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Title: The IL-6 family of cytokines modulates STAT3 activation by desumoylation of PML through SENP1 induction

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Running title: IL-6 desumoylates PML via SENP1

Abstract

Post-translational modification by small ubiquitin-like modifier (SUMO) plays an important role in the regulation of different signaling pathways and is involved in the formation of promyelocytic leukemia (PML) protein nuclear bodies following sumoylation of PML. In the present study, we found that IL-6 induces desumoylation of PML and dissociation between PML and SUMO1 in hepatoma cells. We also found that IL-6 induces mRNA expression of SENP1, a member of the SUMO-specific protease family. Furthermore, wild-type SENP1 but not an inactive SENP1 mutant restored the PML-mediated suppression of STAT3 activation. These results indicate that the IL-6 family of cytokines modulates STAT3 activation by desumoylation and inactivation PML through SENP1 induction.

Keywords: IL-6; LIF; SENP1; SUMO; STAT3; PML; transcriptional regulation

Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates immune and inflammatory responses, and which is implicated in the onset and maintenance of several diseases [1, 2]. The IL-6 receptor is a heterodimeric complex, consisting of an IL-6-specific ligand-binding subunit, the □ chain, and a signal-transducing subunit, gp130, which is shared by the receptors for ciliary neurotrophic factor, leukemia inhibitory factor (LIF), oncostatin M, and cardiotropin 1[3, 4]. The binding of IL-6 to the □ chain leads to the formation of receptor complexes, followed by tyrosine phosphorylation and activation of Janus protein tyrosine kinases (Jaks) and various cellular proteins, including gp130 itself. The activated Jaks, in turn, phosphorylate and activate latent signal transducer and activator of transcription (STAT) transcription factors [5, 6]. One member of the STAT family of proteins is STAT3, which is mainly activated by IL-6 family cytokines, epidermal growth factor, and leptin [3, 4]. Like other members of the STAT family, STAT3 is tyrosine-phosphorylated by Jaks, upon which it dimerizes, and translocates into the nucleus to activate target genes [5, 6].

The promyelocytic leukaemia (PML) protein was originally characterized as part of a fusion protein with RAR□ (PML-RAR□) that results from a chromosomal translocation

in patients with acute promyelocytic leukaemia (APL) [7]. PML has been implicated in many functions, including apoptosis, transcriptional and translational regulation, senescence and cell proliferation [8]. PML belongs to the TRIM protein family, which is an expanding family of RING proteins, also known as RBCC proteins as they contain an RBCC motif, which comprises a RING domain, one or two B-boxes and a predicted coiled-coil region [9]. The above PML functions epitomize the PML-nuclear body (PML-NB), also called nuclear domain 10, or PML oncogenic domains. PML-NBs are dynamic structures that favour the sequestration and release of proteins, mediate their post-translational modifications and promote specific nuclear events in response to various cellular environments [8]. Importantly, it has been shown that PML, but not PML-RAR□, interacts with STAT3 and suppresses its transactivation [10]. Recently, aberrant STAT-3 activation in PML-deficient mouse embryonic fibroblasts has been also described [11]

In the present study, we showed that IL-6 induces desumoylation of PML, and dissociation between PML and SUMO1 in hepatoma cells. We also demonstrated that IL-6 induces mRNA expression of SENP1, a SUMO-specific protease. Furthermore, an inactive SENP1 mutant restored the PML-mediated suppression of LIF-induced STAT3 activation. These results indicate that the IL-6 family of cytokines modulates STAT3

activation by desumoylation and inactivation of PML through SENP1 induction.

Materials and Methods

Reagents and antibodies, Human recombinant IL-6 was a kind gift from Ajinomoto (Tokyo, Japan). Human recombinant LIF was purchased from INTERGEN (Purchase, NY). Arsenic trioxide (As_2O_3) was purchased from Wako Chemicals (Osaka, Japan). Expression vectors, PML, SUMO1, Ubc9 and STAT3-LUC were described previously [10,12,13]. Expression vectors, SENP1 wild-type (wt) and catalytically inactive mutant SENP1 (R630L, K631M) (mut) were kindly provided from E. T. H. Yeh (UT M. D. Anderson Cancer Center)[14]. Anti-PML monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG antibody was purchased from Sigma (St Louis, MO). Anti-T7 antibody was purchased from MBL (Nagoya, Japan). Anti-Actin antibody from Chemicon International (Temecula, CA). Anti-SUMO1 antibody was prepared by immunization of GST-SUMO1 and following by affinity-purification with GST-SUMO1-conjugated Sepharose.

Cell culture, transfection, small interfering RNA (siRNA), luciferase assays and immunoblotting, The IL-3-dependent murine pro-B cell line, Ba/F-G133 expressing the chimeric receptor composed of extracellular domain of G-CSF receptor and cytoplasmic domain of gp130, in which gp130-mediated growth is essentially dependent on STAT3

activity [15], was maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) and 10% conditioned medium from WEHI-3B cells as a source of IL-3. The human hepatoma cell line Hep3B was cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FCS. Hep3B cells were treated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37 °C for 4 hrs, followed by addition of fresh medium containing 10% FCS as described previously [16]. siRNAs targeting human PML and SENP1 used in this study was as follows: PML, 5'-GGAAGGUCAUCAAGAUGGATT-3'; SENP1, 5'-GGUCAUAAAUUAACUGAUATT-3'. Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected by the standard calcium precipitation protocol. Luciferase assay was performed as described [17]. The cells were harvested 36 hrs after transfection and lysed in Reporter Lysis Buffer (Promega, Madison, WI) and assayed for luciferase and β-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. Three or more independent experiments were carried out for each assay. Western blotting assays were performed as described previously [17].

RNA isolation, RT-PCR and quantitative real-time PCR, Cells were harvested and total RNAs were prepared by using Iso-Gen (Nippon Gene, Tokyo, Japan) and used in RT-PCR. RT-PCR was performed using RT-PCR high -Plus- Kit (TOYOBO, Tokyo, Japan) [18]. The following primers were used for amplification of human (hu) SENP1, hu SENP2, hu PML, hu SUMO1, murine (mu) SENP1, mu SENP2, or mu Bcl-2 mRNA: hu SENP1, 5'-AAAAGCCAGATTCCTCAGCA-3' (forward) and 5'-GAGCAGCTGTTTCCAAGGTC-3' (reverse); hu SENP2, 5'-CCTCTAGCTGCTGGTGGTTC-3' (forward) and 5'-AGGGAGTGGCAGCTTGAGTA-3' (reverse); hu PML, 5'-CCTCTAGCTGCTGGTGGTTC-3' (forward) and 5'-AGGGAGTGGCAGCTTGAGTA-3' (reverse); hu SUMO1, 5'-GGGGGATAAGAAGGAAGGTG -3' (forward) and 5'-TCCATTCCCAGTTCTTTTGG -3' (reverse); mu SENP1, 5'-GCATTGGCACTACAGCTTCA-3' (forward) and 5'-CAGCGTTTCACTGCCTGATA-3' (reverse); mu SENP2, 5'-AAACCCATGGTGTCTTCTGC-3' (forward) and 5'- TCTGTCACACCTTGCTCCTG -3' (reverse); mu Bcl-2, 5'-GTCGCTACCGTCGTCACTTC-3' (forward) and 5'-ACAGCCAGGAGAAATCAAAC-3' (reverse). Quantitative real-time PCR analyses of

SENP1 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

Indirect immunofluorescence microscopy, Immunofluorescence stainings were performed as described [8]. The following primary antibodies were used: mouse anti-PML and rabbit anti-SUMO1 antibodies. Two secondary antibodies were used: fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG or rhodamine-conjugated anti-mouse IgG (Chemicon International). DNA was visualized by DAPI (Wako Chemicals) staining. Confocal laser scanning microscopy was performed with a LSM510 microscope (Carl Zeiss, Thornwood, NY) with an Apochromat x63/1.4 oil immersion objective, using excitation wavelengths of 543 nm (rhodamine red) and 488 nm (FITC).

Enzyme-linked immunosorbent assay (ELISA) for sumoylated PML, The ELISA assay was performed as described previously [19]. Briefly, ELISA 96-well, high-binding plates (Corning, Corning, NY) were coated with anti-SUMO1 antibody (1 □g/ml) overnight at 4 °C. After washing with phosphate buffer saline (PBS)-0.05% Tween 20, (PBS-T) blocking with PBS-T and 1% BSA at room temperature (RT) for 1 hr, the

plates were incubated with the total cell lysates for 1 hr at RT. After 3 times washes, the plates were incubated for 1 hr with anti-PML antibody for 1 hr, and washed 3 times with PBS-T. After incubation for 1 hr with horseradish peroxidase-labeled anti-mouse IgG diluted to 1:1000, (Amersham Pharmacia Biotech) followed by 3 washes, the SUMO1-PML binding was visualized using the TMB substrate solution (Pierce, Rockford, IL). After incubation for 15 min, the color development was stopped with 1 M phosphoric acid. The absorbance was measured at a test wavelength of 450 nm and a reference wavelength of 650 nm using a microplate reader (Bio-Rad Laboratories).

Results and Discussion

IL-6 induces desumoylation of PML

The crucial role of IL-6 in the initiation of the acute-phase response in hepatocytes has been well documented [20]. IL-6 is also thought to be an important factor contributing to optimizing the processes in the early stage of liver regeneration through STAT3 [21, 22]. Recently, it was shown that PML-RAR transgenic mice exhibit hepatic preneoplasia and neoplasia, including foci of basophilic hepatocytes, dysplasia and carcinoma [23]. Overexpression of PML-RAR also induces proliferation of hepatocytes [23]. Importantly, PML, but not PML-RAR, regulates STAT3 activation [10]. These facts allow us to examine whether IL-6 signalling affects PML function, which is mainly regulated by post-translational modification. The best-known post-translational modification of PML is sumoylation [8, 24]. Sumoylation of PML is critical for the formation of PML-NBs, because a PML mutant that cannot be modified by SUMO fails to form PML-NBs. We then tested whether IL-6 affects the sumoylation of PML in human hepatoma Hep3B cells, using a sumoylated PML (SUMO1-PML)-specific ELISA assay. As shown in Fig. 1A, sumoylation of PML decreased in a time-dependent manner in Hep3B cells after IL-6 stimulation. A potent

sumolyation agent for PML, arsenic trioxide (As_2O_3) induced marked sumoylation of PML in Hep3B cells (Fig. 1A) [24]. We also examined the co-localization of PML with SUMO1 in the nuclei of Hep3B cells using confocal microscopy. Interestingly, enhanced PML accumulation (dotted structures) in the nucleus was observed in IL-6-treated Hep3B cells (Fig. 1B). However, IL-6 stimulation clearly induced dissociation between PML and SUMO in the nucleus (arrowheads shown in Fig. 1B). To further investigate this effect on sumoylated PML proteins, we transiently expressed PML, SUMO1 and the SUMO-conjugating E2 enzyme Ubc9 in 293T cells, and the transfected cells were treated with LIF. As shown in Fig. 1C, the levels of sumoylated PML proteins were markedly decreased after 36 hrs of LIF stimulation, although PML, SUMO1 and Ubc9 proteins were well expressed at that time point. These results indicate that the IL-6 family of cytokines induces desumoylation of PML in Hep3B and 293T cells.

IL-6 induces mRNA expression of SENP1

Recently, it was shown that androgen induces SENP1 mRNA expression in prostate cancer cells and modulates their proliferation [25]. We examined whether IL-6 induced SENP1 mRNA expression in Hep3B cells. Total RNA isolated from IL-6-treated Hep3B

cells was subjected to RT-PCR analysis. As shown in Fig. 2A, SENP1, but not SENP2 mRNA expression, increased after 3 hr of IL-6 stimulation. The mRNA expression of C/EBP, an IL-6-induced gene was also observed in IL-6-treated Hep3B cells. The IL-6-induced SENP1 mRNA expression was also confirmed by quantitative real-time PCR (Fig. 2B). Interestingly, PML mRNA expression increased in Hep3B cells after IL-6 stimulation. This finding is coincident with enhanced accumulation of PML in the nuclei of Hep3B cells after IL-6 stimulation (Fig. 1B). Importantly, siRNA-mediated reduced expression of endogenous PML resulted in a significant enhancement of IL-6-induced SOCS3 and C/EBP mRNA expressions in Hep3B cells (Fig. 2C), indicating that PML regulates IL-6/STAT3-gene expression in Hep3B cells. These results may also suggest a novel mechanism for the negative regulation of IL-6/STAT3 signalling by PML using a negative feedback loop, although further detailed study will be required. We also tested whether the gp130-mediated signal induces SENP1 mRNA expression in hematopoietic cells. To this end, we used Ba/F-G133 cells and treated cells with G-CSF. G-CSF/gp130 signal induced Bcl-2 mRNA expression in Ba/F-G133 cells (Fig. 2D). Importantly, SENP1 but not SENP2 mRNA expression was induced by G-CSF treatment of Ba/F-G133 cells, suggesting that gp130 signal mediates SENP1 mRNA expression in hematopoietic cells.

SENP1 regulates STAT3 transactivation through PML

SENP1 was also demonstrated to enhance androgen receptor (AR)-mediated transactivation through desumoylation of histone deacetylase 1 (HDAC1) [26]. To assess the functional relevance of SENP1 in IL-6/STAT3-mediated signalling, we first used siRNA to reduce the endogenous expression of SENP1 in Hep3B cells. Hep3B cells were transfected with a specific siRNA for SENP1 or a control siRNA, and total RNA isolated from the transfected cells was subjected to RT-PCR analysis, which confirmed the reduction in SENP1 mRNA expression. As shown in Fig. 3A, a marked reduction of SENP1 mRNA expression was observed in Hep3B cells following SENP1 siRNA expression. We then examined the effect of SENP1 knockdown on IL-6/STAT3-mediated gene expression by RT-PCR. Both C/EBP and SOCS3 mRNA expression were altered in SENP1 siRNA-treated Hep3B cells. These results indicate that SENP1 regulates IL-6/STAT3-mediated gene expression. We also tested the effect of SENP1 knockdown on IL-6-induced decrease of sumoylation of PML in Hep3B cells using a SUMO1-PML-specific ELISA assay. As shown in Fig. 3B, IL-6-induced decrease of sumoylation of PML was restored by SENP1 knockdown, suggesting that IL-6-induced SENP1 affects sumoylation of PML in Hep3B cells.

We finally tested whether SENP1 affects STAT3-mediated transcriptional activation using transient transfection experiments in 293T cells. The STAT3-mediated transcriptional responses were measured using STAT3-LUC, in which the 2-macroglobulin promoter drives expression of a luciferase reporter gene [13]. 293T cells were transfected with STAT3-LUC together with or without PML or SENP1 wt or an inactive SENP1 mut, treated with LIF, and LUC activities were determined. When cells were co-transfected with PML, the transcriptional activation of STAT3-LUC induced by LIF stimulation was reduced (Fig. 3B). Importantly, co-expression of SENP1 wt, but not SENP1 mut restored PML-mediated suppression of STAT3 activation (Fig. 3B), suggesting that SENP1 activity affects STAT3-mediated transcriptional activation via PML.

Concluding remarks

In the present study, we have demonstrated that IL-6 induces desumoylation of PML and dissociation between PML and SUMO1 in Hep3B cells. Furthermore, IL-6 induces SENP1 mRNA expression in Hep3B cells. Importantly, siRNA-mediated reduction of endogenous SENP1 expression enhanced IL-6-induced gene expression and sumoylation of PML in Hep3B cells. Furthermore, overexpression of SENP1 restored

the PML-mediated suppression of STAT3 transactivation. Thus, the results of the present study indicate that SENP1 may play a regulatory role in STAT3-mediated signalling pathways by inducing desumoylation of PML in the nucleus.

Recently, it was demonstrated that AR-mediated transcription is markedly enhanced by SENP1, although SENP1's ability to enhance AR-dependent transcription is not mediated through desumoylation of AR, but rather, through its ability to desumoylate HDAC1 [26]. Therefore, as described here, PML might be another target of SENP1 to regulate the activity of a variety of transcription factors, including STAT3. Dysregulation of IL-6/STAT3-mediated signalling contributes to the onset and maintenance of cancer and autoimmune diseases, such as multiple myeloma, Castleman's disease, mesanginal proliferative glomerulonephritis, and rheumatoid arthritis (RA) [1, 2]. Interestingly, a previous study demonstrated increased levels of SUMO1 in synovial fibroblasts from patients with RA, but not in patients with osteoarthritis, indicating that increased expression of SUMO1 may contribute to the activated phenotype of RA synovial fibroblasts [27]. Furthermore, increased expression of SUMO1 in RA synovial fibroblasts has been shown to affect the resistance of these cells against Fas-induced apoptosis through increased sumoylation of PML and increased recruitment of the proapoptotic protein, Daxx to PML NBs [28]. SENP1 can

mediate the anti-apoptotic effects of SUMO1 by releasing Daxx from PML NBs, and was also found to be expressed at a lower level in RA synovial fibroblasts, suggesting that SENP1 may contribute to the pathogenesis of inflammatory diseases such as RA. Therefore, IL-6 may modulate the transcriptional activation and apoptosis in RA synovial fibroblasts through SENP1 induction.

Taken together, these studies provide insights into the potential role of desumoylation by SENP1 in the regulation of IL-6/STAT3-mediated signalling. A more detailed understanding of SENP1 expression by IL-6 family of cytokines is therefore important, as this new information may lead to new therapeutic approaches for IL-6/STAT3-mediated pathological conditions.

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Figure legends

Fig. 1. IL-6 influences sumoylation of PML

(A) Hep3B cells in a 12-well plate were stimulated with IL-6 (10 ng/ml) for the indicated periods, or As_2O_3 (1 □M) for 1 hr. Total cell lysates isolated from these cells were subjected to an ELISA assay specific for sumoylated PML as described in Materials and Methods. Fold induction was expressed as $OD_{450\ nm}$ of treatment compared with that of total cell lysates untreated with IL-6 as 1.0. Data represent the mean of duplicate determinations, which in general varied by <10%. Shown is a representative experiment, which was repeated at least three with similar results.

(B) 293T cells in a 12-well plate were transfected with FLAG-PML (3 □g), FLAG-Ubc9 (1 □g), and T7-SUMO1 (1 □g). At 24 h after transfection, the cells were treated with LIF (100 ng/ml) for the indicated periods. Cells were lysed, and an aliquot of total cell lysates was blotted with anti-FLAG, anti-T7 or anti-Actin antibody.

(C) Hep3B cells in a 6-well plate were treated or untreated with IL-6 (10 ng/ml) for 6 hrs, and cells were fixed and reacted with mouse anti-PML and rabbit anti-SUMO-1 antibodies, 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 arrowheads indicate co-localization of PML with SUMO1. Approximately 100 cells were estimated according green (FITC), red (rhodamine) and yellow (merge) signals in PML-NBs. More than 90% of PML co-localized with SUMO1 in PML-NBs without IL-6 stimulation, whereas approximately 85% of PML was disassociated with SUMO1 after IL-6 stimulation.

Fig. 2. IL-6/gp130 signal induces mRNA expression of SENP1

(A) Hep3B cells in a 24-well plate 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 SENP1, SENP2, PML, C/EBPd or G3PDH primers.

(B) SENP1 expression level was also quantified by quantitative real-time PCR analysis using the assay-on-demand™ gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system. Data represent the levels of SENP1 mRNA normalized to that of a G3PDH internal control and are expressed relative to the value at time zero. Results are representative of three independent experiments, and the error bars represent the SD.

(C) Hep3B cells in a 24-well plate were treated with control siRNA or PML 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 C/EBP□, SOCS3,

PML or G3PDH primers.

(D) Ba/F-G133 cells ($5x10^6$) were treated with or without G-CSF (30 ng/ml) for the indicated periods. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using SENP1, SENP2, Bcl-2 or G3PDH primers.

Fig.3. SENP1 regulates STAT3 transactivation through PML

(A) Hep3B cells in a 24-well plate were treated with control siRNA or SENP1 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 C/EBP□, SOCS3, SENP1, SUMO1 or G3PDH primers.

(B) Hep3B cells in a 12-well plate were treated with control siRNA or SENP1 siRNA, and cells were stimulated with IL-6 (10 ng/ml) for the indicated periods. Total cell lysates isolated from these cells were subjected to an ELISA assay specific for sumoylated PML. Fold induction was expressed as $OD_{450\ nm}$ of treatment compared with that of total cell lysates untreated with IL-6 as 1.0. Data represent the mean of duplicate determinations, which in general varied by <10%. Shown is a representative experiment, which was repeated at least three with similar results.

(C) 293T cells in a 24-well plate were transfected with or without PML (1 □g), SUMO1 (0.5 □g), Ubc9 (0.5 □g) and STAT3-LUC (0.5 □g) and/or SENP1 wt or mut (0.3, 1.0

□g). At 24h after transfection, the cells were harvested, and luciferase activities were measured.

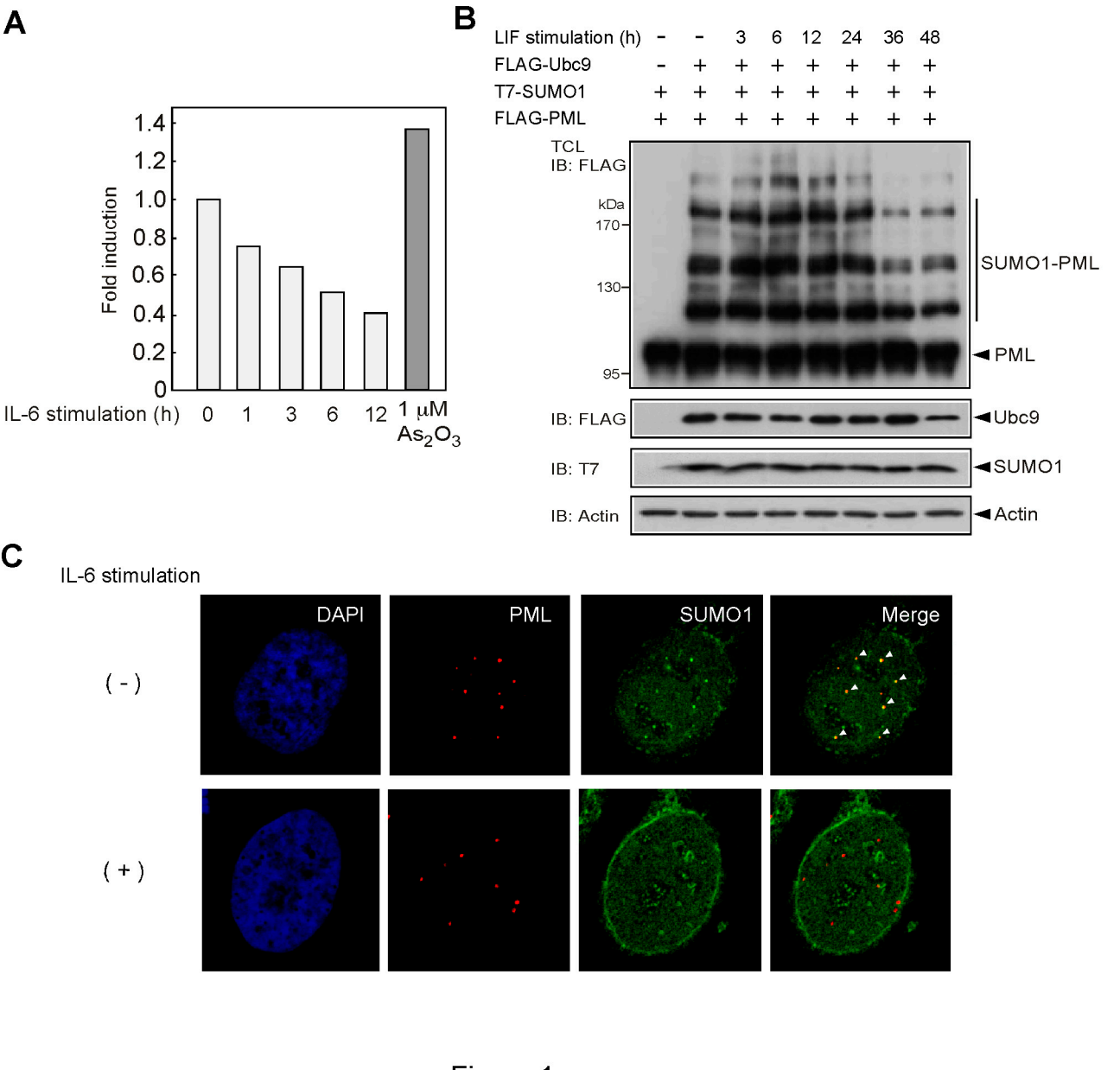

A
Fold induction
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
IL-6 stimulation (h) 0 1 3 6 12 1 μM As2O3
B
LIF stimulation (h) - - 3 6 12 24 36 48
FLAG-Ubc9 - + + + + + + +
T7-SUMO1 + + + + + + + +
FLAG-PML + + + + + + + +
TCL
IB: FLAG
kDa
170
130
95
SUMO1-PML
PML
IB: FLAG
Ubc9
IB: T7
SUMO1
IB: Actin
Actin
C
IL-6 stimulation
DAPI
PML
SUMO1
Merge
(-)
(+)

Figure 1

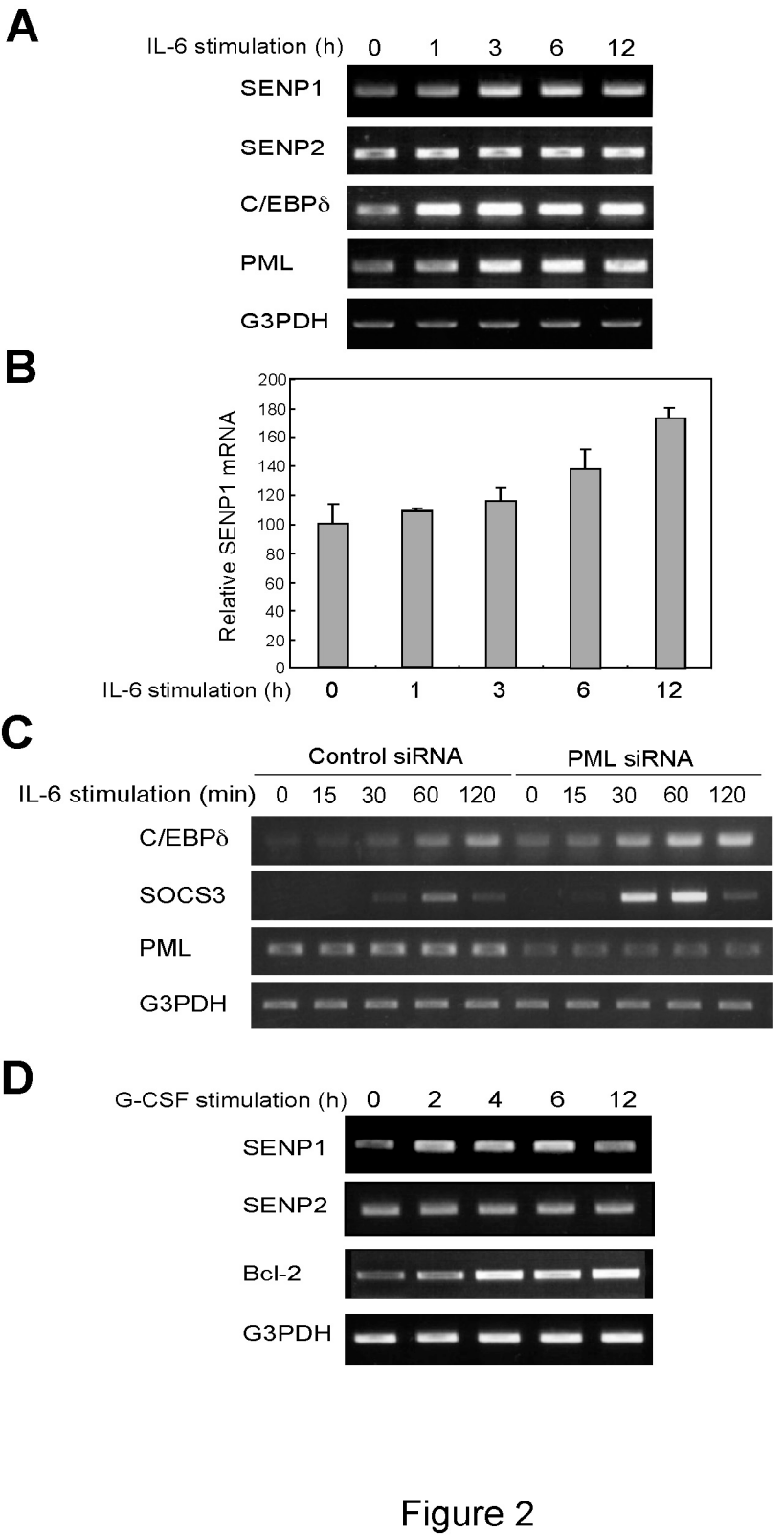
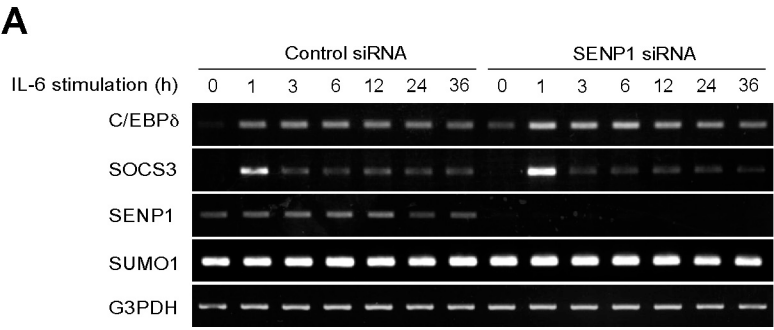
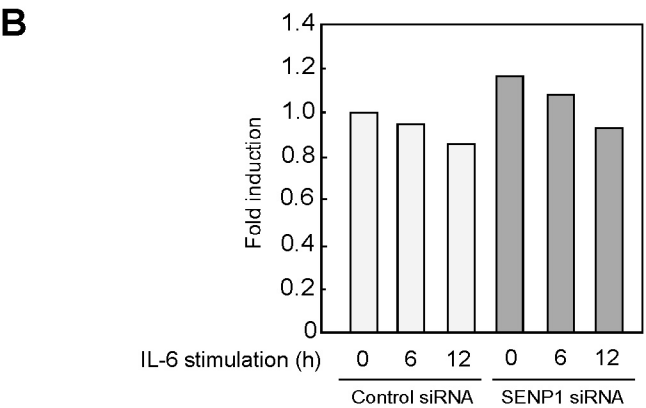

A
IL-6 stimulation (h) 0 1 3 6 12
SENP1
SENP2
C/EBPδ
PML
G3PDH
B
Relative SENP1 mRNA
200
180
160
140
120
100
80
60
40
20
0
IL-6 stimulation (h) 0 1 3 6 12
C
Control siRNA
PML siRNA
IL-6 stimulation (min) 0 15 30 60 120 0 15 30 60 120
C/EBPδ
SOCS3
PML
G3PDH
D
G-CSF stimulation (h) 0 2 4 6 12
SENP1
SENP2
Bcl-2
G3PDH

Figure 2


A
Control siRNA
SENP1 siRNA
IL-6 stimulation (h)
0 1 3 6 12 24 36 0 1 3 6 12 24 36
C/EBPδ
SOCS3
SENP1
SUMO1
G3PDH


B
Fold induction
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
IL-6 stimulation (h)
0 6 12 0 6 12
Control siRNA
SENP1 siRNA

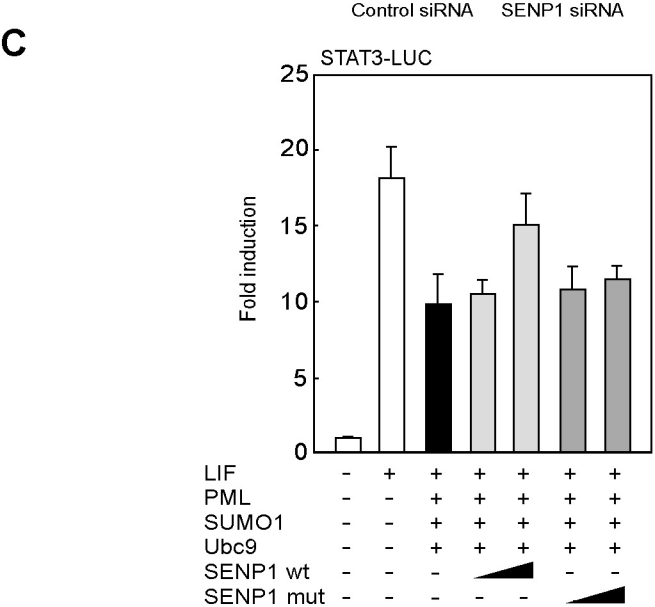

C
STAT3-LUC
Fold induction
25
20
15
10
5
0
LIF
PML
SUMO1
Ubc9
SENP1 wt
SENP1 mut

Figure 3