Supporting Information

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SI Materials and Methods

Confirming the Presence of the Mutation in ES Clones. To confirm the presence of the mutation substituting the aspartic acid at position 838 (equivalent to the human aspartic acid residue at position 835) with a tyrosine (referred to as D835Y hereafter), a PCR product (primers: forward, CAGGGATGCCTCAGATGACT; reverse, CCCCCTCACATTCTCTTTGA) was generated to include the area of the D835Y mutation, isolated on a 1% (wt/vol) agarose gel, purified using a gel extraction kit (Qiagen), and submitted to the sequencing core at Johns Hopkins.

The construct was linearized with NotI and electroporated into S129 embryonic stem (ES) cells. ES clones were selected for using G418 and ganciclovir and then screened by Southern blot, using Spe1 digestion (wild-type band at 4.5 kb and D835Y band at 5 kb) and sequencing. The clones were then further screened for normal karyotype before being injected into blastocysts, which were subsequently implanted into pseudopregnant female mice by the transgenic core at Johns Hopkins. Chimeric mice were generated from the ES clones, as described in the text, at the Johns Hopkins transgenic core, screened for the mutation, and then backcrossed with C57BL/6 mice (National Cancer Institute-Fredrick) to >98% C57BL/6 background, using speed congenics (National Institutes of Health-Genomics Core). Mice were further bred to CMV-CRE mice [B6.C-Tg(CMV-cre)1Cgn/J, Jackson Lab] to excise the phosphoglycerate kinase (PGK)-neomycin resistance cassette at the loxP sites (Fig. 1*A*). All experiments and data in this report were generated using these backcrossed and floxed mice.

Genotyping of the FLT3/D835Y Mouse. Genotyping was done using a custom Taqman SNP 2 color assay (Applied Biosystems) using quantitative reverse transcription–PCR. Forward and reverse primers are placed at either end of the point mutation, with additional primers conjugated to a fluorophore covering the mutation [forward, GTGGTG AAGATCTGTGACTTTGGA; reverse, GTTGCCCCTGACGACGTA; FAM, CCCGA**T**ACATCCTG (FLT3/D835Y) and VIC - CCCGA**G**ACATCCTG (wild-type)].

Harvesting of Tissues, Microscopic Examination, and Immunohistochemistry. Murine tissues for histopathologic examination were fixed in 10% buffered formalin, embedded in paraffin blocks, and stained with H&E. Representative images from 3- and 12-mo-old mice were acquired using a Zeiss Axioskop upright microscope system. Moribund mice were killed for our survival curve. Peripheral blood counts were assayed using a Hemavet950 (Drew Scientific) and modified Wright-Gimsea stained smears of the peripheral blood and bone marrow (BM) cytospins. Bone marrow and spleen cells were made into liquid suspension of PBS/2.5%FBS and analyzed by FACS, using a combination of surface antibodies. Antibody clones used were as follows: Lineage (clone no. MLM20), CD135 (A2F10.1), Sac1 (D7), c-Kit (2B8), CD41 (MWReg30), Ter119 (TER-119). Mac1 (M1/70), Gr1 (RB6-8C5), CD24 (M1/69), CD43 (S7), B220 (RA3-6B2), IgM (II/41), CD4 (L3T4), CD3 (17A2), CD8a (Ly-2), CD90 (53-2.1), CD86 (GL1), CD11c (HL3). All antibodies were obtained from BD Biosciences except the lineage mixture (Invitrogen) and sca-1 (eBioscience).

In addition, H&E slides from formalin-fixed tissue were made from the cohort of sick mice and further examined by immunohistochemistry, using myeloperoxidase (Thermo Scientific), CD3 (AbD Serotec), and paired box 5 (Pax5) (Santa Cruz), using a sodium citrate pH 6 buffer and high heat as the method of antigen retrieval.

Flow Cytometry Analysis. Flow cytometry was preformed on either a FACS Calibur or LSRII (BD Biosciences). Cell sorting was performed on a FACS Aria (BD Biosciences). Fluorescent surface antibodies used are listed above. FlowJo (9.3.3) was used for data analysis of acquired FACS data. Cells (BM and spleen) were suspended in a solution of 1 × PBS/2.5% FBS and kept on ice.

Competitive Engraftment Experiment. Lethally irradiated CD45.1$^+$ recipient mice were injected with a mixture of 75,000 Lin$^-$ BM cells from D835Y (CD45.1$^+$/CD45.2$^+$) mice at a 1:1 ratio, and then with an increasing ratio of lineage-negative BM cells from either wild-type (CD45.2$^+$) or internal tandem duplication (CD45.2$^+$) mice.

BM Cell Viability Assay. Whole BM cells were isolated from 6-mo-old age-matched preleukemic donors. Cells were plated in 96-well plates at a density of 2.5 × 10^4 per well in triplicate in RPMI-1640 and incubated with 5–100 nM Sorafenib or Lestaurtinib at 37 °C for 48 h. Cell viability was measured by CCK-8 cell counting assay (Dojindo Molecular Technologies) by adding 10 μL of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt to each well and incubating plates for an additional 4 h at 37 °C. Optical density (OD) at 450 nm was measured on a microplate reader (680 Bio-Rad) and analyzed using the Microplate Manager software (Bio-Rad). The average OD of the reference wells (media only) was subtracted from each sample OD, and data were represented as %OD relative to DMSO control. Error bars indicate average ± SD.

Western Blot. Lysates were prepared from suspended cells and lysed using CellLyticM (Sigma), using protease inhibitor mixture (Sigma). Lysates were run on a 10% acrylamide gel and transferred to a PVDF membrane (Immobilon-P;Millipore). Antibodies used (Cell Signaling Technologies) were phospho-Stat5 (Tyr694, 9351) and Stat5 (9363). The secondary antibody used was goat anti-rabbit horseradish peroxidase (GE). Detected by enhanced chemiluminescence (ECL; Amersham Biosciences/GE Healthcare).

Quantitative RT-PCR. BM cells were harvested and either sorted for proB cells (B220$^+$CD43$^+$CD19$^-$, Fig. 4*F*) or stained with surface markers for Lin$^-$ (BD biotin lineage panel), incubated with biotin magnetic beads (Miltenyi), and then collected using a LD column (Miltenyi; Fig. 5*C*); RNA was isolated using a RNeasy kit (Qiagen) and reverse-transcribed into cDNA using SSIII RT (Invitrogen). Reactions were run on a iCycler iQ multicolor RT-PCR instrument (Bio-Rad), using SyberGreen Mastermix (Biorad). S-16 was amplified as an internal control. Primers used for each gene are listed here:

Pax5: forward: 5′-GTCAGCCATGGTTGTGTCAG-3′; reverse: 5′-TGTCCGAATGATCCTGTTGA-3′

Pu.1: forward: 5′-ATGCACGTCCTCGATACTCC-3′; reverse: 5′-GGCGAATCTTTTTCTTGCTG-3′

Gata1: forward: 5′-GAAGCGAATGATTGTCAGCA-3′; reverse: 5′-TTCCTCGTCTGGATTCCATC-3′

CEPBα: forward: 5′-TGGACAAGAACAGCAACGAG-3; reverse: 5′-TTGTCACTGGTCAGCTCCAG-3

CISH: forward: 5′-TGTGCATAGCCAAGACGTTC-3′; reverse: 5′-GGGTGCTGTCTCGAACTAGGR-3′

Pim-1: reverse: 5′-CCGAGCTCACCTTCTTCAAC-3′; forward: 5′-ATGGGCTCTCCTGTCAACAC-3′

Pim-2: forward: 5′-AGCACCTCCTCCATGTTGAC-3′; reverse: 5′-GTGCCAAAGCCTCCCTTAC-3′

Socs1: forward: 5′-CCGGAGCATGCGCGACAG-3′; reverse: 5′-GCGTGCTACCATCCTACTCG-3′

Socs3: forward: 5′-GAGATTTCGCTTCGGGACT-3′; reverse: 5′-AACTTGCTGTGGGTGACCAT-3′

S16: forward: 5′-CACGAACCACGGCACTGATT-3′; reverse: 5′-TTTTCTTGCTGCCAGTCTGGAC-3′

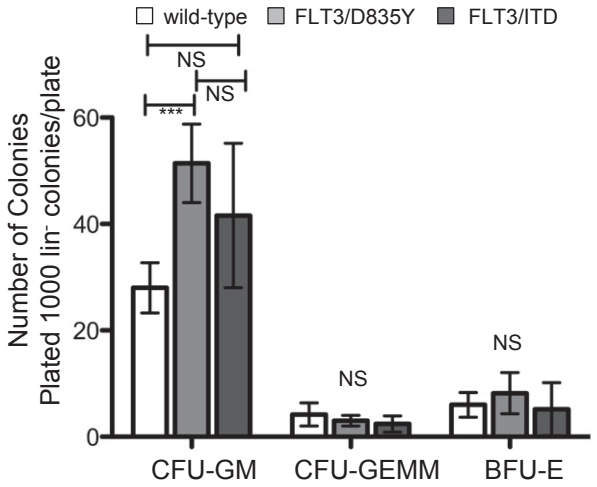

Fig. S1. Both the FLT3/D835Y and FLT3/internal tandem duplication Lin$^-$ BM cells have the ability to form more colony forming unit-granulocyte/monocyte colonies than that of wild-type BM cells. A thousand lineage-depleted BM cells from 12-wk-old mice were cultured in methylcellulose medium (Methocult M3434 from StemCell Technologies). Colonies were counted 11 d after methylcellulose culture. Data are expressed as mean ± SEM (error bars). *P* values on the graph: ***$P < 0.001$; NS, $P > 0.05$.