EUROPEAN HEMATOLOGY ASSOCIATION
haematologica
Journal of the European Hematology Association

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Haematologica 2016 [Epub ahead of print]

Citation: Dovey OM, Chen B, Mupo A, Friedrich M, Grove CS, Cooper JL, Lee B, Varela I, Huang Y, and Vassiliou GS. Identification of a germline F692L drug resistance variant in cis with Flt3-ITD in knock-in mice. Haematologica. 2016; 101:xxx
doi:10.3324/haematol.2016.146159

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

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Running title: Flt3 F692L variant in Flt3-ITD knock-in mice.

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Article word count: 1260

Acknowledgements

We thank Professor Gary Gilliland for careful review of the manuscript and for helpful discussions. We thank Drs Adam Mead and Wallace Langdon for providing us with tail DNA from their *Flt3-ITD* (*Flt3*^{tm1Dgg}) homozygous mice. We thank Dr Brian Huntly for providing us with the *MSCV-CFP* retroviral backbone. OMD, JLC and GSV are funded by a Wellcome Trust Senior Fellowship in Clinical Science (WT095663MA). BC is funded by the "China Scholarship Council" for his visiting studies in UK. AM is funded by the Kay Kendall Leukaemia Fund project grant. CG was funded by a Bloodwise Clinical Research Training Fellowship. IV is funded by Spanish Ministerio de Economía y Competitividad subprograma Ramón y Cajal. We thank Servicio Santander Supercomputación for their support. GSV is a consultant for and holds stock in Kymab Ltd, and receives an educational grant from Celgene. BL is an employee of Takeda Pharmaceuticals International. The authors have no conflicts of interest.

Internal tandem duplication (ITD) mutations in the juxtamembrane domain of the fms-like tyrosine kinase 3 (*FLT3*) gene occur in approximately one quarter of cases of acute myeloid leukemia (AML)[1], are associated with constitutive activation of the kinase[2] and confer a poor prognosis[3]. Our understating of the molecular consequences of these mutations has benefited from studies of bespoke mouse models[4-6]. Herein we report the identification of the germline variant F692L in cis with the *Flt3-ITD* allele of the widely studied *Flt3*^{tm1Dgg} mouse[4]. As this variant is analogous to the human FLT3 F691L "gatekeeper" mutation[3] we investigated this finding further. We found that primary AML cells from *Npm1*^{cA}*; Flt3*^{tm1Dgg} double mutant mice are resistant to Sorafenib and Quizartinib (AC220), but sensitive to Ponatinib. The same pattern of sensitivity was observed when we tested these tyrosine kinase inhibitors (TKIs) against isogenic Ba/F3 cells transfected with the murine *Flt3-ITD* F692L or the reverted *Flt3-ITD* wildtype cDNAs, confirming that *Flt3-ITD* F692L is responsible for recapitulating the resistance pattern of the human F691L mutation. The presence of this *Flt3* TKI resistant variant needs to be considered when interpreting data using this model, but also makes the model an extremely useful tool for studying TKI resistance.

The molecular consequences of *FLT3-ITD* are pleiotropic and include activation of STAT5 via the SRC kinase, phosphorylation of the transcription factor FOXO3A with associated activation of Akt and induction of reactive oxygen species, amongst others (reviewed by Yeung et al[3]). Knock-in mouse models have been instrumental in deciphering the hematopoietic and leukemogenic effects of *FLT3-ITD* alone[4-6] and in combination with mutations co-occurring with *FLT3-ITD* in human AML[7-12]; as well as in studying the effectiveness of different therapeutic approaches against *FLT3-ITD* positive leukaemias [11, 13]. As part of these efforts, we studied the powerful collaboration between *FLT3-ITD* and mutant *NPM1*, by crossing our conditional *Npm1*$^{flox\text{-}cA}$ knock-in[14] with the *Flt3-ITD* knock-in mice (*Flt3*^{tm1Dgg}) generated by Lee et al [4]. Triple transgenic *Npm1*$^{flox\text{-}cA/+}$; *Flt3*$^{ITD/+}$; *Mx1-Cre*$^{+}$ rapidly succumbed to AML with a median latency of 49 days, exhibiting full recombination of the conditional *Npm1*$^{flox\text{-}cA}$ allele without the need to induce *Mx1-Cre* expression with polyinosinic-polycytidylic acid as previously published [8]. To identify or exclude additional cooperating somatic mutations involved in leukemic progression, we performed whole exome sequencing of 5 *Npm1*$^{cA/+}$; *Flt3*$^{ITD/+}$; *Mx1-Cre*$^{+}$ AMLs. Comparison with constitutional (tail) exome sequence from C57BL/6N mice identified a common variant located at Chr5:147349699 (GRCm38), within exon 17 of the *Flt3* gene. The variant, *Flt3* c.2076 T>A (p.F692L), was present at VAFs of 0.55-0.94. Using PCR-amplification followed by MiSeq sequencing we found that these VAFs correlated strongly with those of the *Flt3-ITD* allele itself (Figure 1A). As the *Flt3-ITD* mutation is located at exon 14 and frequently exhibits loss of heterozygosity in our model[8] this indicated that the F692L variant is present *in cis* with the knock-in ITD. We went on to confirm this by genotyping germline DNA (tail) from *Flt3*$^{ITD/+}$ and *Flt3*$^{ITD/ITD}$

single transgenic mice from our cohort (Figure 1B). Furthermore, we identified the same variant in germline DNA from the cohorts of two separate research groups who obtained the *Flt3-ITD* mouse model independently (Supplementary Figure 1).

As the murine F692L variant is equivalent to the human F691L gatekeeper mutation, which confers AML resistance to multiple FLT3 tyrosine kinase inhibitors (TKI)[3, 15], we proceeded to test whether this was also true of the murine variant. To do this we cultured AML cells from two independent *Npm1*$^{cA/+}$; *Flt3*$^{ITD/+}$; *Mx1-Cre*$^{+}$ mice[8] in X-VIVO media (Lonza, Switzerland) supplemented with murine IL-3, IL-6 and SCF (Miltenyi, Germany). After 72hr in culture, we removed IL-3 and exposed the cells to varying concentrations of Quizartinib (AC220), Sorafenib and Ponatinib for three days, using the human FLT3-ITD positive AML cell line MV4-11 as a positive control. After three days of treatment, cell viability was assessed using the CellTiter 96® AQueous assay (Promega, USA). We found that, compared to MV4-11, *Npm1*$^{cA/+}$; *Flt3*$^{ITD/+}$; *Mx1-Cre*$^{+}$ AML cells exhibit resistance to Quizartinib and Sorafenib, but not to Ponatinib which is known to retain activity against the human FLT3-ITD F691L mutant isoform[15, 16] (Figure 2A)

To exclude the possibility that our murine AMLs and MV4-11 displayed different resistance patterns for reasons other than the F692L mutation, we amplified the full open-reading frame of the *Flt3* cDNA from bone marrow mRNA of homozygous *Flt3*$^{ITD/ITD}$ mice and cloned this into an MSCV-CFP retroviral backbone (kind gift of Dr Brian Huntly, University of Cambridge) (Figure 2B). We then also generated a reverted c.2076A>T (*Flt3-ITD*^{F692F}) construct in the same MSCV-CFP retroviral backbone using Gibson assembly (see Supplementary Methods). The two retroviruses were each used to transduce IL-3 dependent CellSensor® irf-bla Ba/F3 cells (Invitrogen, Carlsbad), which were subsequently sorted to >95% purity (Supplemental Methods and Supplementary Figure 2A). The *Flt3-ITD*^{F692L} cDNA- and the *Flt3-ITD*^{F692F} cDNA-transfected cells displayed comparable IL-3 independent growth (Figure 2B) and FRET assays showed concomitant activation of the JAK/STAT pathway, recapitulating the effects of human *FLT3-ITD* mutants (Supplementary Figure 2B). Next, we treated the transformed Ba/F3 cell lines as described above for the primary AML cells. This demonstrated that cells transformed with *Flt3-ITD*^{F692L} were resistant to AC220 and Sorafenib, but not Ponatinib, unlike wild-type *Flt3*$^{ITDp.F692F}$ cells that were sensitive to all three compounds (Figure 2C). These data, taken together with our results with the primary AML cells, confirm that the murine F692L mutation mimics the properties of the human F691L mutant FLT3-ITD and confers a similar profile of resistance to FLT3 inhibitors[16].

Here, we show that the *Flt3*^{tm1Dgg} knock-in mice carry the Flt3 F692L resistance variant *in cis* with the human W51 *Flt3-ITD* mutation. The variant corresponds to the human F691L and shares at

least some of its drug sensitivity/resistance profile. The origin of the c.2076A>T (p.F692L) variant within the *Flt3*^{tm1Dgg} allele is not clear. Examination of the strategy and design of the original gene targeting construct revealed that this did not extend to exon 17, which contains codon F692. Therefore, it appears unlikely that the c.2076T>A SNV was introduced into the mouse Flt3 locus during gene targeting[4]. Also, the presence of the variant in 3/3 independent *Flt3*^{tm1Dgg} mouse colonies tested, rules out the possibility that the SNV was uniquely acquired in our cohort. Therefore, the SNV was either present in the original embryonic stem cells used to generate the knock-in mice or was acquired in the germline during the expansion of the original colony. Notably the SNV is not present in any of the 48 sequenced mouse strains according to the mouse genome informatics (MGI) database (http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=snpQF). Nevertheless, regardless of its origin, the presence of this variant needs to be taken into account when interpreting the findings of the many studies using *Flt3*^{tm1Dgg} mice[7-13] particularly those investigating therapeutic approaches[11, 13]. Additionally, our findings make the *Flt3*^{tm1Dgg} model particularly useful in the study of novel TKIs such as Ponatinib and PLX3397, which retain activity against the human F691L gatekeeper mutation[15]. Moreover, our findings emphasize the importance of careful characterisation of genetically modified mice before drawing conclusions about the pathologies they model, particularly when targeted therapeutic interventions are being investigated. Whilst in our manuscript the genetic variant influencing drug response was not present in wild-type mice, in other cases it can represent a consistent human - mouse difference, as was recently described for Lenalidomide and a mouse-specific variant in Cereblon (*Crbn*) that makes mouse cells insusceptible to the drug[17]. Given the availability and relative affordability of next generation sequencing, we propose that genomic characterisation such as exome sequencing, of frequently-used or novel mouse models should be used to identify coding variants in order avoid misinterpretation of findings from these otherwise highly valuable biological reagents.

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Figure Legends

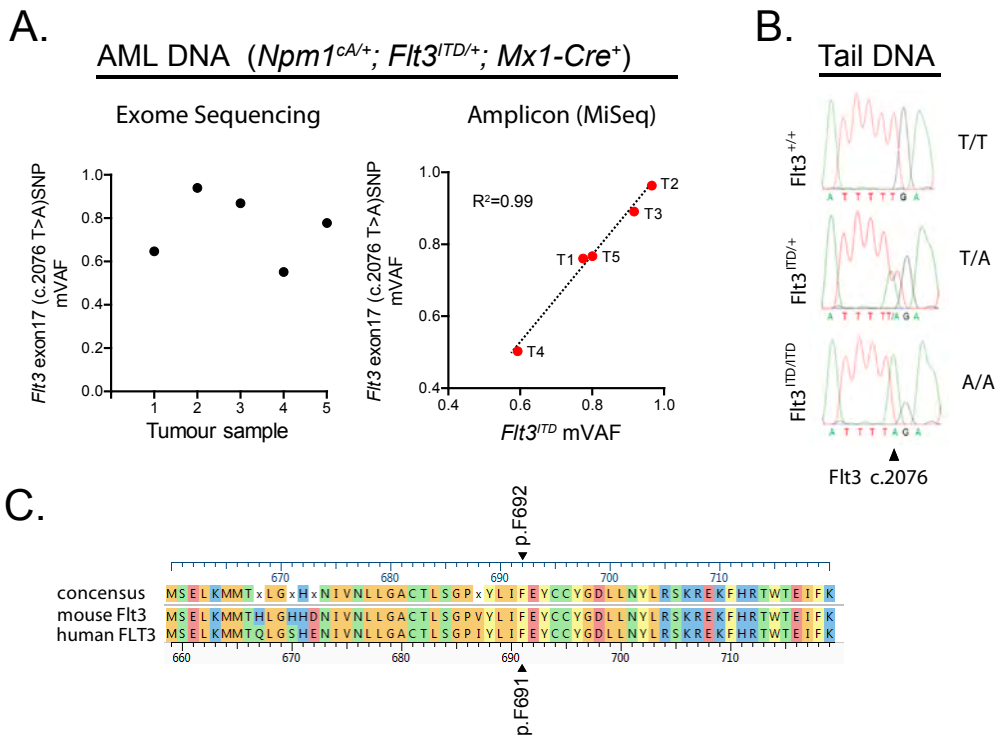
Figure 1. Identification of the *Flt3* c.2076 T>A (F692L) variant *in cis* in *Flt3*-ITD knock-in mice

(A) The *Flt3* c.2076 T>A exon 17 single nucleotide variant (SNV) was identified in 5/5 exomes of *Npm1*$^{cA/+}$; *Flt3*$^{ITD/+}$; *Mx1-Cre*$^{+}$ acute myeloid leukemia (AML) samples at varying variant allele fractions (VAF) (left panel). These results were confirmed by PCR-amplification followed by MiSeq sequencing of the 5 available AML DNA samples (T1-T5) and this also revealed a close correlation between the SNV with the ITD VAFs (middle panel). (B) Sequencing of tail DNA from *Flt3*$^{+/+}$, *Flt3*$^{ITD/+}$ and *Flt3*$^{ITD/ITD}$ mice confirmed that the SNV is present *in cis* with the *Flt3*^{tm1Dgg} allele and not particular to the *Npm1*^{cA}; *Flt3*^{tm1Dgg} double mutant cohort. (C) Protein sequence alignment of mouse and human FLT3 protein demonstrates mouse p.F692 corresponds to human p.F691, target of the F691L gatekeeper TKI resistance mutations.

Figure 2. The *Flt3* c.2076 T>A (F692L) variant confers resistance to Quizartinib and Sorafenib

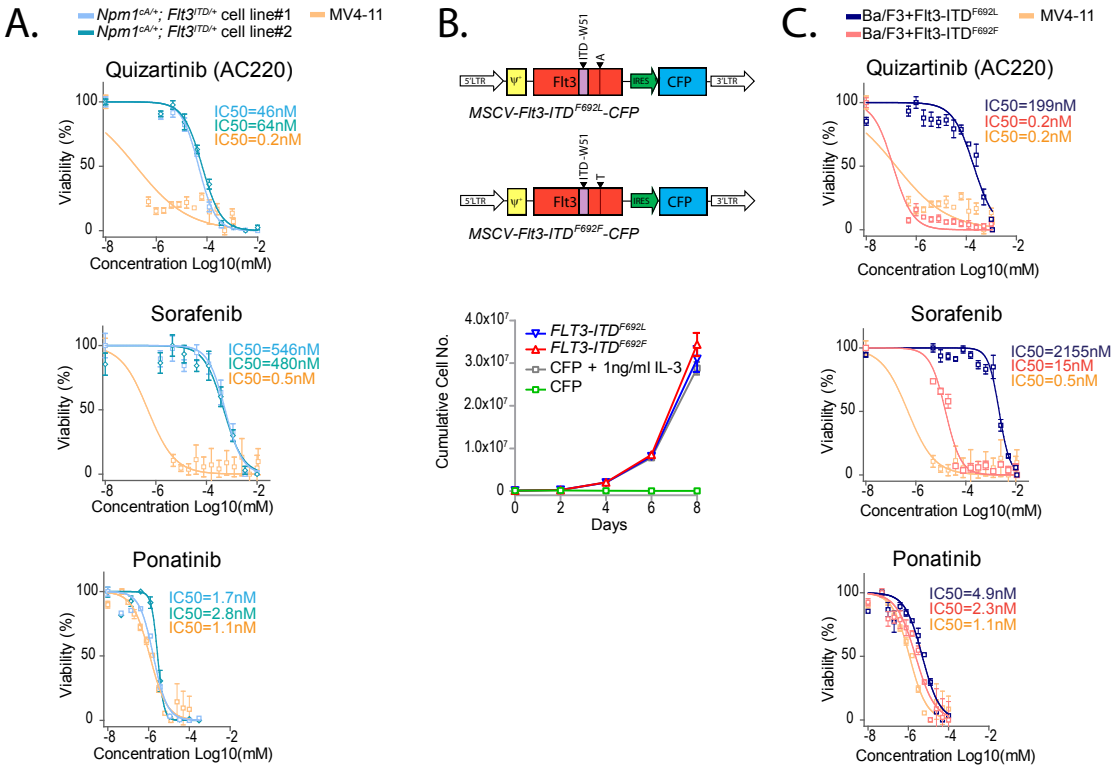
(A) Two independent leukemic cell lines, derived from Npm1$^{cA/+}$; Flt3$^{ITD/+}$ mice, were cultured in X-VIVO-20 medium (Lonza) (supplemented with 5% FCS, 10ng/ml mIL-6 and 50ng/ml mSCF) and incubated with Flt3 TKIs over a range of concentrations. IC50 values for each murine leukemia and for the control cell line MV4;11, in response to each treatment are displayed. (B) *MSCV-Flt3-ITD-CFP* retroviral constructs used to stably transduce CellSensor® irf-bla Ba/F3 cells. Both the *Flt3ITD*^{F692L} and the reverted *Flt3*^{F692F} cDNAs transformed the Ba/F3 cells to IL-3 independent growth with growth kinetics similar to those of IL-3-stimulated cells transduced with an MSCV-CFP (empty) vector (also see Supplementary Figure 2). (C) Stably transduced mutant Ba/F3 cell lines and the *FLT3-ITD*-positive AML cell line MV4;11 were incubated with Flt3 inhibitors at a range of concentrations. The *MSCV-Flt3ITD*^{F692L}*-CFP* construct conferred Ba/F3 resistance to both Quizartinib and Sorafinib, but not Ponatinib; whilst cells transduced with the *MSCV-Flt3ITD*^{F692F}*-CFP* construct remained sensitive to all three inhibitors. IC50 values for Flt3ITDp.F692L (blue) or the corrected Flt3ITDp.F692L cDNA (red) transduced cells are displayed inset for each drug. All assays were performed in triplicate.

Figure 1


A.
AML DNA (Npm1cA/+; Flt3ITD/+; Mx1-Cre+)
Exome Sequencing
Amplicon (MiSeq)
Flt3 exon17 (c.2076 T>A)SNP mVAF
Tumour sample
R²=0.99
T1
T2
T3
T4
T5
Flt3ITD mVAF
B.
Tail DNA
Flt3+/+
Flt3ITD/+
Flt3ITD/ITD
T/T
T/A
A/A
Flt3 c.2076

C.
p.F692
p.F691
concensus
mouse Flt3
human FLT3

Figure 2


A.
Npm1cA/+; Flt3ITD/+ cell line#1
Npm1cA/+; Flt3ITD/+ cell line#2
MV4-11
Quizartinib (AC220)
IC50=46nM
IC50=64nM
IC50=0.2nM
Viability (%)
Concentration Log10(mM)
Sorafenib
IC50=546nM
IC50=480nM
IC50=0.5nM
Ponatinib
IC50=1.7nM
IC50=2.8nM
IC50=1.1nM
B.
ITD -W51
ITD -A
5'LTR
Ψ
Flt3
IRES
CFP
3'LTR
MSCV-Flt3-ITDF692L-CFP
MSCV-Flt3-ITDF692F-CFP
Cumulative Cell No.
FLT3-ITDF692L
FLT3-ITDF692F
CFP + 1ng/ml IL-3
CFP
Days
C.
Ba/F3+Flt3-ITDF692L
Ba/F3+Flt3-ITDF692F
MV4-11
Quizartinib (AC220)
IC50=199nM
IC50=0.2nM
IC50=0.2nM
Sorafenib
IC50=2155nM
IC50=15nM
IC50=0.5nM
Ponatinib
IC50=4.9nM
IC50=2.3nM
IC50=1.1nM

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

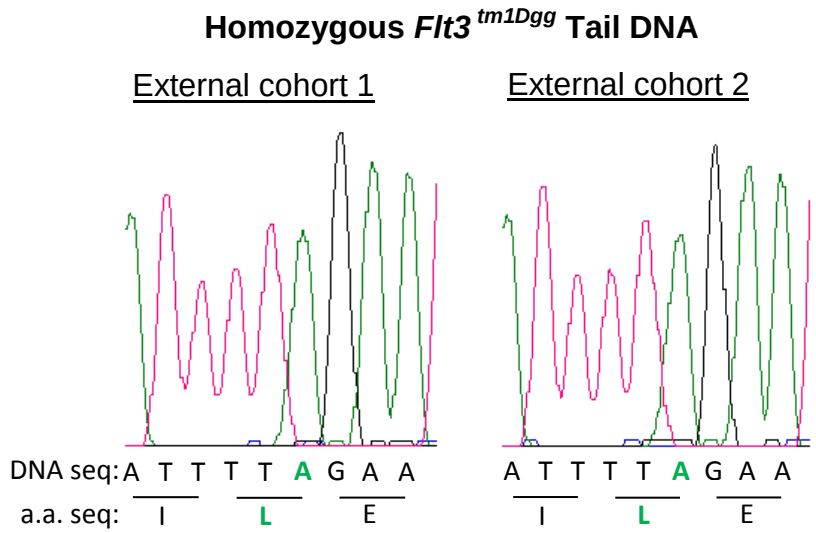
Gibson-Frag2-Rev: ttccaagcggcttcggccagtaacgttagg

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).

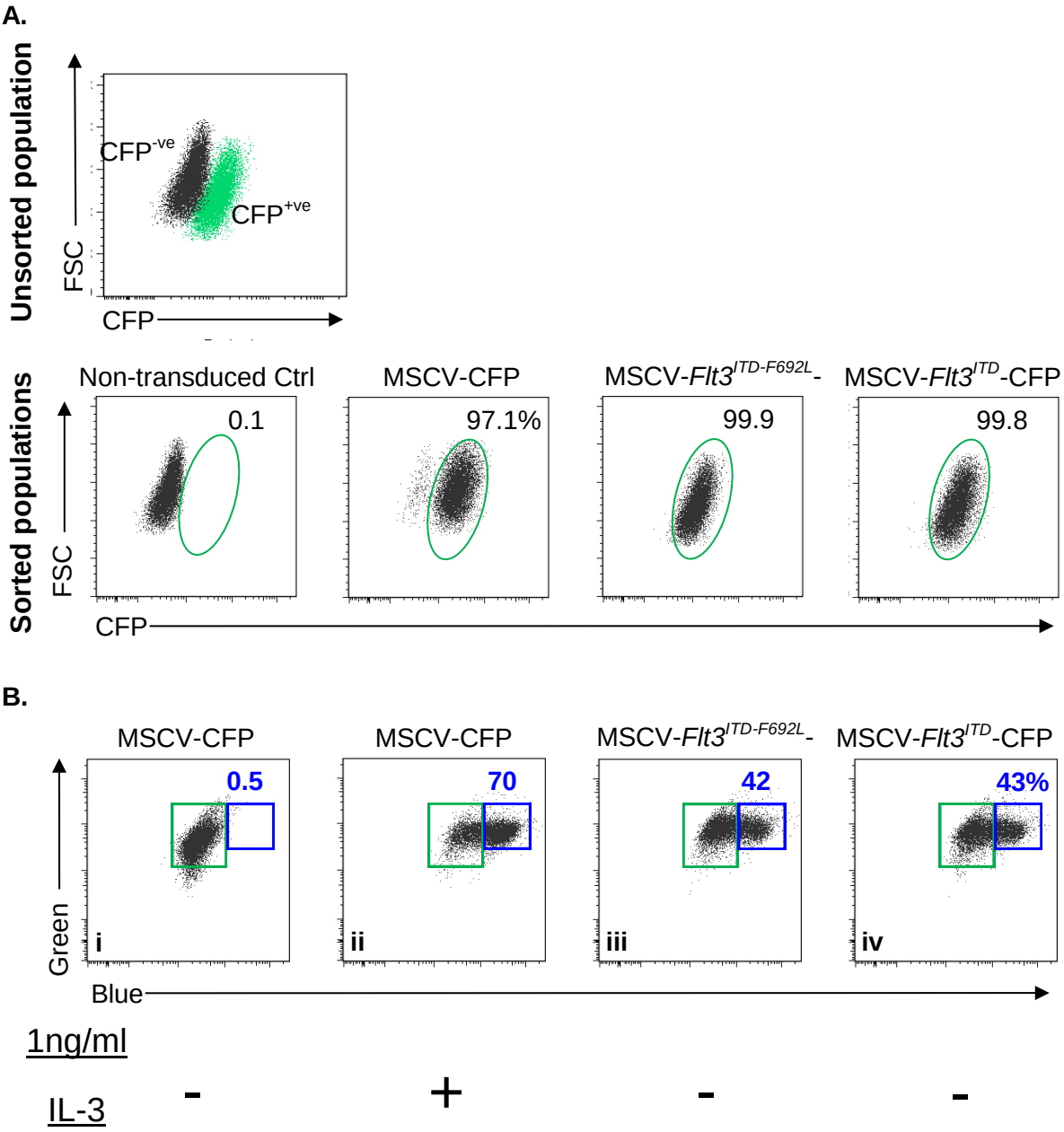
Like other Ba/F3 cell lines, the CellSensor® irf-bla Ba/F3 cell line is an IL-3 dependent cell line. However, it also contains an additional beta-lactamase reporter gene under the control of Stat5 response elements from the IRF1 gene promoter [1]. Of note, activation of STAT5 is a well reported molecular 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.

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