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**Title: STAP-2 is phosphorylated at tyrosine-250 by Brk and modulates Brk-mediated STAT3 activation**

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Running title: Brk phosphorylates and regulates STAP-2 functions

Keywords: Brk, STAP-2, phosphorylation, STAT3, transcription

**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. STAP-2 is also known as breast tumor kinase (Brk) substrate (BKS). Our previous studies revealed that STAP-2 binds to signal transducer and activator of transcription 3 (STAT3) and STAT5, and regulates the signaling pathways downstream of them. In the present study, we identified tyrosine-250 (Tyr250) in STAP-2 as a major site of phosphorylation by Brk, using a series of STAP-2 YF mutants and anti-phospho-STAP-2 Tyr250 antibody. Furthermore, overexpression of the STAP-2 Y250F mutant protein affected Brk-mediated STAT3 activation. Importantly, small-interfering RNA-mediated reduction of endogenous STAP-2 expression decreased Brk-mediated STAT3 activation. Taken together, our findings demonstrate that STAP-2 is phosphorylated at Tyr250 by Brk, and plays an important role in Brk-mediated STAT3 activation.

## Introduction

Protein-tyrosine kinases (PTKs) play critical roles in regulating cell growth, differentiation and transformation. Tyrosine kinases themselves become autophosphorylated within the activation segment of their kinase domains, thereby inducing conversion to a more active state. However, a frequent consequence of tyrosine phosphorylation is the creation of specific binding sites for adaptor proteins that contain Src homology (SH) 2 domains. Such phosphotyrosine-dependent protein-protein interactions serve to recruit regulatory proteins to phosphorylated receptors and other adaptor proteins, and thereby activate signaling pathways that control numerous aspects of cellular functions [1, 2]. The non-receptor tyrosine kinase breast tumor kinase (Brk) was originally isolated from a human breast carcinoma cells [3]. Brk is also known as PTK6, having been identified as a highly expressed PTK in human melanocytes [4], and a cDNA for its mouse homolog, Sik, which has 80% amino acid identity to Brk/PTK6, was cloned from mouse intestinal crypt cells [5]. Brk contains an SH3 domain, an SH2 domain, and a tyrosine kinase catalytic domain, but it lacks an N-terminal myristoylation site for membrane targeting. Subsequent characterization of Brk showed it to be present in approximately 60% of human breast tumors, yet absent in normal or fibrocystic mammary tissues. Brk has also been shown to be expressed in other cancer cells, including metastatic melanomas and colon and prostate tumors [6, 7, 8, 9]. However, the molecular mechanism by which Brk participates in tumorigenesis remains poorly characterized. One substrate of Brk is BKS (Brk substrate)/signal-transducing adaptor protein-2 (STAP-2), which has also been implicated in modulating

the activity of STAT3 and STAT5 [10, 11, 12]. STAP-2 was identified as a c-*fms*-interacting protein, and contains an N-terminal pleckstrin homology (PH) domain and a region distantly related to the SH2 domain [11]. The central region of STAP-2, which is distantly related to the SH2 domain, shares 29% sequence identity with the SH2 domain of human PLC$\gamma$2. Furthermore, STAP-2 possesses a C-terminal proline-rich region and a STAT3-binding YXXQ motif [11].

In the present study, we identified tyrosine-250 (Tyr250) as the major site of phosphorylation of STAP-2 by Brk. We also show that the kinase activity of Brk is required for a direct interaction with STAP-2. Furthermore, we demonstrate that a reduction of endogenous STAP-2 expression decreases Brk-mediated STAT3 activation.

## Materials and Methods

*Reagents and antibodies,* Expression vectors, STAP-2 and its YF (substitution of Tyr to Phe) mutants were described previously [11]. Expression vectors for wild-type Brk (Brk WT), Brk K219M and STAT3-LUC were provided by Dr. A. Harvey (Brunel University, Middlesex, UK) and Dr. T. Hirano (Osaka University, Osaka, Japan), respectively [3, 13]. Anti-Myc and -GST antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FLAG antibody was obtained from Sigma-Aldrich (St. Louis, MO); anti-phosphotyrosine monoclonal antibody (PY20) from Cosmobio (Tokyo, Japan). Anti-STAP-2 antibody was purchased from Everest Biotech (Oxfordshire, UK). Anti-phosphoSTAP-2 Tyr250 was prepared as previously described [14].

*Cell culture, transfection, small interfering RNA (siRNA), RT-PCR and luciferase assays,* Human embryonic kidney carcinoma cell line, 293T, was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and transfected by the standard calcium precipitation protocol. Luciferase assay was performed as described [15]. Human breast cancer cell line, MCF-7 and human cervix carcinoma cell line HeLa were maintained in DMEM containing 10 % FCS. siRNAs targeting human STAP-2 used in this study were as follows: STAP-2#1, 5’-

GCAGGGUCUCACCAUUUAUTT-3'; STAP-2#2, 5'-GGUGCUAGGCUACGUGGAATT-3'. HeLa cells were plated on a 24-well plate at 2 x $10^4$ cells/well, and then incubated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37 °C for 4 h, followed by addition of fresh medium containing 10% FCS [16]. Twenty-four hrs after transfection, the cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. MCF-7 and HeLa cells were transfected with STAT3-LUC using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instruction. Three or more independent experiments were carried out for each assay. Total RNA samples were extracted using Iso-Gen (Nippon Gene, Tokyo, Japan) and subjected to RT-PCR using an RT-PCR High -Plus- Kit (TOYOBO, Tokyo, Japan)[15].

*Immunoprecipitation and immunoblotting,* The immunoprecipitation and Western blotting assays were performed as described previously [15]. 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).

## Results and Discussion

### Brk phosphorylates STAP-2 at Tyr250

STAP-2/BKS was originally identified as a substrate for Brk [10]. However, the tyrosine residue in STAP-2 that undergoes phosphorylation by Brk remained unknown. In the present study, we attempted to identify the site of Brk-mediated tyrosine phosphorylation in STAP-2. We first confirmed tyrosine phosphorylation of STAP-2 by Brk *in vivo*. Myc-tagged STAP-2 was expressed without or with FLAG-tagged wild-type Brk (Brk WT) or a kinase inactive form of Brk, Brk K219M, in 293T cells. The cells were lysed, and lysates were immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-phosphotyrosine (PY) or anti-Myc antibody. As shown in Fig. 1A, significant tyrosine phosphorylation of STAP-2 by Brk was observed, as described previously [10]. We next utilized a series of STAP-2 YF mutants in which four potential tyrosine phosphorylation sites were mutated to phenylalanine (Fig. 1B). To further probe the phosphorylation status of STAP-2 Tyr250, we also used a phospho-specific antibody against the Tyr250 site, designated anti-pSTAP-2 Tyr250, as described previously [14]. Myc-tagged wild-type STAP-2 (STAP-2 WT) or a series of STAP-2 YF mutants were expressed with or without Brk in 293T cells. The expressed STAP-2 WT and YF mutant proteins were immunoblotted with anti-pSTAP-2 Tyr250, anti-phosphotyrosine (PY) and anti-Myc antibodies. As shown in Fig. 1C, the STAP-2 WT and YF mutant proteins, with or without Brk co-expression, were expressed at equivalent protein levels. The anti-pSTAP-2 Tyr250 or anti-PY antibody failed to

recognize the STAP-2 Y250F mutant co-expressed with Brk (Fig. 1C), indicating that Tyr250 is the major site of tyrosine phosphorylation by Brk.

**The kinase activity of Brk is required for a direct interaction with STAP-2**

We next tested whether activation of Brk is required for a direct interaction with STAP-2. We first investigated whether the kinase activity of Brk is necessary for the interaction between Brk and STAP-2. Following transfection of 293T cells with expression vectors for FLAG-tagged STAP-2 and Myc-tagged Brk WT or Brk K219M, the cells were lysed, and lysates were immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-Myc antibody. As shown in Fig. 2A, a significant interaction of STAP-2 with Brk WT was observed, although STAP-2 failed to interact with Brk K219M, suggesting that the kinase activity of Brk is required for the interaction with STAP-2. We further tested whether phosphorylation of STAP-2 at Tyr250 is required for a direct interaction with Brk. Myc-tagged STAP-2 WT or a series of STAP-2 YF mutants were expressed with FLAG-tagged Brk in 293T cells. The cells were lysed, and lysates were immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-Myc antibody. As shown in Fig. 2B, the STAP-2 WT and YF mutant proteins interacted with Brk, although the STAP-2 Y250F mutant showed a slightly lower binding potential than WT and other mutants. Therefore, STAP-2 Tyr 250 is a major site of phosphorylation by Brk, but is not required for a direct interaction with Brk. These results indicate that the kinase activity of Brk rather than phosphorylation of

Y250 in STAP-2 is responsible for STAP-2/Brk interaction.

**Substitution of Tyr250 to Phe in STAP-2 affects Brk-mediated STAT3 activation**

In a previous study we demonstrated that ectopic expression of STAP-2 enhanced LIF-mediated STAT3 activation by transient transfection experiments using STAT3-LUC, in which the 2-macroglobulin promoter drives expression of a luciferase (LUC) reporter gene. We also demonstrated that STAP-2 Tyr250 is involved in LIF-induced STAT3-LUC activation in 293T and MCF-7 cells. In the present study we focused on the involvement of STAP-2 Tyr250 in Brk-mediated STAT3 activation, because STAP-2 Tyr250 is the major site of phosphorylation by Brk, as shown in Fig. 1. To confirm the effect of STAP-2 Y250F on Brk-mediated STAT3 activation, we transfected 293T cells with STAP-2 WT or STAP-2 Y250F together with Brk and STAT3-LUC. After 48 h, the cells were harvested and the STAT3-LUC activities were determined. As shown in Fig. 3A, STAP-2 WT markedly up-regulated Brk-mediated STAT3 activation. By contrast, the STAP-2 Y250F transfectant failed to show enhanced Brk-mediated STAT3 activation, while protein expression levels of STAP-2 WT and STAP-2 Y250F were similar. We also examined this effect using the human breast cancer cell line MCF-7. STAP-2 WT or STAP-2 Y250F together with STAT3-LUC was transfected into MCF-7 cells. After 48 h, the cells were harvested and the STAT3-LUC activities were determined. As shown in Fig. 2B, STAP-2 WT, but not Y250F, positively stimulated Brk-mediated STAT3 activation, while protein expression levels of STAP-2 WT and STAP-2 Y250F were similar. Taken together, these results suggest that phosphorylation

of Y250 in STAP-2 affects but is not required for Brk-mediated STAT3 activation. To further assess the functional relevance of STAP-2 in Brk-mediated STAT3 activation, we examined whether siRNA-mediated reduction of endogenous STAP-2 affects Brk-mediated STAT3 activation. HeLa cells were transfected with a specific siRNA for STAP-2 (#1 or #2), or a control siRNA as previously described [15]. Total cellular proteins or RNA extracted from the transfected cells was subjected to Western blot or RT-PCR analysis, which confirmed a reduction of STAP-2 expression. As shown in Fig. 3C, Western blot analysis revealed that STAP-2 protein expression was reduced by approximately 40-50% by STAP-2 siRNA treatment. Importantly, a reduction of STAP-2 expression in HeLa cells decreased Brk-mediated STAT3-LUC activation, indicating that endogenous STAP-2 is involved in the regulation of Brk-mediated STAT3-LUC activation in HeLa cells.

## Concluding remarks

We here demonstrate that Brk activity is required for STAT3 activation of STAP-2 and phosphorylation of Tyr250, but phosphorylation of Tyr250 is not absolutely required for the binding of STAP-2 to Brk and activation of STAT3. Although this differences are strange, we recently found that the N-terminal PH domain of STAT-2 is required for the binding of STAP-2 to Brk and activation of STAT3 (data not shown). Thus, STAP-2 is likely to interact with Brk and STAT3 via the N-terminal PH domain and to be phosphorylated at Tyr250 in the C-terminal region by Brk. Additionally, unidentified phosphorylation sites involved in Brk-mediated STAT3 activation may exist in the PH domain of STAP-2.

Importantly, recent studies have shown that STAT3 is a Brk substrate and that Brk activates STAT3 [17]. STAT3 is known to act as an oncogene in a constitutively active form, and phosphorylation and activation of STAT3 is correlated with breast cancer [18, 19, 20]. Brk function and interacting partners remain largely undefined. Therefore, it is important to better understand the contribution of Brk kinase activity and protein interactions to the STAT3-mediated signal transduction pathways in breast cancer. Moreover, both Brk and STAP-2 are highly expressed in breast cancer cells, suggesting that this linkage may play a role in the dysregulated activation of STAT3 in breast cancer. Further detailed work will be required to clarify the molecular mechanisms underlying Brk/STAP-2-mediated modification of STAT3 and will provide insights toward the development of a novel therapeutic strategy for breast cancer.

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**Figure legends**

Fig. 1. Phosphorylation of Tyr250 in STAP-2 by Brk

A. 293T cells ($1x10^7$) were transfected with Myc-tagged STAP-2 WT (10 μg) and FLAG-tagged Brk (5 μg) or Brk K219M (5 μg). Forty-eight hrs after transfection, the cells were lysed. An aliquot of each total cell lysates (TCL) was immunoblotted with an anti-PY or anti-Myc antibody. The cell lysates were also immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-PY or anti-FLAG antibody.

B. Domain structure of STAP-2 is schematically shown. Four predicted tyrosine residues are also shown.

C. 293T cells ($1x10^7$) were transfected with or without Myc-tagged STAP-2 WT (10 μg) or STAP-2 YF mutants (10 μg) and/or Brk (5 μg). Forty-eight hrs after transfection, the cells were lysed. An aliquot of each TCL was immunoblotted with an anti-PY, anti-pSTAP-2 Tyr250, anti-Myc or anti-FLAG antibody.

Fig.2. The kinase activity of Brk is required for a direct interaction with STAP-2

A. 293T cells ($1x10^7$) were transfected with Myc-tagged STAP-2 WT (10 μg) and FLAG-tagged Brk (5 μg) or Brk K219M (5 μg). Forty-eight hrs after transfection, the cells were lysed, immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-Myc or anti-FLAG antibody. An aliquot of each TCL was immunoblotted with an anti-Myc or anti-FLAG antibody.

B. 293T cells ($1x10^7$) were transfected with or without Myc-tagged STAP-2 WT (10 μg)

or STAP-2 YF mutants (10 □g) and/or Brk (5 □g). Forty-eight hrs after transfection, the cells were lysed, immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-Myc or anti-FLAG antibody. An aliquot of each TCL was immunoblotted with an anti-Myc antibody.

Fig. 3. Substitution of Tyr250 to Phe in STAP-2 and reduction of endogenous STAP-2 expression decreases Brk-mediated STAT3 activation

A. 293T cells in a 24-well plate were transfected with STAT3-LUC (200 ng) and/or FLAG-tagged Brk (100 ng), and the indicated amounts (1, 10, 100 ng) of expression vector for Myc-tagged STAP-2 WT or STAP-2 Y250F. Forty-eight hrs after transfection, the cells were harvested and the luciferase activities were measured. At least three independent experiments were carried out for each assay. *$p<0.01$. An aliquot of each total cell lysate (TCL) was analyzed by immunoblotting with an anti-Myc or anti-FLAG antibody.

B. MCF-7 cells in a 24-well plate were transfected with STAT3-LUC (100 ng) and/or FLAG-tagged Brk (100 ng), and the indicated amounts (30, 300 ng) of expression vector for Myc-tagged STAP-2 WT or STAP-2 Y250F. Forty-eight hrs after transfection, the cells were harvested and the luciferase activities were measured. At least three independent experiments were carried out for each assay. *$p<0.0005$. An aliquot of each total cell lysate (TCL) was analyzed by immunoblotting with an anti-Myc or anti-FLAG antibody.

C. HeLa cells in a 24-well plate were transfected with control or STAP-2 (#1 and #2) siRNA, and cells were then transfected with STAT3-LUC and/or FLAG-tagged Brk (300 ng) using jetPEI. Total cellular proteins or RNA extracted from the transfected cells was subjected to Western blot (WB) or RT-PCR analysis, which confirmed reductions in STAP-2 expression. Densitometric quantification of the above results of WB was also shown. Relative intensity of STAP-2 protein was normalized to total actin protein of the same sample. Thirty-six hrs after transfection, the cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D. *, $p< 0.0001$. An aliquot of each total cell lysate (TCL) was analyzed by immunoblotting with an anti-FLAG antibody.

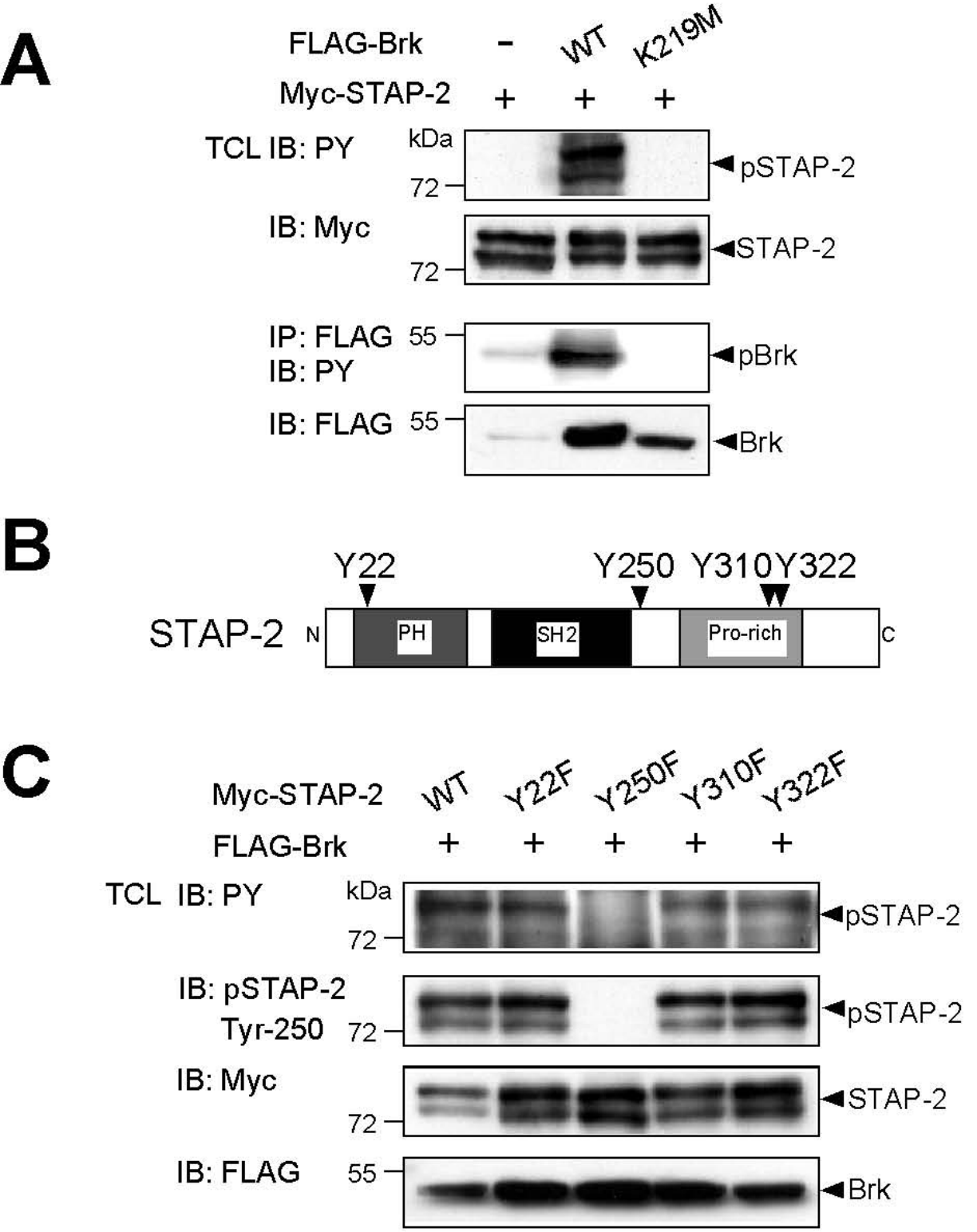
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**Figure 1**

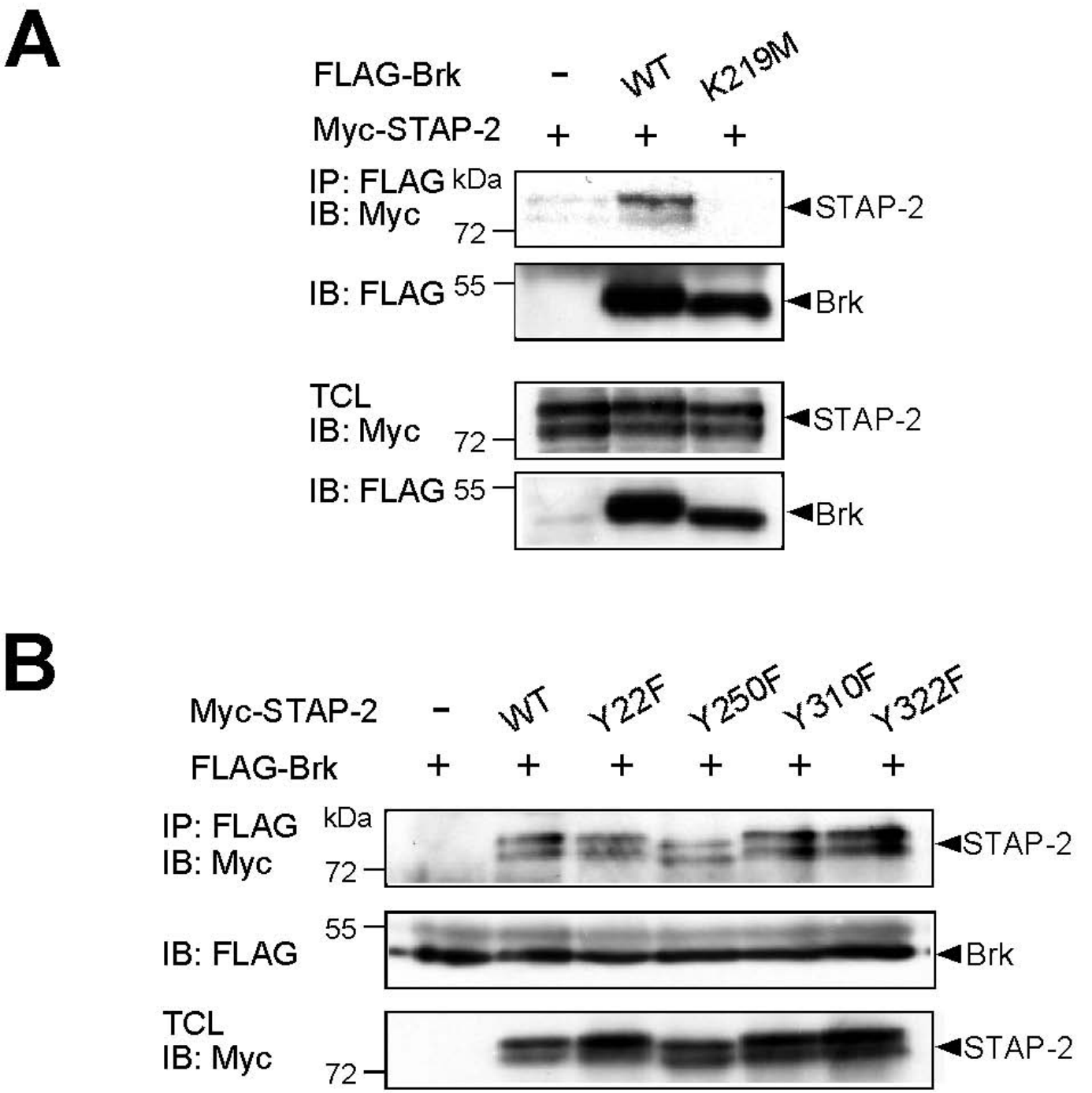
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**Figure 2**

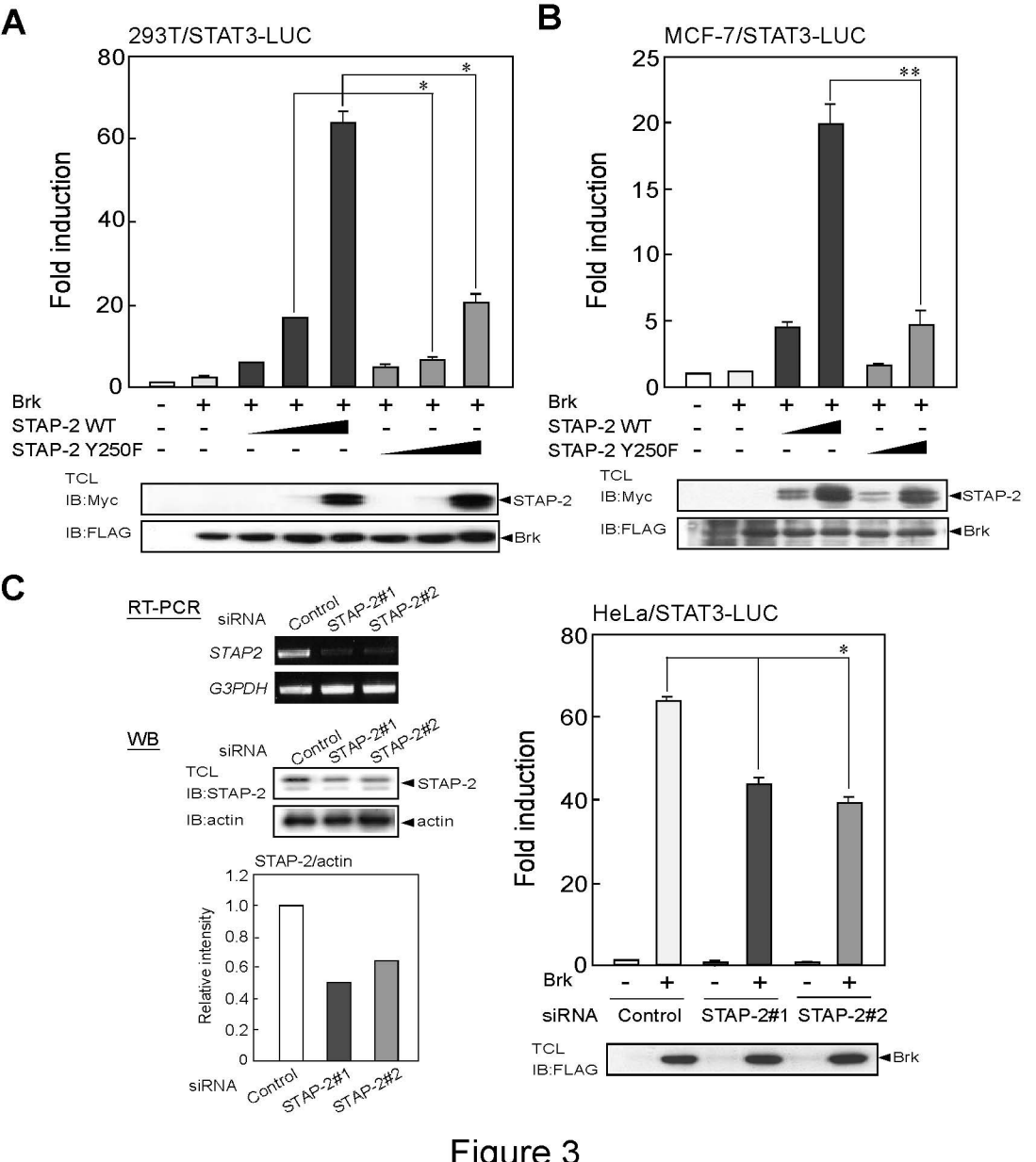
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Figure 3