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Title: The protein content of an adaptor protein, STAP-2 is controlled by E3 ubiquitin ligase Cbl

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Running title: Cbl controls STAP-2 activity through protein degradation

Keywords: STAP-2, Cbl, protein degradation

Abstract

Signal transducing adaptor protein-2 (STAP-2) is a recently identified adaptor protein that contains pleckstrin and Src homology 2 (SH2)-like domains as well as a YXXQ motif in its C-terminal region. Our previous study in T cells demonstrated that STAP-2 influences FAK protein levels through recruitment of E3 ubiquitin ligase, Cbl, to FAK. In the present study, we found that Cbl directly controls the protein levels and activity of STAP-2. STAP-2 physically interacted with Cbl through its PH and SH2-like domains. Small-interfering RNA-mediated reduction of endogenous Cbl restored STAP-2 protein levels. In contrast, over-expression of Cbl induced STAP-2 degradation. Importantly, Cbl-mediated regulation of STAP-2 protein levels affected Brk/STAP-2-induced STAT3 activation. These results indicate that Cbl regulates STAP-2 protein levels and Brk/STAP-2-mediated STAT3 activation.

Introduction

Signal transducing adaptor protein-2 (STAP-2), whose human homolog is identical to a recently cloned adaptor molecule, BKS, a substrate of Brk (breast tumor kinase) is expressed in a variety of tissues and cells, such as lymphocytes, macrophages and hepatocytes, and its abundant expression pattern has suggested a wide range of functions *in vivo* [1; 2]. STAP-2 contains an N-terminal pleckstrin homology (PH) region, a YXXQ motif in its C-terminal region and a region distantly related to the SH2 domain and the proline-rich, tyrosine phosphorylation motifs. Through these functional domains, STAP-2 can bind to a number of signaling molecules and regulate the activities of its binding partners. For example, STAP-2 interacts with STAT3 through its YXXQ motif [1] and with STAT5 through its PH and SH2-like domains [3], resulted in influencing their transcriptional activity. The SH2-like domain of STAP-2 binds to MyD88 and IKK- α/β , thereby modulating TLR4-mediated cytokine production and NF- κ B activation [4]. Both the SH2-like domain and the C-terminal proline-rich region of STAP-2 are required for the binding to PLC- β 1, and the interaction leads to negative regulation of Fc γ RI-mediated signals [5]. Although STAP-2-knockout mice did not show severe abnormalities, LPS- or IL-6-stimulated acute phase protein gene induction was significantly decreased in STAP-2-deficient hepatocytes. In addition, STAP-2-deficient T cells showed the increased FAK protein content and the enhanced adhesion to fibronectin [6]. The above information is likely to indicate the critical involvement of STAP-2 in the processes of immune and inflammatory responses.

Despite of clarifying STAP-2 as the characteristics of an adaptor protein, the regulatory mechanisms for STAP-2 itself are largely unknown. In this manuscript, we

have found that STAP-2 protein is degraded dependently of Casitas B-lineage Lymphoma (Cbl) protein. Cbl is composed of an N-terminal tyrosine-kinase-binding domain, ring-finger motif, a proline-rich region and a C-terminal ubiquitin-associated domain. Cbl is believed to function mainly as a negative regulator of signal transduction pathways, largely via its E3 ubiquitin ligase activity. Cbl mediates degradation of various receptors, kinases and signaling molecules, such as EGF receptor [7], STAT5 [8] and Syk [9].

In the present study, we found that STAP-2 physically interacted with Cbl through its PH and SH2-like domains. Small-interfering RNA (siRNA)-mediated reduction of endogenous Cbl elevated STAP-2 protein levels. In contrast, over-expression of Cbl induced STAP-2 degradation. Furthermore, Cbl-mediated regulation of STAP-2 protein levels affected Brk/STAP-2-induced STAT3 activation. Therefore, our findings propose a novel regulatory mechanism of STAP-2, an important modulator of both immune and inflammatory systems.

Material and Methods

Reagents and antibodies

The proteasome inhibitor MG132 was purchased from Peptide Institute (Osaka, Japan). Cycloheximide (CHX) was purchased from WAKO Chemicals (Osaka, Japan). FLAG-tagged Brk and STAT3-LUC were provided by Dr. A. Harvey (Brunel University, Middlesex, UK), Dr. T. Hirano (Osaka University, Osaka, Japan), respectively [10; 11]. Expression vectors for epitope-tagged STAP-2, glutathione S-transferase (GST)-fusion STAP-2 mutants (GST-STAP-2 PH (1-147 aa), GST-STAP-2 SH2 (148-243 aa), and GST-STAP-2 C (244-403 aa)), Myc-tagged Cbl constructs were described previously [6, 12]. Anti-Cbl, anti-GST and anti-STAT3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin, anti-Myc, anti-FLAG M2 antibody and rabbit polyclonal anti-FLAG antibody were purchased from Sigma (St Louis, MO).

Cell culture, transfection and luciferase assays

Human T-cell leukemia cell line, Jurkat was maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS). Human embryonic kidney carcinoma cell line 293T, human hepatoma cell line Hep3B and human cervix carcinoma cell line HeLa were maintained in DMEM containing 10% FCS. Stable transformants expressing Myc-tagged STAP-2 in Jurkat [3], HeLa [13] and Hep3B [14] were established as described previously and maintained in the above medium in the presence of G418 (0.5 mg/ml). 293T was transfected by the standard calcium precipitation protocol. Luciferase assay was performed as described [3]. At 36 h after transfection, the cells were lysed in 50 μ l of Reporter Lysis Buffer (Promega, Madison, WI) and assayed for luciferase

activities according to the manufacturer's instructions. Luciferase activities were normalized to the β -galactosidase activities. Three or more independent experiments were carried out for each assay. HeLa was transfected using jetPEI (Polytransfection, Illkirch, France). At 36 h after transfection, the cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Three or more independent experiments were carried out for each assay.

Transfection of Small Interfering RNA (siRNA)

HeLa or Hep3B cells were plated on a 24-well plate at 2×10^4 cells/well, and then incubated with control or Cbl siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37 °C for 4 hrs [15]. The HeLa cells were then transfected with or without STAT3-LUC together with or without respective plasmid using jetPEI. Jurkat T cells were nucleofected with control or Cbl siRNA using the Cell Line Nucleofector Kit V (Amaxa Biosystems, Gaithersburg, MD) [6].

Immunoprecipitation and immunoblotting

The immunoprecipitation and Western blotting assays were performed as described previously [3]. The cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride). 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).

Indirect Immunofluorescence

HeLa cells were maintained in DMEM containing 10% FCS transfected with FLAG-STAP-2 and Myc-Cbl by jetPEI. At 48h after transfection, cells were fixed with a solution containing 4% paraformaldehyde and reacted with anti-FLAG and anti-Myc antibodies. The cells were then reacted with FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG antibodies (CHEMICON, Temecula, CA) and observed under a confocal laser fluorescent microscope. DNA was visualized by DAPI (Wako Chemicals, Osaka, Japan) staining. Images were obtained by using a Zeiss LSM 510 laser scanning microscope with an Apochromat x63/1.4 oil immersion objective and x4 zoom.

Statistical methods

The significance of differences between group means was determined by Student's *t*-test.

Results and discussion

Cbl directly interacts with STAP-2

We previously demonstrated that STAP-2-expressing Jurkat T cells show an enhanced degradation of FAK proteins, resulting in reduced T cell adhesion to fibronectin. We have also shown that this effect of STAP-2 is mediated by the formation of a complex containing FAK and Cbl. This finding led us to investigate whether STAP-2 directly interacts with Cbl *in vivo*. Myc-tagged Cbl was expressed with or without FLAG-tagged STAP-2 in 293T cells. The cells were lysed, and the lysates were immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-Myc antibody. As shown in Fig. 1A, association of STAP-2 with Cbl was observed in 293T cells. We also examined cellular co-localization of STAP-2 and Cbl *in vivo* using confocal microscopy. HeLa cells were transfected with FLAG-tagged STAP-2 and Myc-tagged Cbl. As shown in Fig. 1B, STAP-2 was localized throughout the cytoplasm and nucleus. Cbl was mainly localized in the cytoplasm. Importantly, cytoplasmic STAP-2 co-localized with Cbl. As described above, the domain structure of STAP-2 contains several potential tyrosine phosphorylation sites, as well as PH- and SH2-like domains, and STAP-2 shows the characteristics of an adaptor protein that can interact with several other signaling molecules. Indeed, STAP-2 binds to not only STAT3 and STAT5, but also to MyD88 and I κ B kinase through these domain structures. To determine the domain of STAP-2 involved in binding to Cbl, a series of STAP-2 deletion mutants fused with GST (GST-STAP-2 PH, GST-STAP-2 SH2-like and GST-STAP-2 C) were used (Fig. 1C). The respective mutant constructs were transiently expressed in 293T cells together

with Myc-tagged Cbl. The binding potential of these mutant proteins with Cbl was examined by pull-down assays with glutathione-sepharose, followed by western blot analysis with an anti-Myc antibody (Fig. 1D). The precipitates of the GST-STAP-2 PH and SH2-like proteins contained Cbl protein, but no Cbl protein was detected in the GST-STAP-2 C precipitate. Therefore, the PH and SH2-like domains of STAP-2 are able to interact with Cbl.

Cbl regulates STAP-2 protein levels

Cbl regulates the levels of various proteins through its E3 ubiquitin ligase activity. Therefore, to assess the functional relevance of interaction between STAP-2 and Cbl, we focused on the proteasome-dependent degradation of STAP-2 by Cbl. To this end, we used siRNA to reduce endogenous expression of Cbl in several STAP-2 expressing transformants (HeLa/STAP-2, Hep3B/STAP-2 and Jurkat/STAP-2). These transformants were transfected with a specific siRNA for Cbl or with a control siRNA. Total cellular proteins from the transfected cells were subjected to western blot analysis, which confirmed a reduction of Cbl protein levels. As shown in Fig. 2A, siRNA-mediated knockdown of Cbl in these cells resulted in a significant increase of STAP-2 protein levels compared with control siRNA-transfected cells. We also tested the effect of CHX, using siRNA-transfected HeLa cells, to confirm whether Cbl is endogenously involved in STAP-2 stability. As shown in Fig. 2B, Cbl siRNA-transfected HeLa cells showed enhanced stability of endogenous STAP-2 even in the presence of CHX. These results indicate that Cbl controls STAP-2 protein levels via proteasomal degradation. Moreover, the treatment of HeLa cells with MG132, an inhibitor of proteasome-dependent

degradation, resulted in an enhancement of STAP-2 protein levels (Fig. 2C). Importantly, efficient co-expression of STAP-2 with Cbl in 293T cells induced a marked decrease of STAP-2 protein levels, which was recovered by the treatment of MG132 (Fig. 2D). These results indicate that the protein level of STAP-2 is endogenously controlled by Cbl via the proteasome degradation pathway.

Cbl negatively regulates Brk/STAP-2-mediated STAT3 activation through degradation of STAP-2

To elucidate whether the degradation of STAP-2 by Cbl has any functional significance, we examined effects on STAP-2/Brk-mediated STAT3 activation. Recently, we have demonstrated that a protein tyrosine kinase, Brk, efficiently induces STAT3 activation in the presence of STAP-2 [16]. The STAP-2/Brk-mediated STAT3 activation was monitored by transient transfection experiments using STAT3-LUC, in which the α 2-macroglobulin promoter drives expression of a luciferase (LUC) reporter gene. We first tested the effect of treatment with MG132 on STAP-2/Brk-mediated STAT3 activation. As shown Fig. 3A, MG132 treatment augmented STAP-2/Brk-mediated STAT3 activation in HeLa cells. MG132 treatment increased STAP-2 protein levels in parallel with an enhancement of STAT3 activity but did not affect Brk protein levels (Fig. 3A, lower panels). We next examined whether Cbl expression had any effects on STAP-2/Brk-mediated STAT3 activation. STAP-2/Brk-mediated STAT3 activation was suppressed by Cbl in a dose-dependent manner (Fig. 3B), in parallel with a reduction of STAP-2 protein levels (Fig. 3B, lower panel), although Cbl expression had no effect on the protein levels of STAT3 (data not shown). Similar results were obtained from the same experiments using 293T cells (data not shown). These data indicate that

degradation of STAP-2 by Cbl resulted in a decrease of STAT3 activation by Brk/STAP-2. We further tested whether this effect was recovered by treatment with MG132. As shown in Fig. 3C, the Cbl-mediated reduction of Brk/STAP-2-mediated STAT3 activation and STAP-2 protein levels were restored by treatment with MG132. To further verify the role of endogenous Cbl in Brk/STAP-2-mediated STAT3 activation, we tested the effect of Cbl siRNA on Brk-induced STAT3-LUC activation in HeLa/STAP-2 transformants. In HeLa/STAP-2 cells, Brk expression induced STAT3 activation in a dose-dependent manner (Fig. 3D). siRNA-mediated reduction of endogenous Cbl expression resulted in a significant enhancement of Brk-induced STAT3-LUC activation in these cells (Fig. 3D, upper panel). Importantly, the protein levels of STAP-2 were also increased by Cbl knockdown (Fig. 3D, lower panel). No significant alteration of Brk protein levels was observed by Cbl knockdown. These results indicate that Cbl regulates STAP-2 activity via proteasomal degradation.

Concluding remarks

Although the biological activities of signaling molecules can be determined, in part, by regulating their levels through protein degradation, activation-induced protein degradation may help to eliminate activated signaling molecules, thus attenuating signaling. Previously, we have shown that STAP-2 shows the characteristics of an adaptor protein that can interact with several other signaling molecules and that it regulates a variety of signaling pathways. However, only tyrosine-phosphorylation of STAP-2 has been demonstrated to modulate its functions [14]. In this study, we demonstrated, for the first time, a mechanism that regulates STAP-2 activity through Cbl-mediated proteasome-dependent degradation. The results of the present study clearly indicate that STAP-2 is constitutively degraded via interaction with Cbl and that a proteasome inhibitor blocks the degradation of STAP-2. Importantly, Cbl-mediated regulation of STAP-2 protein levels controls Brk/STAP-2-induced STAT3 activation.

We have previously shown that STAP-2 induces proteasomal degradation of FAK by recruiting Cbl, resulting in a decrease of T cell adhesion to fibronectin [6]. This finding suggests that the STAP-2-Cbl axis may regulate T cell receptor (TCR)-mediated signaling, although, we do not know at present whether STAP-2 interacts with other signaling molecules downstream of the TCR. Another adaptor protein, Src-like adaptor protein-2 (SLAP-2), which possesses a short amino-terminal region followed by SH3 and SH2 domains, is reported to be involved in the regulation of T cell receptor signaling through Cbl [17, 18]. SLAP-2 interacts with Cbl through its carboxy-terminal region and negatively regulates tyrosine kinase activity of ZAP-70 in a proteasome degradation-dependent manner [18].

STAP-2 is tyrosine-phosphorylated by epidermal growth factor (EGF) in NIH-3T3 cells [1]. Cbl also plays a critical role in the EGF/EGF receptor signaling pathway [7, 19]. Therefore, STAP-2 may participate in the regulation of EGF/EGF receptor signaling pathways through Cbl. We have also previously demonstrated that STAP-2 negatively regulates the c-Fms-mediated signaling pathway in macrophages [20]. Cbl terminates c-Fms signaling by binding to phosphorylated Tyr-973 in the C-terminus of activated c-Fms and by recruiting E2 ubiquitin ligases to c-Fms [21, 22]. Subsequent polyubiquitination of c-Fms by the E2 ubiquitin ligases targets it for internalization and degradation [23]. It is possible that STAP-2 interacts with Cbl to regulate c-Fms-mediated signaling in macrophages.

It is still unclear whether Cbl effectively induces ubiquitination of STAP-2, because co-expression of STAP-2 and ubiquitin produced aberrant ubiquitination of STAP-2 in the absence of Cbl. Therefore, further detailed study will be required to elucidate the regulatory mechanisms of STAP-2 ubiquitination. Determining the degradation mechanisms of STAP-2 will provide novel functions of STAP-2 in several signaling pathways.

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References

- [1] M. Minoguchi, S. Minoguchi, D. Aki, A. Joo, T. Yamamoto, T. Yumioka, T. Matsuda, and 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) 11182-9.
- [2] P.J. Mitchell, E.A. Sara, and M.R. Crompton, A novel adaptor-like protein which is a substrate for the non-receptor tyrosine kinase, BRK. *Oncogene* 19 (2000) 4273-82.
- [3] Y. Sekine, T. Yamamoto, T. Yumioka, K. Sugiyama, S. Tsuji, K. Oritani, K. Shimoda, M. Minoguchi, A. Yoshimura, and T. Matsuda, Physical and functional interactions between STAP-2/BKS and STAT5. *J Biol Chem* 280 (2005) 8188-96.
- [4] Y. Sekine, T. Yumioka, T. Yamamoto, R. Muromoto, S. Imoto, K. Sugiyama, K. Oritani, K. Shimoda, M. Minoguchi, S. Akira, A. Yoshimura, and T. Matsuda, Modulation of TLR4 signaling by a novel adaptor protein signal-transducing adaptor protein-2 in macrophages. *J Immunol* 176 (2006) 380-9.
- [5] T. Yamamoto, T. Yumioka, Y. Sekine, N. Sato, M. Minoguchi, A. Yoshimura, and 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) 767-73.
- [6] Y. Sekine, S. Tsuji, O. Ikeda, K. Sugiyama, K. Oritani, K. Shimoda, R. Muromoto, N. Ohbayashi, A. Yoshimura, and T. Matsuda, Signal-transducing adaptor protein-2 regulates integrin-mediated T cell adhesion through protein degradation of focal adhesion kinase. *J Immunol* 179 (2007) 2397-407.
- [7] G. Levkowitz, H. Waterman, S.A. Ettenberg, M. Katz, A.Y. Tsygankov, I. Alroy, S. Lavi, K. Iwai, Y. Reiss, A. Ciechanover, S. Lipkowitz, and Y. Yarden, Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol Cell* 4

- (1999) 1029-40.
- [8] E.L. Goh, T. Zhu, W.Y. Leong, and P.E. Lobie, c-Cbl is a negative regulator of GH-stimulated STAT5-mediated transcription. *Endocrinology* 143 (2002) 3590-603.
- [9] N. Rao, A.K. Ghosh, S. Ota, P. Zhou, A.L. Reddi, K. Hakezi, B.K. Druker, J. Wu, and H. Band, The non-receptor tyrosine kinase Syk is a target of Cbl-mediated ubiquitylation upon B-cell receptor stimulation. *Embo J* 20 (2001) 7085-95.
- [10] P.J. Mitchell, K.T. Barker, J.E. Martindale, T. Kamalati, P.N. Lowe, M.J. Page, B.A. Gusterson, and M.R. Crompton, Cloning and characterisation of cDNAs encoding a novel non-receptor tyrosine kinase, brk, expressed in human breast tumours. *Oncogene* 9 (1994) 2383-90.
- [11] K. Nakajima, Y. Yamanaka, K. Nakae, H. Kojima, M. Ichiba, N. Kiuchi, T. Kitaoka, T. Fukada, M. Hibi, and T. Hirano, A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1 leukemia cells. *Embo J* 15 (1996) 3651-8.
- [12] M. Yokouchi, T. Wakioka, H. Sakamoto, H. Yasukawa, S. Ohtsuka, A. Sasaki, M. Ohtsubo, M. Valius, A. Inoue, S. Komiya, and A. Yoshimura, APS, an adaptor protein containing PH and SH2 domains, is associated with the PDGF receptor and c-Cbl and inhibits PDGF-induced mitogenesis. *Oncogene* 18 (1999) 759-67.
- [13] O. Ikeda, Y. Sekine, T. Yasui, K. Oritani, K. Sugiyama, R. Muromoto, N. Ohbayashi, A. Yoshimura, and 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) 5027-42.
- [14] Y. Sekine, S. Tsuji, O. Ikeda, M. Kakisaka, K. Sugiyama, A. Yoshimura, and 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) 517-22.
- [15] Y. Sekine, O. Ikeda, Y. Hayakawa, S. Tsuji, S. Imoto, N. Aoki, K. Sugiyama, and T. Matsuda,

- DUSP22/LMW-DSP2 regulates estrogen receptor-alpha-mediated signaling through dephosphorylation of Ser-118. *Oncogene* 26 (2007) 6038-49.
- [16] O. Ikeda, Y. Miyasaka, Y. Sekine, A. Mizushima, R. Muromoto, A. Nanbo, A. Yoshimura and T. Matsuda, STAP-2 is phosphorylated at tyrosine-250 by Brk and modulates Brk-mediated STAT3 activation. *Biochem. Biophys. Res. Commun.* In press.
- [17] T. Sosinowski, N. Killeen, and A. Weiss, The Src-like adaptor protein downregulates the T cell receptor on CD4+CD8+ thymocytes and regulates positive selection. *Immunity* 15 (2001) 457-66.
- [18] M.P. Loreto, D.M. Berry, and C.J. McGlade, Functional cooperation between c-Cbl and Src-like adaptor protein 2 in the negative regulation of T-cell receptor signaling. *Mol Cell Biol* 22 (2002) 4241-55.
- [19] M.H. Schmidt, and I. Dikic, The Cbl interactome and its functions. *Nat Rev Mol Cell Biol* 6 (2005) 907-18.
- [20] O. Ikeda, Y. Sekine, M. Kakisaka, S. Tsuji, R. Muromoto, N. Ohbayashi, K. Oritani, A. Yoshimura, and T. Matsuda, STAP-2 regulates c-Fms/M-CSF receptor signaling in murine macrophage Raw 264.7 cells. *Biochem Biophys Res Commun* 358 (2007) 931-7.
- [21] K. Wilhelmsen, S. Burkhalter, and P. van der Geer, C-Cbl binds the CSF-1 receptor at tyrosine 973, a novel phosphorylation site in the receptor's carboxy-terminus. *Oncogene* 21 (2002) 1079-89.
- [22] A. Mancini, A. Koch, R. Wilms, and T. Tamura, c-Cbl associates directly with the C-terminal tail of the receptor for the macrophage colony-stimulating factor, c-Fms, and down-modulates this receptor but not the viral oncogene v-Fms. *J Biol Chem* 277 (2002) 14635-40.
- [23] P.S. Lee, Y. Wang, M.G. Dominguez, Y.G. Yeung, M.A. Murphy, D.D. Bowtell, and E.R. Stanley, The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation. *Embo J* 18 (1999) 3616-28.

Figure legend

Figure 1. Molecular interactions between STAP-2 and Cbl

A. 293T cells (1×10^5 cells) were transfected with Myc-tagged Cbl (0.3 μ g) with or without FLAG-tagged STAP-2 (5 μ g). At 24 h after transfection, cells were treated with MG132 (10 μ M) for additional 12h. The cells were lysed and immunoprecipitated (IP) with an anti-FLAG antibody and immunoblotted (IB) with anti-Myc and anti-FLAG antibodies. An aliquot of each total cell lysate (TCL) was immunoblotted with anti-Myc and anti-FLAG antibodies.

B. HeLa cells in a 6-well plate were transfected with FLAG-tagged STAP-2 (1 μ g) and Myc-tagged Cbl (1 μ g), using jetPEI. At 36 h after transfection, the cells were fixed, incubated with anti-FLAG and anti-Myc antibodies and visualized with FITC- and rhodamine-conjugated secondary antibodies. The same slide was also stained with DAPI for the nuclei staining. These figures were also merged.

C. Schematic diagram of the domain structures of STAP-2 and its GST-fused mutant fragments.

D. 293T cells (1×10^7 cells/well) were transfected with Myc-tagged Cbl (15 μ g) with or without GST-fused STAP-2 deletion mutants (10 μ g). At 36 h after transfection, the cells were lysed and pulled down with glutathione-Sepharose, and immunoblotted with anti-Myc and anti-GST antibodies. An aliquot of each TCL was immunoblotted with anti-Myc and anti-GST antibodies.

Figure 2. Cbl induces degradation of STAP-2

C. Hep3B/STAP-2 or HeLa/STAP-2 cells in a 24-well plate were transfected with control or Cbl siRNA (15 pmol). Jurkat/STAP2 cells were nucleofected with control or Cbl siRNA (200 pmol) as described in Material and Methods. At 36 h after transfection, cells were lysed, and an aliquot of total cell lysates (TCL) was immunoblotted with anti-Myc, anti-Cbl or anti-actin antibody.

B. HeLa/STAP-2 cells in a 24-well plate were transfected with a control or Cbl siRNA. At 36 h after transfection, cells were treated with CHX (10 μ g/ml) for the indicated periods. Then cells were lysed, and an aliquot of TCL was immunoblotted with anti-Myc (upper panel), anti-Cbl (middle panel) or anti-actin (lower panel) antibody.

C. HeLa/STAP-2 cells in a 12-well plate were treated with DMSO or MG132 for the indicated times. Then cells were lysed, and an aliquot of TCL was immunoblotted with anti-Myc (upper panel) or anti-actin (lower panel) antibody.

D. 293T cells in a 12-well plate were transfected with FLAG-tagged STAP-2 (0.5 μ g) and increasing amounts of Myc-tagged Cbl (0, 0.1 and 0.3 μ g). At 24 h after transfection the cells were treated with DMSO or MG132 (10 μ M) for an additional 12 hours. The cells were lysed, and an aliquot of TCL was immunoblotted with anti-FLAG (upper panel), anti-Myc (middle panel) or anti-actin (lower panel) antibody.

Figure 3. Cbl negatively regulates Brk/STAP-2-mediated STAT3 activation through degradation of STAP-2

A. HeLa cells in a 24-well plate were transfected with STAT3-LUC (0.1 μ g) and/or FLAG-tagged Brk (0.1 μ g), and pcDNA3 empty vector or Myc-tagged STAP-2 (0.1 μ g). Thirty-six hours after transfection, the cells were treated with DMSO or MG132 (10 μ M) for additional 12 h. The cells were then harvested, and luciferase activities were

measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the SD. Total cellular protein collected from parallel cultures was analyzed by Western blot with anti-FLAG, anti-Myc or anti-actin antibody (lower panels).

B. HeLa cells in a 24-well plate were transfected with STAT3-LUC (0.1 μ g) and/or FLAG-tagged Brk (0.1 μ g), and pcDNA3 empty vector or Myc-tagged STAP-2 (0.1 μ g), and increasing amount of Myc-tagged Cbl (0, 0.03 and 0.1 μ g). At 36 h after transfection, the cells were harvested, and luciferase activities were measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the SD. Total cellular protein collected from parallel cultures was analyzed by Western blot with anti-Myc, anti-FLAG or anti-actin antibody (lower panels).

C. HeLa cells in a 24-well plate were transfected with STAT3-LUC (0.1 μ g) and/or FLAG-tagged Brk (0.1 μ g), and pcDNA3 empty vector or Myc-tagged STAP-2 (0.1 μ g), and/or Myc-tagged Cbl (0.1 μ g). At 36 h after transfection, the cells were treated with DMSO or MG132 (10 nM) for additional 12 h. The cells were then harvested, and luciferase activities were measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the SD. Total cellular protein collected from parallel cultures was analyzed by Western blot with anti-Myc, anti-FLAG or anti-actin antibody(lower panels).

D. HeLa/STAP-2 cells in a 24-well plate were transfected with a control or Cbl siRNA. The cells were then transfected with STAT3-LUC (0.1 μ g) and increasing amount of FLAG-tagged Brk (0, 0.1 and 0.3 μ g). At 36 h after transfection, the cells were harvested, and luciferase activities were measured. The results are indicated as fold

induction of luciferase activity from triplicate experiments, and the error bars represent the SD. Total cellular protein collected from parallel cultures was analyzed by Western blot with anti-Cbl, anti-Myc, anti-FLAG or anti-actin antibody (lower panels).

Figure 1

A **B** **C** **D** **Figure 2**

Figure 3