Flt3-dependent transformation by inactivating c-Cbl mutations in AML

Running Title: Role of Cbl mutations in myeloid transformation

Bülent Sargin1*, Chunaram Choudhary1*, Nicola Crosetto2, Mirko H.H. Schmidt2, Marion Rensinghoff1, Christine Thiessen1, Lara Tickenbrock1, Joachim Schwäble1, Christian Brandts1, Benjamin August1, Steffen Koschmieder1, Srinivasa Rao Bandi1, Wolfgang E. Berdel1, Carsten Müller-Tidow1, Ivan Dikic2, Hubert Serve1

1Department of Medicine, Hematology and Oncology and the Interdisciplinary Center for Clinical Research, University Hospital Münster, D-48149 Münster, Germany
2Institute of Biochemistry II, Goethe University Medical School, Theodor-Stern-Kai 7, D-60590 Frankfurt, Germany

*BS and CC contributed equally to the work presented here

Key Words: AML, Flt3, Flt3-ITD, Cbl

Correspondence to: Hubert Serve MD, Department of Medicine, Hematology and Oncology, University of Münster, Albert-Schweitzer-Strasse 33, 48129 Münster, Germany
phone +49-251-835-2671    fax +49-251-835-2673
E-mail: serve@uni-muenster.de

Word count:  Abstract: 199; Text: 4820    Heading: Neoplasia
Abstract

In acute myeloid leukemia (AML), mutational activation of the receptor tyrosine kinase (RTK) Flt3 is frequently involved in leukemic transformation. However, little is known about a possible role of highly expressed wild-type Flt3 in AML. The proto-oncogene c-Cbl is an important regulator of RTK signaling, acting through its ubiquitin ligase activity and as a platform for several signaling adaptor molecules. Here, we analyzed the role of c-Cbl in Flt3 signal transduction and myeloid transformation. C-Cbl physically interacted with Flt3 and was tyrosine phosphorylated in the presence of Flt3-ligand (FL). Overexpression of a dominant negative form of c-Cbl (Cbl-70Z) inhibited FL-induced Flt3 ubiquitylation and internalization, indicating involvement of c-Cbl in Flt3 signaling. DNA sequencing of AML bone marrow revealed a case with a c-Cbl point mutation (Cbl-R420Q). Cbl-R420Q inhibited Flt3 internalization and ubiquitylation. Co-expression of Cbl-R420Q or Cbl-70Z with Flt3 induced cytokine-independent growth and survival of 32DcI3 cells in the absence of FL. Also, the mutant Cbl proteins altered the amplitude and duration of Flt3-dependent signaling events. Our results indicate an important role of Cbl proteins in Flt3 signal modulation. Also, the data suggest a novel mechanism of leukemic transformation in AML by mutational inactivation of negative RTK regulators.
Introduction

Receptor tyrosine kinases (RTKs) bind extracellular growth factors and activate intracellular signaling networks. The magnitude and kinetics of RTK activation are tightly regulated, since they determine the quality and extent of the biological response.\textsuperscript{1} Attenuation of RTK signaling occurs by endocytosis and subsequent protein degradation.\textsuperscript{2-4} Cbl proteins have been shown to be central players in these processes.\textsuperscript{5-7} The members of the Cbl family, c-Cbl, Cbl-b, and Cbl-3 contain an N-terminal phosphotyrosine binding (PTB) domain that allows direct interaction with activated RTKs, and a RING finger domain that classifies Cbl proteins as E3 ubiquitin ligases.\textsuperscript{5,8-10} Ubiquitylation of RTKs is important for their internalization, endocytic sorting, and targeting for degradation.\textsuperscript{2} In addition to their ubiquitin E3 ligase activity, Cbl proteins also associate with the endocytic machinery via their C-terminus by recruiting proteins like CIN85 and endophilins.\textsuperscript{11,12} However, Cbl proteins are not only important for RTK signal termination, but also mediate positive RTK signaling events to downstream effectors. Upon phosphorylation, Cbl molecules bind signaling molecules including SHP-2, Gab2 and PI3-kinase.\textsuperscript{13} In animal models, but not in human cancers, oncogenic forms of Cbl have been described that are characterized by loss of the E3 ubiquitin ligase activity.\textsuperscript{14,15} It has been reported that these oncogenic mutants of Cbl interact with activated RTKs and function in a dominant negative fashion.\textsuperscript{16,17}

Aberrant signaling by the type III RTK Flt3 is an important event in the pathogenesis of AML. Flt3 strongly influences hematopoietic progenitor cell homeostasis and is highly expressed in AML.\textsuperscript{18-21} Also, about one third of AML cases harbour somatic, activating Flt3 mutations that cause myeloid transformation.\textsuperscript{21-24} In contrast to activating mutations,
little is known about the potential functions of Flt3 in AML cases lacking Flt3 mutations. Also, the mechanisms of Flt3 signal regulation and the role of Cbl proteins in these processes remain undetermined, although it has been shown that Flt3 activation is followed by Cbl phosphorylation.25

Here, we analyzed the role of c-Cbl in the internalization, ubiquitylation and biological functions of wild-type Flt3 (Flt3-WT) and the most commonly described Flt3 mutations in AML, internal tandem duplication (Flt3-ITD). We found that the inhibition of Cbl function severely disturbed Flt3 signal transduction kinetics by blocking Flt3 internalization and ubiquitylation. As a consequence, interference with Cbl function induced ligand-independent, autoactive biological effects of Flt3. Also, we describe a novel E3-ligase inactivating c-Cbl mutation isolated from the blasts of one AML patient. This mutant Cbl protein interfered with the function of endogenous c-Cbl and displayed in vitro transforming activity in myeloid cells that was dependent on the presence of Flt3. To our knowledge, this represents the first case of a transforming Cbl mutation in a human cancer specimen, pointing towards a novel mechanism of disturbed RTK signal termination in human cancers that could be amenable to pharmacological kinase inhibition.
Materials and methods

Reagents and antibodies

Recombinant human Flt3 ligand (FL) and recombinant murine IL-3 were purchased from PeproTech (Rocky Hill, NJ). EGF and PDGF ([125I]-labelled and unlabelled) were purchased from GE Healthcare (Munich, Germany). Polyclonal rabbit anti–phospho–Erk-1/2 and anti-phospho-Akt antibodies were obtained from Cell Signaling Technology (Beverly, MA). The antibodies for GST, 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, anti-phosphotyrosine (4G10) 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). Purified anti-human-Flt3 antibody (clone SF1.340) was purchased from Beckman Coulter (Fullerton, CA). Phycoerythrin (PE)–labeled monoclonal mouse anti–human Flt3 antibodies as well as appropriate isotype controls were obtained from PharMingen (San Diego, CA). The Flt3-specific inhibitor SU11248 was a kind gift from Sugen (San Francisco, CA). Cycloheximide was purchased from Sigma (Taufkirchen, Germany).

Cell lines

The IL-3 dependent murine myeloid cell line 32Dcl3 (subsequently referred to as 32D) was kindly provided by Dr. Felicitas Rosenthal (Freiburg, Germany) and was cultured as described.23,26 COS-7 and 293T cells were cultured in Dulbecco’s modified Eagle’s
medium (Invitrogen GmbH, Karlsruhe, Germany). Cells were maintained in a humidified incubator at 37°C and 5% CO₂.

**Generation of stable cell lines**

The generation of 32D cells stably expressing wild-type Flt3 or Flt3-ITD has been previously described.²³,²⁴ For generating Cbl overexpressing 32D cell lines, parental 32D or 32D cells stably expressing Flt3 receptors were electroporated with different Cbl constructs (c-Cbl-WT, Cbl-70Z, Cbl-R420Q), as described.²³ The stable Cbl expressing bulk cultures were selected using neomycin (800 µg/mL). To avoid possible clonal selection, for each cell line, at least two bulk cultures were generated. All cell lines were generated and maintained in medium supplemented with WEHI conditioned medium as source of IL-3 until being used for the experiments.

**Transient transfections**

For transient expression experiments, COS-7 cells were transfected using SuperFect™ (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. At 48 hours post transfection, cells were lysed and analyzed for expression of indicated proteins.

**Patient samples**

The patient samples were collected from patients enrolled in a treatment optimization trial in Germany.²⁷ Control total bone marrow samples were obtained from healthy donors. Written informed consent was obtained from all individuals. The use of human material for scientific purposes was approved by the ethics committee of each participating institution.
RNA isolation, cDNA preparation and sequencing

RNA was isolated from AML blasts using TRIzol reagent (Invitrogen, San Diego, CA). A total of 1 µg RNA from each sample was reverse-transcribed using oligo-d(T) primer and Moloney murine leukemia virus reverse transcriptase (Clontech, Palo Alto, CA). DNA generated by RT-PCR was amplified with the sense primer 5’-CCTGGCTACATGGCTTTTTTG-3’ and the antisense primer 5’-GTCAAGTCTGGTGCTGCCCTAC-3’, yielding a 708-bp PCR product corresponding to nucleotides 796 – 1503 of the published sequence of human c-Cbl. An ABI PRISM BigDye Terminator kit (Applied Biosystems, Darmstadt, Germany) was used according to the manufacturer’s instructions. A standard cycle sequencing protocol with Taq polymerase was performed and analyzed on an automated sequencing system (ABI Prism 3700, Perkin-Elmer). For direct sequencing of the PCR products, the PCR primers were used.

Immunoprecipitations and Western blot analyses

COS-7 or 32D cells transfected with Flt3 and Cbl constructs were washed twice with PBS and starved overnight in medium containing 0.5% FCS and were then stimulated for 10 min at 37°C either with 40 ng/mL FL or 1 ng/mL IL-3. Flt3 immunoprecipitations were performed as described.23,26 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 by SDS-PAGE and probed with the indicated antibodies overnight at 4°C. Lysate preparation and Western blot analyses were performed as described earlier.23,26
GST pull-down and Far Western assays

293T cells were transfected with the indicated constructs and lysed 48 hours after transfection. For GST pull-down assays, lysates were incubated overnight with equal amounts of either GST alone, GST-Cbl-PTB or GST-Cbl-PTB-G306E coupled to sepharose beads. The beads were subsequently washed five times with lysis buffer and the precipitated proteins were then subsequently resolved by SDS-PAGE. After transfer to a PVDF membrane, the membrane was probed for Flt3 and signals were detected by Western Blot.

For Far Western analyses lysates were resolved by SDS-PAGE and the proteins transferred to PVDF. The membranes were then incubated overnight with TBS buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0) containing 5% BSA and then incubated for two hours at room temperature, with purified GST-Cbl-PTB in TBS-T buffer (TBS buffer containing 0.05% Tween-20) containing 2 mM CaCl₂, at a final concentration of 1 µg/mL. After washing the membrane three times with TBS-T buffer, the GST fusion proteins were detected by western blotting using anti-GST antibodies (Santa Cruz, CA).

Flt3 protein half-life degradation analyses

32D cells expressing the indicated Flt3 proteins were cytokine deprived overnight in medium containing 0.5% FCS. Subsequently, cells were then exposed to 50 µg/mL of Cycloheximide for 2 hours at 37°C. Cells were then incubated with 100 ng/mL FL at 37°C. The experiment was stopped by cell lysis at the indicated time points. For densitometry, blots were scanned and the bands were quantified using the GelPro Analyser software (INTAS, Göttingen, Germany) according to the manufacturer’s instructions.
Analysis of cell growth

The 32D cells expressing Flt3 or Cbl constructs were washed twice and resuspended in RPMI 1640 with 10% FCS alone or supplemented with the indicated growth factors at a concentration of $2 \times 10^5$ cells/mL. Viable cells, determined by trypane blue exclusion, were counted daily until day 4. [³H]-thymidine incorporation assays were performed as described previously.²³ Experiments were repeated at least 3 times. Each data point represents the mean ± standard deviation of 3 wells.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuickChange™ site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands) according to the manufacturer’s instructions. To construct Cbl-R420Q, the point mutation was introduced in the c-Cbl cDNA. For generating kinase deficient Flt3 (Flt3-K644A) lysine 644 of Flt3-WT was replaced with alanine. Both mutations were confirmed by sequencing.

Internalization assays

A total of $5 \times 10^5$ 32D cells expressing HA-tagged Flt3 alone or in combination with the indicated Cbl constructs were incubated with FL for the indicated time points. Internalization of the receptor was stopped by quickly 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-HA 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). For ligand-binding assays of the EGFR and PDGFR, CHO cells were transfected with EGFR or PDGFR and c-Cbl-WT, Cbl-R420Q or Cbl-70Z or with GFP as a control. After 48 hours cells were serum deprived for 6 hours and
receptor internalization was induced with 50 ng/mL EGF/PDGF for the indicated times. Cells were transferred on ice and washed with PBS plus 0.1% BSA (pH 3.4) to remove surface-bound EGF/PDGF. Receptors remaining at the cell surface were labeled with 1 ng/mL $[^{125}\text{I}]-\text{EGF}$ or $[^{125}\text{I}]-\text{PDGF}$ for one and a half hours. After rigid washing for five times cells were lysed and analyzed in a gamma counter (1470 Wizard, Perkin Elmer). Each time point was measured in triplicate and receptor downregulation was determined by comparison to the EGFR/PDGFR level on the surface of non-stimulated cells.

**In vitro ubiquitylation**

293T cells were transfected with HA-tagged Flt3 and lysed as described above. HA-tagged Flt3 was then immunoprecipitated, extensively washed with lysis buffer and washed three times with ubiquitylation buffer (UB) containing 50 mM Tris-\(\text{HCl}\) (pH 7.5), 2.5 mM Mg\(\text{Cl}_2\) and 1 mM DTT. 10 µg of immunoprecipitated HA-Flt3 was diluted in UB buffer with 25 µM His-Ub, 100 nM E1 enzyme (Boston Biochem, Cambridge, MA), 500 nM E2 enzyme (Boston Biochem), 4 mM ATP, 2 µg of either purified GST-Cbl-RING, GST-Cbl-70Z-RING or GST-Cbl-R420Q-RING, in a final volume of 25 µL. The reaction mixture was incubated at 30°C for four hours and then analyzed by SDS-PAGE followed by western blotting.
Results

Cbl associates with and is phosphorylated by Flt3

In order to analyze the effects of Cbl on Flt3 signal mitigation, we generated 32D cell lines that stably overexpressed Flt3 together with c-Cbl or a published dominant negative version of c-Cbl, Cbl-70Z. First, we analyzed whether ligand-induced Flt3 activation led to enhanced phosphorylation of c-Cbl. As shown in Figure 1A, Flt3 activation led to marked enhancement of c-Cbl phosphorylation, regardless of activation of Flt3 by mutation or Flt3 ligand (FL), while the dominant negative c-Cbl mutant was significantly phosphorylated even in the absence of exogenously added FL.

Next, we analyzed whether c-Cbl interacted with Flt3-WT and Flt3-ITD. Western blot analyses of Flt3 immunoprecipitates revealed the physical association of c-Cbl with both receptor types (Figure 1B). Furthermore, immunoprecipitation of exogenously overexpressed, hemagglutinin (HA)-tagged c-Cbl constructs with anti-HA antibodies resulted in immunocomplexes that contained Flt3 (Figure 1C). The association of c-Cbl with Flt3-WT was apparently enhanced by the presence of FL, whereas Cbl-70Z, a dominant negative form, strongly associated with Flt3 receptors even in the absence of FL.

We then analyzed whether Flt3 could directly interact with c-Cbl in vitro. GST pull-down experiments revealed that the Cbl phosphotyrosine binding (PTB) domain associated with exogenously overexpressed Flt3 from cellular lysates (Figure 1D). A mutation that is known to interfere with the function of the Cbl-PTB domain (G306E) strongly inhibited its association with Flt3 (Figure 1D). By far western blot experiments, we readily detected
the association of immunoprecipitated Flt3 separated by SDS-PAGE with purified GST-Cbl-PTB fusion proteins, indicating the direct nature of the interaction (Figure 1E).

These data show that activated Flt3 physically associates with c-Cbl and induces its phosphorylation. Interestingly, Cbl-70Z associated strongly with Flt3 receptors and was significantly phosphorylated in Flt3-WT expressing cells even in the absence of FL.

**Cbl facilitates ubiquitylation of Flt3-WT and Flt3-ITD**

Under physiological conditions, activation of RTKs induces receptor internalization and degradation that are important for RTK signal mitigation. Cbl proteins play an important role in these processes.5-7 Thus, we examined whether these mechanisms were intact for constitutively active Flt3-ITD and whether they involved Cbl function. We analyzed the degradation of Flt3-WT and Flt3-ITD in cycloheximide pre-treated cells in the presence or absence of Flt3-ligand (Figure 2A). Previously, it has been shown that Flt3 migrates in SDS-PAGE as two bands that differ in their glycosylation pattern.23,26 These bands correspond to an immature intracellular receptor of 130 kD and the maturely glycosylated receptor of 155 kD on the cell surface. Recently, it has been shown that intracellular activation of Flt3-ITD in the endoplasmic reticulum delayed exit of the receptor from the ER.29 As shown in Figure 2A, degradation of the mature form of Flt3-ITD protein was slightly enhanced, if compared to wild-type Flt3. However, our results indicate that degradation of the intracellular form of Flt3-ITD, but not of Flt3-WT occurred slower than degradation of the presumably cell-surface localized receptors (Figure 2A).

Next, we analyzed whether Cbl proteins are involved in Flt3 ubiquitylation (Figure 2B). For this purpose, HA-tagged ubiquitin was coexpressed with Flt3-WT or Flt3-ITD in COS-7 cells. In immunoprecipitates of the Flt3 and upon overexpression of HA-tagged
ubiquitin (Ubq), we detected the ubiquitylated form of both Flt3-WT and Flt3-ITD. Ubiquitylation of wild-type Flt3 was increased by FL, while the addition of FL to COS-7 cells that expressed Flt3-ITD did not result in an enhancement of Flt3 ubiquitylation. Importantly, coexpression of the dominant negative Cbl-70Z almost abolished ubiquitylation of the receptor, suggesting Cbl proteins as the major E3 ubiquitin ligases for Flt3.

Hence, Cbl protein function is necessary for Flt3 ubiquitylation. The degradation kinetics of Flt3-WT and Flt3-ITD are not grossly different. These data suggest that c-Cbl is involved in the degradation of wild-type and mutationally activated Flt3 proteins.

**Cbl-70Z induces ligand-independent growth in cooperation with Flt3**

Having observed critical importance of Cbl proteins for Flt3 ubiquitylation, we examined whether interference with Cbl function would alter the biological outcome of Flt3 activation. Therefore, we coexpressed the receptor with Cbl-70Z in 32D cells and analyzed their growth and survival. To exclude clonal evolution of the cultures that may lead to a selection of cells with auto-activated Flt3 receptors, we analyzed bulk cultures instead of single clones. We starved cells from IL-3 that stably expressed different c-Cbl forms and/or Flt3 receptors and assessed the transforming potential of Cbl in the presence or absence of Flt3 by analyzing cellular proliferation and viability. Interestingly, cells coexpressing Flt3-WT and Cbl-70Z rapidly proliferated in the absence of exogenous growth factors. However, in the absence of Cbl-70Z, cells expressing Flt3-WT alone proliferated only in the presence of FL (Figure 3A and data not shown). Cultures expressing only Cbl-70Z (without Flt3 receptors) survived longer after cytokine withdrawal (Figure 3B). However, these cultures failed to proliferate in a cytokine
independent manner and died off after a few days of culture in cytokine free media (Figure 3A). These findings suggest that Cbl-70Z conferred apoptosis resistance to these cells but was incapable of inducing significant proliferative activity. Interestingly, coexpression of Flt3-WT and Cbl-70Z rescued proliferation and these cells were able to grow cytokine independently for extended time periods. In contrast, expression of Flt3-WT (in the absence of FL) alone did not lead to proliferation. Finally, we analyzed the effects of Cbl-70Z on cytokine-independent and Flt3-dependent proliferation by directly comparing it with IL-3-dependent proliferation in thymidine incorporation assays (Figure 3C). In the absence of Flt3, Cbl-70Z induced only minimal proliferation (Figure 3C). Coexpression of Cbl-70Z with Flt3-WT induced a robust proliferative response that was further enhanced by Flt3 activation. Importantly, a Flt3-specific inhibitor, SU11248, suppressed proliferation in a dose-dependent manner. Notably, the major effects of Cbl-70Z were again independent from the presence of FL, although they required the presence of the receptor. In contrast to wild-type Flt3, we did not observe any significant synergistic effects of the coexpression of Flt3-ITD with Cbl-70Z, presumably due to full activation of proliferative signals by the constitutively active Flt3-ITD alone (data not shown).

Identification and characterization of a novel Cbl point mutation in AML

The data presented above suggested that interference with Cbl function may be involved in myeloid transformation. In animal models, several oncogenic Cbl mutations have been described, and all are clustered in the RING domain or in the linker region.\textsuperscript{15,30} Therefore, we analyzed the coding sequences of the corresponding exons in c-Cbl and Cbl-b from the blasts of 150 AML patients by direct cDNA and genomic DNA
sequencing. The cDNA and genomic DNA of c-Cbl from one AML patient with a normal karyotype and no Flt3 mutation contained a G to A substitution at position 1259 of the published human c-Cbl sequence that results in the substitution of the charged arginine residue at position 420 with glutamine (R420Q) (Figure 4A). The presence of the mutation was independently verified in the cDNA and the genomic DNA in both directions, using different PCR products and different sequencing primers. Arginine 420 is evolutionary highly conserved and has been suggested to be involved in the contact site of Cbl with the E2 ubiquitin-conjugation enzyme UbcH7.31 We cloned this c-Cbl mutant (Cbl-R420Q) into an expression vector and analyzed its association with Flt3 and the consequences of its presence for Flt3 ubiquitylation and internalization. As shown in Figure 4B, Cbl-R420Q associates with Flt3-WT. Flt3 was readily ubiquitylated after ligand-activation in the presence of wild-type c-Cbl, but not in the presence of Cbl-R420Q (Figure 4B and C). In vitro ubiquitylation assays revealed that in contrast to the wild-type RING domain, the RING domain containing the R420Q mutation was unable to function as an E3 ubiquitin ligase for Flt3 (Figure 4D).

RTK signal mitigation has been shown to be dependent on Cbl mediated receptor internalization and ubiquitylation.5,7,31,32 Therefore, we analyzed the consequences of Cbl-R420Q overexpression for RTK internalization. Interestingly, Cbl-R420Q strongly inhibited ligand-induced internalization of EGFR and PDGFR, a process dependent on intact Cbl function (Figure 4E). Furthermore, Cbl-R420Q inhibited Flt3 internalization to a similar extent as Cbl-70Z.

In conclusion, we have identified a Cbl mutation in primary blasts from an AML patient that effectively inhibits ubiquitylation and internalization of RTKs upon overexpression.
Cbl-R420Q has transforming capacity in the presence of Flt3

We next analyzed the biological significance of the AML-associated Cbl mutation (Cbl-R420Q) in myeloid cells. Similar to Cbl-70Z, Cbl-R420Q mutant induced proliferation and survival of 32D cells (Figure 5A, B and C) and induced long-term, cytokine-independent proliferation of the cultures exclusively in the presence of Flt3. The effects of SU11248 on the biological effects of concomitant Cbl-70Z and Flt3 expression (Figure 3D) indicated that the Flt3 kinase activity was necessary for the observed synergistic biological effects. To exclude the possibility that off-target effects of chemical kinase inhibitors might be responsible for growth inhibition of the cells, we coexpressed a kinase-inactive Flt3 mutant with Cbl-R420Q in 32D cells. As expected from the inhibitor experiments, kinase-dead Flt3 (Flt3-K644A) failed to induce autonomous growth of 32D cells, even in the presence of Cbl-R420Q (Figure 5A, B, and C). We then analyzed the effects of Cbl proteins on the strength, the quality, and the duration of Flt3 signaling (Figure 5D and E). Coexpression of wild-type Cbl with Flt3 receptors led to diminished activation of Akt and Erk signaling. In contrast, coexpression of Cbl-70Z (Figure 5D) or Cbl-R420Q (Figure 5E and data not shown) induced a stronger activation of the Akt and Erk pathways as indicated by phosphorylation of the respective signaling intermediates after 10 minutes. In time course experiments, we observed a significantly prolonged activation of Erk activity up to 60 minutes after FL stimulation in cells coexpressing Cbl-70Z or Cbl-R420Q (Figure 5E). In summary, our data show that the newly identified AML-associated Cbl mutation R420Q disturbs Flt3 signal mitigation comparable to Cbl-70Z, and likewise affects 32D cell growth in an Flt3 kinase dependent manner.
**Discussion**

In human cancers, deregulated activation of receptor tyrosine kinases is a frequent event. Aberrant activation of RTKs can be induced by inappropriate ligand-stimulation, by overexpression due to genetic amplification, or by activating or sensitizing mutations in the coding region of the RTKs.\(^{33-35}\) Also, 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.\(^{36-39}\) The RTK Flt3 is highly expressed in AML and it is widely accepted that deregulated Flt3 activity has important functions in AML pathogenesis. To understand the mechanisms that control Flt3 signaling, we have analyzed the role of c-Cbl for Flt3 regulation. We show that c-Cbl physically associates with Flt3-WT as well as with the most common activating Flt3 mutation, Flt3-ITD. We also show that upon ligand-stimulation, Flt3 is rapidly ubiquitylated and that a known dominant-negative mutant of c-Cbl (Cbl-70Z) inhibits Flt3 internalization and ubiquitylation (Figure 2) with the consequence of a longer duration of Flt3 signaling (Figure 5). Also, we confirm previous results that tyrosine phosphorylation of c-Cbl is enhanced by Flt3 activation.\(^{25}\)

Sequencing analysis of Cbl in blasts from a cohort of AML patients revealed a single case of a somatic mutation in the RING domain of c-Cbl (Cbl-R420Q). Analysis of the amino acid substitution and comparison of the resulting mutant protein with the published structure of the c-Cbl ubiquitin ligase domain suggests that this mutant residue is critically involved in the binding of c-Cbl with the E2 ubiquitin-conjugating enzyme Ubc-H7 and suggested that a mutation at this position may inhibit the E3 ligase activity of c-Cbl.\(^{31}\) Indeed, we present biochemical evidence that the R420Q mutation
strongly inhibits the ubiquitin ligase activity of c-Cbl, the essential function of c-Cbl required for RTK signaling mitigation. Our data also provide evidence that Cbl-mediated regulation of Flt3 is biologically important. Coexpression of Cbl-70Z or Cbl-R420Q with Flt3 readily transformed 32D cells, even in the absence of Flt3-ligand. It has been previously reported that dominant negative Cbl mutants enhance the survival of myeloid cells. However, as in our report, they alone did not support cellular proliferation. Here, we observed a strong synergism between Cbl-70Z or Cbl-R420Q and Flt3 receptors in myeloid transformation. Its exact molecular mechanisms remain to be elucidated. However, our data obtained with an Flt3-specific inhibitor (Figure 3D) and the kinase-inactive Flt3 receptor (Figure 5A) suggest that Flt3 kinase activity is required for the observed synergism. It appears likely that Cbl-70Z and Cbl-R420Q cause a low but constitutive activation of the Flt3 receptor. Since Cbl proteins have been shown to also associate with c-kit and influence c-kit activity, it may well be that the observed synergism not only relates to Flt3, but also to other hematopoietic type III RTKs. Further work will be required to analyze the effects of dominant-negative Cbl proteins in cells expressing various RTKs. Taken together, these data show that Cbl proteins are directly involved in Flt3 signal control and suggest that interference with the function of negative regulators of RTK signaling may play a role in AML transformation.

To our knowledge the novel Cbl-R420Q mutation identified here is the first reported Cbl mutation with obvious transforming ability in human disease. Although we identified this mutation in a single patient, it is possible that alterations in the negative regulatory machinery of RTKs are more frequently involved in leukemogenic signal transduction. First, Cbl proteins consist of a family of several members with presumably redundant
function. Dominant negative mutations in any Cbl family member may induce similar effects as observed for Cbl-R420Q. Also, termination of RTK signaling is a complex multi-step process, which requires several sorting, segregation, and degradation steps, each of which involve many different proteins. 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. Furthermore, inappropriate expression of proteins involved in endocytic sorting (i.e. Hrs and Tsg101) have also been implicated in human cancers. The potent effects of the point mutation of c-Cbl on myeloid cell proliferation and survival warrants further search for such mutations in clonal myeloid disorders, especially in patients without known activating mutations in signaling intermediates.

Originally, c-Cbl was described as a proto-oncogene of an animal virus that induced lymphoma in mice. It has been shown that the corresponding viral oncogene, v-Cbl, is a relatively weak oncogene. V-Cbl is a truncated protein, consisting of the N-terminal half of Cbl, retaining not much more than the PTB domain of c-Cbl. Later, the description of other, more potent Cbl oncogenes has led to the definition of requirements for the transforming potential of Cbl proteins. Presence of the PTB domain and simultaneous interference with the E3 ligase function of c-Cbl have been defined to be essential minimal requirements for Cbl-mediated transformation, presumably through dominant-negative interference with the normal Cbl function. Our biochemical analyses here show that Cbl-R420Q fulfils these requirements.

However, it is not quite clear, whether Cbl-mediated transformation is solely due to dominant-negative effects of oncogenic Cbl proteins, or whether the mutations also
provide a gain of function. Homozygous c-Cbl knockout mice do not display an obvious tumor phenotype,\textsuperscript{5,49} suggesting that the c-Cbl mutations that we analyzed here are not only dominant-negative over c-Cbl. One explanation could be that they are dominant negative over several Cbl family members, circumventing redundancy that could explain the lack of the knockout phenotype.

Alternatively, the transforming phenotype of oncogenic Cbl mutants could be due to an additional gain of function, which provides activating signals to the cells. For example, it has been shown that c-Cbl binds to and activates Cool-1, which on the one hand inhibits Cbl-mediated receptor endocytosis and degradation, and on the other hand functions as a regulated guanine nucleotide exchange factor (GEF) for Cdc42. This (presumably proto-oncogenic) GEF activity is activated by its phosphorylation in Cbl-Cool-1-tyrosine kinase complexes.\textsuperscript{50,51} Furthermore, phosphorylated c-Cbl forms complexes with PI3-K leading to Akt and Erk activation.\textsuperscript{52} The Src family kinases Fyn and Lyn associate more strongly with increasingly phosphorylated c-Cbl.\textsuperscript{53-55} Recruitment of the CrkL adaptor protein by c-Cbl has been reported to facilitate activation of Jnk and Rap1. All these examples illustrate the extensive positive involvement of Cbl proteins in signaling.\textsuperscript{56-58}

Most of these activities have been mapped to the C-terminal multi-adaptor domain, which contains phosphotyrosine sites that can bind to positive signal regulators like SHP2 and PI3-K.\textsuperscript{13,52,56,59} Consequently, oncogenic Cbl proteins that retain the C-terminal part have been shown to be more potent than Cbl oncogenes lacking this domain.\textsuperscript{5,15} Furthermore, the phosphotyrosine sites in the C-terminus are highly phosphorylated in Cbl-70Z, suggesting that this region of Cbl may play an important role in oncogenic properties at least of Cbl-70Z. Interestingly, the novel Cbl-R420Q mutation fulfils all the requirements of potent Cbl oncogenes, since it only consists of a point
mutation in the E3 ligase domain of c-Cbl, maintaining all other protein domains that are potentially involved in positively mediating oncogenic RTK signaling. Importantly, Cbl mutant-mediated transformation depended on the presence of Flt3 and can be blocked by Flt3 kinase inhibitors. Given the near universal importance of the Cbl-associated machinery in RTK signal mitigation, it is very likely that mutations in the involved proteins (like Cbl R420Q) are influenced in their transforming activity by different RTKs. Thus, our data provide an important example for the critical involvement of tyrosine kinases in a transformation mechanism, where the primary genetic defect is localized in the negative regulatory machinery. Hence, patients without activating mutations in RTKs may suffer from RTK-driven disease and may benefit from kinase inhibitor treatment, similar to patients harbouring activating RTK mutations.

Collectively, the data presented here illustrate a novel mechanism of Flt3 mediated transformation in AML through somatic mutation in a protein involved in the termination of RTK signaling. We show that dominant-negative forms of the E3 ligase c-Cbl induce autonomous growth in cytokine-dependent myeloid progenitor cells. However, this effect is strictly dependent on the presence of Flt3 kinase activity and can be targeted by Flt3 kinase inhibitors. Our results justify further analyses of negative tyrosine kinase regulatory networks that may be dysfunctional to cause oncogenic signal transduction in AML.
Acknowledgements

We thank Marion Baas for her excellent technical assistance and the AML Cooperative Group (AMLCG) for providing samples from leukemia patients. We thank Dr. Dirk Bohmann, Dr. Wallace Y. Langdon and Dr. Hamid Band their generous gifts of plasmid constructs used in this study.

BS, CC and HS conceptualized the idea and designed the research. NC and MHHS performed in-vitro GST pull-downs, far Western blot analyses, in-vitro ubiquitylation assays, and internalization assays for PDGF and EGF receptors. BS, CC, MR and CT performed most of the other work presented here. BS, CC, NC, MHHS, LT, JS, CB, BA, SK, SRB, WEB, CMT, ID and HS were involved in data analyses and discussions. BS, CC, BA and HS wrote the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft (Se 600/3-1), the Deutsche Krebshilfe (10-6697), the Medical Faculty of the University of Münster (IMF Sa 110404), and the Fritz Thyssen Stiftung (10.05.2.178).
References


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


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


41. Masson K, Heiss E, Band H, Ronnstrand L. 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. 2006;399:59-67


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


Figure Legends

Figure 1: Flt3 phosphorylates and physically interacts with c-Cbl.

(A) c-Cbl is phosphorylated upon Flt3 activation. The 32D-Flt3-WT or 32D-Flt3-ITD cells stably transfected with HA-tagged Cbl-WT or Cbl-70Z were starved from IL-3 for 12 hours, and were subsequently exposed to Flt3-ligand (FL) for 10 min where indicated. Cbl proteins were immunoprecipitated by anti-HA antibodies and immunoprecipitates were resolved on SDS-PAGE. The phosphorylation of c-Cbl was analyzed using a phospho-tyrosine specific antibody (4G10) and the amount of total immunoprecipitated c-Cbl was analyzed by re-probing the membranes with an anti-HA antibody. (B) and (C) Flt3 physically interacts with c-Cbl. 32D-Flt3-WT or 32D-Flt3-ITD cells expressing endogenous c-Cbl were starved overnight (B). On the next day, cells were exposed to FL as indicated. Flt3 was immunoprecipitated using anti-Flt3 antibody (as control rabbit IgG was used). Coimmunoprecipitation of Cbl was analyzed using anti-Cbl antibody. (C) 32D-Flt3-WT or 32D-Flt3-ITD cells were stably transfected with HA-tagged c-Cbl or Cbl-70Z. Cells were treated as in (B). Cbl was immunoprecipitated using anti-HA antibody (as control mouse IgG was used). Coimmunoprecipitation of Flt3 was analyzed using anti-Flt3 antibodies. (D) and (E) Cbl phosphotyrosine binding domain (PTB) interacts with Flt3-WT in vitro. In Figure D 293T cells were transfected with Flt3-WT and lysed after 48 hours. The lysates were incubated with the indicated GST-fusion constructs coupled to sepharose beads. The precipitated proteins were then resolved by SDS-PAGE and immunoblotted with anti-Flt3 antibodies. Equal loading of the GST-fusion proteins was confirmed by staining the membranes with Ponceau S. In Figure E, cell lysates prepared from 293T cells transfected with the indicated plasmid constructs were
resolved by SDS-PAGE and transferred on to PVDF membranes. The membranes were then incubated with purified GST-Cbl-PTB fusion protein and the binding of GST-fusion proteins was detected with anti-GST antibodies. EGFR was used as positive control.

**Figure 2: c-Cbl facilitates ubiquitylation of Flt3.**

(A) Flt3 receptor is rapidly degraded in 32D cells. The 32D cells expressing Flt3-WT or Flt3-ITD receptors were starved overnight, pre-treated for 2 hours with cycloheximide before incubating with FL for the indicated time points. Cells were harvested at the indicated time points, cell lysates were resolved on SDS-PAGE and Flt3 protein levels were determined using anti-Flt3 antibodies. The densitometric analyses were performed as described in the materials and methods section. The closed circles ("upper") depict degradation of the presumably mature form of Flt3 (160 kDa), while the open circles ("lower") show degradation of the immature form of Flt3 (130 kDa). (B) Ubiquitylation of Flt3 is inhibited by Cbl-70Z. COS-7 cells were transiently transfected with plasmid constructs encoding Flt3-WT or -ITD with or without Cbl-70Z, together with a plasmid encoding HA-tagged ubiquitin. At 48 hours after transfection cells were left unstimulated or were exposed to FL as indicated. Flt3 immunoprecipitates were resolved on SDS-PAGE and ubiquitylation of Flt3 was analyzed by immunoblotting the membranes with an anti-HA antibody.

**Figure 3: Cbl-70Z and Flt3 synergize to induce autonomous growth.**

(A) and (B) Cbl-70Z induces ligand-independent proliferation and survival of 32D-Flt3-WT cells. The 32D cells overexpressing the indicated Flt3 and or Cbl proteins were grown in the presence of 10% FCS alone or supplemented with FL or IL-3. Cells were counted at the indicated time points by the trypane blue exclusion method. The data are
shown as fold change of the cell number compared to the start of the experiment (A). In (B), the proportion of cells in the culture that was alive is shown. The data represent the average and standard deviation of three independent experiments. (C) Ligand-independent DNA synthesis of Flt3-WT cells coexpressing Cbl-70Z. The 32D cells overexpressing Flt3-WT and/or Cbl constructs were starved from IL-3 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. (D) The Flt3-specific inhibitor SU11248 inhibits proliferation of Cbl-70Z coexpressing 32D-Flt3-WT cells. Proliferation of 32D cells expressing Flt3-WT with or without Cbl-70Z was analyzed as described above.

**Figure 4: Cbl-R420Q inhibits ubiquitylation and endocytosis of RTKs.**

(A) Identification of a novel Cbl mutant from an AML patient. The cDNA from 150 AML patients were screened for Cbl mutations by direct sequencing as described in material and methods. c-Cbl of one patient showed a heterozygous mutation at position 1259 (G-A), resulting in replacement of arginine 420 by glutamine of c-Cbl. (B) and (C) Cbl-R420Q binds to Flt3 and inhibits ubiquitylation of Flt3. COS-7 cells were transiently transfected with the indicated plasmids together with a plasmid for HA-tagged ubiquitin. At 48 hours later, cell lysates were prepared and equal amounts of lysate were immunoprecipitated using anti-Flt3 or anti-HA antibodies. The immunoprecipitates were resolved on SDS-PAGE and analyzed with anti-HA or anti-Cbl (B) or anti-Flt3 (C) antibodies. (D) The R420Q mutation results in loss of Cbl E3 ubiquitin ligase activity. The 293T cells were transiently transfected with Flt3 and Flt3 was immunoprecipitated. The immunoprecipitated Flt3 was incubated with His-tagged ubiquitin and the indicated
Cbl RING finger constructs and *in vitro* ubiquitylation was performed as described in the materials and methods section. The reaction mixtures were separated on SDS-PAGE and ubiquitylation of Flt3 was analyzed by immunoblotting the membrane with anti-His antibodies. (E) Internalization of EGFR, PDGFR and Flt3 is inhibited by the mutant Cbl. The surface level of receptors following time-lapse (up to 60 minutes) after EGF, PDGF or FL-stimulation was analyzed by a receptor downregulation assays. The results are expressed as a percentage of the \[^{125}\text{I}]\)-labelled EGF or PDGF bound to cell surface receptor after stimulation by non-labelled EGF or PDGF for the indicated times. For Flt3, the surface expression of HA-tagged Flt3 was analyzed by flow cytometry after staining with an anti-HA antibody. The total level of steady state surface EGFR after 2.5 days of transfection was detected using \[^{125}\text{I}]\)-EGF without preceding incubation with non-labelled EGF.

**Figure 5: Cbl-R420Q induces ligand-independent growth in cooperation with Flt3 and potentiates Flt3-induced signaling.**

(A, B and C) Synergistic induction of ligand-independent proliferation and survival by Flt3-WT + Cbl-R420Q depends on Flt3 kinase activity. The 32D cell lines expressing the indicated constructs were starved overnight and proliferation, viability and thymidine incorporation was analyzed as described in Figure 3. (D) Cbl influence Flt3 dependent signaling. The 32D-Flt3-WT cells 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. (E) Cbl proteins change the kinetics of Flt3-induced Erk activity. Cells were treated as described in (D) with the exception that they were exposed to FL for the indicated time
periods. Western blot analyses using phospho-specific antibodies for Erk1/2 was performed.
Sargin et al., Figure 1
A

<table>
<thead>
<tr>
<th>Time FL (h)</th>
<th>Flt-WT</th>
<th>Flt-ITD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>3</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>6</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>8</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

αFlt3

αActin

Optical Density

<table>
<thead>
<tr>
<th>Signal (% of 0 h)</th>
<th>Flt3-WT</th>
<th>Flt3-ITD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>20</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>40</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>60</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>80</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

Time CHX + FL (h)

B

**IP: αFlt3**

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>Vector</th>
<th>Clb-70Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>− FL</td>
<td>− FL</td>
</tr>
</tbody>
</table>

αHA (Ubq)

Flt3-WT-Ubq

Flt3-ITD-Ubq

Lysates

αHA (Ubq)

Flt3-WT

-175 kDa

Flt3-ITD

-175 kDa

Sargin et al. Figure 2
Sargin et al., Figure 5
Flt3-dependent transformation by inactivating c-Cbl mutations in AML

Bulent Sargin, Chunaram Choudhary, Nicola Crosetto, Mirko H.H. Schmidt, Marion Rensinghoff, Christine Thiessen, Lara Tickenbrock, Joachim Schwable, Christian Brandts, Benjamin August, Steffen Koschmieder, Srinivasa Rao Bandi, Wolfgang E. Berdel, Carsten Muller-Tidow, Ivan Dikic and Hubert Serve