Regulation of FBI-1 Activity by Sumoylation

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Regulation of FBI-1 Activity by Sumoylation

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Abstract

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FBI-1 is a transcription regulator with POZ domain at N-terminus and four Kruppel-like zinc fingers at C-terminus. It regulates transcription of the *ADH5/FDH* promoter and HIV-1 LTR promoter. Recently, it was suggested that it plays important role in adipogenesis, osteogenesis, oncogenesis, and transcription of NF-kB responsive genes.

Sumoylation modulates cellular functions of many proteins by influencing the activity of transcription regulators, subcellular localization, and stability. Unlike ubiquitination, sumoylation does not lead to protein degradation by proteasome. In general, SUMO seems to decrease transcription while ubiquitin increases transcription. The sumoylation of many transcription factors are reported recently.

We investigated whether FBI-1 could be post-translationally modified by sumoylation and the modification could affect the transcription properties of FBI-1. Upon careful analysis of amino acid sequence of FBI-1, we found 10 potential sumoylation sites located at lysine 61, 354, 371, 379, 383, 396, 486, 487, 536 and 539. We mutated each these amino acids into arginine and tested whether the mutant FBI-1 could affect the transcription properties of FBI-1 on the FBI-1 responsive gene, such as *ADH5/FDH*. Wild type of FBI-1 potently represses transcription of *ADH5/FDH*, but some mutants were much weaker transcription repressors (more than 2.2 fold to 3.3 fold than wild type, respectably mutants K379R, K396R, K536R, and K539R). And other mutants also showed weak repression than wild type (more than 1.3 fold to 1.8 fold than wild type). It suggests that sumolyation might be important in transcription repression by FBI-1.

We also found that FBI-1 is sumoylated *in vivo* and FBI-1 activity as transcription factor may be regulated by sumoylation *in vivo*.

Key Words : FBI-1, SUMO-1, transcriptional repression, sumoylaion.

Regulation of FBI-1 Activity by Sumoylation

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I. Introduction

FBI-1 (Factor that Binds to Inducer of Short Transcripts or also called Pokemon: POK erythroid myeloid ontogenic factor; LRF10: leukemia/lymphoma related factor LRF; OCZF: Osteoclast-derived zinc finger protein) is a member of the POK (POZ and Krüppel) family of transcriptional repressor, which has a POZ domain at N terminus and four Krűppel-like zinc fingers at C-terminus[1-3]. The POZ/BTB domain mediates homodimerization and heterodimerization, and

recruitment of corepressors/HDAC complexes to these proteins. The COOH-terminal zinc fingers mediate sequence specific DNA recognition and binding. Recent reports have uncovered essential roles for POK proteins in development, differentiation, and oncogenesis[3].

FBI-1 was originally identified as a protein that regulates the transcription by binding to the IST (inducer of short transcripts) element of the HIV-1 (human immunodeficiency virus, type 1) LTR promoter. FBI-1 activates the binding activity of Tat to HIV-1 LTR[3], and binds to the proximal promoter of *ADH5/FDH* gene and represses the transcription by interacting with Sp1 zinc finger DNA binding domain[4]. Also it facilitates adipogenesis of preadipocyte[6]. Also, rat homologue, OCZF (osteolast-derived zinc finger) is a transcriptional repressor and appears to be involved in osteogenesis[9]. SAGE (Serial Analysis of Gene Expression) analysis showed that the expression of FBI-1 is high in cancer, suggesting that FBI-1 may play a role as a proto-oncogene (available at www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Hs&CID=104640). Inactivation of FBI-1 in mouse resulted in embryonic lethality due to severe anemia and profoundly impaired cellular differentiation in multiple tissues. And overexpression of FBI-1 caused cancer in multiple tissues[3]. So FBI-1 could be an attractive therapeutic target for human cancer therapy in view of its essential role in oncogenic transformation[3].

SUMO (small ubiquitin-related modifier) is a protein of 97 amino acids that has been called by other names including Smt3p, Pmp2p, PIC-1, GMP-1, Ubl1, and Sentrin[8]. It has only 18% of low amino acids sequence homology but the folding structure of SUMO is very similar to that of ubiquitin (Fig.1), and recognizes the same lysine residue of target protein. SUMO-1 has long and flexible N-terminal domain, which is absent in ubiquitination. Despite the similar protein folding, the distribution of charged residues on the surface is very different from that of ubiquitination. This difference may attribute the unique activities to SUMO[9]. Unlikely ubiquitin, SUMO does not lead to protein degradation by proteasome. SUMO reversibly modifies many proteins important for regulated gene expression, including transcription factors, cofactors, and regulators of chromatin structure, and signal transduction[8]. 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 this function is potentially regulated by SUMO modification (Table 1)[10, 11].

Like ubiquitination, SUMO is covalently conjugated to target proteins. The SUMO-1 interacting amino acid sequence motif comprises the sequence ψKXE (ψ, a large hydrophobic residue, K, lysine to which SUMO-1 is conjugated, X, any amino acid, and E, glutamic acid; http://www.elm.eu.org/ elmPages/MOD_SUMO.html).

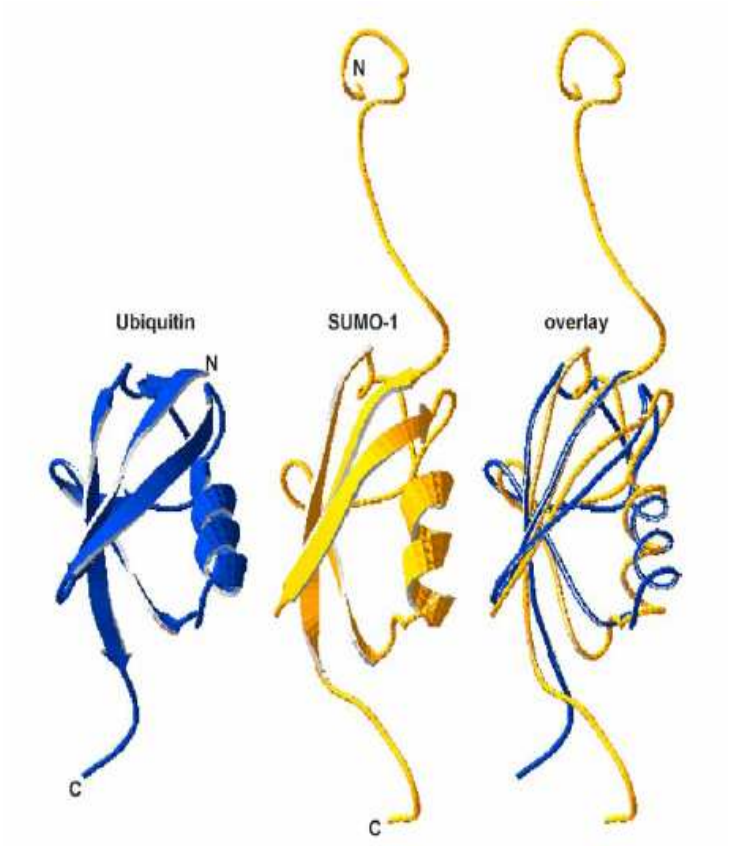

Figure 1. Structure comparison of ubiquitin and SUMO-1[10]. Ubiquitin is composed of C-terminus β-sheets half and N-terminus α-helix half. SUMO is structurally very similar to ubiquitin but has additional N-terminal amino acids stretch. Both proteins share a characteristic tightly packed ββαββαβ fold, and a C-terminal di-glycine motif. SUMO is distinguished by a long and flexible N-terminal extension.

Table 1. Examples of proteins modified by SUMO and ubiquitin with different consequences.[7]

Protein	Function	Role of SUMOylation	Role of ubiquitination	Common lysine?
IKBα	Signal transduction/ Inhibition of NFκB	Stabilizes IkBa by competition with ubiquitin	Promotes proteasome-mediated degradation	Yes
NEMO	Signal transduction/ IKK regulation	Promotes nuclear localization	Required for IKK activation	
PML	Tumor supressor	Regulates subnuclear localization, required for integrity of nuclear bodies	Promotes proteasome-mediated degradation	Yes
P53	Transcription factor tumor supressor	Variable effects observed; reduces transcriptional activation in some contexts	Promotes proteasome-mediated degradation	
Glococorticoid receptor (GR)	Transcription factor	Reduces transcriptional activation dependent on promoter context	Promotes proteasome-mediated degradation	
HDAC	Histone deacetylase /transcriptional corepressor	Promotes deacetylase activity and transcriptional repression	Promotes proteasome-mediated degradation	

The glutamic acid is the most highly conserved position other than the lysine. Remarkably, a ψKXE sequence and a nuclear localization sequence (NLS) are requirements for SUMO conjugation.

The sumoylation process is similar to that of ubiquitination, and involves a cascade action of three enzymes (E1, E2, and E3), and forms a bond between the carboxyl terminal group of SUMO and a side chain amino group of lysine of the target protein (Fig. 2). 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 between E2 and the substrates by bring them in close proximity[13].

Post-translational modification by SUMO-1 has diverse effect on substrate activity, but, SUMOylation of transcriptional regulators correlates with inhibition of transcription. In general, ubiquitin increases gene expression at transcriptional level, and SUMO decreases the transcription (Table 2)[10-13.] SUMO modification of a large number of transcriptional regulators supports SUMO plays an important role in the regulation of gene expression.

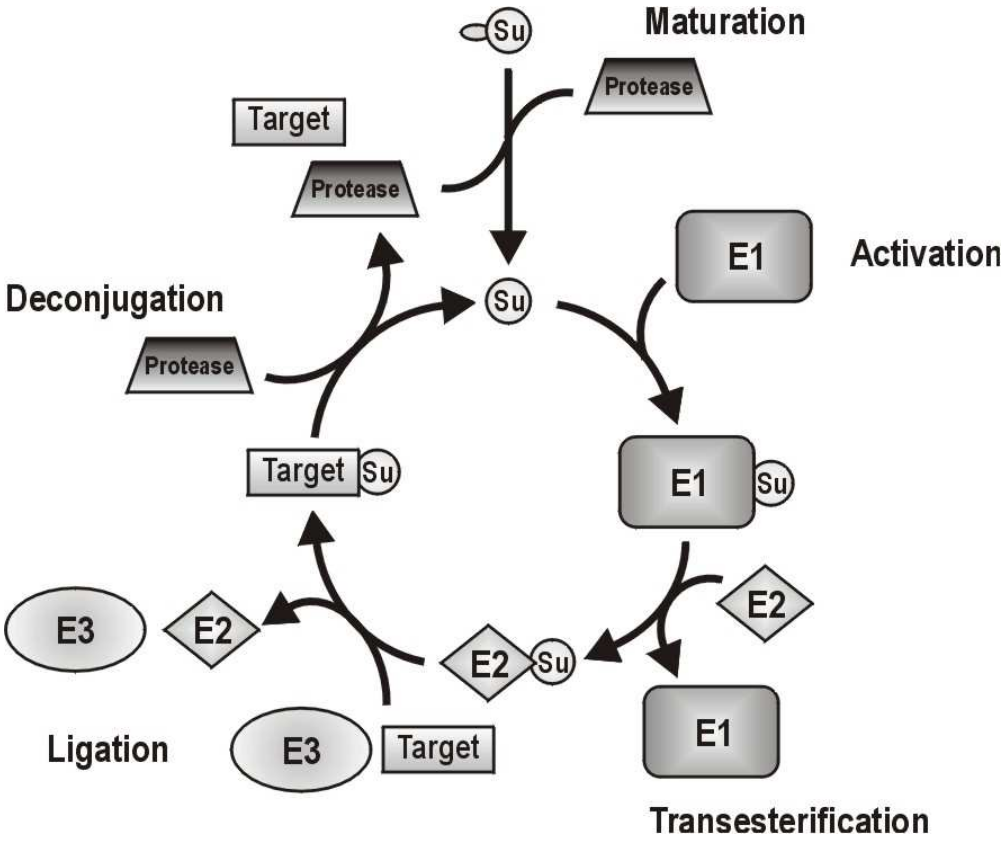

Figure 2. Conjugation pathway of SUMO-1[12]. SUMO is matured through C-terminal processing by protease. The conjugation involves SUMO activating E1 enzyme and E2 conjugating enzyme that form thioester bond with the modifier. E3 ligase enzyme stimulates the attachment of specific lysine residues within a target protein and SUMO.

Table 2. SUMO conjugation and effect on the transcription regulation.[13]

Transcription co-factor	Effect on transcription
Tcf - 4	Positive
HSF - 1	Positive
HSF - 2	Positive
CREB	Positive
APA - 1	Positive
C/EBP	Stabilisation
ELK - 1	Negative
SREBPs	Negative
Sp3	Negative
IRF-1	Negative
ARNT	Negative
AP-2	Negative
Jun	Negative
Lef1	Negative
SRF	Negative
GRIP	Negative
HDAC1	Enhanced repression
HDAC4	Enhanced repression
Histone H4	Negative
CtBP1	Negative
PLZF	Negative

Although post-translational modification by SUMO has diverse effects on transcription factor activity, in most cases SUMOylation has been found to inhibit transcription[10]. Thus, removal of SUMO by mutation of SUMO-acceptor lysines or by overexpression of a deSUMOylating enzyme, such as SENP1(sentrin-specific protease 1) has been shown to increase activities of dozens of transcription factors, including the androgen receptor (AR), the 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 coreressors[10].

Recently PLZF, transcriptional regulator with POZ domain and zinc finger was reported to be conjugated with SUMO-1 and transcriptional activity of it is regulated by sumoylation[12]. FBI-1 has the same structural features with PLZF and contains a POZ domain and zinc fingers. So, we assumed that FBI-1 could be a target protein of sumoylation. We analyzed the amino acids sequence of FBI-1 for the motif which is highly similar to SUMO-1 conjugate consensus motif. We found ten lysine residues with possibility to be sumoylated and lysine 486 and lysine 539 are more likely to be modified by SUMO-1. When we mutated two lysines (K486, K539) of FBI-1 into arginine, the mutation significantly affected transciptinal activity of FBI-1 on the *ADH5/FDH* promoter.

We also investigated whether FBI-1 is sumoylated and found that FBI-1 is modified by SUMO-1 conjugation *in vivo* and *in vitro*. Our results indicate that FBI-1 is sumoylated *in vivo* and *in vitro* and FBI-1 activity as transcription factor may be regulated by sumoylation *in vivo*.

II. Materials and Methods

1. Reagents

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

2. Plasmids preparation

The expression plasmid of human FBI-1 was described in Lee et al [4]. Human FBI-1 (1755 bps, NM_015989) was amplified from human cDNA library using sense primer (5'-GGA TCG AAT TCA CCA TGG ACT ACA AGG ACG ACG ATG ACA AGG CCG GCG GCGTGG ACG GC-3') and anti-sense primer (5'-GGA TCT CTA GAT CAT TAG GCG AGT CCG GCT GT-3') and inserted in *EcoR I* and *Xba I* site of pcDNA3.0. The FBI-1 mutant K61R, K354R, K371R, K379R, K383R, K396R, K486R, K487R, K536R, K539R and SUMO-1 mutant SUMO-1 G97A

were generated with the using a QuickChnage site-directed mutagenesis kit (Stratagene, CA, USA). The mutants were verified by DNA sequencing using ABI-prism automatic sequencer (Applied Biosystems, CA, USA).

The POZ domain of FBI-1 was amplified by PCR from pcDNA 3.0 mammalian expression plasmid as a template using oligonucleotides (sense primer; 5'- GTT AAC GAA TTC ACC ATG GCC GGC GGC GTG GAC GGC CCC ATC GGG AT -3', antisense primer; 5' - GTT AAC GCG GCC GCT TAG ATC TGC CGG TCC AGG AGG TCG GCG CAC - 3'.). The PCR cycling conditions were 95°C for 3min. followed by 30 cycles of 95°C for 30 sec., 60°C for 30 sec., 72°C for 30 sec., followed by 72°C for 5min.. The PCR products were digested with *Eco*RI and *Hpa*I then cloned into pGEX4T-3 vector (Amersham Pharmacia, Buckinghamshire, UK).

His-tagged Human SUMO-1 expression plasmid was kindly provided by Dr. I.Y. Kim (Korea Univ., Seoul, Korea). HA-tagged Human SUMO-1 (active form) expression plasmid was kindly provided by Dr. K.C. Jeong (Yonsei Univ., Seoul, Korea).

The *pG5*-FRE-*Luc* was prepared by cloning oligonucleotides composing FRE (FBI-1 site F; 5'-GAT CTG CAG CTG CTT TTC TCG AGT ACT GGG TCT CTC TAG GGA ACC CAC TGC TTA AGC CTC AAT AAA GCT A-3' and FBI-1 site R; 5'-GAT CTA GCT TTA TTG AGG CTT AAG CAG TGG GTT CCC TAG AGA

GAC CCA GTA CTC GAG AAA AGC AGC TGC A-3') into *pG5* luciferase mammalian expression vector by *EcoR1* and *BamH1*.

3. 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. 1 x 10^6 cells were inoculated into each well of 6 well culture dishes, and then allowed to grow for 16 to 20 hrs. At 80% confluency, cells were transfected with the plasmids mixture (0.2 μg of pGL2-basic ADH/FDH – luciferase reporter plasmid, 0.4 μg of pcDNA 3.0 Wild-type FBI-1, K486R, K539R, K486R/K539R expression plasmids, and 0.1 μg of pCMV-LacZ plasmid) , by using LipofectAMINE PLUS (Promega, WI, USA) according to the manufacturer's protocols. Briefly, the plasmid DNA and 3 μl of PLUS (Promega, WI, USA) reagent were mixed in 100 μl of OPTI-MEM and then added to 100 μl of OPTI-MEM containing 3 μl of LipofectAMINE reagent. The total amount of DNA in each transfection was adjusted to the same amount by addition of pcDNA 3.0. The cells were washed with PBS twice and incubated with 800 μl of OPTI-MEM. After 15 min of incubation, LipofectAMINE-DNA complex was added into wells. The cells were transfected for 3 hrs with the plasmid mixture, then washed twice with PBS, and then grown in MEM supplemented with 10% FBS and 100 μg/ml

antibiotics/antimycotics. After 48 hrs, the cells were harvested and lysed with 100 µl of reporter lysis buffer (Promega, WI, USA), and cell debris was removed by centrifugation. Luciferase activities were measured using 10 µl of cell lysates and 50 µl Luciferase Assay reagent (Promega, WI, USA). Protein concentrations of cell lysates were determined by Bradford method. Luciferase activities were normalized with amount of total protein used in luciferase assay because the CMV promoter-driven expression of β-galactosidase which we used as transfection efficiency normalization control is suppressed by over expression of SUMO-1 and FBI-1.

4. Site-directed mutagenesis

To prepare the FLAG-FBI-1 mutant form and SUMO-1 mutant form, we used the QuickChange site-directed mutagenesis kit (Stratagene, CA, USA). We amplified wild type FLAG-FBI-1 expression vector as template with specific oligonucleotide primers (K61R FBI-1 F', 5'-AGC CAG TAC TTC AAG AGG CTG TTC ACG TCG GGC-3'; K61R FBI-1 R', 5'-GCC CGA CGT GAA CAG CCT CTT GAA GTA CTG GCT-3'; K354R FBI-1 F', 5'-ATG GAC TAC CTG AGG TAC TTC AGC GGC GCC-3'; K354R FBI-1 R', 5'-GGC GCC GCT GAA GTA CCT CAG GTA GTA GTC CAT-3'; K371R FBI-1 F', 5'-TCG CAG AAG GTG GAG AGG AAG ATC CGA GCC AAG-3'; K371R FBI-1 R', 5'-CTT GGC

TCG GAT CTT CCT CTC CAC CTT CTG CGA-3'; K379R FBI-1 F', 5'-AAG AAG ATC CGA GCC AGG GCC TTC CAG AAG TGC-3'; K379R FBI-1 R', 5'-GCA CTT CTG GAA GGC CCT GGC TCG GAT CTT CTT-3'; K383R FBI-1 F', 5'-GCC AAG GCC TTC CAG AGG TGC CCC ATC TGC GAG-3'; K383R FBI-1 R', 5'-CTC GCA GAT GGG GCA CCT CTG GAA GGC CTT GGC-3'; K396R FBI-1 F', 5'-ATC CAG GGC GCC GGC AGG CTG CCG CGA CAC ATC-3'; K396R FBI-1 R', 5'-GAT GTG GCT CGG CAG CCT GCC GGC GCC CTG GAT-3'; K486R FBI-1 F', 5'-CTG CAC AGA CAC CTC AGG AAA GAC GGC TGC AAC-3'; K486R FBI-1 R', 5'-GTT GCA GCC GTC TTT CCT GAG GTG TCT GTG CAG-3'; K487R FBI-1 F', 5'-CAC AGA CAC CTC AAG AGG GAC GGC TGC AAC GGC-3'; K487R FBI-1 R', 5'-GCC GTT GCA GCC GTC CCT CTT GAG GTG TCT GTG-3'; K536R FBI-1 F', 5'-CGC AAC GGC CAG GAG AGG CAC TTT AAG GAC GAG-3'; K536R FBI-1 R', 5'-CTC GTC CTT AAA GTG CCT CTC CTG GCC GTT GCG; K539R FBI-1 F', 5'-CAG GAG AAG CAC TTT AGG GAC GAG GAC GAG GAC-3'; K539R FBI-1 R', 5'-GTC CTC GTC CTC GTC CCT AAA GTG CTT CTC CTG-3'; SUMO-1 G97A 5', 5'-CAG GAA CAA ACG GGG GCT CAT TCA ACA GTT TAG-3'; SUMO-1 G97A 3', 5'-CTA AAC TGT TGA ATG AGC CCC CGT TTG TTC CTG-3'). The PCR mixture has 1 µl of Ultra pfu polymerase, 25 µl of GC buffer (TAKARA BIO Inc., Shiga, Japan), 10 ng of template, 1.25 µl of sense and anti-sense primers (10 pmol) and 2 µl of dNTPs

(2.5 mM). Initial denaturation at 95°C for 30 sec. followed by 16 cycle of denaturation at 95°C for 30 sec., primer annealing at 55°C for 1 min. and extension at 68°C for 10 min. An additional extension at 68°C for 10 min. is followed by holding at 4°C for 10 min. After finishing the PCR, the samples are incubated with DpnI at 37°C for 1 hr. Then 20 µl of the sample were transformed into *E.coli* DH5α.

Multiple mutagenesis was performed with QuickChange® Multi Site- Directed Mutagenesis Kit (Stratagene, CA, USA). We amplified the FBI-1 K379R mutant expression plasmid with specific primers (K396R FBI-1 F', K536R FBI-1 F', K539R FBI-1 F'). The PCR mixture has 1 µl of QuickChange Multi enzyme blend, 2.5 µl of 10x Reaction Buffer, 210 ng of the template, 1.25 µl of sense and anti-sense primers (10 pmol), 1 µl of dNTPs (10 mM) and 0.5 µl of QuickSolution. Initial denaturation at 95°C for 1 min. followed by 30 cycle of denaturation at 95°C for 1 min., primer annealing at 55°C for 1 min. and extension at 65°C for 16 min. Holding at 4°C for extended period of time is followed. After finishing the PCR, the samples are incubated with DpnI at 37°C for 1 hr. Then 10 µl of the sample were transformed into *E.coli* XL-10 Gold Ultracompetent Cells (Stratagene, CA, USA).

5. Western blot analysis

Immunoprecipitants were separated in 8% SDS-polyacrylamide gel (135 voltages for 1 hr) and the acrylamide gel was equilibrated for 20-60 min. in transfer

buffer (25 mM Tris base, 0.2 mM glycine, 20% methanol, pH 8.5). Then the equilibrated gel was transferred to Immuno Blot™ PVDF Membrane (Bio Rad, CA, USA) with TRANS-Blot® Semi-Dry Transfer cell (Bio-Rad, CA, USA) at 15 voltages for 40 min. After completing electrophoretic transfer, the membrane was blocked in 5% Non Fat Dry Milk (Invitrogen Life Technologies, CA, USA) in TBST (25 mM Tris, 140 mM NaCl, 0.05% Tween-20, pH 8.0) for 2 hrs. Then, briefly washed with TBST and the membrane was carried out with primary antibody (polyclonal anti-FLAG antibody or anti-FBI-1 antibody, 1:2000 dilution) at 4°C for overnight. After the incubation, the membrane was washed three times with TBST for 10 min. And the membrane was incubated with secondary antibody (horse radish peroxidase-conjugated anti-rabbit antibody or horse radish peroxidase-conjugated anti-goat antibody, 1:5000 dilution) at room temperature for 45 min. and washed with TBST for 10 min. in three times. Then the membrane was visualized using ECL Kit (PerkinElmer life sciences, MA, USA) with LAS 3000 image reader (Fujifilm, Tokyo, Japan).

6. *In vitro* transcription and translation

The *in vitro* translated Wild-type FBI-1 and sumoylation site mutant forms of FBI-1(K61R, K354R, K371R, K379R, K383R, K396R, K486R, K487R, K536R and K539R) were prepared by incubating the pcDNA3.0 Wild-type FBI-1 and

mutant forms of FBI-1 expression plasmids (1 μg) with T_NT T7 Quick-coupled Transcription/Translation System (Promega, Madison, WI, USA), containing 40 μl of T_NT Quick Master Mix, 2 μl of [^{35}S-]methionine (PerkinElmer Life Sciences, MA, USA), at 30°**C** for 90 min. The translated polypeptides were then analyzed by 2.5 μl of the total mixture with 10% SDS-PAGE and analyzed by autoradiography.

7. *In Vitro* sumoylation assay

Sumoylaion reactions were performed with SUMOylation kit (LAE , MD, USA) according to manufacturer's instruction in a total volume of 20 μl containing 5 μl of [^{35}S-]methionine coupled protein prepared from in vitro transcription and translation reactions at 50 μl scale. In each reaction, add E2 (50 μg/ml) to substrate first, then SUMO-1 (50 μg/ml), 10x reaction buffer (200 mM Hepes pH7.5, 50 mM $MgCl_2$, and 20 mM ATP), E1 (7.5 μg/ml) and 2 μl of 20 mM ATP. Finally, bring up to a final volume of 20 μl by adding H_2O. After the mixture was incubated at 37°C for 90 min. and the reactions was subjected to 10% SDS-PAGE and analyzed by autoradiography.

8. Purification of GST fusion protein

To generate recombinant Glutathione S-transferase (GST) fusion FBI-1 POZ protein, the POZ domain (amino acids 1-133) of FBI-1 was amplified by PCR

cloned into pGEX4T3 expression vectors. The GST-POZ_{FBI-1} expression plasmid was over-expressed in *E. coli*. BL-21 DE3. Bacteria cells were grown in 500 ml of LB broth to A_{600} = 0.8 at 37°C and induced overnight with 0.2 mM isopropyl-D-thiogalactopyranoside at 18°C for overnight. Bacteria cells were collected by centrifugation at 3,000 rpm for 20 min. and resuspended in cold lysis buffer (1x PBS, 2 mM EDTA, and 0.1 mg of lysozyme/ml, 1 mM phenylmethylsulfonyl fluoride.) and sonicated to disrupt the cells at 50 dB for 5 min. The lysates were centrifuged at 12,000 rpm and 4°C for 30 min. to collect soluble supernatant. The lysates were incubated with glutathione Sepharose 4B beads (Peptron, Daejeon, Korea) for 30 min. at 4°C, and the beads were colleted and washed four times with 10x volumes of lysis buffer at 4°C. The protein bound to the resin were eluted in elution buffer (50 mM Tris, 15 mM reduced glutathione , pH9.6, (Sigma, MO, USA) 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA).

9. Preparation of rabbit polyclonal antibody against GST-POZ_{FBI} protein

To generate antibody against FBI-1, purified GST-POZ domain of FBI-1 was used as an antigen. One milligram of antigen was mixed with equal volume of complete Freund's adjuvant (CFA, Sigma, MO, USA), and injected into white rabbit subcutaneously. At two weeks intervals, 1 mg of antigen in incomplete Freund's adjuvant (CFA, Sigma, MO, USA) was injected to rabbit to boost immune

reaction. A week after the 4th injection, rabbit was sacrificed and whole blood was collected. The blood was incubated for 30 min. at 37°C to clot, and placed at 4°C overnight for contraction of clot. The serum was harvested from the clot and any remaining insoluble materials were removed by centrifugation at 12,000 rpm for 10 min. at 4°C.

10. Ab purification by affinity chromatography using CNBr-coupled beads

The purified protein was dialyzed with coupling buffer (0.1 M $NaHCO_3$. pH 8.3, 0.5 M NaCl). CNBr-activated Sepharose 4B beads (1 g will swallow to 3.5 ml of resin) were suspend on 1 mM HCl solution. The swelled beads washed with 20 volumes of 1 mM HCl solution and then HCl solution was replaced with coupling buffer. The protein and the sepharose beads were mixed in a small tube and rotated at 4°C overnight. The coupling sample was washed excess protein ligand away with 5 volumes of coupling buffer. Remained active group were blocked by using 0.1 M Tris·Cl, pH 8.0 buffer. Equal amount of Tris·Cl buffer was added to the beads and incubate at room temperature for 2 hrs with rotation. After the incubation, the beads were washed with 5 volumes of 0.1 M acetate buffer, pH4.0 containing 1 M NaCl followed by 5 volumes of coupling buffer. The beads were equilibrated with buffer containing 20 mM Hepes, pH 7.6, 150 mM KCl, 10% glycerol, 2 mM DTT, 0.1% NP-40, and 0.1 mM PMSF.

The beads were washed with buffer containing 10 mM Tris·Cl, pH 7.5. The immunized rabbit serum (5 ml) was centrifugated at top speed for 10–20 min. to remove any debris. The serum supernatant was diluted with 10 mM Tris·Cl, pH 7.5 and incubated with the beads at cold room for 2-3 hrs with rotation. The beads were packed to the column and washed with 20 volume of 10 mM Tris·Cl, pH 7.5 and 20 volume of 500 mM NaCl, 10 mM Tris·Cl, pH 7.5. The antibody was eluted by passing 2 volume of 100 mM glycine (pH 2.5) through the column. The first eluate was collected in a tube containing 0.2 volume of 1M Tris·Cl, pH 8.0. The column was washed with 10mM Tris·Cl, pH 8.8 until the pH rise to 8.8. The antibody was eluted by passing 2 volume of 100 mM triethylamine (pH11.5, prepared fresh) through the column. The second eluate was collected in a tube containing 0.2 volume of 1M Tris·Cl, pH8.0. Both antibody eluates were combined and dialyzed against 1x PBS with 0.02% sodium azide.

11. Immunofluorescence assay

For Immunostaining, CV-1 cells were grown on coverslips (Sunshine Woks, Korea) washed with cold PBS and fixed in cold methanol:formaldehyde(99:3) for 20 min. at -20℃. The fixed cells were permiabilized in 0.2% Triton X-100 in PBS for 10 min. at room temperature. Then the cells were incubated in blocking solution (5% Goat serum in PBS). After blocking, cells were incubated in the wet chamber

with primary antibody (mouse anti-FLAG antibody, to final 5 μg/ml) in incubation solution (1% BSA, 0.02% sodium azide in PBS) for 2 hours at room temperature. Cells were then rinsed in incubation solution three times for 10 min. at room temperature and further incubated with secondary antibody (anti-mouse antibody conjugated with FITC, to final 5 μg/ml, Jackson Immunoresearch Lab, West Grove, PA, USA) in 50% glycerol for 1 hour at room temperature. The cells were washed with PBS 5 times for 5 min. by low speed shaking.

For double staining after washing the cells with PBS, cells were incubated with 3.7% formaldehyde for 10min. at room temperature. Then the cells were incubated with blocking solution for 30min. at room temperature. Cells were then incubated with different primary antibody (rabbit anti-HA antibody, to final 5 μg/ml) in incubation solution for 2 hours at room temperature and rinsed in incubation solution three times for 10 min. at room temperature. After that, cells were incubated with secondary antibody (anti-rabbit antibody conjugated with rhodamine, to final 5 μg/ml, Jackson Immunoresearch Lab, West Grove, PA, USA) in 50% glycerol for 1 hour at room temperature. Cells were then washed three times with PBS for 5 min. at room temperature and mounted with mounting medium (SIGMA DIAGNOSTICS, St. Louis, MO, USA) 2drops on the cover slip. The excess mounting medium was removed with Kim’s wipe and sealed the margin of the cover slip with transparent nail polish. Cells were examined with a Carl Zeiss

LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany). All images were processed for presentation using Zeiss LSM program.

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III. Results

1. Identification of sumoylaion target site of FBI-1

To identify which amino acid residues of FBI-1 that could be modified by SUMO-1, we analyzed the entire amino acid sequence of FBI-1 protein for the presence of the consensus sequence ψKXE (ψ, a hydrophobic amino acid, K, lysine, X, to 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. 3A), and the ten sites are ordered by their score of possibility to be sumoylated. As their score is high, the lysine residue has high possibility to be sumoylated. The prediction of SUMOplot™ program is like this; 0.91 for lysine 486, 0.85 for lysine 539, 0.57 for lysine 396, 0.56 for lysine 354, 0.50 for lysine 371, 0.44 for lysine 383, 0.39 for lysine 379, 0.31 for lysine 487, 0.15 for lysine 536, 0.13 for lysine 61 (Fig. 3B). They are expected as sumoylation site *in vivo* and *in vitro*.

Interestingly, the lysine residue at a.a. 61 position from the N-terminus are located within the BTB/POZ-domain (a.a. 7-131, underlined by thick solid line) which was shown previously to be important in the transcription repression and interaction with corepressors (unpublished data). The sumolylation at the K61 residue can be important in the transcription repression via sumolylation. Also six potential sumolyation sites are located within or at the position really proximal to the C2H2 type zinc-finger DNA binding domain (a.a. 382-490, double underlined).

The sumolyation of the sites may be important in the recognition event of the FBI-1 target binding site (FRE). Also we noticed that sumolyation site 7, 8 (K487, K486) is partially overlapping with nucelar localization sequence (a.a. 487-505, waved underline) of FBI-1 and the modification might affect the nuclealr localization event of the factor.

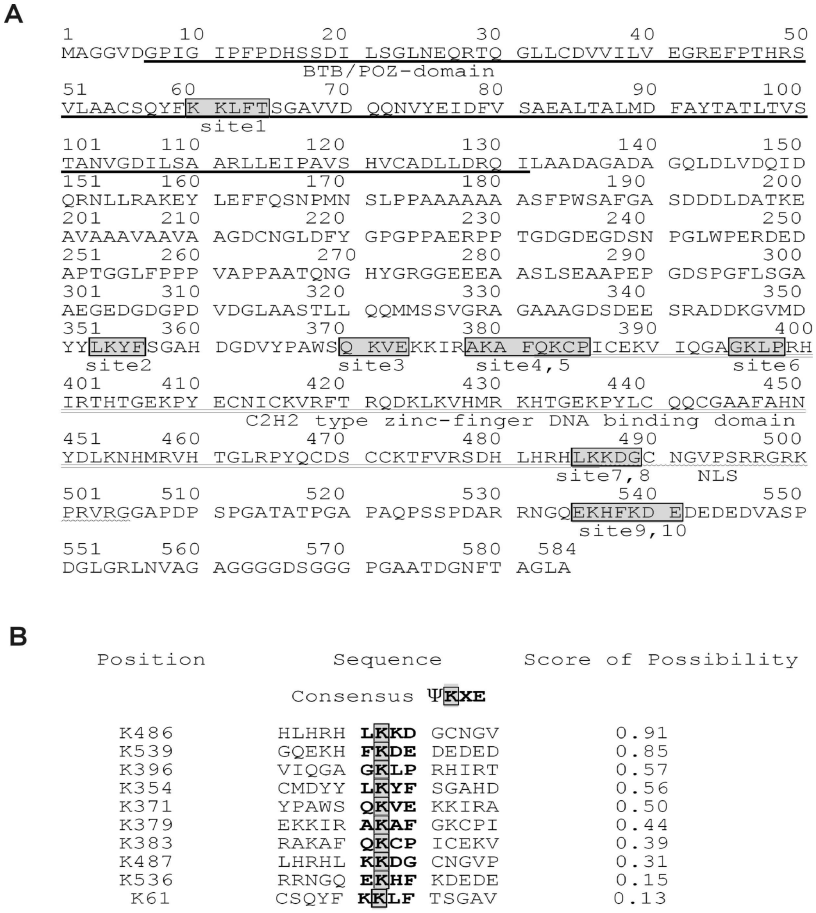

Figure 3. Identification sumoylaion target site of FBI-1. (A) SUMOplot™ analysis results of FBI-1 amino sequences (GI:O95365). The gray box indicates potential sumoylation site. **(B)** The potential sumoylation sites of FBI-1 are ordered by the score of possibility. The gray box indicates the target lysine residue of potential sumoylation site with bold character. (http://www.abgent.com/tool/sumoplot).

2. The preparation of polyclonal antibody against FBI-1

Overexpressed GST-POZ domain of FBI-1 was purified by GSH-agarose affinity column chromatography and analyzed by SDS-PAGE. The predicted size of GST-POZ_{FBI-1} is 38 kDa. SDS-PAGE analysis shows that GST-POZ_{FBI-1} was purified (Fig. 4A). The purified GST-POZ_{FBI-1} was injected into rabbit and the immunized rabbit serum was collected. To purify GST-POZ_{FBI-1} antibody from the whole rabbit serum, we used affinity chromatography with CNBr-activated beads. GST-POZ_{FBI-1} protein was coupled to the CNBr-activated beads and used as a ligand. Then GST-POZ_{FBI-1} coupled beads were incubated with the rabbit serum. By changing pH of elution buffer, GST-POZ_{FBI-1} antibody was eluted.

To confirm the activity of the anti-GST-POZ_{FBI-1} antibody, FLAG-tagged FBI-1 or pcDNA3.0 mammalian expression vectors were transiently transfected into CV-1 cells (Fig. 4B). The transfected cells were collected and lysed. The lysates were separated by 10% SDS-PAGE and transferred on to a PVDF membrane and subjected to Western blot analysis. Western blot analysis shows that both anti-GST-POZ_{FBI-1} and commercial anti-human FBI-1 (ABCAM, CA, USA), are detected FLAG-FBI-1 successfully (about 80 kDa).

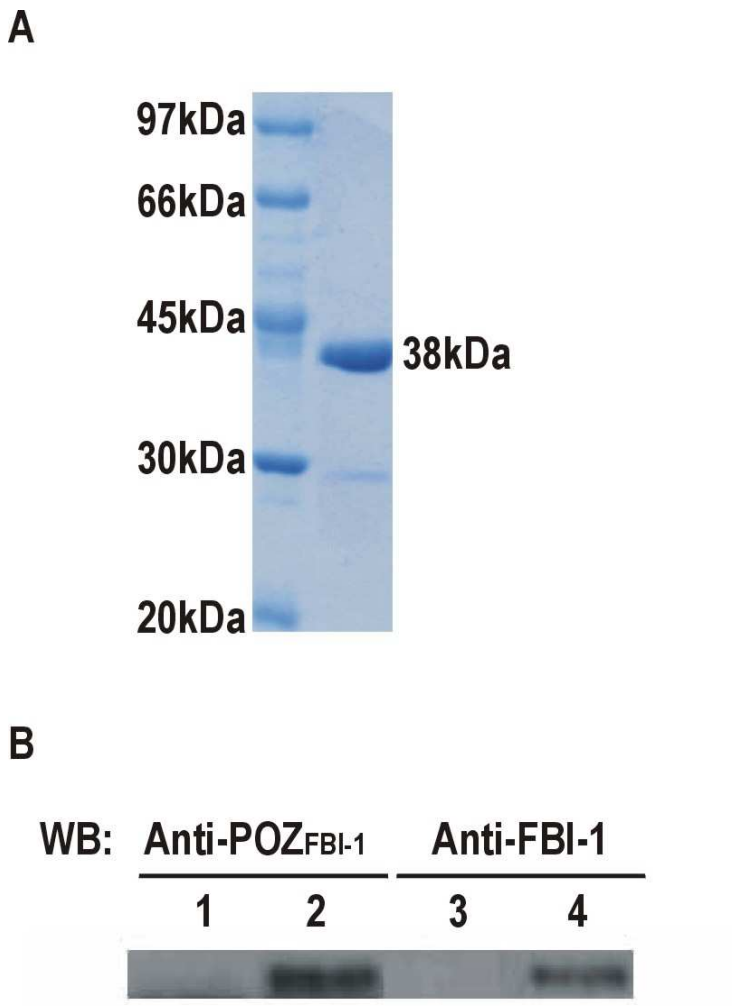

Figure 4. Preparation of rabbit polyclonal antibody against FBI-1. (A) SDS-PAGE of purified GST-POZ_{FBI} protein. **(B)** Western blot analysis of CV-1 cell extracts preparated from the cells transcfected with FLAG-FBI-1 expression vector. Lane 1 and 3 are non-transfected CV-1 cell lysates and lane 2 and 4 are transfected CV-1 cell lysates with FLAG-FBI-1 expression plasmid. Lane 1 and 2 were detected by affinity purified rabbit polyclonal anti GST-POZ_{FBI} antibody and lane 3 and 4 were detected by commercial anti-human FBI-1 antibody.

3. FBI-1 is modified by SUMO-1 *in vivo*.

To test whether FBI-1 is modified by sumoylation, we used 293 TREX cells in which FBI-1 is stably expressed. 293T cells do not express endogenous FBI-1 at a detectable level (Fig 5A. lanes 1, 2). So we prepared FBI-1 stable cells integrating FBI-1 in chromosome and have FBI-1 be over-expressed by inducer, doxicycline

As add of doxycycline, FLAG-FBI-1 was induced in FBI-1 stable cells much more than control TREX cells or non-induced FBI-1 stable cells (Fig 5A. lanes 3, 4). The cell lysates were subjected to Western blot analysis with anti-FLAG antibody and anti-SUMO-1 antibody.

The results of Western blot showed control cell did not show any difference by add of doxycycline (Fig 5A. lanes 1, 2). But FBI-1 expression of stable cells was induced by doxycyline (Fig 5A. lanes 3, 4). The expression of FBI-1 was detected about 80kDa (despite of a predicted molecular weight of 64kDa, FBI-1 has been reported to run significantly higher). Without add of doxycycline, FBI-1 expression of FBI-1 was increased a little than control 293TREX cells (Fig 5A. lane 3). When FBI-1 was over-expressed, we could see interesting four upper bands as indicated with arrows. Among them, we found two bands which are agreed with *in vitro* sumoylation assay as indicated with asterisks.

To confirm whether the upper band indicates SUMO-1 conjugated FBI-1, we performed Western blot analysis with anti-SUMO-1 antibody (Fig. 5B). Anti-SUMO-1 antibody couldn’t detect the significant band as anti-FLAG antibody gave. But it showed very slight band which is agreed with lower asterisks is indicated in

Fig. 5A. In some case such as BMAL1[29], long exposure reveals bands of sumoylated target protein. We might need to extend exposure time.

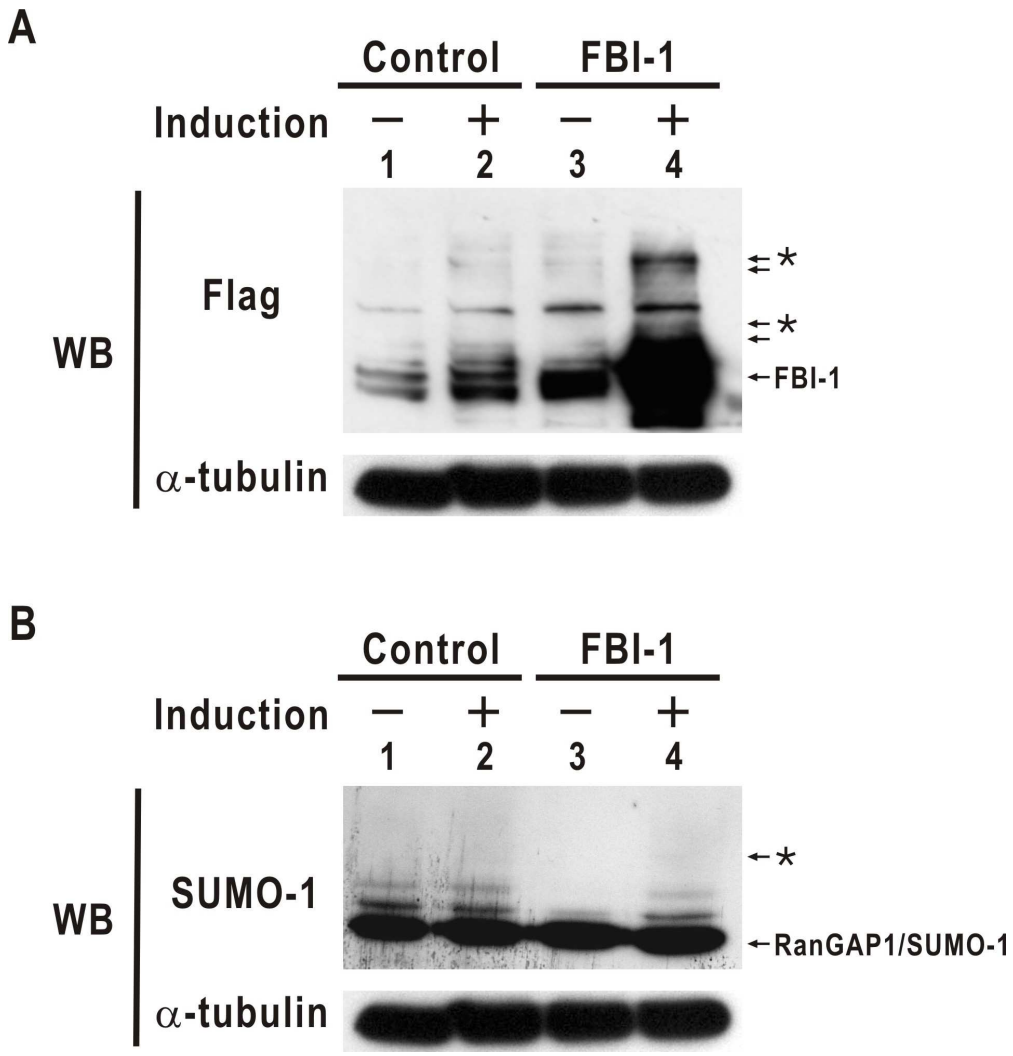

Figure 5. FBI-1 is modified by SUMO-1 *in vivo*. Western blot analysis of human kidney 293TREX cell extracts which overexpressing FBI-1 by induction of doxucycline with anti-FLAG antibody **(A)** and anti-SUMO-1 antibody **(B)** as described. α-tubulin was detected as a loading control. The asterisk indicates FBI-1-SUMO-1 complex.

4. FBI-1 is modified by SUMO-1 *in vitro.*

To test whether FBI-1 can be sumoylated *in vitro*, we prepared the wild-type FBI-1 and the sumoylation mutant FBI-1K61R, K354R, K371R, K379R, K383R, K396R, K486R, K487R, K536R and K539R by *in vitro* translation system in the presence of [^{35}S-]methionine. The translated proteins used for *in vitro* sumoylation assay are shown in Fig. 6A. Sumoylation assay was performed by the addition of SUMO-1, E1 (SAE1/2), E2 (Ubc9) to [^{35}S]-labeled FBI-1 or SUMO-mutant form of FBI-1 proteins in a buffer containing an ATP. As shown in Fig. 6, several bands of the modified FBI-1 were detected. The additional bands of slower mobility were observed when all proteins required for sumoylation were present, providing the evidence that FBI-1 was a substrate for sumoylation.

To ascertain whether the lysines (those have possibility of being sumoylated) are in fact subject to sumoylation, ^{35}S- labeled constructs containing lysine-to-arginine mutations at the position (lysine 61, 354, 371, 379, 383, 396, 486, 487, 536 and 539) used for *in vitro* sumoylation assays (Fig. 6A). When FBI-1 was subjected to sumoylation, a new band was generated. The prominent new band (about 150 kDa) migrated more slowly than unmodified FBI-1 (about 80 kDa). FBI-1 with mutation at lysines still showed an upper band with slow mobility, which indicates that the lysine of mutants of FBI-1 are still sumoylated and they are not sufficient to abolish sumoylation completely. This result suggests that FBI-1 with mutation at

only one lysine is not enough to inhibit the sumoylation of FBI-1 completely. FBI-1 with mutations at two or three lysines could be abolish the upper bands and inhibit the conjugation with SUMO-1 completely.

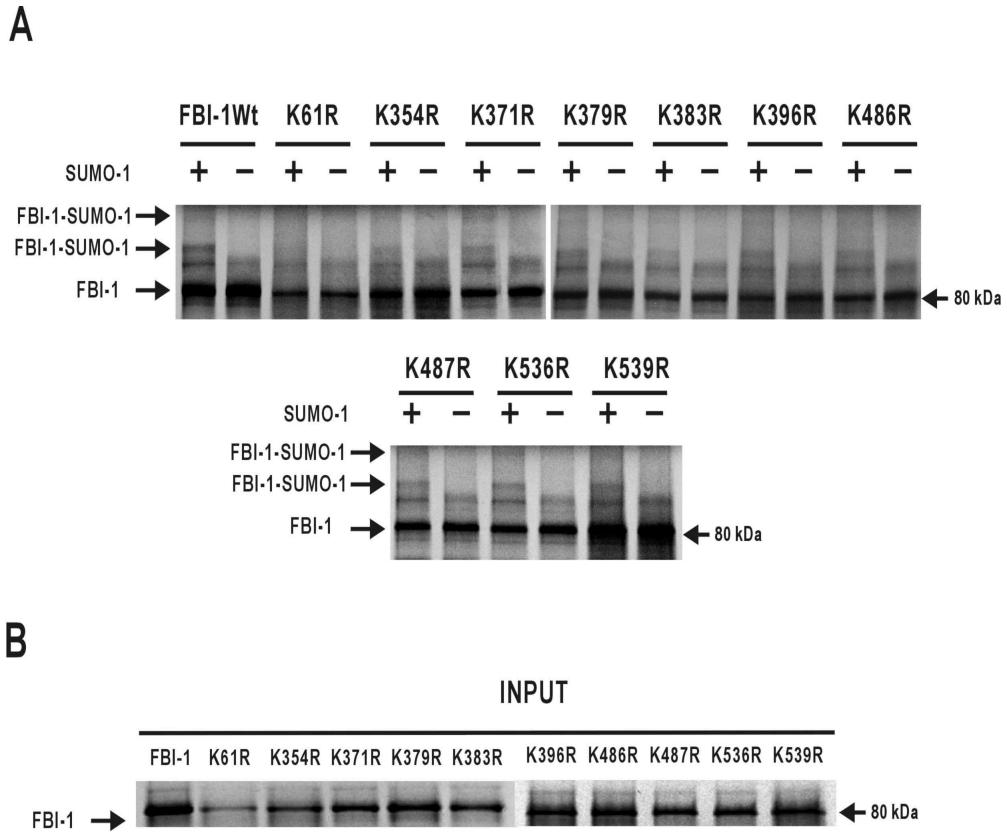

Figure 6. FBI-1 is modified by SUMO-1 *in vitro*. In vitro sumoylation assay was performed with a wild type or mutants of the FBI-1. **(A)** SUMO modification was assayed in the presence or absence of purified E1, E2, SUMO-1 and as indicated. Full-length FBI-1 and FBI-1 – SUMO-1 conjugates are indicated with an arrow. **(B)** [^{35}S]-methionine-labeled FBI-1 was prepared by *in vitro* translation.

5. Sumoylation affected the transcriptional activity of FBI-1.

To examine whether SUMO-1 modification affects the transcriptional regulatory activity of FBI-1, we compared the transcription repression activity of wild-type FBI-1 with those of the sumoylation mutant FBI-1K61R, K354R, K371R, K379R, K383R, K486R, K487R, K536R and K539R. We used *ADH5/FDH–Luc* gene fusion reporter plasmid which was reported to be repressed by FBI-1[4] (Fig. 7A). CV-1 cells were transfected with *ADH5/FDH–Luc* reporter plasmid and wild-type FLAG-FBI-1 or the sumoylation mutant FBI-1 plasmids (Fig. 7B). The potent repression of luciferase gene transcription was apparent in the CV-1 cells expressing wild-type FBI-1 (90%). FBI-1 mutants were less effective in repression compared to wild type FBI-1. Some FBI-1 mutants showed 2.2 fold to 3.3 fold more than wild type FBI-1 (K379R for 2.2 fold, K396R for 3.3 fold, K536R for 2.5 fold and K539R for 2.4 fold). Other FBI-1 mutants also showed weaker repression effect than wild type but not significantly (K61R for 1.8 fold, K354R for 1.6 fold, K371R for 1.4 fold, K383R for 1.5 fold, K486R for 1.1 fold, and K 487R for 1.4 fold). These weak repressions of mutant FBI-1s suggest that sumoylation at lysines which have a possibility to be sumoylated is also required for transcriptional repression by FBI-1.

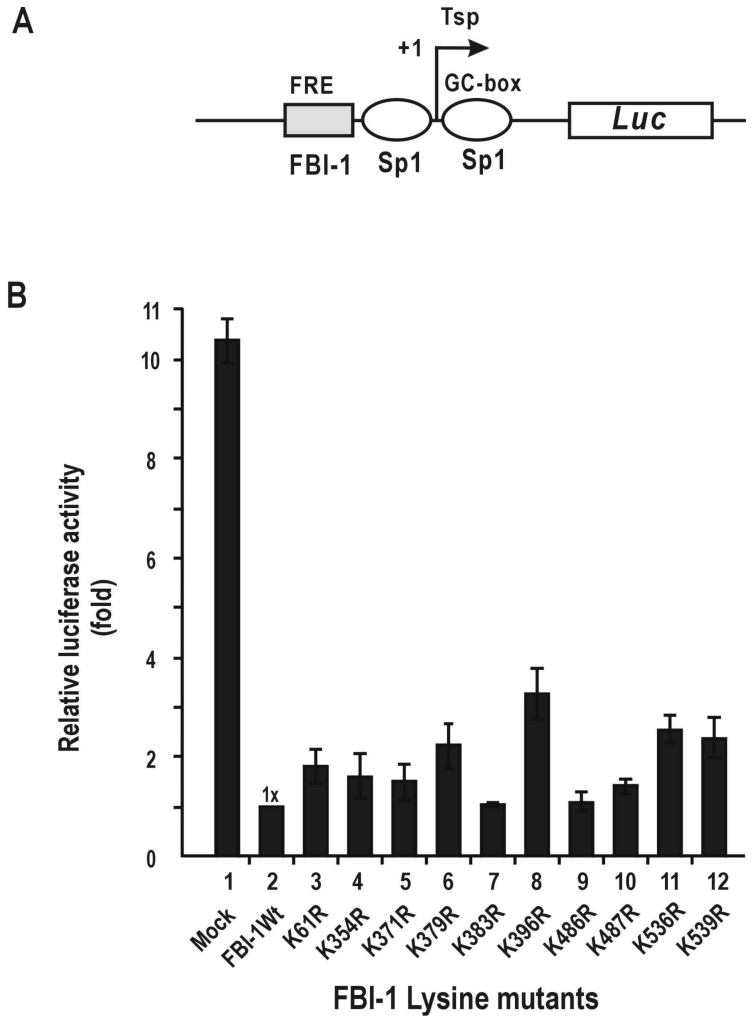

Figure 7. Modulation of the transcriptional regulatory activity of FBI-1 by sumoylation. (A) Schematic representation of *ADH5/FDH-Luc* reporter plasmid. FRE, FBI-1 responsive element; GC-box, Sp1 binding GC-box; Tsp, transcriptional start point; Luc, Luciferase gene. **(B)** CV-1 cells were co-transfected with the luciferase reporter plasmid (*ADH5/FDH-Luc*) and wild-type FLAG-FBI-1 and sumoylation mutant FLAG-FBI-1(K61R, K354R, K371R, K379R, K383R, K396R, K486R, K487R, K536R and K539R).

6. Influence of the cell context on FBI-1 repression activity.

To investigate whether the sumoylation has any effect on FBI-1 activity in different cell types, we performed transient transfection with *pADH5/FDH-Luc* reporter plasmid, wild-type FBI-1 expression plasmid, and His-SUMO-1 expression plasmid. In CV-1 cell, FBI-1 repressed luciferase gene transcription by 60%. When SUMO-1 expression plasmid was co-tranfected, SUMO-1 did not show significant effect on FBI-1 repression (Fig. 8A. lane 4). However, when 293T cells were co-transfected with the same mixture of plasmids, SUMO-1 did not show significant effect on the transcription as in CV-1 cells.

Because the above promoters contain binding sites for many transcription factors and general transcription factors and mediators are expected to work together on the promoter, showing the effect of SUMO-1 modification on the transcription can be very difficult. Accordingly, to demonstrate the effect of sumoylation on FBI-1, we prepared the reporter plasmid containing FRE (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 FBI-1 binding site located in the IST of HIV-1 LTR promoter.

FBI-1 activated the transcription of *pG5*-FRE-*Luc* plasmid by 5 fold and cotransfected SUMO-1 repressed transcription of the reporter gene by 60%. In the reporter expression experiment carried out in the absence of FBI-1, the transcription of *pG5*-FRE-*Luc* was not affected by SUMO-1 (Fig. 9B. lane 1, 2).

Our data suggest that probably SUMO-1 modify the FBI-1 and the sumolylation of FBI-1 could potentiate repression activity of FBI-1 on this particular promoter with FRE.

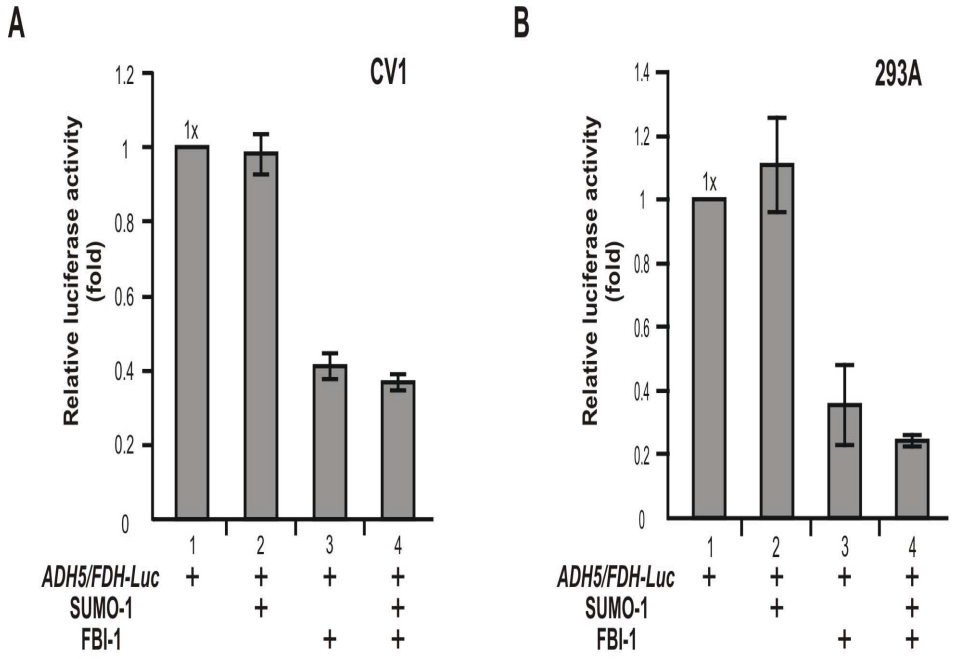

Figure 8. Influence of cell type on FBI-1 repression activity. CV-1 cells **(A)** and 293A cells **(B)** were co-transfected with the luciferase reporter plasmid (*ADH5/FDH-Luc*) and wild-type FLAG-FBI-1 in the absence and the presence of expression vectors for His-SUMO-1 as indicated.

7. SUMOylation affected the transcriptional activity of FBI-1 on *pG5-FRE-Luc* and triple lysine mutants showed less potent transcription repression activity .

To investigate the effect of sumoylation on FBI-1, we prepared the reporter plasmid, pG5-FRE-Luc, containing FRE (FBI-1 response element) inserted in front of adenovirus major late minimal promoter of *pG5-Luc* (Fig. 9A). The FRE site is from the well characterized FBI-1 binding site located in the IST of HIV-1 LTR promoter. We co-transfected expression plasmid of the FBI-1wild type and SUMO-1 into CV-1 cells. The enforced sumolyation of FBI-1 by ectopic SUMO-1 increased the repressor potential on the test promoter (Fig. 9B). Alternatively, to demonstrate the cumulative effect of lysine mutation into arginine at four key potential sumolylation sites, we prepared the mammalian expression plasmids of FBI-1 mutants at K379, K369, K536, K539 and cotransfected with *pG5-FRE-Luc* reporter plasmid. The mutations increased the transcription of the reporter gene by 2.5 fold and 2 fold for FBI-1K379/K369/K539 and FBI-1K379, K369, K536, respectively. The data potentially suggest that profer sumolyation is importaant in transcription repression of by FBI-1 (Fig. 9C).

FBI-1 activated the transcription of pG5-FRE-Luc plasmid by 5 fold and cotransfected SUMO-1 repressed transcription of the reporter gene by 60%. In the reporter expression experiment carried out in the absence of FBI-1, the transcription of pG5-FRE-Luc was not affected by SUMO-1 (Fig. 9B. lane 1, 2). Our data suggest that probably SUMO-1 modify the FBI-1 and the sumolylation of FBI-1 could potentiate repression activity of FBI-1 on this particular promoter with FRE.

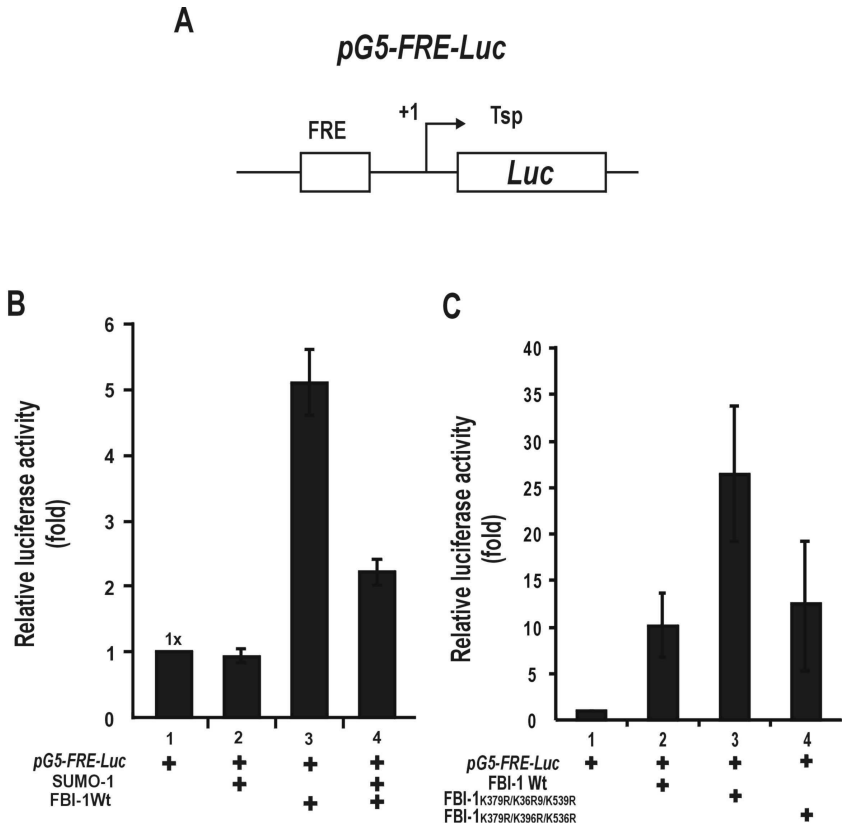

Figure 9. Sumolyation affected the transcriptional activity of FBI-1 on the artificial FBI-1 target *pG5-FRE-Luc* and triple lysine mutants showed less potent transcription repression activity. (A) Schematic representation of *pG5*-FRE-*Luc*. **(B)** CV-1 cells were co-transfected with the luciferase reporter plasmid (*pG5-FRE-Luc*) and wild-type FLAG-FBI-1 and in the absence and the presence of expression vectors for HA-SUMO-1 as indicated. **(C)** The luciferase reporter plasmid (*pG5-FRE-Luc*) and wild-type FLAG-FBI-1 and mutant FBI-1 with mutations at the key sumolyation sites as indicated (FBI-1K379/K369/K539 and FBI-1K379/K369/K536) into CV-1 cells. FRE, FBI-1 responsive element; Tsp, transcriptional start point marked as +1; Luc, Luciferase gene.

8. Sumolyation does not interfere with localization of FBI-1.

We next determined whether FBI-1 colocalized with SUMO-1. CV-1 cells were cotransfected with plasmids encoding FLAG-FBI-1 and HA-tagged SUMO-1. FLAG-FBI-1 was labeled with primary anti-FLAG mouse antibody and secondary anti-mouse antibody-FITC conjugated, and HA-SUMO-1 was labeled with primary anti-HA rabbit antibody and anti-rabbit antibody-rhodamine conjugated. Immunofluorescence analysis revealed that FLAG-FBI-1 immunofluorescence (*green fluorescence*) and HA-SUMO-1 fluorescence *(red fluorescence)* were colocalized (*yellow*) in nuclear (Fig. 10A). Again, it is indicating that FBI-1 is modified by conjugation with SUMO-1 *in vivo*.

Since sumoylation site 7, 8 is overlapped with NLS(nuclear localization ssequence ; a.a. 487-505, waved underline in Fig. 1A), the mutant FBI-1 of those sites may have possibilities to affect the localization of FBI-1. We transfected the wild type and mutants (K486R and K487R) of FLAG-FBI-1 in the absence(Fig.10B) and presence(Fig.10C) of SUMO-1. The wild-type and mutant form of FLAG-FBI-1 (*green fluorescence*) were localized in nucleus. The results of immunofluorescence assay are indicating that sumoylation is not required for localizing FBI-1 to the nucleus. However, this result cannot rule out the possibility that sumoylation can affect the subnuclear localization of FBI-1.

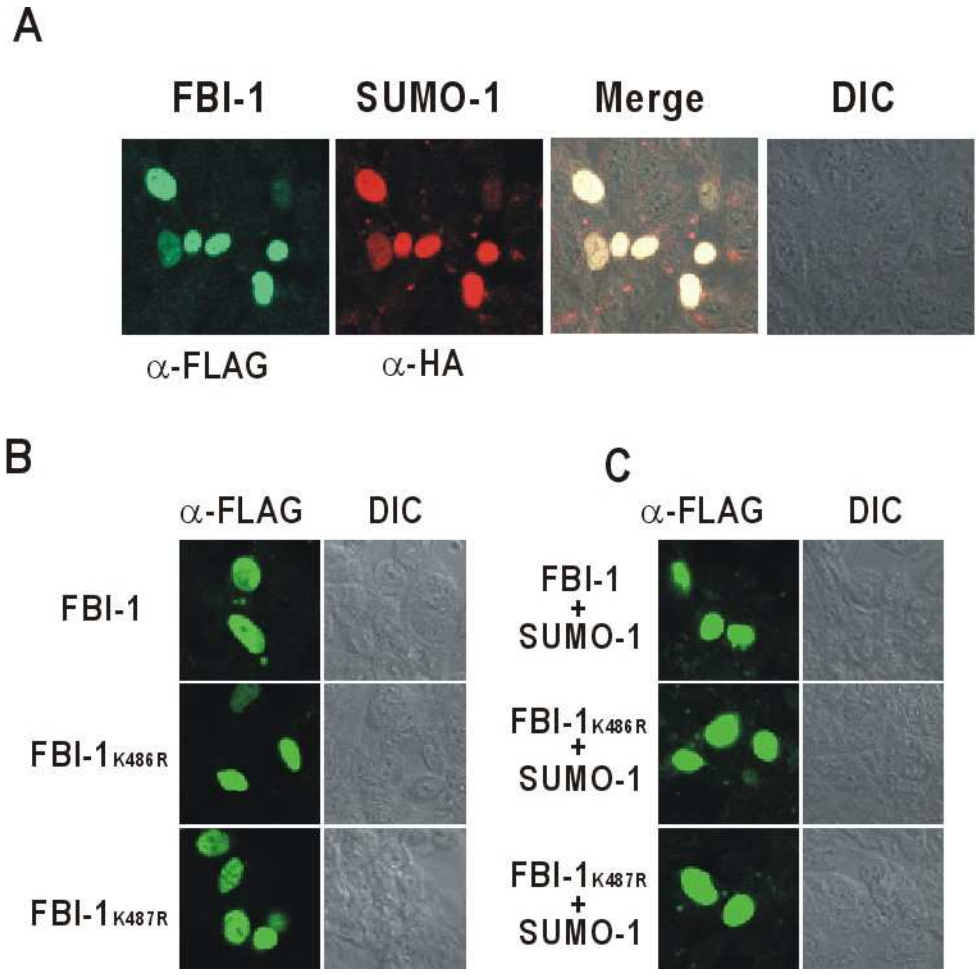

Figure 10. Localization of FBI-1 and its mutants in cells . (A) CV-1 cells were transfected with wild type FLAG-FBI-1 and HA – SUMO-1. FBI-1 and SUMO-1 were labeled with FITC (*green*) and rhodamine (*red*) respectively. Colocalization of the two types of fluorescence is indicated in *yellow* in the merged image. **(B)** CV-1 cells were transfected with wild type FLAG-FBI-1 and mutant FLAG-FBI-1(K486R and K487R in the absent **(B)** or present **(C)** of HA-SUMO-1. FBI-1 was visualized with FITC.

IV. Discussion

In this work we have shown post-translational modification of the transcription factor FBI-1. FBI- has many functions in various cellular processes. It regulates transcription of ADH5/FDH promoter and HIV-1 LTR promoter also involves in cellular development and differentiation such as adipogenesis, osteogenesis and oncogenesis[1-6].

SUMOylation has recently emerged as an important mechanism in transcriptional control. According to those reports, over half of the SUMO target proteins are transcription co-activator and co-repressor and SUMO leads the transcriptional repression in most cases. By reporter gene assay, it is confirmed Gal4-SUMO represses the transcription and SUMO itself has negative effect on the transcription[12]. Since SUMO recognizes same lysine residue as ubiquitin does, it increases the stability of protein in case if CREBP (cAMP-response element-binding protein) or inhibitor of NFκB, IκBα[14]. It also increases DNA binding activity of HSF1 and HSF2, and decreases transcriptional activity of Sp3, c-JUN, c-Myb, AP2. And it promotes formation of PML body and influences to subcellular localization of protein[11, 16]. Consequently, sumoylation plays a role in multiple vital cellular processes.

SUMO-1 modification of most proteins appears to be regulated by the

requirement of the substrate to be targeted to the nucleus and by the possession of a SUMO-1 recognition motif displayed on the surface of the target protein. Since FBI-1 has a nuclear localization signal (a.a. 494-507; *CXXVXXRXXRKXXX*)[4] which suffices for SUMO conjugation *in vivo*[17] with only a few exceptions[18] and also has 10 lysine residues which lie within typical consensus motifs for sumoylation ψKXE (Fig. 3), FBI-1 has possibility of modification by SUMO-1. In particular, sumolylation site 1 is located in the middle of the BTB/POZ-domain, which is important in the transcription repression by interaction with corepressors and other regulatory proteins. Also, most of the sumolylation sites are located within or proximal to the domains critical in the transcription faction of the protein, zinc-finger DNA binding domain (site 3, 4, 5, 6, 7, 8) and nuclear localization sequence (site 7, 8)(Fig. 10). To determine the sumoylation site in FBI-1, we mutated each lysine of 10 lysines where has possibility to be sumoylated to arginine.

To test our hypothesis that FBI-1 is modified by SUMO-1 *in vivo*, we overexpressed FBI-1 in 293T cells with TREX system (Invitrogen, CA, USA). As add of doxycycline, FBI-1 is overexpressed stably. After 36 hrs later of the induction, the cell lysates were subjected to Western blot with anti-FLAG antibody (Fig. 5). The western blot analysis with anti-SUMO-1 antibody show very slight upper band of the sumoylated FBI-1. We thought that is the first evidence for the sumoylation of FBI-1. But still we need to show the same band pattern in the

Western blot with anti-SUMO-1 antibody. One of the reasons why we couldn't show the bands with anti-SUMO-1 antibody, might be that we need more exposure time. According the research about sumoylation of BMAL1, longer exposure only shows the sumoylated BMAL1 bands[29]. They didn't mention about exact exposure time, since little amount of target protein is detected as sumoylated form in other studies[29], longer exposure would be the solution.

Even our *in vivo* sumoylation assay results are not clear, our in vitro sumoylation assay results clearly show that FBI-1 is sumoylated (Fig. 6). ^{35}S-labeled FBI-1 protein and sumoylation mutant form of FBI-1(K61R, K354R, K371R, K379R, K383R, K396R, K486R, K487R, K536R and K539R) were used for *in vitro* sumoylation assays in the presence or absence of SUMO-1. In the presence of SUMO-1, a wild type and mutants of FBI-1 protein show many upper bands which are thought as sumoylated FBI-1. Interestingly, the sumoylated band pattern was different with *in vivo* results. Even the quantity of input of wild type and mutants used for *in vitro* sumoylation assay, the sumoylation bands are clear. Since FBI-1 has many potential sumoylation sites, even we made one mutation at arginine to block sumoylation, the other sumoylation sites are still sumoylated. Because of their high score of possibility to be sumoylated, we thought lysine 486 and lysine 539 may act as a major sumoylation site. But the score means only sumoylation possibility, we couldn't say they should be major sumoylation sites.

Despite the mutation of those residues cannot abolish any sumoylation band in vitro sumoylation assay, they may are sumoylation sites in vivo. We mutated each of these amino acids into arginine and tested whether the mutant FBI-1 could repress transcription of FBI-1 responsive gene, *ADH5/FDH*. FBI-1 potently represses transcription of p*ADH5/FDH-Luc*, but the mutants were weaker transcription repressors. It is suggesting that sumolyation might be important in transcription repression by FBI-1 (Fig. 7). As The transcriptional inhibition of their mutants show little effect, to identify the major sumoylation target site of FBI-1, the research with mutants of double or triple lysines need to be followed. And we also tested Sumoylation can regulate the transcriptional activity of FBI-1 in different cells and different gene. When we compare the results of luciferase activity assay in CV-1 cells (green monkey kidney cells) and 293A cells (human kidney cells) with same setting of transient transfection, we found SUMO-1 showed similar effect in both cells (Fig. 8). Because the promoter that we used such as *ADH5/FDH* has many binding sites of other transcription factors, the sumoylation effect on FBI-1 could be difficult to see with these genes. So we made *pG5*-FRE-*Luc* and demonstrate SUMO-1 repress the transcription of FBI-1 (Fig. 9). SUMO-1 didn't affect the transcription of *pG5*-FRE-*Luc*, but repressed the transcription which is activated by FBI-1. And also FBI-1 mutants with 3-4 lysines mutated showed much weaker transcription repressors on the *pG5*-FRE-*Luc* (Fig. 9). The results suggested

that SUMO-1 could sumolyate the FBI-1 at various lysine residues and the sumolation potentiated repressor activity of FBI-1.

To determine whether sumoylation affects the cellular localization of FBI-1, we carried out indirect immunolocalization experiments with wild-type FBI-1 in the presence of absence of the SUMO-1. As we expected, FBI-1 and SUMO-1 were colocalized in the nucleus. Since lysine 486 and 487 are overlapped with NLS, we compared the nuclear localization of wild-type FBI-1 with the K486R, K487R form of FBI-1. The results showed that wild type and mutant FBI-1 were located in the nucleus (Fig. 10) and it is indicating that sumoylation was not required for localizing FBI-1 to the nucleus.

How sumoylation alters the activity of FBI-1 is not known. It is likely that SUMO-1 modification of FBI-1 may alter its conformation and thus regulate the interactions of binding partners. In some cases the recruitment of HDACs has been shown to depend on the sumoylation of partner proteins[22, 23]. But we have not found evidence of it. Experiments are presently under way to establish whether sumoylated FBI-1 may specifically recruit new co-regulatory partners.

In summary, we have demonstrated that FBI-1 is sumoylated on ten lysine residues located at a conserved sumoylation motif. This modification modulates the transcriptional activity of FBI-1. Therefore, this study identifies sumoylation as a novel regulatory mechanism for FBI-1.

V. Conclusion

1. FBI-1 is modified by SUMO-1 *in vivo* and *in vitro*.
2. FBI-1 has ten potential sumoylation sites.
3. Lysine residues at 61, 354, 371, 379, 383, 396, 486, 487, 536 and 539 of FBI-1are the target for SUMO-1 modification.
4. SUMOylation affected the transcriptional activity of FBI-1 on the FBI-1 target *ADH5/FDH* gene.
5. Modulation of SUMO-1 on the transcriptional properties of FBI-1 is not depending on the cell type.
6. SUMOylation affected the transcriptional activity of FBI-1 on the artificial FBI-1 target *pG5-FRE-Luc* and triple lysine mutants showed less potent transcription repression activity.
7. Sumolyation does not interfere with localization of FBI-1.

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국문요약

수모화에 의한 FBI-1의 기능 조절

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노희은

FBI-1(Factor that Binds to Inducer of Short Transcripts)은 HIV-1(human immunodeficiency virus, type 1) LTR 프로모터의 IST(inducer of short transcripts) element에 결합하여 전사를 조절하며 N-말단에 POZ 도메인과 C-말단에 4개의 Krűppel-like zinc finger를 갖는 단백질이다. 이미 알려진 대로 ADH5/FDH 프로모터와 HIV-1 LTR 프로모터에서 전사를 조절하는 역할 뿐만 아니라 지방세포로의 분화와 골세포 형성과정에도 관여하는 것으로 밝혀져 세포의 발달과 분화 과정에도 작용함을 알 수 있다.

SUMO (small ubiquitin-related modifier)는 ubiquitin과 18%의 낮은 아미노산 서열 유사성을 갖지만, 단백질 3차 구조가 거의 유사하며 target 단백질의 동일한 lysine 잔기를 인식한다. 하지만 ubiquitin과는 다르게 proteasome에 의한 proteolytic protein degradation을 유도하지 않으며 translation의 조절과 세포 내 단백질의 이동에 영향을 미친다. 일반적으로 ubiquitin이 전사 수준에서 유전자 발현을 증가시키는 경향이 있는 반면에 SUMO는 전사를 감소시키는 경향을 보인다.

최근 많은 전사인자들의 sumoylation 이 보고되고 있다. 연구된 바에

따르면 절반이 넘는 SUMO의 target 단백질이 transcription co-activator와 co-repressor이고 대부분의 경우, SUMO가 전사억제를 유도하는 것으로 밝혀졌다.

우리는 FBI-1과 동일하게 POZ 도메인과 zinc finger DNA 결합 부위를 갖는 전사 조절 인자인 PLZF가 sumoylation된다는 사실을 바탕으로 FBI-1의 sumoylation 가능성을 예상, FBI-1에 consensus motif의 존재여부를 확인해보았다. 그 결과, 10개의 sumoylation 가능성이 있는 lysine 잔기가 존재함을 발견했다.

우리는 Sumoylation assay를 통해, FBI-1이 SUMO-1과의 공유결합에 의해 wild type의 FBI-1보다 더 큰 band가 생성되는 결과를 통해, FBI-1이 *in vitro*에서 sumoylation 됨을 확인하였다. 또한 SUMO가 결합하지 못하도록 만든 mutant가 FBI-1의 전사 억제 작용에 미치는 영향을 알아보기 위하여 FBI-1이 전사억제자로 작용하는 것으로 알려진 *ADH5* minimal 프로모터에서 mutant의 효과를 알아보았다. *ADH5* 프로모터의 존재 하에 FBI-1을 처리하였을 경우 잘 알려진 바와 마찬가지로 전사가 80%이상 억제되는 것을 볼 수 있다. FBI-1의 lysine 잔기를 arginine으로 각각 치환한 mutant (K61R, K383R, K371R, K379R, K383R, K486R, K497R, K536R, K539R)를 처리한 경우 FBI-1의 repression 효과가 조금씩 유의성 있게 감소됨을 보였다. 특히 K379R, K396R, K536R, K539R mutant의 경우 wild type의 FBI-1 보다 2배 이상 repression 효과를 감소시켰다. 이러한 sumoylation에 의한 효과는 FRE에 결합하여 전사를 증가시킨 FBI-1의 전사활성 기능을 억제시킴으로써 확인되었다. 이 결과들을 통해, 우리는 FBI-1이 SUMO-1과 결합하며 SUMO-1에 의해 전사조절능력에 영향을 받는다는 사실을 발견하였다.

핵심되는 말: FBI-1, SUMO-1, transcription repression, sumoylation