Mll partial tandem duplication and *Flt3* internal tandem duplication in a double knock-in mouse recapitulates features of counterpart human acute myeloid leukemias

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The *MLL*-partial tandem duplication (PTD) associates with high-risk cytogenetically normal acute myeloid leukemia (AML). Concurrent presence of *FLT3*-internal tandem duplication (ITD) is observed in 25% of patients with *MLL*-PTD AML. However, mice expressing either *Mll*-PTD or *Flt3*-ITD do not develop AML, suggesting that 2 mutations are necessary for the AML phenotype. Thus, we generated a mouse expressing both *Mll*-PTD and *Flt3*-ITD. *Mll*^PTD/WT^:*Flt3*^ITD/WT^ mice developed acute leukemia with 100% penetrance, at a median of 49 weeks. As in human *MLL*-PTD and/or the *FLT3*-ITD AML, mouse blasts exhibited normal cytogenetics, decreased *Mll*-WT-to-*Mll*-PTD ratio, loss of the *Flt3*-WT allele, and increased total *Flt3*. Highlighting the adverse impact of *FLT3*-ITD dosage on patient survival, mice with homozygous *Flt3*-ITD alleles, *Mll*^PTD/WT^:*Flt3*^ITD/ITD^, demonstrated a nearly 30-week reduction in latency to overt AML. Here we demonstrate, for the first time, that *Mll*-PTD contributes to leukemogenesis as a gain-of-function mutation and describe a novel murine model closely recapitulating human AML. (*Blood*. 2012;120(5): 1130-1136)

Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous disease. Recurrent cytogenetic and molecular gene aberrations have been used to classify AML patients into distinct subsets that differ in biologic, clinical, and prognostic characteristics. The *MLL* gene, located at chromosome band 11q23, encodes for a protein involved in epigenetic regulation of gene expression.[1] In AML, this gene is frequently involved in chromosome translocations at 11q23 and, at the molecular level, is fused with one of more than 50 different partners.[2] We first reported an internal duplication of *MLL*, an in-frame repeat producing an elongated protein retaining all functional domains, in cytogenetically normal (CN)–AML.[3] Approximately 5% to 7% of CN-AML patients have a *MLL*-partial tandem duplication (PTD) mutation, which is associated with unfavorable prognosis,[3-5] but if and how it contributes to myeloid leukemogenesis remain to be elucidated. Approximately 25% of CN-AML patients with the *MLL*-PTD had constitutive activation of FLT3, a tyrosine kinase receptor regulating proliferation and survival of hematopoietic cells, via an internal tandem duplication (*FLT3*-ITD), and have a very poor prognosis.[6] This suggests that both *MLL*-PTD and *FLT3*-ITD mutations are necessary for an AML phenotype, as supported by the broadly accepted 2-hit model.[7] Indeed, a *Mll*-PTD mouse, created by knocking in exons 5 to 11 in-frame and driven off of the endogenous *Mll* promoter, did not develop AML.[8] Similarly, the *Flt3*^ITD/Wildtype(WT)^ or *Flt3*^ITD/ITD^

knock-in mice do not develop AML.[9] Thus, we crossed the heterozygous *Mll*^PTD/WT^ and *Flt3*^ITD/WT^ knock-in mouse strains to create *Mll*^PTD/WT^:*Flt3*^ITD/WT^ double knock-in mice.[8,9] This model, which develops AML, is the first that requires the *MLL*-PTD. In this model, the 2 mutated genes are regulated by their respective endogenous promoters to recapitulate the *Mll*^PTD/WT^:*Flt3*^ITD/WT^ AML found in humans. This differs from some other double-mutant mouse models of human AML that carry one mutation driven by the endogenous promoter and the other mutation driven by transgenes,[10] or introduced via viral transduction,[11] or 2 mutations driven off 2 endogenous promoters but requiring BM transplantation.[12]

Methods

Mouse strains

The *Mll*^PTD/WT^, *Flt3*^ITD/WT^, and *Flt3*^ITD/ITD^ mice were generated and genotyped as previously described.[8,9] Male *Flt3*^ITD/WT^ Balb/c mice were backcrossed onto the C57Bl/6J strain to purity and then bred with *Mll*^PTD/WT^ mice to generate *Mll*^PTD/WT^:*Flt3*^ITD/WT^ double knock-in offspring. Genotyping was performed as previously described.[8,9] All animals studied were compared within litters and/or age- and sex-matched. All experiments were conducted under an approved The Ohio State University Institutional

Figure 1. $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice develop a predominance of AML. (A) Frequencies of acute leukemia immunophenotypes identified in $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice (n = 33). (B) Representative flow cytometry plots from spleen and BM samples from age-matched controls and moribund $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice killed with elevated WBC counts. AML with maturation (myeloperoxidase [MPO$^+$]/CD3$^-$/CD19$^-$/B220$^-$/CD11b$^+$/Gr1$^+$/CD117$^-$; 15%), AML without maturation group A (MPO$^+$/CD3$^-$/B220^{lo}/CD19$^-$/CD11b$^{lo/-}$/Gr1$^-$/CD117$^{+/-}$; 34%), AML without maturation group B (MPO$^+$/CD3$^-$/B220$^+$/CD19$^-$/CD11b$^{+/-}$/Gr1$^-$/CD117$^{+/-}$; 21%). Regions highlighted in the red rectangle emphasize key differences in populations for each of the AML immunophenotypes. MPO staining by IHC and flow cytometry results for B-cell and biphenotypic leukemias are not shown. (C) WBC counts of leukemic $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice are significantly elevated at the time of death. Figure shows WBC counts from 3 age-matched cohorts of $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice with AML and controls. (D) $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice with leukemia exhibit significant splenomegaly at the time of death. Figure shows weights from 3 age-matched cohorts of $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice with AML and controls. (E) Representative image of spleens from a $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mouse dying of AML with age-matched controls. (F) Representative blood smear stained with Wright-Giemsa and hematoxylin and eosin-stained organ preparations from a $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mouse dying of AML. Micrographs demonstrate blasts in peripheral blood (original magnification ×1000) and extensive infiltrations of myeloid blasts in liver (original magnification ×400), and adrenal gland (original magnification ×400). Micrograph images were recorded using a Zeiss Axioskop, Olympus Magnafire digital camera, using a 10× eyepiece, 40×/0.65 NA Acroplan objective lens or 100×/1.40 NA Plan-Apochromat and Zeiss Axiovision Version 4.7.2 software.
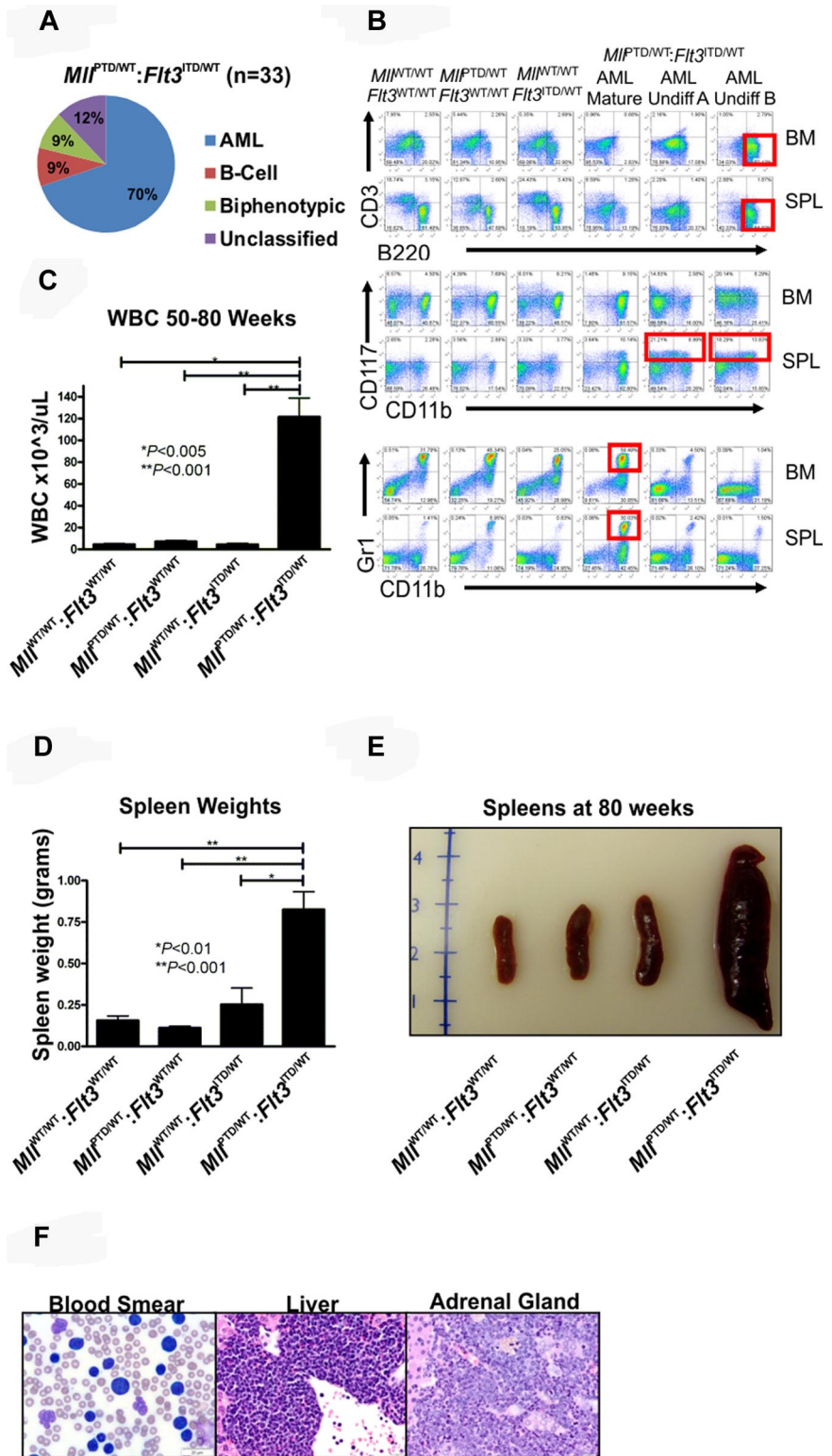
Animal Care and Use Committee protocol and following national guidelines and regulations.

Immunophenotypic analysis

For multiparameter flow cytometry, single-cell suspensions of BM and spleen were prepared from age- and sex-matched littermates. Red blood cells were lysed on ice with red blood cell lysis buffer (StemCell Technologies), washed in RPMI 1640 containing 10% FBS, resuspended in PBS with 0.5% FBS, and stained with the following monoclonal antibodies that were conjugated to V450, V500, FITC, PE, peridinin chlorophyll protein, allophycocyanin, or PE-Cy7: Gr-1, Mac-1, CD117, F4/80, CD3, B220, CD19, IgM, CD45.1, CD45.2, CD71, CD4, and CD8 (BD Biosciences) in PBS plus 0.5% FBS for 30 minutes. Analysis was performed using a BD LSRII cytometer or an Aria II cell sorter Version 6.1.2 (BD Biosciences) and FlowJo Version 7.6.5 (TreeStar) software programs. CD117$^+$ cells were sorted into 50% FBS/RPMI 1640 for subsequent assays.

Pathologic examination, immunohistochemistry, and cytochemistry

Animals were monitored daily for the presence of disease by general inspection. White blood cell (WBC) counts and differentials were obtained biweekly from mice beginning at 35 weeks or when moribund. Peripheral blood was collected from maxillary vein puncture, and total and differential blood cell counts were determined using Hemavet 950 (Drew Scientific). Two consecutive WBC counts of more than 45 000 cells/μL together with an increase in neutrophil/lymphocyte ratio and/or morbidity defined as lethargy, enlarged spleen on palpation, and/or loss of 20% body weight were used as the criteria for death. Blood smears were prepared before death, followed by Wright-Giemsa staining. Killed animals were examined for the presence of tumors or other abnormalities, and organs were collected for further cell and histopathologic cellular analyses. Tissues were fixed for at least 72 hours in 10% neutral buffered formalin (Sigma-Aldrich), dehydrated in ethanol, cleared in xylene, and infiltrated with paraffin on an automated processor (Leica). For solid tissue samples, 4-μm-thick tissue sections were placed on charged slides, deparaffinized with xylene, rehydrated through graded alcohol washes, and stained with hematoxylin and eosin.

G-banded karyotype analysis

Cytogenetic analysis was done on BM cells obtained from the long bones of the animals. The cells were cultured overnight in RPMI 1640 medium with 2% L-Glutamine (Invitrogen), supplemented with 20% FBS (Hyclone Laboratories), and 2% penicillin and streptomycin (Invitrogen) at 37°C in an atmosphere of 5% CO_2. Colcemid (Invitrogen) at a final concentration of 0.1 μg/mL was added for 1 hour. The cells were treated with hypotonic solution (0.075M KCl) for 15 minutes, fixed in 3:1 methanol:acetic acid, and dropped onto warm slides. Chromosomes were G-banded using trypsin and Wright stain by standard laboratory procedures. Twenty metaphases from each animal were completely analyzed.

Serial hematopathologic examinations, flow cytometric analysis, and serial transplantation

For transplantation, Ly5.2 spleen cells isolated from leukemic $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice were resuspended in PBS. Cells (5×10^6) were injected through the lateral tail vein into sublethally irradiated (450 cGy), syngenic Ly5.1 recipient mice. When moribund, mice were killed and hematopoietic tissues harvested and FACS analyses carried out. Spleen cells were serially transplanted up to 4 times.

Quantitative real-time RT-PCR

Predeveloped mouse TaqMan primer/probe sets for *ActB* (Mm01205647_g1), *HoxA9* (Mm00439364_m1), and *Flt3* (Mm00439016_m1) were purchased from Applied Biosystems. An allele-specific TaqMan assay for measuring mouse *Mll*-PTD and *Mll*-WT mRNA levels was performed as previously described.[8] Real-time RT-PCR was carried out using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Relative and absolute quantification assays were carried out as previously described.[13,14] Mouse *Mll* primer and probe sequences are available on request.

Statistical analyses

The log-rank test was used to evaluate overall survival between mouse groups, and Kaplan-Meier survival curves were used to display the results. Statistical significance of differences in parameters measured between WT, single knock-in mutant animals and double knock-in mutant mice were assessed using linear mixed-effects models if the data are correlated or ANOVA if the data are independent with *P* values less than .05 considered to be significant. Statistical analyses were conducted using SAS Version 9.2 (SAS Institute).
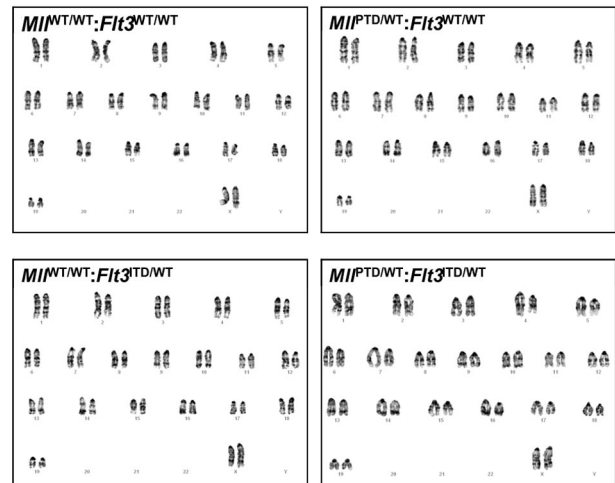

Figure 2. BM samples from $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice dying of AML are cytogenetically normal as are the age-matched, WT, and single-mutant controls. G-banded karyotype analysis of WT, single-mutant, and leukemic $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice was conducted on whole BM samples.

Results

The double knock-in $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice developed acute leukemia with 100% penetrance. Seventy percent of the $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice developed 1 of 3 main subtypes of AML, as defined previously for murine leukemias (Figure 1A-B).[15] The remaining mice developed biphenotypic (12%), B-cell (9%), or unclassifiable (9%) acute leukemia (Figure 1A). Compared with the single mutant knock-ins and WT mice, $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice developed significant leukocytosis (Figure 1C) and splenomegaly (Figure 1D-E). Additional criteria were met by the presence of more than or equal to 20% blasts in blood and blasts in nonhematopoietic organs, including liver and adrenal glands (Figure 1F), and thrombocytopenia and/or anemia (data not shown). Gross structural aberrations in chromosomes were not found with G-banding karyotype analysis of the age-matched single mutant knock-ins or in the leukemic double knock-in mice (Figure 2). $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice died with significantly reduced life spans and a median survival of 49 weeks versus 75 to 94 weeks for WT and single mutants ($P < .0003$; Figure 3A). Leukemia cells were transplantable; and with each serial passage of the cells, transplant recipients showed a progressively shorter period of time to overt morbidity resulting from AML (Figure 3B).

As reported previously for human *MLL*-PTD AML,[14] we observed a nearly 60% decrease of the *Mll*-WT transcript copy number and a decrease in the *Mll*-WT-to-*Mll*-PTD ratio in the $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ AML mice compared with CD117+ BM samples from age-matched controls (Figure 4A). To obtain further support that the *Mll*-PTD is a gain-of-function allele,[14,16] we assessed the impact on a known transcriptional target of Mll, the homeobox gene *HoxA9*. In leukemic $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ BM cells, *HoxA9* expression was 25-fold higher ($P < .05$) versus similarly aged $Mll^{WT/WT}$:$Flt3^{WT/WT}$ mice and higher compared with the levels measured in the $Mll^{PTD/WT}$:$Flt3^{WT/WT}$ single knock-ins ($P < .05$; Figure 4B).

In agreement with previous findings that *MLL*-PTD patient blasts exhibit increased *FLT3* expression,[17] total *Flt3* expression in BM of leukemic $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice also increased over time and was found to be 10-fold higher ($P < .05$) than controls (Figure 4C). Because the Real time RT-PCR conditions used did
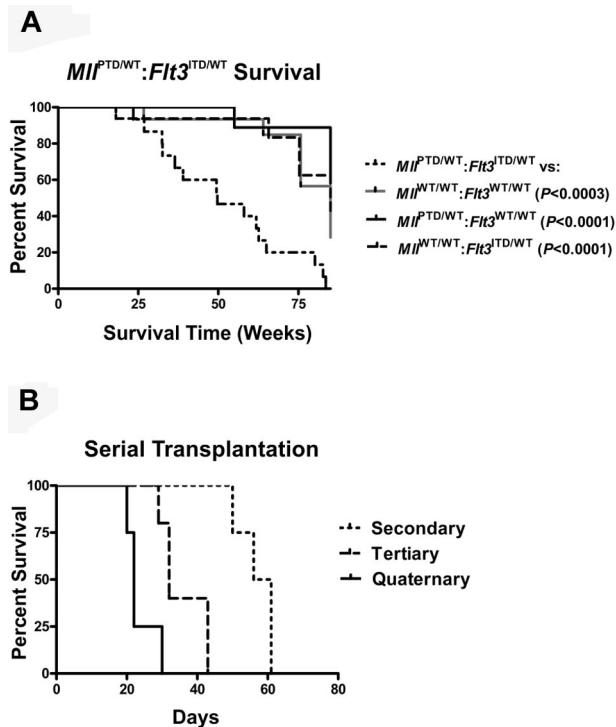
Figure 3. $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice develop a fatal AML that is transplantable.
(A) Survival of WT and single knock-in ($Mll^{PTD/WT}$:$Flt3^{WT/WT}$ or $Mll^{WT/WT}$:$Flt3^{ITD/WT}$) control mice and mice with the heterozygous alleles of $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$. (B) $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ cells from spleen containing AML are serially transplantable and lethal. Serially transplanted cells develop into progressively more aggressive AML with each successive passage.

not distinguish between the $Flt3$-ITD and $Flt3$-WT transcripts, it was not clear if a selective increase in one or the other or both transcripts was occurring. In the human AML harboring $FLT3$-ITD, absence of the $FLT3$-WT allele at diagnosis occurs in approximately one-third of patients via a mechanism of uniparental disomy,[18] and $FLT3$-ITD AML patients with an absent $FLT3$-WT allele at diagnosis have a worse prognosis as the result of more aggressive disease.[13] We found that 9 of 10 $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice that were leukemia-free at 4 weeks of age carried the $Flt3$-WT allele. However, at the time of AML diagnosis, genomic PCR analyses of whole BM and transplantation-derived Ly5.2-sorted leukemic blasts revealed a dramatic reduction in $Flt3$-WT relative to $Flt3$-ITD or absence of the $Flt3$-WT allele (Figure 4D). The implication of these findings is that the "total $Flt3$" expression detected in the majority of leukemic samples is the result of up-regulation of expression of the remaining $Flt3$-ITD allele.

To test the contribution of this phenomenon (ie, loss of the $Flt3$-WT allele) to leukemogenesis, we crossed $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice with $Mll^{WT/WT}$:$Flt3^{ITD/ITD}$ mice to generate $Mll^{PTD/WT}$:$Flt3^{ITD/ITD}$ mice, which are homozygous for $Flt3$-ITD. $Mll^{PTD/WT}$:$Flt3^{ITD/ITD}$ mice had a higher incidence (89%) of AML compared with $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice (70%; Figure 5A vs Figure 1A). B-cell leukemia, which had been previously reported in one patient with MLL-PTD,[19] developed in 11% of $Mll^{PTD/WT}$:$Flt3^{ITD/ITD}$ animals (Figure 5A). More than 95% of $Mll^{PTD/WT}$:$Flt3^{ITD/ITD}$ mice die with leukemic features similar to those described for $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice (Figure 5B-C). The disease was more aggressive with a significantly shorter median life span of $Mll^{PTD/WT}$:$Flt3^{ITD/ITD}$ mice compared with that of the $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice (19 vs 49 weeks, respectively; $P < .0001$; Figure 5D), supporting a

dosage effect by the $Flt3$-ITD, as previously reported both in human and other mouse models.[13,20,21]

As in the older aged $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ leukemias, the Mll-WT-to-Mll-PTD transcript ratio was reduced in CD117+ BM cells from 15- to 25-week-old leukemic $Mll^{PTD/WT}$:$Flt3^{ITD/ITD}$ mice compared with that measured in age-matched, preleukemic $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice ($P < .05$; Figure 5E). In our model, dosage effects of Mll-PTD were not feasible to study because of embryonic lethality of the homozygous $Mll^{PTD/PTD}$ mice.[8] However, the expression data showing Mll-WT reduction in the leukemic samples regardless of the $Flt3$-ITD dosage are supportive that this phenomenon, reduced Mll-WT expression, contributes to the development of murine AML disease.

Discussion

Here we report the first animal model demonstrating that the Mll-PTD defect directly contributes to acute myeloid leukemogenesis in cooperation with a second mutation, the $Flt3$-ITD. First,
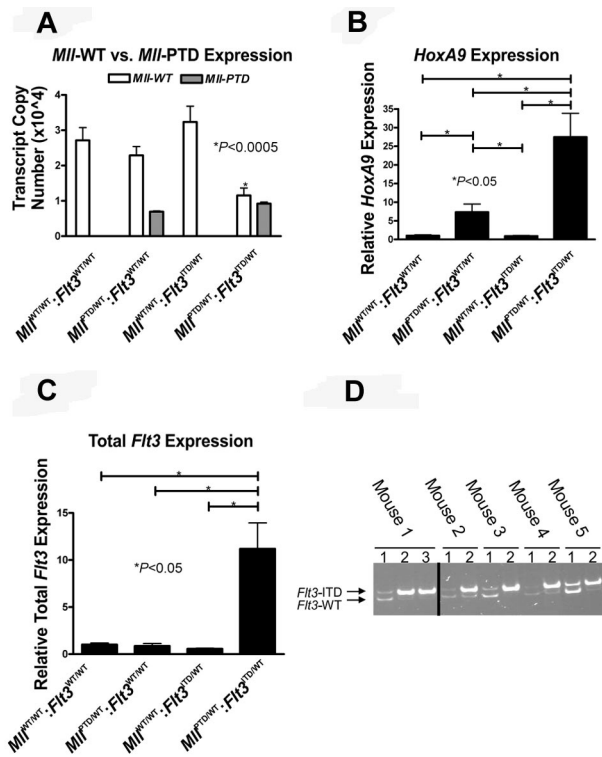

Figure 4. AML from $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice have molecular features similar to those reported for human MLL-PTD+ and/or $FLT3$-ITD+ AML. (A) Quantitative real-time RT-PCR was performed using CD117+ BM cells sorted to > 95% purity from age-matched mice ≥ 50 weeks of age to measure the copy numbers of the mouse Mll-PTD and the Mll-WT transcripts. Results demonstrate reduced Mll-WT copy number only in leukemic $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ mice. Figure is representative of 2 age-matched cohorts of mice. (B) Relative real-time RT-PCR measuring $HoxA9$ expression in whole BM cells from age-matched mice > 50 weeks of age. (C) Total $Flt3$ mRNA was measured by relative real-time RT-PCR using BM cells of age-matched mice ≥ 50 weeks of age. (D) Genomic PCR reactions using 150 ng DNA and genotyping primers to amplify both the $Flt3$-ITD and $Flt3$-WT loci were performed to demonstrate a reduction or loss of $Flt3$-WT in primary $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ AML samples taken from moribund mice. Multiple samples taken from each animal are defined as follows: lane 1, tail DNA from genotyping at 4 weeks; lane 2, whole BM DNA from the time of death when moribund with AML (50-80 weeks); and lane 3, sorted leukemic Ly5.2 $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ AML blasts from secondary transplant of $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ AML blasts into Ly5.1 recipients. The Ly5.2 → Ly5.1 adoptive transfer was used to obtain a pure population of $Mll^{PTD/WT}$:$Flt3^{ITD/WT}$ AML blasts for analysis. Five representative samples are shown. Reactions that did not produce any bands for $Flt3$-WT and $Flt3$-ITD were removed as noted by the black bar.
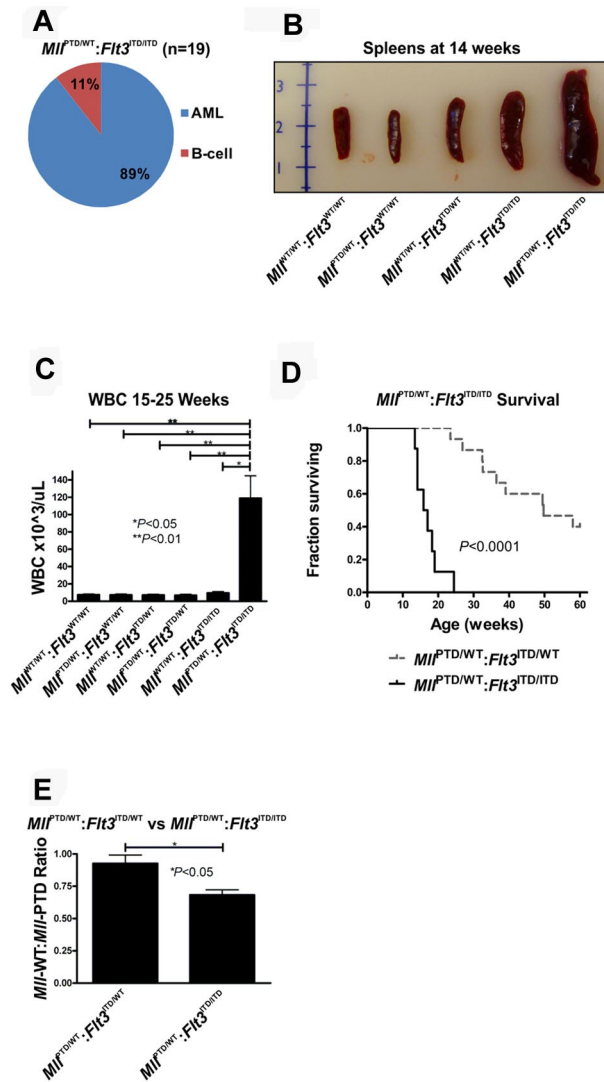
Figure 5. Acute leukemia in *Mll*^{PTD/WT}:*Flt3*^{ITD/ITD} mice. (A) Only AML and B-cell leukemias developed in *Mll*^{PTD/WT}:*Flt3*^{ITD/ITD} mice. Nineteen mice were analyzed. (B) *Mll*^{PTD/WT}:*Flt3*^{ITD/ITD} mice with leukemia exhibited significant splenomegaly at time of death. (C) WBC counts (median ± SEM) for leukemic *Mll*^{PTD/WT}:*Flt3*^{ITD/ITD} and age-matched control genotypes at the time of death. (D) *Mll*^{PTD/WT}:*Flt3*^{ITD/ITD} mice died of AML with a significantly shorter latency than *Mll*^{PTD/WT}:*Flt3*^{ITD/WT} mice. (E) Copy number ratio of *Mll*-WT:*Mll*-PTD was calculated for age-matched nonleukemic 15- to 25-week-old *Mll*^{PTD/WT}:*Flt3*^{ITD/WT} and leukemic *Mll*^{PTD/WT}:*Flt3*^{ITD/ITD} mice. Results are representative of 2 age-matched cohorts of mice.

compared with other in vivo modeling approaches, our model harbors both mutations in the germline, and each gene mutation is under the control of the respective normal endogenous promoter. Thus, expression of the mutant alleles would occur in the proper physiologic and temporal context during hematopoietic development and aging. Furthermore, the knock-in approach was pursued in our model to eliminate the potential for artifacts inherent with other methodologies, such as retroviruses, which induce nonphysiologic overexpression of the gene of interest and/or chromosomal integration of targeting constructs leading to activation of a proto-oncogene.[22-24]

To assess the relevance of this novel mouse model to human AML, we evaluated several phenotypic and molecular features for which published data exist for the human counterpart AML. The development of several AML subtypes in our double mutation knock-in model is consistent with earlier reports of the wide range of French-American-British morphologic subtypes of human AML that carry the *MLL*-PTD and/or *FLT3*-ITD.[4,6,25] The diagnosis of AML in our model associates with significantly reduced life span, and the disease is also characterized by extensive extramedullary involvement and increased disease aggressiveness in serial transplantation assays. Similarly, the presence of *MLL*-PTD and/or *FLT3*-ITD in AML patients treated with cytarabine-based chemotherapy regimens associates with early relapses and reduced survival times compared with patients with AML lacking these defects.[26-29]

We previously reported that human leukemic blasts taken from patients with *MLL*-PTD CN-AML express the mutant *MLL* without concurrently expressing the *MLL*-WT from the nonaffected chromosome.[14] This silencing is the result, at least in part, of epigenetic alterations within the 5′ regulatory region of the WT *MLL* allele.[14] Likewise, at the molecular level in AML blasts from our *Mll*^{PTD/WT}:*Flt3*^{ITD/WT} mice, the expression of the *Mll*-WT, but not the *Mll*-PTD, is consistently and significantly reduced. Notably, this is an acquired event, as the young, preleukemic *Mll*^{PTD/WT}:*Flt3*^{ITD/WT} mice express *Mll*-WT transcript at levels comparable with those in the single heterozygous mutant *Mll*^{PTD/WT} and *Mll*^{WT/WT} control mice that do not develop AML. Whether this reduction in the WT *Mll* allele of leukemic mice is also related to altered epigenetics is under investigation, but considering that the leukemic BM samples of *Mll*^{PTD/WT}:*Flt3*^{ITD/WT} mice remain karyotypically normal, one would consider this to be highly probable for at least a large fraction of the CD117⁺ BM cells studied.

Indeed, our model with its long latency period to overt disease lends itself to studying the repercussions and mechanisms of the *Mll*-PTD in the presence or absence of *Mll*-WT. In the future, this model will further our understanding of the *Mll*-PTD compared with *Mll* chimeric fusions, such as *Mll*-AF9, which appear to require *Mll*-WT for full, abnormal function involved in regulation of at least some target genes, such as *HoxA9*.[30] Our results showing increased *HoxA9* transcript levels in the leukemic *Mll*^{PTD/WT}:*Flt3*^{ITD/WT} BM cells that exhibit reduced *Mll*-WT expression strongly supports a gain of function for the *Mll*-PTD that does not appear to require a full complement of *Mll*-WT to function at the *HoxA9* gene.

Additional similarities to human *FLT3*-ITD–positive AML demonstrated by our model include loss of *Flt3*-WT expression. Based on our findings, *Flt3*-WT is not silenced by epigenetic mechanisms, as PCR using genomic DNA demonstrates loss of the *Flt3*-WT allele rather than a loss at the mRNA transcript level. Given the inbred nature of the mouse model presented, more detailed studies using our *Mll*^{PTD/WT}:*Flt3*^{ITD/WT} crossed with another strain carrying divergent single nucleotide polymorphisms will be required to determine whether the loss of the *Flt3*-WT allele at the genomic level is the result of an intrachromosomal microdeletion or partial or full uniparental disomy. The information gleaned from studies, such as these, will probably provide important information affecting a greater number of patients, particularly the 30% of cytogenetically normal AML patients with the *FLT3*-ITD mutation.

In conclusion, the *Mll*^{PTD/WT}:*Flt3*^{ITD/WT} double knock-in novel mouse model provides what we believe to be the first experimental proof that the *MLL*-PTD directly contributes to myeloid leukemogenesis, when concurrently present with a second mutation, such as *FLT3*-ITD. The *Mll*^{PTD/WT}:*Flt3*^{ITD/WT} AML recapitulates its human AML counterpart with regard to several phenotypic, cytogenetic, and molecular features. Indeed, reduced *Mll*-WT expression, increased total *Flt3* expression and loss of *Flt3*-WT in our mouse

model are features that have been associated with human *MLL*-PTD and/or *FLT3*-ITD AML.[13,27,28,31,32] In addition, the absence of gross structural chromosomal aberrations in the double knock-in mouse mimics the normal karyotypes at diagnosis that constitutes nearly 45% of human AML; both *MLL*-PTD and *FLT3*-ITD are most frequently present in human CN-AML.[25,29,33,34] This study supports our previous findings that the *MLL*-PTD is a gain-of-function mutation.[14,16] Despite the germline nature of the 2 mutations in our mouse model, the AML that develops does so with relatively long latency, implying that the reduction in *Mll*-WT expression and the loss of the *Flt3*-WT are important events, among others that remain to be defined, for full transformation. This model thus affords us the opportunity to carry out in-depth studies to elucidate the underlying mechanisms involved in leukemic transformation that must occur in a more physiologically relevant time-frame and cellular context. It also provides a feasible and relevant model to investigate not only novel leukemia prevention strategies, such as enhancement of innate and/or antigen-specific immunity via vaccination, but also opportunities to explore novel, targeted antileukemia therapeutics. For example, we can envision that the reduction in the *Mll*-WT expression levels along with the up-regulation of the *Flt3*-ITD concurrent with the loss/reduction of *Flt3*-WT should merit assessment of epigenetic modifying agents combined with tyrosine kinase inhibitors. This is a potential therapeutic strategy to combat *MLL*-PTD/*FLT3*-ITD–positive AML in our preclinical model as well as in patients with AML marked by these identical mutations. By virtue of its resemblance to the phenotypic, cytogenetic, and molecular features of human disease, this new AML model carrying the *Mll*-PTD mutation represents a useful tool for studying underlying mechanisms of leukemogenesis and for development and testing of novel target therapies to improve the outcome of this poor-risk AML subtype.

Authorship

Contribution: N.A.Z., K.M.B., S.P.W., R.F.S., E.H.A., G.G.M., D.A.Y., K.K.M., C.M., C.K., A.M.D., N.A.H., and B.H.L. performed the experiments; N.A.Z., K.M.B., S.P.W., R.F.S., A.M.D., B.H.L., G.H., G.M., and M.A.C. designed the experiments; N.A.Z., K.M.B., S.P.W., R.F.S., X.Z., D.J., A.M.D., N.A.H., B.H.L., G.H., G.M., and M.A.C. analyzed the data; S.P.W., A.M.D., G.M., and M.A.C. conceived the original project idea; and N.A.Z., S.P.W., G.M., and M.A.C. wrote the manuscript.

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