

FLT3 Mutations Confer Enhanced Proliferation and Survival Properties to Multipotent Progenitors in a Murine Model of Chronic Myelomonocytic Leukemia

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DOI 10.1016/j.ccr.2007.08.031

SUMMARY

Despite their known transforming properties, the effects of leukemogenic FLT3-ITD mutations on hematopoietic stem and multipotent progenitor cells and on hematopoietic differentiation are not well understood. We report a mouse model harboring an ITD in the murine *Flt3* locus that develops myeloproliferative disease resembling CMML and further identified *FLT3*-ITD mutations in a subset of human CMML. These findings correlated with an increase in number, cell cycling, and survival of multipotent stem and progenitor cells in an ITD dose-dependent manner in animals that exhibited alterations within their myeloid progenitor compartments and a block in normal B cell development. This model provides insights into the consequences of constitutive signaling by an oncogenic tyrosine kinase on hematopoietic progenitor quiescence, function, and cell fate.

INTRODUCTION

The FMS-like tyrosine kinase 3 (*FLT3*) gene encodes a class III receptor tyrosine kinase (RTK) that shares strong sequence and structural similarities to other family members including FLT1, FMS, PDGF β R, and c-KIT (Rosnet and Birnbaum, 1993). FLT3 plays a critical role in normal hematopoiesis (for reviews see Gilliland and Griffin, 2002; Stirewalt and Radich, 2003), and within the hematopoietic system, its expression occurs primarily in imma-

ture myeloid and lymphoid progenitors, including CD34⁺ cells with high levels of CD117 (c-KIT) expression (Rasko et al., 1995; Rosnet et al., 1996), but not in erythroid cells (Gabbianelli et al., 1995), megakaryocytes (Ratajczak et al., 1996), or mast cells (Hjertson et al., 1996). Although targeted disruption of *Flt3* results in healthy adult mice with normal mature hematopoietic populations, these animals demonstrate deficiencies in primitive pro-B and pre-B cell lymphoid compartments (Mackarechtschian et al., 1995). Moreover, bone marrow (BM) reconstitution

SIGNIFICANCE

Activating FLT3 mutations are among the most common genetic events in AML and confer a poor clinical prognosis. Essential to our understanding of how these lesions contribute to myeloid leukemia is the development of a *Flt3*-ITD "knockin" murine model that has allowed examination of the consequences of constitutive FLT3 signaling on primitive hematopoietic progenitors when expressed at appropriate physiologic levels. These animals informed us to the existence of *FLT3*-ITD-positive human CMML, which has clinical importance given the availability of FLT3 small molecule inhibitors. This model will not only serve as a powerful biological tool to identify mutations that cooperate with FLT3 in leukemogenesis, but also to assess molecular therapies that target either FLT3 or components of its signaling pathways.

experiments revealed a reduced ability of cells lacking *Flt3* to reconstitute both T cells and myeloid cells (Mackarehtschian et al., 1995), together indicating an important role for FLT3 in the development of multipotent hematopoietic cells and lymphoid cells.

High levels of wild-type FLT3 expression have been detected in a number of hematologic malignancies including the vast majority of patients with AML (70% to >90%) (Carow et al., 1996; Rosnet et al., 1996) and a large proportion of precursor B cell acute lymphoblastic leukemia (ALL) including a subset of ALL carrying a chromosomal translocation involving the 11q23 locus (Armstrong et al., 2002; Drexler, 1996; Rosnet et al., 1996). Congruent with these findings, FLT3 is also expressed at high levels in both leukemia and lymphoma cell lines (DaSilva et al., 1994; Meierhoff et al., 1995) including pre-B, myeloid, and monocytic cell lines.

Internal tandem duplications (ITD) within the juxtamembrane (JM) domain of FLT3 in patients with AML were first reported in 1996 (Nakao et al., 1996) and occur in approximately 25% of patients, making it one of the most single common mutations in adult AML (Frohling et al., 2002; Kiyoi et al., 1999; Kottaridis et al., 2001; Schnittger et al., 2002; Whitman et al., 2001). FLT3-ITD mutations result in ligand-independent receptor dimerization (Kiyoi et al., 1998), constitutive FLT3 signaling, and activation of the STAT5, RAS/MAPK, and PI3K pathways and confer factor-independent growth to 32D and Ba/F3 cells (Hayakawa et al., 2000; Mizuki et al., 2000). Another major class of FLT3 mutations that also cause constitutive FLT3 activation and induce autonomous proliferation of cytokine-dependent cell lines occurs within the activation loop (AL) of the second kinase domain (Yamamoto et al., 2001). This group of mutations is comprised of substitutions, small deletions, or insertions most commonly involving codons 835 and 836 and is detected in approximately 5%–10% of patients with AML. More recently, AL mutations in FLT3 have also been described in cases of ALL that harbor rearrangements of the *Mixed Lineage Leukemia* (*MLL*) gene on chromosome 11q23 (Armstrong et al., 2003) as well as AL and ITD mutations in a small subset of T-ALL (Paietta et al., 2004) implicating FLT3 in the pathogenesis of both lymphoid and myeloid disease. Although both FLT3-AL and FLT3-ITD mutations cause constitutive activation of the receptor tyrosine kinase, their signal transduction properties and transforming abilities appear to differ considerably from one another (Choudhary et al., 2005; Grundler et al., 2005) arguing for differential roles of these classes of mutations in AML pathogenesis. Lastly, a group of point mutations within the JM domain have also recently been described in approximately 1% of AML cases involving several amino acids including residues 579, 590, 591, 592, and 594 (Reindl et al., 2006; Stirewalt et al., 2004).

Gain-of-function mutations in FLT3, in particular FLT3-ITD mutations, are of significant clinical consequence, and a number of studies have shown that FLT3-ITD mutations may be associated with disease progression (Horiike et al., 1997; Ishii et al., 1999) and are associated with an

increased risk of relapse and shorter overall survival (Frohling et al., 2002; Kiyoi et al., 1999; Kottaridis et al., 2001; Schnittger et al., 2002; Thiede et al., 2002; Whitman et al., 2001). Moreover, patients with low or absent levels of wild-type (WT) FLT3, consistent with homozygosity for the FLT3-ITD allele, appear to have a particularly dismal outcome (Thiede et al., 2002; Whitman et al., 2001), suggesting FLT3-ITD gene dosage has biologic and prognostic significance.

Given their frequency and relevance to AML disease pathogenesis and prognosis, there is a compelling basis for understanding the role of activating FLT3 mutations in leukemogenesis and critical to this advancement is the development of accurate leukemogenic FLT3 animal models. In this study, we have generated a murine model whereby we have introduced an internal tandem duplication (ITD) mutation in the endogenous murine *Flt3* locus. This model has distinct advantages over prior retroviral transduction and transgenic platforms employing heterologous promoters where differences in expression levels of activated FLT3 may affect disease phenotype and avoids potential cooperating mutations introduced by retroviral integration (Baldwin et al., 2007; Kelly et al., 2002b; Lee et al., 2005). Employing this model, we have explored the biological impact of constitutive FLT3 signaling on primitive hematopoietic stem and progenitor populations, providing significant mechanistic insights into how FLT3-ITD mutations contribute to myeloid leukemogenesis and demonstrate that FLT3-ITD expression results in the development of a fully penetrant myeloproliferative disorder within these animals. The murine phenotype closely resembles human chronic myelomonocytic leukemia (CMML), and genomic analysis of a large series of primary patient samples led to the identification of a subset of FLT3-ITD-positive CMML. This mouse model provides an accurate framework for understanding the biological mechanisms governing the genesis and progression of FLT3-associated myeloid disease. Moreover, not only can it serve as a powerful system to identify cooperating mutations with FLT3 in leukemogenesis, but it also allows the ability to assess the therapeutic efficacy of molecular agents that target FLT3 or downstream signaling pathways activated by the FLT3-ITD allele in these cooperative models of acute leukemia.

RESULTS

Generation of a FLT3-ITD “Knockin” Murine Model

To better understand the role of FLT3-ITD mutations in disease pathogenesis when expressed physiologically from its endogenous promoter, we engineered a human ITD (W51) (Kelly et al., 2002b) into exon 14 of the murine *Flt3* locus by homologous recombination in embryonic stem (ES) cells (Figures 1A–1C). Correctly targeted ES cells were used to produce viable mice carrying mutant ITD alleles. Heterozygous (*Flt3*^{+/ITD}) mice were intercrossed to produce homozygous *Flt3*^{ITD/ITD} animals that were viable and fertile with predicted Mendelian ratios (data not shown). Both heterozygous and homozygous

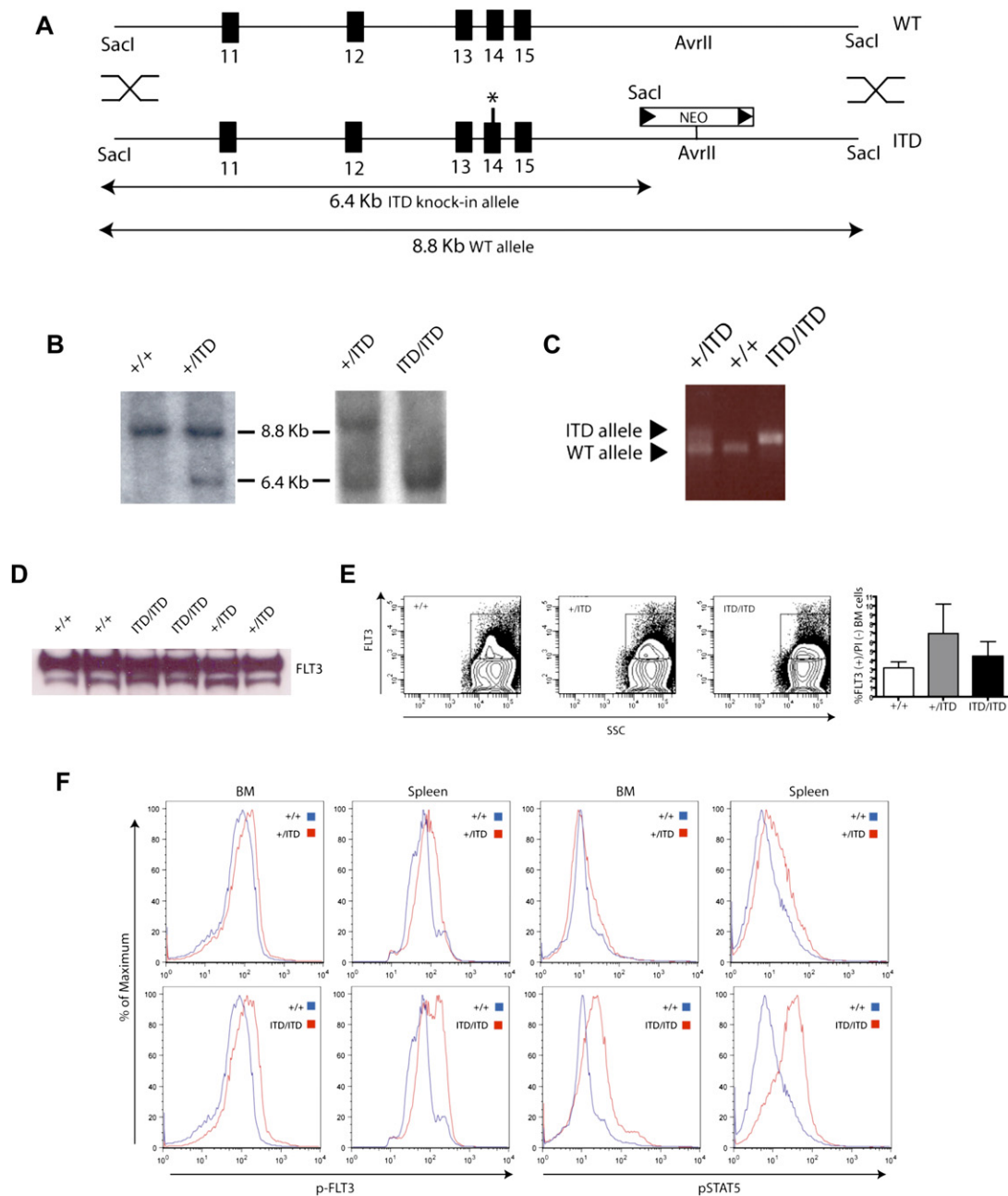


Figure 1. "Knockin" of an Internal Tandem Duplication Mutation in the Murine *Flt3* Gene

(A) Schematic of the *Flt3* locus (WT) and targeting vector (ITD) comprised of murine genomic sequences encompassing exons 11–15 and an in-frame human W51 ITD mutation (*) within exon 14 and a neomycin resistance cassette flanked by two loxP sites (arrowheads) downstream of exon 15.

(B) Southern blot analysis with the 5' probe of *SacI*-digested DNA from *Flt3*^{+/+}, *Flt3*^{+/ITD}, and *Flt3*^{ITD/ITD} mice.

(C) PCR analysis of mouse tail DNA from *Flt3*^{+/+}, *Flt3*^{+/ITD}, and *Flt3*^{ITD/ITD} animals.

(D) Immunoblot of Flt3 expression in primary spleen cells from mutant and WT animals. Equivalent amounts of protein were loaded from spleen protein lysates from two independent animals with the indicated genotype and blotted with anti-Flt3 antibody.

(E) Representative flow cytometric analysis of Flt3 expression in total, live (PI negative) BM cells in *Flt3*^{+/+}, *Flt3*^{+/ITD}, and *Flt3*^{ITD/ITD} mice. Data in bar graph represent the percentage of Flt3 positive in total live BM cells (mean ± SEM) *Flt3*^{+/+} (n = 3), *Flt3*^{+/ITD} (n = 4), *Flt3*^{ITD/ITD} (n = 5) (p = NS), although the cell numbers which express FLT3 are increased in FLT3-ITD mice, the mean fluorescence intensity range which correlates with FLT3 protein expression is similar for all 3 genotypes.

(F) Flow cytometric analysis of levels of phospho-Flt3 (p-FLT3) and phospho-Stat5 (pSTAT5) in whole BM and spleen cells from mutant mice and WT littermate controls. Data are representative of two independent experiments.

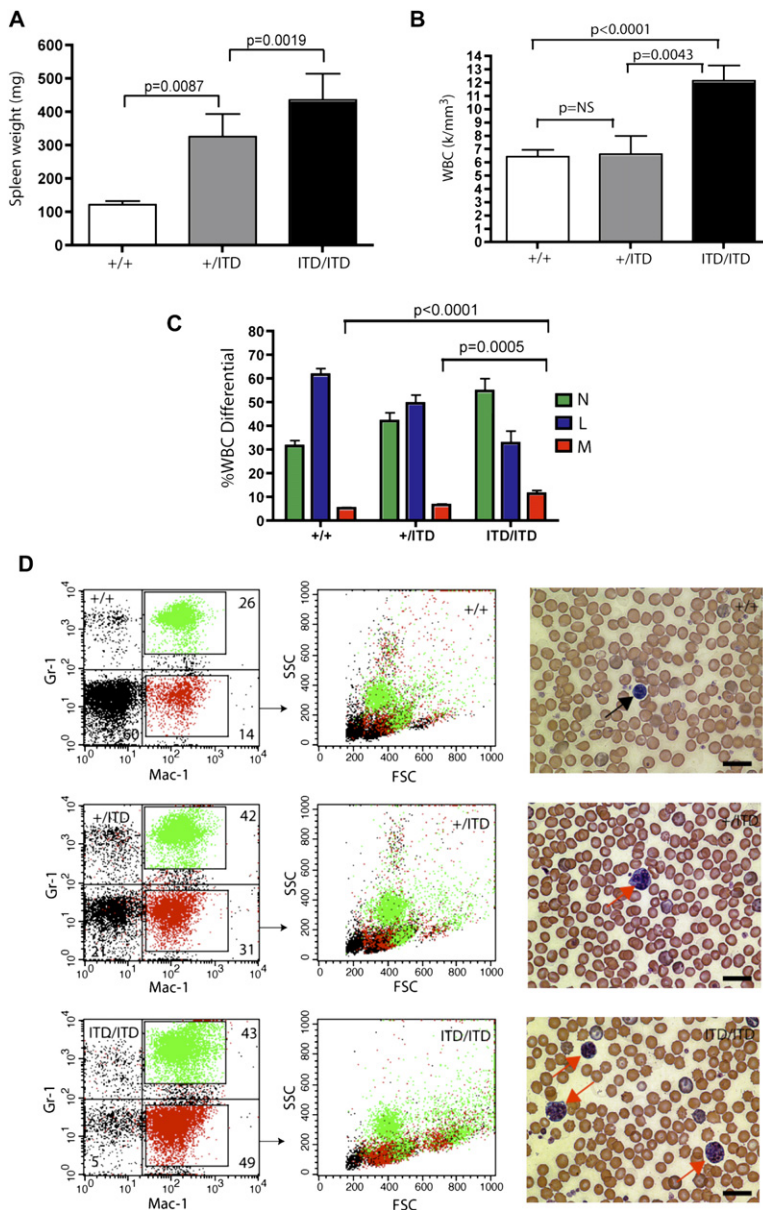


Figure 2. Myelomonocytic Expansion in *Flt3*^{+/ITD} and *Flt3*^{TD/ITD} Animals Versus Wild-Type *Flt3*^{+/+} Mice

(A) Composite data from age-matched littermates of indicated genotypes in the C57BL/6 background (mean \pm SEM; *Flt3*^{+/+}, n = 20; *Flt3*^{+/ITD}, n = 21; *Flt3*^{TD/ITD}, n = 18) demonstrating ITD dose-dependent splenomegaly. (B) Total white blood cell (WBC) counts and (C) WBC differential values from age-matched littermates of mutant and WT animals (mean \pm SEM). (D) Representative flow cytometric analysis of peripheral blood from *Flt3*^{+/+}, *Flt3*^{+/ITD}, and *Flt3*^{TD/ITD} mice for Gr1 and Mac1 highlights expanded granulocytic (green) and monocytic (red) populations, the former exhibiting high forward (FSC^{hi}) and side (SSC^{hi}) scatter properties and the latter exhibiting characteristic intermediate forward (FSC^{int}) and side (SSC^{int}) scatter properties. Peripheral blood images illustrate an unremarkable smear and lymphocyte (black arrow) in a WT animal, but progressively increased numbers of monocytes (red arrows) in mutant animals (Wright-Giemsa; scale bars, 20 μ m).

animals expressed comparable levels of Flt3 protein to wild-type (WT) littermates (Figures 1D and 1E). Compared to WT BM and spleen cells, *Flt3*^{TD/ITD} mutant mice also demonstrated a consistent increase in the levels of phospho-Flt3 and the known downstream signaling intermediate, phospho-Stat5 (Rocnik et al., 2006). This was also observed to a lesser degree in *Flt3*^{+/ITD} animals (Figure 1F).

***Flt3*^{+/ITD} and *Flt3*^{TD/ITD} Animals Develop a Myeloproliferative Disease with Monocytic Features**

Comparative analysis of *Flt3*^{+/ITD} and *Flt3*^{TD/ITD} with age-matched WT littermates demonstrated dose-dependent development of progressive splenomegaly (Figure 2A and Figure S1A in the Supplemental Data available with this article online). Mutant animals showed mildly de-

creased liver size, hemoglobin levels, and platelet counts (Figures S1A–S1D), and *Flt3*^{TD/ITD} animals exhibited significant leukocytosis and monocytosis with a reversal of neutrophil to lymphocyte ratios seen in both hetero- and homozygous animals compared to *Flt3*^{+/+} WT controls (Figures 2B and 2C). Morphologic and flow cytometric analysis of mutant animals further confirmed the expanded granulocyte (Gr1⁺/Mac1⁺/FSC^{hi}/SSC^{hi}) and monocyte (Gr1^{int}/Mac1⁺/FSC^{int}/SSC^{int}) populations in their peripheral blood (PB) (Figure 2D). Histopathology demonstrated an expansion of splenic red pulp in mutant animals comprised mainly of mature myeloid and admixed erythroid elements as well as a marked white pulp expansion by cells resembling maturing monocytes. These cells were also present in mutant BM and livers in a background of increased numbers of maturing myeloid

forms (Figure 3A) and exhibited variably positive nonspecific esterase (NSE), but negative sudan black B (SBB) staining, confirming their monocytic nature (Figure 3D). Flow analysis of mutant BM and spleens demonstrated significantly increased mature myeloid (Gr1⁺/Mac1⁺) and monocytic (Gr1^{-lo}/Mac1⁺/F4-80⁺) populations as well as immature (Mac1⁺/c-kit⁺) cells versus WT controls (Figure 3B and data not shown), which were accompanied by a concomitant decrease in the percentage of B and T lymphoid cells (Figure 3B). These findings were supported by B220, CD3, and Mac1 immunohistochemistry of mutant and WT spleens, which also demonstrated an absence of staining for MPO, CD34, and Mac3 within the expanded splenic white pulp (Figure 3C and data not shown). Additional analysis of the B cell population indicated that there was a significant loss of mature B cells (B220⁺IgM⁺; data not shown) in both the BM and the spleen from mutant animals, and further examination indicated a specific block of B cell development at the pre-B stage (B220⁺CD43⁻; Figure 3E).

Flt3-ITD myeloproliferative disease, including this block in B cell development, was transplantable into secondary recipients as determined by spleen weight, histopathologic examination, and flow cytometric studies performed on lethally irradiated (2 × 550 rad) wild-type recipient animals, receiving 1 × 10⁶ BM cells from either Flt3^{+/+} or Flt3^{ITD/ITD} animals (Figure S2), at experimental endpoint, supporting the notion that the phenotypic effects of the ITD allele were cell autonomous.

Cell Biological and Developmental Impact of FLT3-ITD Expression in the Hematopoietic Stem and Progenitor Compartment

When plated in methylcellulose cultures in the presence of growth factors, BM cells from Flt3^{ITD/ITD} mice demonstrated increased colony numbers overall, with proportionately greater numbers of monocyte colony forming units (CFU-M; Figure 4A and 4B) as well as fewer erythroid burst forming units (BFU-E) and granulocyte-erythroid-monocyte-megakaryocyte units (CFU-GEMM) (Figure 4A) compared to Flt3^{+/ITD} and Flt3^{+/+} littermates. In addition, colonies derived from Flt3^{+/ITD} or Flt3^{ITD/ITD} BM did not show any enhanced serial replating activity and consistently gave rise to fewer numbers of colonies in the second round of replating (Figure 4C), demonstrating that the ITD allele does not confer properties of self-renewal to hematopoietic progenitors in vitro.

Evaluation of the Lin⁻Sca1⁺c-kit⁺ (LSK) compartment (containing long-term and short-term HSC, as well as multipotent progenitors cells) and myeloid progenitor (Lin⁻Sca1⁻c-kit⁺) populations of Flt3^{+/ITD} and Flt3^{ITD/ITD} BM demonstrated an ~2-fold increase in the number of LSK cells ($p = 0.0034$) and a mild trend of increased myeloid progenitors in Flt3^{ITD/ITD} mice over heterozygous and WT littermates (Figure 4D). Although total numbers of common lymphoid progenitors (CLP) and total myeloid progenitors were not significantly different between genotypes (Figure S3 and Figure 4F), the ITD allele affected subpopulations within the myeloid progenitor compart-

ment, with an expansion in the relative percentage of immunophenotypic granulocyte-monocyte progenitors (GMP) and a corresponding decrease in megakaryocyte-erythroid progenitors (MEP) (Figure 4D), consistent with the myeloproliferative phenotype observed in the mice and enhanced CFU-M and reduced BFU-E. We also observed significantly reduced size and numbers of CFU-S in mice transplanted with Flt3^{ITD/ITD} LSK compared with WT LSK (Figure 4G). As CFU-S activity typically reflects primitive multipotent progenitors or ST-HSCs with considerable megakaryocyte-erythroid potential, these observations further support the erythroid potential of ST-HSCs being negatively affected in Flt3^{ITD/ITD} mice.

Analysis of the effects of the Flt3-ITD allele on cell cycle of LSK and myeloid progenitor compartments demonstrated an ITD dose-dependent increase in the proportion of LSK cells in S/G2/M with a corresponding decrease in the proportion in G0/G1 (Figures 5A and 5B). This effect appeared specific to LSK cells as no discernible differences in cell-cycle distributions were detected in myeloid progenitors from WT and mutant animals (Figures 5A and 5C). In contrast, both LSK and myeloid progenitor populations demonstrated an ITD dose-dependent decrease in the number of cells undergoing apoptosis (Figures 5D–5F), suggesting that constitutive FLT3 signaling confers differential proliferative and antiapoptotic effects within these two compartments. Similar results in cell-cycle, survival, stem cell, and progenitor analyses were observed in a younger cohort of animals (~1 month) (Figure S4), further supporting the cell-intrinsic nature of the effects conferred by the ITD allele in these animals.

FLT3-ITD Mutations Are Present in a Subset of Human CMML

FLT3 mutations have been primarily found in patients with AML. However, the hematopoietic phenotype resembling human CMML that was observed in our murine model, the fact that approximately 20% of CMML patients develop secondary AML (Fenaux et al., 1988; Onida et al., 2002), and the high prevalence of FLT3 mutations in myelomonocytic and monocytic variants of de novo AML (subtypes M4 and M5 according to the French-American-British [FAB] classification) (Kottaridis et al., 2001; Schnittger et al., 2002; Thiede et al., 2002) led us to hypothesize that activating FLT3 mutations may also be present in patients with CMML. We therefore analyzed BM samples from 194 patients with newly diagnosed CMML for the presence of FLT3-ITD mutations and FLT3-AL mutations involving codons D835 and I836. This analysis identified FLT3-ITD mutations in 6 (3.1%) of 194 cases (Table 1).

Of the 6 FLT3 mutant patients, 4 presented with “proliferative CMML,” as defined by the FAB classification, which was associated with constitutional symptoms, involvement of extramedullary organs (hepatomegaly, splenomegaly, pleural effusion; Table 2), anemia requiring transfusions, and thrombocytopenia. Furthermore, 5 of the 6 FLT3 mutant cases had a short (less than 3 months) history of an antecedent hematologic disorder. Kaplan-Meier plots indicated that the median survival of CMML

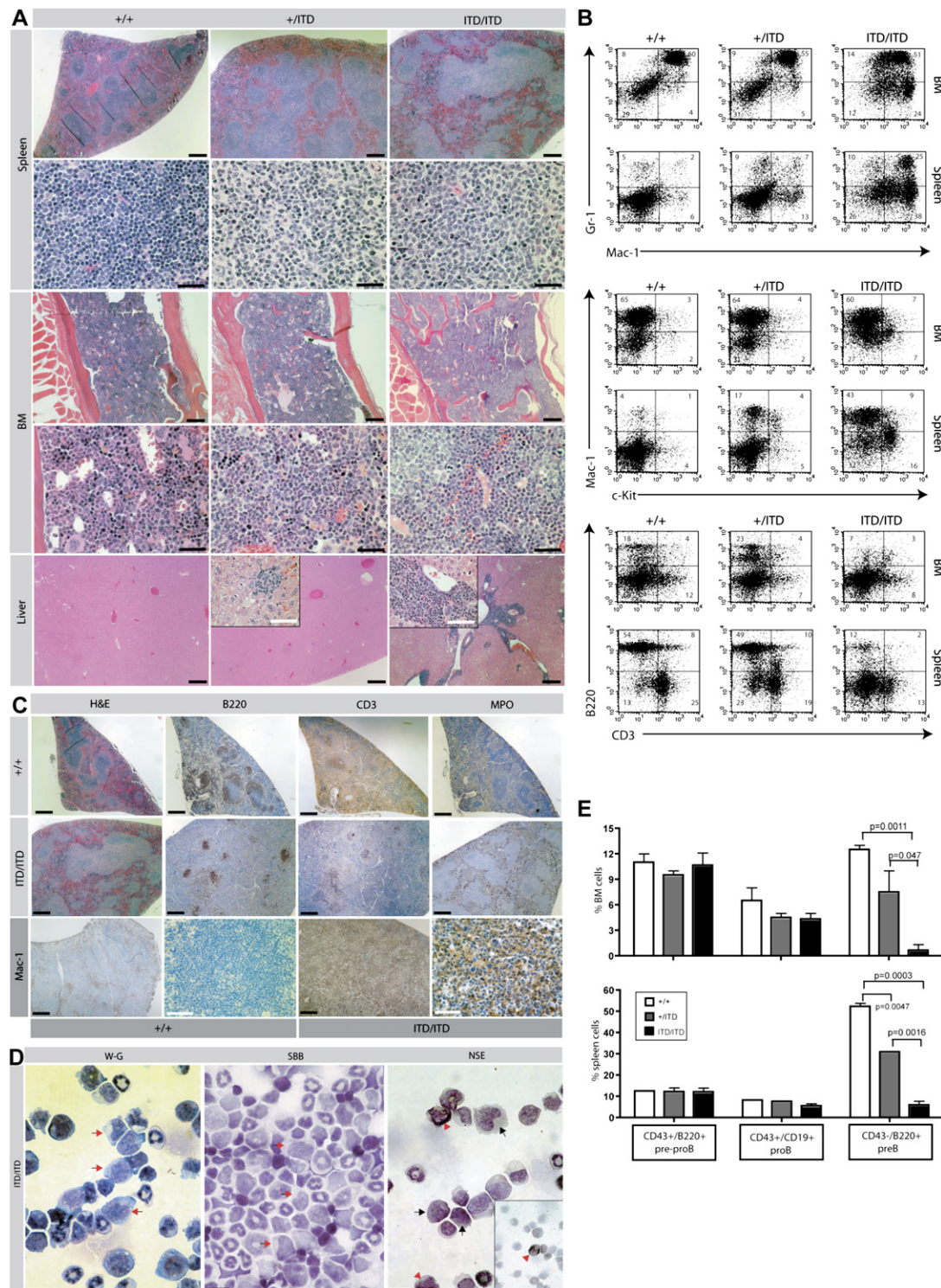


Figure 3. *Flt3*^{+/ITD} and *Flt3*^{ITD/ITD} Mice Develop a Chronic Myeloproliferative Disease with a Prominent Monocytic Component

(A) Histopathologic sections of spleen, bone marrow (BM), and liver from representative *Flt3*^{+/+} (+/+), *Flt3*^{+/ITD} (+/ITD), and *Flt3*^{ITD/ITD} (ITD/ITD) animals in the C57BL/6 background. Splenic red pulp is expanded largely by maturing myeloid and erythroid elements in both hetero- and homozygous animals that also display a prominent white pulp expansion comprised largely of intermediate sized cells with moderate amounts of pale cytoplasm (monocytes) compared to the normal small lymphocyte populations seen in WT control white pulp (spleen: first row [H&E; scale bars, 500 μ m], second row [H&E; scale bars, 50 μ m]). Clusters of morphologically similar pale cells are observed in BM and liver of *Flt3*^{ITD/ITD} mice in a background of significant maturing myeloid hyperplasia (also seen in heterozygous animals to a lesser degree) when compared with unremarkable BM from WT

patients with FLT3-ITD (8.5 months) was shorter than that of CMML patients with FLT3 wild-type (18.2 months) ($p = 0.09$, log-rank test; Figure 6). Univariate Cox proportional hazards models confirmed a significant association of FLT3-ITD with survival ($p = 0.021$, hazard ratio (HR) = 2.66, 95% confidence interval (CI) 1.56–6.13), however such an association was not significant in a multivariate Cox model, presumably due to limited cohort size.

Demographic information and comprehensive laboratory data were available for 168 of the 194 patients (Table S1). While no appreciable differences were observed in the majority of parameters, we noted that median WBC counts and serum LDH levels were modestly higher in FLT3 mutant patients than in patients with FLT3 wild-type. Although small sample size precluded attainment of statistically significant WBC and LDH parameters available for FLT3-ITD ($n = 6$) with those of FLT3 wild-type ($n = 185$), comparison between these two groups displayed trends for increased frequency of features associated with proliferative type CMML (FAB classification) (including an abnormal elevated LDH > 700 U/L, WBC count $> 13.0 \times 10^9$ /L, and the presence of organomegaly) in FLT3-ITD positive versus FLT3 wild-type patients (data not shown).

FLT3-ITD-associated CMML displayed no evidence of eosinophilia in both human cases and in our murine model (Table S2) unlike CMML associated with PDGFR β fusion proteins. Chromosome banding analysis revealed normal cytogenetics in 4 of the 6 FLT3 mutant cases, whereas the remaining 2 cases exhibited a complex karyotype and isolated monosomy 7, respectively. Of note, none of the 6 FLT3 mutant cases carried a mutation in *K-RAS* or *N-RAS*, as compared to 38 (20.2%) of the 188 cases of the FLT3 wild-type patients (Table 1).

DISCUSSION

Murine models of activating tyrosine kinase mutations and fusion proteins implicated in leukemia provide instructive platforms to examine their effects in vivo. While informative, the use of retroviral transduction or transgenic systems employing exogenous promoters in many of these models precludes accurate physiologic expression levels of these oncogenes and may ultimately affect the resultant biological and phenotypic outcome (Ren, 2004). This concept is supported in results from prior bone marrow trans-

plant studies (Kelly et al., 2002b), and transgenic animals modeling activated FLT3 mutations including our *vav* FLT3-ITD mice (Lee et al., 2005) and a recently reported Tel-FLT3 model (Baldwin et al., 2007), wherein the expression of a constitutively activated FLT3 in the form of a Tel-FLT3 fusion protein is driven by an exogenous cytomegalovirus (CMV) promoter. While all of these models have provided valuable insights into FLT3-mediated leukemogenesis, the variable phenotypes observed among these aforementioned systems support the notion that promoter choice (e.g., FLT3-ITD: *vav* and *LTR*; Tel-FLT3: CMV) plays an important role in both the nature and severity of disease phenotype.

To more precisely assess the effects of activated FLT3 expression at more appropriate physiologic levels, we have generated a murine model in which expression of a constitutively activated form of the FLT3 tyrosine kinase is under the control of the endogenous murine *Flt3* promoter, representing, to our knowledge, one of the few “knockin” leukemogenic tyrosine kinase models.

Examination of these animals has revealed insights into the in vivo effects on immature hematopoietic stem and progenitor cell populations of the FLT3-ITD, one of the most common activating tyrosine kinase mutations implicated in leukemia. Our murine data demonstrate that the leukemogenic properties conferred by FLT3-ITD are due, in part, to increased multipotent stem and progenitor cell cycling and enhanced survival properties within these compartments. These findings are supported by recent lentiviral-based FLT3-ITD expression studies in human hematopoietic stem and progenitor cells that reported increased survival and proliferation in FLT3-ITD transduced human cell populations (Li et al., 2007).

While enhanced cell survival was observed in both FLT3-ITD-positive myeloid progenitor ($\text{Lin}^- \text{Sca1}^- \text{ckit}^+$) and more primitive LSK ($\text{Lin}^- \text{Sca1}^+ \text{ckit}^+$) populations containing long-term and short-term HSC, as well as multipotent progenitors cells, increased cell cycling appeared specific to the LSK population (Figure S5). Interestingly, this LSK-specific cell-cycle aberrancy is nearly identical to that observed in triply null *FoxO1*, *FoxO3*, *FoxO4* animals when conditionally deleted in the hematopoietic system (Tothova et al., 2007). Prior reports have demonstrated that activated FLT3 receptor signaling induces phosphorylation of FoxO family members (FoxO3a) in

controls displaying normal maturing trilineage hematopoiesis (BM: first row [H&E; scale bars, 200 μm], second row [H&E; scale bars, 50 μm]; Liver [H&E; scale bars, 200 μm ; insets, 60 μm]).

(B) Flow cytometric analysis of single cell suspensions of BM and spleen of representative *Flt3*^{+/+} (+/+), *Flt3*^{+/ITD} (+/ITD), and *Flt3*^{ITD/ITD} (ITD/ITD) mice demonstrates a progressive increase in both mature granulocytic ($\text{Gr1}^+ \text{Mac1}^+$) and monocytic ($\text{Gr1}^- \text{Mac1}^+$) populations as well as immature myelomonocytic ($\text{Mac1}^{+/-} \text{ckit}^+$) cells with a corresponding decrease in the amount of B (B220^+) and T (CD3^+) lymphoid cell populations.

(C) Immunohistochemistry staining of paraffin-embedded spleen sections from *Flt3*^{+/+} and *Flt3*^{ITD/ITD} mice for B220, CD3, and Mac1 illustrate similar reductions in the B and T lymphoid populations and an expanded Mac1 population in mutant versus WT control, respectively, for both *Flt3*^{+/+} and *Flt3*^{ITD/ITD} mice. Scale bars; 500 μm (black); 50 μm (white). Stains for myeloperoxidase (MPO) highlight increased myeloid populations confined within splenic red pulp of homozygous mutant animals.

(D) Cytospins of BM cells from *Flt3*^{ITD/ITD} mice demonstrates variably weak (black arrows; third panel) to strong (red arrowheads; third panel) staining of the expanded monocyte population [first panel: red arrows, Wright-Giemsa (W-G)]; for naphthol AS-D chloroacetate esterase (third panel: NSE), but negative staining for Sudan black B (middle panel: red arrows, SBB). Scale bars, 20 μm .

(E) Flow cytometric analysis of percentage (mean \pm SEM) of pre-proB ($\text{CD43}^+ \text{B220}^-$), proB ($\text{CD43}^+ \text{CD19}^+$), and preB ($\text{CD43}^- \text{B220}^+$) cells in BM and spleen from *Flt3*^{+/+}, *Flt3*^{+/ITD}, and *Flt3*^{ITD/ITD} mice indicates an ITD allelic dose-dependent block at the preB stage of development.

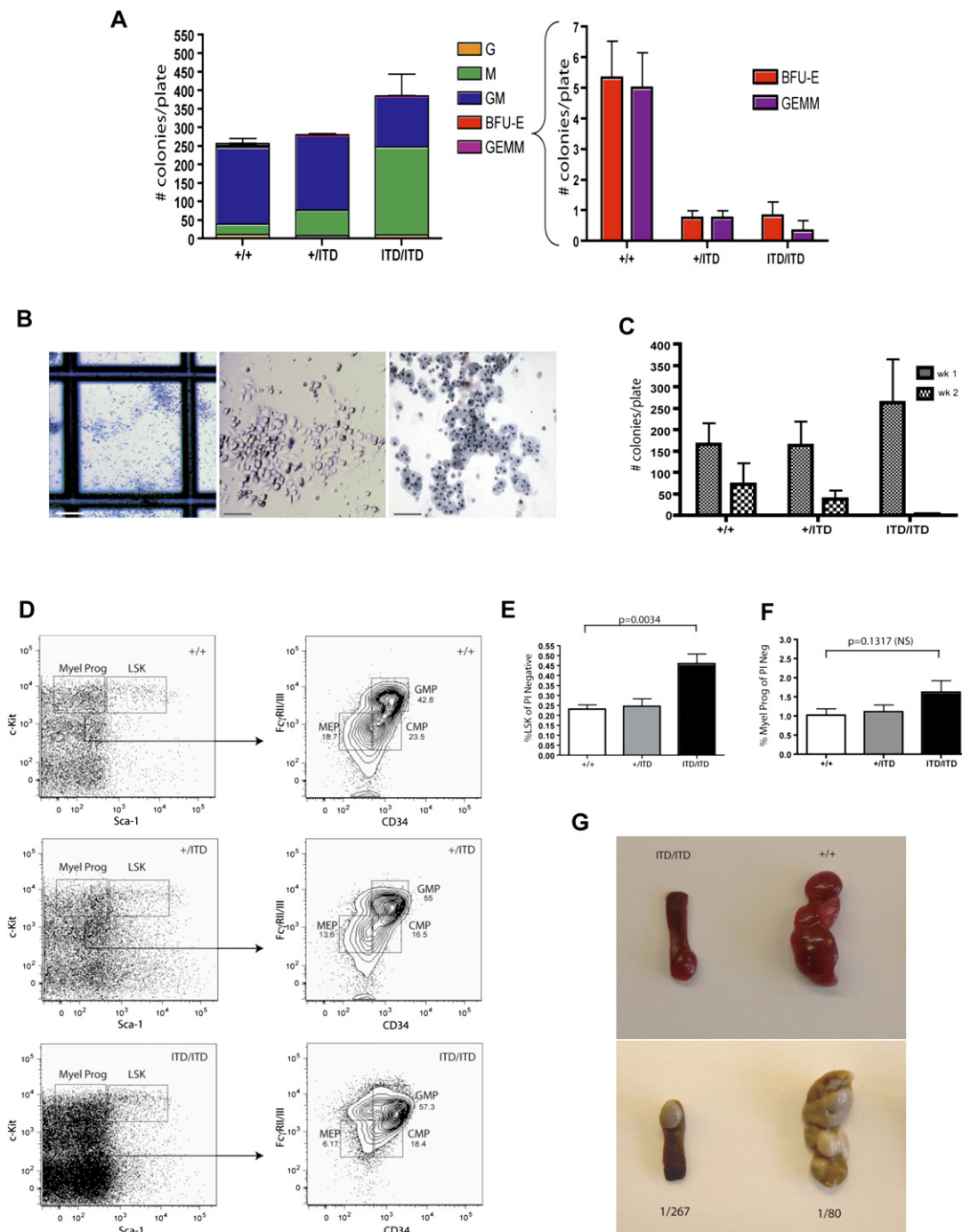


Figure 4. Colony-Forming Assays and Hematopoietic Stem Cell and Progenitor Analysis of Mutant Flt3 Animals

(A) BM cells from $Flt3^{+/+}$, $Flt3^{+/ITD}$, and $Flt3^{ITD/ITD}$ mice were plated on M3434 methylcellulose medium (containing SCF, IL-3, IL-6, EPO) and scored for colony formation 7–10 days later (G, granulocyte; M, macrophage; GM, granulocyte macrophage; BFU-E, burst-forming unit-erythroid; GEMM, granulocyte, erythroid, macrophage, megakaryocyte). Results are the average of three independent experiments performed in duplicate (mean \pm SEM are shown).

(B) Morphology of increased numbers of CFU-M colonies [left panel, scale bar, 500 μ m; middle panel; scale bar, 200 μ m, right panel; scale bar, 100 μ m (Wright-Giemsa)].

(C) Bone marrow cells harboring the mutant Flt3-ITD allele do not demonstrate serial replating capability and exhibit a dose-dependent decreased replating potential capacity. Results presented are the mean \pm SEM of two independent experiments performed in duplicate.

(D–F) Multiparameter flow cytometry demonstrates significantly increased numbers of Lin $^{-}$ Sca1 $^{+}$ ckit $^{+}$ cells and a trend toward increased myeloid progenitors in $Flt3^{ITD/ITD}$ mice over heterozygous and WT animals with a progressive increase in the relative proportions of granulocyte-monocyte

Ba/F3 cells, with resultant nuclear exclusion of FoxO proteins and transcriptional repression of FoxO-target genes critical to normal cell survival and proliferation (Brandts et al., 2005; Scheijen et al., 2004). Taken together, our data suggest that enhanced cell proliferation within the LSK compartment of our mutant FLT3 mice is mediated, at least in part, through inactivation of the FoxO transcription factor family, although we have not formally examined if there are alterations among FoxO family members within these mice.

Our studies also illustrate that constitutive signaling by FLT3-ITD at physiologic levels directs hematopoietic differentiation toward the myeloid/monocytic lineage with concomitant suppression of megakaryocyte-erythroid development (Figure S5). Moreover, while the levels of common lymphoid progenitors (CLP) appear largely indiscernible between mutant FLT3 animals and their wild-type littermates, constitutive FLT3 signaling appears to induce a block in normal B cell development, which is consistent with the prevalence of FLT3-ITD mutations associated with acute myeloid versus acute lymphoid leukemia. This finding is also intriguing given the fact that absence of *Flt3* also leads to deficiencies in normal B cell development, indicating that constitutive FLT3-ITD signaling likely interferes with normal FLT3 gene function (Mackarehtschian et al., 1995).

The phenotypic consequences of the aforementioned alterations in hematopoietic stem and progenitor cell populations results in a chronic MPD in *Flt3^{+/-ITD}* and *Flt3^{ITD/ITD}* animals resembling human CMML, which prompted us to look for the presence of activating FLT3 mutations in patient samples. Our findings along with previous reports (Lin et al., 2006; Pardananani et al., 2003), indicate that although the incidence of *FLT3* mutations is relatively uncommon in chronic MPD, in the instances where isolated FLT3 lesions in chronic MPD are detected, these patients may frequently present with CMML. Although it would be premature to conclude that CMML with FLT3-ITD is a “unique” subtype of this disease that is associated with particular clinical and laboratory features at the current time, our data suggest that this molecular feature may represent an independent negative prognostic factor in CMML. Clearly, analysis of larger cohorts will need to be examined to see whether this notion bears out in multivariate analysis models. Importantly, however, our findings support that molecular assessment for the presence of FLT3 mutations should be performed in individuals with CMML, as their survival may be improved by inhibition of FLT3-ITD with available small molecule compounds, which have shown efficacy in AML (Smith et al., 2004; Stone et al., 2005; Wadleigh et al., 2005). As imatinib mesylate has demonstrated considerable clinical efficacy in the small subset of CMML associated with PDGFR β rear-

rangements involving chromosome 5q31–q33 (Apperley et al., 2002; David et al., 2007), similar effects might be predicted with the use of available FLT3 small molecule inhibitors in patients with FLT3-ITD associated CMML.

The disease phenotype findings in prior FLT3 murine models (Baldwin et al., 2007; Kelly et al., 2002a, 2002b; Lee et al., 2005) and our current “knockin” model suggest that in patients with isolated FLT3 mutations, progression to AML is unlikely in the absence of preexisting or the subsequent development of additional genetic lesions. These data also suggest that subsequent mutations must complement constitutive FLT3 activation to ensure full transformation of hematopoietic cells and that certain combinations of mutations are not only cooperative but also interdependent in the pathogenesis of AML (Figure S5). Our model will serve as a valuable biological tool to explore the individual contributions of these different classes of leukemogenic mutations in the development of AML and molecular therapeutic regimens that target them.

EXPERIMENTAL PROCEDURES

Transgenic Mice

Gene targeting and chimera formation were used to generate an ITD “knockin” allele in exon 14 of the murine *Flt3* locus. A high fidelity PCR-based cloning strategy was employed to construct a targeting vector encompassing intronic murine genomic sequences upstream of exon 11 and downstream of exon 15 and harboring previously described human internal tandem duplication (ITD) mutation W51 (Kelly et al., 2002b) in exon 14 and a neomycin (NEO) selection cassette flanked by two loxP sites downstream of exon 15 at the *AvrII* restriction site (Figure 1A). In brief, W51 results in duplication of human FLT3 amino acids 596–602 (R-E-Y-E-Y-D-L). Human exon 14 DNA sequences harboring the W51 mutation were cloned from pGEM-FLT3 W51 (Kelly et al., 2002b) by PCR as a *BstXI*-*AvaI* fragment that was subsequently subcloned into murine exon 14 between the *BstXI* and *AvaI* restriction sites. All intronic sequences (including splice acceptor and donor sites), exons, and the W51 ITD mutation were verified by DNA sequencing. Correctly targeted ES cells were confirmed by Southern blot using probes lying beyond the regions of homology contained within the targeting vector, and chimeric mice were subsequently generated by standard methods (Brigham and Women's Hospital Transgenic Core Facility). Germline transmission of the ITD allele was achieved by crossing highly chimeric mice to wild-type C57BL/6 or Balb/c mice. Analyses were performed on animals in both the C57BL/6 and Balb/c background which showed similar phenotypes overall (data not shown). All data displayed in this report are from animals in the C57BL/6 background. *Flt3^{+/-ITD}* and *Flt3^{ITD/ITD}* and WT littermate mice were genotyped by PCR with primers *Flt3*14F (5'-AGG TACGAGAGTCAGCTGCAGATG-3') and *Flt3*14R (5'-TGTAAGATGG AGTAAGTCGCGGT-3') using the following parameters: 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 50 s. Animals were monitored two to three times a week for the presence of disease by general inspection and palpation. Peripheral blood was collected from the retro-orbital cavity using an EDTA-treated glass capillary and automated total and differential blood cell counts were determined using Hemavet 950 (Drew Scientific).

progenitors (GMP) in these mice. Plotted in (E) and (F) are the values from two independent experiments (mean \pm SEM; *Flt3^{+/-}*, n = 5; *Flt3^{+/-ITD}*, n = 6; *Flt3^{ITD/ITD}*, n = 6).

(G) Spleen colony forming unit (CFU-S) ability is diminished in *Flt3^{ITD/ITD}* HSC (Lin⁻Sca1⁺ckit⁺) cells. Duplicate WT C57BL/6 recipient mice were transplanted with 500 Lin⁻Sca1⁺ckit⁺ cells sorted from two independent *Flt3^{+/-}* or *Flt3^{ITD/ITD}* donor mice and spleens from representative recipient animals are shown. Frequency of CFU-S is calculated as 1/(no. cells transplanted/no. colonies observed).

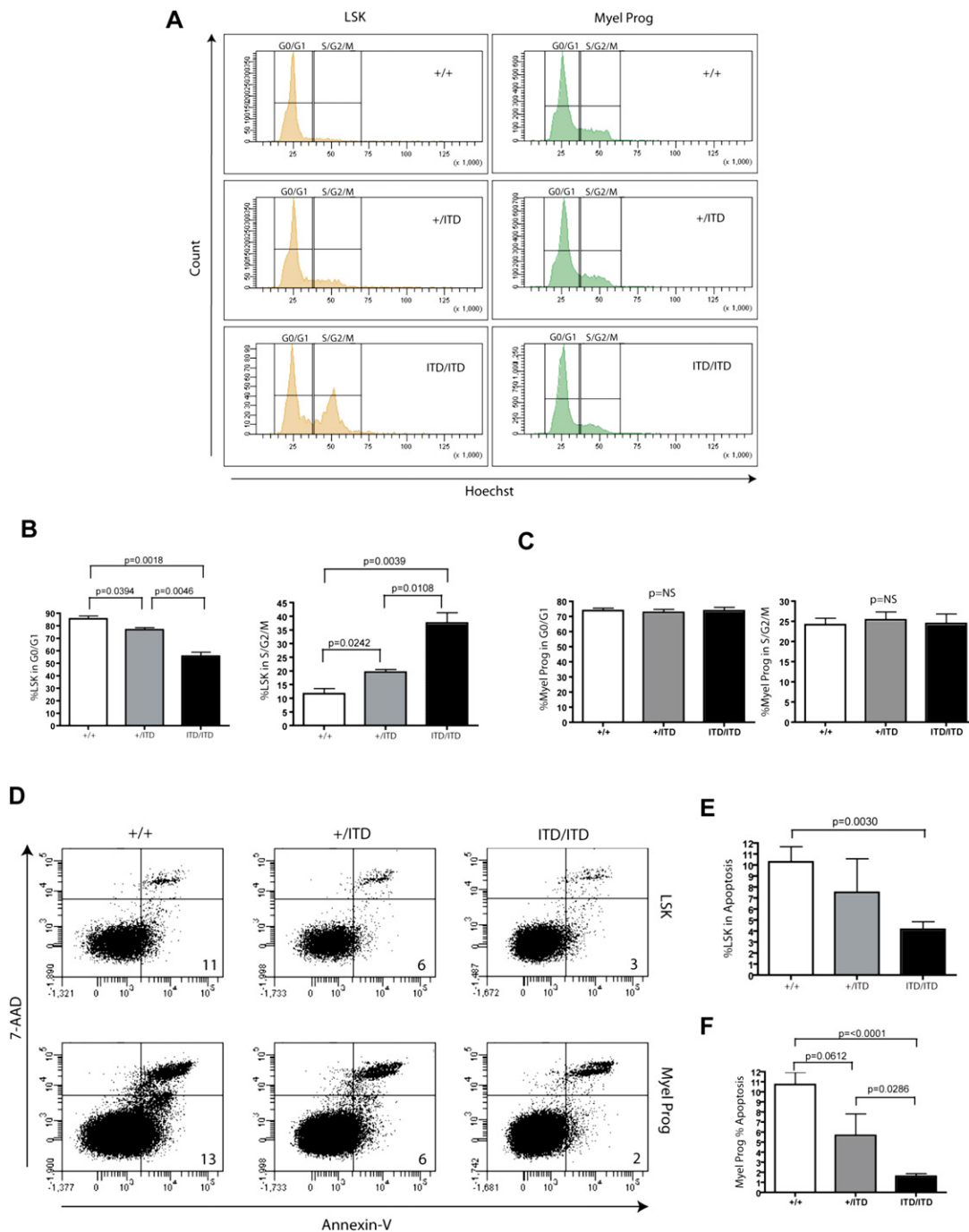


Figure 5. Effects of Activated Flt3 Expression on Cell-Cycle Distribution and Survival Within Multipotent Hematopoietic Stem and Progenitor Cell Compartments

(A–C) Multiparameter flow cytometry of primitive stem and progenitor ($\text{Lin}^{-}\text{Sca1}^{+}\text{ckit}^{+}$) and myeloid progenitor ($\text{Lin}^{-}\text{Sca1}^{-}\text{ckit}^{+}$) populations demonstrate an LSK-specific increase of cells in S/G2/M and proportional decrease in G0/G1 in an ITD dose-dependent manner. (B and C) Representative cell cycle data from one of two independent experiments (mean \pm SEM; $\text{Flt3}^{+/+}$, $n=3$; $\text{Flt3}^{+/ITD}$, $n=3$; $\text{Flt3}^{ITD/ITD}$, $n=3$; differences were statistically significant in both independent experiments, Student's t test).

(D–F) Survival analysis of HSC ($\text{Lin}^{-}\text{Sca1}^{+}\text{ckit}^{+}$) and myeloid progenitor ($\text{Lin}^{-}\text{Sca1}^{-}\text{ckit}^{+}$) cells show a dose-dependent decrease in the percentage of apoptotic cells (7-AAD $^{+}$ /Annexin-V $^{+}$) in both compartments. (E and F) Representative apoptosis data from one of two independent experiments (mean \pm SEM; $\text{Flt3}^{+/+}$, $n=3$; $\text{Flt3}^{+/ITD}$, $n=3$; $\text{Flt3}^{ITD/ITD}$, $n=3$; differences were statistically significant in both independent experiments, Student's t test).

Table 1. Mutations Identified in CMML Patients

Mutation	No. of patients ¹	Percentage (%)
FLT3-ITD ²	6	3.1
FLT3-AL ³	0	0
N-RAS, K-RAS	38	19.6

FLT3-ITD and N-RAS/K-RAS mutations identified were mutually exclusive.

¹Total number of patients analyzed (n = 194).

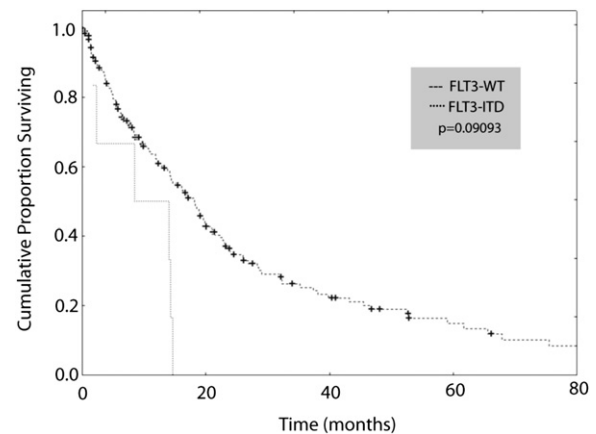
²ITD, internal tandem duplication.

³AL, activation loop.

Collected blood was also used to prepare blood smears, which were stained with Wright and Giemsa. Following sacrifice, mice were examined for the presence of tumors or other abnormalities, and organs were collected for further cell and histopathologic analysis. Single cell suspensions were made from hematopoietic organs, washed; red blood cells were lysed (Puregene) and frozen in 10% dimethylsulfoxide/90% fetal bovine serum (FBS) until analysis. All mice were housed in a pathogen-free animal facility in microisolator cages, and experiments were conducted with the ethical approval of the Harvard Medical Area Standing Committee on Animals.

Flow Cytometry and Expression Analysis

For multiparameter flow cytometry, bone marrow mononuclear cells were isolated from age-matched littermates between 1–4 months of age backcrossed to C57BL/6 at least six generations and were flushed from hind leg bones with RPMI (Cambrex, Biowhittaker) + 10% FCS (GIBCO) + Penicillin/Streptomycin (Cambrex, Biowhittaker), lysed on ice with red blood cell lysis solution (Puregene), and washed in PBS (GIBCO) + 2% FBS. HSC (LSK), CMP, GMP, MEP, and CLP populations were analyzed and sorted using a FACSAria instrument (Becton Dickinson, Mountain View, CA) as previously reported (Akashi et al., 2000; Kondo et al., 1997). Apoptosis was determined by staining freshly harvested bone marrow mononuclear cells with lineage, stem, and progenitor markers, followed by Annexin-V and 7-AAD staining. Cell-cycle analysis was carried out as previously reported (Cheng et al., 2000). Additional analyses were carried out on a 4-color FACSCalibur cytometer (Becton Dickinson, Mountain View, CA) using samples that were washed with PBS and 0.1% bovine serum albumin (BSA, Sigma), blocked with Fc-block (BD-PharMingen) for 10 min, and stained with the following monoclonal antibodies that were conjugated to either PE, FITC, PerCP, or APC: Gr-1, Mac-1, c-kit, F4/80, CD3, B220, IgM, CD45.1, CD45.2 in PBS + 0.1% BSA for 30 min. Viable cells were assessed with staining for 7-AAD. Antibodies for phospho-FLT3 (pFLT3) and phospho-Stat5 were obtained from Cell Signaling, and

**Figure 6. Kaplan-Meier Survival Plots of FLT3-ITD-Positive versus FLT3 Wild-Type Patients with CMML**

Survival data available from 193 newly diagnosed patients with CMML at the M.D. Anderson Cancer Center (MDACC) were plotted, including FLT3-ITD positive (n = 6) and FLT3-ITD negative (wild-type FLT3; n = 187) patients. FLT3-ITD positive patients exhibited a median survival time of 8.5 months compared to 18.2 months for FLT3 wild-type CMML patients (p = 0.09093; log-rank analysis). Median survival times and statistical analyses were calculated using STATISTICA data analysis software.

flow cytometry was carried out on permeabilized and fixed murine bone marrow and spleen cells as previously described (Wernig et al., 2006). A minimum of 10,000 events was acquired and analyzed using CellQuest and FlowJo software. Immunoblot expression analysis for murine Flt3 protein on primary bone marrow and spleen cells was carried out as previously described (Lee et al., 2005) using anti-Flt3 rabbit polyclonal antibody (M20) (Santa Cruz Biotechnologies).

Colony-Forming Assays

Myeloid colony-plating assays were performed in methylcellulose-based medium (M3434) containing 3 U/ml erythropoietin, 10 ng/ml recombinant murine interleukin-3 (rIL-3), 10 ng/ml rIL-6, and 50 ng/ml rmSCF (Stem Cell Technologies, Vancouver, BC, Canada). For primary methylcellulose cultures, 1×10^4 or 1×10^5 cells were seeded in duplicate and scored for colony formation 7–10 days later. Serial replating was carried out as previously described (Deguchi et al., 2003) in three independent experiments with 10^4 cells replated in duplicate for each round of replating and colony counts performed on

Table 2. Characteristics of CMML Patients with FLT3-ITD Mutations

UPIN	WHO	MDAPS	Karyotype	N-, K-RAS	Organ Involve.	Cause of Death
5886	CMML-2	Int-2	45,XX,-13[1]*	WT	pleura, liver	progressive disease
4536	CMML-1	Int-1	46,XY	WT	spleen	progressive disease
1883	CMML-1	Int-1	46,XX	WT	none	AML, sepsis
7054	CMML-1	Int-2	46,XY	WT	liver, spleen	cardiac, post chemo
1339	CMML-1	Int-2	46,XY	WT	liver, spleen	progressive disease
0729	CMML-1	Int-1	-7	WT	none	AML, sepsis

UPIN, unique patient identifier number; WHO, World Health Organization classification (CMML-1 or 2); MDAPS, M.D. Anderson Prognostic Scoring System based upon a 4-scale system of Low, Intermediate-1 (Int-1), Intermediate-2 (Int-2), or High (Beran et al., 2007; Onida et al., 2002); N-, K-RAS = genotypic status.

* 42-46,XX,+1,-3,-7,-8,del(1)(q21q42),t(6;11)(q21;q23),del(7)(p22),+mar[20].

day 7. For CFU-S assays, 500 HSC (Lin⁻Sca1⁺ckit⁺) cells were sorted from BM harvested on a FACSAria instrument (Becton Dickinson, Mountain View, CA) from two *Flt3*^{+/+} or *Flt3*^{ITD/ITD} littermate mice each and injected into lateral tail veins of lethally irradiated WT C57/BL6 recipient mice in duplicate. Day 12 CFU-S assay was carried out as previously described (Morrison and Weissman, 1994).

Histopathology, Immunohistochemistry and Cytochemistry analysis

Tissues were fixed for at least 72 hr in 10% neutral buffered formalin (Sigma), dehydrated in alcohol, cleared in xylene, and infiltrated with paraffin on an automated processor (Leica Bannockburn, IL, USA). Tissue sections (4 μ m thick) were placed on charged slides, deparaffinized in xylene, rehydrated through graded alcohol solutions, and stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed on paraffin-embedded tissue sections using primary antibodies to B220 (PharMingen), myeloperoxidase (MPO; Dako), CD3 (Dako), and Mac1 (AbCam). Cytochemical staining for Napthol AS-D Chloroacetate Esterase (NSE) (Sigma-Aldrich) and Sudan Black B (SBB) (Sigma-Aldrich) were performed on cytospin preparations from bone marrow cells according to manufacturer's protocols.

Sample Collection and DNA Sequence Analysis

Bone marrow samples from 194 patients with chronic myelomonocytic leukemia (CMML) were collected from the M.D. Anderson Cancer Center between 1999 and 2005 and analyzed for both internal tandem duplication (ITD) and activation loop (AL) mutations. PCR amplification and DNA sequencing of exons 14 and 20 of human FLT3 was performed using M13-tailed primer as previously described (Levine et al., 2005). Sequence analysis of bidirectional sequence traces was performed using Mutation Surveyor version 2.28 (SoftGenetics, State College, PA, USA). Candidate mutations were reamplified and sequenced from original DNA for independent verification. Studies involving patient samples were approved by the M.D. Anderson Ethics Committee and all subjects gave informed consent in accordance with the Declaration of Helsinki.

Supplemental Data

The Supplemental Data include five supplemental figures and two supplemental tables and can be found with this article online at <http://www.cancer-cell.org/cgi/content/full/12/4/367/DC1/>.

ACKNOWLEDGMENTS

We would like to thank C. Scholl and other members of the Gilliland laboratory for support and helpful discussions and the Dartmouth and Brigham and Women's Hospital Transgenic core facilities for technical assistance with generation of mice. This work supported by grants from the National Institutes of Health CA113434 (B.H.L.), CA66996, CA105423 (D.G.G.), and the Leukemia and Lymphoma Society (D.G.G.). S.F. was supported by grant FR 2113/1-1 from the Deutsche Forschungsgemeinschaft. B.J.P.H. is a senior clinical fellow of the Medical Research Council (UK). R.L.L. is a Basic Science Fellow of the American Society of Hematology, a Young Investigator Award recipient of the American Society of Clinical Oncology, and a Doris Duke Charitable Foundation Clinical Scientist Development Award recipient. D.G.G. is a Doris Duke Charitable Foundation Distinguished Clinical Scientist and an Investigator of the Howard Hughes Medical Institute. The Lund Stem Cell Center is supported by a Center of Excellence grant from the Swedish Foundation for Strategic Research.

Received: April 26, 2007

Revised: July 9, 2007

Accepted: August 29, 2007

Published: October 15, 2007

REFERENCES

- Akashi, K., Traver, D., Miyamoto, T., and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 404, 193–197.
- Apperley, J.F., Gardembas, M., Melo, J.V., Russell-Jones, R., Bain, B.J., Baxter, E.J., Chase, A., Chessells, J.M., Colombat, M., Dearden, C.E., et al. (2002). Response to imatinib mesylate in patients with chronic myeloproliferative diseases with rearrangements of the platelet-derived growth factor receptor beta. *N. Engl. J. Med.* 347, 481–487.
- Armstrong, S.A., Kung, A.L., Mabon, M.E., Silverman, L.B., Stam, R.W., Den Boer, M.L., Pieters, R., Kersey, J.H., Sallan, S.E., Fletcher, J.A., et al. (2003). Inhibition of FLT3 in MLL. Validation of a therapeutic target identified by gene expression based classification. *Cancer Cell* 3, 173–183.
- Armstrong, S.A., Staunton, J.E., Silverman, L.B., Pieters, R., den Boer, M.L., Minden, M.D., Sallan, S.E., Lander, E.S., Golub, T.R., and Korsmeyer, S.J. (2002). MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. *Nat. Genet.* 30, 41–47.
- Baldwin, B.R., Li, L., Tse, K.F., Small, S., Collector, M., Whartenby, K.A., Sharkis, S.J., Racke, F., Huso, D., and Small, D. (2007). Transgenic mice expressing Tel-FLT3, a constitutively activated form of FLT3, develop myeloproliferative disease. *Leukemia* 21, 764–771.
- Beran, M., Wen, S., Shen, Y., Onida, F., Jelinek, J., Cortes, J., Giles, F., and Kantarjian, H. (2007). Prognostic factors and risk assessment in chronic myelomonocytic leukemia: Validation study of the M.D. Anderson Prognostic Scoring System. *Leuk. Lymphoma* 48, 1150–1160.
- Brandts, C.H., Sargin, B., Rode, M., Biermann, C., Lindtner, B., Schwable, J., Buerger, H., Muller-Tidow, C., Choudhary, C., McMahon, M., et al. (2005). Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. *Cancer Res.* 65, 9643–9650.
- Carow, C.E., Levenstein, M., Kaufmann, S.H., Chen, J., Amin, S., Rockwell, P., Witte, L., Borowitz, M.J., Civin, C.I., and Small, D. (1996). Expression of the hematopoietic growth factor receptor FLT3 (STK-1/Flk2) in human leukemias. *Blood* 87, 1089–1096.
- Cheng, T., Rodrigues, N., Shen, H., Yang, Y., Dombkowski, D., Sykes, M., and Scadden, D.T. (2000). Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* 287, 1804–1808.
- Choudhary, C., Schwable, J., Brandts, C., Tickenbrock, L., Sargin, B., Kindler, T., Fischer, T., Berdel, W.E., Muller-Tidow, C., and Serve, H. (2005). AML-associated Flt3 kinase domain mutations show signal transduction differences compared with Flt3 ITD mutations. *Blood* 106, 265–273.
- DaSilva, N., Hu, Z.B., Ma, W., Rosnet, O., Birnbaum, D., and Drexler, H.G. (1994). Expression of the FLT3 gene in human leukemia-lymphoma cell lines. *Leukemia* 8, 885–888.
- David, M., Cross, N.C., Burgstaller, S., Chase, A., Curtis, C., Dang, R., Gardembas, M., Goldman, J.M., Grand, F., Hughes, G., et al. (2007). Durable responses to imatinib in patients with PDGFRB fusion gene-positive and BCR-ABL-negative chronic myeloproliferative disorders. *Blood* 109, 61–64.
- Deguchi, K., Ayton, P.M., Carapeti, M., Kutok, J.L., Snyder, C.S., Williams, I.R., Cross, N.C., Glass, C.K., Cleary, M.L., and Gilliland, D.G. (2003). MOZ-TIF2-induced acute myeloid leukemia requires the MOZ nucleosome binding motif and TIF2-mediated recruitment of CBP. *Cancer Cell* 3, 259–271.
- Drexler, H.G. (1996). Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells. *Leukemia* 10, 588–599.
- Fenaux, P., Beuscart, R., Lai, J.L., Jouet, J.P., and Bauters, F. (1988). Prognostic factors in adult chronic myelomonocytic leukemia: An analysis of 107 cases. *J. Clin. Oncol.* 6, 1417–1424.
- Frohling, S., Schlenk, R.F., Breitnick, J., Benner, A., Kreitmeier, S., Tobis, K., Dohner, H., and Dohner, K. (2002). Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with

acute myeloid leukemia and normal cytogenetics: A study of the AML Study Group Ulm. *Blood* 100, 4372–4380.

Gabbianelli, M., Pelosi, E., Montesoro, E., Valtieri, M., Luchetti, L., Samoggia, P., Vitelli, L., Barberi, T., Testa, U., Lyman, S., et al. (1995). Multi-level effects of flt3 ligand on human hematopoiesis: Expansion of putative stem cells and proliferation of granulomonocytic progenitors/monocytic precursors. *Blood* 86, 1661–1670.

Gilliland, D.G., and Griffin, J.D. (2002). The roles of FLT3 in hematopoiesis and leukemia. *Blood* 100, 1532–1542.

Grundler, R., Miething, C., Thiede, C., Peschel, C., and Duyster, J. (2005). FLT3-ITD and tyrosine kinase domain mutants induce 2 distinct phenotypes in a murine bone marrow transplantation model. *Blood* 105, 4792–4799.

Hayakawa, F., Towatari, M., Kiyoi, H., Tanimoto, M., Kitamura, T., Saito, H., and Naoe, T. (2000). Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines. *Oncogene* 19, 624–631.

Hjertson, M., Sundstrom, C., Lyman, S.D., Nilsson, K., and Nilsson, G. (1996). Stem cell factor, but not flt3 ligand, induces differentiation and activation of human mast cells. *Exp. Hematol.* 24, 748–754.

Horiike, S., Yokota, S., Nakao, M., Iwai, T., Sasai, Y., Kaneko, H., Taniwaki, M., Kashima, K., Fujii, H., Abe, T., and Misawa, S. (1997). Tandem duplications of the FLT3 receptor gene are associated with leukemic transformation of myelodysplasia. *Leukemia* 11, 1442–1446.

Ishii, E., Zaitzu, M., Ihara, K., Hara, T., and Miyazaki, S. (1999). High expression but no internal tandem duplication of FLT3 in normal hematopoietic cells. *Pediatr. Hematol. Oncol.* 16, 437–441.

Kelly, L.M., Kutok, J.L., Williams, I.R., Boulton, C.L., Amaral, S.M., Curley, D.P., Ley, T.J., and Gilliland, D.G. (2002a). PML/RAR α and FLT3-ITD induce an APL-like disease in a mouse model. *Proc. Natl. Acad. Sci. USA* 99, 8283–8288.

Kelly, L.M., Liu, Q., Kutok, J.L., Williams, I.R., Boulton, C.L., and Gilliland, D.G. (2002b). FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. *Blood* 99, 310–318.

Kiyoi, H., Naoe, T., Nakano, Y., Yokota, S., Minami, S., Miyawaki, S., Asou, N., Kuriyama, K., Jinnai, I., Shimazaki, C., et al. (1999). Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood* 93, 3074–3080.

Kiyoi, H., Towatari, M., Yokota, S., Hamaguchi, M., Ohno, R., Saito, H., and Naoe, T. (1998). Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. *Leukemia* 12, 1333–1337.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 91, 661–672.

Kottaridis, P.D., Gale, R.E., Frew, M.E., Harrison, G., Langabeer, S.E., Belton, A.A., Walker, H., Wheatley, K., Bowen, D.T., Burnett, A.K., et al. (2001). The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: Analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood* 98, 1752–1759.

Lee, B.H., Williams, I.R., Anastasiadou, E., Boulton, C.L., Joseph, S.W., Amaral, S.M., Curley, D.P., Duclos, N., Huntly, B.J., Fabbro, D., et al. (2005). FLT3 internal tandem duplication mutations induce myeloproliferative or lymphoid disease in a transgenic mouse model. *Oncogene* 24, 7882–7892.

Levine, R.L., Wadleigh, M., Cools, J., Ebert, B.L., Wernig, G., Huntly, B.J., Boggon, T.J., Wlodarska, I., Clark, J.J., Moore, S., et al. (2005). Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell* 7, 387–397.

Li, L., Piloto, O., Kim, K.T., Ye, Z., Nguyen, H.B., Yu, X., Levis, M., Cheng, L., and Small, D. (2007). FLT3/ITD expression increases expansion, survival and entry into cell cycle of human haematopoietic stem/progenitor cells. *Br. J. Haematol.* 137, 64–75.

Lin, P., Jones, D., Medeiros, L.J., Chen, W., Vega-Vazquez, F., and Luthra, R. (2006). Activating FLT3 mutations are detectable in chronic and blast phase of chronic myeloproliferative disorders other than chronic myeloid leukemia. *Am. J. Clin. Pathol.* 126, 530–533.

Mackarehtschian, K., Hardin, J.D., Moore, K.A., Boast, S., Goff, S.P., and Lemischka, I.R. (1995). Targeted disruption of the flk2/flt3 gene leads to deficiencies in primitive hematopoietic progenitors. *Immunity* 3, 147–161.

Meierhoff, G., Dehmel, U., Gruss, H.J., Rosnet, O., Birnbaum, D., Quentmeier, H., Dirks, W., and Drexler, H.G. (1995). Expression of FLT3 receptor and FLT3-ligand in human leukemia-lymphoma cell lines. *Leukemia* 9, 1368–1372.

Mizuki, M., Fenski, R., Halfter, H., Matsumura, I., Schmidt, R., Muller, C., Gruning, W., Kratz-Albers, K., Serve, S., Steur, C., et al. (2000). Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. *Blood* 96, 3907–3914.

Morrison, S.J., and Weissman, I.L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* 1, 661–673.

Nakao, M., Yokota, S., Iwai, T., Kaneko, H., Horiike, S., Kashima, K., Sonoda, Y., Fujimoto, T., and Misawa, S. (1996). Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia* 10, 1911–1918.

Onida, F., Kantarjian, H.M., Smith, T.L., Ball, G., Keating, M.J., Estey, E.H., Glassman, A.B., Albitar, M., Kwari, M.I., and Beran, M. (2002). Prognostic factors and scoring systems in chronic myelomonocytic leukemia: A retrospective analysis of 213 patients. *Blood* 99, 840–849.

Paietta, E., Ferrando, A.A., Neuberg, D., Bennett, J.M., Racevskis, J., Lazarus, H., Dewald, G., Rowe, J.M., Wiernik, P.H., Tallman, M.S., and Look, A.T. (2004). Activating FLT3 mutations in CD117/KIT positive T-Cell acute lymphoblastic leukemias. *Blood* 104, 558–560.

Pardanani, A., Reeder, T.L., Kimlinger, T.K., Baek, J.Y., Li, C.Y., Butterfield, J.H., and Tefferi, A. (2003). Flt-3 and c-kit mutation studies in a spectrum of chronic myeloid disorders including systemic mast cell disease. *Leuk. Res.* 27, 739–742.

Rasko, J.E., Metcalf, D., Rossner, M.T., Begley, C.G., and Nicola, N.A. (1995). The flt3/flk-2 ligand: Receptor distribution and action on murine haemopoietic cell survival and proliferation. *Leukemia* 9, 2058–2066.

Ratajczak, M.Z., Ratajczak, J., Ford, J., Kregenow, R., Marlicz, W., and Gewirtz, A.M. (1996). FLT3/FLK-2 (STK-1) Ligand does not stimulate human megakaryopoiesis in vitro. *Stem Cells* 14, 146–150.

Reindl, C., Bagrintseva, K., Vempati, S., Schnittger, S., Ellwart, J.W., Wenig, K., Hopfner, K.P., Hiddemann, W., and Spiekermann, K. (2006). Point mutations in the juxtamembrane domain of FLT3 define a new class of activating mutations in AML. *Blood* 107, 3700–3707.

Ren, R. (2004). Modeling the dosage effect of oncogenes in leukemogenesis. *Curr. Opin. Hematol.* 11, 25–34.

Rocnik, J.L., Okabe, R., Yu, J.C., Lee, B.H., Giese, N., Schenkein, D.P., and Gilliland, D.G. (2006). Roles of tyrosine 589 and 591 in STAT5 activation and transformation mediated by FLT3-ITD. *Blood* 108, 1339–1345.

Rosnet, O., and Birnbaum, D. (1993). Hematopoietic receptors of class III receptor-type tyrosine kinases. *Crit. Rev. Oncog.* 4, 595–613.

Rosnet, O., Buhring, H.J., Marchetto, S., Rappold, I., Lavagna, C., Sainty, D., Arnoulet, C., Chabannon, C., Kanz, L., Hannum, C., and Birnbaum, D. (1996). Human FLT3/FLK2 receptor tyrosine kinase is expressed at the surface of normal and malignant hematopoietic cells. *Leukemia* 10, 238–248.

- Scheijen, B., Ngo, H.T., Kang, H., and Griffin, J.D. (2004). FLT3 receptors with internal tandem duplications promote cell viability and proliferation by signaling through Foxo proteins. *Oncogene* 23, 3338–3349.
- Schnittger, S., Schoch, C., Dugas, M., Kern, W., Staib, P., Wuchter, C., Löffler, H., Sauerland, C.M., Serve, H., Buchner, T., et al. (2002). Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: Correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood* 100, 59–66.
- Smith, B.D., Levis, M., Beran, M., Giles, F., Kantarjian, H., Berg, K., Murphy, K.M., Dausers, T., Allebach, J., and Small, D. (2004). Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia. *Blood* 103, 3669–3676.
- Stirewalt, D.L., Meshinchi, S., Kussick, S.J., Sheets, K.M., Pogossova-Agadjanian, E., Willman, C.L., and Radich, J.P. (2004). Novel FLT3 point mutations within exon 14 found in patients with acute myeloid leukaemia. *Br. J. Haematol.* 124, 481–484.
- Stirewalt, D.L., and Radich, J.P. (2003). The role of FLT3 in haematopoietic malignancies. *Nat. Rev. Cancer* 3, 650–665.
- Stone, R.M., DeAngelo, D.J., Klimek, V., Galinsky, I., Estey, E., Nimer, S.D., Grandin, W., Lebwohl, D., Wang, Y., Cohen, P., et al. (2005). Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. *Blood* 105, 54–60.
- Thiede, C., Steudel, C., Mohr, B., Schaich, M., Schakel, U., Platzbecker, U., Wermke, M., Bornhauser, M., Ritter, M., Neubauer, A., et al. (2002). Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: Association with FAB subtypes and identification of subgroups with poor prognosis. *Blood* 99, 4326–4335.
- Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C., et al. (2007). FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell* 128, 325–339.
- Wadleigh, M., DeAngelo, D.J., Griffin, J.D., and Stone, R.M. (2005). After chronic myelogenous leukemia: Tyrosine kinase inhibitors in other hematologic malignancies. *Blood* 105, 22–30.
- Wernig, G., Mercher, T., Okabe, R., Levine, R.L., Lee, B.H., and Gilliland, D.G. (2006). Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. *Blood* 107, 4274–4281.
- Whitman, S.P., Archer, K.J., Feng, L., Baldus, C., Becknell, B., Carlson, B.D., Carroll, A.J., Mrozek, K., Vardiman, J.W., George, S.L., et al. (2001). Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of FLT3: A cancer and leukemia group B study. *Cancer Res.* 61, 7233–7239.
- Yamamoto, Y., Kiyoi, H., Nakano, Y., Suzuki, R., Kadera, Y., Miyawaki, S., Asou, N., Kuriyama, K., Yagasaki, F., Shimazaki, C., et al. (2001). Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood* 97, 2434–2439.