**Sre- Like Adaptor Protein (SLAP) differentially regulates normal and oncogenic c-Kit signaling**

Julhash U. Kazi¹, Shruti Agarwal¹, Jianmin Sun¹, Enrico Bracco² and Lars Rönnstrand¹

¹Translational Cancer Research, Department of Laboratory Medicine, Lund University, Medicon Village, Lund, Sweden.

²Department of Oncology, University of Turin, Turin, Italy.

Corresponding author: Lars Rönnstrand, Translational Cancer Research, Lund University, Medicon Village, building 404:C3, 223 81 Lund, Sweden Tel.: +46 76 887 02 63

E-mail: Lars.Ronnstrand@med.lu.se

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Summary:

The Src-Like Adaptor Protein (SLAP) is an adaptor protein sharing considerable structural homology with Src. SLAP is expressed in variety of cells regulating receptor tyrosine kinase signaling by direct association. In this report, we show that SLAP associates with both wild-type and oncogenic c-Kit (c-Kit-D816V). The association involves SLAP SH2 domain and receptor phosphotyrosine residues different from those mediating Src interaction. Association of SLAP triggers c-Kit ubiquitination which, in turn, is followed by receptor degradation. Although SLAP depletion potentiates c-Kit downstream signaling by stabilizing the receptor, it remains non-functional in c-Kit-D816V signaling. Ligand-stimulated c-Kit or c-Kit-D816V did not alter membrane localization of SLAP. Interestingly oncogenic c-Kit-D816V, but not wild-type c-Kit, phosphorylates SLAP on Y120, Y258 and Y273 residues. Physical interaction between c-Kit-D816V and SLAP is mandatory for the phosphorylation to take place. Although tyrosine phosphorylated SLAP does not affect c-Kit-D816V signaling, mutation of these tyrosine sites to phenylalanine can restore SLAP activity. Taken together the data demonstrate that SLAP negatively regulates wild-type c-Kit signaling, but not its oncogenic counterpart, indicating a possible mechanism by which the oncogenic c-Kit bypasses the normal cellular negative feedback control.
Introduction

The Src-like adapter protein (SLAP) is an adaptor protein containing both Src homology 2 (SH2) and SH3 domains. SLAP displays considerable structural homology with Src but lacks its kinase domain. Similar to Src, SLAP is myristoylated, thus localizing to the cell membrane (Manes et al., 2000). Furthermore, the presence of an SH2 domain facilitates association of SLAP with many receptors through phosphotyrosine residues and thereby impairs Src-mediated signaling by competing with Src itself.

SLAP is primarily implicated in negative regulation of receptor signaling by facilitating the recruitment of E3 ubiquitin ligases. Since SLAP is capable of association with E3 ubiquitin ligase Cbl through its C-terminal tail in a phosphorylation independent manner, it can form multi-proteins complex (Tang et al., 1999). Ability of self-dimerization also allows SLAP to recruit many proteins at the same time (Tang et al., 1999).

Despite its role in negative regulation of receptor signaling, SLAP is also capable of potentiating signals from certain receptors through unknown mechanism. SLAP transduces mitogenic signals from Syk to NAFT-AP1 and depletion of SLAP reduces FLT3 ligand-induced Akt and Erk1/2 phosphorylation (Kazi and Rönnstrand, 2012; Tang et al., 1999) suggesting that SLAP plays differential roles depending on its kinase partner.

Type III receptor tyrosine kinase (RTK) c-Kit is of importance in variety of physiological conditions and is frequently mutated in many cancers (Kazi et al., 2008; Lennartsson and Rönnstrand, 2012). Whereas wild-type c-Kit receptor requires ligand stem cell factor (SCF) to trigger its dimerization and activation, the oncogenic c-Kit mutants display ligand-independent activation. One of the very first manifestations of an active c-Kit receptor is the auto-phosphorylation on multiple tyrosine residues. These phosphotyrosine residues facilitate docking
of multiple SH2 domain containing proteins which, in turn, further propagate or counteract receptor signaling by distinct mechanisms (Masson and Rönnstrand, 2009). For example, association of Cbl through Y568 directs c-Kit to the lysosomal degradation (Masson et al., 2006) while association of Src through the same tyrosine residue potentiates downstream signaling (Lennartsson et al., 1999). Many receptor interacting proteins tightly regulate receptor stability, phosphorylation and signaling by linking kinases, ubiquitin ligases and other signaling molecules (Kazi and Rönnstrand, 2013a; Kazi and Rönnstrand, 2013b; Kazi et al., 2012; Kazi et al., 2013b; Lin et al., 2012).

Here, we describe that SLAP associates with both normal and oncogenic mutants of c-Kit and its association displays differential impact on receptor downstream signaling.

**Results**

*SLAP associates with SCF-stimulated wild-type c-Kit as well as with oncogenic c-Kit-D816V*— An increasing number of evidence suggests that SLAP regulates receptor signaling. We and other groups recently showed that SLAP associates with a variety of receptors including EphA (Semerdjieva et al., 2013) and FLT3 (Kazi and Rönnstrand, 2012) in a phosphorylation dependent manner. FLT3 is a type III RTK that is found to be frequently mutated in hematopoietic malignancies (Kabir et al., 2013). In our previous report we also showed that SLAP is highly expressed in hematopoietic cells. Since c-Kit belongs to the same RTK family with FLT3 and is highly expressed in hematopoietic malignancies, we tested whether SLAP correspondingly associates with c-Kit. We co-transfected FLAG-tagged SLAP with wild-type c-Kit or an oncogenic version, c-Kit-D816V. We were only able to pull-down c-Kit with an anti-FLAG antibody from cells which were stimulated with SCF prior to lysis (Fig. 1A, lane 3). The
oncogenic c-Kit-D816V mutant is characterized by constitutive tyrosine kinase activity. In the case of the c-Kit-D816V mutant, a strong ligand-independent interaction between SLAP and c-Kit-D816V was detected (Fig. 1A, lane 4). These data suggest that SLAP associates with c-Kit only when c-Kit is tyrosine phosphorylated. The efficiency of c-Kit pull-down from cell lysates was independent of c-Kit expression but was significantly affected by SLAP expression (Fig. 1B and C) indicating that association is dependent on availability of the SLAP protein. To explore endogenous binding, we checked SLAP expression in the human mastocytoma HMC-1.2, the human erythroleukemia TF-1 and the mouse mastocytoma P815 cell lines. While TF-1 cell line endogenously expresses wild-type c-Kit, both HMC-1.2 and P815 cell lines express the oncogenic mutant, c-Kit-D816V. We observed that P815 cell line expresses higher levels of SLAP when compared to the other two cell lines (Fig. 1D). We then pulled-down SLAP from P815 cell lysate using an anti-SLAP antibody. TF-1 cell lysate was used as a negative control. We were able to detect c-Kit-SLAP complexes in P815 cells (Fig. 1E). We were also able to detect endogenous c-Kit and SLAP interaction in HL60 cells that expresses wild-type c-Kit and the interaction was ligand dependent (Fig. 1F). In addition, c-Kit-SLAP interaction displayed a linear decrease over the time but complexes were detectable until 4 hours of SCF stimulation suggesting that SLAP stably associates with c-Kit (Fig. 1G).

SLAP associates with c-Kit through its SH2 domain through a site different from the Src binding site- SLAP associates only with the activated form of c-Kit suggesting that the association is mediated through phosphotyrosine residues of c-Kit and the SLAP SH2 domain. To test this hypothesis, we generated the SLAP-R111E mutant where we replaced the positively charged critical arginine residue in the phosphotyrosine binding pocket of the SH2 domain with the negatively charged glutamic acid. Thereby, this mutant has negatively charged phosphotyrosine binding pocket that will repulse phosphotyrosine residue. We co-expressed c-
Kit-WT and SLAP-WT-FLAG or SLAP-R111E-FLAG in COS-1 cells. While wild-type SLAP efficiently pulled down c-Kit, the interaction was completely abrogated when using the SLAP SH2 domain mutated form SLAP-R111E (Fig. 1H) confirming that SLAP associates with c-Kit through its SH2 domain. Since SLAP shares considerable structural similarity with Src, we also checked whether SLAP associates with c-Kit through the same tyrosine phosphorylation sites as Src. For this purpose we used the c-Kit-Y568F, c-Kit-Y570F and c-Kit-Y567F/Y570F mutants that fail to associate with Src. The results indicated that this mutant of c-Kit is unable to block the association with SLAP, although the binding was diminished due to the lower total tyrosine phosphorylation of these mutants (Fig. 1I). According to these data we propose that SLAP associates with c-Kit through its SH2 domain and that the binding site is different from the one mediating binding to the structurally related protein Src.

**SLAP depletion increases c-Kit tyrosine phosphorylation**- The association of adaptor proteins to the RTKs affects receptor auto-phosphorylation (Lin et al., 2012). We transfected COS-1 cells with increasing amounts of SLAP plasmid while keeping the amount of c-Kit plasmid constant. Cells were serum-starved overnight and stimulated with SCF for a fixed period of time before lysis. We observed a linear decrease of c-Kit tyrosine phosphorylation as well as c-Kit expression with increasing SLAP expression but the net c-Kit tyrosine phosphorylation remained unchanged (Fig. 2A). Since overexpression of exogenous proteins can affect cellular behavior, we intended to check the effect of SLAP on c-Kit phosphorylation using endogenous proteins. Ba/F3 cells express a decent level of SLAP but lack c-Kit expression (Kazi and Rönnstrand, 2012). Thus, we used stably transfected Ba/F3-c-Kit cells (Kazi et al., 2013b) with SLAP shRNA. We were able to achieve a 77.3% SLAP knockdown while keeping the c-Kit expression unchanged (Fig. 2B). Ba/F3-c-Kit-control and Ba/F3-c-Kit-SLAP-shRNA cells were stimulated with SCF for different periods of time and then subjected to immunoprecipitation with
an anti-c-Kit antibody. Upon SLAP depletion c-Kit tyrosine phosphorylation was significantly increased (Fig. 2C) suggesting that SLAP is required in order to maintain the normal level of receptor tyrosine phosphorylation.

**SLAP depletion decreases c-Kit ubiquitination and degradation-** SLAP association with FLT3 increases receptor ubiquitination by recruiting the Cbl E3 ubiquitin ligase to the receptor (Dragone et al., 2006; Kazi and Rönnstrand, 2012). We used Ba/F3-c-Kit-control and Ba/F3-c-Kit-SLAP-shRNA cells to evaluate the effect of SLAP on c-Kit ubiquitination. Cells were serum-starved and treated with MG132 and chloroquine diphosphate prior to SCF stimulation. We observed that depletion of SLAP significantly decreases c-Kit ubiquitination in response to SCF (Fig. 3A), which is in line with previous finding that SLAP potentiates ubiquitination of RTKs (Dragone et al., 2006; Kazi and Rönnstrand, 2012). Since SLAP expression resulted in increased ubiquitination of c-Kit, we then tested whether increased ubiquitination leads to increased degradation of the receptor. We observed that while SLAP expression significantly increased c-Kit degradation in COS-1 cells (Fig. 3B), SLAP depletion significantly stabilized c-Kit in Ba/F3 cells (Fig. 3C).

**SLAP depletion increases SCF-induced Akt and Erk1/2 phosphorylation-** SCF stimulation activates PI3K and MAPK pathways resulting in phosphorylation of Akt, Erk1/2 and p38 (Lennartsson and Rönnstrand, 2012; Masson and Rönnstrand, 2009). To assess the role of SLAP in c-Kit downstream signaling, we stimulated Ba/F3-c-Kit-control shRNA and Ba/F3-c-Kit-SLAP shRNA cells with SCF after 4 hours starvation. SCF stimulation significantly increased Akt phosphorylation at 5 minutes (Fig. 4A). Erk1/2 phosphorylation was decreased at 2 minutes but increased at 5 minutes (Fig. 4B). Phosphorylation of p38 remained mostly unchanged (Fig. 4C). Furthermore, SCF stimulation for an extended time period also followed
similar trends of Akt (Fig. 4D) and Erk1/2 (Fig. 4E) phosphorylation. Thus, we conclude that SLAP negatively regulates SCF-induced c-Kit downstream signaling in a selective manner.

**SLAP controls Akt and Erk1/2 phosphorylation through SHC and Gab2** – Stimulation with SCF induces Akt and Erk1/2 phosphorylation through multiple pathways. To understand which pathways are affected by SLAP, we investigated the phosphorylation status of SHC, SHP2 and Gab2. SHC phosphorylation was significantly decreased after 2 minutes of SCF stimulation while it was increased after 5 minutes of SCF-stimulation in SLAP depleted cells (Fig. 5A). SLAP depletion did not alter SHP2 phosphorylation (Fig. 5B) but enhanced Gab2 phosphorylation after 5 minutes of SCF stimulation (Fig. 5C).

**SLAP did not block c-Kit-D816V-induced Akt and Erk phosphorylation** – Normal c-Kit and its oncogenic mutant c-Kit-D816V display significant differences in cellular signaling (Lennartsson et al., 2003; Sun et al., 2009). Since SLAP associates with c-Kit-D816V, we hypothesized that SLAP might negatively regulate c-Kit-D816V signaling in a similar fashion to wild-type c-Kit. We transfected wild-type SLAP or empty vector into the HMC-1.2 cells which harbor the oncogenic mutant of c-Kit. Surprisingly, SLAP expression did not block SCF-induced Akt and Erk1/2 phosphorylation (Fig. 6A). Similar results were obtained from SLAP-depleted Ba/F3-c-Kit-D816V cells (Fig. 6B). Furthermore, while SLAP depletion accelerated SCF-induced cell proliferation, it did not affect c-Kit-D816V-induced cell proliferation (Fig. 6C). Therefore, we suggest that SLAP negatively regulates wild-type c-Kit signaling but not oncogenic c-Kit-D816V signaling.

**SLAP shows similar sub-cellular localization patterns with c-Kit-WT and c-Kit-D816V** – Since SLAP has different impact on c-Kit signaling, we checked whether this is due to the differential sub-cellular localization of SLAP. We transfected COS-1 cells with SLAP-FLAG and c-Kit-WT or c-Kit-D816V. Cells were serum-starved overnight and the c-Kit-WT transfected
cells were stimulated with SCF before para-formaldehyde fixation. Cells were then stained with Alexa flour 647-conjugated anti-FLAG (DYKDDDDK) and PE-conjugated c-Kit antibodies. Subcellular localization was visualized with a laser scanning confocal microscope. As expected SLAP was found to be localized near the inner surface of the cell membrane in both unstimulated (Fig. 7A) and SCF-stimulated cells (Fig. 7B) as well as in c-Kit-D816V expressing cells (Fig. 7C). In addition, SCF stimulation and expression of c-Kit-D816V significantly increased co-localization of SLAP with c-Kit, but co-localization of SLAP and c-Kit-WT or SLAP and c-Kit-D816V did not display any difference (Fig. 7D).

**Oncogenic c-Kit-D816V but not SCF stimulated wild-type c-Kit induces SLAP tyrosine phosphorylation** – To understand the mechanism of differential regulation of normal and oncogenic c-Kit signaling, we then checked whether SLAP was differentially modified by wild-type and oncogenic c-Kit. We observed that only c-Kit-D816V but not wild-type c-Kit was able to phosphorylate SLAP on tyrosine residues (Fig. 8A). Thus, we suggest that SLAP-mediated differential regulation of c-Kit downstream signaling might be due to the differential posttranslational modification of SLAP by oncogenic c-Kit.

**Oncogenic c-Kit-D816V phosphorylates SLAP on Y120, Y258 and Y273 residues** – SLAP tyrosine phosphorylation sites have not been studied well. The PhosphoSitePlus database (www.phosphosite.org) describes three different predicted phosphorylation sites of SLAP in three different species (Fig. 8B). To explore SLAP tyrosine phosphorylation sites in living cells, we generated Y to F mutants of the corresponding human SLAP tyrosine residues and observed that mutation in any of those three sites reduced the total tyrosine phosphorylation of SLAP and that Y120 and Y258 sites are the major tyrosine phosphorylation sites in SLAP phosphorylated by c-Kit-D816V (Fig. 8C). Then we generated a SLAP-Y120F-Y258F-Y273F (SLAP-YYYYFF) mutant to check whether SLAP has any other minor tyrosine phosphorylation sites other than
those predicted. The triple mutant completely abolished c-Kit-D816V-mediated tyrosine phosphorylation (Fig. 8D) of SLAP indicating the presence of only three tyrosine phosphorylation sites (pY120, pY258 and pY273) in SLAP.

**Association of SLAP with c-Kit-D816V is required for tyrosine phosphorylation of SLAP** – We then checked whether association of SLAP with c-Kit-D816V is required for c-Kit-D816V-mediated tyrosine phosphorylation of SLAP. We transfected cells with wild-type SLAP and non-functional SH2 domain mutant SLAP-R111E together with c-Kit-D816V. We observed that while c-Kit-D816V strongly phosphorylates wild-type SLAP, SLAP-R111E remains mostly non-tyrosine-phosphorylated (Fig. 8E) indicating that association of SLAP with c-Kit-D816V is required for tyrosine phosphorylation of SLAP.

**Mutation of the SLAP tyrosine phosphorylation sites rescues its activity** – Since we observed that c-Kit and its oncogenic counterpart c-Kit-D816V differentially mediate SLAP tyrosine phosphorylation, and SLAP differentially regulates wild-type and mutant c-Kit signaling, we suggest that tyrosine phosphorylation of SLAP inactivates SLAP. To verify this hypothesis we transfected wild-type SLAP, SLAP-YYYFFF and empty vector into the HMC-1.2 cell line. We observed that SLAP-YYYFFF significantly decreased SCF-induced Akt and Erk1/2 phosphorylation (Fig. 9A and 9B) suggesting that tyrosine phosphorylation of SLAP disrupts SLAP activity. Single tyrosine mutant (SLAP-Y120F or -Y258F or –Y273F) also displayed similar reduction in phosphorylation (Fig. 9C). These results are further supported by the observation that expression of SLAP-YYYFFF but not SLAP-WT significantly decreased c-Kit-D816V mediated cell growth (Fig. 9D).
Discussion

Adaptor proteins control RTK signaling either by recruiting ubiquitin ligases, phosphatases, kinases and other signaling proteins, or competing with kinases or phosphatases for association with receptors. Although the adaptor protein SLAP negatively regulates RTK signaling by reducing receptor turnover by influencing degradation (Dragone et al., 2006; Semerdjieva et al., 2013; Sosinowski et al., 2000), a couple of reports suggest that SLAP propagates additional receptor signaling through poorly understood mechanisms (Kazi and Rönnstrand, 2012; Tang et al., 1999). In this report, we show that SLAP depending on its tyrosine phosphorylation status plays dual roles in RTK signaling.

SLAP interacts with ligand stimulated c-Kit as well as with its oncogenic form c-Kit-D816V. This association is mediated through the SLAP SH2 domain and results in accelerated ubiquitination followed by receptor degradation. These findings indicate that the c-Kit-SLAP interaction is dependent on c-Kit tyrosine phosphorylation and that SLAP recruits ubiquitin ligases which tag c-Kit for degradation. This is the most common mechanism through which adaptor proteins regulate receptor stability as well as downstream signaling (Dragone et al., 2006; Dragone et al., 2009). Although, SLAP displays a statistically significant effect on c-Kit ubiquitination, it is not the only factor that regulates c-Kit stability. Several studies suggest that c-Kit directly interacts with multiple ubiquitin ligases such as Cbl (Masson et al., 2006) and SOCS6 (Bayle et al., 2004) or through indirect association of Cbl through Grb2 (Sun et al., 2007). Thus, SCF-induced c-Kit degradation is controlled through multiple pathways and SLAP contributes to these pathways by accelerating ubiquitination.

Since SLAP accelerates receptor ubiquitination followed by degradation, it was expected that SLAP depletion would positively regulate c-Kit downstream signaling. The fact that SLAP depletion enhanced Akt, Erk1/2, SHC and Gab2 phosphorylation at 5 minutes partially supports
the idea that this effect is mediated through alteration of receptor stability, but early decrease of Erk1/2 or SHC phosphorylation and equal phosphorylation of p38 or SHP2 cannot be explained by the same hypothesis. Therefore, it is of interest to identify how SLAP selectively controls c-Kit signaling. Enhanced Akt, Erk1/2 phosphorylation also correlates with increased receptor activation that was observed at 5 min of SCF stimulation in SLAP depleted cells. It is not clear why SLAP depletion increases c-Kit tyrosine phosphorylation. One possible explanation could be that SLAP is regulating ubiquitination and degradation of another kinase that is involved in c-Kit phosphorylation. SFKs promote c-Kit tyrosine phosphorylation and SLAP is known to associate with SFKs (Lennartsson et al., 2003; Sosinowski et al., 2000). Another explanation could be that SLAP competes with SFKs for the same binding site. However, we showed that SLAP does not associate with the known Src binding sites in c-Kit, it is possible that other SFKs associates with c-Kit through different tyrosine residues which overlaps with SLAP binding sites. The non-receptor protein tyrosine kinase Syk is another candidate (Tang et al., 1999). If there is a depletion of SLAP, maybe more of the candidate tyrosine kinase will bind to c-Kit and be activated, and this would then contribute to c-Kit phosphorylation as well as downstream signaling. We know from previous studies that other tyrosine kinases can associate to and phosphorylate c-Kit (Lennartsson et al., 2003).

The inability of SLAP to elicit a negative regulation of c-Kit-D816V-mediated signaling is probably due to the tyrosine phosphorylation SLAP, since the effect was restored in the triple tyrosine mutant of SLAP. Since Cbl-N interacts with SLAP through the C-terminus of SLAP and SLAP also forms homo-dimers through its C-terminus (Tang et al., 1999), tyrosine phosphorylation of SLAP in the C-terminus might disrupt homo-dimerization as well as interaction with Cbl and thereby disable the block of c-Kit-D816V-mediated signaling.
In conclusion we propose that SLAP negatively regulates signal transduction events triggered by activated wild-type c-Kit. In contrast, the oncogenic c-Kit-D816V form bypasses the negative control of SLAP by phosphorylating SLAP on tyrosine residues. Taken together we demonstrate that an adapter protein, SLAP, is able to dramatically influence signaling downstream of c-Kit despite of the fact that it lacks enzymatic activity by itself.

Experimental procedures

*Plasmids, antibodies and reagents*- The pcDNA3-c-Kit-WT, pcDNA3-c-Kit-D816V, pMSCV-c-Kit-WT, pMSCV-c-Kit-D816V and pcDNA3-SLAP-WT plasmids were described elsewhere (Kazi and Rönnstrand, 2012; Kazi et al., 2013b). The pc-DNA3-c-Kit-Y568F, pcDNA3-c-Kit-Y570F, pcDNA3-c-Kit-Y568F/Y570F, pcDNA3-SLAP-Y120F, pcDNA3-SLAP-Y258F, pcDNA3-SLAP-Y273F, pcDNA3-SLAP-Y120F/Y258/Y273F and pcDNA3-SLAP-R111E plasmids were generated using QuikChange site-directed mutagenesis Kit (Stratagene). All reagents and antibodies used in this study were described previously (Kazi et al., 2013b).

*Cell lines and culture conditions*- COS-1 and P815 cells were cultured in DMEM supplemented with 10% FBS. Ba/F3-c-Kit-WT and Ba/F3-c-Kit-D816V cells (Kazi et al., 2013b) were cultured in RPMI 1640 medium supplemented with 10% FBS and 10 ng/ml recombinant murine IL-3 as recommended (Kazi et al., 2013a). HMC-1.2 cells were cultured in IMDM supplemented with 10% FBS. TF-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 10 ng/ml recombinant human IL-3.

*Electroporation, transient transfection and Western Blotting*- Electroporation, transient transfection and Western Blotting methods were described previously (Kazi and Rönnstrand, 2012; Kazi et al., 2012; Kazi et al., 2013b). For electroporation of HMC-1.2 cells 4D-
Nucleofector (Lonza) was used. Cells were washed with PBS before electroporation in SF solution (Lonza) using DS104 program. Where statistical analysis applied, minimum three replicates were used to calculate mean value and standard error of the mean (SEM) was used to calculate the error bars.

Confocal microscopy- Cells were fixed with 4% para-formaldehyde and then permeabilized and blocked with a mixture of 0.5% Triton X100 and 5% goat serum. Cells were then stained with fluorophore-conjugated anti-c-KIT and anti-FLAG antibodies for 1 hour. Nuclei were stained with DAPI before mounting. Subcellular localization of c-Kit and SLAP-FLAG was visualized with a Carl Zeiss LSM 710 Laser Scanning Microscope. CoLocalizer Pro 2.7.1 was used to measure the co-localization of green and red signals.

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Authors’ contributions
JUK conceived, designed and performed the experiments, analyzed the data and wrote the manuscript. SA contributed in the c-Kit-D816V signaling experiments in Ba/F3 cells. JS generated c-Kit mutants. EB conceived the experiments and wrote the manuscript. LR conceived and designed the experiments, discussed data and wrote the manuscript. All authors read and approved the final manuscript.
Competing interests
Authors declare no conflict of interest.

References


**Figure Legends**

Figure 1. SLAP associates with c-Kit-WT and c-Kit-D816V through SLAP SH2 domain.

(A) COS-1 cells were transfected with SLAP-FLAG and c-Kit-WT or c-Kit-D816V constructs. Cells were stimulated with SCF for 10 min where required and immediately lysed.
Cell lysates were immunoprecipitated with an anti-FLAG antibody and then processed for Western blotting analysis. (B) COS-1 cells were transfected with c-Kit-WT and SLAP-FLAG and/or empty vector. Cells were stimulated with SCF for 10 min and processed for immunoprecipitation followed by Western blotting. (C) Western blotting of multiple experiments with different concentration of SLAP and immunoprecipitated c-Kit were quantified and plotted using GraphPad Prism 5.0. (D) Cell lysates from HMC1.2, TF-1 and P815 cells were immunoprecipitated with an anti-SLAP antibody and then analyzed by Western blotting. (E) Cell lysates from TF-1 and P815 cells were immunoprecipitated with an anti-SLAP antibody and then analyzed by Western blotting. (F) Cell lysates from HL60 cells stimulated with SCF or not were immunoprecipitated with an anti-SLAP antibody and then analyzed by Western blotting. (G) COS-1 cells were transfected with SLAP-FLAG and c-Kit-WT constructed. Cells were stimulated with SCF for different time points followed by immunoprecipitation and Western blotting analysis. (H) COS-1 cells were transfected with c-Kit-WT and FLAG-tagged SLAP-WT or SLAP-R111E constructs. Cells were stimulated with SCF or not before lysis. Lysates were immunoprecipitated with an anti-FLAG antibody and then analyzed by Western blotting. (I) COS-1 cells were transfected with SLAP-FLAG and c-Kit-WT or mutants. Cells were stimulated with SCF or not before lysis. Lysates were immunoprecipitated with an anti-FLAG antibody and then analyzed by Western blotting. Relative binding was calculated by normalizing against total pY-c-Kit.

Figure 2. SLAP depletion increases c-Kit phosphorylation in Ba/F3 cells.

(A) COS-1 cells were transfected with c-Kit-WT and different amount of SLAP-FLAG and/or empty control vector. Cells were stimulated for 5 min before lysis and subjected to immunoprecipitation and Western blotting analysis. (B) Ba/F3 cells stably transfected with SLAP
shRNA or empty control vector were lysed and processed for immunoprecipitation with an anti-SLAP antibody followed by Western blotting. (C) Ba/F3-c-Kit-WT/SLAP shRNA and Ba/F3-c-Kit-WT/control shRNA were stimulated with SCF for different time points before lysis. Lysates were processed for immunoprecipitation with an anti-c-Kit antibody followed by Western blotting. Blots from multiple experiments were quantified using Multi Gauge software. ns, not significant; **, p<0.01.

Figure 3. SLAP depletion decreases c-Kit ubiquitination and degradation.

(A) Ba/F3-c-Kit-WT/SLAP shRNA and Ba/F3-c-Kit-WT/control shRNA were stimulated with SCF for different time points before lysis. Lysates were processed for immunoprecipitation with an anti-c-Kit antibody followed by Western blotting. (B) COS-1 cells were transfected with c-Kit-WT and different amount of SLAP or empty control vector. Cells were incubated with cycloheximide for 30 min before 30 min of SCF stimulation. Then cells were lysed and lysates were processed for immunoprecipitation with an anti-c-Kit antibody followed by Western blotting. (C) Ba/F3-c-Kit-WT/SLAP shRNA and Ba/F3-c-Kit-WT/control shRNA were incubated with cycloheximide for 30 min before 30 and 60 min of SCF stimulation. Cells were then lysed and lysates were processed for immunoprecipitation with an anti-c-Kit antibody followed by Western blotting. Blots from multiple experiments were quantified using Multi Gauge software. ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001.

Figure 4. SLAP depletion accelerates Akt and Erk1/2 phosphorylation.

Ba/F3-c-Kit-WT/SLAP shRNA and Ba/F3-c-Kit-WT/control shRNA cells were serum starved for 4 hours before SCF stimulation. Cells were then lysed and lysates were subjected for Western blotting analysis using (A) anti-phospho Akt, anti-Akt and anti-β-Actin, (B) anti-
phospho Erk1/2, anti-Erk and anti-β-Actin, and (C) anti-phospho p38, anti-p38 and anti-β-Actin. (D) Cell lysates were subjected to Western blotting analysis using anti-phospho Akt, anti-Akt and anti-β-Actin. (E) Cell lysates were subjected to Western blotting analysis using anti-phospho Erk1/2, anti-Erk1/2 and anti-β-Actin. Signal intensities were quantified using Multi Gauge software from multiple experiments. ns, not significant; *, p<0.05.

Figure 5. SLAP depletion accelerates SHC and Gab2 phosphorylation.

Ba/F3-c-Kit-WT/SLAP shRNA and Ba/F3-c-Kit-WT/control shRNA cells were serum starved for 4 hours before SCF stimulation followed by lysis. (A) Cell lysates were immunoprecipitated with an anti-SHC antibody and then analyzed by Western blotting. (B) Cell lysates were subjected to Western blotting analysis using anti-phospho SHP2, anti-SHP2 and anti-β-Actin. (C) Cell lysates were subjected to Western blotting analysis using anti-phospho Gab2, anti-Gab2 and anti-β-Actin. Signal intensities were quantified using Multi Gauge software from multiple experiments. ns, not significant; *, p<0.05.

Figure 6. SLAP expression did not alter c-Kit-D816V mediated Akt or Erk1/2 phosphorylation

(A) HMC-1.2 cells transfected with SLAP-WT or empty control vector were serum starved for 4 hours before SCF stimulation. Cells were then lysed and lysates were processed for Western blotting analysis. (B) Ba/F3-c-Kit-D816V/SLAP shRNA and Ba/F3-c-Kit-D816V/control shRNA cells were serum starved for 4 hours before SCF stimulation. Cells were then lysed and lysates were subjected for Western blotting analysis using anti-phospho Akt, anti-Akt, anti-phospho Erk1/2, anti-Erk and anti-β-Actin. Signal intensities were quantified using Multi Gauge software from multiple experiments. ns, not significant; *, p<0.05; **, p<0.01.

Figure 7. SCF stimulation increases co-localization of SLAP
(A-C) COS-1 cells were co-transfected with SLAP-FLAG and c-Kit-WT or c-Kit-D816V plasmids. Cells were serum starved overnight before stimulation and then fixed, permeabilized and stained with fluorophore-conjugated anti-FLAG antibody, anti-c-Kit antibody and DAPI. Subcellular localization was visualized with a Carl Zeiss LSM 710 Laser Scanning Microscope. Blue, DAPI; Red, c-Kit; Green, SLAP-FLAG. (D) Co-localization was quantified using CoLocalizer Pro 2.7.1. Pearson’s R value was used to measure co-localization of red and green colors from 5 cells. ns, not significant; *, p<0.05.

Figure 8. c-Kit-D816V induces tyrosine phosphorylation of SLAP

(A) Cells were transfected with SLAP-FLAG and c-Kit-WT or c-Kit-D816V. Cells were serum starved overnight before SCF stimulation. Cells were then lysed and lysates were immunoprecipitated with an anti-FLAG antibody and then analyzed by Western blotting. (B) Conserved tyrosine residues in human, mouse and rat. (C-D) Cells were transfected with FLAG tagged SLAP mutants and c-Kit-D816V. Cells were serum starved overnight before lysis and lysates were immunoprecipitated with an anti-FLAG antibody and analyzed by Western blotting. (E) Cells were transfected with c-Kit-D816V and FLAG tagged SLAP-WT or SLAP-R111E mutant. Cells were serum starved overnight before lysis. Lysates were immunoprecipitated with an anti-FLAG antibody and then analyzed by Western blotting. pY-SLAP, tyrosine phosphorylated SLAP.

Figure 9. SLAP mutant negatively regulates c-Kit-D816V mediated Akt or Erk1/2 phosphorylation

(A) HMC-1.2 cells transfected with SLAP-WT, SLAP-YYYFFF or empty control vector were serum starved for 4 hours before SCF stimulation. Cells were then lysed and lysates were
processed for Western blotting analysis. (B) Signal intensities were quantified using Multi Gauge software from multiple experiments. (C) HMC-1.2 cells transfected with SLAP-WT, SLAP-Y120F, SLAP-Y258F, SLAP-Y273F or empty control vector were serum starved for 4 hours before SCF stimulation. Cells were then lysed and lysates were processed for Western blotting analysis. Signal intensities were quantified using Multi Gauge software from multiple experiments. (D) HMC-1.2 cells transfected with SLAP-WT, SLAP-YYYYFFF or empty control vector were seeded in 96-well plate and cultured for different time periods. Cell viability measured by PrestoBlue (Life technologies) assays using manufacturers’ protocol. ns, not significant; *, p<0.05; **, p<0.01.
Figure 1

A. c-Kit WT D816V
SLAP-FLAG - + + +
SLAP - + - -
c-Kit - + - -
SLAP - + - -
pY-c-Kit - + - -
c-Kit - + - -
SLAP - + - -
IP: FLAG

B. SLAP-FLAG 0 0.5 1 2 4 8 12 (μg)
pY-c-Kit - - - - - -
c-Kit - - - - - -
SLAP - - - - - -
IP: FLAG

C. r^2=0.89

D. HMC-1.2 TF-1 P815
TCL - - - - - -
IP: SLAP - - - - - -
SLAP - - - - - -
β-Actin - - - - - -

E. TF-1 P815 SCF - + - +
c-Kit - - - - - -
SLAP - - - - - -
IP: SLAP - - - - - -

F. SCF - + - +
c-Kit - - - - - -
SLAP - - - - - -
IP: SLAP - - - - - -

G. Log (c-Kit intensity)
Log (Time)

H. SLAP-WT SLAP-R111E
SCF - - + +
c-Kit - - - -
SLAP - - - -
IP: FLAG - - - -

I. KIT WT Y568F Y570F Y568/570F
Relative Binding 1.0 0.98 1.18 0.57
IB: KIT
IB: FLAG
IB: 4G10
IB: KIT
IB: FLAG
TCL
Figure 2
Figure 3
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9