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The Menin Tumor Suppressor Protein Is an Essential Oncogenic Cofactor for MLL-Associated Leukemogenesis

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Summary

The Mixed-Lineage Leukemia (MLL) protein is a histone methyltransferase that is mutated in clinically and biologically distinctive subsets of acute leukemia. MLL normally associates with a cohort of highly conserved cofactors to form a macromolecular complex that includes menin, a product of the *MEN1* tumor suppressor gene, which is mutated in heritable and sporadic endocrine tumors. We demonstrate here that oncogenic MLL fusion proteins retain an ability to stably associate with menin through a high-affinity, amino-terminal, conserved binding motif and that this interaction is required for the initiation of MLL-mediated leukemogenesis. Furthermore, menin is essential for maintenance of MLL-associated but not other oncogene induced myeloid transformation. Acute genetic ablation of menin reverses aberrant *Hox* gene expression mediated by MLL-menin promoter-associated complexes, and specifically abrogates the differentiation arrest and oncogenic properties of MLL-transformed leukemic blasts. These results demonstrate that a human oncoprotein is critically dependent on direct physical interaction with a tumor suppressor protein for its oncogenic activity, validate a potential target for molecular therapy, and suggest central roles for menin in altered epigenetic functions underlying the pathogenesis of hematopoietic cancers.

Introduction

MLL is a protooncogene that is targeted by chromosomal translocations in a diverse subset of hematologic disorders including acute lymphoid, myeloid, and biphenotypic leukemia and myelodysplastic syndrome, all of which are generally associated with a poor prognosis (DiMartino and Cleary, 1999 for review). As a consequence of acquired genetic aberrations in these disorders, the MLL protein is fused with one of more than 40 different partners to yield a diverse collection of chimeric fusion proteins (Mitterbauer-Hohendanner and Mannhalter, 2004 for review). Despite their remarkable diversity, many fusion partners share an ability to constitutively activate novel transcriptional effector properties of MLL, which correlates with its oncogenic potential in experimental models of acute leukemia (Daser and Rabbitts, 2004; Hess, 2004 for review). The key transcriptional pathways that are subordinate to both wild-type and oncogenic MLL proteins include *Hox* genes, which are master regulators of cell fate, proliferation, and morphogenesis (Owens and Hawley, 2002 for review). During embryogenesis, MLL is required for maintenance of *Hox* gene expression to establish proper body segment identity (Yu et al., 1995; 1998). In the hematopoietic compartment, lack of MLL is associated with reduced expansion of progenitors and decreased *Hox* gene expression (Ernst et al., 2004a, 2004b; Hess et al., 1997; Yagi et al., 1998). Conversely, hematopoietic cells transformed by MLL oncoproteins consistently hyperexpress several *Hoxa* cluster genes, some of which have been shown to be direct targets of MLL and key contributors to the pathologic features of MLL-associated leukemia (Ayton and Cleary, 2003; Kumar et al., 2004; Nakamura et al., 2002; So et al., 2004; Wang et al., 2005; Zeisig et al., 2004). Thus, MLL is an important regulator of *Hox* gene expression, particularly during hematopoiesis, and its malfunction is associated with disordered hematopoiesis and acute leukemogenesis.

Wild-type MLL is a large, structurally complex protein whose transcriptional role is mediated in part through an ability to covalently modify chromatin, thus distinguishing it as one of only a few currently known chromatin-modifying enzymes that are directly mutated in human cancers (Schneider et al., 2002 for review). MLL possesses inherent histone methyltransferase (HMT) activity, which is specific for lysine 4 of histone H3 (H3-K4) (Milne et al., 2002; Nakamura et al., 2002), a mark that is generally associated with transcriptionally active states of chromatin (Noma et al., 2001; Strahl et al., 1999), thus providing a molecular basis for the characteristic role of MLL in maintenance, not initiation, of embryonic *Hox* gene expression (Yu et al., 1998). In this capacity, MLL associates through its carboxy-terminal methylase domain with a cohort of proteins that are highly similar to components of the SET1 histone methyltransferase complexes of yeast and human, suggesting that all three macromolecular complexes employ an evolutionarily conserved biochemical mechanism to mediate their H3-K4-specific HMT activities (Yokoyama et al., 2004). However, oncogenic mutations that create MLL fusion proteins in human leukemia result in disruption of the MLL macromolecular complex with consequent loss of the HMT domain and its associated conserved factors.

Menin is a highly specific partner for MLL proteins, as it is the only known member of the MLL macromolecular complex that is not shared with the SET1 complexes. Menin-containing MLL family HMT complexes were identified independently by immunopurification of menin and of MLL (Hughes et al., 2004; Yokoyama et

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al., 2004). Strikingly, menin remains complexed with oncogenic MLL fusion proteins in leukemia cells while other complex members do not associate with these fusion proteins (Yokoyama et al., 2004).

Menin was initially identified as a product of the *MEN1* tumor suppressor gene (Chandrasekharappa et al., 1997), and its loss of function due to a variety of missense or truncating mutations is observed in heritable as well as sporadic neoplasms of multiple endocrine organs (Chandrasekharappa and Teh, 2003 for review). *MEN1* is an essential gene whose absence results in embryonic lethality at midgestation in the mouse, whereas heterozygous mice develop a variety of endocrine tumors associated with somatic loss of the remaining wild-type allele, consistent with its role as a classic tumor suppressor gene for the endocrine lineage (Bertolino et al., 2003a, 2003b; Crabtree et al., 2001).

Although menin lacks identifiable functional motifs, it reportedly interacts with a variety of transcriptional proteins (Chandrasekharappa and Teh, 2003 for review) in addition to MLL and MLL2. Menin is a postivie regulator of *Hox* and CDK inhibitor (*Cdki*) gene expression and is associated with chromatin at both *Hox* and *Cdki* loci (Hughes et al., 2004; Milne et al., 2005; Yokoyama et al., 2004). A subset of missense mutations in *MEN1* patients abrogates the ability of menin to coprecipitate with HMT activity, suggesting a possible role for this activity in tumor suppression (Hughes et al., 2004). Conversely, the role, if any, of this lineage-specific tumor suppressor protein in hematopoietic malignancies involving gain-of-function mutations of the MLL HMT machinery has not been determined.

Here, we demonstrate that menin associates with MLL fusion proteins on *HOX* promoters in human leukemia cells, and is necessary for maintenance of *HOX* gene expression by MLL oncoproteins. Furthermore, menin is required for MLL-mediated transformation, and targeted acute inactivation of menin specifically reverses the differentiation arrest and oncogenic potential of MLL-transformed blasts. These studies support a unique and novel function for a tumor suppressor protein as a direct, essential cofactor for a distinctive group of oncoproteins in their roles as transcriptional misregulators of critical *HOX* target genes in acute leukemogenesis.

Results

MLL Oncoproteins Associate with Menin on *HOX* Gene Promoters in Human Leukemia Cells

To establish whether MLL oncoproteins associate with menin on target genes that are critical for MLL-mediated transformation, chromatin immunoprecipitation (ChIP) assays were performed using human ML-2 leukemia cells. These cells lack a normal *MLL* gene and thus exclusively express an MLL-AF6 fusion protein as a consequence of t(6;11) chromosomal translocation (Tanabe et al., 1996), in contrast to control cells (REH) that carry no *MLL* chromosomal translocation and only express the processed MLL^N and MLL^C subunits (Figure 1A). Immunoprecipitation of MLL-AF6 from ML-2 cells resulted in the coprecipitation of menin demonstrating that the two proteins associate with each other in the absence of wild-type MLL (Figure 1A, lane 4). ChIP analysis using anti-MLL^N and anti-menin antibodies demonstrated that MLL-AF6 and menin were both present at upstream sites of the *HOXA7*, *HOXA9*, and *HOXA10* promoters, but neither protein was associated with the *β-ACTIN* or *GAPDH* promoters (Figure 1B). *HOXA9* in particular is highly expressed in MLL-associated leukemias (Armstrong et al., 2002; Rozovskaia et al., 2001; Yeoh et al., 2002) and has major influence on their incidence and/or phenotype in mouse models (Ayton and Cleary, 2003; Kumar et al., 2004; So et al., 2004; Wang et al., 2005). Previous studies showed that the expression of *HOXA9* in HeLa cells is dependent on the presence of both wild-type MLL and menin (Yokoyama et al., 2004). The current results demonstrate that *HOXA7*, *HOXA9*, and *HOXA10* are direct target genes for MLL oncoproteins in association with menin, which colocalize on these promoters in human leukemia cells.

MLL Fusion Proteins Interact with Menin through Multiple Domains

To establish the structural requirements for MLL-menin interaction, a series of deletion mutations (Figure 2A) was introduced into the MLL portion of MLL-ENL (ME) and evaluated in IP-Western blot analysis for their effects on the ability of ME to interact with endogenous menin. Immunoprecipitation of an initial series of FLAG-tagged mutant ME proteins from nuclear extracts of transiently transfected cells revealed that a domain conferring strong interaction with endogenous menin resided within the first 331 amino acids of MLL (Figure 2B). A second, much weaker interaction domain was contained in a region spanning residues 640–1251 of MLL. More refined mutational analysis showed that the high-affinity interaction domain for menin localized within the first 35 amino acids of MLL (Figure 2C).

Identification of a Conserved, High-Affinity Menin Binding Motif (hMBM) in MLL

To further define the high-affinity menin binding motif, a series of GAL4 fusion proteins containing different 10-amino-acid segments of MLL were tested for their ability to associate with menin. Immunoprecipitation of the FLAG-tagged GAL4 fusion proteins from transiently transfected cells revealed that three peptides (spanning amino acids 1–10, 4–13, and 6–15 of MLL, respectively) coprecipitated endogenous menin (Figure 2D). Sequence alignment (Figure 2E) revealed that the region of overlap (MLL amino acids 6–10) among all three peptides was conserved in pufferfish (fugu) MLL (Caldas et al., 1998) as well as MLL2 (amino acids 17–21, Huntsman et al., 1999), a homolog of MLL that biochemically associates with menin (Hughes et al., 2004). To confirm that this consensus sequence (RXRFP) is a high-affinity menin binding motif, it was removed by interstitial deletion of amino acids 6–10 within the ME and MLL-GAS7 (MG) oncoproteins (deletion mutants MEd and MGd, respectively). Immunoprecipitation analysis revealed that neither mutant oncoprotein was capable of high-affinity interaction with menin, in contrast to the respective intact fusion proteins (Figure 2F). Thus, the consensus RXRFP sequence near the amino terminus of MLL constitutes a high-affinity menin binding motif (hMBM).

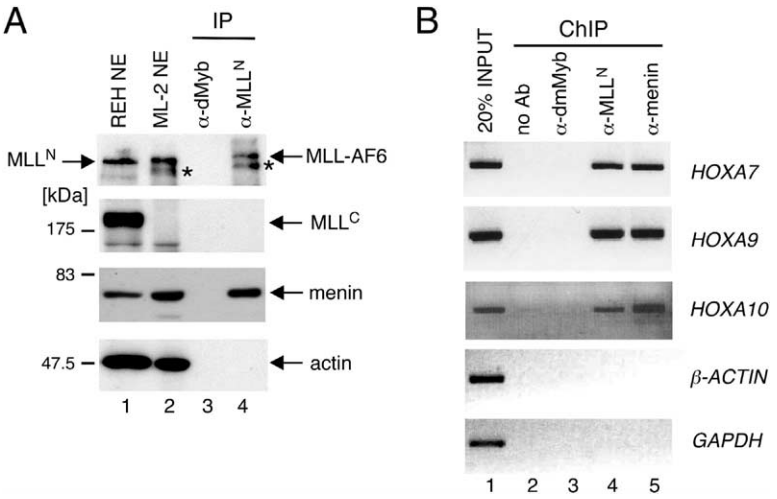

Figure 1. MLL Oncoproteins Associate with Menin on *HOX* Gene Promoters In Vivo

(A) Proteins present in nuclear extracts (lanes 1 and 2) or immunoprecipitates (lanes 3 and 4) were separated by SDS-PAGE and immunoblotted with antibodies specific for MLL^N (top panel), MLL^C (upper middle panel), menin (lower middle panel), or actin (bottom panel). REH cells (lane 1) contain wild-type MLL^N and MLL^C subunits, whereas ML-2 cells (lane 2) contain only the MLL-AF6 fusion protein due to presence of a translocated *MLL* gene and deletion of the wild-type *MLL* gene. Immunoprecipitation with an anti-MLL^N antibody (rpN1) demonstrates co-IP of menin with MLL-AF6 (lane 4) compared to the control IP with anti-dmMyb antibody (lane 3). Molecular sizes of protein markers are shown on the left. Asterisk indicates a degradation product of MLL-AF6.

(B) Chromatin immunoprecipitation assay was performed to assess the coassociation of menin and MLL oncoproteins on various *HOX* gene promoters of human ML-2 leukemia cells. Chromatin was immunoprecipitated with antibodies specific for dmMyb (lane3), MLL^N (lane 4) and menin (lane 5) as well as a no antibody control (lane 2). The presence of *HOX* promoter DNA in the chromatin precipitates was assessed by PCR along with those of *β-ACTIN* and *GAPDH.*

MLL Oncoproteins Are Dependent on Interaction with Menin for Transformation of Myeloid Progenitors

To determine if association with menin is required for MLL-associated leukemogenesis, an MLL fusion protein lacking the hMBM (MEd) was tested for its oncogenic potential in vitro and in vivo. Primary myeloid stem/progenitor cells were harvested from mouse bone marrow, transduced with MLL fusion proteins, and then assessed for their clonogenic properties in methylcellulose serial replating assays and ability to induce leukemia in syngeneic recipient mice (Figure 3A). Cells transduced with the intact ME oncogene displayed enhanced clonogenic activity as evidenced by their ability to generate colonies through three rounds of plating in methylcellulose medium, a hallmark feature of MLL-transformed cells (Lavau et al., 1997). In contrast, progenitors transduced with MEd, which is incapable of binding to menin with high affinity, exhausted their clonogenic activity by the second round of plating (Figure 3B), similar to normal progenitors or those transduced with vector alone. Comparable expression of the transduced genes was confirmed by Western blot analysis of cells from first-round cultures (Figure 3C). Transduced cells established long-term engraftment of syngeneic recipient mice following transplantation (Figure 3D), but MEd-expressing cells did not induce acute myeloid leukemia (Figure 3E), in contrast to ME-transduced cells, which induced leukemia similar to previous studies (Ayton and Cleary, 2003; Lavau et al., 1997). Thus, high-affinity interaction with menin is required for MLL oncoproteins to induce oncogenic transformation of myeloid progenitors.

Menin Is Specifically Required for Maintenance of Transformation Mediated by MLL Oncoproteins

A general role for menin in myeloid transformation and the need for its continuous presence in the maintenance of MLL-associated leukemogenesis were investigated. Myeloid progenitors were isolated from the bone marrow of wild-type (wt) mice or mice homozygous for a floxed *Men1* gene (f/f) and then transduced with various MLL oncogenes including ME, MG, and MA (MLL-AF10) that transform hematopoietic cells by diverse molecular mechanisms (So et al., 2003; Nie et al., 2003; Okada et al., 2005). The unrelated *E2A-HLF* (EH) oncogene was employed for comparison (Figure 4A). All of the genes induced continuous clonogenic activity of myeloid progenitors in serial replating assay and oncoprotein expression was confirmed by Western blot analysis (Figure 4B). After establishment of stable transformation following more than three replatings in methylcellulose medium, the cells were transduced with a retrovirus (MSCV-puro) expressing a conditional Cre-ER^{tam} fusion protein (CER) whose recombinase activity is activated by 4-hydroxy-tamoxifen (4-OHT) to acutely inactivate the *Men1* gene. Secondarily transduced cells were selected in the presence of puromycin and then relative clonogenic activity compared to vector control was assessed in the subsequent round of plating in the presence of 4-OHT to activate the Cre recombinase (Figure 4A). The genotype and menin expression with or without Cre recombinase induction 48 hr after 4-OHT addition were assessed by PCR and Western blotting, respectively (Figures 4C and 4D). Acute loss of menin in ME-transformed menin-floxed progenitors abrogated their clonogenic activity, whereas ME-transformed wild-type cells were unaffected (Figure 4E). The few colonies present in cultures of ME-transformed/CER-transduced *Men1* floxed cells were small with diffuse morphology suggestive of a more differentiated phenotype in contrast to the large blast-type control colonies (Figure 4F). Other MLL oncogene-transformed cells (MA and MG) lost their clonogenic activity upon deletion of menin, as well (Figure 4E). Conversely, EH-transformed cells were unaffected by acute loss of menin and continued to produce colonies in methylcellulose medium (Figure 4E) with morphologies that were indistinguishable with or without Cre activation (data not shown). Therefore, menin is necessary for maintenance of transformation induced by MLL

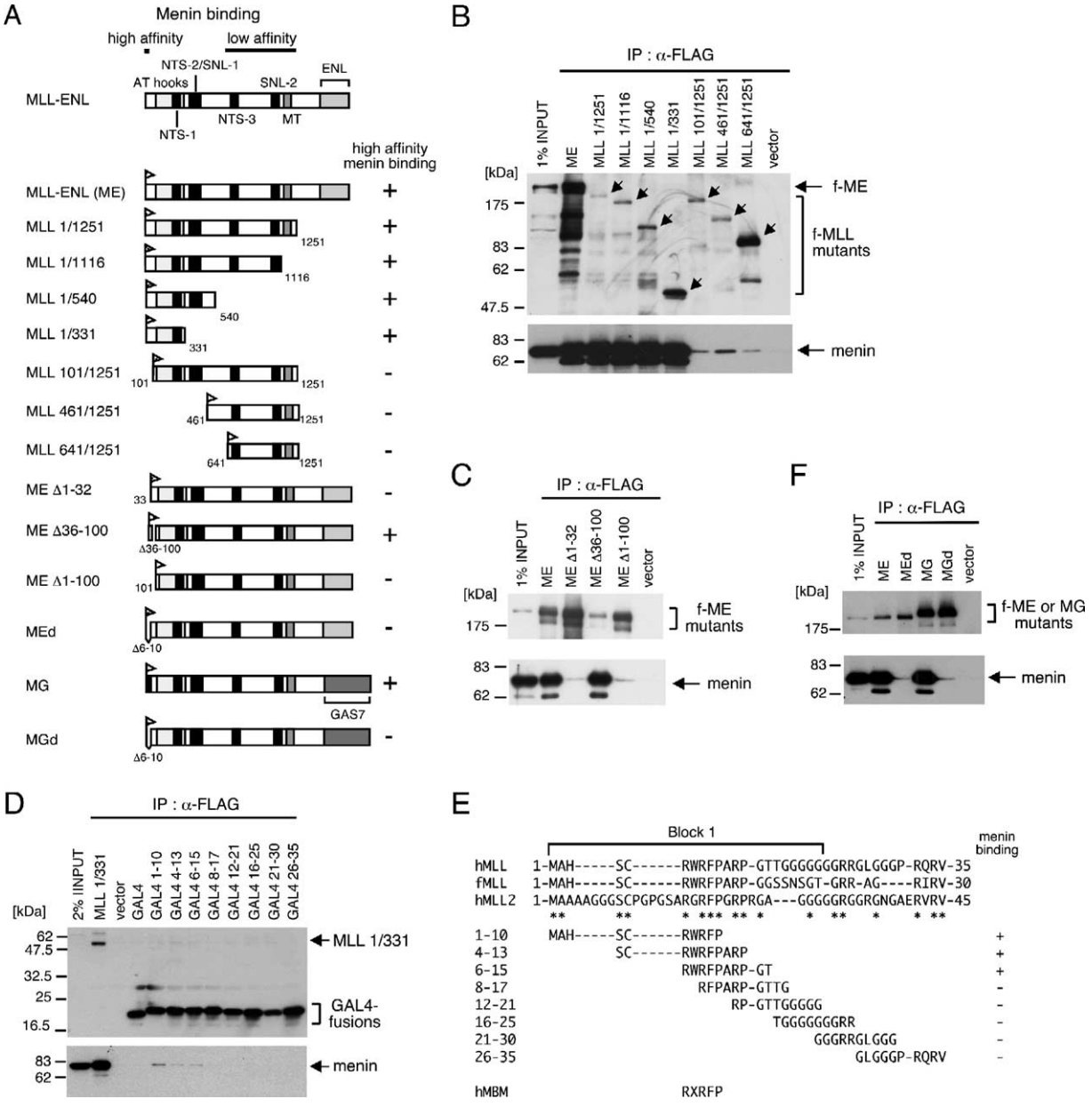

Figure 2. A Conserved, High-Affinity Menin Binding Motif Resides at the Amino Terminus of MLL

(A) Various ME or MG deletion mutants (shown schematically) containing a FLAG epitope tag at their N termini were expressed in 293T cells by transient transfection. Nuclear extracts prepared from transfectants were subjected to immunoprecipitation with anti-FLAG antibody affinity beads. High-affinity menin binding capacities are indicated on the right.

(B and C) Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with anti-FLAG (upper panel) or anti-menin (bottom panel) antibodies. Lane 1 in each panel contains 1% input of ME transfectant. Molecular sizes of protein markers are shown on the left. Arrows in panel (B) denote mutant MLL proteins.

(D) GAL4 fusion proteins containing different 10 amino acid segments of the first 35 residues of human MLL (and a FLAG epitope tag) were analyzed for their binding capacity with menin (MLL 1/331 served as a positive control, lane 2). The immunoprecipitates were separated in SDS-PAGE gel and immunoblotted with anti-FLAG (upper panel) or anti-menin (bottom panel) antibodies. Lane 1 contains 2% input of MLL 1/133. Molecular sizes of protein markers are shown on the left.

(E) The amino acid sequences employed in panel (D) are aligned with fugu MLL and human MLL2. Binding capacity with menin is shown as + or – on the right. A consensus high-affinity menin binding motif (hMBM) is indicated at the bottom.

(F) Internal deletion mutants (shown schematically in panel [A]) of ME and MG lacking the hMBM (amino acids 6–10) were analyzed for their high-affinity binding capacity (+ or –) with menin using methods as described above. Immunoprecipitates were separated in SDS-PAGE gel and immunoblotted with anti-FLAG (upper panel) or anti-menin (bottom panel) antibodies. Lane 1 contains 1% input of ME transfectant. Molecular sizes of protein markers are shown on the left.

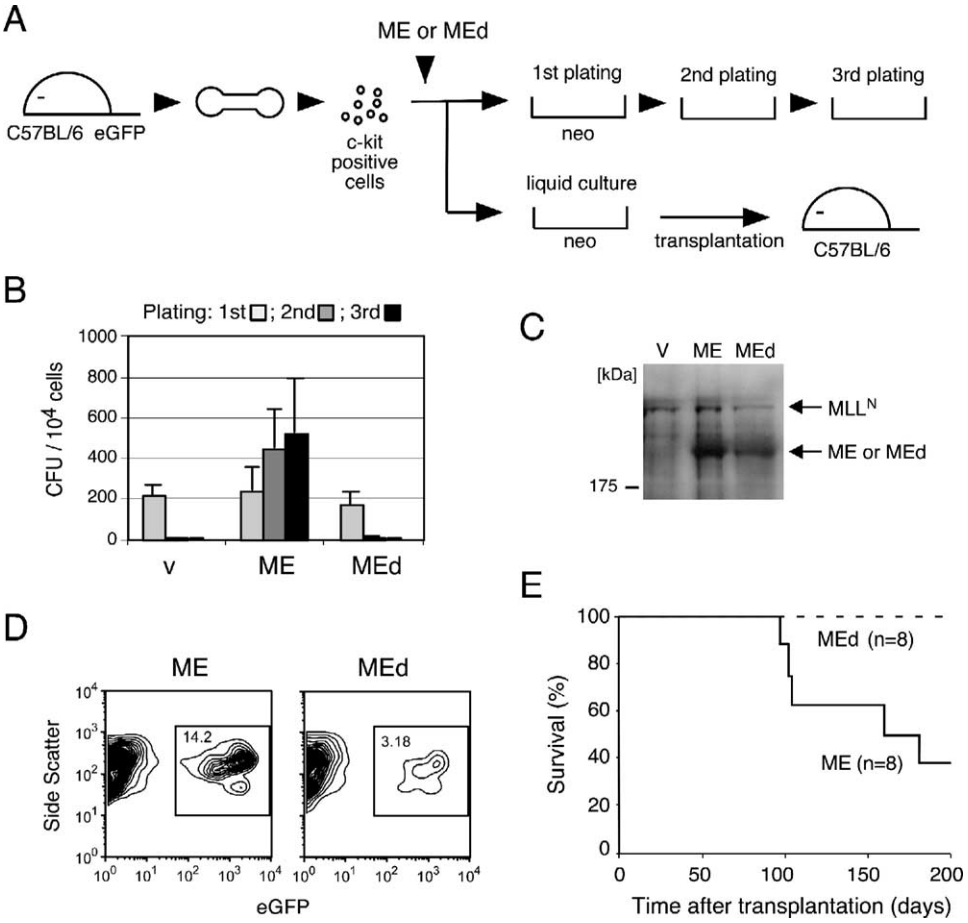

Figure 3. Interaction with Menin Is Required for Transformation by MLL-ENL

(A) Schematic depiction of experimental scheme. BM progenitor/stem cells (c-kit$^+$) from C57BL/6 mice transgenic for eGFP were transduced with ME or MEd and used for serial myeloid replating assays, or cultured in liquid media containing G418 for 5 days, and then transplanted into lethally irradiated recipient mice.

(B) The results of myeloid progenitor transformation assays through three rounds of serial methylcellulose culture are shown as CFU (colony forming unit) per 10^4 plated cells. Error bars indicate standard deviations of three independent experiments.

(C) Western blot analysis shows expression of ME and MEd in lysates of transduced cells from first round colonies. Endogenous wild-type MLL^N served as a loading control.

(D) FACS analyses of cells from bone marrow aspirates performed 61 days posttransplantation demonstrate the presence of eGFP-expressing TER119-negative cells in mice transplanted with ME- or MEd-transduced hematopoietic progenitors, confirming their successful engraftment. The average percentage of eGFP positive cells in the entire ME cohort (17.4 ± 5.6%) was higher than the MEd cohort (3.6 ± 1.7%) indicative of an expanding population of preleukemic cells in the ME-transduced recipients.

(E) Survival curves for mice transplanted with ME (solid line)- or MEd (dashed line)-transduced cells are shown. Presence of acute myeloid leukemia was confirmed by peripheral blood leukocyte counts, FACS analyses, and/or necropsy.

oncoproteins, in contrast to the EH oncoprotein, which appeared to be entirely independent of menin. Taken together, our results indicate a specific, as opposed to general, menin requirement for both the initiation and maintenance of transformation by MLL oncogenes.

Loss of Menin Relieves the Differentiation Block of MLL-Transformed Leukemic Blasts

The consequences of acute menin loss in MLL-transformed cells were further investigated in myeloid progenitors that were transformed by either the *ME* or *MA* oncogenes and coexpressed CER. Without inactivation of the *Men1* gene, the cultures were comprised predominantly of $Mac1^{int}$ cells with the morphologic features of blasts (Figures 5A–5C). Within 48 hr following acute *Men1* deletion, the proportion of blasts was markedly reduced (e.g., 48% to 8% for ME/CER), and the cultures contained mostly $Mac1^{hi}$ cells with mature or maturing cytologic features (Figures 5A–5C). DNA content analysis showed that within 48 hr of *Men1* deletion, there was an increase of the G0/G1 population and a reduction in S-G2/M phase cells (Figure 5D) and cells with metaphase chromatin (data not shown), consistent with the inability of *Men1* null MLL-transformed cells to proliferate and generate CFU in culture (Figure 4E). MA-transformed cells also showed accumulation at G0/G1 phase while the vector control or wild-type cells did not (data not shown). Taken together, these analyses

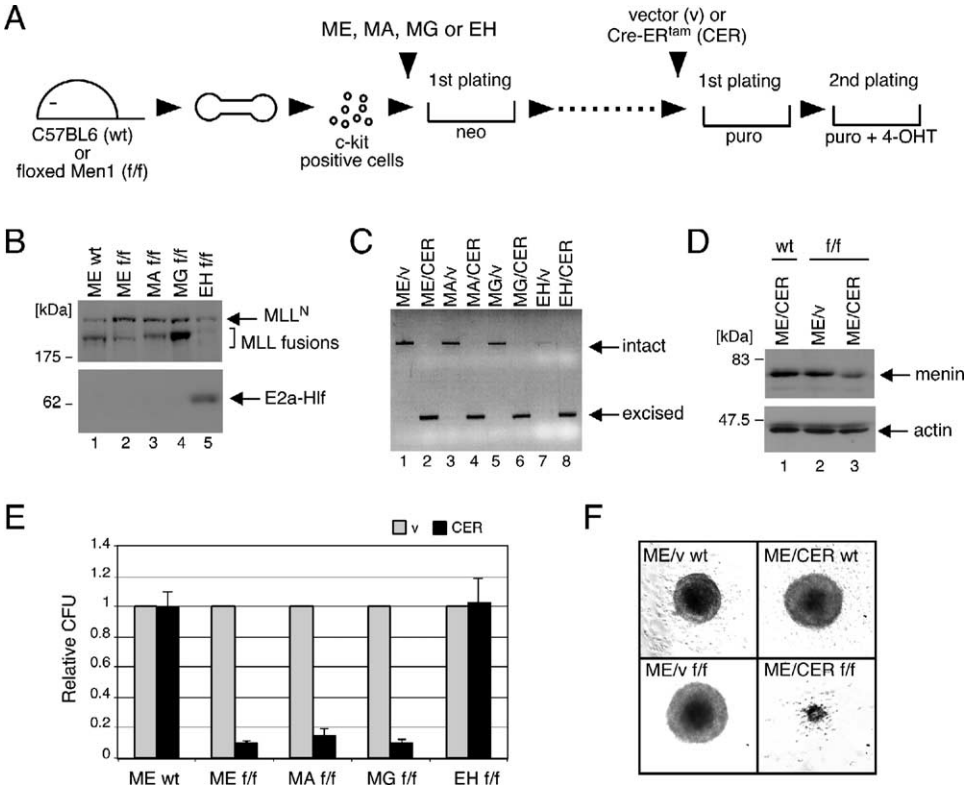

Figure 4. Menin Is Required to Maintain the MLL-Transformed Phenotype of Myeloid Progenitors

(A) BM progenitor/stem cells harvested from wild-type mice (wt) or mice homozygous for a floxed *Men1* allele (f/f) were transduced with various MLL oncogenes (ME, MA, and MG) or the EH oncogene. Immortalized cells from the third to seventh round of in vitro plating were then transduced a second time with either vector alone (v) or a Cre-ER^{tam}-expressing (CER) virus, and selected in the presence of puromycin for one round. The resistant cells were then assessed for their clonogenic potentials in the subsequent round of plating in methylcellulose in the presence of 1 nM 4-OHT.

(B) Oncoprotein expression was confirmed by Western blotting using anti-MLL^N (upper panel) or anti-E2a (lower panel) antibodies.

(C) The *Men1* genotypes of cells present in methylcellulose cultures following secondary retroviral transductions were determined by PCR.

(D) Menin levels were assessed by Western blot analysis using an anti-menin antibody, which demonstrated the rapid reduction of menin in *Men1* floxed (f/f) cells 48 hr after activation of CER when compared with wt cells or vector-transduced cells.

(E) Relative numbers of colonies generated by CER-transduced cells compared to vector control-transduced cells cultured in the presence of 4-OHT for 5 days were examined for each construct (indicated below the panel). Error bars denote standard deviations of three independent experiments. MLL oncogene-transformed, menin-deleted cells show severely reduced clonogenic activity in contrast to EH-transformed cells.

(F) Representative morphologies are shown for colonies obtained for ME-transformed/CER (or empty vector)-transduced wt or *Men1* floxed (f/f) cells following the second round of plating in the presence of 4-OHT.

indicate that myeloid blasts transformed by MLL oncoproteins are critically dependent on menin to effectively maintain their undifferentiated state, as well as their enhanced cycling and clonogenic properties. These features are similar to those associated with acute inactivation of ME in transformed myeloid progenitors (Ayton and Cleary, 2003; Zeisig et al., 2004), providing further support that MLL oncoproteins work together with menin in myeloid leukemogenesis.

Menin Is Required for Maintenance of *Hox* Gene Expression by MLL Fusion Proteins

Given the specific dependence on menin for MLL-mediated myeloid transformation and the coassociation of menin with MLL oncoproteins on *HOX* gene promoters in human leukemia cells, we investigated the contributions of menin to *Hox* gene expression in MLL-transformed murine cells. *HOXA* cluster genes, particularly *HOXA7* and *HOXA9*, are consistently and highly expressed in MLL-associated human and mouse leukemia, and their expression is dependent on functional MLL fusion proteins (Armstrong et al., 2002; Ayton and Cleary, 2003; Kumar et al., 2004; Rozovskaia et al., 2001; Yeoh et al., 2002; Zeisig et al., 2004). Therefore, *Hoxa* gene expression was evaluated by quantitative real-time PCR in murine myeloid progenitors one week following transduction with the ME oncogene or a mutant version lacking the hMBM (MEd). *Hoxa* genes were expressed at 3- to 10-fold higher levels in ME- compared to MEd-transduced cells (Figure 6A), suggesting that maintenance of high-level *Hox* gene expression by ME required its association with menin. As shown previously (Figure 3B), MEd-transduced cells were unable to maintain clonogenic activity when plated in methylcellulose medium, consistent with the association of sustained *Hox* gene expression with MLL-mediated transformation (Ayton and Cleary, 2003). Expression of the same *Hoxa* genes was also analyzed in ME-transformed *Men1* floxed cells 48 hr after *Men1* was acutely deleted by activation of Cre recombinase. The high

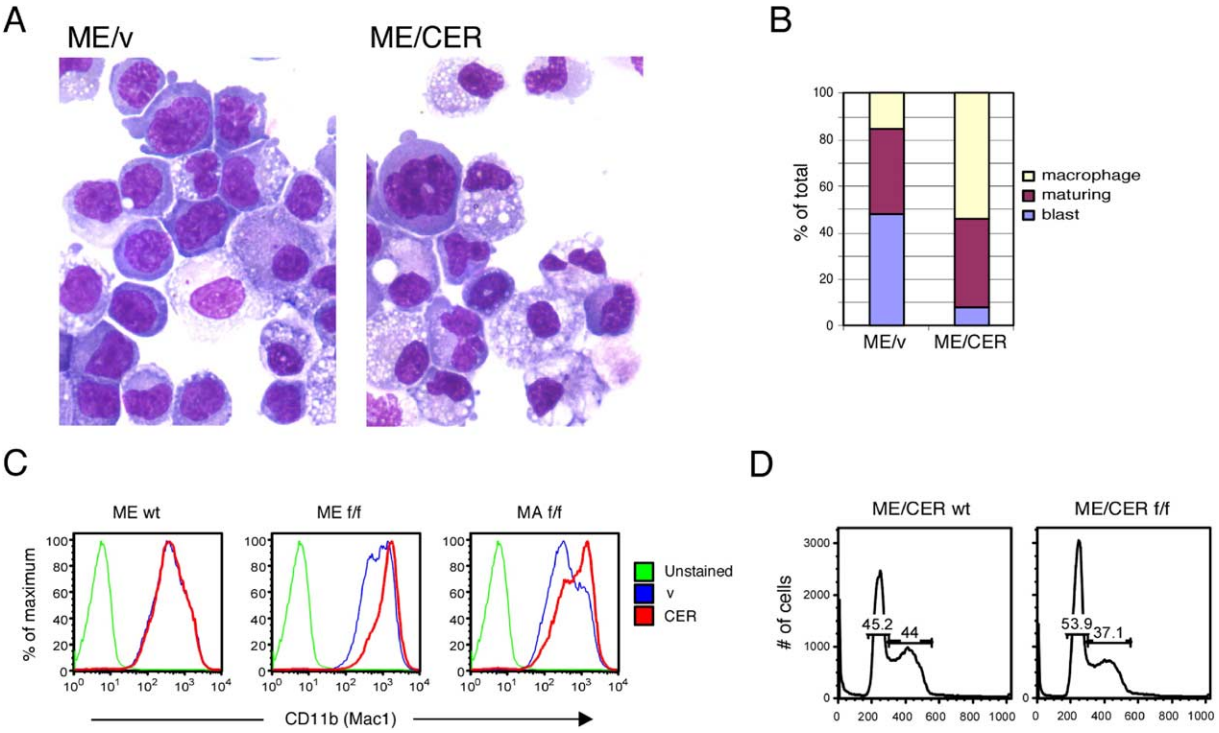

Figure 5. Acute Loss of Menin Results in Differentiation and Reduced Cycling of MLL-Transformed Myeloid Leukemia Blasts

(A) ME-transformed cells homozygous for a floxed *Men1* gene (f/f) were secondarily transduced with a retrovirus expressing CER or empty vector (v) and cultured in the presence of 4-OHT. Cytopreparations of cells harvested after 48 hr in 4-OHT were stained with May Grunwald-Giemsa.

(B) The percentages of cells with morphologic features of blasts, maturing cells, or macrophages are indicated as determined by microscopic evaluation. The differentiation of wt cells was unaffected by CER (data not shown).

(C) FACS analysis of CD11b (Mac-1) expression was performed on ME or MA-transformed/CER-transduced *Men1* floxed (f/f) cells 48 hr after addition of 4-OHT. ME-transformed wt cells served as a negative control. Each panel contains data for unstained (green), empty vector-transduced (blue), or CER-transduced (red) cells.

(D) DNA content of ME-transformed/CER-transduced cells with wild-type (wt) or *Men1* floxed (f/f) genotype was analyzed 48 hr after addition of 4-OHT. Percentages of G0/G1 population (2n) and S-G2/M population (2n < 4n) are indicated (10^5 cells analyzed). ME-transformed cells accumulate at G0/G1 after loss of menin. Representative data are shown for three independent experiments.

levels of expression for *Hoxa7* and *Hoxa9* in ME-transformed cells rapidly decreased to approximately 40% of their original levels within 48 hr following acute loss of menin (Figure 6C), demonstrating its requirement for maintenance of *Hox* gene expression. ChIP performed on ME-transformed cells confirmed that *Hoxa9* is a direct target gene of menin in this context (Figure 6C). On the other hand, expression of the CDK inhibitor $p27^{Kip1}$, reported as a direct target gene of MLL and menin in fibroblasts (Milne et al., 2005), was not affected by loss of menin in myeloid progenitors (Figures 6A and 6B), indicating that it is not a target gene of MLL oncoproteins in myeloid progenitors. Therefore, menin is required for *Hoxa* gene expression mediated by oncogenic MLL fusion proteins in myeloid progenitors, providing a molecular basis for its essential role in MLL-mediated transformation.

Discussion

The Menin Tumor Suppressor Protein Is an Essential Leukemogenic and Transcriptional Cofactor for MLL Oncoproteins

Menin is a product of the *MEN1* tumor suppressor gene, whose inactivation is associated with a variety of heritable and sporadic endocrine tumors (Chandrasekharappa and Teh, 2003 for review). It functions as a classic tumor suppressor protein for the endocrine lineage as confirmed by genetic studies in humans and mice (Bertolino et al., 2003b; Chandrasekharappa et al., 1997; Crabtree et al., 2001). We have previously shown that menin is a component of a macromolecular complex containing MLL, which is a leukemic protooncogenic protein, and that this complex participates in maintenance of *Hox* gene expression (Yokoyama et al., 2004). The unexpected biochemical association of a tumor suppressor protein with a protooncogenic protein raised important questions regarding their respective roles in endocrine and hematologic cancers. The issue specifically addressed in the current study was whether MLL oncoproteins require, or alternatively bypass, menin molecular function in leukemia pathogenesis. Our results demonstrate that MLL oncoproteins are critically dependent on menin, indicating that in addition to its tumor suppressor role, menin also provides an essential function on an oncogenic pathway. Loss of the ability to associate with menin abrogates the oncogenic properties of MLL in vitro and in vivo, and MLL-transformed cells require the continued presence of menin, indicating that it is necessary for both the initia-

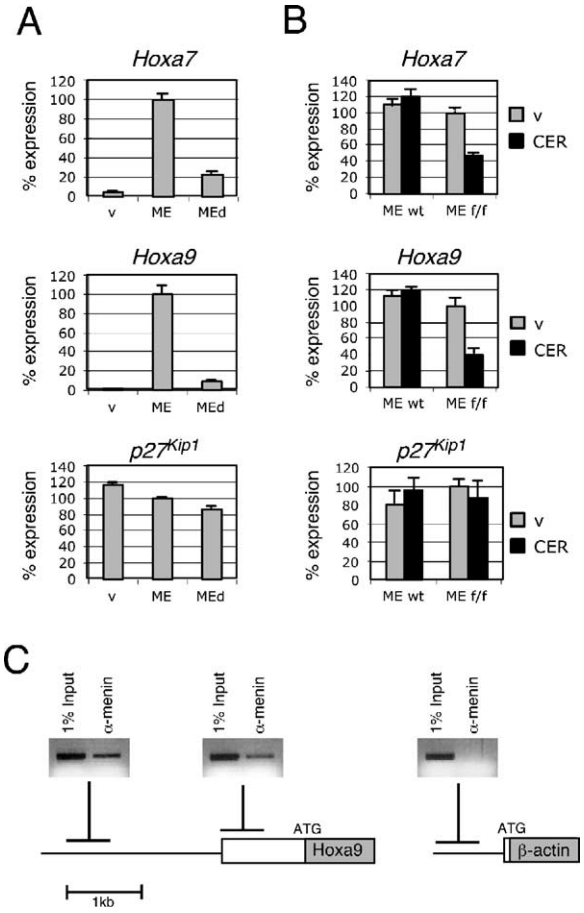

Figure 6. Menin Is Required for Maintenance of *Hox* Gene Expression in MLL-Transformed Cells

(A) Expression of the *Hoxa7, Hoxa9* and *p27^{KIP1}* genes (indicated at the tops of the respective panels) normalized to *β-actin* was determined by quantitative real-time PCR analysis of cells that had been transduced with vector (v), ME, or MEd and cultured in methylcellulose medium for 1 week. Percent expression relative to ME-transduced cells is depicted; error bars represent the standard deviations of triplicate analyses. *Hox* gene expression was not maintained in cells transduced by MLL fusion proteins unable to bind menin.

(B) ME-transformed wt or *Men1* floxed (f/f) cells transduced with empty vector (v) or CER were analyzed 48 hr after addition of 4-OHT for expression levels of *Hoxa7*, *Hoxa9*, and *p27^{KIP1}* transcripts (indicated at the tops of the respective panels) relative to *β-actin* by quantitative real time PCR. Percent expression is depicted relative to ME-transformed/vector-transduced *Men1* floxed (f/f) cells with error bars representing the standard deviation of triplicate analyses. *Hox* gene expression was not maintained in cells lacking menin.

(C) ChIP was performed using ME-transformed cells. DNA fragments upstream of the first ATG of the *Hoxa9* and *β-actin* genes were amplified from anti-menin (BL342) immunoprecipitates (or 1% of input DNA as a control). Menin localized at the *Hoxa9* but not the *β-actin* promoter.

tion and maintenance of MLL-mediated oncogenic transformation. This does not reflect a generalized requirement for menin in myeloid progenitor transformation since the unrelated E2A-HLF oncoprotein is not dependent on menin for its oncogenic effects under identical experimental conditions. Therefore, menin is a specific and essential oncogenic cofactor for MLL-mediated leukemogenesis.

The demonstration that menin associates with MLL oncoproteins on the *HOXA9* promoter in leukemia cells suggested that they work together for transcriptional regulation of biologically relevant target genes, thereby providing a molecular basis for a menin requirement in MLL-mediated oncogenesis. Their colocalization on *Hox* promoters suggests that the menin requirement for transformation is unlikely to simply reflect its dominant-negative sequestration by MLL oncoproteins, although we cannot unequivocally rule out more complex scenarios that may in part involve menin sequestration. In support of a cofactor role for menin, expression of several *Hoxa* cluster genes is dramatically reduced in cells transduced with MLL oncoproteins lacking a menin binding motif and in MLL-transformed cells acutely depleted of menin. *Hoxa9* in particular influences the incidence and/or phenotype of MLL leukemias, demonstrating that constitutive expression of *Hox* genes by menin/MLL fusion protein complexes is an important aspect of MLL leukemogenesis. Furthermore, a simple dominant-negative mechanism should not be affected by menin deletion, which would otherwise be redundant with MLL-mediated sequestration. Thus, menin appears to be a transcriptional and oncogenic cofactor that is required for transformation of myeloid progenitors as well as transcriptional misregulation of *Hox* genes by MLL oncoproteins.

Dual Roles for Menin as a Tumor Suppressor and an Oncogenic Cofactor

Although there are clear examples of oncoproteins (e.g., Mdm2) whose transforming activities are mediated by functional inactivation of subordinate tumor suppressor proteins (Vargas et al., 2003 for review), the dependence of an oncoprotein on direct physical interaction with a tumor suppressor protein as a necessary cofactor for its oncogenic activity has no precedent to our knowledge. Current data support a provisional model for menin as a component of the MLL macromolecular HMT complex, which localizes to *Hox* and other critical target genes to participate in their normal dynamic regulation in response to upstream growth and differentiation signals. MLL oncoproteins harboring gain-of-function mutations retain a critical dependence on menin but are devoid of other known MLL-associated factors and may no longer respond to upstream signals, thus maintaining constitutively high levels of subordinate gene expression likely through transcriptional effector functions of their fusion partners (Figure 7). Misregulation of genes such as *HoxA9*, which has oncogenic properties when hyperexpressed in hematopoietic progenitors (Kroon et al., 1998; Schnabel et al., 2000), would complete an oncogenic circuit.

In endocrine cancers, on the other hand, menin loss of function results from truncating or missense mutations, several of which have been shown to abrogate the ability of menin to coprecipitate with HMT activity (Hughes et al., 2004). Loss of menin function, as opposed to MLL gain of function in leukemias, has been shown to compromise subordinate *Hox* gene expression, such as *Hoxc8* and *HOXA9* in menin-deficient MEFs and HeLa cells, respectively (Hughes et al., 2004;

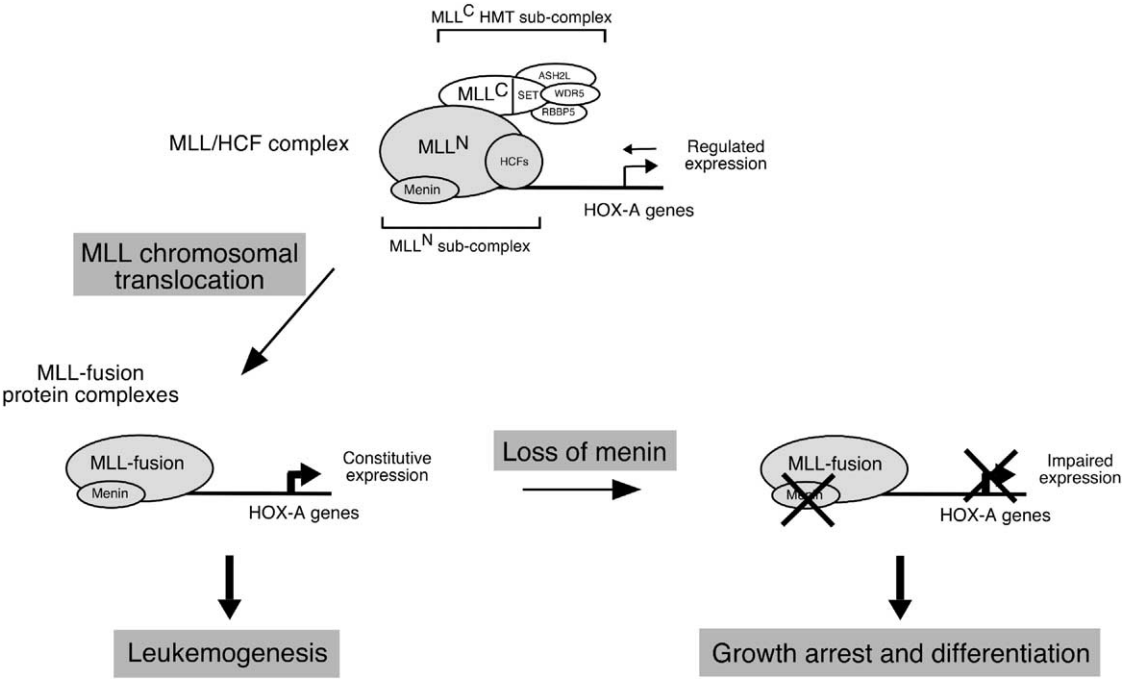

Figure 7. Menin-Dependent Mechanisms for Target Gene Regulation by Wild-Type and Oncogenic MLL Fusion Proteins Provide a Pathogenic Model for MLL-Associated Leukemogenesis

Yokoyama et al., 2004). Although *HOX* gene misregulation may contribute to endocrine neoplasia, menin-MLL HMT complexes also positively regulate genes encoding anti-proliferation factors, such as $p27^{Kip1}$ and $p18^{Ink4c}$. Their expression is compromised in the absence of menin in immortalized fibroblasts (Milne et al., 2005), although not in myeloid progenitors transformed by MLL oncoproteins (current study). Menin is also implicated in negative gene regulation through association with the Sin3/HDAC corepressor complex. Menin-mediated tethering of this chromatin-modifying machinery to JunD alters its transcriptional properties, converting JunD from a growth promoter to a growth suppressor (Agarwal et al., 1999, 2003; Kim et al., 2003). Thus, the consequences of menin loss are complex and apparently cell context, target gene, and coregulator dependent. Taken together, these observations suggest the broader hypothesis that contrasting functions of menin as a tumor suppressor versus oncogenic cofactor may be different manifestations of its critical roles in tethering or targeting a subset of chromatin modifying complexes to specific promoters.

Acute Loss of Menin Specifically Targets Leukemia Blasts Transformed by MLL Oncoproteins and Provides a Rationale for Molecular Therapy

Our studies show that menin is specifically required for MLL-transformed blasts to maintain their oncogenic properties raising the possibility that molecular targeting of MLL-menin interaction may be an effective therapeutic approach. Unlike conventional protein-protein interactions, the MLL-menin interaction appears to involve multiple MLL surfaces with at least two widely separate menin interaction domains. A small highly conserved N-terminal MLL motif (hMBM) mediates high-affinity binding with menin, whereas a second, less well-defined domain that resides within residues 640–1251 mediates low-affinity interaction. Although the physiological significance of low-affinity binding with menin is unclear, it is not sufficient for maintenance of high-level target gene expression or transformation by MLL oncoproteins. Conversely, deletion of a mere five amino acids of the hMBM abrogates the ability of MLL oncoproteins to maintain *HOX* gene expression and induce acute myeloid leukemia. Importantly, menin is required for long-term maintenance of MLL-mediated oncogenesis. Its acute inactivation in MLL-transformed cells results in the rapid loss of leukemia blasts, which lose their clonogenic potential, and undergo differentiation, a phenotype similar to that associated with conditional inactivation of MLL-ENL itself (Ayton and Cleary, 2003; Zeisig et al., 2004). These results credential the hMBM as a therapeutic target and suggest that leukemias that are dependent on MLL function for misregulation of multiple subordinate *HOX* genes should be amenable to selective targeting by small molecule antagonists of MLL-menin interaction. Efforts to isolate such compounds seem well warranted and may provide a treatment modality for a subset of leukemia that currently responds poorly to available therapies.

Experimental Procedures

Cell Culture

Phoenix and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). REH and ML-2 cells were cultured in RPMI 1640 medium supplemented with 10% FCS.

Immunoprecipitation and Western Blotting

Preparation of nuclear extracts, immunoprecipitation analysis, and Western blotting were performed as previously described (Yokoyama et al., 2002, 2004). Primary antibodies included: mouse monoclonals specific for MLL^N (mmN4.4), MLL^C (mmC2.1) and E2A (YAE); rabbit anti-MLL^N (rpN1) (Smith et al., 1999; Yokoyama et al., 2002); rabbit anti-*Drosophila* Myb (generous gift from J. Lipsick); mouse monoclonal anti-actin (C4) (Chemicon International); goat anti-menin (C19) (Santa Cruz Biotechnology, Inc.); and rabbit anti-menin (BL342) (Bethyl Laboratories, Inc.). Rabbit anti-FLAG (F-7425) antibody and agarose affinity beads coupled to mouse anti-Flag (M2) monoclonal antibody were purchased from Sigma.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation was performed as previously described (Weinmann and Farnham, 2002). After one round of immunoprecipitation with anti-MLL^N (rpN1), anti-*Drosophila* Myb or anti-menin (BL342) antibodies, the precipitates were reverse-cross-linked and treated with proteinase K. The DNA was purified by phenol/chloroform extraction, precipitated, and dissolved in TE. PCR was performed for 35 cycles using primer pairs specific for upstream genomic regions of each gene. Primer sequences and PCR conditions are available upon request.

Vector Construction

The expression vectors for various MLL-ENL mutants were generated by restriction enzyme digestion or PCR-based mutagenesis using pMSCV-FLAG-MLL-ENL^C (Ayton and Cleary, 2003) as template. The expression vectors for GAL4 fusion proteins were constructed by PCR using pM (Clontech) as template and cloned into pMSCV-puro vector (Clonetech). pMSCV-ires-puro was created by assembling the IRES element from pMYs-IG (generous gift from T. Kitamura) into pMSCV-puro vector. The Cre-ER^{tam} cDNA fragment from pBSIIKS+ K14CreERtam vector (generous gift from E. Fuchs) was then inserted into pMSCV-ires-puro to create pMSCV-Cre-ER^{tam}-ires-puro. pMSCV-neo constructs encoding MLL-GAS7, MLL-AF10, and E2A-HLF were described elsewhere (Ayton and Cleary, 2003; DiMartino et al., 2002; So et al., 2003).

Myeloid Progenitor Transformation Assays

Mouse bone marrow cells were harvested from leg bones, and cells expressing surface c-kit were purified by auto-MACS using a PE-conjugated anti-c-kit antibody (2B8, BD) and anti-PE magnetic beads (Miltenyi Biotech). Purified cells were used for myeloid progenitor transformation assays as previously described (Lavau et al., 1997) with minor modifications. For secondary transductions, 10^5 cells were infected with retrovirus by spinoculation and cultured overnight in 2 ml of methylcellulose medium (M3231, Stemcell Technologies) containing SCF, IL-3, IL-6, and GM-CSF. Puromycin (1 μg/ml) was added to the culture on the next day and the cells were plated in 6-well dishes. After 4–6 days, puromycin-resistant cells were replated in the presence of 1 nM of 4-OHT (Sigma) for induction of Cre-ER^{tam} for further analysis. Transplantation of transduced progenitors into lethally irradiated, syngeneic recipients was performed as described previously (Lavau et al., 1997), with the exception that donor C57BL/6 mice were transgenic for eGFP (Wright et al., 2001) and progenitors were incubated in RPMI 1640 medium supplemented with 20% FCS, 20% WEHI supernatant, 20 ng/ml SCF, 10 ng/ml IL-6, and 1mg/ml G418 for 5 days following spinoculation prior to transplantation.

Genotyping

Mice containing a floxed *Men1* gene (Hughes et al., 2004) were maintained as homozygotes through intercross matings. Cre-mediated excision of the *Men1* gene was assessed by PCR using a primer pair (5′-CTTGGCTGGACGTAAACTCCTCTTCAGACC-3′/5′-GATGCTAAAGGGTCCTCCCTGTCT-3′).

Flow Cytometry and DNA Content Analysis

For FACS analysis, cells were obtained by bone marrow aspirate from transplanted mice as described (Verlinden et al., 1998), or resuspension in PBS from methylcellulose culture, and then washed and suspended in 3% FCS in PBS. Antibodies against Mac-1 [Cy7APC conjugated (M1/70, BD)] or TER119 (PE conjugated, BD) were added to the cell suspension at a dilution of 1:200 and 1:100, respectively. After 30 min of incubation on ice, cells were washed, suspended in 3% FCS in PBS containing 1μg/ml of propidium iodide (PI, Sigma), and analyzed by flow cytometry.

For DNA content analysis, ice-cold 70% ethanol (5 ml) was added with vortexing to cells suspended in PBS (100 μl) followed by incubation on ice for at least 30 min. Cells were washed with PBS, suspended in 200 μl, mixed with 10 μl of 10 mg/ml RNase A, and then incubated at 37°C for 20 min. Following addition of 500 μl of PI solution (0.1% trisodium citrate, 50 μg/ml PI) and incubation for 10 min at 4°C, the cells were analyzed by flow cytometry.

Real-Time Quantitative PCR Analysis of Gene Expression

Total RNA was purified using an RNeasy mini kit (Qiagen), and 1 μg was reverse-transcribed using a oligo-dT primer and Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. The reaction products were diluted (10×) with TE, and 2 μl was subjected to real-time PCR, which was performed in triplicate using Taqman probes and the ABI Prism 7700 sequence detection system. Taqman probes for *Hoxa7* (Mm00657963_m1), *Hoxa9* (Mm00439364_m1), *$p27^{Kip1}$* (Mm00438167_g1), and *β-Actin* (Mm00607939_m1) were purchased from Applied Biosystems. Expression levels of *Hoxa7*, *Hoxa9,* and *$p27^{Kip1}$* relative to that of *β-Actin* were calculated using a standard curve and the relative quantitation method as described in ABI User Bulletin #2.

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