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Title	Physical and functional interactions between STAT3 and ZIP kinase.
Author(s)	Sato, Noriko; Kawai, Taro; Sugiyama, Kenji et al.
Citation	International Immunology, 17(12), 1543-1552 https://doi.org/10.1093/intimm/dxh331
Issue Date	2005-12
Doc URL	https://hdl.handle.net/2115/28251
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Type	journal article
File Information	II17-12.pdf

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Title: **Physical and functional interactions between STAT3 and ZIP kinase**

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Running title: Interactions between STAT3 and ZIP kinase

Number of pages & figures: 26 pages & 5 figures

Transmitting editor: Dr. Toshio Hirano

Key words: IL-6, LIF, signal transduction, STAT3, ZIP kinase

Abstract

Signal transducer and activator of transcription 3 (STAT3) is a latent cytoplasmic transcription factor that can be activated by cytokines and growth factors. It plays important roles in cell growth, apoptosis and cell transformation, and is constitutively active in a variety of tumor cells. In this study, we provide evidence that ZIP kinase (ZIPK) interacts physically with STAT3. ZIPK specifically interacted with STAT3, and did not bind to STAT1, STAT4, STAT5a, STAT5b or STAT6. ZIPK phosphorylated STAT3 on Ser727 and enhanced STAT3 transcriptional activity. Small-interfering RNA-mediated reduction of ZIPK expression decreased leukemia inhibitory factor (LIF)- and IL-6-induced STAT3-dependent transcription. Furthermore, LIF- and IL-6-mediated STAT3 activation stimulated ZIPK activity. Taken together, our data suggest that ZIPK interacts with STAT3 within the nucleus to regulate the transcriptional activity of STAT3 via phosphorylation of Ser727.

Introduction

Cytokines exert multiple biological responses through interactions with their specific receptors, resulting in the activation of Janus kinase (Jak)-signal transducer and activator of transcription (STAT) pathways (1). STATs comprise a family of latent cytoplasmic transcription factors that are activated by recruitment to cytokine receptors and subsequent phosphorylation by the receptor-associated Jaks. STATs form homo- or heterodimers by reciprocal interaction between their SH2 domains and phosphorylated tyrosine residues and translocate to the nucleus, where they bind to DNA and regulate the expressions of their target genes (2,3). Seven mammalian STAT genes have been identified to be activated by various cytokines and certain growth factors, and exhibit distinct and overlapping functions (4,5).

STAT3 was originally cloned as an acute-phase response factor activated by interleukin-6 (IL-6) in the mouse liver, and also from its homology to STAT1 (6,7). Growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and colony-stimulating factor-1 (CSF-1), can also stimulate STAT3 activity (8). STAT3 is known to play crucial roles in early embryonic development as well as in other biological responses, including cell growth and apoptosis (9-11). Furthermore, it is constitutively activated in oncogenic tyrosine kinase v-src- or v-abl-transformed cells and various primary tumors and cell lines (12,13). STAT3 itself acts as an oncogene in NIH-3T3 cells (14), and plays an anti-apoptotic role in IL-6/gp130-mediated survival in B cells as well as myeloma and head and neck tumor cells (12,13,15,16). Recent studies have demonstrated that the STAT-mediated regulation of apoptotic pathways is largely due to transcriptional induction of genes related to cell death, such as caspases, Fas and TRAIL, as well as cell cycle progression, such as $p21^{WAF1}$ (8,10,17). Interestingly, STATs also seem to regulate apoptosis through a non-transcriptional mechanism by inhibiting activation of the anti-apoptotic protein NF-□B (17). Since dysregulation of STAT3-mediated signaling pathways

is frequently found in clinical tumor samples, understanding the mechanisms that underlie STAT3 regulation of cell survival may lead to successful strategies for targeting STAT3 in cancer therapy (18).

Initially, it appeared that just a single STAT tyrosine residue became phosphorylated as a consequence of cytokine or growth factor stimulation. However, all STATS, except for STAT2 and STAT6, are also phosphorylated on serine residues in response to ligand stimulation (19,20). Phosphorylation of a single serine (residue 727 in both STAT1 and STAT3) in the transcriptional activation domain is required for the maximal transcriptional activation of STATs, and a STAT3 S727A mutant, in which serine 727 (Ser727) is replaced by alanine, was estimated to be about 50% less effective for transcriptional stimulation than wild-type STAT3 (20). To examine the function of Ser727 phosphorylation *in vivo*, SA mutant mice, in which Ser727 is substituted with alanine, have been produced (21). Embryonic fibroblasts from SA/SA mice showed approximately 50% of the transcriptional response of equivalent cells from wild-type mice, indicating that Ser727 phosphorylation is also required for maximal transcriptional activation of STAT3 *in vivo*. Serine phosphorylation is most likely to increase STAT3 activity through association with other cofactors, such as p300 (22). Several different kinases have been implicated in serine phosphorylation, implying an interaction between STAT signaling and serine kinase signaling pathways (19). However, the kinase responsible for Ser727 phosphorylation has not yet been identified.

In this study, we demonstrate that ZIP kinase (ZIPK) binds and phosphorylates Ser727 of STAT3 in the nucleus. ZIPK, a member of the death-associated protein (DAP) kinase family, was originally identified to bind the transcription factor ATF4 and has been implicated in apoptosis induction in the nucleus (23,24). ZIPK enhanced IL-6/leukemia inhibitory factor (LIF)/STAT3-mediated transcription, while small-interfering RNA (siRNA)-mediated reduction in endogenous ZIPK expression revealed that ZIPK was required for LIF/STAT3-mediated transcriptional activation and expression of target genes.

Thus, ZIPK associates with and phosphorylates STAT3 within the nucleus to form an efficient transcription complex.

Methods

Reagents, antibodies and GST fusion protein.

Expression vectors for Jak1, STAT1, STAT4, STAT5a, STAT5b and STAT6 were kindly provided by Dr. J. N. Ihle (St. Jude CRH, Memphis, TN). Expression vectors for epitope-tagged ZIPK, ZIPK K42A, ZIPK ΔLZ, DAPK2, DRAK1, DRAK2, STAT3, STAT3YF, and STAT3-C were described previously (14,23-28). DAPK kinase domain was generated by PCR methods and sequenced (primer sequences are available upon request). Anti-Myc, -HA, -STAT1, -STAT3, -STAT4, -STAT5a/b and -STAT6 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 monoclonal antibody, rabbit polyclonal anti-FLAG antibody and anti-phosphotyrosine (PY) monoclonal antibody (PY20) were purchased from Sigma (St Louis, MO). Anti-phosoho-STAT3 (pSTAT3) Ser727 and anti-pSTAT3 Tyr705 were purchased from Cell Signaling (Beverly, MA). Anti-ZIPK antibody was purchased from BIOMOL Research Laboratories (Plymouth, PA). Anti-actin antibody was purchased from CHEMICON (Temecula, CA). Peptide for STAT3 (residues 720-735) was synthesized and obtained from Operon Technologies (Tokyo, Japan). The full-length cDNA of ZIPK was inserted in frame into pGEX4 vector. Recombinant proteins were expressed in Escherichia coli DH5α. The cells were grown for 6 h at 37 ℃, and the protein was induced with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30 ℃ for 15 h. E. coli cells were harvested and then lysed by sonication. Glutathione S-transferase (GST) fusion proteins were purified by affinity chromatography on glutathione-Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions.

Yeast two-hybrid screen.

Gal4-STAT3/C and -STAT4/C were constructed by fusing the C-terminal domain of STAT3 (494-750) or STAT4 (483-748) in-frame into the Gal4 DNA-binding domain in

BamH I/EcoR I site in the pGBKT7 vector and used as baits. Saccharomyces cerevisiae, AH109 cells transformed with pGal4-STAT3/C, followed by mating with a pretransformed mouse 11-day embryo MATCHMAKER cDNA library in Y187 cells (Clontech, Palo Alto, CA), were plated onto media that lacked tryptophan, leucine and histidine and had been supplemented with 5 mM 3-amino-1, 2, 4-triazole (Sigma, MO). Approximately $2.6x10^6$ colonies were screened for growth in the absence of histidine. Plasmid DNAs derived from positive clones were extracted from yeasts, and sequenced. Clones were re-introduced into AH109 along with either empty pGBKT7, pGBKT7-STAT3/C, pGBKT7-STAT4/C to verify the STAT/clone interaction.

Cell culture, transfection, luciferase assays and RT-PCR analysis.

Human B lymphoblastoid cell line, SKW6-CL4 was maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) (29). Rat hepatoma cell line H35 and human cervix carcinoma cell line, HeLa were maintained in DMEM containing 10 % FCS (24,30). Stable H35 transformants expressing empty vector, ZIPK WT or ZIPK K42A was established as described previously (31) and maintained in the above medium in the presence of G418 (0.5 mg/ml). H35 or HeLa cells were transfected with STAT3-LUC (32) using Metafectene (Biontex Laboratories GmbH, München, Germany) according to the manufacturer's instructions. Thirty hours after transfection, the cells were treated with IL-6 or LIF (100 ng/ml) for 12 h. 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. At least three independent experiments were carried out for each assay. Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10 % FCS and transfected by the standard calcium precipitation protocol (25). The cells were harvested 36 h after transfection and lysed in 50 □l of PicaGene Reporter Lysis Buffer (Toyo Ink, Tokyo, Japan) and assayed for luciferase and □-galactosidase activities according to the manufacturer's instructions. Luciferase activities were normalized

to the β-galactosidase activities. Three or more independent experiments were carried out for each assay. RT-PCR analysis was performed using RT-PCR high -Plus- Kit (TOYOBO, Tokyo, Japan). The primers were used as described previously (33).

Transfection of siRNAs.

siRNAs targeting ZIPK used in this study were as follows: siZIPK#1, 5'-AGGCGGGGAACGAGUUCAAGAACAU-3'; siZIPK#2, 5'-GUCAAAGAUCCCAAGCGGAGAAUGA-3'. HeLa cells were plated on a 6-well plate at 2 x 10^5 cells/well 12 h before transfection, washed with serum-free Opti-MEM, and then incubated with an siRNA-Oligofectamine (Invitrogen, Carlsbad, CA) mixture at 37 ℃ for 4 hrs, followed by addition of 1 ml of fresh medium containing 10 % fetal calf serum. The cells were then transfected with STAT3-LUC using Metafectene as described the above. Twenty-four hrs after transfection, the cells were treated with LIF (100 ng/ml) for an additional 12 h.

Immunoprecipitation, immunoblotting and *in vitro* phosphorylation.

The immunoprecipitation and Western blot analysis were performed as described previously (25). The cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 % NP-40, 1 μM sodium orthovanadate, 1 μM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, pepstatin and leupeptin). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to Immobilon filter (Millipore; Bedford, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). We also stained the gel after electrical transfer with Coomassie blue solution to confirm the amount of loading samples. *In vitro* kinase reactions were preformed as described (34,35). Briefly, immune complex of ZIPK was washed in kinase buffer (10 mM HEPES, pH7.4, 50 mM NaCl, 0.1 mM sodium

orthovanadate, 5 mM $MnCl_2$, 5 mM $MgCl_2$) and mixed with 5 □Ci/□l [□-^{32}P]-ATP at 25 ℃ for 30 min. The products of these reactions were separated by SDS-PAGE.

Indirect Immunofluorescence microscopy.

HeLa cells ($5x10^4$) seeded on a glass plate were transfected using Metafectene. Forty-eight hours after transfection, cells 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, Temecula, CA) and observed under a confocal laser fluorescent microscope. 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

Identification of ZIPK as a STAT3-interacting protein

To study the regulation and function of STAT3, we sought to identify proteins that interacted with STAT3 using yeast two-hybrid screening. We used the C-terminal region of STAT3 containing the SH2 and C-terminal transactivation domains (494-750), which are known to be important for its protein-protein interactions, as bait. More than 20 positive clones were identified to encode a part of mouse ZIPK (161-261). The specificity of the STAT3 and ZIPK interaction in yeast was further confirmed by survival assays in selection medium. As shown in Fig. 1A, the C-terminal region of STAT3, but not that of STAT4, specifically interacted with ZIPK in yeast.

Association of STAT3 with ZIPK

To verify the interaction of STAT3 and ZIPK in mammalian cells, we transfected 293T cells with Myc-tagged ZIPK or a control plasmid together with a variety of STATs. Cell lysates were immunoprecipitated with anti-STAT1, anti-STAT3, anti-STAT4, anti-STAT5 and anti-STAT6 antibodies, and the immunoprecipitates obtained were analyzed by western blotting with an anti-Myc antibody to detect the presence of bound ZIPK. As shown in Fig. 1B, ZIPK was detected in immunoprecipitates after cotransfection of STAT3, but not the other STAT proteins. Next, we determined the regions responsible for the interaction between STAT3 and ZIPK. STAT3 contains several functional domains, as illustrated in Fig 1C. In addition to the C-terminal region, which was used in the two-hybrid screening, other regions may also interact with ZIPK. To explore this possibility, we generated a series of Myc-tagged STAT3 mutants, and co-transfected them into 293T cells with hemagglutinin A epitope (HA)-tagged ZIPK. Co-immunoprecipitation experiments indicated that ZIPK interacted strongly with STAT3 (320-493), which contains the DNA-binding domain. STAT3 (494-750) also interacted with ZIPK. We further delineated the STAT3-interacting

domains in ZIPK by similar co-immunoprecipitation experiments. We transfected a series of FLAG-tagged ZIPK mutants together with Myc-tagged STAT3 into 293T cells. As shown in Fig. 1D, deletion of the ZIPK C-terminal region that contains the leucine zipper domain had no effect on the interaction. Furthermore, we assessed whether the kinase activity of ZIPK was necessary for the interaction with STAT3. For this purpose, we used a ZIPK K42A mutant, in which a conserved lysine residue in kinase subdomain II was changed to alanine, thereby creating a kinase-negative mutant (23). A deletion mutant encoding only the kinase domain of ZIPK with the K42A mutation still interacted with STAT3. These results suggest that the kinase domain of ZIPK interacts with STAT3 through the DNA binding and C-terminal domains, and that its kinase activity is not required for the interaction.

Next, we investigated the interaction between endogenous STAT3 and ZIPK in mammalian cells. For this purpose, we examined the ZIPK protein expression levels in a variety of cell lines that respond to IL-6 stimulation. Several cell lines, including the human B lymphoblastoid cell line SKW6-CL4, were found to contain high levels of ZPIK protein (data not shown). In parallel with the above transfection studies, co-immunoprecipitation experiments were performed using cell lysates obtained from SKW6-CL4 cells that were either left untreated or treated with IL-6. Similar to the results obtained in the transfected 293T cells (Fig. 1E), we found that the STAT3 immunoprecipitated from SKW6-CL4 cells was in a complex with ZIPK and that this interaction was enhanced by IL-6 stimulation.

We also examined whether STAT3 interacts with other DAP kinase family in mammalian cells. To this end, we transfected 293T cells with FLAG-tagged STAT3 or a control plasmid together with a variety of DAP kinase family. Cell lysates were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates obtained were analyzed by western blotting with an anti-Myc antibody to detect the presence of bound DAP kinase family. As shown in Fig. 1F, DAPK kinase domain and ZIPK was detected in immunoprecipitates after cotransfection of STAT3, but not other DAP kinase family proteins. We do not know

the physiological significance on the interaction between STAT3 and DAPK kinase domain. The further work will be required for this issue. The DAPK kinase domain could also interact with STAT1 in 293T cells (data not shown), suggesting that DAPK is involved in the IFN-γ signaling. In fact, introduction of a DAPK antisense was demonstrated to inhibit IFN-γ-induced apoptosis in HeLa cells (36).

ZIPK phosphorylates STAT3 on Ser727

The finding that STAT3 interacted with ZIPK suggested that it was a ZIPK substrate. The major serine/threonine (Ser/Thr) kinase phosphorylation site in STAT3 is known to be Ser727, which is important for maximal activation of STAT3, although the kinase responsible for Ser727 phosphorylation has not yet been identified (19). To investigate the possibility that Ser727 phosphorylation is mediated by ZIPK, we examined whether ZIPK phosphorylated Ser727 using a pSTAT3 Ser727-specific antibody. As shown in Fig 2A, Ser727 phosphorylation increased dose-dependently in response to ZIPK expression. To further examine this finding, we performed *in vitro* kinase assays using a recombinant GST-fusion ZIPK protein. As shown in Fig 2B, ^{32}P incorporation into GST-ZIPK, but not GST, was observed. In a previous report, a Ser/Thr kinase inhibitor, H7, was shown to abolish IL-6-induced gene expression and transcription of a STAT3-driven reporter gene (19,37). Consistent with these observations, serine phosphorylation of STAT3 in response to IL-6 resulted in the generation of a slowly migrating form of STAT3 in an H7-sensitive manner. Therefore, we examined whether or not ZIPK was an H7-sensitive Ser/Thr kinase. Strikingly, the kinase activity of GST-ZIPK was completely inhibited following H7 addition (Fig. 2B). We further confirmed this observation using a synthetic peptide for STAT3 (residues 720-735). As shown in Fig 2C, the STAT3 peptide was phosphorylated by GST-ZIPK and ^{32}P incorporation into the peptide was inhibited by H7 in a dose-dependent manner. We also tried the effect of ZIPK siRNA on IL-6/LIF-induced Ser727 phosphorylation in HeLa cells. However, we could not detect any Ser727 phosphorylation

by stimuli in HeLa cells using a pSTAT3 Ser727-specific antibody. Therefore, we do not know the effect of ZIPK siRNA on IL-6/LIF-induced Ser727 phosphorylation in HeLa cells.

ZIPK activates STAT3-dependent transcription

Next, we measured the effects of ZIPK expression on STAT3 activity in reporter gene assays. The STAT3-mediated transcriptional responses were measured by using STAT3-LUC, in which the 2-macroglobulin promoter (32) drives the expression of the LUC gene. First, 293T cells were transiently transfected with STAT3-LUC and treated with increasing amounts of LIF. As shown in Fig. 3A, LIF stimulated STAT3-mediated transcription in a dose-dependent manner. Overexpression of wild-type ZIPK (ZIPK WT) markedly enhanced STAT3-mediated transcriptional activity to a greater extent than overexpression of ZIPK K42A. We further examined this effect using H35 hepatoma transfectants expressing an empty vector, FLAG-tagged ZIPK WT or ZIPK K42A. Similar results were observed in the H35 transfectants expressing ZIPK WT (Fig. 3B), although the effect of ZIPK K42A expression was a little different. This difference may be due to constitutive expression of Large T antigen, which stimulates STAT3-LUC in 293T cells (data not shown). These data suggest that ZIPK positively regulates the transcriptional activity of STAT3.

We further used RT-PCR to investigate the endogenous STAT3-mediated SOCS3 mRNA expression in H35 cells after stimulation with IL-6. As shown in Fig. 3C, IL-6-mediated endogenous SOCS3 mRNA expression was markedly increased in the H35 transfectants expressing ZIPK WT, but not in ZIPK K42A, suggesting that ZIPK positively regulates IL-6/STAT3-mediated gene expression.

To further explore whether ZIPK is necessary for STAT3-mediated transcriptional activation, we used siRNA to reduce the endogenous expression of ZIPK in HeLa cells. HeLa cells were transfected with a specific siRNA for ZIPK or a control siRNA, and aliquots of the cell lysates were subjected to western blot analyses, which confirmed reduced

expression of ZIPK. We did not find any difference in expression of other Ser/ Thr kinases such as Erk1/2, PKC by siRNA for ZIPK (data not shown). Subsequently, we determined the effects of these siRNAs on LIF-induced STAT3-LUC activation in HeLa cells. As shown in Fig. 3C, siRNA-mediated reduced expression of ZIPK resulted in significant reductions in LIF-induced STAT3-LUC activation, suggesting that ZIPK is necessary for STAT3-mediated transcriptional activation in HeLa cells.

Activation of ZIPK by the IL-6 family of cytokines or oncogenes

To further examine the functional relevance of the STAT3/ZIPK interaction, we examined whether ZIPK expression was induced by IL-6/STAT3-dependent signals. To achieve this, we analyzed the ZIPK mRNA level when IL-6-responsive Hep3B cells were either left untreated or treated with IL-6. No significant alterations in the ZIPK mRNA and protein levels were observed after IL-6 treatment (data not shown). Next, we examined whether IL-6/STAT3 signals had any effects on ZIPK activity. For this purpose, we performed *in vitro* kinase assays on ZIPK immunoprecipitates. First, we tested the effect of Jak1 on ZIPK activity. Expression vectors for Jak1 and/or Myc-tagged ZIPK WT were transfected into 293T cells and immunoprecipitates obtained with an anti-Myc antibody were subjected to *in vitro* kinase assays. As shown in Fig 4A, overexpression of Jak1 resulted in a marked enhancement of ZIPK activity. Furthermore, stimulation with IL-6 or LIF also enhanced the ZIPK activity (Fig. 4B). Interestingly, overexpression of oncogenic proteins, such as Bcr-Abl and v-src, which are known as activators of STAT3 (12,13), remarkably enhanced the ZIPK activity (Fig. 4C). We further examined whether an active form of STAT3, STAT3-C, enhances ZIPK activity. Strikingly, overexpression of STAT3-C resulted in a marked enhancement of ZIPK activity compared to overexpression of STAT3 WT or a dominant-negative STAT3 (STAT3YF). These results suggest that IL-6/LIF/STAT3 activation stimulates ZIPK activity. Furthermore, we could observe the enhanced protein stability of ZIPK in a dose-dependent manner of STAT3 (data not shown), suggesting STAT3

enhances the efficiency of its own Ser 727 phosphorylation by acting as a scaffold for ZIPK.

To further characterize the nature of the interaction between STAT3 and ZIPK, we attempted to determine where their interaction occurs in cells. First, we examined LIF-induced translocation of STAT3 in HeLa cells. We observed that Myc-STAT3 was predominantly distributed in the cytoplasm in unstimulated HeLa cells, consistent with previous findings in other cell lines (38). LIF-stimulation induced translocation of STAT3 to the nucleus (Fig. 5A). We then analyzed where STAT3 was co-localized with ZIPK in HeLa cells. For this experiment, we used ZIPK K42A since overexpression of ZIPK WT together with STAT3 remarkably enhanced the ZIPK activity as described above and caused a loss of cells expressing both molecules due to cell death (data not shown). ZIPK WT and ZIPK K42A were each detected exclusively in the nucleus as described previously (23). Similar results were obtained for Myc-ZIPK, FLAG-ZIPK and HA-ZIPK in HeLa cells, as well as in COS-7 cells (data not shown). Next, the cellular localization of STAT3 and its interaction with ZIPK were examined. We here used ZIPK K42A, which can interacts with STAT3, to avoid a loss of cells due to cell death. The cytoplasmic STAT3 did not co-localize with ZIPK K42A. However, after LIF stimulation, STAT3 translocated to the nucleus where it co-localized with ZIPK K42A (Fig. 5B). Consistent with the *in vivo* interaction data presented above, these results suggest that activated STAT3 interacts with ZIPK in the nucleus.

Discussion

To further understand the regulation and function of STAT3, we searched for STAT3-interacting proteins by yeast two-hybrid screening, and identified ZIPK as a protein that specifically interacts with STAT3. We then demonstrated that ZIPK phosphorylates Ser727 of STAT3 in the nucleus, and enhances IL-6/LIF/STAT3-mediated transcription. SiRNA-mediated reduction in endogenous ZIPK expression revealed that ZIPK is required for LIF/STAT3-mediated transcriptional activation and expression of target genes.

ZIPK was originally identified as a Ser/Thr kinase that binds ATF4, a member of the activating transcription factor/cyclic AMP-responsive element binding protein family of transcription factors (23). ZIPK aggregates through its C-terminal leucine zipper structure, thereby becoming an active enzyme. Ectopic expression of ZIPK in NIH 3T3 cells was reported to induce apoptosis. In contrast, the kinase-inactive ZIPK K42A mutant failed to induce apoptosis, indicating that ZIPK stimulates apoptosis via its catalytic activity (23). The kinase domain of ZIPK shows strong homology to that of death-associated protein kinase (DAPK), and these two enzymes are members of a family of related kinases including DAPK2/DRP-1, DRAK1 and DRAK2 (39,40), all of which are implicated in the execution of apoptosis. Introduction of a DAPK antisense was shown to block IFN-γ-induced apoptosis in HeLa cells (37), indicating that DAPK is required for IFN-γ-induced cell death. Furthermore, evidence that the gene encoding DAPK may function as a tumor suppressor has been presented (41,42). It was also demonstrated that ZIPK, in collaboration with Daxx and Par-4, induced apoptosis from nuclear promyelocytic leukemia protein (PML) oncogenic domains (PODs) (24). However, the mechanisms responsible for the activation of these kinases and their downstream substrates that mediate the apoptotic activity remain unknown.

In the present study, we demonstrated that IL-6/LIF-mediated STAT3 activation enhanced ZIPK activity. This finding may suggest that IL-6/LIF/STAT3 signaling mediates

apoptotic activity by stimulating ZIPK. Indeed, co-expression of ZIPK with STAT3 induced a massive cell death in 293T and HeLa cells (data not shown). We also tested the effect of ZIPK overexpression in M1 cells, whose apoptosis and growth arrest are induced by IL6 or LIF. However, we could not observe its effect, because overexpression of ZIPK induced massive cell death without stimuli. We also demonstrate that Bcr-Abl, v-src, or Jak1 can activate ZIPK in 293T cells. ZIPK activation by Jak1, Bcr-Abl or v-src was detected only in a transient experiments in 293T cells. However, we were unable to generate 293T cells stably expressing ZIPK due to a massive cell death after ZIPK transfection. It is well known that establishment of cell lines over-expressing DAPK family is difficult due to induction of massive cell death. It therefore seems impossible to show effects of ZIPK on Bcr-Abl- or v-src-mediated cellular trasnformation and tumor generation. It would be interesting to know the contribution of ZIPK in cytokine-induced apoptosis and the involvement of Bcr-Abl- or v-src-mediated tumor generation in vivo using ZIPK knockout mice.

On the other hand, ZIPK enhances STAT3-mediated transcription by phosphorylation of STAT3 Ser727. However, ZIPK K42A expression did not suppress this phosphorylation in the late phase of IL-6 stimulation. This finding suggests that other STAT3 Ser727 kinases may be also involved in IL-6 signaling. During the preparation of this manuscript, it has been reported that transforming growth factor-□-activated kinase 1 (TAK1) interacts with STAT3, that the TAK1-Nemo-like kinase (NLK) pathway is efficiently activated by IL-6 through the YXXQ motif, and that this is the YXXQ-mediated H7-sensitive pathway that leads to STAT3 Ser727 phosphorylation (43). Furthermore, STAT3 enhances the efficiency of its own Ser727 phosphorylation by acting as a scaffold for the TAK1/NLK, specifically in the YXXQ motif-derived pathway. Thus, TAK1/NLK and ZIPK might coordinately associate in cells or synergistically activates STAT3-dependent transcription. Alternatively, TAK1/NLK might stimulate Ser727 phosphorylation independently of ZIPK in cells.

We found that endogenous ZIPK associates with endogenous STAT3 in response to IL-6 and regulates IL-6/LIF/STAT3 signaling by enhancing ZIPK catalytic activity. Consistent with these observations, the kinase-negative ZIPK K42A mutant specifically suppressed IL-6/LIF-induced STAT3-dependent transcription and Ser727 phosphorylation. Furthermore, siRNA-mediated reduction of ZIPK expression decreased LIF-induced STAT3-dependent transcription. Therefore, our data suggest that ZIPK interacts with STAT3 within the nucleus to regulate the transcriptional activity of STAT3 by phosphorylation of Ser727.

Acknowledgements

We thank T. Hirano, J. N. Ihle and J. Bromberg for their kind gifts of reagents. We also thank J. Akiyama for encouraging our work. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government, the AstraZeneca Research Grant 2003, the Osaka Foundation for Promotion of Clinical Immunology, the Naito Foundation, the Ichiro Kanehara Foundation and the Sasakawa Scientific Research Grant from the Japan Science Society.

Abbreviations

ATF4	activating transcription factor 4
DAPK	death-associated protein kinase
GST	glutathione S-transferase
HA	hemagglutinin
Jak	Janus kinase
LIF	leukemia inhibitory factor
NLK	Nemo-like kinase
RT	reverse transcription
Ser727	serine 727
Ser/Thr	serine/threonine
siRNA	small interfering RNA
STAT	signal transducer and activator of transcription
TAK1	transforming growth factor-ß-activated kinase 1
TNF	related apoptosis-inducing ligand
ZIPK	zipper-interacting protein kinase
ZIPK WT	wild-type ZIPK

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Figure legend

Figure 1. Physical interactions of ZIPK with STAT3

(A)Interactions between STAT and ZIPK in a yeast two-hybrid assay. Growth of transformed S. cerevisiae demonstrating an interaction between either STAT3/C or STAT4/C and ZIPK. pGBKT7-STAT3/C, pGBKT7-STAT4/C or empty pGBKT7 in AH109 were mated with pACT2-ZIPK(residues 161-261) or empty pACT2 in Y187 as indicated. Colonies were then re-streaked onto high-stringency plates.

(B) 293T cells ($5x10^6$) were transfected with Myc-tagged ZIPK (5 □g) and/or STAT1, STAT3, STAT4, STAT5a, STAT5b or STAT6 (10 □g). Forty-eight hours after transfection, the cells were lysed, immunoprecipitated with respective anti-STAT antibodies and immunoblotted with anti-Myc antibody (upper panel) or respective anti-STAT antibodies (middle panel). Total cell lysates (1 %) were blotted with anti-Myc antibody (bottom panel) to monitor the expression of ZIPK.

(C) Domain structure of STAT3 and mutant fragments are schematically shown. 293T cells ($5x10^6$) were transfected with Myc-tagged STAT3 deletion mutants (10 □g) and/or HA-tagged ZIPK (10 □g). Forty-eight hours after transfection, the cells were lysed, and immunoprecipitated with anti-HA antibody and blotted with anti-Myc (upper panel) or anti-HA antibody (middle panel). Total cell lysates (1 %) were blotted with anti-Myc (bottom panel) to monitor the expression of STAT3 mutant fragments. The asterisks indicate the migration position of STAT3 mutants. IgH, immunoglobulin heavy chain.

(D) Domain structure of ZIPK and mutant fragments are schematically shown. 293T cells ($5x10^6$) were transfected with FLAG-tagged ZIPK mutants (10 □g) and/or Myc-tagged STAT3 (10 □g). Forty-eight hours after transfection, the cells were lysed, and immunoprecipitated with anti-FLAG antibody and blotted with anti-Myc (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1 %) were blotted with anti-FLAG (bottom panel) to monitor the expression of ZIPK mutant fragments.

(E) SKW6Cl4 cells ($5x10^7$) were treated without or with IL-6 (100 ng/ml) for 30 min. The cells were lysed, and immunoprecipitated with control or anti-STAT3 antibody and immunoblotted with anti-ZIPK antibody (upper panels) or anti-STAT3 antibody (lower panels). Total cell lysates (1 %) were blotted with anti-ZIPK or STAT3 antibody (left panels) to monitor the endogenous expression of ZIPK or STAT3.

(F) 293T cells ($5x10^6$) were transfected with FLAG-tagged STAT3 (10 □g) and/or DAPK kinase domain, DAPK2, DRAK1, DRAK2 or ZIPK (10 □g). Forty-eight hours after transfection, the cells were lysed, immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc antibody (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1 %) were blotted with anti-Myc antibody (bottom panel) to monitor the expression of DAPK family protein. The asterisks indicate the migration position of each DAPK family ptotein.

Figure 2. Phosphorylation of STAT3 Ser727 by ZIPK

(A) 293T cells ($5x10^6$) transfected with Myc-tagged STAT3 (10 □g) together with the increasing amount of HA-tagged ZIPK as indicated. Forty-eight hours after transfection, the cells were lysed, immunoprecipitated with anti-Myc antibody and immunoblotted with anti-pSTAT3 Ser727 (upper panel) or anti-Myc antibody (middle panel). Total cell lysates (1 %) were blotted with anti-HA antibody (bottom panel) to monitor the expression of ZIPK.

(B) Recombinant GST or GST-fused ZIPK in the absence or presence of the indicated concentration of H7, was subjected to *in vitro* kinase reaction and resolved by SDS-PAGE. ^{32}P-labeled protein was visualized by autoradiography.

(C) Recombinant GST or GST-fused ZIPK together with the synthetic peptide for STAT3 (residues 720-735), in the absence or presence of the indicated concentration of H7, was subjected to *in vitro* kinase reaction and resolved by SDS-PAGE. ^{32}P-labeled protein was visualized by autoradiography.

Figure 3. Involvement of ZIPK in STAT3-mediated transcription.

(A) 293T cells seeded on a 12-well plate were transfected with STAT3-LUC (0.4 μg) and/or the increasing amounts of ZIPK WT or ZIPK K42A as indicated. Twenty-four hours after transfection, cells were stimulated with the increasing amount of LIF for additional 12 h. The 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.

(B) Stable H35 transfectant expressing empty vector, ZIPK WT or ZIPK K42A was established. Total extracts of each transfectant were examined by Western blot using anti-FLAG antibody. H35 transfectant cells in a 12-well plate were transfected with STAT3-Luc (0.4 μg). Twenty-four hours after transfection, the cells were stimulated with IL-6 (100 ng/ml) and sIL-6Rα (100 ng/ml) for 12 h. The cells were then 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.

(C) H35 transfectants were stimulated with or without IL-6 (50 ng/ml) and sIL-6Rα (100 ng/ml) for the indicated periods. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using SOCS3 (upper panels), glyceraldehydes-3-phosphate dehydrogenase (G3PDH). This figure is representative of three separate experiments.

(D) HeLa cells were transfected with siRNA #1 or siRNA #2 targeting ZIPK and cells were analyzed by immunoblotting using anti-ZIPK or anti-Actin antibody, verifying siRNA-mediated reduction in endogenous ZIPK. HeLa cells were treated with siRNA#2. After 4 h, the cells were transfected with STAT3-LUC (0.4 μg). Twenty-four hours after transfection, the cells were stimulated with LIF for 12 h. The cells were then 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.

Figure 4. Activation of ZIPK activity by an IL-6 family of cytokine or oncogenes.

(A) 293T cells ($5x10^6$) transfected with empty vector or Myc-tagged ZIPK (1□g) together with or without Jak1(1 □g). Forty-eight hours after transfection, the cells were lysed, and immunoprecipitated with anti-Myc antibody, subjected to *in vitro* kinase reaction and resolved by SDS-PAGE. ^{32}P-labeled protein was visualized by autoradiography.

(B) 293T cells ($5x10^6$) transfected with empty vector or FLAG-tagged ZIPK (5 □g). Forty-eight hours after transfection, the cells were treated with or without LIF (100 ng/ml) or IL-6 (100 ng/ml) for 30 min. The cells were then lysed, immunoprecipitated with anti-FLAG antibody, subjected to *in vitro* kinase reaction and resolved by SDS-PAGE. ^{32}P labeled protein was visualized by autoradiography.

(C) 293T cells ($5x10^6$) transfected with Myc-tagged ZIPK (1 □g) together with empty vector or, Bcr-Abl or v-src as indicated. Forty-eight hours after transfection, the cells were lysed, and immunoprecipitated with anti-Myc antibody, subjected to *in vitro* kinase reaction and resolved by SDS-PAGE. ^{32}P-labeled protein was visualized by autoradiography.

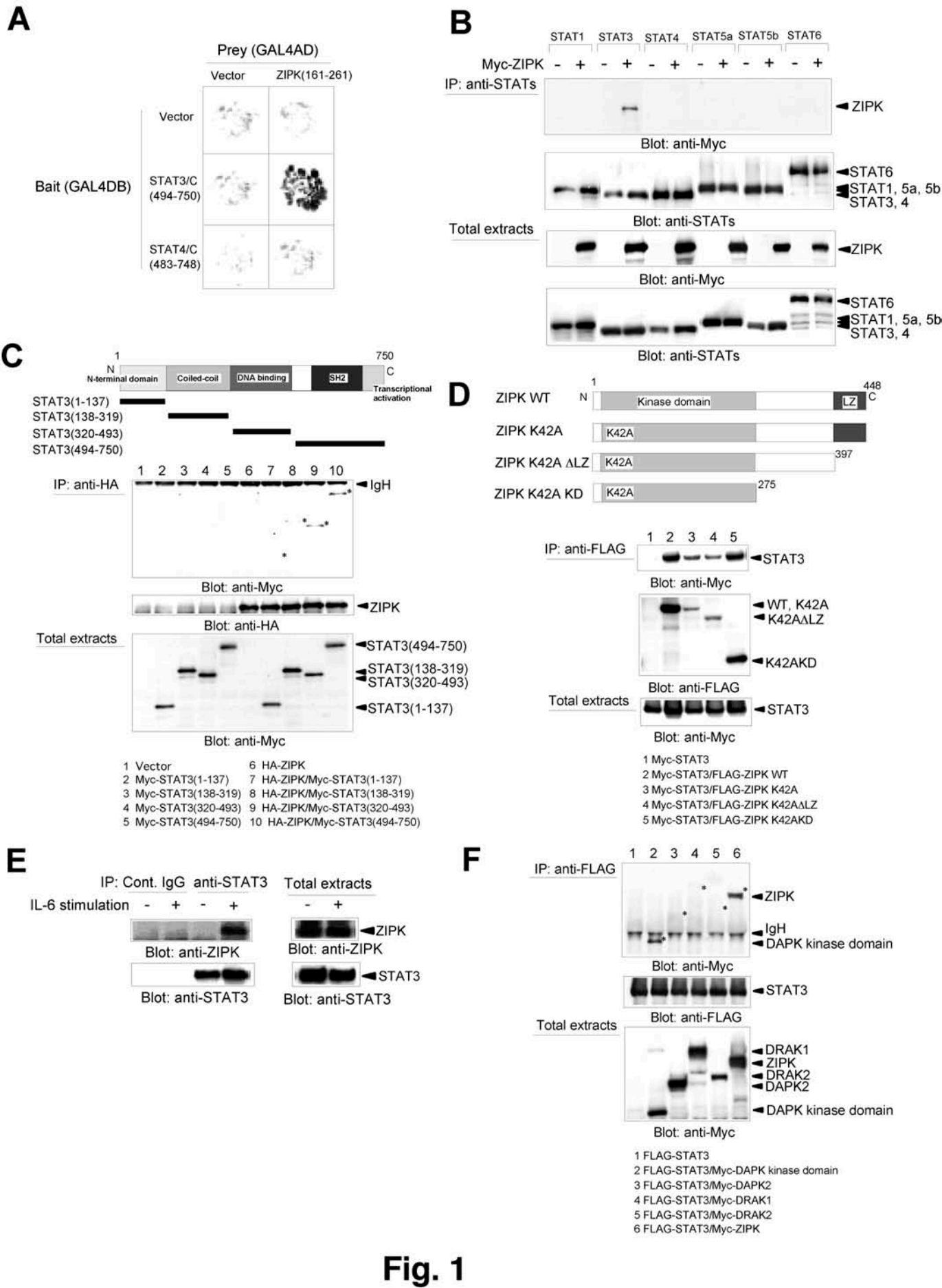
(D) 293T cells ($5x10^6$) were transfected with empty vector or FLAG-tagged STAT3-C, STAT3 WT (3 or 10 □g) together with Myc-tagged ZIPK (1 □g). Forty-eight hours after transfection, the cells were lysed, immunoprecipitated with anti-Myc antibody, subjected to *in vitro* kinase reaction and resolved by SDS-PAGE. ^{32}P-labeled protein was visualized by autoradiography.

Figure 5. Co-localization of STAT3 and ZIPK in the nucleus.

(A) HeLa cells ($2x10^5$) were transfected with Myc-tagged STAT3 (1□g). Forty-eight hours after transfection, the cells were treated or untreated with LIF for 30 min. The cells were fixed and stained with anti-Myc antibody followed by rhodamine-conjugated anti-mouse IgG (red signal). Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (blue signal).

(B) HeLa cells ($2x10^5$) were transfected with FLAG-tagged ZIPK K42A (1 □g) and Myc-

tagged STAT3 (1 μg). Forty-eight hours after transfection, the cells were treated or untreated with LIF for 30 min. The cells were fixed and stained with anti-FLAG or anti-Myc antibody followed by FITC-conjugated anti-rabbit IgG (green signal) orrhodamine-conjugated anti-mouse IgG (red signal). Nuclei were counterstained with DAPI (blue signal).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ZIPK(161-261)
Bait (GAL4DB)
Vector
STAT3/C (494-750)
STAT4/C (483-748)
B
STAT1
STAT3
STAT4
STAT5a
STAT5b
STAT6
Myc-ZIPK - + - + - + - + - + - +
IP: anti-STATs
ZIPK
Blot: anti-Myc
STAT6
STAT1, 5a, 5b
STAT3, 4
Blot: anti-STATs
Total extracts
ZIPK
Blot: anti-Myc
STAT6
STAT1, 5a, 5b
STAT3, 4
Blot: anti-STATs
C
1
750
N
C
N-terminal domain
Coiled-coil
DNA binding
SH2
Transcriptional activation
STAT3(1-137)
STAT3(138-319)
STAT3(320-493)
STAT3(494-750)
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IP: anti-HA
IgH
Blot: anti-Myc
ZIPK
Blot: anti-HA
Total extracts
STAT3(494-750)
STAT3(138-319)
STAT3(320-493)
STAT3(1-137)
Blot: anti-Myc
1 Vector
2 Myc-STAT3(1-137)
3 Myc-STAT3(138-319)
4 Myc-STAT3(320-493)
5 Myc-STAT3(494-750)
6 HA-ZIPK
7 HA-ZIPK/Myc-STAT3(1-137)
8 HA-ZIPK/Myc-STAT3(138-319)
9 HA-ZIPK/Myc-STAT3(320-493)
10 HA-ZIPK/Myc-STAT3(494-750)
D
ZIPK WT
N
1
Kinase domain
LZ
448
C
ZIPK K42A
K42A
ZIPK K42A ΔLZ
K42A
397
ZIPK K42A KD
K42A
275
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STAT3
Blot: anti-Myc
WT, K42A
K42AΔLZ
K42AKD
Blot: anti-FLAG
Total extracts
STAT3
Blot: anti-Myc
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3 Myc-STAT3/FLAG-ZIPK K42A
4 Myc-STAT3/FLAG-ZIPK K42AΔLZ
5 Myc-STAT3/FLAG-ZIPK K42AKD
E
IP: Cont. IgG
anti-STAT3
Total extracts
IL-6 stimulation - + - + - +
ZIPK
Blot: anti-ZIPK
Blot: anti-ZIPK
STAT3
Blot: anti-STAT3
Blot: anti-STAT3
F
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IP: anti-FLAG
ZIPK
IgH
DAPK kinase domain
Blot: anti-Myc
STAT3
Blot: anti-FLAG
Total extracts
DRAK1
ZIPK
DRAK2
DAPK2
DAPK kinase domain
Blot: anti-Myc
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2 FLAG-STAT3/Myc-DAPK kinase domain
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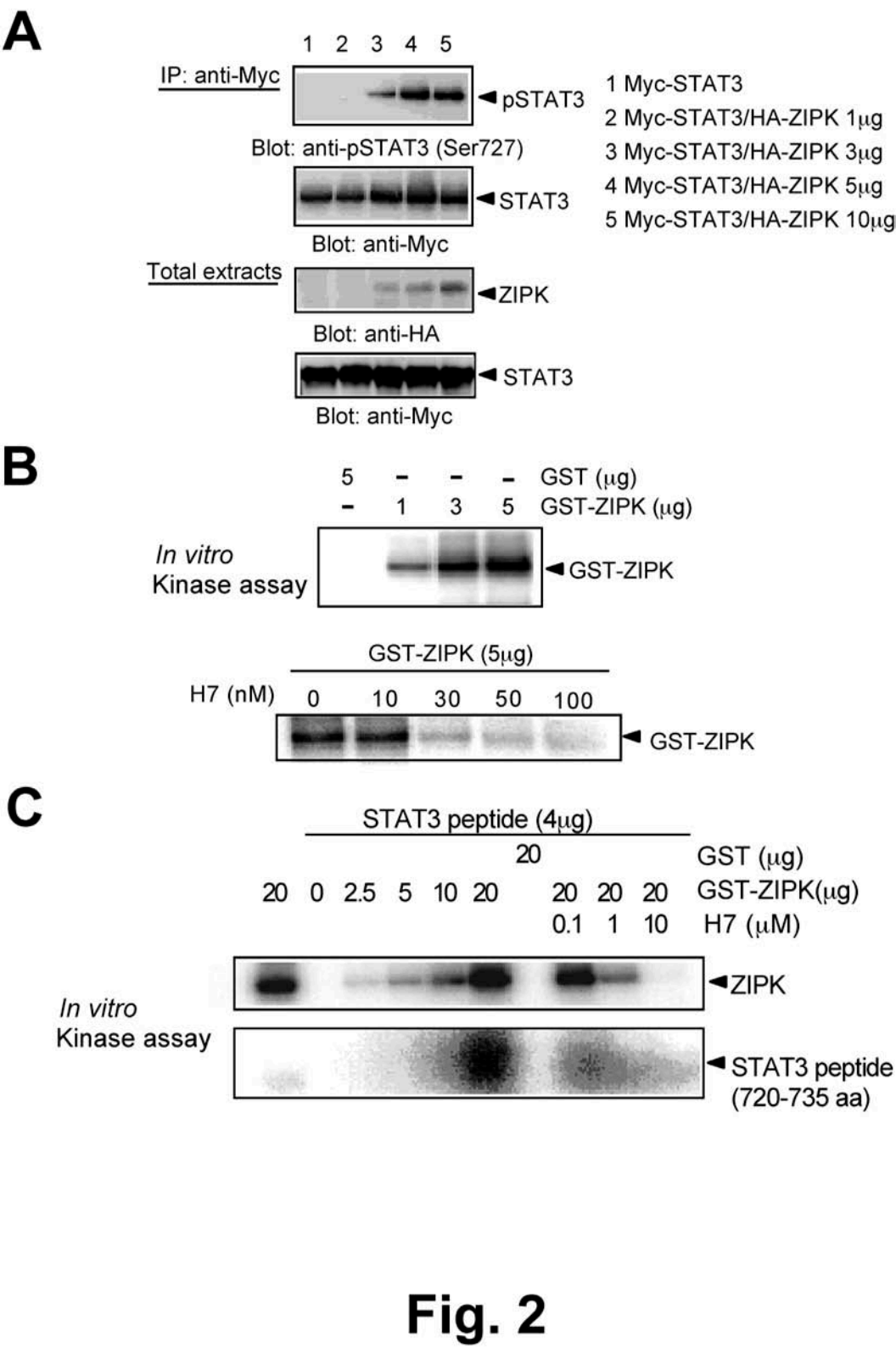
5 FLAG-STAT3/Myc-DRAK2
6 FLAG-STAT3/Myc-ZIPK

Fig. 1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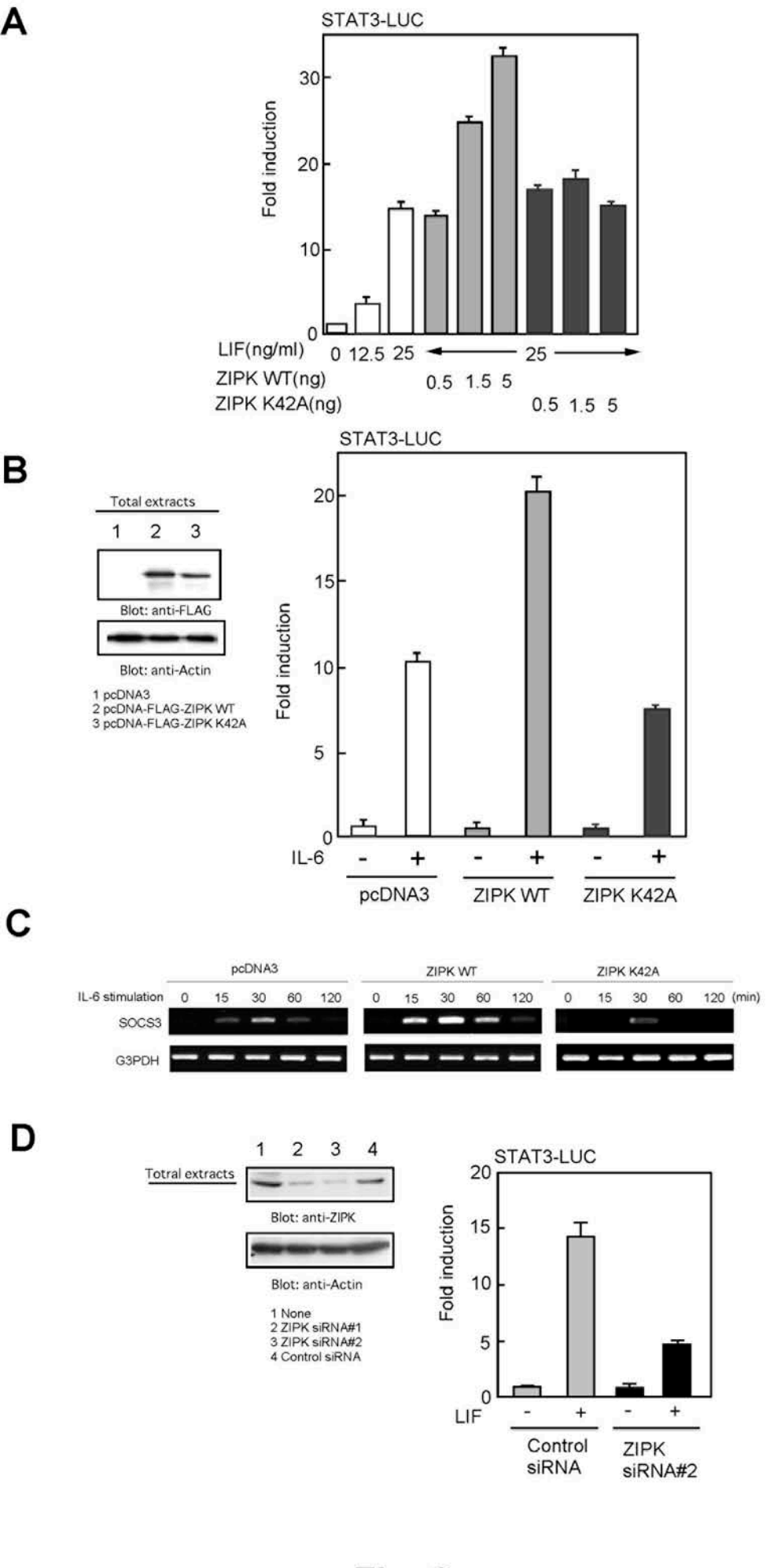
5 Myc-STAT3/HA-ZIPK 10μg
Blot: anti-pSTAT3 (Ser727)
STAT3
Blot: anti-Myc
Total extracts
ZIPK
Blot: anti-HA
STAT3
Blot: anti-Myc
B
5 - - - GST (μg)
- 1 3 5 GST-ZIPK (μg)
In vitro
Kinase assay
GST-ZIPK
GST-ZIPK (5μg)
H7 (nM) 0 10 30 50 100
GST-ZIPK
C
STAT3 peptide (4μg)
20
GST (μg)
20 0 2.5 5 10 20 20 20 20 GST-ZIPK(μg)
0.1 1 10 H7 (μM)
ZIPK
In vitro
Kinase assay
STAT3 peptide
(720-735 aa)

Fig. 2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ZIPK WT(ng) 0.5 1.5 5
ZIPK K42A(ng) 0.5 1.5 5
B
Total extracts
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Blot: anti-Actin
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3 pcDNA-FLAG-ZIPK K42A
STAT3-LUC
Fold induction
20
15
10
5
0
IL-6 - + - + - +
pcDNA3
ZIPK WT
ZIPK K42A
C
pcDNA3
ZIPK WT
ZIPK K42A
IL-6 stimulation 0 15 30 60 120 0 15 30 60 120 0 15 30 60 120 (min)
SOCS3
G3PDH
D
1 2 3 4
Totral extracts
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Blot: anti-Actin
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2 ZIPK siRNA#1
3 ZIPK siRNA#2
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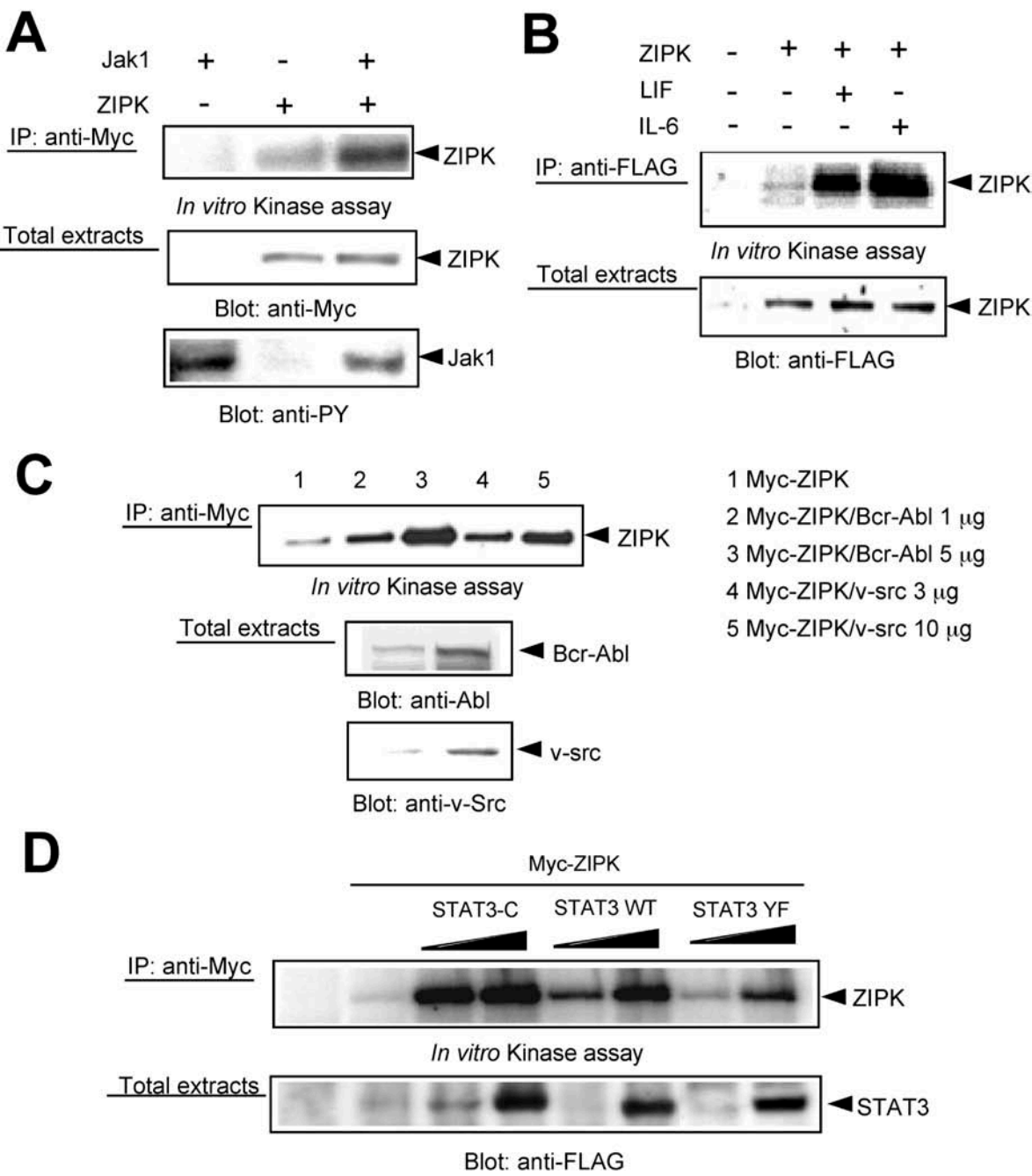
LIF - + - +
Control siRNA
ZIPK siRNA#2

Fig. 3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B
ZIPK - + + +
LIF - - + -
IL-6 - - - +
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In vitro Kinase assay
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In vitro Kinase assay
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Bcr-Abl
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v-src
Blot: anti-v-Src
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5 Myc-ZIPK/v-src 10 μg
D
Myc-ZIPK
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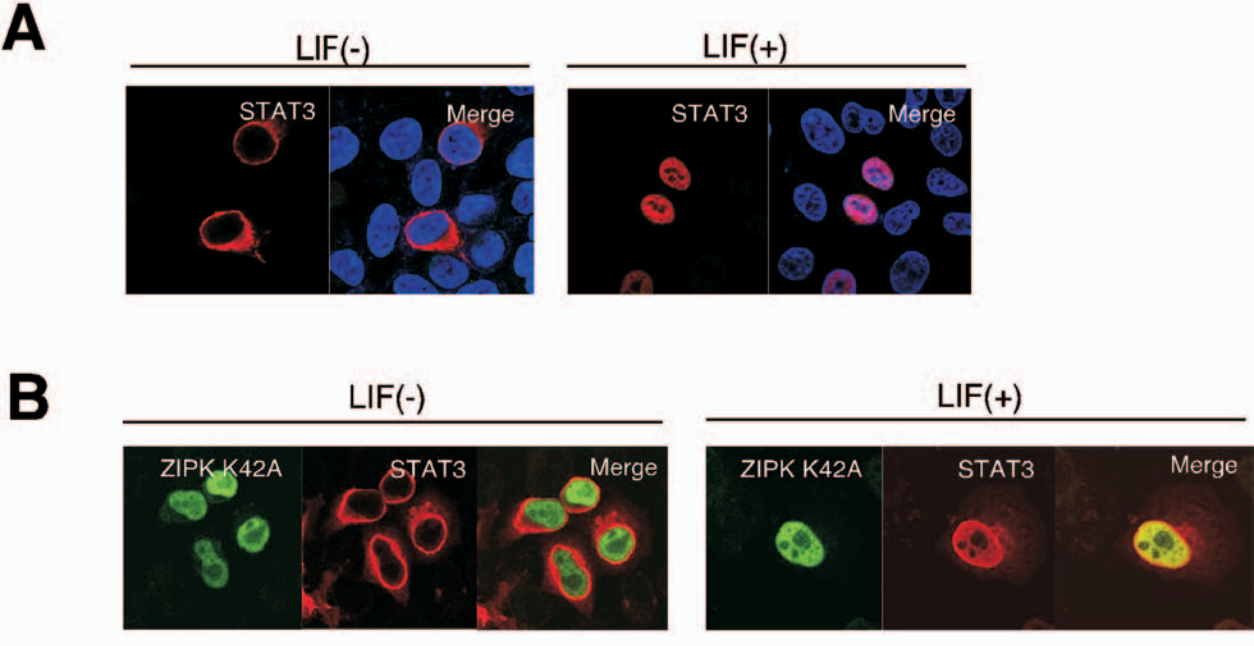
Fig. 4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STAT3
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B
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LIF(+)
ZIPK K42A
STAT3
Merge
ZIPK K42A
STAT3
Merge

Fig. 5