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Title: Positive interactions between STAP-1 and BCR-ABL influence chronic myeloid leukemia cell proliferation and survival

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Abstract

Chronic myeloid leukemia (CML) is a clonal disease characterized by the presence of the Philadelphia chromosome and its oncogenic product, BCR-ABL, which activates multiple pathways involved in cell survival, growth promotion, and disease progression. We recently reported that signal-transducing adaptor protein 1 (STAP-1) is upregulated in CML stem cells (LSCs) and functions to reduce the apoptosis of CML LSCs by upregulating the

STAT5-downstream anti-apoptotic genes. In this study, we demonstrate the detailed molecular interactions among BCR-ABL, STAP-1, and signal transducer and activator of transcription 5 (STAT5). Studies with deletion mutants have revealed that STAP-1 interacts with BCR-ABL and STAT5a through its SH2 and PH domains, respectively, suggesting the possible role of STAP-1 as a scaffold protein. Furthermore, the binding of STAP-1 to BCR-ABL stabilizes the BCR-ABL protein in CML cells. Since STAP-1 is highly expressed in CML cells, we also analyzed the STAP-1 promoter activity using a luciferase reporter construct and found that NFATc1 is involved in activating the STAP-1 promoter and inducing STAP-1 mRNA expression. Our results demonstrate that STAP-1 contributes to the BCR-ABL/STAT5 and BCR-ABL/Ca²⁺/NFAT signals to induce proliferation and STAP-1 mRNA expression in CML cells, respectively.

Keywords: CML; BCR-ABL; STAP-1; STAT5; NFAT

1. Introduction

The BCR-ABL fusion oncogene is involved in chronic myeloid leukemia (CML) pathogenesis [1,2]. CML is characterized by the premature release of leukemia cells from bone marrow, and their substantial accumulation with a potential of differentiation in the hematologic organs. Owing to its autonomous tyrosine kinase activity, BCR-ABL activates several signals, such as the Ras, PI3K/AKT, Janus kinase (JAK)/signal transducer and activator of transcription (STAT), and NF- κ B signaling pathways, leading to peripheral granulocytosis, splenomegaly, and thrombocytosis [1,2]. Since the approval of tyrosine kinase inhibitors (TKIs) for the management of patients with CML, clinical results have successfully improved. However, each TKI has specific off-target adverse effects, which affect the quality of life in patients. In addition, TKI therapy is not completely successful in most patients, and the condition relapses in approximately half of patients within 12 months after discontinuing TKI therapy, even after achieving complete molecular remission [3,4,5].

The proteins in the signal-transducing adaptor proteins-1 (STAP-1) family have a typical structure of adaptor proteins, namely pleckstrin homology (PH) and Src-homology 2 (SH2)-like domains in the N-terminal and C-terminal regions, respectively [6]. Moreover, STAP proteins interact with several tyrosine phosphorylated proteins during inflammatory/immune responses and tumorigenesis [6,7,8,9]. We previously reported that STAP-2, another member of the STAP family, binds to BCR-ABL via its SH2-like domain, and enhances CML cell proliferation and its resistance to imatinib [10 11]. Recently, we also reported that STAP-1 acts as a novel BCR-ABL binding partner and inhibits the apoptosis of CML stem cells (LSCs) by upregulating anti-apoptotic genes, such as BCL-2 and BCL-xL via enhanced STAT5 activity [12]. Notably, STAP-1 mRNA expression in the stem cell subpopulation is significantly higher in patients with CML than that in healthy individuals [9].

In the present study, we have explored the possible molecular mechanisms of STAP-1-induced enhanced BCR-ABL activity and increased STAP-1 expression in CML cells.

2. Materials and Methods

2.1. Reagents and antibodies

Cycloheximide (CHX) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Cyclosporin A (CyA) was purchased from Tokyo Chemical Industries (Tokyo, Japan). Ionomycin was purchased from EMD Millipore (Bedford, MA). Expression vectors for BCR-ABL and STAT5a and their mutant constructs were described previously [8,10]. Myc-tagged STAP-1 was described previously [13]. FLAG-tagged PU.1, RUNX3, NFATc1, and Myc-tagged-STAP-1 deletion mutants were generated by PCR methods and sequenced (primer sequences are available upon request). Anti-Myc, anti-FLAG and anti- β -actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Anti-pSTAT5 (Tyr694) and STAT5 antibodies were purchased from Cell signaling Technologies (Beverly, MA). Anti-ABL antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture and siRNA treatment

Human CML cell line, KU812 was maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS). STAP-1 knockdown KU812 cell lines were established as described previously [12] 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 [14]. For siRNA experiment, KU812 cells were transfected with siRNA by electroporation (950 μ F, 130 V) with a Genepulser II (Bio-Rad Laboratories, Hercules, CA) as previously described [14]. The siRNAs used in this study were as follows: siScramble, 5'-UUCUCCGAACGUGUCACGUTT-3', siNFATc1, 5'-GGUCAUUUUCGUGGAGAAATT-3'.

2.3. RNA isolation and quantitative real-time PCR (qPCR)

Cells were harvested and total RNA was prepared using the TRI Reagent (Molecular Research

Center, Cincinnati, OH, USA). First-strand cDNA was synthesized from 1 µg of total RNA with ReverTra Ace (TOYOBO, Osaka, Japan). Quantitative real-time PCR analysis of transcripts was carried out using a combination of a KAPA SYBR FAST qPCR master mix (KAPA Biosystems, Woburn, MA, USA) with a Mx3005P real-time PCR system (Stratagene, Santa Clara, CA, USA)[15]. qPCR primers used in this study were: *STAP-1*: 5'-TGAGGCCTGGTAGTGACAGTAG-3' (sense), 5'-AGTGCTTGATTCTTGGAATGTCT-3' (antisense); *BCL-2*: 5'-CATGTGTGTGGAGAGCGTCAA-3' (sense), 5'-GCCGGTTCAGGTACTCAGTCA-3' (antisense); *MYC*: 5'-GCTGCTTAGACGCTGGATTTTT-3' (sense), 5'-TCGAGGTCATAGTTCCTGTTGGT-3' (antisense); *NFATc1*: 5'-TCCTCTCCAACACCAAAGTC-3' (sense), 5'-ATGTCCGTCTCTCCTTTCC-3' (antisense); *GAPDH*: 5'-GAAATCCCATCACCATCTTCCAGG-3' (sense), 5'-CAGTAGAGGCAGGGATGATGTTCC-3' (antisense).

2. 4. Immunoprecipitation and western blotting

The immunoprecipitation and western blotting assays were performed as described previously [13]. 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).

2. 5. Luciferase assay

For analysis of human *STAP-1* promoter activity, the *STAP-1*-promoter luciferase reporter (*STAP-1-LUC*) was constructed. In brief, the *STAP-1* promoter region between positions -500 and +x was amplified from human DNA by PCR (primer sequences are available upon request) and inserted into *XhoI*-*HindIII* restriction sites of pGL3-Basic vector (Promega, Madison, WI, USA).

HEK293T cells were grown in a 24 plate semi-confluent and then transfected with expression vectors for FLAG-tagged PU.1, RUNX3 or NFATc1 and STAP-1-LUC using Lipofectamine 2000 according to the manufacturer's instructions. The cells were lysed at 48 h post transfection and then luciferase activity was determined using Dual-Luciferase reporter assay system (Promega, Madison, WI, USA)[15]. Expression of each protein was confirmed by western blotting with anti-FLAG antibody.

2. 6. Statistical analysis

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

3. Results

3. 1. STAP-1 physically interacts with STAT5a and BCR-ABL through its PH and SH2 domains, respectively, to form an effective complex

We recently reported that STAP-1 positively regulates the self-renewal ability of CML LSCs by suppressing apoptosis [12]. STAP-1 deficiency reduced the phosphorylated status of STAT5a and downregulated anti-apoptotic genes, such as BCL-2 and BCL-xL. In addition, BCR-ABL interacts with STAT5a and phosphorylates STAT5a. Thus, STAP-1 directly interacts with both BCR-ABL and STAT5a. However, the detailed molecular mechanisms underlying these interactions are still unclear. Hence, we tried to identify the domain(s) responsible for the interactions among STAP-1, STAT5a, and BCR-ABL.

Myc-tagged STAP-1 deletion mutants (STAP-1 PH and STAP-1 SH2; Fig 1A) were employed to determine the STAP-1 domain(s) involved in the association with BCR-ABL. The respective mutants, together with BCR-ABL, were transiently expressed in 293T cells. The binding potential of these mutants with BCR-ABL was examined by immunoprecipitation with an anti-ABL antibody followed by western blot with an anti-Myc antibody. As shown in Fig 1B, both STAP-1 WT and STAP-1 SH2 strongly interacted with BCR-ABL, whereas STAP-1 PH showed a very weak BCR-ABL binding capacity. Thus, the SH2 domain of STAP-1 is likely to be involved in binding to BCR-ABL. We also determined the STAP-1 domain(s) involved in the association with STAT5a. The respective mutants, together with FLAG-tagged STAT5a, were transiently expressed in 293T cells. As shown in Fig 1C, STAT5a immunoprecipitated in the presence of wild-type STAP-1 and STAP-1 PH, whereas STAP-2 SH2 failed to interact with STAT5a. These results confirmed that the PH domain of STAP-1 is involved in binding to BCR-ABL.

We then determined the STAP-1-interacting domains of STAT5 using several STAT5a deletion mutants (Fig 1D). 293T cells were transfected with Myc-tagged STAP-1 and/or several FLAG-tagged STAT5a deletion mutants. The transfected cells were lysed and then

immunoprecipitated and blotted with anti-Myc and anti-FLAG antibodies, respectively. As shown in Fig 1E, the immunoprecipitants for STAP-1 contained STAT5a (341–496) as well as full-length STAT5a proteins. Thus, the DNA binding domain of STAT5a interacts with STAP-1.

The above data indicate that STAP-1 interacts with BCR-ABL and STAT5a DNA binding domain through its SH2 and PH domains, respectively. These three molecules are likely to form a complex, where STAP-1 may act as a scaffold protein for BCR-ABL and STAT5a. To confirm this possibility, FLAG-tagged STAT5a and/or BCR-ABL with or without Myc-tagged STAP-1 were transiently expressed in 293T cells. The BCR-ABL binding potential of STAT5a was examined using immunoprecipitation with an anti-ABL antibody followed by western blot analysis with an anti-FLAG or anti-Myc antibody. As shown in Fig 1F, STAP-1 co-expression with STAT5a and BCR-ABL enhanced the binding between STAT5a and BCR-ABL. These findings suggest that STAP-1 plays an important role in STAT5a and BCR-ABL complex formation.

3. 2. STAP-1 enhances BCR-ABL/STAT5-downstream gene expression

To assess the physiological roles of the association among STAP-1, STAT5a, and BCR-ABL, we investigated the effects of STAP-1 knockdown on the gene expression of the STAT5-downstream molecules. When STAP-1 mRNA expression in KU812 CML (KU812 shSTAP-1) cells was knocked down to 60% (Fig 2A), STAT5a phosphorylation decreased (Fig 2B). In parallel, the anti-apoptotic BCL-2 and growth-related MYC mRNAs were significantly reduced in STAP-1-knockdown KU812 CML cells (Fig 2C). These results suggest that STAP-1 association with BCR-ABL and STAT5 enhances BCR-ABL/STAT5-induced signals.

3.3. STAP-1 stabilizes BCR-ABL proteins

To examine whether STAP-1 influences BCR-ABL protein stability, we conducted a CHX chase experiment by blocking the *in vivo* protein synthesis. Treatment of the KU812 CML (KU812 shControl or shSTAP-1) cells with CHX led to a time-dependent decrease in the BCR-ABL

protein content (Fig 2D). Notably, the BCR-ABL proteins disappeared quickly in STAP-1 knockdown KU812 cells with (half-life: 16 and 4 hours in the presence and absence of STAP-1, respectively). This suggests that STAP-1 association with BCR-ABL protects BCR-ABL from degradation in CML cells.

3.4. NFATc1, a transcription factor, induces STAP-1 mRNA expression in CML cells

STAP-1 mRNA or protein expression is aberrantly high in CML LSCs [12]. To examine STAP-1 expression regulation in CML cells, we carried out promoter analysis of human STAP-1. Using the USCS genome browser computer analysis (<https://genome.ucsc.edu/>), we identified several putative transcription factor-binding sites for PU.1, RUNX3, and NFATc1 within the 0.5-kb fragment of the 5'-flanking region of human STAP-1 gene (Fig 3A). The genomic DNA of human STAP-1 promoter region was subcloned into a promoter-less luciferase reporter vector pGL3 (STAP-1-LUC). KU812 CML cells were co-transfected with STAP-1-LUC and PU.1, RUNX3, or NFATc1 expression plasmids (Fig 3B). The luciferase activity was greatly induced by ectopic NFATc1, but not by PU.1 or RUNX3. In addition, KU812 CML cells with siRNA-mediated NFATc1 knockdown had impaired STAP-1 mRNA expression (Fig 3C), although the NFATc1 expression was reduced by only 40%. Therefore, NFATc1 positively regulates STAP-1 promoter activation and STAP-1 mRNA expression induction in KU812 CML cells.

NFAT is regulated by Ca^{2+} and calcineurin, a Ca^{2+} /calmodulin-dependent serine phosphatase. Thus, STAP-1 mRNA expression in KU812 CML cells was examined in the presence or absence of ionomycin, a calcium ionophore, as a NFAT activator [17,18]. As shown in Fig 3D, ionomycin treatment significantly induced STAP-1 mRNA expression. Cyclosporin A (CyA), an immunosuppressant, inhibits calcineurin/NFAT signaling [17,18]. Further CyA treatment reverted the increased STAP-1 mRNA expression in ionomycin-treated KU812 CML cells. Taken together, this demonstrated that calcineurin/NFAT signaling controls endogenous STAP-1 mRNA expression in CML cells.

4. Discussion

In this study, we demonstrated that STAP-1 acts as a scaffold protein, which interacts with BCR-ABL and STAT5a through its SH2 and PH domains, respectively. STAP-1 enhanced BCR-ABL/STAT5-mediated gene expression and upregulated BCR-ABL protein levels in CML cells. Notably, BCR-ABL/Ca²⁺/NFATc1 signals induced STAP-1 gene expression. As summarized in Fig 4, our results represent that STAP-1 builds a positive loop with BCR-ABL and STAT5 in CML cells.

Human STAP-1 has been identified to be a Tec-interacting protein, termed as BRDG1 [19], while murine STAP-1 has been identified to be a c-kit-interacting protein [6]. Several reports have suggested the possible involvement of STAP-1 in human diseases. STAP-1 mRNA expression is induced in pro-inflammatory microglia and macrophages that contribute to neuronal apoptosis and degeneration [20]. STAP-1 mutations have been identified in DNA samples from patients with autosomal dominant hypercholesterolemia [21,22], although the effects of STAP-1 in cholesterol homeostasis remain controversial [23,24]. STAP-1 is also involved in autoimmune hepatitis pathogenesis through the regulation of iNKT cell maintenance and/or activation [13]. Microarray data from 572 patients with CML have showed that STAP-1 expression is increased in about 20% samples including ALL with or without BCR-ABL fusion gene [25]. STAP-1 expression is aberrantly upregulated in CML LSCs. In experimental model mice, STAP-1 deletion prolonged CML mice survival by inducing apoptosis in CML LSCs [12]. The transcriptome analyses indicated that STAP-1 affects several BCR-ABL, JAK2, and PPAR γ -related signaling pathways [12].

We have also shown the precise molecular mechanisms of STAP-1-influenced CML characteristics. First, the STAP-1 SH2 domain binds to BCR-ABL and STAP-1 PH domain recognizes STAT5. Notably, STAP-1 expression enhances the binding between BCR-ABL and STAT5a, upregulating the STAT5-downstream signals. Thus, STAP-1 likely acts as a scaffold

protein to bring BCR-ABL and STAT5 close or strengthen the bond between these two proteins. Second, STAP-1 expression upregulates BCR-ABL protein content because STAP-1 protects BCR-ABL protein from degradation. Third, BCR-ABL induces intracellular Ca^{2+} elevation and NFATc1 activity, followed by induction of STAP-1 mRNA expression. STAP-1 in turn induces the BCR-ABL protein stabilization and BCR-ABL-downstream signal activation. Thus, there seems to be a positive loop between STAP-1 and BCR-ABL. In general, some patients with CML acquire resistance against TKIs via problems of compliance [3,4,5], BCR-ABL mutation or duplication, alternative signaling pathway activation, and problems of drug import or export. Since our data indicate that STAP-1 overexpression induces the proliferation and survival of CML cells, high STAP-1 expression may be another reason of TKI-resistance in patients with CML.

In summary, STAP-1 enhances BCR-ABL-induced STAT5 activation that affects cell cycle, anti-apoptotic mechanism, and the Ca^{2+} /NFAT signaling pathway upregulation. The positive loop between BCR-ABL and STAP-1 upregulates the BCR-ABL protein level, an oncogenic fusion protein for CML. STAP-1 does not affect the normal hematopoiesis, and as such can act as a potential target for the treatment of CML by inhibiting the BCR-ABL-STAT5 axis. Alternatively, targeting the Ca^{2+} /NFAT signal might present another way to inhibit STAP-1 expression in the BCR-ABL-STAP-1 loop in CML. From the clinical point of view, our report suggests STAP-1 to be a novel target in CML treatment for overcoming resistance and disease persistence.

Conflict of interest

The authors have no conflicting financial interests.

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Figure legends

Figure1. Molecular interactions among BCR-ABL, STAP-1, and STAT5.

(A) Domain structure of STAP-1 and mutant fragments are schematically shown. WT, wild-type.

(B) HEK293T cells in a 6 well-plate were transfected with FLAG-tagged STAT5a (5 μ g) and/or a series of Myc-tagged STAP-1 mutants (5 μ g). Forty-eight hrs after transfection, the cells were lysed, and immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc antibody (upper panel) or anti-FLAG antibody (bottom panel). Total cell lysates (TCL) were blotted with anti-Myc and anti-FLAG antibodies to monitor the expression of each protein. Shown is a representative experiment, which was repeated at least three times with similar results.

(C) HEK293T cells in a 6 well-plate were transfected with BCR-ABL (5 μ g) and/or a series of Myc-tagged STAP-1 mutants (5 μ g). Forty-eight hrs after transfection, the cells were lysed, and immunoprecipitated with anti-ABL antibody and immunoblotted with anti-Myc antibody (upper panel) or anti-ABL antibody (bottom panel). TCL were blotted with anti-Myc and anti-ABL antibodies to monitor the expression of each protein. Shown is a representative experiment, which was repeated at least three times with similar results.

(D) Domain structure of STAT5a and mutant fragments are schematically shown. FL, full-length.

(E) HEK293T cells in a 6 well-plate were transfected with Myc-tagged STAP-1 (5 μ g) and/or a series of FLAG-tagged STAT5a mutants (5 μ g). Forty-eight hrs after transfection, the cells were lysed, and immunoprecipitated with anti-Myc antibody and immunoblotted with anti-FLAG antibody (upper panel) or anti-FLAG antibody (bottom panel). TCL were blotted with anti-FLAG and anti-Myc antibodies to monitor the expression of each protein. Shown is a representative experiment, which was repeated at least three times with similar results.

(F) HEK293T cells in a 6 well-plate were transfected with FLAG-tagged STAT5a and/or BCR-ABL (5 μ g) together with or without Myc-tagged STAP-1 mutants (5 μ g). Forty-eight hrs after transfection, the cells were lysed, and immunoprecipitated with anti-Myc antibody and immunoblotted with anti-FLAG antibody (upper panels), anti-FLAG antibody (middle panels) or

anti-ABL antibody (bottom panel). TCL were blotted with anti-FLAG, anti-Myc or anti-ABL antibody (lower panels) to monitor the expression of each protein. Shown is a representative experiment, which was repeated at least three times with similar results.

Figure 2. STAP-1 enhances the BCR-ABL/STAT5-downstream gene expression and stabilizes BCR-ABL.

(A) STAP-1 mRNA expression was measured by qPCR in control (Ctrl) or STAP-1 shRNA expressing KU812 CML cells. Shown is a representative experiment, which was repeated at least three times with similar results, and the error bars represent the SD. $**p < 0.01$. (B) STAT5a phosphorylation was measured by western blotting in control (Ctrl) or STAP-1 shRNA expressing KU812 CML cells. An aliquot of total cell lysates (TCL) was immunoblotted by anti-phospho STAT5 (Tyr694) or anti-STAT5 antibody. (B) BCL-2 or MYC mRNA expression was measured by qPCR in Ctrl or STAP-1 shRNA expressing KU812 CML cells. Shown is a representative experiment, which was repeated at least three times with similar results, and the error bars represent the SD. $*p < 0.05$. (C) Ctrl or STAP-1 shRNA expressing KU812 CML cells were treated with or without cycloheximide (CHX, 10 $\mu\text{g/ml}$) for the indicated periods. The cells were lysed and immunoblotted with anti-ABL or anti-Actin antibody. Relative intensity of BCR-ABL protein was normalized to the Actin protein of the TCL sample. Results are representative of three independent experiments, and the error bars represent the SD. $*p < 0.05$, $**p < 0.01$.

Figure 3. NFATc1 induces STAP-1 mRNA expression in CML cells.

(A) Schematic representation of luciferase reporter construct ligated to the STAP-1 promoter region (STAP-1-Luc). Putative transcription binding site are annotated. LUC, luciferase.

(B) HEK293T cells in a 24 well-plate were transfected with expression vectors for FLAG-tagged PU.1, RUNX3 or NFATc1 and STAP-1-LUC. The cells were lysed at 48 h post transfection and then luciferase activity was determined. Shown is a representative experiment, which was repeated

at least three times with similar results, and the error bars represent the SD. * $p < 0.05$.

(C) KU812 CML cells were transfected with siScramble or siNFATc1. STAP-1 or NFATc1 mRNA expression was measured by qPCR in siScramble- or siNFATc1- treated KU812 CML cells. Shown is a representative experiment, which was repeated at least three times with similar results, and the error bars represent the SD. * $p < 0.05$, ** $p < 0.01$.

Figure 4. The Ca^{2+} /NFAT signals to induce STAP-1 mRNA expression in CML cells.

(A) KU812 CML cells were treated with or without ionomycin (1 $\mu\text{g}/\text{ml}$) together with or without CyA (1 μM). At 24 h after treatment, STAP-1 or NFATc1 mRNA expression was measured by qPCR. Shown is a representative experiment, which was repeated at least three times with similar results, and the error bars represent the SD. * $p < 0.05$.

(B) Schematic representation of the STAP-1-mediated positive loop with BCR-ABL and STAT5 in CML cells.

Figure 1

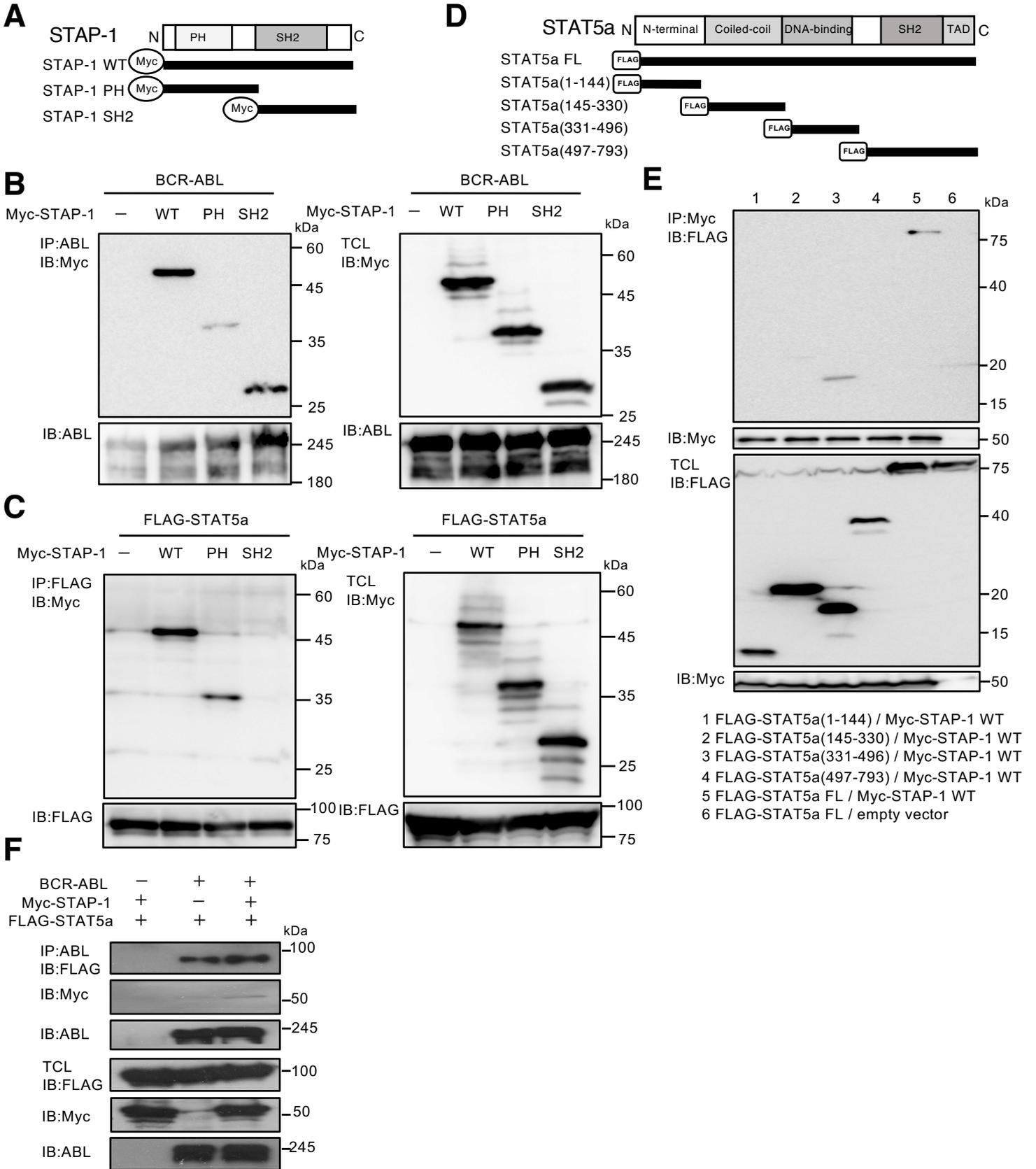


Figure 2

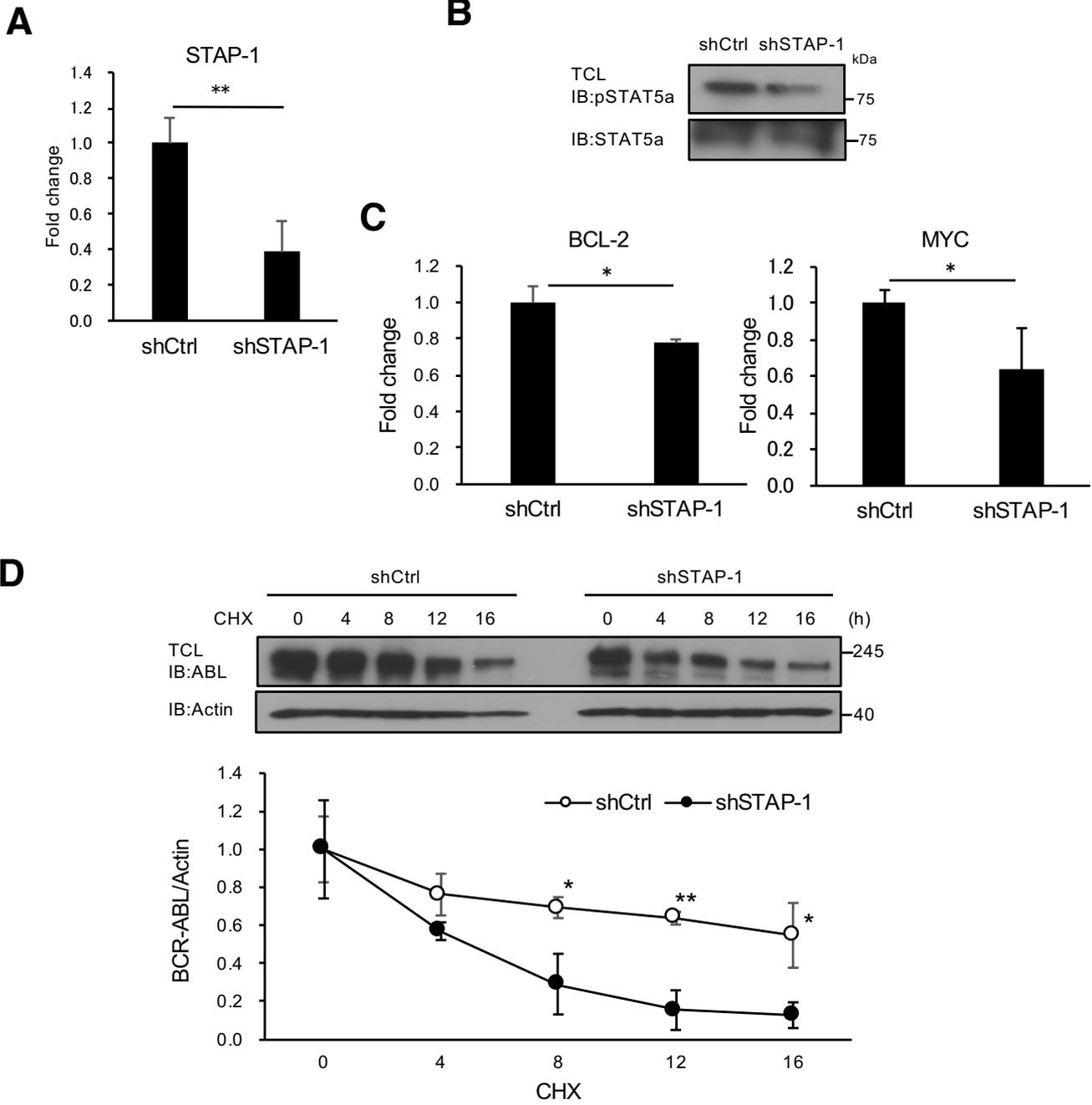


Figure 3

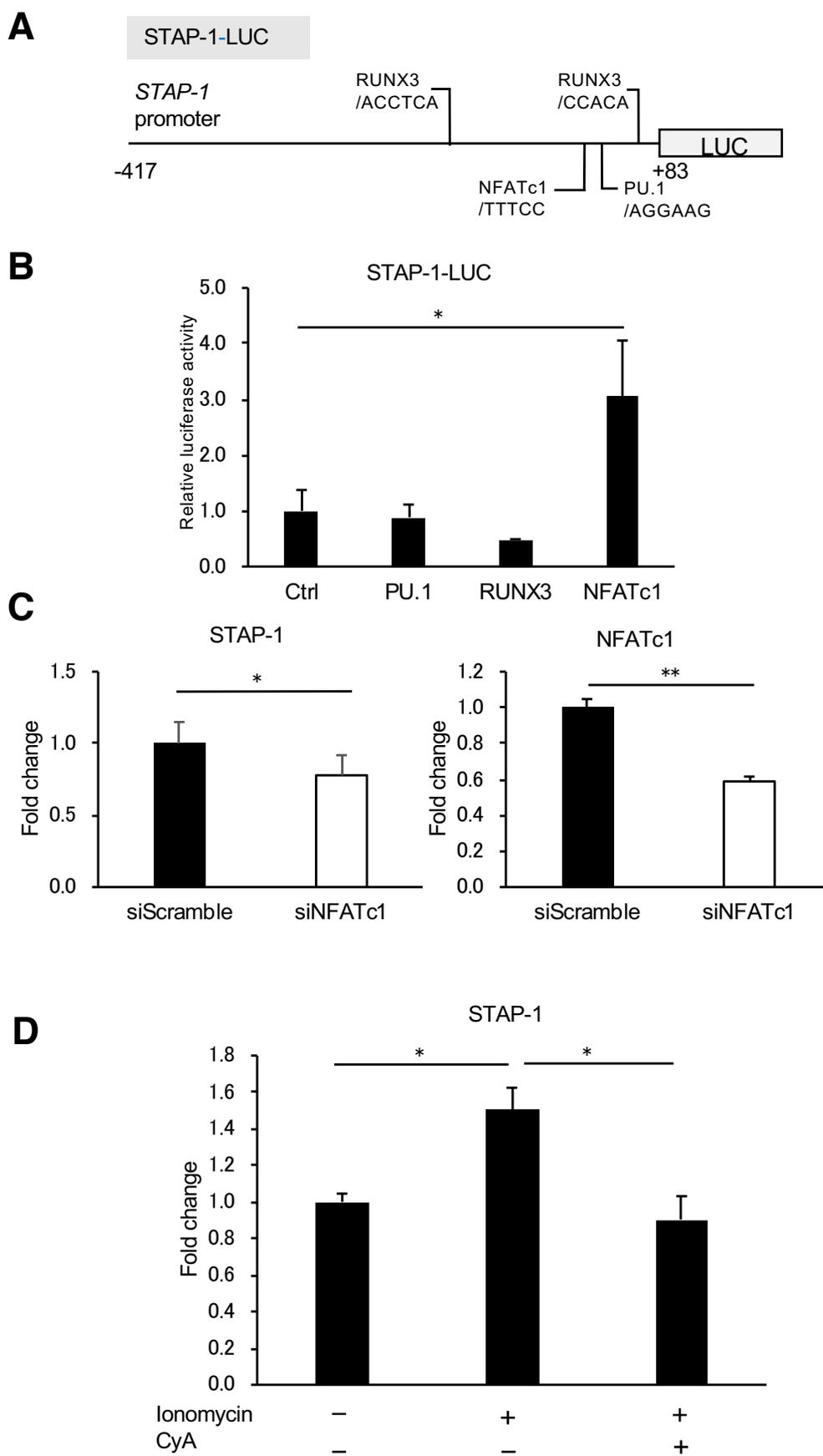


Figure 4

