Myeloid transformation by MLL-ENL depends strictly on C/EBP

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Chromosomal rearrangements of the mixed-lineage leukemia gene MLL are the hallmark of infant acute leukemia. The granulocyte-macrophage progenitor stem cell forms the epigenetic basis for myelomonocytic leukemia stemness and transformation by MLL-type oncoproteins. Previously, it was shown that the establishment of murine myelomonocytic MLL-ENL transformation, but not its maintenance, depends on the transcription factor C/EBPα, suggesting an epigenetic hit-and-run mechanism of MLL-driven oncogenesis. Here, we demonstrate that compound deletion of Cebpa/Cebpb almost entirely abrogated the growth and survival of MLL-ENL-transformed cells. Rare, slow-growing, and apoptosis-prone MLL-ENL-transformed escapees were recovered from compound Cebpa/Cebpb deletions. The escapees were uniformly characterized by high expression of the resident Cebpe gene, suggesting inferior functional compensation of C/EBPβ deficiency by C/EBPα. Complementation was augmented by ectopic C/EBPβ expression and downstream activation of IGF1 that enhanced growth. Cebpe gene inactivation was accomplished only in the presence of complementing C/EBPβ, but not in its absence, confirming the Cebpe dependency of the Cebpa/Cebpb double knockouts. Our data show that MLL-transformed myeloid cells are dependent on C/EBPs during the initiation and maintenance of transformation.

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Introduction

Mixed-lineage leukemia (MLL) represents an aggressive pediatric cancer of the blood, with features of acute lymphoblastic leukemia and acute myeloid leukemia (AML). Chromosomal translocations at 11q23 are predominant in MLL and fuse the N-terminal part of the Trithorax-like MLL1/KMT2A methyltransferase to multiple partner proteins (Shilatifard, 2012; Slany, 2016). MLL-induced AML may originate from hematopoietic stem cells (HSCs) and/or an early progenitor state and involve the establishment of leukemic stem cells (LSCs) that maintain lineage plasticity and an intermediate lymphoid–myeloid immunophenotype (Daigle et al, 2011; Goardon et al, 2011; Chen et al, 2013; Krivtsov et al, 2013). Despite recent advances in understanding the molecular mechanism of the disease, therapy of MLL translocation-induced leukemia remains a clinical challenge.

The prevalent leukemic MLL translocations entai compositions of the super elongation complex, including ENL, AF9, and AF4. Both MLL-ENL and MLL-AF4 represent potent fusion oncoproteins that experimentally transform murine bone marrow cells in vitro (Smith et al, 2011). Mechanistically, MLL fusion oncoproteins stimulate the expression of target genes, including critical genes of the HOXA cluster, by co-recruiting the DOT1L complex and by promoting DNA polymerase II pause release and the elongation phase of gene transcription (Okada et al, 2005; Krivtsov & Armstrong, 2007; Krivtsov et al, 2013). Deregulated expression of the MLL target genes Hoxa9 and Meis1 partially recapitulate leukemogenic self-renewal and eventually cause experimental leukemogenesis (Collins & Hess, 2016b).

C/EBP (CCAAT enhancer–binding protein) family members are transcription factors that may function as activators and repressors depending on the cellular and molecular context and the expression status of the C/EBP protein isoforms (Zahnow, 2002; Nerlov, 2004; Johnson, 2005). C/EBPα, a master regulator of granulocyte-macrophage progenitor (GMP) biology, is also of central importance to leukemic myelomonocytic transformation. C/EBPα controls the transition from common myeloid progenitors to GMPs and prevents exhaustion of the HSC compartment (Zhang et al, 2004). C/EBPα-deficient progenitors resist transformation by MLL-ENL, MLL-AF9, MOZ-TIF2, and Hoxa9/Meis1. Interestingly, after the establishment of MLL-ENL transformation, C/EBPα can be removed genetically, whereas the malignant phenotype persists (Ohlsson et al, 2014). These findings suggest that, in the presence of C/EBPα, a “hit-and-run”–type MLL transformation consolidates an epigenetic state that is maintained in the absence of the initial

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inducing transcription factor C/EBPa (Roe & Vakoc, 2014). Alternatively, other C/EBP family members that have not been examined or that have remained undetected may be involved in maintaining the myelomonocytic and transformed state.

Here, we considered the role of C/EBPβ in maintaining the myeloproliferative MLL-ENL–transformed state. Using a somatic genetics approach, we show that the viability and proliferation of MLL-ENL–transformed mouse myeloblasts were impaired by removing C/EBPβ and were almost entirely abrogated by compound deletion of the Cebpa and Cebpb genes. Strikingly, all of the few surviving Cebpα/Cebpb–deficient MLL-ENL–transformed clones consistently expressed C/EBPα. Our data suggest that both the initiation and the maintenance of MLL-ENL transformation depend on transcription factors of the C/EBP family and imply a therapeutic opportunity in interfering with the C/EBP dependency of MLL transformation.

## Results

### Removing C/EBPβ slows the growth of MLL-ENL–transformed cells

Experimentally induced murine leukemogenesis and acquisition of the LSC state by the MLL-ENL, MLL-AF9, and MOZ-TIF2 oncogenes depend on C/EBPα that induces myeloid commitment, or in the absence of C/EBPα, on the alternative establishment of the GMP state (Ye et al, 2015). This suggests that establishment of the GMP phenotype is sufficient and subsequently epigenetically memo-

![Figure 1. Cebpb Deletion in MLL-ENL–Transformed Cells.](https://doi.org/10.26508/lsa.202000709)

**Figure 1.** Cebpb Deletion in MLL-ENL–Transformed Cells.

(A) Schematic illustration of murine tissue culture MLL-ENL leukemia model. Top to bottom: Murine fetal liver cells from WT^fl^ animals were transduced with MLL-ENL and selected with G418 for 14 d in liquid culture. Subsequently, the cells were seeded in semi-solid methylcellulose medium. Single colonies were isolated, expanded in liquid culture, and assessed for MLL-ENL integration. Next, the cells were treated with TAT-Cre recombinase to remove floxed Cebp alleles. Gene excision was determined by PCR (see Fig S1B).

(B) Top: representative microscopic scans of semi-solid methylcellulose cultures with WT^fl^ and Cebpb KO colonies. Cells were seeded at a density of 5,000 cells per 35-mm well, and colonies were scored after 10 d. Bottom: colony size distribution (see also Fig S1C).

(C) Top: WT^fl^ and Cebpb KO colonies in semi-solid methylcellulose medium. Middle: Growth curves of WT^fl^ and Cebpb KO colonies. Bottom: WST-1 assay showing the effect of Cebpb removal on MLL-ENL–transformed cells: Values are the mean ± SD (two-tailed Mann–Whitney U test, **P < 0.005).
little effect on total colony numbers; however, the secondary colonies derived from MLL-ENL C/EBPβ KO cells were smaller than the colonies derived from MLL-ENL cells with an intact Cebpb allele (Figs 1B and S1C). Cells derived from Cebpb KO cells showed decreased growth rates and diminished metabolism and viability in culture medium containing IL-3 (Fig 1C; cell counts and WST-1 to formazan conversion) as compared with the wild-type WTFL MLL-ENL–transformed cells, in accordance with attenuated growth in semi-solid and liquid cultures. We conclude that, although Cebpb is not essential for the clonogenicity of MLL-ENL–transformed cells, it enhances their proliferation and viability.

Simultaneous deletion of Cebpa/Cebpb inhibits MLL-ENL–transformed cell proliferation

To generate compound Cebpa/Cebpb KO cells, two parental Cebpfl/flCebpbfl/fl MLL-ENL clones were infected with a retrovirus encoding a conditional Cre recombinase, and Cebp allele deletion was monitored by PCR. Cre-mediated gene deletion was readily observed; however, even after prolonged Cre recombinase activation over 5 d, we failed to delete all four Cebp alleles over numerous attempts (data not shown). In an alternative approach, four parental Cebpαfl/fl/Cebpβfl/fl MLL-ENL clones were treated with recombinant Tat-Cre, which also resulted in allelic Cebp heterozygosity (Brown & Byersdorfer, 2017). Clones with partial Cebpb deletions were then pooled, expanded in mass culture (to ~4 × 10⁶ cells), and re-treated with cell-permeable Tat-Cre recombinase to force deletion of the remaining Cebpa/Cebpb alleles. The final round of Cre treatment resulted in a widespread crisis of cell growth and cell death. All surviving cells were directly seeded in semi-solid medium for scoring potential Cebpa/Cebpb double KO (dKO) on a clonal basis. In total, 1,056 colonies (from ~1,200 to 1,300 colonies) were isolated and examined for Cebpa/Cebpb deletion. Only 14 Cebpa/Cebpb dKO subclones from two mice (2 and 12 subclones, respectively) could be identified, suggesting that the transformed MLL-ENL cells were strongly dependent on Cebpa/Cebpb gene expression.

We next performed phenotypical analysis of WTfl, Cebpb KO, and Cebpa/Cebpb dKO cells by flow cytometry (Fig 2). We detected no differences in the frequencies of CD11b+ or Ly6C+ cells between the WTfl and Cebpb KO cells, suggesting that the deletion of Cebpb was compatible with maintaining the progenitor phenotype. Cebpb KO cells, however, showed diminished development of mature Ly6G+ neutrophils, whereas differentiation into CD115+ monocytes or macrophages in vitro was unaffected (Fig 2A). In contrast, compound deletion of Cebpa and Cebpb led to reduced CD11b and Ly6C reactivity, indicating a more immature or neomorphic phenotype (Fig 2A). Accordingly, neither Ly6G+ neutrophils nor CD115+ monocytes or macrophages could be derived from the Cebpa/Cebpb dKO cells (Fig 2A). Analysis of the early and late apoptotic stages in the WTfl, Cebpb KO, and Cebpa/Cebpb dKO clones showed that deleting Cebpb and Cebpa/Cebpb partially and severely enhanced the apoptosis rate, respectively (Fig 2A, right). Next, we analyzed the cell proliferation rate by fluorescent CFSE dye dilution using flow cytometry. As shown in Fig 2B, cells from all genotypes were characterized by high proliferation rates. However, both single Cebpb and compound Cebpa/Cebpb dKO cells showed reduced proliferation as compared with the WTfl cells.

Histological staining of cytopsins of WTfl, Cebpb KO, and Cebpa/Cebpb dKO cells are shown in Fig 2C. WTfl cells characterized by a predominant monoblastic/myeloblastic appearance, sometimes with kidney-shaped nuclei, relatively pale cytoplasm, and few, mostly diffuse cytoplasmatic granules. Cebpb KO cells displayed a similar cytoplasm/nucleus ratio to WTfl cells, with early monocyctic/myelocytic appearance, some vacuoles, and most typically, azurophilic granules in the cytoplasm. The Cebpa/Cebpb dKO cells had a smaller cytoplasm/nucleus ratio, with more darkly stained cytoplasm and frequently hyposegmented nuclei. These data suggest that the different genotypes are also reflected in distinct early myelomonocytic phenotypes.

Comparative serial replating of the WTfl, Cebpb KO, and Cebpa/Cebpb dKO cells revealed a steady increase in the clonogenicity of the WTfl and Cebpb KO cells and a decline in that of the Cebpa/Cebpb dKO cells (Fig 2D), suggesting enrichment for transformed stem cells in the WTfl and Cebpb KO cells and the loss of stemness in the Cebpa/Cebpb dKO cells during replating. The characteristic heterogeneous appearance of the compact and disperse WTfl colonies and more uniform, round, and compact Cebpb KO colonies was maintained during replating, whereas Cebpa/Cebpb dKO colonies continuously declined during replating and ceased growth beyond the fourth replating (Fig 2E). However, differences in the colony-forming capacity between the WTfl and Cebpb KO cells became evident after partial withdrawal of cytokines (Fig 2F, growth in IL-3 medium), revealing that the Cebpb KO cells were more dependent on the standard cytokine mix (IL-3, SCF, IL-6, and GM-CSF) than the WTfl cells.

Endogenous C/EBPα compensates for C/EBPα and C/EBPβ deficiency in MLL-ENL–transformed cells

Related gene products may functionally compensate for distinct deleted genes, and obscure otherwise severe phenotypes. In particular, this can be observed in gene families that share evolutionarily conserved origins, such as the C/EBP family (El-Brolosy & Stainier, 2017). Therefore, we wondered about the compensatory mechanisms that may have occurred in the Cebpa/Cebpb dKO MLL-ENL clones to permit their survival. RNA sequencing (RNA-seq) analysis of two randomly chosen dKO clones derived from different animals confirmed the absence of Cebpa and Cebpb expression (Fig 3A). Strikingly, both dKO MLL-ENL–transformed clones showed up-regulated Cebpα gene expression, whereas Cebpd expression remained largely unchanged in the WTfl and dKO cells. These data suggest that the activation of Cebpa may compensate for the loss of Cebpa/Cebpb. Accordingly, we examined all 14 recovered dKO clones for expression of the C/EBP family members by protein blotting. Fig 3B shows that all dKO clones strongly expressed the C/EBPα protein, whereas only small and inconsistent changes were observed in C/EBPβ, C/EBPδ, and C/EBPζ protein levels. These data are in line with previous findings showing that ectopic expression of C/EBPα, similarly to C/EBPβ, but unlike C/EBPδ, is compatible with the proliferation of GMP-like progenitors (Cirovic et al, 2017).
All 14 recovered Cebpα/Cebpb dKO clones grew slowly in liquid culture and had increased apoptosis, as compared with the WT14 MLL-ENL–transformed cells (compare with Fig 2A, right), suggesting incomplete functional compensation by C/EBPε. To determine whether distinct C/EBP isoforms could rescue MLL-ENL–transformed cell growth, isoforms of C/EBPα (p42 and p30) or C/EBPβ (LAP*, LAP, and LIP) were retrovirally transferred and subsequently enriched for co-expression of GFP, as shown in Fig 4A.

As expected, growing cells that expressed the proliferation-suppressive C/EBPα p42 isoform could not be recovered, and the few GFP+ sorted cells that grew out failed to express the p42 protein, confirming successful retroviral transduction but failure of genetic complementation by C/EBPα p42. Growing cells were obtained for all other constructs (Fig 4A), and their proliferation rates, clonogenicity, and viability were assessed. Colony formation of the parental dKO cells was partially rescued by the C/EBPα p30 isoform (Fig 4B–D), and to a higher extent by the C/EBPβ LAP* isoform, whereas the C/EBPβ LAP and C/EBPβ LIP isoforms displayed intermediate and no discernible complementation capacity, respectively (Figs 4E–G and S2). In summary, the C/EBPβ LAP* isoform, and in part, the C/EBPβ LAP or C/EBPα p30 isoforms, restored dKO MLL-ENL–transformed cell proliferation (Fig 4B and E) and viability (Fig 4C and F).

Next, we examined the role of C/EBPε in Cebpα/Cebpb dKO MLL-ENL cells that expressed high levels of endogenous C/EBPε, termed C/EBPε*, by RNA interference strategies. However, the fragile dKO MLL-ENL C/EBPε* cells did not tolerate the small interfering RNA treatment, and various conditionally inducible, retrovirally delivered short hairpin RNAs failed to down-regulate C/EBPε reproducibly (data not shown). The data may suggest that triple deletion of the Cebpα, Cebpb, and Cebpe genes is incompatible with cell survival. Interestingly, we observed that expression of the endogenous C/EBPε ceased after extended cultivation of MLL-ENL dKO cells that were complemented with the C/EBPβ isoforms LAP*, LIP, and LAP.
and to a lesser extent with LAP, but not with LIP (Fig 5A). Based on the observation of an inverse correlation between resident C/EBPε protein levels before and after ectopic C/EBPβ LAP* expression (Fig 5A), we explored the mutability of Cebpe in dKO MLL-ENL cells in the presence or absence of ectopically expressed C/EBPβ LAP*, as outlined in Fig 5B. Briefly, targeted deletion of resident Cebpe by Cas9 was examined after transduction of dKO cells with either empty control or C/EBPβ LAP* retrovirus in parallel, both expressing GFP as a marker. GFP+ cells from both approaches were sorted, expanded, and infected with the same batch of a genome editing vector, encoding blue fluorescent protein (BFP) as a marker and Cas9 plus guide RNAs (Li et al, 2016; Henriksson et al, 2019), targeting Cebpe exon 1. After infection, sorted BFP+ cells were expanded, seeded in semi-solid medium, and individual colonies were isolated from both vector control and C/EBPβ LAP*-complemented cells (Fig 5B).

In comparison to vector control cultures that showed many abortive small colonies, augmented colony formation and proliferation were already discernible at an early stage during colony formation in C/EBPβ LAP*-complemented dKO cells (data not shown). After single colonies had been isolated, proliferation in liquid culture was discontinued in 35.9% of the vector control clones (N = 79/220 clones), but only 17.8% (N = 32/180 clones) of the C/EBPβ LAP* supplemented dKO clones were abortive (Fig 5C). Only 12.3% (27/220) of the vector controls, yet 48.9% (88/180) of the C/EBPβ-LAP* dKO clones showed robust growth in liquid culture. Among the vector control clones, all 27 properly growing control clone isolates and 31 (out of 88) randomly chosen C/EBPβ-LAP*-complemented clones were expanded in liquid culture. As shown by protein blotting (Fig 5D), each of the five LAP*-dKO and five vector controls showed reciprocal expression of LAP* and C/EBPε. Analysis of the genomic Cebpe status (Fig 5E) showed that 80% (16/27 clones)
of the vector control clones retained the WT Cebpe genotype, and only two showed indel frameshift mutations (see below). In contrast, 74% (20/31 clones) of the C/EBPβ LAP*-complemented clones showed biallelic frameshift indels and/or large deletions. Importantly, 7/27 vector control clones but only 2/31 of LAP* clones had in-frame mutations. Both C/EBPβ LAP*-complemented in-frame mutations were associated with frameshift indels in the second Cebpe allele, whereas both frameshift indels of the vector control cells retained either a WT or an in-frame deletion in the second allele, respectively. Most remarkably, all in-frame mutations affected negative regulatory C/EBPε regions I or II (Fig 5F), previously described to restrain C/EBPε activity (Angerer et al., 1999). Taken together, these data show that biallelic Cebpe inactivation in dKO cells occurred only in the presence of C/EBPβ LAP*, but not in its absence. In the absence of ectopically expressed C/EBPβ LAP*, either the WT genotype persisted, or alternatively, in-frame dele-
tions affecting negative regulatory C/EBPε regions were selected, strongly supporting the requirement of functional C/EBPε in the absence of Cebpa/Cebpb. Accordingly, we conclude that mye-
lo-monocytic MLL-ENL transformation remains dependent on C/EBPβ and that C/EBPε may partially compensate for the loss of Cebpa/ Cebpb.

C/EBPs coordinate the expression of the MLL-ENL/Hoxa target genes

The combined deletion of Cebpa and Cebpb led to widespread leukemic cell death and selected for compensatory expression of C/EBPβ in dKO cells, suggesting that maintenance of the trans-
formed cell identity is C/EBP-dependent. To test this hypothesis, we performed RNA-seq (two clones, #5, #13, in triplicates) of the MLL-
ENL–transformed cell transcriptomes before and after compound Cebpa/Cebpb deletion. Deleting Cebpa/Cebpb resulted in the up-regulation of 2066 (clone #5) and 2517 (clone #13) genes (755 genes overlapping), respectively, and the down-regulation of 2447 (clone #5) and 2702 (clone #13) genes (1024 genes overlapping),...
respectively, when a corrected P-value < 0.05 was used as the cutoff (Fig 6A and Table S1).

We first investigated the impact of the combined loss of C/EBPa and C/EBPB on the direct and indirect targets of MLL-ENL (Collins et al, 2014; Garcia-Cuellar et al, 2016). Of the 166 direct/core MLL-ENL target genes, a significant 70 genes (P = 2.97 × 10⁻³⁴) were the DOT1L/transcription activator of these genes (Zhang et al, 2013).

For the indirect MLL-ENL targets, removing C/EBPa and C/EBPB affected the genes downstream of the transcriptional control of HOXA9/MEIS1 that had been identified after the removal of Cebpa in HOXA9/MEIS1-transformed cells (Collins et al, 2014; Collins & Hess, 2016a, 2016b). Gene set enrichment analysis (GSEA) (Liberzon et al, 2011; Wu & Smyth, 2012) revealed significantly deregulated HOXA9-mediated gene suppression (P < 0.05) (Fig 6C), including Cpa3, Gzmb, Peg13, Mycn, Hifac, Alox5, Gata2, Nkg7, Stx3, Cdh17, Scin, Rgs10, Havcr2, and Col18a1, but excluding the self-renewal inhibitor Cdh2b (gene set extracted from Collins et al [2014]). The entire HOXA9/C/EBPa co-activated gene set was not significantly deregulated, yet the dKO cells showed increased Adra2a, Pcp4a1, Itsn1, and Igfzr expression from the HOXA9/C/EBPa co-activated set, whereas Adam17, Gm1110, Pde7a, Pdcd4, and Nrg2 expression were reduced.

Next, we investigated lineage restriction in the transformed cells by comparing the transcriptional landscape of WT1 and dKO cells to the ImmGen database (Heng & Painter, 2008). Compared with the WT1 cells, the dKO cells lost some of their myeloid expression...
Figure 6. Molecular Genetic Profiling of MLL-ENL–Transformed dKO Cells.
(A) Venn diagrams showing the overlap between up-regulated (Benjamini–Hochberg corrected $P$-value < 0.05) and down-regulated (Benjamini–Hochberg corrected $P$-value < 0.05) genes in both dKO clones as determined by RNA-seq. N = 3 per genotype.
(B) Scatter plot showing dysregulation of MLL-ENL target genes, which are colored according to $P$-value and fold change. All remaining genes are colored according to their $P$-value only (see figure key). Two genes, Igf1 and Bcl11a, are included in the plot, as they are known MLL translocation targets.
(C) Gene set enrichment analysis showing significant enrichment of genes co-repressed by Hoxa9/C/EBPα among the deregulated genes. Most of these genes were up-regulated in the dKO cells.
(D) Comparison of expression patterns to that of ImmGen data shows a shift in overall gene expression.

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characteristics and showed less similarity to monocytes and macrophages (Fig 6D). Specifically, Csf1r, Rel family members (Rel, Rela, and Relb), and Tgfb2 levels were decreased in the dKO cells, supporting the pioneering role of C/EBPα and C/EBPβ in myeloid commitment (Wang et al, 2006; Jaitin et al, 2016; Tamura et al, 2017). However, the WT Fl and dKO cells were similarly enriched for genes shared with HSC, highlighting the LSC state of the cells.

For a more general/unbiased approach, we performed GSEA on the hallmark gene sets of MSigDB to examine the enrichment of functional terms in the group of differentially expressed genes between the WT Fl and dKO genotypes (Liberzon et al, 2011; Wu & Smyth, 2012). Consistent with the reduced myeloid commitment observed in the dKO cells (Fig 2A), we detected strong dysregulation of the inflammatory genes, documented by significant enrichment of “hallmark inflammatory response” and “hallmark Tnfα signaling via Nfkb” (P < 0.05) with partially overlapping genes, in addition to an increase in stem cell transcripts (see the corresponding heat maps in Fig 6E). Altogether, these results support the premise that C/EBPα partially compensates for the loss of Cebpa/Cebpβ by SECuring a MLL-ENL and Hoxa9 regulated core gene expression program and shifts the transformed phenotype towards an immature phenotype.

The superior growth and viability of the WT Fl and dKO-LAP* cells over Cebpa/Cebpβ dKO cells was evident, affirming that compensation by C/EBPβ remains incomplete. To identify ancillary MLL-ENL targets that may be involved in restoring the WT phenotype, we compared differential gene expression with the genome accessibility status using Assay for Transposase-Accessible Chromatin with sequencing (ATAC-seq) (Buenrostro et al, 2013) in the three genotypes, that is, WT Fl, Cebpa/Cebpβ dKO, and Cebpa/Cebpβ dKO complemented with C/EBPβ LAP*. Initial inspection of chromatin accessibility around the Cebpa/Cebpβ loci confirmed the dKO status, whereas the ATAC peak pattern around the Cebpβ locus remained unchanged, ruling out the possibility that a recombination event at the Cebpβ locus may have contributed to enhanced Cebpβ expression in the dKO cells (Fig 6H). Focusing on genes that exhibited both, re-establishing gene expression and restoring chromatin status in dKO-LAP* cells towards WT Fl status yielded a list of genes (highly correlated gene expression and chromatin status), part of which is shown in Fig 6F. Importantly, known motif enrichment search showed significant enrichment of the CEBP, ETS, and RUNX binding motifs (Fig 6G) in the genomic regions determined by the full list of ATAC peaks, as described in Fig 6F. Fig 6H shows that, in dKO cells, the enhanced Sox4 and Hoxa9 gene expression (Fig 6B) was associated with increased chromatin accessibility, whereas decreased Bcl11a and Igf1 gene expression was associated with decreased ATAC peak sizes, whereas ectopic expression of LAP* restored ATAC peaks to the WT conditions (Fig 6H).

Open chromatin regions that were present in WT Fl cells, absent in dKO cells, and restored in dKO-LAP* cells were identified in an intronic region of the Igf1 gene (Fig 6H) that overlapped with a super-enhancer region previously identified in monocytes and liver cells (Jiang et al, 2019). Moreover, IGFI has been described as a growth-regulatory MLL-ENL/Hoxa9/Mefi1 target and as a direct C/EBPβ target gene involved in autocrine stimulation of transformed murine monocytes and in neoplasia (Pollak et al, 2004; Wessells et al, 2004; Steger et al, 2015; Collins & Hess, 2016b). Comparison of the Igf1 RNA-seq reads confirmed Igf1 gene expression in the WT Fl and dKO-LAP* cells, but not in the dKO cells (Fig 6I, top panel), and quantification by ELISA in tissue culture supernatants (Fig 6I, bottom panel) showed that IGFI was produced by the WT Fl and dKO-LAP* cells, but not by dKO cells. Adding IGFI (10 ng/ml) to the culture medium increased the cell number, viability (toluidine exclusion), and metabolism (WST-1 conversion) of the dKO culture (Fig 6I) but not that of the WT Fl or dKO-LAP* cultures (Fig 6K). Taken together, these data suggest that C/EBP family member- and isoform-specific regulation can differentially affect several MLL-ENL target genes. The data also confirm a C/EBPβ co-regulated autocrine function of IGFI in MLL-ENL transformation.

**Discussion**

Myelomonocytic commitment and differentiation into the GMP state depends on C/EBP transcription factors and is a prerequisite for the emergence of AML and LSC. Our data show that the continuous presence of C/EBP transcription factors is essential for maintaining leukemic myelomonocytic transformation by the MLL-ENL oncogene product of fetal liver hematopoietic progenitor cells in tissue culture.

A current concept of myeloid leukemogenesis suggests that LSC arise in committed progenitors that resemble GMP-like cells that acquire self-renewal capacity (Cozzo et al, 2003; Huntly et al, 2004; Jamieson et al, 2004; Kvitvskv et al, 2006). As an inducer of the GMP-like state, it has been suggested that C/EBPα plays a critical role in leukemic myelomonocytic transformation. Intriguingly, once leukemic progenitor transformation is established, C/EBPα can be genetically removed, whereas the leukemic state persists (Ohlsson et al, 2014; Ye et al, 2015). Along the same lines, the initiation of oncogene-induced leukemic transformation in the absence of C/EBPα expression patterns, with loss of myeloid characteristics and maintenance of stem cell patterns in dKO cells indicated by Spearman correlation. (E) Heatmap of inflammatory and Tnfα/NF-κB hallmark genes (gene sets derived from MSigDB) in WT Fl and dKO cells. Both terms are significantly enriched (adjusted P-value < 0.05, according to Gene set enrichment analysis). The heat maps show genes of the respective gene sets exhibiting an adjusted P-value < 0.05 and an absolute fold change ≥ 2. Row z-score of normalized log counts. (F) Subset of the co-regulated transcriptome and genome structure as determined by RNA-seq and ATAC-seq. The whole list comprises genes that exhibit both the re-establishment of gene expression and restoration of the chromatin status in dKO-LAP* cells to WT Fl status. Row z-score of normalized log, counts. (G) Known motif prevalence in dKO-LAP* cells in the full set of genes shown in (F) as determined by HOMER. (H) Examples of replicate ATAC-seq data from WT Fl, dKO, and dKO-LAP* MLL-ENL-transformed cells indicating gain of peaks (left, blue outline) in dKO at the Sox4 and Hoxa9 loci, and loss of peaks at the Bcl11a and Igf1 loci (right, red outline). The top three lanes show C/EBPα and C/EBPβ chromatin immunoprecipitation (ChIP) data from MLL-AP9-transformed cells (Roe et al, 2016); the bottom three lanes show Hoxa9 CHIP data from Zhong et al (2018). (I) Igf1 gene expression (top: RNA-seq data from triplicates) and IGFI secretion into the growth medium (bottom: ELISA data; ELISA was repeated 3 times yielding similar results) by MLL-ENL-transformed cells. (J) Response of MLL-ENL-transformed dKO cells to the addition of Igf1 to the culture medium as determined by cell counts, viability, and metabolic activity. (K) Comparison of response to Igf1 of WT Fl, dKO, and dKO-LAP*-complemented cells.
can still be achieved, if concomitant emergency granulopoiesis is induced (Ye et al., 2015). These data suggest the achievement of the GMP-like state as the most critical event in leukemic transformation, regardless of how the GMP state is achieved. However, emergency granulopoiesis elicits C/EBPα expression (Hirai et al., 2006), and gene replacement experiments have shown that C/EBPα expressed from the C/EBPa locus can fully complement the hematopoietic functions of C/EBPa, including differentiation into GMP (Jones et al., 2002). The alternative explanation for the maintenance of the leukemic state after removing C/EBPa, therefore, is that C/EBPa may initially pass the “GMP baton” to C/EBPβ, which in turn supports proliferation and maintains the transformed myeloid state.

Our data show that maintaining myeloid MLL-ENL transformation strongly depends on combined C/EBPβ and C/EBPα expression. The non-oncogenic dependence (Solimini et al., 2007; Nagel et al., 2016) on C/EBPα transcription factors became evident from the analysis of the dKO mutants, which yielded very few clonogenic cells that consistently expressed resident C/EBPε. Genetic removal of both Cebpα and Cebpβ consequently revealed a surrogate function of C/EBPε in myeloid MLL-ENL transformation. This finding is in line with several KO studies involving various members of the C/EBP family and isoforms that revealed redundant C/EBP functions only in compound deletion mutants. Indeed, C/EBPε partially compensated for the loss of C/EBPα during murine myelopoiesis and the innate immune response and was also compatible with the proliferative progenitor state (Akagi et al., 2010; Cirovic et al., 2017). Whether the rare event of C/EBPα expression in the MLL-ENL–transformed cultures may have occurred before or after Cebpα or Cebpβ deletion, potentially reflecting MLL-ENL progenitor plasticity, or as a regulatory event that occurred under the selective pressure of Cebpα/Cebpβ removal, remains to be determined.

The data suggest a compensatory backup function of C/EBPα in the surviving Cebpα/Cebpβ dKO MLL-ENL cells that may maintain the minimal essential GMP condition in the absence of Cebpα/Cebpβ and serve as a proxy in regulating the most critical genes involved in MLL-ENL transformation. Consistently, genetic removal of C/EBPε from dKO cells by gene editing was only possible after complementation with C/EBPβ LAPε, whereas uncomplemented cells only tolerated C/EBPε gene editing as in-frame mutations that removed parts of the negative regulatory regions of the C/EBPε protein (Angerer et al., 1999). Taken together, the results suggest the essential functions of C/EBP family members, and in particular, a privileged function of C/EBPβ LAPε in the proliferation, survival, and maintenance of the myeloid cell fate of transformed MLL-ENL cells.

Regulation of the Hox cluster target genes, including Hoxa9 and Meis1, previously emerged as a hallmark of MLL translocation leukemia, and the combined expression of Hoxa9 and Meis1 was sufficient for initiating a leukemogenic progenitor self-renewal program (Armstrong et al., 2002; Ayton & Cleary, 2003; Ferrando et al., 2003; Huang et al., 2012; De Braekeleer et al., 2014; Garcia-Cuellar et al., 2016; Collins & Hess, 2016a). C/EBPβ is also essential in Hoxa9/Meis1-induced leukemic transformation (Collins et al., 2014), yet HOXA9/HOXA10/Meis1 were only partially down-regulated in the Cebpα/Cebpβ dKO C/EBPε cells. We believe that the expression of resident C/EBPε in the dKO cells compensated further down-regulation beyond a lower limit, thereby functionally substituting for the absence of Cebpα/Cebpβ, whereas cells that failed to express C/EBPε were lost.

Accordingly, the most strongly affected genes in the dKO cells may therefore represent secondary functional targets, such as autocrine IGF1 stimulation, which contributed to accelerated growth and prevention of apoptosis, but may not be entirely central to leukemic transformation.

Previous research suggesting a C/EBPα-induced epigenetic state that is subsequently maintained by the MLL-ENL oncoprotein (Ohiisson et al., 2014; Roe & Vakoc, 2014) is contrasted by various lines of evidence suggesting that, in higher organisms, maintenance of the epigenetic state relies on the continuous presence of priming master regulatory transcription factors (Blau et al., 1985; Blau & Baltimore, 1991; Holmberg & Perlmann, 2012). Our data support the latter concept and suggest a prominent role for distinct C/EBP family members and isoforms in MLL-ENL transformation, reflecting a type of lineage- and differentiation state–specific non-oncogenic dependence on transformation. Pharmacological interference with the non-oncogenic C/EBP dependence on acutely transforming myelomonocytic oncoproteins could potentially extend a future clinical repertoire of treatment regimens with the aim of eradicating devastating infant AML.

### Materials and Methods

#### Generation of MLL-ENL–transformed cells

Viral supernatants were produced by transfecting Plat-E cells with pMIG-GFP retroviral vector or pMSCV-MLL-ENL construct. Infectious supernatant was collected 48 and 72 h post-transfection. Mouse embryos (13.5 d) were dissected and genotyped. Hematopoietic stem and progenitor cells were isolated from the mouse fetal livers and transduced during two consecutive days by centrifugation (twice: 1 h, 900g) with viral supernatant and 8 μg/ml hexadimethrine bromide. After the second infection, the cells were recovered in IMDM supplemented with murine IL-3 (10 ng/ml), IL-6 (10 ng/ml), and SCF (50 ng/ml) (STEMCELL Technologies). Infected cells were selected with 500 μg/ml G418 (Sigma-Aldrich). After 14-d antibiotic selection, the cells were seeded in MethoCult M3434 (STEMCELL Technologies). Colonies formed from single cells were isolated, expanded, and analyzed. Flow cytometry was performed with LSR II or Fortessa machines (Becton Dickinson). The following antibodies from BioLegend or eBioscience were used: CD11b (M1/70), CD117 (2B8), Ly6C (AL-21), Ly6G (1A8), CD115 (AF598), Sca1 (D7), and CD66a (54-5/7.1).

### Cell culture

The MLL-ENL–transformed cell lines were cultured in IMDM (10% FCS, 1% penicillin/streptomycin [Gibco]) supplemented with 10 ng/ml mouse IL-3 (STEMCELL Technologies). Cells were split every 2–3 d to 1 × 10⁶ cells/ml. Plat-E cells were cultured in DMEM (10% FCS, 1% Heps, and 1% penicillin/streptomycin [Gibco]). Transient transfections were performed using polyethyleneimine (Polysciences) according to the manufacturer’s protocol.

### TAT-Cre treatment and selection of recombinant clones

Before the treatment, TAT-Cre recombinase (Millipore) was diluted to a final concentration of 4 μM in IMDM and filtered (0.2-μm
low-protein-binding filter). Cell pellets of 4 × 10^5 MLL-ENL–transformed cells were resuspended in 1 ml TAT-Cre solution, transferred to a 24-well plate, and incubated at 37°C for 20 h. The cells were washed with PBS and seeded in MethoCult M3134 (STEMCELL Technologies) supplemented with 10 ng/ml mouse IL-3. Colonies were transferred to liquid medium, expanded, and analyzed by PCR for excision of Cebpa and/or Cebpb.

**Retroviral transduction with C/EBP isoforms, GFP vector control, or BFP Cas9-sgRNA C/EBP**

Viral supernatants were produced by transfecting Plat-E cells with empty pMIG-GFP retroviral vector or pMIG-GFP constructs containing p42, p30, LAP*, LAP, or LIP, or pMSCV-BFP constructs containing Cas9 and sgRNAs targeting Cebpe. The cells were centrifuged with infectious supernatant collected 72 h after transfection and 8 μg/ml hexamethylenimine (1 h, 900g), and left for recovery overnight.

**Plasmids and retroviral constructs**

The MLL-ENL retroviral construct has been described previously (Slany et al., 1998). C/EBPa, β isoform expression constructs, amino acid sequence, and retroviral construction have been published previously and are available on request (Kowenz-Leutz et al., 1994; Stoilova et al., 2013; Cirovic et al., 2017). pMSCV-Cas9-2A-GFP-sgRNA (#124889; Addgene) and pMSCV-U6sgRNA(BbsI)-PGKpuro2A-BFP was amplified by PCR, the fragments were purified, cloned by BbsI into the pMSCV_Cas9-2A-BFP-sgRNA vector. The edited sequences were analyzed using modified CRISPR/Cas9 and C/EBP isoform expression constructs, amino acid sequence, and retroviral construction have been published previously and are available on request (Kowenz-Leutz et al., 1994; Stoilova et al., 2013; Cirovic et al., 2017). pMSCV-Cas9-2A-GFP-sgRNA (#124889; Addgene) and pMSCV-U6sgRNA(BbsI)-PGKpuro2A-BFP (#102796; Addgene) were purchased (Li et al., 2016; Henriksson et al., 2019). The T2A-GFP fragment of the pMSCV GFP-Cas9–containing construct was excised by BamHI/NotI restriction digest. The T2A-BFP marker of pMSCV-U6sgRNA(BbsI)-PGKpuro2A-BFP was amplified by PCR and cloned as a BamHI/NotI fragment into the pMSCV Cas9 construct. BFP primer: 5′-BamHI, T2A-BFP 5′-CGCGATACCCAGAGGCGAGAAGGCTTGCATTGC-3′; 5′-BFP: NotI: 5′-ATAGTTACCGCCTGCTCAATAGCTTGC-3′.

Cebpe sgRNAs were selected using CrispR Gold (https://crisprgold.mdc-berlin.de) and CRISPOR (http://crispor.tefor.net). The sgRNAs were cloned by BbsI into the pMSCV_Cas9-2A-BFP-sgRNA vector. The C/EBPε sgRNA oligos were used 5′-CTACCTGTAGGACGCCGAA-3′, 5′-AGGGATAGGCGAATGGCCGA-3′, 5′-CTGGTCTCTCACTAACCCTGC-3′, and 5′-CGCACCATTAGGATCCCGG-3′.

**Analysis of C/EBPε genome editing by CRISPR/Cas9 and C/EBPε sgRNAs**

Total DNA was isolated from single cell clones using QuickExtract DNA Extraction Solution (Epicentre). Cebpe genomic exon 1 was amplified by PCR, the fragments were purified (Invitrogen #1020300300; Invitron), and analyzed by Sanger sequencing (LGC Genomics). The edited sequences were analyzed using modified ICE software (Hsiue et al, 2018 Preprint), available from Synthego (https://www.synthego.com/products/bioinformatic/crispr-analysis). The PCR primers used for Cebpe exon 1 analysis were 5′-CAGGACA-CAGCGAGGATTCTCTA-3′ and 5′-CTAGGCAAAATCCTAGGGCGG-3′.

**Genotyping**

Total DNA was isolated using QuickExtract DNA Extraction Solution (Epicentre). Cebpa excision was evaluated by two separate PCR for the Cebpa floxed allele and Cebpa deletion. The Cebpa floxed allele primers were 5′-TGCGCTGGAGACACAATTGA-3′ and 5′-CGCAGAGATTCTGCCTTT-3′; the expected product was 269 bp. The Cebpa deletion primers were: 5′-GCTCTTGAGGCTAATTCTC-3′ and 5′-TGGAACATTGGTGCTGT-3′; the expected product was 380 bp. Cebpb excision was determined by competitive PCR using the following primers: 5′-GAGCCACCGTCTCTCCAGC-3′, 5′-GGTCGGTCGCTGATTGCC-3′, and 5′-AGCAGACTGCCCCGCCAAA-3′; the reaction produced bands of 253 bp for the flox allele and 610 bp for the deleted allele.

**Immunoblotting**

Total protein lysates were prepared by lysing fresh cell pellets with 0.5 M NaOH with subsequent neutralization with 0.5 M HCl. The samples were sonicated, mixed with SDS sample buffer and glyc erol, and heated (3 min at 95°C). After centrifugation, the protein lysates were separated by electrophoresis and transferred to a nitrocellulose membrane using a Trans-Blot Turbo System (Bio-Rad). Protein signals were detected after incubation with antibodies against C/EBPa (14AA; Santa Cruz Biotechnology), C/EBPβ (C-19; Santa Cruz Biotechnology), C/EBPφ (C-22; Santa Cruz Biotechnology), C/EBPε (NB1P-85446; Novus Biologicals), Flag-M2 (F-1804; Sigma-Aldrich), and GAPDH (ab9484; Abcam).

**Apoptosis assay**

The level of apoptosis was determined with a FITC–Annexin V Apoptosis Detection Kit I (BD Pharmingen). After washing twice with cold PBS, 1 × 10^6 cells were resuspended in 1 ml 1× binding buffer. Cell suspension (100 μl) was transferred to a new tube and mixed with 5 μl FITC–annexin V. The samples were incubated for 15 min at room temperature in the dark. Afterward, 5 μl 7-AAD (BD Pharmingen) was added and the samples were subsequently analyzed using an LSR II flow cytometer (Becton Dickinson).

**Proliferation and metabolism assays**

The MLL-ENL–transformed cells were seeded at 5 × 10^5 cells per well in 100 μl complete IMDM in 96-well plates in triplicate. The cells were incubated for 48, 72, or 96 h at 37°C. The cell proliferation reagent WST-1 (10 μl/well; Roche) was added, gently mixed, and incubated at 37°C for 1 h. The absorbance of the samples was measured at 450 nm using an iMark microplate reader (Bio-Rad). A murine IGF-1 ELISA kit (BKGP9PU89; BioGems) was applied according to the manufacturer’s protocol. For the CFSE proliferation assay, 1 × 10^6 cells were resuspended in 1 ml 5 μM CFSE solution (BioLegend) for 20 min in the dark at room temperature. Afterward, the reaction was quenched using 5 ml cell culture medium plus 10% FCS. The cells were washed and cultured for the indicated time.

** Colony formation assay, determination of colony size and number, serial replating**

The MLL-ENL–transformed cells were added to complete (10% FCS, 1% penicillin/streptomycin [Gibco]) and IL-3–supplemented (10 ng/ml) MethoCult 3134 (STEMCELL Technologies) at a final concentration of 5,000 cells per ml (if not indicated otherwise). The cell mix (1 ml) was seeded in six-well meniscus-free plates (SmartDish;
STEMCELL Technologies) in triplicate. High-resolution microscopic scans were taken after 7, 10, and 14 d using an EVOS imaging system (Thermo Fisher Scientific). The number, size, and approximate cell numbers of the colonies were calculated using ImageJ software with the ColonyArea plugin. Serial repling experiments were set up with 5,000 cells per mL in MethoCult 3134 supplemented with IL-3 (10 ng/ml), IL-6 (5 ng/ml), SCF (25 ng/ml) and GM-CSF (5 ng/ml). After 7 d in culture, colony numbers were determined using the EVOS imaging system, colonies were harvested by centrifugation in culture medium, cell numbers and viability were determined, and comparable numbers of cells were re-seeded in MethoCult 3134 under the conditions described above.

ATAC-seq

To prepare the ATAC-seq libraries, 50,000 cells were sorted into 500 μl PBS. The libraries were prepared as previously described (Buenrostro et al, 2013), with slight modifications (Lara-Astiaso et al, 2014). Briefly, the cells were lysed with 25 μl cold lysis buffer (10 mM Tris–HCl [pH 7.4], 10 mM MgCl₂, and 0.1% Igepal CA-630) and the nuclei were pelleted by centrifugation in culture medium, cell numbers and viability were determined, and comparable numbers of cells were re-seeded in MethoCult 3134 under the conditions described above.

Sequencing data analysis

RNA-seq libraries were prepared using Illumina TruSeq stranded mRNA kit starting with 500 ng of input total RNA. Libraries included dual indices and were equimolar pooled. Paired-end 76 nt sequencing was performed on Illumina NextSeq 500 High Output v.2 flowcell. RNA-seq samples were aligned to the reference genome (mm10) using STAR (Dobin et al, 2013). Expression was quantified using HTSeq (Anders et al, 2015); downstream analysis was done with DESeq2 using the included normalization strategy (for details see Love et al [2014]) and a Benjamini–Hochberg–corrected P-value of 0.05 as a significance cutoff. Alterations in function induced by the different expression patterns in the conditions were assessed using GSEA using gene sets by MSigDB (Liberzon et al, 2011) and Camera (Wu & Smyth, 2012). ATAC-seq samples were preprocessed with PiQx pipelines (Wurmus et al, 2018), including alignment to a reference with Bowtie 2 (Langmead & Salzberg, 2012), to genome version mm10 and peak-calling using MACS (Zhang et al, 2008). Differential peaks were detected with DiffBind (2.14) using a corrected P-value of 0.05 as a cutoff (Ross-Ines et al, 2012). Known motif detection was performed using findMotifsGenome.pl in HOMER (v4.10.3) ($ findMotifsGenome.pl XXX.bed mm10 homer / -size 200 -mask) (Heinz et al, 2010). The set of genomic regions used for motif discovery comprises the regions of those genes, that exhibited both, re-established gene expression (uncorrected P-value < 0.05) and restored chromatin status (corrected P-value < 0.05) in dKO-LAP* cells toward the WT T status.

Data Availability

Data generated during this study have been deposited in Gene Expression Omnibus with the accession codes GSE153622, GSE153623, and GSE153624.

Supplementary Information

Supplementary Information is available at https://doi.org/10.26508/Isa.202000709.

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Author Contributions

R Wesolowski: data curation and writing—original draft, review, and editing. E Kowenz-Leutz: resources, data curation, formal analysis, validation, investigation, methodology, and writing—review and editing. K Zimmermann: data curation, software, formal analysis, and writing—review and editing. D Dör: investigation, methodology, and writing—review and editing. M Hofstätter: validation, investigation, and methodology. RK Slany: resources, methodology, and writing—review and editing. A Mildner: data curation, formal analysis, investigation, and writing—review and editing. A Leutz: conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, project administration, and writing—original draft, review, and editing.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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