C/EBPα 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/EBPα) as a critical collaborator required for Hoxa9/Meis1-mediated leukemogenesis. We show that C/EBPα is required for the proliferation of Hoxa9/Meis1-transformed cells in culture and that loss of C/EBPα 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/EBPα, 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/EBPα to overcome a block in G1 cell cycle progression. Together, our results suggest a previously unidentified role for C/EBPα 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/EBPα), which coimmunoprecipitates with Hoxa9. In addition, C/EBP recognition motifs are enriched at Hoxa9 binding sites.

C/EBPα 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/EBPα 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/EBPα 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/EBPα 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/EBPα 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/EBPα 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/EBPα), 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/EBPα complex. These findings suggest a novel functional interaction between two leukemic transcription factors, HOXA9 and C/EBPα, 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/EBPα complex, whose repression likely contributes to the aberrant proliferation required for Hoxa9-mediated leukemogenesis.

Results

C/EBPα Is Required for Hoxa9/Meis1-Mediated Transformation. We previously identified the lineage-specific transcription factor C/EBPα as a member of the myeloid Hoxa9 complex (9). To determine if C/EBPα 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*$^{f/f}$;*CreERT*$^{+/-}$ mice with Hoxa9/Meis1-GFP (Fig. 1*A*). 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*$^{f/f}$;*CreERT*$^{+/-}$ (C/EBPα HM) cells with 4-hydroxytamoxifen (4OHT) leads to near complete loss of C/EBPα over the course of 8 d (Fig. 1*B*). Loss of C/EBPα leads to a dramatic decrease in cellular proliferation, whereas Cre induction in Hoxa9/Meis1-transformed *WT;CreERT*$^{+/-}$ (WT HM) cells has no effect (Fig. 1 *C* and *D*).

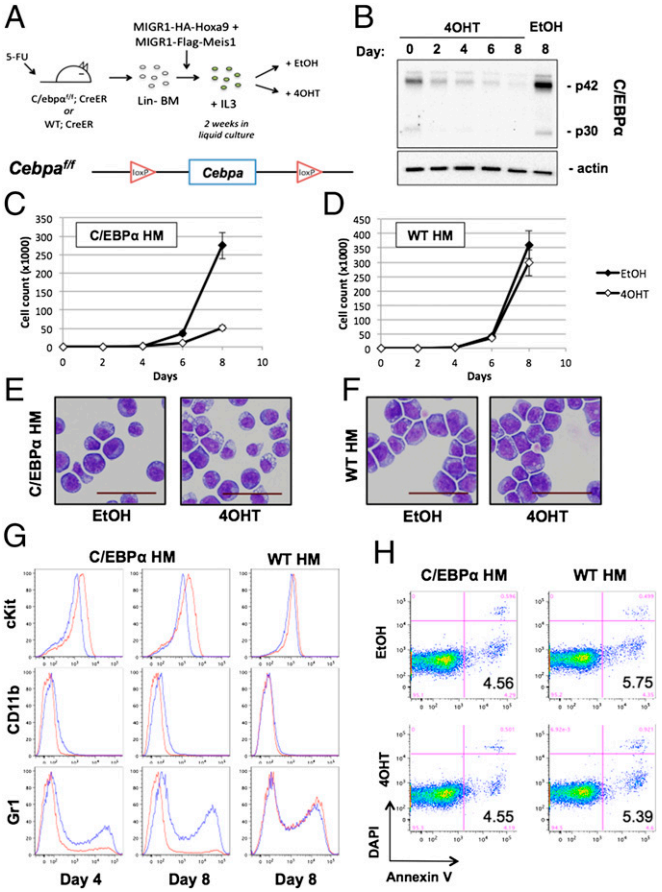

Fig. 1. C/EBPα is required for Hoxa9/Meis1-mediated transformation. (*A*) Schematic of cell line generation and *Cebpa*-targeted allele. 5-FU, 5-flurouracil. (*B*) C/EBPα HM and WT HM cells were treated for an 8-d time course with 5 nM 4OHT or EtOH, and protein levels in the C/EBPα HM cells were assessed using Western blotting. Cellular proliferation of both C/EBPα HM (*C*) and WT HM (*D*) cells was determined by cell counting; data represent mean ± SD of two independent experiments. Cell morphology of C/EBPα HM (*E*) and WT HM (*F*) cells was assessed after 8 d. (Scale bars: 50 μm.) (*G*) Surface expression of c-Kit, CD11b, and Gr1 at days 4 and 8 in C/EBPα 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 ± SD values are as follows: C/EBPα HM EtOH, 4.56 ± 3.62; C/EBPα HM 4OHT, 4.54 ± 1.23; WT EtOH, 5.74 ± 5.31, and WT 4OHT, 5.38 ± 3.75. Flow cytometry plots from one representative experiment are shown.

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/EBPα also leads to a decrease in cellular proliferation, we did not observe terminal differentiation of these cells or induction of apoptosis (Fig. 1 *E–H*). Loss of C/EBPα leads to the accumulation of cytoplasmic vacuoles at day 8 (Fig. 1*E*); 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. 1*F*). Analysis of cell surface marker expression after loss of C/EBPα 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. 1*G* and Fig. S2). This phenotype persists across a 15-d time course, whereas no changes were seen in the WT HM control cells (Fig. 1*G*). These results are consistent with the known importance of C/EBPα in promoting myeloid differentiation, whereby cells lacking C/EBPα cannot initiate the full myeloid differentiation program (18). Furthermore, loss of C/EBPα does not lead to up-regulation of C/EBPβ and C/EBPε, which are also important mediators of myeloid differentiation (Fig. S3). We also tested whether loss of C/EBPα leads to induction of apoptosis by flow cytometry. No significant increase of apoptosis was seen after loss of C/EBPα compared with WT HM controls (Fig. 1*H*). Thus, loss of C/EBPα in Hoxa9/Meis1-transformed cells leads to a decrease in cellular proliferation in the absence of differentiation or apoptosis.

Loss of C/EBPα Impairs Hoxa9-Mediated Leukemogenesis. Given the importance of C/EBPα in maintaining rapid proliferation of Hoxa9/Meis1-transformed cells, we next examined if C/EBPα is required for Hoxa9/Meis1-leukemogenesis in vivo. We transplanted freshly transduced C/EBPα 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/EBPα 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. 2 *A* and *B*). Vehicle-treated mice from the C/EBPα 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/EBPα 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. 2*C*). 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/EBPα levels in the bone marrow at the time of death indicates that there is strong selective pressure for maintaining high levels of C/EBPα in Hoxa9/Meis1-transformed leukemias. One C/EBPα 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/EBPα HM vehicle-treated mouse that died on the same day (day 35; "early") (Fig. 2 *C–E*). On the other hand, the remainder of the C/EBPα 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. 2 *C–E* and Fig. S4). This selective pressure for the presence of C/EBPα can also be seen in cell culture, because cells eventually regain C/EBPα expression in

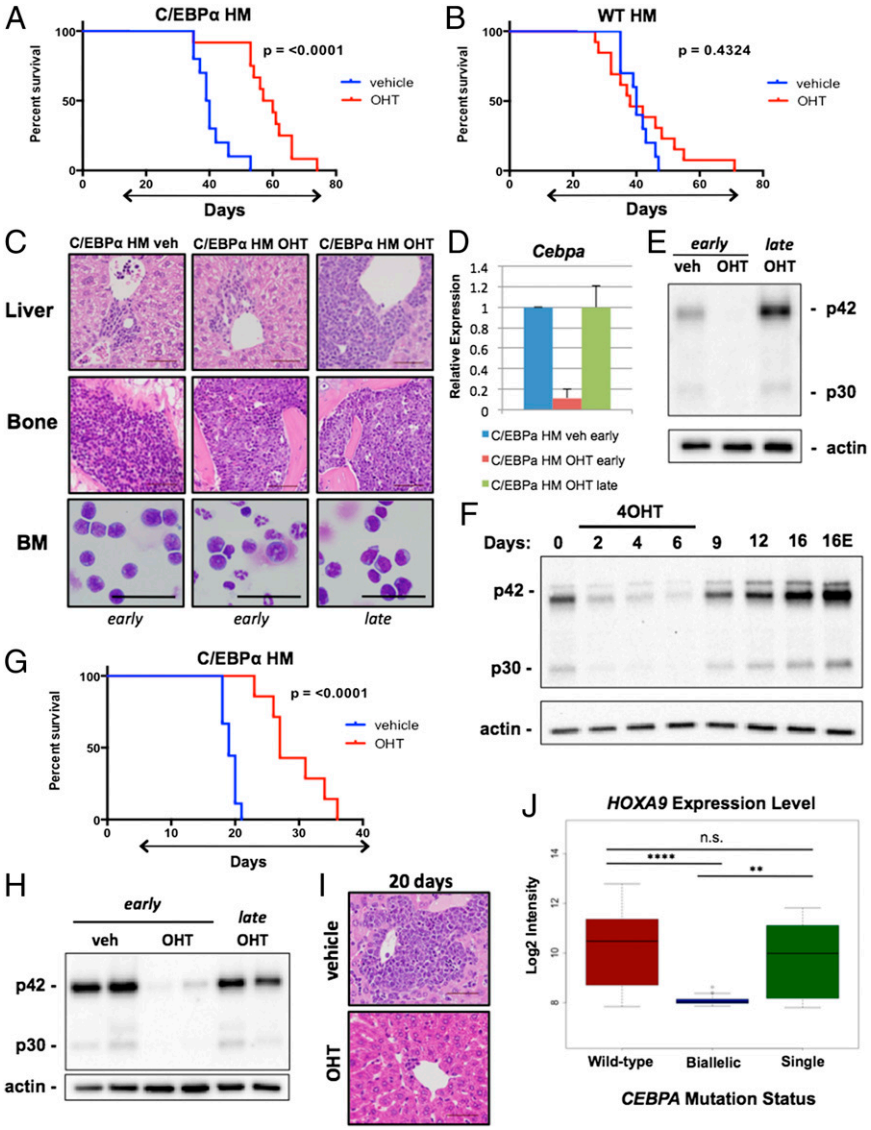

Fig. 2. Loss of C/EBPα impairs Hoxa9-mediated leukemogenesis. Survival curves for mice transplanted with C/EBPα HM cells [*A*; $n = 10$(veh), 12(OHT); $P < 0.0001$ by log rank] or WT HM cells [*B*; $n = 10$(veh), 14(OHT); $P = 0.4324$ by log rank]. The treatment period with OHT (red) or vehicle (blue) is indicated by the arrow below the graphs. (*C*) Tissue histology of liver and bone, and bone marrow (BM) cytospins 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 sigificant.

the absence of 4OHT treatment despite genomic deletion of *Cebpa* and loss of protein levels after 1 wk of 4OHT treatment (Fig. 2*F*). 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. 2*G*). 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. 2 *H* 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. 2 *H* 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. 2*J*). 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-sequencing (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/EBPα, the majority of which occur at promoter distal regions (Fig. 3*A*). Notably, a remarkable proportion (54%) of Hoxa9 binding sites are cobound by C/EBPα ($P < 0.001$) (Fig. 3*B*). C/EBPα does not appear to be absolutely required for Hoxa9 binding, however, because cases of Hoxa9 binding sites with very low levels of C/EBPα can be found even in the vicinity of strong cobound peaks (Fig. 3*C*, *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. 3*D*).

Finally, we examined the biological pathways enriched for putative targets of Hoxa9–C/EBPα-cobound enhancers. Sites cobound by Hoxa9 and C/EBPα 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/EBPα functionally interacts with Hoxa9 at enhancers to facilitate Hoxa9/Meis1-mediated transformation.

C/EBPα and Hoxa9 Coregulate Expression of Cdkn2a/b. Given the requirement for C/EBPα in Hoxa9/Meis1-mediated leukemic transformation and the colocalization of C/EBPα with Hoxa9 at enhancers in transformed cells, we next sought to identify target genes potentially important for leukemogenesis. Using the C/EBPα HM and HerM cells, we performed RNA-sequencing (RNA-seq) at 72 h after loss of C/EBPα or Hoxa9. We identified 31 genes that were coactivated more than 1.5-fold by Hoxa9 and C/EBPα, 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/EBPα target *Sox4*, suggesting a competitive mechanism between C/EBPα 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/EBPα-cobound site in an intergenic region ~50 kb downstream of the *Cdkn2a/b* locus (Fig. 4*A*). Loss of either C/EBPα or Hoxa9 binding at this locus (Fig. 4 *B* and *C*) results in a corresponding increase in *Cdkn2a/b* expression (Fig. 4 *D* and *E*). In addition, loss of either C/EBPα 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. 4 *B* and *C*). Cell cycle analysis performed on C/EBPα HM and HerM cells showed that both loss of C/EBPα and loss of Hoxa9 in Hoxa9/Meis1-transformed cells leads to G1 cell cycle blockade (Fig. 4 *F* and *G*). These data suggest that corepression of the *Cdkn2a/b* locus by C/EBPα 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. 4*H*).

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/EBPα as a critical collaborator protein of

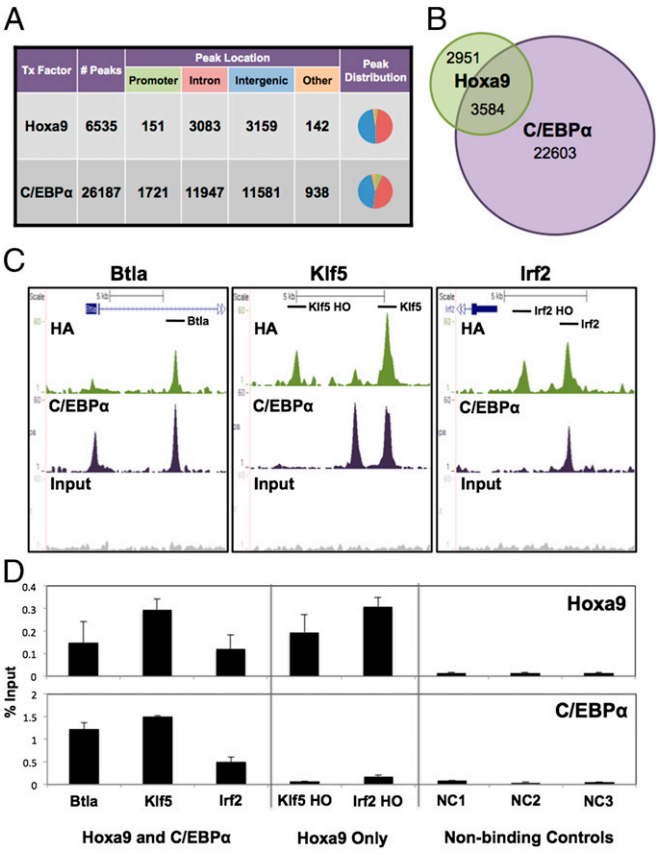

Fig. 3. C/EBPα colocalizes with Hoxa9 at promoter distal enhancers. (*A*) Peak number and distribution of Hoxa9 and C/EBPα ChIP-seq in a Hoxa9/Meis1-transformed cell line ("Other" category contains 5′/3′ UTR and exons). Tx, transcription. (*B*) Peak overlap between Hoxa9 and C/EBPα ChIP-seq. (*C*) Representative Hoxa9 (HA)/C/EBPα-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/EBPα (*Lower*) binding at Hoxa9–C/EBPα-cobound sites, Hoxa9 only, and nonbinding controls; bars indicate mean ± 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/EBPα 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/EBPα is required for the initiation of leukemias transformed by MLL-ENL, a fusion protein that directly up-regulates *HOXA9* expression (30, 31). Conversely, C/EBPα was shown to be dispensable for E2A-HLF–mediated transformation, which has undetectable levels of *HOXA9* (31). This same study also found that C/EBPα is not required for the maintenance of MLL-ENL–induced leukemias. Taken together with our finding that C/EBPα 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

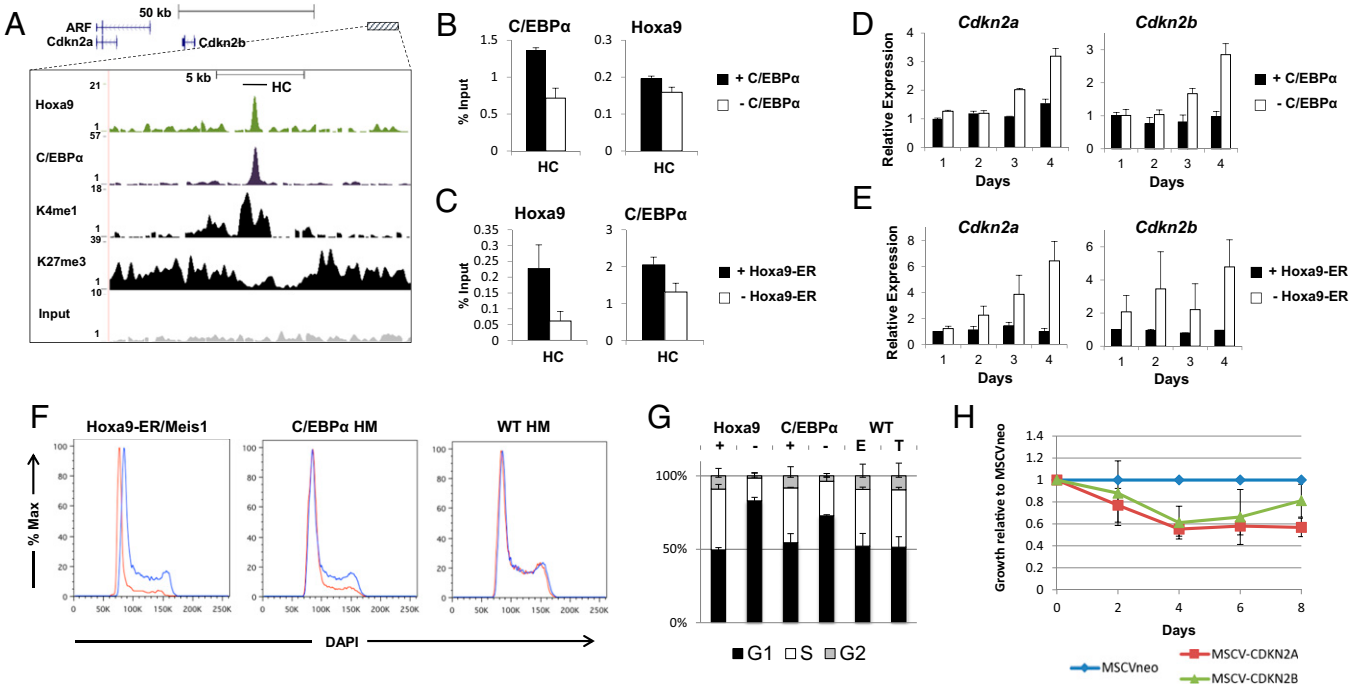

Fig. 4. C/EBPα and Hoxa9 coregulate expression of *Cdkn2a/b*. (*A*) ChIP-seq tracks for Hoxa9, C/EBPα, H3K4me1, and H3K27me3 at the Hoxa9–C/EBPα binding site 50 kb downstream of the *Cdkn2a/b* locus. Bars indicate the location of the qPCR primer set. HC, Hoxa9/C/EBPα cobound site. ChIP-qPCR for C/EBPα 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 ± 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*$^{f/f}$*;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/EBPα 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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