C/EBPα Is Required for Long-Term Self-Renewal and Lineage Priming of Hematopoietic Stem Cells and for the Maintenance of Epigenetic Configurations in Multipotent Progenitors

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Abstract

Transcription factors are key regulators of hematopoietic stem cells (HSCs) and act through their ability to bind DNA and impact on gene transcription. Their functions are interpreted in the complex landscape of chromatin, but current knowledge on how this is achieved is very limited. C/EBPα is an important transcriptional regulator of hematopoiesis, but its potential functions in HSCs have remained elusive. Here we report that C/EBPα serves to protect adult HSCs from apoptosis and to maintain their quiescent state. Consequently, deletion of Cebpa is associated with loss of self-renewal and HSC exhaustion. By combining gene expression analysis with genome-wide assessment of C/EBPα binding and epigenetic configurations, we show that C/EBPα acts to modulate the epigenetic states of genes belonging to molecular pathways important for HSC function. Moreover, our data suggest that C/EBPα acts as a priming factor at the HSC level where it actively promotes myeloid differentiation and counteracts lymphoid lineage choice. Taken together, our results show that C/EBPα is a key regulator of HSC biology, which influences the epigenetic landscape of HSCs in order to balance different cell fate options.

Introduction

Hematopoietic stem cells (HSCs) are responsible for the maintenance of a constant production of blood cells throughout life. To achieve this, HSCs have to tightly regulate their different fate options including self-renewal, proliferation, differentiation and apoptosis, as alterations in any of these may lead to HSC exhaustion, expansion or leukemia [1]. HSC fate options are controlled by a number of different pathways and are influenced both by the microenvironment and by the actions of cell-autonomous regulators such as transcription factors (TFs) and chromatin-interacting proteins [2].

Given their impact on gene expression, the influence of TFs on HSC properties has been the focus of several studies. Indeed, factors such as C-MYB, ERG, and PU.1 are all essential for preserving HSC self-renewal and their deletion have dramatic impact on hematopoietic maintenance both during fetal and adult life [3,4,5,6]. Other factors, as exemplified by SOX17, are required exclusively for the maintenance of fetal HSCs, whereas GFI-1 and ETV6 only appear to play a role in an adult setting [7,8,9]. TF function is interpreted in a chromatin context and, accordingly, chromatin readers and writers have been shown to be important for HSC function and maintenance. Examples include the PRC1 component BMI-1 [10,11], the maintenance DNA methyltransferase DNMT1 [12,13] as well as the H3K4 methyltransferase MLL1 [14]. Despite the importance of both TFs and chromatin context for HSC function, our knowledge on how TF binding is interpreted within an epigenetic landscape, and how they may influence epigenetic configurations remains limited. Importantly, given their inherent developmental plasticity, stem cells have been reported to exhibit unique epigenetic signatures of which the so-called bivalent configuration is the best characterized. Work in ES cells has shown that bivalently marked genes are lowly expressed, enriched in genes involved in development/differentiation, and display active (H3K4me3) as well as repressive
Hematopoietic stem cells (HSCs) are required for the lifelong generation of blood cells. To fulfill this requirement HSCs carefully balance cell fate decisions such as self-renewal, differentiation, quiescence, proliferation and death. These features are regulated in part by transcription factors, which act by controlling the expression of genes important for the functional properties of HSCs. C/EBPα is a well-known inducer of myeloid differentiation. It is lowly expressed in HSCs and its potential function in these cells has been extensively debated. Here, we demonstrate that Cebpa deletion impacts on HSC self-renewal, differentiation, quiescence and survival. Through gene expression and ChIP-seq analyses of stem and progenitor cell-enriched cell populations, we further show that C/EBPα binds to regulatory regions of genes that are induced during granulocytic differentiation, suggesting that C/EBPα acts to prime HSCs for differentiation along the myeloid lineage. Finally, we demonstrate that C/EBPα loss leads to genomic changes at genes central to HSC biology, which implies that it may act to recruit chromatin writers/erasers through mechanisms that remain to be characterized. In conclusion, our work identifies C/EBPα as a central hub for HSC function and highlights how a single transcription factor may coordinate several HSC fate options.

Author Summary

Loss of C/EBPα Compromises HSC Activity and Leads to Their Exhaustion

The reduction of absolute HSC numbers suggests an uncharacterized function of C/EBPα in HSC biology. To test this functionally, we first transplanted BM from Cebpaα−/− and Cebpa−/−;Mx1Cre mice (hereafter termed Cebpaα−/− and Cebpa−/−, respectively) and injected them with pLPC. Mice were analyzed 18–21 days later at which time point the Cebpa allele was completely recombined in Cebpaα−/− mice (Figure S1A). Of note, bone marrows (BM) of Cebpa−/− mice had an approximately 30% reduction in cellularity compared to Cebpa−/− littermates due to the loss of neutrophil granulocytes (Figure S1B, C). In line with its requirement for myeloid differentiation [21] publically available gene expression profiles (http://servers.binf. ku.dk/haemaoexplorer/) [24] revealed a distinct expression pattern in which Cebpa is lowly expressed in the stem cell compartment and becomes transiently upregulated during commitment to the granulocytic monocytic lineage (Figure 1A). Flow cytometry-based analyses of the myelo-erythroid progenitor compartment verified the reported block in monocytic and granulocytic differentiation at the preGM to GMP transition in Cebpaα−/− BMs and the concomitant increase in erythroid and megakaryocytic progenitors (Figure S1D, E) [17,21]. Moving further upstream in the hematopoietic hierarchy, we found a prominent expansion of the MPP compartment (LSK, CD150−, CD48+) whereas the frequency of immunophenotypic HSCs (LSK, CD150+, CD48−) was unaltered. However, given the reduction in BM cellularity in Cebpaα−/− mice this translates into a 50% reduction in the absolute numbers of HSCs upon loss of C/EBPα (Figure 1B, C).
Competitive BM transplantation remains the gold standard for the assessment of HSC function and we therefore transplanted CD45.2 donor cells along with equal amounts of CD45.1 competitor cells. Despite the comparable HSC frequencies in Cebpa\textsuperscript{fl/fl} and Cebpa\textsuperscript{Δ/Δ} donors, loss of Cebpa leads to a marked reduction in donor contribution both to PB and to the HSC compartment of the recipients at 18-week post-transplantation (Figure 2A–C). Importantly, the competitive disadvantage of Cebpa\textsuperscript{Δ/Δ} BM cells was further exacerbated upon re-transplantation of 1 million donor cells from primary recipients having equal donor contributions, and resulted in their complete failure to contribute to the HSPC compartment of secondary recipients (Figure 2D). Next, we transplanted 20 highly purified (LSK, CD150\textsuperscript{+}, CD48\textsuperscript{2}, CD34\textsuperscript{2}) HSCs from Cebpa\textsuperscript{fl/fl} or Cebpa\textsuperscript{Δ/Δ} BM together with 200,000 unfractionated competitor BM cells. In accordance with our previous findings, Cebpa\textsuperscript{Δ/Δ} HSCs were markedly impaired in hematopoietic reconstitution and their contribution to the recipient HSC compartment was negligible (Figure 2E–G). Furthermore, upon re-transplantation of the BM from primary recipients, Cebpa\textsuperscript{Δ/Δ} donor cells were unable to contribute to hematopoietic reconstitution in secondary recipients (Figure 2H, I). Finally, by transplanting 3000 CFSE-labeled HSCs (LSK, CD150+, CD48−, CD34−) and assessing their homing to hematopoietic organs we could exclude any obvious role of CEBPa in HSC trafficking during hematopoietic reconstitution (Figure 2J).

In conclusion, loss of Cebpa has profound effects on hematopoietic reconstitution both in competitive and non-competitive settings and collectively our functional analyses demonstrate an essential role of CEBPa in HSC self-renewal and in preventing HSC exhaustion.

Cebpa Maintains HSC Quiescence and Protects Them from Cell Death

Several reports have demonstrated that CEBPa acts to restrict proliferation and a number of distinct mechanisms have been proposed (reviewed in [25]).

To test to what extent the anti-proliferative properties of CEBPa may explain the loss of self-renewal in Cebpa deficient HSCs, we first analyzed the frequencies of replicating HSCs by in vivo
BrdU incorporation. Surprisingly, we were unable to detect any major differences in the amounts of BrdU+ HSCs within Cebpafl/fl and CebpaΔ/Δ BMs (Figure 3A), suggesting that C/EBPa does not affect DNA synthesis in HSCs per se. In contrast, we found that loss of Cebpa lead to a marked exit from quiescence as evidenced by a reduction of CebpaΔ/Δ HSCs in G0 (Ki67 negative) and a compensatory increase of CebpaΔ/Δ HSCs in G1 (Figure 3B). Finally, we also found that the exit from quiescence was accompanied by a marked increase in CebpaΔ/Δ HSCs that underwent cell death and that this effect was restricted to immunophenotypic HSCs (Figure 3C).

We next tested the behavior of HSCs in vitro by first evaluating proliferation and cell death of single-sorted HSCs. Consistent with our in vivo analysis we found that loss of Cebpa resulted in both fewer and smaller colonies accompanied by an increase in cell death (Figure 3D, E). Secondly, we used single-cell tracking of HSCs in vitro to monitor the cell cycle length during the first three cell divisions [26]. Consistently, we detected no differences between the average cell cycle lengths by comparing Cebpafl/fl and CebpaΔ/Δ HSCs (Figure 3F). Thus both our in vivo and in vitro analysis demonstrated that C/EBPa does not regulate HSC proliferation per se but rather acts to maintain HSC quiescence and to protect HSCs from cell death.

Collectively, our phenotypic profiling of Cebpa deficient HSCs demonstrates that C/EBPa is a crucial regulator of the balance between quiescence, proliferation and cell death in HSCs. Hence, loss of C/EBPa leads to cell cycle entry, loss of HSC self-renewal and cell death.
Loss of Cebpa Affects Gene Expression Programs Associated with Stem Cell Functions and Differentiation

In order to get insights into the molecular mechanisms by which C/EBPa controls HSC self-renewal, we performed microarray-based gene expression analysis of Cebpa<sup>fl/fl</sup> and Cebpa<sup>D/D</sup> HSCs (LSK, CD150<sup>+</sup>, CD48<sup>2</sup>). In total, we found 110 and 261 genes to be more than 1.5x down- and upregulated, respectively, in Cebpa<sup>D/D</sup> HSCs (P < 0.05) (Figure 4A, B, Table S1, Figure S2A for verification by qRT-PCR). Consistent with our phenotypic analysis, gene set enrichment analysis (GSEA) demonstrated a marked negative correlation between HSC signature genes and the Cebpa<sup>D/D</sup> genotype (Figure 4C). Among the gene sets correlating positively with the Cebpa<sup>D/D</sup> genotype we found numerous signatures involved in quiescence/proliferation, which are likely to underlie the loss of quiescence that we observed in Cebpa<sup>D/D</sup> HSCs. Similarly, we also found a positive correlation between the expression of apoptotic signatures and the Cebpa<sup>D/D</sup> genotype as well as an up-regulation of gene sets involved in the DNA damage response pathway. The relevance of the latter is supported by the 10-fold increase of cH2AX (a marker of DNA damage) positive HSCs that we observe in Cebpa<sup>D/D</sup> BMs (Figure 4D). Thus the increased levels in cell death and DNA damage observed upon loss of Cebpa are supported both by phenotypic and gene expression analyses.

Finally, we noted that a number of gene signatures representing distinct hematopoietic lineages were selectively deregulated in Cebpa<sup>D/D</sup> HSCs. Thus whereas the expression of granulocytic-monocytic gene sets were downregulated, their lymphoid and erythroid counterparts correlated positively with the Cebpa<sup>D/D</sup> genotype. These findings strongly suggest that C/EBPa primes HSCs towards the granulocytic-monocytic lineage at the expense of lymphoid and erythroid differentiation and therefore plays a key role in specifying the lineage output from HSCs (Figure 4C).

Identification of C/EBPa Binding Sites in HSPCs

The gene expression analysis reported above identified numerous genes that were de-regulated upon loss of C/EBPa, however whether these are direct or indirect targets remained to be determined. To assess this we performed ChiP-seq analysis for C/EBPa binding in LSK cells, which contains a mixture of HSCs and, predominantly, MPPs.

Despite its low expression, we identified 397 high-confidence C/EBPa bound regions of which selected examples of ChiP-seq coverage are shown in Figure 5A (replicate 1) and verified by qPCR (Figure 5B, Table S2 for the full dataset and Figure S2B-D for data concerning the second replicate). In general, the C/EBPa bound regions were located closer to the transcription start site (TSS) of the
Figure 4. Gene expression profiling of Cebpα<sup>ΔA/ΔA</sup> HSCs shows loss of stemness and biased lineage differentiation. (A) HSCs from Cebpα<sup>ΔA/ΔA</sup> and Cebpα<sup>fl/fl</sup> mice were subjected to gene expression analysis. The table shows selected genes downregulated in Cebpα<sup>ΔA/ΔA</sup> compared to Cebpα<sup>fl/fl</sup> HSCs. (B) Selected genes upregulated in Cebpα<sup>ΔA/ΔA</sup> compared to Cebpα<sup>fl/fl</sup> HSCs. (C) Gene set enrichment analysis of Cebpα<sup>ΔA/ΔA</sup> HSCs compared to Cebpα<sup>fl/fl</sup> HSCs. (D) DNA damage as assessed by γH2AX staining of Cebpα<sup>ΔA/ΔA</sup> (n = 9) and Cebpα<sup>fl/fl</sup> (n = 8) HSCs. Data are represented as mean±SEM. doi:10.1371/journal.pgen.1004079.g004
C/EBPα Is a Key Regulator of HSC Biology

A

![Graphs showing gene expression and regulation](image)

B

![Bar graph showing enrichment](image)

C

![Density plot indicating regulatory elements](image)

D

![Graphs illustrating regulatory elements and binding sites](image)

E

![Graphs depicting PU.1 and EGR2 targets](image)

F

| GREAT GO Terms                                      | P-value | Hits |
|----------------------------------------------------|---------|------|
| Biological Process:                                 |         |      |
| Granulocyte differentiation                         | 1.31E-05| 6    |
| Myeloid cell differentiation                        | 7.80E-05| 16   |
| Myeloid leukocyte differentiation                   | 2.65E-04| 11   |
| Mouse Phenotype:                                    |         |      |
| Abnormal granulocyte morphology                     | 3.03E-11| 41   |
| Abnormal myeloid leukocyte morphology               | 2.71E-10| 56   |
| Increased granulocyte number                        | 3.57E-09| 28   |
| Pathway Commons:                                    |         |      |
| C-MYC pathway                                       | 1.10E-07| 21   |
| Validated targets of C-MYC transcriptional repression| 1.42E-05| 12   |
| Immune System                                       | 8.72E-05| 28   |

G

![Bar graph showing gene expression](image)

H

![Bar graph showing gene expression](image)

I

![Venn diagram illustrating C/EBPα peak overlap](image)
Figure 5. ChIP-seq analysis of C/EBPα binding in LSK cells. (A) Selected examples of ChIP-seq coverage of C/EBPα peaks in LSK cells. (B) ChiP-qPCR for IgG and C/EBPα. Enrichment was determined based on a positive vs. negative primer pair. (C) The distance from all C/EBPα peaks, the 25% highest C/EBPα peaks, or a random set, to the nearest TSS. The dashed lines represent the median. (D) Enriched TF binding motifs at C/EBPα-bound regions. P: P-value, AF: affinity score. See also Table S3. (E) Gene set enrichment analysis of Cebpα+/− HSCs compared to Cebpα−/− HSCs for PU.1 and EGR2 target genes. (F) GREAT analysis of C/EBPα and HSCs. We correlated changes in gene expression in GMPs, we find all regions bound by C/EBPα (Figure 5H). Finally, when we compare the overlap between C/EBPα and HSCs with the nearest C/EBPα bound region identified by ChIP-seq in LSK cells. In total 6/102 genes (6%) and 21/257 (8%) of the selected genes are depicted in Figure 6 (See Figure S3A–C for replicate data).

To systematically analyze these data, we used hierarchical clustering to separate the epigenetic profiles into distinct clusters and subsequently combined these with gene expression and C/EBPα binding data (Figure 7A). From this analysis it became evident that the vast majority of genes were highly similar in terms of their expression and H3K4me3/H3K27me3 modification patterns when Cebpα+/− and Cebpα−/− LSK cells were compared (Figure 7A-C). Although some changes in histone modification between the Cebpα+/− and Cebpα−/− genotypes reached statistical significance, they were overall small and are therefore unlikely to have major phenotypic consequences. One exception was the lowly expressed cluster 6 carrying the bivalent chromatin configuration (originally identified in ES cells [33]), which is characterized by intermediate H3K4me3/high H3K27me3 at the TSS and low H3K27me3 in the gene body. This cluster displayed a specific downregulation of the H3K4me3 mark (p = 8.93 × 10−24) around the TSS in Cebpα+/− LSKs (Figure 7D, E, Table S7) and similar to previous studies in ES cells, GO-analysis identified pathways involved in cell differentiation, regulation of cell differentiation and cell development to be enriched among the group of bivalently marked genes (Figure S4, Table S8). Strikingly, C/EBPα-bound regions were not over-represented in the bivalent cluster and the majority of genes in this cluster did not have a C/EBPα bound region within 100 kb of their TSS (data not shown). Thus, whereas C/EBPα is dispensable for the maintenance of the overall H3K4me3 and H3K27me3 modification patterns, it appears to affect the H3K4me3 status of the bivalent cluster through indirect mechanisms. This may in turn interfere with their expression later in differentiation, or alternatively, interfere with the ability of Cebpα−/− HSPCs to initiate differentiation.

We next took a pathway-centric approach and assessed the epigenetic changes in a selection of the gene sets identified in the GSEA analysis described above. Interestingly, for the stemness signature we observed marked changes in histone modifications in the regions flanking the TSS (Figure 8A, B). Specifically, we found the H3K4me3 (p = 1.83 × 10−7) and H3K27me3 (p = 1.41 × 10−5) marks to be reduced and increased, respectively, in Cebpα−/− LSKs.
cells consistent with the changes in gene expression in HSCs. The alterations in H3K4me3 and H3K27me3 identified by ChIP-seq were verified by qPCR on selected genes as well as genes, which are part of the bivalent cluster and/or the stemness signature (Figure 8C and Figure S3D). Similar to our findings for the bivalent cluster, the majority of the genes constituting the stemness signature did not have a C/EBPα bound region within 100 kb of their TSS (Table S9), again suggesting that C/EBPα most likely affects the epigenetic marks through indirect mechanisms. For the remaining signatures we do see minor changes but none of them reached statistical significance (Figure S5).

We hypothesized that the reduced level of H3K4me3 in CebpaΔ/Δ LSK cells could, in part, be explained by altered levels of H3K4me3 readers or writers. To test this we first scrutinized the HSC gene expression data for changes in the expression of candidate genes and noted that three probesets for Tet1 were marginally downregulated (1.4-fold in all three probesets) in CebpaΔ/Δ HSCs (Table S1). TET1 belongs to the TET family of 5’-methylcytosine hydroxylases, which have emerged as important epigenetic regulators of both normal and malignant hematopoiesis [34,35,36]. TET1 has been shown to bind CpG-islands of TSSs marked either by H3K4me3 or the bivalent H3K4me3/H3K27me3 signature in ES cells [37,38]. To extend these data to a hematopoietic setting we used a published TET1 dataset [37] and found that genes bound by TET1 in ES cells were enriched in the group of bivalently marked genes (cluster 6) in LSK cells (Figure 8D; p<2.2×10^{-16}). Moreover, when TET1 occupancy was used to stratify the bivalent genes, the TET1 bound fraction (56%) showed a marked decrease in H3K4me3 levels in CebpaΔ/Δ LSKs (Figure 8E; p=9.6×10^{-20}). Interestingly, this effect was restricted to the bivalent genes as CebpaΔ/Δ and control LSKs showed similar H3K4me3 levels irrespectively of the binding of TET1 (Figure 8E).

Collectively, whereas C/EBPα is largely dispensable for H3K4me3 and H3K27me3 modification patterns in HSC/MPP, its loss do lead to specific changes of the epigenetic states of bivalently marked genes as well as genes associated with stem cell function.

### Discussion

Proper control of gene transcription is crucial for essentially all biological processes and is orchestrated through a complex interplay between TFs, chromatin structure and epigenetic regulators. This also extends to the complex biology of HSCs as evidenced by the fact that mice deficient for TFs or epigenetic regulators are frequently associated with HSC phenotypes [2]. However, the mechanisms by which TFs act in a chromatin context to regulate transcription and facilitate epigenetic changes of HSCs are largely unknown.

In the present work we therefore analyzed the impact of deleting C/EBPα, one of the key transcriptional regulators of hematopoiesis, on HSC function, gene expression and the epigenetic status of key histone modifications. We find that conditional acute deletion of Cebpa leads to a 2-fold reduction of immunophenotypic HSCs and that Cebpa deficient HSCs were severely compromised. Furthermore, loss of C/EBPα promoted their exit from quiescence and was associated with a marked increase in cell death. These findings were supported by gene expression analyses, which revealed prominent transcriptional downregulation of genes associated with HSC function and quiescence as well as upregulation of genes associated with DNA damage and apoptosis. Among the pathways that displayed transcriptional changes upon loss of Cebpa, we find noticeable changes in the H3K4me3 content and H3K27me3 marks flanking the TSS for genes involved in stem cell function (stemness signature). These genes are expressed at intermediate level (data not shown) and their epigenetic changes may reflect a drift away from an epigenetic state favoring self-renewal. In addition, whereas the overall distribution of H3K4me3 and H3K27me3 marks are largely unaltered in C/EBPα-deficient LSKs, we find that loss of C/EBPα specifically affects the H3K4me3 status of bivalent genes. Interestingly, TET1 was marginally downregulated in CebpaΔ/Δ HSCs and the H3K4me3 levels were selectively reduced in CebpaΔ/Δ LSKs at bivalent genes bound by TET1. Recently it was suggested that TET1, together with the epigenetic regulators CFP1 (a recruiter of the H3K4me3 methylase SETD1) and KDM2A (a H3K36 demethylase), work synergistically to...
maintain a distinct epigenetic configuration on their target genes [39]. Thus in this context, our findings suggest that downregulation of TET1 following loss of Cebpa may affect H3K4me3 status on selected genes. Whether C/EBPa affects other histone modifications such as H3K4me1 or H3K9Ac remains to be elucidated.

The importance of C/EBPa for HSC function has recently been analyzed in an adult setting. Specifically, Ye et al. [23] found that C/EBPa acts to restrict the numbers of HSCs by inhibiting their proliferation, however the effects on HSC self-renewal was not tested in a serial transplantation set-up. Furthermore, they found N-Myc to be bound by C/EBPa in c-Kit+ cells and

Figure 7. Bivalent genes have lower H3K4me3 level in Cebpa^+/Δ^- HSPCs. (A) Clustering analysis of the H3K4me3 data at TSS and the H3K27me3 data at TSS and gene bodies. Gene expression of Cebpa^+/Δ^- and Cebpa^fl/fl HSCs (FC = Fold change) and C/EBPa-binding within 100.000 kb from TSS (column 1) and within 100.000 kb to nearest gene (column 2) are included. (B) Coverage density plot of the data in (A). (C) The gene expression in HSCs (log2, arbitrary units) of clusters 1–6 was investigated. (D) The coverage sums of the clusters in (A) in 10 kb windows centered on the TSS and across the gene body is shown. In cluster 2 and 5, the yellow, green and red curves are covered by the blue, pink and turquoise curves, respectively. (E) P-values of the difference in H3K4me3 in TSS, H3K27me3 in TSS and GB of clusters identified in (A). Black line indicates P = 0.01.

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suggested that loss of C/EBPα de-repressed N-Myc and promoted HSC expansion through an N-Myc-driven increase in proliferation. These findings contrast those reported in the present study, where we find C/EBPα to be important for the maintenance of a functional HSC pool as defined by serial transplantation and quantification of immunophenotypic HSCs. Moreover, in our HSC gene expression analysis, we do not find N-Myc to be upregulated, and C/EBPα does not bind the N-Myc promoter before the GMP state (data not shown), suggesting that C/EBPα regulates N-Myc in progenitors rather than in HSCs.

A number of methodological differences are likely to explain at least some of the differences between the findings in our study and those reported by Ye et al. First of all, whereas Ye et al. performed their analyses shortly (4–7 days after the last pIpC injection), we waited until 14–17 days after the last pIpC injection to avoid any confounding issues with the pIpC induced interferon response. Indeed, it has previously been reported that interferons influence key HSC parameters such as proliferation, apoptosis and self-renewal [40,41], and therefore, it is likely that the HSC compartment is affected differentially 4 and 14 days after treatment with pIpC. We therefore speculate that the effect of C/EBPα loss on HSCs might evolve over time, from an initial expanding phase to a strongly compromised state ultimately resulting in increased cell death and loss of self-renewal. This would explain the increased proliferation and expansion observed early after deletion of Cebpa [23], the balanced HSC numbers three weeks after deletion, the loss of HSCs in primary recipients transplanted with C/EBPα-deficient HSCs/BM and the loss of repopulation in secondary recipients.. It is recognized that HSC phenotypes can progress over time from proliferative to exhaustion especially under stress such as serial transplantations as shown for PTEN-deficiency [42,43]. Moreover, although both studies used a conditional model that facilitates the full ablation of Cebpa, the two models are not completely identical, which could potentially explain some of the diverging results.

The functional consequences of Cebpa loss have also been assessed in other developmental settings. Thus, in a pre-leukemic setting, mutation of Cebpa leads to increased HSC proliferation but its impact on HSC function was obscured by transformation to AML [22] and in a fetal setting, E.14.5 LSK cells were reported to have a competitive advantage although their proliferative status was unaltered [21]. Together with our demonstration that loss of

Figure 8. Correlation of gene expression signatures and epigenetic alterations in CebpaΔ/Δ HSPCs. (A) Stemness genes were investigated for histone modifications. Coverage density plot of H3K4me3 at TSS, H3K27me3 at TSS and Gene Body of stemness genes in CebpaΔ/Δ compared to Cebpafl/fl LSK cells. Gene expression of stemness genes in CebpaΔ/Δ compared to Cebpafl/fl HSCs was included. (B) Coverage sums of the data in (A) in 10 kb windows centered on the TSS and across the gene body. (C) ChiP-qPCR for IgG, H3K4me3 and H3K27me3 on selected genes (examples from Figure 6) and genes which are part of both the bivalent cluster 6 (Figure 7A) and the stemness signature (A). For visualization purposes the H3K27me3 enrichment was normalized to IgG. (D) Overlap of TET1 bound regions in ES cells with bivalently marked genes in LSK cells. (E) H3K4me3 level in TSS of all or bivalent marked genes in CebpaΔ/Δ and Cebpafl/fl LSK cells.

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C/EBPα Is a Key Regulator of HSC Biology
Cebpa has dramatic consequences for HSC self-renewal, these observations suggest that different mechanisms are governing HSC self-renewal during embryonic development, in the adult, and in a disease context. While this concept is not novel, and is supported by the selective importance of transcriptional regulators such as SOX17 [7] and BMI-1 [11] in fetal versus adult HSCs, C/EβPα is to our knowledge the first factor that impact oppositely on fetal and adult HSCs as well as being de-regulated in disease. Thus, our work highlights a key role of C/EβPα in HSC biology in different developmental contexts.

TFs often operate in combinations and our data suggest that at least some of the functional deficits of Cebpa<sup>−/−</sup> HSCs may be explained through crosstalk between C/EβPα and PU.1 - two TFs, which have been reported to act both synergistically and antagonistically in progenitor cells [44,45]. Our findings that ETS binding sites are overrepresented in C/EβPα bound regions in LSK cells and that PU.1 target genes are downregulated in Cebpa<sup>−/−</sup> HSCs suggest that C/EβPα may collaborate with PU.1 to activate a transcriptional program already in HSCs. PU.1 is crucial for the development of almost all blood cells and even minor alterations in its expression level have been found to impair HSC self-renewal and lead to HSC exhaustion [46]. In this context it is tempting to speculate that C/EβPα regulates HSC self-renewal through crosstalk with PU.1 and that loss of C/EβPα reduces the output of PU.1 target genes, ultimately resulting in exhaustion of the stem cell pool.

In order to maintain hematopoietic homeostasis, HSCs have to carefully balance the choice between a number of cell fate options. Whereas early reports suggested that HSCs sampled different lineage-affiliated transcriptional programs uniformly, more recent data suggest a considerable degree of heterogeneity within the HSC compartment [47,48,49]. Moreover, data from cell line experiments suggest that stochastic upregulation of key regulatory molecules including lineage-instructive TFs drives differentiation along their respective lineages [50]. However, due to the technical difficulties in assessing TF binding in low-abundant cell types it is not clear whether these factors are occupying their targets already in the HSC compartment.

When analyzing the C/EβPα bound regions in the HSC/MPP-enriched LSK population we find these to be enriched for genes that were upregulated during granulocytic-monocytic differentiation. In addition, gene sets associated with myeloid differentiation are downregulated in Cebpa<sup>−/−</sup> HSCs, suggesting that the sampling of myeloid transcriptional programs is in part controlled by C/EβPα in HSCs perhaps involving PU.1 crosstalk. Hence, in addition to its accepted role as a lineage-instructive factor required for the transition from preGMPs to GMPs [51], our data demonstrate an additional role of C/EβPα in controlling the sampling of myeloid gene programs already in HSCs through its binding to the regulatory regions of myeloid genes.

Lineage choice may be viewed as a competition between distinct lineage-affiliated programs. Interestingly, we find that loss of Cebpa leads to upregulation of lymphoid gene expression programs in Cebpa<sup>−/−</sup> HSCs, which suggests that C/EβPα may actively repress lymphoid differentiation perhaps through the inhibition of the transcriptional activities of EGR family members [52,53]. This model is also compatible with the ability of C/EβPα to transdifferentiate lymphoid cell types into macrophages [54,55], with the observation that a subclass of leukemia patients with silenced C/EβPα expression develops AML associated with distinct T-cell characteristics [56] and with the finding that preleukemic Cebpa mutant GMPs readily differentiate into T-cells [57].

In conclusion, we find that C/EβPα regulates HSC functions at several distinct levels. Not only is its loss associated with HSC exhaustion, but C/EβPα also appear to be involved in the selection of the myeloid vs. lymphoid lineage suggesting that C/EβPα primes lineage choice at the HSC level. Thus, our work highlight the complexities by which transcriptional circuits are operating to maintain functional HSCs by balancing key cell fate options such as quiescence, cell death, self-renewal and differentiation.

Materials and Methods

Mouse Colony

Animals were housed according to institutional guidelines at the University of Copenhagen and housed according to institutional guidelines. Excision of the Cebpa allele was achieved by subjecting 10–12 weeks old Cebpa<sup>−/−</sup> or Cebpa<sup>−/−;Mx1Cre</sup> mice to 3 injections (day 0, 2 and 4) with 200 µl polyinosinic-polycytidylic acid (pIpC) (1.5 mg/ml in PBS, GE Healthcare) as described previously [58]. After conditional deletion of Cebpa, the mice were treated with ciprofloxacin (100 mg/L, Actavis) in the drinking water. Mice were analysed 18–21 days after first pIpC injection.

Ethics Statement

All animal work was done with approval from the Danish Animal Ethical Committee. This study was approved by the review board at the Faculty of Health Sciences, University of Copenhagen.

Flow Cytometry

BM and PB was stained with antibodies and run on a LSRII (Becton Dickinson) or FACSaria (Becton Dickinson) and analysed using the FlowJo software. Student two-tailed T-test was used to test for significance.

Transplantation Assays

All reconstitution assays were performed using the Ly-5 congenic mouse system. Briefly, whole BM or HSCs (LSK, CD150<sup>+</sup>, CD48<sup>−</sup>) from Ly-5.2 (CD45.2) donors were transplanted by tail vein injection into 10–12 week old lethally irradiated (900 cGy) Ly-5.1 (CD45.1) mice to 3 injections (day 0, 2 and 4) with 200 µl polyinosinic-polycytidylic acid (pIpC) (1.5 mg/ml in PBS, GE Healthcare) as described previously [58]. After conditional deletion of Cebpa, the mice were treated with ciprofloxacin (100 mg/L, Actavis) in the drinking water. For all transplantation assays, PB was analysed 3–4 weeks, 8 weeks and 16–18 weeks after transplantation. The BM from the recipients was analyzed at the experimental endpoint, i.e. 16–18 weeks after transplantation. Student two-tailed T-test was used to test for significance.

Homing Assay

HSCs (LSK, CD150<sup>+</sup>, CD48<sup>−</sup>) were isolated as described above and stained with carboxyfluorescein diacetate succinimidyl ester (CFSE, CellTrace, Invitrogen) according to manufacturer’s protocol. Next, HSCs (3000/mouse) were resuspended in PBS+3% FCS and transplanted by tail vein injection into irradiated (900 cGy) Ly-5.1 (CD45.1) mice. Twelve hours later BM and spleen from recipients were isolated, enriched for c-Ki<sup>+</sup> cells, and analyzed on a LSRII for CFSE<sup>+</sup> cells. Student two-tailed T-test was used to test for significance.

In Vitro Cell Culture

Single HSCs (LSK, CD150<sup>+</sup>, CD48<sup>−</sup>) were sorted directly to round-bottom 96-well plates and cultured in serum-free medium (StemSpan SFEM, StemCell Technologies) supplemented with...
100 ng/ml SCF and 100 ng/ml TPO (Peprotech). Each well was examined by microscopy and cell numbers were recorded every other day for a week. P-values for differences in colony size were calculated using chi-square test.

Continuous Live Imaging of HSCs

HSCs (LSK, CD150+, CD48−) were sorted and cultured in serum-free medium (StemSpan SFEM, StemCell Technologies) supplemented with SCF (100 ng/ml), IL-3 (10 ng/ml), IL-6 (10 ng/ml), TPO (100 ng/ml) (Peprotech), EPO (5 U/ml) (Promocell), penicillin (50 U), streptomycin (50 µg/ml) and Hepes (10 ng/ml), TPO (100 ng/ml) (Peprotech), EPO (5 U/ml) (Propeh). Methylcellulose (mocell), penicillin (50 U/ml), streptomycin (50 µg/ml) and Hepes (10 mM) (Gibco) in T12.5 flasks coated with fibronectin (Innovative Research). Using cell observer (Zeiss), images were acquired every 90 seconds using a VBA module controlling Zeiss AxioVision 4.8 software and analyzed using TTI software [59]. Student two-tailed T-test was used to test for significance.

Gene Expression Profiling

RNA was purified from sorted HSCs (LSK, CD150+, CD48−), amplified, labeled and hybridized to the Mouse Gene 1.0 ST GeneChip Array (Affymetrix, Santa Clara, CA, USA). Raw gene expression data are available at the Gene Expression Omnibus (GEO) online database under ID GSE42498.

ChIP-seq Analysis

ChIP-seq was performed using LSK or GMP cells from Cebpa<sup>fl/fl</sup> or Cebpa<sup>ΔΔ</sup> mice. Chromatin from 100,000 or 500,000 cells was incubated with antibodies for histone marks (H3K4me3 and H3K27me3, Cell Signaling) or C/EBPα (Santa Cruz Biotechnology), respectively. The antibody-bound chromatin was captured with Protein-A sepharose beads, washed, de-cross-linked and precipitated. Precipitated DNA were mixed with 2 ng E. Coli DNA and amplified using NEB Next ChIP-seq sample prep reagent set 1 (New England Biolabs) according to manufacturer’s protocol. Libraries were sequenced on an Illumina Genome Analyzer IIx or Illumina Hiseq2000. Data was deposited in the NCBI Gene Expression Omnibus online database under ID GSE43007. See Table S10 for primers.

Bioinformatic Analyses

All reads were mapped using bowtie 0.12.7 [60] using standard parameters (Figure S3B). C/EBPα peaks were called with MACS, v. 1.4 [61] with an IgG mock sample as control and using 1×10<sup>-5</sup> as the p-value threshold. Clusters in both positional and regular heatmaps were identified by k-means clustering using the heatmaps library R package. Expressed genes were identified in C/EBPα <sup>ΔΔ</sup> samples using log2 fold change < 1.5 and p-value < 0.01. Additional and detailed Materials and Methods are available as Supporting Information (See Text S1).

Supporting Information

Figure S1  Cebpa<sup>ΔΔ</sup> mice have reduced cellularity within the BM and altered distribution of myeloid progenitors. (A) PCR on whole BM and spleen for wt, floxed (fl) and deleted (del) Cebpa allele from Cebpa<sup>fl/fl</sup> and Cebpa<sup>ΔΔ</sup> mice. (B) Cytospins of whole BM from Cebpa<sup>fl/fl</sup> and Cebpa<sup>ΔΔ</sup> mice. (C) BM cell number in femur and tibia of Cebpa<sup>fl/fl</sup> (n = 7) and Cebpa<sup>ΔΔ</sup> (n = 7) mice. (D) Distribution of myeloid progenitors in Cebpa<sup>fl/fl</sup> and Cebpa<sup>ΔΔ</sup> BM. (E) Mean of (D). Cebpa<sup>fl/fl</sup> (n = 6) and Cebpa<sup>ΔΔ</sup> (n = 3). Data are represented as mean±SEM. (EPS)

Figure S2  Gene expression analysis in Cebpa<sup>fl/fl</sup> and Cebpa<sup>ΔΔ</sup> HSCs and ChIP-seq analysis of C/EBPα binding in LSK stem and progenitor cells. (A) Gene expression of selected genes in Cebpa<sup>fl/fl</sup> (n = 3) and Cebpa<sup>ΔΔ</sup> (n = 3) HSCs was quantified using qPCR. (B) Selected examples of ChIP-seq coverage of C/EBPα peaks in LSK cells (replicate 2). (C) Correlation of C/EBPα ChIP-seq replicates. (D) qPCR of selected genes in IgG and C/EBPα ChIP (replicate 2). (EPS)

Figure S3  Analysis of replicate H3K4me3 and H3K27me27 samples. (A) Correlation of replicates in TSS +/− 1000 bp for H3K4me3 and in gene bodies for H3K27me3. (B) Mapped reads of each of the ChIP-seq samples. (C) Selected examples of ChIP-seq coverage in LSK cells. (D) qPCR of selected genes (examples from Figure S3C) and genes which are part of both bivalent and stemness genes in IgG, H3K4me3 and H3K27me3 ChIP samples. For visualization purposes the H3K27me3 enrichment was normalized to IgG. (EPS)

Figure S4  GO-term analysis of the bivalently marked cluster. (EPS)

Figure S5  Analysis of H3K4me3 and H3K27me3 level in Cebpa<sup>fl/fl</sup> and Cebpa<sup>ΔΔ</sup> in signatures identified in GSEA. The corresponding p-values were determined. The black line in the p-value histogram indicates P = 0.01. (TIF)

Table S1  Gene expression analysis of Cebpa<sup>ΔΔ</sup> HSCs and Cebpa<sup>fl/fl</sup> HSCs. (XLS)

Table S2  Genes associated with C/EBPα bound regions in LSKs. C/EBPα peaks were associated to nearest TSS. Shown are the peak positions, the distance from the C/EBPα peaks to the nearest gene TSS, the gene expression fold changes between Cebpa<sup>fl/fl</sup> and Cebpa<sup>ΔΔ</sup> HSCs and p-values for gene expression changes. NA designates genes not assigned on the microarray. (XLSX)

Table S3  Motif analysis at C/EBPα bound regions in LSKs. Pos hit and pos_no_hits designates the number of hits and the number with no hits in the tested dataset, respectively. Neg hit and neg_no_hits designates the number of hits and the number with no hits in the background dataset, respectively. (XLSX)

Table S4  Pathways, GO-terms and mouse phenotypes identified via GREAT analysis of C/EBPα bound regions in LSK cells. (XLSX)

Table S5  Genes associated with C/EBPα bound regions in GMPs. C/EBPα peaks were associated to nearest TSS. Shown are the peak positions and the distance from the C/EBPα peaks to the nearest gene TSS. (XLSX)

Table S6  De-regulated genes in Cebpa<sup>ΔΔ</sup> HSCs and their association with nearest C/EBPα bound region. The TSSs of deregulated (>1.5 fold) genes were correlated to nearest C/EBPα bound region. Shown are gene expression (log2) in HSCs, fold change (FC), p-value and distance to nearest C/EBPα bound region. (XLSX)

Table S7  Distance from the TSS of genes in cluster 6 to nearest C/EBPα bound region. Shown are normalized and scaled values for peak coverage at the TSS (+/− 2000 bp) and in gene bodies.
corrected by Bonferroni correction. In order to account for multiple testing, p-values were

Table S8 GO-term analysis of bivalent genes in cluster 6. The
metric tests. In order to account for multiple testing, p-values were

Table S9 Genes constituting the stemness signature analyzed for
gene expression in Cebpα−/− and Cebpβ−/− HSCs and distance to nearest
C/EBPβ bound region.

Table S10 Primer sets used for qPCR.

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Author Contributions
Conceived and designed the experiments: MSH TS BTP. Performed the experiments: MSH AKF MBS PSF JSJ. Analyzed the data: MSH FKBL. JW NR FOB PSH BTP. Wrote the paper: MSH BTP.
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