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Significance of Protein Interactions in Mediating AF9 Function

Bhavna Malik
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

SIGNIFICANCE OF PROTEIN INTERACTIONS IN MEDIATING AF9 FUNCTION

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BHAVNA MALIK

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LIST OF ABBREVIATIONS

MLL	Mixed Lineage Leukemia
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
PML	Promyelocytic Leukemia
SNL	Speckled Nuclear Localization
RD	Repression Domain
HDAC	Histone Deacetylase
CtBP	C-terminal binding protein
PHD	Plant Homeodomain
RRM	RNA recognition Motif
SET	Su(var)3-9, enhancer of zeste, trithorax
TAD	Trancriptional Activation Domain
PTD	Partial Tandem Duplication
HSC	Hematopoietic stem cell
MPN	Myeloproliferative neoplasia
TALE	Three –amino acid loop extension
ShRNA	short hairpin RNA
Rb	Retinoblastoma
HLH	helix-loop-helix

MBI	Myc box I
MBII	Myc box II
SEC	Super Elongation Complex
PAFc	Polymerase Associated Factor complex
ANC1	Actin non-complementing gene 1
AHD	ANC1 homology domain
ENaCα	Epithelial sodium channel gene α
Sgk1	Serum- and glucocorticoid induced kinase-1
SVZ	Subventricular zone
PTEFb	Positive transcription elongation factor b
CDK9	Cyclin dependent kinase 9
CTD	C-Terminal domain
DNMTs	DNA methyltransferases
HPLC	High performance liquid chromatography
LC-ESMS	Liquid chromatography electrospray ionization mass spectrometry
DSBs	Double strand breaks
OFCD	Oculofaciocardiodental
PRC	Polycomb Repressive Complex
ChIP-Seq	Comparative genome-wide chromatin immunoprecipitation
ESC	Embryonic stem cell
DTT	Dithiothreitol
Rpm	Rotations per minute

FPLC	Fast Pressure Liquid Chromatography
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
qRT-PCR	Quantitative Real time Polymerase Chain Reaction

ABSTRACT

Rearrangements of the *MLL* gene at chromosome band 11q23 have been associated with a heterogeneous group of lymphoid, myeloid and mixed lineage leukemias. *MLL* rearrangements occur approximately in 70% of infant leukemias and are also common in therapy-related leukemias where patients were previously treated with topoisomerase II inhibitors. Unfortunately, these patients have a poor prognosis. *MLL* gene rearrangements give rise to chimeric proteins that contain the N-terminal portion of MLL fused to the C-terminal portion of over 50 different fusion partners. The chimeric proteins cause constitutive expression of some MLL target genes such as *HOXA9* and *MEIS1*, and enhanced proliferation of hematopoietic progenitors. MLL chimeras do not retain the histone methyltransferase activity of wild type MLL and it is unclear how they deregulate the expression of MLL target genes.

AF9 is one of the most common MLL fusion partners encountered in patients with leukemias. Moreover, Af9 is known to negatively regulate the expression of the epithelial sodium channel α (*ENaCα*) gene in murine renal collecting ducts by modulating the activity of the Dot1l. It has been shown that Af9-Dot1l promotes H3K79 methylation at specific sites in the *ENaCα* promoter, which then contributes to its repressed state. A number of reports have described the direct or indirect association of

the C-terminus of AF9 with several distinct proteins including activators as well as repressors of transcription apart from DOT1L. Our data indicate that other AF9 binding proteins compete with DOT1L thereby diminishing its activity. Specifically, we show that AF9 is part of a protein multimer containing members of Polycomb group PRC1 complex, CBX8, RING1B, and BMI1. The interaction of AF9 with other PRC1 proteins is directly mediated by CBX8 and precludes AF9-DOT1L binding. Knockdown of *CBX8* with shRNA leads to decreased *ENaCα* mRNA levels. In contrast, *CBX8* overexpression results in increased *ENaCα* mRNA levels and this effect can be partially overcome by co-overexpression of *AF9*. As predicted, expression of *CBX8* is accompanied by decreased H3K79 trimethylation at *ENaCα* promoter regions. Thus, our data suggest that changing the abundance of different AF9 binding partners shifts the equilibrium of functionally distinct AF9 complexes and alters the regulation of target genes. Moreover, although CBX8 is part of the repressive PRC1 Polycomb complex, its increased expression may actually increase the expression of genes normally repressed by DOT1L through its interaction with AF9.

Furthermore, it has been shown that AF4 and AF9 exist in a large multiprotein complex containing positive transcription elongation factor b (P-TEFb) and therefore are linked to transcriptional elongation. To understand the significance of the AF9-AF4 interaction, our laboratory has mapped the domains involved in their binding and has developed synthetic peptides (PFWT and SPK-111) capable of disrupting their interaction *in vitro* and *in vivo.* The peptides mimic the amino acid sequence of the AF9 binding domain within AF4. These peptides are toxic to MLL-AF4 and MLL-AF9 fusion

harboring leukemic cell lines, suggesting the importance of the AF9-AF4 interaction for the survival of these leukemic cells. However, the molecular mechanism of action of peptide is still not known.

We showed that after peptide treatment, MV4-11 (MLL-AF4) and MOLM13 (MLL-AF9) leukemic cells have reduced levels of MEIS1 and MYC expression both at the transcript levels and protein levels. MEIS1 and c-MYC are known targets of MLL fusion proteins. Moreover, the reduced *MYC* transcript levels correlate with the decreased recruitment of cyclin dependent kinase 9 (CDK9), a component of P-TEFb and phosphorylation of Ser2 of C-terminal domain (CTD) of RNA PolII. Hence, our data suggest that peptide mediated disruption of AF9-AF4 interaction interferes with the stable complex formation involving P-TEFb, which further impairs the productive elongation of the transcripts.

Therefore, modulation of protein-protein interactions involving AF9 would be predicted to have important effects on disease processes that subvert AF9, including MLL-AF9 leukemias.

CHAPTER 1

INTRODUCTION

Leukemia is a cancer of the hematopoietic tissue that arises because of an uncontrolled increase in the number of immature blood cells. Leukemia can be classified as acute or chronic clinically and pathologically, and can be further subdivided as Myelogenous or Lymphoblastic depending upon the blood cell lineage that is being affected. Mixed lineage leukemia (MLL) is characterized by rearrangement of the *MLL* gene at chromosome band 11q23. In patients, about 5-10% of both acute lymphoblastic (ALL) and acute myeloid leukemias (AML) show rearrangement of the *MLL* gene, and some of these leukemias are associated with poor prognosis (Daser and Rabbitts, 2004; Krivtsov and Armstrong, 2007). To date, many studies have been focused on MLL; however, molecular mechanisms underlying MLL rearranged leukemias have yet to be determined. In this dissertation, we focus on one of the most common fusion partners of MLL, AF9. Since AF9 has been shown to associate with a wide variety of proteins including activators and repressors of transcription (Biswas et al., 2011), we sought to determine the significance of protein interactions in mediating AF9 function.

MLL

The *MLL* (a.k.a *ALL-1, MLL1, HRX, Htrx*) gene is approximately 89kb long, and encodes the MLL protein. MLL is a massive protein of 3969 amino acids and is a human

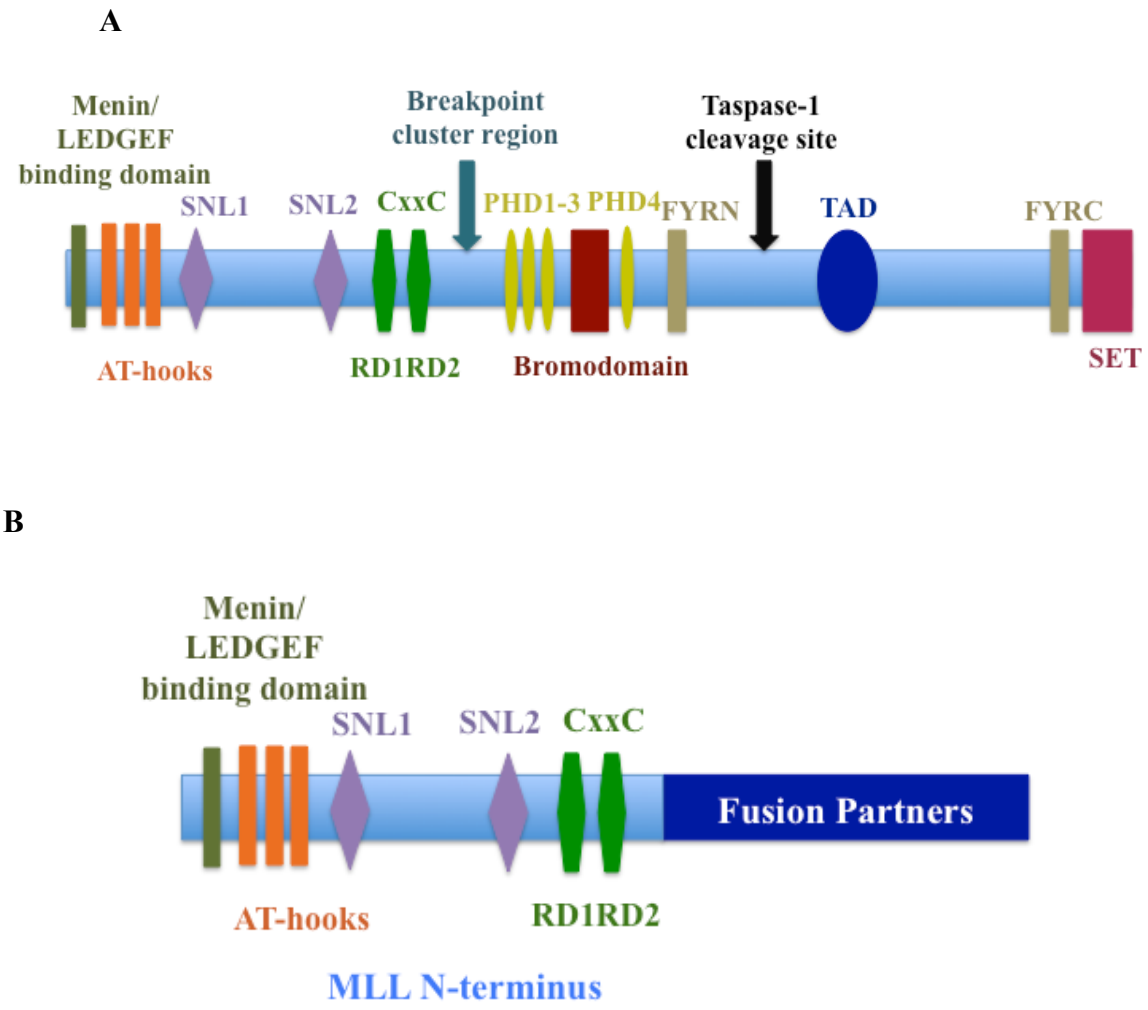
homologue of the Drosophila *Trithorax* gene (Tkachuk et al., 1992). It has been shown using immunohistochemistry that the MLL protein localizes in punctate nuclear structures and does not colocalize with nuclear PML bodies (Butler et al., 1997). Rearrangements of the *MLL* gene at chromosome band 11q23 have been associated with a heterogeneous group of lymphoid, myeloid and mixed lineage leukemias (Hess, 2004). *MLL* rearrangements occur in approximately 70% of infant leukemias and in 10% of adults as well as in therapy-related leukemias where patients are previously treated with topoisomerase II inhibitors (Felix et al., 1995; Mrozek et al., 1997; Rubnitz et al., 2002). All such patients have a poor prognosis (Felix et al., 1995; Krivtsov and Armstrong, 2007; Mrozek et al., 1997; Rubnitz et al., 2002). Infant patients with ALL have five-year event-free survival of approximately 34% in cases with *MLL* rearrangements compared to 60% or more with germline *MLL* (Hilden et al., 2006; Tomizawa et al., 2007). On the other hand, in infants, cases of acute myeloid leukemia (AML) associated with *MLL* rearrangements versus germline do not vary much in terms of their outcome (Chowdhury and Brady, 2008; Pui et al., 2002).

The MLL protein is a multidomain molecule that is cleaved by an aspartate protease called taspase into a larger 320 kDa N-terminal (MLL-N) and a smaller 180kDa C-terminal fragment (MLL-C) and both the fragments remain non-covalently bound in a tight complex (Yokoyama et al., 2002). The interaction of MLL-N and MLL-C termini is dependent on the presence of phenylalanine (F)- and tyrosine (Y)-rich motifs in both the fragments termed FYRN and FYRC domains, respectively, as well as on the SET domain present in the C-terminus (Hsieh et al., 2003b). Moreover, it has been reported that by

disrupting the non-covalent association of MLL-N and MLL-C termini, there is reduction in the protein levels of the MLL N-terminus protein and diffuse nuclear localization of MLL C-terminus (Hsieh et al., 2003b). Furthermore, it has been shown that the proteolytic cleavage of MLL is important for the proper expression of its target Homeobox (*HOX)* genes and for cell cycle regulation (Hsieh et al., 2003a; Takeda et al., 2006).

The structure of the multidomain MLL protein is depicted in Figure 1. At the very N-terminus is a domain for menin/LEDGF binding (Caslini et al., 2007; Yokoyama and Cleary, 2008). Menin was first identified as a tumor suppressor protein (Chandrasekharappa et al., 1997) but later on it was shown to be a MLL associated cofactor whose association is necessary for MLL functions such as maintenance of *HOX* gene expression as well as MLL fusion associated leukemic transformation (Caslini et al., 2007; Yokoyama et al., 2005; Yokoyama et al., 2004). Subsequently, it has been shown that menin binds LEDGF, which recruits the MLL complex to its target gene via its DNA binding PWWP domain (Engelman and Cherepanov, 2008; Yokoyama and Cleary, 2008). Downstream of the menin/LEDGF-binding domain, there are three AT hook motifs that interact with A/T rich scaffold attachment regions (SAR) in the minor groove of DNA (Broeker et al., 1996; Krivtsov and Armstrong, 2007). AT hooks are followed by two speckled nuclear localization signal domains (SNL1 and SNL2) and two repression domains (RD1 and RD2) (Krivtsov and Armstrong, 2007). Nuclear localization signals are responsible for MLL localization to sub-nuclear regions that show specific punctate nuclear distribution mentioned earlier (Caslini et al., 2000; Yano et al., 1997). MLL

Figure 1. Domains of MLL and MLL fusion proteins



(A) MLL is a multidomain protein that is cleaved by a taspase into bigger N-terminal and smaller C-terminal fragments. At the N-terminus MLL has menin-LEDGF binding domain responsible for chromatin recruitment followed by two repression domains, RD1 and RD2, and four PHD fingers. At the C-terminus, MLL has a catalytic SET domain that is responsible for H3K4 methylation. (B) In MLL fusion proteins, the N-terminus of MLL is fused to the C-terminus of over 50 different fusion partners.

fusion proteins retain the two transcriptional repression domains (Zeleznik-Le et al., 1994). Many repressor proteins have been shown to interact with the RD, including, histone deacetylases (HDACs), C-terminal binding protein (CtBP) and Polycomb group proteins HPC2 (CBX8) and BMI1 (Xia et al., 2003), which I will explain later in the introduction. The MLL RD1 contains a CxxC domain rich in cysteine residues, which is involved in the binding to non-methylated CpG islands of DNA and maintains *HOXA9* gene expression (Erfurth et al., 2008). It has been reported that binding of the MLL CxxC domain to non-methylated DNA is necessary for leukemic transformation by MLL fusion proteins (Ayton et al., 2004; Cierpicki et al., 2010).

There are four plant homeodomain (PHD) fingers that are involved in protein-protein interactions following RDs. Specifically, the third PHD finger, which is not present in MLL fusion proteins associates with cyclophilin CYP33 (Fair et al., 2001). CYP33 has a prolyl-*cis*-*trans*-isomerase (PPIase) activity that has been shown to be important for the binding between the RNA recognition motif (RRM) of CYP33 and MLL PHD3 (Birney et al., 1993; Fair et al., 2001; Wang et al., 2010). In addition, CYP33 overexpression results in the decreased expression of MLL target genes, including *HOXA9, HOXC8, c-MYC* and *CDKN1B* that is most likely due to the reduced levels of H3K4 trimethylation (H3K4me3) catalyzed by MLL at the promoter region of these genes (Park et al., 2010). Apart from binding to CYP33, PHD3 is also known to bind in a mutually exclusive manner to trimethylated H3K4. Based on these studies, it was speculated that depending upon the binding partner (H3K4me3 or CYP33), the association of MLL PHD3 could act as a molecular switch between the activated and repressed state of target genes (Park et al., 2010). MLL also has an atypical bromodomain

located between third and forth PHD finger, which is predicted to associate with acetylated lysines of histones like other bromodomain containing proteins, thereby resulting in chromatin remodeling (Jeanmougin et al., 1997; Popovic and Zeleznik-Le, 2005). In the C-terminal fragment, MLL contains a transcriptional activation domain (TAD) and highly conserved SET (Su(var)3-9, enhancer of zeste, trithorax) domain (Krivtsov and Armstrong, 2007). The TAD domain is known to interact directly with CREB-binding protein (CBP), thereby facilitating MLL transcriptional activity (Ernst et al., 2001). The SET domain is responsible for H3K4 methylation and is known to associate with proteins responsible for chromatin remodeling to promote efficient transcription (Milne et al., 2002; Slany, 2009). Although the promoter regions of active genes have shown co-localization of MLL and RNA PolII using genome wide sequencing, only <5% of promoters require MLL for H3K4 methylation (Wang et al., 2009).

Homozygous (-/-) null mutation of Mll in mice has been shown to cause lethality in the embryonic stage, and mice die by E10.5-11.5. These mice embryos also exhibit defects in yolk sac hematopoiesis (Hess et al., 1997; Yu et al., 1995). On the other hand, Mll heterozygous (+/-) mice showed abnormalities in the axial skeleton, hematopoiesis and *Hox* gene expression (Hess et al., 1997; Yu et al., 1995). Importantly, phenotypes of *Mll* null embryos and certain *Hox* knockout mouse models resemble one another closely (Yu et al., 1995). The expression of *Hox* genes is not maintained in *Mll* null embryos during later timepoints, although *Hox* genes are expressed prior to E9.0 stage, suggesting the requirement of MLL for the maintenance of *HOX* gene expression and not for its initiation (Yu et al., 1998). It has been shown that bone marrow cells from conditional

knockout *Mll* mouse are unable to reconstitute the bone marrow of lethally irradiated mice and have reduced ability to form myeloid and lymphoid colonies in methyl-cellulose medium (McMahon et al., 2007). Other studies have shown that in the adult mouse, conditional knockout of *Mll* causes rapid depletion of hematopoietic cells of all lineages, because of depletion of hematopoietic stem cells and reduced proliferative capacity of progenitor cells (Jude et al., 2007). Therefore, the *MLL* gene product is known to play an essential role in embryogenesis and adult hematopoiesis by maintaining the expression of *HOX* genes.

In MLL rearranged leukemias, the 5' coding region of *MLL* is fused to the 3' coding region of over 50 different translocation partner genes thereby giving rise to chimeric proteins that retain the N-terminus of MLL fused to the C-termini of different fusion partners (Huret et al., 2001). MLL chimeras can no longer methylate H3K4 but can still result in the increased expression of wild type MLL target genes such as *HOX* and *MEIS1* genes [1]. Several mechanisms have been proposed which could explain the leukemogenic properties of MLL fusion proteins: one mechanism could be where MLL fusion partners contain transactivation domains that are important for oncogenic transformations by causing constitutive expression of MLL target genes. Another model depends on the ability of the fusion partner to directly oligomerize with a protein that has leukemogenic properties (Bernard et al., 1994; Megonigal et al., 2000; Prasad et al., 1995; Slany et al., 1998; So et al., 2003). Moreover, it has been shown that in some cases, transformation might be mediated by internal tandem duplication within the MLL coding region where the N-terminal amino acid sequence of the MLL protein is duplicated and fused to itself and is called partial tandem duplication (MLL-PTD) (Caligiuri et al., 1997;

So et al., 1997; Yu et al., 1996).

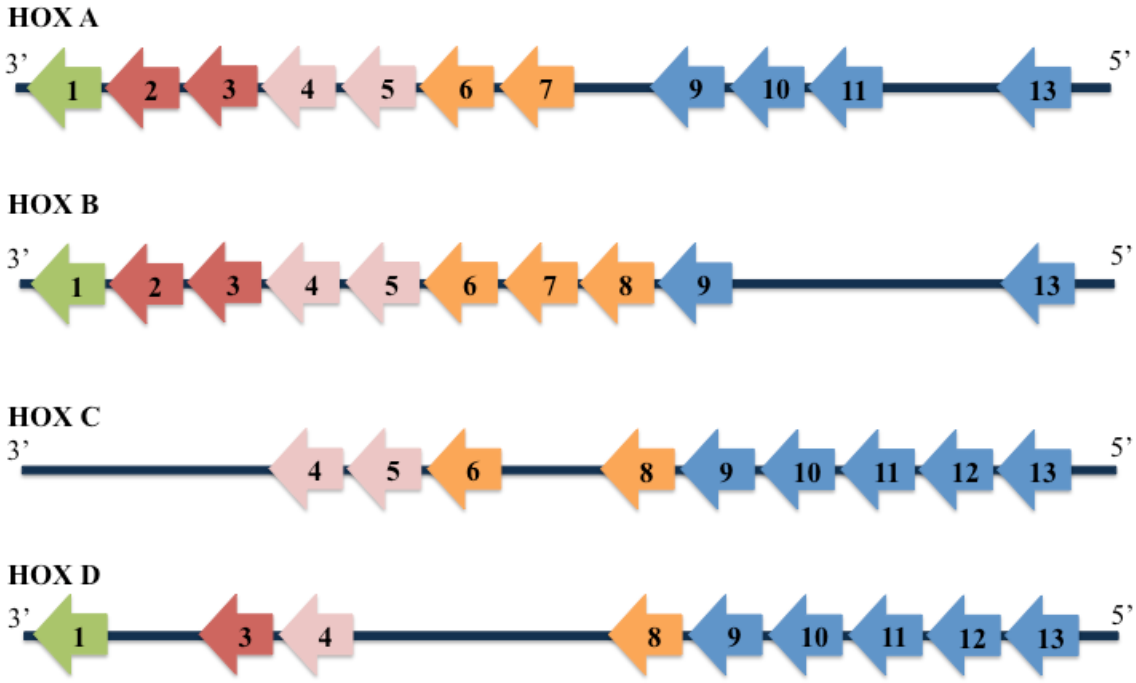
The two most frequently encountered MLL fusion partners seen in patients with leukemias are AF4 and AF9 family members, both of which possess transcriptional activation domains (Huret et al., 2001; Ma and Staudt, 1996; Prasad et al., 1995). All these fusions have shown to cause persistent expression of MLL target genes in leukemias and whose expression is otherwise supposed to decrease with the normal maturation of hematopoietic cells. This persistent gene expression results in differentiation arrest of hematopoietic progenitor cells.

MLL target genes

Homeobox (*HOX*) genes

HOX genes were first identified because of their homology to *HOMC* genes of Drosophila. There are four clusters of *HOX* genes comprising 39 genes in mammals present on different chromosomes (Eklund, 2006, 2007). These four clusters designated as HOXA-D. They contain 13 paralogous groups with, no cluster having all 13 genes, as depicted in Figure 2. HOX proteins have a homeodomain (HD) at its C-terminus responsible for its DNA binding and are highly conserved from Drosophila to humans (Eklund, 2011). The presence of conserved tyrosine residues in the HD is important for the regulation of HOX protein target genes (Eklund et al., 2000; Lindsey et al., 2007). It has been shown that phosphorylation of conserved tyrosine residues in HD affect protein-protein and DNA-protein interactions, which in turn alter the expression profile of target genes (Eklund et al., 2002). At the N-terminus of the HD, HOX proteins have a conserved hexapeptide domain, which interacts with PBX family proteins (Chang et al.,

Figure 2. *HOX* gene cluster



The four mammalian *HOX* gene clusters are present on four different chromosomes and comprised of 39 genes. These four clusters designated as *HOXA-D* have 13 paralogous groups and contain 8-11 genes. Figure is adapted from the review by Argiropoulos and Humphries (Argiropoulos and Humphries, 2007).

1995). HOX and PBX proteins exist as heterodimers and it is speculated that PBX proteins help HOX proteins in the selection of DNA binding sites (Chang et al., 1995; Eklund, 2011).

To understand the functional significance of various HOX proteins using knock out studies is difficult because of high degree of functional similarity and redundancy between members of the same group and other groups. Therefore, most of the studies have focused on overexpression and knock in models. *HOX* genes were initially reported to be important for anterior-posterior body positioning in embryos (Krumlauf, 1994). Later studies showed the importance of *HOX* genes in hematopoiesis. More specifically, gene expression profiling of both human and mouse bone marrow showed that A, B and C clusters of *HOX* genes are mainly expressed in hematopoietic stem cells (HSCs) and in immature progenitor cells, and their expression is downregulated during differentiation and maturation (Giampaolo et al., 1995; Giampaolo et al., 1994; Kawagoe et al., 1999; Moretti et al., 1994; Pineault et al., 2002; Sauvageau et al., 1994). There is very limited information available from knock out studies. *Hoxa9*-/- null mice do not show profound changes, except that there is some small decrease in the repopulating capacity of bone marrow cells from these mice (Lawrence et al., 1997). In contrast, overexpression studies *in vitro* and *in vivo* gave more information. Knock in model techniques used for overexpression of *HOX* genes in mouse bone marrow (HSCs) have shown the importance of *HOX* genes in HSCs proliferation, differentiation and self-renewal (Argiropoulos and Humphries, 2007). It has been shown *in vitro* that overexpression of either HOXA9 or HOXA10 in human or mouse bone marrow cause immortalization of the cells and an increase in the population of granulocyte/monocyte progenitor cells (Bjornsson et al.,

2001; Thorsteinsdottir et al., 2002; Thorsteinsdottir et al., 1997). Moreover, transplantation of HOXA9 overexpressing bone marrow into mice resulted in myeloproliferative neoplasia (MPN), which after long latency advanced to AML (Lawrence et al., 1997). In addition, HOXB3, HOXB6 and HOXA10 overexpression in the bone marrow of mouse have been shown to block differentiation of B and T cells, which then leads to MPN followed by leukemia (Argiropoulos and Humphries, 2007).

During hematopoiesis, transcription of various *HOX* genes depends on the differentiation stage of HSCs, which further regulate the HOX protein activity. However, the mechanisms that regulate the transcription of *HOX* gene clusters during specific differentiation stage are not completely determined. In leukemias with MLL rearrangements, there is constitutive expression of *HOX* genes, suggesting the regulation of *HOX* genes by MLL (Armstrong et al., 2002; Drabkin et al., 2002; Kawagoe et al., 1999). It has been shown that MLL associates with the promoter region of *HOX* genes through its N-terminus (Milne et al., 2002). This association of MLL to *HOX* promoters results in aberrant expression of *HOX* genes, which eventually blocks the differentiation of HSCs. The SET domain, which is not present in MLL fusion proteins, is responsible for initiating the differentiation of *HOX* genes at specific stages (Milne et al., 2002). Thus in MLL fusion proteins because of the absence of SET domain there is aberrant proliferation of immature hematopoietic cells (Eklund, 2011). Gene expression profiling studies of various MLL rearranged leukemias, including T- and B-cell acute leukemias, showed dysregulation of *HOX* genes (Armstrong et al., 2002; Ferrando et al., 2003; Rozovskaia et al., 2001; Yeoh et al., 2002). In addition to *HOX* genes, specifically *HOXA9*, frequent coactivation of *MEIS1* (another MLL target gene) has also been

reported in MLL fusion leukemias (Fine et al., 2004; Kohlmann et al., 2003; Yeoh et al., 2002). Moreover, the importance of Hoxa9 and Meis1 in leukemogenesis has been shown by their absolute requirement for the initiation and maintenance of myeloid progenitors in MLL-ENL immortalized mouse bone marrow cells (Ayton and Cleary, 2003; Zeisig et al., 2004). Furthermore, it has been shown that overexpression of HOXA9 together with MEIS1 could substitute for the immortalization of mouse bone marrow cells by MLL-ENL (Zeisig et al., 2004).

MEIS1

Like *HOXA9*, *MEIS1* is also a transcriptional target of the MLL (Milne et al., 2005). MEIS1 belongs to the three –amino acid loop extension (TALE) class of homeodomain containing proteins and is a cofactor for HOX proteins (Wong et al., 2007). It has been shown that MEIS1 exists in dimeric complex with HOXA9 in myeloid cells in order to stabilize HOX-DNA binding, since HOX proteins by themselves bind very weakly to DNA (Shen et al., 1997). MEIS1 also exists in trimeric complex with another HOX cofactor, PBX1 (HOXA9-PBX1-MEIS1) which enhances the affinity of HOX-DNA binding, which further increases their transcriptional activity (Shen et al., 1999).

The significance of MEIS1 in normal hematopoiesis is not completely understood, but several studies have been done demonstrating that MEIS1 does play a role in normal hematopoiesis. Like HOXA9, MEIS1 is expressed in the immature hematopoietic progenitor cells and its expression decreases as the cells undergo differentiation and maturation (Imamura et al., 2002; Pineault et al., 2002). *Meis1* homozygous -/- null mice are embryonically lethal and die at E14.5 day. These mice

exhibit loss of megakaryocytes leading to severe hemorrhages (Hisa et al., 2004). Thus, these studies suggest the important role of MEIS in normal hematopoiesis.

As previously mentioned, along with HOXA9, MEIS1 is also frequently overexpressed in MLL rearranged leukemias, suggesting the critical role of MEIS1 in MLL fusion associated leukemogenesis. It has been shown that MEIS1 overexpression along with overexpression of several *HOX* or along with the NUP98-HOX fusion gene accelerates the onset of leukemia in the transplanted mouse bone marrow (Calvo et al., 2002; Fischbach et al., 2005; Kroon et al., 1998; Kroon et al., 2001; Pineault et al., 2004; Pineault et al., 2003). Subsequently, Wong et al have shown the involvement and

quirement of MEIS1 in AML using various genetic techniques. They showed the importance of MEIS1 in the function of MLL fusion proteins by knocking down MEIS1 either by short hairpin RNA (shRNA) or by expressing a dominant negative form of MEIS1 with impaired MEIS1 activity. Loss of MEIS1 activity using both these techniques resulted in reduced transforming capability of several MLL-fusion proteins as evidenced by decreased colony formation capacity *in vitro* and ability to initiate leukemia in recipient mice. These experiments convincingly support that MEIS1 expression is rate limiting for MLL fusion mediated leukemogenesis (Wong et al., 2007). Moreover, knockdown of MEIS1 using shRNA in MLL-transduced mouse bone marrow cells has shown the downregulation of genes associated with cell cycle entry, which correlates with the altered growth of these cells (Kumar et al., 2009). Therefore, it is of importance to understand the potential targets of MEIS1 in MLL fusion leukemias. The same report has also shown that MEIS1 shRNA transduced cells undergo G_0/G_1 cell cycle arrest and apoptosis, which was further evidenced by the reduced

expression of cell cycle associated genes (Kumar et al., 2009). Subsequently, one year later, another group investigated further the role of MEIS1 in cell cycle regulation. This group designed a genetically engineered form of mutant MEIS1 that results in the transcriptional repression of MEIS1 target genes to better understand their regulation by MEIS1. These investigators confirmed the G_0/G_1 cell cycle arrest of the transduced bone marrow cells using a repressive mutant form of MEIS1. In addition, they showed that MEIS1 associates with cyclin D3, which further controls the phosphorylation status of Retinoblastoma (Rb) protein (Argiropoulos et al., 2010). The retinoblastoma protein inhibits transactivation by E2F transcription factors that are required for the synthesis of cyclin dependent kinases for entry into S phase of the cell cycle. Phosphorylation of Rb protein releases E2F transcription factors, which then results in transition from G_1 to S phase of cell cycle. Therefore, these studies show the role of MEIS1 in hematopoietic and leukemic stem cell proliferation by regulating G_1 to S phase transition of the cell cycle via regulating phosphorylation of the Rb protein mediated by cyclin D3 (Argiropoulos et al., 2010).

c-MYC

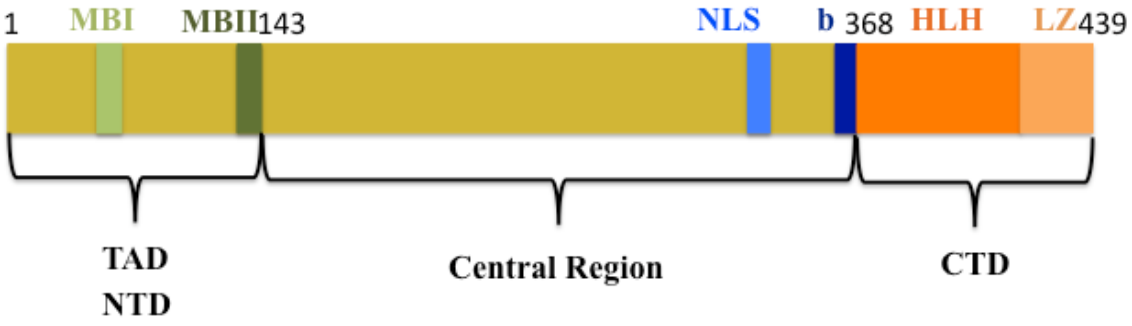
c-Myc is a transcription factor and belongs to a family of proteins that also include L-Myc and N-Myc (Dang, 2012). The role of L-Myc is not well determined, whereas, the expression of N-Myc is tissue restricted with abundant expression in lung, gut, cranial and spinal ganglia, and hematopoietic cells. In murine development N-Myc can also substitute for the expression of c-Myc (Malynn et al., 2000). Like many characterized transcription factors, the C-terminus of c-Myc has a domain of 13 basic amino acids, a

helix-loop-helix motif (HLH) and leucine zipper domain as depicted in Figure 3 (Blackwell et al., 1990; Hoffman et al., 2002). The N-terminus of c-Myc has a conserved region containing Myc box I (MBI) and Myc box II (MBII) found in all Myc family members (Hoffman et al., 2002). It has been shown that the basic amino acids region promotes binding to specific DNA sequences and the HLH-leucine zipper domain mediates protein-protein interactions, suggesting the importance of the C-terminus in transcriptional regulation (Blackwell et al., 1990; Hoffman et al., 2002).

Myc is known to play an important role in many cellular processes including, cell growth, differentiation, metabolism and apoptosis (Askew et al., 1991; Evan and Littlewood, 1993; Hoffman and Liebermann, 1998; Shi et al., 1992). Additionally, c-Myc has been shown to play a major role in hematopoietic cell development and differentiation (Boxer and Dang, 2001; Hoffman et al., 2002; Nesbit et al., 1999). It has been reported that dysregulation of c-Myc expression in either leukemic or normal myeloid cells of mouse bone marrow resulted not only in growth arrest and terminal differentiation but also promoted p53-independent apoptosis (Amanullah et al., 2000). Subsequently, the molecular mechanisms responsible for c-Myc-mediated p53-independent apoptosis unveiled the role of CD95/Fas pathway in c-Myc-induced apoptosis (Amanullah et al., 2002).

Along with HOXA9 and MEIS1, MYC is upregulated in MLL rearranged leukemias (Armstrong et al., 2003; Armstrong et al., 2002). It has been shown that Myc plays an important role in MLL-ENL mediated transformation of murine bone marrow cells (Schreiner et al., 2001). Importantly, the same study showed that a dominant negative form of Myc abrogated the effect of MLL-ENL transduction. Conversely,

Figure 3. Domains of c-MYC protein



MBI - MYC box I
MBII - MYC box II
TAD - Transcriptional Activation Domain
NTD - N-terminal Domain
CTD - C-terminal Domain
NLS - Nuclear Localization Signal
b - Stretch of basic amino acids
HLH - Helix-loop-helix
LZ - Leucine-Zipper
Numbers represent the number of amino acids.

overexpression of Myc cooperated with the MLL fusion proteins, resulting in transformation of bone marrow cells exhibiting irreversible maturation arrest (Schreiner et al., 2001). Moreover, MYC is also overexpressed in HSCs transduced with internal tandem duplicated FLT3, receptor tyrosine kinase that is frequently mutated in AML (Li et al., 2007). Furthermore, the expression of MYC is differentially regulated by FLT3 in hematopoiesis and leukemogenesis (Takahashi, 2011). Recently, Jiang et al have shown MYC as a significant downstream target of MLL fusion proteins. Specifically, this group has shown that MLL fusion proteins upregulated the MYC/LIN28 axis, which further blocks the maturation of micro RNA miR-150 (Jiang et al., 2012). LIN28, an RNA binding protein plays an important role in the maturation of micro RNAs (miRNAs). As a result, the inhibited miR-150 is not able to inhibit the expression of FLT3/HOXA9/MEIS1, which eventually results in leukemogenesis (Jiang et al., 2012).

MLL Fusion Partners

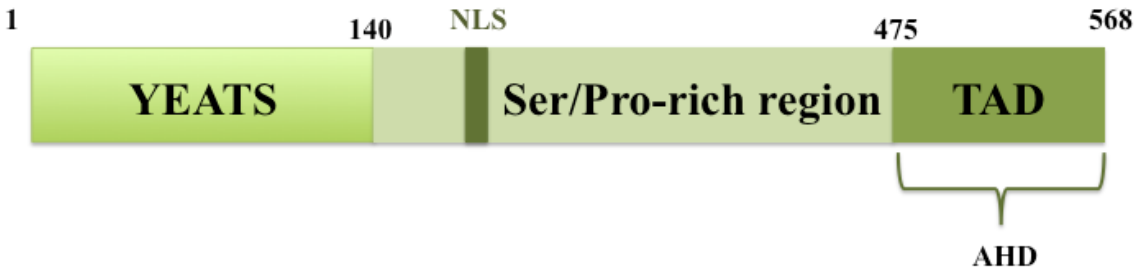
AF9

AF9/MLLT3, located on chromosome band 9p22, was first identified as a fusion partner of the *Mixed Lineage Leukemia* (*MLL*) gene in acute myeloid leukemias (Nakamura et al., 1993). AF9 contains a serine/proline rich region and nuclear localization sequence AKKQK, characteristic of many transcription factors (Iida et al., 1993). AF9 belongs to the highly conserved YEATS domain family of proteins with the YEATS domain sequence conserved from yeast to humans. The name YEATS is derived from the first five proteins that were discovered to contain this domain (Yaf9, ENL, AF9, Taf14 and

Sas5). Yaf9, Taf14 and Sas5 are yeast proteins and ENL and AF9 are human proteins. The YEATS domain, located at the N-terminus of the proteins as depicted in Figure 4, is found in components of chromatin-modifying and transcription-activating complexes (Schulze et al., 2009b). For instance, the AF9/ENL YEATS domain has been shown to play a role in the recruitment of the Super Elongation Complex (SEC) to the chromatin through interaction with the Polymerase Associated Factor complex (PAFc) (Kim et al., 2010; Shi et al., 1996). It has been shown that the YEATS domain is required for binding to histone H3, suggesting its role in the chromatin recruitment (Zeisig et al., 2005). At the C-terminus, AF9 has a hydrophobic transcriptional activation domain that shares a high sequence homology with another mammalian member of the YEATS family, *ENL/MLLT1* (Nakamura et al., 1993). This hydrophobic C-terminus domain of AF9 that is retained in the MLL fusion has an ANC1 (actin non-complementing gene 1, budding yeast protein) homology domain (AHD) comprising of approximately 94 amino acids that is involved in protein-protein interactions as shown in Figure 4.

The function of AF9 has not been completely characterized, but a homozygous null mutation of *Af9* in mice is known to cause lethality in the perinatal period. These mice have axial skeleton defects suggesting the importance of Af9 in normal embryogenesis through interplay with *HOX* gene function (Collins et al., 2002). Apart from its role in development, recent studies indicate that AF9 is an important factor for the normal expression of genes that regulate normal hematopoiesis including *GATA1*. It has been shown that AF9 is highly expressed in hematopoietic stem cells and shRNA-mediated knock down of AF9 in cord blood (CB) CD34+ CD38- cells results in complete abrogation of erythrocyte colony formation (Pina et al., 2008). Overexpression of AF9 in

Figure 4. Domains of AF9 protein



TAD – Transcriptional Activation Domain
AHD – ANC1 Homology Domain
NLS – Nuclear Localization Signal

AF9 has a YEATS domain at the N-terminus from amino acids 1-140, followed by Ser/Pro-rich region. The C-terminal ≈ 94 amino acids of AF9 have a transcriptional activation AHD domain involved in protein-protein interactions.

the same cells resulted in the increased expression of *GATA1, GATA2, SCL,* and *GFI1b* genes that are associated with the development of erythrocytes and megakaryocytes. On the other hand, the expression of *PU.1, CEBPA*, and *GFI* genes that are associated with granulocyte and monocyte development are decreased (Pina et al., 2008). Moreover, in hematopoietic precursor cells, expression of a chimeric protein comprised of the C-terminus of AF9 fused to the N-terminus of MLL leads to leukemic transformation (Chen et al., 2008; Cozzio et al., 2003). Interestingly, the fusion of the minimal transactivation domain of ENL (homologous to AF9) to MLL has been shown to be necessary and sufficient for leukemic transformation (Slany et al., 1998). It has been shown that retroviral transduction of c-kit$^+$ hematopoietic precursor cells with MLL-AF9 leads to its immortalization. Furthermore, these immortalized c-kit$^+$ cells that are able to form colonies in semi-solid methylcellulose media resulted in leukemic transformation when injected into mice (Chen et al., 2008). Thus AF9 has a role in both normal as well as neoplastic blood cell development.

Earlier studies have also shown that Af9 is important in renal salt homeostasis, neuronal development, and HIV gene transcription by regulating the expression of *ENaCα* and *Tbr* genes and by recruitment of HIV Tat protein (Buttner et al., 2010; He et al., 2010; Sobhian et al., 2010; Zhang et al., 2006). Perhaps the best-characterized target of Af9 is the epithelial sodium channel gene *α* (*ENaCα*). The majority of epithelial Na^+ and fluid absorption is regulated by aldosterone via induction of the *ENaCα* gene, which is the rate-limiting step (Eaton et al., 2001; Thomas and Itani, 2004). Aldosterone, which is the major regulator of epithelial Na^+ absorption regulates the transcription, degradation and trafficking to the cell membrane of *ENaCα* (Eaton et al., 2001; Thomas and Itani,

2004). It has been shown that aldosterone negatively regulates the expression of both Af9 and the histone H3 lysine 79 (H3K79) methyltransferase Dot1l (Zhang et al., 2006). Zhang et al. also reported in murine renal collecting ducts that Af9 together with the Dot1l results in H3K79 hypermethylation at the *ENaCα* promoter region that then contributes to a repressed state of gene expression. A year later the same group showed that serum- and glucocorticoid-induced kinase-1 (Sgk1) phosphorylates Af9 and reduces the physical interaction between Af9 and Dot1l. The decreased interaction between Af9 and Dot1l then results in H3K79 hypomethylation at the *ENaCα* promoter region, which then results in its increased expression (Zhang et al., 2007). Later, it was reported that AF9 and AF17, which is another MLL fusion partner, compete with each other for binding with the same domain of Dot1l. AF17 causes the nuclear export of Dot1l, which leads to decreased H3K79 methylation at the *ENaCα* promoter region and its increased gene expression (Reisenauer et al., 2009).

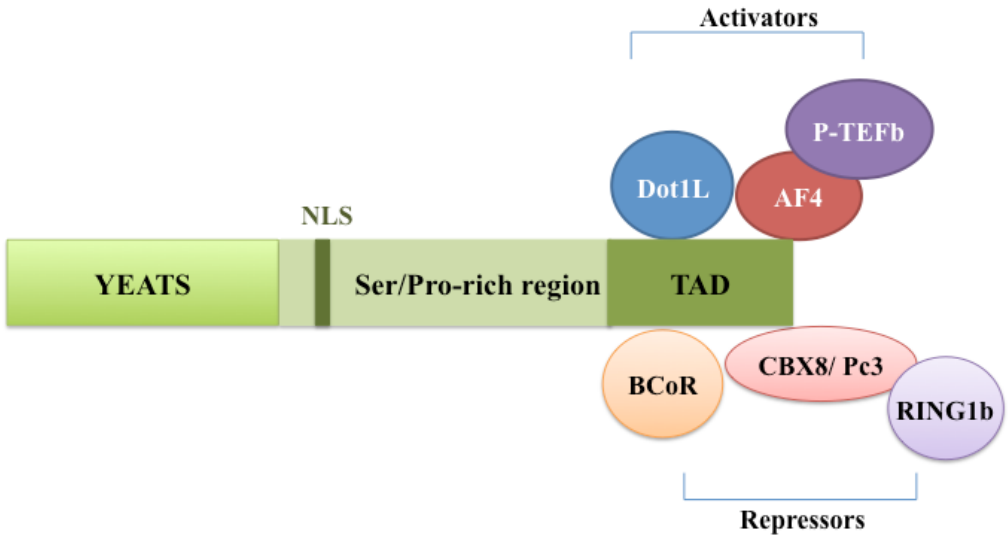
Similarly, Af9 in conjunction with Dot1l negatively regulates the expression of the *Tbr* gene in the subventricular zone (SVZ) of mouse forebrain by governing the status of H3K79 methylation at the gene's transcription start site (Buttner et al., 2010). The same group has also shown that the expression of Af9 blocks premature removal of progenitors during cortical development. The presence of Af9 at the transcription start site of the *Tbr* gene is associated with decreased recruitment of RNA polymerase II (RNA PolII) and increased recruitment of Dot1l, which results in the suppression of the gene. This repressive activity of the H3K79 methylation mark by Dot1l is in contrast to canonical H3K79 methylation that is predominantly associated with actively transcribed genes (Steger et al., 2008).

In addition to AF9-DOT1L complexes, AF9 has also been found in other distinct multiprotein complexes. Affinity purification has revealed that AF9, via its C-terminal domain, associates with at least four functionally and structurally unrelated proteins as shown in Figure 5A (Biswas et al., 2011). AF9 has been shown to exist in a large multiprotein complex containing AF4 (another commonly encountered MLL fusion partner), and the positive transcription elongation factor b (PTEFb) composed of cyclin T1 and CDK9 (Bitoun et al., 2007; Erfurth et al., 2004). AF9 and AF4 form a stable protein complex in the nucleus. Importantly, the interaction domains of both these proteins are retained even after their fusion with MLL. This complex containing AF9-AF4-PTEFb results in phosphorylation of Ser2 of the C-Terminal domain (CTD) of RNA Pol II which promotes productive transcriptional elongation of initiated and paused mRNA transcripts (Mueller et al., 2009). It is through this process that AF9 is required for optimal transcription of HIV genes in conjunction with HIV Tat protein as depicted in Figure 5B. DOT1L is not a part of the AF9-AF4-PTEFb complex (Biswas et al., 2011). Apart from transcriptional activators P-TEFb and DOT1L, AF9 is also known to interact with two repressors of transcription, the Polycomb protein CBX8, and the BCL6 co-repressor BCoR (Hemenway et al., 2001; Srinivasan et al., 2003). More recently, it has been reported that the C-terminal ANC1 homology domain (AHD) of AF9 is intrinsically disordered but acquires a unique structural conformation after binding to each of its binding partners (AF4, DOT1L, CBX8 and BCoR) and binding of one protein to AF9 block binding of the others (Leach et al., 2013).

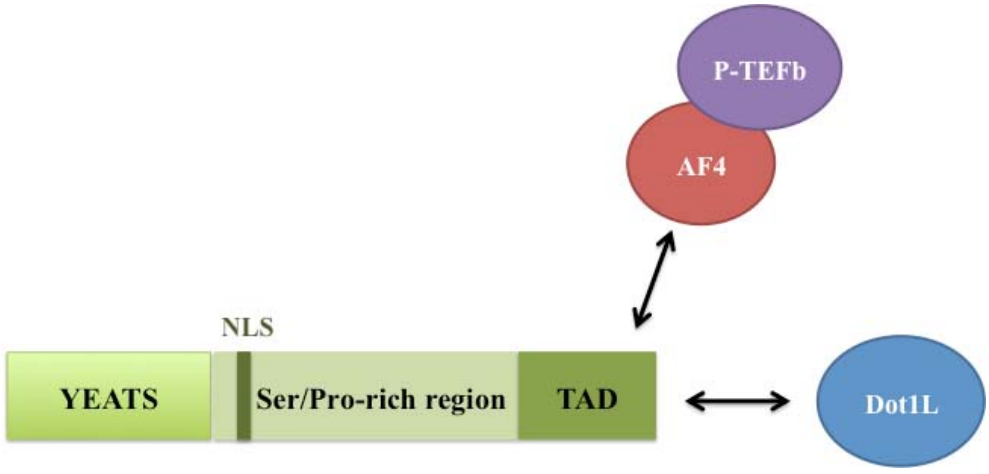
AF9 has shown to interact with a wide variety of proteins, including activators (AF4 and DOT1L) and repressors (CBX8 and BCoR) of transcription. Therefore, rather

Figure 5. AF9 protein interactions

A



B



(A) Interactions of wide variety of proteins including activators (AF4 and DOT1L) and repressors (CBX8 and BCoR) of transcription with the C-terminus ANC1 Homology domain of AF9. (B) AF9 exist in a separate complex with AF4-P-TEFb and DOT1L

than forming one macromolecular complex, we hypothesize that AF9 is an important component of several distinct macromolecular complexes, each of which has a unique function. In this dissertation, as part of one of my projects, we focused on the complex involving AF9 and CBX8.

AF9 associated proteins

AF4 (ALL1-fused gene from chromosome 4):

In cases of MLL rearranged leukemias, the AF4 protein is the most common MLL fusion partner, arising when the *AF4* (FEL) gene fuses with *MLL* in the balanced translocation t(4;11)(q21;q23) (Domer et al., 1993; Parkin et al., 1982). The MLL-AF4 fusion is most frequently associated with acute lymphoid leukemia (ALL) (De Zen et al., 2003). In infants less than one year of age, MLL-AF4 fusion is an indicator of adverse prognosis and over 80% of infant leukemias carry the MLL-AF4 translocation (Bueno et al., 2011; Heerema et al., 1999).

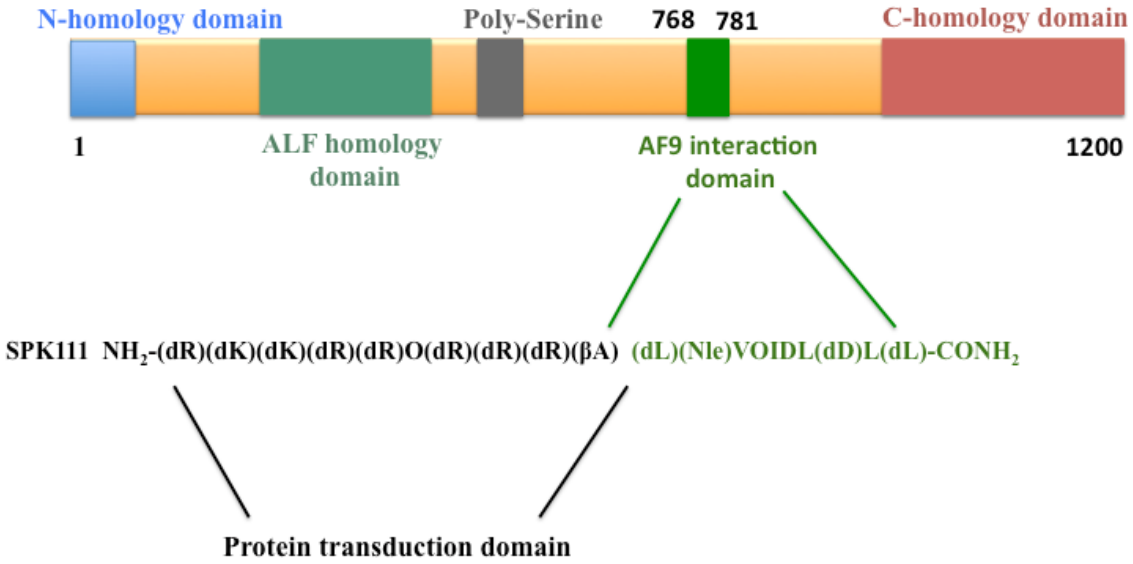
The *AF4* (4q21) gene consists of 23 exons that encode for a 1210 amino acid protein. AF4 belongs to the AF4/LAF4/FMR2 family of nuclear proteins and LAF4 and AF5q31 rarely but recurrently fuse with MLL to cause leukemia (Nilson et al., 1997; Taki et al., 1999; von Bergh et al., 2002). The members of this protein family share a common organization of domains. They have N-terminal and C-terminal homology domains; the C-terminal domain has recently been shown to be involved in homo and hetero-dimerization with its respective family members with preference for hetero-dimerization (Yokoyama et al., 2010). They also have a highly conserved serine/proline rich transactivation domain as well as an ALF (AF4/LAF4/FMR2) homology domain as

shown in Figure 6 (Ma and Staudt, 1996; Prasad et al., 1995).

The ALF domain has been shown to interact with SIAH ubiquitin ligases to promote proteasome-mediated degradation, thereby regulating the stability and turnover of ALF domain-containing proteins (Bursen et al., 2004; Oliver et al., 2004). It has been shown that a single amino acid mutation in the ALF domain of murine Af4 that disrupts binding with SIAH ubiquitin ligases results in accumulation of mutant Af4 (Oliver et al., 2004). Increased levels of Af4 result in the Purkinje cell degeneration in the cerebellum with the loss of motor coordination and balance, a neurodegenerative disease termed "robotic mouse" (Isaacs et al., 2003). Of special relevance to the study of leukemia, AF4 has also been implicated in hematopoiesis. An Af4 knockout mouse has been shown to have severe defects in B and T cell maturation, suggesting an important role of AF4 in lymphoid development (Isnard et al., 2000). AF4 is highly expressed in the brain and in the active areas of hematopoiesis in the embryo, whereas, with the development of tissues expression of AF4 decreases (Baskaran et al., 1997).

As previously mentioned, our lab detected the interaction between AF4 and AF9 at subnuclear foci. The proteins bind one another via discreet domains and both proteins retain their interaction domains after fusion with MLL. To understand the significance of their interaction, our laboratory has mapped the domains involved in the AF4-AF9 interaction and has developed synthetic peptides (PFWT and SPK111) capable of disrupting their interaction *in vitro* and *in vivo* (Srinivasan et al., 2004). The N-terminus of each peptide is fused to a protein transduction domain (Figure 6) to facilitate its entry into the cell. The peptide mimics the amino acid sequence of the minimal AF9 binding domain within AF4 and has shown cytotoxicity towards leukemia cell lines harboring

Figure 6. Domains of AF4 protein and sequence of SPK-111



AF4 has N- and C- terminal homology domains. The ALF homology domain of AF4 is responsible for its stability. The minimal AF9 interacting domain within AF4 has ≈ 10 amino acids and a synthetic peptide (SPK-111) was designed based on these amino acids. SPK-111 has a protein transduction domain at the N-terminus to facilitate its cell entry, followed by AF9 interacting sequence of AF4. SPK-111 has dextro stereo-isomeric (d) form of amino acids compared to *in vivo* levo (l) stereo-isomeric form, in order to make them less susceptible to peptidases. SPK-111 also has ornithine at position 4 instead of lysine in order to make a stronger salt bridge with a negatively charged residue of AF9.

MLL-AF4 and MLL-AF9 fusion proteins, suggesting the importance of the AF4-AF9 interaction for the survival of these leukemia cells (Palermo et al., 2008; Srinivasan et al., 2004). However, the molecular mechanism by which the peptides work is still not known. Based on the importance of AF4-AF9 interaction, our lab has shown through yeast two-hybrid screening that most of the amino acids in the minimal AF9 interacting domain of AF4 are indispensible. The minimal AF9 interacting domain of AF4 consists of 10 amino acids that are highly conserved among AF4 family members. It was further shown that the AF9 interaction was dependent on the lysine 764 (positively charged residue) and many hydrophobic amino acids at the adjacent positions of AF4 (Srinivasan et al., 2004). Moreover, our lab has also shown that the D544 residue in AF9 is important for itsinteraction with AF4 (unpublished data). AF4 has also been shown to interact with positive transcription elongation factor b (P-TEFb), a complex of cyclin dependent kinase 9 and cyclin T1 and exists in a large multiprotein complex containing P-TEFb and AF9/ ENL, thereby linking AF4 and AF9/ ENL in the regulation of transcriptional elongation (Bitoun et al., 2007; Mueller et al., 2007). Mutational analyses of the residues important for AF4-AF9 interaction provide a useful method to determine the functional importance of AF4-AF9 interaction in MLL leukemogenesis. Furthermore, delineating the molecular mechanism of cytotoxic peptides is important for use as potential therapies against MLL leukemias.

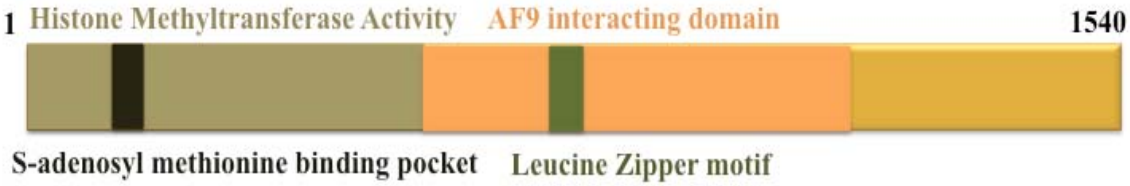
DOT1L (Disruptor of Telomere Silencing)

DOT1L is the only known H3K79 histone methyltransferase. It was first identified in yeast as “disruptor of telomere silencing” (Feng et al., 2002; Singer et al., 1998). The

protein is designated as DOT1L in humans and Dot1l in mice and is highly conserved from yeast to mammals (Sawada et al., 2004). DOT1L does not have a SET domain, which is the major methyltransferase domain in other histone lysine methyltransferases, but has a catalytic domain with a conserved sequence motif at the N-terminus (Figure 7) that is characteristic of class I methyltransferases such as DNA methyltransferases (DNMTs) (Feng et al., 2002). Moreover, unlike other histone methyltransferases, DOT1L methylates lysine 79 residue of histone H3 (H3K79) that is located in the globular domain of the protein and not in the tail (Lacoste et al., 2002; Ng et al., 2002a; van Leeuwen et al., 2002). Based on the structural similarities between yeast Dot1l and arginine methyltransferases, it was proposed that Dot1l could also methylate arginine. However, it has not been possible to detect arginine methyltransferases activity associated with Dot1l using various technologies, including tandem mass spectrometry (MS/MS) and reverse phase high performance liquid chromatography (HPLC) coupled with nano-liquid chromatography electrospray ionization mass spectrometry (LC-ESMS) (van Leeuwen et al., 2002). In mice, five different isoforms of Dot1l have been identified (Dot1a-e) and Dot1a, which is the longest transcript, has been shown to have the greatest similarity with human DOT1L (Zhang et al., 2004).

Regulation of DOT1L mediated H3K79 methylation by histone H2B lysine 123 (H2B-K123) ubiquitination has been suggested (Nguyen and Zhang, 2011). In yeast, histone H2B-K123 is monouboquitinated by Rad6 (ubiquitin-conjugating E2 enzyme) and Bre1 (E3 ubiquitin ligase) (Robzyk et al., 2000; Wood et al., 2003). Moreover, Rad6 deletion blocks H2B ubiquitination as well as H3K4 and H3K79 methylation, suggesting the importance of H2B ubiquitination in mediating H3K79 methylation (Ng et al., 2002b;

Figure 7. Domains of DOT1L protein



Histone Methyltransferase Activity domain (amino acids 1-478)
AF9 interacting domain (amino acids 479-1222)
Leucine Zipper motif (amino acids 576-597)

DOT1L has histone methyltransferase activity at the N-terminus, unlike other histone methyltransferases that has SET domain at the C-terminus. The catalytic domain of DOT1L is followed by AF9 interaction domain.

Sun and Allis, 2002). Three different mechanisms have been proposed which are involved in the regulation of DOT1L mediated H3K79 methylation by H2B-K123 ubiquitination (Nguyen and Zhang, 2011). These observations come from the fact that DOT1L specifically methylates H3K79 only in the presence of nucleosomes and not in the presence of core histones or recombinant H3, suggesting the involvement of trans-histone cross talk in the regulation of DOT1L activity (Feng et al., 2002). According to the first mechanism, there is direct recruitment of DOT1L to ubiquitinated H2B-K123, which then results in H3K79 methylation at intranucleosomal levels. A second model proposes the involvement of other factors like, COMPASS complex containing Set1. Rtf1, a member of the Paf1 complex, recruits the COMPASS complex, which then results in H3K4 methylation. Furthermore, there is direct interaction between COMPASS complex subunit, Cps35 with H2B-K123 and DOT1L, finally bridging DOT1L to the nucleosomes for H3K79 methylation. Finally, it has been suggested that H2B-K123 ubiquitination causes conformational changes in the chromatin structure making it easily accessible to DOT1L for H3K79 methylation (Nguyen and Zhang, 2011).

Studies on Dot1 in yeast have shown its importance in telomere silencing, meiotic checkpoint control, and DNA damage response (Ng et al., 2002a; San-Segundo and Roeder, 2000; van Leeuwen et al., 2002; Wysocki et al., 2005). Sir proteins are involved in the silencing of telomeres. Specifically, Sir3 protein through its N-terminal BAH domain interacts with H3K79 and competes for Dot1 binding. However, Sir3 binding is blocked by H3K79 methylation, which consequently affects heterochromatin spreading (Altaf et al., 2007; Fingerman et al., 2007; Katan-Khaykovich and Struhl, 2005; Onishi et al., 2007; van Welsem et al., 2008). In addition, Dot1 has been shown to play a vital role

in the regulation of cell cycle progression during DNA damage. It was reported that dmc1 (meiotic recombination protein) and zip1 (synaptonemal complex protein) mutants are unable to arrest at pachytene checkpoint when Dot1 is absent (San-Segundo and Roeder, 2000). Dot1 mediated H3K79 methylation has also been reported to have a role in the regulation of cell cycle in yeast. Deletion of Dot1 is associated with failed G1/S DNA damage checkpoint, suggesting the importance of H3K79 methylation for appropriate entry into S phase (Levesque et al., 2010; Wysocki et al., 2005).

In mammals DOT1L has been shown to play important roles in heterochromatin formation and in embryonic development (Jones et al., 2008). Homozygous null mutation of Dot1l is embryonically lethal and mice die at E10.5. These embryos exhibited cardiovascular defects displaying enlargement of heart, shortening of vasculature and anemia (Jones et al., 2008). Thus Dot1l has a role in erythropoiesis apart from its role in cardiovascular functions. Specifically, it has been shown that Dot1l deficient hematopoietic cells derived from yolk sac have reduced expression of the *Gata2* gene. The Gata2 down-regulation is further associated with G_0/G_1 cell cycle arrest and apoptosis of erythroid progenitor cells, whereas there is no effect on the growth and differentiation of myeloid cells (Feng et al., 2010). Moreover, in mammals, 53BP1 protein (tumor suppressor p53 binding protein 1) is known to bind methylated H3K79, which then promotes its association with DNA double strand breaks (DSBs) (Huyen et al., 2004). In addition, the role of Dot1l mediated chromatin modification has also been linked to Wnt/Wingless signaling. This group showed the presence of a DotCom complex comprised of many proteins, including Dot1l, AF10, AF17, AF9, ENL, Skp1, TRRAP, and β-catenin. Since this complex also contains the proteins involved in Wnt signaling

(Skp1, TRRAP, and β-catenin), they showed that by knocking down the *Drosophila* homolog of Dot1l, *Grappa*, there is reduction in the expression of Wingless target genes, including *frizzled 3, senseless* and *homothorax*. Moreover, they also confirmed the requirement of H2B-K123 monoubiquitination for DotCom mediated H3K79 methylation (Mohan et al., 2010).

DOT1L, the only known H3K79 methyltransferase can result in mono-, di-, and tri-methylation. However, the function associated with H3K79 methylation status has not been completely characterized. Interestingly, there exists a strong correlation between H2B-K123 monoubiquitination and H3K79 trimethylation; however, there is no overlap between H2B-K123 monoubiquitination and H3K79 dimethylation (Schulze et al., 2009a). It has been shown in mouse embryos that the presence of H3K79 dimethylation could be detected from the 4-cell until the blastocyst stage whereas H3K79 trimethylation was not detectable even at the blastocyst stage (Ooga et al., 2008). Therefore, it is possible that H3K79 di- and tri-methylation play distinct roles in transcriptional regulation and chromatin remodeling. DOT1L mediated H3K79 methylation is predominately associated with actively transcribed genes (Steger et al., 2008). On the other hand, H3K79 methylation mediated by Dot1l has also been associated with the repressed state of genes as mentioned before in case of *ENaCα* and *Tbr* genes.

The role of DOT1L in MLL rearranged leukemias was first shown after identification of DOT1L interacting protein, AF10, which is also a MLL fusion partner. It was further demonstrated that DOT1L enzymatic activity is important for MLL-AF10 mediated leukemogenesis (Okada et al., 2005). In addition, DOT1L has also been shown to be necessary for the leukemogenic activity of other MLL fusions, including MLL-ENL

(Mueller et al., 2007), MLL-AF4 (Krivtsov et al., 2008), and MLL-AF9 (Chang et al., 2010; Nguyen et al., 2011). Several authors have shown the presence of DOT1L in elongation protein complexes, including EAP (Mueller et al., 2007), SEC (Lin et al., 2010), and AEP (Yokoyama et al., 2010), thereby linking DOT1L to the regulation of transcriptional elongation. The components of these elongation complexes are frequent fusion partners of MLL (Ayton and Cleary, 2001). The mechanism of DOT1L mediated gene activation of MLL target genes is still not clear. Nevertheless, it has been proposed that DOT1L is recruited to MLL target genes by one of the MLL fusion partners, which then results in aberrant H3K79 methylation and constitutive activation of MLL target genes (Nguyen and Zhang, 2011).

Previously it has been shown that DOT1L existed in the same complex with ENL (a close homologue of AF9) family proteins, AF4 family proteins and P-TEFb such that recruitment of DOT1L was important for transformation by the MLL-ENL fusion proteins (Mueller et al., 2007). However, a more recent report has shown that DOT1L is not found in AF4 purified complexes from leukemic cell line which otherwise contains AF4 family members, ENL family members and P-TEFb (Yokoyama et al., 2010). Nevertheless, the presence of H3K79 methylation marks has been detected on MLL-AF4 target genes such as *HOXA9* and *MEIS1*. Moreover, the same report proposes that the recruitment of AF4, ENL and P-TEFb to the MLL target genes is the initial step in transformation by MLL fusion proteins followed later by DOT1L recruitment. More recently, it has been confirmed that the AF9-DOT1L complex is distinct from AF9-AF4-PTEFb complex (Biswas et al., 2011). As mentioned previously, apart from interacting with DOT1L and AF4, AF9 also interacts with the known repressors of transcription,

including BCoR and CBX8.

BCL-6 co-repressor (BCoR)

BCoR was first identified as a co-repressor that interacts specifically with the POZ domain of the BCL6 protein and augments the transcriptional repressive activity of BCL6 (Huynh et al., 2000). BCL6, a transcriptional repressor, was initially discovered in chromosomal translocations resulting in non-Hodgkin's lymphoma (Pasqualucci et al., 2003). Studies have shown the recurrent mutations of BCoR in patients with AML (Grossmann et al., 2011). The *BCoR* gene is located on the X-chromosome and heterozygous X-linked BCoR mutations in females are associated with Oculofaciocardiodental (OFCD) syndrome (Ng et al., 2004). The protein encoded by *BCoR* gene is a large nuclear protein and has been shown to be expressed ubiquitously in human tissues (Huynh et al., 2000). Several studies have shown the important role of BCoR in regulating hematopoiesis, embryonic stem cell development and differentiation, and mesenchymal stem cell function through epigenetic mechanisms (Fan et al., 2009; Wamstad et al., 2008).

Importantly, BCoR is known to potentiate the repressive activity of BCL-6, probably because of its ability to interact with both class I and class II HDACs (Huynh et al., 2000). Previously, our laboratory for the first time demonstrated the interaction between BCoR and AF9 (Srinivasan et al., 2003). This study identified four alternatively spliced transcripts of BCoR in the mouse designated as BCoR a-c. Only isoforms BCoR a and b have been shown to interact with AF9, although all the isoforms have a role in transcriptional repression. Domain mapping studies identified the minimal AF9 binding

domain in BCoR consisting of amino acid residues 1127-1251, which is retained in both a and c isoforms of BCoR. Moreover, AF9 through its C-terminus has been shown to interact with BCoR, which is retained in MLL-AF9 leukemias. Apart from BCoR, the C-terminus of AF9 has also been shown by our laboratory to interact with another repressor protein, CBX8 (Hemenway et al., 2001). CBX8 is a component of the repressive Polycomb PRC1 complex. Like BCoR isoforms a and b, only CBX8 and not its other homologs have been shown to interact with AF9 as described below, suggesting the specificity of these interactions.

Polycomb Group Proteins (PcG)

PcG proteins were first identified in *Drosophila melanogaster* as regulators of homeotic (*HOM-C*) genes, which are important for the pathways associated with the development of the body axis. Similarly, PcG proteins in mammals are known to play a role in the anterior-posterior positioning axis by repressing the expression of *HOX* genes during development (Konuma et al., 2010). Trithorax family proteins, the homologs of human MLL, counteract the repression of *HOX* genes by PcG proteins (Schuettengruber et al., 2007). PcG proteins consist of two Polycomb Repressive Complexes (PRCs) (Konuma et al., 2010):

1) PRC2, an initiation complex
2) PRC1, a maintenance complex

In humans, the PRC2 complex consists of three core proteins, including EZH2, SUZ12 and EED. EZH2 posses a catalytic SET domain and causes trimethylation of histone 3 lysine 27 (H3K27) (depicted in Table 1), which is normally associated with the repressed

state of the gene (Bracken et al., 2006; Cao and Zhang, 2004; Schuettengruber and Cavalli, 2009). Both EED and SUZ12 are known to stimulate histone methyltransferase activity of EZH2 (Konuma et al., 2010). EZH2 is also known to bind DNA methyltransferases (DNMTs), including DNMT1, DNMT3A and DNMT3B, and can result in DNA methylation and gene repression (Rush et al., 2009; Vire et al., 2006). PRC2 complex has also been shown to bind histone deacetylases (HDACs) via EED, resulting in a coordinated repression of target genes (van der Vlag and Otte, 1999). H3K27 trimethylation marks serve as a docking site for proteins containing chromodomains and have been shown to be responsible for the recruitment of PRC1 complexes to the chromatin (Wang et al., 2004).

The PRC1 complex consists of four core proteins, including CBX family members, RING1A/B, BMI1 and HPH1/2 as shown in Table 1. As previously mentioned, EZH2 of the PRC2 complex trimethylates H3K27, which then results in the recruitment of the PRC1 complex to this mark via the chromodomain of CBX family members (Kaustov et al., 2011; Konuma et al., 2010). In flies, PcG proteins are recruited to the Polycomb responsive elements of chromatin, which results in the repression of target gene. On the other hand, in mammals the mechanism of PcG proteins recruitment to the chromatin is still not clear. Nevertheless, a general hierarchical model is approved according to which DNA-binding accessory proteins such as YY1 are involved in the recruitment of PRC2 complex to the promoter region of target genes (Klymenko et al., 2006; Wang et al., 2004). In addition, PcG proteins have been shown to interact with several transcription factors such as Jarid2/Jumonji that mediate recruitment of PRC2 (Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). Moreover, long non-coding RNAs have been

Table 1: Components of the Polycomb Repressive Complexes 1 and 2 and their functions

Polycomb repressive complex2 (Initiation Complex)	**Functions**
EZH2	Methylation of histone H3 at lysine K27
SUZ12	Stimulates H3K27 methyltransferase activity of EZH2
EED	Stimulates H3K27 methyltransferase activity of EZH2
Polycomb Repressive Complex 1 (Maintenance Complex)	**Functions**
CBX8	Binds to trimethylated H3K27 via its chromodomain
RING1a/RING1b	E3 ubiquitin ligase for histone H2A at lysine K119
BMI1	Co-factor of RING1a/RING1b
HPH	Unknown

Modified from review by Konuma et al., *Develop. Growth Differ*. (2010) 52, 505-516

suggested to associate and recruit PRC2 to the target genes (Rinn et al., 2007; Zhao et al., 2008). Therefore, there is an important role of two opposing epigenetic histone modifications in controlling proliferation and differentiation of HSCs, H3K27 trimethylation by PRC2 and H3K4 trimethylation by MLL.

HSCs can undergo self-renewal and differentiation into all lineages of mature blood cell. Several signaling pathways have been identified that are important for the regulation of self-renewal property of HSCs (Zon, 2008). Recently, epigenetic modifications involving PcG proteins have also been shown to play a role in regulating HSCs self-renewal. It has been reported that Eed acts as negative regulator of lymphoid and myeloid progenitor cells using Eed heterozygous +/- mice (Lessard et al., 1999). The hypomorphic mutant of Suz12 has been shown to competitively repopulate the hematopoiesis of recipient mice because of its increased HSC activity (Micklem et al., 1972). In addition, mice lacking thrombopoietin receptor exhibiting reduced platelet numbers and HSC defects were sufficiently improved with the loss of Suz12 (Majewski et al., 2008).

The function of Ezh2 in hematopoiesis has been shown with the use of a conditional knock out system in mice. These mice could not undergo early B cell differentiation and showed defective rearrangement of the immunoglobulin heavy chain gene. Moreover, these mice also exhibited a T cell differentiation block in the thymus at the early CD4-/- and CD8-/- stage, but not much effect on the cells of other lineages (Su et al., 2003; Su et al., 2005). EZH2 has a closely related homolog, EZH1, which provides functional redundancy in the absence of EZH2. In adult hematopoiesis, EZH1 has been

shown to compensate for the function of EZH2 but not in the fetal liver because of lower expression levels of EZH1 in that tissue (Mochizuki-Kashio et al., 2011).

Several reports have demonstrated the oncogenic role of EZH2 as shown by its overexpression in variety of cancers, including prostrate, bladder, breast, pancreatic, lung, colon and lymphomas (Arisan et al., 2005; Kleer et al., 2003; Matsukawa et al., 2006; Varambally et al., 2002; Watanabe et al., 2008). In breast cancer, expression of EZH2 is strongly correlated to poor prognosis and is very highly expressed in invasive and metastatic carcinoma. Similarly, in prostrate cancer, high levels of EZH2 expression are associated with metastatic and aggressive cancer. The oncogenic property of EZH2 has been evidenced by EZH2 overexpression in the H16N2 breast epithelial cell line, which produced increased invasive properties and anchorage-independent colony growth (Kleer et al., 2003). In contrast, EZH2 has also been demonstrated as a tumor suppressor in a variety of T cell Acute Lymphoblastic Leukemia (T-ALL) patients as evidenced by their loss of function mutation (deletion or missense mutations) leading to complete loss of EZH2 protein expression (Ntziachristos et al., 2012).

BMI1, one of the core components of PRC1 complexes, has been shown to play an important role in the maintenance of self-renewal property of HSCs, hepatic stem cells and neural stem cells (Iwama et al., 2004; Lessard and Sauvageau, 2003; Park et al., 2003). Ink4a, a cyclin-dependent kinase inhibitor and Arf, a tumor suppressor, which are critical regulators of senescence pathway, are major targets of BMI1 (Jacobs et al., 1999). It has been reported that defects in the self-renewal capability of HSCs in the Bmi1 null mice can be restored after deletion of both Ink4a and Arf (Oguro et al., 2006). Besides HSCs, BMI1 has also been suggested to have a role in the maintenance of the self-

renewal capacity of leukemic stem cells (LSCs). This was evidenced by the fact that HOXA9 and MEIS1 transformed Bmi1 null fetal liver cells were able to cause leukemia in the primary recipient mice but were unable to cause leukemia in the second recipient mice (Lessard and Sauvageau, 2003).

In mammals five different CBX proteins have been identified, including CBX2, CBX4, CBX6, CBX7 and CBX8. All of the proteins share a homologous N-terminal chromodomain and a C-terminal COOH box (Morey et al., 2012). The N-terminal chromodomain is responsible for binding to methylated histones and it has been shown that the different CBX proteins recognize different methylated histone marks (Bernstein et al., 2006). Recently, Morey et al have shown using comparative genome-wide chromatin immunoprecipitation (ChIP-Seq) that different CBX proteins associated with PRC1 complexes have different roles in embryonic stem cell (ESC) pluripotency and differentiation. Specifically, association of CBX7 with the PRC1 complex is responsible for the pluripotency of ESCs, whereas association of CBX2, CBX4 and CBX8 are responsible for differentiation of ESCs (Morey et al., 2012). Another conserved C-terminal COOH box promotes the binding of CBX proteins to RING1A and RING1B, which are further responsible for histone H2A lysine K119 (H2AK119) monoubiquitination and repression of a target genes (de Napoles et al., 2004).

RING1B, an E3 ubiquitin ligase responsible for H2AK119 monoubiquitination, has also been shown to play an important role in PcG mediated gene repression. There is evidence that RING1B mediated H2AK119 monoubiquitination might interfere with the mobility of RNA polymerase on the target gene (Stock et al., 2007; Zhou et al., 2008). It is also suggested that RING1B mediated H2AK119 monoubiquitination results in the

compaction of nucleosomes that might prevent their displacement by RNA polymerase (Francis et al., 2004). Therefore, the two PcG complexes play an essential role in the maintenance of the repressed state of a gene.

Among all the CBX proteins, only CBX8 has been shown to interact with AF9 (Hemenway et al., 2001). Furthermore, affinity purification of AF9 and its homolog ENL complexes has shown the presence of only CBX8 but not other CBX proteins (Biswas et al., 2011; Mueller et al., 2007). Interestingly, CBX8 binds to AF9 through a non-conserved central region. Similarly, other CBX proteins have also shown to have differential binding with other proteins. CBX4, through a minimal six amino acid motif, has been shown to interact with C-terminal binding protein (CtBP), transcriptional repressor, whereas CBX2 that lacks the six amino acid motif cannot bind (Sewalt et al., 1999). In addition, CBX8 has been shown to simultaneously bind with both AF9 and RING1B, forming trimeric complex (Hemenway et al., 2001). Therefore, differential binding of CBX8 to a transcriptional activator, AF9, suggested a role for CBX8 that potentially is not associated with the repression of target genes. Interestingly, although CBX8 is a component of repressive PcG complexes, it has also been shown to play an important role in MLL-AF9 mediated leukemogenesis. It has been demonstrated that CBX8, independent of the PRC1 complex, acts as an essential transcriptional cofactor for MLL-AF9 mediated leukemic transformation (Tan et al., 2011).

Therefore, although CBX8 is a part of repressive PRC1 complex, it can also act as a transcriptional coactivator in a highly context dependent manner. Collectively, AF9 can interact with wide variety of proteins, including activators and repressors of transcription. Therefore, in this dissertation our goal is to look for the significance of protein

interactions in mediating AF9 function. We propose following AIMS:

AIM1: To determine distinct sub-complexes formed by AF9 and the effect of AF9-CBX8 association on AF9 target gene, *ENaCα*.

AIM2: To determine the molecular mechanism of SPK-111 cytotoxicity

AIM3: To look at the effect of various point mutants of AF9 on the interaction status between AF9 and its binding partners.

CHAPTER 2

MATERIALS AND METHODS

Cell Culture and transfection

MV4-11 (MLL-AF4), MOML13 (MLL-AF9) and K562 (BCR-ABL) human leukemic cell lines (ATCC) were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS), 1.1% Penicillin-Streptomycin (Pen/Strep) and 2.2% Glutamine. HEK293T cells (Clontech) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1.1% Pen/Strep. Cells were incubated at 37^{o} C in room air plus 5% carbon dioxide. For transient transfections of DNA expression vectors and shRNA, Lipofectamine^{TM}2000 reagent (Life technologies # 11668-019) was used according to the manufacturer's protocol. GFP-RI3A-(AF4), GFP-DOT1L, pCMV3XFLAG-AF9, GFP-Pc3 (CBX8) and HA-BCoR expression vectors were described previously (Erfurth et al., 2004; Srinivasan et al., 2003). Myc-DDK-CBX8, Myc-DDK-BMI1 expression vectors and CBX8 shRNA were purchased from OriGene.

Transfection of plasmid DNA or shRNA using Lipofectamine^{TM} 2000 Reagent

Transfections were performed on HEK293T cells cultured in 10-cm dishes. A total of 24μg DNA and 50μl of Lipofectamine^{TM} 2000 Reagent was used for transfections

in 10-cm dishes. In 2 separate microcentrifuge tubes added 1ml of DMEM without serum and added 24μg DNA in one tube and 50μl of Lipofectamine^{TM} 2000 Reagent in another tube. The tubes were allowed to sit at room temperature for 5 mins. After 5 mins, DMEM containing Lipofectamine^{TM} 2000 Reagent was added to the DNA containing tube and the mixture was incubated for 20 mins at room temperature. The medium was aspirated from the dishes to be transfected and replaced with fresh 8μl DMEM with serum. Following incubation, DNA/transfection complexes reagent was added dropwise to the different areas of the dish and gently mixed by rocking back and forth and side to side. The plates were incubated at 37^0C for 48 or 72 hours depending on the experiment.

Size Exclusion Chromatography

1.0 x 10^9 MV4-11 cells were washed once with phosphate buffer saline (PBS) and lysed by resuspension in 1ml of lysis buffer (20mM HEPES pH7.2, 150mM KCl, 2mM EDTA, 1mM DTT [Dithiothreitol], 1X protease inhibitor cocktail, 1X PMSF). The lysate was then passaged through a dounce homogenizer (40 strokes) for further lysis. Lysate was then centrifuged at 13,000rpm (rotations per minute) at 4^0C for 20 mins to remove insoluble debris. The protein concentration of the lysate was checked using Bradford protein assay according to manufacturer's protocol (Bio-Rad). Briefly, BSA standards were made ranging from 0.05mg/ml to 0.5mg/ml. The lysate was also diluted to make 3-5 different dilutions. BSA standards and lysate dilutions (10μl each) were separately loaded into triplicate wells of a microtiter plate. Bio-Rads dye reagent was diluted by adding one part of Dye Reagent Concentrate and four parts of double distilled water. The diluted dye

reagent (200µl) was added into each well and incubated on microplate mixer at room temperature for 5 mins. After 5 mins, the absorbance was measured at 595nm and the protein concentration was calculated by plotting the OD value against protein concentration using BSA values as the standard curve. Cleared cell lysate (250µl, approximately 1.5 mg total protein) was then applied to a Superose 6 10/300 GL column (GE Healthcare) with a fractionation range of 5KDa-5MDa. Subsequently, 96 250µl fractions were collected using Fast Pressure Liquid Chromatography (FPLC) at a flow rate of 0.35 ml/min and a maximum pressure of 1.5MPa. The fractions were then analyzed by 4-12% SDS-PAGE followed by western blotting against AF9 (Novus Biologicals # NB100-1566), RING1b (Santa Cruz # sc-101109) and CBX8 (Bethyl # A300-882A) antibodies.

Immunoprecipitation

HEK293T cells transiently transfected with FLAG-AF9 or left untransfected were washed once with ice-cold PBS after 48 hours of transfection. The cells were lysed in 1 ml lysis buffer (30mM Tris pH7.4, 150mM NaCl, 0.5% Triton-X 100 (v/v), 1X protease inhibitor cocktail and 1mM DTT) and were collected in the microcentrifuge tube using cell scrapper. Cells were then sonicated, centrifuged at 13,000rpm at 4^0C for 20 mins and cleared cell lysates were incubated with anti-FLAG M2 agarose beads (Sigma # A2220) for 1-2 hours or 3µg/ml of an endogenous BMI1 antibody (Cell Signaling # 5856) or isotype control antibody overnight at 4^0C. The BMI1 immunoprecipitate was pulled down after incubation with Protein A agarose beads at 4^0C for 1 hour. Following incubation, both M2 agarose and Protein A beads were washed three times with lysis

buffer and finally mixed with 2X SDS (sodium dodecyl sulfate) sample buffer (0.75M TrisCl pH6.5, 10% SDS, 10% Glycerol, 5% β-mercaptoethanol and 0.005% Bromophenolblue) to elute immune-complexes from the beads. The beads were boiled for 10mins and immunoprecipitates were separated by 4-12% SDS-PAGE. The FLAG-AF9 immunoprecipitate was blotted for endogenous CBX8, RING1b, BMI1 and RING1a (Cell Signaling # 2820). The BMI1 pulled down immunoprecipitate was immunoblotted with anti-FLAG antibody (Sigma # F1804) to detect the presence of AF9.

To examine the interaction status between BMI1 and other AF9 interacting proteins, HEK293T cells co-expressing MYC-DDK-BMI1, GFP-AF4 (RI3A-), GFP-DOT1L, or HA-BCoR were washed once in ice cold PBS after 48 hours of transfection and lysed in 1ml of lysis buffer. Cells were then sonicated, centrifuged at 13,000rpm at 4^0C for 20 mins and the cleared lysate was immunoprecipitated-using 3µg/ml of either anti-Myc (Upstate # 06-549) or anti-DDK (Origene # TA50011) antibodies. Following immunoprecipitation for overnight at 4^0C, lysates were incubated with Protein A agarose beads for one hour at 4^0C. Beads were washed three times with lysis buffer and finally mixed with 2X SDS sample buffer. The beads were boiled for 10mins and immunoprecipitates were separated by 4-12% SDS-PAGE followed by western blotting to detect GFP (Life Technologies # A11122) or HA (Sigma # H3663-200UL) epitope tags that were fused to AF4, DOT1L and BCoR.

To look for the effect of CBX8 knock down on the interaction status between AF9 and PRC1 complex proteins, HEK293T cells were co-transfected with FLAG-AF9 and CBX8 shRNA or scrambled shRNA. Forty-eight hours after transfection, cells were washed once with ice cold PBS and lysed in 1ml lysis buffer. Cells were then sonicated;

centrifuged and cleared lysate was immunoprecipitated overnight at 4^0C with anti-FLAG M2 agarose beads. Beads were washed three times with lysis buffer and mixed with 2X SDS sample buffer. The beads were then boiled to elute the bound proteins and immunoprecipitates were separated by 4-12% SDS-PAGE followed by immunoblotting to detect the presence of CBX8, BMI1, RING1a and RING1b. Whole cell lysate was also immunoblotted with the same antibodies to confirm that total protein remain unaffected after CBX8 knock down.

Co-Immunoprecipitation was also performed to look for the interaction status between point mutants of AF9 and GFP-AF4 (RI3A-), GFP-DOT1L or GFP-CBX8. AF9 point mutants (E506R, I538A, D544R, K557E, L562A and S565D) predicted to disrupt binding with AF4 using structural studies done by Dr. John Bushweller lab in the University of Virginia, were introduced by site directed mutagenesis using Gene Taylor Reagents from Invitrogen according to manufacturer's protocol. HEK293T cells were co-transfected with AF9 wild type (WT) or point mutant and GFP-AF4 (RI3A-), GFP-DOT1L or GFP-Pc3 (CBX8). After 48 hours of transfection, cells were washed once with ice cold PBS and lysed in 1ml of lysis buffer. Cells were then sonicated, centrifuged at 13,000rpm at 4^0C for 20 mins and clear lysate was collected. Cleared lysate was immunoprecipitated with 3μg/ml of anti-GFP antibody or isotype control antibody overnight at 4^0C. The immunoprecipitates were pulled down using Protein A agarose beads after incubation at 4^0C for one hour followed by washing with lysis buffer three times. Beads were mixed with 2X SDS sample buffer and boiled for 10 mins. The immunoprecipitates were separated by 4-12% SDS-PAGE followed by immunoblotting against FLAG antibody to detect the presence of WT AF9 or mutant AF9.

Western Blotting

To look at the effect of SPK-111 on the protein levels of MEIS1, MYC and Retinoblastoma (Rb), we treated 50 million MV4-11, MOLM13 and K562 cells with 6μM of SPK-111 or vehicle control (DMSO) for 6 and 24 hours. After treatment, cells were washed once with PBS and the number of viable cells was counted using trypan blue dye. An equal number of viable cells in both SPK-111 and vehicle control treatment were then lysed in lysis buffer (30mM Tris pH7.4 with HCl, 150mM NaCl, 0.5% Triton-X 100 (vol/vol), 1X Protease inhibitor cocktail and 1mM DTT) followed by sonication. After sonication, lysate was centrifuged at 13,000rpm for 20 mins at 4^0C to remove insoluble debris. The cleared lysate was transferred into a new tube and mixed with an equal volume of 2X SDS sample buffer. Samples were boiled for 10 mins and 10μl was loaded onto 4-12% Bis tris gel and immunoblotted for anti-MEIS1 (Novus Biologicals # NBP1-95898), anti-MYC (Milipore # 06-549), anti-Rb (Novus Biologicals # NBP1-19490) and anti-actin antibodies (Sigma # A5441).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

HEK293T cells were transiently transfected with the designated gene expression vectors and/or shRNA. Cells were harvested after 72 hours and total RNA was extracted using RNeasy kit according to manufacturer's protocol (Qiagen # 74106). 2ug RNA was then reverse transcribed into cDNA using SuperScript III First Strand Synthesis Super Mix kit (Life Technologies # 11752-050). Briefly, a master mix was prepared, which contained 20ul of 2X RT Reaction Mix, 4ul RT Enzyme Mix, 2ug RNA and DEPC-treated water to make final volume of 40ul. All of the components in the tube were gently

mixed and incubated at the following conditions in a PCR Thermocycler (Eppendorf AG 22331 Hamburg).

The incubation protocol for cDNA preparation was as follows:

1) Incubation at 25^0C for 10 mins
2) Incubation at 50^0C for 30 mins
3) Termination of the reaction by incubating at 85^0C for 5 mins
4) Hold/ Chill at 4^0C
5) Addition of 1µl of *E.Coli* RNase H and incubation at 37^0C for 20 mins and followed by storing cDNA at -20^0C until used.

SYBR Green Supermix with ROX (BioRad # 172-5851) was used to perform quantitative RT-PCR using 1:5 diluted cDNA and primers for the human *ENaCα* gene (F: ATGACTTCATTCCCCTGCTG, R: CATCACTGCCATTCTTGGTG) and house keeping *GAPDH* gene (primers provided in the EZ-Magna ChIP™ A/G protocol) [Milipore # 17-10086]). The following PCR Reaction Mix for qRT-PCR was used: SYBR Green with ROX – 12.5ul, Primers (Forward/Reverse) – 0.5ul, cDNA (1:5 diluted) – 5ul and Water – 7ul to make final volume of 25ul. Samples were run on a 7300 Real Time PCR System from Applied Biosystems at an annealing temperature of 60^0C under the following Thermocycler conditions: One cycle of 50^0C for 2 mins, one cycle of 95^0C for 10 mins and forty-five cycles of 95^0C for 15 seconds and 60^0C for 1 min. The relative *ENaCα* gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with *GAPDH* gene expression levels.

To determine the effect of SPK-111 treatment on *HOXA9*, *MEIS1* and *MYC* mRNA levels, 5 million MV4-11, MOLM13 and K562 leukemic cells were treated with

6μM SPK-111 or vehicle control (DMSO) for 6 and 24 hours. Following treatment, cells were washed once with PBS and the number of viable cells was counted using trypan blue dye. An equal number of viable cells in both SPK-111 and DMSO treated samples were collected and the total RNA was extracted using RNeasy kit from Qiagen. cDNA was then reverse transcribed from 2ug of RNA using SuperScript III First Strand Synthesis Super Mix kit (Invitrogen) as mentioned previously. Quantitative RT-PCR using 1:5 diluted cDNA was performed using SYBR Green Supermix with ROX (Bio-Rad) and primers against human *HOXA9* gene (F: TCCCACGCTTGACACTCACACTTT, R: AGTTGGCTGCTGGGTTATTGGGAT), *MEIS1* gene (F: CAGAAAAAGCAGTTGGCACA, R: TCATGCCCATTCCACTCATA), *MYC* gene (F: CCTACCCTCTCAACGACAGC, R: CTCTGACCTTTTGCCAGGAG) and housekeeping *β2M* gene (F: TGCTGTCTCCATGTTTGATGTATCT, R: TCTCTGCTCCCCACCTCTAAGT) using same Thermocycler conditions as mentioned above. Relative *HOXA9, MEIS1* and *MYC* gene expressions were calculated using $2^{-\Delta\Delta Ct}$ methods after normalization with *β2M* gene expression levels.

Chromatin Immunoprecipitation (ChIP)

HEK293T cells were transiently transfected with Myc-DDK-CBX8 or left untransfected. After 48 hours, cells were fixed with 1% formaldehyde, and chromatin immunoprecipitation was performed using the EZ-Magna ChIP™ A/G protocol (Milipore). Briefly, after fixation for 10 mins at room temperature, the reaction was quenched with 10X glycine solution at room temperature for 5 mins. Cells were washed

twice with ice cold PBS and were collected in PBS containing 1X protease inhibitor cocktail II. Cells were pelleted out by spinning at 800g at 4^0C for 5mins. The cell pellet was then resuspended in 0.5ml of Cell Lysis Buffer containing 1X protease inhibitor cocktail II. Cells were incubated on ice for 15 mins followed by centrifugation at 800g at 4^0C for 5mins to pellet the cells. The cell pellet was resuspended in 0.5ml of Nuclear Lysis Buffer containing protease inhibitor cocktail II. The lysate was then sonicated while on ice 5 times for 6 seconds each at an output 4 using Branson Sonifier 250 with an interval of 50-60 seconds between each run of sonication. The cleared supernatant was removed from insoluble materials by centrifugation at 13,000g for 10mins at 4^0C. The supernatant (5µl) was used to check the sheared chromatin. The remaining supernatant was used for immunoprecipitation with four antibodies. 50µl of the supernatant was used for each immunoprecipitation and was diluted 10X with dilution buffer containing 1X protease inhibitor cocktail II. One percent of the input was saved for both the samples. The supernatant was immunoprecipitated by incubating overnight at 4^0 C with 20µl fully resuspended protein A/G magnetic beads and anti-H3 (abcam # ab12079), anti-tri-methylated H3K79 (abcam # ab2621), anti-Myc tag for CBX8 or isotype control antibodies at concentrations of 3µg/ml. After incubation, Protein A/G magnetic beards were pelleted using a magnetic separator and the supernatant was removed. Beads were washed by resuspending in 0.5ml of the following ice cold buffers in the order below and incubating for 5 mins between each wash: Low Salt Wash Buffer, High Salt Wash Buffer, LiCl Wash Buffer and TE Buffer. To elute protein-DNA complexes and reverse cross link protein-DNA complexes to free DNA, beads and input samples were incubated with 100µl ChIP elution buffer containing Proteinase K at 62^0C for 2 hours with constant

shaking. Beads were then incubated at 95^0C for 10 mins and samples were cool down to room temperature. Beads were separated using a magnetic separator and the supernatant was carefully removed and transferred to a new eppendorf tube. Supernatant from the separated beads and input samples were then processed for DNA purification using spin columns. To each 100µl of DNA sample tube, 0.5ml of Bind Reagent A was added and mixed well by pipetting up and down. The mixture was transferred to a spin filter in a collection tube and centrifuged for 30 seconds at 13,000g. The spin filter in the collection tube was washed with 500µl of Wash Reagent B once and centrifuged twice in order to remove any residual wash reagent. The spin filters were put into new collection tube, and the DNA was eluted with 50µl of Elution Buffer and stored at -20^0C. The eluted DNA (1:4 dilution) was then subjected to quantitative RT-PCR using SYBR Green with ROX as mentioned above. Two different sets of primers for the *ENaCα* promoter regions (primer sequences listed in Table 2) were used and enrichment was normalized to input chromatin and IgG control.

To look at the effect of SPK111 treatment on the recruitment of RNA Polymerase II, the phosphorylated form of Ser 2 of CTD of RNA PolII (pS2) and Cyclin Dependent Kinase 9 (CDK9), 10 million MOLM13 cells were treated with 6µM SPK111 or vehicle control (DMSO) for 6 hours. After 6 hours, the cells were washed once with PBS and the number of viable cells was counted using trypan blue stain. An equal number of viable cells were resuspended in 20ml of RPMI media and fixed using 1% formaldehyde and chromatin immunoprecipitation was performed using the EZ-Magna ChIP^{TM} A/G protocol (Milipore) as described above. The lysate was immunoprecipitated overnight at 4^0C using anti-PolII (control antibody from EZ-Magna ChIP^{TM} A/G), anti-pS2 (abcam #

ab5095), anti-CDK9 (Santa Cruz # sc-484) and isotype control antibodies at concentrations of 3µg/ml. Finally DNA was eluted in 50µl of elution buffer and diluted 1:4 for quantitative RT-PCR using primers against *MEIS1* and *MYC* promoter and downstream regions. The fold enrichment was normalized to input chromatin and IgG control.

Table 2 : Primers used for Chromatin Immunoprecipitation Assay

Amplicon	Primers (5' to 3')
ENaCα Promoter P1a	Forward: ACCTCGAGCTGTGTCCTGAT Reverse: GCCCTGCTCACCTTTAATTG
ENaCα Promoter P1b	Forward: GTACTGGACCTGAGAAGGCG Reverse: CTTCTCCTTGTGTTGCCCTC
MEIS1 Promoter	Forward:CGGCGTTGATTCCCAATTTATTTCA Reverse: CACACAAACGCAGGCAGTAG
MEIS1 Downstream Region	Forward: TCTCAGCGCCTCCAAATCTTG Reverse:TTTGTGTGTGTGAAATTTAGCTATTTAGGTTTT
MYC Promoter	Forward: TCCGCCCACCGGCCCTTTAT Reverse: TCAGCGCGATCCCTCCCTCC
MYC Downstream Region	Forward: CGGGGTCTCTGGCGCAGTTG Reverse: GAGCTCAGCCGCGGGCTTTA

Cell Viability Assay

The cell viability assay, Cell Titer Glo Reagent (Promega # G7571), works on the basis of quantification of total ATP present in the sample, which accounts for the presence of metabolically active cells. MV4-11, MOLM13 and K562 leukemic cell lines were used at a concentration of 0.5 x 10^6 cells/ml. Each sample (500µl) was added to a microcentrifuge tube and treated with the various concentrations of SPK111 (4µM-10µM) with the same final amount of DMSO in each treatment or vehicle control (DMSO). The samples were mixed gently by pipetting up and down, and 100µl of cells aliquotted in quadruplicates in a 96 well plate. RPMI media (100µl) without cells was

added in the four wells of microtiter plate as blank samples. The plate was incubated at 37^0C for 6 and 24 hours. After incubation, the plate was kept at room temperature for 30 mins for equilibration of the cells and to let cell titer Glo Reagent thaw at room temperature as well. An equal volume of cell titer Glo Reagent (100µl) was added into each well containing samples or blank samples. The plate was incubated at the rotating platform for 2 mins to induce cell lysis. The plates were then kept at room temperature for 10 mins to stabilize the luminescent signal. Luminescence was recorded using POLAR Star Omega plate reader from BMG Labtech. Cell viability was calculated as percentage of control (DMSO) treated cells.

Cell Cycle Analysis

MOLM13 cells (2.0 X 10^6) were treated with 6µM SPK111 or vehicle control (DMSO) or left untreated and incubated at 37^0C for 6 hours. After treatment, cells were washed once with 3ml of ice cold PBS containing 5% FBS. The cell pellet was collected after centrifugation at 1000rpm for 5 mins. Cells were then fixed by adding 600µl of 70% ethanol to the cell pellet while constantly vortexing at low speed. Cells were incubated overnight at 4^0C and then washed once with 3ml of ice cold PBS containing 5% FBS. The cell pellet was resuspended in 250µl of 10µg/ml of RNase A (Fermentas # EN0531, diluted 1:1000 from a 10mg/ml RNase A stock) in cold PBS and incubated at 37^0C for 15 mins. After incubation, cells were stained with propidium iodide (PI) (Invitrogen # P3566) by adding 250µl of 100µg/ml PI (diluted 1:10 of 1mg/ml stock in cold PBS) and incubating in the dark at room temperature for 2 hours followed by Flowcytometry

Analysis (FACS-CANTO; Becton-Dickinson). The raw data was analyzed using Watson Pragmatic Model for cell cycle analysis by FlowJo v.9.3.

CHAPTER 3

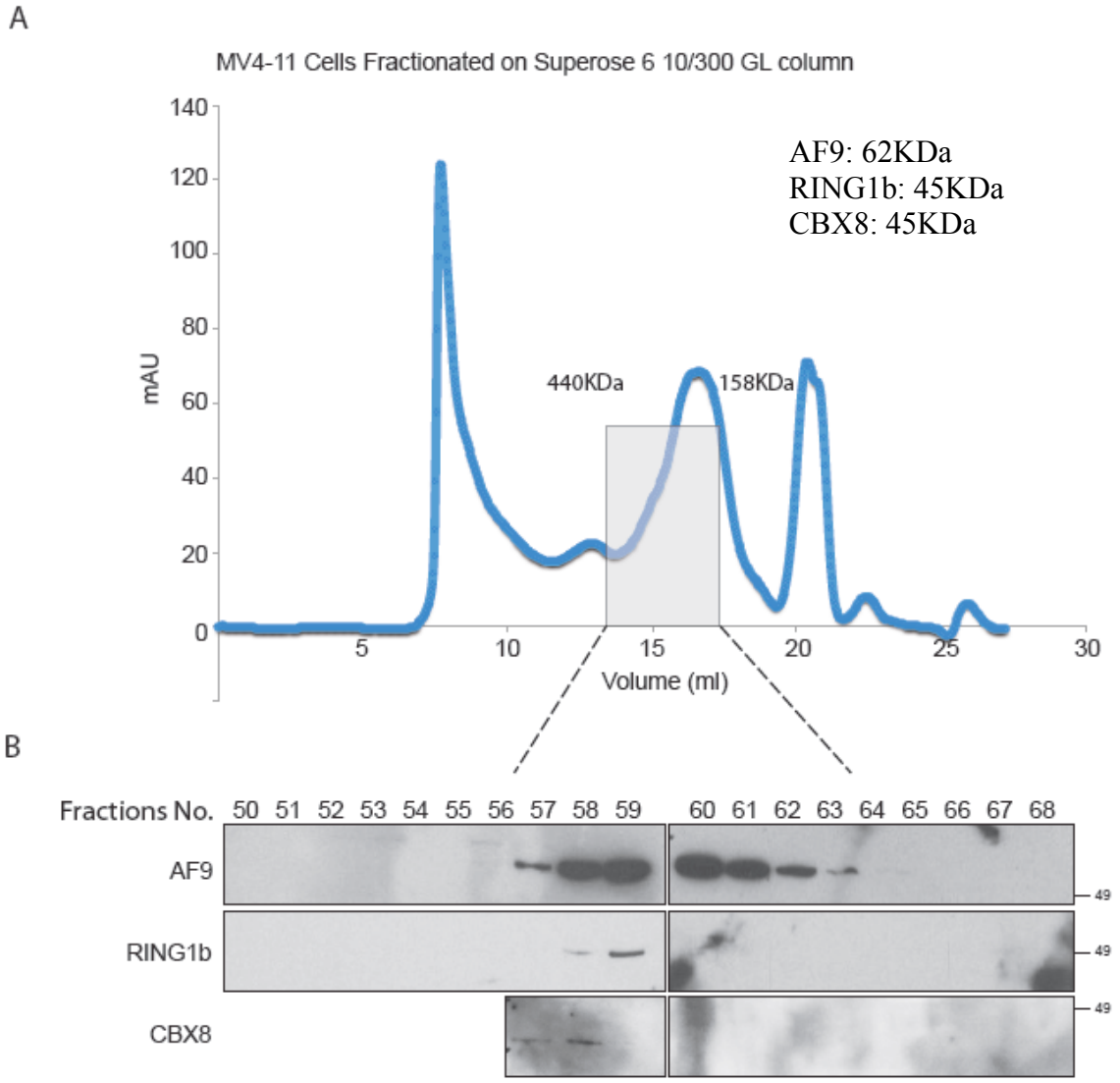
RESULTS

AIM1: Determination of distinct sub-complexes formed by AF9 and the effect of AF9-CBX8 association on AF9 target gene, *ENaCα*

Separation of AF9 complexes using Size Exclusion Chromatography

Affinity purification of AF9 and its homolog ENL has shown that they associate, directly or indirectly, with a large number of proteins including activators and repressors of transcription (Biswas et al., 2011; Mueller et al., 2007). However, it has not been determined whether AF9 forms mega-Dalton complexes collectively comprised of most of these proteins or whether it forms smaller distinct complexes each of which contain a specific subset of AF9 interacting proteins. Here, we sought to determine the presence of discrete subsets of AF9 interacting proteins by size exclusion chromatography. Whole cell lysate was prepared from the MV4-11 leukemia cell line that is known to express wild type AF9 and harbors a MLL-AF4 fusion gene. The lysate was then applied to a Superose 6 10/300GL column and fractions were collected using FPLC. The individual fractions were then subjected to SDS-PAGE and probed for the presence of AF9 by western blot. As shown in Figure 8, AF9 is present in higher molecular weight fractions ranging from 158-440KDa. We then looked for the presence of other known AF9 interacting proteins focusing on members of Polycomb repressive complex 1 (PRC1)

Figure 8. AF9 complexes range in mass from 158 to 440 KDa



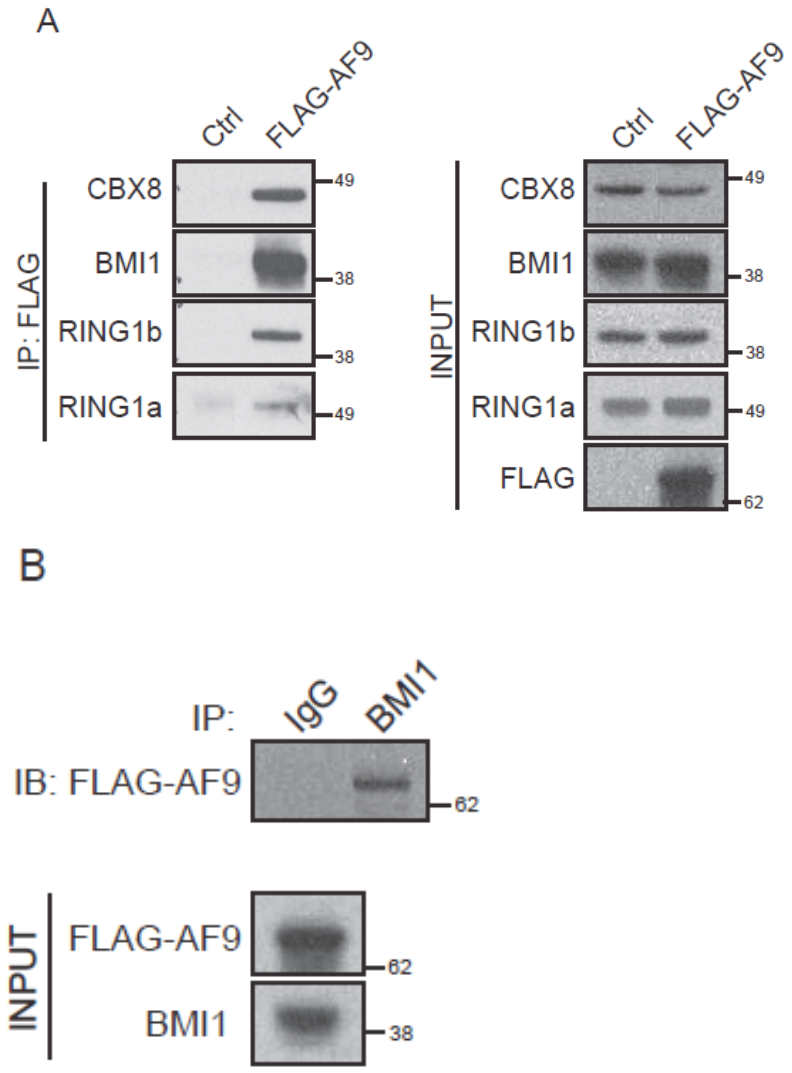
(A) MV4-11 whole cell extract was subjected to size exclusion chromatography using a Superose 6 10/300 GL column and fractions were collected using FPLC. Molecular weight corresponding to the volume of the fractions was detected using Calibration kit standards. (B) Individual fractions were subjected to SDS- 4-12% PAGE and immunoblotted to detect the presence of AF9. Fractions containing AF9 were also immunoblotted with RING1b and CBX8 antibodies.

RING1b and CBX8. As shown, RING1b is present in fractions 58 and 59, and CBX8 is present in fractions 57 and 58. These results indicate that PRC1 proteins are found with AF9 as components of complexes restricted in mass to 300-400KDa and that PRC1 proteins do not promiscuously associate with AF9.

AF9 associates with PRC1 complex proteins

AF9 has been shown previously to interact directly with the non-conserved central region of CBX8 and indirectly with RING1b through RING1b-CBX8 binding (Hemenway et al., 2001). We examined whether AF9 interacts with other PRC1 components, including RING1a, BMI1, and HPH1. HEK293T cells expressing FLAG-AF9 efficiently precipitated endogenous CBX8, RING1a, RING1b and BMI1 using an anti-FLAG antibody (Figure 9A). In contrast, we did not detect HPH1 in the immunoprecipitate (data not shown). We next assessed whether the reciprocal is true, namely that immunoprecipitation of BMI1 can also capture AF9. We have previously demonstrated that (1) BMI1 binds RING1b through the RING finger domains of the two proteins. Furthermore, (2) CBX8 directly binds RING1b through its C-terminal COOH-box. Finally, (3) CBX8 binds the C-terminal domain of AF9 (Hemenway et al., 2001). We predicted that these sequential protein interactions would link AF9 to BMI1. As shown in Figure 9B, HEK293T cells expressing FLAG-AF9 contain AF9 in anti-BMI1 immunoprecipitates. These findings support a model in which a fraction of the total cellular AF9 protein exists within complexes containing at least four Polycomb group proteins of the PRC1 complex.

Figure 9. AF9 interacts with components of the PRC1 complex



(A) HEK293T cells were transiently transfected with FLAG-AF9. Whole cell lysate was then immunoprecipitated with an anti-FLAG antibody. The immunoprecipitate was then analyzed by western blot for PRC1 proteins. (B) HEK293T cells were transiently transfected with FLAG-AF9 and whole cell lysate was immunoprecipitated with an anti-BMI1 antibody. The immunoprecipitate was then analyzed by western blot to detect AF9 with an anti-FLAG antibody. The figures are representative of three independent experiments.

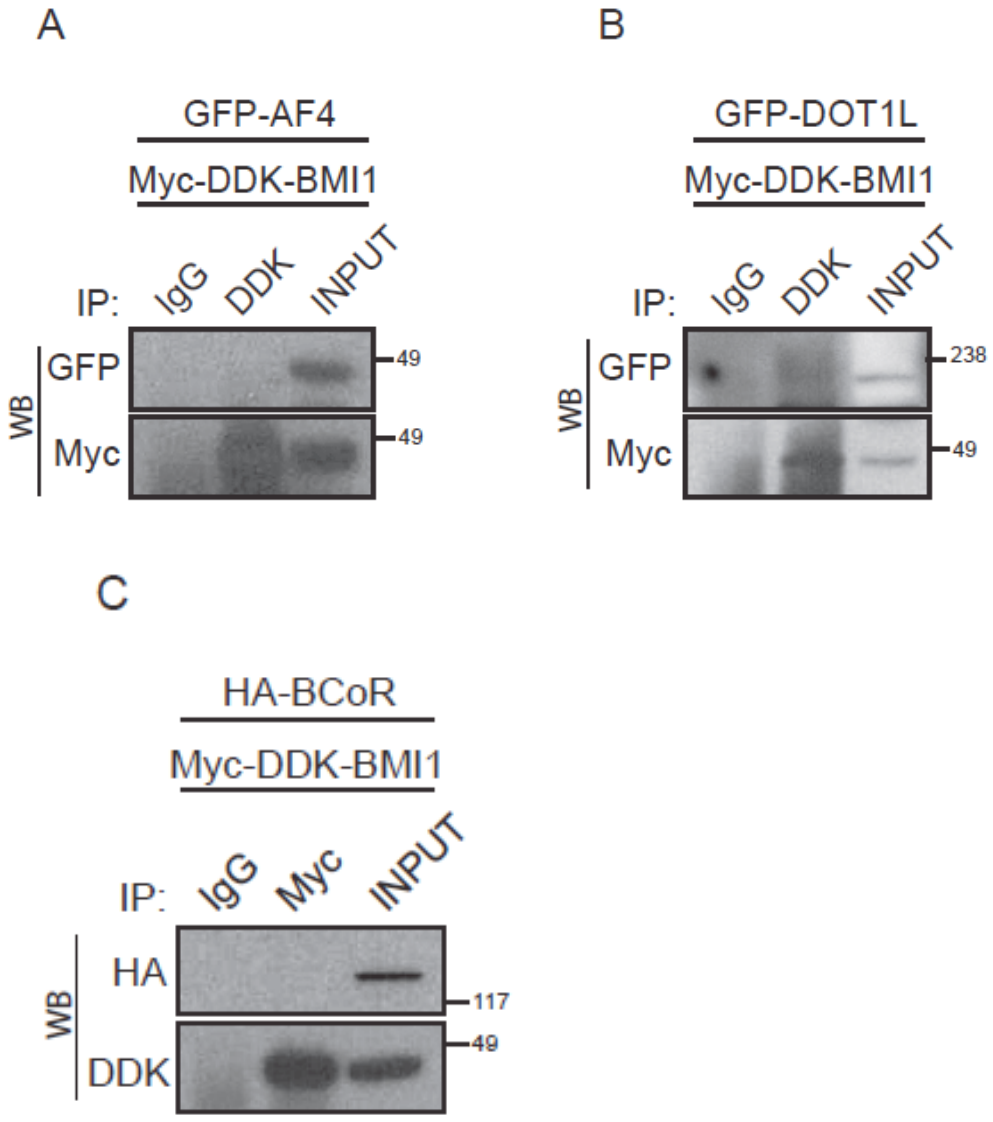
AF9-PRC1 complexes do not contain other known AF9 binding partners

Our gel filtration and immunoprecipitation experiments suggest that AF9 is a component of several physically distinct complexes. In addition to CBX8, it has been shown that AF9 directly binds to AF4-P-TEFb, BCoR and DOT1L (Bitoun et al., 2007; Srinivasan et al., 2003; Zhang et al., 2006). In order to determine whether AF9-PRC1 proteins exist as separate complexes that exclude the other known AF9-binding proteins, we performed co-immunoprecipitation experiments using HEK293T cells co-expressing Myc-DDK-BMI1 in conjunction with GFP-AF4 (RI3A-) (Figure 10A), GFP-DOT1L (Figure 10B) or HA-BCoR (Figure 10C). Since Myc-DDK-BMI1 efficiently precipitated AF9, we wished to analyze whether other AF9 interacting proteins could also be recovered with Myc-DDK-BMI1. As shown in Figure 10A, 10B and 10C, Myc-DDK-BMI1 was not able to immunoprecipitate GFP-AF4, GFP-DOT1L or HA-BCoR. As a control to verify that proteins were effectively immunoprecipitated in the process, blots were probed with DDK (Figure 10A and B) or Myc (Figure 10C) tag antibodies to detect the presence of BMI1. Therefore, these findings suggest that AF9-PRC1 complexes do not contain other proteins that are known to directly bind AF9.

The association between AF9 and PRC1 proteins is mediated by CBX8

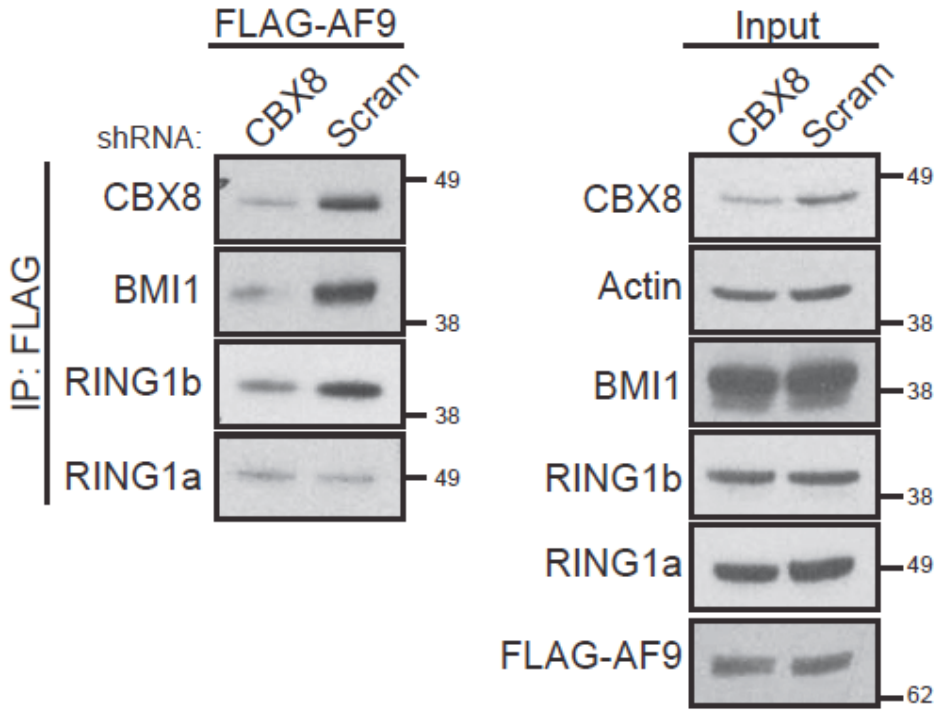
As indicated earlier, using a yeast two-hybrid assay, we found that AF9 and RING1b can simultaneously bind CBX8 to form a ternary complex (Hemenway et al., 2001). In order to determine whether CBX8 is also required for the association between AF9 and other PRC1 components, we knocked down CBX8 using shRNA in HEK293T

Figure 10. Other AF9 binding partners are not detected in AF9-PRC1 complexes



(A) HEK293T cells co-expressing Myc-DDK-BMI1 and GFP-RI3A- (AF4: 647-871aa), GFP-DOT1L (B) or HA-BCoR (C) were immunoprecipitated with an anti-DDK antibody (A and B) or an anti-Myc antibody (C) to capture BMI1. Immunoprecipitates were then analyzed by western blot using an anti-GFP antibody to detect the presence of AF4 or DOT1L (A and B), or an anti-HA antibody for the presence of BCoR (C). The figures are representative of three independent experiments.

Figure 11. The AF9 association with PRC1 complex is CBX8-dependent



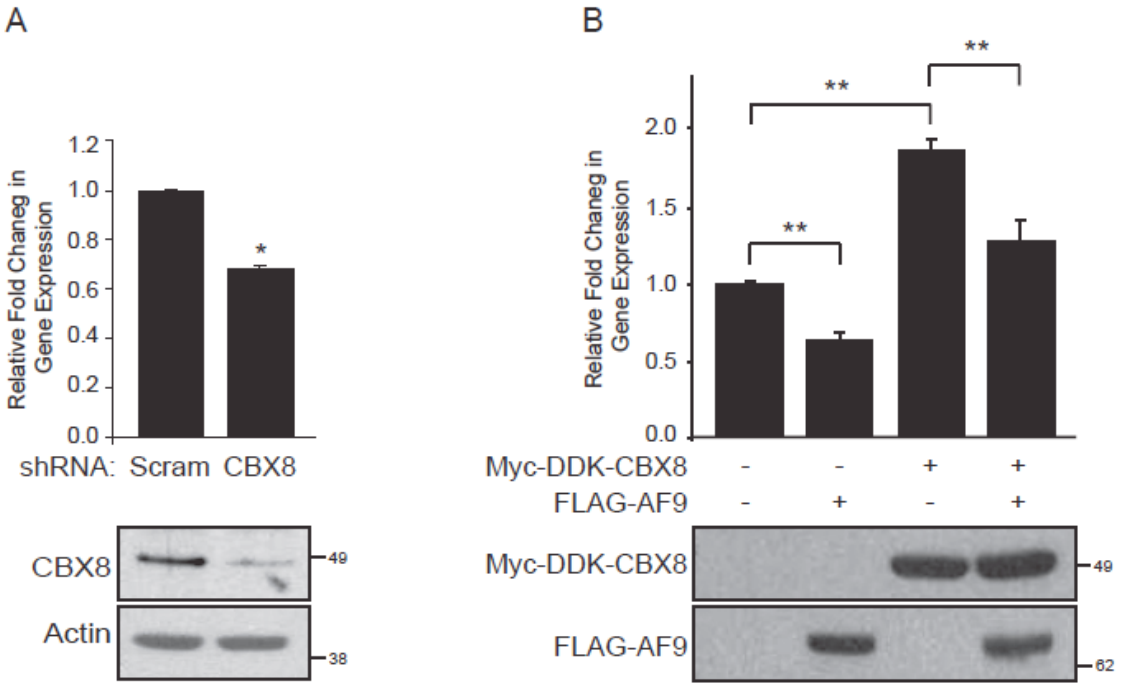
HEK293T cells were co-transfected with FLAG-AF9 and CBX8 shRNA or Scrambled/ Non-targeting (NT) shRNA. Whole cell lysate was immunoprecipitated with an anti-FLAG antibody and then analyzed by western blot to detect the presence different PRC1 proteins. The figure is representative of three independent experiments.

cells and determined the effect on the interaction status between AF9-PRC1 proteins. As shown in Figure 11, knocking down CBX8 with shRNA impaired the ability to co-precipitate FLAG-AF9 and RING1b as well as BMI1, without affecting the total levels of BMI1, RING1b or FLAG-AF9. Scrambled shRNA showed no effect on the interaction status between AF9 and PRC1 proteins. We conclude that CBX8 functions, in part, as a scaffold to support the assembly of AF9-PRC1 complexes.

The association between AF9-CBX8 affects *ENaCα* gene expression

To examine the effect of AF9-CBX8 interactions on a known AF9 target gene, we performed quantitative RT-PCR to measure the gene expression levels of *ENaCα*. We first knocked down CBX8 using shRNA in HEK293T cells with scrambled shRNA used as a control. Knocking down CBX8 decreased *ENaCα* gene expression approximately 25% compared to control-scrambled shRNA (Figure 12A). Conversely, overexpression of CBX8 in HEK293T cells resulted in an increase in *ENaCα* gene expression of approximately 80% compared to control-scrambled shRNA (Figure 12B). Moreover, this increase was partially blocked by the co-expression of AF9. These findings suggest that although CBX8 is a part of the repressive Polycomb PRC1 complex, it acts as an activator of *ENaCα* gene expression by shifting the equilibrium from AF9-DOT1L complexes that repress *ENaCα* gene expression (Zhang et al., 2006) to AF9-PRC1 complexes.

Figure 12. Modulating AF9-CBX8 affects *ENaCα* gene expression

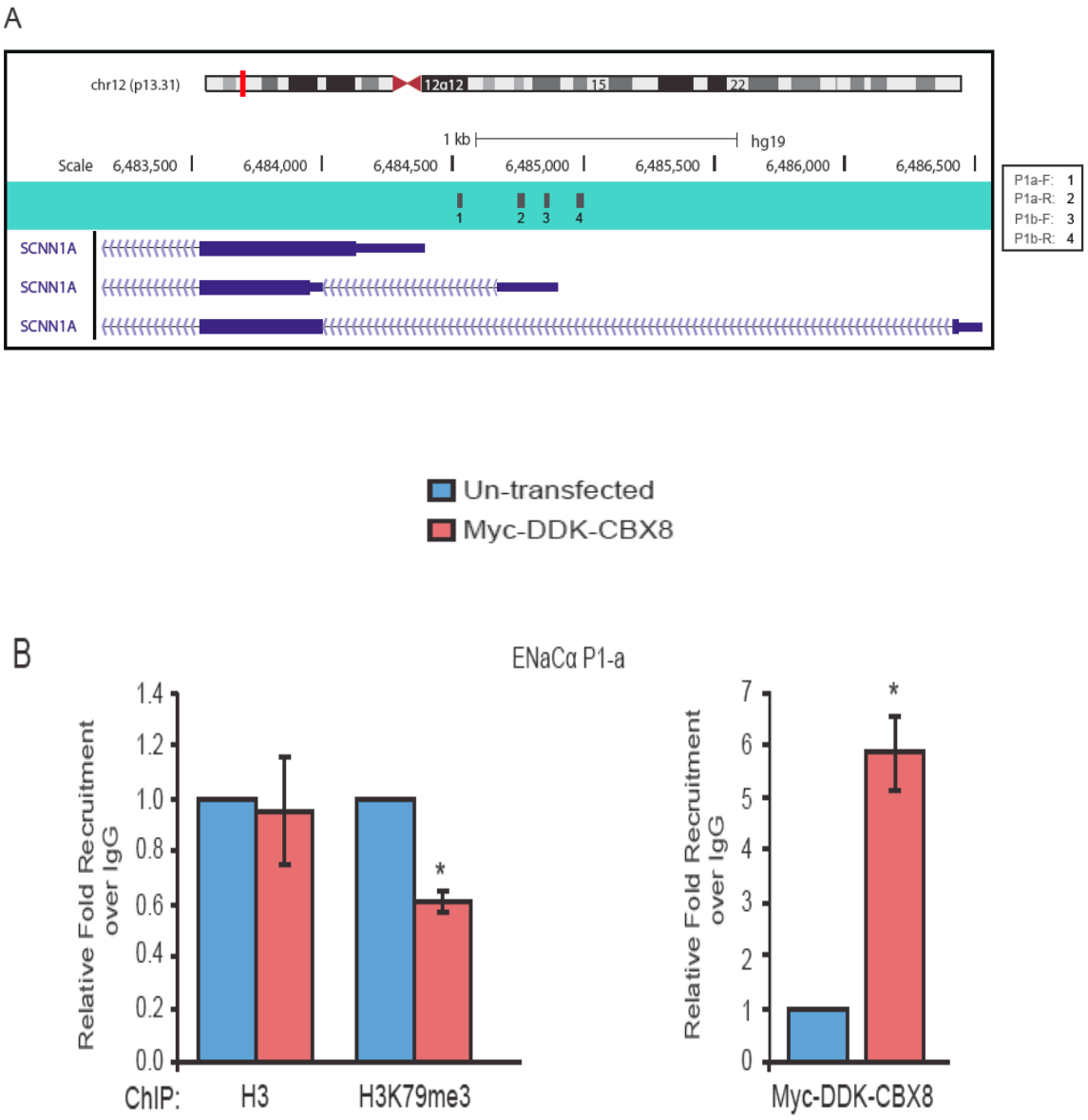


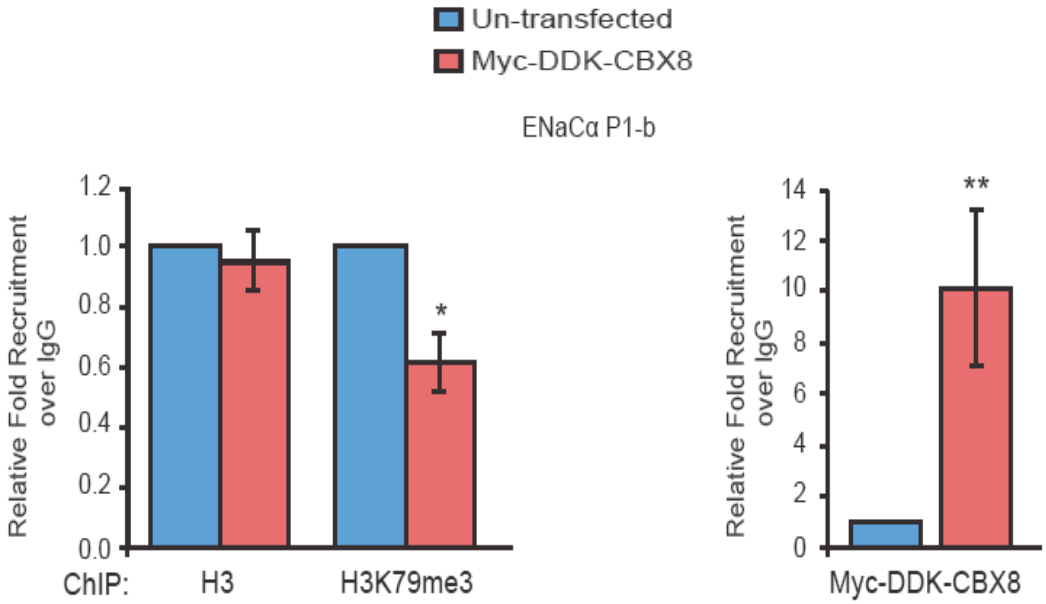
(A) CBX8 knock down with shRNA resulted in decreased *ENaCα* mRNA levels as shown by qRT-PCR. HEK293T cells were transiently transfected with CBX8 or scrambled/NT shRNA. Total RNA was analyzed for *ENaCα* mRNA levels after normalization to the *GAPDH* gene expression. Western blot analysis was done to determine the degree of CBX8 knock down. (B) CBX8 overexpression resulted in increased *ENaCα* mRNA levels. HEK293T cells were transiently transfected with FLAG-AF9, Myc-DDK-CBX8 or FLAG-AF9 and Myc-DDK-CBX8. Again total RNA was analyzed for *ENaCα* mRNA levels after normalization to the *GAPDH* gene. Western blot analysis of the same samples confirmed the presence of FLAG-AF9 and Myc-DDK-CBX8. The experiments were performed in triplicate and each experiment was independently repeated three times. Error bars are the standard deviations of the mean from the three independent experiments. (*,$p = 0.0001$; **, $p < 0.004$)

Increased expression of CBX8 impedes the recruitment of DOT1L to the *ENaCα* promoter

We hypothesized that overexpression of CBX8 increases *ENaCα* gene expression by reducing the recruitment of DOT1L to *ENaCα* promoter regions. To test this hypothesis, we performed chromatin immunoprecipitation using HEK293T cells transfected with Myc-DDK-CBX8. Antibodies recognizing tri-methylated H3 at lysine 79 (H3K79) were used to identify the DOT1L-mediated chromatin mark. We used two primer sets for promoter regions as shown in Figure 13A. Overexpression of CBX8 resulted in decreased H3K79 tri-methylation at both promoter regions without significantly affecting total histone H3 levels. This was accompanied by increased recruitment of CBX8 to these regions (Figure 13B). These findings suggest that a shift in the equilibrium of physically and functionally distinct AF9 complexes alters the regulation of AF9 target genes.

Figure 13. Overexpression of CBX8 decreases histone H3 lysine K79 tri-methylation (H3K79me3) at *ENaCα* promoter regions





(A) Schematic diagram showing the location of primers in the *ENaCα* (*SCNN1A*) promoter region. (B) ChIP assay showing decreased H3K79me3 at the *ENaCα* promoter region with CBX8 overexpression. HEK293T cells were transiently transfected with Myc-DDK-CBX8 and ChIP assays were performed with the indicated antibodies. Immunoprecipitated DNA was amplified by q-PCR using the primer sets shown in panel A. The experiments were performed in triplicate and independently repeated three times. Error bars are the standard deviations of the mean for three different experiments.(*, p= 0.008; **, $p < 0.03$).

AIM2: To determine the molecular mechanism of SPK-111 cytotoxicity

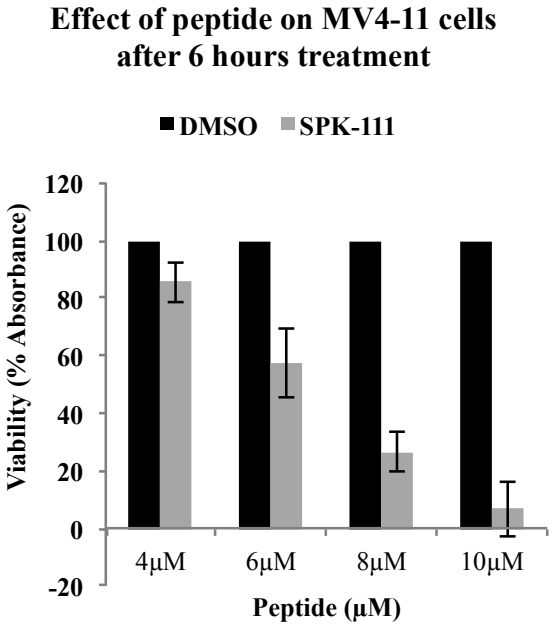
It has been shown previously by our lab that AF9 and AF4 interact at subnuclear foci and that the interaction domains of both the proteins are retained in MLL-AF4 and MLL-AF9 fusion proteins (Erfurth et al., 2004). In order to understand the significance of AF9-AF4 interaction, our lab has developed synthetic peptides (PFWT and SPK-111) that mimic the AF9 binding domain of AF4 and has been shown to disrupt AF4 and AF9 binding both *in vitro* and *in* vivo (Srinivasan et al., 2004). Moreover, the peptide has shown toxicity towards MLL rearranged leukemic cells lines carrying MLL-AF4 and MLL-AF9 fusions, suggesting the importance of AF4-AF9 interaction in the survival of these leukemic cells (Bennett et al., 2009). Hence, we wanted to determine the significance of AF9-AF4 interaction in MLL-rearranged leukemic cell lines by looking for the effect of SPK-111 on MLL target genes, including *HOXA9*, *MEIS1* and *MYC*. We also looked for the mechanism by which the peptide affected the expression of MLL fusion protein target genes.

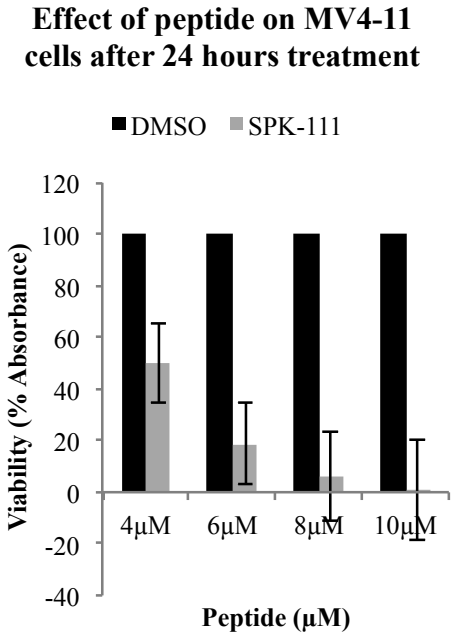
MLL rearranged leukemic cells lines are more sensitive to SPK-111 than non-MLL rearranged

We tested the cell viability of MLL rearranged human myeloid leukemia cell lines, MV4-11 and MOLM13 expressing MLL-AF4 and MLL-AF9 fusion genes, respectively, and the MLL germline human myeloid leukemia cell line, K562 carrying BCR-ABL fusion gene. Cell viability was measured by Cell Titer Glo assay (Promega).

Figure 14. Cell Viability Assay using Cell Titer Glo Luminescence

A





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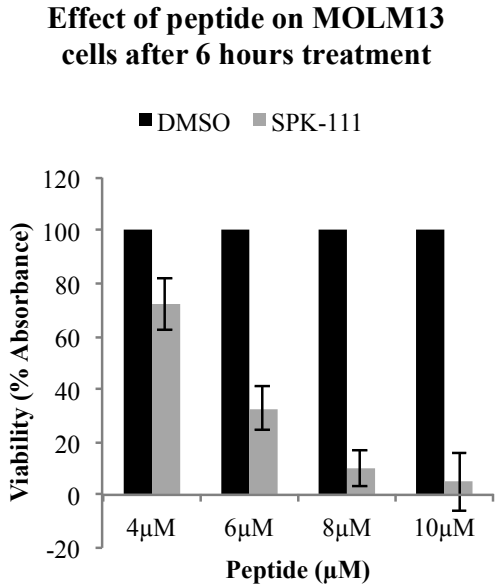
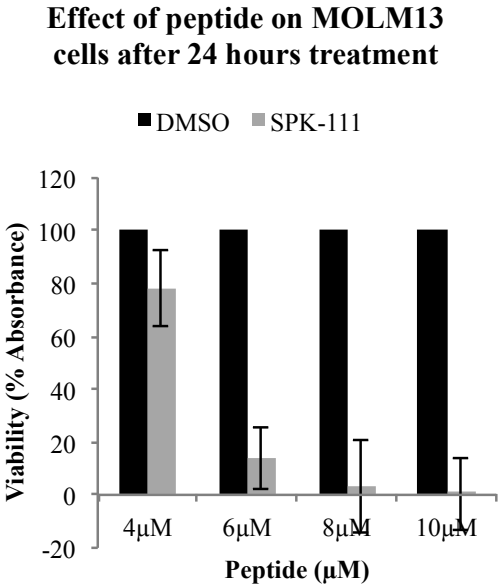
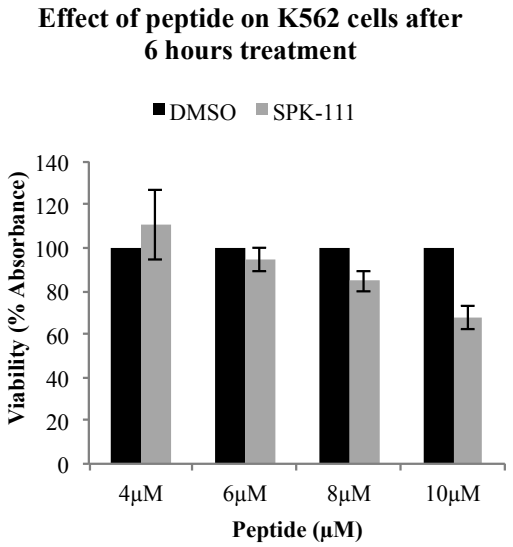
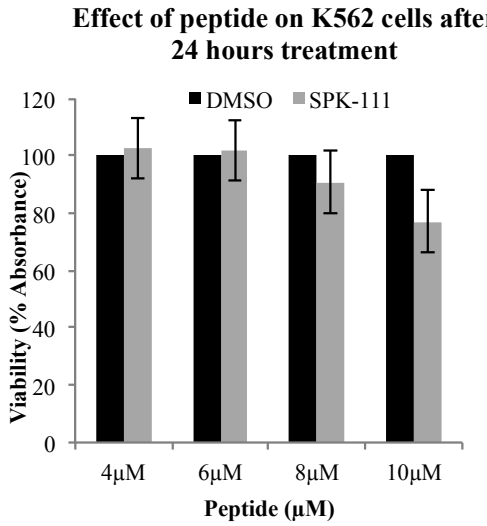




C





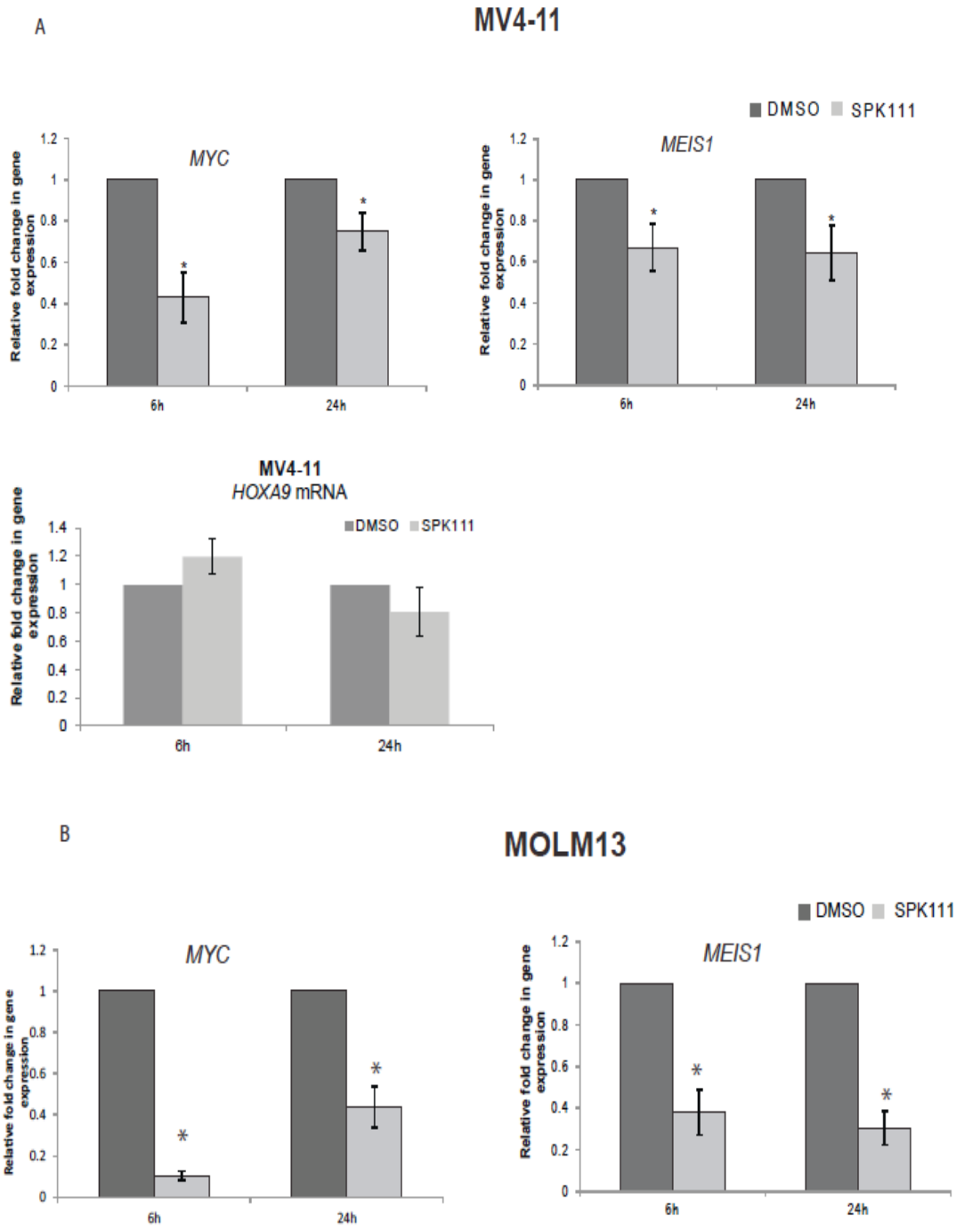
(A) MV4-11, (B) MOLM13 and (C) K562 leukemic cell lines were plated at a concentration of 0.5 x 10^6 cells/ml and treated with 4, 6, 8 and 10μM of SPK-111or DMSO control. Cells were incubated at 37^0C for 6 and 24 hours. Cell viability was assessed using Cell Titer Glo Luminescence reagent.

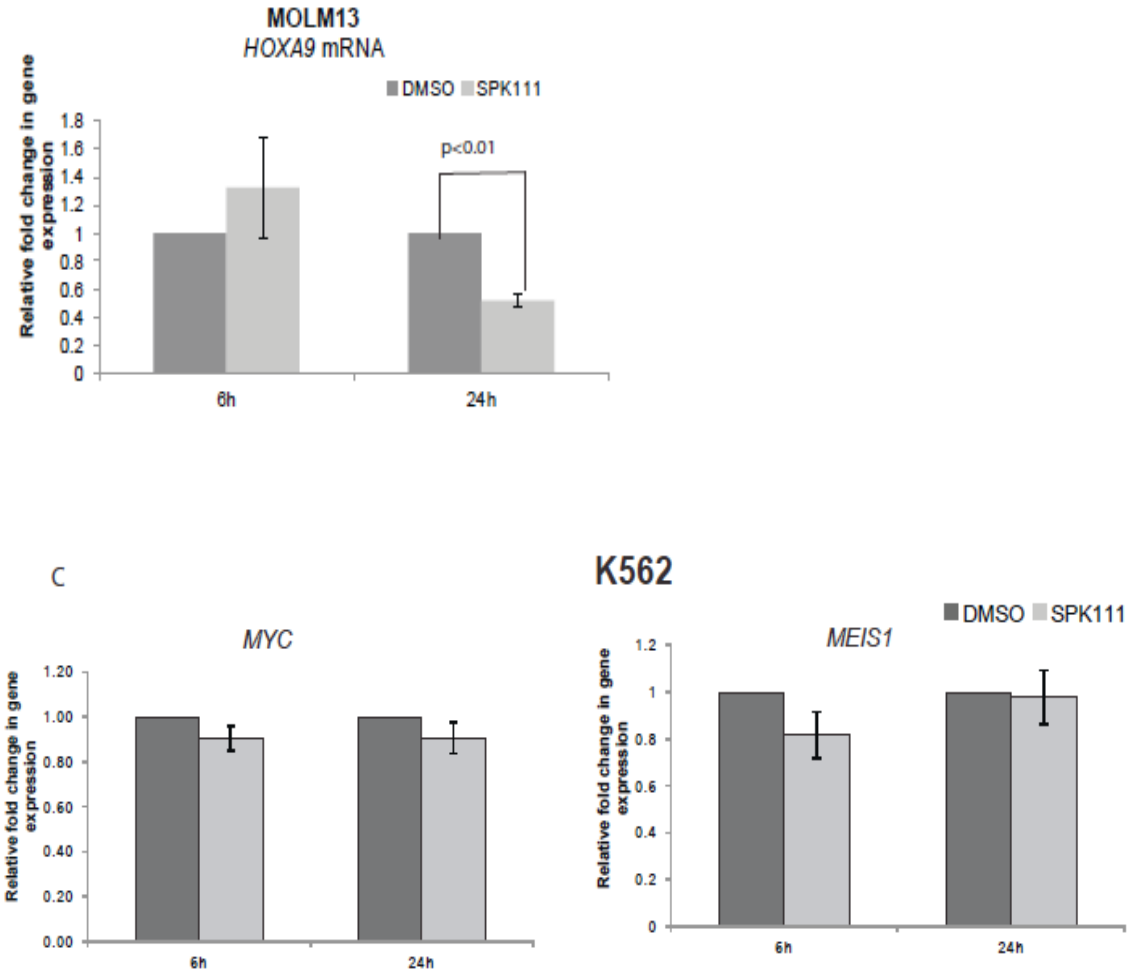
Each cell line was treated with varying concentrations of SPK-111 for 6 and 24 hours. Cell viability was calculated as the average luminescence compared to DMSO controls. As shown in Figure 14A and B, MV4-11 and MOLM13 cell lines are sensitive to SPK-111 with an IC_{50} of approximately 6μM, whereas, the K562 cell line was relatively resistant to SPK-111 (Figure 14C).

SPK-111 treatment inhibits the expression of MLL fusion protein target genes

Since SPK-111 showed cytotoxicity specifically against MLL rearranged leukemias, we next wanted to test whether SPK-111 treatment causes any changes in the expression levels of MLL fusion protein target genes, including *HOXA9, MEIS1* and *MYC*. We treated the leukemia cells lines, MV4-11, MOLM13 and K562 with 6μM SPK-111 for 6 and 24 hours. After treatment, total RNA was extracted and reverse transcribed to cDNA. cDNA was used to perform qRT-PCR using SYBR Green. As shown in Figure 15A and B, there was significant reduction in the mRNA levels of MLL fusion protein target genes, *MEIS1* and *MYC* in both MV4-11 and MOLM13 cell lines after 6 and 24 hours of treatment with SPK-111. On the other hand, mRNA levels of *MEIS1* and *MYC* were not significantly altered in K562 cell line after SPK-111 treatment for 6 and 24 hours (Figure 15C). However, mRNA levels of another well-characterized MLL target gene, *HOXA9* were only marginally affected by SPK-111 in MV4-11 and MOLM13 cell lines (Figure 15A and B), suggesting a differential effect of the peptide on MLL fusion protein target genes. Surprisingly, *HOXA9* transcripts could not be detected in the K562 cell line by qRT-PCR, probably because *HOXA9* gene is not well expressed in this cell

Figure 15. Effect of SPK-111 treatment on the mRNA levels of MLL fusion protein target genes





(A) MV4-11, (B) MOLM13 and (C) K562 cell lines were treated with 6μM SPK-111 or DMSO control for 6 and 24 hours. After treatment equal number of viable cells were collected and total RNA was extracted. RNA was reversed transcribed to cDNA and analyzed for *MEIS1, MYC* and *HOXA9* mRNA levels after normalization to the *β2M* gene expression. The experiments were performed in triplicate and each experiment was independently repeated three times. Error bars are the standard deviations of the mean from the three independent experiments. (*$p < 0.05$)

line.

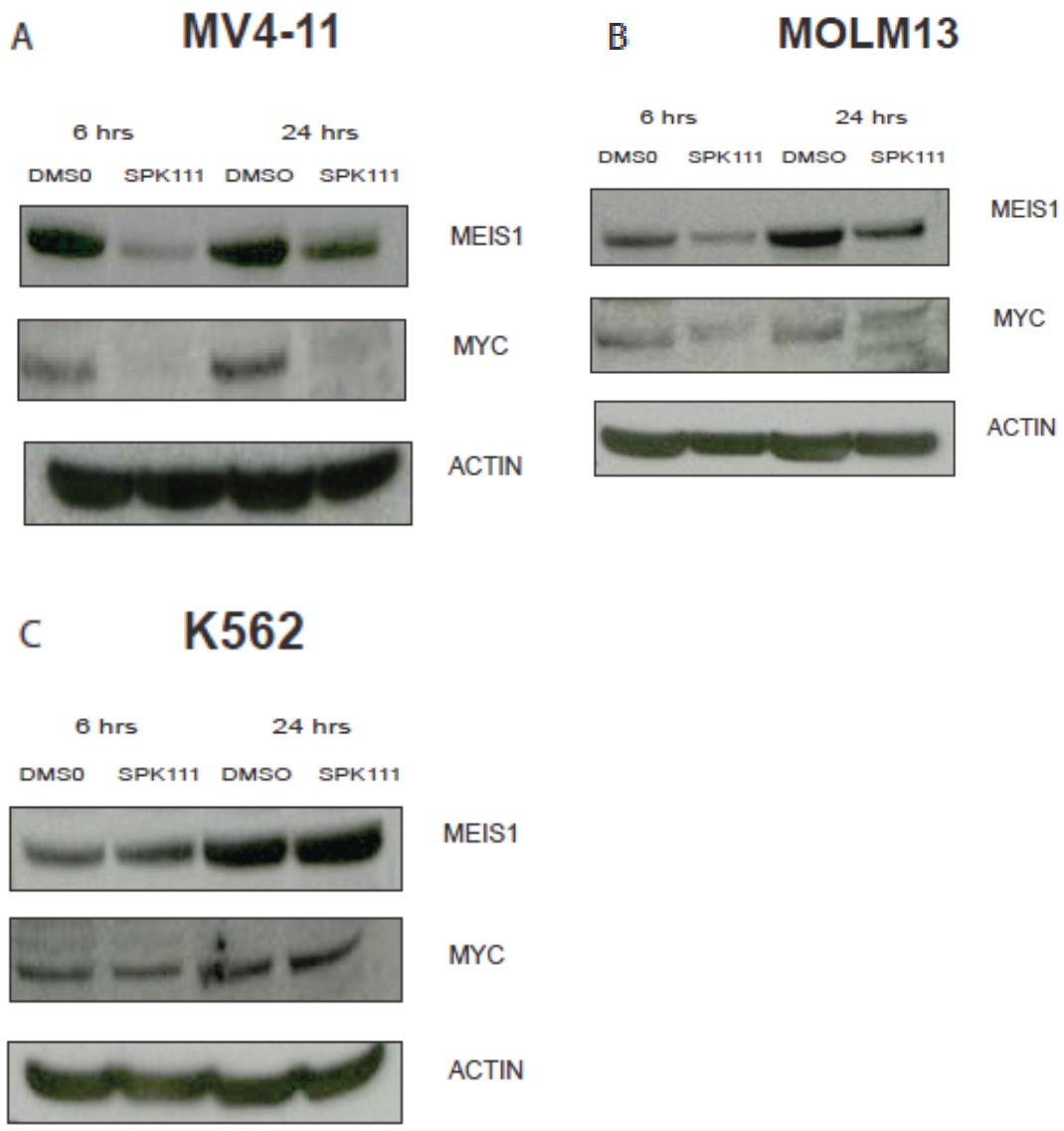
SPK-111 treatment decreases the protein expression of MLL fusion protein targets

As for mRNA analysis, we treated MV4-11, MOLM13 and K562 cell lines with 6μM of SPK-111 for 6 and 24 hours to look for its effect on protein expression levels of MEIS1 and MYC. After treatment, equal number of viable cells were lysed and analyzed for protein expression by western blotting for MEIS1 and MYC. As shown in Figure 16A and B, protein levels of both MEIS1 and MYC were reduced after SPK-111 treatment in MV4-11 and MOLM13 cell lines. On the other hand, protein levels of MEIS1 and MYC were unaffected in the germline MLL K562 cell line after SPK-111 treatment (Figure 16C). β-actin was used as a loading control.

SPK-111 treatment induces cell cycle arrest in S phase

It has previously been reported that Meis1 knock down induces G_0/G_1 cell cycle arrest in a cell line derived from leukemic Mll-Af9 mouse (Kumar et al., 2009). As we have seen that SPK-111 treatment results in the reduced MEIS1 expressions both at the transcript and protein levels in human leukemic cell lines, we decided to look at the effect of SPK-111 treatment on the cell cycle. As shown in Figure 17, instead of G_0/G_1 cell cycle arrest, MOLM13 cells undergo S phase arrest after treatment with 6μM of SPK-111 for 24 hours. The observed changes in cell cycle dynamics are unlikely to be directly attributable to diminished levels of MEIS1.

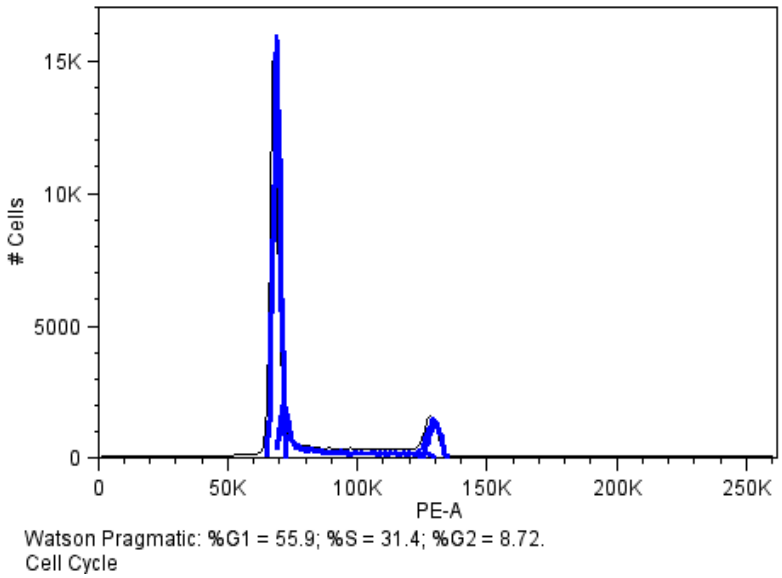
Figure 16. Effect of SPK-111 treatment on the protein expression of MLL fusion protein targets.



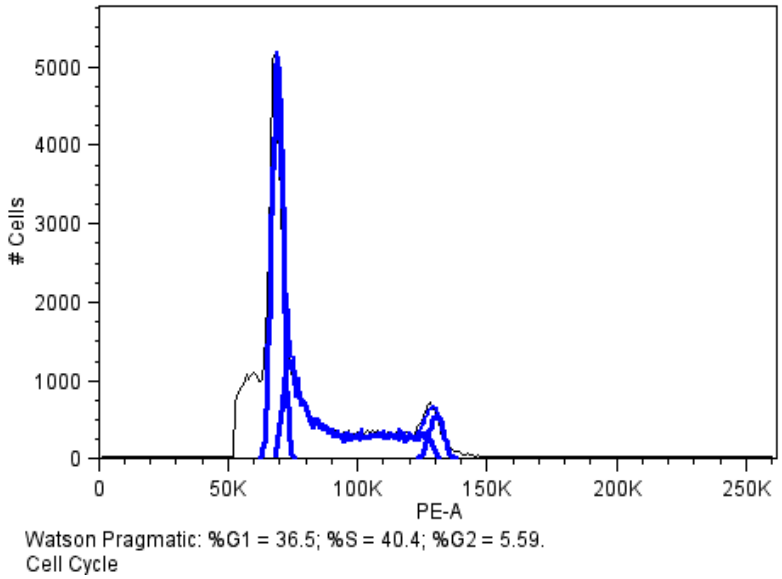
(A) MV4-11, (B) MOLM13 and (C) K562 cell lines were treated with 6μM SPK-111 or DMSO control for 6 and 24 hours. After treatment equal number of viable cells were lysed and analyzed for the protein expression of MLL fusion protein targets by western blotting against MEIS1 and MYC. β-actin was used as a loading control.

Figure 17. Effect of SPK-111 treatment on the cell cycle of MOLM13 cells

A



B



MOLM13 cells were treated with DMSO (A) control or 6μM of SPK-111 (B) for 24 hours. Cells were then fixed with ethanol and stained with propidium iodide for cell cycle analysis using FACS. The percentage of cells in G1, S and G2 phase were determined by Watson Pragmatic cell cycle analysis model.

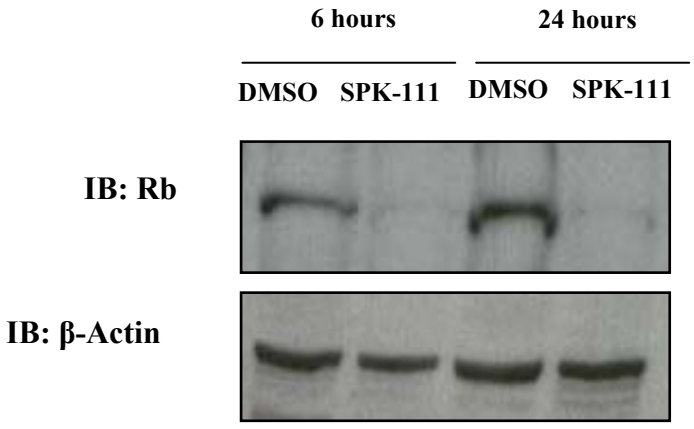
SPK-111 treatment causes reduced levels of Retinoblastoma protein

Argiropoulos et al have reported that G_0/G_1 cell cycle arrest induced by Meis1 knock down is accompanied by decreased phosphorylation of retinoblastoma protein (Argiropoulos et al., 2010). Moreover, phosphorylation of retinoblastoma protein is known to be required for the release of E2F transcription factors that then drive the exit from G_0/G_1 phase to enter S phase of the cell cycle. Furthermore, we have seen S phase cell cycle arrest suggesting that the retinoblastoma protein may no longer be able to inhibit E2F transcription factors required for S phase entry. Therefore, we tested whether S phase arrest caused by SPK-111 treatment was also accompanied by any changes in retinoblastoma protein levels or its phosphorylation status. As shown in Figure 18, there is marked reduction in the total levels of retinoblastoma protein after 6μM SPK-111 treatment for 6 and 24 hours. The absence of retinoblastoma protein may be responsible for promoting cells to enter S phase.

SPK-111 treatment decreases the phosphorylation of serine 2 of RNA PolII C-terminal domain at the *MYC* promoter and downstream regions

Our lab has previously shown that AF4 and AF9 form a stable protein complex in the nucleus and that the interaction domains of both the proteins are retained even after their fusion with the MLL protein in acute leukemias (Erfurth et al., 2004). It has subsequently been shown that AF4 and AF9 exist in a large multiprotein complex containing P-TEFb and therefore are linked to transcriptional elongation (Bitoun et al., 2007). Moreover, our lab has shown that the AF4 mimetic peptide, SPK-111 is capable

Figure 18. Effect of SPK-111 treatment on the protein expression of Retinoblastoma protein

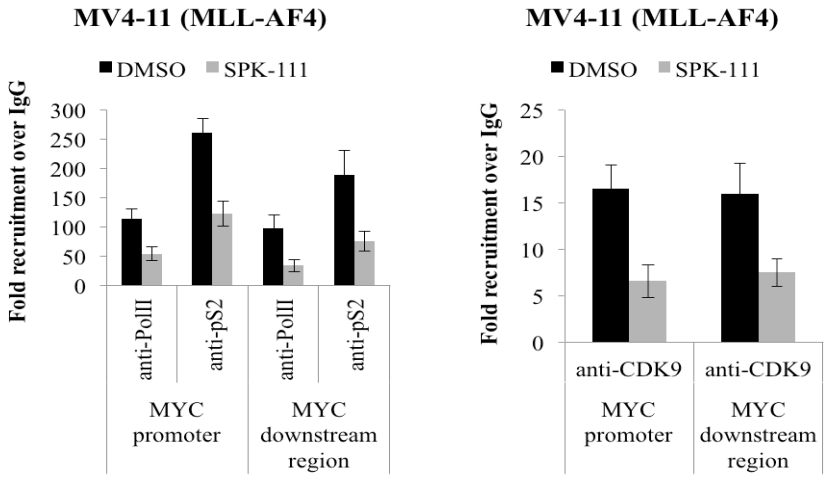


MOLM13 cells were treated with 6μM of SPK-111 or DMSO control for 6 and 24 hours. Equal numbers of viable cells in both the samples were lysed after treatment and SDS-PAGE was run. Samples were analyzed for the expression of retinoblastoma protein by western blotting.

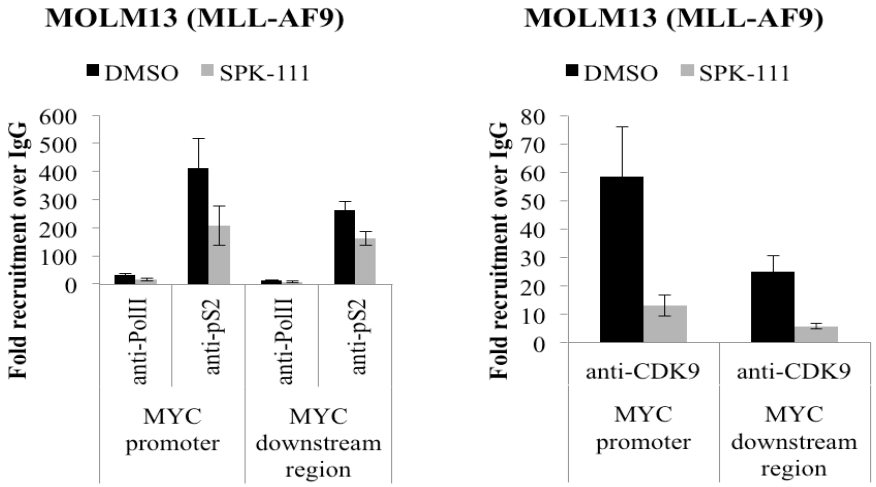
of disrupting AF4-AF9 interactions *in vitro* and *in vivo* (Srinivasan et al., 2004). Based on these results, we hypothesized that SPK-111 affects the transcriptional elongation complex in leukemia cell lines. P-TEFb is composed of CDK9 and Cyclin T1/2, and phosphorylates the Ser 2 residue of the large subunit of RNA PolII, which then results in the productive elongation during transcription. Thus, we analyzed the effects of SPK-111 on the recruitment of CDK9 and phosphorylated status of Ser2 of RNA PolII at *MEIS1* and *MYC* promoters and downstream regions using ChIP. We did not analyze *HOXA9*, since there were no significant changes in the mRNA levels following treatment with SPK-111. As shown in Figure 19A and B, SPK-111 treatment resulted in the decreased recruitment of CDK9 and phosphorylated form of Ser2 of RNA PolII at the *MYC* promoter and downstream regions in both MV4-11 and MOLM13 cell lines, respectively. On the other hand, there was no significant difference in the recruitment of CDK9 and phosphorylation status of Ser2 of CTD of RNA PolII at *MEIS1* promoter and downstream regions in both MV4-11 and MOLM13 cell lines (Figure 19C and D). Taken together, these data suggest that SPK-111 affects the expression of MLL target genes by at least two mechanisms, one of which appears to involve the process of transcriptional elongation.

Figure 19. Effect of SPK-111 treatment on the recruitment of CDK9 and phosphorylation of Ser 2 of CTD of RNA PolII at *MYC* and *MEIS1* promoter and downstream region

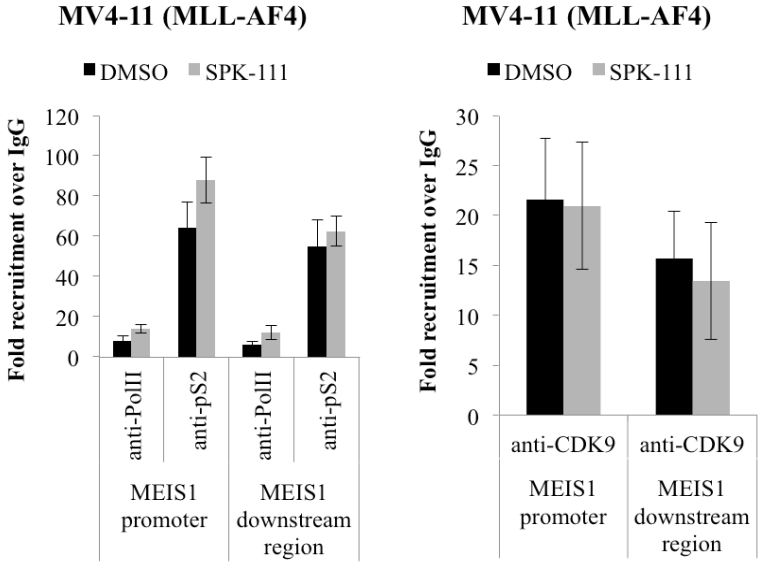
A



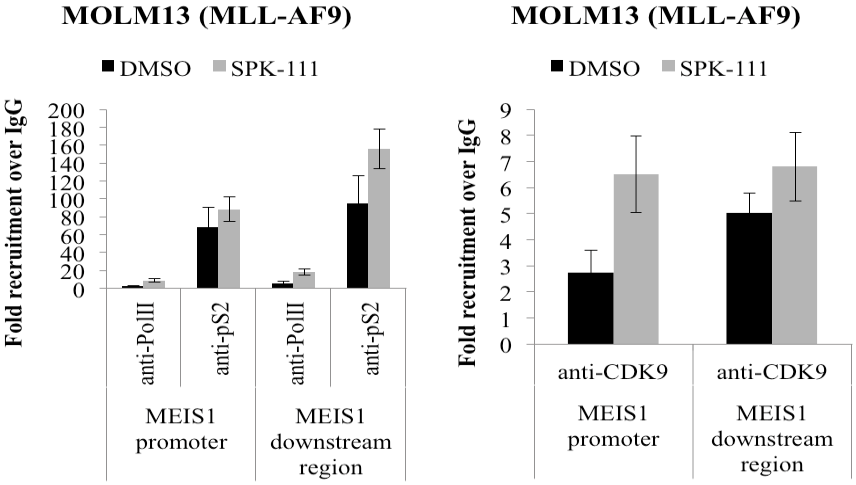
B



C



D



ChIP analysis: Cells were treated with 6μM SPK-111 or vehicle (DMSO) control for 6 hours. After treatment equal number of viable cells were fixed using formaldehyde and ChIP assays were performed with an indicated antibodies. Immunoprecipitated DNA was then amplified by qRT-PCR using primers for *MYC* promoter and downstream region in MV4-11 (A), and MOLM13 (B) cells, and *MEIS1* promoter and downstream region in MV4-11 (C) and MOLM13 (D) cells.

AIM3: To determine the effect of various AF9 point mutants on the interaction status between AF9 and its binding partners, including AF4, DOT1L and CBX8

Mutants of AF9 predicted to disrupt binding

Structural studies done by the laboratory of Dr. John Bushweller at the University of Virginia and genetic studies done previously in our lab have identified residues within the C-terminal domain of AF9, which might be important for the interaction between AF9 and its binding partners, including AF4, DOT1L and CBX8. They include

1) E506R – Introduction of a positive charge in an acidic grove
2) I538A – Predicted to disrupt folding
3) D544R – Disruption of a salt bridge formed between AF4 and AF9
4) K557E – Charge reversal on a surface formed by two parallel helices
5) L562A – Predicted to disrupt local fold in helix 3
6) S565D – Phosphorylation mimic of a possible phosphorylation site

We used the above-mentioned AF9 mutants in co-immunoprecipitation experiments with an aim of determining the effects on interactions with AF4, DOT1L or CBX8. The results are predicted to help determine the importance of the protein interactions in MLL-AF9 mediated leukemogenesis. In these interaction studies we used a C-terminal fragment of AF9 (aa 376-568) that is most commonly found in leukemia patients with MLL-AF9 translocations. We used a FLAG tagged fragment of wild type (WT) or mutant AF9.

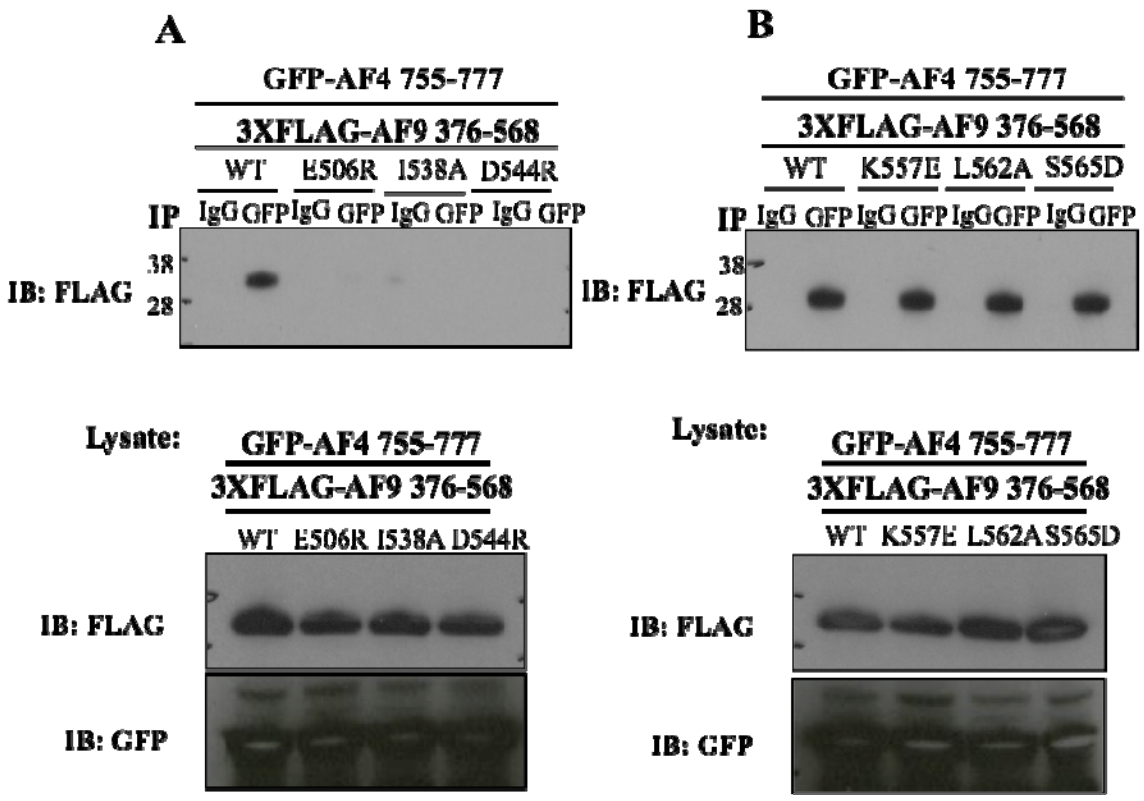
Interaction studies using AF4 (aa755-777) and mutants of AF9 (aa 376-568)

Although the minimal AF9 binding region of AF4 includes amino acids from 768-778, additional upstream residues located in a conserved motif from amino acids 755-777 have been shown to stabilize the interaction (unpublished data from our lab). Therefore, we performed co-immunoprecipitation experiments using HEK293T cells transiently co-transfected with gene expression vectors to look for the effect of various AF9 (aa 376-568) point mutantions on AF9's interaction with the fragment of AF4 containing residues 755-777. As shown in Figure 20A and B, AF9 (aa 376-568) mutants E506R, I538A and D544R completely abrogated its binding with AF4 (755-777), whereas K557E, L562A and S565D have no measurable effect on the interaction with AF4 (755-777) by co-immunoprecipitation followed by western blotting.

Interaction studies using AF4 (RI3A-) and mutants of AF9 (aa 376-568)

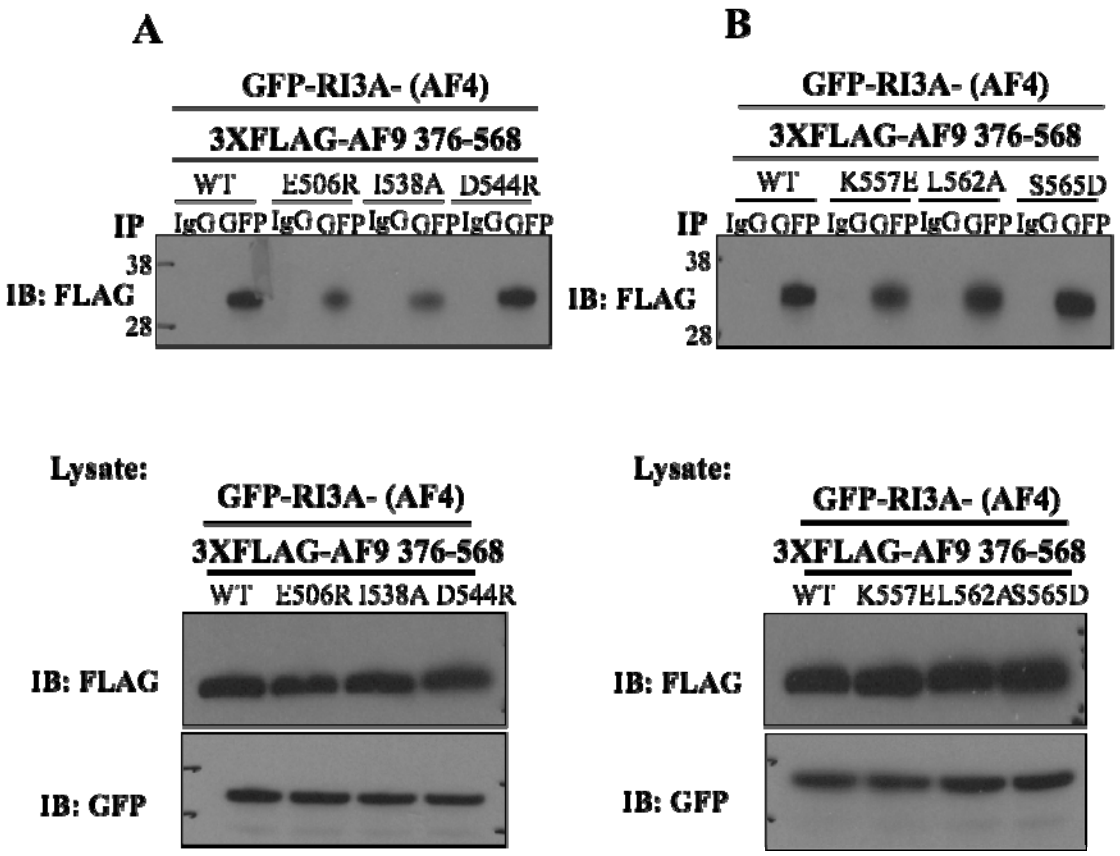
Similarly, using co-immunoprecipitation experiments we tested the effect of AF9 mutants on the association between AF9 (aa 376-568) and a larger fragment of AF4 (RI3A-) from amino acids 647-871, lacking the nuclear localization signal. HEK293T cells were transiently co-transfected with wild type FLAG-AF9 (aa 376-568) or mutants and AF4 (RI3A-) for 48 hours. After transfection, cells were lysed and co-immunoprecipitation was performed. As shown in Figure 21A and B, only E506R and I538A mutants of AF9 showed reduced binding with AF4 (RI3A-), whereas, other mutants have no observable effect on the association between AF9 and AF4 (RI3A-). These results suggest that although smaller fragment of AF4 (755-777) is sufficient for

Figure 20. Interaction studies between AF4$_{755-777}$ and mutants of AF9.



(A) and (B). HEK293T cells co-expressing WT FLAG-AF9$_{376-568}$ or mutant FLAG-AF9$_{376-568}$ and GFP-AF4$_{755-777}$ were immunoprecipitated using an anti-GFP antibody. Immunoprecipitates were analyzed for the presence of WT or mutant AF9 using an anti-FLAG antibody.

Figure 21. Interaction studies between AF4 (RI3A-) and mutants of AF9



(A) and (B). HEK293T cells co-expressing WT FLAG-AF9$_{376-568}$ or mutant FLAG-AF9$_{376-568}$ and GFP-RI3A (AF4) were immunoprecipitated using an anti-GFP antibody. Immunoprecipitates were analyzed for the presence of WT or mutant AF9 using an anti-FLAG antibody.

binding with AF9, the larger fragment of AF4 (RI3A-) enhances or stabilizes the interaction with AF9 such that binding is restored to AF9 D544R mutant.

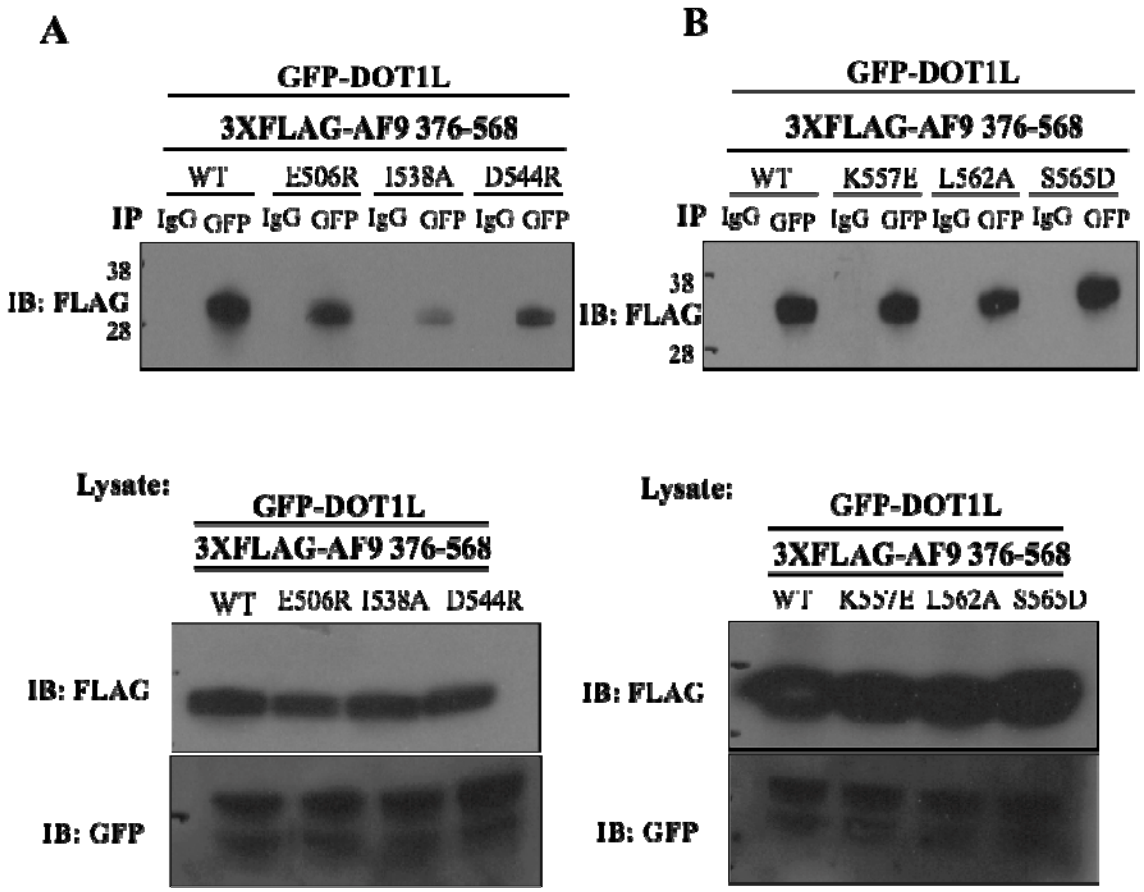
Interaction studies using DOT1L and mutants of AF9 (aa 376-568)

AF9 has been shown to interact with Dot1l and is known to recruit Dot1l at the cis-elements of *ENaCα* promoter regions, which then results in repression due to H3K79 hypermethylation (Zhang et al., 2006; Zhang et al., 2013). In contrast to *ENaCα*, association of AF9 with DOT1L has been reported to be important for MLL-AF9 mediated gene expression and leukemogenesis in which H3K79 methylation is associated with an active state of gene expression (Chang et al., 2010; Nguyen et al., 2011). Therefore, in order to have in depth knowledge of the importance of the AF9-DOT1L association in MLL-AF9 leukemia, we also looked for the effect of various AF9 (aa 376-568) point mutants on their interaction with DOT1L. Similarly, HEK293T cells co-expressing wild type FLAG-AF9 (aa 376-568) or mutants and GFP-DOT1L were lysed after 48 hours of transfection to perform co-immunoprecipitation. As shown in Figure 22A and B, only the I538A mutant of AF9 interfered with the association with DOT1L, whereas the other mutations had little measurable affect on the association with DOT1L.

Interaction studies using CBX8 and mutants of AF9 (aa 376-568)

Among all the CBX family members, only CBX8 has been previously shown by our laboratory to interact with AF9 (Hemenway et al., 2001). Moreover, others have also shown the presence of CBX8 in the AF9 purified complexes using affinity purification

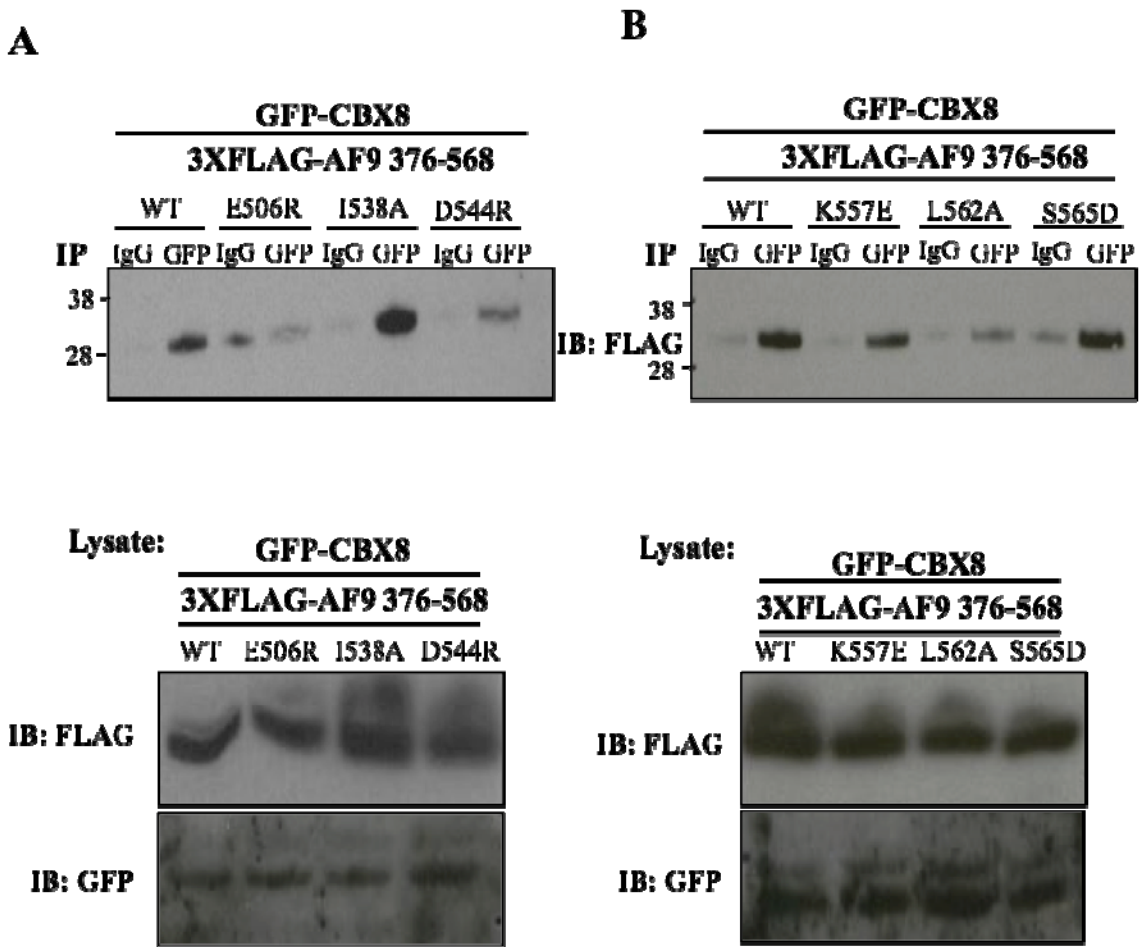
Figure 22. Interaction studies between DOT1L and mutants of AF9



(A) and (B). HEK293T cells co-expressing WT FLAG-AF9$_{376-568}$ or mutant FLAG-AF9$_{376-568}$ and GFP-DOT1L full length were immunoprecipitated using an anti-GFP antibody. Immunoprecipitates were analyzed for the presence of WT or mutant AF9 using an anti-FLAG antibody.

studies among all the CBX family members (Biswas et al., 2011). Although CBX8 belongs to the repressive Polycomb PRC1 complex, it has recently been shown to be important for MLL-AF9 mediated leukemogenesis (Tan et al., 2011). Hence, better understanding of the importance of AF9-CBX8 association is required. With that in mind, we determined the effect of various AF9 (aa376-568) point mutations on the association between AF9 and CBX8. HEK293T cells were co-transfected with wild type FLAG-AF9 (aa 376-568) or mutants and GFP-CBX8 for 48 hours. After transfection cells were lysed and co-immunoprecipitation experiments were performed. As shown in Figure 23A and B, AF9 mutants E506R, D544R and L562A exhibited reduced interactions with CBX8, whereas, I538A, K557E and S565D showed no measurable effect on the interaction between AF9 and CBX8. These results showed that the L562A mutant of AF9 specifically disrupts the association between AF9 and CBX8 and not other AF9 binding proteins. This mutant could be beneficial to look for the importance of the association of AF9 and CBX8 in MLL-AF9 leukemogenesis and normal hematopoiesis. The results of all of the mutational analyses are summarized in Table 3.

Figure 23. Interaction studies between CBX8 and mutants of AF9



(A) and (B). HEK293T cells co-expressing WT FLAG-AF9$_{376-568}$ or mutant FLAG-AF9$_{376-568}$ and GFP-CBX8 full length were immunoprecipitated using an anti-GFP antibody. Immunoprecipitates were analyzed for the presence of WT or mutant AF9 using an anti-FLAG antibody.

Table 3. Summary of Co-IP studies using various mutants of AF9

AF9 376-568	RI3A- (AF4)	AF4 755-777	DOT1L	CBX8
WT	+++	+++	+++	+++
E506R	+	-	++	-
I538A	+	-	+	+++
D544R	+++	-	+++	+
K557E	+++	+++	+++	++
L562A	+++	+++	+++	+
S565D	+++	+++	+++	+++

- No binding
+ Weak binding
++ Moderate Binding
+++ Strong Binding

CHAPTER 4

DISCUSSION

In MLL rearranged leukemias, over 50 different fusion partners have been reported for the *MLL* gene. *MLL* gene rearrangements give rise to chimeric proteins that contain the N-terminus of MLL and C-terminus of the fusion partner. Among all partners, *AF9* is one of the most common (Krivtsov and Armstrong, 2007). AF9 was first shown by our laboratory to interact with another common MLL fusion partner, *AF4* (Erfurth et al., 2004). Moreover, the interaction domains of both proteins are retained even after their fusion with MLL. Subsequently, others have shown that AF4 and AF9 exist in a multiprotein complex containing positive transcription elongation factor b (P-TEFb), thereby linking both AF4 and AF9 to the regulation of transcription elongation (Bitoun et al., 2007). Furthermore, expression of either MLL-AF4 or MLL-AF9 causes the constitutive expression of MLL target genes and bypasses the requirement of normal regulatory mechanisms (Yokoyama et al., 2010). In addition, AF9 has been shown to bind with H3K79 methyltransferase, DOT1L that predominately associates with the actively transcribed genes (Steger et al., 2008; Zhang et al., 2006). Recently, AF9-DOT1L interaction has been shown to be important for MLL-AF9 mediated leukemogenesis (Chang et al., 2010; Nguyen et al., 2011). On the contrary, Af9-Dot1l interaction has been shown to be associated with the repressed state of the *ENaCα* gene in the murine renal collecting duct and *Tbr1* gene in the subventricular zone of mouse

forebrain where H3K79 hypermethylation of the promoter regions of the associated genes is found (Buttner et al., 2010; Zhang et al., 2006).

Apart from interacting with the activators of transcription, including AF4-P-TEFb and DOT1L, AF9 has also been shown by our laboratory to directly interact with two known putative repressors of transcription, CBX8 and BCoR (Hemenway et al., 2001; Srinivasan et al., 2003). Affinity purification studies have also confirmed the association of AF9 with AF4, DOT1L, CBX8 and BCoR (Biswas et al., 2011). Moreover, AF9 through its minimal C-terminal domain of 94 amino acids (called AHD domain) interacts with all of these proteins. The minimal C-terminal domain of AF9, when fused to MLL, has been shown to be sufficient to cause leukemia in mouse models (Chen et al., 2008; Cozzio et al., 2003). This indicates the importance of the protein interactions involving the C-terminal domain of AF9 in both normal and abnormal cellular processes.

Since AF9 can interact with wide variety of proteins, including activators and repressors of transcription, in AIM1, we hypothesized that AF9 is an important component of several distinct sub-complexes, each of which has a unique function. Here, we show through size exclusion chromatography that AF9 elutes in molecular weight fractions ranging in size from 158-400KDa. Polycomb-group repressive complex PRC1 components, including CBX8 and RING1b are also found in these fractions. These results suggest that instead of forming one megadalton complex that includes all AF9 binding proteins, AF9 must form distinct sub-complexes containing only discrete subsets of proteins. Similarly using mass spectroscopy analysis, ENL, a close homolog of AF9 and another fusion partner of MLL has also been shown to interact with the same array of

proteins (Mueller et al., 2007). Moreover, the same group showed through gel filtration studies that distinct ENL containing complexes range in size from 230-600KDa, thus again suggesting the occurrence of discrete sub-complexes of these proteins rather than a megadalton complex (Mueller et al., 2007).

We further showed the association of AF9 with components of the Polycomb-group repressive complex PRC1, including CBX8, BMI1, RING1b and RING1a. Interestingly, we also showed that the AF9-PRC1 complex did not contain other known AF9 interacting proteins, including AF4, DOT1L or BCoR, suggesting the presence of distinct AF9-PRC1, AF9-AF4-P-TEFb, and AF9-DOT1L complexes. Moreover, in the context of MLL, a three-step model has been proposed for the activation of MLL target genes. In the first step, wild type MLL is recruited at the target genes and results in transcriptionally poised state. In the second step, the AF9-AF4-P-TEFb complex is recruited by an unknown mechanism, which then facilitates transcriptional elongation. In the final third step, DOT1L displaces AF4 and binds to AF9, leading to H3K79 methylation, which results in the maintenance of transcriptional memory. According to this model, sequential recruitment of AF4 and DOT1L mediated by AF9 plays a major role in the activation of MLL target genes (Yokoyama et al., 2010). Our results show that AF9 is in distinct complexes supporting the model that it serves as a scaffold protein capable of the sequential recruitment of its binding proteins to target genes.

More specifically, we showed through knock down studies that CBX8 is required for the interaction between AF9 and other PRC1 components. It was also initially reported by our laboratory through a yeast two-hybrid assay that AF9-CBX8-RING1b

forms a trimeric complex and that CBX8 bridges interaction between AF9 and RING1b (Hemenway et al., 2001). Five different CBX proteins have been identified in mammals- CBX2, CBX4, CBX6, CBX7, and CBX8. The proteins share a homologous N-terminal chromodomain and a C-terminal COOH-box (Morey et al., 2012). The N-terminal chromodomain mediates interactions with methylated histone H3 and the different CBX proteins recognize different methylation marks (Bernstein et al., 2006). The COOH-box promotes binding to the Ring-finger proteins RING1a and RING1b, which, in turn, mediate monoubiquitination of histone H2A at lysine 119- a repressive mark (de Napoles et al., 2004). Of the five CBX proteins, only CBX8 is found in complexes containing AF9 or its homolog ENL (Biswas et al., 2011; Mueller et al., 2007). Our laboratory has reported that the AF9 binding domain of CBX8 is not conserved among the CBX proteins likely accounting for the specificity of the CBX8-AF9 interaction (Hemenway et al., 2001).

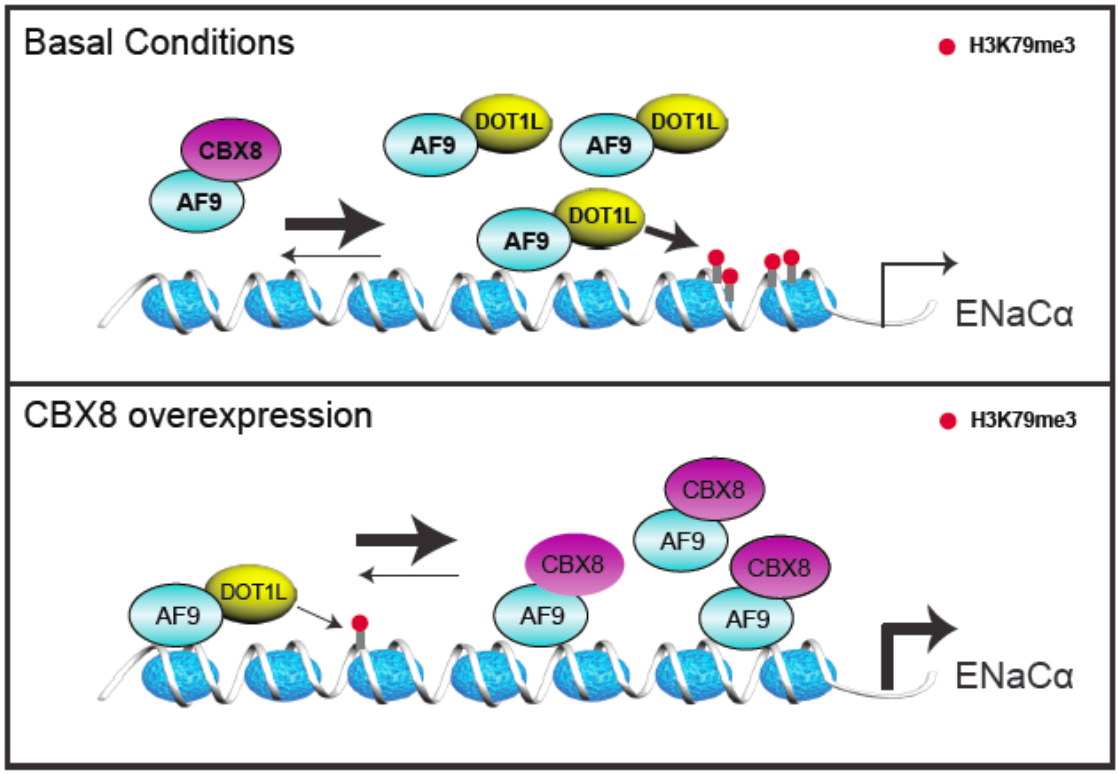
While CBX8 is the only CBX protein that binds AF9, AF9 in turn binds directly to at least three other proteins, AF4, DOT1L, and BCoR. Remarkably, all of these proteins bind to a 94 amino acid domain at the C-terminus of AF9 designated the AHD. Recently, our collaborators have shown that the binding of CBX8, AF4, DOT1L, and BCoR to AF9 is mutually exclusive. Moreover, the AHD itself is intrinsically disordered but adopts a unique structural conformation upon binding to each of the four proteins (Leach et al., 2013). In this report, we provide evidence that shifts in the equilibrium of different AF9 complexes directly affects AF9 function. Specifically, we show that changes in the expression of CBX8 alters the activity of another AF9-binding protein,

DOT1L, whose enzymatic activity is dependent on AF9 binding. In human embryonic kidney cells methylation of H3K79 by DOT1L at the promoter regions represses *ENaCα* gene expression, a known target of AF9-DOT1L (Zhang et al., 2006). It has recently been reported that AF9 binds to the cis-elements on the promoter regions of *ENaCα* and recruits DOT1L, thereby leading to H3K79 hypermethylation and repression (Zhang et al., 2013). We have shown here that CBX8, by competing for AF9 binding, reduces the DOT1L interaction and the associated H3K79 hypermethylation at the *ENaCα* promoter. The reduced H3K79 methylation is then associated with the enhanced expression of the *ENaCα* gene. This leads to the seemingly paradoxical finding that overexpression of a repressive Polycomb-group protein actually up-regulates the expression of a gene. These findings suggest that AF9 is a pleiotropic regulatory protein. By changing the levels of AF9 binding proteins, a shift in the equilibrium of the AF9 complexes differently affects the overall activity of the bound genes.

Based on these results, we propose a model in which, under basal conditions, equilibrium favors the formation of AF9-DOT1L complexes leading to increased H3K79 tri-methylation at the *ENaCα* promoter regions. The increased H3K79 tri-methylation then results in decreased *ENaCα* gene expression. Under conditions in which CBX8 levels increase, equilibrium is shifted to form AF9-CBX8 complexes at the expense of AF9-DOT1L complexes. This results in increased recruitment of CBX8 to the *ENaCα* promoter regions and lower levels of H3K79 methylation. Decreased H3K79 methylation of the *ENaCα* promoter is associated with increased gene expression.

Although we focus on the effects that CBX8 exerts on AF9-DOT1L complexes, it

Figure 24. Model demonstrating the regulation of *ENaCα* gene by modulating the equilibrium between different AF9 complexes.



Under basal conditions, equilibrium favors AF9-DOT1L complexes, causing H3K79 hypermethylation at *ENaCα* promoter regions and their subsequent repression. Under CBX8 overexpression conditions, equilibrium is shifted towards AF9-CBX8 complex, reducing H3K79 methylation at *ENaCα* promoter regions, leading to its increased expression.

is likely that similar effects are exerted on AF9-AF4 and AF9-BCoR complexes. By way of a dynamic equilibrium with its four binding partners, AF9 serves to mediate the activities of protein complexes that function in essential cellular processes ranging from transcription elongation to epigenetic regulation.

Furthermore, to understand the significance of AF4-AF9 interaction, our laboratory mapped domains of AF4 and designed synthetic peptides (PFWT and SPK-111) mimicking the minimal AF9 binding region within AF4. As mentioned before, this peptide initially named as PFWT, showed cytotoxicity against MLL-AF9 and MLL- AF4 expressing leukemic cells lines, suggesting the importance of the AF4-AF9 interaction in the survival of these leukemic cells (Bennett et al., 2009; Srinivasan et al., 2004). Here we show that a newer version peptide named SPK-111 specifically down-regulates the mRNA and protein levels of both MEIS1 and MYC in MLL rearranged leukemic cell lines with no significant effect on the MLL germline leukemic cell line, K652. Moreover, SPK-111 only marginally affected the mRNA levels of the *HOXA9* gene. Although, *HOXA9* is a well-known downstream target of MLL fusion proteins (along with *MEIS1*), it is possible that the peptide only affects a specific subset of MLL target genes.

Since *Meis1* knockdown has been shown to cause G_0/G_1 cell cycle arrest and decreased phosphorylation of the retinoblastoma protein (Argiropoulos et al., 2010), we also looked for the effect of the peptide on the cell cycle. Phosphorylation of the retinoblastoma protein is a pre-requisite for the release of E2F transcription factors which are required for the synthesis of cyclin dependent kinases responsible for entry into the S phase of the cell cycle. Here, we showed that SPK-111 treatment resulted in a S phase

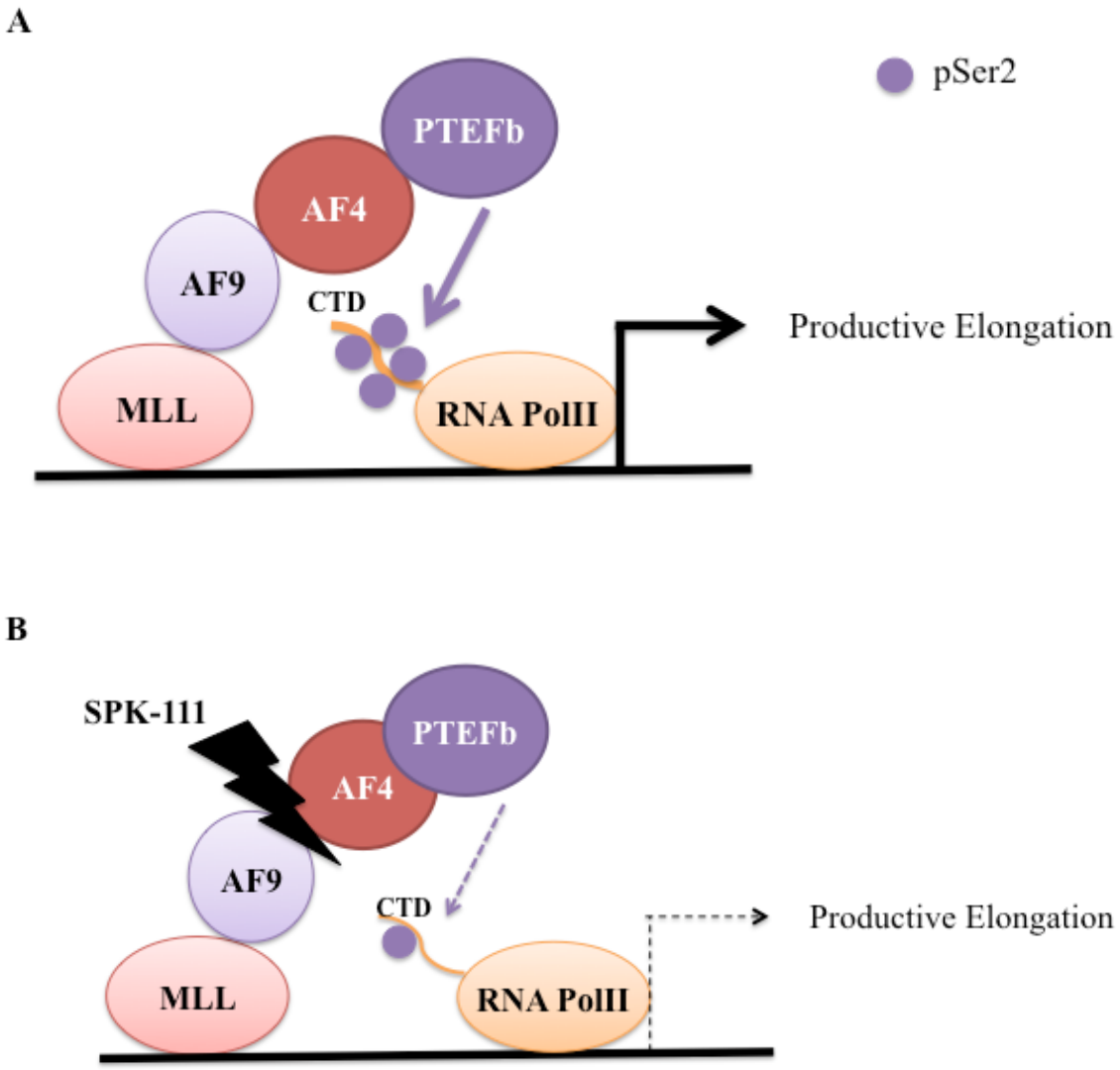
cell cycle arrest that correlated with reduced levels of the retinoblastoma protein. This suggests that because of the reduced levels of the retinoblastoma protein, E2F transcription factors can no longer be inhibited. In turn, uninhibited E2F results in the synthesis of cyclin-dependent kinases those are required for the transition from G_0/G_1 phase to S phase of the cell cycle. Therefore, these data suggest that SPK-111 specifically acts on MLL target genes, including *MEIS1*, which then interferes with the cell cycle progression. It is also equally possible that rather than S phase arrest there may be simply more cells in S phase. Moreover, reduction in the Rb levels would be consistent with the more cells entering the cell cycle. Growth curve would have been important to interpret the results. Nevertheless, the observed cell death may complicate the interpretation even for a growth curve.

To further look for the mechanisms regulating the expression levels of *MEIS1* and *MYC* genes after SPK-111 treatment, we performed ChIP analysis. We hypothesized that SPK-111 disrupts the interaction between AF9 and AF4, which, in turn, can no longer recruit P-TEFb to target genes that are regulated by transcriptional pausing. As mentioned earlier, P-TEFb is composed of CDK9 and Cyclin T1/2 and catalyzes the phosphorylation of Ser2 of the CTD of RNA PolII, which then promotes the elongation of the stalled mRNA transcripts. Therefore, we looked for CDK9 and Ser2 phosphorylation of the CTD of RNA PolII at *MYC* and *MEIS1* promoter and downstream regions. Here, we showed that after SPK-111 treatment, there is reduction in the recruitment of CDK9 and phosphorylated form of the Ser2 of the CTD of RNA PolII. These data suggest that SPK-111 treatment interferes with the stable AF9-AF4-P-TEFb

complex formation at MLL target genes, including *MYC*, which then prevent the productive elongation of the gene, thereby leading to its decreased expression. Similarly, others have also shown the direct correlation between the recruitment of CDK9 and phosphorylated RNA PolII at the promoter regions of MLL target genes. It has been shown that the increased expression of MLL target genes, including *HOXA9* correlates with the increased recruitment of CDK9 and phosphorylated RNA PolII (Mueller et al., 2009; Yokoyama et al., 2010). Surprisingly, we did not see any effect on the fold enrichment of the phosphorylated form of Ser2 of CTD of RNA PolII at the *MEIS1* promoter or downstream regions. One possible explanation is that the promoter or downstream regions of *MEIS1* that we tested were not at the site for PolII stalling. Another explanation could be that other factors are involved in the decreased expression levels of *MEIS1* apart from transcription elongation initiated by P-TEFb. For instance, SPK-111 could be affecting the formation of other stable protein complexes involving AF9 apart from the AF9-AF4-P-TEFb complex that are important for *MEIS1* gene activation.

These results support a model whereby, in MLL-AF9 or MLL-AF4 expressing leukemias, there is aberrant recruitment of P-TEFb via AF4 at MLL target genes. This results in increased phosphorylation of Ser2 of the CTD of RNA PolII. This increased Ser2 phosphorylation further results in uninterrupted productive elongation accounting for constitutive expression of MLL target genes. On the other hand, SPK-111 treatment interferes with the formation of stable transcriptional elongation protein complexes involving AF9-AF4-P-TEFb by competing with AF9 for binding with AF4. Hence, there

Figure 25. Model demonstrating the mechanism of action of SPK-111



(A) MLL-AF9 fusions form a stable transcriptional elongation complex consisting of AF9-AF4-P-TEFb at the MLL target genes, leading to increased pSer2 of RNA PolII and hence increased expression of the target genes. (B) SPK-111 interferes with the transcriptional elongation complex formation by disrupting binding between AF9 and AF4. Hence, results in decreased pSer2 and expression levels of MLL target genes.

is decreased recruitment of P-TEFb at the promoter and downstream regions of MLL fusion protein target genes, which accounts for their decreased expression levels.

Finally, we tested various point mutants of AF9 for their interaction with known-binding partners, including AF4, DOT1L and CBX8 to better understand the functional significance of these interactions. Interactions of AF9 with DOT1L and CBX8 have been shown to play a major role in the MLL-AF9 mediated leukemogenesis (Chang et al., 2010; Nguyen et al., 2011; Tan et al., 2011). Moreover, disrupting AF9-AF4 interactions also affected the transforming capability of MLL-AF9 transduced mouse bone marrow cells (unpublished data from our lab). Thus, these interaction studies of AF9 mutants need to be further explored for their effects on MLL-AF9 mediated leukemogenesis and on normal hematopoiesis.

Moreover, the interaction studies involving the C-terminus of AF9 and CBX8 has been shown by us and others to have important implications on the regulation of *ENaCα* gene and on MLL leukemias, it would be interesting to know the expression status of CBX8 in various cancers. Microarray studies could be done to look for the CBX8 gene expression in various cancer cell lines including MLL leukemic cell lines. This would give us better understanding of the oncogenic activity of CBX8 and help us to understand the various regulatory networks involved in the regulation of CBX8 expression. Although, CBX8 is part of a repressive PRC1 complex, it is involved in the positive regulation of *ENaCα* gene. Therefore, it would be interesting to look for the presence of RING1a/b and its associated repressive chromatin mark, H2AK119 monoubiquitination at the promoter regions of *ENaCα* gene.

Moreover, we have shown that in the presence of CBX8, the DOT1L mediated H3K79 tri-methylation goes down at the promoter regions of *ENaCα*, which is associated with its increased expression levels. On the contrary, DOT1L mediated H3K79 tri-methylation at the body of the gene has been shown to be associated with the active state of the gene. Therefore, it would be important to look for the presence of H3K79 tri-methylation at the body of *ENaCα* gene in the presence and absence of CBX8.

Hence, drug-like molecules that are able to either promote or block the binding of specific AF9 interacting proteins would be predicted to have important effects on disease processes that subvert AF9.

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VITA

The author, Bhavna Malik was born in Ludhiana, India on February 10, 1982 to Sarvish and Rajinder Paul Lumba. Bhavna has two younger brothers, Bharat and Sharad Lumba. She received a Master of Science in Microbiology from Punjab University (Chandigarh, India) in May of 2005 and soon after joined as Pharmacovigilance scientist at Quantum Solutions, India.

In August of 2008, Bhavna joined the Department of Molecular and Cellular Biochemistry at Loyola University Medical Center (Maywood, IL). Shortly thereafter, she joined the laboratory of Dr. Charles Hemenway, where she studied the significance of protein interactions involving AF9 protein, which is one of the most common fusion partners of Mixed Lineage Leukemia (MLL) in acute leukemias.

While studying at Punjab University, Bhavna met Rohit and got married to him in June 2007 and had a baby boy Armaan in July 2011.

After completing her Ph.D., Bhavna will begin a post-doctoral position in the labs of Dr. Tomaz Cierpicki and Dr. Jolanta Grembecka at the University of Michigan, Ann Arbor, where she will focus on mechanistic studies of small molecule inhibitors targeting protein-protein interactions in various cancers.