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**Title: Leukemia inhibitory factor-induced phosphorylation of STAP-2 on tyrosine-250 is involved in its STAT3-enhancing activity**

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*Running title:* LIF induces STAP-2 phosphorylation

*Keywords:* STAP-2, LIF, phosphorylation

## **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 studies revealed that STAP-2 binds to signal transducer and activator of transcription 3 (STAT3) and STAT5, and regulates their signaling pathways. In the present study, we identified tyrosine-250 (Tyr250) in STAP-2 as a major site of phosphorylation by v-Src and Jak2, using a phospho-specific antibody against STAP-2 phosphorylated at Tyr250. Mutational analyses revealed that Tyr250 was involved in the STAT3-enhancing activity of STAP-2. We further found that leukemia inhibitory factor (LIF) or Jak2 expression stimulated STAP-2 Tyr250 phosphorylation in 293T and Hep3B cells. Moreover, endogenous STAP-2 was phosphorylated at Tyr250 following LIF stimulation of murine M1 cell line. Taken together, our findings demonstrate that endogenous STAP-2 is phosphorylated at Tyr250 and that this phosphorylation is involved in its function.

## **Introduction**

Tyrosine kinases 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 to create specific binding sites for adaptor proteins that contain Src homology 2 (SH2) 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].

Recently, we cloned two novel adaptor proteins, designated signal transducing adaptor protein (STAP)-1 and STAP-2 [3,4]. STAP-1 was identified as a c-kit-interacting protein, while STAP-2 interacted with c-fms. Human STAP-2 is identical to the recently cloned adaptor molecule BKS, which is a substrate of breast tumor kinase (Brk) tyrosine kinase [5]. Both STAP-1 and STAP-2 contain an N-terminal Pleckstrin homology (PH) and a region distantly related to the SH2 domain (33% overall amino acid identity). The N-terminal PH domains of STAP-2 and STAP-1 share

36% identity and 58% similarity. The central region of STAP-2, which is distantly related to the SH2 domain, shares 40% sequence identity with the corresponding region of STAP-1 and 29% sequence identity with the SH2 domain of human PLC $\gamma$ 2. However, STAP-2 possesses a C-terminal proline-rich region and a YXXQ motif, neither of which are present in STAP-1. We previously reported that STAP-2 interacts with STAT3 through its YXXQ motif and enhances STAT3 transcriptional activity [4]. STAP-2 also interacts with STAT5 through its PH and SH2-like domains [6]. Notably, our previous studies demonstrated that STAP-2 also binds to MyD88 and IKK- $\alpha/\beta$ , and modulates NF- $\kappa$ B signaling in macrophages [7].

In the present study, we identified tyrosine-250 (Tyr250) as a major phosphorylation site of STAP-2. We further found that mutation of Tyr250 suppressed STAP-2-mediated enhancement of leukemia inhibitory factor (LIF)-induced STAT3 activation. Furthermore, LIF stimulation induced phosphorylation of endogenous STAP-2 at Tyr250, as evaluated using a phospho-specific antibody, suggesting that LIF mediates activation of STAP-2 through phosphorylation of Tyr250.

## **Materials and Methods**

### *Reagents and antibodies*

Recombinant human LIF was purchased from INTERGEN (Purchase, NY). Expression vectors, v-src, GST-Jak2JH1, epitope-tagged STAP-2 and its YF (substitution of Tyr to Phe) mutants were described previously [4]. Expression vectors for STAT3-LUC was provided by Dr. T. Hirano (Osaka University, Osaka, Japan) [8]. Anti-Myc and -GST antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Anti-v-Src antibody was from Oncogene Research Products (Darmstadt, Germany).

Anti-actin antibody was purchased from Chemicon (Temecula, CA).

Anti-phosphotyrosine monoclonal antibody (PY20) was purchased from Cosmobio (Tokyo, Japan). The phosphopeptide used as immunogen for anti-phosphoSTAP-2 Tyr250 (anti-pSTASP-2 Tyr250) was PFLLEDpYEKVLGF, corresponding to residues 243 to 256 of murine STAP-2. The phospho-peptide was conjugated to keyhole limpet hemocyanin as an antigen. Rabbits were immunized and boosted three times at monthly intervals with the conjugate, before blood was collected and immune serum

was obtained. Anti-mouse STAP-2 antibody was also prepared by immunization of the conjugate with the synthetic peptide KVLGFVDSRENAGE corresponding to residues 252 to 265 of murine STAP-2.

*Cell culture, transfection, luciferase assays and cell growth assays*

Murine myeloid leukemia cell line, M1 was cultured in RPMI 1640 containing 10% fetal calf serum (FCS) [9]. Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected by the standard calcium precipitation protocol. Luciferase assay was performed as described [10]. At 36 h after transfection, the cells were stimulated with LIF (50 ng/ml) for additional 6 h and lysed in 50  $\mu$ 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  $\beta$ -galactosidase activities. Three or more independent experiments were carried out for each assay. Human hepatoma cell line, Hep3B, was maintained in DMEM containing 10% FCS and cells were transfected using jetPEI

(PolyPlus-transfection). At 36 h after transfection, the cells were stimulated with LIF (50 ng/ml) for additional 8 h. The stimulated 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. Cell growth was determined by Cell Counting Kit-8 (Wako Chemicals, Tokyo, Japan) according to manufacturer's instructions.

#### *Immunoprecipitation and immunoblotting*

The immunoprecipitation and Western blotting assays were performed as described previously [11]. Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1  $\mu$ M sodium orthovanadate, 1  $\mu$ M phenylmethylsulfonyl fluoride and 10  $\mu$ g/ml each of aprotinin, pepstatin and leupeptin). An aliquot of total cell lysates were resolved on SDS-PAGE and transferred to PVDF transfer membrane (PerkinElmer; Boston, MA, USA). The filters were then immunoblotted with the respective antibody.

## **Results and Discussion**

### *Characterization of a phospho-specific antibody recognizing the Tyr250 phosphorylation site*

Previous studies have demonstrated that STAP-2 is tyrosine-phosphorylated by several protein tyrosine kinases (PTKs), including Brk, v-src, Jak2, Syk and EGFR [4,5,12]. We identified four potential phosphorylation sites via substitution of tyrosine residues with phenylalanine (Fig. 1A). Among these, Tyr250 was found to be phosphorylated by several PTKs. To further probe the phosphorylation status of STAP-2 Tyr250, we generated a phospho-specific antibody against the Tyr250 site (Fig. 1A), designated anti-pSTAP-2 Tyr250, and confirmed its specificity by immunoblotting of intact recombinant STAP-2 proteins. Myc-tagged wild-type STAP-2 (STAP-2 WT) or a series of STAP-2 YF mutants were expressed with or without v-Src in 293T cells. The expressed STAP-2 WT and YF mutant proteins were immunoblotted with preimmune serum, anti-pSTAP-2 Tyr250, anti-phosphotyrosine (PY) or anti-Myc antibodies. As shown in Fig. 1B, the STAP-2 WT and YF mutant proteins, with or without v-Src coexpression, were expressed at equivalent protein levels. The anti-pSTAP-2 Tyr250

antibody only failed to recognize the STAP-2 Y250F mutant coexpressed with v-Src (Fig. 1B, lane 11), indicating that the anti-pSTAP-2 Tyr250 antibody specifically recognizes STAP-2 at Tyr250. Furthermore, the STAP-2 Y250F mutant protein coexpressed with v-Src showed a faster mobility than STAP-2 WT and the other YF mutant proteins. This mobility shift of STAP-2 Y250F may be an effect of phosphorylation, suggesting that Tyr250 is a major phosphorylation site.

Next, we examined whether STAP-2 Tyr250 represented a major site of phosphorylation by Jak2. To achieve this, Myc-tagged STAP-2 WT and the same series of YF mutants were expressed with or without a GST-Jak2 kinase domain fusion protein (GST-JH1) in 293T cells. As shown in Fig. 1C, the anti-pSTAP-2 Tyr250 antibody only failed to recognize the STAP-2 Y250F mutant coexpressed with GST-JH1, indicating that Jak2 also phosphorylates STAP-2 at Tyr250. Furthermore, STAP-2 Y250F also showed a shift to a faster mobility in this experiment (Fig. 1C, lane 4).

#### *Substitution of Tyr250 to Phe in STAP-2 reduces its STAT3-enhancing activity*

In a previous study, we demonstrated that ectopic expression of STAP-2 enhanced

STAT3 activation, using a STAT3-dependent acute phase protein responsive element (APRE)-reporter assay involving STAT3-LUC in HEK-293 cells [4]. The results revealed that STAP-2 WT enhanced EGF- or LIF-induced STAT3-LUC activation in HEK-293 cells. We also demonstrated that both STAP-2 Tyr250 and Tyr322 are involved in v-src-induced enhanced STAT3-LUC activation in MCF7 cells. We here focused the role of Tyr250 phosphorylation in STAP-2, because STAP-2 Tyr250 is the major phosphorylation site in 293T cells by v-src and Jak2 as shown in Fig. 1. To first confirm the effect of substituting STAP-2 Tyr250 with Phe on LIF-induced STAT3-mediated signaling, we transfected STAP-2 WT or STAP-2 Y250F together with STAT3-LUC into 293T cells. After 36 h, the cells were treated with LIF for 6 h and the STAT3-LUC activities were determined. As shown in Fig. 2A, STAP-2 WT positively regulated the LIF-induced STAT3 activation. In contrast, the STAP-2 Y250F transfectant failed to show enhanced LIF-induced STAT3 activation. We also examined this effect using human hepatoma cell line, Hep3B. STAP-2 WT or STAP-2 Y250F together with STAT3-LUC were transfected into Hep3B cells. After 36 h, the cells were treated with LIF for 8 h and the STAT3-LUC activities were determined. As shown in

Fig. 2B, STAP-2 WT but not Y250F positively stimulated the LIF-induced STAT3 activation. These results suggest that Tyr250 in STAP-2 is important for its STAT3-enhancing activity.

*LIF stimulates phosphorylation of STAP-2 Tyr250 in 293T and Hep3B cells*

We further assessed the phosphorylation state of Tyr250 in STAP-2 after LIF stimulation using the anti-pSTAP-2 Tyr250 antibody. To this end, Myc-tagged STAP-2 WT was expressed in 293T cells. After 36 h, the cells were treated with LIF for 8 h and then lysed. Next, aliquots of the total cell lysate were immunoblotted with anti-pSTAP-2 Tyr250, anti-PY or anti-Myc antibodies. As shown in Fig. 3A, the anti-pSTAP-2 Tyr250 antibody only recognized the STAP-2 protein in the LIF-treated 293T transfectant. In contrast, the anti-PY antibody failed to recognize the STAP-2 protein in the LIF-treated 293T transfectant. We also tested LIF-induced phosphorylation of STAP-2 Tyr250 in Hep3B cells expressing STAP-2 WT. As shown in Fig. 3B, LIF stimulation enhanced phosphorylation of STAP-2 Tyr250 in Hep3B cells, although a faint phosphorylation of STAP-2 was observed in Hep3B cells in the absence of LIF stimulation, suggesting that

endogenous unknown tyrosine kinases may phosphorylate STAP-2 Tyr250 in Hep3B cells. In Hep3B cells, the anti-PY antibody could not recognize the STAP-2 protein. Therefore, we could detect phosphorylation of STAP-2 Tyr250 in 293T and Hep3B cells using the anti-pSTAP-2 Tyr250 antibody. Furthermore, the anti-pSTAP-2 Tyr250 antibody recognizes phosphorylated STAP-2 more specifically or sensitively than the anti-PY antibody.

*LIF stimulates phosphorylation of Tyr250 in endogenous STAP-2*

To verify that Tyr250 in endogenous STAP-2 is phosphorylated in response to LIF stimulation, we first used the LIF-responsive murine leukemia cell line M1, which can be induced to undergo growth arrest and terminal differentiation into macrophages in response to LIF [8,13]. In fact, LIF was found to induce growth arrest of M1 cells at 48 h after stimulation (Fig. 4A). Previously, we demonstrated mRNA expression of endogenous STAP-2 in LIF-treated M1 myeloid cells and murine macrophages [4]. However, since an anti-murine STAP-2 antibody was not available, we could not detect the phosphorylation of the endogenous STAP-2 protein in that study. In the present

study, we successfully generated an anti-murine STAP-2 antibody by immunization with a synthetic peptide conjugate. Using the anti-pSTAP-2 Tyr250 and anti-murine STAP-2 (MuSTAP-2) antibodies, we examined the phosphorylation of endogenous STAP-2 by LIF in M1 cells. At 24 h after LIF stimulation, endogenous STAP-2 was phosphorylated at Tyr250 in M1 cells (Fig. 4B). Similar phosphorylation of STAP-2 was observed at 48 h after LIF stimulation, although its protein level was clearly decreased compared with that of actin (Fig. 4B). These data indicate that LIF stimulation results in phosphorylation of endogenous STAP-2 at Tyr250. Moreover, these findings suggest that phosphorylation of STAP-2 may induce its degradation by currently unknown mechanisms.

#### *Concluding remarks*

The results of the present study provide evidence that Tyr250 in STAP-2 is a major phosphorylation site and involved in its STAT3-enhancing activity. We further clarified that LIF induces phosphorylation of ectopically-expressed STAP-2 at Tyr250 in 293T and Hep3B cells using an anti-pSTAP-2 Tyr250 antibody. Furthermore, we demonstrated that

LIF stimulates phosphorylation of endogenous STAP-2 in murine M1 cells and that phosphorylation-dependent STAP-2 degradation is observed in cells. These data indicate the possibility that STAP-2 recruits other regulatory molecules, such as ubiquitin ligases, which may accelerate the degradation of STAP-2 by the ubiquitin-proteasome pathway. Recently, adaptor molecule containing PH and SH2 domains (APS) was shown to be tyrosine-phosphorylated by Jak2 at its C-terminal tyrosine residue and to interact with c-Cbl [14]. STAP-2 may also recruit ubiquitin-proteasome signaling molecules, such as c-Cbl, to suppress its STAT3-enhancing activity. Indeed, c-Cbl was found to interact with STAP-2 in 293T cells (Y. Sekine and T. Matsuda, unpublished data). Further detailed studies are necessary to elucidate the molecular mechanisms of STAP-2 regulation by degradation. However, the anti-pSTAP-2 Tyr250 antibody produced in this study will represent a powerful tool for clarifying the physiological roles of STAP-2 in normal and diseased conditions.

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## Figure legends

### Fig. 1. Phosphorylation of Tyr250 in STAP-2 by v-Src or Jak2

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

B. 293T cells ( $1 \times 10^7$ ) were transfected with or without Myc-tagged STAP-2 WT (10 $\mu$ g) or STAP-2 YF mutants (10 $\mu$ g) and/or v-Src (5 $\mu$ g). Forty-eight hrs after transfection, the cells were lysed. Total cell lysates (1%) were blotted with preimmune antibody, anti-pSTAP-2 Tyr250, anti-PY or anti-v-Src antibody.

C. 293T cells ( $1 \times 10^7$ ) were transfected with or without Myc-tagged STAP-2 WT (10 $\mu$ g) or STAP-2 YF mutants (10 $\mu$ g) and/or GST-JH1 (5 $\mu$ g). Forty-eight hrs after transfection, the cells were lysed. Total cell lysates (1%) were blotted with anti-pSTAP-2 Tyr250, anti-PY or anti-GST antibody.

### Fig.2. Substitution of Tyr250 to Phe in STAP-2 reduces its STAT3 enhancing activity

A. 293T cells in a 12-well plate were transfected with STAT3-LUC (0.4  $\mu$ g) and/or indicated amounts (3, 30 ng) of expression vector for STAP-2 WT or STAP-2 Y250F.

Thirty-six hrs after transfection, the cells were stimulated with LIF (50 ng/ml) for additional 6 hrs. The stimulated 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 S.D. Total cellular protein collected from parallel cultures was analysed for STAP-2 by Western blot analysis (lower panel).

B. Hep3B cells in a 12-well plate were transfected with STAT3-LUC (0.2  $\mu$ g) and/or indicated amounts (30 ng) of expression vector for STAP-2 WT or STAP-2 Y250F. Thirty-six hrs after transfection, the cells were stimulated with LIF (50 ng/ml) for additional 8 hrs. The stimulated 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 S.D. Total cellular protein collected from parallel cultures was analysed for STAP-2 by Western blot analysis (lower panel).

Fig. 3. LIF stimulates phosphorylation of STAP-2 Tyr250 in 293T and Hep3B cells

A. 293T cells ( $1 \times 10^7$ ) were transfected with or without Myc-tagged STAP-2 WT ( $10 \mu\text{g}$ ).

Thirty-six hrs after transfection, the cells were stimulated with LIF (100 ng/ml) for additional 8 hrs. The cells were then lysed. Total cell lysates (1%) were blotted with anti-pSTAP-2 Tyr250, anti-PY or anti-Myc antibody.

B. Hep3B cells ( $1 \times 10^7$ ) expressing Myc-tagged STAP-2 WT. The cells were stimulated with LIF (100 ng/ml) for the indicated periods. The cells were then lysed and immunoprecipitated with anti-Myc antibody, and immunoblotted anti-pSTAP-2 Tyr250, anti-PY or anti-Myc antibody.

Fig. 4. LIF stimulates phosphorylation of Tyr250 in endogenous STAP-2 in murine cell lines

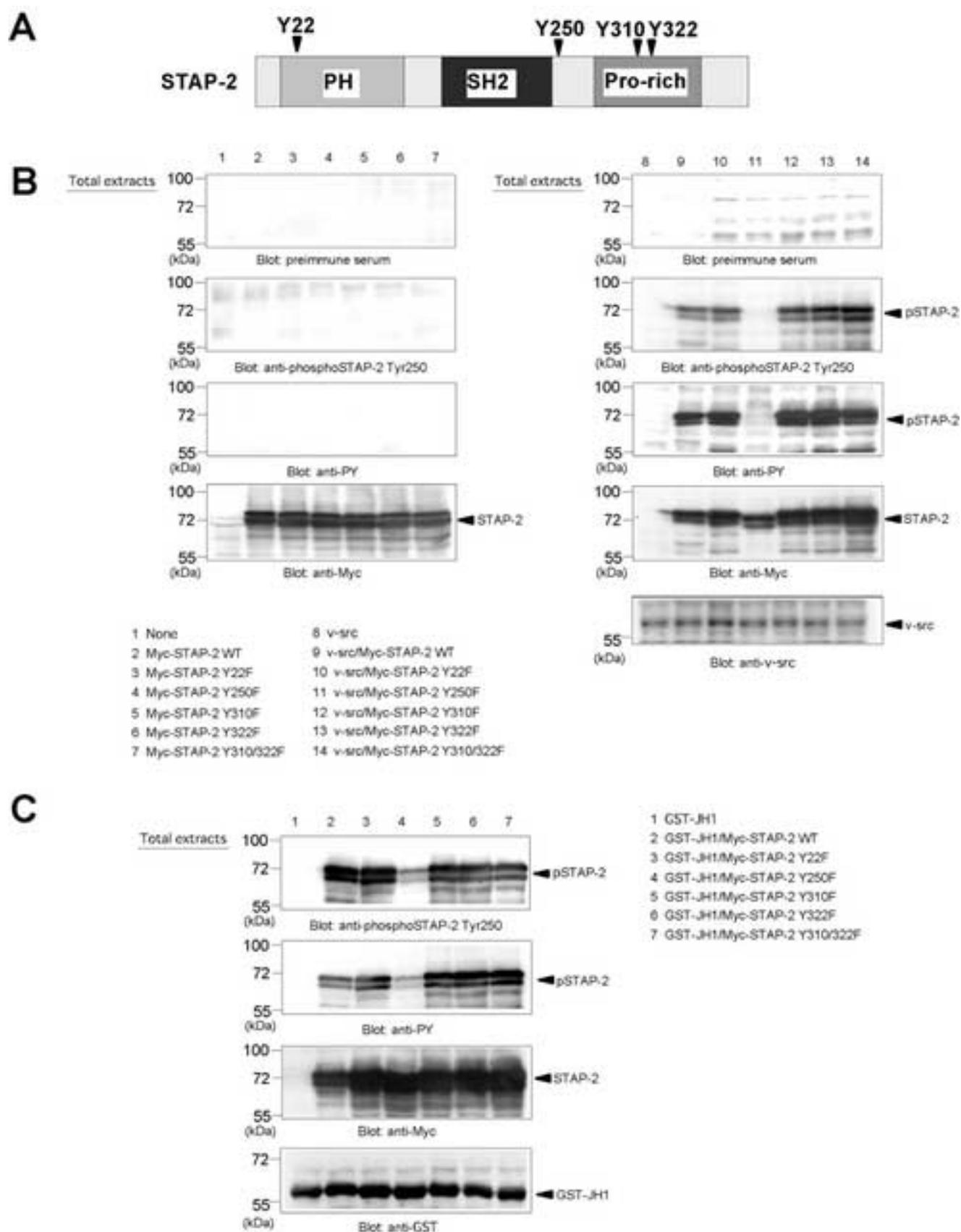
A. M1 cells ( $1 \times 10^4$ /well) were cultured with or without LIF as indicated periods. Cell growth was determined by Cell Counting Kit-8 as described in Materials and methods.

The results are presented from three independent experiments and the error bars represent the standard deviations.

B. M1 cells ( $1 \times 10^6$ ) were cultured with or without LIF as indicated periods. The cells

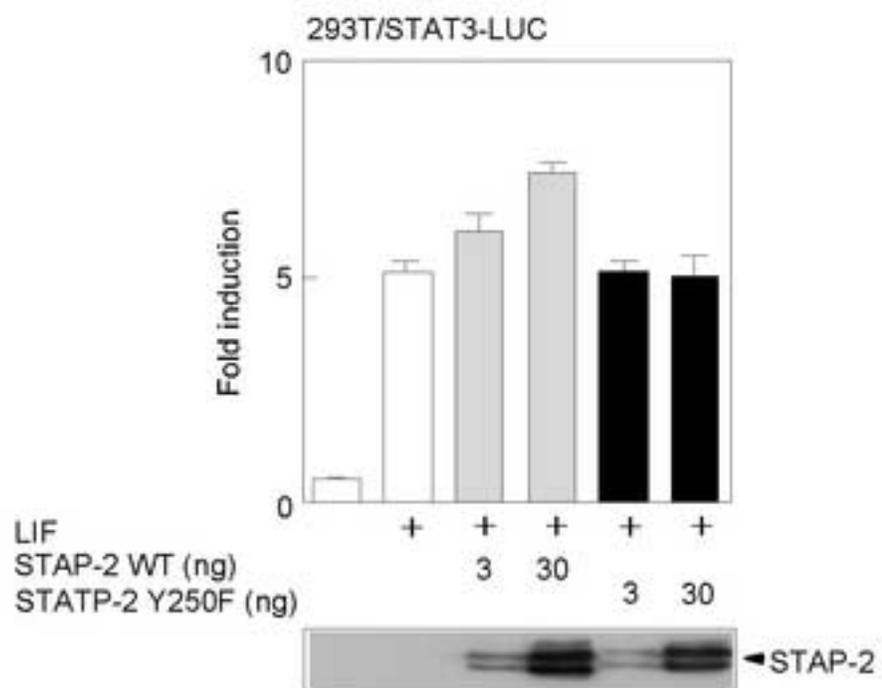
were then lysed. An aliquot of total cell lysates was blotted with anti-pSTAP-2 Tyr250, anti-murine STAP-2 (MuSTAP-2) or anti-actin antibody.

**Figure 1**  
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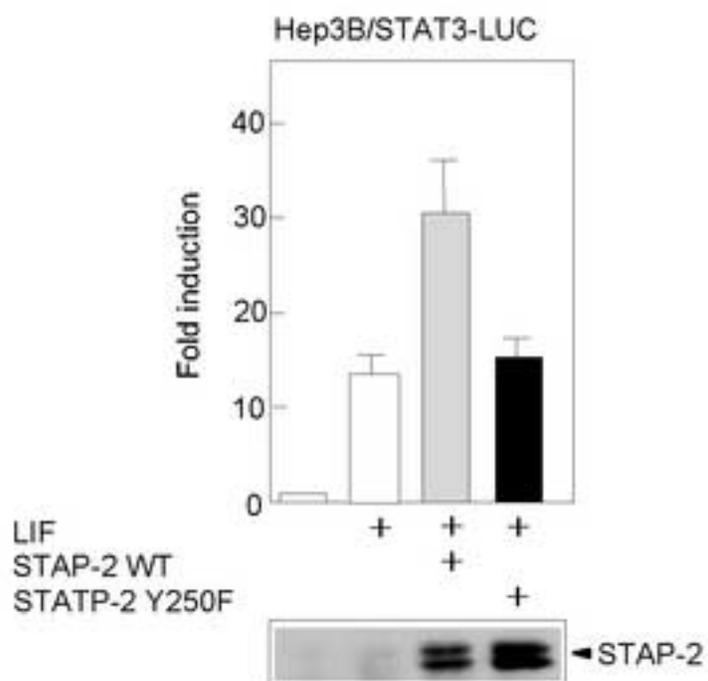


**Fig. 1**

**A**



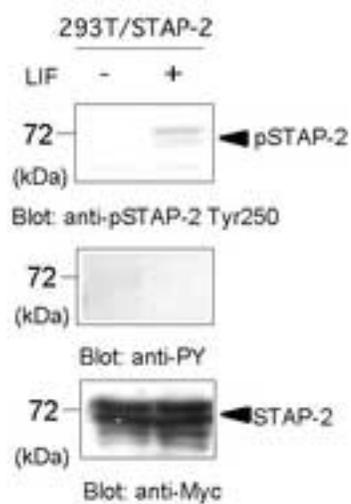
**B**



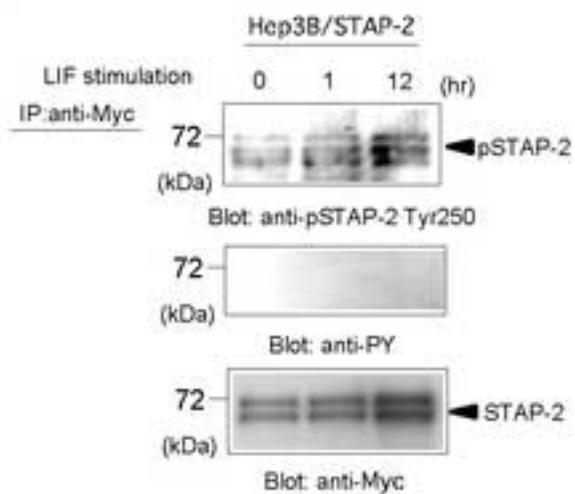
**Fig.2**

**Figure 3**  
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**A**



**B**



**Fig. 3**

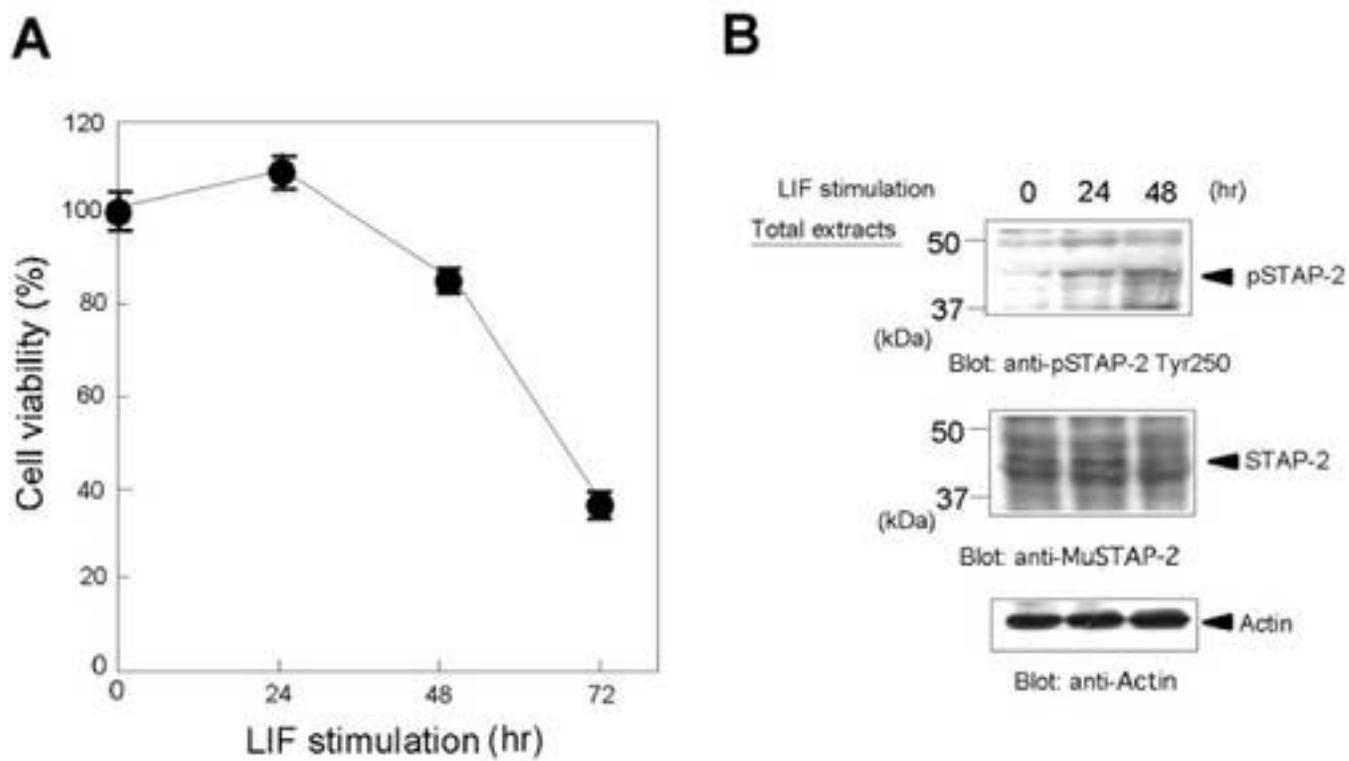


Fig. 4