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

3
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15
16 **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 p110 δ 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 p110 δ plays a lipid-kinase-independent role in c-Kit/D816V-mediated cell transformation. We furthermore demonstrate that p110 δ is phosphorylated at residues Y524 and S1039 and that phosphorylation requires an intact binding site for PI3-kinase in c-Kit/D816V. Overexpression of p110 δ 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 p110 δ in c-Kit/D816V-mediated cell transformation. This furthermore suggests that p110 δ 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, p110 δ , phosphorylation, cell transformation

54 **Introduction**

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

62 PI3-kinase is a lipid kinase that can phosphorylate the 3'-hydroxyl group of
63 phosphoinositides. The lipid products of PI3-kinase act as second messengers activating
64 downstream signaling cascades that mediate multiple cellular responses such as cell
65 survival, proliferation and differentiation. Based on the structure and substrate specificity,
66 PI3-kinase can be divided into three groups: class I, II and III. Among them, the class I
67 PI3-kinases are the most studied. Based on their activation mechanisms, class I PI3-
68 kinases are further divided into class IA and class IB. Class IA PI3-kinase is composed of
69 a regulatory subunit and a catalytic subunit that are constitutively associated (3). To date,
70 p85 α , p55 α , p50 α , p85 β and p55 γ have been identified as regulatory subunits and p110 α ,
71 p110 β and p110 δ as catalytic subunits of the class IA PI3-kinases. P110 α and p110 β are
72 broadly expressed across tissues whereas p110 δ is mainly found in lymphocytes (4) and
73 to a lesser extent expressed in neurons (5). In addition, p110 δ has also been detected in
74 various cancer forms including lung cancer, neuroblastoma, breast cancer and melanoma
75 (6-8). The only member of class IB PI3-kinases that does not bind to p85 is p110 γ (9).

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

97 PI3-kinase plays a key role in transformation mediated by the D816V mutant of c-Kit
98 (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 p110 δ at Y524 and S1039. We furthermore demonstrate that this phosphorylation of p110 δ can promote cell transformation independent of the lipid kinase activity. These results suggest that tyrosine phosphorylation of p110 δ 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 p110 δ -selective inhibitor IC87114 (since p110 δ 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.

P110 δ plays a key role in c-Kit/D816V mediated cell transformation

Since p110 δ is the predominant isoform of class I PI3-kinase in hematopoietic cells, we sought to investigate the role of p110 δ in c-Kit/D816V-induced cell transformation. To this end we used siRNA-mediated knockdown of p110 δ . We could demonstrate that knockdown of p110 δ 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 p110 δ led to the formation of far fewer as well as smaller colonies compared to control siRNA (Fig. 2C). These results clearly demonstrate that p110 δ is important for c-Kit/D816V-mediated cell transformation. To further test if the effect of p110 δ knockdown on cell transformation was maintained *in vivo*, cells pre-treated with p110 δ 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 p110 δ were much smaller than tumors formed from control cells. In addition, knockdown of p110 δ negatively affected tumor weight (Fig. 2D). There was also a clear difference in tumor vascularization as judged by ocular observation, where knockdown of p110 δ led to tumors with markedly reduced blood vessel formation (Fig. 2E). Collectively, these results suggest an important role for p110 δ in c-Kit/D816V-mediated tumor formation.

C-Kit/D816V induces tyrosine phosphorylation of p110 δ independent of its lipid kinase activity

To further identify how PI3-kinase contributes to c-Kit/D816V mediated cell transformation, the regulatory subunit p85 α of PI3-kinase was immunoprecipitated and probed with antibodies against phosphotyrosine and c-Kit, respectively. As expected, the binding of p85 α 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 p85 α 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, p85 α 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 p110 δ subunit of PI3-kinase. The three known isoforms of the catalytic subunit of class IA PI3-kinase, p110 α , p110 β and p110 δ , all have a molecular weight of 110 kDa, and are known to associate with p85. To investigate whether p110 α and p110 β are also phosphorylated by c-Kit/D816V, immunoprecipitation with antibodies against p110 α , p110 β or p110 δ was performed. Probing with a phosphotyrosine antibody demonstrated that only p110 δ 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 p110 δ , indicating that the phosphorylation is independent of the lipid kinase activity of PI3-kinase (Fig. 3D).

P110 δ is phosphorylated by c-Kit/D816V at Y524 and S1039

In order to identify the tyrosine phosphorylation site(s) in p110 δ , cell lysates of c-Kit/D816V expressing Ba/F3 cells were subjected to immunoprecipitation with a p110 δ antibody and the 110 kDa band was excised and analyzed by mass spectrometry. The identity of the protein as p110 δ 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, p110 δ was FLAG-tagged and the Y524F mutation was introduced. This construct was transfected into Ba/F3/c-Kit or Ba/F3/c-

191 Kit/D816V cells. Immunoprecipitation with a FLAG antibody followed by
192 immunoblotting with a phospho-specific antibody against pY524 in p110 δ , revealed that
193 Y524 is a phosphorylation site induced by the c-Kit/D816V but not by ligand-stimulated
194 wild-type c-Kit (Fig. 4C). Furthermore, phosphorylation of Y524 can be inhibited by the
195 Kit/D816V inhibitor Dasatinib at similar concentrations that lead to inhibition of
196 phosphorylation of c-Kit as well to inhibition of proliferation (Fig. 4D). The other two
197 isoforms of class IA PI3 kinases p110 α and p110 β can't get phosphorylated by c-
198 Kit/D816V (Fig. 3B), alignment of p110 α , p110 β and p110 δ showed that p110 α and
199 p110 β have no tyrosine residue that is identical to Y524 of p110 δ (Fig. 4E), which could
200 explain why p110 δ is the only class IA PI3 kinase that is phosphorylated by c-
201 Kit/D816V. We have previously demonstrated that the D816V mutation of c-Kit not only
202 activates the kinase activity but also leads to altered substrate specificity. The substrate
203 specificity of c-Kit/D816V is similar to the specificity of Src and Abl kinases (22). The
204 amino acid sequencing surrounding Y524 in p110 δ is characterized by the presence of an
205 acidic amino acid C-terminal to the tyrosine residue (YEHEKDL) which is known to
206 characterize tyrosine residues phosphorylated by Src. Furthermore, a query of the
207 sequence in PhosphoMotif Finder (<http://www.hprd.org>) revealed that the tyrosine
208 residue is a predicted Src substrate. These results indicate that the D816V mutation of c-
209 Kit gains a function not possessed by the wild-type receptor allowing it to phosphorylate
210 p110 δ .

211 Apart from D816V, which is the most common activating c-Kit mutation found in human
212 malignancies, several other mutations at aspartic acid 816 have been described in the
213 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 p110 δ was phosphorylated at Y524 by all three mutants (Fig. 4F). The difference in the intensity of p110 δ 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 p110 δ is phosphorylated at Y524 in P815 cells as well (Fig. 4G).

Phosphorylation of p110 δ 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 p110 δ plays a role in c-Kit/D816V mediated cell transformation independent of the lipid kinase activity, wild-type p110 δ , mutants that block the phosphorylation of p110 δ (Y524F and S1039A) and the kinase dead mutant D911A of p110 δ 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 p110 δ displayed a significantly lower cell proliferation and survival compared to cells expressing wild-type p110 δ (Fig. 5A) although the phosphorylation of Akt remained intact (Fig. 5C). The kinase dead mutant of p110 δ 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 p110 δ , 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 p110 δ did not block phosphorylation of p110 δ at Tyr 524 (Fig. 5C), which confirms that the tyrosine phosphorylation of p110 δ 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, p110 δ 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 p110 δ 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 p110 α and p110 β , 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 p110 δ 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 p110 α and amplification of p110 β have been found in tumors. Mutations of p110 α lead to increased catalytic activity, and to growth factor-independent cell growth and cell transformation (28). Studies on p110 δ in AML patients have failed to detect any activating mutations (29). In this study, we present data showing a totally new function of p110 δ which is independent of its lipid kinase activity. In contrast to p110 α mutations and p110 β amplification that leads to higher lipid kinase activity of PI3-kinase and thereby contributes to transformation, the c-Kit/D816V phosphorylates p110 δ 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

that utilizes PI3-kinase in a manner independent of its lipid kinase activity. Currently, targeting PI3-kinase therapy focuses on the inhibition of its lipid kinase activity. From our results, we conclude that it will be necessary to block both the lipid kinase-dependent and -independent signal transduction to get a good effect on c-Kit/D816V-expressing malignancies. Phosphorylation at serine residues residing in carboxyterminus of the catalytic subunits p110 β and p110 δ (S1070 and S1039, respectively) have been reported and was found to inhibit the lipid kinase activity of PI3-kinase (30, 31). Our results confirmed the inhibitory function of phosphorylation of S1039 in p110 δ . In addition, blockage of S1039 phosphorylation led to a decreased cell survival although the lipid kinase activity of PI3-kinase was increased, suggesting that phosphorylation of S1039 might have additional functions.

Gain-of-function mutations of c-Kit in cancer have been widely studied. These include in-frame deletions, insertions and substitutions that through different mechanisms lead to autoactivation. Such mutations have been identified in patients with GISTs, AML, mastocytosis and testicular seminomas. 70–80% of GISTs carry an activating c-Kit mutation that renders the receptor constitutively active, thus making c-Kit a clinically important therapeutic target in GISTs (32). Most c-Kit mutations in GISTs are mapped to exon 11, which disrupts the normal interaction between the juxtamembrane domain and the kinase domain leading to activation of the receptor kinase activity (33). More than 80% of mastocytosis patients have a mutation in exon17 that affects aspartic acid 816 in the second part of the kinase domain of c-Kit, with D816V being the most frequently identified mutation (34). Some AML patients also carry c-Kit/D816V (35). The reason why GISTs usually carry mutations in exon 11 and the hematologic malignancies usually

306 carry exon 17 mutations is not known. One possibility might be that the exon17 mutants,
307 such as c-Kit/D816V depend on p110 δ for transformation and since p110 δ is
308 predominantly expressed in hematopoietic cells, this might be at least part of the
309 explanation.

310 The constitutively active mutants of c-Kit have been successfully targeted with selective
311 tyrosine kinase inhibitors. In particular, imatinib has been successful used for the
312 treatment of cancers carrying certain mutants of c-Kit, in particular the exon 11 mutants
313 commonly found in GISTs. However, other mutants of c-Kit such as D816V are resistant
314 to imatinib. Since the advent of imatinib, numerous tyrosine kinase inhibitors have been
315 developed and found to inhibit also the imatinib-resistant mutants of c-Kit, such as
316 D816V. These molecules include dasatinib and nilotinib. However, despite the promising
317 results in the laboratory setting, none of these molecules have given any beneficial
318 response when given to patients (36, 37). Thus, there is a need to find novel therapeutic
319 target for the treatment of these patients. Ideally, a signal transduction molecule that is
320 activated solely by the oncogenic mutant of c-Kit but not by wild-type c-Kit would be a
321 suitable target. This would potentially minimize the risk for unwanted side effect due to
322 inhibition of normal, physiological signaling. In this study, our data suggest that the
323 phosphorylation of p110 δ might be a good target for treatment of c-Kit/D816V-
324 expressing malignancies, since this phosphorylation does not occur in normal cells.
325 Combination treatment against several targets has in many cases improved the outcome
326 of treatment dramatically and it can also avoid development of drug resistance and
327 overcome unresponsiveness (38, 39). Resistance to tyrosine kinase inhibitors could be
328 overcome by targeting multiple levels of the signal transduction cascades. Thus, targeting

p110 δ 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 p110 δ .

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 p110 δ was generated by immunizing a rabbit with the peptide CWLAHNVSKDNRQ conjugated to KLH. Antibodies recognizing pY524 of p110 δ 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, p110 α , p110 β were from Santa Cruz Biotechnology (Santa Cruz, CA). P85 α 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 p110 δ 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 Na₃VO₄ 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 Anova.

397

398 **SiRNA transfection**

399 5 million Ba/F3 cells were washed once with RPMI 1640, mix with 3 μ g siRNA and
400 transfected by 4D-nucleofector (Lonza), program CM150, solution SG.

401

402 **Colony assay**

403 Ba/F3 cells were washed 3 times with PBS, resuspended in IMDM medium, mix with
404 semi-solid colony assay medium MethoCult® M3231 (Stem cell technologies) according
405 to the manufacturer's instruction. Cell mixture was seeded in 24-well plates with either
406 100 ng/ml SCF, 10 ng/ml IL-3 or no cytokine as control. After 4 days incubation,
407 colonies were counted and photographed. Statistical significance was calculated by *t* test.

408

409 **Animal experiment**

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

418

419 **Immunoprecipitation of p110 δ 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 p110 δ 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 p110 δ 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 (10 μ m 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

References

1. Lennartsson J, Rönstrand L. Stem Cell Factor Receptor/c-Kit: From Basic Science to Clinical Implications. *Physiol Rev.* 2012 Oct;92(4):1619-49.
2. Serve H, Yee NS, Stella G, Sepp-Lorenzino L, Tan JC, Besmer P. Differential roles of PI3-kinase and Kit tyrosine 821 in Kit receptor-mediated proliferation, survival and cell adhesion in mast cells. *Embo J.* 1995 Feb 1;14(3):473-83.
3. Jia S, Roberts TM, Zhao JJ. Should individual PI3 kinase isoforms be targeted in cancer? *Curr Opin Cell Biol.* 2009 Apr;21(2):199-20.
4. Vanhaesebroeck B, Welham MJ, Kotani K, Stein R, Warne PH, Zvelebil MJ, et al. P110delta, a novel phosphoinositide 3-kinase in leukocytes. *Proc Natl Acad Sci U S A.* 1997 Apr 29;94(9):4330-5.
5. Eickholt BJ, Ahmed AI, Davies M, Papakonstanti EA, Pearce W, Starkey ML, et al. Control of axonal growth and regeneration of sensory neurons by the p110delta PI 3-kinase. *PLoS One.* 2007;2(9):e869.

- 464 6. Sawyer C, Sturge J, Bennett DC, O'Hare MJ, Allen WE, Bain J, et al. Regulation
465 of breast cancer cell chemotaxis by the phosphoinositide 3-kinase p110delta. *Cancer Res.*
466 2003 Apr 1;63(7):1667-75.
- 467 7. Arcaro A, Khanzada UK, Vanhaesebroeck B, Tetley TD, Waterfield MD, Seckl
468 MJ. Two distinct phosphoinositide 3-kinases mediate polypeptide growth factor-
469 stimulated PKB activation. *Embo J.* 2002 Oct 1;21(19):5097-108.
- 470 8. Boller D, Schramm A, Doepfner KT, Shalaby T, von Bueren AO, Eggert A, et al.
471 Targeting the phosphoinositide 3-kinase isoform p110delta impairs growth and survival
472 in neuroblastoma cells. *Clin Cancer Res.* 2008 Feb 15;14(4):1172-81.
- 473 9. Stoyanov B, Volinia S, Hanck T, Rubio I, Loubtchenkov M, Malek D, et al.
474 Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase.
475 *Science.* 1995 Aug 4;269(5224):690-3.
- 476 10. Ning ZQ, Li J, Arceci RJ. Activating mutations of c-kit at codon 816 confer drug
477 resistance in human leukemia cells. *Leuk Lymphoma.* 2001 May;41(5-6):513-22.
- 478 11. Beghini A, Peterlongo P, Ripamonti CB, Larizza L, Cairoli R, Morra E, et al. C-
479 kit mutations in core binding factor leukemias. *Blood.* 2000 Jan 15;95(2):726-7.
- 480 12. Tian Q, Frierson HF, Jr., Krystal GW, Moskaluk CA. Activating c-kit gene
481 mutations in human germ cell tumors. *Am J Pathol.* 1999 Jun;154(6):1643-7.
- 482 13. Longley BJ, Tyrrell L, Lu SZ, Ma YS, Langley K, Ding TG, et al. Somatic c-KIT
483 activating mutation in urticaria pigmentosa and aggressive mastocytosis: establishment of
484 clonality in a human mast cell neoplasm. *Nat Genet.* 1996 Mar;12(3):312-4.
- 485 14. Sakuma Y, Sakurai S, Oguni S, Satoh M, Hironaka M, Saito K. c-kit gene
486 mutations in intracranial germinomas. *Cancer Sci.* 2004 Sep;95(9):716-20.

- 487 15. Hongyo T, Li T, Syaifudin M, Baskar R, Ikeda H, Kanakura Y, et al. Specific c-
488 kit mutations in sinonasal natural killer/T-cell lymphoma in China and Japan. *Cancer*
489 *Res.* 2000 May 1;60(9):2345-7.
- 490 16. Orfao A, Garcia-Montero AC, Sanchez L, Escribano L. Recent advances in the
491 understanding of mastocytosis: the role of KIT mutations. *Br J Haematol.* 2007
492 Jul;138(1):12-30.
- 493 17. Schittenhelm MM, Shiraga S, Schroeder A, Corbin AS, Griffith D, Lee FY, et al.
494 Dasatinib (BMS-354825), a dual SRC/ABL kinase inhibitor, inhibits the kinase activity
495 of wild-type, juxtamembrane, and activation loop mutant KIT isoforms associated with
496 human malignancies. *Cancer Res.* 2006 Jan 1;66(1):473-81.
- 497 18. Hantschel O, Rix U, Superti-Furga G. Target spectrum of the BCR-ABL
498 inhibitors imatinib, nilotinib and dasatinib. *Leuk Lymphoma.* 2008 Apr;49(4):615-9.
- 499 19. Hashimoto K, Matsumura I, Tsujimura T, Kim DK, Ogihara H, Ikeda H, et al.
500 Necessity of tyrosine 719 and phosphatidylinositol 3'-kinase-mediated signal pathway in
501 constitutive activation and oncogenic potential of c-kit receptor tyrosine kinase with the
502 Asp814Val mutation. *Blood.* 2003 Feb 1;101(3):1094-102.
- 503 20. Backer JM, Myers MG, Jr., Shoelson SE, Chin DJ, Sun XJ, Miralpeix M, et al.
504 Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin
505 stimulation. *Embo J.* 1992 Sep;11(9):3469-79.
- 506 21. Sun J, Pedersen M, Rönnstrand L. Gab2 is involved in differential
507 phosphoinositide 3-kinase signaling by two splice forms of c-Kit. *J Biol Chem.* 2008 Oct
508 10;283(41):27444-51.

509 22. Sun J, Pedersen M, Rönnstrand L. The D816V mutation of c-Kit circumvents a
510 requirement for Src family kinases in c-Kit signal transduction. *J Biol Chem*. 2009 Apr
511 24;284(17):11039-47.

512 23. Longley BJ, Jr., Metcalfe DD, Tharp M, Wang X, Tyrrell L, Lu SZ, et al.
513 Activating and dominant inactivating c-KIT catalytic domain mutations in distinct
514 clinical forms of human mastocytosis. *Proc Natl Acad Sci U S A*. 1999 Feb
515 16;96(4):1609-14.

516 24. Pardanani A. Systemic mastocytosis in adults: 2012 Update on diagnosis, risk
517 stratification, and management. *Am J Hematol*. 2012 Apr;87(4):401-11.

518 25. Chian R, Young S, Danilkovitch-Miagkova A, Rönnstrand L, Leonard E, Ferrao
519 P, et al. Phosphatidylinositol 3 kinase contributes to the transformation of hematopoietic
520 cells by the D816V c-Kit mutant. *Blood*. 2001 Sep 1;98(5):1365-73.

521 26. Harir N, Boudot C, Friedbichler K, Sonneck K, Kondo R, Martin-Lannerée S, et
522 al. Oncogenic Kit controls neoplastic mast cell growth through a Stat5/PI3-kinase
523 signaling cascade. *Blood*. 2008 Sep 15;112(6):2463-73.

524 27. Ogita S, Lorusso P. Targeting phosphatidylinositol 3 kinase (PI3K)-Akt beyond
525 rapalogs. *Target Oncol*. 2011 Jun;6(2):103-17.

526 28. Miller TW, Rexer BN, Garrett JT, Arteaga CL. Mutations in the
527 phosphatidylinositol 3-kinase pathway: role in tumor progression and therapeutic
528 implications in breast cancer. *Breast Cancer Res*. 2011;13(6):224.

529 29. Cornillet-Lefebvre P, Cuccuini W, Bardet V, Tamburini J, Gillot L, Ifrah N, et al.
530 Constitutive phosphoinositide 3-kinase activation in acute myeloid leukemia is not due to
531 p110delta mutations. *Leukemia*. 2006 Feb;20(2):374-6.

- 532 30. Czupalla C, Culo M, Müller EC, Brock C, Reusch HP, Spicher K, et al.
533 Identification and characterization of the autophosphorylation sites of phosphoinositide 3-
534 kinase isoforms beta and gamma. *J Biol Chem*. 2003 Mar 28;278(13):11536-45.
- 535 31. Vanhaesebroeck B, Higashi K, Raven C, Welham M, Anderson S, Brennan P, et
536 al. Autophosphorylation of p110delta phosphoinositide 3-kinase: a new paradigm for the
537 regulation of lipid kinases in vitro and in vivo. *Embo J*. 1999 Mar 1;18(5):1292-302.
- 538 32. Corless CL, Barnett CM, Heinrich MC. Gastrointestinal stromal tumours: origin
539 and molecular oncology. *Nat Rev Cancer*. 2011 Dec;11(12):865-78.
- 540 33. Mol CD, Dougan DR, Schneider TR, Skene RJ, Kraus ML, Scheibe DN, et al.
541 Structural basis for the autoinhibition and STI-571 inhibition of c-Kit tyrosine kinase. *J*
542 *Biol Chem*. 2004 Jul 23;279(30):31655-63.
- 543 34. Sadrzadeh H, Abdel-Wahab O, Fathi AT. Molecular alterations underlying
544 eosinophilic and mast cell malignancies. *Discov Med*. 2011 Dec;12(67):481-93.
- 545 35. Boissel N, Leroy H, Brethon B, Philippe N, de Botton S, Auvrignon A, et al.
546 Incidence and prognostic impact of c-Kit, FLT3, and Ras gene mutations in core binding
547 factor acute myeloid leukemia (CBF-AML). *Leukemia*. 2006 Jun;20(6):965-70.
- 548 36. Aichberger KJ, Sperr WR, Gleixner KV, Kretschmer A, Valent P. Treatment
549 responses to cladribine and dasatinib in rapidly progressing aggressive mastocytosis. *Eur*
550 *J Clin Invest*. 2008 Nov;38(11):869-73.
- 551 37. Verstovsek S, Tefferi A, Cortes J, O'Brien S, Garcia-Manero G, Pardanani A, et
552 al. Phase II study of dasatinib in Philadelphia chromosome-negative acute and chronic
553 myeloid diseases, including systemic mastocytosis. *Clin Cancer Res*. 2008 Jun
554 15;14(12):3906-15.

38. Cohen SM, Mukerji R, Timmermann BN, Samadi AK, Cohen MS. A novel combination of withaferin A and sorafenib shows synergistic efficacy against both papillary and anaplastic thyroid cancers. *American journal of surgery*. 2012 Dec;204(6):895-901.
39. Nijenhuis CM, Haanen JB, Schellens JH, Beijnen JH. Is combination therapy the next step to overcome resistance and reduce toxicities in melanoma? *Cancer Treat Rev*. 2013 Jun;39(4):305-12.
40. Blume-Jensen P, Siegbahn A, Stabel S, Heldin CH, Rönstrand L. Increased Kit/SCF receptor induced mitogenicity but abolished cell motility after inhibition of protein kinase C. *Embo J*. 1993 Nov;12(11):4199-209.
41. Lundby A, Olsen JV. GeLCMS for in-depth protein characterization and advanced analysis of proteomes. *Methods Mol Biol*. 2011;753:143-55.
42. Olsen JV, Schwartz JC, Griep-Raming J, Nielsen ML, Damoc E, Denisov E, et al. A dual pressure linear ion trap Orbitrap instrument with very high sequencing speed. *Mol Cell Proteomics*. 2009 Dec;8(12):2759-69.
43. Cox J, Matic I, Hilger M, Nagaraj N, Selbach M, Olsen JV, et al. A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nat Protoc*. 2009;4(5):698-705.

Figure legends

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 μ M), or p110 δ inhibitor IC87114 (10 μ M) or no inhibitor 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. * $P < 0.05$, *** $P < 0.001$, ns: no significance.

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 μ M) or p110 δ inhibitor IC87114 (10 μ M) 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 c-Kit was probed with pY antibody to evaluate the phosphorylation of c-Kit. Signals of pY blot were quantified and normalized by c-Kit blot to decide the relative phosphorylation of c-Kit. * $P < 0.05$, ns: no significance.

Figure 2. P110 δ plays a key role in c-Kit/D816V mediated cell transformation

A. 5 million Ba/F3/c-Kit and Ba/F3/c-Kit/D816V cells were transfected with 3 μ g p110 δ siRNA by 4D-nucleofector (Lonza), program CM150, solution SG. After 48 hours, cells were washed and starved as above, followed by stimulation with SCF for 2 minutes, and lysed in a lysis buffer. The total cell lysates were probed with Akt, pAkt, Erk and pErk antibodies. Knockdown of p110 δ expression was assessed by probing with p110 δ antibody.

B. Ba/F3/c-Kit and Ba/F3/c-Kit/D816V cells were transfected with control siRNA or p110 δ 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 p110 δ 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 p110 δ siRNA as above. After 24 hours, 6 million cells were washed once with PBS and collected in 100 μ l 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 p110 δ subunit of PI3-kinase is tyrosine-phosphorylated by c-Kit/D816V

644 **A.** Ba/F3/c-Kit, Ba/F3/c-Kit/M724A, Ba/F3/c-Kit/D816V and Ba/F3/c-
645 Kit/D816V/M724A cells were starved for 4 hours in medium without serum and IL-3
646 followed by SCF stimulation (100 ng/ml) for 2 minutes. Cells were washed once in ice-
647 cold PBS and lysed in the lysis buffer. P85 α was immunoprecipitated from total cell
648 lysate and the precipitate was probed with pY, c-Kit and p85 α antibody respectively.

649
650 **B.** Ba/F3/c-Kit/D816V cells were starved and stimulated as above and
651 immunoprecipitated with p110 α , p110 β or p110 δ antibody respectively and probed with
652 pY antibody.

653
654 **C.** Ba/F3/c-Kit, Ba/F3/c-Kit/M724A, Ba/F3/c-Kit/D816V and Ba/F3/c-
655 Kit/D816V/M724A cells were starved and stimulated as above and p110 δ was
656 immunoprecipitated. The precipitate was probed with pY, c-Kit, p110 δ and p85 α
657 antibody respectively.

658
659 **D.** Ba/F3/c-Kit and Ba/F3/c-Kit/D816V cells were starved for 4 hours in medium without
660 serum and IL-3, incubated with general PI3-kinases inhibitor LY294002 (10 μ M) or
661 p110 δ inhibitor IC87114 (10 μ M) at the same time, followed by SCF stimulation
662 (100 ng/ml) for 2 minutes. Total cell lysate was probed with pAkt antibody to test if the
663 inhibitors affect the downstream signaling of PI3-kinases. Immunoprecipitated p110 δ was
664 probed with pY antibody to evaluate the phosphorylation of p110 δ .

665
666 **Figure 4. P110 δ becomes phosphorylated at Y524 and S1039 by c-Kit/D816V.**

667 **A.** Tandem mass spectrometry identified the protein of the 110KD band as p110 δ . The
668 amino acid sequence of the protein is shown, and in red are highlighted peptides covered
669 by the mass spectrometry experiments. A total of 114 unique peptides aligning with
670 p110 δ were identified, and 81% of the amino acid sequence was covered in the
671 experiment, thereby accurately determining the protein as p110 δ .

672

673 **B.** Tyrosine 524 and serine 1039 of p110 δ was identified to be phosphorylated. Identified
674 peptides covering Y524 and S1039 are shown with the detected fragment ions indicated.
675 The fragment ions are highlighted in the MS/MS spectrum. Each peptide contained one
676 phosphate group, and due to the fragmentation pattern the localization of the
677 phosphorylation site could be localized to tyrosine 524 and serine 1039.

678

679 **C.** Ba/F3/c-Kit/FLAG-p110 δ , Ba/F3/c-Kit/FLAG-p110 δ /Y524F, Ba/F3/c-Kit/D816V/
680 FLAG-p110 δ and Ba/F3/c-Kit/D816V/FLAG-p110 δ /Y524F cells were starved,
681 stimulated with SCF for 2 minutes and lysed as above, and p110 δ was
682 immunoprecipitated with FLAG antibody, and probed with p110 δ pY524 antibody to test
683 the Y524 phosphorylation of transfected p110 δ .

684

685 **D.** Ba/F3/c-Kit/D816V cells were incubated with indicated concentration of Dasatinib
686 overnight, p110 δ and c-Kit were immunoprecipitated from the total cell lysates.
687 Immunoprecipitated proteins were separated by SDS-polyacrylamide electrophoresis,
688 electrotransferred to Immobilon filter and probed with antibodies against pY524, p110 δ ,

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 p110 α , p110 β and p110 δ showed that p110 α and p110 β have no tyrosine residue that is identical to Y524 of p110 δ .

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, p110 δ and c-Kit was immunoprecipitated respectively. The p110 δ precipitate was probed with pY524 antibody to evaluate the phosphorylation of p110 δ , 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 p110 δ or c-Kit was immunoprecipitated and probed with pY523, pY, c-Kit, p110 δ and p85 α antibodies respectively.

Figure 5. Phosphorylation of p110 δ at Y524 and S1039 contributes to c-Kit/D816V mediated cell survival and proliferation.

A. Ba/F3/c-Kit/D816V cells expressing FLAG tagged p110 δ 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

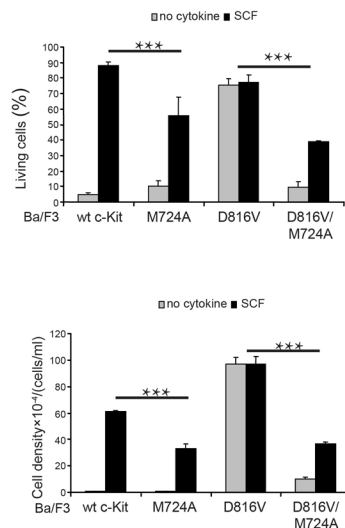
apoptotic cells were counted by flow cytometry. For the cell proliferation assay, living cells were counted under the microscope. *** $P < 0.001$, ns: no significance.

B. Ba/F3/c-Kit cells expressing FLAG tagged p110 δ 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.

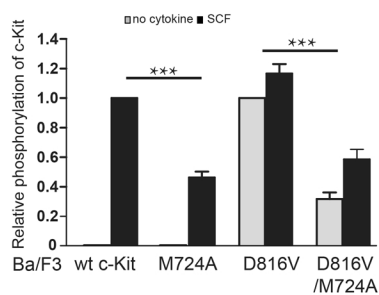
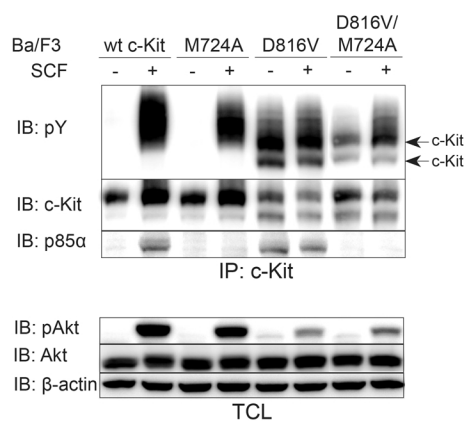
C. Ba/F3/c-Kit/D816V cells expressing FLAG tagged p110 δ were starved for 4 hours in medium without serum and IL-3 followed by SCF stimulation (100 ng/ml) for 2 minutes. Total cell lysate was probed with pAkt antibody, c-Kit or FLAG was immunoprecipitated.

D. Ba/F3/c-Kit cells expressing FLAG tagged p110 δ 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.

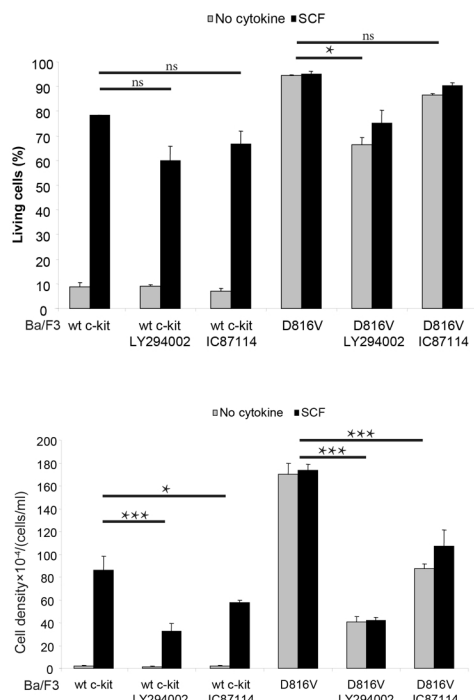
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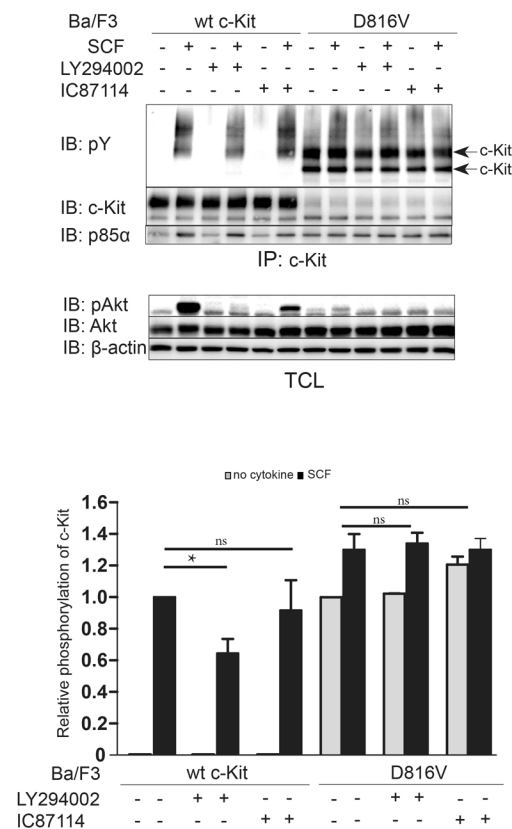
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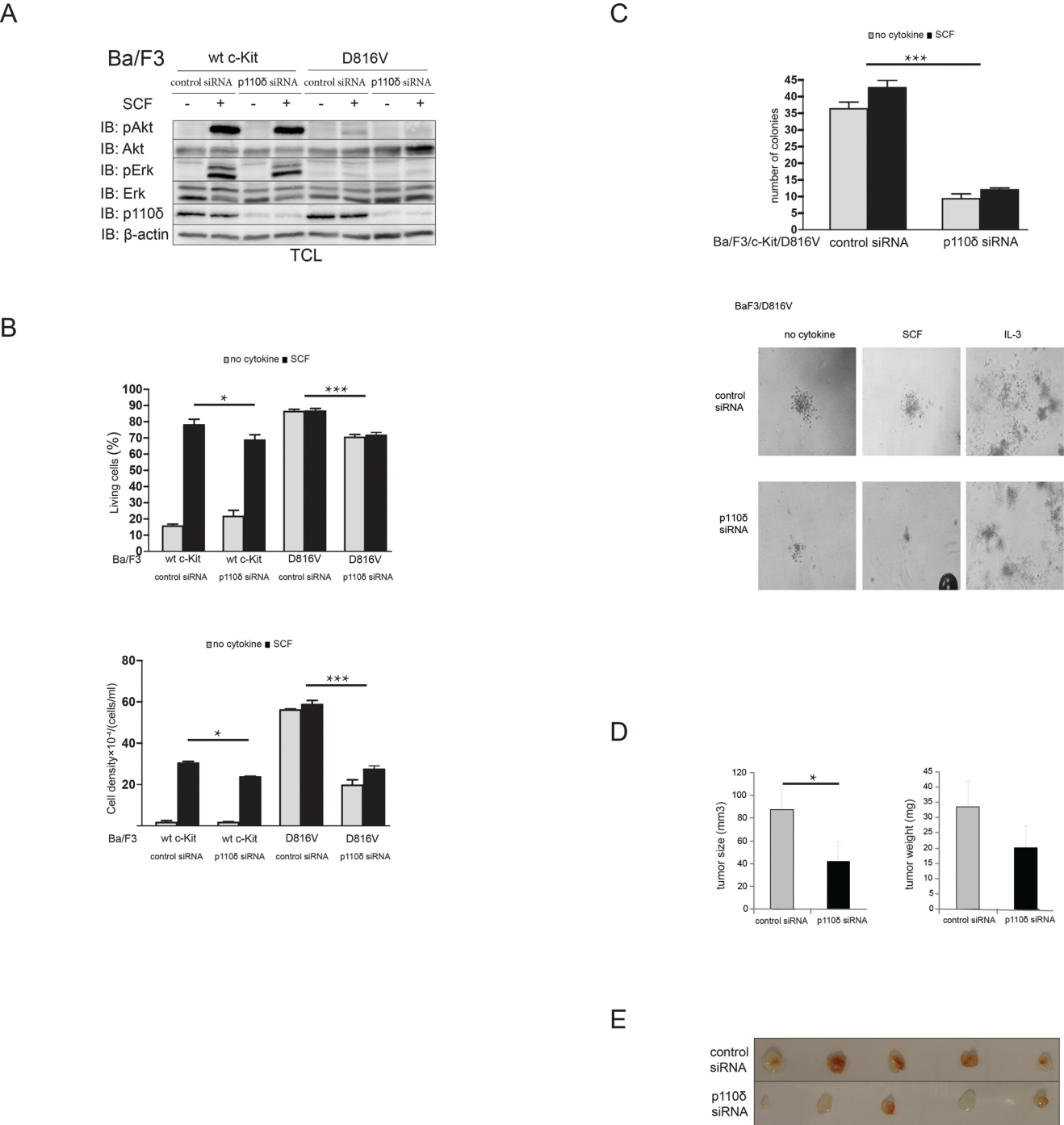
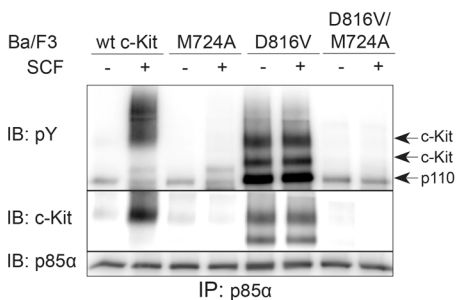
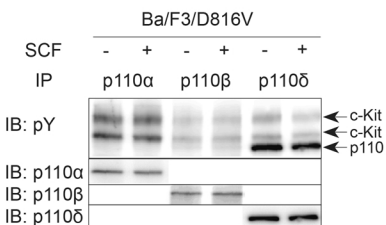


Figure 2

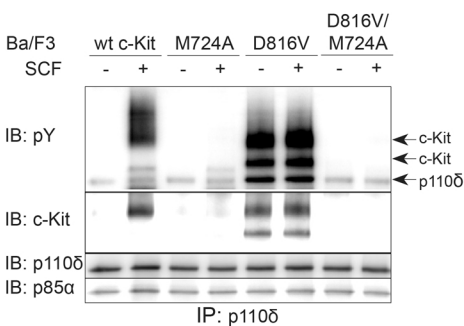
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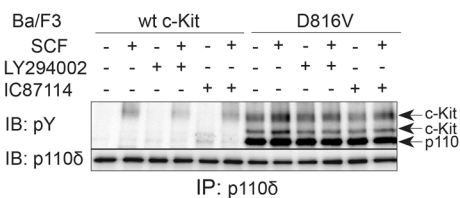
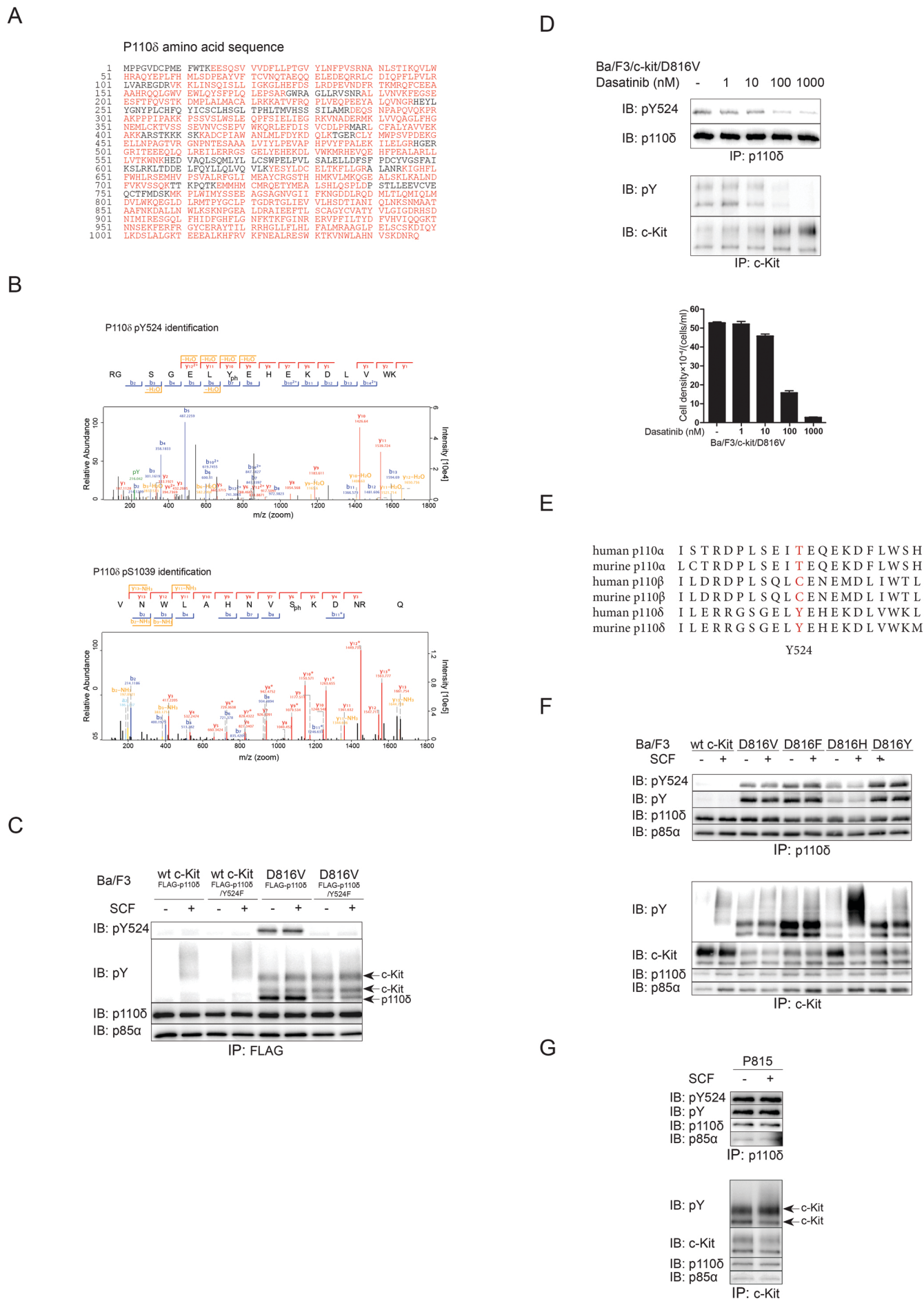


Figure 3



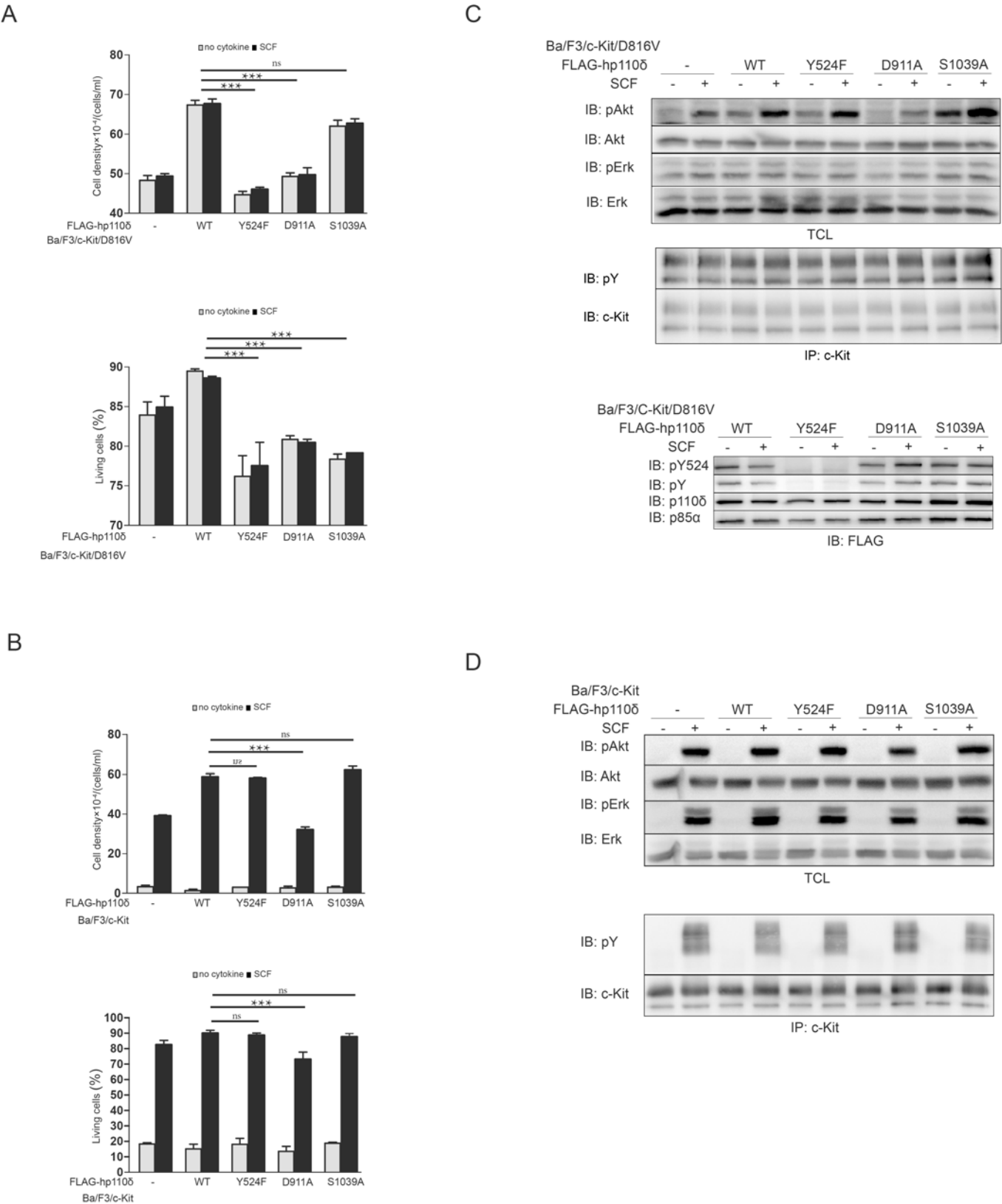


Figure 5

1 **Supplementary MS methods**

2

3 **In-gel digestion**

4 Proteins in the excised band was in-gel digested with trypsin as previously described (1).
5 Briefly, excised gel bands were minced, destained, reduced by DTT and alkylated with
6 chloroacetamide. Proteins were extracted and digested with sequencing grade trypsin
7 (Promega) overnight at room temperature. Tryptic activity was quenched by TFA
8 acidification. Peptides were extracted by acetonitrile/water and desalted and concentrated
9 on C₁₈ STAGE tips (2).

10

11 **Phosphopeptide enrichment**

12 Phosphopeptides were enriched using titanium dioxide beads (10µm titansphere, GL
13 Sciences, Japan) pre-coated with 2,5-dihydroxybenzoic acid (2,5-DHB). 1mg beads were
14 suspended in 5µl 2,5-DHB (0.02g DHB/ml, 80% acetonitrile (ACN), 0.5% acetic acid
15 (AcOH) and added to each sample, which were then incubated with gentle rotation for
16 15min (30rpm). The beads were washed with 100µl 5mM KH₂PO₄, 30% ACN, 350mM
17 KCl followed by 100µl 40% ACN, 0.5% AcOH, 0.05% TFA and then re-suspended in
18 50µl 80% ACN, 0.5% AcOH. The resuspended beads were loaded onto preconditioned
19 in-house packed C₈ STAGE tips, washed with 80% ACN, 0.5% AcOH, eluted with
20 2x10µl 5% ammonia and 2x10µl 10% ammonia, 25%ACN. Ammonia and organic
21 solvents were evaporated using a vacuum centrifuge. Peptides were acidified in 1% TFA,
22 5% ACN and loaded onto in-house packed C₁₈ STAGE tips, preconditioned with 20µl
23 MeOH, 20µl 80% ACN, 0.5% AcOH, 2x20µl 1% TFA, 3% ACN. Following loading, the
24 STAGE tips were washed with 2x20µl 8% ACN, 0.5% AcOH, and 1x50µl 0.5% AcOH.

1

2 **LC-MS/MS**

3 Peptides were eluted with 2x10 μ l 40% MeCN, 0.5% AcOH, organic solvents were
4 removed in a vacuum centrifuge. Peptides were reconstituted in 2% MeCN, 0.5% AcOH,
5 0.1% TFA and analyzed by online reversed-phase C₁₈ nanoscale liquid chromatography
6 tandem mass spectrometry. The experiments were performed on an EASY-nLC™ system
7 (Proxeon Biosystems, Odense, Denmark) connected to the LTQ-Orbitrap Velos (Thermo
8 Electron, Bremen, Germany) through a nano-electrospray ion source, essentially as
9 described previously (REF: Olsen et al, Mol Cell Proteomics 2009). Briefly, the peptide
10 mixtures were separated in a 15 cm analytical column (75 μ m inner diameter) in-house
11 packed with 3 μ m C₁₈ beads (Reposil-AQ Pur, Dr. Maisch) with a 90 min gradient from
12 5% to 30% ACN in 0.5% AcOH. The effluent from the HPLC was directly
13 electrosprayed into the mass spectrometer. The MS instrument was operated in data-
14 dependent mode to automatically switch between full scan MS and MS/MS acquisition.
15 Survey full scan MS spectra (from m/z 300 – 1500) were acquired in the orbitrap with
16 resolution R=60K at m/z 400 after accumulation to a ‘target value’ of 1e6 in the linear
17 ion trap. The ten most intense peptide ions with charge states ≥ 2 were sequentially
18 isolated to a target value of 5e4 and fragmented with Higher-energy Collisional
19 Dissociation (HCD) (3) in the octopole collision cell and analyzed in the orbitrap with a
20 resolution of 7,500. For all full scan measurements with the orbitrap detector a lock-mass
21 ion from ambient air (m/z 445.120024) was used as an internal calibrant as described (4).
22 Typical mass spectrometric conditions were: spray voltage, 2.1 kV; no sheath and
23 auxiliary gas flow; heated capillary temperature, 275°C; 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-pyroglutamine (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).

Reference

1. Lundby A, Olsen JV. GeLCMS for in-depth protein characterization and advanced analysis of proteomes. *Methods Mol Biol* 2011; **753**: 143-155.
2. Rappsilber J, Mann M, Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* 2007; **2**(8): 1896-1906.
3. Olsen JV, Macek B, Lange O, Makarov A, Horning S, Mann M. Higher-energy C-trap dissociation for peptide modification analysis. *Nat Methods* 2007 Sep; **4**(9): 709-712.
4. Olsen JV, de Godoy LM, Li G, Macek B, Mortensen P, Pesch R, *et al.* Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. *Mol Cell Proteomics* 2005 Dec; **4**(12): 2010-2021.

1

2 5. Cox J, Matic I, Hilger M, Nagaraj N, Selbach M, Olsen JV, *et al.* A practical
3 guide to the MaxQuant computational platform for SILAC-based quantitative
4 proteomics. *Nat Protoc* 2009; **4**(5): 698-705.

5

6 6. Cox J, Mann M. MaxQuant enables high peptide identification rates,
7 individualized p.p.b.-range mass accuracies and proteome-wide protein
8 quantification. *Nat Biotechnol* 2008 Dec; **26**(12): 1367-1372.

9

10 7. Olsen JV, Blagoev B, Gnäd F, Macek B, Kumar C, Mortensen P, *et al.* Global, in
11 vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* 2006
12 Nov 3; **127**(3): 635-648.

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14

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