Supporting Information

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SI Materials and Methods

Animals. All animal experiments were performed as approved by the University of Michigan Committee on the Use and Care of Animals and Unit for Laboratory Animal Medicine. *Cebpa*$^{f/f}$ mice (1, 2) (kindly provided by Daniel Tenen, Harvard University, Cambridge, MA) or C57BL/6 *WT* mice (JAX no. 000664; The Jackson Laboratory) were crossed with B6;129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT)Nat}$ mice (JAX no. 004847; The Jackson Laboratory) to obtain *Cebpa*$^{f/f}$;*CreERT*$^{+/-}$ and *WT;CreERT*$^{+/-}$ strains.

Antibodies. For Western blot analysis, anti-CCAAT/enhancer binding protein alpha (C/EBPα) (2295S; Cell Signaling Technology) and anti–β-actin (A2228; Sigma) were used. For ChIP, anti-HA (ab9110; Abcam), anti-C/EBPα (sc-61X; Santa Cruz Biotechnology), anti-H3K4me1 (ab8895; Abcam), anti-H3K27me3 (07-449; Millipore), and IgG (sc-2027; Santa Cruz Biotechnology) were used. For flow cytometry, allopycocyanin (APC)–anti-cKit (105812; Biolegend), APC–anti-CD11b (47-0112-80; eBioscience), APC–anti-Gr1 (108412; Biolegend), APC–anti-F4/80 (123115; Biolegend), APC–anti-Ly6C (128015; Biolegend), APC–anti-AnnexinV (88-8007-74; eBioscience), and DAPI (Sigma) were used.

Cell Lines. Bone marrow from 6- to 10-wk-old *Cebpa*$^{f/f}$;*CreERT*$^{+/-}$, *WT;CreERT*$^{+/-}$, or *WT* mice was harvested 5 d after treatment with 5-flurouracil (150 mg/kg) and Lin^{-}cKit^{+} cells were isolated using the EasySep Mouse Hematopoietic Progenitor Cell Enrichment Kit (Stem Cell Technologies). Cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 15% FBS, 10 ng/mL IL-3, and 100 ng/mL stem cell factor (SCF). One day after harvest, cells were retrovirally transduced on two consecutive days with MIGR1-HA-Hoxa9 or MIGR1-HA-Hoxa9-estrogen receptor tag (ER) and with MIGR1-Flag-Meis1 retrovirus expressing murine proteins [plasmids previously described by Huang et al. (3)]. For cyclin-dependent kinase inhibitors CDKN2A/B overexpression studies, human CDKN2A or CDKN2B was cloned from pCMV6 entry vectors (RC211784 and RC204895; Origene) using FseI (blunted) and EcoR1 excision ligated with MSCVneo linearized with EcoR1/Hpa1 to generate MSCVneo-Myc-Flag-CDKN2A and MSCVneo-Myc-Flag-CDKN2B. Retroviral supernatant was generated by transfecting PlatE packaging cells with the appropriate plasmids using Fugene 6 (Promega). Stable cell lines were established by gradually withdrawing SCF from the cells over the course of 10 d, with additional antibiotic selection with 500 μg/mL G418/Geneticin (Invitrogen) in CDKN2A/B overexpression studies. One hundred percent GFP positivity was subsequently verified using flow cytometry, and coexpression of Hoxa9 and Meis1 was confirmed by Western blot analysis. Cell lines transduced with MIGR1-HA-Hoxa9-ER/MIGR1-Flag-Meis1 (HerM) were cultured in continuous 100 nM 4-hydroxytamoxifen (4-OHT) to maintain transformation.

Cellular Assays. For loss of C/EBPα studies, Hoxa9/Meis1-transformed *Cebpa*$^{f/f}$;*CreERT*$^{+/-}$ (C/EBPα HM) cells or Hoxa9/Meis1-transformed *WT;CreERT*$^{+/-}$ (WT HM) cells were treated continuously with ethanol (EtOH) or 5 nM 4OHT (H7904; Sigma). For the 4OHT withdrawal experiment, after 1 wk of continuous culturing in EtOH or 5 nM 4OHT, C/EBPα HM cells were washed once with culture media and continued in culture with EtOH only. For loss of Hoxa9 studies, HerM cells were washed three times with culture media and then maintained in either EtOH or 100 nM 4OHT. For CDKN2A/B overexpression studies, 2 d after spinoculation, cells were treated with 500 μg/mL G418 for 3 d and subsequently seeded (with continuous 500 μg/mL G418 treatment) at equal cell numbers to assess cellular proliferation. Cellular proliferation was assessed by trypan blue dye exclusion and cell counting (all experiments performed in at least biological replicates). Cellular morphology was assessed using cytospin and heme staining. Whole-cell lysates were collected by directly lysing washed cells in SDS loading buffer plus β-mercaptoethanol. Protein levels were visualized using SDS/PAGE and Western blotting on PVDF membranes. RNA was collected and purified using a Qiagen RNeasy Kit with on-column DNase treatment. cDNA was generated using SuperScript II RT (Invitrogen), and target gene expression was determined relative to B-actin using Invitrogen TaqMan primer-probe sets [*Cdkn2a* (Mm00494449_m1), *Cdkn2b* (Mm00483241_m1), *Cebpa* (Mm00514283_s1), and *Bactin* (Mm00607939_s1)].

Flow Cytometry. For surface marker expression, cells were washed and resuspended in recommended media (2% FBS in PBS) and then incubated for 30 min on ice with 0.2 μg of the appropriate antibody. For apoptosis assays, cells were washed and resuspended in binding buffer, and subsequently incubated at room temperature for 15 min with 0.6 μg APC–anti-AnnexinV and DAPI. For cell cycle analysis, cells were washed, resuspended in ice-cold Dulbecco's phosphate buffered saline (DPBS), and added drop-wise to cold 70% EtOH. Cells were stored for at least 24 h at −20 °C. After storage, cells were washed with cold DPBS, rehydrated for 30 min on ice in DPBS, and subsequently treated with RNase A (19101; Qiagen) and DAPI at room temperature for 20 min. All samples were collected on a Becton Dickinson LSR II. Data collected from at least 20,000 events from biological replicate experiments were analyzed using FlowJo (TreeStar).

Bone Marrow Transplantation. For primary leukemia assays, freshly transduced C/EBPα HM and WT HM cells (described above) were injected by tail vein in cohorts of lethally irradiated (900 rad) ∼8-wk-old female C57BL/6 mice (1.5×10^5 cells per mouse). Mice were maintained on antibiotics for 2 wk post-irradiation. At 2 wk, mice were treated with biweekly i.p. injections of tamoxifen (OHT; 200 mg/kg, T5648; Sigma) or corn oil until death. For secondary leukemia assays, spleen cells harvested from primary leukemic mice in the C/EBPα HM corn oil-treated cohort were injected by tail vein in cohorts of sub-lethally irradiated (600 rad) ∼8-wk-old female C57BL/6 mice (1.5×10^5 cells per mouse). After 5 d, mice were treated for 5 consecutive days with i.p. injections of OHT (200 mg/kg) or corn oil and continued on twice-weekly injections until death. Mice were killed after becoming moribund. Liver, spleen, and bone were harvested from control and leukemic mice at the time of death for paraffin embedding and H&E staining. Bone marrow was flushed for collecting RNA, whole cell lysate, and cytospin samples. Survival curves were plotted in Prism (GraphPad), and statistical significance was evaluated by log rank test.

SI Gene Expression Analysis in Patient Samples

Analysis of *CEBPA* and *HOXA9* expression in patient samples was performed on data previously described and published by Figueroa et al. (4) and Verhaak et al. (5). Briefly, gene expression microarrays on Affymetrix Human Genome 133 Plus2.0 GeneChips were generated for 344 acute myeloid leukemia cases

collected at Erasmus University Medical Center (Rotterdam, The Netherlands) between 1990 and 2008 (Gene Expression Omnibus accession no. GSE6891). Raw data were processed using the GC-RMA package (version 2.16.0) from BioConductor. Statistical analysis was performed using R 2.8.1 and BioConductor. All comparisons were done with a two-sample *t* test using the R Stats Package.

ChIP. A total of $30 \times 10^{\wedge 6}$ cells were fixed for 15 min at room temperature with 1% paraformaldehyde in IMDM, washed two times with cold PBS, and snap-frozen on dry ice. Cells were then lysed in 1.5 mL of SDS lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris·HCl (pH 8)], sheared two times through a 27-gauge needle, and sonicated to achieve a majority of DNA fragment distribution below 500 bp. Samples were centrifuged for 20 min at maximum speed to remove debris, and supernatant was collected and diluted at a 1:10 ratio with dilution buffer [0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris·HCl (pH 7.5), 167 mM NaCl]. Diluted chromatin (1.5 mL) was incubated with 2.5 μg of appropriate antibody overnight at 4 °C with rotation. Immunoprecipitation was then performed by adding 30 μL of BSA-blocked protein G Dynabeads (Invitrogen) to each sample for 1 h at 4 °C with rotation. Immunoprecipitates were washed for 5 min in low-salt (150 mM), high-salt (500 mM), and lithium chloride (0.25 M) buffers, and twice with Tris/EDTA buffer. Captured chromatin was eluted by incubating beads in 250 μL of elution buffer (1% SDS, 100 mM $NaHCO_3$) for 30 min at 42 °C. Cross-linking was reversed by the addition of NaCl (final 50 μM) and overnight incubation at 65 °C. Chromatin was then RNase A-treated and purified using a Qiagen PCR purification kit. Binding was quantified relative to input by quantitative PCR (7500 PCR System; Applied Biosystems) using SYBR green fluorescent labeling and primers designed using the Integrated DNA Technologies PrimerQuest program (genes coactivated by Hoxa9 and C/EBPα are listed in a display table in the main text).

ChIP-Sequencing and RNA-Sequencing. For ChIP-sequencing (ChIP-seq) analysis, 10 ng of immunoprecipitated DNA was processed for library generation using a ChIP-seq Library Preparation Kit (Illumina) following the manufacturer's protocol. For RNA-sequencing (RNA-seq), RNA was extracted using a Qiagen RNeasy kit with on-column DNase treatment following the protocol described by the manufacturer. Biological replicate cDNA libraries were generated using a TruSeq RNA Sample Preparation Kit (Illumina). For both ChIP-seq and RNA-seq, sequencing was performed on an Illumina HiSeq2000 at University of Michigan DNA sequencing core, and raw RNA-seq data were processed using the Illumina software pipeline.

Peak Calling. Sequenced reads were preprocessed to remove contamination of adaptor sequences and then aligned to mouse reference genome (mm9) using BWA software (version 0.6.2). Model-based Analysis for ChIP-seq (model-based analysis of ChIP-seq) was used for peak calling with the following parameters (default values were used if not specified otherwise): format = BED – g·mm–nomodel–shiftsize 75 –w –S (generate single wig file profile). Peak tracks were displayed in USCS Genome Browser (University of California, Santa Cruz). Distribution of peaks in the promoter (−1 kb to +100 bp of TSS), exon, intron, and intergenic regions, for example, was estimated using HOMER software (6). Peaks were annotated to their nearest gene using CisGenome software. Peak overlap was calculated with the criterion that there is at least a 1-bp overlap between tested peaks. The significance of peak overlap was calculated using a hypergeometric test with the background (total number of tests) set to 159,029, as an estimation of total transcription factor binding sites obtained from K526 leukemia cells (7). Pathway analysis was performed using the Genomic Regions Enrichment of Annotations Tool Web tool based on a binomial test P value <0.05. A Venn diagram was made using the R program.

Differential Gene Analysis. Sequenced reads were aligned to mouse reference genome (mm9) using Bowtie and Tophat (version 2.0.3) software. The software program Cuffdiff was used for differential gene expression analysis.

1. Ye M, et al. (2013) C/EBPa controls acquisition and maintenance of adult haematopoietic stem cell quiescence. *Nat Cell Biol* 15(4):385–394.
2. Zhang DE, et al. (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(2):569–574.
3. Huang Y, et al. (2012) Identification and characterization of Hoxa9 binding sites in hematopoietic cells. *Blood* 119(2):388–398.
4. Figueroa ME, et al. (2010) DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. *Cancer Cell* 17(1):13–27.
5. Verhaak RG, et al. (2009) Prediction of molecular subtypes in acute myeloid leukemia based on gene expression profiling. *Haematologica* 94(1):131–134.
6. Heinz S, et al. (2010) Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell* 38(4):576–589.
7. Cui L, et al. (2013) [Expression of long non-coding RNA HOTAIR mRNA in ovarian cancer]. *Sichuan Da Xue Xue Bao Yi Xue Ban* 44(1):57–59, Chinese.

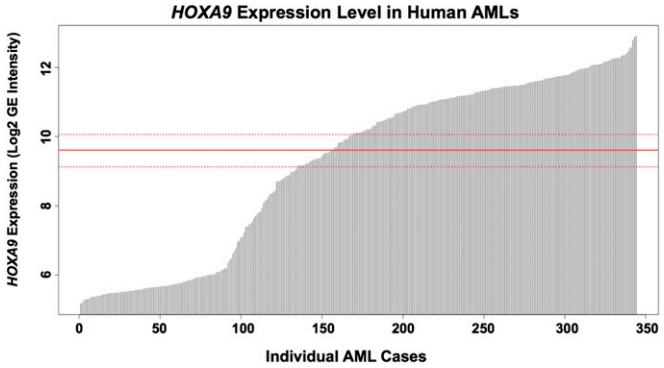

Fig. S1. HOXA9 expression in acute myeloid leukemias (AMLs) compared with healthy controls. In a cohort of 344 human AMLs, 50.87% (n = 175) have HOXA9 expression levels 2 SDs higher than healthy controls (n = 11) (mean = 9.6, SD = 0.23, mean + 2 SD = 10.06). A plot of individual patient sample HOXA9 expression levels with mean of healthy controls (solid line) and ±2 SD (dotted line) is shown. Data from studies by Figueroa et al. (4) and Verhaak et al. (5) were reanalyzed. GE, gene expression.

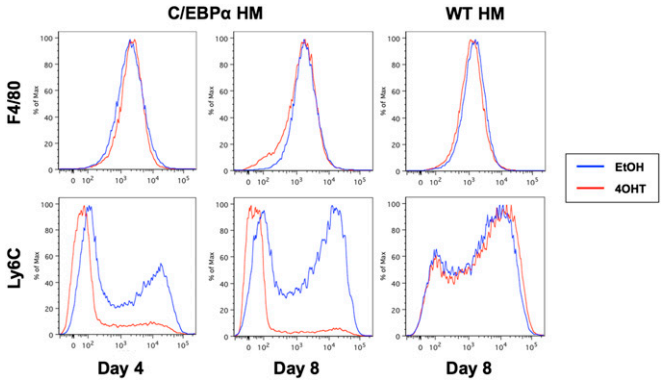

Fig. S2. Surface marker changes after loss of C/EBPα. C/EBPα HM and WT HM cells were treated for an 8-d time course with 5 nM 4OHT (red) or EtOH (blue), and surface expression of F4/80 and Ly6C at days 4 and 8 in C/EBPα HM cells (*Left* and *Center*) and WT HM cells (*Right*) was assessed using flow cytometry. Data are representative of at least two independent experiments.

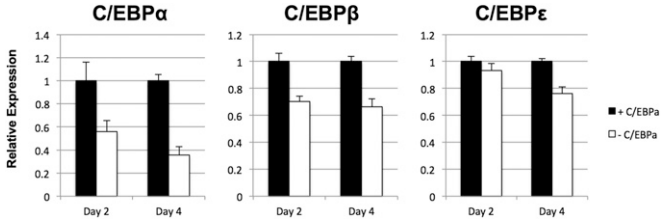

Fig. S3. Change in C/EBP isoform expression after loss of C/EBPα. Expression of C/EBPα, C/EBPβ, and C/EBPε was determined by RT-PCR of cDNA generated from C/EBPα HM cells after 2 and 4 d of treatment with 4OHT or EtOH. Expression was normalized to β-actin and calculated relative to the EtOH-treated sample at each time point. Bars indicate mean ± SD of three independent experiments. C/EBPa, C/EBPα.

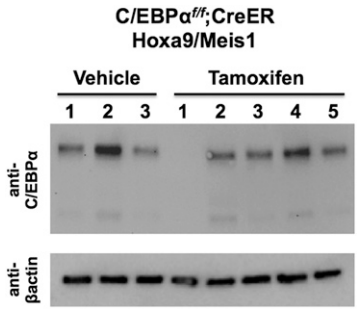

Fig. S4. C/EBPα protein level in primary murine Hoxa9/Meis1 leukemias. C/EBPα protein level was determined in whole-cell lysates of bone marrow from leukemic mice transplanted with C/EBPα HM cells treated with vehicle or tamoxifen. Tamoxifen-treated mouse 1 succumbed to leukemia before day 40 and shows complete loss of C/EBPα, whereas tamoxifen-treated mice 2–5 succumbing to leukemia later than day 50 show escape of Cre-mediated deletion of C/EBPα.

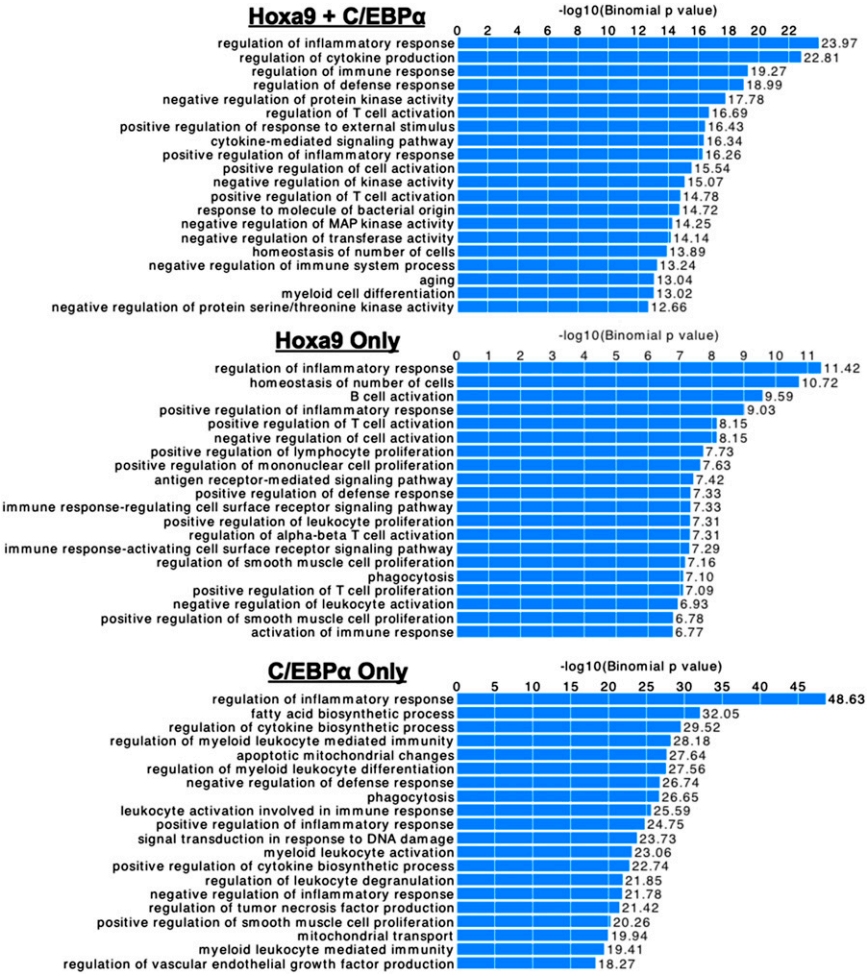

Fig. S5. Genomic Regions Enrichment of Annotations Tool (GREAT) pathway analysis of Hoxa9–C/EBPα-cobound regions. Pathway analysis of regions cobound by Hoxa9 and C/EBPα (*Top*), regions bound only by Hoxa9 (*Middle*), or regions bound only by C/EBPα (*Bottom*) was performed. Analysis was performed using the GREAT against the biological pathways database. The *x* axis indicates the *P* value (−10 * log).

Table S1. Genes coactivated by Hoxa9 and C/EBPα

Genes coactivated by Hoxa9 and C/EBPα			
Adam17	Cpe	Itsn1	Pde7a
Adra2a	D1Ertd622e	Kcnq3	Serpine2
Adrb2	Ern1	Lbp	Sorcs2
Aldh1a3	Galnt3	Nrg2	Tgm3
Appl2	Gcnt4	P2rx3	Tpst1
Bcas1	Gm1110	Pcp4l1	Tulp3
Ccdc85a	Igf2r	Pcsk9	Vit
Clca3	Il2ra	Pdcd4	

RNAseq was performed 72 h after loss of either Hoxa9 (in HerM cell line) or C/EBPα (in C/EBPα HM cell line). Genes that decreased expression more than 1.5-fold in both the Hoxa9 and the C/EBPα knockdown experiments are listed.

Table S2. Genes corepressed by Hoxa9 and C/EBPα

Genes corepressed by Hoxa9 and C/EBPα				
Aim1	Eya2	Hgfac	Mycn	Ralgps2
Alox5	Fyb	Il1rl1	Nat6	Rgs10
Cd74	Gata2	Il6	Nek6	Rnf144a
Cdh1	Gch1	Inpp4b	Nkg7	Scin
Cdh17	Gcnt2	Irs2	Peg13	Sema7a
Cdkn2b	Gfi1b	Itpr2	Plxdc2	Siglec5
Col18a1	Gzmb	Kif17	Prkcq	Stx3
Cpa3	Havcr2	Lat2	Ptms	Trp53inp1
Dock10	Hemgn	Mrc1	Ptprg	Txk

RNAseq was performed 72 h after loss of either Hoxa9 (in HerM cell line) or C/EBPα (in C/EBPα HM cell line). Genes that increased expression more than 1.5-fold in both the Hoxa9 and the C/EBPα knockdown experiments are listed.

Table S3. Primers used for ChIP-quantitative PCR experiments

Primer	Sequence
Btla Fwd	GTGGTTGGAGCTATCACAGAATA
Btla Rev	CAAGCCCACTCCTAGAGAAATC
Klf5 Fwd	CTGCCATAAACCTCTCCCTTT
Klf5 Rev	CTGAGCCAAAGTCCAGTATGT
Irf2 Fwd	CCTTTAGCAGCCGATGACTTTA
Irf2 Rev	GAGTCCCGAGTTCAACAAGAA
Klf5 HO Fwd	CAGGCTACGGAGAAGATGAAAG
Klf5 HO Rev	GTGACCCAGTGGTAACAAAGA
Irf2 HO Fwd	CACCAGGAAGCCAAACAAAC
Irf2 HO Rev	CACACACAGAGGCCTACTAAAT
NC1 Fwd	CCTTTGTGTGTAGGGTGTATGG
NC1 Rev	ATCATTGTCCTCGGGTAGGT
NC2 Fwd	TGGCATCCAGGAGAGTCTTA
NC2 Rev	CAGCCACTAGAACTGTGGTAAA
NC3 Fwd	CACTCCCGTTCTAGGATTTCAG
NC3 Rev	GGGTTGGCCTTCTCATCTATT
Cdkn2 HC Fwd	AGTGAAGGACTGCAGGAAAC
Cdkn2 HC Rev	CAATTGCAGAGGCCACAAAC

Primers designed against the mouse reference genome (mm9) using the Primer 3 web tool. Btla, B and T lymphocyte associated; Cdkn2, cyclin-dependent kinase inhibitor 2; Fwd, forward; HC, Hoxa9/C/EBPα cobound site; HO, Hoxa9 only bound site; Irf2, interferon regulatory factor 2; Klf5, Kruppel-like factor 5; NC, negative control; Rev, reverse.