Title: Loss of c-Cbl E3 ubiquitin ligase activity enhances the development of myeloid leukemia in FLT3-ITD mutant mice

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Abstract: Mutations in the FLT3 receptor tyrosine kinase (RTK) occur frequently in acute myeloid leukemia (AML), with the most common involving internal tandem duplication (ITD) within the juxtamembrane domain. FLT3-ITD mutations result in a mislocalized and constitutively activated receptor, which aberrantly phosphorylates STAT5 and upregulates the expression of its target genes. c-Cbl is an E3 ubiquitin ligase that negatively regulates RTKs, including FLT3, but whether it can downregulate mislocalized FLT3-ITD remains to be resolved. To help clarify this we combined a FLT3-ITD mutation with a loss-of-function mutation in the RING finger domain of c-Cbl that abolishes its E3 ligase activity. Mice transplanted with hematopoietic stem cells expressing both mutations rapidly develop myeloid leukemia indicating strong cooperation between the two. Although the c-Cbl mutation was shown to cause hyper-activation of another RTK, c-Kit, it had no effect on enhancing FLT3-ITD protein levels or STAT5 activation. This indicates that c-Cbl does not downregulate FLT3-ITD, and that the leukemia is driven by independent pathways involving FLT3-ITD’s activation of STAT5 and mutant c-Cbl’s activation of other RTKs, such as c-Kit. This study highlights the importance of c-Cbl’s negative regulation of wild-type RTKs in suppressing FLT3-ITD-driven myeloid leukemia.
Schematic of the potential cellular mechanisms driving myeloid leukemia development in the FLT3-ITD; c-Cbl RING finger (A5-) double mutant mouse. The combination of FLT3-ITD-induced STAT5 activation together with mutant c-Cbl-induced PI 3-kinase hyperactivity leads to perturbed expression of distinct sets of genes, which cooperate to drive leukemia development.
HIGHLIGHTS

- FLT3-ITD and c-Cbl RING finger mutations cooperate to generate myeloid leukemia.
- The c-Cbl mutation does not enhance FLT3-ITD activity.
- FLT3-ITD leukemia is enhanced by the additional activity of non-FLT3 c-Cbl targets.
- These findings suggest FLT3-ITD escapes c-Cbl-directed downregulation.
Loss of c-Cbl E3 ubiquitin ligase activity enhances the development of myeloid leukemia in FLT3-ITD mutant mice

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ABSTRACT

Mutations in the FLT3 receptor tyrosine kinase (RTK) occur frequently in acute myeloid leukemia (AML), with the most common involving internal tandem duplication (ITD) within the juxtamembrane domain. FLT3-ITD mutations result in a mislocalized and constitutively activated receptor, which aberrantly phosphorylates STAT5 and upregulates the expression of its target genes. c-Cbl is an E3 ubiquitin ligase that negatively regulates RTKs, including FLT3, but whether it can downregulate mislocalized FLT3-ITD remains to be resolved. To help clarify this we combined a FLT3-ITD mutation with a loss-of-function mutation in the RING finger domain of c-Cbl that abolishes its E3 ligase activity. Mice transplanted with hematopoietic stem cells expressing both mutations rapidly develop myeloid leukemia indicating strong cooperation between the two. Although the c-Cbl mutation was shown to cause hyper-activation of another RTK, c-Kit, it had no effect on enhancing FLT3-ITD protein levels or STAT5 activation. This indicates that c-Cbl does not downregulate FLT3-ITD, and that the leukemia is driven by independent pathways involving FLT3-ITD’s activation of STAT5 and mutant c-Cbl’s activation of other RTKs, such as c-Kit. This study highlights the importance of c-Cbl’s negative regulation of wild-type RTKs in suppressing FLT3-ITD-driven myeloid leukemia.
INTRODUCTION

Since the discovery of the FLT3 receptor tyrosine kinase (RTK) as the most frequently mutated kinase in hematological malignancies there has been intense study into how it causes leukemia and into the development of compounds that target its activity. Approximately 30% of acute myeloid leukemia (AML) patients harbour FLT3 mutations, with the most frequent occurring as internal tandem duplications (ITD) of sequences within the juxtamembrane domain [1,2]. FLT3-ITD mutations are associated with poor survival and a high rate of relapse, an event that often occurs with the acquisition of FLT3-ITD homozygosity [3].

One of the striking features that distinguish FLT3-ITD from wild-type (WT) FLT3 is its impaired intracellular trafficking that results in the retention of FLT3-ITD in the endoplasmic reticulum (ER) causing aberrant signaling and the constitutive activation of STAT5 [4-7]. It has been proposed that the retention of FLT3-ITD in the ER also results in an escape from degradation by the c-Cbl E3 ubiquitin ligase [8], a fate assigned to mature WT FLT3 at the plasma membrane when it is activated by FLT3 ligand [9,10]. c-Cbl is a RING finger based E3 ubiquitin ligase that has been well characterized as a key negative regulator of mammalian and invertebrate RTKs [11,12].

Recently c-Cbl mutations have been identified in a range of hematopoietic neoplasms, the most prominent being chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML) [13]. These mutations are located either in the RING finger or adjacent linker domain, both of which are essential for c-Cbl to associate with E2 ubiquitin conjugating enzymes and therefore to function as an E3 ligase. Mice with a knock-in mutation in the RING finger domain of c-Cbl develop a severe myeloproliferative disease (MPD) that is characterized by an
expanded population of FLT3⁺ multi-potent progenitors (MPPs) [14]. When these mice were mated to FLT3 ligand deficient mice, or treated with the FLT3 inhibitor AC220, the mice did not develop a MPD [14,15]. This indicated that deregulated FLT3 signaling promotes disease development, and that c-Cbl E3 ligase activity maintains a level of FLT3 signaling that prevents MPD development. To test whether c-Cbl E3 ligase activity is similarly involved in suppressing MPD caused by FLT3-ITD we studied mice repopulated with hematopoietic stem cells doubly mutant for the c-Cbl RING finger domain and FLT3-ITD. We find a robust but indirect cooperation between the two mutations resulting in the rapid development of an aggressive myeloid leukemia.

MATERIALS and METHODS

Mice
The generation of c-Cbl(C379A) RING finger mutant mice (i.e. c-Cblᴬᶜ) and FLT3-ITD mice have previously been described [16,17] C57BL/6.CD45.1 congenic mice were purchased from the Animal Resources Centre (Canning Vale, Western Australia). Mouse experiments were approved and performed in accordance with the guidelines and regulations of the Animal Ethics Committee at the University of Western Australia (approval 100/1169). Mice were housed under pathogen-free conditions in micro-isolator cages at the animal facilities of the University of Western Australia.

Analysis of peripheral blood, bone marrow and spleen by flow cytometry
Blood was collected from the tail vein or heart and differential cell counts determined using a Hemavet HV950FS blood analyzer (Drew Scientific, Waterbury, CT). Bone marrow suspensions were obtained from 2 pairs of leg bones (tibia and femur). Bone marrow, spleen and white blood cells were analyzed by flow cytometry (FACS Canto, BD Biosciences). All monoclonal antibodies for flow cytometry are from BD Biosciences, except where noted otherwise: CD3-biotin, CD11b-
FITC, -PECy7 or -biotin, CD19-PE or -PerCP-Cy5.5, B220-biotin or -FITC, TER119-biotin or -FITC, Gr-1- FITC or -biotin, TCR-APC, MHC II-PE, CD11c-biotin or -APC, IgM-APC, CD24-PECy7, CD43-PE, c-Kit-FITC, -APC (eBiosciences) or –PE (eBiosciences), Sca-1-PE or PE-Cy7 (eBiosciences), Flt3-PE (eBiosciences), Rat IgG2ak-PE (eBiosciences), CD48-FITC and CD150-PECy7 (eBiosciences). Cells incubated with biotinylated antibodies were treated with streptavidin conjugated with APC-Cy7 (BD Biosciences). Cells stained for pSTAT5 analysis were fixed with Cytofix™ (BD) and permeabilized with ice-cold methanol then stained with phospho-STAT5 – AF647 (BD). The data was collected using FACSDIVA™ software (BD Biosciences) and analyzed using FlowJo 9.4.11 software (Tree Star, Inc., Ashland, OR).

**SCF stimulation of lineage negative bone marrow cells**

Lineage negative bone marrow was obtained using a magnetic lineage depletion kit (BD) following the manufacturer’s instructions. The lineage negative bone marrow was incubated for 1hr at 37°C before stimulation with mouse SCF (2.5 ng/ml or 25 ng/ml; from R&D Systems) for 5 or 30 minutes. Following fixation/permeabilization with Cytofix solution (BD) and ice-cold methanol, intra-cellular staining for phospho-S6 ribosomal protein-AF647 (Cell Signaling Technology), phospho-Erk-AF647 (Cell Signaling Technology) and c-Kit-PE (eBiosciences) was performed for flow cytometry analysis.

**Immunohistochemistry**

Dewaxed, rehydrated, and permeabilized tissue samples were blocked for 1hr (20% normal goat serum, 1% bovine serum albumin in TBS pH 7.4) at room temperature and immersed in rabbit anti-human myeloperoxidase polyclonal antibody (Dako) at 10.6 ug/ml or rabbit IgG isotype control overnight at 4°C. The next day samples were washed in TBS and quenched for endogenous peroxidase (3% H₂O₂ in TBS) for 15 min at room temperature before application of EnVision
polymer (Dako) for 40 min at room temperature. Slides were developed in 3% H₂O₂ and diaminobenzidine, rinsed, counterstained with Mayer's hematoxylin, rinsed in Scott's Tap Water Substitute then dehydrated and coverslipped.

**Immunoblotting**

Lineage-negative BM cells were purified using a hematopoietic progenitor cell enrichment kit (BD Biosciences) and were lysed in SDS sample buffer. Cell lysates were examined by immunoblotting with anti-phospho-Akt(S473), anti-phospho-c-Cbl(Y731) and anti-phospho-STAT5(Y694) (all from Cell Signaling Technology), anti-c-Cbl (7G10, Millipore), anti-Akt (clone 7, BD Biosciences) and anti-STAT5 (3H7, Cell Signaling Technology).

**Statistical analyses**

To validate the significance of the observed differences between the various genotypes of mice we used unpaired two-sided t-tests (Prism 5, GraphPad Software). P values less than 0.05 were considered statistically significant. All statistical data are presented as means ± standard errors, unless otherwise stated in figure legends.

**RESULTS**

**Co-expression of FLT3-ITD and c-Cbl RING finger mutations causes embryonic lethality**

The generation and phenotype of c-Cbl(C379A) RING finger knock-in mice (i.e. c-Cbl<sup>AV</sup> mice) has previously been described [16]. Since homozygous c-Cbl RING finger mutant mice die in utero, we analyze mice that express a single mutant c-Cbl RING finger allele and a c-Cbl null allele (i.e. c-Cbl<sup>AV</sup>). FLT3-ITD knock-in mice breed successfully as homozygotes and develop a MPD that resembles human CMML [17]. To generate c-Cbl RING finger mutant mice that additionally carry either homozygous or heterozygous FLT3-ITD mutations we mated FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>AV+</sup> with
FLT3\textsuperscript{+ITD};c-Cbl\textsuperscript{+} mice. These matings can produce 4 genotypes: FLT3\textsuperscript{+ITD};c-Cbl\textsuperscript{+}, FLT3\textsuperscript{+ITD};c-Cbl\textsuperscript{A/-}, FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/-}, or FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-}, with a predicted frequency of 25% each. However, the frequency of births was much lower for mice with combined c-Cbl RING finger and FLT3-ITD mutations, an effect that was most severe with the homozygous FLT3-ITD mutation, i.e. 2 of 80 births for FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} mice compared to 8 FLT3\textsuperscript{+ITD};c-Cbl\textsuperscript{A/-} mice (Fig. 1A).

Analysis of fetal development at day E14 found that Mendelian frequencies were maintained, with the FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} genotype accounting for 11 of 38 fetuses. This allowed cohorts of mice to be generated by transplanting fetal liver cells from day 14 embryos into lethally irradiated (2x 5.5 Gy) C57BL/6.CD45.1 recipient mice. FLT3-ITD and c-Cbl RING finger mutations cooperate to promote the rapid development of myeloid leukemia

Cohorts of 10 irradiated mice were repopulated with fetal liver cells from each of the four genotypes described above, and monitored for 24 weeks. Over this period all mice receiving FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} fetal liver cells required euthanasia, whereas no mice in the other cohorts succumbed to disease (Fig. 1B). The FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} mice showed markedly larger spleens compared to mice transplanted with either FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/-} fetal liver cells or c-Cbl\textsuperscript{A/-} bone marrow (Fig. 1, C and D). Furthermore the FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} phenotype was markedly more severe than that observed in FLT3\textsuperscript{+ITD};c-Cbl\textsuperscript{A/-} mice (Fig. 1, A, B and C), a finding consistent with previous studies where homozygous FLT3-ITD is significantly more potent than the effect seen in mice that retain a WT FLT3 allele [18,19]. Whether this affect is entirely due to gene dosage differences, or whether the WT allele actively suppresses the oncogenic potential of FLT3-ITD remains to be determined [18]. Histopathological analysis of peripheral organs from FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} mice revealed extensive infiltration of cells into the liver and lungs and disruption of the
splenic architecture (Fig. 1E). Examination by immunohistochemistry revealed many of the invasive cells were positive for myeloperoxidase (MPO), suggesting a myeloid leukemia (Fig. 1E).

A comparison of white blood cell (WBC) counts at 12 weeks from mice transplanted with fetal liver or bone marrow cells from 7 different c-Cbl and FLT3 genotype combinations showed the striking effect of the FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>A/</sup>- double mutation; an effect that was markedly more potent than either of the single mutations (Fig. 2A). Included in this analysis were mice transplanted with FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>A/-</sup> fetal liver cells which showed a similar phenotype to mice transplanted with FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>+/</sup>- fetal liver (Fig. 2A and B). This finding indicated that the complete loss of c-Cbl does not enhance leukemia development in FLT3-ITD mice, possibly because of compensation by Cbl-b (which would be blocked in cells expressing the c-Cbl<sup>A/-</sup> protein), and also because of the loss of c-Cbl’s positive role as an adaptor in cell signaling [11].

The marked increase in WBC counts in FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>A/-</sup> mice at 12 weeks was also accompanied by a higher proportion of neutrophils compared to other FLT3<sup>ITD/ITD</sup> transplanted mice, thus indicating that the RING finger mutation enhances the myeloid bias of the FLT3<sup>ITD/ITD</sup> phenotype (Fig. 2B). A time-course analysis of WBCs at 9, 12 and 18 weeks (6 of the 10 FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>A/-</sup> mice survived to 18 weeks), showed a large increase in the WBC counts between weeks 12 and 18, whereas the counts from FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>+/</sup>- mice remained stable at ~16 x 10<sup>9</sup>/L (Fig. 2C). This large increase in WBCs is suggestive of the acquisition of additional mutations, although this possibility was not examined here. Furthermore by 18 weeks we observed a decrease in the proportion of neutrophils compared to 12 weeks (Fig. 2D), an increase in monocytes and c-Kit<sup>+</sup> cells (Fig. 2E and F), a marked decrease in the proportion of CD19<sup>+</sup> B cells (Fig. 2G), and a 10-fold increase in the proportion of CD19<sup>-</sup> B220<sup>+</sup> cells (Fig. 2G), a population we further characterize in Figure 6. These phenotypic characteristics, and the presence of blast cells in
blood films from FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} mice (Fig. 2I and J), are consistent with the development of myeloid leukemia with maturation [20].

The c-Cbl RING finger mutation promotes a greater loss of long-term hematopoietic stem cells in FLT3-ITD mice

A striking phenotype of bone marrow (BM) from FLT3-ITD mice is the marked decrease in long-term hematopoietic stem cells (LT-HSCs) and the expansion of multi-potent progenitors (MPPs) [21]. This effect on LT-HSCs indicates that FLT3-ITD perturbs hematopoiesis at an earlier point than had previously been thought. To examine the effects of the c-Cbl RING finger mutation on LT-HSCs and MPPs in FLT3-ITD mice we examined CD48 and CD150 markers within the lineage negative, Sca-1\(^+\), c-Kit\(^+\) (LSK) population, where LT-HSCs are defined as CD48\(^-\) CD150\(^+\), and MPPs as CD48\(^+\) CD150\(^-\) [22]. By comparing BM cells from B6 mice with mice transplanted with FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} or FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} fetal liver cells (at 14 – 22 weeks post-transplantation) we found that the c-Cbl RING mutation significantly exacerbated the severity of the FLT3-ITD phenotype with LT-HSC numbers reduced to nearly undetectable levels and MPPs increasing (Fig. 3A). In contrast, the proportion of lineage negative cells, which is markedly expanded in FLT3-ITD mice, was not further altered by the RING finger mutation (Fig. 3A).

The marked effects of the c-Cbl RING finger mutation on FLT3-ITD BM cells were also evident by phenotyping with lineage antibodies. Notably, the proportion of CD11b\(^+\) Gr-1\(^-\) myeloid cells was significantly expanded at the expense of more differentiated CD11b\(^+\) Gr-1\(^+\) cells, and there was a substantial decrease in the proportion of CD19\(^+\) B lineage cells (Fig. 3B). As seen in the blood, the two mutations also resulted in a marked expansion of CD19\(^-\) B220\(^+\) cells. This phenotype was mirrored in the spleen with the double mutation promoting a considerable reduction
in the percentage of CD19+ cells and an expansion of CD19+ B220+ and CD11b+ Gr-1+ cells (Fig. 3C).

All FLT3ITD/ITD;c-CblA−/− mice in this cohort were analyzed at their endpoints of disease, a time where the large increase in WBC counts suggested additional genetic changes may have occurred. This raises the possibility that phenotypic characteristics that are exclusively driven by the combination of FLT3-ITD and c-Cbl RING finger mutations may be masked or altered by subsequent mutational events. For this reason we carried out further analysis at earlier times after fetal liver transplantation.

The c-Cbl RING finger mutation in FLT3-ITD mice promotes a marked expansion of MPPs and a displacement of HSCs into the periphery

Detailed flow cytometric analyses of a cohort of mice 6-10 weeks post-transplantation with c-Cbl+/− or c-CblA−/− BM, or FLT3ITD/ITD;c-CblA+/− or FLT3ITD/ITD;c-CblA−/− fetal liver cells verified the phenotype found in the older cohort. Overall the BM from FLT3ITD/ITD;c-CblA+/− mice exhibited a large loss of LT-HSCs and short-term (ST)-HSCs (defined as LSK CD48− CD150+) [22], and a profound expansion of MPPs, all indicating an exaggeration of the FLT3-ITD phenotype (Fig. 4A). Of note was the large expansion of LT-HSCs in the BM of c-CblA−/− mice, a previously described phenotype that was unable to rescue the lack of LT-HSCs in FLT3-ITD mice (Fig. 4A, left panel) [23].

To investigate whether HSCs are displaced from the BM into the periphery we examined spleen and blood. We found that the blood of FLT3ITD/ITD;c-CblA−/− mice exhibited a significant increase in LT-HSCs compared to FLT3ITD/ITD;c-CblA+/− mice (Fig. 4B, left panel). The ST-HSC population was also increased in FLT3ITD/ITD;c-CblA−/− mice relative to their c-CblA+/− counterpart (Fig. 4B, middle
However, the largest perturbation occurred within the MPP population where the combination of both mutations led to an expansion that was ~10-fold greater than that observed with the single FLT3-ITD mutation (Fig. 4B, right panel). Significantly, the MPPs accounted for ~14% of the undifferentiated c-Kit+ blast cells in the peripheral blood of FLT3ITD/ITD;c-CblAv− mice (data not shown).

Examination of spleens also identified an increase in LT-HSCs, ST-HSCs and MPPs in FLT3ITD/ITD;c-CblAv− mice relative to FLT3ITD/ITD;c-CblAv/c mice (Fig. 4C). Thus the loss of c-Cbl E3 ligase activity in the FLT3-ITD mouse promotes a marked displacement of these three populations into the periphery. However, from our estimates, the greater numbers of LT-HSCs in FLT3ITD/ITD;c-CblAv− blood and spleen makes up for the acute loss in the BM when compared to the FLT3ITD/ITD;c-CblAv/c mice such that there is no significant difference in the total numbers of LT-HSCs (Fig. 4D, left panel). It should also be noted that the high level of peripheral hematopoiesis in FLT3ITD/ITD;c-CblAv− mice also exacerbates the vast expansion of MPPs and ST-HSCs (Fig. 4D).

**The FLT3-ITD block in B-cell development is not altered by the c-Cbl RING finger mutation**

Mice repopulated with FLT3ITD/ITD;c-CblAv/c or FLT3ITD/ITD;c-CblAv− fetal liver cells have markedly reduced proportions of CD19+ B cells in peripheral blood, and this perturbation is most severe in FLT3ITD/ITD;c-CblAv− mice (Fig. 5A). However, due to higher WBC counts in FLT3ITD/ITD;c-CblAv− mice, the total numbers of B cells do not differ between the two (Fig. 5B). Further, analysis of BM showed a marked deficiency in IgM+ B cells in both FLT3-ITD genotypes (Fig. 5, C and D). This was caused by a severe block in differentiation at the pre-B stage, which resulted in a corresponding accumulation of pro- and prepro-B cells (Fig. 5, C and F-H). Since the proportions of all three B cell precursor populations were equivalent between the FLT3ITD/ITD;c-CblAv/c and FLT3ITD/ITD;c-CblAv− mice it appears that the c-Cbl RING finger mutation does not affect the FLT3-
ITD block in B cell development.

**FLT3-ITD and c-Cbl RING finger mutations combine to cause a marked expansion in dendritic cells**

As previously mentioned, WBCs from mice repopulated with FLT3<sup>ITD/ITD</sup>;Cbl<sup>A</sup>−/− fetal liver cells exhibit a marked increase in B220<sup>+</sup> CD19<sup>−</sup> cells (Fig. 5A), a population that is also expanded within the bone marrow and spleen (Fig. 6A). To further characterize these cells we examined peripheral blood for the expression of additional surface markers which revealed that the majority expressed high levels of CD11c and MHC II, markers for plasmacytoid dendritic cells (pDCs) (Fig. 6B). Another large proportion of B220<sup>+</sup> CD19<sup>−</sup> cells also co-expressed CD11b (Fig. 6B).

Earlier studies have identified expansions in DC-like (CD11c<sup>+</sup>) populations in FLT3-ITD mice [19,24], however our investigation found this expansion to occur in all mature DC subsets. The numbers of plasmacytoid (p)DCs and classical (c)DCs (both CD11b<sup>+</sup> and CD11b<sup>−</sup>) were expanded in the blood of all of three mutant genotypes compared to c-Cbl<sup>A</sup>−/− mice, however this expansion was most profound in FLT3<sup>ITD/ITD</sup>;Cbl<sup>A</sup>−/− mice (Fig. 7, A and B). Similar perturbations in all DC populations were found in the BM and spleen (Fig. 7, C and D), with FLT3<sup>ITD/ITD</sup>;Cbl<sup>A</sup>−/− mice exhibiting the greatest expansion. Thus the two mutations work synergistically to create a massive increase in all DC populations.

**The c-Cbl RING finger mutation does not alter FLT3-ITD protein expression or enhance STAT5 signaling**

As previously established, the c-Cbl RING finger mutant mouse has an enhanced level of surface FLT3 expression within the LSK population whereas the FLT3-ITD protein fails to reach the surface in detectable amounts (Fig. 8A) and [14,18,25]. A current point of conjecture revolves
around whether c-Cbl can negatively regulate the mislocalized FLT3-ITD protein [8-10]. To investigate whether the level of FLT3-ITD protein was altered by the loss of c-Cbl E3 ligase activity we measured total FLT3 protein in fixed and permeabilized LSK cells. As found with surface expression the c-CblA/− LSK cells had an increased proportion of FLT3hi cells, however no difference was evident between the flow cytometry profiles of LSK cells from FLT3ITDITD;c-Cbl+/− and FLT3ITDITD;c-CblA/− mice (Fig. 8B). Examination of the lineage negative (Lin−) compartment for FLT3 expression detected an abnormally high proportion of cells expressing FLT3 protein in the FLT3-ITD mice, i.e. FLT3 expression was expanded beyond the LSK population (Fig. 8C). However, consistent with LSK cells, the RING finger mutation did not alter FLT3-ITD protein levels in the Lin− compartment (Fig. 8C). These findings imply that c-Cbl is not negatively regulating FLT3-ITD levels.

To further clarify whether c-Cbl has an effect on FLT3-ITD we measured a readout of FLT3-ITD activity, i.e. the levels of phosphorylated (p) STAT5, a protein that is constitutively activated by FLT3-ITD [4-7]. It was found that the level of pSTAT5 was not altered in Lin− cells from FLT3ITDITD;c-CblA/− mice indicating that c-Cbl does not negatively regulate this aspect of FLT3-ITD signaling (Fig. 8D). The pSTAT5 signal was validated by an immunoblot (Supplementary Fig. 1).

c-Cbl RING finger mutant-induced hyperactivity of the PI 3-kinase pathway is retained in FLT3ITDITD;c-CblA/− mice

Following the finding that STAT5 signaling in FLT3-ITD mice was unaffected by the c-Cbl RING finger mutation we investigated additional signaling events that involve the PI 3-kinase and Ras/MAP kinase pathways. As previously established [14], c-CblA/− mice exhibit constitutive activity in the PI 3-kinase pathway of Lin− cells as illustrated by enhanced levels of pS6 (Fig. 9A,
left panel). In contrast there was minimal constitutive S6 activity in Lin\textsuperscript{−} cells from c-Cbl\textsuperscript{+/−} or FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/−} mice (Fig. 9A). Interestingly constitutive S6 activity was not evident in the Lin\textsuperscript{−} c-Kit\textsuperscript{+} (LK) population of c-Cbl\textsuperscript{+/−} and FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/−} mice. This indicates that the c-Cbl RING finger mutation drives constitutive S6 activity in more committed progenitors. The activation of the PI 3-kinase pathway was also shown by a pAkt immunoblot of Lin\textsuperscript{−} cells where a FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/−} mouse showed higher levels of pAkt compared to a B6 and a FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/−} mouse (Supplementary Fig. 1). In contrast none of the genotypes displayed constitutive activation of the Ras/MAP kinase pathway as evidenced by pErk levels in LK and Lin\textsuperscript{−} cells (Fig. 9A, right panels).

To further characterize the activity of these pathways in the four genotypes we examined the effects of stimulating LK cells with stem cell factor (SCF). SCF is the ligand for c-Kit, an RTK that is negatively regulated by c-Cbl. Stimulation for 5 min caused a large induction of pS6 in LK cells from all four genotypes (Fig. 9, B and C), however by 30 min the pS6 response had significantly diminished in the c-Cbl\textsuperscript{+/−} and FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/−} LK cells. In contrast pS6 levels were sustained in LK cells from both c-Cbl\textsuperscript{+/−} and FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/−} mice, i.e. there is no decrease in the activation of S6 by SCF between 5 and 30 minutes (Fig. 9, B and C). Stimulation with SCF also caused an induction of pErk at 5 min that was similar for all four genotypes (Fig. 9, B and D), and after 30 min all four returned to near basal levels (Fig. 9, B and D). These findings indicate that enhanced PI 3-kinase activity caused by the c-Cbl RING finger mutation is likely to be a key factor that contributes to the development of myeloid leukemia in the FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/−} mouse.

**DISCUSSION**

This study has demonstrated that FLT3-ITD and c-Cbl RING finger mutations cooperative to promote the rapid induction of myeloid leukemia. The single mutation knock-in mice develop less
severe diseases where FLT3-ITD causes an MPD with slightly elevated WBC counts and a bias towards myeloid cells [17,24], and the c-Cbl RING finger mutation leads to an MPD with a more heterogeneous involvement of lineages [14]. However, in combination, these two mutations promote the development of a very aggressive leukemia with an average survival of ~20 weeks.

The strong cooperation between these two mutations raises the question of whether leukemia development is a direct effect because c-Cbl can no longer function as an E3 ligase for FLT3-ITD; or whether the two mutant proteins act independently of each other and remain unaffected by their dual presence in the same cell. Data from a number of studies on the in vitro functions of these proteins indicate that a probable cause could be the hyper-activation of FLT3-ITD signaling because c-Cbl was shown to associate with FLT3-ITD and promote its polyubiquitination and degradation [9,10]. However, another study found that c-Cbl was only able to target mature fully glycosylated forms of FLT3-ITD that could reach the cell surface, and that the abundant immature intracellular pool was resistant to c-Cbl-mediated degradation [8]. From our analysis of c-Cbl+/−, c-CblA−/−, FLT3ITD/ITD;c-Cbl+/− and FLT3ITD/ITD;c-CblA−/− mice we conclude that the development of the more aggressive leukemia is not due to a loss of the negative regulation of FLT3-ITD but rather from the combination of separate signaling pathways that are activated by the individual mutations. The evidence for this is the equivalent level of FLT3-ITD protein in both FLT3ITD/ITD;c-Cbl+/− and FLT3ITD/ITD;c-CblA−/− mice, and the lack of enhanced activity of STAT5 in FLT3ITD/ITD;c-CblA−/− mice. Deregulated STAT5 signaling is a hallmark of FLT3-ITD-expressing cells, and if the E3 ligase that directs its degradation were lost then enhancement of STAT5 signaling would be anticipated.

As previously shown [14,16], we found that the most profound signaling effect caused by the c-Cbl RING finger mutation was the enhanced activation of the PI 3-kinase pathway, and importantly
this enhancement was found to be equivalent between c-Cbl<sub>AV</sub>- and FLT3<sub>ITD/ITD</sub>;c-Cbl<sub>AV</sub> mice. This indicates that c-Cbl RING finger mutant signaling, as with FLT3-ITD signaling, is not heightened in the double mutant mouse, yet the combination of both has a profound effect in enhancing leukemia development. Interestingly the absence of WT FLT3 protein in FLT3<sub>ITD/ITD</sub>;c-Cbl<sub>AV</sub> mice did not noticeably affect the activity of the PI 3-kinase pathway. This indicates that additional c-Cbl targets, such as c-Kit, that become hyper-responsive as a result of the RING finger mutation, are capable of contributing to the enhanced PI 3-kinase signaling. It has previously been identified that the combination of PI 3-kinase and STAT5 signaling can lead to acute forms of leukemia, for example with CRLF2-rearranged B-precursor acute lymphoblastic leukemia [26]. Thus leukemia development from the constitutive activation of STAT5 by FLT3-ITD, and enhanced PI 3-kinase activation by the c-Cbl RING finger mutation, has a precedent.

There are a number of studies that have investigated mutational cooperation with FLT3-ITD in murine models of myeloid leukemia. Interestingly most of these are type II mutations that are presumed to affect differentiation. These include the transcription factors CBF, CEBPA and HOXD13, the histone methyltransferase MLL, the multi-function protein chaperon NPM1, and the metabolic enzyme IDH2 [19,27-31]. This is the first study to our knowledge to identify FLT3-ITD working in combination with an additional type I (proliferative) mutation, i.e. the c-Cbl RING finger mutation is involved in enhancing tyrosine kinase signaling.

Both the FLT3-ITD and c-Cbl RING finger mutations lead to altered but unique gene expression profiles, which when combined would contribute to the more aggressive disease in the doubly mutant mice (Graphical Abstract). FLT3-ITD drives the up-regulation of many genes including Id1, Cish, Pim1, SOCS1/2, ENPP and MRC1 [6,18,32,33]. On the other hand, the activation of RTKs by the c-Cbl RING finger mutation is associated with the up-regulation of many
transcription factors (including Id1, Hoxb5, Rorc and Meis1) and down-regulation of many suppressor genes (including JunB, EgR1 and GATA1) [14]. The majority of these genes are involved in regulating hematopoiesis, and perturbations within these are commonly found in AML [34-40]. Therefore, by combining the FLT3-ITD and c-Cbl RING finger mutations a potent transcriptional environment is likely to be formed that drives the development of myeloid leukemia.

The present study further highlights the ability of mutant and mislocalized RTKs to evade ubiquitin ligases, and in this case within an in vivo model [41-44]. It would therefore be interesting to examine the effects of c-Cbl on FLT3 with activating mutations that involve point mutations in the second tyrosine kinase domain (TKD). Gain-of-function FLT3-TKD mutations range between surface and non-surface localizations [32,45], hence the importance of cellular location upon c-Cbl mediated regulation could be further examined. Furthermore it will be of interest to clarify the role of other ubiquitin ligases including Nedd4 and SIAH1 [8,9], which have been shown to target FLT3-ITD in transfected cell lines, and to determine if this is the case in vivo.

In summary these studies have revealed that c-Cbl’s negative regulation of tyrosine kinases involved in hematopoietic stem and progenitor cell maintenance is critical for suppressing the development of an aggressive myeloid leukemia by FLT3-ITD. These findings therefore raise the previously unrealized possibility that other deregulated tyrosine kinases may cooperate with FLT3-ITD to promote the development of myeloid leukemia.

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hematological diagnoses. This work was supported by the National Health and Medical Research Council (project grants 572516 an 634414), the Medical and Health Research Infrastructure Fund (Health Department of Western Australia), the L.T Thean Memorial Scholarship, and the Cancer Council of Western Australia.

**Competing interests statement:** The authors declare no competing financial interest.

**REFERENCES**


FIGURE LEGENDS

Figure 1. Mice transplanted with FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A-} fetal liver cells rapidly succumb to myeloid leukemia. (A) Frequencies of naturally born offspring and day E14 fetuses from matings of FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A+} and FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{-} parents (n=80 and n=38 respectively). (B) Kaplan-Meier plot of survival of C57BL/6.CD45.1 mice transplanted with E14 fetal liver cells from each of the four genotypes from the above breeding (n=9-10 for each genotype). (C) Spleen weights of mice 14-24 weeks post transplantation (n=4-6). (D) Spleens from FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A+/A-} and FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A-} mice 18 weeks post transplantation. (E) Lung, spleen and liver sections stained with hematoxylin and eosin (H&E) or myeloperoxidase (MPO) antibodies. The sections are from 22 weeks post transplantation of FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A-/A-} and FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A-} mice. The scale bars are 200µm for H&E and 100µm for MPO images. The images were acquired at room temperature using an Olympus BX51 microscope with a 60X/0.09 objective and photographed with an SIS 3VCU Olympus digital camera.

Figure 2. Characterization of acute myeloid leukemia in FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A-} transplanted mice. (A) White blood cell (WBC) counts and (B) proportions of neutrophils of C57BL/6.CD45.1 mice 12 weeks after transplantation with BM or fetal liver cells from the 6 indicated genotypes (n=9-10). (C) WBC counts from mice 9, 12 and 18 weeks after transplantation with FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A+/A-} or FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A-} fetal liver cells (n=6-10). (D) Proportions neutrophils, (E) monocytes, (F) c-Kit\textsuperscript{+}, (G) CD19\textsuperscript{+} and (H) CD19\textsuperscript{B220+} cells from mice 9, 12 and 18 weeks after transplantation with FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A+/A-} or FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A-} fetal liver cells. Data in graphs are expressed as means ± SEM. (I) Representative blood films from mice after transplantation with FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A+/A-} and FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A-} fetal liver cells (scale bar 50µm), including higher power images of the FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A-} blood at 18 weeks. The images were acquired at room temperature using an Olympus BX51 microscope with a 60X/0.09 objective and photographed with an SIS 3VCU Olympus digital camera. High power images were acquired at room temperature using an Olympus U-TV0.SXC-3 microscope with either a 60x/0.9 or 100x/1.4 (oil) objective and photographed with a Pixera Pro 600Es-CU camera. (J) Percentages of blast cells 18 weeks after transplantation. Percentages are from blood films from 8 mice transplanted with FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A+/A-} fetal liver cells and 6 mice transplanted with FLT3\textsuperscript{ITD/ITD} c-Cbl\textsuperscript{A-} fetal liver cells.
Figure 3. Phenotyping of moribund FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>Av</sup> transplanted mice demonstrates perturbations in many BM populations. (A) Flow cytometry profiles of bone marrow cells from a WT B6 mouse and mice transplanted with FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>Av</sup>/c-Cbl<sup>Av</sup> fetal liver cells. LT-HSCs are defined as CD150<sup>+</sup>CD48<sup>-</sup> LSK cells (n=4-5). (B) Flow cytometry profiles of bone marrow cells showing an expansion in CD11b<sup>+</sup>Gr1<sup>-</sup> (immature myeloid) and B220<sup>+</sup>CD19<sup>-</sup> cells, along with a profound decrease in CD19<sup>+</sup> (B-lineage) cells in the bone marrow of mice transplanted with FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>Av</sup>/c-Cbl<sup>Av</sup> fetal liver cells (n=4-5). (C) Flow cytometry profiles of spleen cells showing similar expansions and reductions of these populations (n=4-5). Data are expressed as means ± SEM and statistics were calculated using Students’ t-tests, *= p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Figure 4. FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>Av</sup> transplanted mice show a significant loss of long term hematopoietic stem cells (LT-HSCs) and expanded numbers of multi-potent progenitors (MPPs). (A) Numbers of LT-HSCs, short-term (ST-) HSCs and MPPs in the bone marrow, (B) peripheral blood and (C) spleen of c-Cbl<sup>Av</sup>/c-Cbl<sup>Av</sup>, FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>Av</sup>/c-Cbl<sup>Av</sup> and FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>Av</sup>/c-Cbl<sup>Av</sup> mice at 6-10 weeks post-transplantation (n=5-7 for each genotype). (D) Estimates of total LT-HSCs, ST-HSCs and MPPs calculated from BM, spleen and blood of c-Cbl<sup>Av</sup>/c-Cbl<sup>Av</sup>, FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>Av</sup>/c-Cbl<sup>Av</sup> mice at 6-10 weeks post-transplantation. Estimates were calculated assuming a blood volume of 2 ml and total number of BM cells of 466 x 10<sup>6</sup> [46]. Data in the graphs are expressed as means ± SEM and statistics were calculated using Students’ t-tests, *= p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Figure 5. FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>Av</sup> transplanted mice show a marked block in B cell differentiation. (A) Representative proportions of cell-surface markers (c-Kit, B220, CD19, CD11b and Gr-1) in peripheral WBCs, excluding T-cells, from mice of each genotype. (B) Numbers of CD19<sup>+</sup> B-cells in blood. (C) Representative flow cytometry profiles showing the IgM and B220 populations, and the proportions of PrePro-, Pro- and Pre-B cells in the four genotypes. (D) Proportion of total BM cells that are B220<sup>+</sup>IgM<sup>+</sup> or, (E) B220<sup>+</sup>IgM<sup>-</sup>. (F) Proportion of B220<sup>+</sup>IgM<sup>-</sup> BM cells that are PrePro-B, (G) Pro-B or (H) Pre-B cells (n=6). All the B-lineage populations were gated on CD11b/CD11c negative BM to eliminate B220<sup>+</sup> cells that express these myeloid/DC markers in FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>Av</sup> mice. Data in the graphs are expressed as means ± SEM and statistics were calculated using Students’ t-tests, *= p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.
Figure 6. FLT3ITD/ITD; c-Chl^A/- mice possess an expanded population of B220^+CD19^- cells that express high levels of CD11b, CD11c and MHC II. (A) Proportion of B220^+CD19^- cells in blood, BM, and spleen of the four genotypes (n=8). (B) Representative single parameter flow cytometry profiles showing the expression of CD11b, CD11c, MHC II, Gr-1 and c-Kit on the B220^+CD19^- population from FLT3ITD/ITD; c-Chl^A/- WBCs. Data in the graphs are expressed as means ± SEM and statistics were calculated using Students’ t-tests, *= p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Figure 7. Dendritic cell populations are markedly expanded in FLT3ITD/ITD; c-Chl^A/- transplanted mice. (A) Representative flow cytometry profiles showing the populations of plasmacytoid dendritic cells (pDCs) and classical (c) DCs (CD11b^+ and CD11b^-) from the peripheral blood of c-Chl^+/-, c-Chl^A/-, FLT3ITD/ITD; c-Chl^+/-, and FLT3ITD/ITD; c-Chl^A/- transplanted mice. (B) Numbers of pDCs and cDCs (CD11b^+ and CD11b^-) in the peripheral blood (n=8), (C) bone marrow (n=8), and (D) spleen (n=5). Data in the graphs are expressed as means ± SEM. All statistics were calculated using Students’ t-tests, *= p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Figure 8. FLT3-ITD expression and constitutive STAT5 activity are unaffected by the c-Chl RING finger mutation. (A) Representative flow cytometry profile of surface FLT3 expression on LSK cells, and the proportion of FLT3^hi cells from c-Chl^+/-, c-Chl^A/-, FLT3ITD/ITD; c-Chl^+/-, and FLT3ITD/ITD; c-Chl^A/- mice 6-10 weeks post-transplantation (n=3-4). (B) Representative flow cytometry profile of total FLT3 levels of LSK cells including proportions of FLT3^hi cells and mean fluorescence intensity (MFI) of total FLT3 expression (n=4). (C) Representative flow cytometry profile of total FLT3 expression from the Lin^-BM cells, including MFI of FLT3 expression from the four genotypes at 6-10 weeks (n=4). (D) Representative flow cytometry profile of intracellular pSTAT5 expression in the Lin^-BM cells, including MFI of pSTAT5 from the four genotypes at 6-10 weeks (n=9). The MFI graphs are expressed as a proportion of c-Chl^+/-. All data are expressed as means ± SEM and statistics were calculated using Students’ t-tests, *= p<0.05.

Figure 9. The c-Chl RING finger mutation enhances constitutive and SCF-induced activation of the PI 3-kinase pathway. (A) Flow cytometry profiles showing the levels of pS6 (left panels)
and pErk (right panels) in unstimulated Lin⁻ and Lin⁺ c-Kit⁺ (LK) BM cells from c-Cbl⁺/−, c-Cbl⁺/−, FLT3⁺/−;c-Cbl⁺/− and FLT3⁺/−;c-Cbl⁺/− mice at 6–10 weeks post-transplantation. (B) pS6 and pErk flow cytometry profiles following ex vivo stimulation with 2.5 ng/ml SCF of Lin⁻ c-Kit⁺ BM cells from the four indicated genotypes. (C) Proportion of pS6⁺ cells over the indicated time course of stimulation with 2.5 ng/ml SCF (n=4). (D) Proportion of pErk⁺ cells over the stimulation time course with 2.5 or 25 ng/ml SCF (n=3). A higher concentration of SCF (25 ng/ml) was found to induce a similar pErk induction to 2.5 ng/ml, hence was included in the pooled analysis. Data in the graphs are expressed as means ± SEM and statistics were calculated using Students’ t-tests, *= p<0.05.
Reviewer #1

The phenotype of the cells is not really suggestive of biphenotypic leukemia. Biphenotypic leukemia is when leukemic cells co-express markers of the myeloid and lymphoid lineage on the same cell. This has not been demonstrated since CD11b and CD11c are not strictly myeloid antigens. The mice described here appear to have an expansion of several different cell types at various stages of maturation. I think the reference to biphenotypic leukemia should be removed.

The reference to biphenotypic leukemia has been removed (page 13).

Figure 8E should be removed or placed in supplementary data. It could be argued that the blot of pSTAT5 supports the flow data, although is hardly necessary, but the pAKT and pc-Cbl add little to nothing, and as they are based on single western blot, lack scientific rigor. Similarly, the results at the end of page 14 and the top of page 15 should also be removed for the same reasons. The activation of PI-3K pathway is shown in a more rigorous manner by the pS6 data and is sufficient to argue activation of the pathway.

We have removed Figure 8E and placed the immunoblot as Supplementary Figure 1. We have also removed the pc-Cbl blot and deleted the accompanying text (previously on page 14). The text referring to the pAkt blot has also been reduced (page 15).
Figure 1

A) Pie chart showing Naturally Born with percentages: 10%, 45%, 42.5%, 2.5%
B) Graph showing percent survival over days for different genotypes: +/ITD, +/ITD; A/-, ITD/ITD, +/ITD, ITD/ITD; A/-
C) Bar graph showing spleen weight (mg) for different genotypes: +/ITD, +/ITD; A/-, ITD/ITD, +/ITD, ITD/ITD; A/-
D) Image showing spleen weights: 177mg, 1662mg
E) H&E and MPO images for lung, spleen, and liver for ITD/ITD; +/-, ITD/ITD; A/-
Figure 2

A and B: 

C: 

D: 

E: 

F: 

G: 

H: 

I: 

J: 

Figure 2
Figure 3

Bone Marrow

A

B6  |  ITD/ITD: +/-  |  ITD/ITD: A-
--- | --- | ---
Lin- | Lin- | Lin-
No. of cells | No. of cells | No. of cells

Scal-1  |  LSK  |  LSK  |  LSK
--- | --- | --- | ---
kit | 4.2 | 1.9 | 2.0

c-Kit

CD150  |  LT-HSC  |  LT-HSC  |  LT-HSC
--- | --- | --- | ---
MPP | 7.0 | 6.0 | 9.0

CD48

Number of Lin+ x 10^7

Number of MPP x 10^7

C

Bone Marrow

B6  |  ITD/ITD: +/-  |  ITD/ITD: A-
--- | --- | ---
Gr-1  |  CD11b  |  CD11b
--- | --- | ---

CD19

B220

% CD11b+  |  % CD19+  |  % B220+ CD19
--- | --- | ---
B6  |  ITD/ITD: +/-  |  ITD/ITD: A-

Spleen

B6  |  ITD/ITD: +/-  |  ITD/ITD: A-
--- | --- | ---
Gr-1  |  CD11b  |  CD11b
--- | --- | ---

CD19

B220

% CD11b+  |  % CD19+  |  % CD19 B220
--- | --- | ---
B6  |  ITD/ITD: +/-  |  ITD/ITD: A-
Figure 4

Click here to download high resolution image
Figure 6

(A) Distribution of B220+ CD19+ cells in Blood, Bone Marrow, and Spleen.

(B) Flow cytometry analysis of B220+ CD19- cells in Blood, showing expression levels of CD11b, CD11c, MHC II, Gr-1, and c-Kit.
Figure 7

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Figure 9

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Supplemental data

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