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ONCOGENIC FLT3 RECEPTORS DISPLAY DIFFERENT SPECIFICITY AND KINETICS OF  
AUTOPHOSPHORYLATION

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## **Abstract**

*Objective.* Fms-like tyrosine kinase-3 (FLT3), a growth factor receptor normally expressed in hematopoietic progenitor cells, has been shown to have an important role in the development of acute myeloid leukemia due to activating mutations. FLT3 mutations are found in approximately one third of AML patients and correlate with a poor prognosis, thus making the FLT3 receptor a potential therapeutic target. The aim of the investigation is to analyze the kinetics and specificity of FLT3 autophosphorylation in wild-type FLT3 as well as in the oncogenic FLT3 mutants.

*Methods.* We have used Ba/F3 cells stably expressing either wild-type, ITD or D835Y mutants of FLT3 in order to compare the site selectivity of tyrosine phosphorylation sites. By the use of a panel of phospho-specific antibodies directed against potential tyrosine phosphorylation sites in FLT3, we identified several novel phosphorylation sites in FLT3 and studied the kinetics and specificity of ligand-induced phosphorylation in living cells.

*Results.* Eight phosphorylated tyrosines (pY589, pY591, pY599, pY726, pY768, pY793, pY842 and pY955) were investigated and shown to be differentially phosphorylated in the wild-type versus the mutated receptors. Furthermore, we show that tyrosines 726, 793 and 842 are novel phosphorylation sites of FLT3 in intact cells.

*Conclusion.* In this study, we have looked at the site-specific phosphorylation in the wild-type FLT3 in comparison to the mutants found in AML. We observed not only quantitative changes but more importantly, qualitative differences in the phosphorylation patterns of the wild-type and the mutated FLT3 receptors, which might contribute to the understanding of the mechanisms by which FLT3 contributes to AML in patients with mutations in FLT3.

**Keywords:** Receptor tyrosine kinase; FLT3; ITD; D835Y; AML; phospho-specific antibodies; phosphorylation

## **Introduction**

In the context of acute myeloid leukemia (AML), Flt3 is one of the most frequently altered genes, resulting in constitutive activation in approximately 30% of all patients, and correlating to a worse prognosis as compared to patients carrying the wild-type gene [1, 2]. Human Flt3 (also known as fetal liver kinase-2 or human stem cell kinase-1) [3] was cloned in 1991 [4, 5] and identified as a member of the class III receptor tyrosine kinase (RTK) family. FLT3 is expressed on the cell surface of immature/ progenitor cells of the myeloid and B-lymphoid lineages and the expression is lost upon differentiation, with the exception of dendritic cells [6, 7]. Recent studies on the FLT3 expression have indicated distinct differences between the human and murine hematopoietic systems. While the expression of murine FLT3 is restricted to multipotent and lymphoid progenitor cells incapable of self-renewal, the human FLT3 is expressed on primitive hematopoietic stem cells and on progenitors along both the lymphoid and the granulocyte/macrophage pathway [8]. The main functions of FLT3 include induction of proliferation, differentiation and survival of normal hematopoietic progenitor cells. The FLT3 receptor also plays a role in the immune system by promoting dendritic cell development when activated by FLT3 ligand (FL) [9-11]. FL is expressed by bone marrow stroma cells and in many other tissues [2, 12]. Activation of the wild-type receptor occurs by FL binding, causing dimerization and autophosphorylation of specific tyrosine residues which in turn trigger signal transduction further into the cell [13, 14].

The first described activating mutation in Flt3 was the so-called internal tandem duplication (ITD). The ITD occurs as an in-frame duplication of a sequence of varying length, leading to release of the negative regulatory constraint that the juxtamembrane domain poses on the kinase domain of FLT3 [15, 16]. This is found in about 17-26% of patients with AML, and is the most common mutation in FLT3 [1, 2]. Another mutation shown to be of much importance is a missense point mutation of D835 (substitution to Y,

V, H, E or N) in the activation loop of the tyrosine kinase domain of FLT3, which is found in approximately 7% of AML patients [17]. Both the ITD and the D835Y mutations cause constitutive activation and ligand-independent phosphorylation of the receptor [18].

Mutations analogous to D835Y have been found in the closely related receptor tyrosine kinase c-KIT at aspartic acid 816, which is mutated in a subset of AML patients [19]. The corresponding site in c-MET receptor (D1264N) has been found to be mutated in papillary renal carcinoma, leading not only to activation of the kinase activity of the receptor, but also altering its substrate specificity [20]. Furthermore, the oncogenic M918T mutant of the receptor tyrosine kinase c-RET (found in multiple endocrine neoplasia type 2B) showed both altered substrate specificity towards exogenous substrates as well as altered pattern of autophosphorylation [21]. This prompted us to investigate whether also the oncogenic mutants of FLT3 showed altered pattern of autophosphorylation, compared to wild-type FLT3 and whether this could explain the phenotypic differences of cells expressing the respective mutant.

In the undertaken study we have identified the tyrosine residues Y726, Y793 and Y842 as novel phosphorylation sites of FLT3 in intact cells. Furthermore, in total eight phosphorylation sites in FLT3 were analyzed and shown to be phosphorylated upon ligand stimulation. Kinetics of the phosphorylation of these sites was compared in Ba/F3 cells stably transfected with either wild-type FLT3, ITD or D835Y mutants. Tyrosine residues Y589, Y591, Y599, Y726, Y768, Y793, Y842 and Y955 were shown to be differentially activated in the wild-type receptor and the mutant receptors, respectively. These data form the basis for a further exploration of the function of the individual tyrosine phosphorylation sites in downstream signaling through the wild-type FLT3 as well as the oncogenic mutants of FLT3 in order to understand the mechanism by which FLT3-ITD and FLT3-D835Y functions in pathological conditions.

## **Materials and Methods**

### *Plasmids, peptides and antibodies*

The pMSCV-puro vector containing human Flt3 cDNA was a kind gift from Dr. D. Gary Gilliland. The anti-FLT3 antibody was raised against synthetic peptides corresponding to amino acids 740-757 of human FLT3 and purified as described [22]. The phospho-specific antibodies against individual tyrosine phosphorylation sites in FLT3 were raised by immunizing rabbits with synthetic peptides (JPT Peptides Technology, Berlin, Germany) corresponding to the peptide sequence surrounding the individual tyrosine residues conjugated to keyhole limpet hemocyanin (KLH) (pY589:CGSSDNEpYFYVDFREY;pY591:CTGSSDNEYFpYVDFREY;pY599:CYVDFREYEpYDLKWEF;pY726:CEHNFSFpYPTFQSHp;pY768:CSEDEIEpYENQKRLEE;pY793:CDLLCFApYQVAKGMEF;pY842:CIMSDSNpYVVRGNAR;pY955:CDAEEAMpYQNVDGRVS;pY969:CESPHTpYQNRPPFSR). All antibodies were affinity purified as described [23]. 4G10 anti-phosphotyrosine antibody was from Upstate Biotechnology and the horse radish peroxidase (HRP)-coupled secondary anti-mouse and anti-rabbit antibodies were from Pierce (Rockford, IL).

### *Site-directed mutagenesis*

To mutate specific tyrosine residues to phenylalanine residues, the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used and all mutations were confirmed by sequencing.

Cell culture-The murine pro-B cell line Ba/F3 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), was cultured in RPMI-1640 medium with L-Glutamine and HEPES (PAA Laboratories GmbH, Pasching, Austria). Medium was supplemented with 10% heat-inactivated FBS (PAA Laboratories GmbH, Pasching, Austria), 10 ng/mL of recombinant murine IL-3 (Prospec Tany, Rehovot, IL), 100 units/mL penicillin and streptomycin (PAA Laboratories GmbH, Pasching, Austria).



COS-1 and EcoPack cells were cultured in Dulbeccos modified essential medium (PAA Laboratories GmbH), supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin. The human AML cell lines MV4-11 and THP-1 were maintained in RPMI-1640 medium with 20% FBS and 100 units/mL penicillin and streptomycin.

#### *Transient and stable transfection*

COS-1 cells were transfected using JetPEI (PolyPlus-transfection, Illkirch, France) according to manufacturer's instructions. To establish cells stably expressing wild-type, ITD or D835Y of FLT3, packaging EcoPack cells were transfected with the corresponding pMSCV-Flt3 constructs and virus-containing supernatants were collected 72 hours after transfection. Retroviral infection of Ba/F3 cells was followed by a 2-week selection in 2 µg/mL puromycin (Sigma-Aldrich, Steinheim, Germany) and Flt3 expression was confirmed using flow cytometry.

#### *Cell stimulation, immunoprecipitation and Western blotting*

Before stimulation Ba/F3 cells were starved for 4 hours in RPMI-1640 medium without serum and cytokines. Treatment of cells with sodium pervanadate for 15 minutes was used as positive control for full kinase activation [24]. Cells were stimulated with 100ng/mL FL (Prospec Tany, Rehovot, Israel) for the indicated periods of time at 37°C, washed once with cold PBS and lysed. Lysates were subjected to immunoprecipitation and Western blotting as described elsewhere [23, 25]. Immunodetection was performed by enhanced chemoluminescence using Immobilon Western chemoluminescent HRP substrate (Millipore Corporation, USA) and a CCD camera (LAS-3000, Fujifilm, Tokyo, Japan). Signal intensity was quantified by MultiGauge software (Fujifilm).

### *Statistical analysis*

Non-parametric Kruskal-Wallis test was performed to observe the difference in each time points between wild-type FLT3, FLT3-ITD and FLT3-D835Y mutated Ba/F3 cell lines. Posthoc analysis using non-parametric unpaired t-test, Mann-Whitney test was performed when the p-value were significant (p-value  $\leq$  0.05) or close to significant (p-value  $<$  0.067) level.

## **Results**

### *Assessment of functionality and specificity of the phospho-specific antibodies against FLT3 and identification of the novel autophosphorylation sites Y726, Y793 and Y842 in living cells*

COS-1 cells were used as a model for assessing the functionality and specificity of the phospho-specific antibodies. Cells were transfected with wild-type FLT3 and each corresponding Y-to-F mutant. Following starvation, cells were stimulated for 5 minutes with FL. Samples were immunoprecipitated with FLT3 antibody and subsequently analyzed by Western blotting with the corresponding affinity purified antibody. As controls for receptor activation and expression, the membranes were stripped and re-probed with antibodies against phosphotyrosine (4G10) and FLT3. Thus, the kinase activity of FLT3 was intact in the individual mutants. The results using the FLT3 antibodies against pY589, pY591, pY599, pY726, pY793 and pY842 are shown in Fig. 1. Thus, in addition to the previously identified phosphorylation sites pY589, pY591, pY599, pY768, pY955 and pY969 [26, 27] we here demonstrate three novel phosphorylation sites: pY726, pY793 and pY842.

### *Comparison of the site-specific activation kinetics between the wild-type FLT3, ITD and D835Y receptors*

Depending on several factors, such as the ligand, signal strength and the duration of activation, receptor tyrosine kinases often show different patterns of autophosphorylation and activation of downstream targets

[28-30]. Correspondingly transfected Ba/F3 cells were used in order to study the kinetic response for the different phosphorylation sites in FLT3 between wild-type, ITD and D835Y receptors. After starvation, cells were stimulated with FL for 0, 2, 5, 15 and 30 minutes, respectively. Western blot analysis with phospho-specific antibodies was used for the comparison between the receptors (Fig. 2). As positive control for the tyrosine phosphorylation and the functionality of the antibodies, cells were treated with sodium pervanadate in parallel, which induces global tyrosine phosphorylation in a non-specific manner [31]. For most of the tyrosine phosphorylation sites in the wild-type receptor, there was a rapid response to FL and the signal decreased after 5-15 minutes, also reflected by the analysis of total tyrosine phosphorylation. The signals were prolonged and enhanced in both FLT3-ITD and D835Y, however with somewhat different site specificity. Y589, Y591, Y599 and Y726 were highly phosphorylated in the FLT3-ITD receptor and Y768, Y793 and Y842 in the FLT3-D835Y receptor. In order to investigate the different phosphorylation patterns for each specific site of FLT3, the signals were quantified and normalized to the total FLT3 expression levels; and acquired data were statistically analyzed. Compared to wild-type FLT3, Y591 showed significant phosphorylation in FLT3-ITD ( $p \leq 0.05$ ) and FLT3-D835Y ( $p \leq 0.05$ ) in the absence of ligand stimulation. However, this significant difference was only present up to 2 and 5 minutes of stimulation with FL, and not present after 15 and 30 minutes of stimulation (data not shown). Y599 (Fig. 3A) and Y726 (Fig. 3B) showed no significant difference in phosphorylation between these three receptor versions at any time point. Y589 also didn't show any significant difference in phosphorylation except after 30 minutes of stimulation with FL, where ITD was significantly phosphorylated ( $p \leq 0.05$ ) as compared to wild-type and D835Y (data not shown). Moreover, similar to Y591, Y768 showed significant phosphorylation in FLT3-ITD ( $p \leq 0.05$ ) and D835Y ( $p \leq 0.05$ ) in the absence of ligand stimulation as compared to wild-type FLT3, but no significant difference was observed at any time point within the groups after stimulation with FL (data not shown). Y793 showed significant phosphorylation in FLT3-D835Y ( $p \leq 0.05$ ) compared to wild-type

FLT3 only, but not to ITD when unstimulated (Fig. 3C). In addition, Y842 showed significant phosphorylation both in the absence or presence of FL and at all time points in FLT3-D835Y ( $p \leq 0.05$ ) compared both to wild-type FLT3 and ITD (Fig. 3D). For a summary of our findings on the site-specific phosphorylation in FLT3 wild-type, ITD and D835Y, see figure 4.

#### *Verification of the functionality of the phospho-specific antibodies in human AML cell lines*

Since Flt3 is the most commonly mutated gene in AML, it is of high relevance to investigate the pattern of site-specific phosphorylation of FLT3 and its downstream signaling in human AML cell lines. For this purpose, THP-1 and MV4-11, which have endogenous expression of wild-type FLT3 and Flt3-ITD, respectively, were used. Cells were stimulated for 5 min with FL followed by Western blot analysis using the different phospho-specific FLT3 antibodies. The total tyrosine phosphorylation levels as well as the total protein levels were detected using 4G10 and anti-FLT3 antibodies, respectively. THP-1 and MV4-11 cells show similar results with phospho-specific antibodies against tyrosine residues 589, 591, 599, 768, 726, 793, 842 and 955, as observed in Ba/F3 cells expressing wild-type FLT3 and FLT3-ITD, respectively (Fig. 5).

#### **Discussion**

Only a few phosphorylation sites of FLT3 have been identified to date, but many are still to be investigated. Single Y-to-F mutations of the cytoplasmic tyrosine residues of murine FLT3 have been analyzed in the context of wild-type versus the corresponding D838V kinase domain mutant of FLT3 [32]. In that study, tyrosines 845, 892 and 922 of the murine FLT3 proved to be critical for the constitutive activation of the mutant receptor, suggesting that these residues are involved in stabilizing the active structure. However, they were not demonstrated to be phosphorylation sites. In addition, it has been shown that mutation of the

critical aspartic acid residue to a valine residue is not equivalent to a tyrosine residue in terms of ability to confer cytokine-independent growth of transfected Ba/F3 cells and in terms of sensitivity to kinase inhibitors [33].

In the present study, we used phospho-specific antibodies to identify Y726, Y793 and Y842 as novel FLT3 phosphorylation sites. In addition, our group recently identified Y768, Y955 and Y969 as phosphorylation sites in living cells by the use of phospho-specific FLT3 antibodies [27]. Previously, we also identified Y572, Y589, Y591 and Y599 of FLT3 to be phosphorylated in 32D cells transfected with wild-type FLT3 using two-dimensional phospho-peptide mapping combined with radio-Edman degradation [26]. In a study using a recombinant intracellular fragment of FLT3, several autophosphorylation sites were identified *in vitro* by mass spectrometry, including Y589, Y591, Y726, Y842, Y955 and Y969 [34] (Table 1). However, in order to draw conclusions from these studies one should consider that the different methods used to identify phosphorylation sites have their individual drawbacks as well as advantages. The receptor in its physiological context can use accessory tyrosine kinases both for its own phosphorylation and for downstream signaling, and thus *in vitro* approaches might miss some physiologically relevant sites. The use of phospho-specific antibodies is a relatively biased approach but it enables fast and reproducible detection of phosphorylation status. It allows the rapid and sensitive analysis of cellular samples treated under different conditions. We have produced a panel of site-specific antibodies in our lab based on the fact that most of the known tyrosine phosphorylation sites in receptor tyrosine kinases reside outside the kinase domains (with exception of the activation loop tyrosines and some other rare cases) (for reviews see [35, 36]). Thus, we generated phospho-specific antibodies against phosphorylated Y572, Y589, Y591, Y599, Y726, Y768, Y842, Y919 and Y955. Of these all gave detectable phosphorylation in at least one of the three variants of FLT3, with the exception of Y572 and Y919 (data now shown). Y572 was identified as a phosphorylation site in 32D cells expressing wild-type FLT3 [26], but we failed to detect any signal in

Ba/F3 cells expressing FLT3. This might be due to the presence or absence of a tyrosine kinase or protein tyrosine phosphatase in either cell type. Y919 is homologous to Y900 in c-KIT [37] and Y934 in the PDGF $\alpha$ -receptor [38] that were identified as low stoichiometry phosphorylation sites whose phosphorylation was mediated by Src. However, we failed to detect phosphorylation of Y919 in FLT3, but we do not know whether this is due to a poor antibody or lack of surface exposure of the tyrosine residue (data not shown).

We have screened through wild-type FLT3 as well as the FLT3-ITD and FLT3-D835Y mutants, with regards to site-specific phosphorylation and kinetics. As expected, most sites were phosphorylated independently of FL in the mutants. Nevertheless, an enhanced response after stimulation with FL was observed with the mutants. Interestingly, several reports have shown that leukemic cell lines express FLT3 and FL simultaneously, which indicates a potential role for the ligand even when the receptor is constitutively activated [39]. In a study by Zheng et al. primary AML blasts were screened for FLT3 and FL protein co-expression, suggesting autocrine or paracrine stimulatory loops [40] and further supporting our results.

The juxtamembrane tyrosines (Y589, Y591 and Y599) show a stronger and more prolonged phosphorylation in the ITD receptor, which at least in part might be a result of a duplication of these sites (the FLT3-ITD used in this study has a duplication containing Y589 and Y591; [41]). Y589 and Y591 have been identified as Src association sites [26] and involved in the activation of STAT5 [34]. Moreover, these two residues were shown to be crucial for the transforming potential of FLT3-ITD and D835Y as well as for the ligand-dependent activation of wild-type FLT3 [42]. Y599 was demonstrated to be involved in binding of the protein tyrosine phosphatase SHP2 [26]. Evidently, in addition to quantitative, a qualitative difference in downstream signaling exists between wild-type and the FLT3-ITD receptors. Furthermore, in a microarray experiment, several genes were found to be selectively regulated by the ITD and not by the

wild-type FLT3, for example Pim-2, and the STAT5 target genes Socs2 and Socs3. Additionally, FLT3-ITD repressed the expression of *C/EBP $\alpha$* , a transcription factor essential for granulocytic differentiation and important roles in down-regulating proteins such as CDK2/4 and c-Myc [43].

Tyrosine 726 is phosphorylated to a similar extent in wild-type FLT3 as in the mutants. However, potential interaction partners for this site have not yet been reported. When performing a database search, no characteristic binding motif was found from the surrounding sequence and additional studies are required. We have previously shown that the autophosphorylation sites Y768, Y955 and Y969 are GRB2 binding sites [27]. Interestingly, these sites demonstrate a similar phosphorylation pattern in response to FL in both wild-type and mutant FLT3.

Strikingly, tyrosines 793 and 842 have a significantly more pronounced phosphorylation in the FLT3-D835Y, as compared to both the wild-type and ITD receptors. Both of these sites are located within the kinase domain and no interacting proteins have so far been identified.

The differences in phosphorylation sites between wild-type FLT3, FLT3-ITD and FLT3-D835Y can have several explanations. The two activating mutations act through two distinctively different mechanisms. The FLT3-ITD mutation activates the kinase activity through releasing the negative constraint that the juxtamembrane region poses on the kinase domain of FLT3 [15]. Thus, the mutation does not involve any structural changes in the kinase domain. In contrast, the FLT3-D835Y mutation resides in the activation loop of the kinase domain. This mutation is thought to mimic the activated state of the kinase domain after ligand-induced activation. From the study of several other receptor tyrosine kinases it is known that mutations in this region not only activate the kinase activity, but also alter its specificity. Thus, in the case of RET the M918T mutation in the kinase domain of patients with MEN2B (multiple endocrine neoplasia 2B) leads to constitutive activation of the kinase and a shift in specificity which in turn leads to an altered pattern of autophosphorylation [44]. Likewise, the activating mutations D1246N and D816V in the

kinase domain of MET and KIT (corresponding to D835 in FLT3) have also been demonstrated to lead to a shift in substrate specificity of the kinase [45, 46].

The phosphorylation sites seen in a living cells could both be due to true autophosphorylation, i.e. a receptor phosphorylates another receptor in a cross-wise manner, but it could also in part be due to the activity of other tyrosine kinases activated downstream of the receptor (such as Src family kinases, Fes etc.). This could explain the discrepancy between the sites found using recombinant FLT3 *in vitro* and the findings in living cells. Yet another factor that could contribute to the differences in phosphorylation sites seen in these experiments is the subcellular localization of the receptor. It is known that the FLT3-ITD, in contrast to wild-type FLT3 and FLT3-D835Y is trapped in the endoplasmic reticulum and does not reach the cell surface to any appreciable extent [47]. Thus, the signal transduction molecules (including kinases and phosphatases) it encounters is likely to be different from those at the cell surface, and this could contribute to the differences in phosphorylation seen in the different forms of the receptor.

Tyrosine kinase domain (TKD) mutations of FLT3, such as D835Y give rise to similar transforming abilities as FLT3-ITD but the downstream signaling and its phenotype more resembles wild-type FLT3. This is also supported by the finding of clonogenic growth of 32D cells when transfected with FLT3-ITD, but not with wild-type FLT3 or FLT3-D835Y mutation [48]. Given the fact that both receptors are constitutively activated but give rise to different outcomes in terms of signaling and biological phenotype, these observations argue for qualitatively different downstream signaling patterns. One well studied example is STAT5 which is activated by the ITD but not the wild-type FLT3 or FLT3-D835Y [49]. Furthermore, the FLT3-ITD causes myeloproliferative disease in mouse models, while the FLT3-D835Y mutant does not [50]. Several clinical studies show that AML patients with ITD belong to a worse prognostic group than those with wild-type FLT3 or D835Y mutations [51]. In line with our findings, this



indicates signaling differences between ITDs and D835Y of FLT3. As observed in the whole population of AML patients, about 7% of AML patients with FLT3-ITD also carry the D835Y mutation, indicating that the ITD and the D835Y mutant receptors are not functionally redundant [48].

RTKs commonly contain one or more tyrosine residues in the kinase activation loop, and the biological function of several RTKs, including insulin-like growth factor-1 (IGF-1) receptor [52], fibroblast growth factor (FGF) receptor [53] and hepatocyte growth factor receptor (Met) [54], depends on the autophosphorylation of these residues. However, similar to our findings that the activation loop tyrosine 842 does not have a critical role in wild-type FLT3 receptor signaling, it has been reported that the corresponding Y823 site of the closely related c-KIT receptor was not required for the kinase activity of the receptor. Phosphorylation of this residue was secondary to the phosphorylation of tyrosines 568 and 570 in the juxtamembrane region [55].

No potential interacting partners to Y842 of FLT3 have been suggested to this point, and there are a few examples from other studies where direct binding partners of an activation loop tyrosine have been found. GRB10 was found to interact with tyrosines 1162 and 1163 of the insulin receptor (IR) [56]. It was also shown that the adapter protein APS associates with the same phosphotyrosine residues within the activation loop of IR [57]. Another example of an SH2-domain protein interacting directly with the activation-loop is the closely related SH2B, also identified as a substrate for IR where mutation of the three activation loop tyrosines 1158, 1162 and 1163 abolished the observed interaction [58]. Moreover, SOCS-1 was shown to interact with JAK2 through binding of the SH2 domain to Y1007 on the activation-loop, a process required for proteasomal degradation of JAK2 [59].

In conclusion, we have compared the site-specific phosphorylation patterns of wild-type FLT3, FLT3-ITD and FLT3-D835Y in Ba/F3 cells using a panel of phospho-specific FLT3 antibodies. Interestingly, there were not only quantitative but also qualitative differences between the receptors. Some

sites are activated in similar fashion, whereas some sites were activated selectively either by the FLT3-ITD or the FLT3-D835Y mutant. As mutations in the FLT3 receptor are found to be a major cause of transformation in AML cells, several different FLT3 inhibitors have been tested in clinical trials [60], but more efficient and specific therapy is required. Future work will focus on the detailed downstream signaling from the newly identified sites presented here. The current findings contribute to the overall understanding of oncogenic FLT3 signaling and enable more specific approaches for targeting mutant receptors and their downstream signal transduction.

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## Figure Legends

### ***Figure 1. Verification of the phospho-specific FLT3 antibodies in COS-1 cells***

COS-1 cells transiently transfected with either the wild-type Flt3 or each of the corresponding Y-to-F mutants, were starved overnight and stimulated with FL for 5 minutes, lysed and subjected to immunoprecipitation with anti- FLT3. The samples were analyzed by Western blotting and the membranes were probed with pY589 (A), pY591 (B), pY599 (C), pY726 (D), pY793 (E) and pY842 (F). All membranes were re-probed with antibodies against phospho-tyrosine (4G10) and total FLT3, as controls. The figure shows one representative experiment out of three.

### ***Figure 2. Kinetic studies on the site specific phosphorylation in Ba/F3 cells***

Ba/F3 cells stably transfected with either Flt3 wild-type, Flt3-ITD or Flt3-D835Y, were starved for 4 hours prior to stimulation with FL for the indicated periods of time. In parallel as a positive control, cells were incubated with sodium pervanadate for 15 minutes, at 37°C, and stimulated for 5 minutes before lysis (denoted “+” in the figures). The analysis was performed using Western blotting and the membranes were probed with pY589 (A), pY591 (B), pY599 (C), pY726 (D), pY768 (E), pY793 (F), pY842 (G) and pY955 (H). As controls, all membranes were re-probed with antibodies against phospho-tyrosine (4G10) and total FLT3. The figure shows one representative experiment out of three.

### ***Figure 3. Statistical analysis of the kinetics and specificity of phosphorylation between wild-type FLT3, FLT3-ITD and FLT3-D835Y.***

Ba/F3 cells expressing wild-type FLT3, FLT3-ITD and FLT3-D835Y were stimulated with FL for 0, 2, 5, 15 and 30 minutes. Western blotting analysis was performed with phospho-specific antibodies against

individual sites of FLT3, and with anti-phospho-tyrosine antibody and anti- FLT3 antibody. Signals were quantified and normalized to the total FLT3 expression level; data obtained were analyzed statistically. Representative graphs shows results obtained for pY599 (A), pY726 (B), pY793 (C) and pY842 (D).

***Figure 4. Schematic picture of the wild-type FLT3, FLT3-ITD and FLT3-D835Y and their phosphorylation sites***

Wild-type FLT3, FLT3-ITD and FLT3-D835Y are pointed out as shown, and the different phosphorylation sites are indicated in the figure (the size indicates the phosphorylation ability). TM (transmembrane domain), TK (tyrosine kinase domain).

***Figure 5. Verification of the phosphorylated tyrosine residues of FLT3 in the AML cell lines THP-1 and MV4-11***

MV4-11 and THP-1 cells were starved overnight and stimulated with FL for 5 minutes, lysed and subjected to immunoprecipitation with anti-FLT3. The samples were analyzed by Western blotting and the membranes were probed with pY726 (A and D), pY793 (B and E), and pY842 (C and F). All membranes were re-probed with antibodies against phosphotyrosine (4G10) and total FLT3, as controls. The figure shows one representative experiment out of three.

***Table 1. Phosphorylation sites in FLT3.*** Identified phosphorylation sites *in vitro* or in intact cells are indicated by “+” and the reference is given within brackets. The phosphorylation sites identified in this work is denoted “\*”.

Figure 1

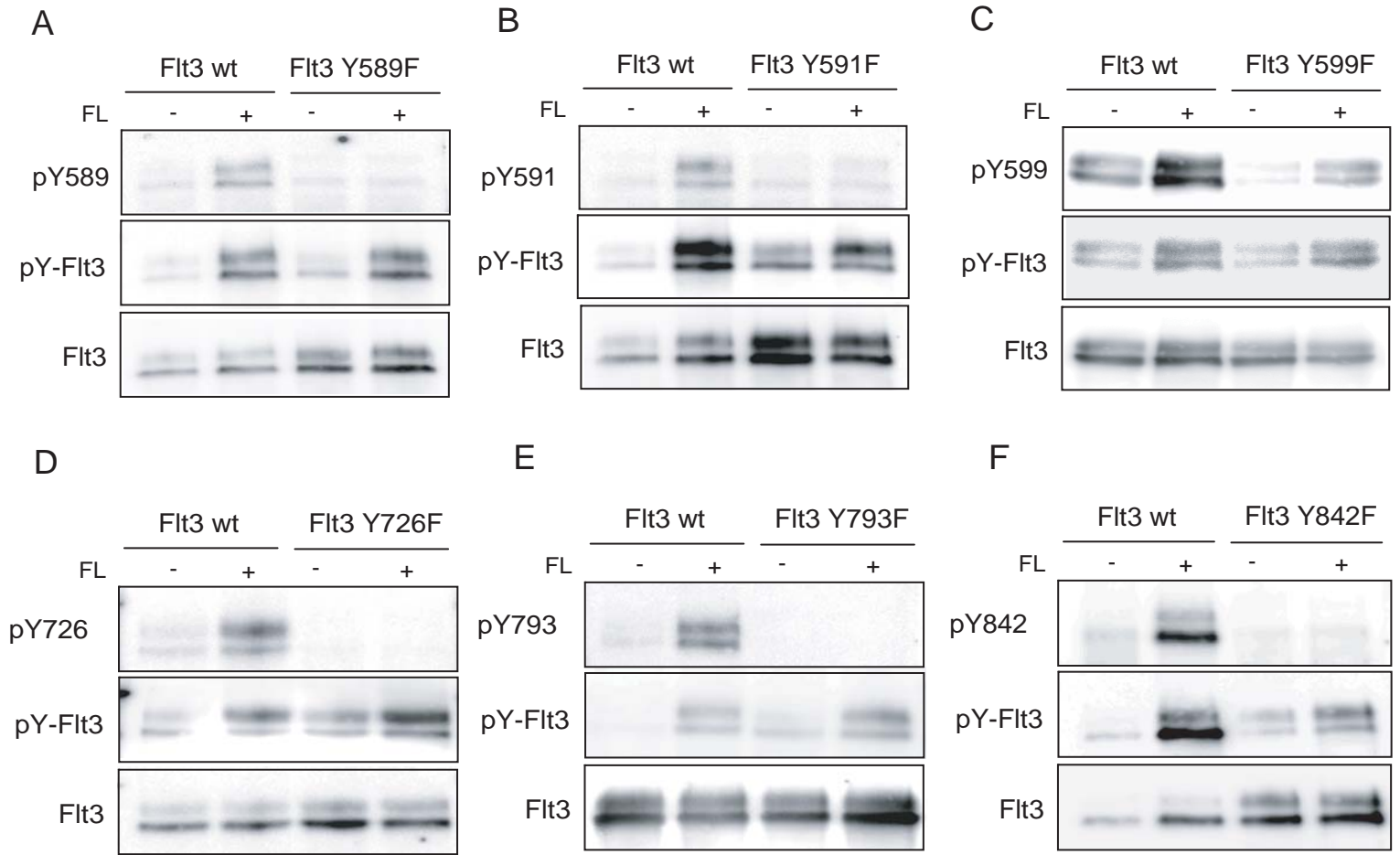




Figure 2

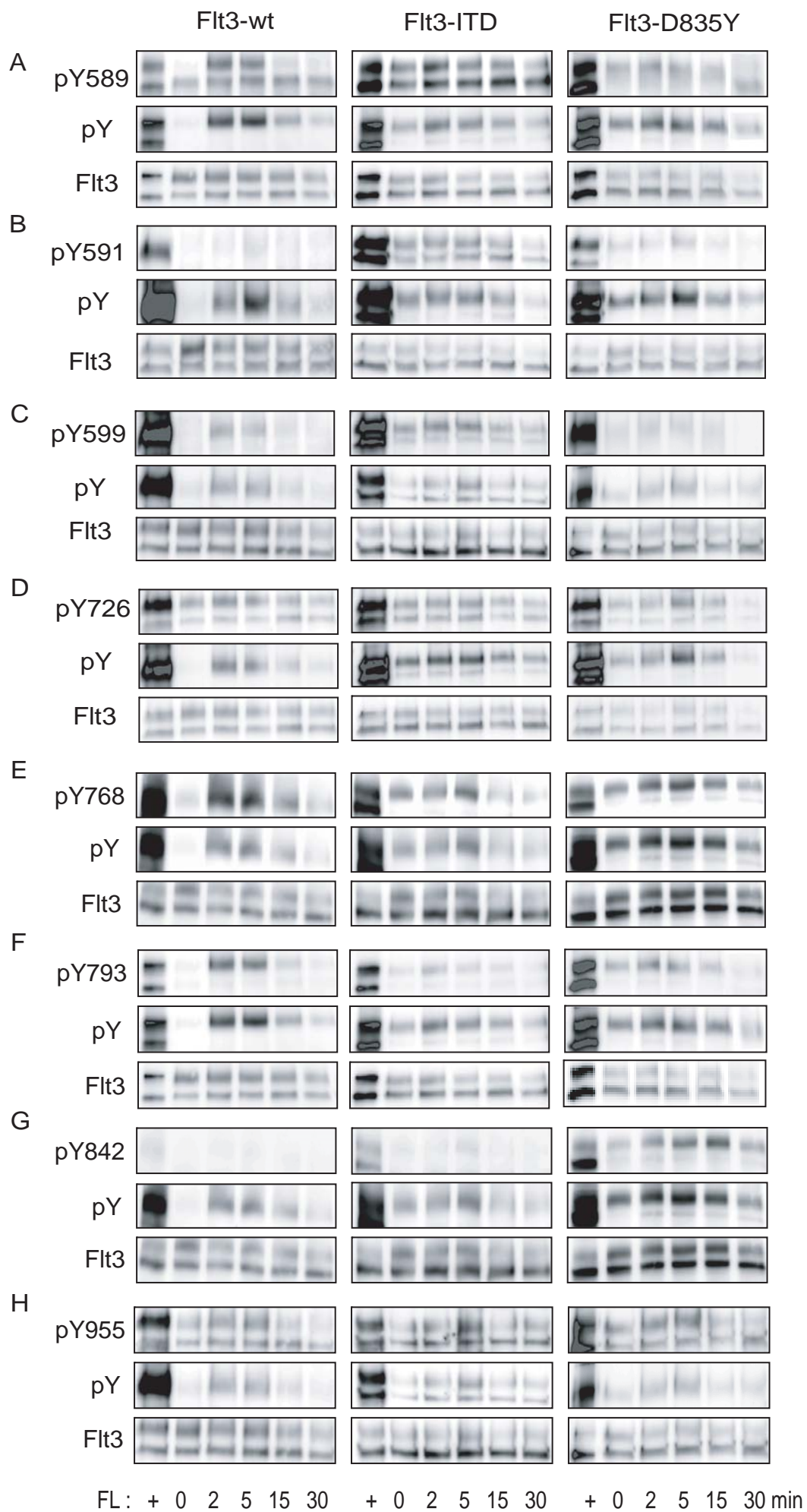


Figure 3

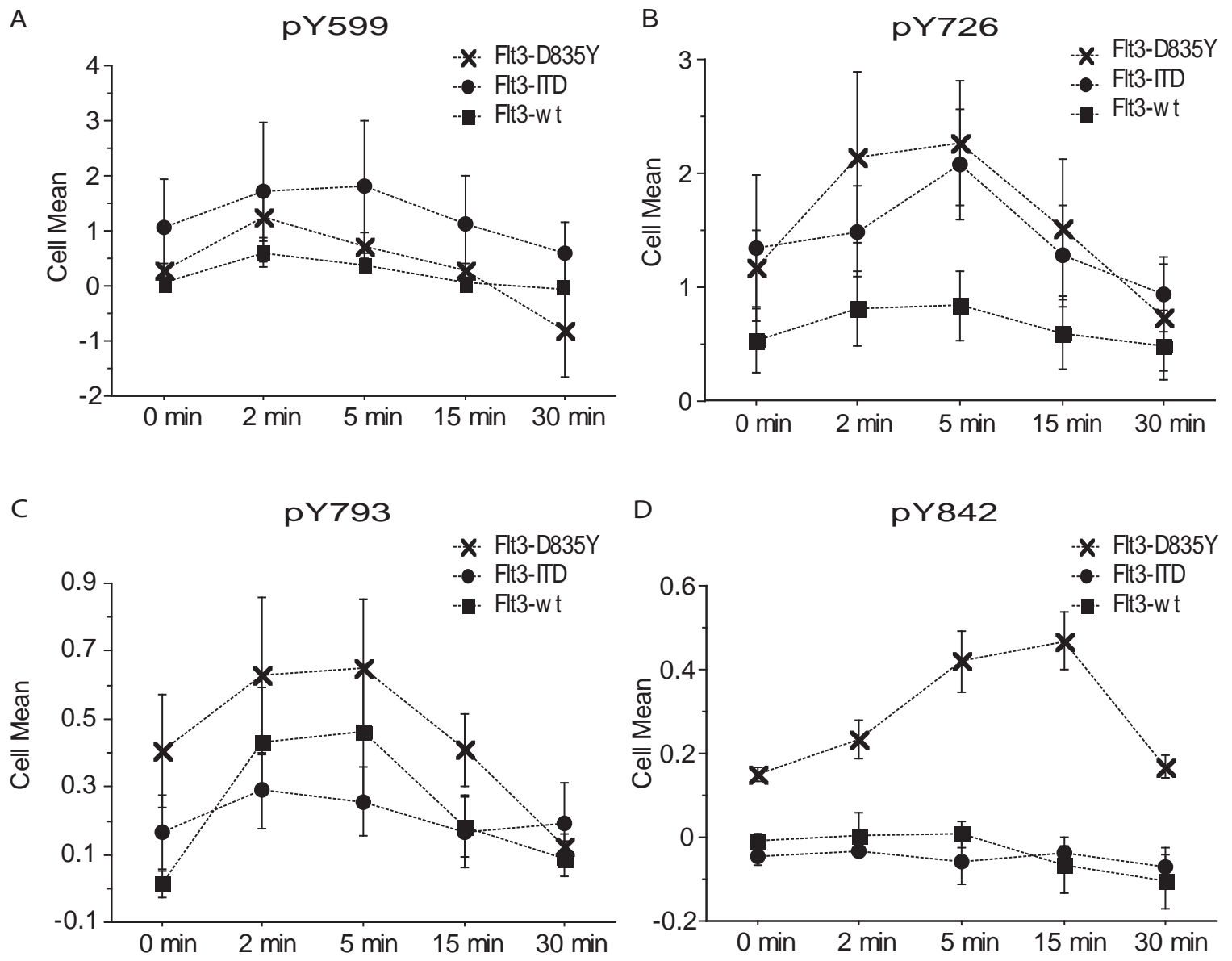


Figure 4

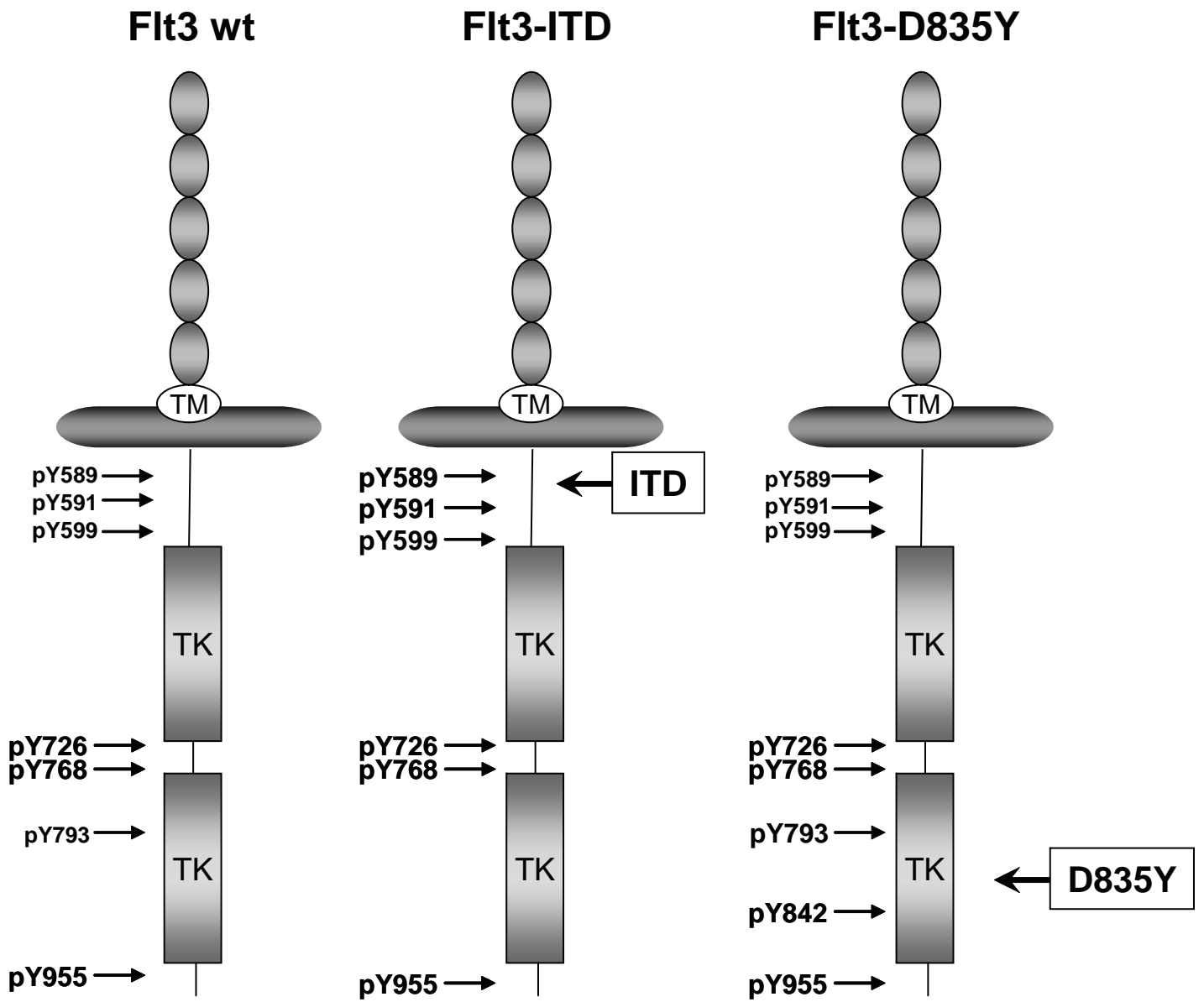
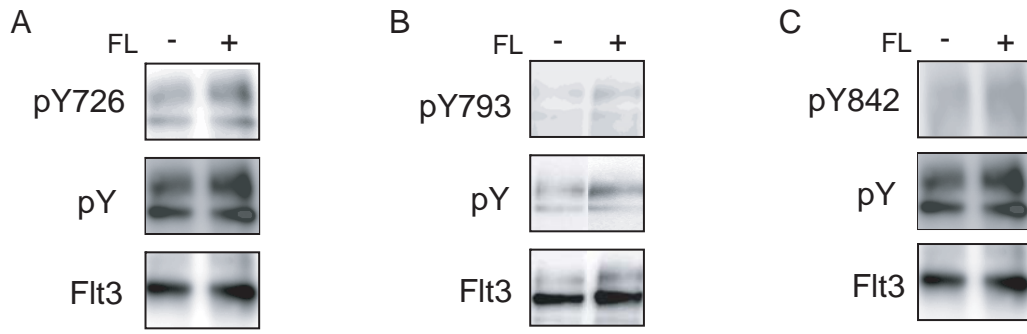
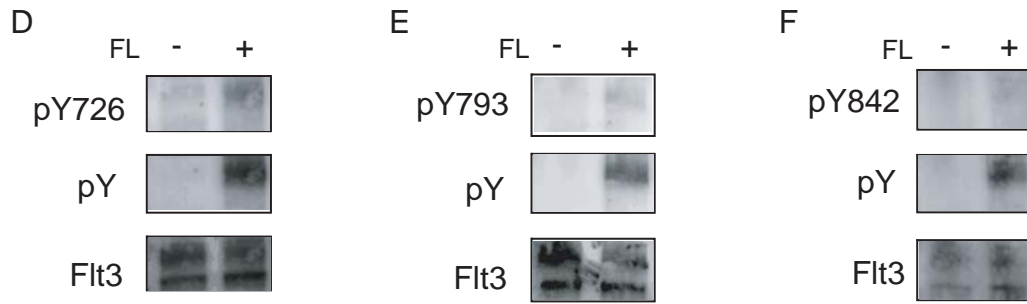


Figure 5

MV4-11 cells



THP-1 cells



Identified phosphorylation sites	in vitro	intact cells
Y572	-	+ (26)
Y589	+ (34)	+ (26)
Y591	+ (34)	+ (26)
Y599	-	+ (26)
Y726	+ (34)	+ *
Y768	-	+ (27)
Y793	-	+ *
Y842	+ (34)	+ *
Y955	+ (34)	+ (27)
Y969	+ (34)	+ (27)