MLL-AF9-Induced Leukemogenesis Requires Coexpression of the Wild-Type Mll Allele

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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 MLL and oncogenic MLL-AF9 fusion protein to the loci of HOX genes to activate their transcription. Wild-type MLL not only catalyzes histone methylation at key target genes but also controls distinct MLL-AF9-induced histone methylation. Notably, the wild-type Mll allele is required for MLL-AF9-induced leukemogenesis and maintenance of MLL-AF9-transformed cells. These findings suggest an essential cooperation between an oncogene and its wild-type counterpart in MLL-AF9-induced leukemogenesis.

INTRODUCTION

Multiple oncogenic fusion proteins resulting from chromosomal translocations are capable of initiating tumorigenesis, but little is known about the role of the remaining wild-type (WT) allele in this process. 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 multiple MLL fusion proteins (MLL-FPs) (Hess, 2004; Krivtsov and Armstrong, 2007). MLL-FPs are capable of leukemic transformation and dysregulation of multiple Hox genes, including Hoxa9. 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 (Okada et al., 2005). The H3K79 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 WT counterparts of additional MLL fusion partners such as AF4 and ENL have been shown to interact with Dot1L in a large protein complex (Bitoun et al., 2007; Mueller et al., 2007), illustrating one common mechanism for 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 (Suv3-9, Enhancer of zeste and Trithorax), which catalyzes histone H3 lysine 4 (H3K4) methylation and upregulates transcription of HOX genes in fibroblasts or epithelial cell lines (Milne et al., 2002; Nakamura et al., 2002). H3K4 trimethylation (H3K4m3) is associated with euchromatin and active genes and specifically recruits chromatin-remodeling proteins to stimulate gene expression (Berger, 2007; Flanagan et al., 2005; Li et al., 2006).

WT MLL forms a large complex with several proteins including menin (Hughes et al., 2004; Yokoyama et al., 2005), a nuclear

Significance

The potential role of wild-type (WT) MLL in the development of mixed lineage leukemia, which is highly aggressive and often refractory to therapy, has been elusive. We demonstrate a crucial role for WT MLL, the common WT precursor of over 60 distinct MLL-FPs, in MLL-AF9-induced leukemogenesis. WT MLL influences MLL-AF9-induced histone methylation and gene expression as well as growth and survival of MLL-AF9-transformed leukemia cells. These findings underscore the importance of WT MLL in the development of MLL-AF9-induced acute leukemia.
DNA-binding 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 (Yokoyama et al., 2005), increases H3K4 trimethylation (H3K4m3) at the Hoxa9 locus, and upregulates its transcription in MLL-FP-transformed hematopoietic cells (Chen et al., 2006; Yokoyama et al., 2005). Moreover, menin is required for proliferation of cells transformed by MLL-AF9 fusion protein (MA9 hereafter) (Chen et al., 2006). However, little is known as to whether menin affects MA9-regulated H3K79 methylation and whether WT MLL is important for MA9-mediated leukemic transformation.

The potential role (or lack thereof) of WT MLL in MLL-FP-induced leukemogenesis has not been addressed. On the one hand, despite a lack of the WT MLL SET domain, MA9 remains capable of initiating leukemogenesis when introduced into WT 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 partly by WT MLL. Furthermore, in MLL-FP-expressing human leukemia cells, which in theory lose one of the two WT MLL alleles in 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 raise the possibility that WT MLL is not crucial for oncogenic transformation by MLL-FPs. On the other hand, WT MLL is crucial for H3K4 methylation and expression of HOX genes in fibroblasts and HeLa cells (Mline et al., 2002; Nakamura et al., 2002). Moreover, WT MLL excision compromises the function of hematopoietic stem cells (HSCs) and expression of 5° HOX genes, including Hoxa9 (Jude et al., 2007; McMahon et al., 2007), yet these HOX genes are upregulated in an MA9-transformed leukemia stem cell (LSC)–enriched population (Krivtsov et al., 2006), raising the possibility that WT MLL is involved in MA9-induced leukemogenesis. Therefore, whether the WT MLL allele is crucial for MA9-induced leukemogenesis remains unresolved.

A better understanding of the function of these WT alleles in tumorigenesis could yield insights into the mechanisms of transformation. Our earlier findings on the role of menin in proliferation and gene transcription of MA9-transformed cells prompted us to investigate the potential role of WT MLL in MA9-induced leukemogenesis.

RESULTS

Menin Is Required for Methylation of Both Histone H3 Lysine 4 and Histone H3 Lysine 79 at the Hoxa9 Locus

The MLL-AF10 fusion protein has been reported to transform BM by increasing Dot1L-catalyzed H3K79 methylation but repressing histone H3 lysine 4 (H3K4) methylation at the Hoxa9 locus, suggesting that histone H3 lysine 79 (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 fusion proteins (Yokoyama et al., 2005), little is known as to whether menin is crucial for H3K79 methylation at Hoxa9 in MA9-transformed BM cells. To address this question, we excised the floxed Men1 gene in MA9-transformed BM cells (AT1 cells), which harbor Men1+/−;Cre-ER, using 4-hydroxy tamoxifen (4-OHT) to induce Cre activity (Figure 1A, lane 2). We performed chromatin immunoprecipitation (ChIP) assays with the control and Men1-excised cells. Our results showed that Men1 excision reduced H3K79 dimethylation in two separate locations at the Hoxa9 locus, as shown by amplicons a and b (Figure 1B). As Dot1L is the only known H3K79-methylating enzyme in mammals, this finding is consistent with the notion that menin is crucial for MA9-induced Dot1L recruitment to the Hoxa9 locus and H3K79 methylation at the locus.

We also noted that Men1 excision reduced H3K4 trimethylation at Hoxa9 (Figure 1C), in agreement with our previous findings (Chen et al., 2006). However, this finding is different from 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 promoter of Hoxa9, and menin binding was abrogated in Men1D/D cells (Figure 1D). Together, these results demonstrate that menin is crucial not only for H3K4 methylation but also for Dot1L-mediated H3K79 methylation at the Hoxa9 locus in the MA9-transformed cells. These results raise 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 to the locus depends on menin in MA9-transformed BM cells. Because MA9 lacks the MLL-C portion of WT MLL, detection of MLL-C at the Hoxa9 locus indicates that WT MLL is recruited to the locus. Hence, we chose to use an antibody that specifically recognizes only MLL-C to detect WT MLL. ChIP assays showed that MLL-C bound the Hoxa9 locus (Figure 2A), but the MLL-C binding was abrogated when Men1 was excised (Men1ΔΔ) in the MA9-transformed BM cells (Figure 2A). These results indicate that menin is required for recruiting WT MLL to Hoxa9 in the MA9-transformed cells.

Because menin interacts with the N terminus of MLL (Yokoyama et al., 2005), we determined whether menin affects recruitment of MA9 (which contains the N terminus of MLL) to Hoxa9 by ChIP assay, using an anti-AF9 antibody that recognizes the AF9 portion of the MA9 fusion protein (Figure 2B).

WT MLL Is Required for Proliferation and Survival of MA9-Transformed Leukemia Cells and for Expression of HOX Genes

It has been unresolved whether WT MLL is crucial for MLL-FP-induced leukemia. On the one hand, H3K4 methylation, which is at least partly mediated by WT MLL, is repressed in MLL-AF10-transformed cells (Okada et al., 2005). On the other hand, WT MLL is crucial for H3K4 methylation and expression of HOX genes (Milne et al., 2002; Nakamura et al., 2002), some of which are crucial for BM transformation by certain MLL-FPs (Ayton and Cleary, 2003). Our data indicate a crucial role for menin in WT MLL recruitment to and H3K4 methylation at Hoxa9locus, thereby increasing WT MLL-mediated H3K4 methylation and Dot1L-mediated H3K79 methylation at the locus, respectively.

Our results indicate that MA9 bound to Hoxa9 and that Men1 excision reduced MA9 binding to the locus (Figure 2B). Because the C-terminal portion (aa 397–557) of AF9 has been reported to bind Dot1L (Zhang et al., 2006), we tested whether the AF9 part of MA9 bound Dot1L using a GST-AF9 pull-down assay and found that the AF9 portion from MA9 bound Dot1L (see Figures S1A–S1C available online). To further evaluate whether menin affects recruitment of MA9 to the Hoxa9 locus in MA9-expressing human leukemia cells, we knocked down menin expression, using lentiviruses expressing MEN1 shRNAs, in THP-1 cells that harbor MA9 (Figures S1D and S1F). MEN1 knockdown (KD) reduced the number of THP-1 cells (Figure S1E), HOXA9 expression (Figure S1F), and menin binding to the HOXA9 locus (Figure S1G). Consistently, MEN1 KD also reduced the binding of both MA9 and Dot1L to the HOXA9 locus (Figure 2C). Together with data from Figure 1, these results indicate that menin promotes recruitment of both WT MLL and MA9/Dot1L to the HOXA9 locus, thereby increasing WT MLL-mediated H3K4 methylation and Dot1L-mediated H3K79 methylation at the locus, respectively.
scrambled vector, reduced the number of AT1 cells (Figure 3B) and the mRNA levels of WT Mll, Hoxa9, and Ccna2, which encodes cyclin A2 (referred to as cyclin A hereafter) (Figure 3C), and the MLL-C and cyclin A proteins (Figure 3D).

To determine whether WT MLL is also crucial for proliferation of human MA9-expressing leukemia cells, we knocked down WT MLL in THP-1 cells by transducing lentiviruses expressing shRNAs that targeted the C terminus of human and murine MLL (Figure 3A). Two independent MLL-C shRNAs, but not the control shRNA, reduced the number of THP-1 cells (Figure 3E). We also detected an increased percentage of dead cells from WT MLL knockdown (KD) THP-1 cells (Figure S2). As expected, WT MLL expression in the shRNA-transduced cells was reduced, as shown by qRT-PCR and western blotting (Figures 3F and 3G). The protein level of MLL-N was also reduced; however, the menin and MA9 levels were not affected (Figure 3H). The expression of HOXA9 and CCNA2 was also reduced in WT MLL KD cells (Figures 3F and 3G). Collectively, these results indicate that WT MLL upregulates expression of HOXA9 and CCNA2 as well as promoting proliferation and survival of the human 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 the Mllf/f mice (Jude et al., 2007) with ubc9-Cre-ER mice (Ruzankina et al., 2007) and demonstrated efficient Mll excision that was induced by 4-hydroxy tamoxifen (4-OHT) in splenocytes from the Mllf/f;Cre-ER mice (Figures 4A and 4B). Mll excision after BM cells were transformed with MA9-expressing retroviruses reduced the number of the MA9-transformed BM cells (Figure 4C) and the expression of Hox genes in these cells (Figure 4D), whereas 4-OHT did not affect proliferation of MA9-transformed cells that did not express Cre-ER (Figures S3A and S3B). Together, these experiments demonstrate that WT MLL is important for proliferation and survival of MA9-transformed leukemia cells and for high-level expression of HOX genes in these cells.

**WT MLL Is Crucial for Maximal Methylation of Both H3K4 and H3K79 at the Target Genes**

On the one hand, the SET domain of WT MLL methylates H3K4 yet is deleted from MA9 fusion protein (Milne et al., 2002). On the
other hand, loss of menin, an MLL-interacting protein, reduced H3K4 methylation at *Hoxa9* in MA9-transformed cells (Figure 1C). It has been unclear whether the remaining WT MLL allele in human MA9-expressing leukemia cells is still crucial for H3K4 methylation. We performed ChIP assays with control and WT MLL KD THP-1 cells. WT MLL KD reduced MLL-C binding to *HOXA9* (Figure 5A), indicating that WT MLL is recruited to its targets in the leukemia cells.

We have shown that menin is crucial for both H3K4 and H3K79 methylation at the *Hoxa9* locus (Figure 1) through recruiting WT MLL and MA9/Dot1L, respectively (Figure 2). WT counterparts of several MLL fusion partners, such as AF4, AF9, and ENL, interact with each other as well as with Dot1L in a transcription-activating complex (Bitoun et al., 2007; Mueller et al., 2007). However, because WT MLL is not known to directly interact with the WT proteins of its fusion partners, it remains elusive whether WT MLL is important for MA9/Dot1L-mediated H3K79 methylation. Notably, H3K79 methylation was also reduced at the *HOXA9* locus in these WT MLL KD cells (Figure 5B), 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 5C), reinforcing the role of WT MLL in both H3K4 and H3K79 methylation. Collectively, these results suggest that WT MLL is crucial for maximal expression of both HSC-enriched HOX genes and proliferating cell cycle genes, such as CCNA2, probably reflecting a dual role in regulating the self-renewal and proliferation of MA9-transformed LSCs.

### Figure 4. WT Mll Excision Reduces the Number of MA9-Transformed Cells 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*. Spleen cells from a *Mllf/f;Cre-ER* mouse were cultured with either DMSO or 4-OHT; this was followed by isolation of the genomic DNA and PCR amplification.

(C) A growth curve for MA9-transformed BM cells with either *Mllf/f* or *MllD/D* (± SD, cells seeded in triplicate).

(D) Quantification of *Hoxa9* and *Hoxa5* mRNAs in either *Mllf/f* or *MllD/D* MA9-transformed cells (± SD). See also Figure S3.

Ablation or Knockdown of WT MLL Reduces Colony Formation of MA9-Transduced BM

Regulation of H3K79 methylation by WT MLL raised an intriguing possibility that WT MLL is important for transformation of BM by MA9 or maintenance of MA9-transformed cells. 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 semisolid 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 the control shRNA or each of the two MLL-C shRNAs into the MA9-transduced BM cells after the second plating, followed by puromycin selection (Figure 6A). 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 6B, Scram). However, WT MLL KD with each of the MLL-C shRNAs reduced colony formation from the MA9-transduced BM (Figures 6B and 6C).

To further confirm the role of WT MLL in MA9-induced colony formation, we used *Mllfl/fl*;Cre-ER BM to excise WT MLL after transformation. BM from control *Mllf/f* or *MllD/D*;Cre-ER mice was transduced with MA9 retroviruses and serially replated on a semisolid medium (Figure 7A). *Mll* excision induced by 4-OHT significantly...

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**Figure 5. WT MLL Is Crucial for Maximal Methylation of Both H3K4 and H3K79 at Key Target Genes**

THP-1 cells were transduced with either control scrambled shRNA or MLL-C shRNA 14 lentiviruses to knock down WT MLL and were evaluated with ChIP assay for MLL-C binding to Hoxa9 (A) and for histone H3K4m3 and H3K79m2 at HOXA9 (B) and CCNA2 (C). The specificity of the anti-H3K4m3 antibody and the anti-H3K79m2 were confirmed using specifically modified peptides and Western blot. Error bars denote ± SD. See also Figure S4.
examination revealed that WT MLL KD reduced engraftment of the human leukemia cells. To confirm these results, we established a mouse leukemia model in which the floxed Mll can be excised in a temporally controllable manner. BM from the Mllf/f;Cre-ER mice was transduced with MA9 retroviruses. These transduced primary BM cells were transplanted into lethally irradiated C57B6 x B6.SJL F1 mice (Figure 8A). Flow cytometry analysis of peripheral blood demonstrated successful engraftment of MA9-transduced donor BM (CD45.1+/2+) and cotransplanted WT BM (CD45.1+/2+) (Figure S5A). The percentage of cells expressing CD11b or CD11b/Gr-1 (myeloid markers) was much higher within the MA9-transduced BM (Figures S5C and S5D) than that from the cotransplanted normal BM (Figure S5B). Overt acute leukemia developed because obvious leukemia cell infiltrations were detectable in various organs, including the femur, liver, and spleen (Figures S5H–S5J).

For examining the impact of WT Mll excision on the development of MA9-induced acute myeloid leukemia (AML), the MA9 transduced BM-transplanted mice were fed with either control corn oil or TAM to excise the floxed Mll from MA9-transduced cells and were monitored for peripheral white blood cell (WBC) number, immunophenotypes of the WBCs, and survival rate of the recipient mice (Figure 8A). 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 control cells (Figures 8B and 8C; p < 0.05). These results suggest that WT Mll KD reduced engraftment of the human leukemia cells.

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 Mllf/f;Cre-ER mice that were treated with tamoxifen (TAM) displayed effective excision of the floxed Mll allele (Figure 7F, bottom) and reduction of colony formation (Figure 7F, top). Moreover, BM with WT Mll or with previous deletion of WT Mll was transduced with retroviruses expressing MA9 and was plated on semisolid medium. We found that previous deletion of WT Mll reduced colony formation at the first plating (Figure 7G). These results, coupled with other results from the colony formation assay, indicated that WT Mll is crucial for survival 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 in mice using a xenotransplantation model. Either scrambled vector or MLL-C shRNA-transduced THP-1 cells were injected into NOD-SCID mice. Six weeks after transplantation, histological examination revealed that WT MLL KD reduced leukemic infiltration in long bones and spleens (Figure S4A). The spleens from mice injected with the control cells were significantly larger than those of the mice injected with the WT MLL KD cells (Figures S4B and S4C; p < 0.05). These results suggest that WT MLL KD reduced engraftment of the human leukemia cells.

Reduced the number of colonies from the MA9-transformed Mllf/f;Cre-ER BM (Figure 7B). As a control, 4-OHT failed to reduce colony formation from MA9 Mllf/f BM (Figure 7C). Tamoxifen (4-OHT)-induced excision of the floxed Mll in MA9-transformed BM from the Mllf/f;Cre-ER mice was confirmed by genomic PCR (Figure 7D, lane 2). These results indicate that the WT Mll alleles are required for maintenance of MA9-transformed cells. Hox9 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 Hox9/Meis1 (Figure 7E). These results indicate that WT Mll is not required for colony formation induced by Hox9/Meis1, likely because both Hox9 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 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 Mllf/f;Cre-ER mice that were treated with tamoxifen (TAM) displayed effective excision of the floxed Mll allele (Figure 7F, bottom) and reduction of colony formation (Figure 7F, top). Moreover, BM with WT Mll or with previous deletion of WT Mll was transduced with retroviruses expressing MA9 and was plated on semisolid medium. We found that previous deletion
H3K4 trimethylation at the loci of leukemic transformation (Popovic and Zeleznik-Le, 2005). unresolved whether WT MLL plays a role in MLL-FP-mediated partly mediated by WT MLL. Therefore, it has been unclear and 5 leukemogenesis by aberrantly upregulating expression of certain tumorigenesis. A prevalent model suggests that MLL-FPs induce with little attention paid to the role of the cognate WT alleles in malignant diseases are often centered on the fusion proteins domain in WT MLL was not thought to be crucial for MLL-FP-trig-
repressing H3K4 methylation (Okada et al., 2005). The SET (Milne et al., 2005), partly through enhancing Dot1L-mediated H3K79 while does not suppress H3K4 trimethylation at (Figures 3 and 4; Figures S2 and S5L), and for maintenance of repression of H3K4 dimethylation (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 H3K4 trimethylation at Hoxa9 (Milne et al., 2005). Additionally, in MLL-AF4-expressing human leukemia cells, both methylated H3K4 and H3K79 are colocalized at large chromatin domains, including the domain harboring the HOXA7 and HOXA9 loci (Guenther et al., 2008).

WT MLL may control MA9-induced leukemogenesis by facilitating the expression of HOX genes and other self-renewal genes and supporting LSC maintenance. Although WT MLL is crucial for preventing HSCs from abnormal entry into cell cycle, it may be particularly important for promoting proliferation and survival of MA9 LSCs (Figures 8D and 8E), a function distinct from that in HSCs (Jude et al., 2007; McMahon et al., 2007). Supporting this explanation, the expression of Hoxa9 is reactivated in MA9-transformed LSCs (Krivtsov et al., 2006), and WT MLL is crucial for growth and survival of MA9-transformed leukemia cells (Figures 3 and 4; Figures S2 and S5L), and for maintenance of MA9 LSCs (Figures 8D and 8E). Therefore, both growth and survival may represent mechanisms by which WT MLL influences MA9 transformed cells. However, our data using Hoxa9/Meis1-mediated BM transformation suggest that this requirement for WT MLL can be replaced by ectopic expression of Hoxa9/Meis1, likely because they are direct targets of MLL and therefore, in this case, act downstream of MLL (Figure 7E).

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 increase H3K79 methylation at target loci (Krivtsov et al., 2008; Okada et al., 2005) and WT MLL is also necessary for maximal H3K79

The WT Mll Allele, the Common Precursor of All MLL-FPs, Controls MA9-Induced Leukemogenesis Oncogenic fusion proteins resulting from chromosomal translocations represent a major molecular lesion in leukemia and certain solid tumors (Nambiar et al., 2008). Studies about these malignant diseases are often centered on the fusion proteins with little attention paid to the role of the cognate WT alleles in tumorigenesis. A prevalent model suggests that MLL-FPs induce leukemogenesis by aberrantly upregulating expression of certain 5′-Hoxa genes (Yokoyama and Cleary, 2008; Yokoyama et al., 2005), partly through enhancing Dot1L-mediated H3K79 while repressing H3K4 methylation (Okada et al., 2005). The SET domain in WT MLL was not thought to be crucial for MLL-FP-triggered leukemogenesis, given that MLL-AF10 even suppresses H3K4 dimethylation (Okada et al., 2005), which could be at least partly mediated by WT MLL. Therefore, it has been unclear and unresolved whether WT MLL plays a role in MLL-FP-mediated leukemic transformation (Popovic and Zeleznik-Le, 2005).

We have shown that WT MLL is crucial for maximal levels of H3K4 trimethylation at the loci of HOXA9 and CCNA2 and for MA9-induced leukemogenesis. Although MLL-AF10 suppresses H3K4 dimethylation, because WT MLL is able to convert dimethylated H3K4 (H3K4m2) to trimethylated H3K4 (H3K4m3), the repression of H3K4 dimethylation (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 H3K4 trimethylation at Hoxa9 (Milne et al., 2005). Additionally, in MLL-AF4-expressing human leukemia cells, both methylated H3K4 and H3K79 are colocalized at large chromatin domains, including the domain harboring the HOXA7 and HOXA9 loci (Guenther et al., 2008).

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methyltransferase (HMT) activity and epigenetic regulation. The WT Mll allele is known to be essential for MA9-induced leukemogenesis, as MA9 is insufficient for maintaining MA9-transformed leukemia cells without the WT Mll allele. Rather, it depends on coexpression of the WT Mll allele, which we have shown to upregulate not only H3K4 methylation but also MA9/Dot1L-mediated H3K79 methylation at HOXA9. Consistent with this model, Men1 depletion reduced recruitment of WT MLL to the Hoxa9 locus and diminished not only H3K4 methylation but also H3K79 methylation at the locus.

These findings have revealed a link between WT MLL and MA9 in upregulating stem cell-related HOX genes and certain cell cycle genes in MA9-transformed leukemia cells. These results are consistent with a model whereby menin, WT MLL, and MA9 converge at the loci of HOXA9 and certain cell cycle genes such as CCNA2. Menin may recruit both WT MLL and MA9 at these target genes to epigenetically promote their transcription, leading to maintenance of LSCs and the development of MA9-induced leukemia (Figure 8G). MLL is also associated with the cyclin E locus in mouse embryonic fibroblasts (Takeda et al.,...
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. One may argue that if the WT MLL allele is required for MLL-FP-induced leukemogenesis, then what is the benefit for the leukemia cells to cripple one of the two WT MLL alleles to generate an MLL-FP? One likely explanation is that, for instance, MA9 or MA9-induced H3K79 dimethylation cooperates with WT MLL or WT MLL-mediated H3K4 trimethylation in activation of HOX genes and epigenetic chromatin reprogramming, offsetting the loss of one of the two WT MLL alleles. This is consistent with the findings that WT MLL and MA9 target genes, HOX genes, are expressed at a higher level in MLL-FP-expressing human leukemia cells than in non-MLL-FP-expressing human leukemia cells (Armstrong et al., 2002). Moreover, WT MLL may also regulate genes controlling proliferation and self-renewal in MA9-transformed leukemia cells or LSCs.

The necessity for WT MLL in multiple processes of leukemogenesis, including MA9/Dot1L-mediated H3K79 dimethylation at HOX genes and maintenance of MA9-expressing LSCs, could render MA9-harbouring leukemia cells selectively sensitive to inhibition of WT MLL. Despite the demonstration that MLL is crucial for HSC and progenitor maintenance (Jude et al., 2007), another Mll knockout model does not display obvious defects in homeostatic hematopoiesis (McMahon et al., 2007). It is possible that MLL-FP-expressing leukemia cells may become particularly “addicted” to WT MLL for expression of stem cell-related HOX genes and certain cell growth- and survival-related genes. This “addiction” may be attributable to the reduced cellular WT MLL levels in human leukemia cells that, in theory, result from disruption of one WT MLL allele in 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 WT HSCs (Chen et al., 2008). Moreover, WT MLL may also be more crucial to the MLL-FP-expressing leukemia cells because these cells rely on WT MLL for MLL-FP-induced H3K79 dimethylation and gene transcription (Figure 8G). However, it cannot be ruled out that WT MLL is also crucial for transformation induced by non-MLL-FPs.

Collectively, our findings support development of strategies to treat MLL-FP-induced leukemia, in part by targeting the pathway of menin and WT MLL, the common WT allele of over 60 MLL-FPs. WT MLL may well serve as a “nononcogene addiction” target for treating MLL-FP-expressing leukemia (Solimini et al., 2007). 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.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Culture

Various plasmids were as described elsewhere: pMX-GFP, pMX-puro, pMX-puro-menin, pMSCV-MLL-AF9 (MA9), pMSCVpGk-Hoxa9-GFP, pMSCVpAc-Meis1A, and pMSCV-GFP (Chen et al., 2006; Jin et al., 2003). Retroviral or lentiviral constructs expressing shRNAs were obtained from Open Biosystems: retroviral constructs for mouse shRNAs, pshRNA 11-MLL-C (mouse, 5′-gctgctgctgctgctg)-3′, and lentiviral constructs expressing MLL-C shRNAs-12 and 14 (5′-cggcggatttacctactactaatttcaattt-3′) and 5′-cggcggatttacctactactaatttcaattt-3′) were highly conserved between the mouse and human WT MLL.
mRNAs, plKO.1 vector (Scram) was from Sigma. Lentiviral packaging plasmids, pMD2G and pAX2G, were purchased from Addgene.

HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% Pen/Strep. AT1 cells were generated from BM cells of Mllf/f;Cre-ER mice by transduction with retroviruses expressing MA9 and cultured in medium with 10 ng/ml IL3 (Chen et al., 2006). In MA9-transformed BM cells, Mllf/f or Mllf/ff was excised by treating the cells with 4-OH tamoxifen (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.

**Packaging of Recombinant Retroviruses and Lentiviruses and Transfection of Cells.**
pMX-GFP, pMX-puro, and pshRNAs were cotransfected with psi-2 helper plasmid into 293T cells using the calcium chloride precipitation method, as described elsewhere (Jin et al., 2003). For packaging lentiviruses, lent-GFP, scrambled plKO.1 vector or specific pshRNAs were cotransfected into 293T cells with pAX2G and pMD2G, using Fugene-mediated transfection. The resulting recombinant retro- or lentiviruses were collected for transduction of cells by spinoculation, followed by selection in 2 μg/ml puromycin for 2 days.

**RNA Extraction and Quantitative RT-PCR**
Total RNA was extracted from the cultured cells with Trizol and an RNeasy extraction kit (QIAGEN) and was used as template (1 μg) to synthesize cDNA for quantitative RT-PCR (qRT-PCR) analysis in a 7500 fast real-time PCR system (Applied Biosystems). Relative mRNA levels of a specific gene were calculated by ΔΔ-CT values calibrated with either GAPDH or beta-actin, using SYBR green dye for detection (QIAGEN, QuantiTect SYBR Green Mastermix). The sequences of primers for qRT-PCR are listed in Supplemental Experimental Procedures.

**Chromatin Immunoprecipitation (ChIP) Assay**
The ChIP assay was performed as described elsewhere, with a Quick ChIP kit (Imgenex) (Chen et al., 2006; Yan et al., 2006). In brief, cells (106) were fixed with 1% formaldehyde and lysed in ChIP lysis buffer with protease inhibitors. The genomic DNA was incubated with either control IgG or a specific primary antibody at 4°C overnight, and collected with protein G agarose beads. The protein-DNA complexes were eluted from the beads and incubated at 65°C overnight for reversal of the protein-DNA crosslinking. Quantity of the precipitated DNA was determined with qPCR (QIAGEN, QuantiTect SYBR Green Mastermix) and normalized with the input genomic DNA. The sequences of the primer pairs for ChIP assay are listed in the Supplemental Experimental Procedures.

**Mice and BM Transformation**
All laboratory mice were maintained on a 12 hr 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. Mllf/f mice (in C57Bl/SJL background) (Jude et al., 2007) were bred with ubc9-Cre-ER mice (in C57Bl background) (Schneppe et al., 2006), and BM from the mice was isolated and transformed, as described elsewhere (Chen et al., 2006). Briefly, C57Bl6 mice or Mllf/f;Cre-ER mice (6–8 weeks old) were injected with 5-fluorouracil, and BM cells were collected from femurs and prestimulated with a cocktail of cytokines and growth factors, as described elsewhere (Chen et al., 2006). The cells were transduced twice with pMSCV-MA9 retroviruses by spinoculation and reaped weekly in MethoCult GF M3434 medium (StemCell Technologies) with 1 mg/ml G418. After the second plating, 2 μg/ml puromycin and scored for colonies with > 50 cells one week after plating. To excise the floxed Mll, MA9-transformed BM with Mllf/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, Mllf/f;Cre-ER mice were treated with corn oil or 200 mg/kg body weight TAM to excise the floxed Mll, as described elsewhere (Schneppe et al., 2006), and then isolated BM was either plated directly in methylcellulose or transduced with MA9 and then plated.

**Leukemia Induction and WT Mll Excision from Leukemic cells in Mice**
The Mllf/f mice were backcrossed with C57Bl/SJL 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 C57Bl background, CD45.2) (Ruzankina et al., 2007). The Mllf/f;Cre-ER mice were intercrossed so that the Mllf/f; Cre-ER genotype with the CD45.2+ marker could be maintained. BM cells from these mice were transduced with MA9-retroviruses and transplanted retro-orbitally into C57Bl6 × C57Bl6-SJL F1 female mice (CD45.1+2+, 8 weeks old, 106 cells per mouse, Taconic), together with 2.5 x 10⁵ BM cells from an F1 mouse. The recipient mice were irradiated with 900 rad, prior to transplantation. The mice were fed with either control corn oil or tamoxifen (TAM) (Sigma) at a dose of 200 mg/kg body weight to excise the floxed Mll. Organs from control and leukemic mice were isolated, fixed, and processed for H&E staining and analyzed under a microscope.

**Flow Cytometry Analysis and Antibodies**
Cells from peripheral blood, BM, or spleen were harvested for analysis of immunophenotypes. After blocking unspecific binding with unlabeled rat plus mouse IgG (Sigma), cells were stained on ice in PBS plus 4% FCS and analyzed on LSR II, FACSCalibur, or FACSaria (Becton Dickinson). Files were analyzed in FlowJo (Tree Star). Antibodies for flow cytometry analysis, immunoblotting, and ChIP assay were described in Supplemental Experimental Procedures. All the biochemical experiments, including the ChIP assay, qRT-PCR, and cell proliferation, were repeated at least twice with consistent results.

**Statistical Analysis**
Microsoft Excel and GraphPad Prism software was used for statistical analysis. Student’s t test was used for determining the significance of the results unless otherwise indicated. Kaplan-Meier statistical analysis was performed with the log rank test.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2009.12.034.

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