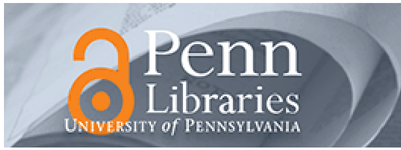

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The Central Role of Menin and Wild-Type MLL in MLL-AF9-Induced Leukemia

Abstract

Chromosomal translocations involving the Mixed Lineage Leukemia (MLL) gene lead to the expression of MLL fusion proteins and acute leukemia. MLL fusion protein-induced leukemia is aggressive, and often refractory to therapy, highlighting the importance of studying the pathogenesis of this disease. MLL fusion proteins upregulate wild-type MLL target genes, including HOX genes, and block hematopoietic differentiation, promoting leukemogenesis. However, the precise mechanism by which MLL fusion proteins upregulate HOX genes and block differentiation has been unclear. My thesis

research shows that leukemia cells expressing the MLL fusion protein MLL-AF9 also express wild-type MLL from the non-translocated MLL allele. Wild-type MLL is required for MLL-AF9-mediated HOX gene upregulation and leukemogenesis. Menin, a

nuclear DNA-binding protein, recruits both wild-type MLL and MLL-AF9 to HOX genes to activate their transcription, highlighting the central role of menin in this disease. We also found that menin blocks MLL-AF9 leukemia cell differentiation by promoting the

expression of the polycomb group protein EZH2. EZH2 represses target genes of the pro-differentiation transcription factor C/EBP&alpha, a previously unknown mechanism for blocking differentiation. The cooperation between EZH2 and trithorax-associated menin is counter to the classical opposing roles of polycomb and trithorax proteins. These findings have uncovered novel insights into how menin and MLL-AF9 upregulate target genes and block leukemia cell differentiation, highlighting novel potential therapeutic targets for this disease.

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THE CENTRAL ROLE OF MENIN AND WILD-TYPE MLL IN MLL-AF9-INDUCED LEUKEMIA

Austin T. Thiel

A DISSERTATION

in

Cell and Molecular Biology

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ABSTRACT

THE CENTRAL ROLE OF MENIN AND WILD-TYPE MLL IN MLL-AF9 LEUKEMIA

Austin T. Thiel

Dr. Xianxin Hua

Chromosomal translocations involving the Mixed Lineage Leukemia (*MLL*) gene lead to the expression of MLL fusion proteins and acute leukemia. MLL fusion protein-induced leukemia is aggressive, and often refractory to therapy, highlighting the importance of studying the pathogenesis of this disease. MLL fusion proteins upregulate wild-type MLL target genes, including *HOX* genes, and block hematopoietic differentiation, promoting leukemogenesis. However, the precise mechanism by which MLL fusion proteins upregulate *HOX* genes and block differentiation has been unclear. My thesis research shows that leukemia cells expressing the MLL fusion protein MLL-AF9 also express wild-type MLL from the non-translocated *MLL* allele. Wild-type MLL is required for MLL-AF9-mediated *HOX* gene upregulation and leukemogenesis. Menin, a nuclear DNA-binding protein, recruits both wild-type MLL and MLL-AF9 to *HOX* genes to activate their transcription, highlighting the central role of menin in this disease. We also found that menin blocks MLL-AF9 leukemia cell differentiation by promoting the expression of the polycomb group protein EZH2. EZH2 represses target genes of the pro-differentiation transcription factor C/EBPα, a previously unknown mechanism for blocking differentiation. The cooperation between EZH2 and trithorax-associated menin

is counter to the classical opposing roles of polycomb and trithorax proteins. These findings have uncovered novel insights into how menin and MLL-AF9 upregulate target genes and block leukemia cell differentiation, highlighting novel potential therapeutic targets for this disease.

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LIST OF ABBREVIATIONS

4-OHT	4-hydroxyl-tamoxifen
ALL	acute lymphoid leukemia
AML	acute myeloid leukemia
BM	bone marrow
C/EBPα	CCAAT enhancer binding protein alpha
CBP	CREB binding protein
CCNA2	cyclin A2
ChIP	chromatin immunoprecipitation
CO	corn oil
Dot1L	disruptor of telomeric silencing 1-like
EZH2	enhancer of zeste homolog 2
GCSFR	granulocyte colony stimulating factor receptor
GMP	granulocyte-macrophage progenitor
GSEA	gene set enrichment analysis
H3K4m2	histone H3 lysine 4 di-methylation
H3K4m3	histone H3 lysine 4 tri-methylation

H3K27m3	histone H3 lysine 27 tri-methylation
H3K79m2	histone H3 lysine 79 di-methylation
HOX	homeobox
HSC	hematopoietic stem cell
IP	immunoprecipitation
KD	knockdown
KO	knockout
LIC	leukemia-initiating cell
LSC	leukemia stem cell
MA9	MLL-AF9
MCSFR	macrophage colony stimulating factor receptor
MLL-FP	MLL fusion protein
MLL	mixed lineage leukemia
PcG	polycomb group
PolII	RNA polymerase II
PRC	polycomb repressive complex
SET domain	Su-(var)3-9 and enhancer of zest domain

TAD	trans-activation domain
TAM	tamoxifen
Trx	trithorax
WBC	white blood cell
WT	wild-type

CHAPTER 1 - INTRODUCTION

Cancers are comprised of a heterogeneous population of cells, and experimental models have demonstrated that only a subset of these cells is capable of propagating oncogenic disease (Visvader, 2011). These tumor-propagating cells must be eradicated in order to effectively treat cancers and prevent recurrence (Mills, 2010). Cancer cells frequently utilize developmental pathways that control the function of normal stem cells, such as self-renewal in order to propagate disease (Mills, 2010). Therefore, understanding how these developmental pathways are deregulated in cancer cells is critical to developing therapies to target these cells in various types of cancer.

Epigenetic regulation of gene expression is critical for development

The development of multicellular organisms is a complex process, requiring cell fate decisions, which result in the generation and maintenance of diverse cell types. The generation of these cell types from stem and progenitor cells that contain the same genetic material is regulated at the level of gene expression (Schuettengruber et al., 2011). The maintenance of an expression pattern specific to a particular cell lineage, and the ability to pass on this expression pattern to daughter cells is critical to maintaining specialized cell types and proper development. The establishment and maintenance of this transcriptional program is governed by epigenetics (Mills, 2010). Epigenetics, broadly defined as the heritable pattern of gene expression and/or cell phenotype not related to changes in DNA sequence, is controlled at least in part through mechanisms that regulate chromatin structure (Krivtsov and Armstrong, 2007).

In the nucleus, DNA is wrapped around histone octamers, which consist of two copies each of the histone proteins H2A, H2B, H3 and H4. The 147 base pairs of DNA wrapped around the histone octamer constitute a nucleosome, the basic subunit of chromatin (Dawson and Kouzarides, 2012). Nucleosomes are packed into higher-ordered structures, with regions of expressed genes, termed euchromatin, being more loosely packaged than non-expressed heterochromatin. The loose packaging of euchromatin allows transcriptional machinery to access DNA, increasing the expression of genes in these regions. Conversely, heterochromatin is resistant to transcription due to its tight packaging, yielding a mechanism for transcriptional regulation based on chromatin organization (Dawson and Kouzarides, 2012).

Enzymes that catalyze chromatin modifications, including DNA methylation and post-translational modification of histones, regulate chromatin structure and gene expression patterns. The N-terminal tails of histones are highly modified, and there is increasing evidence for a complex "histone code", in which combinations of these modifications dictate whether, and to what extent, a gene is activated or repressed (Lee et al., 2010). Various histone modifications are specifically recognized by "reader" proteins, which can remodel chromatin as a means of regulating transcription (Dawson and Kouzarides, 2012).

Initially, these modifications were considered to be long-lived, if not permanent. While this seems to be the case for the long-term silencing of transposons and imprinted genes, the regulation of many genes through chromatin modification is a dynamic process

(Dawson and Kouzarides, 2012). For example, certain genes that are expressed in progenitor cells may need to be repressed, while others must be activated during differentiation and cell type specification. Two major groups of proteins that govern transcriptional programs leading to cell fate decisions are polycomb (PcG) and trithorax (Trx) group proteins (Mills, 2010).

PcG and Trx proteins have opposing roles in transcriptional regulation

PcG and Trx group proteins have antagonizing roles in the transcriptional regulation of common target genes, many of which are critical for development (Figure 1.1). The regulation of these genes is tightly controlled, and an imbalance in this regulation can lead to the improper expression of target genes and cancer (Mills, 2010). One set of genes that is regulated by PcG/Trx, that becomes dysregulated in cancer are *Hox Genes* (Argiropoulos and Humphries, 2007; Schuettengruber et al., 2007). *Hox* genes were first discovered due to their role in determining positional identity along the anterior-posterior axis of developing animals, which is critical for proper body patterning during development (Krumlauf, 1994). *Hox* genes are highly conserved across species and encode DNA-binding transcription factors. In mammalian cells, there are 39 *Hox* genes residing in 4 distinct clusters (A-D) on 4 respective chromosomes (He et al., 2011).

PcG genes were initially discovered in *Drosophila* through genetic screens that caused homeotic transformations through the upregulation of *Hox* genes. This inappropriate activation of *Hox* genes led to the understanding that PcG proteins are involved in transcriptional repression (Mills, 2010). Further investigation into how these proteins

mediate repression led to the discovery that PcG proteins repress target genes through chromatin modification (Schuettengruber et al., 2007). In mammals, there are two PcG complexes, polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2). EZH2, the catalytic component of PRC2, tri-methylates histone H3 at lysine 27 (H3K27m3), leading to repression (Figure 1.1) (Margueron and Reinberg, 2011).

In contrast to the repressing function of PcG proteins, Trx proteins activate the expression of target genes, and share many of the same targets as PcG proteins. Trx group genes were discovered due to their ability to counteract PcG function, which led to the subsequent finding that Trx proteins activate gene transcription (Mills, 2010). The *Drosophila* trithorax protein and its mammalian homologs methylate histone H3 at lysine 4 (H3K4), which is associated with transcriptional activation (Figure 1.1) (Schuettengruber et al., 2011). Common PcG/Trx target genes are frequently enriched for both H3K4 tri-methylation (H3K4m3) and H3K27m3, and termed "bivalent" (Fisher and Fisher, 2011). These genes remain repressed, but are poised for activation, and the balance between H3K4m3 and H3K27m3 determines whether, and to what extent, these genes are expressed (Fisher and Fisher, 2011). The mammalian Trx group protein MLL is a critical regulator of this balance in hematopoietic cells.

MLL maintains *Hox* gene expression and is essential for hematopoiesis

Like other organ systems, hematopoiesis, or blood cell development, must be properly regulated to ensure the maintenance of a stem cell population and proper differentiation, as well as prevent tumorigenesis. Blood cells arise from the hematopoietic stem cell

(HSC), which has the ability to give rise to an identical daughter cell, termed self-renewal, or more differentiated progenitor cells that give rise to further differentiated lineages. Hematopoietic differentiation is regulated through both extracellular signals and the transcription of developmentally relevant genes, such as *Hox* genes (Argiropoulos and Humphries, 2007). The maintenance of *Hox* gene transcription in hematopoietic stem/progenitor cells is regulated at least in part by the Trx group protein MLL (Krivtsov and Armstrong, 2007).

MLL is expressed as a large ~4,000 amino acid protein, which is post-translationally cleaved by the protease taspase-1 into N-terminal (MLL-N) and C-terminal (MLL-C) fragments. These fragments re-associate to form the MLL complex (Figure 1.2) (Hsieh et al., 2003). Knockout studies have demonstrated a critical role for MLL in hematopoiesis, as MLL is required for the expansion of hematopoietic stem and progenitor cells during development (Ernst et al., 2004a; Ernst et al., 2004b; Hess et al., 1997; Yu et al., 1995).

MLL is required for the maintenance of *Hox* gene expression in hematopoietic stem and progenitor cells. *Hox* genes are expressed at high levels in these cells, and decreased expression of *Hox* genes correlates with cell differentiation (Maillard et al., 2009; Pineault et al., 2002). The hematopoietic deficiency imparted by MLL KO can be partially rescued by the ectopic expression of *Hox* genes, demonstrating the importance of *Hox* genes for the maintenance of hematopoietic stem and progenitor cells (Ernst et al., 2004b).

Menin is required for MLL complex recruitment to target genes

The MLL complex is recruited to target genes, such as *Hox* genes, via its N-terminal domains, including its menin-interacting domain (Yokoyama et al., 2004). Menin, encoded by the *Men1* gene, was first characterized as a bona-fide tumor suppressor in endocrine organs, due to frequently occurring *Men1* mutations in multiple endocrine neoplasia (Lemmens et al., 1997). However, in the hematopoietic system, menin was found to activate the expression of *Hox* genes, which can promote tumorigenesis (Yokoyama et al., 2004). Menin, a DNA-binding protein, directly interacts with MLL-N and is required for MLL recruitment to target genes (La et al., 2004; Yokoyama et al., 2004). Menin also provides a bridge for interactions with LEDGF, a chromatin binding protein, and c-myb, a specific DNA-binding transcription factor, both of which are necessary for proper targeting of MLL to gene loci (Figure 1.2) (Jin et al., 2010; Nakata et al., 2010; Yokoyama and Cleary, 2008). In addition, menin is required for MLL target gene transcription (Yokoyama et al., 2004). These findings place menin as a central scaffold protein linking MLL with chromatin that is essential for MLL function.

Chromosomal Translocations Disrupt MLL Function and Cause Leukemia

MLL function becomes disrupted through chromosomal translocations involving the *MLL* gene, which cause leukemia. These translocations occur with one of multiple partner genes, leading to the expression of MLL fusion proteins (MLL-FPs) and the development of acute leukemias. These leukemias can be lymphoid (ALL), myeloid (AML), or biphenotypic in nature (Daser and Rabbitts, 2004). *MLL* translocations are

found in ~10% of all leukemias and the majority of infant leukemia cases, and patients harboring this genetic abnormality have a particularly poor prognosis (Holleman et al., 2004; Liu et al., 2009), highlighting the importance of studying the pathogenesis of this disease.

MLL-FPs consist of an N-terminal portion of MLL fused to one of over 60 proteins (Figure 1.3). Some less frequently occurring MLL-FPs have cytosolic fusion partners, such as AF6, GAS7, EEN, and Septin proteins, and the mechanism by which these fusion proteins induce leukemia is not well understood (Krivtsov and Armstrong, 2007; So et al., 2004). However, the most common MLL-FPs have nuclear fusion partners, and these MLL-FPs upregulate *Hox* genes to promote leukemogenesis (Krivtsov and Armstrong, 2007; Lavau et al., 1997; Wei et al., 2008).

Hox genes are critical downstream targets of MLL-FPs, as overexpression of *Hoxa9* in combination with its cofactor *Meis1* is able to transform mouse bone marrow (Kroon et al., 1998). As *Hox* genes are important for the maintenance of stem and progenitor cells in normal bone marrow (Ernst et al., 2004b), it is likely that MLL-FP-driven *Hox* gene overexpression inappropriately activates stem cell-associated programs to promote leukemogenesis.

MLL-FPs retain the N-terminal menin-interaction domains, and the oncogenic potential of nuclear MLL-FPs is dependent on the MLL-FP interaction with menin (Caslini et al., 2007). These findings have led to a model where menin interacts with and recruits MLL-FPs to MLL target genes, leading to the upregulation of these genes, and leukemia (Figure 1.3) (Okada et al., 2005). However, MLL-FPs are lacking a large C-terminal portion of WT MLL that is normally required for MLL target gene upregulation (Figures 1.2 and 1.3). Although it has been unclear how these fusion proteins compensate for the loss of MLL-C, MLL-FPs gain the ability to promote gene expression through their interaction with complexes containing pTEFb and Dot1L.

MLL-FPs upregulate MLL target genes and can cause leukemia through their interaction with Dot1L and pTEFb

Many MLL-FPs recruit the histone H3 lysine 79 (H3K79) methyltransferase Dot1L to target genes, promoting leukemogenesis (Figure 1.3A). MLL translocation partners, including AF9, ENL, and AF10, exist in a complex with Dot1L, and MLL-FPs containing these fusion partners retain the ability to interact with Dot1L, leading to increased H3K79 methylation at target genes (Bitoun et al., 2007; Mohan et al., 2010). Although it is not yet known how H3K79 methylation regulates transcription, this modification is frequently found at active genes (Steger et al., 2008).

Enhanced transcription of MLL targets is associated with MLL-FP recruitment of Dot1L to these genes. *Hox* gene upregulation and leukemic transformation is contingent upon the interaction between MLL-AF10 and Dot1L (DiMartino et al., 2002; Okada et al., 2005). Additionally, direct fusion of Dot1L to MLL-N transforms mouse BM, while a Dot1L catalytic mutant fused to MLL-N fails to do so (Okada et al., 2005). These findings demonstrate that Dot1L catalytic activity is necessary for MLL-FP-mediated target gene upregulation and leukemogenesis.

In addition to Dot1L, MLL-FPs recruit the pTEFb complex to target genes to promote transcriptional elongation, providing another mechanism for transformation by MLL-FPs (Figure 1.3B). Some of the same MLL fusion partners that interact with Dot1L, such as AF9 and ENL, as well as other fusion partners, including AF4, are part of a distinct complex containing pTEFb, a kinase consisting of the Cyclin T and CDK9 proteins, that promotes transcriptional elongation (Bitoun et al., 2007; Dahmus, 1996; Liao et al., 1995). Transcriptional elongation is regulated at many developmentally relevant genes that have an initiated or "poised" RNA polymerase II (Pol II) resting at their promoters (Muse et al., 2007). Initiated Pol II is phosphorylated by pTEFb at serine 2 of its C-terminal domain (CTD), causing Pol II to be released from the promoter, and allowing transcriptional elongation (Peterlin and Price, 2006).

The expression of MLL-FPs, including MLL-AF9 (MA9), -ENL, and –AF4, leads to pTEFb recruitment to MLL target genes and enhanced transcriptional elongation (Figure 1.3B) (Mueller et al., 2009; Yokoyama et al., 2010). MLL-FP leukemia cell lines are more sensitive to the CDK9 inhibitors flavopiridol and alsterpaullone than non-MLL-FP cell lines, suggesting that dysregulated transcriptional elongation at MLL targets is at least one mechanism for leukemic transformation by MLL-FPs (Mueller et al., 2009). The finding that MLL-FPs recruit Dot1L and/or pTEFb to target genes, leading to their upregulation, has provided insight into the mechanism by which MLL-FPs cause leukemia. However, this gain of function due to chromosomal translocation also results in the loss of MLL C-terminal domains that are normally required for transcriptional activation of MLL target genes, and it remains unresolved whether MLL-FPs can activate target genes in the absence of MLL-C function.

It is unknown whether WT MLL has a role in MLL-FP-induced leukemia

MLL C-terminal domains that are lacking in MLL-FPs, and are important for the normal activation of MLL target genes include the su(var)3-9 and enhancer of zeste (SET) domain and trans-activation domain (TAD) (Figures 1.2 and 1.3). The WT MLL SET domain works in concert with the cofactors Wdr5, Ash2L and Rbbp5 to catalyze H3K4m3, which is associated with transcriptional activation (Figure 1.2) (Dou et al., 2005; Nakamura et al., 2002). Mice expressing MLL lacking the SET domain are viable, but exhibit skeletal defects and decreased expression of *Hox* genes (Terranova et al.,

2006), suggesting that the SET domain and H3K4m3 are normally required for the optimal expression of MLL target genes. In addition, the WT MLL TAD interacts with the histone acetyltransferase (HAT) enzymes MOF and CREB binding protein (CBP) (Figure 1.2) (Dou et al., 2005; Ernst et al., 2001). CBP directly interacts with the WT MLL TAD, and is required for MLL transactivation activity in normal cells (Ernst et al., 2001), suggesting that histone acetylation mediated by the WT MLL TAD also plays a role in target gene upregulation.

Although MLL-FPs do not contain C-terminal domains of WT MLL that are normally critical for target gene upregulation, they are able to enhance *Hox* genes to a greater extent than WT MLL. These findings suggest that WT MLL C-terminal domains are not required for *Hox* gene upregulation in MLL-FP-expressing cells. Along these lines, a prevalent model for MLL-FP-driven leukemogenesis suggests that MLL-FPs enhance H3K79m2 and transcriptional elongation, while WT MLL-mediated H3K4 methylation is reduced at the *Hoxa9* promoter (Okada et al., 2005). However, one allele of *MLL* remains intact in these cells, and enrichment for H3K4m3 has been reported at *Hox* genes in MLL-FP-expressing cells (Chen et al., 2006; Krivtsov et al., 2008), raising the possibility that residual expression of WT MLL from the non-translocated *MLL* allele promotes *Hox* gene expression and MLL-FP-mediated leukemogenesis.

The mechanism by which MLL-FPs block differentiation is not well understood

One of the consequences of MLL-FP expression, and a hallmark of leukemia, is a block in differentiation (Figure 1.4) (Lavau et al., 1997). As HSCs differentiate along various lineages, their self-renewal capability is lost (Seita and Weissman, 2010). However, ectopic expression of the MLL-FP MA9 in HSCs, common myeloid progenitors, or granulocyte-macrophage progenitors (GMPs) leads to the generation of cells that have a block in myeloid differentiation, and have gained the ability to self-renew (Figure 1.4) (Krivtsov et al., 2006).

These cells have been termed leukemia stem cells (LSCs) due to their ability to propagate leukemic disease. (Figure 1.4) (Krivtsov et al., 2006). In contrast to normal stem cells, which are few in number, LSCs may make up as much as 25% of the total leukemia cell population in MA9-expressing leukemias (Somervaille and Cleary, 2006). The eradication of these self-renewing LSCs is key to the effective treatment of MLL-FP leukemia, and understanding how MLL-FPs, such as MA9, block differentiation and promote self-renewal may provide novel therapeutic targets for the treatment of this disease. Menin is critical for MLL-FP-mediated leukemogenesis (Yokoyama et al., 2005). However, little is known as to the effect of menin depletion on MLL-FP leukemia cell differentiation *in vivo*, or the mechanism by which menin may block leukemia cell differentiation. Uncovering the mechanism by which menin/MLL-FPs block differentiation could lead to the discovery of novel therapeutic targets for this disease.

C/EBPα is critical for normal myeloid differentiation and its function is suppressed by various leukemogenic oncogenes

In many types of leukemia, differentiation is suppressed by inhibiting the function of the C/EBPα protein (Koschmieder et al., 2009). C/EBPα is a leucine zipper transcription factor that promotes myeloid differentiation through the repression of proliferation and the activation of target genes involved in differentiation, such as the cytokine receptors G-CSF receptor (GCSFR) and M-CSF receptor (MCSFR) (Heath et al., 2004; Zhang et al., 1996; Zhang et al., 1997). C/EBPα knockout mice completely lack granulocytes (Zhang et al., 1997), and are deficient in the ability to produce macrophages (Heath et al., 2004) demonstrating the critical role of C/EBPα in myeloid differentiation.

Many leukemogenic fusion proteins arising from chromosomal translocations block C/EBPα function as a means of suppressing leukemia cell differentiation. For example, the AML1-ETO fusion protein represses *CEBPA* transcription, Bcr-Abl blocks *CEBPA* translation, and PLZF-RAR represses C/EBPα binding to target genes (Koschmieder et al., 2009). However, although there is a block in myeloid differentiation in MA9-expressing LSCs, it is not known whether C/EBPα suppression contributes to this block, opening up the possibility for investigating how C/EBPα might be repressed by MA9 and menin.

MLL-FP-induced leukemia is highly aggressive, and patients with this disease have a poor prognosis (Holleman et al., 2004). It is therefore critical to understand the pathogenesis of this disease. MLL-FPs cooperate with menin to upregulate *Hox* genes and promote leukemic transformation (Yokoyama et al., 2005). However, little is known about how MLL-FPs might compensate for the lack of WT MLL domains normally required to upregulate *Hox* genes. In addition, it is unknown how menin, likely in combination with MLL-FPs, might act to block leukemia cell differentiation. A greater understanding of the mechanism by which menin and MLL-FPs upregulate target genes and block differentiation could identify additional targets for the treatment of this disease.

Project Aims

Aim 1:

MLL-FPs cause leukemic transformation at least in part through the upregulation of *Hox* genes (Zeisig et al., 2004). However, MLL-FPs lack a large C-terminal region of WT MLL that is normally required to *Hox* gene upregulation, including the SET domain, which catalyzes H3K4m3. Intriguingly, H3K4m3 is even more enriched at *Hox* gene loci in MLL-FP leukemia cells than normal hematopoietic cells (Krivtsov et al., 2008). It is possible that WT MLL expression from the non-translocated *Mll* allele is responsible for H3K4m3 at *Hox* loci in MLL-FP leukemia cells. These findings lead to the hypothesis that WT MLL is required for MA9-induced *Hox* gene upregulation leukemogenesis. To determine the role of WT MLL in MA9 leukemia, we will use shRNAs to knock down (KD) WT MLL in MA9-expressing cell lines and test the effect of WT MLL KD on

Hoxa9 transcript levels and cell growth. We will also establish a murine model with the ability to acutely deplete WT *Mll*. Using this model, we will test the role of WT MLL in MA9-mediated transformation *in vitro* and the effect of WT MLL depletion on MA9 leukemia *in vivo*. The demonstration of a role for WT MLL in MLL-FP leukemia could pave the way to developing inhibitors of MLL C-terminal domains to treat this disease.

Aim 2:

A consequence of MLL-FP expression is a block in differentiation, which is critical for maintaining the LSC pool and propagating leukemic disease (Cleary, 2009; Lavau et al., 1997). Menin is an essential cofactor for MLL-FP function, and is critical for MLL-FP-mediated leukemogenesis, but it is unclear how menin might suppress leukemia cell differentiation. Differentiation is blocked in many types of leukemia through suppression of C/EBPα function. However, there is no known mechanism for C/EBPα inhibition in MLL-FP leukemias, leading to the hypothesis that menin blocks MA9 leukemia cell differentiation by suppressing C/EBPα function. To investigate the potential interplay between menin and C/EBPα in blocking MA9 leukemia cell differentiation, we will use a murine model for MA9-induced leukemia, and an MA9-transformed cell line, with the ability to acutely deplete menin. Investigating how menin suppresses MLL-FP leukemia cell differentiation may reveal novel potential targets for the treatment of MLL-FP leukemias.

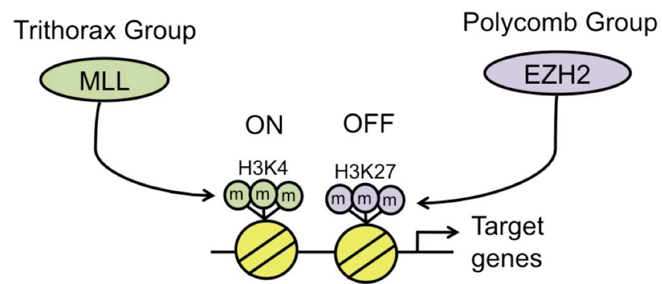


Figure 1.1. The classical opposing roles of PcG and Trx proteins in the regulation of common target genes. Trx group proteins, such as MLL catalyze H3K4m3 and activate target genes. PcG proteins, such as EZH2 methylate H3K27, and repress target genes. One group of genes commonly regulated by PcG and Trx proteins are *Hox* genes.

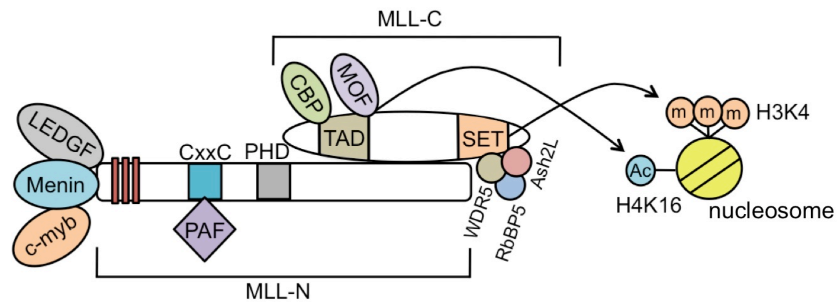
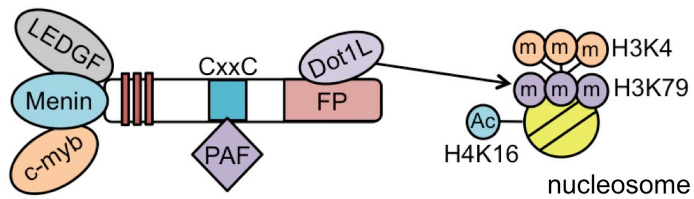


Figure 1.2. The MLL complex, and chromatin modifications catalyzed by this complex. MLL-N recruits the MLL complex to target genes through multiple interactions. Menin directly interacts with the extreme N-terminus of MLL-N and DNA, as well as c-myb and LEDGF, coordinating multiple linkages between MLL-N and chromatin. The MLL-C SET domain catalyzes H3K4m3. The MLL-C TAD interacts with the histone acetyltransferases CBP and MOF. These chromatin modifications are associated with transcriptional activation.



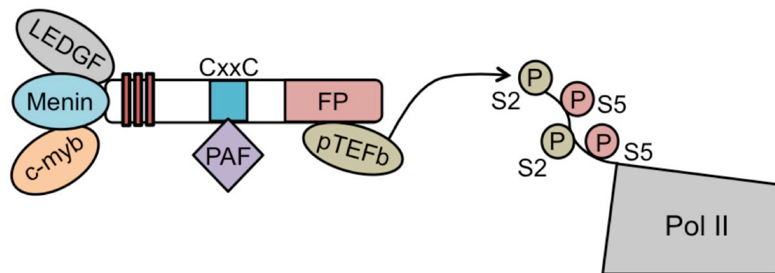


Figure 1.3. Menin/MLL-FP complexes, and chromatin modifications catalyzed by these complexes. (A) Many MLL-FPs interact with and recruit the H3K79 methyltransferase Dot1L to MLL target genes. **(B)** Many MLL-FPs also recruit the pTEFb (cyclin T/CDK9) kinase complex to MLL target genes, leading to dysregulated transcriptional elongation. MLL-FPs retain N-terminal domains required for recruitment to MLL target genes, including the menin-interaction domain.

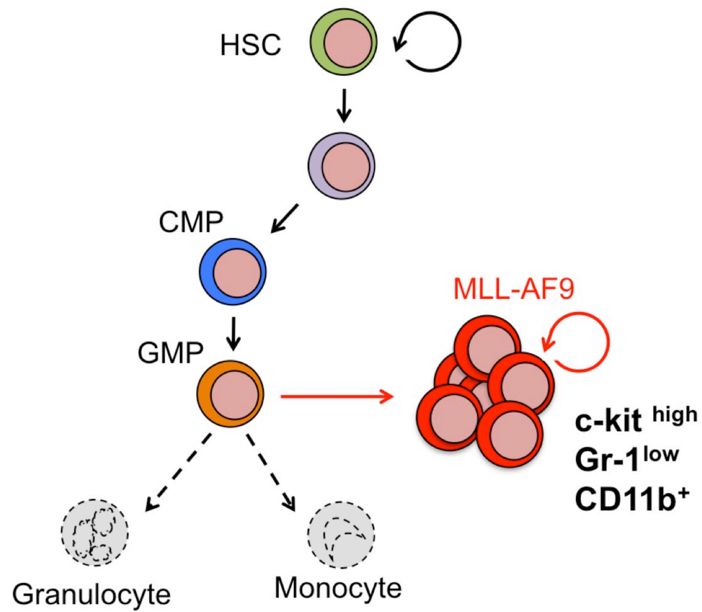


Figure 1.4. MLL-AF9 LSCs have a block in differentiation at the GMP stage of myeloid differentiation. Expression of MLL-AF9 in HSCs, CMPs, or GMPs leads to the generation of GMP-like cells that have improper self-renewal and the ability to propagate leukemic disease.

CHAPTER 2 - MLL-AF9-MEDIATED LEUKEMOGENESIS REQUIRES CO-EXPRESSION OF THE WILD-TYPE *MLL* ALLELE

The data in Chapter 2 have been published in *Cancer Cell* (Thiel et al., 2010).

Summary

Oncogenic fusion proteins are capable of initiating tumorigenesis, but the role of their wild-type counterparts in this process is poorly understood. The mixed lineage leukemia (*MLL*) gene undergoes chromosomal translocations, resulting in the formation of oncogenic MLL fusion proteins (MLL-FPs). Here we show that menin recruits both wild-type (WT) MLL and MLL-AF9 to the loci of *Hox* genes to activate their transcription. WT MLL not only catalyzes histone methylation at key target genes, but also controls distinct MLL-AF9-induced histone methylation. Notably, the WT *Mll* allele is required for MLL-AF9-induced leukemogenesis and the maintenance of MLL-AF9-transformed cells. These findings suggest an essential cooperation between an oncogene and its WT counterpart in MLL-AF9-induced leukemogenesis.

Introduction

Multiple oncogenic fusion proteins resulting from chromosomal translocations are capable of initiating tumorigenesis. The mixed lineage leukemia gene (*MLL*) is fused with one of over 60 distinct partner genes through chromosomal translocations in various human acute leukemias, resulting in the formation of MLL fusion proteins (MLL-FPs) (Hess, 2004; Krivtsov and Armstrong, 2007). MLL-FPs enhance *HOX* gene expression and cause leukemic transformation. In one well-characterized example, MLL-AF10 directly interacts with Dot1L, the only known H3K79-specific methyltransferase, via the AF10 moiety, and recruits Dot1L to the *Hoxa9* locus to aberrantly increase H3K79 dimethylation (H3K79m2) (Okada et al., 2005). The methyltransferase activity of Dot1L is required for enhanced transcription of certain *Hox* genes and for MLL-AF10-induced bone marrow (BM) transformation. MLL-AF4 also enhances Dot1L-mediated H3K79 methylation at *Hox* genes (Krivtsov et al., 2008), and the wild type counterparts of additional MLL fusion partners such as AF9 and ENL have been shown to interact with Dot1L in a large protein complex (Bitoun et al., 2007; Mueller et al., 2007), illustrating a common mechanism for leukemic transformation.

WT *MLL* is homologous to the *Drosophila* trithorax gene, a positive regulator of gene expression. WT MLL is proteolytically cleaved into two parts, MLL-N and MLL-C, by the protease Taspase 1 (Hsieh et al., 2003). MLL-C contains a conserved SET domain, which catalyzes histone H3K4 methylation and upregulates the transcription of *HOX* genes in fibroblasts and epithelial cell lines (Milne et al., 2002; Nakamura et al., 2002).

H3K4m3 is associated with euchromatin and active genes, and specifically recruits chromatin-remodeling proteins to stimulate gene expression (Berger, 2007; Flanagan et al., 2005).

WT MLL forms a large complex with several proteins including menin (Hughes et al., 2004; Yokoyama et al., 2005), a DNA-binding scaffold protein that is mutated in an inherited human endocrine tumor syndrome (La et al., 2004). Menin interacts with the N-terminus of both MLL and MLL-FPs through their identical N-terminal sequences (Yokoyama et al., 2005), promotes H3K4m3 at the *Hoxa9* locus, and upregulates *Hoxa9* transcription in MLL-FP-transformed hematopoietic cells (Chen et al., 2006; Yokoyama et al., 2005). Moreover, menin is required for the proliferation of cells transformed by MLL-AF9 fusion protein (MA9 hereafter) (Caslini et al., 2007; Chen et al., 2006). However, little is known as to whether menin affects MA9-regulated H3K79 methylation and whether WT MLL, potentially expressed from the remaining non-translocated *MLL* allele, is important for MA9-mediated leukemic transformation.

A potential role for WT MLL in MA9 leukemogenesis has been unclear. Despite lacking the WT MLL SET domain, MA9 remains capable of initiating leukemogenesis when introduced into murine or human hematopoietic progenitors (Barabe et al., 2007; Krivtsov et al., 2006; Somervaille and Cleary, 2006; Wei et al., 2008). Moreover, MLL-AF10 reduces H3K4 dimethylation at the *Hoxa9* locus (Okada et al., 2005), which is mediated at least in part by WT MLL. Further, in MLL-FP-expressing human leukemia

cells, which lose one of the two WT *MLL* alleles via chromosomal translocation, expression of WT MLL target genes such as *HOXA9* is even higher than in non-MLL-FP-leukemia cells (Armstrong et al., 2002). These studies suggest that WT MLL may not be involved in oncogenic transformation mediated by MLL-FPs. On the other hand, WT MLL promotes H3K4 methylation and *Hox* gene expression in fibroblasts and HeLa cells (Milne et al., 2002; Nakamura et al., 2002). Also, WT *Mll* excision compromises the expression of 5' *Hoxa* genes including *Hoxa9* and hematopoietic stem cell (HSC) function (Jude et al., 2007; McMahon et al., 2007). The role of WT MLL in *Hox* gene upregulation, coupled with the fact that *Hox* genes are upregulated in MA9-transformed leukemia cells, raises the possibility that WT MLL is involved in MA9-induced leukemogenesis.

Many oncogenic fusion proteins resulting from chromosomal translocations have been identified in various leukemias and solid cancers (Nambiar et al., 2008). However, it is poorly understood whether the WT alleles of genes involved in these translocations influence tumorigenesis induced by the majority of known oncogenic fusion proteins. A better understanding of the function of these WT alleles in tumorigenesis could yield new insights into transformation mechanisms. Our earlier findings on the role of menin in proliferation and gene transcription in MA9-transformed cells prompted us to investigate a potential role for WT MLL in MA9-induced leukemogenesis.

Results

Menin is required for methylation of both histone H3 lysine 4 (H3K4) and histone H3 lysine 79 (H3K79) at the *Hoxa9* locus

The MLL-AF10 fusion protein has been reported to transform mouse BM by promoting Dot1L-catalyzed H3K79 methylation, but repressing H3K4 methylation at the *Hoxa9* locus, suggesting that H3K79-methylating Dot1L, but not H3K4-methylating WT MLL, is crucial for MLL-FP-induced leukemic transformation (Okada et al., 2005). Although menin interacts with the N-terminus of WT MLL and MLL-FPs (Yokoyama et al., 2005), it has been unclear whether menin promotes H3K79 methylation at *Hoxa9* in MA9-transformed BM cells. To address this question, we excised the floxed *Men1* gene in MA9-transformed BM cells (AT-1 cells), which harbor *Men1*$^{f/f}$*;Cre-ER*, using 4-hydroxyl tamoxifen (4-OHT) to induce Cre activity (Figure 2.1A, lane 2). We then performed chromatin immunoprecipitation (ChIP) assays to determine the effect of menin depletion on H3K79 methylation. *Men1* excision reduced H3K79 dimethylation (H3K79m2) in two separate locations at the *Hoxa9* locus (Figure 2.1B). As Dot1L is the only known H3K79 methyltransferase in mammals, this finding is consistent with the notion that menin is crucial for MA9-induced Dot1L recruitment to the *Hoxa9* locus, enhancing H3K79 methylation.

Men1 excision also reduced H3K4 trimethylation (H3K4m3) at the *Hoxa9* promoter (Figure 2.1C), in agreement with our previous findings (Chen et al., 2006). However, this finding contrasts with the proposed role of MLL-AF10 in reducing H3K4

methylation at the *Hoxa9* locus (Okada et al., 2005), which could be at least partly mediated by WT MLL. As a control, ChIP assays showed that menin bound the *Hoxa9* promoter, and menin binding was abrogated in *Men1*$^{\Delta/\Delta}$ cells (Figure 2.1D). Together, these results demonstrate that menin is crucial not only for Dot1L-mediated H3K79 methylation, but also H3K4 methylation at the *Hoxa9* locus in the MA9-transformed cells. As WT MLL catalyzes H3K4 methylation at *HOX* genes, these results raised the possibility that WT MLL is crucial for MA9-induced *HOX* gene expression.

Menin recruits both WT MLL and MA9 to the *Hoxa9* locus

To explore whether WT MLL participates in upregulating *Hox* gene expression in MA9-transformed cells, we first examined whether WT MLL binds the *Hoxa9* locus, and if so, whether WT MLL binding is dependent on menin in MA9-transformed BM cells. Since MA9 lacks the MLL-C portion of WT MLL, detection of MLL-C at the *Hoxa9* locus indicates WT MLL enrichment. Hence, we chose to use an antibody that specifically recognizes MLL-C to detect WT MLL. ChIP assays showed that MLL-C bound the *Hoxa9* promoter, and this binding was dependent on menin (Figure 2.2A). These results indicate that menin is required for recruiting WT MLL to *Hoxa9* in MA9-transformed cells.

As menin interacts with the N-terminus of MLL (Yokoyama et al., 2005), we determined if menin affects the recruitment of MA9 (which contains the N-terminus of MLL) to

Hoxa9 by ChIP assay, using an anti-AF9 antibody that specifically recognizes the AF9 portion of the MA9 fusion protein (Figure 2.2B). Our results show menin-dependent MA9 enrichment at the *Hoxa9* promoter (Figure 2.2B). As the C-terminal portion (residues 397-557) of AF9 has been reported to bind Dot1L (Zhang et al., 2006), we decided to test whether the AF9 part of MA9 interacted with Dot1L using a GST-AF9 pull-down assay, and found that the AF9 portion from MA9 bound Dot1L (Figure 2.3 A-C). To further evaluate whether menin affects the recruitment of MA9 to the *HOXA9* locus in human MA9-expressing leukemia cells, we knocked down menin expression in THP-1 cells (Figure 2.3D, F). Menin knockdown (KD) reduced the number of THP-1 cells (Figure 2.3E), *HOXA9* expression (Figure 2.3F), and menin binding to the *HOXA9* promoter (Figure 2.3G). Menin KD also reduced enrichment for both MA9 and Dot1L at *HOXA9* (Figure 2.2C). Together with the data from Figure 2.1, these results indicate that menin promotes the recruitment of both WT MLL and MA9/Dot1L to the *HOXA9* locus, thereby increasing WT MLL-mediated H3K4 methylation and Dot1L-mediated H3K79 methylation.

WT MLL is required for MA9-transformed leukemia cell growth and *HOX* gene expression

It has been unclear whether WT MLL has a role in MLL-FP-induced leukemia. H3K4 methylation, which is at least partly mediated by WT MLL, has been reported to be repressed in MLL-AF10-transformed cells (Okada et al., 2005). However, WT MLL is crucial for H3K4 methylation at *HOX* gene loci and the expression of *HOX* genes (Milne

et al., 2002; Nakamura et al., 2002), some of which are critical for BM transformation by MLL-FPs (Ayton and Cleary, 2003). Our data indicate a crucial role for menin in WT MLL recruitment to, and H3K4 methylation at, *HOXA9* (Figure 2.2A and 2.1C). To determine if WT MLL is important for the expression of *HOX* and cell cycle genes and for the growth of MA9-transformed BM cells, we transduced AT-1 cells with either control scrambled or MLL-C-targeting shRNAs (Figure 2.4A, shRNA 11). The rationale for targeting the C-terminus was to avoid affecting the mRNA encoding MA9, which lacks the MLL-C sequence. The MLL-C shRNAs, but not the scrambled vector, reduced the expression of WT *Mll*, *Hoxa9*, and *Ccna2*, which encodes cyclin A2 (referred to as cyclin A hereafter) (Figure 2.4C, D), and AT-1 cell growth (Figure 2.4B).

To determine if WT MLL is also crucial for proliferation of human MA9-expressing leukemia cells, we knocked down WT MLL in THP-1 cells using shRNAs that targeted the C-terminus of human MLL (Figure 2.4A). Two independent MLL-C shRNAs, but not the control scrambled shRNA, reduced the number of THP-1 cells (Figure 2.4E). As expected, WT MLL expression was reduced in the shRNA-transduced cells, as shown by qRT-PCR and Western blotting (Figure 2.4F, G). The protein level of MLL-N was also reduced. However, menin and MA9 levels were not affected (Figure 2.4H). *HOXA9* and *CCNA2* were also reduced in WT MLL KD cells (Figure 2.4F, G). Collectively, these results indicate that WT MLL upregulates the expression of *HOXA9* and *CCNA2* as well as promoting the proliferation/survival of human MA9 leukemia cells.

To further confirm the impact of WT *Mll* on primary MA9-transformed BM cells, we used a genetically tractable mouse model to specifically excise the WT *Mll* gene. We bred *Mll*$^{f/f}$ mice (Jude et al., 2007) with ubc9-*Cre-ER* mice (Ruzankina et al., 2007), and demonstrated efficient *Mll* excision induced by 4-hydroxyl tamoxifen (4-OHT) treatment in splenocytes from the resulting *Mll*$^{f/f}$*;Cre-ER* mice (Figure 2.5A, B). *Mll* excision after BM cells were transformed with MA9-expressing retrovirus reduced the number of the MA9-transformed BM cells (Figure 2.5C) and the expression of *Hox* genes in these cells (Figure 2.5D). Together, these experiments demonstrate that WT MLL is required for the optimal expression of *HOX* genes and the proliferation/survival of MA9-transformed leukemia cells.

WT MLL is required for maximal methylation of both H3K4 and H3K79 at target genes

The SET domain of WT MLL methylates H3K4, yet is lacking in the MA9 fusion protein (Milne et al., 2002). However, loss of menin, an MLL-interacting protein, reduced H3K4 methylation at *Hoxa9* in MA9-transformed cells (Figure 2.1C). It has been unclear whether the remaining WT *MLL* allele has a role in promoting H3K4 methylation in human MA9-expressing leukemia cells. To determine whether WT MLL promotes H3K4m3 at its target genes in MA9 leukemia cells, we performed ChIP assays with control and WT MLL KD THP-1 cells. WT MLL KD reduced both MLL-C and H3K4m3 enrichment at *HOXA9* (Figure 2.6A, B), indicating that WT MLL catalyzes H3K4m3 at *HOX* genes in MA9 leukemia cells.

Notably, H3K79 methylation was also reduced at the *HOXA9* locus in these WT MLL KD cells (Figure 2.6B), indicating a role for WT MLL in MA9/Dot1L-mediated H3K79 methylation. Similarly, WT MLL is also required for maximal methylation of both H3K4 and H3K79 at the *CCNA2* locus (Figure 2.6C), reinforcing the role of WT MLL in both H3K4 and H3K79 methylation. Collectively, these results suggest that WT MLL controls both H3K4 and H3K79 methylation at MA9 target genes in leukemia cells.

WT MLL depletion reduces the colony formation of MA9-transformed bone marrow

Regulation of H3K79 methylation by WT MLL raised an intriguing possibility that WT MLL is important for the maintenance of MA9-mediated BM transformation. Therefore, we examined the impact of WT MLL knockdown on colony formation of MA9-transduced BM using a colony formation assay. Plating of MLL-ENL-transduced BM in a semi-solid medium for three consecutive rounds leads to immortalization and transformation of the hematopoietic progenitors (Lavau et al., 1997). To examine the impact of WT MLL KD on MA9-induced BM transformation, we transduced either control scrambled shRNA or each of the two MLL-C shRNAs into MA9-transduced BM cells after the second plating, followed by puromycin selection (Figure 2.7A). The titers of these distinct shRNA and control lentiviruses were comparable, and WT *Mll* KD was efficient in mouse cells (data not shown). At the fourth plating, numerous colonies appeared from the control cells (Figure 2.7B, Scram). However, WT MLL KD with each of the MLL-C shRNAs reduced colony formation from the MA9-transduced BM,

suggesting that WT MLL is required to maintain MA9-mediated leukemogenesis (Figure 2.7B, C).

To further confirm the role of WT MLL in maintaining MA9-induced BM transformation, we used *Mll*$^{f/f}$*;Cre-ER* BM to excise WT *Mll* after transformation. BM from control *Mll*$^{f/f}$ or *Mll*$^{f/f}$*;Cre-ER* mice was transduced with MA9 retrovirus and serially replated on a semi-solid medium (Figure 2.8A). *Mll* excision induced by 4-OHT significantly reduced the number of colonies from the MA9-transformed *Mll*$^{f/f}$*;Cre-ER* BM (Figure 2.8B). As a control, 4-OHT failed to reduce colony formation from MA9 *Mll*$^{f/f}$ BM (Figure 2.8C). 4-OHT-induced excision of the floxed *Mll* in MA9-transformed BM from the *Mll*$^{f/f}$*;Cre-ER* mice was confirmed by genomic PCR (Figure 2.8D, lane 2). These results demonstrate that WT MLL is required for the maintenance of MA9-transformed cells.

Hoxa9 and Meis1 have previously been shown to have the ability to transform primary BM (Kroon et al., 1998). WT *Mll* excision did not inhibit BM colony formation induced by Hoxa9/Meis1 (Figure 2.8E). These results indicate that WT *Mll* is not required for colony formation induced by Hoxa9/Meis1, likely because both *Hoxa9* and *Meis1* are direct MLL targets and act downstream of MLL (Guenther et al., 2005; Nakamura et al., 2002). This finding is consistent with the notion that WT MLL is essential for the maintenance of MA9-transformed cells at least partly through upregulating certain *HOX* genes.

We further examined the effect of pre-existing WT *Mll* excision on colony formation from normal BM as well as MA9-transduced BM. BM from *MLL*$^{f/f}$*; Cre-ER* mice that were treated with tamoxifen (TAM) displayed effective excision of the floxed *Mll* allele (Figure 2.8F, bottom), and *Mll* excision reduced the ability of normal BM to form colonies (Figure 2.8F, top). Moreover, BM with WT *Mll* or with previous deletion of WT *Mll* was transduced with MA9-expressing retrovirus, and plated on semi-solid medium. We found that previous deletion of WT *Mll* reduced colony formation at the first plating (Figure 2.8G). These results, coupled with other results from the colony formation assay, indicate that WT *Mll* is crucial for survival and/or proliferation of BM progenitors and the maintenance of MA9-transformed cells, but not necessarily for MA9-induced transformation. From a standpoint of leukemia therapy, inhibiting the maintenance of MA9-transformed cells is more important than inhibiting MA9-induced transformation, because failure in maintaining MLL-FP-transformed cells could lead to eradication of the leukemia cells.

***Mll* excision in MA9-transformed cells inhibits the development of MA9-induced leukemia in mice**

We next determined the role of WT MLL in MA9-induced leukemogenesis using the murine leukemia model with *Mll*$^{f/f}$*;Cre-ER* BM. BM from *Mll*$^{f/f}$*;Cre-ER* mice was transduced with MA9 retrovirus and transplanted into lethally irradiated recipient mice (Figure 2.9A). Flow cytometry analysis of peripheral blood demonstrated successful engraftment of MA9-transduced donor BM (CD45.2+ only) and co-transplanted wild-

type BM (CD45.1+/2+) (Figure 2.9B). The percentage of cells expressing CD11b or CD11b/Gr-1 (myeloid markers) was much higher in the MA9-transduced BM (Figure 2.9D) than the co-transplanted normal BM (Figure 2.9C). Overt acute leukemia developed, indicated by obvious leukemia cell infiltrations in various organs including the femur, liver, and spleen (Figure 2.9H-J).

To examine the impact of WT *Mll* excision on the development of MA9-induced acute myeloid leukemia (AML), the mice transplanted with MA9-overexpressing BM were treated with either control corn oil (CO) or TAM to excise the floxed *Mll* from MA9-transduced cells, then monitored for peripheral white blood cell (WBC) number, WBC immunophenotype, and survival rate (Figure 2.9A). As expected, effective *Mll* excision in peripheral WBCs in TAM-treated mice was observed (data not shown). The number of total peripheral WBCs and the percentage of MA9-transduced BM-derived CD11b+ myeloid cells were significantly lower in TAM-treated mice than in the corn oil-fed control mice (Figure 2.10A, B, $p<0.001$ and 0.011, respectively).

In splenocytes from terminally ill CO- or TAM-treated mice, WT *Mll* excision reduced the percentage of cells bearing the markers for L-GMP (Figure 2.10C, D), namely c-kit+/Sca-1-/FcRgII/III+/CD11b+/CD34+, which have been reported to be enriched in MA9-induced leukemia stem cells (LSCs) (Krivtsov et al., 2006). WT *Mll* excision from the MA9-expressing cells also significantly increased the survival rate of recipient mice, based on Kaplan-Meier analysis (Figure 2.10E, $p<0.001$). Together, these results suggest

that WT *Mll* is critical for the development of MA9-induced leukemia, partly through enhancing leukemia stem cell proliferation and/or survival.

Discussion

WT MLL controls MA9-induced leukemogenesis

Oncogenic fusion proteins resulting from chromosomal translocations represent a major molecular lesion in leukemia and certain solid tumors. Studies about these malignant diseases are often focused on the fusion protein, with little attention paid to the potential role of the remaining WT alleles of the translocated genes in tumorigenesis. The WT MLL SET domain was not thought to be crucial for MLL-FP-triggered leukemogenesis, as MLL-AF10 even suppresses H3K4 dimethylation. Therefore, it has been unclear whether WT MLL plays a role in MLL-FP-mediated leukemic transformation.

We have shown that WT MLL is required for MA9-mediated leukemogenesis. WT MLL may control MA9-induced leukemogenesis by facilitating the expression of *HOX* genes and other self-renewal genes, supporting LSC maintenance. Although WT MLL is crucial for preventing HSCs from abnormal cell cycle entry, it may be particularly important for promoting the proliferation and survival of MA9 LSCs (Figure 2.10C, D), a function distinct from that in HSCs (Jude et al., 2007; McMahon et al., 2007). In support of this thoery, *Hoxa9* expression is reactivated in MA9-transformed LSCs (Krivtsov et al., 2006), and WT MLL promotes MA9 LSC maintenance (Figure 2.10C, D).

There are over 60 distinct MLL-FPs, and further work remains to determine whether our findings are generally applicable to other MLL-FPs. Given that MLL-AF10 and MLL-AF4 enhance H3K79 methylation at target gene loci (Krivtsov et al., 2008; Okada et al., 2005), and WT MLL is also necessary for maximal H3K79 methylation (Figure 2.6B), it is likely that many MLL-FPs require WT MLL for leukemogenesis. On the other hand, deletion of the WT *Mll* allele does not change *Hoxa9* expression, but reduces the colony forming activity (CFU-GEMM) of mouse fetal liver cells expressing MLL-PTD (partial tandem duplication) (Dorrance et al., 2008). MLL-PTD retains the C-terminal SET domain, yet WT MLL is silenced in leukemia cells expressing MLL-PTD (Whitman et al., 2005), raising the possibility that MLL-PTD-initiated leukemia arises through a distinct mechanism.

The mechanism for WT MLL-dependent epigenetic regulation and MLL-AF9-induced leukemogenesis

WT MLL promotes H3K4m3 at the *HOXA9* and *CCNA2* loci in MA9 leukemia cells, which is associated with transcriptional activation. Although MLL-AF10 suppresses H3K4 dimethylation (H3K4m2), as WT MLL is able to convert dimethylated H3K4m2 to H3K4m3, the decrease in H3K4m2 by MLL-AF10 could actually result from increased WT MLL-mediated conversion of H3K4m2 to H3K4m3. This explanation is consistent with the observation that MLL-ENL induces H3K79 methylation, but does not suppress H3K4m3 at *Hoxa9* (Milne et al., 2005a). Additionally, in MLL-AF4-expressing human leukemia cells, H3K4 and H3K79 methylation are colocalized at large chromatin

domains, including the domain harboring the *HOXA7* and *HOXA9* loci (Guenther et al., 2005).

WT MLL controls not only H3K4m3, but also H3K79m2, two distinct positive histone H3 modifications, at *HOXA9* and *CCNA2* loci in MA9-transformed cells. It is likely that menin recruits WT MLL and MLL-FPs/Dot1L to target genes, enhancing H3K4m3 and H3K79m2, because menin physically interacts with the N-terminus of both WT MLL and MLL-FPs (Yokoyama et al., 2005). A combination of H3K4m3 and H3K79m2 may cooperatively activate the transcription of *HOX* genes and cell cycle genes, triggering leukemic transformation and supporting the maintenance of LSCs (Figure 2.10F).

It remains unclear how WT MLL controls H3K79m2. Several WT counterparts of MLL fusion partners, such as AF4, AF9 and ENL, form a transcriptional elongation complex containing RNA polymerase II transcription elongation factor b (pTEFb) and Dot1L to increase gene expression (Bitoun et al., 2007; Mueller et al., 2007). It is unknown whether WT MLL is also in this complex and crucial for the function of this transcription elongation complex or Dot1L-mediated H3K79 methylation. It is also possible that WT MLL-mediated H3K4m3 is important for the role of WT MLL in MA9-induced H3K79m2. In this regard, methylated H3K4 serves as a docking site to recruit various transcription-activating proteins such as WDR5 and BPTF (Wysocka et al., 2005; Wysocka et al., 2006). It is possible that H3K4m3-binding proteins may affect the recruitment or activity of Dot1L and subsequent H3K79 methylation.

Contrary to WT MLL-dependent H3K79 dimethylation at *HOXA9*, H3K4m3 does not appear to rely on Dot1L-mediated H3K79 methylation. MLL-ENL enhances H3K79m2 at *Hoxa9*, but is dispensable for H3K4m3 at the locus (Milne et al., 2005b). Moreover, Dot1L ablation from cells abrogates H3K79m2 but does not affect H3K4m3 (Steger et al., 2008), suggesting a uni-directional order of H3K4 and H3K79 methylation that is controlled by WT MLL (Figure 2.10F).

WT MLL as a potential target for leukemia therapy

Our findings demonstrate that WT MLL is crucial for maximal expression of *HOXA9* and MA9-induced leukemogenesis. The necessity for WT MLL in multiple processes of leukemogenesis, including MA9/Dot1L-mediated H3K79m2 at *HOX* genes and maintenance of MA9-expressing LSCs, could render MLL-FP-containing leukemia cells selectively sensitive to inhibition of WT MLL. Despite our finding that WT *Mll* excision decreases colony formation in normal BM progenitors, WT *Mll* deletion in adult mice is well tolerated and does not adversely affect homeostatic hematopoiesis (McMahon et al., 2007). MLL-FP-expressing leukemia cells may become particularly "addicted" to WT MLL for the expression of stem cell-related *HOX* genes and certain cell growth/survival-related genes. This addiction may be attributable to the reduced cellular WT MLL levels in human leukemia cells that result from disruption of one WT *MLL* allele due to the chromosomal translocation. In agreement with this interpretation, in murine HSCs harboring endogenous knockin MA9, the amount of WT MLL is only half that found in normal HSCs (Chen et al., 2008). WT MLL may also be more important in MLL-FP-

expressing leukemia cells because these cells rely on WT MLL for MLL-FP-induced H3K79m2 and enhanced target gene transcription (Figure 2.10F). However, it cannot be ruled out that WT *MLL* also plays a role in transformation induced by non-MLL-FPs.

Collectively, our findings support the development of strategies to treat MLL-FP-induced leukemia, in part by targeting WT MLL. These studies raise the possibility for developing lead compounds that specifically inhibit WT MLL, its interacting proteins, processing enzymes, or its methyltransferase activity to treat MLL-FP-induced acute leukemia.

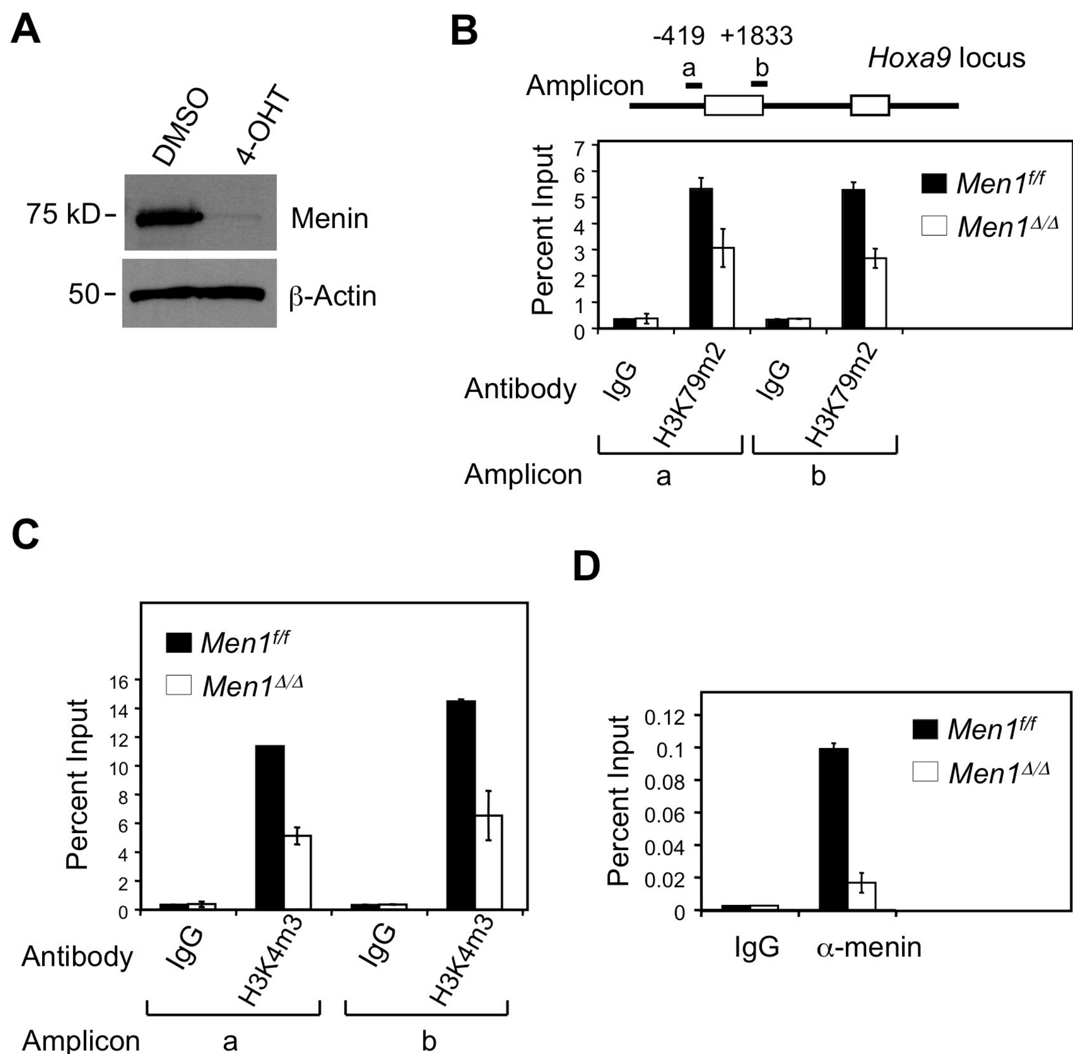


Figure 2.1. Menin is required for both H3K4m3 and H3K79m2 at *Hoxa9* in MA9-transformed cells. (A) Western blot for menin in control or *Men1* excised MA9-transformed AT-1 cells, which harbor *Men1*$^{f/f}$*;Cre-ER*. The cells were treated with either control DMSO (*Men1*$^{f/f}$) or 4-OHT (*Men1*$^{\Delta/\Delta}$) to excise the floxed *Men1*. **(B-D)** ChIP assay, with two distinct amplicons, for detecting H3K79m2 **(B)**, H3K4m3 **(C)**, and menin binding **(D)** at *Hoxa9* in *Men1*$^{f/f}$ and *Men1*$^{\Delta/\Delta}$ AT-1 cells.

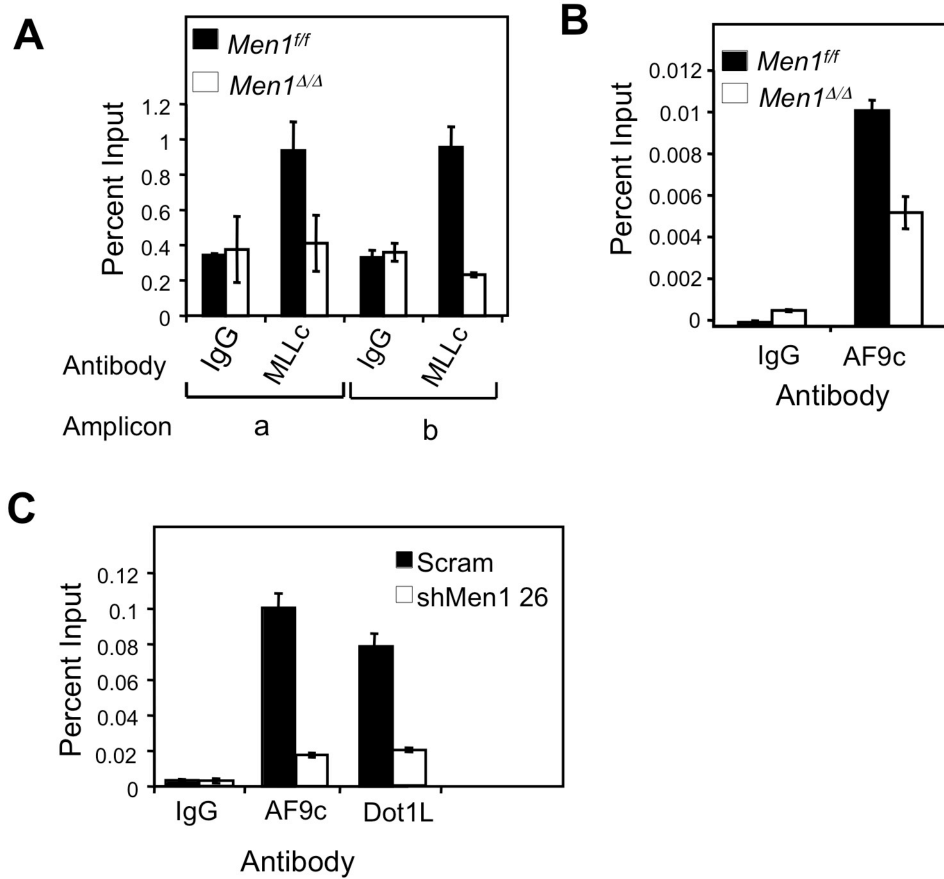


Figure 2.2. WT MLL and MA9 are recruited to *Hoxa9* in a menin-dependent manner. AT-1 cells were treated with either DMSO (*Men1*$^{f/f}$) or 4-OHT (*Men1*$^{\Delta/\Delta}$) and processed for ChIP assay with either **(A)** anti-MLL-C or **(B)** anti-AF9c antibodies. **(C)** THP-1 cells were transduced with either control scrambled or *Men1* shRNA-expressing lentivirus, and used for ChIP assay with anti-AF9c or anti-Dot1L antibodies.

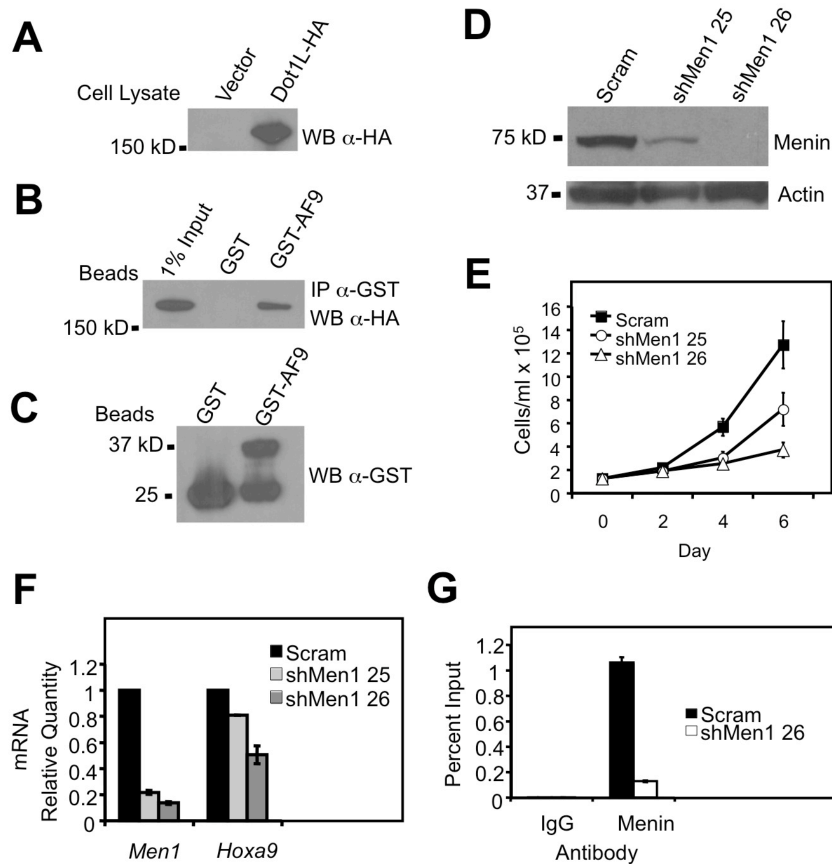


Figure 2.3. Menin KD in THP-1 cells reduces *HOXA9* expression and cell growth. **(A-C)** Immunoprecipitation of HA-tagged Dot1L expressed in 293T cells with recombinant GST-tagged AF9c purified from *E. coli*. **(D)** Western blot for menin in menin KD THP-1 cells. **(E)** Growth curve of control and menin KD THP-1 cells. **(F)** The mRNA levels of *MEN1* and *HOXA9* genes in the control and menin KD THP-1 cells. **(G)** ChIP assay for menin binding to the *HOXA9* promoter in the control and menin KD THP-1 cells.

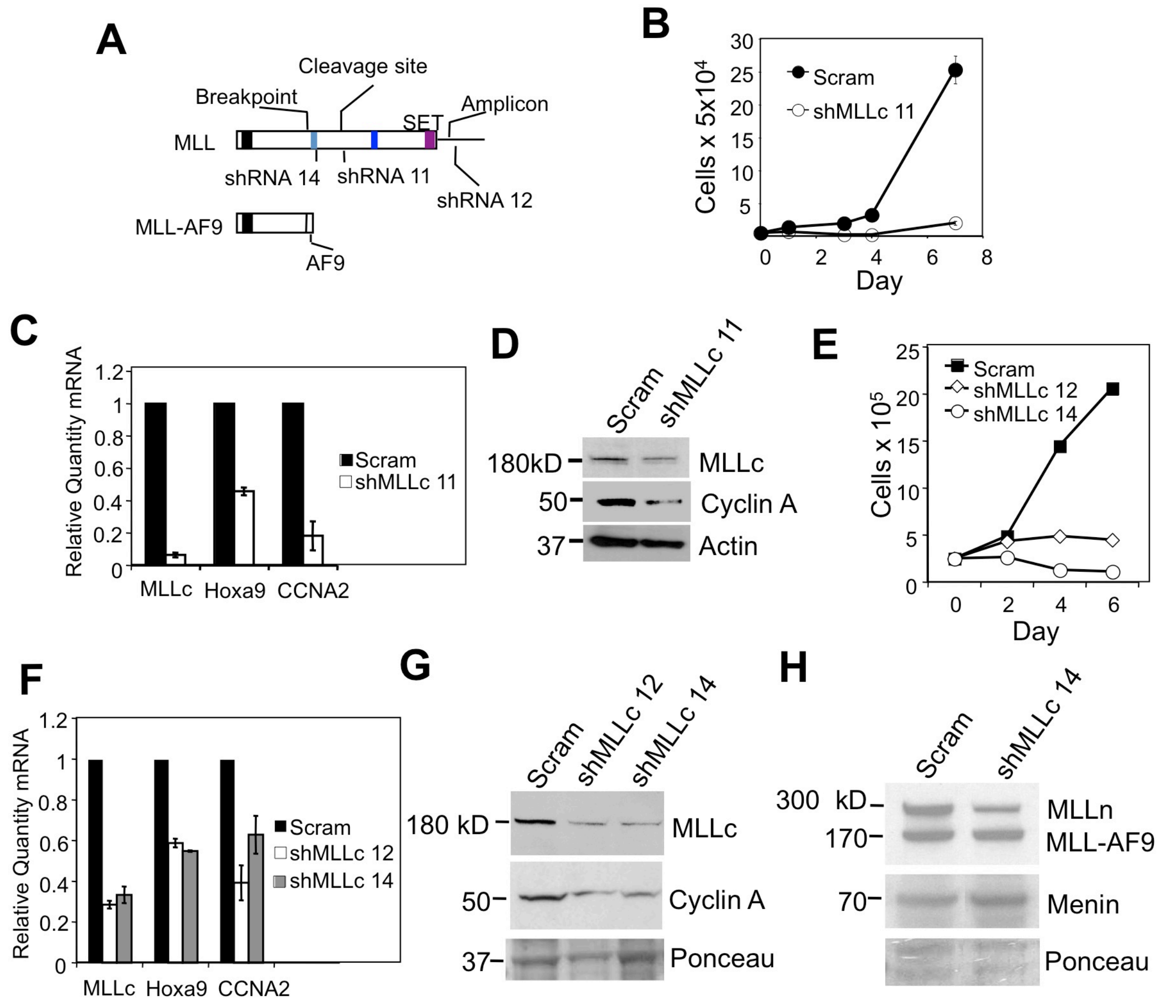


Figure 2.4. WT *Mll* is required for the growth of MA9-transformed leukemia cells and expression of *Hoxa9* and *CCNA2*. (A) A diagram for the structure of WT MLL, MA9 fusion protein, and shRNAs targeting various parts of MLL-C, but not MA9. **(B)** AT-1 cells were transduced with retrovirus containing either vector or MLL-C shRNA 11 and monitored for cell number. **(C, D)** WT *Mll*, *Hoxa9*, and *cyclin A* (*CCNA2*) mRNA **(C)** and MLL-C/cyclin A protein levels **(D)** in control of WT MLL KD AT-1 cells. THP-1 cells were transduced with either control scrambled shRNA lentivirus (Scram) or MLL-C shRNAs. The resulting cells were monitored for change in number **(E)**, mRNA levels of WT *Mll*, *Hoxa9*, and *CCNA2* **(F)** and the protein levels of MLL-C, cyclin A **(G),** MLL-N, MA9, and menin **(H)**.

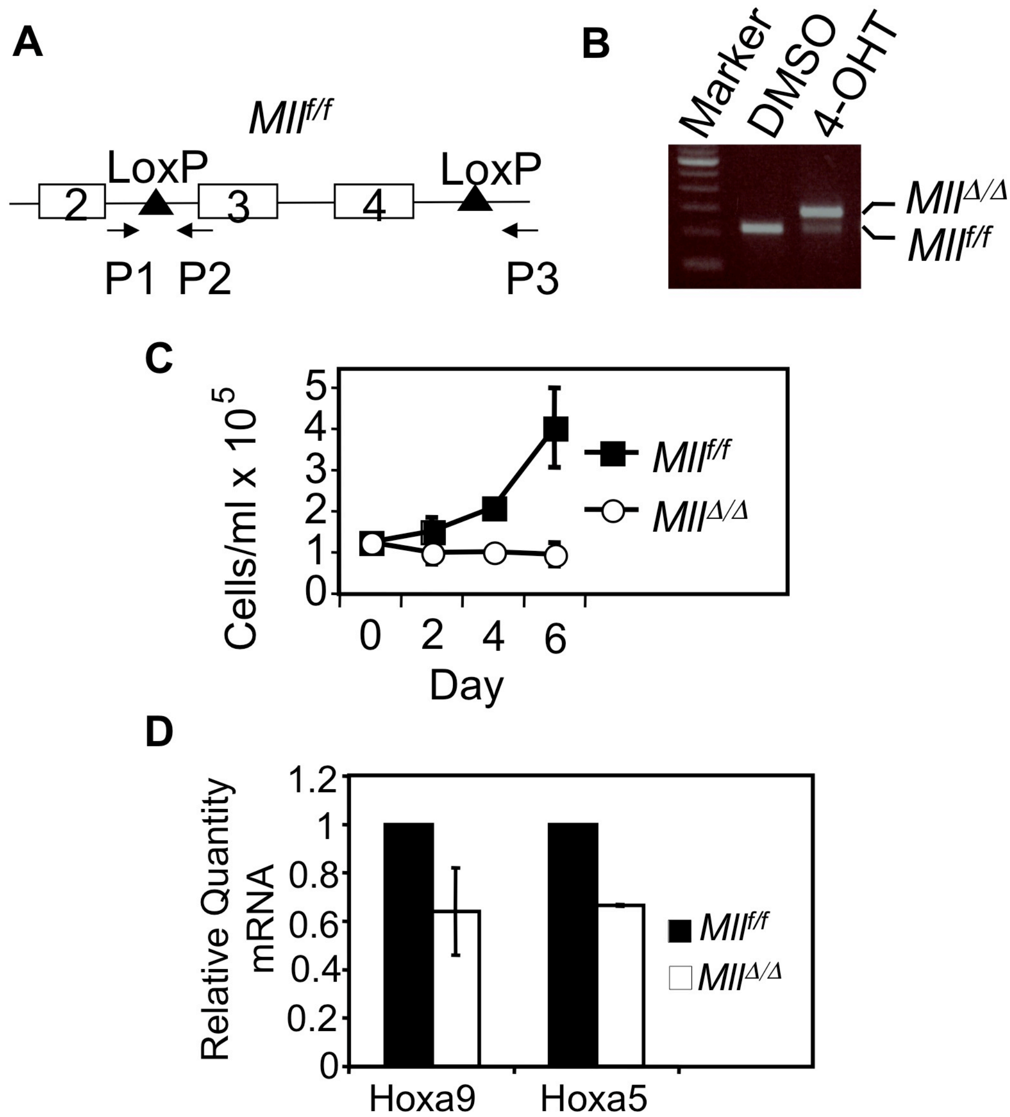


Figure 2.5. WT MLL depletion reduces MA9-transformed cell number and *Hox* gene expression. **(A)** A diagram for the floxed *Mll* and the primers used to detect the intact or excised *Mll*. **(B)** 4-OHT induced excision of the floxed *Mll*. Splenocytes from an $Mll^{f/f}$*;Cre-ER* mouse were cultured with either DMSO or 4-OHT, followed by the isolation of genomic DNA and PCR amplification. **(C)** A growth curve for MA9-transformed BM cells with either $Mll^{f/f}$ or $Mll^{\Delta/\Delta}$. **(D)** Quantification of *Hoxa9* and *Hoxa5* transcript levels in either $Mll^{f/f}$ or $Mll^{\Delta/\Delta}$ MA9-transformed cells.

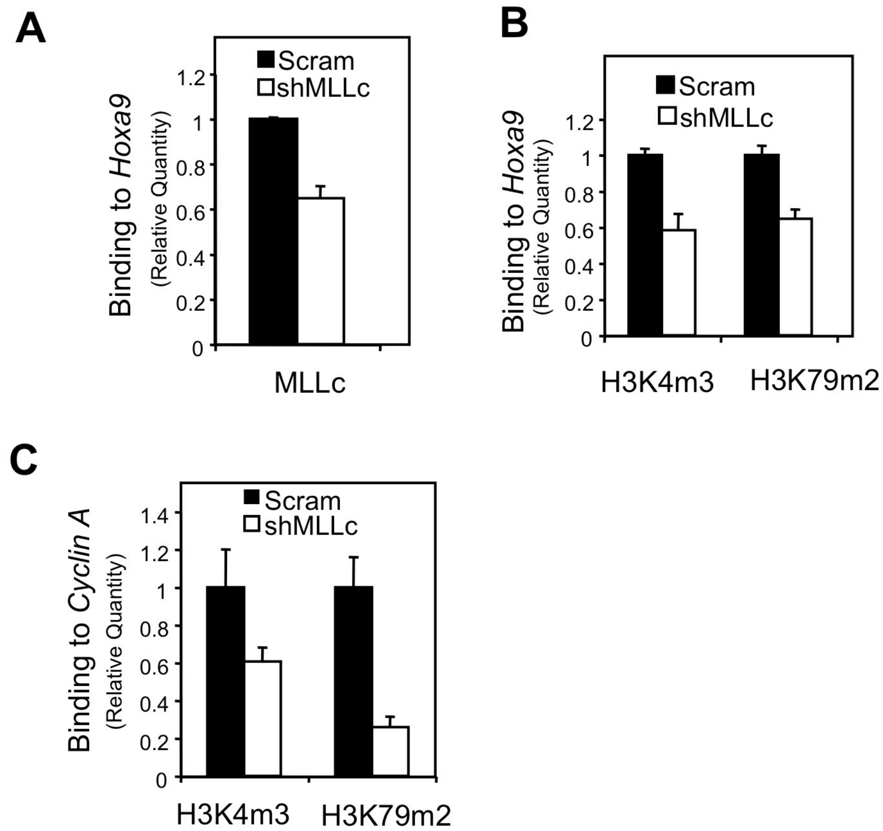


Figure 2.6. WT MLL promotes H3K4 and H3K79 methylation at key target genes. ChIP assay in control or WT MLL KD (shRNA 14) THP-1 cells for MLL-C enrichment at *Hoxa9* **(A)** and for histone H3K4m3 and H3K79m2 at *HOXA9* **(B)** and *CCNA2* **(C)**.

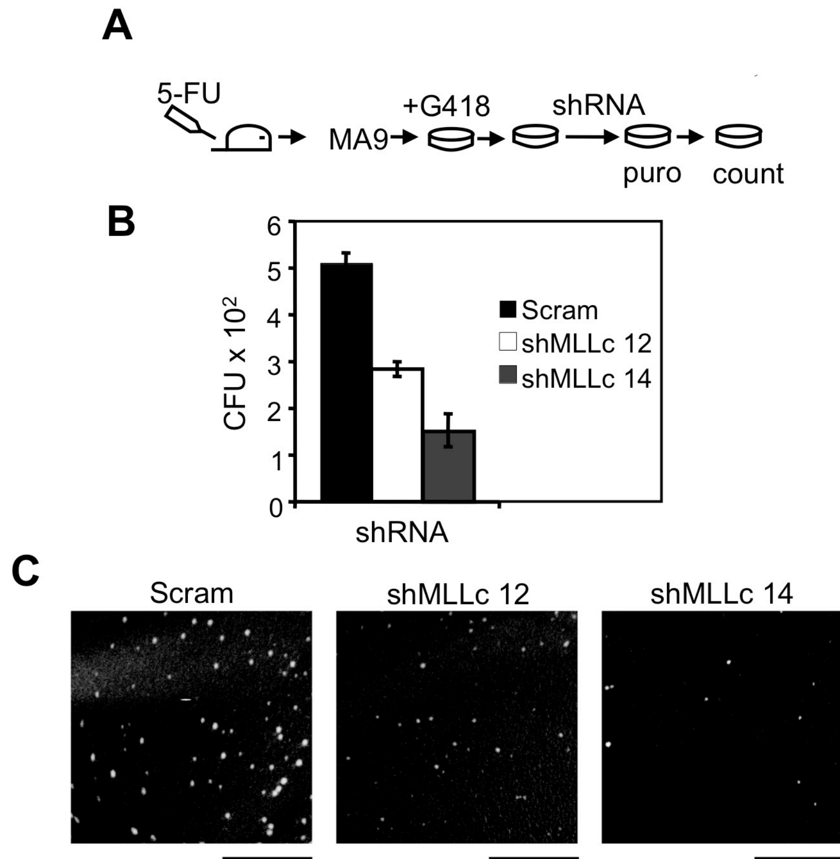


Figure 2.7. WT MLL KD suppresses colony formation of MA9-transduced BM. (A) Procedure for the colony formation assay. BM cells from a C57B6 mouse were transduced with pMSCV-MA9 retrovirus, and replated in triplicate weekly in methylcellulose medium with G418. After the second plating, surviving MA9 cells were transduced with each of the MLL-C shRNAs (12 and 14) or scrambled vector. **(B)** A summary of colony numbers for control or *Mll* shRNA-transduced BM. **(C)** Representative colonies from the culture plates (Scale bars 5mm).

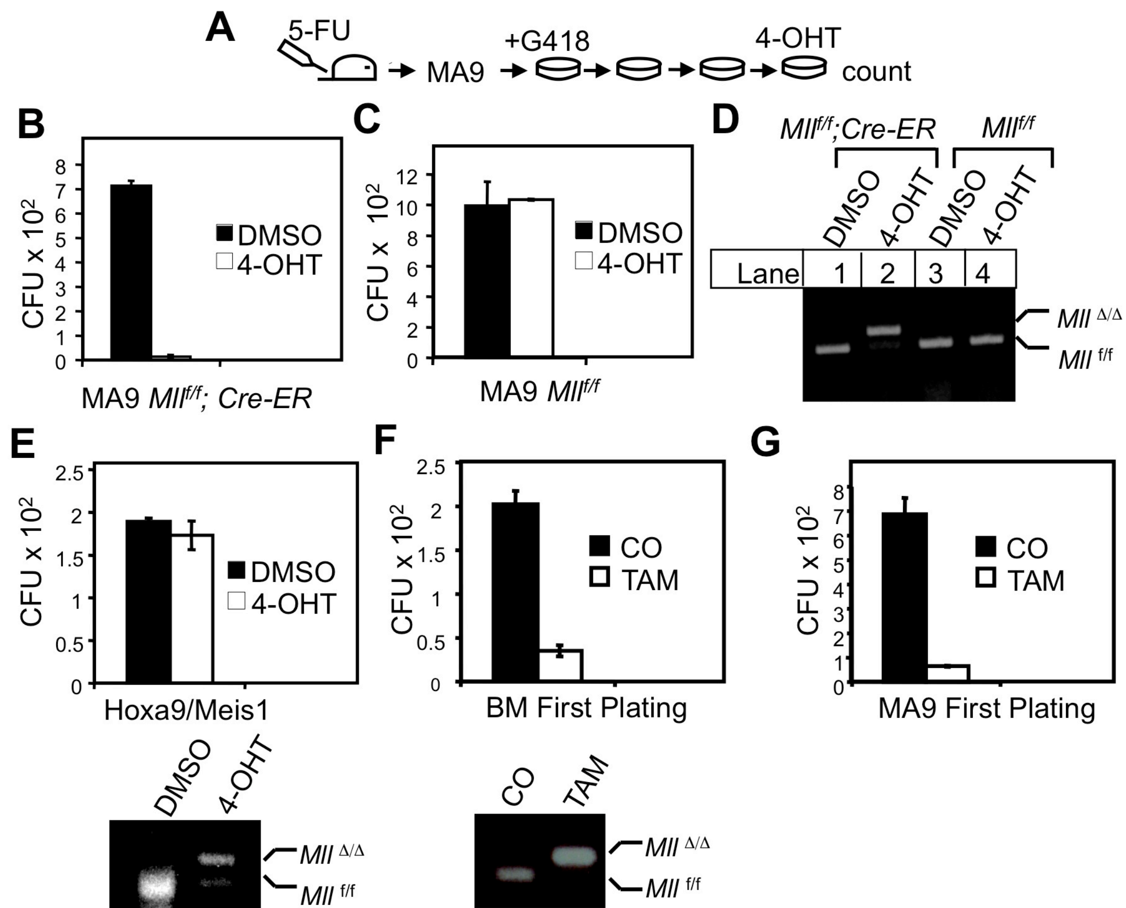


Figure 2.8. WT *Mll* is required for colony formation of MA9-induced BM. (A) A flowchart for procedures of MA9-induced transformation and 4-OHT-induced *Mll* excision. **(B)** *Mll* excision reduced the number of colonies formed by MA9-transduced BM from the *Mll*$^{f/f}$*;Cre-ER* mice. **(C)** 4-OHT failed to reduce colony formation of MA9 retrovirus-transduced BM from *Mll*$^{f/f}$ mice. **(D)** Genotyping with genomic DNA showed that 4-OHT induced *Mll* excision in MA9-transformed cells with the *Mll*$^{f/f}$*;Cre-ER* genotype (lane 2) but failed to induce *Mll* excision in MA9-transformed BM cells with *Mll*$^{f/f}$ but without the *Cre-ER* transgene (lane 4). **(E)** WT *Mll* excision failed to reduce Hoxa9/Meis1-induced BM colony formation (Top). 4-OHT-induced WT *Mll* excision in Hoxa9/Meis1-transformed BM (Bottom). **(F)** WT *Mll* excision reduced colony formation from normal BM. BM from CO or TAM-treated *Mll*$^{f/f}$*;Cre-ER* mice was plated on methylcellulose medium and the colony number was scored at the first plating (Top). WT *Mll* was excised in BM from TAM-treated, but not from corn oil-fed, *Mll*$^{f/f}$*;Cre-ER* mice (Bottom). **(G)** BM from corn oil or TAM-treated *Mll*$^{f/f}$*;Cre-ER* mice was first transduced with MA9, followed by plating on methylcellulose medium, and the colony number was scored at the first plating.

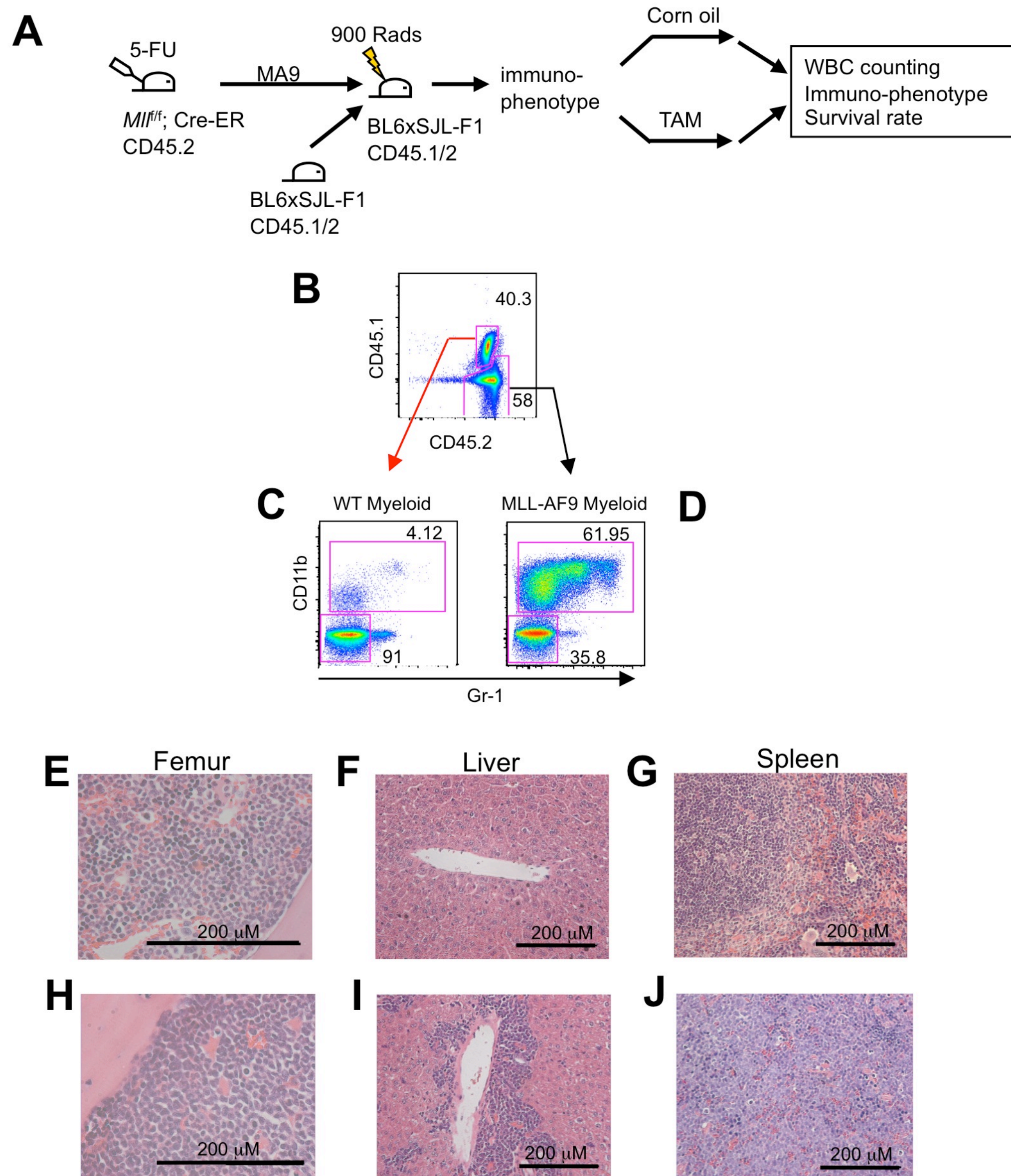


Figure 2.9. Tamoxifen-induced WT *Mll* excision in MA9-transduced cells in recipient mice. **(A)** A diagram for MA9-induced leukemogenesis and WT *Mll* excision in mice. **(B)** Peripheral white blood cells from MA9-BM-transplanted mice were isolated seven weeks after transplantation and stained fluorescence-labeled antibodies, as indicated. The donor cells were only CD45.2+ (lower right quadrant), while recipient or normal co-transplanted BM-derived cells were CD45.1+/2+ (upper right quadrant). A small percentage (4%) of cells in WT recipient PB WBCs were myeloid (CD11b+/Gr1+) **(C)**, but a large percentage (~62%) of the MA9-transformed BM-derived cells were CD11b+ **(D)**. **(E-G)** H&E stained sections of normal BM transplanted mice. **(H-J)** H&E stained sections from MA9-transduced BM transplanted mice.

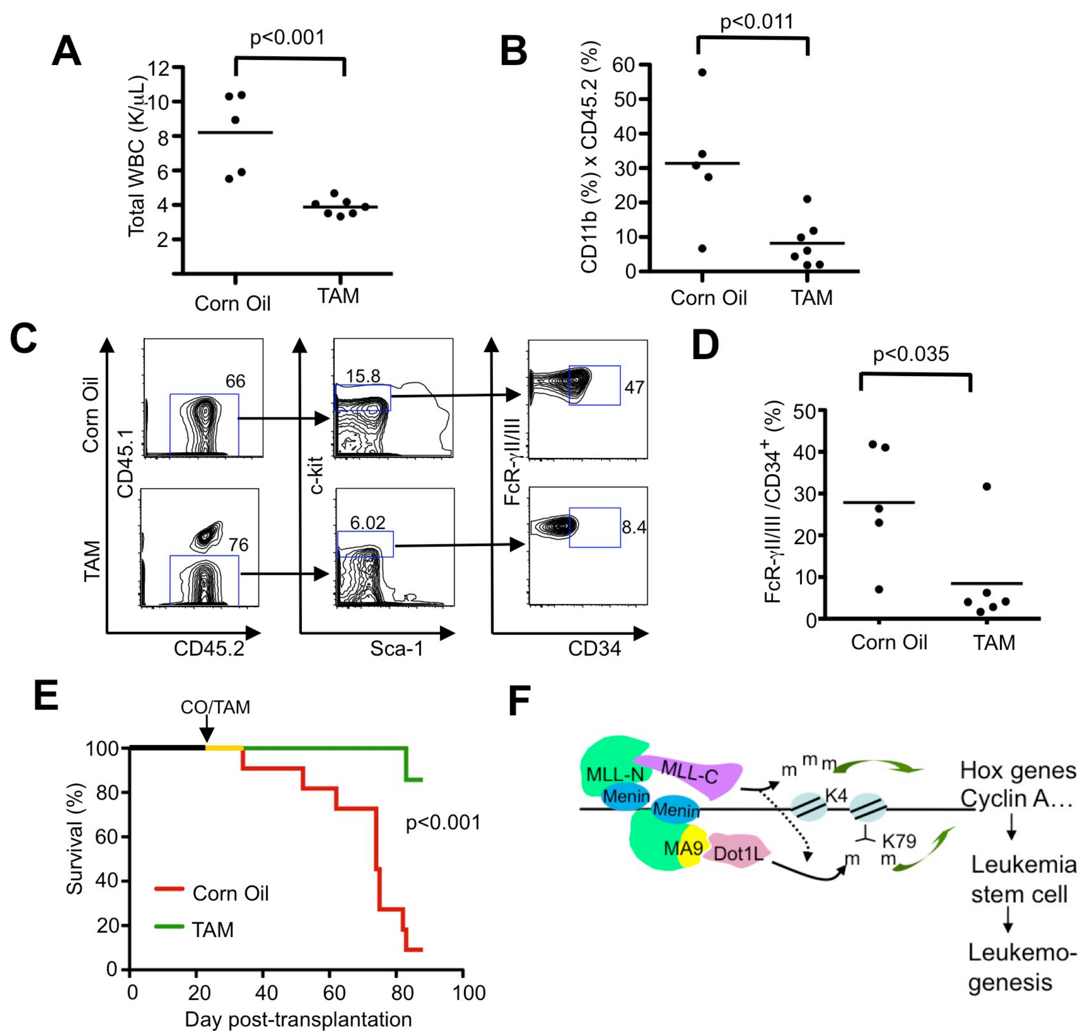


Figure 2.10. WT MLL is required for MA9-induced leukemogenesis in mice. (A) The total peripheral white blood cells (WBCs) in mice transplanted with MA9-transduced *Mll$^{f/f}$;Cre-ER* BM measured five weeks after CO or TAM feeding. **(B)** Flow cytometry analysis of MA9-transformed donor cells from transplanted mice, five weeks after the mice were fed with CO or TAM. **(C)** Flow cytometry analysis of CD45.2+ splenocytes from terminally ill CO- or TAM-fed mice. **(D)** A summary of the percentage of c-kit high cells that were FcRgII/III+/CD34+ from CO or TAM-fed mice. **(E)** Kaplan-Meier curve for mice transplanted with MA9-transduced *Mll$^{f/f}$;Cre-ER* BM that were fed with either CO (n=11) or TAM (n=7), 3 weeks after MA9 BM transplantation. **(F)** A model for menin, WT MLL, and MA9/Dot1L-controlled regulation of coupled yet distinct histone methylation events, enhancing target gene transcription, leading to leukemogenesis.

CHAPTER 3 – THE TRITHORAX PROTEIN PARTNER MENIN ACTS IN TANDEM WITH EZH2 TO SUPPRESS C/EBPα AND DIFFERENTIATION IN MLL-AF9 LEUKEMIA

Summary

Trithorax (Trx) and Polycomb (PcG) proteins antagonistically regulate the transcription of many genes, and cancer can result from the disruption of this regulation. Disruption of Trx function occurs through chromosomal translocations involving the Trx group gene *MLL,* which lead to the expression of MLL fusion proteins and acute leukemia. It is poorly understood how MLL fusion proteins block differentiation, a hallmark of leukemogenesis. Here we show that acute depletion of the Trx protein MLL, or menin, a close partner of MLL that is critical for MLL and MLL-AF9 recruitment to target genes, triggers MLL-AF9 cell differentiation. Differentiation occurs independent of *Hoxa9/ Meis1*, known MLL-AF9 target genes. We have found that menin binds the promoter of the polycomb gene *Ezh2*, and promotes its expression, also independent of *Hoxa9/Meis1*. EZH2 interacts with the differentiation-promoting transcription factor C/EBPα, and specifically represses C/EBPα target genes. Menin depletion reduces EZH2 expression, EZH2 binding and repressive H3K27 methylation at C/EBPα target genes, and induces the expression of pro-differentiation C/EBPα targets. In contrast to its classical role antagonizing Trx function, the PcG protein EZH2 collaborates with Trx-associated menin to block MLL-AF9 leukemia cell differentiation, uncovering a novel mechanism for suppression of C/EBPα and leukemia cell differentiation.

Introduction

Trithorax (Trx) and polycomb (PcG) group proteins have opposing roles in the regulation of key genes involved in development and stem cell maintenance (Schuettengruber et al., 2011). The expression of these common target genes is tightly regulated during cell differentiation, with PcG complexes repressing and Trx promoting transcription (Mills, 2010). PcG and Trx proteins regulate transcription by influencing chromatin structure, in part through covalent modification of histones. One group of genes regulated in this manner is *HOX* genes (Hanson et al., 1999). *HOX* gene expression is maintained at high levels in hematopoietic progenitors, and is coordinately decreased during blood cell differentiation (Pineault et al., 2002).

The Trx group protein MLL maintains *HOX* gene expression in hematopoietic progenitors, at least in part through trimethylation of histone H3 at lysine 4 (H3K4m3) via its C-terminal SET domain (Terranova et al., 2006). Chromosomal translocations involving the *MLL* gene result in the formation of MLL fusion proteins (MLL-FPs), which disrupt normal MLL function, causing acute leukemia. *MLL* translocated leukemias represent ~10% of adult acute leukemias and the majority of infant leukemia cases, and these patients have a poor prognosis (Daser and Rabbitts, 2004; Holleman et al., 2004).

MLL-FPs promote the expression of a subset of wild-type (WT) MLL target genes, including *HOX* genes, through the recruitment of the histone H3K79 methyltransferase Dot1L and the pTEFb complex, enhancing transcriptional elongation (Bernt et al., 2011; Mueller et al., 2009; Nguyen et al., 2011; Okada et al., 2005; Wang et al., 2011). MLL-FPs lack a large C-terminal portion of the WT MLL protein, including the histone H3 lysine 4 (H3K4)-methylating SET domain. This functional deficiency is remedied by expression of WT MLL from the non-translocated *MLL* allele. WT MLL works in concert with MLL-FPs to upregulate *HOX* genes and promote leukemia, highlighting a critical role for WT MLL in this disease (Chapter 2) (Thiel et al., 2010).

WT MLL and MLL-FPs are recruited to target genes through their interaction with menin, a protein encoded by the *Men1* gene (Figure 2.2) (Chen et al., 2006; Hughes et al., 2004; Thiel et al., 2010; Yokoyama et al., 2004). X-ray crystallographic studies have recently shown that menin interacts with the identical N-terminal sequences of both WT MLL and MLL-FPs via a deep central pocket, demonstrating that menin is a close partner of these proteins (Huang et al., 2012; Murai et al., 2011). This interaction is required for leukemic transformation, demonstrating a central role for menin in MLL-FP leukemia (Caslini et al., 2007).

A major hallmark of leukemia and consequence of MLL-FP expression is a block in hematopoietic differentiation (Huntly and Gilliland, 2005). MLL-AF9 (MA9) leukemia

cells have a block in the myeloid lineage at the granulocyte-macrophage progenitor stage, with cells expressing high levels of the cell surface receptor c-kit being enriched for leukemia-initiating cells (LICs) (Chen et al., 2008; Krivtsov et al., 2006; Somervaille and Cleary, 2006; Wang et al., 2010). While *HOX* genes are at least partially responsible for this suppression of differentiation, it remains unclear how MLL-FP leukemia cells are blocked at the progenitor stage (Faber et al., 2009; Kroon et al., 1998). In addition, global analysis has identified over 200 direct MLL-FP target genes, some of which could also have a role in blocking differentiation (Wang et al., 2011).

C/EBPα is a leucine zipper transcription factor that promotes myeloid differentiation in part through the activation of differentiation-associated genes. Many leukemogenic oncogenes and pathways inhibit the expression/function of C/EBPα as a means of blocking differentiation, including Bcr-Abl, AML-ETO, and Notch/Trib2 (Koschmieder et al., 2009). However, little is known as to whether repression of C/EBPα is involved in blocking differentiation in MLL-FP leukemias.

Polycomb repressive complex 2 (PRC2) consists of Suz12, EED, RbAp46/48, and the catalytic component EZH2, which methylates H3K27, leading to transcriptional repression. EZH2 point mutations are found in about 10% of MDS cases, suggesting that PRC2 acts as a tumor suppressor in the myeloid lineage (Ernst et al., 2010; Nikoloski et al., 2010). However, recent work has demonstrated that ectopic expression of EZH2

causes a block in myeloid differentiation, leading to the development of myeloproliferative disease (Herrera-Merchan et al., 2012). Thus, the role of EZH2 in hematopoietic development and leukemia is not well understood. Utilizing murine models for MA9 leukemia, we set out to examine the role of the Trx protein MLL and its partner menin in regulating MA9 cell differentiation *in vivo*, which led to the finding that the polycomb protein EZH2 is a collaborating factor in suppressing C/EBPα and differentiation in MA9-induced leukemia.

Results

Acute menin depletion causes MA9 cell differentiation in culture

To determine the effect of acute menin depletion on MA9 cell differentiation, we utilized the murine MA9 cell line AT-1, which contains floxed *Men1* alleles and the *Cre-ER* transgene, allowing *Men1* excision by addition of 4-hydroxytamoxifen (4-OHT) to the culture medium (Chen et al., 2006). 4-OHT treatment effectively excised the floxed *Men1* alleles within 2 days (Figure 3.1A), and markedly reduced menin protein expression by day 4 (Figure 3.1B). We measured cell differentiation by flow cytometry using the terminal myeloid differentiation marker Gr-1, and found little effect on Gr-1 cell surface expression 4 days after *Men1* excision, but a marked increase in the percentage of cells expressing high levels of Gr-1 by day 6 (Figure 3.1C), indicating that terminal myeloid differentiation follows reduced menin expression. This increase in Gr-1

high cells also corresponded with a cell morphology change consistent with myeloid differentiation (Figure 3.1D, right).

The ability to observe cell differentiation in this setting allowed us to next explore how menin depletion affects genes that regulate this process over time. The direct menin/MA9 target gene *Hoxa9*, which is at least partially responsible for the MA9-mediated differentiation block (Faber et al., 2009; Kroon et al., 1998), was decreased as early as day 4 post 4-OHT treatment, prior to cell differentiation (Figure 3.1E). However, overexpression of Hoxa9 in combination with its cofactor Meis1 did not completely rescue differentiation caused by menin depletion (Figure 3.1F), suggesting an additional function for menin in suppressing MA9 leukemia cell differentiation. Interestingly, *Mcsfr* and *Gcsfr*, cytokine receptors important for myeloid differentiation, were upregulated by day 6 post 4-OHT treatment (Figure 3.1G, H). To determine if menin is also important for blocking the differentiation of human MLL-FP-expressing leukemia cells, we used shRNAs targeting *MEN1* in the human MA9 cell line THP-1. Menin knockdown (KD) increased cell surface expression of CD11b, a prominent myeloid differentiation marker in human leukemia cells, and also increased *MCSFR* transcript levels (Figure 3.2 A, B). Together, these results indicate that menin depletion upregulates genes associated with myeloid differentiation and causes MA9 cell differentiation.

Acute menin depletion causes MA9 cell differentiation *in vivo*

To determine if menin depletion is pathologically relevant to MA9-induced leukemia, we transduced *Men1$^{f/f}$; Cre-ER* bone marrow (BM) with MA9-ires-GFP retrovirus and transplanted these cells into lethally irradiated recipient mice (Figure 3.3A). When we observed substantial GFP^{+} cells in the peripheral blood of recipient mice, we treated the mice with either corn oil (CO) control or CO with tamoxifen (TAM) by oral gavage to excise *Men1* in the transplanted cells. At days 4 and 7 post-initial gavage, we isolated splenocytes from each treatment group for analysis by flow cytometry. Flow cytometry analysis revealed a striking increase in the percentage of Gr-1^{high} MA9 (GFP^{+}) cells in *Men1*-excised splenocytes as early as 4 days post-initial gavage, and this effect became even more pronounced at day 7 (Figure 3.3B, D). Consistent with this increased Gr-1^{high} population, we observed mature differentiated myeloid cells in H&E-stained spleen sections from mice treated with TAM to deplete menin (Figure 3.3C). In contrast to the immediate increase in the percentage of Gr-1^{high} cells, the c-kit^{high} population, which is enriched for LICs, was unchanged at day 4 post-treatment, but decreased significantly by day 7 (Figure 3.3E). Further analysis of the relationship between c-kit and Gr-1 revealed an increase in the c-kit^{high}/Gr-1^{high} population at day 4 (Figure 3.4A), followed by a loss of this population at day 7 (Figure 3.4B). We also observed increased staining for the apoptotic marker Annexin V at day 7 (Figure 3.4C). These results suggest that as a consequence of menin depletion, c-kit high cells first gain expression of Gr-1, then either lose c-kit expression during the process of differentiation or undergo cell death, leading to the depletion of c-kit high MA9 LICs.

WT MLL depletion causes MA9 cell differentiation *in vivo*

Due to the observed effect of menin depletion on MA9 cells, we hypothesized that acute depletion of WT MLL, which interacts with menin and is required to maintain MA9-mediated transformation (Chapter 2), would also cause MA9 cell differentiation *in vivo*. To this end, we isolated control and WT *Mll*-excised MA9 (GFP^+) Splenocytes for plating in methylcellulose and performed flow cytometry to evaluate leukemia cell differentiation status. WT MLL-depleted MA9 splenocytes were deficient in colony formation in methylcellulose as compared to their WT MLL-containing counterparts, suggesting that MA9 cells lacking WT MLL are deficient in LICs when derived from an *in vivo* setting (Figure 3.5A). Consistent with the effect of menin depletion, flow cytometry analysis of MA9 splenocytes lacking WT MLL demonstrated a significant increase in the percentage of $Gr\text{-}1^{high}$ cells, and a significant decrease in the $c\text{-}kit^{high}$ population compared to controls, suggesting that MA9 cells also undergo differentiation in response to WT MLL depletion (Figure 3.5C-E). These findings demonstrate that a key Trx group gene, *Mll*, is critical for blocking MA9 leukemia cell differentiation.

Given that WT MLL depletion had a less pronounced increase in $Gr\text{-}1^{high}$ MA9 cells than that of menin, we wondered whether Gr-1 low cells lacking WT MLL were defective in propagating leukemic disease. To address this question, we isolated control and WT *Mll*-excised MA9 splenocytes from primary recipients, sorted into either $Gr\text{-}1^{low}$ or $Gr\text{-}1^{high}$ MA9 cell populations, and transplanted these cells into lethally irradiated secondary

recipient mice. WT MLL-depleted Gr-1^{low} recipients had a significantly longer survival rate than the WT MLL-containing Gr-1^{low} cohort (Figure 3.5F), likely due to the decreased population of c-kit^{high} cells in this group (Figure 3.5E). This survival effect may have been less pronounced due to the eventual outgrowth of MA9 cells resistant to *Mll* excision in about half of the TAM-treated Gr-1^{low} recipients (data not shown). Consistent with previously published results, Gr-1^{high} recipient mice from each treatment group had significantly longer survival rates than their Gr-1^{low} counterparts (Figure 3.5F) (Wang et al., 2010). Together, these data demonstrate a role for WT MLL in maintaining a population of Gr-1^{low}/c-kit^{high} MA9 cells, which possess the ability to propagate leukemic disease.

Menin depletion leads to the upregulation of C/EBPα target genes in MA9 cells *in vivo*

To investigate a potential mechanism for the menin/WT MLL-mediated block in myeloid differentiation, we performed a cDNA microarray using primary GFP^{+} MA9 splenocytes from either control or *Men1*-excised recipient mice. We isolated cells at day 4 post-initial gavage to determine genes affected by menin depletion, and performed gene set enrichment analysis (GSEA) of the microarray data to identify groups of genes regulated by menin that are associated with differentiation. GSEA revealed a significant overlap of menin-regulated genes with C/EBP transcription factor target genes (Figure 3.6A),

consistent with upregulation of C/EBPα target genes *Mcsfr* and *Gcsfr* in AT-1 cells, leading us to investigate a role for menin in repressing C/EBPα function.

As multiple leukemogenic pathways affect the expression level of C/EBPα p42 or increase the ratio of the dominant negative p30 isoform over p42 (Koschmieder et al., 2009), we first explored whether menin depletion affected C/EBPα expression or target gene binding in AT-1 cells. Although overexpression of C/EBPα-ER followed by activation via addition of 4-OHT to the culture medium was able to drive differentiation (Figure 3.6B), we failed to observe a menin-dependent effect on C/EBPα protein levels (Figure 3.6C). In addition, there was no detectable expression of C/EBPα p30, which has been reported to promote leukemia development (Figure 3.6C) (Kirstetter et al., 2008). Chromatin immunoprecipitation (ChIP) assay indicated that C/EBPα was also able to bind the promoter of its target gene *Mcsfr* regardless of menin expression (Figure 3.6D). The lack of a direct effect on C/EBPα protein led us to investigate a potential role for menin in actively repressing C/EBPα target genes.

Menin promotes EZH2 and Evi-1 expression in MA9-expressing leukemia cells

In exploring potential repressors of C/EBPα function that may be regulated by menin/WT MLL, we observed that *Mecom*, which encodes the transcription factor Evi-1, was the most down-regulated gene in response to acute menin depletion *in vivo* and was

also decreased in *Men1*-excised AT-1 cells (Figure 3.7A). Evi-1 is directly activated by MA9, and is required for the maintenance of MLL-ENL transformed cells (Goyama et al., 2008). An Evi-1 oncogenic fusion protein has been shown to repress C/EBPα at target genes, and Evi-1 has been reported to mediate transcriptional repression of the tumor suppressor PTEN through its interaction with PcG proteins, including EZH2 (Arai et al., 2011; Tokita et al., 2007; Yoshimi et al., 2011).

Surprisingly, we found that expression of EZH2, the catalytic component of polycomb repressive complex 2 (PRC2), was also decreased upon menin depletion in MA9-expressing cells (Figure 3.7 A, B), while PTEN expression, which is suppressed by EZH2 in Evi-1-induced leukemia cells (Yoshimi et al., 2011), was unaffected (data not shown). This effect on EZH2 occurs independent of *Hoxa9/Meis1*, as overexpression of these genes is unable to rescue the decrease in *Ezh2* transcript levels due to menin depletion (Figure 3.7C). These findings suggest that in addition to *Mecom*, *Ezh2* is a direct target of menin/MA9. Along these lines, ChIP assay showed enrichment for menin and the portion of AF9 found in MA9 (AF9c) at the *Ezh2* and *Mecom* promoters. As expected, this enrichment was diminished by menin depletion (Figure 3.7 D, E). H3K4m3 and H3K79m2, which are associated with upregulation of gene transcription (Schuettengruber et al., 2011; Steger et al., 2008), were also substantially decreased at the *Mecom* locus in response to menin depletion (Figure 3.7F). *Men1* excision also reduced these marks at certain part of the *Ezh2* locus (Figure 3.7G, amplicon 1), albeit with a milder effect than at the *Mecom* locus. Together, these results demonstrate that menin promotes EZH2 and

Evi-1 expression, suggesting that menin/MA9 promote EZH2-mediated repression of C/EBPα target genes, leading to a block in MA9 leukemia cell differentiation.

EZH2 interacts with C/EBPα and represses its transcriptional activity

To test whether EZH2 inhibits C/EBPα function, we first determined whether EZH2 could repress C/EBPα-mediated transcriptional activation. To this end, we transfected 293T cells with a C/EBPα binding site-containing promoter-driven luciferase reporter (Keeshan et al., 2006), C/EBPα, and increasing amounts of EZH2 expression plasmids. C/EBPα robustly activated the luciferase reporter, and EZH2 repressed C/EBPα-mediated activation in a dose-dependent manner (Figure 3.8A). As a control, EZH2 was unable to repress Gli1-activated luciferase (Figure 3.8B). As EZH2 could repress C/EBPα-mediated activation, we decided to test the possibility that EZH2 physically interacts with C/EBPα in MA9 cells by performing immunoprecipitation (IP) experiments using endogenously expressed proteins in THP-1 cells. IP of C/EBPα was able to bring down EZH2 (Figure 3.8C), and conversely, IP of EZH2 pulled down C/EBPα (Figure 3.8D), showing that these proteins physically interact at endogenous levels in MA9 leukemia cells.

EZH2 binds C/EBPα target genes and suppresses MA9 cell differentiation

Since EZH2 interacts with C/EBPα in MA9 cells and represses its transcriptional activity, we reasoned that EZH2 and its interacting protein Evi-1 could bind C/EBPα target genes to mediate direct repression. To explore this possibility, we performed ChIP to determine whether Evi-1 and EZH2 bind to the promoter of the C/EBPα target gene *Mcsfr* in AT-1 cells. ChIP assay showed that Evi-1 and EZH2 binding were enriched at the *Mcsfr* promoter, and menin depletion by addition of 4-OHT led to decreased promoter occupancy of both Evi-1 and EZH2 (Figure 3.9A). As EZH2 mediates transcriptional repression through catalysis of histone H3 lysine 27 trimethylation (H3K27m3), we also tested whether H3K27m3 is reduced at the *Mcsfr* promoter in response to *Men1* excision. Consistent with EZH2 ChIP results, H3K27m3 was enriched at the *Mcsfr* promoter, and was decreased in menin-depleted cells (Figure 3.9B), supporting a model where EZH2 interacts with C/EBPα at target gene promoters, leading to increased H3K27m3 and transcriptional repression of C/EBPα target genes in MA9 cells.

Since EZH2 occupies the *Mcsfr* promoter in MA9 cells and inhibits C/EBPα−mediated transcriptional activation, we decided to test whether loss of EZH2 causes upregulation of C/EBPα target genes and MA9 cell differentiation by using shRNAs to knock down EZH2 in THP-1 cells. Transduction of THP-1 cells with each of three different shRNAs resulted in a reduction of EZH2 expression compared to the scrambled (Scr) control (Figure 3.9C, D). EZH2 KD led to a dose-dependent increase in *MCSFR* transcript

levels, but did not affect *HOXA9* expression (Figure 3.9D). EZH2 KD also caused a dose-dependent increase in the percentage of CD11b positive cells (Figure 3.9E). Taken together, these results highlight EZH2 as a necessary component for the MA9-mediated block in myeloid differentiation, revealing a novel mechanism by which myeloid differentiation is inhibited via EZH2-mediated repressive histone methylation.

Discussion

Depletion of the Trx protein MLL or its partner menin triggers MA9 cell differentiation

One of the major mechanisms for MA9-mediated leukemogenesis is causing a block in mature myeloid differentiation (Schreiner et al., 2001), but it is poorly understood how MA9 cells are blocked in their mature differentiation. While Trx and PcG proteins are well known for their antagonizing function in regulating gene expression, little has been known as to whether and how they might work together to regulate differentiation in acute myeloid leukemia.

We found that excision of either *Men1* or *Mll* in MA9-expressing leukemia cells *in vivo* significantly increased the mature myeloid differentiation of the MA9 leukemia cells and decreased the population of c-kit^{high} cells, which are enriched for LICs. In addition to c-kit, Gr-1 expression is a functional indicator, as Gr-1^{high} cells are deficient in the ability to cause transplantable leukemic disease (Figure 3.5F).

Interestingly, menin depletion increased the Gr-1^{high} population as early as 4 days after the initial TAM treatment, before the c-kit^{high} population was diminished (Figure 3.3D, E). Closer examination of flow cytometry data showed an increase in c-kit^{high} cells expressing Gr-1 at the day 4 time point, and this population was lost at day 7, either due to decreased c-kit expression or apoptotic cell death, as Annexin V staining was also increased as a result of menin loss at day 7 (Figure 3.4C). These results suggest that increased Gr-1 expression is an early indicator of MA9 cell differentiation, followed by c-kit loss and/or cell death following *Men1* deletion. While Hoxa9 and Meis1 are well known for their crucial role in MLL-FP-induced leukemia (Faber et al., 2009; Kroon et al., 1998; Zeisig et al., 2004), we found that menin blocks the mature differentiation of MA9 leukemia cells independent of Hoxa9/Meis1 (Figure 3.1F).

The acute effect of menin depletion in MA9 cells was more drastic than that of WT MLL depletion on differentiation and apoptosis. WT MLL KD in human MA9 cells causes decreased H3K79m2 at target genes, suggesting that WT MLL is required for MA9 function (Figure 2.6B, C) (Thiel et al., 2010). Additionally, in MLL-null MEF cells, MA9 is unable to bind the *Hoxa9* promoter (Milne et al., 2010). However, there is no physical interaction between WT MLL and MA9, suggesting that MA9 recruitment resulting from WT MLL function at target gene promoters is an indirect process. In contrast, menin directly binds both WT MLL and MA9 via their common N-terminal domains and is required for their recruitment to target genes. The acute effect of menin loss could be more drastic than WT MLL loss alone, because menin is directly

responsible for the recruitment of both WT MLL and MA9 to target genes to enhance their transcription.

Menin promotes EZH2 transcription in MA9 leukemia cells

Little has been known about the role of Trx complex components menin and MLL in regulating the expression of their rival polycomb genes. We have found that menin binds the *Ezh2* promoter (Figure 3.7D), and menin promotes EZH2 expression in MA9 leukemia cells. We have observed a substantial reduction in EZH2 expression following *Men1* excision and modest decrease in H3K4m3 and H3K79m2 at the locus in response to menin loss (Figure 3.7G), indicating a crucial role for menin in promoting the EZH2 expression (Figure 3.7A, B). It is not yet clear whether menin regulates the expression of EZH2 in other leukemias or normal hematopoietic cells, or why menin might promote the expression of protein complexes that oppose its function at common target genes. One possibility is that during development or in stem/progenitor cells, menin/MLL promote the expression of PRC components to preserve "bivalent" histone methylation, with both H3K4m3 and H3K27m3 at relevant promoters, leaving these genes repressed, but poised for activation (Mills, 2010). The maintenance of bivalency is critical for the regulated expression of these genes, and may be the rationale behind menin/MLL-mediated activation of PRC protein expression in a developmental context.

EZH2 represses C/EBPα target genes in MA9 leukemia cells

C/EBPα is a critical transcription factor that controls myeloid differentiation (Nerlov, 2004), and its normal function is inhibited through mutation, aberrant expression, or oncogene-mediated suppression, contributing to a block in myeloid differentiation in various leukemias (Nerlov, 2004; Reckzeh and Cammenga, 2010). Little has been known about whether and/or how C/EBPα is regulated in MA9 leukemia cells. Forced expression of C/EBPα drives MLL-FP cell differentiation (Figure 3.6B) (Matsushita et al., 2008), but C/EBPα protein levels and target gene promoter binding are unchanged in response to menin depletion (Figure 3.6C, D), suggesting an alternative method for menin-mediated repression of C/EBPα target genes in MA9 leukemia cells.

We have found that EZH2 physically associates with C/EBPα, binds to the promoter of the C/EBPα target gene *Mcsfr* in MA9 cells, and represses C/EBPα target genes (Figure 3.8, 3.9A). The direct MA9 target Evi-1 is required for MLL-ENL-mediated transformation, is found in polycomb complexes, and also binds the *Mcsfr* promoter (Figure 3.9A) (Arai et al., 2011; Goyama et al., 2008). Menin does not affect C/EBPα expression. Rather, menin induces expression of EZH2, which then suppresses the expression of C/EBPα targets and blocks the differentiation of MA9 cells. These findings reveal a previously unappreciated mechanism for suppressing C/EBPα and MA9 leukemia cell differentiation (Figure 3.9F).

Consistent with our findings, recent reports show that EZH2 depletion causes primary MLL-FP leukemia cell differentiation (Neff et al., 2012; Shi et al., 2012). The role of EZH2 in regulating C/EBPα function during normal hematopoiesis and in other leukemia types remains to be examined. Although EZH2 is required for stem/progenitor cell expansion in developmental hematopoiesis, EZH2 depletion in adult BM is less severe, with no effect on LSK cells, but impaired differentiation of lymphoid cells, and increased myelo-erythroid progenitors, suggesting that EZH2 does regulate at least some stages of myeloid differentiation (Mochizuki-Kashio et al., 2011). Future studies will clarify whether EZH2 specifically regulates C/EBPα function in MA9 cells or in a broader spectrum of cell types.

A context-dependent role for EZH2 in hematopoietic malignancies

The role of EZH2 in hematopoietic malignancies is complex, and seems to be context dependent. In myelodysplastic/ myeloproliferative disorders, mono- or bi-allelic mutations were found in 12% of patients, suggesting EZH2 may be a tumor suppressor in the myeloid lineage (Ernst et al., 2010; Nikoloski et al., 2010). However, in lymphomas of germinal center origin, a specific recurring point mutation in the SET domain of EZH2, converting tyrosine 646 to cytosine has been observed (Morin et al., 2010; Ryan et al., 2011). This mutation causes increased H3K27 methyltransferase activity, suggesting that EZH2 function promotes tumorigenesis in this disease (Sneeringer et al., 2010; Yap et al., 2011). EZH2 is involved in suppressing PTEN expression and

promoting AKT signaling in Evi-1-induced leukemia cells (Yoshimi et al., 2011), yet we found that EZH2 knockdown did not affect PTEN expression in MA9 leukemia cells, suggesting an alternative mechanism for EZH2 in regulating MA9 cell differentiation.

Consistent with the role of EZH2 in regulating differentiation, EZH2 has been reported to promote MA9 leukemias (Neff et al., 2012; Shi et al., 2012). It has been unclear as to how mature differentiation of MA9-induced leukemia is blocked. We found for the first time that in MA9-induced leukemia, menin plays a key role in promoting the expression of the polycomb protein EZH2. EZH2 cooperates with menin to epigenetically suppress the expression of pro-differentiation C/EBPα targets and block the mature differentiation of MA9 leukemia cells (Figure 3.9F). These findings unravel a novel mechanism for EZH2 in blocking MLL-AF9 leukemia cell differentiation.

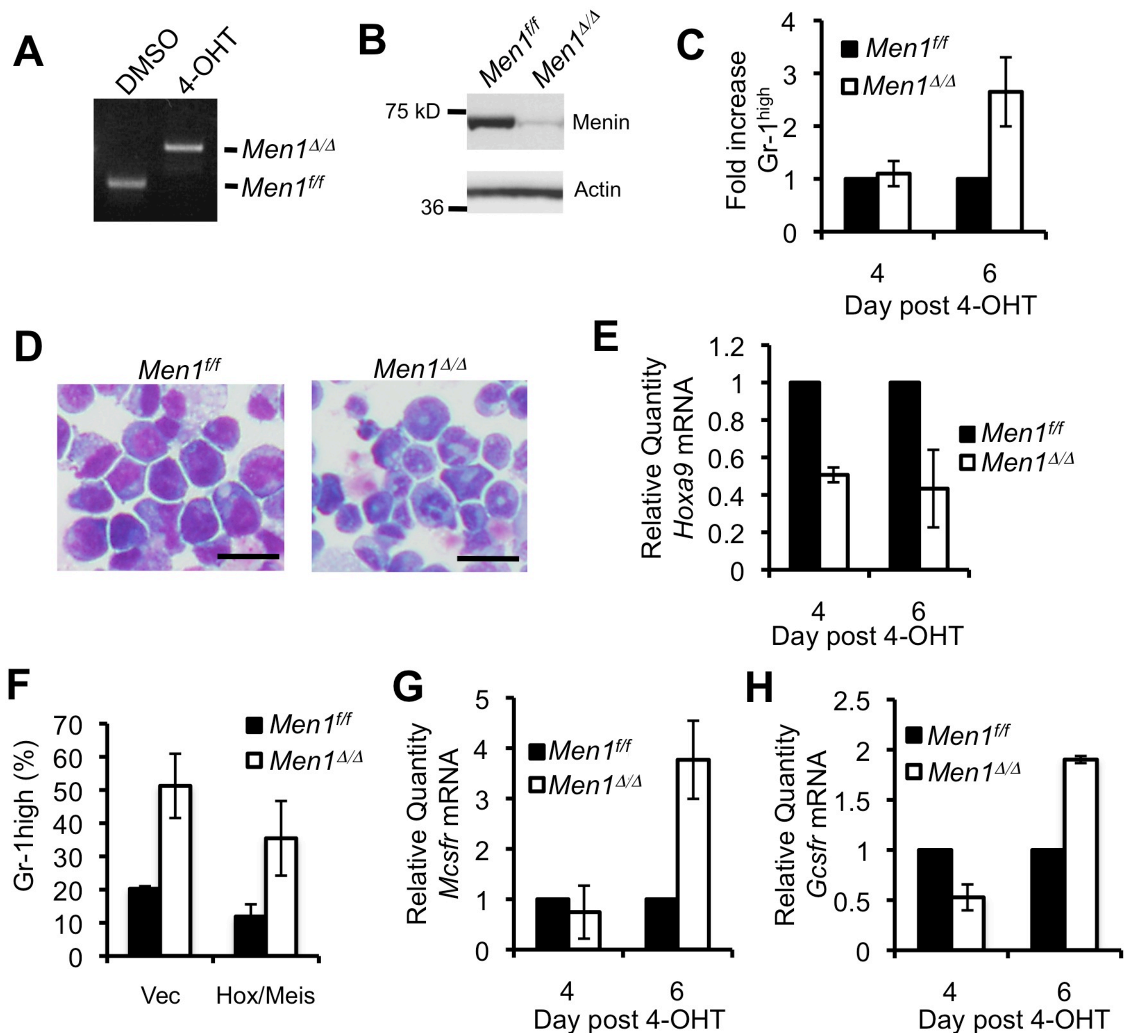


Figure 3.1. Acute menin depletion leads to MA9 cell differentiation in culture. (A) Genotype for *Men1* excision in AT-1 cells 2 days post 4-OHT treatment. **(B)** Western blot for menin in control and *Men1*-excised AT-1 cells 4 days post 4-OHT treatment. **(C)** Flow cytometry for Gr-1 cell surface expression in control and *Men1*-excised AT-1 cells. **(D)** Wright-Giemsa staining of control and *Men1*-excised AT-1 cells 6 days post 4-OHT treatment. **(E)** Real-Time PCR examining *Hoxa9* transcript levels in control and *Men1*-excised AT-1 cells. **(F)** Flow cytometry for Gr-1 cell surface expression in control and *Men1*-excised AT-1 cells with or without Hoxa9/Meis1 overexpression day 6 post 4-OHT treatment. **(G, H)** Real-Time PCR examining *Mcsfr* **(G)** *and Gcsfr* **(H)** transcript levels in control and *Men1*-excised AT-1 cells.

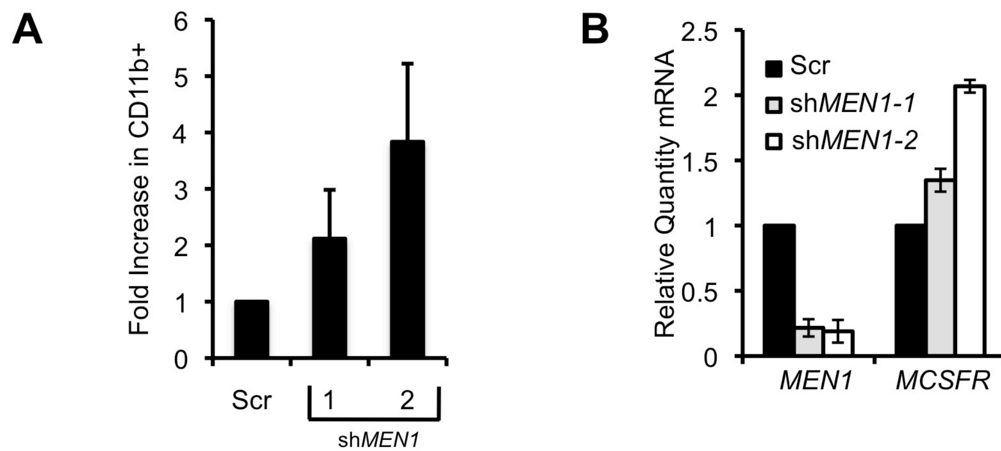


Figure 3.2. Menin KD causes human MLL-AF9 leukemia cell differentiation. (A) Flow cytometry analysis of CD11b^{+} population in control and menin KD THP-1 cells. **(B)** Real-Time PCR analysis of *MCSFR* transcript levels in control and menin KD THP-1 cells.

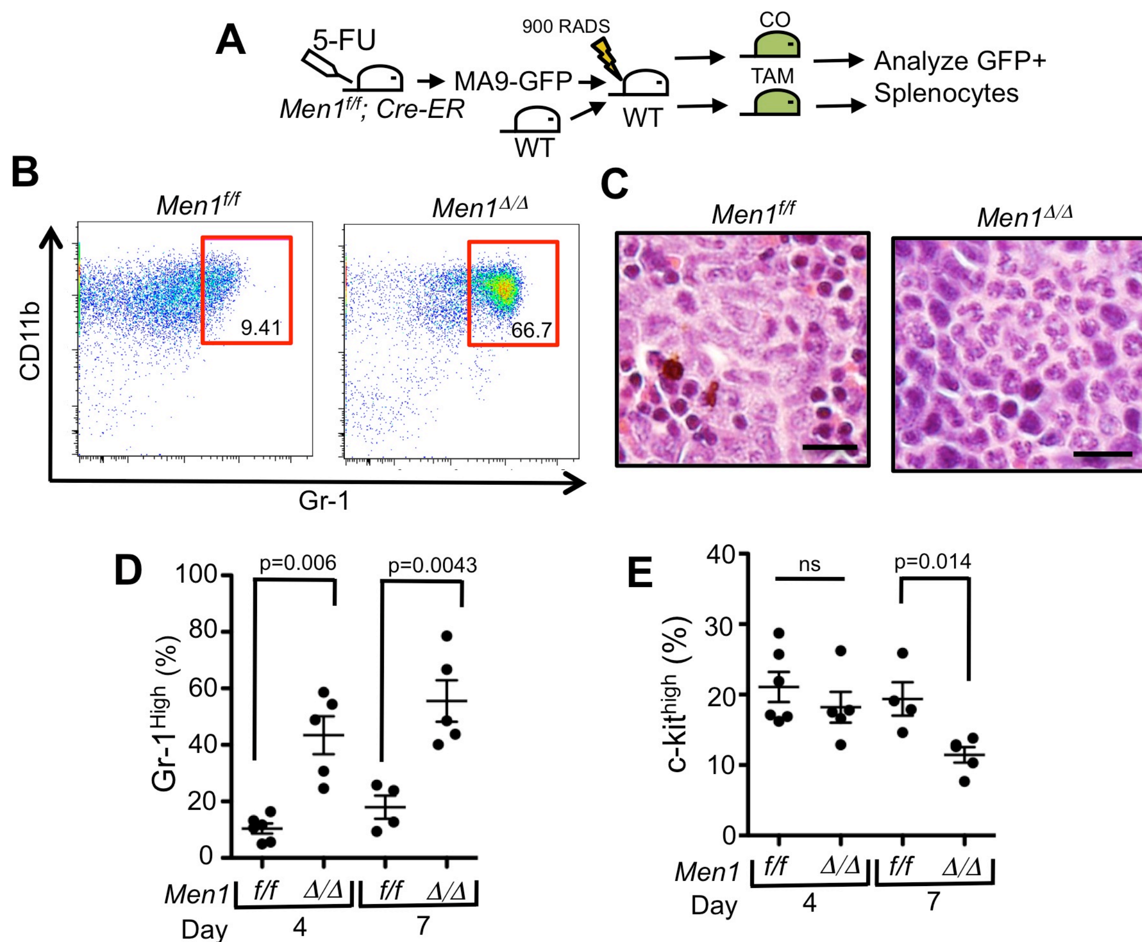


Figure 3.3. Menin depletion results in MA9 cell differentiation *in vivo*. (A) A schematic for examining the acute effect of menin depletion on MA9 cells *in vivo*. *Men1$^{f/f}$; Cre-ER* BM was transduced with MA9-ires-GFP and transplanted into lethally irradiated recipients. Recipient mice were treated with CO control or TAM to excise the floxed *Men1* allele. GFP^{+} cells were analyzed for MA9 cell immunophenotype. **(B)** FACS plot showing increased Gr-1^{high} percentage in *Men1*-excised MA9 primary cells 7 days post-initial TAM treatment. **(C)** H&E staining of spleen sections from control (left) and TAM-treated (right) *Men1$^{f/f}$; Cre-ER* MA9 primary recipients 7 days post-initial TAM treatment. **(D, E)** A summary of the Gr-1^{high} **(D)** and c-kit^{high} **(E)** MA9 cell population in response to menin depletion 4 and 7 days post-initial TAM treatment.

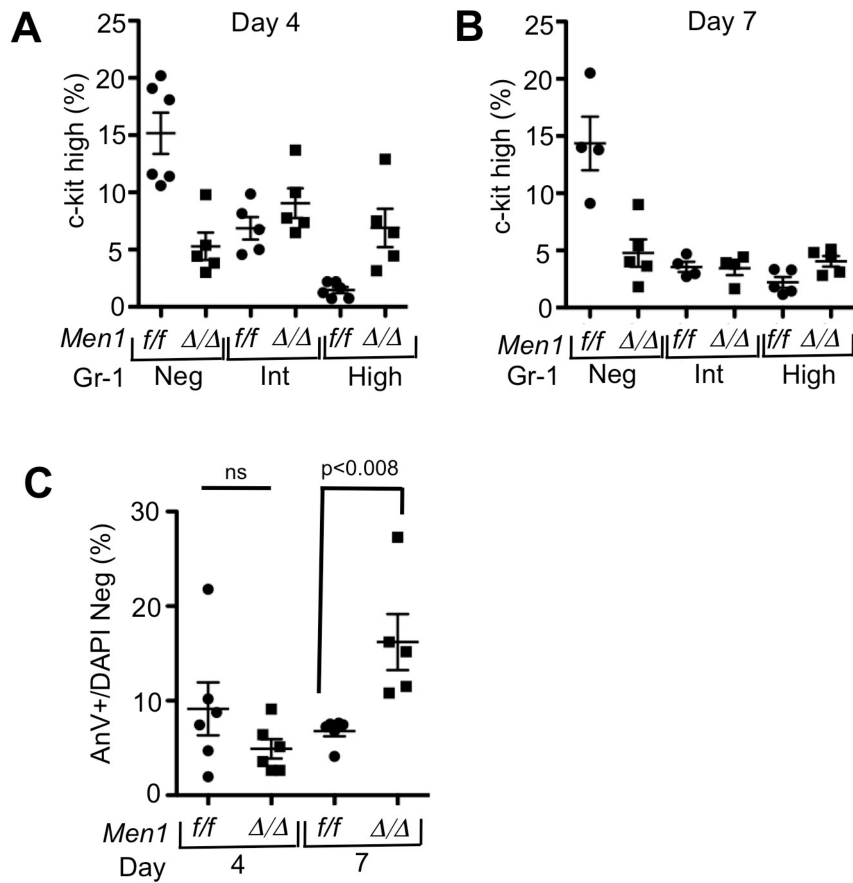


Figure 3.4. Menin depletion causes MA9 cell differentiation *in vivo*. (A, B) Flow cytometry analysis of control and *Men1*-excised MA9 primary cells, examining c-kit^{high} cells in different Gr-1 populations 4 and 7 days post-TAM treatment. **(C)** Flow cytometry analysis of control and *Men1*-excised MA9 primary cells for Annexin V and DAPI staining.

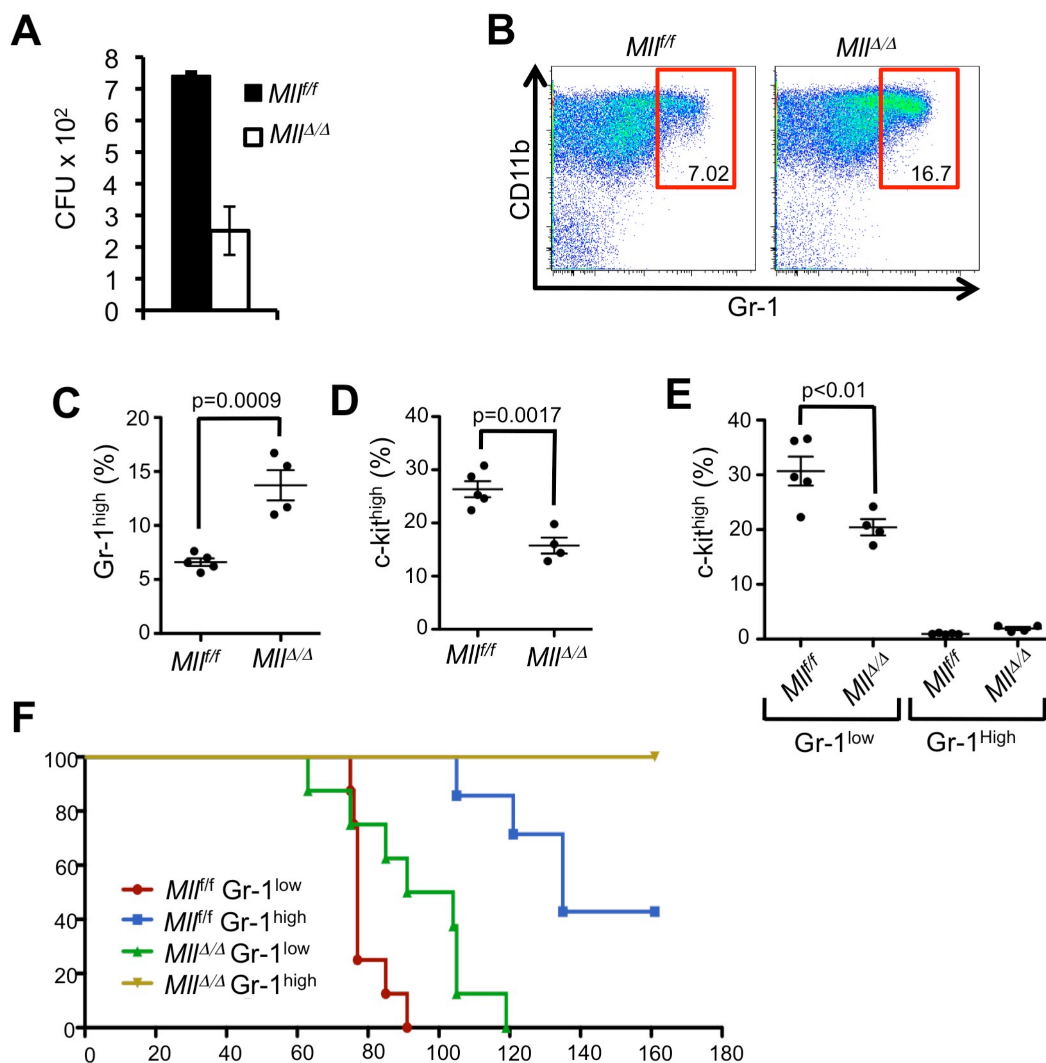


Figure 3.5. WT MLL depletion causes MA9 cell differentiation *in vivo*. (A) Methylcellulose plating of GFP$^+$ MA9 cells treated with CO or TAM *in vivo* 7 days post-initial treatment. **(B)** FACS plot for CD11b/Gr-1 in control or WT MLL-depleted MA9 cells *in vivo* 7 days post-initial TAM treatment. **(C, D)** A summary of flow cytometry for Gr-1^{high} **(C)** or c-kit^{high} **(D)** in control or WT MLL-depleted MA9 cells 7 days post-initial TAM treatment. **(E)** Analysis of c-kit^{high} cells in different Gr-1 populations in control or WT MLL-depleted MA9 cells *in vivo* 7 days post-initial TAM treatment. **(F)** Kaplan-Meier analysis of Gr-1^{low} and Gr-1^{high} secondary recipient mice with or without WT MLL.

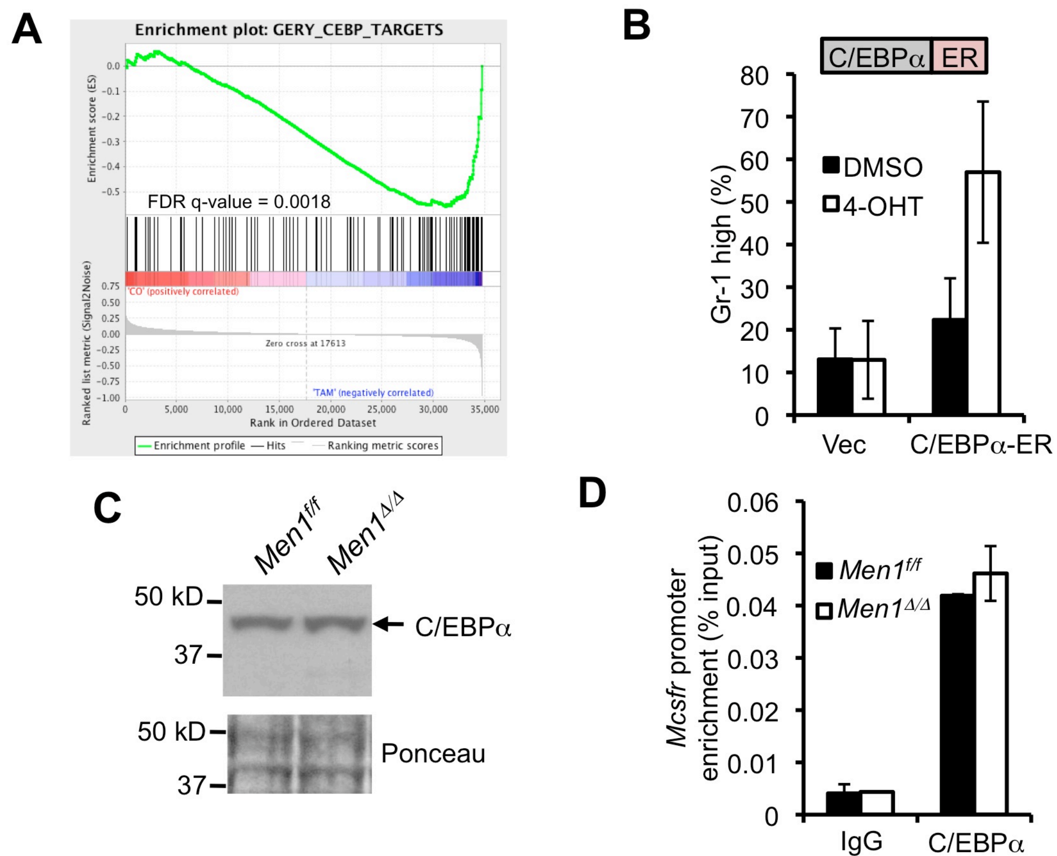


Figure 3.6. ***Men1*** **excision leads to C/EBPα target gene upregulation but does not affect C/EBPα expression. (A)** GSEA analysis comparing *Men1$^{f/f}$; Cre-ER* MA9 primary cells treated with TAM *in vivo* to the C/EBP targets data set. **(B)** Flow cytometry analysis of Gr-1 cell surface expression in vector or C/EBPα-ER transduced AT-1 cells 2 days post 4-OHT treatment. **(C)** Western blot for C/EBPα expression in control or *Men1*-excised AT-1 cells 6 days post 4-OHT treatment. **(D)** ChIP for C/EBPα enrichment at the *Mcsfr* promoter in control or *Men1*-excised AT-1 cells 6 days post 4-OHT treatment.

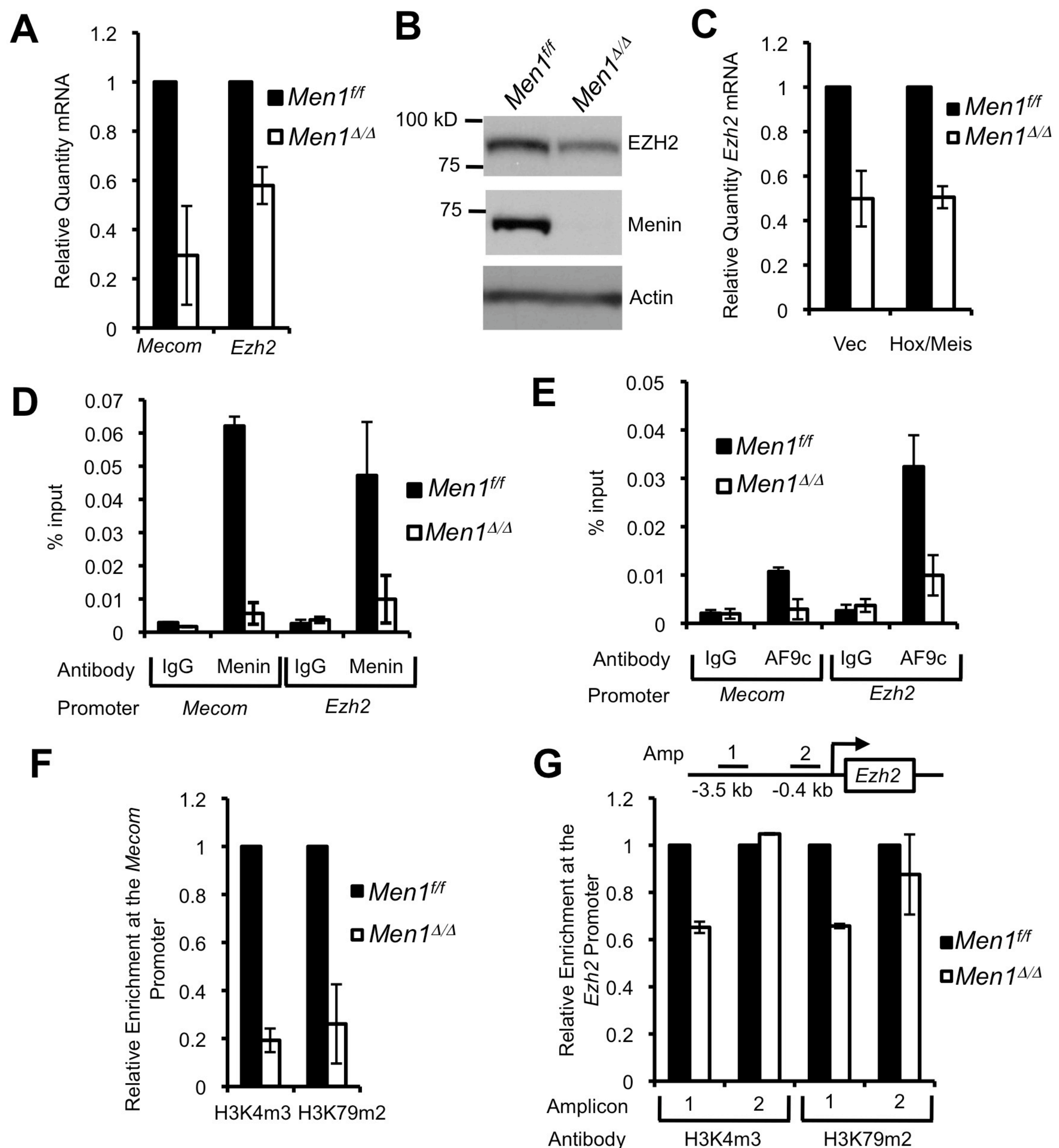


Figure 3.7. Menin promotes EZH2 and Evi-1 expression in MA9 cells. (A) Real-time PCR analysis of *Mecom* and *Ezh2* transcript levels in control or *Men1*-excised AT-1 cells. **(B)** Western blot for EZH2 in control or *Men1*-excised AT-1 cells. **(C)** Real-Time PCR analysis of *Ezh2* transcript levels in control and *Men1*-excised AT-1 cells with or without Hoxa9/Meis1 overexpression. **(D, E)** ChIP assays for menin **(D)** and AF9c **(E)** binding at the *Mecom* and *Ezh2* promoters in control or *Men1*-excised AT-1 cells. **(F, G)** ChIP assays for H3K4m3 and H3K79m2 at the *Mecom* **(F)** and *Ezh2* **(G)** promoters in control or *Men1*-excised AT-1 cells.

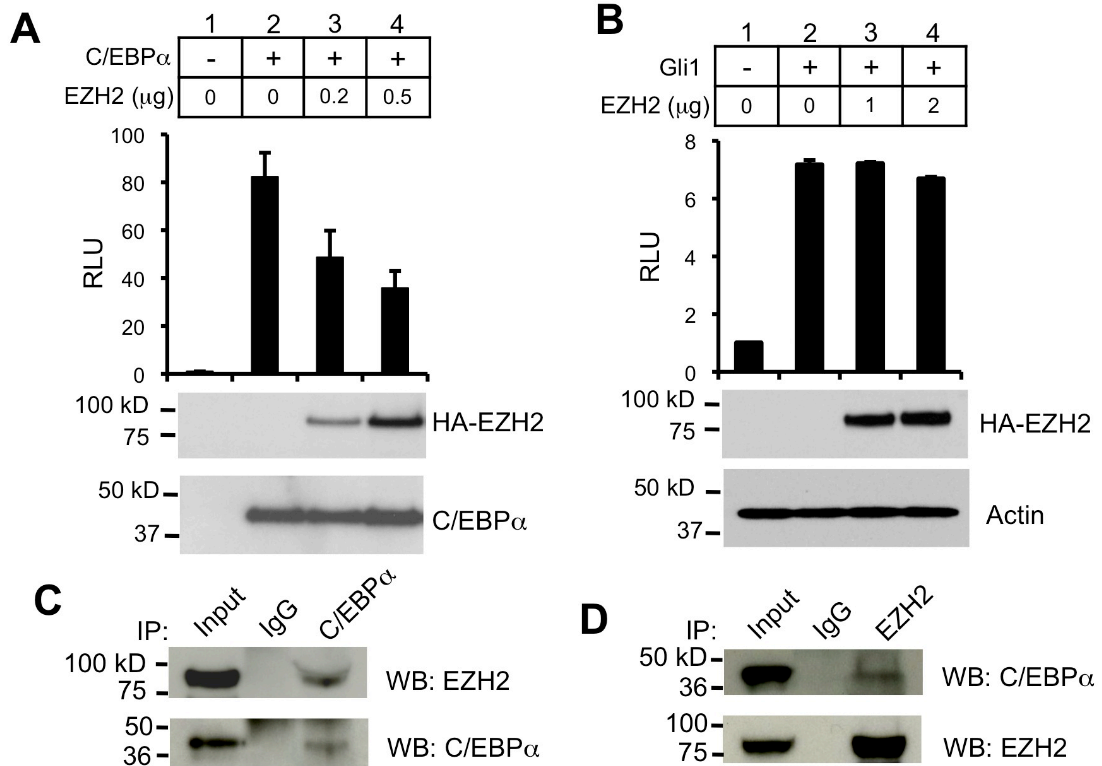


Figure 3.8. EZH2 interacts with C/EBPα in MA9 cells and represses C/EBPα target genes. (A) Luciferase assay in HEK 293T cells with a C/EBPα binding site-containing promoter-driven luciferase plasmid, C/EBPα and increasing amounts of EZH2. **(B)** Luciferase assay in HEK 293T cells with a Gli-1 binding site-containing promoter-driven luciferase plasmid, Gli-1 and increasing amounts of EZH2. **(C)** IP for C/EBPα followed by western blotting for EZH2 (top) or C/EBPα (bottom). **(D)** IP for EZH2 followed by western blotting for C/EBPα (top) and EZH2 (bottom) in THP-1 cells.

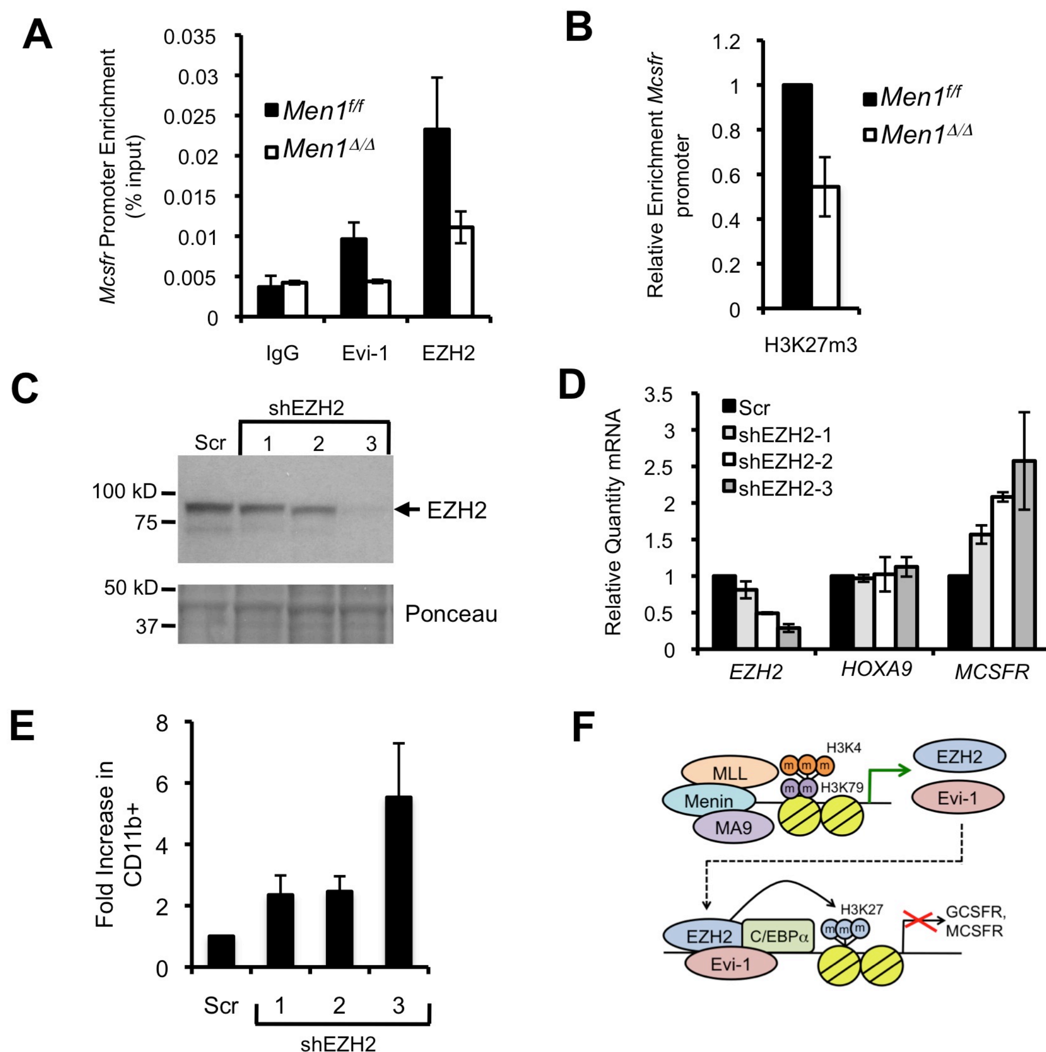


Figure 3.9. EZH2 knockdown induces MA9 cell differentiation. (A) ChIP assay for Evi-1 and EZH2 enrichment at the *Mcsfr* promoter in control or *Men1*-excised AT-1 cells. **(B)** ChIP assay for H3K27m3 enrichment at the *Mcsfr* promoter in control or *Men1*-excised AT-1 cells. **(C)** Western blot for EZH2 expression in Scr control and EZH2 KD THP-1 cells. **(D)** Real-Time PCR analysis of Scr control and EZH2 KD THP-1 cells for *EZH2, HOXA9,* and *MCSFR* transcript levels. **(E)** Flow cytometry analysis of CD11b cell surface expression in Scr and EZH2 KD THP-1 cells. **(F)** A model for the role of EZH2 in MA9 leukemia.

CHAPTER 4 – CONCLUSIONS AND FUTURE DIRECTIONS

MLL-FP-induced acute leukemia is highly aggressive and often refractory to therapy (Holleman et al., 2004). The studies described in my thesis provide insights into the mechanism by which MLL-FPs promote leukemogenesis. We have shown that menin recruits both WT MLL and MLL-FPs to target genes, and WT MLL is required for MLL-FP target gene upregulation and leukemogenesis (Chapter 2). The role of menin in recruiting both WT MLL and MLL-FPs to target genes highlights menin as a central scaffold protein controlling this type of leukemia (Figure 4.1). One consequence of MLL-FP expression is a block in differentiation (Figure 1.4) (Lavau et al., 1997), and we have shown that depletion of menin alleviates this differentiation block (Figure 3.1-3.3). We found that this block in differentiation is at least partially controlled through menin-mediated up regulation of EZH2, which interacts with C/EBPα and represses C/EBPα target genes (Figure 3.9F). The findings that WT MLL is required for MLL-FP leukemia cell maintenance, and that EZH2 is an important downstream target of menin, have revealed new potential therapeutic targets to improve the treatment of MLL-FP leukemias.

WT MLL is required for MA9-induced leukemogenesis

MLL-FPs lack a large C-terminal portion of WT MLL that is normally necessary for target gene activation (Figures 1.2 and 1.3). However, we have shown that in MLL-FP-expressing cells, WT MLL function is retained through expression of full-length WT MLL from the non-translocated allele. We found that WT MLL is critical for

maintaining the transformed state of MA9-induced leukemia, and the survival of MA9 leukemic mice is prolonged by WT MLL knockout (Figures 2.8B and 2.10E), demonstrating the critical role of WT MLL in MA9-induced leukemogenesis.

It remains to be tested whether WT MLL is required for transformation mediated by other MLL-FPs. It is likely that the most common MLL-FPs, which contain nuclear translocation partners, require WT MLL for transformation, as these MLL-FPs have a common transformation mechanism (Krivtsov and Armstrong, 2007). It is also unknown if WT MLL has a role in promoting leukemias that do not have MLL-FPs. The role of WT MLL in these other leukemia types could be tested in a manner similar to the experiments performed to demonstrate a role for WT MLL in MA9 leukemia, which are described in Chapter 2.

WT MLL is required for *Hox* gene expression in MA9 leukemia cells

We found that WT MLL is required for the maintenance of MA9-mediated transformation, at least in part through promoting *Hox* gene transcription, as overexpression of *Hoxa9* and *Meis1* is able to compensate for the abrogation of colony formation due to WT MLL depletion (Figure 2.8B, E). However, it remains unclear exactly how WT MLL upregulates *Hox* genes in MLL-FP leukemia cells. MLL C-terminal domains, which are lacking in MLL-FPs, are normally required for MLL target

gene activation, and may be important for *Hox* gene upregulation in MLL-FP-expressing cells.

The WT MLL TAD, which promotes histone acetylation, and the SET domain, which catalyzes H3K4m3, are critical for target gene upregulation in non-MLL-FP containing cells. Therefore, it is likely that the WT MLL TAD and SET domain also have a role in upregulating MLL-FP target genes and promoting leukemogenesis. The potential roles of these domains could be evaluated by creating MLL conditional knockout models to remove the TAD, the SET domain, or both domains while retaining the rest of the MLL protein. The experiments described in Chapter 2 could then be performed to determine the role of each of these domains in MLL-FP-induced *Hox* gene upregulation and leukemogenesis.

In addition to its potential role in promoting target gene expression through its normal function in transcriptional regulation, WT MLL also controls the function of MA9. We found that WT MLL depletion in MA9-expressing cells leads to a decrease in MA9-mediated H3K79m2 at the *Hoxa9* promoter (Figure 2.6B). In addition, WT MLL is necessary for MA9 recruitment to *Hox* genes in MEF cells (Milne et al., 2010), providing evidence that WT MLL is involved in MLL-FP recruitment to target genes in leukemia cells. However, WT MLL does not directly recruit MLL-FPs to target genes, as there is no physical interaction between MA9 and WT MLL (Milne et al., 2010). Therefore, WT

MLL may indirectly lead to MLL-FP recruitment through functions that are lacking in the MLL-FP. The role of each of these domains in MLL-FP recruitment and H3K79 methylation at *Hox* genes could be determined by establishing MA9-transformed cell lines with the ability to conditionally remove each domain of WT MLL individually, as described above, followed by ChIP assay. Future studies will further elucidate the mechanism by which WT MLL promotes MLL-FP-induced *Hox* gene upregulation and leukemogenesis.

WT MLL as a therapeutic target to treat MLL-FP leukemias

As we showed that WT MLL is essential for maintaining MA9-mediated transformation, inhibiting WT MLL function could hold therapeutic value for patients with this disease. Specific inhibitors of the WT MLL SET domain could inhibit WT MLL function in MLL-FP-mediated leukemias. In addition, it might be possible to target the WT MLL TAD or HAT proteins associated with the TAD to treat this disease. However, the exact mechanism by which WT MLL promotes MLL-FP-mediated leukemogenesis remains unknown, and a detailed understanding of how WT MLL promotes MLL-FP-induced leukemia is necessary before inhibitors can be developed. Also, while pharmacological inhibition rarely completely inhibits the function of a protein, deletion of WT MLL causes hematopoietic defects, suggesting that inhibition of WT MLL function could have deleterious effects on normal BM (Gan et al., 2010; Jude et al., 2007; McMahon et al., 2007). Future studies will determine which domains of WT MLL are important for

MLL-FP-induced leukemogenesis, and the feasibility of targeting these domains for therapy.

Menin is essential for MLL-FP-mediated leukemogenesis and a potential therapeutic target

Menin directly interacts with WT MLL and MLL-FPs, and we found that menin is necessary for the recruitment of these two components required for leukemogenesis to *HOX* genes, establishing menin as a central player in this disease (Figure 4.1) (Chen et al., 2006; Grembecka et al., 2012; Huang et al., 2012; Murai et al., 2011; Thiel et al., 2010; Yokoyama et al., 2004). Since menin is involved in the recruitment of both WT MLL and MA9, two critical proteins involved in leukemogenesis, inhibiting menin would likely be more effective than blocking a single function of either WT MLL or MLL-FPs (Figure 4.1). In addition, WT MLL and MLL-FPs interact with menin via identical N-terminal motifs, raising the possibility of disrupting the menin-WT MLL and menin-MLL-FP interactions with a single inhibitor.

Recently, the structure of menin in complex with MLL N-terminal domains has been elucidated, revealing potential targets for inhibition of menin function with small molecules (Huang et al., 2012; Murai et al., 2011). Menin interacts with the N-terminus (residues 6-25) of MLL (Huang et al., 2012), a region that is found in both WT MLL and MLL-FPs. MLL-N adopts a highly coiled conformation and plugs into a deep

hydrophobic pocket in menin. The hydrophobic menin pocket that interacts with MLL-N is specifically shaped for the phenyl ring of phenylalanine (Huang et al., 2012), suggesting that small aromatic compounds could inhibit the menin-MLL-N interaction. Along these lines, a potent inhibitor of the menin/MLL-N interaction has recently been developed. This thienopyromidine class compound, MI-2, inhibits the interaction between MLL-FPs and menin, and blocks MLL-FP induced transformation (Grembecka et al., 2012), establishing the menin-MLL-N interaction as a druggable target, and a potential therapy for MLL-FP leukemias. Future studies will focus on the development of similar inhibitors, and the testing of these inhibitors for their efficacy *in vivo*.

Acute menin depletion causes MA9 leukemia cell differentiation

As menin is a central mediator of MLL-FP leukemia and potential therapeutic target, it is critical to understand the effect of menin depletion/inhibition *in vivo*. In addition, characterization of pathways regulated by menin that are involved in leukemogenesis may lead to the discovery of previously unappreciated therapeutic targets for this disease. We found that the function of the pro-differentiation transcription factor C/EBPα is suppressed through menin-mediated upregulation of the polycomb group protein EZH2. EZH2 interacts with C/EBPα and represses C/EBPα target genes. Our findings provide a novel mechanism for blocking C/EBPα function and MA9 leukemia cell differentiation, and potential targets for the treatment of this disease downstream of menin.

Menin does not directly regulate C/EBPα in MA9 leukemia cells

We found that acute menin depletion causes C/EBPα target gene upregulation and MA9 leukemia cell differentiation. As C/EBPα is a transcription factor that promotes myeloid differentiation, we sought to determine how menin regulates C/EBPα function. Many leukemogenic oncogenes and pathways repress C/EBPα expression to block differentiation. The Bcr-Abl fusion protein inhibits C/EBPα translation, reducing its protein levels. In addition, the Notch signaling pathway leads to degradation of the pro-differentiation C/EPBα p42 isoform to the p30 isoform, which suppresses differentiation. These observations led us to investigate whether C/EBPα p42 expression is regulated by menin in MA9 leukemia cells. However, we found that C/EBPα p42 is expressed in MA9 leukemia cells, while p30 levels were not detectable by Western blot, and menin depletion had no effect on C/EBPα expression (Figure 3.6C).

The lack of an effect on C/EBPα expression led us to test whether menin depletion had an effect on C/EBPα target gene binding, as the PLZF-RARα leukemogenic fusion protein has been shown to block C/EBPα localization to target genes to suppress differentiation. In contrast to PLZF-RARα leukemia, we found that C/EBPα was already bound to the promoter of one of its key target genes, *Mcsfr*, in the presence of menin, and menin depletion had no effect on C/EBPα target gene binding (Figure 3.6D). Together, these results show that menin does not affect C/EBPα expression or target gene binding, two mechanisms that are utilized in other types of leukemia to block C/EBPα function. However, menin depletion leads to the upregulation of the C/EBPα target gene *Mcsfr*,

and MA9 leukemia cell differentiation, suggesting that menin might block C/EBPα function through a previously uncharacterized mechanism. The lack of a direct effect on C/EBPα led us to investigate how menin might act to repress C/EBPα target genes.

Menin promotes EZH2 expression in MA9 leukemia cells

Our search for transcriptional repressors that are regulated by menin and could potentially repress C/EBPα target genes led to the finding that that menin promotes the expression of the polycomb group protein EZH2. Menin depletion leads to a decrease in EZH2 transcript and protein levels (Figure 3.7A, B). In addition, EZH2 expression is reduced when *Hoxa9* and *Meis1*, known menin targets that are critical for MA9-induced leukemia, are overexpressed (Figure 3.7C). These results suggest that menin directly regulates EZH2, and ChIP assay showed that menin binds the *Ezh2* promoter (Figure 3.7D). Menin depletion led to a decrease in AF9c the portion of AF9 found in MA9 at the *Ezh2* promoter, and a modest decrease in H3K79m2, suggesting that menin recruits MA9 to the *Ezh2* promoter, leading to its upregulation (Figure 3.9E, G). As EZH2 is a transcriptional repressor, our finding that menin regulates EZH2 expression raised the possibility that independent of its role in upregulating *HOX* genes to promote leukemogenesis, menin upregulates EZH2 expression to repress C/EBPα target genes and block MA9 leukemia cell differentiation.

EZH2 represses C/EBPα target genes and suppresses MA9 leukemia cell differentiation

We found that EZH2 does in fact repress C/EBPα target genes and block MA9 leukemia cell differentiation. EZH2 interacts with C/EBPα, binds C/EBPα target genes, and represses their transcription (Figures 3.8 and 3.9). Menin depletion leads to a decrease in EZH2 binding and EZH2-mediated H3K27m3 at the *Mcsfr* promoter (Figure 3.9A, B). In addition, EZH2 depletion caused *MCSFR* upregulation and differentiation (Figure 3.9D, E), demonstrating a role for EZH2 in blocking MA9 leukemia cell differentiation.

Various oncogenes and pathways inhibit C/EBPα function as a mechanism for blocking differentiation and maintaining leukemogenesis (Reckzeh and Cammenga, 2010). These previously characterized mechanisms directly inhibit C/EBPα either at the level of expression or target gene binding. In the case of MA9 leukemia, the block in C/EBPα function is indirect, through menin, and likely MA9-mediated upregulation of EZH2 (Figure 3.9F). EZH2 then interacts with C/EBPα, catalyzing H3K27m3 at C/EBPα target genes and repressing these genes (Figure 3.9F). These findings highlight a previously uncharacterized mechanism for blocking leukemia cell differentiation.

Cooperation between polycomb and trithorax-related proteins to block MA9 cell differentiation

Polycomb and trithorax proteins have classically opposing roles in the transcriptional regulation of common target genes (Mills, 2010). Therefore, the finding that trithorax-

related menin promotes EZH2 expression was unexpected. It is unclear whether menin promotes EZH2 expression specifically in MLL-FP leukemia cells, or menin regulates EZH2 expression in a broader context. It is possible that in normal cells, menin promotes both MLL function through its recruitment to target genes and EZH2 expression as a mechanism for maintaining bivalent chromatin. The maintenance of bivalent chromatin, containing both MLL-mediated H3K4m3 and EZH2-catalyzed H3K27m3 is essential for the proper regulation of genes during development and differentiation (Mills, 2010).

We could test the role of menin in regulating EZH2 expression in other contexts using our model for acute *Men1* depletion. Cell populations could be isolated from various tissues in developing and adult mice to determine how menin affects EZH2 expression. We could also test whether menin depletion affects EZH2 expression in other types of leukemia by transforming mouse BM with various leukemic oncogenes and examining EZH2 expression levels in menin-depleted cells.

In addition, menin and EZH2 cooperate to block differentiation in MA9-expressing leukemia cells. We initially thought that EZH2 might function as a tumor suppressor in MA9 leukemia, due to its classical role in repressing *Hox* genes, downstream targets of menin and MA9 that are critical for leukemogenesis. In addition, loss of function EZH2 mutations are prevalent in myelodysplastic syndrome and myeloproliferative disorders (Ernst et al., 2010; Nikoloski et al., 2010), providing further evidence the EZH2 is a tumor suppressor in the myeloid lineage. Loss of EZH2 in the context of

myelodysplastic syndrome and myeloproliferative disorders may lead to *HOX* gene upregulation and enhanced self renewal.

However, in MA9-expressing leukemia cells, EZH2 depletion has no effect on *HOXA9* expression (Figure 3.9D). It is possible that in the context of MLL-FP leukemia, *HOX* gene expression is driven to such high levels by the MLL-FP that loss of EZH2 does not further increase *HOX* gene levels. The lack of an effect on *HOX* gene expression by EZH2 depletion in MLL-FP cells may provide a context for observing previously uncharacterized functions for EZH2 in blocking differentiation.

It is not yet known whether EZH2-mediated suppression of C/EBPα target genes is a more general mechanism for blocking differentiation, or specific to MLL-FP leukemias. Determining whether EZH2 interacts with C/EBPα and binds C/EBPα target genes, followed by determining the effect of EZH2 depletion on C/EBPα target gene expression in other types of leukemia and normal cells will give further insight into the context in which EZH2 blocks C/EBPα function.

Targeting EZH2 to treat MLL-FP leukemia

The findings presented here and other recently published results demonstrate that EZH2 depletion causes MA9 leukemia cell differentiation in cell culture (Figure 3.9E) (Neff et al., 2012; Tanaka et al., 2012). However, EZH2 knockout seems to have a less severe effect on MA9 cells *in vivo* (Neff et al., 2012). One potential reason for this more mild

effect is partial compensation for loss of EZH2 function by the closely related EZH1 protein, which can also be found in the PRC2 complex and catalyzes H3K27 methylation. In fact, depletion of the core PRC2 component EED, which is essential for both EZH1 and EZH2 function, more effectively extends the life span of MA9 leukemic mice than EZH2 knockout (Neff et al., 2012). In addition, combined knockdown of EZH1 and EZH2 more effectively reduces MA9 cell growth than EZH2 knockdown alone (Shi et al., 2012).

These results suggest that inhibition of EZH2 would be insufficient to effectively treat MLL-FP leukemia patients. However, EZH1 and EZH2 SET domains, which catalyze H3K27 methylation to repress target genes, are highly homologous (Laible et al., 1997), and it may be possible to design small molecule inhibitors that can simultaneously block EZH1 and EZH2 catalytic activity. However, the finding that loss of function EZH2 mutations are prevalent in myelodysplastic and myeloproliferative diseases suggests that inhibition of EZH2 could itself lead to hematopoietic disorders in patients (Ernst et al., 2010; Nikoloski et al., 2010). Therefore, caution should be exercised when developing EZH2 inhibitors. Nonetheless, these studies have highlighted EZH2 and PRC2 as potential targets for the treatment of MLL-FP leukemia.

Concluding Remarks

Recently, significant progress has been made in understanding how MLL-FPs cause leukemia. The findings presented in my thesis provide insights into the molecular pathways governing MLL-FP-mediated upregulation of target genes, and downstream

effectors that are critical for blocking differentiation and promoting leukemogenesis. The lack of MLL C-terminal domains in MLL-FPs is remedied by expression of WT MLL from the non-translocated *Mll* allele, and WT MLL is required for MLL-FP target gene upregulation and leukemogenesis. These findings highlight the possibility for targeting WT MLL to treat MLL-FP leukemias.

Notably, menin is involved in the recruitment of both WT MLL and MLL-FPs to target genes in MLL-FP leukemia cells, placing menin as a central mediator of two critical components required for leukemogenesis and candidate for therapeutic targeting (Chapter 2). Inhibition of the menin/MLL-N interaction with the small molecule MI-2 is effective in blocking MLL-FP-induced transformation (Grembecka et al., 2012), providing proof of principle for targeting this interaction to treat patients with this disease.

Investigating the mechanism by which menin/MLL-FPs block leukemia cell differentiation led to our discovery that menin promotes the expression of the PcG protein EZH2, which interacts with C/EBPα at target genes, suppressing C/EBPα target gene upregulation and differentiation. This cooperation between Trx-related menin and the PcG protein EZH2 is counter their classical opposing functions. As EZH2 depletion causes MLL-FP leukemia cell differentiation, inhibition of EZH2 could potentially be used as a therapy for patients with this disease. These studies have uncovered novel mechanisms and pathways that promote MLL-FP target gene upregulation and block

leukemia cell differentiation, and could lead to novel therapies and a more favorable prognosis for MLL-FP leukemia patients in the future.

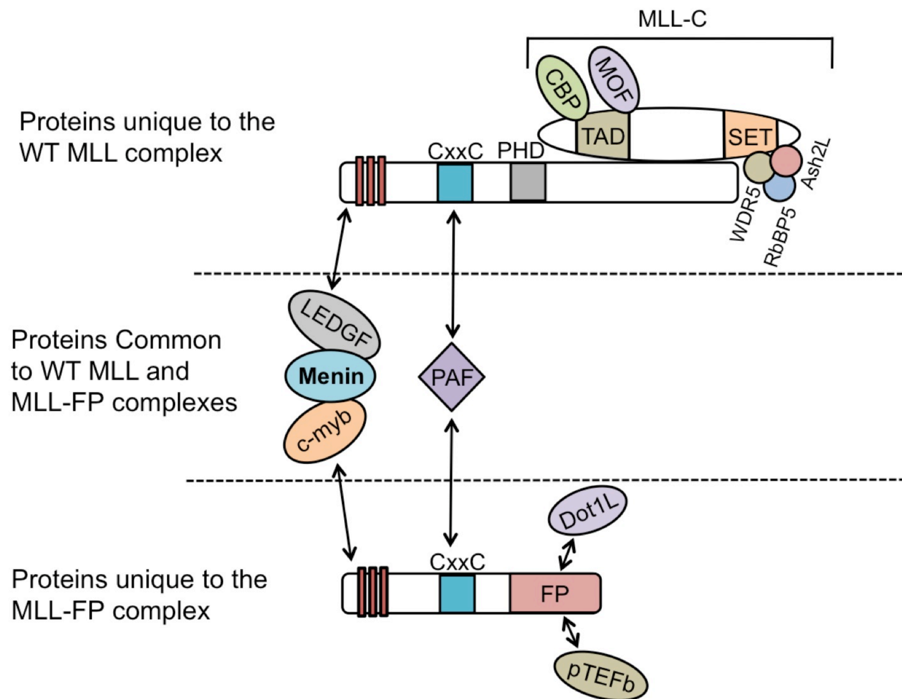


Figure 4.1. Menin as a central hub regulating WT MLL and MLL-FPs. Menin binds the N-terminus of either WT MLL or MLL-FPs, together with C-Myb and LEDGF (middle). The interaction between menin and LEDGF requires the MLL-N. The PAF complex interacts with the CxxC domain of both WT MLL and MLL-FPs. The proteins interacting with the C-terminal part of WT MLL or MLL-FPs are distinct (top and bottom). WT MLL and MLL-FPs are recruited to many of the same target genes.

CHAPTER 5 – MATERIALS AND METHODS

Plasmids and cell culture

Various plasmids were as previously described: pMX-GFP, pMX-puro, pMX-puro-menin, pMSCV-MLL-AF9, pMSCVpgk-Hoxa9-GFP, pMSCVpacMeis1A, pMSCV-GFP, pMIG-MLL-AF9-ires-GFP, and pcDNA3-CEBPA-HA (Chen et al., 2006; Jin et al., 2003). Dr. Alan Friedman provided MigRI-CEBPA-ER. pCMV-HA-EZH2 and lentiviral packaging plasmids, pMD2G and pAX2G were obtained from Addgene. Retroviral or lentiviral constructs expressing shRNAs were obtained from Open Biosystems (Huntsville, AL) or Sigma (Cambridge, MA).

AT-1 cells were generated from BM cells of *Men1*$^{f/f}$*;Cre-ER* mice by transduction with retrovirus expressing MA9 and cultured in medium with 15% FBS for LT myeloid culture (Cat #06500, Stem Cell Technologies) and 10 ng/ml IL3. In AT-1 cells, *Men1*$^{f/f}$ was excised by treating the cells with 4-OHT (200 nM). THP-1 cells were maintained in RPMI-1640 containing 10% FBS and 1% Pen/Strep supplemented with 0.05 mM 2-mercaptoethanol. HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% Pen/Strep.

Packaging of recombinant retrovirus and lentivirus and transduction of cells

Plasmids for retroviral packaging were co-transfected with psi-2 helper plasmid into 293T cells using the calcium chloride precipitation method. For lentiviral shRNA packaging, scrambled pLKO.1 vector or specific shRNA in pLKO.1 vector were co-transfected into 293T cells with pAX2G and pMD2G. The resulting recombinant virus

was collected for transduction of cells by spinoculation, followed by selection in 2 μg/ml puromycin for 3 days.

RNA isolation and quantitative real-time PCR

RNA was isolated using Trizol (Ambion) and the RNeasy kit (Qiagen). 1μg of total RNA was used to make cDNA with the SS III RT system (Invitrogen), and real-time PCR was performed using the 7500 fast real-time PCR system (Applied Biosystems) and Quantitect Sybr Green kit (Qiagen). Transcript levels were normalized to GAPDH between samples, and relative quantity was calculated using the ΔΔCt method.

Immunoprecipitation and Western blotting

Cells were transfected, and the resulting cells were lysed in RIPA buffer supplemented with 1x protease inhibitor cocktail (Sigma) for IP. For Western blot, cell lysates were separated through SDS-PAGE and processed for detection with ECL Western blotting detection reagents (GE Healthcare).

ChIP assay

ChIP assays were performed as previously described using the Imgenex kit (Chen et al., 2006). Briefly, 10^6- 10^7 formaldehyde crosslinked cells were lysed and sonicated to obtain sheared DNA. This lysate was then incubated with control IgG or an antigen-specific antibody overnight, then bound to beads and washed. Samples were eluted from

the beads and incubated at 65°C overnight to reverse crosslinking. Eluted DNA was quantified using the 7500 fast real-time PCR system (Applied Biosystems) with the Quantitect Sybr Green kit (Qiagen), and normalized to input DNA, as well as total H3 for histone modifications.

Mice and bone marrow transformation

All laboratory mice were maintained on a 12-h light-dark cycle in the animal facility at the University of Pennsylvania. All experiments on mice in our research protocol were approved by Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania and were performed in accordance with relevant institutional and national guidelines and regulations. $Mll^{f/f}$ mice (in C57B6-SJL background) (Jude et al., 2007) were bred with ubc9-Cre-ER mice (in C57B6 background) (Schnepp et al., 2006), and BM from the mice was isolated and transformed, as previously described (Chen et al., 2006). C57B6 mice or $Mll^{f/f}$*;Cre-ER* mice (6-8 weeks old) were injected with 5-FU, and BM cells were collected from femurs and prestimulated with a cocktail of cytokines and growth factors, as previously described (Chen et al., 2006). The cells were transduced with pMSCV-neo-MA9 retrovirus by spinoculation and replated weekly in MethoCult GF M3434 medium (StemCell Technologies) with 1mg/ml G418. After the second plating, surviving cells from C57B6 or $Mll^{f/f}$*;Cre-ER* mice were transduced twice with either scrambled control retrovirus or lentivirus or their counterparts expressing one of the MLL-C shRNAs. The transduced cells (2 x10^4) were seeded in a 35-mm Petri dish with methylcellulose-based medium containing 2μg/ml puromycin and scored for

colonies with >50 cells one week after plating. To excise the floxed *Mll*, MA9-transformed BM with *Mll$^{f/f}$;Cre-ER* were treated with either DMSO or 4-OHT (400 nM) at the fourth plating. For *in vivo Mll* excision prior to the first plating, *Mll$^{f/f}$;Cre-ER* mice were treated with CO or 200 mg/kg body weight TAM to excise the floxed *Mll*, then isolated BM was either plated directly in methylcellulose, or transduced with MA9, followed by plating.

Leukemia induction and WT *Mll* excision from leukemic cells in mice

The *Mll$^{f/f}$* mice were backcrossed with C57B6sjl mice (CD45.1+) for over nine generations (Jude et al., 2007), and the mice were then bred with transgenic mice expressing *ubc9-Cre-ER* (in C57B6 background, CD45.2+) (Ruzankina et al., 2007). The *Mll$^{f/f}$*; Cre-ER or *Men1$^{f/f}$;* Cre-ER mice were intercrossed to maintain the CD45.2+ marker. BM cells from these mice were transduced with MA9-retrovirus, and transplanted retro-orbitally into C57B6 x C57B6-SJL F1 female mice (CD45.1+/2+, 6-8 weeks old, 10^6 cells per mouse, Taconic), together with $2.5x10^5$ BM cells from an F1 mouse. The recipient mice were irradiated with 900 rad prior to transplantation. The mice were fed with either control CO or TAM (Sigma, St. Louis, MO) at a dose of 200 mg/kg body weight to excise the floxed *Mll* or *Men1*. Organs from control and leukemic mice were isolated, fixed, and processed for H & E staining and analyzed under microscope.

Engraftment of human leukemia cells into NOG mice

NOD/Shi-*scid*/IL-2Rγ^{null} mice, 6-8 weeks old, were obtained at the Stem Cell Core Facility at the University of Pennsylvania and irradiated at 275 rad 24 hrs prior to tail vein injection with 1.5 $x10^6$ scrambled vector control THP-1 cells or cells transduced with shRNA 14 targeting MLL-C. Mice were weighed weekly starting from the time of injection. The recipient mice were sacrificed 6 weeks after injection, and the long bones (femurs) and spleens were collected for histological analysis by H&E staining.

Isolation of Peripheral Blood Cells

Blood samples collected from submandibular bleeds were stored in Microtainer tubes with EDTA (BD Biosciences). The hematology profile of each mouse was analyzed immediately on a Hemavet blood cell counter (CDC Technologies). For flow cytometry or genotyping of peripheral white blood cells, mice were bled into 4% Sodium Citrate in FACS tubes and IMDM (1ml) with 2% FBS was added to the tube, followed by addition of an underlay of 1ml Ficoll solution (GE healthcare). The samples were centrifuged at 1,000 x g for 20 min, and the white layer was collected, washed once in 1x PBS, and used for further analysis.

Flow cytometry analysis

Cells from peripheral blood, bone marrow or spleen were harvested for analysis of immunophenotypes. After blocking unspecific binding with unlabeled Rat+Mouse IgG

(Sigma), cells were stained on ice in PBS + 1% FBS and analyzed on LSR II, FACSCalibur or FACSAria (Becton Dickinson). Files were analyzed in FlowJo (Tree Star).

Antibodies for Western Blotting, ChIP, and IP

Cyclin A2 (Cat #ab7596, Abcam), Cyclin E2 (Cat#ab40890, Abcam), HA (Cat#ab1893, Abcam), Dot1L (Cat#A300-953A, Bethyl), MLL-N (Cat#A300-086A, Bethyl), MLL-C (Cat#A300-374A, Bethyl), ß-actin (Cat#A-4700, Sigma), GST (Cat#13-6700, Zymed), EZH2 (Cat#612666, BD or Cat#17-662, Millipore), C/EBPα (Cat#2295, Cell Signaling or Cat# SC-61, Santa Cruz), IgG (Abcam Cat#ab46540), Evi-1 (Cat #C50E12 Cell Signaling), Menin (Cat#A300-105A, Bethyl), AF9c (Cat#A300-597A, Bethyl), H3K4m3 (Cat #ab8580, Abcam), H3K79m2 (Cat #ab3594, Abcam), H3K27m3 (Cat #17-622 Millipore), and total H3 (Cat #ab1791, Abcam).

Antibodies used for Flow Cytometry

Pharmingen (San Diego, CA): anti-CD45.2 (104), c-Kit (2B8), CD34 (RAM34), B220 (RA3-6B2), Gr1 (RB6-8C5), CD4 (RM4-5), and CD8 (53-6.7); from eBioscience (San Diego, CA): CD45.1 (A20), Sca-1 (E13-161.7), and CD11b (M1/70). Biotinylated antibodies were revealed with Streptavidin-Pacific Blue (Molecular Probes, Eugene, OR) or PE-Texas Red (Caltag, Burlingame, CA). Lineage^{+} cells were defined with anti-Gr1, TER119, B220, CD19, CD8, CD4, CD3, and CD127 (IL-7R).

shRNA sequences (antisense sequence):

MLL-C 11: GCTGGCCTCCCATAATTTAT, *MLL-C* 12: CGCGGTATTATCCTAATT TAA, *MLL-C* 14: CGCCTTCACTTGACCATAATT, *MEN1*-1(25): TATGATCCTTTC AGGTACAGC, *MEN1*-2(26): TTTCTGCTTCTTC ATCTGCAC, *EZH2*-1: TTTGGTC CCAATTAACCTAGC, *EZH2*-2: ATTTGGTCC ATCTATGTTGGG, *EZH2*-3: TGATC ACCGTTAACCATCATA

Primer sequences for mouse real-time PCR to amplify cDNA

Mll-C: F: 5'-TGAGCCGTGAGGGTTCAAG-3', R: 5'-GTGAACGGTTTGCGGATG-3' *Hoxa5*:F: 5'-TTCCACTTCAACCGCTACCT -3', R: 5'-CGGCCATACTCATGCTTTT C-3', *Hoxa9*: F: 5'-CCCCGACTTCAGTCCTTGC-3', R: 5'-GATGCACGTAGGGGTG GTG-3' or F: 5'-CCACGCTTGACACTCACACT-3', R: 5'-CAGCGTCTGGTGTTTTG TGT-3', *Meis1* F: 5'-AAGGTGATGGCTTGGACAAC-3', R: 5'-TGTGCCAACTGCTT TTTCTG-3', *Ccna2*: F: 5'-GCCTTCACCATTCATGTGGAT-3', R: 5'-TTGCTGCGGG TAAAGAGACAG-3', *Gcsfr* F: 5'-CCCACCAGCTTCATCCTAAA-3', R: 5'-ACTCGC TGGACCCTAGCATA-3', *Mcsfr* F: 5'-AACACTGGGACCTACCGTTG-3', R: 5'-ACC GTTTTGCGTAAGACCTG-3', *Mecom* F: 5'-GGAGGAGGACTTGCAACAAA-3', R: 5'-GACAGCATGTGCTTCTCCAA-3', *Ezh2* F: 5'-GGGACTGAAACTGGGGGAGA-3', R: 5'-CATGGAGGCTTCAGCACCAC-3'

Primer sequences for human real-time PCR to amplify cDNA

MLL-C F: 5'-GGCCTGAATTTCTCCACAGA-3', R: 5'-TTCGACAGACGCTGTAGGTG-3', *HOXA9*: F: 5'-AGACCGAGCAAAAGACGAG-3', R: 5'-CTGAGGTTTAGAGCCGCTTT-3', *CCNA2*: F: 5'-CGCTGGCGGTACTGAAGTC-3', R: 5'-AAGGAGGAACGGTGACATGC-3', *CCNE2:* F: 5'-AAGTAGCCGTTTACAAGCTAAGC-3', R: 5'-TGATGTTTCTTGGTGACCTCC-3', *EZH2* F: 5'-CGATGATGATGATGGAGACG-3', R: 5'-GCTGTGCCCTTATCTGGAAA-3', *HOXA9* F: 5'-CACGCTTGACACTCACACT-3', R: 5'-CGCTCTCATTCTCAGCATTG-3', *MCSFR* F: 5'-GGACATTCATCAACGGCTCT-3', R: 5'-GCTCAGGACCTCAGGGTATG-3', *MEN1* F: 5'-CGCAAAGGCCTCTGAACTAC-3', R: 5'-GGAGAAAATCGTGGGTTTGA-3'

Primer sequences for murine genes for ChIP assay

Hoxa9 A: F: 5'-TGGAAGGCACAAAATTCACA-3', R: 5'-AATTAACCCGGGAGGAACAC-3', *Hoxa9* B: F: 5'-CATCGATCCCAGTAAGTGTCTC-3', R: 5'-CCGCCCCCTCACTGCAGCAGC-3', *Mecom* F: 5'-GTACCACCCACATTTCTTTCTCTC-3', R: 5'-CCAAAATGAATTAGTCACCACCTC-3', *Ezh2-1* F: 5'-TCCTGGAAATCCCTATGTGG-3', R: 5'-TAGATCCTGGCTGCTGACCT-3', *Ezh2-2* F: 5'-TCGCCTTTTCTTCCGTCGTC-3', R: 5'-CACTTTTGTTGGCGCCACTG-3', *Mcsfr* F: 5'-TTACCAGTTGGTCCCAGAGG-3', R: 5'-AGCAGCAACTGGAAGTCTCC-3'

Primer sequences for human genes for ChIP assay

HOXA9 F: 5'-AGTGGCGGCGTAAATCCT-3', R: 5'-TGATCACGTCTGTGGCTTATT TGAA-3' or F: 5'-CCGCCTTTATTCCTCTCTCC-3', R: 5'-AGTGCAACAGAGTGCC C-3', *CCNA2*: F: 5'-CCAGCCAGTTTGTTTCTCCC-3', R: 5'-GACCAATGAAAGCGC TCG -3'

cDNA microarray and GSEA

Seven control (CO) samples and six *Men1-excised* (TAM) samples were hybridized on an Affymetrix Mouse Gene 1.0 ST chip. The data analysis was done in the statistical environment R for the quality analysis the affyPLM library available through Bioconductor (www.bioconductor.org). The gene expression normalization and summarization was done using RMA from the same library mentioned above. A principal components analysis (PCA) was done to visually assess similarities and differences among the samples. For the identification of differentially expressed genes, we used the Cyber-T method. Multiple testing correction was applied using the p.adjust function. GSEA was performed to identify gene sets that were enriched in the microarray data. Gene sets were taken from the MSigDB database.

Statistical analysis

Microsoft Excel and GraphPad Prism software was used for statistical analysis. Student's t test was used to determine the significance of the results unless otherwise indicated.

Kaplan-Meier statistical analysis was performed using the log rank test. Statistical analysis of microarray results is detailed above.

CHAPTER 6 - REFERENCES

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