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Title: HDAC3 influences phosphorylation of STAT3 at serine 727 by interacting with PP2A

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Running title: Interactions among STAT3, HDAC3 and PP2A

Abstract

Signal transducer and activator of transcription 3 (STAT3), which mediates biological actions in many physiological processes, is activated by cytokines and growth factors, and has been reported to be involved in the pathogenesis of various human diseases. Here, we show that treatment of HeLa cells with a histone deacetylase (HDAC) inhibitor, trichostatin A, or small-interfering RNA (siRNA)-mediated repression of HDAC3, enhances phosphorylation of STAT3 at Ser727. Furthermore, dephosphorylation of STAT3 at Ser727 by protein phosphatase 2A (PP2A) was restored by treatment of cells with HDAC3 siRNA. We further found that formation of a complex between STAT3 and PP2A was enhanced in the presence of HDAC3. Importantly, small-interfering RNA-mediated repression of both HDAC3 and PP2A effectively enhanced leukemia inhibitory factor (LIF)-induced STAT3 activation. These results indicate that HDAC3 may act as a scaffold protein for PP2A to regulate the LIF/STAT3-mediated signaling pathway.

Keywords: STAT3; PP2A; HDAC3; dephosphorylation; transcriptional regulation

Introduction

Signal transducer and activator of transcription 3 (STAT3) was originally cloned as an acute-phase response factor activated by interleukin (IL)-6 in the mouse liver, and based on its homology with STAT1 [1,2]. Growth factors, such as epidermal growth factor and platelet-derived growth factor, can also stimulate STAT3 activity [3]. STAT3 is known to play crucial roles in early embryonic development as well as in other biological responses, including cell growth and apoptosis [4-6]. STAT3 is constitutively activated in v-src- or v-abl-transformed cells and various primary tumors and cell lines [7,8]. Moreover, STAT3 itself acts as an oncogene in NIH-3T3 cells [9]. Since dysregulation of the STAT3-mediated signaling pathway is frequently detected in clinical tumor samples, understanding the mechanisms underlying STAT3 regulation of cell survival may lead to successful strategies for targeting STAT3 in cancer therapy [5,7, 8].

A single tyrosine residue in all STATs is phosphorylated as a consequence of cytokine or growth factor stimulation and this phosphorylation is essential for the activation of these STATs. In addition, all STATs, except for STAT2 and STAT6, are phosphorylated on serine residues in response to ligand stimulation [10]. In the case of STAT3, phosphorylation of a single serine residue (Ser727) in the transcriptional activation domain is required for maximal transcriptional activation [11]. A STAT3 S727A mutant, in which Ser727 is replaced with an alanine, exhibited a marked reduction in transcriptional activation in vivo [12]. Serine phosphorylation most likely increases STAT3 activity by increasing its association with other cofactors, such as p300 [13]. Several different

kinases, including ZIP kinase, have been implicated in serine phosphorylation, implying an interaction between STAT3 signaling and serine kinase signaling pathways [10,14]. However, the regulatory mechanisms underlying Ser727-phosphorylation of STAT3 have not yet been identified. A recent study demonstrated that STAT3 is also acetylated on a single lysine residue, Lys685 [15]. Histone acetyltransferase p300-mediated STAT3 acetylation on Lys685 was restored by type I histone deacetylase (HDAC). This effect was reversible by treatment of cells with trichostatin A (TSA), a broad inhibitor of HDACs. STAT3 interacted with HDAC1, HDAC2 and HDAC3. Among them, HDAC3 displayed the strongest inhibitory effect on STAT3 deacetylase activity and bound to STAT3 through its C-terminal region, which plays a regulatory role in HDAC catalytic activity.

In the present study, we found that treatment of HeLa cells with TSA, or small-interfering RNA (siRNA)-mediated repression of HDAC3, enhances phosphorylation of STAT3 at Ser727. Furthermore, dephosphorylation of STAT3 at Ser727 by protein phosphatase 2A (PP2A) was restored by treatment of HeLa cells with HDAC3 siRNA. We further found that formation of a complex between STAT3 and PP2A was enhanced in the presence of HDAC3. Moreover, siRNA-mediated repression of both HDAC3 and PP2A effectively enhanced leukemia inhibitory factor (LIF)-induced STAT3 activation. These results indicate that HDAC3 regulates both the phosphorylation and acetylation of STAT3 in the STAT3-mediated signaling pathway.

Materials and methods

Reagents and antibodies, Trichostatin A (TSA) was purchased from Wako Pure Chemicals(Osaka, Japan). Recombinant human LIF was purchased from INTERGEN (Purchase, NY). Expression vectors for STAT3, HDAC3, PP2A and STAT3-LUC were described previously [16, 17]. Anti-Myc, anti-STAT3 and anti-HDAC3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-FLAG antibody from Sigma-Aldrich (St. Louis, MO); anti-pSTAT3(Tyr705), anti-pSTAT3(Ser727) and anti-PP2A antibodies from Cell Signaling Technologies (Beverly, MA); anti-actin antibody from Chemicon International (Temecula, CA).

Cell culture, transfection, small interfering RNA (siRNA) and luciferase assays, Human cervix carcinoma cell line HeLa and human embryonic kidney carcinoma cell line 293T were maintained in DMEM containing 10 % FCS. 293T cells were transfected with the standard calcium precipitation protocol [18]. siRNAs targeting human HDAC3 and PP2A (catalytic subunit) used in this study were as follows: HDAC3, 5'-GCCGGUUAUCAACCAGGUATT-3'; PP2A, 5'-GAUACAAAUUACUUGUUUATT-3'. HeLa cells were plated on a 24-well plate at 2×10^4 cells/well, and then incubated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37 °C for 4 h, followed by addition of fresh medium containing 10% FCS [19]. Twenty-four hrs after transfection, the cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. HeLa cells were then transfected with STAT3-LUC using jetPEI (PolyPlus-

transfection, Strasbourg, France) according to the manufacturer's instruction. The cells were harvested 36 h after transfection and lysed in Reporter Lysis Buffer (Promega) and assayed for luciferase and β -galactosidase activities according to the manufacturer's instructions. Three or more independent experiments were carried out for each assay.

Immunoprecipitation and immunoblotting. The immunoprecipitation and Western blotting assays were performed as described previously [20]. The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to PVDF transfer membrane (PerkinElmer; Boston, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore; Bedford, MA).

Results and Discussion

HDAC3 influences phosphorylation of STAT3 at Ser727

It has recently been reported that HDAC3 interacts with and deacetylates STAT3 at Lys685, and that treatment of cells with TSA enhances STAT3-mediated transcriptional activity [15]. These findings led us to examine whether HDAC3 is also involved in the regulation of phosphorylation of STAT3. To examine the involvement of HDAC3 in the phosphorylation of STAT3, we first tested the effects of TSA on the phosphorylation of STAT3. 293T or HeLa cells were pretreated with TSA for 12 h, stimulated with LIF for 30 min and analyzed for their levels of endogenous STAT3 phosphorylation. As shown in Fig. 1A and B, Ser727-phosphorylation, but not Tyr705-phosphorylation, was markedly enhanced by TSA pretreatment in both types of cells, suggesting that type I HDAC may be involved in regulating Ser727-phosphorylation, but not Tyr705-phosphorylation, of STAT3. We further investigated whether HDAC3 is involved in the regulation of STAT3 Ser727-phosphorylation using an siRNA to reduce endogenous HDAC3 expression in HeLa cells. HeLa cells were transfected with a specific siRNA against HDAC3, or a control siRNA, and aliquots of cell lysates were analyzed by Western blotting to confirm reductions in HDAC3 protein levels (Fig. 1C). Importantly, siRNA-mediated reduced expression of HDAC3 significantly enhanced Ser727-phosphorylation, but not Tyr705-phosphorylation, of STAT3 (Fig. 1C), further indicating that HDAC3 regulates Ser727-phosphorylation, but not Tyr705-phosphorylation, of STAT3.

HDAC3 affects phosphorylation of STAT3 at Ser727 by interacting with PP2A

In a previous study, inhibition of a major cellular serine/threonine-specific protein phosphatase, PP2A was found to induce serine phosphorylation and activation of STAT3 [21]. Furthermore, expression of PP2A antisense constructs in human hepatoma Hep3B cells was shown to enhance IL-6-induced transcription [22]. Interestingly, IL-6 stimulation activated the phosphatase activity of PP2A in Hep3B cells. Therefore, we examined whether overexpression or reduction of PP2A affects Ser727-phosphorylation of STAT3. 293T cells were transfected with expression vectors for STAT3 and PP2A, and stimulated with LIF for 30 min. As shown in Fig. 2A, Ser727-phosphorylation, but not Tyr705-phosphorylation, of STAT3, was markedly decreased by overexpression of PP2A in 293T cells. Importantly, siRNA-mediated repression of PP2A by treatment of HeLa cells with a PP2A siRNA significantly enhanced Ser727-phosphorylation, but not Tyr705-phosphorylation, of endogenous STAT3 (Fig. 2B), confirming that PP2A regulates Ser727-phosphorylation, but not Tyr705-phosphorylation, of STAT3. Recent studies have demonstrated that another major cellular serine/threonine-specific protein phosphatase, PP1, interacts with HDACs [23], and that a PP1-HDAC1 complex controls the functions of the transcription factor, cAMP-responsive element-binding protein (CREB) [24]. To further examine the involvement of PP2A in the HDAC3 siRNA-mediated Ser727-dephosphorylation of STAT3, we tested the effect of reducing the levels of endogenous HDAC3 on the PP2A-mediated decrease of Ser727-phosphorylation of endogenous STAT3. Expression vector for PP2A was transfected

into HeLa cells treated with an siRNA for HDAC3, or a control siRNA, and stimulated with LIF for 30 min. As shown in Fig. 2C, Ser727-dephosphorylation of STAT3 by PP2A was restored by reduction of endogenous HDAC3 expression. These results indicated that HDAC3 is involved in PP2A-mediated Ser727-dephosphorylation of STAT3.

Cooperative interactions among STAT3, HDAC3 and PP2A

One of the mechanisms consistent with the above-described data is a direct interaction among STAT3, HDAC3 and PP2A. To clarify this possibility, co-immunoprecipitation experiments were performed using 293T cells, although a recent study revealed a direct interaction between STAT3 and HDAC3 [15]. Following transfection of 293T cells with expression vectors for FLAG-tagged STAT3 and Myc-tagged PP2A, the cells were lysed, and lysates were immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-Myc antibody. As shown in Fig. 3A, a significant interaction of STAT3 with PP2A was observed, as described previously [22, 25]. When expression vectors for FLAG-tagged HDAC3 and Myc-tagged PP2A were transfected into 293T cells and the cells were lysed, immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-Myc, the immunoprecipitates were found to contain PP2A (Fig. 3A), indicating that direct interactions among STAT3, HDAC3 and PP2A occur in 293T cells. Next, we examined whether HDAC3 influences effective complex formation between STAT3 and PP2A to dephosphorylate STAT3. Following transfection of 293T cells with expression vectors for FLAG-tagged STAT3 and Myc-tagged PP2A, with or without vector for FLAG-tagged HDAC3, the cells

were lysed, and the lysates were immunoprecipitated with an anti-STAT3 antibody and immunoblotted with an anti-Myc antibody. As shown in Fig. 3B, a significantly enhanced interaction between STAT3 and PP2A was observed in the presence of HDAC3. These results indicate that HDAC3 may act as an effective reservoir for STAT3 and PP2A.

HDAC3 and PP2A are involved in regulating LIF/STAT3-mediated transactivation

To further assess the functional relevance of HDAC3 and PP2A in STAT3-mediated signaling, we examined whether siRNA-mediated reduction of endogenous HDAC3 or PP2A affects STAT3-mediated transcriptional activation by transient transfection experiments using STAT3-LUC, in which the α 2-macroglobulin promoter drives expression of a luciferase (LUC) reporter gene. HeLa cells were transfected with a specific siRNA for HDAC3 or PP2A, or a control siRNA, and aliquots of cell lysates from the transfected cells were subjected to Western blot analyses, which confirmed reductions in the PP2A and HDAC3 protein contents (Fig. 4, lower panel). As shown in Fig. 4 (upper panel), a reduction in the level of HDAC3 or PP2A expression in HeLa cells resulted in enhanced LIF/STAT3-induced STAT3-LUC activation, indicating that both HDAC3 and PP2A are involved in the regulation of LIF/STAT3-induced STAT3-LUC activation in HeLa cells. We also examined the effect of combined knockdown of HDAC3 and PP2A on LIF/STAT3-induced STAT3-LUC activation in HeLa cells. Importantly, a significant enhancement of LIF/STAT3-induced STAT3-LUC activation was observed in HDAC3- and PP2A-knocked down HeLa cells. These results indicate that PP2A cooperatively acts with HDAC3 in the repression of LIF/STAT3-

induced STAT3-LUC activation in HeLa cells.

Concluding remarks

In the present study, we have shown that HDAC3 inhibition enhances Ser727-phosphorylation of STAT3, and that STAT3, HDAC3 and PP2A form a complex. This complex formation results in Ser727-dephosphorylation of STAT3, followed by regulation of STAT3-mediated transcriptional activation.

A serine/threonine-specific protein phosphatase, PP1, has been shown to form complexes with HDAC1, HDAC6 and HDAC10 in vivo [23]. Interestingly, TSA treatment disrupted all of these cellular HDAC-PP1 complexes, suggesting that this HDAC inhibitor elicits coordinated changes in cellular protein phosphorylation and acetylation, followed by suppression of cellular functions such as cell growth and transformation. PP1 has been shown to be involved in Ser133-dephosphorylation of CREB [24]. Ser133-phosphorylation of CREB promotes the recruitment of its coactivators CBP and p300, which in turn stimulate the acetylation of promoter-bound histones to trigger further transcriptional activation. Indeed, TSA treatment enhances CREB activity by sustaining Ser133-phosphorylation via cAMP stimulation, suggesting a potential role for HDAC complexes in silencing CREB activity. Importantly, HDAC1 associates with and inhibits Ser133-phosphorylation of CREB by Ser133-dephosphorylation via an interaction with PP1. Therefore, complex formation between HDACs and PP1/2 is an important mechanism by which signaling and chromatin-modifying activities act coordinately to repress phosphorylation-dependent

activation of transcription factors.

Based on the present data, we propose HDAC3 as a novel player that forms an effective complex with PP2A to regulate STAT3-mediated transactivation. Further detailed studies of STAT3-related corepressor complexes will be very important for clarifying the mechanism underlying 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 therapeutic strategies aimed at the treatment of cancer and autoimmune diseases.

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Figure legends

Figure 1. HDAC3 influences phosphorylation of STAT3 at Ser727

(A) 293T cells in a 12-well plate were pretreated with TSA (100, 200 ng/ ml) for 12 h, cells were stimulated with LIF (100 ng/ml) for an additional 30min. The cells were lysed, and an aliquot of total cell lysates (TCL) was analyzed by immunoblotting using anti-pSTAT3 (Ser727) or anti-pSTAT3 (Tyr705) or anti-STAT3 antibody. (B) HeLa cells in a 12-well plate were pretreated with TSA (100, 200 ng/ ml) for 12 h, cells were stimulated with LIF (100 ng/ml) for an additional 30min. The cells were lysed, and an aliquot of TCL was analyzed by immunoblotting using anti-pSTAT3 (Ser727) or anti-pSTAT3 (Tyr705) or anti-STAT3 antibody. (C) HeLa cells in a 24-well plate were treated with control or HDAC3 siRNA, and cells were lysed, and an aliquot of TCL was analyzed by immunoblotting using anti-HDAC3 or anti-actin antibody. HeLa cells treated with control or HDAC3 siRNA were also stimulated with LIF (100 ng/ml) for the indicated periods. The cells were lysed, and an aliquot of TCL was analyzed by immunoblotting using anti-pSTAT3 (Ser727) or anti-pSTAT3 (Tyr705) or anti-STAT3 antibody.

Figure 2. HDAC3 affects phosphorylation of STAT3 at Ser727 by interacting with PP2A

(A) HeLa cells in a 24-well plate were transfected with with Myc-STAT3 (0.2 µg) and/or Myc-PP2A (0.2 µg). At 36 h after transfection, the cells were stimulated with LIF (100ng/ml) for 30min, and lysed. An aliquot of total cell lysates (TCL) was analyzed by immunoblotting using anti-pSTAT3

(Ser727) or anti-pSTAT3 (Tyr705), anti-Myc antibody. (B) HeLa cells in a 24-well plate were treated with control or PP2A siRNA, and cells were stimulated with LIF (100 ng/ml) for 30 min. The cells were then lysed, and an aliquot of TCL was analyzed by immunoblotting using anti-pSTAT3 (Ser727) or anti-pSTAT3 (Tyr705), anti-STAT3 or anti-PP2A antibody. (B) HeLa cells treated with control or HDAC3 siRNA in a 24-well plate were transfected with Myc-PP2A (0.2 μ g). At 36 h after transfection, the cells were then stimulated with LIF (100 ng/ml) for 30 min. The cells were lysed, and an aliquot of TCL was analyzed by immunoblotting using anti-pSTAT3 (Ser727), anti-pSTAT3 (Tyr705), anti-STAT3, anti-HDAC3 or anti-PP2A antibody. Densitometric quantification of the above results was also shown. Relative intensity of pSTAT3 (Ser727) was normalized to the STAT3 protein of the same sample.

Figure 3. Cooperative interactions among STAT3, HDAC3 and PP2A

(A) 293T cells (1×10^7) were transfected with Myc-PP2A (10 μ g) and/or FLAG-STAT3 (5 μ g) or FLAG-HDAC3 (5 μ g). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-Myc (upper panel), anti-FLAG (middle panel). An aliquot of TCL was blotted with anti-Myc antibody (lower panel). (B) 293T cells (1×10^7) were transfected with Myc-PP2A (10 μ g) and/or Myc-STAT3 (5 μ g) or FLAG-HDAC3 (5 μ g). At 48 h after transfection, the cells were lysed, and immunoprecipitated with anti-STAT3 antibody and immunoblotted with anti-Myc or anti-FLAG antibody. An aliquot of TCL was analyzed by immunoblotting using anti-Myc or anti-FLAG antibody.

Figure 4. PP2A and HDAC3 are involved regulating LIF/STAT3-mediated transactivation

HeLa cells in a 24-well plate were transfected with control, PP2A and/or HDAC3 siRNA, and cells were then transfected with STAT3-LUC using jetPEI. At 36 h after transfection, cells were treated with LIF (100 ng/ml) for an additional 8 h. The cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D. *, $p < 0.05$ **, $p < 0.01$ An aliquot of TCL was analyzed by immunoblotting using anti-PP2A, anti-HDAC3 or anti-actin antibody.

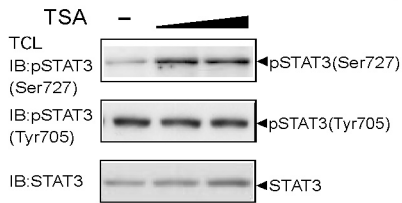
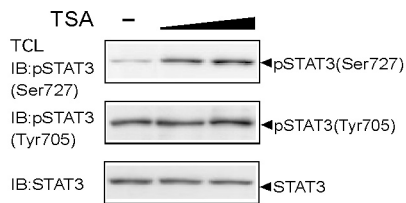
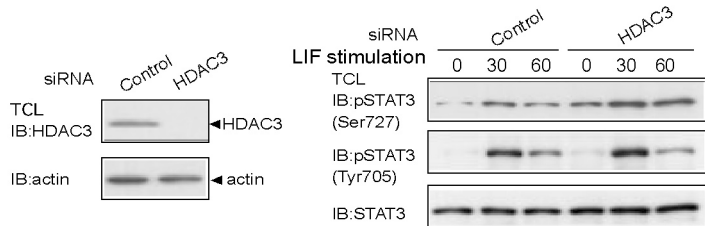
A**B****C**

Figure 1

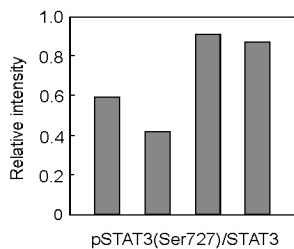
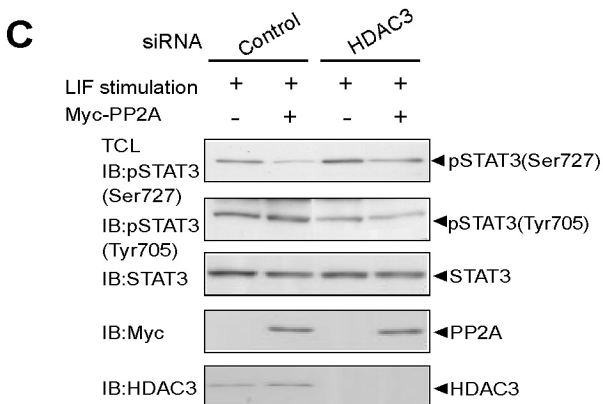
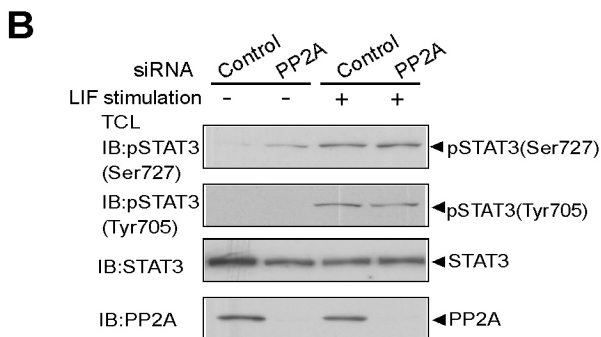
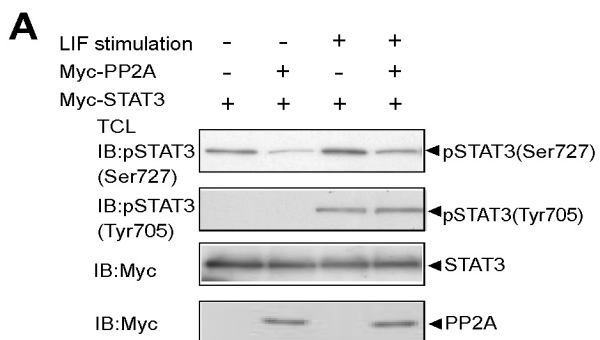
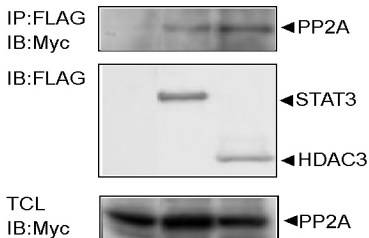


Figure 2

A

Myc-PP2A	+	+	+
FLAG-STAT3	-	+	-
FLAG-HDAC3	-	-	+

**B**

Myc-PP2A	+	+	+
FLAG-STAT3	-	+	+
FLAG-HDAC3	-	-	+

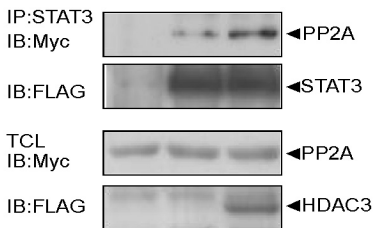


Figure 3

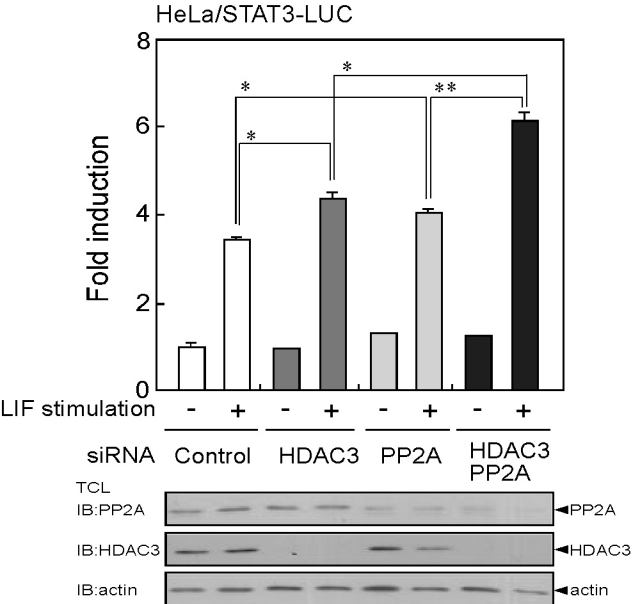


Figure 4