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Exome Sequencing Identifies Recurring *FLT3* N676K Mutations in Core-Binding Factor Leukemia

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Key Points

- *FLT3* N676K mutations without concurrent internal tandem duplication (ITD) are associated with core-binding factor leukemia
- N676K activates FLT3 and downstream signaling pathways

Abstract

The t(8;21) and inv(16)/t(16;16) rearrangements affecting the core-binding factors, RUNX1 and CBFB, respectively, are found in 15-20% of adult *de novo* AML cases and are associated with a favourable prognosis. Since the expression of the fusion genes *CBFB*/*MYH11* or *RUNX1*/*RUNX1T1* alone is not sufficient to cause leukemia, we performed exome sequencing of an AML sample with an inv(16) to identify mutations, which may collaborate with the CBFB/MYH11 fusion during leukemogenesis. We discovered an N676K mutation in the ATP-binding domain (TKD1) of the *fms-related tyrosine kinase 3* (*FLT3*) gene. In a cohort of 84 *de novo* AML patients with a *CBFB/MYH11* rearrangement and in 36 patients with a *RUNX1/RUNX1T1* rearrangement, the *FLT3* N676K mutation was identified in 5 and 1 patients, respectively (5/84, 6%; 1/36, 3%). The FLT3-N676K mutant alone leads to factor-independent growth in Ba/F3 cells and, together with a concurrent FLT3-ITD, confers resistance to the FLT3 PTK inhibitors PKC412 and AC220. Gene expression analysis of AML patients with *CBFB*/*MYH11* rearrangement and *FLT3* N676K mutation showed a trend towards a specific expression profile. Ours is the first report of recurring *FLT3* N676 mutations in CBF leukemias and suggests a defined subgroup of CBF leukemias.

Registered at www.clinicaltrials.gov: AMLCG-1999 trial (NCT00266136)

Introduction

The inversion inv(16)(p13;q22), the translocation t(16;16)(p13;q22) and the translocation t(8;21)(q22;q22) are recurring rearrangements in acute myeloid leukemia (AML), which result in the fusion genes *CBFB*/*MYH11* or *RUNX1/RUNX1T1*, respectively. These rearrangements are found in 15-20% of adult *de novo* AML cases and represent recognized WHO entities that are associated with a favorable prognosis.[1,2]

CBFB and RUNX1 form the core-binding factor (CBF), a heterodimeric transcription factor essential for normal hematopoiesis. The CBFB/MYH11 and RUNX1/RUNX1T1 fusion proteins disrupt the physiological activity of CBF, leading to the repression of CBF target genes resulting in a block of differentiation and impaired haematopoiesis. Since knock-in mouse models have demonstrated that the expression of CBFB/MYH11 or RUNX1/RUNX1T1 by themselves is not sufficient to cause leukemia, it is highly likely that additional mutations are required for malignant transformation.[1,3,4] Leukemogenesis is a multistep process. Mutations associated with myeloid malignancies have been found in genes involved in several functional classes: signaling pathways (e.g. *FLT3*, *KIT*, *RAS*), transcription factors (e.g. *RUNX1/RUNX1T1*, *CBFB/MYH11*), epigenetic regulators (e.g. *DNMT3A*, *IDH1*, *IDH2*, *TET2*), tumor suppressors (e.g. *TP53*, *WT1*), and splicing (e.g. *SF3B1*, *SRSF2*).[5] In CBF leukemia, mutations in genes coding for signaling proteins (so-called proliferation drivers) are commonly found to collaborate with the core-binding factor fusion genes.[6] Mutations in *KIT*, *FLT3* or *NRAS/KRAS* have been frequently detected in CBF leukemia.[7-9] Up to 90% of AML patients with a CBFB/MYH11 fusion either have a mutation in a receptor tyrosine kinase (RTK) or in RAS.[10,11] In general, these signaling pathway mutations are mutually exclusive.[5] However, about 10% of

AML patients with a CBFB/MYH11 fusion do not carry any of the currently known mutations.

To systematically identify additional collaborating mutations in *CBFB*/*MYH11* positive AML patients we performed exome sequencing of an AML with an inv(16) without any additional known genetic alterations. Using this approach, we identified a *FLT3* N676K mutation. Screening a cohort of 120 CBF AML patients, we discovered the FLT3 N676K mutation to be present in 6 of these patients. Mutations affecting the ATP-binding pocket, in particular position N676 resulting in variable amino acid changes (N676D or N676S) were initially discovered in a screen for resistance to tyrosine kinase inhibitors (TKI) in *FLT3* internal tandem duplication (ITD) expressing Ba/F3 cells.[12,13] To our knowledge a *FLT3* N676K point mutation has been reported just once before in a cytogenetically normal (CN) AML patient with a *FLT3*-ITD mutation who was screened for the cause of the acquired TKI-resistance after PKC412 therapy.[14] In the present study we report recurring *FLT3* N676K mutations at first diagnosis of CBF AML without concurrent *FLT3*-ITD. Importantly, Ba/F3 cells expressing the FLT3 N676K mutation show factor independent growth and reduced sensitivity towards commonly used TKIs suggesting that the presence of the *FLT3* N676K mutation in CBF leukemia patients might be an important predictor for resistance towards TKI therapy.

Materials and methods

Patient samples

A diagnostic bone marrow sample was collected from an 18-year-old patient, diagnosed with AML M4eo according to standard FAB (French-American-British) and WHO (World Health Organization) criteria in December 2003. The inv(16)(p13;q22) was detected by standard cytogenetics analysis (karyotype: 46,XY,inv(16)(p13;q22)[10]). The CBFB-MYH11 fusion transcript was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR). No additional genetic alterations were detected at this time. The patient was enrolled in the AMLCG-1999 trial of the German AML Cooperative Group (NCT00266136), and written informed consent was obtained in accordance with the Declaration of Helsinki. The samples were obtained under AMLCG study protocols approved by the ethics committees of the participating centers. After induction chemotherapy and autologous peripheral blood stem cell transplantation (auto-PBSCT), complete remission was achieved (<5% bone marrow blasts; *CBFB/MYH11* transcripts no longer detectable by RT-PCR). A bone marrow sample at complete remission was used as normal control for exome sequencing.

In total, bone marrow or peripheral blood samples from 84 adult patients with newly diagnosed and untreated AML M4eo (*CBFB*/*MYH11* fusion positive; including the case analyzed by exome sequencing), from 36 patients with a t (8;21) and from 90 patients with cytogenetic normal (CN) AML were used for targeted mutation screening.

Sample preparation and high-throughput sequencing

Genomic DNA was extracted from patients' bone marrow or peripheral blood samples using QIAcube technology (QIAGEN, Hilden, Germany). For exome sequencing of the index patient 3 µg of genomic DNA was fragmented to an average size of 150 bp using the Bioruptor sonicator (Diagenode, Liège, Belgium). Paired-end sequencing libraries were prepared using DNA sample prep reagent set 1 (NEB Next). Library preparation included end repair, adapter ligation, and PCR enrichment and was carried out as recommended by Illumina protocols. Exon-coding sequences were then captured using SureSelect human all exon 50Mb kit version 3 (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. Exome libraries were sequenced by performing 76-bp paired end reads on a Genome Analyzer IIx platform (Illumina, San Diego, CA, USA). Sequence alignment and variant detection was performed as described previously.[15]

Sanger sequencing

The non-synonymous somatic variant in the *FLT3* gene (detected in the AML but not in the remission sample) was verified by sequencing both DNA strands using ABI 3100-Avant technology (Applied Biosystems) after PCR amplification of *FLT3* exon 16. PCR and sequence analysis of genomic DNA was performed with forward primer 5'-TGCAGATTGACTCTGAGCTG-3' and reverse primer 5'-CACTGTGACTGAGAAAAGACAAAG-3', located in the 5' and 3' flanking introns, spanning the complete exon 16, yielding a 327 bp PCR product corresponding to AA 649 to 685 of the human FLT3 protein (genebank accession number NM_004119). The same assay was used on a total of 209 *de novo* AML patients (*84 CBFB*/*MYH11* rearranged, 36 *RUNX1/RUNX1T1* rearranged and 90 CN-AML cases). Routine

diagnostic tests included mutation analysis at defined positions of *FLT3, KIT, KRAS, NRAS, NPM1, MLL* and *WT1* of all 84 AML M4eo samples (supplemental table S3).

DNA constructs and vectors

The human FLT3-WT and the FLT3-ITD-NPOS construct containing a 28 AA duplicated sequence (CSSDNEYFYVDFREYEYDLKWEFPRENL) inserted between AA 611/612 of human FLT3-WT were kindly provided by Gary Gilliland (Boston Harvard Medical School, MA, USA). The FLT3 constructs were subcloned into the MSCV-IRES-EYFP retroviral expression vector (kindly provided by R. K. Humphries, The Terry Fox Laboratory, Vancouver, University of British Columbia).

***In vitro* mutagenesis**

The N676K mutation was introduced into the FLT3-WT and the FLT3-ITD-NPOS vector by using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The mutant FLT3 D835Y construct was generated using the QuikChange Site-Directed Mutagenesis Kit.[16] The correct sequence of all constructs was confirmed by sequencing.

Cell lines, reagents and antibodies

Phoenix Eco cells were purchased from Orbigen (San Diego, CA, USA) and cultured in Dulbecco´s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.5% Penicillin/Streptomycin. Low-passage murine Ba/F3 WEHI-3B cells were obtained from the DSMZ (Braunschweig, Germany) and maintained in RPMI-1640 medium containing 10% FBS, 0.5% Penicillin/Streptomycin and 10% WEHI-3B conditioned medium as source of IL-3. Recombinant human FLT3 ligand

and recombinant murine IL-3 were obtained from Immunotools (Friesoythe, Germany). FLT3 inhibitor PKC412 was obtained from Novartis (Basel, CH) and AC220 from SYNthesis Med Chem (Cambridge, UK).

The following antibodies were used: anti-AKT (9272), anti-pAKT (4060), anti-MAPK (9107), anti-pMAPK (9101), and anti-pSTAT5 (9351) [Cell Signaling Technology (Danvers, MA, USA)]; anti-FLT3 (sc-480), anti-pTyr (sc-7020), anti-STAT5 (sc-835), and anti-GAPDH (sc-32233) [all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA)]; CD135-PE (IM2234U) and IgG1-PE isotype control (A07796) [Immunotech (Marseille, FRA)]. Stable transduction of Ba/F3 cells, western blot analysis and detection of surface markers was performed as described previously.[17,18]

Proliferation and apoptotic cell death of Ba/F3 cells

Proliferation and apoptosis assays were carried out as described previously.[17] For long term proliferation assays, cells were seeded at a density of 2 x 10^5/ml cells in growth medium containing 0.1% WEHI conditioned medium as source of murine IL-3 and as control in the presence of 10 ng/ml IL-3. After 72 hours, Ba/F3 cells were cleared from IL-3 by two centrifugation steps with PBS and resuspended in medium without IL-3. As control cells were cultivated in presence of 10 ng/ml IL-3. Viable cells were counted every day and a cell density of 2.5 x 10^6 was not exceeded.

Gene expression profiling / Microarray Analyses

Pretreatment bone marrow samples of 33 patients (data deposited in GSE37642) were analyzed using Affymetrix HG-U133 A/B oligonucleotide microarrays (Affymetrix, Santa Clara, CA) as described previously.[19,20] For probes to probe set

annotation we used custom chip definition files (CDFs) based on GeneAnnot version 2.0, synchronized with GeneCards Version 3.04 (available at http://www.xlab.unimo.it/GA_CDF/). [21] Normalization was carried out by the Robust Multichip Average (RMA) method as described by Irizarry et al 2003.[22] The Linear Models for Microarray Data (Limma) package was used to compute differentially regulated probe sets by comparing patients with *CBFB*/*MYH11* rearrangement and one of the following mutations affecting *FLT3* D835, *NRAS*, *KRAS*, *KIT* or *FLT3*-ITD to patients with *CBFB*/*MYH11* rearrangement and the *FLT3* N676K. Gene set enrichment analysis (GSEA) was performed with GSEA software (MIT) to assess significant changes in gene expression levels.[23] The GSEA was run with 1,000 permutations and compared to the ‘‘c2_kegg’’ collection from the Molecular Signatures Database MsigDB 3.0 consisting of 186 gene sets. All statistical analyses were performed using the R 3.0.1 software and routines from the biostatistics software repository Bioconductor.

Results

Exome sequencing of an AML M4eo case

To systematically identify mutations, which may collaborate with CBFB/MYH11 during leukemogenesis, we performed exome sequencing of an AML sample with inversion (16). The sample was selected based on sample availability and the absence of additional genetic alterations (*FLT3*-ITD, *MLL-PTD, FLT3*-TKD, *NPM1, NRAS, KRAS KIT* and *WT1* mutation negative). We sequenced the exome (protein coding regions) of the diagnostic sample and a remission sample from the same patient, generating at least 4 Gbp of sequence from each exome. This allowed us to cover more than 80% of RefSeq coding exon positions with a minimum read depth of 10 (supplemental table S1). By comparing both exome sequences and excluding known polymorphisms we were able to identify somatically acquired, leukemia-specific sequence variants. Non-synonymous coding mutations were confirmed using Sanger sequencing. We found a total of 2 somatic mutations, namely a N676K missense mutation in the ATP-binding domain (TKD1) of *FLT*3 (NM_004119.2:c.2028C>A; figure 1 A, B and C) and an A251V missense mutation in the *CAT* gene, which encodes the cytoplasmic enzyme catalase (supplemental table S2).

Recurring *FLT3* N676K mutations in CBF AML

We sequenced *FLT3* exon 16 (containing the codon of N676) in a cohort of 84 AML patients with CBFB/MYH11 rearrangement [71 inv(16) patients and 13 patients with t(16;16)]. Strikingly, we detected heterozygous missense mutations (N/K) at position 676 of *FLT3* in 5 patients (6%) with inv(16) or t(16;16) (4/71, 6% and 1/13 8%, respectively). Thus, in AML with a CBFB/MYH11 fusion *FLT3* N676K mutations have

a similar frequency as *FLT3* D835 mutations (figure 1 D).

In 36 AML samples with a t(8;21)(q22;q22) and a RUNX1/RUNX1T1 fusion one patient with a *FLT3* N676K mutation could be identified (1/36, 3%). None of the CBF AML patients with a *FLT3* N676K mutation had an additional *FLT3*-ITD or a D835 mutation. In contrast, in 90 AML patients with normal karyotype we detected only a single case with a *FLT3* N676K mutation, and this patient had a concurrent *FLT3*-ITD similar to the patient described by Heidel and colleagues.[14] The incidence of *FLT3* N676K without concurrent ITD in CBF AML (6/120) was compared with the incidence in CN-AML (0/90) using a two-tailed Fisher's exact test (p=0.039). These results suggest a specific association between *FLT3* N676K mutations and CBF leukemias.

To determine whether the *FLT3* N676K mutations are somatically acquired, we sequenced remission samples where available. Paired diagnostic and remission material was available only from the N676K positive patient with the t(8;21) and from one inv(16) patient. In both cases the N676K mutation could be detected at diagnosis but not in the remission sample (supplemental figure S1). Deep amplicon sequencing of N676K positive cases confirmed variable allele frequencies ranging from 11% to 44 % indicating clonal heterogeneity (supplemental table S5).

Additional mutations in *CBFB/MYH11*- rearranged AML

We analyzed mutational hotspots (see Methods) of several commonly mutated genes (*FLT3, KIT, KRAS, NRAS, NPM1, MLL* and *WT1*) in our 84 *CBFB*/*MYH11*-positive cohort: The mutation frequency of these genes in our cohort (figure 1 D) was similar to previous reports.[7,8,24-26] We found *KIT* mutations in 18% of *CBFB*/*MYH11*-positive AMLs (14% exon 8 frame shift mutations, known to be frequent in inv(16) AML,[7,9] and 4% D816 missense mutations). RAS missense mutations were present in 51% of the

cases (14% *KRAS*, 37% *NRAS*). As expected, the *FLT3*-ITD mutation was rare in our cohort (2%), whereas the *FLT3*-TKD (D835) mutation had a frequency of 10%. Together with the 6% *FLT3* N676K mutated patients, a total of 18% (15/84) of the CBFB/MYH11-positive patients had a *FLT3* mutation.

In addition to the common signaling pathway mutations we found 7% of samples with WT1 mutations causing a frame shift in exon 7. *MLL* partial tandem duplications (PTD) (1%) and *NPM1* mutations (0%) were rare in our *CBFB*/*MYH11*–positive patients. In 18% of the patients no mutation was detected in the mutational hotspots analyzed. In 12 patients (14%) more than one mutation was present. 79% of the patients (66/84) carried a mutation in *FLT3*, *KRAS*, *NRAS* or *KIT*.

FLT3 N676K is strongly expressed on the cell surface of Ba/F3 cells

To analyze the transforming potential of the FLT3 N676K mutant, Ba/F3 cell lines stably expressing various FLT3 constructs were established. The expression of the wild type and mutant FLT3 receptors was confirmed by immunoblotting or flow cytometry (supplemental figure S2). Like FLT3-WT the FLT3 N676K receptor was highly expressed on the cell surface (mature receptor, 160 kDa) compared to FLT3-ITD and FLT3 D835Y. The FLT3-N676K-ITD double mutant showed the weakest cell surface expression. A weak cell surface expression was correlated with an enhanced expression of the immature receptor with a molecular weight of 130 kDa.[27]

The FLT3 N676K mutant receptor leads to cytokine independent growth and resistance to apoptosis

Proliferation assays of Ba/F3 cells expressing FLT3 mutant receptors revealed a cytokine independent growth. As described before, FLT3-ITD was able to fully

transform Ba/F3 cells reaching 100% of IL-3-mediated growth. FLT3 D835Y expressing cells reached 41%. The mutant FLT3 N676K receptor led to IL-3 and FLT3-ligand (FL) independent cell growth and the Ba/F3 cells reached about 25% of the IL-3 reference proliferation rate at 72 h culture time (figure 2 A). This pro-proliferative phenotype increased over time, and eventually FLT3 N676K expressing cells reached a similar proliferation rate to FLT3 D835Y expressing cells (figure 2 B). In addition to an enhanced proliferation, Ba/F3 cells expressing the various FLT3 mutants showed a strong resistance to apoptosis after cytokine deprivation (figure 2 C). This anti-apoptotic phenotype was strongest in FLT3-ITD expressing cells (only 4.5% apoptotic cells) followed by FLT3 D835Y (7%) and FLT3 N676K (10%) expressing cells.

Constitutive activation of FLT3 signaling in FLT3 N676K expressing cells

To determine critical pathways for the transforming potential of the FLT3 mutants, we analyzed the activation of three key signaling molecules downstream of FLT3: The mitogen-activated protein kinase (MAPK), protein kinase B (AKT), and signal transducer and activator of transcription 5 (STAT5). Protein lysates of unstimulated and FLT3 ligand (FL) stimulated Ba/F3 cells expressing FLT3 and the mutants were immunoblotted (figure 3 A). MAPK was strongly activated in all FL stimulated FLT3 or FLT3 mutant expressing cells. In contrast to FLT3-ITD expressing cells, STAT5 was not phosphorylated in FLT3-WT or in the FLT3 N676K or the FLT3 D835Y mutant expressing cells. MAPK was constitutively phosphorylated in unstimulated cells of the two TKD mutants N676K and D835Y compared to WT and ITD cells**.** To determine the activation of FLT3 N676K mutant receptor, the protein was immunoprecipitated and analyzed for tyrosine phosphorylation by immunoblotting. The FLT3 N676K

receptor showed a 5-fold stronger constitutive phosphorylation as well as a 2-fold stronger phosphorylation after ligand stimulation compared to FLT3-WT taking into account the total protein loaded on the gel (figure 3 B). In conclusion, FLT3 N676K mutant expressing cells showed an enhanced signaling through the MAPK pathway but no aberrant activation of STAT5. Thus, the increased MAPK activation is most likely responsible for the mutant phenotypes.

FLT3 N676K induced proliferation can be abrogated by selective PTK inhibition

The *FLT3* N676K mutation was previously described only in combination with a *FLT3*-ITD to mediate resistance to protein tyrosine kinase inhibitors (PTKI).[12] It was not tested, whether *FLT3* N676K alone might be sufficient to confer PTKI resistance. To address this question, we used PKC412 and AC220 as selective FLT3 inhibitors in increasing nontoxic concentrations (figure 4). Non-toxicity of the inhibitors was confirmed in FLT3-WT expressing Ba/F3 cells (supplemental figure S5). Both compounds potently inhibited FLT3-ITD expressing cells with an IC(50) of 13 nM for PKC412 and 2.5 nM for AC220, respectively. FLT3 N676K expressing cells were also sensitive to FLT3 inhibitors with an IC(50) of 7.5 nM for PKC412 and 3 nM for AC220. FLT3-ITD-N676K double mutants showed a strong resistance to both inhibitors (IC(50) greater than 80 nM for PKC412, and greater than 16 nM for ACC220). Taken together, the FLT3 N676K mutation together with an ITD is very resistant to FLT3 inhibitors. However, cell proliferation driven by FLT3 N676K alone can be inhibited rather effectively.

Differential gene expression in *FLT3* N676K mutated *CBFB/MYH11* rearranged AML

To assess the impact of *FLT3* N676K mutations on gene expression, we analyzed the gene expression profiles of 33 patients with *CBFB*/*MYH11* rearranged AML. Four patients with *FLT3* N676K mutations were compared to 29 patients with *FLT3* D835 (n=4), *NRAS* (n=15), *KRAS* (n=3), *KIT* (n=3) and *WT1* (n=1) and *FLT3*-ITD (n=2) mutations or no mutations in any of these genes (n=5). Some patients had no (n=5) or more than one (n=3) mutation. Finally, all unique probe sets with p<.005 and log fold change >1.5 were selected for unsupervised clustering (n=18). Interestingly, all cases with *FLT3* N676K clustered together (supplemental figure S3). Of these 18 genes, six were highly correlated with sex, because all cases with N676 mutation in our analysis were discovered in male patients. Interestingly, genes with high association and elevated levels in the N676K cluster were CCNA1 (cell cycle), PRG3 (immune response) and HLA-DQA1 (immune response). Genes with negative correlation to the N676K cluster were MEST (imprinting) and ARG1 (metabolism). To evaluate which pathways were associated with *FLT3* N676K mutations we compared the 4 patients with this mutation to 29 patients without this mutation. Nine gene sets were significantly enriched at FDR <25% and p<.05 including metabolic, inflammation and degradation pathways (supplemental table S4).

Structural mapping of the FLT3 N676K receptor mutation

Since we could demonstrate that a single point mutation in the ATP binding domain is sufficient to constitutively activate the receptor and increase downstream signaling, we performed structural modelling of the FLT3 N676K mutant to gain further insights into the consequences of the mutation (figure 5).

Mapping of the FLT3 N676K onto the crystal structure of FLT3 showed that this mutation destabilizes the fold of the kinase domain between the juxtamembrane domain (JMD) and a hydrophobic pocket that is the target of FLT3 inhibitors. The crystal structure of the inactive conformation of FLT3 showed that the JMD serves as a key autoinhibitory element regulating the kinase activity.[28,29] N676K mutations might therefore interfere with the FLT3 autoinhibition by reducing the stability of the JMD, hence, suggesting a structural basis for the transforming activity observed in our experiments with Ba/F3 cells.

Clinical characteristics associated with *FLT3* N676K mutations

Fifty-six of the 84 *CBFB*/*MYH11* rearranged AML patients screened for mutations in the present study were enrolled in the multicenter AMLCG-1999 trial of the German AML Cooperative Group (NCT00266136). Among these patients five carried *FLT3* N676K mutations. In this cohort, which was homogeneous both with regard to treatment and cytogenetics, the mutation was significantly associated with higher leukocyte counts (p=.02), elevated LDH (p=.02) and male sex (p=.02) (table 1). There was no significant difference in survival of patients with a *FLT3* N676K (n=4) compared to patients with *FLT3* N676 wild type (n=47) (supplemental figure S4 A)**.** However, there was a trend towards reduced complete remission rates associated with *FLT3* N676K mutations (table 1).

We also compared *CBFB*/*MYH11* rearranged AML patients with *FLT3* point mutations affecting residues N676 or D835 (n=9) to all other CBFB/MYH11 rearranged patients (n=42) and did not observe a significant difference in survival (supplemental figure S4 B).

Discussion

Ours is the first report of recurring *FLT3* N676K mutations in CBF leukemia. Despite the overall rather favorable prognosis associated with CBF rearrangements, almost one third of patients relapses within the first year after intensive chemotherapy and only 60% of CBF AML patients are still alive after 5 years. This heterogeneous clinical outcome of CBF AML patients may reflect the heterogeneity of additional genetic lesions in this subgroup and underscores the need for further investigation. Understanding the pathogenesis of AML is challenging due to the multitude of genetic events. By sequencing of known mutational targets we and others have demonstrated that between 80 and 90% of *CBFB*/*MYH11* rearranged patients have mutations that activate either RAS signaling or receptor tyrosine kinase signaling (*FLT3* and *KIT*), while other common AML-related gene mutations (e.g. in *NPM1*, *WT1* and *MLL*) are rarely found.[7-11] The discovery of *FLT3* N676K mutations adds a further piece to the puzzle of CBF related leukemogenesis, suggesting that the proportion of CBF leukemia with activating receptor tyrosine kinase mutations has been underestimated. Our observation of concurrent activating mutations in different genes (e.g. *KRAS* and *NRAS* or *NRAS* and *KIT*; figure 1 D) suggests either clonal heterogeneity or multiple additive hits in synergistic pathways within *CBFB*/*MYH11* rearranged AML.

The specific occurrence of recurring *FLT3* N676K mutations in the *CBFB*/*MYH11*-rearranged AML subgroup, which accounts for only 6% of AML, might explain why *FLT3* N676K mutations have remained undetected in previous full-length *FLT3* mutation screens of unselected AML patients.[30] Other large studies limited *FLT3* mutational screening to *FLT3*-ITD mutations (exon 14/15) and TKD2 mutations (exon 20, e.g. D835) and thus would have missed *FLT3* N676K mutations (exon 16).[7,25]

Even though the FLT3 N676K in combination with a FLT3-ITD had previously been shown to lead to PTK-inhibitor resistance,[12,14] we show in this report that the FLT3 N676K mutant on its own exhibits gain of function properties. Notably, FLT3 N676K alone has direct transforming potential in Ba/F3 cells through increased downstream signaling similar to the FLT3 D835 mutant, but weaker than FLT3-ITD (figure 3 and 4). The power of the gene expression analysis of the *FLT3* N676K mutated patients is limited by the small sample size. However, *FLT3* N676K mutated cases clustered together after unsupervised clustering analysis of gene expression in *CBFB*/*MYH11* rearranged AML with different mutations affecting *FLT3*, *NRAS*, *KRAS*, *KIT* and *WT1* (supplemental figure S3). These findings suggest a distinct biological subgroup within *CBFB*/*MYH11* rearranged AML characterized by *FLT3* N676K mutations.

Based on the crystal structure of FLT3, Cools and colleagues proposed that mutation of N676 destabilizes the conformation of the hinge segment, which makes H-bonds with the lactam ring of the PTK inhibitor PKC412.[12] We suggest that a mutation at position N676 may also activate FLT3 by disturbing its autoinhibition capacity (figure 5). Taken together, mutations at position N676 most probably have two consequences: activating FLT3 and, together with a concurrent FLT3-ITD, conferring PTK inhibitor resistance. The additional N676K mutation on the ITD background might change the conformation of the FLT3-ITD protein in a way that the binding site of the PTK inhibitors is masked since the ITD results in an extension of the juxtamembrane domain, which leads to a conformational change of the kinase domain. The fact that the FLT3 N676K alone (without concurrent ITD) does not confer PTK inhibitor resistance, might also be related to its localization on the cell surface, in contrast to the mostly intracellular localization of the ITD-N676K double mutant. Hence, N676K mutated FLT3 might be exposed to higher inhibitor

concentrations at the cell surface possibly allowing efficient inhibition of the tyrosine kinase domains directly beneath the cell membrane. It was shown by others that there is higher intracellular accumulation of PTK inhibitors in the more sensitive AML cells lines than in the less sensitive ones.[31]

In contrast to the initial report of a *FLT3* N676K mutation as a late arising, disease modifying event, detected at the time of clinical relapse while on PKC412 monotherapy,[14] all of our N676K mutations were detectable at initial diagnosis. Since cell proliferation driven by FLT3 N676K alone could be greatly reduced by FLT3 inhibitors, N676K positive patients without concurrent ITD may actually benefit from treatment with FLT3 inhibitors.

Our clinical data did not show a significant impact of *FLT3* N676K mutations on survival within *CBFB*/*MYH11* rearranged AML patients, but there was a trend towards reduced complete remission rates (table 1). The significant association of *FLT3* N676K mutations with higher leukocyte counts, elevated LDH levels and male sex (table 1), suggests a distinct biology of these leukemias. Given the small number of *FLT3* N676K positive patients (n=4) in our patient cohort, the prognostic significance of the *FLT3* N676K mutation needs to be investigated in larger patient cohorts.

The varying allele frequencies of the FLT3 N676K mutation ranging from 14 to 44% in those cases in which the presence of the CBFB/MYH11 rearrangement was detected by fluorescence in situ hybridization (FISH) in the majority of the bone marrow cells (supplemental table S5) indicate that the FLT3 N676K mutation did not always represent the dominant leukemic clone at diagnosis. It would be interesting to study the clonal evolution in those cases by assessing the *FLT3* N676 status at relapse, unfortunately, no relapse samples from our patients were available.

Although *FLT3* has been known for more than a decade to be mutated in about one

third of AML patients, it appears that the spectrum of *FLT3* mutations is still not fully understood. In particular, defined genetic subgroups of AML might harbor specific *FLT3* mutations. Unbiased mutation screening using exome sequencing allows the detection of novel sequence variations even in extensively studied genes such as *FLT3*.

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Authorship

S.O., H.P., K.S., S.K.B. and. P.A.G. conceived and designed the experiments. S.O., H.P., N.P.K., B.K., E.Z., and S.K. performed experiments. S.O., H.P., and T.H. analyzed data. K.P.H. performed structural modelling. S.V. and A.G. provided bioinformatics support. H.B. managed the Genome Analyzer IIx platform. S.O., B.K.., A.D., E.Z., P.M.K., S.S., J.B., S.K.B. and K.S. characterized patient samples; M.C.S., J.B., W.E.B., T.B., B.J.W. and W.H. coordinated the AMLCG clinical trial. P.A.G., K.S., and S.K.B. supervised the project. S.O., H.P., T.H., S.K.B. and P.A.G. wrote the manuscript.

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Table 1 Characteristics of *CBFB*/*MYH11* rearranged patients

Variable	***FLT3 N676* wt**	***FLT3 N676K* mut.**	**P-value**
No. of patients	51	5	
Median age, years (range)	42 (20-75)	54 (18-61)	n.s.
Male sex, no. (%)	22 (43.1)	5 (100)	**0.02**
White-cell count, G/l, median(range)	38.5 (1.3-316)	134 (54.1-259)	**0.02**
Hemoglobin, g/dl, median(range)	8.6 (4.4-14.6)	9.1 (8-14.1)	n.s.
Platelet count, G/l ,median(range)	32.0 (0.01-370)	44 (32-47)	n.s.
LDH (U/l), median(range)	666 (143-1870)	1326 (717-2508)	**0.02**
Bone marrow blasts, %, median(range)	80 (25-95)	60 (10-90)	n.s.
Performance Status (ECOG) ≥ 2 (%)	14 (29.2)	1 (20)	n.s.
de novo AML (%)	46 (90.2)	3 (75)	n.s.
NPM1 mut., no. (%)	0 (0)	0 (0)	n.s.
FLT3-ITD, no. (%)	2 (3.9)	0 (0)	n.s.
FLT3-D835	5 (9.8)	0 (0)	n.s.
MLL-PTD, no. (%)	1 (2)	0 (0)	n.s.
KRAS mut., no. (%)	7 (13.7)	0 (0)	n.s.
NRAS mut., no. (%)	21 (41.2)	0 (0)	0.15
KIT mut., no. (%)	10 (19.6)	0 (0)	n.s.
WT1 mut., no. (%)	4 (7.8)	0 (0)	n.s.
Complete remission, no. (%)	39 (76)	2 (40)	0.11
Deceased, no. (%)	19 (37.2)	2 (40)	n.s.

All patients were enrolled in the AMLCG-99 trial and received intensive induction treatment. Categorical clinical variables of the *FLT3* N676K mutated and *FLT3* N676 wt cohorts were compared by Fisher's exact test. The continuous variables were compared by Mann-Whitney U-Test. A p-value of <.05 was considered significant.

Figure legends

Figure 1 *FLT3* N676K mutations identified in *CBFB*/*MYH11* rearranged AML.

(**A**) Exome data sets of a *CBFB*/*MYH11* positive AML sample (upper panels) and the corresponding follow up sample from the same patient (lower panels) are displayed using the integrative genomics viewer.[32] Horizontal grey bars symbolize the 76 bp reads aligned to the reference sequence. The frequency of 25% of the mutant nucleotide T in the diagnostic leukemia sample indicates a heterozygous point mutation causing an amino acid substitution (NM_004119.2:c.2028C>A; p.N676K), whereas in the follow up sample only the wild-type nucleotide G is detected at this position. Read depth and base count is indicated for the affected positions, respectively.

(**B**) Sanger sequencing confirmed the *FLT3* N676K mutation found initially by exome sequencing. Chromatograms are shown for both the diagnostic AML sample and the corresponding follow-up sample at complete remission from the same patient.

(**C**) The structure of the human FLT3 protein includes the transmembrane domain (TM) the juxtamembrane domain (JM) and the tyrosine kinase domains (TKD1 and TKD2). Amino acid positions targeted by known recurrent mutations in AML are indicated in green colour above the corresponding domains. N676 is indicated in blue colour below the TKD1 domain.

(**D**) Frequency distribution of additional genetic aberrations in 84 *CBFB*/*MYH11* rearranged patients. Each column indicates one patient. Dark grey boxes are indicative for patients who are positive for the respective mutation; light grey boxes indicate wild type status. Missing information is shown as a white space. Gene names and types of mutations are indicated on the left. Mutation frequencies are

indicated on the right. Abbreviations: *FLT3*-ITD, internal tandem duplications in the *FLT3* gene; *MLL*-PTD, partial tandem duplications in the MLL gene.

Figure 2 Transforming potential of FLT3 mutants in Ba/F3 cells.

All experiments were performed in triplicates. Error bars represent standard deviation of the mean. (**A**) Ba/F3 cells expressing indicated FLT3-constructs were seeded at a density of 4 x 10^4 cells/ml in the presence or absence of 10 ng/ml IL-3 and 100 ng/ml FL. Viable cells were counted by trypan blue exclusion after 72 h. (**B**) Ba/F3 cells transduced with the indicated FLT3 constructs were seeded at a density of 2 x 10^5 cells/ml in 0.1 % WEHI conditioned medium and cultured for 10 days. After 72 hours cells were cleared from previous medium and resuspended in 0 % WEHI conditioned medium. As control cells were cultured in 10 ng/ml IL-3 supplemented medium. (**C**) Cells were cultured in the presence or absence of 10 ng/ml IL-3 for 72 h and stained with Annexin V and 7-aminoactinomycin D. The percentage of apoptotic cells was determined by FACS analysis.

Figure 3 Constitutive activation of FLT3 signaling by the FLT3 N676K mutant.

Ba/F3 cells expressing indicated constructs were starved for 24 h in media containing 0.3 % FCS. Cells were left untreated or were stimulated with 100 ng/ml FL for 10 min. Crude cell lysates were separated by SDS-PAGE and analyzed by western blot for phosphorylation of signaling molecules. (**A**) The activation of STAT5, AKT and MAPK were analyzed by using phosphor-specific antibodies, stripped and reprobed with antibodies against total STAT5, AKT and MAPK. Ba/F3 native cells were stimulated with 100 ng/ml IL-3 for 5 min as control and an antibody against GAPDH was used as loading control. (**B**) FLT3 receptor was immunoprecipitated with polyclonal FLT3

antibody and analyzed for the tyrosine phosphorylation status by immunoblotting with a phospho-tyrosin antibody, stripped, and reprobed with FLT3 antibody.

Figure 4 FLT3 N676K but not FLT3-ITD N676K is sensitive to AC220 and PKC412.

Ba/F3 cells expressing indicated FLT3 variants were seeded at a density of 4 x 10^4 cells/ml and counted by trypan blue exclusion after 72 h. All experiments were performed in triplicates. Error bars represent standard deviation of the mean. (**A**) Cells were treated with increasing non-toxic concentrations of selective tyrosine kinase inhibitor AC220. (**B**) Cells were treated with increasing non-toxic concentration of tyrosin kinase inhibitor PKC412.

Figure 5 Structural mapping of N676K.

Structure of the autoinhibited FLT3 kinase (PDB accession number 1RJB) is shown as ribbon model with highlighted secondary structure and color coded domains. N676 forms hydrogen bonds to the backbone of H671, stabilizing a loop at the back of the substrate and inhibitor binding pocket (asterisk). N676K will remove these hydrogen bonds, likely destabilizing the loop and the nearby substrate binding pocket. This structural effect can explain resistance against tyrosine kinase inhibitors which target the nearby pocket. However, the mutation could also lift the autoinhibition of FLT3, providing a possible explanation for the observation that this mutation alone shows transforming potential.

Fig. 1 A Exome sequencing

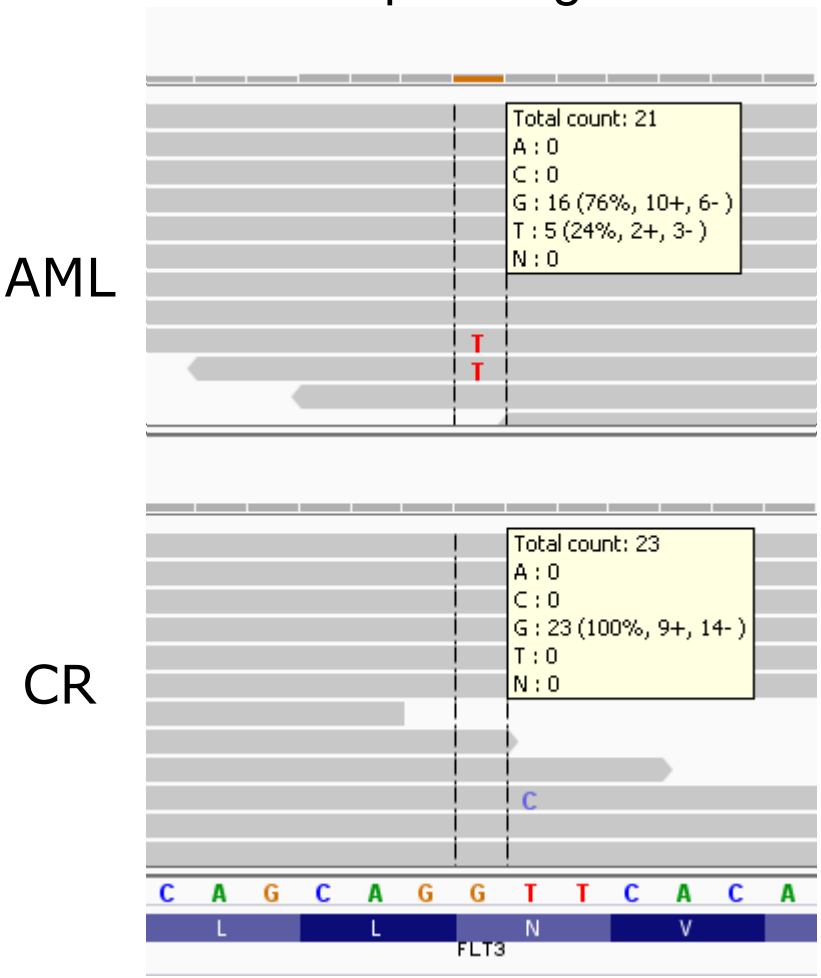

Fig. 1 B Sanger sequencing

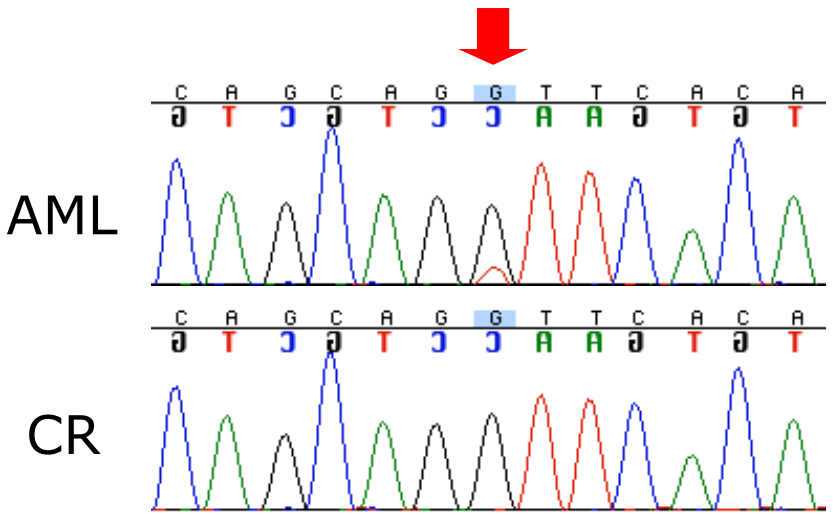

Fig. 1 C Distribution of FLT3 mutations

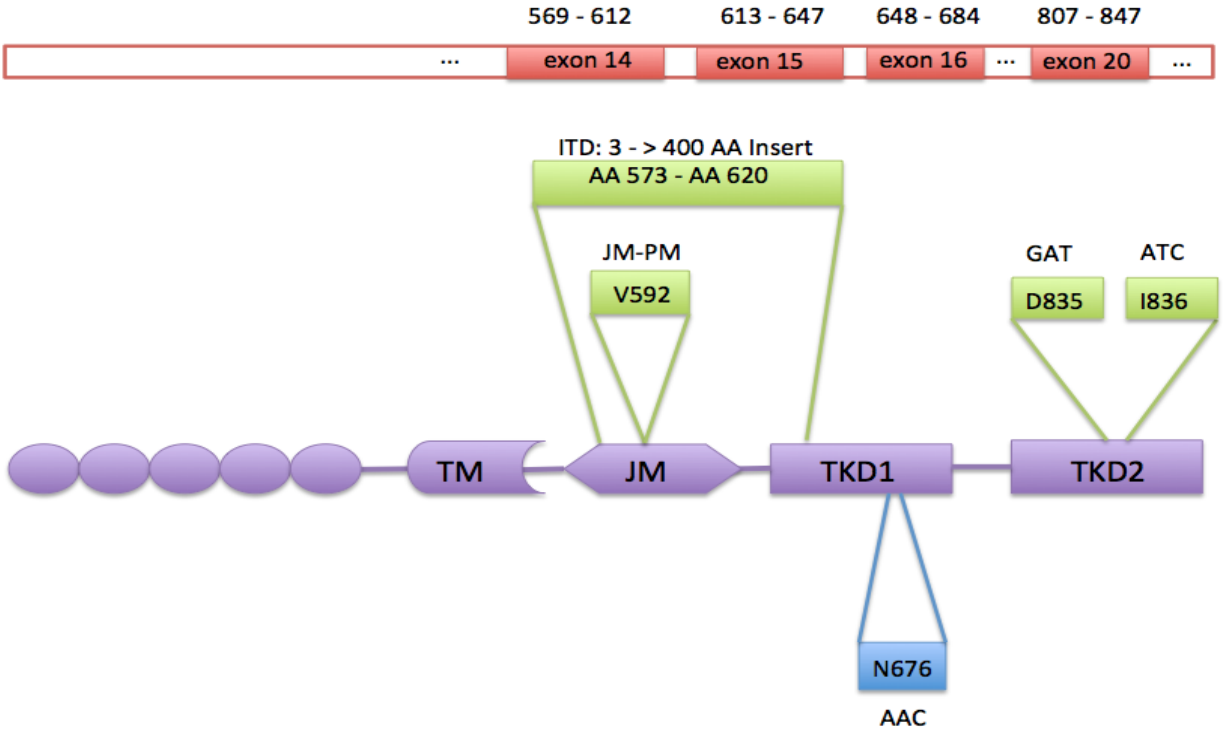

Fig. 1 D Frequency of additional mutations in *CBFB/MYH11* positive AML

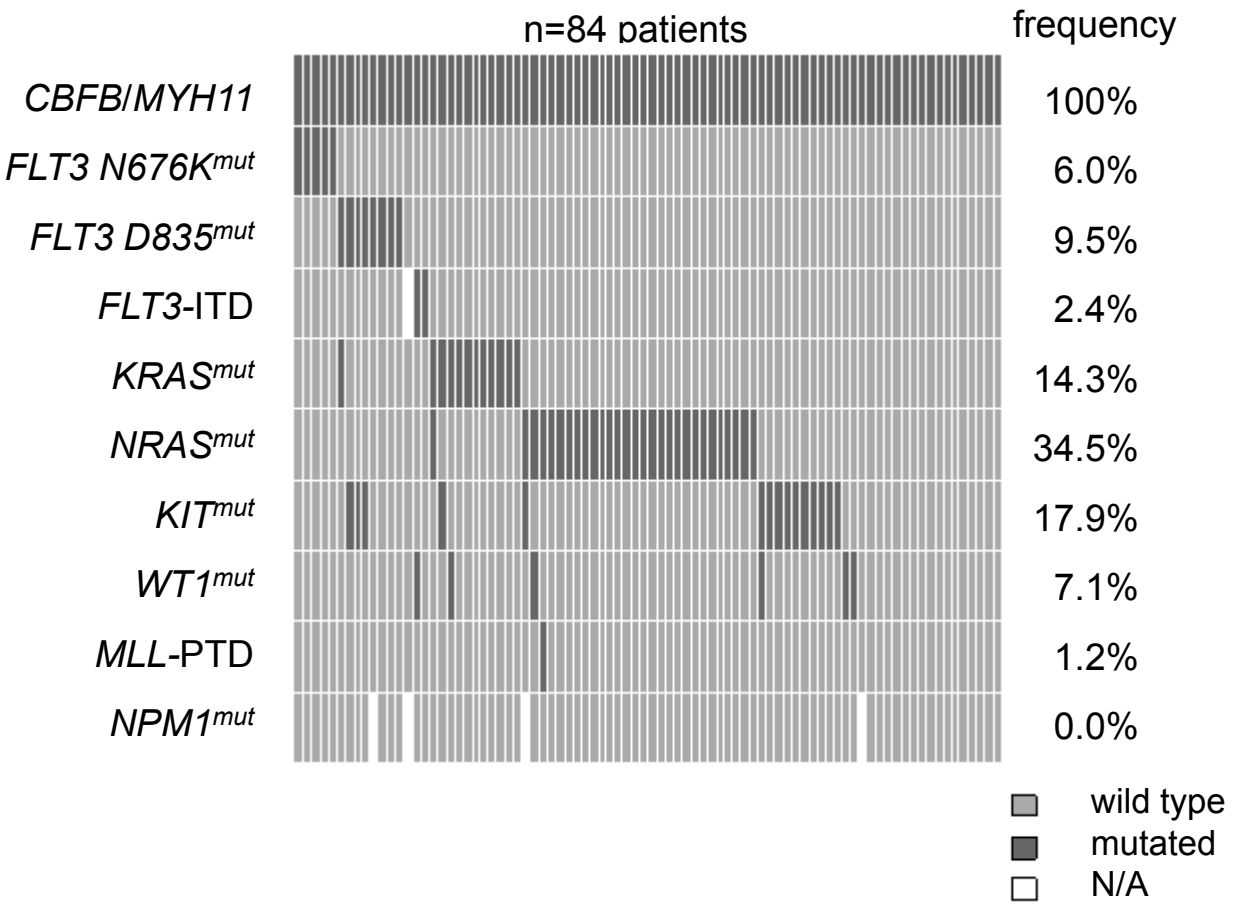

Fig. 2 Transforming potential of FLT3 mutants in Ba/F3 cells

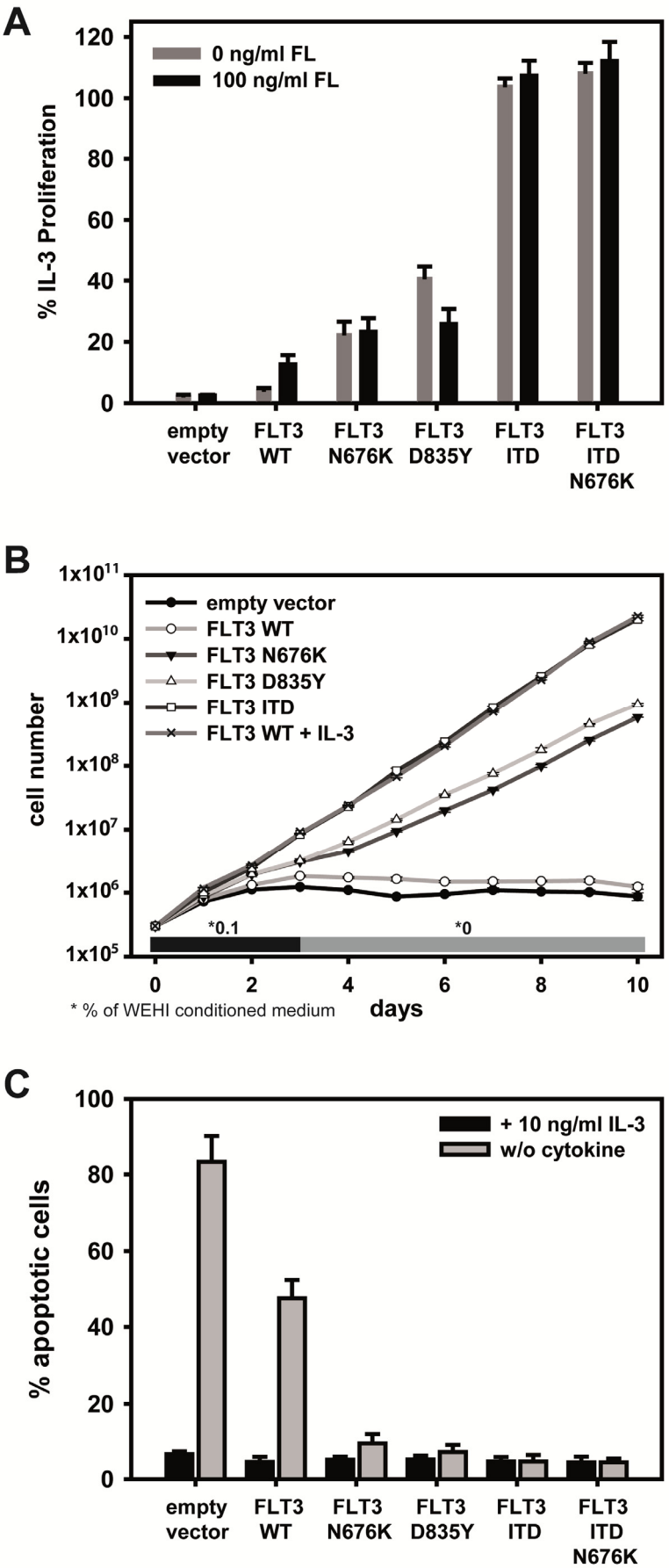

Fig. 3 FLT3 N676K mutant shows constitutive activation of FLT3 signaling

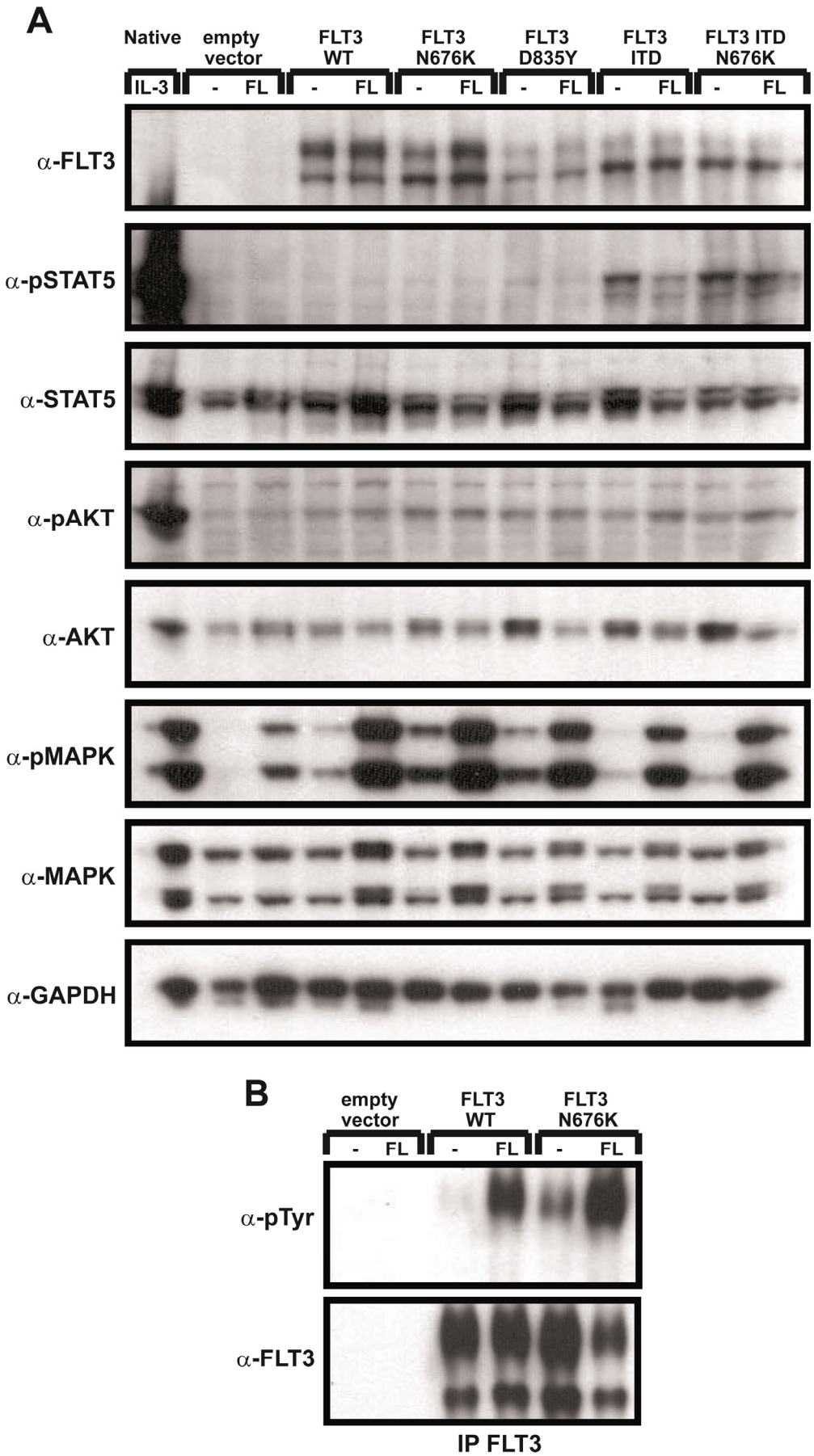

Fig. 4 FLT3 N676K but not FLT3-ITD N676K is sensitive to AC220 and PKC412

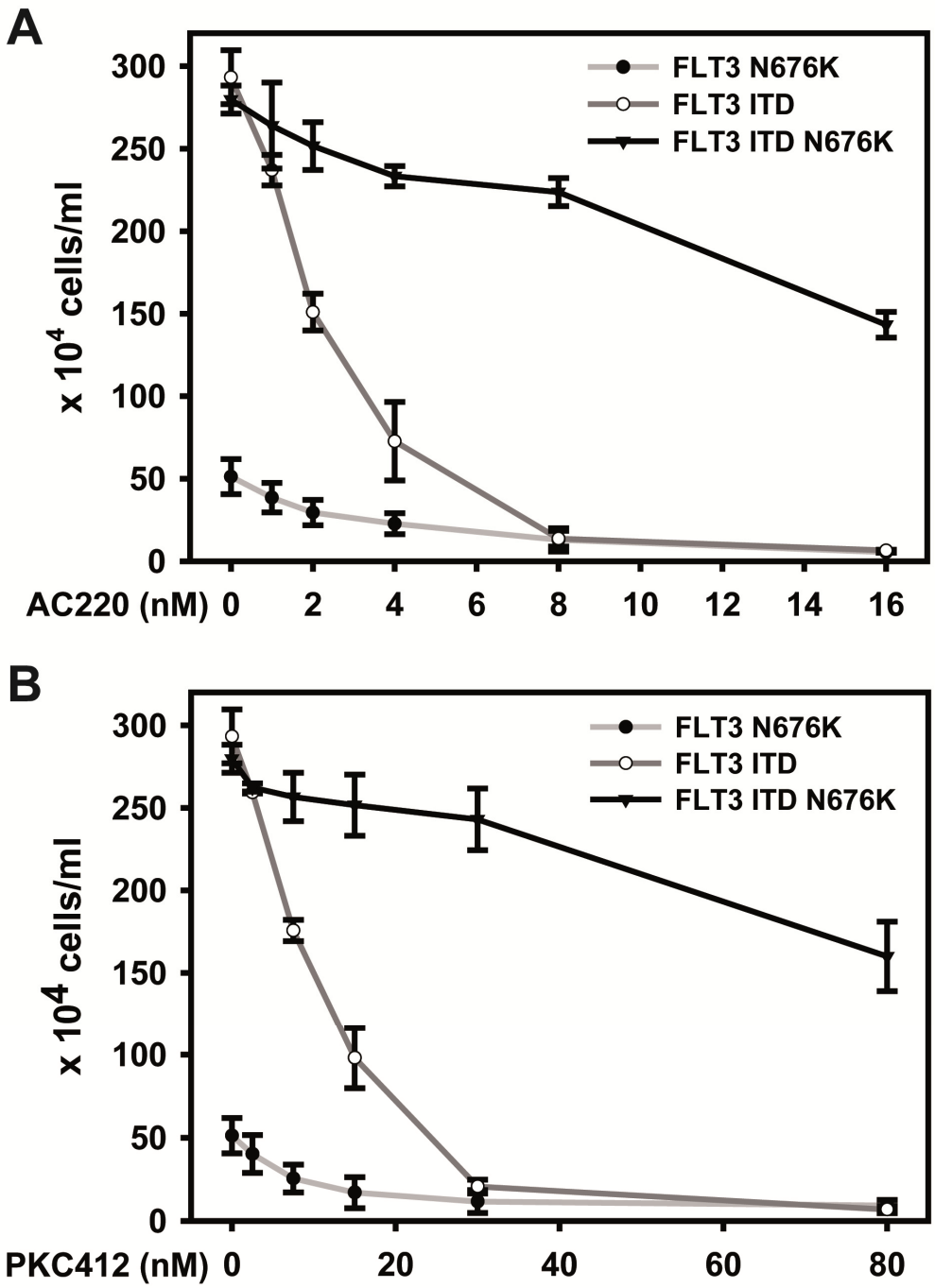

Fig. 5 Structural mapping of N676K