Phosphorylation of Serine 248 of C/EBPα Is Dispensable for Myelopoiesis but Its Disruption Leads to a Low Penetrant Myeloid Disorder with Long latency

Marie S. Hasemann1,2,3,*, Mikkel B. Schuster1,2,3,*, Anne-Katrine Frank1,2,3, Kim Theilgaard-Mönch1,2,4, Thomas Å. Pedersen5,*, Claus Nerlov5,6, Bo T. Porse1,2,3

1 The Finsen Laboratory, Rigshospitalet, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark, 2 Biotech Research and Innovation Center (BRIC), University of Copenhagen, Copenhagen, Denmark, 3 Danish Stem Cell Centre (DanStem) Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark, 4 Department of Hematology, Skanes University Hospital, University of Lund, Lund, Sweden, 5 European Molecular Biology Laboratory (EMBL) Mouse Biology Unit, Monterotondo, Italy, 6 Medical Research Council (MRC) Center for Regenerative Medicine, Institute for Stem Cell Research, University of Edinburg, Edinburg, United Kingdom

Abstract

Background: Transcription factors play a key role in lineage commitment and differentiation of stem cells into distinct mature cells. In hematopoiesis, they regulate lineage-specific gene expression in a stage-specific manner through various physical and functional interactions with regulatory proteins that are simultaneously recruited and activated to ensure timely gene expression. The transcription factor CCAAT/enhancer binding protein α (C/EBPα) is such a factor and is essential for the development of granulocytic/monocytic cells. The activity of C/EBPα is regulated on several levels including gene expression, alternative translation, protein interactions and posttranslational modifications, such as phosphorylation. In particular, the phosphorylation of serine 248 of the transactivation domain has been shown to be of crucial importance for granulocytic differentiation of 32Dc3 cells in vitro.

Methodology/Principal Findings: Here, we use mouse genetics to investigate the significance of C/EBPα serine 248 in vivo through the construction and analysis of CebpaS248A/S248A knock-in mice. Surprisingly, 8-week old CebpaS248A/S248A mice display normal steady-state hematopoiesis including unaltered development of mature myeloid cells. However, over time some of the animals develop a hematopoietic disorder with accumulation of multipotent, megakaryocytic and erythroid progenitor cells and a mild impairment of differentiation along the granulocytic-monocytic lineage. Furthermore, BM cells from CebpaS248A/S248A animals display a competitive advantage compared to wild type cells in a transplantation assay.

Conclusions/Significance: Taken together, our data shows that the substitution of C/EBPα serine 248 to alanine favors the selection of the megakaryocytic/erythroid lineage over the monocytic/granulocytic compartment in old mice and suggests that S248 phosphorylation may be required to maintain proper hematopoietic homeostasis in response to changes in the wiring of cellular signalling networks. More broadly, the marked differences between the phenotype of the S248A variant in vivo and in vitro highlight the need to exert caution when extending in vitro phenotypes to the more appropriate in vivo context.

Citation: Hasemann M5, Schuster MB, Frank A-K, Theilgaard-Mönch K, Pedersen TÅ, et al. (2012) Phosphorylation of Serine 248 of C/EBPα Is Dispensable for Myelopoiesis but Its Disruption Leads to a Low Penetrant Myeloid Disorder with Long Latency. PLoS ONE 7(6): e38841. doi:10.1371/journal.pone.0038841

Editor: Michael Alexander Rieger, Georg Speyer Haus, Germany

Received December 22, 2011; Accepted May 11, 2012; Published June 8, 2012

Copyright: © 2012 Hasemann et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Lundbeck Foundation, The NovoNordisk Foundation and the Danish Cancer Society. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: bo.porse@finsenlab.dk

† These authors contributed equally to this work.

¤ Current address: Novo Nordisk A/S, Målev, Denmark

Introduction

The human body contains trillions of blood cells that are continuously replaced through normal cell turnover. Hematopoiesis is the highly orchestrated process responsible for regulating the generation of mature blood cells from a rare population of hematopoietic stem cells (HSC). The HSCs possess the ability to self-renew and differentiate into all blood lineages and are the ultimate reservoir for maintaining the supply of blood cells throughout life. Multiple mechanisms are required in order to meet both the changing demands from the body and to maintain steady-state hematopoiesis [1]. In particular, many transcription factors have been shown to modulate key events in differentiation and proliferation and their function in hematopoiesis has been investigated thoroughly through the examination of knockout mice [2]. One of these transcription factors is CCAAT/enhancer binding protein alpha (C/EBPα), which is not only involved in regulation of hematopoiesis, but also exerts its function in other
tissues such as lung, liver and adipose tissue through the induction of lineage-specific gene programs in combination with an ability to promote cell cycle exit [3,4,5]. Within the hematopoietic system, C/EBPα has been shown to be important for the myeloid lineage, since conditional deletion of the Cebpα allele in the hematopoietic compartment of adult mice blocks the transition from common myeloid progenitors (CMPs) to granulocyte-monocyte progenitors (GMPs), thus resulting in complete loss of granulocytes, monocytes and eosinophils [6,7]. Besides this late granulocytic-monocytic differentiation block, fetal livers of newborn Cebpα-null mice display increased numbers of progenitors and mature cells of the erythroid lineage, suggesting that C/EBPα might play a role in repressing erythroid differentiation [7]. In line with this, overexpression of C/EBPα in erythroid progenitor cells, redirects the differentiation potential in a granulocytic direction resulting in an increased level of mature granulocytes and granulocyte-monocytic progenitors with a concomitant decrease of erythroid progenitors [8].

The activity of C/EBPα is tightly controlled through multiple layers of regulation. First of all, timely expression is required and involves regulation of gene transcription, mRNA translation and protein degradation [9,10]. Secondly, protein interactions have a major impact on the ability of C/EBPα to induce or repress gene transcription [11,12,13]. Thirdly, C/EBPα activity can be altered by posttranslational modifications such as sumoylation and phosphorylation [14,15]. The phosphorylation status of serine 21 (S21) has been shown to have a major impact on the decision to differentiate towards the monocytic or granulocytic lineage in vitro. Upon phosphorylation of S21 or expression of a phosphomimicking mutant in K-562 cells, granulopoiesis is inhibited, thereby favoring monocytic differentiation at the cost of granulocytic differentiation [15,16]. Furthermore, the phosphorylation of serine 248 in the transactivation domain has been suggested to be required for myeloid differentiation since mutating it to an alanine residue abrogates the capacity of C/EBPα to induce granulocytic differentiation of 32Dc3 cells in vitro [17]. Serine 248 (S248) is phosphorylated by activated Ras signaling and this phosphorylation increases the ability of C/EBPα to promote expression from the G-CSF receptor promoter. Therefore, it appears that phosphorylation and dephosphorylation of C/EBPα at distinct serine residues can directly push the cells towards a specific myeloid branch. However, these phosphorylation sites have only been investigated in an in vitro context and what the functions in vivo is therefore unknown.

We and others have previously reported on several Cebpα knock-in mouse models [18,19,20,21,22,23], which have provided valuable information pertaining the role of C/EBPα in myeloid differentiation and in the development of leukemia. In this study, we use knock-in mutagenesis to elucidate the importance of S248 phosphorylation for myeloid differentiation by introducing an allele of Cebpα with an alanine substituted for serine 248, thereby abrogating phosphorylation of this residue. Surprisingly, we could show that whereas myeloid differentiation of cells expressing C/EBPα-S248A is blocked in vitro, this is not the case in vivo. Thus, young CebpαS248A/S248A mice display no phenotypic alterations in the hematopoietic compartment or other tissues. In contrast, aged CebpαS248A/S248A animals develop a low-penetrant myeloid disorder characterized by a mild impairment of differentiation along the granulocytic-monocytic lineage and by the accumulation of HSCs, multipotent progenitor cells (MPPs), as well as megakaryocytic and early erythroid progenitors.

**Results**

S248 is required for C/EBPα to promote granulocytic differentiation in vitro

The murine myeloid 32Dc3 cell line has long been considered a suitable in vitro model system for analyzing myelopoiesis, since it is one of the few cell lines that can terminally differentiate into mature neutrophils. The cell line is diploid and non-leukemic in syngeneic murine recipients [24,25]. It proliferates in media containing IL-3 however, upon removal of this cytokine and addition of G-CSF, proliferation ceases and differentiation into neutrophil granulocytes proceeds. It is well documented that ectopic expression of C/EBPα in 32Dc3 cells is sufficient to induce terminal granulocytic differentiation even in the presence of IL-3, making this a suitable differentiation assay to analyze the effect of C/EBPα mutations on this process [24,25]. In order to investigate if C/EBPα-S248A is defective in granulocytic-mono-cytic in vitro differentiation as previously reported [17], 32Dc3 clones expressing either a wild type C/EBPα-estrogen receptor ligand-binding domain fusion protein (C/EBPα-ER) or the C/EBPα-S248A-ER variant were constructed and clones expressing an equal amount of protein were selected for further analysis (Figure 1A).

To test whether S248 is required for the ability of C/EBPα to promote granulocytic differentiation C/EBPα-ER was translocated to the nucleus by addition of 4-hydroxytamoxifen (4-OHT). Cells were monitored for three days, and samples were collected each day and analyzed by flow cytometry to assess proliferation (Figure 1D) and granulocytic differentiation (Figure 1B, C). As expected 32Dc3-C/EBPα-ER cells exited cell cycle within a few days, the G1/S ratio increased 15 fold (Figure 1D) and cells became positive for the granulocytic-monocytic marker Mac-1 and later for Gr-1 (Figure 1B, C). On the other hand, nuclear translocation of C/EBPα-S248A-ER did not lead to growth arrest and cells failed to express the two differentiation markers.

Thus in agreement with previous findings [17], these data show that S248 is necessary for C/EBPα to induce growth arrest and differentiation of neutrophil granulocytes in vitro.

**Initial analysis of CebpαS248A/S248A knock-in mice**

In order to investigate the importance of C/EBPα-S248 in vivo, we generated a knock-in mouse line, in which the wild type Cebpα gene was replaced with an allele expressing C/EBPα-S248A. CebpαS248A/S248A knock-in mice were born and weaned in Mendelian ratios, were physically indistinguishable from both wild type littermates and the more appropriate CebpαKI/KI controls [20,26], showed no visible signs of illness and were fully fertile (data not shown), thus demonstrating that S248 of C/EBPα is fully dispensable for embryonic survival. Furthermore, inspection of tissues in which C/EBPα function has previously been demonstrated to be important, such as liver, lung, spleen and white adipose tissue revealed no obvious abnormalities neither in terms of morphology nor size in the CebpαS248/248 animals (Figure S1 and data not shown). These findings suggest that S248 is dispensable for the development and maintenance of these tissues.

**Young CebpαS248A/S248A mice are phenotypically normal in the hematopoietic system**

C/EBPα is a key regulator of myeloid differentiation and altered C/EBPα activity has been shown to affect hematopoietic development and predispose to myeloid malignancies such as AML [3,21,22]. Moreover, the finding that C/EBPα-S248A was unable to direct granulocytic-monocytic differentiation in vivo prompted us to investigate the hematopoietic system of CebpαS248A/S248A mice.
S248A/S248A mice. Bone marrows (BM) from 8 week-old Cebpa S248A/S248A and Cebpa KI/KI mice were collected and stained with antibodies for cell-surface markers specific for the different mature lineages and analyzed by flow cytometry. Surprisingly, in contrast to the in vitro data reported above, mutation of S248 did not alter the frequency of mature granulocytes (Mac-1+, Gr-1+) and the prevalence of other BM populations such as B cells (B220+), T cells (CD4+ and/or CD8+), erythroid cells (Ter119+, CD71+/int) was also unaffected (Figure 2A, B). Furthermore, examination of cytospins prepared from BM or spleen from 8 week-old mice showed no aberrations in cellular morphologies or distributions (Figure 2C, D). These data suggest, that S248 of C/EBPα is dispensable for the in vivo development of mature hematopoietic lineages.

Since Cebpa null mice have previously been shown to accumulate myeloid progenitors and to harbor a differentiation block upstream of the GMP [6], we analyzed the myeloid progenitor compartment using the antibody panel reported by Pronk et al. [27]. However, we were unable to detect any major alterations in the cellular distributions of myeloid progenitors or HSCs/MPPs (Figure 3A, B), suggesting that S248 of C/EBPα is not required for steady state lineage commitment or differentiation of myeloid progenitors in young mice.

To evaluate the myeloid progenitors functionally, we next plated BM cells from 8 week-old Cebpa S248A/S248A and Cebpa KI/KI animals in semisolid media to allow for the outgrowth of all myeloid colonies. In contrast to previous studies involving BM progenitors derived from other Cebpa mutant mouse lines [19,21], Cebpa S248A/S248A BMs yielded similar distributions of BFU-E, CFU-GM and CFU-GEMM colonies as the Cebpa KI/KI controls (Figure 3C). Furthermore, when we analyzed the proliferative consequences of mutating S248 by performing serial replating of colonies derived from the first round of plating, Cebpa S248A/S248A progenitors displayed the same limited proliferative potential as progenitors from the Cebpa KI/KI controls when counted either as colonies (Figure 3D) or as total cell number (data not shown). In conclusion, at eight weeks of age Cebpa S248A/S248A mice display no overt phenotype in the hematopoietic compartment.

A fraction of Cebpa S248A/S248A mice develop a low-penetrant hematopoietic disorder with long latency

Many hematopoietic diseases are particularly prevalent in the elderly population, but the mechanisms involved are not resolved but may be related to an increase in myeloid-based HSCs upon ageing or accumulation of acquired genetic mutations [28]. We therefore analyzed a cohort of Cebpa S248A/S248A animals at one year of age (12–14 months) to detect whether mutation of S248 led to any phenotypic changes in the hematopoietic compartment in older animals. At this age, several of the Cebpa S248A/S248A mice (4/
23) had enlarged spleen (Figure S2D), whereas all of the knock-in wild type controls were phenotypically indistinguishable from the young wild type controls.

Next, we analyzed the distribution of the mature hematopoietic populations within the BM of cohorts of Cebpa<sup>KI/KI</sup> control and Cebpa<sup>S248A/S248A</sup> mice. In the BM from Cebpa<sup>S248A/S248A</sup> mice there was a slight reduction in mature neutrophil granulocytes (Mac-1⁺, Gr-1⁺), erythroid cells (E = CD71⁺, Ter119⁺), B cells (B = B220⁺) and T cells (T = CD4⁺ or CD8a⁺). (B) Quantification of the data from (A), Cebpa<sup>KI/KI</sup> (n = 6) and Cebpa<sup>S248A/S248A</sup> (n = 6) (mean ±/− standard deviation). (C) Cytospin analysis of BM and spleen cells from Cebpa<sup>KI/KI</sup> and Cebpa<sup>S248A/S248A</sup> mice. (D) Differential counts of the cytospins in (C) from Cebpa<sup>S248A/S248A</sup> (n = 4) (mean ±/− standard deviation). Abbreviations indicate the granulocytic (Myeloblasts (MB); Promyelocytes (PM); Myelocytes (My); Metamyelocytes (MM); Band cells (Band) and Polymorphonuclear Granulocytes (PMN)) monocytic (Promonocytes (Pmo); Monocytes (Mo)), erythroid (Proerythroblasts (Pery); Erythroblasts (ery); basophilic normoblast (bNB); polychromatic normoblast (pNB)); lymphocytes (Ly); eosinophils (Eo) and plasma cell (PC) types.
reduced GMP/preMegE ratio, approximately 30% (7 out of 23) of the Cebpa^S248A/S248A animals displayed a GMP/preMegE ratio lower than the mean of the Cebpa^KI/KI animals minus one standard deviation (Figure 5B and Figure S2). These phenotypically progressed Cebpa^S248A/S248A mice had a significant increase in the preGMs (Figure 5C), indicating partially impaired differentiation towards the granulocyte-monocytic compartment at the preGM to GMP transition, where C/EBPα executes its lineage-instructive function [6,7].

Furthermore, the partly impaired preGM to GMP transition was accompanied by increased levels of megakaryocytic and erythroid progenitors (preMegE, MkP, preCFU-E), suggesting that
the partial block in granulocytic-monocytic differentiation directs the cells towards the erythroid lineage (Figure 5C). This lineage-skewing was also detected in colony assays, where \textit{Cebpa}^{S248A/S248A} BM gave rise to significantly more BFU-Es than the \textit{Cebpa}^{KIKI} control (Figure 4C).

In addition to the observed changes in the committed progenitor compartments, a fraction of the one year-old \textit{Cebpa}^{S248A/S248A} animals with biased lineage choice displayed increased frequencies of the HSC/MPP-enriched Lineage negative, Sca-1^[+], c-Kit^[+], (LSK) population compared to \textit{Cebpa}^{KIKI} mice. Further resolution of this compartment showed that the progressed \textit{Cebpa}^{S248A/S248A} BM had a two-fold expansion of MPPs compared to \textit{Cebpa}^{KIKI} controls as well as a trend towards increased numbers of HSCs (Figure 5C). Notably, we observed only a partial overlap between delayed preGM to GMP transition and expansion of the stem and multipotent progenitor compartment (Figure S2), suggesting that these events occur by different mechanisms.

Next we wanted to assess in more detail whether there existed any correlations between the different aberrant phenotypes (enlarged spleen, expanded LSK compartment, erythroid-biased differentiation, etc) that we observed in old \textit{Cebpa}^{S248A/S248A} animals with biased lineage choice displayed increased frequencies of the HSC/MPP-enriched Lineage negative, Sca-1^[+], c-Kit^[+], (LSK) population compared to \textit{Cebpa}^{KIKI} mice. Further resolution of this compartment showed that the progressed \textit{Cebpa}^{S248A/S248A} BM had a two-fold expansion of MPPs compared to \textit{Cebpa}^{KIKI} controls as well as a trend towards increased numbers of HSCs (Figure 5C). Notably, we observed only a partial overlap between delayed preGM to GMP transition and expansion of the stem and multipotent progenitor compartment (Figure S2), suggesting that these events occur by different mechanisms.

Next we wanted to assess in more detail whether there existed any correlations between the different aberrant phenotypes (enlarged spleen, expanded LSK compartment, erythroid-biased differentiation, etc) that we observed in old \textit{Cebpa}^{S248A/S248A} animals with biased lineage choice displayed increased frequencies of the HSC/MPP-enriched Lineage negative, Sca-1^[+], c-Kit^[+], (LSK) population compared to \textit{Cebpa}^{KIKI} mice. Further resolution of this compartment showed that the progressed \textit{Cebpa}^{S248A/S248A} BM had a two-fold expansion of MPPs compared to \textit{Cebpa}^{KIKI} controls as well as a trend towards increased numbers of HSCs (Figure 5C). Notably, we observed only a partial overlap between delayed preGM to GMP transition and expansion of the stem and multipotent progenitor compartment (Figure S2), suggesting that these events occur by different mechanisms.

Next we wanted to assess in more detail whether there existed any correlations between the different aberrant phenotypes (enlarged spleen, expanded LSK compartment, erythroid-biased differentiation, etc) that we observed in old \textit{Cebpa}^{S248A/S248A} animals with biased lineage choice displayed increased frequencies of the HSC/MPP-enriched Lineage negative, Sca-1^[+], c-Kit^[+], (LSK) population compared to \textit{Cebpa}^{KIKI} mice. Further resolution of this compartment showed that the progressed \textit{Cebpa}^{S248A/S248A} BM had a two-fold expansion of MPPs compared to \textit{Cebpa}^{KIKI} controls as well as a trend towards increased numbers of HSCs (Figure 5C). Notably, we observed only a partial overlap between delayed preGM to GMP transition and expansion of the stem and multipotent progenitor compartment (Figure S2), suggesting that these events occur by different mechanisms.

Next we wanted to assess in more detail whether there existed any correlations between the different aberrant phenotypes (enlarged spleen, expanded LSK compartment, erythroid-biased differentiation, etc) that we observed in old \textit{Cebpa}^{S248A/S248A} animals with biased lineage choice displayed increased frequencies of the HSC/MPP-enriched Lineage negative, Sca-1^[+], c-Kit^[+], (LSK) population compared to \textit{Cebpa}^{KIKI} mice. Further resolution of this compartment showed that the progressed \textit{Cebpa}^{S248A/S248A} BM had a two-fold expansion of MPPs compared to \textit{Cebpa}^{KIKI} controls as well as a trend towards increased numbers of HSCs (Figure 5C). Notably, we observed only a partial overlap between delayed preGM to GMP transition and expansion of the stem and multipotent progenitor compartment (Figure S2), suggesting that these events occur by different mechanisms.

Next we wanted to assess in more detail whether there existed any correlations between the different aberrant phenotypes (enlarged spleen, expanded LSK compartment, erythroid-biased differentiation, etc) that we observed in old \textit{Cebpa}^{S248A/S248A} animals with biased lineage choice displayed increased frequencies of the HSC/MPP-enriched Lineage negative, Sca-1^[+], c-Kit^[+], (LSK) population compared to \textit{Cebpa}^{KIKI} mice. Further resolution of this compartment showed that the progressed \textit{Cebpa}^{S248A/S248A} BM had a two-fold expansion of MPPs compared to \textit{Cebpa}^{KIKI} controls as well as a trend towards increased numbers of HSCs (Figure 5C). Notably, we observed only a partial overlap between delayed preGM to GMP transition and expansion of the stem and multipotent progenitor compartment (Figure S2), suggesting that these events occur by different mechanisms.

Next we wanted to assess in more detail whether there existed any correlations between the different aberrant phenotypes (enlarged spleen, expanded LSK compartment, erythroid-biased differentiation, etc) that we observed in old \textit{Cebpa}^{S248A/S248A} animals with biased lineage choice displayed increased frequencies of the HSC/MPP-enriched Lineage negative, Sca-1^[+], c-Kit^[+], (LSK) population compared to \textit{Cebpa}^{KIKI} mice. Further resolution of this compartment showed that the progressed \textit{Cebpa}^{S248A/S248A} BM had a two-fold expansion of MPPs compared to \textit{Cebpa}^{KIKI} controls as well as a trend towards increased numbers of HSCs (Figure 5C). Notably, we observed only a partial overlap between delayed preGM to GMP transition and expansion of the stem and multipotent progenitor compartment (Figure S2), suggesting that these events occur by different mechanisms.

Next we wanted to assess in more detail whether there existed any correlations between the different aberrant phenotypes (enlarged spleen, expanded LSK compartment, erythroid-biased differentiation, etc) that we observed in old \textit{Cebpa}^{S248A/S248A} animals with biased lineage choice displayed increased frequencies of the HSC/MPP-enriched Lineage negative, Sca-1^[+], c-Kit^[+], (LSK) population compared to \textit{Cebpa}^{KIKI} mice. Further resolution of this compartment showed that the progressed \textit{Cebpa}^{S248A/S248A} BM had a two-fold expansion of MPPs compared to \textit{Cebpa}^{KIKI} controls as well as a trend towards increased numbers of HSCs (Figure 5C). Notably, we observed only a partial overlap between delayed preGM to GMP transition and expansion of the stem and multipotent progenitor compartment (Figure S2), suggesting that these events occur by different mechanisms.

Next we wanted to assess in more detail whether there existed any correlations between the different aberrant phenotypes (enlarged spleen, expanded LSK compartment, erythroid-biased differentiation, etc) that we observed in old \textit{Cebpa}^{S248A/S248A} animals with biased lineage choice displayed increased frequencies of the HSC/MPP-enriched Lineage negative, Sca-1^[+], c-Kit^[+], (LSK) population compared to \textit{Cebpa}^{KIKI} mice. Further resolution of this compartment showed that the progressed \textit{Cebpa}^{S248A/S248A} BM had a two-fold expansion of MPPs compared to \textit{Cebpa}^{KIKI} controls as well as a trend towards increased numbers of HSCs (Figure 5C). Notably, we observed only a partial overlap between delayed preGM to GMP transition and expansion of the stem and multipotent progenitor compartment (Figure S2), suggesting that these events occur by different mechanisms.

Next we wanted to assess in more detail whether there existed any correlations between the different aberrant phenotypes (enlarged spleen, expanded LSK compartment, erythroid-biased differentiation, etc) that we observed in old \textit{Cebpa}^{S248A/S248A} animals with biased lineage choice displayed increased frequencies of the HSC/MPP-enriched Lineage negative, Sca-1^[+], c-Kit^[+], (LSK) population compared to \textit{Cebpa}^{KIKI} mice. Further resolution of this compartment showed that the progressed \textit{Cebpa}^{S248A/S248A} BM had a two-fold expansion of MPPs compared to \textit{Cebpa}^{KIKI} controls as well as a trend towards increased numbers of HSCs (Figure 5C). Notably, we observed only a partial overlap between delayed preGM to GMP transition and expansion of the stem and multipotent progenitor compartment (Figure S2), suggesting that these events occur by different mechanisms.

Next we wanted to assess in more detail whether there existed any correlations between the different aberrant phenotypes (enlarged spleen, expanded LSK compartment, erythroid-biased differentiation, etc) that we observed in old \textit{Cebpa}^{S248A/S248A} animals with biased lineage choice displayed increased frequencies of the HSC/MPP-enriched Lineage negative, Sca-1^[+], c-Kit^[+], (LSK) population compared to \textit{Cebpa}^{KIKI} mice. Further resolution of this compartment showed that the progressed \textit{Cebpa}^{S248A/S248A} BM had a two-fold expansion of MPPs compared to \textit{Cebpa}^{KIKI} controls as well as a trend towards increased numbers of HSCs (Figure 5C). Notably, we observed only a partial overlap between delayed preGM to GMP transition and expansion of the stem and multipotent progenitor compartment (Figure S2), suggesting that these events occur by different mechanisms.
bances in the myeloid compartment and/or expansion of HSCs and MPPs.

The slowly developing hematopoietic disorders in old Cebpa<sup>S248A/S248A</sup> animals are cell-intrinsic

To investigate whether the above-described phenotypes are cell-intrinsic to the hematopoietic compartment, we reconstituted lethally irradiated recipients (CD45.1) with whole BM from 8 week-old Cebpa<sup>S248A/S248A</sup> and Cebpa<sup>KI/KI</sup> donors (CD45.2), and analyzed recipient BM 16 weeks post-transplantation.

Similar to what was observed in one-year-old Cebpa<sup>S248A/S248A</sup> mice, a fraction (4/7) of the recipients transplanted with Cebpa<sup>S248A/S248A</sup> BMs displayed diminished levels of GMPs and accumulation of megakaryocytic and erythroid progenitors (pre-MegE and pre-CFU-E) in comparison to recipient mice transplanted with Cebpa<sup>KI/KI</sup> control BM (Figure 7 and Figure S5). Consistent with the phenotype in the Cebpa<sup>S248A/S248A</sup> mice, there was only a partial overlap between the disturbance in the myeloid compartment and increased numbers of LSK cells (Figure S5), which supports the notion that the partly halted granulocytic-monocytic differentiation and the accumulation of HSCs and MPPs occur by two distinct mechanisms.

To test whether the S248A allele results in a stem cell repopulation advantage or disadvantage compared to the wild type knock-in allele, we performed serial competitive BM transplantations, in which whole BM (CD45.2) from eight week-old Cebpa<sup>S248A/S248A</sup> and Cebpa<sup>KI/KI</sup> mice was mixed in a 1:1 ratio with competitor BM (CD45.1) and transplanted into lethally irradiated CD45.1 recipients. Recipient BM was harvested 18 weeks post-transplantation and donor (CD45.2) versus competitor

Figure 5. A fraction of the one-year old Cebpa<sup>S248A/S248A</sup> mice develops a myeloid disorder with biased lineage choice. (A) Flow cytometry analysis of the myeloerythroid progenitor compartment in BMs from Cebpa<sup>KI/KI</sup> (n = 17) and Cebpa<sup>S248A/S248A</sup> (n = 23) mice. (B) Seven out of 23 Cebpa<sup>S248A/S248A</sup> mice (termed “progressed”) had a skewed lineage distribution with a decreased GMP/preMegE ratio compared to Cebpa<sup>KI/KI</sup>. Black line indicates cut-off. Cut-off was defined as mean of Cebpa<sup>KI/KI</sup>—standard deviation. (C) Quantification of the data from (A). Numbers of mice in each of the groups were as follows: Cebpa<sup>KI/KI</sup> (n = 17), Cebpa<sup>S248A/S248A</sup> (n = 16) and progressed Cebpa<sup>S248A/S248A</sup> (n = 7) mice. P values designate significance between progressed Cebpa<sup>S248A/S248A</sup> and Cebpa<sup>KI/KI</sup>, ns = not significant (mean ± standard deviation).

doi:10.1371/journal.pone.0038841.g005
(CD45.1) contribution was analyzed. As shown in figure 8A, there were no significant differences in these ratios between recipients transplanted with \( \text{Cebpa}^{\text{N248A/S248A}} \) and \( \text{Cebpa}^{\text{K1/K1}} \) donor cells, respectively. To test whether a repopulation phenotype could be uncovered when further proliferative stress was applied to the hematopoietic system, we transplanted pooled whole BM from 6–7 primary recipients (Figure 8B) into secondary recipients, which were analyzed 21 and 34 weeks post-transplantation. At 21 weeks after secondary transplantation, the LSK compartment of mice transplanted with \( \text{Cebpa}^{\text{S248A/K248A}} \) was significantly increased compared to mice transplanted with \( \text{Cebpa}^{\text{K1/K1}} \) BM and this increased contribution in the stem cell compartment of \( \text{Cebpa}^{\text{S248A/K248A}} \) mice resulted in a significant increase of donor BM cells at 34 weeks post-transplantation (Figure 8C, D). It should be noted that a selective advantage of donor-versus competitor cells was also observed in mice transplanted with \( \text{Cebpa}^{\text{K1/K1}} \) secondary donors, since the donor/competitor ratio had also increased relative to input cells in these animals. Therefore, some selective advantage must be assigned to the genetic background of the cells used. However, the effect was significantly higher in the \( \text{Cebpa}^{\text{S248A/K248A}} \) secondary recipients suggesting that the S248A mutation confer a mild selective advantage to the HSC/MPP compartment.

In summary, the \( \text{Cebpa}^{\text{S248A}} \) allele results in a low-penetrant cell-intrinsic partly impairment of granulocytic-monocytic differentiation accompanied by skewing towards the megakaryocytic and erythroid compartment in a fraction of aged animals. Furthermore, these data show that \( \text{Cebpa}^{\text{S248A/S248A}} \) HSCs have a competitive advantage compared to their wild type counterparts resulting in the accumulation of HSCs and MPPs in some of the \( \text{Cebpa}^{\text{S248A/S248A}} \) mice.

**Discussion**

Protein phosphorylation represents an important layer of cellular regulation, which may affect the stability, activity and functional property of the modified protein. Whether phosphorylation of key regulators plays a role in normal hematopoiesis has only been addressed to a limited extent and mainly in a cell culture context. The key hematopoietic regulator C/EBP\( \alpha \) contains a number of phosphorylation sites that confer regulation under a variety of conditions [15,29,30]. Previously, Ras-dependent phosphorylation of C/EBP\( \alpha \) at S248 was reported to increase its transactivational activity and promote increased granulocytic-monocytic differentiation in vitro, whereas Erk-dependent phosphorylation of S211 inhibited the same process [15,17]. Furthermore, it has been shown that S21 must be dephosphorylated in order to induce not only expression of granulocytic-monocytic markers but also the erythroid-specific CD71 [16]. These findings suggest that the phosphorylation status of C/EBP\( \alpha \) is potentially involved in regulating both granulopoiesis and erythropoiesis.

In order to extend this analysis to the proper in vivo context, we decided to generate mice, in which the wild type \( \text{Cebpa} \) gene was replaced by an allele containing a S248A substitution. Before initiating the phenotypic analysis of these animals we verified the previous reports on the requirement of S248 for in vivo differentiation of granulocytic-monocytic cell lines [17,31]. Indeed, by introducing C/EBP\( \alpha \)-ER and C/EBP\( \alpha \)-S248A-ER into 32Dcl3 cells, we could show that the S248A variant is unable to promote myeloid differentiation. Whereas C/EBP\( \alpha \)-ER induces the expression of myeloid markers such as Mac-1 and Gr-1 accompanied by cell cycle exit, 32Dcl3 cells expressing C/EBP\( \alpha \)-S248A-ER fail to induce expression of these markers and

**Table 1. Overview of the phenotypes of \( \text{Cebpa}^{\text{S248A/S248A}} \) and \( \text{Cebpa}^{\text{K1/K1}} \) mice.**

| Genotype | 8 weeks | 1 year | 1,5–2 years |
|----------|---------|--------|-------------|
|          | KI/S248A | KI/S248A | KI/S248A   |
| Low GMP/preMegE ratio | 0/6 | 0/6 | 0/8 | 6/21 (29%) |
| Expanded LSK compartment | 0/6 | 0/6 | 2/17 (12%) | 7/23 (30%) | 1/8 (13%) | 6/21 (29%) |
| Enlarged spleen | 0/6 | 0/6 | 0/17 | 4/23 (17%) | 0/8 | 6/21 (29%) |

doi:10.1371/journal.pone.0038841.g006
continue to proliferate. This suggests that phosphorylation of S248 is required in order for the cells to differentiate into mature granulocytic-monocytic cells in vitro.

Given the strong requirement for S248 for in vitro granulocytic-monocytic differentiation, we were surprised to find that young Cebpa<sup>S248A/S248A</sup> mice and their relevant controls contain a similar number of mature and immature myeloid cells. In line with this, BM cells plated in semi-solid media result in outgrowth of an equal amount of colonies with similar distributions of myeloid progenitors from Cebpa<sup>S248A/S248A</sup> and Cebpa<sup>KI/KI</sup> mice. This suggests that in young mice, S248 is dispensable for the induction of granulocytic-monocytic differentiation, and consequently that phosphorylation of S248 is neither required for the preGM to GMP transition nor for the differentiation into mature neutrophil granulocytes in mice at 8 weeks of age.

Interestingly, a fraction of older Cebpa<sup>S248A/S248A</sup> mice develop a myeloid disorder in which differentiation towards the megakaryocytic and erythroid lineage is promoted at the expense of granulocytic-monocytic differentiation as evident by an increase in early megakaryocytic and erythroid progenitors and a corresponding decrease in their granulocytic-monocytic equivalents. Another, partly overlapping, fraction of older Cebpa<sup>S248A/S248A</sup> mice presents with a mild expansion of the HSC-containing LSK compartment.

Collectively, these data contribute to the increasing body of evidence pointing towards C/EBPα as an important regulator of cell fate decisions in progenitors more primitive than GMPs. Conditional Cebpa knockout in the hematopoietic system in adult mice blocks the transition from CMP to GMP resulting in loss of granulocytes and monocytes but increases the numbers of myeloid...
cells compared to Cebpa aged genetic lesions this model also explains why only a fraction of the C/EBP lineage to a myeloid proliferative condition and later to an AML-like disease, showing first of all, that mutations in C/EBP result in a predisposition to myeloid diseases. Similarly, expression of C/EBP induces granulocyte-monocytic differentiation and inhibits erythroid development resulting in an increase in mature granulocytes and loss of megakaryocytic and erythroid progenitors [33]. In accordance, it has been shown that mice with mutations in the CD45.2) was mixed in a 1:1 ratio with whole competitor BM (CD45.1) and transplanted into lethally irradiated mice. BM donor contribution was assessed 18 weeks post-transplantation. Cebpa KI/KI (n = 7) and Cebpa S248A/S248A (n = 6) BM cells. (B) Input BM for the secondary transplantation show similar levels of Cebpa S248A/S248A and Cebpa KI/KI contribution in the two input samples. (C, D) BM donor contribution in whole BM (wBM) or in the LSK compartment of the secondary recipients was analyzed after 21 (C) and 34 (D) weeks. Cebpa 248A/S248A transplanted mice (n = 4; black bars); Cebpa S248A transplanted mice (n = 4; white bars).

doI:10.1371/journal.pone.0038841.g008

Figure 8. Cebpa S248A/S248A BM cells have a competitive advantage in comparison to Cebpa KI/KI BM cells. (A) Whole BM from Cebpa S24-AK and Cebpa S248A/S248A mice (CD45.2) was mixed in a 1:1 ratio with whole competitor BM (CD45.1) and transplanted into lethally irradiated mice. BM donor contribution was assessed 18 weeks post-transplantation. Cebpa KI/KI (n = 7) and Cebpa S248A/S248A (n = 6) BM cells. (B) Input BM for the secondary transplantation show similar levels of Cebpa S248A/S248A and Cebpa KI/KI contribution in the two input samples. (C, D) BM donor contribution in whole BM (wBM) or in the LSK compartment of the secondary recipients was analyzed after 21 (C) and 34 (D) weeks. Cebpa 248A/S248A transplanted mice (n = 4; black bars); Cebpa S248A transplanted mice (n = 4; white bars).

doI:10.1371/journal.pone.0038841.g008

Growing evidence suggests that tight regulation of lineage-specific transcription factors plays a major role in the HSC compartment. In accordance, it has been shown that mice with various mutations in Cebpa have deregulated stem cell pools [19,20,21,23]. The data presented in this work also support a functional role for C/EBPα in HSCs since a fraction of the 1–2 years old Cebpa S248A/S248A mice present with an expanded LSK compartment. Furthermore, upon serial whole BM transplantation we observed a competitive advantage of Cebpa S248A/S248A BM cells compared to Cebpa KI/KI controls and after the second round of transplantation the Cebpa S248A/S248A BM cells have overtaken the recipient BM. Formally, we cannot exclude the possibility that the donor-derived cells in one or more of the primary recipients had an expansion of the LSK compartment. Therefore it is unclear whether the observed increased selective advantage of Cebpa S248A/S248A BM cells is due to an acquired event leading to an increase of functional stem cells or an increase of self-renewal per se in older Cebpa S248A/S248A HSCs. In either case, our data demonstrates that the S248A mutation confers a competitive advantage to HSCs, when these are subjected to proliferative stress.

The development of hematopoietic disorders in older Cebpa S248A/S248A animals may in principle be explained by two non-exclusive models: One formal possibility is that C/EBPα-S248 is receiving input from a signalling transduction pathway that changes its activity in an age-dependent manner. Precedence for such a model comes from studies of rodent livers, where C/EBPα display different phosphorylation patterns in young and old animals. Specifically, the proportion of C/EBPα that is phosphorylated on S193 by cyclin D3-cdk4/6 increase with age, which correlates with the expression pattern of cyclin D3, and results in a reduced ability to eliminate the growth repressive potential of C/EBPα through dephosphorylation of S193 after partial hepatectomy [34,35,36].

Alternatively, the hematopoietic disorders in the old Cebpa S248A/S248A animals arise as a result of different additional genetic/epigenetic events in the Cebpa S248A/S248A mice, which facilitate the progression into an erythroid-biased condition, the expansion of the LSK compartment or both. We favour this second model as the Cebpa S248A/S248A mice with an expanded LSK compartment and an erythroid-biased lineage choice only partially overlap, which suggests that these phenotypes are driven by distinct
molecular mechanisms. However, this does not exclude the possibility that mutation of S248 makes hematopoietic cells more susceptible to age-dependent changes in signal transduction pathways operating through C/EBPα.

In conclusion, our data show that S248 is dispensable for normal steady-state hematopoiesis, and that Cebpa<sup>S248A/S248A</sup> mice develop a low-penetrant myeloid disorder with age associated with a mild skewing towards the erythroid lineage and a partial differentiation block at the preGM to GMP transition. Additionally, Cebpa<sup>S248A/S248A</sup> BM display a competitive advantage during serial transplantation suggesting that phosphorylation of S248 may normally serve to restrict HSC self-renewal in ageing mice.

In more general terms, the stark difference between the in vitro and in vivo phenotypes of the S248A mutant highlights the need to exert caution when extrapolating in vitro data to a more appropriate in vivo setting. Moreover, the phenotypic progression in old Cebpa<sup>S248A/S248A</sup> mice to a condition with a partial resemblance to the in vitro phenotype of mutating S248 may suggest that granulocytic-monocytic cell lines could be wired in a manner—either genetically or in terms of active signal transduction networks—that render them more relevant as models for an aged hematopoietic system.

Materials and Methods

**Ethic statement**

All mouse work was performed according to national and international guidelines and approved by the Danish Animal Ethical Committee. This study was approved by the review board at the Faculty of Health Sciences, University of Copenhagen (P10-014).

**Cell culture**

All cell lines were grown at 37°C at 5% CO<sub>2</sub>. 32Dc13 cells were kindly provided by A. Friedmann [24,25]. The cells were grown in IMDM with 1-L-glutamine and 25 mM Hepes (Gibco) supplemented with 10% FBS (HyClone), 100 µg/ml penicillin/streptomycin (Gibco) and 1 µg/ml IL3 (Stem cell Technologies). For culturing stable transfectants 1 µg/ml puromycin (Sigma) was added. Differentiation of 32Dc13 cells expressing ER<sup>TM</sup> fusion protein was induced by addition of 4-hydroxytamoxifen (4-OHT, Sigma) in the presence of IL3. Phoenix-E cells (obtained from ATCC) were cultured in DMEM (Gibco) supplemented with 10% FBS (HyClone) and 100 µg/ml penicillin/streptomycin (Gibco).

**Generation of C/EBPα-expressing cells**

A plasmid expressing rat C/EBPα was kindly provided by G. Behre. pBabePuro-C/EBPα-S248A-ER<sup>TM</sup> was constructed by ligating a Sfi I/EcoRI fragment containing the S248A substitution in place of the identical wild type fragment in phabePuro-C/EBPα-ER<sup>TM</sup>. Phoenix-E cells were transiently transfected with phabePuro-C/EBPα-ER<sup>TM</sup> or phabePuro-C/EBPα-S248A-ER<sup>TM</sup> and virus-containing supernatant was collected after two and three days. 32Dc13 cells were infected and selected by addition of puromycin as described in [18]. Cells were limited-diluted in 96-well culture dishes to obtain clones, which were expanded and tested for expression of C/EBPα or C/EBPα-S248A by western blotting.

**Western Blotting**

Western blotting was performed as previously described [18] and probed with antibodies against C/EBPα (14AA, sc-61) and Cdk2 (Sc-163) from Santa Cruz Biotechnology.

**Cell cycle analysis**

The cell cycle distribution was analyzed at day 0–3 after addition of 4-OHT as previously described [18].

**Mouse work and procedures**

The Cebpa-S248A mutant was generated by using the Quickchange mutagenesis kit (Stratagene) and confirmed by sequencing. Cloning of the targeting construct, electroporation, selection of E14.1 ES cells, blastocyst injection and breeding of chimera was performed as described previously [20]. The Cebpa<sup>S248A</sup> and Cebpa<sup>KI</sup> alleles were backcrossed to C57BL/6 for at least 6 generations [20].

BM transplantations were carried out by tail vein injection of whole CD45.2 BM cells into CD45.1 recipients, which had been subjected to a lethal dose (900 rad) of gamma-irradiation 16 hours prior to transplantation. In the non-competitive setup, 1 to 2 million donor cells were used. In the competitive setup, primary transplantations were carried out by co-injecting 500,000 donor cells and 500,000 competitor cells (CD45.1). For secondary transplantations, BM cells from 6–7 primary recipients from the competitive transplantations were pooled, and 5 million of these cells were used for as secondary donor cells.

**Flow cytometry**

C/EBPα-ER and C/EBPα-S248A-ER expressing cell lines were stained with Mac-1 and Gr-1 and analyzed on a FACSCalibur. Briefly, 500,000 cells were incubated with 1 µl Fc receptor block (anti-CD16/32, BD) in 20 µl PBS%FCS on ice for 15 min. The cells were washed with cold PBS%FCS and stained in the dark on ice with antibodies against Mac-1 and Gr-1 or corresponding isotype controls (eBioscience) for 20 min. Cells were resuspended in PBS%FCS and run on a FACSCalibur (BD).

The hematopoietic compartment was analyzed as follows: Femurs and tibiae were collected and crushed in PBS+3% FCS. The BM cells were stained for mature cells using antibodies against Ter119, CD71, Mac-1, Gr-1, B220, CD4, and CD8a (eBioscience) and stem and progenitor cells using antibodies against Lineage (CD3e, B220, Mac-1, Gr-1), Sca-1, c-Kit, CD105, CD41, FcgRII/III, Ter119 (eBioscience) and stem and progenitor cells using antibodies against Mac-1 and Gr-1 or corresponding isotype controls (eBioscience) for 20 min. Cells were resuspended in PBS%FCS and run on a FACSCalibur (BD).

The hematopoietic compartment was analyzed as follows: Femurs and tibiae were collected and crushed in PBS+3% FCS. The BM cells were stained for mature cells using antibodies against Ter119, CD71, Mac-1, Gr-1, B220, CD4, and CD8a (eBioscience) and stem and progenitor cells using antibodies against Lineage (CD3e, B220, Mac-1, Gr-1), Sca-1, c-Kit, CD105, CD41, FcgRII/III, Ter119 (eBioscience) and CD150 (Biolegend). For the analysis of transplanted animals, antibody cocktails were supplemented with CD45.1 and CD45.2 antibodies (eBioscience). After wash the mature stained cells were resuspended in PBS+3% FCS containing DAPI (0.2 µg/ml, Invitrogen) and cells stained for stem and progenitor cells were resuspended in PBS+3% FCS containing 7AAD (1 µg/ml, Invitrogen). The samples were run on a LSRII and analyzed using FlowJo software.

**Cytospins, colony assays and serial replating experiments**

For the analysis of colony-forming potential, BM cells (5,000–20,000 cells/35-mm dish) were seeded in methylcellulose-based medium (M3434, StemCell Technologies Inc.) supplied with erythropoietin, IL-3, IL-6, and stem cell factor. After 10–12 d in culture, the colonies were scored as CFU-GM, BFU-E, or CFU-GEMM. In the serial replating experiments, a similar number of BM cells were seeded in M3434 medium, cultured for 7 days, and the number of colonies was counted. The cells were harvested, washed with PBS, diluted and replated in fresh M3434 medium and cultured for an additional 7 d. This procedure was repeated for 5 weeks.

Preparation of cytospins was performed as described in [21].
Supporting Information

Figure S1 Liver, lung and spleen tissue sections. Tissue sections from Cebpa+/+ and CebpaS248A/S248A mice were stained with a Hematoxylin/Eosin solution. There were no changes in morphology of the tissues. (TIF)

Figure S2 Expanded stem and progenitor compartment in a fraction of the one-year old CebpaS248A/S248A mice. (A) Seven out of 23 CebpaS248A/S248A mice had a skewed lineage distribution with a decreased GMP/preMegE ratio compared to CebpaKI/KI. Black line indicates cut-off. Cut-off was defined as mean of CebpaKI/KI + standard deviation. Asterisks show the mice analyzed in figure 4B and 4C. (B) Seven out of 23 CebpaS248A/S248A mice had an expanded LSK compartment compared to CebpaKI/KI. Black line indicates cut-off. Cut-off was defined as mean of CebpaKI/KI + standard deviation. Asterisks show the mice analyzed in figure 4B and 4C. (C) Partial overlap of mice with expanded LSK compartment and low GMP/preMegE ratio. (D) Enlarged spleen from a one-year old CebpaS248A/S248A mouse. (TIF)

Figure S3 Correlation of mice with enlarged spleen, enhanced LSK compartment and myeloid-biased differentiation in 18–24 months old CebpaS248A/S248A mice. (A) Six out of 21 CebpaS248A/S248A mice had a skewed lineage distribution with a decreased GMP/preMegE ratio compared to CebpaKI/KI. Black line indicates cut-off. Cut-off was defined as mean of CebpaKI/KI + standard deviation. (B) Six out of 21 CebpaS248A/S248A mice had an expanded LSK compartment compared to 1 out of 8 CebpaS248A/S248A mice. Black line indicates cut-off. Cut-off was defined as mean of CebpaKI/KI + standard deviation. (C) Six out of 21 CebpaS248A/S248A mice had an enlarged spleen compared to CebpaKI/KI (n = 6) were termed “progressed”. Numbers of mice in the other groups were as follows: CebpaKI/KI (n = 8) and CebpaS248A/S248A (n = 15) mice. Triangle designates enhanced LSK compartment, white color designates enlarged spleen, ns = not significant (mean +/− standard deviation). (G) Correlation plot of mice with enlarged spleen, expanded LSK compartment and low GMP/preMegE ratio. (TIF)

Figure S4 Erythroid-biased differentiation of progressed CebpaS248A/S248A mice. (A) Progressed CebpaS248A/S248A mice (n = 6) had increased level of erythroid cells in the BM and (B) displayed enhanced BFU-E colony formation. Numbers of mice in the other groups were as follows: CebpaKI/KI (n = 15) and CebpaS248A/S248A (n = 8) mice. Triangle designates enhanced LSK compartment, white color designates enlarged spleen, P values designate significance between progressed CebpaS248A/S248A and CebpaKI/KI, ns = not significant (mean +/− standard deviation). (TIF)

Figure S5 Expanded stem and progenitor compartment in some of the recipients receiving CebpaS248A/S248A BM. (A) Four out of seven recipients of CebpaS248A/S248A BM had a decreased GMP/preMegE ratio compared to CebpaKI/KI. Black line indicates cut-off. Cut-off was defined as mean of CebpaKI/KI + standard deviation (B) Four out of seven recipient mice with BM from CebpaS248A/S248A had an expanded LSK compartment. Black line indicates cut-off. Cut-off was defined as mean of CebpaKI/KI + standard deviation. (C) Partial overlap of mice with expanded LSK compartment and low GMP/preMegE ratio. (TIF)

Acknowledgments
We thank Inge Damgaard for expert technical assistance and I.B. Christensen for statistical advice.

Author Contributions
Conceived and designed the experiments: MSH MBS AKF KTM TA˚P. Performed the experiments: MSH MBS AKT TAP. Analyzed the data: MSH MBS AKT KTM BTP. Contributed reagents/materials/ analysis tools: CN BTP. Wrote the paper: MSH MBS BTP.

References
1. Bryder D, Rossi DJ, Weissman IL (2006) Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. Am J Pathol 169: 338–546.
2. Rosenthaler F, Tremain DG (2007) Transcription factors in myeloid development: balancing differentiation with transformation. Nat Rev Immunol 7: 260–271.
3. Schuster MB, Porre BT (2006) C/EBPalpha: a tumour suppressor in multiple tissues? Biochem Biophys Acta 1766: 88–103.
4. Johnson PF (2005) Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors. J Cell Sci 118: 2545–2555.
5. McKnight SL (2001) McBindall—a better name for CCAAT/enhancer binding proteins? Cell 107: 259–261.
6. Zhang P, Iwasaki-Arai J, Iwasaki H, Fenyus ML, Dayaram T, et al. (2004) 2. Zhang P, Iwasaki-Arai J, Iwasaki H, Fenyus ML, Dayaram T, et al. (2004) Granulocyte inducer C/EBPalpha inactivates the myeloid master regulator PU.1: possible role in lineage commitment decisions. Blood 100: 483–490.
7. Pedersen TA, Kownez-Leutz E, Leutz A, Nerlof C (2001) Cooperation between C/EBPalpha TBP/TFIIB and SWI/SNF recruiting domains is required for adipocyte differentiation. Genes Dev 15: 3208–3216.
8. Subramanian L, Benson MD, Iniguez-Lluhi JA (2005) A synergy control motif within the attenuator domain of CCAAT/enhancer-binding protein alpha inhibits transcriptional synergy through its PIAAy-enhanced modification by SUMO-1 or SUMO-3. J Biol Chem 280: 9134–9141.
9. Ross SE, Radomska HS, Wu B, Zhang P, Winny JN, et al. (2004) Phosphorylation of C/EBPalpha inhibits granulopoiesis. Mol Cell Biol 24: 675–686.
10. Twu YC, Hsieh CY, Lin M, Tseung CH, Sun CF, et al. (2010) Phosphorylation status of transcription factor C/EBPalpha determines cell-surface poly-Lys-Nacetylhexadecane (L antigen) formation in erythropoiesis and granulopoiesis. Blood 115: 2491–2499.
11. Behre G, Singh SM, Liu H, Bertolin LT, Christopeit M, et al. (2002) Ras signaling enhances the activity of C/EBP alpha to induce granulocytic differentiation by phosphorylation of serine 240, 246. J Biol Chem 277: 26293–26299.
12. Reddy VA, Iwama A, Bozoga V, Schultz M, Ehsasser A, et al. (2002) Granulocyte inducer C/EBPalpha inactivates the myeloid master regulator PU.1: possible role in lineage commitment decisions. Blood 100: 483–490.
13. Porse BT, Pedersen TA, Hasemann MS, Schuster MB, Kirstetter P, et al. (2006) granulopoiesis. Mol Cell Biol 26: 1028–1037.
14. Kirstetter P, Schuster MB, Bereshchenko O, Moore S, Dvinge H, et al. (2008) Phosphorylation of C/EBPalpha mutant acute myeloid leukemia reveals a common expression signature of committed myeloid leukemia-initiating cells. Cancer Cell 13: 299–310.
20. Porse BT, Pedersen TA, Xu X, Lindberg B, Wever UM, et al. (2001) E2F repression by C/EBPα is required for adipogenesis and granulopoiesis in vivo. Cell 107: 247–258.

21. Porse BT, Bryder D, Theilig-Monch K, Hasemann MS, Anderson K, et al. (2005) Loss of C/EBP α α cell cycle control increases myeloid progenitor proliferation and transforms the neutrophil granulocyte lineage. J Exp Med 202: 85–96.

22. Hasemann MS, Dungaard I, Schuster MB, Theilig-Monch K, Sorensen AB, et al. (2008) Mutation of C/EBPα predisposes to the development of myeloid leukemia in a retroviral insertional mutagenesis screen. Blood 111: 4309–4321.

23. Bereshchenko O, Mancini E, Moore S, Bilhao D, Mannson R, et al. (2009) Hematopoietic stem cell expansion precedes the generation of committed myeloid leukemia-initiating cells in C/EBPα mutant AML. Cancer Cell 16: 390–400.

24. Wang X, Scott E, Swevers CL, Friedman AD (1999) C/EBPα bypasses granulocyte colony-stimulating factor signals to rapidly induce PU.1 gene expression, stimulate granulocytic differentiation, and limit proliferation in 32D cl3 myeloblasts. Blood 94: 560–571.

25. Guchhait P, Tosi MF, Smith CW, Chakaraborty A (2003) The murine myeloid cell line 32Dcl3 as a model system for studying neutrophil functions. J Immunol Methods 283: 195–204.

26. Pedersen TA, Bereshchenko O, Garcia-Silva S, Ermakova O, Kurz E, et al. (2007) Distinct C/EBPα motifs regulate lipogenic and gluconeogenic gene expression in vivo. EMBO J 26: 1081–1093.

27. Pröhl CJ, Rossi DJ, Mannson R, Attema JL, Nordhalm GL, et al. (2007) Elucidation of the phenotypic, functional, and molecular topography of a myeloid-myeloid progenitor cell hierarchy. Cell Stem Cell 1: 426–442.

28. Warren LA, Rossi DJ (2009) Stem cells and aging in the hematopoietic system. Mech Ageing Dev 130: 46–53.

29. Ross SF, Erickson RL, Hemati N, MacDougald OA (1999) Glycogen synthase kinase 3 is an insulin-regulated C/EBPα kinase. Mol Cell Biol 19: 8433–8441.

30. Mahoney CW, Shuman J, McKnight SL, Chen HC, Huang KP (1992) Phosphorylation of CCAAT-enhancer binding protein by protein kinase C attenuates site-selective DNA binding. J Biol Chem 267: 19396–19403.

31. Singh SM, Trivedi AK, Rehre G (2008) C/EBPα mutation reduces granulocytic differentiation in human leukemia K562 cells. Biochem Biophys Res Commun 369: 692–694.

32. Stuh LC, Geysy J, Renn K, Friedman AD, Johnson PF, et al. (2006) C/EBPα determines hematopoietic cell fate in multipotent progenitor cells by inhibiting erythroid differentiation and inducing myeloid differentiation. Blood 107: 4308–4316.

33. Fukuchi Y, Ito M, Shirata F, Kitamura T, Nakajima H (2006) Activation of CCAAT/enhancer-binding protein alpha or PU.1 in hematopoietic stem cells leads to their reduced self-renewal and proliferation. Stem Cells 26: 3172–3181.

34. Iakova P, Awad SS, Timchenko NA (2003) Aging Reduces Proliferative Capacities of Liver by Switching Pathways of C/EBP [alpha] Growth Arrest. Cell 113: 495–506.

35. Wang G-L, Shi X, Salisbury E, Sun Y, Albrecht JH, et al. (2006) Cyclin D3 Maintains Growth-Inhibitory Activity of C/EBP[alpha] by Stabilizing C/EBP[alpha]-cdk2 and C/EBP[alpha]-Brm Complexes. Mol Cell Biol 26: 2570–2582.

36. Wang G-L, Timchenko NA (2005) Dephosphorylated C/EBP[alpha] Accelerates Cell Proliferation through Sequestering Retinoblastoma Protein. Mol Cell Biol 25: 1325–1338.