# A murine *Mll-AF4* knock-in model results in lymphoid and myeloid deregulation and hematologic malignancy

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**The 2 most frequent human *MLL* hematopoietic malignancies involve either *AF4* or *AF9* as fusion partners; each has distinct biology but the role of the fusion partner is not clear. We produced *Mll-AF4* knock-in (KI) mice by homologous recombination in embryonic stem cells and compared them with *Mll-AF9* KI mice. Young *Mll-AF4* mice had lymphoid and myeloid deregulation manifest by increased lymphoid and myeloid cells in hematopoietic organs. In vitro, bone marrow cells from young mice formed unique mixed pro-B lymphoid (B220$^+$CD19$^+$CD43$^+$sIgM$^-$, PAX5$^+$, TdT$^+$, IgH rearranged)/myeloid (CD11b/Mac1$^+$, c-fms$^+$, lysozyme$^+$) colonies when grown in IL-7– and Flt3 ligand-containing media. Mixed lymphoid/myeloid hyperplasia and hematologic malignancies (most frequently B-cell lymphomas) developed in *Mll-AF4* mice after prolonged latency; long latency to malignancy indicates that *Mll-AF4*–induced lymphoid/myeloid deregulation alone is insufficient to produce malignancy. In contrast, young *Mll-AF9* mice had predominately myeloid deregulation in vivo and in vitro and developed myeloid malignancies. The early onset of distinct mixed lymphoid/myeloid lineage deregulation in *Mll-AF4* mice shows evidence for both "instructive" and "noninstructive" roles for *AF4* and *AF9* as partners in *MLL* fusion genes. The molecular basis for "instruction" and secondary cooperating mutations can now be studied in our *Mll-AF4* model. (Blood. 2006; 108:669-677)**



## Introduction

The myeloid/mixed lymphoid leukemia gene *(MLL)* on human chromosome 11 was first described from a cell line derived from a patient with a hematologic malignancy that resulted from a reciprocal translocation involving chromosome 4.[1] *MLL* was subsequently shown to partner with many other genes to result in hematologic malignancy.[2] The fusion of *MLL* to *AF4* family members, *LAF4* and *AF5*, results in malignancies that are the most common and unique among the *MLL* fusion gene malignancies in that they are generally lymphoid or lymphoid/myeloid in type but rarely purely myeloid. They are also unique because of the high frequency in infants, extensive spread beyond the hematopoietic compartment, and a poor outcome with treatment.[3-10] In contrast to *MLL-AF4*, the fusion of *MLL* with most other partners, including the second most common partner *AF9,*[8] results in myeloid malignancies.

To date, no murine model of *MLL-AF4* translocation has been reported and thus neither the premalignant early events nor the eventual malignancies have been defined. In this study, we produced *Mll-AF4* knock-in (KI) mice and compare them with *Mll-AF9* mice developed previously.[11] The KI models, which have the advantage of having a single copy of the fusion gene in all stem/progenitor cells, permit control of bias introduced by a variable number of gene copies in the various progenitor populations in other models.

The mechanisms for the association between the *MLL* partner gene and type of malignancy have not been elucidated. The *MLL* partner may be "instructive" in directing the selective expansion and transformation of cells toward fusion gene-specific lineages.[12] Some evidence derives from studies in which *MLL-AF4* and *MLL-AF9* had differing effects when introduced into *Drosophila*[13] or a human cell line.[14] Instructed multipotent stem/progenitor cells may have the capacity for either lymphoid or myeloid expansion depending on the *MLL* fusion gene partner. However, it is also likely that some of the effects of the *MLL* fusion genes are partner independent.

Our new *Mll-AF4* model produced deregulation of mixed-lineage progenitor cells (in vitro) and lymphoid and myeloid proliferation (in vivo) in young mice. In contrast, myeloid deregulation predominated in *Mll-AF9* mice. After a long latency period, *Mll-AF4* mice developed mixed lymphoid/myeloid hyperplasia and hematologic malignancies (most frequently B-cell

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J.H.K. designed and directed the research; W.C. was involved in experimental design, colony studies, FACS, and RT-PCR; Q.L. developed the *Mll-AF4* knock-in ES cells and detected IgH rearrangements with PCR and Southern blotting; W.A.H. was involved in mouse genotyping, necropsy, and FACS; A.K. performed real-time quantitative RT-PCR; N.K. conducted pathologic analysis; W.C., Q.L., and W.A.H. performed all the other experiments; and J.H.K., W.C., and Q.L. analyzed data. All the authors were involved in manuscript writing and checking.

W.C. and Q.L. contributed equally to this study.

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An Inside *Blood* analysis of this article appears at the front of this issue.

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lymphomas), whereas myeloid malignancies predominated in *Mll-AF9* mice.

## Materials and methods

### Construction of *Mll-AF4*–targeting vector and generation of targeted ES cells

Mouse gDNA containing the *Mll* locus was isolated from a P1 library by polymerase chain reaction (PCR) screening (Genome Systems, St Louis, MO). Human *MLL-AF4* cDNA (GenBank accession no. L22179) was cloned using standard methods.[15,16] Because the exon 7 of mouse *Mll* gene shares 100% homology with the exon 7 of human *MLL* between the PflM1 site and the breakpoint in RS4;11 cells, the PflM1 site in *Mll* exon 7 was used to generate the in-frame fusion of mouse *Mll* with human *AF4* cDNA. The KI construct contains a 5′ and a 3′ arm from *Mll*, the fragment of human *AF4* cDNA from the breakpoint to the 3′ end, a 953-bp fragment of SV40 with stop signals, and a PGK-Neo cassette (Figure 1A). The targeting vector was linearized by *Xho*I, electroporated into passage 10 of CJ7 embryonic stem (ES) cells (derived from 129/sv mouse strain) obtained from the Mouse Genetics Laboratory, University of Minnesota, Cancer Center, and selected by G418. gDNA of ES cells was purified and digested with *Bam*HI. The N-P1.6 probe used for Southern blotting detects only an 8-kb fragment in wild-type ES cells but both the 12-kb and 8-kb fragment in ES cells carrying the *Mll-AF4* fusion gene.

### Generation of heterozygous *Mll-AF4*$^{+/-}$ mice

Two correctly targeted ES-cell clones were used for injection into C57Bl/6 blastocysts to produce chimeric mice. Before injections, a complete cytogenetic analysis showed a normal karyotype of the ES cells. Male chimeric mice were bred to C57Bl/6 females. Heterozygous offspring were selected by PCR (Table S1, available on the *Blood* website; see the Supplemental Tables link at the top of the online article). These germline offspring were bred on a C57Bl/6 background for 2 generations and then bred to FVB mice to improve fertility and viability of offspring.

The *Mll-AF9* mice were originally produced in the laboratory of Dr Terence Rabbitts (Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom)[11] using the same homologous recombination approach. CCB ES cells (derived from mouse strain of 129/sv) were targeted to produce *Mll-AF9* mice that comprise a KI of the human *AF9* short form (breakpoint to 3′ end) into exon 8 of the mouse *Mll* gene. In summary, both CJ7 ES cells used for *Mll-AF4* and CCB ES cells used for *Mll-AF9* were derived from 129/sv strain. As with the *Mll-AF4* mice, the *Mll-AF9* mice originally were on a C57Bl/6 background. To permit *Mll-AF4* versus *Mll-AF9* experiments controlled for strain differences, we recently crossed *Mll-AF9* C57Bl/6 with FVB mice. There were no significant differences in hematologic analysis (including white blood cell [WBC] count, absolute numbers of immature cells, monocytes, neutrophils, and lymphocytes in blood), colony numbers, and percentage of different type of colonies in colony-forming assays, or malignancies; thus the results reported were combined from C57Bl/6 and C57Bl/FVB hybrid mice.

### Hematologic analysis

Young *Mll-AF4* and *Mll-AF9* mice (5-8 weeks old) and their wild-type littermates were humanely killed. The total WBC counts in peripheral blood were determined by Unopette Micro Collection System (BD Biosciences, San Diego, CA). Blood and bone marrow smears were stained with Wright-Giemsa stain. Differential counts were determined by counting 200 nucleated cells under a microscope. Statistical significance between means was determined by the Student *t* test.

### Bone marrow cell cultures

Single bone marrow cell suspension was cultured in methylcellulose medium under either lymphoid (pro-B) conditions using Methocult 3630 (StemCell Technologies, Vancouver, BC, Canada) supplemented with 50 ng/mL flt3 ligand (R&D Systems, Minneapolis, MN) for the growth of pro-B colonies, or myeloid conditions using Methocult 3534 (StemCell Technologies) supplemented with 10 ng/mL GM-CSF (R&D Systems) for the growth of myeloid colonies. The cells were cultured in triplicate for 3 consecutive generations of 7 to 8 days each. The myeloid colonies were counted and classified into 3 types (I, II, and III), as previously described.[17] Briefly, type I colonies are compact colonies with a dense center and a smooth edge, type II colonies have a compact center and a halo of loose cells, and type III colonies have dispersed cells but no center. Type I colonies are composed mostly of immature myeloid precursors, whereas the cells in type II and type III colonies are progressively more differentiated. The colony containing more than 50 cells was scored. The total number of pro-B colonies was scored and counted as previously reported.[18]

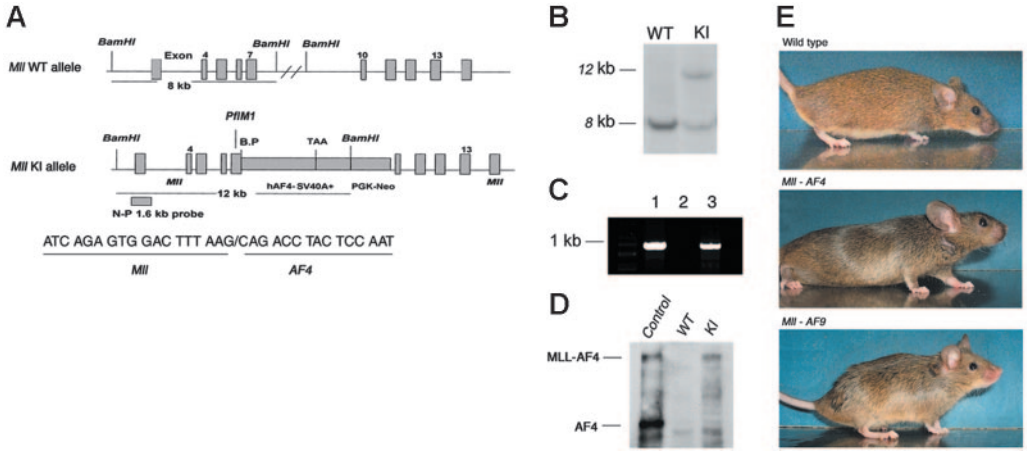


**Figure 1. Construction of the *Mll-AF4* KI allele and characterization of targeted ES cells.** (A) A diagrammatic description of the KI allele. The top map represents the *Mll* wild-type allele, indicating the 8-kb fragment detected by N-P1.6 probe. The bottom map represents the *Mll*-exon7-*AF4*–targeting allele, indicating the position of the *Mll-AF4* breakpoint, PGK-Neo cassette, and a 12-kb fragment detected by N-P1.6 probe in Southern blotting. The *Mll-AF4* breakpoint and junction sequence are also shown. (B) Southern blotting analysis of wild-type and targeted ES cells. The 8-kb wild-type (WT) band and 12-kb KI band are indicated. (C) Detection of *Mll-AF4* fusion gene by PCR. PCR was performed with gDNA from ES cells with KI allele (lane 1), wild-type ES cells (lane 2), and a representative *Mll-AF4* mouse (lane 3). The 5′ primer of *Mll* exon 6 and 3′ primer of human *AF4* amplified a 930-bp *Mll-AF4* fragment in lanes 1 and 3. (D) Expression of Mll-AF4 fusion protein in targeted ES cells detected by Western blotting. The 240-kDa Mll-AF4 fusion protein was detected in targeted ES cells. A cell line transfected with *MLL-AF4* DNA was used as positive control. (E) *Mll-AF4*, *Mll-AF9*, and wild-type mouse at the age of 5 weeks. *Mll-AF4* mice have a shortened face and large ears. *Mll-AF9* mice have a more pointed face and large ears.

### Histopathology and multiparameter FACS analysis

For histopathologic analysis, mice that were terminally ill were humanely killed. Tissues were fixed with 10% formalin. Hematoxylin and eosin (H&E) staining and immunochemistry were performed according to standard protocols. Primary antibodies used here were antimyeloperoxidase (DakoCytomation, Carpinteria, CA), anti-B220/CD45R, anti-CD19 (PharMingen, San Diego, CA), anti-IgM (BioGenex, San Ramon, CA), and anti-PAX5 and anti–Bcl-6 (Santa Cruz Biotechnology, Santa Cruz, CA). Images were processed using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA). After staining with primary antibodies, sections were incubated with biotinylated secondary antibodies and the streptavidin HRP enzyme conjugate (DakoCytomation) or the alkaline phosphatase enzyme system (Biogenex), followed by 3,3′-diaminobenzidine tetrahydrochloride (Dakocytomation), and were counterstained with Mayer hematoxylin. Photomicrographs were taken using a SPOT Insight digital camera along with SPOT Advanced software version 4.0.9 (Diagnostic Instruments, Sterling Heights, MI) under a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). Nikon Plan 2 ×/0.08 numeric aperture (NA) and Plan 40 ×/0.75 NA objectives were used for Figures 7A and 7B-H, respectively.

For flow cytometric analysis, cells were stained with FITC- and PE-conjugated antibodies to CD11b/Mac1, Gr-1, CD43, B220/CD45R, CD19, IgM, and CD3 (PharMingen). Stained samples were acquired by fluorescence-activated cell sorting with a BD FACSCalibur using Cell Quest Pro software. Multiparameter analysis of the data was done with FloJo software (Tree Star, San Carlos, CA).

### Gene expression by RT-PCR

RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA). cDNA was synthesized with SuperSpript first-strand synthesis system for reverse transcription-PCR kit (RT-PCR; Invitrogen) according to the manufacturer's instruction. PCR primers are described in Table S1.

### *Mll-AF4* and *Mll-AF9* expression by real-time quantitative RT-PCR

Quantitative RT-PCR was performed to detect the expression of *Mll-AF4* and *Mll-AF9* gene in young and old diseased mice. Total RNA was isolated from bone marrow cells using the RNeasy kit (Qiagen, Valencia, CA), incorporating on-column DNase digestion. For real-time quantitative RT-PCR, *Taq*Man primer and probe sets were obtained from Applied Biosystems (ABI, Foster City, CA). The probes spanned the *Mll-AF4* and *Mll-AF9* junctions. Quantitative PCR was performed using an ABI prism 7500 sequence detection system. Quantitative analysis was performed using REST (Relative Expression Software Tool).[19]

### Immunoglobulin heavy-chain rearrangements by PCR or Southern blotting

$DJ_H$ rearrangements of the immunoglobulin heavy chain in the cultured cells from colonies under pro-B condition were determined by PCR using 2 upstream degenerate primers (DFL/DSP and DQ52) and one reverse primer. All 3 primers were used in a single PCR to detect rearrangements from $DJ_H1$ to $DJ_H4$.[20]

Immunoglobulin heavy-chain rearrangements in the organs from sick *Mll-AF4* mice were determined by Southern blotting. gDNA was digested with *Eco*RI. Plasmid pBR322 containing mouse IgH J1-J4 sequences was used as the probe.[21] gDNA was also digested with *Hin*dIII followed by Southern blotting to confirm IgH rearrangements.

All the experiments using mice were conducted after approval by the Institutional Animal Care and Research Committee, University of Minnesota.

## Results

### Generation of *Mll-AF4* mice

We constructed a KI vector to fuse the 5′ portion of the murine *Mll* with the 3′ portion of the human *AF4*. Blastocyst injection with targeted ES cells resulted in 14 *Mll-AF4* chimeric mice with more than 70% chimerism as judged by fur color. Two founders (A and D1) showed germline transmission. However, offspring of the founder D1 were not fertile; therefore, all *Mll-AF4* germline mice used in this study were derived from founder A. The organization of the wild-type *Mll* allele and the *Mll-AF4* KI allele were shown (Figure 1A). The breakpoint and junction sequence was confirmed by DNA sequencing (Figure 1A). Southern analysis showed a specific 12-kb band derived from the *Mll-AF4* gene in KI ES clones, different from the 8-kb band from wild-type *Mll* (Figure 1B). The *Mll-AF4* fusion gene was also detected by PCR (Figure 1C). The 240-kDa Mll-AF4 fusion protein was detected in the KI ES cells, as shown by Western blotting (Figure 1D), indicating expression of full-length Mll-AF4 protein.[22]

Expression of the *Mll-AF4* or *Mll-AF9* fusion gene in bone marrow from randomly selected young and old diseased mice was tested using real time quantitative RT-PCR. As expected, all samples tested showed expression of the appropriate fusion gene (Table S2). In both *Mll-AF4* and *Mll-AF9* mice that were older and diseased with lymphoma and myeloid hyperplasia, relative expression levels were lower than in young mice ($P < .01$). The difference was larger among *Mll-AF4* and smaller among *Mll-AF9* mice. Overall, there are no significance differences between *Mll-AF4* and *Mll-AF9* ($P > .05$). However, differences between *Mll-AF4* and *Mll-AF9* may be masked by the "interaction" between age/disease and type of gene as described in Table S2. Additional studies using purified cell populations and larger sample sizes will be necessary to further evaluate potential differences in detail.

### Young *Mll-AF4* and *Mll-AF9* mice have distinctive phenotypic features and different lymphoid and myeloid expansion in vivo

*Mll-AF4* and *Mll-AF9* mice were small at birth compared to wild-type littermates. At 5 weeks of age, *Mll-AF4* mice weighed a mean of 13.0 g, *Mll-AF9* mice 13.6 g, and wild-type littermates 17.4 g. *Mll-AF4* mice had a distinctive appearance, including a shortened blunt face, large ears, and a high-set tail (Figure 1E). *Mll-AF9* mice had faces that were also shortened but pointed rather than square. The ears were also enlarged but less pronounced than those of the *Mll-AF4* animals.

We evaluated the effects of the 2 fusion genes on hematopoietic differentiation in young (5–8-week-old) mice. Elevated WBC counts were found in both *Mll-AF4* mice ($7.2 \times 10^3 \pm 1.2 \times 10^3/\mu L$; n = 10) and *Mll-AF9* ($7.3 \times 10^3 \pm 1.3 \times 10^3/\mu L$; n = 8) as compared to wild-type controls ($3.9 \times 10^3 \pm 1.0 \times 10^3/\mu L$; n = 9). *Mll-AF4* and *Mll-AF9* mice, but not wild-type mice, showed immature cells in blood ($P < .001$; Figure 2A). Notably, *Mll-AF4* mice showed the highest total number of lymphocytes (*Mll-AF4* versus wild type, $P < .01$). A statistically insignificant increase also observed in *Mll-AF9*. The highest number of monocytes was found in *Mll-AF9* mice, although *Mll-AF4* mice also showed an increase compared to wild-type mice ($P < .01$ for all the compared groups). Neutrophils were increased in both *Mll-AF9* and *Mll-AF4* ($P < .001$) but differences between *Mll-AF4* and *Mll-AF9* were not significant.

To further define the role of the *Mll* fusion partners, *AF4* and *AF9,* in hematopoietic differentiation, multiparameter FACS analysis was used to characterize the leukocytes in bone marrow (Figure 2B). The highest percentage of $B220^+$ or $CD19^+$ B-lymphoid lineage cells was found in *Mll-AF4* marrow ($P < .01$, *Mll-AF4* versus wild type), with intermediate level in wild-type and lowest in *Mll-AF9* marrow ($P < .001$, *Mll-AF9* versus wild type). In contrast, $CD11b^+Gr1^+$ cells predominated in *Mll-AF9* marrow ($P < .001$, *Mll-AF9* versus wild type).

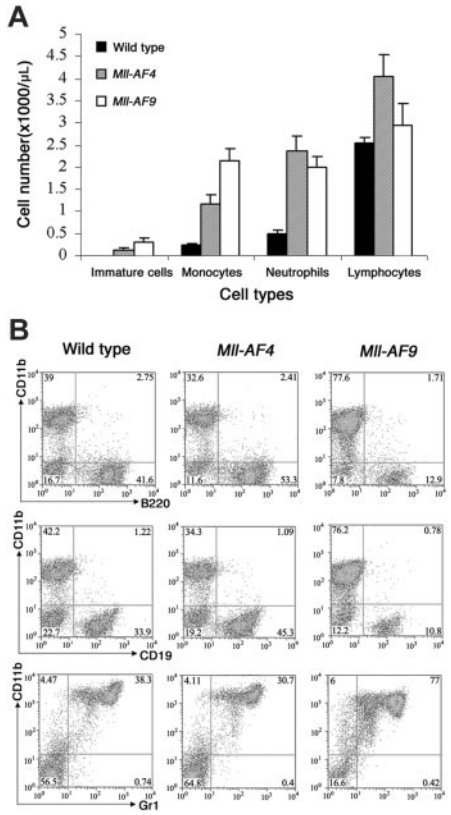


**Figure 2. Cell types in blood and bone marrow of young wild-type, *Mll-AF4*, and *Mll-AF9* mice.** (A) The total numbers of immature cells, monocytes, neutrophils, and lymphocytes in the blood of young wild-type, *Mll-AF4*, and *Mll-AF9* mice. The numbers were calculated by multiplying total WBC counts with percentage of each cell type and expressed as cell numbers per microliter peripheral blood. The error bars represent SEMs. (B) Immunophenotype of nucleated cells in the bone marrow of young *Mll-AF4, Mll-AF9*, and wild-type mice. Phenotypic analysis of bone marrow cells from representative 5- to 8-week-old *Mll-AF4, Mll-AF9*, and wild-type mice was performed by FACS.

### *Mll-AF4* bone marrow from young mice shows lymphoid progenitor deregulation in vitro

Colony assays, which allowed direct observation of cells that have undergone deregulation based on plating abilities over several generations, were performed to evaluate potential differences in young *Mll-AF4* and *Mll-AF9* compared to wild-type mice. Pro-B colonies grown in methylcellulose containing Flt3 ligand and IL-7[23,24] were studied. Results are shown in Figure 3A. Pro-B colony-forming units (CFUs) from *Mll-AF4* mice were significantly higher than those from wild-type and *Mll-AF9* mice beginning in the first generation of plating ($P < .01$). As expected, pro-B colonies were almost undetectable in wild-type mice by the third generation. However, significant numbers of *Mll-AF4* and fewer *Mll-AF9* pro-B colonies were found in the third generation of plating. As shown, the number of pro-B CFUs of the *Mll-AF4* mice was always significantly higher than the one of *Mll-AF9* mice (Figure 3A).

### Mixed pro-B lymphoid ($B220^+$ $CD19^+CD43^+sIgM^-$, $PAX5^+$, $TdT^+$, IgH rearranged)/myeloid ($CD11b/Mac1^+$, $c\text{-}fms^+$, $lysozyme^+$) progenitor cells are produced from marrow culture of young *Mll-AF4* mice

Multiparameter FACS analysis was carried out on pooled cells from the methylcellulose culture of the third generation grown under pro-B lymphoid conditions. Cells from *Mll-AF4* colonies were $B220^+CD43^+$ and coexpressed lymphoid and myeloid-associated markers, $B220^+CD11b^+$ (a representative experiment is shown in Figure 3B). The percentage of mixed-lineage $B220^+$ $CD43^+$ $CD11b^+$ cells in the *Mll-AF4* colonies was reproducibly higher than wild-type and *Mll-AF9* mice.

To further characterize the $B220^+CD43^+CD11b^+$ population from the bone marrow culture of *Mll-AF4* mice, the B-cell–associated markers CD19 and sIgM were also examined by FACS. The result indicated that these cultured bone marrow cells were sIgM negative and about 50% of them were $B220^+CD19^+$ (Figure 4A). RT-PCR was conducted to detect the expression of B-cell markers (PAX5, TdT) and myeloid markers (c-fms, lysozyme). As shown in Figure 4B, these cultured cells were positive for PAX5, TdT, c-fms, and lysozyme. DJH1-DJH4 rearrangements of immunoglobulin heavy chain were also found in this population (Figure 4C). All of these data indicated that this expanded population from *Mll-AF4* bone marrow grown under pro-B condition was positive for both pro–B cell markers ($B220^+$ $CD19^+CD43^+sIgM^-$, $PAX5^+$, $TdT^+$, IgH rearranged) and myeloid cell markers ($CD11b/Mac1^+$, $c\text{-}fms^+$, $lysozyme^+$).

Cells from the third-generation culture were subsequently put in IMDM supplemented with fetal bovine serum, Flt3 ligand, and IL-7 to evaluate the growth potential of the mixed-lineage *Mll-AF4* cells. None of the cells from beyond the third-generation *Mll-AF4* lymphoid cultures could grow for more than 2 months, suggesting that these cells had not attained a fully malignant phenotype. Similarly, no cells from *Mll-AF9* or wild-type mice survived under these lymphoid growth conditions.

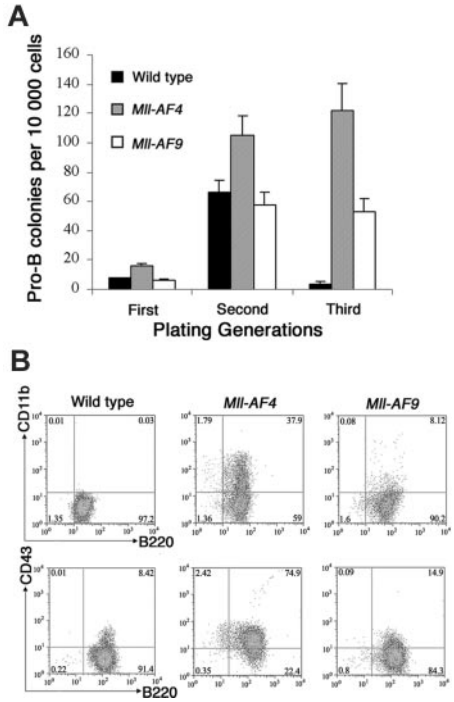


**Figure 3. *Mll-AF4* marrow progenitor cells expressed myeloid and lymphoid markers (B220, CD43, and CD11b) when grown under pro-B lymphoid conditions.** (A) Colonies from young (5- to 8-week-old) mice. Bone marrow cells from wild-type (n = 8), *Mll-AF4* (n = 7), and *Mll-AF9* (n = 8) mice were plated in methylcellulose medium under lymphoid conditions for 3 generations. The error bars represent SEMs. (B) Representative results of multiparameter FACS analysis on pooled cells from the methylcellulose culture of the third generation under lymphoid conditions.

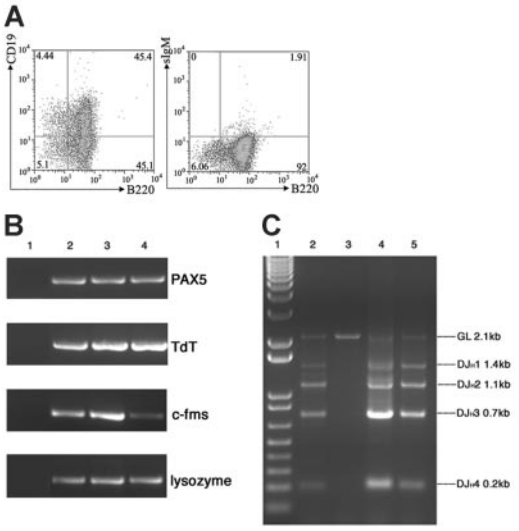


**Figure 4. Marrow cells from *Mll-AF4* mice expressed additional lymphoid (CD19, PAX5, TdT, $D_H$-$J_H$ but not sIgM) and myeloid markers (c-fms and lysozyme) when cultured under pro-B culture conditions.** (A) FACS analysis of B220/CD19 and B220/sIgM. (B) PAX5, TdT, c-fms, and lysozyme expression detected by RT-PCR. Lane 1, $H_2O$ control; lane 2, mouse spleen cells as control; lanes 3-4, bone marrow cells from 2 *Mll-AF4* mice cultured for 21 days. (C) Immunoglobulin $D_H$-$J_H$ heavy-chain rearrangement detected by PCR. Lane 1, molecular weight marker; lane 2, mouse spleen DNA as control; lane 3, mouse kidney DNA as control; and lanes 4-5, DNA of bone marrow cells from 2 *Mll-AF4* mice cultured for 21 days.

### In contrast to *Mll-AF4, Mll-AF9* bone marrow shows predominately myeloid progenitor cell expansion in colony assays

We also studied myeloid colony formation of young mouse marrow in methylcellulose supplemented with GM-CSF, IL-3, SCF, and IL-6 (Figure 5A). Myeloid CFUs from *Mll-AF9* marrow were significantly higher in number than from wild-type and *Mll-AF4* mice beginning in the first generation ($P < .01$). We observed no increase in *Mll-AF4* myeloid colonies in the first generation compared to wild-type mice. As expected, few myeloid colonies persisted in control cultures by the third generation. However, significant expansion of myeloid CFUs persisting through 3 generations was clearly evident in the *Mll-AF9* cultures. Some evidence of myeloid deregulation of *Mll-AF4* marrow was found but much less than that of *Mll-AF9* marrow.

To further characterize cells from myeloid cultures, multiparameter FACS analysis of third-generation cells was carried out (Figure 5B). *Mll-AF9* colonies contained the highest number of $CD11b^+$. Minorities of the cells were also $Gr1^+$. As expected, cells from myeloid cultures were B220 negative in all 3 types of mice (data not shown).

### Type I myeloid colonies are found predominately in *Mll-AF9* mice

Under myeloid conditions *Mll* fusion gene–expressing marrow cells form 3 types of distinct colonies as described previously.[17,25,26] The colony distribution of bone marrow cultures from young *Mll-AF4*, *Mll-AF9*, and wild-type mice under myeloid conditions was compared (Figure 5C). Type I colonies were found in the culture of *Mll-AF9* marrow and these colonies persisted through all generations. The distribution of *Mll-AF4* colonies was similar to wild-type mice in 3 plating generations. Thus, although *Mll-AF4* had some ability to deregulate myeloid cells, the type I colonies, which were primarily composed of immature myeloid cells, were found only in *Mll-AF9* mice.

### *Mll-AF4* mice develop a complex and variable mix of lymphoid and myeloid hyperplasia, B-cell lymphoma, and myeloid malignancies

We observed *Mll-AF4* mice up to 22 months for development of malignancy. Moribund *Mll-AF4* mice showed typical features of severe hematologic disease, rough coat, hunched posture, and abnormal behaviors. Mice that were terminally ill were humanely killed. Kaplan-Meier analysis of the proportion of animals alive and free of disease are shown (Figure 6). The median time to development of hematologic malignancy was 520 days (17 months) for *Mll-AF4* and 220 days (7 months) for *Mll-AF9* mice. No wild-type littermate mice died during the 22-month observation.

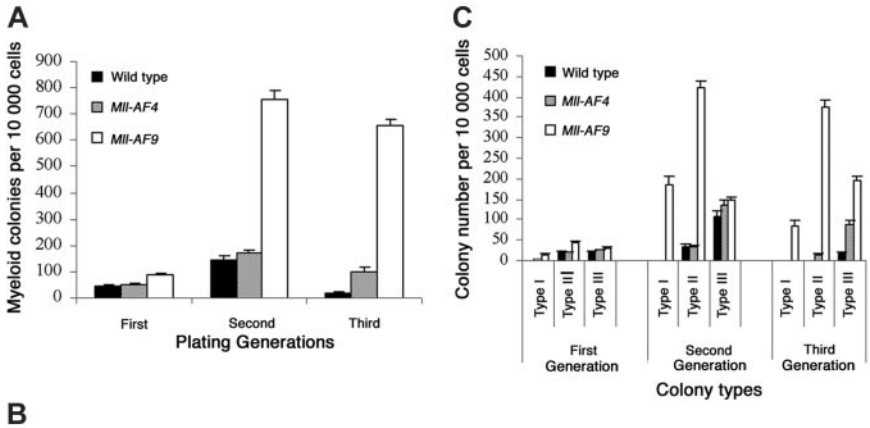


**Figure 5. Bone marrow cultures under myeloid growth conditions.** (A) Myeloid colonies from young mice. Cells from wild-type (n = 6), *Mll-AF4* (n = 7), and *Mll-AF9* (n = 12) mice were plated in methylcellulose medium under myeloid conditions for 3 generations. (B) Multiparameter FACS analysis on pooled cells from the methylcellulose culture of third-generation cells grown under myeloid conditions. (C) Colony distribution of murine bone marrow cultures under myeloid growth conditions. The frequency of myeloid type I, II, and III colonies was determined from young wild-type, *Mll-AF4*, and *Mll-AF9* mice for 3 generations. The error bars represent SEMs.

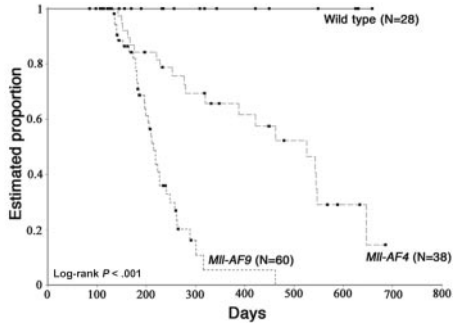


**Figure 6. Kaplan-Meier analysis of the estimated proportion of animals alive and free of hematologic malignancy.** The analysis was done on *Mll-AF4* (n = 38), *Mll-AF9* animals (n = 60), and WT (n = 28). Overall log-rank $P < .001$.

A total of 20 of the 38 *Mll-AF4* mice became sick and died or were killed. WBC counts, cell types in blood, spleen weights, FACS analysis of spleen, and immunohistochemistry were evaluated. Histopathology was reviewed independently by 3 experts in murine hematopathology. Immunoglobulin gene rearrangements and Pax5 expressions were also tested. The Bethesda classification was used for diagnosis in all cases.[27,28] Results of each mouse, including the final diagnosis, are shown in Table S3. The mean WBC count of *Mll-AF4* mice with malignancy was higher than the one of wild-type mice ($10.4 \times 10^3 \pm 0.6 \times 10^3/\mu L$ [n = 15] versus $2.4 \times 10^3 \pm 0.2 \times 10^3/\mu L$ [n = 10]; $P = .01$). When compared to wild-type mice, *Mll-AF4* mice notably showed the presence of blasts and increases in the total numbers of monocytes and neutrophils in blood. All 20 mice had evidence of "benign" lymphoid and/or myeloid hyperplasia in spleen and bone marrow. Spleens were significantly enlarged with a mean 7.3-fold increase in size (means of *Mll-AF4* = 0.51 g compared to 0.07 g in wild-type mice). The histopathology and immunohistochemistry of the spleen and lymph nodes were abnormal. These animals had lymphoid tumors arising from follicular centers (Table S3). Spleen histopathology from one mouse with lymphoid malignancy is shown (Figure 7A-B). The lymphoid malignancy affected spleen, lymph nodes, and often liver, lung, intestine, or kidney but not blood; cells from the spleen and other sites studied by immunohistochemistry had a follicular center B-cell phenotype: $B220^+$ (Figure 7C), Bcl-$6^+$ (Figure 7D), $Pax5^+$ (Figure 7E), $sIgM^+$ (Figure 7F), $CD19^+$. Monoclonal or oligoclonal IgH rearrangements were detected in mice with lymphoma. The rearrangement in the spleen and lymph node from one representative mouse is shown in Figure 7I. The IgH rearrangements were also confirmed by *Hin*dIII digestion followed by Southern blotting (data not shown). The morphologic features combined with the immunophenotypic characteristics of blood and hematopoietic organs and immunoglobulin gene rearrangements resulted in the diagnosis of follicular B-cell lymphoma.[28] Erythroid leukemia was present in one animal (FM1, Table S3). Spleen from mouse ChG2 is shown in H&E-stained (Figure 7G) and myeloperoxidase-stained sections (Figure 7H). This $MPO^+$ myeloid malignancy and that of ChB were classified as MPD-like myeloid leukemia.[29] Myeloid hyperplasia with no evidence of frank malignancy was present in 3 mice (ChD2, FM6m, and FM105). In summary, FACS of splenic cells, WBCs, cell morphology, and histopathology of different organs showed alterations in both lymphoid and myeloid cells in most of the 20 animals that developed fatal disease, indicating these *Mll-AF4* mice had a complex, variable mixed lymphoid (most frequently B-cell lymphoma) and myeloid disease.

Transplantation of spleens and lymph nodes from *Mll-AF4* mice with lymphoma and myeloid hyperplasia resulted in 2 distinct types of fatal malignancy. One was B-cell lymphoma in hematopoietic organs with IgH rearrangements, identical to that in donor *Mll-AF4* mice with lymphoma. The second was fatal myeloid disease, characterized by increased number of $CD11b^+$ $Gr1^+$ $F4/80^+$ myeloid cells in hematopoietic organs.

### *Mll-AF9* mice develop predominately myeloid malignancies

Thirty-seven *Mll-AF9* mice developed hematologic malignancies at a median of 7 months (Figure 6). These malignancies were MPD-like

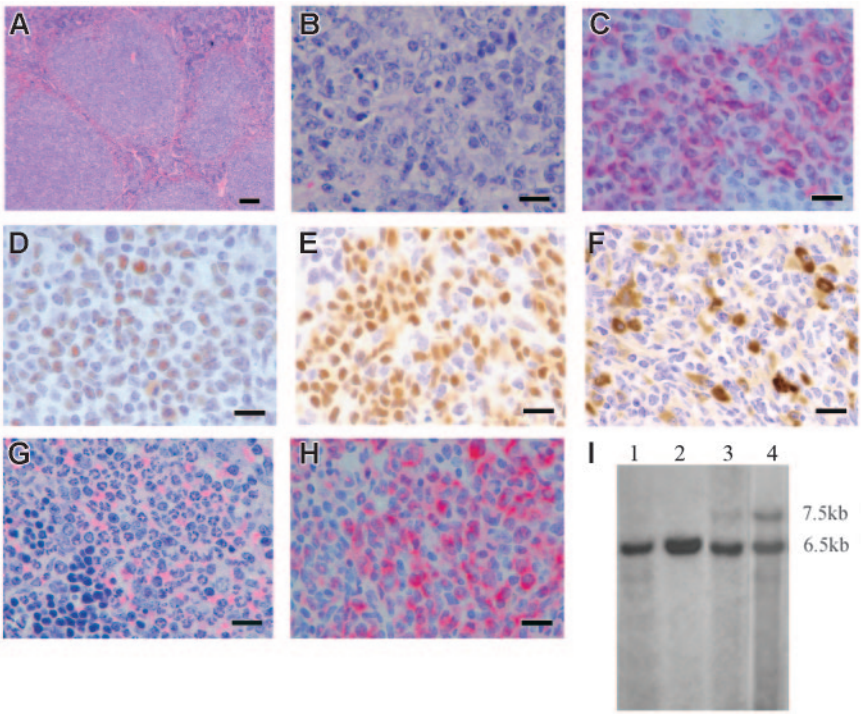


**Figure 7. Histopathology and immunohistochemistry of *Mll-AF4* mice with leukemia or lymphoma.** (A) Spleen section from a representative *Mll-AF4* mouse with lymphoma stained with H&E and shown at low magnification demonstrates loss of normal architecture resulting from follicular B-cell lymphoma. Bar represents 200 μm. (B) The same spleen section is shown at high magnification. Bar represents 50 μm. (C) Liver section indicates $B220^+$ lymphoma cell infiltration. Bar represents 50 μm. (D) Liver section from the same specimen shows Bcl-$6^+$ lymphoma. Bar represents 50 μm. (E) Spleen section shows $PAX5^+$ lymphoma cells. Bar represents 50 μm. (F) Small intestine section shows $IgM^+$ lymphoma cells. Bar represents 50 μm. (G) Spleen section from an *Mll-AF4* mouse with MPD-like myeloid leukemia stained with H&E indicates loss of normal architecture and heavy infiltration of myeloid cells at all stage of maturation. Bar represents 50 μm. (H) Spleen section from a *Mll-AF4* mouse with MPD-like myeloid leukemia stained with myeloperoxidase. Bar represents 50 μm. (I) IgH rearrangements in the spleen and lymph nodes of an *Mll-AF4* mouse with follicular B-cell lymphoma. Lane 1, spleen from a wild-type mouse; lane 2, kidney from the same wild-type mouse; lane 3, spleen from an *Mll-AF4* mouse; lane 4, lymph node from the same mouse. gDNA was digested by *Eco*RI. Germline band, 6.5 kb; additional band in lane 2 and lane 3, 7.5 kb.

myeloid leukemias in 36 mice, similar to earlier reports,[11,25,30] and follicular B-cell lymphoma with MPD in one mouse. The mean WBC count of *Mll-AF9* mice with myeloid disease was dramatically increased compared to wild-type littermates ($225.1 \times 10^3 \pm 42.4 \times 10^3/\mu L$ [n = 17] versus $2.4 \times 10^3 \pm 0.2 \times 10^3/\mu L$ [n = 10]; $P < .01$) and higher than *Mll-AF4* mice. *Mll-AF9* mice also had significant leukemic infiltration of bone marrow, spleen, liver, lymph nodes, and Peyer patches. Malignant cells showed variable degrees of differentiation, ranging from blasts to cells with maturation into segmented neutrophils. Macrophages with a striated eosinophilic cytoplasm were frequently noted. Tumors were immunopositive for myeloperoxidase. FACS analysis demonstrated these malignancies to be $CD11b^+Gr1^+B220^-$ as reported earlier.[17,30] Because the number of immature/blast cells were increased, but less than 10%, these malignancies, previously termed $Gr1^+$ myeloid leukemias,[17] were reclassified as MPD-like myeloid leukemia.[29]

## Discussion

In this report, we describe the first model of murine *Mll-AF4* disease. *Mll-AF4* mice had abnormalities in the hematopoietic and skeletal systems. Skeletal and hematologic changes included distinctive craniofacial features that differed from both *Mll-AF9* and wild-type mice. *Mll-AF4* mice were small with reduced fertility. The effects of *Mll-AF4* on lymphohematopoiesis were found early after birth and progressed to a fatal, complex, and variable mixed lymphoid (most frequently B-cell lymphoma)/myeloid disease in most mice after a long latency period.

*MLL* is known to be important in mammalian embryonic skeletal and hematopoietic development.[31,32] *AF4* is expressed in brain and multiple other organs.[21,33] *AF4*-deficient mice have defective B-lymphoid development,[34] and *LAF4* is highest in B-lymphoid progenitor cells.[35] In humans, *MLL* fusions with *AF4* family members (*AF4, LAF4*, and *AF5q31*) result in lymphoid or mixed/multilineage malignancies.[3,4,6,8-10] *MLL-AF9* fusions most often result in myeloid malignancies.[5,36] Our data showing preferential effects of *Mll-AF4* on lymphoid and mixed-lineage cells are consistent with the patterns of *MLL-AF4*.

Our results showed distinctive effects of *Mll-AF4* on lymphoid and myeloid populations of young premalignant mice. These differences were evident both in culture systems and in mice. Mixed-lineage pro-B ($B220^+CD19^+CD43^+sIgM^-$, $PAX5^+$, $TdT^+$, IgH rearranged)/myeloid ($CD11b/Mac1^+$, $c\text{-}fms^+$, $lysozyme^+$) colonies were frequent in *Mll-AF4* bone marrow grown under lymphoid conditions (Figures 3B and 4), which may represent multilineage capability possibly contributing to both lymphoid and myeloid proliferation in vivo. The existence of cells with mixed-lineage markers and multipotential for growth in normal marrow has been reported previously in normal marrow.[37] Previously, murine marrow, retrovirally transfected with *Mll-ENL* or *Mll-GAS7* and cultured under the similar conditions, grew mixed-lineage/multipotential cells.[20,24] Depending on their self-renewal potential, increased progenitor cells in our cultures are also likely to increase the number of cellular targets at risk for secondary mutations. The increased numbers of premalignant *Mll-AF4* progenitor cells could also be the result of reduced apoptosis. Previous studies have demonstrated resistance to apoptosis in *MLL-AF4* leukemia[38] or in cells transfected with *MLL-AF4*.[14]

Our *Mll-AF4* KI murine model, in its current form, results in a long latency period before fatal lymphoid or myeloid disease rather than B-lineage leukemias found in humans. Thus, the model is not fully penetrant and indicates probably replicating the first does not replicate later steps in evolution of human *Mll-AF4* disease. Whereas rarely are *MLL-AF4* lymphomas found in humans,[39] most human *MLL-AF4* malignances are pro-B or pro-B/myeloid mixed-lineage leukemias. The most likely explanation for the differences with human disease follows from an absence of secondary mutations that are present in human *Mll-AF4* disease. There may also be species differences, chromatin-limited accessibility of mutable genes, and lack of haploinsufficiency in the murine model or other to-be-identified factors. In other models of *Mll* fusion genes, including *Mll-ENL* and *Mll-CBP*, cooperating secondary mutations have been suggested to play a key role in development of frank malignancy, although in these models, as well as the *Mll-AF4* model, these mutations remain largely undefined.[40,41] The development of a fully penetrant model through induction of important additional cooperating mutations in the *Mll-AF4* model is now a high priority for our laboratory. As in humans with *MLL* fusion gene leukemias, *Flt3* might be one of a number of candidates for secondary mutations in transgenic mice with *Mll* fusions genes.[3,42]

In the *Mll-AF4* and *Mll-AF9* models, all hematopoietic progenitors and stem cells contain the fusion gene and yet different cellular effects are observed. The results show that both *Mll-AF4* and *Mll-AF9* fusion partners are active in deregulating hematopoiesis in young mice long before the development of hematologic malignancy. Our data provide evidence that some of cell type–preferential downstream effects are fusion partner dependent. Our findings showing that at least some of the effects are partner dependent suggest that the "hits" resulting from chromosomal translocation are not required to be in different cell types to obtain differing effects on cellular proliferation and differentiation.

Is *Mll* partner-dependent "instruction" a consequence of varying levels of expression of the fusion oncogene? We studied the expression of the *Mll-AF4* or *Mll-AF9* gene in selected bone marrows of young and older diseased mice using quantitative RT-PCR. All mice had expression of the appropriate gene. To date, no significant overall differences have been seen when *Mll-AF4* and *Mll-AF9* mice are compared. This result might be expected in the KI model where the 5′ region of *Mll* of *Mll-AF4* or *Mll-AF9* is not disrupted and under the control of the endogenous promoter. However, there are likely to be cell type–specific differences in expression levels. This possibility is suggested from the result of mice tested with differing age and disease status. Older diseased mice had lower levels of gene expression than young mice in both the *Mll-AF4* and *Mll-AF9* groups. Additional studies are under way to study these differences in purified populations of cells.

*Mll* fusion partner-independent effects were also found in our experiments with lymphoid and myeloid abnormalities in both *Mll-AF4* and *Mll-AF9* mice. Thus, while preferential, the effects of *AF4* and *AF9* were partially overlapping to some extent. Myeloid and lymphoid expansions were enhanced by both fusion genes. This crossover suggests that there are common features to both fusion genes that could result from effects of shared functional domains either in *MLL* or in the partners or both. Gain-of-function models proposed by many investigators for *MLL* fusion oncogenes have mostly focused on "partner-independent" characteristics.[2,43]

How do *Mll-AF4* and *Mll-AF9* produce preferential "instructive" expansion of mixed-lineage or myeloid cells, respectively, and set in motion the processes that eventually result in leukemia? Both *AF4* and *AF9* contain critical 3′ transactivation sequences that are retained in the fusion genes.[2,41-43] The 5′ DNA-binding sequences of *Mll*, when fused to the 3′ portion of *AF4* or *AF9*, may

change the normal transcriptional regulation of downstream genes, such as *Hox* genes.[17,20] *Hox* genes have been identified as the downstream targets of the *MLL* gene in both *Drosophila* and mammals.[30,31,44,45] Up-regulation of *Hox* genes has been reported in both *MLL-AF4* and *MLL-AF9* leukemia[3,46,47] in human and *Mll-AF9* mice.[17] *Hox* genes can alter the phenotype of leukemias but there is apparent redundancy in *Mll* fusion gene leukemogenesis.[17,48] The differing abnormalities of facial morphology in *Mll-AF4* and *Mll-AF9* mice, which results in part from skeletal defects, may be the results of altered expression patterns of *Hox* genes during early development. Further studies of *Hox* and other genes in these mice are under way in our laboratory.

Partner-dependent "instructive" expression of other genes is likely to be important in the differing effects of *Mll-AF4* and *Mll-AF9*. Milne et al[49] have shown that the cyclin-dependent kinase inhibitor, $p27^{kip1}$, is regulated by *MLL* through binding to the p27 promoter. Xia et al[50] showed that *MLL-AF4* binds to the CDKN1B promoter and p27 is highly expressed in *MLL-AF4* but not *MLL-AF9* leukemic cell lines. *Menin* is likely to play an important role in the effects of *MLL* fusion genes, as Yokoyama et al[51] showed *Menin* to be an essential oncogenic cofactor in *MLL* fusion gene leukemia.

The fact that *Mll-AF4* malignancies differ in phenotype from most other *Mll* fusion-induced malignancies in both mice and humans suggests that the "downstream" cells responsible for development of malignancy may be different in *Mll-AF4* family member malignancies compared to most other *MLL* malignancies. Currently, the phenotype of the malignancy-initiating stem cells[52] in *Mll-AF4* versus *Mll-AF9* malignancies remains to be determined. These important cells may differ from the bulk of cells found in the diverse malignancies that range from follicular B-cell to immature myeloid and mixed lineage in our *Mll-AF4* transgenic animals. Additional studies of the progression and evolution of these important malignancy-inducing stem cells are now possible with the *Mll-AF4* KI mice.

The *Mll-AF4* KI model will also provide mice and cells for molecular studies of the *AF4* partner in *Mll* fusion gene-induced lymphoid/myeloid deregulation and the later development of malignancy. Molecular studies of promoter transactivation, protein-protein interactions, and signal transduction pathways now can be done with this model.

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