Title: **Physical and functional interactions between Daxx and STAT3**

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Running title: Interaction between STAT3 and Daxx

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Abstract

Signal transducer and activator of transcription 3 (STAT3) plays key roles in the intracellular signaling pathways of the interleukin (IL)-6 family of cytokines, which exhibits a diverse set of cellular responses, including cell proliferation and differentiation. Dysregulated IL-6/STAT3 signaling is involved in the pathogenesis of several diseases, for examples autoimmune diseases and tumors. Type I interferon (IFN) induces the expression of proapoptotic genes and has been used in the clinical treatment of several tumors. In the present study, we found that type I IFN suppressed IL-6/STAT3-mediated transcription and gene expression. Furthermore, a type I IFN-induced protein, Daxx, also suppressed STAT3-mediated transcriptional activation, while overexpression of Daxx inhibited IL-6/STAT3-mediated gene expression. Importantly, small-interfering RNA-mediated reduction of Daxx expression enhanced IL-6/leukemia inhibitory factor (LIF)-induced STAT3-dependent transcription. Co-immunoprecipitation studies revealed a physical interaction between Daxx and STAT3 in transiently transfected 293T cells. We further found that Daxx and STAT3 were co-localized in the nucleus. These results indicate that Daxx may serve as a transcriptional regulator of type I IFN-mediated suppression of the IL-6/STAT3 signaling pathway.

Main Text

STAT3, a member of the STAT protein family, is mainly activated by the IL-6 family of cytokines, epidermal growth factor and leptin (Hirano *et al.*, 1997). Similar to other members of the STAT family, STAT3 is tyrosine-phosphorylated by Janus kinases (Jaks) and subsequently dimerizes and translocates into the nucleus to activate its target genes (Darnell *et al.*, 1994; Ihle, 1995). Constitutive activation or dysregulated expression of STAT3 has been identified in cancer cells and oncogene-transfected cells and also shown to be involved in a wide range of other diseases, such as autoimmune diseases (Bromberg, 2000; Levy and Lee, 2002).

Type I IFNs have been widely used in the treatment of human solid and hematologic malignancies. Type I IFN appears to block cell proliferation, at least in part, through the induction of apoptotic effects (Platanias, 2005). It has been also reported that IFN-□ treatment leads to STAT3 inactivation in human melanoma precursor lesions and in myeloma cell lines (Jelinek *et al.*, 1997; Kirkwood *et al.*, 1999). However, the molecular mechanisms how type I IFNs act on the suppression of IL-6/STAT3 signaling still remain unknown.

Our previous studies of *Tyk2*-deficient mice revealed that Tyk2 is essential for type I IFN-induced inhibition of B cell colony formation through the IFN-induced Daxx (Shimoda *et al.*, 2002), suggesting that Daxx is one of the key molecules, which mediate biological functions by type I IFNs. Daxx is also known to possess strong transcriptional repression activity (Ishov *et al.*, 1999; Li *et al.*, 2000a; Torii *et al.*, 1999; Zhong *et al.*, 2000). In addition, Daxx binds to the transcriptional activating factors such as Ets and Pax family proteins and inhibits their

transcriptional activities leading to the down-regulation of their target genes (Hollenbach *et al.*, 1999; Li *et al.*, 2000b). We also found that Daxx forms a functional complex composed of DNA methyltransferase 1, DNA methyltransferase 1 associated protein, and tumor susceptibility gene 101 (Muromoto *et al.*, 2004a; Muromoto *et al.*, 2004b).

In this study, we focused on an IFN-inducible protein, Daxx as a transcriptional regulator for STAT3 and demonstrated a novel functional link between IFN/Daxx- and IL-6/LIF/STAT3-mediated signaling.

To examine the molecular basis of the cross-talk between IL-6/STAT3 and IFN signaling pathways, we performed transient transfection assays using HeLa or Hep3B cells and measured the IL-6/STAT3-mediated transcriptional responses using STAT3-LUC, in which the □2-macroglobulin promoter drives expression of a luciferase (LUC) reporter gene (Nakajima *et al.*, 1996). Following transfection with STAT3-LUC, HeLa cells were pretreated with IFN for 14 h, and then stimulated with LIF or IL-6 for a further 12h. IFN pretreatment caused a decrease in the transcriptional activation of STAT3-LUC (Figure 1a). Similar results were obtained in Hep3B cells (Figure 1b). We also examined the effect of the IFN pretreatment on the STAT3-mediated endogenous gene expression of suppressor of cytokine signaling 3 (SOCS3). As shown in Figure 1c, IL-6-induced SOCS3 mRNA expression was suppressed by the pretreatment with IFN. These results suggest that IFN-mediated signaling negatively regulates IL-6/STAT3-mediated transcription and gene expression.

Previously, we and others have demonstrated that Daxx modulates transcriptional activity through direct interactions with transcription factors in the nucleus. Therefore, we examined the IFN-induced translocation of Daxx protein within nucleus in HeLa or Hep3B cells by confocal microscopy. As shown in Figure 1d, IFN treatment induced Daxx accumulation into small punctate structures within the nucleus in both of them. Next, we investigated whether Daxx affects the STAT3-mediated transcriptional activity. After transfection with STAT3-LUC with or without Daxx, 293T cells were treated with LIF and the LUC activities were determined. When cells were co-transfected with Daxx, the transcriptional activation of STAT3-LUC decreased in a dose-dependent manner (Figure 2a). In order to evaluate the possibility of a direct interaction between STAT3 and Daxx, we cotransfected 293T cells with STAT3-LUC and expression vectors for Daxx and/or STAT3-C, a constitutively active form of STAT3 (Bromberg *et al.*, 1999). STAT3-C induces STAT3-LUC without ligand stimulation in 293T cells. However, its STAT3-LUC activation requires a large amount of plasmids to transfect, since its main cellular localization is cytoplasm. As shown in Figure 2b, STAT3-LUC activation by STAT3-C was decreased by Daxx in a dose-dependent manner. These results indicate that Daxx suppresses STAT3-mediated transcriptional activity through a direct interaction. We further examined the effect of Daxx expression on the STAT3-mediated gene expression of endogenous SOCS3. An expression vector for Daxx or an empty vector was transfected into HeLa cells and stimulated with LIF. Aliquots of the total RNAs extracted from the transfected cells were subjected to RT-PCR analysis, which confirmed reduced mRNA expression of SOCS3. As shown in Figure 2c, IL-6-induced SOCS3 mRNA expression

was suppressed by Daxx expression.

To examine the effect of Daxx on the IL-6/STAT3-mediated signaling pathway under more physiological conditions, we established a stable Hep3B transfectant that expressed Daxx and designated it Hep3B/Daxx (Figure 2d, left panel). Since IL-6 is known to stimulate STAT3-mediated SOCS3 mRNA expression in Hep3B cells, we used RT-PCR to investigate the endogenous SOCS3 mRNA expression in Hep3B/Daxx cells after stimulation with IL-6. As shown in Figure 2d, IL-6-mediated endogenous SOCS3 mRNA expression was markedly decreased in Hep3B/Daxx cells, but not in parental Hep3B cells, suggesting that Daxx negatively regulates IL-6/STAT3-mediated transcription and gene expression.

To further explore whether Daxx represses STAT3-mediated transcriptional activation, we used small interfering RNA (siRNA) to reduce endogenous expression of Daxx in HeLa cells. A specific siRNA for Daxx or a control siRNA was transfected into HeLa cells, and aliquots of the cell lysates were analyzed by western blotting, which confirmed reduced expression of Daxx. Next, we determined the effects of these siRNAs on IL-6- or LIF-induced STAT3-LUC activation in HeLa cells. As shown in Figure 2e and f, siRNA-mediated reduced expression of Daxx resulted in a significant enhancement of IL-6- or LIF-induced STAT3-LUC activation and LIF-induced SOCS3 mRNA expression, strongly indicating that Daxx regulates IL-6/LIF/STAT3-mediated transcriptional activation and gene expression in HeLa cells.

One of the mechanisms consistent with the above-described data is a direct interaction between STAT3 and Daxx. Therefore, we tested this possibility by co-immunoprecipitation experiments. As

shown in Figure 3a, STAT3 interacted with Daxx in transient transfected 293T cells.

To delineate the domains in STAT3 that mediate the protein-protein interactions between STAT3 and Daxx, pull-down experiments were performed with a series of mutant GST-STAT3 proteins. As shown in Figure 3b, only the DNA-binding domain of STAT3 (320-493) showed a significant interaction with Daxx.

Next, we determined the Daxx domain involved in the interaction with the STAT3 DNA-binding domain. As shown in Figure 3c, the N-terminal domain of Daxx interacted with the DNA-binding domain of STAT3, while the central domain of Daxx showed weak binding to that of STAT3. These data suggest that the DNA-binding domain of STAT3 is required for the interaction with the N-terminal domain of Daxx.

To further characterize the nature of the interaction between STAT3 and Daxx, we attempted to determine where their interaction occurs in cells, by examining IL-6-induced translocation of STAT3 in Hep3B cells. While the cytoplasmic STAT3 did not co-localize with Daxx, STAT3 translocated into the nucleus and co-localized with Daxx in the nucleus after IL-6 stimulation (Figure 3d). Moreover, we could observe co-localization of endogenous Daxx and STAT3 in the nucleus (Figure 3e). Consistent with the data for their interaction in 293T cells presented above, these results suggest that activated STAT3 interacts with Daxx in the nucleus.

We have shown here that Daxx affects the transcriptional activation of STAT3 through a direct interaction. The transcriptional activation of STAT3 is regulated by four major steps, which are

tyrosine 705-phosphorylation, a dimer formation, nuclear translocation/retention and DNA binding activity. However, these were not affected by Daxx expression (data not shown). There may be another mechanism for Daxx-mediated suppression of STAT3 activity.

The direct interaction of Daxx with HDACs is proposed to be a mechanism for the transcriptional repression by Daxx (Hollenbach *et al.*, 2002). Previous studies have demonstrated that treatment with a HDAC inhibitor, Trichostatin A (TSA), efficiently reverses the repressive effect of Daxx (Kim *et al.*, 2003; Li *et al.*, 2000a), suggesting that HDACs may be involved in Daxx-mediated transcriptional repression. Indeed, TSA treatment relieved Daxx-mediated transcriptional repression of STAT3 in 293T cells, but not in HeLa cells (data not shown). These findings suggest that TSA-sensitive factors may be involved in Daxx-mediated suppression of STAT3 activity in 293T cells, but not in HeLa cells. Therefore, other factors except for HDACs may participate in Daxx-mediated suppression of STAT3 activity suppression in HeLa cells.

Type I IFN-inducible SOCS family proteins may be also involved in suppression of IL-6/LIF-mediated STAT3 activation. IFN-□ is shown to induce SOCS proteins in human T cells and melanoma cells (Brender *et al.*, 2001; Li *et al.*, 2004). Indeed, expression of both Daxx and SOCS1 in 293T cells inhibited LIF-induced STAT3 activation more efficiently than Daxx alone (data not shown).

In the present study, we have shown that type I IFN interferes with IL-6/STAT3-mediated transcription and gene expression through a protein-protein interaction with Daxx. These results strongly suggest that Daxx plays important roles in the cross-talk between IFNs and IL-6 family

cytokines by interacting with STAT3. The identification of Daxx as an effector of IFN responses in IL-6/STAT3-mediated signaling provides insights toward the development of a novel therapeutic strategy for STAT3-mediated malignancies and autoimmune diseases.

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Titles and legends for Figure

Figure 1. *IFN-signal suppresses the IL-6/STAT3-mediated signaling*

a. Human cervix carcinoma cell line, HeLa, was maintained in DMEM containing 10 % FCS. HeLa cells in a 12-well plate were transfected with STAT3-LUC (0.4 μg) using jetPEI (PolyPlus-transfection) according to the manufacturer's instructions. The cells were treated with IFN-β (150 U/ml) for 14 h and followed by stimulation for 12 h with LIF (INTERGEN) (100 ng/ml). The cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). At least three independent experiments were carried out for each assay.

b. Human hepatoma cell line Hep3B were maintained in DMEM containing 10 % FCS. Hep3B cells in a 12-well plate were transfected with STAT3-LUC (0.4 μg) as described the above. The cells were treated with IFN-β (150 U/ml) for 14 hrs and followed by stimulation for 12 hrs with IL-6 (a kind gift from Ajinomoto Co., 10 ng/ml). The cells were harvested and assayed as described the above.

c. HeLa cells in a 6-well plate were treated with IFN-β (150 U/ml) for 14 h and followed by stimulation 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 and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) primers as described previously (Shimoda *et al.*, 2002). RT-PCR products were separated on a 1% agarose gel.

d. HeLa or Hep3B cells were treated or untreated with IFN-β (a gift from Sumitomo Pharmaceuticals, 150 U/ml) for 12 hrs. The cells were fixed with a solution containing 4% paraformaldehyde and

reacted with rabbit anti-Daxx antibody (Santa Cruz) as described previously (Muromoto *et al.*, 2004a). At the same time, the nuclei in the cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) (Wako).

Figure 2. *Daxx suppresses the STAT3-mediated gene expression*

a. Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected 293T cells in a 12-well plate were transfected with STAT3-LUC (0.2 μg) and/or indicated amounts (5–50 ng) of empty vector or expression vector for Daxx. Twenty-four hrs after transfection, the cells were stimulated with LIF (100 ng/ml) for additional 12 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.

b. 293T cells in a 12-well plate were transfected with STAT3-LUC (0.2 μg) and/or indicated amounts (5–50 ng) of empty vector, expression vector for Daxx together with or without STAT-C (0.2 μg). At 36 h after transfection, the cells were harvested, and luciferase activities were measured as described the above.

c. HeLa cells in a 12-well plate were transfected with empty vector or expression vector for Daxx (2 μg). At 36 h after transfection, the cells were stimulated with LIF (100 ng/ml) for the indicated periods.

Total RNA samples isolated from these cells were subjected to RT-PCR analysis as described the above.

d. A stable Hep3B transfectant expressing Daxx was established as described previously (Muromoto *et al.*, 2004a). Transfectants were screened for protein expression of Daxx or actin by western blot with anti-Daxx or anti-actin antibody (Chemicon). Hep3B or Hep3B/Daxx cells in a 12-well plate were stimulated with IL-6 (100 ng/ml) for the indicated periods. Total RNA samples isolated from these cells were subjected to RT-PCR analysis as described the above.

e. Human Daxx-specific stealth siRNA or control scrambled siRNA used in this study were purchased from Invitrogen. The sense strand sequences of the oligonucleotides are as follows: hDaxx siRNA, 5'-GGAGCAGGCAUGGUCUCUUCUACUU-3', and control siRNA, 5'-GGACGGAGGUACUCUUCUUACGCUU-3'. HeLa cells were transfected with siRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After trasnfection, the cells were then washed twice with DMEM containing 10% FCS and then transfected with STAT3-LUC using Jet-PEI (Polytransfection, Illkirch, France). HeLa cells were transfected with control siRNA or Daxx siRNA and cells were analyzed by immunoblotting using anti-Daxx or anti-STAT3 antibody (Santa Cruz), verifying siRNA-mediated reduction in endogenous Daxx. At 24 h after transfection, the cells were treated with IL-6 (10 ng/ml) or LIF (30 ng/ml) for an additional 12 hrs. The cells were then harvested, and luciferase activities were measured. f. HeLa cells were treated with control siRNA or Daxx siRNA

as described the above and cells were stimulated with LIF (100 ng/ml) for the indicated periods. Total RNA samples isolated from these cells were subjected to RT-PCR analysis as described the above.

Figure 3. *Daxx physically interact with STAT3 in vivo and co-localization of Daxx with STAT3 in the nucleus*

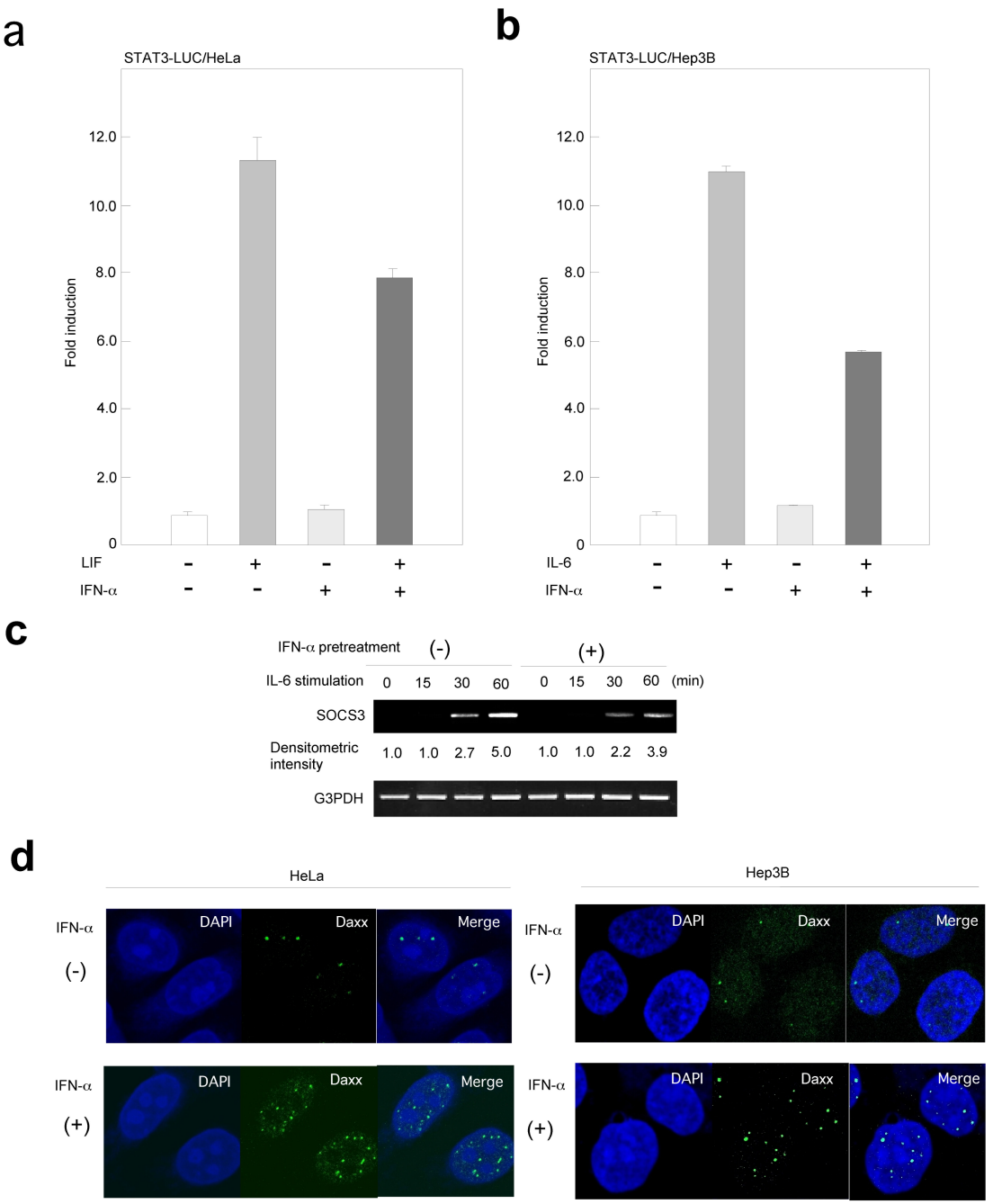
a. 293T cells ($1x10^7$ cells) were transfected with Myc-tagged STAT3 (7.5 □g) together with or without FLAG-Daxx (10 □g). At 48 h after transfection, the cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 □M sodium orthovanadate and 1 □M phenylmethylsulfonyl), and immunoprecipitated with anti-FLAG antibody (Sigma) and blotted with anti-Myc (Santa Cruz) (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc (bottom panel).

b. Domain structure of STAT3 and GST-fused mutant fragments are schematically shown. 293T cells ($1x10^7$ cells) were transfected with GST, GST-STAT3 (1-137), GST-STAT3 (138-319), GST-STAT3 (320-493) and GST-STAT3 (494-750) (10 □g) together with or without FLAG-tagged Daxx (10 □g) . At 48h after transfection, the cells were lysed, and pull down with glutathione-Sepharose beads (Amersham)(GSH bound), followed by immunoblotting with anti-FLAG (upper) or anti-GST antibody (Santa Cruz)(middle). Total cell lysates (1%) were blotted with anti-FLAG antibody (lowers) to monitor the expression of Daxx.

c. Domain structure of Daxx and mutant fragments are schematically shown. 293T cells ($1x10^7$) were transfected with a series of Myc-tagged Daxx mutants (10 μg) and GST-STAT3 (30-493)(10 μg). At 48 h after transfection, cells were lysed and immunoprecipitated with an anti-FLAG antibody, and pull down with glutathione-Sepharose beads (GSH bound), followed by immunoblotting with anti-Myc (upper) or anti-GST antibody (middle). Total cell lysates (1%) were blotted with anti-Myc antibody (lower panel). The asterisks indicate the migration positions of Daxx mutants.

d. Hep3B cells were transfected with Myc-tagged STAT3 (2 μg) and FLAG-tagged Daxx (1 μg). Thirty minutes after stimulation with IL-6 (100 ng/ml), the cells were fixed with 4% paraformaldehyde and reacted with rabbit anti-Daxx antibody and mouse anti-Myc antibody as described the above.

e. Hep3B cells were stimulated with IL-6 (100 ng/ml) for 30 min, the cells were fixed with 4% paraformaldehyde and reacted with rabbit anti-Daxx antibody and mouse anti-pSTAT3 (Tyr705) antibody (Cell signaling Technologies) as described the above. The arrows point the merge images.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STAT3-LUC/Hep3B
Fold induction
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IFN-α
c
IFN-α pretreatment
(-)
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SOCS3
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(-)
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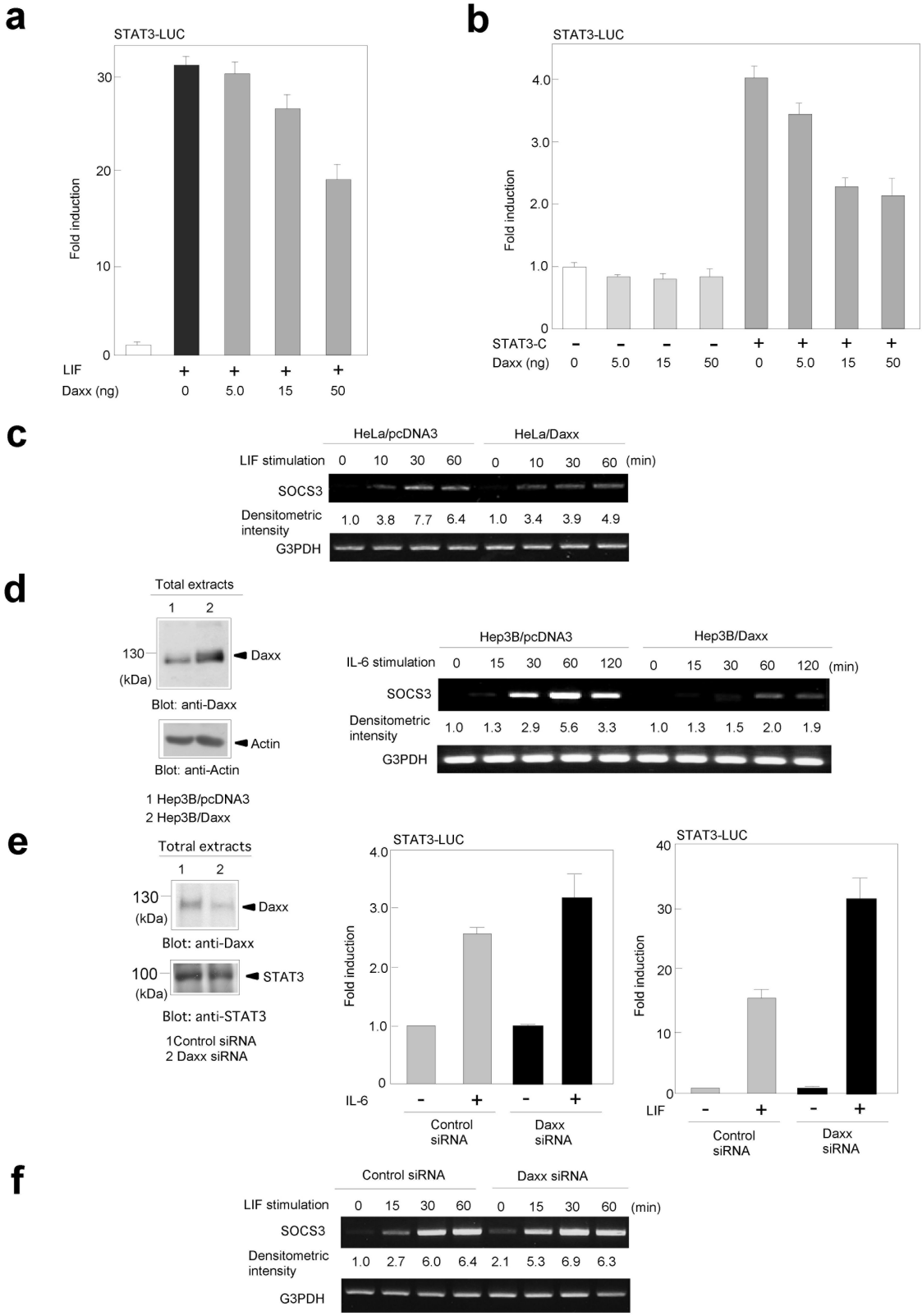
Fig. 1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HeLa/Daxx
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SOCS3
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d
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Blot: anti-Actin
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2 Hep3B/Daxx
Hep3B/pcDNA3
Hep3B/Daxx
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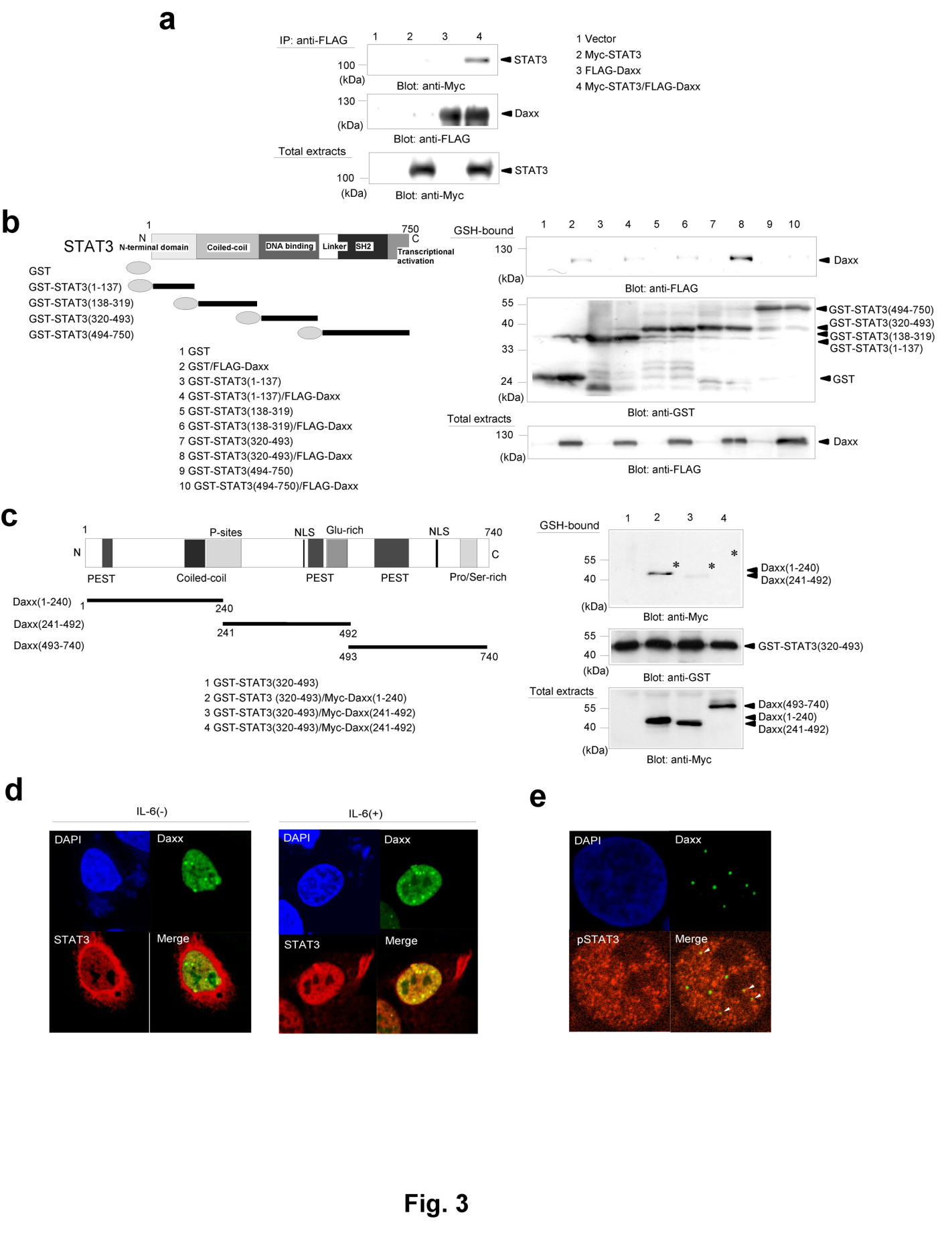
f

Fig. 2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130
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STAT3
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2 Myc-STAT3
3 FLAG-Daxx
4 Myc-STAT3/FLAG-Daxx
b
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N
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C
N-terminal domain
Coiled-coil
DNA binding
Linker
SH2
Transcriptional activation
GST
GST-STAT3(1-137)
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GST-STAT3(320-493)
GST-STAT3(494-750)
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2 GST/FLAG-Daxx
3 GST-STAT3(1-137)
4 GST-STAT3(1-137)/FLAG-Daxx
5 GST-STAT3(138-319)
6 GST-STAT3(138-319)/FLAG-Daxx
7 GST-STAT3(320-493)
8 GST-STAT3(320-493)/FLAG-Daxx
9 GST-STAT3(494-750)
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GSH-bound
1 2 3 4 5 6 7 8 9 10
130
(kDa)
Daxx
Blot: anti-FLAG
55
40
33
24
(kDa)
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GST-STAT3(320-493)
GST-STAT3(138-319)
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GST
Blot: anti-GST
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(kDa)
Daxx
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c
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N
P-sites
NLS
Glu-rich
NLS
740
C
PEST
Coiled-coil
PEST
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Pro/Ser-rich
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Daxx(493-740)
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740
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3 GST-STAT3(320-493)/Myc-Daxx(241-492)
4 GST-STAT3(320-493)/Myc-Daxx(241-492)
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(kDa)
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IL-6(-)
IL-6(+)
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Daxx
STAT3
Merge
DAPI
Daxx
STAT3
Merge
e
DAPI
Daxx
pSTAT3
Merge

Fig. 3