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Lindblad, Oscar; Kazi, Julhash U.; Rönstrand, Lars; Sun, Jianmin

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PO Box 117
221 00 Lund
+46 46-222 00 00

PI3 kinase is indispensable for oncogenic transformation by the V560D mutant of c-Kit in a kinase independent manner

Oscar Lindblad, Julhash U. Kazi, Lars Rönnstrand, Jianmin Sun

Division of Translational Cancer Research and Lund Stem Cell Center, Department of Laboratory Medicine, Lund University, Sweden

Corresponding author:

Jianmin Sun, Jianmin.sun@med.lu.se

Division of Translational Cancer Research and Lund Stem Cell Center,

Department of Laboratory Medicine, Lund University

Medicon Village 404C3, Scheelevägen 8, Lund 22363, Sweden

Abstract

Oncogenic mutants of c-Kit are often found in mastocytosis, gastrointestinal stromal tumors (GISTs) and acute myeloid leukemia (AML). The activation mechanism of the most commonly occurring mutation, D816V in exon 17 of c-Kit, has been well-studied while other mutations remain fairly uncharacterized in this respect. In this study, we show that the constitutive activity of the exon 11 mutant V560D is weaker than the D816V mutant. Phosphorylation of downstream signaling proteins induced by the ligand for c-Kit, stem cell factor (SCF), was stronger in c-Kit/V560D expressing cells than in cells expressing c-kit/D816V. Although cells expressing c-Kit/V560D showed increased ligand-independent proliferation and survival compared to wild-type c-Kit expressing cells, these biological effects were weaker than in c-Kit/D816V expressing cells. In contrast to cells expressing wild-type c-Kit, cells expressing c-Kit/V560D were independent of Src family kinases for downstream signaling. However, the independence of Src family kinases was not due to a Src-like kinase activity that c-Kit/D816V displayed. Point mutations that selectively block the association of PI3 kinase with c-Kit/V560D inhibited ligand-independent activation of the receptor, while inhibition of the kinase activity of PI3 kinase with pharmacological inhibitors didn't affect the kinase activity of the receptor. This suggests a lipid-kinase independent key role of PI3 kinase in c-Kit/V560D-mediated oncogenic signal transduction. Thus, PI3 kinase is an attractive therapeutic target in malignancies induced by c-Kit mutations independent of its lipid kinase activity.

Key words: receptor tyrosine kinase, Ba/F3, cancer, LY294002, GDC0941, signaling

Introduction

The type III receptor tyrosine kinase c-Kit belongs to the same subfamily as the platelet-derived growth factor (PDGF) receptors, Fms-like tyrosine kinase 3 (FLT3), and the macrophage colony stimulating factor (M-CSF) receptor. C-Kit plays important roles in hematopoiesis, melanogenesis, and gametogenesis under normal physiological conditions [1]. Binding of its ligand, stem cell factor (SCF) induces dimerization of c-Kit followed by activation of its intrinsic tyrosine kinase activity. This leads to phosphorylation of key tyrosine residues that constitute binding sites for Src homology 2 domain containing signaling proteins. These events result in the phosphorylation and activation of downstream signaling molecules that mediate the biological effects, such as cell proliferation, survival and migration.

Mutations of c-Kit have been identified in around 90% of mastocytosis and 80% of gastrointestinal stromal tumors (GISTs) cases. It also exists, less commonly, in sub-group of acute myeloid leukemia (AML), in melanoma and in germ cell tumors such as seminomas and ovarian dysgerminomas [1]. Ligand-independent activation of oncogenic mutants of receptor tyrosine kinases has long been recognized as important mediators of oncogenesis. However, we and others have shown that some oncogenic mutants of receptor tyrosine kinases, in addition to being constitutively active, also gain altered substrate specificity [2, 3].

The Src family kinases are a group of non-receptor tyrosine kinases that are important signal mediators in receptor signaling. Activation of wild-type c-Kit is partially dependent on the activity of Src family kinases, and inhibition of Src family kinases leads to decreased c-Kit activation [4]. The D816V mutation is the most commonly identified and well characterized mutation of c-Kit in malignancies. We have previously found that c-Kit/D816V gains a Src family kinase-like activity and thus circumvents a requirement of Src family kinases for its activation and cell transformation [2]. This suggests that oncogenic c-Kit mutations, in addition to being ligand-independent, have different mechanisms of activation and signal transduction compared to wild-type c-Kit.

PI3 kinases are intracellular lipid kinases that are involved in receptor signal transduction under both normal and pathological situations. Unlike Src family kinases, PI3 kinases play an important role in c-Kit/D816V-mediated cell transformation which has been shown to be independent of its lipid kinase activity [5].

Since there is a difference between the way how wild-type c-Kit and c-Kit-D816V utilize Src family kinases and PI3 kinases for receptor activation and downstream cellular signaling, it is of interest to investigate the role of these two kinase families in the signal transduction and cell transformation mediated by other oncogenic c-Kit mutants. By introducing mutations in c-Kit and by using selective inhibitors, we observed that the commonly identified exon 11 mutation V560D of c-Kit is not dependent on Src family kinases for its signaling. In contrast, the ligand-

independent activation of c-Kit/V560D is totally dependent on an intact binding site for PI3 kinase in c-Kit but is independent of its lipid kinase activity. These results confirm that oncogenic c-Kit mutants have different mechanisms of both activation and signaling than wild-type c-Kit.

Materials and methods

Kits and reagents

The PI3-kinase inhibitor LY294002 and Src Family Kinase inhibitor SU6656 were from Sigma-Aldrich (St. Louis, MO), another PI3 kinase inhibitor GDC0941 was from Apexbio (Boston, MA). Lipofectamine 2000 was from Life Technologies (Carlsbad, CA). Chemiluminescent HRP substrate was from Millipore (Billerica, MA). QuikChange mutagenesis kit was from Agilent Technologies (Santa Clara, CA). Annexin V-PE apoptosis detection kit was from BD Biosciences (San Jose, CA). [γ - 32 P]ATP and protein G-Sepharose beads were from GE Healthcare (Little Chalfont, UK). All the reagents and kits were used according to the manufacturer's instructions.

Cytokines and antibodies

Recombinant human SCF was purchased from Prospec Tany (Rehovot, Israel). The rabbit antibody KitC1, recognizing the C-terminal tail of human c-Kit, was purified as described [6]. Antibodies recognizing pTyr-568, pTyr-703, pTyr-Y721, and pTyr-936 in human c-Kit were previously described [2]. Antibodies against Akt, pErk and SHP2 were from Santa Cruz Biotechnology (Santa Cruz, CA). pAkt antibody was from Epitomics (Burlingame, CA). β -actin antibody were from Sigma-Aldrich. SHC antibody was from BD Biosciences. Phospho-tyrosine antibody 4G10 and Gab2 antibody was from Millipore. PE labeled c-Kit antibody (104D2) was from Biolegend (San Diego, CA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from Life Technologies (Carlsbad, CA).

Cell culture

EcoPack virus packaging cell line (Clontech, Mountainview, CA) was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. Ba/F3 cells (DSMZ, Braunschweig, Germany) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin, and 10 ng/ml recombinant murine IL-3 as recommended previously [7]. In order to establish Ba/F3 cell lines expressing c-Kit, EcoPack cells were transfected with either wild-type or mutant of c-Kit constructs in pMSCVpuro vector. Supernatants were collected to infect Ba/F3 cells followed by 2-weeks selection in 1.2 μ g/ml puromycin. Expression levels of c-Kit were confirmed by flow cytometry and immunoblotting. C-Kit expressing Ba/F3 cells were grown in the same medium as untransfected Ba/F3 cells.

Cell survival and proliferation assay

Cell survival and proliferation assay was performed as described [5].

Cell stimulation, immunoprecipitation and Western blotting

Cell stimulation, immunoprecipitation and Western blotting was performed as described [5].

In vitro kinase activity assay

Ba/F3 cells were starved for 4 hours in medium without serum and IL-3 and subsequently stimulated with SCF (100 ng/ml) for 5 minutes. Thereafter cells were washed once in ice-cold PBS, lysed, immunoprecipitated with the KitC1 antibody, and processed for *in vitro* kinase assay as described [8] with the exception that Src optimal peptide [9] was used as substrate.

Results

The V560D mutant is less oncogenic than the D816V mutant of c-Kit

The exon 11 mutation V560D of c-Kit is frequently identified in GISTs [10] while the exon 17 mutation D816V of c-Kit is frequently identified in mastocytosis and less frequently in certain types of AML [1]. In order to compare the transforming ability of these two types of mutants, we transfected Ba/F3 cells, which lack endogenous c-Kit expression, with either wild-type c-Kit or c-Kit mutants. Cell surface expression of c-Kit was shown to differ between the three different mutant cell lines. While cells expressing wild-type c-Kit displayed the highest level of cell surface expression, c-Kit/V560D had intermediate surface expression, and cells expressing c-Kit/D816V displayed the lowest cell surface expression (Fig. 1A). C-Kit is expressed as a mature, fully glycosylated 145KD and an immature, partially glycosylated 125 kD protein [11], The ratio between the two c-Kit protein bands are also different between wild-type c-Kit and the two c-Kit mutants. The 145 kD band is dominant in cells expressing wild-type c-Kit while the two bands are more equal in intensity in the mutants (Fig. 1B). In order to investigate the oncogenic potential of these mutants, we examined cell survival and cell proliferation of transfected Ba/F3 cells in the presence or absence of SCF. Cell survival (Fig. 1C) and cell proliferation (Fig. 1D) of cells expressing wild-type c-Kit were dependent on the presence of SCF. In contrast, c-Kit/D816V supported both cell survival and cell proliferation independent of SCF stimulation. In comparison, Ba/F3 cells expressing c-Kit/V560D showed moderate cell survival and weak cell proliferation regardless of SCF-stimulation, suggesting a weaker transforming ability of c-Kit/V560D compared with c-Kit/D816V.

The activation kinetics of c-Kit/V560D is different from that of c-Kit/D816V

In order to investigate the mechanism behind the weak transforming ability of c-Kit/V560D, c-Kit activation was compared between wild-type c-Kit, c-Kit/V560D and c-Kit/D816V expressing cells. Both c-Kit/V560D and c-Kit/D816V can induce ligand-independent activation of the receptor (Fig. 2A). It is well established that Src Family Kinases play a crucial role in the activation of wild-type c-Kit. The binding site of Src Family Kinases in c-Kit is Tyr568 [12]. By using a phospho-specific antibody, we show that Tyr568 is strongly phosphorylated in cells expressing the V560D independent of SCF stimulation. In contrast, phosphorylation of the Grb2 binding sites Tyr703 and Tyr936 [13], and PI3 kinase binding site Tyr721 [14] was weaker in c-Kit/V560D than in c-Kit/D816V. In addition, the total phosphorylation of c-Kit/V560D was weaker than that of c-Kit/D816V. Similar to the receptor activation, the ligand-independent phosphorylation of downstream signaling molecules such as Akt and Erk was also weaker in c-Kit/V560D expressing cells compared to c-Kit/D816V expressing cells (Fig. 2B), although ligand stimulation could transiently enhanced phosphorylation further in c-Kit/V560D expressing cells. These results suggest that c-Kit/V560D can weakly activate downstream signaling through Grb2 and PI3 kinase leading to weaker activation of Akt and Erk signaling as well as weaker cell proliferation and survival. The adapter proteins Gab2 and SHC are important intermediates in the activation of both Akt and Erk [15, 16], and the protein tyrosine phosphatase SHP2 is involved in Erk activation [17]. By using phospho-tyrosine antibodies, c-Kit/V560D was shown to be able to phosphorylate Gab2, SHC and SHP2 even in the absence of ligand stimulation and similar to Akt and Erk phosphorylation, phosphorylation of these three proteins was weaker than that of c-KIT/D816V expressing cells (Fig. 2C).

The kinase activity of c-Kit/V560D is not dependent on Src family kinases

Src family kinases are important for the activation of wild-type c-Kit [4]. We have previously found that the D816V mutation of c-Kit circumvents a requirement of Src family kinases for its activation of c-Kit and downstream signal transduction [2]. Thus, we sought to investigate whether the V560D mutant of c-Kit is still dependent on Src family kinases. By using a selective Src family kinase inhibitor, SU6656, we showed that both ligand-independent and ligand-dependent activation of c-Kit/V560D was independent on the kinase activity of Src family kinases (Fig. 3A). Likewise, phosphorylation of downstream signaling molecules such as Akt and Erk were not inhibited by the Src family kinase inhibitor SU6656 (Fig. 3B).

C-Kit/V560D has not gained Src-like substrate specificity

We have previously found that c-Kit/D816V has gained substrate specificity similar to Src family kinases and that it thereby loses its dependence on Src family kinases for its activation and downstream signaling. Similar to c-Kit/D816V, the V560D mutant of c-Kit has also lost its dependence on Src family kinases for its activation and downstream signaling (Fig. 3). We therefore wanted to investigate whether also c-Kit/V560D, similar to c-Kit/D816V, has gained altered substrate specificity. Using an *in vitro* kinase assay utilizing the Src optimal peptide as a

substrate, we could show that c-Kit/D816V displays substrate specificity similar to Src family kinases while both wild-type c-Kit and c-Kit/V560D do not show any such activity even in the presence of ligand (Fig. 4). These results indicate that the mechanism of activation and signaling is different between c-Kit/V560D and c-Kit/D816V.

3.5. Ligand-independent activation of c-Kit/V560D is dependent on PI3 kinase in a lipid kinase independent manner

PI3 kinase plays an important role in the ligand-independent signaling by c-Kit/D816V, and c-Kit/D816V-mediated cell transformation is independent of its lipid kinase activity [5]. PI3 kinase binds to pY721 in c-Kit [18] and the binding requires the motif pYXXM in c-Kit [19]. By introducing the M724A mutation in c-Kit/V560D to block the direct PI3 kinase binding without affecting c-Kit tyrosine phosphorylation, we show that the ligand-independent activation of c-Kit/V560D is totally blocked although ligand can still activate the receptor (Fig. 5A). Ligand-dependent phosphorylation of PI3 kinase downstream signaling component Akt was partially reduced other than complete diminution, while PI3 kinase independent component Erk phosphorylation remained unchanged (Fig. 5B). These findings suggest that the c-Kit/V560D mutant can transduce signals without recruiting PI3 kinase but PI3 kinase association is required for constitutive activation of the receptor. In order to elucidate whether this effect is dependent on the lipid kinase activity of PI3 kinase, the effect of a PI3 kinase inhibitor on the activation of c-Kit/V560D was examined. The PI3 kinase inhibitor LY294002 did not block the activation of c-Kit/V560D independent of SCF stimulation (Fig. 5C). Furthermore, Akt phosphorylation was inhibited in the presence of LY294002 (Fig. 5D) indicating that PI3 kinase is activated by c-Kit/V560D. A cell proliferation assay in the presence of Src family kinase and PI3 kinase inhibitors further confirmed lipid kinase independent role of PI3 kinase in c-Kit/V560D signaling (Fig. 6).

Discussion

Mutations in c-Kit have been identified in exon 9, 11, 13 and 17 in different malignancies. In GISTs, most of c-Kit mutations were mapped to exon 11, and to a lesser extent also to exon 9 and 13 [20]. In mastocytosis, the D816V mutation in exon 17 of c-Kit is the most commonly identified mutation, found in about 80-90 % of patients [1]. The different mutants of c-Kit have different sensitivity to the current clinically approved c-Kit inhibitors. Mutations in exon 11 of c-Kit usually respond well to imatinib while c-Kit/D816V is insensitive to imatinib. After treatment with imatinib, relapsed GISTs many times gain secondary mutations in exon 13 and 17 of c-Kit that are resistant to imatinib [20, 21]. The difference in response of c-Kit mutations to inhibitors is most likely partially due to differences in activation mechanism. It is known that imatinib binds to the inactive kinase conformation of c-Kit [22], while c-Kit/D816V is constitutively in its active conformation [23] and thus does not bind imatinib. Moreover, different c-Kit mutants display considerable difference in intrinsic kinase activity. For example,

c-Kit/D816V has a higher intrinsic kinase activity than c-Kit/V560D [21]. Therefore it is important to elucidate the detailed activation mechanism of these mutants in order to design more efficient drugs.

Since Src family kinases are very important in wild-type c-Kit signaling, it is tempting to consider them as therapeutic targets in tumors driven by mutant c-Kit. However, we have previously found that the c-Kit/D816V mutant circumvents a requirement for Src family kinases by displaying altered substrate specificity. For that reason, it is likely that Src family kinase inhibitor will probably not be useful for the treatment of malignancies expressing c-Kit/D816V. It is of importance to investigate whether the independency on Src family kinases of receptor activation and signaling is a common mechanism among other activating c-Kit mutants. By using inhibitors and generating oncogenic mutants of c-Kit, we demonstrate that the commonly occurring c-Kit mutation V560D in GISTs also is able to circumvent a requirement of Src family kinases for its signal transduction. These results further suggest that the use of Src family kinase inhibitors in the treatment of cancers carrying c-Kit mutations will not be a viable option.

PI3-kinases are lipid kinases that play important roles in cell survival, proliferation and metabolism. Overactivation of PI3 kinase contributes to transformation in several malignancies. As with Src family kinases, PI3-kinases are also attractive treatment targets for cancer. Various small molecule inhibitors have been developed and taken into clinical trials [24].

PI3 kinases contribute to wild-type c-Kit-mediated cell survival and proliferation. Ligand-dependent activation of wild-type c-Kit is not dependent on PI3-kinases [25]. In contrast, the most commonly identified c-Kit mutant, D816V, has been shown to be dependent on PI3 kinase for its transforming ability [18], thus making PI3-kinase an attractive target in the treatment of malignancies carrying the c-Kit/D816V mutant. In addition to its lipid kinase activity, we have recently demonstrated that the important role of PI3-kinase in c-Kit/D816V mediated cell transformation is independent on its lipid kinase activity [5]. Since currently available PI3-kinase inhibitors block the lipid kinase activity and no other functions of PI3-kinase, such as binding to other signal transduction molecules, they are probably of low clinical efficacy in this type of malignancies.

Mutations in the c-Kit gene lead to activation of its kinase activity by different mechanisms and display differential sensitivity to c-Kit inhibitors. It is interesting to know whether the dependence on PI3 kinases is a common mechanism among all c-Kit mutants. By introducing mutations in c-Kit that block the association of PI3-kinase with c-Kit, we showed that the ligand-independent activation of c-Kit/V560D is totally dependent on PI3-kinase. The loss of PI3-kinase binding completely abolishes ligand independent activation of c-Kit/V560D. As is the case with c-Kit/D816V, the contribution of PI3-kinases to ligand independent activation of c-Kit/V560D is independent of its lipid kinase activity. These results suggest a new activity or function of PI3-kinase in addition to its lipid kinase activity.

Currently available PI3 kinase inhibitors are based on the inhibition of its lipid kinase activity. However, as we showed in this study, PI3-kinase inhibitors cannot inhibit the activation of c-Kit mutants and downstream signaling pathways and they will probably not be efficient in the treatment of malignancies carrying c-Kit mutations. To identify the novel activity of PI3 kinase and develop new inhibitors against the non-lipid kinase activity will be a new direction. The novel inhibitors of PI3 kinases might be useful in the treatment of malignancies driven by oncogenic mutants of c-Kit.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Figure legends

Fig. 1 Cell surface expression of c-Kit in Ba/F3 cells, cell survival and cell proliferation of BaF3 cells expressing wild-type c-Kit and c-Kit mutants. (A) Cell surface expression of c-Kit in Ba/F3 cells stably transfected with either wild-type c-Kit, c-Kit/V560D or c-Kit/816V was measured by flow cytometry (grey curve: isotype control, black curve: anti-c-Kit). (B) Ba/F3 cells expressing c-Kit were lysed and subjected to immunoprecipitation using a c-Kit antibody, and thereafter subjected to Western blotting using c-Kit antibody. The signal intensity of the 145 kD band and 125 kD band were quantified, the ratio between the 145 kD band and 125 kD band was calculated. (C and D) Ba/F3 cells expressing wild-type c-Kit, c-Kit/V560D or c-Kit/816V were analyzed for their proliferative response to SCF. Cells were washed free from IL-3, and subsequently seeded at a density of 70,000 cells/mL in 6-well plates. Thereafter cells were SCF-stimulated for 48 h (black column) or left untreated (empty column) as control. Living cells were counted under the microscope to examine cell proliferation (C) or stained with the apoptosis (D) detection kit (BD biosciences) and subsequently subjected to flow cytometry.

Fig. 2 Activation of wild-type c-Kit and c-Kit mutants, and downstream signaling pathways. (A) Western blot analysis using phosphospecific antibodies against known c-Kit phosphorylation sites. Ba/F3 cells expressing c-Kit were starved of serum and IL-3 for 4 hours followed by SCF (100ng/ml)-stimulation for 0, 2, 5, 15 or 30 minutes. Lysates were subjected to immunoprecipitation using a c-Kit antibody, and thereafter subjected to Western blotting using the following antibodies: pY568, pY703, pY721, pY936 and anti-phospho-tyrosine antibodies. C-Kit antibody was used as control. (B) Phosphorylation Akt and Erk in whole cell lysates (from the experiment 2A) was investigated by Western blot analysis using the following antibodies: anti-phosphoAkt (Ser473), anti-Akt, anti-phosphoErk (Thr202/Tyr204), anti-Erk and anti- β -actin. (C) Ba/F3 cells expressing c-Kit were starved of serum and IL-3 for 4 hours followed by stimulation with SCF(100ng/ml) for 2 minutes or left untreated. Lysates were subjected to immunoprecipitation using antibody against Gab2, SHC and SHP2 respectively. Tyrosine phosphorylation of Gab2, SHC and SHP2 was evaluated by Western blot analysis using anti-phosphotyrosine antibodies. Phosphorylation of Gab2, SHC and SHP2 was quantified and normalized against total protein.

Fig. 3 Src family kinases are not required for the activation of c-Kit/V560D and downstream signal transduction. (A) Ba/F3 cells expressing wild-type c-Kit, c-Kit/V560D and c-Kit/816V were starved of serum and IL-3 for 4 hours, thereafter cells were pre-incubated with the Src inhibitor SU6656 (2 μ m) for 30 minutes before SCF stimulation (100 ng/ml) for 2 minutes followed by immunoprecipitation using a c-Kit antibody. After SDS-PAGE and electrotransfer, membranes were probed with phosphotyrosine antibody and reprobed with c-Kit antibody. (B) Phosphorylation Akt and Erk in whole cell lysates was investigated by Western blot analysis as in Fig. 2.

Fig. 4 C-Kit/V560D does not gain Src family kinase activity. Ba/F3 cells expressing wild-type c-Kit, c-Kit/V560D or c-Kit/816V were starved of serum and IL-3 for 4 hours, stimulated with SCF (100 ng/ml) for 2 minutes. Cells were then lysed and immunoprecipitated using a c-Kit antibody. The immunoprecipitated c-Kit proteins were used in a kinase assay together with Src optimal peptide as substrate. the phosphorylation of the substrate by wild-type c-Kit or c-Kit/D816V was detected by Fuji FLA 3000. Signal intensity from multiple blots was quantified by the Multigauge software. *Error bars* indicate standard deviation.

Fig. 5 Ligand independent activation of c-Kit/V560D is dependent on PI3 kinase in a lipid kinase independent manner. (A) Ba/F3 cells expressing wild-type c-Kit, c-Kit/M724A, c-Kit/V560D or c-Kit/V560D/M724A were starved of serum and IL-3 for 4 hours followed by SCF(100ng/ml)-stimulation for 0, 2, 5, 15 or 30 minutes. Lysates were subjected to immunoprecipitation using a c-Kit antibody, and thereafter subjected to Western blotting using anti-phosphotyrosine antibodies and c-Kit antibodies. (B) Phosphorylation of Akt and Erk in whole cell lysates was investigated by Western blot analysis using the following antibodies: anti-phosphoAkt (Ser473), anti-Akt, anti-phosphoErk (Thr202/Tyr204), anti-Erk and anti- β -actin. (C) Ba/F3 cells expressing wild-type c-Kit or c-Kit/V560D were starved of serum and IL-3 for 4 hours, thereafter cells were pre-incubated with the PI3 kinase inhibitor PY294002 (2 μ M) for 30 minutes before SCF stimulation (100 ng/ml) for 2 minutes followed by immunoprecipitation using a c-Kit antibody. After SDS-PAGE and electrotransfer, membranes were probed with phosphor-tyrosine antibody and reprobed with c-Kit antibody. (D) Phosphorylation Akt and Erk in whole cell lysates were investigated using Western blotting.

Fig. 6 Ba/F3 cells expressing wild-type c-Kit, c-Kit/V560D or c-Kit/816V were analyzed for their proliferative response in the presence of Src family kinase inhibitor or PI3 kinase inhibitor. Cells were washed free from IL-3, and subsequently seeded at a density of 70,000 cells/mL in 6-well plates. Thereafter cells were SCF-stimulated for 48 h (black column) or left untreated (empty column) as control in the presence or absence of Src family kinase inhibitor SU6656 (2 μ M) or PI3 kinase inhibitor LY294002 (10 μ M) or GDC0941 (0.5 μ M). Living cells were counted under the microscope to examine cell proliferation.

Figure

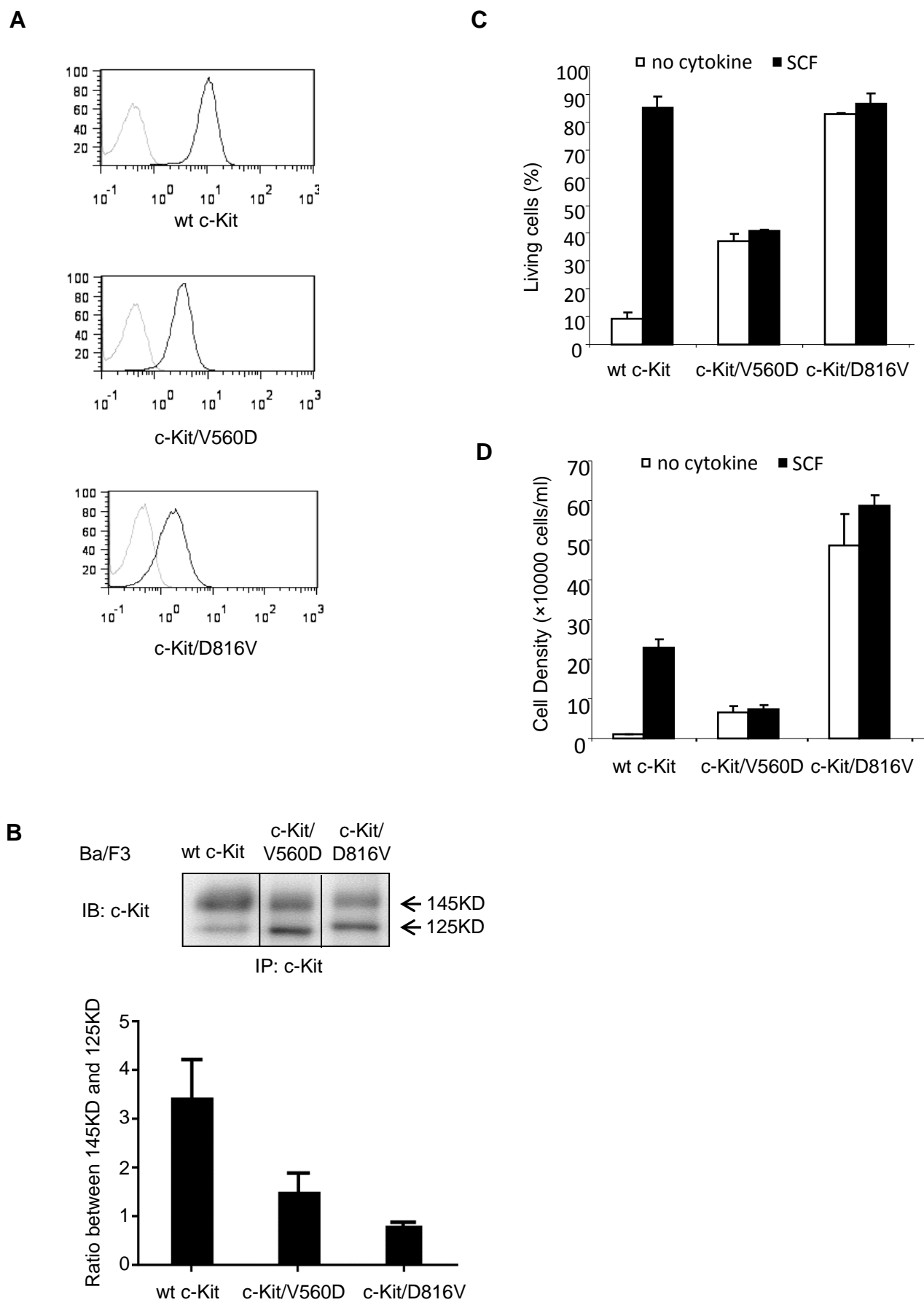
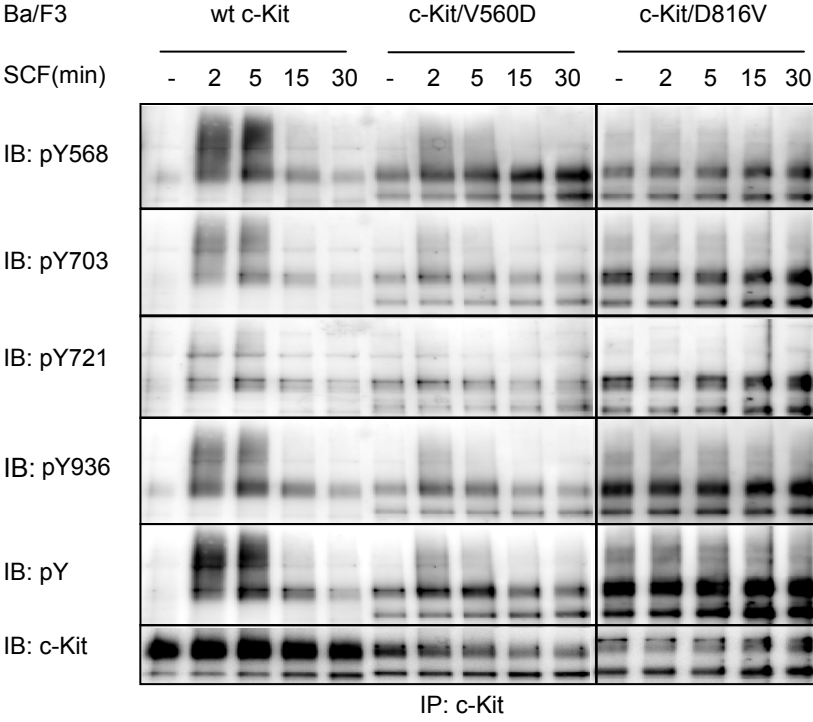
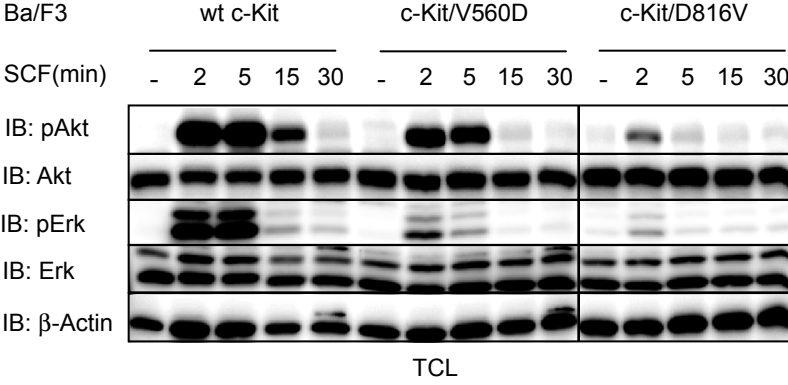
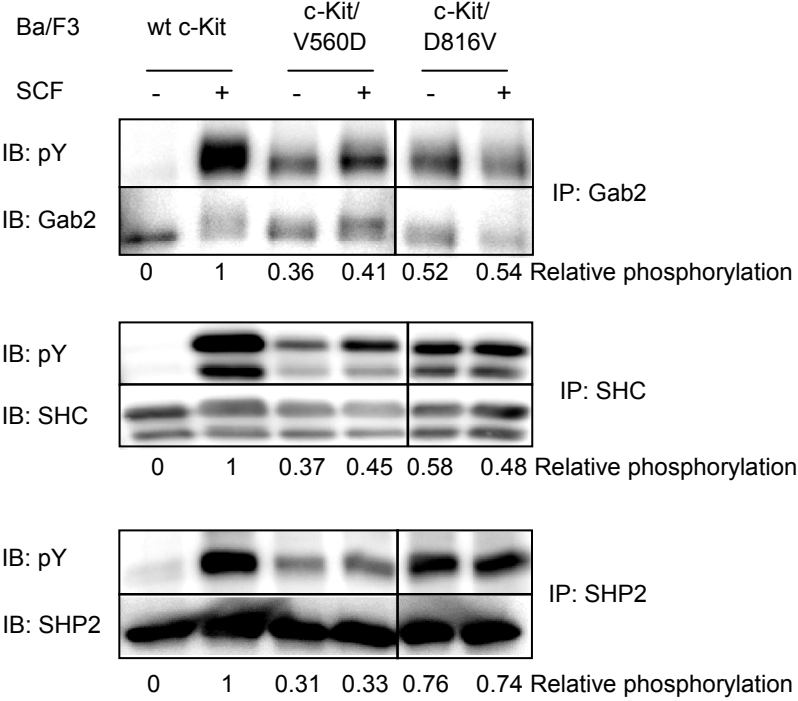
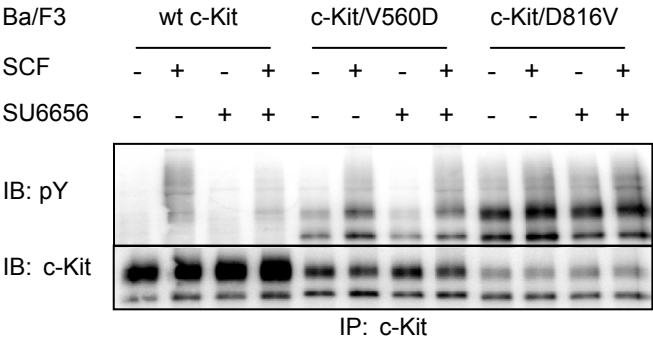


Figure 1

A**B****C****Figure 2**

A



B

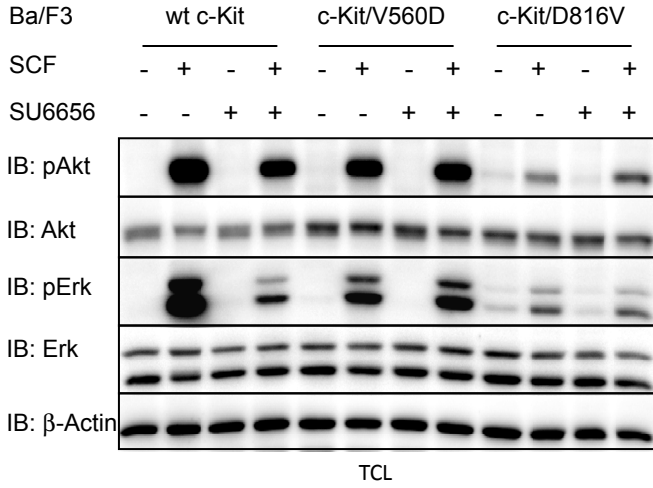


Figure 3

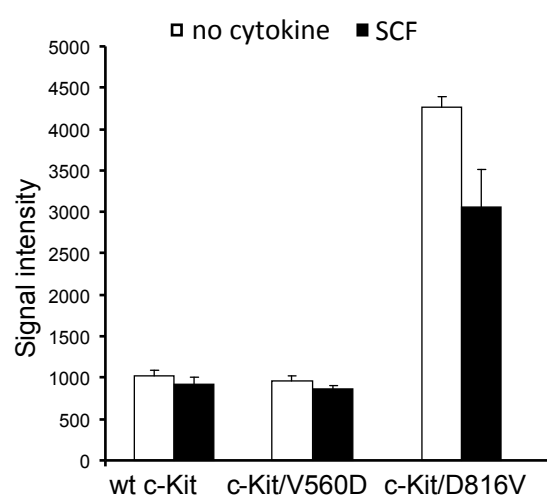
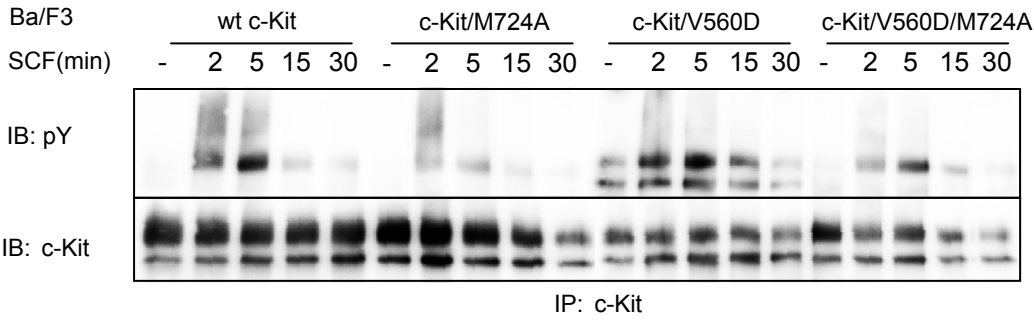
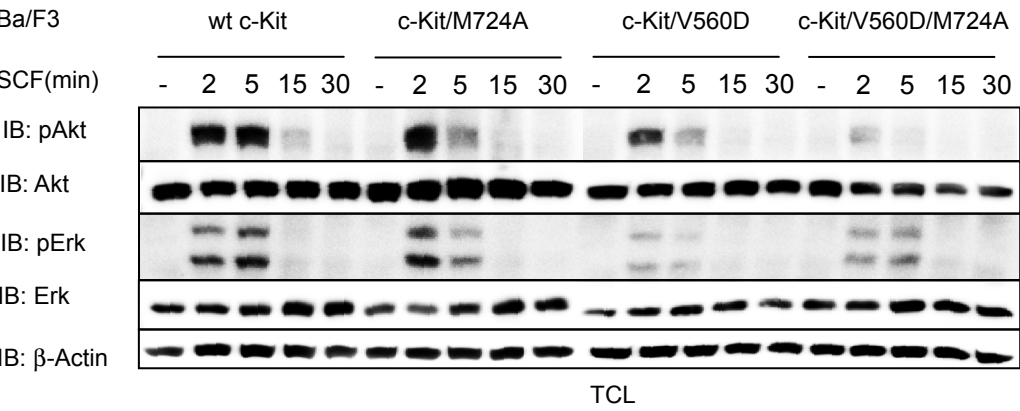


Figure 4

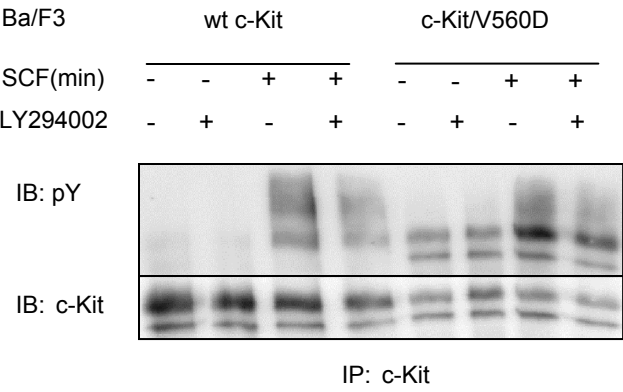
A



B



C



D

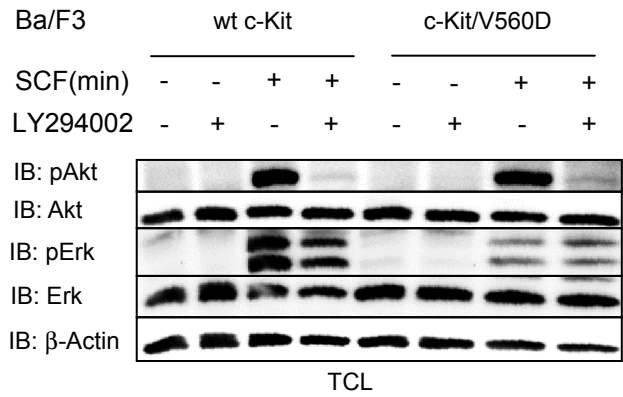


Figure 5

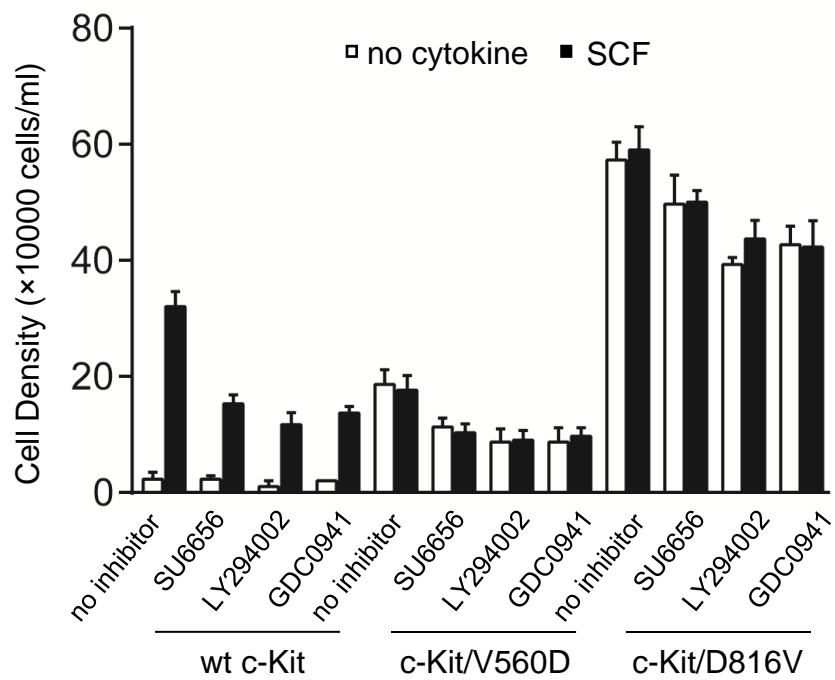


Figure 6