Chapter 5. Loss of c-Cbl ubiquitin ligase activity enhances the development of myeloid leukaemia in FLT3-ITD mutant mice

5.1 Introduction

Since the discovery of the FLT3 receptor tyrosine kinase (RTK) as the most frequently mutated kinase in haematological malignancies there has been intense study into how it causes leukaemia and into the development of compounds that target its activity. Approximately 30% of acute myeloid leukaemia (AML) patients harbour FLT3 mutations, with the most frequent occurring as internal tandem duplications (ITD) of sequences within the juxtamembrane domain (Kottaridis et al., 2001; Schnittger et al., 2002). 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 (Raghavan et al., 2008).

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 signalling and the constitutive activation of STAT5 (Choudhary et al., 2005; Hayakawa et al., 2000; Mizuki et al., 2000; Yoshimoto et al., 2009). 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 (Buchwald et al., 2010), a fate assigned to mature WT FLT3 at the plasma membrane when it is activated by FLT3 ligand (Oshikawa et al., 2011; Sargin et al., 2007). c-Cbl is a RING finger based E3 ubiquitin ligase that has been well characterised as a key negative regulator of mammalian and invertebrate RTKs (Mohapatra et al., 2013; Thien and Langdon, 2005a).

Recently c-Cbl mutations have been identified in a range of haematopoietic neoplasms, the most prominent being chronic myelomonocytic leukaemia (CMML) and juvenile myelomonocytic leukaemia (JMML) (Kales et al., 2010). 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 characterised by an expanded population of FLT3\(^+\) multi-potent progenitors (MPPs) (Rathinam et al., 2010). When these mice were mated to FLT3 ligand deficient mice, or treated with the FLT3 inhibitor AC220, the mice did not develop a MPD (Rathinam et al., 2010; Taylor et al., 2012). This indicated that deregulated FLT3 signalling promotes disease development, and that c-Cbl E3 ligase activity maintains a level of FLT3 signalling 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 haematopoietic 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 leukaemia.

5.2 Results

5.2.1 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\(^{A/-}\) mice) has previously been described (Thien et al., 2005). Since homozygous c-Cbl RING finger mutant mice die in utero, we analyse mice that express a single mutant c-Cbl RING finger allele and a c-Cbl null allele (i.e. c-Cbl\(^{A/-}\)). FLT3-ITD knock-in mice breed successfully as homozygotes and develop a MPD that resembles human CMML (Lee et al., 2007). To generate c-Cbl RING finger mutant mice that additionally carry either homozygous or heterozygous FLT3-ITD mutations we mated FLT3\(^{ITD/ITD}\);c-Cbl\(^{A/+}\) with FLT3\(^{+/ITD}\);c-Cbl\(^{A/-}\) mice. These matings can produce 4 genotypes: FLT3\(^{+/ITD}\);c-Cbl\(^{+/+}\), FLT3\(^{+/ITD}\);c-Cbl\(^{A/+}\), FLT3\(^{ITD/ITD}\);c-Cbl\(^{+/+}\), or FLT3\(^{ITD/ITD}\);c-Cbl\(^{A/-}\), 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\(^{ITD/ITD}\);c-Cbl\(^{A/-}\) mice compared to 8 FLT3\(^{+/ITD}\);c-Cbl\(^{A/-}\) mice (Figure 5.1A). Analysis of foetal development at day E14 found that Mendelian frequencies were maintained, with the FLT3\(^{ITD/ITD}\);c-Cbl\(^{A/-}\) genotype accounting for 11 of 38 foetuses. This allowed cohorts of mice to be generated by transplanting foetal liver cells from day 14 embryos into lethally irradiated (2x 5.5 Gy) C57BL/6.CD45.1 recipient mice.
5.2.2 FLT3-ITD and c-Cbl RING finger mutations cooperate to promote the rapid development of myeloid leukaemia

Cohorts of 10 irradiated mice were repopulated with foetal liver cells from each of the four genotypes described above, and monitored for 24 weeks. Over this period all mice receiving FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>A/</sup> foetal liver cells required euthanasia, whereas no mice in the other cohorts succumbed to disease (Figure 5.1B). The FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>A/</sup> mice showed markedly larger spleens compared to mice transplanted with either FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>+/</sup> foetal liver cells or c-Cbl<sup>+</sup> bone marrow (Figure 5.1C and D). Furthermore the FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>A/</sup> phenotype was markedly more severe than that observed in FLT3<sup>+/ITD</sup>;c-Cbl<sup>A/</sup> mice (Figure 5.1A, 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 (Greenblatt et al., 2012; Kharazi et al., 2011). Whether this effect is entirely due to gene dosage differences, or whether the WT allele actively suppresses the oncogenic potential of FLT3-ITD remains to be determined (Kharazi et al., 2011). Histopathological analysis of peripheral organs from FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>A/</sup> mice revealed extensive infiltration of cells into the liver and lungs and disruption of the splenic architecture (Figure 5.1E). Examination by immunohistochemistry revealed many of the invasive cells were positive for myeloperoxidase (MPO – a marker of myeloid cells), suggesting a myeloid leukaemia (Figure 5.1E).

A comparison of white blood cell (WBC) counts at 12 weeks from mice transplanted with foetal 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 (Figure 5.2A). Included in this analysis were mice transplanted with FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>+/</sup> foetal liver cells which showed a similar phenotype to mice transplanted with FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>+/</sup> foetal liver (Figure 5.2A and B). This finding indicated that the complete loss of c-Cbl does not enhance leukaemia development in FLT3-ITD mice, possibly because of compensation by Cbl-b (which would be blocked in cells expressing the c-Cbl RING-finger mutant protein), and also because of the loss of c-Cbl’s positive role as an adaptor in cell signalling (Thien and Langdon, 2005a).
The marked increase in WBC counts in FLT3<sup>ITD/ITD</sup>:c-Cbl<sup>V/−</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 (Figure 5.2B). A time-course analysis of WBCs at 9, 12 and 18 weeks (6 of the 10 FLT3<sup>ITD/ITD</sup>:c-Cbl<sup>V/−</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>V/−</sup> mice remained stable at ∼16 x 10<sup>9</sup>/L (Figure 5.2C). 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 (Figure 5.2D), an increase in monocytes and c-Kit<sup>+</sup> cells (Figure 5.2E and F), a marked decrease in the proportion of CD19<sup>+</sup> B cells (Figure 5.2G), and a 10-fold increase in the proportion of CD19<sup>−</sup> B220<sup>+</sup> cells (Figure 5.2G), a population we further characterised in Figure 5.6. These phenotypic characteristics, and the presence of blast cells in blood films from FLT3<sup>ITD/ITD</sup>:c-Cbl<sup>V/−</sup> mice (Figure 5.2I and J), are consistent with the development of myeloid leukaemia with maturation (Kogan et al., 2002).

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

A striking phenotype of bone marrow (BM) from FLT3-ITD mice is the marked decrease in long-term haematopoietic stem cells (LT-HSCs) and the expansion of multipotent progenitors (MPPs) (Chu et al., 2012). This effect on LT-HSCs indicates that FLT3-ITD perturbs haematopoiesis 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<sup>+</sup>, c-Kit<sup>+</sup> (LSK) population, where LT-HSCs are defined as CD48<sup>−</sup> CD150<sup>+</sup>, and MPPs as CD48<sup>+</sup> CD150<sup>−</sup> (Reynaud et al., 2011). By comparing BM cells from B6 mice with mice transplanted with FLT3<sup>ITD/ITD</sup>:c-Cbl<sup>V/−</sup> or FLT3<sup>ITD/ITD</sup>:c-Cbl<sup>V/−</sup> foetal 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 (Figure 5.3A). 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 (Figure 5.3A).
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 (typically monocytes) was significantly expanded at the expense of more differentiated CD11b+ Gr-1+ cells (typically neutrophils), and there was a substantial decrease in the proportion of CD19+ B lineage cells (Figure 5.3B). As seen in the blood, the two mutations also resulted in a marked expansion of CD19+ B220+ cells (a population described in more detail in 5.2.6). 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 (Figure 5.3C).

All FLT3ITDITD;c-CblA/- mice in this cohort were analysed at their endpoints of disease, a time when 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 foetal liver transplantation.

5.2.4 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 FLT3ITDITD;c-Cbl+/- or FLT3ITDITD;c-CblA/- foetal liver cells verified the phenotype found in the older cohort. Overall the BM from FLT3ITDITD;c-CblA/- reconstituted mice exhibited a large loss of LT-HSCs and short-term (ST)-HSCs (defined as LSK CD48- CD150-) (Reynaud et al., 2011), and a profound expansion of MPPs, all indicating an exaggeration of the FLT3-ITD phenotype (Figure 5.4A). Of note was the large expansion of LT-HSCs in the BM of c-CblA/- reconstituted mice, a previously described phenotype that was unable to rescue the lack of LT-HSCs in FLT3-ITD mice (Figure 5.4A, left panel) (Rathinam et al., 2008).

To investigate whether HSCs are displaced from the BM into the periphery we examined spleen and blood. We found that the blood of FLT3ITDITD;c-CblA/-
reconstituted mice exhibited a significant increase in LT-HSCs compared to the FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/−} reconstituted mice (Figure 5.4B, left panel). The ST-HSC population was also increased in FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A+/−} reconstituted mice relative to their c-Cbl\textsuperscript{+/−} counterpart (Figure 5.4B, middle panel). 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 (Figure 5.4B, right panel). Significantly, the MPPs accounted for ~14% of the undifferentiated c-Kit\textsuperscript{+} blast cells in the peripheral blood of FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A+/−} reconstituted mice (data not shown).

Examination of spleens also identified an increase in LT-HSCs, ST-HSCs and MPPs in FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A+/−} reconstituted mice relative to FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/−} reconstituted mice (Figure 5.4C). 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 FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A+/−} blood and spleen makes up for the acute loss in the BM when compared to the FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/−} reconstituted mice such that there is no significant difference in the total numbers of LT-HSCs (Figure 5.4D, left panel). It should also be noted that the high level of peripheral haematopoiesis in FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A+/−} reconstituted mice also exacerbates the vast expansion of MPPs and ST-HSCs (Figure 5.4D).

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

Mice repopulated with FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/−} or FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A+/−} foetal liver cells have markedly reduced proportions of CD19\textsuperscript{+} B cells in peripheral blood, and this perturbation is most severe in FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A+/−} mice (Figure 5.5A). However, due to higher WBC counts in FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A+/−} mice, the total numbers of B cells do not differ between the two groups of mice (Figure 5.5B). Further, analysis of BM showed a marked deficiency in IgM\textsuperscript{+} B cells in both FLT3-ITD genotypes (Figure 5.5C 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 propro-B cells (Figure 5.5C and F-H). Since the proportions of all three B cell precursor populations were equivalent between the FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/−} and FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A+/−} reconstituted mice it appears that the c-Cbl RING finger mutation does not affect the FLT3-ITD block in B cell development.
5.2.6 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\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/c} foetal liver cells exhibit a marked increase in B220\textsuperscript{+} CD19\textsuperscript{−} cells (Figure 5.5A), a population that is also expanded within the bone marrow and spleen (Figure 5.6A). To further characterise 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) (Figure 5.6B). Another large proportion of B220\textsuperscript{+} CD19\textsuperscript{−} cells also co-expressed CD11b (Figure 5.6B), a phenotype that is suggestive of biphenotypic acute leukaemia, however additional markers would be required to confirm this possibility.

Earlier studies have identified expansions in DC-like (CD11c\textsuperscript{+}) populations in FLT3-ITD mice (Greenblatt et al., 2012; Li et al., 2008), 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\textsuperscript{+} and CD11b\textsuperscript{−}) were expanded in the blood of all of three mutant genotypes compared to c-Cbl\textsuperscript{+/-} reconstituted mice, however this expansion was most profound in FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/c} reconstituted mice (Figure 5.7A and B). Similar perturbations in all DC populations were found in the BM and spleen (Figure 5.7C and D), with FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/c} reconstituted mice exhibiting the greatest expansion. Thus the two mutations work synergistically to create a massive increase in all DC populations.

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

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 (Figure 5.8A) and (Kharazi et al., 2011; Rathinam et al., 2010; Schmidt-Arras et al., 2005). A current point of conjecture revolves around whether c-Cbl can negatively regulate the mislocalised FLT3-ITD protein (Buchwald et al., 2010; Oshikawa et al., 2011; Sargin et al., 2007). 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 permeabilised LSK cells.
As found with surface expression the c-Cbl<sup>−/−</sup> LSK cells had an increased proportion of FLT3<sup>hi</sup> cells, however no difference was evident between the flow cytometry profiles of LSK cells from FLT3<sup>ITD/ITD;Cbl<sup>−/−</sup></sup> and FLT3<sup>ITD/ITD;Cbl<sup>−/+</sup></sup> mice (Figure 5.8B). Examination of the lineage negative (Lin<sup>−</sup>) 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 (Figure 5.8C). However, consistent with LSK cells, the RING finger mutation did not alter FLT3-ITD protein levels in the Lin<sup>−</sup> compartment (Figure 5.8C). 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 (Choudhary et al., 2005; Hayakawa et al., 2000; Mizuki et al., 2000; Yoshimoto et al., 2009). It was found that the level of pSTAT5 was not altered in Lin<sup>−</sup> cells from FLT3<sup>ITD/ITD;Cbl<sup>−/−</sup></sup> mice indicating that c-Cbl does not negatively regulate this aspect of FLT3-ITD signalling (Figure 5.8D). The pSTAT5 signal was validated by an immunoblot (Figure 5.8E). In addition Lin<sup>−</sup> cells from FLT3<sup>ITD/ITD;Cbl<sup>−/−</sup></sup> reconstituted mice showed markedly higher levels of pAkt compared to both FLT3<sup>ITD/ITD;Cbl<sup>−/+</sup></sup> and B6 mice (Figure 5.8E). This phenotype is consistent with our previous studies showing that the c-Cbl RING finger mutation promotes a prominent activation of PI 3-kinase pathway that is mediated by the interaction between pY731 and the SH2 domains of the p85 regulatory subunit of PI 3-kinase (Rathinam et al., 2010; Thien et al., 2005). Because of the prominent activation of Akt in FLT3<sup>ITD/ITD;Cbl<sup>−/−</sup></sup> reconstituted mice we carried out a more detailed examination of the PI 3-kinase pathway.

**5.2.8 c-Cbl RING finger mutant-induced hyperactivity of the PI 3-kinase pathway is retained in FLT3<sup>ITD/ITD;Cbl<sup>−/−</sup></sup> reconstituted mice**

Following the finding that STAT5 signalling in FLT3-ITD reconstituted mice was unaffected by the c-Cbl RING finger mutation we investigated additional signalling events that involve the PI 3-kinase and Ras/MAP kinase pathways. As previously established (Rathinam et al., 2010), c-Cbl<sup>−/−</sup> mice exhibit constitutive activity in the PI 3-kinase pathway of Lin<sup>−</sup> cells as illustrated by enhanced levels of pS6 (Figure 5.9A, left panel). In contrast there was minimal constitutive S6 activity in Lin<sup>−</sup> cells from c-
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Cbl\textsuperscript{+/+} or FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/+} reconstituted mice (Figure 5.9A). Interestingly constitutive S6 activity was not evident in the Lin\textsuperscript{−} c-Kit\textsuperscript{+} (LK) population of c-Cbl\textsuperscript{A−/A−} and FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A−/A−} reconstituted mice. This indicates that the c-Cbl RING finger mutation drives constitutive S6 activity in more committed progenitors. 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 (Figure 5.9A, right panels).

To further characterise 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 (Figure 5.9B 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{A−/−} and FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A−/−} reconstituted mice, i.e. there is no decrease in the activation of S6 by SCF between 5 and 30 minutes (Figure 5.9B and C). Stimulation with SCF also caused an induction of pErk at 5 min that was similar for all four genotypes (Figure 5.9B and D), and after 30 min all four returned to near basal levels (Figure 5.9B 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 leukaemia in the FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A−/−} reconstituted mouse.

5.2.9 AC220 reverses the development of myeloid leukaemia in FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A−/−} reconstituted mice

An additional cohort of FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A−/−} reconstituted mice was generated to examine the effect of daily dosing with the FLT3 inhibitor AC220. After completion of this study it was revealed that the FLT3-ITD mouse has a F691L mutation in the FLT3 gene, conferring resistance to AC220 (Albers et al., 2013; Dovey et al., 2016). Thus, the observations from this chapter have been interpreted accordingly.

Dosing FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A−/−} reconstituted mice with AC220 caused a marked reduction in WBC counts over a 36 day time-course (Figure 5.10A and B). Further analysis at day 36 revealed that the characteristically high proportion of neutrophils evident in FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A−/−} reconstituted mice was reversed, the RBC counts were significantly reduced, and the platelet counts were trending lower (Figure 5.10C, D and E).
Flow cytometric analyses of the blood also identified profound changes in the blood following 36 days of AC220 dosing, with increases in B-lineage cells (CD19⁺B220⁺) and decreases in myeloid cells (CD11b⁺Gr1⁺ and CD11b⁺Gr1⁻) (Figure 5.10F and G). Additional analyses of the blood identified significant reductions in c-Kit⁺ blast cells and DCs (both cDCs and pDCs) in (Figure 5.10H and I). Overall the FLT3<sup>ITD/ITD; c-Cbl<sup>A⁻</sup></sup> blood phenotype reverted more towards a normal B6 phenotype following AC220 treatment. AC220 exerted additional benefits with significant reductions to spleen weights and total bone marrow counts in the FLT3<sup>ITD/ITD; c-Cbl<sup>A⁻</sup></sup> reconstituted mice after AC220 dosing (Figure 5.10J and K).

It was surprising that AC220 exerted such profound beneficial effects upon the phenotype of FLT3<sup>ITD/ITD; c-Cbl<sup>A⁻</sup></sup> reconstituted mice given the presence of the F691L resistance-mediating mutation. This finding supports the idea that non-FLT3 pathways, which are inhibited by AC220 (namely c-Kit), are highly important for leukaemia development in these mice.

5.2.10 AC220 dosing corrects the myeloid differentiation bias in FLT3<sup>ITD/ITD; c-Cbl<sup>A⁻</sup></sup> progenitor cells

Consistent with the AC220-mediated increase in peripheral B-lineage cells, it was identified that the proportion of IgM⁺B220⁺, pre- and prepro- B cells in the BM were significantly increased by 36 days of AC220 dosing (Figure 5.11A and B). Furthermore, common lymphoid progenitors (CLPs) were expanded ~4-fold, whereas as the common myeloid and granulocyte macrophage progenitors (CMPs and GMPs) were significantly reduced (Figure 5.11C, D and E). The megakaryocyte erythroid progenitor (MEP) compartment did not change following AC220 dosing (Figure 5.11F). This identifies that AC220 dosing reverses the strong myeloid bias in FLT3<sup>ITD/ITD; c-Cbl<sup>A⁻</sup></sup> reconstituted mice at an early stage of haematopoietic development.

Interestingly, the proportion of LSK cells rose in the FLT3<sup>ITD/ITD; c-Cbl<sup>A⁻</sup></sup> reconstituted mice dosed with AC220 (Figure 5.11G), however, the proportions of LT-HSCs, ST-HSCs and MPPs did not change significantly (Figure 5.11H). This suggests that the markedly expanded MPP population in FLT3<sup>ITD/ITD; c-Cbl<sup>A⁻</sup></sup> reconstituted mice is resistant to the inhibitory effects of AC220, likely because of the F691L mutation. Thus
it appears that FLT3-ITD receptor signalling is the key pathway driving proliferation and survival of MPPs, with other WT signalling pathways proving to be less important. This contrasts with FLT3^{ITD/ITD};c-Cbl^{A/-} downstream progenitors (i.e. CMPs/GMPs) that appear to be highly dependent upon non-FLT3-ITD signalling pathways that are sensitive to AC220 inhibition. An analysis of the AC220 response observed here compared to inhibition of c-Kit or FLT3-ITD F691L signalling would further clarify the role of these receptors in disease development of FLT3^{ITD/ITD};c-Cbl^{A/-} reconstituted mice.

5.3 Discussion

This study has demonstrated that FLT3-ITD and c-Cbl RING finger mutations cooperative to promote the rapid induction of myeloid leukaemia. 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 (Lee et al., 2007; Li et al., 2008), and the c-Cbl RING finger mutation leads to an MPD with a more heterogeneous involvement of lineages (Rathinam et al., 2010). However, in combination, these two mutations promote the development of a very aggressive leukaemia with an average survival of ~20 weeks.

The strong cooperation between these two mutations raises the question of whether leukaemia 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 signalling because c-Cbl was shown to associate with FLT3-ITD and promote its polyubiquitination and degradation (Oshikawa et al., 2011; Sargin et al., 2007). 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 (Buchwald et al., 2010). From our analysis of c-Cbl^{+/+}, c-Cbl^{A/-}, FLT3^{ITD/ITD};c-Cbl^{+/-} and FLT3^{ITD/ITD};c-Cbl^{A/-} reconstituted mice we conclude that the development of the more aggressive leukaemia is not due to a loss of the negative regulation of FLT3-ITD but rather from the combination of separate signalling pathways that are activated by the individual mutations. The evidence for this is the
equivalent level of FLT3-ITD protein in both FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>+/−</sup> and FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>A/+</sup> reconstituted mice, and the lack of enhanced activity of STAT5 in FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>A/+</sup> mice. Deregulated STAT5 signalling is a hallmark of FLT3-ITD-expressing cells, and if the E3 ligase that directs its degradation were lost then enhancement of STAT5 signalling would be anticipated.

As previously shown (Rathinam et al., 2010; Thien et al., 2005), we found that the most profound signalling 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<sup>A/+</sup> and FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>A/+</sup> reconstituted mice. This indicates that c-Cbl RING finger mutant signalling, as with FLT3-ITD signalling, is not heightened in the double mutant mouse, yet the combination of both has a profound effect in enhancing leukaemia development. Interestingly the absence of WT FLT3 protein in FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>A/+</sup> reconstituted 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 signalling. It has previously been identified that the combination of PI 3-kinase and STAT5 signalling can lead to acute forms of leukaemia, for example with CRLF2-rearranged B-precursor acute lymphoblastic leukaemia (Tasian et al., 2012). Thus leukaemia 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 leukaemia. 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 multifunction protein chaperon NPM1, and the metabolic enzyme IDH2 (Greenblatt et al., 2012; Kats et al., 2014; Kim et al., 2008; Mupo et al., 2013; Reckzeh et al., 2012; Stubbs et al., 2008). 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 signalling.

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
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disease in the doubly mutant mice (Figure 5.12). FLT3-ITD drives the up-regulation of many genes including Id1, Cish, Pim1, SOCS1/2, ENPP and MRC1 (Choudhary et al., 2005; Janke et al., 2014; Kharazi et al., 2011; Reddy et al., 2012). 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) (Rathinam et al., 2010). The majority of these genes are involved in regulating haematopoiesis, and perturbations within these are commonly found in AML (Grubach et al., 2008; Joslin et al., 2007; Neben et al., 2005; Passegue et al., 2004; Shimamoto et al., 1995; Tang et al., 2009; Yu et al., 2014). 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 leukaemia.

The present study further highlights the ability of mutant and mislocalised RTKs to evade ubiquitin ligases, and in this case within an in vivo model (Mak et al., 2007; Padron et al., 2007; Toffalini et al., 2009; Yang et al., 2006). 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 (Janke et al., 2014; Mizuki et al., 2000), 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 (Buchwald et al., 2010; Oshikawa et al., 2011), 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 haematopoietic stem and progenitor cell maintenance is critical for suppressing the development of an aggressive myeloid leukaemia by FLT3-ITD. These findings therefore raise the previously unrealised possibility that other deregulated tyrosine kinases may cooperate with FLT3-ITD to promote the development of myeloid leukaemia.
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Figure 5.1. Mice transplanted with FLT3 ITD/ITD; c-CblA/− foetal liver cells rapidly succumb to myeloid leukaemia.
(A) Frequencies of naturally born offspring and day E14 fetuses from matings of FLT3 ITD/ITD; c-CblA/+ and FLT3 ITD/ITD; c-Cbl−/+ parents (n=80 and n=38 respectively). (B) Kaplan-Meier plot of survival of C57BL/6;CD45.1 mice transplanted with E14 foetal 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 ITD/ITD; c-CblA/+ and FLT3 ITD/ITD; c-CblA/− mice 18 weeks post transplantation. (E) Lung, spleen and liver sections stained with haematoxylin and eosin (H&E) or myeloperoxidase (MPO) antibodies. The sections are from 22 weeks post transplantation of FLT3 ITD/ITD; c-CblA/+ and FLT3 ITD/ITD; c-CblA/− mice. The scale bars are 200 µm for H&E and 100 µm 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.
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Figure 5.2. Characterization of acute myeloid leukaemia 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 foetal 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{+/} or FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} foetal liver cells (n=6-10). (D) Proportions neutrophils, (E) monocytes, (F) c-Kit\textsuperscript{+}, (G) CD19\textsuperscript{+} and (H) CD19\textsuperscript{-}B220\textsuperscript{+} cells from mice 9, 12 and 18 weeks after transplantation with FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{+/-} or FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} foetal 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{+/-} and FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} foetal liver cells (scale bar 50\textmu m), 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/0.90 objective and photographed with an SIS 3VCU Olympus digital camera or an Olympus U-TV0.SXC-3 microscope (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{+/-} foetal liver cells and 6 mice transplanted with FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} foetal liver cells.
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Figure 5.3. Phenotyping of moribund FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>–/–</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>–/–</sup> or FLT3<sup>ITD/ITD</sup>;c-Cbl<sup>–/–</sup> foetal 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>–/–</sup> foetal 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.

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Figure 5.4. FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} transplanted mice show a significant loss of long term haematopoietic 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\textsuperscript{+/+}, c-Cbl\textsuperscript{A/-}, FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} and FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} 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\textsuperscript{+/+}, c-Cbl\textsuperscript{A/-}, FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} and FLT3\textsuperscript{ITD/ITD};c-Cbl\textsuperscript{A/-} 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\textsuperscript{6} (Colvin et al., 2004). 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.