Transformation by Oncogenic Mutants and Ligand-Dependent Activation of FLT3 Wild-type Requires the Tyrosine Residues 589 and 591

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Abstract

Purpose: Mutations in the receptor tyrosine kinase FLT3 are found in up to 30% of acute myelogenous leukemia patients and are associated with an inferior prognosis. In this study, we characterized critical tyrosine residues responsible for the transforming potential of active FLT3-receptor mutants and ligand-dependent activation of FLT3-WT.

Experimental Design: We performed a detailed structure-function analysis of putative autophosphorylation tyrosine residues in the FLT3-D835Y tyrosine kinase domain (TKD) mutant. All tyrosine residues in the juxtamembrane domain (Y566, Y572, Y589, Y591, Y597, and Y599), interkinase domain (Y726 and Y768), and COOH-terminal domain (Y955 and Y969) of the FLT3-D835Y construct were successively mutated to phenylalanine and the transforming activity of these mutants was analyzed in interleukin-3-dependent Ba/F3 cells. Tyrosine residues critical for the transforming potential of FLT3-D835Y were also analyzed in FLT3 internal tandem duplication mutants (FLT3-ITD) and the FLT3 wild-type (FLT3-WT) receptor.

Result: The substitution of the tyrosine residues by phenylalanine in the juxtamembrane, interkinase, and COOH-terminal domains resulted in a complete loss of the transforming potential of FLT3-D835Y-expressing cells which can be attributed to a significant reduction of signal transducer and activator of transcription 5 (STAT5) phosphorylation at the molecular level. Reintroduction of single tyrosine residues revealed the critical role of Y589 and Y591 in reconstituting interleukin-3-independent growth of FLT3-TKD-expressing cells. Combined mutation of Y589 and Y591 to phenylalanine also abrogated ligand-dependent proliferation of FLT3-WT and the transforming potential of FLT3-ITD with a subsequent abrogation of STAT5 phosphorylation.

Conclusion: We identified two tyrosine residues, Y589 and Y591, in the juxtamembrane domain that are critical for the ligand-dependent activation of FLT3-WT and the transforming potential of oncogenic FLT3 mutants.

FLT3 is a member of the class III protein receptor tyrosine kinase family (RTK) that is characterized by five extracellular immunoglobulin-like domains, a juxtamembrane domain (JM), and two protein tyrosine kinase domains (TKD) split by an interkinase domain (IK; ref. 1). The class III receptors also include KIT, FMS, platelet-derived growth factor receptor-α (PDGFRα), and platelet-derived growth factor receptor-β (PDGFRβ). Binding of FLT3 ligand (FL) to its receptor induces dimerization, phosphorylation, and subsequent activation of downstream signaling pathways such as signal transducer and activator of transcription 5 (STAT5), Ras/mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase/AKT (2–6). FLT3 has been shown to play an important role in normal hematopoiesis and is highly expressed in CD34+ hematopoietic progenitor cells (2, 7–9).

Activating mutations of FLT3 are found in 30% of patients with acute myelogenous leukemia (AML) and are associated with an inferior clinical outcome (10–12). FLT3 internal tandem duplications (FLT3-ITD) represent one of the most frequent genetic alterations and occur in ~20% to 25% of patients. These mutations have a variable length resulting in an elongated FLT3 protein with constitutive kinase activity and are associated with higher leukocyte counts at diagnosis (13). A second class of FLT3 mutations primarily occurs at the highly conserved residue D835 in the TKD and is present in 7% to 8% of all patients with AML (14, 15).
We and others have recently shown that FLT3-ITD mutants enhance the proliferative potential of hematopoietic progenitor cells and collaborate with fusion oncogenes, such as the AML1-ETO or PML-RARα oncoproteins, to induce AML in vivo (16, 17). FLT3 selective tyrosine kinase inhibitors are currently in clinical trials for combined treatment with conventional chemotherapy (18, 19). Nevertheless, the underlying mechanism of transformation that is exerted by constitutively activated FLT3 remains elusive.

In the present study, we performed a detailed structure-function analysis of FLT3-TKD and FLT3-ITD receptor mutants to characterize the molecular mechanisms of FLT3-induced transformation. We identified two tyrosine residues, 589 and 591, in the juxtamembrane region of FLT3 that are indispensable for the transforming potential of both FLT3-ITD and FLT3-TKD mutants and ligand-dependent activation of FLT3 wild-type (FLT3-WT).

**Materials and Methods**

*Reagents and cell lines.* Low-passage murine Ba/F3 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and maintained in RPMI 1640 with 10% fetal bovine serum and 10% WEHI conditioned media.
medium as a source of murine interleukin-3 (IL-3) when indicated. Recombinant human FL was purchased from Promokine and recombinant murine IL-3 from Biosource (Solingen, Germany).

**DNA constructs and vectors.** The FLT3-TKD-W51 construct contains a 7-amino acid duplicated sequence (REYELYD) inserted between amino acids 601 and 602 of human FLT3-WT, and the FLT3-TKD-NPOS contains a 28-amino acid duplicated sequence (CSSDNFVYDVFREYELYDKWEFPRNL) inserted between amino acids 611 and 612 of FLT3-WT (17). The FLT3-TKD carries substitution (point mutation) of aspartic acid to tyrosine at position 835 of FLT3-WT. All FLT3 constructs were subcloned in the MSCV-IRES-EYFP retroviral expression vector (kindly provided by R.K. Humphries, The Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, Canada).

**In vitro site directed mutagenesis, DNA sequencing, and nomenclature.** Point mutations were introduced into FLT3-WT cDNA, FLT3-TKD (FLT3-W51 and FLT3-NPOS), and FLT3-TKD by site-directed mutagenesis using the QuickChange kit from Stratagene as described previously (20). The correct sequence of all constructs was confirmed by complete nucleotide sequencing. Mutants with successive single tyrosine residues at the FLT3-D835Y background were named F1 to F8 (Fig. 1A), the mutant 4F contains four tyrosine-to-phenylalanine (Y→F) mutations in the JM domain (Y→F) mutations in the JM domain (YF589/591/597/599). FLT3-D835Y-kinase dead (KD) indicates the mutant containing the K644R mutation leading to the complete loss of kinase activity as described previously (16). Mutations in the JM domain of FLT3-W51 and FLT3-NPOS were named according to the modified positions. All modifications of FLT3-TKD constructs were done in the wild-type, not the duplicated, DNA stretch (Fig. 3A).

**Expression of CD135 by flow cytometry.** Determination of FLT3 expression by FACS analysis was carried out as described previously (20).

**Cell proliferation of Ba/F3 cells and assessment of apoptotic cell death by flow cytometry.** IL-3-dependent Ba/F3 cells stably expressing the indicated constructs were seeded at a concentration of 4 × 10⁴/mL in the presence or absence of IL-3 and FL as described previously (21). Assessment of apoptotic cells was carried out by Annexin V/7-aminoactinomycin D staining as recommended by the manufacturer (Annexin V-phycocyanin apoptosis detection kit; Becton Dickinson) using a FACSCalibur flow cytometer (Becton Dickinson).

**Antibodies.** The following antibodies were used: anti-FLT3 antibody (Santa Cruz), anti-phosphorylated STAT5-Tyr (New England Biolabs), anti-STAT5 (Santa Cruz), anti-pY (Santa Cruz), anti-phosphorylated p44/42 MAPK (New England Biolabs), and anti-p44/42 MAPK (New England Biolabs). Transient transfection of 293 cells and stable transduction of Ba/F3 cells (22), immunoprecipitation and Western blot analysis (22), and GST pull-down (23) were done as described previously.

**Results**

**Structure-function analysis of successive Y→F mutations in the FLT3-TKD background.** In a screening approach to study the contribution of single tyrosine residues to the transforming potential of the FLT3 receptor, we successively mutated the tyrosine residues 566, 572, 589, 591, 597, 599, 725, 768, 955, and 969 of the intracelullar domain of FLT3-TKD-D835Y to phenylalanine and named the constructs as D835Y-F1 to D835Y-F8 and D835Y-4F (Fig. 1A). The tyrosine residues in TKD1 (Y630, Y688, Y693, Y696, and Y702) and TKD2 (Y793, Y842, Y865, Y874, Y889, Y899, Y913, and Y919) are likely to be indispensable for the enzyme function as shown previously for the related KIT receptor (24); hence, these residues were not mutated. IL-3-dependent Ba/F3 cells were retrovirally transduced with the indicated constructs (F1-F8 and 4F) and stable expression was confirmed by CD135 staining as described in Materials and Methods (data not shown). The transduced cell lines were characterized by short-term and long-term proliferation assays. In short-term proliferation assays, the cell lines carrying constructs D835Y-F1 (Y597F and Y599F) or D835Y-F2 (Y572F, Y597F, and Y599F) showed comparable or even increased proliferation rates compared with cell lines expressing the parental FLT3-TKD construct after IL-3 withdrawal for 72 h (Fig. 1B). In detail, the proliferation rates were 90% (D835Y-F1) and 117% (D835Y-F2) compared with cells expressing FLT3-TKD-D835Y (74%; Fig. 1B). The cell lines expressing the D835Y-F3 construct showed a significantly reduced proliferation after 72 h, suggesting that substitution of Y589 and Y591 impaired the transforming potential of FLT3-TKD (Fig. 1B). All the cell lines that harbor the substitutions of Y589 and Y591 (D835Y-F3 to D835Y-F8) showed a reduced transforming potential of 2.7% to 25.7% (Fig. 1B). The lowest transforming potential was observed in cell lines expressing D835Y-4F (data not shown) and D835Y-F8 (Fig. 1B). The effect was even more pronounced in long-term proliferation assays as cell lines expressing constructs D835Y-F3 to D835Y-F8 and D835Y-4F showed a very low transforming potential or completely died after 10 days in
culture, whereas the transforming potential of cell lines expressing D835Y-F2 did not significantly differ from cells expressing FLT3-TKD (Fig. 1C). Next, we analyzed the phosphorylation status of FLT3. Cells expressing FLT3-D835Y-F3 to FLT3-D835Y-F7 showed a successive reduction of the amount of phosphorylated FLT3 when compared with cells expressing nonmanipulated FLT3-TKD-D835Y (Supplementary Fig. S1), whereas no FLT3 phosphorylation was observed in D835Y-F8.

FLT3-TKD mutants carrying the Y\textsuperscript{589}!F substitutions of 589 and 591 show reduced STAT5 phosphorylation and increased apoptotic cell death. An important downstream signaling pathway of activated FLT3 is the STAT5 pathway. STAT5 has been shown to contribute essentially to the transforming potential of the activated FLT3 receptor \textit{in vitro} and \textit{in vivo} (25–27). To investigate the activation of the STAT5 signaling pathway, we prepared crude cell lysates of serum-starved Ba/F3 cells transduced with either vector control (mock), FLT3-WT, FLT3-D835Y, D835Y-F1, D835Y-F2, and D835Y-F3. Immunoprecipitated STAT5 was analyzed with specific antibody against phosphotyrosine (pY). We could clearly show that the expression of D835Y-F3 did not show any phosphorylation of STAT5 compared with FLT3-TKD-D835Y, which is in accordance with the proliferation data (Fig. 2A). Conversely, the level of phosphorylated STAT5 was higher in the D835Y-F2 mutant that showed enhanced proliferation when compared with FLT3-D835Y (Fig. 2A).

Having shown that Y589 and Y591 play an important role in the transforming potential and STAT5 phosphorylation of FLT3-TKD mutants, we next analyzed the cell lines expressing D835Y-F8-F566Y, D835Y-F8-F572Y, D835Y-F8-F589Y, D835Y-F8-F591Y, D835Y-F8-FF589/591YY, or mock-transduced cells were seeded at a density of $4 \times 10^4/mL$ in the absence or presence of IL-3. Viable cells were counted after 72 h by trypan blue exclusion. The growth of cells with IL-3 was defined as 100% (control). SE of three independent experiments is indicated.

Fig. 2. Re-mutation of both the residues 589 and 591 from F\textsuperscript{589}\!Y reconstitutes the transforming potential of the D835Y-F8 mutant. A, overview of phenylalanine to tyrosine re-mutations at the FLT3-D835Y-F8 background. B, Ba/F3 cells expressing the FLT3-D835Y, D835Y-F8, F8-F566Y, F8-F572Y, F8-F589Y, F8-F591Y, F8-FF589/591YY, or mock-transduced cells were seeded at a density of $4 \times 10^4/mL$ in the absence or presence of IL-3. Viable cells were counted after 72 h by trypan blue exclusion. The growth of cells with IL-3 was defined as 100% (control). SE of three independent experiments is indicated.
phenylalanine residues were re-mutated to tyrosine in the D835Y-F8 mutant (Fig. 3A). Reintroduction of tyrosine at amino acid 566 (F8-F566Y) or amino acid 572 (F8-F572Y) induced only negligible IL-3-independent proliferation rates of 3.6% and 1.2%, respectively, when compared with unmanipulated FLT3-TKD-D835Y (Fig. 3B). In contrast, the reintroduction of tyrosine residues at amino acid 589 (F8-F589Y) or amino acid 591 (F8-F591Y) led to a partial reconstitution of the transforming potential of the mutated FLT3 receptor by 30% and 42%, respectively (Fig. 3B). Reintroduction of tyrosine at positions 589 and 591 (F8-FF589/591Y) completely reconstituted the transforming potential of the D835Y-F8 mutant (Fig. 3B).

Single Y→F mutations of Y589, Y591, or Y597 in the FLT3-ITD background do not reduce the transforming potential. To further characterize the roles of tyrosine residues 589 and 591 in the FLT3-ITD receptor, single Y→F mutations were introduced at amino acid 589 (FLT3-ITD-Y589F) or amino acid

![Diagram](image_url)

**Fig. 4.** Functional analysis of the role of tyrosine residues Y589, Y591, Y597, and Y599 in the FLT3-ITD background. A, overview of Y→F mutations generated in the FLT3-ITD background. B, Ba/F3 cells expressing the FLT3-W51, FLT3-W51-Y589F or FLT3-W51-Y591F, FLT3-W51-Y597F, or mock-transduced cells were seeded at a density of 4 × 10^4/mL in the absence or presence of IL-3. Viable cells were counted after 72 h by trypan blue exclusion. The proliferation of cells with IL-3 was defined as 100% (control). SE of three independent experiments is indicated. C and D, FLT3-ITD mutants were stably expressed in Ba/F3 cells and analyzed as described in B.
591 (FLT3-ITD-Y591F) in the FLT3-ITD-W51 and FLT3-ITD-NPOS constructs (Fig. 4A). All manipulations were done in the wild-type, not in the duplicated DNA stretch. As shown in Fig. 4B and D, cell lines expressing FLT3-ITD-Y589F or FLT3-ITD-Y591F showed no significant reduction of their transforming potential after IL-3 withdrawal when compared with unmanipulated FLT3-ITD cells. To extend this finding that single substitutions of tyrosine residues have no effect on transforming potential of FLT3-ITD-expressing cells, we generated another Y-F substitution mutant at amino acid 597 (FLT3-W51-Y597F; Fig. 4B). Cells expressing FLT3-ITD-Y597F show a comparable transforming potential to unmanipulated FLT3-ITD-W51 by phenylalanine.

Combined substitution of both Y589 and Y591 abrogates by phenylalanine the transforming potential of FLT3-ITD. As single Y-F mutants of amino acids 589, 591, and 597 had no effect on the transforming potential of FLT3-ITD-expressing cells, we generated a variety of double substitution mutants of JM domain tyrosine residues 589, 591, 597, and 599: FLT3-W51-YY589/591FF, FLT3-W51-YY589/597FF, and FLT3-W51-YY589/599FF in addition to FLT3-W51-YY591/597FF, FLT3-W51-5991/99FF, and FLT3-W51-YY597/599FF (Fig. 4A). In proliferation assays, Ba/F3 cells expressing FLT3-W51-YY589/591FF were unable to proliferate in the absence of IL-3 (Fig. 4C). Except for FLT3-W51-YY597/599FF cell lines expressing W51-YY589/597FF, W51-YY589/599FF, W51-YY591/599FF, or W51-YY591/599FF showed a significant reduction in the transforming potential by 58% to 82% when compared with unmanipulated FLT3-ITD-W51 cells (Fig. 4C). Substitution of both Y589 and Y591 with phenylalanine (FLT3-NPOS-YY589/591FF) in a structurally different ITD (FLT3-ITD-NPOS) induced a phenotype identical to FLT3-W51-YY589/591FF-expressing cells (Fig. 4D).

Western blot analysis revealed a slight reduction of FLT3 phosphorylation in FLT3-W51-YY589/591FF cells when compared with FLT3-ITD-W51 cells (Supplementary Fig. S1).

Double Y-F substitution mutants of 589, 591, 597, and 599 show reduced activation of STAT5 and MAPK pathways. To investigate the activation of the STAT5 signaling pathway, we prepared whole-cell lysates of serum-starved Ba/F3 cells transduced with either vector control (MIY) or FLT3-WT, FLT3-ITD-W51, FLT3-W51-YY589F, FLT3-W51-YY591F, FLT3-W51-YY597F, FLT3-W51-YY589/591FF, FLT3-W51-YY589/597FF, FLT3-W51-YY589/599FF, FLT3-W51-YY591/599FF, or FLT3-W51-YY597/599FF. Single Y-F substitution and FLT3-ITD-W51-expressing cells showed comparable levels or slightly reduced levels (FLT3-W51-YY591F) of phosphorylated STAT5. The double substitution mutants FLT3-W51-YY589/591FF, FLT3-W51-YY589/599FF, FLT3-W51-YY591/599FF, and FLT3-W51-YY597/599FF showed a significantly reduced STAT5 phosphorylation. FLT3-W51-YY597/599FF and FLT3-W51-YY589/597FF showed a slightly reduced STAT5 phosphorylation, when compared with nonmanipulated FLT3-ITD-W51 or single Y-F substitution mutants (FLT3-W51-YY589F, FLT3-W51-YY591F, and FLT3-YY597F) cells (Fig. 5A).

Cells expressing FLT3-W51-YY589/599FF, FLT3-W51-YY591/597FF, and FLT3-W51-5991/99FF showed a transforming potential of ~20% but did not show any STAT5 phosphorylation. To further analyze this phenomenon, we measured MAPK phosphorylation, another important downstream signaling pathway of the activated FLT3 receptor. Analysis of the lysates by immunoblotting with a specific antibody against phosphorylated MAPK showed that all the cells with double Y-F substitution mutants, except FLT3-W51-YY589/591FF, showed phosphorylation of MAPK but at a reduced rate compared with nonmanipulated FLT3-ITD-W51 or single Y-F substitution mutant cells (Fig. 5B).

These results show that the residues Y589 and Y591 play an important role in STAT5 and MAPK activation by the FLT3-ITD receptor.

Y589 and Y591 are indispensable for ligand-dependent signaling of the FLT3-WT receptor. Because combined Y-F mutations of Y589 and Y591 had severe effects on the transforming potential of FLT-TKD and FLT3-ITD expressing cells, we analyzed the role of Y589 and Y591 in the signaling properties of the FLT3-WT receptor. We generated the Y-F substitution mutant of both Y589 and Y591 (FLT3-WT-YY589/591FF) in FLT3-WT (Fig. 6A). Overexpression of FLT3-WT-YY589/591FF did not induce any IL-3-independent growth, but proliferation was totally abrogated in the presence of FL when compared with cells expressing FLT3-WT (Fig. 6B).

Discussion

In contrast to the studies published to date on structural motifs critical for the transformation mediated by FLT3 mutant receptors (28–30), our study presents data not only on FLT3-WT but also on FLT3-TKD and FLT3-ITD receptor mutants. Because the structure of the JM domain is altered by the insertion of an ITD (31), the study was focused on a FLT3 receptor with an activating mutation in the TKD domain that allows the analysis of all critical tyrosine residues. During the course of our experiments, we identified amino acids Y589 and Y591 as critical residues for the transformation exerted by FLT3-TKD. The essential role of tyrosine residues YY589/591 in mediating transformation was then confirmed in ITD mutants and the FLT3-WT receptor.
Our results indicate that a combined Y→F substitution of amino acids Y597, Y599, and Y572 in FLT3-TKD (F1 and F2) induce a higher proliferation rate in IL-3-deprived Ba/F3 cells compared with unmanipulated FLT3-TKD (Fig. 1B and C). The FLT3 crystal structures shows that amino acids Y572 and Y599 are the only two tyrosine residues that form a tight pocket in FLT3 and might be responsible for maintenance of the inactive state of FLT3-WT (31). This finding is also in line with previous reports on the homologous Y533 in KIT showing the negative regulatory function of this residue (32). Mutation of Y533 (Y572 in FLT3) to alanine in wild-type KIT led to spontaneous phosphorylation of the receptor. The lack of phenolic hydroxyl oxygen in Y→F substituted 572 and 599 probably disrupts the interactions formed by these amino acids and therefore could account for the increased transforming potential of D835Y-F1 and D835Y-F2 compared with FLT3-TKD-D835Y.

Our results clearly show that Ba/F3 cell lines carrying FLT3-TKD constructs with Y→F substitution of Y589 and concomitantly Y591 (D835Y-F3-F8 and D835Y-4F) lead to a reduced transforming potential in short-term cultures and total loss of transforming potential in long-term cultures (Fig. 1B and C). To determine whether loss of FLT3 phosphorylation results in concomitant loss of transforming potential, we analyzed the FLT3 phosphorylation in FLT3-TKD-D835Y mutants. All the mutants D835Y-F3 to D835Y-F7 with little or no transforming potential in short-term and long-term assays exhibited FLT3 phosphorylation but at a reduced level when compared with nonmanipulated FLT3-TKD-D835Y (Supplementary Fig. S1). There was a total lack of phosphorylation in D835Y-F8 mutant in which all tyrosines in the JM, Ki and C-terminal region were mutated. The loss of the transforming potential in mutants D835Y-F3 and D835Y-4F, even in the presence of FLT3 phosphorylation, suggests that Y589 and Y591 act as docking sites for downstream signaling molecules independent of FLT3 phosphorylation.

Y589 and Y591 have been shown to be conserved in the related tyrosine kinases KIT (Y568, Y570; ref. 33), PDGFRα (Y572, Y576; refs. 34, 35), and PDGFRβ (Y579;Y581; ref. 36), and substitution of homologous residues with phenylalanine in these RTK’s reduced their kinase activity (37–41). Previous studies on PDGFRβ have shown that Y579 and Y581 homologous to Y589 and Y591 in FLT3 bind STAT5 (36). STAT5 is an important downstream signaling pathway of FLT3 and phosphorylated STAT5 has been detected in blasts of 20% to 80% of patients with AML (42–45). Our data clearly show that Y589 and Y591 play an important role in STAT5 autophosphorylation and apoptosis. All cell lines expressing activated FLT3 with Y→F substitutions of both Y589 and Y591 showed reduced STAT5 phosphorylation and an increased rate of apoptotic cell death after cytokine withdrawal (Fig. 2A and B). This information is in contradiction to a recent report by Rocnik et al., in which no difference in the STAT5 phosphorylation was observed between FLT3-TKD-D835Y and FLT3-D835Y-YY589/591FF (29). A possible explanation for this discrepancy might be the use of different cell culture systems, like the usage of 32D cells by Rocnik et al. instead of Ba/F3 cells and starving of the cells for only 4 h, compared to 24 h starvation period in this report. STAT5 has been shown to induce the expression of the antiapoptotic protein BCL-XL (46) thereby protecting the cells from apoptosis (47). D835Y-F1 and D835Y-F2 cells showed high levels of phosphorylated STAT5 corresponding to a low rate of apoptosis after IL-3 withdrawal (5–8%). In contrast, the D835Y-F7 and D835Y-F8, which showed no STAT5 autophosphorylation (data not shown), displayed a higher apoptotic rate after IL-3 withdrawal (32–40%; Fig. 2B).

To further confirm the role of Y589 and Y591 for the transforming potential of FLT3-TKD, we re-mutated single phenylalanine residues to tyrosine in the D835Y-F8 background (Fig. 3A). Single re-mutation of 566 or 572 (F8-F566Y or F8-F572Y) did not induce any significant IL-3-independent proliferation in Ba/F3 cells. The reintroduction of tyrosine at amino acid 589 (F8-F589Y) or amino acid 591 (F8-F591F) partially reconstituted the transforming potential of the D835Y-F8 mutant to 30% to 42% (Fig. 3B). Interestingly, reintroduction of tyrosines at both 589 and 591 (F8-FF589/591YY) totally reconstituted the transforming potential of the D835Y-F8 mutant. These results suggest that Y589 and Y591 are two critical amino acids for the transforming potential of FLT3-TKD. FLT3 phosphorylation studies in the F8-FF589Y, F8-FF591Y, and FLT3-FF589/591YY showed weak FLT3 phosphorylation (data not shown). This result points to a role of Y589 and Y591 as docking sites for downstream signaling molecules independent of FLT3 phosphorylation.

Next, we analyzed the role of Y589 and Y591 for the transforming potential of FLT3-ITD mutants in Ba/F3 cells. Single Y→F mutation of 589 (ITD-Y589F) and 591 (ITD-Y591F) in two structurally different FLT3-ITDs (W51/NPOS) did not significantly affect the transforming potential of cell lines expressing these constructs (Fig. 4B and D). However, Y→F substitution of both Y589 and Y591 (ITD-YY589/591FF) totally

![Fig. 6. Substitution of both Y589 and Y591 by phenylalanine abrogates the ligand-dependent activation of FLT3-WT in Ba/F3 cells. A, overview of Y→F substitutions of both Y589 and Y591 generated in FLT3WT & Ba/F3 cells expressing the FLT3-WT, FLT3-WT-YY589/591FF, or mock-transduced cells were seeded at a density of 4 × 10^4/mL in the absence or presence of IL-3 or FL (60 ng/mL). Viable cells were counted after 72 h trypan blue exclusion. The proliferation of cells with FL was defined as 100% (control). SE of three independent experiments is indicated.](image-url)
abolished the transforming potential of FLT3-ITD (Fig. 4C and D). Our data are discrepant to the results published by Kiyoi et al., where a FLT3-ITD carrying Y589, Y591, Y597, and Y599 mutated to phenylalanine did not show any difference in the transforming potential in 32D cells when compared with nonmanipulated FLT3-ITD (28). However, a similar study by Rocnik et al. in 32D cells expressing the mutant FLT3-ITD-YY589/591FF showed a reduced transforming potential when compared with nonmanipulated FLT3-ITD (29). To address these discrepancies, we expressed the mutants FLT3-ITD-W51/NPOS, FLT3-W51-YY589/591FF and NPOS-YY589/591FF in 32D cells. No factor-independent growth was observed in 32D cells expressing FLT3-W51/±NPOS-YY589/591FF confirming our data observed in Ba/F3 cells (Supplementary Fig. S2). Moreover, our results are in line with the study by Rocnik et al. reporting that transfected mice with bone marrow cells carrying a FLT3-ITD-YY589/591FF construct showed no difference in survival compared with mice transfected with FLT3-WT-expressing bone marrow. In contrast, transplantation with FLT3-ITD-transduced bone marrow cells led to a lethal myeloproliferative disease with short latency (29).

Next, we analyzed other combinations of tyrosine residues in the JM domain responsible for the transforming potential of FLT3-ITD. Hence, we analyzed the effect of Y→F substitution of different combinations of JM domain tyrosines (30) (Y589, Y591, Y597, and Y599). Cell lines expressing FLT3-W51-YY589/597FF, FLT3-W51-YY589/599FF, FLT3-W51-YY591/597FF, and FLT3-W51-YY591/99FF conferred a low transforming potential (Fig. 4C). FLT3-W51-YY597FF-expressing cells did not show an impaired IL-3-independent growth. These results were further supported by analyses of STAT5 phosphorylation (Fig. 5A). We observed a slightly reduced STAT5 phosphorylation in the cells expressing FLT3-W51-YY591F mutant when compared with FLT3-W51-YY589F, FLT3-W51-YY597F, and nonmanipulated FLT3-ITD-W51 (Fig. 5A). This observance could be explained by a recent study, in which in vitro mapping of autophosphorylated tyrosine sites in FLT3-ITD revealed Y591 as the sole autophosphorylated site in the JM domain. Also, Y591 along with Y589 were reported to be the putative binding sites for STAT5 in another study (29).

In accordance with the proliferation data, cell lines expressing FLT3-W51-YY589/591FF showed no STAT5 phosphorylation. Cell lines expressing FLT3-W51-YY589/597FF and FLT3-W51-YY597/599FF showed a reduced STAT5 phosphorylation when compared with unmanipulated FLT3-ITD (Fig. 5A).

Surprisingly, cell lines expressing FLT3-W51-YY589/599FF, FLT3-W51-YY591/597FF, and FLT3-W51-591/99FF, which showed transforming potential of ~20%, did not show any STAT5 phosphorylation (Fig. 5A). Therefore, we further analyzed phosphorylation of MAPK, another important FLT3 downstream signaling molecule. All the cells expressing double Y→F substitution mutants of FLT3-ITD-W51, except FLT3-W51-YY589/591FF, showed MAPK phosphorylation but at lower rate compared with unmanipulated FLT3-ITD cells (Fig. 5B). These results suggest that combination of amino acid residues Y589 and Y591 in FLT3-ITD is important for STAT5 and MAPK phosphorylation, whereas residues Y597 and Y599 might be important for the structural maintenance of FLT3-ITD for STAT5 recruitment. Furthermore, these results suggest that multiple signaling pathways play a role for transformation of cells by FLT3 mutants. To analyze the mechanism of STAT5 phosphorylation, we performed coimmunoprecipitation assays and GST pull-down of FLT3 with SH2 domain of STAT5. We could not detect any direct interaction of STAT5 with FLT3 or FLT3 mutants (data not shown). In line with the proliferation data, FLT3-ITD-W51 showed an interaction with the SH2 domain of Src, whereas the FLT3-W51-YY591/599FF showed no interaction (Supplementary Fig. S3). In GST pull-down experiments, we confirmed direct interaction of STAT5 with Src-SH2 domain (Supplementary Fig. S3). Recent studies have shown the interaction of Src kinases with Y589 of FLT3-WT (30). Src kinases direct the phosphorylation of STAT5 (48). These data show that Y589 binds Src kinases, which can interact with an activated STAT5.

Y→F substitution of both 589 and 591 in the FLT3-WT background (FLT3-WT-YY589/591FF) totally abrogated the proliferation of Ba/F3 cells upon stimulation with FL (Fig. 6B) indicating that Y589 and Y591 in FLT3-WT are essential for FL-mediated proliferation.

In conclusion, we have identified Y589 and Y591 as the critical tyrosine residues required for STAT5 signaling and for the transforming phenotype of active FLT3-receptor mutants and FL-mediated proliferation of FLT3-WT.

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References


Tyrosine Residues 589 and 591

Transformation by Oncogenic Mutants and Ligand-Dependent Activation of FLT3 Wild-type Requires the Tyrosine Residues 589 and 591

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