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The PI3-kinase isoform p110δ is essential for cell transformation induced by the D816V mutant of c-Kit in a lipid-kinase independent manner

Jianmin Sun[1], Sofie Mohlin[2], Alicia Lundby[3], Julhash U. Kazi[1], Ulf Hellman[4], Sven Påhlman[2], Jesper V. Olsen[3], Lars Rönnstrand[1,5]

[1]Experimental Clinical Chemistry, Department of Laboratory Medicine, Lund University Skåne University Hospital, Malmö, Sweden;

[2]Center for Molecular Pathology, Department of Laboratory Medicine, CREATE Health, Lund University, Skåne University Hospital, Malmö, Sweden;

[3]Novo Nordisk Foundation Center for Protein Research, Department of Proteomics, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark;

[4]Ludwig Institute for Cancer Research, Uppsala, Sweden.

[5] corresponding author, Lars Rönnstrand. Tel: +46-40-337222, Fax: +46-40-331104

Lars.Ronnstrand@med.lu.se

Running title: p110δ is essential for cell transformation

Abstract

PI3-kinase plays a crucial role in transformation mediated by the oncogenic c-Kit mutant D816V. In this study we demonstrate that the c-Kit/D816V-mediated cell survival is dependent on an intact direct binding of PI3-kinase to c-Kit. However, mutation of this binding site had little effect on the PI3-kinase activity in the cells, suggesting that c-Kit/D816V-mediated cell survival is dependent on PI3-kinase but not its kinase activity. Furthermore, inhibition of the lipid kinase activity of PI3-kinase led only to a slight inhibition of cell survival. Knockdown of the predominant PI3-kinase isoform p110δ in c-Kit/D816V-expressing Ba/F3 cells led to reduced cell transformation both *in vitro* and *in vivo* without affecting the overall PI3-kinase activity. This suggests that p110δ plays a lipid-kinase-independent role in c-Kit/D816V-mediated cell transformation. We furthermore demonstrate that p110δ is phosphorylated at residues Y524 and S1039 and that phosphorylation requires an intact binding site for PI3-kinase in c-Kit/D816V. Overexpression of p110δ carrying the Y523F and S1038A mutations significantly reduced c-Kit/D816V-mediated cell survival and proliferation. Taken together, our results demonstrate an important lipid-kinase-independent role of p110δ in c-Kit/D816V-mediated cell transformation. This furthermore suggests that p110δ could be a potential diagnostic factor and selective therapeutic target for c-Kit/D816V-expressing malignancies.

Key words: c-Kit, c-Kit/D816V, PI3-kinase, p110δ, phosphorylation, cell transformation

Introduction

C-Kit is a type III receptor tyrosine kinase that belongs to the same subfamily as the platelet-derived growth factor (PDGF) receptors, FLT3, and the macrophage colony stimulating factor (M-CSF) receptor (1). When c-Kit binds its ligand stem cell factor (SCF), it dimerizes and its intrinsic tyrosine kinase is activated. Specific tyrosine residues are phosphorylated and act as docking sites for adaptors or activate signaling molecules such as Src family kinases and PI3-kinase. Of these, PI3-kinase has been shown to play an important role in c-Kit-induced cell survival, proliferation and cell adhesion (2).

PI3-kinase is a lipid kinase that can phosphorylate the 3′-hydroxyl group of phosphoinositides. The lipid products of PI3-kinase act as second messengers activating downstream signaling cascades that mediate multiple cellular responses such as cell survival, proliferation and differentiation. Based on the structure and substrate specificity, PI3-kinase can be divided into three groups: class I, II and III. Among them, the class I PI3-kinases are the most studied. Based on their activation mechanisms, class I PI3-kinases are further divided into class IA and class IB. Class IA PI3-kinase is composed of a regulatory subunit and a catalytic subunit that are constitutively associated (3). To date, p85α, p55α, p50α, p85β and p55γ have been identified as regulatory subunits and p110α, p110β and p110δ as catalytic subunits of the class IA PI3-kinases. P110α and p110β are broadly expressed across tissues whereas p110δ is mainly found in lymphocytes (4) and to a lesser extent expressed in neurons (5). In addition, p110δ has also been detected in various cancer forms including lung cancer, neuroblastoma, breast cancer and melanoma (6-8). The only member of class IB PI3-kinases that does not bind to p85 is p110γ (9).

C-Kit is frequently found mutated and autoactivated in human malignancies. Mutations in gastrointestinal stromal tumors (GISTs) mainly reside in exon 11 which encodes the juxtamembrane region of c-Kit. In other malignancies mutation at codon 816 in exon 17 is the most frequent seen mutation. This type of mutation involves the substitution of aspartic acid 816 with either a valine, tyrosine, phenylalanine, asparagine or histidine residue. Irrespective of substituting amino acid, these mutants are all constitutively active and transforming. This type of mutation has been found in mastocytosis, certain subtypes of acute myeloid leukemia (AML), germ cell tumors of the seminoma or dysgerminoma types, sinonasal nasal NK/T-cell lymphomas, intracranial teratomas and malignant melanoma (10-15). D816 mutations in c-Kit lead to constitutive, ligand-independent activation of its intrinsic kinase activity and thereby constitutively activates downstream signaling pathways leading to cell transformation (16). Imatinib (Gleevec) is a well-known inhibitor of c-Kit and has been used in the treatment of GISTs. However, c-Kit/D816V is resistant to imatinib while the so-called Src/Abl dual inhibitor dasatinib can inhibit c-Kit/D816V (17). Since all c-Kit inhibitors available in the clinic also targets wild-type c-Kit and its normal physiological functions, there are unwanted side effects of treatment. Furthermore, these inhibitors can also inhibit other tyrosine kinases and some even other enzymes (18) leading to additional side effects. Therefore, there is a need to identify signaling pathways that are selectively activated by the oncogenic mutant of c-Kit but not by wild-type c-Kit, in order to provide a specific target for pharmacological intervention. These proteins could also constitute valuable diagnostic markers.

PI3-kinase plays a key role in transformation mediated by the D816V mutant of c-Kit (19). In this study we show that the PI3-kinase has a role in c-Kit/D816V-mediated cell

transformation that is independent of its lipid kinase activity. The D816V mutant of c-Kit, unlike the wild-type c-Kit, is able to phosphorylate p110δ at Y524 and S1039. We furthermore demonstrate that this phosphorylation of p110δ can promote cell transformation independent of the lipid kinase activity. These results suggest that tyrosine phosphorylation of p110δ is a specific signaling event in c-Kit/D816V expressing cells.

Results

The direct association of PI3-kinase with c-Kit/D816V but not its lipid kinase activity, is important for receptor activation as well as for cell survival

The role of PI3-kinase in c-Kit/D816V-mediated cell transformation has previously been investigated by mutating the PI3-kinase binding site Y721 of c-Kit (Y719 in mouse c-Kit) to block the direct binding of PI3-kinase to c-Kit/D816V (19). The results indicated that Y721 in c-Kit is important for c-Kit/D816V-induced cell survival and proliferation. Since the direct binding of PI3-kinase to c-Kit requires the presence of an intact YXXM motif (20), we introduced the M724A mutation into either wild-type c-Kit or c-Kit/D816V and transfected into Ba/F3 cells. Ligand-independent cell survival and proliferation was greatly diminished in cells expressing the c-Kit/D816V/M724A mutant compared to c-Kit/D816V (Fig. 1A), which is in agreement with previous studies (19). In contrast, phosphorylation of Akt, a target downstream of PI3-kinase, was almost unchanged (Fig. 1B). We have previously demonstrated that PI3-kinase activation by c-Kit is not solely mediated by the direct interaction with c-Kit, but also through the scaffolding protein Gab2 (21). In other words, the biological phenotype in terms of survival does not match the PI3-kinase signaling activity as measured by AKT phosphorylation, suggesting that

lipid kinase-independent pathways are responsible for the effect seen. In order to further clarify the role of PI3-kinase enzymatic activity in these biological responses, we used the general PI3-kinase inhibitor LY294002 and the p110δ-selective inhibitor IC87114 (since p110δ is abundantly expressed in hematopoietic cells). Neither LY294002 nor IC87114 were able to block c-Kit/D816V induced-cell survival and proliferation, although a partial effect was seen on proliferation (Fig. 1C). This suggests either that PI3-kinase has a lipid-kinase independent role or that another important signaling protein binds to the same site, Y721. However, since we used the M724A rather than the Y721F mutant, the latter is unlikely given that there is no other SH2 domain containing protein that requires a methionine residue in position +3. To our surprise, the activation of c-Kit/D816V was also affected by loss of the PI3-kinase binding site, while PI3-kinase inhibitors were without effect (Figs 1B and 1D). The activation of ligand-stimulated wild-type c-Kit was dramatically reduced by both the loss of PI3-kinase binding and inhibition of PI3-kinase activity (Figs.1B and 1C). These data suggest that PI3-kinase can affect the signaling capability of c-Kit/D816V in a lipid-kinase independent fashion.

P110δ plays a key role in c-Kit/D816V mediated cell transformation

Since p110δ is the predominant isoform of class I PI3-kinase in hematopoietic cells, we sought to investigate the role of p110δ in c-Kit/D816V-induced cell transformation. To this end we used siRNA-mediated knockdown of p110δ. We could demonstrate that knockdown of p110δ had marginal effects on phosphorylation of Akt in both wild-type c-Kit and c-Kit/D816V expressing Ba/F3 cells (Fig. 2A). In contrast, both cell survival and cell proliferation were inhibited (Fig. 2B). In order to further elucidate the role of PI3-

kinase in c-Kit/D816V-mediated transformation, we performed colony formation assays in semi-solid culture. SiRNA-mediated knockdown of p110δ led to the formation of far fewer as well as smaller colonies compared to control siRNA (Fig. 2C). These results clearly demonstrate that p110δ is important for c-Kit/D816V-mediated cell transformation. To further test if the effect of p110δ knockdown on cell transformation was maintained *in vivo*, cells pre-treated with p110δ siRNA, or control siRNA, were injected subcutaneously into nude mice. Five days later tumors were excised and measured. Tumors formed from cells with decreased levels of p110δ were much smaller than tumors formed from control cells. In addition, knockdown of p110δ negatively affected tumor weight (Fig. 2D). There was also a clear difference in tumor vascularization as judged by ocular observation, where knockdown of p110δ led to tumors with markedly reduced blood vessel formation (Fig. 2E). Collectively, these results suggest an important role for p110δ in c-Kit/D816V-mediated tumor formation.

C-Kit/D816V induces tyrosine phosphorylation of p110δ independent of its lipid kinase activity

To further identify how PI3-kinase contributes to c-Kit/D816V mediated cell transformation, the regulatory subunit p85α of PI3-kinase was immunoprecipitated and probed with antibodies against phosphotyrosine and c-Kit, respectively. As expected, the binding of p85α to c-Kit was blocked by introducing the M724A mutation into wild-type c-Kit as well as to c-Kit/D186V (Fig. 3A). This is in agreement with previous studies (20). Unexpectedly however, we noticed a heavily tyrosine-phosphorylated 110 kDa protein that co-immunoprecipitated with p85α in Ba/F3 cells expressing c-Kit/D816V but

not in cells expressing wild-type c-Kit. In order to identify the tyrosine-phosphorylated 110 kDa protein, p85α was immunoprecipitated from cells expressing either wild-type c-Kit or c-Kit/D816V. Immunoprecipitated proteins were eluted by boiling and the released proteins were subjected to immunoprecipitation with a phosphotyrosine antibody. The 110 kDa protein was identified by mass spectrometry as the p110δ subunit of PI3-kinase. The three known isoforms of the catalytic subunit of class IA PI3-kinase, p110α, p110β and p110δ, all have a molecular weight of 110 kDa, and are known to associate with p85. To investigate whether p110α and p110β are also phosphorylated by c-Kit/D816V, immunoprecipitation with antibodies against p110α, p110β or p110δ was performed. Probing with a phosphotyrosine antibody demonstrated that only p110δ was tyrosine-phosphorylated (Fig. 3B), and furthermore, the phosphorylation was dependent on the direct binding of p85 to c-Kit (Fig. 3C). PI3-kinase inhibitors LY294002 and IC87114 did not inhibit the tyrosine phosphorylation of p110δ, indicating that the phosphorylation is independent of the lipid kinase activity of PI3-kinase (Fig. 3D).

P110δ is phosphorylated by c-Kit/D816V at Y524 and S1039

In order to identify the tyrosine phosphorylation site(s) in p110δ, cell lysates of c-Kit/D816V expressing Ba/F3 cells were subjected to immunoprecipitation with a p110δ antibody and the 110 kDa band was excised and analyzed by mass spectrometry. The identity of the protein as p110δ was confirmed, and we found that Y524 and S1039 were phosphorylated in c-Kit/D816V expressing Ba/F3 cells independent of SCF stimulation (Fig. 4A, B). To investigate this finding further, p110δ was FLAG-tagged and the Y524F mutation was introduced. This construct was transfected into Ba/F3/c-Kit or Ba/F3/c-

Kit/D816V cells. Immunoprecipitation with a FLAG antibody followed by immunoblotting with a phospho-specific antibody against pY524 in p110δ, revealed that Y524 is a phosphorylation site induced by the c-Kit/D816V but not by ligand-stimulated wild-type c-Kit (Fig. 4C). Furthermore, phosphorylation of Y524 can be inhibited by the Kit/D816V inhibitor Dasatinib at similar concentrations that lead to inhibition of phosphorylation of c-Kit as well to inhibition of proliferation (Fig. 4D). The other two isoforms of class IA PI3 kinases p110α and p110β can't get phosphorylated by c-Kit/D816V (Fig. 3B), alignment of p110α, p110β and p110δ showed that p110α and p110β have no tyrosine residue that is identical to Y524 of p110δ (Fig. 4E), which could explain why p110δ is the only class IA PI3 kinase that is phosphorylated by c-Kit/D816V. We have previously demonstrated that the D816V mutation of c-Kit not only activates the kinase activity but also leads to altered substrate specificity. The substrate specificity of c-Kit/D816V is similar to the specificity of Src and Abl kinases (22). The amino acid sequencing surrounding Y524 in p110δ is characterized by the presence of an acidic amino acid C-terminal to the tyrosine residue (YEHEKDL) which is known to characterize tyrosine residues phosphorylated by Src. Furthermore, a query of the sequence in PhosphoMotif Finder (http://www.hprdg.org) revealed that the tyrosine residue is a predicted Src substrate. These results indicate that the D816V mutation of c-Kit gains a function not possessed by the wild-type receptor allowing it to phosphorylate p110δ.

Apart from D816V, which is the most common activating c-Kit mutation found in human malignancies, several other mutations at aspartic acid 816 have been described in the literature (23). In order to investigate whether these D816X mutants behaved in a similar

fashion to D816V, the c-Kit mutants D816F, D816H and D816Y were transfected into Ba/F3 cells, and p110δ was phosphorylated at Y524 by all three mutants (Fig. 4F). The difference in the intensity of p110δ phosphorylation was a reflection of the overall kinase activity of the individual c-Kit mutants. The P815 cell line is a murine mast cell line that carries the D816V mutation of c-Kit endogenously, and p110δ is phosphorylated at Y524 in P815 cells as well (Fig. 4G).

Phosphorylation of p110δ on Y524 and S1039 contributes to c-Kit/D816V mediated cell survival and proliferation independent of the lipid kinase activity of PI3-kinase

In order to know whether the phosphorylation of p110δ plays a role in c-Kit/D816V mediated cell transformation independent of the lipid kinase activity, wild-type p110δ , mutants that block the phosphorylation of p110δ (Y524F and S1039A) and the kinase dead mutant D911A of p110δ were introduced into Ba/F3/c-Kit or Ba/F3/c-Kit/D816V cells. Ba/F3/c-Kit/D816V cells expressing the Y524F mutant of p110δ displayed a significantly lower cell proliferation and survival compared to cells expressing wild-type p110δ (Fig. 5A) although the phosphorylation of Akt remained intact (Fig. 5C). The kinase dead mutant of p110δ inhibited proliferation of both Ba/F3/c-Kit and Ba/F3/c-Kit/D816V cells while survival was only marginally affected (Fig. 5A, B). This was concomitant with an inhibition of Akt phosphorylation (Fig. 5C, D). Both Y524 and S1039 are important for cell survival mediated by c-Kit/D816V (Fig. 5A) although phosphorylation of S1039 seems to negatively regulate Akt phosphorylation (Fig. 5C). In Ba/F3 cells expressing wild-type c-Kit, Y524 and S1039 is not important for survival and proliferation compared to wild-type p110δ, since mutation of Y524 or S1039 made no

significant difference in either survival or proliferation assays. These results suggest that the Y524 and S1039 phosphorylation sites are more important in the oncogenic D816V mutant of c-Kit. In addition, the D911A mutant of p110δ did not block phosphorylation of p110δ at Tyr 524 (Fig. 5C), which confirms that the tyrosine phosphorylation of p110δ is not dependent on its lipid kinase activity.

Discussion

Mastocytosis is a clonal disease of mast cells characterized by abnormal accumulation of mast cells in tissues including the skin, bone marrow, liver and the lymph nodes. Treatment of mastocytosis is palliative and mainly focuses on the relief of symptoms (24). The D816V mutation is the most commonly found c-Kit mutation in mastocytosis patients and has been extensively studied. Several papers have demonstrated PI3-kinase to be a key mediator in c-Kit/D816V-induced cell transformation (25, 26). In this study we show that c-Kit/D816V-induced cell transformation is dependent on PI3-kinase but independent of its lipid kinase activity, suggesting that PI3-kinase might have other functions. We have previously shown that the D816V mutation endows c-Kit with a Src-like kinase activity (22), indicating that the mutation does not only induce constitutive activation of the receptor, but also adds functionality to the receptor. In order to further investigate this mechanism, we tested whether PI3-kinase can be modified by c-Kit/D816V and thereby contribute to transformation.

We found that in cells transformed by c-Kit/D816V, p110δ associated with c-Kit and was strongly phosphorylated at tyrosine residues. This phosphorylation was dependent on an intact direct physical association between PI3-kinase and c-Kit. Furthermore,

phosphorylation of p110δ was only seen in c-Kit/D816V-expressing cells and not in cells expressing wild-type c-Kit. Thus, this phosphorylation is unique to c-Kit/D816V-transformed cells. We could furthermore demonstrate that other isoforms of PI3-kinase, such as p110α and p110β, were not tyrosine phosphorylated by c-Kit/D816V. Mass spectrometry analysis identified the site of tyrosine phosphorylation to Y524 and additionally a serine phosphorylation site, S1039, was identified.

In wild-type c-Kit expressing cells, PI3-kinase can either be activated by direct association with phosphorylated Y721 of c-Kit or indirectly by binding to the scaffolding protein Gab2 (21). However, in terms of cells expressing c-Kit/D816V, it has great functional influence whether the binding is direct or indirect. In this study we show that phosphorylation of p110δ by c-Kit/D816V is abolished when the direct association to c-Kit is blocked.

The signaling pathways downstream of PI3-kinase are well studied, and dysregulation of these pathways contribute to oncogenesis (27). Activating mutations of p110α and amplification of p110β have been found in tumors. Mutations of p110α lead to increased catalytic activity, and to growth factor-independent cell growth and cell transformation (28). Studies on p110δ in AML patients have failed to detect any activating mutations (29). In this study, we present data showing a totally new function of p110δ which is independent of its lipid kinase activity. In contrast to p110α mutations and p110β amplification that leads to higher lipid kinase activity of PI3-kinase and thereby contributes to transformation, the c-Kit/D816V phosphorylates p110δ at Y524 and S1039 which contributes to c-Kit/D816V-mediated cell transformation independent of its lipid kinase activity. This is the first report of an oncogenic mutant of receptor tyrosine kinase

283 that utilizes PI3-kinase in a manner independent of its lipid kinase activity. Currently,
284 targeting PI3-kinase therapy focuses on the inhibition of its lipid kinase activity. From
285 our results, we conclude that it will be necessary to block both the lipid kinase-dependent
286 and -independent signal transduction to get a good effect on c-Kit/D816V-expressing
287 malignancies. Phosphorylation at serine residues residing in carboxyterminus of the
288 catalytic subunits p110β and p110δ (S1070 and S1039, respectively) have been reported
289 and was found to inhibit the lipid kinase activity of PI3-kinase (30, 31). Our results
290 confirmed the inhibitory function of phosphorylation of S1039 in p110δ. In addition,
291 blockage of S1039 phosphorylation led to a decreased cell survival although the lipid
292 kinase activity of PI3-kinase was increased, suggesting that phosphorylation of S1039
293 might have additional functions.
294 Gain-of-function mutations of c-Kit in cancer have been widely studied. These include
295 in-frame deletions, insertions and substitutions that through different mechanisms lead to
296 autoactivation. Such mutations have been identified in patients with GISTs, AML,
297 mastocytosis and testicular seminomas. 70–80% of GISTs carry an activating c-Kit
298 mutation that renders the receptor constitutively active, thus making c-Kit a clinically
299 important therapeutic target in GISTs (32). Most c-Kit mutations in GISTs are mapped to
300 exon 11, which disrupts the normal interaction between the juxtamembrane domain and
301 the kinase domain leading to activation of the receptor kinase activity (33). More than
302 80% of mastocytosis patients have a mutation in exon17 that affects aspartic acid 816 in
303 the second part of the kinase domain of c-Kit, with D816V being the most frequently
304 identified mutation (34). Some AML patients also carry c-Kit/D816V (35). The reason
305 why GISTs usually carry mutations in exon 11 and the hematologic malignancies usually

carry exon 17 mutations is not known. One possibility might be that the exon17 mutants, such as c-Kit/D816V depend on p110δ for transformation and since p110δ is predominantly expressed in hematopoietic cells, this might be at least part of the explanation.

The constitutively active mutants of c-Kit have been successfully targeted with selective tyrosine kinase inhibitors. In particular, imatinib has been successful used for the treatment of cancers carrying certain mutants of c-Kit, in particular the exon 11 mutants commonly found in GISTs. However, other mutants of c-Kit such as D816V are resistant to imatinib. Since the advent of imatinib, numerous tyrosine kinase inhibitors have been developed and found to inhibit also the imatinib-resistant mutants of c-Kit, such as D816V. These molecules include dasatinib and nilotinib. However, despite the promising results in the laboratory setting, none of these molecules have given any beneficial response when given to patients (36, 37). Thus, there is a need to find novel therapeutic target for the treatment of these patients. Ideally, a signal transduction molecule that is activated solely by the oncogenic mutant of c-Kit but not by wild-type c-Kit would be a suitable target. This would potentially minimize the risk for unwanted side effect due to inhibition of normal, physiological signaling. In this study, our data suggest that the phosphorylation of p110δ might be a good target for treatment of c-Kit/D816V-expressing malignancies, since this phosphorylation does not occur in normal cells. Combination treatment against several targets has in many cases improved the outcome of treatment dramatically and it can also avoid development of drug resistance and overcome unresponsiveness (38, 39). Resistance to tyrosine kinase inhibitors could be overcome by targeting multiple levels of the signal transduction cascades. Thus, targeting

p110δ combined with targeting of c-Kit might improve the treatment efficacy of malignancies carrying the D816V mutation of c-Kit. Future studies will aim at elucidating the signaling pathways downstream of p110δ.

Materials and methods

Cytokines, antibodies and peptides

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 (40). The antibody against p110δ was generated by immunizing a rabbit with the peptide CWLAHNVSKDNRQ conjugated to KLH. Antibodies recognizing pY524 of p110δ were generated by immunizing rabbits with synthetic phosphopeptides corresponding to the phosphorylation sites and purified extensively by affinity chromatography. All peptides were synthesized by JPT Peptide Technology (Berlin, Germany). FLAG and β-actin antibody were from Sigma (St. Louis, MI). Antibodies against Akt, pErk, p110α, p110β were from Santa Cruz Biotechnology (Santa Cruz, CA). P85α antibody was from US Biological (Swampscott, MA). pAkt antibody was from Epitomics (Burlingame, CA). Phospho-tyrosine antibody 4G10 was from Upstate Biotechnology (Charlottesville, VA). PE labeled c-Kit antibody (104D2) was from Biolegend (San Diego, CA).

Kits and reagents

QuikChange mutagenesis kit was from Stratagene (La Jolla, CA) and used according to the manufacturer's instructions. The PI3-kinase inhibitor LY294002 was from Sigma (St. Louis, MI) and the p110δ inhibitor IC87114 was from Symansis (Timaru, New Zealand).

Lipofectamine 2000 was from Life Technologies (Sweden). Chemiluminescent HRP substrate was from Millipore (Billerica, MA). Annexin V-PE apoptosis detection kit was from BD Bioscience (San Diego, CA).

Cell culture

EcoPack virus packaging cell line (Clontech), COS-1 and P815 cell line (ATCC) was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Ba/F3 cells (DSMZ) 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 IL-3. 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 μg/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. To establish Ba/F3/c-Kit or Ba/F3/c-Kit/D816V cells expressing p110δ, FLAG tagged p110δ constructs in pMSCVneo vector were transfected into EcoPack cells, supernatants were collected to infect Ba/F3/c-Kit or Ba/F3/c-Kit/D816V cells followed by 2-weeks selection in 0.8 mg/ml G418. Expression levels of p110δ were confirmed by western blot.

Cell stimulation, immunoprecipitation and western blotting

Ba/F3 cells were starved for 4 hours in medium in the absence of serum and IL-3 followed by SCF stimulation (100 ng/ml) for the indicated periods of time. Cells were washed once in ice-cold PBS and lysed in a lysis buffer containing 1% Triton X-100, 25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 2 μg/ml Trasylol, 1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 14,000 × *g* for 15 minutes at 4 °C and supernatants were incubated end-over-end with the indicated antibody for 1 hour followed by incubation with protein G-Sepharose beads for 30 minutes at 4 °C. The immunoprecipitates were washed three times in lysis buffer, boiled for 5 min in SDS sample buffer and separated by SDS-PAGE, followed by electrotransfer to Immobilon P membranes (Millipore). Membranes were blocked with 0.2% Tween-20 in PBS for 1 hour at room temperature and then incubated with primary antibody overnight at 4 °C, followed by washing with 0.05% Tween-20 in PBS. Incubation with secondary horseradish peroxidase-conjugated antibody was done for 1 hour at room temperature, followed by washing with 0.05% Tween 20 in PBS. The immunodetection was performed by Millipore ECL reagent.

Cell survival and proliferation assay

Ba/F3 cells were washed 3 times with PBS, resuspended in Ba/F3 complete medium without IL-3, seeded in 24-well plates with either 100 ng/ml SCF, 10 ng/ml IL-3 or no cytokine as control. After 48 hours incubation, cells were stained with Annexin V-PE apoptosis detection kit. The living cells and apoptotic cells were counted by flow cytometry. For the cell proliferation assay, living cells were counted under the microscope. Statistical significance was calculated by *t* test or one-way Annova.

SiRNA transfection

5 million Ba/F3 cells were washed once with RPMI 1640, mix with 3 µg siRNA and transfected by 4D-nucleofector (Lonza), program CM150, solution SG.

Colony assay

Ba/F3 cells were washed 3 times with PBS, resuspended in IMDM medium, mix with semi-solid colony assay medium MethoCult® M3231 (Stem cell technologies) according to the manufacturer's instruction. Cell mixture was seeded in 24-well plates with either 100 ng/ml SCF, 10 ng/ml IL-3 or no cytokine as control. After 4 days incubation, colonies were counted and photographed. Statistical significance was calculated by *t* test.

Animal experiment

Female athymic mice (NMRI-Nu/Nu strain, 4 weeks of age at arrival) were used and housed in a controlled environment and all procedures were approved by the regional ethics committee for animal research (approval no. M69/11). For subcutaneous xenograft tumors, cells (6×10^6) were collected in 100 µl Matrigel:PBS (2,3:1) and kept on ice until injection on the right flank. Mice (n=5 for each group) were monitored daily and tumors were excised, measured and weighed 5 days after injection. Tumor volume is calculated by $(\pi \times l \times s^2)/6$, where l=long side and s=short side. Statistical significance was calculated by *t* test.

Immunoprecipitation of p110δ for mass spectrometry

300 million Ba/F3/c-Kit and Ba/F3/c-Kit/D816V cells, respectively, were starved, stimulated with SCF and lysed as above. After centrifugation for 15 minutes, supernatants were incubated with p110δ antibody for 1 hour followed by incubation end-over-end with Dyna protein G beads (Invitrogen) for 30 minutes at 4 °C. The immunoprecipitates were washed three times with lysis buffer, boiled for 5 min in SDS sample buffer and separated by SDS-PAGE, followed by Coomassie staining. A 110 kDa band was excised and analysed by mass spectrometry.

Identify the phosphorylation site in p110δ by mass spectrometry

For full description of the mass spectrometry experiments, see Supplementary Methods Mass Spectrometry. Briefly, proteins in excised bands were in-gel digested with trypsin, essentially as previously described (41). Phosphopeptides were enriched by use of titanium dioxide beads (10µm titansphere, GL Sciences, Japan) pre-coated with 2,5-dihydroxybenzoic acid. All experiments were performed on an EASY-nLC™ system (Proxeon Biosystems, Odense, Denmark) connected to a LTQ-Orbitrap Velos (Thermo Electron, Bremen, Germany) through a nano-electrospray ion source, essentially as described previously (42). Acquired data was processed by MaxQuant 1.0.14.7 as described (43).

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Supplementary Information accompanies the paper on the Oncogene website

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573

574 **Figure legends**

575

Figure 1. Mutation of the PI3-kinase binding site in c-Kit, but not the PI3-kinase inhibitors, block c-Kit/D816V induced ligand-independent cell survival with almost unaltered Akt phosphorylation.

A. Ba/F3/c-Kit, Ba/F3/c-Kit/M724A, Ba/F3/c-Kit/D816V and Ba/F3/c-Kit/D816V/M724A cells were washed 3 times with PBS, resuspended in Ba/F3 complete medium without IL-3, seeded in 24-well plates with either 100 ng/ml SCF, 10 ng/ml IL-3 or no cytokine as control. After 48 hours incubation, cells were stained with Annexin V-PE apoptosis detection kit and the living cells and apoptotic cells were counted by flow cytometry. For the cell proliferation assay, living cells were counted under the microscope. *** $P<0.001$

B. Ba/F3/c-Kit, Ba/F3/c-Kit/M724A, Ba/F3/c-Kit/D816V and Ba/F3/c-Kit/D816V/M724A cells were starved for 4 hours in medium without serum and IL-3 followed by SCF stimulation (100 ng/ml) for 2 minutes. Cells were washed once in ice-cold PBS and lysed in the lysis buffer. Total cell lysate (TCL) was probed with pAkt antibody, or c-Kit was immunoprecipitated and then probed with 4G10 (pY) antibody to evaluate c-Kit activation. Signals of pY blot were quantified and normalized by c-Kit blot to decide the relative phosphorylation of c-Kit. *** $P<0.001$

C. Ba/F3/c-Kit and Ba/F3/c-Kit/D816V cells were washed 3 times with PBS, resuspended in Ba/F3 complete medium without IL-3 and seeded in 24-well plates with either 100 ng/ml SCF or no cytokine as control. The media either contained PI3-kinases inhibitor LY294002 (10 μM), or p110δ inhibitor IC87114 (10 μM) or no inhibitor as

599 control. After 48 hours incubation, cells were stained with Annexin V-PE apoptosis
600 detection kit. The living cells and apoptotic cells were counted by flow cytometry. For
601 the cell proliferation assay, living cells were counted under the microscope. * $P<0.05$,
602 *** $P<0.001$, ns: no significance.
603
604 **D.** Ba/F3/c-Kit and Ba/F3/c-Kit/D816V cells were starved for 4 hours in medium without
605 serum and IL-3, incubated with general PI3-kinases inhibitor LY294002 (10 μM) or
606 p110δ inhibitor IC87114 (10 μM) at the same time, followed by SCF stimulation
607 (100 ng/ml) for 2 minutes. Total cell lysate was probed with pAkt antibody to test if the
608 inhibitors affect the downstream signaling of PI3-kinases. Immunoprecipitated c-Kit was
609 probed with pY antibody to evaluate the phosphorylation of c-Kit. Signals of pY blot
610 were quantified and normalized by c-Kit blot to decide the relative phosphorylation of c-
611 Kit. * $P<0.05$, ns: no significance.
612
613 **Figure 2. P110δ plays a key role in c-Kit/D816V mediated cell transformation**
614 **A.** 5 million Ba/F3/c-Kit and Ba/F3/c-Kit/D816V cells were transfected with 3 ug p110δ
615 siRNA by 4D-nucleofector (Lonza), program CM150, solution SG. After 48 hours, cells
616 were washed and starved as above, followed by stimulation with SCF for 2 minutes, and
617 lysed in a lysis buffer. The total cell lysates were probed with Akt, pAkt, Erk and pErk
618 antibodies. Knockdown of p110δ expression was assessed by probing with p110δ
619 antibody.
620

B. Ba/F3/c-Kit and Ba/F3/c-Kit/D816V cells were transfected with control siRNA or p110δ siRNA as above. After 24 hours, cells were washed and seeded in 24-well plates with either 100 ng/ml SCF, 10 ng/ml IL-3 or no cytokine as control. After 48 hours incubation, cells were stained with Annexin V-PE apoptosis detection kit. The living cells and apoptotic cells were counted by flow cytometry. For the cell proliferation assay, living cells were counted under the microscope. * $P<0.05$, *** $P<0.001$.

C. Ba/F3/c-Kit and Ba/F3/c-Kit/D816V cells were transfected with control siRNA or p110δ siRNA as above. After 24 hours, cells were washed and seeded in semi-solid medium in 24-well plates. The number of colonies was counted after 6 days of incubation. The photos show colonies for the D816V mutant after 4 days of incubation. *** $P<0.001$.

D. Ba/F3/c-Kit and Ba/F3/c-Kit/D816V cells were transfected with control siRNA or p110δ siRNA as above. After 24 hours, 6 million cells were washed once with PBS and collected in 100 µl Matrigel:PBS (2,3:1) and injected subcutaneously into mice. 5 days after injection, tumors were excised, measured and weighed. Tumor volume was calculated as $(\pi*l*s^2)/6$, where l=long side and s=short side. * $P<0.05$

E. Photos of the tumors.

Figure 3. The p110δ subunit of PI3-kinase is tyrosine-phosphorylated by c-Kit/D816V

A. Ba/F3/c-Kit, Ba/F3/c-Kit/M724A, Ba/F3/c-Kit/D816V and Ba/F3/c-Kit/D816V/M724A cells were starved for 4 hours in medium without serum and IL-3 followed by SCF stimulation (100 ng/ml) for 2 minutes. Cells were washed once in ice-cold PBS and lysed in the lysis buffer. P85α was immunoprecipitated from total cell lysate and the precipitate was probed with pY, c-Kit and p85α antibody respectively.

B. Ba/F3/c-Kit/D816V cells were starved and stimulated as above and immunoprecipitated with p110α, p110β or p110δ antibody respectively and probed with pY antibody.

C. Ba/F3/c-Kit, Ba/F3/c-Kit/M724A, Ba/F3/c-Kit/D816V and Ba/F3/c-Kit/D816V/M724A cells were starved and stimulated as above and p110δ was immunoprecipitated. The precipitate was probed with pY, c-Kit, p110δ and p85α antibody respectively.

D. Ba/F3/c-Kit and Ba/F3/c-Kit/D816V cells were starved for 4 hours in medium without serum and IL-3, incubated with general PI3-kinases inhibitor LY294002 (10 μM) or p110δ inhibitor IC87114 (10 μM) at the same time, followed by SCF stimulation (100 ng/ml) for 2 minutes. Total cell lysate was probed with pAkt antibody to test if the inhibitors affect the downstream signaling of PI3-kinases. Immunoprecipitated p110δ was probed with pY antibody to evaluate the phosphorylation of p110δ.

Figure 4. P110δ becomes phosphorylated at Y524 and S1039 by c-Kit/D816V.

A. Tandem mass spectrometry identified the protein of the 110KD band as p110δ. The amino acid sequence of the protein is shown, and in red are highlighted peptides covered by the mass spectrometry experiments. A total of 114 unique peptides aligning with p110δ were identified, and 81% of the amino acid sequence was covered in the experiment, thereby accurately determining the protein as p110δ.

B. Tyrosine 524 and serine 1039 of p110δ was identified to be phosphorylated. Identified peptides covering Y524 and S1039 are shown with the detected fragment ions indicated. The fragment ions are highlighted in the MS/MS spectrum. Each peptide contained one phosphate group, and due to the fragmentation pattern the localization of the phosphorylation site could be localized to tyrosine 524 and serine 1039.

C. Ba/F3/c-Kit/FLAG-p110δ, Ba/F3/c-Kit/FLAG-p110δ/Y524F, Ba/F3/c-Kit/D816V/ FLAG-p110δ and Ba/F3/c-Kit/D816V/FLAG-p110δ/Y524F cells were starved, stimulated with SCF for 2 minutes and lysed as above, and p110δ was immunoprecipitated with FLAG antibody, and probed with p110δ pY524 antibody to test the Y524 phosphorylation of transfected p110δ.

D. Ba/F3/c-Kit/D816V cells were incubated with indicated concentration of Dasatinib overnight, p110δ and c-Kit were immunoprecipitated from the total cell lysates. Immunoprecipitated proteins were separated by SDS-polyacrylamide electrophoresis, electrotransferred to Immobilon filter and probed with antibodies against pY524, p110δ,

4G10 or c-Kit, respectively. Cell proliferation was calculated after Ba/F3/c-Kit/D816V cells were incubated with Dasatinib for 48 hour in the absence of IL-3.

E. Alignment of human and murine p110α, p110β and p110δ showed that p110α and p110β have no tyrosine residue that is identical to Y524 of p110δ.

F. Ba/F3/c-Kit, Ba/F3/c-Kit/D816V, Ba/F3/c-Kit/D816F, Ba/F3/c-Kit/D816H and Ba/F3/c-Kit/D816Y cells were starved, stimulated with SCF for 2 minutes and lysed as above, p110δ and c-Kit was immunoprecipitated respectively. The p110δ precipitate was probed with pY524 antibody to evaluate the phosphorylation of p110δ, and the c-Kit precipitate was probed with pY antibody to test the activation of c-Kit.

G. P815 cells were starved for 4 hours in medium without serum followed by SCF stimulation for 2 minutes. Cells were washed once in ice-cold PBS and lysed in the lysis buffer, and p110δ or c-Kit was immunoprecipitated and probed with pY523, pY, c-Kit, p110δ and p85α antibodies respectively.

Figure 5. Phosphorylation of p110δ at Y524 and S1039 contributes to c-Kit/D816V mediated cell survival and proliferation.

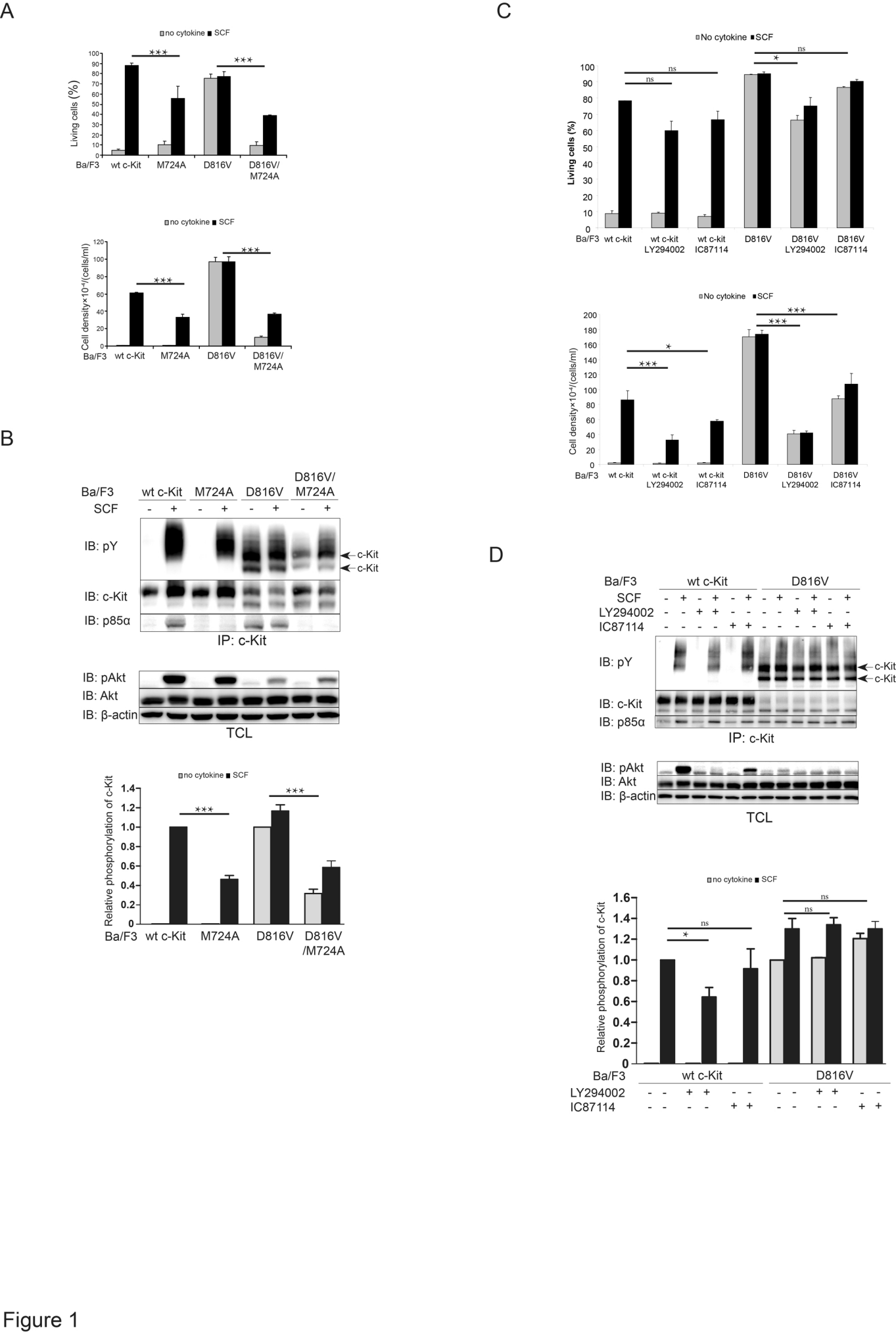
A. Ba/F3/c-Kit/D816V cells expressing FLAG tagged p110δ were washed 3 times with PBS, resuspended in Ba/F3 complete medium without IL-3, seeded in 24-well plates with either 100 ng/ml SCF, 10 ng/ml IL-3 or no cytokine as control. After 48 hours incubation, cells were stained with Annexin V-PE apoptosis detection kit and the living cells and

apoptotic cells were counted by flow cytometry. For the cell proliferation assay, living cells were counted under the microscope. *** P<0.001, ns: no significance.

B. Ba/F3/c-Kit cells expressing FLAG tagged p110δ were washed 3 times with PBS, resuspended in Ba/F3 complete medium without IL-3, seeded in 24-well plates with either 100 ng/ml SCF, 10 ng/ml IL-3 or no cytokine as control. After 48 hours incubation, cells were stained with Annexin V-PE apoptosis detection kit and the living cells and apoptotic cells were counted by flow cytometry. For the cell proliferation assay, living cells were counted under the microscope. *** P<0.001, ns: no significance.

C. Ba/F3/c-Kit/D816V cells expressing FLAG tagged p110δ were starved for 4 hours in medium without serum and IL-3 followed by SCF stimulation (100 ng/ml) for 2 minutes. Total cell lysate was probed with pAkt antibody, c-Kit or FLAG was immunoprecipitated.

D. Ba/F3/c-Kit cells expressing FLAG tagged p110δ were starved for 4 hours in medium without serum and IL-3 followed by SCF stimulation (100 ng/ml) for 2 minutes. Total cell lysate was probed with pAkt antibody, c-Kit was immunoprecipitated.


A
no cytokine SCF
Living cells (%)
***
***
Ba/F3
wt c-Kit
M724A
D816V
D816V/
M724A
no cytokine SCF
Cell density×10⁻⁴/(cells/ml)
***
***
Ba/F3
wt c-Kit
M724A
D816V
D816V/
M724A
B
Ba/F3
wt c-Kit
M724A
D816V
D816V/
M724A
SCF
IB: pY
c-Kit
c-Kit
IB: c-Kit
IB: p85α
IP: c-Kit
IB: pAkt
IB: Akt
IB: β-actin
TCL
no cytokine SCF
Relative phosphorylation of c-Kit
***
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Ba/F3
wt c-Kit
M724A
D816V
D816V
/M724A
C
No cytokine SCF
Living cells (%)
ns
ns
*
ns
Ba/F3
wt c-kit
wt c-kit
LY294002
wt c-kit
IC87114
D816V
D816V
LY294002
D816V
IC87114
No cytokine SCF
Cell density×10⁻⁴/(cells/ml)
*
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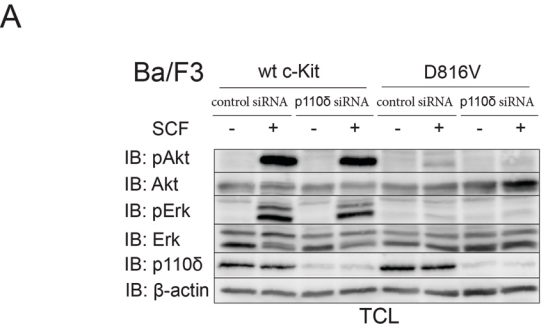
***
***
Ba/F3
wt c-kit
wt c-kit
LY294002
wt c-kit
IC87114
D816V
D816V
LY294002
D816V
IC87114
D
Ba/F3
wt c-Kit
D816V
SCF
LY294002
IC87114
IB: pY
c-Kit
c-Kit
IB: c-Kit
IB: p85α
IP: c-Kit
IB: pAkt
IB: Akt
IB: β-actin
TCL
no cytokine SCF
Relative phosphorylation of c-Kit
ns
*
ns
ns
Ba/F3
wt c-Kit
D816V
LY294002
IC87114

Figure 1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Ba/F3
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D816V
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p110δ siRNA
control siRNA
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IB: Erk
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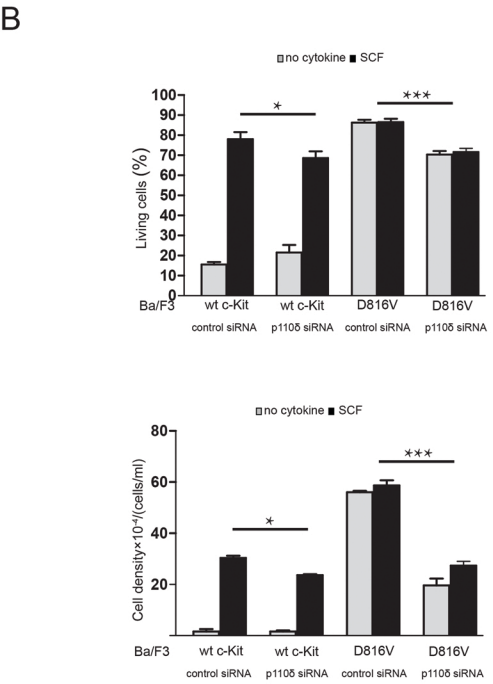
TCL


B
no cytokine ■ SCF
Living cells (%)
*
***
Ba/F3
wt c-Kit
wt c-Kit
D816V
D816V
control siRNA
p110δ siRNA
control siRNA
p110δ siRNA
no cytokine ■ SCF
Cell density×10⁻⁴/(cells/ml)
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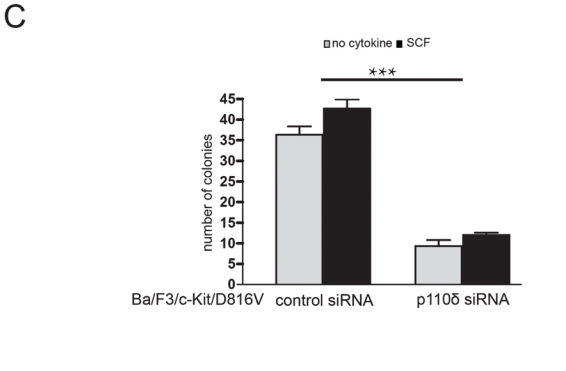
p110δ siRNA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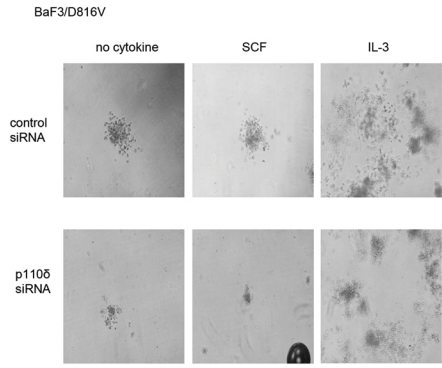
no cytokine ■ SCF
***
number of colonies
Ba/F3/c-Kit/D816V
control siRNA
p110δ siRNA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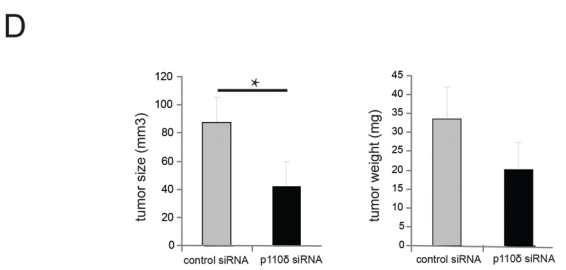
SCF
IL-3
control siRNA
p110δ siRNA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tumor size (mm3)
*
control siRNA
p110δ siRNA
tumor weight (mg)
control siRNA
p110δ siRNA

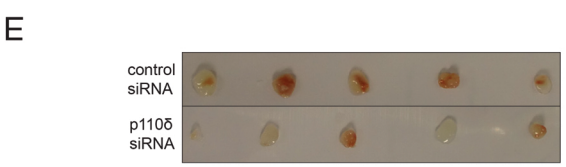
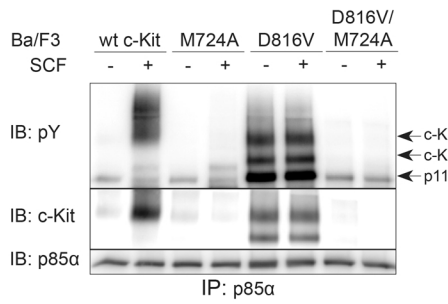

E
control siRNA
p110δ siRNA

Figure 2

A



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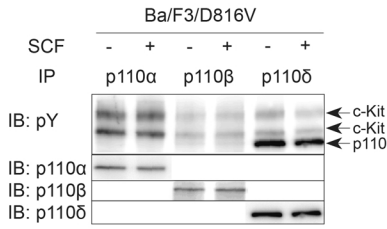

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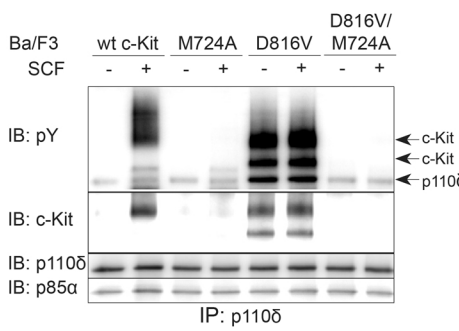

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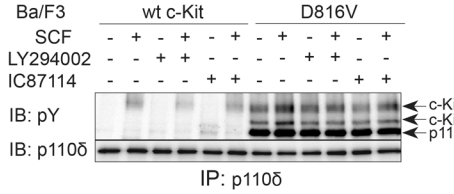

Figure 3

A

P110δ amino acid sequence

```
   1  MPPGVDCPME FWTKEESQSV VVDFLLPTGV YLNFPVSRNA NLSTIKQVLW
  51  HRAQYEPLFH MLSDPEAYVF TCVNQTAEQQ ELEDEQRRLC DIQPFLPVLR
 101  LVAREGDRVK KLINSQISLL IGKGLHEFDS LRDPEVNDFR TKMRQFCEEA
 151  AAHRQQLGWV EWLQYSFPLQ LEPSARGWRA GLLRVSNRAL LVNVKFEGSE
 201  ESFTFQVSTK DMPLALMACA LRKKATVFRQ PLVEQPEEYA LQVNGRHEYL
 251  YGNYPLCHFQ YICSCLHSGL TPHLTMVHSS SILAMRDEQS NPAPQVQKPR
 301  AKPPPIPAKK PSSVSLWSLE QPFSIELIEG RKVNADERMK LVVQAGLFHG
 351  NEMLCKTVSS SEVNVCSEPV WKQRLEFDIS VCDLPRMARL CFALYAVVEK
 401  AKKARSTKKK SKKADCPIAW ANLMLFDYKD QLKTGERCLY MWPSVPDEKG
 451  ELLNPAGTVR GNPNTESAAA LVIYLPEVAP HPVYFPALEK ILELGRHGER
 501  GRITEEEQLQ LREILERRGS GELYEHEKDL VWKMRHEVQE HFPEALARLL
 551  LVTKWNKHED VAQLSQMLYL LCSWPELPVL SALELLDFSF PDCVVGSFAI
 601  KSLRKLTDDE LFQYLLQLVQ VLKYESYLDC ELTKFLLGRA LANRKIGHFL
 651  FWHLRSEMHV PSVALRFGLI MEAYCRGSTH HMKVLMKQGE ALSKLKALND
 701  FVKVSSQKTT KPQTKEMMHM CMRQETYMEA LSHLQSPLDP STLLEEVCVE
 751  QCTFMDSKMK PLWIMYSSEE AGSAGNVGII FKNGDDLRQD MLTLQMIQLM
 801  DVLWKQEGLD LRMTPYGCLP TGDRTGLIEV VLHSDTIANI QLNKSNMAAT
 851  AAFNKDALLN WLKSKNPGEA LDRAIEEFTL SCAGYCVATY VLGIGDRHSD
 901  NIMIRESGQL FHIDFGHFLG NFKTKFGINR ERVPFILTYD FVHVIQQGKT
 951  NNSEKFERFR GYCERAYTIL RRHGLLFLHL FALMRAAGLP ELSCSKDIQY
1001  LKDSLALGKT EEEALKHFRV KFNEALRESW KTKVNWLAHN VSKDNRQ
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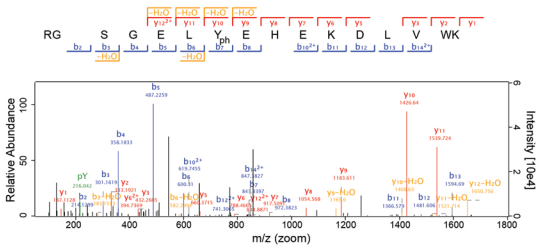
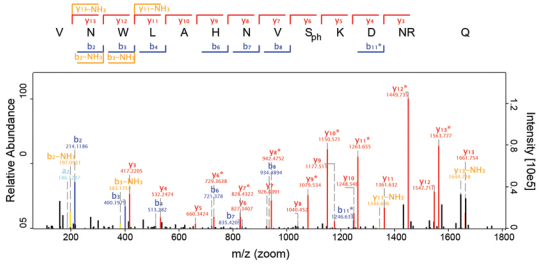
B

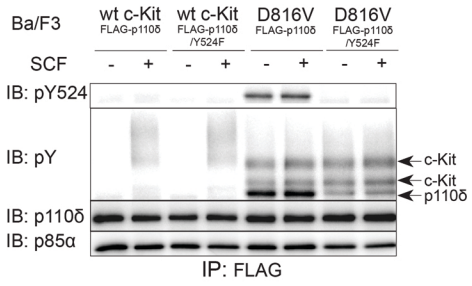
P110δ pY524 identification



P110δ pS1039 identification



C



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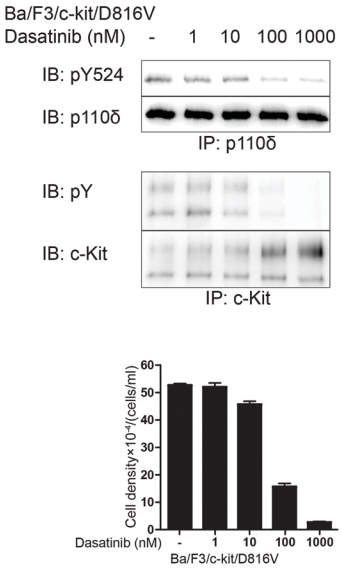
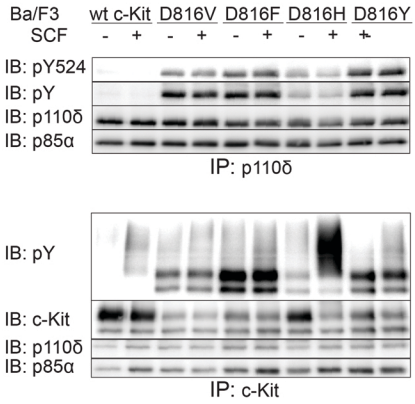


E

F



G

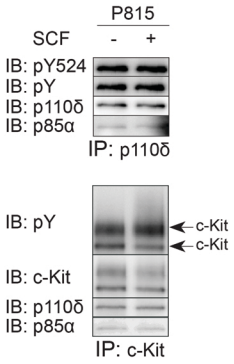


Figure 4

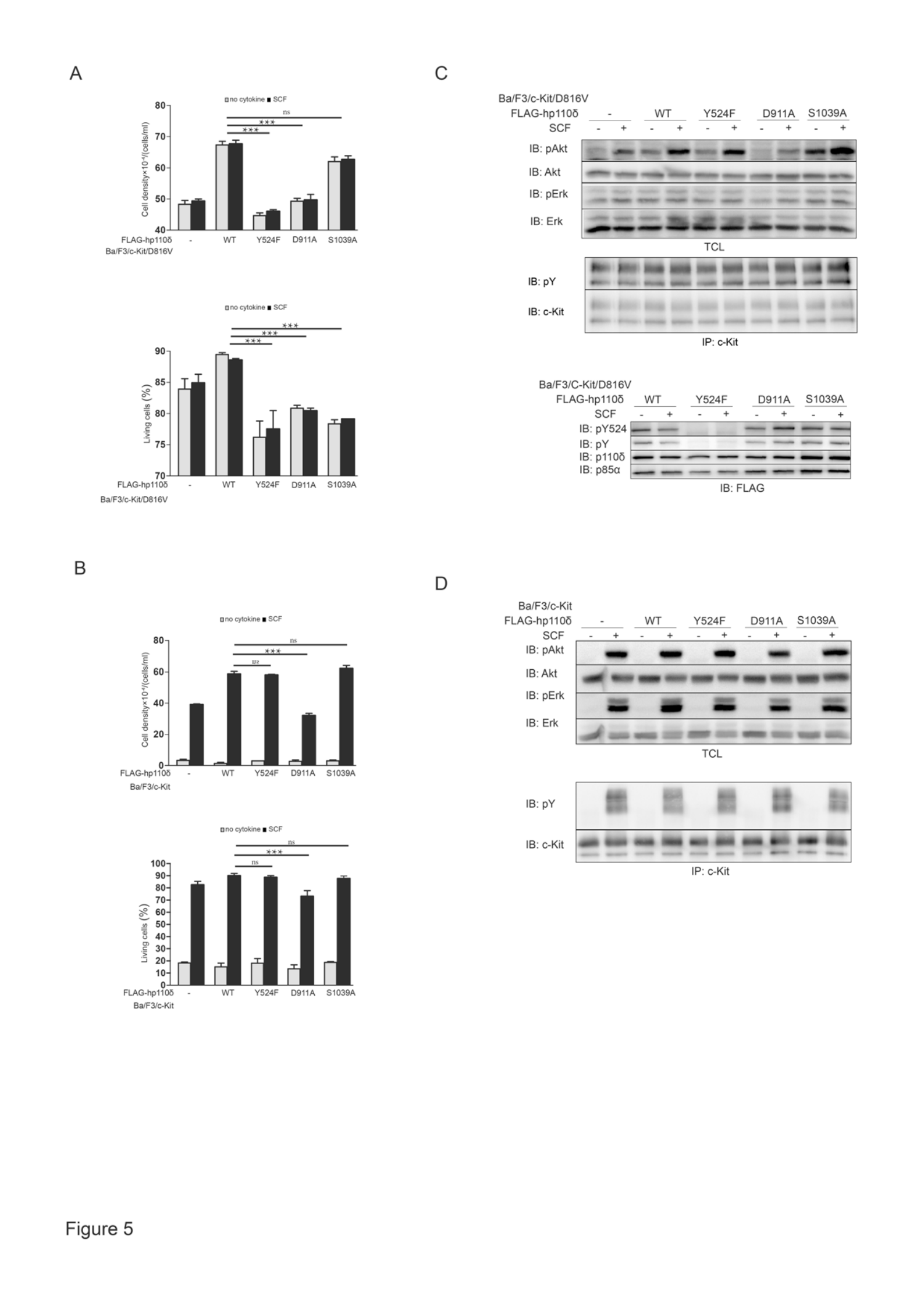

A
no cytokine SCF
Cell density×10⁻³/(cells/ml)
ns
***
***
***
FLAG-hp110δ
Ba/F3/c-Kit/D816V
-
WT
Y524F
D911A
S1039A
Living cells (%)
B
Ba/F3/c-Kit
C
Ba/F3/c-Kit/D816V
IB: pAkt
IB: Akt
IB: pErk
IB: Erk
TCL
IB: pY
IB: c-Kit
IP: c-Kit
Ba/F3/C-Kit/D816V
IB: pY524
IB: p110δ
IB: p85α
IB: FLAG
D
Ba/F3/c-Kit

Figure 5

Supplementary MS methods

In-gel digestion

Proteins in the excised band was in-gel digested with trypsin as previously described (1). Briefly, excised gel bands were minced, destained, reduced by DTT and alkylated with chloroacetamide. Proteins were extracted and digested with sequencing grade trypsin (Promega) overnight at room temperature. Tryptic activity was quenched by TFA acidification. Peptides were extracted by acetonitrile/water and desalted and concentrated on C_{18} STAGE tips (2).

Phosphopeptide enrichment

Phosphopeptides were enriched using titanium dioxide beads (10µm titansphere, GL Sciences, Japan) pre-coated with 2,5-dihydroxybenzoic acid (2,5-DHB). 1mg beads were suspended in 5µl 2,5-DHB (0.02g DHB/ml, 80% acetonitrile (ACN), 0.5% acetic acid (AcOH) and added to each sample, which were then incubated with gentle rotation for 15min (30rpm). The beads were washed with 100µl 5mM KH_2PO_4, 30% ACN, 350mM KCl followed by 100µl 40% ACN, 0.5% AcOH, 0.05% TFA and then re-suspended in 50µl 80% ACN, 0.5% AcOH. The resuspended beads were loaded onto preconditioned in-house packed C_8 STAGE tips, washed with 80% ACN, 0.5% AcOH, eluted with 2x10µl 5% ammonia and 2x10µl 10% ammonia, 25%ACN. Ammonia and organic solvents were evaporated using a vacuum centrifuge. Peptides were acidified in 1% TFA, 5% ACN and loaded onto in-house packed C_{18} STAGE tips, preconditioned with 20µl MeOH, 20µl 80% ACN, 0.5% AcOH, 2x20µl 1% TFA, 3% ACN. Following loading, the STAGE tips were washed with 2x20µl 8% ACN, 0.5% AcOH, and 1x50µl 0.5% AcOH.

LC-MS/MS

Peptides were eluted with 2x10µl 40% MeCN, 0.5% AcOH, organic solvents were removed in a vacuum centrifuge. Peptides were reconstituted in 2% MeCN, 0.5% AcOH, 0.1% TFA and analyzed by online reversed-phase C_{18} nanoscale liquid chromatography tandem mass spectrometry. The experiments were performed on an EASY-nLC™ system (Proxeon Biosystems, Odense, Denmark) connected to the LTQ-Orbitrap Velos (Thermo Electron, Bremen, Germany) through a nano-electrospray ion source, essentially as described previously (REF: Olsen et al, Mol Cell Proteomics 2009). Briefly, the peptide mixtures were separated in a 15 cm analytical column (75 µm inner diameter) in-house packed with 3µm C_{18} beads (Reprosil-AQ Pur, Dr. Maisch) with a 90 min gradient from 5% to 30% ACN in 0.5% AcOH. The effluent from the HPLC was directly electrosprayed into the mass spectrometer. The MS instrument was operated in data-dependent mode to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 300 – 1500) were acquired in the orbitrap with resolution R=60K at m/z 400 after accumulation to a 'target value' of 1e6 in the linear ion trap. The ten most intense peptide ions with charge states ≥ 2 were sequentially isolated to a target value of 5e4 and fragmented with Higher-energy Collisional Dissociation (HCD) (3) in the octopole collision cell and analyzed in the orbitrap with a resolution of 7,500. For all full scan measurements with the orbitrap detector a lock-mass ion from ambient air (m/z 445.120024) was used as an internal calibrant as described (4). Typical mass spectrometric conditions were: spray voltage, 2.1 kV; no sheath and auxiliary gas flow; heated capillary temperature, 275ºC; normalized collision energy 40%

for HCD. The ion selection threshold was 5000 counts for HCD and the maximum allowed ion time were 500 ms for full scans and 250 ms HCD-MS/MS.

Peptide identification by MASCOT and MaxQuant

Acquired data was processed by MaxQuant 1.0.14.7 as described (5). MaxQuant determined the accurate precursor masses for all peptide peaks using the entire LC elution profiles and MS/MS spectra were merged into peak-list files (*.msm). Peptides and proteins were identified by Mascot (Matrix Science, London, UK) via automated database matching of all tandem mass spectra against an in-house curated concatenated target/decoy database; a forward and reversed version of the International Protein Index (IPI) sequence database (version 3.37; 138,632 forward and reversed protein sequences from EBI (http://www.ebi.ac.uk/IPI/)) supplemented with common contaminants such as human keratins, bovine serum proteins and porcine trypsin. Tandem mass spectra were initially matched with a mass tolerance of 7 ppm on precursor masses and 0.02 Da for fragment ions, and strict trypsin specificity and allowing for up to 3 missed tryptic cleavage sites. Cysteine carbamidomethylation (Cys +57.021464 Da) was searched as a fixed modification, whereas N-acetylation of protein (N-term +42.010565 Da), N-pyro-glutamine (Gln –17.026549), oxidized methionine (+15.994915 Da) and phosphorylation of serine, threonine and tyrosine (Ser/Thr/Tyr +79.966331 Da) were searched as variable modifications.

Peptide filtering and phosphosite localization

The resulting Mascot result files (*.dat) were loaded into the MaxQuant software suite for further processing. In MaxQuant we fixed the estimated false discovery rate (FDR) of all

peptide and protein identifications at 1%, by automatically filtering on peptide length, mass error precision estimates and Mascot score of all forward and reversed peptide identifications (6). Finally, to pinpoint the actual phosphorylated amino acid residue(s) within all identified phosphopeptide sequences in an unbiased manner, MaxQuant calculated the localization probabilities of all putative serine, threonine and tyrosine phosphorylation sites using the PTM score algorithm as described (7).

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