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Title: An RNA biding protein, Y14 interacts with and modulates STAT3 activation

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Running title: Interactions between STAT3 and Y14

Abstract

Signal transducer and activator of transcription 3 (STAT3), which mediates biological actions in many physiological processes, is activated by cytokines and growth factors via specific tyrosine phosphorylation, dimerization and nuclear translocation. To clarify the molecular mechanisms underlying the regulation of STAT3 activation, we performed yeast two-hybrid screening. We identified Y14, an RNA-binding protein, as a novel STAT3-binding partner. Y14 bound to STAT3 through the C-terminal region of STAT3 *in vivo*. Importantly, small-interfering RNA-mediated reduction of endogenous Y14 expression decreased IL-6-induced tyrosine-phosphorylation, nuclear accumulation and DNA binding activity of STAT3, as well as IL-6/STAT3-dependent gene expression. These results indicate that Y14 interacts with STAT3 and regulates the transcriptional activation of STAT3 by influencing the tyrosine-phosphorylation of STAT3.

Key words: Cytokine, STAT3, Y14, RNA-binding protein, gene expression, signal transduction

Introduction

The Jak/STAT pathways are utilized by a wide range of cytokines to regulate gene expression. Cytokines activate members of the Jak family of protein tyrosine kinases, which in turn activate, by tyrosine phosphorylation, one or more members of the STAT family of transcription factors. STATs are unusual among transcription factors in that they have the characteristics of cytoplasmic signaling molecules, such as a Src-homology 2 domain and tyrosine phosphorylation sites. Upon tyrosine phosphorylation, the STATs dimerize through their phosphorylated SH2 domains and translocate to the nucleus [1, 2, 3]. In recent years, constitutive or dysregulated expression of STAT3 has been found in cancer cells and oncogene-transfected cells and shown to be involved in a wide range of diseases including autoimmune diseases [4-7]. Studies in *Dictyostelium*, *Drosophila* and zebrafish have also shown that the Jak/STAT pathway is required for a broad set of developmental processes, including cell proliferation, cell fate determination, cell migration, planar polarity, convergent extension and immunity [8, 9]. These findings imply that STAT activation must be

tightly regulated.

To investigate the specific regulatory mechanisms of STAT3, we sought to identify STAT3-interacting proteins using yeast two-hybrid screening with the C-terminal region of STAT3 as bait. We identified Y14, an RNA-binding protein, as a novel binding partner of STAT3. Y14 is an RNA-binding protein that binds preferentially to spliced mRNAs immediately upstream of the exon–exon junctions, and remains bound to the mRNA after nuclear export [10, 11]. Y14 binds to STAT3 through the C-terminal region of STAT3. Furthermore, small-interfering RNA-mediated reduction of endogenous Y14 expression decreased IL-6-induced tyrosine-phosphorylation, nuclear accumulation and DNA binding activity of STAT3, as well as IL-6/STAT3-dependent gene expression. Here, we show that Y14 is a STAT3 binding partner with a critical role in STAT3-mediated transcription.

Materials and Methods

Reagents and antibodies, Recombinant human IL-6 was a kind gift from Ajinomoto Co. (Tokyo, Japan). Recombinant human LIF was purchased from INTERGEN (Purchase, NY). Epipote-tagged STAT3 and its mutants were previously described [12, 13]. Epitope-tagged Y14 was generated by PCR and sequenced (primer sequences are available upon request). Anti-Myc, anti-GST, anti-STAT3, and Y14 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-STAT3 Tyr705 (pSTAT3 Tyr705) was purchased from Cell Signaling Technologies (Beverly, MA). Anti-Actin antibody from Chemicon (Temecula, CA).

Yeast two-hybrid screen, Gal4-STAT3 was constructed by fusing the coding sequence for the C-terminal region (amino acids 494-750) of STAT3 in-frame to the Gal4 DNA-binding domain in the pGBKT7 vector (Clontech, Palo Alto, CA). *S. cerevisiae* AH109 cells were transformed with pGal4-STAT3, then mated with Y187 cells containing a pretransformed mouse 11-day embryo MATCHMAKER cDNA library (Clontech, Palo Alto, CA) and approximately 2.6 x 10^6 colonies were screened as

previously described [14]. Plasmid DNAs derived from positive clones were extracted from the yeast and sequenced.

Cell culture, transfection, siRNA, RT-PCR and quantitative real-time PCR, Human embryonic kidney carcinoma cell line, 293T, was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS and transfected by the standard calcium precipitation protocol. The human hepatoma cell line Hep3B was cultured in DMEM, supplemented with 10% fetal bovine serum (FBS). Hep3B cells were treated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37 °C for 4 h, followed by addition of fresh medium containing 10% FCS as previously described [15]. siRNAs targeting human Y14 used in this study was follows: siY14, 5'-GGGGUAUACUCUAGUUGATT-3'. Total RNAs were prepared by using Iso-Gen (Nippon Gene, Tokyo, Japan) and used in reverse transcription (RT)-PCR. RT-PCR was performed using RT-PCR high -Plus- Kit (TOYOBO, Tokyo, Japan) [18]. Primers used for RT-PCR were: Y14: 5'-AAGATTTCGCCATGGATGAG-3' (sense), 5'-ATCAAATCCTGGCCATTGAG-3' (antisense). Quantitative real-time PCR analyses of SOCS3 and C/EBP□ as well as the control G3PDH mRNA transcripts were 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 [16]. 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 or mutant oligonucleotide-Sepharose conjugate (Santa Cruz) as described previously [15]. The precipitates were subjected to Western blot analysis using anti-pSTAT3 antibody. An aliquot of TCL was blotted with anti-pSTAT3, anti-STAT3 or anti-Y14 antibody.

Indirect Immunofluorescence microscopy, Hep3B 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 [17]. 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

Molecular interactions between STAT3 and Y14

To investigate the regulatory mechanisms of STAT3, we sought to identify STAT3-interacting proteins. We performed a yeast two-hybrid screen of a mouse embryo cDNA library using the C-terminal region of STAT3 (amino acids 494-750) as bait. From a screen of about 2.6 x 10^6 transformants, we identified several positive clones. Sequence analysis revealed that one of them encoded the entire Y14 protein (amino acids 1-204).

We first examined whether Y14 binds STAT3 in mammalian cells. 293T cells were transfected with various deletion constructs of GST-fused STAT3 (Fig. 1A) together with Myc-tagged Y14. Total cell lysates from the transfected cells were subjected to pull-down assays. As shown in Fig. 1B, the DNA-binding and C-terminal domains of STAT3 interacted with Y14 as well as full-length STAT3 in 293T cells. We next examined whether LIF stimulation enhances the binding of Y14 to STAT3 in 293T cells. As shown in Fig. 1C, Y14 bound to STAT3 without LIF stimulation, although STAT3

was tyrosine-phosphorylated by LIF stimulation, indicating that binding of Y14 to STAT3 occurs independently of activation of STAT3. Moreover, an inactive form of STAT3, STAT3 YF mutant, bound to Y14 like wild-type STAT3 did (Fig. 1C), indicating that unphoshorylated and monomer STAT3 can interact with Y14.

To characterize further the nature of the interaction between STAT3 and Y14, we attempted to determine where this interaction occurs in cells. Y14 predominantly localized in the nucleus in the presence or absence of IL-6-stimulation, while IL-6 stimulation induced translocation of STAT3 into the nuclei of Hep3B cells (Fig. 1D). However, after IL-6 stimulation, STAT3 translocated into the nucleus and co-localized with Y14. Consistent with the *in vivo* interaction data presented above, these results suggest that activated STAT3 mainly interacts with Y14 in the nucleus.

Y14 is involved in tyrosine-phosphorylation, nuclear accumulation and DNA binding activity of STAT3

To assess the functional relevance of the interaction between STAT3 and Y14, we first examined whether reduction of Y14 expression affects IL-6-induced tyrosine-

phosphorylation of STAT3. As shown in Fig. 2A, tyrosine-phosphorylation of STAT3 decreased following a reduction in Y14 expression in Hep3B cells. We also observed the effects of a reduction in Y14 expression on IL-6-induced nuclear accumulation of STAT3 in control and Y14 siRNA-treated Hep3B cells. When we observed nuclear accumulation of STAT3 in Y14 siRNA-treated Hep3B cells, nuclear accumulation of STAT3 was significantly reduced in Y14 knockdown cells after 90 min of IL-6 stimulation. These findings suggest that the loss of Y14 affects IL-6-induced tyrosine phosphorylation of STAT3 following the decreased nuclear accumulation of STAT3.

We further examined whether a reduction in Y14 expression affects the DNA binding activity of STAT3. As shown in Fig. 3A and B, IL-6-induced DNA binding activity of STAT3 was slightly affected by Y14 siRNA treatment, suggesting that Y14 has an effect on STAT3's DNA binding activity. This may be due to the decreased IL-6-induced tyrosine-phosphorylation of STAT3 by Y14 siRNA treatment, although there is the possibility that Y14 may stabilize STAT3-DNA interactions to keep STAT3 in the nucleus, because Y14 interacts with STAT3 through the DNA-binding domain of STAT3.

Y14 is involved in IL-6/STAT3-mediated gene expression

To finally clarify the physiological significance of the molecular interactions between STAT3 and Y14, we examined the effect of Y14 on STAT3 activity. To test whether Y14 affects STAT3-mediated gene expression, we used siRNA to reduce the endogenous expression of Y14 in Hep3B cells. A specific siRNA for Y14 or a control siRNA was transfected into Hep3B cells. Total RNA from the transfected cells was subjected to RT-PCR analysis, confirming the reduction in Y14 mRNA expression. As shown in Fig. 3C and D, IL-6/STAT3-mediated SOCS3 and C/EBP mRNA expressions were markedly reduced in Y14 siRNA-treated Hep3B cells, indicating that Y14 plays a critical role in the regulation of STAT3 activity *in vivo*.

Concluding remarks

We here demonstrated that Y14 is a STAT3 binding partner and that a reduction in the levels of endogenous Y14 decreases STAT3-mediated transactivation by influencing tyrosine-phosphorylation and nuclear accumulation of STAT3.

Recently, Y14 has been shown to form the *Drosophila* oskar mRNA localization complex and act as the nuclear shuttling protein for oskar mRNA cytoplasmic localization [18, 19]. In Drosophila, localization of oskar mRNA at the posterior pole is essential and sufficient for the specification of the germline and the abdomen. Its posterior transport along the microtubules is mediated by kinesin I and several other proteins, such as Y14 and Mago-nashi, which, together with oskar mRNA, form a posterior localization complex. Human Y14 has also been shown to associate with mRNAs upon splicing and shuttles to the cytoplasm where it interacts with MAGOH, the human homolog of Mago-nashi[20, 21]. At the present time, there has been no report of an mRNA shuttling protein involved in the regulation of transcriptional factors, such as the nuclear shuttling of STATs.

Although the STATs show similar structural features, their nuclear–cytoplasmic localization has been shown to be differently regulated. After the importin-STAT1 complex enters the nucleus, importin is released from the complex and STAT1 can then bind to DNA [22,23]. In contrast to STAT1, STAT3 has been shown to accumulate in the nucleus independent of its phosphorylation state [24], although a reduction in

importin-β3 expression by siRNA inhibits the nuclear localization of STAT3. Therefore, STAT3 continuously shuttles between nuclear and cytoplasmic compartments, and can thereby respond to extracellular stimuli. The nuclear accumulation of unphosphorylated STAT3 might assist a rapid response of activated STAT3 by extracellular or intracellular stimuli. Y14 may interact with unphosphorylated STAT3 and assist rapid STAT3 activation. In Y14 knockdown cells, nuclear accumulation decreased after 90 min of IL-6 stimulation, suggesting that Y 14 may also play a role in the nuclear retention of STAT3. Recently, we have identified BART as a novel STAT3 binding partner critical for the nuclear retention for STAT3 [25]. Therefore, it will be noteworthy to clarify the detailed interactions among STAT3, BART and Y14.

It will be very important to discover every step in the STAT3-specific regulation of gene transcription, since STAT3 may be a key player in the pathogenesis of diverse human diseases and an important target for novel therapies. Further understanding of the detailed molecular interactions between STAT3 and Y14 may provide a novel therapeutic strategy for STAT3-related human diseases.

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References

[1] J. E. Darnell Jr., I. M. Kerr, G. R. Stark, Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264 (1994) 1415-1421.

[2] J. N. Ihle, STATs: signal transducers and activators of transcription. Cell 84 (1996) 331-334.

[3] J. J. O'Shea, Jaks, STATs, cytokine signal transduction, and immunoregulation: are we there yet? Immunity 7 (1997) 1-11.

[4] T. Bowman, R. Garcia, J. Turkson and R. Jove, STATs in oncogenesis. Oncogene 19 (2000) 2474-2488.

[5] D. E. Levy, C.K. Lee, What does Stat3 do? J. Clin. Invest. 109 (2002) 1143-1148

[6] J. Bromberg, J.E. Darnell Jr. The role of STATs in transcriptional control and their impact on cellular function. Oncogene 19 (2000) 2468-2473.

[7] T. Hirano, K. Ishihara, M. Hibi, Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene 19 (2000) 2548-2556.

[8] S.X. Hou, Z. Zheng, X. Chen, N. Perrimon, The Jak/STAT pathway in model organisms: emerging roles in cell movement. Dev. Cell 3 (2002) 765-778

[9] S. Yamashita, T. Hirano, in: P.B. Sehgal, D. E. Levy, T. Hirano (Eds), STATs in cell mobility and polarity during morphogenetic movement in Signal Transducers and Activators of Transcription (STATs): Activation and Biology, Kluwer Academic, Dordrecht. 2003, pp.595-607.

[10] N. Kataoka , J. Yong, V.N. Kim, F. Velazquez, R.A. Perkinson, F. Wang, G. Dreyfuss., Pre-mRNA splicing imprints mRNA in the nucleus with a novel RNA-binding protein that persists in the cytoplasm, Mol Cell. 6 (2000) 673-682.

[11] V.N. Kim, J.Yong, N. Kataoka, L. Abel, M.D. Diem, G. Dreyfuss,. The Y14 protein communicates to the cytoplasm the position of exon-exon junctions. EMBO J. 20 (2001) 2062-2068.

[12] R. Muromoto, K. Nakao, T. Watanabe, N. Sato, Y. Sekine, K. Sugiyama, K. Oritani, K. Shimoda, T. Matsuda, Physical and functional interactions between Daxx and STAT3. Oncogene 25 (2006) 2131-2136.

[13] Y. Sekine, S. Tsuji, O. Ikeda, N. Sato, N. Aoki, K. Aoyama, K. Sugiyama, T.

Matsuda, Regulation of STAT3-mediated signaling by LMW-DSP2. Oncogene 25 (2006) 5801-5806.

[14] N. Sato, T. Kawai, K. Sugiyama, R. Muromoto, S. Imoto, Y. Sekine, M. Ishida, S. Akira, T. Matsuda, Physical and functional interactions between STAT3 and ZIP kinase, Int. Immunol. 17 (2005) 1543-1552.

[15] Y. Sekine, O. Ikeda, Y. Hayakawa, S. Tsuji, S. Imoto, N. Aoki, K. Sugiyama, T. Matsuda, DUSP22/LMW-DSP2 regulates estrogen receptor-alpha-mediated signaling through dephosphorylation of Ser-118, Oncogene 26 (2007) 6038-6049

[16] T. Matsuda, T. Yamamoto, A. Muraguchi, F. Saatcioglu, Cross-talk between transforming growth factor-beta and estrogen receptor signaling through Smad3, J. Biol. Chem. 276 (2001) 42908-42914.

[17] R. Muromoto, M. Ishida, K. Sugiyama, Y. Sekine, K. Oritani, K. Shimoda, T. Matsuda, Sumoylation of Daxx regulates IFN-induced growth suppression of B-lymphocytes and the hormone receptor-mediated transactivation, J. Immunol. 177 (2006) 1160-1170.

[18] I.M. Palacios, RNA processing: splicing and the cytoplasmic localisation of mRNA,

Curr Biol. 12 (2002) R50-R52.

[19] O. Hachet, S. Ephrussi, Splicing of oskar RNA in the nucleus is coupled to its cytoplasmic localization, Nature 428 (2004) 959-963.

[20] X.F. Zhao, N.J. Nowak, T.B. Shows, P.D. Aplan, MAGOH interacts with a novel RNA-binding protein. Genomics 63 (2000) 145-148.

[21] J.M. Mingot, S. Kostka, R. Kraft, E. Hartmann, D. Gorlich, Importin 13: a novel mediator of nuclear import and export. EMBO J. 20 (2001) 3685–3694.

[22] T. Sekimoto, N. Imamoto, K. Nakajima, T. Hirano and Y. Yoneda, Extracellular signal-dependent nuclear import of Stat1 is mediated by nuclear pore-targeting complex formation with NPI-1, but not Rch1. EMBO J. 16 (1997) 7067-7077.

[23] K.M. McBride, G. Banninger, C. McDonald, N. C. Reich, Regulated nuclear import of the STAT1 transcription factor by direct binding of importin-β. EMBO J. 21 (2002) 1754-1763

[24] L. Liu, K.M. McBride, N. C. Reich, STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin-β3. Proc. Natl. Acad. Sci. USA 102 (2005) 8150–8155.

[25] R. Muromoto, Y. Sekine, S. Imoto, O. Ikeda, T. Okayama, N. Sato, T. Matsuda, BART is essential for nuclear retention of STAT3. Int. Immunol. 20 (2008) 395-403.

Figure legends

Fig. 1. Molecular interactions between STAT3 and Y14

(A) Domain structure of STAT3 and GST-fused mutant fragments are schematically shown. (B) 293T cells ($1x10^7$ cells) were transfected with GST, GST-STAT3 (1-137), GST-STAT3 (138-319), GST-STAT3 (320-493), GST-STAT3 (494-750) and GST-STAT3 (1-750) (10 μg) together with Myc-tagged Y14 (10 μg). At 48 h after transfection, the cells were lysed, and pull down with glutathione-Sepharose beads (GSH bound), followed by immunoblotting with anti-Myc antibody. TCL (1%) was blotted with anti-Myc antibody. (C) 293T cells in a 6 cm dish were transfected with Myc-STAT3 WT (WT) or STAT3 YF (YF) (5 μg) with Myc-Y14 (5 μg). At 48 h after transfection, cells were stimulated or unstimulated with LIF for 30 min. The cells were and lysed, immunoprecipitated with anti-STAT3, and blotted with anti-Myc antibody. TCL (1%) was blotted with anti-Myc or anti-pSTAT3 (Tyr705) antibody. (D) Hep3B cells were treated with or without IL-6 (10 ng/ml) for 30 min, and fixed with 4% paraformaldehyde and reacted with mouse anti-Y14 monoclonal antibody and rabbit

anti-STAT3 antibody, and visualized with FITC- or rhodamine-conjugated secondary antibodies. These figures were merged. The same slide was also stained with DAPI for the nuclei staining.

Fig. 2. Y14 is involved in tyrosine-phosphorylation and nuclear accumulation of STAT3

(A) Hep3B cells in a 24-well plate were treated with control or Y14 siRNA, and cells were stimulated with IL-6 (10 ng/ml) for the indicated periods. The cells were lysed, and an aliquot of TCL was blotted with anti-pSTAT3 (Tyr705), anti-STAT3, anti-Actin or anti-Y14 antibody. (B) Densitometric quantification of the above results was also shown. Relative intensity of pSTAT3 (Tyr705) was normalized to the STAT3 protein of the same sample. (C) Hep3B cells in a 6-well plate were treated with control or Y14 siRNA, and cells were stimulated with IL-6 (10 ng/ml) for the indicated periods, and fixed with 4% paraformaldehyde and reacted with anti-Y14 and anti-STAT3 antibody, and visualized with FITC- or rhodamine-conjugated secondary antibodies. These figures were merged. The same slide was also stained with DAPI for the nuclei staining. The representative data (0, 30 and 90 min) are shown. The dashed white lines mark Y14

knockdown Hep3B cells. In approximately 70 % of Y14 siRNA-treated Hep3B cells, Y14 protein was knocked-down by confirming with immunostaining. (D) Quantitative analysis of the subcellular localization of STAT3. Approximately one hundred of control or Y14 knocked-down Hep3B cells were classified according fluorescein signals in the cytoplasm, cytoplasm and nucleus (N/C) and nucleus.

Fig. 3. Y14 is involved in DNA binding activity of STAT3, and IL-6/STAT3-dependent gene expression

(A) Hep3B cells in a 6 cm dish were treated with control or Y14 siRNA, and cells were stimulated with IL-6 (10 ng/ml) for 30 min. To measure STAT3 DNA binding, cell extracts were treated with the immobilized STAT3 consensus or mutant oligonucleotide-Sepharose conjugate. The precipitates were subjected to Western blot analysis using anti-pSTAT3 antibody. An aliquot of TCL was blotted with anti-STAT3, anti-pSTAT3 anti-Y14 or anti-Actin antibody. (B) Densitometric quantification of the above results was also shown. Relative intensity of pSTAT3 (Tyr705) was normalized to the STAT3 protein of the total cell lysates. (C) Hep3B cells in a 24-well plate were treated with

control or KAP1 siRNA, and cells were stimulated with IL-6 (10 ng/ml) for the indicated periods. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using SOCS3, C/EBP, Y14 or G3PDH primers. (D) SOCS3 and C/EBP expression levels were also quantified by reverse transcription and quantitative real-time PCR analysis using the assay-on-demandTM gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system (Applied Biosystems). Data represent the levels of SOCS3 and C/EBP mRNA normalized to that of a G3PDH internal control and are expressed relative to the value of control siRNA-treated samples without IL-6-stimulation. Results are representative of three independent experiments, and the error bars represent the S.D.

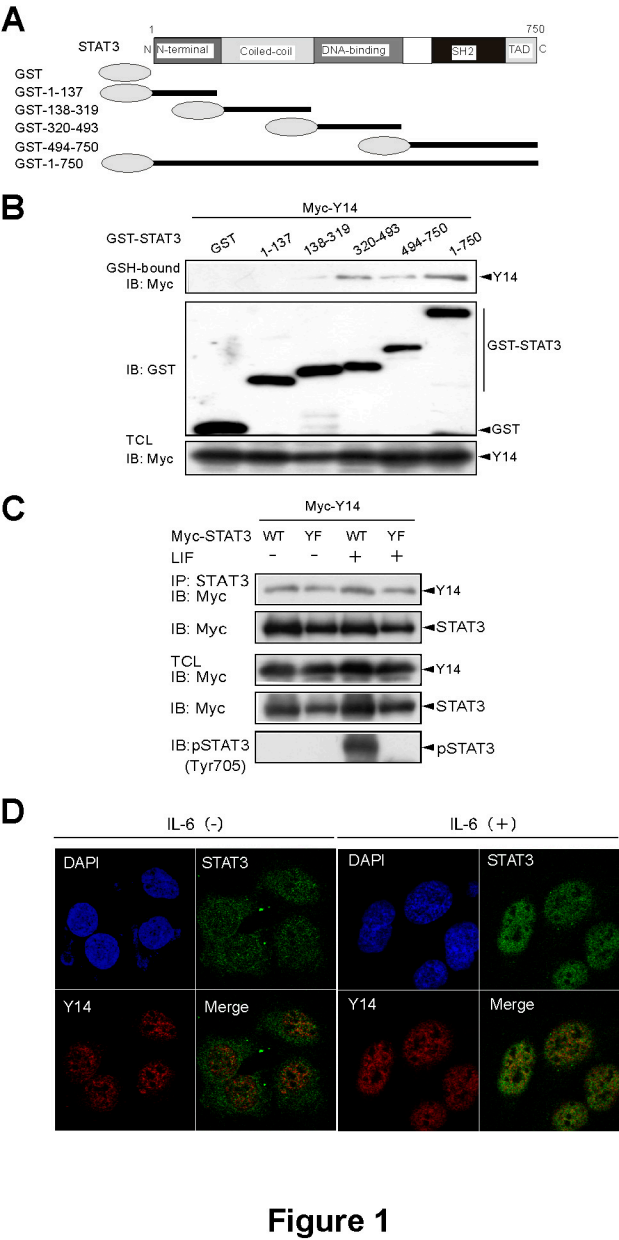
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Figure 1

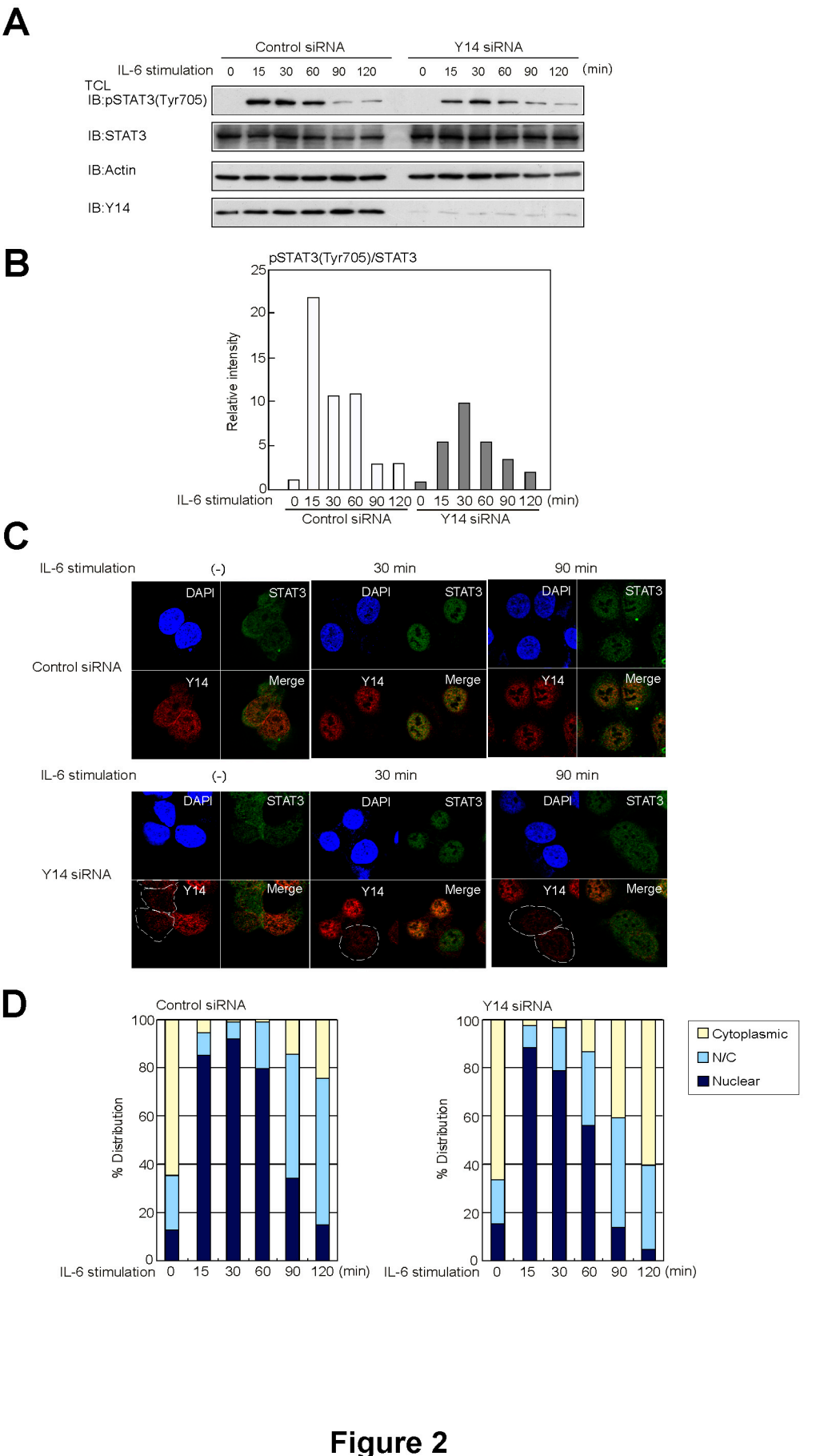
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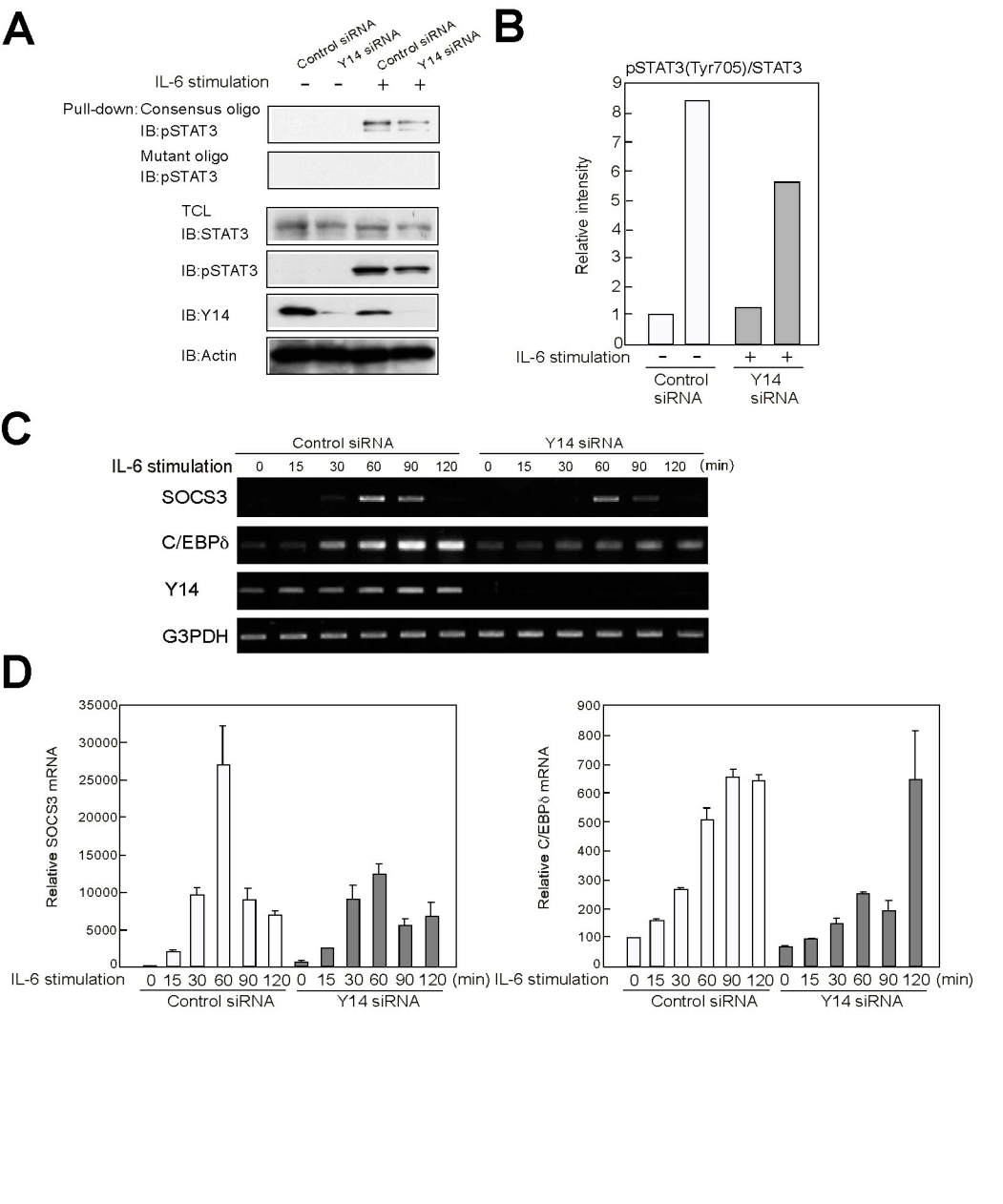
Figure 2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TCL
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IB:pSTAT3
IB:Y14
IB:Actin
B
pSTAT3(Tyr705)/STAT3
Relative intensity
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IL-6 stimulation
- - + +
Control siRNA
Y14 siRNA
C
Control siRNA
Y14 siRNA
IL-6 stimulation
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SOCS3
C/EBPδ
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G3PDH
D
Relative SOCS3 mRNA
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IL-6 stimulation
0 15 30 60 90 120 0 15 30 60 90 120 (min)
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Relative C/EBPδ mRNA
0 100 200 300 400 500 600 700 800 900
IL-6 stimulation
0 15 30 60 90 120 0 15 30 60 90 120 (min)
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Figure 3