THE BIOLOGICAL EFFECTS OF C/EBPα IN K562 CELLS DEPEND ON THE POTENCY OF THE N-TERMINAL REGULATORY REGION NOT ON SPECIFICITY OF THE DNA BINDING DOMAIN.

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The transcription factor C/EBPα induces granulocytic differentiation and inhibits proliferation of BCR/ABL-expressing cells very effectively whereas C/EBPβ, which is highly homologous to C/EBPα in the C-terminal DNA binding domain (DBD) but not in the N-terminal transactivation domain (TAD), is much less potent.

We took a “domain swapping” approach to assess biological effects, modulation of gene expression and binding to C/EBPα-regulated promoters by wild-type and chimeric C/EBPα/C/EBPβ proteins. Wild-type and N-C/EBPα+C/EBPβ-DBD induced transcription of the granulocyte-colony stimulating factor receptor (G-CSFR) gene, promoted differentiation and suppressed proliferation of K562 cells vigorously; instead, wild-type C/EBPβ and N-C/EBPβ+C/EBPα-DBD had modest effects, although they bound the G-CSFR promoter like wild type C/EBPα and N-C/EBPβ+C/EBPβ-DBD. Chimeric proteins consisting of the TAD of VP16 and the DBD of C/EBPα or C/EBPβ inhibited proliferation and induced differentiation of K562 cells as effectively as wild-type C/EBPα, indicating that the potency of the TAD and/or unique protein interactions with the N-terminus determine the transcription-dependent effects of C/EBPα.

Gene expression profiles induced by C/EBPα resembled those modulated by N-C/EBPα+C/EBPβ-DBD whereas C/EBPβ induced a pattern similar to that of N-C/EBPβ+C/EBPα-DBD, although each protein modulated specific gene subsets.

C/EBPα activation induced changes in the expression of more cell cycle- and apoptosis-related genes than the other proteins and enhanced Imatinib-induced apoptosis of K562 cells. Expression of FOXO3a, a novel C/EBPα-regulated gene, was required for apoptosis but not for differentiation induction or proliferation inhibition of K562 cells.

C/EBPα and C/EBPβ, two transcription factors of the CAAT/Enhancer Binding Protein family, regulate the proliferation and differentiation of various cell types (1). In hematopoietic cells, C/EBPα is expressed by myeloid progenitors and precursors but not by monocytes (2, 3) and induces granulopoiesis and blocks monocytic differentiation, upon expression in bipotential myeloid progenitors (4); consistent with this effect, loss of C/EBPα in vivo led to mice capable of producing monocytes but not mature granulocytes (5), and conditional knockout of C/EBPα in hematopoietic stem cells/early progenitors increased the frequency of hematopoietic stem cells and blocked the transition of common myeloid progenitors (CMPs) into granulocyte-monocyte precursors (GMPs) (6).

The requirement of C/EBPβ in hematopoietic cells is less well defined; C/EBPβ knockout mice exhibit a lymphoproliferative disorder and defective splenic macrophage activation but no apparent changes in granulopoiesis (7, 8). Nevertheless, the knock-in of the C/EBPβ gene at the C/EBPα locus restored granulocytic differentiation of C/EBPα knockout mice, but did not rescue their metabolic disorder, suggesting that
C/EBPβ has overlapping but not entirely redundant functions with C/EBPα (9).

While C/EBPα expression is finely regulated in normal hematopoietic cells, its expression/activity is consistently altered in myeloid leukemias (10), suggesting that genetic or functional inactivation of C/EBPα is an important event in leukemogenesis (10,11). For example, transformation of myeloid precursor cells by p210BCR/ABL and chronic phase-to-blast crisis transition in chronic myelogenous leukemia (CML) is associated with decreased expression of C/EBPα by a mechanism that depends, in part, on inhibition of c/ebpα mRNA translation (12,13). C/EBPα is mutated in approximately 10% of acute myelogenous leukemia (AML) patients (14-16); the identified mutants have reduced DNA binding and/or transactivation activity, suggesting that they suppress differentiation of blast cells through a dominant-negative effect or that reduced levels of functional protein are insufficient to promote blast cell differentiation. Genetic evidence indicates that expression of p30 C/EBPα (an isoform lacking the N-terminal transactivation domain) or of a DNA binding deficient C-terminus mutant in the absence of wild-type C/EBPα promotes the development of acute myeloid leukemia in mice (17,18). In AML patients with the t(8;21) translocation, the AML-1-ETO fusion protein appears to block differentiation by physically interacting with C/EBPα and suppressing its activity and by transcriptional repression of C/EBPα expression (19,20). C/EBPα expression is also downregulated via enhanced degradation in AML overexpressing Trib2 (21) and via translation inhibition in AML with the inv16 fusion protein and the AML1-MDS1-EVI1 fusion gene (22, 23). In AML with mutant FLT3, C/EBPα expression is, in some cases, downmodulated, but its function may also be inhibited by MAP kinase-dependent phosphorylation in the N-terminus (24, 25). Treatment of FLT3-ITD myeloid leukemia lines with a MAP kinase inhibitor induced partial differentiation and was associated with C/EBPα activation (25), and ectopic expression of C/EBPα in the t(8;21)-positive Kasumi cell line and in BCR/ABL-expressing cells induced granulocytic differentiation and suppressed proliferation in vitro and in leukemic mice (19, 26, 27), emphasizing the therapeutic potential of restoring expression of functional C/EBPα in leukemic cells.

Unlike C/EBPα, much less is known about potential mechanisms of structural or functional inactivation of C/EBPβ in AML and CML-BC cells. We found that expression of C/EBPβ was induced in Imatinib (IM)-treated BCR/ABL-expressing cells and that levels of full-length C/EBPβ were lower in CML-BC than in CML-CP or healthy individuals CD34+ progenitors (28). The effect of IM in 32D-BCR/ABL-expressing cells is reminiscent of that of All Trans Retinoic Acid (ATRA) in acute promyelocytic leukemia (APL) cells. In APL cells, C/EBPβ expression was induced by ATRA treatment and was required for ATRA-dependent differentiation (29).

Consistent with these findings, ectopic expression of C/EBPβ inhibited proliferation, induced differentiation and suppressed leukemogenesis of 32D-BCR/ABL cells (28). However, C/EBPβ was considerably less potent than C/EBPα (27), perhaps because C/EBPα exerts its biological functions not only via DNA binding and transcription activation of C/EBP-regulated genes, but also via protein-protein interactions involving cell cycle regulators and chromatin remodeling proteins (30-33). In addition, the less potent effects of C/EBPβ may also reflect the subset of genes transcriptionally regulated by C/EBPα, C/EBPβ or both.

C/EBPα and C/EBPβ have a global homology at the protein level of 34%, but the homology in their TAD at the N-terminus is only 24%, whereas it increases to 71% in the C-terminus, where the DBD, the leucine-zipper domain (LZD) and the basic region (BR) reside (34).

To obtain additional insights on the mechanisms underlying the different potency of C/EBPα and C/EBPβ in p210BCR/ABL expressing cells, we assessed the biological effects of 4-hydroxytamoxifen (4-HT)-regulated proteins consisting of C/EBPα-C/EBPβ hybrids or of VP16-C/EBPα or C/EBPβ DBD chimera in K562 cells. We report here that the potent effect of C/EBPα in inhibiting proliferation and inducing differentiation of BCR/ABL-expressing cells depends on the strong activity of its TAD more than on different specificities of the DBDs; in addition to a subset of genes whose expression is modulated by C/EBPα, C/EBPβ and the chimeric proteins, expression of most C/EBP-regulated genes is influenced by their respective TAD or appears to be C/EBPα- or C/EBPβ-specific. In particular, C/EBPα modulates the expression of a subset of apoptosis and cell
cycle-regulatory genes which includes FOXO3a; expression of FOXO3a is essential for the apoptosis-enhancing effect of C/EBPα but not for its roles in cell proliferation and differentiation.

**EXPERIMENTAL PROCEDURES**

**Plasmids.** C/EBPα-ER and C/EBPβ-ER plasmids were previously described (26,27). N-C/EBPα + C/EBPβ-DBD-ER and N-C/EBPβ + C/EBPα-DBD-ER plasmids were generated as follows: a NotI site was inserted in C/EBPα-ER plasmid with the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) at nucleotide 623 upstream of the DBD; a NotI site was eliminated in C/EBPβ-ER plasmid at nucleotide 460 and a NotI site was inserted at nucleotide 810 upstream of the DBD. Each plasmid (sequenced to verify presence of mutations) was digested with NotI and BamHI to release the C/EBPα or C/EBPβ DBDs, leaving the MigRI plasmids with the N-terminus of C/EBPα or C/EBPβ and the ER cassette. The C/EBPα DBD was directionally cloned into the NotI/BamHI-digested N-terminus-C/EBPβ-ER-MigRI to obtain N-C/EBPβ+C/EBPα-DBD-ER plasmid, whereas the C/EBPβ DBD was directionally cloned into the NotI/BamHI-digested N-terminus-C/EBPα-ER-MigRI to obtain N-C/EBPα+C/EBPβ-DBD-ER plasmid.

VP16+C/EBPα-DBD-ER, VP16+C/EBPβ-DBD-ER and VP16+K298E-DBD-ER plasmids were generated as follows: the VP16 fragment was PCR-amplified from a pVP16-Nedd4 plasmid with 5’ and 3’ primers containing flapping XhoI and NotI sites, respectively; C/EBPα-DBD-ER, C/EBPβ-DBD-ER and K298E-DBD-ER fragments were generated by PCR from N-C/EBPβ-DBD-ER, N-C/EBPα-DBD-ER and K298E-ER (27) plasmids, respectively; the VP16 and C/EBPα-DBD-ER, C/EBPβ-DBD-ER or K298E-DBD-ER products were directionally cloned into the XhoI/EcoRI-digested MigRI vector. FOXO3a A-B-C pshRNA and scramble vectors were previously described (35).

**Cell culture, retroviral infection and shRNA transfection.** K562 and derivative cell lines were cultured in Iscove modified Dulbecco medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM L-glutamine. 293T and Phoenix cells were cultured in Dulbecco modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM L-glutamine. For retroviral infections, amphotropic Phoenix cells were transiently transfected with the indicated plasmids. The infectious supernatant was collected 48 hours later and used to infect (a 48-hour procedure) K562 cells. Twenty-four hours later, cells were sorted (EPICS Profile Analyzer; Coulter, Hialeah, FL) for green fluorescent protein (GFP) expression.

For shRNAs transfection, K562 cells (100 μL at a density of 10⁶/mL) were resuspended in nucleofector V (Amaza, Gaithersburg, MD) solution and mixed with 5 μg shRNA. The solution was added to Amaza electrode cuvettes and electroporated in Amaza Nucleofector II, using program T-16. Then, cells were diluted in 2 mL IMDM and 24h later seeded at 10⁷/mL and treated with 0.2 μM IM. 12h later, 250nM 4-HT was added and samples collected after 24, 48 and 72 hours for cell cycle and apoptosis analyses.

**Cell proliferation, cell cycle analysis, apoptosis and differentiation assays.** For proliferation and cell cycle analyses, K562 cells were washed with phosphate-buffered saline (PBS) and treated with 4-HT (250 nM; Sigma, St Louis, MO). Viable cells were counted by trypan blue exclusion.

For colony formation assays, cells were pre-treated (1h) with 250 nM 4-HT and plated in methylcellulose (5 x 10²/plate) in presence of 250 nM 4-HT. Colonies were counted 6 days later. Cell cycle distribution was determined by DNA content analysis of propidium iodide-stained nuclei, as described (27). Differentiation was monitored by May-Grunwald/Giemsa staining and by detection of CD11b and CD15 differentiation markers, as described (27). Images were visualized using an Olympus CK2 microscope with a 40×/0.65 numeric aperture objective, and were photographed using an Olympus SC35 type 12 camera (Olympus, Melville, NY). JPEG images were viewed using Adobe Photoshop (Adobe Systems, San Jose, CA), and contrast adjustments were made.

For induction of apoptosis, K562 cells were seeded at 10⁴/ml and pre-treated with 0.2 μM IM for 12h,
then 250nM 4-HT was added to IM-treated cells and percentage of apoptotic cells evaluated 24, 48 and 72 hours after 4-HT addition.

Reverse transcription-PCR analysis and Real-Time Quantitative PCR.

For reverse-transcription (RT)–PCR analysis, RNA of untreated or 4-HT–treated cells was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. Extracted RNA was digested with DNase-RNase free (Roche) for 1h at 37 C, and deactivated 15 minutes at 65 C. RT-PCR was performed with 200 ng RNA using the ONE STEP RT-PCR KIT (Qiagen) and oligodeoxynucleotides specific for granulocyte colony-stimulating factor (G-CSF) receptor and for FOXO3a cDNA (Supplementary Table 1). cDNA samples were adjusted to yield relatively equal amplification of HPRT transcripts.

For real time quantitative (RT-Q)-PCR, total RNA was isolated using the RNeasy Mini kit (Qiagen). Extracted RNA was digested with DNase-RNase free (Roche) for 1h at 37 C, and deactivated 15 minutes at 65 C. 2 µg of total mRNA was reverse transcribed and the resulting first-strand cDNA used as PCR template. All reactions were done in triplicates. Primer pairs for all analyzed genes (Supplementary Table 1) were designed using the ABI Primer Express software. RT-Q-PCR was performed using iQ SYBR Green supermix (Bio-Rad) on a MyIQ thermocycler (Bio-Rad) and quantified using MyIQ software (Bio-Rad). HPRT, a housekeeping gene with constant expression, was used as an internal control to normalize input cDNA. For relative comparison of each gene, we used the MyIQ software that analyze the Ct value of real-time PCR data with delta delta Ct method.

Luciferase assay.

293T cells were transiently transfected using ProFection Mammalian Transfection System-Calcium Phosphate (Promega, Madison, WI) with 3 µg reporter plasmid pTK-G-CSFR-luciferase (which contains 4 C/EBPα binding sites from the G-CSF receptor promoter), 3 µg of the indicated C/EBP expression plasmid, and 1/50 Renilla luciferase plasmid to account for variation in transfection efficiencies. 24h after co-transfection, 293T were treated with 250 nM 4-HT for 12h. Firefly and renilla luciferase activity was recorded on a luminometer using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Results are expressed as fold activation relative to empty vector after correction for Renilla luciferase activity and are representative of 3 independent experiments (performed in triplicate).

Microarray analysis.

RNA was isolated from 4-HT- or vehicle-treated C/EBP-ER K562 cells and purified with RNeasy Column (Qiagen, CA). 5ug total RNA were used to generate biotin labeled cRNA using an oligo T7 primer in a reverse transcription reaction followed by in vitro transcription reaction with biotin labeled UTP and CTP. Ten micrograms of cRNA were fragmented and hybridized to HGU133 2.0 Plus arrays (Affymetrix, Santa Clara, CA) representing nearly 50,000 RNA transcripts and variants. Hybridized arrays were stained according to the manufactures’ protocols on a Fluidics Station 450 and scanned on an Affymetrix scanner 3000. All array images were visually inspected for defects and quality. Signal values were determined using Gene Chip Operating System 1.0 (GCOS, Affymetrix). For each array, all probe sets were normalized to a mean signal intensity value of 100. A qualifier was considered detectable if the mean expression was greater than 50 signal units and the percentage of samples with a Present (P) call as determined by GCOS default settings was greater than or equal to 25%. A qualifier was considered to be regulated if the difference between 4-HT- and vehicle-treated samples met the following criteria; (1) the qualifier was detected in at least 25% of the samples in either the vehicle or 4-HT-treated samples, (2) the fold change was at least 2, and (3) the p-value based on an t-test was ≤0.05. For identification of gene associated with specific biological processes we used EPLtool, an automatic gene clustering tool developed by Wyeth Bioinformatics for Affymetrix qualifiers.

Chromatin immunoprecipitation.

(ChIP) assays were performed using the EZ-ChIP Assay Kit (Upstate). Briefly, 3 x 10^7 exponentially growing K562 cells (untreated and 4-HT-treated, 24 h) were cross-linked with 1% formaldehyde, incubated for 15 min and treated with glycine at a final concentration of 125 mM for 5 minutes at room temperature. Cells were then washed with ice-cold PBS and resuspended in 1mL of lysis buffer with a protease inhibitor containing cocktail and sonicated at 24% power for 12 pulses of 10 seconds each in a Branson Sonifier 450 (Branson Ultrasoundics, Danbury, CT). Chromatin was precleared with 50 µL protein A-agarose beads for
60 min at 4°C with rotation and pre-cleared lysates were immunoprecipitated with the anti-Estrogen Receptor alpha antibody (12 μg; SRA-1010 Stressgen) at 4°C overnight with rotation. Immune precipitations without antibody (no antibody control) and an anti-rabbit IgG were included with each experiment. Immune complexes were collected with 50 μL protein A-agarose beads for 60 min at 4°C with rotation (except for 10 μL of supernatant of the no antibody control saved as Input) and washed with the buffer recommended in the ChIP protocol. Immune complexes were next eluted using freshly prepared elution buffer (1% SDS and 0.1 M NaHCO3). Cross-links were reversed by heating at 65°C overnight in the presence of 0.2 M NaCl. ChIP DNA (2 μL) was next used as a template for RT-Q-PCR using the following primers: human FOXO3a promoter primers (-655/-453) which encompasses the putative C/EBP binding site TATTTCCACA at nucleotides -608 to -599 identified through both the AliBaba2 and PATCH programs; human FOXO3a promoter primers (-960/-756) which does not contain putative C/EBP binding sites as negative control (Supplementary Table 1); and human G-CSFR promoter primers which includes a canonical C/EBP binding site TGTTGCAATC at nucleotide -55 as positive control(Supplementary Table 1).

Recovered DNA was analyzed by RT-Q-PCR with the primers in Supplementary Table 1; to normalize ChIP-qPCR data were analyzed with the Percent Input Method; percent input was calculated by 100 x 2^(CtInput-CtEnriched).

Statistical Analyses. Data (presented as means ± SD of two or three experiments) were analyzed for statistical significance by the unpaired, 2-tailed Student’s t-test. p values of less than 0.05 were considered statistically significant.

RESULTS

Biological effects of wild type and chimeric C/EBPα/β proteins in K562 cells.

Activation of C/EBPα-ER in K562 cells induces granulocytic differentiation rapidly and efficiently while C/EBPβ-ER had no effects (33,36). To assess the mechanisms underlying such distinct effects, we generated retroviruses for two chimeric proteins by swapping the C-terminus of C/EBPα and C/EBPβ (Figure 1A), and stably expressed wild-type and chimeric proteins into K562 cells (Figure 1B). After treatment with 4-HT to activate ectopic C/EBP proteins, we first assessed the effects on K562 cell proliferation by cell counts and DNA content analyses. Compared to empty vector transduced cells, activation of C/EBPα-ER or N-C/EBPα+C/EBPβ-DBD-ER led to statistically significant inhibition of proliferation (Figure 1C). Likewise, DNA content analysis showed a statistically significant increase of G1 phase cells, and a decrease of S and G2/M phase cells only after a 72 h activation of C/EBPα-ER or N-C/EBPα+C/EBPβ-DBD-ER (Figure 1D).

We also tested the effects of the C/EBPα/β chimeric proteins on colony formation of K562 cells. Consistent with the effects on cell proliferation, activation of C/EBPα-ER or N-C/EBPα+C/EBPβ-DBD-ER markedly suppressed (approximately 90% inhibition compared to controls) K562 colony formation (Figure 1E); activation of C/EBPβ-ER or N-C/EBPβ+C/EBPα-DBD-ER also suppressed K562 colony formation but the effect was considerably less striking (40-50% inhibition compared to controls) (Figure 1E). The effects on differentiation were assessed by morphology and expression of granulocytic markers. Activation of C/EBPα-ER or N-C/EBPα+C/EBPβ-DBD-ER promoted granulocytic differentiation of K562 cells, whereas activation of C/EBPβ-ER or N-C/EBPβ+C/EBPα-DBD-ER induced the early appearance of metamyelocytes, as indicated by the presence of cells with “doughnut”-shaped nuclei, but the vast majority of these cells failed to undergo terminal differentiation (Figure 2A, Table 1). Analysis of granulocytic differentiation markers confirmed these morphological changes; compared to empty vector-transduced cells, activation of C/EBPα-ER or N-C/EBPα+C/EBPβ-DBD-ER led to a statistically significant induction of CD11b and CD15, whereas expression of these two markers did not increase in cells expressing wild-type C/EBPβ-ER or N-C/EBPα+C/EBPβ-DBD-ER (Figure 2B and 2C, respectively). Moreover, G-CSFR mRNA transcripts were readily detected by real-time or by semi-quantitative RT-PCR upon activation of wild-type C/EBPα-ER or the N-C/EBPα+C/EBPβ-DBD-ER chimera (Figure 2D and 2E, respectively).

Effects of wild type and chimeric C/EBPα/β
proteins on the GCSF-R promoter.
To further assess the effects of wild type and chimeric C/EBPα and C/EBPβ proteins, we performed luciferase assays on the C/EBPα-responsive G-CSFR promoter. After co-transfection of 293T cells with the G-CSFR promoter-luciferase reporter (G-CSFR/Luc) plasmid, the Renilla luciferase control plasmid to account for variation in transfection efficiencies and C/EBP plasmids or the MigRI empty vector, cells were treated with 4-HT for 12 h and luciferase activity measured thereafter. Results are expressed as fold activation relative to vector alone after normalization for Renilla luciferase activity (Figure 3A). C/EBPα and N-C/EBPα+C/EBPβ-DBD transactivated the G-CSFR/Luc plasmid more effectively than C/EBPβ or the N-C/EBPβ+C/EBPα-DBD chimeras; however, the results of these assays do not allow to exclude that differences in DNA binding could account for the distinct effects of the chimeric proteins. Thus, quantitative ChIP assays were performed upon activation of C/EBP-ER proteins in K562 cells. As shown in Figure 3B, quantitative PCR showed comparable binding of C/EBPα, C/EBPβ and the chimeric proteins to a segment of the G-CSFR promoter containing a canonical C/EBPα binding site, in sharp contrast with the inability of C/EBPβ N-terminus containing proteins to activate G-CSFR expression (Figure 2D and E).

Effects of VP16-C/EBPα/β-DBD-ER chimeric proteins on the GCSF-R promoter and the proliferation and differentiation of K562 cells.
To further address the possibility that the more potent biologic effects of C/EBPα depend on the stronger activity of its TAD more than on different specificities of the DBD, we generated K562 cell lines expressing chimeric proteins consisting of the VP16 TAD juxtaposed to the DBD of C/EBPα or C/EBPβ fused to the tamoxifen-responsive estrogen receptor ligand binding domain (ER\textsuperscript{TAM}) (Figure 4A and B). The herpes simplex virus type 1 (HSV-1) tegument protein VP16 is a structural protein of HSV-1 which activates transcription of the immediate early promoters of the virus (37,38). Although VP16 specifically activates promoters containing the so called TAATGARAT element, specificity is conferred by the cellular DNA binding protein(s) fused to the N-terminal domain of VP16 (39, 40). To assess the transactivation ability of the two VP16-C/EBPs-DBD-ER chimeric proteins, we performed luciferase assays on the C/EBPα responsive G-CSFR promoter. After co-transfection of 293T cells with the G-CSFR/Luc plasmid, the Renilla luciferase control plasmid to account for variation in transfection efficiency, VP16-C/EBPs-DBD-ER expression vectors or the MigRI empty vector, cells were treated with 4-HT for 12 h and luciferase activity was measured thereafter. Results are expressed as fold activation relative to vector only-transfected cells after normalization for Renilla luciferase activity. The assays show that the two chimeric proteins are essentially undistinguishable in the ability to transactivate the C/EBPα-responsive G-CSFR promoter (Figure 4C).

To analyze the biological effects of these chimeric proteins, differentiation and proliferation were analyzed upon 4-HT treatment of VP16-C/EBPα/β-DBD-ER retrovirally transduced K562 cells (Figure 4B). Upon 4-HT activation, both chimeric proteins rapidly induced granulocytic differentiation as indicated by morphology (Figure 5A), expression of CD15 and CD11b differentiation markers (Figure 5B) and G-CSFR mRNA levels (Figure 5C). We also assessed cell cycle distribution and methylcellulose colony formation of untreated and 4-HT-treated VP16-C/EBPα/β-DBD-ER/K562 cells. After a 72-h treatment with 4-HT, either chimeric protein induced a marked increase in the number of G1 cells and a decrease in the fraction of S and G2/M phase cells (Figure 5D); likewise, both proteins markedly suppressed methylcellulose colony formation of K562 cells (Figure 5E). Of note, the effects of the VP16-C/EBPα/β-DBD-ER proteins appear to be undistinguishable from those of wild-type C/EBPα-ER and N-C/EBPα+C/EBPβ-DBD-ER (compare Figure 1 and Figure 5). As control, we also generated a VP16-C/EBPα K298E-DBD-ER expression plasmid in which the K298E mutation prevents DNA binding (27) to assess whether the effects of the chimeric VP16-C/EBP DBD-ER proteins were DNA binding-dependent. As shown in Supplementary Figure 1, activation of this chimeric protein failed to induce granulocytic differentiation or to suppress colony formation of K562 cells. Together, these studies further confirm that the markedly different biological effects of C/EBPα
and C/EBPβ in K562 cells depend on the potency of their transactivation domain.

Gene expression profiles regulated by wild-type and chimeric C/EBPα/β proteins

The different transactivation activities of wild-type and chimeric C/EBPα/β proteins should be reflected in the modulation of distinct patterns of gene expression; thus, we performed microarray hybridization assays using RNA of untreated and 4-HT-treated (12h) C/EBP-ER K562 cells to assess overall pattern of gene expression and specific gene subsets regulated by wild-type and chimeric C/EBPα/β proteins.

An overview of the genes differentially regulated by wild-type and chimeric C/EBPα/β proteins in K562 cells is shown in Supplementary Figure 2 (panel A). Upon 4-HT treatment, C/EBPα-ER and N-C/EBPα-C/EBPβ-DBD-ER regulated the expression of a larger number of genes, 3498 (1644↓;1854↑) respectively, than C/EBPβ-ER and N-C/EBPβ-C/EBPα-DBD-ER, 1387 (744↓;643↑) and 1316 (663↓;653↑), respectively.

The different transactivation activities of wild-type and chimeric C/EBPα/β proteins, in agreement with the results of the microarray hybridization data.

We then asked to which functional groups the C/EBP-regulated genes belonged in order to correlate gene expression profiles and biological effects induced by wild-type and chimeric C/EBPα/β proteins.

Within the group of cell cycle related genes, C/EBPα and N-C/EBPα+C/EBPβ-DBD modulated the expression of 114 and 81 cell cycle related genes, respectively; of these, 58 were unique for C/EBPα and 35 for N-C/EBPα+C/EBPβ-DBD chimeric protein, whereas 26 were modulated by both proteins. C/EBPβ and N-C/EBPβ+C/EBPα-DBD regulated 56 and 61 cell cycle related genes, respectively; of these, 20 were unique for C/EBPβ and 37 for the N-C/EBPβ+C/EBPα-DBD chimeric protein, whereas 5 were regulated by both proteins, suggesting that the more potent inhibitory effects of C/EBPα and the N-C/EBPα+C/EBPβ-DBD chimeric protein in proliferation and colony formation of K562 cells probably depend on a more complex pattern of cell cycle-related gene regulation.

The same analysis was performed on genes involved in apoptosis. C/EBPα regulated 80 apoptosis-related genes, 40 of which were unique, whereas C/EBPβ, N-C/EBPα+C/EBPβ-DBD and N-C/EBPβ+C/EBPα-DBD modulated 40, 51 and 40 apoptosis-related genes, respectively; of these, 10, 17 and 18 were unique.

The effect of C/EBPα in apoptosis is, in part, dependent on FOXO3a expression. Although a pro-apoptotic effect was not discerned upon C/EBPα activation in BCR/ABL-expressing cells in vitro and in leukemic mice (27), the more complex subset of apoptosis-related genes regulated by C/EBPα raised the possibility that, under certain circumstances, C/EBPα activation could also inhibit cell survival, possibly enhancing its anti-leukemia effects.

To address this possibility, K562 cells expressing C/EBPα, C/EBPβ or the chimeric proteins were pre-treated for 12 h with a suboptimal concentration of IM (0.2 μM), followed by incubation with 250 nM 4-HT and apoptosis was assessed by propidium iodide staining to detect
cells with hypodiploid DNA content. As shown in Figure 6A, only activation of C/EBPα led to a statistically significant increase in the number of apoptotic cells. Among apoptosis-related genes modulated by C/EBPα, it seemed especially interesting to further assess the role of FOXO3a, a transcription factor of the FoxO family that appears to play an important role in cell cycle arrest and apoptosis of normal and BCR/ABL-expressing hematopoietic cells (41-43). From the microarray data, FOXO3a was differentially regulated by C/EBPα, C/EBPβ, and N-C/EBPα+C/EBPβ-DBD, but only C/EBPα was able to enhance the expression of 5 different FOXO3a qualifiers, one of which showing a 2.5 fold increase. Indeed, upon a 12h 4-HT treatment of K562 cells expressing C/EBPα, C/EBPβ or the chimeric proteins, expression of FOXO3a mRNA was readily induced only by C/EBPα (Figure 6B); the increased expression of FOXO3a was confirmed by anti-FOXO3a Western blotting (Figure 6C).

Of interest, both VP16-C/EBPα/β-DBD-ER chimeric proteins failed to activate FOXO3a mRNA expression (Supplementary Figure 4). To assess whether FOXO3a is directly regulated by C/EBPα, FOXO3a mRNA levels were measured in K562 cells treated with 250nM 4-HT (to activate C/EBPα), in the presence of the protein synthesis inhibitor cycloheximide (CHX). In control experiments using K562 cells transduced with the MigRI empty vector, co-treatment with 4-HT and CHX had no effect on FOXO3a mRNA levels (not shown); by contrast, expression of FOXO3a mRNA was rapidly induced after C/EBPα-activation and this early increase was not prevented by a 4-h treatment with CHX (Figure 6D), consistent with FOXO3a being a direct target of C/EBPα.

Additional proof in support of FOXO3a being a transcriptional target of C/EBPα was obtained by ChIP assays; a search for C/EBP binding sites in the 5` flanking region of the human FOXO3a gene identified a putative binding site at nucleotides -608 to -599 (underlined in Figure 6E). PCR amplification of a segment including this site after anti-ER ChIP from 4-HT-treated K562 cells expressing wild type or chimeric C/EBPα-C/EBPβ proteins demonstrated that each of these proteins was able to interact with the a segment (nucleotides -655 to -453) of the FOXO3a promoter (Figure 6E), although only C/EBPα was able to activate FOXO3a mRNA expression (Figure 6B). C/EBP proteins did not interact with a more distal segment of the FOXO3a promoter lacking putative C/EBP binding sites (not shown).

To assess if the enhanced propensity of IM-treated C/EBPα-expressing K562 cells to undergo apoptosis was associated with the ability of C/EBPα to induce FOXO3a expression, levels of FOXO3A were downregulated by RNAi in empty vector-transduced or C/EBPα-ER expressing K562 cells (Fig 7A) and the frequency of apoptotic cells was assessed after treatment with a suboptimal concentration of IM (0.2 μM). Downregulation of FOXO3a expression suppressed the increase in apoptosis induced by co-treatment of C/EBPα-ER expressing K562 cells with 4-HT and IM (Figure 7B). Thus, under certain conditions (i.e. IM or chemotherapy activation of apoptotic pathways), C/EBPα may enhance apoptosis through FOXO3a-dependent mechanisms.

FOXO3a expression is not required for C/EBPα-dependent granulocytic differentiation and proliferation inhibition in K562 cells.

Since expression of constitutively active FOXO3a induces erythroid differentiation of K562 cells (44), we assessed also the role of C/EBPα-dependent FOXO3a expression in C/EBPα-induced granulocytic differentiation of K562 cells. Thus, FOXO3a expression was downregulated by RNAi in C/EBPα-ER expressing K562 cells (Supplementary Figure 5A) and the induction of granulocytic differentiation was assessed upon 4-HT treatment. K562 cells transfected with scramble or FOXO3a specific shRNAs underwent granulocytic differentiation as indicated by morphology (Supplementary Figure 5B) and expression of the myeloid differentiation marker CD11b (Supplementary Figure 5C). The role of FOXO3a expression for the proliferation inhibitory effects of C/EBPα were tested by assessing cell counts, cell cycle distribution, and methylcellulose colony formation of untreated and 4-HT-treated K562 cells transfected with scramble or FOXO3a specific shRNAs. Activation of C/EBPα suppressed proliferation (Supplementary Figure 5D) and induced a marked increase in the number of G1 cells and a decrease in the fraction of S and G2/M phase cells.
(Supplementary Figure 5E) as effectively in scrambled and in FOXO3a shRNA-transfected cells; likewise, downregulation of FOXO3a expression did not rescue the inhibition of methylcellulose colony formation induced by expression of functional C/EBPα in K562 cells (Supplementary Figure 5F).

**DISCUSSION**

Expression of C/EBPα and C/EBPβ is reduced in BCR/ABL transformed cell lines and in blast cells from patients with CML-blast crisis (BC) (27, 28), suggesting that loss of C/EBP activity is necessary to suppress the differentiation potential of CML-BC progenitors. Consistent with this hypothesis, ectopic expression of C/EBPα or C/EBPβ induced granulocytic differentiation and inhibited proliferation of BCR/ABL-expressing cells in vitro and in mice, but the effects of C/EBPβ were considerably less potent than those of C/EBPα (27, 28). This contrasts with the apparent complete rescue of granulocytic differentiation in mice in which the C/EBPα gene was replaced by C/EBPβ (9) and is probably due to the severe block of differentiation induced by BCR/ABL. Since C/EBPα and C/EBPβ are highly homologous in their N-terminus where basic region, DNA binding and leucine zipper domain reside, but are highly divergent in the N-terminus where the TAD is located, we took a “domain swapping” approach to evaluate biological effects and gene expression profiles in K562 cells expressing 4-HT-regulated C/EBPα-C/EBPβ chimeric proteins.

Activation of C/EBPα or of the chimeric protein N-C/EBPα+C/EBPβ-DBD that shares the TAD of wild type C/EBPα, suppressed proliferation and promoted an increase in the number of G1 phase cells, whereas C/EBPβ or N-C/EBPβ+C/EBPα-DBD-ER that shares the TAD of wild type C/EBPβ did not. Likewise, activation of C/EBPα and N-C/EBPα+C/EBPβ-DBD led to efficient granulocytic differentiation of K562 cells while C/EBPβ and N-C/EBPβ+C/EBPα-DBD-ER did not. These effects correlated with those on the transactivation of the G-CSFR promoter. However, ChIP assays did not reveal any difference in the ability of C/EBPα, C/EBPβ or the chimeric proteins to interact with the G-CSFR promoter, suggesting that the TAD of C/EBPα is more potent of that of C/EBPβ perhaps through the recruitment of interacting proteins capable of enhancing its transcription activation function.

In an attempt to distinguish between the role of C/EBPα N-terminus specific interactions versus TAD strength, we assessed biological effects (induction of differentiation and inhibition of proliferation) and transcription activation of chimeric proteins consisting of the same N-terminus (the VP16 TAD) fused to the C/EBPα or C/EBPβ DBD. The effects of these two chimeric proteins were undistinguishable, confirming that the DBD does not confer specificity in modulating the transcription-dependent program required for proliferation inhibition and differentiation induction in K562 cells. Of equal importance, the effects of the VP16-C/EBPα/β-DBD chimeric proteins were identical to those of wild-type C/EBPα- or N-C/EBPα+C/EBPβ-DBD, indicating that the gene expression program responsible for the ability of C/EBPα to induce granulocytic differentiation and to inhibit proliferation of K562 cells can be faithfully recapitulated by a chimeric protein in which the C/EBP DBD (which determines specificity of gene targets) is under the control of a strong TAD. Of interest, mutation of the DNA binding domain suppressed differentiation induction and colony formation inhibition by the VP16-C/EBP-DBD chimera suggesting that C/EBPα can suppress proliferation through both transcriptional mechanisms and the interaction with cell cycle regulatory proteins (30-33).

The differences in the biological and biochemical effects of C/EBPα, C/EBPβ and chimeric proteins were reflected in the activation of distinct gene expression profiles, although a subset of genes was modulated by each protein.

The gene expression profile modulated by C/EBPα and N-C/EBPα+C/EBPβ-DBD was more complex of that regulated by C/EBPβ and N-C/EBPβ+C/EBPα-DBD; most likely reflecting the higher transactivation activity of C/EBPα and N-C/EBPα+C/EBPβ-DBD; however, the number of C/EBPα-regulated genes was higher of that modulated by N-C/EBPα+C/EBPβ-DBD, raising the possibility that certain changes in gene expression may be due to a DBD specific effect or to loss of C/EBPα N-terminus protein-protein interactions in the N-C/EBPα+C/EBPβ-DBD chimeric protein.
Since gene expression profiles modulated by C/EBP proteins were analyzed 12 hours post 4-HT treatment, the difference in the number of C/EBPα- and N-C/EBPα+C/EBPβ-DBD-regulated genes might have been magnified by secondary changes possibly due to protein-protein interactions in the N-terminus or DBD target specificity, although this would be inconsequential for the ability of N-C/EBPα+C/EBPβ-DBD to inhibit proliferation and induce differentiation of K562 cells as effectively as wild-type C/EBPα.

Functional analysis of the gene subsets regulated by C/EBPα, C/EBPβ and the two chimeric proteins revealed that genes in the apoptosis pathway were especially modulated by C/EBPα; this finding was of interest because activation of C/EBPα, per se, does not promote apoptosis of BCR/ABL-expressing cells (27), but it enhanced IM-induced cell death of K562 cells.

One of the genes regulated by C/EBPα and potentially required for its biological effects is FOXO3a, a transcription factor of the FoxO family with crucial roles in regulating the proliferation and survival of hematopoietic stem cells (41). Of interest, expression of FOXO3a was not activated by N-C/EBPα-C/EBPβ-DBD or by the VP16-C/EBP-DBD chimeric proteins; this suggests that the transcription of some C/EBPα targets depends not only on the potency of the transactivation domain but also on specific protein interactions that may not be limited to the N-terminal region, although it cannot be excluded that changes in conformation of the chimeric proteins may have affected the interaction with cofactors necessary for FOXO3a transcription. Although we did not demonstrate that C/EBPα transactivates the FOXO3a promoter, a direct role of C/EBPα in enhancing FOXO3a expression is suggested by ChIP assays demonstrating interaction with a C/EBP binding site in the human FOXO3a promoter and by the finding that C/EBPα induced FOXO3a mRNA expression in cells treated with cycloheximide to block de novo protein synthesis.

Recent studies have revealed that BCR-ABL inhibits FOXO3a activity via Akt-dependent phosphorylation, which promotes interaction with 14-3-3 protein and sequestration of FOXO3a into the cytoplasm (42, 43), leading to lower expression of pro-apoptotic genes such TRAIL and BIM (44); furthermore, FOXO3A inhibition by BCR-ABL also affects the expression of cyclin D1 and D2 (45, 46).

Consistent with the pro-apoptotic effects of FOXO3a, down-regulation of FOXO3A expression by RNAi blocked the increase in apoptosis induced by C/EBPα activation in IM-treated K562 cells. Based on the finding that expression of a constitutively active mutant of FOXO3a induced erythroid differentiation of K562 cells (47) and that proliferation regulatory genes are among the FOXO3A targets (45, 46), we suspected that FOXO3a was also involved in C/EBPα-regulated granulocytic differentiation and proliferation of K562 cells; however, FOXO3a silencing did not rescue either effect, indicating that distinct C/EBPα-regulated genes function as essential downstream effectors to enhance apoptosis susceptibility, promote differentiation and inhibit proliferation. It remains to be established whether differentiation induction and proliferation inhibition are regulated by common C/EBPα target genes or are under the control of separate effectors. In summary, we have used a C/EBPα-C/EBPβ “domain swapping” approach to demonstrate that: i) a strong TAD is responsible for the biological effects of C/EBPα in K562 cells; and ii) the effects correlate with C/EBPα-dependent activation of gene expression profiles which include many cell cycle and apoptosis regulatory genes.

Although activation of C/EBPα is insufficient to induce apoptosis of BCR/ABL-expressing cells, it enhanced IM-induced cell death via expression of FOXO3a, further emphasizing the therapeutic potential of restoring functional C/EBPα, alone or together with other anti-cancer agents, in CML-BC and, perhaps, other types of acute myeloid leukemia.
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FOOTNOTES

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The abbreviations used are: DBD, DNA binding domain; TAD, transactivation domain; CML, chronic myelogenous leukemia; CML-BC, chronic myelogenous leukemia blast crisis; CML-CP, chronic...
Fig. 1 Effects of wild type and chimeric C/EBP proteins on proliferation of K562 cells. (A) Schematic diagram of C/EBPs-ER chimeric proteins; (B) Western blot shows levels of C/EBP-ER proteins in K562 cells detected by anti-estrogen receptor α monoclonal antibody (Stressgene); expression of GRB2, as loading control, was detected by anti-GRB2 monoclonal antibody (610112, BD Transduction Laboratories). (C) Cell counts and (D) cell cycle distribution at 72h of 4-HT–treated MigRI- or C/EBP-ER–transduced K562 cells; values represent the mean ± SD of 3 independent experiments. * p<0.05 relative to MigRI transduced K562 cells. (E) Methylcellulose colony formation of 4- HT–treated MigRI- or C/EBP-ER–transduced K562 cells. Colonies were scored 6 days after seeding 5 x 10^2 cells/plate; values (mean ± SD of 2 independent experiments performed in duplicate) are expressed as the percentage of colonies from 4-HT-treated cells compared to those derived from the corresponding untreated cells taken as 100%. p values indicates statistical significance of the difference in colony number of untreated MigRI- or C/EBP-ER–transduced K562 cells versus 4-HT treated calculated using unpaired, 2-tailed Student’s t-test.

Fig. 2. Effects of wild type and chimeric C/EBP proteins on differentiation of K562 cells. (A) Morphology; light microscopy images of May-Grünwald–stained, untreated or 4-HT-treated K562 cells; original magnification x40; counts of differentiated cells, summarized in Table 1, were performed in at least 10 fields. (B) CD11b and (C) CD15 positivity; values represent the mean ± SD of 3 independent experiments. * p<0.05 relative to MigRI transduced K562 cells. GCSF-R mRNA expression assessed by real time-Q-PCR (D) or semiquantitative RT-PCR (E) in 4-HT-treated (24 hours in panel D; 24, 48 and 72 hours in panel E) K562 cells retrovirally transduced with MigRI or cDNAs encoding wild type or chimeric C/EBP proteins. HPRT expression was used as internal control in both PCR. In panel D, results are reported as normalized fold variation of the expression of G-CSFR mRNA; values are expressed as fold variation relative to expression in the 4-HT treated empty vector transduced sample taken as 1. HPRT expression was used as internal loading control. Error bars denote standard deviations of normalized means of two independent experiments performed in triplicate.

Fig. 3. Effect of wild type and chimeric C/EBP proteins on the G-CSFR promoter. (A) Histogram shows luciferase activity in 293T cells co-transfected with pTK-G-CSFR-luciferase and C/EBP plasmids or the MigRI empty vector after treatment (12 hours) with 4-HT. Results (three independent experiments) are expressed as fold activation over that in empty vector-transfected cells after normalization for Renilla luciferase activity and are reported as the means of triplicate determinations; error bars represent the standard deviation of the mean; p values indicate statistical significance of the difference in transactivation activity of C/EBPα-ER versus C/EBPβ-ER or N-C/EBPβ-C/EBPα-DBD-ER calculated using unpaired, 2-tailed Student’s t-test. (B) Quantitative ChIP assays show interaction of C/EBPα, C/EBPβ and C/EBPα-C/EBPβ chimeric proteins with a segment of the G-CSFR promoter (nucleotides -147 to +25) containing a functional C/EBPα binding site detected by real time Q-PCR. Error bars denote standard deviations of the means of one representative experiment (of two) performed in triplicate. Western blot shows total and immunoprecipitated C/EBPα, C/EBPβ and chimeric proteins from K562 cell lysates used for ChIP assays. Chimeric proteins were immunoprecipitated and detected by Western blot with the anti-estrogen receptor α monoclonal antibody (Stressgene)

Fig. 4. Effect of VP16-C/EBP-DBD chimeric proteins on the G-CSFR promoter activity. (A) Schematic representation of the VP16-C/EBP-DBD-ER chimeric proteins; (B) Expression of VP16-C/EBP-DBD-ER chimeric proteins in retrovirally transduced K562 cells; chimeric proteins were detected
by Western blot with the anti-estrogen receptor α monoclonal antibody (Stressgene); expression of GRB2 was detected by anti-GRB2 monoclonal antibody (610112, BD Transduction Laboratories). (C) Luciferase activity of the GCSF-R promoter in 293T cells transfected with the VP16-C/EBP-DBD-ER expression plasmids and the GCSF-R-LUC reporter after treatment with 4-HT (12 h, 250 nM). Histogram shows fold activation of luciferase activity over that in cells transfected with the MigRI empty vector after normalization for Renilla; error bars represent the standard deviation of the mean of three independent experiments performed in triplicates. NS indicates that the difference in transactivation between N-VP16-C/EBPβ-DBD-ER and N-VP16-C/EBPα-DBD-ER is not significant; statistical significance was calculated using unpaired, 2-tailed Student’s t-test.

Figure 5. Effect of VP16-C/EBP-DBD chimeric proteins on proliferation and differentiation of K562 cells. (A) Morphology; light microscopy images of May-Grünwald–stained untreated or 4-HT-treated MigRI-transduced or VP16-C/EBP-DBD-expressing K562 cells; original magnification x40. (B) CD11b (upper panel) and CD15 (lower panel) expression detected by flow cytometry with phycoerythrin (PE)-conjugated monoclonal antibodies (PharMingen, San Diego, CA); values represent the mean ± SD of 3 independent experiments. * p<0.03 relative to MigRI transduced K562 cells. (C) GCSF-R expression assessed by semiquantitative RT-PCR; HPRT expression was used as internal loading control; representative of two independent experiments (D) Cell cycle distribution (DNA content analysis of propidium-iodide-stained nuclei) of 4-HT-treated (72 h) K562 cells; values represent the mean ± SD of 3 independent experiments. (E) Colony formation assay of 4-HT–treated MigRI- or VP16-C/EBP-DBD-ER–transduced K562 cells. Values (mean ± SD of 2 independent experiments performed in duplicate) are expressed as the percent of colonies of 4-HT–treated versus untreated cells taken as 100%. p values indicate statistical significance of the difference in colony number of untreated MigRI- or VP16-C/EBP-DBD-ER–transduced K562 cells versus 4-HT treated calculated using unpaired, 2-tailed Student’s t-test.

Figure 6. Activation of C/EBPα enhances IM-induced apoptosis of K562 cells. (A) Number of apoptotic cells (mean plus SD, three experiments) in MigRI-transduced and C/EBP-ER-expressing K562 cells after treatment with 0.2 μM IM alone or with 4-HT; * p<0.05 relative to corresponding 4-HT-untreated sample. (B) Histogram shows FOXO3a mRNA levels, assessed by real time Q-PCR, in 4-HT-treated C/EBP-ER-transduced K562 cells; HPRT expression was used as internal control; error bars denote standard deviations of the normalized means of one representative (of two) experiment performed in triplicate. (C) Expression of FOXO3a in 4-HT-treated C/EBPα-ER K562 cells; expression of FOXO3a was detected by anti-FKHRL/FOXO3a rabbit polyclonal antibody (07-702, UBI) (D) FOXO3A mRNA levels, assessed by semiquantitative RT-PCR, in C/EBPα-ER-expressing K562 cells after treatment with 4-HT, alone or in the presence of CHX; HPRT expression was used as internal loading control; representative of two independent experiments. (E) Quantitative ChIP assays show binding of C/EBPα, C/EBPβ and C/EBPα-C/EBPβ chimeric proteins to a segment (nucleotides -655 to -453) of the FOXO3a promoter containing a putative C/EBP binding site (nucleotides -608 to -599) detected by real time Q-PCR. Error bars denote standard deviations of the means of one representative experiment (of two) performed in triplicate.

Figure 7. Suppression of FOXO3a expression blocks the apoptosis-inducing effect of C/EBPα. (A) FOXO3A mRNA levels, assessed by real time Q-PCR, in 4-HT-treated scramble or FOXO3A shRNA-transfected K562 cells (MigRI-transduced or C/EBPα-ER-expressing); HPRT expression was used as internal control; error bars denote standard deviations of normalized means of one representative experiment (of three) performed in triplicate. (B) Frequency of apoptotic cells (mean plus SD, three experiments) in scrambled or shRNAFOXO3A-transfected K562 cells (MigRI-transduced or C/EBPα-ER-expressing) after treatment with 0.2 μM IM alone or with 4-HT (250 nM) assessed by propidium iodide percentage after propidium iodide staining. * p<0.05 relative to corresponding 4-HT-untreated sample.
Table 1. Frequency of undifferentiated and differentiated myeloid cells in 4-HT treated C/EBP-ER transduced K562 cells.

|              | C/EBPα-ER | T0  | 24h | 48h | 72h |
|--------------|------------|-----|-----|-----|-----|
| Blasts       | 75.7% (318)| -   | -   | -   | -   |
| Promyelocytes| 15.7% (66) | 13.9% (36) | - | - | - |
| Myelocytes   | 8.5% (36)  | 30.3% (78) | 12% (25) | - | - |
| Metamyelocytes| -         | 25.6% (66) | 26% (54) | 20.3% (66) | - |
| Bands        | -          | 21.3% (55) | 37.5% (78) | 45.5% (148) | - |
| Segments     | -          | 8.9% (23)  | 24.5% (51) | 34.2% (111) | - |
| Total # of cells | 100% (420) | 100% (258) | 100% (208) | 100% (325) | - |

|              | C/EBPβ-ER | T0  | 24h | 48h | 72h |
|--------------|------------|-----|-----|-----|-----|
| Blasts       | 73.2% (238)| 14.3% (49) | 14.2% (39) | 34.3% (141) | - |
| Promyelocytes| 16.6% (54) | 46.5% (159) | 30.4% (90) | 44% (181) | - |
| Myelocytes   | 9.6% (31)  | 28.4% (97) | 39% (117) | 12.9% (53) | - |
| Metamyelocytes| 0.6% (2)  | 8.8% (30)  | 14% (43)  | 8.8% (36) | - |
| Bands        | -          | 2% (7)     | 1.7% (5)  | - | - |
| Segments     | -          | -          | 0.7% (2)  | - | - |
| Total # of cells | 100% (325) | 100% (342) | 100% (296) | 100% (411) | - |

|              | N-C/EBPα+ C/EBPβ-DBD-ER | T0  | 24h | 48h | 72h |
|--------------|-------------------------|-----|-----|-----|-----|
| Blasts       | 69.2% (275)            | -   | 0.3% (1) | - | - |
| Promyelocytes| 17.9% (71)             | 5.3% (15) | 0.3% (1) | 0.9% (2) | - |
| Myelocytes   | 11.9% (47)             | 16.4% (47) | 5.5% (16) | 0.4% (1) | - |
| Metamyelocytes| 1% (4)               | 30.6% (88) | 45.3% (132) | 10% (23) | - |
| Bands        | -                      | 35.9% (103) | 31.8% (93) | 55.7% (128) | - |
| Segments     | -                      | 11.8% (34) | 16.8% (49) | 33% (76) | - |
| Total # of cells | 100% (397)          | 100% (287) | 100% (292) | 100% (230) | - |

|              | N-C/EBPα+ C/EBPβ-DBD-ER | T0  | 24h | 48h | 72h |
|--------------|-------------------------|-----|-----|-----|-----|
| Blasts       | 62.4% (164)            | 16.8% (55) | 28.8% (79) | 38.4% (119) | - |
| Promyelocytes| 25.8% (68)             | 32.4% (106) | 35% (96) | 46.3% (144) | - |
| Myelocytes   | 10.7% (28)             | 34.6% (113) | 26.6% (73) | 9.7% (30) | - |
| Metamyelocytes| 1.1% (3)              | 12.5% (41) | 8.4% (23) | 5.6% (17) | - |
| Bands        | -                      | 3.7% (12)  | 1.2% (3)  | - | - |
| Segments     | -                      | -          | -          | - | - |
| Total # of cells | 100% (263)           | 100% (327) | 100% (274) | 100% (310) | - |
Figure 3

A

Fold activation on GCSF-R reporter plasmid (relative to MigR1 vector)

C/EBPα-ER  C/EBPβ-ER  N-C/EBPβ+  C/EBPβ-DBD-ER

p<0.05  p<0.05

B

C/EBPα-ER

C/EBPβ-ER

N-C/EBPα+  C/EBPβ-DBD-ER

N-C/EBPβ+  C/EBPα-DBD-ER

% of Input

IgG  NoAb  α-ER

IgG  NoAb  α-ER

IgG  NoAb  α-ER

IgG  NoAb  α-ER

% of Input

Control  4-HT

Control  4-HT

Control  4-HT

Control  4-HT

IP  α-ER  Total lysate

C/EBPα-ER  C/EBPβ-ER  N-C/EBPα+  N-C/EBPβ+
Figure 4

A

N-VP16-C/EBPα-DBD
N-VP16-C/EBPβ-DBD

B

C

Fold activation on GCSF-R reporter plasmid (relative to MigRI basic vector)
The biological effects of C/EBPα in K562 cells depend on the potency of the N-terminal regulatory region not on specificity of the DNA binding domain.

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