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Isolated Hoxa9 overexpression predisposes to the development of lymphoid but not myeloid leukemia

Sarah H. Beachy[1], **Masahiro Onozawa**[1], **Deborah Silverman**[1], **Yang Jo Chung**[1], **Mariela Martinez Rivera**[1], and **Peter D. Aplan**[1]

[1]Genetics Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

Abstract

Hoxa9 is expressed in hematopoietic stem and progenitor cells although this expression is usually diminished as these cells undergo differentiation. In addition, aberrant expression of *Hoxa9* is strongly associated with both T-cell and myeloid leukemia in mice and humans. Despite this strong association, enforced expression of *Hoxa9* in murine bone marrow or thymus has only shown a modest ability to transform cells. To investigate this question, we used *Vav* regulatory elements to generate a transgenic mouse that targets *Hoxa9* overexpression to all hematopoietic tissues. High level expression of the Hoxa9 transgene in the hematopoietic compartment was associated with embryonic lethality, as no pups from founders that expressed high levels of the transgene were born live. However, offspring of an additional founder line, which expressed lower levels of *Hoxa9*, developed a precursor T cell lymphoblastic leukemia/lymphoma (pre-T LBL), accompanied by spontaneous *Notch1* mutations. In contrast to most murine models of leukemia associated with *Hoxa9* overexpression, the *Vav-Hoxa9* mice did not overexpress other *Hoxa* cluster genes, mir196b (a microRNA which is embedded in the *Hoxa* locus), *Meis1*, or *Pbx3*. The *Hoxa9* transgenic mouse reported here provides a suitable system for the study of *Hoxa9* collaborators that drive myeloid and lymphoid malignant transformation.

Keywords

Hoxa9; pre-T LBL; embryonic lethal; *Notch1*

Introduction

The clustered homeobox (*HOX*) genes encode a family of evolutionarily conserved transcription factors that are required for assigning segment identity during embryonic development [1, 2]. The *Hox* genes are organized into four clusters (A–D) which contain up to 11 genes each. A subset of *Hox* genes, including *Hoxa9*, are expressed in hematopoietic stem and progenitor cells (HSPCs); this expression is typically extinguished as these cells differentiate and mature [3–6]. Experiments with *Hoxa9*-deficient mice have implicated *Hoxa9* in HSC self-renewal and in lympho- and myelo-poiesis, as disruption of this gene impairs normal hematopoietic proliferation and repopulation [7, 8].

Corresponding author: Peter D. Aplan, MD, Genetics Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, 41 Center Drive, Bethesda, MD 20892, USA, Telephone: 301-435-5005, Fax: 301-496-0047, aplanp@mail.nih.gov.

A role for *Hoxa9* in the development of acute myeloid leukemia (AML) was initially recognized in mice. Using retroviral insertional mutagenesis, *Hoxa9*, along with *Meis1*, which encodes a homeodomain protein that binds Hoxa9 [9], were identified as common retroviral insertion sties in mice that developed AML [10]. Subsequently, clinical studies showed that a NUP98-HOXA9 fusion protein was expressed as a result of a t(7;11) translocation that is present in a subset of AML patients [11]. In addition, several studies have noted that *HOXA9* is overexpressed in about half of all AML patients, and is correlated with poor prognosis [11, 12]. Finally, it has recently been demonstrated that several classes of leukemogenic fusion genes, including those involving the *MLL* [13] and *NUP98* genes [14], as well as the CALM-AF10 fusion [15], lead to over-expression of *HOXA9*, that is often, but not invariably, accompanied by overexpression of *MEIS1*.

In addition to myeloid diseases, *HOXA9* expression is also involved in a subset of lymphoid malignancies, most prominently precursor T-cell lymphoblastic leukemia/lymphoma (pre-T LBL). *HOXA9*, along with *MEIS1*, is overexpressed as a target gene in pre-T LBL samples bearing *MLL* translocations [16–18]. Similarly, *HOXA9* and *MEIS1* are overexpressed in patients with pre-T LBL and a CALM-AF10 fusion [19] and the *HOXA* cluster is deregulated in patients that have a chromosome translocation involving the *HOXA* locus [20]. In addition, *NUP98-HOXD13* (*NHD13*) and *NUP98-PHF23* transgenic mice that develop pre-T LBL also overexpress *Hoxa9* [21, 22].

Given the strong correlation between *Hoxa9* overexpression and malignant transformation, it is somewhat surprising that mice that overexpress *Hoxa9* demonstrate only a modest predisposition to AML. Bone marrow transduction experiments that used retroviral constructs to overexpress *Hoxa9*, showed that overexpression of *Hoxa9* alone was only weakly transforming, and that Hoxa9 cofactors E2a-Pbx1a or Meis1a were needed to accelerate the onset of AML [9, 23]. An impact on hematopoiesis was noted however, in that transplantation of bone marrow transduced with a *Hoxa9* construct resulted in increased myelopoiesis and decreased B cell lymphopoiesis [24]. Transgenic mice that expressed *Hoxa9* targeted to B and T lymphocytes through the use of the T cell receptor (TCR) V_{β} promoter and an immunoglobulin enhancer did not develop hematologic malignancy over an 18 month observation period [24]. It is not clear why the transforming ability of *Hoxa9* in these assays was modest. One possibility is that the *in vitro* culture of bone marrow during the transduction procedure may have selected against transformed cells, which died prior to transplantation. However, this possibility would not explain an absence of leukemia after targeting expression of *Hoxa9* to T lymphocytes in transgenic mice.

Because of the strong correlation between *HOXA9* expression and both AML and pre-T LBL, but a lack of acute malignancy observed in mouse models that overexpress *Hoxa9*, we sought to further investigate the role of this gene in mice. Using *Vav* regulatory elements, we generated a transgenic mouse that targets *Hoxa9* overexpression to all hematopoietic tissues. This study reveals that *Hoxa9* overexpression in mice leads to pre-T LBL that is accompanied by mutations in *Notch1* and a lack of overexpression of other *Hoxa* genes, making this an attractive system for the study of *Hoxa9* collaborators that drive malignant transformation.

Materials and Methods

Transgenic mice

To establish targeted expression in hematopoietic tissues, a murine *Hoxa9* cDNA was generated by RT-PCR using primers designated "*Hoxa9* cDNA generation" and listed in Table S1, corresponding to nucleotides 1189-2272 of NM_010456, plus 821 additional nucleotides of *Hoxa9* 3′UTR from BC055059. The resultant *Hoxa9* cDNA was completely

sequenced to verify the absence of PCR errors, and inserted into the HS 21/45-vav vector which contains 3′ and 5′ *Vav* regulatory elements [25] (Figure 1A), as previously described [26]. The purified *Vav-Hoxa9* construct was microinjected into zygotes from C57BL/6 mice. Malignant tumors that developed in the mice were diagnosed using the Bethesda proposal for lymphoid neoplasms in mice classification [27]. Complete blood counts (CBCs) were obtained every 2–4 months using blood collected from the tail vein. All animal studies were approved by the NCI Intramural Animal Care and Use Committee guidelines.

Flow cytometry, immunohistochemistry, and immunoblots

Single cell suspensions of 1×10^6 cells were prepared in Hank's Balanced Salt Solution supplemented with 2% fetal bovine serum (HF2) and incubated with antibodies to B220 (RA3-6B2, BD Pharmigen, BD, San Jose, CA USA), CD3 (145-2C11, eBioscience, eB, San Diego, CA, USA), CD4 (GK1.5, BD), CD8 (52-6.7, BD), CD25 (3C7, BD), CD44 (IM7, BD), CD71 (R17217, eB), c-Kit (2B8, BD), Gr-1 (RB 6-8C5, BD), Mac1 (M1/70, eB), Sca-1 (D7, BD), StemSep Mouse Hematopoietic Progenitor Cell Enrichment Cocktail-biotin (Stem Cell Technologies, Vancouver, BC, Canada), and Ter-119 (TER-119, BD) that were conjugated to FITC, PE or APC for 30 min on ice, washed with PBS, resuspended in 1μg/ml propidium iodide to assess viability, and analyzed on a FACScan (BD). Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin (H&E), CD3 (MCA1477, AbD Serotec, Raleigh, NC, USA), or B220 (553086, BD). For immunoblots, 40μg of whole protein extract (RIPA lysis buffer, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were separated on a 10% Tris-glycine gel (Life Technologies, Grand Island, NY, USA) and transferred to 0.2μm PVDF membrane (Millipore). The membrane was blocked with 5% non-fat dry milk in PBS-Tween-20 (0.05%), incubated with primary [Hoxa9 (Millipore 07-178, Billerica, MA, USA) or beta-Actin (Cell Signaling 4967, Danvers, MA, USA)], and secondary antibodies and then with ECL substrate (Pierce, Rockford, IL, USA) before exposure to film.

Gene expression assays

Total RNA was isolated using TRIzol (Invitrogen, Grand Island, NY, USA) reagents and protocols. First strand cDNA for reverse transcriptase polymerase chain reaction (RT-PCR) and real time quantitative PCR (RQ-PCR) was generated from 1 ug of total RNA using SuperScript III (Invitrogen) reagents and protocols. Expression of the *Hoxa9* transgene was confirmed by conventional RT-PCR (see Table S1 for primers). Quantitative expression of *Hoxa5* (Mm00439362_m1) *Hoxa7* (Mm00657963_m1) *Hoxa9* (Mm00439364_m1), *Hoxa10* (Mm00433966_m1), *Meis1* (Mm00487664_m1), and *Pbx3* (Mm00479413_m1) was measured using TaqMan primer-probe sets and the 7500 Fast Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA). For *Hoxa9* TaqMan assays, the RNA was DNase-treated with a DNA-free kit (Ambion/Life Technologies, Grand Island, NY, USA). Samples were normalized to 18S rRNA endogenous control (4308329) and calibrated to a wild type tissue unless otherwise indicated. TaqMan microRNA assays (Applied Biosystems) were used to assess miR-196b (121219) expression. cDNA was transcribed from 10ng RNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and U6 snRNA (001973) was used as an endogenous control.

OP9-DL1 co-culture

The $CD4^-CD8^-$ subset of thymocytes was isolated by negative selection from the whole thymus using biotinylated antibodies (BD Biosciences) and anti-biotin microbeads with LS columns (Miltenyi Biotec, Auburn, CA, USA). 1×10^5 double negative thymocytes were plated onto an OP9-DL1 monolayer and cultured in αMEM media with 20% FBS, 5ng/ml Flt3L and 1ng/ml IL7 (PeproTech, Rocky Hill, NJ, USA). Cultures were fed every 2 days and passaged every 4 days as previously reported [28].

Myeloid CFC culture

Bone marrow was harvested from mouse femorae and tibiae by flushing the bones with HF2 and plating 3×10^4 cells per 35mm dish in MethoCult M3231 (StemCell Technologies) supplemented with 100ng/ml GM-CSF, 10ng/ml IL3, 10ng/ml IL6, and 20ng/ml SCF (PeproTech). Colonies were counted and replated every 7 days at 1×10^4 cells per plate.

Meis1 transduction of bone marrow

Lineage negative (Lin-) cells were isolated from bone marrow using the StemSep Mouse Hematopoietic Progenitor Cell Enrichment Kit (StemCell Technologies) and used as target cells. Lin- cells were prestimulated *in vitro* for 2 days at 37°C in IMDM with 15% FBS and 6ng/ml mIL3, 10ng/ml hIL6 and 100ng/ml mSCF (PeproTech) until infection. To generate retroviral stocks, Phoenix-Eco cells were transfected using calcium phosphate precipitation with the Meis1/MIYR (MSCV-IRES-YFP-Retrovirus) or control (MIYR) vector plasmids as described in Pineault et al [29]. Virus-containing media was collected 48–72 hrs post transfection and used to infect Lin- bone marrow cells 3 times at 12 hr intervals. Two days following the first infection, infected Lin- bone marrow was harvested and plated in Methocult media (M3231, Stemcell Technologies) supplemented with mSCF (20ng/mL), hIL-6 (10ng/mL), mGM-CSF (10ng/mL) and mIL-3 (10ng/mL) for colony forming cell (CFC) assays. To evaluate the self renewal activity of the transduced cells, CFC cells were harvested and re-plated in M3231 supplemented with the same cytokine cocktail. Colonies were counted and the immunophenotype of the CFC were determined by FACS every 7 days after re-plating (5×10^3 or 1×10^4 cells per 35mm dish were re-plated each time).

Tcrb gene rearrangements and candidate gene resequencing

The *Tcrb* region was amplified from cDNA with using a constant (C) region primer and a degenerate variable (V) region primer (Table S1) and previously reported cycling parameters [21]. PCR products were gel purified (Qiagen, Valencia, CA, USA), subcloned into a pGEM T-easy vector (Promega, Madison, WI, USA), and transformed into competent DH5α *E. coli*. Plasmid DNA was isolated using Qiagen reagents and protocols and sequenced with an ABI 3130 Genetic Analyzer. The sequences were compared to reference mouse *Tcrb* genomic sequences (GenBank ID MMAE00063.1, MMAE00064.1, MMAE00065.1); V, D (diversity), J (joining) and "N" (non-templated) region nucleotides were identified to determine clonality. *Notch1*, *Bcl11b*, *Fbxw7*, and *Pten* were examined for mutations using the primers listed in Table S1. Notch1 sequences were compared with GenBank AL732541.11. Genomic DNA was used as a sequencing template for all genes except *Pten* when a cDNA template was used.

Statistical analysis

The Student's t test was used to determine statistical significance unless stated otherwise.

Results

Enforced expression of *Hoxa9* leads to perinatal death in mice

Following pronuclear injection and implantation into pseudopregnant mothers, potential *Hoxa9* founders were identified by Southern blotting, and bred with WT mice. Four independent lines (H6, I2, F7 and X1) were studied. Surprisingly, the F7 and H6 founders did not transmit the transgene despite delivering 3 litters each, containing a combined total of 16 and 15 pups, respectively. Litters from the H6 and F7 lines were smaller than those from either the X1 line or from an independent line of mice that was also generated using the *Vav* vector backbone (*NHD13*; Figure 1B left panel) [30]. Furthermore, the pups from the H6 and F7 litters were all negative for the *Hoxa9* transgene, whereas the X1 and I2 lines

did transmit the transgene to offspring, suggesting that the *Hoxa9* transgene was embryonic lethal in the H6 and F7 lines (Figure 1B, right panel). The H6 and F7 founders (males) were bred with WT female mice, and the pregnant mice were euthanized. Although H6 did not produce any additional litters during these timed matings, three timed matings from the F7 founder were analyzed. Between days E8 and E9.5, 38% and 18% of the litters, respectively, were positive for the *Hoxa9* transgene, however, none of seven mice analyzed at E16 were positive for the transgene, consistent with the hypothesis that expression of the *Hoxa9* transgene resulted in embryonic lethality (Figure 1C).

Despite the observation that transmission of the *Hoxa9* transgene was associated with embryonic lethality in offspring of the F7 and H6 founders, the I2 and X1 founders did successfully transmit the transgene to viable pups. In order to determine if there was a correlation between expression level of *Hoxa9* and the ability of the founders to transmit the transgene, expression of the *Hoxa9* transgene was assessed by RQ-PCR. Interestingly, *Hoxa9* expression level for the F7 founder was the highest among the lines assayed, and at least 24 times higher than that of the X1 line, indicating that higher levels of *Hoxa9* expression was associated with embryonic lethality (Figure 1D).

Expression of *Hoxa9* in hematopoietic tissues (bone marrow (BM), spleen and thymus) in offspring of the X1 line was confirmed by the presence of a 271bp *Hoxa9* band, indicating that a splicing event took place to remove the SV40 intron (Figure 1E, arrowhead). Western blot analysis demonstrated clear expression of the Hoxa9 protein in the F7 thymus, lesser expression in the X1 thymus, and no expression in the WT thymus, WT BM, or X1 BM (Figure 1F). These levels of Hoxa9 protein expression were entirely consistent with the *Hoxa9* mRNA expression shown in Figure 1D, in which the ratio of F7 thymus: X1 thymus: X1 BM was 330: 9: 1 (Figure 1D and 1F).

Hematologic characterization of tissues from *Hoxa9* transgenic mice

Complete blood counts (CBC) were monitored over 10 months for indications of malignant transformation in the I2 and X1 lines, however, these offspring were healthy and had normal CBCs (Supplemental Figure S1A). Although the I2 line transmitted the transgene, the level of expression was lowest of the lines studied (Figure 1D) and offspring from this line did not demonstrate any clinical signs of illness during the 18 month observation period. Therefore, we focused our subsequent studies on the X1 line. Immunophenotypic analysis of peripheral blood and spleen showed no differences in granulocyte, $CD4^+$ T, $CD8^+$ T, or B lymphoctes between the Hoxa9 (X1 line) and WT mice (Supplemental Figure S1B–C). To determine whether any differences in precursor cell populations could be identified, we assayed thymocyte subsets and BM cells, and again detected no differences between the Hoxa9 and WT mice (Supplemental Figure S1D–E).

Hoxa9 has been implicated in the regulation of granulocyte-macrophage progenitor proliferation [8], and retroviral transduction of a *Hoxa9* cDNA has been reported to immortalize hematopoietic precursors *in vitro* [31–33]. To investigate the possibility that *Hoxa9* overexpression from the X1 BM would result in increased colony formation and re-plating ability *in vitro*, whole BM was cultured in methylcellulose media under conditions to promote myeloid colony growth. In 2 of 3 independent experiments, the *Hoxa9* bone marrow re-plated one more time than the WT BM; however, these cells were mast cells as defined by expression of CD16 and c-Kit (Supplemental Figure S2 and data not shown). These observations demonstrated that expression of the *Hoxa9* transgene, driven by *Vav* regulatory elements, did not increase the re-plating potential in this assay.

Hoxa9 mice develop pre-T LBL

The X1 mice were observed over a period of 18 months along with age-matched WT littermates. During that time, 4 of 18 (22%) mice in the *Hoxa9* group developed a T cell malignancy with the initial signs of illness characterized by hunched posture and weight loss (Figure 2A). These findings were statistically significant and the malignancies were classified as pre-T LBL according to the Bethesda protocols for lymphoid leukemia in mice [27]. The mean age of death among the 4 mice with pre-T LBL was 13.4 months and all exhibited clonal *Tcrb* gene rearrangements (Tables 1 and 2). The disease presentation varied; mouse 1077 had pleural effusion, thymoma and a CD4 single positive clonal population, whereas 1118 and 1138 had hepatosplenomegaly and lymphadenopathy but no pleural effusion (Table 1). Mouse 1125 had a thymoma and an expansion of single positive CD8 cells (Table 1). Common to all of these mice were heavily-infiltrated areas of the lung, often perivascular as revealed with immunohistochemistry for CD3 (Figure 2B). Of the mice that presented with thymomas, 1077 and 1125 demonstrated a sheet of monomorphic cells in the thymus with no demarcation between cortex and medulla (Figure 2B). Hoxa9 protein in these tumors was expressed at high levels (Figure 2C). The bone marrow from mice 1118 and 1138 was infiltrated with CD3 positive T cells, with the BM of mouse 1118 being more heavily infiltrated than that of 1138.

Due to the latency period prior to disease presentation, we hypothesized that there may be collaborative mutations in the pre-T LBL tumors that contribute to malignant transformation. Therefore, these tumors were assessed for *Notch1, Bcl11b, Fbxw7*, and *Pten* mutations as these are commonly associated with T cell leukemias. All 4 tumors had a mutation in either the heterodimerization or PEST domains of *Notch1*, consistent with the diagnosis of pre-T LBL (Table 3). Additionally, mouse 1138 had a *Pten* mutation that resulted in a hydrophobic amino acid substitution for a hydrophilic amino acid (Table 3).

Clinically healthy *Hoxa9* mice from the X1 line were assessed for evidence of T cell abnormalities, including clonal *Tcrb* gene rearrangements and impaired thymocyte differentiation. Mice ranging from 2 to 8 months of age showed no evidence of clonal Tcrb gene rearrangements (Supplemental Table S2). When $CD4^{-}CD8^{-}$ thymocytes were isolated from *Hoxa9* transgenic mice and cultured on a layer of OP9-DL1 cells [28], their differentiation was not distinct from that of WT thymocytes (Supplemental Figure S3).

Expression of Hoxa cluster genes in the pre-T LBL samples

A number of genetically engineered mice that express leukemic fusion genes, such as *MLL-AF9, CALM-AF10*, *NUP98-HOXD13*, and *NUP98-PHF23* [16, 21, 26] (S. Gough and P.D. Aplan, unpublished observations) overexpress genes from the *Hoxa* cluster in concert, most commonly *Hoxa5-11*. In addition, mir 196b, located between *Hoxa9* and *Hoxa10*, has been noted to be upregulated in human and murine leukemias associated with *Hoxa9* overexpression [34, 35]. In order to determine whether the pre-T LBL tumors demonstrated a global upregulation of the *Hoxa* cluster genes, expression of *Hoxa5-10* and mir196b was assessed. As shown in Figure 3, the *Hoxa9* pre-T LBL samples invariably showed an increase in *Hoxa9* compared to WT thymus, but did not show an increase of any of the other *Hoxa* cluster genes or mir196b, with one exception (1138 *Hoxa10* expression).

Role of *Meis1* in pre-T LBL and myeloid immortalization

In order to determine the potential involvement of known *Hoxa9* cofactors in the pre-T LBL tumors, the expression of *Meis1* and *Pbx3* was analyzed. *Meis1* expression was either similar to or lower than that of WT thymus, suggesting that *Meis1* over-expression does not play a crucial role in *Hoxa9*-induced pre-T LBL (Figure 4A) [9]. *Pbx3* overexpression has been associated with myeloid and thymocyte transformation driven by a *NUP98-HOXD13*

transgene [36, 37]. Tumors 1118 and 1138 expressed modestly elevated levels of *Pbx3* compared with the WT control, but the other 2 tumors did not (Figure 4B).

Surprisingly, *Hoxa9* mice developed only pre-T LBL and not AML during the 18 month study. In order to determine whether this could be attributed in part to insufficient expression of Meis1, a Hoxa9 binding partner that is often co-expressed in AML samples that express Hoxa9 and has been shown to collaborate with Hoxa9 during leukemic transformation [3, 38], an *in vitro* immortalization assay was performed [39]. *Hoxa9* or WT BM were infected with a *Meis1* or control retrovirus, and the cells were subjected to a re-plating assay as previously described [39]. Consistent with our experiments using non-transduced *Hoxa9* BM, the *Hoxa9* bone marrow transduced with a control vector re-plated only 4 times, whereas the *Hoxa9* + *Meis1* cells continued to re-plate until the experiment ended following the 7th replating (Figure 4C). The *Hoxa9* + *Meis1* cells that continued to re-plate displayed morphology and immunophenotype (Mac1^{+}CD16^{+}Gr1^{-}cKit^{-}) that is consistent with a mixed population of monoblasts and macrophages (Figure 4D). The immortalization of the *Hoxa9* BM cells by the addition of *Meis1* expression suggests that transformation to AML via *Hoxa9* overexpression requires an additional co-factor.

Discussion

Overexpression of *Hoxa9* has been implicated in both AML and pre-T LBL, both in human patients as well as animal models of leukemia [9–11, 30, 40, 41]. Indeed, several specific recurrent chromosomal rearrangements have been associated with in pre-T LBL in humans, including *CALM-AF10, SET-NUP214, MLL-ENL*, and *TCRB-HOXA9* [42–45]. To further delineate the role of *Hoxa9* in malignant transformation, we generated transgenic mice that overexpressed *Hoxa9* in hematopoietic tissues through the use of *Vav* regulatory elements [25]. Unexpectedly, AML was not detected in any of the transgenic mice. Furthermore, although it has previously been reported that overexpression of *Hoxa9* is sufficient to immortalize BM myeloid progenitors [31–33], we were unable to replate the *Hoxa9* BM more than four times (Fig. S2 and 4), indicating that BM from the *Hoxa9* transgenic mice was not immortalized. There are several possible interpretations of this apparent discrepancy. For instance, it may be due to mouse strain differences, the use of similar but not identical cytokine cocktails, or possibly differences in expression levels of *Hoxa9* in those experiments compared to the current study. In addition, these previous reports have assessed the *Hoxa9* effect by introducing *Hoxa9* using retroviral vectors. It is possible that retroviral integrations activated host genes that collaborated with *Hoxa9* to allow immortalization. Consistent with this hypothesis, one of these previous reports [31] demonstrated that progenitors immortalized by *Hoxa9* were clonal as opposed to polyclonal; if *Hoxa9* was sufficient to immortalize the progenitors, then the immortalized culture might be expected to be polyclonal.

A subset of the *Hoxa9* mice from the X1 line developed a pre-T LBL that was characterized by clonal *Tcrb* gene rearrangements, perivascular infiltration of parenchymal tissues, and invasion of the bone marrow and thymus, the latter leading to disruption of the histological architecture that defines the corticomedullary regions (Table 2 and Figure 2B). In addition, all of the Hoxa9 pre-T LBL samples had spontaneously acquired *Notch1* mutations, which are commonly associated with pre-T LBL in both mice and humans [46–49] (Table 3). Given that the pre-T LBL only arose in one line, it is possible that a transgene integration effect could have contributed to the pre-T LBL phenotype. However, the observation that the *Hoxa9* transgene collaborates with *Meis1* to generate an AML-like phenotype (Fig 4) suggests that the X1 integration site did not predispose to a T-cell phenotype. An alternate explanation is that the X1 founder was the only founder that expressed adequate levels of

Hoxa9 to predispose to pre-T LBL, as the only other founder that transmitted the transgene (I2) expressed 10.7-fold lower levels of *Hoxa9* in the thymus (Fig 1D).

A phenomenon by which *Hoxa* genes (*Hoxa5, Hoxa6, Hoxa7, Hoxa9* and *Hoxa10*) are upregulated in concert is characteristic of acute leukemias with *MLL* rearrangements, and has been referred to as the "*Hox* code" [50]. In addition to leukemias with MLL fusions, *Hoxa* cluster overexpression, often, but not invariably accompanied by *Meis1* overexpression, is seen in patients with other recurrent genetic lesions, including *NUP98* fusions, monosomy 7, and *CALM-AF10* fusions. The overexpression of *Hoxa* cluster genes is thought to be a result of the fusion gene driving expression of *Hoxa5-10* genes as downstream targets; in some cases, this has been shown to be associated with persistent epigenetic changes, such as persistent H3K4me3 at the *Hoxa5-10* cluster [51]. In addition, some *MLL* and *NUP98* fusions also upregulate miR196b [35, 37], and S. Gough unpublished observations), a microRNA located between *Hoxa9* and *Hoxa10*; the role of miR-196b overexpression in AML or pre-T LBL remains unclear. In contrast, although *Hoxa9* is reported to positively regulate its own expression, Hoxa9 does not regulate expression of other *Hoxa* genes [50, 52, 53]; this is the most likely explanation for the lack of *Hoxa* cluster gene and miR 196b upregulation in the *Hoxa9* pre-T LBL samples.

We noted a correlation between the level of *Hoxa9* expression, embryonic lethality and incidence of pre-T LBL. Mice from the F7 line expressed the highest level of *Hoxa9* (more than 20 times higher than the X1 line) but died in utero between E9.5 and E16, resulting in an absence of transgenic mice in the litters (Figure 1B–D). Mice from the X1 line expressed the next highest level of the transgene; this line developed pre-T LBL (Figure 1D and Figure 2). The I2 line expressed the lowest level of *Hoxa9*; none of the transgenic mice from this line developed pre-T LBL during the 18 month study (Figure 1D). These observations suggest that there are at least 3 levels of *Hoxa9* expression (e.g. high, moderate, low) that may be used to predict compatibility with life and progression to disease. Above a certain threshold (i.e. I2), *Hoxa9* mice have the potential to develop leukemia (X1) but overexpression beyond that level interferes with embryonic development resulting in embryonic lethality (F7).

Hoxa9 overexpression was not sufficient to cause AML, consistent with findings from retroviral transduction and transplantation experiments [9, 24]. However, in contrast to the retroviral transduction and transplantation experiments, isolated overexpression of *Hoxa9* does predispose mice to develop pre-T LBL. We suspect that the lack of transformation to AML was due to the lack of enforced *Meis1* overexpression in the *Hoxa9* mice. In the case of *MLL* fusions, it has been shown that Meis1 is a critical co-factor for transformation to AML, and *MLL* fusions with the highest levels of *Meis1* transformed significantly more rapidly than did *MLL* fusions which produced lower levels of *Meis1* expression [54]. *NUP98-HOXD13* transgenic mice overexpress *Hoxa* cluster genes; however, they have lower expression of *Meis1* compared to WT mice [37]. Approximately one-third of the *NUP98-HOXD13* mice that transform to AML spontaneously upregulate *Meis1* (34), or undergo spontaneous mutations of *Ras* pathway genes, therefore, we anticipated that some *Hoxa9* mice might likewise up-regulate *Meis1* or other, undefined complementary genes and transform to AML. However, this was not the case, as we detected no cases of AML despite an observation period of 18 months. Transduction of *Hoxa9* bone marrow with *Meis1* led to immortalization in a CFC re-plating assay, indicating that *Meis1* can complement *Hoxa9* in transgenic mice, as has been shown previously in a retroviral transduction and transplantation model [9].

This report demonstrates that overexpression of *Hoxa9* alone, in the absence of overexpression of additional *Hoxa* cluster genes, miR 196b, or Meis1 led to pre-T LBL. The

reason for the prolonged latency and modest penetrance is unclear; perhaps the penetrance would be greater if additional *Hoxa* cluster genes were overexpressed, however, this possibility remains speculative. This *Hoxa9* transgenic mouse, unique in its absence of overexpression of other *Hoxa* cluster genes and *Hoxa9* cofactors, may provide a useful tool for the assessment of required complementary cofactors and evaluation of potential therapeutic targets for AML and pre-T LBL.

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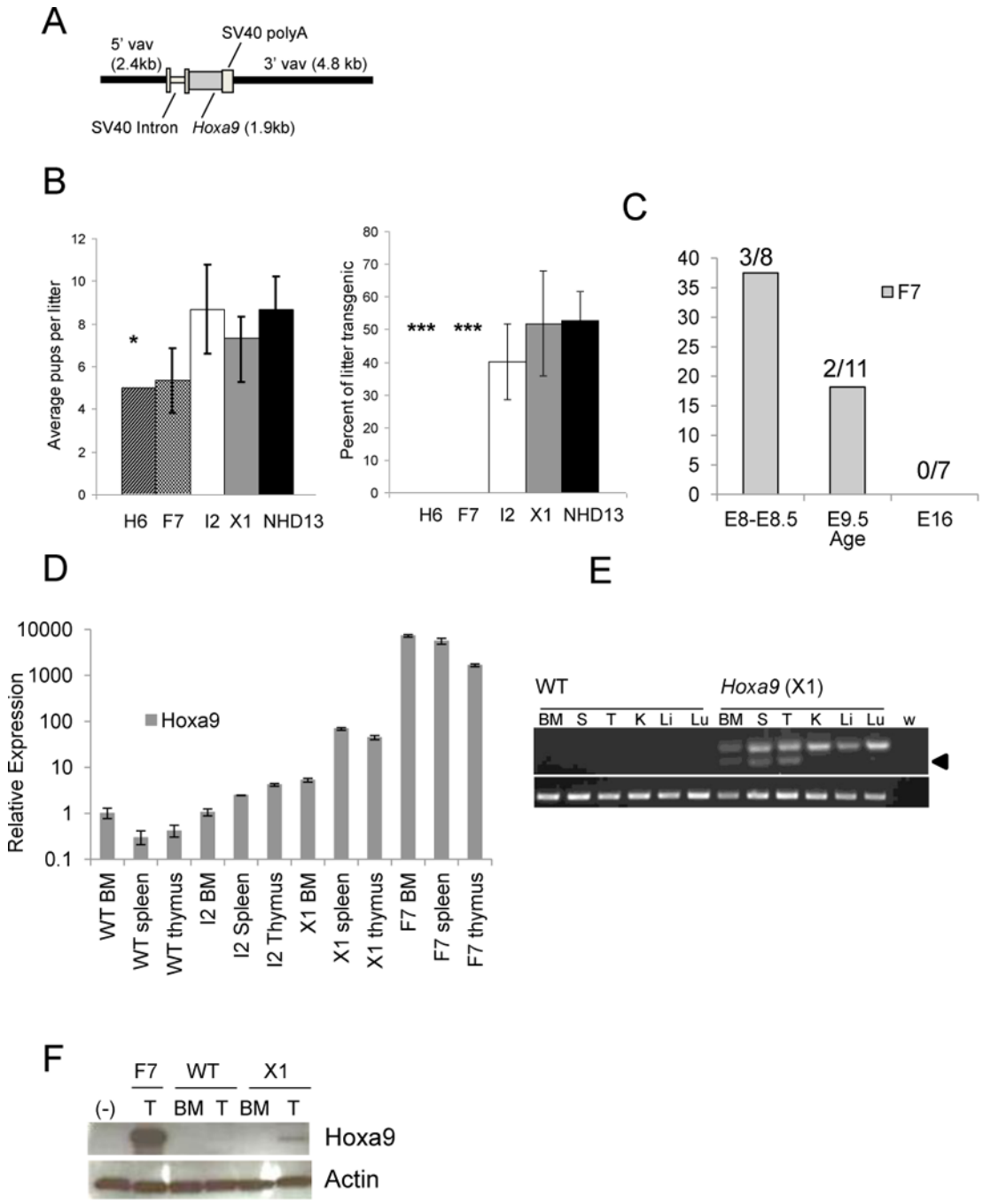

Figure 1. Level of *Hoxa9* transgene expression correlates with transmission of the transgene

A) Schematic of the *Vav* vector containing the mouse *Hoxa9* cDNA sequence. B) Average number of pups born per litter from *Hoxa9* (H6, F7, I2, X1) and *NHD13* [30] WT × transgenic breeding pairs (left panel) and the percent of those litters that had transgenic pups (right panel); statistics as compared with NHD13 group. N = 3 litters per genotype. C) Timed matings of the F7 founder. The female mate was euthanized and embryos were genotyped at the indicated days post conception. Each bar represents a separate litter. D) TaqMan assays were used to determine Hoxa9 expression of mouse tissues from the I2, X1 and F7 lines. Assays were performed in triplicate, normalized to the 18S rRNA, and calibrated to WT BM. E) *Hoxa9* transgene expression in hematopoietic tissues from the X1

line. Upper band (368bp)-contaminating genomic DNA or unspliced mRNA and lower band (271bp)- spliced (indicated with arrowhead) product without the SV40 intron. *Actb* was used as a loading control (bottom panel). BM, bone marrow; S, spleen; T, thymus; K, kidney; Li, liver; Lu, lung; w, water. F) Western blot for Hoxa9 (36kDa) with Actin (45kDa) as a loading control. From the far left, (−) WT brain, F7 thymus (T), WT BM and thymus, X1 BM and thymus. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$.

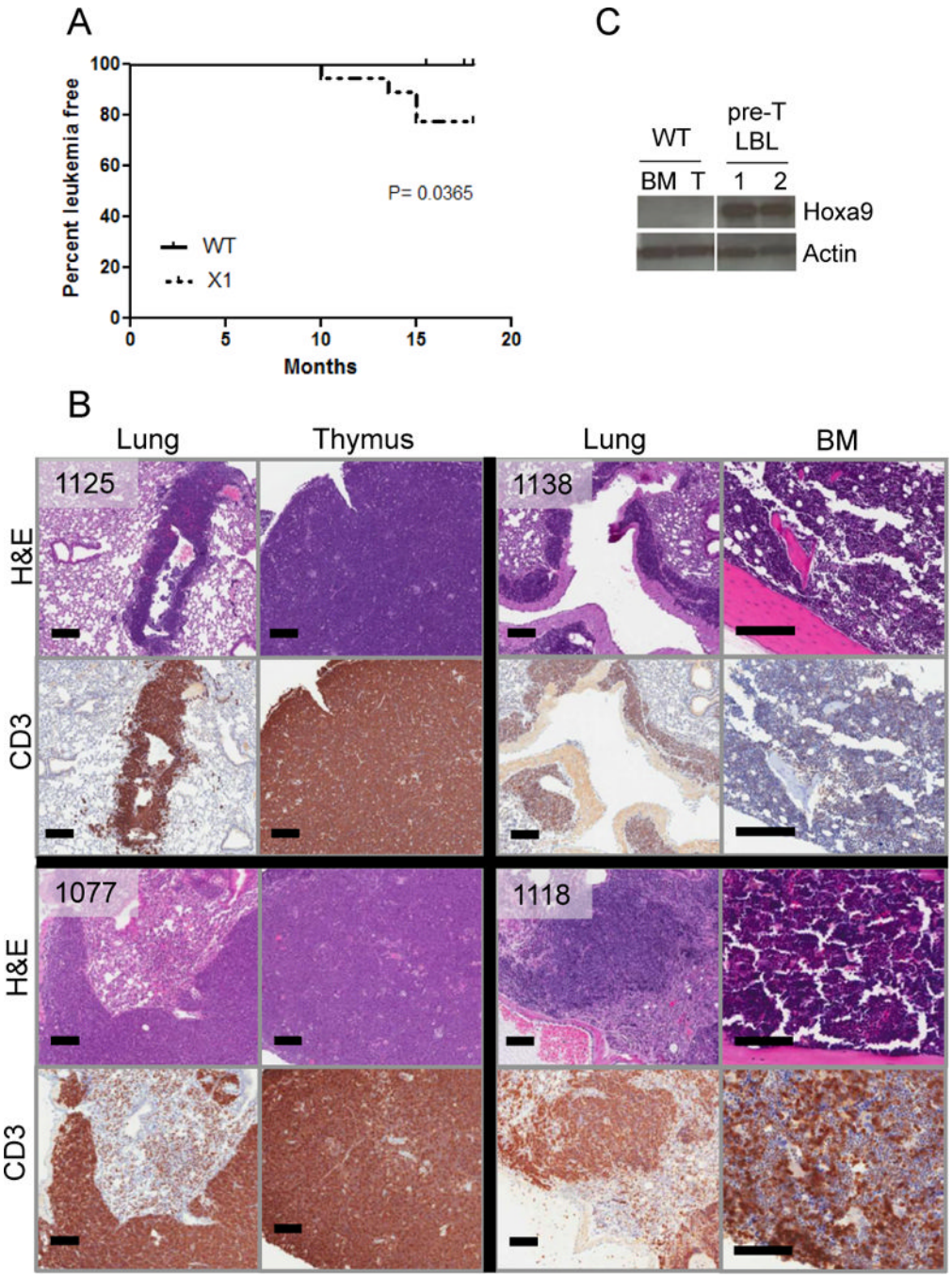

Figure 2. Overexpression of *Hoxa9* results in increased incidence of pre-T LBL in mice

A) A cohort of X1 mice were monitored for disease development over the course of an 18-month period. WT, N = 18 and *Hoxa9*, N = 18. Statistics determined by Log-rank Test. B) H&E staining and immunohistochemistry for CD3. Scale bar indicates 200μm. C) Western blot for Hoxa9 (36kDa) with Actin (45kDa) as a loading control. From the far left, WT BM and thymus, 1–1077 thymus and 2–1125 thymus.

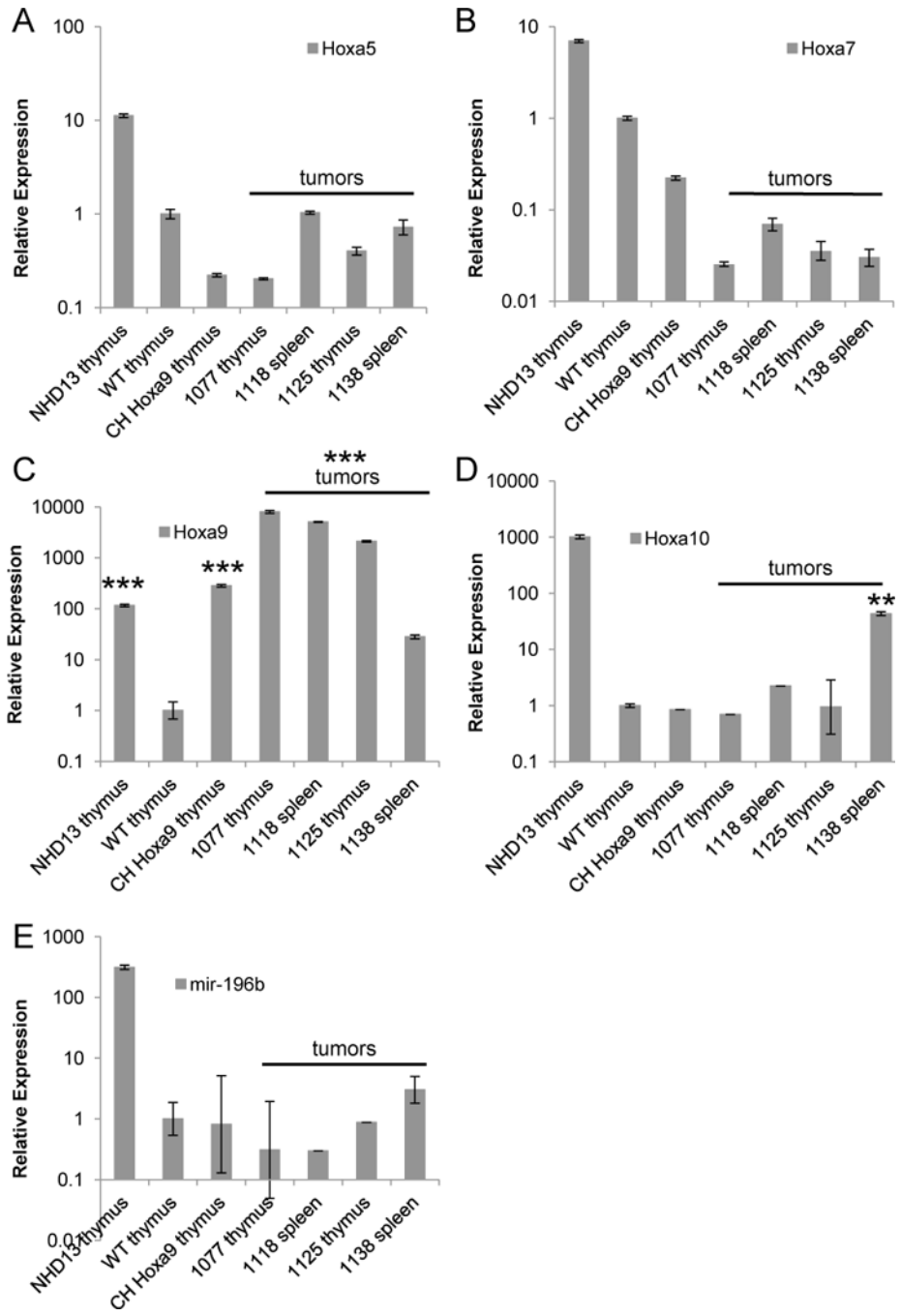

Figure 3. *Hoxa9* T cell tumors lack global overexpression of the *Hoxa* cluster

Quantitative RT-PCR for A) *Hoxa5*, B) *Hoxa7*, C) *Hoxa9*, D) *Hoxa10*, $p \leq 0.01$ compared with WT thymus and E) mir-196b. Thymus from a healthy *NHD13* mouse was used as a positive control and all samples were calibrated to WT thymus set to 1. CH, clinically healthy. Mice 1077, 1118, 1125 and 1138 were diagnosed with pre-T LBL. **$p \leq 0.01$, ***$p \leq 0.005$ by Student's t test.

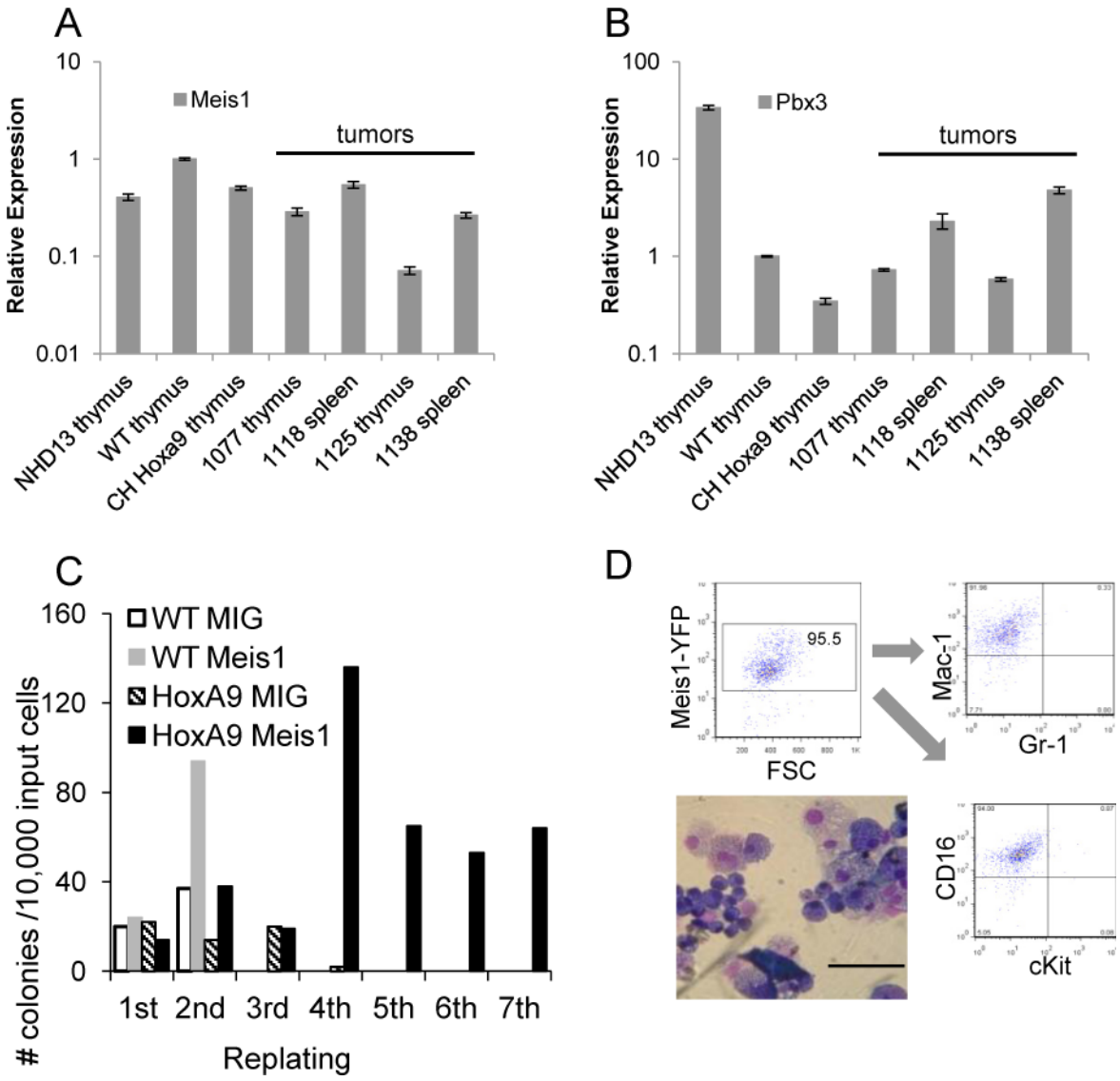

Figure 4. Potential role of *Hoxa9* co-factors in leukemic transformation

Quantitative RT-PCR for A) *Meis1* and B) *Pbx3*. Thymus from a healthy *NHD13* mouse was used as a negative control for *Meis1* and a positive control for *Pbx3*. All samples were calibrated to WT thymus set to 1. CH, clinically healthy. Mice 1077, 1118, 1125 and 1138 were mice with pre-T LBL. C) CFC re-plating assay with WT or *Hoxa9* Lin- bone marrow transduced with empty vector (MIG) or *Meis1*. D) FACS analyses of re-plated transduced cells from (C) to assess for transduction efficiency and immunophenotype at 7th re-plating. Inset photomicrograph of *Hoxa9* + *Meis1* at 7th re-plating stained with May-Giemsa, scale bar = 100μm.

Table 1

Mice from the X1 line with pre-T LBL.

ID	Sex	Age	Gross Findings	IHC Findings	Infiltration (H&E)	CD3	B220	FACS
1077	F	15	PE, S, T	liver	++	++	ND	thymus
				spleen	+++	+++		73% CD4+
				kidney	++	++		
				lung	+++	+++		
				thymus	++	+++		
				bone	+	+		
1118	F	13.5	H, L, S	liver	+++	+++	++	LN
				spleen	+++	+++	+	68% CD3+B220+
				kidney	+	+	−	
				lung	++	++	+	
				LN	+++	++	++	
				bone	++	++	+	
1138	M	10	H, L, S, K	liver	+++	faint staining	ND	spleen
				spleen	+++	across		58% CD4+8+
				kidney	++	all		
				lung	++	tissues		
				bone	+++	examined		
1125	F	15	T	liver	−	−	ND	thymus
				spleen	−	++		61% CD8+
				kidney	−	−		
				lung	++	++		
				thymus	+++	+++		
				bone	+	+		

H, hepatomegaly; K, enlarged/pale kidneys; L, lymphadenopathy; PE, pleural effusion; S, splenomegaly; T, thymoma, ND, not determined

Table 2

Clonal *Tcrb* gene rearrangements in tumors from *Hoxa9* mice.

Subclone	V segment	V	N	D	N	J	J segment
1138spl-4	***V11S1***	TGCAAGCAGCTTAga*	ATCT	GGGGC	GCT	AGTCAAAACACCTTG	***J2S4***
1138spl-5	***V11S1***	TGCAAGCAGCTTAga	ATCT	GGGGC	GCT	AGTCAAAACACCTTG	***J2S4***
1138spl-6	***V11S1***	TGCAAGCAGCTTAga	ATCT	GGGGC	GCT	AGTCAAAACACCTTG	***J2S4***
1138spl-7	***V11S1***	TGCAAGCAGCTTAga	ATCT	GGGGC	GCT	AGTCAAAACACCTTG	***J2S4***
1138spl-10	***V11S1***	TGCAAGCAGCTTAga	ATCT	GGGGC	GCT	AGTCAAAACACCTTG	***J2S4***
1077thy-4	***V6S1***	GTGCCAGCAGTATAg	AGGGTA	GGGA		ctCCTATGAACAAGT	***J2S7***
1077thy-7	***V6S1***	GTGCCAGCAGTATAg	AGGGTA	GGGA		ctCCTATGAACAAGT	***J2S7***
1077thy-3	***V6S1***	GTGCCAGCAGTATAg	AGGGTA	GGGA		ctCCTATGAACAAGT	***J2S7***
1077thy-5	***V6S1***	GTGCCAGCAGTATAg	AGGGTA	GGGA		ctCCTATGAACAAGT	***J2S7***
1077thy-10	***V6S1***	GTGCCAGCAGTATAg	AGGGTA	GGGA		ctCCTATGAACAAGT	***J2S7***
1118LN-1	***V6S1***	GTGCCAGCAGTatag	CCA	GGACAGGGG	TA	TTCTGGAAAATACGC	***J1S3***
1118LN-2	***V8S2***	GTGCCAGCGGTGAtg	AG			ctcctATGAACAGTA	***J2S7***
1118LN-3	***V2S1***	CACCTGCAGTGcaga	GACA	GGGA	GGG	ctcctatGAACAGTA	***J2S7***
1118LN-9	***V8S2***	GTGCCAGCGGTGAtg	AG			ctcctATGAACAGTA	***J2S7***
1118LN-10	***V8S3***	GTGCCAGCAgtgatg	CC	GGACTGGGGGGGC	GC	agtGCAGAAACGCTG	***J2S3***
1118LN-11	***V8S2***	GTGCCAGCGGTGAtg		GG		ctccTATGAACAGTA	***J2S7***
1118LN-12	***V8S2***	GTGCCAGCGGTGAtg	AG			ctcctATGAACAGTA	***J2S7***
1118LN-13	***V2S1***	CACCTGCAGTGCAGa	TCC	GGGACAGGG	TGG	ttcctataATTCGCC	***J1S6***
1118LN-14	***V6S1***	GTGCCAGCAGTAtag	GC	GACTGGGGGGGG		ctccTATGAACAGTA	***J2S7***
1118LN-15	***V8S2***	GTGCCAGCGGTGAtg	AG			ctcctATGAACAGTA	***J2S7***
1125thy-1	***V6S1***	GTGCCAGCAGTATAG	A	GGGA	GG	cAAACACCGGGCAGC	***J2S2***
1125thy-2	***V6S1***	GTGCCAGCAGTATAG	A	GGGA	GG	cAAACACCGGGCAGC	***J2S2***
1125thy-5	***V6S1***	GTGCCAGCAGTATAG	A	GGGA	GG	cAAACACCGGGCAGC	***J2S2***
1125thy-6	***V6S1***	GTGCCAGCAGTATAG	A	GGGA	GG	cAAACACCGGGCAGC	***J2S2***
1125thy-8	***V6S1***	GTGCCAGCAGTATAG	A	GGGA	GG	cAAACACCGGGCAGC	***J2S2***

Table 3

Targeted gene re-sequencing in tumors from *Hoxa9* mice.

Sample ID	Tissue	*Notch1* 5′ del mutation	*Notch1* HD Exon 26	*Notch1* HD Exon 27	*Notch1* JM Exon 28	*Notch1* PEST domain Exon 34	*Fbxw7* Exon 8	*Fbxw7* Exon 9	*Bcl11b* del mutation	*Pten* (RT-PCR)
1077	Thy	GL	GL	GL	GL	**66353CC > TTTG**	GL	GL	GL	GL
1118	Sp	GL	GL	**59855AAGA > TAAG (Lys-Ser > STOP-Gly)**	GL	**66336C > GG**	GL	GL	GL	GL
1125	Thy	**P**	GL	GL	GL	**66282CT > GCG**	GL	GL	GL	GL
1138	Sp	GL	GL	**59724T > C(Leu > Pro)**	GL	GL	GL	GL	GL	**388C > T, Arg > Trp**

Sp: spleen, Thy: thymus

HD: heterodimerization domain, JM: juxtamembrane domain

PEST: Proline/Glutmaic Acid/Serine/Threonine domain

GL, germline; P, positive for 5′ del mutation