**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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**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 chromosomal 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 **11q23** and, at the molecular level, is fused with one of more than 50 different partners.2 We first reported an internal duplication of **11q23** and, at the molecular level, is fused with one of more than 50 different partners.2 We first reported an internal duplication of **11q23** and, at the molecular level, is fused with one of more than 50 different partners.2 We first reported an internal duplication of **11q23** and, at the molecular level, is fused with one of more than 50 different partners.2 We first reported an internal duplication of **11q23** and, at the molecular level, is fused with one of more than 50 different partners.2 We first reported an internal duplication of **11q23** and, at the molecular level, is fused with one of more than 50 different partners.2

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 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**-ITD-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)

### 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/ITD 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 Animal Care and Use Committee protocol. **N.A.Z., K.M.B., and S.P.W. contributed equally to this study.**


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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, CD19, IgM, CD19, 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.

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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 moribidity 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

Cyto genetic 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% CO2. 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 MllPTD/WT, Flt3ITD/WT mice were resuspended in PBS. Cells (5 × 10⁶) were injected through the lateral tail vein into sublethally irradiated (450 cGy), syngeneic Ly5.1 recipient mice. When moribund, mice were killed and hematopoietic tissues were collected. The cells were cultured overnight in RPMI 1640 medium with 10% FBS (Hyclone Laboratories), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were treated with hypotonic solution (0.075M KCl) for 1 hour. The cells were processed for G-banded karyotype analysis as previously described.10

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 Flt3 expression was 25-fold higher (P < .05) versus controls (Figure 4C). Because the Real time RT-PCR conditions used did not Hallucinate.

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).

Results

The double knock-in MllPTD/WT, Flt3ITD/WT mice developed acute leukemia with 100% penetrance. Seventy percent of the MllPTD/WT, Flt3ITD/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, MllPTD/WT, Flt3ITD/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 thrombocytope-

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up-regulation of expression of the remaining allele) to leukemogenesis, we crossed MllITD/WT:Flt3ITD/WT mice compared with that measured in age-matched, preleukemic MllITD/WT:Flt3ITD/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 MllITD/ITD 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-ITD 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,}

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 MllITD/WT:Flt3ITD/WT mice that were leukemia-free at 4 weeks of age carried the deletion.13 We found that 9 of 10 MllITD/WT:Flt3ITD/WT mice that were leukemia-free at 4 weeks of age carried the deletion.13 We found that 9 of 10 MllITD/WT:Flt3ITD/WT mice that were leukemia-free at 4 weeks of age carried the deletion.13 We found that 9 of 10 MllITD/WT:Flt3ITD/WT mice that were leukemia-free at 4 weeks of age carried the deletion.13 We found that 9 of 10 MllITD/WT:Flt3ITD/WT mice that were leukemia-free at 4 weeks of age carried the deletion.13
of French-American-British morphologic subtypes of human AML that carry the MLL-PTD and/or FLT3-ITD. 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.

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. This silencing is the result, at least in part, of epigenetic alterations within the S′ regulatory region of the WT MLL allele. Likewise, at the molecular level in AML blasts from our Mll<sup>PTD/WT</sup>; Flt3<sup>ITD/WT</sup> 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<sup>PTD/WT</sup>; Flt3<sup>ITD/WT</sup> mice express MLL-WT transcript at levels comparable with those in the single heterozygous mutant Mll<sup>PTD/WT</sup> and Mll<sup>ITD/WT</sup> control mice that do not develop AML. Whether this reduction in the WT Mll allelic of leukemic mice is also related to altered epigenetics is under investigation, but considering that the leukemic BM samples of Mll<sup>PTD/WT</sup>; Flt3<sup>ITD/WT</sup> mice remain karyotypically normal, one would consider this to be highly probable for at least a large fraction of the CD117<sup>+</sup> 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. Our results showing increased HoxA9 transcript levels in the leukemic Mll<sup>PTD/WT</sup>; Flt3<sup>ITD/WT</sup> 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<sup>PTD/WT</sup>; Flt3<sup>ITD/WT</sup> 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<sup>PTD/WT</sup>; Flt3<sup>ITD/WT</sup> 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<sup>PTD/WT</sup>; Flt3<sup>ITD/WT</sup> 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 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.

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
model are features that have been associated with human MLL-PTD and/or FLT3-ITD AML. 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. This study supports our previous findings that the MLL-PTD is a gain-of-function mutation. 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-IT 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-IT 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.

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Authorship


Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


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