The role of oncogenic Cbl mutants in Kit signaling and myeloid transformation

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Contents

Summary

Acute myeloid leukemia (AML) is a hematopoietic cell disorder characterized by a block in differentiation and increased proliferation and survival of malignant blasts. Expansion of the malignant cell clone effects the normal production of blood cells and – if left untreated – leads to death. Receptor tyrosine kinases (RTKs) play an important role in the pathogenesis of AML, as they are either often mutated or overexpressed. In normal hematopoiesis, RTK signal termination is tightly controlled, and involves ubiquitination, internalization, endocytosis and degradation. Cbl proteins are E3 ligases and have been shown to ubiquitinate several activated RTKs, including Flt3 and Kit, targeting them for degradation. Recently, several Cbl mutations have been identified: Cbl-R420Q was identified in an AML patient and Cbl-70Z was identified in a mouse lymphoma model. In this thesis work, the role of these Cbl mutants in Kit signaling and in a mouse transplantation model was studied.

Cbl mutants (Cbl-R420Q, Cbl-70Z) have the ability to transform the myeloid 32D cell line in cooperation with Kit WT. Cbl mutants along with Kit promoted interleukin-3 (IL3)-independent proliferation and enhanced the cell survival of 32D cells. In contrast, expression of the Cbl mutants alone did not confer IL3-independent growth. Stem cell factor (SCF, the Kit ligand) dependent growth was enhanced in the presence of Cbl mutants and Cbl mutants promoted colonogenic growth in the presence of Kit. Furthermore, Cbl mutants inhibited the ubiquitination of the activated Kit receptor. In addition, Cbl mutants inhibited the endocytosis of the activated Kit receptor. Retroviral expression of Cbl mutants in transplanted bone marrow induced a generalized mastocytosis, a myeloproliferative disease and, in rare care cases, myeloid leukemia. Splenomegaly was observed in the presence of Cbl mutants. Furthermore, mast cells with variable range of infiltration were noticed in all the vital organs (spleen, liver, bone marrow, lung, kidney, heart) of Cbl (mutant) transplanted mice. Almost all recipients of bone marrow cells transduced with Cbl mutants developed a lethal hematologic disorder with a mean latency of 341 days in the Cbl-R420Q group and 395 days in the Cbl-70Z group. This is the first published report on a hematological disease with Cbl mutants in a mouse model. Co-immunoprecipitation studies indicated that Cbl-70Z binds to Kit, even in the absence of Kit ligand. Cbl-R420Q also bound to Kit in the absence of SCF, albeit to a lesser extent. Association of Cbl mutants to Kit was enhanced in the presence of SCF.

Signaling studies demonstrated the constitutive activation of Akt and Erk in the presence of Cbl mutants and Kit. In addition, Cbl mutants enhanced the SCF-dependent Kit, Akt and Erk activation. Cbl-70Z, in association with kinase-dead Kit (Kit-KD) or kinase-dead Flt3 (Flt3-KD), conferred IL3-independent growth and survival to the myeloid 32D cell line. Cbl-R420Q provided only a slight growth advantage in the presence of Kit-KD. As demonstrated by pharmacological inhibition studies, Akt activation was necessary for the transformation mediated by Cbl-70Z and Kit-KD / Flt3-KD.

Cbl mutants enhanced the Src family kinases (SFKs) activity. The pharmacological inhibition of SFK activity inhibited the proliferation and colonogenic growth. Interaction was found between Cbl-70Z, SFKs and Kit-KD. The SFK member Fyn was identified to bind to Cbl. In addition, kinase activity of SFKs was necessary for binding to Cbl, since SFKs inhibition by PP-2 abolished the binding between the complex-binding partners. Dasatinib and PP-2, both SFK inhibitors, inhibited the Cbl and Akt phosphorylation indicating that Fyn acts upstream of Akt.

Inhibition of Kit with imatinib reduced the proliferation of cells overexpressing Kit WT and Cbl-70Z much stronger compared with cells expressing Kit-KD and Cbl-70Z, but much less than the dual KIT/SFK inhibitor dasatinib. This indicated that Kit kinase activity was required but not essential. The data presented in this thesis work implies that both RTK and SFK inhibition may have to be targeted, in order to effectively prevent transformation. In summary, the present thesis work indicates an important role of Cbl, Kit and SFKs in myeloid transformation and deregulated signal transduction.

1 Introduction

1.1 Hematopoiesis

Hematopoiesis is the process of blood cell formation. All cellular components of blood are derived from hematopoietic stem cells (HSC). The adult hematopoietic system produces one trillion blood cells every day. Hematopoietic stem cells possess the unique capability of self-renewal (Figure 1). The self-renewal property allows HSC to divide into two identical daughter cells, which have the same capability to produce blood cells as their parent HSC. Self-renewal allows HSC to maintain their number throughout life time. HSC can also differentiate to committed progenitor cells, which finally differentiate into mature blood cells. The majority of HSC reside in the bone marrow with a few cells circulating in the peripheral blood (Cantor and Orkin, 2001;Orkin, 1995;Orkin, 1996;Orkin and Zon, 2002). During embryogenesis, hematopoiesis occurs in different waves. Mammalian hematopoiesis starts in the yolk sac, continues in the fetal liver and finally occurs in the bone marrow (Orkin, 1995).

Mature blood cells have a limited life span and must continuously replaced by new cells. In the presence of cytokines HSCs proliferate and differentiate to common myeloid and lymphoid progenitors. Under control of appropriate cytokines, common myeloid progenitor cells further differentiate to form erythrocytes, granulocytes, monocytes, dendritic cells and megakaryocytes (Figure 1). Lymphoid progenitors, on the other hand, differentiate into T and B lymphocytes and natural killer (NK) cells (Figure 1) (Cantor and Orkin, 2001;Orkin, 1995).

1.2 Leukemia

Leukemia is a cancer of the blood or bone marrow and characterised by abnormal proliferation of white blood cells (WBC). These abnormal WBCs overcrowd the bone marrow and blood stream and are functionally impaired in fighting infectious agents. As leukemia progresses, it affects the formation of normal hematopoiesis, leading to decreased number of red blood cells (called anemia) and platelets (called thrombocytopenia).

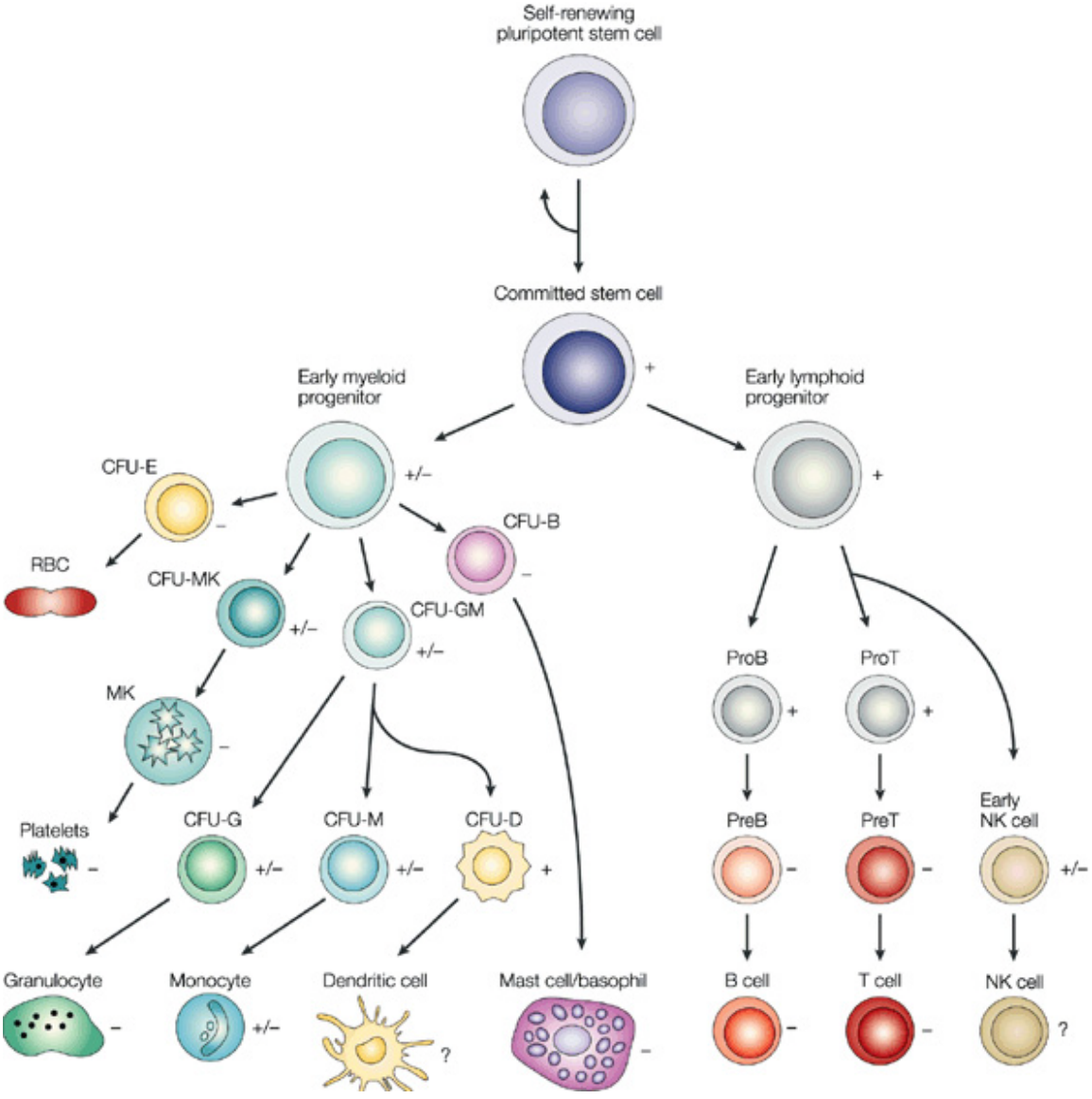


Figure 1. Hematopoiesis. The maturation and differentiation of cells during normal hematopoiesis is shown. Colony-forming units for the erythroid (CFU-E), megakaryocytic (CFU-MK), granulocytic–monocytic (CFU-GM), basophilic (CFU-B), granulocytic (CFU-G), monocytic (CFU-M), and dendritic (CFU-D) lineages are shown. NK cell, natural killer cell; RBC, red blood cell. Taken from (Stirewalt and Radich, 2003)

1.2.1 Classification of leukemia

Leukemias are classified based on two characteristics:

1 Based on the rate of disease progression, leukemias are either acute (rapidly developing) or chronic (slowly developing).

2 Based on the type of bone marrow cell involved, leukemias are either of lymphoid or myeloid origin.

Therefore, leukemias are classified into 4 types based on the above factors.

- Acute myeloid (or myelogenous) leukemia (AML)
- Chronic myeloid (or myelogenous) leukemia (CML)
- Acute lymphocytic (or lymphoblastic) leukemia (ALL)
- Chronic lymphocytic leukemia (CLL)

This thesis work focuses on the pathogenesis of AML.

1.2.2 Acute myeloid leukemia (AML)

AML is characterized by a differentiation block and expansion of early myeloid progenitor cells. This results in the accumulation of functionally impaired myeloblasts (called leukemic blasts) in the bone marrow and blood. The incidence of AML increases with age and less common in children and young adults (Appelbaum et al, 2001;Lowenberg et al, 1999). Numerous balanced translocations and mutations have been identified to date in AML (Brandts et al, 2007a). Genetic alterations found in AML have been divided into two classes of mutations (Kelly and Gilliland, 2002a). The first class of mutations, also termed class I mutations, confer a proliferative and/or survival advantage to the hematopoietic cells. Examples include activating mutations in the receptor tyrosine kinases Flt3 and Kit, as well as oncogenic mutations in N- and K-Ras (Brandts et al, 2007a). The second classes of mutations, termed class II mutations, involve myeloid transcription factors and impair the differentiation of leukemic blasts. Important examples are the recurrent balanced translocations identified in AML, such as the translocation between chromosomes 8 and 21 (t(8;21) leading to the fusion protein AML1-ETO, the t(15;17) leading to PML/RARα, as well as MLL fusion proteins (Kelly and Gilliland, 2002a).

Mouse models have demonstrated that both class I and class II mutations cooperate in the development of AML (Brandts et al, 2007b;Kelly and Gilliland, 2002b). Furthermore, data from AML patients confirm that class I and class II mutations often occur simultaneously.

Among the class I mutations, activating mutations in receptor tyrosine kinases (RTKs) have been extensively studied (Stirewalt and Radich, 2003).

1.3 Receptor tyrosine kinases (RTKs)

RTKs are cell surface signaling molecules and are often mutated in human cancers (Blume-Jensen and Hunter, 2001a). The Human Genome Project has determined that approximately 20% of human genes encode proteins which are involved in signal

transduction. These proteins are transmembrane receptors, G-protein subunits and signal generating enzymes. More than 520 protein kinases and 130 protein phosphatases are known to date (Blume-Jensen and Hunter, 2001b). Based on their catalytic specificity for tyrosine or serine/threonine residues, these kinases are subdivided into tyrosine kinases and serine/threonine kinases. To date, around 90 human tyrosine kinases have been reported. Of, 58 encode for RTKs and 32 encode cytoplasmic, non-receptor protein tyrosine kinases (PTKs) (Figure 2). RTKs are divided into 20 subfamilies(Blume-Jensen and Hunter, 2001b).

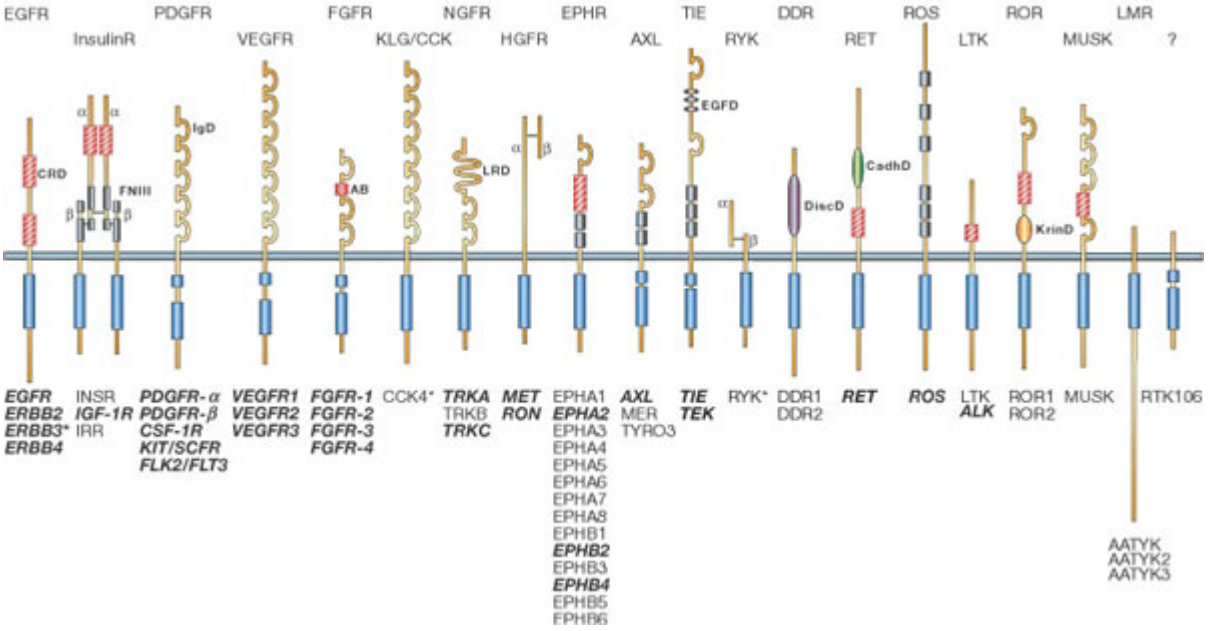


Figure 2. Human receptor tyrosine kinases. The prototypic receptor for each family is indicated above the receptor, and the known members are listed below. Abbreviations of the prototypic receptors: EGFR, epidermal growth factor receptor; InsR, insulin receptor; PDGFR, platelet-derived growth factor receptor; VEGFR; vascular endothelial growth factor receptor;FGFR, fibroblast growth factor receptor; KLG/CCK, colon carcinoma kinase; NGFR, nerve growth factor receptor; HGFR, hepatocyte growth factor receptor, EphR, ephrin receptor; Axl, a Tyro3 PTK; TIE, tyrosine kinase receptor in endothelial cells; RYK, receptor related to tyrosine kinases; DDR, discoidin domain receptor; Ret, rearranged during transfection; ROS, RPTK expressed in some epithelial cell types; LTK, leukocyte tyrosine kinase; ROR, receptor orphan; MuSK, muscle-specific kinase; LMR, Lemur. Other abbreviations: AB, acidic box; CadhD, cadherin-like domain; CRD, cysteine-rich domain; DiscD, discoidin-like domain; EGFD, epidermal growth factor-like domain; FNIII, fibronectin type III-like domain; IgD, immunoglobulin-like domain; KrinD, kringle-like domain; LRD, leucine-rich domain. The symbols □ and □ denote distinct RPTK subunits. RPTK members in bold and italic type are implicated in human malignancies. An asterisk indicates that the member is devoid of intrinsic kinase activity adopted from (Blume-Jensen and Hunter, 2001b).

1.3.1 Activation of RTKs

RTK signaling is initiated by binding of its respective ligand. Upon ligand binding, RTKs undergo dimerization, followed by tyrosine autophosphorylation of the receptor subunits. In the unstimulated state, the activation loop of the RTK occludes substrate tyrosine binding to the active site. In the presence of ligand, one or several tyrosine residues of the activation loop become phosphorylated in *trans* by the dimeric receptor partner (Figure 3) (Blume-Jensen and Hunter, 2001a). This leads to the repositioning of the activation loop away from the active site and allows substrate access. The importance of this mechanism is underlined by several examples of oncogenic mutations in the activation loop which cause constitutive RTK activation (Blume-Jensen and Hunter, 2001a). Once the RTK is autophosphorylated, cytoplasmic signaling molecules having Src homology-2 (SH2) and protein tyrosine binding domains bind to the RTK, activating specific signaling cascades.

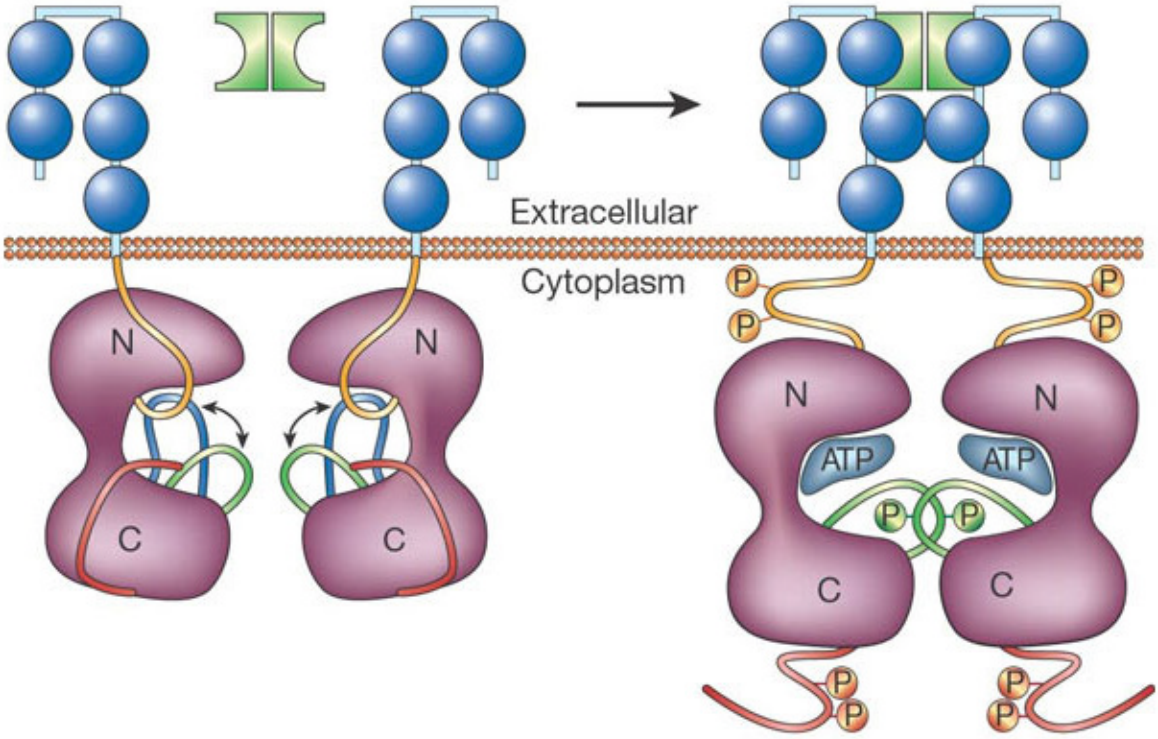


Figure 3. RTK activation. RTK kinase activity is tightly repressed in the unstimulated state (left). The activation and catalytic loops exist in an equilibrium between a substrate-precluding (blue) and substrate-accessible (green) conformation. In addition, the juxtamembrane region (orange) and C-terminal region (red) might interfere with the conformation of the N-terminal kinase lobe ('N') and/or substrate access. Ligand-induced receptor dimerization and tyrosine autophosphorylation result in relief of the inhibitory constraints exerted by the activation loop, and the juxtamembrane and C-terminal regions (right). Adopted from (Stirewalt and Radich, 2003)

It has been shown that more than half of known human RTKs are found to be mutated or overexpressed in human malignancies (Blume-Jensen and Hunter, 2001b). Kit and Flt3 belong to class III RTK sub family, which also includes PDGFRα, PDGFRβ, and CSF1R.

1.4 FMS-like tyrosine kinase 3 (Flt3)

1.4.1 Flt3 receptor expression and activation

FMS-like tyrosine kinase 3 (Flt3), is a membrane-bound RTK with an important role in hematopoiesis. It has 993 amino acids and consists of five immunoglobulin-like extracellular domains, a transmembrane domain, a juxtamembrane domain and two intracellular tyrosine-kinase domains (TKDs) linked by a kinase-insert domain (Figure 4) (Agnes et al, 1994). In human cells, Flt3 is expressed on lymphoid, myeloid progenitor cells (Rosnet et al, 1996), but not on erythroid cells (Gabbianelli et al, 1995), megakaryocytes (Ratajczak et al, 1996) and mast cells (Hjertson et al, 1996) . Flt3 mRNA can be detected in lympho-hematopoietic organs, such as liver, spleen, thymus and placenta, but this may be due to contamination with hematopoietic cells (Rosnet et al, 1993).

Flt3 receptor is activated by binding of its ligand called Flt3 ligand (FL). FL is not only expressed in hematopoietic organs such as spleen, thymus, peripheral blood and bone marrow but also in the prostate, ovary, kidney, lung, colon, small intestine, testis, heart and placenta. Upon binding of FL to the Flt3 receptor, Flt3 dimerizes. Subsequently, the activation loop is removed from the substrate binding site and the receptor becomes autophosphorylated in *trans* (Figure 4) (Turner et al, 1996;Weiss and Schlessinger, 1998). Flt3 receptor upon activation activates several signaling pathways (MAP and PI-3-Kinase pathways). Later, activated Flt3 is endocytosed and degraded in order to terminate the signaling.

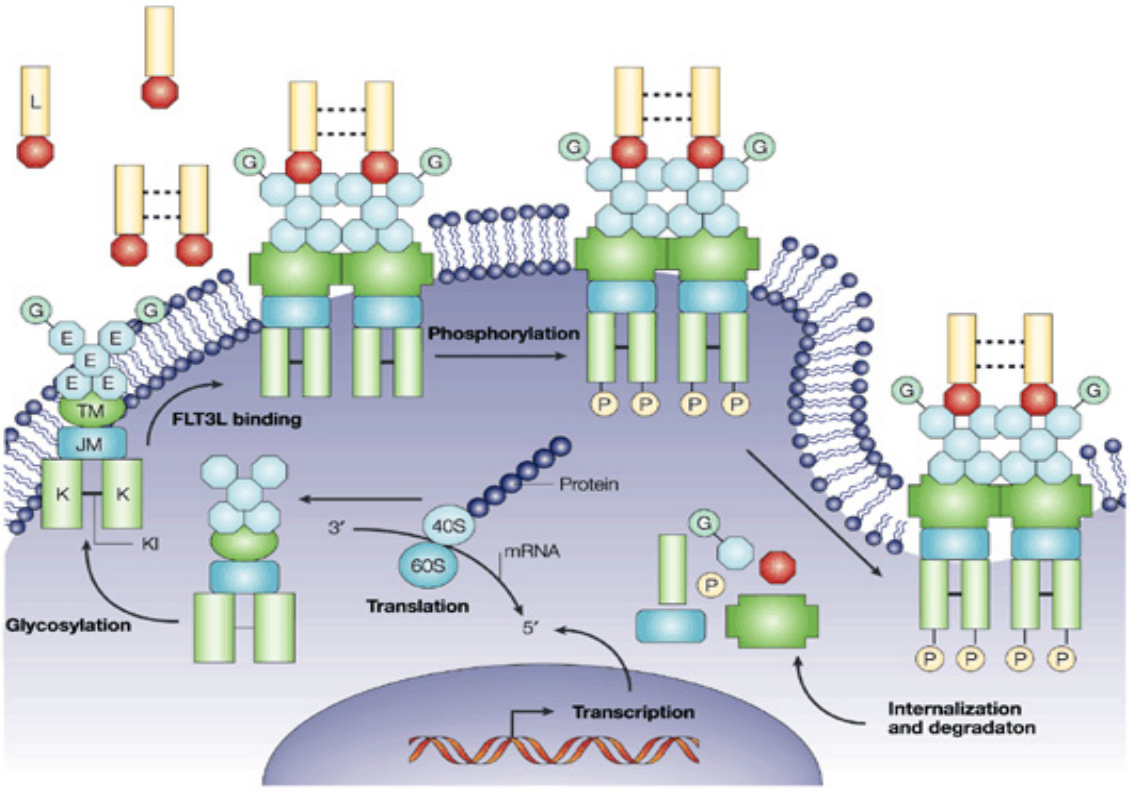


Figure 4. Structure and activation of wild-type Flt3. Transcription of the *Flt3* gene produces *Flt3* mRNA, which is translated to Flt3 protein. Flt3 contains five extracellular immunoglobulin-like domains (E), a transmembrane domain (TM), a juxtamembrane domain (JM) and two tyrosine-kinase domains (K) that are linked through the tyrosine-kinase insert (KI). Cytoplasmic Flt3 undergoes glycosylation (G), which promotes localization of the receptor to the membrane. Wild-type Flt3 remains as a monomeric, inactivated protein on the cell surface until Flt3 ligand (L), probably in a dimeric form, binds the receptor and induces receptor dimerization. Flt3 dimerization promotes phosphorylation (P) of the tyrosine-kinase domains, thereby activating the receptor and downstream effectors. The dimerized receptors are quickly internalized and degraded. Adopted from (Stirewalt and Radich, 2003)

1.4.2 Flt3 mutations

The most commonly occurring Flt3 mutations are known as Flt3 internal tandem duplication (Flt3-ITD) and they occurs as a result of an internal tandem duplication (ITD) in exons 14 and 15 (previously known as exons 11 and 12). ITD mutations account for 15–35% of patients with AML (Abu-Duhier et al, 2000;Meshinchi et al, 2001;Schnittger et al, 2002;Thiede et al, 2002) and 5–10% of patients with myelodysplasia (Horiike et al, 1997). The other most common Flt3 mutation is a missense point mutation in exon 20 of the TKD. These mutations occur in AML (5–10%), MDS (2–5%) and ALL (1–3%) patients (Thiede et al, 2002;Yamamoto et al, 2001;Yokota et al, 1997). Flt3-ITD and Flt3-TKD have been shown to confer factor independent growth to the myeloid cell lines and involved in deregulated signaling pathways (Choudhary et al, 2005).

1.5 Kit

1.5.1 Kit structure

Kit is a class-III RTK and a cellular homolog of v-Kit oncogene, which was identified in 1986 as the transforming gene in the Hardy-Zuckerman 4 feline sarcoma virus (HZ4-FeSV) (Besmer et al, 1986;Yarden et al, 1987). Kit protein is encoded by W locus of mice. “Loss-of-function mutations” have been reported at the W locus. In mice, complete loss of Kit expression (homozygous) results death in utero or prenatally, and it is likely because of severe anemia. In the heterozygous state, mice suffer from anemia, pigmentation defects and reduced fertility (Edling and Hallberg, 2007;Kitamura and Hirotab, 2004;Lennartsson et al, 2005). Kit has an extracellular (EC) domain which consists of five immunoglobulin (Ig)-like domains, a single transmembrane domain and an intracellular tyrosine kinase domain, which splits into two by an insert region (Figure 5) (Edling and Hallberg, 2007;Kitamura and Hirotab, 2004;Lennartsson et al, 2005). Studies have shown that the first three N-terminal Ig-like domains of Kit are involved in ligand binding.

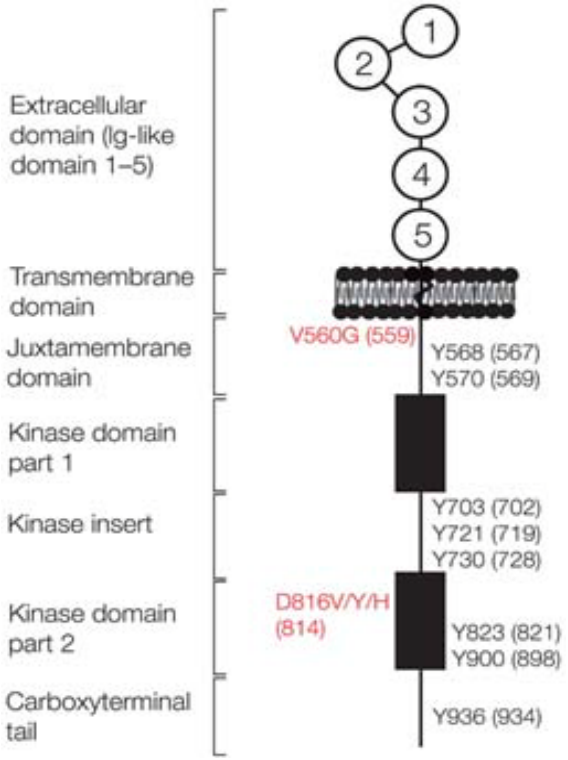


Figure 5. Schematic illustration of the Kit receptor. The receptor is drawn to indicate the domain structure of Kit. Phosphorylated tyrosine residues (in black) in the human Kit and their number are indicated. The corresponding numbers in the murine receptor are designated in parenthesis. Activating mutations are shown in red. Abbreviation: Ig, immunoglobulin. Adopted from (Lennartsson et al, 2005)

1.5.2 Stem cell factor

The product of the steel locus *(SL)* was shown to be the Kit receptor ligand also known as stem cell factor (SCF). SCF is a heavily glycosylated transmembrane protein. The active form of SCF is a non-covalently associated dimer. Kit signaling is important for the normal hematopoiesis, gametogenesis and melanogenesis (Lennartsson et al, 2005).

1.5.3 Kit expression

Hematopoietic stem cells express Kit, suggesting an important role of Kit in primitive hematopoiesis (Simmons et al, 1994). Kit is also expressed on myeloid, erythroid, megakaryocytic progenitor cells (Lennartsson et al, 2005). During hematopoietic cell differentiation, Kit expression is usually lost, but mast cells retain this RTK. SCF supports proliferation, survival and maturation of mast cells and enhances chemotaxis and adhesion of mature mast cells (Oliveira and Lukacs, 2003). “Loss-of-function mutations” of Kit cause deficiency of mast cells. Kit expression is observed on lymphoid progenitors, but not on differentiated lymphocytes (Lennartsson et al, 2005).

1.5.4 Kit mutations

“Loss-of-function mutations” in Kit protein are associated with autosomal-dominant piebaldism which involves deafness, megacolon and abnormalities in pigmentation of skin and hair (Spritz, 1994). “Gain-of-function mutations” in Kit are often associated with gastrointestinal stromal tumors (GISTs), mastocytosis and AML (Edling and Hallberg, 2007;Kitamura and Hirotab, 2004;Lennartsson et al, 2005). GISTs accounts for less than 1% of all the gastrointestinal (GI) tract tumors.
Most of the GIST patients (approximately 90%), express Kit and many of them contain activating mutations in the juxtamembrane domain (JMD). Specifically, exon11 mutations account for 57%-71% of the GIST patients. In rare cases, mutations in the kinase domain are observed (GIST patients). Mutations in the JMD abrogate, the negative regulatory function of JMD and responsible for the constitutive activation of the Kit receptor (Ma et al, 1999).
Normal wild type Kit (80%-90%) is over-expressed on blasts of most AML patients and in some cases of CML blast crisis (Heinrich et al, 2002). In contrast, Kit is rarely expressed on the leukemic blasts of lymphoid origin. Most of them are deletions and

insertions in exon 8 that code for the Kit extra cellular domain (Gari et al, 1999). Mutations at codon 416 was observed and this is believed to be “gain-of-function mutation” (Gari et al, 1999). Kit mutations (D816Y or D816V), at codon 816 in the kinase domain are also observed in the patients of core binding factor leukemias (Beghini et al, 2000a;Paschka et al, 2006a).

1.6 Kit signaling

It is known that SCF/Kit signaling play a role in proliferation, maturation/differentiation, and survival. Upon binding of SCF to the Kit receptor, Kit undergoes dimerization, followed by transphosphorylation of two tyrosine residues (Y568 and Y570) in the auto inhibitory juxtamembrane segment (Figure 5) (Hubbard, 2004;Mol et al, 2004). This leads to a conformational modification of the activation loop and results in the intrinsic tyrosine kinase activity of the Kit (Figure 5) (Mol et al, 2004). Finally, activated Kit autophosphorylates the other tyrosine residues that serve as docking sites for signal transduction molecules containing Src homology 2 (SH2) or phospho tyrosine binding domains (Figure 6).

1.7 Activation of signal transduction pathways

Stimulation of the Flt3 and Kit receptor with their respective ligands activates several downstream signaling pathways (Figure 6).

1.7.1 MAP kinase pathway

Kit and Flt3 have been shown to activate the MAP (mitogen activated protein) kinase pathway (Choudhary et al, 2005;Edling and Hallberg, 2007). In short, the MAP kinase pathway involves, activated receptor recruits and activates the Grb2 -SOS complex. This complex activates RAF kinase via Ras. RAF kinase activates MEK by phosphorylation. MEK activates a mitogen-activated protein kinase (MAPK). MAPK also called as extracellular signal regulated kinase (ERK) and microtubule-associated protein kinase (MAPK). MAPK activates several transcription factors, including c-myc, CREB and c-Fos. Map kinase pathway is involved in a wide range of cellular functions like proliferation (Yoon and Seger, 2006).

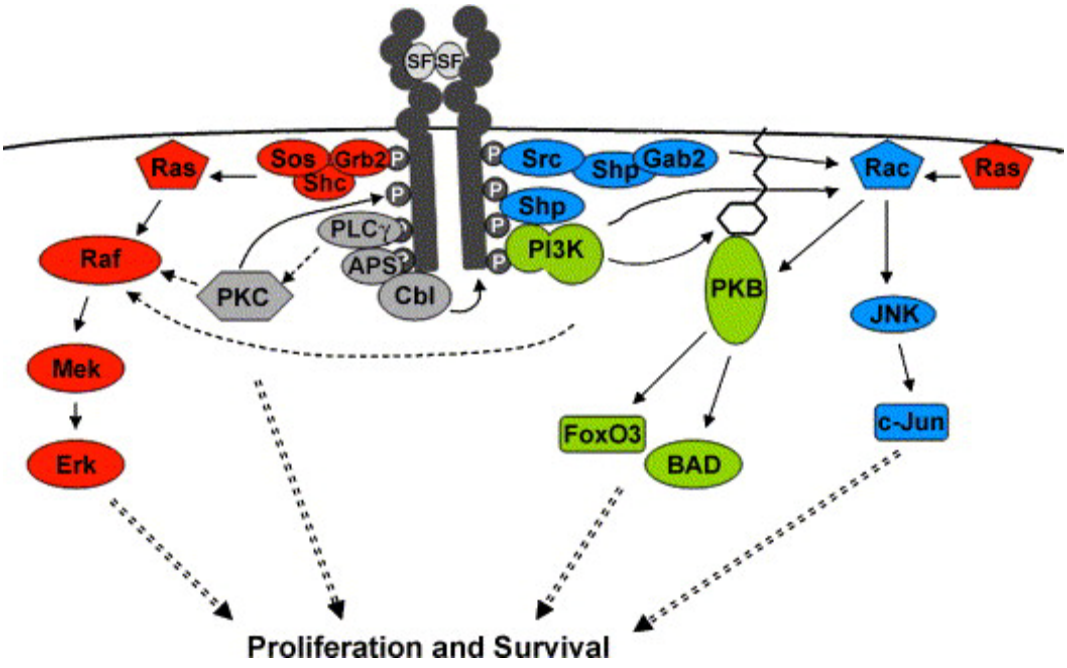


Figure 6. Overview of the signaling pathways activated upon SF ligation to Kit. Ras is activated via the adaptor complex Sos/Grb2/Shc, Ras in turn activates the MAPK pathway (indicated in red). PLCγ leads to activation of PKC (grey). Activation of Cbl via APS leads to downregulation of the receptor (grey). Src, Shp and Gab2 are involved in activating Rac in mast cells, which results in activation of the transcription factor c-Jun (blue). PI-3-Kinase binds the receptor directly and activates PKB via phospholipids at the membrane, which subsequently leads to inactivation of pro-apoptotic proteins (green). Adopted from (Edling and Hallberg, 2007)

1.7.2 PI-3-Kinase pathway

The other signaling pathway activated upon ligand binding of Flt3 and Kit is PI-3(phosphoinositide-3)-Kinase (Choudhary et al, 2005;Vosseller et al, 1997). PI3-Kinase is a heterodimeric complex composed of the p85 regulatory and the p110 catalytic subunit. Activated PI-3 kinase phosphorylates phosphatidylinositol-4, 5-bisphosphate (PtdIns4, 5P2), and subsequently generates PtdIns (3, 4, 5) P3. The lipid intermediate PtdIns (3, 4, 5) P3, activates Akt (protein kinase B). PI3-kinase binds to tyrosine residue 721 in the kinase insert of human Kit (Figure 5-6). PI3-kinase has been shown to be involved in DNA synthesis, cell survival, membrane ruffling, chemotaxis, receptor and vesicular trafficking (Fresno Vara et al, 2004;Linnekin, 1999).

1.7.3 JAK/STAT pathway

The Janus kinases (JAKs) are tyrosine kinases and localized in the cytoplasm. They are rapidly activated by ligand binding to RTKs or cytokine receptors. JAKs activate signal transducers and activators of transcription (STAT). STATs are transcription

factors and have a DNA binding domain, SH2 domain and a C-terminal transactivation domain. JAKs, activate cytoplasmic STATs by phosphorylation, which undergo dimerization through the SH2 domain and subsequent translocation to the nucleus. STATs control expression of responsive genes by binding to their promoters. Phosphorylation of serine residues in STATs (at least 1, 3, and 5), regulate their transcriptional activity. SCF activates JAK-STAT pathway (Weiler et al, 1996). JAK2 constitutively associates with Kit and Flt3 and are transiently activated in response to FL/SCF (Linnekin et al, 1996). JAK2 siRNA studies suggest that it is important for SCF-induced proliferation (Linnekin et al, 1996). FL/SCF stimulation leads to the association of STAT1, STAT5A, and STAT5B with Flt3 and Kit and become tyrosine phosphorylated (Brizzi et al, 1999;Zhang et al, 2000). SCF, but not FL activates STAT3 by serine phosphorylation (Lennartsson et al, 2005;Zhang et al, 2000).

1.8 Src family tyrosine kinases (SFKs)

The Src family tyrosine kinases (SFKs) are named after its prototypic family member c-Src, which is a cellular homolog of v-Src (the transforming protein of Rous sarcoma virus). SFKs play a role in a wide variety of cellular functions including adhesion, chemotaxis, survival, proliferation and protein trafficking. Src family members, such as Src, Yes and Fyn, are expressed ubiquitously, whereas expression of Lck, Hck, Fgr, Lyn and Blk is restricted to particular cell types (Abram and Courtneidge, 2000). SFKs have an N-terminal sequence, which is responsible for myristoylation and palmitoylation, both of which mediate anchoring to the plasma membrane. SFKs also contain a SH3 domain, a SH2 domain and a tyrosine kinase domain. In general, the kinase activity of SFKs is tightly controlled through intermolecular interactions (Figure 7).

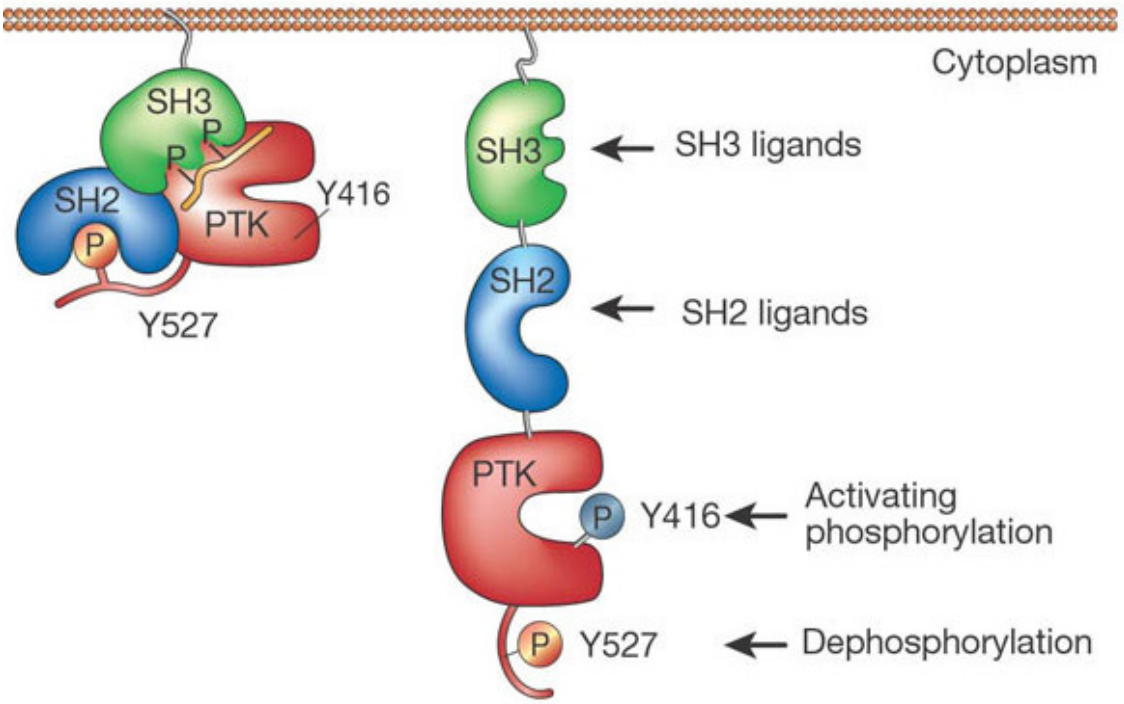


Figure 7. Src activation. Left: c-Src kinase activity is tightly repressed in the unstimulated state. The SH2 domain interacts with phospho-Tyr 527 in the C terminus and the SH3 domain with the polyproline type II helix in the linker region between the SH2 and kinase domain. This causes misalignment of residues that are critical for kinase activity. Right: binding of molecules to the SH2 or SH3 domain and/or dephosphorylation of phospho-Tyr527 by phosphatases relieves the inhibitory constraints on the kinase, resulting in its activation. Adopted from (Blume-Jensen and Hunter, 2001b).

These SH2 and SH3 intermolecular interactions inhibit kinase activity. SFKs can be activated by dephosphorylation of Tyr527, through by binding of its SH2 domain to the autophosphorylated tyrosine residues in the ligand-stimulated RPTKs, or by binding of the SH3 domain to Pro-X-X-Pro motifs present in the target molecules. Phosphorylation at tyrosine 416, (present in the activation loop) also stabilizes the active conformation of SFKs. Kit and Flt3 activates SFKs by recruiting them to the phosphorylated tyrosine residues 568 and 570 (Y589 and Y599 in the case of Flt3) (Heiss et al, 2006;Lennartsson et al, 1999;Timokhina et al, 1998). Kit activated SFKs play role in ligand-induced internalization of Kit and chemotaxis in primary hematopoietic cells. Furthermore, studies indicate that, Kit receptor lacking SFK binding site (Kit Y567F) resulted in decreased autophosphorylation of the receptor and defects in mast cell and lymphocyte development (O'Laughlin-Bunner et al, 2001;Ware et al, 1997).

1.9 Regulation of Flt3 and Kit

After activation, Kit and Flt3 are degraded via the E3 ubiquitin–protein ligase Cbl. Cbl promotes the degradation of Kit and Flt3 proteins by ubiquitinating the receptors (Sargin et al, 2007;Zeng et al, 2005). Furthermore, Kit activity is down regulated via Shp1 (SH2 domain-containing phosphatase 1) protein, which is a cytosolic phosphotyrosyl phosphatase. Studies indicate that Kit binds to Shp1 directly, which dephosphorylates the receptor, or indirectly by dephosphorylating other receptor-associated protein-tyrosine kinases (Kozlowski et al, 1998). Recent data suggests that protein kinase C (PKC) can downregulate Kit activity by phosphorylating Kit on specific serine residues leading to negative regulation of Kit activation (Edling et al, 2007). In summary, the signaling pathways downstream of the Kit and Flt3 receptor are required for controlling proliferation, survival and other functions in early hematopoietic cells.

1.10 Ubiquitination

Ubiquitination occurs in a multistep process. Initially, the E1 ubiquitin-activating enzyme forms a thioester bond with ubiquitin molecule. Then the ubiquitin molecule is transferred to an E2 conjugase, which has some substrate specificity. Ubiquitin-conjugating enzymes interact with the RING fingers of Cbl proteins (Pickart, 2001). Finally, the transfer of ubiquitin from the E2 molecule to target protein is catalysed by an E3 ligase. E3 ligases are of mainly two types (RING or HECT). E3 ligases of the RING type form no covalent bond between ubiquitin and ligase. E3 ligases of the HECT type form a covalent bond between ubiquitin and E3 ligases. E3 ubiquitin ligases are the proteins that have the ability to transfer ubiquitin molecules to the target protein. Cbl proteins are RING finger type E3 ligases. TKB and RING domain are interconnected by a short linker sequence which is also crucial for ubiquitin ligase activity of Cbl. Mutations in linker region render Cbl transforming (Thien et al, 2001). TKB and RING facilitate ubiquitination and degradation of activated PTKs (Swaminathan and Tsygankov, 2006). TKB domain confers substrate specificity where as the RING finger helps in bringing an E2 ubiquitin-conjugating enzyme.

1.11 Cbl (Casitas B-lineage lymphoma)

1.11.1 Cbl homologues and structure

v-Cbl was the first member of the Cbl family proteins and was cloned from the Cas NS-1 retrovirus in 1989. V-Cbl induces pre-B-cell lymphomas and myelogenous leukemia in mice (Langdon et al, 1989a). Subsequently, it was noticed that v-Cbl is a truncated form of a cellular homologue known as c-Cbl (Langdon et al, 1989b). The full-length form of Cbl consists of an N-terminal tyrosine-kinase-binding (TKB) domain, a RING (interesting new gene) finger motif, a proline-rich region and a c-terminal ubiquitin-associated (UBA) domain and leucine zipper (LZ) motif (Figure 8). Cbl has been shown to have three mammalian homologues c-Cbl, Cbl-b (Keane et al, 1995) and Cbl-c (Figure 8) (Keane et al, 1999;Kim et al, 1999). C-Cbl and Cbl-b are expressed ubiquitously and have orthologues in *Drosophila melanogaster* and *Caenorhabditis elegans*. Cbl-c is the shorter isoform of Cbl and mainly expressed in epithelial cells. From here onwards, c-Cbl is termed Cbl, unless indicated.

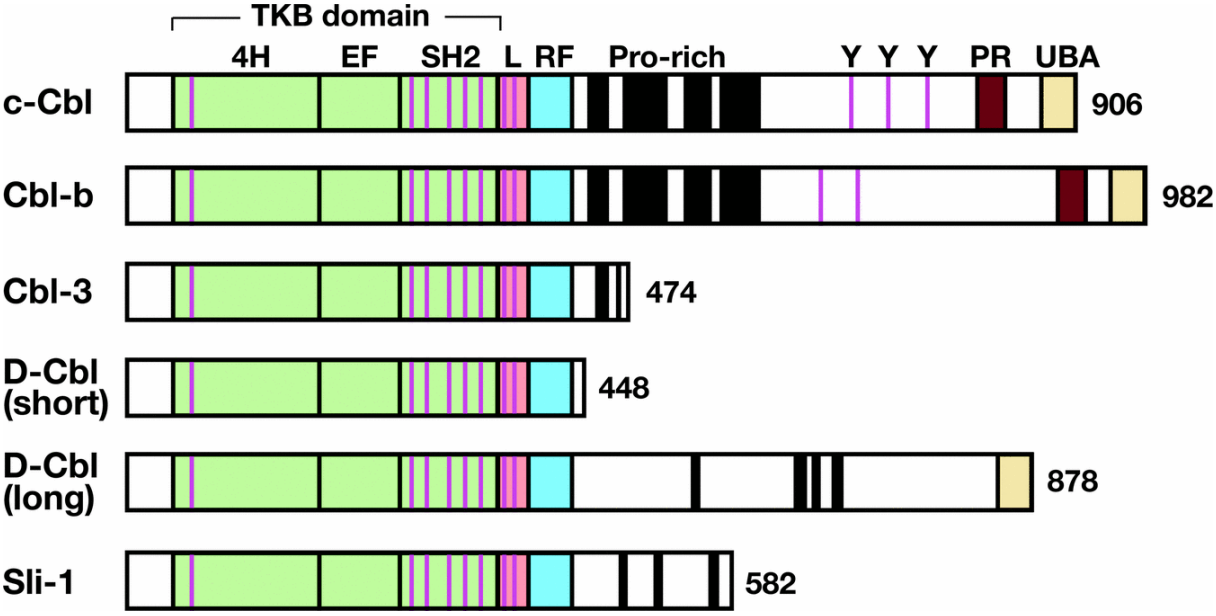


Figure 8. Cbl protein family in mammals. All Cbl proteins share a high level of sequence conservation between their TKB, linker (L) and RING finger (RF) domains. Cbl and Cbl-b have extensive proline-rich regions (black) in their C-terminal halves that mediate interactions with numerous SH3-domain-containing proteins. The TKB domain is composed of three interacting regions comprising a four-helix bundle (4H), a calcium-binding EF hand and a variant SH2 domain that is connected to the RING finger by a short linker domain. The PR domain in the C-terminus of Cbl and Cbl-b refers to a PX(P/A)XXR motif that binds SH3 domains of the CIN85/RUK (regulator of ubiquitous kinase)/CD2AP (C2-associated protein) family of proteins. The UBA domain at the C-terminus of Cbl, Cbl-b and D-Cbl (long) refers to a region with homology to UBA domains. Conserved tyrosine residues are shown in purple. Adopted from (Thien and Langdon, 2005a)

The tyrosine kinase-binding (TKB) domain is homologous to v-Cbl. TKB domain contains a four-helix bundle (4H), a calcium-binding EF hand and a modified SH2 domain. The TKB domain binds to phospho-tyrosine residues in multiple tyrosine kinases (Meng et al, 1999a). The preferred sequence for binding of TKB to the target molecules is (N/D) XpY(S/T) XXP. The RING finger domain is a highly conserved region of Cbl, which plays an important role towards conferring an E3 ubiquitin ligase activity to the Cbl (Joazeiro et al, 1999;Levkowitz et al, 1999).

1.11.2 RTK regulation by Cbl

Activated RTKs mediate downstream signaling and duration of their downstream response is tightly controlled. Receptor deactivation takes place by dephosphorylation of the receptor or by removing the receptors from the membrane by endocytosis, followed by degradation or recycling. Cbl plays an important role in the RTK ubiquitination. Epidermal growth factor receptor (EGFR) trafficking and degradation by Cbl is one of the best-studied mechanisms in RTK degradation. RTK degradation is a multistep process and was described initially in *C. elegans*. The Cbl orthologue SLI-1 regulates the signaling downstream of LET-23 receptor (EGFR orthologue) (Jongeward et al, 1995;Sternberg et al, 1995;Yoon et al, 1995). RTK degradation involves the following steps. Upon binding of EGFR with its ligand EGF, the receptor is dimerized and activated. Autophosphorylation of the receptor creates docking sites for suitable proteins and initiates the formation of a multiprotein-receptor complex. Grb2 (growth-factor-receptor bound-2) is one of the first proteins to be recruited to the complex. Grb2 binds to proline-rich region of the Cbl and recruits it to the complex (Fukazawa et al, 1996;Jiang et al, 2003). During EGFR activation, tyrosine 1045 becomes phosphorylated, creating a direct Cbl binding site. As a consequence, the Cbl molecule mediates the ubiquitination of EGFR. Following the ubiquitination of the EGFR by Cbl, EGFR is recruited to clathrin-coated pits by adaptors, such as Eps15. These adaptors possess ubiquitin-binding domains and motifs that bind to clathrin adaptors (AP2) (de Melker et al, 2004a;de Melker et al, 2004b). AP2 complexes help in the formation of clathrin-coated vesicles. They eventually fuse with internal vesicles to form an early endosome (Figure 9). They progress to become multi-vesicular bodies. In multi-vesicular bodies, receptors are sorted for either recycling or degradation, which takes place in the lysosomes.

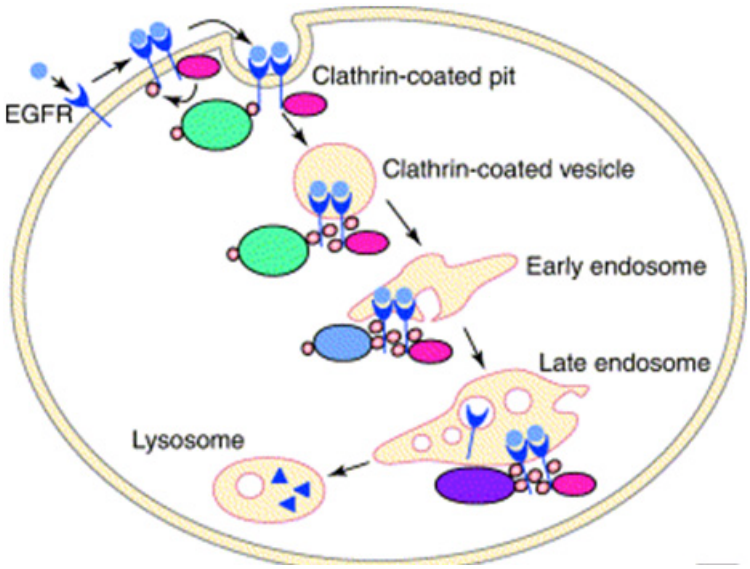


Figure 9. Ubiquitination in receptor endocytosis and degradation. Monoubiquitin (orange circles) appears to carry both internalization and degradation signals. Depicted is the current view on how interactions between monoubiquitinated cargo [e.g. EGFR] and sorting Ub-receptors [e.g. Eps15, hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs; blue oval) and tumor-susceptibility gene product 101 (TSG101; purple oval)] might control RTK downregulation.

Cbl has been shown to down regulate several RTKs, including platelet-derived growth factor receptor (PDGFR), colony-stimulating factor (CSF-1) receptor, hepatocyte growth factor (HGF) receptor (Met), neurotrophin receptor (p75NTR), Kit (Zeng et al, 2005), vascular endothelial growth factor (VEGF) receptor (Flk-1), Flt-1 macrophage-stimulating protein (MSP) receptor, glial cell-derived neurotrophic factor (GDNF) receptor (Ret), fibroblast growth factor receptor (FGFR), and the insulin receptor (IR) (Fukazawa et al, 1996;Jiang et al, 2003;Swaminathan and Tsygankov, 2006). Furthermore, Cbl has also been shown to down-regulate non-receptor protein tyrosine kinases including Syk, Fyn, Lck, Hck, Lyn, and c-Abl (Kyo et al, 2003;Yokouchi et al, 2001).

1.11.3 Phosphorylation of Cbl

Cbl is phosphorylated by a wide variety of molecules. Cbl is a substrate for oncogenic PTKs such as Bcr-Abl, v-Abl and v-Src (Andoniou et al, 1994a;Andoniou et al, 1996). T-cell receptor/CD3 (TCR/CD3), B-cell receptor (BCR), as well as other multi chain immune recognition receptors induce Cbl phosphorylation upon activation (Cory et al, 1995). Furthermore, several RTKs, such as Kit, EGFR, and PDGFR, CSF-1, and insulin receptor have been shown to induce Cbl phosphorylation (Bonita et al, 1997a;Kanagasundaram et al, 1996). Thus, Cbl becomes phosphorylated in

response to cytokines such as, thrombopoietin (TPO), erythropoietin (EPO), interleukin 3 (IL-3), IL-4 and IFN-α (Barber et al, 1997;Gesbert et al, 1998). Cbl tyrosines 700, 731 and 774 have been identified as important sites of c-Cbl. These are phosphorylated by non-receptor PTKs (Andoniou et al, 1994a;Feshchenko et al, 1998). It appears that all non-receptor PTKs phosphorylate Cbl at these sites, showing that no significant individual specificity. When Cbl is phosphorylated by non-receptor PTKs at tyrosine 731, it recruits and activates PI-3-kinase (Hunter et al, 1999a).

1.11.4 Cbl as a multivalent adaptor protein

Numerous reports have shown that Cbl acts as multivalent adaptor protein. Cbl is rapidly phosphorylated upon several external or oncogenic signals and is able to associate with a wide range of intracellular signaling partners. PTB domain of Cbl interacts with phosphorylated protein tyrosine kinases, such as RTKs, Src and Zap70 (Meng et al, 1999b;Sanjay et al, 2001;Schmidt and Dikic, 2005). The RING domain mainly interacts with components of the ubiquitin system including E2 proteins (UbcH7) and sprouty proteins (Wong et al, 2002). N-terminal sequences of Cbl proteins are typically followed by proline-rich regions, which appear to mediate binding with SH3 domain proteins. Grb2, which is an adaptor protein constitutively, associates with Cbl via its amino terminal SH3 domain (Meng et al, 1999b;Sanjay et al, 2001;Schmidt and Dikic, 2005). Furthermore, several proteins such as Nck, p85, Cap, ArgBp2, CIN85, CMS, SFKs, Abl and CrkL have been shown to bind proline rich regions of the Cbl (Dikic et al, 2003). Thus, Cbl can integrate several signaling pathways and act as a scaffold.

1.11.5 Cbl mutations in cancer

Mutations in the tyrosine kinase binding domain, linker, or RING finger domain of Cbl result in deregulation of RTKs (Andoniou et al, 1994b;Peschard et al, 2001a;Peschard and Park, 2003). Most of these mutants are thought to act in a dominant-negative manner by competing with the wild-type receptor. v-Cbl, a mutant form of Cbl, has been found in Cas NS-1 retrovirus as a fusion protein. It lacks the C-terminus part of wild-type Cbl and can induce pre–B-cell lymphomas and leukemia in mice (Langdon et al, 1989b). Cbl-70Z was a 17-amino acid deletion mutant and isolated from 70Z/3 mouse pre–B-cell lymphoma (Andoniou et al, 1994b) (deletion

occurs between amino acids 366 and 382, at the boundary of the linker and RING finger domain).

We recently reported the first Cbl oncogenic mutation in a single case of 150 *de novo* AML patients, Cbl-R420Q, and analyzed the role of this mutant in Flt3 signaling (Sargin et al, 2007). Cbl-R420Q and Cbl-70Z confer IL-3–independent growth to the 32Dcl3 cell line in the presence of wild-type Flt3 receptor (Sargin et al, 2007). C-Cbl physically interacts with Flt3 and is tyrosine phosphorylated in the presence of Flt3-ligand (FL). Overexpression of a dominant-negative form of c-Cbl (Cbl-70Z) inhibited FL-induced Flt3 ubiquitination and internalization, indicating involvement of c-Cbl in Flt3 signaling. Cbl-R420Q also inhibited Flt3 internalization and ubiquitination. In addition, the mutant Cbl proteins altered the amplitude and duration of Flt3-dependent signaling events.

Subsequently, several additional mutations have been found in Cbl and Cbl-b (Abbas et al, 2008;Dunbar et al, 2008;Grand et al, 2009;Loh et al, 2009;Makishima et al, 2009;Sanada et al, 2009;Sargin et al, 2007). In a separate study, missense mutations in exon 8 were reported in 3 out 12 AML cases (Caligiuri et al, 2007a). Further studies have shown Cbl exon 8 splice variant mutations associated with CBF leukemias (Abbas et al, 2008;Reindl et al, 2009). Several reports have also shown somatic CBL mutations in the critical linker and ring finger domains (exons 8 and 9). (Makishima et al, 2009;Sanada et al, 2009). These mutations are frequent in myeloid malignancies, particularly (but not exclusively) in myelodysplasia/myeloproliferative neoplasms (MDS/MPN) with acquired uniparental disomy (aUPD) at chromosome 11q (Dunbar et al, 2008;Grand et al, 2009;Loh et al, 2009).

2 Aims of the study

In AML, Flt3 and Kit are frequently mutated, leading to constitutive activation. However, Flt3 and Kit are not mutated in the majority of AML patients, but found highly expressed on the cell surface of myeloids blasts. The mechanisms leading to Flt3 and Kit overexprssion are unknown. The magnitude and kinetics of RTKs activation is tightly regulated which determines the quality and quantity of the biologic response. Termination of RTK signaling occurs by endocytosis and subsequent RTK degradation or recycling. Cbl proteins have been shown to be central players in the Ubiquitination, internalization and endocytosis of RTKs, but their role for Flt3 and Kit in myeloid transformation has not been determined. During this thesis work, we were specifically interested in the following questions:

- Do Cbl mutants synergize with Kit receptor to give cytokine independent growth in a myeloid cell line system, as an indication for transformation?
- Do Cbl mutants inhibit the ubiquitination and endocytosis of the activated Kit receptor?
- Is kinase activity of Kit and Flt3 required in the context of Cbl mutants?
- Do Cbl mutants alter the quality and quantity of Kit receptor signaling?
- Do Cbl mutants cause a hematological disease *in vivo*?
- What is the functional consequence of over-expressed Kit receptor?

3 Materials and methods

3.1 Reagents and antibodies

Recombinant human Flt3 ligand (FL), murine SCF and murine IL-3 were purchased from PeproTech (Rocky Hill, NJ). Polyclonal rabbit anti-phospho-Erk-1/2, anti-phospho-Kit (Tyr-719), anti-phospho-Src family (Tyr-416) and anti-phospho-Akt antibodies were obtained from Cell Signaling Technology (Beverly, MA). The antibodies for Kit, Flt3, HA-tag, and signal transducer and activator of transcription 5a/b (anti-STAT5a/b) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal anti-c-Cbl antibody was obtained from BD Biosciences (San Jose, CA). Antibodies for anti-Erk-1/2, and anti-phospho-STAT5a/b were purchased from Upstate Biotechnology (Lake Placid, NY). The mouse monoclonal anti-actin antibody was purchased from Sigma (Taufkirchen, Germany). Phycoerythrin (PE)-labeled murine anti-Kit antibody was obtained from BD Pharmingen (San Jose, CA). The Src family inhibitors PP-1 and PP-2 were purchased from Calbiochem (San Diego, CA). The Horseradish peroxidase conjugated goat-anti-mouse, goat-anti-rabbit, and red-X conjugated goat-anti-mouse antibodies were obtained from Jackson Immunoresearch (West Grove, PA).

3.2 Methods

3.2.1 Generation of plasmid constructs

pAL-Kit wt (murine and human hybrid) was a kind gift of Dr. Klaus-Michael Debatin. pCDNA3 Kit WT and pCDNA3 Kit-D790N (Kit-KD) were generated by sub cloning from pAL-Kit WT and pAL-Kit-KD using EcoRI and Not1 restriction enzymes. pMY Cbl wt, pMY Cbl-R420Q, pMY Cbl-70Z were generated by sub cloning from pSRα vector based plasmids using Not1 restriction enzyme. HA (Hemagglutinin) tagged Cbl constructs were sub-cloned to pMY vector using BamH1 restriction enzyme from pSRα based plasmids.

3.2.2 Site-directed mutagenesis

Site-directed mutagenesis was performed using the Quick-Change site directed mutagenesis Kit (Stratagene, Amsterdam, Netherlands) according to the manufacturer's instructions. For generating kinase dead Kit (Kit-D790N), aspartic acid 790 of Kit-WT was replaced with asparagine. Mutation was confirmed by sequencing.

3.2.3 Cell lines

The IL-3-dependent murine myeloid cell line 32Dcl3 (subsequently referred to as 32D) was cultured in RPMI 1640, supplemented with 10% WEHI-conditioned medium as a source of IL-3, 10% fetal calf serum (FCS) and antibiotics as described. COS-7 cells were cultured in Dulbecco modified Eagle medium (Invitrogen, Karlsruhe, Germany). Cells were maintained in a humidified incubator at 37°C and 5% CO_2.

3.2.4 Retrovirus preparation

Platinum-E (Plat-E) ecotropic packaging cells (Morita et al, 2000) and NIH3T3 cells were maintained in Dulbecco modified Eagle medium (DMEM; GIBCO-BRL, Karlsruhe, Germany) supplemented with 10% FCS. Plat-E cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) with pMY (vector alone)/pMY Cbl-R420Q/pMY Cbl-70Z retroviral plasmids. Viral supernatants were collected 36 hours post transfection at 12 hours interval, sterile filtered using 0.45 µM syringe filters and stored at 4°C for short-term storage or snap frozen and stored at -80^{o}C until use. Retrovirus was titered by transduction of 5 × 10^4 NIH3T3 cells with serial dilutions of retrovirus in the presence of 4 µg/mL polybrene (Sigma, Deisenhofen, Germany). Forty-eight-hours after transduction, percentage of infected cells were determined by flow cytometric analysis of EGFP expression. The titer was calculated by multiplication of the total number of EGFP-positive cells with the dilution factor of the retroviral supernatant. We tested for the presence of replication-competent viral particles by transferring supernatant onto NIH3T3 cells. The NIH3T3 cells were subsequently analyzed for EGFP expression by fluorescence-activated cell scanner (FACS) analysis.

3.2.5 Transduction and transplantation of murine bone marrow

Murine bone marrow was harvested from female Balb/C donor mice 4 days after injection of 150 mg/kg 5-fluorouracil (Ribosepharm) and prestimulated overnight in Iscove modified Dulbecco medium/20% FCS supplemented with growth factors (10 ng/mL murine IL-3 [mIL-3], 10 ng/mL mIL-6, 50 ng/mL mSCF). Cells were transduced by 4 rounds of spin infection (1200*g*, 32°C, 90 minutes) every 12 hours in retroviral supernatant supplemented with growth factors and 4 µg/mL polybrene (Sigma-Aldrich). Retroviral experiments were performed using the murine stem cell virus-based retroviral construct pMY (Kitamura et al, 2003a). Subsequently, cells were

resuspended in Hanks balanced salt solution (Sigma-Aldrich) and injected into the tail vein of lethally irradiated (8 Gy) female Balb/C recipient mice. Transplanted animals were monitored for signs of disease development and by serial measurement of peripheral blood counts. All procedures were reviewed and approved by the University of Frankfurt supervisory animal care committee.

3.2.6 Flow cytometric immunophenotyping (FACS analysis)

Single-cell suspensions of indicated tissue samples were prepared, and red blood cells of peripheral blood were lysed before analysis. Cells were subsequently stained with PE-conjugated anti-CD117 (Kit), anti-CD11b (Mac-1), anti–Gr-1, anti-CD45R/B220, or anti-CD3 antibodies. All antibodies were purchased from BD Biosciences PharMingen.

3.2.7 Histopathology

Complete necropsies were performed and histopathology examinations were carried out on spleen, liver, Bone marrow, Kidney and heart from both Cbl mutants transplanted and control mice. Tissue samples were fixed by immersion in 10% neutral-buffered formalin, embedded in paraffin, and 5-µm tissue sections were stained with hematoxylin and eosin (H&E). Representative images of H&E-stained sections were acquired using a microscope digital camera.

3.2.8 Generation of stable cell lines

For the generation of stable cell lines 32D cells were electroporated with 10 µg of pAL Kit WT, pAL Kit-D790N (Kit-KD) or pAL Flt3-K644A (Flt3-KD) together with 1 µg of pMAM/BSD (Blasticidin) (Kaken Pharmaceutical, Japan) as selection marker in 0.4 cm cuvettes at 280 V and 975 µF. After 48hrs of culturing, cells were selected with 15 µg/mL Blasticidin (Invitrogen, Groningen, Netherlands) in IL-3 supplemented culture. In order to generate stable cell lines with Cbl, 32D or 32D cells stably expressing Kit-WT, Kit-D790N or Flt3-K644A were infected with retroviral supernatants of pMY Cbl-R420Q, pMY Cbl-70Z or pMY (empty vector). For immunoprecipitation experiments, cell lines were transduced with different HA-tagged Cbl retroviral supernatants. Green fluorescent protein (GFP) marker was used for the sorting of the transduced cells with the help of Flow cytometry. To avoid possible clonal selection, at least 2 bulk cultures were generated for each cell line.

3.2.9 Analysis of cell growth

The 32D cells expressing indicating constructs were washed twice with phosphate buffered saline (PBS) and re-suspended in RPMI 1640 with 10% FCS alone or supplemented with the indicated growth factors or with the inhibitors Imatinib, dasatinib, or Akt inhibitor. Cell viability was determined by Trypan blue exclusion until day 3. Cells were seeded at a concentration of $2x10^5$ cells/mL.

3.2.10 ^{3}[H]-thymidine incorporation

A total of 3×10^4 32D cells with indicated constructs were starved of IL-3 in 10% FCS for 12 hours and subsequently placed in 200 µl of medium containing indicated cytokines/nothing/15 µM of PP-1/PP-2. After 6 hours of incubation at 37°C with 5% CO_2, 0.037 MBq (1 µCi) ^{3}[H]-thymidine was added to each well and cells were incubated for an additional 15 hours. Cells were harvested on glass fiber filters, and emission of the bound DNA was analyzed with a scintillation counter. Experiments were repeated at least 3 times. Each data point represents the mean and the standard deviation of triplicates from one of at least three independent experiments.

3.2.11 Ubiquitination assays

For transient expression experiments COS-7 cells were used. For transfection, Nanofectin (Q051-005; PAA Laboratories, Cölbe, Germany) was used. Transfection was done according to the manufacturer's protocol. A total of 2×10^6 cells were seeded in 10-centimeter dish. On the following day cells were transfected with pCDNA3 Kit WT or Kit-KD in the presence or absence of Cbl mutants along with HA tagged Ubiquitin (pCMV-HA-Ubiquitin). After 48hrs of transfection, cells were serum starved for 12 hours and then stimulated with 50 ng/mL SCF for 10 minutes. Subsequently, cells were washed once with ice-cold PBS and lysed with buffer containing 50 mM HEPES (pH 7.4), 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 µM $ZnCl_2$, 25 mM NaF, proteinase inhibitors (Complete; Boehringer Mannheim, Germany), 1 µM pepstatin, and 1 mM sodium orthovanadate. After incubation on ice for 10 minutes, cell lysates were clarified at 20,000g for 10 minutes at 4^{o}C. For immunoprecipitation, the cell lysates were incubated either with control rabbit polyclonal serum or with an antibody to Kit overnight and then protein A/G-Plus-sepharose beads (Santa Cruz Biotechnology) were added for an additional 2 hours. The immunoprecipitates were washed 4 times with lysis buffer, resolved by

SDS-PAGE, transferred to Immobilon P membranes (Millipore, Bedford, MA) and probed either with anti HA (detects exogenous Ubiquitin) or with anti-Kit (an antibody that recognizes total Kit present in the cell) or with Cbl antibodies overnight at 4^{o}C. Immunodetection was performed using ECL-Plus (Amersham Pharmacia, Uppsala, Sweden).

3.2.12 Immunoprecipitation and Western blot analyses

32D cells transfected with either Kit-WT or Kit-D790N (Kit-KD) or Flt-3-KD and Cbl constructs were washed twice with PBS and starved overnight in the medium containing 10% FCS or cells were starved in the medium containing 10% FCS with inhibitors PP-1, PP-2, or dasatinib for 12hrs. They were then stimulated for 10 minutes at 37°C with either 50 ng/mL SCF or 1 ng/mL IL-3 or nothing. Cells were lysed in RIPA lysis buffer (It has 150 mM NaCl, 1% NP40, 0.5% deoxycholicacid, 0.1% SDS, 50 mM Tris pH 8, 1µM pepstatin, and 1 mM sodium orthovanadate) or in the buffer (which was used for all immunoprecipitation experiments) containing 50 mM HEPES (pH 7.4), 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 µM ZnCl2, 25 mM NaF, proteinase inhibitors (Complete; Boehringer Mannheim, Germany), 1 µM pepstatin, and 1 mM sodium orthovanadate. For the immunoprecipitation of HA-tagged proteins, cell lysates were incubated overnight with an anti-HA antibody conjugated affinity matrix (Roche, Mannheim, Germany). The immunoprecipitates were washed 4 times with lysis buffer, resolved on SDS-PAGE, and probed with the indicated antibodies overnight at 4°C.

3.2.13 Internalization assays

Internalization assays were carried out in both 32D and COS-7 cells. For transfection of COS-7 cells, nanofectin (Q051-005; PAA Laboratories, Cölbe, Germany) was used as a transfection reagent. Transfection was done according to the manufacturer's protocol. 2 × 10^6 cells were seeded in 10-centimeter dish. On the next day, cells were transfected with pCDNA3 Kit WT in the presence or absence of Cbl mutants. A total of 5 × 10^5 32D or Cos-7 cells expressing Kit-WT alone or in combination with the indicated Cbl constructs were washed with PBS and then stimulated with SCF for 0, 5, 10, and 30 mins. Internalization of the receptor was stopped by placing cells on ice and adding ice-cold PBS containing 0.4% sodium azide. After washing, the cells were incubated with a PE-labeled anti-Kit antibody for 30 minutes at 4°C. The mean

fluorescence intensity of the stained cells was analyzed by flow cytometry using CellQuest software (BD Biosciences, Palo Alto, CA).

3.2.14 Clonal growth in methylcellulose

To analyze clonal growth, 1 mL of a culture mixture containing Iscove's Modified dulbecco medium (IMDM; Life Technologies, Grand Island, NY), 1% methylcellulose, and 10% FCS was plated on a 35-mm culture dish in the presence of either with no cytokines or with 15μM PP-2. Stably transfected 32D cells expressing Kit wt/Kit-KD/Flt3-KD in the presence or absence of Cbl mutants were seeded at a concentration of $1x10^3$ cells per dish. The assays were carried out in triplicates, and colonies were photographed and counted on day 6. The results shown are representative of one of at least 3 independent experiments per construct.

4 Results

In order to address the questions outlined in the Aims section (section 2) of this thesis, the role of the Cbl mutants Cbl-R420Q and Cbl-70Z *in vitro* and *in vivo* was analysed. For many experiments, we used the myeloid 32D cell line, unless otherwise indicated.

4.1 Cbl mutants (Cbl-R420Q and Cbl-70Z) confer ligand-independent growth in cooperation with Kit

4.1.1 Surface expression of Kit receptor

Firstly, stable 32D cell lines expressing Kit wild-type (WT) were generated. Cells were analyzed for surface expression of Kit by FACS analysis. Flow cytometric analysis revealed surface expression of these proteins (Figure 10). Western blot analyses showed two different forms of the Kit protein with different molecular weights: the heavily glycosylated (mature) form with a molecular weight of 145 kD and the hypoglycosylated (immature) form with a molecular weight of 130 kD (data not shown).

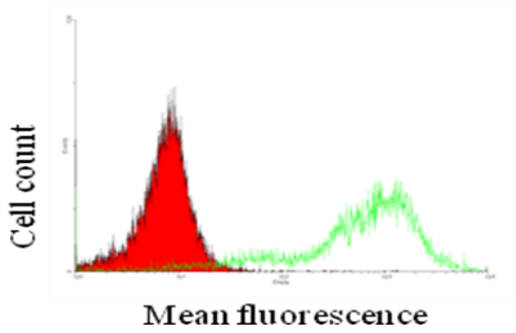


Figure 10. Expression of Kit proteins. Flow cytometric analysis of Kit surface expression on a stably expressing 32D cell line. The green areas show staining with anti-Kit antibody. The red areas show the isotype control.

4.1.2 Cbl mutants confer ligand-independent growth in association with Kit

Next, it was examined whether Cbl mutants expression lead to increased proliferation by performing growth curves of stable 32D cell lines. Cells expressing either Cbl mutant in the presence of Kit-WT rapidly proliferated in the absence of exogenous SCF or IL-3 (Figure 11A). After 72 hours, Cbl-70Z led to more than fivefold proliferation in the presence of Kit WT, compared to a 3-fold increase observed in the cell lines both Cbl-R420Q and Kit WT (Figure 11A). In contrast, in the absence of Cbl

mutants, cells co-expressing Kit WT alone did not proliferate in the absence of cytokines. Cultures expressing one of the Cbl mutants with Kit-WT survived for extended time periods in the absence of any growth factors (Figure 11B). As reported earlier, 32D cells expressing Cbl-70Z have shown moderate growth advantage and cell survival compared to cells expressing Cbl-R420Q (Hamilton et al, 2001). After 72 hours, a less significant fraction of cells was alive in the case of cells expressing Cbl-R420Q or Kit WT (Figure 11B).

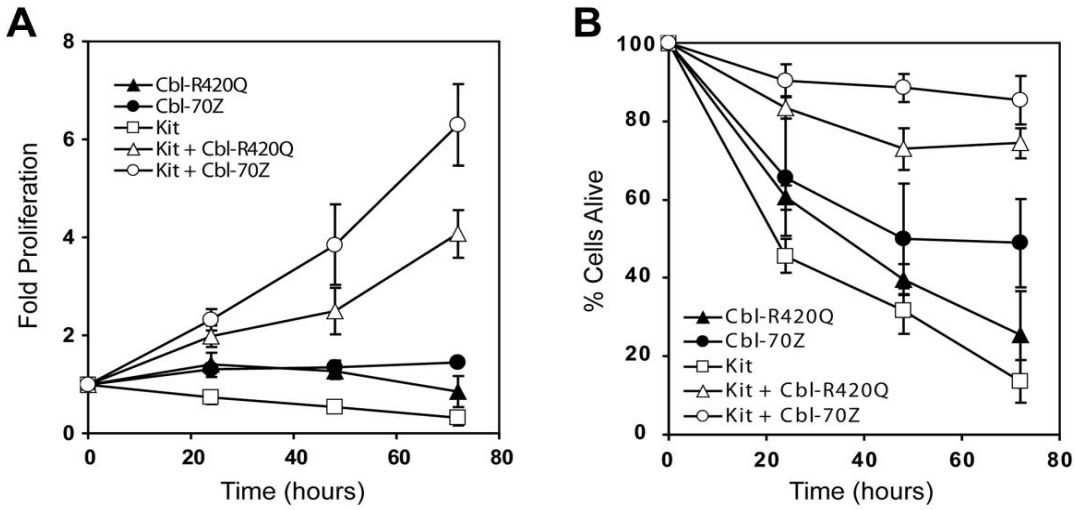


Figure 11. Cbl mutants synergize with Kit to induce autonomous growth and *in vitro* colony formation. (A) The 32D cells stably overexpressing Kit-WT and/or indicated Cbl mutants were starved from IL-3, and cells were grown in the presence of 10% FCS. Cells were counted at the indicated time points by the trypan blue exclusion method, and the data are shown as fold change of the cell number compared with the start of the experiment. (B) The percentage of cells in the culture that was alive at the indicated time points. The data represent the average and standard deviation of 3 independent experiments.

4.1.3 Enhanced ^{3}[H]-thymidine incorporation in the presence of Cbl mutants and Kit WT

The effect of Cbl mutants on IL-3 independent and SCF-dependent proliferation was analysed in ^{3}[H]-thymidine incorporation assays. As in proliferation assays, co-expression of Cbl mutants with Kit WT induced a proliferative growth advantage in the absence of SCF and this growth was further enhanced after SCF-stimulation compared to cells expressing Kit WT (Figure 12). Cells expressing Cbl-70Z and Kit WT showed more SCF-dependent and independent growth compared to cells expressing Cbl-R420Q and Kit WT. Cbl-R420Q alone did not show SCF dependent or independent growth. Kit WT cells proliferated only in the presence of SCF.

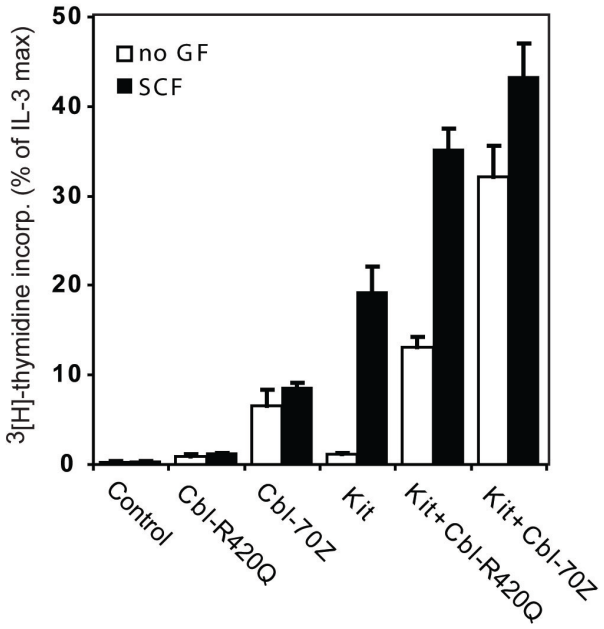


Figure 12. Enhanced ^{3}[H]-thymidine incorporation in the presence of Cbl mutants and Kit WT. The 32D cells stably expressing Kit-WT and/or Cbl constructs were starved from IL-3 for 12 hours and then cultured in the presence or absence of SCF or IL-3. Proliferation was measured by ^{3}[H]-thymidine incorporation assays. Data are shown as percentage of thymidine incorporation relative to the thymidine incorporation of the respective cell line with IL-3 supplementation. The data represent the average and standard deviation of 3 independent experiments.

4.1.4 Cbl mutants confer clonogenic growth in cooperation with Kit WT

To further investigate the transforming potential of the two mutant Cbl proteins, we

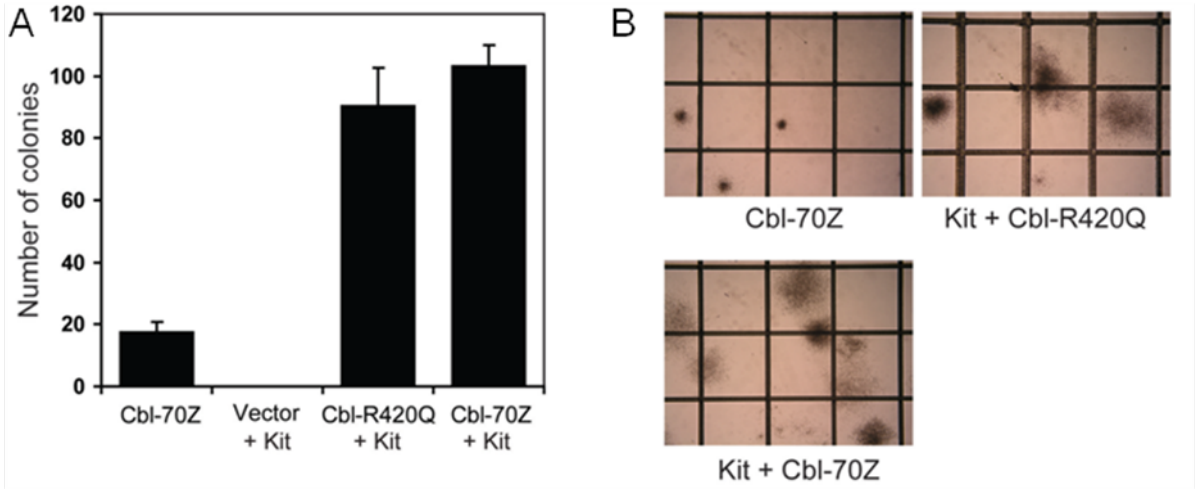


Figure 13. Cbl mutants lead to cytokine-independent colony growth. (A) The 32D cells stably overexpressing Kit-WT and/or indicated Cbl mutants were serum starved for 12 hours and then plated at a concentration of 1.000 cells per dish in the absence of any growth factors. Colonies were counted on day 6. The assays were plated as triplicates. The numbers given show the results of one of at least 3 independent experiments per construct, which all gave similar results. (B) Photographs of the dishes were taken on day 6 and show morphologic differences in clonal growth.

examined their ability to confer clonogenic growth to 32D cells (Figure 13). When expressed in 32D cells, Cbl-70Z induced the formation of very few, small colonies in

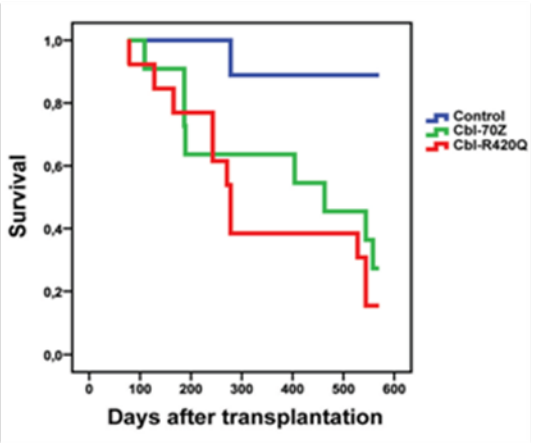
the absence of growth factors (Figure 13B). In contrast, expression of Cbl mutants together with Kit induced the growth of numerous large and dispersed colonies (Figure 13A).

4.2 Cbl mutants induce a generalized mastocytosis and a myeloproliferative disease in a murine bone marrow transplantation model

4.2.1 Cbl mutants induce hematologic disorder

To investigate the transforming capability of different Cbl mutants *in vivo*, murine ecotropic retrovirus (pMY) expressing Cbl-R420Q or Cbl-70Z or vector alone were generated. A bicistronic retroviral vector co-expressing the Cbl mutants together with the enhanced green fluorescent protein (EGFP) via an internal ribosomal entry site (IRES) was used (Kitamura et al, 2003b). Bone marrow was transduced with each retrovirus at a titer of 4×10^5 retroviral particles per milliliter, analyzed by flow cytometry for EGFP expression and the total cell number was determined. 150.000 EGFP positive cells were injected into the tail vein of lethally irradiated (8 Gy [800 rad]) female Balb/C recipient mice. Initial control experiments showed a similar level of expression between Cbl-R420Q and Cbl-70Z (data not shown). Almost all recipients of bone marrow cells transduced with Cbl mutants developed a lethal hematologic disorder with a mean latency of 341 days in the Cbl-R420Q group and 395 days in the Cbl-70Z group (Figure 14). Eleven of 13 mice in the Cbl-R420Q group and eight of 11 mice in the Cbl-70Z group died (Figure 14).

A



B

Group	No. mice	Survival time d(mean)	Disease manifestation
Control	9	278-570 (538)	None
Cbl-70Z	11	109-570 (375)	Spleen PB BM
Cbl-R420Q	13	76-579 (341)	Spleen PB BM

Figure 14. Cbl mutants induce hematologic disorder. (A) Kaplan-Meier survival curve for recipients of bone marrow transduced with Cbl-70Z, Cbl-R420Q or empty vector (Control). (B) Analysis of mice; BM, bone marrow; PB, peripheral blood.

4.2.2 Cbl-R420Q mutant induce myeloid leukemia

Two animals in the Cbl-R420Q group succumbed to a “myeloid leukemia with maturation” matching all 5 criteria for nonlymphoid leukemia of the Bethesda proposals (Kogan et al, 2002). These 2 mice showed leukocytosis of up to 140.000/µL (Figure 19B) with more than 20% blasts in the peripheral blood (Figure. 19A) and up to 70% blast infiltration in the spleen (Figure 18). Splenomegaly (Figure 15) developed with large differences in spleen weights and expansion of myeloid cells in liver and spleen were observed (Figure 18).

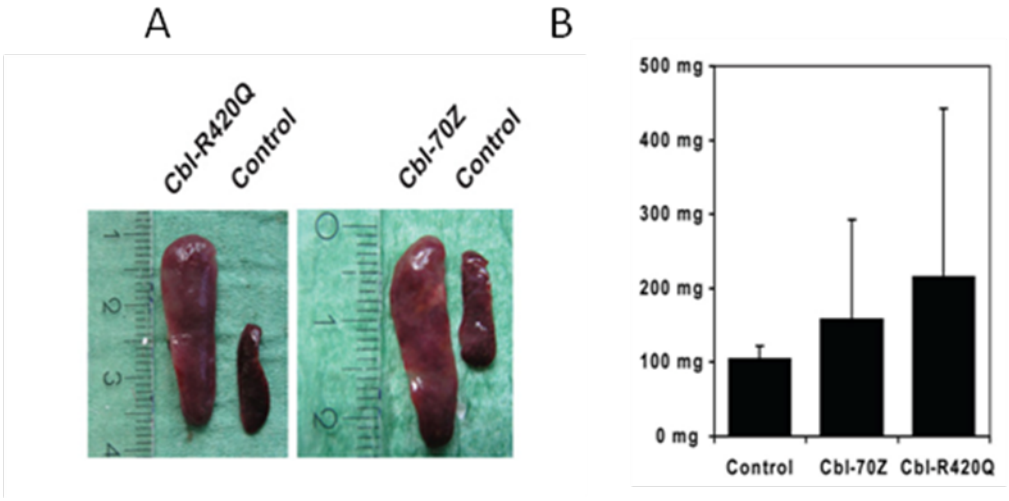


Figure 15. Cbl mutants cause splenomegaly. (A) Splenomegaly in mice that received transplants of Cbl mutant transduced bone marrow cells; empty vector (Control). (B) Comparison of spleen weight from mice receiving bone marrow transduced with Cbl mutants.

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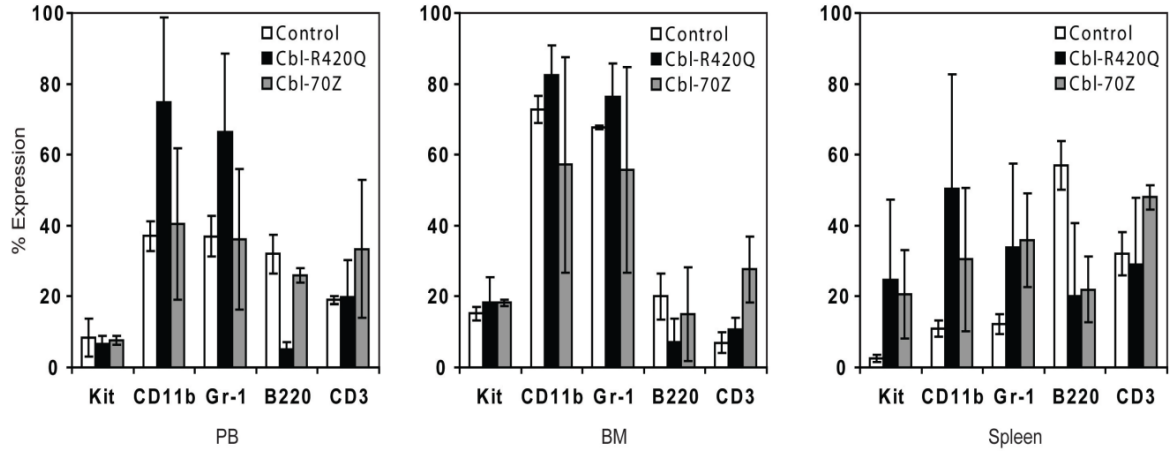

Figure 16. Immunophenotype of peripheral blood, bone marrow and spleen. Comparison of immunophenotype of peripheral blood (PB), bone marrow (BM) and spleen cells from mice receiving bone marrow transduced with Cbl mutants representing lineage-specific antigens (Kit, CD-11b, Gr-1, B220, CD3). The data represent the average and the standard deviation.

Histology sections of spleen, liver and bone marrow and FACS analyses of spleen, bone marrow and peripheral blood showed extensive infiltration of blasts and mature myeloid cells (Figures 16-18 and 19A)

4.2.3 Cbl mutants induce a generalized mastocytosis

Importantly, all the animals in the Cbl-R420Q and Cbl-70Z group showed a diffuse organ infiltration (spleen, liver, bone marrow, lung, kidney, heart) of mast cells with a very variable range of mast cell infiltration (Figures 17-18 and 20). Six mice showed increased mast cell infiltration, reminiscent of mast cell sarcoma. These cells have clear mast cell morphology with numerous granules in Giemsa staining and are highly positive in NACE (naphthol AS-D chloroacetate esterase) staining. The lesions have characteristic cytology with uniform, closely packed cells with round nuclei and clear, abundant cytoplasm with granules, which are characteristic features of mast cells (Figures 17-18 and 20-21)

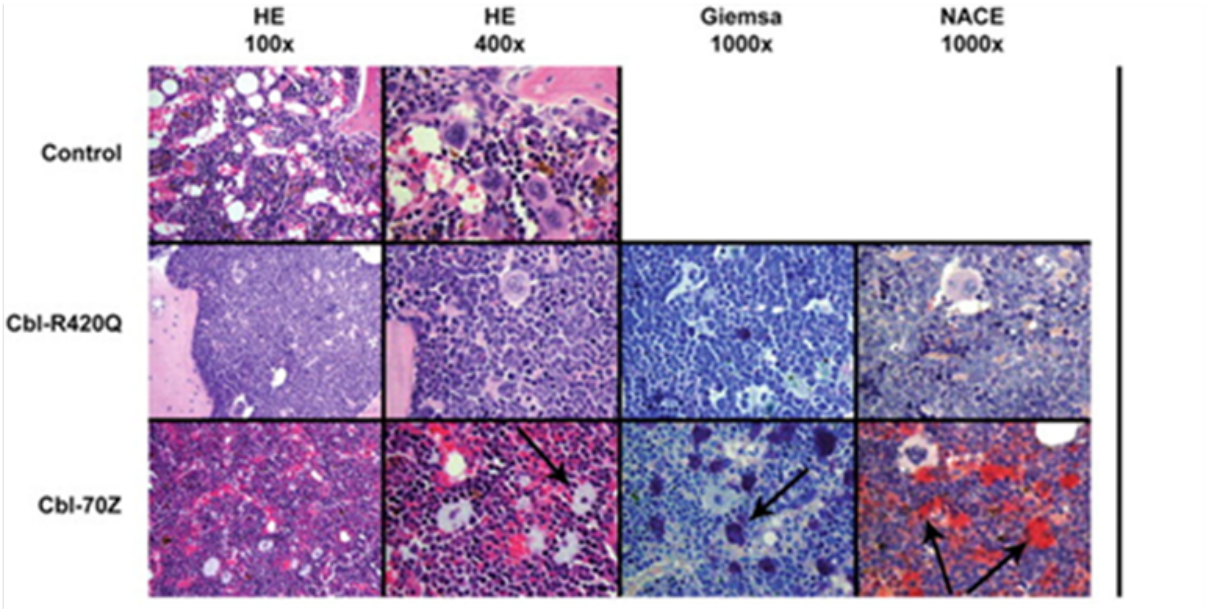

Figure 17. Cbl mutants induce a myeloid leukemia or mast cell sarcoma. Histopathology of the bone marrow (BM) from mice transplanted with Cbl mutant transduced bone marrow cells. Bone marrow sections were stained with hematoxylin and eosin [HE], Giemsa, naphthol AS-D chloroacetate esterase [NACE]). They are from a representative leukemic mouse (Cbl-R420Q) and a mouse with mast cell sarcoma (Cbl-70Z), which received bone marrow cells transduced with Cbl-R420Q or Cbl-70Z, respectively. Normal organ architecture is visible in a control mouse.

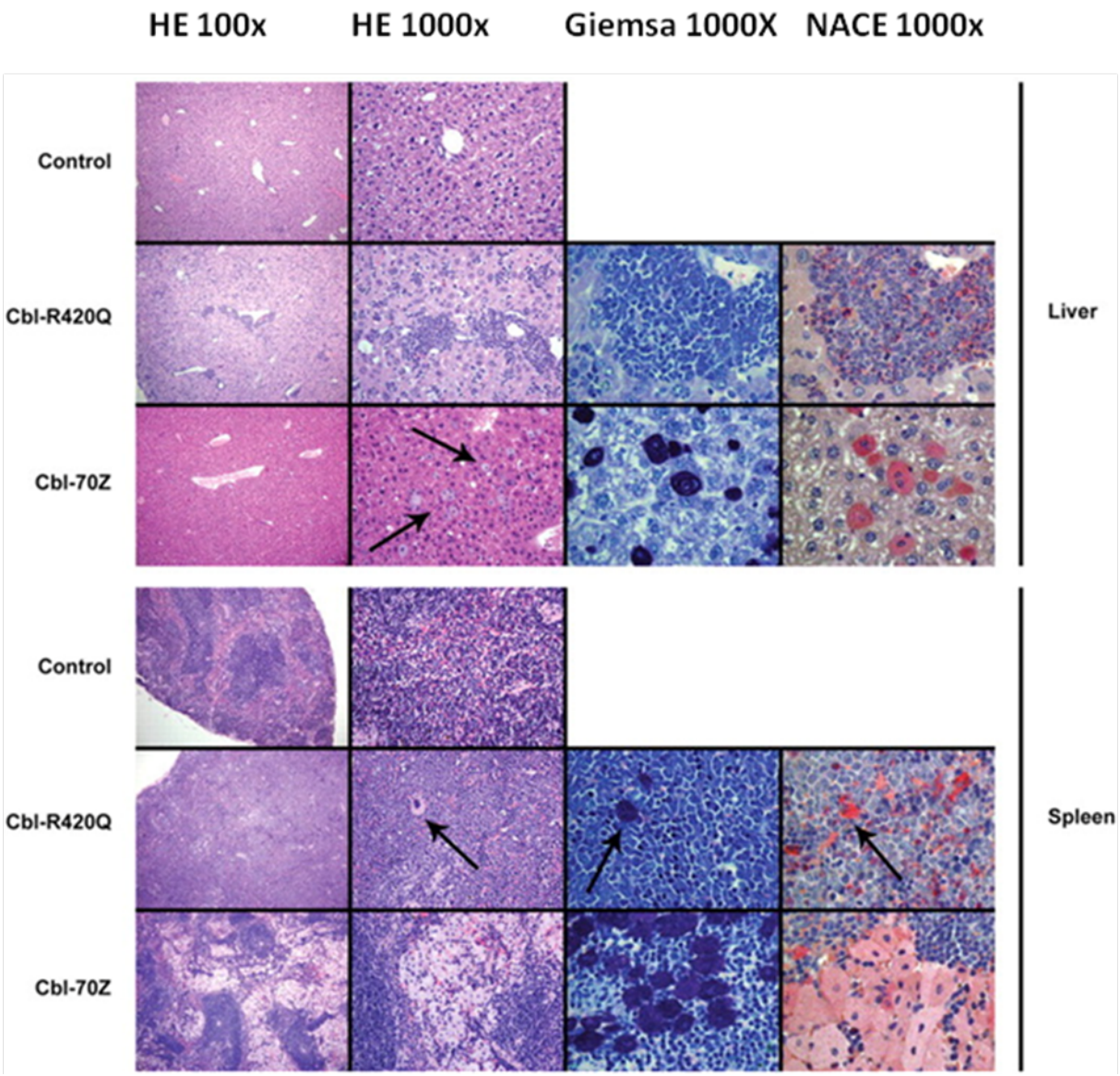

Figure 18. Histopathology of the liver and spleen from mice transplanted with Cbl mutant transduced bone marrow cells. Spleen, liver sections stained with hematoxylin and eosin [HE], Giemsa, naphthol AS-D chloroacetate esterase [NACE]). They are from a representative leukemic mouse (Cbl-R420Q) and a mouse with mast cell sarcoma (Cbl-70Z), which received bone marrow cells transduced with Cbl-R420Q or Cbl-70Z, respectively. Normal organ architecture is visible in a control mouse. The leukemic mouse (Cbl-R420Q) reveals a massive infiltration of immature myeloid cells with its maximum in the spleen and consecutive destruction of the normal organ structure. There are also mast cells sporadically visible in between the immature cell infiltration (see arrow in the spleen pictures and Giemsa staining). In contrast mast cells in the mice with mast cell sarcoma have characteristic cytology with uniform, closely packed cells with round nuclei and clear, abundant cytoplasm with granules and the typical features of mast cells with numerous granules in Giemsa staining and are highly positive in NACE (naphthol AS-D chloroacetate esterase) staining.

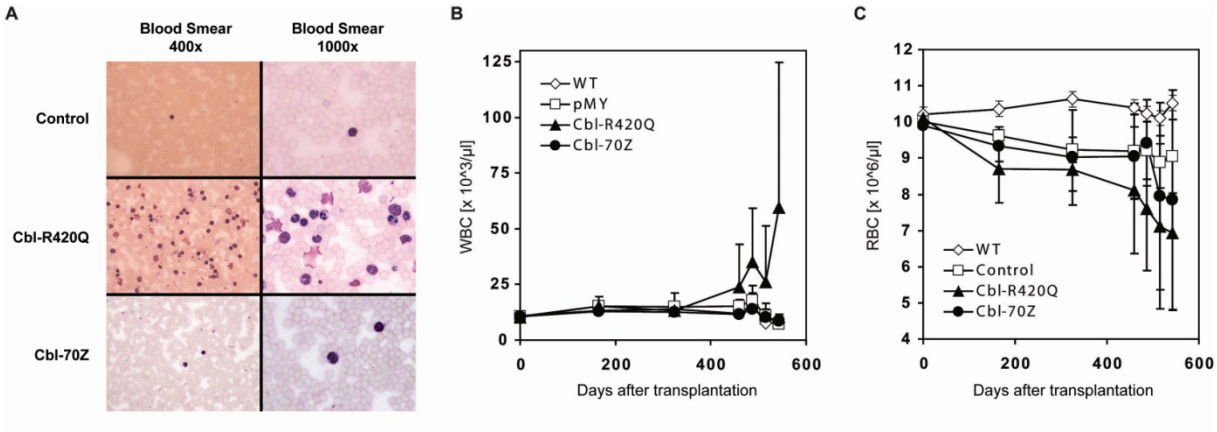

Figure 19. Blood smear, white and red blood cell count, and immunophenotype of cells of peripheral blood of mice that received a transplant of Cbl mutant-transduced bone marrow. (A) Blood smear of peripheral blood with hyperleukocytosis in leukemic Cbl-R420Q mice (Pappenheim's staining). (B–C) Leukemic Cbl-R420Q mice develop leukocytosis (B) and anemia (C). Peripheral blood leukocyte (WBC) and erythrocyte (RBC) counts for recipients of bone marrow transduced with Cbl mutants.

One mouse had an organ infiltration of mast cells together with mature myeloid cells and 5-10% myeloid blast cells. Importantly, none of the control mice had histological evidence of mastocytosis or other hematological disease (Figure 17-18).

4.2.4 Granules in mast cells

Electron microscopic pictures reveal large cytoplasmic granules in the mast cells. These granules are round, oval or irregular bodies of 0.5 to 1.0 µm in size, having granules sharp boundaries which are sometimes wavy at points of contact with other granules and thus present the appearance of an interlocking of the surfaces of the granules. Inside the granules is found a reticular or vacuolar structure consisting of thread-like material which is similar to, but finer than, that of the interior of the nucleus (Figure 21). These features resemble granules of mast cells.

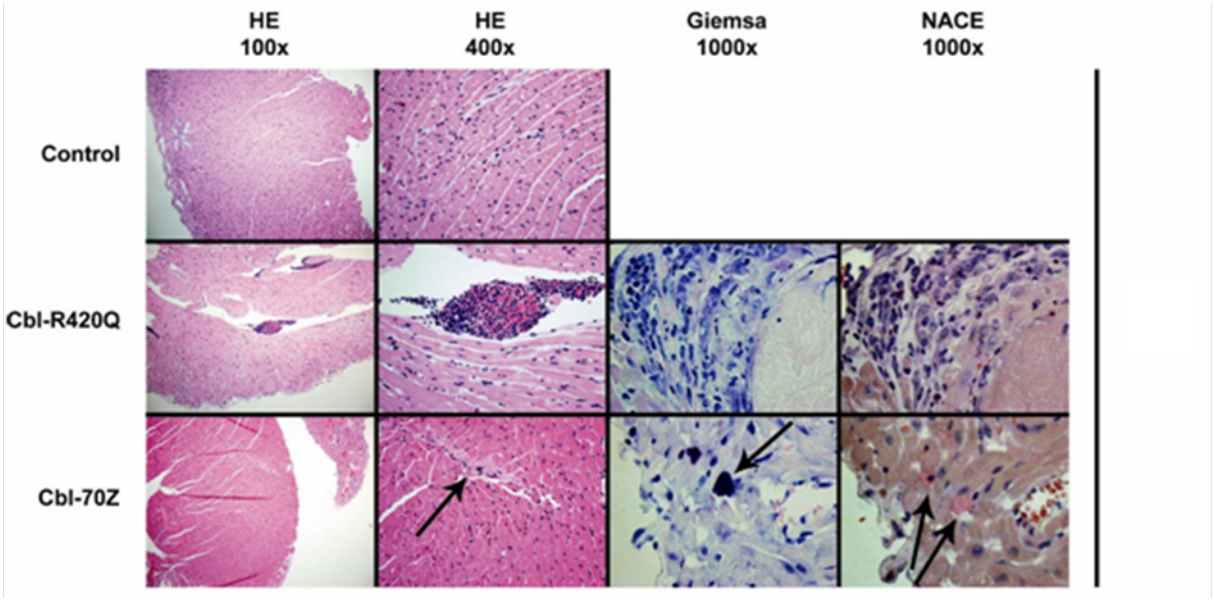

Figure 20. Histopathology of the heart from mice transplanted with Cbl mutant transduced bone marrow cells. Heart sections (hematoxylin and eosin HE, Giemsa, naphthol AS-D chloroacetate esterase NACE, original magnification 100–1000×) from a representative leukemic mouse (Cbl-R420Q) and a mouse with mast cell sarcoma (Cbl-70Z), which received bone marrow cells transduced with Cbl-R420Q or Cbl-70Z, respectively. Normal organ architecture is visible in a control mouse (vector alone). A leukemic mouse (Cbl-R420Q) reveals a massive infiltration of immature myeloid cells in the heart. A mouse with generalized mastocytosis and mast cell sarcoma shows diffuse mast cell infiltration in between the heart muscle cells (see arrows).

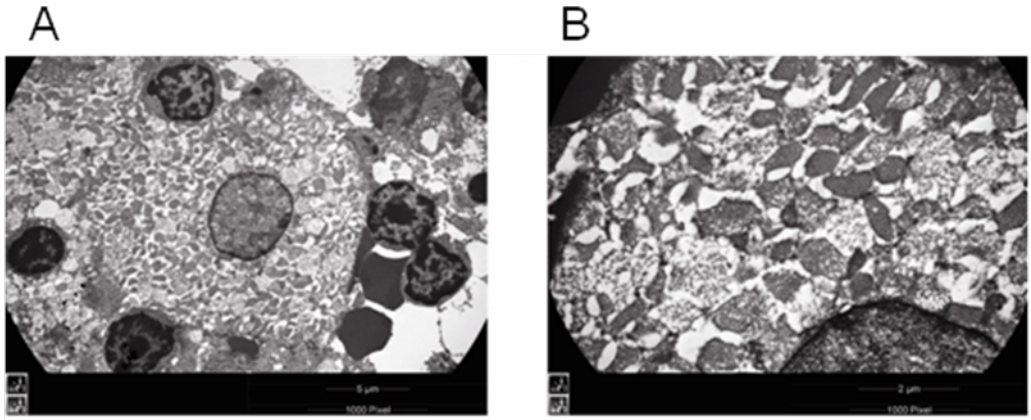

Figure 21. Electron microscopy of mast cells (magnification see photo)

Thus, bone marrow transplantation of cells expressing Cbl mutants led to a generalized mastocytosis (in extreme cases mast cell sarcoma), a myeloproliferative disorder and myeloid leukemia with a long latency and at high penetrance.

4.3 Cbl mutants inhibit ubiquitination of Kit

The observation that Flt3 and Kit proteins cooperated with Cbl mutants to transform myeloid cells *in vitro* prompted us to study the underlying mechanisms. Under physiological conditions, ligand activation of RTKs induces receptor internalization and degradation that are important for RTK signal mitigation. Cbl proteins play an important role in these processes (Thien and Langdon, 2001;Waterman and Yarden, 2001;Zeng et al, 2005). Therefore, we analyzed whether Cbl mutants can abolish ubiquitination of the activated Kit receptor. COS-7 cells were transiently transfected with Kit WT and HA-tagged ubiquitin (Ubq) in the presence or absence of Cbl mutants.

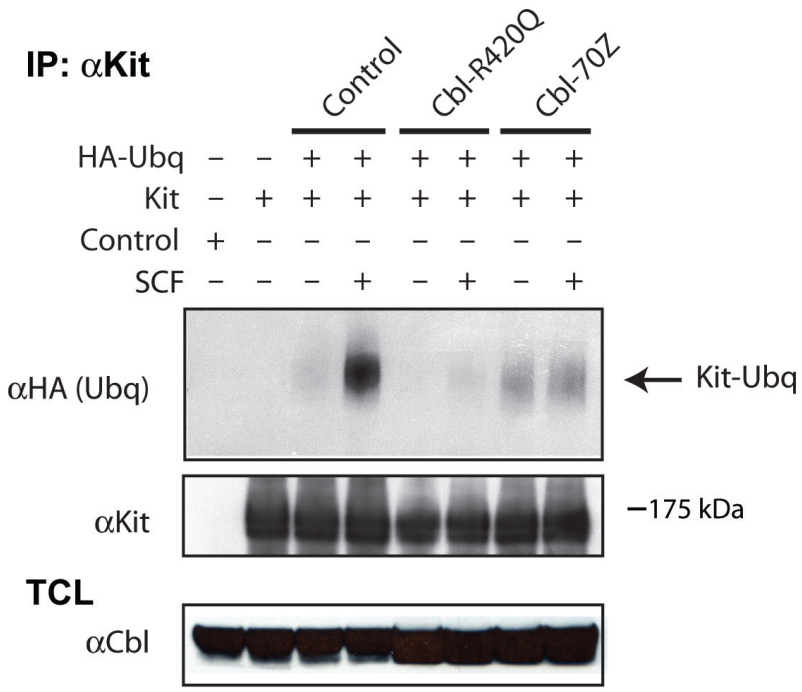

Figure 22. Cbl mutants inhibit ubiquitination of Kit. COS-7 cells were transiently transfected with the indicated constructs together with a plasmid for HA-tagged ubiquitin (Ubq). After 48 hours of transfection cells were serum-starved for 12 hours and stimulated with 50 ng/mL SCF for 10 minutes or left unstimulated. Cell lysates were prepared and equal amounts of lysates were immunoprecipitated using anti-Kit antibody. The immunoprecipitates were resolved on SDS-page and analyzed with anti-HA or anti-Kit antibodies. Expression of overexpressed Cbl mutants is shown in total cell lysates (TCL) using anti-Cbl antibody.

After transfection, cells were stimulated with SCF. Kit was effectively ubiquitinated in the presence of endogenous Cbl WT after stimulation with SCF (Figure 22), but the presence of Cbl-R420Q completely abolished ubiquitination of the activated receptor. Cbl-70Z also repressed ubiquitination, although not to the same extent as in the case of Cbl-R420Q. In the control cells ubiquitination was not observed (Figure 22). The expression of Kit receptor and the Cbl mutants was similar in all cases.

4.4 Cbl mutants inhibit internalization of ligand-activated Kit

As ubiquitination often leads to endocytosis of the activated receptor (Ceresa and Schmid, 2000;Haglund et al, 2003;Le and Wrana, 2005), the effect of Cbl mutants on endocytosis of activated Kit receptor in 32D and COS-7 cells was examined. To avoid the end point differences in signal termination, between different cell types, COS-7 cells were used for both ubiquitination and endocytosis experiments.

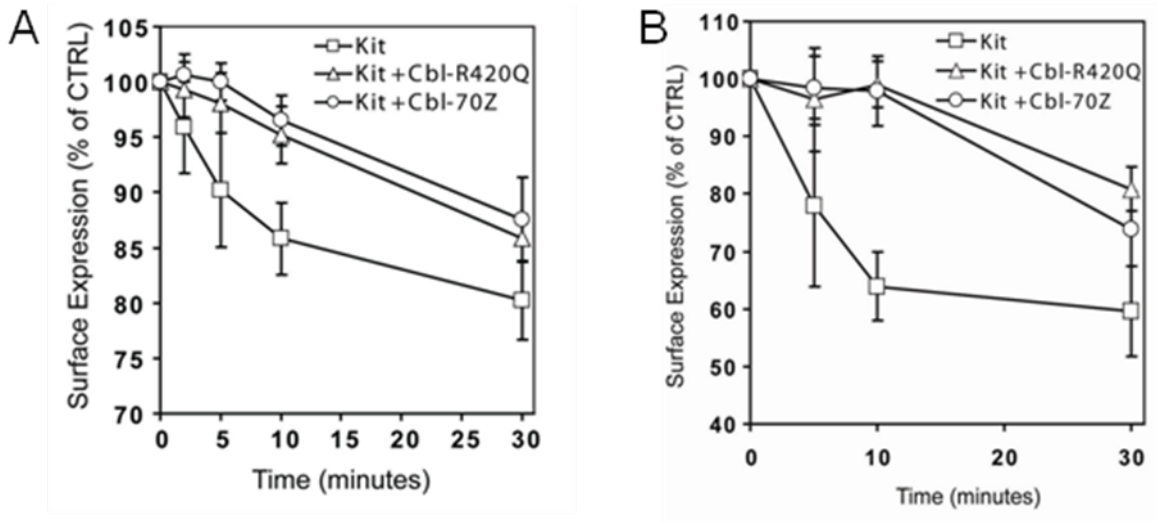

Figure 23. Internalization of Kit is inhibited by Cbl mutants. 32D (A) or Cos-7 (B) cells expressing Kit in the presence or absence of different Cbl mutants were washed with PBS and then stimulated with SCF for the indicated time points. Subsequently the amount of Kit remained on the cell surface was measured by flow cytometry after staining with a PE-labelled anti-Kit antibody. Sodium azide was used to stop the internalization. Results are expressed as mean (+/- standard deviation) of 3 independent experiments.

COS-7 cells transfected with Kit WT in the presence or absence of Cbl mutants or 32D stable cell lines stimulated with SCF and the amount of Kit receptor at the surface was analyzed by FACS by using PE labeled Kit antibody, at the indicated time points. During first 10 mins of stimulation, approximately 15% of Kit protein was internalized, compared to 5% in the presence of Cbl mutants. Cbl mutants quite effectively inhibited the initial rapid rate of ligand-induced receptor internalization (Figure 23).

4.5 Cbl mutants associate with Kit

The physical interaction between the Cbl mutants and Kit was analysed. 32D cell lines stably expressing Kit WT in the presence of HA-tagged Cbl mutants were serum starved and stimulated with SCF.

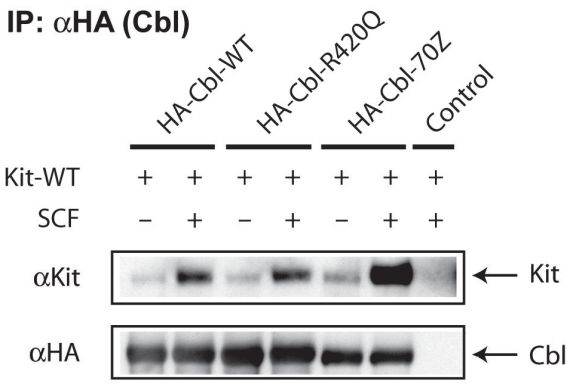

Figure 24. Kit physically interacts with Cbl. 32D-Kit-WT cells stably transfected with HA-tagged Cbl proteins (Cbl-WT, Cbl-R420Q, Cbl-70Z) were deprived from cytokines overnight and subsequently exposed to the indicated cytokines for 10 minutes. Cbl proteins were immunoprecipitated by anti-HA antibodies and immunoprecipitates were resolved on SDS-PAGE. Co-immunoprecipitation of Kit was analyzed using anti-Kit antibodies.

Immunoprecipitation of exogenously overexpressed, hemagglutinin (HA)–tagged c-Cbl with anti-HA antibodies resulted in immunocomplexes that contained Kit (Figure 24). The association of Cbl-WT and Cbl-R420Q with Kit was apparently enhanced by the presence of SCF, whereas Cbl-70Z strongly associated with Kit receptor in the presence of SCF and a slight association was present even in the absence of ligand (Figure 24). A slightly reduced level of Cbl expression was observed in the presence of Cbl-70Z.

4.6 Akt and Erk activation in the presence of Cbl mutants and Kit WT

The effects of Cbl mutants on receptor mediated downstream signaling pathways was analysed (Figure 25). 32D cells expressing Cbl mutants alone or in combination with Kit WT were serum starved and stimulated with either SCF or IL-3.

Co-expression of Cbl-70Z or Cbl-R420Q with Kit receptors induced a stronger activation of the Akt and Erk pathways compared to cells expressing Kit WT alone as indicated by phosphorylation of the respective signaling intermediates after 10 minutes (Figure 25B). Further, in the cells expressing Cbl mutants and Kit WT, Akt and Erk (to a lesser extent) were phosphorylated (i.e. activated) even in the absence of growth factors (Figure 25B). No activation of intracellular signaling mediators was observed when the Cbl mutants were overexpressed alone, except when cells were stimulated with IL-3 (Figure 25A).

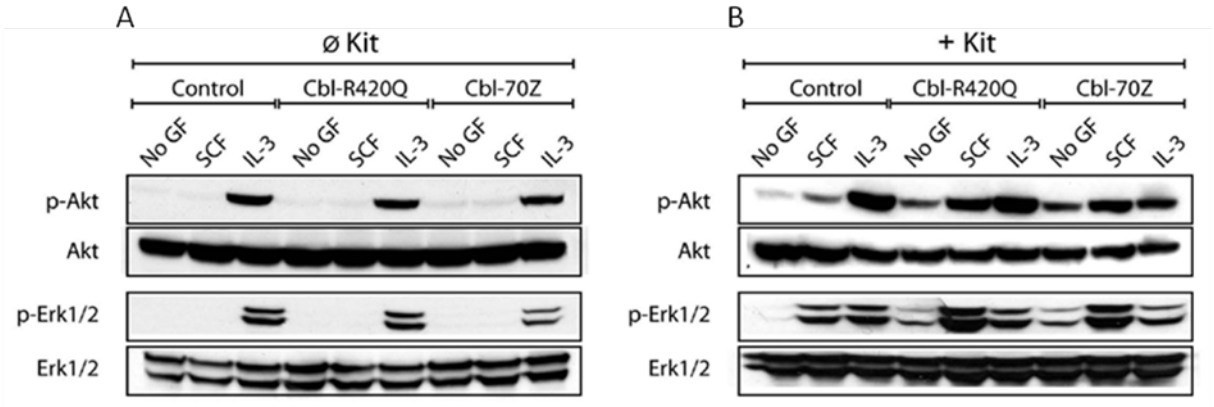

Figure 25. Cbl influences Kit-mediated signaling. 32D cells with (B) and without Kit-WT (A) were engineered to express the indicated Cbl proteins, deprived from cytokines overnight and subsequently exposed to the indicated cytokines for 10 minutes. Western blot analyses with the indicated antibodies were performed.

4.7 Cbl mutants prolonged Kit mediated signaling

Next, the effects of Cbl proteins on the strength, quality, and duration of Kit signaling were analysed. 32D cells expressing Kit WT in the presence or absence of Cbl mutants were serum starved and then stimulated with SCF for 240 mins. In the cells expressing Kit alone, maximum stimulation of Kit receptor was observed after 10mins and the signal intensity was decreased thereafter. Nevertheless, cells expressing Kit and Cbl mutants showed maximum stimulation of Kit receptor after 10mins (higher than in the cells expressing Kit alone) followed by persistent activation of Kit. A prolonged activation of Kit, Akt and Erk activity up to 240 minutes after SCF stimulation in the cells expressing Cbl-70Z or Cbl-R420Q compared to the cells expressing Kit WT was observed (Figure 26)..

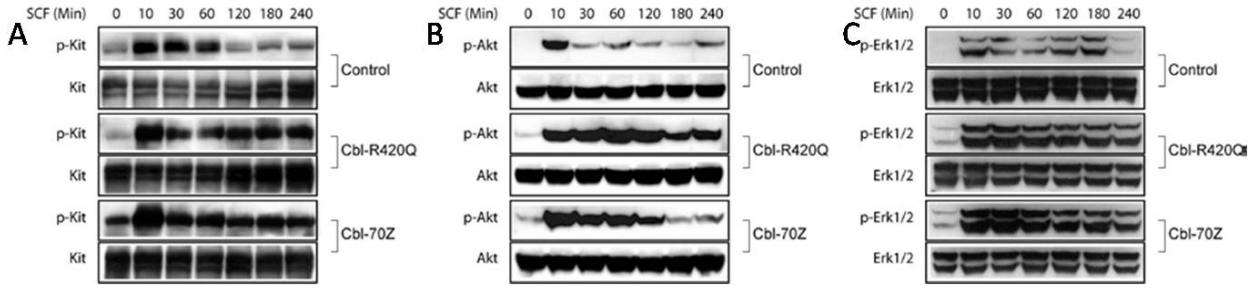

Figure 26. Cbl proteins change the kinetics of Kit-phosphorylation and Kit-induced Akt and Erk activity. 32D-Kit-WT cells stably transfected with the indicated Cbl proteins were serum starved and then exposed to SCF for the indicated time periods. Western blot analyses using phospho-specific antibodies for Kit (A), Akt (B) and Erk1/2 (C) were performed.

In addition, Cbl-70Z expression, but not Cbl-R420Q or Kit WT alone, led to autophosphorylation of the Kit receptor even in the absence of any ligand (Figure

26A). Kit receptor was maximally stimulated in the presence of Cbl mutants compared to cells expressing Kit alone (Figure 26A).

4.8 Kinase activity of Kit and Flt3 is dispensable for Cbl-70Z mediated transformation

In order to determine the requirement of RTK kinase activity during the transformation by Cbl mutants and Kit receptor, 32D cells stably expressing kinase-dead (KD) mutants of Kit (Kit-D790N, Kit-KD) in the presence or absence of Cbl mutants were generated.

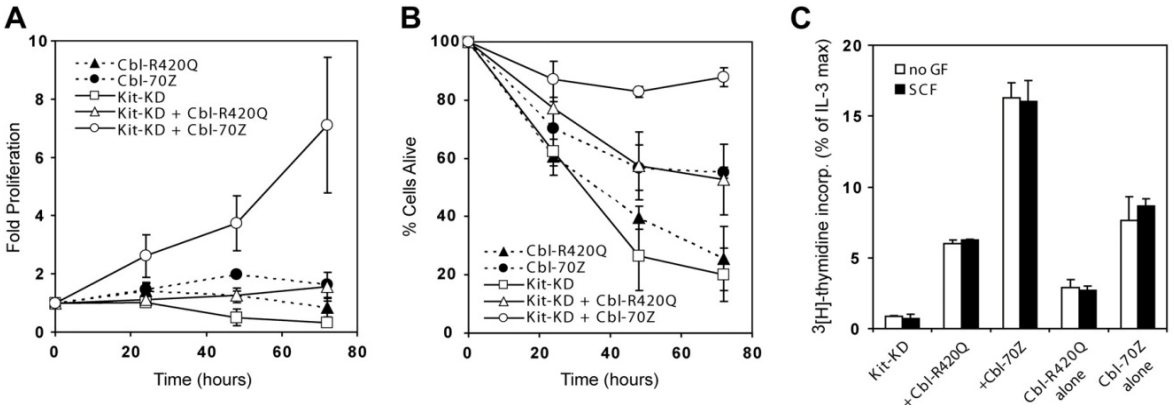

Figure 27. Kinase activity of Kit is dispensable for Cbl-70Z mediated transformation. (A) The 32D cells stably overexpressing Kit-KD and/or indicated Cbl mutants were starved from IL-3 and cells were grown in the presence of 10% FCS. Cells were counted at the indicated time points by the trypan blue exclusion method and the data are shown as fold change of the cell number compared with the start of the experiment. (B) The percentage of cells in the culture that were alive at the indicated time points are shown. (C) 32D-Kit-KD cells stably overexpressing the indicated constructs were starved from IL-3 for 12 hours, treated with the indicated cytokines and proliferation was measured in ^{3}[H]-thymidine incorporation assays.

Analyzing the proliferative capacity of cells that co-express Kit-KD and Cbl-R420Q, it was found a surprising growth and survival advantage when compared to Kit-KD alone (Figure 27A-B). This effect was more pronounced with Cbl-70Z than with Cbl-R420Q. Interestingly, cells co-expressing Kit-KD and Cbl-70Z rapidly proliferated and survived for extended time periods in the absence of exogenous growth factors (Figure 27). Kinase-dead Flt3 (Flt3-K644A, Flt3-KD) expressing 32D cells in the presence and absence of Cbl mutants were generated. Cells co-expressing Flt3-KD and Cbl-70Z also proliferated and survived in the absence of growth factors (Figure 28).

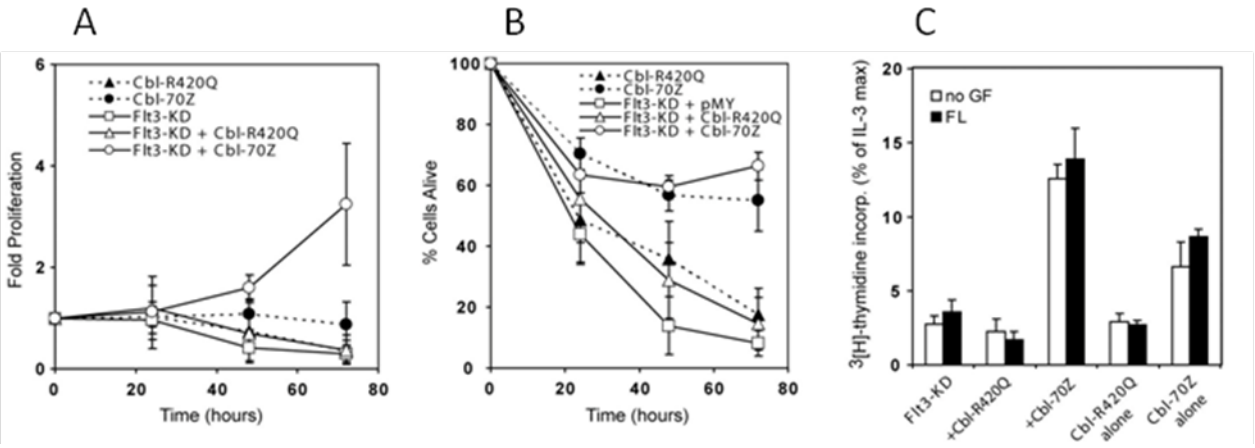

Figure 28. Kinase activity of Flt3 is dispensable for Cbl-70Z mediated transformation. (A) The 32D cells stably overexpressing Flt3-KD and/or indicated Cbl mutants were starved from IL-3 and cells were grown in the presence of 10% FCS. Cells were counted at the indicated time points by the trypan blue exclusion method and the data are shown as fold change of the cell number compared with the start of the experiment. (B) The percentage of cells in the culture that were alive at the indicated time points are shown. (C) 32D Flt3-KD cells stably overexpressing the indicated constructs were starved from IL-3 for 12 hours, treated with the indicated cytokines and proliferation was measured in ^{3}[H]-thymidine incorporation assays. Data are shown as percentage of thymidine incorporation relative to the thymidine incorporation of the respective cell line with IL-3 supplementation. The data (panel A) represent the average and standard deviation of at least 3 independent experiments.

Viability assays (data not shown) and ^{3}[H]-thymidine incorporation assays confirm that both kinase-dead receptors were not responsive to their respective ligands (Figure 27 and 28).

4.9 Role of Akt in Cbl-70Z transformed Kit kinase dead cells

To determine what signaling molecules supported the transforming effect of Cbl-70Z, Akt, Erk and Stat pathways were analysed in 32D cell lines co-expressing Kit-KD or Flt3-KD and Cbl-70Z or Cbl-R420Q. Surprisingly, it was found ligand independent activation of Akt in the cell lines expressing Cbl-70Z in the presence of either of the KD receptors, but not in the cells expressing Cbl-R420Q and kinase-dead receptors or control cells (Figure 29A-B). No differences were found in other pathways (Stat/Erk) (Figure 29A).

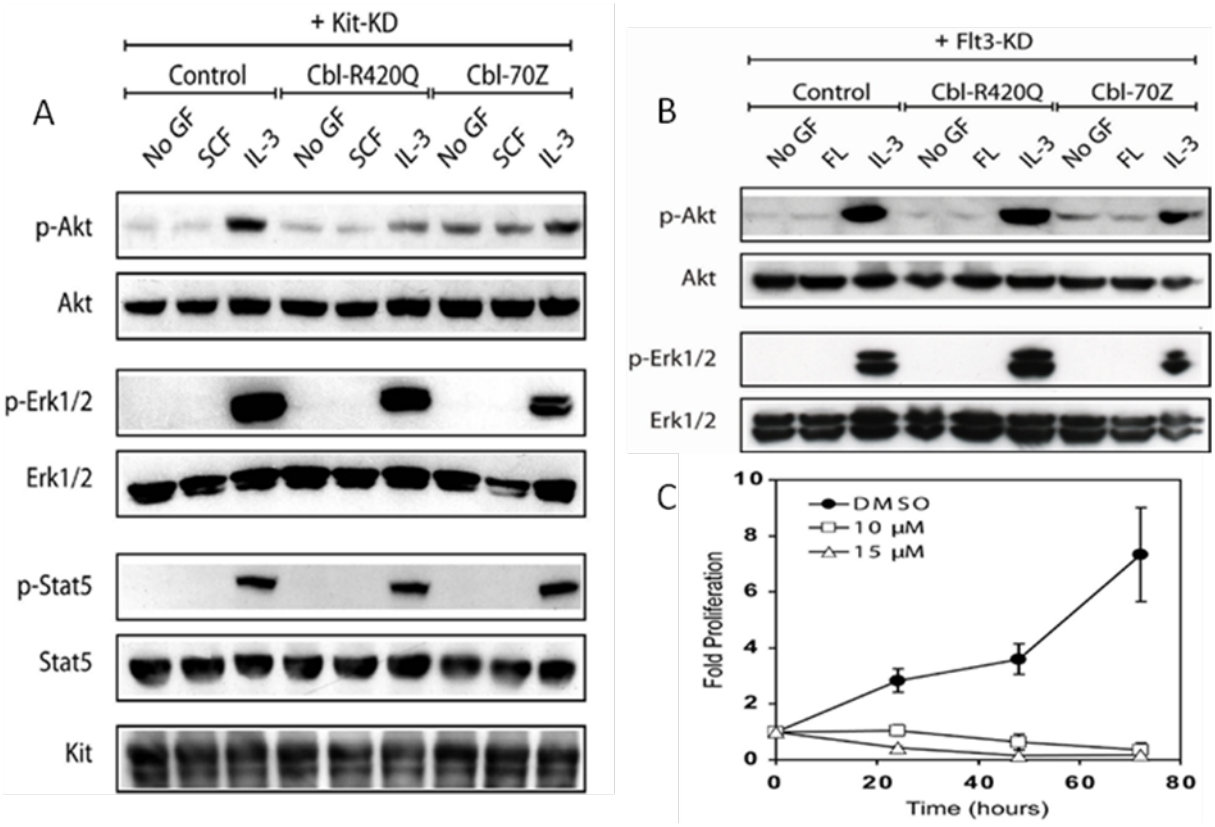

Figure 29. Constitutive Akt activation in Kit-KD cells expressing Cbl-70Z. (A) 32D cells with Kit-WT or Kit-KD were engineered to express the indicated Cbl proteins, deprived from cytokines overnight and subsequently exposed to the indicated cytokines for 10 minutes. Western blot analyses with the indicated antibodies were performed. (B) 32D cells overexpressing Kit-KD and Cbl-70Z were deprived from cytokines overnight and subsequently exposed to the indicated cytokines for 10 minutes. Western blot analyses with the indicated antibodies were performed. (C) Kit-KD cells stably overexpressing Cbl-70Z were starved from IL-3 and cells were grown in the presence of 10% FCS and 10 to 15 µM Akt inhibitor or DMSO as solvent. Cells were counted at the indicated time points by the trypan blue exclusion method and the data are shown as fold change of the cell number compared with the start of the experiment.

4.9.1 Akt inhibition abolished transformation

Importantly, inhibition of Akt activity abolished the cytokine-independent growth of these cells (Figure 29C). Growth was inhibited in dose dependent manner. After 72hrs, almost all cells were died in the presence of Akt inhibitor. Cells expressing Flt3-KD and Cbl-70Z also died in the presence of Akt inhibitor (data not shown).

4.9.2 Cbl-70Z do not confer autophosphorylation to kinase-dead (KD) receptors

The presence of Cbl-70Z conferred ligand-independent autophosphorylation to Kit WT (Figure 26A), whilst autophosphorylated kinase-dead receptors in the absence or presence of any dominant-negative Cbl mutant was undetectable (Figure 30).

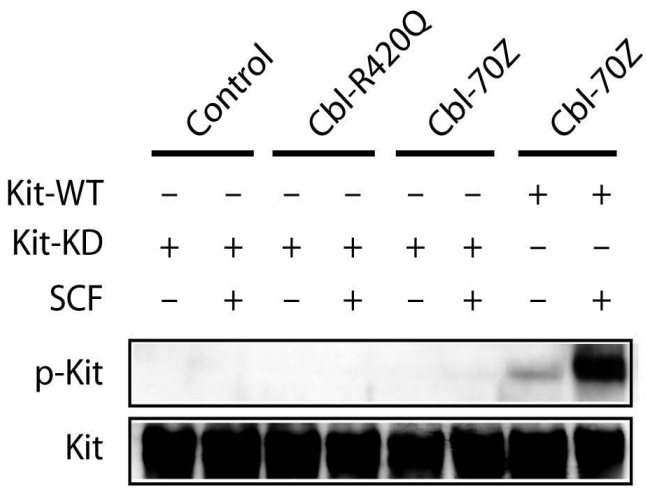

Figure 30. Cbl mutants do not confer autophosphorylation to the Kinase-dead receptors. 32D cells with Kit WT or Kit-KD were engineered to express the indicated Cbl proteins, deprived from cytokines overnight and subsequently exposed to the indicated cytokines for 10 minutes. Western blot analyses with the indicated antibodies was performed.

4.9.3 Kinase-dead Kit receptor does not undergo ubiquitination

In order to understand the basic mechanism of degradation of this receptor the turnover of the kinase-dead Kit receptor by ubiquitination experiments was analysed. No degree of ubiquitination of the receptor, compared with the basic level of the unstimulated Kit WT receptor was observed (Figure 31).

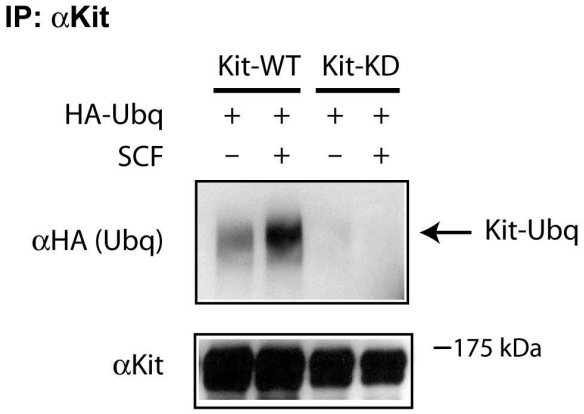

Figure 31. Kit-KD is not ubiquitinated. COS-7 cells were transiently transfected with the indicated constructs together with a plasmid for HA-tagged ubiquitin. 48 hours post-transfection cells were serum-starved for 12 hours and stimulated with 50 ng/mL SCF for 10 minutes or left unstimulated. Cell lysates were prepared and equal amounts of lysates were immunoprecipitated using anti-Kit antibody. The immunoprecipitates were resolved on SDS-PAGE and analyzed with anti-HA or anti-Kit antibodies.

Taken together, Cbl-70Z and Cbl-R420Q not only strongly synergized with unstimulated receptor tyrosine kinases with intact kinase function, but surprisingly also induce biologically meaningful signaling events emanating from kinase-inactive class III receptor tyrosine kinases.

4.10 Src family tyrosine kinases (SFKs) are necessary for Cbl-70Z-mediated transformation

4.10.1 Enhanced SFKs activity in the presence of Cbl mutants

SFKs play an important role in intracellular signaling processes, for example of cytokine receptors. These receptors do not have tyrosine kinase activity (Miyajima et al, 1993;Parsons and Parsons, 2004). Based on the observation of RTK independent proliferation in the cells expressing RTKs and Cbl mutants, it was speculated that whether SFKs could play a role in Cbl-70Z mediated transformation. First, SFKs activation status in the presence of Cbl mutants was analysed. In 32D cells, it was observed that some SFKs were already constitutively active in the absence of any growth factors; this activation was not further enhanced by IL-3 stimulation but was strongly enhanced in the presence of both Cbl mutants (data not shown). In addition, the overexpression of Kit in the presence of Cbl mutants showed a similar phosphorylation of SFKs irrespective of the kinase activity of the RTK (Figure 32).

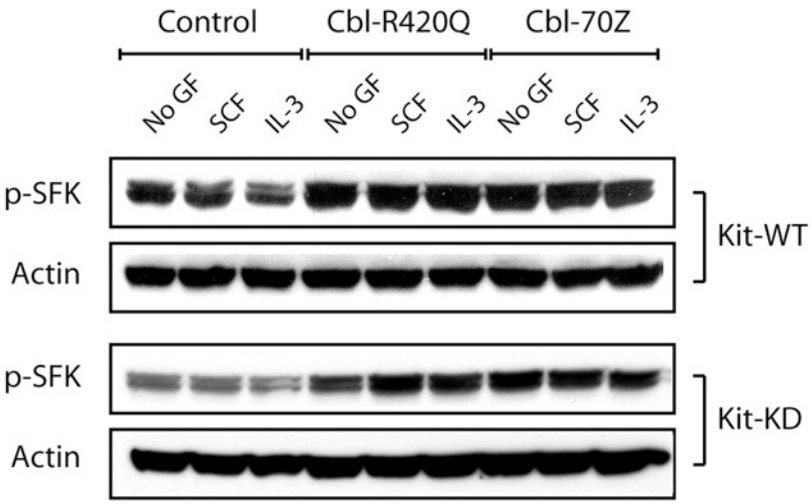

Figure 32. Phosphorylation of SFKs is enhanced by Cbl mutants. The 32D-Kit-WT (top 2 blots) or 32D-Kit-KD (bottom 2 blots) cells stably overexpressing the indicated Cbl proteins were starved from IL-3 overnight and subsequently exposed to the indicated cytokines for 10 minutes. Western blot analyses using the phosphospecific antibody recognizing the activated form of SFK members (anti-pSrc Y_{416}) and anti–β-actin antibody were performed as described.

4.10.2 Constitutive association of Kit-KD, Cbl and SFKs

The binding of Kit-KD, Cbl proteins and SFKs was examined. Immunoprecipitation experiments showed a constitutive association between SFKs and Cbl proteins in the absence of Kit (Figure 33). Surprisingly, immunoprecipitation of Cbl mutants also resulted in immunocomplexes with Kit-KD (Figure 33). Differences in the expression

levels of Cbl mutants can be excluded as reason for the different phenotypical behavior of these mutants (Figure 33 and data not shown). The association of Cbl mutants with Kit-KD was not enhanced by the presence of SCF.

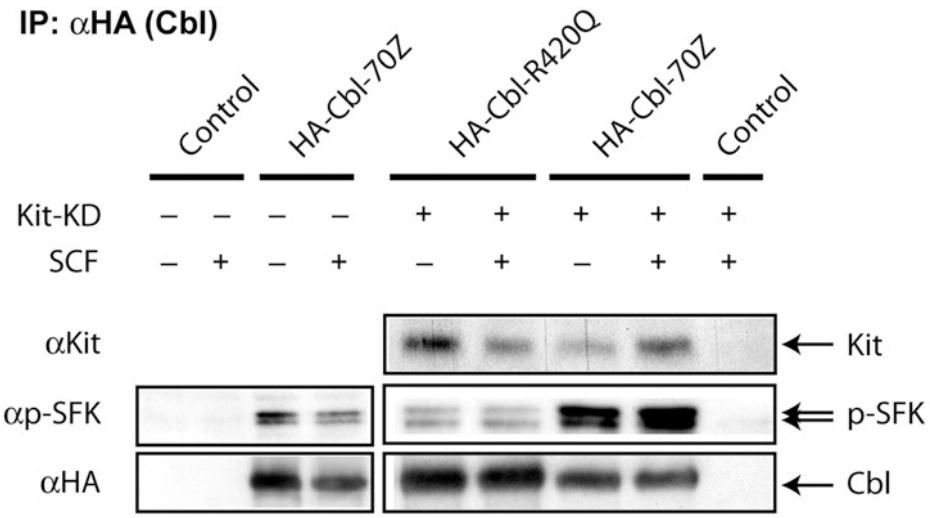

Figure 33. Cbl mutants physically interact with the KD Kit receptor. The 32D cells with or without Kit-KD stably transfected with HA-tagged Cbl proteins (Cbl-R420Q and Cbl-70Z) were starved from IL-3 overnight and subsequently exposed to the indicated cytokines for 10 minutes. Cbl proteins were immunoprecipitated by anti-HA antibodies, and immunoprecipitates were resolved on SDS-PAGE. Coimmunoprecipitation of Kit and p-SFKs was analyzed using anti-Kit or anti–phospho-SFK antibodies.

4.10.3 SFK inhibitors inhibit SFK phosphorylation

Cbl mutants associated with the kinase-dead Kit receptor which could be a prerequisite for the transformation potential of Kit-KD together with Cbl mutants. To test this hypothesis, the well established Src family inhibitors PP-1, PP-2 and dasatinib (Choudhary et al, 2007;Ramirez and DiPersio, 2008) were used. In the presence of Src inhibitors, the phosphorylation of SFKs was completely inhibited (Figure 34).

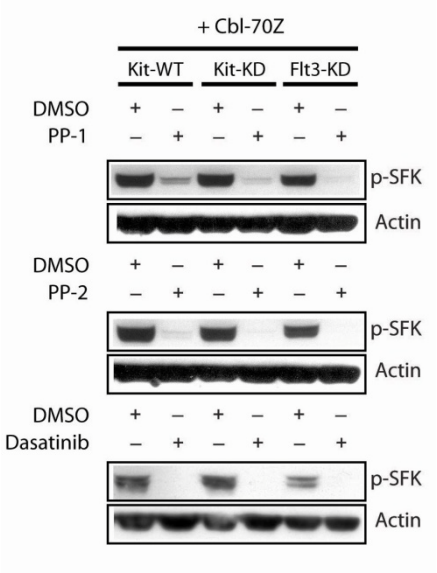

Figure 34. SFK phosphorylation is inhibited by Src inhibitors PP-1, PP-2, and dasatinib. 32D cells stably expressing the indicated constructs were starved in 10% FCS in the presence of PP-1, PP-2, Dasatinib or DMSO as control. Cell lysates were prepared as described, resolved on SDS-PAGE, and membranes were blotted for phospho-SFK and β-Actin.

4.10.4 Role of Fyn

Furthermore, PP-2 completely abrogated the interaction between activated SFKs and Cbl (Figure 35A), indicating a central role of activated SFKs. In order to identify the SFK involved in this process, Cbl was immunoprecipitated and blotted for each individual Src family members. These experiments revealed that Fyn, a SFK, binds to Cbl in a phospho-dependent manner, but not Lck, Hck, Src, Yes, and Lyn (Figure 35B, and data not shown).

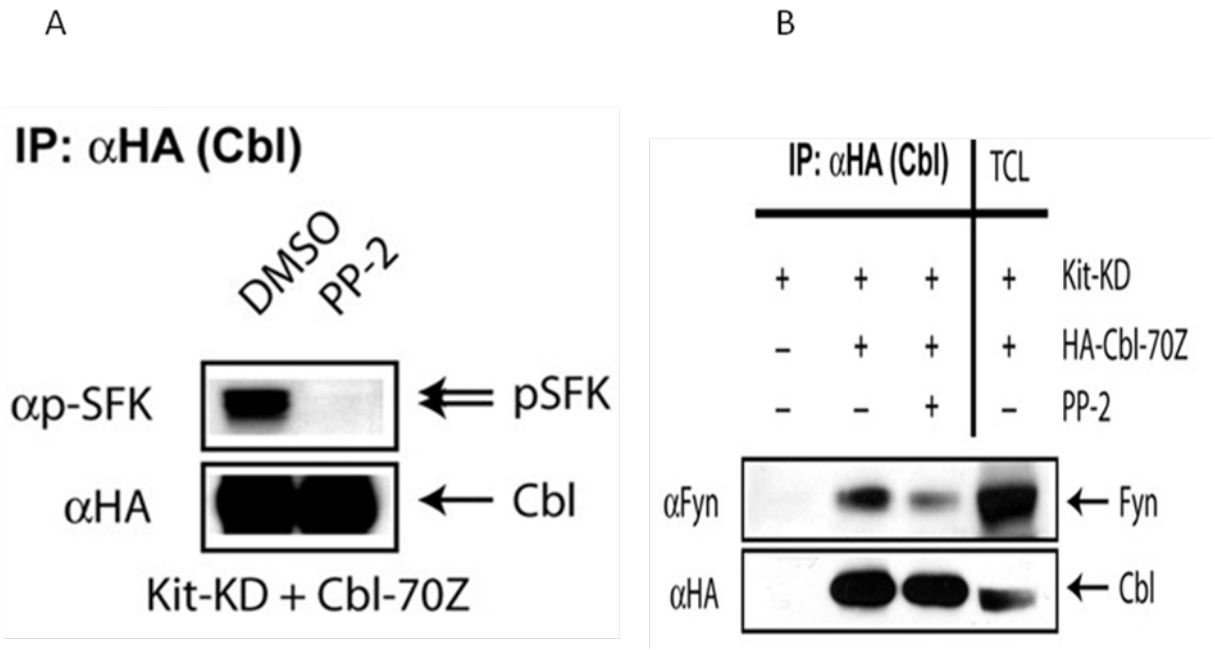

Figure 35. Interaction of SFKs and Cbl proteins is completely inhibited by the Src inhibitor PP-2. (A) The 32D-Kit-KD cells stably overexpressing HA-tagged Cbl-70Z were starved from IL-3 in the presence of DMSO or PP-2 (15 µM) for 12 hours. Cbl proteins were immunoprecipitated by anti-HA, antibodies and immunoprecipitates were resolved on SDS-PAGE. Western blot analyses were performed with anti–phospho-SFK and anti-HA antibodies. (B) The 32D-Kit-KD cells stably overexpressing HA-tagged Cbl-70Z were starved from IL-3 in the presence of DMSO or PP-2 (15 µM) for 12 hours. Cbl proteins were immunoprecipitated by anti-HA antibodies and immunoprecipitates were resolved on SDS-PAGE. Coimmunoprecipitation of Fyn was analyzed using anti-Fyn antibody.

4.10.5 SFKs inhibition abolished Cbl-70Z transformation

Proliferation assays of cells co-expressing Kit-KD or Flt3-KD together with Cbl-70Z were unable to grow in the presence of PP-1 or PP-2 (Figure 36A). Finally, the effect of the Src inhibitors on the clonogenic growth mediated by Cbl mutants was investigated. Importantly, the presence of Src inhibitor PP-2 significantly inhibited the clonogenic growth in the presence of Cbl mutants and Kit or Flt3 receptor (Figure 36B).

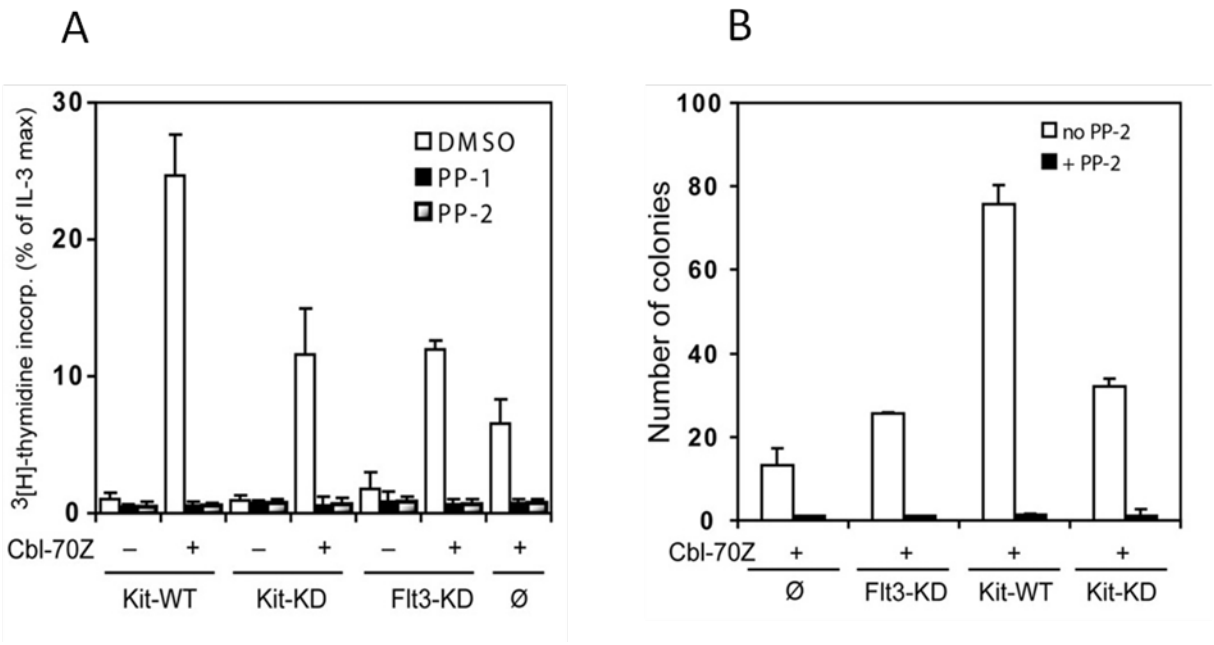

Figure 36. Cbl-70Z–mediated ligand-independent DNA synthesis is blocked by Src inhibitors. (A) 32D cells stably expressing Kit, Flt3 and/or Cbl-70Z were starved from IL-3 for 12 hours, treated with the indicated cytokines and PP-1/PP-2 (15 µM) or DMSO for 6 hours, and incubated with ^{3}[H]-thymidine. After 15 hours of incubation, proliferation was measured in ^{3}[H]-thymidine incorporation assays. Data are shown as percentage of thymidine incorporation relative to the thymidine incorporation of the respective cell line with IL-3 supplementation. The data represent the average and SD of 3 independent experiments. (B) The 32D cells stably overexpressing Kit or Flt3 constructs and/or Cbl-70Z were serum starved for 12 hours and then plated at a concentration of 1000 cells per dish in the presence or absence of PP-2 (15 µM) and in the absence of any growth factors. Colonies were counted on day 6. The assays were plated as triplicates. The numbers given show the results of 1 of at least 3 independent experiments per construct, which all gave similar results.

4.10.6 Src inhibitors inhibit Akt pathway

After determining a prominent role of Fyn, we hypothesized that Fyn might be activating the PI-3-Kinase/Akt pathway either directly or by phosphorylating Cbl at tyrosine 731, since tyrosine 731 is a PI-3-Kinase binding site. Surprisingly, dasatinib and PP-2 completely inhibited Akt activation and Cbl phosphorylation at 731 (Figure 37).

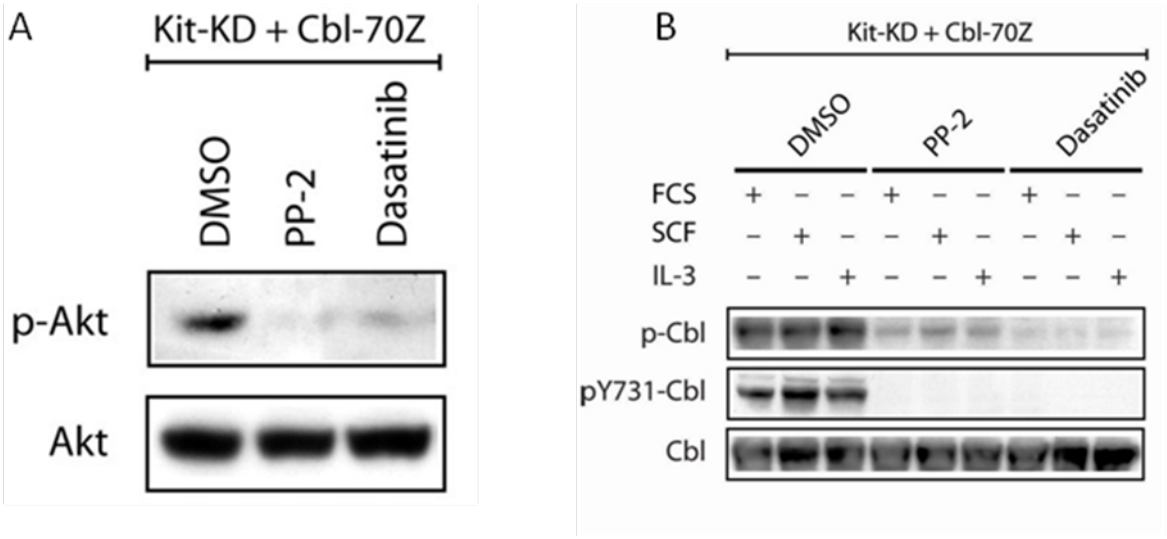

Figure 37. Src inhibitors inhibit phosphorylation of Akt. (A) The 32D-Kit-KD cells stably overexpressing HA-tagged Cbl-70Z were starved from IL-3 in the presence of DMSO or PP-2 (15 µM) or dasatinib (150 nM) for 12 hours. Western blot analyses were performed with anti–phospho-Akt and anti-Akt antibodies. (B), 32D cells stably expressing the indicated constructs were starved in 10% FCS in the presence of PP-1 (only panel B), PP-2, Dasatinib or DMSO as control. Cell lysates were prepared as described, resolved on SDS-PAGE, and membranes were blotted for phospho-SFK and β-Actin (panel B) or for pan phospho-Cbl, phospho-Y731-Cbl, and total Cbl.

4.10.7 Kinase activity is required, but not essential

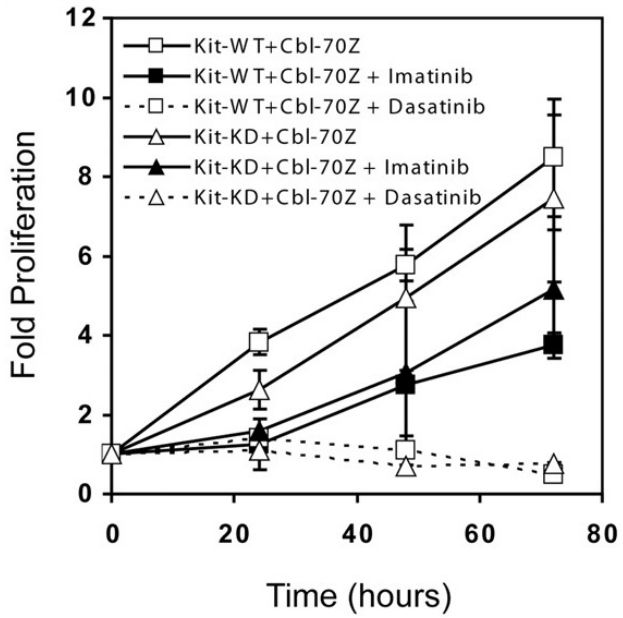

Figure 38. Imatinib and dasatinib differently inhibit cell proliferation. 32D cells stably overexpressing Kit WT or Kit-KD along with Cbl-70Z were starved from IL-3, and cells were grown in the presence of 10% FCS or along with Kit inhibitor imatinib (2.5 µM) or dual kinase inhibitor (Kit and SFKs) dasatinib (150 nM). Cells were counted at the indicated time points by trypan blue exclusion method, and the data are shown as fold change of the cell number compared with the start of the experiment. The data represent the average and SD of 3 independent experiments.

Furthermore, imatinib (as inhibitor of Kit) and dasatinib (as an inhibitor of both Kit and SFKs) was used (Ramirez and DiPersio, 2008) to demonstrate that the growth and

survival mediated by Cbl mutants is SFK dependent. As shown in Figure 38, dasatinib strongly inhibited the proliferation of Kit-WT (or Kit-KD) cells overexpressing Cbl-70Z, whereas imatinib reduced proliferation to a much lesser extent, most probably because imatinib does not inhibit SFKs (Figure 38).

5 Discussion

Receptor tyrosine kinases (RTKs) play a pivotal role in embryonic development and in hematopoiesis. Extracellular binding of a ligand to its respective RTK induces dimerization followed by intracellular signaling (Schlessinger, 1988). The amplitude and duration of RTK signaling is tightly controlled; termination of RTK signaling occurs by receptor ubiquitination, internalization and degradation (Ceresa and Schmid, 2000;Haglund et al, 2005;Le and Wrana, 2005). Aberrant activation of RTKs can be induced by inappropriate ligand stimulation or by overexpression due to genetic amplification or by activating or sensitizing mutations in the coding region of the RTKs (Basson et al, 2005;Peschard et al, 2001b;Peschard and Park, 2003;Zheng et al, 2004). In addition, inappropriate activation of RTKs by loss of negative regulators is increasingly recognized as a possible alternative mechanism to lead to aberrant RTK activity in cancer cells (Basson et al, 2005;Peschard et al, 2001b;Peschard and Park, 2003;Zheng et al, 2004). The RTKs Flt3 and Kit play an important role in leukemogenesis (Blume-Jensen and Hunter, 2001b;Brandts et al, 2007b;Stirewalt and Radich, 2003). Approximately 25% of all AML patients carry mutations in Flt3, whereas Kit mutations are found less frequently. Kit mutations have been preferentially associated with the core-binding factor leukemias (Beghini et al, 2000b). Activating mutations of Flt3 (such as Flt3-ITD and activating point mutations in the tyrosine kinase domain, Flt3-TKD) lead to aberrant signal transduction. ITD mutations lead to constitutive receptor dimerization and activation of receptor as well as the activation of the STAT5, RAS/MAPK and PI3K pathway. These mutations have spurred considerable interest in the development of specific tyrosine kinase inhibitors for these RTKs (Beghini et al, 2000a;Brandts et al, 2007b;Paschka et al, 2006b).

Cbl mutants confer factor-independent growth in association with Kit

Our group was the first to report a Cbl oncogenic mutation in a single case of 150 *de novo* AML patients (Sargin et al, 2007). The identified point mutation Cbl-R420Q has also been found by others (Grand et al, 2009;Sanada et al, 2009). Our group have previously analyzed the role of this Cbl mutant in Flt3 signaling (Sargin et al, 2007). The Cbl mutant Cbl-70Z was identified in a mouse B-cell lymphoma. Cbl-R420Q and Cbl-70Z were able to confer IL-3-independent growth to the 32D cell line in the presence of wild-type Flt3 receptor (Sargin et al, 2007). Several more additional

mutations were subsequently found in Cbl and Cbl-b (Abbas et al, 2008;Dunbar et al, 2008;Grand et al, 2009;Loh et al, 2009;Makishima et al, 2009;Sanada et al, 2009;Sargin et al, 2007) suggesting that Cbl mutations are recurring events in AML. Cbl mutations probably occur in other hematologic and solid tumors as well.

Cbl has been shown to degrade several RTKs, including the EGFR and PDGFR (Schmidt and Dikic, 2005;Thien and Langdon, 2001;Thien and Langdon, 2005a;Thien and Langdon, 2005b). Recently, it was showed that Flt3 is degraded in a Cbl-dependent manner (Sargin et al, 2007). Hematopoietic stem cells (HSC) and its progenitor cells express Kit. In this study, it was studied, if Cbl mutants alter the Kit signaling. To address this question, a myeloid cell line 32D was used, to study the signaling events of Kit in the presence or absence of Cbl mutants. 32D cells are an immortalized myeloblast like cell line originally derived from long-term cultures of murine bone marrow supplemented with WEHI-3B conditioned medium as an IL-3 source. Removal of IL-3 arrests 32D cells in the G0/G1 phase of the cell cycle and prolonged removal leads to cell death. As shown here, the expression of Kit in myeloid 32D cells does not lead to cytokine-independent growth. In the present study, over-expression of Cbl mutants in the 32D cell line does not lead to factor-independent growth. As previously reported, Cbl-70Z expression (alone) enhanced cell survival, but does not lead to factor-independent growth (Hamilton et al, 2001). It has been reported that dominant-negative Cbl mutants enhance the survival of myeloid cells (Hamilton et al, 2001). However, as in this report, they alone did not support cellular proliferation (Hamilton et al, 2001). However, in the presence of Kit, Cbl-R420Q or Cbl-70Z expression promotes cytokine-independent growth. This effect can be enhanced after SCF stimulation. Similarly, coexpression of Kit with either Cbl-R420Q or Cbl-70Z promotes survival and proliferation. In addition to trypan blue assays, thymidine incorporation assays also show that Cbl mutants synergize with Kit in order to give factor-independent to the myeloid cell line. Our results indicate that Cbl-70Z is stronger than Cbl-R420Q towards the transformation of myeloid cell line. We observed similar effects with Flt3, (Sargin et al, 2007) suggesting that this may be a general mechanism in most RTKs. It is of future interest to determine these types of synergistic events in other cancer types.

Colony formation assays, which involve growing of cells in a semi solid media, are generally used to study the transformation property of cells. As shown here, co-expression of Kit and either of the Cbl mutants leds to colony growth in the absence

of growth factors. Cbl-70Z expression alone, but not Cbl-R420Q formed few and small colonies. This indicates that Kit and Cbl mutant expression are essential for complete transformation. The data presented here provides evidence that Cbl-mediated regulation of Kit is biologically important. In summary, coexpression of Cbl-70Z or Cbl-R420Q with Kit readily transformed 32D cells, even in the absence of Kit ligand. Many of the AML cases overexpress Kit or Flt3 without harboring any mutations (Blume-Jensen and Hunter, 2001b). Our results may explain the possibility of these types of synergistic events in AML patients expressing Flt3 WT and Kit WT.

Cbl mutations are rare, but recurring events in hematological disease

Recently, missense mutations in exon 8 were reported in three out 12 AML cases (Caligiuri et al, 2007a). Other studies have also shown Cbl exon 8 splice variant mutations; these mutations were associated with CBF leukemias (Abbas et al, 2008;Reindl et al, 2009). In addition, several reports have shown somatic Cbl mutations in the critical linker and ring finger domains (exons 8 and 9) (Makishima et al, 2009;Sanada et al, 2009). These are frequent in myeloid malignancies, particularly (but not exclusively) in myelodysplasia/myeloproliferative neoplasms (MDS/MPN) with acquired uniparental disomy (aUPD) at chromosome 11q (Dunbar et al, 2008;Grand et al, 2009;Loh et al, 2009). It is not yet clear how frequently Cbl mutations occur in AML, but probably less than 2% of cases. No Cbl mutations were observed in mature lymphoid malignancies.

Cbl mutants induce a generalized mastocytosis and a myeloproliferative disease in a murine bone marrow transplantation model

So far, the role of Cbl mutations in mouse models had not been determined. In *in vivo* study, the role of Cbl mutants in a bone marrow transplantation model was addressed. Two distinct diseases such as a generalized mastocytosis and a myeloproliferative disease with high penetrance and long latency were observed. In addition, two mice developed a myeloid leukemia with high leukocyte counts, splenomegaly, and organ infiltration by myeloid immature cells. Six mice developed mast cell sarcomas. Mastocytosis is defined as heterogeneous disorder, characterized by the expansion and accumulation of mature mast cells (MC) in a different organs and tissues, such as skin, gastrointestinal tract, liver, spleen, bone

marrow and lymphoid organs (Edling and Hallberg, 2007;Kitamura and Hirotab, 2004;Lennartsson et al, 2005).

Mast cells were identified in spleen, liver, bone marrow, lung, kidney, heart, indicating that it is generalized mastocytosis. Activating mutations of Kit have been identified in mastocytosis patients. Mostly, these mutations (D816V, D816Y and D820G) were found in the kinase domain of Kit (Orfao et al, 2007). Some JMD mutations (at codons 509, 522 and 533) were also found in mastocytosis patients. Kit mutation D816V has been first reported in the mastocytosis cell line HMC-1 (Furitsu et al, 1993). A tantalizing explanation for the increased mast cell proliferation could be the typical expression of Kit in these cells. Future studies are warranted to test if Cbl mutations occur in mastocytosis patients with or without Kit mutations. It is also possible, that Cbl mutations synergize with Kit mutations more strongly when compared to normal Kit WT.

Mast cell activation plays a significant role in allergy. IgE receptor aggregation on mast cells releases inflammatory mediators by degranulation leading to the production of the multiple cytokines and leukotrienes which cause allergic symptoms. Furthermore, upon activation of the IgE receptor the SFK Lyn is activated, which in turn phosphorylates the IgE receptor (Kyo et al, 2003). Over-expression of Cbl (membrane-anchored form of Cbl) inhibits the IgE-receptor-mediated degranulation and cytokine production by the degradation of Lyn through enhanced ubiquitination (Kyo et al, 2003). Here, Cbl mutants might have interfered WT Cbl function and finally led to the mastocytosis. An inhibitor of the mast cell activation might have therapeutic potential. Cbl may be the candidate molecule for developing novel anti-allergic drugs. In conclusion, our results indicate that Cbl mutations might contribute to the pathogenesis of mastocytosis and related diseases.

The long latency observed in mouse model is very suggestive of secondary events, such mutations and epigenetic changes. Different secondary events may have occured in different animals, at different times with different rates, explaining the variability of disease onset and death. Further evidence for the requirement of cooperating genetic (or epigenetic) events comes from published mouse studies in which either complete Cbl knockout or heterozygous knock in of the RING finger inactivating mutant C379A (mouse C379 is equivalent to human C381) did not show any evidence of an MPN or other malignancy (Thien et al, 2005). Here, postmortem analysis of Cbl expression was not performed, as endogenous Cbl is widely

expressed. As the Cbl mutants affect the stability of several intracellular and surface molecules, including Flt3 and Kit in these *in vivo* experiments, we were unable to pinpoint the contribution of the Cbl targets to the disease phenotype. Although unproven, the similarity of Cbl-70Z and Cbl-R420Q in disease development suggests a similar mechanism for both mutants. A recent report showed that hematopoietic stem cells from $Cbl^{-/-}$ mice have an increased pool size, are hyperproliferative, and show enhanced long-term repopulating capacity (Rathinam et al, 2008). It is tempting to speculate that Cbl-inactivating mutations may promote similar function in leukemia-initiating cells. Importantly, $Cbl^{-/-}$ hematopoietic stem cells expanded more in the presence of SCF and thrombopoietin, which is explained by the fact that Cbl is the negative regulator of Kit and the thrombopoietin receptor (cMpl) and supports the validity of increased SCF responsiveness we observed in cells coexpressing Kit and Cbl mutants. NUP98-HOXD13 (NHD13) fusion gene plays an important role in myelodysplastic syndrome and acute nonlymphocytic leukemia. Recently, an acquired Cbl mutation was shown in a Nup98-HoxD13 mouse model, which strongly supports the prominent role of Cbl mutations *in vivo* (Slape et al, 2008). Another report (Sanada et al, 2009) shows that Cbl can act as tumorsuppressor. In that report, $Cbl^{-/-}$ hematopoietic stem/progenitor cells (HSPCs) show enhanced sensitivity towards different kinds of cytokines, compared to $Cbl^{+/+}$ HSPCs, and further transduction of gain-of-function Cbl mutants into $Cbl^{-/-}$ HSPCs augmented their sensitivities to a broader spectrum of cytokines, including SCF, TPO, IL3 and FL, indicating that function-gain-mutations in Cbl perform drastically in the absence of Cbl WT. Acquired uniparental disomy (aUPD) is frequently observed in cancer genomes and leads to loss of heterozygosity. aUPD is associated with loss-of-function mutations of tumor suppressor genes (Knudson, 2001) and also with gain-of-function mutations of proto-oncogenes (James et al, 2005). aUPD, which is more common in myeloproliferative neoplasms (MPN), generally associated with the following chromosomes, 1p, 1q, 4q, 7q, 11p, 11q, 14q, 17p and 21q. Cbl mutations were identified in most of the 11q aUPD cases (MPN) (Sanada et al, 2009). Out of these Cbl mutations, most of them were found to be homozygous, indicating the loss of Cbl WT and gain-of-function of the Cbl mutants. Furthermore our experiments, including bone marrow transplantation experiments, were carried out in the presence of endogenous Cbl WT. Cbl mutants (Cbl-R420Q and Cbl-70Z), would have shown more drastic effects (reduced latency), if the experiments were carried out in the

absence of Cbl WT. Therefore, it is interesting to conduct the experiments in the absence of endogenous Cbl.

The *in vivo* data presented in this report is the first evidence that a Cbl mutant, identified from human malignancy, is involved in disease pathogenesis. The minor differences observed between Cbl-R420Q and Cbl-70Z are within the variability of animal experimentation.

Cbl mutants abolished the ubiquitination and endocytosis of the Kit receptor

It is well established that an intact RING domain is required for the E3 ligase activity of Cbl (Swaminathan and Tsygankov, 2006;Waterman et al, 1999). Several ubiquitin ligases, including Nedd4, have been involved in the ubiquitination of RTKs in mammalian cells (Dikic and Giordano, 2003). However, it is generally believed that the Cbl family ubiquitin ligases play most prominent role in mediating ligand activated RTKs. RTKs, such as EGFR, ErbB2, PDGF, colony-stimulating factor 1 receptor and hepatocyte growth factor receptor undergo ubiquitination upon interaction with Cbl (Dikic and Giordano, 2003). Generally, Cbl binds to RTKs (EGFR) at the plasma membrane and is continuously associated in a complex throughout the endocytic route. Furthermore, Cbl is also recruited from the cytoplasmic pool into the endosome, for the continuous ubiquitination and subsequent degradation of RTKs in the lysosomes.

Cbl-70Z and Cbl-R420Q have a disrupted RING domain and are E3 ligase defective (Sargin et al, 2007). In our observations, Cbl-R420Q and Cbl-70Z prevented ubiquitination and endocytosis of Kit. Cbl-R420Q has suppressed the ubiquitination more effectively than Cbl-70Z. Termination of RTK signaling is a complex multistep process, which requires several sorting, segregation, and degradation steps, each of which involve many different proteins (Marmor and Yarden, 2004;Mousavi et al, 2004;Seto et al, 2002). It may well be that other proteins involved in these processes may be mutated more frequently in AML and possibly in other human cancers. This notion, at least in part, is also supported by data showing that proteins involved in the endocytosis machinery are often constituents of oncogenic fusion proteins (Crosetto et al, 2005;Magnusson et al, 2001). Furthermore, inappropriate expression of proteins involved in endocytic sorting (i.e., Hrs and Tsg101) has also been implicated in human cancers. The potent effects of the point mutation of Cbl on myeloid cell proliferation and survival warrant further search for such mutations in clonal myeloid

disorders, especially in patients without known activating mutations in signaling intermediates.

Cbl mutants constitutively activate Akt and MAPK pathways

Originally, Cbl was described as a proto-oncogene of an animal virus that induced lymphoma in mice (Langdon et al, 1989c). It has been shown that the corresponding viral oncogene, v-Cbl, is a relatively weak oncogene (Thien and Langdon, 2001). v-Cbl is a truncated protein, consisting of the N-terminal half of Cbl, retaining not much more than the PTB domain of Cbl. Subsequently, the description of other, more potent Cbl oncogenes led to the definition of requirements for the transforming potential of Cbl proteins (Peschard et al, 2001a;Thien and Langdon, 2001). Presence of the PTB domain and simultaneous interference with the E3 ligase function of Cbl has been defined to be essential minimal requirements for Cbl-mediated transformation. Most of these mutants are thought to act in a dominant-negative manner by competing with the wild-type receptor. Our biochemical analyses here show that Cbl mutants fulfill these requirements. We were interested to further clarify the mechanisms of transformation by Cbl mutants and performed a careful analysis of the signaling properties conferred by Kit in the presence or absence of Cbl mutants. These experiments revealed enhanced basal RTK autophosphorylation and prolonged ligand-activated MAPK and PI3K/Akt signaling in the cells expressing Cbl mutants and Kit, which is in line with published data for EGFR (Thien and Langdon, 1997) PDGFR (Bonita et al, 1997b) and Flt3 (Sargin et al, 2007). However, we also observed basal activation of Akt and Erk, which contrasts with data for Flt3, where no differences in Akt basal signaling were identified (Sargin et al, 2007). The reasons for this difference are unknown but may be the result of differences in Kit and Flt3 expression. In any case, as Akt activation has been shown to cause cytokine-independent colony growth (Brandts et al, 2005), this may partly explain why coexpression of Cbl mutants with Kit (in the absence of ligand) led to colony growth.

Cbl-70Z associates with Kit constitutively

Several reports have shown that Cbl and RTKs can interact either directly or indirectly through adaptor proteins such as Grb2, APS or the p85 subunit of PI3K. Cbl binds to Kit directly and indirectly via Grb2 (Masson et al, 2006). Cbl has been shown to interact with Kit in a direct manner at position Tyr^{568} and Tyr^{936}, and that this

interaction is dependent on a leucine or isoleucine residue in position +3. Cbl is known to be phosphorylated by Src family kinases (SFKs) and mediates ubiquitination of the Kit receptor on activation (Masson et al, 2006). In this study, the binding of Cbl mutants to the Kit protein was addressed. Here, our coimmunoprecipitation experiments showed that Cbl mutants are part of a complex with Kit after ligand stimulation. Importantly, Cbl-70Z (and to a lesser extent Cbl-R420Q) coimmunoprecipitated with Kit in the absence of ligand. This suggests a constitutive complex formation between Cbl mutants and Kit, which was similarly observed for EGFR and Flt3 (Sargin et al, 2007;Thien and Langdon, 1997).

AML blasts express Kit significantly higher compared with normal bone marrow (Scolnik et al, 2002) and about one-third of AML blasts co-express Kit and SCF (Pietsch, 1993) indicating paracrine and autocrine growth mechanisms of human stem cell factor in myeloid leukemia. Here, Cbl mutants have enhanced SCF dependent growth and signaling. It is tempting to speculate that AML patients harboring Kit expression and Cbl mutations might have worse prognosis.

The data reveal functional differences between Cbl-70Z and Cbl-R420Q. The loss of E3 ligase activity is more pronounced in cells expressing Cbl-R420Q than in those expressing Cbl-70Z, whereas the consequences of expression of either Cbl mutant appear to be very similar in both the *in vitro* and *in vivo* experiments with primary murine bone marrow. However, the biologic consequences of Cbl-70Z are more dramatic in cell culture experiments performed in 32D cells. Further studies are necessary to decipher these differences between Cbl mutants, including the novel mutations that have been described since our initial observation and have not yet been molecularly characterized (Abbas et al, 2008;Caligiuri et al, 2007b;Dunbar et al, 2008;Grand et al, 2009;Makishima et al, 2009;Reindl et al, 2009;Sanada et al, 2009). However, it is possible that conformational changes or differences in sub cellular localization are involved.

Kinase activity of Kit is not required for Cbl-70Z mediated transformation

It was of interest, in defining the kinase requirements of the Cbl mutants/RTK interaction. Importantly, the data revealed that the biologic functions mediated by Kit and Cbl-70Z were in large part dependent on the receptor kinase activity. Biologically dominant effect also observed in the presence of kinase-inactive Kit and Flt3 cells expressing Cbl mutants. Although it was not sufficient for cytokine-independent

growth that the cells expressed Cbl-70Z, KD Kit, and Flt3 synergized with Cbl mutants. It could be speculated that Kit or Flt3, which can be found to be in complex with the Cbl proteins, may act as a necessary scaffold. This is in line with a recent report that survival of cancer cells was maintained by EGFR independently of its kinase activity (Weihua et al, 2008). The yet unimpressive clinical outcomes of tyrosine kinase inhibitors for treatment of AML may be indicative that kinase-independent functions of RTKs may contribute to transformation.

The potential signaling molecules that were assumed to be responsible for the Cbl-70Z mediated transformation in the presence of Kit-KD were anlysed. Cbl-70Z has conferred autophosphorylation to the Kit WT receptor in the absence of SCF, in contrast to Ki-KD.

Akt activation mediates the Cbl-70Z transformation

It seems that kinase activity of the Kit is required, in order to undergo ubiquitination, since Kit-KD was not ubiquitinated in response to SCF. Finally, it was noticed that Akt activation is the driving force, for the proliferation of Kit kinase dead cells in the presence of Cbl-70Z. Generally, growth factor receptors upon activation activate PI-3-kinase, which leads to accumulation of phosphatidylinositol trisphosphate at the cell membrane and binding of the serine-threonine kinase Akt via its pleckstrin homology domain. At the cell membrane, Akt undergoes a conformational change and is phosphorylated on Thr^{308} by PDK1 and Ser^{478} by an unknown kinase, leading to Akt activation (Vivanco and Sawyers, 2002). Akt activation has been shown to mediate survival and proliferation (Vivanco and Sawyers, 2002). Here, it was showed that Akt inhibition by its inhibitor abolishes transformation mediated by Cbl-70Z and Kit-KD. In addition, SFK's inhibitors (PP-2 and dasatinib), inhibited the Akt activation. It indicates that SFKs are acting upstream to PI-3-Kinase/ Akt pathway as previously reported (Karnitz et al, 1994;Pleiman et al, 1994). Our data also shows that SFKs can activate Cbl by phosphorylating the tyrosine residue at 731, which is a PI-3-kinase binding site. In summary, SFKs can activate Akt either directly or by phosphorylating Cbl at tyrosine 731 (Thien and Langdon, 2001;Hunter et al, 1999b). Complete inhibition of proliferation and colonogenic growth of the cells expressing kinase dead receptors and Cbl-70Z has occurred in the presence of SFKs inhibitors (PP-1, PP-2 and dasatinib).

Role of Src family kinases (SFKs) in Cbl-70Z mediated transformation

Cbl has been shown to degrade SFKs by ubiquitination (Kyo et al, 2003;Yokouchi et al, 2001). Increased SFK activity in the presence of Cbl mutants may be explained by lack of their degradation. This needs to be addressed in the future. In addition, we found, that Cbl-R420Q and Cbl-70Z in complex with Kit-KD and SFKs. Here Kit-KD might be playing scaffold role in assembling other potential molecules, besides to SFKs and Cbl-70Z. Previously, Fyn has been shown to bind (Hunter et al, 1999b) and phosphorylate Cbl, which leads to the activation of PI-3-Kinase. Our data shows that Fyn can bind Cbl. However, its role is yet to be confirmed in the phosphorylation of Cbl.

Kit inhibition by imatinib reduced the proliferation of cells over expressing Kit WT and Cbl-70Z stronger compared with cells expressing Kit-KD and Cbl-70Z, but much lesser than dasatinib, which inhibits both Kit and SFKs. This indicates that Kit kinase activity is required but not essential. These findings are extending and complementing our previous findings (Sargin et al, 2007). SFKs therefore are crucial components of the complex formed by kinase dead RTKs and Cbl mutants, and their inhibition prevents transformation. Generally, it is believed that the kinase activity of BCR-ABL is responsible for activation of all its downstream signaling pathways. Therefore, BCR-ABL kinase activity inhibitors should completely inhibit BCR-ABL functions and cure the disease. However, reports have shown that Src kinases remain active following imatinib inhibition of BCR-ABL kinase activity in leukemic cells (Hu et al, 2006). These results explain the importance of signaling events that come from the kinase-dead receptors and the role of SFKs. Other mechanisms of getting resistance to the tyrosine kinase inhibitors are acquiring secondary mutations in the kinase domain of the receptors. Secondary mutations were noticed in the kinase domain of Kit or EGFR in 50% of the patients with GIST or non-small cell lung cancer that have acquired resistance to imatinib or gefitinib/erlotinib respectively (Kobayashi et al, 2005;Tamborini et al, 2004). Secondary mutations in the kinase domain are called gatekeeper mutations. These gate keeper mutations prevent the binding of receptor to the kinase inhibitor (by preventing the hydrogen bond formation between inhibitor and receptor (Daub et al, 2004). Another mechanism that contributes the resistance to kinase inhibitors is a “kinase switch”. This mechanism involves activation of another kinase that is other than primarily targeted. Different

studies have shown activation of PDGFR (due to secondary mutations) in GIST patients, where primarily Kit was targeted (Debiec-Rychter et al, 2005).

Therefore, RTKs may not be the ideal target kinases for tyrosine kinase inhibitors in a Cbl mutation–driven disease. Instead, SFKs may be interesting target kinases, for which inhibitors are readily available. Dasatinib, for example, is a multitargeted kinase inhibitor of BCR-ABL, SFKs, Kit, PDGFR, and ephrin A receptor kinases (Lombardo et al, 2004;Nam et al, 2005;Schittenhelm et al, 2006) . It also emerged as a potent inhibitor of imatinib-resistant protein tyrosine kinase activation loop mutants of Kit, and it is able to induce apoptosis in mast cell and leukemic cell lines expressing these mutations (Shah et al, 2006) .

The data presented here demonstrate that SFKs play a critical role in the mechanism of transformation of Cbl mutants. AML blasts expressing Cbl mutants (or dysregulated Cbl function by other mechanisms) might be resistant to tyrosine kinase inhibitors targeted for Flt3 or Kit. Although direct Cbl mutations may be rare in AML, similar mechanisms in the RTK degradation machinery may be operational in a large proportion of AMLs. Further studies are required to determine whether kinase inhibitors targeted at both RTKs and SFKs may overcome this primary resistance to RTK inhibitors.

6 Zusammenfassung

Die akute myeloische Leukämie (AML) ist eine Erkrankung hämatopoetischer Zellen, die durch einen Differenzierungsblock und die unkontrollierte Bildung unreifer hämatopoetischer Zellen oder Blasten charakterisiert ist. Dabei kommt es zu einer starken Einschränkung der normalen Hämatopoese, was letztlich ohne Behandlung zum Tod des Patienten führt. Die Entstehung einer AML wird grundsätzlich als Entwicklung angesehen, die in mehreren Schritten vonstatten geht. Genetische Veränderungen, wie Mutationen, Translokationen, Amplifikationen und Deletionen sind an der Pathogenese der AML beteiligt. Diese genetischen Veränderungen können in zwei Gruppen eingeteilt werden: solche die zu einem Proliferations- oder Überlebensvorteil führen (Klasse I Mutationen) und andere, die einen Differenzierungsblock hervorrufen (Klasse II Mutationen). Eine einzelne Mutation dieser Art ist nicht in der Lage alleine einer AML auszulösen. Während Kit-Mutationen relativ selten sind, wird bei der Mehrzahl der AML-Patienten eine Überexpression des Kit Rezeptors gefunden. Der Kit-Rezeptor wird durch seinen Liganden „Stem Cell Factor“ (SCF) aktiviert. Die Aktivierung von Kit führt zur Rekrutierung von Signalmolekülen. Sog. „Gain-of-function“-Mutationen von Kit führen zu einer konstitutiven Aktivierung des Rezeptors. Cbl-Proteine degradieren bekanntermaßen aktivierte Rezeptortyrosinkinasen (RTKs) durch Ubiquitinierung. Die Untersuchung der Signaltransduktion von RTKs wie Kit oder Flt3 und deren Degradation, ist für die Entwicklung neuer therapeutischer Strategien zu von enormer Wichtigkeit. Gegenstand der vorliegenden Untersuchung ist der Einfluss von Cbl-Mutanten (Cbl-R420Q und Cbl-70Z) auf die Kit–vermittelte Signaltransduktion. Cbl-R420Z wurde bei einem AML-Patienten gefunden, während Cbl-70Z in Maus-Myelom-Studien identifiziert wurde. Diese Cbl Mutanten haben die E3-Ligase Aktivität, die eine wichtige Rolle beim Transfer der Ubiquitin Moleküle zum Zielprotein spielt, verloren. Aus der vorliegenden Studie können folgende Schlussfolgerungen gezogen werden.

Schlussfolgerung 1: Cbl Mutationen vermitteln Faktor-unabhängiges Wachstum in Kooperation mit Kit.

Um die Funktion der Cbl Mutanten zu untersuchen, führten wir Proliferationsassays durch. Wir konnten zeigen, dass Cbl Mutanten (Cbl-R420, Cbl-R70Z) in Kooperation mit Wildtyp-Kit in der Lage sind, eine myeloische Zellinie (32D) zu transformieren.

Ebenso führen die Cbl-Mutanten zusammen mit Kit zu einem verstärkten Überleben von 32D Zellen. Die Expression der Cbl Mutanten alleine führte in diesen Zellen zu einem leicht erhöhten Überlebensvorteil, ohne jedoch eine Faktor-unabhängige Proliferation zu vermitteln. Ferner wurde in dieser Zelllinie das SCF-Abhängige Wachstum durch die Cbl-Mutanten verstärkt. Außerdem vermittelten die Cbl Mutationen klonales Wachstum in Kooperation mit Kit. Die akute myeloische Leukämie entsteht nicht aufgrund einer einzelnen Mutation, sondern aufgrund mehrerer kooperiernder Ereignisse oder Mutationen. Unsere Ergebnisse zeigen, dass überexprimierte RTKs mit mutierten untergeordneten Signalmolekülen in der Transformation myeloischer Zellen kooperieren.

Schlussfolgerung 2: Cbl Mutanten verhindern die Ubiquitinierung und Endozytose des Kit Rezeptors.

Es ist bekannt, dass Cbl-Proteine negative Regulatoren von RTKs einschließlich Kit und Flt3 sind. Funktionell inaktivierende Mutanten von Cbl (RING oder linker domain) setzen die E3-Ligase Aktivität von Cbl außer Kraft. In der vorliegenden Arbeit konnte gezeigt werden, dass Cbl Mutanten die Ubiquitinierung des aktivierten Kit-Rezeptors hemmen können. Die Ubiquitinierung von RTKs führt oft zur Internalisierung des Rezeptors. Es konnte gezeigt werden, dass Cbl-Mutanten die Endozytose Kit Rezeptors hemmt. Cbl-R420Q hemmte die Ubiquitinierung und Endozytose effektiver als Cbl-70Z.

Schlussfolgerung 3: Cbl Mutanten führen zu einer generalisierten Mastozytose und einer myeloproliferativen Erkrankung im Knochenmark.

Die Cbl-R420Q-Mutante wurde in einem AML-Patienten gefunden. Deshalb wurde untersucht, ob diese Cbl-Mutante eine hämtologische Erkrankung auslösen kann. Die retrovirale Überexpression der Cbl-Mutanten in murinem Knochenmark führte nach Transplantation zu einer generalisierten Mastozytose, einer myeloproliferativen Erkrankung sowie in seltenen Fällen einer myeloische Leukämie. Ferner kam es durch die Cbl-Mutanten zu einer Splenomegalie. Außerdem wurde in allen Organen der mit den Cbl Mutanten transplantieren Mäusen (Milz, Leber, Knochenmark, Lunge, Niere, Herz), eine Mastzellinfiltration unterschiedlichen Ausmaßes gefunden. Fast alle Empfänger von mit Cbl-Mutanten transduzierten Knochenmarkzellen entwickelten eine tödliche hämatologische Erkrankung, mit einer mittleren Latenzzeit

von 341 Tagen in der Cbl-R420Q Gruppe und 395 Tagen in der Cbl-70Z Gruppe. Die lange Latenzzeit könnte durch die Notwendigkeit des Erwerbs weiterer Mutationen für die Ausbildung des Phänotyps bedingt sein. Nach unserem Kenntnisstand ist dieses der erste Bericht, eines in-vivo Modells zur Untersuchung von Cbl Mutationen in der malignen Transformation.

Schlussfolgerung 4: Cbl-70Z bindet konstitutiv an Kit.

Für die Funktion der Cbl-Mutanten ist eventuell eine direkte Bindung an Kit notwendig. Unsere Ko-Immunopräzipitationsstudien zeigten, dass Cbl-70Z an Kit, selbst in Abwesenheit des Kit-Liganden (SCF) bindet. Cbl-R420Q zeigt ebenfalls eine schwache Bindung an Kit in Abwesenheit von SCF. Die Stimulation mit SCF führte zu einer verstärkten Bindung der Cbl-Mutanten an Kit.

Schlussfolgerung 5: Cbl Mutanten führen zu einer konstitutiven Aktivierung der Akt- und Erk-Signalwege.

Die Überexpression von Kit WT mit den Cbl-Mutanten führt zu einer Faktor-unabhängigen Proliferation. Dies deutet darauf hin, dass die Expression von Cbl-Mutanten zu einer Deregulation Wachstum-kontrollierender Signaltransduktionswege führt. Unsere Untersuchungen zeigten eine konstitutive Aktivierung des Akt- und Erk-Signalwegs in Gegenwart der Cbl-Mutanten und Kit. Diese Signalwege wurden in 32D-Zellen durch die Cbl-Mutatanten alleine in Abwesenheit von IL3 nicht aktiviert.

Schlussfolgerung 6: Cbl Mutanten verlängern SCF-Abhängige Signale.

Die Art und Stärke der Aktivierung von RTKs wird durch deren Dergadation genau kontrolliert. Veränderungen oder Beeinträchtigungen der RTK-Degradation wirken sich auf Qualität und Stärke des RTK-Signals aus. In Unseren Untersuchungen verstärkten die Cbl-Mutatuinen das SCF-abhängige Wachstum. Ferner wurde die SCF-abhängige Aktivierung von Kit, Akt und Erk durch die Cbl-Mutationen verlängert.

Schlussfolgerung 7: Kit ist in der Gegenwart von Cbl-70Z autophosphoryliert.

In unseren Untersuchen führte die Expression von Cbl-70Z, nicht jedoch die von Cbl-R420Q zu einer SCF-unabhängigne Autophosphorylierung von Kit. Diese Ergebnisse deuten auf funktionelle Unterschiede zwischen den beiden Cbl-Mutanten hin.

Schlussfolgerung 8: Für die Cbl-70Z vermittelte Transformation ist keine Kinase-Aktivität von Kit erforderlich.

In der vorliegenden Untersuchung wurde analysiert, ob für die durch Kit und die Cbl-Mutanten synergistisch vermittelte Transformation myeloischer Zellen, Kinase-Aktivität des Kit-Rezeptors notwendig ist. Cbl-70Z zusammen mit Kinase-defizientem Kit (Kit-KD) vermittelt in der myeloischen Zelllinie 32D Faktor-unabhängiges Wachstum, während Cbl-R420Q zusammen mit Kit-KD nur einen schwachen Wachstumsvorteil vermittelte. Cbl-70Z vermittelte ferner im Zusammenhang mit Kit-KD ein vermehrtes Faktor-unabhängiges Überleben der Zellen. In diesen Versuchen zeigte eine SCF-Stimulation keine Wirkung auf die Kinase-defizienten Kit-Rezeptoren.

Schlussfolgerung 9: Die Kinase-Aktivität von Flt3 ist für die Cbl-70Z vermittelte Transformation nicht notwendig.

Cbl-70Z führt zusammen mit Kinase-defizientem Flt3 (Flt3-KD) zu Faktor-unabhängigem Wachstum in 32D Zellen. Cbl-R420Q vermittelte zusammen mit Flt3-KD keinen Wachstumsvorteil. Cbl-70Z führte zusammen mit Flt3-KD zu einem verstärkten Faktor-unabhängigen Überleben der Zellen. Ähnlich wie beim Kit-Rezeptor, zeigte Flt3-KD keine Phosphorylierung nach Ligandenstimulation. Zusammen mit den unter Schlussfolgerung 8 dargelegten Ergebnissen zeigen unsere Daten, dass für die Cbl-70Z vermittelte Transformation keine RTK Kinase-Aktivität erforderlich ist.

Schlussfolgerung 10: Die durch Cbl-70Z induzierte Transformation wird durch Akt vermittelt.

Die Tatsache, dass die Cbl-70Z induzierte Transformation keine Kinase-Aktivität der kooperiertenden Rezeptortyrosinkinase erfordert, legt nahe, dass die Transformation in diesen Zellen durch andere Signalwege vermittelt wird. Unsere Ergebnisse deuten darauf hin, dass die Cbl-70Z induzierte Transformation nicht durch Erk- oder STAT-abhängige Signalwerge vermittelt wird. Außerdem führte Cbl-70Z zu keiner Autophosphorylierung der Kinase-defizienten Rezeptoren. Jedoch zeigte sich, dass die Aktivierung von Akt für die Cbl-70Z-induzierte Transformation im Zusammenspiel mit Kit-KD bzw. Flt3-KD verantwortlich ist. Unsere Untersuchungen zeigten ferner, dass die Kinase-Aktivität von Kit für dessen Ubiquitinierung notwendig ist, da keine

Ubiuquitinierung des Kinase-defizienten Rezeptors in Anwesenheit von SCF beobachtet wurde.

Schlussfolgerung 11: Die Rolle von „Src-Family"-Kinasen (SFKs) in der Cbl-70Z vermittelten Transformation.

SFKs spielen bekanntermaßen eine wichtige Rolle bei der Vermittlung intrazellulärer Signale, insbesondere von Zytokinrezeptoren. Diese Rezeptoren haben keine Tyrosin-Kinase Aktivität. Da wir bei der durch die Cbl-Mutanten im Synergismus mit RTKs vermittelten Proliferation eine Tyrosin-Kinase-Unabhängikeit beobachteten, könnten SFKs in der Cbl-70Z vermittelten Transformation eine Rolle spielen. In unseren Untersuchungen führten die Cbl-Mutanten zu einer erhöhten Aktivität von SFKs. Die Hemmung von SFKs resultierten in einer Verminderung von durch Cbl-Mutanten induzierter Proliferation bzw. klonalem Wachstum. Ferner konnten wir eine Bindung von Fyn an Cbl nachweisen. Es konnte weiterhin eine Interaktion zwischen Cbl-70Z, SFKs und Kit-KD gezeigt werden. Außerdem ist die Kinase-Aktivität von SFKs für die Bindung an Cbl notwendig, da diese Bindung durch PP-2, eine SFK-Inhibitor gehemmt werden kann.

Schlussfolgerung 12: Kinase-Aktivität ist nicht notwendig.

Im Zusammenhang mit Krebserkrakungen wird oft eine Deregulation von Rezeptortyrosinkinasen beobachtet und zwischenzeitlich ist eine große Anzahl von Tyrosinkiaseionhibitoren entwickelt worden. In der vorliegenden Studie wurden, zur Untersuchung der Notwendigkeit der Tyrosinkinase-Aktivität für die Cbl-70Z vermittelte Transformation, die Tyrosinkinase-inhibitoren Imatinib und Dasatinib verwendet. Imatinib hemmt Bcr-abl, Kit und den PDGFR, während Dasarinib zusätlich zu den genannten RTKs SFKs hemmt. Imatinib hemmte die Proliferation von Zellen mit Kit WT und Cbl-70Z deutlich stärker als in Zellen mit Kinase-defizientem Kit (Kit-KD) und Cbl-70Z, aber insgesamt in deutlich geringerem Ausmaß als Dasatinib. Dies deutet an, dass die Kit-Aktivität wichtig, aber nicht unabdingbar für die Cbl-70Z induzierte Transformation ist. Dasatinib und PP-2 (ein SFK-Inhibitor), hemmten die Phosphorylierung voin Cbl und Akt, was auf eine Rolle der SFKs „upstream" von Cbl und Akt hinweist. SFKs sind deshalb ein wichtiger Bestandteil des Komplexes aus Kinase-defizienten RTKs und Cbl-Mutanten und ihre Hemmung verhindert die Transformation durch Cbl-Mutanten.

Unsere Untersuchungen zeigen, dass Rezeptortyrosinkinasen nicht in jedem Fall die besten Ansatzpunkte für therapeutische Interventionen bei transformierten myeloischen Zellen sind. Eine konstitutive Aktivierung von Src-Family-Kinases wurde in AML-Zellinien und in frisch isolierten primären Zellen von AML-Patienten beobachtet. Deshalb könnten SFKs attraktive Zielmoleküle zukünftiger Therapien sein, für die es bereits Inhibitoren, wie Dasatinib, gibt. Zusammenfassend zeigt die vorliegende Studie eine wichtige Rolle von Cbl, Kit und SFKs bei der Transformation myeloischer Zellen.

7 References

Abbas S, Rotmans G, Lowenberg B, Valk PJ (2008). Exon 8 splice site mutations in the gene encoding the E3-ligase CBL are associated with core binding factor acute myeloid leukemias. Haematologica 93:1595-1597.

Abram CL, Courtneidge SA (2000). Src family tyrosine kinases and growth factor signaling. Exp Cell Res 254:1-13.

Abu-Duhier FM, Goodeve AC, Wilson GA, Gari MA, Peake IR, Rees DC, Vandenberghe EA, Winship PR, Reilly JT (2000). FLT3 internal tandem duplication mutations in adult acute myeloid leukaemia define a high-risk group. Br J Haematol 111:190-195.

Agnes F, Shamoon B, Dina C, Rosnet O, Birnbaum D, Galibert F (1994). Genomic structure of the downstream part of the human FLT3 gene: exon/intron structure conservation among genes encoding receptor tyrosine kinases (RTK) of subclass III. Gene 145:283-288.

Andoniou CE, Thien CB, Langdon WY (1994a). Tumour induction by activated abl involves tyrosine phosphorylation of the product of the cbl oncogene. EMBO J 13:4515-4523.

Andoniou CE, Thien CB, Langdon WY (1994b). Tumour induction by activated abl involves tyrosine phosphorylation of the product of the cbl oncogene. EMBO J 13:4515-4523.

Andoniou CE, Thien CB, Langdon WY (1996). The two major sites of cbl tyrosine phosphorylation in abl-transformed cells select the crkL SH2 domain. Oncogene 12:1981-1989.

Appelbaum FR, Rowe JM, Radich J, Dick JE (2001). Acute myeloid leukemia. Hematology Am Soc Hematol Educ Program62-86.

Barber DL, Mason JM, Fukazawa T, Reedquist KA, Druker BJ, Band H, D'Andrea AD (1997). Erythropoietin and interleukin-3 activate tyrosine phosphorylation of CBL and association with CRK adaptor proteins. Blood 89:3166-3174.

Basson MA, Akbulut S, Watson-Johnson J, Simon R, Carroll TJ, Shakya R, Gross I, Martin GR, Lufkin T, McMahon AP, Wilson PD, Costantini FD, Mason IJ, Licht JD (2005). Sprouty1 is a critical regulator of GDNF/RET-mediated kidney induction. Dev Cell 8:229-239.

Beghini A, Peterlongo P, Ripamonti CB, Larizza L, Cairoli R, Morra E, Mecucci C (2000a). C-kit mutations in core binding factor leukemias. Blood 95:726-727.

Beghini A, Peterlongo P, Ripamonti CB, Larizza L, Cairoli R, Morra E, Mecucci C (2000b). C-kit mutations in core binding factor leukemias. Blood 95:726-727.

Besmer P, Murphy JE, George PC, Qiu FH, Bergold PJ, Lederman L, Snyder HW, Jr., Brodeur D, Zuckerman EE, Hardy WD (1986). A new acute transforming feline retrovirus and relationship of its oncogene v-kit with the protein kinase gene family. Nature 320:415-421.

Blume-Jensen P, Hunter T (2001a). Oncogenic kinase signalling. Nature 411:355-365.

Blume-Jensen P, Hunter T (2001b). Oncogenic kinase signalling. Nature 411:355-365.

Bonita DP, Miyake S, Lupher ML, Jr., Langdon WY, Band H (1997a). Phosphotyrosine binding domain-dependent upregulation of the platelet-derived growth factor receptor alpha signaling cascade by transforming mutants of Cbl: implications for Cbl's function and oncogenicity. Mol Cell Biol 17:4597-4610.

Bonita DP, Miyake S, Lupher ML, Jr., Langdon WY, Band H (1997b). Phosphotyrosine binding domain-dependent upregulation of the platelet-derived growth factor receptor alpha signaling cascade

by transforming mutants of Cbl: implications for Cbl's function and oncogenicity. Mol Cell Biol 17:4597-4610.

Brandts CH, Berdel WE, Serve H (2007a). Oncogenic signaling in acute myeloid leukemia. Curr Drug Targets 8:237-246.

Brandts CH, Berdel WE, Serve H (2007b). Oncogenic signaling in acute myeloid leukemia. Curr Drug Targets 8:237-246.

Brandts CH, Sargin B, Rode M, Biermann C, Lindtner B, Schwable J, Buerger H, Muller-Tidow C, Choudhary C, McMahon M, Berdel WE, Serve H (2005). Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. Cancer Res 65:9643-9650.

Brizzi MF, Dentelli P, Rosso A, Yarden Y, Pegoraro L (1999). STAT protein recruitment and activation in c-Kit deletion mutants. J Biol Chem 274:16965-16972.

Caligiuri MA, Briesewitz R, Yu J, Wang L, Wei M, Arnoczky KJ, Marburger TB, Wen J, Perrotti D, Bloomfield CD, Whitman SP (2007a). Novel c-CBL and CBL-b ubiquitin ligase mutations in human acute myeloid leukemia. Blood 110:1022-1024.

Caligiuri MA, Briesewitz R, Yu J, Wang L, Wei M, Arnoczky KJ, Marburger TB, Wen J, Perrotti D, Bloomfield CD, Whitman SP (2007b). Novel c-CBL and CBL-b ubiquitin ligase mutations in human acute myeloid leukemia. Blood 110:1022-1024.

Cantor AB, Orkin SH (2001). Hematopoietic development: a balancing act. Curr Opin Genet Dev 11:513-519.

Ceresa BP, Schmid SL (2000). Regulation of signal transduction by endocytosis. Curr Opin Cell Biol 12:204-210.

Choudhary C, Brandts C, Schwable J, Tickenbrock L, Sargin B, Ueker A, Bohmer FD, Berdel WE, Muller-Tidow C, Serve H (2007). Activation mechanisms of STAT5 by oncogenic Flt3-ITD. Blood 110:370-374.

Choudhary C, Schwable J, Brandts C, Tickenbrock L, Sargin B, Kindler T, Fischer T, Berdel WE, Muller-Tidow C, Serve H (2005). AML-associated Flt3 kinase domain mutations show signal transduction differences compared with Flt3 ITD mutations. Blood 106:265-273.

Cory GO, Lovering RC, Hinshelwood S, MacCarthy-Morrogh L, Levinsky RJ, Kinnon C (1995). The protein product of the c-cbl protooncogene is phosphorylated after B cell receptor stimulation and binds the SH3 domain of Bruton's tyrosine kinase. J Exp Med 182:611-615.

Crosetto N, Tikkanen R, Dikic I (2005). Oncogenic breakdowns in endocytic adaptor proteins. FEBS Lett 579:3231-3238.

Daub H, Specht K, Ullrich A (2004). Strategies to overcome resistance to targeted protein kinase inhibitors. Nat Rev Drug Discov 3:1001-1010.

de Melker AA, van der Horst G, Borst J (2004a). c-Cbl directs EGF receptors into an endocytic pathway that involves the ubiquitin-interacting motif of Eps15. J Cell Sci 117:5001-5012.

de Melker AA, van der Horst G, Borst J (2004b). Ubiquitin ligase activity of c-Cbl guides the epidermal growth factor receptor into clathrin-coated pits by two distinct modes of Eps15 recruitment. J Biol Chem 279:55465-55473.

Debiec-Rychter M, Cools J, Dumez H, Sciot R, Stul M, Mentens N, Vranckx H, Wasag B, Prenen H, Roesel J, Hagemeijer A, Van OA, Marynen P (2005). Mechanisms of resistance to imatinib mesylate in gastrointestinal stromal tumors and activity of the PKC412 inhibitor against imatinib-resistant mutants. Gastroenterology 128:270-279.

Dikic I, Giordano S (2003). Negative receptor signalling. Curr Opin Cell Biol 15:128-135.

Dikic I, Szymkiewicz I, Soubeyran P (2003). Cbl signaling networks in the regulation of cell function. Cell Mol Life Sci 60:1805-1827.

Dunbar AJ, Gondek LP, O'Keefe CL, Makishima H, Rataul MS, Szpurka H, Sekeres MA, Wang XF, McDevitt MA, Maciejewski JP (2008). 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. Cancer Res 68:10349-10357.

Edling CE, Hallberg B (2007). c-Kit--a hematopoietic cell essential receptor tyrosine kinase. Int J Biochem Cell Biol 39:1995-1998.

Edling CE, Pedersen M, Carlsson L, Ronnstrand L, Palmer RH, Hallberg B (2007). Haematopoietic progenitor cells utilise conventional PKC to suppress PKB/Akt activity in response to c-Kit stimulation. Br J Haematol 136:260-268.

Feshchenko EA, Langdon WY, Tsygankov AY (1998). Fyn, Yes, and Syk phosphorylation sites in c-Cbl map to the same tyrosine residues that become phosphorylated in activated T cells. J Biol Chem 273:8323-8331.

Fresno Vara JA, Casado E, De CJ, Cejas P, Belda-Iniesta C, Gonzalez-Baron M (2004). PI3K/Akt signalling pathway and cancer. Cancer Treat Rev 30:193-204.

Fukazawa T, Miyake S, Band V, Band H (1996). Tyrosine phosphorylation of Cbl upon epidermal growth factor (EGF) stimulation and its association with EGF receptor and downstream signaling proteins. J Biol Chem 271:14554-14559.

Furitsu T, Tsujimura T, Tono T, Ikeda H, Kitayama H, Koshimizu U, Sugahara H, Butterfield JH, Ashman LK, Kanayama Y, . (1993). Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product. J Clin Invest 92:1736-1744.

Gabbianelli M, Pelosi E, Montesoro E, Valtieri M, Luchetti L, Samoggia P, Vitelli L, Barberi T, Testa U, Lyman S, . (1995). Multi-level effects of flt3 ligand on human hematopoiesis: expansion of putative stem cells and proliferation of granulomonocytic progenitors/monocytic precursors. Blood 86:1661-1670.

Gari M, Goodeve A, Wilson G, Winship P, Langabeer S, Linch D, Vandenberghe E, Peake I, Reilly J (1999). c-kit proto-oncogene exon 8 in-frame deletion plus insertion mutations in acute myeloid leukaemia. Br J Haematol 105:894-900.

Gesbert F, Garbay C, Bertoglio J (1998). Interleukin-2 stimulation induces tyrosine phosphorylation of p120-Cbl and CrkL and formation of multimolecular signaling complexes in T lymphocytes and natural killer cells. J Biol Chem 273:3986-3993.

Grand FH, Hidalgo-Curtis CE, Ernst T, Zoi K, Zoi C, McGuire C, Kreil S, Jones A, Score J, Metzgeroth G, Oscier D, Hall A, Brandts C, Serve H, Reiter A, Chase AJ, Cross NC (2009). Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. Blood 113:6182-6192.

Haglund K, Schmidt MH, Wong ES, Guy GR, Dikic I (2005). Sprouty2 acts at the Cbl/CIN85 interface to inhibit epidermal growth factor receptor downregulation. EMBO Rep 6:635-641.

Haglund K, Sigismund S, Polo S, Szymkiewicz I, Di Fiore PP, Dikic I (2003). Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol 5:461-466.

Hamilton E, Miller KM, Helm KM, Langdon WY, Anderson SM (2001). Suppression of apoptosis induced by growth factor withdrawal by an oncogenic form of c-Cbl. J Biol Chem 276:9028-9037.

Heinrich MC, Blanke CD, Druker BJ, Corless CL (2002). Inhibition of KIT tyrosine kinase activity: a novel molecular approach to the treatment of KIT-positive malignancies. J Clin Oncol 20:1692-1703.

Heiss E, Masson K, Sundberg C, Pedersen M, Sun J, Bengtsson S, Ronnstrand L (2006). Identification of Y589 and Y599 in the juxtamembrane domain of Flt3 as ligand-induced autophosphorylation sites involved in binding of Src family kinases and the protein tyrosine phosphatase SHP2. Blood 108:1542-1550.

Hjertson M, Sundstrom C, Lyman SD, Nilsson K, Nilsson G (1996). Stem cell factor, but not flt3 ligand, induces differentiation and activation of human mast cells. Exp Hematol 24:748-754.

Horiike S, Yokota S, Nakao M, Iwai T, Sasai Y, Kaneko H, Taniwaki M, Kashima K, Fujii H, Abe T, Misawa S (1997). Tandem duplications of the FLT3 receptor gene are associated with leukemic transformation of myelodysplasia. Leukemia 11:1442-1446.

Hu Y, Swerdlow S, Duffy TM, Weinmann R, Lee FY, Li S (2006). Targeting multiple kinase pathways in leukemic progenitors and stem cells is essential for improved treatment of Ph+ leukemia in mice. Proc Natl Acad Sci U S A 103:16870-16875.

Hubbard SR (2004). Juxtamembrane autoinhibition in receptor tyrosine kinases. Nat Rev Mol Cell Biol 5:464-471.

Hunter S, Burton EA, Wu SC, Anderson SM (1999a). Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase. J Biol Chem 274:2097-2106.

Hunter S, Burton EA, Wu SC, Anderson SM (1999b). Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase. J Biol Chem 274:2097-2106.

James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, Garcon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villeval JL, Constantinescu SN, Casadevall N, Vainchenker W (2005). A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature 434:1144-1148.

Jiang X, Huang F, Marusyk A, Sorkin A (2003). Grb2 regulates internalization of EGF receptors through clathrin-coated pits. Mol Biol Cell 14:858-870.

Joazeiro CA, Wing SS, Huang H, Leverson JD, Hunter T, Liu YC (1999). The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science 286:309-312.

Jongeward GD, Clandinin TR, Sternberg PW (1995). sli-1, a negative regulator of let-23-mediated signaling in C. elegans. Genetics 139:1553-1566.

Kanagasundaram V, Jaworowski A, Hamilton JA (1996). Association between phosphatidylinositol-3 kinase, Cbl and other tyrosine phosphorylated proteins in colony-stimulating factor-1-stimulated macrophages. Biochem J 320 (Pt 1):69-77.

Karnitz LM, Sutor SL, Abraham RT (1994). The Src-family kinase, Fyn, regulates the activation of phosphatidylinositol 3-kinase in an interleukin 2-responsive T cell line. J Exp Med 179:1799-1808.

Keane MM, Ettenberg SA, Nau MM, Banerjee P, Cuello M, Penninger J, Lipkowitz S (1999). cbl-3: a new mammalian cbl family protein. Oncogene 18:3365-3375.

Keane MM, Rivero-Lezcano OM, Mitchell JA, Robbins KC, Lipkowitz S (1995). Cloning and characterization of cbl-b: a SH3 binding protein with homology to the c-cbl proto-oncogene. Oncogene 10:2367-2377.

Kelly LM, Gilliland DG (2002a). Genetics of myeloid leukemias. Annu Rev Genomics Hum Genet 3:179-198.

Kelly LM, Gilliland DG (2002b). Genetics of myeloid leukemias. Annu Rev Genomics Hum Genet 3:179-198.

Kim M, Tezuka T, Suziki Y, Sugano S, Hirai M, Yamamoto T (1999). Molecular cloning and characterization of a novel cbl-family gene, cbl-c. Gene 239:145-154.

Kitamura T, Koshino Y, Shibata F, Oki T, Nakajima H, Nosaka T, Kumagai H (2003a). Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. Exp Hematol 31:1007-1014.

Kitamura T, Koshino Y, Shibata F, Oki T, Nakajima H, Nosaka T, Kumagai H (2003b). Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. Exp Hematol 31:1007-1014.

Kitamura Y, Hirotab S (2004). Kit as a human oncogenic tyrosine kinase. Cell Mol Life Sci 61:2924-2931.

Knudson AG (2001). Two genetic hits (more or less) to cancer. Nat Rev Cancer 1:157-162.

Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, Halmos B (2005). EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 352:786-792.

Kogan SC, Ward JM, Anver MR, Berman JJ, Brayton C, Cardiff RD, Carter JS, de CS, Downing JR, Fredrickson TN, Haines DC, Harris AW, Harris NL, Hiai H, Jaffe ES, MacLennan IC, Pandolfi PP, Pattengale PK, Perkins AS, Simpson RM, Tuttle MS, Wong JF, Morse HC, III (2002). Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice. Blood 100:238-245.

Kozlowski M, Larose L, Lee F, Le DM, Rottapel R, Siminovitch KA (1998). SHP-1 binds and negatively modulates the c-Kit receptor by interaction with tyrosine 569 in the c-Kit juxtamembrane domain. Mol Cell Biol 18:2089-2099.

Kyo S, Sada K, Qu X, Maeno K, Miah SM, Kawauchi-Kamata K, Yamamura H (2003). Negative regulation of Lyn protein-tyrosine kinase by c-Cbl ubiquitin-protein ligase in Fc epsilon RI-mediated mast cell activation. Genes Cells 8:825-836.

Langdon WY, Hartley JW, Klinken SP, Ruscetti SK, Morse HC, III (1989a). v-cbl, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas. Proc Natl Acad Sci U S A 86:1168-1172.

Langdon WY, Hartley JW, Klinken SP, Ruscetti SK, Morse HC, III (1989b). v-cbl, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas. Proc Natl Acad Sci U S A 86:1168-1172.

Langdon WY, Hartley JW, Klinken SP, Ruscetti SK, Morse HC, III (1989c). v-cbl, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas. Proc Natl Acad Sci U S A 86:1168-1172.

Le RC, Wrana JL (2005). Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. Nat Rev Mol Cell Biol 6:112-126.

Lennartsson J, Blume-Jensen P, Hermanson M, Ponten E, Carlberg M, Ronnstrand L (1999). Phosphorylation of Shc by Src family kinases is necessary for stem cell factor receptor/c-kit mediated activation of the Ras/MAP kinase pathway and c-fos induction. Oncogene 18:5546-5553.

Lennartsson J, Jelacic T, Linnekin D, Shivakrupa R (2005). Normal and oncogenic forms of the receptor tyrosine kinase kit. Stem Cells 23:16-43.

Levkowitz G, Waterman H, Ettenberg SA, Katz M, Tsygankov AY, Alroy I, Lavi S, Iwai K, Reiss Y, Ciechanover A, Lipkowitz S, Yarden Y (1999). Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol Cell 4:1029-1040.

Linnekin D (1999). Early signaling pathways activated by c-Kit in hematopoietic cells. Int J Biochem Cell Biol 31:1053-1074.

Linnekin D, Weiler SR, Mou S, Deberry CS, Keller JR, Ruscetti FW, Ferris DK, Longo DL (1996). JAK2 is constitutively associated with c-Kit and is phosphorylated in response to stem cell factor. Acta Haematol 95:224-228.

Loh ML, Sakai DS, Flotho C, Kang M, Fliegauf M, Archambeault S, Mullighan CG, Chen L, Bergstraesser E, Bueso-Ramos CE, Emanuel PD, Hasle H, Issa JP, van den Heuvel-Eibrink MM, Locatelli F, Stary J, Trebo M, Wlodarski M, Zecca M, Shannon KM, Niemeyer CM (2009). Mutations in CBL occur frequently in juvenile myelomonocytic leukemia. Blood 114:1859-1863.

Lombardo LJ, Lee FY, Chen P, Norris D, Barrish JC, Behnia K, Castaneda S, Cornelius LA, Das J, Doweyko AM, Fairchild C, Hunt JT, Inigo I, Johnston K, Kamath A, Kan D, Klei H, Marathe P, Pang S, Peterson R, Pitt S, Schieven GL, Schmidt RJ, Tokarski J, Wen ML, Wityak J, Borzilleri RM (2004). Discovery of N-(2-chloro-6-methyl- phenyl)-2-(6-(4-(2-hydroxyethyl)- piperazin-1-yl)-2-methylpyrimidin-4- ylamino)thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. J Med Chem 47:6658-6661.

Lowenberg B, Downing JR, Burnett A (1999). Acute myeloid leukemia. N Engl J Med 341:1051-1062.

Ma Y, Cunningham ME, Wang X, Ghosh I, Regan L, Longley BJ (1999). Inhibition of spontaneous receptor phosphorylation by residues in a putative alpha-helix in the KIT intracellular juxtamembrane region. J Biol Chem 274:13399-13402.

Magnusson MK, Meade KE, Brown KE, Arthur DC, Krueger LA, Barrett AJ, Dunbar CE (2001). Rabaptin-5 is a novel fusion partner to platelet-derived growth factor beta receptor in chronic myelomonocytic leukemia. Blood 98:2518-2525.

Makishima H, Cazzolli H, Szpurka H, Dunbar A, Tiu R, Huh J, Muramatsu H, O'Keefe C, Hsi E, Paquette RL, Kojima S, List AF, Sekeres MA, McDevitt MA, Maciejewski JP (2009). Mutations of E3 Ubiquitin Ligase Cbl Family Members Constitute a Novel Common Pathogenic Lesion in Myeloid Malignancies. J Clin Oncol.

Marmor MD, Yarden Y (2004). Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. Oncogene 23:2057-2070.

Masson K, Heiss E, Band H, Ronnstrand L (2006). Direct binding of Cbl to Tyr568 and Tyr936 of the stem cell factor receptor/c-Kit is required for ligand-induced ubiquitination, internalization and degradation. Biochem J 399:59-67.

Meng W, Sawasdikosol S, Burakoff SJ, Eck MJ (1999a). Structure of the amino-terminal domain of Cbl complexed to its binding site on ZAP-70 kinase. Nature 398:84-90.

Meng W, Sawasdikosol S, Burakoff SJ, Eck MJ (1999b). Structure of the amino-terminal domain of Cbl complexed to its binding site on ZAP-70 kinase. Nature 398:84-90.

Meshinchi S, Woods WG, Stirewalt DL, Sweetser DA, Buckley JD, Tjoa TK, Bernstein ID, Radich JP (2001). Prevalence and prognostic significance of Flt3 internal tandem duplication in pediatric acute myeloid leukemia. Blood 97:89-94.

Miyajima A, Mui AL, Ogorochi T, Sakamaki K (1993). Receptors for granulocyte-macrophage colony-stimulating factor, interleukin-3, and interleukin-5. Blood 82:1960-1974.

Mol CD, Dougan DR, Schneider TR, Skene RJ, Kraus ML, Scheibe DN, Snell GP, Zou H, Sang BC, Wilson KP (2004). Structural basis for the autoinhibition and STI-571 inhibition of c-Kit tyrosine kinase. J Biol Chem 279:31655-31663.

Morita S, Kojima T, Kitamura T (2000). Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther 7:1063-1066.

Mousavi SA, Malerod L, Berg T, Kjeken R (2004). Clathrin-dependent endocytosis. Biochem J 377:1-16.

Nam S, Kim D, Cheng JQ, Zhang S, Lee JH, Buettner R, Mirosevich J, Lee FY, Jove R (2005). Action of the Src family kinase inhibitor, dasatinib (BMS-354825), on human prostate cancer cells. Cancer Res 65:9185-9189.

O'Laughlin-Bunner B, Radosevic N, Taylor ML, Shivakrupa, DeBerry C, Metcalfe DD, Zhou M, Lowell C, Linnekin D (2001). Lyn is required for normal stem cell factor-induced proliferation and chemotaxis of primary hematopoietic cells. Blood 98:343-350.

Oliveira SH, Lukacs NW (2003). Stem cell factor: a hemopoietic cytokine with important targets in asthma. Curr Drug Targets Inflamm Allergy 2:313-318.

Orfao A, Garcia-Montero AC, Sanchez L, Escribano L (2007). Recent advances in the understanding of mastocytosis: the role of KIT mutations. Br J Haematol 138:12-30.

Orkin SH (1995). Hematopoiesis: how does it happen? Curr Opin Cell Biol 7:870-877.

Orkin SH (1996). Development of the hematopoietic system. Curr Opin Genet Dev 6:597-602.

Orkin SH, Zon LI (2002). Hematopoiesis and stem cells: plasticity versus developmental heterogeneity. Nat Immunol 3:323-328.

Parsons SJ, Parsons JT (2004). Src family kinases, key regulators of signal transduction. Oncogene 23:7906-7909.

Paschka P, Marcucci G, Ruppert AS, Mrozek K, Chen H, Kittles RA, Vukosavljevic T, Perrotti D, Vardiman JW, Carroll AJ, Kolitz JE, Larson RA, Bloomfield CD (2006a). Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study. J Clin Oncol 24:3904-3911.

Paschka P, Marcucci G, Ruppert AS, Mrozek K, Chen H, Kittles RA, Vukosavljevic T, Perrotti D, Vardiman JW, Carroll AJ, Kolitz JE, Larson RA, Bloomfield CD (2006b). Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study. J Clin Oncol 24:3904-3911.

Peschard P, Fournier TM, Lamorte L, Naujokas MA, Band H, Langdon WY, Park M (2001a). Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. Mol Cell 8:995-1004.

Peschard P, Fournier TM, Lamorte L, Naujokas MA, Band H, Langdon WY, Park M (2001b). Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. Mol Cell 8:995-1004.

Peschard P, Park M (2003). Escape from Cbl-mediated downregulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases. Cancer Cell 3:519-523.

Pickart CM (2001). Mechanisms underlying ubiquitination. Annu Rev Biochem 70:503-533.

Pietsch T (1993). Paracrine and autocrine growth mechanisms of human stem cell factor (c-kit ligand) in myeloid leukemia. Nouv Rev Fr Hematol 35:285-286.

Pleiman CM, Hertz WM, Cambier JC (1994). Activation of phosphatidylinositol-3' kinase by Src-family kinase SH3 binding to the p85 subunit. Science 263:1609-1612.

Ramirez P, DiPersio JF (2008). Therapy options in imatinib failures. Oncologist 13:424-434.

Ratajczak MZ, Ratajczak J, Ford J, Kregenow R, Marlicz W, Gewirtz AM (1996). FLT3/FLK-2 (STK-1) Ligand does not stimulate human megakaryopoiesis in vitro. Stem Cells 14:146-150.

Rathinam C, Thien CB, Langdon WY, Gu H, Flavell RA (2008). The E3 ubiquitin ligase c-Cbl restricts development and functions of hematopoietic stem cells. Genes Dev 22:992-997.

Reindl C, Quentmeier H, Petropoulos K, Greif PA, Benthaus T, Argiropoulos B, Mellert G, Vempati S, Duyster J, Buske C, Bohlander SK, Humphries KR, Hiddemann W, Spiekermann K (2009). CBL exon 8/9 mutants activate the FLT3 pathway and cluster in core binding factor/11q deletion acute myeloid leukemia/myelodysplastic syndrome subtypes. Clin Cancer Res 15:2238-2247.

Rosnet O, Buhring HJ, Marchetto S, Rappold I, Lavagna C, Sainty D, Arnoulet C, Chabannon C, Kanz L, Hannum C, Birnbaum D (1996). Human FLT3/FLK2 receptor tyrosine kinase is expressed at the surface of normal and malignant hematopoietic cells. Leukemia 10:238-248.

Rosnet O, Schiff C, Pebusque MJ, Marchetto S, Tonnelle C, Toiron Y, Birg F, Birnbaum D (1993). Human FLT3/FLK2 gene: cDNA cloning and expression in hematopoietic cells. Blood 82:1110-1119.

Sanada M, Suzuki T, Shih LY, Otsu M, Kato M, Yamazaki S, Tamura A, Honda H, Sakata-Yanagimoto M, Kumano K, Oda H, Yamagata T, Takita J, Gotoh N, Nakazaki K, Kawamata N, Onodera M, Nobuyoshi M, Hayashi Y, Harada H, Kurokawa M, Chiba S, Mori H, Ozawa K, Omine M, Hirai H, Nakauchi H, Koeffler HP, Ogawa S (2009). Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. Nature 460:904-908.

Sanjay A, Houghton A, Neff L, DiDomenico E, Bardelay C, Antoine E, Levy J, Gailit J, Bowtell D, Horne WC, Baron R (2001). Cbl associates with Pyk2 and Src to regulate Src kinase activity, alpha(v)beta(3) integrin-mediated signaling, cell adhesion, and osteoclast motility. J Cell Biol 152:181-195.

Sargin B, Choudhary C, Crosetto N, Schmidt MH, Grundler R, Rensinghoff M, Thiessen C, Tickenbrock L, Schwable J, Brandts C, August B, Koschmieder S, Bandi SR, Duyster J, Berdel WE, Muller-Tidow C, Dikic I, Serve H (2007). Flt3-dependent transformation by inactivating c-Cbl mutations in AML. Blood 110:1004-1012.

Schittenhelm MM, Shiraga S, Schroeder A, Corbin AS, Griffith D, Lee FY, Bokemeyer C, Deininger MW, Druker BJ, Heinrich MC (2006). Dasatinib (BMS-354825), a dual SRC/ABL kinase inhibitor, inhibits the kinase activity of wild-type, juxtamembrane, and activation loop mutant KIT isoforms associated with human malignancies. Cancer Res 66:473-481.

Schlessinger J (1988). Signal transduction by allosteric receptor oligomerization. Trends Biochem Sci 13:443-447.

Schmidt MH, Dikic I (2005). The Cbl interactome and its functions. Nat Rev Mol Cell Biol 6:907-918.

Schnittger S, Schoch C, Dugas M, Kern W, Staib P, Wuchter C, Loffler H, Sauerland CM, Serve H, Buchner T, Haferlach T, Hiddemann W (2002). Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. Blood 100:59-66.

Scolnik MP, Morilla R, de Bracco MM, Catovsky D, Matutes E (2002). CD34 and CD117 are overexpressed in AML and may be valuable to detect minimal residual disease. Leuk Res 26:615-619.

Seto ES, Bellen HJ, Lloyd TE (2002). When cell biology meets development: endocytic regulation of signaling pathways. Genes Dev 16:1314-1336.

Shah NP, Lee FY, Luo R, Jiang Y, Donker M, Akin C (2006). Dasatinib (BMS-354825) inhibits KITD816V, an imatinib-resistant activating mutation that triggers neoplastic growth in most patients with systemic mastocytosis. Blood 108:286-291.

Simmons PJ, Aylett GW, Niutta S, To LB, Juttner CA, Ashman LK (1994). c-kit is expressed by primitive human hematopoietic cells that give rise to colony-forming cells in stroma-dependent or cytokine-supplemented culture. Exp Hematol 22:157-165.

Slape C, Liu LY, Beachy S, Aplan PD (2008). Leukemic transformation in mice expressing a NUP98-HOXD13 transgene is accompanied by spontaneous mutations in Nras, Kras, and Cbl. Blood 112:2017-2019.

Spritz RA (1994). Molecular basis of human piebaldism. J Invest Dermatol 103:137S-140S.

Sternberg PW, Lesa G, Lee J, Katz WS, Yoon C, Clandinin TR, Huang LS, Chamberlin HM, Jongeward G (1995). LET-23-mediated signal transduction during Caenorhabditis elegans development. Mol Reprod Dev 42:523-528.

Stirewalt DL, Radich JP (2003). The role of FLT3 in haematopoietic malignancies. Nat Rev Cancer 3:650-665.

Swaminathan G, Tsygankov AY (2006). The Cbl family proteins: ring leaders in regulation of cell signaling. J Cell Physiol 209:21-43.

Tamborini E, Bonadiman L, Greco A, Albertini V, Negri T, Gronchi A, Bertulli R, Colecchia M, Casali PG, Pierotti MA, Pilotti S (2004). A new mutation in the KIT ATP pocket causes acquired resistance to imatinib in a gastrointestinal stromal tumor patient. Gastroenterology 127:294-299.

Thiede C, Steudel C, Mohr B, Schaich M, Schakel U, Platzbecker U, Wermke M, Bornhauser M, Ritter M, Neubauer A, Ehninger G, Illmer T (2002). Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood 99:4326-4335.

Thien CB, Blystad FD, Zhan Y, Lew AM, Voigt V, Andoniou CE, Langdon WY (2005). Loss of c-Cbl RING finger function results in high-intensity TCR signaling and thymic deletion. EMBO J 24:3807-3819.

Thien CB, Langdon WY (1997). Tyrosine kinase activity of the EGF receptor is enhanced by the expression of oncogenic 70Z-Cbl. Oncogene 15:2909-2919.

Thien CB, Langdon WY (2001). Cbl: many adaptations to regulate protein tyrosine kinases. Nat Rev Mol Cell Biol 2:294-307.

Thien CB, Langdon WY (2005a). c-Cbl and Cbl-b ubiquitin ligases: substrate diversity and the negative regulation of signalling responses. Biochem J 391:153-166.

Thien CB, Langdon WY (2005b). Negative regulation of PTK signalling by Cbl proteins. Growth Factors 23:161-167.

Thien CB, Walker F, Langdon WY (2001). RING finger mutations that abolish c-Cbl-directed polyubiquitination and downregulation of the EGF receptor are insufficient for cell transformation. Mol Cell 7:355-365.

Timokhina I, Kissel H, Stella G, Besmer P (1998). Kit signaling through PI 3-kinase and Src kinase pathways: an essential role for Rac1 and JNK activation in mast cell proliferation. EMBO J 17:6250-6262.

Turner AM, Lin NL, Issarachai S, Lyman SD, Broudy VC (1996). FLT3 receptor expression on the surface of normal and malignant human hematopoietic cells. Blood 88:3383-3390.

Vivanco I, Sawyers CL (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2:489-501.

Vosseller K, Stella G, Yee NS, Besmer P (1997). c-kit receptor signaling through its phosphatidylinositide-3'-kinase-binding site and protein kinase C: role in mast cell enhancement of degranulation, adhesion, and membrane ruffling. Mol Biol Cell 8:909-922.

Ware MF, Tice DA, Parsons SJ, Lauffenburger DA (1997). Overexpression of cellular Src in fibroblasts enhances endocytic internalization of epidermal growth factor receptor. J Biol Chem 272:30185-30190.

Waterman H, Levkowitz G, Alroy I, Yarden Y (1999). The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. J Biol Chem 274:22151-22154.

Waterman H, Yarden Y (2001). Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases. FEBS Lett 490:142-152.

Weihua Z, Tsan R, Huang WC, Wu Q, Chiu CH, Fidler IJ, Hung MC (2008). Survival of cancer cells is maintained by EGFR independent of its kinase activity. Cancer Cell 13:385-393.

Weiler SR, Mou S, DeBerry CS, Keller JR, Ruscetti FW, Ferris DK, Longo DL, Linnekin D (1996). JAK2 is associated with the c-kit proto-oncogene product and is phosphorylated in response to stem cell factor. Blood 87:3688-3693.

Weiss A, Schlessinger J (1998). Switching signals on or off by receptor dimerization. Cell 94:277-280.

Wong ES, Fong CW, Lim J, Yusoff P, Low BC, Langdon WY, Guy GR (2002). Sprouty2 attenuates epidermal growth factor receptor ubiquitylation and endocytosis, and consequently enhances Ras/ERK signalling. EMBO J 21:4796-4808.

Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, Asou N, Kuriyama K, Yagasaki F, Shimazaki C, Akiyama H, Saito K, Nishimura M, Motoji T, Shinagawa K, Takeshita A, Saito H, Ueda R, Ohno R, Naoe T (2001). Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood 97:2434-2439.

Yarden Y, Kuang WJ, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, Chen E, Schlessinger J, Francke U, Ullrich A (1987). Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J 6:3341-3351.

Yokota S, Kiyoi H, Nakao M, Iwai T, Misawa S, Okuda T, Sonoda Y, Abe T, Kahsima K, Matsuo Y, Naoe T (1997). Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. Leukemia 11:1605-1609.

Yokouchi M, Kondo T, Sanjay A, Houghton A, Yoshimura A, Komiya S, Zhang H, Baron R (2001). Src-catalyzed phosphorylation of c-Cbl leads to the interdependent ubiquitination of both proteins. J Biol Chem 276:35185-35193.

Yoon CH, Lee J, Jongeward GD, Sternberg PW (1995). Similarity of sli-1, a regulator of vulval development in C. elegans, to the mammalian proto-oncogene c-cbl. Science 269:1102-1105.

Yoon S, Seger R (2006). The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors 24:21-44.

Zeng S, Xu Z, Lipkowitz S, Longley JB (2005). Regulation of stem cell factor receptor signaling by Cbl family proteins (Cbl-b/c-Cbl). Blood 105:226-232.

Zhang S, Fukuda S, Lee Y, Hangoc G, Cooper S, Spolski R, Leonard WJ, Broxmeyer HE (2000). Essential role of signal transducer and activator of transcription (Stat)5a but not Stat5b for Flt3-dependent signaling. J Exp Med 192:719-728.

Zheng R, Levis M, Piloto O, Brown P, Baldwin BR, Gorin NC, Beran M, Zhu Z, Ludwig D, Hicklin D, Witte L, Li Y, Small D (2004). FLT3 ligand causes autocrine signaling in acute myeloid leukemia cells. Blood 103:267-274.

8 Abbreviations

+	Plus
_	Minus
%	Percent
4H	Four-Helix-bundle
A	Alanine
AML	Acute Myeloid Leukemia
ALL	Acute Lymphocytic Leukemia
aUPD	acquired Uniparental Disomy
BCR	B-Cell Receptor
BM	Bone Marrow
BSD	Blasticidine
CBF	Core Binding Factor
CBL	Casitas B-lineage Lymphoma
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
CSF1R	Colony Stimulating Factor 1Receptor
C	Cysteine
°C	Centigrade
CO_2	Carbon dioxide
D	Aspartic acid
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl Sulfoxide
eGFP	Enhanced Green Fluorescent Protein
EC	Extracellular
ECL	Enhanced chemiluminiscence
EGFR	Epidermal Growth Factor Receptor
EDTA	Ethylene Diamine Tetra acetic Acid
EGTA	Ethylene Glycol Tetra acetic Acid
EPO	Erythropoietin
Erk	Extracellular regulated kinase
et. al.	Et alia
FACS	Fluorescence Activated Cell Sorter

FCS	Fetal Calf Serum
FGFR	Fibroblast Growth Factor Receptor
FL	Flt3 Ligand
Flt3	FMS-like Tyrosine Kinase 3
Flt3-ITD	FMS-like Tyrosine Kinase 3- Internal Tandem Duplication
FLt3-KD	FMS-like Tyrosine Kinase 3 Kinase-Dead
G	Glycine
GFP	Green Fluorescent Protein
GIST	Gastrointestinal Stromal Tumors
Gy	Gray
Grb2	Growth factor receptor-bound protein 2
^{3}H	Tritium
HA	Hemagglutinin
HE	Hematoxylin and Eosin
HECT	Homologous to the E6-AP Carboxyl Terminus
HGF	Hepatocyte Growth Factor
Hrs	Hours
HSC	Hematopoietic Stem Cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSPC	Hematopoietic Stem and Progenitor Cell
HZ4-FeSV	Hardy-Zuckerman 4 Feline Sarcoma Virus
Ig	Immunoglobulin
IL-3	Interleukin-3
IMDM	Iscove's Modified Dulbecco's Medium
IRES	Internal Ribosome Entry Site
ITAM	Immunoreceptor tyrosine based activation motif
JAK	Janus Kinases
JMD	Juxtamembrane domain
K	Lysine
KD	Kilo Dalton
Kg	Kilogram
Kit-KD	Kit Kinase-Dead
LZ	Leucine Zipper

MAPK	Mitogen-Activated Protein Kinase
MBq	Megabecquerel
mg	Milligram
Min	Minutes
ml	Milliliter
µCi	Microcurie
µg	Microgram
ml	Milliliter
µF	Microfarad
µl	Microliter
µM	Micromolar
mM	MilliMolar
mm	Millimeter
mg	Milligram
MC	Mast Cells
MDS	Myelodysplasia
MPD	Myeloproliferative diseases
MPP	Multipotent progenitor cells
MPN	Myeloproliferative Neoplasams
N	Asparagine
NACE	Naphthol AS-D Chloroacetate Esterase
NaCl	Sodium Chloride
NaF	Sodium Fluoride
ng	nano gram
nM	nano Molar
NP-40	nonyl phenoxylpolyethoxylethanol
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PI-3-Kinase	Phosphoinositide-3-Kinase
PDGFR	Platelet Derived Growth Factor Receptor
p^H	potential Hydrogen
PLAT-E	Platinum-E
PKC	Protein Kinase C
PTKs	Protein Tyrosine Kinases

Q	Glutamine
R	Arginine
RING	Really Interesting New Gene
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RTKs	Receptor Tyrosine Kinases
RBC	Red Blood Cell
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SFKs	Src Family Kinases
SH2	Src Homology 2
SH3	Src Homology 3
SHP1	SH2 domain containing phophatase-1
SL	Steel Locus
SCF	Stem Cell Factor
Ser	Serine
STAT	Signal transducer and activator of transcription
TCL	Total Cell Protein
TCR	T-Cell Receptor
Thr	Threonine
TKB	Tyrosine Kinase Binding
TKD	Tyrosine Kinase Domain
TPO	Thrombopoietin
Tyr	Tyrosine
UBA	Ubiquitin Binding Associated
V	Valine
V	Volts
VEGFR	Vascular Endothelial Growth Factor Receptor
WEHI	Walter and Elisa Hall Institute
Y	Tyrosine
$ZnCl_2$	Zinc Chloride

9 Eidesstattliche Erklärung

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre hiermit eidesstattlich, daß ich die dem Fachbereich Biochemie, Chemie und Pharmazie zur Promotionsprüfung eingereichte Arbeit mit dem Titel

Die Rolle von onkogenen Cbl Mutanten in der Kit-vermittelten Signaltransduktion und myeloischen Transformation

im Zentrum der Inneren Medizin, Medizinische Klinik II, Hämatologie und Onkologie des Universitätsklinikums Frankfurt bei Prof. Dr. Hubert Serve und unter Leitung von Prof. Dr. Rolf Marschalek mit Unterstützung von Dr. med. Christian Brandts ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.
Ich habe bisher an keiner in- oder ausländischen Biologischen Fakultät ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende Arbeit als Dissertation vorgelegt.
Inhalte der vorliegenden Arbeit wurden in *Blood* 2009 Nov 5;114(19):4197-208 und *Blood* 2007 Aug 1;110(3):1004-12 publiziert.

Frankfurt, den 21.01.2010

Srinivasa Rao Bandi

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11 Curriculum vitae

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2003-2005	Master of Science (M.Sc) Studied Biochemistry and Molecular biology University of Hyderabad, Hyderabad, India
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- Junior Research Fellow (JRF) Council of Scientific and Industrial Research (CSIR), Govt. of India, Dec 2005
- Junior Research Fellow (JRF) Indian Council of Medical Research (ICMR), Govt. of India, Dec 2005
- Merit cum Means scholarship from University of Hyderabad while studying Master of science (M.Sc)
- Qualified GATE-2005 in life science stream with 99.61 percentile.

Publications

1 **Srinivasa Rao Bandi**,* Christian Brandts,* Marion Rensinghoff, Rebekka Grundler, Lara Tickenbrock, Gabriele Kohler, Justus Duyster, Wolfgang E. Berdel, Carsten Muller-Tidow, Hubert Serve, and Bülent Sargin
E3 ligase-defective Cbl mutants lead to a generalized mastocytosis and a myeloproliferative disease. (**Blood, 5 November 2009, Vol. 114, No. 19, pp. 4197-4208**) * equal contribution

2 Bülent Sargin, Chunaram Choudhary, Nicola Crosetto, Mirko H. H. Schmidt, Rebekka Grundler, Marion Rensinghoff, Christine Thiessen, Lara Tickenbrock, Joachim Schwäble, Christian Brandts, Benjamin August, Steffen Koschmieder, **Srinivasa Rao Bandi**, Justus Duyster, Wolfgang E. Berdel, Carsten Müller-Tidow, Ivan Dikic, and Hubert Serve
Flt3-dependent transformation by inactivating c-Cbl mutations in AML (Blood, Aug 2007; 110: 1004 – 1012)

Conferences and Poster presentation

1, 9th DGZ Young Scientist Meeting “Signalling Cascades in Development and Disease“. September 20 - 21, 2007 Max-Planck-Institute for Molecular Biomedicine, Münster.

Synergism between c-Kit and c-Cbl mutants leads to factor independent growth and transformation of a myeloid cell line 32D (poster presented).

2, DGHO conference Gemeinsame Jahrestagung '09 der Deutschen, Österreichischen und Schweizerischen Gesellschaften für Hämatologie und Onkologie. 2. bis 6. October 2009 Heidelberg / Mannheim.

Cbl mutants interact with c-Kit independent of its kinase activity and lead to factor independent growth and transformation of a myeloid cell line 32D (poster presented).

3, UCT-Forschungstag/UCT Science Day Frankfurt Freitag 6. November 2009.

E3 ligase-defective Cbl mutants lead to a generalized mastocytosis and a myeloproliferative disease.

4, Several Scientific conferences were attended as a participant in and around of Frankfurt and Muenster Germany.