C/EBPγ deregulation results in differentiation arrest in acute myeloid leukemia

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C/EBPs are a family of transcription factors that regulate growth control and differentiation of various tissues. We found that C/EBPγ is highly upregulated in a subset of acute myeloid leukemia (AML) samples characterized by C/EBPα hypermethylation/silencing. Similarly, C/EBPγ was upregulated in murine hematopoietic stem/progenitor cells lacking C/EBPα, as C/EBPα mediates C/EBPγ 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/EBPα-C/EBPγ balance and upregulated the expression of myeloid differentiation markers. Our results indicate that C/EBPγ mediates the myeloid differentiation arrest induced by C/EBPα deficiency and that targeting the C/EBPα-C/EBPγ 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/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ (5–8). In the hematopoietic system, C/EBPα plays a crucial role in the commitment of multipotent progenitor cells into the myeloid lineage, and mice deficient in C/EBPα 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/EBPα function in acute myeloid leukemia (AML) (11–17). C/EBPβ 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/EBPε, C/EBPδ, and C/EBPζ 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/EBPγ is the only C/EBP member that has not been studied in the context of myeloid differentiation or human AML.

C/EBPγ 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/EBPγ 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/EBPγ alone neither activates nor represses transactivation; however, C/EBPγ can inhibit transcriptional activation by other C/EBP members in a cell-specific manner (27, 29). C/EBPγ-KO mice show a high mortality rate within 48 hours after birth, and analysis of the lymphoid lineage demonstrated that C/EBPγ plays a critical role in the functional maturation of natural killer cells (30). In addition, overexpression of C/EBPγ 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/EBPγ is a suppressor of myeloid differentiation in these AML samples and that targeting the C/EBPα-C/EBPγ 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*

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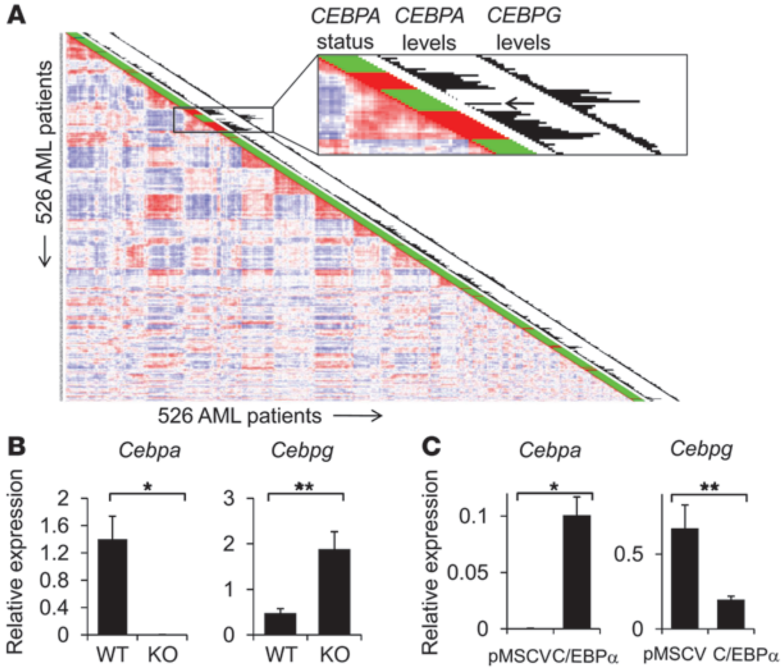

Figure 1

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-6r*α (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

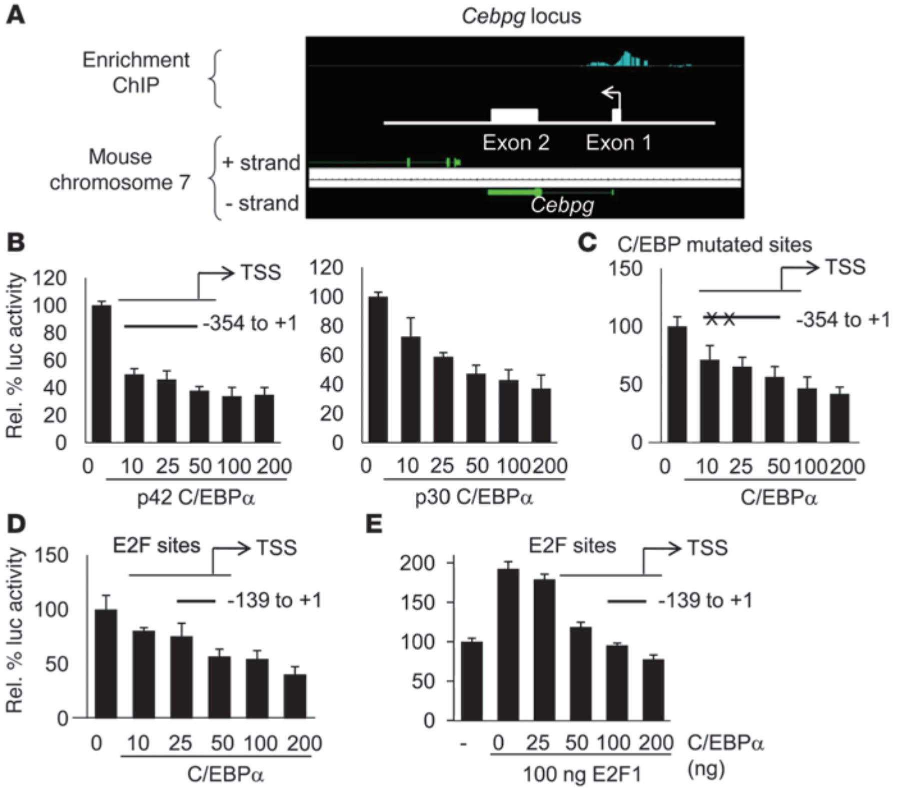

Figure 2

C/EBPα interacts with the *Cebpg* promoter and mediates *Cebpg* repression. (**A**) ChIP on DNA promoter microarrays (ChIP on chip). 32D/G-GSF–R-C/EBPα-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/EBPα represses *CEBPG* promoter–driven luciferase activity. 293T cells, which endogenously express C/EBPγ, were cotransfected with 100 ng *CEBPG* luciferase reporter vector (–354 to +1) and increasing amounts of a C/EBPα 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/EBPα repression. 100 ng of the mutated reporter vector were cotransfected with increasing amounts of p42 C/EBPα plasmid. (**D**) C/EBPα 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/EBPα expression plasmid. (**E**) C/EBPα reduces E2F1 transactivation of the C/EBPγ promoter. 100 ng of the short C/EBPγ reporter construct was cotransfected with 100 ng E2F1 expression construct and increasing amounts of C/EBPα 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/EBPα could immunoprecipitate *Cebpg* promoter. Similarly to the C/EBPα-ER overexpressing system, we observed immunoprecipitation of *Cebpg* promoter by endogenous C/EBPα (Supplemental Figure 2A), but not of a control upstream region (data not shown). To further investigate the binding of C/EBPα to the *CEBPG* promoter, we used K562 cells stably transfected with C/EBPα-ER as a model for human granulocytic differentiation (39). We identified a putative C/EBPα-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/EBPα 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/EBPα can bind to the proximal promoter of *CEBPG* and potentially downregulate *CEBPG* expression.

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

To identify the *cis*-acting elements on the *CEBPG* promoter that respond to C/EBPα, 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/EBPα expression vector showed similar C/EBPα-mediated repression in both C/EBPγ 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

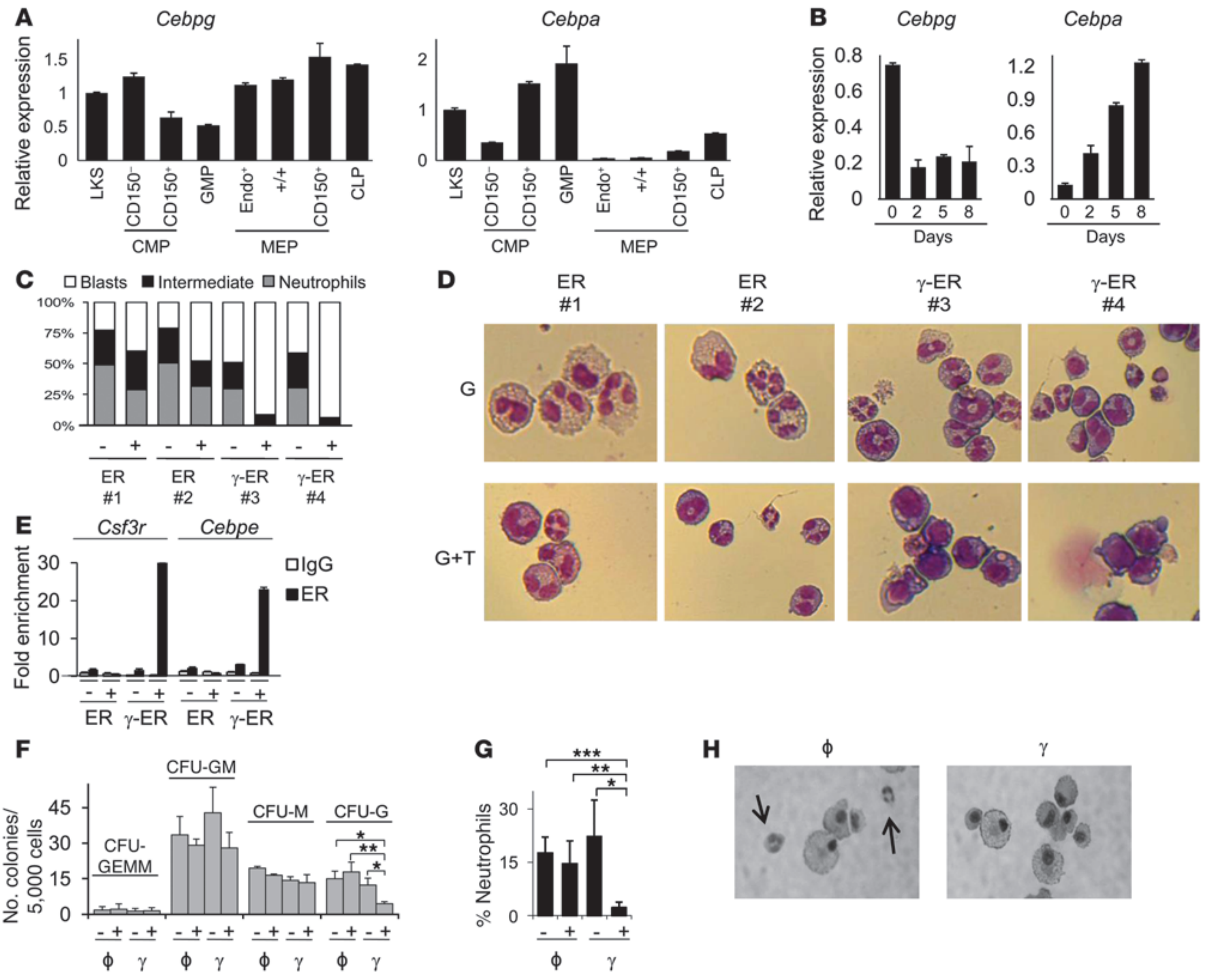

Figure 3

C/EBPγ is downregulated with neutrophilic differentiation, whereas constitutive expression of C/EBPγ 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/EBPγ-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 cytospins of 2 ER and 2 C/EBPγ-ER clones (day 8) cultured in G-CSF (G) with or without 4-hydroxytamoxifen (T). (**E**) C/EBPγ binds to the proximal promoter of *Csf3r* (encoding G-CSF–R) and *Cebpe*. ChIP on C/EBPγ-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/EBPγ 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 cytospun 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 cytospins of sorted GFP$^+$ infected cells. Original magnification, ×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/EBPα was still able to repress the luciferase activity (Figure 2C). Thus C/EBPα 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/EBPα can act as a transcriptional repressor through E2F-binding sites (40, 41). We determined the presence of 3 E2F-binding sites in the C/EBPγ luciferase constructs (Supplemental Figure 2B). To determine whether the E2F sites were required for C/EBPα repression, we generated a luciferase construct containing solely these 3 sites (–139 bp to +1 bp from TSS) and observed that C/EBPα 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

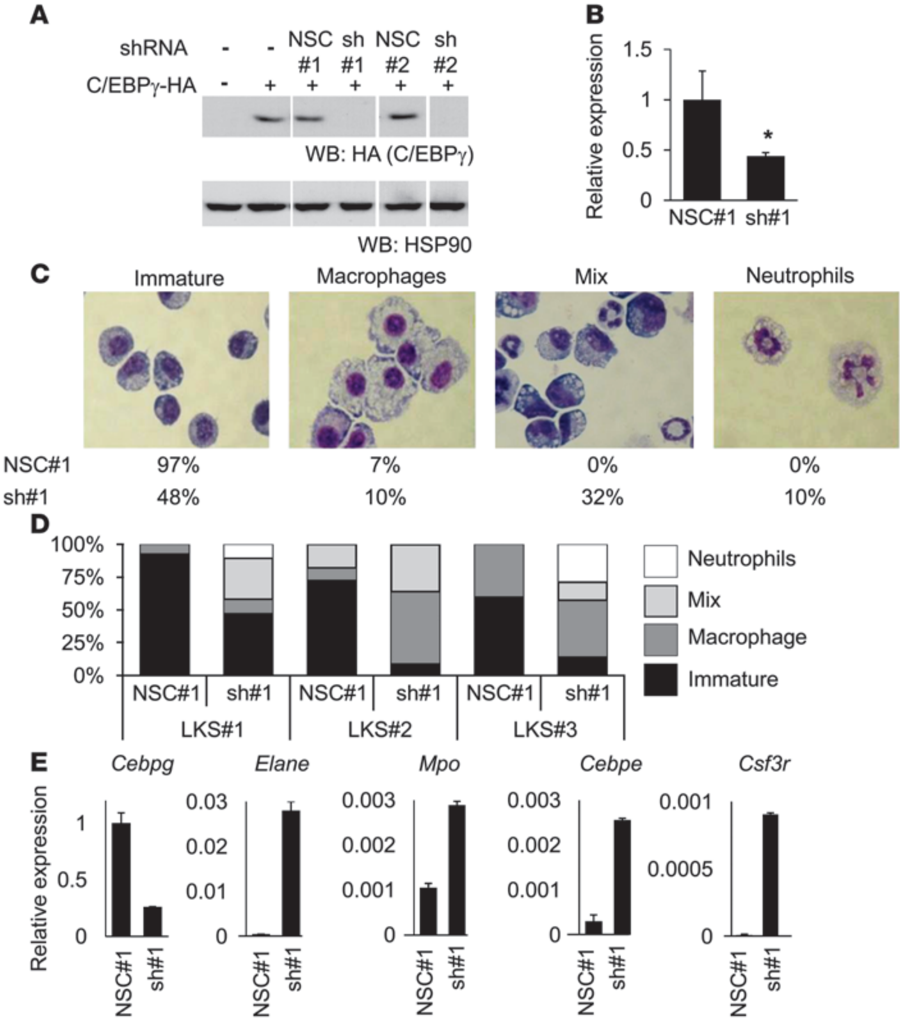

Figure 4

Downregulation of C/EBPγ in murine C/EBPα-KO LKS cells restores neutrophilic differentiation in cell culture. (**A**) Western blot analysis on 293T cells transfected with or without C/EBPγ-HA in combination with shRNA-expressing constructs. sh, shRNA targeting C/EBPγ. Blots were stained with HA and HSP90 (loading control) antibodies. (**B**) LKS cells from pI:pC-treated *Cebpa*$^{Flox/Flox}$ cre^{+} (KO) mice were transduced with either NSC#1 or C/EBPγ 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/EBPα-KO LKS cells were infected with NSC#1 or C/EBPγ 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, monocyte-macrophages, and neutrophils; and neutrophilic colonies had only neutrophils. Original magnification, ×40. (**D**) Percentage of the distinct colony types determined by cell morphology analysis in individual colonies from NSC#1- or sh#1-infected C/EBPα-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/EBPα-KO LKS cells 12 days after semi-solid culture (cultures shown in **D**). Expression of *Cebpg*, neutrophil elastase (*Ela2*), *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/EBPα regulates *CEBPG* through these E2F-binding sites. First, we assessed whether E2F binding to the *CEBPG* promoter could be affected by C/EBPα. ChIP performed on K562-C/EBPαER cells at distinct time points upon E_2 treatment showed that C/EBPα 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/EBPα had no effect on E2F1 binding to a consensus E2F DNA–binding site (40). Alternatively, C/EBPα 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/EBPα expression plasmid (Figure 2E). Together, these results indicate that C/EBPα can bind to *CEBPG* proximal promoter and downregulate *C/EBPγ* expression by affecting E2F1 transcriptional activity.

Next, we investigated whether changes in chromatin structure, which regulate gene expression, would be present in the C/EBPα-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/EBPα-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/EBPα-KO cells.

Cebpg is expressed in murine stem/progenitor cells and downregulated in committed myeloid cells. To understand the role of C/EBPγ 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

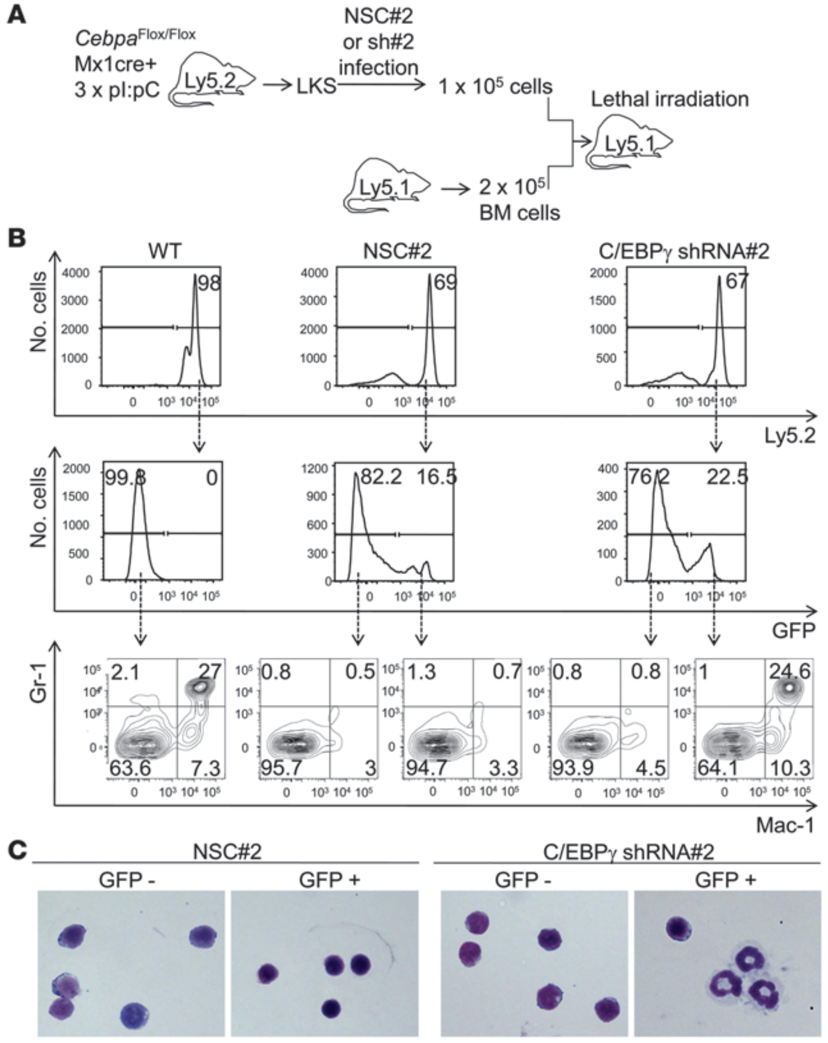

Figure 5

Downregulation of C/EBPγ in murine C/EBPα-KO LKS cells restores neutrophilic differentiation in vivo. (**A**) Schematic representation of the bone marrow transplantation experiment. (**B**) Sorted LKS cells from C/EBPα-KO mice were infected with either NSC#2 or C/EBPγ 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, ×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/EBPα 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/EBPγ is downregulated in G-CSF–induced neutrophilic differentiation. To further investigate C/EBPγ 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/EBPγ 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/EBPα 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/EBPγ and C/EBPα levels are inversely correlated during neutrophilic differentiation and that C/EBPγ is more abundant in immature cells, similar to the bone marrow–sorted subsets.

Overexpression of C/EBPγ induces a block of granulocytic differentiation. To investigate whether downregulation of C/EBPγ is required for granulocytic differentiation, we overexpressed C/EBPγ in the 32D/G-CSF–R system described above. We introduced a tamoxifen-inducible form of C/EBPγ (C/EBPγ-ER) into 32D/G-CSF–R cells. ER control clones (containing the estrogen receptor peptide without C/EBPγ) and C/EBPγ-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/EBPγ-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/CEBPγ-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/EBPγ-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/EBPγ promotes a block of granulocytic differentiation, we made use of the 32D/G-CSF–R cell line expressing either C/EBPγ-ER or the ER control alone. Cells were treated for 4 hours with 4-hydroxytamoxifen

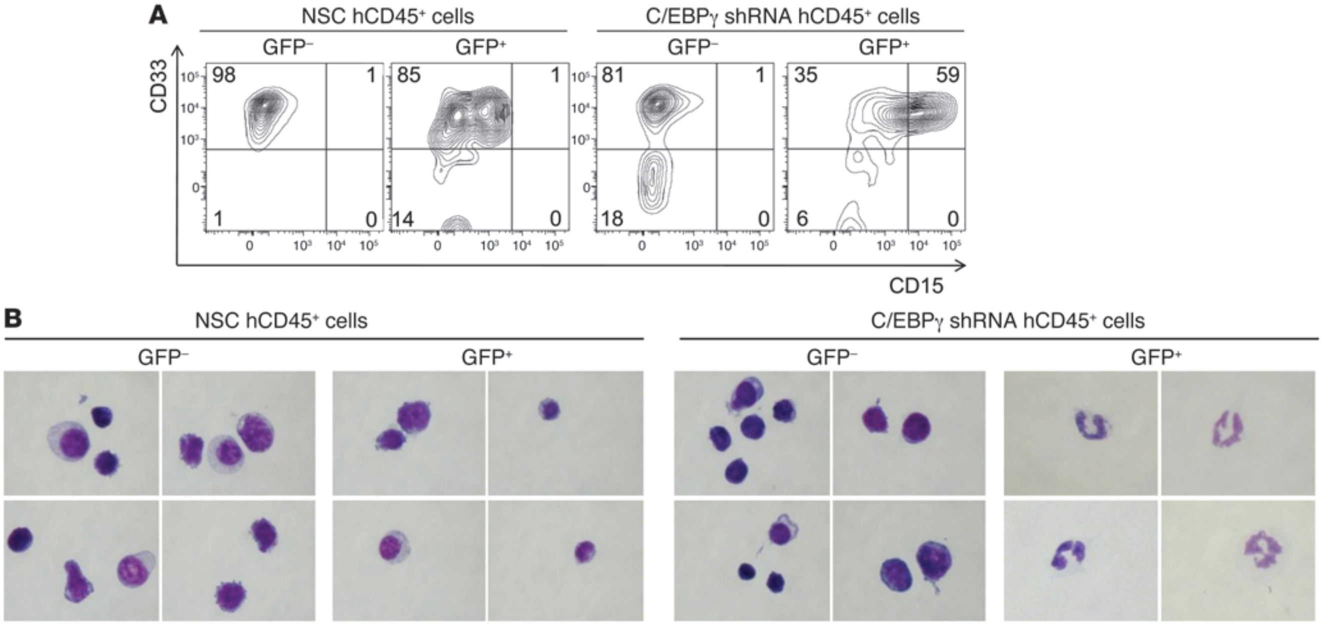

Figure 6

Downregulation of C/EBPγ in human AML cells restores neutrophilic differentiation in vivo. (**A**) Bone marrow analysis of NSG recipient mice 16 weeks after transplantation of human AML cells. Plots represent 2 individual mice transplanted with 1.25 × 10^6 cells infected with either NSC (nonsilencing control) shRNA lentivirus ($n = 2$) or a specific human C/EBPγ shRNA lentivirus ($n = 2$). Plots are gated for human CD45+ cells and divided by means of GFP: GFP− (noninfected) and GFP+ (infected cells). Images show human CD33 and CD15 expression and indicate the percentage of cells in each quadrant. (**B**) Cell morphology on Wright-Giemsa–stained cytospins of sorted human CD45+ mononuclear cells according to GFP levels. Original magnification, ×100.

and ChIP was performed using an ER-specific antibody or an IgG control antibody. Oligos were designed on the proximal promoter of putative target genes, such as *Csf3r* (44) and *Cebpe* (45), which presented potential C/EBP-binding sites and were downregulated upon 4-hydroxytamoxifen treatment. Quantitative RT-PCR showed enrichment of the *Csf3r* and *Cebpe* promoters upon 4-hydroxytamoxifen treatment in the C/EBPγ-ER–expressing cells, but not in the absence of 4-hydroxytamoxifen or the ER control cells (Figure 3E). Further, enrichment was not observed in the IgG antibody control (Figure 3E) or in deserted regions (data not shown).

Next, we investigate whether enforced C/EBPγ expression would affect neutrophilic differentiation of murine bone marrow cells. *Cebpg* and pMSCV empty vector control were introduced into WT lineage depleted bone marrow cells, and overexpression was verified by quantitative RT-PCR (Supplemental Figure 3F). GFP− (noninfected) and GFP+ (infected) cells were cultured in semi-solid medium, and morphological analysis determined that the C/EBPγ overexpressing cells had reduced number of granulocytic-like colonies (G-CFU), whereas the ability to form other colony types was not affected (Figure 3F). Morphological analysis of cytocentrifuged cells from the semi-solid cultures demonstrated that overexpression of C/EBPγ resulted in a lack of mature neutrophils (Figure 3, G and H). Together, these experiments demonstrate that C/EBPγ overexpression induces a block in granulocytic differentiation, suggesting that downregulation of C/EBPγ is required for myeloid differentiation.

Downregulation of C/EBPγ rescues granulopoiesis in murine C/EBPα-KO stem/progenitor cells. C/EBPα-KO mice are characterized by the lack of mature neutrophils in the bone marrow and blood (9, 10). Our observations that LKS from this mouse model have high levels of *Cebpg* mRNA and that sustained C/EBPγ expression blocks granulocytic differentiation (Figure 1B and Figure 3, C–H) led us to hypothesize that downregulation of C/EBPγ is required for neutrophilic differentiation in vivo. To study the contribution of C/EBPγ to the block in neutrophilic differentiation, we designed several shRNA constructs specifically targeting *Cebpg* (and not other C/EBPs) and demonstrated their efficiency at protein and RNA levels (Figure 4A and Supplemental Figure 4, A and B). C/EBPα-KO LKS cells were infected with a lentivirus expressing either a C/EBPγ-specific shRNA (sh#1) or a nonsilencing control shRNA (NSC#1). *Cebpg* was significantly downregulated in sh#1-infected LKS cells in comparison with cells infected with the NSC#1 (Figure 4B). Furthermore, *Cebpa* expression could not be detected in these cells (data not shown). Each individual colony in semi-solid culture was picked, and morphological analysis of cytocentrifuged cells revealed that downregulation of C/EBPγ gave rise to neutrophil-containing colonies (Figure 4, C and D). Quantitative RT-PCR of these cultures showed upregulation of granulocyte-specific genes such as neutrophil elastase (*Elane*), *Mpo*, *Cebpe*, and G-CSF–R (*Csf3r*) in C/EBPγ shRNA-infected cells (Figure 4E). Serial replating experiments demonstrated that while C/EBPα-KO NSC#1-infected cells could be replated for up to 4 times, downregulation of C/EBPγ in these cells abolished their replating abilities (Supplemental Figure 4C). Further, C/EBPα-KO infected cells were also placed in liquid culture, and flow cytometric analysis revealed a decrease in immature cell-surface markers such as c-kit and Sca-1 and an increase in mature markers such as Gr-1 and Mac-1 in sh#1-infected cells in comparison with NSC#1-infected cells (Supplemental Figure 4D).

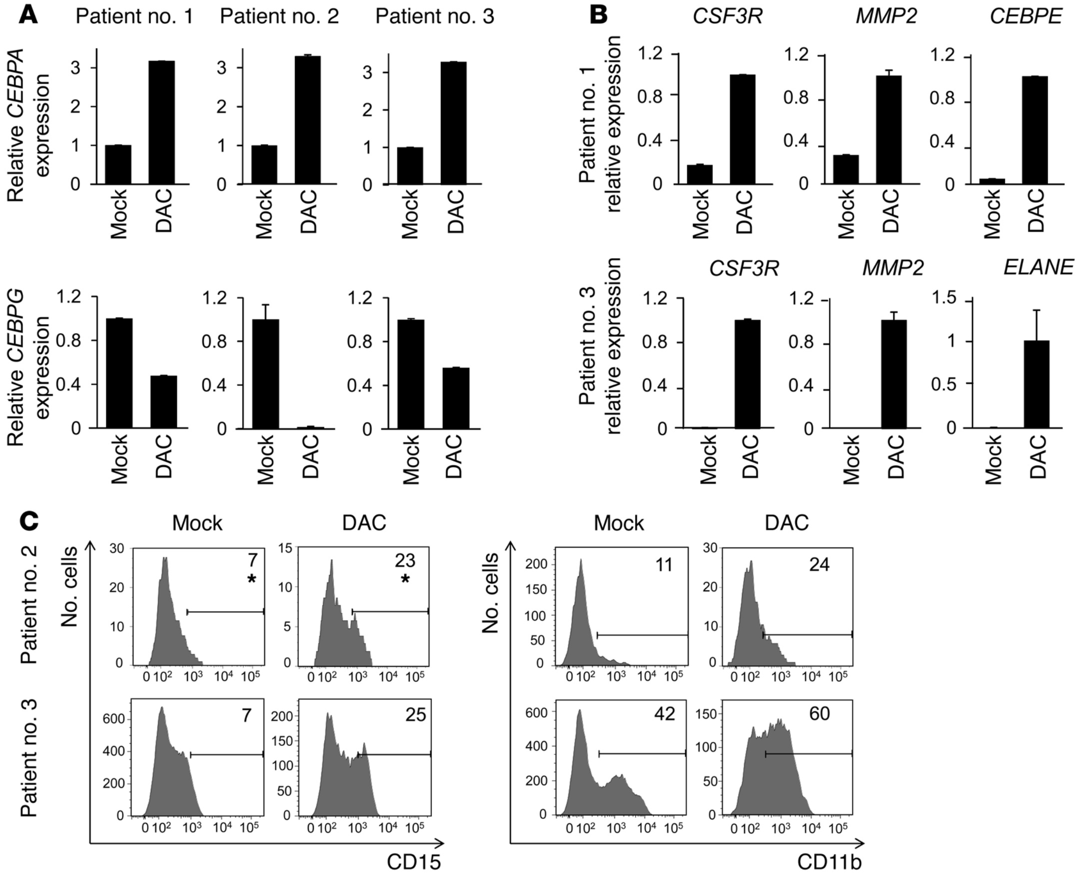

Figure 7

DAC treatment restores the C/EBPα-C/EBPγ balance and promotes differentiation of primary human AML samples characterized by C/EBPα silencing and C/EBPγ upregulation in vitro. (**A**) Quantitative RT-PCR in 3 independent AML patient samples treated with either mock control or 1 μM 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 μM 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/EBPα 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 cytospins showed a more differentiated phenotype in cells lacking C/EBPα and downregulation of C/EBPγ (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/EBPγ was downregulated in C/EBPα-deficient LKS cells in comparison with the NSC#2 colonies (Supplemental Figure 4, H and I). Together, these results show that downregulation of C/EBPγ levels in murine C/EBPα-KO LKS cells is sufficient to restore neutrophilic differentiation in vitro. In addition, serial replating experiments suggest that downregulation of C/EBPγ restricts the increased self-renewal properties of C/EBPα-KO cells (10).

We next studied the effects of knocking down C/EBPγ in the absence of C/EBPα in vivo. For these experiments we made use of the shRNA retroviral vector containing a GFP reporter. C/EBPα-KO LKS cells were isolated and infected with retrovirus containing either shRNA#2, targeting C/EBPγ, 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/EBPα-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/EBPγ shRNA#2). Of note, 9 out of 11 mice transplanted with C/EBPγ 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

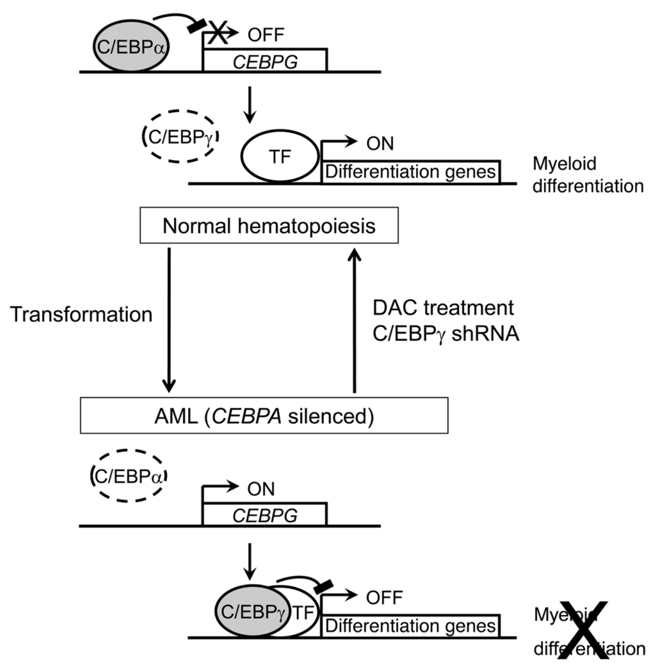

Figure 8
Proposed model. The upper panel summarizes the proposed model during normal hematopoiesis, and the lower panel represents the situation in a specific subset of AML patients (*CEBPA* silenced). In normal hematopoiesis, C/EBPα binds to the proximal promoter of *CEBPG* and represses its expression. 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′-deoxycitidine (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/EBPγ 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/EBPγ levels. We also demonstrate that treatment of these AML patient samples with demethylating agents such as DAC results in hypomethylation and reexpression of C/EBPα, which, as we predicted based in our findings in the murine and cell line models, contributes to the downregulation of C/EBPγ 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/EBPα-C/EBPγ 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/EBPα-C/EBPγ 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/EBPα, we might expect that other AML subtypes characterized by C/EBPα 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/EBPα mutants and the C/EBPα 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/EBPα 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/EBPα inactivation is the absence of C/EBPα versus low or mutated C/EBPα. 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/EBPγ 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/EBPα is involved in the active repression of *Cebpg* during myeloid differentiation. Accordingly, C/EPPγ-KO murine models have shown that C/EBPγ is relevant for proper lymphoid differentiation (30). Together, these observations highlight the possibility that C/EBPγ 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/EBPα and downregulation of C/EBPγ, 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/EBPγ expression is reduced during myeloid differentiation (53). The reciprocal expression of C/EBPα and C/EBPγ suggests that these 2 transcription factors may regulate each other. In fact, our data demonstrate that C/EBPα 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/EBPα p42 isoform represses E2F transcriptional activity (41), it has been reported that C/EBPα p30 can also suppress E2F activity (54). In fact, the luciferase reporter assays using the C/EBPγ proximal promoter indicate that both C/EBPα isoforms can repress C/EBPγ transactivation, consistent with our previously published studies demonstrating that p30 and p42 C/EBPα 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/EBPα-C/EBPγ 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/EBPγ 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/EBPγ. 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/EBPα-C/EBPγ regulation. In fact, since C/EBPα levels are reduced at the later stages of differentiation and C/EBPγ levels need to be low in order to accomplish neutrophilic differentiation, we hypothesize that other transcription factors may be involved in C/EBPγ downregulation. Further, we previously reported that C/EBPα-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/EBPγ expression during myeloid differentiation and in the C/EBPα 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/EBPα or C/EBPβ, can induce granulocytic differentiation of cell lines or primary cells in vitro (56–59). In contrast, our experiments demonstrate that C/EBPγ overexpression, either induced by ectopic expression in cell culture or by excision of C/EBPα in KO mice, results in a block of neutrophilic differentiation. Therefore, the effect of C/EBPγ 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/EBPγ resembles the C/EBPβ isoform liver inhibitory protein (LIP) (7). C/EBPγ 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/EBPγ acts as a transdominant negative inhibitor of other C/EBP members (27, 29). In addition, C/EBPγ could also functionally mimic the C/EBPα 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 μM 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 μM 17β-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 $MgCl_2$, 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 (Dynabeads; Invitrogen). IP was carried out overnight at 4°C using 20 μg rabbit polyclonal IgG anti-ERα (sc543X; Santa Cruz Biotechnology Inc.). Samples were incubated for 2.5 hours at room temperature in the presence of 200 μl 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 μl 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 μl 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 μl 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 μM E_2 or 50 nM 4-hydroxytamoxifen (Sigma-Aldrich) as indicated. IP was carried out using 20 μg rabbit polyclonal IgG anti-ERα (sc543X; Santa Cruz Biotechnology Inc.), 10 μg anti-C/EBPα (14AA) (Santa Cruz Biotechnology Inc.), 10 μg anti–E2F-1 (clones KH20 and KH95; Millipore), 10 μg anti-H3K4me3 (clone MC315; Millipore), or 10 μg anti-H3K27me3 (6002; Abcam). Oligonucleotide sequences are listed below.

Whole-genome amplification, promoter array hybridization, and promoter array data analysis. 10 μl 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 μg 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/EBPα-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/EBPβ 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/EBPγ, 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/EBPβ and increasing amounts of C/EBPγ 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/EBPα (14AA, 1:1000; Santa Cruz Biotechnology Inc.), rabbit C/EBPγ (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/EBPα was cloned into the MSCV-IReS-GFP retroviral vector (17), and p30 C/EBPα 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/EBPγ-specific shRNA#2 were cloned into the retroviral vector RSG-SIN (46). C/EBPβ 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/EBPγ-specific shRNA#1 into pLKO lentiviral vector were purchased from Open Biosystems. Human C/EBPγ-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 μg/ml) (Sigma-Aldrich). Puromycin (2 μg/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 μg/ml) (Invivogen). Single colonies were picked up 12 days after culture and cytospun; 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^{a}Pep3^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 ^{137}Cs 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-$Prkdc^{scid}$ $IL2rg^{tm1Wjl}$/szJ) 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): ATCTCGCTTGGGCGAGAGTAAG; sequence shRNA#1 (in pLKO vector): GGAAGAGAATGAACGGTTGGAA; sequence NSC#2 (in RSG-SIN vector): TCGCTTGGGCGAGAGTAAG; 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′-GACCATTAGCCTTGTGTGTACTGTATG-3′; murine endogenous C/EBPα reverse (R): 5′-TGGATCGATTGTGCTTCAAGTT-3′; murine C/EBPα overexpression F: 5′-ACGAGTTCCTGGCCGACCT-3′; murine C/EBPα overexpression R: 5′-GGGCTCCCGGGTAGTCAAAG-3′; murine C/EBPγ F: 5′-GCGCAGAGAGCGGAACAA-3′; murine C/EBPγ R: 5′-GTATCTTGAGCTTTCTGCTTGCT-3′; murine ELA 2 F: 5′-CACCATCAGTCAGGTCTTCC-3′; murine ELA 2 R: 5′-AGTCTCCGAAGCATATGCC-3′; murine MPO F: 5′-ATGCAGTGGGGACAGTTTCTG-3′; murine MPO R: 5′-GTCGTTGTAGGATCGGTACTG-3′; murine C/EBPε F: 5′-AAGGCCAAGAGGCGCATT-3′; murine C/EBPε R: 5′-CGCTCGTTTTCAGCCATGTA-3′; murine G-CSF–R F: 5′-TCCGTCACCCTAAACATCTC-3′; murine G-CSF–R R: 5′-TGGAAGGTTTCCTCTGTCAT-3′; murine Trib2 F: 5′-AGCCCGACTGTTCTACCAGA-3′; murine Trib2 R: 5′-AGCGTCTTCCAAACTCTCCA-3′; murine GAPDH F: 5′-CCAGCCTCGTCCCGTAGAC-3′; murine GAPDH R: 5′-CCCTTGACTGTGCCGTTG-3′; 18S F: 5′-ACTGGAATTACCGCCTGGCAC-3′; 18S R: 5′-CGGCTACCACATCCAAGGAAG-3′; human GAPDH F: 5′-CCACATCGCTCAGACACCAT-3′; human GAPDH R: 5′-CCAGGCGCCCAATACG-3′; human G-CSF–R F: 5′-TTTCAGGAACTTCTCTTGACGAGAA-3′; human G-CSF–R R: 5′-CGAGCCGAGCCTCAGTTTC-3′; human C/EBPε F: 5′-CTCCGATCTCTTTGCCGTGAA-3′; human C/EBPε R: 5′-TGGGCCGAAGGTATGTGGA-3; human C/EBPα F: 5′-TCGGTGGACAAGAACAG-3′; human C/EBPα R: 5′-GCAGGCGGTCATTG-3′; human C/EBPα taqman probe: 5′-TGGAGACGCAGCAGAAGGTG-3′; human C/EBPγ F: 5′-GGCTTGAATGTTAAAGGTGTGACC-3′; human C/EBPγ R: 5′-TTGAGTCATGGAAATGGACAACTT-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′-CCGACACCACTCATGTCAATGGCTG-3′; human gelatinase A F: 5′-GTGGGACAAGAACCAGATCACAT-3′; human gelatinase A R: 5′-GTCTGCCTCTCCATCATGGATT-3′; human neutrophil elastase F: 5′-CCACCCGGCAGGTGTTC-3′; and human neutrophil elastase R: 5′-GTGGCCGACCCGTTGAG-3′.

ChIP oligonucleotides. Sequences were as follows: murine oligos set 1 F: 5′-CTTCCCCGACTGTGGTGAG-3′; murine oligos set 1 R: 5′-GGCGTCTGATGCAACCTG-3′; murine oligos set 2 F: 5′-CGAAAGGTTTGATTGCTTC-3′; murine oligos set 2 R: 5′-TATGTGGAGAGCTGCTCT-3′; murine oligos Csf3r F: 5′-TTGCATTACAGGGTCATAGCAC-3′; murine oligos Csf3r R: 5′-TCCTAGGGGTTCCTGGTTTT-3′; murine oligos Cebpe F: 5′-TGGCTTGACACCTCACTCTG-3′; murine oligos Cebpe R: 5′-GGAGGGGTGCTTAGCAGTTA-3′; human Oligos A F: 5′-CCATTGTTCACCGTTGTGACC-3′; human oligos A R: 5′-AGCGTTTTAACACCAGGAAGC-3′; human oligos B F: 5′-CTGTGCCAATCCAAGAGTCCT-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′-GTTAGTTGTTTGGTTTTATTTTTTT-3′; C/EBPα F2: 5′-CCTAAAACAAACAAAAAAAAAAAAC-3′; C/EBPα R2: 5′-GGTTTTGTAGGTGGTTGTTTAT-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).

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