

Amendment history:

- [Corrigendum](#) (January 2013)

C/EBP γ deregulation results in differentiation arrest in acute myeloid leukemia

Meritxell Alberich-Jordà, ... , Ruud Delwel, Daniel G. Tenen

J Clin Invest. 2012;122(12):4490-4504. <https://doi.org/10.1172/JCI65102>.

Research Article

Oncology

C/EBPs are a family of transcription factors that regulate growth control and differentiation of various tissues. We found that C/EBP γ is highly upregulated in a subset of acute myeloid leukemia (AML) samples characterized by C/EBP α hypermethylation/silencing. Similarly, C/EBP γ was upregulated in murine hematopoietic stem/progenitor cells lacking C/EBP α , as C/EBP α mediates C/EBP γ suppression. Studies in myeloid cells demonstrated that *CEBPG* overexpression blocked neutrophilic differentiation. Further, downregulation of *Cebpg* in murine *Cebpa*-deficient stem/progenitor cells or in human *CEBPA*-silenced AML samples restored granulocytic differentiation. In addition, treatment of these leukemias with demethylating agents restored the C/EBP α -C/EBP γ balance and upregulated the expression of myeloid differentiation markers. Our results indicate that C/EBP γ mediates the myeloid differentiation arrest induced by C/EBP α deficiency and that targeting the C/EBP α -C/EBP γ axis rescues neutrophilic differentiation in this unique subset of AMLs.

Find the latest version:

<https://jci.me/65102/pdf>





C/EBP γ deregulation results in differentiation arrest in acute myeloid leukemia

Meritxell Alberich-Jordà,^{1,2} Bas Wouters,³ Martin Balastik,^{2,4} Clara Shapiro-Koss,^{1,3} Hong Zhang,^{1,2} Annalisa DiRuscio,^{1,2} Hanna S. Radomska,² Alexander K. Ebralidze,^{1,2} Giovanni Amabile,^{1,2} Min Ye,^{1,2} Junyan Zhang,^{1,2} Irene Lowers,³ Roberto Avellino,³ Ari Melnick,⁵ Maria E. Figueroa,⁶ Peter J.M. Valk,³ Ruud Delwel,³ and Daniel G. Tenen^{1,7}

¹Harvard Stem Cell Institute, Harvard Medical School, Boston, Massachusetts, USA. ²Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA. ³Erasmus University Medical Center, Rotterdam, The Netherlands.

⁴Institute of Molecular Genetics of the ASCR, Prague, Czech Republic. ⁵Weill Cornell Medical College, New York, New York, USA.

⁶University of Michigan, Ann Arbor, Michigan, USA. ⁷Cancer Science Institute, National University of Singapore, Singapore.

C/EBPs are a family of transcription factors that regulate growth control and differentiation of various tissues. We found that C/EBP γ is highly upregulated in a subset of acute myeloid leukemia (AML) samples characterized by C/EBP α hypermethylation/silencing. Similarly, C/EBP γ was upregulated in murine hematopoietic stem/progenitor cells lacking C/EBP α , as C/EBP α mediates C/EBP γ suppression. Studies in myeloid cells demonstrated that *CEBPG* overexpression blocked neutrophilic differentiation. Further, downregulation of *Cebpg* in murine *Cebpa*-deficient stem/progenitor cells or in human *CEBPA*-silenced AML samples restored granulocytic differentiation. In addition, treatment of these leukemias with demethylating agents restored the C/EBP α -C/EBP γ balance and upregulated the expression of myeloid differentiation markers. Our results indicate that C/EBP γ mediates the myeloid differentiation arrest induced by C/EBP α deficiency and that targeting the C/EBP α -C/EBP γ axis rescues neutrophilic differentiation in this unique subset of AMLs.

Introduction

The temporal and spatial control of cell-specific transcription factors and their abundance determines to a large extent the cell fate. The hematopoietic system, due to its well characterized cell hierarchy and well defined cell types, has been extensively used to study the function of transcription factors in cell renewal and differentiation (1, 2). Balancing these 2 processes is crucial for homeostasis, and defects in lineage-specific transcription factors, often caused by translocations or mutations, have been described in human leukemia (3–5).

CCAAT/enhancer-binding proteins (C/EBPs) are a family of basic region leucine zipper DNA-binding proteins of which 6 core members have been identified: C/EBP α , C/EBP β , C/EBP γ , C/EBP δ , C/EBP ϵ , and C/EBP ζ (5–8). In the hematopoietic system, C/EBP α plays a crucial role in the commitment of multipotent progenitor cells into the myeloid lineage, and mice deficient in C/EBP α are characterized by a specific block in the transition from common myeloid progenitors (CMP) to granulocyte-macrophage progenitors (GMP), resulting in the lack of mature and functional granulocytes (9, 10). Defects in *CEBPA*, such as differentiation-deficient mutations, posttranscriptional modifications, posttranslational inhibition, and epigenetic regulation, have been shown to inhibit C/EBP α function in acute myeloid leukemia (AML) (11–17). C/EBP β plays a role in macrophage activation (18) and is required during emergency granulopoiesis following administration of cytokines, a process that involves rapid production of granulocytes (19). C/EBP ϵ , C/EBP δ , and C/EBP ζ have also been implicated at different stages of granulocytic development (20, 21), and aberrant expression of these C/EBP members has been observed in AML and secondary granule deficiency syndromes (22–25). Thus, C/EBP γ is the only C/EBP member that has not been studied in the context of myeloid differentiation or human AML.

C/EBP γ is ubiquitously expressed and was first identified by its affinity for *cis*-regulatory sites in the immunoglobulin heavy chain promoter and enhancer (26). Structurally, C/EBP γ is characterized by the absence of transactivation domains, although it retains the basic region and the leucine zipper domains, required for DNA binding and homo/heterodimerization, respectively (27, 28). C/EBP γ alone neither activates nor represses transactivation; however, C/EBP γ can inhibit transcriptional activation by other C/EBP members in a cell-specific manner (27, 29). C/EBP γ -KO mice show a high mortality rate within 48 hours after birth, and analysis of the lymphoid lineage demonstrated that C/EBP γ plays a critical role in the functional maturation of natural killer cells (30). In addition, overexpression of C/EBP γ in transgenic mice causes a block in definitive erythropoiesis, leading to severe anemia and fetal lethality (31).

In the present study, we identify a unique subset of AML patients with increased *CEBPG* expression levels and methylation/silencing of the *CEBPA* gene. We demonstrate that C/EBP γ is a suppressor of myeloid differentiation in these AML samples and that targeting the C/EBP α -C/EBP γ axis represents a novel therapeutic approach in this particular subtype of AML.

Results

CEBPG is upregulated in a specific subset of AML patients with *CEBPA* promoter hypermethylation and silencing. In order to determine whether *CEBPG* is aberrantly expressed in certain cases of AML, we analyzed the gene expression profile (GEP) of 526 AML cases. This analysis revealed that *CEBPG* mRNA was significantly upregulated in a small subset of patients ($n = 8$) (Figure 1A). The 526 AML cases include a previously reported cohort of $n = 285$ samples (32), and as found in the original study, unsupervised clustering of the 526 cases defined a group of patients characterized by defects in *CEBPA* (Figure 1A). The defects in *CEBPA* were caused by either *CEBPA* biallelic mutations ($n = 21$) or *CEBPA*

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J Clin Invest.* 2012;122(12):4490–4504. doi:10.1172/JCI65102.

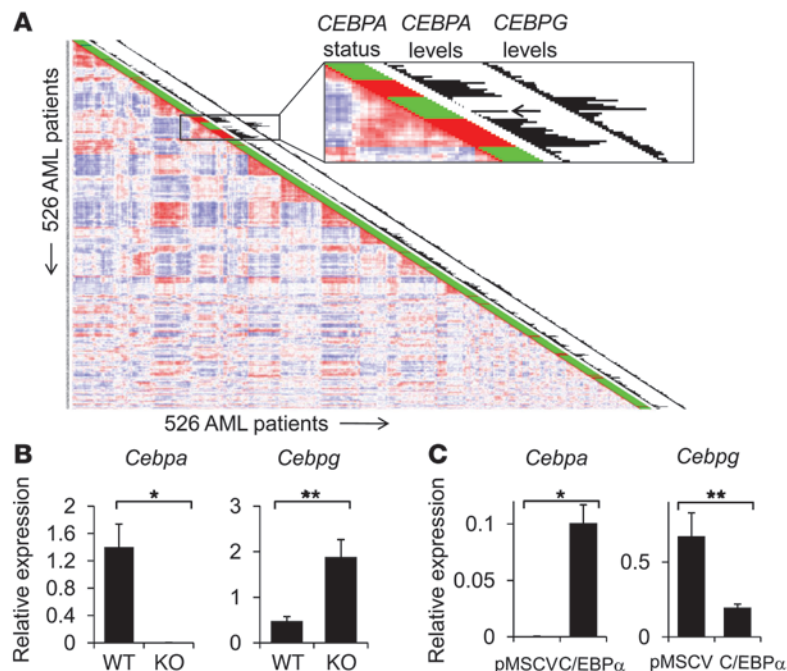


Figure 1
CEBPG RNA is upregulated in the absence of **CEBPA** in a subset of human AML and in murine **C/EBPα**-deficient hematopoietic stem/progenitor cells. **(A)** Pairwise correlations between GEPs of 526 AML samples hybridized to the Affymetrix HGU133Plus 2.0 GeneChips identifies a subset with high **CEBPG** and low **C/EBPA** levels. The rectangle amplifies a cluster that gathers samples with defects in **CEBPA**. The bar and histograms next to each sample indicate the following: **CEBPA** status (mutations are shown in red and silencing in green), **CEBPA**, and **CEBPG** expression levels. In total, 8 cases present high **CEBPG** levels and **CEBPA** silencing due to hypermethylation. The single case with a blue arrow is a patient without **CEBPA** mutation but with very high **CEBPA** mRNA expression levels and without **CEBPG** mRNA expression. As this case shows a very similar gene expression signature, we propose this represents another defect within the **C/EBPα/C/EBPγ** “pathway.” **(B)** Quantitative RT-PCR analysis in murine sorted LKS cells from **Cebpa^{Flox/Flox} cre⁻** (WT) and **Cebpa^{Flox/Flox} cre⁺** (KO) mice. **Cebpa** and **Cebpg** mRNA expression levels were determined as percentage of *Gapdh* (relative expression) and represent the average value plus SD of at least 5 mice in each group. * $P = 1.1 \times 10^{-7}$; ** $P = 1.6 \times 10^{-6}$. **(C)** **C/EBPα**-KO LKS cells were transduced with either pMSCV empty vector or **C/EBPα** pMSCV expression construct. **Cebpa** and **Cebpg** mRNA expression levels were determined in GFP⁺ cells 1 days after transduction. Data are the average of 3 independent experiments expressed as percentage of *GAPDH* plus SD. * $P = 0.00045$; ** $P = 0.0065$.

silencing, primarily due to hypermethylation ($n = 9$) (Figure 1A and refs. 17, 33). These later cases showed very low or no **CEBPA** mRNA levels compared with all other AML cases (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI65102DS1), and **C/EBPα** protein could not be detected (17). In addition, it was reported that these leukemias highly express *Trib2*, a protein that degrades **C/EBPα** p42 protein, contributing to the absence of **C/EBPα** in the silenced cases (34). Interestingly, **CEBPG** expression levels were particularly high in 8 out of 9 cases characterized by **CEBPA** silencing in comparison with the other AML samples, normal bone marrow, and CD34⁺ specimens (Figure 1A). We verified these changes in **CEBPG** RNA levels by quantitative RT-PCR (Supplemental Figure 1B). In summary, we identified a subset of AML patients characterized by silencing of **CEBPA** and upregulation of **CEBPG**.

Loss of C/EBPα in vivo results in upregulation of Cebpg in early stem/progenitor cells. Since we identified a subgroup of AML patients characterized by silencing of **C/EBPα** and upregulation of **CEBPG**, we asked whether ablation of *Cebpa* in vivo would lead to an increase of *Cebpg* expression, in particular in the early hematopoietic stem/progenitor cells (lineage⁻c-kit⁺ Sca-1⁺ [LKS]). For these experiments we used a well defined *Cebpa* conditional KO model previously developed in our laboratory (10). *Cebpa^{Flox/Flox} Mx1Cre⁺* conditional KO mice and *Cebpa^{Flox/Flox} Mx1Cre⁻* control mice (WT) were treated with polyinosinic-polycytidylic acid (pI:pC) to induce excision of the single exon *Cebpa* gene (10). Quantitative RT-PCR analysis revealed that KO LKS cells lost detectable expression of *Cebpa* ($P = 1.1 \times 10^{-7}$) and that the *Cebpg* transcripts had increased significantly compared with control LKS ($P = 1.6 \times 10^{-6}$) (Figure 1B). Further, we determined that LKS cells from *Cebpa* heterozygous mice (*Cebpa^{Flox/+} Mx1Cre⁺*) did not present increased *Cebpg* levels compared with the *Cebpa^{Flox/+} Mx1Cre⁻* control mice upon pI:pC treatment (Supplemental Figure 1C). Reintroduction of **C/EBPα** into **C/EBPα**-KO LKS cells using retroviral infection significantly reduced *Cebpg* expression ($P = 0.0065$) (Figure 1C). Similarly, reintroduction of the **C/EBPα** p30 isoform also diminished the *Cebpg* mRNA levels ($P = 0.034$) (Supplemental Figure 1D). In summary, our data suggest that **C/EBPα** negatively regulates *Cebpg* expression not only during later stages of granulopoiesis, but also in murine hematopoietic stem/progenitor cells. Further, the *Cebpa^{Flox/Flox} Mx1Cre⁺* conditional KO mice mimic the human AMLs characterized by silencing of **C/EBPα** and upregulation of *Cebpg*, providing an excellent model to study the contribution of **C/EBPγ** to the development of those leukemias.

C/EBPα binds to the CEBPG promoter. Since we observed that **CEBPG** and **C/EBPA** expression levels are inversely correlated in a subset of AML patient samples and in the *Cebpa* conditional KO mice, we questioned whether **CEBPG** repression is directed by **C/EBPα**. First, we studied **C/EBPα** interaction with *Cebpg* regulatory regions in murine 32D/G-CSF-R cells transduced with a β-estradiol-inducible (E_2)

C/EBPα-ER expression construct. When stimulated with E_2 to induce nuclear translocation of **C/EBPα**, these cells differentiate toward neutrophils within 3–4 days of culture, even in the presence of IL-3 (data not shown). After 4 hours of treatment, we performed ChIP followed by promoter array analysis and identified 4826 chromosomal regions, sized between 310 and 7399 nucleotides (median 1084), enriched in **C/EBPα**-ER cells compared with ER control cells at $P < 1 \times 10^{-5}$ (false discovery rate 2.3%). The transcriptional start site of 1064 unique RefSeq genes was located within 1 kb downstream of 1 or more of the 4826 fragments. Among those, we identified previously reported genes to be directly or indirectly regulated by **C/EBPs**, such as *Mpo* (35), *Hp* (36), *C3* (36), *PU.1* (37), and *IL-6α* (38) (data not shown). A strong interaction with the *Cebpg* promoter was observed (Figure 2A), whereas almost none of the other loci in that region appeared to

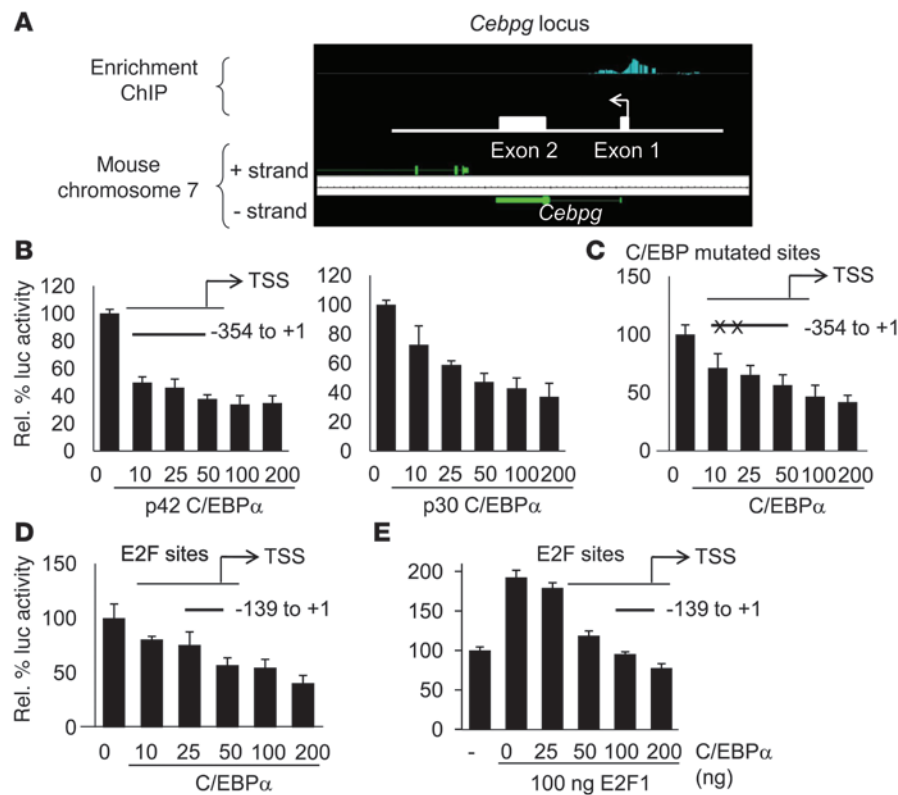


Figure 2 C/EBP α interacts with the *Cebpg* promoter and mediates *Cebpg* repression. (A) ChIP on DNA promoter microarrays (ChIP on chip). 32D/G-GSF-R-C/EBP α -ER cells or ER-expressing cells were treated with E $_2$ for 4 hours. The diagram shows an amplified view of the *Cebpg* locus in chromosome 7. Genes (including *Cebpg*) are indicated in green, either on the plus or on the minus strand. Specific hybridization was observed (multiple probes in blue) to the representative *Cebpg* promoter region (– strand), whereas almost none of the other loci in that region appeared to be enriched by ChIP. (B) C/EBP α represses *CEBPG* promoter-driven luciferase activity. 293T cells, which endogenously express C/EBP γ , were cotransfected with 100 ng *CEBPG* luciferase reporter vector (–354 to +1) and increasing amounts of a C/EBP α expression plasmid, encoding for either the p42 or p30 form. (C) Mutation of the C/EBP-binding sites in the *CEBPG* luciferase reporter vector does not affect C/EBP α repression. 100 ng of the mutated reporter vector were cotransfected with increasing amounts of p42 C/EBP α plasmid. (D) C/EBP α suppresses *CEBPG* promoter activity of a short *CEBPG* reporter construct. 100 ng of a reporter construct containing 3 E2F-binding sites were cotransfected with different amounts of C/EBP α expression plasmid. (E) C/EBP α reduces E2F1 transactivation of the C/EBP γ promoter. 100 ng of the short C/EBP γ reporter construct was cotransfected with 100 ng E2F1 expression construct and increasing amounts of C/EBP α expression plasmid. The y axis indicates the relative percentage of luciferase activity. Results represent the average of at least 3 independent experiments; bars indicate SD.

be enriched by ChIP. Next, ChIP was performed in 32D/G-CSF-R cells to determine whether endogenous C/EBP α could immunoprecipitate *Cebpg* promoter. Similarly to the C/EBP α -ER over-expressing system, we observed immunoprecipitation of *Cebpg* promoter by endogenous C/EBP α (Supplemental Figure 2A), but not of a control upstream region (data not shown). To further investigate the binding of C/EBP α to the *CEBPG* promoter, we used K562 cells stably transfected with C/EBP α -ER as a model for human granulocytic differentiation (39). We identified a putative C/EBP α -binding site in the human *CEBPG* proximal promoter, which was highly conserved between mouse and human (–871 bp to –762 bp, oligos A, corresponding to the region amplified by oligos no. 2 in the murine promoter, Supplemental Figure 2B). ChIP assays showed binding of C/EBP α to this region (oligos A),

but not to a control upstream region (oligos B) (Supplemental Figure 2, C and D). Together, these results demonstrate that C/EBP α can bind to the proximal promoter of *CEBPG* and potentially down-regulate *CEBPG* expression.

C/EBP α mediates *CEBPG* repression by affecting *E2F1* transcriptional activity. In order to determine whether C/EBP α could negatively regulate *CEBPG* promoter, we performed luciferase reporter assays. We generated a human *CEBPG* promoter construct (–354 bp to +1 bp from TSS, Supplemental Figure 2B) in the pXP2 reporter vector and performed luciferase assays to analyze *CEBPG* promoter activity. Cotransfection of the *CEBPG* reporter construct with increasing amounts of a C/EBP α expression plasmid showed that C/EBP α was able to repress *CEBPG* promoter reporter gene activity (Figure 2B). Of note, both a p42-C/EBP α plasmid and a p30-C/EBP α plasmid inhibited the *CEBPG* reporter gene activity in a dose-dependent manner and with similar efficiency (Figure 2B). As a positive control for C/EBP α transactivation, we cotransfected the C/EBP α expression vector with a reporter for the gene encoding the G-CSF receptor (*CSF3R*), denoted here as the G-CSF-R reporter. Luciferase assays showed that C/EBP α transactivated the G-CSF-R reporter gene, indicating that negative regulation by C/EBP α is specific to the *CEBPG* promoter (data not shown). Based on the AML microarray (Figure 1A) C/EBP γ is only upregulated in the C/EBP α silenced leukemias, suggesting that mutated C/EBP α proteins are still capable of repressing C/EBP γ expression. Luciferase assays with the C/EBP γ reporter construct and increasing amounts of distinct mutated C/EBP α expression plasmids showed that the N-terminal mutants repress C/EBP γ luciferase activity (Supplemental Figure 2E). These results demonstrate that WT p42 and p30 C/EBP α forms as well as N-terminal mutant C/EBP α proteins are able to negatively regulate the C/EBP γ promoter.

To identify the *cis*-acting elements on the *CEBPG* promoter that respond to C/EBP α , we first compared 2 human *CEBPG* reporter constructs: one corresponding to –957 bp to +1 bp of the *CEBPG* proximal promoter and the other corresponding to a shorter version (from –354 bp to +1 bp), which is missing the first 2 potential C/EBP-binding sites (Supplemental Figure 2B). Luciferase assays with increasing amounts of C/EBP α expression vector showed similar C/EBP α -mediated repression in both C/EBP γ reporter constructs, indicating that the first 2 potential C/EBP-binding sites are not necessary for the repression (data not shown). Next, we investigated whether the other 2 potential C/EBP-binding sites present in

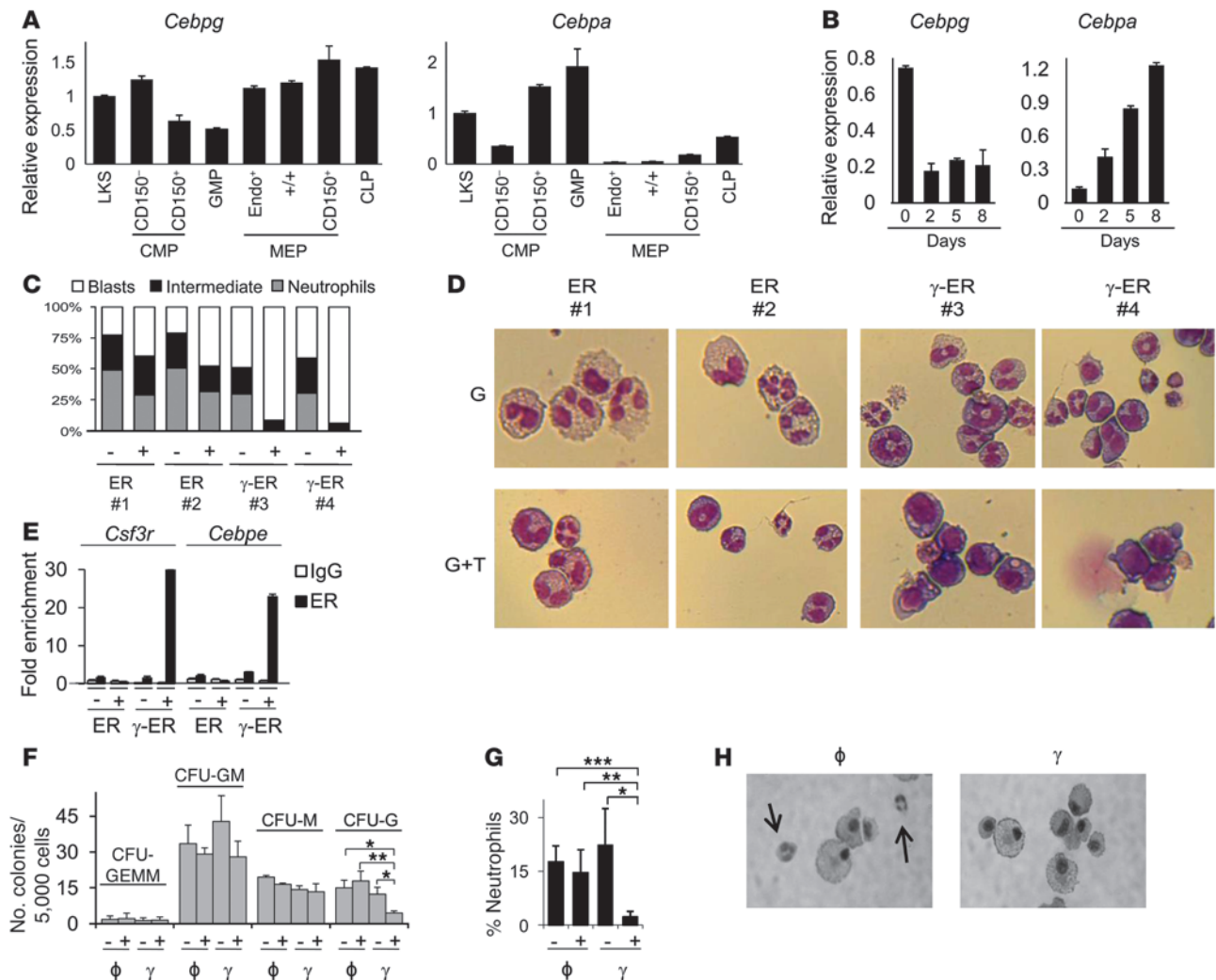


Figure 3

C/EBP γ is downregulated with neutrophilic differentiation, whereas constitutive expression of *C/EBP γ* blocks G-CSF–induced neutrophilic differentiation. (A) *Cebpg* and *Cebpa* RNA in murine bone marrow–sorted populations: LKS, CD150⁺CMP, CD150⁻CMP, GMP, MEP endo⁺ (endoglin⁺ CD150⁻), MEP ^{+/+} (endoglin⁺ CD150⁺), MEP CD150⁺ (endoglin⁻ CD150⁺) CLP(78). Average and SD of 3 independent experiments. Relative expression calculated as percentage of *Gapdh*. (B) *Cebpg* and *Cebpa* expression in 32D/G-CSF–R cells during G-CSF–induced differentiation. The y axis indicates the relative expression as percentage of *Gapdh*. (C) Differential counts of 2 ER and 2 *C/EBP γ -ER* 32D/G-CSF–R– expressing clones cultured in G-CSF with (+) or without (–) 4-hydroxytamoxifen (8 days). (D) Morphologic analysis of May-Grunwald-Giemsa–stained cytopins of 2 ER and 2 *C/EBP γ -ER* clones (day 8) cultured in G-CSF (G) with or without 4-hydroxytamoxifen (T). (E) *C/EBP γ* binds to the proximal promoter of *Csf3r* (encoding G-CSF–R) and *Cebpe*. ChIP on *C/EBP γ -ER* or ER cells stimulated with (+) or without (–) 4-hydroxytamoxifen. The y axis represents binding fold enrichment. (F) Quantification of CFU-GEMM, CFU-GM, CFU-M, and CFU-G in semi-solid cultures (day 12). Lineage-depleted murine bone marrow cells infected with MSCV empty vector (ϕ) or *C/EBP γ* MSCV (γ) were sorted into GFP⁻ (–) or GFP⁺ (+). The y axis indicates the number of colonies per 5000 input cells. Average and SD of 3 independent experiments. **P* = 0.001; ***P* = 0.0004. (G) Differential counting of cytopun semi-solid cultures. The y axis indicates the percentage of mature neutrophils present in each condition. Average and SD of 3 independent experiments. **P* = 0.028; ***P* = 0.031, ****P* = 0.005. (H) Cell morphology on cytopins of sorted GFP⁺ infected cells. Original magnification, \times 100.

the shorter reporter construct were necessary to repress the luciferase activity. We therefore generated a *CEBPG* reporter construct with mutated *C/EBP*-binding sites in the context of the shorter reporter. These 2 *C/EBP* sites appeared not to be required, since *C/EBP α* was still able to repress the luciferase activity (Figure 2C). Thus *C/EBP α* represses either through other binding sites and/or in an indirect manner, through interaction with other transcriptional regulators. In fact, we and others have previously reported that *C/EBP α* can act as a transcriptional repressor through E2F-

binding sites (40, 41). We determined the presence of 3 E2F-binding sites in the *C/EBP γ* luciferase constructs (Supplemental Figure 2B). To determine whether the E2F sites were required for *C/EBP α* repression, we generated a luciferase construct containing solely these 3 sites (–139 bp to +1 bp from TSS) and observed that *C/EBP α* could still significantly repress luciferase activity (Figure 2D). Deletion or mutation of these sites (Supplemental Figure 2F) resulted in a lack of reporter activity, pointing to a critical role of E2F in transcriptional control of *CEBPG* (data not shown). Several

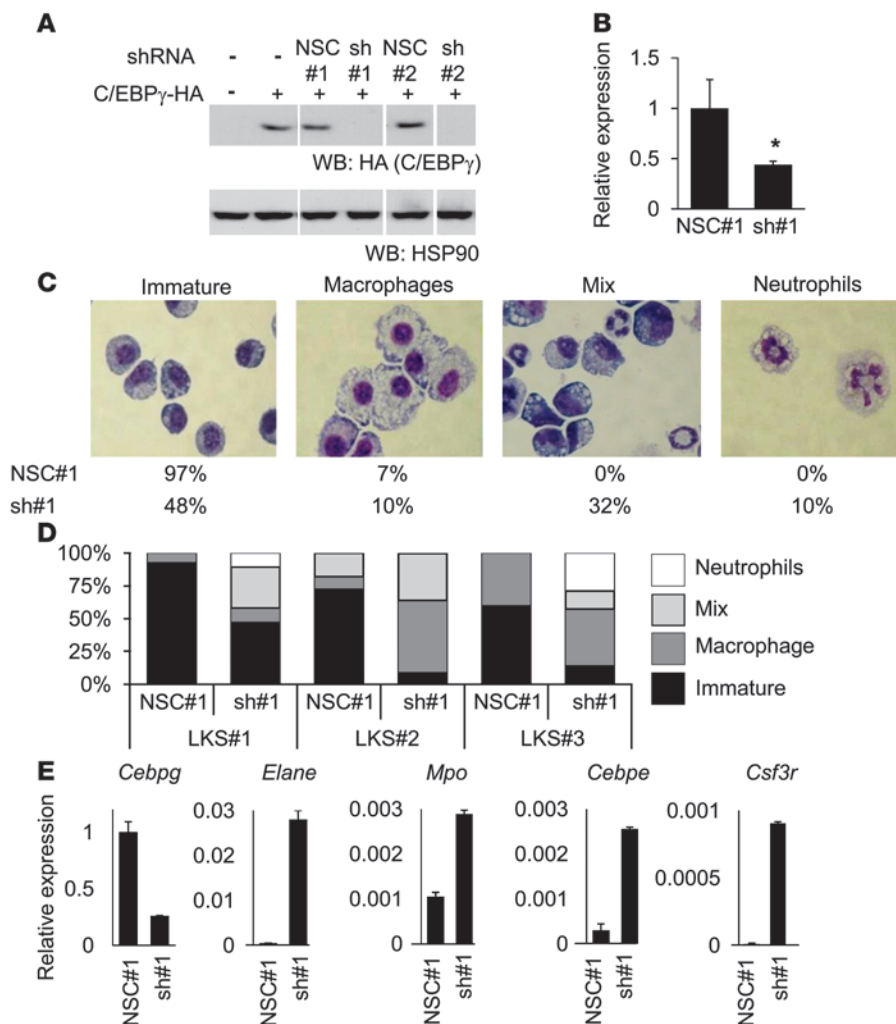


Figure 4

Downregulation of C/EBP γ in murine C/EBP α -KO LKS cells restores neutrophilic differentiation in cell culture. (A) Western blot analysis on 293T cells transfected with or without C/EBP γ -HA in combination with shRNA-expressing constructs. sh, shRNA targeting C/EBP γ . Blots were stained with HA and HSP90 (loading control) antibodies. (B) LKS cells from pl:pC-treated *Cebpa*^{Flox/Flox} cre⁺ (KO) mice were transduced with either NSC#1 or C/EBP γ shRNA#1 (sh#1). *Cebpg* mRNA expression was determined 2 days after infection. The y axis indicates relative *Cebpg* levels expressed as percentage of *Gapdh* plus SD in 2 independent mice, $P = 0.0042$. (C) C/EBP α -KO LKS cells were infected with NSC#1 or C/EBP γ shRNA#1 (sh#1) and seeded in semi-solid medium in the presence of puromycin. 12 days after culture, individual colonies were picked up and cell morphology was analyzed. Immature colonies contained blast and myeloblast; macrophage colonies contained mainly monocytes and macrophages; mix colonies contained immature cells, monocytemacrophages, and neutrophils; and neutrophilic colonies had only neutrophils. Original magnification, $\times 40$. (D) Percentage of the distinct colony types determined by cell morphology analysis in individual colonies from NSC#1- or sh#1-infected C/EBP α -KO LKS cells 12 days after culture. 3 independent experiments are shown (LKS#1, 2, and 3). (E) Quantitative RT-PCR was performed in NSC#1- and sh#1-infected C/EBP α -KO LKS cells 12 days after semi-solid culture (cultures shown in D). Expression of *Cebpg*, neutrophil elastase (*Elane*), *Mpo*, *Cepbe*, and G-CSF-R (*Csf3r*) was determined in 2 independent mice. The y axis indicates relative expression in 1 representative mouse as percentage of *Gapdh* plus SD.

mechanisms may explain how C/EBP α regulates *CEBPG* through these E2F-binding sites. First, we assessed whether E2F binding to the *CEBPG* promoter could be affected by C/EBP α . ChIP performed on K562-C/EBP α ER cells at distinct time points upon E₂ treatment showed that C/EBP α binds to the proximal promoter of

CEBPG without displacing E2F1 (Supplemental Figure 2G). This result is in agreement with a previous publication by Johansen et al. demonstrating that C/EBP α had no effect on E2F1 binding to a consensus E2F DNA-binding site (40). Alternatively, C/EBP α may interfere with E2F transcriptional activity and repress the *CEBPG* promoter. We demonstrated that E2F1 protein (but not other E2F family members) could transactivate the *CEBPG* promoter and that E2F1-induced reporter activity could be reduced by increasing amounts of C/EBP α expression plasmid (Figure 2E). Together, these results indicate that C/EBP α can bind to *CEBPG* proximal promoter and downregulate C/EBP γ expression by affecting E2F1 transcriptional activity.

Next, we investigated whether changes in chromatin structure, which regulate gene expression, would be present in the C/EBP α -KO cells versus WT. DNA methylation HpaII tiny fragment enrichment by ligation mediated PCR (HELP) arrays in lineage-c-kit⁺ murine bone marrow cells demonstrated no differences in the *Cebpg* DNA methylation status from KO versus WT mice (Supplemental Figure 2H). Similarly, HELP arrays in human AML patient samples showed no differences in the *CEBPG* DNA methylation profile of samples with *CEBPA* silencing and other AML subtypes (data not shown). Further, ChIP using H3K4me3 and H3K27me3 antibodies in lineage-c-kit⁺ cells demonstrated increased H3K4me3 enrichment in the *Cebpg* promoter of C/EBP α -KO compared with WT cells (Supplemental Figure 2I), whereas no difference was observed in the enrichment of the H3K27me3 mark (data not shown). Together, these experiments indicate that the active histone modification mark H3K4me3, but not changes in DNA methylation, contributes to the elevated *Cebpg* levels observed in the C/EBP α -KO cells.

Cebpg is expressed in murine stem/progenitor cells and downregulated in committed myeloid cells. To understand the role of C/EBP γ in the pathogenesis of AML, we first investigated the expression of

this transcription factor in normal hematopoiesis and determined *Cebpg* expression levels in distinct hematopoietic progenitor cell populations. RT-PCR showed that *Cebpg* transcripts are expressed in the LKS (lineage-c-kit⁺ Sca-1⁺) population, which is enriched in both long- and short-term HSCs. *Cebpg* expression is still high in

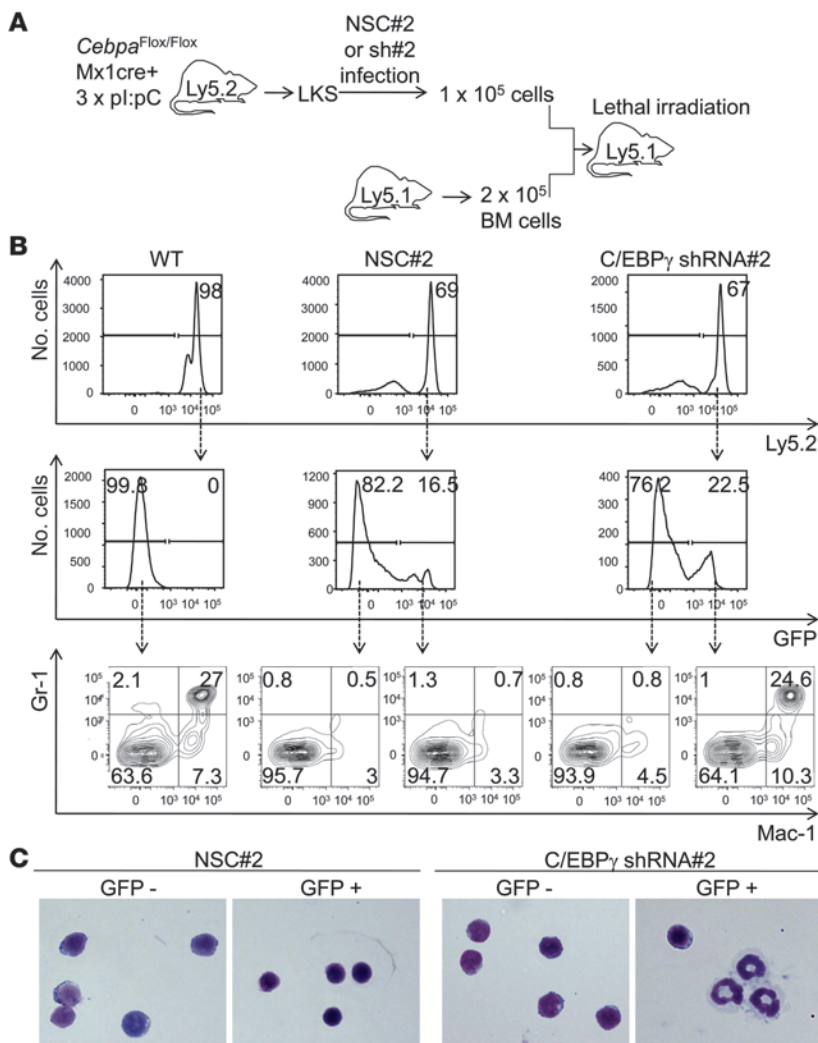


Figure 5 Downregulation of C/EBP γ in murine C/EBP α -KO LKS cells restores neutrophilic differentiation in vivo. **(A)** Schematic representation of the bone marrow transplantation experiment. **(B)** Sorted LKS cells from C/EBP α -KO mice were infected with either NSC#2 or C/EBP γ shRNA#2 (sh#2) and transplanted into lethally irradiated mice. 6 weeks after transplantation, recipient mice were bled and flow cytometry analysis was performed. Upper histogram panels are gated on Ly5.2⁺ cells (donor cells) and further divided in GFP⁻ (noninfected) and GFP⁺ (infected cells). Lower dot plot images show Gr-1/Mac-1 expression and indicate the percentage of double-positive cells. See also Supplemental Figure 5. **(C)** Cell morphology of peripheral blood mononuclear cells sorted according to Ly5.2⁺ and GFP levels. Original magnification, $\times 63$.

the CD150-CMP subset, but decreases significantly as cells commit to the myeloid lineage and reach the CD150⁺ CMP and GMP stages (Figure 3A). *Cebpg* mRNA levels are also high in megakaryocyte-erythroid progenitors (MEP), independently of endoglin and CD150 markers, and in common lymphoid progenitors (CLP).

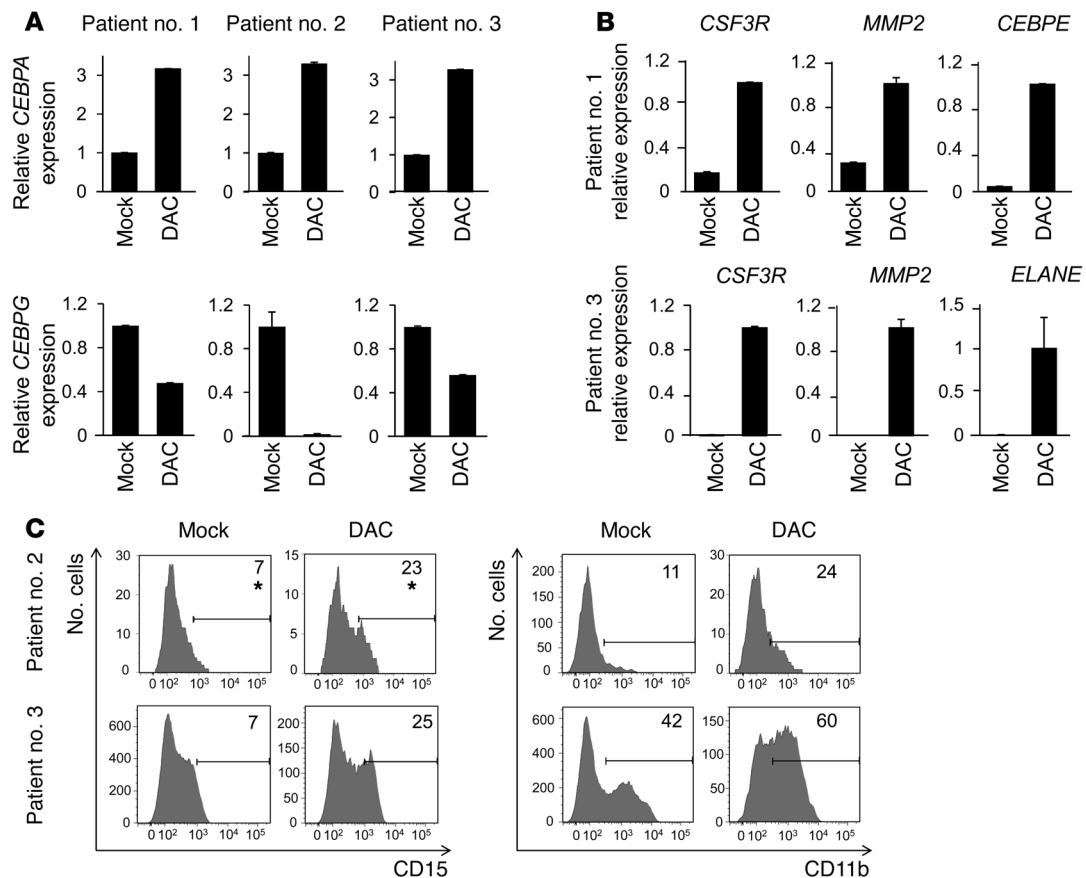
As C/EBP α is a crucial factor for myelopoiesis, we analyzed *Cebpa* expression in parallel in the same sorted populations. We observed that *Cebpa* is also expressed in LKS cells, however, with further lineage commitment, the expression of *Cebpa* and *Cebpg* followed a reciprocal pattern, showing upregulation of *Cebpa* in CD150⁺ CMP and GMP cells (Figure 3A and Supplemental Figure 3A). This inverse correlation was also seen in CD150-CMP and MEP, as well

as CLP, where *Cebpg* levels were increased and *Cebpa* reduced (Figure 3A and Supplemental Figure 3A). In summary, *Cebpg* is expressed at the earliest hematopoietic stem/progenitor stage and at highest levels in MEP and CLP, with relatively lower levels in myeloid cells.

C/EBP γ is downregulated in G-CSF-induced neutrophilic differentiation. To further investigate C/EBP γ expression during granulocytic differentiation, we made use of the 32D/G-CSF-R murine cell line model. 32D/G-CSF-R cells proliferate in the presence of IL-3 and can fully differentiate toward mature neutrophils upon G-CSF stimulation (42, 43). Quantitative RT-PCR and Western blot analysis demonstrated that C/EBP γ is highly expressed in 32D/G-CSF-R cells while proliferating under the control of IL-3 (day 0) (Figure 3B and Supplemental Figure 3B). This expression level rapidly decreases as cells differentiate in the presence of G-CSF (day 2 to 8). In contrast, C/EBP α expression levels are low in the presence of IL-3, but gradually increase during G-CSF-induced differentiation (Figure 3B and Supplemental Figure 3C). These data demonstrate that C/EBP γ and C/EBP α levels are inversely correlated during neutrophilic differentiation and that C/EBP γ is more abundant in immature cells, similar to the bone marrow-sorted subsets.

Overexpression of C/EBP γ induces a block of granulocytic differentiation. To investigate whether downregulation of C/EBP γ is required for granulocytic differentiation, we overexpressed C/EBP γ in the 32D/G-CSF-R system described above. We introduced a tamoxifen-inducible form of C/EBP γ (C/EBP γ -ER) into 32D/G-CSF-R cells. ER control clones (containing the estrogen receptor peptide without C/EBP γ) and C/EBP γ -ER-expressing clones were cultured in the presence of G-CSF with or without 4-hydroxytamoxifen. All clones cultured in the presence of G-CSF fully differentiated toward granulocytes; however, a complete block of differentiation was observed in the C/EBP γ -ER-expressing cells when cultured in the presence of G-CSF and 4-hydroxytamoxifen (Figure 3, C and D). Accordingly, upregulation of granulocytic markers such as myeloperoxidase (*Mpo*), *Cebpe*, and *Csf3r* (G-CSF-R) was observed when 32D/G-CSF-R/C/EBP γ -ER cells were cultured with G-CSF, but not when 4-hydroxytamoxifen was added to the cultures (Supplemental Figure 3D). ER control clones differentiated toward neutrophils when stimulated with G-CSF+4-hydroxytamoxifen and stopped proliferating after 4–5 days of culture, whereas the C/EBP γ -ER-expressing clones remained proliferative in the same conditions, with no signs of differentiation observed at any given time point (cultures were kept till day 19) (Supplemental Figure 3E).

To investigate the mechanism by which C/EBP γ promotes a block of granulocytic differentiation, we made use of the 32D/G-CSF-R cell line expressing either C/EBP γ -ER or the ER control alone. Cells were treated for 4 hours with 4-hydroxytamoxifen

**Figure 7**

DAC treatment restores the *C/EBP α -C/EBP γ* balance and promotes differentiation of primary human AML samples characterized by *C/EBP α* silencing and *C/EBP γ* upregulation in vitro. **(A)** Quantitative RT-PCR in 3 independent AML patient samples treated with either mock control or 1 μ M DAC. *CEBPA* and *CEBPG* expression are shown relative to *18S* expression. The y axis indicates fold change as compared with mock treatment. **(B)** DAC treatment promotes expression of myeloid differentiation markers in AML patient samples. Quantitative RT-PCR in 2 AML patient samples treated with either mock control or 1 μ M DAC. The y axis indicates relative expression of G-CSF-R (*CSF3R*), gelatinase A (*MMP2*), *CEBPE*, and neutrophil elastase (*ELANE*), calculated as relative to *GAPDH* expression. **(C)** *C/EBP α* silenced patient samples treated with DAC or mock control, and analyzed by flow cytometry. Histograms represent expression of CD15 and CD11b after 8 days of treatment (except those indicated by asterisk, which were analyzed after 6 days). Numbers indicate percentage of positive cells.

Morphological analysis on Wright-Giemsa-stained cytopins showed a more differentiated phenotype in cells lacking *C/EBP α* and downregulation of *C/EBP γ* (Supplemental Figure 4E). Quantitative RT-PCR was used to verify absence of *Cebpa* in those liquid cultures and downregulation of *Cebpg* in sh#1-infected cells in comparison with NSC#1-infected cells (Supplemental Figure 4, F-G, and data not shown). Similar experiments were performed using a retroviral vector (46) coexpressing another shRNA specifically targeting *Cebpg*, shRNA#2, and a GFP reporter (Figure 4A and Supplemental Figure 4, A and B). Semi-solid cultures of sorted GFP⁺ infected cells showed the presence of neutrophil-containing colonies when *C/EBP γ* was downregulated in *C/EBP α* -deficient LKS cells in comparison with the NSC#2 colonies (Supplemental Figure 4, H and I). Together, these results show that downregulation of *C/EBP γ* levels in murine *C/EBP α* -KO LKS cells is sufficient to restore neutrophilic differentiation in vitro. In addition, serial replating experiments suggest that downregulation of *C/EBP γ* restricts the increased self-renewal properties of *C/EBP α* -KO cells (10).

We next studied the effects of knocking down *C/EBP γ* in the absence of *C/EBP α* in vivo. For these experiments we made use of the shRNA retroviral vector containing a GFP reporter. *C/EBP α* -KO LKS cells were isolated and infected with retrovirus containing either shRNA#2, targeting *C/EBP γ* , or a control scrambled shRNA (NSC#2) and transplanted into lethally irradiated recipient mice (Figure 5A). Figure 5B shows flow cytometry data from 2 representative mice, transplanted with either sh#2- or NSC#2-infected cells. Plots were gated on Ly5.2⁺ cells (*C/EBP α* -KO cells), and further divided into GFP⁻ (noninfected) and GFP⁺ (infected) cells. We observed that mice transplanted with sh#2 had a high level of Gr-1/Mac-1⁺ cells (neutrophils) in the GFP⁺ fraction, whereas an absence of Gr-1/Mac-1⁺ cells was observed in the sh#2 GFP⁻ fraction or in the NSC#2 transplanted mice (Figure 5, B and C, and Supplemental Figure 5A) ($n = 8$ for NSC#2 and $n = 11$ for *C/EBP γ* shRNA#2). Of note, 9 out of 11 mice transplanted with *C/EBP γ* shRNA#2 contain granulocytes in the GFP⁺ fraction, whereas 0 out of 8 control mice did. In Supplemental Figure 5A, we measured the percentage of Gr-1/Mac-1⁺ cells present in blood from

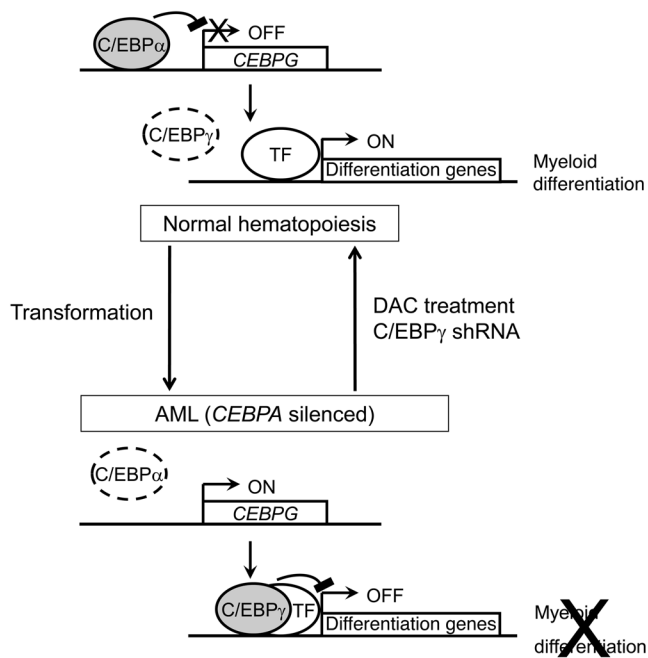


Figure 8

Proposed model. The upper panel summarizes the proposed model during normal hematopoiesis, and the lower panel represents the situation in a specific subset of AML patients (*CEBPA* silenced). In normal hematopoiesis, C/EBPα binds to the proximal promoter of *CEBPG* and represses its expression. Consequently, myeloid transcription factors drive expression of crucial genes required for neutrophilic differentiation, such as *CSF3R* (encoding for G-CSF-R) and *CEBPE*. In contrast, in a subset of AML patient samples characterized by *CEBPA* silencing due to promoter hypermethylation, we observed that absence of C/EBPα leads to upregulation of C/EBPγ. High C/EBPγ levels prevent transactivation of genes such as *CSF3R* and *CEBPE*, resulting in a neutrophilic differentiation block. DAC treatment to restore the C/EBPα-C/EBPγ balance or shRNA approaches to silence C/EBPγ can be applied and restore myeloid differentiation in these particular AMLs.

reconstituted mice 6 weeks after transplantation. At this time point, Ly5.2⁺ blood cells were sorted into GFP⁻ and GFP⁺ cells, and genomic DNA was extracted. Complete excision of *Cebpa*^{Flox/Flox} alleles was observed by PCR of genomic DNA in all transplanted animals and, accordingly, a PCR-specific excision band was detected (Supplemental Figure 5B). Further, GFP⁻ and GFP⁺ cells from NSC#2 and sh#2 transplanted mice gave rise to B220/IgM⁺ cells, indicating that downregulation of C/EBPγ in the absence of C/EBPα does not affect the production of B-lymphocytes (Supplemental Figure 6A). Animals were sacrificed 6–10 weeks after transplantation, and the absence of *Cebpa* was determined by RT-PCR in peripheral blood, total bone marrow, and LKS cells (Supplemental Figure 6, B and C, and data not shown). In summary, these transplantation experiments demonstrate that downregulation of C/EBPγ can rescue granulopoiesis in C/EBPα-KO mice, demonstrating that upregulation of C/EBPγ contributes to the myeloid differentiation arrest.

Downregulation of C/EBPγ rescues granulocytic differentiation in primary human AML cells. Since we have identified a subset of patient samples characterized by silencing of *CEBPA* and upregulation of *CEBPG* (Figure 1 and Supplemental Figure 1), we next investigated whether downregulation of C/EBPγ in human *CEBPA*-silenced AML samples would restore granulocytic differentiation. First, we designed and examined the effect of an shRNA sequence specifically targeting human *CEBPG* (Supplemental Figure 7, A and B). A *CEBPA*-silenced AML patient sample was then infected with either this C/EBPγ shRNA or an NSC shRNA lentivirus, and cells were transplanted into sublethally irradiated NSG mice. Analysis of the recipient bone marrow cells 16 weeks after transplantation revealed the presence of human CD15-positive cells exclusively in the C/EBPγ shRNA-infected GFP⁺ cells (Figure 6). Further, analysis of the GFP⁻ fraction in these recipients and the NSC transplanted mice showed absence of CD15 expression, although myeloid identity was observed by CD33 expression (Figure 6A). Morphological analysis of human CD45⁺GFP⁻ and CD45⁺GFP⁺ sorted cells showed the presence of mature neutrophils only in

the C/EBPγ shRNA GFP⁺ infected cells (Figure 6B). Similarly to the flow cytometry data, differential counting of the hCD45⁺ C/EBPγ infected cytospun cells showed 39% versus 1% mature neutrophils in the GFP⁺ and GFP⁻ fractions, respectively. These experiments indicate that downregulation of C/EBPγ in human *CEBPA*-silenced AML samples restores granulocytic differentiation in vivo.

DAC treatment restores the C/EBPα-C/EBPγ balance and promotes expression of myeloid markers in AML samples. Since a subset of the AML patient samples we identified are characterized by silencing of *CEBPA* due to promoter hypermethylation (refs. 17, 33, Figure 1, and Supplemental Figure 1), we next investigated whether these AML cases could benefit from treatment with demethylating agents. We observed that *CEBPA*-silenced AML samples treated with 1 μM 5-aza-2'-deoxycytidine (DAC) showed upregulation of *CEBPA* mRNA expression in comparison with mock control treatment (Figure 7A). In line with our observations above, we determined that this *CEBPA* upregulation results in a significant reduction of the *CEBPG* levels in DAC-treated cells (Figure 7A). Further, we observed that these changes in *CEBPA* and *CEBPG* expression in C/EBPα-silenced AML upon DAC treatment result in upregulation of myeloid differentiation markers such as *CSF3R*, Gelatinase A, *CEBPE*, and neutrophil elastase (Figure 7B). Similarly, flow cytometric analysis showed upregulation of mature granulocytic markers such as CD15 and CD11b upon DAC treatment in the C/EBPα-silenced AML (Figure 7C). In addition, AML patient samples lacking *CEBPA* promoter hypermethylation did not respond to DAC treatment, and neither *CEBPA* nor *CEBPG* levels significantly changed (Supplemental Figure 7C). Bisulfite sequencing demonstrated DAC induced demethylation of the *CEBPA* promoter (Supplemental Figure 7, D and E). Together, these data indicate that demethylating agents can restore the C/EBPα-C/EBPγ balance and promote myeloid differentiation in certain AML samples.

Discussion

Here, in a unique subset of AML defined by the absence of C/EBPα, we found increased *CEBPG* expression levels. Similar to these human AML cases, in *Cebpa* conditional KO mice, *Cebpa* ablation caused an upregulation of *Cebpg*, demonstrating a direct relation between *Cebpg* expression and absence of C/EBPα. We showed that overexpression of C/EBPγ in a cell-culture model of granulopoiesis and in murine bone marrow cells induces a neutrophilic differentiation arrest. Accordingly, in C/EBPα-KO mice and in *CEBPA* silenced human AML, both characterized by a block of neutrophilic development, downregulation of C/EBPγ was sufficient to



completely restore granulocytic differentiation (Figure 8). These data point to a role for C/EBP γ in impairment of granulocytic development in a subset of human AML. We therefore hypothesize that these leukemia patients could benefit from therapeutic approaches meant to reduce C/EBP γ levels. We also demonstrate that treatment of these AML patient samples with demethylating agents such as DAC results in hypomethylation and reexpression of C/EBP α , which, as we predicted based in our findings in the murine and cell line models, contributes to the downregulation of C/EBP γ and upregulation of myeloid differentiation markers. This specific subset of AML cases is characterized by an aberrant DNA hypermethylation signature (17, 33), and therefore we assume that the beneficial effect of DAC is not restricted to restoring the C/EBP α -C/EBP γ balance. However, our data support an important role of this balance in myeloid differentiation. Currently, demethylating agents are the standard of care for patients with high-risk myelodysplastic syndromes (MDS) (47, 48), reducing the risk of transformation to AML and improving overall survival. But although these agents are being used as therapeutic agents, very few specific gene targets have been identified, and certainly it is difficult to explain the effect on AML cells based on previously identified methylation targets (49, 50). Therefore, identification of the C/EBP α -C/EBP γ axis as a specific target of DAC contributes to our understanding of the mechanisms of action of these drugs.

It is intriguing to ask why *CEBPG* upregulation is restricted to AML patients with *CEBPA* methylation/silencing. If *CEBPG* upregulation is a direct result of defects in C/EBP α , we might expect that other AML subtypes characterized by C/EBP α inactivation would also present elevated *CEBPG* levels. On one hand, AML cases harboring differentiation-deficient mutations in *CEBPA* have increased expression of *CEBPA* and low *CEBPG* RNA levels. Here, we demonstrate that the N-terminal C/EBP α mutants and the C/EBP α p30 isoform, which is expressed and active in leukemias with mutations in *CEBPA* (14, 51), are able to suppress *Cebpg* upregulation and transactivation. On the other hand, FLT3-ITD- and AML1-ETO-positive leukemias present reduced C/EBP α levels and activity (14, 52), but do not show upregulation of *CEBPG*. Our data indicate that elevated *CEBPG* levels are directly related to the total absence of *CEBPA* expression exclusive of *CEBPA*-silenced leukemias, whereas in the FLT3-ITD and AML1-ETO cases, the *CEBPA* mRNA levels are reduced, but still present. Accordingly, we demonstrate that in *Cebpa*^{WT/Flox} Mx1Cre⁺ LKS cells, which harbor reduced *Cebpa* levels, *Cebpg* levels are not increased. Therefore, the critical difference between this particular subtype of AML and the rest of leukemias with C/EBP α inactivation is the absence of C/EBP α versus low or mutated C/EBP α . Nevertheless, we cannot exclude that a yet-unidentified factor in *CEBPA*-silenced leukemias also contributes to *CEBPG* upregulation. Our data support that modifications at the chromatin level also regulate murine C/EBP γ expression. It would be of interest to study whether regulation at this level also occurs in the distinct AML types.

We previously reported that activating mutations in *NOTCH1* are a key hallmark in *CEBPA*-silenced leukemias (17). These leukemias are phenotypically characterized by the simultaneous expression of myeloid as well as T-lymphoid markers. Our expression data indicate that *Cebpg* is highly expressed in lymphoid progenitor cells and that C/EBP α is involved in the active repression of *Cebpg* during myeloid differentiation. Accordingly, C/EBP γ -KO murine models have shown that C/EBP γ is relevant for proper lymphoid differentiation (30). Together, these observations highlight

the possibility that C/EBP γ might be directly involved in the development of these biphenotypic leukemias, either by upregulating lymphoid markers and/or by repressing myeloid differentiation.

Our experiments indicate that granulocytic differentiation requires gradual upregulation of C/EBP α and downregulation of C/EBP γ , as seen in defined progenitor bone marrow populations and in a murine myeloid cell model. These results are in agreement with previous observations showing that C/EBP γ expression is reduced during myeloid differentiation (53). The reciprocal expression of C/EBP α and C/EBP γ suggests that these 2 transcription factors may regulate each other. In fact, our data demonstrate that C/EBP α represses *Cebpg* expression by affecting E2F1 transcriptional activity, which is in agreement with previous publications (40, 41). Although several reports state that only the C/EBP α p42 isoform represses E2F transcriptional activity (41), it has been reported that C/EBP α p30 can also suppress E2F activity (54). In fact, the luciferase reporter assays using the C/EBP γ proximal promoter indicate that both C/EBP α isoforms can repress C/EBP γ transactivation, consistent with our previously published studies demonstrating that p30 and p42 C/EBP α retain the ability to interact with E2F proteins in myeloid cell extracts (39). These different observations could be explained by a cell-type-dependent effect (54), and indeed the C/EBP α -C/EBP γ ratio seems to correlate with lineage commitment, rather than with cell-cycle activity, suggesting that other mechanisms yet to be identified, and that could work in a cell-specific manner, may also be involved in C/EBP γ repression. In contrast, our preliminary data (unpublished observations) suggest that this regulation is not reciprocal, since we do not observe changes in *Cebpa* in LKS cells that overexpress C/EBP γ . However, we cannot rule out the possibility that this regulation is cell-type- or differentiation-stage specific. Additionally, other factors could be involved in this C/EBP α -C/EBP γ regulation. In fact, since C/EBP α levels are reduced at the later stages of differentiation and C/EBP γ levels need to be low in order to accomplish neutrophilic differentiation, we hypothesize that other transcription factors may be involved in C/EBP γ downregulation. Further, we previously reported that C/EBP α -deficient progenitor/stem cells have increased *Bmi-1* expression (10). *Bmi-1* is a component of the polycomb group complex and regulates hematopoietic stem cell self renewal by repressing differentiation-related gene expression (55). Given the similarity between *Bmi-1* and C/EBP γ expression during myeloid differentiation and in the C/EBP α conditional KO murine model, it will be intriguing to determine how these components interact during differentiation and stem cell self renewal.

It has previously been shown that ectopic expression of other C/EBP members, such as C/EBP α or C/EBP β , can induce granulocytic differentiation of cell lines or primary cells in vitro (56–59). In contrast, our experiments demonstrate that C/EBP γ overexpression, either induced by ectopic expression in cell culture or by excision of C/EBP α in KO mice, results in a block of neutrophilic differentiation. Therefore, the effect of C/EBP γ overexpression is opposite to the one observed with other C/EBP members, suggesting different mechanisms of action and activation of distinct target genes. Structurally, C/EBP γ resembles the C/EBP β isoform liver inhibitory protein (LIP) (7). C/EBP γ and LIP retain the basic region-leucine zipper DNA-binding domain, characteristic of the C/EBP family of transcription factors, but lack the transactivation domains. Similarly to LIP, C/EBP γ acts as a transdominant negative inhibitor of other C/EBP members (27, 29). In addition, C/EBP γ could also functionally mimic the C/EBP α mutant proteins identified in AML patient



samples. We and others have shown that loss of *C/EBPα* is not the key event in leukemogenesis, but rather the synergistic activity of *C/EBPα* p30 and C-terminal mutants (14, 51). Therefore, *C/EBPγ* could contribute to the granulocytic differentiation arrest characteristic of AML by performing as a *C/EBPα* mutant protein. The results reported here demonstrate that downregulation of *C/EBPγ* by an shRNA approach can induce *C/EBPα*-independent granulopoiesis in vitro and in vivo. This is in agreement with previous publications in which we and others have shown that *C/EBPα*-KO cells can generate granulocytes in response to cytokines (19, 38, 60) and that this effect was mediated by *C/EBPβ* (19). Further, expression of *C/EBPβ* from the *Cebpa* locus results in normal granulopoiesis, indicating that *C/EBPβ* can drive granulopoiesis in the absence of *C/EBPα* (61). In the present study, we indeed observed that *C/EBPγ* can regulate *C/EBPβ* levels in murine LKS (Supplemental Figure 8A), and in line with these results, we observed low *C/EBPβ* mRNA levels in the AML cases with increased *C/EBPγ* expression (Supplemental Figure 8B). In addition, *C/EBPγ* modulates *C/EBPβ* transactivation activity (Supplemental Figure 8C). Next, we demonstrated that expression of *C/EBPβ* was able to drive neutrophilic differentiation of *C/EBPα* KO LKS cells (Supplemental Figure 8, D and E). Thus, we hypothesize that the rescue we observed upon *C/EBPγ* knockdown in *C/EBPα*-KO mice as well as in *CEBPA*-silenced leukemias might be partially mediated by *C/EBPβ*. Supporting our hypothesis, it was previously reported that *C/EBPγ* forms heterodimers with *C/EBPβ* and acts as a dominant negative inhibitor of *C/EBPβ* (27, 29). Further, since *C/EBPγ* can bind to G-CSF promoter elements (62), we hypothesize that *C/EBPγ* could also directly regulate G-CSF production and consequently neutrophilic differentiation. In line with this hypothesis, our experiments demonstrate that *C/EBPγ* binds to the proximal promoters of *Csf3r* and *Cebpe* and that enforced *C/EBPγ* expression prevents upregulation of these myeloid differentiation factors, which in normal hematopoiesis contribute to the granulocytic differentiation.

In summary, we have here identified *C/EBPγ* as a protooncogene in AML. Sustained *C/EBPγ* expression in myeloid precursors prevents myeloid differentiation toward mature granulocytes, and downregulation of *C/EBPγ* can restore neutrophilic differentiation in a *C/EBPα*-independent manner. Finally, we demonstrated that DAC treatment restores the *C/EBPα*-*C/EBPγ* balance affected in a subset of AML cases, contributing to the upregulation of myeloid differentiation markers in vitro (Figure 8). To date, treatment results with demethylating agents in AML have not been overwhelmingly positive (63, 64). However, these disappointing results could be explained if only certain subsets of AML patients are responsive. A precedent for this is found in treatment of AML patients with retinoids, in which only a specific subset of patients, those harboring the *PML/RARα* translocation, are responsive (65, 66). Our data supports DAC treatment and *C/EBPα*-*C/EBPγ* axis target approaches as a therapy in AML patients with *CEBPA* hypermethylation and upregulation of *CEBPG*.

Methods

AML patients, GEP, and data analysis. We made use of the leukemic cell specimens of 2 independent and representative cohorts of AML patients (17, 32, 67). The first cohort included 264 samples and the second 262. Patients not treated according to the Hovon study were excluded from this analysis. GEP of 526 AML cases was carried out on Affymetrix HGU133A Plus2.0 GeneChips. Further details on data analysis have previously been described (17, 32, 67, 68) (GEO GSE6891).

Cell lines. 32D/G-CSF-R cells were cultured as previously described (42, 43). K562 and 32D/G-CSF-R cell lines stably expressing *C/EBPα*-ER, *C/EBPγ*-ER, or ER alone were maintained in phenol red-free RPMI medium supplemented with 10% charcoal-stripped FBS. Independent clones were analyzed, and induction of nuclear localization of *C/EBPα*-ER or *C/EBPγ*-ER fusion protein was achieved by addition of 1 μ M β -estradiol or 50 nM 4-hydroxytamoxifen (Sigma-Aldrich), as indicated, into the culture medium.

ChIP assay. For ChIP on DNA promoter microarrays (ChIP on chip), 50 to 100 million cells were cultured in the presence of 1 μ M 17 β -estradiol (E2) (Sigma-Aldrich) for 4 hours. Protein and DNA were crosslinked by incubation with formaldehyde (final concentration 1%; Sigma-Aldrich) for 10 minutes, followed by quenching with glycine (final concentration 0.125 M; Sigma-Aldrich) for 5 minutes. Cells were pelleted, washed twice with ice-cold PBS and 3 times with lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂, 0.5% IGEPAL (Sigma-Aldrich), 1 mM PMSF), and then resuspended in pre-IP dilution buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂, 1 mM CaCl₂, 4% IGEPAL, 1 mM PMSF; Sigma-Aldrich). Material was sonicated in a total volume of 1 ml using a Soniprep 150 device (MSE) for 6 cycles of 20 seconds pulse/90 seconds rest at an amplitude of 6 microns. Cellular debris were removed by pelleting; 10% of the supernatant was kept aside as input control, and the remainder was used for IP after addition of 5 volumes of IP dilution buffer (20 mM Tris-HCl, 2 mM EDTA, 1% Triton X-100 [Roche], 150 mM NaCl and fresh protease inhibitor [Roche]). Chromatin was precleared by incubation for 30 minutes at 4°C with preequilibrated protein G-coupled magnetic beads (Dyna-beads; Invitrogen). IP was carried out overnight at 4°C using 20 μ g rabbit polyclonal IgG anti-ER α (sc543X; Santa Cruz Biotechnology Inc.). Samples were incubated for 2.5 hours at room temperature in the presence of 200 μ l of preequilibrated magnetic beads. The beads were washed twice with ChIP wash 1 (20 mM Tris-HCl, 2 mM EDTA, 1% Triton X-100, 150 mM NaCl, and 1 mM PMSF), once with ChIP wash 2 (20 mM Tris-HCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 500 mM NaCl, and 1 mM PMSF), once with ChIP wash 3 (10 mM Tris-HCl, 1 mM EDTA, 0.25 M LiCl, 0.5% IGEPAL, and 0.5% deoxycholate), and twice with TE. Protein/DNA complexes were eluted into 400 μ l elution buffer (25 mM Tris-HCl, 10 mM EDTA, 0.5% SDS) by heating at 65°C for 30 minutes. Crosslinks were reversed overnight at 65°C in the presence of 20 μ l proteinase K (20 mg/ml; New England Biolabs). The non-IP 10% input control sample was processed similarly. De-crosslinked material was purified using a QIAGEN PCR Purification Kit and eluted into 20 μ l buffer EB.

Other ChIP experiments were performed using ChIP assay kits (Upstate Biotechnology). Briefly, 1 million lineage⁻c-kit⁺ bone marrow cells from WT or *C/EBPα* KO mice were sorted, or 10 million 32D/G-CSF-R and K562 cells stably transfected with *C/EBPα*-ER, *C/EBPγ*-ER, or control ER were treated with 1 μ M E₂ or 50 nM 4-hydroxytamoxifen (Sigma-Aldrich) as indicated. IP was carried out using 20 μ g rabbit polyclonal IgG anti-ER α (sc543X; Santa Cruz Biotechnology Inc.), 10 μ g anti-*C/EBPα* (14AA) (Santa Cruz Biotechnology Inc.), 10 μ g anti-E2F-1 (clones KH20 and KH95; Millipore), 10 μ g anti-H3K4me3 (clone MC315; Millipore), or 10 μ g anti-H3K27me3 (6002; Abcam). Oligonucleotide sequences are listed below.

Whole-genome amplification, promoter array hybridization, and promoter array data analysis. 10 μ l of DNA isolated by ChIP in 32D/G-CSF-R cells was used for amplification using a Whole Genome Amplification (WGA2) kit (QIAGEN). From 5 to 10 μ g of amplified DNA was fragmented following the protocol of Affymetrix SNP array kits, which was confirmed on an Agilent Bioanalyzer. Labeling, hybridization to Affymetrix Mouse Promoter 1.0R arrays, staining, washing, and scanning were performed according to the manufacturer's recommendations. Raw promoter array data were processed using Model based Analysis of Tiling arrays (MAT) software (69). In these analyses, 2 ER samples



and 3 C/EBP α -ER samples were included. Enriched fragments at the 0.0001 significance level were considered to be target regions at a bandwidth of 300, maxgap of 300, and minprobe of 8. The enriched chromosomal regions were mapped to NCBI murine Genome Build 36 (assembled February 2006) and annotated using Cis-regulatory Element Annotation System (CEAS) (<http://ceas.cbi.pku.edu.cn/>) (70). CEAS was also used to search for transcription factor-binding motifs from the TRANSFAC and JASPAR databases (70–72). Visualization of enriched regions was carried out using Affymetrix Integrated Genome Browser (IGB) software.

Processing and normalization of DNA methylation HELP arrays. High-molecular-weight DNA was isolated from sorted lineage-ckit⁺ murine bone marrow cells ($n = 3$ animals per group) using the PureGene kit from QIAGEN and the HELP assay was carried out as previously described (73, 74). All samples for microarray hybridization were processed at the Roche NimbleGen Service Laboratory. Samples were labeled using Cy-labeled random primers (9 mers) and then hybridized onto a mouse custom-designed oligonucleotide array (50 mers) covering 25,720 HpaII amplifiable fragments (HAF) (>50,000 CpGs), annotated to 15,465 unique gene symbols (Roche NimbleGen, Design name: 2006-10-26_MM5_HELP_Promoter Design ID=4803). HpaII amplifiable fragments are defined as genomic sequences contained between 2 flanking HpaII sites found within 200–2,000 bp of each other, and each one is represented on the array by 15 individual probes randomly distributed across the microarray slide. Scanning was performed using a GenePix 4000B scanner (Molecular Devices) as previously described (75). Quality control and preprocessing of HELP microarrays was performed as described in Thompson et al. (76). Analysis of normalized data revealed the presence of a bimodal distribution. For each sample, a cutoff was selected at the point that more clearly separated these 2 populations, and the data were centered around this point. Each fragment was then categorized as either methylated, if the centered log HpaII/MspI ratio was less than zero, or hypomethylated, if on the other hand the log ratio was greater than zero.

Plasmids, transient transfections, and luciferase assays. Distinct fragments of the human CEBPG promoter were cloned into the pXP2 firefly luciferase reporter vector: long construct (from –957 bp to +1 bp), short construct (from –354 bp to +1 bp), and E2F-binding sites construct (from –139 to +1 bp). All distances are relative to transcriptional start site. The C/EBP binding sites were mutated into TTATAATA (5' site) and TAGTTACC (3' site), using a QuikChange Site-Directed Mutagenesis Kit and following manufacturer's instructions (Stratagene). C/EBP β pCDNA3 plasmid was donated by Peter F. Johnson (Center for Cancer Research, National Cancer Institute, Frederick, Maryland, USA). Luciferase experiments were done as previously described (40). Briefly, 1.5×10^5 293T cells, which endogenously express C/EBP γ , were cotransfected with the reporter construct (100 ng) and increasing amounts of C/EBP plasmids (10, 25, 50, 100, and 200 ng). When a luciferase reporter vector containing 4 C/EBP binding sites (77) was used, reporter activity was upregulated by 1 ng C/EBP β and increasing amounts of C/EBP γ expression plasmid were added to the luciferase reporter assay as indicated. DNA input was equalized by addition of pDNA3 plasmid. Transfections were normalized with the 20 ng of renilla luciferase vector as an internal control.

Quantitative RT-PCR. RNA was isolated by TriReagent (MRC Inc.), treated with DNaseI, and reverse-transcribed into cDNA (Invitrogen). Quantitative RT-PCR was performed using iQ Sybr Green supermix (Bio-Rad). Amplification was done with a Corbett Rotor Gene 6000 (QIAGEN) using the following parameters: 95°C (10 minutes), 45 cycles of 95°C (15 seconds), and 60°C (1 minute). For Taqman Analysis, Hotstart Probe One-step qRT-PCR Master Mix was used. RNA was reverse transcribed for 10 minutes at 50°C, followed by 2 minutes at 95°C, and 45 cycles of 95°C (15 seconds) and 60°C (1 minute). Primer and probe sequences are depicted below.

Western blot analysis and antibodies. Briefly, single-cell suspensions were lysed with modified RIPA buffer and whole-cell lysates separated on 10% SDS-PAGE gels. Immunoblots were stained with the following primary antibodies: rabbit C/EBP α (14AA, 1:1000; Santa Cruz Biotechnology Inc.), rabbit C/EBP γ (1:1000; donated by Peter F. Johnson), ER (HC-20, 1:1000; Santa Cruz Biotechnology Inc.), HA (Y-11, 1:1000; Santa Cruz Biotechnology Inc.), HSP90 (1:2000, BD Bioscience), and β -actin (1:10,000; Sigma-Aldrich). All secondary antibodies were HRP conjugated (Santa Cruz Biotechnology Inc.) and diluted 1:5000 for rabbit-HRP and 1:3000 for mouse-HRP.

Flow cytometry and cell sorting. LKS and progenitor cells were isolated from murine bone marrow as described previously (78). For analysis of peripheral blood and bone marrow, lysis of red blood cells was followed by staining with PE-conjugated anti-CD45 (Ly5.2), Pacific blue-conjugated anti-Gr1, and allophycocyanin-conjugated anti-Mac-1 or allophycocyanin-conjugated anti-CD45 (Ly5.2), PE-conjugated anti-IgM and Pacific blue-conjugated anti-B220. Detection and analysis of human cells in murine bone marrow NSG recipient mice was performed 16 weeks after transplantation by the following staining: PE-conjugated anti-human CD45, PE-Cy7-conjugated anti-human CD33 and Pacific blue-conjugated anti-human CD15. Staining of liquid cultures was done using fluorescein isothiocyanate-conjugated Gr-1 and allophycocyanin-conjugated Mac-1 or allophycocyanin-conjugated Sca-1 and PE-conjugated CD117/c-kit. All antibodies were from eBioscience. Exclusion of dead cells was done by addition of DAPI. Cell sorting was performed using FACSAria and immunophenotyping was done on an LSRII flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (Treestar Inc.).

Viral vectors and transduction. Murine p42 C/EBP α was cloned into the MSCV-IRES-GFP retroviral vector (17), and p30 C/EBP α MSCV was donated by Ting Xi Liu's lab (Shanghai Stem Cell Institute, Shanghai Jiao Tong University School of Medicine, Shanghai, China). NSC#2 control shRNA and C/EBP γ -specific shRNA#2 were cloned into the retroviral vector RSG-SIN (46). C/EBP β was cloned into the MIGR1 retroviral vector. Retrovirus was produced as previously described (17) and concentrated using a Centricon Plus-70 100000 MWCO column (Millipore). Retroviral transduction was performed as described (17). For in vitro cultures, infected GFP⁺ cells were sorted prior to culture. NSC#1 control shRNA and murine C/EBP γ -specific shRNA#1 into pLKO lentiviral vector were purchased from Open Biosystems. Human C/EBP γ -specific shRNA and an NSC shRNA were cloned into the lentiviral vector pGhU6. 293T cells were cotransfected using lipofectamine 2000 with lentiviral constructs (Gag-Pol and Env). Virus was harvested and concentrated as mentioned above. A single lentiviral transduction was performed in culture dishes (Falcon 1008; BD) in the presence of polybrene (8 μ g/ml) (Sigma-Aldrich). Puromycin (2 μ g/ml) was added to the cultures 2 days after infection with pLKO constructs. Human AML samples were infected with an MOI = 20 for 6 hours, washed, and transplanted into NSG mice (1.25×10^6 cells/recipient).

Methylcellulose colony assay. Colony assay and replating (79) of sorted lineage-negative BM cells or infected LKS cells was performed with Methocult M3434 medium (Stem Cell Technologies). One day after infection, retrovirally infected GFP⁺ cells were sorted and placed in Methocult, and lentivirally infected cells were placed in medium supplemented with puromycin (2 μ g/ml) (Invivogen). Single colonies were picked up 12 days after culture and cytopun; slides were stained with Wright-Giemsa (Diff-Quick; Baxter Healthcare). 10,000 cells/dish were used for replating assays.

Mice and transplantation experiments. *Cebpa*^{Flox/Flox} Mx1-Cre⁻, *Cebpa*^{Flox/Flox} Mx1-Cre⁺, *Cebpa*^{WT/Flox} Mx1-Cre⁻, *Cebpa*^{WT/Flox} Mx1-Cre⁺, C57BL/6J (CD45.2⁺), and congenic B6.SJL-Ptprc^aPep3^b/BoyJ (CD45.1⁺ congenic C57BL/6J) mice were bred and maintained in the Animal Research Facility at the Center for Life Sciences in accordance with institutional guidelines. *Cebpa*^{Flox/Flox} Mx1-Cre mice were genotyped as previously described (10).



Cebpa excision was induced by 3 poly I:C injections (600 µg/mouse, every other day) (Sigma-Aldrich). For adult bone marrow transplantation, 8- to 12-week-old CD45.1⁺ congenic C57BL/6J recipient mice were lethally irradiated with 9.5 Gy from a ¹³⁷Cs irradiator (Gammacell 40 Exactor; Nordion International). Cells were transplanted by retroorbital injection. Peripheral blood samples were obtained from facial vein at different time points. Human AML patient samples were transplanted into sublethally irradiated (1.5 Gy) NSG (NOD.Cg-Prkdc^{scid} IL2rg^{tm1Wjl/sz}) mice (Jackson Laboratory).

DAC treatment and bisulfite sequencing. AML patient samples were cultured for 96 hours (or as indicated) in BSF60 medium supplemented with 50 ng/ml hSCF, 50 ng/ml hFLT3L, 10 ng/ml hIL-3, 10 ng/ml hTPO and 10% FBS, and in the presence of 1 µM DAC or 0.005% acetic acid. Medium was refreshed 48 hours after culture. RNA (for RT-PCR) and gDNA (for bisulfite sequencing) were isolated after 96 hours of treatment. Bisulfite sequencing was performed following the manufacture's instructions. Briefly, genomic DNA was isolated, treated with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research) according to the manufacturer's recommendations, and used as a template for methylation analysis of the *Cebpa* promoter region (-428 to +64 relative to ATG) in a nested PCR approach, as described previously (17). Sequencing results were analyzed using BiQ analyzer software (80). Primer sets used are indicated below.

shRNA sequences. Sequences were as follows: sequence NSC#1 (in pLKO vector): ATCTCGCTTGGGCGAGAGTAAAG; sequence shRNA#1 (in pLKO vector): GGAAGAGAATGAACGGTTGGAA; sequence NSC#2 (in RSG-SIN vector): TCGCTTGGGCGAGAGTAAAG; sequence shRNA#2 (in RSG-SIN vector): GGATCGGAATAGTGACGAA; sequence NSC (in pGhU6 vector): ATCTCGCTTGGGCGAGAGTAA; sequence C/EBPγ shRNA (in pGhU6 vector): GAAGAGAATGAACGGTTGGAA.

Quantitative RT-PCR oligonucleotides, forward and reverse. Sequences were as follows: murine endogenous C/EBPα forward (F): 5'-GACCATTAGCCTTGTGTACTGTATG-3'; murine endogenous C/EBPα reverse (R): 5'-TGGATCGATTGTGCTTCAAGTT-3'; murine C/EBPα overexpression F: 5'-ACGAGTTCCTGGCCGACCT-3'; murine C/EBPα overexpression R: 5'-GGGCTCCCGGTAGTCAAAG-3'; murine C/EBPγ F: 5'-GCG-CAGAGAGCGGAACAA-3'; murine C/EBPγ R: 5'-GTATCTTGAGCTTCT-GCTTGCT-3'; murine ELA 2 F: 5'-CACCATCAGTCAGTCTTCC-3'; murine MPO F: 5'-ATGCAGTGGGAGCATTTCTG-3'; murine MPO R: 5'-GTCGTTG-TAGGATCGGTACTG-3'; murine C/EBPε F: 5'-AAGGCCAAGAGGCG-CATT-3'; murine C/EBPε R: 5'-CGCTCGTTTTCAGCCATGTA-3'; murine G-CSF-R F: 5'-TCCGTCACCCTAAACATCTC-3'; murine G-CSF-R R: 5'-TGGAAGGTTTCTCTGTGCAT-3'; murine Trib2 F: 5'-AGCCCGACT-GTTCTACCAGA-3'; murine Trib2 R: 5'-AGCGTCTTCCAACTCTCCA-3'; murine GAPDH F: 5'-CCAGCTCGTCCCGTAGAC-3'; murine GAPDH R: 5'-CCCTTACTGTGCCGTTG-3'; 18S F: 5'-ACTGGAATTACCGCCTG-GCAC-3'; 18S R: 5'-CGGCTACCACATCCAAGGAAG-3'; human GAPDH F: 5'-CCACATCGCTCAGACACCAT-3'; human GAPDH R: 5'-CCAGGC-GCCCAATACG-3'; human G-CSF-R F: 5'-TTTCAGGAACTTCTTT-GACGAGAA-3'; human G-CSF-R R: 5'-CGAGCCGAGCCTCAGTTTC-3'; human C/EBPε F: 5'-CTCCGATCTCTTTCGCGTGA-3'; human C/EBPε R: 5'-TGGGCCGAAGGTATGTGGA-3'; human C/EBPα F: 5'-TCGGTGG-CAAGAACAG-3'; human C/EBPα R: 5'-GCAGGCGGTCATTG-3'; human C/EBPα taqman probe: 5'-TGGAGACGCAGCAGAAGGTG-3'; human C/EBPγ F: 5'-GGCTTGAATGTTAAAGTGTGACC-3'; human C/EBPγ R: 5'-TTGAGTCATGGAATGGACAACCTT-3'; human C/EBPγ F (to verify microarray data): 5'-GGCTAGAGGAGCAGGTACAT-3'; human C/EBPγ R

(to verify microarray data): 5'-GCCTGGGTATGGATAACACTA-3'; human C/EBPγ TaqMan probe: 5'-CCGACCACTCATGTCAATGGCTG-3'; human gelatinase A F: 5'-GTGGGACAAGAACCAGATCACAT-3'; human gelatinase A R: 5'-GTCTGCCTTCCATCATGGATT-3'; human neutrophil elastase F: 5'-CCACCCGGCAGGTGTTTC-3'; and human neutrophil elastase R: 5'-GTGGCCGACCCGTTGAG-3'.

ChIP oligonucleotides. Sequences were as follows: murine oligos set 1 F: 5'-CTTCCCGACTGTGGTGAG-3'; murine oligos set 1 R: 5'-GGCGTCT-GATGCAACCTG-3'; murine oligos set 2 F: 5'-CGAAAGGTTTGATT-GCTTC-3'; murine oligos set 2 R: 5'-TATGTGGAGAGCTGCTCT-3'; murine oligos Csf3r F: 5'-TTGCATTACAGGGTCATAGCAC-3'; murine oligos Csf3r R: 5'-TCCTAGGGGTTCTGGTTTT-3'; murine oligos Cebpε F: 5'-TGGCTTGACACCTCACTCTG-3'; murine oligos Cebpε R: 5'-GGAGGGGTGCTTAGCAGTTA-3'; human Oligos A F: 5'-CCATT-GTTCACCGTTGTGACC-3'; human oligos A R: 5'-AGCGTTTTAACAC-CAGGAAGC-3'; human oligos B F: 5'-CTGTGCCAATCCAAGAGTCT-3'; human oligos B R: 5'-TATGCTTTCTGCTGGCACAT-3'.

Bisulfite sequencing oligonucleotides. Sequences were as follows: C/EBPα F1: 5'-AAACAAACCTAATTCTAACTTAAA-3'; C/EBPα R1: 5'-GTTAGTTGTTTGGTTTTATTTTTT-3'; C/EBPα F2: 5'-CCTA-AAACAAACAAAAAAAAAAC-3'; C/EBPα R2: 5'-GGTTTTGTAG-GTGGTTGTTTAT-3'.

Statistics. We used 2-sided unpaired Student's *t* test to determine the statistical significance of experimental results. When indicated, a Wilcoxon or a Mann-Whitney 1-sided test analysis was applied. *P* value < 0.05 was considered significant.

Study approval. Patients' informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board: Committee on Clinical Investigations of Beth Israel Deaconess Medical Center (Boston, Massachusetts, USA).

Acknowledgments

We thank Britta Will, David Gonzalez, Hideo Hirai, and all members of the Tenen laboratory for helpful discussions; Pu Zhang, and Susumu S. Kobayashi for careful reading of the manuscript and suggestions; Carol Stocking and Peter F. Johnson for kindly providing us with the RSG-SIN retroviral vector (Stocking), the murine C/EBPγ antibody (Johnson), and the C/EBPβ pCDNA3 plasmid (Johnson); Heike Pahl for providing us with the pGhU6 lentiviral vector; and Li Chai for her generous donation of the NSG mice. We thank Min Deng for providing us with the p30 MSCV plasmid; and John Tigges, Vasilis Toxavidis, and Heidi Mariani from the Harvard Stem Cell Institute/Beth Israel Deaconess Center flow cytometry facility for their help and assistance during cell sorting. This work was supported by NIH R01 grants CA118316 to D.G. Tenen and R. Delwel, HL56745 to D.G. Tenen, a Collegio Ghislieri's fellowship to G. Amabile, and an EHA research fellowship from the European Hematology Association to M. Alberich-Jordà.

Received for publication May 30, 2012, and accepted in revised form September 20, 2012.

Address correspondence to: Daniel G. Tenen, Center for Life Sciences, 3 Blackfan Circle, Room 437, Boston, Massachusetts 02115, USA. Phone: 617.735.2235; Fax: 617.735.2222; E-mail: dtenen@bidmc.harvard.edu.

1. Hock H, Orkin SH. Zinc-finger transcription factor Gfi-1: versatile regulator of lymphocytes, neutrophils and hematopoietic stem cells. *Curr Opin Hematol.* 2006;13(1):1-6.

2. Rosenbauer F, Tenen DG. Transcription factors in myeloid development: balancing differentiation with transformation. *Nat Rev Immunol.* 2007; 7(2):105-117.

3. Brown D, et al. A PMLRARA transgene initiates murine acute promyelocytic leukemia. *Proc Natl Acad Sci USA.* 1997;94(6):2551-2556.
4. Peterson LF, Zhang DE. The 8;21 translocation in



- leukemogenesis. *Oncogene*. 2004;23(24):4255–4262.
5. Tenen DG. Disruption of differentiation in human cancer: AML shows the way. *Nat Rev Cancer*. 2003; 3(2):89–101.
6. Lekstrom-Himes J, Xanthopoulos KG. Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J Biol Chem*. 1998; 273(44):28545–28548.
7. Nerlov C. The C/EBP family of transcription factors: a paradigm for interaction between gene expression and proliferation control. *Trends Cell Biol*. 2007;17(7):318–324.
8. Poli V. The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *J Biol Chem*. 1998;273(45):29279–29282.
9. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proc Natl Acad Sci U S A*. 1997;94(2):569–574.
10. Zhang P, et al. Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. *Immunity*. 2004;21(6):853–863.
11. Chim CS, Wong AS, Kwong YL. Infrequent hypermethylation of CEBPA promoter in acute myeloid leukaemia. *Br J Haematol*. 2002;119(4):988–990.
12. Cilloni D, et al. Down-modulation of the C/EBPalpha transcription factor in core binding factor acute myeloid leukemias. *Blood*. 2003;102(7):2705–2706.
13. Leroy H, Roumier C, Huyghe P, Biggio V, Fenaux P, Preudhomme C. CEBPA point mutations in hematologic malignancies. *Leukemia*. 2005;19(3):329–334.
14. Pabst T, et al. Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat Genet*. 2001;27(3):263–270.
15. Perrotti D, et al. BCR-ABL suppresses C/EBPalpha expression through inhibitory action of hnRNP E2. *Nat Genet*. 2002;30(1):48–58.
16. Preudhomme C, et al. Favorable prognostic significance of CEBPA mutations in patients with de novo acute myeloid leukemia: a study from the Acute Leukemia French Association (ALFA). *Blood*. 2002;100(8):2717–2723.
17. Wouters BJ, et al. Distinct gene expression profiles of acute myeloid/T-lymphoid leukemia with silenced CEBPA and mutations in NOTCH1. *Blood*. 2007;110(10):3706–3714.
18. Tanaka T, et al. Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. *Cell*. 1995;80(2):353–361.
19. Hirai H, et al. C/EBPbeta is required for 'emergency' granulopoiesis. *Nat Immunol*. 2006;7(7):732–739.
20. Huang AM, Montagna C, Sharan S, Ni Y, Ried T, Sterneck E. Loss of CCAAT/enhancer binding protein delta promotes chromosomal instability. *Oncogene*. 2004;23(8):1549–1557.
21. Yamanaka R, et al. Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. *Proc Natl Acad Sci U S A*. 1997;94(24):13187–13192.
22. Agrawal S, et al. The C/EBPdelta tumor suppressor is silenced by hypermethylation in acute myeloid leukemia. *Blood*. 2007;109(9):3895–3905.
23. Gombart AF, Koefler HP. Neutrophil specific granule deficiency and mutations in the gene encoding transcription factor C/EBP(epsilon). *Curr Opin Hematol*. 2002;9(1):36–42.
24. Lekstrom-Himes JA, Dorman SE, Kopar P, Holland SM, Gallin JI. Neutrophil-specific granule deficiency results from a novel mutation with loss of function of the transcription factor CCAAT/enhancer binding protein epsilon. *J Exp Med*. 1999; 189(11):1847–1852.
25. Qian J, Chen Z, Lin J, Wang W, Cen J. Decreased expression of CCAAT/enhancer binding protein zeta (C/EBPzeta) in patients with different myeloid diseases. *Leuk Res*. 2005;29(12):1435–1441.
26. Roman C, Platero JS, Shuman J, Calame K. Ig/EBP-1: a ubiquitously expressed immunoglobulin enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP. *Genes Dev*. 1990; 4(8):1404–1415.
27. Cooper C, Henderson A, Artandi S, Avitahl N, Calame K. Ig/EBP (C/EBP gamma) is a transdominant negative inhibitor of C/EBP family transcriptional activators. *Nucleic Acids Res*. 1995;23(21):4371–4377.
28. Thomassin H, Hamel D, Bernier D, Guertin M, Belanger L. Molecular cloning of two C/EBP-related proteins that bind to the promoter and the enhancer of the alpha 1-fetoprotein gene. Further analysis of C/EBP beta and C/EBP gamma. *Nucleic Acids Res*. 1992;20(12):3091–3098.
29. Parkin SE, Baer M, Copeland TD, Schwartz RC, Johnson PF. Regulation of CCAAT/enhancer-binding protein (C/EBP) activator proteins by heterodimerization with C/EBPgamma (Ig/EBP). *J Biol Chem*. 2002;277(26):23563–23572.
30. Kaisho T, et al. Impairment of natural killer cytotoxic activity and interferon gamma production in CCAAT/enhancer binding protein gamma-deficient mice. *J Exp Med*. 1999;190(11):1573–1582.
31. Zafarana G, Rottier R, Grosveld F, Philipsen S. Erythroid overexpression of C/EBPgamma in transgenic mice affects gamma-globin expression and fetal liver erythropoiesis. *EMBO J*. 2000; 19(21):5856–5863.
32. Valk PJ, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med*. 2004;350(16):1617–1628.
33. Figueroa ME, et al. Genome-wide epigenetic analysis delineates a biologically distinct immature acute leukemia with myeloid/T-lymphoid features. *Blood*. 2009;113(12):2795–2804.
34. Keeshan K, et al. Transformation by Tribbles homolog 2 (Trib2) requires both the Trib2 kinase domain and COP1 binding. *Blood*. 2006;116(23):4948–4957.
35. Wang W, Wang X, Ward AC, Touw IP, Friedman AD. C/EBPalpha and G-CSF receptor signals cooperate to induce the myeloperoxidase and neutrophil elastase genes. *Leukemia*. 2001;15(5):779–786.
36. Ramji DP, Foka P. CCAAT/enhancer-binding proteins: structure, function, and regulation. *Biochem J*. 2002;365(pt 3):561–575.
37. Kummalur T, Friedman AD. Cross-talk between regulators of myeloid development: C/EBPalpha binds and activates the promoter of the PU.1 gene. *J Leukoc Biol*. 2003;74(3):464–470.
38. Zhang P, Iwama A, Datta MW, Darlington GJ, Link DC, Tenen DG. Upregulation of interleukin 6 and granulocyte colony-stimulating factor receptors by transcription factor CCAAT enhancer binding protein alpha (C/EBP alpha) is critical for granulopoiesis. *J Exp Med*. 1998;188(6):1173–1184.
39. D'Alò F, et al. The amino terminal and E2F interaction domains are critical for C/EBP alpha-mediated induction of granulopoietic development of hematopoietic cells. *Blood*. 2003;102(9):3163–3171.
40. Johansen LM, et al. c-Myc is a critical target for C/EBPalpha in granulopoiesis. *Mol Cell Biol*. 2001; 21(11):3789–3806.
41. Porse BT, et al. E2F repression by C/EBPalpha is required for adipogenesis and granulopoiesis in vivo. *Cell*. 2001;107(2):247–258.
42. Dong F, Brynes RK, Tidow N, Welte K, Lowenberg B, Touw IP. Mutations in the gene for the granulocyte colony-stimulating-factor receptor in patients with acute myeloid leukemia preceded by severe congenital neutropenia. *N Engl J Med*. 1995; 333(8):487–493.
43. Jorda MA, Lowenberg B, Delwel R. The peripheral cannabinoid receptor Cb2, a novel oncoprotein, induces a reversible block in neutrophilic differentiation. *Blood*. 2003;101(4):1336–1343.
44. Ito Y, et al. Structural analysis of the functional gene and pseudogene encoding the murine granulocyte colony-stimulating-factor receptor. *Eur J Biochem*. 1994;220(3):881–891.
45. Yamanaka R, et al. CCAAT/enhancer binding protein epsilon is preferentially up-regulated during granulocytic differentiation and its functional versatility is determined by alternative use of promoters and differential splicing. *Proc Natl Acad Sci U S A*. 1997;94(12):6462–6467.
46. Niebuhr B, Iwanski GB, Schwieger M, Roscher S, Stocking C, Cammenga J. Investigation of C/EBPalpha function in human (versus murine) myelopoiesis provides novel insight into the impact of CEBPA mutations in acute myelogenous leukemia (AML). *Leukemia*. 2009;23(5):978–983.
47. Garcia-Manero G. Demethylating agents in myeloid malignancies. *Curr Opin Oncol*. 2008; 20(6):705–710.
48. Gotze K, et al. Azacitidine for treatment of patients with myelodysplastic syndromes (MDS): practical recommendations of the German MDS Study Group. *Ann Hematol*. 2010;89(9):841–850.
49. Kuang SQ, et al. Aberrant DNA methylation and epigenetic inactivation of Eph receptor tyrosine kinases and ephrin ligands in acute lymphoblastic leukemia. *Blood*. 2010;115(12):2412–2419.
50. Mestre-Escorihuela C, et al. Homozygous deletions localize novel tumor suppressor genes in B-cell lymphomas. *Blood*. 2007;109(1):271–280.
51. Kirstetter P, et al. Modeling of C/EBPalpha mutant acute myeloid leukemia reveals a common expression signature of committed myeloid leukemia-initiating cells. *Cancer Cell*. 2008;13(4):299–310.
52. Radomska HS, et al. Block of C/EBP alpha function by phosphorylation in acute myeloid leukemia with FLT3 activating mutations. *J Exp Med*. 2006; 203(2):371–381.
53. Bjerregaard MD, Jurlander J, Klausen P, Borregaard N, Cowland JB. The in vivo profile of transcription factors during neutrophil differentiation in human bone marrow. *Blood*. 2003;101(11):4322–4332.
54. Cleaves R, Wang QF, Friedman AD. C/EBPalpha30, a myeloid leukemia oncoprotein, limits G-CSF receptor expression but not terminal granulopoiesis via site-selective inhibition of C/EBP DNA binding. *Oncogene*. 2004;23(3):716–725.
55. Iwama A, et al. Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. *Immunity*. 2004;21(6):843–851.
56. Duprez E, Wagner K, Koch H, Tenen DG. C/EBPbeta: a major PML-RARA-responsive gene in retinoic acid-induced differentiation of APL cells. *EMBO J*. 2003; 22(21):5806–5816.
57. Popernack PM, Truong LT, Kamphuis M, Henderson AJ. Ectopic expression of CCAAT/enhancer binding protein beta (C/EBPbeta) in long-term bone marrow cultures induces granulopoiesis and alters stromal cell function. *J Hematother Stem Cell Res*. 2001;10(5):631–642.
58. Ross SE, et al. Phosphorylation of C/EBPalpha inhibits granulopoiesis. *Mol Cell Biol*. 2004; 24(2):675–686.
59. Wang QF, Friedman AD. CCAAT/enhancer-binding proteins are required for granulopoiesis independent of their induction of the granulocyte colony-stimulating factor receptor. *Blood*. 2002; 99(8):2776–2785.
60. Collins SJ, Ulmer J, Purton CE, Darlington G. Multipotent hematopoietic cell lines derived from C/EBPalpha(-/-) KO mice display granulocyte macrophage-colony-stimulating factor, granulocyte-colony-stimulating factor, and retinoic acid-induced granulocytic differentiation. *Blood*. 2001;98(8):2382–2388.
61. Jones LC, et al. Expression of C/EBPbeta from the C/ebpalpha gene locus is sufficient for normal



- hematopoiesis in vivo. *Blood*. 2002;99(6):2032–2036.
62. Nishizawa M, Wakabayashi-Ito N, Nagata S. Molecular cloning of cDNA and a chromosomal gene encoding GPE1-BP, a nuclear protein which binds to granulocyte colony-stimulating factor promoter element 1. *FEBS Lett*. 1991;282(1):95–97.
63. Thepot S, et al. Treatment of progression of Philadelphia negative myeloproliferative neoplasms to myelodysplastic syndrome or acute myeloid leukemia by azacitidine: a report on 54 cases on the behalf of the Groupe Francophone des Myelodysplasies. *Blood*. 2010;116(19):3735–3742.
64. Zhu X, Ma Y, Liu D. Novel agents and regimens for acute myeloid leukemia: 2009 ASH annual meeting highlights. *J Hematol Oncol*. 2010;3:17.
65. Chen SJ, Wang ZY, Chen Z. Acute promyelocytic leukemia: from clinic to molecular biology. *Stem Cells*. 1995;13(1):22–31.
66. Chen Z, Chen SJ, Wang ZY. Retinoic acid and acute promyelocytic leukemia: a model of targetting treatment for human cancer. *C R Acad Sci III*. 1994;317(12):1135–1141.
67. Wouters BJ, Lowenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. *Blood*. 2009;113(13):3088–3091.
68. Verhaak RG, et al. Prediction of molecular subtypes in acute myeloid leukemia based on gene expression profiling. *Haematologica*. 2009;94(1):131–134.
69. Johnson WE, et al. Model-based analysis of tiling-arrays for ChIP-chip. *Proc Natl Acad Sci U S A*. 2006;103(33):12457–12462.
70. Ji X, Li W, Song J, Wei L, Liu XS. CEAS: cis-regulatory element annotation system. *Nucleic Acids Res*. 2006;34(Web Server issue):W551–W554.
71. Matys V, et al. TRANSFAC: transcriptional regulation, from patterns to profiles. *Nucleic Acids Res*. 2003;31(1):374–378.
72. Sandelin A, Alkema W, Engstrom P, Wasserman WW, Lenhard B. JASPAR: an open-access database for eukaryotic transcription factor binding profiles. *Nucleic Acids Res*. 2004;32(Database issue):D91–D94.
73. Figueroa ME, Melnick A, Greal JM. Genome-wide determination of DNA methylation by Hpa II tiny fragment enrichment by ligation-mediated PCR (HELP) for the study of acute leukemias. *Methods Mol Biol*. 2009;538:395–407.
74. Khulan B, et al. Comparative isoschizomer profiling of cytosine methylation: the HELP assay. *Genome Res*. 2006;16(8):1046–1055.
75. Selzer RR, et al. Analysis of chromosome breakpoints in neuroblastoma at sub-kilobase resolution using fine-tiling oligonucleotide array CGH. *Genes Chromosomes Cancer*. 2005;44(3):305–319.
76. Thompson RF, et al. An analytical pipeline for genomic representations used for cytosine methylation studies. *Bioinformatics*. 2008;24(9):1161–1167.
77. Smith LT, Hohaus S, Gonzalez DA, Dziennis SE, Tenen DG. PU.1 (Spi-1) and C/EBP alpha regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. *Blood*. 1996;88(4):1234–1247.
78. Pronk CJ, et al. Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. *Cell Stem Cell*. 2007;1(4):428–442.
79. Okuda T, et al. Expression of a knocked-in AML1-ETO leukemia gene inhibits the establishment of normal definitive hematopoiesis and directly generates dysplastic hematopoietic progenitors. *Blood*. 1998;91(9):3134–3143.
80. Bock C, Reither S, Mikeska T, Paulsen M, Walter J, Lengauer T. BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics*. 2005;21(21):4067–4068.