The tumor suppressor menin regulates hematopoiesis and myeloid transformation by influencing Hox gene expression

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Menin is the product of the tumor suppressor gene *Men1* that is mutated in the inherited tumor syndrome multiple endocrine neoplasia type 1 (MEN1). Menin has been shown to interact with SET-1 domain-containing histone 3 lysine 4 (H3K4) methyltransferases including mixed lineage leukemia proteins to regulate homeobox (*Hox*) gene expression *in vitro*. Using conditional *Men1* knockout mice, we have investigated the requirement for menin in hematopoiesis and myeloid transformation. *Men1* excision causes reduction of Hoxa9 expression, colony formation by hematopoietic progenitors, and the peripheral white blood cell count. Menin directly activates Hoxa9 expression, at least in part, by binding to the *Hoxa9* locus, facilitating methylation of H3K4, and recruiting the methylated H3K4 binding protein chd1 to the locus. Consistent with signaling downstream of menin, ectopic expression of both Hoxa9 and Meis1 rescues colony formation defects in *Men1*-excised bone marrow. Moreover, *Men1* excision also suppresses proliferation of leukemogenic mixed lineage leukemia-AF9 fusion-protein-transformed myeloid cells and *Hoxa9* expression. These studies uncover an important role for menin in both normal hematopoiesis and myeloid transformation and provide a mechanistic understanding of menin's function in these processes that may be used for therapy.

multiple endocrine neoplasia type 1 | mixed lineage leukemia | *Men1* gene | histone lysine methylation

Menin is a tumor suppressor encoded by *Men1* that is mutated in the human inherited tumor syndrome multiple endocrine neoplasia type 1 (MEN1) (1, 2). This syndrome is characterized by tumor development in multiple endocrine organs (3, 4). Although the primary sequence of menin is highly conserved from fly to human, it does not bear obvious homology to known protein motifs, making it difficult to elucidate its biochemical function. Recent progress showed that menin regulates cell proliferation (5–7), apoptosis (8, 9), and genome stability (10–12). Many of these functions rely on the ability of menin to regulate transcription of various genes, including cyclin-dependent kinase inhibitors and caspase 8 (9, 13). Although menin directly binds DNA and associates with chromatin, no evidence shows that it binds to specific DNA sequences to regulate gene transcription (14).

Menin interacts with several transcription factors such as Jun D and NF-κB and a yeast SET1-like complex containing mixed-lineage leukemia (MLL) protein (15–19). MLL is a large protein with a highly conserved SET domain that specifically methylates histone 3 lysine 4 (H3K4) (20, 21). This protein is often fused with other proteins in MLL cells because of chromosomal translocations (22–24). These MLL fusion proteins play a crucial role in up-regulating the homeobox (Hox) genes, including *Hoxa9*, and the development of MLL. Wild-type MLL up-regulates transcription of *Hox* genes, presumably by binding to the promoters of these genes and methylating H3K4 (20, 21). Both MLL and its target *Hoxa9*, a Hox-containing transcription factor, are required for normal he-

matopoiesis (25, 26), and MLL fusion proteins may lead to dysregulated expression of Hoxa9 and its cofactor Meis1, resulting in the development of MLL (27).

Although menin and its interaction with MLL are implicated in regulating several *Hox* genes (18, 19), it is unclear whether menin plays a role in either hematopoiesis or MLL oncoprotein-induced myeloid transformation. To examine the potential role of menin in hematopoiesis, we generated a system that can excise *Men1* in a temporally controlled manner. Our studies revealed an important role for menin in bone marrow (BM) hematopoiesis and proliferation of MLL-AF9-transformed myeloid cells. These menin functions are executed, at least in part, by binding to the *Hoxa9* locus and up-regulating *Hoxa9* expression, via methylation of H3K4 at the *Hoxa9* locus. These data provide a molecular model whereby menin-dependent modification of histones at certain *Hox* gene loci plays an important role in hematopoiesis and suggest that menin is required for proliferation of some MLL fusion protein-transformed cells.

Results

Men1 Excision from Men1[l/l];Cre-ER Mice Reduces the Peripheral White Blood Cell (WBC) Count. Since homozygous *Men1* deletion in mice leads to early embryonic lethality ~day 11 because of unknown causes (28, 29), it is difficult to assess the role of menin in hematopoiesis. Although mice harboring conditional *Men1* alleles (floxed *Men1*) and tissue-specific Cre were successfully used to determine the role of menin in tumor suppression in endocrine and liver cells (30–33), these models were unable to address menin function in hematopoiesis. Thus, we used a conditional system to delete *Men1* in a temporally controlled manner via tamoxifen (TAM or 4-OHT) treatment. The floxed *Men1* (*Men1[l/l]*) mice (30) were bred with mice expressing the Cre-ERT2 fusion protein that can be activated by TAM, and the transgene expressing Cre-ERT2 was driven by a ubiquitously active UBC9 promoter (34, 35) (E.J.B., unpublished data). To determine whether oral ingestion of TAM effectively excises the floxed *Men1* from the *Men1[l/l];Cre-ER* mice, BM genomic DNA was prepared from either untreated *Men1[l/l];Cre-ER* mice or TAM-treated *Men1^{+/+};Cre-ER* and *Men1[l/l];Cre-ER* mice for PCR genotyping (Fig. 1 *A* and *B*). Treating the *Men1[l/l];Cre-ER* mice with TAM effectively excised the floxed *Men1* from BM (Fig. 1*B*, lane 2), whereas the floxed *Men1* was not excised in the
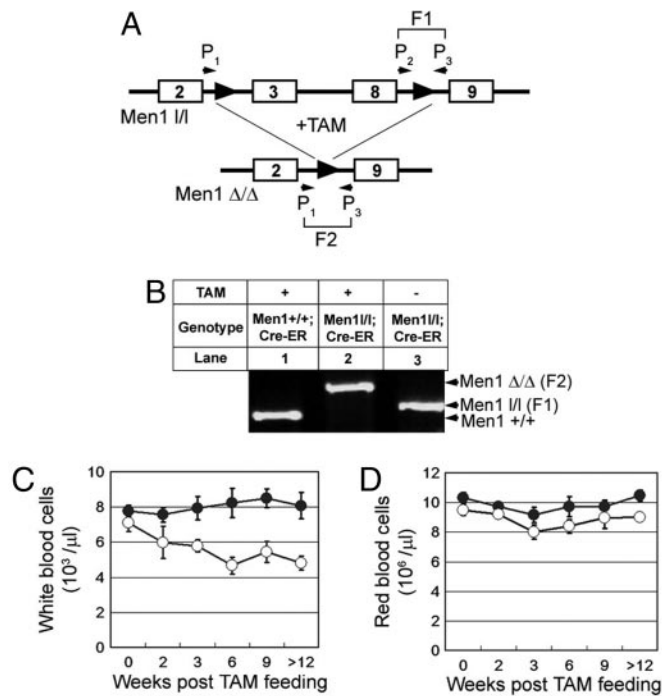
Fig. 1. Excision of floxed *Men1* in mice reduces the peripheral WBC counts. (*A*) A diagram for the floxed *Men1* and primers for detecting excision of the floxed *Men1* in genome. P1, P2, and P3 denote primers 1, 2, and 3, respectively. F1 and F2 are DNA fragments representing the unexcised and excised floxed *Men1* loci, respectively. The numbered boxes denote exon numbers. (*B*) Tight control of TAM induced the floxed *Men1* excision. *Men1^{+/+}*;*Cre-ER* mice (lane 1) and *Men1^{l/l}*;*Cre-ER* mice (lane 2) were treated with TAM, whereas *Men1^{l/l}*;*Cre-ER* mice (lane 3) were treated with corn oil. BM genomic DNA from each of the mice was used for genotyping. The results are representative of three mice for each condition. *Men1^{Δ/Δ}* (F2), excised *Men1* locus; *Men1^{l/l}* (F1), unexcised floxed *Men1*. (*C*) Total peripheral WBC of control *Men1^{+/+}*;*Cre-ER* mice (●) and *Men1^{l/l}*;*Cre-ER* mice (○) are plotted, after treatment with TAM for various periods of time (weeks). (*D*) Time course of RBC counts in mice of both distinct genotypes. Five to 20 mice for each group were used for each time point over 12 weeks.

Fig. 2. *Men1* excision *in vitro* reduces colony formation of BM. (*A–D*) Representative plates with visible colonies from colony formation assays. BM pooled from *Men1^{+/+}*;*Cre-ER* mice or *Men1^{l/l}*;*Cre-ER* mice were plated (2.5 × 10^4 cells per plate) in complete medium supplemented with either DMSO or 4-OHT. The genotype of BM cells and treatment applied to the plate are indicated. (*E*) PCR genotyping with genomic DNA from the above plates as templates. (*F*) Summary of colony numbers from three independent experiments with duplicate plates. Asterisks indicate statistically significant differences between the adjacent groups ($P < 3 \times 10^{-4}$). (*G*) Average numbers of CFU-granulocytes (G), CFU-macrophages (M), CFU-granulocytes/macrophages (GM), and CFU-subset of B-lymphoid progenitors (PreB) were classified and recorded for each group from three independent experiments with duplicate plates as described in *F*. Identities of representative colonies were confirmed with Wright-Giemsa staining.

untreated mice (Fig. 1*B*, lane 3), indicating a tight control of *Men1* excision.

With this system, we first sought to determine whether *Men1* excision caused abnormalities in the peripheral blood of these mice. Both *Men1^{+/+}*;*Cre-ER* control mice and *Men1^{l/l}*;*Cre-ER* mice were treated with TAM, and then monitored for changes in the numbers of peripheral WBC and red blood cells (RBC) for >12 weeks. The total WBC counts in the control and experimental mice were similar before TAM treatment, ~7–8 × 10^3/μl (Fig. 1*C*). Excision of *Men1* gradually decreased the total WBC, from 7.1 × 10^3/μl to 4.5 × 10^3/μl at the sixth week after TAM treatment, a 40% reduction (Fig. 1*C*, ○, $P < 0.004$), remaining low after >12 weeks, whereas the total WBC remained unchanged in control *Men1^{+/+}*;*Cre-ER* mice after TAM treatment (Fig. 1*C*, ●). However, *Men1* excision did not significantly reduce RBC numbers (Fig. 1*D*) over 12 weeks. Collectively, these results indicate that menin is important for maintenance of steady-state peripheral WBC counts.

***Men1* Excision *in Vitro* and *in Vivo* Reduces BM Colony Formation.** We reasoned that the reduced peripheral WBC counts might be caused by dysfunction of BM progenitors, and thus sought to compare the function of hematopoietic progenitors from *Men1*-excised and *Men1*-intact mice by using colony formation assays, which are widely used to assess BM hematopoiesis (36, 37). BM
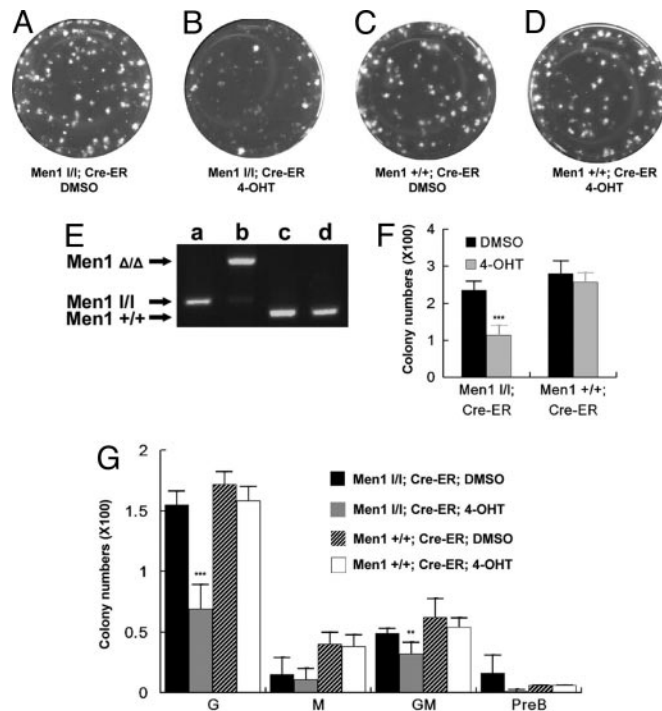
was collected from *Men1^{+/+}*;*Cre-ER* mice and *Men1^{l/l}*;*Cre-ER* mice 7 days after TAM treatment and plated in methylcellulose medium for colony formation. BM cells from TAM-treated *Men1^{+/+}*;*Cre-ER* mice formed 251 colonies, whereas *Men1* excision in *Men1^{l/l}*;*Cre-ER* mice markedly reduced colony formation to nine colonies, a reduction of 96% (Fig. 5 *A–C*, which is published as supporting information on the PNAS web site, $P < 10^{-4}$). Notably, expression of Hoxa9, Hoxa7, Hoxc8, and Hoxc6, but not the control gene *GAPDH*, was also reduced, as shown by RT-PCR (Fig. 5*D*). As Hoxa9 is important for normal hematopoiesis (25), these data not only indicate that menin is important for BM colony formation, but also suggest that reduction of menin-dependent expression of *Hox* genes, including *Hoxa9*, may contribute to the defects in colony formation by the *Men1*-excised BM.

We sought to test whether the defective BM colony formation is caused by loss of an autonomous role of *Men1* in BM cells. Thus, BM cells from both control *Men1^{+/+}*;*Cre-ER* mice and *Men1^{l/l}*;*Cre-ER* mice were plated in the methylcellulose-based medium with either DMSO or 4-OHT. After 12 days of culture, 4-OHT-mediated *Men1* excision reduced colony formation of the *Men1^{l/l}*;*Cre-ER* BM cells, from 235 colonies to 113 colonies, a 52% reduction (Fig. 2 *A*, *B*, and *F*, $P < 0.001$). It appeared that excision of menin preferentially reduced the number of big colonies. In contrast, the colony numbers from *Men1^{+/+}*;*Cre-ER* BM treated with either DMSO or 4-OHT were 280 and 256,

respectively, not significantly different (Fig. 2 *C*, *D*, and *F*, *P* >
0.2). The cells in those plates were harvested to determine the
status of *Men1* excision. Floxed *Men1* was almost completely
excised by 4-OHT treatment in *Men1*^{l/l}*;Cre-ER* cells (Fig. 2*E*,
lane b), but remained intact in the DMSO-treated cells
(Fig. 2*E*, lane a). As expected, 4-OHT did not excise *Men1* in
Men1^{+/+}*;Cre-ER* cells (Fig. 2*E*, lane d). Thus, we conclude that
menin plays an autonomous role in maintaining the function of
BM progenitors in colony formation. *Men1* excision *in vivo* led
to a 96% reduction in colony formation (Fig. 5*C*), but excision
of the locus *in vitro* caused only 52% reduction (Fig. 2*F*). This
difference is likely caused by the slow disappearance of the
menin protein within the initial several days after the *in vitro*
4-OHT treatment, because low levels of menin are still detect-
able by Western blotting on day 4 after the 4-OHT treatment
(data not shown). In addition, the total numbers of BM cells
from the femur and tibias did not appear to be markedly
different between the *Men1*-excised mice and *Men1*^{+/+} mice
(data not shown, Y.-X.C., unpublished work), although the total
progenitors from the BM were decreased when the floxed *Men1*
was excised, based on colony formation assays (Fig. 2 *F* and *G*).

BM colony formation units (CFU) in the above plates were
also quantified. The average numbers of CFU-granulocytes
decreased from 155 to 69 (*P* < 0.0006) after *Men1* excision. In
addition, CFU-granulocytes/macrophages decreased from 15 to
11 (Fig. 2*G*, *P* < 0.01). Numbers of CFU-subset of B-lymphoid
progenitors and CFU-macrophages were not markedly affected
by *Men1* excision (*P* > 0.075 and 0.65, respectively). However, we
cannot rule out that the relatively low numbers of the two types
of colonies masked a potential difference.

Ectopic Expression of Menin Rescues Defective Colony Formation of
***Men1*-Excised BM Cells and *Hoxa9* Expression.** To further establish
an essential and autonomous role for menin in maintaining
normal BM precursors, we sought to "rescue" the colony
formation defect in *Men1*-excised BM by ectopically expressing
menin. BM cells from the *Men1*^{l/l}*;Cre-ER* mice were transduced
with either control vector retrovirus or retrovirus expressing
menin. The transduced cells were assayed for colony formation
in the presence of either DMSO or 4-OHT. Transduction by the
control vector virus did not affect the reduction of colony
formation caused by 4-OHT-mediated *Men1* excision (Fig. 6 *A*,
B, and *E*, which is published as supporting information on the
PNAS web site, *P* < 0.001). However, the colony numbers from
ectopic menin-expressing cells were quite similar between
DMSO-treated and 4-OHT-treated *Men1*^{l/l}*;Cre-ER* cells, i.e., 249
and 255, respectively (Fig. 6 *C–E*, *P* > 0.3). These studies indicate
that exogenous menin genetically complements the functions of
excised endogenous *Men1* in colony formation of hematopoietic
progenitors, whereas menin overexpression alone is insufficient
to increase BM colony formation (Fig. 6*E*).

Real-time RT-PCR confirmed that 4-OHT treatment reduced
expression of the endogenous *Men1* but not ectopically trans-
duced menin (Fig. 6*F*). Hoxa9 expression is reduced by 4-OHT
treatment in vector-transduced BM cells by 85% (Fig. 6*G Left*),
consistent with reduced Hoxa9 expression in primary BM cells
with *Men1*-excised *in vivo* (Fig. 5*D*). Ectopic menin expression
increased Hoxa9 mRNA levels by ~70% and maintained this
level even after the endogenous *Men1* was excised (Fig. 6*G*
Right).

Overexpression of Hoxa9 and Meis1 Rescues the Deficient Colony
Formation of *Men1*-Excised BM. We explored the possibility that
Hoxa9 is a downstream factor that mediates menin's function in
hematopoiesis. Because Hoxa9 and its cofactor Meis1 together
strongly stimulate proliferation of myeloid cells (27), we tested
whether CFU activity of *Men1*-excised hematopoietic precursors
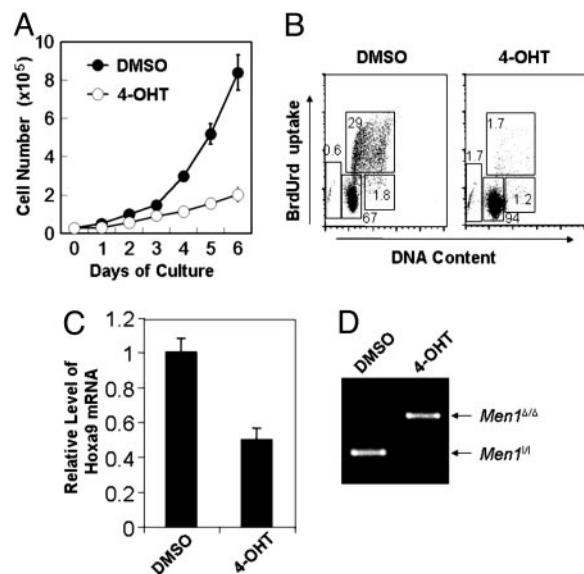can be maintained by overexpression of Hoxa9 and/or Meis1.

Fig. 3. *Men1* excision inhibits proliferation of MLL-AF9-transformed
myeloid cells and suppresses Hoxa9 expression. (*A*) BM from multiple
Men1^{l/l}*;Cre-ER* mice was transformed by transduction with retroviruses ex-
pressing MLL-AF9. The transformed BM cells from a representative mouse,
AT-1, were plated in triplicate at 2.5×10^4 cells per well, treated with either
DMSO or 4-OHT, and counted daily for 6 days. (*B*) The myeloid cells treated
with either DMSO or 4-OHT for 4 days were pulsed with BrdUrd for 90 min, and
then stained with an anti-BrdUrd antibody and 7-amino-actinomycin D, fol-
lowed by flow cytometric analysis. (*C*) The transformed *Men1*^{l/l}*;Cre-ER* cells
were treated with either DMSO or TAM for 4 days, and then harvested for RNA
isolation and real-time PCR to quantitate Hoxa9 mRNA. (*D*) Genomic DNA
from the cells treated with either DMSO or 4-OHT was used for PCR to
determine *Men1* excision. Two independent experiments were performed for
each of the above experiments.

BM cells from *Men1*^{l/l}*;Cre-ER* mice were infected with vector
retrovirus, virus expressing Hoxa9, virus expressing Meis1, or a
combination of viruses expressing either Hoxa9 or Meis1. The
transduced cells were selected and assayed for colony formation.
Men1 excision by 4-OHT treatment reduced colony formation in
vector-infected cells by 40% (Fig. 7 *A*, *B*, and *I*, which is published
as supporting information on the PNAS web site, *P* < 0.001).
Ectopic expression of Hoxa9 alone modestly increased colony
formation from both *Men1*-intact cells and *Men1*-excised cells, by
24% and 41%, respectively (Fig. 7 *C*, *D*, and *I*, *P* < 0.07 and *P* <
0.02), whereas ectopic expression of Meis1 alone did not increase
colony formation (Fig. 7 *E*, *F*, and *I*, *P* > 0.7 and 0.9, respec-
tively). Importantly, coexpression of Hoxa9 and Meis1 not only
markedly enhanced colony formation of the cells with or without
menin expression, to 407 and 382 colonies, respectively, but also
negated the dependence on menin in colony formation from
4-OHT-treated cells (Fig. 7 *G–I*, *P* > 0.30).

Real-time RT-PCR results confirmed that both Hoxa9 and
Meis1 were overexpressed (Fig. 7 *J* and *K*). In addition, floxed
Men1 excision in the 4-OHT-treated cells was confirmed by PCR
genotyping and real-time RT-PCR (data not shown). Together,
these results show that coexpression of both Hoxa9 and Meis1
prevents reduction of colony formation that results from loss of
menin expression. Coupled with the finding that menin is
important for Hoxa9 expression in BM (Fig. 6*G*), these results
suggest that Hoxa9 and Meis1 are among the major effectors of
menin in hematopoiesis. Because *Men1* excision reduced expres-
sion of multiple Hox genes, including *Hoxa9* (Fig. 5*D*) but not
Meis1 (Fig. 7*K*), and Meis1 is a cofactor for Hoxa9, it is
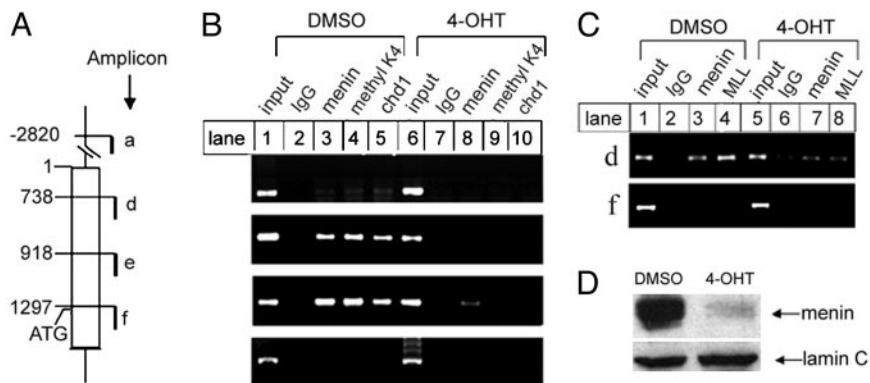conceivable that coexpression of both Hoxa9 and Meis1 at an

Fig. 4. Menin is important for methylation of H3K4 in the *Hoxa9* locus and for binding of MLL and cdh1 to the locus. (*A*) A diagram of the murine Hoxa9 locus. (*B* and *C*) Myeloid cells harboring *Men1^{l/l};Cre-ER* were treated with DMSO or 4-OHT, and then processed for ChIP assays by using the indicated antibodies. Results are representative of two independent experiments. (*D*) *Men1* excision induced by 4-OHT was confirmed by Western blotting using an antimenin antibody. Equal loading of the samples was revealed by blotting for Lamin C.

elevated level is necessary to compensate for the reduced colony formation in the absence of *Men1*.

Menin Is Required for Optimal Proliferation of MLL-AF9-Transformed Myeloid Cells and Hoxa9 Expression. As our data indicate that menin is important for normal BM colony formation, we sought to determine whether menin is also important for growth of MLL oncoprotein-transformed myeloid cells. BM from *Men1^{l/l};Cre-ER* mice was transformed by transduction with retroviruses expressing oncogenic MLL-AF9, an MLL fusion protein (23). Treatment of the cells with 4-OHT, but not DMSO, effectively excised floxed *Men1* (Fig. 3*D*). Equal numbers of DMSO-treated or 4-OHT-treated cells were plated and cells were counted. By day 6 of culture, DMSO-treated cells reached a much higher number (8.7×10^5 cells) than the 4-OHT-treated cells (2.1×10^5 cells), a 4-fold difference (Fig. 3*A*). To determine whether loss of menin expression affected cell proliferation, apoptosis, or differentiation, the DMSO- or 4-OHT-treated cells were pulsed with BrdUrd, and BrdUrd-positive cells were determined by flow cytometry analysis. *Men1*-excised cells showed a marked decrease in cell proliferation (1.7% BrdUrd-positive cells) as compared with DMSO-treated cells (29% BrdUrd-positive cells) (Fig. 3*B*). Up to 10 days after 4-OHT treatment, the treated cells did not show either significant differentiation, by staining for the myeloid differentiation markers Mac-1 and Gr-1, or apoptosis, by staining for Annexin V (data not shown). Together, these results indicate that *Men1* excision significantly inhibits proliferation but does not enhance apoptosis and differentiation of MLL-AF9-transformed cells. Moreover, *Men1* excision in the cells also reduced expression of Hoxa9 mRNA (Fig. 3*C*).

Menin Is Important for Methylation of H3K4 and Recruitment of Methylated H3K4-Binding Protein Chd1 to the Hoxa9 Locus. Regulation of Hoxa9 by menin is an attractive explanation for the effect of menin on normal hematopoiesis and myeloid transformation, because menin has been reported to bind MLL and been implicated in regulating Hoxa9 expression (19). Thus, we tested whether menin directly binds the *Hoxa9* locus and is essential for methylation of H3K4 by using chromatin immunoprecipitation (ChIP) assays. Seven independent primer pairs for seven distinct amplicons (a–g) spanning over 5 kb in the *Hoxa9* locus were synthesized to detect the potential location of menin in the locus *in vivo* (Fig. 8 *Left*, which is published as supporting information on the PNAS web site). ChIP assays with the transformed myeloid cells showed that menin primarily associated with the *Hoxa9* locus from −1,221 to +1,109 bp (Fig. 8 *Center*, lane 3, amplicons a–e), but not with the coding sequence surveyed by

the amplicons (Fig. 8 *Center*, lane 3, amplicons f and g). Almost superimposable on the pattern of menin association with the locus was the methylated H3K4, as detected by the antibody specific for the trimethylated lysine 4 of histone 3 (38, 39) (Fig. 8 *Center*, lane 4). Similarly, chd1, which specifically binds the methylated H3K4, remodels chromatin, and recruits histone acetylases (40), also overlaps with the binding patterns of menin and methylated H3K4 (Fig. 8 *Center*, lane 5). Because menin specifically binds serine 5-phosphorylated RNA polymerase II (18), we further tested whether RNA polymerase II associates with the *Hoxa9* locus and its pattern of association with the locus. The ChIP assay showed that phosphorylated RNA polymerase II associated with the *Hoxa9* locus in a pattern mimicking menin's distribution (Fig. 8 *Right*, lane 4). Because menin interacts with MLL and MLL2 that specifically methylates H3K4, our data suggest that menin and a H3K4 methyltransferase complex are recruited to the *Hoxa9* locus at a 2-kb region that includes the proximal promoter and 5′ UTR region, but not the coding region, leading to H3K4 methylation.

To determine whether menin is required for H3K4 methylation at the *Hoxa9* locus and binding of the methylated H3K4 binding protein chd1 to the locus, menin expression was markedly reduced in MLL-AF9-transformed *Men1^{l/l};Cre-ER* myeloid cells with 4-OHT treatment, as shown by Western blotting (Fig. 4*D*). ChIP assays show that menin, methylated H3K4, and chd1 bind the *Hoxa9* locus in menin-expressing myeloid cells, in a region covered by amplicons d and e (Fig. 4*B*, lanes 3–5); *Men1* excision led to the marked reduction of menin binding to the *Hoxa9* locus (Fig. 4*B*, lane 8). Additionally, association of methylated H3K4 and chd1 to the *Hoxa9* locus was markedly reduced after *Men1* excision (Fig. 4*B*, lanes 9 and 10). ChIP assays also showed that both menin and MLL bound to the *Hoxa9* locus (Fig. 4*C*, lanes 3 and 4), but *Men1* excision markedly reduced the binding of both menin and MLL to the locus (Fig. 4*C*, lanes 7 and 8). Together, these data indicate that menin is important for binding of MLL, its substrate H3K4, and chd1 to the *Hoxa9* locus and suggests a molecular link between these proteins in the *Hoxa9* locus.

Discussion

We identified an important and autonomous role for menin in BM hematopoiesis, at least in part, through regulating Hox genes, including *Hoxa9*. We also found that menin is important for the proliferation of MLL oncoprotein-transformed myeloid cells, pointing to a paradoxically oncogenic role for the tumor suppressor menin in proliferation of transformed myeloid cells. This finding is consistent with a recent report showing that

menin is required for maintenance of myeloid transformation by the oncogenic MLL-ENL (41). Moreover, our studies uncovered a molecular link between menin, methylation of H3K4, and the recruitment of chd1 to the *Hoxa9* locus, suggesting a role of this cascade in hematopoiesis and myeloid transformation.

Because modification or a combination of modifications of histones may serve as a "code" to regulate transcription from the genome (42), menin-dependent modification of H3K4 may serve as a platform for maintenance of *Hoxa9* transcription. Thus, MLL may require menin for binding to the *Hoxa9* locus and to methylate the local H3K4. The methylated H3K4 may further recruit chd1, a protein that specifically binds the methylated H3K4 via its second chromodomain and is also a component of large chromatin remodeling and acetylation complexes such as SAGA and SLIK (40). Our data indicate that chd1 associates with the *Hoxa9* locus, overlapping with the region bound by methylated H3K4 (Fig. 8), and chd1 binding to the locus also depends on the presence of menin (Fig. 4*B*). This finding suggests an important role for menin in lysine 4 methylation by MLL and/or other H3K4 methyltransferases and subsequent binding of the methylated H3K4 by chd1. A recent report shows that WDR5, a component of the MLL complex, binds the methylated H3K4 and activates Hoxa9 transcription (43). Our finding, together with that report, highlights the importance of the methylated H3K4 in regulating *Hox* gene expression and hematopoiesis. The relative contribution of either chd1 or WDR5 to hematopoiesis is unclear. Because menin does not bind to specific DNA sequence but is recruited specifically to the *Hoxa9* locus (Fig. 4), it is likely that menin is recruited either directly or indirectly by a DNA sequence-specific factor to the *Hoxa9* locus to promote *Hoxa9* transcription. Further work is required to decipher the detailed mechanisms governing the specific recruitment of menin, MLL complex, and the methylated H3K4 proteins to the *Hoxa9* locus and their role in hematopoiesis and myeloid transformation.

Although we observed an important role for menin in hematopoietic CFU activity and peripheral WBC, hematological abnormalities in either MEN1 patients or *Men1*^{+/−} mice have not yet been reported to our knowledge. It is possible that MEN1 patients or *Men1*^{+/−} mice have mild blood abnormalities or alternatively are more vulnerable to BM stress. Mutations of *Men1*, a bona fide tumor suppressor, result in development of tumors in multiple endocrine organs (4). Yet *Men1* promotes proliferation of MLL-AF9-transformed cells (Fig. 3). Thus, the role of menin in tumorigenesis is context-dependent; it serves as a tumor suppressor in endocrine organs but a tumor promoter in myeloid cells. It remains to be delineated what determines the tissue-specific activities of menin and whether the menin/MLL/Hox gene pathway plays a role in suppressing tumorigenesis in endocrine organs.

Men1 excision potently inhibits proliferation of MLL-AF9-transformed cells and Hoxa9 expression (Fig. 3). Hoxa9 is often up-regulated in MLL cells or by retroviral insertion-induced leukemias (44), and it is also required for MLL-ENL-mediated myeloid transformation (45). Menin may promote proliferation of MLL-AF9-transformed cells by up-regulating *Hoxa9* and other genes through recruiting MLL, because loss of menin markedly reduces binding of MLL to the *Hoxa9* locus (Fig. 4*C*). This result is also consistent with a recent report showing that MLL-AF6 is also recruited to the Hoxa9 locus (41). Hence, menin may cooperate with MLL to control expression of a group of genes, including *Hoxa9*. Thus menin may act as a convergent point in regulating a group of *Hox* genes to control normal hematopoiesis and myeloid transformation, which raises the interesting possibility that blocking menin's function in MLL cells may lead to a novel therapy for this leukemia.

Materials and Methods

Mice, Genotyping, and Treatment with TAM. All laboratory mice were maintained on a 12-h light-dark cycle in the animal facility at the University of Pennsylvania in compliance with related guidelines. *Men1*[l/l]*;Cre-ER* mice, in a mixed genetic background, were generated by crossing floxed *Men1* mice (Sv129) and *Cre-ER* transgenic mice (C57BL/6). Floxed *Men1* mice were kindly provided by Francis Collins (National Institute for Human Genome Research, Bethesda) (30), in which exons 3–8 of *Men1* were flanked by two loxP sites. Mice expressing a transgene, Cre-ERT2 driven by the pan-active UBC9 promoter, were generated by lentitransgenesis, as described (34, 35) (E.J.B., unpublished data). The mice were genotyped by PCR using the tail genomic DNA; the primer sequences will be provided on request. Genotyping and excision of the floxed *Men1* are described in *Supporting Text*, which is published as supporting information on the PNAS web site.

Analysis of Peripheral Blood Cells and Statistic Analysis. Blood samples collected from the retroorbital sinus of mice were stored in Microtainer tubes with EDTA (BD Biosciences, Franklin Lakes, NJ). The hematology profile of each mouse was analyzed immediately on a Hemavet blood cell counter (CDC Technologies, Oxford, CT). Student's t tests were used to analyze the differences in blood cell counts and BM colony formation.

Generation of Recombinant Retroviruses, Infection of Primary BM Cells, and Transformation of Myeloid Cells. Plasmids for generating retroviruses, pMX-Vector-puro, pMX-menin, pMSCVpgk-Hoxa9-GFP, pMSCV-MLL-AF9, and pMSCVpacMeis1A were generated as described (11, 20, 27). The viruses were packaged in 293T cells as described (9). *Men1*[l/l]*;Cre-ER* and *Men1*^{+/+}*;Cre-ER* mice were injected with 5-fluorouracil (American Pharmaceutical Partners, Schaumburg, IL) i.p. at a dose of 50 mg/kg body weight. Four days later, BM cells were collected from femurs and tibias of the mice and prestimulated with 10 ng/ml IL-3, 10 ng/ml IL-6, and 100 ng/ml stem cell factor (PeproTech, Rocky Hill, NJ) overnight, as described (46). *In vitro* infection was performed on 2 consecutive days by incubating BM cells with retroviral supernatant with 4 μg/ml polybrene and spinoculating at 1,300 × *g* for 90 min at 25°C. The cells were seeded at 1×10^5 cells per 35-mm dish in methylcellulose-based medium supplemented with IL-3, IL-6, and stem cell factor (MethoCult GF M3534; StemCell Technologies, Vancouver). The cells infected with MSCV-MLL-AF9 viruses were replated weekly in the same medium with 1 mg/ml G418. After the third plating, the transformed myeloid cells were transferred to liquid medium (Iscove's modified Dulbecco's medium supplemented with 15% fetal calf medium, 1% Pen/Strep, 1% L-glutamine, and 10 ng/ml IL-3) as described (46).

Colony Formation Assay and Analysis of Cell Growth. BM cells used for colony formation assays were harvested from BM as described above or retrovirally infected myeloid cells expressing ectopic genes. Cells (2.5×10^4) were seeded in a 35-mm Petri dish in 1.1 ml of MethoCult GF M3534 methylcellulose medium (StemCell Technologies). 4-OH (Sigma) dissolved in DMSO was added to the medium at 1:500 dilution to reach 200 nM to excise floxed *Men1*, and an equal volume of DMSO was added to control dishes. After 12-day incubation at 37°C, the dishes were photographed, and the total colony numbers and types of colonies in plates were evaluated under a microscope, and colonies with >50 cells were scored. Criteria for classifying various CFU were as described and confirmed by Wright-Giemsa staining (47). For evaluating cell growth, MLL-AF9-transformed *Men1*[l/l]*;Cre-ER cells* were maintained in Iscove's modified Dulbecco's medium with 15% FCS and 10 ng/ml IL-3 and treated with either 200 nM 4-OHT or DMSO for 4 days. PCR genotyping was performed to confirm *Men1* excision. *Men1*-intact and *Men1*-excised cells were seeded in 6-well plates in the

same medium at a cell density of 2.5×10^4 cells per well in triplicate and counted in a hemacytometer daily.

PCR, RT-PCR, Taqman Real-Time RT-PCR, Western Blotting, and Flow Cytometry Analysis. Total RNA from tissues or cells was prepared by using an RNeasy extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. For classical RT-PCR, 1 mg of RNA from each sample was amplified by using the Titan One-Tube RT-PCR system (Roche Applied Science). For real-time RT-PCR, 5 μg of total RNA from each sample was used to synthesize cDNA by using SuperScript II RNase H reverse transcriptase (Invitrogen) with oligo(dT) primers. Real-time PCR was performed in triplicate tubes on the AB 7500 real-time PCR system (Applied Biosystems) by using the indicated FAM primer pairs (Applied Biosystems). The average Δ-Δ cycle threshold of different samples was analyzed to calculate the relative quantities of mRNA levels, using either actin or GAPDH mRNAs as control. Western

blot and flow cytometry analysis were performed as described (14). All of the PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA).

ChIP. ChIP assays were performed as described with certain modifications (48). The detailed procedures are described in *Supporting Text*.

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