

C/EBP α is an essential collaborator in Hoxa9/Meis1-mediated leukemogenesis

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Homeobox A9 (HOXA9) is a homeodomain-containing transcription factor that plays a key role in hematopoietic stem cell expansion and is commonly deregulated in human acute leukemias. A variety of upstream genetic alterations in acute myeloid leukemia (AML) lead to overexpression of HOXA9, almost always in association with overexpression of its cofactor meis homeobox 1 (MEIS1). A wide range of data suggests that HOXA9 and MEIS1 play a synergistic causative role in AML, although the molecular mechanisms leading to transformation by HOXA9 and MEIS1 remain elusive. In this study, we identify CCAAT/enhancer binding protein alpha (C/EBP α) as a critical collaborator required for Hoxa9/Meis1-mediated leukemogenesis. We show that C/EBP α is required for the proliferation of Hoxa9/Meis1-transformed cells in culture and that loss of C/EBP α greatly improves survival in both primary and secondary murine models of Hoxa9/Meis1-induced leukemia. Over 50% of Hoxa9 genome-wide binding sites are cobound by C/EBP α , which coregulates a number of downstream target genes involved in the regulation of cell proliferation and differentiation. Finally, we show that Hoxa9 represses the locus of the cyclin-dependent kinase inhibitors *Cdkn2a/b* in concert with C/EBP α to overcome a block in G1 cell cycle progression. Together, our results suggest a previously unidentified role for C/EBP α in maintaining the proliferation required for Hoxa9/Meis1-mediated leukemogenesis.

enhancer | gene regulation

Homeobox A9 (HOXA9) is a member of the highly conserved HOX protein family of transcription factors, which play key roles in both development and hematopoiesis (1, 2). HOXA9 is most highly expressed in long-term hematopoietic stem cells (HSCs) and early progenitors, where it promotes cellular proliferation and is subsequently down-regulated during differentiation (3). Nearly one-half of acute myeloid leukemia (AML) cases show up-regulation of HOXA9, which correlates strongly with poor prognosis (4–8) (Fig. S1). In most cases, up-regulation of HOXA9 is accompanied by up-regulation of its cofactor MEIS1, which colocalizes with HOXA9 at enhancers (9, 10). Although overexpression of HOXA9 alone is sufficient for transformation of HSCs in culture, coexpression with MEIS1 increases transformation efficiency and results in rapidly fatal leukemias in transplanted animals (11).

A variety of upstream genetic alterations, including mixed-lineage leukemia (MLL) translocations, NPM1 mutations, NUP98 translocations, and CDX2 overexpression, lead to HOXA9 up-regulation in AML; however, the mechanisms through which high levels of HOXA9 contribute to leukemic transformation are not known (12–16). It has been suggested that lineage-specific “collaborator” proteins bind at relevant loci along with HOXA9 and its cofactors, PBX and MEIS proteins, to confer both site specificity and transcriptional activity of the HOXA9 complex (17). Recently, our group identified a number of potential Hoxa9 collaborators by characterizing the genome-wide binding sites of Hoxa9 and Meis1 in a murine myeloblastic cell line and by identifying proteins that interact with the Hoxa9 complex (9). One of these putative collaborators is CCAAT/enhancer binding protein alpha (C/EBP α), which coimmunoprecipitates

with Hoxa9. In addition, C/EBP recognition motifs are enriched at Hoxa9 binding sites.

C/EBP α is a basic leucine-zipper transcription factor that plays a critical role in lineage commitment during hematopoietic differentiation (18). Whereas *Cebpa*^{-/-} mice show complete loss of the granulocytic compartment, recent work shows that loss of C/EBP α in adult HSCs leads to both an increase in the number of functional HSCs and an increase in their proliferative and repopulating capacity (19, 20). Conversely, *CEBPA* overexpression can promote transdifferentiation of a variety of fibroblastic cells to the myeloid lineage and can induce monocytic differentiation in MLL-fusion protein-mediated leukemias (21, 22).

Although C/EBP α binds directly to target gene promoters, increasing evidence suggests that it also regulates gene expression through binding at promoter distal regulatory elements. For example, C/EBP α has been reported to colocalize with Pu.1, another critical regulator of hematopoiesis, at myeloid-specific enhancers, where it acts to establish areas of chromatin accessibility and facilitate the recruitment of signal-dependent transcription factors (23). The ability of CEBPA to act as a pioneer transcription factor at enhancers suggests that it may play a similar role in HOXA9-driven leukemogenesis.

To test this hypothesis, we used models allowing for conditional deletion of *Cebpa* in Hoxa9-transformed cells. We found that C/EBP α is critical for maintaining cellular proliferation in vitro and is a significant contributor to the severity of Hoxa9-mediated leukemia in vivo. Using genome-wide analysis, we found that C/EBP α colocalizes with Hoxa9 at promoter distal

Significance

Acute myeloid leukemia (AML) is a highly heterogeneous form of cancer that results from the uncontrolled proliferation of primitive immune cells. Homeobox A9 (HOXA9) is an evolutionarily conserved transcription factor that is overexpressed in a large percentage of AML cases and is associated with a poor prognosis. Here, we show that CCAAT/enhancer binding protein alpha (C/EBP α), a transcription factor involved in immune cell development that is commonly mutated in AML, is a critical collaborator required for HOXA9-mediated leukemic transformation. We also establish that the cell cycle regulator cyclin-dependent kinase inhibitors *Cdkn2a/b* are corepressed by the Hoxa9–C/EBP α complex. These findings suggest a novel functional interaction between two leukemic transcription factors, HOXA9 and C/EBP α , that is altered in a large percentage of AML cases.

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enhancers, resulting in both target gene activation and repression. Finally, we identified the cyclin-dependent kinase inhibitors *Cdkn2a/b* as critical targets of the Hoxa9-C/EBP α complex, whose repression likely contributes to the aberrant proliferation required for Hoxa9-mediated leukemogenesis.

Results

C/EBP α Is Required for Hoxa9/Meis1-Mediated Transformation. We previously identified the lineage-specific transcription factor C/EBP α as a member of the myeloid Hoxa9 complex (9). To determine if C/EBP α is required for transformation by Hoxa9 and Meis1, we generated cell lines that allow for conditional deletion of *Cebpa* by retrovirally transducing bone marrow from *Cebpa*^{fl/fl}; *CreERT*^{+/-} mice with Hoxa9/Meis1-GFP (Fig. 1A). We also generated control cell lines from *WT*; *CreERT*^{+/-} mice to control for the effects of tamoxifen (OHT) treatment and Cre-mediated toxicity. Continuous treatment of the Hoxa9/Meis1-transformed

Cebpa^{fl/fl}; *CreERT*^{+/-} (C/EBP α HM) cells with 4-hydroxytamoxifen (4OHT) leads to near complete loss of C/EBP α over the course of 8 d (Fig. 1B). Loss of C/EBP α leads to a dramatic decrease in cellular proliferation, whereas Cre induction in Hoxa9/Meis1-transformed *WT*; *CreERT*^{+/-} (WT HM) cells has no effect (Fig. 1C and D).

Our previously published work using a Hoxa9-estrogen receptor tag (ER)/Meis1 conditional cell line established that loss of Hoxa9 leads to a reduction in cellular proliferation, which is accompanied by differentiation of the cells into macrophages and induction of apoptosis (9). Although loss of C/EBP α also leads to a decrease in cellular proliferation, we did not observe terminal differentiation of these cells or induction of apoptosis (Fig. 1E–H). Loss of C/EBP α leads to the accumulation of cytoplasmic vacuoles at day 8 (Fig. 1E); however, this morphology did not progress with continued treatment of cells for 15 d. No changes in cellular morphology were observed in the WT HM cells under the same treatment conditions (Fig. 1F). Analysis of cell surface marker expression after loss of C/EBP α shows an increase in the immature cell surface marker c-Kit and either stable or decreasing expression in the myeloid surface markers CD11b, Gr1, F4/80, and Ly6C (Fig. 1G and Fig. S2). This phenotype persists across a 15-d time course, whereas no changes were seen in the WT HM control cells (Fig. 1G). These results are consistent with the known importance of C/EBP α in promoting myeloid differentiation, whereby cells lacking C/EBP α cannot initiate the full myeloid differentiation program (18). Furthermore, loss of C/EBP α does not lead to up-regulation of C/EBP β and C/EBP ϵ , which are also important mediators of myeloid differentiation (Fig. S3). We also tested whether loss of C/EBP α leads to induction of apoptosis by flow cytometry. No significant increase of apoptosis was seen after loss of C/EBP α compared with WT HM controls (Fig. 1H). Thus, loss of C/EBP α in Hoxa9/Meis1-transformed cells leads to a decrease in cellular proliferation in the absence of differentiation or apoptosis.

Loss of C/EBP α Impairs Hoxa9-Mediated Leukemogenesis. Given the importance of C/EBP α in maintaining rapid proliferation of Hoxa9/Meis1-transformed cells, we next examined if C/EBP α is required for Hoxa9/Meis1-leukemogenesis in vivo. We transplanted freshly transduced C/EBP α HM or WT HM cells into lethally irradiated C57B6 mice. After 2 wk, the mice were treated with biweekly injections of OHT to induce deletion of *Cebpa* in the transplanted cells.

Loss of *Cebpa* significantly improved survival of the C/EBP α HM-transplanted mice [$n = 10$ (veh), 12(OHT); $P < 0.0001$], whereas there was no survival difference seen in the vehicle- or OHT-treated WT HM cohort [$n = 10$ (veh), 14(OHT); $P = 0.4324$] (Fig. 2A and B). Vehicle-treated mice from the C/EBP α HM cohort, as well as all mice in the WT HM groups, developed myeloblastic leukemia in an average of 40 d showing extensive liver, spleen, and peripheral blood infiltration. Conversely, C/EBP α HM mice treated with OHT developed leukemia in an average of 60 d, also with infiltration of the liver and spleen in late stages (Fig. 2C). This delay in leukemia is even more impressive given that *Cebpa*^{-/-} bone marrow is reported to have enhanced repopulating activity and faster proliferation than WT cells (19).

Examination of C/EBP α levels in the bone marrow at the time of death indicates that there is strong selective pressure for maintaining high levels of C/EBP α in Hoxa9/Meis1-transformed leukemias. One C/EBP α HM OHT-treated mouse died early and showed complete loss of *Cebpa* expression, although this mouse presented with a lower blast count and a more mature phenotype than a C/EBP α HM vehicle-treated mouse that died on the same day (day 35; “early”) (Fig. 2C–E). On the other hand, the remainder of the C/EBP α HM OHT-treated cohort that succumbed to leukemia at later time points showed recovery of *Cebpa* expression and a phenotype similar to vehicle-treated mice (day 60; “late”) through outgrowth of clones that had escaped Cre-mediated deletion (Fig. 2C–E and Fig. S4). This selective pressure for the presence of C/EBP α can also be seen in cell culture, because cells eventually regain C/EBP α expression in

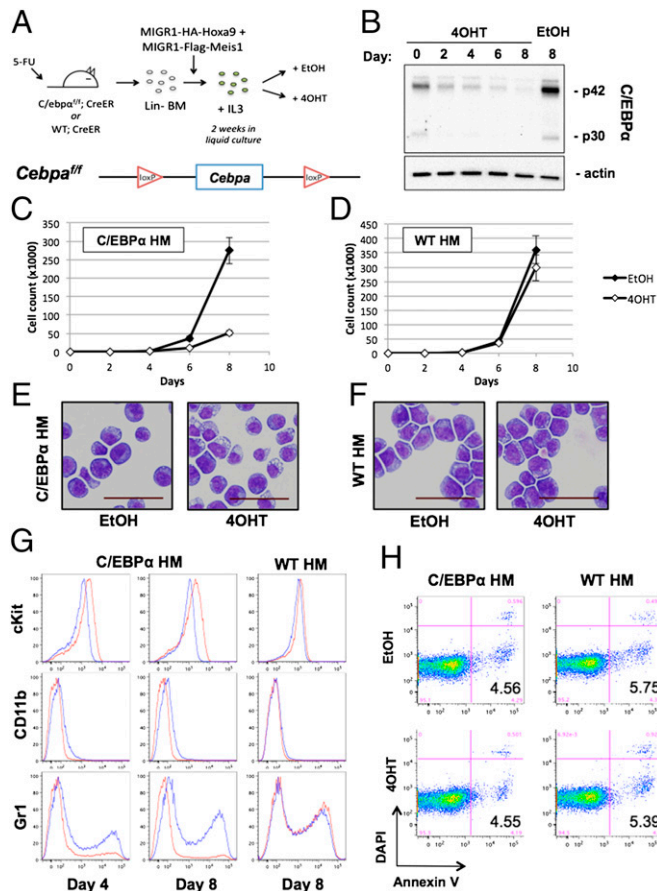


Fig. 1. C/EBP α is required for Hoxa9/Meis1-mediated transformation. (A) Schematic of cell line generation and *Cebpa*-targeted allele. 5-FU, 5-fluorouracil. (B) C/EBP α HM and WT HM cells were treated for an 8-d time course with 5 nM 4OHT or EtOH, and protein levels in the C/EBP α HM cells were assessed using Western blotting. Cellular proliferation of both C/EBP α HM (C) and WT HM (D) cells was determined by cell counting; data represent mean \pm SD of two independent experiments. Cell morphology of C/EBP α HM (E) and WT HM (F) cells was assessed after 8 d. (Scale bars: 50 μ m.) (G) Surface expression of c-Kit, CD11b, and Gr1 at days 4 and 8 in C/EBP α HM cells (Left and Center) and WT HM cells (Right) after continuous treatment with 4OHT (red) or EtOH (blue). (H) Annexin V and DAPI staining for apoptotic cells at day 8 of treatment with 4OHT or EtOH. Black numbers in the lower right corner represent the mean number of apoptotic cells (AnnexinV⁺/DAPI⁻) from four independent experiments. Mean \pm SD values are as follows: C/EBP α HM EtOH, 4.56 \pm 3.62; C/EBP α HM 4OHT, 4.54 \pm 1.23; WT EtOH, 5.74 \pm 5.31, and WT 4OHT, 5.38 \pm 3.75. Flow cytometry plots from one representative experiment are shown.

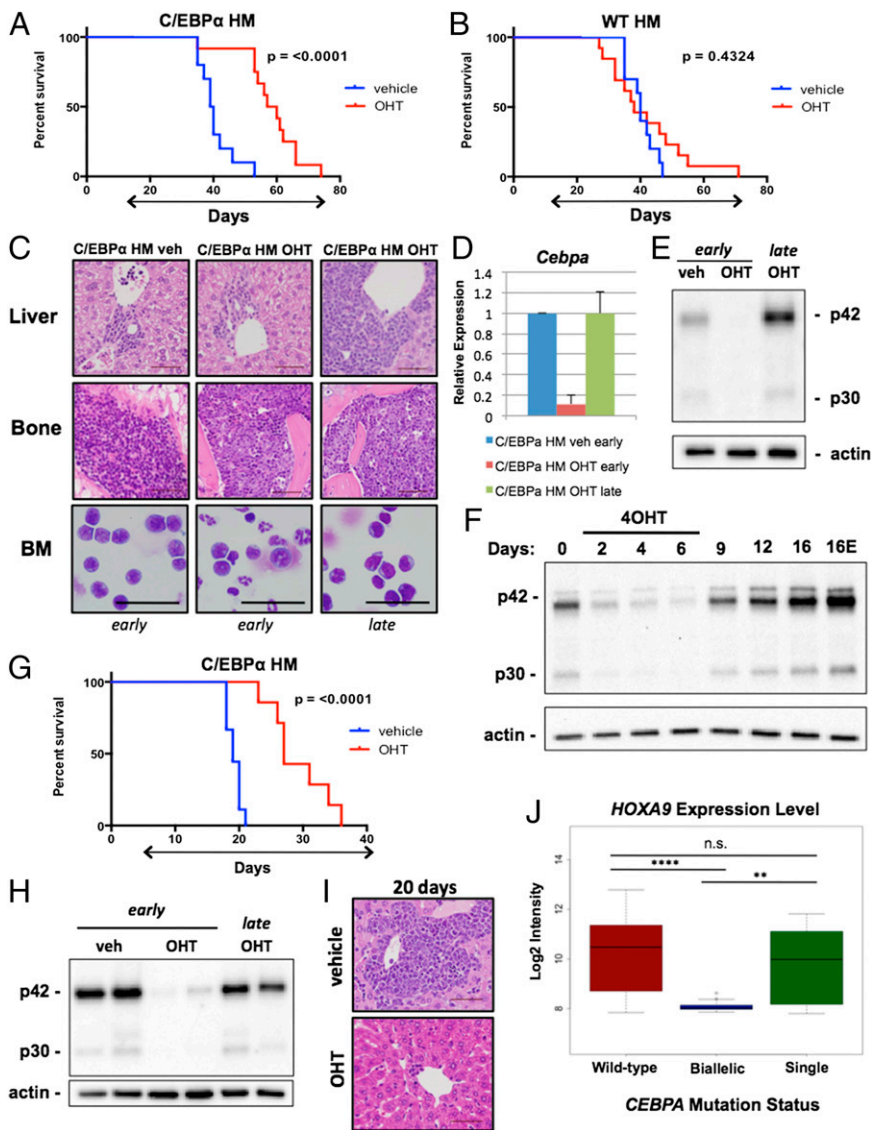


Fig. 2. Loss of *C/EBPα* impairs *Hoxa9*-mediated leukemogenesis. Survival curves for mice transplanted with *C/EBPα* HM cells [A; *n* = 10(veh), 12(OHT); *P* < 0.0001 by log rank] or WT HM cells [B; *n* = 10(veh), 14(OHT); *P* = 0.4324 by log rank]. The treatment period with OHT (red) or vehicle (blue) is indicated by the arrow below the graphs. (C) Tissue histology of liver and bone, and bone marrow (BM) cytopins for *C/EBPα* HM vehicle- and OHT-treated mice that died before 40 d (Left and Center; “early”) and an OHT-treated mouse that died at 60 d posttransplantation (Right; “late”). (Scale bars: 50 μm.) (D and E) RT-PCR expression of *Cebpa* and Western blot analysis of *C/EBPα* protein levels corresponding to samples shown in C (mean ± SD). veh, vehicle. (F) *C/EBPα* protein levels in cells treated with 4OHT for 6 d and subsequently maintained in the absence of 4OHT for an additional 10 d. The rightmost lane (16E) corresponds to cells treated continuously with EtOH for 16 d. (G) Survival curve of mice transplanted with primary leukemic spleen cells from a *C/EBPα* HM vehicle-treated mouse [*n* = 9(veh), 7(OHT); *P* < 0.0001 by log rank]. The treatment period with OHT (red) or vehicle (blue) is indicated by the arrow below the graph. (H) *C/EBPα* protein levels in vehicle-treated leukemic mice (Left) compared with OHT-treated mice preleukemic controls (Center) and leukemic OHT-treated mice (Right). (I) Liver histology of leukemic vehicle- and preleukemic OHT-treated mice at 20 d. (Scale bars: 50 μm.) (J) *HOXA9* expression level in a cohort of patients with AML subdivided by *CEBPA* mutation status (*n* = 344) *****P* < 0.0005; ***P* < 0.005; ns, not significant.

the absence of 4OHT treatment despite genomic deletion of *Cebpa* and loss of protein levels after 1 wk of 4OHT treatment (Fig. 2F). The *C/EBPα* HM OHT-treated mouse that succumbed to leukemia in the absence of *C/EBPα* may be explained by the acquisition of cooperating mutations or activation of compensatory pathways to escape the requirement for *C/EBPα* expression.

We also examined the role of *C/EBPα* in secondary *Hoxa9*/*Meis1*-induced leukemias. Spleen cells harvested from a *C/EBPα* HM vehicle-treated primary leukemia mouse were injected into the tail vein of sublethally irradiated C57B6 mice to establish secondary leukemias, and the mice were then treated with OHT to induce *Cebpa* excision. Loss of *C/EBPα* led to prolonged survival of the mice with secondary leukemia [*n* = 9(veh), 7(OHT); *P* < 0.0001] (Fig. 2G). OHT-treated mice that were killed as controls alongside leukemic vehicle-treated mice showed significantly reduced *C/EBPα* levels and tissue infiltration compared with the vehicle-treated mice, confirming the efficacy of the OHT treatment (Fig. 2H and I). Conversely, OHT-treated mice that eventually succumbed to leukemia regained high *C/EBPα* levels and showed liver infiltration similar to vehicle-treated mice, again displaying strong selective pressure for *Cebpa* reexpression (Fig. 2H and I). Taken together, these results show that *C/EBPα* is required for *Hoxa9*/*Meis1*-mediated leukemogenesis.

Given these findings, we analyzed the relationship between *CEBPA* and *HOXA9* expression in a cohort of 344 human acute leukemias [data reanalyzed from a study by Figueroa et al. (24)]. Although there was no significant correlation between the expression level of *CEBPA* and *HOXA9*, this cohort included leukemias with single and biallelic mutations in *CEBPA*, resulting in both loss of function and dominant negative activities of the protein. Interestingly, when we analyzed expression of *HOXA9* relative to *CEBPA* mutational status, we found that leukemias with at least one WT copy of *CEBPA* express high levels of *HOXA9*, whereas cases with biallelic mutations of *CEBPA* are associated with much lower levels of *HOXA9* (Fig. 2J). These data provide further support for a requirement for WT *CEBPA* in human leukemias with high levels of *HOXA9* expression.

***C/EBPα* Colocalizes with *Hoxa9* at Promoter Distal Enhancers.** We sought to establish a molecular mechanism for the functional interplay between *Hoxa9* and *C/EBPα* in leukemogenesis. Given that *Hoxa9* and *C/EBPα* physically interact and that the *C/EBP* motif is enriched at *Hoxa9* binding sites (9), we next determined if *C/EBPα* cobinds with *Hoxa9* on a genome-wide level. We performed ChIP-seq for *Hoxa9* and *C/EBPα* in a mouse myeloblastic cell line transformed with HA-*Hoxa9*-ER

and Flag-Meis1 (HerM). Because there are currently no antibodies against Hoxa9 suitable for ChIP-seq, we used an HA antibody to immunoprecipitate Hoxa9. We identified 6,535 peaks that are bound by Hoxa9 and 26,187 peaks that are bound by C/EBP α , the majority of which occur at promoter distal regions (Fig. 3A). Notably, a remarkable proportion (54%) of Hoxa9 binding sites are cobound by C/EBP α ($P < 0.001$) (Fig. 3B). C/EBP α does not appear to be absolutely required for Hoxa9 binding, however, because cases of Hoxa9 binding sites with very low levels of C/EBP α can be found even in the vicinity of strong cobound peaks (Fig. 3C, *Center and Right*). To validate our ChIP-seq results, multiple sites of each class of Hoxa9-bound enhancer were confirmed using ChIP-quantitative PCR (Fig. 3D).

Finally, we examined the biological pathways enriched for putative targets of Hoxa9–C/EBP α -cobound enhancers. Sites cobound by Hoxa9 and C/EBP α showed an enrichment for genes critical for hematopoietic pathways, including the regulation of myeloid differentiation, regulation of the inflammatory response, and regulation of cytokine production (Fig. S5). In addition, cobound sites showed enrichment for pathways involved in signal transduction, including the regulation of protein kinase activity, MAP kinase activity, and serine/threonine kinase activity. Collectively, these results suggest that C/EBP α functionally interacts with Hoxa9 at enhancers to facilitate Hoxa9/Meis1-mediated transformation.

C/EBP α and Hoxa9 Coregulate Expression of *Cdkn2a/b*. Given the requirement for C/EBP α in Hoxa9/Meis1-mediated leukemic transformation and the colocalization of C/EBP α with Hoxa9 at enhancers in transformed cells, we next sought to identify target genes potentially important for leukemogenesis. Using the C/EBP α HM and HerM cells, we performed RNA-sequencing (RNA-seq) at 72 h after loss of C/EBP α or Hoxa9. We identified 31 genes that were coactivated more than 1.5-fold by Hoxa9 and C/EBP α , including *Adam17*, *Igf2r*, *Il2ra*, and *Cpe* (Table S1). In addition, 45 genes were corepressed more than 1.5-fold, including *Gata2*, *Gfi1b*, *Prkca*, and *Cdkn2b* (Table S2).

Interestingly, a number of genes were antagonistically regulated by the two proteins, including the known C/EBP α target *Sox4*, suggesting a competitive mechanism between C/EBP α and Hoxa9 at some targets.

Two genes that were significantly repressed by both Hoxa9 and C/EBP were *Cdkn2a/b* (*INK4a/b*). *Cdkn2a/b* are critical regulators of HSC self-renewal, apoptosis, and oncogene-induced senescence whose expression leads to a block in the cell cycle at the G1 phase (25). In addition, *Cdkn2a/b* are commonly deleted in acute lymphoid leukemias (26–28). Our ChIP-seq studies identified a Hoxa9–C/EBP α -cobound site in an intergenic region ~50 kb downstream of the *Cdkn2a/b* locus (Fig. 4A). Loss of either C/EBP α or Hoxa9 binding at this locus (Fig. 4B and C) results in a corresponding increase in *Cdkn2a/b* expression (Fig. 4D and E). In addition, loss of either C/EBP α or Hoxa9 leads to only a slight reduction in the binding of the other protein, suggesting that the cobinding of both proteins is necessary for repression of the *Cdkn2a/b* locus (Fig. 4B and C). Cell cycle analysis performed on C/EBP α HM and HerM cells showed that both loss of C/EBP α and loss of Hoxa9 in Hoxa9/Meis1-transformed cells leads to G1 cell cycle blockade (Fig. 4F and G). These data suggest that corepression of the *Cdkn2a/b* locus by C/EBP α and Hoxa9 contributes to the rapid proliferation induced with Hoxa9-mediated transformation. Indeed, overexpression of *CDKN2A* or *CDKN2B* in cells transformed by Hoxa9/Meis1 leads to a decrease in proliferation relative to control cells transduced with an empty vector (Fig. 4H).

Discussion

Although a variety of upstream genetic alterations in AML are known to work through increased expression of *HOXA9*, the downstream mechanisms through which high levels of *HOXA9* mediate transformation are yet to be fully elucidated. In this study, we identify C/EBP α as a critical collaborator protein of

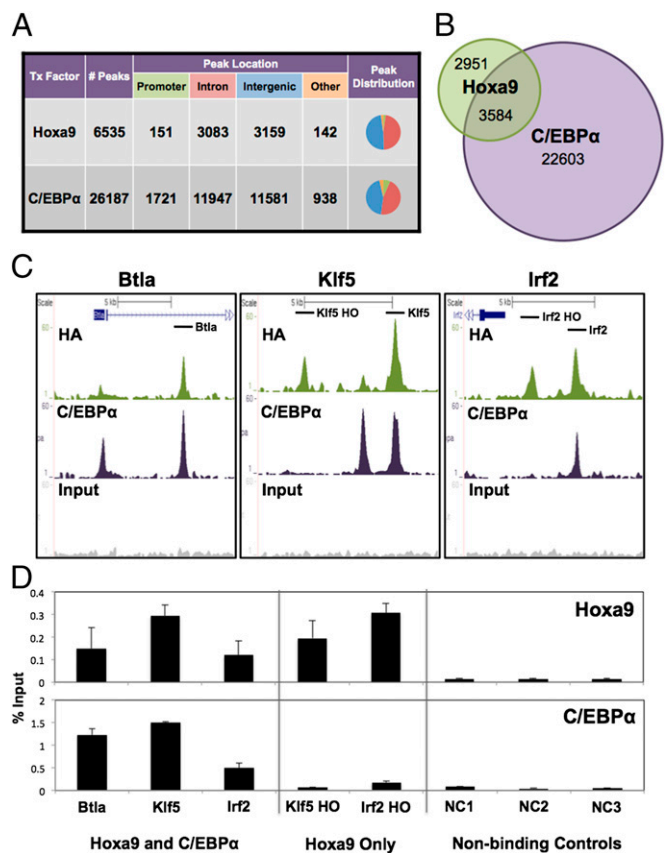


Fig. 3. C/EBP α colocalizes with Hoxa9 at promoter distal enhancers. (A) Peak number and distribution of Hoxa9 and C/EBP α ChIP-seq in a Hoxa9/Meis1-transformed cell line (“Other” category contains 5’/3’ UTR and exons). Tx, transcription. (B) Peak overlap between Hoxa9 and C/EBP α ChIP-seq. (C) Representative Hoxa9 (HA)/C/EBP α -cobound loci. Bars indicate location of quantitative PCR (qPCR) primer pairs. (D) Independent ChIP-qPCR validation of ChIP-seq data for Hoxa9 (*Upper*) and C/EBP α (*Lower*) binding at Hoxa9–C/EBP α -cobound sites, Hoxa9 only, and nonbinding controls; bars indicate mean \pm SD of at least two independent experiments. Btla, B and T lymphocyte associated; Klf5, Kruppel-like factor 5; Irf2, interferon regulatory factor 2.

Hoxa9 in myeloid leukemia. Our work shows that C/EBP α is required for the rapid proliferation of Hoxa9/Meis1-transformed cells in culture and for aggressive disease in primary and secondary murine models of Hoxa9/Meis1-induced leukemia. In this context, it is noteworthy that human AMLs with high *HOXA9* expression almost always retain one WT copy of *CEBPA*. Taken together with the observation that null mutations of *CEBPA* are almost never seen in AML, our data suggest that some residual function of *CEBPA* is required for transformation (29). Although these findings are surprising, given that high levels of *CEBPA* have been shown to promote myeloid differentiation (22), it is most likely that a moderate level of *CEBPA* is required for *HOXA9*-mediated AML.

The requirement for *CEBPA* in AML may be specific to leukemias with high levels of *HOXA9*. Recent work shows that C/EBP α is required for the initiation of leukemias transformed by MLL-ENL, a fusion protein that directly up-regulates *HOXA9* expression (30, 31). Conversely, C/EBP α was shown to be dispensable for E2A-HLF-mediated transformation, which has undetectable levels of *HOXA9* (31). This same study also found that C/EBP α is not required for the maintenance of MLL-ENL-induced leukemias. Taken together with our finding that C/EBP α is required for the maintenance of Hoxa9-mediated transformation, these data suggest that MLL-ENL activates alternative pathways to compensate for changes in Hoxa9 target gene

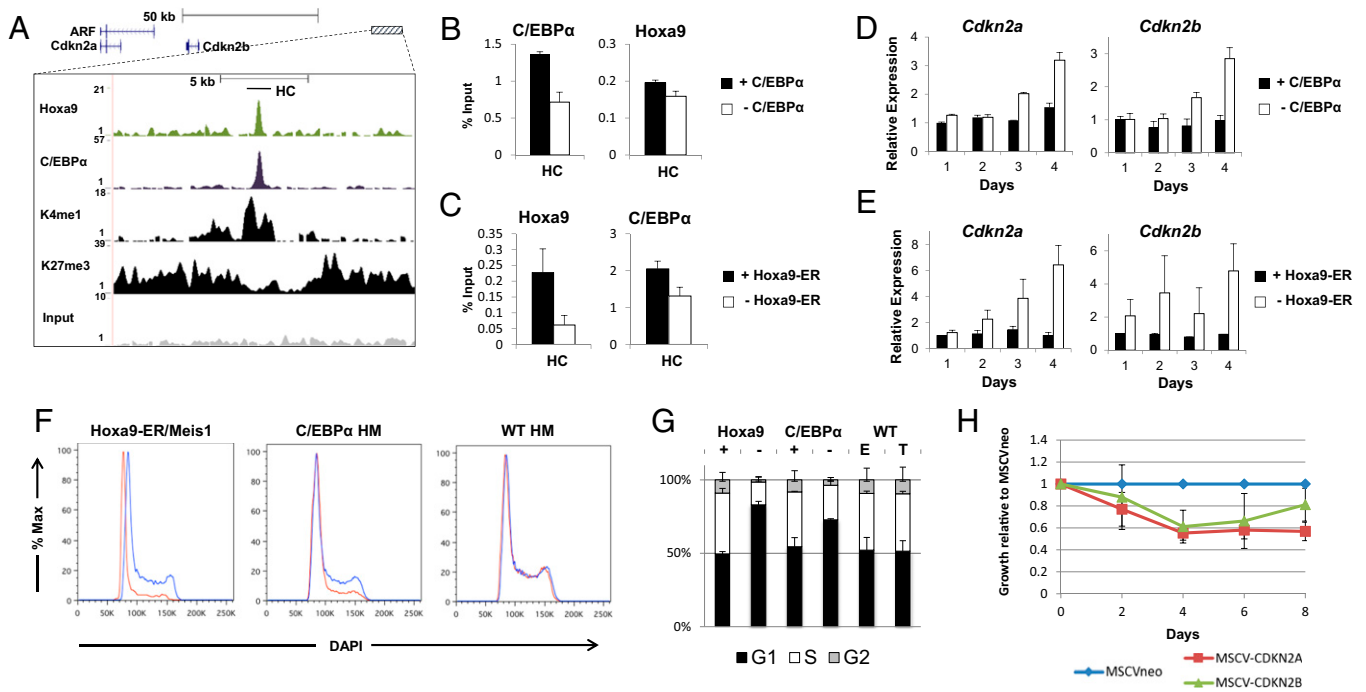


Fig. 4. *C/EBPα* and *Hoxa9* coregulate expression of *Cdkn2a/b*. (A) ChIP-seq tracks for *Hoxa9*, *C/EBPα*, H3K4me1, and H3K27me3 at the *Hoxa9*–*C/EBPα* binding site 50 kb downstream of the *Cdkn2a/b* locus. Bars indicate the location of the qPCR primer set. HC, *Hoxa9/C/EBPα* cobound site. ChIP-qPCR for *C/EBPα* and *Hoxa9* binding at the HC binding site after 3-d loss of *C/EBPα* (B) or *Hoxa9* (C). RT-PCR expression of *Cdkn2a* and *Cdkn2b* over a 4-d time course after loss of *C/EBPα* (D) or loss of *Hoxa9* (E). (F) Cell cycle analysis at day 6 after loss of *Hoxa9* (Left) or *C/EBPα* (Center), or in EtOH- or 4OHT-treated WT HM cells (Right) (blue, control; red, loss of *Hoxa9* or *C/EBPα*). (G) Quantification of cell cycle profiles analyzed using FlowJo (TreeStar). E, EtOH; T, 4OHT. (H) Relative cellular proliferation of *Hoxa9/Meis1*-transformed cells coexpressing MSCVneo, MSCV-CDKN2A, or MSCV-CDKN2B. All data are expressed as mean \pm SD of at least two independent experiments.

regulation after loss of *C/EBPα*. Further characterization of the downstream gene expression changes after loss of *C/EBPα* in *MLL*-rearranged leukemias and other *HOXA9*-high leukemias, especially in comparison to leukemias with low expression of *HOXA9*, will help elucidate these alternative pathways.

Given that our work implicates a requirement for WT *CEBPA* in the development of leukemia with a high level of *HOXA9*, it is also interesting to speculate how mutant forms of *CEBPA* in AML may functionally interact with *HOXA9*. About 10% of AMLs carry mutations in *CEBPA*, two-thirds of which are biallelic mutations, where one allele carries a mutation in the C-terminal DNA binding domain and the other allele carries an N-terminal mutation that leads to transcription of the short p30 isoform (29, 32, 33). These mutations affect not only the binding and localization of *CEBPA* but also the recruitment of coactivator and corepressor complexes to *CEBPA*-bound loci (29, 34). In these cases, expression of *HOXA9* is very low, suggesting oncogene incompatibility between high expression of *HOXA9* and biallelic mutations of *CEBPA*. In addition, the genetic signature of *CEBPA* mutant leukemias is distinct from that of *MLL*-rearranged and other *CEBPA* WT leukemias (35). Conversely, our work shows that single mutant cases of *CEBPA* do occur in the presence of high levels of *HOXA9*, potentially affecting target gene regulation. Further study on the effect of various mutant forms of *CEBPA* on regulation of *HOXA9* target genes could provide valuable insight into mechanisms of transformation in these cases.

In addition to uncovering an unexpected requirement of *C/EBPα* in *Hoxa9*-mediated leukemogenesis, our work implicates *C/EBPα* in the control of cell cycle and senescence in cells transformed by *Hoxa9/Meis1*. Recent work has shown that *Hoxa9* inhibits *Cdkn2a* expression in *Bmi1*^{-/-} *MLL*-AF9 cells, allowing for escape of the oncogene-induced senescence that is seen in *Bmi1*^{-/-} cells transformed with other oncogenes (36). Although *Hoxa9* was found to suppress *Cdkn2a* expression through direct binding at the promoter, Smith et al. (36) suggest that other

non-*HOX* proteins may be involved in achieving full repression of this locus. Our work implicates *C/EBPα* as a cofactor aiding in *Hoxa9* repression of both *Cdkn2a* and *Cdkn2b*, which is potentially mediated through looping of the downstream *Hoxa9*–*C/EBPα*-cobound site to the promoters. The silencing of both *CDKN2A/B* through deletion or promoter methylation is known to play critical roles in AML; thus, repression of this locus may also be central to *HOXA9/MEIS*-mediated leukemogenesis (26–28). Derepression of *Cdkn2a/b* may contribute to the G1 cell cycle blockade and decreased proliferation that occurs in the absence of *C/EBPα*, although overexpression of *CDKN2A* or *CDKN2B* in cells transformed by *Hoxa9/Meis1* does not fully recapitulate this phenotype. Therefore, it remains possible that additional *Hoxa9*–*C/EBPα*-regulated target genes, in concert with *Cdkn2a/b*, are critical to maintaining rapid proliferation in cells transformed by *Hoxa9/Meis1*. Additional experiments are required to identify these factors and their relationship with *Cdkn2a/b* repression in promoting leukemogenesis downstream of *Hoxa9* and *C/EBPα*.

Finally, although *C/EBPα* is required for *Hoxa9*-mediated leukemogenesis at essential coregulated targets, such as *Cdkn2a/b*, we also suggest that high levels of *Hoxa9* may antagonize *C/EBPα* at genes associated with myeloid differentiation. Consistent with this idea, we find that *Hoxa9* and *C/EBPα* have antagonistic effects on *Sox4*, which is reported to be a direct target of *C/EBPα* and whose repression is required for normal hematopoietic differentiation (35). Additional study of antagonistically regulated *HOXA9/CEBPA* target genes may provide further insight into the mechanisms through which high levels of *HOXA9* expression lead to transformation in AML.

Materials and Methods

Cell Lines. Lineage-depleted bone marrow from *Cebpa*^{fl/fl}; *CreERT*^{+/+}, *WT*; *CreERT*^{+/+}, or *WT* mice was retrovirally transduced with MIGR1-HA-*Hoxa9* or MIGR1-HA-*Hoxa9*-ER and MIGR1-Flag-*Meis1* [plasmids previously described

by Huang et al. (9)]. The detailed protocol and culture conditions are provided in *SI Materials and Methods*.

Cellular Assays. For loss of C/EBP α studies, cells were treated continuously with ethanol (EtOH) or 5 nM 4OHT (H7904; Sigma). For the 4OHT withdrawal experiment, after 1 wk of continuous culture in EtOH or 5 nM 4OHT, cells were washed once with culture media and continued in culture with EtOH only. For loss of Hoxa9 studies, cells were washed three times with culture media and then maintained in either EtOH or 100 nM 4OHT. Detailed information on proliferation, morphology, Western blotting, RNA extraction, and cDNA generation is provided in *SI Materials and Methods*.

Flow Cytometry. For apoptosis assays, cells were stained with allophycocyanin (APC)-anti-AnnexinV and DAPI. For cell cycle analysis, cells were fixed in 70% (vol/vol) EtOH, and treated with RNase A (19101; Qiagen) and DAPI. Detailed protocol and antibody information is provided in *SI Materials and Methods*.

Bone Marrow Transplantation. The detailed protocol is provided in *SI Materials and Methods*. Briefly, for primary leukemia assays, freshly transduced cells were injected by tail vein in cohorts of lethally irradiated ~8-wk-old female C57BL/6 mice. At 2 wk, mice were treated with biweekly i.p. injections of OHT (200 mg/kg, T5648; Sigma) or corn oil. For secondary leukemia assays, spleen cells harvested from primary leukemic mice in the corn oil-treated cohort were injected by tail vein in cohorts of sublethally irradiated

~8-wk-old female C57BL/6 mice. 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.

ChIP. The detailed protocol is provided in *SI Materials and Methods*. Briefly, cells fixed with 1% paraformaldehyde were lysed in SDS lysis buffer and sonicated to produce chromatin shearing below 500 bp. Diluted chromatin was incubated overnight with antibody. Antibody was captured using Protein G Dynabeads (Invitrogen) and washed with low-salt, high-salt, lithium chloride, and Tris/EDTA buffers. Eluted chromatin was reverse-cross-linked overnight, RNase A-treated, and purified. Primers used in qPCR are listed in Table S3.

ChIP-Seq and RNA-Seq. ChIP-seq and RNA-seq libraries were generated using an Illumina ChIP-seq Library Preparation Kit and Illumina TruSeq RNA Sample Preparation Kit, respectively. Sequencing was performed on an Illumina HiSeq2000. Data analysis was performed as described in *SI Materials and Methods*. ChIP-seq and RNA-seq datasets have been deposited in the Gene Expression Omnibus database.

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