Initiation of MLL-rearranged AML is dependent on C/EBPα

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MLL-fusion proteins are potent inducers of oncogenic transformation, and their expression is considered to be the main oncogenic driving force in ∼10% of human acute myeloid leukemia (AML) patients. These oncogenic fusion proteins are responsible for the initiation of a downstream transcriptional program leading to the expression of factors such as MEIS1 and HOXA9, which in turn can replace MLL-fusion proteins in overexpression experiments. To what extent MLL fusion proteins act on their own during tumor initiation, or if they collaborate with other transcriptional regulators, is unclear. Here, we have compared gene expression profiles from human MLL-rearranged AML to normal progenitors and identified the myeloid tumor suppressor C/EBPα as a putative collaborator in MLL-rearranged AML. Interestingly, we find that deletion of *Cebpa* rendered murine hematopoietic progenitors completely resistant to MLL-ENL–induced leukemic transformation, whereas C/EBPα was dispensable in already established AMLs. Furthermore, we show that *Cebpa*-deficient granulocytic-monocytic progenitors were equally resistant to transformation and that C/EBPα collaborates with MLL-ENL in the induction of a transcriptional program, which is also apparent in human AML. Thus, our studies demonstrate a key role of C/EBPα in MLL fusion–driven transformation and find that it sharply demarcates tumor initiation and maintenance.

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Abbreviations used: 4-OHT, 4-hydroxy tamoxifen; AML, acute myeloid leukemia; ChIP-seq, chromatin immunoprecipitation sequencing; GMP, granulocytic monocytic progenitor; GSEA, gene set enrichment analysis; HSC, hematopoietic stem cells; HSPC, hematopoietic stem and progenitor cells; LSK, Lin$^-$, Sca-1$^+$, c-Kit$^+$; pIpC, polyinosinic-polycytidylic acid; preGM, pre-granulocytic monocytic progenitor; TSS, transcriptional start site.

AML is associated with several genetic and epigenetic events that result in malignant transformation of hematopoietic cells. In particular, transcription factors and epigenetic regulators involved in normal hematopoiesis are often found to be mutated, leading to the formation of leukemic stem cells and the accumulation of immature blasts (Estey and Döhner, 2006). Translocations involving the mixed lineage leukemia (MLL) gene at chromosome band 11q23 are among the most frequent lesions (∼10%) in AML and are associated with poor prognosis. More than 50 genes that fuse with MLL have been identified, of which ENL, ELL, AF6, AF9, and AF10 are the most frequent partners (Krivtsov and Armstrong, 2007; Muntean and Hess, 2012). Specifically, these fusions result in the expression of chimeric proteins in which the N terminus of the MLL protein is fused in-frame to the C terminus of the partner protein, thereby destroying the H3K4 histone methyltransferase activity of the full-length MLL, while retaining its target selectivity for a subset of MLL-targets genes (Ayton et al., 2004; Slany, 2009). In line with this, the constitutive recruitment of the chimeric fusion proteins is believed to facilitate sustained expression of a subset of genes normally targeted by wild-type MLL resulting in leukemic transformation (Wang et al., 2011). The oncogenic potential of MLL-fusion proteins is driven, in part, by the selective recruitment of DOT1L and subsequent methylation of H3K79, which leads to the formation of H3K79me2/me3 i.e., histone marks normally associated with activated gene expression (Nguyen and Zhang, 2011). This, in turn, drives the induction of an MLL-fusion–dependent program involving *Hox* genes of which *Hoxa9* and its cofactor *Meis1* have been reported to be

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central to the oncogenic process (Kroon et al., 1998; Zeisig et al., 2003). In contrast to these well-studied transcriptional networks operating downstream of MLL-fusion proteins, we have very little insights into potential pathways or factors that may synergize with the oncogenic driver during the initial phases of leukemic transformation and to what extent tumor initiation and maintenance can be separated.

C/EBPα is a key myeloid transcription factor, which is absolutely required for the formation of granulocytic monocytic progenitors (GMPs) during normal hematopoiesis (Zhang et al., 2004). *CEBPA* is frequently mutated in AML, but surprisingly, none of the observed mutations result in the full ablation of the gene (Nerlov, 2004). This suggests that residual activity of C/EBPα is required for leukemogenesis, and the current notion is that C/EBPα activity is required for AMLs to attain their myeloid identity (Wagner et al., 2006). Thus, in addition to working as a tumor suppressor, as indicated by disabling mutations, C/EBPα appears to be required for the development of at least some AML subtypes suggesting a peculiar dual function for C/EBPα in AML etiology.

In the present work, we identified C/EBPα as a key collaborating factor uniquely required during the initial phases of MLL-ENL–driven leukemic transformation. We show that this requirement is independent of differentiation stage and identify a C/EBPα–dependent, MLL-ENL–driven transcriptional program. Collectively, our data shows that C/EBPα collaborates with MLL-ENL to activate a group of genes that, together with *Hoxa9* and *Meis1*, are responsible for the early events that transform normal hematopoietic cells into malignant cancer cells.

RESULTS AND DISCUSSION

We hypothesized that transcriptional regulators that collaborate with MLL-fusion proteins during leukemic initiation would generate a transcriptional footprint in MLL-rearranged AML. To identify such a footprint we used gene set enrichment analysis (GSEA) to compare the publically available gene expression profiles of AML blasts from patients with MLL-rearrangements to those of normal healthy GMPs. Interestingly, when we scrutinized the data for transcriptional regulators, we found several signatures for C/EBPα target genes to be up-regulated in MLL-rearranged AML (Fig. 1 A), suggesting that C/EBPα is a potential collaborator in MLL fusion–induced malignant transformation.

C/EBPα is required for MLL-ENL–induced malignant transformation

To test if C/EBPα is indeed required for MLL-fusion driven transformation, we first generated *Cebpa*$^{fl/fl}$;*Mx1Cre* mice and used polyinosinic-polycytidylic acid (pIpC) to facilitate ablation of C/EBPα in the hematopoietic compartment (Kühn et al., 1995; Lee et al., 1997). 2 wk after deletion, c-Kit$^+$ hematopoietic stem and progenitor cells (HSPCs) from control (*Cebpa*$^{fl/fl}$) and *Cebpa*-deleted (*Cebpa*$^{\Delta/\Delta}$) animals were transduced with either the empty pMIG retroviral vector or a pMIG-MLL-ENL construct. Transduced cells were subsequently serially replated in methylcellulose medium (Somervaille and Cleary, 2006) or cultured in liquid medium. Interestingly, whereas *Cebpa*$^{fl/fl}$ cells transduced with MLL-ENL had a high replating efficiency, MLL-ENL was unable to provide *Cebpa*$^{\Delta/\Delta}$ cells with an increased colony forming ability compared with *Cebpa*$^{fl/fl}$ or *Cebpa*$^{\Delta/\Delta}$ cells transduced with empty vector (EV; Fig. 1 B). Furthermore, whereas MLL-ENL–transduced *Cebpa*$^{fl/fl}$ cells selectively accumulated in liquid culture, the MLL-ENL fusion protein did not provide the *Cebpa*-deficient cells with any proliferative advantage (Fig. 1 C). Analysis of the *Cebpa*$^{fl/fl}$ and *Cebpa*$^{\Delta/\Delta}$ HSPCs showed that whereas *Cebpa*$^{fl/fl}$ cells lost c-Kit expression and up-regulated Mac-1 expression when transduced with MLL-ENL, the *Cebpa*$^{\Delta/\Delta}$ cells did not alter the expression of these cell surface markers at day 18 after transduction (Fig. 1 D). Collectively, these findings demonstrate that C/EBPα is required for MLL-ENL-induced transformation in vitro.

To test if C/EBPα was also required for MLL-ENL–mediated development of AML in vivo, we next transplanted freshly transduced *Cebpa*$^{fl/fl}$ or *Cebpa*$^{\Delta/\Delta}$ HSPCs into irradiated recipients. As expected, mice reconstituted with *Cebpa*$^{fl/fl}$ cells expressing MLL-ENL developed a lethal form of myeloid leukemia with a median latency of 10 wk, accumulation of GFP$^+$-expressing blast cells in several hematopoietic tissues, pale bones, and splenomegaly (unpublished data). In contrast, MLL-ENL–transduced *Cebpa*$^{\Delta/\Delta}$ HSPCs did not give rise to leukemia or other forms of dysplasia in transplanted mice (Fig. 1 E). Importantly, this was not because of poor reconstitution as we could detect GFP$^+$ *Cebpa*$^{\Delta/\Delta}$ cells in the periphery of transplanted mice at 13 wk post-transplant (Fig. 1 F). Moreover, the requirement of C/EBPα for myeloid transformation was not a general feature as the oncogenic fusion protein E2A-HLF was able to provide *Cebpa*$^{\Delta/\Delta}$ HSPCs with a proliferative advantage (Fig. 1 G). Collectively, these results demonstrate that C/EBPα is required for MLL-ENL dependent transformation both in vitro and in vivo.

C/EBPα is dispensable for leukemic maintenance

The aforementioned data could, in principle, be explained by a requirement for C/EBPα during leukemic initiation, leukemic maintenance, or both. To distinguish between these possibilities, we crossed the conditional *Cebpa* allele into mice expressing a tamoxifen-regulated Cre recombinase estrogen receptor fusion protein (*R26-Cre-ER*; Ventura et al., 2007) and established MLL-ENL–transformed *Cebpa*$^{fl/fl}$;*Cre-ER* cells. We next used 4-hydroxytamoxifen (4-OHT) to delete *Cebpa* and tested the requirement for C/EBPα in sustaining serial replating of the MLL-ENL–expressing cells. Surprisingly, deletion of *Cebpa* in this context did not affect colony numbers, suggesting that the transformed cells grow independently of C/EBPα (Fig. 1 H). To asses this in an in vivo situation, we generated *Cebpa*$^{fl/fl}$;*Mx1Cre* and *Cebpa*$^{fl/fl}$ MLL-ENL expressing primary AMLs, transplanted these into sublethally irradiated secondary recipients, and treated the resulting mice with pIpC 2 wk later. Intriguingly, we found that ablation of *Cebpa* in an already established leukemia affected neither the

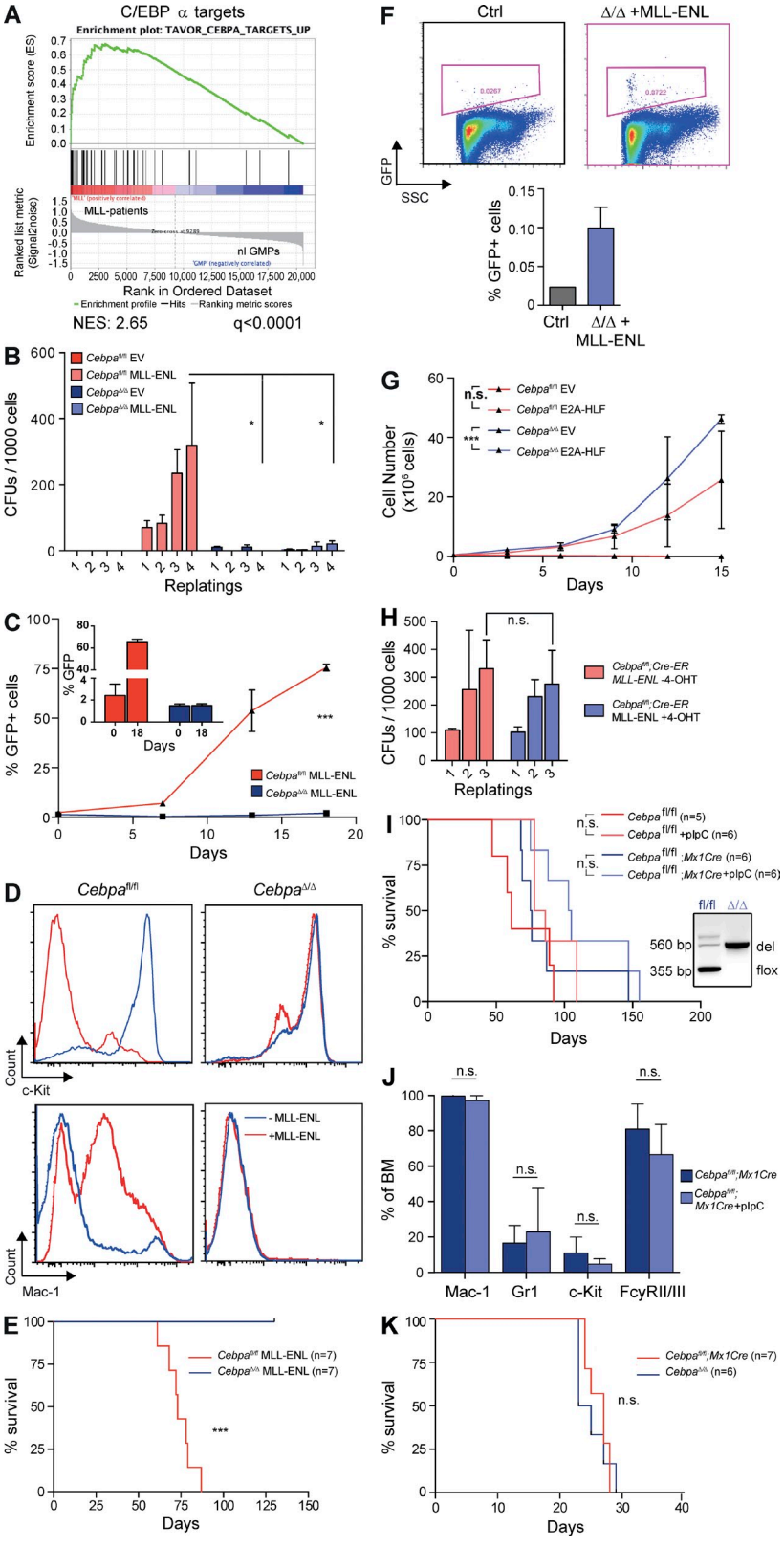

Figure 1. Initiation of MLL-rearranged AML is dependent on C/EBPα activity. (A) GSEA analysis of gene expression profiles from patients with MLL-rearranged leukemias compared with those from GMPs identified a C/EBPα target signature to be up-regulated in the former. (B) Serial replating of *Cebpa*$^{\Delta/\Delta}$ and *Cebpa*$^{fl/fl}$ BM cells transduced with MLL-ENL or EV ($n = 3$). (C) Frequency of GFP$^+$ cells cultured in liquid R20/20 medium. (D) Analysis of cell surface markers in BM cells 18 d after transduction with MLL-ENL. (E) Survival of mice transplanted with MLL-ENL–transduced *Cebpa*$^{\Delta/\Delta}$ ($n = 7$) or *Cebpa*$^{fl/fl}$ ($n = 7$) HSPCs. (F) Analysis of PB 13 wk after transplantation with *Cebpa*$^{\Delta/\Delta}$ -MLL-ENL HSPCs. (G) Growth of *Cebpa*$^{\Delta/\Delta}$ and *Cebpa*$^{fl/fl}$ HSPCs transduced with EV or E2A-HLF ($n = 2$). (H) Serial replating of established MLL-ENL–transformed *Cebpa*$^{fl/fl}$; *Cre-ER* cells ± 4-OHT ($n = 3$). (I) Survival of mice transplanted with an established MLL-ENL AML and injected with pIpC 2 wk after transplantation ($n = 5$–6). Insert: recombination of *Cebpa* in secondary AMLs. (J) Analysis of cell surface markers in secondary *Cebpa*$^{fl/fl}$; *Mx1Cre* AMLs with or without pIpC injections. (K) Survival of mice transplanted with equal doses of AML cells with (*Cebpa*$^{\Delta/\Delta}$; $n = 6$) or without (*Cebpa*$^{fl/fl}$;*Mx1Cre*; $n = 7$) deletion of *Cebpa*. Data are represented as mean ± SD. Statistics were determined by Student's two-tailed *t* test or log-rank test (survival). n.s., not significant; *, $P < 0.05$; ***, $P < 0.001$; $P < 0.0001$. One representative experiment out of one (G and H), two (K), and three (B– F, I, and J) are shown.

overall survival nor the immunophenotype of the leukemic cells (Fig. 1, I and J). Moreover, transplantation of an equal dose of *Cebpa*$^{fl/fl}$;*Mx1Cre* and the *Cebpa*$^{\Delta/\Delta}$ leukemic cells into sublethally irradiated tertiary recipients resulted in the development of AMLs with similar latencies, suggesting that C/EBPα is dispensable for the maintenance of leukemic stem

cells (Fig. 1 K). Collectively, these results therefore demonstrate that C/EBPα is dispensable for both the in vitro and in vivo maintenance of fully transformed MLL-ENL cells and point to a specific role of C/EBPα during the initial transformation process.

MLL-rearranged leukemia is dependent on C/EBPα activity irrespective of differentiation state

C/EBPα drives the transition from pregranulocytic-monocytic progenitors (preGMs) to GMPs and deletion of *Cebpa* in the adult murine hematopoietic system results in a complete differentiation block between these two progenitors. In line with the notion that a differentiation block is an initiating step during leukemogenesis, *CEBPA* is mutated in ∼10% of all cases of AML (Reckzeh and Cammenga, 2010). However, no mutations result in the complete ablation of C/EBPα suggesting that residual C/EBPα activity is required for development of AML and it has therefore been hypothesized that C/EBPα is necessary for the leukemic cells to gain their myeloid identity at the GMP stage (Nerlov, 2004; Wagner et al., 2006). To test if deletion of *Cebpa* at the GMP stage was able to rescue the C/EBPα dependence of MLL-ENL–induced transformation, we FACS sorted *Cebpa*$^{fl/fl}$;*Cre-ER* and *Cebpa*$^{fl/fl}$ GMP cells and cultured them in presence of 4-OHT to facilitate deletion of *Cebpa*. *Cebpa* was fully excised after 2 d of 4-OHT induction (Fig. 2 A) and the cells were further transduced with pMIG-empty or pMIG-MLL-ENL and serially replated in semisolid medium. Cells expressing C/EBPα and MLL-ENL formed increasing numbers of dense colonies and gave rise to leukemia when transplanted into irradiated mice, whereas MLL-ENL–expressing *Cebpa*$^{\Delta/\Delta}$ GMPs were unable to form colonies or give rise to leukemia (Fig. 2, A–D). These results demonstrate that ablation of *Cebpa* downstream of the preGM to GMP differentiation block also abrogates the transformation process, suggesting that it is not the differentiation state, per se, but rather a specific C/EBPα-driven transcriptional program that is required for the initiation of MLL-rearranged leukemia.

C/EBPα regulates a transcriptional program upstream of HOXA9/MEIS1 that is required for MLL-rearranged leukemia

Transformation by MLL-fusion proteins is associated with an up-regulation of the transcription factor *Hoxa9* and its cofactor

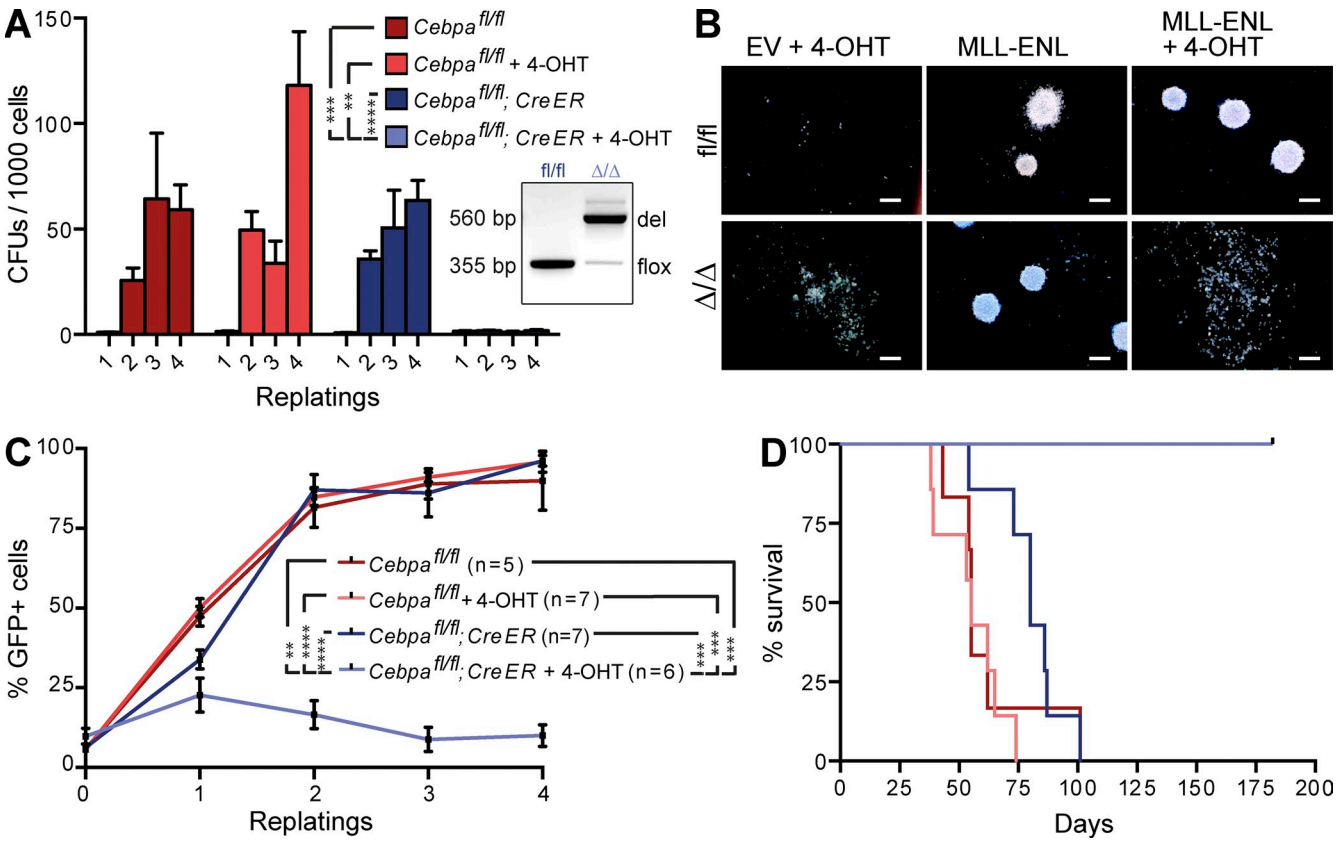

Figure 2. C/EBPα is required for MLL-rearranged leukemia independent of differentiation stage. (A) Serial replating experiment of *Cebpa*$^{fl/fl}$;*Cre-ER* and *Cebpa*$^{fl/fl}$ GMPs ± 4-OHT (n = 3). P-values indicate significance at fourth replating. Recombination of *Cebpa* in *Cebpa*$^{fl/fl}$;*Cre-ER* GMPs 2 d after addition of 4-OHT. (B) Examples of colonies from A. Bars, 200 µm. (C) Frequency of GFP$^+$ cells at each replating. P-values indicate significance at fourth replating. (D) Survival of mice transplanted with MLL-ENL–transduced GMPs from *Cebpa*$^{fl/fl}$;*Cre-ER* and *Cebpa*$^{fl/fl}$ grown in the presence or absence of 4-OHT (n = 5–7). Statistics were determined by Student's two-tailed t test or log-rank test (survival). **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. One representative experiment out of one (D) and two (A– C) are shown.

Meis1, and overexpression of these genes in HSPCs efficiently transforms the cells and gives rise to a myeloid leukemia that mimics MLL-rearranged AML (Kroon et al., 1998; Wilhelm et al., 2011). To test if these two key downstream targets were affected by *Cebpa* deletion, we assessed their expression changes during initial transformation (i.e., 72 h after MLL-ENL–mediated transformation of $Cebpa^{fl/fl}$ and $Cebpa^{\Delta/\Delta}$ preGMs), as well as in established MLL-ENL–transformed cells, or leukemias, after deletion of *Cebpa*. Loss of *Cebpa* leads to a pronounced down-regulation of *Hoxa9* levels in untransduced preGMs, but leaves the expression of *Meis1* unaffected (Fig. 3 A). Moreover, we found that whereas C/EBPα was required for the up-regulation of *Hoxa9* and *Meis1* during initial transformation (Fig. 3 A; P = 0.06 and P = 0.05 for *Meis1* and *Hoxa9*, respectively), it was completely dispensable for the maintenance of the high expression levels of these genes in both transformed cells and established leukemias (Fig. 3, B and C). In addition, to underline the specific importance of C/EBPα during initial transformation, these results raised the possibility that the resistance of *Cebpa*-deficient cells to undergo *MLL-ENL*–mediated transformation might be caused by their failure to up-regulate the expression of *Hoxa9/Meis1*. This, in turn, suggested that this phenotype may be rescued by overexpression of these two genes. To test this, we retrovirally transduced $Cebpa^{fl/fl}$ and $Cebpa^{\Delta/\Delta}$ c-Kit$^+$ HSPCs with Hoxa9-IRES-Meis1-PGK-neo or a control EV selected with G418 for 7 d in semisolid medium and further serially replated cells in methylcellulose medium. In addition, the cells were directly injected into irradiated recipient mice along with support cells. However, whereas $Cebpa^{fl/fl}$ cells expressing *Hoxa9* and *Meis1* were efficiently immortalized in semisolid medium (Fig. 3 D) and gave rise to a myeloid leukemia with a mean latency of 7 wk (Fig. 3 E), the $Cebpa^{\Delta/\Delta}$ cells were unable to form colonies in vitro and failed to give rise to AML in transplanted mice (Fig. 3, D and E). These findings not only demonstrate that C/EBPα acts upstream of HOXA9/MEIS1 but also imply that C/EBPα plays a role in the activation of a larger number of genes that are responsible for the malignant transformation in MLL-rearranged leukemia. Furthermore, because C/EBPα is dispensable for tumor maintenance, our findings

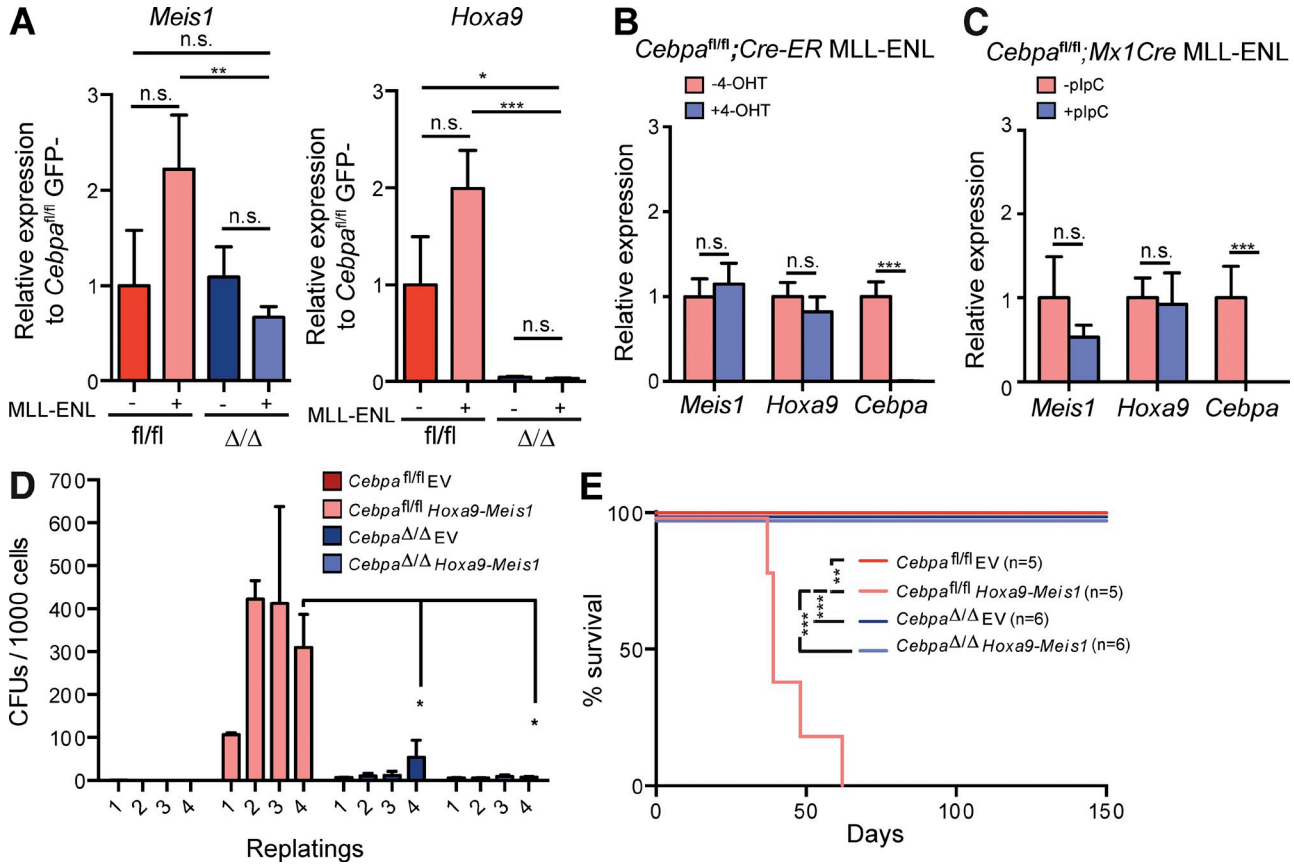

Figure 3. C/EBPα acts upstream of HOXA9/MEIS1 in the transformation process. (A) Gene expression analysis of *Hoxa9* and *Meis1* in $Cebpa^{\Delta/\Delta}$ and $Cebpa^{fl/fl}$ preGMs transduced with EV or MLL-ENL. ($n = 3$). (B) Gene expression analysis of *Hoxa9*, *Meis1*, and *Cebpa* in established MLL-ENL–transformed $Cebpa^{fl/fl}$;*Cre-ER* cells ± 4-OHT. (C) Gene expression analysis of *Hoxa9*, *Meis1*, and *Cebpa* in established $Cebpa^{\Delta/\Delta}$ and $Cebpa^{fl/fl}$;*Mx1Cre* leukemia ($n = 2$–3). (D) Serial replating of $Cebpa^{\Delta/\Delta}$ ($n = 3$) and $Cebpa^{fl/fl}$ ($n = 3$) BM cells transduced with *Hoxa9/Meis1* or EV. Data are represented as mean ± SD. (E) Survival of mice transplanted with *Hoxa9/Meis1*- or EV-transduced $Cebpa^{fl/fl}$ or $Cebpa^{\Delta/\Delta}$ HSPCs ($n = 5$–6). Statistics were determined by Student's two-tailed *t* test or log-rank test (survival). n.s., not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001. One representative experiment out of one (A–C) and two (D and E) are shown.

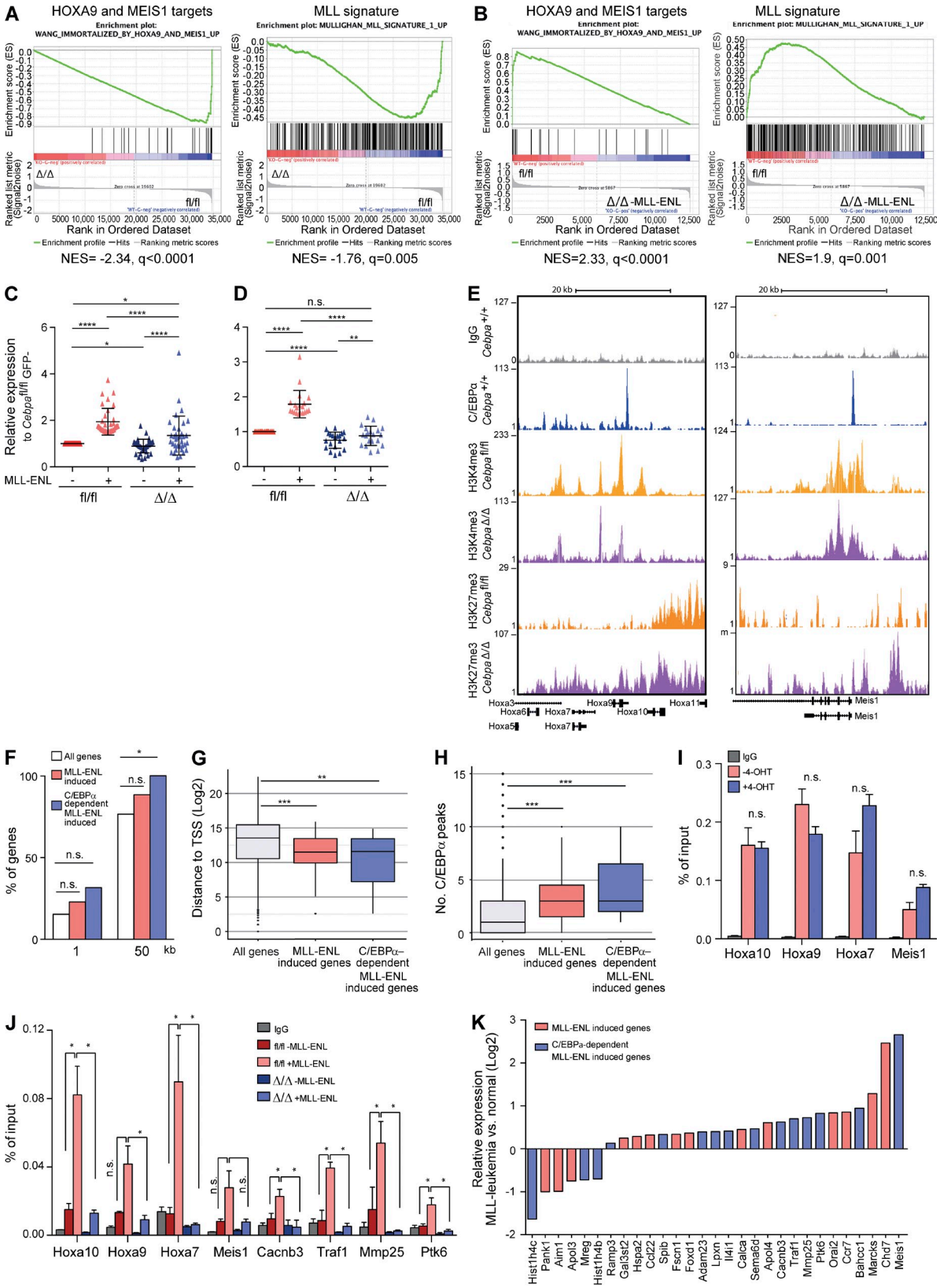

Figure 4. C/EBPα regulates the expression of genes important for initiation of MLL fusion protein leukemias. (A) GSEA analysis of untransduced *Cebpa*$^{\Delta/\Delta}$ versus *Cebpa*$^{fl/fl}$ identified several MLL-signatures down-regulated in untransduced *Cebpa*$^{\Delta/\Delta}$ preGMs ($n = 3$). (B) GSEA analysis of MLL-ENL–transduced *Cebpa*$^{\Delta/\Delta}$ versus untransduced *Cebpa*$^{fl/fl}$ identified several MLL signatures down-regulated in MLL-ENL–transduced *Cebpa*$^{\Delta/\Delta}$ preGMs.

suggest that C/EBPα regulates several genes that are specifically important for initiation of MLL-rearranged leukemia.

To identify such genes, we next performed gene expression analysis of *Cebpa*$^{fl/fl}$ and *Cebpa*$^{\Delta/\Delta}$ preGMs transduced with MLL-ENL for 72 h (Table S1). Using GSEA, we find that not only are several MLL leukemia signatures down-regulated in untransduced (GFP$^-$) *Cebpa*$^{\Delta/\Delta}$ versus *Cebpa*$^{fl/fl}$ preGMs (Fig. 4 A) but also that MLL-ENL (GFP$^+$) fail to induce their expression in *Cebpa*$^{\Delta/\Delta}$ preGMs to levels exceeding those of untransduced *Cebpa*$^{fl/fl}$ preGMs (Fig. 4 B).

Next, we focused our analysis on the 35 genes that were significantly up-regulated (FC > 1.5; P < 0.05) in *Cebpa*$^{fl/fl}$ preGMs as a result of MLL-ENL expression (Fig. 4 C) and, as such, represents genes important for the early MLL-ENL–induced transformation process. Interestingly, 60% (21/35) of these genes were not up-regulated to the same level in *Cebpa*$^{\Delta/\Delta}$-expressing MLL-ENL preGMs (Fig. 4 D and Table S1) and, moreover, 26% (9/35) of the MLL-ENL–induced genes had a significantly lower basal expression in un-transduced *Cebpa*$^{\Delta/\Delta}$ versus *Cebpa*$^{fl/fl}$ preGMs (Table S1). This shows that MLL-ENL is dependent on C/EBPα activity to initiate transcription of genes during early MLL-ENL–induced transformation.

To gain further insights into the role of C/EBPα in this process, we next performed ChIP-seq analysis for C/EBPα binding in WT GMPs as well as for the histone marks H3K4me3 and H3K27me3 in *Cebpa*$^{\Delta/\Delta}$ and *Cebpa*$^{fl/fl}$ preGMs (Fig. 4 E; see Materials and methods). This analysis reveals a strong trend toward more C/EBPα binding in the immediate vicinity of MLL-ENL–induced genes (Fig. 4 F; P = 0.06 for the comparison between the white and blue bars at the 1-kb threshold; Table S2). Moreover, when we compare the distances from the TSS to the nearest C/EBPα peak, we found these to be shorter for MLL-ENL–induced genes and the subset of genes that are dependent on C/EBPα for their expression (Fig. 4 G, see Materials and methods for definitions). In line with this, the MLL-ENL–induced genes have significantly more C/EBPα peaks in close proximity to the TSS (Fig. 4 H). We do see minor differences in the abundance of the active chromatin mark, H3K4me3, and the repressive chromatin mark, H3K27me3, in *Cebpa*$^{fl/fl}$ and *Cebpa*$^{\Delta/\Delta}$ preGM cells at some of the MLL-ENL–induced loci (Fig. 4 E), but this is not a general feature and we do not observe any overall differences between these epigenetic marks on MLL-ENL–induced genes in *Cebpa*$^{fl/fl}$ and *Cebpa*$^{\Delta/\Delta}$ preGM (not depicted). We therefore hypothesized that C/EBPα could be required for the MLL-ENL–dependent recruitment of the methyl transferase DOT1L (Nguyen and Zhang, 2011). This would in turn result in the deposition of the active H3K79me2/me3 mark and in the transcriptional activation of the MLL-ENL–immediate genes. To test this, we performed H3K79me2 ChIP in *Cebpa*$^{fl/fl}$ and *Cebpa*$^{\Delta/\Delta}$ preGMs ± MLL-ENL, as well as in established MLL-ENL–expressing *Cebpa*$^{fl/fl}$;*Cre-ER* cells. Intriguingly, we find that whereas the high levels of the H3K79me2 mark at key *Hox* genes were unaffected by the C/EBPα status in established leukemic cells (Fig. 4 I), the deposition of H3K79me2 at key MLL-ENL–immediate genes during initial transformation was strongly dependent on C/EBPα (Fig. 4 J; P < 0.05 for all *Cebpa*$^{fl/fl}$ vs. *Cebpa*$^{fl/fl}$ + MLL-ENL comparisons except for *Hoxa9* [P = 0.05] and *Meis1* [P = 0.05]). These observations are consistent with C/EBPα being involved in the recruitment of MLL-ENL/DOT1L during the initial phases of leukemic transformation.

On a final note, we examined whether the aforementioned MLL-ENL–induced genes may also play a role in patients with MLL-rearranged leukemia. Here, we found that the majority of these genes displayed higher expression in leukemic cells compared with GMPs (Fig. 4 K), which suggests that the transcriptional network we have identified is highly relevant for patients with MLL-fusion protein leukemia and that C/EBPα plays a similar role in a human setting.

Collectively, we have shown that C/EBPα collaborate with MLL-ENL to activate a group of genes that, together with *Hoxa9* and *Meis1*, are responsible for the transcriptional changes that underlie the transformation of normal hematopoietic cells into leukemic cells. We have demonstrated a critical role for C/EBPα in MLL-ENL–dependent transformation but not in the maintenance of established MLL-ENL–driven tumors. We could show that this was independent of the C/EBPα-mediated differentiation block upstream of GMPs, but rather depended on the ability of C/EBPα to collaborate with MLL-ENL in the induction of a transcriptional transformation program that includes *Hoxa9* and *Meis1*. Thus, our findings place C/EBPα upstream of the MLL-ENL fusion protein and suggest that C/EBPα facilitates the binding of MLL-ENL/DOT1L, and the subsequent deposition of H3K79me2 at MLL-ENL target genes, during the initial phases of leukemic transformation. This promotes the formation of a stable transcriptional network, which depends on the sustained expression of MEIS1 and HOXA9, but is independent of C/EBPα activity.

Importantly, our findings also have implications for human AML. Thus, we find MLL-rearranged AMLs to be correlated

(C) Gene expression analysis of genes up-regulated (Fold change >1.5; P < 0.05) in *Cebpa*$^{fl/fl}$ preGMs 72 h after transduction with MLL-ENL. (D) Analysis of C/EBPα-dependent MLL-ENL–induced genes. (E) Selected examples of ChIP-seq coverage of C/EBPα (GMPs), as well as H3K4me3 and H3K27me3 (both preGMs). Shown are sequencing tracks for one of two replicates for each sample. (F) Percentage of genes with a C/EBPα peak within 1 or 50 kb from TSS. (G) Distances from the TSS of either all genes in the genome, MLL-ENL–induced genes, or the C/EBPα-dependent MLL-ENL–induced genes to the nearest C/EBPα peaks. (H) Number of C/EBPα peaks within 50 kb from TSS. (I) H3K79me2 ChIP in established MLL-ENL transformed *Cebpa*$^{fl/fl}$;*Cre-ER* ± 4-OHT (n = 3; mean ± SEM) (J) H3K79me2 ChIP in *Cebpa*$^{\Delta/\Delta}$ and *Cebpa*$^{fl/fl}$ preGMs 72 h after transduction with MLL-ENL (n = 3; mean ± SEM). (K) Median gene expression of MLL-ENL–induced genes in MLL-rearranged leukemic patients (n = 45) compared with normal healthy GMPs (n = 4). Statistics were determined by Wilcoxon signed-rank test (C and D), Fisher's exact test (F), Wilcoxon rank-sum test (G and H), and Student's two-tailed t test (I and J). n.s., not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

both with a general C/EBPα transcriptional signature, as well as with the immediate C/EBPα-dependent MLL-ENL transformation signature identified in the present work. Our work provides a mechanistic explanation for the lack of *CEBPA*-null mutations in human AML despite its established role as a myeloid tumor suppressor. Thus, C/EBPα impacts in a dichotomous manner on the development and maintenance of human AML.

MATERIALS AND METHODS

Mouse colony and transplantation experiments

Animals were maintained at the Department of Experimental Medicine at University of Copenhagen and housed according to institutional guidelines. All animal work was performed under the approval of Danish Animal Ethical Committee. All mouse lines (*Cebpa*^{fl}, *Mx1-Cre*, and *R26-Cre-ER*) were backcrossed for at least eight generations onto the C57BL/6 background. Excision of the *Cebpa* allele was achieved by subjecting 10–12-wk-old *Cebpa*$^{fl/fl}$ or *Cebpa*$^{fl/fl}$;*Mx1Cre* mice to 3 injections with 300 µg pIpC, as described previously (Weischenfeldt et al., 2008). Excision of *Cebpa* was evaluated by competitive PCR using the primers 5′-GTCCTGCAGCCAGGCAGTGTCCCCACTCACCGCCTTGGAAAGTCACA-3′ and 5′-CCGCGGCTCCACCTCGTAGAAGTCG-3′, which give rise to 355-bp and 560-bp products for the floxed and deleted allele, respectively.

All transplantation assays were performed using the Ly-5 congenic mouse system. Generally, 10,000 GFP-expressing *Cebpa*$^{fl/fl}$ or *Cebpa*$^{\Delta/\Delta}$ Ly-5.2 (CD45.2) HSPCs or primary leukemic *Cebpa*$^{fl/fl}$;*Mx1Cre* or *Cebpa*$^{fl/fl}$ cells were transplanted by tail vein injection into 10–12-wk-old lethally (900 cGy) or sublethally (500 cGy) irradiated Ly-5.1 (CD45.1) mice. 2.5×10^5 Ly-5.1 whole BM cells/mouse were given as support to lethally irradiated mice. *Cebpa* excision in leukemic cells was induced by 3 injections of pIpC at 2 wk after transplantation. BMs from leukemic animals were analyzed at the experimental endpoint. Log-rank test was used to evaluate significance of the survival differences.

Retroviral transduction and culture of HSPCs

The MLL-ENL-IRES-GFP, Hoxa9-IRES-Meis1-neo, and E2A-HLF-neo retroviral constructs were provided by D. Bryder (Lund University, Lund, Sweden), G. Sauvageau (University of Montreal, Montreal, Canada), and R. Slany (University of Erlangen-Nuremberg, Erlangen, Germany), respectively. Viral supernatants were produced by transfection of Phoenix-Eco cells. BM cells were enriched for c-Kit-expression using CD117 MicroBeads according to the manual MACS Cell Separation system (Miltenyi Biotec). c-Kit$^+$ HSPCs were prestimulated in RPMI 1640 with 20% fetal calf serum, 20% WEHI-conditioned medium, 20 ng/ml SCF (PeproTech), and 10 ng/ml IL-6 (R20/20; PeproTech). Cells were transduced during two consecutive days by incubating retronectin (Takara Bio)-coated wells with viral supernatant for 2 h (3 times, 40 min) and followed by seeding of 5×10^5 cells/ml in R20/20 medium supplemented with 4 µg/ml protamine sulfate. Transduced cells were plated in methylcellulose medium M3231 (Stem Cell Technologies) supplemented with 1% penicillin-streptomycin (PAA Laboratories), 20 ng/ml SCF (PeproTech), 10 ng/ml human IL-6 (PeproTech), 10 ng/ml GM-CSF (PeproTech), and 10 ng/ml IL-3 (PeproTech). Cells expressing the Hoxa9-IRES-Meis1-PGK-neo construct were selected in 750 µg/ml G418 for 7 d. Colonies were counted every seventh day, and GFP expression was quantified by flow cytometry. Student's two-tailed *t* test was used to test for significance.

Cebpa$^{fl/fl}$;*Cre-ER* c-Kit$^+$ HSPCs were transduced with MLL-ENL-IRES-GFP, plated in methylcellulose medium (M3231, see above), and replated three times. *Cebpa* excision was induced in the fully transformed cells by supplementing with 0.1 µM 4-OHT to the methylcellulose medium.

Cebpa$^{fl/fl}$;*Cre-ER* and *Cebpa*$^{fl/fl}$ GMP (Lin$^-$ Sca1$^-$ Kit$^+$ CD150$^-$ CD41$^-$ FcγRII/III$^+$) were sorted by FACS and prestimulated in R20/20 medium (Somervaille and Cleary, 2006) supplemented with 1 µM 4-OHT. *Cebpa* was fully excised after 2 d of 4-OHT treatment, and cells were further transduced with pMIG-empty or pMIG-MLL-ENL, serially replated in semisolid medium, and harvested after three passages.

Flow cytometry

BM and peripheral blood were stained with antibodies and run on a FACSCalibur (BD), LSRII (BD), or FACSAria (BD) and analyzed using the FlowJo software. For analyzing and sorting of hematopoietic progenitors B220, CD3, CD11b, Gr1, Ter119, CD105, FcgRII/III, CD41, Sca-1, c-Kit (eBioscience), and CD150 (BioLegend) were used as described previously (Hasemann et al., 2012). Student's two-tailed *t* test was used to test for significance.

Expression analysis

Hoxa9, *Meis1*, and *Cebpa* expression was quantified relative to β-actin in sorted GFP$^+$ *Cebpa*$^{fl/fl}$ or *Cebpa*$^{\Delta/\Delta}$ preGM cells transduced with empty vector or MLL-ENL. Each sample was analyzed in triplicates on the same 96-well plate using the LightCycler 480 (Roche). Expression levels were determined by SYBR Green (Roche; Table S3).

Gene expression profiling

Murine data. c-Kit$^+$ *Cebpa*$^{fl/fl}$ or *Cebpa*$^{\Delta/\Delta}$ cells were transduced over two consecutive days, and GFP$^+$ and GFP-preGM cells (Lin$^-$ Sca1$^-$ Kit$^+$ CD150$^-$ CD41$^-$ FcgRII/III$^-$ CD105$^-$) were sorted 72 h after the first transduction. Total RNA was subsequently purified, amplified using the Ovation Pico WTA system (NuGen), labeled, and hybridized to the Mouse Gene 1.0 ST GeneChip Array (Affymetrix). After RMA normalization, the four sample types were subjected to Limma analysis. For each pairwise phenotype comparison, genes were selected using the following criteria: $\log_2$ fold change > 0.58; $P < 0.05$, moderated t-statistics, corrected for multiple testing (Bonferroni), Limma package (Smyth, 2004). We defined MLL-ENL–induced genes based on differences between the GFP$^+$ and GFP$^-$ *Cebpa*$^{fl/fl}$ samples. For the C/EBPα-dependent subset, we also required these to be significantly down-regulated ($P < 0.05$) in the GFP$^+$ *Cebpa*$^{\Delta/\Delta}$ vs. GFP$^+$ *Cebpa*$^{fl/fl}$ samples. Raw gene expression data are available at the Gene Expression Omnibus online database under accession no. GSE46534.

Patient data. Microarray dataset from patients with AML with t(11q23)/MLL were downloaded from GEO (accession nos. GSE13159 and GSE14468; Wouters et al., 2009; Haferlach et al., 2010). Microarrays from normal healthy human GMPs were downloaded from GEO accession no. GSE24006 (Gentles et al., 2010). Datasets were normalized using RMA (Irizarry et al., 2009). The median gene expression for the MLL-ENL–induced genes in cells from leukemic patients was compared with median gene expression in healthy normal GMPs.

We used GSEA (Subramanian et al., 2005) to identify gene signatures that were altered in murine GFP$^-$ *Cebpa*$^{\Delta/\Delta}$ preGMs versus GFP$^-$ *Cebpa*$^{fl/fl}$ preGMs, MLL-ENL–transduced GFP$^+$ *Cebpa*$^{\Delta/\Delta}$ preGMs versus GFP$^-$ *Cebpa*$^{fl/fl}$ preGMs, and human AML-MLL phenotype versus normal healthy GMPs. Gene sets originated from the MSigDB (www.broadinstitute.org/gsea/msigdb).

ChIP analysis

ChIP-seq was performed in replicates using BM cells from *Cebpa*$^{fl/fl}$ or *Cebpa*$^{\Delta/\Delta}$ mice, and ChIP-qPCR was performed in triplicate in sorted preGM cells from *Cebpa*$^{fl/fl}$ or *Cebpa*$^{\Delta/\Delta}$ ± MLL-ENL. Chromatin from 100,000 preGMs or 500,000 GMPs was incubated with antibodies for histone marks (H3K79me2,ab3594 [Abcam]; H3K4me3, C42D8 [Cell Signaling Technology]; and H3K27me3, C36B11 [Cell Signaling Technology]) or C/EBPα (14AA ; Santa Cruz Biotechnology, Inc.), respectively. The antibody-bound chromatin was captured with Protein A–Sepharose beads, washed, de-cross-linked, and precipitated. Precipitated DNA was quantified with qPCR (see primers in Table S3) or mixed with 2 ng *Escherichia Coli* DNA and amplified using NEB Next ChIP-seq sample prep reagent set 1 (New England Biolabs) according to the manufacturer's protocol. Libraries were sequenced on an Illumina Hiseq2000. Data were deposited in the GEO under accession no. GSE47003.

Bioinformatic analyses

All reads were mapped using bowtie 0.12.7 (Langmead et al., 2009) using standard parameters. The C/EBPα, H3K4me3, and H3K27me3 ChIP-seqs

were performed as biological replicates, and the correlation coefficients, r^2, were calculated to the following: *Cebpa*$^{fl/fl}$ H3K27me3, 0.938; *Cebpa*$^{\Delta/\Delta}$ H3K27me3, 0.901; *Cebpa*$^{fl/fl}$ H3K4me3, 0.896; *Cebpa*$^{\Delta/\Delta}$ H3K4me3, 0.922; and *Cebpa*$^{+/+}$ C/EBPα, 0.805. Replicate C/EBPα peaks were called using macs2 with the following settings: macs2 callpeak -t GMP_peak_file.tagAlign -c IgG.tagAlign -f BED -g mm -p 1e-3–to-large. Overlapping peaks ($P < 10^{-14}$ in both peak callings) were further filtered based on peak height and regions with high enrichment in the IgG samples removed. Genes were defined using the mm9 RefSeq set, taking the longest supported isoform for genes with multiple isoforms. Distances were calculated from TSS to the summit of the closest C/EBPα peaks. P-values were calculated using the Wilcoxon rank sum test.

Online supplemental material. Table S1 shows gene expression of GFP$^+$ and GFP$^-$ *Cebpa*$^{\Delta/\Delta}$ and *Cebpa*$^{fl/fl}$ preGM cells transduced for 72 h with MLL-ENL–IRES-GFP. Table S2 shows the distance to closest C/EBPa peak of MLL-ENL immediate genes. Table S3 shows the primers used for qPCR. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20130932/DC1.

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