Please cite this article in press as: Cirovic et al., C/EBP-Induced Transdifferentiation Reveals Granulocyte-Macrophage Precursor-like Plasticity of B Cells, Stem Cell Reports (2016), http://dx.doi.org/10.1016/j.stemcr.2016.12.015

Stem Cell Reports
Article

ISSCR

OPEN ACCESS

C/EBP-Induced Transdifferentiation Reveals Granulocyte-Macrophage Precursor-like Plasticity of B Cells

Branko Cirovic,[1,2] Jörg Schönheit,[1] Elisabeth Kowenz-Leutz,[1] Jelena Ivanovska,[1] Christine Klement,[1] Nina Pronina,[1] Valérie Bégay,[1] and Achim Leutz[1,2,*]

[1]Department of Tumorigenesis and Cell Differentiation, Max-Delbrueck-Center for Molecular Medicine, Robert-Roessle-Straße10, 13125 Berlin, Germany
[2]Humboldt-University of Berlin, Institute of Biology, 10115 Berlin, Germany
*Correspondence: aleutz@mdc-berlin.de
http://dx.doi.org/10.1016/j.stemcr.2016.12.015

SUMMARY

The lymphoid-myeloid transdifferentiation potentials of members of the C/EBP family (C/EBPα, β, δ, and ε) were compared in v-Abl-immortalized primary B cells. Conversion of B cells to macrophages was readily induced by the ectopic expression of any C/EBP, and enhanced by endogenous C/EBPα and β activation. High transgene expression of C/EBPβ or C/EBPε, but not of C/EBPα or C/EBPδ, also induced the formation of granulocytes. Granulocytes and macrophages emerged in a mutually exclusive manner. C/EBPβ-expressing B cells produced granulocyte-macrophage progenitor (GMP)-like progenitors when subjected to selective pressure to eliminate lymphoid cells. The GMP-like progenitors remained self-renewing and cytokine-independent, and continuously produced macrophages and granulocytes. In addition to their suitability to study myelomonocytic lineage bifurcation, lineage-switched GMP-like progenitors could reflect the features of the lympho-myeloid lineage switch observed in leukemic progression.

INTRODUCTION

Hematopoiesis is thought to begin from stem cells that progress through consecutive precursor cell stages in a hierarchical fashion whereby lineage commitment precludes alternative cell differentiation. However, there has been increasing evidence of hematopoietic plasticity and cell lineage conversion, particularly during leukemogenesis (Cobaleda and Busslinger, 2008; Graf, 2008; Greaves et al., 1986; Regalo and Leutz, 2013). The transcription factors C/EBPα and C/EBPβ are potent inducers of myelomonocytic genes in heterologous cell types (Ness et al., 1993), and the experimental conversion of lymphoid cells to myeloid cells modulated by both C/EBPs has highlighted their lympho-myeloid transdifferentiation potential (Graf and Enver, 2009).

The C/EBP family members C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε are expressed in myeloid cells (Cloutier et al., 2009; Scott et al., 1992). Loss-of-function studies in genetically modified mice suggested combinatorial and partially redundant functions of C/EBPs in myelopoiesis (Tsukada et al., 2011). Knockout studies showed that deletion of *Cebpa* has the strongest impact on myelopoiesis, resulting in an almost complete loss of neutrophils and impaired development of granulocyte-macrophage progenitor (GMP) cells (Zhang et al., 1997, 2004). However, cytokines could compensate for the lack of *Cebpa* by the concomitant activation of *Cebpb*, and genetic replacement of *Cebpa* with *Cebpb* in the *Cebpa* locus compensates for the *Cebpa* requirement in hematopoiesis and liver functions (Chen et al., 2000; Hirai et al., 2006; Jones et al., 2002). Individual deletions of C/EBPβ, δ, and ε evoke milder and gene-specific phenotypes, such as susceptibility to infections, failure of emergency granulopoiesis, impaired cytokine production, and partial granulocyte deficiency that is intensified by compound C/EBP gene deletions. For example, compound *Cebpb*/*Cebpe* deletion mutants display impaired granulopoiesis, defective macrophage functions, and a disrupted innate immune regulatory gene expression network, confirming the compensatory and redundant functions of the C/EBPs (Akagi et al., 2010; Hirai et al., 2006; Litvak et al., 2009; Tanaka et al., 1995; Yamanaka et al., 1997).

C/EBPα can stimulate the transdifferentiation of B and T cells and, together with PU.1, even fibroblasts into macrophages (Bussmann et al., 2009; Feng et al., 2008; Ness et al., 1993; Xie et al., 2004). Conversion of B cells into inflammatory-type macrophages occurs rapidly after C/EBP expression, with high efficiency and through a direct route (Bussmann et al., 2009; Di Tullio et al., 2011; Xie et al., 2004). An experimental transdifferentiation system based on an estrogen-responsive, conditional C/EBPα protein in the *v-H-ras*-transformed pre-B cell line HAFTL1 (Holmes et al., 1986) has served as a tool to examine the mechanistic aspects of lympho-myeloid lineage conversion, including alterations of chromatin occupancy, gene expression kinetics, non-coding RNA expression, and DNA methylation (Barneda-Zahonero et al., 2013; Di Tullio et al., 2011; Kallin et al., 2012; Krijger et al., 2016; Rodriguez-Ubreva et al., 2012, 2014; van Oevelen et al., 2015).

We recently found that structural alterations and post-translational modification sites of C/EBPβ may determine the path of transdifferentiation of primary progenitor B cells toward distinct myeloid cell fates (including

Please cite this article in press as: Cirovic et al., C/EBP-Induced Transdifferentiation Reveals Granulocyte-Macrophage Precursor-like Plasticity of B Cells, Stem Cell Reports (2016), http://dx.doi.org/10.1016/j.stemcr.2016.12.015

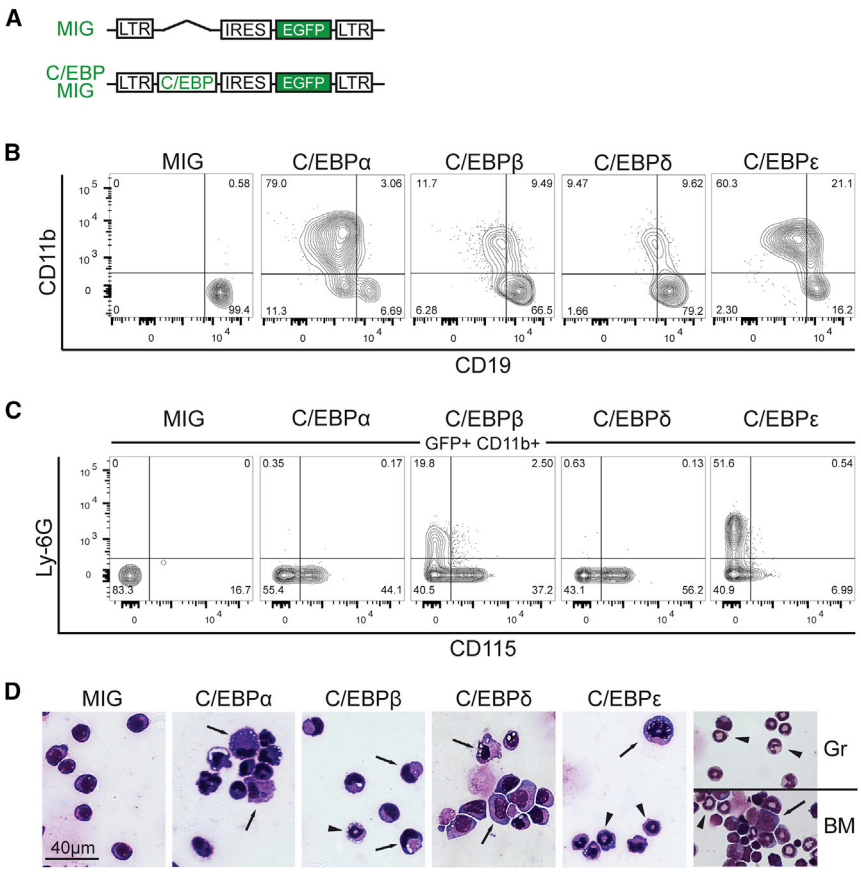


Figure 1. C/EBPα, β, δ, and ε Transdifferentiate B Cells to Myeloid Cells

(A) pMSCV-based vectors containing an internal ribosomal entry site (IRES) and EGFP (MIG) marker used for retroviral transduction. Full-length open reading frames of *Cebpa*, *Cebpb*, *Cebpd*, or *Cebpe* were inserted upstream of the IRES element. Empty vector (MIG) served as control.

(B) Flow cytometric analysis of GFP^+-infected B cells 4 days after transduction with individual C/EBPs.

(C) Analysis of granulocyte (Ly-6G) and macrophage (CD115) cell-surface markers in GFP^+CD11b^+ transdifferentiated cells 6 days after transduction.

(D) May-Grünwald staining of GFP^+-sorted B cells 4 days after transduction. Arrows indicate cells with typical macrophage morphology and arrowheads mark granulocyte-like cells. Primary bone marrow (BM) and sorted granulocytes (Gr) were used as references (far right).

See also Figure S1.

granulocytes and dendritic cells), suggesting that epigenetic instructions beyond the inflammatory macrophage cell fate are encoded in the C/EBP structure and could account for cell-type specification (Stoilova et al., 2013). This observation has prompted us to compare the lineage conversion capacity of all transactivator C/EBP family members and to develop a lympho-myeloid transdifferentiation system that is amenable to targeted mouse genetics and cell-culture manipulation.

In this study, we generated murine v-Abl-immortalized B cells from wild-type and genetically altered mice to compare the lympho-myeloid transdifferentiation potential of the C/EBP family members C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε. Our data showed that C/EBPβ and C/EBPε readily induce a granulocytic fate in addition to macrophage formation. Granulocytic conversion largely depended on transgene dosage. In addition, efficient transdifferentiation required endogenous *Cebpa*/*Cebpb*. Importantly, applying selective pressure on immortalized B cells expressing C/EBPβ by depriving β-mercaptoethanol resulted in the rapid extinction of B cells and massive expansion of stable myeloid cells. These myeloid progenitors displayed bipotential GMP-like properties and continuously produced macrophages and granulocytes. This process could suggest a link between C/EBP-induced lympho-myeloid lineage switch and a B cell-derived leukemic myelomonocytic GMP-like phenotype (Slamova et al., 2013).

RESULTS

Transdifferentiation Potential of Various C/EBP Family Members

v-Abl-immortalized pre-B cells derived from primary mouse bone marrow cells (hereafter termed "B cells") were used to assess the ability of individual C/EBP family members to induce B-lymphoid to myeloid transdifferentiation (Rosenberg et al., 1975; Shore et al., 2002). pMSCV-based retroviral expression vectors (MIG) encoding full-length C/EBPα-, C/EBPβ-, C/EBPδ-, or C/EBPε-IRES-EGFP were constructed (Figure 1A). Transgene expression was confirmed in the retrovirus-packaging cell line (Figure S1A). As shown in Figure 1B, each C/EBP family member could induce expression of the myeloid marker CD11b (Mac-1) and caused the transdifferentiation of B cells into $CD11b^+CD19^{dim}$

Please cite this article in press as: Cirovic et al., C/EBP-Induced Transdifferentiation Reveals Granulocyte-Macrophage Precursor-like Plasticity of B Cells, Stem Cell Reports (2016), http://dx.doi.org/10.1016/j.stemcr.2016.12.015

myeloid cells. Additional myeloid surface markers indicative of dendritic cells ($CD11c^+$), macrophages ($CD115^+$), or granulocytes ($Ly\text{-}6G^+$), were included to assess myeloid subsets in the $CD11b^+$ population (Figure S1B). While no $CD11c^+$ cells were found, a fraction of transdifferentiated cells showed upregulated CD115 (MCSF-R), a macrophage marker and C/EBP target gene. A $CD11b^+Ly\text{-}6G^+$ population, characteristic of granulocytes, was detected with C/EBPε expression, consistent with observations of strong mRNA expression of C/EBPε in bone marrow-derived granulocytes (Figures S1B and S1C). C/EBPβ expression also caused the formation of a $CD11b^+Ly\text{-}6G^+$ population after prolonged transdifferentiation for 6 days, whereas C/EBPα and C/EBPδ expression did not (Figures 1C and S1B). Interestingly, the expression of Ly-6G and CD115 appeared to be mutually exclusive, as no double-positive cells could be detected, indicating a stringent mechanism of cell-fate decision (Figure 1C). Cytospin preparations of GFP^+ cells confirmed that all C/EBPs stimulated the formation of myeloid cells with monocyte/macrophage features (Figure 1D). The C/EBPβ- and C/EBPε-transdifferentiated cell population contained small cells with segmented or indented nuclei, strongly resembling bone marrow-derived granulocytes and suggesting that C/EBPε or C/EBPβ may induce granulocyte/macrophage (G/M) cell fates. Transdifferentiation into granulocytes by C/EBPβ and C/EBPε was not restricted to the v-Abl B cell system, but also occurred in the fetal liver-derived *H-ras*-transformed HAFTL1 pre-B cell line (Figure S1D) (Bussmann et al., 2009). These findings suggest that all the tested C/EBP transcription factors could cause the conversion of B cells to myeloid cells and that C/EBPβ and C/EBPε could stimulate macrophage and granulocyte transdifferentiation of B cells.

Quantitative gene expression analysis was performed by NanoString hybridization 1 day after transduction with individual C/EBPs, at the earliest emergence of GFP-positive cells (Figures 2A and 2B; Table S1). A predefined mouse immunology code set covering 547 probes, including key transcription factors, was used. Genes with 2-fold altered expression levels, compared with empty vector-transduced B cell controls, were considered. As shown in Figures 2A and 2B, all C/EBPs upregulated and downregulated a core set of 22 and 12 genes, respectively. Each C/EBP family member also displayed additional and partial overlapping regulatory specificity. The core transdifferentiation signature of 22 upregulated genes included myeloid factors, such as *Csf1r*, *Csf2rb*, *Fcgr1*, and *Gfi1*, and several chemokine CC-family members (Figure 2C). The 12 downregulated genes included lymphocyte genes, such as *Il7r*, *Cd2*, and the *Rag* genes (Figure 2D). These data suggest that C/EBPα, β, δ, and ε likely suppress the B cell program and induce lympho-myeloid conversion.

Deletion of Endogenous *Cebpa* and *Cebpb* Impairs Transdifferentiation but Has No Impact on Cell-Type Outcome

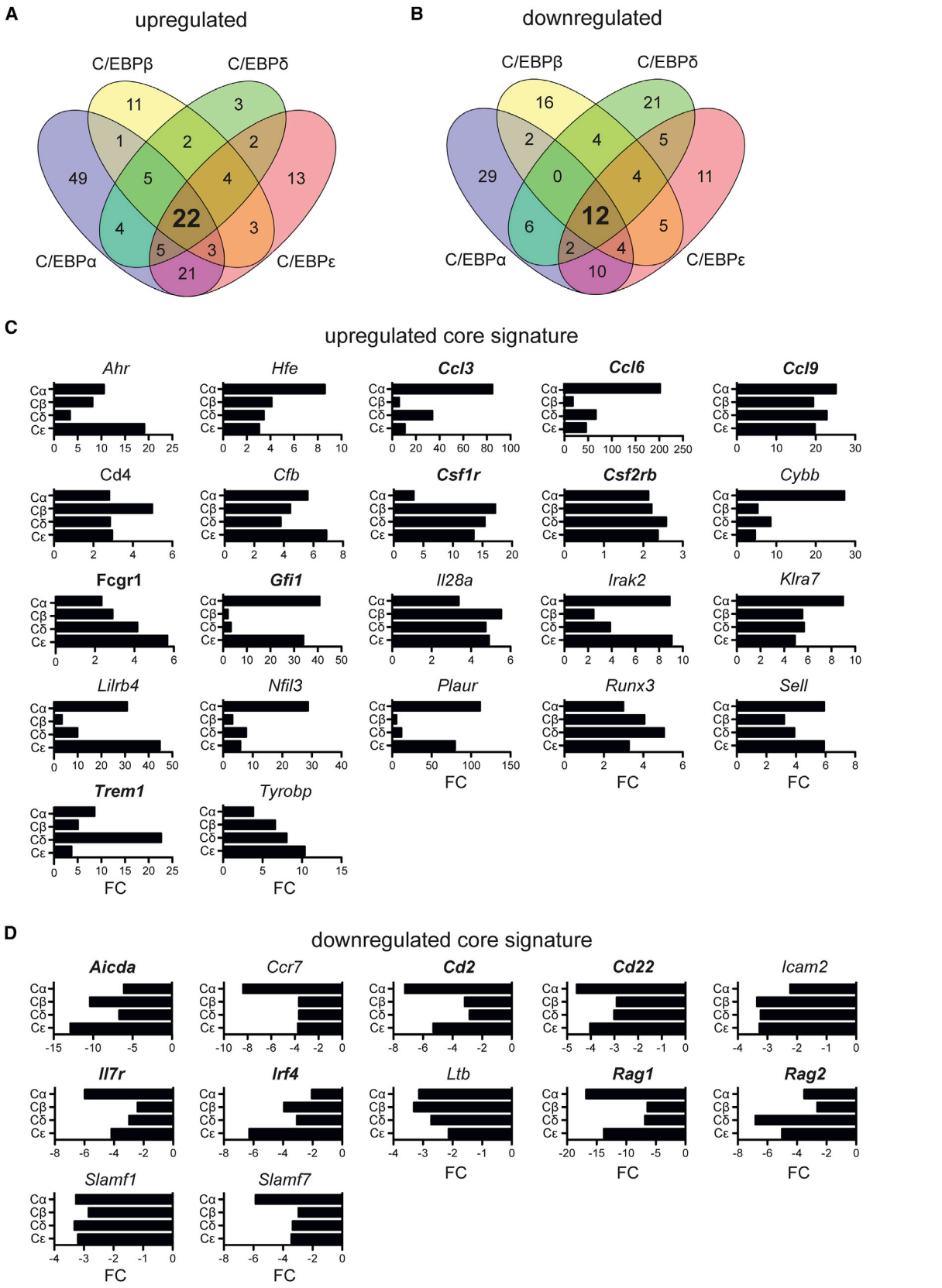
As previously reported by Bussmann et al. (2009) and shown in Figure S2A, activation of conditional C/EBPα-ER induced endogenous *Cebpb* and *Cebpd* gene expression in HAFTL1 B cells. We induced myeloid transdifferentiation via C/EBPε expression in HAFTL1 cells and analyzed endogenous C/EBPα and C/EBPβ protein expression. Interestingly, the expression of C/EBPε also led to a marked upregulation of endogenous C/EBPα after 16 hr, while only low levels of C/EBPβ were detected. However, after 24 hr, C/EBPβ protein expression increased while C/EBPα expression was diminished. A surge of C/EBPα and C/EBPβ expression was observed after 120 hr, indicating crosstalk between transgenic and endogenous C/EBPs during transdifferentiation (Figure S2B).

Synergistic collaboration between C/EBP family members was previously described as a key element to specify myeloid differentiation (Akagi et al., 2010). To examine the contribution of endogenous C/EBPs to lympho-myeloid transdifferentiation, we treated B cells generated from a homozygous $Cebpa^{f/f}$;$Cebpb^{f/f}$ mouse with cell-permeable Cre-recombinase to generate double-knockout derivatives (Figure S2C). Verified $Cebpa^{\Delta/\Delta}$;$Cebpb^{\Delta/\Delta}$ double-knockout B cell clones were designated as "B-DKO" (Figure S2D).

B-DKO cells or isogenic controls (not treated with Cre-recombinase) were infected with the G/M-transdifferentiation proficient retroviral constructs that co-expressed C/EBPβ and GFP (MIG) or C/EBPε and BFP (MIB) (Figures 3A and 3B). GFP^+BFP^+ gated cell populations, indicative of C/EBPβ and C/EBPε expression, were analyzed (Figure 3C). Deletion of endogenous *Cebpa* and *Cebpb* strongly impaired transdifferentiation induced by C/EBPε or C/EBPβ after 4 days, in comparison with isogenic controls (Figures 3C and S2E). Transdifferentiation efficacy in C/EBPε-expressing B-DKO cells could be partially rescued by retroviral co-expression of C/EBPβ. These data suggest synergistic effects between endogenous and exogenous C/EBPs during transdifferentiation (Figures 3C and S2E).

Comparison of gene expression in C/EBPε-transduced wild-type or B-DKO cells revealed genes that were dependent on endogenous *Cebpa* and *Cebpb* (Figure S2F and Table S1). The list of refractory genes included *Csf1r*, *Fcgr1*, *Itgam* (CD11b), and the transcription factor *Gfi1*, known to promote granulocyte differentiation. These genes represent important myeloid cell factors, and failure of induction may explain the impaired transdifferentiation (Figures 3C and S2E). In addition, *Pax5* or *Pou2f2* (Oct-2) were refractory to downregulation, which could contribute to the persistence of the B cell phenotype (Figure S2G and Table S1).

Please cite this article in press as: Cirovic et al., C/EBP-Induced Transdifferentiation Reveals Granulocyte-Macrophage Precursor-like Plasticity of B Cells, Stem Cell Reports (2016), http://dx.doi.org/10.1016/j.stemcr.2016.12.015



(legend on next page)

To determine whether the G/M cell-type outcome was affected by deletion of endogenous *Cebpa* and *Cebpb*, we analyzed C/EBPε-transdifferentiated GFP$^+$CD11b$^+$ cells for Ly-6G or CD115 surface marker expression after 7 days of culture. Comparison of transdifferentiated B-DKO and control cells did not reveal differences in the frequency of Ly-6G$^+$ versus CD115$^+$ cells in the CD11b$^+$-gated cell population (Figure 3D), suggesting that the continuously expressed transgene product but not endogenous *Cebpa* and *Cebpb* predominantly affected myeloid cell lineage choice.

C/EBP Dosage and Granulocyte-Macrophage Lineage Choice

Transcription factor dosage is an important determinant in hematopoietic lineage choice (Dahl et al., 2003; DeKoter et al., 2007; DeKoter and Singh, 2000; Di Tullio and Graf, 2012; Ma et al., 2014; Rosenbauer et al., 2004; Simmons et al., 2012). We therefore examined how the level of C/EBP transgene expression would alter lympho-myeloid cell fate. As shown in Figures S3A and S3B, GFP intensity directly correlated with retroviral C/EBP protein expression and enabled us to examine transcription factor dosage effects on lympho-myeloid transdifferentiation. Based on increasing GFP intensity, C/EBPβ-transduced B cells were divided into six fractions, GFP1–6 (Figure 4A). Macrophage and granulocyte surface marker analysis of the CD11b$^+$ cells revealed a bipartite profile when all GFP$^+$ cells were included in the analysis (as in Figure 1C). As shown in Figures 4B and 4C, the distribution of CD11b$^+$Ly-6G$^+$ versus CD11b$^+$CD115$^+$ shifted according to the GFP intensity. GFP^{dim} fractions were preferentially CD11b$^+$CD115$^{-/+}$ and Ly-6G$^-$. The CD11b$^+$Ly-6G$^+$ cell population expanded with increasing GFP intensity, while the CD11b$^+$CD115$^+$ cell fraction diminished. The fraction with the highest GFP intensity (GFP6) consisted mostly of CD11b$^+$Ly-6G$^+$ cells (Figures 4B and 4C). Cytospin preparations of GFP^{dim}- and GFP^{high}-sorted cells showed that the GFP^{dim} fraction consisted of large cells, characteristic of monocyte/macrophages, while the GFP^{high} fraction contained cells with indented and segmented nuclei—typical features of mature granulocytes (Figure 4D). Finally, B cells transduced with C/EBPε were separated by flow cytometry according to their GFP intensity 24 hr post infection. Six days later, cell-surface marker expression analysis indicated that the GFP^{high} cells were enriched for Ly-6G$^+$ versus CD115$^+$ cells when compared with the GFP$^+$ culture. These data indicate a prospective cell-type outcome according to the initial C/EBP dosage (Figure 4E). To further exclude artifacts produced by the bicistronic C/EBP-IRES-EGFP MIG constructs, we examined the dosage effect using retroviral vectors with direct C/EBPε-GFP fusion. As shown in Figure S3C, higher GFP intensity correlated with the G/M surface marker profile. Taken together, these findings show that the dosage of C/EBPβ or C/EBPε transcription factors determines granulocyte versus macrophage cell-fate outcome during lympho-myeloid transdifferentiation.

Long-Term Proliferating C/EBPβ-Lympho-Myeloid Progeny Display a GMP-like State and Spontaneously Differentiate into Granulocytes and Macrophages

To assess the proliferation and lineage-forming capacity of C/EBPβ-transdifferentiated B cells, we aimed to establish a stably converted B cell line. After the removal of β-mercaptoethanol, an essential culture supplement for B cell maintenance, all B cells perished within a few days in C/EBPβ-transduced and empty vector-transduced cells. In contrast to the controls, however, proliferating GFP$^+$ cells emerged from C/EBPβ-transduced B cells within 2 weeks after infection. Comparison of surface marker expression after 8 days and 6 weeks of cell culture showed that the long-term proliferating cells maintained both macrophage and granulocyte differentiation potential (Figures 5A and 5C). Cytospin preparations from CD11b$^+$Ly-6G$^+$ and CD11b$^+$CD115$^+$ sorted cells resembled typical granulocytes and macrophages, respectively (Figures 5B and 5D). Genotyping of rearranged immunoglobulin heavy chain (IgH) locus of C/EBPβ-transduced cells ruled out the possibility of a contamination with myeloid remnants from initiating cultures (Figure 5E). Immunoblot analysis showed that transgenic C/EBPβ protein is present and that endogenous C/EBPβ protein isoforms are also upregulated in transdifferentiated cells (Figure 5F). In addition, C/EBPβ-transduced cells grew in a semi-adherent fashion and showed irregular morphological features typical of myeloid progenitor cells, in contrast to round, refractile B cells. Upon treatment with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) the proliferation rate decreased and cells became adherent, large, and motile, resembling activated macrophages and suggesting that the transdifferentiated cells respond to myeloid signaling pathway activation (Figures S4A and S4B).

The G/M bilineage phenotype of the transdifferentiated cells was maintained during more than 6 months of continuous cell culture. Nevertheless, C/EBPβ may

Figure 2. Transdifferentiation Core Gene Signatures of B Cells Induced with C/EBP Family Members

Quantification of mRNA from GFP$^+$-sorted B cells 24 hr after transduction with individual C/EBPs. Venn diagram of (A) upregulated and (B) downregulated genes, compared with empty vector (MIG) control. Overlapping core signatures of (C) 22 upregulated or (D) 12 downregulated genes. Key myeloid (in C) and lymphoid (in D) genes are shown in bold. The fold change (FC) relative to empty vector (MIG)-transduced B cells is shown. See also Table S1.

Please cite this article in press as: Cirovic et al., C/EBP-Induced Transdifferentiation Reveals Granulocyte-Macrophage Precursor-like Plasticity of B Cells, Stem Cell Reports (2016), http://dx.doi.org/10.1016/j.stemcr.2016.12.015

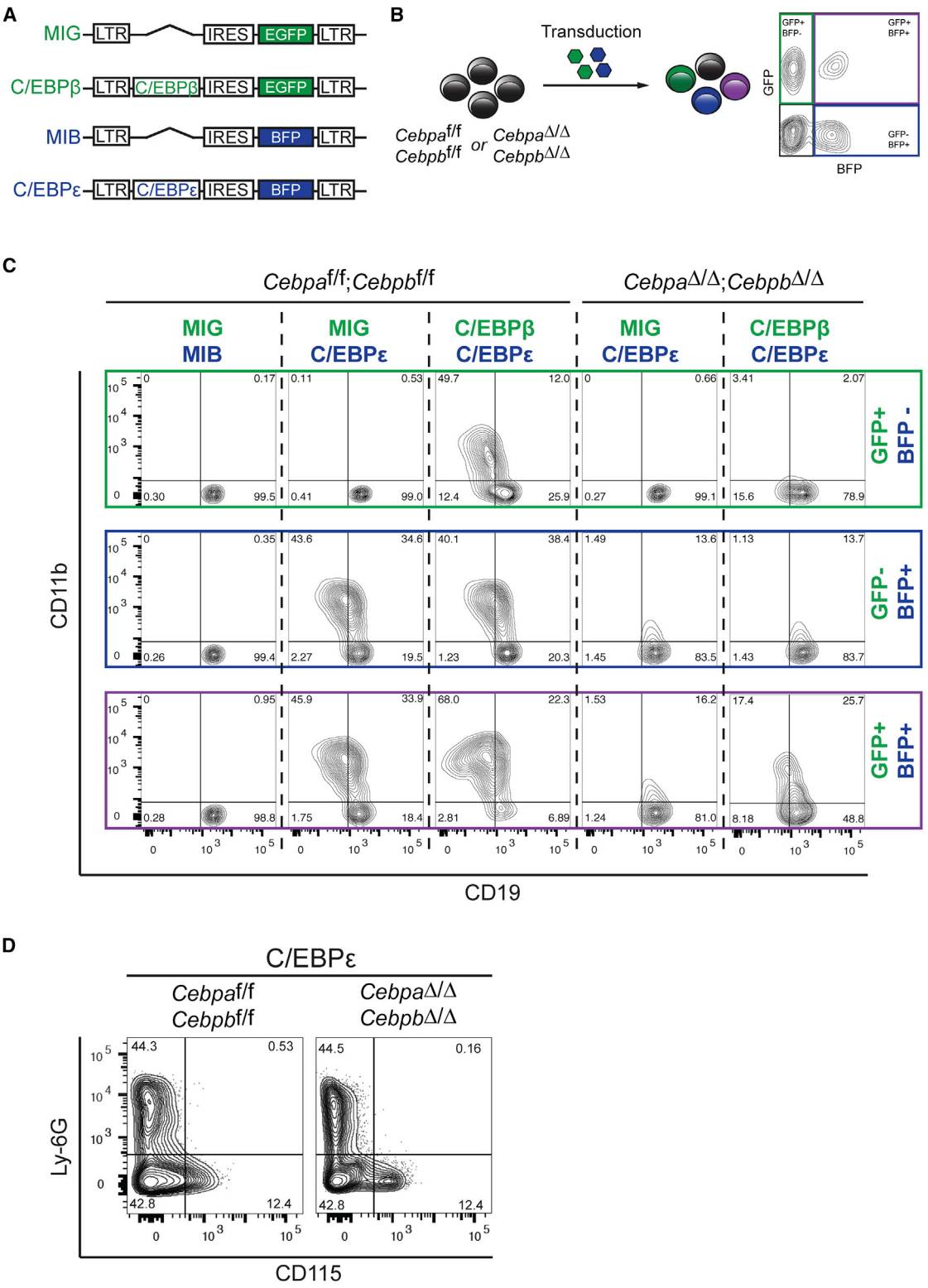


Figure 3. Endogenous *Cebpa* and *Cebpb* Promote Transdifferentiation but Do Not Affect Lineage Outcome

(A) pMSCV-based vectors for the expression of C/EBPβ linked to EGFP marker or C/EBPε linked to BFP. Empty vectors (MIG/MIB) were used as controls.

(legend continued on next page)

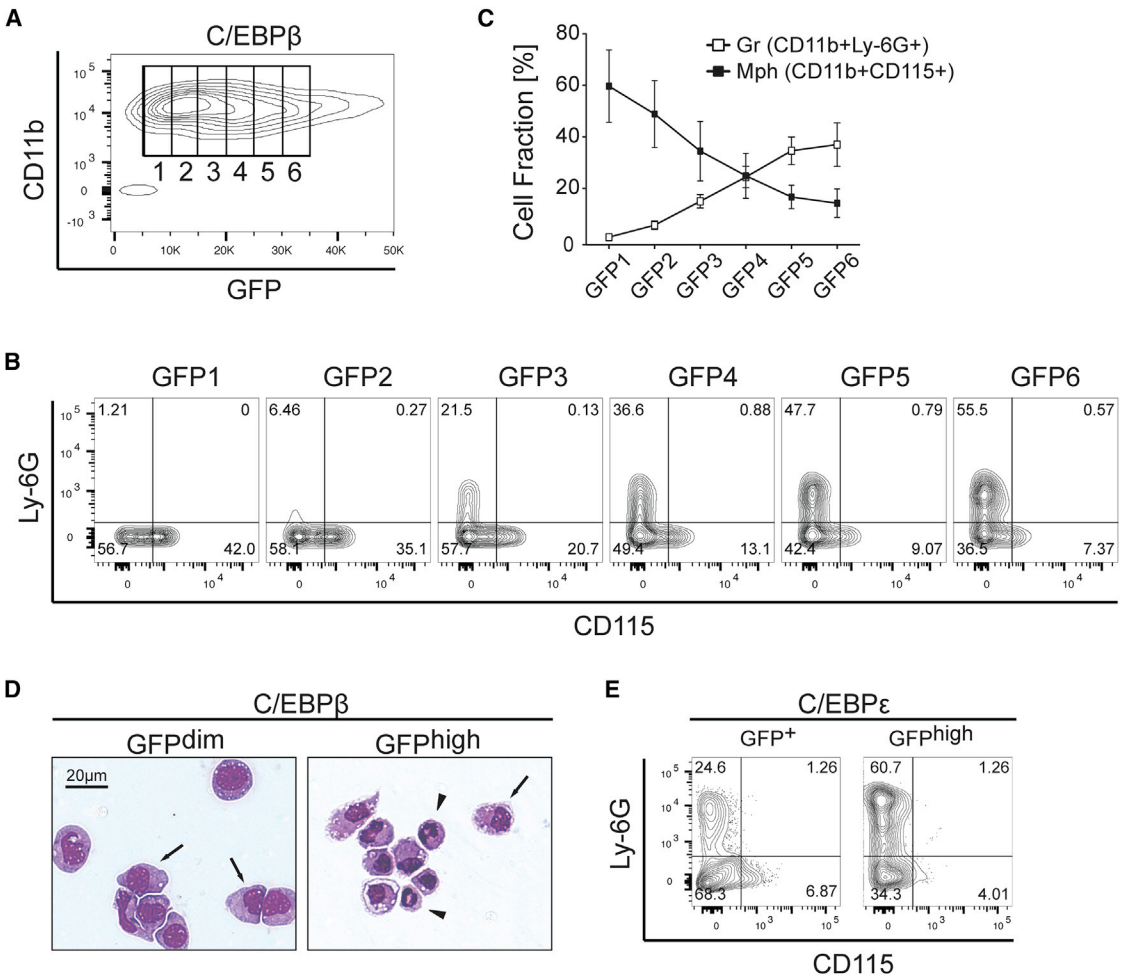


Figure 4. C/EBPβ Transgene Dosage Affects Macrophage versus Granulocyte Lineage Outcome

(A) Data binning of C/EBPβ-transduced B cells according to GFP intensity (GFP1–6).

(B) Flow cytometric analysis of GFP1–6 bins (CD11b + gated) 6 days after transduction.

(C) Quantification from five independent experiments. Data are presented as mean ± SEM.

(D) Cytospin preparation and May-Grünwald staining of GFP^{dim}- and GFP^{high}-sorted C/EBPβ-transduced B cells at day 7. Arrows indicate macrophage and arrowheads granulocyte-like cells.

(E) B cells were transduced with C/EBPε, sorted after 24 hr as GFP^+ or GFP^{high}, and recultured. Flow cytometric analysis of granulocyte ($Ly\text{-}6G^+$) versus macrophage ($CD115^+$) markers in GFP^+CD11b^+-gated populations 6 days post sorting.

See also Figure S3.

stimulate B cells to transdifferentiate into distinct G and M progenitors either directly or through a common GMP cell type. To distinguish between these possibilities, we seeded 4,500 of the stably transdifferentiated cells into semi-solid medium to derive colonies from single cells. Approximately 40–70 colonies were obtained in a single experiment. Individual GFP^+ colonies were inspected from day 6 to day 18 and displayed a compact core and a periphery of larger motile, oval cells, reminiscent of colony-forming unit GM colonies (Figure 5G). Two colonies were isolated

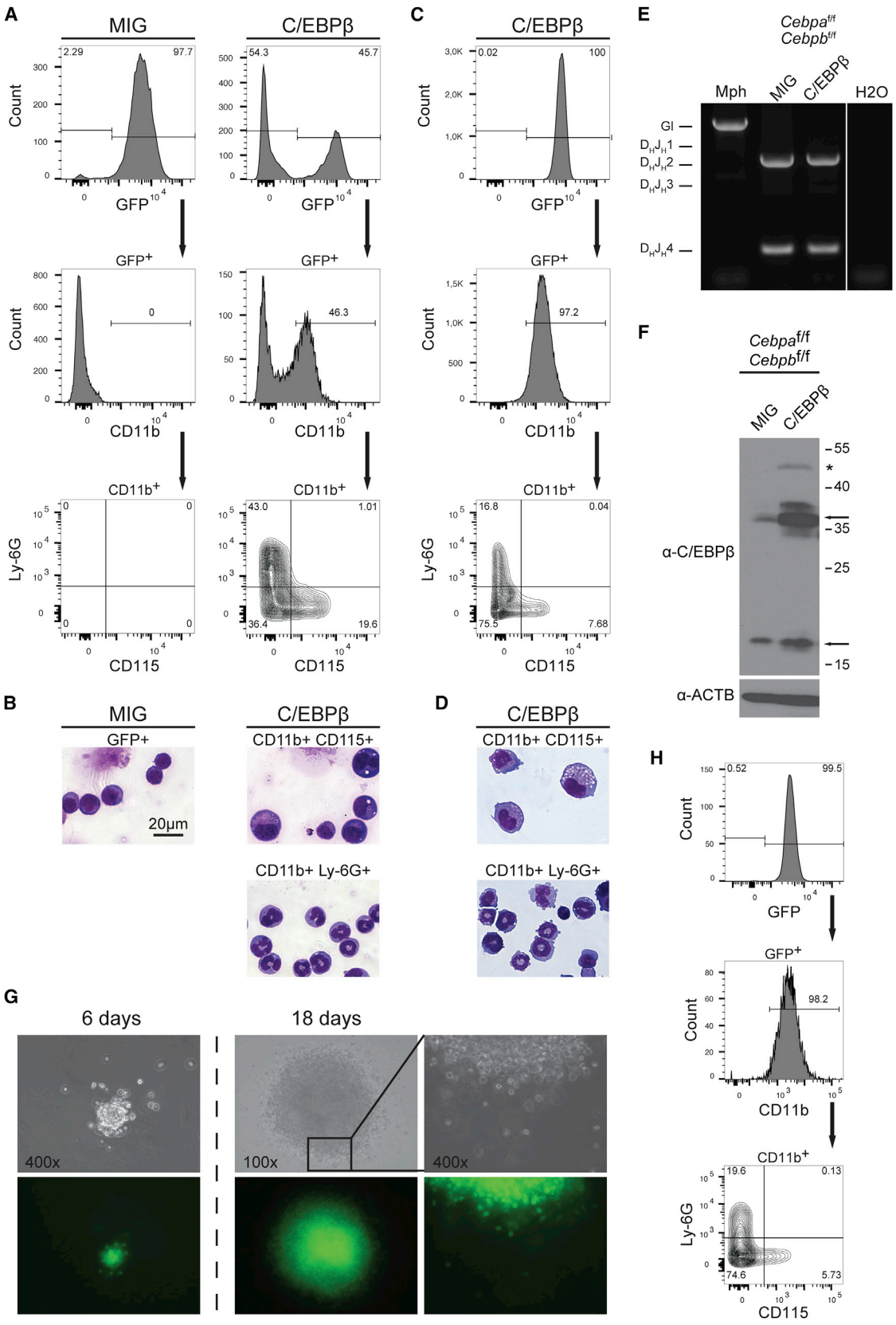
(B) $Cebpa^{\Delta/\Delta}$;$Cebpb^{\Delta/\Delta}$ or isogenic control B cells ($Cebpa^{f/f}$;$Cebpb^{f/f}$) were transduced with combinations of the vectors shown in (A).

(C) The GFP^+BFP^- fraction (top panels), GFP^-BFP^+ fraction (middle panels), and GFP^+BFP^+ fraction (bottom panels) were individually analyzed for myeloid (CD11b) and B cell (CD19) markers by flow cytometry after 4 days.

(D) $Cebpa^{\Delta/\Delta}$;$Cebpb^{\Delta/\Delta}$ B cells and isogenic controls were transduced with C/EBPε, GFP^+-sorted after 24 hr, and recultured. Flow cytometric analysis of granulocyte ($Ly\text{-}6G^+$) and macrophage ($CD115^+$) cell fractions in GFP^+CD11b^+-gated populations 7 days after transduction.

See also Figure S2.

Please cite this article in press as: Cirovic et al., C/EBP-Induced Transdifferentiation Reveals Granulocyte-Macrophage Precursor-like Plasticity of B Cells, Stem Cell Reports (2016), http://dx.doi.org/10.1016/j.stemcr.2016.12.015



Please cite this article in press as: Cirovic et al., C/EBP-Induced Transdifferentiation Reveals Granulocyte-Macrophage Precursor-like Plasticity of B Cells, Stem Cell Reports (2016), http://dx.doi.org/10.1016/j.stemcr.2016.12.015

and propagated in liquid culture and both displayed a Ly-6G$^+$ and CD115$^+$ phenotype indicative of GMP-like potential (Figure 5H, result for one clone is shown). Subsequent recloning of one of the isolates in semi-solid medium resulted in stably growing secondary subclones (cloning efficiency between ~4% and 6%) and persistence of the GMP-like phenotype of all 17 subclones examined, as shown in Figure S5.

We conclude that C/EBPβ-induced lympho-myeloid conversion confers a selective advantage by abrogating the dependence on B cell survival conditions. These C/EBPβ-converted cells acquire cytokine-independent long-term proliferation capacity and a robust GMP-like potential.

DISCUSSION

Transcription factor-mediated conversion of one cell type into another is an important avenue by which we can advance our understanding of instructive mechanisms of cellular lineage commitment and differentiation. The primary B cells immortalized by v-Abl provide an experimental system to combine B cell mouse genetics and ectopic gene expression for the exploration of progenitor biology, lympho-myeloid lineage plasticity, maintenance of cell identity, and regulatory mechanisms of myeloid lineage commitment.

Experimental transdifferentiation of B cells by ectopic expression of conditional C/EBPα has shed light on the molecular dynamics of the conversion of lymphoid cells into inflammatory macrophages (Bussmann et al., 2009; Di Tullio et al., 2011; Krijger et al., 2016; Laiosa et al., 2006; Rodriguez-Ubreva et al., 2012, 2014; van Oevelen et al., 2015; Xie et al., 2004). However, lympho-myeloid transdifferentiation may not be restricted to activated macrophages but may yield other myeloid cell types depending on the C/EBP structure and post-translational modification pattern (Stoilova et al., 2013). Here, we assessed the lympho-myeloid transdifferentiation potential of C/EBP α, β, δ, and ε in primary v-Abl-immortalized B cells. Our data show that v-Abl B cells represent a robust in vitro system for experimental cell lineage switch and may also be used for targeted murine genetics and in vitro genetic manipulation at the B cell stage.

Lineage conversion could be achieved using any of the four C/EBP members, revealing a common gene signature, and their common potential to extinguish B cell identity and induce the myeloid cell fate. C/EBPβ and C/EBPε induced granulocytic differentiation in addition to monocytic transdifferentiation. Granulopoietic capacity of C/EBPβ has previously been documented in emergency granulopoiesis (Hirai et al., 2006) and by restoring *Cebpa* deficiency (Jones et al., 2002). The lack of granulopoietic transdifferentiation potential of C/EBPα was surprising and has been discussed previously (Xie et al., 2004), because C/EBPα induced granulocyte differentiation in a myeloid progenitor cell line (Radomska et al., 1998; Suh et al., 2006). Although we do not know why C/EBPα and C/EBPδ fail to induce granulocyte formation, the strong proliferation arrest elicited by C/EBPα could account for the failure of granulocytic conversion. On the other hand, our data may also raise the possibility that some of the functions of C/EBPα reported in established progenitor cell lines may depend on the activation of endogenous C/EBPβ. Nevertheless, the v-Abl B cell system could be useful to resolve quantitative and qualitative C/EBP functions and help uncover mechanisms of lineage specification, e.g., by C/EBP domain-swap experiments and mutational analysis.

Our findings support the concept that subtle variations in transcription factor levels have an impact on hematopoietic cell lineage decisions. In addition to transcription factor dosage, specificity and functional redundancy play major roles; high levels of C/EBPβ or C/EBPε consistently increased the granulocyte over macrophage ratio. The exact interplay between C/EBP quantity and qualitative

Figure 5. Long-Term Proliferating GMP-like Cells Derived from C/EBPβ-Transduced B Cells

(A) *Cebpa*$^{f/f}$;*Cebpb*$^{f/f}$ B cells transduced with C/EBPβ or vector control (MIG), sorted after 24 hr, and recultured. Flow cytometric analysis of myeloid surface markers was performed after 6 days. Gating strategy is indicated by arrows.

(B) May-Grünwald staining of macrophage (CD11b$^+$CD115$^+$) or granulocyte (CD11b$^+$Ly-6G$^+$) marker-sorted C/EBPβ-transduced cells from (A) and GFP$^+$-purified empty vector (MIG)-transduced cells as control.

(C and D) Flow cytometry analysis (C) and May-Grünwald staining (D) according to (A) and (B) of non-purified C/EBPβ-transduced cells 6 weeks after removal of β-mercaptoethanol.

(E) Analysis of immunoglobulin heavy chain (IgH) rearrangement of long-term C/EBPβ-transduced cells or MIG-transduced controls. Monocytic Raw264.7 (Mph) cells served as a control for IgH germ line (Gl) configuration.

(F) Immunoblot analysis of C/EBPβ from protein lysates of long-term C/EBPβ- or empty vector (MIG)-transduced B cells. Arrows indicate C/EBP isoforms; asterisk indicates the size of transgene.

(G) Colony formation from single cells. Phase contrast (top) and fluorescent signal (bottom) micrographs of a C/EBPβ-transdifferentiated B cell colony. Clonogenic growth is shown at days 6 and 18. Magnification of inset area focuses on the border of the colony.

(H) Flow cytometric characterization of the recultured cell clone derived from (G) using myeloid markers.

See also Figure S5.

specificity remains to be explored, but is likely similar to graded PU.1 expression that has an impact on lympho-myeloid specification by occupancy of low-affinity binding sites only at high transcription factor concentrations (DeKoter and Singh, 2000; Pham et al., 2013). Relative expression levels of PU.1 and C/EBPα are also key determinants in granulocyte versus macrophage specification. Higher PU.1 levels promote macrophage differentiation, while higher C/EBPα levels lead to granulopoiesis (Dahl et al., 2003; Ma et al., 2014). Therefore, less accessible "granulocytic genes" may require higher C/EBP concentrations in addition to other features of C/EBPβ and C/EBPε proteins.

Activation of endogenous C/EBP genes and impaired lineage conversion after their removal suggests that autoregulatory loops are involved in the kinetics of lineage conversion (Bussmann et al., 2009; Lu et al., 2009). However, deletion of endogenous C/EBPα and C/EBPβ appears to affect the conversion efficiency but not the lineage outcome, suggesting a dominant and distinguished qualitative role for the constitutively expressed C/EBP transgene.

Leukemia with indifferent or promiscuous lineage association is frequently resistant to standard chemotherapy and may show changed lineages during relapse (Dorantes-Acosta and Pelayo, 2012; Golemovic et al., 2006; Matutes et al., 1997). Inter-myeloid and lympho-myeloid lineage ambiguity therefore emerges as an important pathological entity (Greaves et al., 1986; Smith et al., 1983). It is therefore conceivable that switching lineages reflect an epigenetic selection process in leukemogenesis associated with gain-of-function survival and proliferation capacity (Greaves et al., 1986; Janz et al., 2006; Regalo and Leutz, 2013). Our experimental data provide direct evidence that enhancing the selective pressure on B cells by removal of β-mercaptoethanol strongly promotes C/EBPβ-induced lympho-myeloid converts that remained immortalized and highly proliferative. We therefore conclude that cancerous B cells may escape from hostile conditions by lineage conversion through C/EBPβ activation. Furthermore, B cells could profit from lineage-foreign survival signals by aberrant expression of growth factor receptors, such as CSF1-R, a target gene of C/EBPβ (Lamprecht et al., 2010). Various C/EBP translocations have been associated with B cell-derived leukemia, implying their cancer-supporting function. However, the role played by transdifferentiation remains unknown (Akasaka et al., 2007; Chapiro et al., 2006; Slamova et al., 2013; Wiemels et al., 2015). Strikingly, DNA demethylation and upregulation of C/EBPα was suspected to be involved in lineage switching during leukemia progression (Slamova et al., 2013). In addition to pre-B cell lymphoma, v-Abl retroviral infection of murine bone marrow cells also evoked a chronic myeloid leukemia-like disease that involved immunoglobulin rearrangements (Kelliher et al., 1990).

In a recent study, pulsed C/EBPα expression was shown to induce a GMP-like state according to gene expression and chromatin accessibility data. However, during prolonged exposure only a macrophage fate was consolidated (Di Stefano et al., 2016). Remarkably, the stable C/EBPβ B cell converts reported here resembled self-renewing bipotential GMP progenitors that were responsive to GM-CSF but not dependent on cytokine signaling, and stably retained G/M differentiation potential. In the absence of cytokines, expression of macrophage- and granulocyte-specific markers emerged in a mutually exclusive fashion. These data suggest the involvement of C/EBPβ in maintenance, commitment, or both at the GMP stage.

The cytokine-independent, self-renewing, spontaneously differentiating GMP-like cells present a valuable experimental tool for the study of determinants of granulocyte versus macrophage specification by genetic manipulation and screening approaches. In addition, maintenance of the transdifferentiation capacity of the v-Abl B cells establishes a sound basis for using targeted genetic mouse models to examine signaling pathways and other instructive cues of cell specification. The fact that v-Abl B cells are amenable to DNA transfection permits experimental alteration at the B cell stage, such as stable transfection, gene deletion, or chromosomal engineering, and is an important technical advance in overcoming the hurdle of difficult genetic manipulation of myelomonocytic cells.

In summary, we present a robust in vitro lympho-myeloid transdifferentiation system to study determinants of lineage promiscuity, GMP progenitor biology, and macrophage versus granulocyte cell-fate decisions. In addition, our findings lend experimental evidence to lympho-myeloid transdifferentiation processes as a trait of lineage ambiguity in leukemia progression.

EXPERIMENTAL PROCEDURES

Expression Constructs

C/EBP genes were inserted into pMSCV-IRES-EGFP (MIG) or pMSCV-IRES-BFP (MIB) retroviral vectors using XhoI/EcoRI restriction sites. C/EBPs lacking stop codons were cloned in-frame with the EGFP open reading frame and inserted into pMSCV for C/EBP-GFP fusion construct. Expression vectors encoding C/EBPα p42 isoform and C/EBPβ LAP* isoform were described previously (Stoilova et al., 2013). Murine C/EBPδ was isolated from pMEX-mC/EBPδ (a gift from Dr. E Sterneck). Codon-optimized human C/EBPε p32 isoform was commercially synthesized (MWG-Biotech) and subcloned from pEX-A2 into MIG or MIB using EcoRI sites flanking C/EBPε. The v-Abl-expressing pMSCV vector was a gift from Dr. F Melchers (Ohnishi and Melchers, 2003). For production of infectious supernatants the retroviral packaging cell line

Plat-E was used. Infectious supernatants were collected 48 hr and 72 hr after transfection.

Generation of v-Abl-Transformed B Cells

Bone marrow cells were isolated from the femur and tibia of 8- to 9-week-old *Cebpa*$^{f/f}$-crossed (Zhang et al., 2004) and *Cebpb*$^{f/f}$-crossed (Sterneck et al., 2006) *Cebpa*$^{f/f}$;*Cebpb*$^{f/f}$ mice and C57BL/6J wild-type controls. These mice were maintained and handled in compliance with protocols approved by the institutional Animal Care and Use Committee. After erythrolysis, bone marrow cells were transduced with v-Abl-expressing retroviral supernatants and 8 μg/mL hexadimethrine bromide in complete DMEM (10% fetal calf serum (FCS), 10 mM HEPES, and penicillin/streptomycin (Gibco)) supplemented with 50 μM β-mercaptoethanol. Bone marrow cells were washed on the following day and medium was changed every other day. A stable cell line with a pre-B cell-like phenotype (CD19$^+$, c-kit$^-$, CD25$^+$, IgM$^-$) emerged after 4 weeks of culture. For *loxP* site recombination, 5×10^5 B cells were washed three times in serum-free DMEM and incubated with purified TAT-Cre protein (50 μg/mL; a gift from Dr. K Rajewsky) in serum-free DMEM at 37°C for 45 min (Peitz et al., 2002). The cells were then washed and cultured for 24 hr and seeded subsequently as single-cell clones (by fluorescence-activated cell sorting (FACS)) into 96-well plates. Deletion of the *Cebpa* and *Cebpb* genes was checked by PCR to identify double-knockout clones. Primer sequences used are shown in Table S2.

Cell Lines

The monocytic/macrophage Raw264.7 cell line was cultured in complete DMEM. The fetal liver-derived pre-B cell line HAFTL1 was cultured in complete RPMI-1640 (10% FCS, 10 mM HEPES, penicillin/streptomycin (Gibco)) supplemented with 50 μM β-mercaptoethanol (Pierce and Aaronson, 1982). The HAFTL1-derived C10 clone (a gift from Dr. T Graf) containing a C/EBPα-ER_IRES_GFP transgene was cultured in complete phenol red-free RPMI-1640 (10% charcoal-stripped FCS (HyClone), 10 mM HEPES, penicillin/streptomycin (Gibco)) supplemented with 50 μM β-mercaptoethanol (Bussmann et al., 2009). Transdifferentiation of C10 cells was induced with 200 nM hydroxytamoxifen (Sigma). 293T-derived Plat-E line (Cell Biolabs) was used for packaging.

Retroviral Transduction

v-Abl-generated stable B cells or HAFTL1 cells (2×10^5/12-well or 1×10^6/6-well) were mixed with freshly prepared retroviral supernatants (1/3 of total volume) in complete medium supplemented with 8 μg/mL hexadimethrine bromide and 50 μM β-mercaptoethanol. Cells were centrifuged at 1,000 rpm without brakes for 10 min at room temperature and incubated overnight. Infected cells were washed and processed for flow cytometry or replated in 6-well plates (BD Falcon). For the generation of the long-term proliferating transdifferentiated cells, β-mercaptoethanol supplementation was omitted after 24 hr. Half of the medium was changed every other day.

Flow Cytometry

For flow cytometry or cell sorting, cells were transferred to 15-mL tubes and washed in cold buffer (2% FCS, 2 mM EDTA in PBS). Before antibody labeling, cells were incubated with Fc-Block (1:200, anti-CD16/32 antibody; 2.4G2, BD Pharmingen) for 10 min at 4°C and washed. Cells were stained in buffer with (1:100) fluorescently labeled antibodies against CD11b (clone M1/70, BD Pharmingen), CD11c (clone N418, Biolegend), CD115 (clone AFS98, eBioscience), CD19 (clone 1D3, BD Pharmingen), and Ly-6G (1A8, Biolegend) for 30 min at 4°C in the dark. 7-AAD (BD Pharmingen) was added before measurement to exclude dead cells. Analysis was performed on LSRFortessa and sorting on FACSAria II or III (BD Pharmingen). Unstained, empty vector-transduced cells or fluorescence-minus-one staining setups served as controls.

PCR

Total RNA was prepared using TriPure (Roche) following the manufacturer's instructions. DNA contamination was removed by DNase I treatment (Roche). For standard cDNA synthesis the RevertAid First Strand Synthesis Kit (Thermo Scientific) was used. Ten nanograms of cDNA was applied to PCR according to the MangoTaq kit protocol (Bioline). PCR reaction was subjected to 94°C for 3 min and 27 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 5 min on a Mastercycler Pro (Eppendorf). For detection of D_H-J_H IgH rearrangements, DNA was prepared as described by Truett et al. (2000) and subjected to PCR conditions as reported by Ehlich et al. (1994), using the combination of primers DQ52 FW1, DFS FW2, and JH4A RV. *Cebpa* and *Cebpb* genotypes were assessed using "-flox" or "-Δ" primer pairs, respectively.

Immunoblotting

Total protein lysates were prepared by incubation of snap-frozen cell pellets with lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 10% glycerol, and proteinase inhibitor cocktail [Roche] in H_2O) for 30 min on ice. After centrifugation protein concentration was measured in supernatants using Pierce Reagent (Thermo Scientific). Protein lysates were subjected to electrophoresis and transferred to a nitrocellulose membrane on the Trans-Blot Turbo System (Bio-Rad). Specific protein signals were detected by incubation with antibodies against C/EBPα (14AA, Santa Cruz Biotechnology), C/EBPβ (C-19, Santa Cruz), C/EBPδ (C-22, Santa Cruz), C/EBPε (C-22, Santa Cruz), GFP (7.1/13.1, Roche), and ACTB (AC-15, Sigma).

NanoString Analysis

Total RNA from freshly FACS-enriched cells or cell cultures was prepared and quality checked on a 2100 Bioanalyzer (Agilent Technologies). Detection of mouse protein-coding transcripts was performed using a predefined panel (GXA-MIM1-12, nCounter), containing 547 probes against immunology-related mRNAs and 14 probes against housekeeping genes (*G6pdx*, *Hprt*, *Gapdh*, *Alas1*, *Oaz1*, *Sdha*, *Rpl19*, *Eef1g*, *Tbp*, *Ppia*, *Polr2a*, *Gusb*, *Tubb5*, and *Polr1b*) for normalization. Primary data were processed using default settings of the nSolver software. Genes were defined as "upregulated" or "downregulated" if normalized expression values were increased or decreased at least 2-fold compared with empty vector-transduced controls.

Statistical Analysis

Processed data were visualized using GraphPad Prism software (Version 5.0a). Error bars represent the SEM. Hypothesis testing was performed from at least three independent experiments using the t-test function (two-tailed) in Rstudio (R version 3.2.1, R Foundation). p Values of <0.05 were defined as significant and $p < 0.01$ highly significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.12.015.

AUTHOR CONTRIBUTIONS

B.C. conceived the methodology, performed the experiments, analyzed the data, and wrote the draft. V.B. provided $Cebpa^{f/f}$; $Cebpb^{f/f}$ mouse resources and experimental advice. E.K.-L. generated CEBP-EGFP fusion construct resources, and J.S. and C.K. validated experiments. V.B., J.I., N.P., J.S., and E.K.-L. were involved in reviewing the manuscript. B.C. and A.L. conceptualized the work and wrote the manuscript. A.L. was responsible for supervision, project administration, and funding acquisition.

ACKNOWLEDGMENTS

We thank Dr. B Niesler and R. Roeth of the nCounter Core Facility, Heidelberg, for providing services; Dr. H.-P. Rahn and K. Rautenberg (MDC, Berlin, Germany) for flow cytometry support; Dr. T. Graf (CRG, Barcelona, Spain) for the C10 cell line; M. Hofstätter and J. Bergemann (MDC, Berlin, Germany) for technical assistance; and Dr. E. Sterneck (NCI, Frederick, MD), Dr. F. Melchers (MPI for Infection Biology, Berlin, Germany), and Dr. K. Rajewsky (MDC, Berlin, Germany) for reagents and mice.

Received: July 16, 2016
Revised: December 14, 2016
Accepted: December 15, 2016
Published: January 19, 2017

REFERENCES

Akagi, T., Thoennissen, N.H., George, A., Crooks, G., Song, J.H., Okamoto, R., Nowak, D., Gombart, A.F., and Koeffler, H.P. (2010). In vivo deficiency of both C/EBPbeta and C/EBPepsilon results in highly defective myeloid differentiation and lack of cytokine response. PLoS One *5*, e15419.

Akasaka, T., Balasas, T., Russell, L.J., Sugimoto, K.J., Majid, A., Walewska, R., Karran, E.L., Brown, D.G., Cain, K., Harder, L., et al. (2007). Five members of the CEBP transcription factor family are targeted by recurrent IGH translocations in B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Blood *109*, 3451–3461.

Barneda-Zahonero, B., Roman-Gonzalez, L., Collazo, O., Rafati, H., Islam, A.B., Bussmann, L.H., di Tullio, A., De Andres, L., Graf, T., Lopez-Bigas, N., et al. (2013). HDAC7 is a repressor of myeloid genes whose downregulation is required for transdifferentiation of pre-B cells into macrophages. PLoS Genet. *9*, e1003503.

Bussmann, L.H., Schubert, A., Vu Manh, T.P., De Andres, L., Desbordes, S.C., Parra, M., Zimmermann, T., Rapino, F., Rodriguez-Ubreva, J., Ballestar, E., et al. (2009). A robust and highly efficient immune cell reprogramming system. Cell Stem Cell *5*, 554–566.

Chapiro, E., Russell, L., Radford-Weiss, I., Bastard, C., Lessard, M., Struski, S., Cave, H., Fert-Ferrer, S., Barin, C., Maarek, O., et al. (2006). Overexpression of CEBPA resulting from the translocation t(14;19)(q32;q13) of human precursor B acute lymphoblastic leukemia. Blood *108*, 3560–3563.

Chen, S.S., Chen, J.F., Johnson, P.F., Muppala, V., and Lee, Y.H. (2000). C/EBPbeta, when expressed from the C/ebpalpha gene locus, can functionally replace C/EBPalpha in liver but not in adipose tissue. Mol. Cell. Biol. *20*, 7292–7299.

Cloutier, A., Guindi, C., Larivee, P., Dubois, C.M., Amrani, A., and McDonald, P.P. (2009). Inflammatory cytokine production by human neutrophils involves C/EBP transcription factors. J. Immunol. *182*, 563–571.

Cobaleda, C., and Busslinger, M. (2008). Developmental plasticity of lymphocytes. Curr. Opin. Immunol. *20*, 139–148.

Dahl, R., Walsh, J.C., Lancki, D., Laslo, P., Iyer, S.R., Singh, H., and Simon, M.C. (2003). Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. Nat. Immunol. *4*, 1029–1036.

DeKoter, R.P., and Singh, H. (2000). Regulation of B lymphocyte and macrophage development by graded expression of PU.1. Science *288*, 1439–1441.

DeKoter, R.P., Kamath, M.B., and Houston, I.B. (2007). Analysis of concentration-dependent functions of PU.1 in hematopoiesis using mouse models. Blood Cells Mol. Dis. *39*, 316–320.

Di Stefano, B., Collombet, S., Jakobsen, J.S., Wierer, M., Sardina, J.L., Lackner, A., Stadhouders, R., Segura-Morales, C., Francesconi, M., Limone, F., et al. (2016). C/EBPalpha creates elite cells for iPSC reprogramming by upregulating Klf4 and increasing the levels of Lsd1 and Brd4. Nat. Cell Biol. *18*, 371–381.

Di Tullio, A., and Graf, T. (2012). C/EBPalpha bypasses cell cycle-dependency during immune cell transdifferentiation. Cell Cycle *11*, 2739–2746.

Di Tullio, A., Manh, T.P., Schubert, A., Mansson, R., and Graf, T. (2011). CCAAT/enhancer binding protein alpha (C/EBP(alpha))-induced transdifferentiation of pre-B cells into macrophages involves no overt retrodifferentiation. Proc. Natl. Acad. Sci. USA *108*, 17016–17021.

Dorantes-Acosta, E., and Pelayo, R. (2012). Lineage switching in acute leukemias: a consequence of stem cell plasticity? Bone Marrow Res. *2012*, 406796.

Ehlich, A., Martin, V., Muller, W., and Rajewsky, K. (1994). Analysis of the B-cell progenitor compartment at the level of single cells. Curr. Biol. *4*, 573–583.

Feng, R., Desbordes, S.C., Xie, H., Tillo, E.S., Pixley, F., Stanley, E.R., and Graf, T. (2008). PU.1 and C/EBPalpha/beta convert fibroblasts into macrophage-like cells. Proc. Natl. Acad. Sci. USA *105*, 6057–6062.

Golemovic, M., Sucic, M., Zadro, R., Mrsic, S., Mikulic, M., Labar, B., Rajic, L.J., and Batinic, D. (2006). IgH and TCRgamma gene

rearrangements, cyclin A1 and HOXA9 gene expression in biphenotypic acute leukemias. Leuk. Res. *30*, 211–221.

Graf, T. (2008). Immunology: blood lines redrawn. Nature *452*, 702–703.

Graf, T., and Enver, T. (2009). Forcing cells to change lineages. Nature *462*, 587–594.

Greaves, M.F., Chan, L.C., Furley, A.J., Watt, S.M., and Molgaard, H.V. (1986). Lineage promiscuity in hemopoietic differentiation and leukemia. Blood *67*, 1–11.

Hirai, H., Zhang, P., Dayaram, T., Hetherington, C.J., Mizuno, S., Imanishi, J., Akashi, K., and Tenen, D.G. (2006). C/EBPbeta is required for 'emergency' granulopoiesis. Nat. Immunol. *7*, 732–739.

Holmes, K.L., Pierce, J.H., Davidson, W.F., and Morse, H.C., 3rd. (1986). Murine hematopoietic cells with pre-B or pre-B/myeloid characteristics are generated by in vitro transformation with retroviruses containing fes, ras, abl, and src oncogenes. J. Exp. Med. *164*, 443–457.

Janz, M., Dorken, B., and Mathas, S. (2006). Reprogramming of B lymphoid cells in human lymphoma pathogenesis. Cell Cycle *5*, 1057–1061.

Jones, L.C., Lin, M.L., Chen, S.S., Krug, U., Hofmann, W.K., Lee, S., Lee, Y.H., and Koeffler, H.P. (2002). Expression of C/EBPbeta from the C/ebpalpha gene locus is sufficient for normal hematopoiesis in vivo. Blood *99*, 2032–2036.

Kallin, E.M., Rodriguez-Ubreva, J., Christensen, J., Cimmino, L., Aifantis, I., Helin, K., Ballestar, E., and Graf, T. (2012). Tet2 facilitates the derepression of myeloid target genes during CEBPalpha-induced transdifferentiation of pre-B cells. Mol. Cell *48*, 266–276.

Kelliher, M.A., McLaughlin, J., Witte, O.N., and Rosenberg, N. (1990). Induction of a chronic myelogenous leukemia-like syndrome in mice with v-abl and BCR/ABL. Proc. Natl. Acad. Sci. USA *87*, 6649–6653.

Krijger, P.H., Di Stefano, B., de Wit, E., Limone, F., van Oevelen, C., de Laat, W., and Graf, T. (2016). Cell-of-origin-specific 3D genome structure acquired during somatic cell reprogramming. Cell Stem Cell *18*, 597–610.

Laiosa, C.V., Stadtfeld, M., Xie, H., de Andres-Aguayo, L., and Graf, T. (2006). Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBP alpha and PU.1 transcription factors. Immunity *25*, 731–744.

Lamprecht, B., Walter, K., Kreher, S., Kumar, R., Hummel, M., Lenze, D., Kochert, K., Bouhlel, M.A., Richter, J., Soler, E., et al. (2010). Derepression of an endogenous long terminal repeat activates the CSF1R proto-oncogene in human lymphoma. Nat. Med. *16*, 571–579, 1p following 579.

Litvak, V., Ramsey, S.A., Rust, A.G., Zak, D.E., Kennedy, K.A., Lampano, A.E., Nykter, M., Shmulevich, I., and Aderem, A. (2009). Function of C/EBPdelta in a regulatory circuit that discriminates between transient and persistent TLR4-induced signals. Nat. Immunol. *10*, 437–443.

Lu, Y.C., Kim, I., Lye, E., Shen, F., Suzuki, N., Suzuki, S., Gerondakis, S., Akira, S., Gaffen, S.L., Yeh, W.C., and Ohashi, P.S. (2009). Differential role for c-Rel and C/EBPbeta/delta in TLR-mediated induction of proinflammatory cytokines. J. Immunol. *182*, 7212–7221.

Ma, O., Hong, S., Guo, H., Ghiaur, G., and Friedman, A.D. (2014). Granulopoiesis requires increased C/EBPalpha compared to monopoiesis, correlated with elevated cebpa in immature G-CSF receptor versus M-CSF receptor expressing cells. PLoS One *9*, e95784.

Matutes, E., Morilla, R., Farahat, N., Carbonell, F., Swansbury, J., Dyer, M., and Catovsky, D. (1997). Definition of acute biphenotypic leukemia. Haematologica *82*, 64–66.

Ness, S.A., Kowenz-Leutz, E., Casini, T., Graf, T., and Leutz, A. (1993). Myb and NF-M: combinatorial activators of myeloid genes in heterologous cell types. Genes Dev. *7*, 749–759.

Ohnishi, K., and Melchers, F. (2003). The nonimmunoglobulin portion of lambda5 mediates cell-autonomous pre-B cell receptor signaling. Nat. Immunol. *4*, 849–856.

Peitz, M., Pfannkuche, K., Rajewsky, K., and Edenhofer, F. (2002). Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes. Proc. Natl. Acad. Sci. USA *99*, 4489–4494.

Pham, T.H., Minderjahn, J., Schmidl, C., Hoffmeister, H., Schmidhofer, S., Chen, W., Langst, G., Benner, C., and Rehli, M. (2013). Mechanisms of in vivo binding site selection of the hematopoietic master transcription factor PU.1. Nucleic Acids Res. *41*, 6391–6402.

Pierce, J.H., and Aaronson, S.A. (1982). Balb- and Harvey-murine sarcoma virus transformation of a novel lymphoid progenitor cell. J. Exp. Med. *156*, 873–887.

Radomska, H.S., Huettner, C.S., Zhang, P., Cheng, T., Scadden, D.T., and Tenen, D.G. (1998). CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. Mol. Cell. Biol. *18*, 4301–4314.

Regalo, G., and Leutz, A. (2013). Hacking cell differentiation: transcriptional rerouting in reprogramming, lineage infidelity and metaplasia. EMBO Mol. Med. *5*, 1154–1164.

Rodriguez-Ubreva, J., Ciudad, L., Gomez-Cabrero, D., Parra, M., Bussmann, L.H., di Tullio, A., Kallin, E.M., Tegner, J., Graf, T., and Ballestar, E. (2012). Pre-B cell to macrophage transdifferentiation without significant promoter DNA methylation changes. Nucleic Acids Res. *40*, 1954–1968.

Rodriguez-Ubreva, J., Ciudad, L., van Oevelen, C., Parra, M., Graf, T., and Ballestar, E. (2014). C/EBPa-mediated activation of miR-34a and miR-223 inhibits Lef1 expression to achieve efficient reprogramming into macrophages. Mol. Cell. Biol. *34*, 1145–1157.

Rosenbauer, F., Wagner, K., Kutok, J.L., Iwasaki, H., Le Beau, M.M., Okuno, Y., Akashi, K., Fiering, S., and Tenen, D.G. (2004). Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. Nat. Genet. *36*, 624–630.

Rosenberg, N., Baltimore, D., and Scher, C.D. (1975). In vitro transformation of lymphoid cells by Abelson murine leukemia virus. Proc. Natl. Acad. Sci. USA *72*, 1932–1936.

Scott, L.M., Civin, C.I., Rorth, P., and Friedman, A.D. (1992). A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. Blood *80*, 1725–1735.

Shore, S.K., Tantravahi, R.V., and Reddy, E.P. (2002). Transforming pathways activated by the v-Abl tyrosine kinase. Oncogene *21*, 8568–8576.

Simmons, S., Knoll, M., Drewell, C., Wolf, I., Mollenkopf, H.J., Bouquet, C., and Melchers, F. (2012). Biphenotypic B-lymphoid/myeloid cells expressing low levels of Pax5: potential targets of BAL development. Blood *120*, 3688–3698.

Slamova, L., Starkova, J., Fronkova, E., Zaliova, M.K., Reznickova, L.R., van Delft, F.W., Vodickova, E., Volejnikova, J., Zemanova, Z., Polgarova, K., et al. (2013). CD2-positive B-cell precursor acute lymphoblastic leukemia with an early switch to the monocytic lineage. Leukemia *28*, 609–620.

Smith, L.J., Curtis, J.E., Messner, H.A., Senn, J.S., Furthmayr, H., and McCulloch, E.A. (1983). Lineage infidelity in acute leukemia. Blood *61*, 1138–1145.

Sterneck, E., Zhu, S., Ramirez, A., Jorcano, J.L., and Smart, R.C. (2006). Conditional ablation of C/EBP beta demonstrates its keratinocyte-specific requirement for cell survival and mouse skin tumorigenesis. Oncogene *25*, 1272–1276.

Stoilova, B., Kowenz-Leutz, E., Scheller, M., and Leutz, A. (2013). Lymphoid to myeloid cell trans-differentiation is determined by C/EBPbeta structure and post-translational modifications. PLoS One *8*, e65169.

Suh, H.C., Gooya, J., Renn, K., Friedman, A.D., Johnson, P.F., and Keller, J.R. (2006). C/EBPalpha determines hematopoietic cell fate in multipotential progenitor cells by inhibiting erythroid differentiation and inducing myeloid differentiation. Blood *107*, 4308–4316.

Tanaka, T., Akira, S., Yoshida, K., Umemoto, M., Yoneda, Y., Shirafuji, N., Fujiwara, H., Suematsu, S., Yoshida, N., and Kishimoto, T. (1995). Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. Cell *80*, 353–361.

Truett, G.E., Heeger, P., Mynatt, R.L., Truett, A.A., Walker, J.A., and Warman, M.L. (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). Biotechniques *29*, 52–54.

Tsukada, J., Yoshida, Y., Kominato, Y., and Auron, P.E. (2011). The CCAAT/enhancer (C/EBP) family of basic-leucine zipper (bZIP) transcription factors is a multifaceted highly-regulated system for gene regulation. Cytokine *54*, 6–19.

van Oevelen, C., Collombet, S., Vicent, G., Hoogenkamp, M., Lepoivre, C., Badeaux, A., Bussmann, L., Sardina, J.L., Thieffry, D., Beato, M., et al. (2015). C/EBPalpha activates pre-existing and de novo macrophage enhancers during induced pre-B cell transdifferentiation and myelopoiesis. Stem Cell Rep. *5*, 232–247.

Wiemels, J.L., de Smith, A.J., Xiao, J., Lee, S.T., Muench, M.O., Fomin, M.E., Zhou, M., Hansen, H.M., Termuhlen, A., Metayer, C., et al. (2015). A functional polymorphism in the CEBPE gene promoter influences acute lymphoblastic leukemia risk through interaction with the hematopoietic transcription factor Ikaros. Leukemia *30*, 1194–1197.

Xie, H., Ye, M., Feng, R., and Graf, T. (2004). Stepwise reprogramming of B cells into macrophages. Cell *117*, 663–676.

Yamanaka, R., Barlow, C., Lekstrom-Himes, J., Castilla, L.H., Liu, P.P., Eckhaus, M., Decker, T., Wynshaw-Boris, A., and Xanthopoulos, K.G. (1997). Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. Proc. Natl. Acad. Sci. USA *94*, 13187–13192.

Zhang, D.E., Zhang, P., Wang, N.D., Hetherington, C.J., Darlington, G.J., and Tenen, D.G. (1997). Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc. Natl. Acad. Sci. USA *94*, 569–574.

Zhang, P., Iwasaki-Arai, J., Iwasaki, H., Fenyus, M.L., Dayaram, T., Owens, B.M., Shigematsu, H., Levantini, E., Huettner, C.S., Lekstrom-Himes, J.A., et al. (2004). Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. Immunity *21*, 853–863.

Stem Cell Reports, Volume *8*

Supplemental Information

C/EBP-Induced Transdifferentiation Reveals Granulocyte-Macrophage Precursor-like Plasticity of B Cells

Branko Cirovic, Jörg Schönheit, Elisabeth Kowenz-Leutz, Jelena Ivanovska, Christine Klement, Nina Pronina, Valérie Bégay, and Achim Leutz

Stem Cell Reports

Supplemental Information

C/EBP-induced transdifferentiation reveals granulocyte-macrophage precursor-like plasticity of B cells

Cirovic, Branko; Schönheit, Joerg; Kowenz-Leutz, Elisabeth; Ivanovska, Jelena; Klement, Christine; Pronina, Nina; Bégay, Valérie; Leutz, Achim

Supplemental Figures

Figure S1

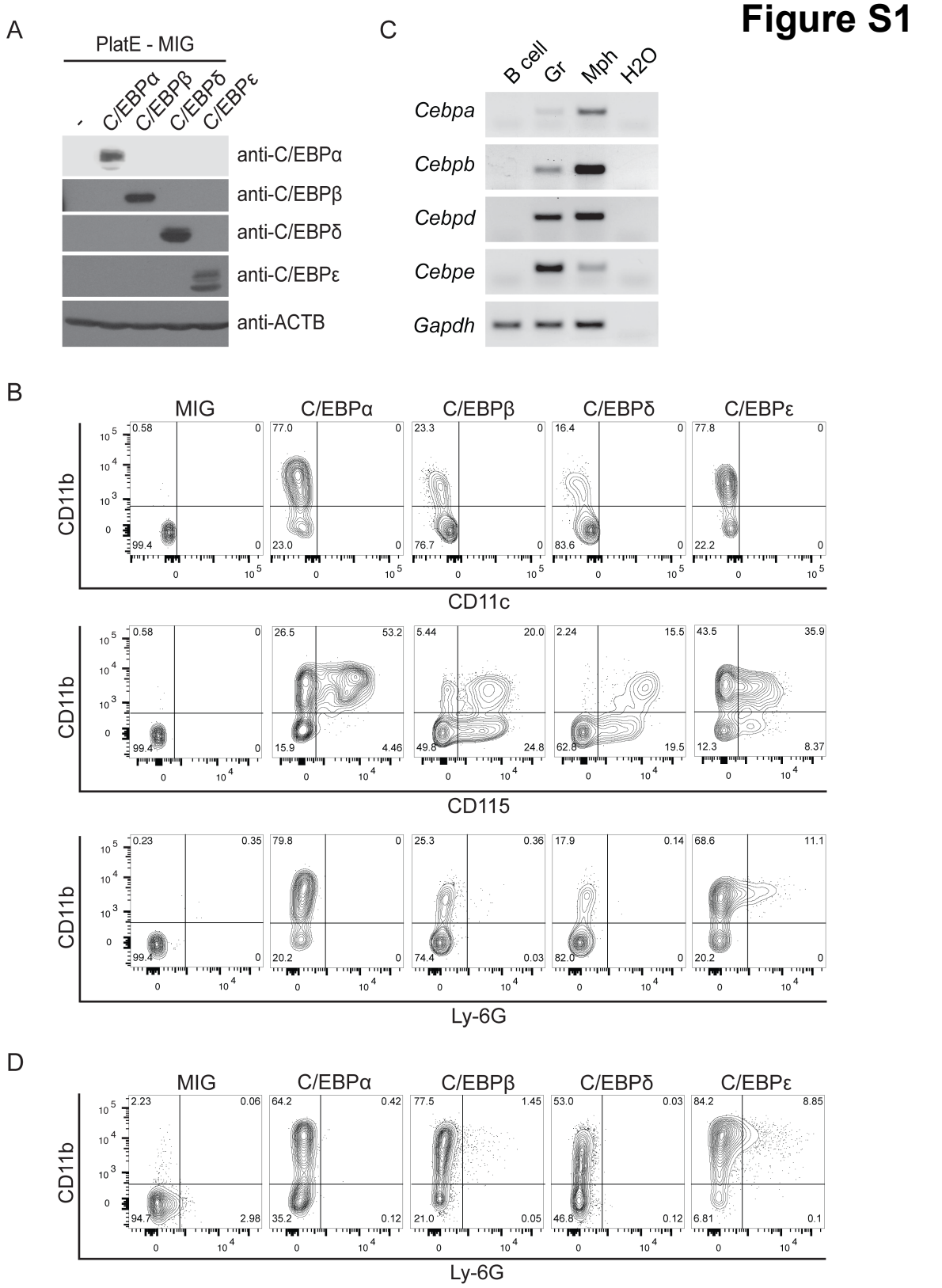
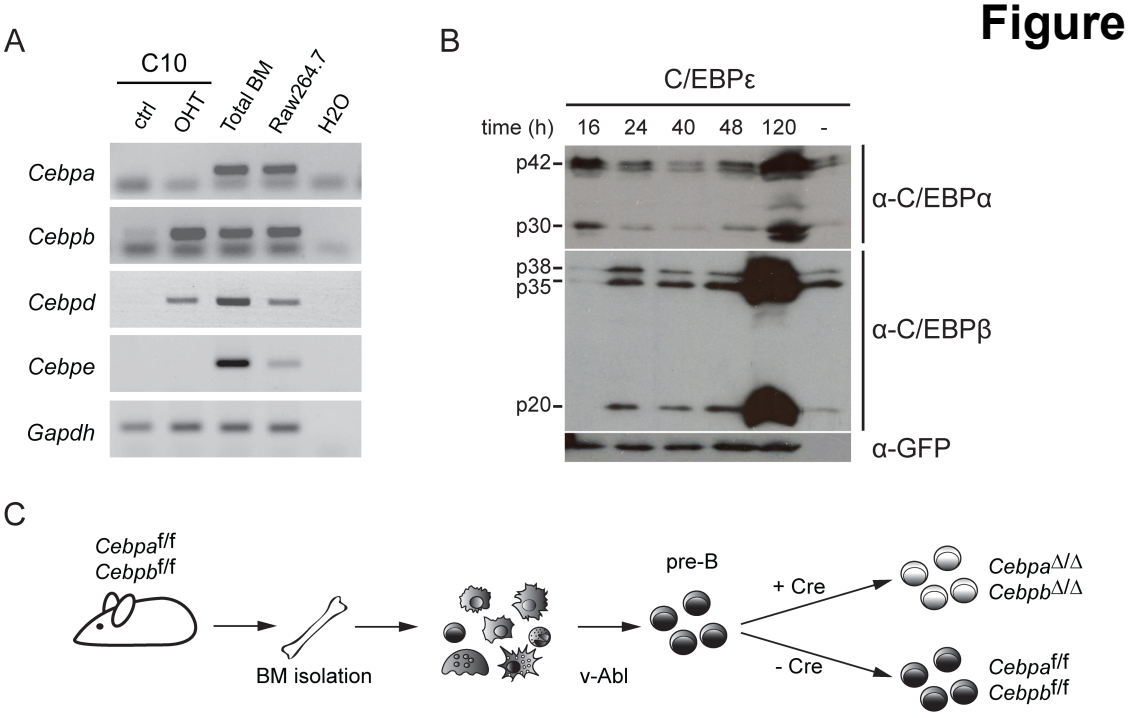
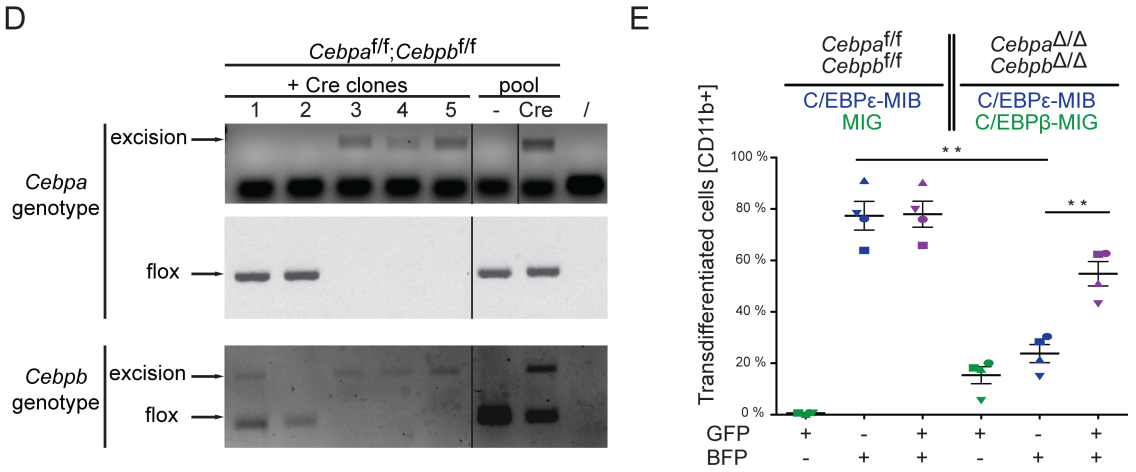


Figure S2





F

upregulated

Ahr	*Csf1r*	*Il18r1*	*Notch2*
Arhgdib	*Csf2rb*	*Il1rn*	*Nt5e*
Btnl1	*Ctsc*	*Il28a*	*Pecam1*
Ccl12	*Cybb*	*Il33*	*Plaur*
Ccl24	*Ddx58*	*Irak2*	*Runx3*
Ccl3	*Entpd1*	*Irak3*	*S100a9*
Ccl6	*Ets1*	*Irf7*	*Sele*
Ccl9	*Fcgr1*	*Itgal*	*Sell*
Ccr5	*Fcgr2b*	*Itgam*	*Ski*
Ccrl2	*Gfi1*	*Kir3dl1*	*Smad3*
Cd4	*Gpi1*	*Klra7*	*Stat3*
Cd44	*Gpr183*	*Lair1*	*Tagap*
Cd55	*H2-Ea-ps*	*Lilrb3*	*Tgfbi*
Cd69	*Hcst*	*Lilrb4*	*Tlr1*
Cdkn1a	*Hfe*	*Ltb4r1*	*Trem1*
Ceacam1	*Hlx*	*Mbl2*	*Tyrobp*
Cfb	*Ifih1*	*Muc1*	*Vcam1*
Csf1	*Ifitm1*	*Nfil3*	*Vtn*

G

downregulated

Aicda	*Ciita*	*Klrc3*	*Tnfrsf13c*
Bcl3	*Cmklr1*	*Lta*	*Traf4*
Btla	*Cxcr2*	*Ltb*	*Xcr1*
C1qa	*Cxcr6*	*Masp1*	
C1s	*Fas*	*Masp2*	
C8a	*Fn1*	*Pax5*	
Ccr6	*Icam2*	*Pdcd1lg2*	
Ccr7	*Ifna2*	*Pla2g2a*	
Ccr8	*Ifng*	*Pou2f2*	
Cd160	*Ikzf4*	*Rag1*	
Cd163	*Il10*	*Rag2*	
Cd2	*Il17b*	*Rorc*	
Cd22	*Il20*	*Slamf1*	
Cd244	*Il22*	*Slamf7*	
Cd40	*Il7r*	*Tfrc*	
Cd74	*Irf4*	*Tgfb3*	
Cd79b	*Irf8*	*Tigit*	
Cfd	*Klrb1*	*Tnfaip6*	

Figure S3

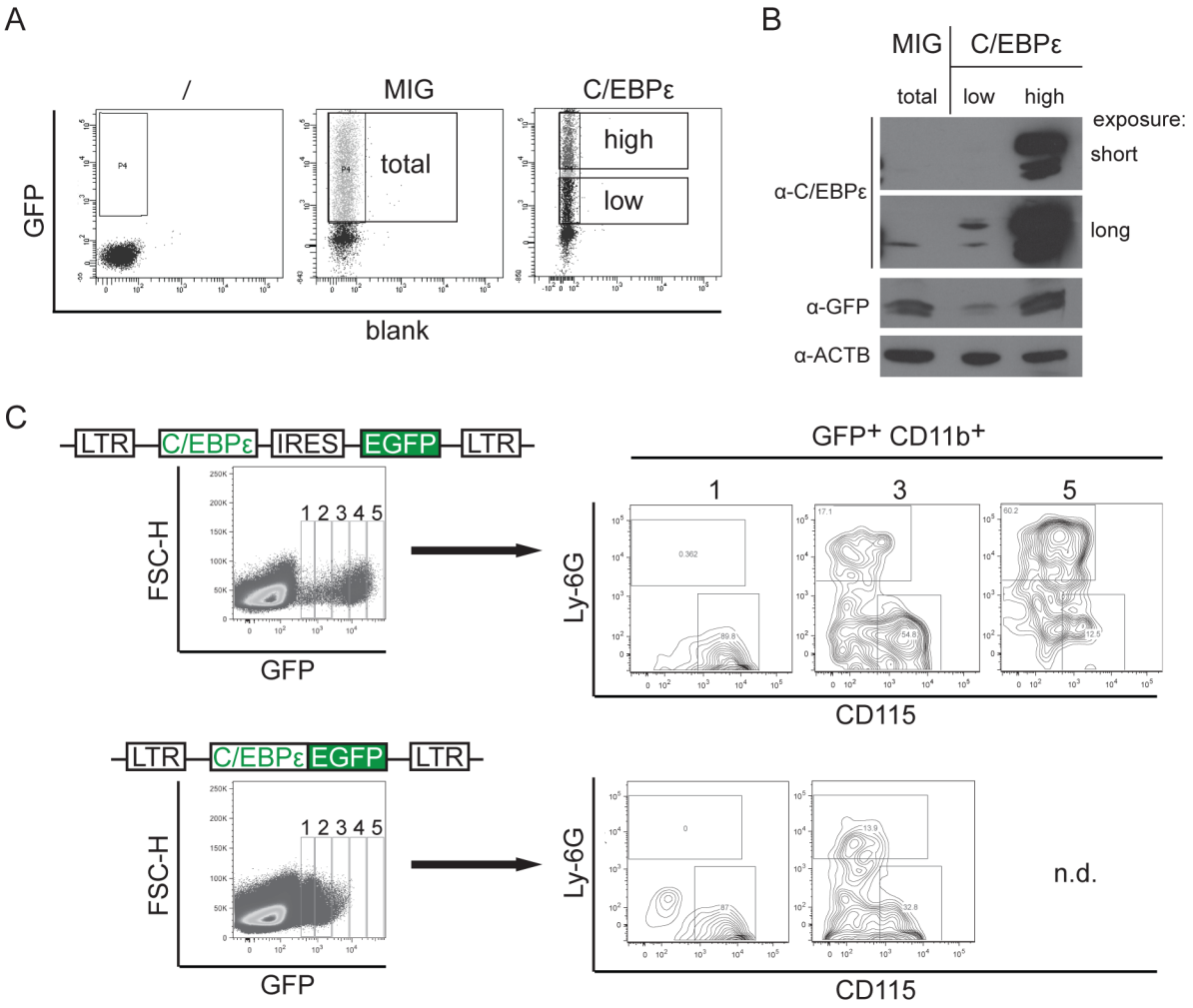

A
/
MIG
C/EBPε
GFP
total
high
low
blank
B
MIG
C/EBPε
total
low
high
exposure:
short
long
α-C/EBPε
α-GFP
α-ACTB
C
LTR
C/EBPε
IRES
EGFP
LTR
FSC-H
GFP
1 2 3 4 5
GFP+ CD11b+
1
3
5
Ly-6G
CD115
n.d.

Figure S4

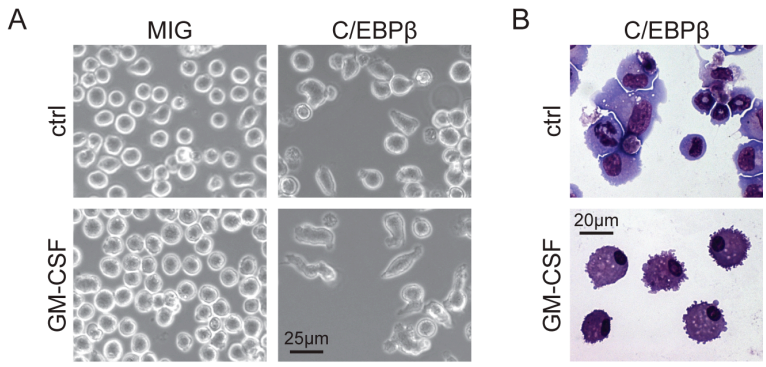


Figure S5

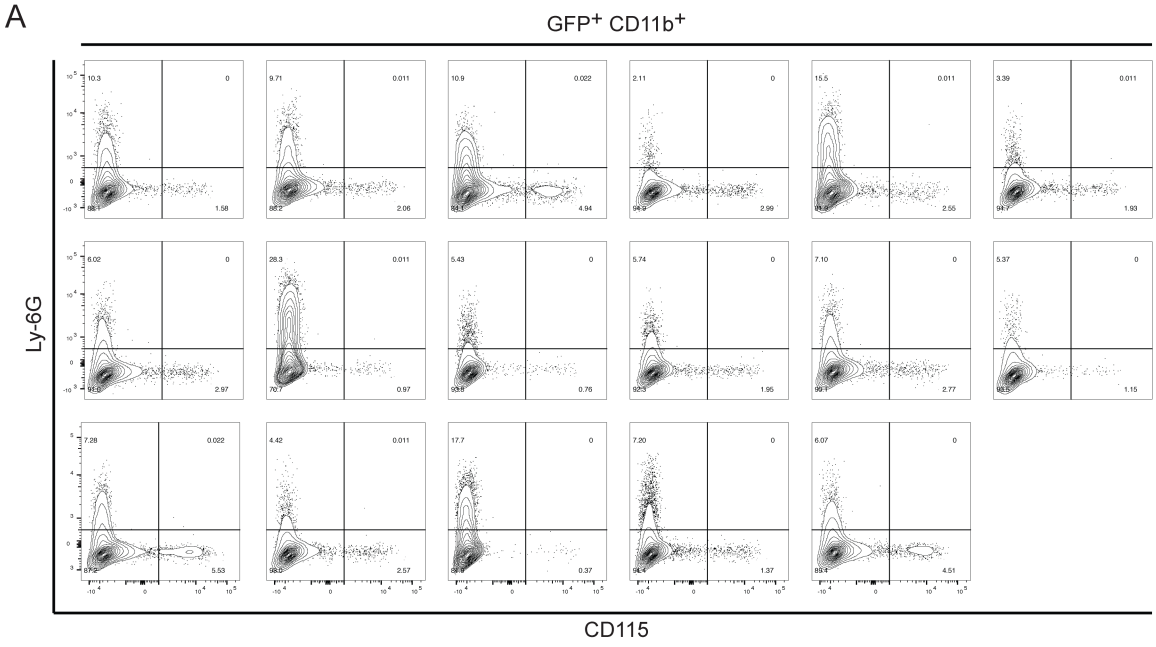


Figure S1: Lympho-myeloid conversion potential of C/EBP family members,

Related to Figure 1

(**A**) Immunoblot analysis of C/EBP family members of total protein lysates from Plat-E cells 72 h after transduction with C/EBP-MIG constructs. Untransduced cells served as control and ACTB as housekeeping gene. (**B**) Flow cytrometric analysis of B cells transduced with individual C/EBPs focusing on macrophage ($CD11b^+CD115^+$), dendritic ($CD11b^+CD11c^+$) and granulocytic ($CD11b^+Ly\text{-}6G^+$) markers 4 days after transduction. (**C**) Semi-quantitative RT-PCR analysis of C/EBP expression in murine primary FACS-purified B cells ($CD19^+$), granulocytes ($CD11b^+Ly\text{-}6G^+$) and macrophages ($CD11b^+CD115^+$). *Gapdh* served as housekeeping gene. (**D**) Flow cytometric analysis of C/EBP-transduced GFP^+-gated HAFTL1 cells 4 days after induction.

Figure S2: The role of endogenous *Cebpa* and *Cebpb* on transdifferentiation,

Related to Figure 3

(**A**) Semi-quantitative RT-PCR expression analysis of endogenous C/EBP family members in C10 cells treated with OHT for 4 days and non-induced controls (Bussmann et al. 2009). RNA from total bone marrow or monocytic Raw264.7 cells served as controls. *Gapdh* was used as housekeeping gene. (**B**) Expression kinetics of endogenous C/EBPα, and β protein in C/EBPε-transduced B cells based on immunoblot analysis. (**C**) Workflow for the generation of *Cebpa* and *Cebpb* double knockout v-Abl immortalized B cells and isogenic controls derived from a mouse with homozygous floxed *Cebpa* and *Cebpb* alleles. Deletion of *Cebpa* and *Cebpb* by incubating cells with recombinant Cre-recombinase. **(D)** *Cebpa* and *Cebpb* genotypes of established Cre-incubated cell clones was assessed by PCR. Pools of Cre-treated and untreated cells served as controls. Clone 4 was selected for further experiments.

(**E**) Quantification of transdifferentiated cells ($CD11b^+$, related to Figure 3C) from four independent experiments. ±SEM, **p<0.01. (**F**) Nanostring analysis showing upregulated genes 24 h after C/EBPε transduction in GFP^+-sorted cells compared to control B cells (MIG-transduced). Genes that were not upregulated in $Cebpa^{\Delta/\Delta}$;$Cebpb^{\Delta/\Delta}$ cells are marked in red. (**G**) Downregulated genes in GFP^+ FACS-purified control cells transduced for one day with C/EBPε compared to empty vector. Genes refractory to down-regulation in $Cebpa^{\Delta/\Delta}$;$Cebpb^{\Delta/\Delta}$ cells are marked in red. See also Table S1.

Figure S3: Effect of C/EBPε dosage on transdifferentiated cell type outcome, Related to Figure 4

(**A**) Sorting scheme of GFP^+ Plat-E cells transduced with empty vector control (MIG) or C/EBPε-MIG for 72 h. (**B**) Immunoblot detection of C/EBPε, GFP and ACTB in protein lysates of 1×10^6 FACS-purified cells from GFP-fractions in (A). Two exposure times (short and long) are shown. (**C**) B cell were transduced with pMSCV-based vectors either containing bi-cistronic C/EBPε-IRES-EGFP (top panels) or C/EBPε-EGFP fusion (bottom panels) transgenes. Transduced cells were classified according to GFP intensity and analysed for $CD11b^+$Ly-$6G^+$ and $CD11b^+CD115^+$ fractions.

Figure S4: Myeloid signalling-response of transdifferentiated bi-potential cells,

Related to Figure 5

(**A**) Bright field images of long-term C/EBPβ-transduced cells, untreated or treated with 10 ng/ml GM-CSF for 24 h. Empty vector (MIG) transduced B cells served as control. (**B**) Subsequent cytospin preparations and May-Grünwald-staining after 16 days of culture.

Figure S5: Persistence of the GMP-like phenotype after serial subcloning,

Related to Figure 5

Analysis of 17 subclones derived from a GMP-like parental clone presented in Figure 5G,H. Flow cytometric analysis of CD115 versus Ly-6G in the GFP+CD11b+-gated population indicates persistence of the GMP-like potential.

Supplemental Tables

Table S1: Gene expression signature in C/EBP-transduced B cells,

Related to Figure 2, 3

Nanostring analysis (gene set GXA-MIM) of GFP^+ B cells (*Cebpa*$^{f/f}$;*Cebpb*$^{f/f}$) transduced with individual C/EBPs for 24 h. C/EBPε-infected *Cebpa*$^{\Delta/\Delta}$;*Cebpb*$^{\Delta/\Delta}$ B cells (B-DKO) were included for comparison. MIG-transduced cells served as control. Normalized gene expression values are shown.

Table S2: List of primer sequences,

Related to Experimental Procedures, Figure 4, Figure S1, S2

Oligonucleotide	Sequence 5'-3'
Cebpa FW	GCCAGTTGGGGCACTGGGTG
Cebpa RV	CCGCGGCTCCACCTCGTAGA
Cebpb FW	GCGTTCATGCACCGCCTGCT
Cebpb RV	TAGGCCAGGCAGTCGGGCTC
Cebpd FW	AGAACCCGCGGCCTTCTAC
Cebpd RV	ATGTAGGCGCTGAAGTCGAT
Cebpe FW	CACACTGCGGGCAGACAG
Cebpe RV	GTGCCTTGAGAAGGGGACT
Gapdh FW	AATGTGTCCGTCGTGGATCTGA
Gapdh RV	GATGCCTGCTTCACCACCTTCT
Cebpa flox FW	TGGCCTGGAGACGCAATGA
Cebpa flow RV	CGCAGAGATTGTGCGTCTTT
Cebpa Δ FW	GCCTGGTAAGCCTAGCAATCCT
Cebpa Δ RV	TGGAAACTTGGGTTGGGTGT
Cebpb flox/Δ FW	GAGCCACCGCGTCCTCCAGC
Cebpb flox RV	GGTCGGTGCGCGTCATTGCC
Cebpb Δ RV	AGCAGAGCTGCCCCGGCAAA
DQ52 FW1	ACGTCGACTTTTGYAAGGGATCTACTACTGT
DFS FW2	ACGTCGACGCGGACGACCACAGTGCAACTG
JH4A RV	GGGTCTAGACTCTCAGCCGGCTCCCTCAGGG