Impact of gene dosage, loss of wild-type allele, and FLT3 ligand on *Flt3-ITD*–induced myeloproliferation

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Acquisition of homozygous activating growth factor receptor mutations might accelerate cancer progression through a simple gene-dosage effect. Internal tandem duplications (ITDs) of FLT3 occur in approximately 25% cases of acute myeloid leukemia and induce ligand-independent constitutive signaling. Homozygous *FLT3-ITD*s confer an adverse prognosis and are frequently detected at relapse. Using a mouse knockin model of *Flt3*–internal tandem duplication (*Flt3-ITD*)–induced myeloproliferation, we herein demonstrate that the enhanced myeloid phenotype and expansion of granulocyte-monocyte and primitive Lin$^-$Sca1$^+$c-Kit$^+$ progenitors in *Flt3-ITD* homozygous mice can in part be mediated through the loss of the second wild-type allele. Further, whereas autocrine FLT3 ligand production has been implicated in FLT3-ITD myeloid malignancies and resistance to FLT3 inhibitors, we demonstrate here that the mouse *Flt3$^{ITD/ITD}$* myeloid phenotype is FLT3 ligand-independent. (*Blood*. 2011;118(13): 3613-3621)

Introduction

Activating mutations of growth factor receptors (GFRs) are frequent events in many tumor types. A common theme of such mutations is the acquisition of mutant allele specific imbalance (MASI) either because of copy number-neutral loss of heterozygosity or mutant allele amplification, particularly during disease progression.[1] For example, MASI affects epidermal GFR (*EGFR*) mutations in lung cancer and glioblastoma,[2] *KIT* mutations in gastrointestinal tumors,[3] and *MPL* mutations in myelofibrosis.[4] MASI of activated GFRs may accelerate disease through a simple gene-dosage effect, although it is also possible that loss of the wild-type (WT) protein enhances the impact of the mutant allele, for example, by enhancing formation of mutant homodimers. Although the impact of mutant GFR gene dosage has been modeled in vivo,[5-8] less is known about the impact of loss of the second WT allele. In the case of activating *RET* mutations in endocrine neoplasia, deletion of the WT allele occurs in connection with tumor progression,[9,10] although in a mouse model (*Ret^{MEN2B}*), hemizygous *Ret* mutations were not biologically distinct from heterozygous mutations.[11]

Although many mutations confer a degree of ligand-independent GFR activation, in vitro studies often observe an additional impact of exogenous ligand. EGFR,[12] platelet-derived GFR,[13] and MET receptor tyrosine kinase mutations[14] are all dependent on their ligands for full transforming activity.

FMS-like tyrosine kinase3 (FLT3) is a receptor tyrosine kinase expressed on normal hematopoietic multipotent progenitors[15] and acute leukemia blast cells.[16] Internal tandem duplications (ITDs) within the juxtamembrane domain of FLT3, inducing ligand-independent dimerization and constitutive signaling, occur in ~ 25% of acute myeloid leukemia (AML).[16] FLT3-ITD is associated with high relapse rate and poor overall survival in AML.[16,17] As with other GFR mutations, MASI at the *FLT3* locus in *FLT3-ITD*$^+$ AML is associated with a particularly poor prognosis[18] and occurs because of copy number-neutral loss of heterozygosity with consequent ITD homozygosity.[19] Although MASI is uncommon at diagnosis (~ 15% of ITD$^+$ cases), it is frequently observed at relapse.[20]

Mice heterozygous for an *ITD* at the endogenous *Flt3* locus develop chronic myeloproliferative disease (MPD) with expanded myeloid progenitor cells.[7,21] Importantly, *ITD* homozygosity results in a more severe MPD phenotype.[7] However, it remains unclear to what degree disease progression only reflects an *ITD* gene dosage effect and/or whether loss of the WT allele itself might accelerate the phenotype of heterozygous *ITD*s.

Although ITDs clearly induce FLT3 ligand (FL)–independent autophosphorylation of FLT3,[16] addition of exogenous FL to FL-deficient ITD cell lines results in enhanced FLT3 receptor activation.[22] Furthermore, FL increases ITD-induced activation of STAT pathways in vitro, a mechanism proposed to mediate resistance to FLT3 inhibitors.[23] It has recently been suggested that increases in FL levels can inhibit the efficiency of FLT3 inhibitors in patients with ITD$^+$ AML.[24] Moreover, some ITD$^+$ AML blast

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cells express FL and also respond to FL in vitro,[22,25,26] implicating autocrine FL production as an important mechanism of activation of FLT3-ITDs. However, the in vivo impact of FL on the ITD-induced MPD phenotype is unclear and needs further investigation.

In the present study, we crossed *Flt3-ITD* knockin mice[7] with *Flt3* receptor[27] and *Fl* knockout mice[28] to specifically address the impact of the loss of the WT allele or the ligand, on the myeloid phenotype induced by FLT3-ITD.

Methods

Mice

Flt3-ITD knockin mice on C57BL/6 background were previously described.[7] $Flt3^{-/ITD}$ mice were generated by cross-breeding of $Flt3^{ITD/ITD}$ and $Flk2^{-/-}$ ($Flt3^{-/-}$) mice (on C57BL/6 background[27]; kindly provided by Dr Ihor Lemischka, Mt Sinai, NY). *Flt3-ITD*x$Fl^{-/-}$ mice were obtained by cross-breeding of either $Flt3^{ITD/ITD}$ or $Flt3^{+/ITD}$ with $Fl^{-/-}$ (on C57BL/6 background[28]) mice. WT ($Flt3^{+/+}$) C57BL/6 mice from The Jackson Laboratory were used as WT controls. Congenic CD45.1 and CD45.2 WT C57BL/6 mice were used as controls and recipients in transplantation experiments. All experiments were approved by the Ethical Committee at Lund University.

Fluorescent antibodies and immunomagnetic beads used for FACS analysis and sorting

Antibodies used for cell surface staining were as follows: CD11b/Mac1 (M1/70), CD4 (H129.19), CD8a (53–6.7), B220/CD45 (RA3–6B2), CD5 (Ly1), Ter119 (Ter-1119), Gr1/Ly6G, and Ly6C (RB6–8C5), CD19 (ID3), CD41/Itga2b (MWReg30), CD135/Flt3 (A2F10.1), CD45.1 (A20), CD45.2 (104; BD Biosciences PharMingen); NK1.1 (PK136), Sca1 (D7), CD117/c-Kit (2B8), CD16/32 (93) CD105/Eng (MJ7/18; eBioscience). Biotinylated antibodies were visualized with streptavidin-QD655 (Invitrogen) or streptavidin-Tricolor (Invitrogen), and purified lineage antibodies were visualized with polyclonal goat anti–rat Tricolor (Invitrogen) or polyclonal goat anti–rat-QD605 (Invitrogen). MACS column enrichment of c-Kit^+ cells was done using anti-CD117 immunomagnetic beads (Miltenyi Biotec) as previously described.[29]

Flow cytometric analysis

B cells were identified as $Ter119^-NK1.1^-CD19^+$ and immature myelomonocytic cells as $Ter119^-CD4/8^-CD19^-NK1.1^-Mac1^{low/+}$c-$Kit^{low/+}$ cells. Hematopoietic stem and progenitor cells were analyzed as previously described.[15,30,31] Briefly, bone marrow (BM) or spleen cells were stained with a cocktail of purified rat antibodies against lineage markers B220, CD4, CD5, CD8α, CD11b, Gr1, and Ter119. $Lineage^+$ cells were visualized with a goat anti–rat-Tricolor or -QD605 staining, followed by c-Kit enrichment for sorting analyses. Thereafter, hematopoietic stem/progenitor cells were defined as Lin^-Sca1^+c-Kit^+ (LSK), pre-granulocyte-monocyte progenitors (pre-GMPs; Lin^-c-$Kit^+Sca1^-[LKS^-]CD41^-CD16/32^{low/-}CD150^-CD105^{low/-}$), GMPs ($LKS^-CD16/32^{hi}CD150^-$), megakaryocyte progenitors (MkP, $LKS^-CD41^+CD150^+$), erythroid colony-forming units (CFU-E; $LKS^-CD41^-CD16/32^{-/low}CD150^-CD105^+$), pre-CFU-E ($LKS^-CD41^-CD16/32^{-/low}CD150^+CD105^+$), and pre-megakaryocyte-erythroid progenitors (Pre-MegE, $LKS^-CD41^-CD16/32^{-/low}CD150^+CD105^{-/low}$). Propidium iodide (Invitrogen) or 7-amino-actinomycin D (Sigma-Aldrich) was used to exclude dead cells. Cell acquisition and analysis were performed on a 4-laser LSRII (BD Biosciences) using FlowJo Version 8.8 software (TreeStar). Cell sorting was done on a FACSAria (BD Biosciences).

Competitive transplantation assay

A total of 3×10^5 $CD45.1^+$ competitor BM cells obtained from WT mice were mixed with 3×10^6 $CD45.2^+$ unfractionated spleen cells from $Flt3^{+/+}$, $Flt3^{+/ITD}$, $Flt3^{ITD/ITD}$, and $Flt3^{-/ITD}$ mice and transplanted into lethally irradiated (900 cGy) recipient mice. Flow cytometric analysis of CD45.1 and CD45.2 reconstitution and donor-derived lineage reconstitution were assessed 32 weeks after transplantation.

Myeloid progenitor assays

To analyze myeloid potential of unfractionated BM cells, a granulocyte-macrophage colony-forming unit (CFU-GM) assay was performed as described before.[32] Briefly, freshly isolated unfractionated BM or spleen cells were plated in methylcellulose (M3134; StemCell Technologies) supplemented with 20% FCS (Thermo Fisher Scientific), 1% L-glutamine, 1% penicillin/streptomycin (PAA), 0.1mM 2-mercaptoethanol (Sigma-Aldrich), human FLT3 ligand (FL, 10 ng/mL, PeproTech), mouse IL-3 (2 ng/mL; PeproTech), human granulocyte-colony stimulating factor (G-CSF, 10 ng/mL; Amgen), and mouse granulocyte-monocyte colony stimulating factor (5 ng/mL; Immunex) in 35-mm Petri dishes. Colonies (> 50 cells) were scored using an Olympus IX70 inverted microscope (Olympus) after 7 and 10 days of incubation at 37°C, 98% humidity, and 5% CO_2.

For evaluation of GM potential, cells were manually plated at a dilution of 1 cell per well with X-vivo 15 (Lonza Walkersville) supplemented with 0.5% detoxified BSA (StemCell Technologies), 10% FCS, 0.1mM 2-mercaptoethanol, 1% penicillin/streptomycin, and 25 ng/mL mouse stem cell factor (PeproTech), 25 ng/mL FL, 25 ng/mL human thrombopoietin (PeproTech), 20 ng/mL GM-CSF, 25 ng/mL G-CSF, and 20 ng/mL IL-3. Wells were scored for clonal growth with an inverted light microscope after 8 days of culture. Percentage of cloning efficiency was calculated according to the Poisson distribution, which predicts that 63% of wells should contain 1 or more cells following manual plating (76 of 120 wells). For the evaluation of in vitro cytokine responses, LSK cells were cultured as described for evaluation of GM potential in the presence or absence of 25 ng/mL FL and/or 10 ng/mL IL-3 as indicated. Wells were scored for clonal growth with an inverted light microscope after 8 days of culture.

In vitro colony replating assay for LSK cells

For primary cultures, 30 sorted LSK cells were cultured in M3134 methylcellulose supplemented with FCS, L-glutamine, penicillin/streptomycin, and 2-mercaptoethanol together with stem cell factor (50 ng/mL), IL-3 (10 ng/mL), and/or FL (50 ng/mL). After 7 days of culture and counting colonies, the secondary clonogenic assay was performed by pooling and counting cells from primary cultures and replating 10 000 cells in M3134 medium with the same conditions. Tertiary replating was done in the same way.

Inhibitor assays

CEP701 (Lestaurtinib) was obtained from Tocris Biosciences and dissolved in DMSO to prepare an initial 4mM stock solution. Serial dilutions were then made just before use to obtain final dilutions for cellular assays. Methylcellulose assay was done as described for myeloid progenitor assays with 0.3×10^6 unfractionated BM cells. Human G-CSF was added to the cell cultures at a concentration of 50 ng/mL.

Gene expression analysis by dynamic arrays

Real-time PCR analysis was performed using the nanofluidic BioMark 48.48 Dynamic Array (Fluidigm) and TaqMan Gene Expression Assays (Applied Biosystems). The gene expression assays used are shown in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). cDNA generation and gene-specific preamplification were carried out using CellsDirect One-Step qRT-PCR Kit (Invitrogen). In brief, 200 cells were sorted directly into 10 μL of reaction buffer containing the 5 μL of CellsDirect 2× Reaction Mix (Invitrogen), 1.2 μL of CellsDirect RT/Taq Mix, 1.2 μL TE buffer, 0.1 μL SUPERase-In RNase Inhibitor (Ambion, AM2694), and 2.5 μL of a mix of 0.2× TaqMan Gene Expression Assays (Applied Biosystems; supplemental Table 1). Reverse transcription and specific target preamplification were carried out with the following conditions: 15 minutes at 50°C followed by 2 minutes at 95°C; then 22 cycles of 95°C for 15 seconds and 60°C for 4 minutes. After this, 40 μL of TE buffer was added to the

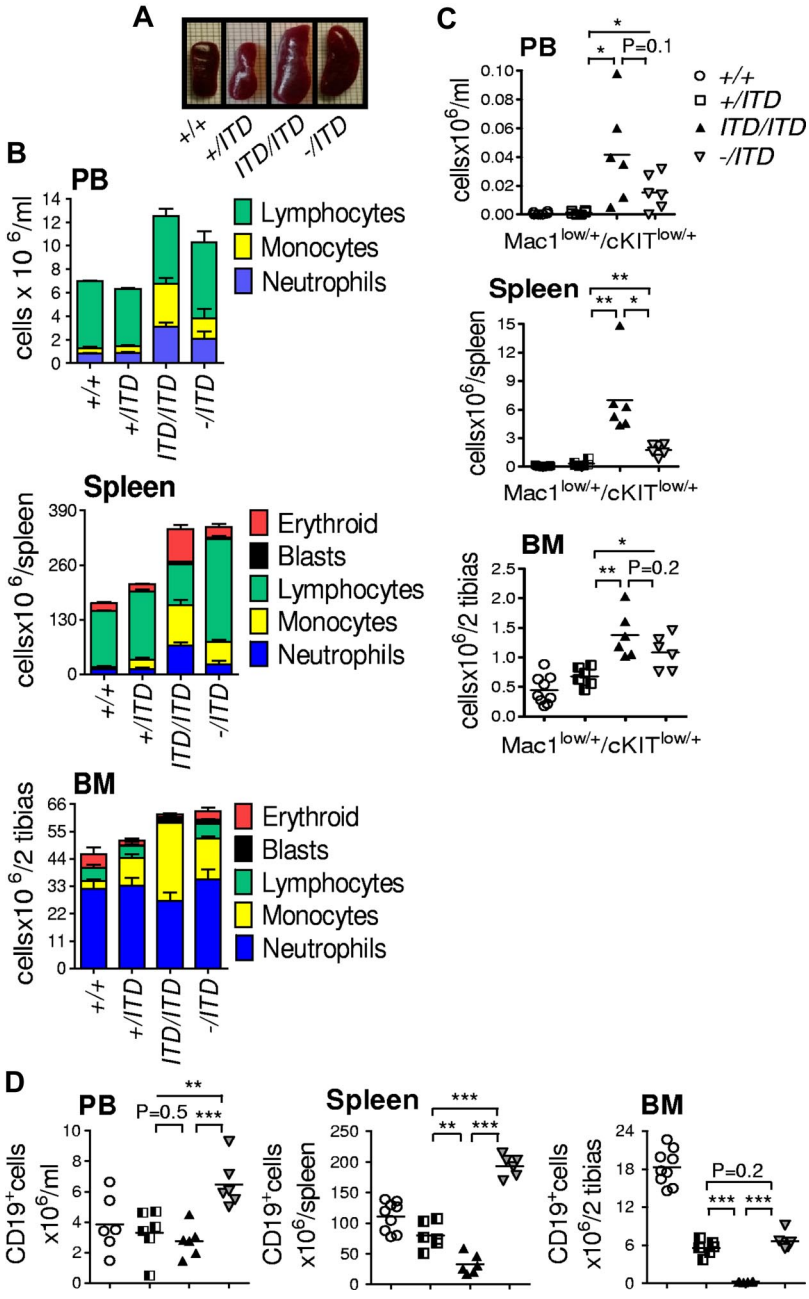

Figure 1. Enhanced expansion of myelomonocytic cells in *Flt3-ITD* hemizygous mice. (A) Typical splenomegaly in *Flt3*$^{ITD/ITD}$ (*ITD/ITD*) and *Flt3*$^{-/ITD}$ (*−/ITD*) but not *Flt3*$^{+/ITD}$ (*+/ITD*) mice. (B) Mean total cellularity of the PB, spleen, and BM for 9 to 18 mice of each genotype. Differential counts were performed on Giemsa-stained smears (PB) and slides after cytospin centrifugation (spleen and BM), on 3 to 5 mice of each genotype, all at 8 to 9 weeks of age. Differential results are displayed as mean ± SD as a proportion of mean total cellularity. (C-D) FACS was used to quantify Mac1$^{low/+}$c-Kit$^{low/+}$ myelomonocytic immature cells (C) and CD19^{+} B cells (D) in PB, spleen, and BM from age-matched littermates of mutant and WT (+/+) animals (mean ± SD; 6-9 male mice at 8-9 weeks of age per genotype) in at least 2 independent experiments. *$P < .05$. **$P < .01$. ***$P < .001$.

preamplified cDNA and stored at −20°C until required. The Dynamic Array PCR cycling condition were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. All reactions were carried out in duplicate or triplicate. Data were analyzed using the $\Delta\Delta C_t$ method. Results from each experiment were normalized to the expression of hypoxanthine guanine phosphoribosyl transferase 1. The mean expression level relative to a specified reference population was then calculated. Results were expressed as the mean normalized expression level (± SD) relative to the reference population derived from 2-4 independent experiments.

Results

Distinct myelopoietic impacts of heterozygous, homozygous, and hemizygous FLT3-ITD expression

To understand the impact of loss of the WT allele and the *ITD* gene dosage effect on myelopoiesis, we crossed *Flt3*$^{ITD/ITD}$ to WT (*Flt3*$^{+/+}$) and to *Flt3*$^{-/-}$[27] mice to derive heterozygous (*Flt3*$^{+/ITD}$) and hemizygous (*Flt3*$^{-/ITD}$) offspring, respectively. To identify differences in the impact on myelopoiesis in *Flt3-ITD* heterozygous, homozygous, and hemizygous mice, we analyzed young adults, at 8-9 weeks of age, when the phenotype of the heterozygous mice remains mild.[7] Analysis of total cell numbers in the peripheral blood (PB), spleen, and BM of 8- to 9-week-old mice demonstrated small changes in *Flt3*$^{+/ITD}$ mice but significant increases in *Flt3*$^{ITD/ITD}$ mice (Figure 1A-B). Notably, a significant cell expansion (and splenomegaly) was also observed in hemizygous (*Flt3*$^{-/ITD}$) mice in the PB ($P < .05$ vs WT; and $P < .01$ vs *Flt3*$^{+/ITD}$), spleen ($P < .001$ vs WT, and $P = .001$ vs *Flt3*$^{+/ITD}$), and BM ($P < .001$ vs WT, and $P = .001$ vs *Flt3*$^{+/ITD}$), in all cases comparable with that observed in homozygous mice (Figure 1A-B).

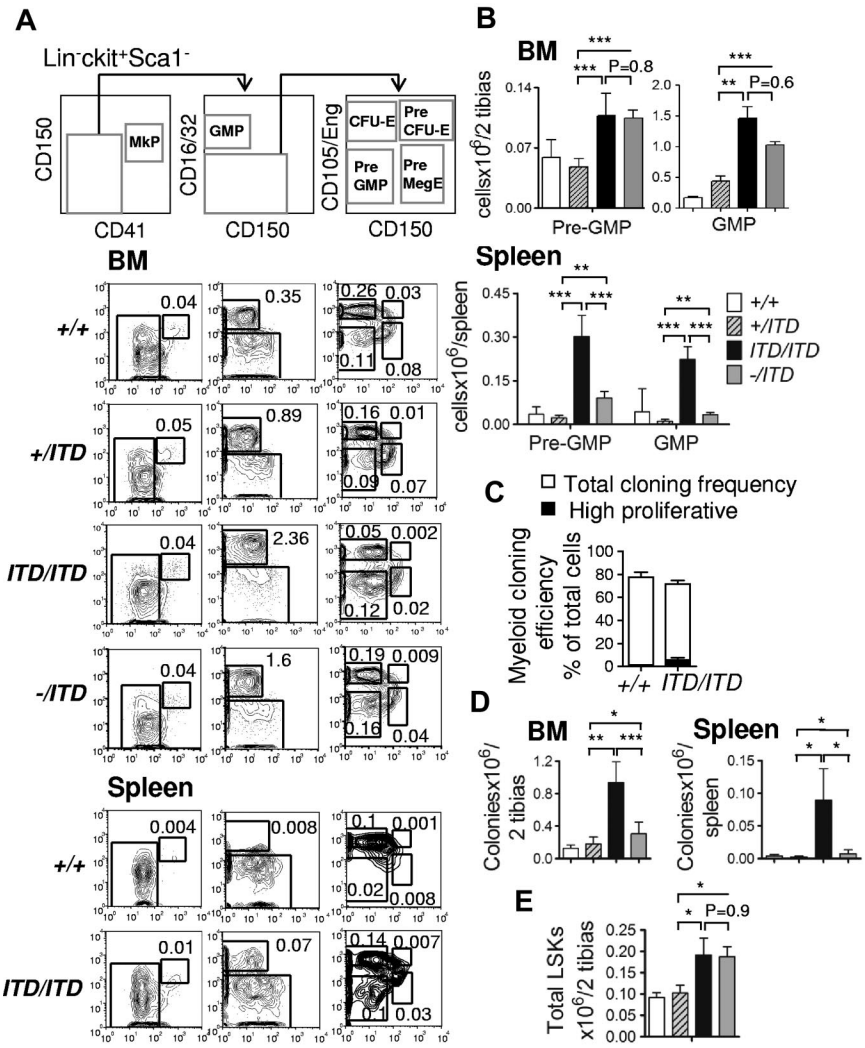

Figure 2. Impact of heterozygous, homozygous, and hemizygous *Flt3-ITD* expression on myeloid progenitor subsets. (A) Representative FACS analysis of MkP ($Lin^-c\text{-}Kit^+Sca1^-$ [LKS^-] $CD41^+CD150^+$), GMPs (LKS^- $CD41^-CD16/32^{hi}CD150^-$), CFU-E ($LKS^-$ $CD41^-CD16/32^{-/low}$ $CD150^-CD105^+$), pre-CFU-E (LKS^- $CD41^-CD16/32^{-/low}$ $CD150^+CD105^+$), pre-GMPs (LKS^- $CD41^-CD16/32^{-/low}$ $CD150^-CD105^{-/low}$), and PreMegE (LKS^- $CD41^-CD16/32^{-/low}CD150^+CD105^{-/low}$) in the BM of *Flt3*$^{+/+}$, *Flt3*$^{ITD/ITD}$, *Flt3*$^{+/ITD}$, and *Flt3*$^{-/ITD}$ and spleen of *Flt3*$^{+/+}$ and *Flt3*$^{ITD/ITD}$ mice. Numbers shown are the mean values for the frequencies (percentage of total BM cells) of each progenitor subset from a total of 4-9 male mice for each genotype. (B) Mean (SD) absolute numbers of pre-GMPs and GMPs in the BM and spleen of *Flt3*$^{+/+}$, *Flt3*$^{ITD/ITD}$, *Flt3*$^{+/ITD}$, and *Flt3*$^{-/ITD}$ mice (4-9 male mice analyzed for each genotype in at least 2 independent experiments) at 8-9 weeks of age. (C) Single-cell GM colony formation of GMPs from *Flt3*$^{+/+}$ and *Flt3*$^{ITD/ITD}$ BM cells. Open bars represent cloning frequencies as established after 8 days of culture; and black bars, frequency of high proliferative clones (covering > 50% of the well). Mean (SD) values from 2 experiments, each in which BM cells from 3-9 mice of each genotype were pooled for FACS sorting. (D) Unfractionated BM and spleen cells from WT, *Flt3*$^{+/ITD}$, *Flt3*$^{ITD/ITD}$, and *Flt3*$^{-/ITD}$ mice were plated on methylcellulose supplemented with myeloid promoting cytokines and scored for GM colony formation after 10 days of incubation. Results are mean (SD) values of at least 3 experiments (5-11 mice of each genotype, each performed in duplicate). $^*P < .05$. $^{**}P < .01$. $^{***}P < .001$. (E) Mean (SD) numbers of LSK cells in BM of *Flt3*$^{+/+}$, *Flt3*$^{+/ITD}$, *Flt3*$^{ITD/ITD}$, and *Flt3*$^{-/ITD}$ mice. A total of 8-14 mice (8-9 weeks old, of each genotype) were investigated. $^*P < .05$.

In agreement with the reported increases in cells of the myelomonocytic lineage in FLT3-ITD patients[33] as well as *Flt3-ITD* knockin mice,[7,21] an increase in monocytes was observed in *Flt3*$^{+/ITD}$ mice in the spleen ($P = .05$) and BM ($P < .01$) compared with WT mice at 8 to 9 weeks of age. This increase in monocytes was, however, much more pronounced in *Flt3*$^{ITD/ITD}$ and, to a smaller degree, in *Flt3*$^{-/ITD}$ mice compared with *Flt3*$^{+/ITD}$ mice (PB, $P = .01$; spleen, $P < .01$; and BM, $P = .01$ for *Flt3*$^{ITD/ITD}$; and PB, $P = .01$; spleen, $P < .01$; and BM $P < .05$ for *Flt3*$^{-/ITD}$; Figure 1B).

Mouse models of myeloid malignancies have demonstrated an expansion of primitive $Mac1^{low/+}c\text{-}Kit^{low/+}$ myeloid precursors/progenitors.[34] Paralleling the expansion of monocytes, we also observed a significant expansion of $Mac1^{low/+}c\text{-}Kit^{low/+}$ cells in the PB, spleen, and BM of *Flt3*$^{ITD/ITD}$ and *Flt3*$^{-/ITD}$ mice compared with *Flt3*$^{+/ITD}$ mice (Figure 1C).

A reduction in B cells has been reported in *Flt3*$^{ITD/ITD}$ mice[7]; and in agreement with this, the number of $CD19^+$ B cells was reduced in the spleen and BM of *Flt3-ITD* heterozygous and, in particular, homozygous mice compared with WT animals; whereas in *Flt3-ITD* hemizygous mice, the number of B cells were expanded in the PB and spleen (Figure 1D).

Long-term myeloid-biased engraftment was achieved after transplantation of unfractionated spleen cells from *Flt3-ITD* donor mice, with the highest levels of engraftment observed with *Flt3*$^{ITD/ITD}$ cells, although also *Flt3*$^{+/ITD}$ and *Flt3*$^{-/ITD}$ transplant recipients showed a myeloid biased reconstitution compared with recipients of WT cells (supplemental Figure 1).

Although previous studies demonstrated an increase in granulocyte-monocyte (GM) colony formation in *Flt3*$^{ITD/ITD}$ mice, no significant expansion of phenotypically defined GMPs was observed.[7] Using a more recent and detailed staging of myeloid progenitors,[30] a marked expansion of GMPs ($Lin^-c\text{-}Kit^+Sca1^-$ [LKS^-] $CD41^-CD16/32^{hi}CD150^-$) and of pre-GMPs ($LKS^-CD41^-$ $CD16/32^{-/low}CD150^-$ $CD105^{low}$) were observed in the BM as well as the spleen of *Flt3*$^{ITD/ITD}$ mice, relative to *Flt3*$^{+/ITD}$ littermates (Figure 2A-B). *Flt3*$^{-/ITD}$ mice showed almost the same expansion as *Flt3*$^{ITD/ITD}$ mice in the numbers of BM GMPs and pre-GMPs. In the spleen, *Flt3*$^{-/ITD}$ mice had an intermediate phenotype with 2.3-fold higher number of GMPs than *Flt3*$^{+/ITD}$ mice, and with 4-fold more pre-GMPs (Figure 2B). GM colony-forming assays confirmed the $LKS^-CD41^-CD16/32^{hi}CD150^-$ GMP phenotype as a reliable marker of GMPs in *Flt3*$^{ITD/ITD}$ mice (Figure 2C). Supporting the phenotypic analysis, numbers of granulocytic/monocytic colony-forming units (CFU-GM) were expanded more extensively in the BM and spleen of *Flt3*$^{ITD/ITD}$ than *Flt3*$^{+/ITD}$ and *Flt3*$^{-/ITD}$ mice. However, CFU-GM numbers were also higher in

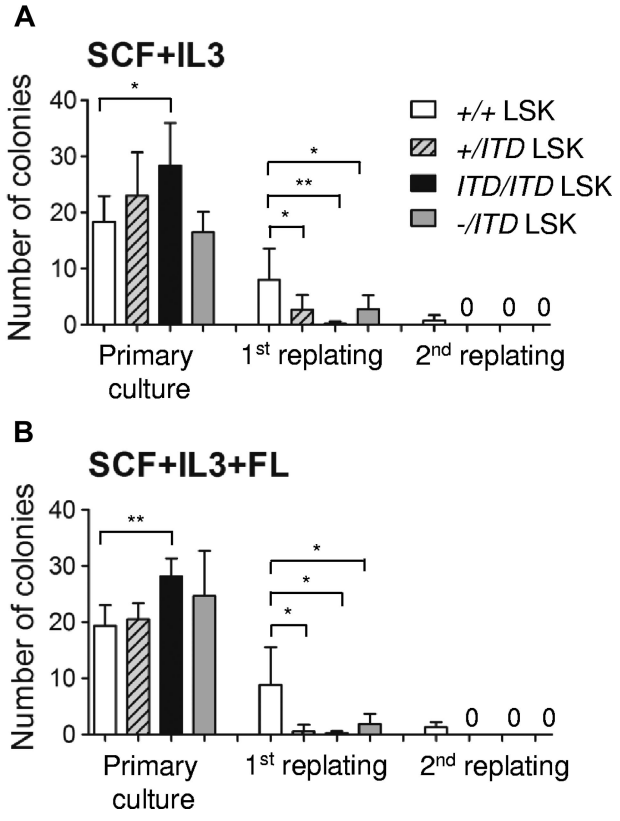

Figure 3. LSK cells from BM of *Flt3-ITD* mice have reduced serial replating capability. (A-B) Thirty sorted LSK cells from BM of WT, $Flt3^{+/ITD}$, $Flt3^{ITD/ITD}$, and $Flt3^{-/ITD}$ mice were plated in methylcellulose containing stem cell factor (SCF), IL-3, and/or FL. The first and secondary replating colony-forming assays were done after 7 days of culture in the presence of the aforementioned cytokines. Results are mean (SD) values of 2 experiments (3 mice of each genotype, each performed in duplicate). $^*P < .05$. $^{**}P < .01$.

$Flt3^{-/ITD}$ than $Flt3^{+/ITD}$ mice in the BM (1.7-fold) and spleen (3.5-fold; Figure 2D).

Previous studies demonstrated that the primitive $Lin^-Sca1^+c\text{-}Kit^+$ (LSK) compartment is expanded in the BM of $Flt3^{ITD/ITD}$ but not $Flt3^{+/ITD}$ mice.[7] In keeping with this, LSK cells were expanded in the BM of both $Flt3^{ITD/ITD}$ and $Flt3^{-/ITD}$ mice (1.9- and 1.8-fold, respectively) compared with $Flt3^{+/ITD}$ mice (Figure 2E). LSK cells were also expanded in the spleen, with a 33- and 4-fold expansion of LSK cells in $Flt3^{ITD/ITD}$ and $Flt3^{-/ITD}$ mice compared with $Flt3^{+/ITD}$ mice (supplemental Figure 2). In agreement with previous studies,[7] colonies derived from LSK cells of FLT3-ITD mice showed decreased serial replating activity (Figure 3A), independently of FL stimulation (Figure 3B).

Taken together, $Flt3^{-/ITD}$ and $Flt3^{ITD/ITD}$ mice have an equivalent expansion of cell numbers in hematopoietic tissues, markedly above that seen in $Flt3^{+/ITD}$ mice. In agreement with this, both $Flt3^{-/ITD}$ and $Flt3^{ITD/ITD}$ have a more pronounced expansion of LSK cells as well as cells of the myelomonocytic lineage, $Mac1^{low/+}c\text{-}Kit^{low/+}$ myeloid precursors and GMPs, than $Flt3^{+/ITD}$ mice. These findings establish that not only an *ITD* mutation in the second *Flt3* allele, but also the deletion of the *Flt3*-WT allele, acts to enhance the myeloid expansion in $Flt3^{+/ITD}$ mice. However, the myeloproliferative effect is much more pronounced in $Flt3^{ITD/ITD}$ than $Flt3^{-/ITD}$ mice in keeping with the importance of a gene dosage effect.

Activation of STAT5 target genes in *Flt3-ITD* expanded GMPs and primitive progenitors

Activation of STAT5 target genes (eg, *Cish*, *Id1*, *Pim1*, and *Pim2*) distinguishes FLT3-ITD from FLT3-WT signaling.[35] Notably, expression of some of these STAT5 target genes (*Cish* and *Id1*) showed a pronounced *Flt3-ITD* dose-dependent up-regulation in the GMP, pre-GMP, and LSK cell populations in *Flt3-ITD* mice compared with WT animals (Figure 4).

$Flt3^{ITD/ITD}$–induced myeloproliferation is FL-independent

To specifically address the potential impact of FL activation on the ITD-induced myeloid phenotype, we crossed $Flt3^{ITD/ITD}$ and $Flt3^{+/ITD}$ littermates with $Fl^{-/-}$ mice,[28] eliminating the possibility of autocrine or paracrine signaling through FL. First, we found that the serum level of FL was significantly increased in $Flt3^{ITD/ITD}$ but not $Flt3^{+/ITD}$ mice (Figure 5A). However, this increase was also seen and even more distinct in $Flk2^{-/-}$ ($Flt3^{-/-}$) mice, suggesting

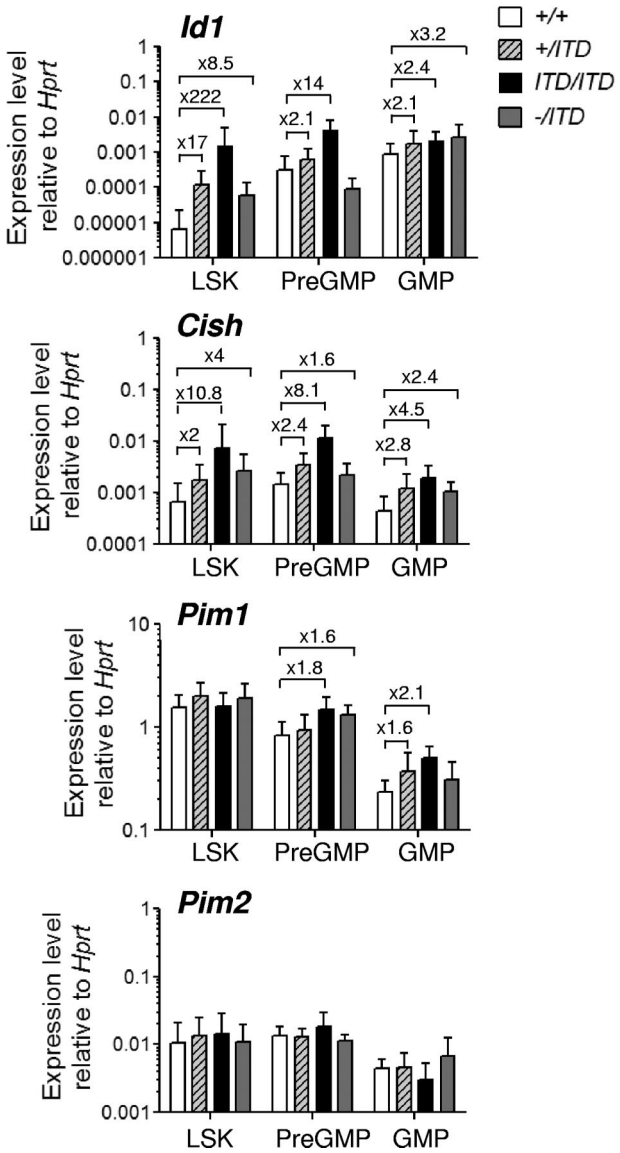

Figure 4. Altered expression of STAT5 target genes in *Flt3-ITD* mice. Transcriptional expression of STAT5 target genes in GMPs, pre-GMPs, and LSKs from $Flt3^{+/+}$, $Flt3^{+/ITD}$, $Flt3^{ITD/ITD}$, and $Flt3^{-/ITD}$ mice. Mean (SD) results (shown as expression relative to *Hprt*) of 2 experiments with 4 replicates analyzed in each experiment.

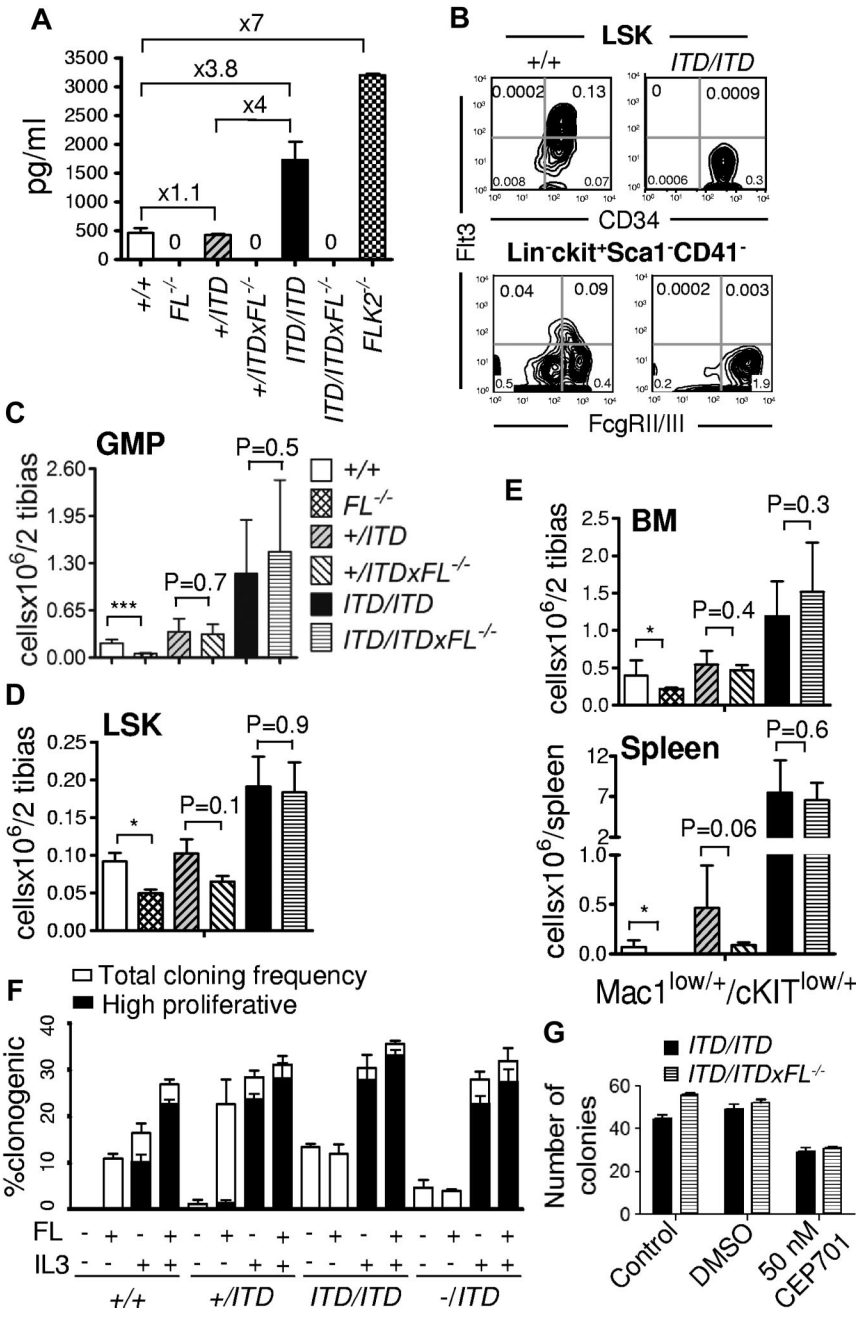

Figure 5. Soluble FLT3 ligand is increased, but the myeloproliferative phenotype of *Flt3*$^{ITD/ITD}$ mice is FL-independent. (A) FL serum levels in *Flt3*$^{+/+}$, *Flt3*$^{+/ITD}$, *Flt3*$^{ITD/ITD}$, and *Flt3*$^{-/-}$ mice, on an *Fl*$^{+/+}$ or *Fl*$^{-/-}$ background, as determined by ELISA. Mean (SD) values of 2-7 mice (8-10 weeks old) investigated per genotype. (B) Representative FACS analysis of FLT3 expression in LSK CD34^{+} lymphoid-primed multipotent progenitors (LMPPs) and LKS^{-} CD41^{-}CD16/32$^{+/-}$ myeloid progenitors in BM of *Flt3*$^{+/+}$ and *Flt3*$^{ITD/ITD}$ mice. Numbers in the graph represent mean frequencies (of total BM cells) of cell populations within indicated quadrants, with 2-5 mice analyzed of each genotype. (C-E) Analysis of BM GMPs (C), BM LSKs (D), and Mac1$^{low/+}$ c-Kit$^{low/+}$ myelomonocytic immature cells in spleen and BM (E) of *Flt3*$^{+/+}$, *Flt3*$^{+/ITD}$, and *Flt3*$^{ITD/ITD}$ mice on a WT *Fl*$^{+/+}$ or *Fl*$^{-/-}$ background. Mean (SD) results from 5-15 mice (8-9 weeks old) of each genotype. *$P < .05$. ***$P < .001$. (F) In vitro cytokine (FL and IL-3) responsiveness of LSK cells purified from BM of WT and FLT3-ITD mice. Clonal growth was scored after 8 days of culture. Mean (SD) values from 2 experiments, with 120 cells plated per group in each experiment. Open bars represent cloning frequencies; and black bars, frequency of high proliferative clones (covering $>$ 50% of the well). (G) A total of 0.3×10^6 unfractionated BM cells from *Flt3*$^{ITD/ITD}$ and *Flt3*$^{ITD/ITD}$x*Fl*$^{-/-}$ mice were cultured in methylcellulose with 50 ng/mL G-CSF, in the absence of any further additions (control), together with the vehicle (dimethyl sulfoxide [DMSO]) or 50nM CEP701, and scored for colony formation 7 days later. Mean (SD) results from 2 independent experiments.

that the enhanced FL serum level could be a result of reduced cell surface FLT3 expression. Indeed, FACS analysis failed to detect cell surface FLT3 expression on either LSK CD34^{+} or LKS^{-}CD41^{-}FcγR$^{+/-}$ myeloid progenitor cells in *Flt3*$^{ITD/ITD}$ mice (Figure 5B), whereas FLT3 cell surface expression was seen on progenitors in *Flt3*$^{+/ITD}$ mice (S.K. and S.E.W.J., unpublished observations, April 2010).

Whereas we, in agreement with recent studies,[36] found LKS^{-}CD41^{-}CD16/32^{hi}CD150^{-} GMPs to be reduced in *Fl*$^{-/-}$ mice, we observed no difference in the GMP and LSK phenotype in *Flt3*$^{ITD/ITD}$ mice on an *Fl*$^{+/+}$ and *Fl*$^{-/-}$ background, demonstrating that, in *Flt3*$^{ITD/ITD}$ mice, the FLT3-ITD-induced expansion of GMPs and LSKs occurs in an FL-independent manner (Figure 5C-D). Similarly, the splenomegaly (data not shown) and increases in the myelomonocytic lineage in *Flt3*$^{ITD/ITD}$ mice were unaffected by FL deficiency (Figure 5E). In contrast, and in agreement with the reductions in myeloid progenitors and LSK cells in *Flt3*$^{+/+}$x*Fl*x$^{-/-}$ mice, these were also slightly reduced in *Flt3*$^{+/ITD}$ mice on an *Fl*$^{-/-}$ background (Figure 5C-E).

That addition of FL has little or no impact on *Flt3*$^{ITD/ITD}$-induced proliferation was further supported by in vitro studies. FL-independent growth was observed of *Flt3*$^{ITD/ITD}$ and *Flt3*$^{-/ITD}$ but not of *Flt3*$^{+/+}$ and only marginally of *Flt3*$^{+/ITD}$ LSK cells (Figure 5F). Furthermore, these experiments also demonstrated that *Flt3*$^{+/ITD}$, *Flt3*$^{ITD/ITD}$, and *Flt3*$^{-/ITD}$ could replace the need for added FL to obtain the growth seen in response to FL in combination with IL-3 on WT LSK cells, and thus the addition of FL to IL-3–supplemented cultures had little or no further impact on clonal growth of *Flt3*$^{ITD/ITD}$, *Flt3*$^{+/ITD}$, or *Flt3*$^{-/ITD}$ LSK cells (Figure 5F). However, in the absence of other cytokines, *Flt3*$^{+/ITD}$, unlike

Flt3$^{ITD/ITD}$ and *Flt3*$^{-/ITD}$ LSK cells showed enhanced growth on addition of FL (Figure 5F). The inhibitory effect of the FLT3 inhibitor CEP701 (lestaurtinib)[37] was comparable on *Flt3*$^{ITD/ITD}$x*Fl*$^{+/+}$ and *Flt3*$^{ITD/ITD}$x*Fl*$^{-/-}$ BM progenitors in vitro (Figure 5G).

Discussion

MASI frequently affects tumors carrying activating GFR mutations, typically in connection with tumor progression.[1] In ITD$^+$ AML, MASI confers a markedly adverse prognosis[18] and is frequently identified at relapse.[20] Thus, understanding the mechanism by which MASI influences the hematopoietic impact of the *FLT3-ITD* mutation has considerable relevance for AML biology and therapy, as well as for other activating GFR mutations in human tumors.[1] A fundamental question, yet to be addressed, is whether the selective advantage conferred to a mutant-positive malignant cell that acquires MASI is exclusively the result of a gene dosage effect or also in part the result of the simultaneous loss of the WT allele. In the present studies, we addressed this using an *Flt3-ITD* knockin model in combination with *Flt3* receptor knockout mice.[7,27] Notably, both *Flt3*$^{ITD/ITD}$ and *Flt3*$^{-/ITD}$ mice developed a more pronounced myeloid expanded phenotype compared with heterozygous (*Flt3*$^{+/ITD}$) mice, with enhanced expansion of monocytic cells as well as LSK and GM progenitors, in keeping with the increased leukocytosis seen in AML patients with a homozygous *FLT3-ITD*.[18] The enhanced malignant phenotype of an oncogenic GFR mutation caused by a deletion of the WT allele without an enhanced dosage of the oncogenic mutation is a novel finding warranting screening for such hemizygous mutations in leukemia and other tumors with GFR mutations. Indeed, isolated loss of the second *RET* WT allele has been reported to occur in association with tumor progression.[9,10] It should be noted, however, that the enhanced myeloid phenotype of *Flt3-ITD* was significantly greater in homozygous than hemizygous mice, suggesting that gene dosage is probably more important than the loss of the WT allele. In keeping with this, hemizygous *FLT3-ITD* mutations have not been reported in patients. Regardless, through acquisition of homozygosity, obviously both a loss of the WT allele and an increased gene dosage are achieved.

The exact mechanism by which loss of the WT allele enhances the oncogenic potential of an activating GFR mutation remains to be established. This may relate to enhanced frequency of mutant homodimers rather than heterodimers between the WT and mutant GFRs. Alternatively, the WT receptor may interfere with mutant signaling through differences in interactions with the ligand or subcellular localization of the mutant GFRs. In that regard, our studies demonstrate that, whereas *Flt3*$^{ITD/ITD}$ and *Flt3*$^{-/ITD}$ LSK cells that both lack expression of the WT FLT3 receptor show little or no response to FL in vitro, *Flt3*$^{+/ITD}$ LSK cells continue to respond to FL probably through sustained WT receptor expression.

FLT3-ITD and -WT signaling differs with regards to activation of STAT5 pathways by mutant but not WT receptors.[35] It is noteworthy, therefore, that some STAT5 target genes were increased in GMP, pre-GMP, and LSK populations in *Flt3-ITD* mice, with evidence of a gene dose–dependent impact on some, but not all of these genes.

For some GFR mutations, their full transforming activity appears ligand-dependent,[14] but this has not been modeled through genetics approaches. This is relevant for *Flt3-ITD* mutations, because although constitutively activated,[16] FL has been demonstrated to enhance FLT3-ITD signaling in vitro,[22,23,26] including in studies in which the cell lines investigated had no WT FLT3 receptor expression,[22,23] suggesting that FL can enhance signaling through the constitutively activated FLT3-ITD receptor. However, in a few patients with homozygous *FLT3*$^{ITD/ITD}$ mutations, addition of FL had no consistent effects on STAT5 phosphorylation or other relevant signaling pathways in primary AML blasts in vitro, but nevertheless, in some cases, slightly enhanced their in vitro survival.[22] Furthermore, some ITD$^+$ AML blast cells coexpress FL and FLT3,[25,26] suggesting that targeting of FL in ITD$^+$ myeloid malignancies might be therapeutically relevant. In this regard, FL has been implicated to enhance resistance to FLT3 inhibitors in FLT3-ITD AML.[23,24] Most notably, it has recently been suggested that relapsed FLT3-ITD$^+$ AMLs, which in general respond more poorly to FLT3 inhibitors than newly diagnosed FLT3-ITD$^+$ AMLs, see a bigger increase in chemotherapy-induced FL levels than at diagnosis.[24] Although this offers one possible explanation for the enhanced FLT3 inhibitor resistance on relapse, there are obviously many others, one being the enhanced acquisition of homozygous *FLT3-ITD* mutations on relapse.[20] Our findings also provide a plausible explanation for how relapsed cases with homozygous *FLT3-ITD* mutations could have enhanced serum levels of FL. Specifically, we could not detect FLT3 expression on the cell surface of hematopoietic cells from *Flt3-ITD* homozygous mice by FACS (whereas *Flt3*$^{+/ITD}$ cells did express cell surface FLT3), probably because of aberrant processing and cell surface expression.[38,39] Thus, in agreement with our demonstration of *Flt3*$^{-/-}$ mice lacking FLT3 receptor expression having further increases in FL levels, the increased FL levels in *Flt3*$^{ITD/ITD}$ mice, and potentially in FLT3-ITD patients on relapse,[24] can potentially be explained, at least in part, by reduced FLT3 cell surface expression. However, we cannot rule out other explanations for the higher FL expression in *Flt3*$^{ITD/ITD}$ mice.

Analysis of *Flt3*$^{ITD/ITD}$x*Fl*$^{-/-}$ mice demonstrated that the expanded myeloid and progenitor phenotypes of *Flt3*$^{ITD/ITD}$ mice is FL-independent, further supported by our finding of a lack of response of *Flt3*$^{ITD/ITD}$ progenitors to endogenous FL. Importantly, these experiments also demonstrated that the enhanced phenotype of *Flt3*$^{-/ITD}$ compared with *Flt3*$^{+/ITD}$ mice was not caused by lack of FLT3-WT interaction with FL, as the phenotype of *Flt3*$^{+/ITD}$ mice was not enhanced in the absence of FL. On the contrary, there was a general tendency toward the myeloid and progenitor phenotype of *Flt3*$^{+/ITD}$ mice being slightly milder on an *Fl*$^{-/-}$ background. Because the *Fl*$^{-/-}$ mice themselves had a mild myeloid phenotype in agreement with recent studies,[36] these findings are collectively most compatible with the milder myeloid phenotype of *Flt3*$^{+/ITD}$ mice on an *Fl*$^{-/-}$ being explained by the presence of WT FLT3 receptors, rather than enhanced signaling through FLT3-ITD. Thus, our mouse model studies in vivo and in vitro would in themselves suggest that FL might potentially enhance the resistance to FLT3 inhibitors in the case of heterozygous, but not homozygous cases of *FLT3-ITD*. Nevertheless, the data suggesting that FL can enhance FLT3-ITD signaling and resistance to FLT3 inhibitors in vitro, also in cells homozygous for *FLT3-ITD*, are compelling and should be explored further. In that regard, our finding that *Fl*$^{-/-}$ progenitors show the same sensitivity to an FLT3 inhibitor as *Fl*$^{+/+}$ progenitors does not contradict the finding that addition of FL to cultures might enhance the FLT3 inhibitor resistance of AML cells homozygous for *FLT3-ITD*. Furthermore, as obviously *FLT3-ITD* mutations are typically secondary events, seen in full-blown AML rather than isolated events in MPD, the impact of *Flt3-ITD* gene dosage, loss of the

Flt3 WT allele, and (not the least) the impact of endogenous FL should be extended to mouse models in which *Flt3-ITD* collaborate with other mutations in the development of AML.[40]

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Authorship

Contribution: S.K. performed and analyzed experiments and wrote the manuscript; A.J.M. contributed to the design, performance, and analysis of experiments and wrote the manuscript; C.B. analyzed data at the end of study and provided technical advice; A.M., A.H., J.C., E.S., K.M., L.R., S.L., and N.B.-V. were involved in clonal assays and sorting experiments; Z.M. and H.F. performed FACS sorting; D.A. provided technical assistance; K.R., A.H., K.M., F.A., T.S., J.C., L.R., C.N., and E.S. were involved in discussions and experimental design; S.E.W.J. conceived and supervised the project, designed and analyzed experiments, and wrote the manuscript; and all authors read and approved of the final manuscript.

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