Meis1 is an essential and rate-limiting regulator of *MLL* leukemia stem cell potential

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Oncogenic mutations of the MLL histone methyltransferase confer an unusual ability to transform non-self-renewing myeloid progenitors into leukemia stem cells (LSCs) by mechanisms that remain poorly defined. Misregulation of *Hox* genes is likely to be critical for LSC induction and maintenance but alone it does not recapitulate the phenotype and biology of *MLL* leukemias, which are clinically heterogeneous—presumably reflecting differences in LSC biology and/or frequency. TALE (three-amino-acid loop extension) class homeodomain proteins of the Pbx and Meis families are also misexpressed in this context, and we thus employed knockout, knockdown, and dominant-negative genetic techniques to investigate the requirements and contributions of these factors in MLL oncoprotein-induced acute myeloid leukemia. Our studies show that induction and maintenance of *MLL* transformation requires *Meis1* and is codependent on the redundant contributions of *Pbx2* and *Pbx3*. *Meis1* in particular serves a major role in establishing LSC potential, and determines LSC frequency by quantitatively regulating the extent of self-renewal, differentiation arrest, and cycling, as well as the rate of in vivo LSC generation from myeloid progenitors. Thus, TALE proteins are critical downstream effectors within an essential homeoprotein network that serves a rate-limiting regulatory role in *MLL* leukemogenesis.

[*Keywords*: Leukemia stem cells; *MLL*; *Meis1*; *Pbx*; TALE homeodomain proteins]

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Leukemia stem cells (LSCs) comprise a functionally distinct subpopulation of leukemic cells with the ability to self-renew extensively, and to initiate, sustain, or regenerate disease. In acute myeloid leukemia (AML), LSCs are generally considered to be rare upstream cells that arise out of the normal hematopoietic stem cell (HSC) or primitive progenitor compartments, and are organized in a hierarchy based on quantitative differences in their self-renewal potentials (Passegue and Weisman 2005). This paradigm, however, may not apply to all myeloid leukemias, as suggested by recent studies using a mouse model of AML induced by the *MLL-AF9* oncogene (Krivtsov et al. 2006; Somervaille and Cleary 2006), which is typically associated with FAB-M4 or M5 subtypes of human AML (Swansbury 1998). LSCs were found to be very frequent and representative of downstream myeloid lineage cells that ectopically acquired extensive self-renewal and other biologic properties more typical of HSCs. The key regulators and subordinate genetic programs by which MLL converts myeloid progenitors into LSCs are of major interest.

MLL is a histone methyltransferase with features suggestive of a general transcriptional role at most promoters (Guenther et al. 2005); however, *Hox* genes appear to be particularly dependent on its function (Yu et al. 1995). In leukemias harboring MLL-activating mutations, several *Hoxa* genes are consistently expressed at high levels, suggesting that MLL oncoproteins inappropriately maintain their expression and prevent their programmed down-regulation that otherwise accompanies terminal myeloid differentiation (Imamura et al. 2002b; Pineault et al. 2002). Since various *Hox* genes have been implicated in the regulation of normal stem cell self-renewal, their misregulation in *MLL* leukemias is likely to be important for LSC maintenance. *Hoxa9*, in particular, has been shown to critically influence *MLL* oncogenesis (Ayton and Cleary 2003; Kumar et al. 2004; So et al. 2004; Okada et al. 2005; J. Wang et al. 2005). However, *Hox* gene misregulation alone does not recapitulate the biological and clinical features of *MLL* leukemia.

Additional candidate factors that may critically regulate LSC potentials in *MLL* leukemias are TALE (three-amino-acid loop extension) class homeodomain proteins

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of the Pbx and Meis families. They enhance the relatively nonspecific DNA-binding properties of Hox transcription factors and regulate gene expression as heterooligomeric complexes with Hox proteins (Mann 1995). TALE proteins are required for the execution of some Hox-dependent developmental programs and are implicated in leukemogenesis (Azpiazu and Morata 1998; Ryoo et al. 1999; Selleri et al. 2001; Manley et al. 2004; Eklund 2007; Rice and Licht 2007). Notably, mutations of Hoxa9 that prevent interactions with Pbx proteins abrogate its oncogenic properties (Schnabel et al. 2000). Furthermore, coexpression of *Meis1* markedly shortens the latency and increases the penetrance of *Hoxa9*-induced myeloid leukemia, and recapitulates some of the features of *MLL* leukemia (Kroon et al. 1998; Calvo et al. 2001; Zeisig et al. 2004; G.G. Wang et al. 2005). Since *Meis1* is also a transcriptional target of MLL oncoproteins and is consistently highly expressed in *MLL* leukemias (Lawrence et al. 1999; Imamura et al. 2002b; Milne et al. 2005), simultaneous misregulation of *Hoxa9* and *Meis1* may be sufficient for *MLL* oncogenesis (Zeisig et al. 2004). However, *MLL* leukemias are biologically and clinically diverse, and the specific roles of TALE proteins in regulating LSC properties that underlie these differences have not been defined.

In this report, we demonstrate that TALE homeodomain proteins are essential for the induction and maintenance of *MLL* leukemogenesis. *Meis1*, in particular, quantitatively regulates the differentiation arrest, cycling activity, in vivo progression, and self-renewal of *MLL* leukemia cells, thereby functioning as a critical and rate-limiting determinant of LSC potential.

Results

The latencies of MLL *leukemias correlate with* Meis1 *endogenous expression levels*

Twelve different MLL fusion oncoproteins were investigated for their *TALE* protein-dependent oncogenic properties. These included MLL oncoproteins containing different classes of fusion partners including cytoplasmic proteins (GAS7, AF1P, AF6, and EB1), nuclear proteins of the forkhead family (FKHRL1 and AFX) or AF4 family/complex (LAF4, AF5, ENL, AF9, and AF10), and histone modifiers (CBP), which induce leukemia with widely varying latencies and morphologies in mice (Lavau et al. 1997, 2000; DiMartino et al. 2002; So et al. 2003, 2004; J. Wang et al. 2005; Somervaille and Cleary 2006; our unpublished observations). *E2A-HLF* served as a control, since it transforms myeloid progenitors through *Hox*-independent pathways (Ayton and Cleary 2003; So et al. 2004). Retroviral constructs for each of the respective *MLL* fusion cDNAs expressed proteins that migrated at their predicted molecular weights in Western blot analysis (Supplementary Fig. 1A).

Primary murine myeloid progenitors (c-kit$^+$) transduced by the 12 *MLL* fusion constructs, but not empty vector, formed colonies in methylcellulose that replated through at least four rounds of culture (data not shown), demonstrating the enhanced self-renewal and impaired differentiation typically induced by *MLL* oncogenes (Lavau et al. 1997, 2000). Real-time quantitative PCR analysis of cells from fourth-round cultures showed that *Hoxa5*, *Hoxa7*, *Hoxa9*, and *Hoxa10* were highly expressed in myeloid cells transduced by *MLL* oncogenes compared with control (*E2A-HLF*) cells under our experimental conditions (Supplementary Fig. 1B,C). The relative and absolute levels of these *Hox* transcripts were fairly uniform, and did not appear to distinguish the different molecular subtypes of *MLL*-transformed cells.

Conversely, absolute *Meis1* expression levels varied considerably among the different *MLL*-transformed cells, from twofold to 40-fold the levels detected in control (*E2A-HLF*) cells (Fig. 1A). *Meis2* and *Meis3* were also expressed, but not consistently increased relative to their levels in cells transformed by *E2A-HLF*, such that *Meis1* accounted for 75%–95% of total *Meis* transcripts in *MLL*-transformed cells (Fig. 1B). Interestingly, *Meis1* transcript levels showed a significant correlation with the latencies for *MLL* leukemia (Fig. 1C; Supplementary Table 1). Cells transformed by MLL fusion proteins that induce a short latency AML in murine models displayed higher levels of *Meis1* expression, whereas those that induce AML with prolonged latencies displayed lower levels of *Meis1* expression. Statistical analysis showed a power relationship, indicating that *Meis1* transcript levels decreased at a specific rate with respect to increasing time required for leukemia development. Conversely, *Hoxa9* expression levels showed no correlation with leukemia latencies (Supplementary Fig. 2). These results suggested that *Meis1* likely serves a critical and potentially rate-limiting role in the pathogenesis of *MLL* leukemia.

Meis1 *is essential for induction and maintenance of* MLL*-mediated transformation*

To specifically address the requirement for *Meis1* in *MLL*-mediated oncogenesis, transformation assays were performed using fetal liver (FL) cells isolated from *Meis1*$^{-/-}$ embryos at embryonic day 13.5 (E13.5), prior to their intrauterine demise at approximately E14 (Hisa et al. 2004). *Meis1*-deficient FL cells were efficiently transformed by the control *E2A-HLF* gene; however, none of the *MLL* oncogenes was capable of inducing sustained replating (Fig. 2A). Rather, the plating capacities of *MLL*-transduced cells were exhausted after the second round. *Meis1*$^{-/-}$ second-round colonies were small in size with a diffuse morphology and contained markedly reduced numbers of myeloid blasts when compared with transformed colonies from wild-type FL (Fig. 2B). Conversely, all tested *MLL* oncogenes induced continued replating of E13.5 FL cells isolated from wild-type mice. Moreover, transformation of *Meis1*$^{-/-}$ FL cells by *MLL-AF9* was rescued by cotransduction of *Meis1* (Fig. 2A), demonstrating that the progenitors susceptible to *MLL* transformation were present in *Meis1*$^{-/-}$ FLs at E13.5. Thus, in the absence of *Meis1*, *MLL* oncogenes are incapable of inducing

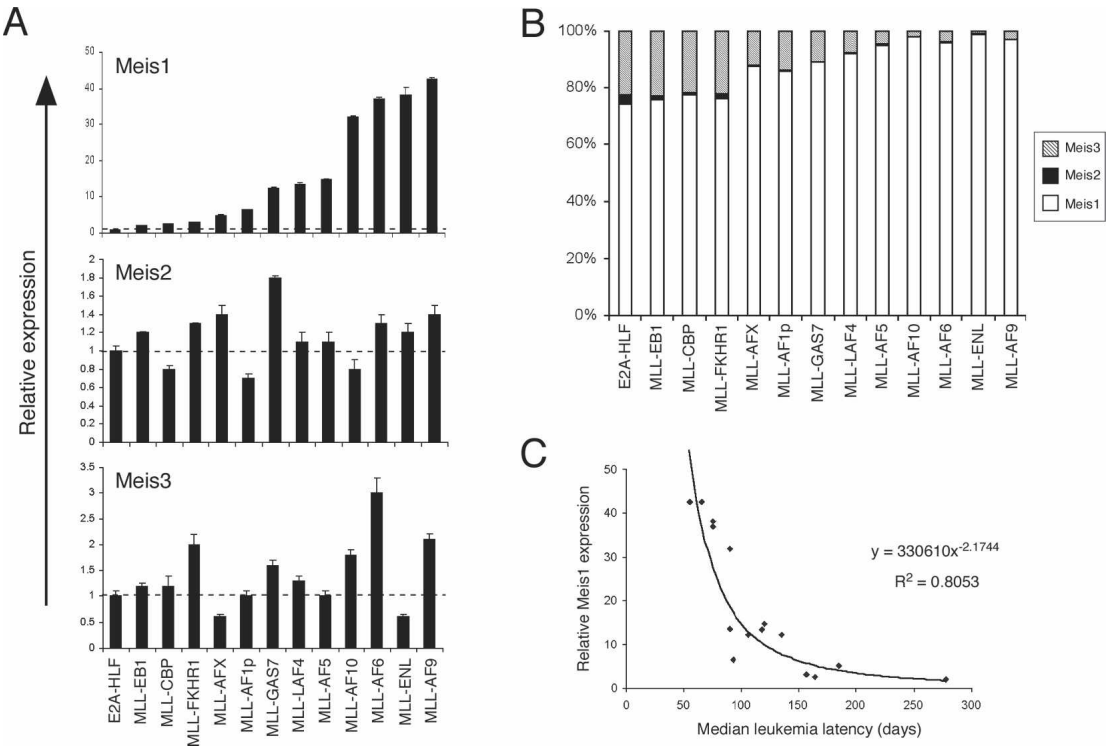

Figure 1. *Meis1* expression levels correlate with the latencies of *MLL* leukemias. (*A*) The expression levels of *Meis1*, *Meis2*, and *Meis3* transcripts were determined by real-time PCR analysis of *MLL*-transformed cells (indicated *below*) from the fourth round of serial replating (error bars indicate standard deviations of triplicate analyses). Results are expressed relative to levels observed in cells transformed by *E2A-HLF* (dashed line). (*B*) Bar graph indicates the total relative levels of *Meis* transcripts expressed in cells transformed by various oncogenes (indicated at *bottom*) from the fourth round of plating in methylcellulose cultures. The relative abundance of *Meis* transcripts was determined using the cycle time (Ct) value method, assuming that all primers were optimized to generate equal PCR efficiencies. (*C*) The relative expression levels of *Meis1* are plotted against median latency times required for leukemia induction by the respective *MLL* oncogenes. Latency data are derived in part from the current study as well as published studies (Supplementary Table 1). The data display a power trend line best-fit ($R^2 = 0.8053$) indicating that *Meis1* expression levels decrease at a specific rate with respect to the time required for leukemia development.

the enhanced self-renewal and impaired differentiation necessary for myeloid transformation.

To assess whether *Meis1* was required for maintenance of *MLL*-mediated transformation, wild-type cells stably transformed by *MLL* oncogenes were secondarily transduced with lentiviral vectors expressing short hairpin RNAs (shRNAs) to specifically silence *Meis1* (Supplementary Fig. 3). Expression of two different *Meis1* shRNAs resulted in substantial impairment (>70%–80%) of clonogenic capacity compared with *MLL*-transformed cells secondarily transduced with lentiviral vector alone (Fig. 2C). Similarly, *MLL*-transformed cells displayed a marked reduction in clonogenic capacity (>80%) when secondarily transduced with a dominant-negative construct that lacked the Meis1 DNA-binding homeodomain, whereas *E2A-HLF*-transformed cells were unaffected (Fig. 2D). Therefore, *Meis1* is essential for both the initiation and maintenance of enhanced self-renewal imposed on myeloid progenitors by *MLL* oncogenes.

MEIS1 has been reported to be consistently expressed in human *MLL* leukemias (Imamura et al. 2002b; Ferrando et al. 2003; Quentmeier et al. 2004), suggesting that it may serve a similar role in their maintenance. Quantitative RT–PCR analysis showed that *MEIS1* was expressed in leukemia cell lines with *MLL* chromosomal translocations (Fig. 2E). However, in contrast with murine *MLL*-transformed cells, *MEIS2* was also substantially expressed and ML2 cells were notable for expressing 10-fold more *MEIS2* versus *MEIS1* (Fig. 2E). Lentiviral transduction of two different *MEIS1* shRNAs resulted in substantial impairment (~40%–60%) of clonogenic capacity for *MLL* leukemia cell lines compared with control K562 cells with the exception of ML2 cells, which were not significantly compromised (Fig. 2F). Thus, despite the redundancy of *MEIS* gene expression, our results suggest that it is nevertheless required to maintain human *MLL* leukemias.

Hyperexpression of Meis1 *accelerates the progression of* MLL *leukemogenesis*

The observed correlation of endogenous *Meis1* expression levels with leukemia latency and the requirement of *Meis1* for *MLL*-mediated stem and progenitor cell transformation raised the possibility that *Meis1* may di-

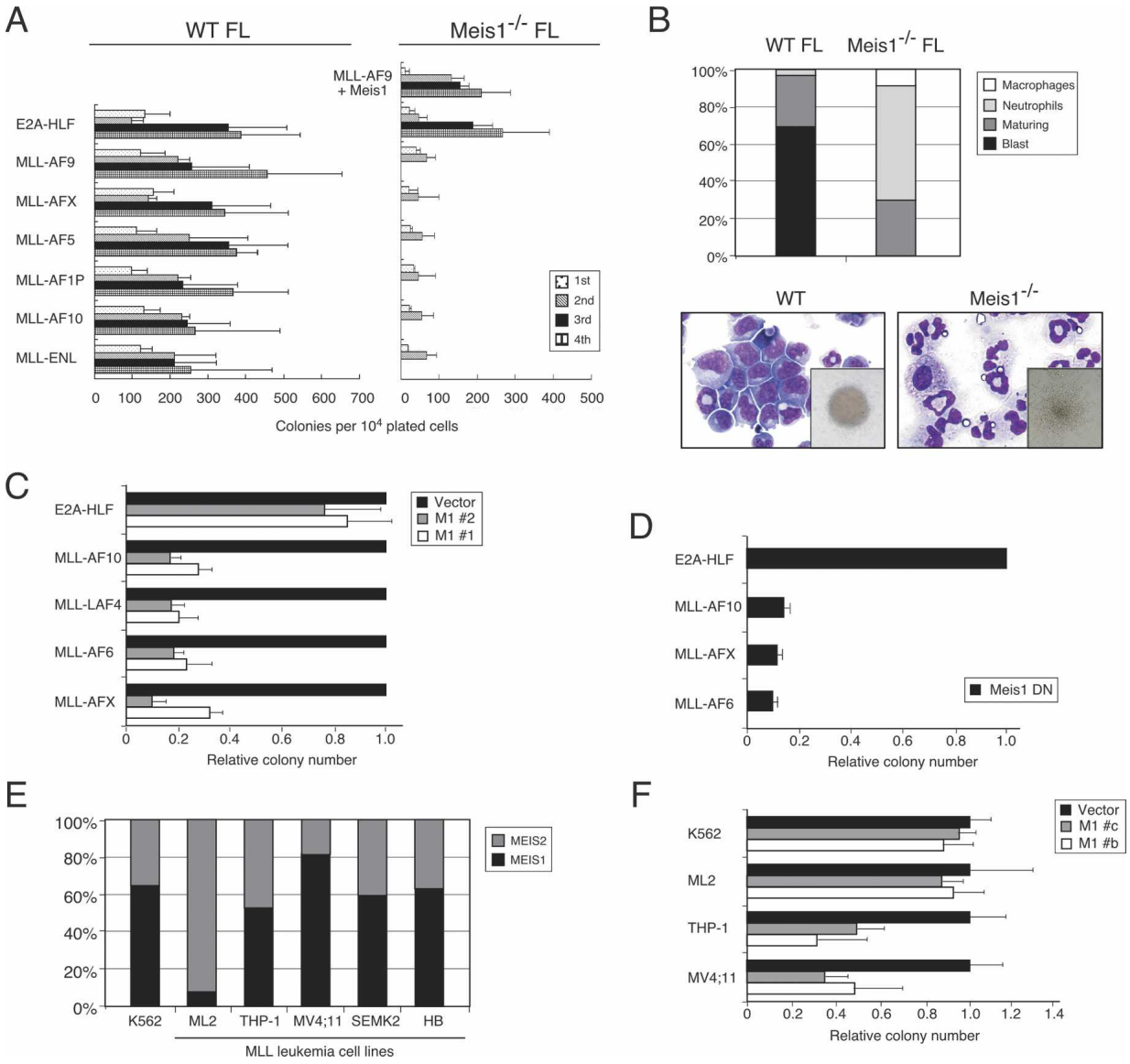

Figure 2. *Meis1* is required for initiation and maintenance of *MLL*-mediated myeloid transformation. (*A*) FL cells from wild-type (*left*) or *Meis1*$^{-/-}$ (*right*) mice were transduced with fusion oncogenes (indicated on *left*) and serially plated in methylcellulose cultures every 5 d. Results are shown as colony numbers per 10^4 plated cells for each round of culture. Data are also shown for *Meis1*$^{-/-}$ cells cotransduced with *MLL-AF9* and *Meis1*. Error bars indicate standard deviations of three independent experiments. (*B*) Cytospin preparations of wild-type (*left*) or *Meis1*$^{-/-}$ (*right*) FL cells transduced with *MLL-AF9* were analyzed for the proportions of cells at the end of the second round of plating in methylcellulose culture with morphological features of blasts versus differentiation (May Grunwald Giemsa staining). Bar graph indicates the mean number of cells with the indicated morphologic features ($n = 4$). *Insets* show colony morphologies at the end of the second round. (*C*) Myeloid progenitors transformed by various *MLL* oncogenes (indicated on *left*) were secondarily transduced with lentiviral vectors encoding shRNAs specific for *Meis1* (M1 #1 or M1 #2). Colony numbers are shown relative to cells secondarily transduced with lentiviral vector alone. Error bars indicate standard deviations of at least two independent experiments. (*D*) Myeloid progenitors transformed by various oncogenes (indicated on *left*) were secondarily transduced with a dominant-negative *Meis1* construct (Meis1 DN). Colony numbers are shown relative to cells transformed by *E2A-HLF*. Error bars indicate standard deviations of at least two independent experiments. (*E*) Bar graph indicates the total relative levels of *MEIS1* and *MEIS2* transcripts expressed in human leukemia cell lines (indicated at *bottom*). The relative abundance of *MEIS* transcripts was determined using the Ct value method, assuming that all primers were optimized to generate equal PCR efficiencies. (*F*) Human leukemia cell lines (indicated on *left*) were transduced with lentiviral vectors encoding shRNAs specific for *MEIS1* (M1 #b or M1 #c). Colony numbers are shown relative to cells transduced with lentiviral vector alone. Error bars indicate standard deviations of at least two independent experiments.

rectly influence the in vivo generation of LSCs from myeloid progenitors, which requires undefined secondary alterations (Lavau et al. 2000; Somervaille and Cleary 2006). To investigate this possibility, *Meis1* was hyperexpressed along with representative *MLL* oncogenes (*MLL-AF10*, *MLL-GAS7*, or *MLL-LAF4*) to determine

whether its enhanced expression may accelerate *MLL* leukemogenesis. Myeloid progenitors (c-kit$^+$) cotransduced with an *MLL* oncogene plus *Meis1* (*MLL/Meis1*) displayed *Meis1* transcript levels that were several-fold higher than progenitors cotransduced with the respective *MLL* oncogene plus empty vector (*MLL/v*) prior to transplantation (Fig. 3A). Following transplantation of equal numbers of cotransduced cells (representing comparable numbers of colony-forming cells [CFCs]) into syngeneic recipient mice, the *MLL/Meis1* cohorts developed leukemia with substantially shortened latencies compared with mice transplanted with *MLL/v* cotransduced cells (Fig. 3B). Leukemias in both cohorts appeared grossly similar pathologically—with effacement of the normal bone marrow architecture, marked splenomegaly, and infiltration of the liver by leukemia cells—and maintained similar levels of *Meis1* when compared with the respective primary cotransduced progenitors (data not shown). Therefore, *Meis1* serves a rate-limiting role in progression of *MLL*-associated leukemia.

Meis1 *modulates the differentiation arrest, self-renewal, and cell cycle activity of* MLL *leukemia cells*

FACS analysis demonstrated that all leukemias were donor-derived (data not shown) and consistently displayed myeloid phenotypes (Mac1$^+$ Gr1$^+$). However, a greater fraction of *MLL/Meis1* leukemia cells expressed high-level c-kit and lower Mac1, suggestive of less differentiation (Fig. 4A). Morphologic assessment of splenocytes obtained at necropsy of *MLL/Meis1* mice revealed a several-fold higher proportion of cells with cytologic features of blasts and substantially fewer differentiating forms (Fig. 4B) compared with splenocytes from *MLL/v* mice. Furthermore, in semisolid culture assays, the frequencies of CFCs in the spleens of leukemic mice were up to sevenfold higher for *MLL/Meis1* leukemias (Fig. 4C) and colony morphologies (Lavau et al. 1997) were predominantly type I for *MLL/Meis1* CFCs, compared with mostly type II/III for *MLL/v* CFC (72% vs. 21% for MLL-AF10 leukemias) (data not shown). Thus, *MLL* leukemias where *Meis1* was overexpressed exhibited a more pronounced block in differentiation as well as a higher frequency of clonogenic leukemia cells.

Cell cycle analysis of explanted leukemia cells growing in methylcellulose cultures revealed a significant increase in the proportion of S/G2/M-phase cells for *MLL/Meis1* versus *MLL/v* leukemias (Fig. 5A). The higher fraction of cells in cycle correlated with substantially lower transcript levels for the CDK inhibitor p16^{Ink4a}, whereas expression of several other cell cycle regulators including p19^{Arf}, an alternatively spliced *Ink4a* transcript that codes for a positive regulator of the p53 pathway, did not differ significantly between *MLL/v* and *MLL/Meis1* leukemias (Fig. 5B). However, expression of *Bmi-1*, a negative regulator of the *Ink4a* locus (Jacobs et al. 1999), was significantly higher in *MLL/Meis1* leukemias (Fig. 5B). The changes in gene expression as well as latency were more pronounced in MLL-AF10 versus MLL-LAF4 leukemias. Therefore, the enabling effects of *Meis1* on proliferation and arrested differentiation of *MLL* leukemia cells correlated with specific perturbations of the *Bmi-1/Ink4a* axis, which is implicated in maintenance of HSC and LSC self-renewal (Lessard and Sauvageau 2003; Park et al. 2003).

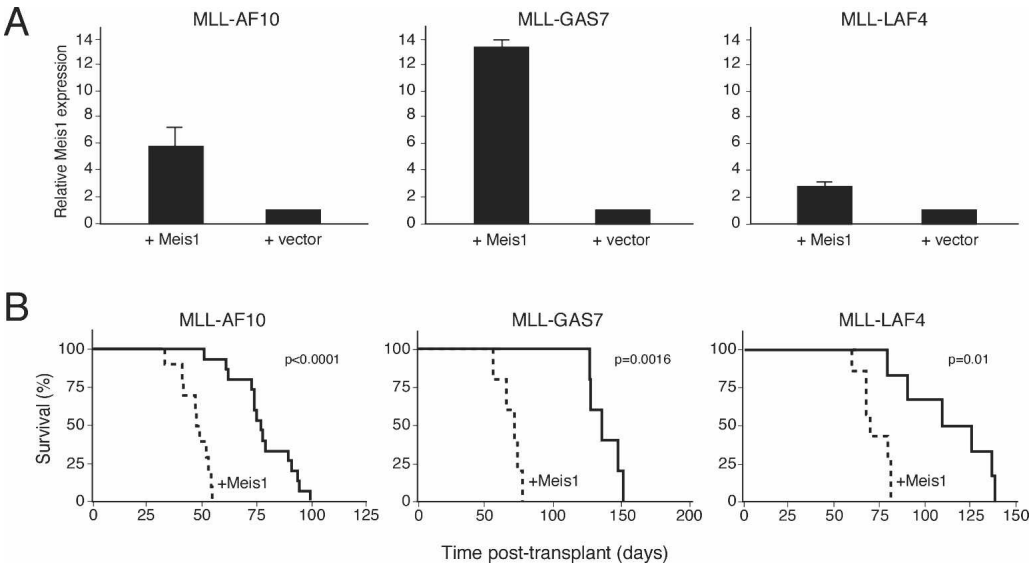

Figure 3. Increased *Meis1* expression accelerates *MLL*-mediated leukemogenesis. (*A*) *Meis1* transcript levels in myeloid progenitors (c-kit$^+$) were determined by quantitative real-time PCR 24 h following transduction with retroviruses encoding the *MLL* oncogenes indicated *above* the panel, with or without cotransduced *Meis1* (indicated *below*). Data are expressed relative to the level observed in cells transduced with *MLL* oncogene alone (bars indicate the average of triplicate analyses). (*B*) Survival curves are shown for cohorts of mice transplanted with cells cotransduced with the indicated *MLL* oncogene and *Meis1* or empty vector. Acute leukemia was confirmed by peripheral blood leukocyte counts, FACS analyses, and/or necropsy.

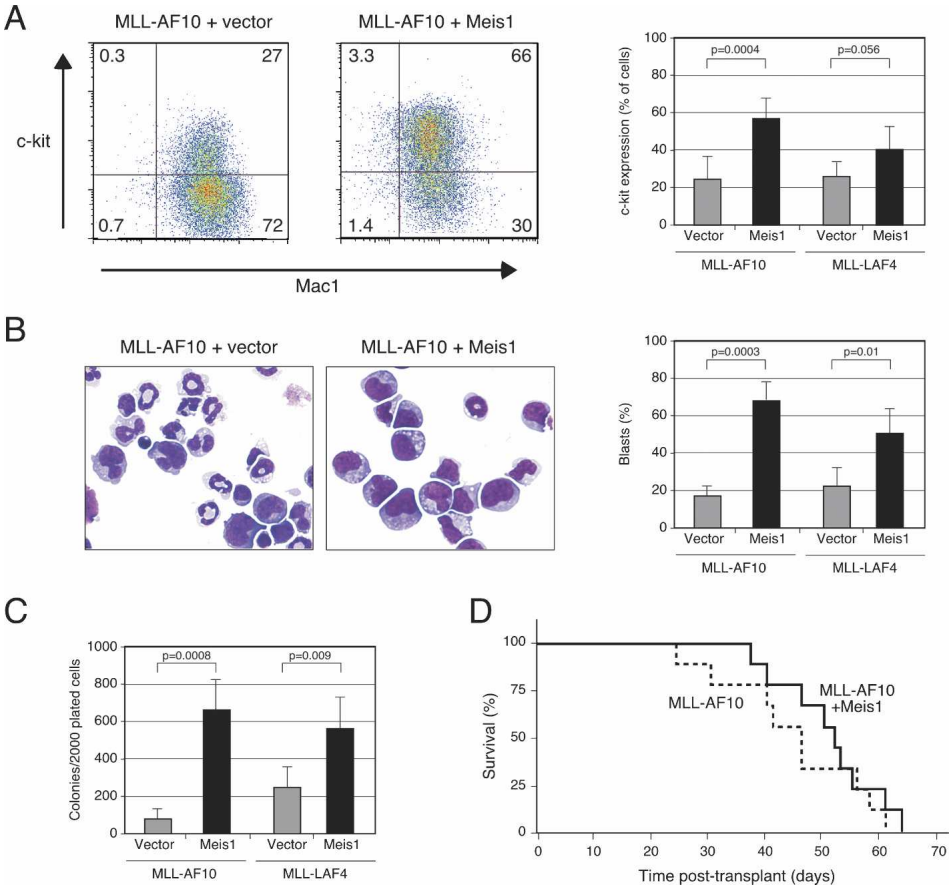

Figure 4. Meis1 modulates myeloid differentiation arrest, as well as CFC and LSC frequencies of *MLL* leukemias. (*A*) FACS profiles demonstrate representative c-kit and Mac1 expression on splenocytes from mice with AML induced by transplantation of progenitors transduced with genes indicated at the *tops* of the panels. Bar graph summarizes data from five mice in each category. (*B*) Splenocytes from leukemic mice were analyzed for the proportion of cells with morphologic features of blasts versus differentiation following May Grunwald Giemsa staining of cytospin preparations. Bar graph indicates the number of cells with the indicated morphologic features for five mice in each cohort. (*C*) FACS-sorted Mac1$^+$ leukemic splenocytes were cultured in methylcellulose medium for 6 d to determine CFC frequencies. Bar graph indicates the numbers of clonogenic cells for each of five leukemias in the respective cohorts. (*D*) Survival curves of sublethally irradiated mice transplanted with single AML colonies (3000 cells) plucked after 7 d in semisolid culture and directly injected into secondary recipients, or expanded in semisolid culture for 5 d prior to injection (2.5×10^5 cells).

Meis1 *determines* MLL *LSC frequency*

Previous studies of *MLL-AF9* AML showed that CFCs present in the spleens of leukemic mice have the biological properties of LSCs (Somervaille and Cleary 2006). To test if the increased CFC frequencies in leukemias induced by coexpressed exogenous *Meis1* also correlated with increased numbers of LSCs, individual CFCs (type I or II) were plucked from methylcellulose media and transplanted into syngeneic mice (or expanded in semisolid culture prior to transplant) to assess their potential to initiate AML in secondary recipients, a hallmark feature of LSCs. All transplanted mice succumbed to AML regardless of whether the transplanted CFCs derived from primary leukemias with or without coexpressed exogenous *Meis1* (Fig. 4D). These results confirmed that CFCs in each cohort were a reliable indicator of LSCs, and demonstrated sevenfold higher LSC frequencies in *MLL/Meis1* leukemias in comparison with *MLL/v* leukemias. Unlike the case for primary transplant recipients, latencies were similar irrespective of *Meis1* status since the cells had achieved full leukemic potential prior to secondary transplant. Nevertheless, the morphologic features and phenotypes of the respective secondary leukemias were similar to those observed in the primary mice—i.e., a less-differentiated phenotype in leukemias coexpressing exogenous *Meis1* (data not shown)—demonstrating that *Meis1* expression levels stably determine LSC properties in *MLL*-associated AML.

Meis1 functions as a DNA-binding transcriptional cofactor in MLL *transformation*

A structure/function analysis was conducted to investigate whether Meis1 may require interactions with Pbx TALE protein partners for *MLL*-mediated transformation. Meis1 constructs containing either deletion or point mutations (in the M1/M2 domains) that abrogate Pbx interaction (G.G. Wang et al. 2005) were incapable of

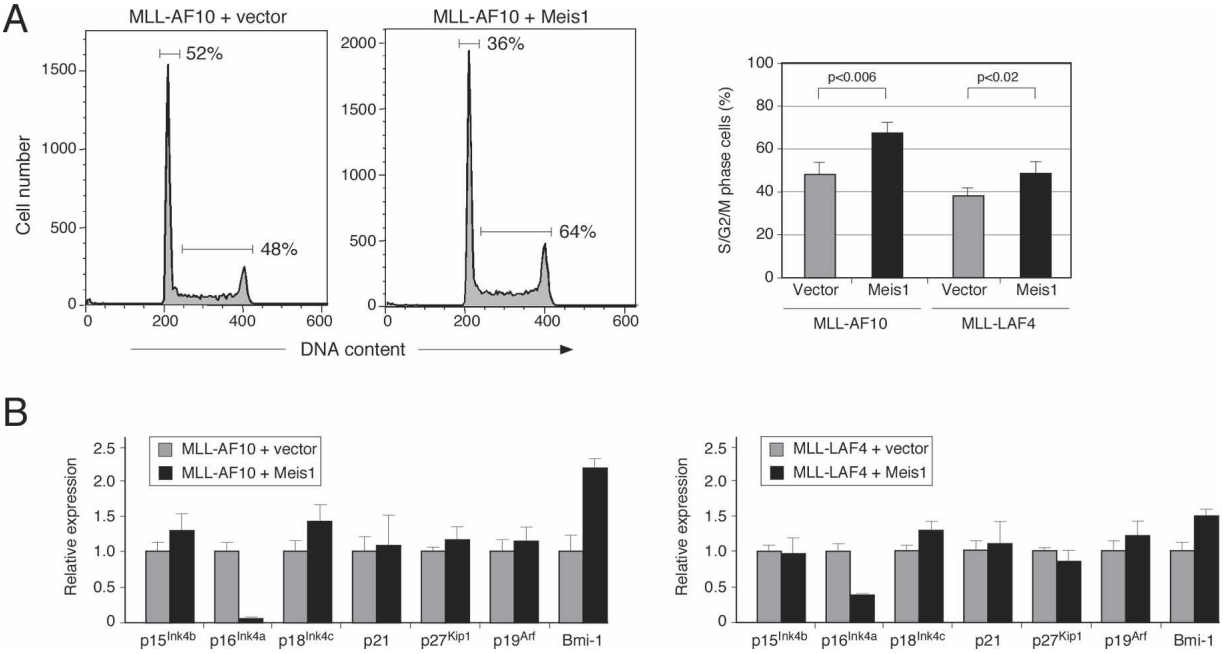

Figure 5. Altered cell cycle activity induced by *Meis1* in *MLL-AF10* leukemia. (*A*) DNA content analysis was determined by FACS analysis of explanted leukemia cells growing in methylcellulose cultures. Bar graph summarizes data for three mice in each cohort. (*B*) Expression levels of the indicated transcripts were determined by quantitative RT–PCR analysis of splenocytes isolated from leukemic mice. Results are the mean of three leukemias in each category, and are expressed relative to transcript levels in leukemia cells transformed by MLL-AF10.

rescuing *MLL* transformation of *Meis1*$^{-/-}$ FL cells (Fig. 6B, constructs #2 and #5), in contrast to wild-type Meis1 (Fig. 6B, construct #1). Meis1 constructs containing homedomain mutations, which have been shown to disrupt Meis1 DNA binding (G.G. Wang et al. 2005), were also unable to complement *Meis1* deficiency (Fig. 6A,B, constructs #3 and #4). Furthermore, deletion of the conserved C-terminal tail of Meis1 (Fig. 6A,B, construct #6), which has been implicated in its transcriptional effector properties, abrogated rescue of *MLL* transformation. Taken together, these analyses suggest that Meis1 associates with Pbx partners in a DNA-binding transcriptional complex to mediate *MLL* transformation.

MLL-*mediated myeloid transformation is codependent on TALE homeodomain proteins* Pbx2 *and* Pbx3

A genetic approach was employed to interrogate the contributions of Pbx proteins in *MLL* leukemia. Real-time quantitative PCR analysis showed that three *Pbx* genes (*Pbx1*, *Pbx2*, and *Pbx3*) were expressed in myeloid progenitors immortalized by *MLL* oncogenes (Fig. 7A; data not shown). *Pbx3* was notable for its significantly increased levels (three- to eightfold) compared with control cells transformed by *E2A-HLF*, consistent with increased *Pbx3* levels in human MLL AMLs (Ross et al. 2004). Together, *Pbx2* and *Pbx3* accounted for >90% of total *Pbx* transcripts, whereas *Pbx1* was minimally expressed (Fig. 7B). To investigate whether specific *Pbx* functions were required for *MLL* transformation, serial replating assays were performed using FL cells harvested from *Pbx3*$^{-/-}$ embryos (E14.5–E18) (Rhee et al. 2004) or bone marrow cells isolated from adult *Pbx2*$^{-/-}$ mice (Selleri et al. 2004; Capellini et al. 2006). *MLL* oncoproteins transformed the *Pbx*-deficient myeloid progenitors as efficiently as wild-type cells (Supplementary Fig. 4), indicating that *MLL*-mediated transformation was not dependent on the presence of *Pbx2* or *Pbx3* alone.

To further reduce *Pbx* levels, *Pbx2*$^{-/-}$ cells transformed by *MLL* oncogenes were secondarily transduced with shRNAs targeting *Pbx3*, resulting in an ~70%–80% reduction of *Pbx3* transcript levels (Supplementary Fig. 3). Following *Pbx3* knockdown, the clonogenic potential of *Pbx2*$^{-/-}$/*Pbx3*^{kd} *MLL*-transformed cells was substantially decreased (>60%) when compared with *Pbx2*$^{-/-}$ transformed cells secondarily transduced with vector alone (Fig. 7C). This effect was specific to *MLL*-mediated transformation since *Pbx2*$^{-/-}$/*Pbx3*^{kd} cells transformed by *E2A-HLF* displayed no significant reduction in clonogenic potential following *Pbx3* silencing (Fig. 7C). These results demonstrate that maintenance of *MLL*-mediated transformation is specifically dependent on *Pbx* activity, and further support that Meis1 functions in complex with Pbx proteins in myeloid progenitors transformed by *MLL* oncogenes.

Discussion

MLL leukemias are invariably associated with expression of *Hox* and *TALE* homeobox genes, but nevertheless

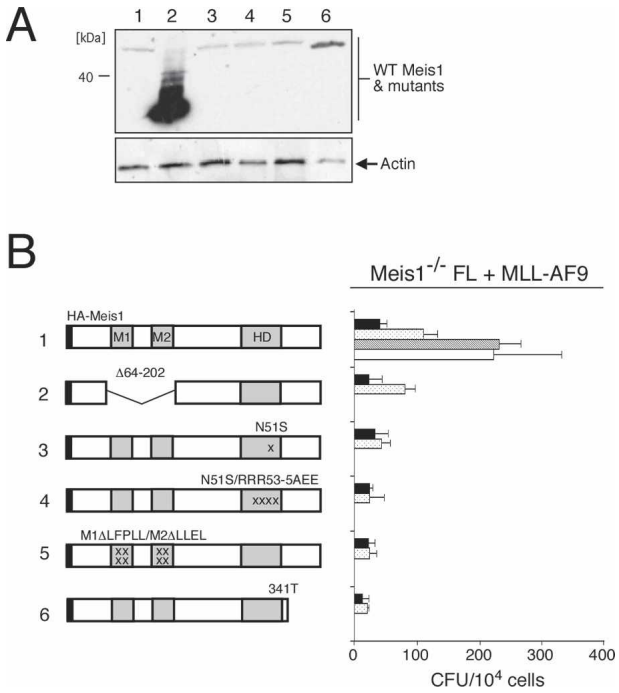

Figure 6. Structure/function analysis of Meis1 requirements in *MLL* transformation. (*A*) Western blot analysis of wild-type and mutant Meis1 proteins expressed in retroviral packaging Phoenix cells and detected with an anti-HA antibody. Identities of constructs are indicated at the *tops* of lanes and correspond to the schematic depictions in *B*. The enhanced stability of construct #2 lacking Pbx interaction motifs M1/M2 has been reported previously (G.G. Wang et al. 2005). (*B*) Myeloid transformation assay was performed using *Meis1*$^{-/-}$ FL cells cotransduced with *MLL-AF9* and various *Meis1* constructs schematically illustrated on the *left*. Results from four rounds of plating are shown with each bar representing the mean ± SD of the total number of myeloid colonies per 10^4 plated cells derived from at least three replicates.

are clinically heterogeneous possibly reflecting differences in LSC biology and/or frequency. Misregulation of *Hox* genes in *MLL* leukemias is likely to be critical for LSC maintenance, as suggested by genetic loss-of-function studies (Ayton and Cleary 2003; Kumar et al. 2004; So et al. 2004; Okada et al. 2005; J. Wang et al. 2005) and consistent with their normal roles in HSC self-renewal (Abramovich and Humphries 2005). However, *Hox* gene misregulation alone does not recapitulate the phenotype and biology of *MLL* leukemias and is unlikely to support the high LSC frequencies recently demonstrated in a murine model of *MLL-AF9* AML that are far in excess of those previously estimated for AML in general (Somervaille and Cleary 2006). Our current studies demonstrate that TALE homeodomain proteins are essential regulators of *MLL* transformation, and that Meis1 in particular determines LSC frequency and potential by quantitatively regulating the extent of self-renewal, differentiation arrest, and cycling, as well as the rate of in vivo LSC generation from myeloid progenitors.

Meis1 *is an essential regulator of LSC biology in* MLL *leukemia*

Genetic analyses revealed a critical dependence on *Meis1* for induction and maintenance of myeloid transformation induced by several molecularly and functionally distinct *MLL* oncogenes. Notably, lack of *Meis1* confers a consistent and severe impairment of *MLL*-mediated transformation in contrast to deficiencies of single *Hoxa* genes, whose requirements vary for different *MLL* oncogenes (Ayton and Cleary 2003; Kumar et al. 2004; So et al. 2004; Okada et al. 2005; J. Wang et al. 2005; our unpublished observations). *Meis1* is the predominant *Meis* family gene expressed in murine myeloid progenitors, and its TALE protein product functions as a transcriptional cofactor for multiple Hox proteins, which are redundantly expressed. Thus, *Meis1* deficiency likely results in a broad compromise of Hox transcriptional activity, in addition to possible impacts on non-Hox-dependent pathways, and consequently a more severe limitation on *MLL* transformation than the loss of single *Hox* genes.

Although *Meis1* is consistently expressed and required in murine myeloid cells transformed in vitro by different MLL oncoproteins, its endogenous expression levels varied by >20-fold and correlated with critical features of leukemia biology and LSC frequency. *Meis1* dictates the extent of myeloid differentiation arrest induced by *MLL* oncogenes as well as the fraction of leukemia cells in cycle, and their clonogenic frequency. The similarity of these effects to those observed for *Meis1* in *Hoxa9*-associated leukemogenesis (Kroon et al. 1998; G.G. Wang et al. 2005) provides strong support for previous suggestions that *Meis1* and *Hoxa9* are major effectors of *MLL* leukemogenesis (Zeisig et al. 2004). Clonogenic frequency is a surrogate measure of *MLL* LSCs (Somervaille and Cleary 2006), which are substantially increased (up to sevenfold) in AMLs associated with hyperexpression of exogenous *Meis1*. This correlates with observations that *Meis1* programs expression of HSC-associated genes in *Hoxa9*-induced leukemia (G.G. Wang et al. 2005). Our studies substantially extend these observations by demonstrating that *Meis1* quantitatively regulates functional properties of LSCs in a model of *MLL* leukemia.

Meis1 *is rate-limiting for* MLL *leukemogenesis*

Meis1 appears to serve two distinct but possibly interrelated roles in *MLL* leukemogenesis. In addition to its requirement to initiate in vitro immortalization, the significant correlation of *Meis1* expression levels with latencies required for development of *MLL* leukemias in our mouse model supports a role for *Meis1* in regulating progression of *MLL*-immortalized cells to LSCs. The genetic and/or epigenetic changes underlying this transition have not yet been defined, although it is characterized in part by acquisition of an enhanced ability to interact with the bone marrow microenvironment (Shah et al. 1998; Somervaille and Cleary 2006). A role in LSC progression is consistent with previous observations that

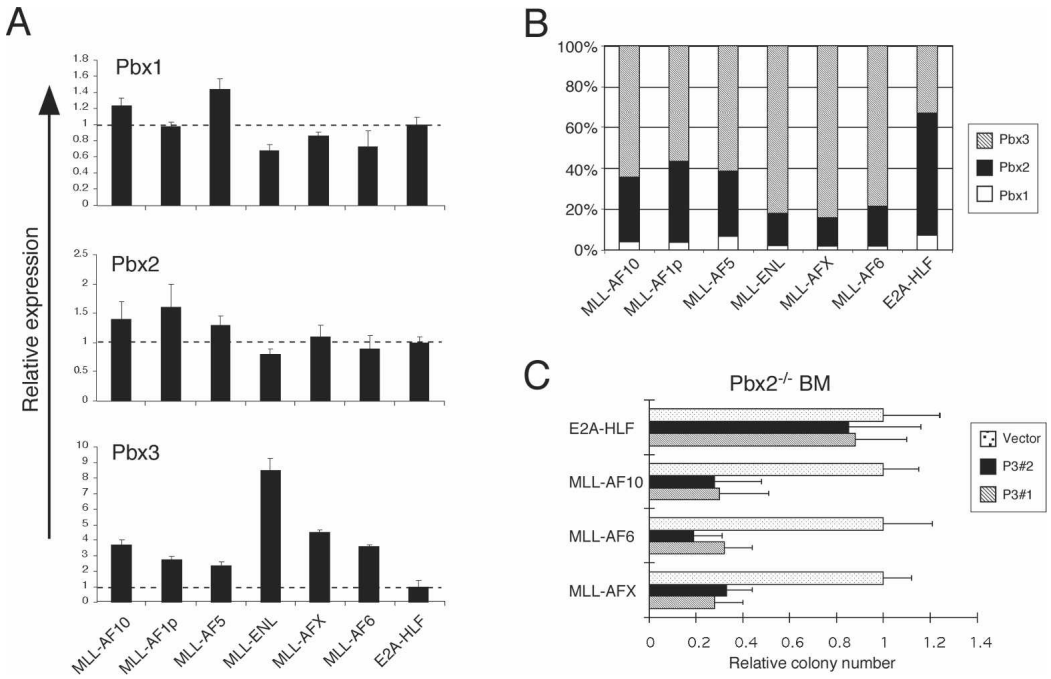

Figure 7. *Pbx2* and *Pbx3* are required for maintenance of *MLL*-mediated transformation. (*A*) The relative expression levels of *Pbx1*, *Pbx2*, and *Pbx3* transcripts were determined by real-time PCR analysis of *MLL*-transformed cells (indicated *below*) from the fourth round of serial replating (error bars indicate standard deviations of triplicate analyses). Results are expressed relative to levels observed in cells transformed by *E2A-HLF*. (*B*) Bar graph indicates the total relative levels of *Pbx* transcripts expressed in cells transformed by various oncogenes (indicated at *bottom*) from the fourth round of plating in methylcellulose cultures. The relative abundance of *Pbx* transcripts was determined using the Ct value method, assuming that all primers were optimized to generate equal PCR efficiencies. (*C*) *Pbx2*$^{-/-}$ myeloid progenitors transformed by various oncogenes (indicated on *left*) were secondarily transduced with lentiviral vectors encoding shRNAs specific for *Pbx3* (P3 #1 or P3 #2). Colony numbers are shown relative to cells secondarily transduced with lentiviral vector alone. Error bars indicate standard deviations of at least two independent experiments.

hyperexpression of *Meis1* markedly increased the penetrance and reduced the latency for development of *Hoxa9*-induced AML (Kroon et al. 1998; G.G. Wang et al. 2005). Thus, *Meis1* is rate-limiting such that low levels temper or abrogate immortalization of myeloid progenitors in vitro, whereas high levels accelerate their in vivo transition to LSCs.

The critical influence of *Meis1* on LSC biology appears to correlate with epigenetic regulation of the RB pathway. Leukemias that developed with shortened latencies following hyperexpression of exogenous *Meis1* were associated with increased expression of the *Bmi-1* gene, which encodes a polycomb group epigenetic repressor protein. This is consistent with previous observations that *BMI-1* levels increased during step-wise transformation and immortalization of human AML (Warner et al. 2005). However, the level of *Bmi-1* mRNA increase observed in our studies was relatively modest, presumably because Bmi-1 genetically opposes MLL function through its antagonistic effects on *Hox* gene expression (Hanson et al. 1999). *Bmi-1* is essential for the maintenance of normal adult HSCs as well as LSCs (Lessard and Sauvageau 2003; Park et al. 2003), consistent with the increased LSC frequencies in *MLL/Meis1* leukemias. In this capacity, Bmi-1 represses transcription of $p16^{Ink4a}$, a CDKI at the apex of the RB pathway. Expression of $p16^{Ink4a}$ inversely correlated with *Meis1* and *Bmi-1* levels as well as the increased fraction of cycling cells in *MLL* leukemias and the frequency of LSCs, which are highly enriched for cycling cells (T.C.P. Somervaille and M.L. Cleary, unpubl.). It was the only CDKI whose expression was consistently altered, supportive of a specific as opposed to secondary role in regulating LSC potential. Preliminary studies suggest that *Bmi-1* is not a direct transcriptional target of Meis1 and is not absolutely essential for *MLL*-mediated transformation in vitro (P. Wong and M.L. Cleary, unpubl.), consistent with an alternative role in regulating long-term self-renewal that will require assessment by long-term transplantation studies. Taken together, our studies suggest that the contributions of *Meis1* in generating *MLL* LSCs are mediated in part through the RB pathway by epigenetic modulation of *Ink4a* expression.

Since MLL fusion proteins, in conjunction with their essential cofactor menin, have been shown to associate with the *Meis1* promoter (Milne et al. 2005; Yokoyama et al. 2005; Caslini et al. 2007), our observations raise the intriguing possibility that they may vary in their abilities to maintain expression of the *Meis1* gene. Consistent with this possibility, MLL-ENL and MLL-FKBP, which differ in their oncogenic potencies, impose different histone modifications at the *Meis1* promoter (Milne et al. 2005). Thus, the absolute levels of *Meis1* expression may be differentially regulated by the transcrip-

tional effector properties of individual MLL oncoproteins, which in turn dictates the rate of progression of immortalized cells to LSCs that initiate and sustain AML. This contrasts with *Hox* gene expression levels, which do not show a similar correlation with leukemia latencies or molecular subtypes (Supplementary Fig. 2).

Our studies suggest that TALE proteins may also critically maintain human *MLL* leukemia, since *MEIS1* knockdown impaired the growth of leukemia cell lines. Interestingly, the relative prevalence of different *MLL* fusion genes in human leukemias is consistent with a potential rate-limiting role for *MEIS* gene expression comparable with mouse leukemias. Commonly recurring *MLL* fusion genes such as *MLL-AF9*, *MLL-ENL*, *MLL-AF6*, and *MLL-AF10*, which were associated with the highest levels of *Meis1* expression in our mouse model, comprise the vast majority of spontaneous *MLL*-associated human AMLs. Conversely, *MLL-EB1* and *MLL-CBP*, which are associated with lower *Meis1* expression in our model, have only rarely been observed in human AML (a single case for *MLL-EB1*) (Fu et al. 2005). Furthermore, *MLL-CBP* is associated with human MDS (Satake et al. 1997) and induces an MDS-like disorder in mice (J. Wang et al. 2005), which are premalignant conditions characterized by altered self-renewal and differentiation potentials, suggesting that *MLL-CBP* initiates transformation but does not readily facilitate progression to AML. Thus, *MEIS* family gene expression levels in subclinical initiated progenitors harboring *MLL* chromosomal translocations may influence the probability of acquiring necessary secondary mutations for their conversion into LSCs capable of inducing clinical leukemia.

MLL-*mediated transformation is dependent on* Pbx *function*

Pbx proteins heterodimerize and bind DNA with Meis proteins, which also regulate Pbx stability and nuclear localization. Consistent with their biochemical interactions, lack of *Meis1* partially phenocopies *Pbx1* deficiency in hematopoietic development (DiMartino et al. 2001; Hisa et al. 2004). In *MLL* leukemias, Meis1 contributions are dependent on the integrity of its Pbx dimerization motifs. Unlike the case for *Meis1*, however, single deficiencies of *Pbx2* or *Pbx3*, the most highly expressed *Pbx* genes in myeloid progenitors, did not abrogate *MLL*-mediated transformation. Nevertheless, the observed reduction of clonogenic activity in *Pbx2/3* compound-deficient cells indicates that transformation of myeloid progenitors by MLL oncoproteins is dependent on *Pbx* function. This is a specific requirement and not reflective of a general suppressive effect of *Pbx* deficiency on myeloid transformation, since no impairment of proliferation or enhanced self-renewal was observed in $Pbx2^{-/-}Pbx3^{KD}$ cells transformed by *E2A-HLF*, which transforms through a *Hox*-independent pathway (Ayton and Cleary 2003; So et al. 2004) and would not be expected to require Hox cofactors such as Pbx for oncogenesis.

Dependence on the combined contributions of Pbx2 and Pbx3 may reflect their redundant properties, consistent with previous studies demonstrating that different Pbx proteins exhibit essentially identical cooperative DNA binding with a subset of Hox proteins in vitro (Chang et al. 1995). Although we cannot exclude that Pbx2 and Pbx3 make isoform-specific contributions to *MLL* oncogenesis, it is more likely that single *Pbx* deficiencies are functionally compensated by other members of the Pbx protein family, whereas compound deficiencies reduce total Pbx dosage below a critical functional threshold. In support of this, novel compound-deficient *Pbx* phenotypes have recently been reported for *Pbx1* and *Pbx2* in limb development (Capellini et al. 2006) despite the fact that *Pbx2* deficiency alone results in no phenotype (Selleri et al. 2004). Our data suggest that reduction of total *Pbx* (*Pbx1*, *Pbx2*, *Pbx3*) expression substantially below half of wild-type levels is limiting for *MLL* transformation, thus establishing a critical role for Pbx TALE proteins.

In summary, TALE homeodomain proteins are rate-limiting for many of the biological properties that define *MLL* LSCs. *Meis1* in particular regulates LSC frequencies and their origin from immortalized myeloid progenitors. Although deregulated *Hox* gene expression is consistently induced by *MLL* fusion oncogenes, Hox protein function may be limited by the availability of TALE protein cofactors, which are more variable in their abundance and correlate with leukemia latency and biologic heterogeneity. The central role of TALE proteins in *MLL* leukemia maintenance suggests their consideration as potential therapeutic targets, which warrants further investigation.

Materials and methods

Mice

Knockout mice deficient for *Pbx2* (Selleri et al. 2004), *Pbx3* (Rhee et al. 2004), and *Meis1* (Hisa et al. 2004) were maintained on a C57BL/6 genetic background. C57BL/6 mice congenic for CD45 (Ly5.1/Ly5.2) were employed for transplant studies to distinguish donor and recipient cells.

Cell lines

The human leukemia cell lines MV4.11, K562, ML2, THP-1, SEMK2, and HB were obtained from the American Type Culture Collection (ATCC) or generated in our laboratory and were maintained under standard conditions.

Retroviral constructs and hematopoietic progenitor transformation assays

Retroviral constructs encoding *MLL-GAS7*, *MLL-AF1P*, *MLL-AF6*, *MLL-ENL*, *MLL-AF9*, *MLL-AF10*, *MLL-FKHRL1*, *MLL-AFX*, *MLL-CBP*, *E2A-HLF*, and *Meis1* have been described previously (Lavau et al. 1997, 2000; DiMartino et al. 2002; So and Cleary 2002, 2003; So et al. 2003, 2004; Fu et al. 2005; J. Wang et al. 2005; Somervaille and Cleary 2006). Retroviral constructs encoding *MLL-LAF4*, *MLL-AF5*, *MLL-AF6*, and *MLL-EB1* were generated by insertion of the respective cDNAs or subtotal frag-

ments with oncogenic potential (encoding LAF4 amino acids 335–1227, AF5 amino acids 727–1163, AF6 amino acids 35–137, and EB1 amino acids 199–269;) (Imamura et al. 2002a; von Bergh et al. 2002; Fu et al. 2005) into the *MLL* 5′ vector using standard cloning techniques. A dominant-negative *Meis1* construct, analogous to known dominant-negative *Drosophila* and *Xenopus Meis* isoforms (Dibner et al. 2001; Inbal et al. 2001), was generated by deletion of C-terminal amino acids 280–390, which encode a portion of the homeodomain and downstream residues. Retroviral constructs for Meis1Δ62–202 (Pbx1), M1ΔLFPLL/M2ΔLLEL (Pbx2), HDΔN51S (HD1), HDΔN51S RRR53-5AEE (HD2), and Meis1 341T with a deletion of a conserved C terminus were reported previously (G.G. Wang et al. 2005). Hematopoietic transformation assays were performed essentially as described previously using primary murine myeloid progenitors harvested from bone marrow or FL (So et al. 2004). Cells transduced with retroviral vectors were selected for stable transduction in methylcellulose medium containing the appropriate antibiotic (250 μg/mL hygromycin, 1 μg/mL puromycin, and/or 1mg/mL neomycin).

Lentivirus generation and secondary transduction

The pSicoR lentiviral vector (Ventura et al. 2004) carrying either puromycin resistance or GFP marker genes was used for knockdown studies. shRNAs (Supplementary Table 2) were designed using pSicoOligomaker 1.5 (developed by A. Ventura, Jacks Laboratory, Cambridge, MA) and cloned into HpaI–XhoI-digested pSicoR. Lentiviral stocks were generated essentially as described previously (Ventura et al. 2004). In brief, DNA constructs encoding lentiviral vectors (5 μg), CMV gag-pol-rev 8.74 (4 μg), and VSVG (1 μg) were cotransfected into 293T cells using FuGENE 6 reagent (Roche Diagnostics). Supernatants were collected 36–48 h after transfection, passed through a 0.4-μm filter, and immediately incubated with immortalized cells (2×10^4 to 4×10^4) or human cell lines (1×10^5) for 12 h at 37°C. Cells transduced with pSicoR-GFP vectors were cultured in liquid medium for 48 h and then purified by FACS sorting for GFP expression. Transduced mouse cells (2000 cells) were plated in methylcellulose medium (M3231; Stem Cell Technologies) supplemented with cytokines (20 ng/mL SCF, 10 ng/mL IL-6, 10 ng/mL IL-3, 10 ng/mL GM-CSF) or in MethoCult (H4236; Stem Cell Technologies) for human cells. Cells transduced with pSicoR carrying the puromycin resistance gene were plated 24 h after transduction in 0.9% methylcellulose medium (M3231) supplemented with cytokines and 1 μg/mL puromycin.

Leukemogenicity and long-term in vivo reconstitution assays

Transplantation experiments were performed as described previously (Lavau et al. 2000) with the following minor modifications. For cotransduction experiments, transduced progenitors were incubated in 0.9% methycellulose medium containing cytokines (20 ng/mL SCF, 10 ng/mL IL-6, 10 ng/mL IL-3) in the presence of puromycin (1 μg/mL) and neomycin (1 mg/mL) for 5 d, and then transplanted (1×10^5 cells) together with a radioprotective dose of total bone marrow cells (2×10^5) into the retro-orbital venous sinus of 6- to 12-wk-old syngeneic C57BL/6 mice that had been lethally irradiated with 9.0 Gy of total body γ irradiation (^{135}Cs). When transplanted mice exhibited signs of ill health (shortness of breath, lethargy, and hunched posture) they were euthanized. Donor and recipient cells were distinguished by FACs analysis of CD45 congenic marker expression. Necropsy tissues were fixed in buffered formalin, sectioned, and stained with hematoxylin and eosin (H&E) for histological analysis. Secondary transplants were performed by retro-orbital injection of leukemia cells (3×10^3 from a single colony or 2.5×10^5 expanded in semisolid medium) into sublethally irradiated (450 cGy) syngeneic C57BL/6 mice.

Flow cytometry analysis

Bone marrow and spleen cells were stained with fluorochrome-conjugated monoclonal antibodies to either c-Kit (2B8 clone), Mac-1 (M1/70 clone), Gr-1 (RB6-8C5 clone), CD19 (1D3 clone), B220 (RA3-6B2 clone), CD45.1 (A20.1.7), or CD45.2 (AL1-4A2). Antibodies were purchased from PharMingen or eBioscience. Procedures employed for cell staining and FACS analysis have been described previously (So et al. 2004). DNA content analysis was performed by PI staining and analyzed by FACS.

Immunoblotting

Transiently transfected Phoenix cells were harvested and lysed in 250 μL of 2× sample buffer. Proteins from ~20 μL of lysate were fractionated by electrophoresis through 4% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Bio-Rad) using Tris-glycine sodium dodecyl sulfate transfer buffer. After blocking with 5% milk, membranes were incubated with monoclonal antibody N4.4 directed against an *MLL* N-terminal epitope, and then processed for chemiluminescent detection. HA-tagged Meis1a proteins were detected using the M2 anti-HA monoclonal antibody (Abcam). Anti-actin (mouse monoclonal C4) was obtained from Chemicon International.

Real-time quantitative PCR analysis of gene expression

cDNA was synthesized and subjected to real-time PCR as described previously (Yokoyama et al. 2005). TaqMan probes for the following mouse and human genes were purchased from Applied Biosystems: *Meis1* (Mm00487664_m1), *Meis2* (Mm00487748_m1), *Meis3* (Mm00485209_m1), *Pbx1* (Mm00435507_m1), *Pbx2* (Mm00479560_m1), *Pbx3* (Mm00479413_m1), *p19Arf* (Mm01257348), *Bmi1* (Mm00776122_gH), *Cdkn1a* (p21) (Mm01303209_m1), *Cdkn1b* (p27) (Mm00438167_g1), *Cdkn2a* (Mm00494449_m1), *Cdkn2c* (Mm00483243_m1), *Cdkn4b* p15 (Mm00483241_m1), β-*Actin* (Mm00607939_s1); *MEIS1* (Hs00180020_m1), *MEIS2* (Hs00542638_m1), *MEIS3* (Hs00911770_g1), and *ACTB* (Hs99999903 _m1). Primers for mouse *p16Ink4a* were designed previously (Zhang et al. 2003) and purchased from Applied Biosystems. Expression levels of target transcripts relative to that of β-*Actin* were calculated using a standard curve and relative quantitation methods as described in ABI User Bulletin #2. Relative dosages of *Meis*, *Pbx*, and *Hox* transcripts were calculated using relative cycle time (Ct) value according to the manufacturer's instructions.

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