5 expression was determined by RT-PCR and Western blotting. Figure 4D shows that WT C/EBP α increases ARL6IP5 mRNA up to 15–20 folds; while R290A mutant has a minor (1.5 fold) induction of ARL6IP5 mRNA. Consistent with these data, examination of protein levels of ARL6IP5 showed a dramatic elevation of ARL6IP5 in NIH3T3 cells by WT C/EBP α and a weak induction by the R290A mutant (Fig 4E). A re-probe of the membrane with antibodies to C/EBP α and to β -actin showed that both WT and R290A mutant are expressed in the cells. To further determine if C/EBP α increases expression of ARL6IP5 in NIH3T3 cells via activation of the promoter, we have performed chromatin immunoprecipitation assay (chip) with NIH3T3 and 3T3-L1 cells. Chromatin solutions were prepared from cells transfected with empty vector, with WT C/EBP α and with C/EBP α -R290A mutant. C/EBP α was immunoprecipitated from these solutions and PCR was performed with primers which cover three C/EBP α sites. The results of these studies are shown in Fig 4F. As one can see, C/EBP α is not bound to the ARL6IP5 promoter in 3T3-L1 cells, but it is associated with the promoter in NIH3T3 cells. Consistent with failure of C/EBP α -R290A mutant to increase levels of ARL6IP5 mRNA, association of this mutant with the promoter is not detectable. Taken together, these studies demonstrated that C/EBP α increases expression of pro-apoptotic protein ARL6IP5 via interaction with the promoter of ARL6IP5 gene and that this activation is specific and takes place only in NIH3T3 cells, but it is not detectable in 3T3-L1 cells.

ARL6IP5 is required for the C/EBP α -mediated apoptosis in NIH3T3 cells

We next asked if the C/EBP α -mediated activation of ARL6IP5 is involved in the apoptosis. To address this question, we have inhibited expression of ARL6IP5 by siRNA (25 nucleotides RNA oligomer, position +80 – +104) in cells transfected with WT C/EBP α and examined number of survival cells. Figure 5A shows that siRNA to ARL6IP5 reduces mRNA levels in un-transfected cells as well as in cells transfected with C/EBP α to the 15–20% of the original levels. Most important, the siRNA completely blocked the C/EBP α -mediated induction of ARL6IP5 mRNA. Consistent with the inhibition of ARL6IP5 mRNA, Western blotting showed that siRNA also inhibits expression of the protein (Fig 5B). This inhibition is specific and does not involve neutralization of C/EBP α activities since EMSA assay with the site 3 of the ARL6IP5 promoter showed a strong DNA binding activity of C/EBP α in cells transfected with siRNA to ARL6IP5 (Fig 5C). We next co-transfected siRNA to ARL6IP5 with C/EBP α and examined survived cells within 5 days after transfection. We have observed that siRNA-mediated inhibition of ARL6IP5 in NIH3T3 cells almost completely blocked pro-apoptotic activities of C/EBP α ; while more than 90% cells transfected with WT C/EBP α alone died at day three (Fig 5D). These results showed that ARL6IP5 is a key mediator of C/EBP α apoptotic activities in NIH3T3 cells. Taken together, investigations of the mechanisms of C/EBP α -mediated apoptosis showed that, in NIH3T3 cells, C/EBP α activates the promoter of ARL6IP5, increases mRNA and protein levels of ARL6IP5 and that this activation causes the apoptosis.

C/EBP α -R290A mutant fails to inhibit proliferation of NIH3T3 cells

Given established conditions for the surviving NIH3T3 cells after transfections with C/EBP α , we have next examined the growth inhibitory activities of the C/EBP α -R290A mutant in NIH3T3 cells. Parallel experiments were performed with 3T3-L1 and HEK293 cells. Figure 6A–B show results of these studies. Consistent with previous finding that DNA binding activity of C/EBP α is not required for growth arrest, C/EBP α -R290A mutant inhibits proliferation of HEK293 and 3T3-L1 cells. On the contrary, no inhibition of NIH3T3 cells was observed with this C/EBP α mutant. Although these initial studies suggested that DNA binding activity of C/EBP α (which is blocked by R290A mutation) might be required for growth arrest in NIH3T3 cells, further experiments revealed that transcriptional activity of C/EBP α is also not required to inhibit NIH3T3 cells since the double R290A-S193D mutant inhibits proliferation of these cells (see below). Thus, these studies suggested that NIH3T3 cells have developed a mechanism which blocks growth inhibitory activity of C/EBP α . Since micro-array studies showed a significant increase in the expression of HDAC family and a down-regulation of the p16INK4 inhibitors by WT C/EBP α , we have examined if the R290A mutant is able to produce these alterations. Micro-array studies were performed with 3T3-L1 and NIH3T3 cells transfected with the R290A mutant. We have found that, although the R290A C/EBP α mutant does not activate HDAC4 and does not inhibit p14, it still up-regulates HDAC1 and HDAC5 and reduces expression of p15, p16 and p19 to the levels observed in cells transfected with WT C/EBP α (see Fig 3). To verify data of micro array analysis, we have determined protein levels of HDAC1, p16 and p21 in NIH3T3 cells transfected with C/EBP α . These studies revealed that HDAC1 and p21 are up-regulated; while p16 is inhibited by both WT C/EBP α and C/EBP α -R290A mutant (Fig 6D). Since the C/EBP α -R290 mutant does not bind to DNA, these data suggest that the differences in the C/EBP α -dependent expression of HDAC4 and p14 are mediated by transcriptional activity of C/EBP α , while alterations of other cell cycle proteins are mediated by protein-protein interactions of C/EBP α .

Low levels of cyclin D3 in NIH3T3 cells are not sufficient to phosphorylate C/EBP α and to support cdk2 and Brm dependent pathways of growth arrest

We have previously shown that cyclin D3 supports growth inhibitory activity of C/EBP α in the liver [12]. Although down-regulation of p16 family proteins in NIH3T3 cells might partially explain the lack of growth arrest, we have examined if cyclin D3 pathways might be also changed in NIH3T3 compared with HEK293, Hep3B2 and 3T3-L1 cells. Figure 7A shows results of these studies. In 3T3-L1 and HEK293 cells, expression of cyclin D3 is relatively high; while levels of cyclin D3 are very low in Hep3B2 and in NIH3T3 cells. This observation suggests that the levels of cyclin D3 in NIH3T3 cells might be not sufficient to support the growth inhibitory activity of C/EBP α . To examine this hypothesis, we have compared the interaction of cyclin D3 with C/EBP α and the formation of growth inhibitory C/EBP α -cdk2 and C/EBP α -Brm complexes in 3T3-L1 and NIH3T3 cells. We have first examined kinase activity of cyclin D3-cdk4 complexes in these cells. Cyclin D3 and cdk4 were immunoprecipitated from nuclear extracts of 3T3-L1 and NIH3T3 cells and examined in an *in vitro* kinase assay with GST-Rb substrate. As can be seen in Figure 7B, both cyclin D3 and cdk4 IPs from 3T3-L1 cells display a significant kinase activity; while cyclin D3 and cdk4 IPs from NIH3T3 cells have much lower activities against Rb. Fig 7B shows results with 2 hours exposure of the film, under which the kinase activity of cdk4 and cyclin D3 IPs from NIH3T3 cells is not detectable. After overnight exposure; however, the activity of these enzymes in NIH 3T3 cells could be detected (data not shown). We next examined the interactions of C/EBP α with cdk2 and Brm in NIH3T3 and in 3T3-L1 cells. WT and mutant R290A C/EBP α proteins were transfected into these cells, nuclear extracts were isolated 16 hours after transfections and C/EBP α was immunoprecipitated. C/EBP α IPs were then examined by Western blotting with antibodies to which specifically recognize Ser193-

phosphorylated isoform of C/EBP α , cyclin D3, cdk2 and Brm. Figure 7C shows that C/EBP α is associated with and phosphorylated by cyclin D3 in 3T3-L1 cells, while no interaction of C/EBP α with cyclin D3 and no phosphorylation of C/EBP α are detected in NIH3T3 cells. Consistent with the role of cyclin D3-mediated phosphorylation of C/EBP α in the formation of growth inhibitory complexes, cdk2 and Brm are observed in complexes with C/EBP α in 3T3-L1 cells, but these proteins are not detectable in C/EBP α IPs from NIH3T3 cells. Thus, these studies showed that the failure of C/EBP α to inhibit NIH3T3 cells correlates with the lack of phosphorylation at Ser193 and with the lack of formation of the C/EBP α -Brm and C/EBP α cdk2 complexes.

Ectopic expression of cyclin D3 and the mutation of Ser193 to aspartate restore growth inhibitory activity of C/EBP α in NIH3T3 cells

To examine if the phosphorylation of C/EBP α in NIH3T3 cells by cyclin D3 might restore inhibitory activity of C/EBP α , cyclin D3 was co-transfected with C/EBP α -R290A into NIH3T3 cells. Examination of green colonies at day 3 after transfections showed that cyclin D3 partially restores growth inhibitory activity of C/EBP α . However, a portion of NIH3T3 cells still proliferate (Fig 7D). Western blotting with protein extracts isolated from experimental plates showed that levels of cyclin D3 are only slightly increased in NIH3T3 cells transfected with pAdTrack cyclin D3 plasmid (see Fig 7D). Although the restoration of growth inhibitory activity of C/EBP α was not complete, these studies clearly demonstrated that cooperation of cyclin D3 and C/EBP α is required for the inhibition of proliferation of NIH3T3 cells. To confirm this conclusion, we have generated a double mutant of C/EBP α (R290A + S193D) which contains negatively charged residue in position of 193 and mimics cyclin D3/cdk4-mediated phosphorylation of C/EBP α . Examination of growth inhibitory activity of this mutant in NIH3T3 cells revealed quite different results which are shown in Fig 7E. Around 80% of NIH3T3 cells are arrested by the double C/EBP α -R290A-S193D mutant; while C/EBP α -R290A mutant does not inhibit proliferation of NIH3T3 cells. Thus, these studies demonstrated that NIH3T3 cells are resistant to C/EBP α growth arrest perhaps due to a low level of cyclin D3 in these cells.

Cyclin D3 cooperates with C/EBP α in the inhibition of HEK293 cells

Given a critical role of cyclin D3 in the growth inhibitory activity of C/EBP α in NIH3T3 cells, we have examined if cyclin D3 is required for growth inhibitory activity of C/EBP α in HEK293 cells. Since under certain settings cyclin D3 promotes proliferation, we have initially examined the effects of overexpression of cyclin D3 on the proliferation of HEK293 cells. For this goal, we have transfected HEK293 cells with cyclin D3-pAdTrack plasmid and examined the formation of single and multi-cells colonies. Western blotting with antibodies to cyclin D3 showed a 5–7 fold elevation of cyclin D3 in cells transfected with cyclin D3 relatively to cells transfected with empty vector (Fig 8A). Colony growth assay showed that cyclin D3 does not inhibit proliferation of C/EBP α -negative HEK293 cells (Fig 8A bottom). Moreover, under our conditions, overexpression of cyclin D3 in C/EBP α -negative HEK293 cells leads to the formation of a larger number (up to 55–60%) of multi-cell colonies, while the amounts of multi-cell colonies in cells transfected with empty vector are less than 8% (Fig. 8A). These observations suggest that cyclin D3 promotes proliferation of C/EBP α -negative HEK293 cells and are consistent with previous observations showing that ectopic expression of cyclin D3 accelerates progression of rat fibroblasts through G1 phase [22].

We have next examined if cyclin D3 is required for C/EBP α -mediated inhibition of HEK293 cells. For this goal, we have inhibited endogenous cyclin D3 in HEK293 cells by siRNA and examined C/EBP α growth arrest in cells with low levels of cyclin D3. C/EBP α (pAdTrack-C/EBP α plasmid) was co-transfected with siRNA to cyclin D3 into HEK293 cells and

proliferation of these cells was examined by colony formation assay. A parallel examination of cyclin D3 levels after transfection showed that siRNA significantly reduces levels of cyclin D3, which represents 10–15% of levels in control cells (Fig 8B, Western). This inhibition of cyclin D3 leads to a significant reduction of C/EBP α growth arrest. Only 35–40% of HEK293 cells transfected with siRNA to cyclin D3 are arrested by C/EBP α . We suggest that the remaining activity of C/EBP α might be because of a non-complete inhibition of cyclin D3 or due to a partial redundancy of D-type cyclins. Taken together, these studies revealed that cyclin D3-C/EBP α pathway is required for the inhibition of cell proliferation.

Cyclin D3 inhibits proliferation of hepatoma cells by activating endogenous C/EBP α

The low levels of cyclin D3 in Hep3B2 cells suggested that this reduction might be important event in the loss of a negative control of proliferation. To examine if cyclin D3 might inhibit proliferation of Hep3B2 cells, pAdTrack-cyclin D3 was transfected into Hep3B2 cells and proliferation of these cells was investigated. Western blotting with antibodies to cyclin D3 revealed a 4.2–4.5 fold inductions of cyclin D3 levels relative to levels of endogenous cyclin D3 (Fig. 8C). Since Hep3B2 cells express endogenous C/EBP α , we have next established conditions for the inhibition of C/EBP α in these cells by siRNA. Western blotting (Fig. 8D) showed that, under our conditions, protein levels of C/EBP α are not detectable in cells transfected with siRNA. We next examined the effects of cyclin D3 on proliferation of Hep3B2 cells which express endogenous C/EBP α and cells in which C/EBP α is inhibited by siRNA as described in our previous papers [1, 11]. Single cells (growth arrested) and colonies of two-three cluster cells were counted at day 3 after transfections with pAdTrack-cyclin D3 plasmid. As one can see in Fig 8E, around 80% of Hep3B2 cells are arrested by cyclin D3. These studies demonstrated that cyclin D3 inhibits proliferation of Hep3B2 cells. Results of the colony growth arrest assay with Hep3B2 cells treated with siRNA to C/EBP α demonstrated that cyclin D3 is not able to arrest proliferation of Hep3B2 cells in which C/EBP α is inhibited by siRNA (Fig 8E). Interestingly, the inhibition of C/EBP α in cells transfected with cyclin D3 resulted in acceleration of cell proliferation. This result confirmed data obtained in C/EBP α -negative HEK293 cells and emphasized that biological activities of cyclin D3 are dependent on C/EBP α . Taken together, these studies show that the elevation of cyclin D3 levels in hepatoma 3B2 cells inhibits proliferation and that this inhibition requires endogenous C/EBP α . Previous studies revealed that cyclin D3-cdk4-mediated phosphorylation of C/EBP α at Ser193 is critical for C/EBP α inhibitory activity and that this phosphorylation activates two pathways of C/EBP α : the inhibition of cdk2 and the inhibition of E2F targets in the complexes with Brm [1, 11]. Therefore, we determined if the ectopic expression of cyclin D3 is sufficient to phosphorylate C/EBP α and to initiate formation of growth inhibitory C/EBP α -cdk2 and C/EBP α -Brm complexes. C/EBP α was immunoprecipitated from cells transfected with empty vector and with cyclin D3 and these IPs were analyzed by Western blotting with antibodies to phosphorylated isoform of C/EBP α (ph-Ser193), cdk2, and Brm. As one can see in Fig. 8F, elevation of cyclin D3 leads to the phosphorylation of C/EBP α at Ser193 and to formation of complexes with cdk2 and Brm. Taken together, these studies revealed that cyclin D3 inhibits proliferation of hepatoma cells via activating C/EBP α -cdk2 and C/EBP α -Brm pathways.

DISCUSSION

Role of cyclin D3-C/EBP α pathway in inhibition of cell proliferation

The major function of D-type cyclins is the activation of cdk4/cdk6 and promotion of cell proliferation. On the contrary to cyclins D1 and D2 which strongly promote cell growth, cyclin D3 is expressed in certain growth arrested cells and is involved in growth arrest and differentiation [12–17, 19]. Data in this paper show that the biological functions of cyclin

D3 depend on the cellular environment. Experiments with C/EBP α -positive Hep3B2 and C/EBP α -negative HEK293 cells revealed that cyclin D3 inhibits proliferation of cells which express C/EBP α and promotes proliferation of cells that lack C/EBP α . The cyclin D3 mediated inhibition of Hep3B2 cells involves activation of C/EBP α and formation of growth inhibitory complexes of C/EBP α with cdk2 and Brm. Examination of NIH3T3 cells showed that, similar to Hep3B2 cells, NIH3T3 cells express very low levels of cyclin D3. As the result, C/EBP α is not able to form growth inhibitory complexes with cdk2 and Brm in NIH3T3 cells. These data are consistent with recently published observations showing that, in NIH3T3 cells, C/EBP α does not interact with cdk2 [23]. The low levels of cyclin D3 in NIH3T3 cells perhaps are the result of a rapid degradation or inhibition of translation of cyclin D3 since the expression of cyclin D3 from a transfected plasmid does not produce protein above the levels of endogenous cyclin D3.

Apoptotic activities of C/EBP α

Among four cell types examined in our experiments, NIH3T3 cells showed quite different responses to the expression of C/EBP α . In these cells, we have observed a strong apoptotic activity of C/EBP α . Several previous studies described C/EBP α -mediated apoptosis in myeloid lineages at days 3–4 after transfections with C/EBP α [24–26]. We have surprisingly found that the C/EBP α initiates apoptosis in NIH3T3 cells at 4–8 hours after transfections and that less than 5% of NIH3T3 cells survived at days 4–5 after transfections. This apoptotic activity was observed in experiments with low density cells; while NIH3T3 cells at high density were less sensitive to the apoptotic activity of C/EBP α . Since growth arrest studies require delivery of the C/EBP α into low density proliferating cells, this situation prevents investigations of growth inhibitory activity of WT C/EBP α in these cells. Therefore, we have investigated in details how C/EBP α causes apoptosis and generated mutants, in which apoptotic and growth inhibitory activities of C/EBP α were separated. Interestingly, several previous publications have examined C/EBP α inhibitory activities in NIH3T3 cells using approaches with a selection of C/EBP α expressing cells for resistance to antibiotics during 2–3 weeks [23, 27, 28]. Because C/EBP α causes a massive cell death in NIH3T3 cells, it is possible that these investigations have examined the survival cells which have developed a resistance to the apoptotic activities of C/EBP α . It is also possible that some of the C/EBP α mutants, previously determined in NIH3T3 cells as defective in growth arrest, might lack apoptotic activity, but possessed growth inhibitory activity which has not been displayed in these cells due to low levels of cyclin D3. In summary, given the C/EBP α apoptotic activity (data in this paper and in other publications [24–26]), we think that the growth inhibitory activities of C/EBP α in cultured cells should be examined within first 2–3 days after transfections and/or should be carefully separated from apoptotic activity of C/EBP α if a longer time is required.

The pro-apoptotic activity of C/EBP α in hematopoietic cells has been initially shown by a work from Tenen's lab [25]; however, pathways by which C/EBP α causes apoptosis have not been elucidated. Searching for the mechanisms of the apoptotic activities of C/EBP α , we have performed careful analyses of gene expression in NIH 3T3 cells and in 3T3-L1 cells after transfections with WT C/EBP α . We have found quite different alterations in the patterns of gene expression after transfections with C/EBP α . The most dramatic differences in the C/EBP α -mediated alterations of gene expression were observed for the apoptotic signaling and for the cell cycle proteins. We have also identified a direct target of C/EBP α which is critical for C/EBP α -mediated apoptosis in NIH3T3 cells. This target is the ARL6IP5 protein which has been previously characterized as an inducer of apoptosis in cultured cells. The promoter region of the ARL6IP5 gene contains two binding sites for C/EBP proteins and potentially might be regulated by C/EBP proteins [21]. Given the ubiquitous expression of another member of C/EBP family, C/EBP α , one would assume that

the ARL6IP5 promoter might be active in many cell types. However, the basal activity of the ARL6IP5 promoter is cell type-dependent with maximum activity in NIH3T3 cells and with a very low activity in 3T3-L1 cells. The C/EBP α -mediated up-regulation of the ARL6IP5 promoter is also cell type specific and takes place in NIH3T3 cells, but not in Hep3B2 cells.

The major result of this paper is that the cellular environment significantly changes biological activities of C/EBP α and that C/EBP α has different targets in NIH3T3 and in 3T3-L1 cells. In addition to known targets of C/EBP α within cell cycle, we have identified genes which are specifically affected only in NIH 3T3 cells. In these cells, C/EBP α increases mRNAs of several members of HDAC family and down-regulates mRNAs of p16INK4 and p19ARF. Interestingly, these new targets of C/EBP α are likely to be controlled through protein-protein interactions since the mutant C/EBP α -R290A (which does not bind to DNA, Fig 2) changes expression of majority of these proteins to the levels observed in transfections with WT C/EBP α . We think that the cell type specific down-regulation of p16INK4 and p19ARF proteins by C/EBP α and low levels of cyclin D3 contribute to the inability of C/EBP α to arrest proliferation of NIH 3T3 cells. The experiments with overexpression of cyclin D3 and with double mutant C/EBP α -S193D-R290A; however, suggest that the reduction of cyclin D3 is the major reason for the lack of growth inhibitory activity of C/EBP α in NIH 3T3 cells. Taken together, data in this paper show that biological functions of C/EBP α are controlled by a complex network of pathways which operate up-stream and downstream of C/EBP α and that these pathways differ in different cell types.

Acknowledgments

This work was supported by National Institutes of Health Grants CA10070, GM55188 and AG025477 (NAT).

References

1. Wang GL, Iakova P, Wilde M, Awad SS, Timchenko NA. Liver tumors escape negative control of proliferation via PI3K/Akt-mediated block of C/EBP α growth inhibitory activity. *Gen & Dev*. 2004; 18:912–925.
2. Tan EH, Hooi SC, Laban M, Wong E, Ponniah AW, Wang N. CCAAT/Enhancer Binding Protein α Knock-in Mice Exhibit Early Liver Glycogene Storage and Reduced Susceptibility to Hepatocellular Carcinoma. *Cancer Res*. 2005; 65:10330–10337. [PubMed: 16288022]
3. Johnson PE. Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors. *J Cell Science*. 2005; 118:2545–2455. [PubMed: 15944395]
4. Birkenmeier EB, Gwynn B, Howard S, Jerry J, Gordon JI, Landschulz WH, McKnight SL. Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes & Dev*. 1989; 3:1146–1156. [PubMed: 2792758]
5. Timchenko NA, Wilde M, Darlington GJ. C/EBP α regulates formation of S-phase specific E2F/p107 complexes in livers of newborn mice. *Mol Cell Biol*. 1999; 19:2936–2945. [PubMed: 10082561]
6. Sato Y, Miyake K, Kaneoka H, Iijima S. Sumoylation of CCAAT/Enhancer Binding Protein α and its functional Role in Hepatocyte Differentiation. *J Biol Chem*. 2006; 281:21629–21639. [PubMed: 16735515]
7. Timchenko NA, Harris TE, Wilde M, Bilyeu TA, Burgess-Beusse BL, Finegold MJ, Darlington GJ. CCAAT/enhancer binding protein α regulates p21 protein and hepatocyte proliferation in newborn mice. *Mol Cell Biol*. 1997; 17:7353–7361. [PubMed: 9372966]
8. Flodby P, Barlow C, Kylefjord H, Ahrlund-Richter L, Xanthopoulos KG. Increased Hepatic Cell Proliferation and Lung Abnormalities in Mice Deficient in CCAAT/Enhancer Binding Protein α . *J Biol Chem*. 1996; 271:42753–42760.

9. Yamasaki H, Sada A, Iwata T, Niwa T, Tomizawa M, Xanthopoulos KG, Koike T, Shiojiri N. Suppression of C/EBP α expression in periportal hepatoblasts may stimulate biliary cell differentiation through increased Hnf6 and Hnf1b expression. *Development*. 2006; 133:4233–4243. [PubMed: 17021047]
10. Wang H, Iakova P, Wilde M, Welm A, Goode T, Roesler WJ, Timchenko NA. C/EBP α arrests cell proliferation through direct inhibition of cdk2 and cdk4. *Molecular Cell*. 2001; 8:817–828. [PubMed: 11684017]
11. Iakova P, Awad SS, Timchenko NA. Aging reduces proliferative capacities of liver by switching pathways of C/EBP α growth arrest. *Cell*. 2003; 113:495–506. [PubMed: 12757710]
12. Wang GL, Shi X, Salisbury E, Sun Y, Albrecht JH, Smith RG, Timchenko NA. Cyclin D3 maintains growth inhibitory activity of C/EBP α by stabilizing C/EBP α -cdk2 and C/EBP α -Brm complexes. *Mol Cell Biol*. 2006; 26:2570–2582. [PubMed: 16537903]
13. Bartkova J, Lukas J, Strauss M, Bartek J. Cyclin D3: requirement for G1/S transition and high abundance in quiescent tissues suggest a dual role in proliferation and differentiation. *Oncogene*. 1998; 17:1027–1037. [PubMed: 9747882]
14. Cenciarelli C, De Santa F, Puri PL, Mattei E, Ricci L, Bucci F, Felsani A, Caruso M. Critical role played by cyclin D3 in the MyoD-mediated arrest of cell cycle during myoblasts differentiation. *Mol Cell Biol*. 1999; 19:5203–5217. [PubMed: 10373569]
15. Doglioni C, Chiarelli C, Macri E, Dei Tos A-P, Meggiolaro E, Dalla Palma P, Barbareschi M. Cyclin D3 expression in normal, reactive and neoplastic tissues. *J Pathol*. 1998; 185:159–166. [PubMed: 9713342]
16. de La Serna IL, Roy K, Carlson KA, Imbalzano AN. MyoD Can Induce Cell Cycle Arrest but Not Muscle Differentiation in the Presence of Dominant Negative SWI/SNF Chromatin Remodeling Enzymes. *J Biol Chem*. 2001; 276:41486–41491. [PubMed: 11522799]
17. Reichert M, Eick D. Analysis of cell cycle arrest in adipocyte differentiation. *Oncogene*. 1999; 18:459–466. [PubMed: 9927202]
18. Sarruf DA, Iankova I, Abella A, Assou S, Miard S, Fajas L. Cyclin D3 promotes adipogenesis through activation of peroxisome proliferator-activated receptor γ . *Mol Cell Biol*. 2005; 25:9985–9995. [PubMed: 16260612]
19. Rickheim DG, Nelsen CJ, Fassett JT, Timchenko NA, Hansen LK, Albrecht JH. Differential regulation of cyclins D1 and D3 in hepatocyte proliferation. *Hepatology*. 2002; 36:30–38. [PubMed: 12085346]
20. Wang GL, Timchenko NA. Dephosphorylated C/EBP α accelerates cell proliferation through sequestering retinoblastoma protein. *Mol Cell Biol*. 2005; 25:1325–1338. [PubMed: 15684384]
21. Mao WG, Liu ZL, Li AP, Zhou JW. JWA is required for the antiproliferative and pro-apoptotic effects of all-trans retinoic acid in Hela cells. *Clin Exp Pharm Physiol*. 2006; 33:816–824.
22. Herzinger T, Reed SI. Cyclin D3 Is Rate-limiting for the G₁/S Phase Transition in Fibroblasts. *J Biol Chem*. 1998; 273:14958–14961. [PubMed: 9614101]
23. Porse BT, Pederson TA, Haseman MS, Schuster MB, Kirsteter P, Luedde T, Damgaard I, Kurtz E, Schjerling XK, Nerlov C. The proline-histidine rich cdk2/cdk4 interaction region of C/EBP α is dispensable for C/EBP α -mediated growth regulation in vivo. *Mol Cell Biol*. 2006; 26:1028–1037.
24. Keeshan K, Santilli G, Corradini F, Perrotui D, Calabretta B. Transcriptional activation function of C/EBP α is required for induction of granulocytic differentiation. *Blood*. 2003; 102:1267–1275. [PubMed: 12702500]
25. D'Alo F, Johansen LM, Nelson EA, Radomska HS, Evans EK, Zhang P, Nerlov C, Tenen DG. The amino terminal and E2F interaction domains are critical for C/EBP α -mediated induction of granulopoietic development of hematopoietic cells. *Blood*. 2003; 102:3163–3171. [PubMed: 12869508]
26. Wang QF, Cleaves R, Kummalu T, Nerlov C, Friedman AD. Cell cycle inhibition by the outer surface of the C/EBP α basic region is required but not sufficient for granulopoiesis. *Oncogene*. 2003; 22:2548–2557. [PubMed: 12730669]
27. Porse BT, Pederson TA, Xu X, Lindbergh B, Wewer UM, Fris-Hansen L, Nerlov C. E2F repression by C/EBP α is required for adipogenesis and granulopoiesis in vivo. *Cell*. 2001; 107:247–258. [PubMed: 11672531]

28. Muller K, Calkhoven CF, Sha X, Leutz A. The CCAAT/Enhancer binding protein α (C/EBP α) requires a SWI/SNF complex for proliferation arrest. *J Biol Chem.* 2004; 297:7353–7358. [PubMed: 14660596]

Figure 1. Expression of C/EBPα in NIH3T3 cells causes a massive death of the cells

A. A diagram showing C/EBPα mutant constructs used in these studies. Mutation of Ser193 to Ala blocks its growth inhibitory activity; while mutation of S193 to Asp (D) creates constitutively active C/EBPα. Mutation of R290 to Ala blocks the ability of C/EBPα to interact with DNA [1]. **B. C/EBPα causes a rapid death in NIH3T3 cells.** pAdTrack-C/EBPα C/EBPα-S193A and an empty vector were transfected into 3T3-L1, NIH3T3, HEK2993 and Hep3B2 cells and a number of survived colonies (multi-cell colonies in proliferating cells and single cell colonies in arrested cells) was calculated at days 2, 3, 4 and 5 as percentage of number of colonies at day 1. **C. Western blotting of proteins isolated from experimental plates in the end of experiments with antibodies to C/EBPα and GFP.** Total protein extracts were examined by Western blotting with antibodies to C/EBPα, GFP and β-actin. C/EBPα and GFP were not detected in NIH3T3 cells since the transfected cells died at earlier time after transfections. β-actin shows that un-transfected NIH3T3 cells on the same plates grow.

Figure 2. DNA binding activity of C/EBP α is required for initiation of apoptosis in NIH3T3 cells
A. C/EBP α initiates apoptosis in NIH3T3 cells at 8 hours after transfections. DNA was isolated from NIH3T3 cells transfected with empty vector and with C/EBP α and examined by a native gel electrophoresis. DNA laddering is observed in cells expressing C/EBP α . **B. C/EBP α -R290A mutant does not bind to DNA.** Nuclear extracts from NIH3T3 cells transfected with WT C/EBP α and with R290A mutant were incubated with bZIP probe (containing binding site for C/EBP α) and binding reactions were separated by a native 5% PAAG. Antibodies to C/EBP α were added into reactions mixtures before the probe addition. **C. Western blotting of nuclear extracts from NIH3T3 cells transfected with WT and R290A C/EBP α .** The membrane was re-probed with β -actin. **D. The R290A mutant of C/EBP α does not cause cell death in NIH3T3 cells.** The experiment was performed as described in the legend to Figure 1.

C/EBP α -mediated alterations of gene expression in NIH3T3 compared to levels of these mRNAs in 3T3-L1 cells

Figure 3. Differential activation of gene expression by C/EBP α in NIH3T3 and 3T3-L1 cells
A. C/EBP α -mediated up-regulation of genes in 3T3-L1 and in NIH3T3 cells. The picture represents data of micro array assay performed as described in Materials and Methods. 14 identical genes activated by C/EBP α in both cell lines are shown below. **B.** C/EBP α -mediated down-regulation of genes in NIH3T3 and 3T3-L1 cells. 5 identical down-regulated genes are shown below. **C.** List of genes which are differentially up or down regulated in NIH3T3 by C/EBP α compared to levels of these genes in 3T3-L1 cells transfected with C/EBP α . Apoptosis signaling and cell cycle pathway are shown. The numbers of fold up or down regulation show the level of genes in NIH3T3 cells compared to the levels in 3T3-L1 cells.

Figure 4. C/EBPα increases expression of a pro-apoptotic protein ARL6IP5 through the activation of ARL6IP5 promoter

A. Nucleotide sequence of the ARL6IP5 promoter. Three C/EBPα binding sites are shown by red. The sequences of oligomers used for the gelshift are underlined. Start position of initiation of transcription is shown. **B. Interaction of C/EBPα with the ARL6IP5 promoter determined by Gelshift analysis.** DNA probes covering C/EBPα sites within ARL6IP5 promoter were incubated with C/EBPα overexpressed in HEK293 cells. Antibodies to C/EBPα were added to the binding reactions. Positions of C/EBPα, supershift and free probes are shown. **C. C/EBPα activates the ARL6IP5 promoter in NIH3T3 cells.** The promoter region of ARL6IP5 was linked to a luciferase reporter gene (dual) and co-transfected with WT and R290A C/EBPα mutant into 3T3-L1, Hep3B2 and NIH3T3 cells. Activity of the ARL6IP5 promoter was determined as ratio to mg of protein. **D. DNA binding activity of C/EBPα is required to increase ARL6IP5 mRNA in NIH3T3 cells.** WT and R290A C/EBPα were delivered in NIH3T3 cells by adenovirus vectors, total RNA was isolated and levels of ARL6IP5 mRNA were examined by RT-PCR. The bar graph picture shows a ratio of ARL6IP5 mRNA to GAPDH mRNA as a summary of three experiments. **E. Levels of ARL6IP5 protein.** Western blotting shows the levels of ARL6IP5 protein determined in protein extracts of cells transfected with WT C/EBPα and C/EBPα-R290A mutant. **F. Occupation of the ARL6IP5 promoter by C/EBPα in NIH3T3 and 3T3-L1 cells.** Chromatin solutions were prepared from cells transfected with empty vector (V), with WT C/EBPα and with C/EBPα-R290A mutant. C/EBPα was immunoprecipitated and PCR was performed with primers covering three C/EBP sites of the ARL6IP5 promoter. Upper panel shows results of PCR with C/EBPα IPs; the bottom panel shows PCR with 1/100 part of input.

Figure 5. Inhibition of ARL6IP5 abolishes apoptotic activities of C/EBP α

A. Inhibition of ARL6IP5 mRNA expression by siRNA. Different amounts of siRNA to ARL6IP5 were co-transfected into NIH3T3 cells with empty vector and with pAdTrack-C/EBP α plasmid and levels of ARL6IP5 mRNA were examined by RT-PCR as ratios to β -actin and as fold change relatively to levels in non-transfected cells. **B. siRNA to ARL6IP5 inhibits expression of ARL6IP5 protein.** Protein levels of ARL6IP5 were examined by Western blotting. The filter was re-probed with antibodies to β -actin. **C. DNA binding activity of C/EBP α in cells transfected with siRNA to ARL6IP5.** Gelshift was performed as described in the legend to figure 4 with a DNA probe (site 3). **D. siRNA-mediated inhibition of ARL6IP5 blocks the ability of C/EBP α to initiate apoptosis in NIH3T3 cells.** Percentage of survival cells was calculated in NIH3T3 cells transfected with WT C/EBP α alone and in cells transfected with C/EBP α and siRNA to ARL6IP5.

Figure 6.

A. C/EBP α does not inhibit proliferation of NIH3T3 cells. Bar graphs show colony growth assay of NIH3T3, 3T3-L1 and HEK293 cells transfected with WT, S193D and R290A C/EBP proteins. **B. Images show typical pictures of transfected cells at day 3 after transfections.** R290A mutant does not inhibit proliferation of NIH3T3 cells, but strongly inhibits proliferation of 3T3-L1 and HEK293 cells. **C. The C/EBP α -R290A mutant activates HDAC proteins and down-regulates p16INK4 and p19ARF mRNAs.** The table shows alterations in the levels of mRNA for cell cycle proteins determined by micro array. Micro array was performed with cells transfected with the C/EBP α -R290A mutant. The levels of mRNA which are changed by WT C/EBP α (see Fig 3) are compared to each other. **D. Examination of protein levels of p21 and p16 in NIH3T3 cells transfected with C/EBP α .** Western blotting was performed with nuclear extracts isolated from un-transfected cells, cells transfected with an empty vector (V), wild type C/EBP α (WT) and with R290A mutant. C/EBP α , HDAC1, p16 and p21 were examined and the filter was re-probed with antibodies to β -actin.

Figure 7. The growth arrest of NIH3T3 cells by C/EBP α requires high levels of cyclin D3

A. A comparison of cyclin D3 levels in HEK293, Hep3B2, 3T3-L1 and NIH3T3 cells. Western blotting was performed with antibodies to cyclin D3 using nuclear extracts isolated from the cells. The filter was re-probed with β -actin and cyclin D3 was calculated as a ratio to β -actin (bar graph). **B. Kinase activity of cyclin D3-cdk4 complexes is much lower in NIH3T3 cells compared to that in 3T3-L1 cells.** Cyclin D3 and cdk4 were immunoprecipitated from protein extracts of 3T3-L1 and NIH3T3 cells and examined in a kinase assay with Rb substrate. Agarose beads (Ag) were incubated with protein extracts and added into kinase assay to monitor a possible absorption. D1/K4; baculovirus expressed cyclin D1-cdk4 kinase which was used as a positive control. Bottom image shows a coomassie stain of the membrane to verify loading of Rb. **C. Co-immunoprecipitation of C/EBP α from 3T3-L1 and NIH3T3 cells transfected with WT and with R290A C/EBP α proteins.** Proteins were isolated 16 hours after transfections, C/EBP α was immunoprecipitated and these IPs were probed with antibodies to cyclin D3, ph-Ser193-C/EBP α , Brm, cdk2 and C/EBP α . NE; nuclear extract from 3T3-L1 cells is a positive control. C/EBP α -cdk2 and C/EBP α -Brm complexes are observed in 3T3-L1 cells, but not in NIH3T3 cells. **D. Overexpression of cyclin D3 in NIH3T3 cells partially restores growth inhibitory activity of C/EBP α .** C/EBP α -R290A was co-transfected with cyclin D3 into NIH3T3 cells and colony growth assay was performed. Western blotting shows levels of cyclin D3 in transfected cells. **E. Constitutively active double mutant C/EBP α (R290A-S193D) inhibits proliferation of NIH3T3 cells.** Bar graphs show a colony growth assay as a summary of three independent experiments. A typical pictures of cells transfected with C/EBP α proteins is shown on the right.

Figure 8. Cyclin D3 and C/EBP α cooperate in the inhibition of HEK293 and Hep3B2 cells
A. Overexpression of cyclin D3 in C/EBP α -negative HEK293 cells accelerates cell proliferation. Left image shows expression of cyclin D3 after transfections with cyclin D3 plasmid. A typical picture of images of transfected cells at day 4 after transfections is shown below. Right part shows a percentage of multi-cell colonies (more than 4 cells/colony) at day 4 after transfections with an empty vector and with pAdTrack-cyclin D3. **B. Inhibition of cyclin D3 in HEK293 cells reduces the ability of C/EBP α to inhibit proliferation.** C/EBP α was transfected into HEK293 cells alone or with a vector expression siRNA to cyclin D3. Bar graphs show results of colony growth assay. Western shows the inhibition of cyclin D3 by siRNA obtained in an independent transfection. Bottom panel shows typical images of transfected cells. Two cells clusters on the plate with C/EBP α + siRNA cyclin D3 are shown by white arrows. **C. Levels of cyclin D3 in C/EBP α -positive Hep3B2 cells transfected with cyclin D3 plasmid.** Nuclear extracts from cells transfected with empty vector (V) and with cyclin D3 plasmid were examined by Western blotting with antibodies to cyclin D3. CRM; cross reactive molecule which interacts with antibodies to cyclin D3 and serves as a loading control. **D. Inhibition of C/EBP α by siRNA.** The membrane was re-probed with antibodies to β -actin. **E. Colony growth assay of Hep3B2 cells transfected with cyclin D3 and with cyclin D3 + siRNA to C/EBP α .** Bar graphs show results of colony growth assay as a summary of three independent experiments. **F. Interactions of C/EBP α with cdk2 and Brm in cells transfected with cyclin D3.** C/EBP α was immunoprecipitated from Hep3B2 cells transfected with empty vector or with pAdTrack-cyclin D3 plasmid. The C/EBP α IPs were probed with antibodies to ph-Ser193, cdk2, Brm, E2F4 and C/EBP α Ag; mock agarose control.