New proto-oncogene FBI-1 (Pokemon/ZBTB7a) inhibits p53 and Sp1 binding to repress the cyclin-dependent kinase inhibitor p21 gene transcription

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Directed by Professor Man-Wook Hur

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This certifies that the Master's Thesis

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New proto-oncogene FBI-1(Pokemon/ZBTB7a) inhibits p53 and Sp1 binding to repress the cyclin-dependent kinase inhibitor p21 gene transcription

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FBI-1 (Pokemon/ZBTB7a) is a ubiquitous POK family oncogenic transcription factor. Virtually every gene in the Arf-Mdm2-p53-p21 regulatory pathway, important in the regulation of cell cycle progression, was found to be a repression target of FBI-1. In this study, the cell cycle regulator gene $p21^{Waf/Cip1}$, which is downstream of Arf and p53, was selected for investigation into the molecular mechanism of transcriptional repression. This repression is known to involve FBI-1, p53, Sp1, the FRE (FBI-1 binding site)/Sp1-3 GC-box, and a distal p53-binding element. The FRE and Sp1-3 GC box sequences were found to overlap, and FBI-1 was found to

represses transcription by molecular competition with Sp1 for binding to the Sp1-3 GC-box, an element critical in transcriptional regulation by Sp1 and synergistic transcriptional activation by Sp1 and p53. FBI-1 was also found to compete with p53 for binding to the distal p53-binding element to repress transcription. FBI-1 bound to sequences from both the proximal and distal regulatory elements. FBI-1 also interacted with corepressors such as mSin3A, N-CoR, and SMRT, and such interactions led to deacetylation of the Ac-H3 and Ac-H4 histones at the proximal promoter, which is likely an important step in transcriptional repression.

FBI-1 expression caused cellular transformation and promoted cell cycle proliferation, as seen by a significant increase in the number of cells in the S-phase. Immunohistochemical staining showed that FBI-1 is prominently detected in adenocarcinoma (particularly in colon cancer) and squamous cell carcinoma cells, which express only low levels of p21. Overall, data from this study suggests that FBI-1 is the major regulator of the Arf-Mdm2-p53-$p21^{Waf/Cip1}$ pathway and that FBI-1 promotes oncogenic transformation and tumor cell growth.

Key Words : FBI-1, Pokemon, ZBTB7a, p21, Sp1, p53, ARF, Arf-Mdm2-p53-p21 pathway, corepressor, SMRT, NcoR, mSIN3A, HDAC

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I. Introduction

FBI-1 (Pokemon/ZBTB7a) is a ubiquitous transcription factor containing a BTB/POZ domain and Krüppel-like zinc fingers at its N-terminus and C-terminus, respectively. FBI-1 was first purified as a cellular factor that binds specifically to the wild-type IST (inducer of short transcript) elements of HIV-1 long terminal repeats (LTR) and the proximal promoter of the *ADH5/FDH* gene (1, 2), thus earning FBI-1 its name: factor that binds to the IST of Human Immunodeficiency Virus-1.

There have been several recent reports on the function of FBI-1. FBI-1 stimulates Tat (transactivator of transcription) activity on the HIV-1 LTR, participates in adipocyte differentiation, enhances the nuclear localization of NF-κB, and represses human *ADH5/FDH* gene expression (3-6). The mouse counterpart of FBI-1, LRF (leukemia/lymphoma-related factor), co-immunoprecipitates and co-localizes with Bcl-6 (7). The rat homolog of FBI-1, OCZF (osteoclast-derived zinc finger), is a transcriptional repressor involved in osteoclastogenesis (8). SAGE analysis and a recent report show that the expression of FBI-1 is increased in multiple cancers and that cells lacking FBI-1 are refractory to oncogenic transformation (9).

FBI-1 was recently shown to have proto-oncogenic activity by repressing the tumor suppressor gene *Arf*, which in turn lowers the expression of the tumor suppressor gene *p53*. While over-expressed FBI-1 causes oncogenesis in the thymus, liver, and spleen, down-regulated FBI-1 causes cellular senescence, apoptosis, and the blockage of cellular differentiation (9). Considering that FBI-1 is also

overexpressed in solid tumors, such as cancers of the colon and bladder, where the normal functions of *Arf* and *p53* are lost (9), it is likely that FBI-1 has additional target genes by which it can exert its oncogenic activity. This study sought to investigate whether genes in the Arf-Mdm2-p53-p21$^{Waf/Cip1}$ pathway, which is important in cell cycle regulation and oncogenesis, are controlled by FBI-1. In particular, the molecular mechanism of repression of *p21*$^{Waf/Cip1}$ (hereafter indicated as *p21*) expression by FBI-1 was characterized in detail.

The cyclin-dependent kinase inhibitor p21 plays a major role in the regulation of cell cycle progression in mammalian cells. Its main function is to inhibit the activities of cyclin/cdk2 complexes and negatively modulate cell cycle progression. *p21* is primarily regulated at the transcriptional level. Whereas induction of *p21* leads predominantly to cell cycle arrest, repression of *p21* may have a variety of outcomes depending on the context (10-11). *p21* is a transcriptional target of p53 and plays a crucial role in mediating growth arrest when cells are exposed to DNA damaging agents such as doxorubicin and γ-irradiation (12, 13). In addition to its role in the DNA damage response, p21 has also been implicated in terminal differentiation, replicative senescence, and protection from p53-dependent and -independent apoptosis (12, 17 and references therein). A variety of other factors, including Sp1/Sp3, Smads, Ap2, STAT, BRCA1, E2F-1/E2F-3, and C/EBPα and ß, also activate the transcription of *p21* (17, 18), and overexpression of *p21* results in G_1-, G_2- (14), or S-phase arrest (15, 16).

The major regulators that affect *p21* gene expression are Sp-family transcription factors bound at the proximal promoter region between base pairs (bp) −120 to −50 upstream of the transcription start site (18-21). Among the six Sp1 binding sites found in the region, the Sp1-3 GC-box has been shown to be particularly important in the transcription of the *p21* gene. Mutation of this particular site not only dramatically reduces transcription but also disrupts the synergistic transcriptional activation by Sp1 and p53, important for *p21* induction under genotoxic stress (18).

Sp1 is a member of a family of transcription factors with zinc finger DNA-binding domains that bind to GC-box sequences in numerous cellular and viral genes (22). Sp1 is one of the best characterized sequence-specific transcription factors, and the domains of Sp1 crucial to its transactivation, DNA binding, and oligomerization functions have been identified. The N-terminal glutamine- and serine/threonine-rich domains are essential for transcriptional activation, the C-terminal ZFDBD (zinc finger DNA-binding domain of Sp1) is involved in interaction with other transcription factors, and the ZFDBD alone is sufficient for efficient recognition of its target gene (23).

Sp1 has been shown to interact directly with proteins of the basal transcription machinery, several sequence-specific activators, coactivators, and corepressors including NF-κB, GATA, YY1, E2F1, Rb, SREBP-1, p300, HDAC, NCoR, SMRT, and BCoR. These interactions are important in the transcriptional regulation of genes with Sp1 binding sites in their promoters (2, 22, 24 and references therein). The direct competition among Sp1, Sp1 family members, and Krüppel-like

transcription factors is important in the transcriptional regulation of the genes with GC-boxes in their promoters (25 and references therein).

The FBI-1 DNA-binding consensus sequence, FRE, is a GC–rich sequence that has similarity to the Sp1 consensus sequence (9, 26). This study revealed that the *p21* promoter contains a potential FRE and that *p21* was potently repressed by FBI-1. Based on this initial finding, the molecular mechanism of transcriptional repression was investigated and found to involve p53, FBI-1, Sp1, the proximal FRE/Sp1-3 GC-box, the distal p53-binding element, and a corepressor-HDAC complex, which is important in oncogenic cellular transformation and tumor growth. These results showed that FBI-1 is the major regulator of the Arf-Mdm2-p53-p21 pathway.

II. Materials and Methods

1. *Plasmids, antibodies, and reagents*

pGL2-p21-Luc, pGL2-p21 Wt-131-Luc and pGL2-p21 Wt-101-Luc plasmids were kindly provided by Dr. Yoshihiro Sowa of Kyoto Perpetual University of Medicine (Kyoto, Japan). Various mutant p21 promoter and reporter gene fusion plasmids with mutations at the FRE and Sp1 binding GC-box were prepared using site-directed mutagenesis kit (Stratagene, CA). Construction of the pcDNA3-FBI-1 and pcDNA3-FBI-1ΔPOZ plasmids was reported elsewhere (2, 4). pTrex-FBI-1 plasmid was cloned into pcDNA5.0/FRT/TO© (Invitrogen, CA) to prepare doxycyclin inducible 293Trex-FBI-1 cell. The pcDNA3.1-p53 expression plasmid was prepared by cloning the human p53 cDNA into pcDNA3.1 (Invitrogen, CA). The expression plasmids for Sp1ZDBD (a.a. 622-778) and FBI-1ZFDBD (a.a. 382-490) were prepared by cloning PCR amplified cDNA fragments into pGEX 4T1 (Amersham Biosciences, NJ). To prepare the GST-POZFBI-1 fusion protein expression plasmid, the cDNA fragment encoding the POZ-domain of FBI-1 was cloned into pGEX4T3 (Amersham Biosciences, NJ) as reported elsewhere (2). Antibodies against FBI-1, p21, p53, His-Tag, FLAG-Tag. Myc-Tag, Ac-Histone 3, Ac-Histone 4, tubulin, SMRT, NCoR, mSin3A, HDACs, and GAPDH were purchased from Upstate (Charlottesville, VA), Chemicon (Temecula, CA), and Calbiochem (San Diego, CA), SantaCruz Biotech (SantaCruz, CA), Abcam

(Abcam, Cambridge, UK). Most of the chemical reagents were purchased from Sigma (St. Louis, MO).

2. Cell culture/stable cell line

HeLa, NIH3T3, HEK 293A, HCT116 $p53^{-/-}$, and HCT116 $p53^{+/+}$ cells were cultured in Dulbecco' modified eagle medium (DMEM)(Gibco-BRL, MD) supplemented with 10% fetal bovine serum (FBS)(Gibco-BRL, MD). Saos-2 cells were cultured in McCoy's 5A medium supplemented with 15% FBS. MB352 MEF and human osteosarcoma Saos-2 cell lines, both lacking endogenous p53, were purchased from American type culture collection (ATCC) (Manassas, VA).

HeLa cells stably overexpressing FBI-1 were prepared via transfection with a recombinant Lenti virus, LentiM1.4-FBI-1-FLAG. 4×10^7 TU/ml of LentiM1.4-FBI-1_FLAG (Vectorcorea, Korea) in a volume of 350 μl were used to infect HeLa cells plated on 12-well plates supplemented with polybrene 8 μg/ml (Sigma, MO) to increase infection efficiency. Infected cells were incubated at 37 in a 5% $CO℃_2$ atmosphere for 6~8 hrs, after which culture media was replaced. After an additional incubation for 2 to 3 days, cells were transferred onto 6-well plates and stable cells were selected with 1 μg/ml puromysin. Control stable cells were prepared by infection with LentiM1.4eGFP and LentiM1.4LacZ recombinant virus over expressing GFP and β-galactosidase, respectively (Vectorcorea, Korea).

NIH3T3, HCT116 $p53^{-/-}$, and HCT116 $p53^{+/+}$ cells were cultured in DMEM supplemented with 10% fetal bovine serum. Stable NIH3T3, HCT116 $p53^{-/-}$, and HCT116 $p53^{+/+}$ cells overexpressing FBI-1 were prepared by transfection of NIH/3T3 cells with a recombinant Lenti virus, LentiM1.4-FBI-1 tagged with His and Myc peptide. After 2~3 days of incubation, cells were transferred onto a 6-well plate and selected stable cells with Puromysin (Sigma, MO). Control stable cells were prepared by infection with LentiM1.4 (Vectorcorea, Korea).

FBI-1 over expression cells inducible by Doxycyclin were prepared by transfection of mammalian Flp-In™ T-REx™ host 293T cells (Invitrogen, CA) with a 9:1 ratio of pOG44:pcDNA5/FRT/TO©-FBI-1 plasmid DNA using Lipofectamin 2000 (Invitrogen, CA). The Flp-In™ T-REx™ FBI-1 stable cells overexpressing FBI-1 was selected by culturing the transfected cells in a medium containing hygromycin (300 μg/mL) and blasticidin (15 μg/mL). To induce expression of the FBI-1, the stable cells were cultured in medium containing 1 μg/mL doxycyclin for intended period of time.

3. *Transcriptional analysis of the pGL2-p21$^{Waf1/cip1}$-Luc, pGL2-ARF-Luc and pGL2-p53-Luc promoter*

pGL2-p21-Luc, pGL2-ARF-Luc, and pGL2-p53-Luc promoter fusion plasmids were generously provided by Dr. Sowa. Various mutant pGL2-p21-Luc promoter and reporter fusion gene plasmids were prepared by site-directed mutagenesis (Stratagene, CA). Wt or mutant p21-Luc reporter plasmids, along

with pcDNA3-FBI-1, pcDNA3.1-p53 and pCMV-LacZ, were transiently transfected into HeLa using Lipopectamine Plus reagent (Invitrogen, CA) according to manufacturer's recommended protocol. After 36 hrs of incubation, cells were harvested and analyzed for luciferase activity. The reporter activity was normalized with cotransfected β-galactosidase activity or protein concentration.

4. ***Knock-down of FBI-1 mRNA and expression by siRNA***

The siRNA for glyceraldehyde-3-phosphate dehydrogenase and three siRNAs of FBI-1 were designed and purchased from Ambion Inc. (Austin, TX). siFBI-1 #1, 5'-GCAGAACGUGUACGAGAUCtt -3', 5'-GAUCUCGUACACGUUCUGCtg-3'; siFBI-1 #2, 5'-CCUUGUAGAUCAAAUUGAUtt-3, 5'-AUCAAUUUGAUCUACAAGGtc-3; siFBI-1 #3, 5'-CGGGUACUACACUUUAUCUtt-3, 5'-AGAUAAAGUGUAGUACCCGtt -3'. The siRNAs of FBI-1 (20 ng of each) were transfected into 6x 10^6 HeLa cells by using Lipofectamine RNAiMAX (Invitrogen, CA). After transfection, the cells were harvested, and total RNA was prepared and RT-PCR of mRNA was performed as described below.

5. ***RT-PCR of FBI-1 mRNA in cells***

Total RNA was isolated from HeLa cells and stable HeLa cells expressing FBI-1, GFP and LacZ using TRIzol reagent (Invitrogen, CA). cDNAs were

synthesized using 5 μg total RNA, random hexamer (10 pmol), and Superscript reverse transcriptase Ⅱ (200 units) in 20 μl using reverse transcription kit (Invitrogen, CA). PCR were performed by following amplification cycling condition: 94℃ denaturation 5 min, 35 cycles of amplification reaction, 94℃ 30 sec, 55℃ 30 sec, 72℃ 1 min, and final extension reaction at 72℃ 5 min. PCR primers used were as follows; FBI-1 primer (forward primer: 5'-GGCCTGCTGTGCGACGTGGT-3', reverse primer: 5'-CAGCAGGCGGGCGGCGCTGA-3'). β-actin primer (forward primer : 5'-ATGGATGACGATATCGCTGC-3', reverse primer: 5'-CACACTGTGCCCATCTACGA-3'). GAPDH primer (forward primer: 5'-ACCACAGTCCATGCCATCAC-3', reverse primer: 5'-TCCACCACCCTGTTGCTGTA -3'). Human p21 primer (forward primer: 5'-ATGTCAGAACCGGCTGGGGATGTCC-3', reverse primer: 5'-TTAGGGCTTCCTCTTGGAGAAGATC-3').

6. ***Western Blot Analysis***

HeLa and NIH3T3 cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl pH8.0, 1% NP-40, 0.25% sodium deoxycholic acid, 150 mM NaCl, 1 mM EGTA, complete mini-protease cocktail), and cellular extracts (40 μg) were separated on a 10% SDS-PAGE gel. Proteins were then transferred to a Immun-Blot^{TM} PVDF Membrane (Bio-Rad, CA) and blocked with 5% skim milk (BD, Biosciences, MD) for 1 hr. Blotted membranes were incubated with antibodies

against FLAG-tag (Abcam, Cambridge, UK), FBI-1 (Abcam, Cambridge, UK), GAPDH (Chemicon, CA), or $p21^{Waf/Cip1}$ (Upstate, NY) diluted 1:2000. Lastly, blots were incubated with HRP conjugated mouse or goat IgG (Vector Laboratory, CA). Protein bands were visualized with an ECL solution (PerkinElmer, CA).

7. *Electrophoretic mobility shift assays (EMSAs)*

EMSAs were carried out as described previously (29). The sequences of FBI-1 binding FRE and mutant FRE probes used in EMSA are as follows. Top strand sequences are shown. FRE, 5'-GATCGAGGCGGGACCCGCGCTCG-3'; mFRE, 5'-GATCGAGGCGGGAAAAGCGCTCG-3'.The sequence of the Sp1 binding GC-box 3 probe is, 5'-GAGGCGGGACCCGCGCTCG-3'.Mutant Sp1 binding GC-box 3 probe, 5'-GAGGCTTTACCCGCGCTCG-3'. Each binding reaction was carried out at room temperature for 30 min in 20$\mu\ell$ of binding buffer (10 mM HEPES pH7.9, 60 mM KCl, 5 μM $ZnCl_2$, 1 mM dithiothreitol, 1% BSA, and 7% glycerol) with 0.1 μg recombinant FBI-1ZFDBD or Sp1ZFDBD and 10,000 cpm labeled probe. Where indicated, antibodies against GST-Tag, FBI-1, or Sp1 was added to EMSA binding reactions. To investigate the binding competition between Sp1 and FBI-1 for FRE, probe was incubated with recombinant Sp1ZFDBD (75 ng) and increasing amounts of FBI-1 ZFDBD (75–675 ng).

8. ***Site-directed mutagenesis of pGL2- p21$^{Waf1/cip1}$-Luc***

To investigate the role of FRE, mutation was introduced into the proximal promoter sequence of the *p21* gene using the QuikChange site-directed mutagenesis kit (Stratagene, CA). To introduce mutation into the core binding sequences of FRE, the following oligonucleotide was used. Only top strand is shown. mFRE5, 5'-CCCGCCTCAAGGAGGCGGGAAAAGCGCTCGGCCC-3'. For site-directed mutagenesis, 18 cycles of PCR with denaturation at 94℃ for 30 sec, hybridization at 55℃ for 1 min, and extension at 68℃ for 10 min per cycle were used. Amplified mixtures were treated with DpnI (Stratagene, CA) at 37℃ for 1 hr and aliquots were used to transform competent *E. coli*. All of the constructs were confirmed by DNA sequencing using an ABI automatic DNA sequencer (Ramlsey, MN).

9. ***Chromatin immunoprecipitation (ChIP) assays***

We investigated whether the molecular interaction between FBI-1 and the FRE is actually occurs *in vivo* using the ChIP assay kit (Upstate Inc., VA). Subconfluent HeLa and Saos-2 cells growing on a 10 cm dish were transfected with pGL2-p21-Luc (1 μg) and either pcDNA3 or pcDNA3-FBI-1-Flag (3 μg) using Lipofectamine Plus reagent and grown for 48 hrs. HeLa or SL2 cells were fixed with formaldehyde (final 1%), washed with a cold phosphate-buffered saline, lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH8.0), and sonicated to shear into DNA fragments of 500~1000 bp. The

sonicated supernatant was diluted 10-fold with ChIP dilution buffer (1% SDS, 1% Triton X-100, 16.7 mM Tris-HCl, pH8.1, 167 mM NaCl, 1.2 mM EDTA) and pre-cleared with protein-A agarose beads (Sigma, MO). The supernatant was then incubated with antibodies mouse M2 ant-FLAG monoclonal antibody (Sigma, MO) or control mouse IgG overnight at 4°C with rotation. Salmon sperm DNA/protein A-agarose slurry was added to the mixture, incubated for 1 h at 4°C, and pelleted DNA-FBI-1/protein A-agarose complex by brief centrifugation (4,000 rpm) at 4°C. After extensive washing of the pellet with various washing buffers, the pellet was dissolved with 500 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) and spun to remove agarose. The supernatant was treated with 20 μl of 5 M NaCl and heated to 65°C for 4 hrs to reverse protein-DNA cross-linking. After treatment with EDTA and proteinase K, the supernatant was extracted with phenol/chloroform and precipitated with ethanol to recover DNA. PCR reactions of immunoprecipitated DNA were carried out using oligonucleotide primers designed to amplify the proximal promoter region or distal p53 binding region of p21 gene. Proximal region ChIP PCR primers (bps -287 to +16, forward primer: 5'-AAAAAAGCCAGATTTGTGGC-3', reverse primer: 5'-TGCTCACACCTCAGCTGGCG-3'). Distal FBI-1/p53 binding region (bp -2307 to - 1930, forward primer: 5'-TGCTTGGGCAGCAGGCTGTG-3', reverse primer: 5'-GCAACCATGCACTTGAATGT-3').

We also investigated whether the acetylation status of histone H3 and H4

tails of nucleosomes at the proximal p21 promoter element is modified by ectopic FBI-1 using antibodies specific to Ac-Histone H3 and Ac-Histone H4 by following the same procedures as described above.

Also to demonstrate the binding competition between Sp1 and FBI-1 in Drosophila SL2 cells, pGL2-p21-Luc 2.4 kb reporter plasmid, and expression vector of pPac-Sp1 and 1-4 fold of pPac-FBI-1 expression vector were contransfected. After 36 hrs, SL2 cells were fixed, immuniprecipitated, and analyzed as described above.

10. ***Immunoprecipitation assays***

Human embryonic kidney 293Trex-FBI-1-FLAG cells were induced with doxycyclin for 12 hrs, 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 M2 anti-FLAG antibody on a rotating platform overnight at 4℃, followed by incubation with protein A-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.

11. ***Fluorescence Activated Cell Sorter (FACS) analysis***

HeLa cells were gently washed with PBS, fixed with ice-cold 70% methanol

at -20°C for 1 hr, washed with cold PBS, and stained with 50 μg/mL Propidium Iodide (Sigma, MO) in the presence of 100 μg/mL ribonuclease A for 30 min at 37°C in the dark. DNA content was analyzed by flow cytometry analyzer FACSCalibur (BD biosciences, NJ). DNA contents were assessed by cell treatment with PI and emission detection at the excitation wavelengths 488 nm and the peak emission 575 nm. The cell cycle profile and forward scatter (FSC) were determined using a Becton Dickinson FACS caliber and the data were analyzed using ModFit LT 2.0 (Verity Software House, Inc., ME) and WindMDI 2.8 (Created by Joseph Trotter, Scripps Research Institute, CA).

12. ***Colony Foci Formation Assay***

The HeLa cells were cultured for 24 hrs in six-well plates at a density of $1x10^5$ cells/well in 2 ml of DMEM, and 0.5 μg/μL pcDNA3 or pcDNA3-FBI-1 was transfected into the cells using Lipofectamine plus reagent (Invitrogen, CA). After transfection, cells were maintained in the medium with 10% FBS for 2 weeks in the presence of G418 (800 μg/mL)(Sigma, MO). Medium containing G418 was changed twice a week. After 2 weeks, G418 resistant HeLa cell colonies were stained with 0.5% Crystal Violet (Sigma, MO) in 20% ethanol.

13. ***BrdU incorporation Assays***

The HeLa, NIH-3T3 and NIH-3T3-FBI-1 stable cells were plated onto glass cover slips at 40% confluency and were transfected with 1.68 μg of siRNA in a

3.5 cm dish or with pcDNA3-FBI-1-FLAG or pcDNA3. After 48 hrs, cells were grown for 4 hrs in DMEM containing 20 μM BrdU. The cells were washed twice in PBS, fixed in a methanol/formaldehyde (99:1) mixture, and permeabilized with PBS containing 0.2% Triton X-100. The cells were incubated with anti-BrdU moncoclonal antibody for 2 hrs, washed with PBS, and further incubated wih Alexa Fluor 488 goat anti-mouse IgG secondary antibody for 1 h. To stain nuclei, cells were soacked with DAPI solution for 10 min. HeLa cells were mounted and analyzed with Radiance 2100 Laser Scanning System (Bio-Rad, UK). Each analysis was performed at least three times.

14. *Immuno-histochemistry*

Serial sections of colonal cancer tissues (4 μM) were applied to silane coated slides (Muto Pure Chemicals, Tokyo, Japan). Deparaffinixation and rehydration were performed using Xylene and graded alcohols. Endogenous peroxydase was blocked with 3% hydrogen peroxide for 10 minutes. For antigen retrieval, the slides were pretreated in a microwave oven for 15 minutes in a pressure cooker filled with citric acid buffer (pH6.0). The slides were then incubated at room temperature for 1 hour with primary antibodies, anti-FBI-1 (Abcam, Cambridge, UK), anti-p53 (SantaCruz, CA), anti-p21 (Dako, CA), followed by a 4^{o}C overnight incubation. After washing, the signals were detected with the Envision kit (Dakocytomation, Glostrup, Denmark) and

diaminobenzidine as a chromogen. In case of FBI-1 Ab, we used biotinylated anti-goat IgG (Vector Laboratory, Burlingame, CA) and streptavidin-HRP as detection system. The slides were also counter stained with hematoxylin. Appropriate positive and negative controls were included in all stains to ensure the quality and consistency of staining results.

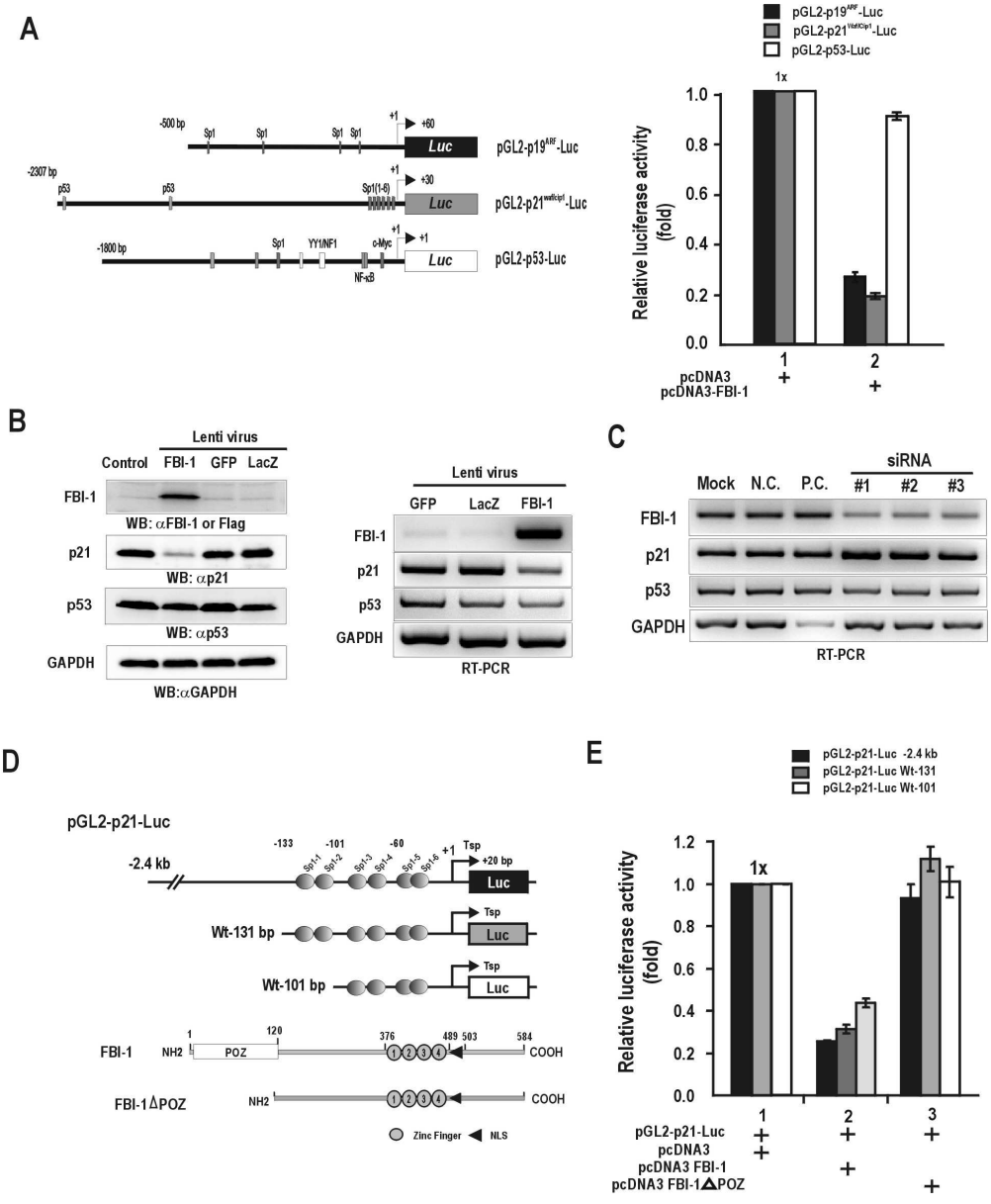
Various cancer anc colon cancer tissue AccuMax arrays® (Cat# A203, A201) were purchased from ISU Abxis (Seoul, South Korea) and were treated the same way as the paraffin embedded tissue samples.

III. Results

1. FBI-1 represses transcription of *p21* gene in HeLa cells

FBI-1 causes multiple cancers by repressing transcription of *Arf*, which results in an inhibition of *p53* gene expression (9). Several groups have shown that FBI-1 can modulate various target genes important in key biological functions (1-9). Although *Arf* was shown to be repressed by FBI-1 and such repression might be important in oncogenesis, FBI-1 might also regulate other genes in the Arf-Mdm2-p53-p21 regulatory pathway that play critical roles in oncogenesis, such as *p21*(10).

To further explore this possibility, FBI-1 regulation of three genes in the pathway (*Arf, p21, p53*) was evaluated in HeLa cells cotransfected with promoter-luciferase plasmids and an FBI-1 expression plasmid (Fig. 1A). FBI-1 potently repressed *Arf* as previously reported (9) and also repressed the *p21*, but not the *p53*, promoter. Further investigation into the transcription of the *p21* gene assessed whether *p21* expression is modulated by either overexpression or knock-down of FBI-1. In the HeLa cells transfected with recombinant lentivirus overexpressing FBI-1, FBI-1 expression reduced protein and mRNA levels of both *p21* and p53, but did not affect levels of the control, GAPDH (Fig. 1B). The weak reduction of p53 appeared to be caused by the transcriptional repression of *Arf*, which is an activator of *p53* transcription. Alternatively, HeLa cells were treated with three siRNAs specific to FBI-1 mRNA. Decreasing FBI-1 expression resulted in increased *p21* expression while the FBI siRNA did not affect p53 or GAPDH expression (Fig. 1C).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pGL2-p21Waf/Cip1-Luc
pGL2-p53-Luc
-500 bp
Sp1
+1
+60
Luc
-2307 bp
p53
+30
Sp1(1-6)
-1800 bp
YY1/NF1
c-Myc
NF-κB
Relative luciferase activity (fold)
1x
pcDNA3
pcDNA3-FBI-1
B
Lenti virus
Control
FBI-1
GFP
LacZ
WB: αFBI-1 or Flag
p21
WB: αp21
p53
WB: αp53
GAPDH
WB:αGAPDH
RT-PCR
C
Mock
N.C.
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siRNA
#1
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D
pGL2-p21-Luc
-2.4 kb
-133
-101
-60
Sp1-1
Sp1-2
Sp1-3
Sp1-4
Sp1-5
Sp1-6
Tsp
+20 bp
Wt-131 bp
Wt-101 bp
FBI-1
NH2
POZ
COOH
FBI-1ΔPOZ
Zinc Finger
NLS
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pGL2-p21-Luc Wt-101
pcDNA3 FBI-1
pcDNA3 FBI-1ΔPOZ

Figure 1. FBI-1 represses transcription of the *p21$^{Waf/Cip1}$* gene in HeLa cells. ***(A)*** Structures of the ARF, p53, and p21 gene promoters fused with luciferase reporter gene cloned in pGL2-Luc. Binding sites for transcription factors are indicated. +1, transcription start site (→). HeLa cells were co-transfected with promoter-Luc fusion palsmid, FBI-1 expression plasmid, and pCMV-LacZ expression plasmid. After 48 hrs, cells were harvested and analyzed for luciferase reporter activity. The data shown are an average of three independent assays. Bars, S.D. ***(B)*** Western blot and RT-PCR analysis of the stable HeLa cells overexpressing FBI-1, GFP, or LacZ established by transfection with recombinant Lenti viruses. GAPDH, control. ***(C)*** Knock-down of FBI-1 mRNA by three different siRNAs and p21 transcription. HeLa cells were transfected with three different siRNAs against FBI-1 mRNA and mRNA expression of FBI-1, p21, p53, and GAPDH was analyzed. NC, negative control scrambled siRNA. PC, positive control siRNA against GAPDH. ***(D)*** Structures of the three p21-Luc plasmids and the two FBI cDNA constructs in pcDNA3.0. Shaded circles, Sp1 binding GC-box; POZ domain, Open box; numbered filled circles, zinc fingers; triangle, nuclear localization sequence. ***(E)*** FBI-1 represses transcription by acting on the proximal promoter of the p21 gene and the POZ-domain is important in transcriptional repression. HeLa cells were transiently co-transfected with plasmids expressing FBI-1 or FBI-1△POZ and pGL2-p21-Luc. Luciferase activity was measured 48 hrs after transfection. The data shown are an average of three independent assays. Bars, S.D.

All together, the data showed that the *p21* gene, a key regulator of the cell cycle and end regulator of the Arf-Mdm2-p53-p21 pathway, is potently repressed by FBI-1.

The mechanism of repression was evaluated by testing which regions of the *p21* promoter and which domains of FBI-1 were important for transcriptional repression. HeLa cells were co-transfected with two different pGL2-*p21*- promoter-luciferase plasmids with a vector expressing wild-type FBI-1 or mutant FBI-1 POZ (Fig. 1D) (2). FBI-1 potently repressed transcription of the three promoter constructs by >60%-70%. FBI-1 most potently repressed the construct lacking 2.4 kb of upstream sequence. Deletion of the POZ domain resulted in no transcriptional repression, suggesting that the POZ domain is important for transcriptional repression (Fig. 1E). The data suggest that FBI-1 represses transcription by acting on the proximal promoter, which is highly loaded with Sp1-binding GC boxes, and potentially also by other mechanisms that involve distal upstream regulatory elements.

2. Identification of proximal FBI-1 binding promoter element (FRE) and overlap of the FRE which the Sp1-3 GC-box

Analysis of the reported FBI-1 binding sites and CAST assay data showed that the FBI-1 binding consensus sequence is 5'-GDGGGYYYY-3' (9, 26). Using the consensus sequence, the *p21* promoter was examined for potential FBI-1 binding sites. Seven potential FBI-1 binding sites (FREs) were found, located proximal to or overlapping with the six well-defined Sp1 binding sites. EMSA and transcription

assays revealed one functionally significant FRE (5'-GCGGGCCCC-3') which overlaps with the Sp1-3 GC-box (bp -82 to -77). Recombinant FBI-1ZFDBD (zinc finger DNA-binding domain) and the FRE probe showed a strong binding interaction, which was inhibited by excess cold probe and super-shifted by antibody. Mutation of the core GGG sequence into TTT completely prevented probe binding to FBI-1 ZFDBD (Fig. 2A, B).

To investigate whether the FRE is important in the transcriptional repression of the *p21* gene by FBI-1 *in vivo*, mutations were introduced into the pGL2-*p21*-Luc Wt-131 bp construct, resulting in the pGL2-*p21*-Luc-mFRE-131 bp construct, where the core GGG was replaced with TTT. In HeLa cells, FBI-1 potently repressed the pGL2-*p21*-Luc Wt-131 bp promoter but could not repress transcription on the mutated promoter, suggesting that FBI-1 binds to the FRE and represses transcription (Fig. 2C).

Furthermore, the interactions between the FRE/Sp1-3 GC-box and FBI-1 on the endogenous *p21* gene *in vivo* were investigated by chromatin immuno-precipitation (ChIP) assays. ChIP assays clearly showed that FBI-1 bound to the proximal promoter region (bp -287 to +16) and that FBI-1 does not bind to the GLUT2 promoter, which was used as a control (Fig. 2D).

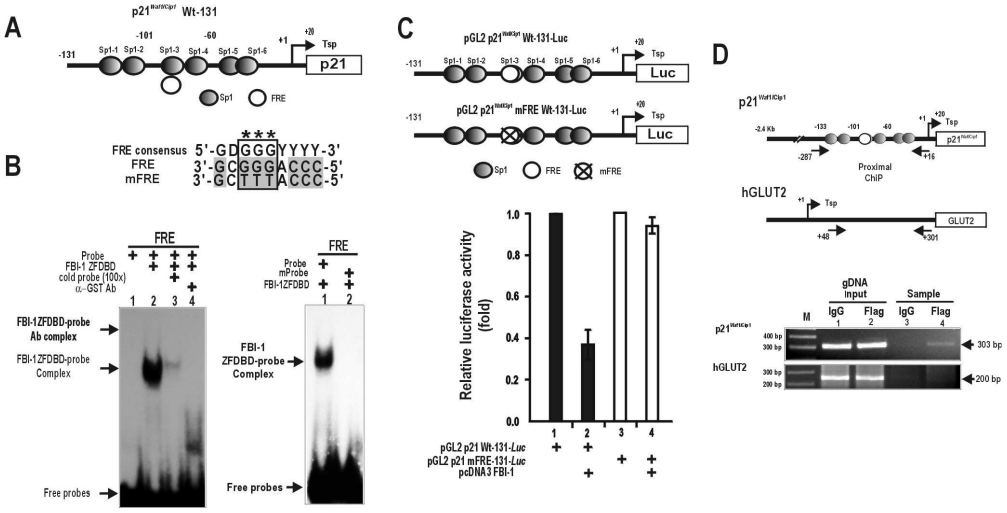


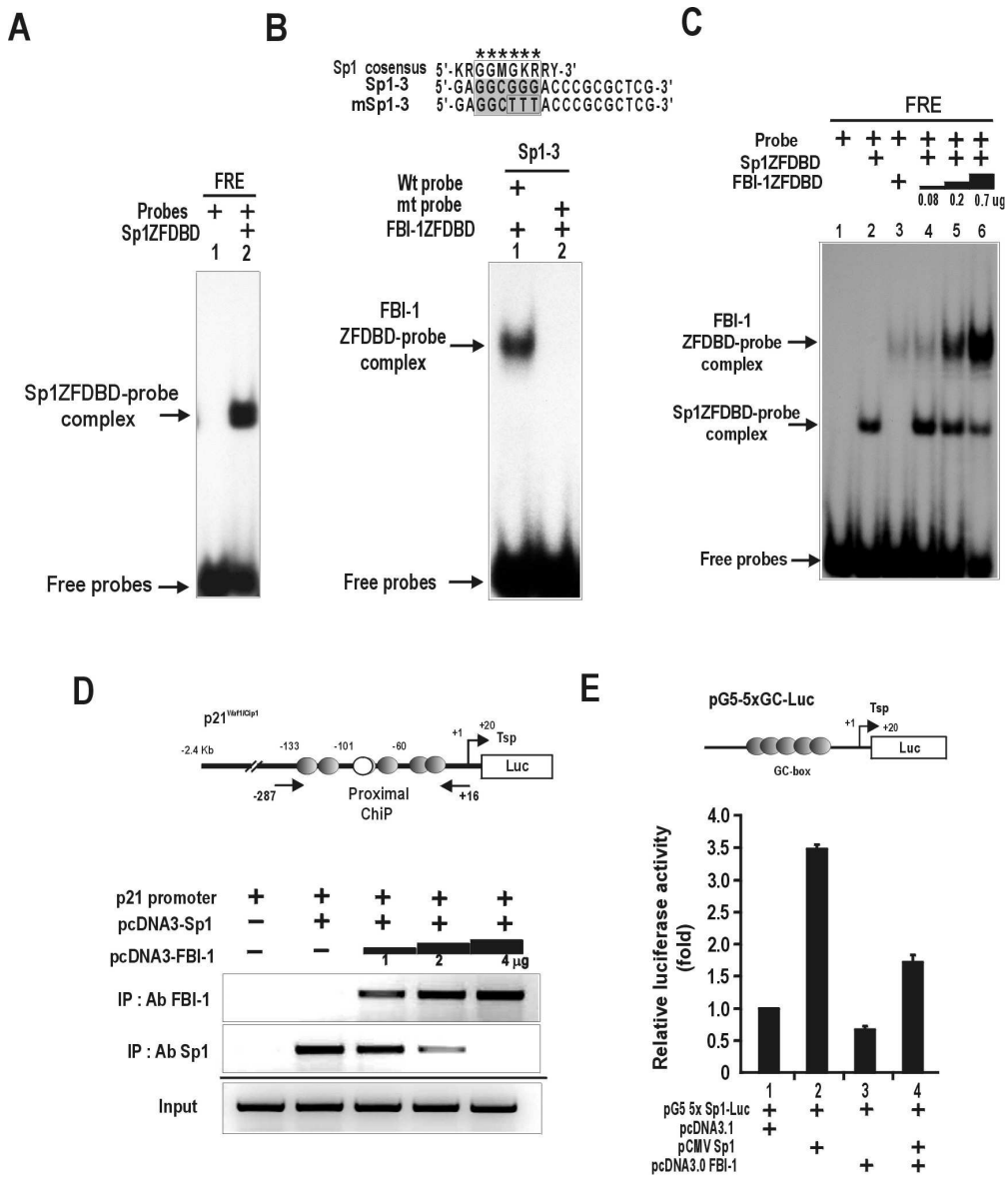
Figure 2. Identification of a functional FRE (bp -97 to -75) in the proximal *p21*$^{Waf/Cip1}$ promoter and overlap of FRE and Sp1-3 GC-box sequences. ***(A)*** Structure of the pGL2-*p21*-Luc-131 bp. GC boxes, shaded filled circles; FRE, open circle. The Sp1-3 GC-box and FRE are overlapping. ***(B)*** EMSA. Recombinant FBI-1 binds the FRE probe, and mutation of the core GGG→TTT (mFRE) abolishes FBI-1 binding. ***(C)*** The FRE is important in transcriptional repression by FBI-1 *in vivo*. HeLa cells were transiently co-transfected with plasmids expressing FBI-1 and pGL2-*p21*-Luc-Wt FRE or pGL2-*p21*-Luc-mFRE. Luciferase activity was measured 36 hrs after transfection. The data shown are an average of three independent assays. Bars, S.D**.** ***(D)*** ChIP assays and structures of two endogenous human gene promoters, *p21* and hGLUT2. The arrows indicate the locations of PCR primers used in ChIP assays. GC boxes, filled circles; FRE, open circle. gDNA, human genomic DNA from HeLa cells; IgG and Flag Ab, antibodies used in immunoprecipitation; Tsp (+1, →), transcription start site.

3. Molecular competition between Sp1 and FBI-1 at the FRE/Sp1-3 GC box is partly responsible for transcription repression

The EMSA data showed that FBI-1 binds to the FRE, which overlaps the Sp1-3 GC-box, raising the possibility that the two overlapping elements might be the center of molecular interactions or competition between FBI-1 and Sp1. Thus, the ability of Sp1ZFDBD to bind the FRE was investigated, and EMSA showed that Sp1ZFDBD binds well to the FRE (Fig. 3A). Alternatively, the ability of FBI-1 to bind to the Sp1-3 GC box probe was also investigated. FBI-1 also binds well to the GC-box probe, and mutation of the GGG core into TTT abolished FBI-1 binding to the probe (Fig. 3B).

The EMSA suggests that molecular competition between FBI-1 and Sp1 for the FRE/Sp1-3 GC box may occur *in vivo*, and that this competition may be important in the transcriptional repression of *p21* by FBI-1.

Competition between Sp1 and FBI-1 for binding to the FRE/Sp1-3 GC-box was evaluated by EMSA using recombinant Sp1ZFDBD (75 ng) in the presence of increasing amounts of FBI-1ZFDBD (75-700 ng). FBI-1 competed well with Sp1 at the FRE (Fig. 3C). The molecular competition was also investigated *in vivo*. The pGL2-*p21*-Luc reporter and pPac expression vectors for Sp1 and FBI-1 were co-transfected into Drosophila SL2 cells. ChIP assays showed that as the amount of FBI-1 expression is increased, Sp1 binding significantly decreased, demonstrating a molecular competition between Sp1 and FBI-1 at the *p21* proximal promoter *in vivo* (Fig. 3D).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FRE
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Sp1ZFDBD +
1 2
Sp1ZFDBD-probe complex
Free probes
B
******
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Sp1-3 5'-GAGGCGGGACCCGCGCTCG-3'
mSp1-3 5'-GAGGCTTTACCCGCGCTCG-3'
Sp1-3
Wt probe +
mt probe +
FBI-1ZFDBD + +
1 2
FBI-1 ZFDBD-probe complex
Free probes
C
FRE
Probe + + + + + +
Sp1ZFDBD + + + +
FBI-1ZFDBD + +
0.08 0.2 0.7 ug
1 2 3 4 5 6
FBI-1 ZFDBD-probe complex
Sp1ZFDBD-probe complex
Free probes
D
p21 Waf1/Cip1
-2.4 Kb
-133
-101
-60
+1
+20
Tsp
Luc
-287
Proximal ChiP
+16
p21 promoter + + + + +
pcDNA3-Sp1 − + + + +
pcDNA3-FBI-1 − − 1 2 4 µg
IP : Ab FBI-1
IP : Ab Sp1
Input
E
pG5-5xGC-Luc
Tsp
+1
+20
Luc
GC-box
Relative luciferase activity (fold)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
1 2 3 4
pG5 5x Sp1-Luc + + + +
pcDNA3.1 +
pCMV Sp1 + +
pcDNA3.0 FBI-1 + +

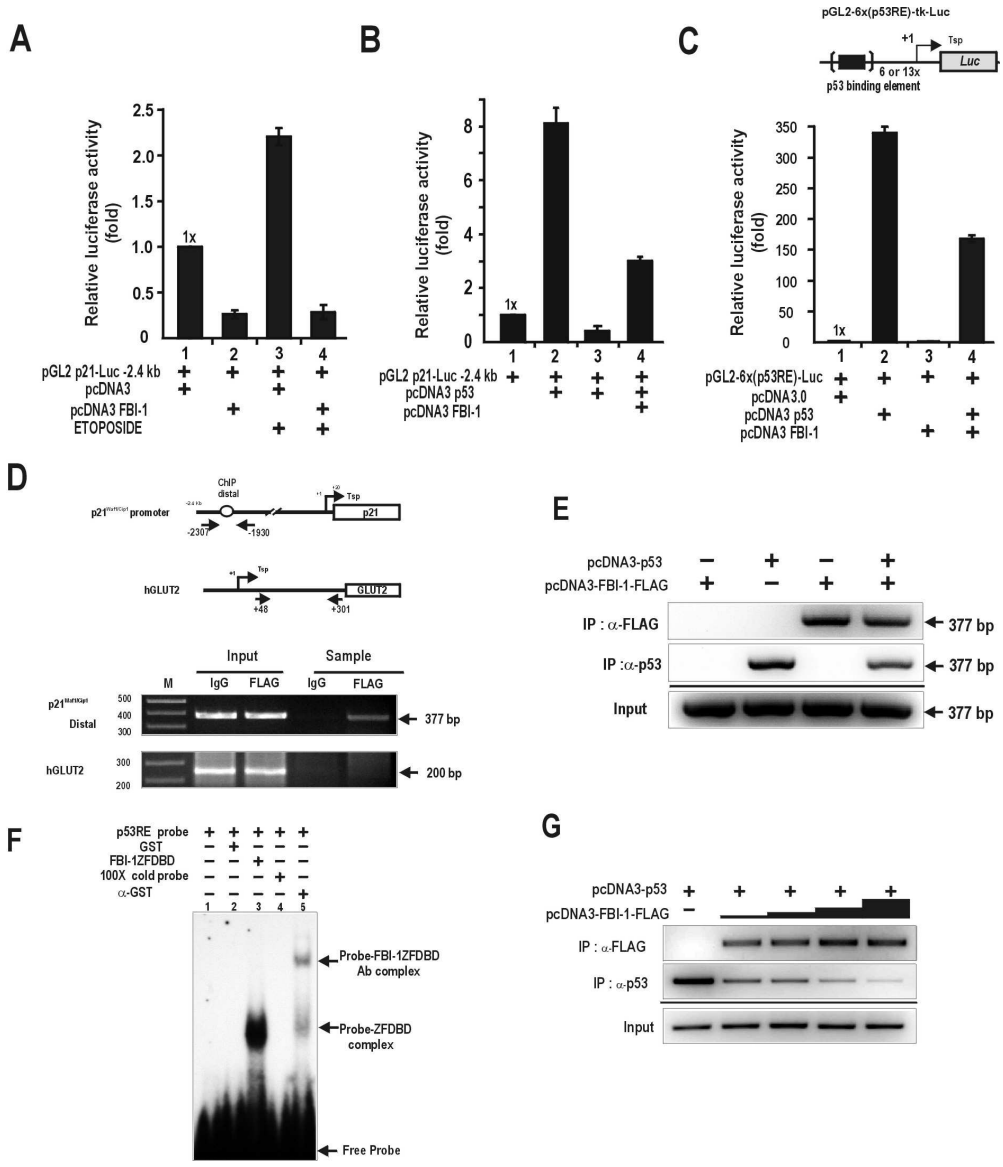
Figure 3. Sp1 and FBI-1 compete with each other in binding to the FRE/Sp1-3 GC-box *in vivo* and *in vitro*. *(A)* EMSA. The Sp1ZFDBD binds to the FRE. ***(B)*** EMSA. Sequence alignment of the Sp1 consensus sequence, Sp1-3 GC box, and mutated Sp1-3 GC box. In mSp1-3 GC-box, the core GGG was mutated into TTT. The FBI-1ZFDBD bound to the Sp1-3 GC-box probe, but was unable to bind the mSp1-3 GC-box probe. ZFDBD, zinc-finger DNA-binding domain. ***(C)*** EMSA and binding competition between FBI-1ZFDBD and Sp1ZFDBD. The Sp1ZFDBD (200 ng) and FRE probes were incubated in the absence or in the presence of increasing amounts of FBI-1ZFDBD (75, 200, 675 ng). ***(D)*** ChIP assays of binding competition between FBI-1 and Sp1 to the *p21* promoter in SL2-cells. SL2 cells were transiently co-transfected with pPac-Sp1 and increasing amounts of pPac-FBI-1, and pGL2-*p21*-Luc -2.4 kb. The cells were analyzed by ChIP using an anti-Sp1 antibody or an anti-FBI-1 antibody. With increasing amounts of pPac-FBI-1, Sp1 binding is decreased. ***(E)*** Transcriptional activation by Sp1 is repressed by FBI-1 on the pG5-5x(GC box)-Luc. HeLa cells were transiently transfected with pG5-5x(GC box)-Luc in the presence or absence of Sp1 and FBI-1 expression vector and analyzed for luciferase activity. The data shown are an average of three independent assays. Bar, S.D.

Furthermore, the transcriptional activation of pG5-5xGC-Luc by Sp1 could be repressed by FBI-1 in HeLa cells. While Sp1 increased the transcription of the reporter gene, FBI-1 repressed the transcriptional activation by Sp1 when co-transfected (Fig. 3E).

These data suggest that FBI-1 can repress transcription of *p21* by acting on the same proximal regulatory element, the FRE/Sp1-3 GC- box, which can be bound not only by Sp1 but also by FBI-1. Previous reports have shown that the Sp1-3 GC-box (or newly defined FRE) is the most critical regulatory element in the transcriptional regulation of the *p21* gene (18).

4. FBI-1 binds to the p53-binding element of *p21* and blocks transcriptional activation by p53.

p53, the expression of which is inducible by DNA damage, was shown to interact with Sp1 bound at the Sp1-3 GC-box and potently activate *p21* transcription (18). Thus, the ability of FBI-1 to block the p53 expression-mediated transcriptional activation of *p21* was investigated by inducing DNA damage with etoposide treatment in HeLa cells. These cells contained reporter constructs bearing a promoter with 2.4 kb of *p21* upstream regulatory sequence, which contains the distal p53-binding element. The transcription of the *p21* gene was activated by induced p53 expression in this system, and such activation was repressed by FBI-1 (Fig. 4A).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2.0
1.5
1.0
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1x
1 2 3 4
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pcDNA3 +
pcDNA3 FBI-1 + +
ETOPOSIDE + +
B
Relative luciferase activity (fold)
8
6
4
2
0
1x
1 2 3 4
pGL2 p21-Luc -2.4 kb +
pcDNA3 p53 + + +
pcDNA3 FBI-1 + + +
C
pGL2-6x(p53RE)-tk-Luc
+1
Tsp
Luc
6 or 13x
p53 binding element
Relative luciferase activity (fold)
350
300
250
200
150
100
50
0
1x
1 2 3 4
pGL2-6x(p53RE)-Luc + + + +
pcDNA3.0 +
pcDNA3 p53 + +
pcDNA3 FBI-1 + +
D
ChIP distal
p21 promoter
Tsp
p21
-2307
-1930
hGLUT2
GLUT2
+48
+301
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Sample
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IgG
FLAG
IgG
FLAG
Distal
500
400
300
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hGLUT2
300
200
200 bp
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pcDNA3-FBI-1-FLAG + − + +
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Free Probe
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pcDNA3-FBI-1-FLAG −
IP : α-FLAG
IP : α-p53
Input

Figure 4. FBI-1 represses transcription of *p21*$^{Waf/Cip1}$ by binding competition with p53 on the distal p53 binding element. ***(A)*** Induction of p53 expression by etoposide and transcriptional repression of *p21* by FBI-1 in HeLa cells. HeLa cells were transfected with pGL2-*p21*-Luc -2.4 kb and FBI-1 expression plasmids. After culturing for 40 hrs, cells were treated with the DNA-damaging agent etoposide for 8 hr, harvested, and analyzed for luciferase reporter activity. The data shown are an average of three independent assays. Bars, S.D. ***(B)*** Transcriptional activation of *p21* gene by p53 is inhibited by FBI-1 in p53-/- Saos-2 cells. Plasmids expressing FBI-1, p53, and pGL2-*p21*-Luc-2.4 kb were transiently co-transfected into Saos-2 cells. After culturing for 48 hrs, cells were harvested and analyzed for luciferase reporter activity. The data shown are an average of three independent assays. Bars, S.D. ***(C)*** Transcriptional activation of pGL2-6x(p53RE)-Luc reporter plasmid by ectopic p53 can be repressed by FBI-1 in Saos-2 cells. Saos-2 cells were co-transfected with pGL2-6x(p53RE)-Luc and p53 expression vectors in the absence or presence of FBI-1 expression vector. ***(D)*** ChIP assay and the structures of the two endogeous human gene promoters and locations of ChIP PCR primer binding sites are shown. HeLa cells were transfected with FBI-1-FLAG expression vector and immunoprecipitated with IgG or anti-FLAG ab. ***(E)*** ChIP assay of FBI-1 binding to the endogenous distal *p21* promoter in Saos-2 cells. Saos-2 cells were co-transfected with p53 expression vector with or without FBI-1 expression vector, and immunoprecipitated with the antibodies indicated. ***(F)*** EMSA. Distal p53 binding probe was incubated with FBI-1ZFDBD and analyzed by 12% nondenaturing PAGE and the gel was exposed to X-ray film. ***(G)*** ChIP assays and binding competition between FBI-1 and p53 on the distal p53-binding element of endogenous *p21* gene in Saos-2 cells. Saos-2 cells were co-transfected with p53 expression vector with increasing amount of FBI-1 expression vector, and immunoprecipitated with the antibodies indicated ChIP assays.

Co-transfection experiments with pGL2-p53-Luc and FBI-1 expression plasmids showed that FBI-1 does not affect transcription of the *p53* gene itself (Fig. 1A).

The ability of FBI-1 to block the transcriptional activation of *p21* by ectopic p53 was examined in Saos-2 cells lacking p53. FBI- 1 alone can repress transcription in Saos-2 cells while ectopic p53 expression increased reporter gene expression by 3.5-fold. When p53 and FBI-1 were co-expressed, transcriptional activation by p53 was potently repressed by FBI-1 (Fig. 4B).

Although FBI-1 inhibits transcriptional activation by p53, it is unclear how FBI-1 represses transcription. Thus, the ability of ectopic FBI-1 to repress transcription on the pGL2-6x(p53RE)-Luc construct, containing six copies of p53-binding element of the *p21* gene in the proximal promoter, was assayed in Saos-2 cells. p53 potently activated transcription on this construct and such activation was repressed by FBI-1 (Fig. 4C). The data suggest that FBI-1 represses transcription of *p21* and that the repression involves not only the proximal FRE/Sp1-3 GC-box but also p53 or the p53-binding element.

Because FBI-1 transcriptional repression involves either p53 or the p53-binding element, the molecular events ocurring between the distal p53-binding element, p53, and FBI-1 were investigated by ChIP assays. ChIP assays from HeLa cells transfected with FBI-1-FLAG expression vectors showed that FBI-1 binds to the distal p53-binding elements of the endogenous *p21* gene (Fig. 4D). ChIP assays were also performed with Saos-2 cells transiently transfected with FBI-1-FLAG expression vectors in the presence or absence of p53 expression vectors. FBI-1 or

p53 independently bound to the distal p53 binding region (bp -2307 to -1947), and the binding of each protein was decreased by co-expression of the other protein (Fig. 4E). These data suggest that FBI-1 might actually bind to the p53-binding element *in vivo* and may compete with p53 in binding to the element to repress transcription. To test whether FBI-1 binds directly to the p53-binding element, *in vitro* EMSA was performed. Indeed, EMSA showed that FBI-1 ZFDBD specifically binds the p53 binding probe. Further, *in vivo* ChiP assays of the endogenous *p21* gene showed that p53 and FBI-1 actually compete with each other in binding to the distal p53-binding element in Saos-2 cells (Fig. 4F, G).

5. The zinc-finger DNA-binding domain of FBI-1 direct interacts with p53 via the p53 DNA binding domain

ChIP assays showed that FBI-1 expression resulted in decreased p53 binding and vice versa. One possibility for this decrease is that the two proteins may physically interact with each other and affect their DNA binding activities. To assess whether FBI-1 is directly interacting with p53, co-immunoprecipitation and GST-fusion protein pull-down assays were performed. The cell extracts, prepared from control cells or cells stably transformed with a FLAB-FBI-1 construct, were immunoprecipitated using an M2 anti-FLAG antibody and analyzed by Western blot assays using an antibody against p53. p53 and FBI-1 were found to interact with each other *in vivo* (Fig. 5A). The molecular interaction between FBI-1 and p53 was investigated using *in vitro* GST-fusion protein pull-down assays.

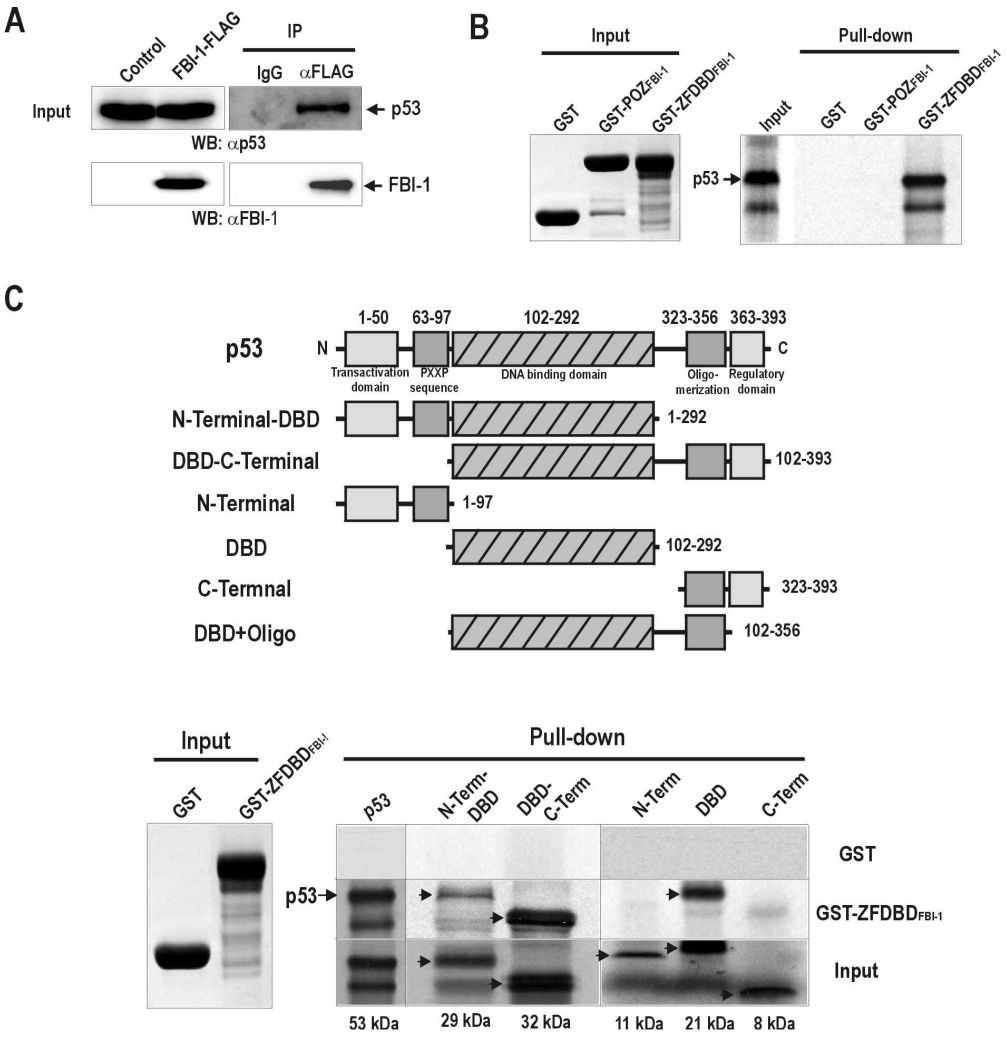


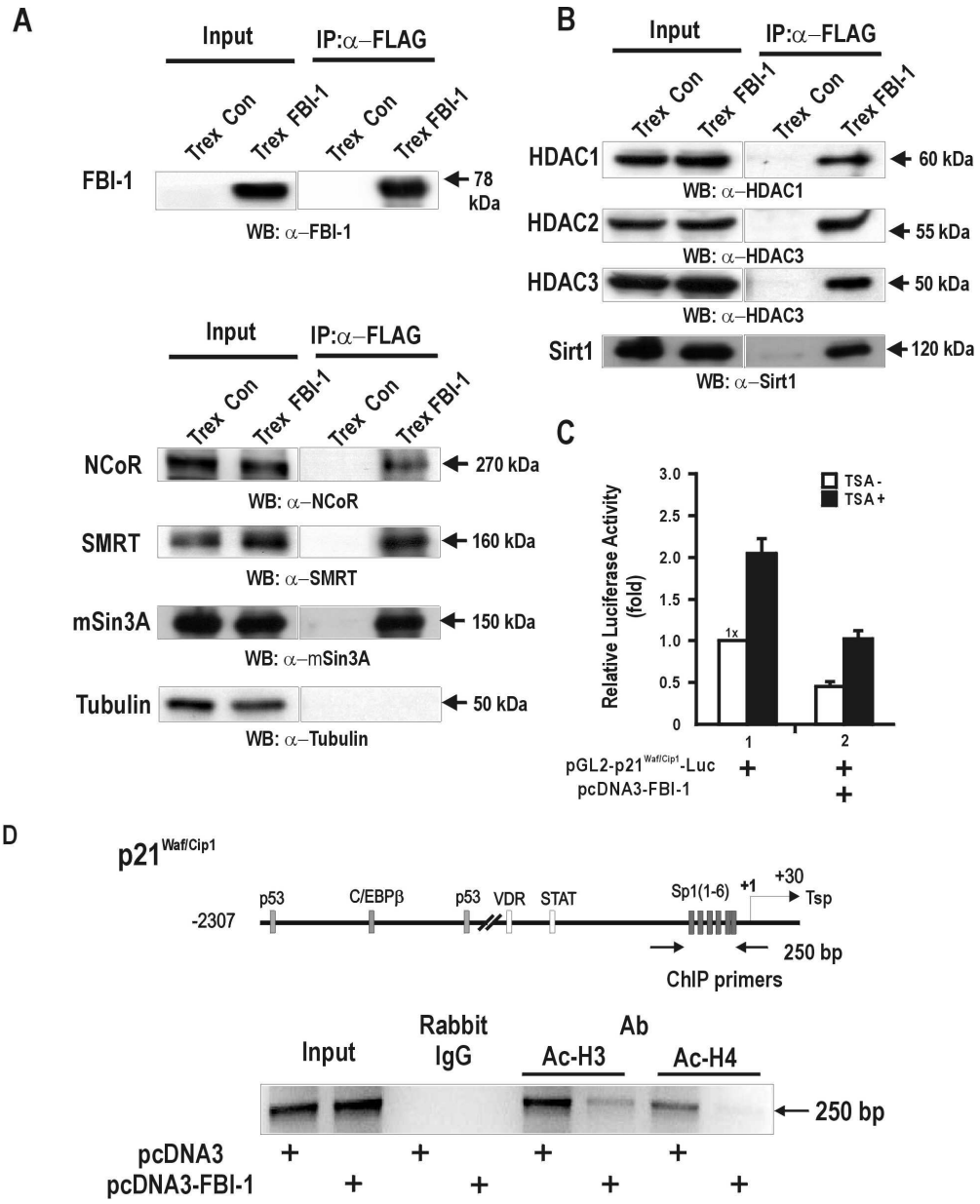
Figure 5. FBI-1 interacts with p53 via the p53 DNA-binding domain. ***(A)*** Co-immunoprecipitation of FBI-1 and p53. Lysates prepared from 293Trex cells and 293Trex-FBI-1–FLAG cells were immunoprecipitated using the anti-FLAG M2-antibody and analyzed by western blotting with anti-p53 antibody. ***(B, C)*** GST-fusion protein pull-down assays. Recombinant GST protein, GST-POZFBI-1, or GST-ZFFBI-1 was incubated with [^{35}S]-methionine labeled p53 or its deletion polypeptides, was pulled down, and was resolved with 15% SDS-PAGE. The gel was then exposed to X-ray film.

Recombinant GST-POZFBI-1 and GST-ZFFBI-1 were allowed to interact with *in vitro* translated [S^{35}]-methionine labeled p53 polypeptides.

The GST-ZF domain, but not the GST-POZ-domain, interacted directly with the polypeptide containing the p53 DNA-binding domain. These data indicate that FBI-1 and p53 directly interact with each other via their DNA-binding domains, and this interaction may affect their DNA binding activity (Fig. 5B, C).

6. The FBI-1 interacts with corepressor-HDAC complexes *in vitro* and *in vivo.* and results in deacetylation of histones Ac-H3 and Ac-H4 at the proximal promoter

FBI-1 can repress transcription by competing with either Sp1 and/or p53 for the FRE/Sp1-3 GC-box and the distal p53-binding element, which are critical for transcription. A question remains as to how FBI-1 can repress transcription other than by binding competition with transcriptional activators such as Sp1 and p53. The data above suggest that the POZ-domain is important for transcriptional repression because the FBI-1ΔPOZ fragment cannot repress transcription (Fig. 1E). Transcriptional repressors such as PLZF and BCl-6 are known to repress transcription by interacting with corepressors such as mSin3A, SMRT, NcoR, and BCoR via their POZ-domains (27, 28).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FBI-1
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WB: α–FBI-1
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Sirt1
120 kDa
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2.0
1.5
1.0
0.5
0
TSA -
TSA +
1x
1
2
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pcDNA3-FBI-1
D
p21Waf/Cip1
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p53
C/EBPβ
p53
VDR
STAT
Sp1(1-6)
+1
+30
Tsp
250 bp
ChIP primers
Input
Rabbit IgG
Ab
Ac-H3
Ac-H4
250 bp
pcDNA3
pcDNA3-FBI-1

Figure 6. FBI-1 interacts with corepressors, and deacetylates histones, AC-H3 and AC-H4 bound at the p21 proximal promoter. ***(A)*** Co-immunoprecipitation of FBI-1 and corepressors, SMRT, NCoR, and mSin3A. Nuclear extracts prepared from 293Trex control and stable 293Trex-FLAG-FBI-1 cells were immunoprecipitated with ant-FLAG M2 antibody, and the precipitates were analyzed by western blot using antibodies against FBI-1 and corepressors. ***(B)*** Co-immunoprecipitation of FBI-1 and HDACs of class 1, 2 and 3. Assays were carried out as in (A). ***(C)*** Transcriptional repression by FBI-1 involves HDACs. HeLa cells were transfected with pGL2-*p21*-Luc with or without FBI-1 expression vector. Cells were treated with TSA for 30 hrs before harvest and analyzed for luciferase activity. ***(D)*** ChIP assays of histone modification at the proximal promoter of endogenous *p21* gene using antibodies against Ac-H3 and Ac-H4. HeLa cells were transfected with FBI-1-FLAG expression plasmid and immunoprecipitated with the antibodies indicated.

Accordingly, interactions beteween FBI-1 and corepressors and functional consequences of such interactions were evaluated in the transcription regulation of *p21* both *in vivo* and *in vitro*. Stable 293 Trex FBI-1 FLAG cells were prepared which overexpress FBI-1 in the presence of an inducer, doxycycline. Co-immunoprecipiation of nuclear extract prepared from 293 Trex-FBI-1 FLAG cells showed that FBI-1 was interacting with corepressors such as NCoR, SMRT, and mSin3A (Fig. 6A).

Often, corepressor complex recruited by transcriptional repressors contain HDACs (histone deacetylases), and these HDACs deacetylate the histones in nearby nucleosomes. Thus, FBI-1-corepresor complex immunoprecipitates were examined for the presence of HDACs of class 1, 2, or 3. Western blots of the immunoprecipitates showed that HDACs are components of the precipitated complex (Fig. 6B). Accordingly, the effects on reporter transcription of HDAC inhibitor treatment on cells cotransfected with pGL2-*p21*-Luc and pcDNA3-FBI-1 were evaluated. Treatment with TSA, an HDAC inhibitor, resulted in a two-fold increase in reporter transcription (Fig. 6C). FBI-1 expression repressed transcription by more than 50%, consistent with results seen in Fig. 1. However, in the presence of FBI-1, TSA treatment did not result in the full activation of transcription. This data suggests that HDACs are at least partly involved in the transcriptional repression and that other mechanisms of transcriptional repression, such as binding competition between transcriptional activators Sp1 or p53 and the repressor, FBI-1, are also contributing to the transcriptional repression (Figs. 3, 5).

Because FBI-1 interacted with a corepressor-HDAC complex, the deacetylation of the Ac-H3 and AcH4 histones by the the FBI-1-corepressor-HDAC complex was investigated using ChIP assays in HeLa cells. FBI-1 decreased the levels of acetylated histones 3 and 4 significantly. This result suggests that histone deacetylation at the proximal promoter is responsible for the transcriptional repression by the FBI-1-corepressor-HDAC complex when recruited to the FRE/Sp1-3 GC-box and the distal p53-binding element (Fig. 6D).

7. FBI-1 induces cellular transformation and stimulates proliferation of HeLa and NIH3T3 cells

As *p21*, an important negative regulator of cell cycle progression, is potently repressed by FBI-1 at the transcriptional level, FBI-1 expression may cause cellular transformation and promote cell cycle progression. The ability of FBI-1 to form foci of transformation was tested in HeLa cells transfected with FBI-1 expression vector. The transfected cells formed a substantial number of foci, suggesting that FBI-1 can cause cellular transformation (Fig. 7A).

FACS analysis was used to investigate whether overexpression of FBI-1 could stimulate cell cycle progression and increase the number of HeLa cells at S phase. Stable HeLa cells overexpressing FBI-1 and LacZ were generated by infection with recombinant lentivirus-LacZ or -FBI-1, and cell cycle progression was analyzed. The number of cells in S phase increased from 28.94% in the control HeLa-LacZ cells to 50.49% in HeLa-FBI-1 cells, suggesting that the cell cycle could not be

arrested at the G1 check point because of transcriptional repression of the *p21* gene by FBI-1. In addition, the number of cells in the G2-M phase decreased from 19.68% to 0.0%. However, in contrast, there was little change in the number of cells in G0-G1 phase (Fig. 7B).

The effect of FBI-1 knock-down or overexpression on cellular proliferation was also examined using BrdU incorporation as an index of cell proliferation. While the percentage of cells incorporating BrdU was significantly increased from 2% to 37% in the NIH3T3 cells stably expressing FBI-1 (Fig. 7D), knock-down of FBI-1 mRNA significantly decreased the number of BrdU incorporation-positive HeLA cells (Fig. 7C, D). A similar BrdU incorporation assay was performed with control and stably-transformed 294Trex-FLAG-FBI-1 cells. Induced expression of FBI-1 by doxycycline significantly increased the percentage of cells with BrdU incorporation while the control cells did not show a significant difference (Fig. 7E). These data, along with the data from FACS analysis, suggest that FBI-1 expression promotes cell proliferation.

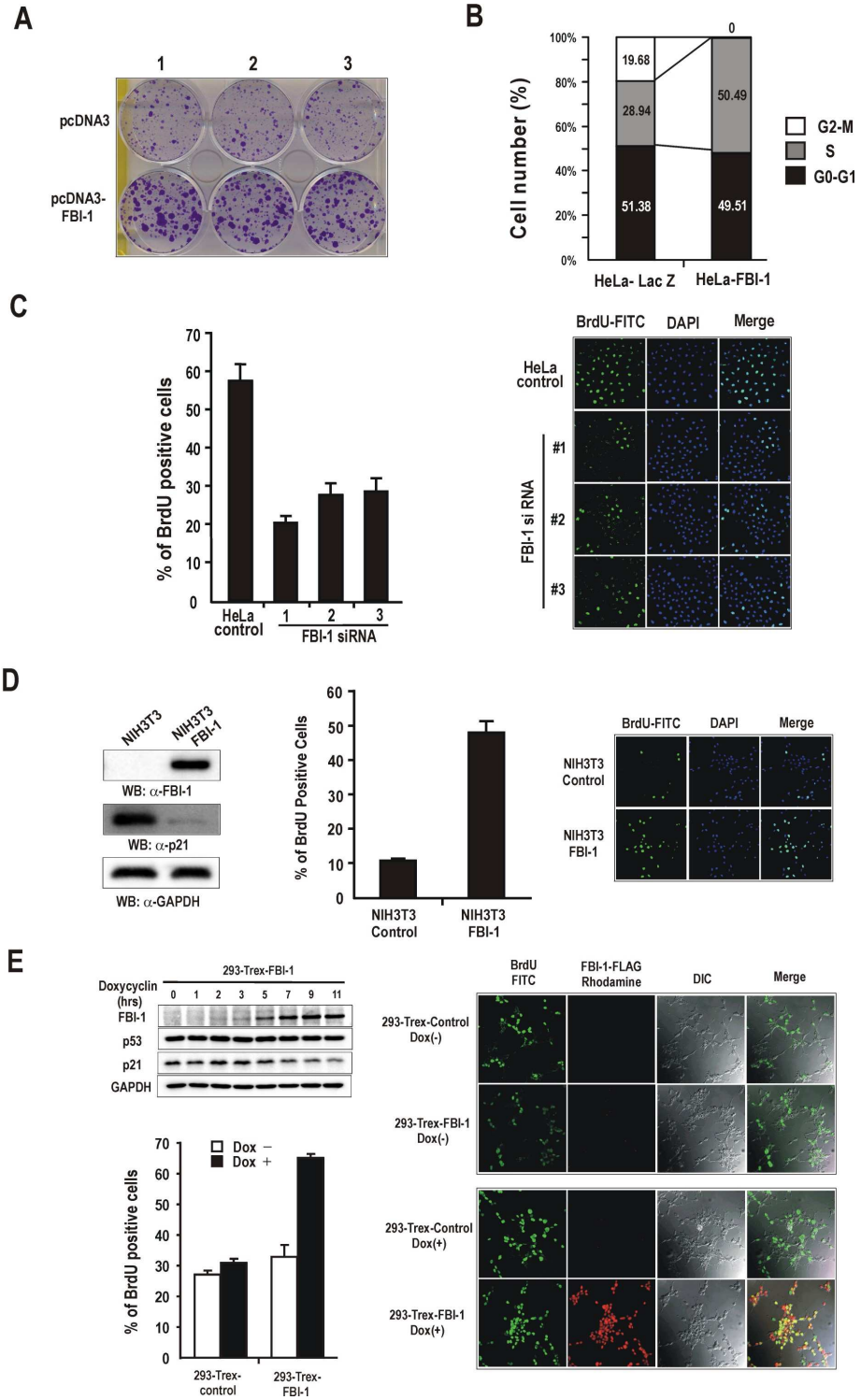

A
1
2
3
pcDNA3
pcDNA3-FBI-1
B
Cell number (%)
100%
80%
60%
40%
20%
0%
19.68
28.94
51.38
0
50.49
49.51
G2-M
S
G0-G1
HeLa- Lac Z
HeLa-FBI-1
C
% of BrdU positive cells
70
60
50
40
30
20
10
0
HeLa control
1
2
3
FBI-1 siRNA
BrdU-FITC
DAPI
Merge
HeLa control
FBI-1 si RNA
#1
#2
#3
D
NIH3T3
NIH3T3 FBI-1
WB: α-FBI-1
WB: α-p21
WB: α-GAPDH
% of BrdU Positive Cells
60
50
40
30
20
10
0
NIH3T3 Control
NIH3T3 FBI-1
BrdU-FITC
DAPI
Merge
NIH3T3 Control
NIH3T3 FBI-1
E
293-Trex-FBI-1
Doxycyclin (hrs)
0
1
2
3
5
7
9
11
FBI-1
p53
p21
GAPDH
% of BrdU positive cells
70
60
50
40
30
20
10
0
Dox −
Dox +
293-Trex-control
293-Trex-FBI-1
BrdU FITC
FBI-1-FLAG Rhodamine
DIC
Merge
293-Trex-Control Dox(-)
293-Trex-FBI-1 Dox(-)
293-Trex-Control Dox(+)
293-Trex-FBI-1 Dox(+)

Figure 7. FBI-1 causes cellular transformation, and promotes cell cycle progression and proliferation. ***(A)*** Foci-formation assays. HeLa cells transfected with the FBI-1 expression vector were cultured in medium containing G418 for two weeks. Cells were fixed with 4% formaldehyde and stained with 0.1% crystal violet. ***(B)*** FACS analysis of cell cycle progression. HeLa cells stably overexpressing FBI-1 and LacZ were established by Lentivirus transfection and G418 selection. Cells were stained with Propidium Iodide and cell proliferation was measured with FACS. ***(C)*** Knock-down of FBI-1 and BrdU incorporation. HeLa cells were transfected with control siRNA or three different FBI-1 siRNA. After transfection, cells were grown in DMEM media containing BrdU and analyzed as described above. ***(D)*** BrdU incorporation assay. NIH3T3 and stable NIH3T3-FBI-1 cells were grown in DMEM media containing BrdU. The cells were fixed in 3.7% formaldehyde, rinsed, and treated with 2N HCl. Cells were then incubated with anti-BrdU antibody, and further incubated with goat Cy2-conjugated anti-mouse secondary antibody. The cells were treated with DAPI, and the cells with incorporated BrdU were counted. ***(E)*** BrdU incorporation assay in 293Trex cells. 293Trex control and stable 293Trex FBI-1-FLAG cells were grown in DMEM media containing BrdU in the presence or absence of inducer Doxycycline. The cells were analyzed as described above.

8. FBI-1 and p53 is highly expressed and p21 expression is low in colorectal cancer tissues

The relationship among p53, FBI-1, and p21 in clonal cancer tissues was evaluated by immunochemical staining of micro-tissue arrays of human normal and caner tissues. FBI-1 was weakly detected in 12 out of 45 non-neoplastic tissues tested (prostate, testis, proeddometrium, sec-ednometrium, paratoid glans, pancrea, fallopian tube, stomach, jejuni, ileum, kidney cortex and gal bladder) . Most positive staining of the normal tissue showed weakly positive reaction in the cytoplasm.

FBI-1 was abundantly expressed in both the nucleus and cytoplasm of various tumor tissues and, interestingly, the majority of tumors showing positive staining reactions were adenocarcinoma and squamous cell carcinomas of stomach, pancreas, lung, and prostate. In rare tumor cases, FBI-1 was seen mainly in the cytoplasm. In an immunohistochemical staining assay for FBI-1 of 45 colon cancer array cores, approximately half (19 out of 45) of the cases showed strong positive nuclear and cytoplasmic staining patterns (Fig. 8E). As reported, p53 was abundantly expressed in the nucleus of cancer cells (Fig. 8G).

In agreement with the above transcription analysis, p21, a target of p53 and FBI-1, was detected in only two cases, both of which showed weak staining in a small number of clonal cancer cells (Fig. 8H), although many cases showed positive p53 nuclear staining. This data suggestes that FBI-1 can contribute to the repressed transcription of *p21* in clonal cancer tissues, which may be important in oncogenesis and tumor growth.

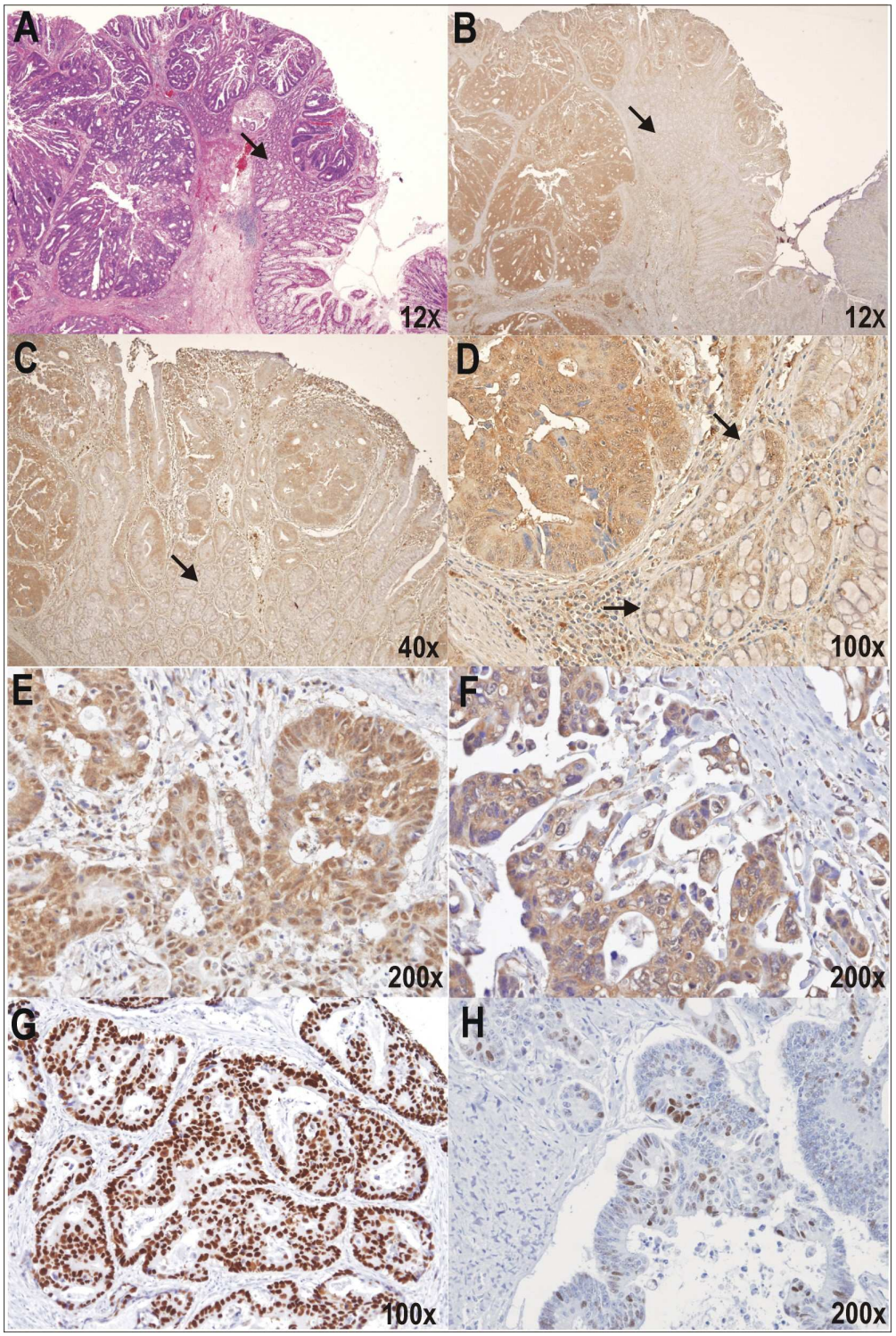

A
12x
B
12x
C
40x
D
100x
E
200x
F
200x
G
100x
H
200x

Figure 8. Immuno-histochemistry of colon cancer tissues for FBI-1, p53 and p21 expression. Low power microscopic images comparison between H&E staining and FBI-1 immunohistochemical staining of colonic adenocarcinoma **(*A*,** H&E, 12x; ***B*,** FBI-1 12x**).** The colonic adenocarcinoma cells show more intense FBI-1 immunoreactivity than adjacent normal colonic mucosa (indicated by arrows →) **(*C*,** FBI-1, 40x; ***D*,** FBI-1 100x**).** Although most of the adenocarcinoma tissues show prominent nuclear and cytoplasmic FBI-1 immunohistochemical staining pattern **(*E*,** FBI-1 200x**),** some colonic adenocarcinoma cells show cytoplasmic FBI-1 immunoreactivity **(*F*,** FBI-1 200x**).** In many cases, p53 shows intense nuclear immunoreactivity **(*G*,** p53 100x**).** But p21 expression is low and, only in rare cases, focal immunoreactivity was observed **(*H*,** p21 200x**).**

IV. Discussion

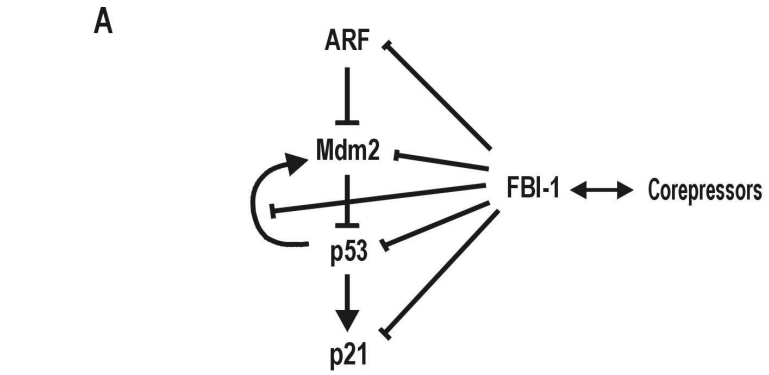
FBI-1 expression was shown to cause cancer in the thymus, liver, and spleen of transgenic mice by repressing mouse *Arf*, a tumor suppressor gene, which in turn lowers expression of the tumor suppressor gene *p53* (9). FBI-1 has broad biological functions outside of its activity on the *Arf* gene: *p53*, *p21*, and, possibly, *Mdm2* of the Arf-Mdm2-p53-p21 pathway, are also targets of FBI-1. Although FBI-1 appears to regulate virtually every gene of the pathway directly or indirectly, we selected a gene which plays a more direct role in controlling cell cycle progression to investigate the molecular mechanism of protooncoprotein FBI-1 action in cellular transformation and oncogenesis. We found that the *p21* gene is a direct target of FBI-1 and the molecular mechanism of its regulation by FBI-1 is complex, involving both the proximal promoter and the distal p53-binding element.

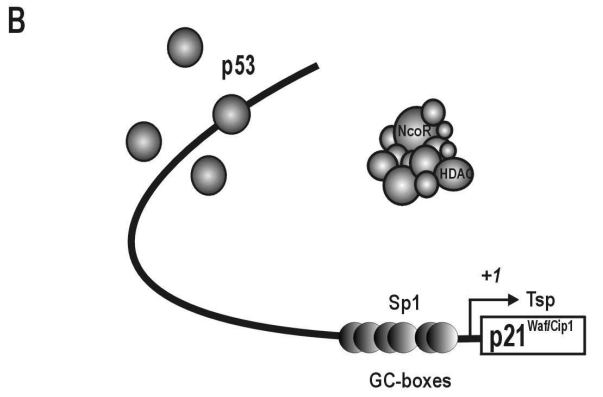
EMSA, mutagenesis, and transfection assays all showed that FBI-1 competes with Sp1 to bind to the proximal FRE/Sp1-3 GC-box, located at -82 to -77 bp. With respect to Sp1, the FRE/Sp1-3 GC-box is probably the most important regulatory element in the transcriptional regulation of *p21*. It is a direct target of regulation by Sp1 and Sp family members, and mutation of the element dramatically lowers transcription, as shown by others (18). The FRE/Sp1-3 GC-box is also essential for the synergistic transcriptional activation by p53-Sp1 interactions. The p53-mediated transactivation of the *p21* promoter is achieved through functional cooperation between p53 and Sp1 bound to the FRE/Sp1-3 GC-box. Intriguingly, FBI-1 competes for this particular site to disrupt both transcriptional activation by Sp1 and

also p53-Sp1 interactions, which are important for inducible expression of *p21* upon p53 induction by genotoxic stress.

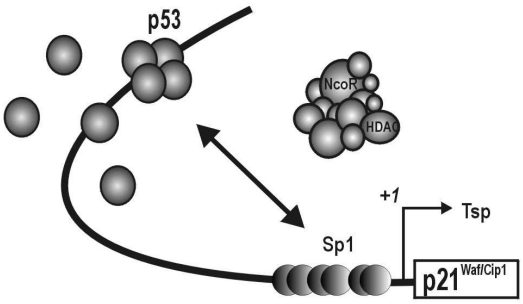
ChIP assays showed that FBI-1 was also cross-linked to the distal p53-responsive region of the *p21* promoter. EMSA and protein-protein interaction assays showed that FBI-1 binds to the p53-binding element by competing with p53 and that FBI-1 interacts with p53 directly to inhibit p53 binding, resulting in reduced transcriptional activation by p53. This site has previously been shown to mediate the induction of *p21* by various agents such as TGF-β, butyrate, the histone deacetylase inhibitor trichostatin A, lovastatin, and Ca^{2+}, among others. Accordingly, not only are DNA-damaging signals that result in p53-medicated induction of *p21* blocked by FBI-1, but the various signals mentioned above are likely to be blocked by FBI-1 as well, since it occupies the FRE/Sp1-3 GC-box by competing with Sp1 and it also binds to the distal p53 binding site.

The molecular mechanism of cell cycle regulation via transcriptional repression of *p21* by FBI-1 is intriguing (Fig. 9). Under normal cellular conditions, where p53 is expressed at a low basal level and FBI-1 is not present or is low compared to Sp1, the *p21* gene is expressed at a low basal level and cells proliferate normally. When cells are challenged with genotoxic stress, the tumor suppressor p53 is induced and activates transcription of *p21* by interacting with the Sp1 bound at the FRE/Sp1-3 GC-box. The induced *p21* arrests cell cycle progression, allowing cells to repair DNA damage.

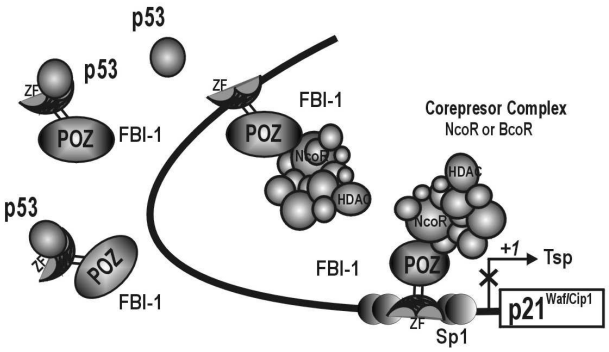




basal p53
no FBI-1
basal p21 transcription



genotoxic stress
induced p53
no FBI-1
induced p21 transcription
p53, as tumor suppressor



genotoxic stress
induced p53, inhibited
high FBI-1
no p21 transcription
cell proliferation
mutation accumulation
oncogenesis
tumour growth

Figure 9. Molecular mechanism of transcriptional regulation of the cell cycle regulator *p21$^{Waf/Cip1}$* gene by proto-oncogene FBI-1. ***(A)*** Regulation of Arf-Mdm2-p53-p21 pathway by FBI-1. FBI-1 represses transcription of Arf, p21 genes. Solid line, transcription repression; double arrow line, protein-protein interaction. ***(B)*** Hypothetical molecular mechanism of transcriptional regulation of the p21 gene in three different physiological states. In the normal state, where p53 and FBI-1 expression are low, transcription of the p21 gene is controlled mainly by Sp1 family transcription factors acting on the proximal GC-boxes and transcription is at basal level. Cells proliferate normally. When cells are challenged by genotoxic stress and the expression of FBI-1 is low, induced tumor suppressor p53 binds to the distal element and communicates with proximal promoter bound Sp1 to activate transcription of p21. The induced p21 arrests cell cycle progression and allowing cells to repair DNA damage. When expression of both p53 and FBI-1 is high, as in cancer cells, FBI-1 binds to the distal p53 binding element by binding competition with p53 and also to the proximal GC-box 3/FRE element by binding competition with Sp1. The FBI-1 on the p53 binding element and proximal promoter, in turn, recruits the corepressor-HDAC complex to deacetylated histones, Ac-H3 and AcH4 at the proximal promoter, which repress transcription. Tsp (+1), transcription start point (→).

In cells where FBI-1 expression is high and with no DNA damage, FBI-1 represses transcription by directly binding to both the proximal FRE/Sp1-3 GC-box and distal p53-binding element. Such DNA-bound FBI-1 can recruit corepressor-HDAC complexes and deacetylate histones of the proximal promoter, which represses transcription.

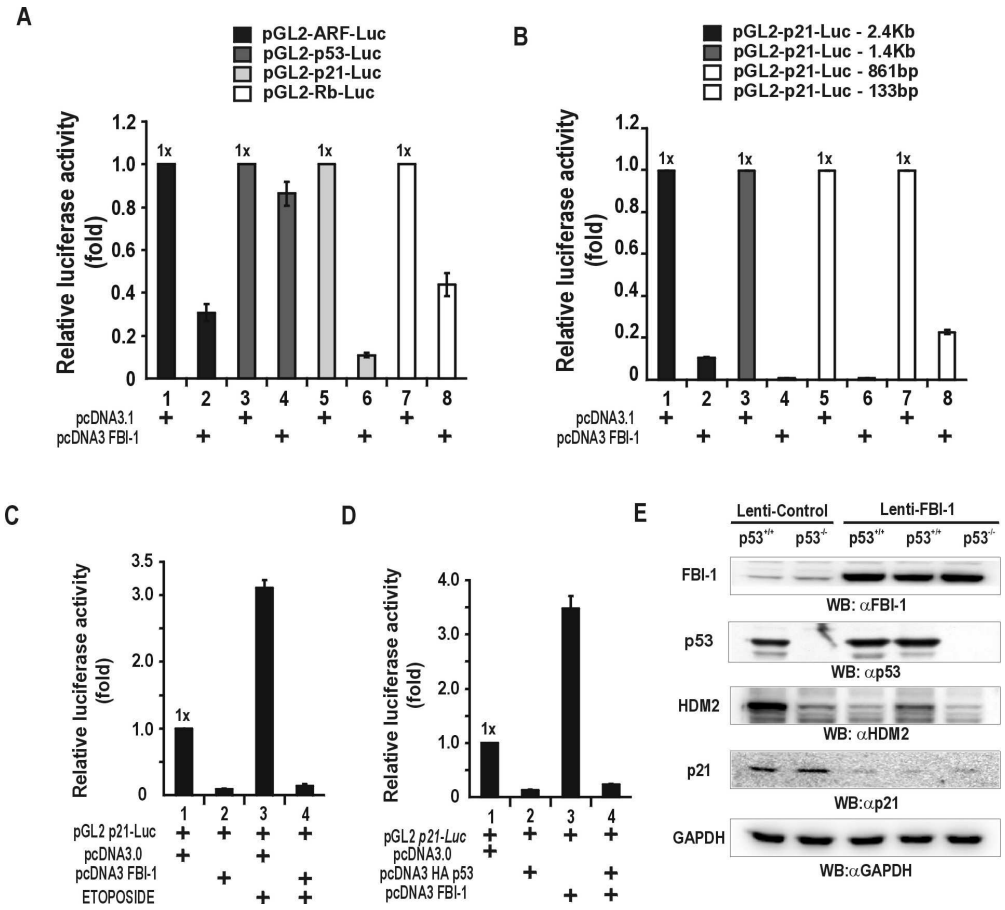

A
pGL2-ARF-Luc
pGL2-p53-Luc
pGL2-p21-Luc
pGL2-Rb-Luc
Relative luciferase activity (fold)
1x
pcDNA3.1
pcDNA3 FBI-1
B
pGL2-p21-Luc - 2.4Kb
pGL2-p21-Luc - 1.4Kb
pGL2-p21-Luc - 861bp
pGL2-p21-Luc - 133bp
C
pGL2 p21-Luc
pcDNA3.0
pcDNA3 FBI-1
ETOPOSIDE
D
pGL2 p21-Luc
pcDNA3.0
pcDNA3 HA p53
pcDNA3 FBI-1
E
Lenti-Control
Lenti-FBI-1
p53+/+
p53-/-
FBI-1
WB: αFBI-1
p53
WB: αp53
HDM2
WB: αHDM2
p21
WB:αp21
GAPDH
WB:αGAPDH

Figure 10. FBI-1 represses transcription of the *p21$^{Waf/Cip1}$* gene in HCT-116 cells. ***(A)*** HCT-116 cells were co-transfected with promoter-Luc fusion palsmid, FBI-1 expression plasmid, and pCMV-LacZ expression plasmid. After 48 hrs, cells were harvested and analyzed for luciferase reporter activity. The data shown are an average of three independent assays. Bars, S.D. ***(B)*** FBI-1 represses transcription by acting on the proximal promoter of p21 gene. HCT-116 cells were transiently co-transfected with plasmids expressing FBI-1 and pGL2-p21-Luc. Luciferase activity was measured 48 hrs after transfection. The data shown are average of three independent assays. Bars, S.D. ***(C)*** Induction of p53 expression by etoposide and transcription repression of p21 by FBI-1 in HCT-116 cells. HCT-116 cells were transfected with pGL2-p21-Luc -2.4 kb and FBI-1 expression plasmid. After culturing 40 hrs, cells were treated with DNA damaging agent etoposide for 8 hr, harvested and analyzed for luciferase reporter activity. The data shown are an average of three independent assays. Bars, S.D. ***(D)*** Transcription activation of p21 gene by p53 is inhibited by FBI-1 in HCT-116 p53-/- cells. Plasmids expressing FBI-1, p53, and pGL2-p21-Luc-2.4 kb were transiently co-transfected into HCT-116 p53-/- cells. After culturing 48 hrs, cells were harvested and analyzed for luciferase reporter activity. The data shown are an average of three independent assays. Bars, S.D. ***(E)*** Western blot and RT-PCR analysis of the stable HCT-116 p53 +/+ and p53-/- cells overexpressing FBI-1 or control vector established by transfection with recombinant Lenti viruses. GAPDH, control.

This mechanism suggests that FBI-1 itself may potently repress *p21* gene expression and promote cell proliferation. This action of FBI-1 may be important in sustained tumor growth, where p53 is no longer induced or p53 expression is low.

In the situation where cells are under genotoxic stress and FBI-1 expression is high or in cancer tissues which have high levels of both p53 and FBI-1, as in some clonal cancer tissues, FBI-1 represses transcription by directly binding to both the proximal FRE/Sp1-3 GC-box and the distal p53-binding element. Although p53 expression is also highly induced, p53 has to compete with FBI-1 to bind to the distal p53-binding element. In addition, molecular interactions between the DNA-binding domains of p53 and FBI-1 affect the binding of p53 to the distal p53-binding element. Also, because the proximal FRE/Sp1-GC box is occupied by FBI-1, p53 cannot exert its transcriptional activation potential. The DNA-FBI-1 complex recruits corepressor-HDAC complexes, which deacetylate histones at the proximal promoter. Although p53 is around, *p21* transcription is potently repressed by FBI-1. In this case, cells proliferate without arrest and mutations accumulate, resulting in cells that are likely to undergo oncogenic transformation.

Molecular interactions occurring in both the proximal and distal promoter are unique and may be applicable to the transcriptional regulation of other genes in the Arf-Mdm2-p53-p21 pathway. Maeda *et al.* found that FBI-1 competed with Sp1 to bind the GC-box regulatory element of *ARF* (18). Recently, Our laboratory found that FBI-1 competed with Sp1 for the proximal GC-box regulatory element of the Rb gene (in preparation). Upon EMSA, FBI-1 shows some characteristics of Sp1-

family Krüppel like zinc-finger proteins and bound to sequences similar to the GC boxes recognized by Sp1 (data not shown). CAST analysis for the FBI-1 DNA binding consensus sequence selects GC–rich sequences that have some similarity to the Sp1 binding consensus sequence (9, 26). Not only does recombinant Sp1 bind most of the FRE probes, some of the Sp1-binding GC-boxes can be bound by FBI-1. This suggests that a subset of GC-boxes recognized by Sp1 may be transcriptional repression targets of FBI-1, and, accordingly Sp1-FBI-1 antagonism may be a general mechanism of transcriptional repression of FBI-1 target genes.

Our findings suggest that FBI-1 plays a critical role in regulating important biological processes such as DNA repair, cell growth, differentiation, and apoptosis by complex molecular interactions that involve FBI-1, Sp1, p53, a distal p53-binding element, and a proximal promoter FRE/Sp1-3 GC-box element of the *p21* gene. It seems that a major mode of *p21* transcriptional repression by the negative regulator FBI-1 is the interference with positive transcription factors, such as constitutitve Sp1 and inducible p53.

V. Conclusion

1. FBI-1 represses transcription of the *p21* gene in HeLa cells

2. Identification of the proximal FBI-1 binding promoter element (FRE) and overlap of the FRE with the Sp1-3 GC-box

3. Molecular competition between Sp1 and FBI-1 at the FRE/Sp1-3 GC box is partly responsible for transcriptional repression

4. FBI-1 binds to the p53-binding element of *p21* and blocks transcriptional activation by p53

5. The Zinc-finger DNA-binding domain of FBI-1 interacts directly with p53 via the p53 DNA-binding domain

6. FBI-1 interacts with corepressor-HDAC complexes *in vitro* and *in vivo* and results in the deacetylation of histones Ac-H3 and Ac-H4 at the proximal promoter

7. FBI-1 induces cellular transformation and stimulates proliferation of HeLa and NIH3T3 cells

8. FBI-1 and p53 are highly expressed and p21 expression is low in colorectal cancer tissues

9. FBI-1 plays a critical role in regulating important biological processes such as DNA repair, cell growth, differentiation, and apoptosis by complex molecular interactions that involve FBI-1, Sp1, p53, a distal p53-binding element, and a proximal promoter FRE/Sp1-3 GC-box element of the *p21* gene.

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국문요약

새로운 proto-oncogene인 FBI-1 (pokemon/ZBTB7a)는 cyclin-dependent kinase 억제자 *p21* 유전자의 전사를 p53과 Sp1의 프로모터 조절 부위에 결합을 방해함으로써 억제한다.

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최 원 일

FBI-1 (Pokemon/ZBTB7a)는 여러 조직에서 발현되는 POK 계열의 전 발암 유전자이다. 우리는 FBI-1의 생체 내 기능을 연구하는 과정에서, 세포 주기 조절에서 중요한 조절 경로인 Arf-Mdm2-p53-p21 (p53 경로라 부름) 의 모든 유전자가 FBI-1에 의하여 그 발현이 억제되는 표적임을 발견하였다. FBI-1이 수행하는 세포 주기 조절 기전을 연구하고자, p53 경로의 Arf와 p53의 하부 유전자로써 세포 주기 조절의 핵심 존절자인 $p21^{Waf/Cip1}$을 선택하여 연구를 진행하였다. 우리는 FBI-1, p53, Sp1, FRE(FBI-1 결합

부위)/Sp1-3 GC-box, 그리고 p53 결합 부위를 포함하는 복잡한 분자적 기전에 의하여 p21 유전자의 발현을 FBI-1이 억제 조절함을 발견하였다. 구체적으로 p21 프로모터에서 FBI-1은 Sp1에 의한 전사 활성화에 중요하다고 보고된 Sp1-3 GC-box (FBI-1 결합 부위와 중첩됨) 에서 전사 활성자인 Sp1과 분자적인 자리 경쟁에 의해 전사를 억제하며, 이를 통해서 Sp1과 p53간의 상호작용에 의한 전사 활성화도 억제한다. 또한 FBI-1이 p21 유전자의 전사 억제를 위해 distal p53 결합 부위에 결합하여 전사 활성화시키는 p53과 경쟁적으로 결합하는 것을 또한 발견하였다. FBI-1은 일단 proximal, distal 조절 부위에 결합하면, 전사 억제에서 중요한 HDAC이 포함된 mSIN3A, N-CoR, SMRT과 같은 보조전사 억제인자들과 상호작용하여, p21 유전자의 proximal 프로모터 부위에서 acetylation된 histon H3과 histon H4를 deacetylation함으로 nucleosome의 구조를 촘촘하게 하여 전사를 억제를 한다.

나아가 우리는 p53 경로, 특히 p21의 전사 억제가 의미하는 생물학적 기능을 연구하고자 세포주기의 FACS 분석, Foci-formation 분석, BrdU-incorporation 속도 등을 조사한 결과, FACS 분석결과, DNA 합성기 (S-상) 세포의 수가 현저히 증가함을 보였고, 이는 BrdU incorporation 속도와 일치하여 FBI-1은 증식이 빠른 세포로의 형질 전환을 유도하고 세포 주기는 촉진시킴을 발견하였다. 여러 병리 암 조직의 면역 화학 염색에서는 FBI-1이 adenocarcinoma와 squamous cell carconoma에서 두드러지게 발현되는 것

을 관찰되었다.

결론적으로 FBI-1은 Arf-Mdm2-p53-p21 (p53 경로) 경로의 중요한 억제 조절 전사 인자이며 이를 통해 세포를 암세포로 형질전환 시키며 세포의 증식을 촉진함을 발견하였다. 우리의 연구는 어떻게 새로운 전 발암 유전자인 FBI-1이 암세포로의 전환을 유발하고, 종양세포의 증식을 유도하는지를 설명해주고 있다.

핵심되는 말: FBI-1, Pokemon, ZBTB7a, p21, Sp1, p53, ARF, Arf-Mdm2-p53-p21 pathway, corepressor, SMRT, NcoR, mSIN3A, HDAC