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Title: Involvement of STAP-2 in Brk-mediated phosphorylation and activation of STAT5 in breast cancer cells.

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Summary

Signal-transducing adaptor protein (STAP)-2 is a recently identified adaptor protein that contains Pleckstrin homology (PH) and Src homology 2 (SH2)-like domains, and is also known to be a substrate of breast tumor kinase (Brk). In a previous study, we found that STAP-2 upregulated Brk-mediated activation of signal transducer and activator of transcription (STAT) 3 in breast cancer cells. Here, we examined the involvement of STAP-2 in Brk-mediated STAT5 activation in breast cancer cells. Ectopic expression of STAP-2 induced Brk-mediated transcriptional activity of STAT5. Furthermore, STAP-2-knockdown in T47D breast cancer cells induced a marked decrease in proliferation that was as strong as that after Brk- or STAT5b-knockdown. Regarding the mechanism, the PH domain of STAP-2 is likely to participate in the process by which Brk phosphorylates and activates STAT5. Taken together, our findings provide insights toward the development of novel therapeutic strategies as well as novel prognostic values in breast carcinomas.

Introduction

A key step in the progression of the majority of breast cancers is the transition to steroid hormone-independent proliferation, and estrogen-independent tumors often have increased levels of total tyrosine kinase activity in both the cytosolic and membrane fractions.[1]

Although breast tumor kinase (Brk), also known as protein tyrosine kinase 6, is undetectable in the normal mammary gland, it is overexpressed in more than 60% of human breast tumors and breast cancer cell lines, with the highest levels in advanced tumors.[2-4] Notably, small-interfering RNA (siRNA)-mediated downregulation of Brk expression in breast cancer cells results in a decrease in their growth capacity,[4] indicating that Brk is one of the major molecules causing excessive proliferation of breast cancer cells. Brk is a nonreceptor tyrosine kinase that activates signal transducer and activator of transcription (STAT) 3 and STAT5.[5,6] Brk phosphorylates STAT3 and STAT5, leading to increases in their transcriptional activity. Furthermore, both STATs play fundamental roles in the normal growth and development of the mammary gland,[7] and are often overexpressed or constitutively activated in breast cancer tumors.[8] Thus, both the Brk/STAT3 and Brk/STAT5 axes are likely to be potential targets for breast cancer therapy.

On the other hand, signal-transducing adaptor protein (STAP)-2, which we isolated as a c-*fms*-interacting protein, is another substrate whose tyrosine residues are phosphorylated by Brk.[9-11] STAP-2 shows high sequence and structural similarities to STAP-1, which we cloned as a c-kit-interacting protein.[12] Both STAP-1 and STAP-2 contain an N-terminal Pleckstrin homology (PH) domain and a region weakly related to a Src homology 2 (SH2) domain (overall amino acid identity, 33%). The N-terminal PH

domains of STAP-2 and STAP-1 share 36% identity and 58% similarity. The central region of STAP-2 is distantly related to the SH2 domain. This region of STAP-2 shares 40% sequence identity with that of STAP-1 and 29% sequence identity with the SH2 domain of human phospholipase C-γ2. However, STAP-2 has a C-terminal proline-rich region and a YXXQ motif, both of which are absent from STAP-1. Although STAP-1 shows a restricted expression pattern in hematopoietic cells,[12] STAP-2 is expressed in a variety of tissues and cells, such as lymphocytes, macrophages and hepatocytes,[10] and its abundant expression pattern suggests that STAP-2 influences a variety of signaling or transcriptional molecules. Indeed, we previously reported that STAP-2 can modulate the transcriptional activity of STAT3 and STAT5, as well as FcεRI- and Toll-like receptor-mediated signals.[10,13-15] It is noteworthy that thymocytes and peripheral T cells from STAP-2-deficient mice show enhanced IL-2- or TCR-dependent cell growth and enhanced integrin-mediated adherence to fibronectin.[13,16] In contrast, the roles of STAP-1 have not been identified.

Recently, we reported essential roles of STAP-2 in Brk-mediated STAT3 activation.[11,17] In the absence of STAP-2, Brk failed to phosphorylate STAT3 in breast cancer cell lines.[17] With regard to Brk-mediated STAT5 activation, the involvement of STAP-2 in this process has not yet been clarified. However, the PH- and SH2-like domains of STAP-2 can recognize the C-terminal region of STAT5.[13] Thus, the ability of STAP-2 to interact with both Brk and STAT5 seems to suggest its possible roles in the Brk/STAT5 axis.

In this study, we focused on STAP-2 as a novel transcriptional regulator of STAT5 and demonstrated a functional link between STAP-2 and Brk/STAT5-mediated transcriptional activation and cell growth in human breast cancer cells.

Materials and methods

Reagents and antibodies. Expression vectors for STAP-2, STAT5a, STAT5b and STAT5-LUC were described previously.[13] Expression vectors for wild-type Brk and an active form of Brk (Brk Y447F) were provided by Dr. A. J. Harvey (Brunel University, Middlesex, UK).[2] Anti-Myc, -Brk, -STAT5b, and -GST antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), anti-FLAG antibody from Sigma-Aldrich (St. Louis, MO); anti-phosphotyrosine monoclonal antibody (PY20) from Cosmobio (Tokyo, Japan); anti-phoshoSTAT5a/b (Tyr694/Tyr699) antibody (Stressgen, Victoria, BC, Canada); and anti-actin antibody (Chemicon International, Temecula, CA); anti-STAP-2 antibody was purchased from Everest Biotech (Oxfordshire, UK).

Cell culture, transfection, small interfering RNA (siRNA) 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.[17] Human breast cancer cell line, MCF7 and T47D, STAT5a/b-deficient mouse embryonic fibroblasts (MEFs) were maintained in DMEM containing 10 % FCS. STAP-2- or Brk-knockdown T47D clones (T47D/shSTAP-2#1 and #2, or T47D/shBrk#1 and #2) were established by transfection of with pGPU6/GFP/Neo vector (Shanghai GenePharm , Shanghai, China) bearing short hairpin RNA (shRNA) targeting STAP-2 (5'-CCAGCTGTTGACTATGAGA-3') or Brk (5'-GGATTCTCCTGCATGAGATGT-3') and then selected with G418 (1 mg/mL; Sigma-Aldrich). Similarly, control shRNA (non-sliencing; 5'-TTCTCCGAACGTGTCACGT-3')-transfected T47D clones (T47D/shControl #1 and

#2) were also established. siRNAs targeting human Brk, STAP-2 and STAT5b used in this study were as follows: STAP-2, 5'-GCAGGGUCUCACCAUUUAUTT-3'; STAT5b, 5'-GUGUGAUGGAAGUGUUAAATT-3'; Brk, 5'-GGGUCCAGGUGGCCAUUAATT- 3'. T47D 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. MCF-7 and T47D cells were transfected with STAT5-LUC using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instruction. At 36 h after the cells were transfected, they were harvested and assayed for their luciferase activities, using a dual-luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's instructions. Three or more independent experiments were carried out for each assay.

Immunoprecipitation and immunoblotting. The immunoprecipitation and Western blotting assays were performed as described previously.[17] 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).

Cell proliferation assay. The numbers of viable T47D cells after the indicated treatments were measured using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay (Cell Counting Kit-8; Wako Pure Chemicals).[17] Briefly, 10 μl of WST-8 solution was added to the cells in each well and incubated for 2 h. The absorbances were measured at a test wavelength of 450 nm and a reference wavelength

of 650 nm using a microplate reader (Bio-Rad, Hercules, CA).

Results

STAP-2 regulates Brk-mediated STAT5 activation in breast cancer cells. First, we attempted to clarify the roles of STAP-2 in Brk-mediated STAT5 activation. We transfected plasmids for STAT5a or STAT5b and STAP-2 together with Brk and STAT5-LUC into 293T cells. At 48 h after transfection, the cells were harvested and the STAT5-LUC activities were evaluated. As shown in Fig. 1A, a significantly enhanced STAT5-LUC activity was observed when both Brk and STAP-2 together with STAT5 were expressed simultaneously. However, separate expression of either Brk or STAP-2 failed to induce STAT5-LUC activation. These findings suggest that Brk-mediated STAT5 activation is dependent on STAP-2. Next, we examined the dependency in breast cancer cell lines. We transfected plasmids for STAT5b and STAP-2 together with Brk and STAT5-LUC into MCF7 cells. Similar to the data in 293T cells, a significantly enhanced luciferase activity was only observed when both Brk and STAP-2 were expressed simultaneously (Fig. 1B). In addition, the Brk-mediated STAT5-LUC activity was increased by STAP-2 in a dose-dependent manner. To further assess the functional relevance of endogenous STAP-2 to Brk-mediated STAT5 activation in breast cancer cells, we established a STAP-2-knockdown cell clone (shSTAP-2) of T47D cells expressing endogenous STAP-2. We transfected plasmids for STAT5b with an active form of Brk (Brk Y447F) and STAT5-LUC into the T47D clones. As shown in Fig. 1C, a reduction in STAP-2 expression in T47D cells decreased Brk-mediated STAT5-LUC activation, and STAP-2-knockdown T47D cells completely lost STAT5-LUC activity after Brk Y447F expression. Therefore, endogenous STAP-2 is involved in the positive

regulation of Brk-mediated STAT5 activation in breast cancer cells.

Molecular interactions among STAP-2, STAT3, STAT5 and Brk, and regulation of breast cancer cell growth by STAP-2. To confirm the functional relationship among Brk, STAT5 and STAP-2, we examined their complex formation. As shown in Fig. 2A, the immunoprecipitates for endogenous Brk contained significant levels of endogenous STAT3, STAT5 and STAP-2 in T47D cells. Therefore, Brk forms an efficient complex for its activation in the presence of STAP-2, STAT3 and STAT5. Since STAP-2 positively regulated Brk-mediated STAT5 activation in breast cancer cells, we examined whether STAP-2 expression affected the cell growth. As shown in Fig. 2B, siRNA-mediated reduction of Brk, STAT5b or STAP-2 expression in T47D cells induced marked decreases in the cell growth, indicating that these molecules play important roles in T47D cell growth. SiRNA-mediated reduction of STAT3 expression in T47D cells induced similar decreased cell growth to STAT5-knockdown (Fig. 2C). These findings suggest that the Brk-mediated growth of T47D cells may be largely dependent on STAT3/5 and STAP-2. Taken together, Brk/STAT3/5-mediated proliferation is a major mechanism for breast cancer cell growth, and STAP-2 plays essential roles in this process. Brk and STAP-2 were highly expressed in breast cancer cells, suggesting that this linkage plays an important role in the dysregulated activation of STAT3/5. Our data concerning Brk/STAP-2-mediated modification of STAT3/5 will provide insights toward the development of novel therapeutic strategies for breast cancers. Therefore, we conducted additional experiments to further clarify the molecular mechanisms underlying the Brk/STAP-2-mediated STAT5 activation in breast cancer cells.

STAP-2 regulates Brk-induced tyrosine-phosphorylation of STAT5b at Tyr699.

Initially, we examined the effects of STAP-2 on Brk-mediated tyrosine-phosphorylation of STAT5, which is an important step for transcriptional activation. STAP-2 and STAT5b were transiently expressed without or with Brk in 293T and MCF7 cells. The cells were lysed, and the lysates were immunoblotted with an anti-phosphoSTAT5a/b Tyr694/Tyr699 (pSTAT5a/b) antibody. As shown in Fig. 3A and 3B, the induction of STAT5 tyrosine-phosphorylation by Brk was significantly enhanced in the presence of STAP-2 in both 293T and MCF7 cells. Phosphorylation of endogenous STAT5 at Tyr699 was also enhanced by expression of Brk and STAP-2 in MCF7 cells (Fig. 3C). Consequently, Brk-mediated tyrosine-phosphorylation of STAT5 was markedly enhanced, probably resulting in the enhancement of STAT5-LUC as well as cell growth in breast cancers. Next, we examined Brk-mediated STAT5a and STAT5b activation separately by using STAT5a/b-deficient MEFs. STAP-2 and Brk together with STAT5a or STAT5b were transfected into STAT5a/b-deficient MEFs. As shown in Fig. 3D, the tyrosine-phosphorylation of both ectopically expressed STAT5a and STAT5b was markedly enhanced in STAT5a/b-deficient MEFs only when Brk and STAP-2 were expressed simultaneously. Therefore, the involvement of STAP-2 in Brk-mediated STAT5 activation can be adaptable for both STAT5a and STAT5b. To examine whether a reduction in endogenous STAP-2 expression affects the tyrosine-phosphorylation of STAT5 in breast cancer cells, we used STAP-2-knockdown T47D cells. Constitutive tyrosine-phosphorylation of STAT5 was observed in control T47D cells. However, the tyrosine-phosphorylation of STAT5 was markedly reduced in STAP-2-knockdown T47D cells as well as in Brk-knockdown T47D cells (Fig. 3E). Therefore, STAP-2 plays important roles in the Brk-mediated tyrosine-phosphorylation of STAT5 in breast cancer cells.

Functional role of the PH domain and Tyr250 of STAP-2 in Brk-mediated STAT5 activation. We previously reported that STAP-2 binds to several functional molecules. For example, the SH2 domain of STAP-2 interacts with MyD88 and IKKs,[15] and the PH and SH2 domains of STAP-2 are responsible for the associations with STAT5, LMP1 and TRAF3.[13,18] To assess the functional relationships among Brk, STAP-2 and STAT5, we determined the domains of STAP-2 responsible for Brk-mediated STAT5 activation. We transfected Brk, STAT5b and STAT5-LUC together with a series of STAP-2 deletion mutants (Fig. 4A) into 293T cells. The Brk/STAT5b-mediated STAT5-LUC activity was significantly enhanced by expression of STAP-2 WT, STAP-2 ΔSH2 and STAP-2 ΔC in dose-dependent manners, although STAP-2 ΔSH2 and STAP-2 ΔC showed slightly lower activation than STAP-2 WT (Fig. 4B). Importantly, STAP-2 ΔPH did not show any enhancing effects on the STAT5-LUC activity mediated by Brk. The involvement of the PH domain of STAP-2 was confirmed by using another series of STAP-2 mutants. As shown in Fig. 4C, enhanced tyrosine-phosphorylation of STAT5b by Brk was observed in the presence of STAP-2 PH as well as STAP-2 WT. However, expression of STAP-2 SH2 or STAP-2 C did not induce Brk-mediated STAT5b tyrosine-phosphorylation. Similarly, enhanced tyrosine-phosphorylation of STAT5b by Brk was observed in the presence of STAP-2 PH as well as STAP-2 WT in STAT5a/b-deficient MEFs (Fig. 4D). These findings indicate that the PH domain of STAP-2 plays an essential role in the Brk-mediated STAT5b activation. However, STAP-2 PH domain did not show full Brk-mediated STAT5 phosphorylation induced by STAP-2 WT, indicating that other domains of STAP-2 may be involved in Brk-mediated STAT5 phosphorylation. We also used a STAP-2 Y250F mutant, in which a major Brk-mediated tyrosine-phosphorylation site (Y250) on STAP-2 is mutated.[11] As shown

in Fig. 4E, STAP-2 Y250F showed lower Brk-mediated STAT5b activation than STAP-2 WT, indicating that phosphorylation of STAP-2 at Tyr250 may partly affect Brk-mediated STAT5b activation. Therefore, Brk-mediated STAT5 activation requires the PH domain of STAP-2 and is partly regulated by the phosphorylated state of STAP-2 Tyr 250.

Discussion

Several Brk-interacting proteins or Brk substrates, such as the RNA-binding protein Sam68 and the polypyrimidine tract-binding protein-associated splicing factor PSF, have been reported.[19,20] However, the molecular mechanisms by which Brk cooperates with these proteins and participates in tumorigenesis remain poorly characterized. Our data will provide insights into the molecular mechanisms for how Brk acts as a tumorigenic protein in breast cancers.

STAT5 has two isoforms, STAT5a and STAT5b, which are encoded by two genes that are closely linked on human chromosome 17.[21] STAT5a and STAT5b share 96% sequence similarity in humans. STAT5a was originally identified as a mammary gland factor that augments milk protein expression, whereas STAT5b was shown to mediate the biological effects of growth hormone in the mammary gland. STAT5 is also activated in the downstream of other cytokines including erythropoietin, IL-2 or IL-3. Although STAP-2 augmented Brk-mediated STAT5 activation in breast cancer cells, it has been shown to negatively regulate STAT5 activation in erythropoietin-, IL-2- and IL-3-induced signaling.[13] At the present time, we cannot explain these different effects of STAP-2 on STAT5 activation, although it is possible that unknown signal-specific or cell type-specific factors may influence the effects of STAP-2. Importantly, both

STAT5a and STAT5b are overexpressed or constitutively activated in cancers, including some breast cancer tumors.[8,22] However, recent evidence indicates that STAT5b, but not STAT5a, has a proproliferative role in breast cancer, head and neck cancer, and prostate cancer.[6, 23-25]

In conclusion, the activation of STAT5 by Brk is one of the critical events during the process of Brk-mediated tumorigenesis in breast cancer cells. Our manipulation of STAP-2 expression revealed essential roles of STAP-2 in this process through complex interactions among Brk, STAP-2 and STAT5. In particular, experiments using deletion mutants indicated that the PH domain of STAP-2 is involved in multiple steps, namely the binding between Brk and STAP-2, the activation and tyrosine-phosphorylation of STAT5, and the activation of Brk. These findings suggest one mechanism for how STAP-2 cooperates with Brk to enhance breast cancer growth. In addition, our recent report described that STAP-2 is essential for Brk-mediated STAT3 activation.[18] Taken together, STAP-2 plays crucial roles in both the Brk/STAT3 and Brk/STAT5 axes, which are major events for Brk-induced breast cancers. Consequently, our information regarding Brk/STAP-2 interactions will be helpful for breast cancer treatment. On the other hand, Brk expression in breast cancer cells has been reported to indicate a poor prognosis.[26] However, the synergistic effects of Brk and STAP-2 on STAT3/5 activation could suggest that expression of Brk together with STAP-2 may provide more valuable prognostic scores for the outcomes of breast carcinomas than individual examinations of Brk expression.

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Disclosure Statement

The authors declare no conflict of interest.

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Figure legends

Fig. 1. STAP-2 regulates Brk-mediated STAT5 activation in breast cancer cells.

(A) 293T cells in a 24-well plate were transfected with STAT5-LUC (100 ng) and FLAG-tagged STAT5a (100 ng) or STAT5b (100 ng) and/or FLAG-tagged Brk (100 ng) and the indicated amounts (30, 300 ng) of expression vector for Myc-tagged STAP-2. At 48 h 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 anti-FLAG or anti-Myc antibody. (B) MCF7 cells in a 24-well plate were transfected with STAT5-LUC (100 ng) and/or FLAG-tagged STAT5b (100 ng) and/or FLAG-tagged Brk (100 ng), and the indicated amounts (30, 300 ng) of expression vector for Myc-tagged STAP-2. At 48 h after transfection, the cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System. At least three independent experiments were carried out for each assay. **$p<0.01$. An aliquot of each TCL was analyzed by immunoblotting with anti-FLAG or anti-Myc antibody. (C) shControl- or shSTAP-2-T47D cells in a 24-well plate were transfected with STAT5-LUC (100 ng) and/or Brk Y447F (300 ng) using jetPEI. At 36 h after transfection, the cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System. At least three independent experiments were carried out for each assay. *$p< 0.05$. An aliquot of each TCL was analyzed by immunoblotting with anti-Brk, anti-FLAG, anti-STAP-2 or anti-actin antibody.

Fig. 2. Molecular interactions among STAP-2, STAT5 and Brk, and regulation of breast

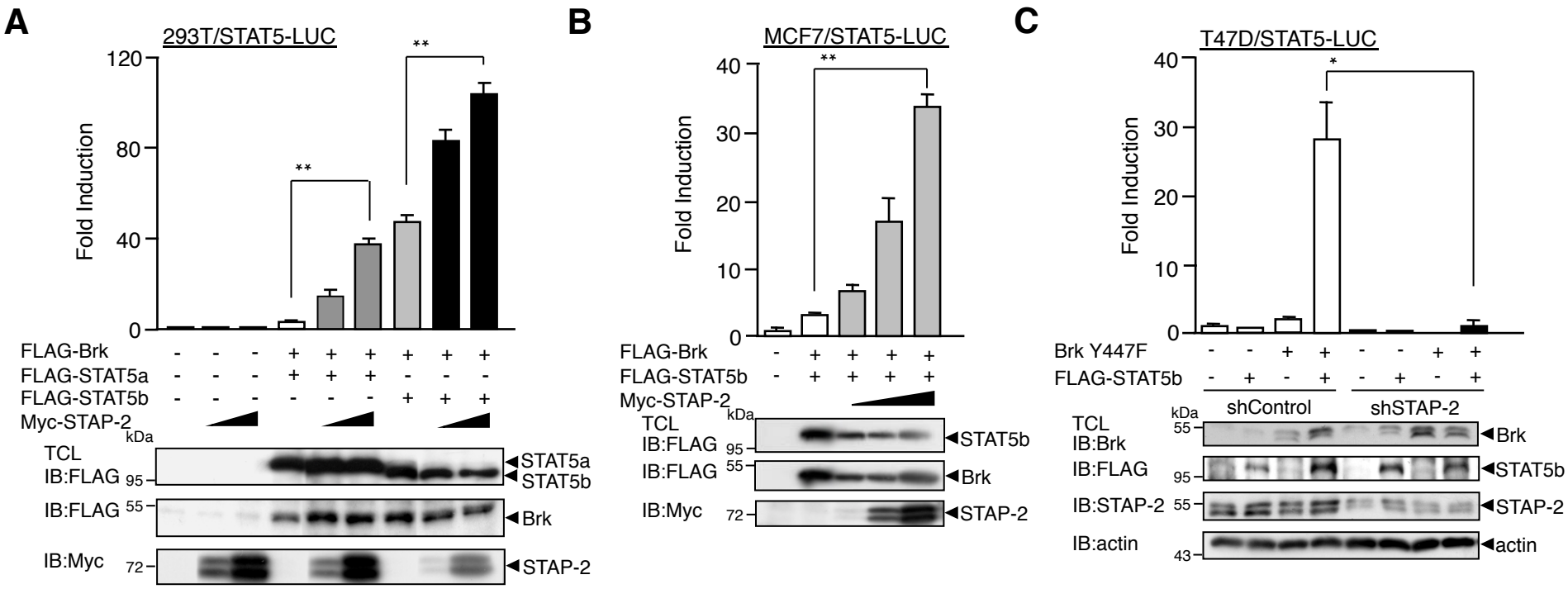
cancer cell growth by STAP-2. (A) T47D cells ($3x10^7$) were lysed and immunoprecipitated with control IgG or anti-Brk antibody and immunoblotted with anti-STAT3, anti-STAT5b or anti-STAP-2 (upper panel). An aliquot of total cell lysate (TCL) was also blotted with anti-Brk antibody (bottom panel). (B) T47D cells were transfected with a control siRNA, Brk siRNA, STAP-2 siRNA or STAT5b siRNA. The cells were then cultured in 96-well plates for the indicated periods. The cell numbers were measured using a WST-8. The data are the means of triplicate experiments, which generally varied by <10%. Similar results were obtained in three independent experiments. An aliquot of each TCL after siRNA transfection (day 3) was analyzed by immunoblotting with an anti-Brk, anti-STAP-2, anti-STAT5b or anti-actin antibody. Knockdown of each protein was confirmed until 6 days after siRNA trasfection. (C) T47D cells were transfected with a control siRNA, STAT3 siRNA or STAT5b siRNA. The cells were then cultured in 96-well plates for the indicated periods. The cell numbers were measured using a WST-8. The data are the means of triplicate experiments, which generally varied by <10%. Similar results were obtained in three independent experiments. An aliquot of each TCL after siRNA transfection (day 3) was analyzed by immunoblotting with an anti-STAT3, anti-STAT5b or anti-actin antibody. Knockdown of each protein was confirmed until 6 days after siRNA trasfection.

Fig. 3. STAP-2 regulates Brk-induced tyrosine-phosphorylation of STAT5b at Tyr699.
(A) 293T cells in a 12-well plate were transfected with FLAG-tagged STAT5b (0.5 μg) and/or FLAG-tagged Brk (1μg), and the increasing amounts of Myc-tagged STAP-2 (0.3, 1 μg). At 48 h after transfection, the cells were lysed, and an aliquot of each total cell lysate (TCL) was analyzed by immunoblotting with anti-pSTAT5a/b (Tyr694/699),

anti-FLAG or anti-Myc antibody. (B) MCF7 cells in a 12-well plate were transfected with FLAG-tagged STAT5b (0.5 μg) and/or FLAG-tagged Brk (1 μg) and the increasing amounts of Myc-tagged STAP-2 (0.3, 1 μg). At 48 h after transfection, the cells were lysed, and an aliquot of each TCL was analyzed by immunoblotting with anti-pSTAT5a/b (Tyr694/699), anti-FLAG or anti-Myc antibody. (C) MCF7 cells in a 12-well plate were transfected with FLAG-tagged Brk (0.5 μg) and the increasing amounts of Myc-tagged STAP-2 (0.3, 1 μg). At 48 h after transfection, the cells were lysed, and an aliquot of each TCL was analyzed by immunoblotting with anti-pSTAT5a/b (Tyr694/699), anti-STAT5b, anti-FLAG or anti-Myc antibody. (D) STAT5a/b-deficient MEFs in a 12-well plate were transfected with Myc-tagged STAP-2 (1 μg) and/or FLAG-tagged STAT5a or STAT5b (0.5 μg) and/or FLAG-tagged Brk (1 μg). At 48 h after transfection, the cells were lysed, and an aliquot of each TCL was analyzed by immunoblotting with anti-pSTAT5a/b (Tyr694/699), anti-FLAG or anti-Myc antibody. (E) T47D/shControl (#1 and #2), T47D/shSTAP-2 (#1 and #2) and T47D/shBrk (#1 and #2) cells were lysed, immunoprecipitated with anti-STAT5b and immunoblotted with anti-pSTAT5a/b (Tyr694/Tyr699) and anti-STAT5b antibody. An aliquot of each TCL was analyzed by immunoblotting with anti-STAP-2, anti-Brk and anti-actin antibody.

Fig. 4. Functional role of the PH domain and Tyr250 of STAP-2 in Brk-mediated STAT5 activation. (A) Schematic diagrams of the domain structures of the STAP-2 deletion mutant fragments. (B) 293T cells in a 24-well plate were transfected with STAT5-LUC (100 ng) and FLAG-tagged STAT5b (100 ng) and/or FLAG-tagged Brk (100 ng), and the increasing amounts (30, 300 ng) of expression vector for Myc-tagged

STAP-2 WT, STAP-2 ΔPH, STAP-2 ΔSH2 or STAP-2 ΔC. At 48 h after transfection, the cells were harvested and the luciferase activities were measured as described the above. At least three independent experiments were carried out for each assay. ***p<0.001. An aliquot of each total cell lysate (TCL) was analyzed by immunoblotting with anti- FLAG or anti- Myc antibody. (C) 293T cells in a 12-well plate were transfected with FLAG-tagged Brk (1 μg) with or without FLAG-tagged STAP-2 deletion mutants (1 μg). At 48 h after transfection, the cells were lysed and immunoblotted with an anti-pSTAT5a/b (Tyr694/699) and anti-FLAG antibody. (D) STAT5a/b-deficient MEFs in a 12-well plate were transfected with FLAG-tagged STAT5b (0.5 μg) with GST or GST-tagged STAP-2 (1 μg) and/or FLAG-tagged Brk (1 μg). At 48 h after transfection, the cells were lysed, and an aliquot of each TCL was analyzed by immunoblotting with anti-pSTAT5a/b (Tyr694/699), anti-FLAG or anti-GST antibody. (E) 293T cells in a 24-well plate were transfected with STAT5-LUC (100 ng) and FLAG-tagged STAT5b (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. At 48 h after transfection, the cells were harvested and the luciferase activities were measured as described the above. At least three independent experiments were carried out for each assay. **p<0.01. An aliquot of each TCL was analyzed by immunoblotting with anti-FLAG or anti-Myc antibody.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293T/STAT5-LUC
Fold Induction
120
80
40
0
**
**
FLAG-Brk - - - + + + + + +
FLAG-STAT5a - - - + + + - - -
FLAG-STAT5b - - - - - - + + +
Myc-STAP-2
kDa
TCL
IB:FLAG 95
STAT5a
STAT5b
IB:FLAG 55
Brk
IB:Myc 72
STAP-2
B
MCF7/STAT5-LUC
Fold Induction
40
30
20
10
0
**
FLAG-Brk - + + + +
FLAG-STAT5b - + + + +
Myc-STAP-2
kDa
TCL
IB:FLAG 95
STAT5b
IB:FLAG 55
Brk
IB:Myc 72
STAP-2
C
T47D/STAT5-LUC
Fold Induction
40
30
20
10
0
*
Brk Y447F - - + + - - + +
FLAG-STAT5b - + - + - + - +
shControl
shSTAP-2
kDa
TCL
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Brk
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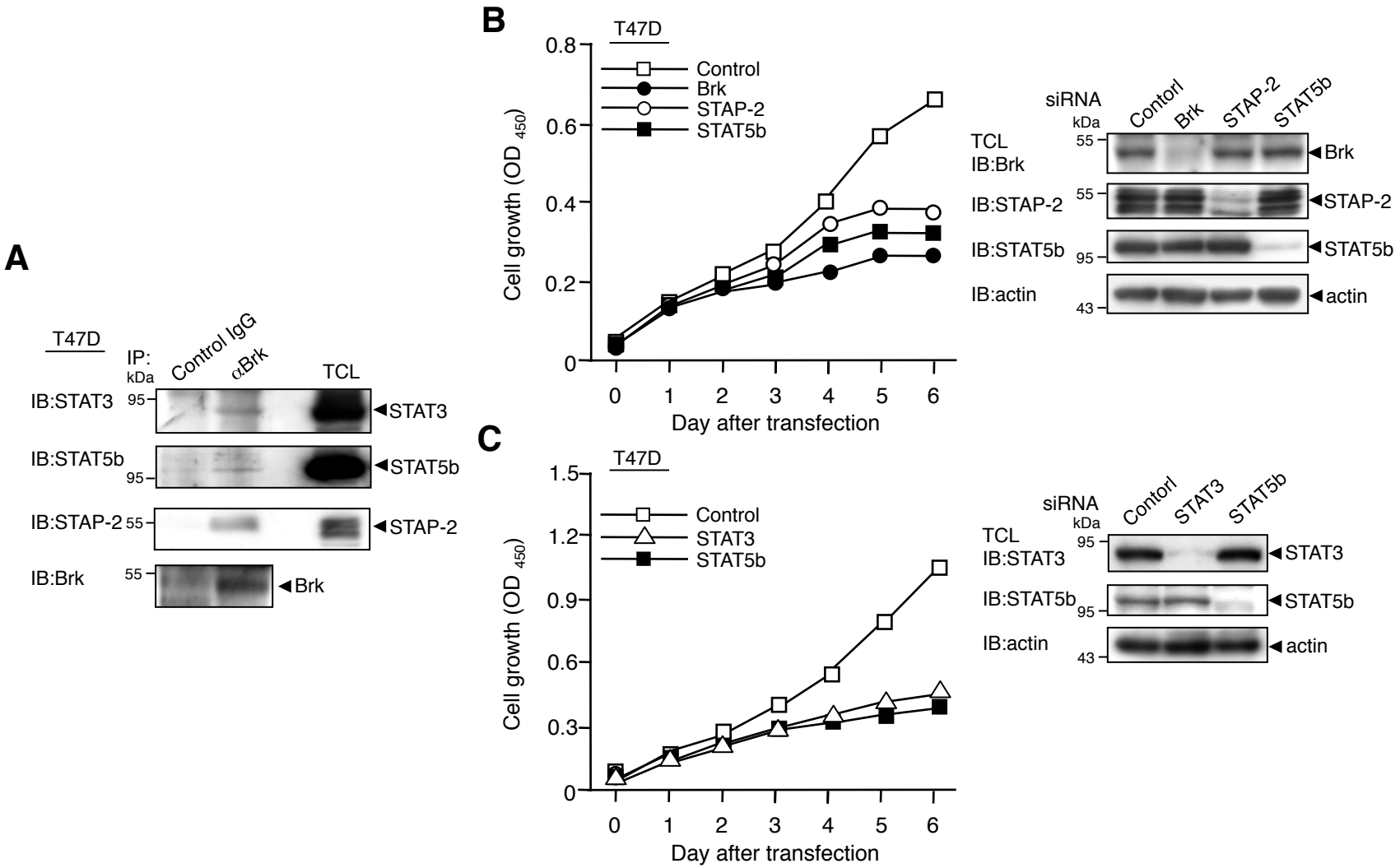
STAT5b
IB:STAP-2 55
STAP-2
IB:actin 43
actin

Figure 1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T47D
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kDa
Control IgG
αBrk
TCL
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95
STAT3
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95
STAT5b
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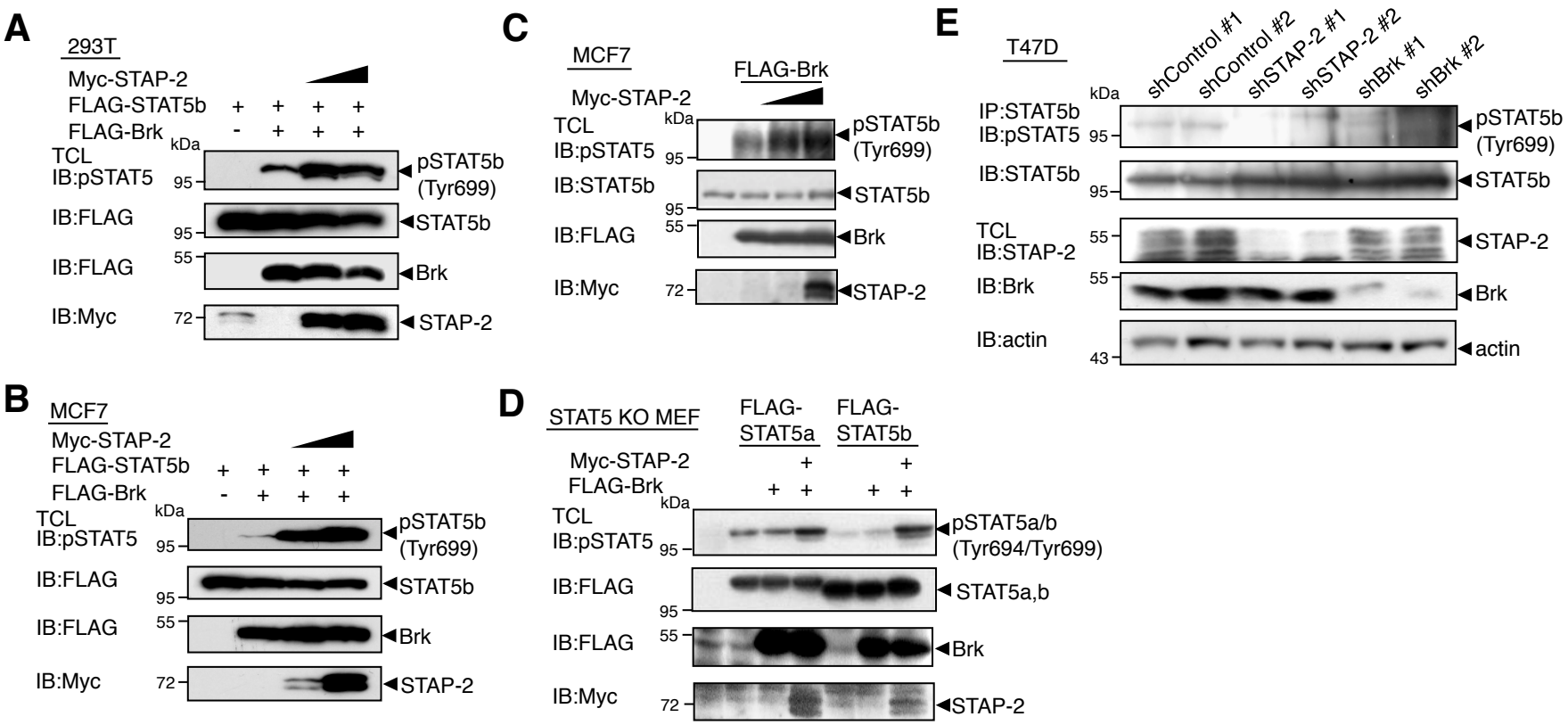
55
STAP-2
IB:Brk
55
Brk
B
T47D
Control
Brk
STAP-2
STAT5b
Cell growth (OD 450)
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6
Day after transfection
siRNA
kDa
Contorl
Brk
STAP-2
STAT5b
TCL
IB:Brk
55
Brk
IB:STAP-2
55
STAP-2
IB:STAT5b
95
STAT5b
IB:actin
43
actin
C
T47D
Control
STAT3
STAT5b
Cell growth (OD 450)
1.5
1.2
0.9
0.6
0.3
0
0 1 2 3 4 5 6
Day after transfection
siRNA
kDa
Contorl
STAT3
STAT5b
TCL
IB:STAT3
95
STAT3
IB:STAT5b
95
STAT5b
IB:actin
43
actin

Figure 2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293T
Myc-STAP-2
FLAG-STAT5b + + + +
FLAG-Brk - + + +
kDa
TCL
IB:pSTAT5
95
pSTAT5b
(Tyr699)
IB:FLAG
95
STAT5b
IB:FLAG
55
Brk
IB:Myc
72
STAP-2
B
MCF7
Myc-STAP-2
FLAG-STAT5b + + + +
FLAG-Brk - + + +
kDa
TCL
IB:pSTAT5
95
pSTAT5b
(Tyr699)
IB:FLAG
95
STAT5b
IB:FLAG
55
Brk
IB:Myc
72
STAP-2
C
MCF7
FLAG-Brk
Myc-STAP-2
kDa
TCL
IB:pSTAT5
95
pSTAT5b
(Tyr699)
IB:STAT5b
95
STAT5b
IB:FLAG
55
Brk
IB:Myc
72
STAP-2
D
STAT5 KO MEF
FLAG-
STAT5a
FLAG-
STAT5b
Myc-STAP-2 + +
FLAG-Brk + + + +
kDa
TCL
IB:pSTAT5
95
pSTAT5a/b
(Tyr694/Tyr699)
IB:FLAG
95
STAT5a,b
IB:FLAG
55
Brk
IB:Myc
72
STAP-2
E
T47D
shControl #1
shControl #2
shSTAP-2 #1
shSTAP-2 #2
shBrk #1
shBrk #2
kDa
IP:STAT5b
IB:pSTAT5
95
pSTAT5b
(Tyr699)
IB:STAT5b
95
STAT5b
TCL
IB:STAP-2
55
STAP-2
IB:Brk
55
Brk
IB:actin
43
actin

Figure 3

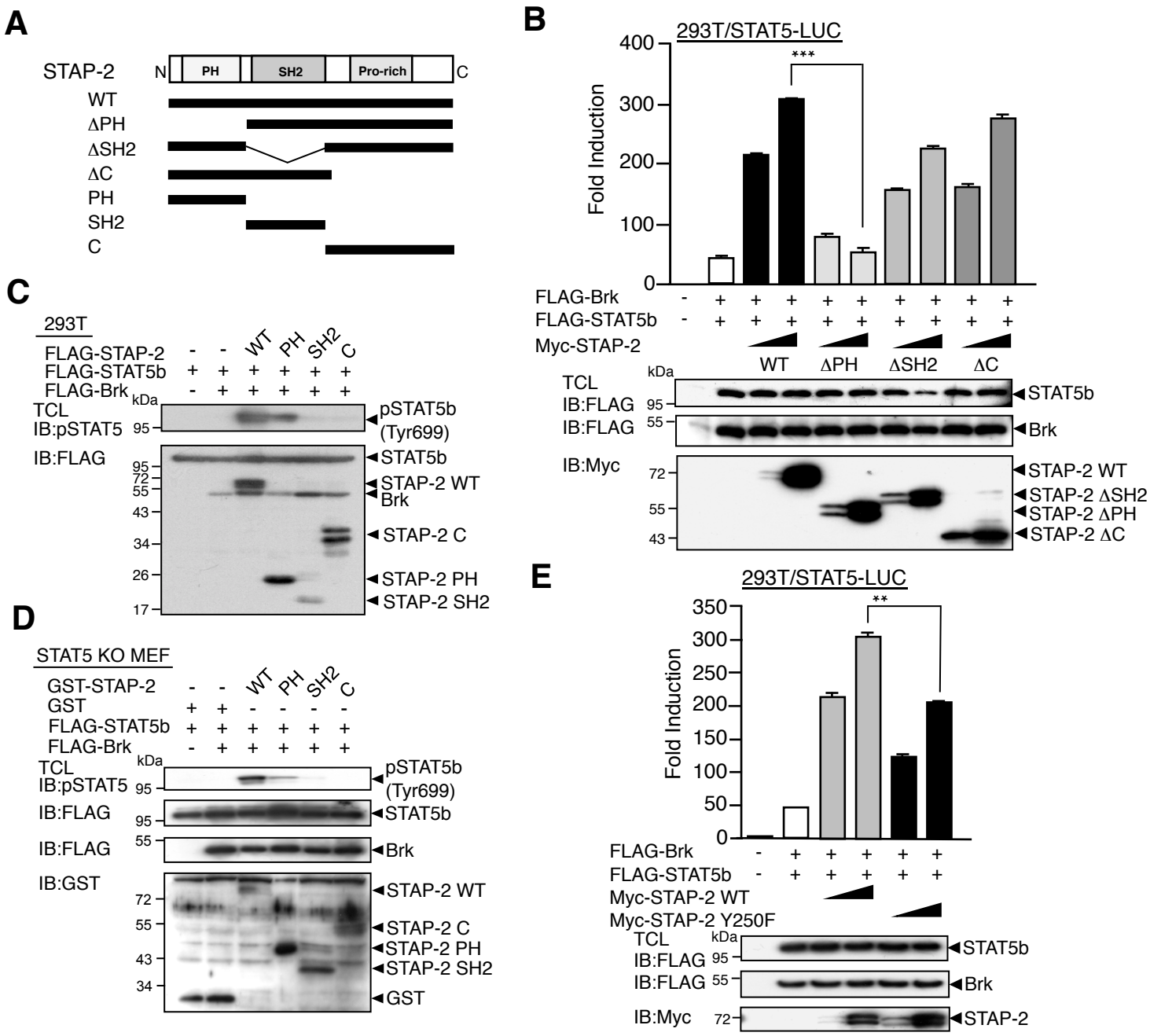
A
STAP-2
N
PH
SH2
Pro-rich
C
WT
ΔPH
ΔSH2
ΔC
PH
SH2
C
B
293T/STAT5-LUC
Fold Induction
400
300
200
100
0
***
FLAG-Brk
FLAG-STAT5b
Myc-STAP-2
WT
ΔPH
ΔSH2
ΔC
TCL
IB:FLAG
IB:FLAG
IB:Myc
kDa
95
55
72
55
43
STAT5b
Brk
STAP-2 WT
STAP-2 ΔSH2
STAP-2 ΔPH
STAP-2 ΔC
C
293T
FLAG-STAP-2
FLAG-STAT5b
FLAG-Brk
WT
PH
SH2
C
TCL
IB:pSTAT5
IB:FLAG
kDa
95
95
72
55
43
34
26
17
pSTAT5b
(Tyr699)
STAT5b
STAP-2 WT
Brk
STAP-2 C
STAP-2 PH
STAP-2 SH2
D
STAT5 KO MEF
GST-STAP-2
GST
FLAG-STAT5b
FLAG-Brk
WT
PH
SH2
C
TCL
IB:pSTAT5
IB:FLAG
IB:FLAG
IB:GST
kDa
95
95
55
72
55
43
34
pSTAT5b
(Tyr699)
STAT5b
Brk
STAP-2 WT
STAP-2 C
STAP-2 PH
STAP-2 SH2
GST
E
293T/STAT5-LUC
Fold Induction
350
300
250
200
150
100
50
0
**
FLAG-Brk
FLAG-STAT5b
Myc-STAP-2 WT
Myc-STAP-2 Y250F
TCL
IB:FLAG
IB:FLAG
IB:Myc
kDa
95
55
72
STAT5b
Brk
STAP-2

Figure 4