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Title: **Epstein-Barr virus-derived EBNA2 regulates STAT3 activation**

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Running title: Physical and functional interactions between STAT3 and EBNA2

Key words: STAT3; EBV; EBNA2; LMP1; LIF; transcription

Abstract

The Epstein-Barr virus (EBV)-encoded latency protein EBNA2 is a nuclear transcriptional activator that is essential for EBV-induced cellular transformation. Here, we show that EBNA2 interacts with STAT3, a signal transducer for an interleukin-6 family cytokine, and enhances the transcriptional activity of STAT3 by influencing its DNA-binding activity. Furthermore, EBNA2 cooperatively acts on STAT3 activation with LMP1. These data demonstrate that EBNA2 acts as a transcriptional coactivator of STAT3.

Introduction

STAT3 was originally cloned as an acute-phase response factor activated by interleukin (IL)-6 in the mouse liver, and by its homology to STAT1 [1,2]. Growth factors, such as epidermal growth factor, platelet-derived growth factor and colony-stimulating factor-1, can also stimulate STAT3 activity [1,2]. The transcriptional factor plays crucial roles in early embryonic development as well as in other biological responses, including cell growth and apoptosis [1,2,3]. Of importance, STAT3 is constitutively activated in oncogene-transformed cells and various primary tumors and cell lines [4]. Several tumor viruses are also associated with STAT3 activation. For example, it is constitutively activated in human T cell lymphotrophic virus I-transformed T cells and Epstein-Barr virus (EBV)-related lymphoma cell lines [5,6,7]. The herpesvirus saimiri tyrosine kinase-interacting protein Tip-484 also activates STAT3 [8].

Epstein-Barr nuclear antigen 2 (EBNA2), one of the six viral nuclear proteins expressed in latently infected B lymphocytes, is essential for immortalization of B cells by EBV [9]. The virus product promotes transcriptional transactivation of viral and cellular genes by acting as an adapter molecule that binds to cellular sequence-specific DNA-binding proteins, JK recombination signal-binding protein and PU.1 and engages multiple members of the RNA polymerase II transcription complex [10-13].

In the present study, we examined the physical and functional interactions between STAT3 and EBNA2. EBNA2 interacted with STAT3 and enhanced its transcriptional activation. Importantly, overexpression of EBNA2 augmented the STAT3's DNA

binding activity. Furthermore, co-expression of LMP1 with EBNA2 cooperatively acts on STAT3 activation. Taken together, these data show that EBNA2 is a transcriptional coactivator of STAT3 by influencing its DNA-binding activity.

Materials and Methods

Reagents and antibodies, Recombinant human IL-6 was kindly provided from Ajinomoto (Tokyo, Japan). Recombinant human LIF was purchased from INTERGEN (Purchase, NY). Expression vectors, epitope-tagged STAT3, STAT3-LUC and STAT3-C were provided by Dr. T. Hirano (Osaka University, Osaka, Japan), Dr. J. F. Bromberg (Rockefeller Univ., New York, NY) and Dr. J. N. Ihle (St. Jude CRH, Memphis, TN), respectively [14,15]. Expression vectors for FLAG-EBNA2 and LMP1 were previously described [16,17]. Anti-EBNA2 mAb was purchased from Advanced Biotechnologies Inc. (Maryland USA). Anti-STAT3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 mAb was purchased from Sigma (St Louis, MO). Anti-phospho-STAT3 Tyr705 (pSTAT3 Tyr705), anti-phospho-STAT3 Ser727 (pSTAT3 Ser727) and anti-Nucleoporin antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG was purchased from Chemicon (Temecula, CA).

Cell culture, transfections, luciferase assays and quantitative real-time PCR, Human cervix carcinoma cell line HeLa and human embryonic kidney carcinoma cell line 293T were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS). HeLa cells were transfected using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instruction. 293T cells were transfected with the standard calcium precipitation protocol [18]. Human embryonic kidney carcinoma cell line, 293T was maintained in DMEM containing 10%

fetal bovine serum (FBS) and transfected by the standard calcium precipitation protocol. Luciferase assay was performed as described [19]. Human Burkitt's lymphoma DG75 B cells were grown in RPMI 1640 medium containing 10% FBS. $5x10^6$ of DG75 cells were nucreofected with 2.5 μg of STAT3-LUC, 0.3 μg of pRL-TK (for internal control) and 2.5 μg of EBNA2 by Human B Cell Nucleofector Kit (Amaxa biosystems, Cologne, Germany)[20]. The stimulated 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. Quantitative real-time PCR analysis of SOCS3 mRNA transcripts was carried out using the assay-on-demandTM gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system.

Immunoprecipitation, immunoblotting and DNA binding assay, Immunoprecipitation and Western blotting were performed as previously described [21]. The cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 mg /ml each of aprotinin, pepstatin, and leupeptin). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to an Immobilon filter (Millipore; Bedford, MA), which was then immunoblotted with antibodies. To measure STAT3 DNA binding, cell extracts were treated with the immobilized STAT3 consensus oligonucleotide-Sepharose conjugate (Santa Cruz) as described previously [22].

Indirect Immunofluorescence microscopy, HeLa cells ($5x10^4$) seeded on a glass plate were fixed with 4 % paraformaldehyde and reacted with respective antibodies. The cells

were then reacted with FITC-conjugated anti-rabbit IgG or rhodamine-conjugated anti-mouse IgG (Chemicon) and observed under a confocal laser fluorescent microscope [19]. Images were obtained by using a Zeiss LSM 510 laser scanning microscope with an Apochromat x63/1.4 oil immersion objective and x4 zoom. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Wako, Osaka, Japan).

Results and Discussion

EBNA2 augments transcriptional activation of STAT3

STAT3 activation has been reported to play roles in the development of human herpesvirus-associated diseases [9,23]. Our previous study demonstrated that a Kaposi's sarcoma-associated herpesvirus (KSHV)-derived nuclear protein, LANA, directly interacts with STAT3 and augments its transcriptional activation [20]. EBV-derived LMP1 has also been shown to mediate STAT3 activation [9,23]. These findings led us to investigate the effects of the EBV-derived nuclear protein EBNA2 on STAT3 activation.

To assess the functional relevance of EBNA2 and STAT3, we examined whether EBNA2 affects STAT3-mediated transcriptional activation by transient transfection experiments using STAT3-LUC, in which the □2-macroglobulin promoter drives expression of a luciferase (LUC) reporter gene. 293T and HeLa cells were transfected with STAT3-LUC with or without EBNA2, then, treated with LIF. When cells were cotransfected with EBNA2, the transcriptional activation of STAT3-LUC was augmented in a dose-dependent manner in both types of cells (Fig. 1A and B). To further confirm the enhancing effect of EBNA2 on STAT3 activation, we overexpressed EBNA2 together with STAT3-LUC in an EBV-negative human B cell line, DG75. As shown in Fig. 1C, ectopically expressed EBNA2 markedly augmented IL-6-induced STAT3-LUC activation in DG75 cells, indicating that EBNA2 also acts as a transcriptional activator of STAT3 in B cells. To further assess the direct interaction between STAT3 and EBNA2, we used a constitutively active form of STAT3, STAT3-C

[15]. 293T cells were transfected with STAT3-LUC and expression vectors for EBNA2 and/or STAT3-C. As shown in Fig. 1D, STAT3-LUC activation by STAT3-C was augmented by EBNA2 in a dose-dependent manner, suggesting that EBNA2 directly interacts with STAT3 and augments its activation. We further examined whether EBNA2 affects LIF/STAT3-mediated gene expression. HeLa cells were transfected with an empty vector or EBNA2. Total RNA from the transfected cells was analyzed by quantitative real-time PCR. As shown in Fig. 1E, LIF/STAT3-mediated SOCS3 mRNA expression was markedly enhanced in EBNA2-transfected HeLa cells, indicating that EBNA2 activates LIF/STAT3-induced gene expression *in vivo*.

STAT3 and EBNA2 physically interact in vivo

One mechanism consistent with the above data may be a direct interaction between STAT3 and EBNA2. To examine this possibility, we performed coimmunoprecipitation experiments in 293T cells transiently transfected with FLAG-STAT3 and EBNA2 expression constructs. The transfected 293T cells were lysed and subjected to immunoprecipitation with an anti-FLAG antibody. The immunoprecipitates were then analyzed by western blotting with an anti-EBNA2 antibody. As shown in Fig. 2A, STAT3 interacted with EBNA2 in 293T cells. To further characterize the nature of the interaction between STAT3 and EBNA2, we attempted to determine where this interaction occurs in cells (Fig. 2B). In HeLa cells, EBNA2 was localized in the nucleus in the presence or absence of LIF stimulation. In the absence of LIF stimulation, STAT3 was located in the cytoplasm independently with EBNA2. However, in the presence of

LIF stimulation, STAT3 was translocated into the nucleus where it colocalized with EBNA2. Consistent with the *in vivo* interaction data presented above, these results suggest that activated STAT3 interacts with EBNA2 in the nucleus.

EBNA2 influences the DNA-binding activity of STAT3

To understand the molecular mechanisms responsible for EBNA2-mediated enhancement of STAT3 activation, we first examined whether EBNA2 expression affects STAT3 phosphorylation. As shown in Fig. 3A, EBNA2 had no significant effect on STAT3 phosphorylation in HeLa cells. Next, we examined the effects of EBNA2 on LIF-induced nuclear accumulation of STAT3 in HeLa cells. We found that the nuclear accumulation of STAT3 was not significantly altered in EBNA2-transfected HeLa cells after LIF stimulation (Fig. 3B). We further examined whether EBNA2 expression affects the DNA-binding activity of STAT3 after LIF stimulation. Importantly, we found that the LIF-induced DNA-binding activity of STAT3 was enhanced by EBNA2 expression (Fig. 3C). Therefore, EBNA2 appears to enhance STAT3 activation by augmenting its DNA-binding activity.

EBNA2 cooperatively acts on STAT3 with EBV-derived LMP1

Numerous studies have also demonstrated that EBV-derived LMP1 can mediate STAT3 activation in EBV infection through several signaling pathways [9,23]. Finally, we examined whether EBNA2 cooperatively acts on STAT3 with EBV-derived LMP1. As shown in Fig. 4, LMP1-induced STAT3-LUC activation was markedly augmented by

EBNA2 expression. Therefore, these results indicate that LMP1 and EBNA2 cooperatively mediate STAT3 activation.

Concluding remarks

STAT3 is constitutively phosphorylated and activated in various primary human tumors and transformed cell lines, and has been implicated in tumorigenesis [2,3,4]. However, the molecular mechanisms of the persistent activation of STAT3 in human tumor cells remain largely unknown. Recently, the hepatitis C virus (HCV) core protein was shown to directly interact with and activate STAT3 through phosphorylation of a critical tyrosine residue [24]. Chronic infection by HCV is associated with the development of liver cirrhosis and hepatocellular carcinoma. The HCV core protein has also been proposed to be involved in virus-induced transformation, indicating that it cooperates with STAT3, thereby leading to cellular transformation. We also demonstrated a novel interaction between STAT3 and KSHV-derived LANA in PEL cells [20]. LANA interacted with STAT3 and augmented its transcriptional activity, suggesting that STAT3-mediated signaling plays crucial roles in the development of KSHV-associated diseases.

In the present study, we have demonstrated a novel interaction between STAT3 and EBV-derived EBNA2. EBNA2 augmented the transcriptional activity of STAT3 by influencing its DNA-binding activity. We also showed that EBNA2 cooperatively acted on STAT3 activation with LMP1. Thus further understanding of the detailed molecular interactions among STAT3, EBNA2 and LMP1 may lead to novel therapeutic strategies

for EBV-associated diseases.

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Figure Legends

Fig. 1. EBNA2 augments transcriptional activation of STAT3

(A) 293T cells in a 12-well plate were transfected with STAT3-LUC (400 ng) and/or increasing amounts of empty vector, expression vector for EBNA2 (100–400 ng). Thirty-six hrs after transfection, the cells were stimulated with LIF (100 ng/ml) for additional 8 hrs. The stimulated cells were harvested, and luciferase activities were measured. (B) HeLa cells in a 12-well plate were transfected with STAT3-LUC (400 ng) and/or increasing amounts of empty vector, expression vector for EBNA2 (100–400 ng). Thirty-six hrs after transfection, the cells were stimulated with LIF (100 ng/ml) for additional 8 hrs. The stimulated cells were harvested, and luciferase activities were measured. (C) Human Burkitt's lymphoma DG75 cells were nucreofected with 2.5 □g of STAT3-LUC, 0.3 □g of pRL-TK and 2.5 □g of EBNA2 by Nucleofector. Thirty-six hrs after transfection, the cells were stimulated with IL-6 (100 ng/ml) for additional 8 hrs. The stimulated cells were harvested, and luciferase activities were measured. (D) 293T cells in a 12-well plate were transfected with STAT3-LUC (400 ng) together withorwithout STAT3-C (500 ng) and/or indicated amounts (100-400 ng) of expression vector for EBNA2. Forty-eight hrs after transfection, the cells were harvested, and luciferase activities were measured. The above results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D. (E) HeLa cells in a 12-well plate were transfected with empty vector, expression vector for EBNA2 (400 ng), and cells were stimulated with LIF (100 ng/ml) for the indicated

periods. SOCS3 expression level were also quantified by quantitative real-time PCR analysis. Data represent the levels of SOCS3 mRNA normalized to that of a G3PDH internal control and are expressed relative to the value of empty vector transfected samples without LIF-stimulation. Data represent the mean of duplicate PCR determinations, which in general varied by <10%. Shown is a representative experiment, which was repeated at least twice with similar results.

Fig. 1. STAT3 and EBNA2 physically interact in vivo

(A) 293T cells ($1x10^7$ cells) were transfected with EBNA2 (10 μg) together with or without FLAG-STAT3 (7.5 μg). Forty-eight hrs after transfection, the cells were lysed, and immunoprecipitated with anti-EBNA2 antibody and immnoblotted with anti-FLAG (upper panel) or anti-EBNA2 antibody (middle panel). Total cell lysates (1%) were blotted with anti-FLAG antibody (lower panels).

(B) HeLa cells were transfected with Myc-STAT3 (1 μg) and EBNA2 (1 μg). Thirty hrs after transfection, cells were treated with or without LIF (100 ng/ml) for 30 min, and then fixed and reacted with rabbit anti-EBNA2 polyclonal and mouse anti-Myc monoclonal antibody and visualized with FITC-conjugated anti-mouse antibody or rhodamine conjugated anti-rabbit antibody.

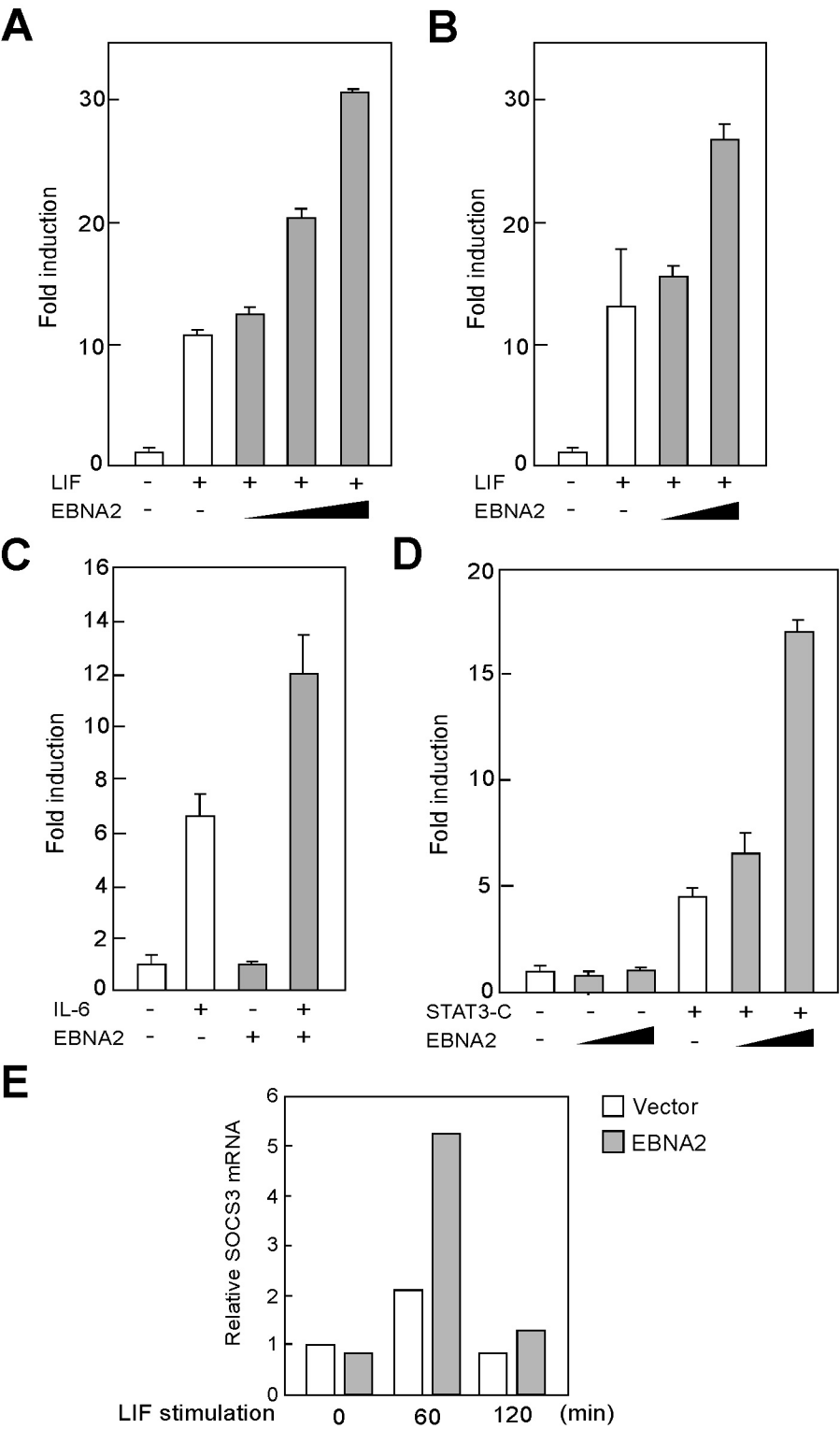
Fig. 3. EBNA2 influences DNA binding activity of STAT3

(A) HeLa cells in a 12-well plate were transfected with empty vector, expression vector for EBNA2 (400 ng). At 48 h after transfection, cells were stimulated or unstimulated

with LIF for the indicated periods. The cells were lysed, and an aliquot of total cell lysate (TCL)(1%) was blotted with anti-pSTAT3 (Tyr705), anti-pSTAT3 (Ser727), anti-STAT3 and anti-EBNA2 antibodies. (B) HeLa cells in a 12-well plate were transfected with empty vector, expression vector for EBNA2 (400 ng). At 48 h after transfection, cells were stimulated or unstimulated with LIF for the indicated periods. The nuclear extracts of cells were prepared, and an aliquot of nuclear extracts (1%) was blotted with anti-STAT3 and anti-EBNA2 and anti-Nucleoporin antibodies. (C) HeLa cells in a 12-well plate were transfected with empty vector, expression vector for EBNA2 (400 ng), and cells were stimulated with LIF (100 ng/ml) for the indicated periods. To measure STAT3 DNA binding, cell extracts were treated with the immobilized STAT3 consensus oligonucleotide-Sepharose conjugate. The precipitates were subjected to Western blot analysis using anti-STAT3 antibody. An aliquot of TCL was blotted with anti-STAT3 and anti-EBNA2 antibodies. Densitometric quantification of the above results was also shown. Relative intensity of DNA-bound STAT3 was normalized to the STAT3 protein of the total cell lysates.

Fig. 4. EBNA2 acts on STAT3 activation in cooperation with LMP1

293T cells in 24-well plates were transfected with STAT3-LUC (200 ng) with or without LMP1 (10 ng) and/or increasing amounts of EBNA2 (10 or 100 ng). At 48 h after transfection, the cells were harvested and the 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.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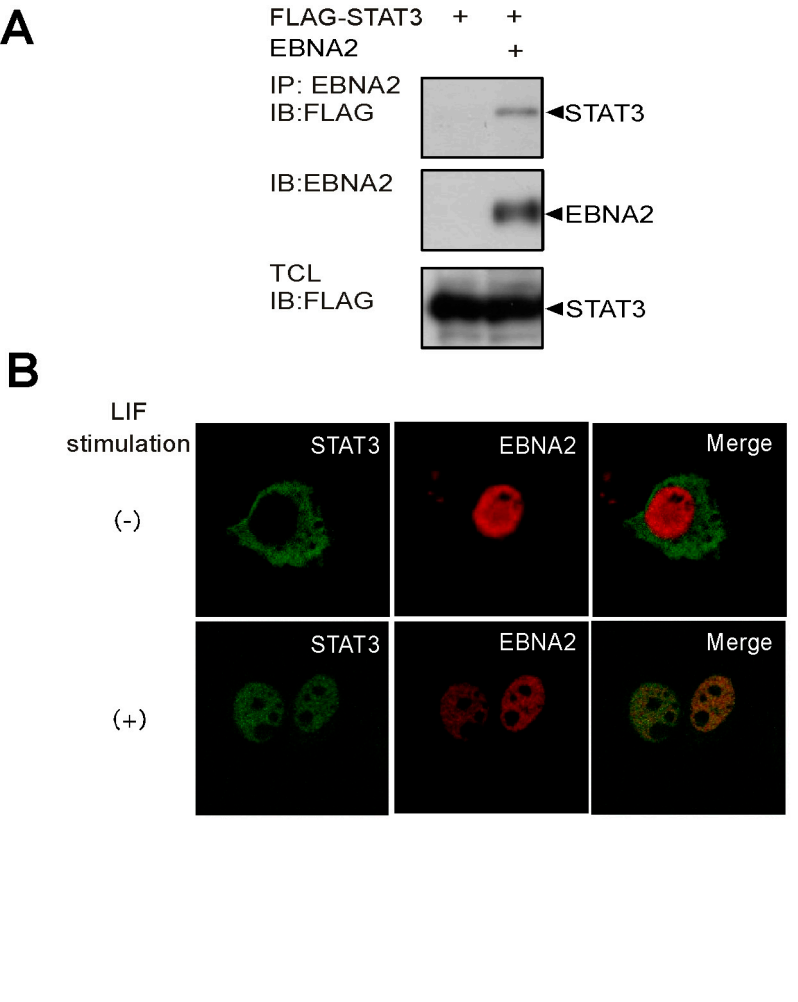
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Figure 2

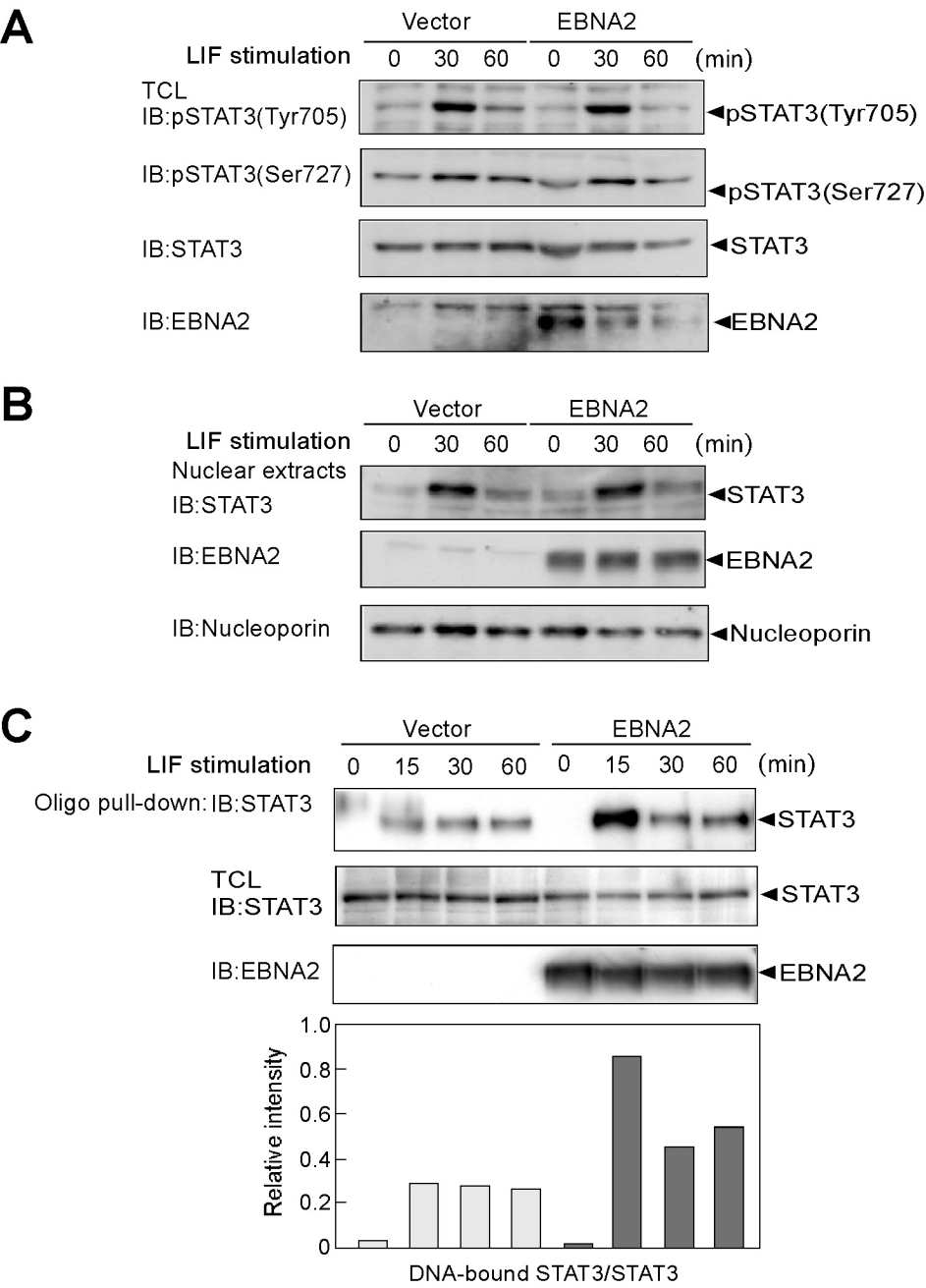
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Figure 3

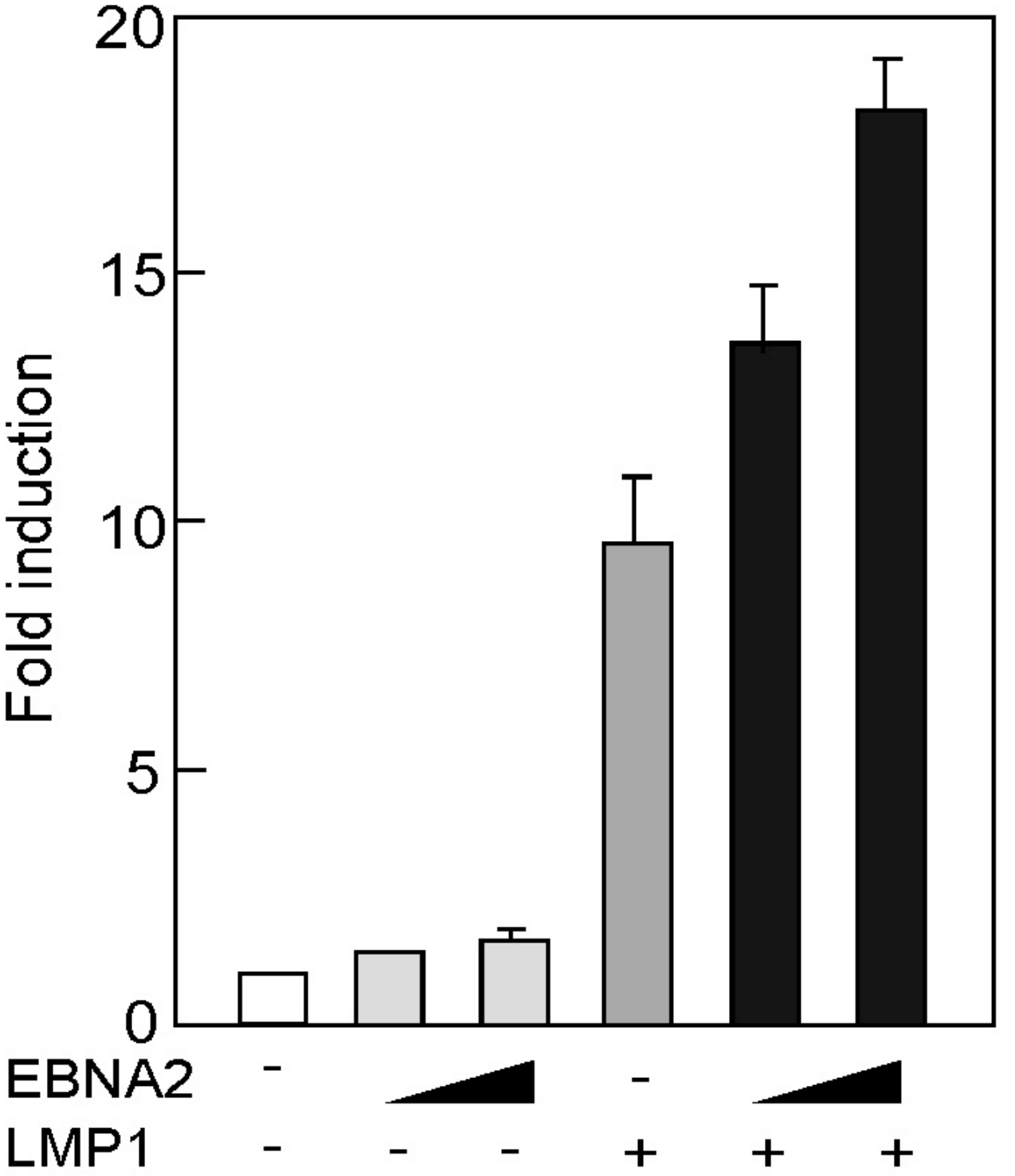

Figure 4