Regulation of Pokemon 1 Activity by Sumoylation

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Key Words

Pokemon • Sumoylation • POZ-Domain • Transcription • Repression

Abstract

Pokemon 1 is a proto-oncogenic transcriptional regulator that contains a POZ domain at the N-terminus and four Kruppel-like zinc fingers at the C-terminus. Pokemon 1 plays an important role in adipogenesis, osteogenesis, oncogenesis, and transcription of NF- κ B responsive genes. Recent reports have shown that biological activities of transcription factors are regulated by sumoylation. We investigated whether Pokemon 1 is post-translationally modified by sumoylation and whether the modification affects Pokemon 1's transcriptional properties. We found that Pokemon 1 is sumoylated *in vitro* and *in vivo*. Upon careful analysis of the amino acid sequence of Pokemon 1, we found ten potential sumoylation sites located at lysines 61, 354, 371, 379, 383, 396, 486, 487, 536 and 539. We mutated each of these amino acids into arginine and tested whether the mutation could affect the transcriptional properties of Pokemon 1 on the Pokemon 1 responsive genes, such as *ADH5/FDH* and *pG5-FRE-Luc*. Wild-type

Pokemon 1 potentially represses transcription of *ADH5/FDH*. Most of the mutants, however, were weaker transcription repressors and repressed transcription 1.3-3.3 fold less effective. Although potential sumoylation sites were located close to the DNA binding domain or the nuclear localization sequence, the mutations did not alter nuclear localization or DNA binding activity. In addition, on the *pG5-FRE-Luc* test promoter construct, ectopic SUMO-1 repressed transcription in the presence of Pokemon 1. The sumoylation target lysine residue at amino acid 61, which is located in the middle of the POZ-domain, is important because K61R mutation resulted in a much weaker molecular interaction with corepressors. Our data suggest that Pokemon 1's activity as a transcription factor may involve sumoylation, and that sumoylation might be important in the regulation of transcription by Pokemon 1.

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Introduction

Pokemon 1 (POK erythroid myeloid ontogenic factor, also called FBI-1) is a member of the POK (POZ and Kruppel) family of transcriptional repressors, which

has a POZ domain at the N terminus and four Krüppel-like zinc fingers at the C-terminus [1, 2]. The POZ/BTB domain mediates homodimerization and heterodimerization, and recruitment of corepressors and HDAC complexes [2]. The C-terminal zinc fingers mediate sequence-specific DNA recognition [2]. Recent reports have uncovered essential roles for POK proteins in development, differentiation, and oncogenesis [3, 4].

Pokemon 1 was originally identified as a protein that regulates transcription by binding to the IST (inducer of short transcripts) element of the HIV-1 (human immunodeficiency virus, type 1) LTR promoter [1]. Pokemon 1 activates binding of Tat to the HIV-1 LTR [5], and binds to the proximal promoter of the *ADH5/FDH* gene and represses transcription by interacting with the Sp1 zinc finger DNA-binding domain [6]. Also, it facilitates differentiation of the preadipocyte [7]. In addition, the rat homologue of Pokemon 1, OCZF (osteoclast-derived zinc finger) is a transcriptional repressor and appears to be involved in osteogenesis [8]. SAGE (Serial Analysis of Gene Expression) analysis showed that the expression of Pokemon 1 is high in cancer, suggesting that Pokemon 1 may play a role as a proto-oncogene. Inactivation of Pokemon 1 in mouse resulted in embryonic lethality due to severe anemia and profoundly impaired cellular differentiation in multiple tissues, and overexpression of Pokemon 1 caused cancer in multiple tissues [4]. For all of these reasons, Pokemon 1 is potentially a therapeutic target for human cancer therapy because of its essential role in oncogenic transformation [4].

SUMO (small ubiquitin-related modifier) is a protein containing 97 amino acids and its folding structure is very similar to that of ubiquitin [9]. Although amino acid sequence homology to ubiquitin is only 18%, SUMO and ubiquitin recognize the same lysine residue on target proteins. SUMO-1 has a long and flexible N-terminal domain, which is absent in ubiquitin. Despite similar protein folding, the distribution of charged residues on the surface of SUMO is very different from that of ubiquitin. This difference may confer upon SUMO unique activities [10]. Unlike ubiquitin, SUMO does not lead to protein degradation by the proteasome. SUMO reversibly modifies many proteins important in gene expression, which include transcription factors, cofactors, and regulators of chromatin structure, and signal transduction [9]. Transcription factors must be stably expressed, translocate to the nucleus, bind DNA or other proteins in order to localize to the target gene, and interact with other factors to regulate transcription. Each of these processes

are potentially regulated by Sumoylation [10, 11].

Like ubiquitination, SUMO is covalently conjugated to target proteins. The SUMO-1 interacting amino acid sequence motif comprises the sequence, ψ KXE (ψ , large hydrophobic residue, K, lysine to which SUMO-1 is conjugated, X, any amino acid, and E, glutamic acid; <http://www.abgent.com/tool/sumoplot>). The glutamic acid residue is the most highly conserved amino acid after lysine. Remarkably, a ψ KXE sequence and a nuclear localization sequence (NLS) are the requirements for SUMO conjugation [10-13].

The sumoylation process is similar to that of ubiquitination and involves an interaction cascade involving three enzymes (E1, E2, and E3), and forms a bond between the C-terminus of SUMO and a side chain amino group of lysine on the target protein. SUMO-activating enzyme (E1) is a heterodimer of SAE1 and SAE2. The SUMO-conjugating enzyme (E2) is UBC9. The protein-Sumo ligases (E3) include members of the PIAS family and RanBP2 [10-13]. SUMO protease cleaves the C-terminus of the nascent SUMO to expose the C-terminal glycine residue. It is subsequently conjugated to the target proteins by means of E1 activating, E2 conjugating and E3 ligating enzymes. The E3-like proteins might serve to increase the affinity of E2 with substrates by bringing the two in close proximity with one another [13, and references therein].

Although post-translational modification by SUMO has diverse effects on transcription factor activity, in most cases sumoylation has been found to inhibit transcription [14-16]. Thus, blocking of SUMO activity by mutation of the SUMO-acceptor lysines or by overexpression of a desumoylating enzyme, such as SENP1 (sentrin-specific protease 1) has been shown to increase activities of transcription factors, including androgen receptor (AR), CAAT/Enhancer binding (C/EBP) proteins, Elk-1, Sp3, and Smad4. Although the molecular mechanisms by which SUMO regulates transcription factor activity are not fully understood, recent advances suggest that one consequence of sumoylation is to promote transcription factor interactions with corepressors [14-16].

Recently PLZF, a transcriptional regulator with a POZ domain and zinc fingers, was reported to be conjugated with SUMO-1, and transcriptional activity of PLZF is regulated by sumoylation [17]. Pokemon 1 has similar structural features as PLZF, such as the POZ domain and zinc fingers. Therefore, we considered the possibility that Pokemon 1 could be targeted for sumoylation. We searched the amino acid sequence of Pokemon 1 for a motif which is highly similar to the

SUMO-1 conjugate consensus motif, ψ KXE, using the SUMOplot™ prediction program [10-13]. We found that ten lysine residues have the possibility to be sumoylated and, in particular, lysines 486 and 539 have a high probability of being modified by SUMO-1. When we mutated the various lysine residues of Pokemon 1 to arginine, we found that the mutations significantly affected transcription activity of Pokemon 1 on the test promoters. We also investigated whether Pokemon 1 is sumoylated and found that Pokemon 1 is modified by SUMO-1 conjugation *in vitro*. Our results indicate that Pokemon 1 activity as a transcription factor may be regulated by sumoylation *in vivo*.

Materials and Methods

Reagents

All reagents for cell cultures and Lipofectamin PLUS were purchased from Invitrogen (CA, USA). Luciferase assay kit and T_NT Quick Coupled Transcription /Translation System kit were purchased from Promega (WI, USA). ECL Kit was purchased from PerkinElmer life sciences (MA, USA). The Bradford assay kit was purchased from Bio-Rad (CA, USA). The sumoylation kit was purchased from LAE (MD, USA). The Quick Change site-directed mutagenesis kit was purchased from Stratagene (CA, USA). Other chemicals or reagents were mostly purchased from Sigma (MO, USA).

Plasmids preparation

To prepare the mammalian expression plasmid of human Pokemon 1, Pokemon 1 cDNA (NM_015989) containing the coding sequence was amplified from a human cDNA library using the oligonucleotide set (forward 5'-GGA TCG AAT TCA CCA TGG ACT ACA AGG ACG ACG ATG ACA AGG CCG GCG GCG TGG ACG GC-3' and reverse 5'-GGA TCT CTA GAT CAT TAG GCG AGT CCG GCT GT-3') and was inserted into pcDNA3.0/EcoRI-XbaI (Invitrogen, CA, USA). The various Pokemon 1 mutant K61R, K354R, K371R, K379R, K383R, K396R, K486R, K487R, K536R, K539R and SUMO-1 mutant SUMO-1 G97A were generated using a Quick Change site-directed mutagenesis kit (Stratagene, CA, USA). The mutants were verified by DNA sequencing using an ABI-prism automatic sequencer (Applied Biosystems, CA, USA).

To prepare the bacterial overexpression plasmid of the GST-POZ domain fusion protein, a cDNA fragment encoding the POZ domain of Pokemon 1 was amplified by PCR from pcDNA3.0-Pokemon 1 using oligonucleotide primers (forward 5'-GTTAACGAATT-CAC ATG CCG GCG GCG TGG ACG GC CCC TCG GGAT-3'; reverse 5'-GTT AAC-GCG GCC GCT TAG TCT GCC GGT CCA GGA GGT CGG CGC AC-3'). The PCR cycling conditions were denaturation at 95°C for 3 min, followed by 30 cycles of an amplification reaction at 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, followed by final extension at 72°C for 5 min. The PCR product was cloned into pGEX4T3

expression plasmid/EcoRI-HpaI (Amersham Pharmacia, Buckinghamshire, UK).

The His-tagged Human SUMO-1 expression plasmid was kindly provided by Dr. Ick Young Kim (Korea Univ., Seoul, Korea). The HA-tagged Human SUMO-1 (active form) expression plasmid was kindly provided by Dr. Kwang-Chul Jeong (Yonsei Univ., Seoul, Korea).

The pG5-FRE-*Luc* was prepared by cloning annealed oligonucleotides composing FRE (Pokemon 1 binding site) of HIV-1 LTR sequence (Sense 5'-GAT CTG CAG CTG CTT TTC TCG AGT ACT GGG TCT CTC TAG GGA ACC CAC TGC TTA A-GCC TCA ATA AAG CTA-3'. Antisense, 5'-GAT CTA CTT T-ATT GAG CTT AAG CAG T-GGG TTC CCT AGA GAG ACC CAG ACT CGA GAA AAG CAG CTG CA-3') into pG5-Luc reporter plasmid/EcoRI-BamHI (Promega, WI, USA).

Cell culture and transient transfection

293A cells and CV-1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 µg/ml antibiotics at 37°C. 1x10⁶ cells were inoculated into each well of 6 well culture dishes, and then allowed to grow for 20 hrs. At 80% confluency, cells were transfected with the plasmid mixture (0.2 µg of pGL2-basic *ADH5/FDH*-luciferase reporter plasmid, 0.4 µg of pcDNA3.0 Pokemon 1, (both the wild-type construct and the lysine mutants K61R, K354R, K371R, K379R, K383R, K396R, K486R, K487R, K536R, K539R expression plasmids), and 0.1 µg of pCMV-*LacZ* plasmid) by using Lipofectamine Plus (Invitrogen, CA, USA). After 48 hrs, the cells were harvested, lysed, and luciferase activities were measured. Luciferase activities were normalized to the total amount of total protein used in the luciferase assay because the CMV promoter-driven expression of β -galactosidase which we used as transfection efficiency normalization control is suppressed by overexpression of SUMO-1 and Pokemon 1.

Site-directed mutagenesis

To prepare the FLAG-Pokemon 1 mutant form and SUMO-1 mutant form, we used the Quick Change site-directed mutagenesis kit (Stratagene, CA, USA). The following oligonucleotide primers were used in mutagenesis (only the sense strand is shown). K61R, 5'-AGC CAG TAC TTC AAG AGG CTG TTC ACG TCG GGC-3'; K354R, 5'-ATG ACT ACC TGAGGT ACT TCA GCG GCG C-3'; K371R, 5'-TCG CAGAAG GTG GAG AGG AAG ATC CGA GCC AAG-3'; K379R, 5'-AAG AAG ATC CGA GCC AGG GCC TTC CAGAAG TGC-3'; K383R, 5'-GCCAAG GCC TTC CAG AGG TGC CCC ATC TGC GAG-3'; K396R, 5'-ATC CAG GGC GCC GGC AGG CTG CCG CGA CAC ATC-3'; K486R, 5'-CTG CAC AGA CAC CTC AGG AAA GAC GGC TGC AAC-3'; K487R, 5'-CAC AGA CAC CTC AAG AGG GAC GGC TGC AAC GGC-3'; K536R, 5'-CGC AAC GGC CAG GAG AGG CAC TTA AGG ACG AG-3'; K539R, 5'-CAG GAG AAG CACT TTA GGG ACG AGG ACG AGG AC-3'; SUMO-1 mutagenesis G97A, 5'-CAG GAA CAA ACG GGG GCT CAT TCA ACA GTT TAG-3'.

Multiple mutagenesis reactions were performed with Quick Change® Multi Site-Directed Mutagenesis Kit (Stratagene, CA, USA). Using pcDNA3.0 Pokemon 1 K379R mutant plasmid, we

prepared the pcDNA3.0 Pokemon 1 K379R, K396R, K536R, K539R, with the mixture of K396R, K536R, K539R specific oligonucleotide mutagenesis primers described above, according to the protocol recommended by the manufacturer.

Western blot analysis

Nuclear extracts prepared from the stable 293T cells overexpressing FLAG tagged Pokemon 1 were immunoprecipitated with anti-FLAG-M2 agarose beads (Sigma, MO). The immunoprecipitates were separated by 8% SDS-polyacrylamide gel (135 volt for 1 hr) and transferred onto the Immuno Blot™ PVDF Membrane (BioRad, CA, USA). Next, the membrane was blocked with 5% Non Fat Dry Milk (Invitrogen Life Technologies, CA, USA) in TBST (25 mM Tris-HCl pH8.0, 140 mM NaCl, 0.05% Tween-20) for 2 hrs. After washing the membrane with TBST, the membrane was incubated with primary antibody (polyclonal anti-FLAG antibody or anti-Pokemon 1 antibody at 1:2000 dilution) at 4°C overnight, which was followed by washing with TBST, and incubation with the HRP conjugated anti-rabbit or anti-goat secondary antibody diluted at 1:5000. The membrane was visualized using the ECL Kit (PerkinElmer life sciences, MA, USA) with the LAS 3000 image reader (Fujifilm, Tokyo, Japan).

Immuno-precipitation assays

293T cells overexpressing FLAG tagged Pokemon 1 were washed, pelleted, and resuspended in lysis buffer supplemented with protease inhibitors (20 mM Tris HCl, pH7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100). Cell lysate was pre-cleared, and supernatant was incubated with anti-Flag antibody on a rotating platform overnight at 4°C, followed by incubation with protein G-Sepharose Fast Flow beads. Beads were collected, washed, and resuspended in equal volume of 5x SDS loading buffer. Immuno-precipitated proteins were separated by 12% SDS-PAGE. Western blot assay was performed as described above.

In vitro transcription and translation to make Pokemon 1 Wt and Sumo mutants

The *in vitro* translated Pokemon 1 Wt and sumoylation site mutant forms of Pokemon 1 (K61R, K354R, K371R, K379R, K383R, K396R, K486R, K487R, K536R and K539R) were prepared by incubating the PCR products (1 µg) with T_NT T7 Quick-coupled Transcription/Translation System (Promega, Madison, WI, USA).

In vitro Sumoylation assays

Sumoylation reactions were performed with Sumoylation kit (LAE, MD, USA) according to manufacturer's protocol in a total volume of 20 µl containing 5 µl of [³⁵S]-methionine labeled protein prepared by *in vitro* transcription and translation reactions at 50 µl scale. In each reaction, E2 (50 µg/ml) was added to substrate first, then SUMO-1 (50 µg/ml), then 10x reaction buffer (200 mM HEPES pH7.5, 50 mM MgCl₂, and 20 mM ATP), then E1 (7.5 µg/ml) and next 2 µl of 20 mM ATP. Finally, the reaction mixture was brought up to a final volume of 20 µl by adding H₂O. After the mixture was incubated at 37°C for 90 min., the reaction was subjected to 10% SDS-PAGE and

analyzed by autoradiography.

Purification of GST fusion protein

To generate the recombinant GST-POZ_{Pokemon 1} fusion protein, a cDNA fragment encoding the POZ domain (a.a.1-133) of Pokemon 1 was amplified by PCR and cloned into the pGEX4T3 expression plasmid. The GST-POZ_{Pokemon 1} protein was overexpressed in *E. coli* BL21 DE3 by incubating overnight with 0.2 mM isopropyl-D-thiogalactopyranoside at 18°C. Bacteria were collected by centrifugation and lysed by sonication in lysis buffer (1x PBS, 2 mM EDTA, and 0.1 mg of lysozyme/ml, 1 mM phenylmethylsulfonyl fluoride). The fusion protein was purified from the lysate by affinity chromatography using glutathione Sepharose 4B beads (Pepton, Daejeon, Korea).

Preparation of rabbit polyclonal antibody against GST-POZ_{Pokemon 1} protein

To generate an antibody against Pokemon 1, recombinant GST-POZ_{Pokemon 1} (1 mg) was mixed with an equal volume of complete Freund's adjuvant (CFA, Sigma, MO, USA), and injected into NAW (KBL) SPF rabbits subcutaneously. At two week intervals, GST-POZ_{Pokemon 1} (1 mg) mixed with complete Freund's adjuvant was injected to rabbits to boost the immune reaction. A week after the 4th injection, the rabbit was sacrificed and blood was collected. The blood was incubated for 30 min. at 37°C to clot, and placed at 4°C overnight for contraction of the clot. The serum was harvested from the clot and the remaining insoluble materials were removed by centrifugation at 12,000 rpm for 10 min. at 4°C.

To purify antibody from serum by affinity chromatography, purified GST-POZ_{Pokemon 1} (1 mg) was cross-linked to CNBr-activated Sepharose 4B beads. After excess proteins were washed away by washing the beads, the beads were incubated with rabbit serum for 3 hrs in the cold room. The beads were packed to the column and washed with a 20x volume of 10 mM Tris-HCl, pH7.5 and a 20x volume of 500 mM NaCl, 10 mM Tris-HCl, pH7.5. The antibody was eluted by passing a 2x volume of 100 mM glycine (pH2.5) through the column. The first eluate was collected in a tube containing 0.2 volume of 1M Tris-Cl, pH8.0. The column was washed with 10mM Tris-HCl, pH 8.8 until the pH rose to 8.8. The antibody was eluted by passing a 2x volume of 100 mM triethylamine (pH11.5, prepared fresh) through the column. The second eluate was collected in a tube containing a 0.2x volume of 1M Tris-HCl, pH8.0. Both antibody eluates were combined and dialyzed against 1x PBS with 0.02% sodium azide.

Mammalian Two-Hybrid assays between the Gal4-POZ_{Pokemon 1}-domain K61R and corepressors

African green monkey kidney cells (CV-1) were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml of streptomycin, and 100 units/ml of penicillin. The cells were inoculated on 6-well tissue culture plates, at a density of 2.5x 10⁵ cells/well in 2 ml of DMEM. After the cells had grown for 16 hrs, they were transiently transfected with the Gal4-POZ-domain from Pokemon 1 Wt or K61R mutant expression plasmid (0.4 µg), the VP16

fusion effector plasmids of corepressors (0.4 µg), and the reporter plasmid pG5-*Luc* (0.4 µg), using Lipofectamine Plus reagent (Invitrogen, CA) in serum-free OPTI-MEM, according to the manufacturer's recommendations. After 3 hrs, the cells were supplied with fresh, complete DMEM, and allowed to grow for 36 additional hrs. The cells were then harvested and lysed in 100 µl of reporter lysis buffer (Promega, WI), vortexed for 1 min., and then centrifuged at 12,000 rpm for 15 min. at 4°C. The luciferase reporter assays were performed with 5 µl of cell extracts, using 50 µl of luciferase assay reagent (Promega, WI) on a luminometer (Microplate Luminometer LB 96V, EG&G Berthold, MD). Luciferase activities were normalized with the amount of protein used in the assay because Pokemon 1 affects β-galactosidase expression.

EMSA of Pokemon 1 zinc-finger DNA binding domain with lysine to arginine mutations

EMSAs were carried out as described previously [6]. The FRE5 probe of *p21^{Waf/Cip1}* that strongly binds Pokemon 1 was used for EMSA and the sequence of the probe is shown below. Annealed oligonucleotide probe was labeled with [³²P]-dATP and Klenow fragment. FRE5, 5'-GAT CCG AGC GCG GGT CCC GCC TC-3'. Antisense, 5'-GAT CGA GGC GGG ACC CGC GCT CG-3'. Each binding reaction was carried out in 20 of binding buffer containing 10 mM HEPES (pH7.9), 60 mM KCl, 5 µM ZnCl₂, 1 mM dithiothreitol, 1% BSA, 7% glycerol, 10 µl of in vitro translated product of Pokemon 1ZFDBD Wt or Sumo mutant, and 50,000 cpm labeled FRE5 probe. The mixture was incubated at room temperature for 30 min.

Preparation of Pokemon 1 zinc-finger DNA binding domain by in vitro transcription and translation

To prepare the various synthetic ZFDBD (a.a. 349-584) of sumoylation mutants (K354R, K371R, K379R, K383R, K396R, K486R, K487R, K536R and K539R) and wild type Pokemon 1 from *in vitro* transcription and translation for EMSA, PCR products of zinc finger DNA binding region tagged with T7 promoter sequence at the 5' end and Flag peptide encoding sequence at the 3'-end were prepared using following oligonucleotides. Forward, 5'-TAA TAC GAC TCA CTA TAG GGA CTT GCA CCATGG ACTACT ACC TGAAGT ACT TC-3'; Reverse, 5'-GTT AAC TCT AGA TTA CTT GTC ATC GTC GTC CTT GTAGTC GGC GAG TCC GGC TGT GAAGTT ACC GTC-3'. PCR was performed by denaturing at 94°C for 5 min, 30 cycles of amplification reaction (94°C for 30 s, 55°C for 30 sec, and 72°C for 1.5 min), and a final extension at 72°C for 4 min.

The *in vitro* -translated ZFDBD of Pokemon 1 Wt and sumoylation site mutant forms of Pokemon 1 (K354R, K371R, K379R, K383R, K396R, K486R, K487R, K536R and K539R) were prepared by incubating the PCR products (800 ng) with 50 µl of total reaction mixture of T_NT T7 Quick-coupled Transcription/Translation System (Promega, Madison, WI, USA).

Immono-histochemistry

CV-1 cells were grown on coverslips (Sunshine Woks, Korea) and transfected with pcDNA3.0 Pokemon 1-Flag alone

or with pcDNA3.0 SUMO-1-HA expression plasmids using Lipofectamine Plus reagent (Invitrogen, CA) and grown for 24 hrs. CV-1 cells were washed with cold PBS and fixed in cold methanol:formaldehyde (99:1) for 20 min at -20°C. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and the cells were incubated in blocking solution (5% goat serum in PBS). Fixed cells were incubated in a wet chamber with the mouse primary antibody against Flag tag (to final 5 µg/ml) in incubation solution (1% BSA, 0.02% sodium azide in PBS) for 2 hrs at room temperature. Cells were then rinsed in incubation solution three times 10 min each at room temperature and incubated further with mouse secondary antibody conjugated with FITC (final 5 µg/ml) (Jackson Immunoresearch Lab, West Grove, PA, USA) in 50% glycerol for 1 hr at room temperature. The cells were washed with PBS 5 times, 5 min each.

For double staining of the cells with the other antibody, the cells were washed with PBS and fixed by incubating with 3.7% formaldehyde solution for 10 min at room temperature. The cells were incubated with blocking solution for 30 min at room temperature. Cells were then incubated with a different primary antibody (rabbit anti-HA antibody, to final 5 µg/ml) in incubation solution for 2 hrs at room temperature and rinsed with incubation solution three times 10 min each at room temperature. After that, cells were further incubated with the secondary antibody (anti-rabbit antibody conjugated with Rhodamine, final 5 µg/ml)(Jackson Immunoresearch Lab, West Grove, PA, USA) in 50% glycerol for 1 hr at room temperature. Cells were then washed three times with PBS 5 min each at room temperature and mounted with mounting medium (Sigma Diagnostics, St. Louis, MO, USA) on the cover slip. Cells were examined with a Carl Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

Results

Identification of potential sumoylation target sites of Pokemon 1

To identify which lysine residues of Pokemon 1 could be modified by SUMO-1, we searched the amino acid sequence of the Pokemon 1 protein for sequences that match the sumoylation consensus sequence, ψKXE (ψ, hydrophobic amino acid, K, lysine, X, any amino acid, E, glutamic acid) using SUMOplot™ program provided by ABGENT (<http://www.abgent.com/tool/sumoplot>). Ten potential sumoylation sites were identified (Fig. 1A) with different likelihoods of being sumoylated (Fig. 1B). Six potential sumoylation sites out of ten sites were either juxtaposed or overlapping with other potential sumoylation sites. According to the program, the lysine residues with high SUMO scores have a high possibility to be sumoylated. The prediction of the SUMOplot™ program shows the following scores for various lysine residues, 0.91, K486; 0.85, K539; 0.57, K396; 0.56, K354; 0.50,

Fig. 1. Identification of potential sumoylation target sites of Pokemon 1. (A) SUMOplot™ analysis results of Pokemon 1 amino sequence (Genbank accession number, GI:O95365). (B) The potential sumoylation sites of Pokemon 1 are ordered by predicted likelihood of sumoylation. The gray boxes indicate the target lysine residues of potential sumoylation sites. (<http://www.abgent.com/tool/sumoplot>). Thick solid line, BTB/POZ-domain; thin double dotted line, zinc-finger DNA binding domains; single waved dotted line, nuclear localization sequence (NLS).

K371; 0.44, K383; 0.39, K379; 0.31, K487; 0.15, K536; 0.13, K61 (Fig. 1B).

Interestingly, the lysine residue 61 amino acids from the N-terminus is located within the BTB/POZ-domain (a.a. 7-131, underlined by thick solid line), which was shown previously to be important in the transcriptional repression and interaction with corepressors [6, 20]. Sumoylation at the K61 residue can be important in the transcriptional repression. Also, six potential sumoylation sites are located within or at the position proximal to the C₂H₂ type zinc-finger DNA binding domain (a.a. 382-490, double underlined). Sumoylation of the sites may be important in Pokemon 1's recognition of target binding sites (FRE). Also, we noticed that sumolyation sites 7 and 8 (K487, K486) are partially overlapping with the nuclear localization sequence (a.a. 487-505, waved underline) of Pokemon 1.

Pokemon 1 is potentially modified by SUMO-1 in vivo

We investigated whether Pokemon 1 is modified by sumoylation. We prepared a human kidney 293T TREX cells in which Pokemon 1 can be expressed upon induction. The 293T TREX Pokemon 1 stable cells have a doxycycline-inducible Pokemon 1 gene integrated into a chromosome and the cells express low levels of Pokemon 1 in the absence of inducer due to leaky expression. 293T cells do not express endogenous Pokemon 1 at high enough level to be detected by Western blot analysis (using the total cell lysate and an anti-FLAG antibody in the absence or presence of Doxycyclin (Fig. 2A. lanes 1, 2). Doxycycline inducer dramatically induces expression of Flag-tagged Pokemon 1 at the position corresponding to 80 kDa (Fig. 2A. lanes 3, 4), despite the predicted molecular weight of 64 kDa. Interestingly,

Fig. 2. Pokemon 1 is sumoylated *in vivo*. (A) Western blot analysis of human kidney 293TREX cells which overexpress Pokemon 1 tagged with Flag. Pokemon 1 proteins running considerably slow are indicated with asterisks. (B) Western blot analysis of Pokemon 1 immunoprecipitate with anti-FLAG antibody shows that Pokemon 1 is sumoylated. GAPDH was used as a loading control to indicate that the same amount of protein is loaded onto the SDS-PAGE gel.

Pokemon 1 runs significantly slower in SDS-PAGE gel. When Pokemon 1 was overexpressed, we detected an additional two upper bands indicated by arrows, which are posttranslationally modified, and are potentially the sumoylated forms of Pokemon 1 (Fig. 2A, lane 4).

To demonstrate that the upper bands are the sumoylated form of Pokemon 1, we prepared nuclear extract of 293T TREX FLAG tagged-Pokemon 1 stable cells induced with doxycyclin treatment. The nuclear extract was immunoprecipitated with anti-FLAG antibody-agarose beads. The precipitates were analyzed by Western blotting using antibodies against SUMO, FLAG tag, and GAPDH. Indeed, Western blot analysis of the immunoprecipitate with anti-SUMO antibody revealed the protein band at 97-100 kDa, a band responsible for sumoylated form of Pokemon 1. Other upper bands may be posttranslationally modified form of Pokemon 1 and appears to be too low in amount to be detected by Western blot analysis (Fig. 2B).

Pokemon 1 is modified by SUMO-1 in vitro

To test whether Pokemon 1 can be directly sumoylated *in vitro*, we prepared wild-type Pokemon 1 and the sumoylation mutant proteins, Pokemon 1 K61R, K354R, K371R, K379R, K383R, K396R, K486R, K487R, K536R and K539R by *in vitro* transcription/translation system in the presence of [³⁵S]-methionine. The *in vitro* synthesized [³⁵S]-labeled Pokemon 1 or the SUMO-mutant forms of Pokemon 1 were used in the *in vitro* sumoylation assay by incubating them with the sumoylation reaction mixture which was comprised of ATP, SUMO-1, E1 (SAE1/2), and E2 (Ubc9). SDS-PAGE of the Pokemon 1 Wt sumoylation reaction mixture and autoradiography showed several bands that moved very slowly, and were not detected in the control reaction. The data clearly show that Pokemon 1 can be a substrate for sumoylation (Fig. 3). To investigate which lysine residues (those that have possibility of being sumoylated)

are, in fact, sumoylated, [³⁵S]-labeled synthetic Pokemon 1 with lysine to arginine mutations at various positions (lysine at 61, 354, 371, 379, 383, 396, 486, 487, 536 and 539) were used for *in vitro* sumoylation assays (Fig. 3).

When Pokemon 1 Wt was subjected to sumoylation, a new band with slow mobility at 150 kDa was detected. The prominent new band migrated more slowly than unmodified Pokemon 1 (about 80 kDa). Pokemon 1 with mutations at various lysine residues also showed upper bands with slow mobility, which indicates that all the lysine mutants of Pokemon 1 are still sumoylated and the single mutation is not sufficient to abolish sumoylation completely in most cases, with the notable exception of K61R (Fig. 3A). This result suggests that when Pokemon 1 is mutated at only one lysine, this alteration is not enough to inhibit the sumoylation of Pokemon 1. It appears that Pokemon 1 might be sumoylated at multiple lysine residues.

Sumoylation of Pokemon 1 affects the transcriptional activation of the ADH5/FDH gene

To examine whether SUMO-1 modification affects the ability of Pokemon 1 to regulate transcription, we compared the transcriptional repression activity of Pokemon 1 Wt with that of various sumoylation mutants K61R, K354R, K371R, K379R, K383R, K486R, K487R, K536R and K539R. We used the *ADH5/FDH-Luc* gene fusion reporter plasmid which was reported to be

Fig. 3. Pokemon 1 is modified by SUMO-1 *in vitro*. (A) *In vitro* sumoylation assay was performed with wild type or mutants of synthetic Pokemon 1. [^{35}S]-methionine-labeled Pokemon 1 was prepared by *in vitro* translation. Sumoylation was assayed in the presence or absence of purified E1, E2, and SUMO-1. Full-length Pokemon 1 and Pokemon 1–SUMO-1 conjugates are indicated with arrows. Input indicate the amount of various [^{35}S]-methionine-labeled Pokemon 1 polypeptides used in sumoylation reaction.

repressed by Pokemon 1 previously (Fig. 4A) (6). CV-1 cells were transfected with the *ADH5/FDH-Luc* reporter plasmid and either the Wt or the sumoylation mutant Pokemon 1 expression plasmids (Fig. 4B). Pokemon 1 Wt potently repressed transcription in CV-1 cells by more than 90%. Pokemon 1 mutants were less effective in transcription repression compared to Pokemon 1 Wt, suggesting sumoylation of Pokemon 1 is important in transcriptional repression. Most of the Pokemon 1 mutants, such as K379R, K396R, K536R and K539R, showed 2.2 fold to 3.3 fold weaker transcriptional repression compared to Pokemon 1 Wt (Fig. 4B). Interestingly, the lysine mutations located within or proximal (K379R, K396R) to the DNA binding domain or C-terminal domain (K536R, K539R) showed greater effect on transcription repression by Pokemon 1 and appear to be important in the transcriptional regulation of this Krüppel-like zinc-finger protein. Other Pokemon 1 mutants (K61R, K354R, K371R, K383R, K487R) also weakly repressed transcription (by between 1.4–1.8x). The decreased ability to repress transcription observed in Pokemon 1 mutants suggests that sumoylation at several lysine residues is required for transcriptional

repression by Pokemon 1. Sumoylation of several lysine residues of Pokemon 1 may be necessary to achieve full repressive activity.

Sumoylation affected the ability of Pokemon 1 to activate the artificial Pokemon 1 target pG5-FRE-Luc and triple lysine mutants were weak transcriptional repressors

To investigate the effect of sumoylation on Pokemon 1, we prepared the reporter plasmid, pG5-FRE-Luc, containing FRE (Pokemon 1 or FBI-1 response element) inserted in front of adenovirus major late minimal promoter of pG5-Luc (Fig. 5A). The FRE site is from the well-characterized Pokemon 1 binding site located in the IST of HIV-1 LTR promoter [2, 18]. First, we cotransfected the Pokemon 1 Wt and SUMO-1 expression plasmids into CV-1 cells. In the absence of Pokemon 1, the transcription of pG5-FRE-Luc was not affected by SUMO-1 (Fig. 5B, lane 1, 2). Interestingly, Pokemon 1 Wt alone transactivated transcription by 5 fold on the pG5-FRE-Luc, suggesting a nascent transcription activation potential of Pokemon 1, as with many other transcription factors that act either as transcription

Fig. 4. K to R mutations derepress the transcription repression by Pokemon 1. (A) Schematic representation of *ADH5/FDH-Luc* reporter plasmid. FRE, Pokemon 1 binding element; GC-box, Sp1 binding GC-box; Tsp, transcriptional start point marked as +1; Luc, Luciferase gene. (B) K to R mutations of potential sumoylation sites weakened transcription repression potential of Pokemon 1. CV-1 cells were co-transfected with the luciferase reporter plasmid (*ADH5/FDH-Luc*) and wild-type Flag-Pokemon 1 or sumoylation mutant Flag-Pokemon 1 (K61R, K354R, K371R, K379R, K383R, K396R, K486R, K487R, K536R and K539R) expression plasmids.

activators or repressors [19]. However, sumoylation of Pokemon 1 by ectopic SUMO-1 increased the repressor potential of Pokemon 1 by 60% on the test promoter, again suggesting the importance of sumoylation of Pokemon 1 in transcriptional repression (Fig. 5B).

To demonstrate the cumulative effect of lysine-to-arginine mutations at four key potential sumoylation sites, we prepared the mammalian expression plasmids of Pokemon 1 mutants at K379, K396, K536, K539 and cotransfected these plasmids with a pG5-FRE-Luc reporter plasmid. The mutations increased the transcription of the reporter gene by 2.5 fold and 2 fold for Pokemon 1 K379/K369/K539 and Pokemon 1 K379/

Fig. 5. Sumoylation site mutations affect the transcriptional activity of Pokemon 1 on the artificial Pokemon 1 responsive pG5-FRE-Luc plasmid and Pokemon 1 triple lysine mutants showed weaker transcription repression activity. (A) Schematic representation of pG5-FRE-Luc. (B) SUMO-1 inhibits transcription in the presence of Pokemon 1. CV-1 cells were co-transfected with the luciferase reporter plasmid (pG5-FRE-Luc) and wild-type Flag-Pokemon 1 and in the absence or presence of mammalian expression vector for His-SUMO-1, as indicated. (C) CV-1 cells were co-transfected with the luciferase reporter plasmid (pG5-FRE-Luc) and wild-type FLAG-Pokemon 1 and mutant Pokemon 1 with mutations at the key sumoylation sites are indicated. Pokemon 1_K379/K369/K539 and Pokemon 1_K379/K369/K536. FRE, Pokemon 1 responsive element; Tsp, transcriptional start point marked as +1; Luc, Luciferase gene.

K369/K536, respectively. The data suggest that proper sumoylation of Pokemon 1 by SUMO-1 is important in transcriptional repression of this particular test promoter with FRE (Fig. 5C).

EMSA showed that mutation of the lysine residues located within or proximal to the zinc-finger DNA binding domain did not affect Pokemon 1 ZFDBD binding to the FRE element

Interestingly, we found that the six potential sumoylation sites are located within or at the position proximal to the C₂H₂ type zinc-finger DNA binding domain (a.a. 382-490). In addition, the mutation of K into

Fig. 6. EMSA of FRE5 probe of the *p21^{Waf/Cip1}* promoter with various the zinc-finger DNA binding domain (ZFDBD) of Pokemon 1. Synthetic ZFDBDs of Pokemon 1 were prepared by *in vitro* transcription/translation and sumoylated *in vitro*.

R significantly affected transcription repression by Pokemon 1. We suspected that potential sumoylation of the lysine residues could affect the DNA binding activity of Pokemon 1. Accordingly, we prepared the zinc-finger DNA binding domain of Pokemon 1 (ZFDBD) by *in vitro* transcription and translation and used the construct for EMSA assays using the recently characterized FRE element of the *p21^{Waf/Cip1}* promoter that binds Pokemon 1 strongly (Choi et al. in preparation). Overall EMSA data showed that mutation of sumoylation sites located within or proximal to the zinc-finger DNA binding domain did not affect the molecular interaction between the Pokemon 1 ZFDBD and the FRE element (Fig. 6).

Sumoylation affected the molecular interaction between the POZ-domain and the corepressors, SMRT/NCoR, and BCoR

We found that the POZ-domain of Pokemon 1 interacts with the corepressors such as SMRT, NCoR, and BCoR (Choi et al. in preparation), which could be important in transcriptional repression, as is the case with Bcl-6 and PLZF [20, 21]. Also, others have shown that interaction with co-repressors can be modulated by sumoylation [14, 22].

Interestingly, the lysine residue at the position 61 amino acids from the N-terminus is located within the BTB/POZ-domain (Fig. 1, a.a. 7-131), which was shown previously to be important in the transcription repression [6] and interaction with corepressors (Choi et al. in preparation). We suspected that the sumoylation at the K61 residue might be important in transcriptional regulation of Pokemon 1 responsive genes by either increasing or

decreasing the molecular interaction between the corepressors and the BTB/POZ-domain. Accordingly, using a mammalian two-hybrid protein-protein interaction assay system, we investigated the effect of K61R mutation on molecular interactions (Fig. 7A). The POZ-domain of Pokemon 1 interacts with corepressors such as SMRT, NCoR and BCoR. Interaction with mSin3A is quite weak. However, molecular interaction with BCoR is strong. Interestingly, the POZ-domain with the K61R mutation showed relatively weak molecular interaction with the corepressors (29-59%) compared to the Wt, suggesting sumoylation at the K61 site is important in molecular interaction with corepressors and eventually in transcription repression by Pokemon 1 (Fig. 7B).

Pokemon 1 and SUMO-1 are co-localized in the nucleus and mutations of the lysines at amino acids 486 and 487, which are juxtaposed to the NLS, does not affect nuclear localization of Pokemon 1

The *in vitro* sumoylation (Fig. 3) evidence above suggests that SUMO-1 and Pokemon 1 likely interact with each other. To further confirm this, we asked whether SUMO-1 and Pokemon 1 are colocalized in cells using confocal microscopy. We co-transfected CV-1 cells with pcDNA3.0 Pokemon 1-Flag or the SUMO-1-HA expression vector. Immunohistochemistry of the transfected cells showed that ectopic SUMO-1 and Pokemon 1 are detected and colocalized in the nucleus, suggesting potential molecular interaction between SUMO-1 and Pokemon 1 (Fig. 8A).

We noticed that sumoylation sites 7 and 8 (K487,

Fig. 7. Sumoylation affected the molecular interaction between the POZ-domain and the corepressors, SMRT/ NCoR, and BCoR. (A) Scheme of Mammalian two-hybrid assays involving the POZ-domain of Pokemon 1 and the corepressors. (B) The Wt POZ-domain interacts with the corepressor BCoR most strongly, which is followed by SMRT. The K61R mutant POZ-domain showed much weaker molecular interaction with the corepressors. X, corepressor.

Fig. 8. Immunohistochemistry. (A) Pokemon 1 and SUMO-1 are co-localized in the nucleus. CV-1 cells were transfected with pcDNA3.0 Pokemon 1-Flag and pcDNA3.0 SUMO-1-HA expression plasmids. Cellular localization of the two proteins were detected by immunohistochemistry using antibody against Flag or HA epitopes. (B) Mutations of the lysines at a.a. 486 and 487, which are juxtaposed to the NLS, do not affect nuclear localization of Pokemon 1. CV-1 cells were transfected with pcDNA3.0 Pokemon 1-Flag Wt or K to R sumoylation mutants and pcDNA3.0 SUMO-1-HA expression plasmids. Cellular localization of Pokemon 1 was monitored by immunohistochemistry using an antibody against the Flag epitope.

K486) partially overlap with the nuclear localization sequence (a.a. 487-505, waved underline) of Pokemon 1 (Fig. 1A). The modification might alter the nuclear localization of the transcription factor. To investigate whether sumoylation affects the nuclear translocation of

Pokemon 1, we co-transfected CV-1 cells with either the pcDNA3.0 Pokemon 1 Wt, Pokemon 1 K486R or K487R mutant expression vector along with the SUMO-1 expression vector. The SUMO-1 expression plasmid was introduced into cells to enforce sumoylation of

Pokemon 1. Pokemon 1 and Pokemon 1 mutants were tagged with the FLAG epitope and SUMO-1 was marked with an HA tag. Immunohistochemistry of the transfected cells showed that the mutation at K486 or K487 located very proximal to the NLS, did not affect the nuclear localization of Pokemon 1 and the colocalization of the two proteins (Fig. 8B, C).

Discussion

Pokemon 1 was shown to regulate transcription of the *ADH5/FDH* promoter, HIV-1 LTR, and ARF promoters [4-6]. Pokemon 1 has many functions and is involved in cellular development and differentiation processes, such as adipogenesis, osteogenesis, and oncogenesis [4, 7, 8]. Frequently, SUMO target proteins are transcription factors, transcription co-activators, and co-repressors [9-16]. Sumoylation has recently emerged as an important mechanism in transcriptional control [15, 16].

Pokemon 1 has as many as ten lysine residues which match closely with the sumoylation consensus motif, ψ KXE, and is likely sumoylated, as suggested by our *in vitro* sumoylation and transcription assays. To test whether Pokemon 1 is modified by SUMO-1 *in vivo*, we overexpressed Pokemon 1 tagged with Flag in 293T cells. Western blot analysis of the total cell lysate with anti-FLAG antibody, showed slow moving upper bands, potential evidence for sumoylation of Pokemon 1. Also more clearly, the *in vitro* sumoylation assay using [³⁵S]-labeled Pokemon 1 Wt and sumoylation mutants, showed that Pokemon 1 can be sumoylated. In addition, it is possible that all sites tested might be sumoylated because none of the mutations resulted in major changes in the sumoylation patterns.

With the pG5-FRE-*Luc* test promoter, we observed that Pokemon 1 alone transactivates by 5 fold, but the transcription was repressed upon cotransfection with SUMO-1 expression plasmids. In addition, SUMO-1 has no effect on the control pG5-FRE-*Luc*, suggesting that SUMO-1 increased repressor activity of Pokemon 1, probably by sumoylating Pokemon 1.

Our mass MALDI-TOF mass spectrometry analysis of immunoprecipitate done with antibody against Pokemon 1 showed that Pokemon 1 was detected as multiple spots at various positions differing greatly in molecular weight and mobility on a two dimensional gel. The data suggest that probably Pokemon 1 is posttranslationally modified in many ways, one of which

may be sumoylation (unpublished data).

How sumoylation alters the activity of Pokemon 1 was not known before. Also, most of the sumoylation sites are located within or proximal to the domains critical for regulating transcription and molecular interactions with other regulatory proteins, and is also in close proximity to the zinc-finger DNA binding domain (sites 3-8) and nuclear localization sequences (sites 7, 8). Considering the locations of the sumoylation sites, the modification could change molecular properties of Pokemon 1 important in carrying out the biological functions by Pokemon 1 such as interaction with other regulatory proteins, recognition of its target DNA sequences, nuclear localization, and homo- and hetero- dimerization.

To determine the significance and biological function of the sumoylation sites in Pokemon 1, we mutated each of the ten lysine residues and investigated the biological function of the protein with the induced mutation. Mutation of any single lysine residue did not drastically alter the overall sumoylation pattern, DNA binding activity, or cellular localization, but did show some effect on the transcriptional repression on the test promoters and on molecular interactions with the corepressors. Overall, most of the the lysine residues appear important in Pokemon 1's ability to regulate transcription.

SUMO-1 modification of most proteins requires a SUMO-1 recognition motif displayed on the surface of the target protein. The two lysine residues at K486 and 539, have the highest probability of being displayed on the surface according to the surface probability prediction made by the MacVector7.2 program analysis. Although the SUMOplot™ prediction program predicted that the two lysine residues at 486 and 539 are the ones most likely to be sumoylated, transcription and *in vitro* sumoylation assays showed mixed results. Interestingly, although the K486 residue has both high probability of being sumoylated and high probability of being on the surface of the folded protein, the K to R mutation does not show any significant effect on sumoylation, transcription repression, or in DNA binding according to the EMSA assay (Figs. 3, 4, 6). However, the K539 residue also had high predicted values for sumoylation and surface presentation, but this mutant did show much weaker transcription repression potential, suggesting sumoylation at this particular site is important in transcription repression. Although we suspected that lysines 486 and 539 may be the major sumoylation sites because of their high predicted value for sumoylation, our transcription, sumoylation and other data suggest that the two sites may not necessarily be the major sumoylation

sites. Sumoylation mutation at K61, 379, 396, 536, and 539 resulted in derepression of transcription driven by the *ADH5/FDH* test promoter. In particular, the K396R mutant showed the strongest effect on transcription derepression. Coincidentally, K396 lies in the middle of the first C₂H₂ zinc-finger motif. Previously, we have shown that zinc-fingers of the Sp1 family interact with the corepressors and the interaction is controlled by MEK, which mediates transcriptional regulation of genes of the Sp1 family by binding to GC boxes in their promoters [23]. Sumoylation of the K396 might be important in interaction with corepressors and thus the DNA binding activity of the Pokemon 1. Multiple mutation, as in the K379R/K369R/K539R mutant, resulted in more potent derepression, suggesting additive effects of multiple sumoylation sites.

Others have shown that sumoylation is important in interactions with corepressors, an important component of transcriptional repression [14, 22]. Also, we found that the POZ-domain of Pokemon 1 plays an important role in transcription repression by interacting with corepressors such as BCoR, NCoR, and SMRT (Choi et al. in preparation). Interestingly, the lysine residue at position 61 lies in the middle of the POZ-domain of Pokemon 1, which is potentially important in the molecular interaction with other molecules and also in dimerization. K61 is not important in the interaction with other molecules or in the overall structure of the domain [24]. However, the K61R mutant of the POZ-domain showed significantly weak (26-59% of the control Wt) molecular interaction with co-repressors, compared to the Wt POZ domain, suggesting sumoylation is important in the molecular interaction with co-repressors and thus in transcription repression.

The data suggest that Pokemon 1 can be sumoylated

at multiple sites due to a conserved sumoylation motif. Sumoylation of some of these sites appear to be important in transcription repression by Pokemon 1. Sumoylation may be one of the novel regulatory mechanisms regulating Pokemon 1 activity.

Abbreviations

ADH5/FDH (alcohol dehydrogenase5/formaldehyde dehydrogenase); bp (base pair); BTB/POZ (bric-à-brac, tramtrack and broad complex and pox virus zinc finger); CMV (cytomegalovirus); CV-1 (African green monkey kidney cell); EMSA (Electrophoretic Mobility Shift Assay); FBI-1 (factor that binds to the inducer of short transcripts of human immunodeficiency virus-1); FBS (fetal bovine serum); FRE (Pokemon 1 binding sites); GST (glutathione S-transferase); HDAC (histone deacetylase); HIV (human immunodeficiency virus); IP (immunoprecipitation); LTR (long terminal repeats); PCR (polymerase chain reaction); PLZF (promyelocytic leukemia zinc finger); Pokemon (POK erythroid myeloid ontogenic factor); SAGE (serial analysis of gene expression); SDS-PAGE (Sodium-dodecyl sulfate-polyacrylamide gel); SUMO (small ubiquitin like modifiers); UAS (upstream activator Gal4 binding sequence); ZFDBD (zinc finger DNA binding domain).

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