Identification of a germline F692L drug resistance variant *in cis* with *Flt3*-internal tandem duplication in knock-in mice

Oliver M. Dovey,[1†] Bin Chen,[2,3,4*] Annalisa Mupo,[1*] Mathias Friedrich,[1] Carolyn S. Grove,[1,5,6] Jonathan L. Cooper,[1] Benjamin Lee,[7] Ignacio Varela,[8] Yue Huang,[2,3] and George S. Vassiliou[1,4†]*

[1]The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, UK; [2]Department of Medical Genetics, School of Basic Medicine, Peking Union Medical College, Beijing, China; [3]State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China; [4]Department of Haematology, Cambridge University Hospitals NHS Trust, UK; [5]School of Pathology and Laboratory Medicine, University of Western Australia, Crawley, Australia; [6]PathWest Division of Clinical Pathology, Queen Elizabeth II Medical Centre, Nedlands, Australia; [7]Takeda Pharmaceuticals International, Cambridge, MA, USA; and [8]Instituto de Biomedicina y Biotecnología de Cantabria, Santander, Spain

**OMD, BC and AM contributed equally to this work.*

Correspondence: od2@sanger.ac.uk/gsv20@sanger.ac.uk
doi:10.3324/haematol.2016.146159

Supplementary Methods.

Sequencing Validation Primers.

MiSeq Amplicon Sequencing.

For generating Variant Allele Frequencies for *Flt3-WT* and *Flt3-ITD* alleles in DNA extracted from AML samples, the following primers were used. MiSeq adapter sequences are underlined; Flt3 locus specific sequences are in bold.

Flt3_Miseq Fwd: acactctttccctacacgacgctcttccgatct**aggtacgagagtcagctgcagatg**

Flt3_Miseq Rev: tcggcattcctgctgaaccgctcttccgatct**tgtaaagatggagtaagtgcgggt**

For generating Variant Allele Frequencies for *Flt3-WT* and *Flt3-ITDF692L* alleles in DNA extracted from AML samples, the following primers were used. MiSeq adapter sequences are underlined; Flt3 locus specific sequences are in bold.

Flt3_Ex17SNP_ Miseq Fwd: acactctttccctacacgacgctcttccgatct**tgtctgcagtctgtctaacct**

Flt3_Ex17SNP_Miseq Rev: tcggcattcctgctgaaccgctcttccgatct**gtgtgtgcagtggtcattct**

Sanger Sequencing.

For generating Flt3 Exon 17 sequencing amplicon;

Flt3_Ex17_Fwd: acagtgctgagatcgagtgt

Flt3_Ex17_Rev: cttaggtctcaggccacaca

For sequencing Exon 17 amplicon;

Flt3_Ex17_2076A>T_Fwd: tgtctgcagtctgtctaaccttgta

Flt3_Ex17_2076A>T_Rev: gtgtgtgcagtggtcattct

Cloning of mouse *Flt3*-ITD$^{p.F692L}$ and *Flt3*-ITD$^{p.F692F}$ cDNA into the MSCV-CFP retroviral backbone.

mRNA extracted from homozygous *Flt3*^{tm1Dgg} mouse bone marrow cells was reverse transcribed using SuperScript III (Invitrogen) and the subsequent cDNA was used as template to amplify the *Flt3*ITDp.F692L cDNA using high fidelity *taq polymerase* (KAPA HiFi HotStart ReadyMix, Kapa Biosystems) and the following primers;

MluI-mFLT3ITD3-XhoI Fwd: aattc***acgcgt***atgcgggcgttggcgcagcgcagcgaccg

MluI-mFLT3ITD3-XhoI Rev: ggatct***ctcgag***ctaacttctttctccgtgaatcttcacc

This fragment was cloned into the MluI/XhoI linearized fragment of the MSCV-CFP retroviral backbone using standard molecular biology techniques.

A three way Gibson assembly was used to clone the corrected *Flt3*ITDp.F692F cDNA into the same MSCV-CFP retroviral backbone. Briefly, this consisted of the linearized MSCV backbone (Fragment 3) and two other fragments generated by PCR from the MSCV-*Flt3*ITDp.F692L -CFP plasmid (Fragment 1 and 2). Fragment 2 used a 5' forward primer complimentary to the c.2076T>A SNP (with the appropriate adenine corrected to thymine, highlighted in bold capitals below). Fragments 1 and 2 were generated using high fidelity taq polymerase and the following primers;

Fragment 1

Gibson-Frag1-Fwd: ccctcactccttctctaggcgccggaattca

Gibson-Frag1-Rev: tgacagtgtgcatgccccca

Fragment 2

Gibson-Frag2-Fwd: tgggggcatgcacactgtcagggccagtgtacttgattttt**T**gaat

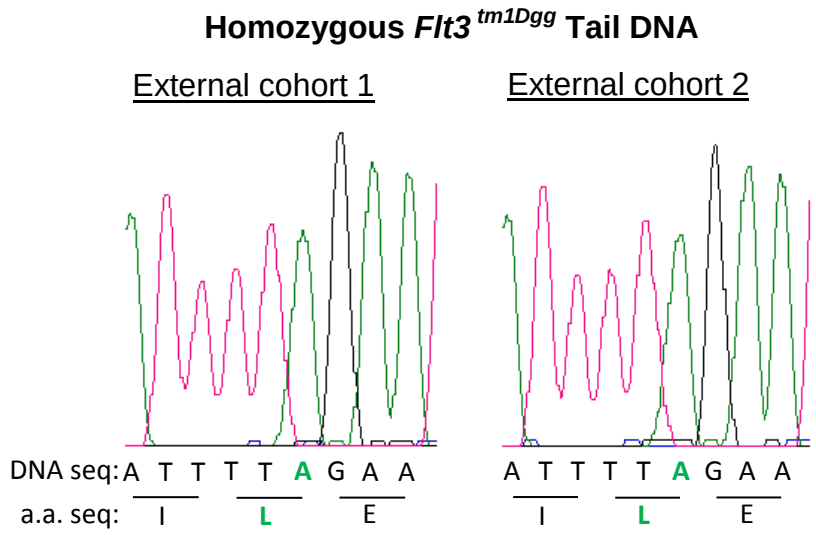
Gibson-Frag2-Rev: ttccaagcggcttcggccagtaacgttagg

We assembled the two insert fragments and the linearized vector in a single reaction by Gibson Assembly® (Gibson Assembly® Master Mix, NEB) and validated the presence or absence of the SNP by Sanger sequencing.

The CellSensor® irf-bla Ba/F3 cell line (#K1654, Invitrogen, Calrsbad).

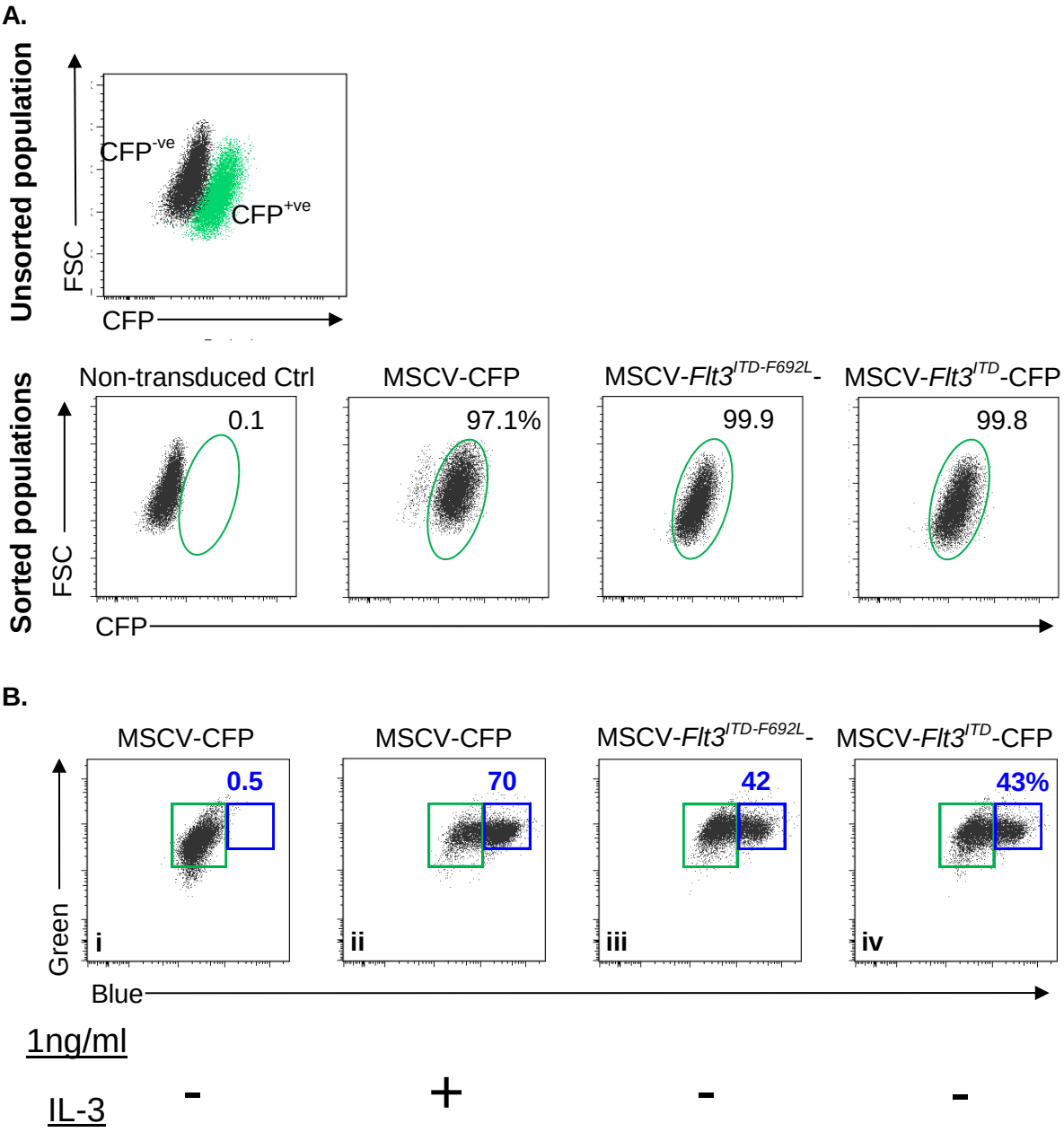
Like other Ba/F3 cell lines, the CellSensor® irf-bla Ba/F3 cell line is an IL-3 dependent cell line. However, it also contains an additional beta-lactamase reporter gene under the control of Stat5 response elements from the IRF1 gene promoter [1]. Of note, activation of STAT5 is a well reported molecular phenomenon downstream of mutant FLT3-ITD protein[2, 3]. Using this cell line, in conjunction with the LiveBlazer™ FRET B/G substrate (Invitrogen, Calrsbad), one can determine the response to a number of agonists (or antagonists) of the IL-3, JAK2 and STAT5 signalling pathways. Addition of the LiveBlazer™ FRET B/G substrate (excited at 409nm) in the absence of an appropriate agonist (such as murine IL-3) results in detection at an emission of 520nm (i.e. green). In the presence of the appropriate agonist, expression of beta-lactamase results in cleavage of the substrate detectable at an emission of 447nm (i.e. blue, Supplementary Figure 2B).

Supplementary Figure 1



Supplementary Figure 1. The Flt3 exon 17 c.2076 T>A SNP is present in other *Flt3*^{tm1Dgg} cohorts.
Sanger sequencing of Homozygous *Flt3*^{tm1Dgg} tail DNA, covering exon 17 of the Flt3 gene, from two independently acquired *Flt3*^{tm1Dgg} cohorts reveals the presence of the c.2076 T>A SNP on the mutant allele. The nucleotide substitution (T>A) and the amino acid (a.a.) substitution (F>L) are highlighted in green.

Supplementary Figure 2



Supplementary Figure 2. Generation of stably expressing *Flt3*$^{ITDp.F692L}$ or *Flt3*$^{ITDp.F692F}$ Ba/F3 cells.
(A) CellSensor® irf-bla Ba/F3 cells were stably transduced with empty MSCV-CFP, *MSCV-Flt3*$^{ITD\ p.F692L}$*-CFP*, or the reverted *MSCV-Flt3*$^{ITD\ p.F692F}$*-CFP* virus. 5 days post transduction cells were sorted for expression of CFP using a Mo-Flo^{TM} XDP (Beckman Coulter UK Ltd). FACS analysis reveals transduced, sorted populations to be >95% CFP positive. (B) FRET assays performed on stably transduced, IL-3 independent transformed cell lines show that both mutant *Flt3* cDNAs activate the Jak/Stat pathway to equivalent levels. FACS shows the shift from single positive Green cells (panel **i**) to the double positive Green/Blue reporter activated populations (panel **iii** and **iv**) in the presence of *Flt3*$^{ITDp.F692L}$, or the corrected *Flt3*$^{ITDp.F692F}$ cDNA.

References

1. Dawson MA, Curry, JE, Barber K, Beer PA, Graham B *et al*. AT9283, a potent inhibitor of the Aurora kinases and Jak2, has therapeutic potential in myeloproliferative disorders. *Br J Haematol* 2010 Jul; **150**: 46-57.

2. Hayakawa F, Towatari M, Kiyoi H, Tanimoto M, Kitamura T et al. Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines. Oncogene 2000; **19**: 624-631.

3. Choudhary C, Schwable J, Brandts C, Tickenbrock L, Sargin B *et al*. AML-associated Flt3 kinase domain mutations show signal transduction differences compared with Flt3 ITD mutations. *Blood* 2005; **106**: 265–273.