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## GAB2 IS INVOLVED IN DIFFERENTIAL PI3-KINASE SIGNALING BY TWO SPLICE FORMS OF C-KIT

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Running title: Splice form specific activation of PI3-kinase by c-Kit

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**The stem cell factor receptor/c-Kit plays an important physiological role in hematopoiesis, melanogenesis and gametogenesis. It has also been implicated in numerous human malignancies. Signal transduction pathways shown to be of importance for c-Kit mediated transformation include the PI3-kinase/Akt pathway. We have previously shown that two alternative splice forms of c-Kit, denoted GNNK- and GNNK+ respectively, mediate distinctively different signals. In this study we find that in the hematopoietic cell line Ba/F3, the GNNK- c-Kit mediates a substantially stronger activation of PI3-kinase/Akt than the GNNK+ c-Kit. This difference in signaling was shown to be dependent on the association of the scaffolding protein Gab2 to c-Kit and Src-mediated phosphorylation of Gab2, to be independent of the direct association of PI3-kinase with c-Kit. Furthermore, proliferation and survival of Ba/F3 cells expressing a mutant of c-Kit that fails to bind to PI3-kinase directly was slightly decreased compared to wild-type c-Kit expressing cells. Using siRNA technology we further verified a role of Gab2 in inducing activation of PI3-kinase/Akt downstream of c-Kit.**

**To summarize, we show that PI3-kinase activation by c-Kit is both splice form dependent and cell type specific. Furthermore, activation of PI3-kinase by c-Kit is dependent both on the direct PI3-kinase binding site in c-Kit as well as on the phosphorylation of Gab2. The fact that c-Kit has been found mutated in numerous human malignancies including acute myeloid leukemia and that Gab2 often is overexpressed in acute myeloid leukemia suggests a potential role of Gab2 mediated PI3-kinase activation in transformation.**

The receptor for stem cell factor (SCF), c-Kit, is a type III receptor tyrosine kinase belonging to the same subfamily as the platelet-derived growth factor (PDGF) receptors, Flt3, and the macrophage colony stimulating factor (M-CSF) receptor (for review see(1)). Furthermore, the c-Kit gene is identical to the white spotting locus (W) in the mouse. Loss-of-function mutations in c-Kit lead to defects in melanogenesis, gametogenesis and hematopoiesis. Stimulation of the c-Kit receptor with its ligand, SCF, leads to dimerization of receptors and activation of its intrinsic tyrosine kinase activity. Specific tyrosine residues are autophosphorylated which results in the activation of downstream signaling pathways including both positive and negative pathways.

As a result of alternative mRNA splicing, four isoforms of c-Kit have been identified in humans and two in mice (2,3). In both mice and humans alternative splicing results in isoforms characterized by the presence or absence of a tetrapeptide sequence GNNK in the extracellular part of the juxtamembrane region. Variants GNNK+ and GNNK- (also denoted Kit and KitA, respectively) are co-expressed in most tissues, with the GNNK- form predominating (4). In NIH3T3 cells transfected with either isoform of c-Kit, it was demonstrated that upon ligand stimulation, the GNNK- isoform was more strongly tyrosine phosphorylated and more rapidly internalized. Furthermore, it activated Erk more strongly than the GNNK+ isoform, while the activation of PI3-kinase was at similar level in the two splice forms (5). However, since c-Kit is not normally expressed in fibroblasts, but rather plays an important role in hematopoietic cells, we chose to study how the two splice forms of c-Kit signal in the pro-B cell line Ba/F3 to determine the role of alternatively spliced c-Kit in a more physiological setting.

Activation of PI3-kinase by c-Kit has been linked to mitogenesis, differentiation, survival, adhesion, secretion and actin cytoskeletal reorganization. In c-Kit, Y721 has been found to directly interact with PI3-kinase (6), and was initially claimed to be essential for PI3-kinase activation (7). However, it was later shown that in addition to the direct recruitment of PI3-kinase to c-Kit, also the indirect recruitment via Gab2 is important for full PI3-kinase activation (8,9). The scaffolding protein Gab2 is expressed in many tissues, including brain, kidney, lung heart, bone marrow, testis and ovary and found to be a positive mediator of cytokine and growth factor signaling (for review, see (10)). It can be activated by c-Kit and was found to be required for mast cell development and function (8,11). SCF-induced Erk and Akt activation was decreased in Gab2-deficient bone marrow-derived mast cells (BMMC) (8), which indicates a role of Gab2 in PI3-kinase/Akt activation.

C-kit promotes cell survival via several mechanisms. PI3-kinase-dependent activation of Akt and phosphorylation of Bad, a pro-apoptotic molecule, at S136 in vivo leads to its binding to 14-3-3 protein and sequestering from the anti-apoptotic molecule Bcl-xl. Akt has also been shown to phosphorylate Foxo transcription factors, leading to their exclusion from the nucleus and inability to act in pro-apoptotic signaling (12). Using immortalized murine progenitor cells transduced with the Y721F mutant D816V c-Kit, Chian et al. (13) showed that transformation by this constitutively active form of c-Kit is dependent on PI3-kinase.

In this study, we have investigated the mechanisms of splice form specific signaling of c-Kit in a hematopoietic cell line, Ba/F3, and found that in contrast to our previous findings in fibroblasts, the GNNK- isoform of c-Kit mediates a markedly stronger activation of PI3-kinase than the GNNK+ isoform. We further demonstrate that this difference arises from differential activation of Src family kinases and subsequent phosphorylation of Gab2. By mutating the direct PI3-kinase binding site in c-Kit, we show that the splice form specific effect is not mediated through differences in direct association between the two splice forms. We further demonstrate that binding and phosphorylation of Gab2 requires interaction with c-Kit through indirect binding via the adapter

protein Grb2 to Tyr703 and Tyr936 in c-Kit. Using siRNA against Gab2 we show that knock down of Gab2 expression leads to a significant reduction in PI3-kinase activation. To summarize, we demonstrate splice form specific differences in PI3-kinase signaling that could have consequences for the mode of activation of PI3-kinase by c-Kit in transformed cells and provides a target for future therapeutic intervention with c-Kit signaling in tumors.

## Experimental Procedures

*Antibodies and peptide-* Recombinant human SCF was purchased from ProspeCTany (Rehovot, Israel). The rabbit antiserum KitC1, recognizing the C-terminal tail of c-Kit, was purified as described (14). Antibodies against Gab2, Akt and SHP2 were from Santa Cruz Biotechnology (Santa Cruz, CA). p85 $\alpha$  antibody was from US Biological (Swampscott, MA). The antibody against p110 $\alpha$  has been described elsewhere (15). pAkt antibody is from Cell Signaling Technology (Danvers, MA). SHC antibody and Grb2 antibody were from BD Biosciences (San Jose, CA). Phosphotyrosine antibody 4G10 was from Upstate Biotechnology (Charlottesville, VA), PE labeled c-Kit antibody (104D2) was from Biolegend (San Diego, CA). The phosphospecific c-Kit antibodies pY568 and pY721 have been described elsewhere(5)

*Kits and Reagents-* Quikchange site directed mutagenesis kit was from Stratagene (La Jolla, CA). Src family kinase inhibitor SU6656 was from Sigma (St. Louis, MI). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Super Signal Dura reagent was from Pierce (Rockford, IL). Gab2 siRNA was from Dharmacon (Lafayette, CO). Annexin V-PE apoptosis detection kit was from BD Biosciences (San Jose, CA).

*Cell Culture-* The virus packaging cell line EcoPack (Clontech, Mountain View, CA) and c-Kit expressing NIH3T3 cells (5) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin; Ba/F3 cells and 32D cells (Deutsche Sammlung von Microorganismen und Zellen, Braunschweig, Germany) were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml

penicillin and 100 µg/ml streptomycin, and 10 ng/ml recombinant murine IL3; In order to establish c-Kit expressing Ba/F3 and 32D cell lines, EcoPack cells were transfected with wild-type and variant mutated c-Kit constructs on pMSCVpuro respectively, supernatants were collected to infect Ba/F3 or 32D cells followed by 2-week selection in puromycin. Expression of c-Kit was confirmed by flow cytometry and immunoblotting. C-kit expressing Ba/F3 and 32D cells were grown in the same media as Ba/F3 and 32D cells supplemented with 1.2 µg/ml puromycin.

*Cell stimulation, immunoprecipitation and Western Blotting-* Ba/F3 cells and 32D cells were starved of IL-3 for 5 hours, stimulated with 100 ng/ml SCF for the indicated times. NIH3T3 cells were starved of serum overnight. Cells were washed once in ice-cold PBS, lysed and processed for immunoprecipitation and Western blotting as described (5). Immunodetection was performed by enhanced chemiluminescence using the Super Signal Dura reagent (Pierce, Rockford, IL) and a CCD camera (LAS3000; Fujifilm).

*Electroporation of siRNA-* Silencing of Gab2 in c-Kit expressing Ba/F3 cells was achieved by electroporation (300 V, 1500 µF) in a Gene Pulser II (Bio-Rad, Hercules, CA) in the presence of 100 nM Gab2 siRNA. Cells were incubated in the Ba/F3 growth medium for 12 hours, starved for 5 hours, and then stimulated with SCF and lysed.

*Cell survival assay-* Cells were washed 3 times, resuspended in Ba/F3 complete medium without IL3, then seeded in 6-well plates containing 100 ng/ml SCF. Cells were seeded with 10 ng/ml IL3 or without any cytokine as control. After 48 hours incubation, cells were stained with Annexin V-PE apoptosis detection kit. Living cells, apoptotic cells and dead cells were quantified using flow cytometry

## RESULTS

*The alternative splice form GNNK- mediates a much stronger activation of PI3-kinase than the GNNK+ isoform in Ba/F3 cells-* In a previous study, we showed in transfected NIH3T3 cells that there was no quantitative difference in

phosphorylation of Y721 of c-Kit, the binding site for PI3-kinase, or in the activation of PI3-kinase between the two splice forms GNNK- and GNNK+, respectively. However, since c-Kit is normally not expressed in fibroblasts, we sought to study the role of the two splice forms in a more relevant cell type. For this purpose we chose the pro-B cell line Ba/F3, which was retrovirally transduced with either splice form of c-Kit. Equal expression of c-Kit was verified by flow cytometry (Fig. 1). Cells were stimulated with SCF, followed by lysis and separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransfer to Immobilon P. The filter was probed with an antibody against phosphorylated Akt (Ser472). In parallel, c-Kit was immunoprecipitated from lysates and subjected to SDS-PAGE and Western blotting with antibodies against phosphotyrosine and c-Kit, respectively. Akt was rapidly and strongly phosphorylated by GNNK- isoform of c-Kit after stimulation with SCF while the Akt phosphorylation was slower and weaker in cells expressing the GNNK+ isoform of c-Kit (Fig. 2A). Similar kinetics of Akt phosphorylation was seen in the myeloid cell line 32D transduced with the GNNK- and GNNK+ isoform, respectively (Fig. 2B). Furthermore, co-immunoprecipitation between the p85 subunit of PI3-kinase and c-Kit was stronger and showed a faster kinetics in cells expressing the GNNK- isoform compared to the GNNK+ isoform (Fig. 2D). This is in strong contrast to the kinetics of Akt phosphorylation in NIH3T3 fibroblasts transduced with the GNNK- and GNNK+ isoform, respectively, where both isoforms of c-Kit elicit a response to the same level, although with slightly different kinetics (Fig. 2C; (5)). However, while the overall tyrosine phosphorylation was higher in the GNNK- isoform as well as the phosphorylation of the Src binding site in c-Kit, Y568, phosphorylation of the direct binding site, Y721, showed similar levels of phosphorylation in both isoforms (Fig. 2D). Thus, the kinetics and magnitude of phosphorylation of individual tyrosine residues in c-Kit follows the same pattern in Ba/F3 cells as in NIH3T3 cells (5). *PI3-kinase activation is dependent on both direct and indirect association to c-Kit-* The p85 subunit of PI3-kinase is known to associate with c-Kit through phosphorylated Y721. In order to evaluate its importance in SCF-stimulated PI3-kinase

activation, we generated two mutants of c-Kit that blocks the direct binding of p85 to c-Kit; Y721F blocks any protein binding to phosphorylated Y721, while the M724A is more selective for PI3-kinase (16). The Y721F and M724A mutants of c-Kit blocked binding of p85 to either isoform of c-Kit, as expected (Fig. 3). However, the M724A mutant of either splice form of c-Kit was able to mediate phosphorylation of Akt despite its inability to associate with PI3-kinase (Fig. 4). This suggests that additional pathways, apart from the direct binding of PI3-kinase to c-Kit, mediate PI3-kinase activation.

*The direct binding of PI3-kinase to Y721 in c-Kit is required for cell proliferation but not for cell survival-* In order to investigate the role of direct versus indirect recruitment of PI3-kinase to c-Kit in the mitogenic and survival response to SCF stimulation, Ba/F3 cells expressing either wild-type or M724 mutant c-Kit of either splice form were incubated in the presence or absence of SCF in medium devoid of IL3 and scored for proliferation by an MTT assay after 48 hours incubation. All cell lines showed SCF-dependent proliferation with the cell lines expressing the GNNK- isoform of c-Kit growing much faster than cells expressing GNNK+ (Fig. 5A). Blocking the direct binding of PI3-kinase by the M724A mutant led to a decreased growth rate.

Cell survival assays were performed on the same cell lines using an Annexin V-PE apoptosis detection kit (Fig 5B). These data show that cells expressing the M724A mutant of c-Kit, despite its inability to bind PI3-kinase, can still survive in the presence of SCF, although the cell death is increased. These data clearly indicate that the two splice forms of c-Kit differ in their ability to mediate cell growth and survival and that the direct binding of PI3-kinase to c-Kit contributes to cell growth, but is not absolutely required for cell survival.

*C-kit activates PI3-kinase/Akt pathway via Gab2-* In order to assess how the two isoforms of c-Kit activate PI3-kinase/Akt pathway without the direct binding to PI3-kinase, Ba/F3 cells expressing wild-type or the M724 mutant of either isoforms of c-Kit were stimulated with SCF, cell lysates were prepared and subjected to immunoprecipitation with antibodies against the regulatory subunit p85 or the catalytic subunit p110 of PI3-kinase, and then probed with the anti-

phospho-tyrosine antibody 4G10 in order to detect binding partners of PI3-kinase other than c-Kit. One potential candidate is the scaffolding protein Gab2. The blotting result shows that both c-Kit and Gab2 can bind to p85 and p110 after SCF-stimulation of c-Kit. Furthermore, the M724A mutation doesn't affect the binding between Gab2 and p85 or p110 (Fig 6). This indicates that PI3-kinase can be activated by c-Kit either through direct recruitment to c-Kit or through indirect binding via Gab2. In order to elucidate the role of Gab2 in SCF-stimulated PI3-kinase/Akt activation, Gab2 siRNA was introduced into Ba/F3 cells expressing c-Kit/M724A to knock down the expression of Gab2. This led to the suppression of Akt phosphorylation (Fig. 7), suggesting again that PI3-kinase activation by c-Kit is mediated both through the direct binding to c-Kit and through the indirect association to Gab2. Filters were also probed with antibodies against Gab1, but it was not found to be negative (data not shown). This is in agreement with previous reports (17).

*Gab2 activation requires Src family kinase activity and Grb2 binding to c-Kit-* Reports from a number of receptor systems have suggested that phosphorylation of Gab2 could be indirect and mediated through activation of Src family kinases. In order to investigate this possibility, Ba/F3 cells expressing either isoform of c-Kit were stimulated with SCF and subjected to immunoprecipitation with an antibody against Gab2 antibody and probed with anti-phosphotyrosine antibodies. It was clearly demonstrated that GNNK- can strongly and rapidly phosphorylate Gab2, while the phosphorylation of Gab2 is much weaker in the GNNK+ isoform of c-Kit (Fig 8). Gab2 is known to be able to complex with both p85, SHP2, Shc and Grb2. Since Gab2 is known to associate to Grb2 via proline-rich regions of Gab2 and the SH3 domains of Grb2, we investigated whether Grb2 could act as an adapter bringing Gab2 to c-Kit. Grb2 binds to c-Kit through Y703 and Y936 (18,19). Using the N705A/N938A mutant of c-Kit, that fails to bind to Grb2, and the Src family kinase selective inhibitor SU6656, we investigated the requirements for c-Kit mediated Gab2 phosphorylation. These experiments showed that phosphorylation of Gab2 is dependent on intact Grb2 binding sites in c-Kit and intact Src family kinase activity (Fig. 9A). In order to assess

the importance of Grb2/Gab2 binding to c-Kit and Src family kinases in activation of PI3-kinase, a parallel experiment was performed probing for phosphoAkt (Fig. 9B). Combination of both Grb2 binding mutant and the Src family kinase inhibitor blocked Gab2 phosphorylation as well as Akt phosphorylation. From this result, we hypothesize that Grb2 can bring Gab2 to c-Kit through the binding of Grb2 to Y703 and Y936 of c-Kit, which then is phosphorylated through the action of Src family kinases. Phosphorylated Gab2 recruits the p85-p110 complex and activates the PI3-kinase/Akt signaling pathway.

## DISCUSSION

The stem cell factor receptor/c-Kit is known to undergo alternative splicing, leading to the existence of at least six different isoforms of c-Kit in mice and humans. Two of these splice forms, GNNK- and GNNK+, exist as a result of alternative splice acceptor site usage and leads to the absence or presence of a tetrapeptide sequence, GNNK, in the extracellular part of the juxtamembrane region. We and others have previously shown that the two splice forms differ dramatically in signaling downstream of c-Kit (5,20,21). While both isoforms are reported to bind SCF with equal affinity, the GNNK- isoform responds to SCF with a rapid and strong activation of its intrinsic kinase activity as well as Src family kinase activity. In contrast, GNNK+ responses are slower and weaker, and this seems to be due to a lower degree of activation of Src family kinases (5). It is both a qualitative and quantitative difference in signaling between the two splice forms. Probing of the receptor isoforms with phosphospecific antibodies revealed considerably higher level of Y568 phosphorylation in the GNNK- isoform, while phosphorylation of Y721 was at similar levels in either isoform (5, Fig. 2C). The mechanisms by which these differences in signaling arise is incompletely known. From the structural data obtained through X-ray crystallography of the extracellular domain of c-Kit, one region has been identified that is involved in stabilization of ligand-induced receptor dimers (22). It is possible, but remains to be shown, that the absence or presence of the GNNK sequence in this region influences dimer stability.

The initial studies on how the two alternative splice forms of c-Kit signal were all performed on transfected NIH3T3 cells. However, c-Kit is normally not expressed in fibroblasts and some important signal transduction molecules might be missing in fibroblasts. In this paper we have studied the signaling of the two alternative splice forms in the pro-B cell line Ba/F3. We demonstrate that, in contrast to what is seen in transfected NIH3T3 cells (5, Fig. 2C), phosphorylation of Akt downstream of c-Kit is considerably stronger in the GNNK- isoform (Fig. 2A). This occurs despite the fact that the phosphorylation of the direct binding site for PI3-kinase, Y721, is phosphorylated to similar extent in cells expressing either isoform of c-Kit (Fig. 2D). We also found the same to be true for the myeloid cell line 32D transfected with either the GNNK- or the GNNK+ isoform of c-Kit (Fig. 2B). This is in contrast to recent findings by Young and co-workers studying c-Kit signaling in the murine FDC-P1 early myeloid cell line and Myb-immortalized hematopoietic cells (21) who found marginal differences in PI3-kinase association with c-Kit as well as in Akt phosphorylation.

Early studies on c-Kit mediated PI3-kinases have indicated the crucial importance of phosphorylated Y721 for activation of PI3-kinase (7). However, these studies were many times performed studying the activity of PI3-kinase physically associated to c-Kit and not the total cellular activity of PI3-kinase. In this study we show that mutation of Y721 only partially reduces SCF-dependent Akt phosphorylation (Fig. 4) in both GNNK- and GNNK+ isoforms. PI3-kinase is known to be important for survival and proliferation of cells in a number settings (23). In order to evaluate the impact of direct PI3-kinase association to c-Kit on proliferation and survival, we performed MTT assays on Ba/F3 cells transfected with either wild-type or M724A mutant c-Kit (which fails to bind PI3-kinase). As can be seen in Fig. 5A, loss of direct binding of PI3-kinase to either splice form of c-Kit had only marginal effect on SCF-mediated cell proliferation. Scoring for SCF-induced survival of these cells under conditions of IL3 starvation demonstrated a partial effect on cell survival in either splice form of c-Kit (Fig. 5B). In contrast, treatment with a pharmacological inhibitor of PI3-

kinase has been shown to efficiently induce apoptosis of c-Kit expressing cells (24).

Given the importance of PI3-kinase in c-Kit mediated survival, alternative pathways for activation of PI3-kinase are likely to exist. Recent studies have reported the importance of the scaffolding protein Gab2 in c-Kit signaling. Gab2 is a scaffolding protein containing a PH domain, proline-rich sequences and several tyrosine residues that are phosphorylated upon growth factor or cytokine stimulation which leads to recruitment of downstream signal transduction molecules (for review, see (25)). Using targeted deletion of the Gab2 locus in mice, Nishida and co-workers (8) noted a decrease in mast cell numbers in Gab2 deficient mice. Furthermore, SCF stimulated Erk phosphorylation was markedly reduced in BMMC from such mice, indicating its importance in c-Kit signaling. Gab2 has been shown to regulate SCF-stimulated mast cell proliferation via SHP2-dependent activation of the Rac/JNK pathway (9). It was further demonstrated that mutation of Y719 (corresponding to Y721 in the human sequence), the direct binding site of PI3-kinase, did not fully inhibit PI3-kinase activation in BMMC, but that Gab2 also could contribute to PI3-kinase activation. In this study we show that in Ba/F3 cells, c-Kit-induced activation of PI3-kinase is mediated to about equal extent through the direct binding to c-Kit through Y721 and through the Src-dependent phosphorylation of Gab2 (Fig.4 and Fig. 9). This is also consistent with data from Yu et al. (9) who demonstrated a dramatic reduction in Gab2 phosphorylation in BMMC expressing the Y567 (corresponding to Y568 of the human sequence) mutant of c-Kit that fails to activate Src family kinases. Association between Gab2 and RTKs has in most cases been shown to occur indirectly through the adapter protein Grb2. Gab2 contains proline-rich sequences to which Grb2 binds through its SH3 domains, and the SH2 domain of Grb2 associates with phosphorylated tyrosine residues on the RTK. We have recently demonstrated that Grb2 in living cells associates with c-Kit through phosphorylated tyrosines 703 and 936 (19). Since binding of Grb2 to phosphorylated tyrosine residues is dependent on an asparagine residue in position +2 to the phosphorylated tyrosine residues, we employed a c-Kit double mutant N705A/N938A to block

binding of Grb2 to c-Kit. It was clearly demonstrated that SCF-induced phosphorylation of Gab2 was dependent on these two Grb2 binding sites (Fig. 9). To verify that indeed Gab2 is important for SCF-stimulated Akt activation, we used siRNA to selectively knock down expression of Gab2 (Fig. 7). Knock down of Gab2 expression in combination with the M724A mutant of c-Kit further decreased Akt phosphorylation in either splice form of c-Kit.

To summarize, we have found that the two splice forms of c-Kit, GNNK- and GNNK+, differ in a cell type specific manner in their mode of activation of PI3-kinase/Akt. In the pro-B cell line Ba/F3 transfected with c-Kit, PI3-kinase activation is dependent on both the direct binding site in c-Kit, Y721, as well as of the association of the scaffolding protein Gab2 to c-Kit through Grb2, and Src-dependent phosphorylation of Gab2, while in NIH3T3 cells it is solely dependent on phosphorylated Y721. The physiological function of PI3-kinase in c-Kit signaling has been studied in mice expressing the Y721F mutant of c-Kit, giving rise to a phenotype with either defective spermatogenesis or both defects in spermatogenesis and oogenesis (26,27) without affecting other c-Kit functions. It is tempting to speculate that these phenotypes might not fully reflect the function of PI3-kinase in c-Kit signaling, but may be the result of tissues not expressing high enough level of Gab2 to rescue the Y721F phenotype. However, this remains to be shown. Other studies targeting various isoforms of PI3-kinase in mice, have demonstrated an importance of the p110 $\delta$  isoform of PI3-kinase in mast cells. Expression of a catalytically compromised mutant of p110 $\delta$  in transgenic mice, led to defective SCF-mediated *in vitro* proliferation, adhesion and migration, and to impaired allergen-IgE-induced degranulation and cytokine release (28). Future studies are aiming at investigating the role of splice-form specific activation of PI3-kinase and other signal transduction molecules in other cell type, such as melanocytes. Given the recently demonstrated occurrence of activating mutations of c-Kit in a subtype of melanoma (29), the role of alternatively splice isoforms of c-Kit in such a setting is important to investigate and will be the basis of future projects.

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## FOONOTES

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<sup>1</sup>Abbreviations used: PI3-kinase, phosphoinositide 3-kinase; SH, Src homology; SCF, stem cell factor; PDGF, platelet-derived growth factor; Flt3, Fms-like tyrosine kinase 3; M-CSF, macrophage colony stimulating factor; BMDC, bone marrow-derived mast cells; SDS-PAGE, SDS polyacrylamide electrophoresis; IL3, interleukin 3

## FIGURE LEGENDS

**Fig. 1.** Surface expression of c-Kit on Ba/F3 cells. Ba/F3 cells expressing either the GNNK- isoform of c-Kit (Kit<sup>-</sup>), the GNNK<sup>+</sup> isoform of c-Kit (Kit<sup>+</sup>) or various mutants of c-Kit were stained with PE conjugated anti-c-Kit antibody or isotype control followed by examination through flow cytometry. All the cell lines showed similar cell surface expression of c-Kit (open curve, anti-c-Kit; filled curve, isotype control).

**Fig. 2.** The GNNK- isoform of c-Kit mediates a stronger association of PI3-kinase and stronger phosphorylation of Akt. A: Ba/F3 cells expressing either GNNK- or GNNK<sup>+</sup> isoforms of c-Kit were starved for 5 hours and stimulated for 0, 2, 5 and 15 minutes respectively. Total cell lysates were separated by SDS-PAGE and electrotransferred to Immobilon P, and probed with a phosphoAkt antibody (Ser473) to show the activation of PI3 kinase/Akt pathway. B: 32D cells expressing either GNNK- or GNNK<sup>+</sup> isoforms of c-Kit were treated as described above. C: NIH3T3 cells expressing either GNNK- or GNNK<sup>+</sup> isoforms of c-Kit were treated as described above. D: SCF-dependent activation of c-Kit was examined by immunoprecipitation of c-Kit and probing of filters with anti-phosphotyrosine antibody, pY568 and pY721 phosphospecific antibodies. Filters were stripped and reprobed with antibodies against c-Kit and the p85 subunit of PI3-kinase, respectively.

**Fig. 3.** Direct binding of PI3-kinase to either splice form of c-Kit is dependent on Y721 and M724. Ba/F3 cells expressing various splice forms of c-Kit and various mutants of c-Kit were starved for 5 hours, stimulated, lysed, and immunoprecipitated with a c-Kit antibody. After SDS-PAGE and electrotransfer, membranes were probed with antibodies against phosphotyrosine, c-Kit and p85 respectively.

**Fig. 4.** Loss of direct binding of PI3-kinase to c-Kit leads to a partial reduction in Akt activation in either splice form of c-Kit. Ba/F3 cells expressing two isoforms of c-Kit with or without p85 binding mutation were starved for 5 hours, stimulated and lysed. Total cell lysates were probed with pAkt and Akt antibodies.

**Fig. 5.** Effect of the direct binding of PI3-kinase to c-Kit on SCF-stimulated Ba/F3 proliferation and survival. A: Cells were washed 3 times to remove IL3 and seeded in 96-wells plates with various concentrations of SCF (20,000 cells per well). After 48 hours incubation, an MTT assay was performed. Growth curves were drawn based on A580 value. ♦ Kit-; ■ M724A-; ▲ Kit+; × M724A+; B: Cells were washed 3 times, resuspended in Ba/F3 complete medium without IL3, and seeded in 6-well plates in the presence of 100 ng/ml SCF. Cells were cultured for 48 hours, stained with Annexin V-PE apoptosis detection kit (BD Biosciences). The number of living cells, apoptotic cells and dead cells were scored by flow cytometry. Control cells kept in 10 ng/ml IL3 were viable and growing, while cells left without any cytokines died (data not shown). Black boxes indicate living cells; grey boxes indicate apoptotic cells and open boxes indicate dead cells.

**Fig. 6.** SCF stimulation leads to direct recruitment of PI3-kinase to c-Kit as well as to binding of PI3-kinase to Gab2. Ba/F3 cells expressing wild-type c-Kit or the M724A mutant of c-Kit in either splice form background were starved for 5 hours, stimulated and lysed, followed by immunoprecipitation of p85 $\alpha$  (A) and p110 $\alpha$  (B) respectively. After SDS-PAGE and electrotransfer, membranes were probed for phosphotyrosine, c-Kit, Gab2, p85 $\alpha$  and p110 $\alpha$  respectively.

**Fig. 7.** Knock down of Gab2 by siRNA leads to reduced SCF-dependent Akt phosphorylation. Ba/F3 cells expressing either of the two isoforms of c-Kit carrying the specific p85 binding mutation M724A were electroporated with Gab2 siRNA to knock down the expression of Gab2. After incubation overnight in cell growth medium, cells were starved followed by stimulation with SCF. Cells were lysed and the total cell lysate was separated by SDS-PAGE and electrotransferred to Immobilon P or subjected to immunoprecipitation with an antibody against Gab2. Filters were probed with antibodies against phosphotyrosine, and Gab2, respectively.

**Fig. 8.** SCF stimulation of either isoform of c-Kit leads to the complex formation between Gab2 and SHP2 and p85, while Grb2 association is constitutive. Ba/F3 cells expressing either isoform of c-Kit were starved for 5 hours and stimulated with SCF for the indicated times. Cell lysates were immunoprecipitated with an antibody against Gab2. After SDS-PAGE and electrotransfer, membranes were probed for phosphotyrosine, Gab2, SHP2, p85 $\alpha$  and Grb2.

**Fig. 9.** SCF-stimulated phosphorylation of Gab2 and Akt phosphorylation requires both intact Grb2 binding sites on c-Kit and the activity of Src family kinases. Ba/F3 cells expressing either splice form of c-Kit with or without Grb2 binding mutation N705A/N938A were starved for 5 hours, incubated with the Src selective inhibitor SU6656 for 30 minutes followed by SCF stimulation. A: Cell lysates were immunoprecipitated with a Gab2 antibody. Following SDS-PAGE and electrotransfer, membranes were probed for phosphotyrosine and Gab2. B: Total cell lysates were separated by SDS-PAGE and electrotransferred to Immobilon P, and probed with a phosphoAkt antibody (Ser473) to show the activation of PI3 kinase/Akt pathway

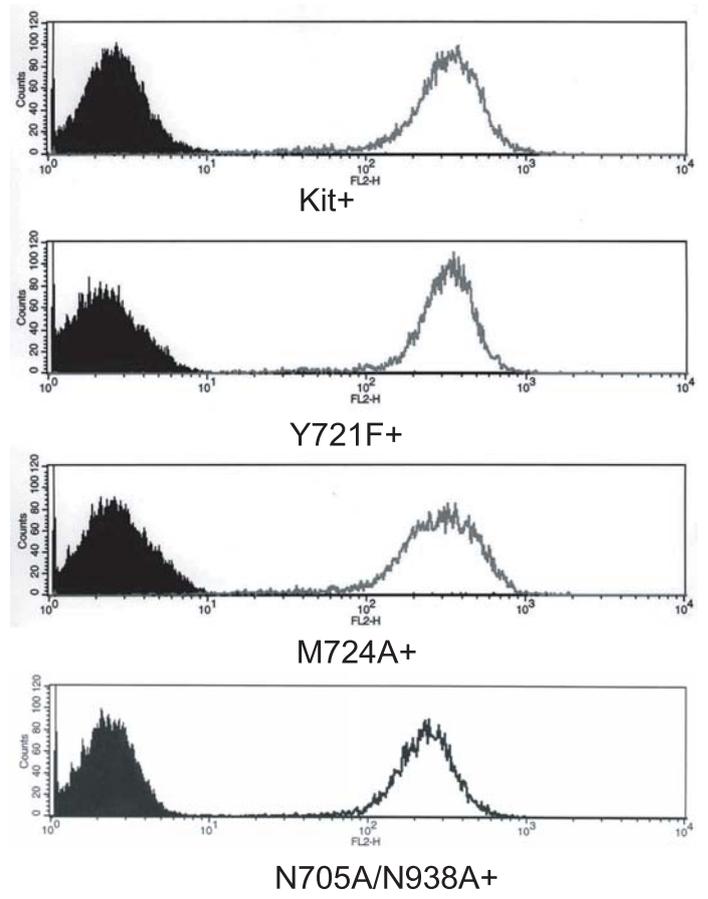
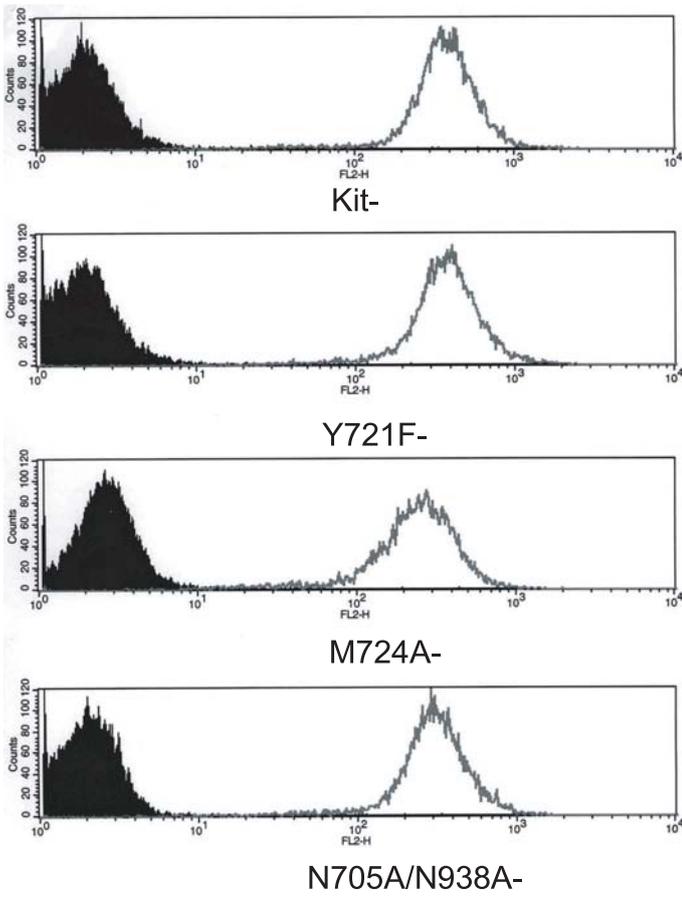
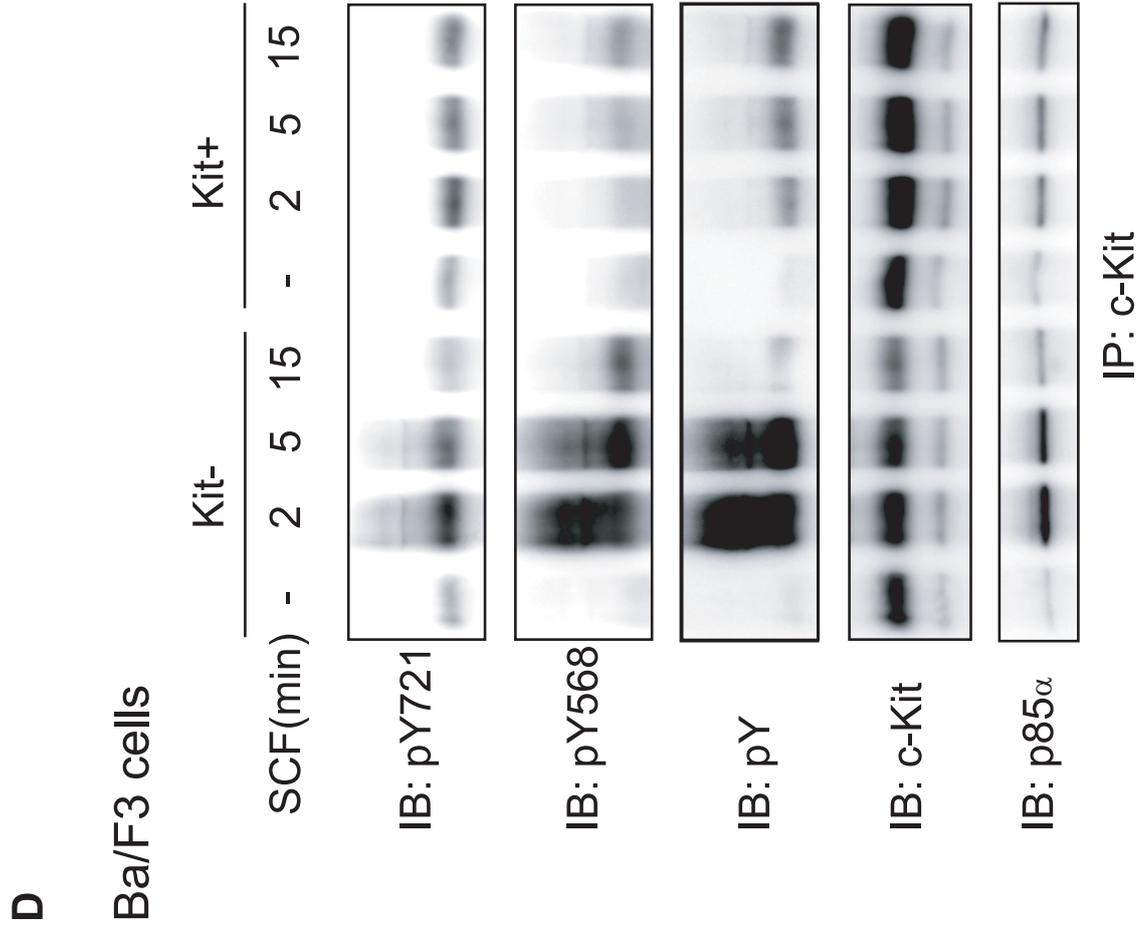
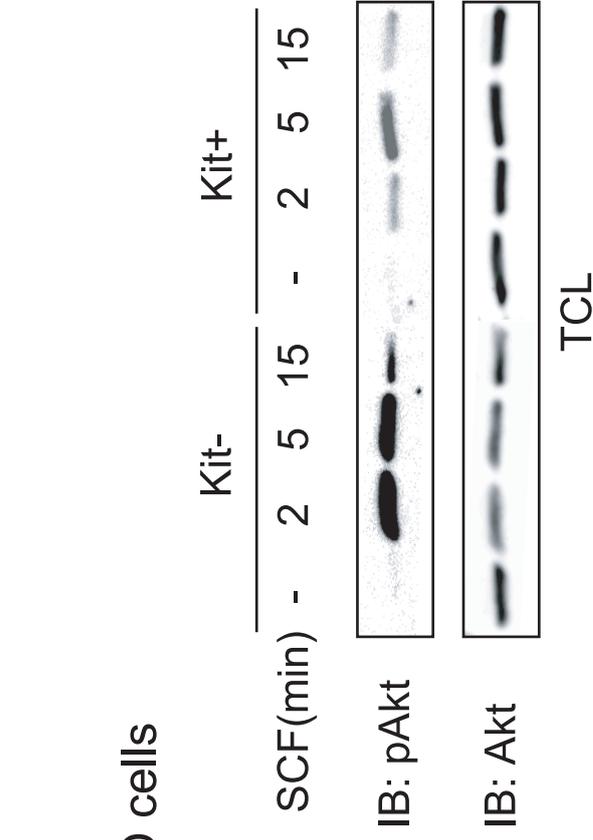
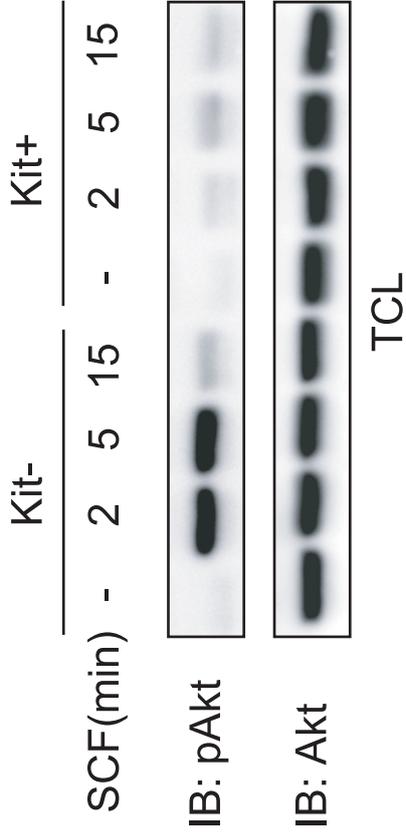


Fig. 1



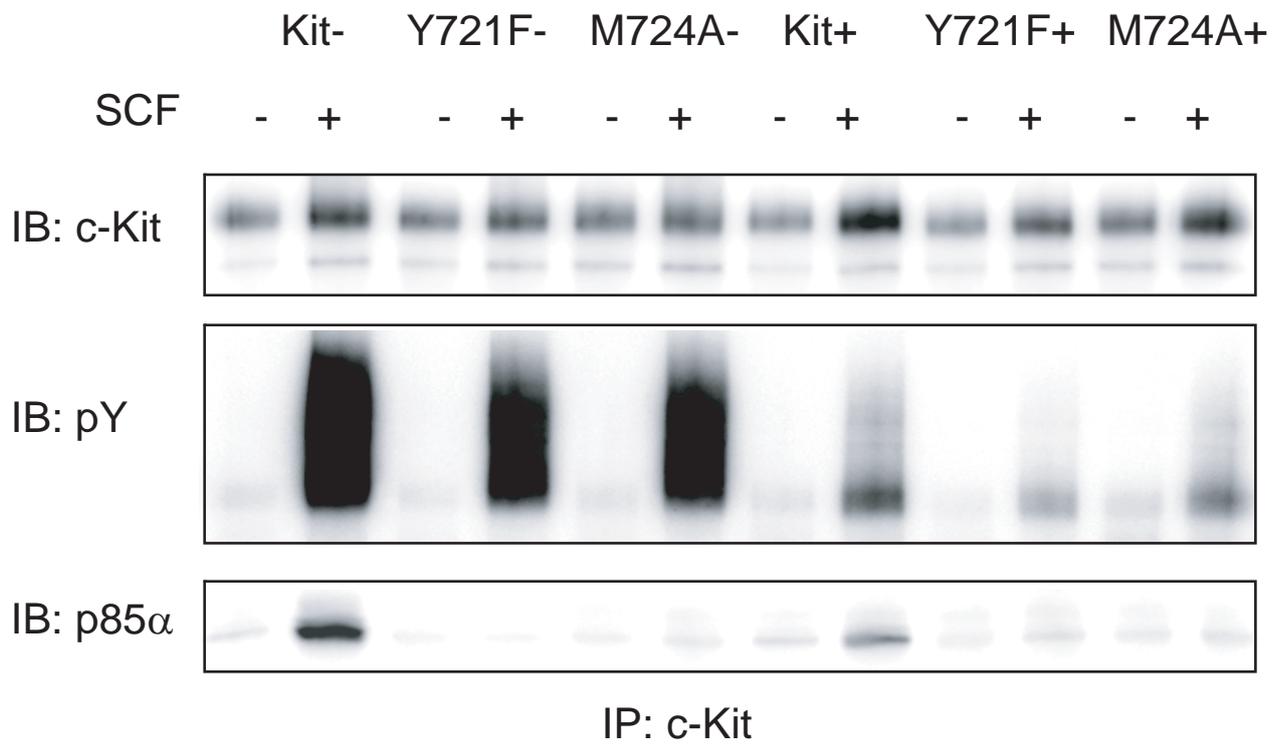


Fig. 3

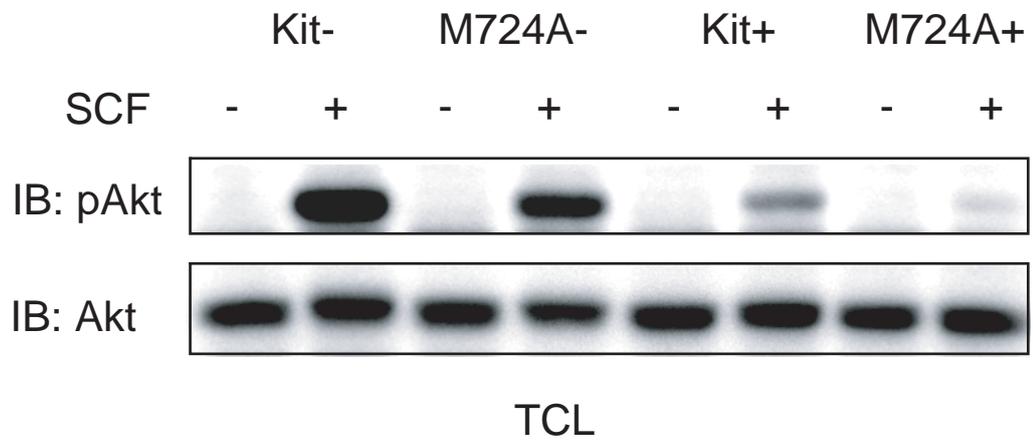


Fig. 4

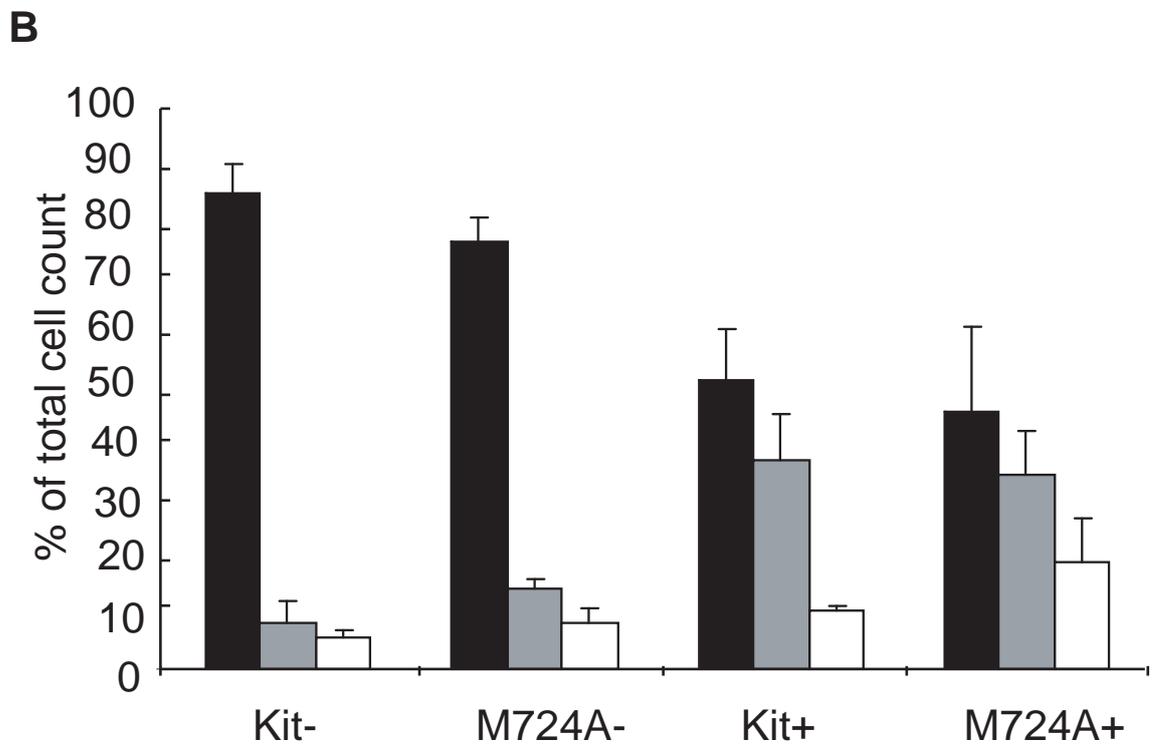
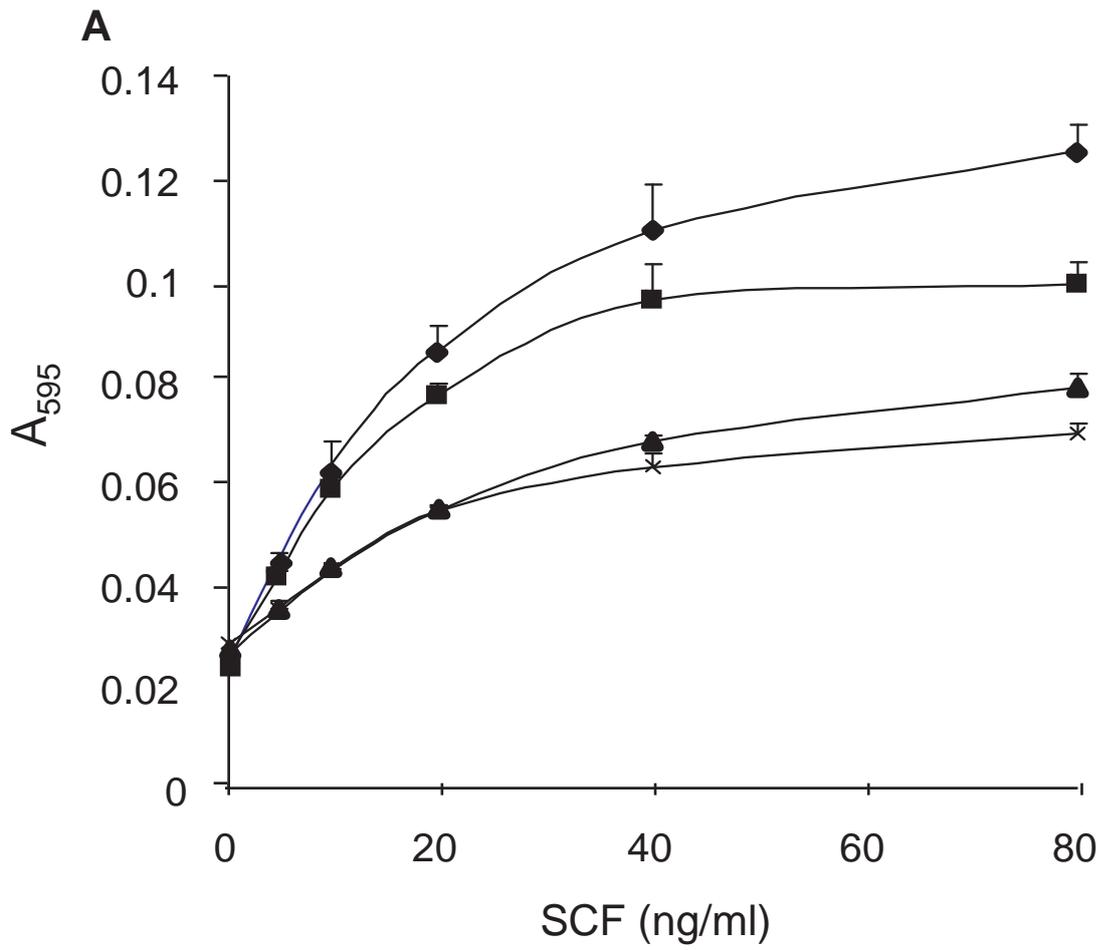


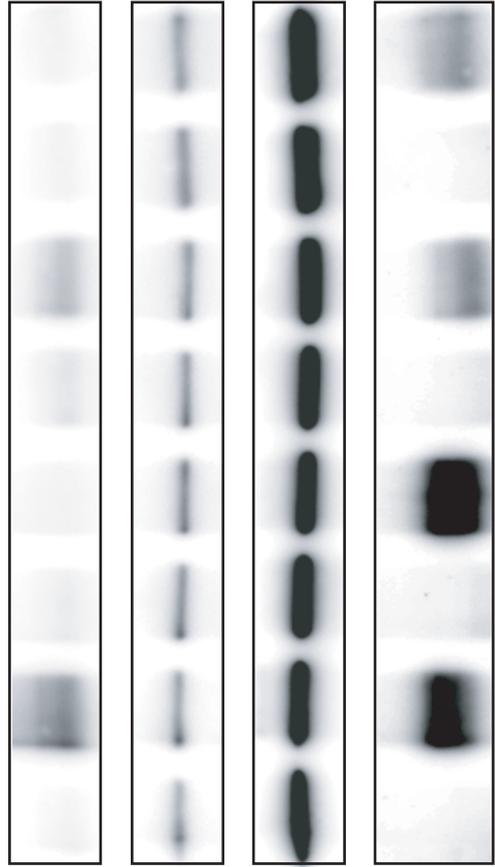
Fig. 5



← c-Kit

← Gab2

IB: pY



IB: c-Kit

IB: p110 $\alpha$

IB: p85 $\alpha$

IB: Gab2

IP: p85 $\alpha$

IP: p110 $\alpha$

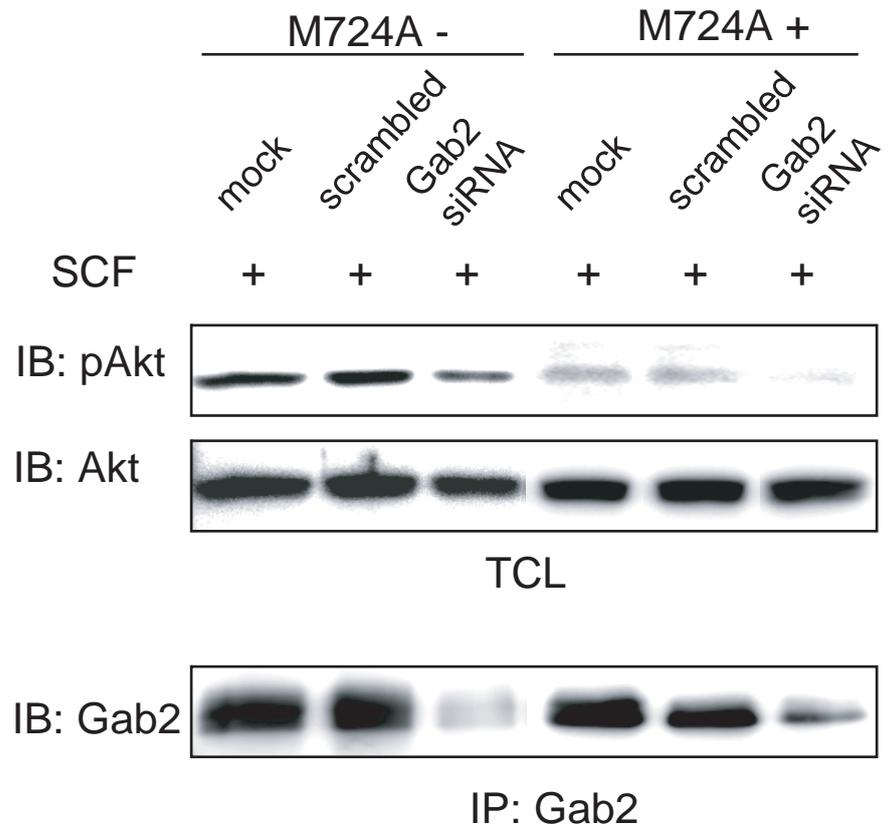


Fig. 7

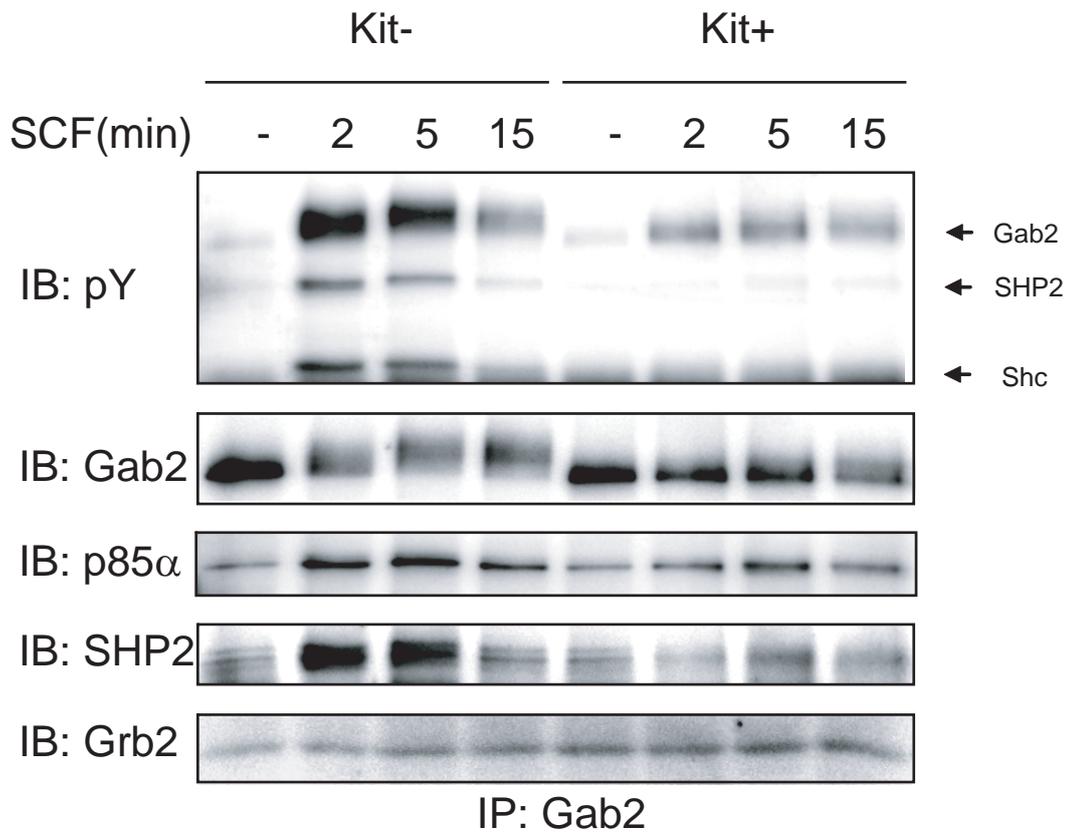


Fig. 8

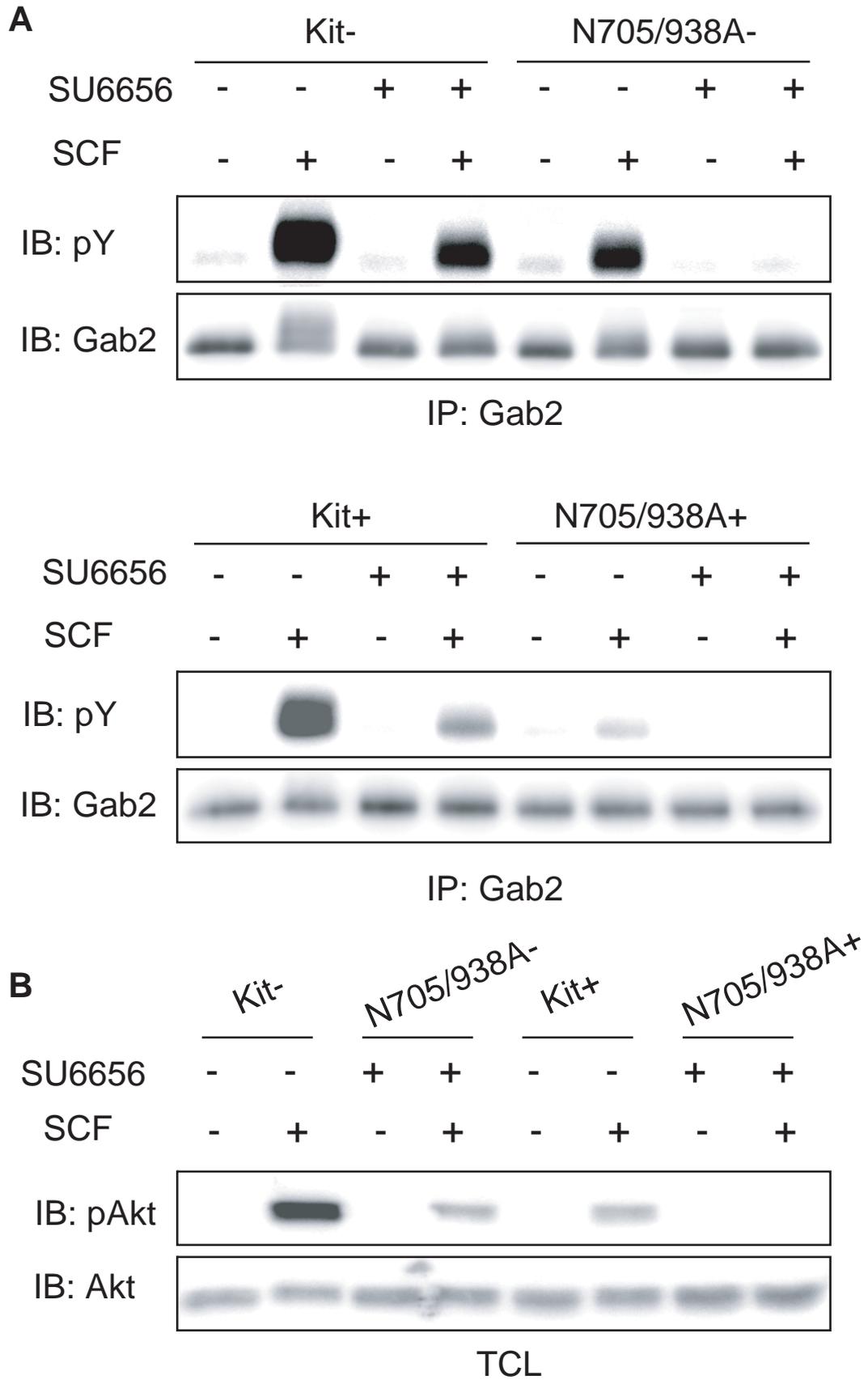


Fig. 9