# CBL Exon 8/9 Mutants Activate the FLT3 Pathway and Cluster in Core Binding Factor/11q Deletion Acute Myeloid Leukemia/ Myelodysplastic Syndrome Subtypes

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**Abstract**

**Purpose:** CBL is a negative regulator of activated receptor tyrosine kinases (RTK). In this study, we determined the frequency of CBL mutations in acute leukemias and evaluated the oncogenic potential of mutant CBL.

**Experimental Design:** The cDNA of 300 acute myeloid leukemia (AML)/myelodysplastic syndrome (MDS) and acute lymphoblastic leukemia (ALL) patients and 82 human leukemic cell lines was screened for aberrations in the linker and RING finger domain of CBL. The oncogenic potential of identified mutants was evaluated in hematopoietic cells.

**Results:** We identified 3 of 279 AML/MDS patients expressing CBL exon 8/9 deletion mutants. Three of four cases at diagnosis expressed deleted transcripts missing exon 8 or exon 8/9. In remission samples a weak or no expression of mutant CBL was detected. No aberrations were found in normal hematopoietic tissues. One of 116 sequenced AML/MDS cases carried a R420G missense mutation. All AML/MDS patients with identified CBL mutants belonged to the core binding factor and 11q deletion AML subtypes. Functionally, CBL negatively regulated FMS-like tyrosine kinase 3 (FLT3) activity and interacted with human FLT3 via the autophosphorylation sites Y589 and Y599 and colocalized *in vivo*. Expression of CBLΔexon8 and CBLΔexon8+9 in FLT3-WT-Ba/F3 cells induced growth factor – independent proliferation associated with autophosphorylation of FLT3 and activated the downstream targets signal transducer and activator of transcription 5 (STAT5) and protein kinase B (AKT). FLT3 ligand – dependent hyperproliferation of CBL mutant cells could be abrogated by treatment with the FLT3 PTK inhibitor PKC412 (midostaurin).

**Conclusion:** CBL exon8/9 mutants occur in genetically defined AML/MDS subtypes and transform hematopoietic cells by constitutively activating the FLT3 pathway. This phenotype resembles the one of mutated RTKs and suggests that CBL mutant AML patients might benefit from treatment with FLT3 PTK inhibitors.

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CBL, a known negative regulator of activated receptor tyrosine kinases (RTK), is localized on human chromosome 11q23, a region frequently associated with chromosomal aberrations. Translocations t(4;11) and t(11;14), and mixed-lineage leukemia fusion genes involving CBL have been described in patients with leukemia and lymphoma (1–3). CBL oncogenes were initially identified in the murine system. CBL-70Z, carrying an internal deletion of 17 amino acids, was isolated from the 70Z/3 mouse pre-B-cell lymphoma cell line (4). CBL-70Z deregulates the cellular tyrosine kinase machinery, as NIH3T3 serum-starved cells expressing CBL-70Z showed significantly increased endothelial growth factor receptor (EGFR) kinase activity after EGF stimulation (5). p95CBL, expressed in the murine reticulum sarcoma cell line J-774, lacks internal 111 amino acids, comprising whole exons 8 and 9 (6). CBL70Z and p95CBL mutations both target the linker and RING finger domain, which points to a mutation-sensitive region within the CBL protein. Recently the first human CBL mutation has been reported in a patient with acute myeloid leukemia (AML; ref. 7). The point mutation R420Q targets a conserved arginine

## Translational Relevance

In this article we provide detailed insight into the role of oncogenic CBL exon8/9 mutations in the pathogenesis of acute myeloid leukemia (AML)/myelodysplastic syndrome (MDS), but we could not find CBL mutations in our acute lymphoblastic leukemia cohort. Analysis of a large cohort of AML/MDS patients of different cytogenetic subgroups revealed an overall low frequency of CBL exon 8+9 deletions of 1.1%. However, we identified preferential association of CBL exon8/9 mutations with core binding factor leukemias and 11q aberrations.

We describe that all known human CBL deletion mutants lead to transformation of wild-type FLT3–expressing hematopoietic cells. This phenotype resembles that seen with activated receptor tyrosine kinases. Therefore, it is of particular note that analysis of CBL should be introduced in routine leukemia diagnostics, as patients who harbor CBL mutations might benefit from treatment with FLT3 protein tyrosine kinase inhibitors.

in the RING finger domain (8, 9) and was shown to inhibit FMS-like tyrosine kinase 3 (FLT3) internalization and ubiquitylation (7). Additional screenings of AML patients revealed more cases with CBL mutations, including internal deletions of exon 8 and point mutations targeting G375 (10, 11). Activating FLT3 mutations represent one of the most common genetic alterations in AML and are associated with a poor prognosis (12). Overexpression of wild-type FLT3 (FLT3-WT) has been shown to synergize with other oncogenic fusions to induce aggressive AML in mice (13). Because of these reasons, FLT3 is a prominent target for therapeutic intervention. Currently two compounds targeting FLT3 kinase activity, PKC412 (midostaurin) and CEP701 (lestaurtinib), have reached clinical phase III (14). As patients that do not carry activating FLT3 mutations benefit from treatment with FLT3 inhibitors (15), we hypothesized that other mechanisms, such as CBL mutations, might contribute to the oncogenic potential of FLT3.

Here, we report a frequency of 1.1% of CBL exon8/9 mutations in AML/ myelodysplastic syndrome (MDS). Three of the four newly diagnosed AML/MDS patients expressed CBL lacking exon 8 or exon 8/9 and one patient carried a R420G missense mutation. Aberrant CBL transcripts were lost in remission samples. CBL is part of the human FLT3 downstream signaling pathway and promoted down-regulation of FLT3 activity. Finally, CBL deletion mutants CBLΔExon8 and CBL exon8/9 transformed Ba/F3 cells in the presence, but not in the absence of FLT3-WT.

## Materials and Methods

***Analysis of cell lines and patient samples.*** The study design adhered to the principles of the Helsinki Declaration and was approved by the ethics committees of participating institutions. Cell culture, cDNA, and genomic DNA synthesis were done as described previously (16). PCR screening of cDNA was done with forward primer 5′-CATCCTGGCTACATGGCTTT-3′, localized in exon 5, and reverse primer 5′-GCAGAAGCACTTGAGGGAAC-3′, localized in exon 10, yielding a 752-bp PCR product corresponding to nucleotides 793 to 1544 of the published human CBL sequence (genebank accession number NM_005188). Sequencing was done using the forward primer. For screening of normal human hematopoietic tissues the Human MTC panel II (Clontech) was used. For analysis of gDNA a 8050-bp fragment was amplified using forward primer 5′-GACTAATGTGGCCTACAAATCCTAA-3′, localized in intron 6, and reverse primer 5′-GTTGGTTAATTCTCTTCCTGTCCA-3′, localized in exon 11. Sequencing was done using the forward and reverse primer as well as the following primers: 5′-CTGTTAACATTTATAATTGCAGT-3′, 5′-GACAGATATTGTGTTAAGCAC-3′, 5′-GAGTGAGTTGTGATTGTACC-3′. 5′-TAGCTGGATGGTAGGTAAGT-3′, 5′-ATAGTAGTATGTGATACCGTG-3′, 5′-TTCCTCATCCTTGATTTGATG-3′, 5′-GCTTGTTAGGGAGTTGTGG-3′, 5′-CAAAGAGTTAAAGATGAGAGC-3′, 5′-GATCTCACTATGTTGACAAGGC-3′. PCR fragment analysis was done as described previously (17) with forward primer 5′-TGGCTTATGTGAACCAACTCC-3′ in exon 7 and reverse primer 5′-CTTGAGGGAACACATACTCG-3′ in exon 10 yielding peaks at 483 (CBL-WT), 353 (CBLΔexon8) and 148 (CBLΔexon8/9) nucleotides.

***Reagents and cell lines.*** The 70Z/3 B-cell lymphoma cell line was kindly provided by Dirk Eick, Helmholtz Center, Munich, and was maintained in RPMI-1640 medium supplemented with 20% fetal bovine serum, 1% Pyruvat, and 50 μmol/L mercaptoethanol. All other cell lines were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) or kindly provided by Hans Drexler, DSMZ. AML cell lines were maintained in RPMI-1640 medium supplemented with 20% fetal bovine serum, murine Ba/F3 cells in RPMI-1640 medium supplemented with 10% fetal bovine serum, and 10% WEHI-3B conditioned medium as a source of murine interleukin 3 (IL-3). PKC412 (midostaurin) was provided by Novartis Pharma AG, FLT3 ligand was purchased from Promocell, and LY294002 and rapamycin came from Calbiochem.

***Antibodies.*** The following antibodies were used: anti-CBL (C-15; sc-170), normal rabbit IgG (sc-2027), anti-phospho-tyrosine (pY99; sc-7020), anti-STAT5 (sc-835), anti-FLT3 (S18; sc-480), anti-hemagglutinin (Y-11; sc-805), all from Santa Cruz Biotechnology; anti-phospho-STAT5-Tyr694 (9351), anti-phospho-AKT (Ser473; 4060), anti-AKT (9272), anti-phsopho-p44/42 MAPK (Thr202/Tyr204; 4370), and anti-p44/42 MAPK (9102), all from New England Biolabs; and anti-V5 (R-960-25) from Invitrogen.

***DNA constructs and vectors.*** The human FLT3-WT construct was kindly provided by Gary Gilliland (Boston, USA). The human hemagglutinin-tagged CBL-WT and hemagglutinin(HA)-tagged CBL-70Z constructs were kindly provided by Ivan Dikic (Frankfurt, Germany). CBLΔexon8 and CBLΔexon8+9 were cloned from the CBL-WT construct and engineered to express a V5/His-tag. Point mutations targeting R420 and G375 were introduced into the CBL-WT cDNA, and K644R, Y589F, and Y599F into the FLT3-WT cDNA using the QuikChange SiteDirected Mutagenesis Kit. FLT3 constructs were subcloned in the MSCV-IRES-EYFP and CBL constructs in the MSCV-IRES-EGFP retroviral expression vectors. For immunofluorescence experiments V5-FLT3-WT and HA-wild-type CBL were subcloned in pcDNA6A (Invitrogen, USA). The correct sequence of all constructs was confirmed by nucleotide sequencing.

***Transient transfection of 293 cells, stable transduction of Ba/F3 cells.*** These experiments were carried out as described previously (16). Expression of all constructs was confirmed by Western Blot analysis. FLT3 expression was additionally verified by fluorescence-activated cell sorting analysis with CD135-phycoerythrin antibody (Immunotech).

***Cell proliferation assays of Ba/F3 cells.*** Trypan blue exclusion was done as described previously (16). For proliferation assays using WST-1 reagent (Roche) 2,000 cells were seeded in 100 μL of medium. After 72 h 10 μL WST-1 were added to the cells. After 4 h at 37°C proliferation was assessed spectophotometrically using an ELISA reader. Each proliferation experiment was carried out at least three times independently.

***TaqMan PCR analysis.*** Quantification of CBL expression in Ba/F3 cells was analyzed by real-time quantitative-PCR with primers from Applied Biosystems as described previously (18).

*Western blot analysis, immunoprecipitation experiments.* These experiments were done as described previously (16).

*Immunostaining and confocal laser scanning fluorescence microscopy.* For intracellular localization studies, U2OS human osteosarcoma cells were grown on coverslips and cotransfected with V5-FLT3 and HA-tagged CBL expression plasmids as described above. After 48 h the cells were stimulated with 100 ng/mL FLT3 ligand while control cells where left untreated, and 30 min after stimulation the cells were fixed with 2% paraformaldehyde in PBS for 10 min, permeabilized with PBS 0.1% Triton X for 10 min, and blocked with PBS 10% FCS for 1 h. Coverslips were incubated overnight with polyclonal rabbit HA antibodies (Santa Cruz) and monoclonal mouse V5 antibodies (Invitrogen). Following extensive washing with PBS, Alexa 555 and Alexa 488 conjugated secondary antibodies were added for 1 h. After further washing steps, the cells were stained with 4′,6-diamidino-2-phenylindole and mounted using Cytomat medium (DAKO). Finally, immunostained species were analyzed in a confocal fluorescence laser scanning system (TCS-SP2 scanning system and DM IRB inverted microscope; Leica).

*FLT3 receptor internalization.* $3 \times 10^5$ cells were stimulated with 100ng/100 μL FLT3 ligand in PBS with 5% fetal bovine serum for indicated time periods. The reaction was stopped by adding 1mL 0.1% sodium azide in PBS on ice. After extensive washing the cells were stained with CD135-phycoerythrin antibody for fluorescence-activated cell sorting analysis.

All conducted experiments were verified by replication.

## Results

***A subset of AML/MDS patients carries mutations in the proto-oncogene CBL.*** Recent reports have indicated a putative contribution of CBL mutations to the pathogenesis of human leukemias (7, 10). To obtain a comprehensive insight into the role of CBL in human acute leukemias, we firstly did a PCR screening of the cDNA of 61 AML and 21 acute lymphoblastic leukemia (ALL) cell lines (Table 1; Supplementary Table S1) for deletions in the linker and RING finger domains. We identified a monoallelic CBL deletion transcript in the AML cell line MOLM-13 and the sister cell line MOLM-14. Sequencing of the aberrant transcripts revealed loss of the complete coding sequence of exon 8. All other analyzed cell lines expressed exclusively wild-type CBL (CBL-WT). Secondly, we used the above PCR screening assay to analyze a well-characterized cohort of AML/MDS and ALL patient samples for the presence of aberrant CBL transcripts (Table 1A). Among 279 AML samples of different cytogenetic subtypes and 21 T-cell ALL samples we detected three AML/MDS patients expressing CBL-WT as well as an aberrant CBL transcript (Table 1B). Patient 1 with a t(8;21) (AML1/ETO positive) and patient 2 with an 11q deletion both expressed a CBL transcript lacking exon 8, and patient 3 with an inv(16) (CBFB/MYH11 positive) expressed CBL lacking exon 8 and 9 (Table 1B; Fig. 1A). By PCR fragment analysis we determined the relative expression of mutant versus CBL-WT. For patient 1 we detected 40% mutant versus 60% wild-type transcript. For patient 2 a 20% mutant versus 80% wild-type transcript was observed, supposedly due to the low blast count (20%) in the patient sample. Patient 3 showed equal ratios of mutant versus wild-type transcripts (data not shown). To verify that the shorter CBL transcripts were expressed in the malignant cell population, we analyzed remission samples in the case of patients 1 and 3. Conventional PCR screening of remission samples from patient 1 showed a weak expression of the transcript lacking exon 8 (Fig. 1B). PCR fragment analysis revealed that still 15% to 20% of the mutant CBL was present in the remission sample (data not shown). In case of patient 3 the PCR screening as well as the fragment analysis showed loss of the mutant CBL transcript lacking exon 8 and 9 in remission. To exclude the possibility of normally occurring splice variants, we analyzed the cDNA of normal human hematopoietic tissues, in detail normal bone marrow, peripheral leukocytes, spleen, and thymus. All of the analyzed tissues expressed exclusively CBL-WT transcripts (data not shown).

**Table 1.**

**A. Analyzed AML/MDS and ALL cell lines and primary patient samples**

| | Cell lines | Patient samples |
|---|---|---|
| AML/MDS | 61* | 170 normal karyotype<br>36 t(15;17)<br>22 t(8;21)<br>21 inv(16)<br>17 complex karyotype<br>13 11q aberrations |
| ALL | 21* | 21 T-ALL |
| Total | 82 | 300 |

**B. Cytogenetic findings in AML/MDS patients positive for CBL mutations**

| Patient | Cytogenetic subgroup | Detected aberrant CBL transcript |
|---|---|---|
| 1 | t(8;21) | Δ Exon 8 |
| 2 | 11q aberration | Δ Exon 8 |
| 3 | inv(16) | Δ Exon 8+9 |
| 4 | 11q aberration | R420G |

NOTE: The cDNA of 82 human leukemic cell lines and 300 ALL and AML/MDS patient samples of different subtypes was analyzed by PCR screening with primers spanning whole exon 7, 8 and 9 of CBL. Four AML/MDS patients were found positive for aberrant CBL transcripts. Two patients belong to CBF leukemias [t(8;21); inv(16)] and two patients harbor 11q aberrations.
*A detailed listing of all cell lines is given in Supplementary Table 1.

In addition to the PCR screening, a subset of 116 PCR products from AML/MDS patients of all subtypes were analyzed by nucleotide sequencing comprising the region of exon 7, 8 and 9. One AML/MDS patient with an 11q aberration showed a substitution of arginine to glycine at position 420, which is located in the RING finger domain (Table 1B, Fig. 1A).

In summary, we identified 2 of 61 AML cell lines [3.3% (0.4%, 11.3%)] and 3 of 279 (1.1%) AML/MDS patients expressing aberrant CBL transcripts deleted in the linker and RING finger domains of CBL. Aberrant CBL transcripts could not be detected in any of the analyzed ALL cell lines and ALL patient samples.

A murine CBL deletion transcript has been described in the 70Z/3 B-cell lymphoma cell line (CBL-70Z lacking the first 17 amino acids of exon 8; ref. 4). In the 70Z/3 line the causative event is a splice site mutation, changing the splice acceptor site AG to CG, generating an alternative splice site, which excludes the first 17 amino acids of exon 8 (Fig. 1A). Analysis of the genomic DNA of MOLM-13 cells detected a deletion of 14 nucleotides at the exon 8/intron 8 boundary eliminating a

splicing element leading to expression of the CBLΔexon8 mutant (data not shown), as had been described previously (10).

By amplifying a 8,050-bp fragment of genomic DNA spanning intron 6 to exon 11 and subsequent nucleotide sequencing, we detected mutations in all three patients expressing aberrant CBL transcripts (data not shown). One allele of patient 1 (CBLΔexon8) harbored a deletion of 43 nucleotides at the exon 8-intron 8 boundary, including the last 36 nucleotides of exon 8 and the first 7 nucleotides of intron 8, thereby losing the whole splice donor site of intron 8. Analysis of the genomic DNA of patient 2 (CBLΔexon8) revealed a point mutation of the splice donor site of intron 8, changing the conserved "GT" to "GC" in about 10% of the amplified DNA. The sample from patient 2 contained only 20% blasts, suggesting that this mutation occurred in one of the CBL alleles of the malignant clone. Patient 3 (CBLΔexon8+9) carries a monoallelic large genomic deletion of 1,680 nucleotides, starting in intron 7 and ending in intron 9 leading to the loss of exon 8 and 9.

In conclusion, all CBL transcripts, which lack whole exons, can be explained by genomic events that target splice donor or acceptor sites by mutations or deletions.

***CBL interacts with the FLT3 receptor and contributes to FLT3 downstream signaling.*** As CBL is known to be involved in the signal transduction of class III RTKs, platelet-derived growth factor receptor A (PDGFRA), FMS (colony forming growth factor 1 receptor), and KIT (stem cell factor receptor) (19–21), we hypothesized that CBL mutants might play a role in human FLT3 downstream signaling. Lysates of FLT3-WT expressing Ba/F3 cells with or without FLT3 ligand stimulation were analyzed for the phosphorylation status of endogenous CBL. We could show that activation of human FLT3 after binding of FLT3 ligand leads to high levels of phosphorylated CBL in FLT3-WT–expressing cells, whereas the kinase-dead mutant receptor (FLT3-R644K) cells do not show phosphorylated CBL (Fig. 2A). To localize the interaction site within the FLT3-WT receptor we substituted two essential *in vivo* autophosphorylation sites of FLT3, tyrosines Y589 and Y599 (22), with phenylalanine. FLT3Y589F and FLT3Y599F receptor–expressing cells show a remarkable decrease of CBL phosphorylation after FLT3 ligand stimulation compared with FLT3-WT–expressing cells (Fig. 2A). We conclude that Y589 and Y599 participate in the interaction of the FLT3 receptor with CBL. To detect interaction of FLT3 and CBL, we did coimmunoprecipitation experiments with lysates from FLT3 ligand–stimulated cells expressing V5-tagged FLT3-WT alone or together with CBL-WT or CBL-70Z. Figure 2B shows that CBL-WT and CBL-70Z, interact with the FLT3 receptor. The FLT3 receptor is generally detected as two distinct bands, the mature glycosylated form at 155 kDa and the immature cytoplasmic form at 130 kDa (23). The interaction between CBL-WT and CBL-70Z with FLT3 occurred predominantly with the mature form of FLT3 (higher band). To study the interaction between the FLT3 receptor and CBL at the cellular level, we did *in vivo* colocalization experiments using immunofluorescence of FLT3 and CBL-WT. In U2OS human osteosarcoma cells transiently transfected FLT3 localizes to the cell membrane and the cytoplasm (Fig. 2C). In the cytoplasm FLT3 accumulates in the perinuclear endoplasmatic reticulum, consistent with recent reports (24). CBL shows a diffuse cytoplasmic distribution. Stimulation of the cells with FLT3 ligand results in internalization of FLT3, which is indicated by loss of FLT3 staining in the cell membrane and formation of endosomal vesicles. Upon addition of FLT3 ligand, CBL clearly colocalizes with internalized FLT3 in the endosomal vesicles.

CBL regulates the function of EGFR via ubiquitin-dependent degradation of EGFR (25, 26). Therefore, we sought to investigate if CBL negatively regulates human FLT3. Two hundred and ninety-three cells were transiently transfected with FLT3-WT alone or FLT3-WT and CBL-WT and stimulated with FLT3 ligand for different time periods. Lysates were analyzed for FLT3 phosphorylation and total levels of FLT3 protein. Figure 2D shows constant phosphorylation and levels of FLT3 protein in FLT3-WT–transfected cells up to 120 minutes,

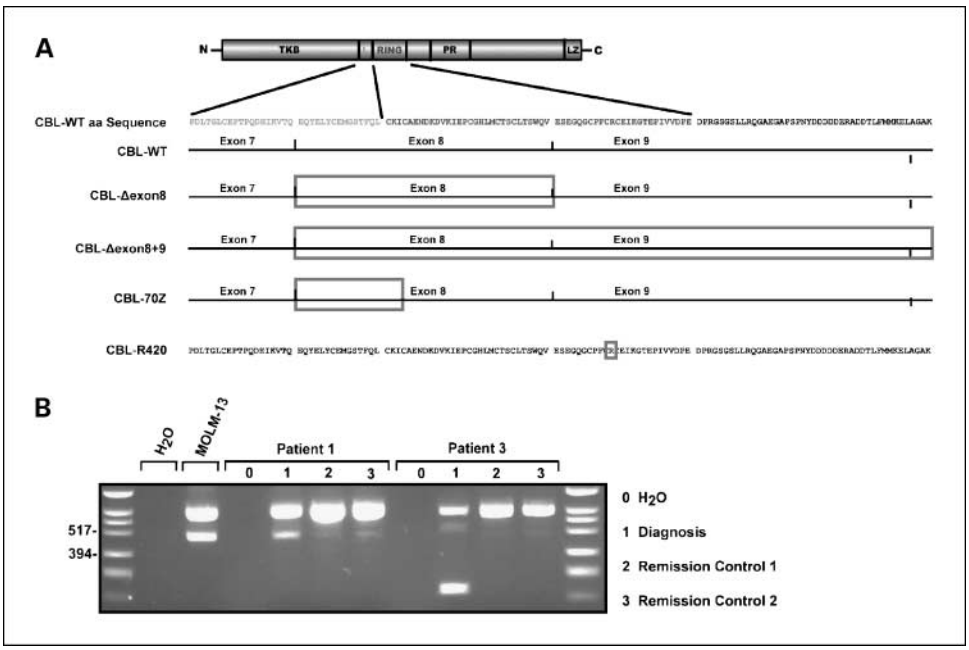


**Fig. 1.** Identified CBL mutants in AML patients. Aberrant CBL transcripts are lost in remission. *A,* the top panel shows the CBL-WT protein sequence from amino acids 346 to 477 comprising the COOH-terminal part of exon 7 and whole exon 8 and 9, which include the linker (L) and RING finger domains. TKB, tyrosine kinase binding domain; PR, proline-rich domain. Shown below are the CBL deletion mutants, CBLΔexon8 and CBLΔexon8+9, found in AML patients, CBL-70Z, a known oncogenic form of CBL, comprising a deletion of the first 17 amino acids within exon 8, and the location of R420, found to be mutated in one AML patient in our study. Red squares, mutated region within the CBL protein. *B,* gel electrophoresis of cDNA PCR products amplified with primers spanning the sequences encoding the linker and RING finger domains of CBL-WT. Patient samples at diagnosis and each two remission controls of patient 1 and patient 3 were analyzed for deletions. MOLM-13 cells served as a positive control.

whereas the phospho-tyrosine signal is comparably less in double-transfected cells after 60 minutes and barely detectable after 120 minutes. The total protein levels of FLT3 are significantly reduced after 90 minutes of FLT3 ligand stimulation in the double-transfected cells compared with FLT3-WT–transfected cells, implicating an enhanced degradation of FLT3 by CBL.

***CBL mutants confer a transforming potential to Ba/F3 cells expressing FLT3-WT.*** In light of the direct interaction between FLT3 and CBL and the modulation of FLT3 signaling by CBL, we hypothesized that CBL mutants could activate the oncogenic potential of FLT3-WT in AML blasts.

The following CBL mutants found in AML/MDS patients and leukemic cell lines, CBLΔexon8, CBLΔexon8+9 and CBLR420 and CBLG375 point mutants, as well as CBL-WT and CBL-70Z as a positive control, were generated and subcloned into MSCV-GFP. FLT3-WT was subcloned into MSCV-YFP vector. All CBL constructs were stably expressed alone or together with FLT3-WT in Ba/F3 cells. Positive selection was done by fluorescence-activated cell sorting. The expression of transduced human CBL in FLT3-WT/CBL coexpressing cells was higher than the expression of endogenous CBL, as analyzed by real time quantitative-PCR (Supplementary Fig. S1A) and verified by Western Blot analysis for the CBLΔexon8+9 mutant (Supplementary Fig. S1B). Expression of any single CBL construct alone or coexpression of CBL-WT and FLT3-WT did not confer growth factor independent proliferation to Ba/F3 cells (data not shown). However, coexpression of CBL-70Z, CBLΔexon8 or CBLΔexon8+9 with FLT3-WT led to significant IL-3 independent proliferation (Fig. 3A). Compared with the CBL deletion mutants, expression of CBL R420G, R420Q, G375S, and G375P point mutants did not confer any proliferative capacity, but enhanced cell survival, as 50% of cells survived for 3 days after IL-3 withdrawal compared with 10% survival of the control cells (data not shown).

The presence of FLT3 ligand induced a strong dose-dependent proliferation of FLT3-WT/CBL-70Z, FLT3-WT/CBLΔexon8, and FLT3-WT/CBLΔexon8+9 cotransduced cells

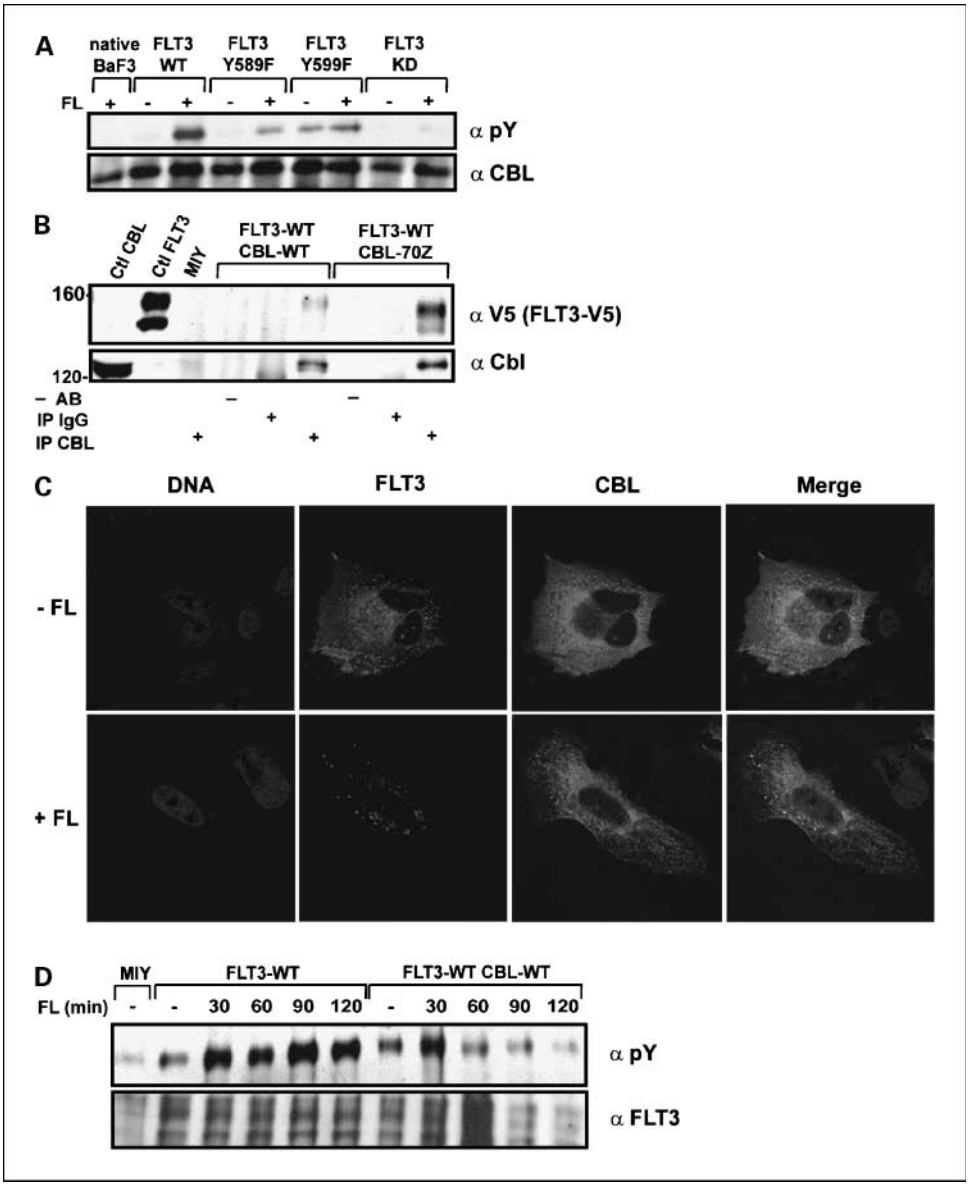


**Fig. 2.** CBL is phosphorylated after activation of human FLT3-WT, directly interacts with FLT3 through tyrosines Y589 and Y599, and negatively regulates FLT3WT. *A,* unstimulated (-) or FLT3 ligand (FL) – stimulated (+; 5 min; 100 ng/mL) Ba/F3 cells expressing FLT3-WT, FLT3Y589F, FLT3Y599F, or FLT3K644R were lysed, and endogenous CBL protein was immunoprecipitated with polyclonal CBL antibody and analyzed for the tyrosine phosphorylation status by immunoblotting with phospho-Tyrosine antibody. *B,* 293 cells were transfected with MIY (control), V5-tagged FLT3-WT CBL-WT or V5-tagged FLT3-WT CBL-70Z and stimulated with 100 ng/mL FLT3 ligand for 5 min. Coimmunoprecipitation of CBL and FLT3 was done with polyclonal CBL antibody. After separation on SDS-PAGE and blotting on nitrocellulose membrane, V5-tagged FLT3-WT was detected with monoclonal V5 antibody. Control samples contained either no or unspecific IgG antibody. *C,* confocal laser scans of immunostained U2OS cells that coexpress FLT3 and CBL. DNA is visualized by 4′,6-diamidino-2-phenylindole counterstain. Upper panel (unstimulated), localization of FTL3 (*red*) in the cell membrane and also in the cytoplasm, whereas CBL (*green*) is diffusely distributed throughout the cytoplasm. Lower panel (stimulated), internalization of FLT3 after stimulation with FLT3 ligand (100 ng/mL for 30 min). Colocalization of CBL and FLT3 is indicated by yellow color of the merged image. *D,* 293 cells were transfected with MIY (control), FLT3-WT, or FLT3-WT and CBL-WT together. After 48 h the cells were unstimulated (-) or stimulated for 30, 60, 90 or 120 min with FLT3 ligand. FLT3 protein was immunoprecipitated from cell lysates, resolved by SDS-PAGE, and blotted for phospho-tyrosine, stripped and reblotted for FLT3.

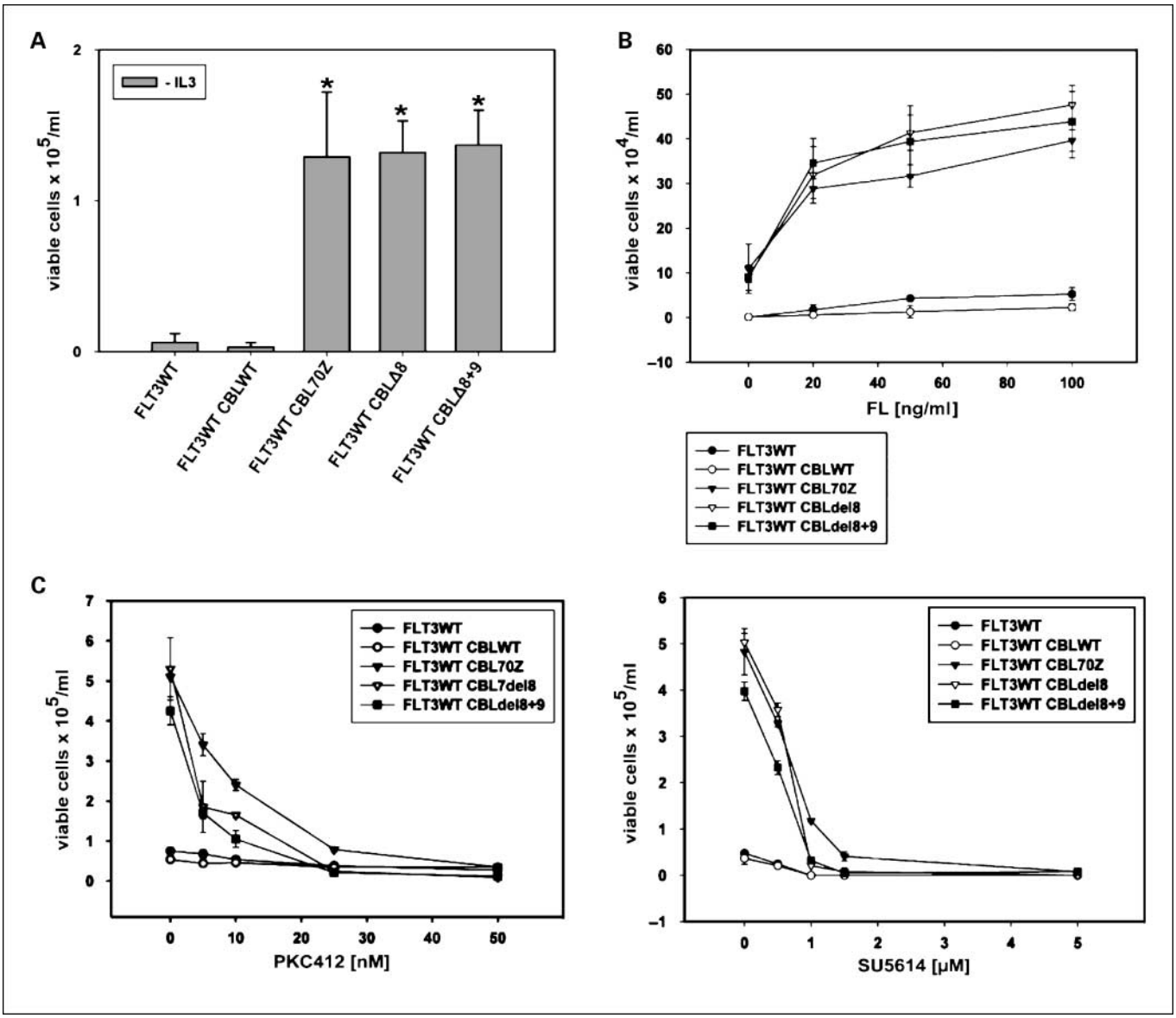


**Fig. 3.** CBL deletion mutants induce a transforming potential in FLT3-WT – expressing Ba/F3 cells and respond to FLT3 PTK inhibitor treatment *A,* cells stably transduced with FLT3-WT alone or FLT3-WT and CBL-WT, CBL-70Z, CBLΔexon8 or CBLΔexon8+9 were cultured in the absence of IL3 for 72 h and viable cells counted after trypan blue exclusion. *B,* cells were analyzed according to *A*, but different concentrations of FL up to 100 ng/mL were added to the culture medium. *C,* cells were analyzed according to *A*, but 50 ng/mL FLT3 ligand and different concentrations of the FLT3 inhibitor PKC412 up to 50 nmol/L or SU5614 up to 5 μmol/L were added to the culture medium. Three independent experiments were done.

with a proliferation rate about 4 to 6 times higher than the control cells (Fig. 3B). The FLT3-WT/CBLG375 cotransduced cells showed no increase in proliferation rate compared with FLT3-WT cells. The FLT3-WT/CBLR420Q mutant cells showed a 2-fold enhanced proliferation rate compared with FLT3-WT cells (data not shown).

Incubation of the FLT3-WT/CBL mutant coexpressing cells with the FLT3 inhibitor PKC412 in increasing nontoxic concentrations up to 50 nmol/L or the FLT3 inhibitor SU5614 up to 5 μmol/L in the presence of 50 ng/mL FLT3 ligand led to a dose-dependent abrogation of the proliferation (Fig. 3C). Coexpression of a kinase-dead FLT3 receptor, FLT3K644R, with CBL-70Z provided further evidence that the FLT3 kinase activity is required for the transformed phenotype, as it did not lead to IL-3 – independent proliferation (data not shown).

Collectively, the overexpression of CBL-70Z, CBLΔexon8 or CBLΔexon8+9 in FLT3-WT – expressing Ba/F3 cells led to factor independent growth and FLT3 ligand – dependent hyperproliferation.

***Coexpression of CBL deletion mutants with FLT3-WT autoactivates the FLT3 receptor.*** The known classes of FLT3 mutations have been shown to autoactivate the kinase activity of FLT3 (16, 27 – 30). We prepared lysates of FLT3 ligand – stimulated and nonstimulated Ba/F3 cells coexpressing FLT3-WT and a CBL deletion mutant, immunoprecipitated the FLT3 receptor, and analyzed it for tyrosine phosphorylation. In contrast to FLT3-WT- or FLT3-WT/CBL-WT – expressing cells, all three CBL mutant – expressing cells displayed autophosphorylated FLT3 in the absence of FLT3 ligand (Fig. 4A). Stimulation with FLT3 ligand induced high phosphorylation levels of FLT3

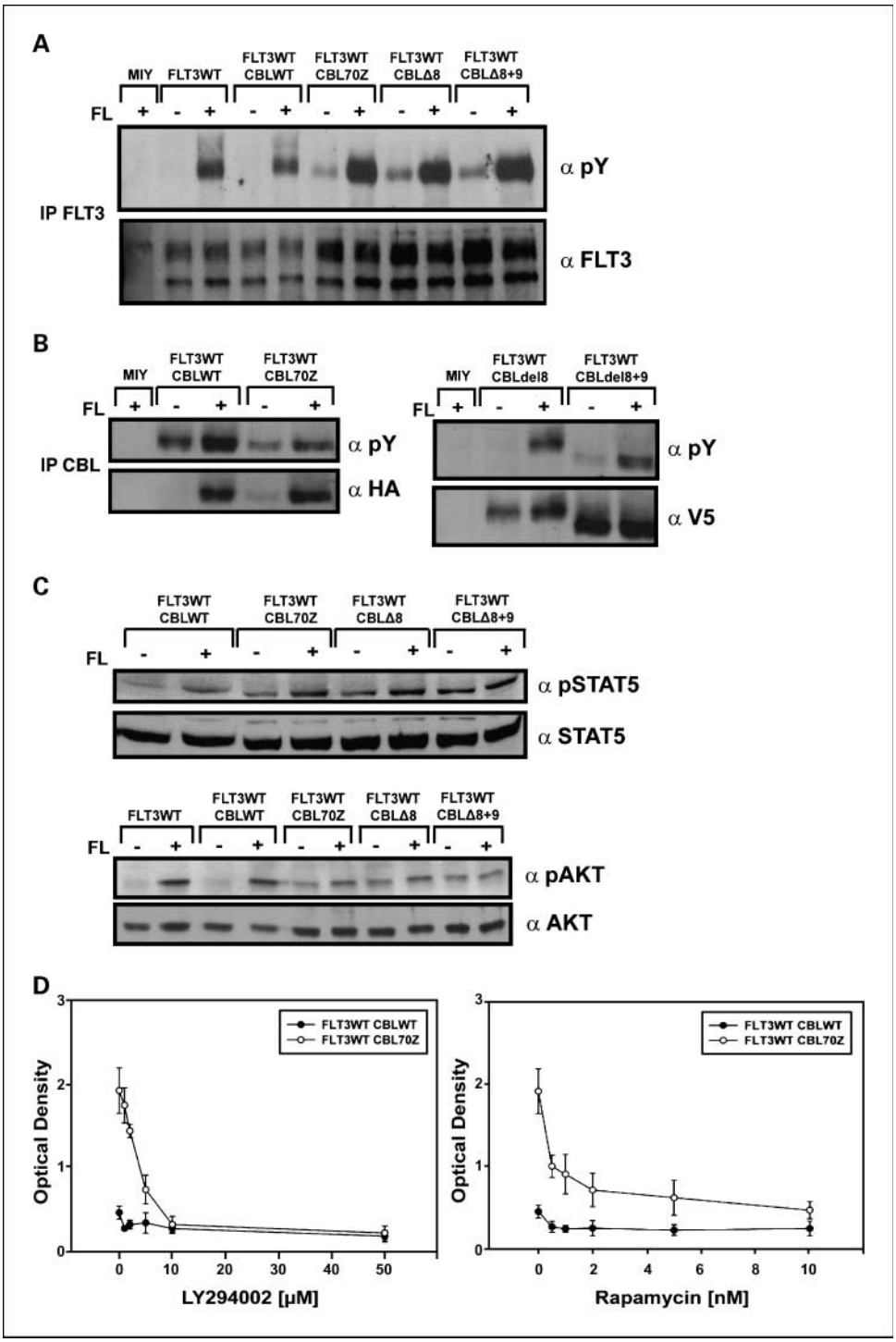


**Fig. 4.** Coexpression of CBL deletion mutants autoactivates FLT3, CBL mutants, and the downstream signaling pathways STAT5 and AKT *A,* Mock, FLT3-WT, FLT3-WT/CBL-WT, FLT3-WT/CBL-70Z, FLT3-WT/CBLΔexon8, and FLT3-WT/CBLΔexon8+9 cells were starved for 24 h in the presence of 0.3% FCS and unstimulated or stimulated with 100 ng/mL FLT3 ligand for 5 min. FLT3 was immunoprecipitated with polyclonal FLT3 antibody and analyzed by immunoblotting with phospho-tyrosine antibody, stripped, and reprobed with FLT3 antibody. *B,* CBL-WT and CBL mutant proteins were immunoprecipitated with hemagglutinin- or V5-antibody and analyzed as described in *A. C,* cells were treated according to *A* and crude cell lysates were separated by SDS-PAGE and analyzed for phosphorylation of STAT5 and AKT using phospho-specific antibodies. *D,* WST proliferation assays were done in presence of FLT3 ligand and the PI3K inhibitor LY294002 or the mTOR inhibitor rapamycin.

in all analyzed cells. The amount of phosphorylated FLT3 in FLT3-WT/CBL mutant–expressing cells was considerably higher than in control cells, as was the amount of total FLT3 protein. This confirms the hypothesis that coexpression of a CBL deletion mutant, but not CBL-WT, leads to a remarkable stabilization of the FLT3 protein within the cells by inhibiting the down-regulation and degradation of the FLT3 protein. Analysis of the phosphorylation status of transduced CBL in FLT3-WT/CBL mutant–expressing cells revealed that all CBL mutants became phosphorylated after stimulation of the cells with FLT3 ligand (Fig. 4B). Of note, we detected a weak constitutive phosphorylation of CBL mutants, but not CBL-WT, in the absence of FL.

Autoactivation of FLT3 results in ligand-independent signaling of the receptor (31, 32). We therefore analyzed the activation of three important downstream signaling effectors of FLT3: signal transducer and activator of transcription 5 (STAT5), phosphoinositide 3-kinase (PI3K)/protein kinases B (AKT), and mitogen-activated protein kinase (MAPK). Activated STAT5 and AKT were detected in unstimulated FLT3-WT/CBL-70Z-,

FLT3-WT/CBLΔexon8-, and FLT3-WT/CBLΔexon8+9–coexpressing cells but not FLT3-WT- or FLT3-WT/CBL-WT–coexpressing cells (Fig. 4C).

However, no differences were observed in MAPK activation, as all unstimulated cells lack phosphorylated MAPK (data not shown).

To evaluate the contribution of the PI3K pathway to the FLT3 ligand–dependent proliferation of FLT3-WT/CBL mutant–expressing cells, we analyzed the proliferative capacity of FLT3-WT/CBL-70Z–coexpressing cells and FLT3-WT/CBL-WT control cells in the presence of the small molecule inhibitors LY294002 and rapamycin. LY294002 is a well known PI3K inhibitor (33) and rapamycin specifically inhibits the mammalian target of rapamycin (mTOR) kinase, a downstream target of PI3K (34, 35). Both compounds were able to significantly, but not totally, inhibit the proliferation of FLT3-WT/CBL-70Z cells (Fig. 4D). The $IC_{50}$ value for FLT3-WT/CBL-70Z–expressing cells were 3.8 μmol/L for LY294002 and 0.5 nmol/L for rapamycin, pointing to a strong contribution of the PI3K/AKT pathway to the transforming phenotype of FLT3/CBL mutant–coexpressing cells. Incubation of the cells with the maximum used concentration of inhibitor in presence of IL-3 was not cytotoxic (data not shown).

***Expression of CBL-70Z in FLT3-WT cells does not alter the internalization of the FLT3 receptor.*** Analysis of EGFR activity has shown that CBL promotes the invagination of the plasma membrane during receptor endocytosis (25, 26, 36). CBL-70Z has been proposed to act in a dominant negative manner preventing binding of CBL-WT to activated RTKs (37).

We intended to analyze whether the expression of CBL-WT or CBL-70Z might alter the rate or timing of FLT3 internalization after activation with FLT3 ligand. We did staining of FLT3 or FLT3-WT/CBL-70Z–expressing cells with CD135 antibody to detect the levels of FLT3 on the surface dependent on the time of FLT3 ligand stimulation up to 60 minutes. In FLT3-WT cells the FLT3 receptor is rapidly internalized after 10 to 20 minutes of FLT3 ligand stimulation (Fig. 5). In contrast to FLT3-WT the kinase-dead FLT3K644R, which served as a control, was not internalized even after 60 minutes of FLT3 ligand stimulation. Coexpression of CBL-WT (data not shown) or the mutant CBL-70Z had no effect on the receptor turnover as after 20 minutes of FLT3 ligand stimulation FLT3 was undetectable on the cell surface in all analyzed cells.

## Discussion

Growth factors and their associated membrane bound receptors play an important role in the initiation and propagation of many types of cancer (38). As the aberrant activation of RTKs by overexpression and activating mutations is a well-investigated topic in oncology research, it is becoming increasingly clear that impaired receptor down-regulation might contribute to the malignant phenotype as well (39). Receptor down-regulation involves ligand-induced endocytosis and subsequent degradation of the proteins in the lysosomes, leading to termination of RTK signaling and, in case of disturbance, to aberrantly extended signaling.

Recent findings have addressed the aberrant down-regulation of FLT3 by CBL in AML (7, 10, 11). In this study we provide a comprehensive insight by analyzing 300 AML/MDS and ALL cases. Four of 279 AML/MDS patients, but no ALL patients, were found positive for expression of aberrant CBL transcripts in the linker and RING finger domains, previously characterized as the critical location for transforming mutations. The equivalently analyzed large number of AML and ALL cell lines revealed two positive AML cell lines, MOLM-13 and the sister cell line MOLM-14, which matches the results obtained in patient material and points to a low frequency of CBL mutations in AML. However, an independent study found 1 of 12 AML patients with a point mutation in CBL-b (10), a CBL family member, highlighting the importance of further studies to determine the contribution of CBL members to the pathogenesis of AML (10). Of more importance was whether other aberrations in other negative regulatory molecules, e.g. SHP phosphatases, lead to deregulated RTK activity.

Three of the described aberrant CBL transcripts lack whole exons, which were significantly decreased in expression or not detectable in the patient's remission controls. Screening of

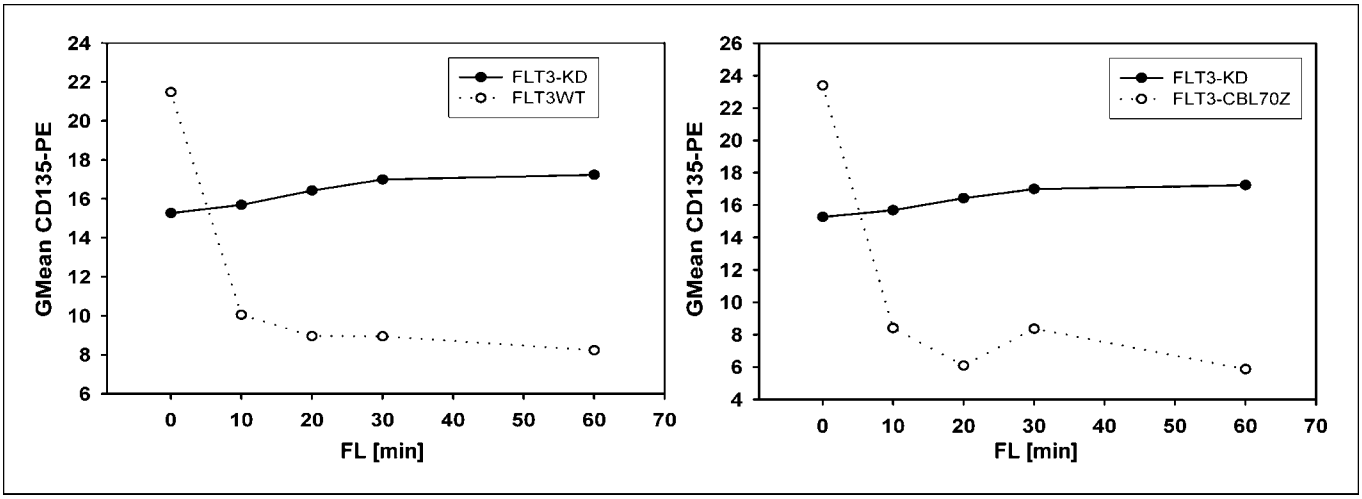


**Fig. 5.** The internalization of FLT3-WT is not disturbed by coexpression of CBL70Z. Ba/F3 cells expressing FLT3-WT, FLT3-WT/CBL-70Z, or FLT3-KD were stimulated with FLT3 ligand for different time periods from 0 min up to 60 min. After stimulation cells were immediately incubated on ice and cell membranes fixed by addition of 0.3% sodium azide. Cells were stained with FLT3-specific surface antibody and analyzed in fluorescence-activated cell sorter for surface expression levels of FLT3. The *Y*-axis represents the Geometric Mean (GMean) of the CD135-phycoerythrin-stained population.

normal hematopoietic tissues did not show aberrant CBL transcripts, further pointing to an oncogenic event in the malignant clone. Indeed, all three patients carried genomic lesions affecting splice donor or acceptor sites by mutation or deletion, so that we can clearly exclude aberrant splicing events within the malignant clone.

Of note, we detected a preferential association of CBL mutations with specific subtypes of AML/MDS. Among 43 cases of core binding factor leukemias [t(8;21) and inv(16)] two patients expressing aberrant CBL transcripts were identified [4.7% (0.6%, 15.8%)]. As core binding factor leukemias show a high expression of the RTK KIT (40), further studies need to address the question of whether CBL mutations might lead to autoactivation of KIT in these patients. Two of 13 patients with 11q deletions were found positive [15.4% (1.9%, 45.4%)]. This AML/MDS subgroup shows the highest FLT3 expression among all AML subgroups (41), so that CBL mutations might be the event causing the high FLT3 expression and an aberrant FLT3 activation in these patients.

CBL was shown to be responsible for ubiquitination of several receptors including EGFR, PDGFRA, and FMS (25, 42, 43). Until now only indirect evidence existed for a role of CBL in human FLT3 downstream signaling. Lavagna-Sevenier et al. reported prominent CBL phosphorylation in human hematopoietic cell lines expressing endogenous FLT3 (44). Recently Sargin et al. reported the physical interaction of CBL with a murine chimeric FLT3 receptor, where the juxtamembrane domain has been replaced by human sequences (7). Their study presents further indirect evidence that CBL is the responsible E3 ligase ubiquitinating the chimeric murine FLT3 receptor after binding of FLT3 ligand. However, this remains to be tested for human FLT3. Here, we provide clear evidence that murine endogenous CBL is rapidly phosphorylated after activation of the human FLT3-WT receptor. As murine and human CBL share 92% homology, we conclude that this signaling event is conserved from mice to humans. Recently Heiss et al. identified two important *in vivo* autophosphorylation sites of human FLT3 after FLT3 ligand stimulation, tyrosines 589 and 599, which associate with important signaling molecules, like Src family members and SHP2 (22). Mutation of Y589 and Y599 to phenylalanine revealed a significantly decreased phosphorylation of CBL in response to FLT3 ligand stimulation of the mutant FLT3 receptors, implicating that these tyrosines serve as docking sites. A similar effect has been seen for the corresponding juxtamembrane tyrosines Y568 and Y570 in KIT and Y572 and Y574 in PDGFRα (21, 45). However, as still small amounts of phosphorylated CBL were detectable, we propose that FLT3 contains more interaction sites for CBL, which might be direct or indirect via adaptor proteins, e.g. GRB2. Additional COOH-terminal association sites of CBL have been described for FMS (Y973) and for KIT (Y936; refs. 19, 46). In coimmunoprecipitation experiments we have shown interaction of overexpressed human FLT3-WT with human CBL-WT after FLT3 ligand stimulation. The interaction of CBL-70Z with FLT3-WT was even more pronounced, indicating that the interaction of mutant CBL and FLT3 might be more stable and might be very efficient in blocking CBL-WT from binding sites on activated FLT3 receptors. *In vivo* colocalization experiments confirmed colocalization of FLT3 and CBL in endosomal vesicles after FLT3 ligand stimulation of cells. It has been suggested that CBL ubiquitylates FLT3 (7). Here, we show that the presence of overexpressed CBL-WT enhances down-regulation of FLT3 after binding of FLT3 ligand, providing direct evidence for a functional role of CBL in down-regulation of FLT3 activity.

To analyze the transforming potential of CBL mutants we have chosen the IL-3–dependent cell line BaF3, as it is a valuable model for the investigation of activated RTKs. We analyzed all human CBL mutations thus far detected and CBL-70Z as a control. Only cells coexpressing FLT3-WT and a CBL deletion mutant were able to proliferate in the absence of IL-3. CBL point mutants coexpressed with FLT3WT showed enhanced survival, but no proliferative capacity in BaF3 cells. This finding is in contrast to the recent study of Sargin et al., in which the R420Q point mutation induced IL-3–independent growth in myeloid 32D cells (7). This discrepancy may arise from the different cell systems used and highlights the need to analyze the phenotype of CBL mutations in primary cells. The addition of exogenous FLT3 ligand induced a rapid proliferative response to FLT3-WT CBL deletion mutant–coexpressing cells. As AML blasts coexpress FLT3 ligand leading to an autocrine stimulation, this phenotype mimics the *in vivo* situation (47, 48). In the case of CBL point mutants only R420Q, but not G375 mutants, show significant growth *in vitro* in presence of FLT3 ligand. Coexpression of a CBL mutant autophosphorylates FLT3 and downstream molecules STAT5 and AKT. Additionally, CBL mutants themselves are constitutively phosphorylated, which points to a so far undescribed mechanism independent from the proposed dominant negative mechanism, in which CBL mutants block CBL-WT from binding sites at activated RTKs (47). Consistent with a dominant negative behavior of CBL mutants is the fact that we detected higher levels of FLT3 protein in FLT3/CBL mutant cells compared with control cells, as the increased stability of the FLT3 protein should be the result of a decreased degradation of FLT3.

Analyzing the internalization rate of FLT3 in FLT3-WT and FLT3-WT/CBL-70Z cells revealed no difference in rate and speed of internalization; thus, early steps of endocytosis of FLT3 seem not to be affected by the presence of a CBL deletion mutant. This is in concert with a study from Jiang et al., in which late stages of endocytosis, but not recruitment of EGFR into coated pits, were affected by CBL-70Z (48). However, Sargin et al. found a slightly reduced internalization of FLT3 in the presence of CBL-70Z and CBLR420Q, so that this information is in conflict and needs to be clarified.

Taken together, aberrant CBL transcripts occur in AML and are associated with core binding factor leukemias and 11q aberrations. The CBL deletion mutants show a transforming phenotype in BaF3 cells in the presence of FLT3, that resembles the phenotype seen in FLT3-activating mutations (FLT3-ITD, FLT3-TKD, FLT3-JM-PM). This new mechanism of activation through mutated negative-regulatory molecules of RTKs opens a door for treating patients with PTK inhibitors, who do not carry mutations in the RTK itself.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# CBL Exon 8/9 Mutants Activate the FLT3 Pathway and Cluster in Core Binding Factor/11q Deletion Acute Myeloid Leukemia/Myelodysplastic Syndrome Subtypes

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