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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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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 (H3K27me3) marks [15,16].

HSCs also harbor unique chromatin configurations, which the so-called bivalent configuration is the best characterized. In this context, the transcription factor C/EBPα is required for the maintenance of the bivalent configuration, and for the differentiation of multipotent progenitors [16]. C/EBPα is a key modulator of hematopoiesis, and for the maintenance of fetal HSCs, whereas GFI-1 and ETV6 only appear to play a role in an adult setting [7,8,9]. 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.
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 epigenetic 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.

(H3K27me3) histone marks [15,16]. As stem cells progress along the path of differentiation the bivalent configuration is resolved into an active or repressed state with a concomitant upregulation or downregulation, respectively, of the expression of previously marked genes [15,16]. To what extent the bivalent signature is influenced by loss of TFs in HSCs has not been characterized.

C/EBPα is an important myeloid TF that functions not only by binding to regulatory DNA elements and directing transcription, but also through its ability to constrain proliferation by inhibiting the transcriptional activity of E2F-complexes [17,18,19,20]. In the hematopoietic system loss of C/EBPα leads to a differentiation block upstream of the Granulocytic Monocytic Progenitor (GMP) compartment, 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.

C/EBPα binding using Chromatin ImmunoPrecipitation coupled to sequencing (ChIP-seq). Our findings demonstrate that C/EBPα modulates key pathways associated with HSC function and differentiation, in part by affecting the epigenetic states of genes associated with some of these.

Loss of C/EBPα Affects the Numbers of Immunophenotypic HSPCs

To investigate the functional importance of C/EBPα in the HSPC compartment, we generated Cebpa<sup>fl/fl</sup> and Cebpa<sup>0/0</sup>;Mx1Cre mice (hereafter termed Cebpa<sup>A</sup> and Cebpa<sup>Δ/A</sup>, respectively) and injected them with pIpC. Mice were analyzed 18–21 days later at which time point the Cebpa allele was completely recombined in Cebpa<sup>Δ/A</sup> mice (Figure S1A). Of note, bone marrows (BM) of Cebpa<sup>Δ/A</sup> mice had an approximately 30% reduction in cellularity compared to Cebpa<sup>A</sup> 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/haematoexplorer/) [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 mononuclear 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 Cebpa<sup>Δ/A</sup> 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<sup>+</sup>, CD48<sup>+</sup>) and the frequency of immunophenotypic HSCs (LSK, CD150<sup>+</sup>, CD48<sup>-</sup>) was unaltered. However, given the reduction in BM cellularity in Cebpa<sup>Δ/A</sup> mice this translates into a 50% reduction in the absolute numbers of HSCs upon loss of C/EBPα (Figure 1B, C).

Loss of Cebpa 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 Cebpa<sup>Δ/A</sup> and Cebpa<sup>0/0</sup> mice non-competitively into lethally irradiated recipient mice. Whereas all mice receiving Cebpa<sup>0/0</sup> BM cells survived, 4/16 of those receiving Cebpa<sup>Δ/A</sup> BM died within 16 weeks (Figure 1D). In the remaining mice Cebpa<sup>Δ/A</sup> donor cells contributed to the peripheral blood to a similar extent as the Cebpa<sup>0/0</sup> control at 8 and 16 weeks post-transplantation, but exhibited a lower level of reconstitution at three weeks post-transplantation due to the requirement of C/EBPα for the production of the early reconstituting neutrophil granulocytes (Figure 1E). Importantly, we find the recipients to be completely devoid of donor-derived granulocytes at all time points (Figure 1F and data not shown) showing that Cebpa is fully recombined in the transplanted cells and that it is equally important for granulocytic differentiation in a transplantation setting. Remarkably, the frequency of Cebpa<sup>Δ/A</sup> HSCs was reduced 20-fold in the recipient BMs 16-weeks post-transplantation, whereas the effects on other HSPC subsets were similar to those observed in non-transplanted animals (Figure 1G).

To assess this further, we serially transplanted BM from primary recipients and found that only 3/17 of the secondary recipients reconstituted with Cebpa<sup>Δ/A</sup> BM survived 16 weeks post-transplantation (Figure 1D).

Results

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To assess this further, we serially transplanted BM from primary recipients and found that only 3/17 of the secondary recipients reconstituted with Cebpa<sup>Δ/A</sup> BM survived 16 weeks post-transplantation (Figure 1D).
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<sup>fl/fl</sup> and Cebpa<sup>Δ/Δ</sup> 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<sup>Δ/Δ</sup> 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<sup>+</sup>, CD48<sup>−</sup>, CD34<sup>−</sup>) HSCs from Cebpa<sup>fl/fl</sup> or Cebpa<sup>Δ/Δ</sup> BM together with 200,000 unfractionated competitor BM cells. In accordance with our previous findings, Cebpa<sup>Δ/Δ</sup> 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<sup>Δ/Δ</sup> donor cells were unable to contribute to hematopoietic reconstitution in secondary recipients (Figure 2H, I). Finally, by transplanting 3000 CFSE-labeled HSCs (LSK, CD150<sup>+</sup>, CD48<sup>−</sup>) and assessing their homing to hematopoietic organs we could exclude any obvious role of C/EBPa 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 C/EBPa in HSC self-renewal and in preventing HSC exhaustion.

Cebpa Maintains HSC Quiescence and Protects Them from Cell Death

Several reports have demonstrated that C/EBPα 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 C/EBPα 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 Cebpa\(^{fl/fl}\) and Cebpa\(^{Δ/Δ}\) BMs (Figure 3A), suggesting that C/EBP\(\alpha\) 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 Cebpa\(^{Δ/Δ}\) HSCs in G0 (Ki67 negative) and a compensatory increase of Cebpa\(^{Δ/Δ}\) HSCs in G1 (Figure 3B). Finally, we also found that the exit from quiescence was accompanied by a marked increase in Cebpa\(^{Δ/Δ}\) 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 Cebpa\(^{fl/fl}\) and Cebpa\(^{Δ/Δ}\) HSCs (Figure 3F). Thus both our in vivo and in vitro analysis demonstrated that C/EBP\(\alpha\) 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/EBP\(\alpha\) is a crucial regulator of the balance between quiescence, proliferation and cell death in HSCs. Hence, loss of C/EBP\(\alpha\) 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/EBPα controls HSC self-renewal, we performed microarray-based gene expression analysis of Cebpa^{fl/fl} and Cebpa^{Δ/Δ} HSCs (LSK, CD150+, CD48−). In total, we found 110 and 261 genes to be more than 1.5x down- and upregulated, respectively, in Cebpa^{Δ/Δ} 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^{Δ/Δ} genotype (Figure 4C). Among the gene sets correlating positively with the Cebpa^{Δ/Δ} genotype we found numerous signatures involved in quiescence/proliferation, which are likely to underlie the loss of quiescence that we observed in Cebpa^{Δ/Δ} HSCs. Similarly, we also found a positive correlation between the expression of apoptotic signatures and the Cebpa^{Δ/Δ} 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 γH2AX (a marker of DNA damage) positive HSCs that we observe in Cebpa^{Δ/Δ} 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^{Δ/Δ} HSCs. Thus whereas the expression of granulocytic-monocytic gene sets were downregulated, their lymphoid and erythroid counterparts correlated positively with the Cebpa^{Δ/Δ} genotype. These findings strongly suggest that C/EBPα 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/EBPα Binding Sites in HSPCs

The gene expression analysis reported above identified numerous genes that were de-regulated in Cebpa^{Δ/Δ} HSCs. Thus whereas the expression of granulocytic-monocytic gene sets were downregulated, their lymphoid and erythroid counterparts correlated positively with the Cebpa^{Δ/Δ} genotype. These findings strongly suggest that C/EBPα 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).

![Figure 3. Cebpa^{Δ/Δ} HSCs do not have a proliferative advantage but are prone to cell death.](image-url)
Figure 4. Gene expression profiling of Cebpa\textsuperscript{A/A} HSCs shows loss of stemness and biased lineage differentiation. (A) HSCs from Cebpa\textsuperscript{D/D} and Cebpa\textsuperscript{fl/fl} mice were subjected to gene expression analysis. The table shows selected genes downregulated in Cebpa\textsuperscript{D/D} compared to Cebpa\textsuperscript{fl/fl} HSCs. (B) Selected genes upregulated in Cebpa\textsuperscript{D/D} compared to Cebpa\textsuperscript{fl/fl} HSCs. (C) Gene set enrichment analysis of Cebpa\textsuperscript{D/D} HSCs compared to Cebpa\textsuperscript{fl/fl} HSCs. (D) DNA damage as assessed by γH2AX staining of Cebpa\textsuperscript{D/D} (n = 9) and Cebpa\textsuperscript{fl/fl} (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 illustrating the expression of C/EBPα and its interactions with other genes.

B. Enrichment plot showing the enrichment of C/EBPα binding sites at various loci.

C. Density plots depicting the distribution of C/EBPα binding sites across different distances from the TSS.

D. Diagrams illustrating the enrichment of C/EBPα binding sites at specific loci.

E. Heatmaps and bar charts showing the enrichment of PU.1 and EGR2 targets.

F. Table listing GREAT GO terms, P-values, and number of hits.

G. Bar charts comparing gene expression in control and mutants.

H. Bar charts comparing gene expression in different cell lineages.

I. Circular diagram illustrating the peak overlap of C/EBPα in different subpopulations.
Cebpa is a key regulator of HSC biology. We characterized C/EBPα binding to regulatory regions identified by ChIP-seq analysis in LSK cells, showing that C/EBPα is a key regulator of HSC biology. C/EBPα binding was found to be enriched in regions with putative targets, and C/EBPα binding was correlated with gene expression changes in HSCs/MPPs. We further found that C/EBPα binding was associated with gene expression changes in HSCs/MPPs, and that C/EBPα binding was correlated with gene expression changes in HSCs/MPPs. These findings suggest a role for C/EBPα in regulating lineage decisions in HSCs/MPPs.

**Loss of Cebpa Affects the Epigenetic Configuration of Bivalent Genes and Pathways Involved in HSC Function**

Transcriptional regulation is a complex process not only involving TFs but also the chromatin context in which their function is exerted. Chromatin structure and accessibility is influenced by post-transcriptional modifications on histone tails, which are dynamically deposited and removed by chromatin modifying enzymes and serves as recruitment platforms for other chromatin binding proteins. To test if loss of Cebpa affected chromatin structure, we performed ChIP-seq analysis for histone marks H3K4me3 and H3K27me3 in LSK cells. ChIP-seq profiles of 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 Cebpafl/fl and Cebpafl/+ LSK cells were compared (Figure 7A–C). Although some changes in histone modification between the Cebpafl/+ and Cebpafl/fl 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), 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 Cebpafl/+ 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 neither in the LSKs (Table S7) nor in the GMPs (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 Cebpafl/+ 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 Cebpafl/+ LSKs compared to Cebpafl/fl LSKs. This is further supported by the ChIP-seq analysis of C/EBPα binding to regulatory regions identified by ChIP-seq analysis in LSK cells, showing that C/EBPα is a key regulator of HSC biology. C/EBPα binding was found to be enriched in regions with putative targets, and C/EBPα binding was correlated with gene expression changes in HSCs/MPPs. We further found that C/EBPα binding was associated with gene expression changes in HSCs/MPPs, and that C/EBPα binding was correlated with gene expression changes in HSCs/MPPs. These findings suggest a role for C/EBPα in regulating lineage decisions in HSCs/MPPs.
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 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/EBPα for HSC function has recently been analyzed in an adult setting. Specifically, Ye et al. [23] found that C/EBPα 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/EBPα in c-Kit+ cells and

Figure 7. Bivalent genes have lower H3K4me3 level in CebpaHSPCs. (A) Clustering analysis of the H3K4me3 data at TSS and the H3K27me3 data at TSS and gene bodies. Gene expression of Cebpa and Cebpa HSCs (FC = Fold change) and C/EBPα-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. doi:10.1371/journal.pgen.1004079.g007
suggested that loss of C/EBPa 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/EBPa 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/EBPa does not bind the N-Myc promoter before the GMP state (data not shown), suggesting that C/EBPa 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/EBPa 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/EBPa-deficient HSCs/BM and the loss of re-population 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 C/EBPa Is a Key Regulator of HSC Biology

Figure 8. Correlation of gene expression signatures and epigenetic alterations in Cebpa 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 Cebpa compared to Cebpa HSCs. Gene expression of stemness genes in Cebpa compared to Cebpa 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 Cebpa and Cebpa HSCs. doi:10.1371/journal.pgen.1004079.g008

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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/EBPα 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/EBPα 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>Δ/Δ</sup> HSCs may be explained through crosstalk between C/EBPα 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/EBPα bound regions in LSK cells and that PU.1 target genes are downregulated in Cebpa<sup>Δ/Δ</sup> HSCs suggest that C/EBPα 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/EBPα regulates HSC self-renewal through crosstalk with PU.1 and that loss of C/EBPα 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/EBPα 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>Δ/Δ</sup> HSCs, suggesting that the sampling of myeloid transcriptional programs is in part controlled by C/EBPα in HSCs perhaps involving PU.1 crosstalk. Hence, in addition to its accepted role as a lineage-instructive factor required for the transition from preGMs to GMPs [51], our data demonstrate an additional role of C/EBPα 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>Δ/Δ</sup> HSCs, which suggests that C/EBPα 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/EBPα to transdifferentiate lymphoid cell types into macrophages [54,55], with the observation that a subclass of leukemia patients with silenced C/EBPα 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/EBPα regulates HSC functions at several distinct levels. Not only is its loss associated with HSC exhaustion, but C/EBPα also appear to be involved in the selection of the myeloid vs. lymphoid lineage suggesting that C/EBPα primes lineage choice at the HSC level. Thus, our work highlight the complexities by which transcriptional circuitries 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>Δ/Δ</sup> or Cebpa<sup>Δ/Δ</sup>Mx1Cre mice to 3 injections (day 0, 2 and 4) with 200 ul 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+, CD48−) from Ly-5.2 (CD45.2) donors were transplant-ed by tail vein injection into 10–12 week old lethally irradiated (900 cGy) Ly-5.1 (CD45.1) mice +/− Ly-5.1 support BM cells. The recipient mice were treated with ciprofloxacin (100 mg/L, Actavis) in the drinking water. For all transplantation assays, PB was analysed at 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+, CD48−) 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-Kit<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+, CD48−) 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 mM) (Gibco) in T12.5 flasks coated with fibronectin (Innovative Research). Using cell observer (Zeiss), images were acquired (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 AxiosVision 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>Δ/Δ</sup> or Cebpa<sup>Δ/Δ</sup> mice. Chromatin from 100,000 or 500,000 cells was incubated with antibodies for histone marks (H3K4me3 and H3K27me3, Cell Signaling) or C/EBP<sub>a</sub> (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 Ix or and 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/EBP<sub>a</sub> peaks were called with MACS, v. 1.4 [61] with an IgG mock sample as control and using 1*10^-5 as the p-value threshold. Clusters in both positional and regular heatmaps were identified by k-means clustering using the biopython Bio Cluster module [62]. In order to identify TF motifs in C/EBP<sub>a</sub> bound regions, sequences below the peak summit +/- 70 bp were analyzed.

Additional and detailed Materials and Methods are available as Supporting Information (See Text S1).

Supporting Information
Figure S1  Cebpa<sup>Δ/Δ</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>Δ/Δ</sup> and Cebpa<sup>Δ/Δ</sup> mice. (B) Cytosins of whole BM from Cebpa<sup>Δ/Δ</sup> and Cebpa<sup>Δ/Δ</sup> mice. (C) BM cell number in femur and tibia of Cebpa<sup>Δ/Δ</sup> (n = 7) and Cebpa<sup>Δ/Δ</sup> (n = 7) mice. (D) Distribution of myeloid progenitors in Cebpa<sup>Δ/Δ</sup> and Cebpa<sup>Δ/Δ</sup> BM. (E) Mean of (D). Cebpa<sup>Δ/Δ</sup> (n = 6) and Cebpa<sup>Δ/Δ</sup> (n = 3). Data are represented as mean±SEM. (EPS)

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

Figure S3  Analysis of replicate H3K4me3 and H3K27me2 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>Δ/Δ</sup> and Cebpa<sup>Δ/Δ</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>Δ/Δ</sup> HSCs and Cebpa<sup>Δ/Δ</sup> HSCs. (XLS)

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

Table S3 Motif analysis at C/EBP<sub>a</sub> 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/EBP<sub>a</sub> bound regions in LSK cells. (XLSX)

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

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

Table S7  Distance from the TSS of genes in cluster 6 to nearest C/EBP<sub>a</sub> bound region. Shown are normalized and scaled values for peak coverage at the TSS (+/−2000 bp) and in gene bodies (EPS)
(GB) in Cluster 6 for H3K4me3 and H3K27me3, gene expression (log2) in HSCs, fold change (FC), p-value and distance to nearest C/EBPα bound region. (XLSX)

Table S8: GO-term analysis of bivalent genes in cluster 6. The significance of enriched GO terms was assessed using hypergeometric tests. In order to account for multiple testing, p-values were corrected by Bonferroni correction. (XLSX)

Table S9: Genes constituting the stemness signature analyzed for gene expression in Cebpα−/− and Cebpα−/− HSCs and distance to nearest C/EBPα bound region. (XLSX)

Table S10: Primer sets used for qPCR.

References


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Author Contributions

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

Text S1: Detailed Materials and Methods.

(DOCX)


