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Title: Physical and functional interactions between STAT3 and KAP1

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Abstract

Signal transducers and activators of transcription (STATs) mediate cell proliferation, differentiation, and survival in immune responses, hematopoiesis, neurogenesis, and other biological processes. For example, STAT3 has been reported to be constitutively activated in numerous cancer cells. To clarify the molecular mechanisms underlying the STAT activation, we performed yeast two-hybrid screening and identified KAP1/TIF1□ as a novel STAT-binding partner. KAP1 is a universal corepressor protein for the KRAB zinc finger protein superfamily of transcriptional repressors. We found endogenous KAP1 associated with endogenous STAT3 in vivo. Importantly, small-interfering RNA-mediated reduction of KAP1 expression enhanced IL-6-induced STAT3-dependent transcription and gene expression. Furthermore, reduction of KAP1 expression resulted in the marked nuclear accumulation of STAT3 phosphorylated on Ser727 in the nucleus, a modification that regulates its transcriptional activation. These results indicate that KAP1 may serve as a transcriptional regulator of the IL-6/STAT3 signaling pathway.

Main Text

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 characteristics of cytoplasmic signaling molecules, such as a Src-homology 2 (SH2) domain and tyrosine phosphorylation sites. Upon tyrosine phosphorylation, the STATs dimerize through their phosphorylated SH2 domains and translocate to the nucleus (Darnell et al., 1994; Ihle, 1996; O'Shea, 1997). In recent years, constitutive or dysregulated expression of STATs has been found in cancer cells and oncogene-transfected cells and shown to be involved in a wide range of diseases (Levy et al., 2002). Therefore, STAT activation is tightly regulated by a variety of mechanisms. The protein inhibitor of activated STAT (PIAS) family of proteins decreases STAT-dependent transcription by blocking STAT-DNA binding in the nucleus (Shuai et al., 2003). Suppressor of cytokine signaling (SOCS) proteins are induced by STATs and play roles in the negative feedback of STAT activation (Yasukawa et al., 2000). Cytoplasmic tyrosine phosphatases, such as SH2-containing phosphatase 1 (SHP1), SHP2 and protein-tyrosine phosphatase 1B (PTP1B), also prevent further STAT activation in the cytoplasm (Shuai et al., 2003; Yasukawa et al., 2000). Nuclear tyrosine phosphatases, such as TC45, dephosphorylate nuclear STATs, thereby allowing them to return to the cytoplasm (Shuai et al., 2003).

KAP1/TIF1 β was initially identified as a co-repressor for the Kruppel-associated box (KRAB)-domain-containing zinc finger proteins (Kim et al., 1996; Friedman et al., 1996; Agata et al., 1999). KAP1 can recruit and coordinate many components involved in gene silencing. KAP1-mediated gene silencing involves the recruitment of the histone deacetylase (HDAC) complex (Underhill et al., 2000; Schultz et al., 2001; Satou et al., 2001), and binding to a histone methyltransferase (Schultz et al., 2002). Therefore, KAP1 orchestrates the function of these co-repressor complexes to inhibit the transcription of its target genes. In this study, we focused on KAP1 as a novel transcriptional regulator of STAT3 and demonstrated a functional link between KAP1 and IL-6/STAT3-mediated signaling.

We performed a yeast two-hybrid screen of a mouse embryo cDNA library using the C-terminal region of STAT4 (amino acids 483-748) as bait. From a screen of about 2.6×10^6 transformants, we identified several positive clones. Sequence analysis revealed that one of them encoded the C-terminal region of KAP1 (amino acids 394-835). We examined the distribution of KAP1 in a variety of human cancer cell lines by Western blot analysis and found it was ubiquitously expressed as described previously (data not shown; Underhill et al., 2000). We first examined whether KAP1 binds STAT4 and/or other STATs in mammalian cells. 293T cells were transfected with a series of FLAG-tagged STAT expression vectors together with Myc-tagged KAP1 (394-835). Western blot analysis of the immunoprecipitates with an anti-Myc antibody revealed that KAP1 interacts with STAT1, STAT3, STAT4 and STAT6, but not with STAT2, STAT5a or STAT5b, in

293T cells (Figure 1a). These results indicate that KAP1 has binding potential for several STAT proteins. STAT3 and STAT4 strongly interacted with KAP1. Although STAT3 and STAT4 are more closely related to each other than to any other STATs, STAT4 expression is restricted to the testis, thymus, and spleen, while STAT3 is ubiquitously expressed (Zhong et al., 1994). These results suggested that the STAT3-KAP1 interaction may have a wider range of biological functions than the STAT4-KAP1 interaction. Therefore, we focused on the functional association between KAP1 and STAT3.

To confirm that endogenous KAP1 interacts with STAT3 *in vivo*, co-immunoprecipitation experiments were performed using cell extracts obtained from Hep3B cells, in which both proteins could be detected using specific antibodies. An anti-KAP1 antibody immunoprecipitated STAT3 when Hep3B cells were stimulated or unstimulated with IL-6, indicating that the binding of KAP1 to unphosphorylated STAT3 occurs at physiological expression levels (Figure 1b).

We further confirmed the involvement of STAT3 phosphorylation state on their interactions. Expression vectors encoding HA-tagged KAP1 and Myc-tagged STAT3 or STAT3 YF mutant were transiently transfected into 293T cells. STAT3 YF failed to be phosphorylated at Tyr705 and dimerized. The transfected 293T cells were lysed and subjected to immunoprecipitation with anti-HA antibody. Immunoprecipitates were then used in Western blot analysis with anti-Myc antibody. As shown in Figure 1c, STAT3 YF showed much stronger binding potential to KAP1 than STAT3 WT, indicating that unphosphorylated monomer STAT3 prefers to bind to KAP1.

Similarly, we tested the interactions between KAP1 and STAT3 SA mutant (a substitution of Ser727 with Ala). Interestingly, STAT3 SA showed a very weak binding potential to KAP1 (Figure 1d), suggesting that STAT3 Ser 727 plays a critical role in the interaction between KAP1 and STAT3.

Next, to delineate the regions of STAT3 involved in the KAP1-STAT3 interaction, various deletion constructs of GST-fused STAT3 (Figure 1e) were subjected to pull-down assays in 293T cells. As shown in Figure 1f, the coiled-coil and the DNA-binding domain of STAT3 interacted strongly with KAP1. We also examined whether KAP1 colocalizes with STAT3 in Hep3B cells. Myc-tagged STAT3 was transiently expressed in Hep3B cells. Unphosphorylated STAT3 has been shown to continuously shuttle between nuclear and cytoplasmic compartments (Liu et al., 2005). Endogenous KAP1 was present within the nucleus without stimulation. As shown in Figure 1g, STAT3 was colocalized with KAP1 in the nucleus without stimulation. These results suggest that nuclear unphosphorylated STAT3 predominantly binds to KAP1.

To clarify the physiological significance of the molecular interactions between STAT3 and KAP1, we examined the effect of KAP1 on STAT3 activity. To explore whether KAP1 affects STAT3-mediated transcriptional activation, we used small interfering RNA (siRNA) to reduce the endogenous expression of KAP1 in Hep3B cells. A specific siRNA for KAP1 or a control siRNA was transfected into Hep3B cells, and an aliquot of cell lysates were analyzed by Western blotting, which confirmed reduced expression of KAP1 (Figure 2 b). First, we determined the effects of

these siRNAs on IL-6/STAT3-mediated transcriptional activation in Hep3B cells. The STAT3-mediated transcriptional responses were measured by using STAT3-LUC, in which the $\alpha 2$ -macroglobulin promoter drives expression of the LUC gene. As shown in Figure 2a, siRNA-mediated reduced expression of KAP1 resulted in a significant enhancement of IL-6-induced STAT3-LUC activation. Furthermore, IL-6-induced SOCS3 and C/EBP β mRNA expressions were markedly enhanced (Figure 2b and c), strongly indicating that KAP1 regulates IL-6/STAT3-mediated transcriptional activation and gene expression in Hep3B cells. We also examined c-myc and Mcl-1 mRNA expression in Hep3B cells. As shown in Figure 2d, IL-6-induced c-myc and Mcl-1 mRNA expressions were also enhanced in KAP1 siRNA-treated Hep3B cells. However, both mRNA expressions were up-regulated by KAP1 siRNA treatment even in the absence of IL-6 stimulation. These results indicate that KAP1 effectively influences the basal level of STAT3 transcriptional activity in some STAT3 target genes. We further examined the effects of KAP1 on IL-10/STAT3-mediated inhibition of LPS-induced macrophage activation through SOCS3 expression in murine macrophage Raw264.7 cells. As shown in Figure 2e and f, LPS plus IL-10 induced SOCS3 mRNA expression in Raw264.7 cells to suppress macrophage activation. Importantly, an enhanced SOCS3 mRNA expression was observed in KAP1 siRNA-treated Raw264.7 cells, indicating that KAP1 participates in the regulation of LPS-induced macrophage activation.

We next examined whether KAP1 affects other STAT-mediated transcriptional activation. We then used the IFN-stimulated responsive element (ISRE)-LUC to monitor IFN/STAT1-mediated

transcriptional activation in HeLa cells. As shown in Figure 2g, siRNA-mediated reduced expression of KAP1 resulted in a significant enhancement of IFN- α -induced ISRE-LUC activation. This result suggests that KAP1 is also involved in the regulation of STAT1 activation.

To further understand the molecular mechanisms responsible for KAP1-mediated repression of STAT3 activation, we examined whether reduction of KAP1 expression affects the nuclear translocation and DNA binding activity of STAT3, however, we observed no significant effect on these functions owing to reduction of KAP1 expression in Hep3B cells (Figure 3a and b). We further examined whether reduction of KAP1 expression affects phosphorylation of STAT3. As shown in Figure 3c and d, we detected IL-6-induced tyrosine (Tyr705)- and serine (Ser727)-phosphorylation of STAT3 in control siRNA- and KAP1 siRNA-treated Hep3B cells. Importantly, serine-phosphorylation but not tyrosine-phosphorylation, of STAT3, was increased by a reduction in KAP1 expression in Hep3B cells. Coincident with the above results, reduction of KAP1 expression resulted in enhanced accumulation of STAT3 phosphorylated on Ser727 in the nucleus (4-fold), with a dot-like structure (Figure 3e and f). These results suggest that KAP1 may trap STAT3 through STAT3 Ser727 to repress STAT3 transcriptional activation, because STAT3 SA mutant fails to interact with KAP1 as shown in Figure 1d. A direct interaction of KAP1 with HDACs is also proposed to be a mechanism for the transcriptional repression by KAP1 (Underhill et al., 2000; Schultz et al., 2001; Satou et al., 2001). STAT3 has been also demonstrated to that STAT3 associates with HDAC3 and trichostatin A, an HDAC inhibitor, restores its transcriptional activity (Yuan et al., 2005). We tested the effects of KAP1 on interactions between STAT3 and

HDAC3. Expression of KAP1 significantly enhanced interactions between HDAC3 and STAT3, (Figure 3g), although expression of HDAC3 had no effect on Tyr705- or Ser727-phosphorylation of STAT3 (data not shown). We further confirmed the effects of KAP1 on acetylation status of STAT3. As shown in Figure 3h, acetylation of STAT3 was markedly enhanced in KAP1 siRNA-treated Hep3B cells. Thus, KAP1 effectively bridges STAT3 and HDAC3, suggesting that HDACs may be involved in KAP1-mediated transcriptional repression. We also examined the effects of KAP1 on interaction STAT3 and PIAS3. Expression of KAP1 had no effect of their interactions (Figure 3i).

In the present study, we have shown that KAP1 regulates IL-6/STAT3-mediated transcription and gene expression via interacting with STAT3. Phosphorylation of STAT3 on Ser727 was selectively modulated by KAP1. STAT3 Ser727 played an important role in the interactions between STAT3 and KAP1. Phosphorylation of STAT3 on Ser727 is required for the maximal transcriptional activation of STAT3 (Wen et al., 1995), and is also shown to increase STAT3 activity through association with other cofactors, such as p300 (Schuringa et al., 2001). Therefore, KAP1 may compete with p300 at STAT3 Ser727. Although these results also indicate that KAP1 may recruit protein phosphatases for phosphorylated STAT3 on Ser727 in the nucleus, we did not succeed in co-precipitating of protein phosphatases with KAP1 in the present study. Alternatively, as shown in Figure 3e and f, STAT3 phosphorylated on Ser727 localized to dot-like structures in the nucleus and accumulated there in the absence of KAP1. Recently, Harrmann et

al reported that activated STAT3 is enriched in dot-like structures within the nucleus (Herrmann et al., 2004). The STAT3-related dot-like structures are accompanied with CREB binding protein and are believed to act as reservoirs of activated STAT3. Thus, KAP1 may play a role in interfering movement of STAT3 phosphorylated on Ser727 into STAT3-related dot-like structures, resulting in early dephosphorylation of STAT3. This hypothesis might be partly supported by the fact that KAP1 is rarely detected in STAT3-related dot-like structures (data not shown). Because KAP1 can bridge STAT3 and HDACs strongly, these interactions may suppress formation of STAT3-related dot-like structures. Further detailed work will be required to clarify the molecular mechanisms of KAP1-mediated modification of Ser727 phosphorylation status in STAT3 and will provide 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. KAP1 physically interact with STAT3 in vivo.

a. Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS. 293T cells (5×10^6) were transfected with Myc-tagged KAP1(394-835) (5 μ g) and/or FLAG-tagged STAT1-6 (10 μ 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 μ M sodium orthovanadate and 1 μ M phenylmethylsulfonyl), and immunoprecipitated with anti-Myc antibody (Santa Cruz) and immunoblotted with anti-FLAG antibody or anti-Myc antibody. Total cell lysates (TCL) (1 %) were blotted with anti-FLAG antibody.

b. Human hepatoma cell line, Hep3B, was maintained in DMEM containing 10 % FCS. Hep3B cells (1×10^7) were treated with IL-6 (a kind gift from Ajinomoto Co., 20 ng/ml) for 30 min. The cells were lysed, and immunoprecipitated with control or anti-KAP1 (Bethyl Laboratories) antibody and immunoblotted with anti-STAT3 (Santa Cruz) or anti-KAP1 antibody. TCL (1 %) was blotted with anti-KAP1, anti-STAT3 or anti-pSTAT3 (Tyr705) (Cell signaling Technologies) antibody.

c. 293T cells (1×10^7) were transfected with Myc-STAT3 WT (WT) or STAT3 YF (YF) (10 μ g) with or without HA-KAP1 (10 μ g). At 48 h after transfection, cells were stimulated or unstimulated with LIF (INTERGEN, 50 ng/ml) for 30 min. The cells were lysed, immunoprecipitated with anti-HA, and blotted with anti-Myc or anti-HA antibody. TCL (1%) was blotted with anti-Myc or anti-pSTAT3 (Tyr705) antibody.

c. 293T cells (1×10^7) were

transfected with HA-STAT3 WT (WT) or STAT3 SA (SA) (10 μ g) with or without FLAG-KAP1 (10 μ g). At 48 h after transfection, cells were lysed, immunoprecipitated with anti-FLAG, and blotted with anti-HA or anti-FLAG antibody. TCL (1%) was blotted with anti-HA or anti-pSTAT3 (Ser727) (Cell signaling Technologies) antibody. e. Domain structure of STAT3 and GST-fused mutant fragments are schematically shown. f. 293T cells (1×10^7 cells) were transfected with GST, GST-STAT3 (1-137), GST-STAT3 (138-319), GST-STAT3 (320-493) and GST-STAT3 (494-750) (10 μ g) together with or without HA-tagged human full-length KAP1 (a kind gift of Dr. H Ariga, Hokkaido Univ.) (10 μ g). At 48h after transfection, the cells were lysed, and pull down with glutathione-Sepharose beads (Amersham)(GSH bound), followed by immunoblotting with anti-HA (Santa Cruz) or anti-GST antibody (Santa Cruz). TCL (1%) was blotted with anti-HA antibody. g. Hep3B cells in a 6-well plate were transfected with Myc-tagged STAT3 (1 μ g). At 36h after transfection, cells were treated with or without IL-6 (20 ng/ml) for 30 min, and fixed with 4% paraformaldehyde and reacted with rabbit anti-KAP1 antibody and mouse anti-Myc antibody as described previously (Sekine et al., 2006).

Figure 2. KAP1 regulates the IL-6/STAT3-mediated transcription and gene expression in hepatoma cells.

a. Hep3B cells in a 24-well plate were transfected with control siRNA or siRNA targeting human KAP1 using Lipofectamine2000 (Invitrogen) as described previously (Sekine et al., 2006).

siRNA targeting human KAP1 used in this study were as follows: 5'-UGACCAAGAUCCAGAAGCATT-3'. The cells were then transfected with STAT3-LUC using jetPEI (PolyPlus-transfection). At 36 h after transfection, cells were treated with IL-6 (20 ng/ml) for an additional 8h. The cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D.

b. Hep3B cells were treated with control siRNA or KAP1 siRNA as described the above, and cells were stimulated with IL-6 (20 ng/ml) for the indicated periods. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using SOCS3, C/EBP β , KAP1 or G3PDH primers as described previously (Sekine et al., 2006). An aliquot of TCL was analyzed by immunoblotting using anti-KAP1 or anti-actin antibody (Chemicon).

c. SOCS3 and C/EBP β 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 β mRNA normalized to that of a G3PDH internal control and are expressed relative to the value at time zero. 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.

d. Hep3B cells were treated with control siRNA or KAP1 siRNA as described the above, and cells were stimulated with IL-6 (20 ng/ml) for the indicated periods. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using c-myc, Mcl-1, KAP1 or

G3PDH primers. e. Murine macrophage cell line, Raw267.4, was maintained in DMEM containing 10 % FCS. Raw264.7 cells in a 24-well plate were transfected with control siRNA or siRNA targeting murine KAP1 using Lipofectamine RNAiMAX (Invitrogen). siRNA targeting murine KAP1 used in this study were as follows: 5'-CCAAAGACAUCGUGGAGAATT-3'. An aliquot of TCL from these cells was analyzed by immunoblotting using anti-KAP1 or anti-actin antibody. At 36 h after transfection, cells were treated with IL-10 (Wako, 10 ng/ml), LPS (Sigma, 10 ng/ml), IL-10 plus LPS or left untreated for an additional 8 h. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using SOCS3, KAP1 or G3PDH primers. f. SOCS3 expression levels were also quantified by reverse transcription and quantitative real-time PCR analysis as described the above. g. Human cervix carcinoma cell line, HeLa, was maintained in DMEM containing 10 % FCS. HeLa cells in a 24-well plate were transfected with control siRNA or siRNA as described the above. An aliquot of TCL from these cells was also analyzed by immunoblotting using anti-KAP1 or anti-actin antibody. The cells were then transfected with ISRE-LUC (Clontech) using jetPEI. At 36 h after transfection, cells were treated with IFN- γ (a kind gift from Dainippon Sumitomo Pharma Co., 1000 U/ml) for an additional 12 h. The cells were harvested and assayed for the luciferase activity as described the above. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D.

Figure 3. Reduction of endogenous KAP1 enhances accumulation of STAT3 phosphorylated on

Ser727 in hepatoma cells.

a. Hep3B cells were treated with control siRNA or KAP1 siRNA as described the above and cells were stimulated with IL-6 (20 ng/ml) for 30 min. Nuclear translocation of STAT3 was estimated by quantitative analysis of the subcellular localization of STAT3 using indirect immunofluorescence microscopy as described the above. Approximately 100 cells were classified according fluorescein signals in the cytoplasm (white) and nucleus (grey). The results represent the means of three individual experiments, in which 100 cells were counted. b. Hep3B cells were treated with control siRNA or KAP1 siRNA as described the above, and cells were stimulated with IL-6 (20 ng/ml) for the indicated periods. To measure STAT3 DNA binding, cell extracts were treated with the immobilized STAT3 Consensus Oligonucleotide (Santa Cruz)-Sepharose conjugate as described previously (Sekine et al., 2007). The precipitates were subjected to Western blot analysis using anti-STAT3 antibody. An aliquot of TCL was blotted with anti-STAT3 or anti-KAP1 antibody. c. Hep3B cells were treated with control siRNA or KAP1 siRNA as described the above, and cells were stimulated with IL-6 (20 ng/ml) for the indicated periods. The cells were lysed, and an aliquot of TCL was blotted with anti-pSTAT3 (Tyr705), anti-pSTAT3 (Ser727), anti-STAT3 or anti-KAP1 antibody. d. Densitometric quantification of the above results was also shown. Relative intensity of pSTAT3 (Tyr705) or pSTAT3 (Ser727) was normalized to the STAT3 protein of the same sample. e. Hep3B cells were treated with control siRNA or KAP1 siRNA as described the above, and cells were stimulated with IL-6 (20 ng/ml) for 30 min, and fixed with 4% paraformaldehyde and reacted with anti-KAP1

antibody and anti-pSTAT3 (Ser727) as described the above. The dotted white circles mark a whole cell area.

f. Quantitative analysis of accumulation of phosphorylated STAT3 on Ser727 using indirect immunofluorescence microscopy as described the above. Approximately 100 cells were classified according the dot-like structure of anti-pSTAT3 (Ser727) staining in Hep3B cells treated with control siRNA or KAP1 siRNA as described the above.

g. 293T cells (1×10^7) were transfected with Myc-STAT3 (10 μ g) with or without FLAG-HDAC3 (a kind gift from Dr. E. Seto, H. Lee Moffitt Cancer Center and Research Institute) (10 μ g) and the increasing amount of HA-KAP1 (5, 10 μ g). At 48 h after transfection, cells were lysed, and immunoprecipitated with anti-FLAG, and blotted with anti-Myc, anti-HA or anti-FLAG antibody. TCL (1%) was blotted with anti-Myc, anti-HA or anti-FLAG antibody.

h. Hep3B cells were treated with control siRNA or KAP1 siRNA as described the above, and cells were stimulated with IL-6 (20 ng/ml) for 30min. The cells were lysed, and immunoprecipitated with anti-STAT3 antibody and immunoblotted with anti-AcSTAT3(Lys685) (Ohbayashi et al., 2007) or anti-STAT3 antibody. TCL (1 %) was blotted with anti-pSTAT3(Tyr705), anti-STAT3 or anti-KAP1 antibody.

i. 293T cells (1×10^7) were transfected with FLAG-STAT3 together with or without Myc-tagged KAP1(394-835)(5 μ g) and/or without Myc-PIAS3 (10 μ g). At 48 h after transfection, cells were lysed, and immunoprecipitated with anti-FLAG, and blotted with anti-Myc or anti-FLAG antibody. TCL (1%) was blotted with anti-Myc or anti-FLAG antibody.

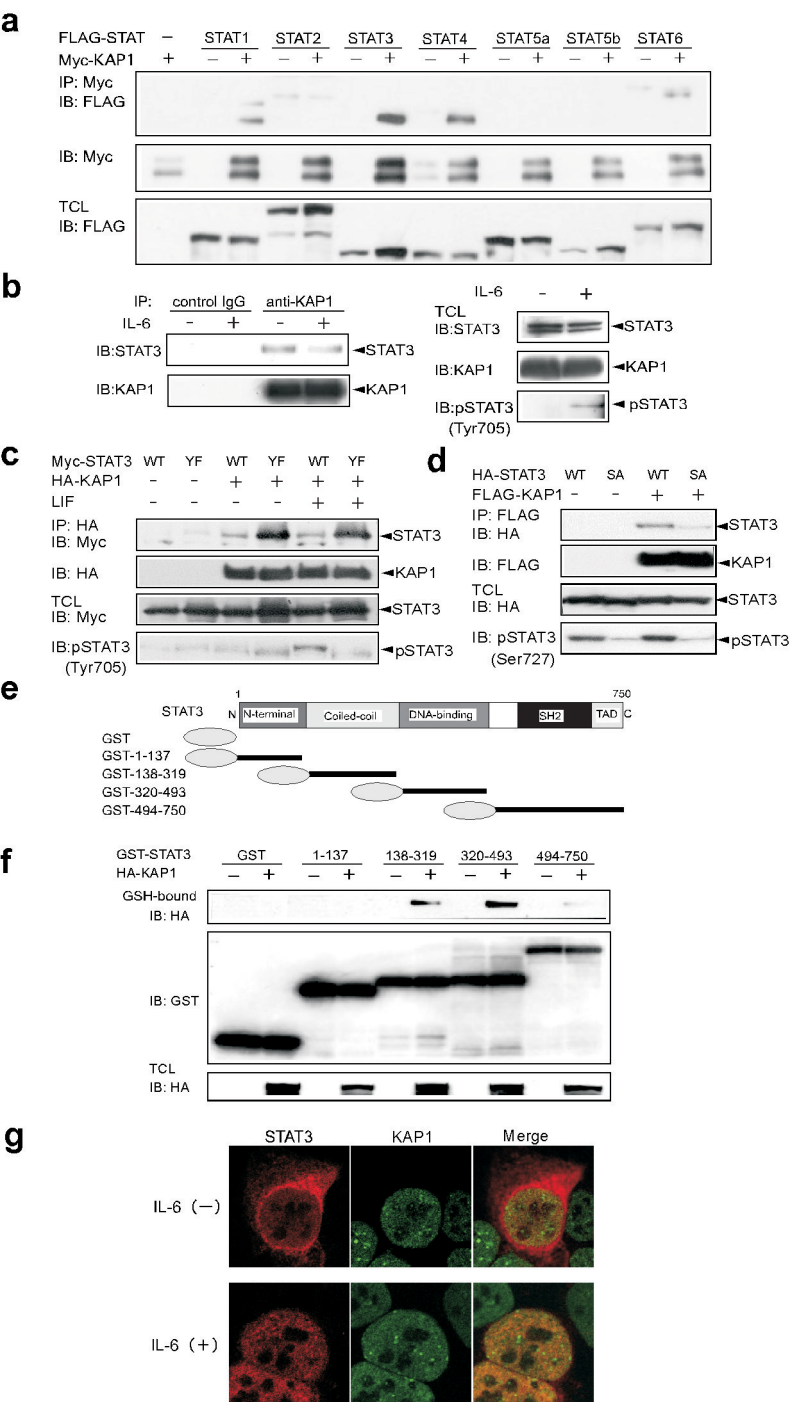


Figure 1

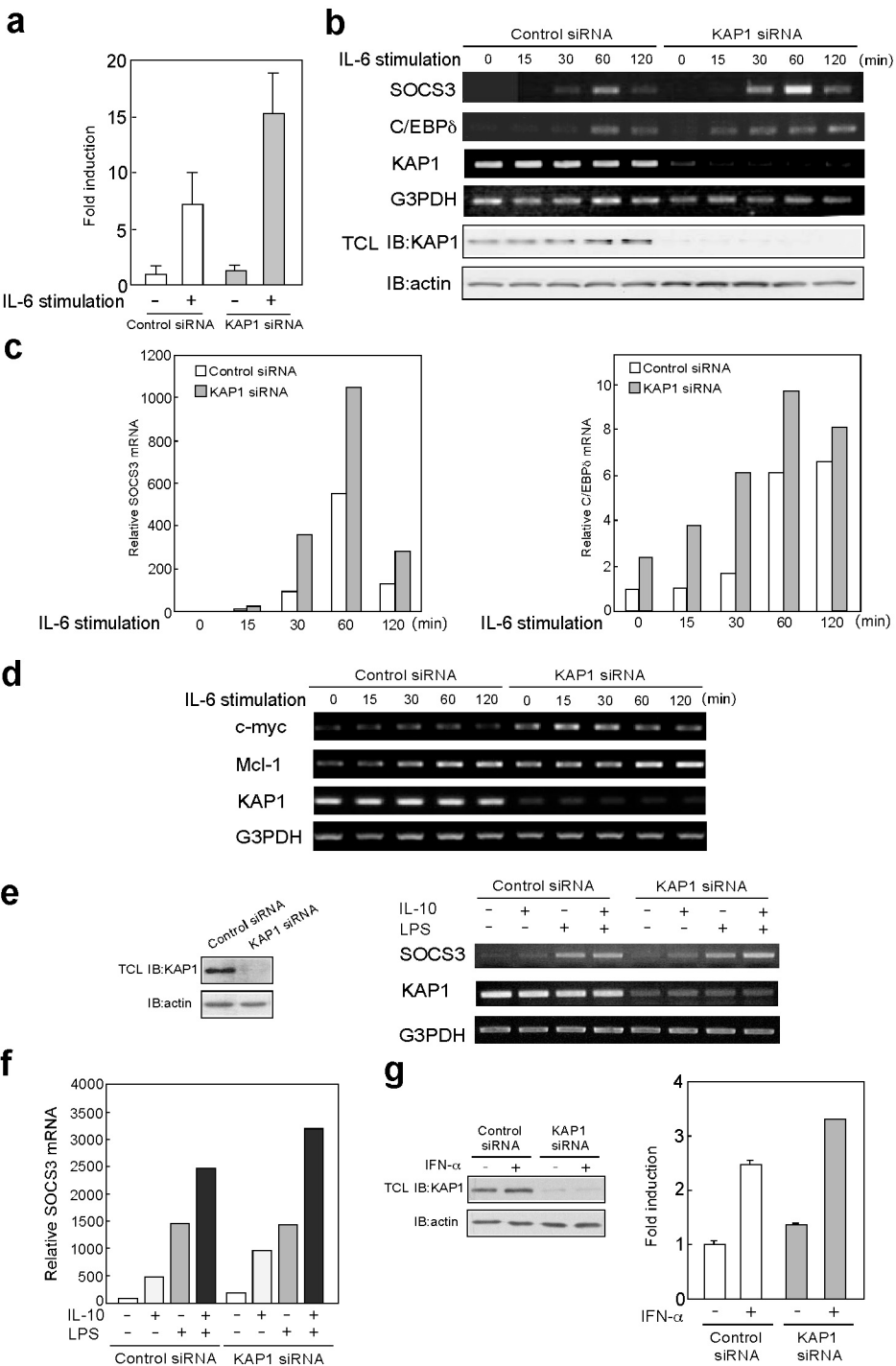


Figure 2

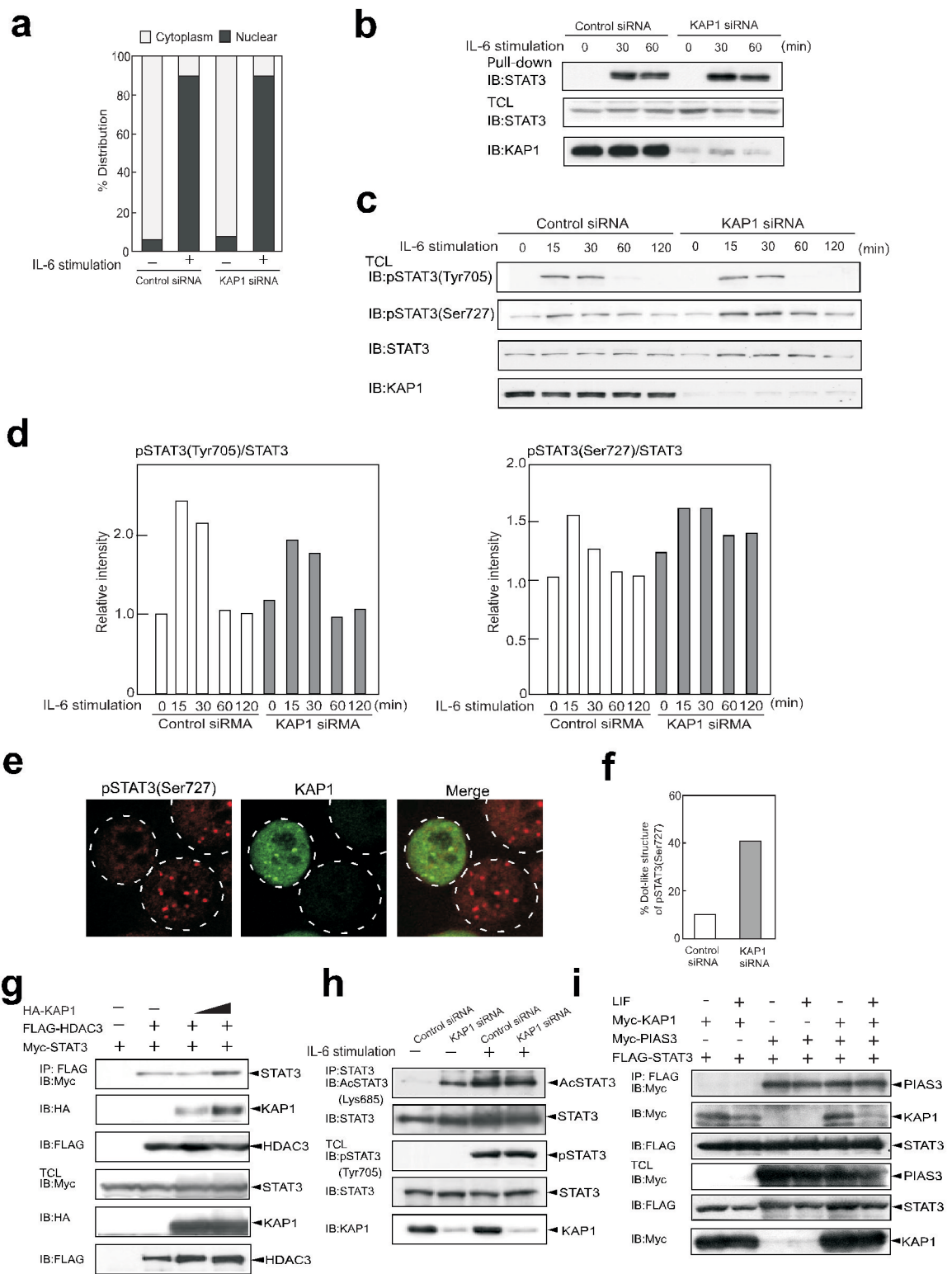


Figure 3