ROLE OF COLLABORATOR PROTEINS IN HOXA9/MEIS1-MEDIATED LEUKEMOGENESIS

by

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DEDICATION

To Mom and Dad:
who provide me with the love, encouragement and drive to dream big and never give up

To Merrin:
who is the best friend anyone could ask for

To Heiko:
who reminds me everyday how lucky we are to live and to love

And to all of my mentors, past and present:
who have inspired me and given me the opportunity to act on that inspiration

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ABSTRACT

HOXA9 is a homeodomain-containing transcription factor that plays important roles in hematopoietic stem cell proliferation and is commonly deregulated in human acute leukemias. More than 50% of acute myeloid leukemia (AML) cases have high expression levels of *HOXA9*, almost always in association with high level expression of its cofactor *MEIS1*. In a study of gene expression in human AMLs, high expression of *HOXA9* was the single most predictive marker for poor prognosis. A wide range of data suggests that HOXA9 and MEIS1 play a synergistic causative role in AML, though the molecular mechanisms leading to transformation by HOXA9 and MEIS1 remain elusive. Understanding HOXA9-mediated leukemogenesis first requires a better understanding of what confers binding specificity of HOX family proteins. All HOX proteins bind a ubiquitous AT-rich DNA motif through their highly homologous homeodomains, which alone cannot account for their tight control of transcriptional activity. Additional sequence specificity is achieved *in vivo* through association with other DNA-binding cofactors, such as MEIS1. Another level of regulation is likely conferred by diverse sets of collaborators that direct HOX protein specificity, but the identity of these proteins and the mechanisms through which they regulate HOX binding have yet to be elucidated.

Our lab has made considerable progress towards identifying potential collaborators by characterizing *in vivo* binding sites of Hoxa9 and Meis1 and by identifying proteins that interact with the Hoxa9 complex. Our studies found that Hoxa9

and Meis1 bind to evolutionarily conserved sites that contain an epigenetic signature consistent with enhancer sequences. De novo motif analysis of the binding regions showed a marked enrichment of motifs for transcription factors (TF) in the C/EBP, Ets, and Stat families. Subsequent mass spectrometry and co-immunoprecipitation experiments confirmed association of the Hoxa9 complex with C/EBPα and Stat5.

In this study I functionally establish C/EBPα as a critical collaborator required for Hoxa9/Meis1-mediated leukemogenesis. I show that C/EBPα is required for the proliferation of Hoxa9/Meis1-transformed cells in culture, and that this decrease of proliferation is not accompanied by an increase in apoptosis or differentiation of cells. Using an *in vivo* murine leukemogenesis assay, I show that loss of C/EBPα greatly improves survival in both primary and secondary models of Hoxa9/Meis1-induced leukemia. In addition, the *in vivo* assay uncovered a strong selective pressure for maintaining high C/EBPα levels in Hoxa9/Meis1-transformed cells, which could be recapitulated in cell culture systems. Finally, I found a requirement for C/EBPα in HOXA9-high human acute leukemias, as cases with double mutant alleles of *CEBPA* do not have high expression of *HOXA9.* These results provide strong evidence for C/EBPα acting as a critical collaborator of HOXA9 in acute leukemia.

To begin to identify the mechanism through which C/EBPα collaborates with Hoxa9 in leukemic transformation, I performed ChIP-seq for Hoxa9 and C/EBPα in our transformed cell lines and RNA-seq after loss of either protein. Over 50% of Hoxa9 genome-wide binding sites are cobound by C/EBPα, which coregulate a number of downstream target genes involved in the regulation of cell proliferation and differentiation. Specifically, I show that Hoxa9 represses expression of *Cdkn2a/b* in

concert with C/EBPα to overcome a block in G1 cell cycle progression. Together these results suggest a novel function for C/EBPα in maintaining the proliferation required for Hoxa9/Meis1-mediated leukemogenesis.

I have also established preliminary data with the aim to further elucidate the mechanism for collaboration between Hoxa9 and C/EBPα in leukemogenesis. Studies include characterization of the physical interaction between Hoxa9 and C/EBPα, as well as determining the functional consequence of *CEBPA* mutations in leukemias with upregulation of Hoxa9. In addition, I aim to characterize the requirement for *Cdk2na/b* repression in Hoxa9-meditated transformation and the mechanism for this co-regulation by Hoxa9 and C/EBPα. Continuing work will also study the functional interaction between Hoxa9 and other putative collaborators, including the SWI/SNF chromatin-remodeling enzyme Brg1, in hopes of gaining additional insight into the transcriptional regulatory mechanisms of HOXA9 in both normal hematopoiesis and leukemogenesis.

CHAPTER 1:

INTRODUCTION

HOX proteins are a family of homeodomain containing transcription factors that were first described in *Drosphila* for their ability to produce homeotic transformations - changing one section of the body into another - when misexpressed during development (*1, 2*). Since this early discovery, an entire field has been devoted to studying these master regulators of developmental processes and their role in disease. The 39 mammalian HOX proteins are highly evolutionarily conserved from the *Drospohila* HOMC ancestors, and are arranged in four paralogous clusters on separate chromosomes (Figure 1-1) (*3, 4*). These clusters are named A, B C and D, and are thought to have been produced through multiple duplication events during evolution (*5*). Specific *HOX* genes are numbered based on their position anteriorly to posteriorly, and paralogs of the same number share the highest degree of homology (*HOXA4*, *HOXB4*, *HOXC4* and *HOXD4*). This precise spatial organization allows for tight transcriptional control of HOX proteins during development, which is critical for establishing the anterior-posterior body plan and assigning tissue fate (*3*). When this control is altered, and *HOX* genes are misexpressed, a variety of diseases can occur. Indeed, more than 20 different malignancies have been found to involve dysregulation of various *HOX* genes.

One challenge to defining the mechanisms through which HOX proteins contribute to disease processes is the relative lack of understanding of how HOX

proteins regulate gene expression. Of critical importance is determining how individual HOX proteins regulate distinct subsets of target genes, despite sharing highly homologous DNA-binding homeodomains. Recent work suggests that HOX binding specificity is achieved through a combination of motif affinity, interactions with cofactor and collaborating proteins, and context-specific chromatin accessibility (*6*-*8*). Many studies have established that HOX proteins can both activate and repress downstream gene expression, though the mechanisms for these actions are relatively unknown. Finally, increasing efforts are being made towards identifying the most important downstream targets of HOX proteins, especially in disease. While significant progress has been made in addressing these aspects of HOX biology, additional studies will provide valuable information to guide future therapies for diseases with dysregulated *HOX* gene expression.

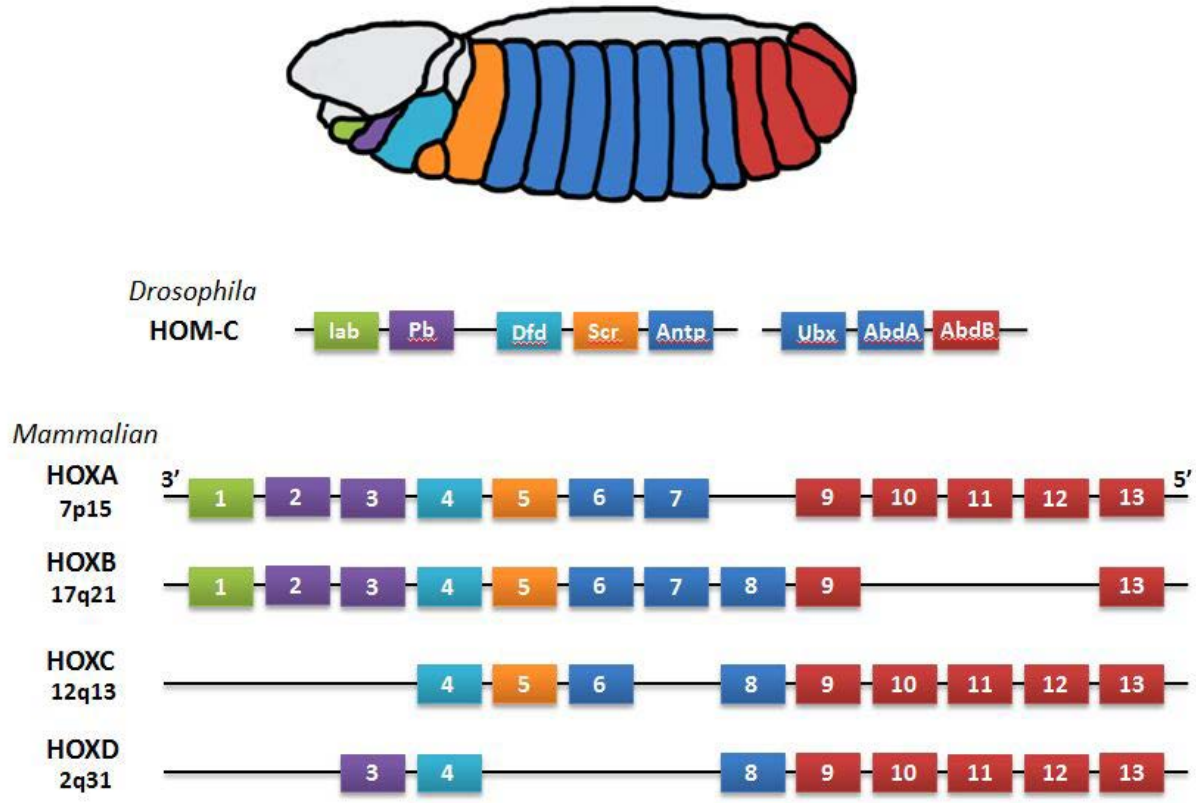

Figure 1-1 - Schematic of HOX gene organization
The 39 mammalian *HOX* genes are organized into four clusters, named A-D that share homology to the *Drosophila HOM-C* genes. These clusters reside on four separate chromosomes: 7p15 (A), 17q21 (B), 12q13 (C) and 2q31 (D). Closest homology exists between *HOX* genes of the same number across the four clusters. During embryonic development, *HOX* genes are spatially restricted in their expression with 3' genes expressed in the anterior regions and 5' genes expressed in the posterior regions. This distribution is represented by the coloration of the *Drosophila* embryo corresponding to the *HOM-C* gene expressed in that region.

NORMAL FUNCTION OF HOX PROTEINS

Regulation of *HOX* Gene Expression

During development, *HOX* genes follow both a temporal and spatial pattern of expression, such that 3' *HOX* genes are expressed earliest in the developing embryo and in the anterior regions, while 5' *HOX* genes are expressed at later stages and more posteriorly

(*3, 4*). This pattern of expression is critical for defining the various segmental identities along the anterior-posterior axis as well as for determining tissue fate. The tight regulation of *HOX* gene expression is the coordinated effort of a variety of processes including the activity of epigenetic regulators, early developmental transcription factors, long non-coding RNAs, and HOX proteins themselves (*9-11*). Additionally, it is becoming clear that the 3D localization of the *HOX* loci within the nucleus also plays a critical role in coordinating expression (*12, 13*).

The two master regulators of *HOX* gene expression belong to the trithorax and the polycomb families of histone methyltransferases, which activate and repress transcription respectively (*14*). The mixed lineage leukemia (MLL) methyltransferase positively regulate *HOX* gene expression by trimethylating histone 3 lysine 4 (H3K4me3) at *HOX* gene promoters (*15*). This activity is directly antagonized by the sequential activity of polycomb repressive complexes PRC1 and PRC2. The silencing process is initiated by PRC2 trimethylating histone 3 lysine 27 (H3K27me3) which then recruits the PRC1 complex to promote complete silencing of the *HOX* locus (*14*). The dynamic interplay between these regulatory complexes is critical for the correct expression patterning of the various *HOX* genes during development, as well as in adult tissues such as the hematopoietic system. Studies in both *Drosophila* and mice have found that, similar to mutations in individual HOX proteins, mutations in trithorax proteins/MLL can lead to homeotic transformations (*16*). In the case of *Drosophila*, these transformations can be rescued by complementary mutations in polycomb proteins (*17*). In addition, loss of MLL in mouse models leads to profound impairment of hematopoiesis (*18, 19*). As such, alterations in the activity or expression of MLL or PRCs can lead to a variety of both developmental disorders and malignancies (*20, 21*).

Along with MLL and PRC methyltransferases, the CDX family of transcription factors

also play an important role in regulating *HOX* gene expression during embryonic hematopoiesis (*22*). CDX1, 2 and 4 are members of the unclustered ParaHox class of homeobox genes that, like HOX proteins, contain a DNA-binding homeodomain (*23*). Studies in various model systems show that CDX proteins activate expression of *HOX* genes primarily in the A and B clusters, though the mechanisms for this regulation are unknown (*24-26*). Knockout mice have been generated for each CDX protein, and both CDX1-null and CDX2-heterozygous mice display homeotic transformations (*27, 28*). While CDX4 mice are phenotypically normal, studies in zebrafish have established a requirement for CDX4 in maintaining *HOX* gene expression during embryonic hematopoiesis (*29, 30*).

The *HOX* loci are also regulated by long non-coding RNAs (lncRNAs), which can also work to either activate or repress hox proteins. Both *HOTTIP*, a lncRNA expressed from the 5' region of *HOXA13* and *Hoxb5b6as,* which is transcribed from the *Hoxb5/6* locus, can interact with trithorax group proteins to maintain active transcription or their respective clusters (*11, 31*). Conversely, *HOTAIR*, *HOX* antisense intergenic RNA, is a long non-coding RNA that is transcribed from the *HOX*C locus that functions to maintain repression of the *HOXD* locus in humans (*32*). This repression is achieved by interaction of the 5' end of *HOTAIR* with PRC2, while the 3' end interacts with the histone demethylase LSD1. Like the epigenetic regulators and CDX proteins, misexpression of lncRNAs is observed in a variety of human malignancies and likely contributes to the pathogenesis of these diseases (*33*). Indeed, dysregulation of *HOTAIR*, and subsequently the expression of *HOXD* genes, is seen in a wide range of malignancies including breast, liver, lung, ovarian, colorectal, gastric, hepatocellular, esophageal and endometrial carcinomas (*32, 34-42*).

HOX Proteins In Development

HOX proteins are most well characterized in their role of defining segmental identity in the developing embryo through temporal and spatial expression patterning known as the 'HOX code' (*3, 43*). In vertebrates, this code is essential for the correct patterning of the axial skeleton (*44*). At very early stages in embryonic development, identical somites become differentiated into various morphologic identities through expression of specific *HOX* genes. HOX proteins also play important roles in patterning of the central nervous system (*45-47*), as well as development of the facial bones and other tissues of neural crest origin (*48, 49*). Furthermore, they are required for development of various tissues such as lung and airways (*50-52*), the reproductive tract (*53, 54*), heart (*55*) and kidney (*56, 57*). The specification of these various tissues requires regulation of a precise subset of target genes that define each cell type. While targeting a distinct subset of genes is a trait common to many transcription factors, HOX proteins maintain this ability despite the striking homology between family members. How HOX proteins achieve this functional diversity is a topic of ongoing research, though a significant amount of progress has been made towards this goal.

HOX Proteins in Adult Tissues

Upon completion of development, most *HOX* genes are transcriptionally silenced, though certain members of the A, B and C clusters are important regulators of adult hematopoiesis (*58, 59*). In addition, HOX proteins are important for maintaining the germline stem cell niche, a process critical to the reproductive fitness of organisms (*60*). HOX proteins have also been found to play important roles in wound healing and tissue regeneration (*61-65*).

Hematopoiesis

In the adult hematopoietic system, *HOX* genes in the A-C clusters are expressed while the *HOXD* cluster is silent (*58, 66*). Of these, *HOXA* cluster are the most highly expressed followed by *B* and much less from *C*. Expression of *HOX* genes follows a similar pattern to that in development such that anterior *HOX* genes (*HOX1-6*) are expressed in early uncommitted progenitors while posterior *HOX* genes (*Hox7-13*) are expressed in myeloid and erythroid committed CD34+ cells (Figure 1-2) (*59*). As cells become fully mature and lose CD34 positivity, *HOX* gene expression is silenced. Studies in cell lines indicate there may also be some lineage-restricted expression of some *HOX* genes. For example, while *HOXA9* and *HOXA10* are expressed primarily in myeloid cell lines, *HOXC4* is restricted to those with lymphoid characteristics (*67*). Similarly, there are multiple *HOXB* genes expressed only in erythroid cell lines (*68*). On the other hand, some genes from *HOXA-C* are expressed in all three hematopoietic lineages.

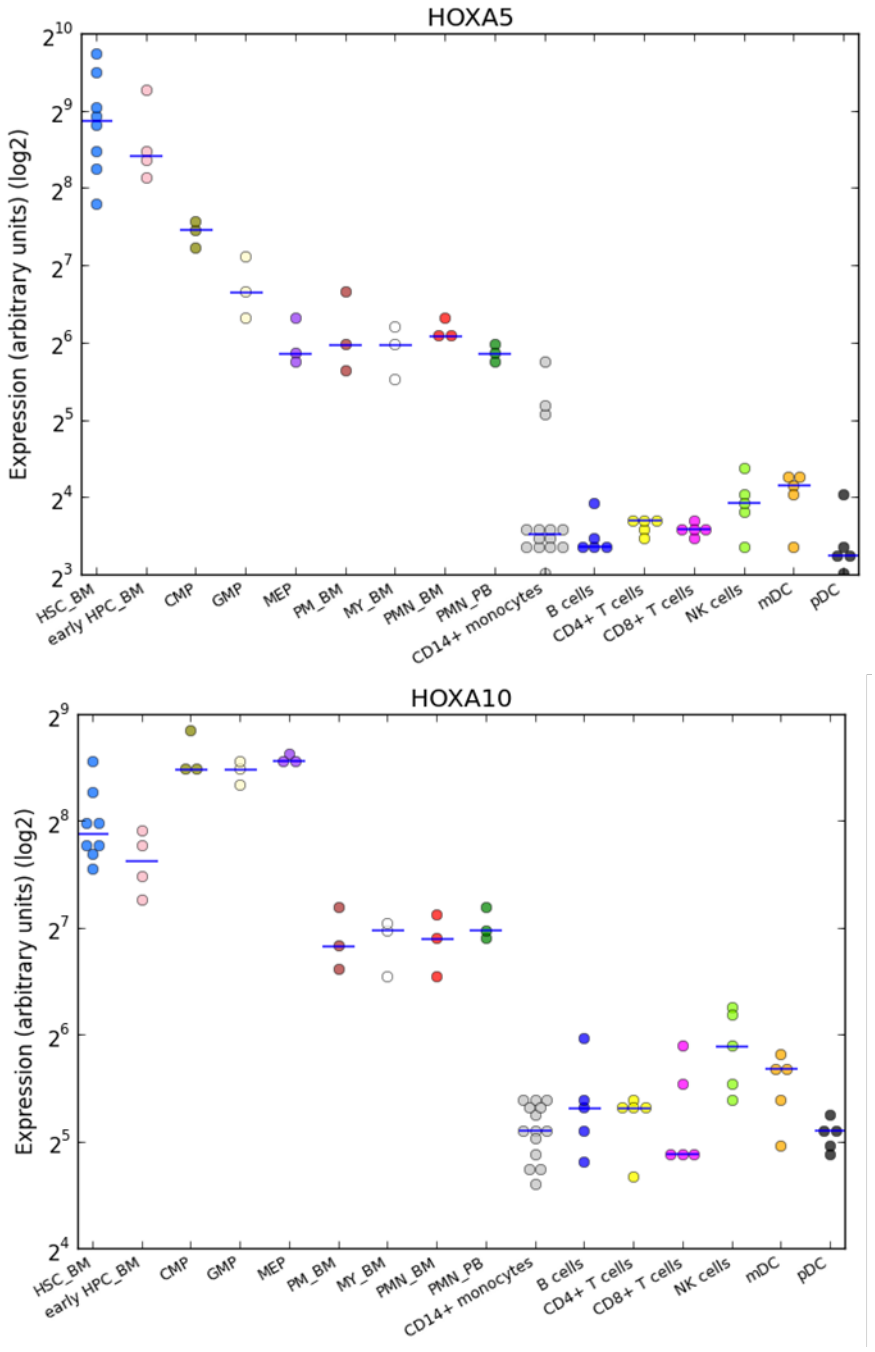

Figure 1-2 - *HOXA5* vs *HOXA10* expression during hematopoiesis
Schematic representation of the expression levels of *HOXA5* and *HOXA10* during normal hematopoietic differentiation, as determined from an online database of multiple gene expression profiles in hematopoietic cells (*69*) [Expression presented in log2 scale]. *HOXA5*, the more anterior *HOX* gene, is expressed most highly in hematopoietic stem cells and decreases rapidly during differentiation. The more posterior gene, *HOXA10*, reaches highest expression in later stages of differentiation and maintains high expression in early committed progenitors.

The concurrent expression of multiple *HOX* genes in various hematopoietic lineages may account for the functional redundancy that has been revealed in the generation of knockout mouse models for various HOX proteins. Knockout mice for *Hoxb3* and *Hoxb4* exhibit a mild reduction of bone marrow cellularity and HSC number without a dramatically changed lineage commitment of downstream cells (*70-72*). Even compound loss of *HoxB3/B4* or complete knockout of the B cluster (B1-9) has a limited effect on the ability of HSCs to repopulate the bone marrow (*70, 73*). In the case of *Hoxb3/b4* knockout mice, an increase in expression of other *Hoxb* genes is observed, while increased levels of *Hoxc* seem to compensate for the lack of *Hoxb1-9*. These studies highlight the complex gene regulatory functions of *HOX* genes in hematopoiesis and suggest that cellular context can change the behavior of these transcriptional regulators. This flexibility allows for redundancy and protection of the hematopoietic compartment. On the other hand, loss of *HOX* genes in the A and C cluster can lead to lineage skewing or more dramatic phenotypes. Loss of HOXA5, a7, b6, C4 or c8 results in defects in erythroid lineages, although loss of A5 and b6 leads to increases in erythroid progenitors while the others lead to decreases (studies done in human cells capitalized; studies done in mice lower case) [reviewed in (*74*)].

HOXA9 is the most highly expressed *HOX* gene in the hematopoietic compartment, and as such *Hoxa9*$^{-/-}$ mice display the most dramatic hematopoietic phenotype (*75, 76*). Still, loss of *Hoxa9* leads to only a mild pancytopenia and a reduction in spleen size and cellularity. Competitive repopulation assays uncover a more significant defect such that *Hoxa9*$^{-/-}$ fetal liver HSCs have reduced repopulation

capacity compared to normal HSCs (*77*).

In addition to knockout mice, overexpression studies in both murine and human systems have helped to define the potential effects of various *HOX* genes during hematopoiesis. Overexpression of *HOXA5, Hoxa9, HOXA10, Hoxb3, HOXB4* and *Hoxb6* can all lead to proliferation of the HSC compartment or early progenitors (*74*). In the case of *Hoxa9/10* and *Hoxb3/4,* overexpression can lead to myeloproliferative phenotypes in mice with progression to AML for *Hoxa9/10*. The aggressiveness of murine leukemias generated by overexpression of *Hoxa9* is increased by coexpression of its cofactor, *Meis1*, which is almost always expressed at high levels along with *HOXA9* in human disease (*78-80*).

ROLE OF HOX PROTEINS IN DISEASE

***HOX* Genes in Acute Leukemias**

The most broadly studied diseases with dysregulation of HOX proteins are acute leukemias [reviewed in (*74, 81, 82*)]. In most cases, HOX proteins are expressed at high levels in acute leukemias, which is associated with an intermediate to unfavorable prognosis in patients (*83*). In one study, the HOX protein HOXA9 was found to be the single strongest predictor of poor prognosis in a cohort of acute myeloid leukemias (*84*). A variety of upstream genetic alterations can lead to dysregulation of *HOX* genes, including *MLL*-translocations, NUP98-fusions, *NPM1* mutations, *CDX* dysregulation and MOZ-fusions.

MLL-Fusion Proteins

The MLL histone-methyltransferase is a critical regulator of hematopoiesis, and

fusion of the N-terminus of MLL with a variety of partners is seen in a subset of human acute leukemias. About 10% of acute leukemias involve chromosomal translocations at the 11q23 locus, and these cases present with aggressive disease and poor prognosis (*85*). There have been over 60 different fusion partners of MLL identified, though 90% of these translocations involve one of nine partners: AF1p, AF4, AF6, AF7, AF10, AF17, ENL, ELL, and SEPT7 (*86*). In addition, a partial tandem duplication event can occur within the N-terminus of MLL, which is observed in about 10% of cytogenically normal AML (*87*). MLL fusion proteins constitutively upregulate *HOX* gene expression, which in the case of *HOXA9* is both required and sufficient for maintaining leukemic transformation (*88*). The upregulation of *HOX* genes is directly linked to promoter trimethylation by MLL-fusion proteins, however there has also been documentation of DNA hypomethylation at HOX promoters in MLL-fusion leukemias (*89*).

NUP98-Fusion Proteins

NUP98 is a member of the nucleoporin family of proteins that complex to form multisubunit channels in the nuclear membranes. These nuclear pore complexes (NPCs) were first described for their role in facilitating transfer of metabolites and molecules between the cytoplasm and nucleus (*90*). Recent work has found that NPCs also play a critical role in defining the chromatin landscape in the nucleus and facilitating gene transcription from euchromatic regions of the genome (*91*). Nucleoporins are involved in chromosomal translocations that can lead to acute leukemias, most commonly involving *NUP98* [reviewed in (*92*)]. The most potent *NUP98* oncogenes are those fused to homeobox partners, of which there are eight (*93*). These fusions in turn lead to general upregulation of *HOX* genes, which contributes to leukemogenesis (*94*).

In addition, fusions with *NSD1* and *JARID1A* upregulate *HOXA* and *HOXB* proteins in AML and AMKL (*95, 96*). It is noteworthy that, aside from increases in *HOX* genes, these leukemias have an expression signature distinct from that of *MLL*-rearranged leukemias (*96*).

NPM1c

One of the most common genetic abnormalities in adult AML is mutation in the chaperone protein Nucleophosmin1 (*97*). While under normal conditions NPM1 resides primarily in the nucleus, mutations seen in AML result in cytoplasmic localization of NPM1 (*98*). Though the mechanisms of action are currently unknown, cytoplasmic NPM1 upregulates HOX gene expression including leukemogenic *HOXA9, HOXA10* and *MEIS1* (*99*). Studies in mice have also established that NPM1c can collaborate with *Flt3*, *Csf2* and *Rasgrp1* in vivo to produce leukemias with long latency (*100*).

Other mechanisms of* HOX *gene dysregulation

There are many other upstream genetic alterations that lead to *HOX* gene dysregulation in acute leukemia. MOZ fusion proteins can directly upregulate *HOXA9/10* and *MEIS1* by colocalizing at promoters with the histone acetyltransferase, BRPF1 (*101*). Chromosomal translocations generating the CALM-AF10 fusion protein lead to *HOX* upregulation in T-ALL (*94*). Deletions or decreased expression of polycomb protein EZH2, which plays a key role in the normal regulation of Hox proteins, can lead to leukemia with upregulation of *HOXA9* (*102*). Due to their normal function in *HOX* regulation, overexpression of Cdx proteins, in collaboration with Meis1, leads to leukemias with high levels of *Hox* expression (*103, 104*). Also, transformation by MN1 requires *HOXA9* and *MEIS1*, though MN1 alone cannot activate expression of these

targets, suggesting that *HOXA9* and *MEIS1* must be expressed in the cell of origin (*105*). Similarly, *Hoxa9* collaborates with *E2A-PBX1* to repress B-cell genes and activate Flt3 in murine B cell leukemia (*106*).

Other Hematopoietic Diseases

HOX proteins are also dysregulated in other hematopoietic diseases. In chronic lymphoid leukemia (CLL) and mantle cell lymphoma (MCL), *HOX* genes are repressed by chromatin and DNA hypermethylation (*107, 108*). In MCL, this repression is the result of increased EZH2 expression, which leads to chromatin methylation and subsequent recruitment of DNA methylation machinery leading to a stable repression of key *HOX* targets (*107*). Finally, *HOXA* genes along with *HOXB7* are expressed at high levels in multiple myeloma (*109*).

***HOX* Genes in Other Malignancies**

In addition to leukemias, there are a variety of other malignancies that have dysregulated *HOX* gene expression. These include prostate, breast, ovarian, pancreatic, colon, and gastric cancers as well as thyroid, bladder, hepatocellular carcinoma and some neurologic tumors (*56, 110-119*). Due to their central role in development, HOX genes are also dysregulated in non-neoplastic and developmental disorders. Mutations in *HOXD13* lead to synpolydactyly, resulting in both the fusion of digits and supernumerary digits (*120, 121*). Furthermore, alterations in *HOXA1* can lead to congenital heart defects, and misexpression of distal *HOX* genes like *HOX10* and *HOX11* can lead to caudal duplication syndrome (*122, 123*).

Prostate, Breast and Ovarian Cancers

A considerable amount of work has been done studying the dysregulation of

HOX genes in prostate and breast cancers. In prostate cancer, expression of a number of different *HOX* genes is increased and use of the HOX-PBX blocking drug HXR9 has been shown to be effective for slowing tumor growth (*124*). On the other hand, expression of HOX cofactors in the MEIS and PBX families are decreased, suggesting that they may act as tumor suppressors in prostate cancers (*125*). Similarly in breast cancer, *HOX* genes can have either increased or decreased expression. Many *HOX* genes are hypermethylated and silenced in breast cancer (*126*). On the other hand, *HOXA7* has been shown to upregulate ER expression and stimulate proliferation (*127*), while *Hoxb13* downregulates ER expression leading to tamoxifen resistance and increased proliferation by activating mTOR via Stat3 (*128*). *HOXD3* is also expressed at high levels in some hormone receptor negative breast cancer and is an unfavorable prognostic marker (*129*). Finally, in ovarian cancer there have been reports of decreased *Hoxd10* expression through upregulation of microRNA mir-10b (*39, 130*).

Cancers of the Gastrointestinal System

HOX genes are involved in cancers throughout the gastrointestinal tract. There are many reports of dysregulated histone and DNA methylation across the *HOX* gene loci in oral squamous cell carcinoma (*131-133*). Increased *HOXD3* expression is observed in some hormone receptor negative breast cancer and is an unfavorable prognostic marker (*134*). In Barrett's esophagus, a precancerous disease that can progress to esophageal cancer, there is increased expression of *HOXB5-7* (*135*). *HOXA13*, *HOXC6* and *PBX3* are all expressed at high levels in gastric cancer, correlating with more aggressive phenotypes and shorter overall survival (*136-138*). On the other hand, there are reports of *HOX* gene silencing in gastric cancer as a result of

promoter hypermethylation (*139*). *HOXA13* has also been shown to be upregulated in hepatocellular carcinoma (*112*), while *HOXA4* and *HOXD10* are upregulated in colon cancer (*140*). Finally, *HOX* genes are known to be dysregulated in pancreatic cancer and other MEN1 tumors (*141, 142*).

It is clear from the wide variety of diseases with dysregulated *HOX* expression that understanding the general mechanisms of downstream transcriptional regulation by HOX proteins will shed light on disease mechanisms and provide novel pathways for therapeutic design. While the dysregulation of *HOX* genes can be achieved through multiple mechanisms, and both loss and gain of HOX function can lead to disease, defining the common and unique characteristics of HOX protein function will undoubtedly shed light on general principles of HOX biology that can be applied to a range of diseases with misregulated *HOX* expression.

MECHANISMS OF HOX-REGULATED GENE TRANSCRIPTION

HOX genes carry out their highly specialized function of developing the body plan by being extraordinarily spatially restricted in their expression, but also by regulating a distinct subset of target genes in tissue-specific manners. It is becoming clear that these functions are most likely achieved through association at promoter distal, lineage specific cis-regulatory elements (*8, 143*). Understanding how HOX proteins can target these sites to allow for precise control of downstream gene expression is challenging. All HOX proteins share a highly homologous DNA binding homeodomain, which alone cannot account for the distinct subpopulations of target genes. Additional sequence specificity is achieved through association with other DNA-binding cofactors, the most

well characterized being members of the three amino acid loop extension (TALE) family. It is true, however, that these factors alone cannot explain the differences in the binding pattern of HOX genes, and indeed some HOX binding is independent of both MEIS and PBX proteins. A final level of regulation is likely conferred by diverse sets of collaborator proteins that direct HOX protein specificity through DNA dependent and independent interactions. These lineage specific collaborator proteins may function to establish areas of chromatin accessibility in a given cell type and recruit and stabilize Hox proteins to various loci. Furthermore, the downstream activity of HOX protein binding to activate or repress target gene expression may be modulated by the association of various cofactors and collaborators. Below we will discuss what is known about DNA binding properties of HOX proteins and known binding partners that confer specificity to HOX proteins, with a focus on recent advances in the field.

Factors contributing to HOX DNA-binding

Homeodomains

The homeobox family of transcription factors is defined by the presence of a DNA binding homeodomain, which is highly homologous within the 39 mammalian HOX proteins and similarly conserved across species. Early studies have found that this 60-amino acid region makes direct contact with DNA via 4 critical amino acids - aa47, 50, 51, and 54 - within the third alpha helix of the homeodomain (*144*). Interestingly, nearly all homeodomains contain the same residues in these critical positions (*145*). Even so, studies have found that the small differences in homeodomains themselves can confer unique properties to HOX proteins (*146*). For example, swapping the homeodomains of Hoxa1 and Hoxa9 conferred leukemogenic properties to Hoxa1 while abolishing those

of Hoxa9 (*147*). This phenomenon required the presence of the N-terminal region and Pbx interaction motif, though these regions were interchangeable between Hoxa1 and Hoxa9. Differences in the homeodomain regions of Hoxd10 and Hoxd11 are also critical for separating their function during motor neuron fate identity (*148*). In addition, a region very close to the homeodomain of HoxD proteins, which may be regulated by phosphorylation, has been found to be required for the rib-repressing function of the HoxD proteins (*149*). Finally, work in Drosophila has characterized specific protein motifs in the homeodomains of Scr, Abd-A and Ubx that account for different DNA binding abilities of these three Hox proteins (*150*). Interestingly, the contributions of the homeodomain to specific phenotypes may also be the result of interaction with different collaborator proteins, as this region has been found to mediate protein-protein interactions in addition to DNA-binding. Indeed Cdx1 and Foxo1a can interact with homeodomain regions of Hox proteins, and the work in this thesis also suggests that C/EBPα interacts with the homeodomain region of Hoxa9 (*151, 152*).

Motif Affinity

Comprehensive studies using both bacterial 1 hybrid approach in *Drosophila* and protein-binding microarray approaches in murine cells established that all HOX homeodomains bind highly similar AT-rich DNA motifs (Figure 1-3) (*153-155*). While differences exist between the *in vitro* and *in vivo* results, this likely reflects the importance of cofactor and collaborator proteins in directing motif-binding preferences. In Drosophila, this TAATNA motif occurs over 100,000 times throughout the genome, and thus cannot explain the distinct subsets of target genes for each HOX protein (*154*). Therefore, cells must employ a variety of mechanisms to help direct HOX proteins to the

appropriate regulatory regions. Some target genes are in fact regulated by all or multiple HOX proteins, allowing for flexibility in binding site affinity for these targets. In other cases, posterior HOX proteins show a competitive advantage in cofactor-dependant DNA binding at shared loci, a property called posterior prevalence (*156*). Conversely, gene regulation specific to a single HOX protein likely results from the combination of motif affinity of that particular homeodomain, as well as the chromatin accessibility landscape and subset of expressed cofactors and collaborators in the cellular context in which that HOX protein is expressed (*157, 158*). Work has successfully characterized cognate binding motifs of HOX proteins complexed with PBX cofactors (*159*). An interesting example of the contribution of the motif itself can be seen in snakes where single nucleotide polymorphisms in a Hox/Pax enhancer changes the affinity of Hox10 vs Hox6 in this region, leading to the continuation of ribs in the thoracic region (*160*).

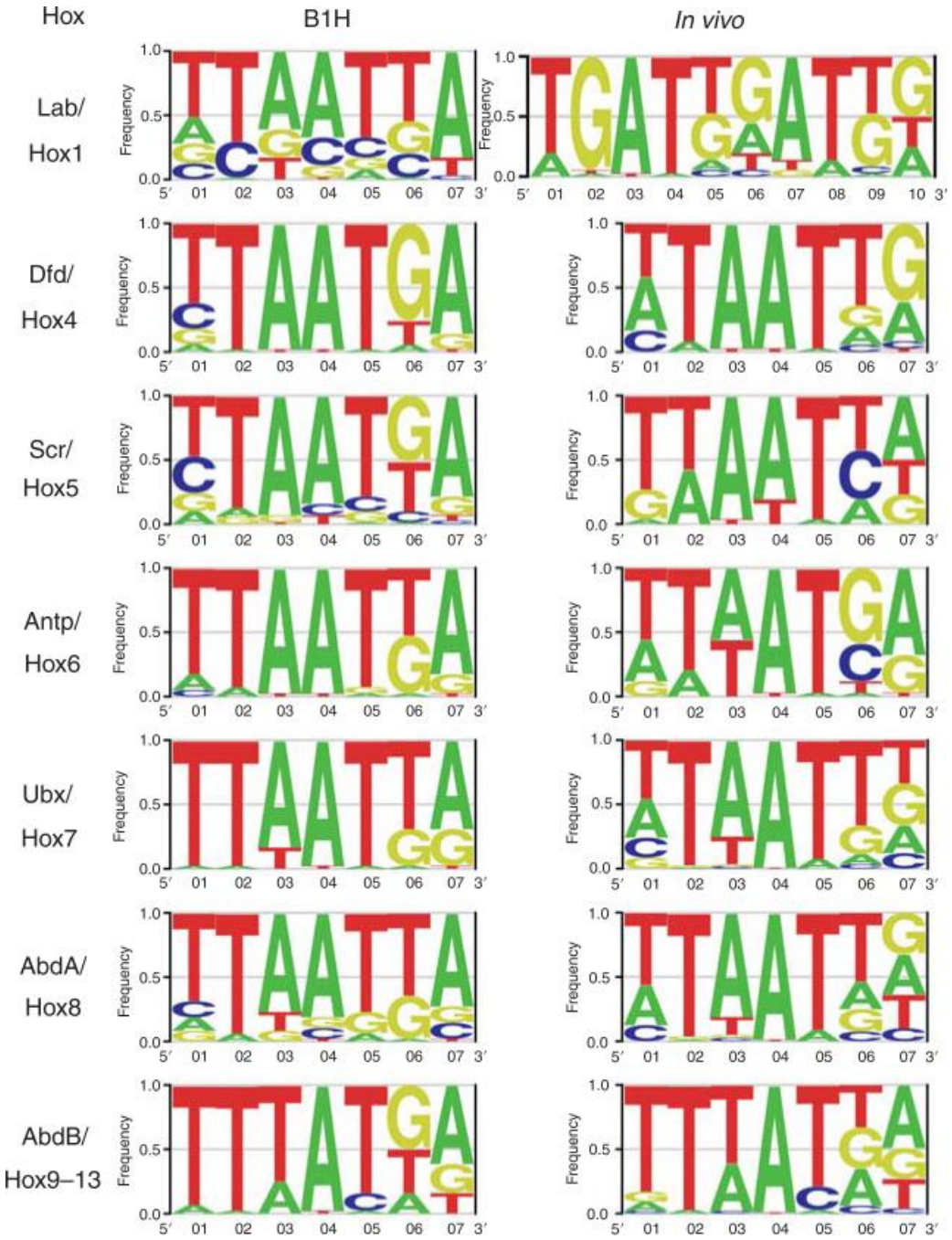

Figure 1-3 - Summary of HOX protein DNA motif recognition
Summary of HOX protein binding site preferences as established by both *in vitro* and cell based (*in vivo*) studies. *In vitro* study performed by yeast 1-hybrid approach (left column) and *in vivo* study generated from literature review of cell based studies of HOX binding motifs. All HOX proteins bind similar “AT”-rich DNA motifs. Original figure and experimental detail in (*154*).

Chromatin Accessibility

There have been recent advances in both HOX biology and general transcription factor biology that suggest that targeting of TFs to specific loci is significantly affected by the chromatin accessibility throughout the genome. In the case of HOX proteins, ChIP of *Drosophila* Hox proteins Utx and Hth across various stages of development indicates that binding profiles are strongly influenced by chromatin accessibility (*157, 158*). In the hematopoietic system, early factors like PU.1 and C/EBPα establish areas of relaxed chromatin that allow for signaling-dependent recruitment of TFs (*161*). This is likely mediated by SWI/SNF chromatin remodelers, which are known to interact with the CEBP proteins (*162*). The binding profiles of HOX proteins in various tissues are probably similarly influenced by the genomic landscape set up by other lineage-specific transcription factors, allowing for a distinct subset of targets to be activated in a given cell type despite the conserved DNA-binding homeodomain and ubiquitous TAATNA motif.

HOX protein interacting partners

Cofactors

It is well established that Hox proteins bind to DNA and regulate downstream gene expression along with a small subset of cofactor proteins (*163*). The best characterized cofactors are members of the Three-amino-acid-loop-extension, or TALE family of proteins (*164*). Mouse TALE proteins include Pbx1-4, Meis1-3 and Prep1-2. In addition, Hox proteins can homo and heterodimerize to aid in diversity and specificity of binding (*165*). Whether Hox proteins bind along with Meis or Prep proteins subdivides clusters of binding sites (*166*). These cofactors may play a role in directly targeting Hox

proteins to specific loci, as opposed to binding in a pre-formed complex and conferring site specificity. Recent work shows that TALE factors bind first at some promoters and promote the deposition of poised chromatin marks, whereby subsequent binding of HOX proteins results in transcriptional activation (*167*). Additional studies have established that while Hox proteins often bind along with TALE cofactors, these interactions are not required at some loci (*168*). In fact, there is new evidence of antagonism between TALE proteins and HOX proteins at specific genomic regions (*169*). In the setting of leukemia, however, the critical targets seem to be those co-regulated by HOXA9 and MEIS1, as cofactor MEIS1 is almost always expressed at high levels along with HOXA9 and is required for producing an aggressive leukemia in mice (*78-80*).

In a comprehensive review of cis-regulatory elements directly regulated by HOX proteins, three interesting generalizations were uncovered about the contribution of different cofactors (*154*). First, the majority of sites cobound by HOX and PBX proteins were activating, suggesting that complexes containing HOX/PBX may be primarily transcriptional activators. Second, anterior HOX proteins (1-5) were more likely to bind along with a PBX cofactor than members in the posterior group (6-13). Third, sites that did not cobind with a PBX protein tended to have more than one HOX binding site, suggesting that HOX proteins may homo or heterodimerize at these sites.

Collaborating Proteins

In a seminal review, Richard Mann proposed that context-specific collaborator proteins may provide a final level of specificity to HOX complexes to allow for their context-specific actions in controlling downstream gene expression (*154*). These tissue

specific interactors would bind along with HOX proteins and TALE cofactors to establish areas of chromatin accessibility, provide stability in DNA binding and help modulate the downstream activity of HOX complexes (*170*). Recent studies have focused on identifying potential collaborator proteins in a variety of systems. In *Drosophila*, a technique called bimolecular fluorescence complementation has been used to identify partners (*170-172*). Yeast two hybrid approaches have also looked at binding partners for Hoxa1 and Hoxa9 ((*173*) and unpublished). In addition, our group has identified interactors of Hoxa9 in transformed myeloblastic cell lines using co-immunoprecipitation followed by mass-spectrometry (*174*). With these various approaches, some themes in collaborator proteins are surfacing. Many seem to be lineage specific factors known for general priming of enhancer regions of the genome. Still others are involved in signal transduction. It is important to highlight that although there may be great diversity in HOX complexes to allow for the distinct functions in various contexts, HOX proteins are central and essential to correct functioning. Loss of a single HOX protein can lead to dramatic changes at the level of the organism, highlighting the fact that the HOX proteins themselves are the master regulators of these transcriptional complexes.

Enzymes/Machinery

HOX proteins and HOX complexes most likely control downstream gene expression through the recruitment of histone modifying machinery. Both Hoxa9 and Meis1 have been shown to recruit the histone acetyltransferase p300/CBP to mediate activation of downstream targets (*175, 176*). Both activation and repression domains in Hoxa10 (and other Hox10 proteins) have been defined that facilitate interaction with CBP and the histone deacetylase HDAC2 respectively (*177, 178*). Recent work also

established that Hoxa9 interacts with the histone methyltransferase G9a, and that this interaction is required for aggressive disease in mouse models of leukemia (*179*). HOX proteins can interact with other enzymes and machinery leading to their own modification and alterations in cellular processes. Both HOXB7 and HOXA7 can bind to PARP-1, leading to their subsequent ADP-ribosylation and a decrease in transcriptional activity (*180*). Other HOX proteins can also be ribosylated in this manner, but without affecting their ability to interact with DNA.

TRANSCRIPTIONAL TARGETS OF HOX PROTEINS

Of equal importance as understanding the mechanisms through which HOX proteins achieve target specificity is determining the critical downstream targets of HOX proteins and the mechanisms through which HOX proteins regulate the downstream transcriptional activity. Many efforts have been made using both genome-wide approaches and site-specific experiments for identifying these important targets in development and disease. Continued work in this area will allow for a deeper understanding of HOX biology and help to guide therapy for diseases in which HOX proteins play a central role.

General Discussion of HOX Target Regulation

Transcriptional regulation of target genes downstream of HOX proteins during development can follow two different patterns. In some cases, HOX proteins first activate transcription factors and members of signalling cascades, which mediate additional downstream gene expression required for tissue specification. In other cases, HOX proteins directly modulate the expression of master regulators of tissue specificity.

The difference in these two modes of regulation is most likely due to the transcriptional landscape in the cell type where each HOX protein is expressed, thus governing their activity. Indeed, in a thorough study of the downstream targets of Ultrabithorax (Ubx) during different stages of *Drosophila* haltere development, authors showed that the majority of Ubx targets were specific to the stage in which Ubx was activated and only about 10% were common between all three stages studied (*181*). In addition, the study found that the majority of expression changes induced by Ubx expression were small (less than 4-fold) suggesting that HOX proteins induce subtle changes in transcriptional networks and may act to set the stage for larger expression changes upon binding of additional activators and repressors. There are certainly cases of the classic recruitment of general transcriptional machinery by Hox complexes to modulate downstream gene expression (*182*). Interestingly, there are reports that suggest HOX proteins may regulate expression through competitive binding to block other TF complexes at shared cis-regulatory regions (*183*).

Specific Targets

While many target genes of HOX proteins have been inferred using microarray analysis of different tissue types, recent advances in genome-wide sequencing techniques have allowed for identification of direct targets of HOX proteins. ChIP-seq of Hoxa2 during embryonic development in mice identified many Wnt target genes (*184*). Our group performed ChIP-seq in a mouse myeloblastic cell line transformed by Hoxa9/Meis1 and identified multiple proleukemic targets, including *Erg, Flt3, Lmo2 and Myb,* that are directly regulated by Hoxa9 (*185*). Regulation of Lmo2 has also been observed during development of the limb bud. Importantly, knockdown of Lmo2 impairs

growth of leukemic cells and high levels of *LMO2* predict poor prognosis in patients (*186*). Additional specific targets of HOX genes have been studied in the context of leukemia. HOXA9 activates *BCL2* expression, which is required for transformation by HOXA9, NUP98-HOXA9 and MLL. Furthermore, loss of *Bcl2* leads to improved survival in mouse model of Hoxa9/Meis1 leukemia (*187*). MLL-ELL upregulates Fgf2 expression in a Hoxa9/a10 dependent fashion, leading to increased proliferation and cytokine hypersensitivity (*101, 188*). Other work has identified a positive feedback loop between Hoxa10 and Cdx4-Fgf2-β-catenin (*189, 190*). Hoxa9 can regulate Ink4a expression to overcome oncogene-induced senescence during transformation by AML1-ETO in *Bmi1*-/- cells (*191*). Finally, Hoxa10 upregulates expression of the E3 ubiquitin ligase Triad1 leading to a decrease in colony forming activity of myeloid cells, thereby preventing hyperproliferation and cellular exhaustion during transformation (*192*).

Some additional targets of HOX proteins have been identified during development processes. Hoxa1 and Hoxa9 regulate Rac1 activity by directly upregulating Vav2 expression (*193*). Hoxa/b/c5 have redundant roles in repressing Shh expression in developing forelimb, mediated by binding at an enhancer and interaction with promyelocytic leukemia zinc finger (*194*). Lastly, Hoxa2 binds at the promoter of Meox1 to activate its transcription during development of the second branchial arch (*195*).

Non-transcriptional roles of HOX Proteins

In addition to acting as classical transcription factors regulating downstream gene expression, HOX proteins may also have non-transcriptional functions that are critical for their role in malignancy (*196*). For example, Hoxa2 can indirectly stabilize p53 by

binding to p53's E3 ubiquitin ligase, RCHY1, leading to the degradation of RCHY1 (*197*). In addition, Hoxb4 and Hoxa9 can act as E3 ligases for Geminin, leading to its degradation, which contributes to Hoxa9-mediated transformation (*198, 199*). Conflicting reports, however, also find that Hoxa9-Geminin binding can sequester Hoxa9 thereby inhibiting its transcriptional activity (*200*). Hoxa7 and Hoxa14 can bind to the initiation factor eIF4E in liver cancer, potentially affecting the nuclear transport of eIF4E-dependent transcripts like *c-myc, fgf2, vegf*, ornithine decarboxylase and cyclin-D1 (*201*). Finally, the yeast-two-hybrid screen of Hoxa1 interactors identified many putative binding partners involved in signal transduction, cell adhesion and vesicular trafficking, pointing to additional non-transcriptional roles for this and other HOX proteins (*173*).

RECENT ADVANCES

As we continue to learn more about the vast number of maliganancies and diseases that involve dysregulation of *HOX* genes, the need for a deeper understanding of the mechanisms through which HOX proteins exert their function is becoming critical. Understanding the mechanisms of action of HOXA9 needs to be at the forefront of these efforts, as increased expression of HOXA9 is seen in over 50% of acute myeloid leukemias and these cases usually carry a poor prognosis (Figure 1-4) (*79, 84*). Our group has made considerable progress towards understanding HOXA9-mediated leukemogenesis through the identification of the genome-wide binding sites of Hoxa9 and Meis1 in transformed myeloblastic cells, as well as the identification of a number of potential collaborators that are members of the myeloid Hoxa9 complex (*174*). ChIP-seq experiments using murine bone marrow transduced with Hoxa9 and Meis1 identified

825 genomic regions that bind Hoxa9, Meis1 or both, and these regions showed a high degree of evolutionary conservation. Over 90% of the binding sites are located in distal intergenic regions (>10kb from transcriptional start sites) or gene introns, while less than 3% are located within 3kb of promoter regions (Figure 1-5a). *De novo* motif analysis of Hoxa9/Meis1 (H/M) binding sites uncovered a striking enrichment for lineage-specific transcription factor motifs in the ETS and C/EBP families near the H/M peaks, and to a lesser extent, STAT and RUNX (Figure 1-5b), suggesting that these proteins might coassociate with Hoxa9/Meis1 at distal regulatory elements.

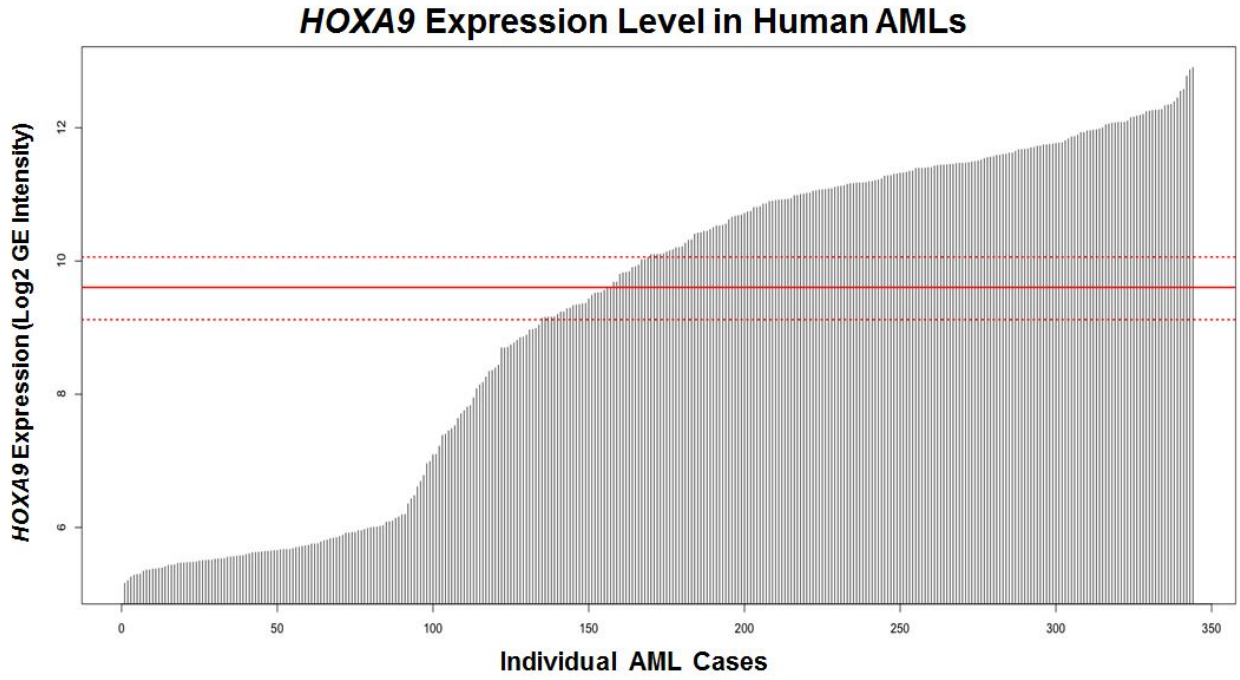

Figure 1-4 - *HOXA9* expression in AMLs compared to healthy controls
In a cohort of 344 human acute leukemias, 50.87% (n=175) have *HOXA9* expression levels two standard deviations higher than healthy controls (n=11) [mean=9.6, SD=0.23, mean+2SD=10.06]. Plot of individual patient sample *HOXA9* expression levels with solid line mean of healthy controls, dotted line ±2SD. Data reanalyzed from (*202*).

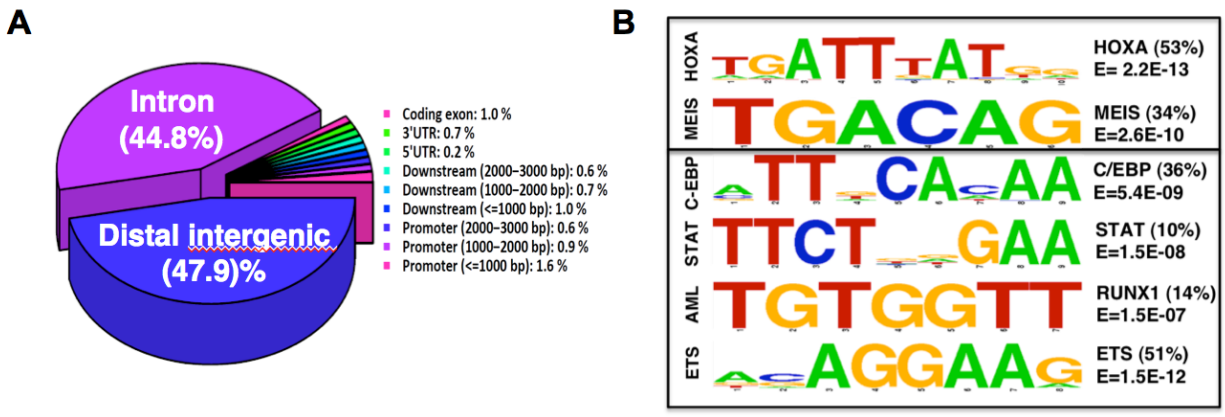

Figure 1-5 - ChIPseq for Hoxa9 and Meis1 in murine myeloblastic cell line
Hoxa9 and Meis1 genome-wide binding sites were determined in replicate ChIP-seq experiments for each protein in murine cells transformed by Hoxa9 and Meis1. (A) Distribution of genomic localization of Hoxa9/Meis1 binding sites. (B) De novo motif analysis of Hoxa9/Meis1 binding sites and corresponding enrichment statistics.

To complement the ChIP-seq data, our group also performed a parallel co-immunoprecipitation and mass spectrometry screen to identify leukemia-specific collaborators of Hoxa9 and Meis1. The transcription factors C/EBPα and Stat5b were both identified in this binding partner screen, along with the chromatin-remodeling enzyme Brg1 and multiple other members of the SWI/SNF complex (Figure 1-6a). These interactions were confirmed by western blot, as well as in overexpression studies in 293 cells (Figure 1-6b and data not shown). Interestingly, each of these putative collaborators are known to be mutated or otherwise dysregulated in leukemia, providing further basis for studying their functional interplay with HOXA9 (*203-205*). In the work presented in this thesis, I used both loss and gain of function approaches to determine the role of C/EBPα in Hoxa9/Meis1-mediated leukemogenesis. I also began preliminary characterization of importance of the SWI/SNF complex and chromatin remodeling in transcriptional regulation by Hoxa9. My work presents significant advances in our understanding of HOX biology in leukemic transformation by establishing a requirement

for a lineage determining transcription factor as well as implicating a role for chromatin remodeling at Hoxa9 target sites. The data presented in this thesis will serve as a foundation for future studies that will continue to increase our understanding of Hoxa9-mediated transcriptional regulation in normal hematopoiesis and leukemia.

A

IP	Protein ID	Pw	Specs	Peps
Hoxa9-1	Stat5b	1.000	6	4
Hoxa9-2	Stat5b	1.000	11	7
Hoxa9-2	C/ebpa	0.919	2	1
Meis1-1	C/ebpa	0.949	3	1

B

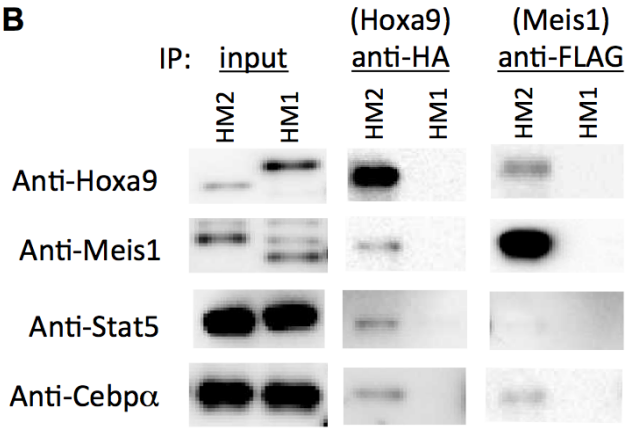

Figure 1-6 - Stat5b and C/EBPα are members of the myeloid Hoxa9/Meis1 complex Identification of putative collaborator proteins using co-immunoprecipitation of Hoxa9 or Meis1 followed by mass spectrometry. (A) Mass spectrometric identification of Stat5b and C/EBPα in replicate immunoprecipitations with Hoxa9/Meis1 (labeled Hoxa9-1/Hoxa9-2 or Meis1-1/Meis1-2). (B) Coimmunoprecipitations of Stat5b and C/EBPα with Hoxa9/Meis1 in cells transformed with HA-Hoxa9/FLAG-Meis1 (HM2) or untagged Hoxa9/Meis1 (HM1) as controls. Abbreviations as follows: IP, Immunoprecipitation; ID, identification; Pw, predicitive value; Specs, spectrums; Peps, peptides.

CHAPTER 2:

C/EBPA IS AN ESSENTIAL COLLABORATOR IN HOXA9/MEIS1-MEDIATED LEUKEMOGENESIS

INTRODUCTION

HOXA9 is member of the highly conserved HOX protein family of transcription factors, which play key roles in both development and hematopoiesis (*3, 81*). *HOXA9* is most highly expressed in long-term hematopoietic stem cells (LT-HSCs) and early progenitors, where it promotes cellular proliferation and is subsequently down-regulated during differentiation (*82*). More than 50% of acute myeloid leukemia (AML) cases show up-regulation of *HOXA9*, which correlates strongly with poor prognosis (*84, 206*). In most cases, up-regulation of *HOXA9* is accompanied by up-regulation of its cofactor *MEIS1*, which co-localizes with Hoxa9 at enhancers (*79, 185*). While HOXA9 alone is sufficient for transformation of hematopoietic stem cells in culture, the addition of MEIS1 increases transformation efficiency and results in rapidly fatal leukemias in transplanted animals (*78*).

A variety of upstream genetic alterations, including MLL translocations, NPM1 mutations, and CDX2 over expression, lead to *HOXA9* up-regulation in AML, however the mechanisms through which high levels of *HOXA9* contribute to leukemic transformation are not known (*207-209*). 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 (*154*). 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 (*185*). One of these putative collaborators is 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 (*210*). While *Cebpa*$^{-/-}$ mice show complete loss of the granulocytic compartment, recent work shows that loss of C/EBPα in adult hematopoietic stem cells (HSCs) leads to both an increase in the number of functional HSCs and an increase in their proliferative and repopulating capacity (*211, 212*). 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 (*213, 214*).

While 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 (*161*). The ability of C/EBPα 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, I used models allowing for conditional deletion of *Cebpa*

in Hoxa9-transformed cells. I 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, I found that C/EBPα co-localizes with Hoxa9 at promoter distal enhancers resulting in both target gene activation and repression. Finally, I 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.

MATERIALS AND METHODS

Animals. All animal experiments were performed as approved by the University of Michigan Committee on Use and Care of Animals and Unit for Laboratory Animal Medicine. *Cebpa*$^{f/f}$ mice ((*211, 212*); kindly provided by Dr. Daniel Tenen, Harvard University) or C57BL/6 *WT* mice (JAX #000664) were crossed with B6;129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT)Nat}$ (JAX #004847) to obtain *Cebpa*$^{f/f}$*;CreERT*$^{+/-}$ and *WT;CreERT*$^{+/-}$ strains.

Antibodies. For western blot, anti-C/EBPα (CST 2295S) and anti-βactin (Sigma A2228) were used. For ChIP, anti-HA (Abcam ab9110), anti-C/EBPα (SCBT sc-61X), anti-H3K4me1 (Abcam ab8895), anti-H3K27me3 (Millipore 07-449) and IgG (SCBT sc-2027) were used. For flow cytometry, APC-anti-cKit (Biolegend 105812), APC-anti-CD11b (eBioscience 47-0112-80), APC-anti-Gr1 (Biolegend 108412), APC-anti-AnnexinV (eBioscience 88-8007-74) and DAPI (Sigma) were used.

Cell Lines. Bone marrow from 6-10 week old *Cebpa*$^{f/f}$*;CreERT*$^{+/-}$*, WT;CreERT*$^{+/-}$*,* or *WT*

mice was harvested 5 days after treatment with 5-flurouracil (150mg/kg) and $Lin^{-}cKit^{+}$ cells were isolated using the EasyStep Mouse hematopoietic progenitor cell enrichment kit (Stem Cell Tech). Cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 15% fetal bovine serum, 10ng/mL IL3 and 100ng/mL SCF. One day after harvest, cells were retrovirally transduced on two consecutive days with MIGR1-HA-Hoxa9 or MIGR1-HA-Hoxa9-ER, and MIGR1-Flag-Meis1 retrovirus expressing murine proteins (plasmids previously described in (*185*)). 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 days. 100% GFP positivity was subsequently verified using flow cytometry and co-expression of Hoxa9 and Meis1 was confirmed by western blot. Cell lines transduced with MIGR1-HA-Hoxa9-ER/MIGR1-Flag-Meis1 (HerM) were cultured in continuous 100nM 4-hydroxytamoxifen (4-OHT) to maintain transformation.

Cellular Assays. For loss of C/EBPα studies, C/EBPα HM or WT HM cells were treated continuously with 5nM 4OHT (Sigma H7904) or EtOH. For the 4OHT withdrawal experiment, after one week continuous culture in EtOH or 5nM 4OHT, C/EBPα HM cells were washed once with culture medium, and continued in cultured with EtOH only. For loss of Hoxa9 studies, HerM cells were washed three times with culture media and then maintained in either EtOH or 100nM 4OHT. Cellular proliferation was assessed by trypan blue dye exclusion and cell counting. Cellular morphology was assessed using cytospin and Wright-Geimsa staining. Whole cell lysates were collected by directly

lysing washed cells in SDS loading buffer + beta-mercaptoethanol. Protein levels were visualized using SDS-polyacrylamide gel electrophoresis and western blotting on PVDF membranes. RNA was collected and purified using the Qiagen RNeasy kit with on-column DNase treatment. cDNA was generated using Superscript II RT and target gene expression was determined relative to β-actin using Invitrogen Taqman primer-probe sets (*Cdkn2a* [Mm00494449_m1]; *Cdkn2b* [Mm00483241_m1]; *Cebpa* [Mm00514283_s1]; *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 mins on ice with 0.2ug of the appropriate antibody. For apoptosis assay, cells were washed and resuspened in binding buffer, and subsequently incubated at room temperature for 15mins with 0.6ug APC-anti-AnnexinV and DAPI. For cell cycle analysis, cells were washed, resuspended in ice cold DPBS and added dropwise to cold 70% EtOH. Cells were stored for at least 24hrs at -20°C. After storage, cells were washed with cold DPBS, rehydrated for 30mins on ice in DPBS, and subsequently treated with RNase A (Qiagen 19101) and DAPI at room temp for 20mins. All samples collected on a Becton Dickinson LSR II. Data collected from at least 20,000 events from biologic replicate experiments were analyzed using FloJo.

BM 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 rads) ~8 week old female C57BL/6 mice ($1.5x10^{\wedge 5}$ cells per mouse).

Mice were maintained on antibiotics for two weeks post irradiation. At two weeks, mice were treated with biweekly intraperitoneal injections of OHT (Sigma T5648; 200mg/kg) or corn oil until sacrifice. 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 sublethally irradiated (600 rads) ~8 week old female C57BL/6 mice ($1.5x10^{\wedge 5}$ cells per mouse). After 5 days, mice were treated for 5 consecutive days with intraperitoneal injections of OHT (200 mg/kg) or corn oil and continued on twice weekly injections until sacrifice. Mice were sacrificed after becoming moribund. Liver, spleen, and bone was harvested from control and leukemic mice at time of sacrifice for paraffin embedding and H&E staining. Bone marrow was flushed for collecting RNA, WCL and cytospin samples. Survival curves plotted in Prism and statistical significance evaluated by log rank test.

Chromatin Immunoprecipitation (ChIP). $30x10^{\wedge 6}$ cells were fixed for 15 mins at room temperature with 1% paraformaldehyde in IMDM, washed 2 times with cold PBS and snap frozen on dry ice. Cells were then lysed in 1.5mL of SDS lysis buffer (1% SDS; 10mM EDTA; 50 mM Tris-HCL pH 8), sheared 2X through a 27G needle, and sonicated to achieve DNA fragmentation distribution below 500bp. Samples were centrifuged for 20 mins at maximum speed to remove debris, and supernatant was collected and diluted 1:10 with dilution buffer (0.01% SDS; 1% TritonX-100; 1.2mM EDTA; 16.7mM Tris-HCL pH 7.5; 167mM NaCl). 1.5mL of diluted chromatin was incubated with 2.5ug of appropriate antibody overnight at 4°C with rotation. Immunoprecipitation (IP) was then preformed by adding 30uL of BSA blocked protein G Dynabeads (Invitrogen) to each

sample for 1 hour at 4°C with rotation. IPs were washed for 5 mins in low salt (150mM), high salt (500mM), and LiCl buffers (0.25M), and twice with TE buffer. Captured chromatin was eluted by incubating beads in 250uL elution buffer (1% SDS, 100mM NaHCO3) for 30mins at 42°C. Crosslinking was reversed by the addition of NaCl (final 50uM) and overnight incubation at 65°C. Chromatin was then RNase A treated and purified using Qiagen PCR purification kit. Binding was quantified relative to input by qPCR (7500 PCR System, Applied Biosystems) using SYBR green fluorescent labeling and primers designed using the IDT primer quest program (Table S1).

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

Peak Calling. Data analysis performed by Jingya Wang. Sequenced reads were pre-processed to remove contamination of adaptor sequences and then aligned to mouse reference genome (mm9) using BWA (version 0.6.2). Model-based Analysis for ChIP-Seq (MACS) was used for peak calling with the parameters as below (default values are used if not specified): format=BED -g mm --nomodel --shiftsize 75 -w –S. Peak tracks

were displayed in USCS genome browser. Distribution of peaks in the promoter (-1kb to +100bp of TSS), exon, intron, and intergenic regions etc, was estimated using HOMER (*161*). Peaks are annotated to their nearest gene using CisGenome. Peak overlap was calculated with the criteria that there is at least 1 basepair overlap between tested peaks. The significance of peak overlap was calculated using 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 (*215*). Pathway analysis was performed using the GREAT web tool based on binomial test p-value <0.05. Venn Diagram was made using R program.

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

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 (*185*). 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 (Figure 2-1a). We also generated control cell lines from *WT;CreERT*$^{+/-}$ mice to control for the effects of tamoxifen treatment and Cre-mediated toxicity. Continuous treatment of the Hoxa9/Meis1-transformed *Cebpa*$^{f/f}$*;CreERT*$^{+/-}$ cells (C/EBPα HM) with 4-hydroxytamoxifen (4OHT) leads to near complete loss of C/EBPα over the course of 8 days (Figure 2-1b). Loss of C/EBPα leads to a dramatic decrease in cellular proliferation, while Cre induction in Hoxa9/Meis1-transformed *WT;CreERT*$^{+/-}$ cells (WT HM) has no effect (Figure 2-1c, upper panels). The decrease in cellular proliferation is similar to the effect seen after loss of Hoxa9 through use of a Hoxa9-ER/Meis1 conditional cell line (HerM). It is also unlikely due to a non-specific effect of loss of a general transcription factor, as cre-mediated excision of Pu.1 in cells transformed by Hoxa9/Meis1 did not lead to a significant decrease in proliferation (Figure 2-1c, lower panel).

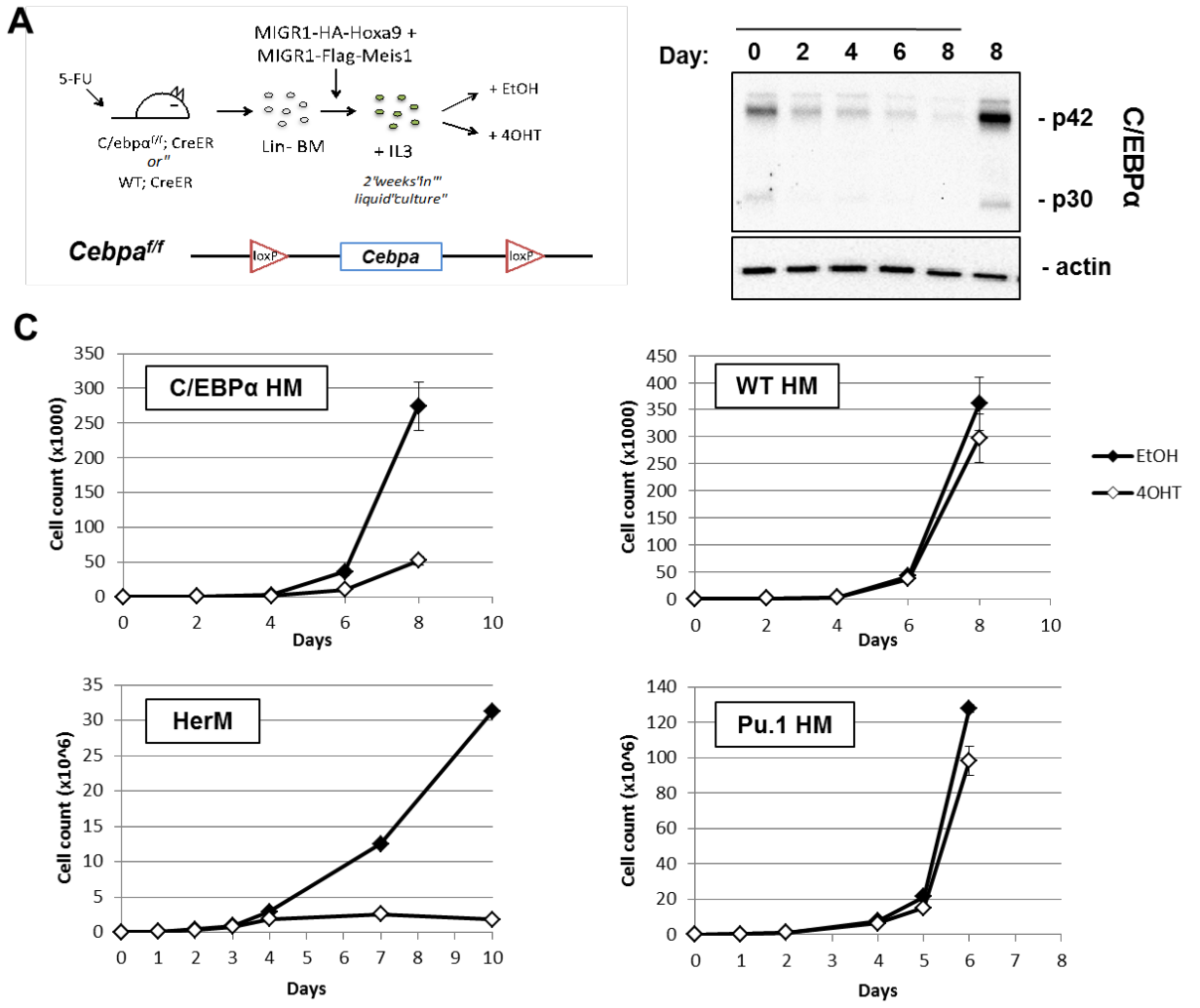

Figure 2-1 - Growth inhibition after loss of C/EBPα mimicks loss of Hoxa9
(A) Schematic of cell line generation and *Cebpa* targeted allele. (B) C/EBPα HM and WT HM cells were treated for an 8-day time course with 5nM 4OHT or EtOH and protein levels in the C/EBPα HM cells was assessed using western blot. (C) Cellular proliferation of C/EBPα HM, WT HM, Hoxa9-ER/Meis1 (HerM) and *Pu.1*$^{f/f}$*;CreERT*$^{+/-}$ /Hoxa9/Meis1 transformed cells (Pu.1 HM) was determined by cell counting; data represent mean ±SD of two independent experiments.

Our previously published work using the Hoxa9-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 (Figure 2-2a,b) (*185*). While loss of C/EBPα also leads to a decrease in cellular

proliferation, we did not observe the morphologic featues of full differentiation of these cells into macrophages or neutrophils or induction of apoptosis. Loss of C/EBPα leads to the accumulation of cytoplasmic vacuoles at day 8 (Figure 2-2a), however no further differentiation occurs after treating the cells for 15 days. No changes in cellular morphology were observed in the WT HM cells under the same treatment conditions. We also characterized changes in cell surface marker expression in these cell lines. Loss of C/EBPα leads to an increase in the immature cell surface marker, c-Kit, and a decrease in the myeloid surface markers CD11b and Gr1 (Figure 2-2b). This phenotype persists across a 15-day time course, while no changes were seen in the WT HM control cells. 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 (*210*). Conversely, loss of Hoxa9 leads to a downregulation of c-Kit and an upregulation of CD11b and Gr1, indicating an induction of a differentiation program in these cells.

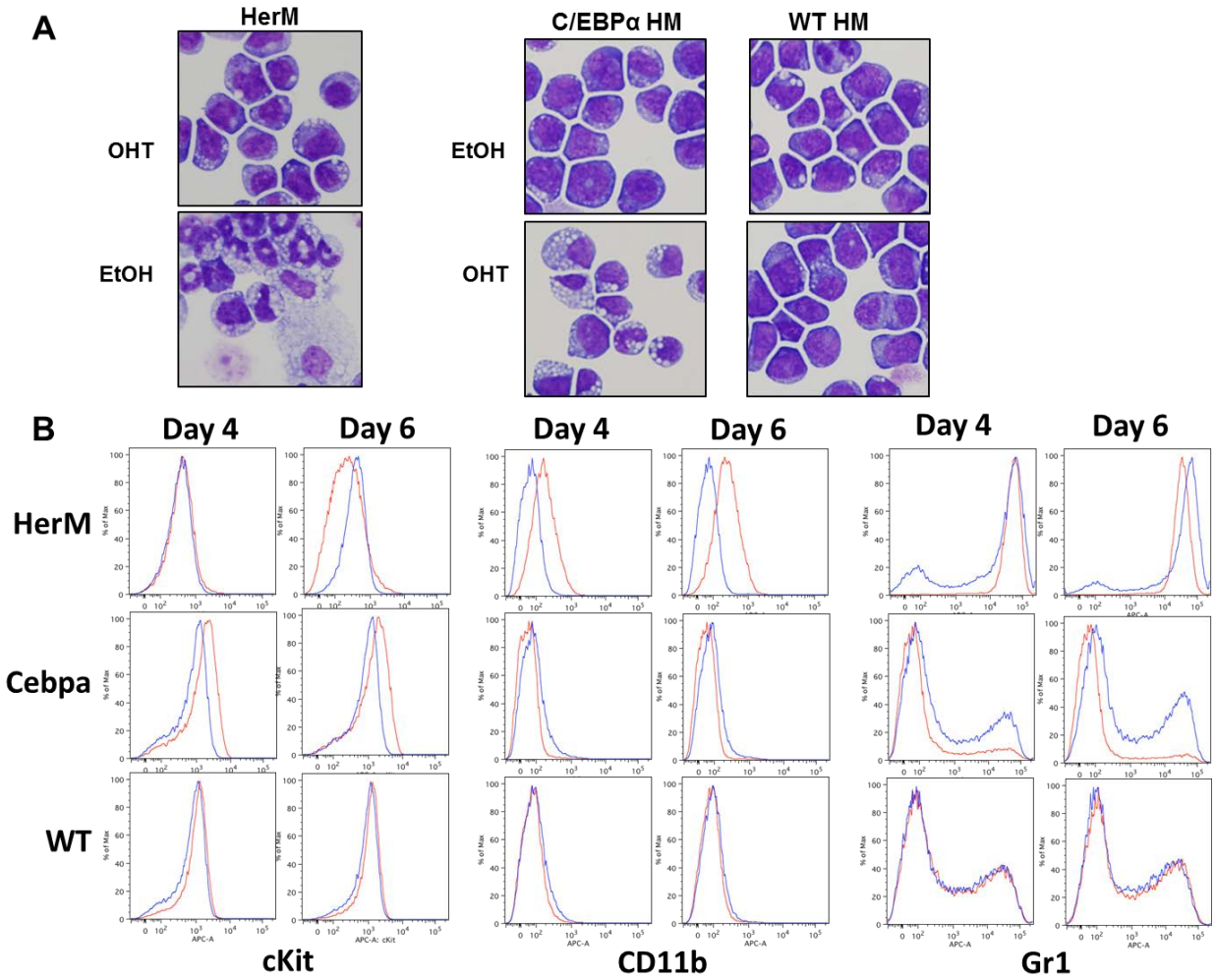

Figure 2-2 Cellular phenotype after loss of Hoxa9 or C/ebpa
(A) Cell morphology of HerM, C/EBPα HM and WT HM was assessed after 8 days in indicated conditions. (B) Surface expression of c-Kit, CD11b, and Gr1 at day 4 and 6 in HerM, C/EBPα HM and WT HM cells. Blue line indicates resting condition and red line indicates loss of Hoxa9 or Cebpa (or 4OHT treatment for WT HM). Images representative of at least two independent experiments.

Finally, we 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 to loss of Hoxa9 in HerM cells (Figure 2-3). Induction of Cre in the WT HM cells also had no effect. Thus, loss of C/EBPα in Hoxa9/Meis1-transformed cells leads to a decrease in cellular proliferation in the absence of differentiation or apoptosis.

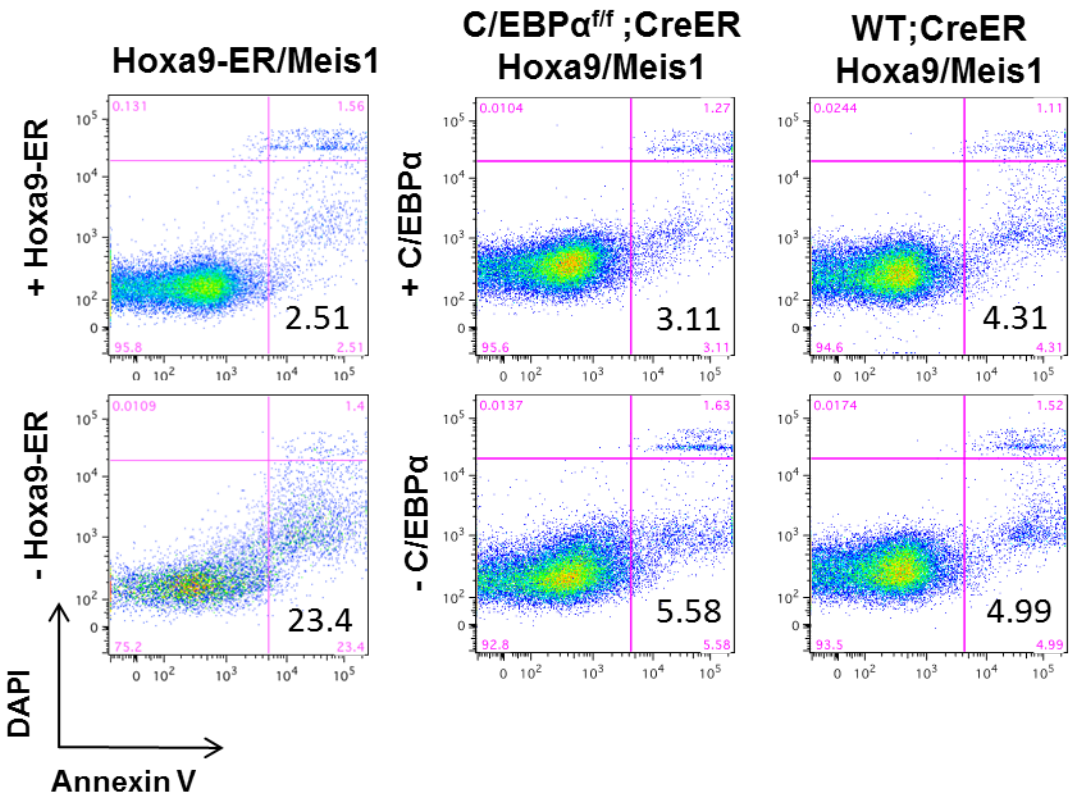

Figure 2-3 - Apoptosis of cells after loss of Hoxa9 or Cebpa
Annexin V and DAPI staining for apoptotic cells at day 6 of indicated treatment. Flow cytometry plots representative of at least two independent experiments.

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 (with the help of Hongzhi Miao). After two weeks, the mice were treated with bi-weekly injections of tamoxifen (*119*) 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(*119*); p<0.0001], while there was no survival difference seen in the vehicle or OHT-treated WT HM cohort [n=10(veh), 14(*119*); p=0.4324] (Figure 2-4a). Vehicle-treated mice from the C/EBPα

HM cohort, as well as all mice in the WT HM groups, developed myeloblastic leukemias in an average of 40 days showing extensive liver, spleen and peripheral blood infiltration (Figure 2-4b). Conversely, C/EBPα HM mice treated with OHT developed leukemia in an average of 60 days, also with infiltration of the liver and spleen in late stages. 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 (*211*).

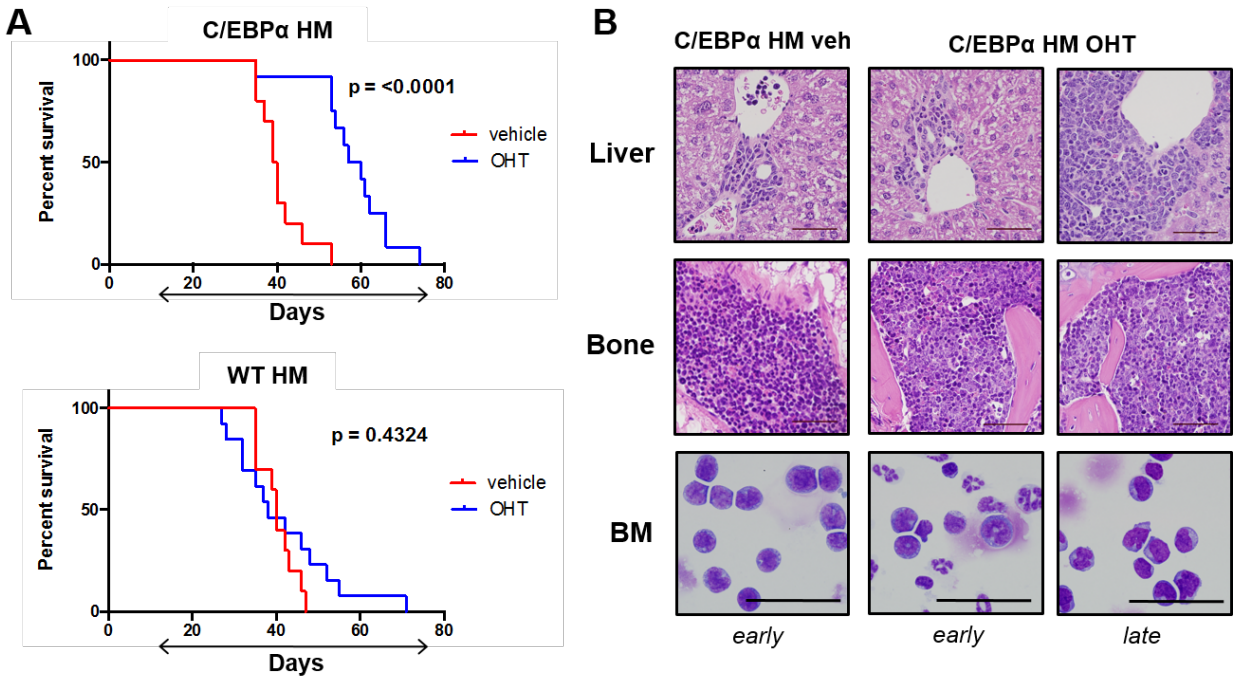

Figure 2-4 – Loss of C/EBPα improves survival in Hoxa9/Meis1 *in vivo* leukemogenesis
(A) Survival curves for mice transplanted with C/EBPα HM [n=10(veh), 12(*119*); P<0.0001 by log rank], or WT HM cells [n=10(veh), 14(*119*); P=0.4324 by log rank]. Treatment period with OHT (blue) or vehicle (red) indicated by arrow below graphs. (B) Tissue histology of liver and bone, and bone marrow cytospins for C/EBPα HM vehicle and OHT-treated mice that died prior to 40 days (left, middle; "early") and an OHT-treated mouse that died at 60 days post transplantation (right; "late") (scale bars = 50μm).

Examination of C/EBPα levels in the bone marrow at time of death indicates that there is strong selective pressure for maintaining high levels of C/EBPα in Hoxa9/Meis1

transformed leukemias. A C/EBPα HM OHT-treated mouse that died early showed complete loss of *Cebpa* expression, 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*") (Figure 2-5a,b). However, C/EBPα HM OHT-treated mice that died later (day 60; "*late*") showed recovery of *Cebpa* expression and a phenotype similar to vehicle-treated mice, likely through outgrowth of clones that had escaped Cre-mediated deletion (Fig 2-5a,b). This selective pressure for the presence of C/EBPα was also seen in cell culture, as cells eventually regain C/EBPα expression in the absence of 4OHT treatment despite genomic deletion of *Cebpa* and loss of protein levels after one week of 4OHT treatment (Figure 2-5c).

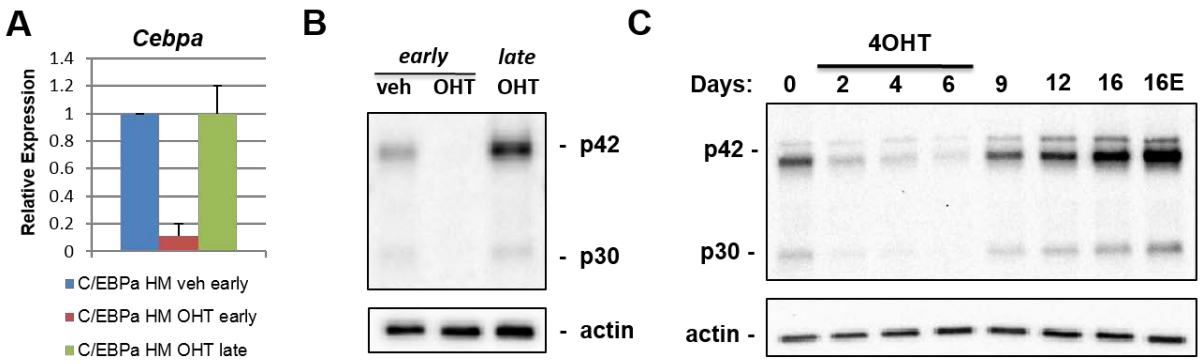

Figure 2-5 - Strong selective pressure for expression of C/EBPa
(A,B) RT-PCR expression of *Cebpa* and western blot analysis of C/EBPα protein levels corresponding to samples shown in (Figure 2-4b) (mean ±SD). (B) C/EBPα protein levels in cells treated with 4OHT for 6 days and subsequently maintained in the absence of 4OHT for an additional 10 days. Rightmost lane (16E) corresponds to cells treated continuously with EtOH for 16 days.

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 C57/B6 mice to establish secondary leukemias, and then mice were 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(*119*); p<0.0001] (Figure 2-6a). OHT-treated mice that were sacrificed as controls alongside leukemic vehicle-treated mice showed significantly reduced C/EBPα levels and tissue infiltration compared to the vehicle-treated mice, confirming the efficacy of the OHT treatment (Figure 2-6b,c). 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 (Figure 2-6b). Taken together, these results show that C/EBPα is required for Hoxa9/Meis1-mediated leukemogenesis.

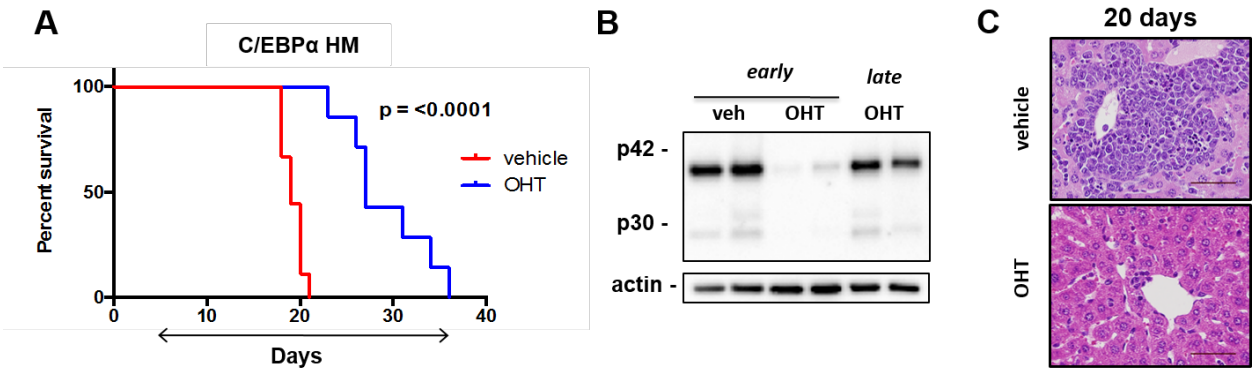

Figure 2-6 - Requirement for C/EBPa in Hoxa9/Meis1 secondary leukemia
(A) Survival curve of mice transplanted with primary leukemic spleen cells from a C/EBPα HM vehicle treated mouse [n=9(veh), 7(*119*); P<0.0001 by log rank]. Treatment period with OHT (blue) or vehicle (red) indicated by arrow below graphs. (B) C/EBPα protein levels in vehicle treated leukemic mice (left) compared to OHT treated mice preleukemic controls (*142*) and leukemic OHT treated mice (*100*). (C) Liver histology of leukemic vehicle and preleukemic OHT treated mice at 20 days (scale bars = 50μm).

Given these findings, it is noteworthy that analysis of gene expression in 344 human leukemias revealed that leukemias with high levels of *HOXA9* retain at least one wild-type copy of *CEBPA* while cases with biallelic mutations of *CEBPA* are associated with much lower levels of *HOXA9* (Figure 2-7) (data reanalyzed from (*202*) with Dr. Maria Figueroa). These data provide further support for the critical importance of

CEBPA for *HOXA9*-mediated transformation in human leukemias.

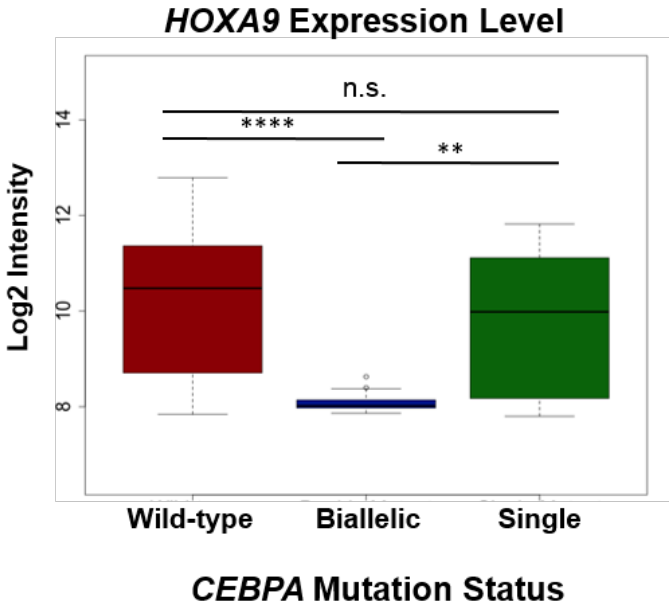

Figure 2-7 - *HOXA9* expression in human leukemias with mutated *CEBPA*
HOXA9 expression level in a cohort of AML patients subdivided by *CEBPA* mutation status [n=344; ****, P<0.0005; **, P<0.005]. Data analyzed with Dr. Maria Figueroa.

C/EBPα co-localizes 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 (*185*), we next determined if C/EBPα co-binds 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 (statistical analysis done by Jingya Wang) (*216*). Since there are currently no antibodies against Hoxa9 suitable for ChIP-seq, we used an HA antibody to immunoprecipitate HA-Hoxa9-ER. We identified 6535 peaks that are bound by Hoxa9 and 26,187 that are bound by C/EBPα, the majority of which occur at promoter distal regions (Figure 2-8a). Notably, a remarkable proportion

(54%) of Hoxa9 binding sites are co-bound by C/EBPα ($p<0.001$) (Figure 2-8b). C/EBPα does not appear to be absolutely required for Hoxa9 binding, however, as cases of Hoxa9 binding sites with very low levels of C/EBPα can be found, even in the vicinity of strong co-bound peaks (Figure 2-8c). To validate our ChIP-seq results, multiple sites of each class of Hoxa9-bound enhancer were confirmed using ChIP-qPCR (Figure 2-9).

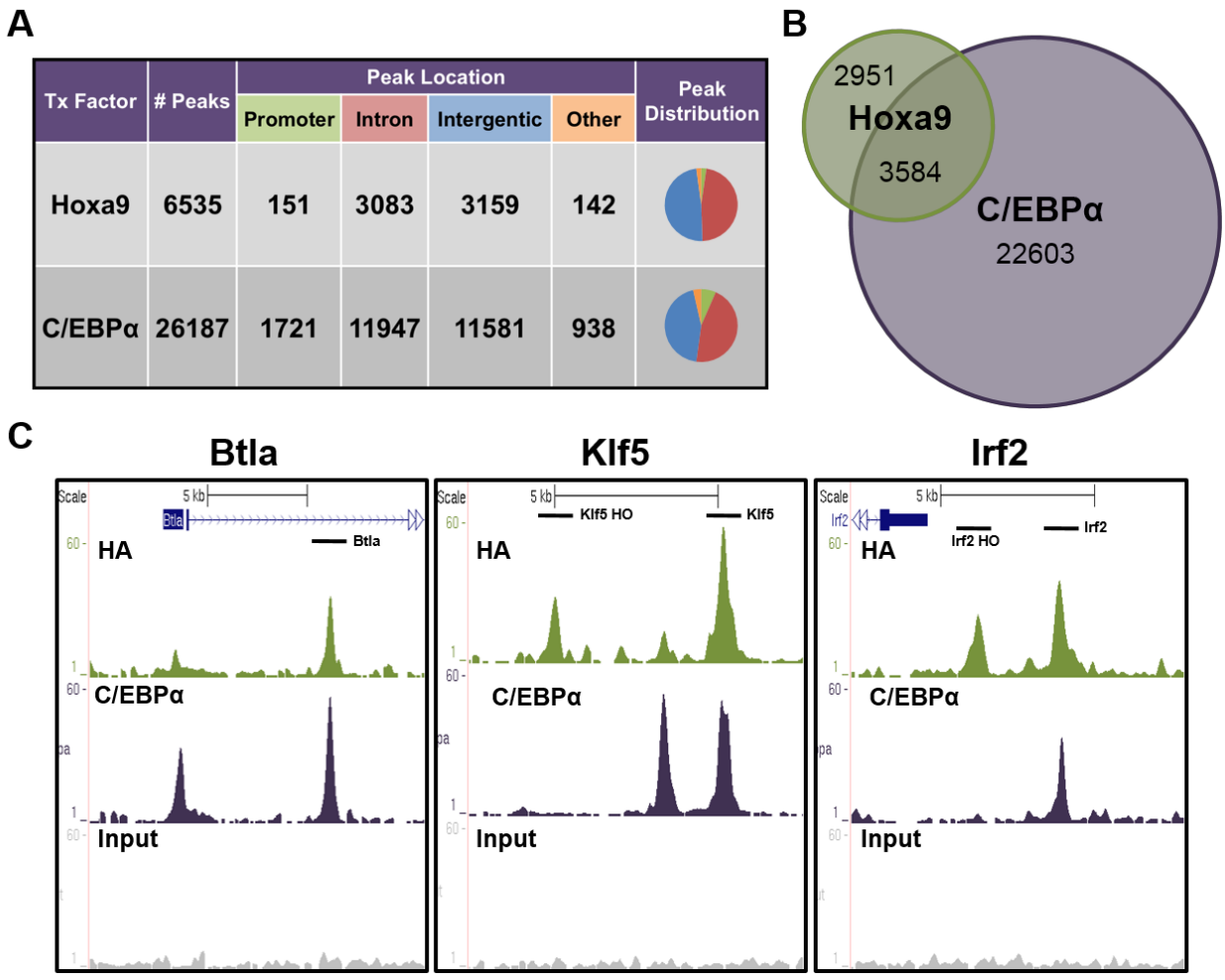

Figure 2-8 – Hoxa9 and Cebpa colocalize at distal regulatory regions
(A) Peak number and distribution of Hoxa9 (HA) and C/EBPα ChIPseq in a Hoxa9/Meis1-transformed cell line (other category contains 5'/3' UTR and exons). (B) Peak overlap between Hoxa9 and C/EBPα ChIPseq. (C) Representative Hoxa9/C/EBPα co-bound loci. Bars indicate location of qPCR primer pairs. Data analyzed by Jingya Wang.

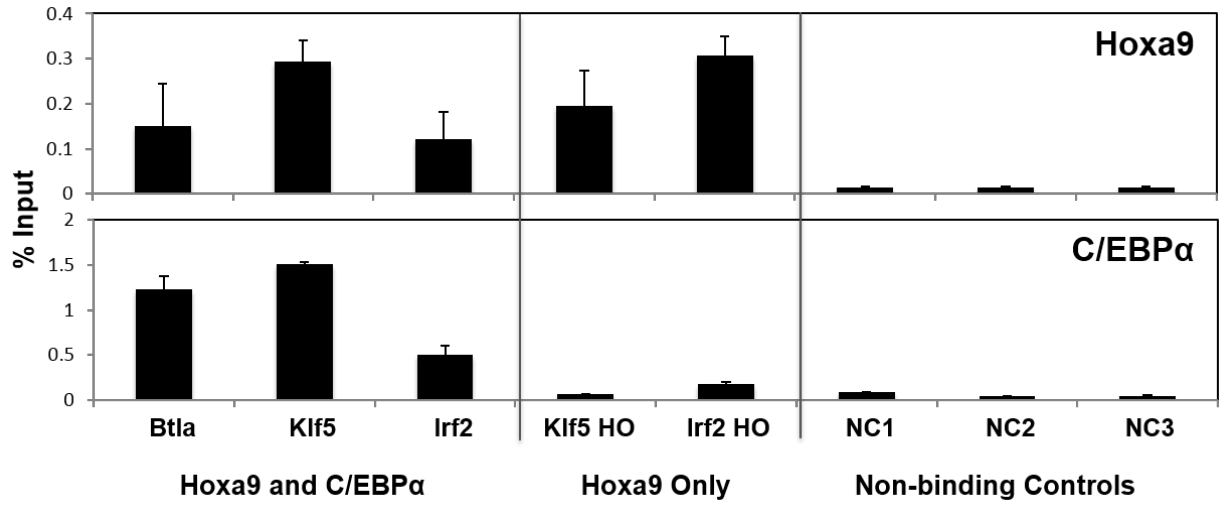

Figure 2-9 - Validation of ChIPseq data with ChIP-qPCR
Independent ChIP-qPCR validation of ChIPseq data for Hoxa9 (HA) and C/EBPα binding at Hoxa9/C/EBPα co-bound sites, Hoxa9 Only, and Non-binding Controls; bars indicate mean ±SD of at least two independent experiments.

Finally, we examined the biological pathways enriched for putative targets of Hoxa9/C/EBPα co-bound enhancers. Sites co-bound by Hoxa9 and C/EBPα showed dramatic enrichment for genes critical for hematopoietic pathways including the regulation of myeloid differentiation, regulation of the inflammatory response and regulation of cytokine production (Figure 2-10). Collectively, these results suggest that C/EBPα functionally interacts with Hoxa9 at enhancers to facilitate Hoxa9/Meis1-mediated transformation.

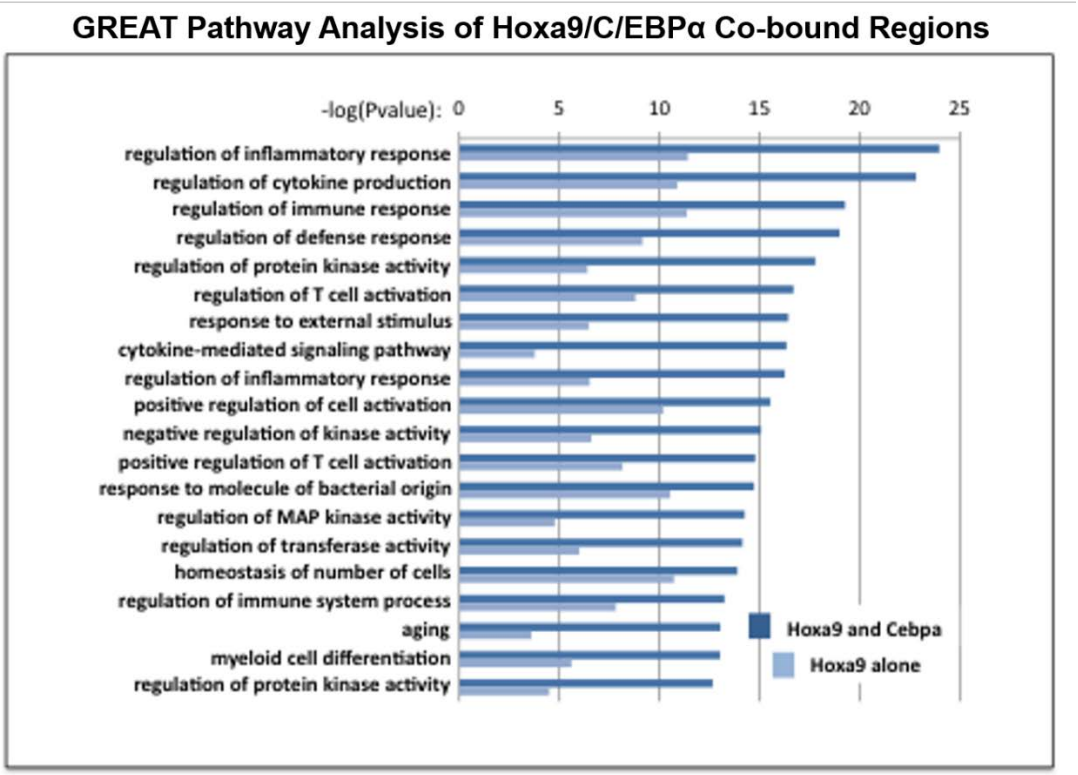

Figure 2-10 – GREAT Pathway analysis of Hoxa9/C/EBPα cobound regions
Pathway analysis of regions co-bound by Hoxa9 and C/EBPα (dark blue) or regions bound only by Hoxa9 (light blue). Analysis was performed using the Genomic Regions Enrichment of Annotations tool (GREAT) against the biological pathways database. X-axis indicates p-value (-10*log).

C/EBPα and Hoxa9 co-regulate expression of Cdkn2a/b

Given the requirement for C/EBPα in Hoxa9/Meis1-mediated leukemic transformation and the co-localization 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 RNAseq at 72hrs after loss of C/EBPα or Hoxa9. We identified 31 genes that were co-activated more than 1.5-fold by Hoxa9 and C/EBPα, including *Adam17*, *Igf2r*, *Il2ra*, and *Cpe* (Figure 2-11). In addition, 45 genes were co-repressed more than 1.5-fold, including *Gata2*, *Gfi1b*, *Prkca*, and *Cdkn2b*. 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 (Figure 3-7).

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	

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

Figure 2-11 - Genes coregulated by Hoxa9 and C/EBPa
Lists of genes that are coactivated and corepressed more than 1.5 fold by Hoxa9 and C/EBPa as determined by overlap of RNAseq 72 hours after loss of Hoxa9 or C/EBPa. Boxes highlighted in red indicate genes associated with the 'regulation of cellular proliferation' GO term.

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 cell cycle at the G1 phase (*217*). In addition, *Cdkn2a/b* are commonly deleted in acute lymphoid leukemias (*218-220*). Our ChIP-seq studies identified a Hoxa9/C/EBPα co-bound site in

an intergenic region approximately 50kb downstream of the *Cdkn2a/b* locus (Figure 2-12a). Loss of either C/EBPα or Hoxa9 binding at this locus (Figure 2-12b,c) results in a corresponding increase in *Cdkn2a/b* expression (Figure 2-12d,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 co-binding of both proteins is necessary for repression of the Cdkn2a/b locus (Fig 4b,c).

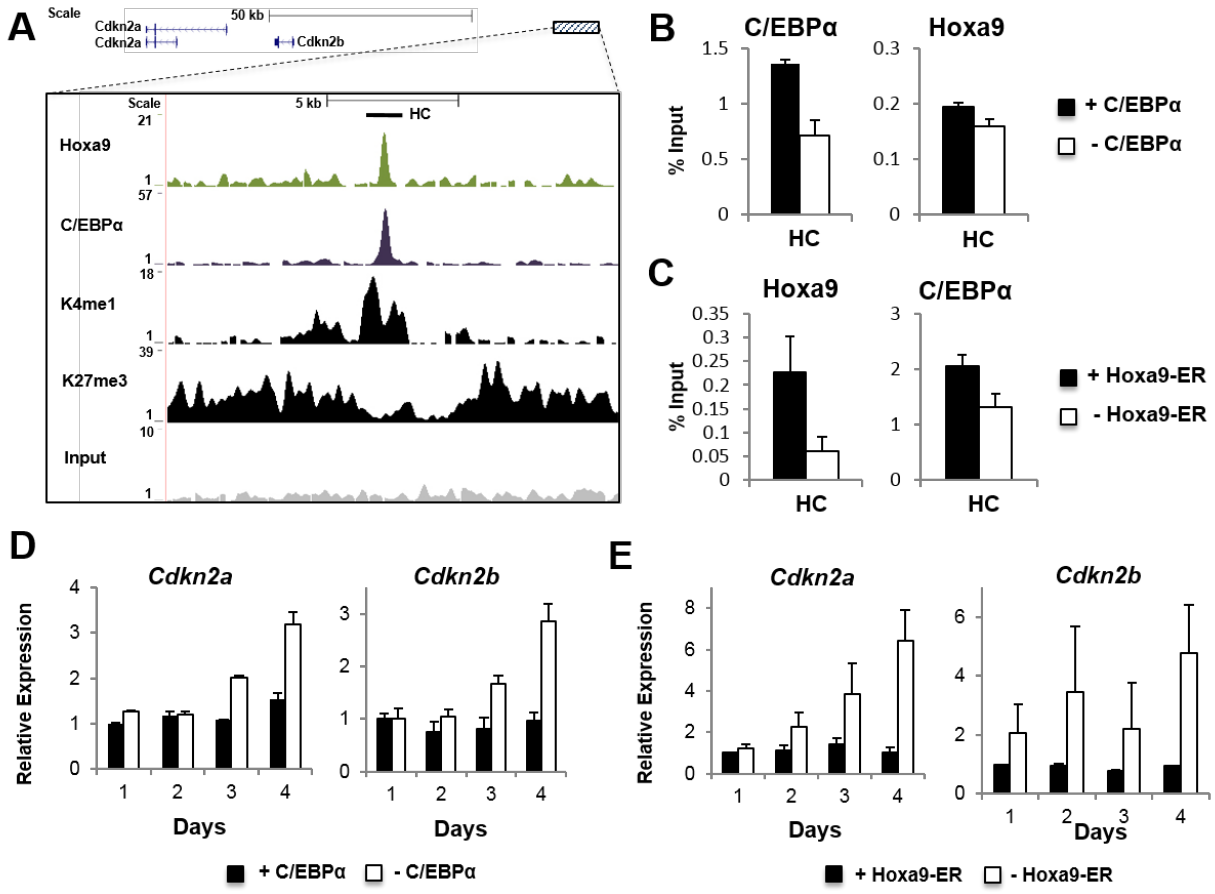

Figure 2-12 - Cdkn2/b locus corepressed by putative Hoxa9/C/EBPa cobound regulatory region
(A) ChIPseq tracks for Hoxa9 (HA), C/EBPα, H3K4me1 and H3K27me3 at the Hoxa9/C/EBPα binding site 50kb downstream of the Cdkn2a/b locus. Bars indicate location of qPCR primer set. (B,C) ChIP-qPCR for C/EBPα and Hoxa9 binding at the HC binding site after 3 days loss of C/EBPα (B) or Hoxa9 (C). (D, E) RT-PCR expression of Cdkn2a and Cdkn2b over a 4-day timecourse after loss of C/EBPα (D) or loss of Hoxa9 (E). All data expressed as mean ±SD of at least two independent experiments.

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 block (Figure 2-13a,b). These data suggest that together, C/EBPα and Hoxa9 repress the *Cdkn2a/b* locus to overcome the G1 cell cycle block that would otherwise occur in the presence of either protein.

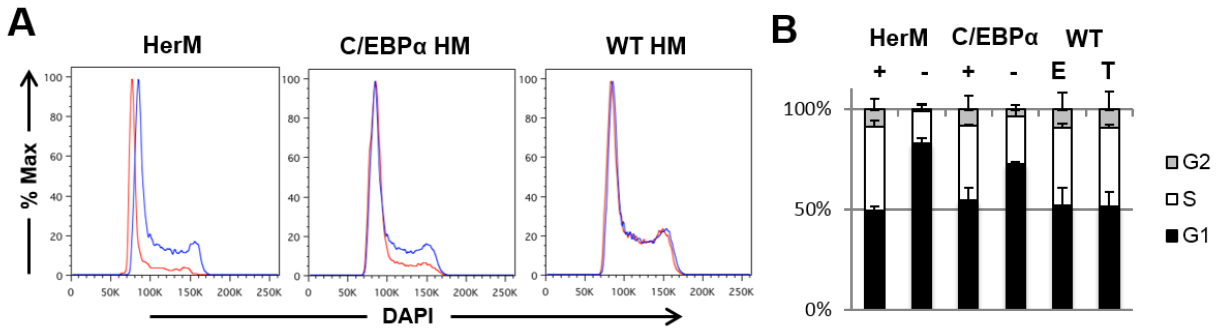

Figure 2-13 - Loss of Hoxa9 or C/EBPa leads to a G1 cell cycle block
(A) Cell cycle analysis at day 6 after loss of Hoxa9 (Left) C/EBPα (Middle) or in EtOH or 4OHT treated WT HM cells (Right) [Blue = control; Red = loss of Hoxa9 or C/EBPα]. (B) Quantification of cell cycle profiles analyzed using FloJo. All data expressed as mean ±SD of at least two independent experiments.

DISCUSSION

While 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 mediates transformation are yet to be fully elucidated. In this study, we identify C/EBPα as a critical collaborator protein of 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. It is noteworthy in this context that human AMLs with high *HOXA9* expression almost always retain one wild-type 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 C/EBPα is required for transformation (*204*). While these findings are surprising given that high levels of C/EBPα have been shown to promote myeloid differentiation (*214*), it is most likely that a moderate level of C/EBPα is required for HOXA9-mediated AML.

It is noteworthy that 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 (*74, 221*). Conversely, C/EBPα was shown to be dispensable for E2A-HLF-mediated transformation, which has undetectable levels of *HOXA9* (*221*). 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 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 wild-type CEBPA in the development of leukemia with high level of HOXA9, it is also interesting to speculate how mutations 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 (*140,*

204, 222). These mutations affect not only the binding and localization of CEBPA, but also the recruitment of co-activator and co-repressor complexes to CEBPA-bound loci (*204, 223*). 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-AF9 translocated and other *CEBPA* WT leukemias (*224*). 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 also implicates C/EBPα in the control of senescence. 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 other *Bmi1-/-* transformed cells (*191*). While Hoxa9 was found to suppress *Cdkn2a* expression through direct binding at the promoter, the authors suggest that other non-Hox factors may be involved for achieving full repression of this locus. Our work identifies C/EBPα as an additional factor aiding in Hoxa9 repression of the entire *Cdkn2a/b* locus, potentially through looping to the downstream Hoxa9/C/EBPα co-bound site to the promoters (Fig 2-14). The silencing of both *CDKN2a/b* through deletion or promoter methylation is known to play critical roles in AML (*218-220*). Our work, together with work from the So lab and others (*191, 225*), suggests that high levels of HOXA9, in concert with CEBPA, results in polycomb protein-mediated

repression of the *CDKN2A/B* locus, thereby employing a fundamental mechanism of leukemogenesis.

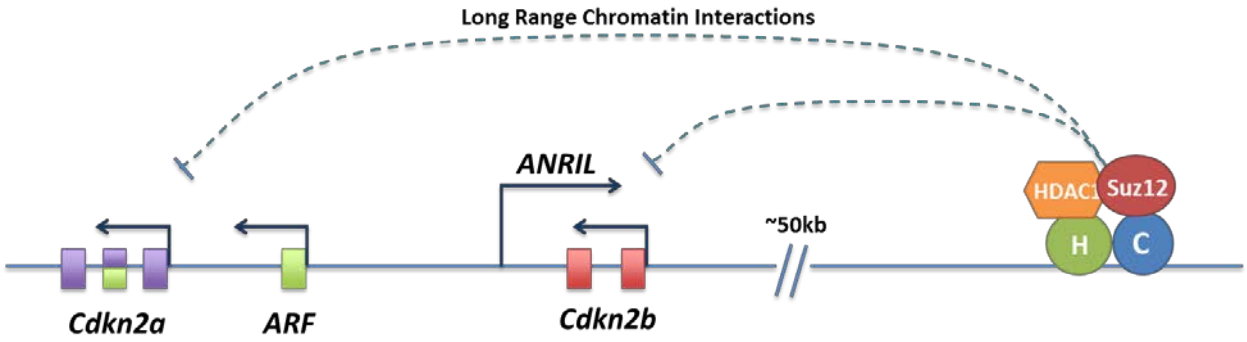

Figure 2-14 - Model for *Cdkn2a/b* regulation by Hoxa9 and C/EBPα
Schematic of Hoxa9 and C/EBPα co-repression of *Cdkn2a/b* locus. Repression of *Cdkn2a/b* may be mediated through long range interactions between the Hoxa9/C/EBPα co-bound *cis*-regulatory element 50kb downstream of the *Cdkn2a/b* locus. Recruitment of histone deacetylase 1 (HDAC1) and polycomb proteins (Suz12) likely contributes to this repression.

Finally, while C/EBPα is required for Hoxa9-mediated leukemogenesis at essential co-regulated 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 (*224*). Additional study of antagonistically regulated HOXA9/ CEBPA target genes may provide further insight into the mechanisms through which high levels of *HOXA9* expression leads to transformation in acute myeloid leukemia.

CHAPTER 3:

OTHER RESULTS AND FUTURE DIRECTIONS

TARGETING THE HOXA9-C/EBPA INTERACTION

We first identified C/EBPα as a potential collaborator of Hoxa9 due to both the enrichment of CEBP motifs at Hoxa9 binding sites and the co-immunoprecipitation of C/EBPα along with Hoxa9 and Meis1 from a myeloblastic cell line. The work presented in this thesis establishes that C/EBPα is required for transformation mediated by Hoxa9 and Meis1, and shows co-association of C/EBPα at over 50% of genome-wide Hoxa9 binding sites. Given the functional requirement for Cebpa and the potential for this requirement to be mediated through physical contacts between Hoxa9 and C/EBPα, characterization of the interaction of Hoxa9 and C/EBPα will provide valuable information for future studies.

Mapping the physical interaction between C/EBPα and Hoxa9

In our preliminary studies, we confirmed the co-immunoprecipitation of C/EBPα in the myeloid Hoxa9/Meis1-complex using coIP followed by western blot analysis (*174*). To determine if this interaction was direct, we purified MBP-tagged Hoxa9, Mocr-tagged Meis1 and Mocr-tagged C/EBPα from bacteria and performed in vitro binding assays with these proteins (in collaboration with Joel Bronstein). As expected, MBP-Hoxa9 and Mocr-Meis1 specifically interact *in vitro*, while there was no non-specific binding seen to

the MBP tag alone (Figure 3-1). MBP-Hoxa9 was also able to interact with Mocr-C/EBPα, suggesting a direct physical interaction between these two proteins. Studies using co-immunoprecipitation from myeloid cells with extended nuclease digestion provide further support of a physical interaction between C/EBPα and Hoxa9 in the absence of DNA.

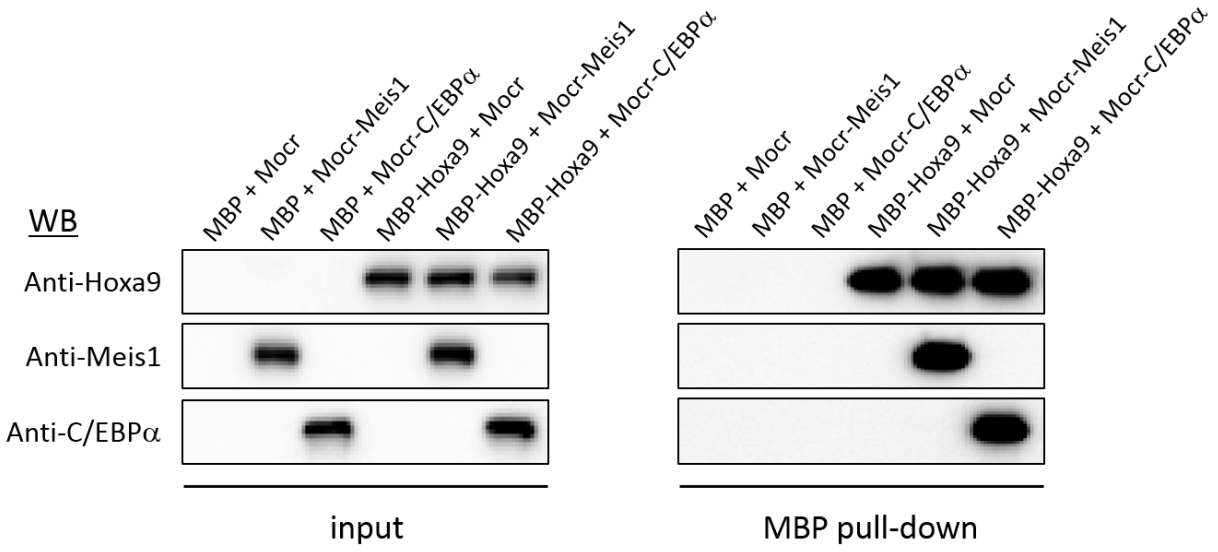

Figure 3-1 - Hoxa9 binds directly with C/EBPα
Recombinant His-tagged MBP and His-tagged MBP fused to Hoxa9 (MBP-Hoxa9) were expressed in E. coli, purified by Ni-NTA Agarose and captured by Amylose Resin. His-tagged Mocr, His-tagged Mocr fused to Meis1 (Mocr-Meis1) and His-tagged Mocr fused to C/EBPa (Mocr-C/EBPa) were similarly expressed and purified and added in combination with either MBP or MBP-Hoxa9. Input and eluted proteins were visualized by coomassie stain and western blot following SDS-PAGE. Meis1 and C/EBPa directly interact with Hoxa9 as indicated by western blotting. (In collaboration with Joel Bronstein).

To more definitively test for a direct interaction between Hoxa9 and C/EBPα, future studies can be performed using surface Plasmon resonance. Surface Plasmon Resonance (SPR) technology has the ability to determine the specificity, affinity, and kinetics of protein-protein interactions (*226*). A bait protein is immobilized on a gold-coated glass surface while a potential interactor protein can be flowed over this surface.

Binding results in reversible changes to the surface resonance or absorbance in real time. Direct physical interactions result in very specific resonance profiles, which are easy to distinguish from non-specific binding. This method is especially versatile because after immobilizing your protein of interest, a variety of mutant proteins can be tested to fully characterize the physical interaction domain.

We have conducted preliminary mapping of the Hoxa9-C/EBPα interaction using co-IP experiments with transiently transfected mutant proteins in 293 cells. Both the full-length and short isoform of C/EBPα preferentially interact with the C-terminal region of Hoxa9, but not the N-terminal region that is known to be required for transformation (Figure 3-2). This C-terminal region contains the homeodomain, which has been shown to mediate protein-protein interactions with Hox proteins in other model systems (*151, 152*). We tested the interaction between the two C/EBPα isoforms and both a homeodomain-deficient mutant of Hoxa9 and the homeodomain alone. Interestingly, C/EBPα p42 interacts more strongly with the homeodomain deletion mutant than with the homeodomain alone. Taken together with the experiments with the C-terminal region, these results suggest that the interaction between Hoxa9 and C/EBPα p42 may be mediated through aa186-205, with some contribution of residues in the homeodomain itself. Conversely, the interaction between Hoxa9 and C/EBPα p30 seems to occur most strongly with the homeodomain alone and to a lesser extent with residues outside the homeodomain. Since both p42 and p30 share the same C-terminal region, it is possible that residues in the N-terminal portion of p42 stabilize an interaction with Hoxa9 via residues outside of the homeodomain, while residues in the C-terminus of C/EBPα make contact with the homeodomain itself. Additional mutant forms of both

C/EBPα and Hoxa9 could potentially narrow these interacting regions.

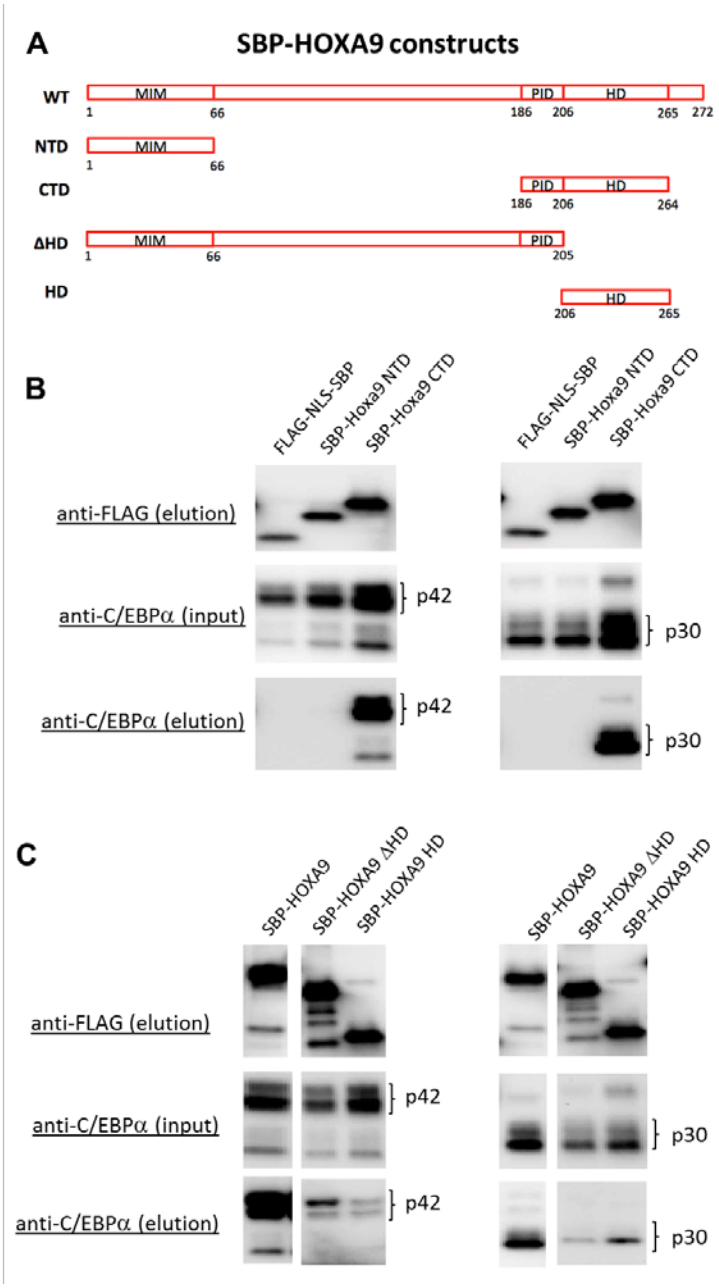

Figure 3-2 - Domain mapping of HOXA9-C/EBPα interaction
(A) Schematic of streptavidin binding protein (SBP)-tagged HOXA9 expression contructs used in biotin immunoprecipitation assays. (B) FLAG-SBP-HOXA9 N-terminal domain (NTD, aa1-66) or C-terminal domain (CTD, aa186-264) were expressed in 293 cells with C/EBPα p42 or p30 isoforms and immunoprecipitation was performed. Western blot was performed for C/EBPα or FLAG. (C) Experiments were repeated with FLAG-SBP constructs expressing full length HOXA9, a homeodomain deletion (ΔHD) or the HOXA9 homeodomain only (HD). Abbreviations: MIM, Meis1 interaction motif; PID, Pbx interaction domain.

Potential avenues for disrupting the HOXA9-C/EBPα interaction

One of the goals for determining the interaction region between Hoxa9 and C/EBPα would be to develop a small molecule inhibitor to disrupt this interaction in cells. This inhibitor could be used to further assess the specific requirement for the Hoxa9-C/EBPα physical interaction in leukemogenesis. In addition, small molecules could be modified to become novel therapeutic strategies for leukemias with high levels of Hoxa9. Developing a small molecule that disrupts a specific protein-protein interaction is a multistep process that can be pursued through a variety of approaches (*227*). A general requirement for screening potential small molecules is a highly sensitive assay to measure the binding between your two proteins of interest. A potential strategy for testing the interaction between Hoxa9 and C/EBPα is fluorescence resonance energy transfer assays (*228*). Directed drug design could also be investigated if the interaction domain between Hoxa9 and C/EBPα can be more specifically defined. Identifying a small peptide that can block the Hoxa9-C/EBPα interaction can be guided by mutational studies and potential peptides can be screened using SPR assays (*226*). This putative peptide could then serve as a backbone for developing peptidomimetic molecules.

FUNCTIONAL CONSEQUENCES OF *CEBPA* MUTATIONS ON HOXA9-MEDIATED TRANSFORMATION

While the work presented in this thesis demonstrates a requirement for wild-type *CEBPA* in the development of leukemia with high *HOXA9* expression, analysis of human leukemias shows that high *HOXA9* expression can occur in leukemia with single mutations in *CEBPA* (Figure 2-7). Mutations in *CEBPA* fall into two categories: N-terminal mutations that lead to transcription of only the short p30 isoform or C-terminal

mutations in the DNA-binding domain (*204*). These mutations affect not only the binding and localization of C/EBPα, but also the recruitment of co-activator and co-repressor complexes to C/EBPα-bound loci (*223*). Though two thirds of leukemias with mutations in *CEBPA* carry biallelic mutations, it is interesting to speculate how single mutations of *CEBPA* may functionally interact with *HOXA9* in AML (*229-231*). Understanding how mutant forms of C/EBPα affect the binding and activity of HOXA9 will not only give insight into potential mechanisms of transformation in *CEBPA* single mutant leukemia, but may also help further characterize the mechanism for requirement of wild-type *CEBPA*.

Functional interplay between Hoxa9 and C/EBPα isoforms

Under normal conditions, the *CEBPA* transcript yields two isoforms: a full-length p42 isoform and a shorter p30 isoform that is transcribed from an alternative start codon. Both isoforms are important for hematopoietic differentiation, but they behave differently with regards to localization throughout the genome and the proteins they interact with (*232, 233*). Both the p42 and p30 isoforms are detectable by western blot in Hoxa9/Meis1 transformed cells (Figure 2-1b), though the full-length p42 is expressed at much higher levels. *CEBPA* is a single exon gene, and the two isoforms are expressed as the result of two alternative start codons. The loxP targeting in our C/EBPα HM cells result in complete excision of this single exon, therefore leading to the loss of both p42 and p30 expression (*212*).

Since Hoxa9 can interact with both p42 and p30, we first wanted to test the effect of expressing each isoform with Hoxa9. We generated cell lines that allow for conditional expression of the C/EBPα isoforms by transducing cells with Hoxa9 and

C/EBPα (p42 or p30)-ER or an empty vector control. Hoxa9 is a weak oncogene in the absence of co-expressed Meis1, allowing for easier detection of phenotypic differences upon induction of C/EBPα. Co-expression of either p42 or p30 leads to a decrease in number and size of colonies transformed by Hoxa9 (Figure 3-3a,b). In liquid culture, induction of p42 or p30 in cells previously transformed by Hoxa9 leads to a reduction in proliferation and differentiation along the myeloid lineage (Figure 3-3c and data not shown). These results are consistent with the critical role that C/EBPα plays in driving myeloid differentiation. Indeed, forced expression of *CEBPA* can promote transdifferentiation of fibroblasts and *MLL*-transformed cell lines into macrophages (*213, 214*).

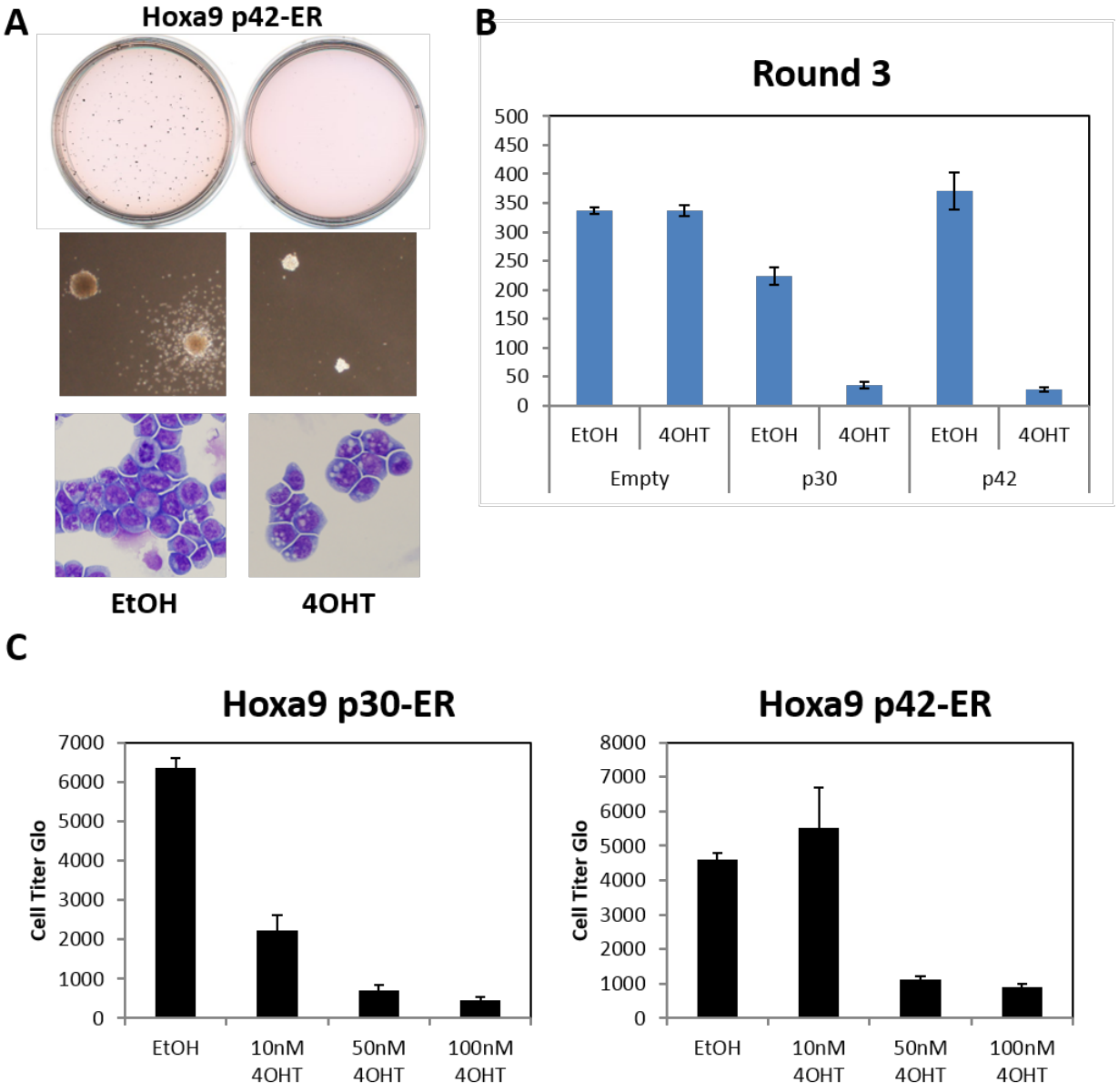

Figure 3-3 - Overexpression of C/EBPα inhibits transformation by Hoxa9
(A) Coexpression of full length C/EBPα during initiation of transformation by Hoxa9 leads to decrease in colony formation size and number. (B) Quantiation of colony number in the third round of replating after expressing Hoxa9 with an empty vector, p42-ER or p30-ER isoforms of C/EBPα. (C) Expression of p42 and p30 lead to decrease in proliferation of cells previously transformed by Hoxa9 in a concentration dependent manner.

The inhibition of Hoxa9-mediated transformation by both p42 and p30 highlights the importance of the balance of C/EBPα-HOXA9 levels in leukemia. Indeed, inhibition of growth in liquid culture seems to have some concentration dependency. At low levels of tamoxifen, there may even be a synergy between C/EBPα p42 and Hoxa9. Further characterization of this phenotype using additional concentrations of tamoxifen will be worthwhile. Future studies using our C/EBPα HM cells to investigate the expression of p42 or p30 on a *Cebpa* null background will also be valuable for more definitively characterizing the phenotype associated with each isoform.

Since C/EBPα is present at over 50% of Hoxa9-binding sites, we next wanted to determine if p42, p30 or both can co-associate with Hoxa9 at these cis-regulatory regions. The antibody used in the ChIPseq studies recognizes the C-terminus of C/EBPα, which is shared between both the p42 and p30 isoforms. N-terminal antibodies exist that are specific to p42, though no p30-specific antibody can be generated due to the homology of the C-terminal region. We performed ChIP-qPCR experiments using two different C/EBPα N-terminal antibodies at a variety of loci in our Hoxa9/Meis1 transformed cells (Figure 3-4). While the N-terminal antibodies both produced lower enrichment than the C-terminal antibody, the ratio of binding between the various peaks is consistent for all three C/EBPα antibodies. The lower enrichment is most likely due to differences in antibody affinity. We can definitively conclude that the p42 isoform of C/EBPα is co-bound with Hoxa9 at the sites tested. While we cannot exclude the possibility that p30 is also present at these sites, this would require the p42/p30 ratio of binding at all sites to be the same. If one site had a preference of binding to p30 vs p42, the relative levels of binding at various loci would be different using the C-terminal or N-

terminal antibodies. Therefore, it is more likely that only the p42 isoform is present at these sites. It is possible that the p30 isoform co-binds with Hoxa9 at a different subset of loci, which could provide different activity of the Hoxa9-complex at these sites. Cell lines with conditional expression of p42 or p30 on a *Cebpa*-null background would be a good system for assessing the coassociation of these three proteins at the genomic level.

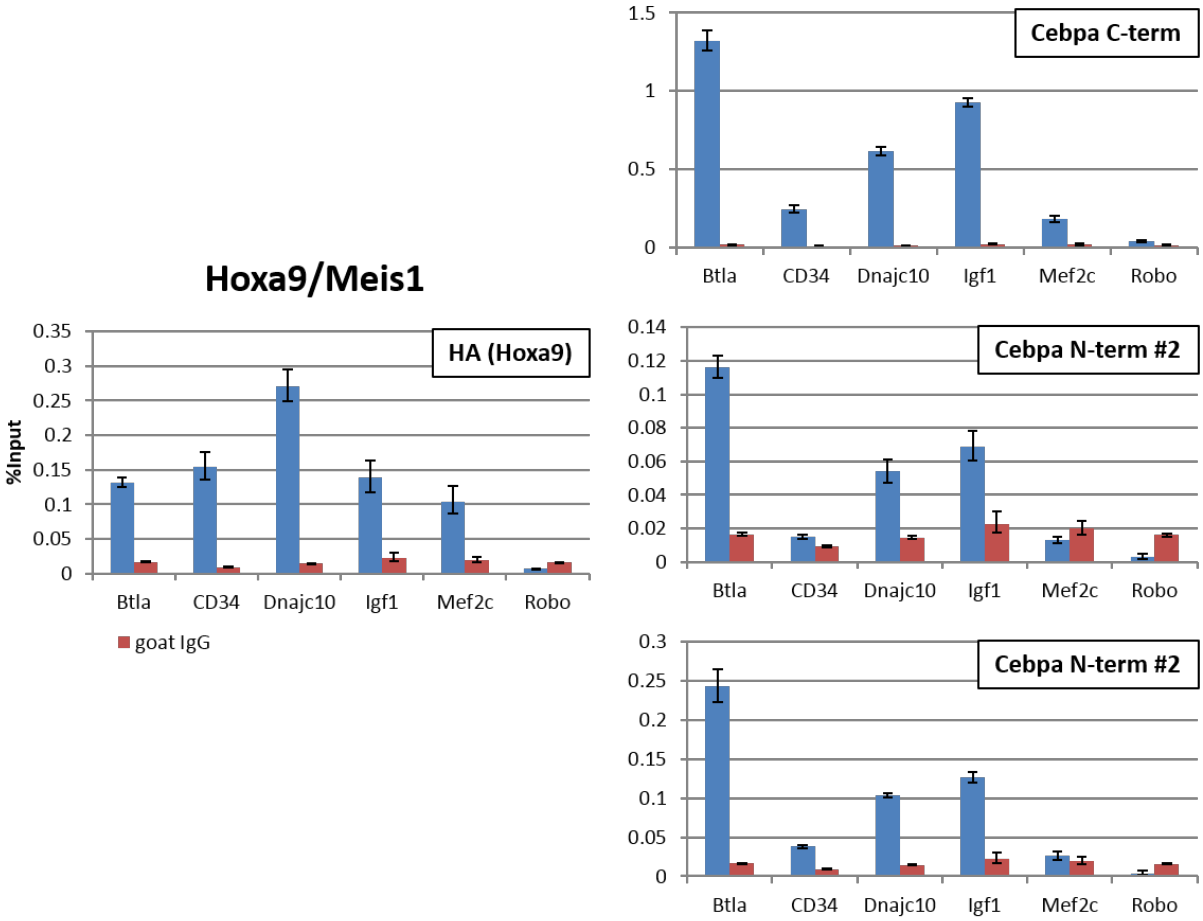

Figure 3-4 - C/EBPα p42 is present at Hoxa9 binding sites
ChIP for C/EBPα C-terminus (specific to p42 only) and N-terminus (common between p42 and p30) show that p42 is present at Hoxa9 binding sites. Similar ratios of C/EBPα association at various binding sites with C-term and N-terminal antibodies suggests that either only p42 is present at these sites, or the ratio between p42 and p30 is the same at all sites. ChIP studies with ER-tagged proteins could aid in further answering this question.

While significant progress has been made in studying *CEBPA* mutant leukemia, many unanswered questions remain. Characterizing the functional interaction between Hoxa9 and C/EBPα p42/p30 may provide valuable insight into the pathogenesis of leukemias with mutant forms of *CEBPA*. These studies may also help to elucidate fundamental mechanisms of function in normal hematopoiesis and leukemia.

REQUIREMENT FOR C/EBPA IN OTHER HOXA9-HIGH OR LOW LEUKEMIAS

A variety of upstream genetic changes lead to high level Hoxa9 expression in leukemia. In our studies we used transformation by Hoxa9 and its cofactor Meis1 as a model for leukemias with high levels of Hoxa9 regardless of the mechanisms leading to this overexpression. To better understand the requirement of C/EBPα in leukemias with high levels of HOXA9 it will be important to test this requirement with a variety of oncogenes that transform with high HOXA9. Recent publications indicate that C/EBPα is required in the initiation of MLL-ENL transformation, but not in the maintenance of these leukemias (*234*). Our results show that C/EBPα is required for both the initiation and maintenance of Hoxa9/Meis1-mediated transformation, suggesting that MLL-ENL activates compensatory mechanisms that allow for maintenance of transformation in the absence of C/EBPα. Testing additional oncogenes for this initiation vs. maintenance phenotype will be valuable in understanding the role of C/EBPα in human disease and also may uncover differences between transforming mechanisms between various oncogenes that upregulate Hoxa9/Meis1.

The recent work also suggested that C/EBPα may not be required for transformation of cells by E2A-HLF, an oncogene that transforms without high levels of Hoxa9/Meis1 (*234*). It should be noted that while E2A-HLF has the ability to transform cells in culture in the

absence of C/EBPα, proliferation of these cells was compromised. In addition, the requirement for C/EBPα in the maintainence of these leukemias was not tested. While we found that the expression of *HOXA9* in one cohort of human leukemias varied depending on the mutational status of *CEBPA*, the expression of *CEBPA* was independent of levels of *HOXA9* expression and of MLL mutational status (Figure 3-5a,b) (data reanalyzed from (*202*)). Looking at expression of *CEBPA* in the entire cohort as a function of the FAB status shows that a majority of leukemias express *CEBPA* to some extent (Figure 3-5c). This brings up the possibility that *CEBPA* may play a role in leukemias without high levels of *HOXA9* as well. To determine HOXA9-independent roles of C/EBPα in leukemia, more in depth study of E2A-HLF transformed cells, as well as a panel of other oncogenes with low HOXA9 including PML-RARA of the M3 subtype will be required. The conditional deletion system for *CEBPA* is a great model for studying the specificity of this requirement of C/EBPα in leukemia. Phenotypes can be characterized using liquid proliferation assays, colony formation assays, and in vivo leukemogenesis assays.

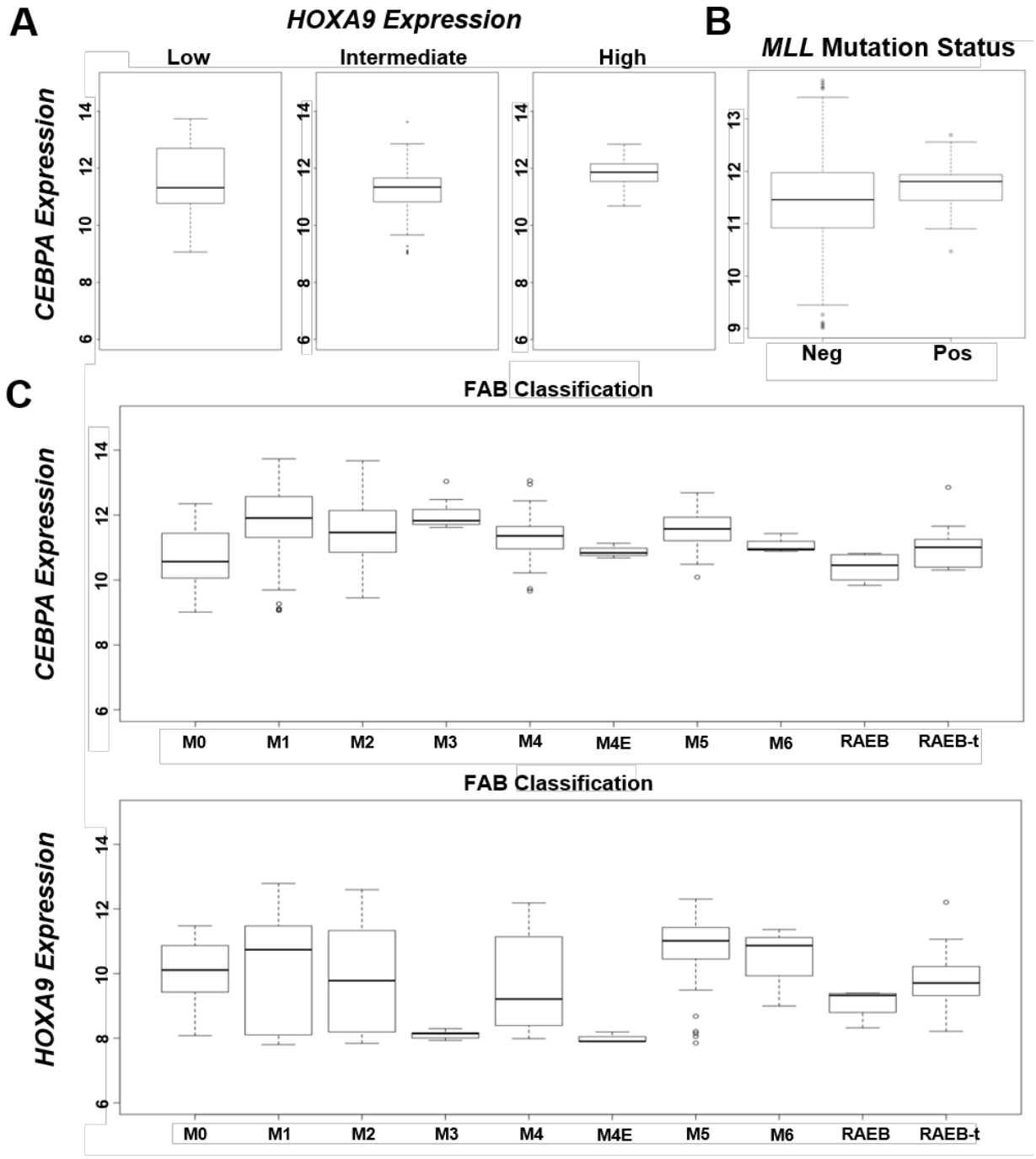

Figure 3-5 - Expression of *CEBPA* and *HOXA9* in human leukemias
(A) *CEBPA* expression in Hoxa9 low, intermediate and high groups as determined by the mean ±2SD. (B) *CEBPA* expression level with respect to MLL translocation status (negative or positive). (C) *CEBPA* and *HOXA9* expression with respect to FAB status. Data reanalyzed from (*202*) with Dr. Maria Figueroa.

HOXA9 AND C/EBPA IN NORMAL HEMATOPOIESIS

Oncogenic transformation can result from three categories of alterations: loss of function, gain of function or misexpression, where a protein retains its normal function but is expressed when it should not be. In the case of HOXA9 activity in acute myeloid leukemia, it is not known whether this is a case of gain of function or misexpression. In other words, does HOXA9 gain new transcriptional targets during leukemic transformation as a result of a change in the transcription factor mileu in malignant cells, or does HOXA9 regulate the same genes in the same way during normal and malignant transformation and it is simply the lack of downregulation of HOXA9 that contributes to transformation? Investigating these questions can provide valuable information with regards to the role of HOXA9 in normal hematopoiesis, as well as guiding potential therapeutics. One challenge in targeted cancer therapy is the effect drugs may have on the normal function of the targeted protein. If leukemia-specific functions of HOXA9 can be identified, these can be specifically targeted for therapeutics.

It is possible that the physical and functional interaction with C/EBPα is an example of a gain of function of HOXA9 in leukemia. We determined that C/EBPα colocalizes at 50% of Hoxa9 binding sites in transformed cells, however it is unknown whether this colocalization also occurs in normal hematopoiesis. ChIPseq studies of HOX proteins and other hematopoietic transcription factors demonstrate that the binding profiles and transcriptional targets of a specific transcription factor can change dramatically depending on the differentiation state of a cell. Since antibodies for ChIP for endogenous HOXA9 are currently unavailable, it is difficult to study the localization of HOXA9 throughout normal hematopoietic differentiation. Continuing efforts to generate a ChIP-grade antibody for

HOXA9 would be extremely useful for studying the normal function of HOXA9 as well as for studying human leukemias with high expression of *HOXA9*. Alternatively, a knockin mouse could be generated to allow for expression of an epitope-tagged Hoxa9 from the endogenous locus, however generation of transgenic mice is challenging and requires significant investment of time and resources.

There are reasons to believe that the interaction between Hoxa9 and C/EBPα may also exist in normal hematopoiesis. While HOXA9 is most highly expressed in hematopoietic progenitors and C/EBPα is expressed during myeloid commitment, both proteins are co-expressed in long-term multipotent progenitors, common myeloid progenitors/pre-granulocytic myeloid progenitors and granulocytic myeloid progenitors (and to a lesser extent pro-B cells) (Figure 3-6). Our studies suggest that a balance of levels of C/EBPα is critical to maintaining a permissive environment for Hoxa9-mediated transformation. The balance of C/EBPα levels has also been established to be critical to lineage fate choices during hematopoietic differentiation (*210*). It is possible that in addition to directing the differentiation of cells, early expression of C/EBPα in LMPP contributes to the activity of HOXA9 in normal hematopoiesis. Since HOXA9 is primarily responsible for maintaining proliferation and C/EBPα drives differentiation, it is also possible that there are antagonistic targets of HOXA9 and C/EBPα where the increasing C/EBPα out-competes HOXA9 to promote decrease in proliferation and differentiation.

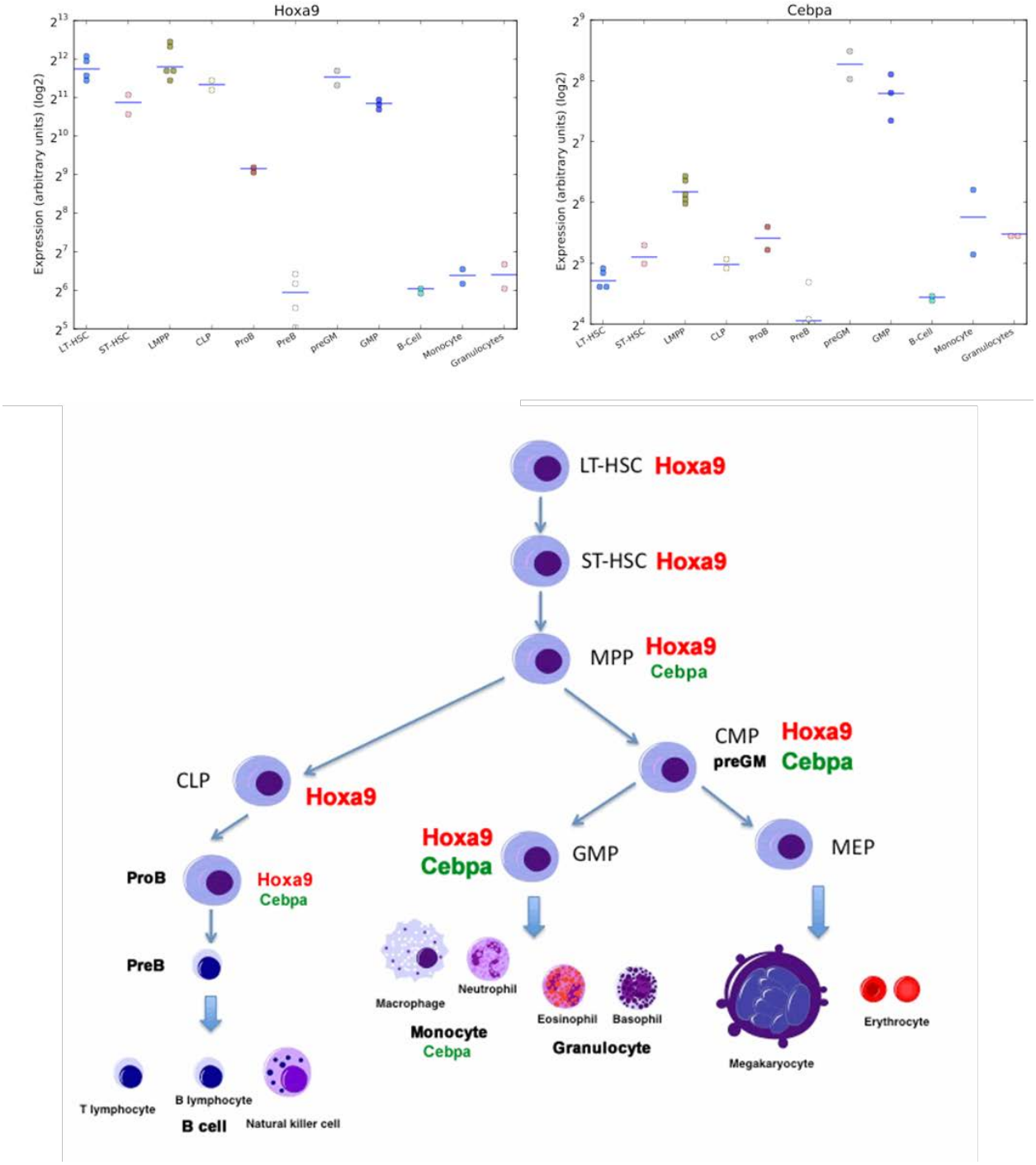

Figure 3-6 - Expression of Hoxa9 and Cebpa during hematopoietic differentiation
Expression levels of *Hoxa9* and *Cebpa* during normal hematopoietic differentiation, as determined from an online database of multiple gene expression profiles in hematopoietic cells (*69*) [Expression presented in log2 scale]. Schematic representation of *Hoxa9* and *Cebpa* expression, where large font and bold typeface indicate higher expression levels. Image modified from (*235*).

ANTAGONISM OF HOXA9 AND C/EBPA

We performed RNA-seq in cells 72 hrs after loss of either C/EBPα or Hoxa9, and looked for co-regulated targets between the two proteins. While some genes were significantly co-activated or co-repressed by Hoxa9 and C/EBPα (Figure 2-11) the majority of targets were antagonistically regulated (Figure 3-7). These gene targets include proleukemic targets such as *Flt3*, *CD34* and *Sox4* that are activated by Hoxa9 and repressed by C/EBPα. In addition, genes involved in myeloid differentiation such as *CD14*, *Csf1* and *Itgam* (*CD11b*) are activated by C/EBPα and repressed by Hoxa9. These results are consistent with our phenotypic characterization of cells after loss of Hoxa9 or C/EBPα, as cells differentiate and upregulate CD14 and CD11b after loss of Hoxa9 but decrease expression of CD14 and CD11b after loss of C/EBPα. In many ways, antagonism between Hoxa9 and C/EBPα is expected, as the two proteins play fundamentally different roles in hematopoietic development. It is reasonable to believe that Hoxa9 and C/EBPα may compete for binding at some targets, and changes in their expression level as differentiation progresses leads to skewing of cell programming from self-renewal to differentiation. Conversely, the collaboration between Hoxa9 and C/EBPα uncovers a novel role for C/EBPα in promoting leukemogenic transformation. This cooperation suggests that the balance of C/EBPα levels is critical for maintaining transformation by Hoxa9/Meis1.

Genes activated by Hoxa9 and repressed by C/EBPα					
Abca4	Ccnjl	Fyn	Lgals9	Podxl	Slc9a9
Akap2	Cd34	Fzd5	Map3k8	Prkch	Slfn5
Als2	Cd48	Gimap1	Map4k4	Psd3	Slfn8
Angpt1	Clnk	Gimap5	Marveld2	Psg17	Snx30
Auts2	Csgalnact1	Gimap6	Mdga1	Rai14	Sord
Axl	Csrp3	Gimap8	Mex3a	Rasgrp1	Sox4
C2cd4a	Ctla2a	Gp5	Msi2	Rgs12	St3gal2
C2cd4b	Dio2	Gpr179	Myct1	Rtn4r	Stxbp6
C530008M17Rik	Dkkl1	Gpr56	Nlrc3	Samd14	Thsd1
Capn5	Epsti1	Hbb-b1	Nrgn	Shank3	Tjp1
Car13	F2rl3	Id3	Pdgfrb	Slc22a3	Tmem176a
Ccdc102a	Fgf3	Iqgap2	Pitpnm2	Slc26a11	Tmem176b
Ccdc112	Flt3	Krt80	Podnl1	Slc45a3	Usp40

Repressed by Hoxa9, Activated by Cebpa						
Abca13	Cd33	Dmkn	Hsd11b1	Mtus1	Prom1	Stx11
Abcd2	Chn2	Dpep2	Idh1	Myo1e	Prtn3	Tex2
Adssl1	Chpf	E2f2	Ikbke	N4bp3	Ptgs2	Tfec
Alas1	Chst13	Ednra	Il1rn	Neb	Rbms1	Tgm2
Als2cl	Chst15	Ehd1	Il4ra	Nedd9	Rhou	Thbs1
Arg1	Ckap4	F13a1	Itgam	Nfil3	Rnd1	Tlr13
Arid3a	Cldn11	F630028O10Rik	Itk	Niacr1	S100a8	Tmem154
Arl11	Cldn15	Fam169b	Kcnj2	Nlrp3	S100a9	Tmem38b
Avil	Clec5a	Fgd4	Kif1b	Nod1	Samd5	Tnfrsf1b
B3gnt8	Clec7a	Fgd6	Klf2	Nos1ap	Sc5d	Tnfrsf26
B430306N03Rik	Clu	Fmnl2	Lcn2	Notch1	Sipa1l2	Trem1
B4galt6	Csf1	G6pdx	Lgals3	Nucb2	Sirpa	Trem3
Basp1	Cstb	Gipr	Lhfpl2	Olfml2b	Slc13a2	Vcam1
Bhlhe40	Cth	Glipr2	Lpl	Olr1	Slc24a6	Xdh
C3	Ctse	Gm7694	Lyst	Peli2	Slc7a11	Zfp36l1
Cass4	Cybb	Gpr84	Mgam	Pgd	Snai3	
Cd14	D930048N14Rik	Gpx3	Mgll	Plod1	Socs3	
Cd177	Dennd2a	Gsr	Mgst1	Pmaip1	Sorl1	
Cd28	Dgat1	Hmox1	Mmp13	Ppm1k	Spp1	
Cd300lb	Dgat2	Hp	Mreg	Pram1	Sqrdl	

Figure 3-7 - Genes antagonistically regulated by Hoxa9 and C/EBPα
Lists of genes that are antagoniscally regulated more than 1.5 fold by Hoxa9 and C/EBPα as determined by overlap of RNAseq 72 hours after loss of Hoxa9 or C/EBPα.

INVESTIGATION OF OTHER POTENTIAL HOXA9 COLLABORATORS

SWI/SNF complex member, Brg1

In our original study identifying potential collaborator proteins of Hoxa9, a number of additional factors were identified using co-immunoprecipitation followed by mass spectrometry. Of particular interest, multiple members of the SWI/SNF chromatin-remodeling complex were identified as potential members of the myeloid Hoxa9/Meis1 complex. The SWI/SNF complex is a multi-subunit protein complex that regulates chromatin organization by modulating nucleosomal positioning in an ATP-dependent manner. The SWI/SNF complex was first identified in *S. cerevisiae* as five genes important for the induction of transcriptional programs during mating and sucrose fermentation (*236*). Further studies established that these proteins physically interact and immunoprecipitate in a single complex that functions to modulate chromatin structure (*237*). Members of the SWI/SNF complex are highly evolutionarily conserved in eukaryotes. Owing to the large number of homologues of SWI/SNF, there are a large number of mammalian SWI/SNF complexes with various compositions of different subunit combinations (*238*). While the composition of mammalian SWI/SNF complexes is tissue specific and multiple complexes can be formed in a single cell type, a core complex exists containing BAF155, BAF170, INI1 and a catalytic subunit of either BRG1 or BRM. BRG1 and BRM are highly homologous ATPases that are mutually exclusive and regulate distinct subsets of target genes (*239*). It has also been established that Brm-containing complexes are more common in cells that are not proliferating while Brg1-containing complexes are found in cells that require replication (*239*).

Using co-immunoprecipitation followed by mass spectrometry, we identified Brg1

as a potential member of the myeloid Hoxa9/Meis1 complex. Brg1 was identified with high predictive scores in immunoprecipitations with both Hoxa9 and Meis1, along with additional SWI/SNF complex members Baf57, Baf170, Baf155, Baf250a and Baf60a/b (Figure 3-8). The interaction between Brg1 and Hoxa9 was subsequently confirmed by co-expressing various mutant forms of Hoxa9 in 293 cells which immunoprecipitated along with endogenous Brg1. BRG1 has been shown to play important roles in malignant transformation, both as a tumor suppressor through the regulation of Rb mediated cell cycle arrest and as an oncogene by suppressing p53 activity (*240, 241*). Most importantly, in a loss of function shRNA screen targeting known chromatin regulators, Brg1/Smarca4 was identified as being required for the maintenance of MLL-AF9-induced leukemic transformation (*242*).

anti-HA (Hoxa9)	Proteins Identified	Immunoprecipitation #1			Immunoprecipitation #2		
		Pw	Specs	Peps	Pw	Specs	Peps
	Hoxa9 Isoform HoxA-9 of Homeobox protein Hox-A9	1.000	38	9	1.000	120	18
	Meis1 Isoform 1/2 of Homeobox protein Meis1	1.000	5	4	1.000	16	9
	Pbx2 Pre-B-cell leukemia transcription factor 2	1.000	9	3	1.000	20	11
	Pbx3 Isoform PBX3a/b of Pre-B-cell leukemia transcription factor 3				1.000	10	4
	Arid1a AT rich interactive domain 1A (Baf250a)				1.000	7	4
	Smarca4 SWI/SNF related (Brg1)				1.000	19	12
	Smarcc1 Isoform 1 of SWI/SNF complex (Baf155)	1.000	3	2			
	Smarcc2 Isoform 2 of SWI/SNF complex (Baf170)				1.000	20	10
	Smarcd1 SWI/SNF related (Baf60a)				0.996	2	2
	Smarce1 SWI/SNF related (Baf57)	0.970	2	1	1.000	18	8

anti-FLAG (Meis1)	Proteins Identified	Immunoprecipitation #1			Immunoprecipitation #2		
		Pw	Specs	Peps	Pw	Specs	Peps
	Hoxa9 Isoform HoxA-9 of Homeobox protein Hox-A9	1.000	3	2			
	Meis1 Isoform 1/2 of Homeobox protein Meis1	1.000	141	30	1.000	41	19
	Pbx2 Pre-B-cell leukemia transcription factor 2	1.000	116	33	1.000	31	11
	Pbx3 Isoform PBX3a/b of Pre-B-cell leukemia transcription factor 3	1.000	56	12	1.000	14	6
	Arid1a AT rich interactive domain 1A (Baf250a)						
	Smarca4 SWI/SNF related (Brg1)	1.000	10	7			
	Smarcc2 Isoform 2 of SWI/SNF complex (Baf170)	1.000	6	3			
	Smarcd2 SWI/SNF related (Baf60b)				0.995	3	2
	Smarce1 SWI/SNF related (Baf57)	0.961	2	1			

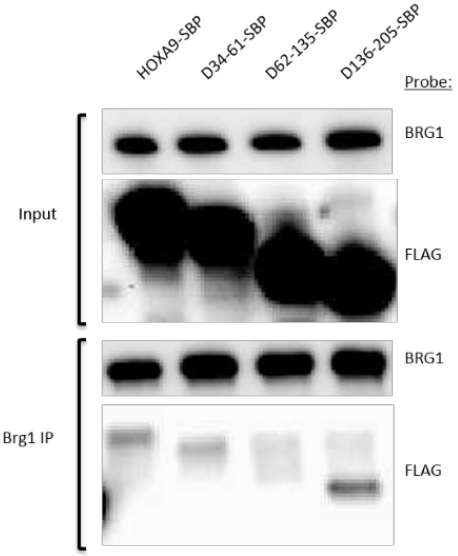

Figure 3-8 - Brg1 physically interacts with Hoxa9 in mouse myeloblastic cells
Co-immunoprecipitation mass spectrometry experiments in cells transformed with Hoxa9/Meis1 identified multiple SWI/SNF family members as potentially interacting with Hoxa9. Co-immunoprecipitation in 293 cells followed by western blot confirmed the interaction between Hoxa9 and SWI/SNF catalytic subunit Brg1.

To test the requirement for Brg1 in Hoxa9/Meis1-mediated transformation, we

generated cell lines that allow for conditional deletion of Brg1 using an inducible shRNA (in collaboration with Surya Nagaraja and Laura Wu). These cells constitutively express the Tetracycline repressor protein (TetR), which mediates silencing of the cotransfected shRNA constructs. In the presence of doxycycline, the TetR dissociates from the DNA allowing for transcription of the shRNA. Using this system, we transduced cells with Hoxa9, Meis1 and shRNAs targeting Brg1 or Renilla (as a control) (*242*). We also generated a control cell line using E2A-HLF, an oncogene that leads to myeloid transformation in the absence of detectable Hoxa9 or Meis1 expression levels. Consistent with results in MLL-AF9 transformed cells, loss of Brg1 dramatically reduces the proliferative capacity of cells transformed with Hoxa9/Meis1 and results in differentiation of the cells along the myeloid lineage (Figure 3-9a-c). Loss of Brg1 also leads to a decrease in proliferation in the E2A-HLF transformed cells, though to a lesser extent than in the Hoxa9/Meis1 transformed cells. These results suggest that Brg1 and the SWI/SNF chromatin-remodeling complex may play a more general role in maintaining the transcriptional program required for leukemic transformation. Future work employing this inducible model system to study the requirement for Brg1 in leukemias initiated by other oncogenes, including those with high and low Hoxa9/Meis1 expression, will help to define general and context specific mechanism for Brg1 in transformation. While recent publications suggest that Brg1 may play a role upstream of Hoxa9 in MLL-AF9 transformed cells by maintaining high expression levels of Hoxa9, our results suggest that Brg1 has additional roles downstream of Hoxa9/Meis1 expression (244). These data provide further support for Brg1 and the SWI/SNF complex acting as collaborating members of the myeloid Hoxa9/Meis1 complex.

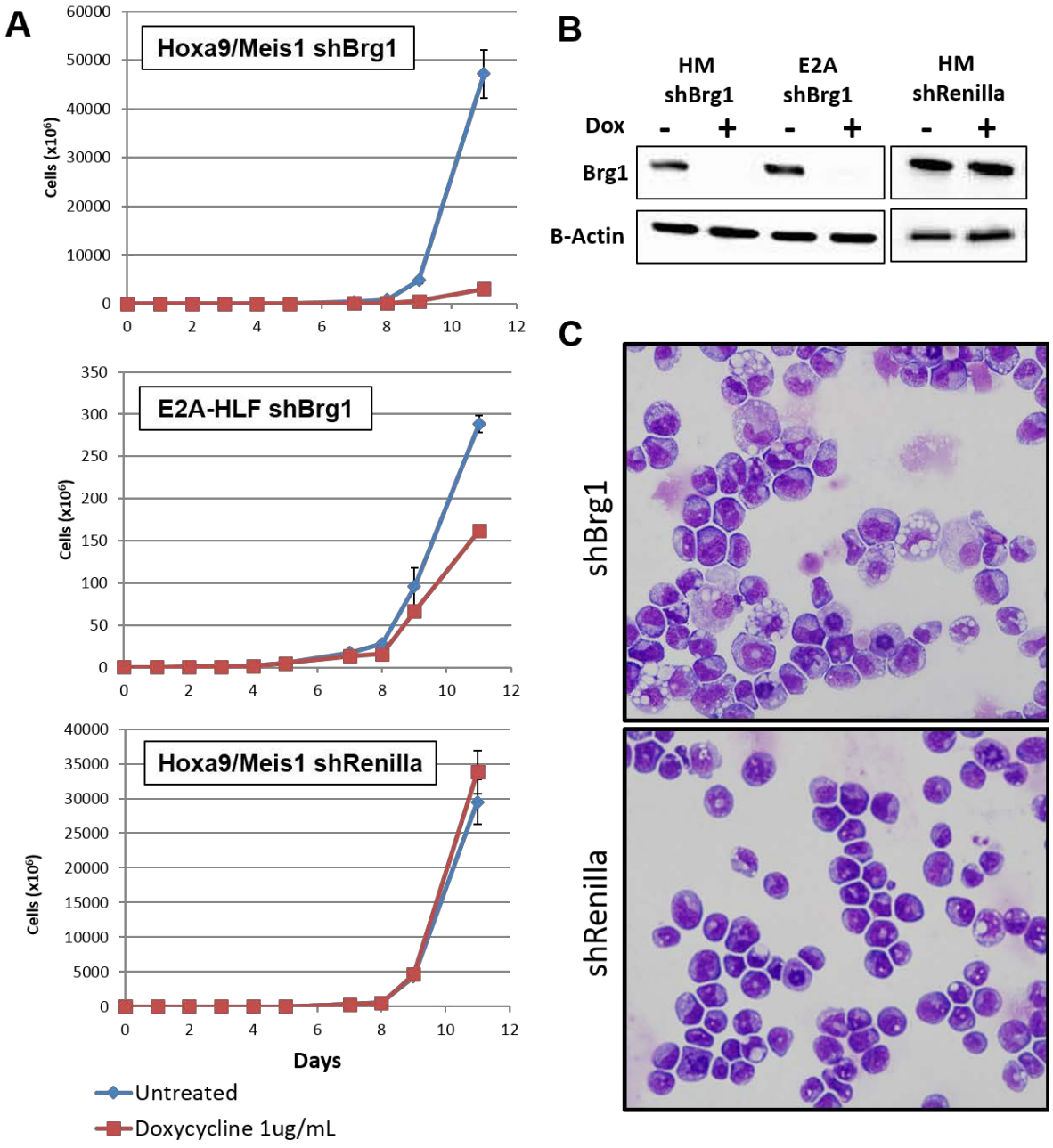

Figure 3-9 - Loss of Brg1 leads to growth inhibition and differentiation of transformed cell lines

(A) Knockdown of Brg1 in Hoxa9/Meis1 and E2A-HLF transformed cells leads to a decrease in cellular proliferation, while induction of shRenilla control lead to no change in proliferation. (B) Protein levels assessed by western blot show efficient knockdown fo Brg1 after 4 days treatment with doxycycline (DOX). (C) Gross morphologic changes in Hoxa9/Meis1 transformed cells consistent with differentiation along the myeloid lineage. (in collaboration with Surya Nagaraja and Laura Wu).

SWI/SNF complexes bind to DNA in a sequence-independent manner, partially mediated by the bromodomain region in the Brg1 or Brm subunit, which binds to acetylated histones (*237*). Transcription factors that interact with various SWI/SNF complexes add targeting specificity for nucleosomal remodeling. In addition, the activity of the SWI/SNF complex to relax or condense chromatin can subsequently make certain genomic regions more or less accessible for the binding of additional transcription factors. To investigate the functional relationship between Brg1 and Hoxa9 at the genomic level, we performed ChIP-qPCR for Hoxa9 and Brg1 in our model cell lines. Brg1 co-binds along with Hoxa9 at a number of cis-regulatory sites identified in our Hoxa9 ChIPseq experiments. Interestingly, Hoxa9 binding at these sites is stable 72hrs after the loss of Brg1 (Figure 3-10). These data suggest that Brg1 may not be playing a role in maintaining chromatin accessibility for Hoxa9 at these particular loci. It should be noted, however, that the reduced proliferative capacity of Hoxa9/Meis1 transformed cells after loss of Brg1 has a long latency, where significant changes in cell number are seen starting around 6 days. It is possible that while Brg1 is required for establishing the areas of chromatin accessibility for Hoxa9 binding, it does not play a role in maintaining this architecture. Thus, changes in nucleosomal positioning at these sites may require additional time or cell divisions. Examining the binding stability of Hoxa9 after extended periods of loss of Brg1 would be a first step in clarifying this result. Similarly, establishing the requirement of Hoxa9 for Brg1 binding at co-bound loci using the Hoxa9-ER/Meis1 tamoxifen-inducible cells can determine if Hoxa9 is playing a role in stabilizing or targeting Brg1 to specific regions of the genome.

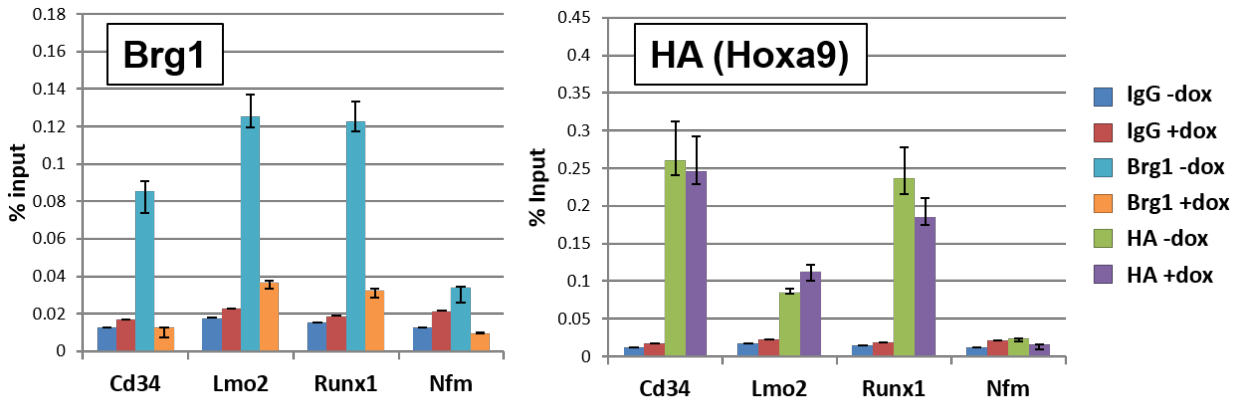

Figure 3-10 - Hoxa9 binding is stable in the absence of Brg1
ChIP performed in Hoxa9/Meis1 transformed cells at 72 hours after loss of Brg1 (+dox) shows that Brg1 is not required for the immediate stability of Hoxa9 at cis-regulatory elements. (in collaboration with Surya Nagaraja and Laura Wu).

While studying the functional interaction between Hoxa9 and Brg1 at a subset of binding sites can give clues to a potential mechanism for the requirement of Brg1 in Hoxa9/Meis1-mediated transformation, employing genome-wide approaches can likely provide more valuable insights. Establishing the co-dependancy of Brg1 and Hoxa9 binding on the genome-wide scale using our two inducible systems will help to define a mechanism for the requirement of Brg1 in Hoxa9/Meis1 transformation. It is possible that Brg1 establishes areas of chromatin accessibility to allow for Hoxa9 binding, but it is also equally likely that Hoxa9 (along with other collaborating factors) recruits Brg1 to specific loci, thereby directing the chromatin landscape in those regions. Investigating the genome-wide chromatin architecture using DNase-hypersensitivity assays after loss of either Brg1 or Hoxa9 will help distinguish between these two possibilities (*243*).

Since the vast majority of Hoxa9 binding sites occur in promoter-distal regions, it is highly likely that looping to facilitate enhancer-promoter interactions is critical to

Hoxa9's role as a transcriptional regulator. Multiple studies have confirmed a requirement for SWI/SNF in chromatin looping, including the recent work in MLL-AF9 cells that shows Brg1 is required for maintaining enhancer-promoter proximity at the *Myc* superenhancer (*244*). Brg1 may be required for maintaining enhancer-promoter looping required for Hoxa9-dependent gene regulation from distal genomic regions. While comprehensive "5C" methods exist for providing full-scale characterization of genome-wide looping interactions, more targeted 4C and 3C approaches can be used to characterize Hoxa9-dependant looping and the requirement for Brg1 in those interactions (*245*).

Given the requirement for Brg1 for the proliferation of Hoxa9/Meis1-transformed cells *in vitro* and for the aggressiveness of MLL-AF9 transformed cells *in vivo*, further characterization of the mechanism for this requirement is critical. Interestingly, 4 out of the 6 other SWI/SNF subunits that were found to be required in MLL-AF9 transformation (BAF60b, BAF250a, BAF60a and BAF155) were also identified in our co-immunoprecipitation studies with Hoxa9/Meis1, providing strong evidence for a specific role for the SWI/SNF complex through physical interaction with the Hoxa9/Meis1 complex (*244*). Understanding this functional interaction will shed light on mechanisms of Hoxa9-dependent gene regulation.

ASSESSING DIRECT TARGETS OF HOXA9

Cdkn2a/b

Of equal interest to characterizing the mechanisms through which Hoxa9 regulates gene expression is the identification of the critical downstream targets that

mediate leukemic transformation. In studying the functional interaction between C/EBPα and Hoxa9, we observed that loss of either C/EBPα or Hoxa9 leads to a decrease in proliferation and a block in cell cycle progression at the G1/S transition. We also identified the cell cycle regulators, *Cdkn2a(p16/INK4a)* and *Cdkn2b(p15/INK4b)*, as targets of repression by Hoxa9 and C/EBPα. Both Cdkn2a and Cdkn2b regulate the G1 to S-phase transition through direct inhibition of cyclin dependent kinases 4 and 6 (Cdk4/6), thereby preventing the phosphorylation of retinoblastoma family members required for cell cycle progression (*246*). An additional cell cycle regulator, *ARF,* is also expressed from an alternative start codon in the *Cdkn2a* locus, and while these two proteins share exons 2 and 3 they are expressed from two different open reading frames and share no sequence homology. *ARF* acts as a cell cycle inhibitor through the Mdm2/p53 pathway, and expression of *Cdkn2a* and *Arf* are tightly co-regulated (*247*). Cdkn2a/Arf/Cdkn2b all act as tumor suppressors in a wide variety of malignancies, where their expression is decreased through both epigenetic silencing and deletions (*217, 248*). Mouse models with deletions of each show increased incidence of spontaneous cancers, many occurring in the hematopoietic compartment, with the most dramatic phenotypes seen in mice with deletion of all three proteins (*249-252*). Indeed, both *Cdkn2a/b* are silenced in hematopoietic neoplasms through DNA hypermethylation, which in the case of *Cdkn2b* can be observed in 70-80% of patients with AML and is associated with poor prognosis (*218, 253, 254*).

Work is currently ongoing to test the requirement of Cdkn2a/b repression for transformation by Hoxa9/Meis1 as well as for mediating the block in cell cycle progression after loss of C/EBPα or Hoxa9. Preliminary results suggest that

overexpression of *CDKN2A* or *CDKN2B* decreased the transforming ability of Hoxa9/Meis1 as evidenced by decrease in colony number and size in the first and second round of plating. Similarly, overexpression of *CDKN2A*/*2B* in cells transformed by Hoxa9/Meis1 leads to a decrease in proliferation in liquid culture. Studies are ongoing to characterize the cell cycle dynamics in these systems. We are also attempting to rescue the cell cycle block after loss of Hoxa9 or C/EBPα using shRNAs targeting *Cdkn2a* or *Cdkn2b*. Given that *Cdkn2b* can compensate for loss of *Cdkn2a* and vice versa, it may be required to target both genes simultaneously to achieve maximum efficiency (*252*). Still, it is likely that repression of *Cdkn2a/b* is one of many of the important actions of Hoxa9 and C/EBPα for leukemic transformation, so only a partial rescue may be observed.

Previous work performed in a fibroblast model of oncogene-induced senescence has implicated HOXA9 in the direct repression of *CDKN2A* through promoter binding and recruitment of polycomb protein SUZ12 and histone deacetylase HDAC1 (*225*). While we did not observe a Hoxa9 binding site at the promoter of either *Cdkn2a/b* in our ChIPseq study or by ChIP-qPCR, we did identify a Hoxa9/C/EBPα cobound region 50kb downstream of the *Cdkn2a/Arf/Cdkn2b* locus (Figure 2-12a). Interestingly, this binding site shows local enrichment of H3K4me1 and depletion of H3K27me3 in a region otherwise broadly modified by H3K27me3. It is possible that Hoxa9 and C/EBPα mediate the repression of *Cdkn2a/b* through recruitment of polycomb and histone-deacetylases to the promoters via chromatin looping interactions. Indeed, C/EBPα has been shown to interact with HDAC1 in liver cells, and HDAC1 was identified in our original coIP/MS screen as interacting with Hoxa9 (*174, 255*). Initial steps towards

testing this mechanism would include performing coimmunoprecipitation to determine if HDAC1 complexes with Hoxa9 and C/EBPα in Hoxa9/Meis1 transformed cells. Additionally, ChIPqPCR for HDAC1 and SUZ12 at both the *Cdkn2a/b* promoters and the downstream Hoxa9/C/EBPα cobound site in both the presence and absence of Hoxa9 or C/EBPα would potentially show a requirement for Hoxa9 and C/EBPα in recruiting polycomb to these promoters. Preliminary attempts in ChIP for HDAC1 and SUZ12 did not show binding at either locus, though positive and negative control regions for both proteins are needed to determine if the immunoprecipitation was successful. Alternatively, characterizing the change in H3K4me3 and H3K27ace, the histone modifications regulated by HDAC1 and SUZ12, can act as a surrogate readout. These studies are currently ongoing and will help shed light on potential mechanism of Hoxa9/C/EBPα mediated repression of *Cdkn2a/b*.

There is additional evidence that Hoxa9 and C/EBPα may regulate *Cdkn2a/b* repression by long-range chromatin interactions. First, the reports of HOXA9 binding to the promoter of *CDKN2A* both in vitro and in cells establishes that this interaction is possible but potentially not strong enough to show direct binding using our ChIP methods (*225*). Furthermore, the SWI/SNF complex is well established to be involved in chromatin looping, and multiple SWI/SNF complex members interact with Hoxa9 including Brg1, which we show is required for Hoxa9-mediated transformation (*256*). C/EBPα also interacts with Brg1, suggesting the three proteins may form a complex that helps mediate repression of this locus (*162*). Using our Hoxa9/Meis1-transformed cells that allow for conditional deletion of Brg1, we can test the requirement for Brg1 in *Cdkn2a/b* repression, as well as in the changes to epigenetic modifications in this

region. Chromatin conformation capture technology can also be used to test either direct interaction between the Hoxa9/C/EBPα binding site and the *Cdkn2a/b* promoters or in a high-throughput manner to determine all of the long-range interactions that occur with this binding site.

It is possible that the repression of *Cdkn2a/b* by Hoxa9 and C/EBPα does not occur via promoter interactions, but instead through the regulation of a long non-coding RNA. Recent studies implicate lncRNA *ANRIL* in the polycomb-mediated repression of the *INK4A/B* locus in humans (*257, 258*). *ANRIL* is transcribed antisense through the *Cdkn2b* locus, extending over 120kb downstream. The Hoxa9/C/EBPα cobound region lies within the mouse homolog of the *ANRIL* transcript, potentially regulating its transcription or activity. Preliminary studies testing the expression of this lncRNA after loss of Hoxa9 or C/EBPα could be performed. If changes in expression level are observed, exogenous overexpression of *ANRIL* or targeting with an shRNA can further characterize the requirement of this transcript for Hoxa9-mediated transformation.

While our work conclusively shows an induction of *Cdkn2a/b* expression after loss of either Hoxa9 or C/EBPα (Fig2-12d,e), more definitive studies are required to establish the requirement of the co-bound cis-regulatory region for *Cdkn2a/b* repression. Recent advances in genome editing technology allows for more efficient targeting of specific loci. A technique called CRISPR (clustered regulatory interspaced short palindromic repeats) uses complementary RNA sequences to target an endonuclease to specific loci, which creates double strand DNA breaks (*259-261*). Genomic editing can be accomplished by coexpressing a complementary oligonucleotide that will be used in homologous repair of the double-strand break. For

the purposes of our studies, this complementary oligo could contain mutated or deleted HOX or CEBP motifs to disrupt binding. Alternatively, the oligo could have inserted loxP sequences flanking the Hoxa9/C/EBPα cobound region to allow for cre-mediated deletion of this binding site. Correctly targeted cells could be used directly in cell based assays, or could subsequently be used for generating a targeted mouse model. As a third approach, CRISPR could be used to disrupt Hoxa9/C/EBPα binding by targeting a catalytically inactive endonuclease with a library of RNAs complementary to the co-bound site, which will remain bound without inducing any genomic breaks and potentially block the Hoxa9/C/EBPα association.

CHAPTER 4:

CONCLUSIONS

With the continuing discovery of diseases that involve the dysregulation of *HOX* genes, it is increasingly more important to understand the mechanisms through which HOX proteins achieve transformation to help guide future therapies. A large number of studies have employed microarray technology to establish transcriptional targets of HOX proteins, but relatively little progress has been made determining which targets are critical for disease processes and how HOX proteins regulate these targets. In this thesis, we focused our studies on HOXA9, whose expression is upregulated in over 50% of AML and correlates strongly with poor prognosis. By combining genome-wide analysis of Hoxa9 binding sites in murine cell lines transformed by Hoxa9/Meis1 and identification of proteins that co-immunoprecipitate with the myeloid Hoxa9 complex, our group identified a number of factors that could potentially collaborate with Hoxa9 during leukemic transformation. Of particular interest were the lineage specific transcription factor, C/EBPα, as well as the SWI/SNF catalytic subunit, Brg1. Using cell based assays, genome-wide approaches, and in vivo studies, I made significant progress in characterizing the functional interaction between these factors and Hoxa9.

In Chapter 2, I show that C/EBPα is required for leukemic transformation mediated by Hoxa9/Meis1. I first generated cell lines that allow for conditional loss of Hoxa9 or C/EBPα and characterized the phenotypes of these cells. Loss of either

Hoxa9 or C/EBPα leads to a decrease of proliferation of cells in liquid culture, which is accompanied by a block in cell cycle at the G1/S phase. Interestingly, cells additionally undergo myeloid differentiation and apoptosis in the absence of Hoxa9 but not after loss of C/EBPα, likely reflecting the important role that C/EBPα plays in promoting these processes. Given the requirement for C/EBPα for Hoxa9/Meis1-transformation in culture, I next tested the requirement of C/EBPα for *in vivo* leukemogenesis. Loss of C/EBPα leads to a significant improvement in survival in both primary and secondary Hoxa9/Meis1 leukemias in mice. Additionally, I observed a strong selective pressure for maintaining high C/EBPα levels in cell culture and in the murine leukemias.

To elucidate the molecular mechanism behind the requirement of C/EBPα in Hoxa9-medated leukemogenesis, I performed ChIPseq for C/EBPα and Hoxa9 in our transformed cells. C/EBPα co-associates at over 50% of Hoxa9 binding sites, and genes downstream of C/EBPα/Hoxa9 co-bound sites are involved in the regulation of critical hematopoietic processes. I also performed RNAseq using our conditional cell lines to identify targets co-regulated by C/EBPα and Hoxa9. Though relatively small number of genes were found to be significantly coactivated or corepressed by C/EBPα and Hoxa9, multiple targets involved in the regulation of cell cycle were among these targets. Of particular interest were the cyclin dependent kinase inhibitors *Cdkn2a* and *Cdkn2b*. I identified a strong Hoxa9/C/EBPα co-bound site 50kb downstream of the Cdkn2a/b locus that could potentially allow for direct regulation of their expression. Studies characterizing the mechanism for Hoxa9/ C/EBPα repression of *Cdkn2a/b* as well as the requirement of this repression for leukemogenesis are ongoing.

Consistent with the differing phenotypes of our cell lines after loss of Hoxa9 or

C/EBPα, a large number of genes were antagonistically regulated by these two transcription factors. Many of these genes are involved in pathways regulating myeloid differentiation while others associated with cellular proliferation. These targets may represent genes normally antagonistically regulated by Hoxa9 and C/EBPα during hematopoietic differentiation. Indeed, Hoxa9 is most highly expressed in early progenitors while C/EBPα is expressed in cells committed to the myeloid lineage. It is reasonable to believe that Hoxa9 and C/EBPα may compete for binding at some targets, and changes in their expression level as differentiation progresses leads to skewing of cell programming from self-renewal to differentiation. Further characterization of this antagonism will likely provide valuable insight into the function of Hoxa9 in both normal and malignant hematopoiesis. In addition, this antagonism suggests that the balance of C/EBPα levels is critical for maintaining transformation by Hoxa9/Meis1. Indeed, both loss of C/EBPα and its overexpression leads to a decrease in proliferation of Hoxa9-transformed cells, suggesting moderate levels of C/EBPα provide an environment that promotes transformation without driving myeloid differentiation (Figure 4-1). Study of the requirement for C/EBPα in other Hoxa9-high and Hoxa9-low models of leukemia will help establish if this is a more general property of lineage-determining transcription factors in leukemia, or if this is specific to the Hoxa9- C/EBPα functional interaction.

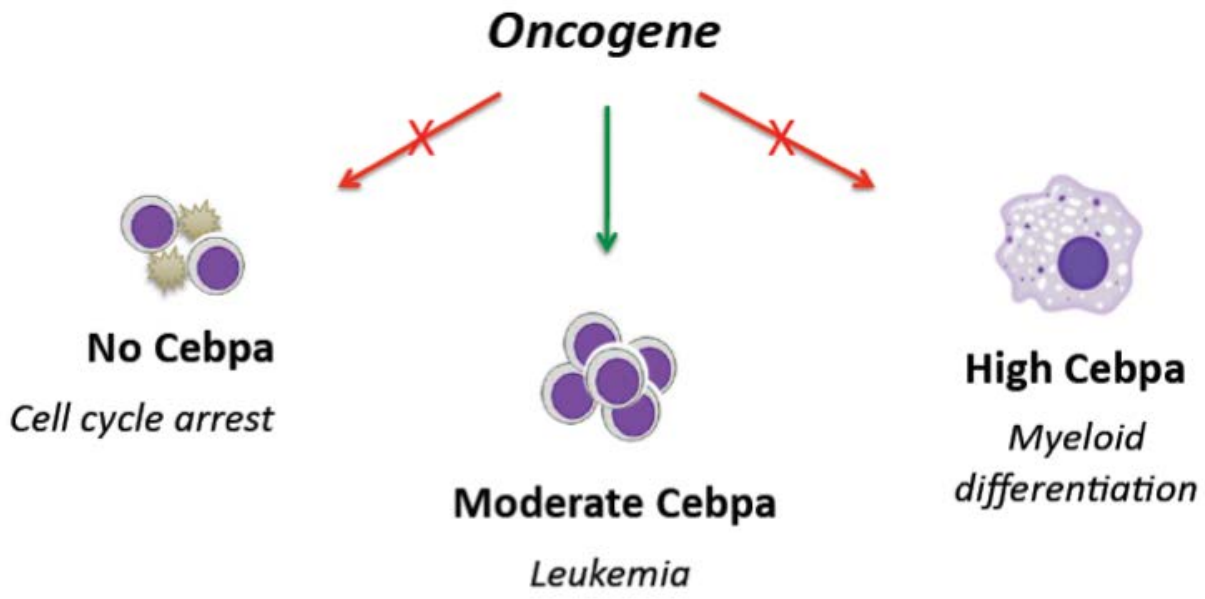

Figure 4-1 - Levels of C/EBPα critical for maintaining transformation
Moderate levels of C/EBPα are required for transformation by Hoxa9/Meis1. Loss of C/EBPα leads to cell cycle arrest while overexpression of C/EBPα leads to induction of myeloid differentiation.

I also made preliminary progress in establishing a role for the SWI/SNF complex, specifically the catalytic subunit Brg1, in Hoxa9-mediated leukemogenesis. Using a cell line allowing for conditional expression of shRNAs targeting Brg1, I found that loss of Brg1 leads to both a decrease in proliferation and induction of differentiation of cells transformed by Hoxa9/Meis1. Given the increasing evidence that both chromatin accessibility and long-range chromatin interactions are critical for transcriptional regulatory abilities of distal enhancers, we hope to use this model system to continue to study the importance of the SWI/SNF complex in Hoxa9-mediated transformation.

There are still many interesting avenues to explore with regards to other proteins that potentially collaborate with Hoxa9. My studies suggest that Hoxa9 targeting and downstream gene regulation depends on both lineage specific transcription factors and chromatin remodeling machinery (Figure 4-2). I can also speculate that the activity of

Hoxa9 transcriptional regulatory complexes may be modulated by upstream signal transduction pathways, including those acting through Stat5. Work is ongoing in attempts to characterize the functional interaction between Hoxa9 and Stat5 in leukemic transformation. It is also interesting to hypothesize about the lineage specificity of Hoxa9-complexes and transcriptional targets. Indeed, Hoxa9 is upregulated in a subset of acute lymphoblastic leukemias as well as in other malignancies including prostate, ovarian and breast cancer. Many of the approaches used in this thesis could be applied to studying Hoxa9 in other model systems as a way to increase our understanding of Hoxa9-mediated transformation in general. I hope that the work presented in this thesis will serve as a solid foundation for future studies that will continue to increase our understanding of HOX biology in disease, and ultimately lead to improved therapeutic strategies and patient outcomes.

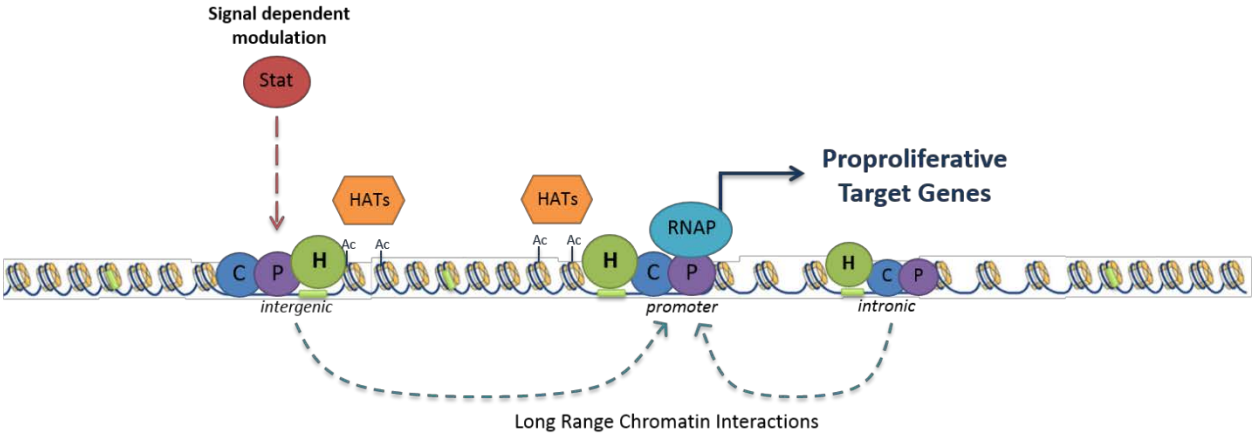

Figure 4-2 - Model for HOX targeting and activity
The work presented in this thesis the combined action of lineage specific transcription factors, chromatin remodeling machinery, chromatin modifying enzymes and upstream signal transduction pathways in mediating transcriptional regulation by Hoxa9 in leukemia.

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