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## **STAP-2 interacts with Pyk2 and enhances Pyk2 activity in T-cells**

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Abbreviations: STAP-2: Signal-Transducing Adaptor Protein-2, Pyk2: Protein tyrosine kinase 2, SDF-1: Stromal cell-derived factor-1

## **Abstract**

STAP-2 is an adaptor molecule regulating several signaling pathways, including TLRs and cytokine/chemokine receptors in immune cells. We previously reported that STAP-2 enhances SDF-1 $\alpha$ -induced Vav1/Rac1-mediated T-cell chemotaxis. However, the detailed mechanisms of STAP-2 involvement in enhancing T-cell chemotaxis remain unknown. In the present study, we demonstrate that STAP-2 directly interacts with Pyk2, which is a key molecule in the regulation of SDF-1 $\alpha$ /CXCR4-mediated T-cell chemotaxis, and increases phosphorylation of Pyk2. Pyk2 itself can induce STAP-2 Y250 phosphorylation, and this phosphorylation is critical for maximal interactions between STAP-2 and Pyk2. Finally, SDF-1 $\alpha$ -induced T-cell chemotaxis is inhibited by treatment with Pyk2 siRNA or AG17, an inhibitor of Pyk2, in Jurkat cells overexpressing STAP-2. Taken together, the Pyk2/STAP-2 interaction is a novel mechanism to regulate SDF-1 $\alpha$ -dependent T-cell chemotaxis.

**Keywords:** Pyk2; STAP-2; SDF-1 $\alpha$ ; T-cell; chemotaxis

## Highlights

- STAP-2 directly interacts with and increases phosphorylation of Pyk2.
- STAP-2 Y250 phosphorylation is critical for maximal interaction with Pyk2.
- Pyk2 siRNA or AG17 inhibits SDF-1 $\alpha$ -induced chemotaxis in Jurkat T-cells overexpressing STAP-2.

## **Introduction**

Chemokines are involved in migration and homing of leukocytes [1,2]. Leukocyte chemotaxis plays an important role in immune surveillance, lymphocyte differentiation and immune responses in the body. Chemokines are divided into four subfamilies based on the position of the first two cysteine residues at the N-terminus [1,2]. Stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) is a member of the CXC chemokine family and binds to its receptors CXCR4 and CXCR7 [3,4]. The SDF-1 $\alpha$ /CXCR4 interaction triggers many signaling events including increased Ca<sup>2+</sup> influx, ERK phosphorylation, PI3K activation, and upregulation of NF- $\kappa$ B activity [3,5]. The SDF-1 $\alpha$ /CXCR4 interaction also induces chemotaxis via several signaling molecules such as protein tyrosine kinase 2 (Pyk2) [6].

Pyk2 is a member of the focal adhesion kinase subfamily and is expressed in endothelium, the central nervous system and hematopoietic cells [7,8,9,10]. It is involved in SDF-1 $\alpha$ -induced chemotaxis via forming signaling complexes with Paxillin, Crk and p130Cas [6]. Pyk2-deficient mice are defective in marginal zone B-cell formation and demonstrate inhibited production of antigen-specific IgM, IgG<sub>2</sub> and IgG<sub>3</sub> after T-cell-independent antigen type II immunization because of defects in migration capacity of B-cells [11]. Pyk2 is also required for migration of several tumor cell types, such as glioblastoma and astrocytoma cells in humans [12,13]. Therefore, detailed analysis of Pyk2-mediated chemotaxis may provide a novel Pyk2-targeting therapeutic strategy in immune disorders and cancers.

Signal-Transducing Adaptor Protein-2 (STAP-2), also known as substrate of Brk (BKS), is an adaptor molecule involved in regulation of several intracellular signaling events in immune cells. STAP-2 contains a Pleckstrin homology (PH) domain

at the N-terminus, a SH2-like domain in the central portion and a proline-rich region at C-terminus. STAP-2 also has a YXXQ motif, which is a potential STAT3-binding site [14]. STAP-2 influences STAT3 and STAT5 activity, FcεRI-mediated mast cell activation, TLR-mediated macrophage functions and Fas-mediated T-cell apoptosis [15,16,17,18,19,20,21,22,23]. We have reported that STAP-2 is involved in SDF-1α-induced T-cell chemotaxis via enhancing the Vav1/Rac1 pathway, which is downstream of Pyk2 signaling [24].

In the current study, we found that STAP-2 interacted directly with Pyk2 and increased its phosphorylation. This interaction was not observed in a STAP-2 Y250F-expressing mutant, or in Pyk2 inhibitor-treated T-cells. Furthermore, T-cell chemotaxis was significantly decreased in Pyk2 inhibitor-treated T-cells. Taken together, our findings indicate that STAP-2 modulates SDF-1α-induced T-cell chemotaxis via enhancing Pyk2 kinase activity.

## **Materials and Methods**

### *Reagents, antibodies and mice*

Recombinant human SDF-1 $\alpha$  was purchased from Wako Pure Chemical Industries (Osaka, Japan). AG17 was purchased from EMD Millipore (Bedford, MA). Expression vectors for Vav1, Pyk2 and a kinase-negative mutant of Pyk2 (PKM) were kindly provided by Dr. X. R. Bustelo (CSIC-Univ. Salamanca, Salamanca, Spain) and Dr. T. Miyazaki (Hokkaido Univ.) [25,26]. Epitope-tagged STAP-2 and STAP-2 mutants constructs were described previously [15]. Anti-Myc, anti-FLAG, anti- $\beta$ -actin and anti-phosphotyrosine (PY20) Abs were purchased from Sigma-Aldrich (St. Louis, MO). Anti-Pyk2 Ab was purchased from Bethyl Laboratories (Montgomery, TX). Anti-phospho-Pyk2 (Tyr402) Ab was purchased from Cell Signaling Technology (Beverly, MA). Anti-Vav Ab was purchased from EPITOMICS (Burlingame, CA). Other Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### *Cell culture and treatment*

Human T-cell leukemia cell line, Jurkat was maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS). Stable Jurkat transformants expressing STAP-2 WT (Jurkat/STAP-2) were established as described previously [24]. Human T-cell lymphoma, HUT78 was maintained in RPMI1640 medium supplemented with 10% (FCS). Human embryonic kidney carcinoma cell line, 293T was maintained in DMEM containing 10% FCS, and the indicated plasmids were transfected with the standard calcium precipitation protocol [16]. For activation of Jurkat T-cells, cells were stimulated with SDF-1 $\alpha$  for indicated periods. In some experiments, Jurkat cells were pretreated with indicated concentration of AG17 for 1.5 h prior stimulation of the cells

with SDF-1 $\alpha$ . For siRNA experiment, Jurkat cells are treated with siRNA as previously described [24]. The siRNAs used in this study were as follows: control siRNA (QIAGEN), Pyk2 siRNA, GCAUAGAGUCAGACAUCUATT.

#### *Immunoprecipitation and western blotting*

The immunoprecipitation and western blotting assays were performed as described previously[16]. Briefly, the immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to PVDF transfer membrane (PerkinElmer; Boston, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore; Bedford, MA).

#### *Chemotaxis assays*

In vitro T-cell migration assay was conducted as previously described [24]. Briefly, the transwell filters (5  $\mu$ M pore filter; BD Falcon, Franklin lakes, NJ, USA) were placed in the lower chamber containing 500  $\mu$ l of complete medium with or without SDF-1 $\alpha$ . After 1-3 hrs of incubation at 37°C in 5% CO<sub>2</sub>, the numbers of migrated cells of the lower chamber were counted with a phase contrast microscope. The results are expressed as the number of cells migrated to the bottom chamber. In some experiments, Jurkat cells were pretreated with AG17 for 1.5 h, then, migration experiments were done.

#### *Statistical analysis*

The significance of differences between group means was determined by Student's

t-test.

## Results

### *Jurkat cells overexpressing STAP-2 show high Pyk2 phosphorylation after SDF-1 $\alpha$ stimulation*

We previously reported that STAP-2 enhanced migration in response to SDF-1 $\alpha$  in Jurkat T-cells [24]. We identified the binding of STAP-2 to Vav1, a guanine-nucleotide exchanging factor for Rac1, as a possible mechanism. Because SDF-1 $\alpha$ /CXCL12 signals play central roles in lymphocyte trafficking, it would be very informative to know precisely how STAP-2 affects the signaling pathway. Pyk2 is a key molecule in the regulation of SDF-1 $\alpha$ /CXCL12 signaling, therefore, we analyzed the effects of STAP-2 on Pyk2 activities after SDF-1 $\alpha$  stimulation.

We first tested the effects of STAP-2 on SDF-1 $\alpha$ -induced phosphorylation of Pyk2. STAP-2 overexpressing Jurkat cells (Jurkat/STAP-2) were stimulated with SDF-1 $\alpha$  at the indicated periods, and phosphorylation of signaling molecules were then evaluated by western blotting. Pyk2 phosphorylation was only slightly induced in control vector-transfected Jurkat cells (Jurkat/vector) after SDF-1 $\alpha$  stimulation (Fig. 1A). When Jurkat/STAP-2 cells were stimulated with SDF-1 $\alpha$ , stronger Pyk2 phosphorylation was induced compared with Jurkat/vector cells. In addition, STAP-2 interacted with Pyk2 in resting Jurkat/STAP-2 cells, and this interaction was strongly enhanced after SDF-1 $\alpha$  stimulation. We also examined the interaction between endogenous STAP-2 and Pyk2 in human T-cell lymphoma HUT78, which expresses both proteins at high levels. An anti-Pyk2 antibody co-immunoprecipitated STAP-2 at physiological expression levels (Fig. 1B). These results suggest that STAP-2 regulates phosphorylation of Pyk2 via the binding of STAP-2 to Pyk2 in T-cells.

*STAP-2 PH and SH2-like regions are necessary for interaction with Pyk2*

We mapped the binding sites of STAP-2 to Pyk2 by immunoprecipitation, employing expression vectors for STAP-2 PH, SH2-like and C-terminal domains (Fig 1C). When Pyk2 was co-expressed, GST-STAP-2 full length (FL), PH and SH2-like fragments, but not the C-terminal region, were co-immunoprecipitated with Pyk2 (Fig 1D), indicating that STAP-2 binds to Pyk2 through PH and SH2-like regions on the STAP-2 molecule. Consistent with this result, STAP-2 FL and  $\Delta$ C mutants, but not  $\Delta$ PH or  $\Delta$ SH2 mutants were co-immunoprecipitated with Pyk2 (Fig 1E).

It has been reported that Pyk2 associates with Zap-70 and Vav in Jurkat cells [25]. To investigate whether STAP-2 enhances the Pyk2/Vav1 association, we performed a co-immunoprecipitation assay using STAP-2/Pyk2/Vav1-transfected 293T cells (Fig. 1F). Immunoprecipitates of Pyk2 contained both Vav1 and STAP-2 proteins. However, the binding of Pyk2 to Vav1 was not changed by overexpression of STAP-2, suggesting that STAP-2 is dispensable for the interaction between Pyk2 and Vav1.

*Pyk2-mediated STAP-2 Y250 phosphorylation is critical for Pyk2/STAP-2 interaction*

STAP-2 contains four putative phosphorylation sites at residues 22, 250, 310, and 322, and phosphorylation of Y250 in particular is required in some cases [17,26]. To evaluate the effects of Pyk2 on STAP-2 phosphorylation, we analyzed STAP-2 YF mutants in Pyk2-transfected 293T cells (Fig. 2A). When 293T cells were transfected with only wild type (WT) STAP-2 or YF mutants, STAP-2 phosphorylation was not observed. When Pyk2 was co-expressed with STAP-2 WT, STAP-2 phosphorylation was clearly detected. STAP-2 phosphorylation was also observed in Pyk2/STAP-2 Y22F/Y310F/Y322F-mutant co-expressed 293T cells. However, when 293T cells were

transfected with Pyk2 and the STAP-2 Y250F mutant, phosphorylation of STAP-2 was not detected. Thus, a tyrosine residue at position 250 on STAP-2 is phosphorylated by Pyk2. To confirm Pyk2-induced STAP-2 phosphorylation, we tested phosphorylation of STAP-2 in Pyk2/STAP-2-transfected 293T cells treated with or without AG17, a Pyk2 inhibitor. Although STAP-2 was phosphorylated in Pyk2/STAP-2-transfected 293T cells, this phosphorylation was abolished by treatment with AG17 in a dose-dependent manner (Fig 2B). We also tested phosphorylation of STAP-2 in a kinase-dead Pyk2 (PKM)/STAP-2-transfected 293T cells. STAP-2 was not phosphorylated in PKM/STAP-2 transfected 293T cells (Fig 2C). These results suggest that STAP-2 Y250 is specifically phosphorylated by Pyk2.

To elucidate the meaning of Pyk2-induced STAP-2 phosphorylation, we analyzed the binding of Pyk2 to the STAP-2 Y250F mutant using a co-immunoprecipitation assay (Fig. 3A). When 293T cells were co-transfected with Pyk2 and STAP-2 WT, an association between STAP-2 and Pyk2 was observed. However, this association band completely disappeared in 293T cells co-transfected with Pyk2 and the STAP-2 Y250F mutant. Furthermore, a kinase-dead Pyk2 PKM failed to interact with STAP-2 in 293T cells (Fig. 3B). These results suggest that Pyk2-mediated STAP-2 phosphorylation is required for maximal interaction between STAP-2 and Pyk2.

#### *Activation of Pyk2 is essential for increased STAP-2-mediated T-cell chemotaxis*

To investigate whether Pyk2 is required for STAP-2-dependent enhancement of T-cell chemotaxis, we analyzed SDF-1 $\alpha$ -induced migration of Jurkat cells treated with Pyk2 siRNA. Pyk2 expression of Jurkat/STAP-2 cells treated with Pyk2 siRNA was decreased compared with control siRNA-treated Jurkat/STAP-2 cells (Fig 4A). When

control siRNA-treated Jurkat/STAP-2 cells were stimulated with SDF-1 $\alpha$ , T-cell migration was increased. This SDF-1 $\alpha$ -induced T-cell migration was significantly inhibited by the treatment of Jurkat/STAP-2 cells with Pyk2 siRNA (Fig 4B). To evaluate the contribution of Pyk2 kinase activity in STAP-2-mediated T-cell chemotaxis, we stimulated Jurkat/STAP-2 cells with SDF-1 $\alpha$  in the presence or absence of a Pyk2 inhibitor, AG17. Phosphorylation of Pyk2 was dramatically reduced by treatment with 200 mM AG17 (Fig 4C). Similarly, the reduction of ERK phosphorylation was observed in AG17-treated Jurkat/STAP-2 cells. In accordance with the reduction of Pyk2 phosphorylation, SDF-1 $\alpha$ -induced STAP-2-dependent enhancement of T-cell migration was significantly inhibited by treatment with AG17 (Fig 3 D). These results suggest that Pyk2 kinase activity is essential for the increase in T-cell migration involving STAP-2.

## Discussions

In this study, we have described how STAP-2 directly interacts with Pyk2 through its PH and SH2-like domains. Pyk2 phosphorylation in Jurkat/STAP-2 cells is increased to a greater extent after SDF-1 $\alpha$  stimulation than in Jurkat/vector cells. Tyrosine 250 of STAP-2 is phosphorylated by Pyk2, and this STAP-2 phosphorylation is important for interactions between Pyk2 and STAP-2. Furthermore, Pyk2 siRNA or AG17, a Pyk2 inhibitor, suppressed the interaction of Pyk2 with STAP-2 and STAP-2-dependent enhancement of SDF-1 $\alpha$ -induced migration. Therefore, the Pyk2/STAP-2 interaction and STAP-2-dependent increase in phosphorylation of Pyk2 are critical for maximal SDF-1 $\alpha$ -induced T-cell chemotaxis.

Both PH and SH2-like domains of STAP-2 are involved in the association with Pyk2. The PH domain of STAP-2 associates with STAT5, Epstein-Barr virus-derived latent membrane protein-1 (LMP1), and the macrophage colony stimulating factor receptor [16,27,28]. The SH2-like domain also associates with many signaling molecules, including STAT5, myeloid differentiation factor 88, E3 ubiquitin ligase CBL, focal adhesion kinase and LMP1, as well as Vav1 [16,22,24,27,29,30,31,32]. In the cases of the association of STAP-2 with STAT5 and LMP1, both PH and SH2-like domains are involved. For the association of STAP-2 with Vav1, both SH2-like and proline-rich domains are involved in the interaction. It is not clear why and how STAP-2 uses plural sites to recognize one molecule. Pyk2 has been shown to be involved in SDF-1 $\alpha$ -induced chemotaxis by forming signaling complexes with Paxillin, Crk and p130Cas in breast cancer cells [6]. Pyk2 is tyrosine-phosphorylated after SDF-1 $\alpha$ /CXCR4 activation and associates with ZAP-70 and Vav in Jurkat cells [27]. Pyk2 also participates in the activation of MAPK signaling. Interestingly, another

adaptor protein, Dok1, is also tyrosine-phosphorylated after SDF-1 $\alpha$ /CXCR4 activation and associates with RasGAP, Nck and Crk-L in Jurkat cells [27]. However, Dok1 plays a negative role in SDF-1 $\alpha$ /CXCR4-mediated chemotaxis and MAPK activation, suggesting that Dok1 shows the opposite effect on the Pyk2–STAP-2 axis compared with SDF-1 $\alpha$ /CXCR4-mediated signaling in T-cells. In the present study, we have described the interactions between STAP-2 and Pyk2 that lead to enhanced SDF-1 $\alpha$ -induced T-cell migration. We also reported that STAP-2 can bind to Vav to enhance T-cell migration in response to SDF-1 $\alpha$ . Taken together, STAP-2 is involved in signals mediated by SDF-1 $\alpha$ /CXCR4 at two steps: Pyk2 and Vav1. Our data show that the binding of Pyk2 to Vav1 was not changed by overexpression of STAP-2 and that the binding of Pyk2 to STAP-2 was not changed by overexpression of Vav1. Thus, although Pyk2, Vav1, and STAP-2 bind to each other, their binding seems to be independent.

In our previous study, we showed that STAP-2 Y250 is a major site for phosphorylation by Brk and critical for Brk-mediated STAT3 transactivation [15]. In chronic myeloid leukemia, BCR-ABL also phosphorylates STAP-2 Y250 and the phosphorylated STAP-2 in turn upregulates BCR-ABL phosphorylation, leading to enhanced activation of downstream signaling molecules including ERK, STAT5, BCL-xL and BCL-2 [28]. We herein identified that Y250 of STAP-2 is also phosphorylated by Pyk2. Of note, STAP-2 phosphorylation has the capacity to enhance Pyk2 phosphorylation, thus, interactions between Pyk2 and STAP-2 are likely to phosphorylate and activate each other. Indeed, STAP-2 phosphorylation upregulates the binding between Pyk2 and STAP-2. Therefore, tyrosine phosphorylation at position 250 on STAP-2 plays a crucial role in the enhancement of SDF-1 $\alpha$ /CXCR4/Pyk2-related signals.

The SDF-1 $\alpha$ /CXCR4 axis plays a role in lymphocyte trafficking and hematopoiesis as well as tumor metastasis. In addition, cancer immunotherapies, such as antibodies targeting immune checkpoints, chimeric antigen receptor T-cells, and anti-cancer vaccines, require efficient homing of endogenous and/or adoptively transferred effector T-cells into target tumor tissues. Thus, understanding of the multi-step lymphocyte trafficking cascades is vital for advances in immunotherapy. We have shown that STAP-2 regulates multiple steps in SDF-1 $\alpha$ /CXCR4-mediated signaling, leading to enhanced lymphocyte migration. Moreover, we have reported that STAP-2 is involved in several events of the immune response, including the decrease in T-cell adhesion to fibronectin and the enhancement of activation-induced cell death [23]. In macrophages, STAP-2 enhanced IL-6 production after lipopolysaccharide-stimulation [22]. Taken together, STAP-2 is likely to control immune responses at multiple steps *in vivo*. Further analysis as well as new findings in this study regarding STAP-2 will provide a novel strategy to improve cancer immunotherapies and/or to modulate infection/inflammation. Therefore, STAP-2 is likely to be a possible candidate for therapeutic drug development.

### **Conflict of interest**

The authors have no conflicting financial interests.

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## References

- [1] J.W. Griffith, C.L. Sokol, A.D. Luster, Chemokines and chemokine receptors: positioning cells for host defense and immunity, *Annu Rev Immunol* 32 (2014) 659-702.
- [2] D. Rossi, A. Zlotnik, The biology of chemokines and their receptors, *Annu Rev Immunol* 18 (2000) 217-242.
- [3] C.C. Bleul, M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski, T.A. Springer, The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry, *Nature* 382 (1996) 829-833.
- [4] K. Balabanian, B. Lagane, S. Infantino, K.Y. Chow, J. Harriague, B. Moepps, F. Arenzana-Seisdedos, M. Thelen, F. Bachelier, The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes, *J Biol Chem* 280 (2005) 35760-35766.
- [5] R.K. Ganju, S.A. Brubaker, J. Meyer, P. Dutt, Y. Yang, S. Qin, W. Newman, J.E. Groopman, The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways, *J Biol Chem* 273 (1998) 23169-23175.
- [6] A.Z. Fernandis, A. Prasad, H. Band, R. Klosel, R.K. Ganju, Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells, *Oncogene* 23 (2004) 157-167.
- [7] S. Avraham, R. London, Y. Fu, S. Ota, D. Hiregowdara, J. Li, S. Jiang, L.M. Pasztor, R.A. White, J.E. Groopman, et al., Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain, *J Biol Chem* 270 (1995) 27742-27751.

- [8] S. Lev, H. Moreno, R. Martinez, P. Canoll, E. Peles, J.M. Musacchio, G.D. Plowman, B. Rudy, J. Schlessinger, Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions, *Nature* 376 (1995) 737-745.
- [9] A. Menegon, F. Burgaya, P. Baudot, D.D. Dunlap, J.A. Girault, F. Valtorta, FAK+ and PYK2/CAKbeta, two related tyrosine kinases highly expressed in the central nervous system: similarities and differences in the expression pattern, *Eur J Neurosci* 11 (1999) 3777-3788.
- [10] Z.Y. Liu, R.K. Ganju, J.F. Wang, K. Schweitzer, B. Weksler, S. Avraham, J.E. Groopman, Characterization of signal transduction pathways in human bone marrow endothelial cells, *Blood* 90 (1997) 2253-2259.
- [11] R. Guinamard, M. Okigaki, J. Schlessinger, J.V. Ravetch, Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response, *Nat Immunol* 1 (2000) 31-36.
- [12] C.A. Lipinski, N.L. Tran, E. Menashi, C. Rohl, J. Kloss, R.C. Bay, M.E. Berens, J.C. Loftus, The tyrosine kinase pyk2 promotes migration and invasion of glioma cells, *Neoplasia* 7 (2005) 435-445.
- [13] A. Gutenberg, W. Bruck, M. Buchfelder, H.C. Ludwig, Expression of tyrosine kinases FAK and Pyk2 in 331 human astrocytomas, *Acta Neuropathol* 108 (2004) 224-230.
- [14] M. Minoguchi, S. Minoguchi, D. Aki, A. Joo, T. Yamamoto, T. Yumioka, T. Matsuda, A. Yoshimura, STAP-2/BKS, an adaptor/docking protein, modulates STAT3 activation in acute-phase response through its YXXQ motif, *J Biol Chem* 278 (2003) 11182-11189.

- [15] Y. Sekine, S. Tsuji, O. Ikeda, M. Kakisaka, K. Sugiyama, A. Yoshimura, T. Matsuda, Leukemia inhibitory factor-induced phosphorylation of STAP-2 on tyrosine-250 is involved in its STAT3-enhancing activity, *Biochem Biophys Res Commun* 356 (2007) 517-522.
- [16] Y. Sekine, T. Yamamoto, T. Yumioka, K. Sugiyama, S. Tsuji, K. Oritani, K. Shimoda, M. Minoguchi, A. Yoshimura, T. Matsuda, Physical and functional interactions between STAP-2/BKS and STAT5, *J Biol Chem* 280 (2005) 8188-8196.
- [17] O. Ikeda, Y. Miyasaka, Y. Sekine, A. Mizushima, R. Muromoto, A. Nanbo, A. Yoshimura, T. Matsuda, STAP-2 is phosphorylated at tyrosine-250 by Brk and modulates Brk-mediated STAT3 activation, *Biochem Biophys Res Commun* 384 (2009) 71-75.
- [18] O. Ikeda, Y. Sekine, A. Mizushima, M. Nakasuji, Y. Miyasaka, C. Yamamoto, R. Muromoto, A. Nanbo, K. Oritani, A. Yoshimura, T. Matsuda, Interactions of STAP-2 with Brk and STAT3 participate in cell growth of human breast cancer cells, *J Biol Chem* 285 (2010) 38093-38103.
- [19] O. Ikeda, A. Mizushima, Y. Sekine, C. Yamamoto, R. Muromoto, A. Nanbo, K. Oritani, A. Yoshimura, T. Matsuda, Involvement of STAP-2 in Brk-mediated phosphorylation and activation of STAT5 in breast cancer cells, *Cancer Sci* 102 (2011) 756-761.
- [20] T. Yamamoto, T. Yumioka, Y. Sekine, N. Sato, M. Minoguchi, A. Yoshimura, T. Matsuda, Regulation of FcepsilonRI-mediated signaling by an adaptor protein STAP-2/BSK in rat basophilic leukemia RBL-2H3 cells, *Biochem Biophys Res Commun* 306 (2003) 767-773.

- [21] Y. Sekine, K. Nishida, S. Yamasaki, R. Muromoto, S. Kon, J. Kashiwakura, K. Saitoh, S. Togi, A. Yoshimura, K. Oritani, T. Matsuda, Signal-transducing adaptor protein-2 controls the IgE-mediated, mast cell-mediated anaphylactic responses, *J Immunol* 192 (2014) 3488-3495.
- [22] Y. Sekine, T. Yumioka, T. Yamamoto, R. Muromoto, S. Imoto, K. Sugiyama, K. Oritani, K. Shimoda, M. Minoguchi, S. Akira, A. Yoshimura, T. Matsuda, Modulation of TLR4 signaling by a novel adaptor protein signal-transducing adaptor protein-2 in macrophages, *J Immunol* 176 (2006) 380-389.
- [23] Y. Sekine, C. Yamamoto, M. Kakisaka, R. Muromoto, S. Kon, D. Ashitomi, N. Fujita, A. Yoshimura, K. Oritani, T. Matsuda, Signal-transducing adaptor protein-2 modulates Fas-mediated T cell apoptosis by interacting with caspase-8, *J Immunol* 188 (2012) 6194-6204.
- [24] Y. Sekine, O. Ikeda, S. Tsuji, C. Yamamoto, R. Muromoto, A. Nanbo, K. Oritani, A. Yoshimura, T. Matsuda, Signal-transducing adaptor protein-2 regulates stromal cell-derived factor-1 alpha-induced chemotaxis in T cells, *J Immunol* 183 (2009) 7966-7974.
- [25] M. Lopez-Lago, H. Lee, C. Cruz, N. Movilla, X.R. Bustelo, Tyrosine phosphorylation mediates both activation and downmodulation of the biological activity of Vav, *Mol Cell Biol* 20 (2000) 1678-1691.
- [26] T. Miyazaki, A. Takaoka, L. Nogueira, I. Dikic, H. Fujii, S. Tsujino, Y. Mitani, M. Maeda, J. Schlessinger, T. Taniguchi, Pyk2 is a downstream mediator of the IL-2 receptor-coupled Jak signaling pathway, *Genes Dev* 12 (1998) 770-775.
- [27] S. Okabe, S. Fukuda, Y.J. Kim, M. Niki, L.M. Pelus, K. Ohyashiki, P.P. Pandolfi, H.E. Broxmeyer, Stromal cell-derived factor-1alpha/CXCL12-induced

- chemotaxis of T cells involves activation of the RasGAP-associated docking protein p62Dok-1, *Blood* 105 (2005) 474-480.
- [28] Y. Sekine, O. Ikeda, A. Mizushima, Y. Ueno, R. Muromoto, A. Yoshimura, Y. Kanakura, K. Oritani, T. Matsuda, STAP-2 interacts with and modulates BCR-ABL-mediated tumorigenesis, *Oncogene* 31 (2012) 4384-4396.
- [29] O. Ikeda, Y. Sekine, T. Yasui, K. Oritani, K. Sugiyma, R. Muromoto, N. Ohbayashi, A. Yoshimura, T. Matsuda, STAP-2 negatively regulates both canonical and noncanonical NF-kappaB activation induced by Epstein-Barr virus-derived latent membrane protein 1, *Mol Cell Biol* 28 (2008) 5027-5042.
- [30] O. Ikeda, Y. Sekine, M. Kakisaka, S. Tsuji, R. Muromoto, N. Ohbayashi, K. Oritani, A. Yoshimura, T. Matsuda, STAP-2 regulates c-Fms/M-CSF receptor signaling in murine macrophage Raw 264.7 cells, *Biochem Biophys Res Commun* 358 (2007) 931-937.
- [31] Y. Sekine, C. Yamamoto, O. Ikeda, R. Muromoto, A. Nanbo, K. Oritani, A. Yoshimura, T. Matsuda, The protein content of an adaptor protein, STAP-2 is controlled by E3 ubiquitin ligase Cbl, *Biochem Biophys Res Commun* 384 (2009) 187-192.
- [32] Y. Sekine, S. Tsuji, O. Ikeda, K. Sugiyma, K. Oritani, K. Shimoda, R. Muromoto, N. Ohbayashi, A. Yoshimura, T. Matsuda, Signal-transducing adaptor protein-2 regulates integrin-mediated T cell adhesion through protein degradation of focal adhesion kinase, *J Immunol* 179 (2007) 2397-2407.

## Figure legends

### Figure 1. Interaction of STAP-2 with Pyk2

(A) Jurkat/vector and Jurkat/STAP-2 cells were stimulated with 50 ng/ml SDF-1 $\alpha$  for indicated period. The cells were lysed and immunoprecipitated with anti-Myc Ab and immunoblotted by anti-Pyk2, anti-phospho Pyk2 (Y402) and anti-Myc. An aliquot of total cell lysates (TCL) was immunoblotted by anti-Myc, anti-Pyk2 and anti-Actin Abs.

(B) HUT78 cells ( $1 \times 10^8$ ) were lysed, immunoprecipitated with control IgG or anti-Pyk2 antibody, and immunoblotted with anti-STAP-2 Ab. An aliquot of TCL was immunoblotted by anti-STAP-2 and anti-Pyk2 Abs.

(C) Scheme of full-length (FL) and truncated forms of GST-STAP-2 or Myc-STAP-2 used for coimmunoprecipitation assay

(D) 293T cells were transfected with Myc-Pyk2 and GST-STAP-2 FL/truncated forms. At 48 h after transfection, the cells were lysed and immunoprecipitated with anti-Myc Ab and immunoblotted with anti-GST and anti-Myc Abs. An aliquot of TCL was blotted with anti-GST and anti-Myc Abs.

(E) 293T cells were transfected with Myc-Pyk2 and Myc-STAP-2 FL/domain-deleted forms. At 48 h after transfection, the cells were lysed and immunoprecipitated with anti-Pyk2 Ab and immunoblotted with anti-Myc Abs. An aliquot of TCL was blotted with anti-Pyk2 and anti-Myc Abs.

(F) 293T cells were transfected with Vav1, GST-STAP-2 and Myc-Pyk2. At 48 h after transfection, the cells were lysed and immunoprecipitated with anti-Myc Ab and immunoblotted with anti-Vav1, anti-GST and anti-Myc Abs. An aliquot of TCL was blotted with anti-Vav1, anti-GST and anti-Myc. All experiments are representative of three individual experiments.

### Figure 2. Contribution of tyrosine at 250 in STAP-2 to the phosphorylation by Pyk2

(A) 293T cells were transfected Myc-STAP-2 WT/YF mutants with or without Myc-Pyk2. At 48 h after transfection, the cells were lysed and immunoblotted with anti-phosphotyrosine (PY) and anti-Myc Abs. (B) 293T cells were transfected Myc-STAP-2 with or without Myc-Pyk2. At 48 h after transfection, the cells were treated with or without AG17 (40  $\mu$ M, 200 $\mu$ M). At 3 h after treatment, the cells were lysed and immunoblotted with anti-PY and anti-Myc mAbs. (B) 293T cells were transfected Myc-STAP-2 with or without Pyk2 or PKM. At 48 h after transfection, the cells were lysed and immunoblotted with anti-PY, anti-Myc, anti-phospho Pyk2(Y402) and anti-Pyk2 Abs. All experiments are representative of three individual experiments.

Figure 3. Contribution of tyrosine at 250 in STAP-2 to the association with Pyk2

(A) 293T cells were transfected with Myc-Pyk2 and FLAG-STAP-2 WT/Y250F mutant. At 48 h after transfection, cells were lysed and immunoprecipitated with anti-Myc Ab and immunoblotted with anti-FLAG and anti-Myc Abs. An aliquot of total cell lysates (TCL) was blotted with anti-FLAG and anti-Myc Abs. (B) 293T cells were transfected with Myc-STAP-2 and Pyk2WT or PKM mutant. At 48 h after transfection, cells were lysed and immunoprecipitated with anti-Pyk2 Ab and immunoblotted with anti-Myc and anti-Pyk2 Abs. An aliquot of TCL was blotted with anti-Myc and anti-Pyk2 Abs. All experiments are representative of three individual experiments.

Figure 4. Involvement of Pyk2 kinase activity in STAP-2-dependent enhancement of SDF-1 $\alpha$ -induced T-cell chemotaxis

(A) Jurkat/STAP-2 cells were transfected with or without Pyk2 siRNA. At 36 h after transfection, the cells were treated with or without SDF-1 $\alpha$  (50 ng/ml) Cells were lysed

and immunoblotted with anti-Pyk2, anti-phospho Erk, anti-Erk and anti-Actin Abs (B) Control siRNA- and Pyk2 siRNA-transfected Jurkat cells were stimulated with 50 mg/ml SDF-1 $\alpha$  and chemotaxis was analyzed using Transwell chamber after 8 h stimulation. These experiments were performed three times independently. Data are shown mean +/- SEM \*, P < 0.01 (C) Jurkat/STAP-2 cells were treated with or without AG17 (40  $\mu$ M, 200 $\mu$ M) for 3 h and stimulated with 50 ng/ml SDF-1 $\alpha$ . The cells were lysed and immunoprecipitated with anti-Pyk2 Ab and immunoblotted with anti-phospho Pyk2 (Y402), anti-Pyk2, anti-phospho Erk, anti-Erk, anti-Myc and anti-Actin Abs (D) Jurkat/STAP-2 cells were pretreated with AG17 (40  $\mu$ M, 200 $\mu$ M) for 3 h and stimulated with 50 ng/ml SDF-1 $\alpha$ . SDF-1 $\alpha$ -induced chemotaxis was analyzed using Transwell chamber after 8 h stimulation. These experiments were performed three times independently. Data are shown mean +/- SEM. \*, P < 0.01

**Figure 1**

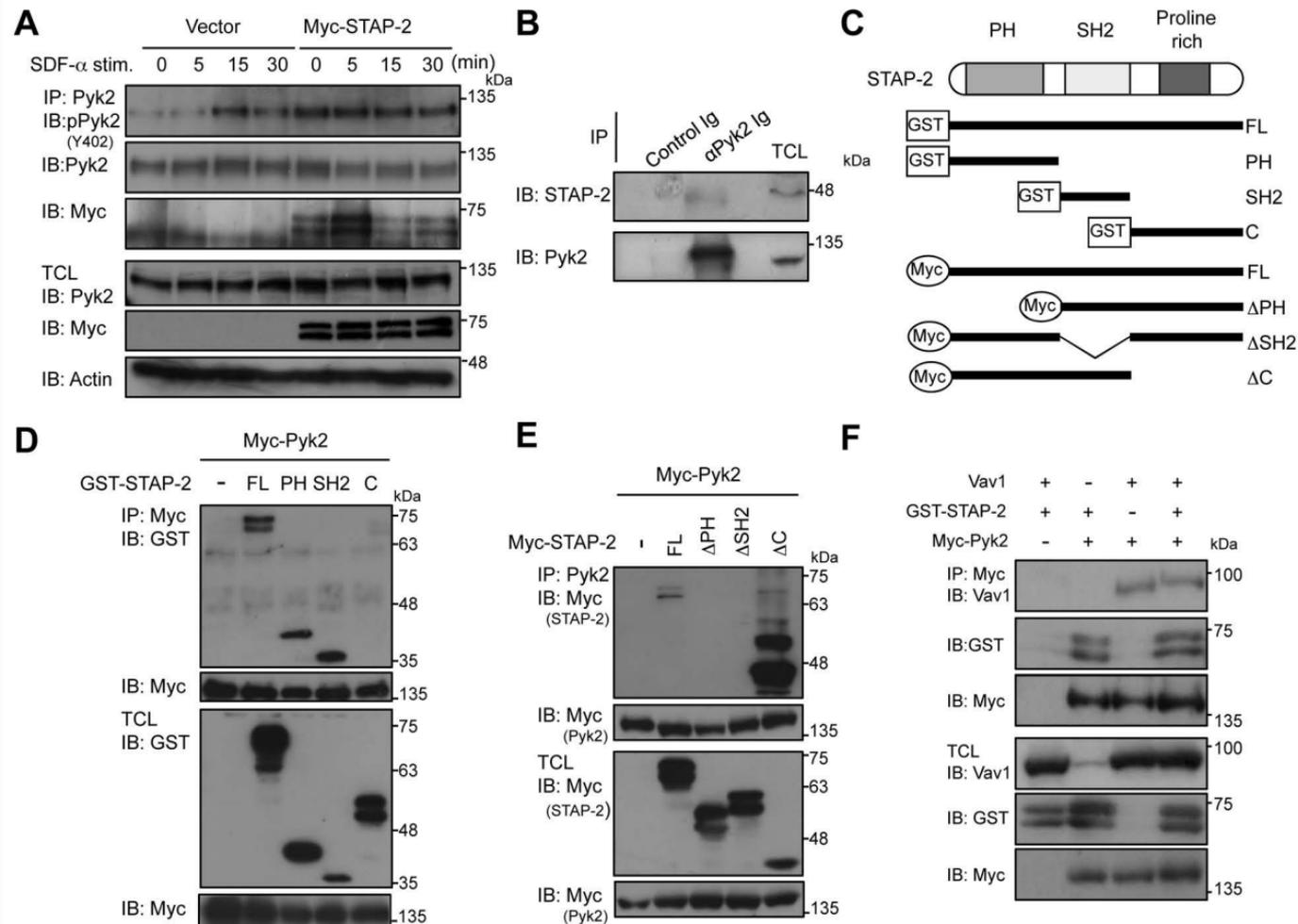
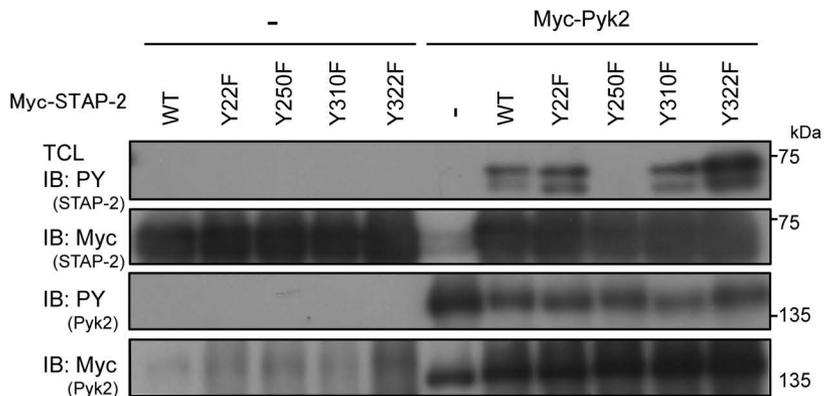
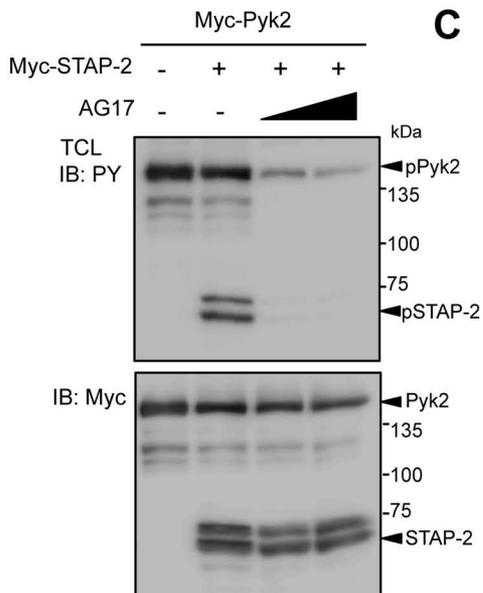


Figure 2

**A**



**B**



**C**

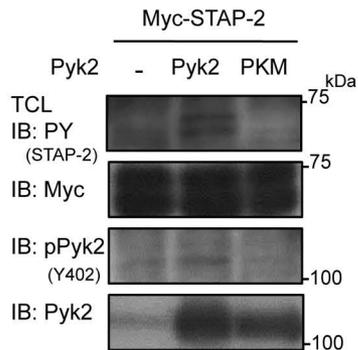


Figure 3

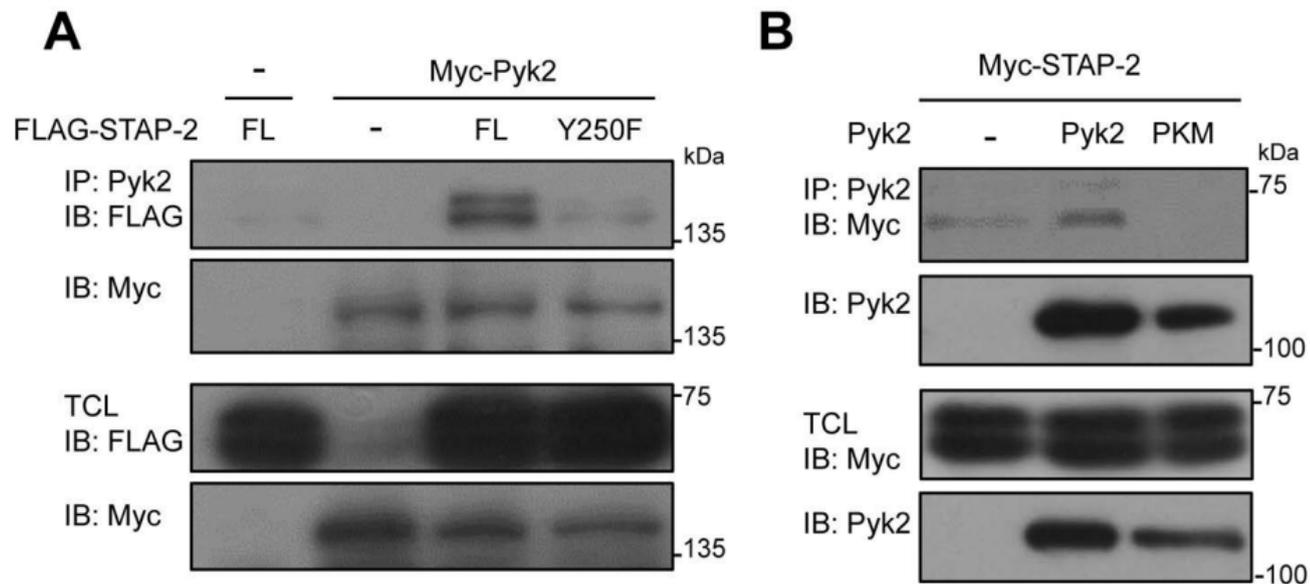


Figure 4

