Regulation of Rb function by FBI-1 through repression of *Rb* gene transcription

Jung-Yoon Yoo

Department of Medical Science

The Graduate School, Yonsei University

Regulation of Rb function by FBI-1 through repression of *Rb* gene transcription

Directed by Professor Man-Wook Hur

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Jung-Yoon Yoo

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Thesis Supervisor : Man-Wook Hur

Jeon-Han Park

Ho-Geun Yoon

The Graduate School

Yonsei University

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Jung-Yoon Yoo

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FBI-1 (also called Pokemon) is a transcription factor with a POZ-domain at N-terminus and four Krüppel-like zinc fingers at C-terminus. FBI-1 regulates transcription of various genes and plays important roles in differentiation, oncogenesis and adipocyte differentiation. Recently, FBI-1 was shown to have a proto-oncogenic activity and its expression is elevated in many cancer tissues. We investigated whether FBI-1 affects tumor suppressor

Retinoblastoma (Rb) gene expression at transcription level. Rb protein plays important roles in the regulation of cell cycle, apoptosis, and cell differentiation and the role is important in tumor suppressor. We found that the several potential FBI-1 binding sites (FRE consensus sequence, GDGGGYYYY) are present in the proximal promoter region of *Rb* gene. The transcription of the *Rb* gene is potently repressed by FBI-1. We found that FBI-1 binds to the four FRE (bp -308 to -300, bp -298 to -290, bp -244 to -236, and bp -188 to -180) by EMSA *in vitro* and *in vivo*, as evidenced by ChIP assays. The transcription of *Rb* gene is regulated by transcription factor such as Sp1, E4TF1, Fli-1, p53, ATF, E2F, CREB1 and CDF-1 by acting on the region from bp -64 to -41, which is essential in transcription. The *Rb* promoter contains two Sp1 sites at bp -64 to -59 and bp -17 to -12, both known to be critical transcription. We found that FBI-1 could bind to one of the Sp1 binding site located at bp -17 to -12. Sp1 can bind three FREs located at bp -308 to -300, bp -244 to -236, and bp -188 to -180. We demonstrate that FBI-1 can repress transcription of *Rb* gene by competitive binding with Sp1 to the Sp1 binding GC-box (bp -17 to -12) and to the FRE (bp -244 to -236). Although the competition by FBI-1 can be important in transcription repression by excluding transcription activator Sp1 from the key regulatory elements, FBI-1 can also repress transcription by having repression domain

called POZ-domain that was shown to repress transcription by recruiting corepressor protein complex, as shown for other POK family proteins, PLZF and Bcl-6. We investigated whether the POZ-domain of FBI-1 can recruit the corepressors such as BCoR, NCoR, mSin3A, and SMRT by mammalian two-hybrid reporter assays, and GST pull-down assays. The BCoR interacted with the POZ-domain of FBI-1 most strongly, which is followed by SMRT, and NCoR. mSin3A did not show significant molecular interaction. Histone deacetylation caused by corepressors might be important in the transcription repression. In conclusion, FBI-1 might repress transcription of *Rb* gene by competitive binding with Sp1 to some key regulatory elements and also by recruiting corepressors to the promoter region and chromatin modification.

Since Rb protein plays an important role in cell cycle arrest and *Rb* gene is repressed by ectopic FBI-1, FBI-1 may influence cell cycle, apoptosis, and cell differentiation by repressing *Rb* gene expression.

Key words: Rb, *Rb* promoter, FBI-1, Pokemon, transcription repression, Sp1, corepressors

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I. Introduction

The tumor suppressor retinoblastoma (Rb) has been one of the main targets for cancer research. The importance of Rb protein in regulating key cellular events was first suggested by the identification of a tumor, retinoblastoma, in which the Rb locus was invariably found to be deleted, and

implicated in the development of various cancers such as small cell lung carcinoma, osteosarcoma, breast cancer, head and neck tumors, and mantle cell lymphomas.[1, 2]

The *Rb* gene functions to suppress tumorigenesis by inhibiting cell cycle progression at the G1/S transcription of several genes.[3, 4] Early studies demonstrated that Rb is phosphorylated in a cell cycle–dependent manner. Accordingly, Rb appears to be predominantly unphosphorylated or hypophosphorylated in the G1 and maximally phosphorylated in G2 phase of the cell cycle (Fig. 1). The critical phosphorylation events regulating the function of the Rb are likely to be mediated at the boundary between the G1 and S phases (called check-point) of the cell cycle by cyclin and cyclin-dependant kinase (Cdk) protein complexes.[3, 5] When it is not phospharylated, Rb forms complexes with proteins in the E2F family and inhibits transcription by recruiting proteins involved in transcriptional repression.[3, 6] And when phosphorylated, Rb can no longer efficiently form complexes with E2Fs. The E2F proteins, when dimerized with their differentiation-regulated transcription factor (DP) partner proteins, are then capable of activating the expression of a number of genes that are likely to regulate or promote entry into S phase, including DNA polymerase, thymidylate synthase, ribonucleotide reductase, cyclin E, and dihydrofolate reductase.[3] The E2F

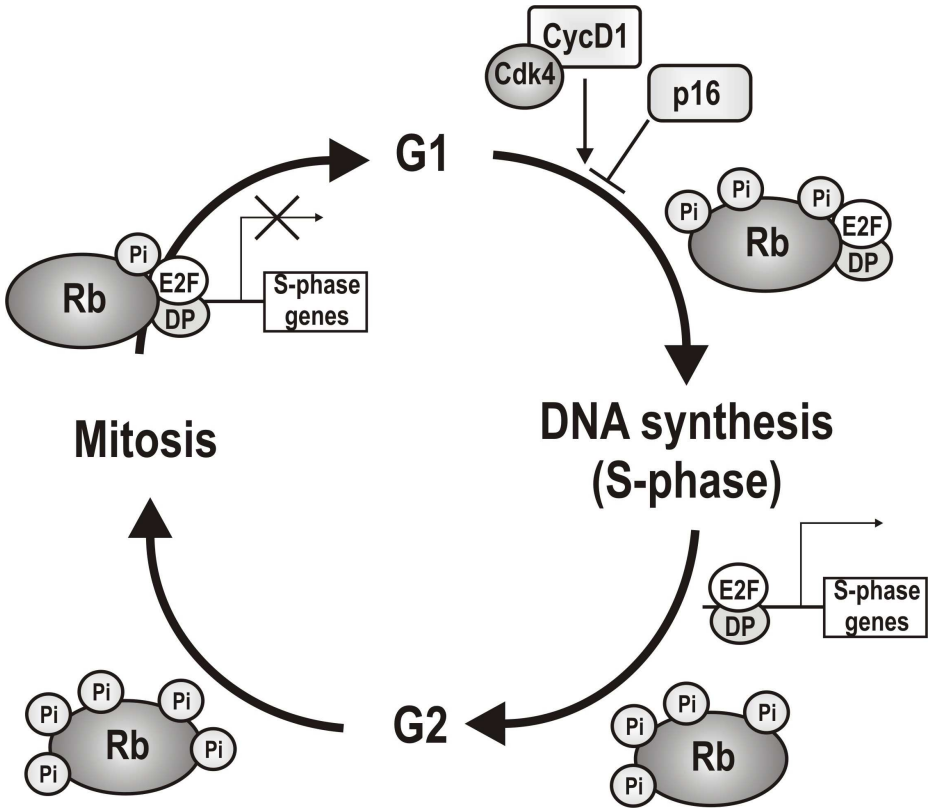

CycD1
Cdk4
p16
G1
Pi
Pi
Pi
Rb
E2F
DP
Pi
Rb
E2F
DP
S-phase
genes
DNA synthesis
(S-phase)
E2F
DP
S-phase
genes
G2
Pi
Pi
Pi
Rb
Pi
Pi
Pi
Rb
Pi
Mitosis

Figure 1. Regulation of the retinoblastoma protein (Rb) inactivating by phosphorylation during cell cycle progression. The Rb protein is hypophosphorylated (~3 Pi) in G1 phase of cell cycle, and phosphorylation (Pi) of specific sites appears to increase during cell cycle progression. A protein complex containing cyclin (Cyc) and cyclin-dependant kinase (Cdk) (e.g., CycD1 and Cdk4) phosphorylates Rb prior to DNA synthesis (S-phase) The CycD1/Cdk4 complex is regulated by p16 inhibitor protein, which is itself the product of a tumor suppressor gene. When Rb in hypophosphorylated state binds with E2F transcription factor and is brought to the E2F/DP target gene promoters and represses transcription. Hyperphosphorylation of Rb releases Rb from the E2F/DP bound protein complex and results in transcription activation of E2F target genes such as those involved in DNA synthesis. The figure indicates that Rb phosphorylation increases in G2 phase while Rb is dephosphorylated at or near anaphase.

family members directly affect cell cycle progression, proliferation and development was recently shown in conditional knockout mouse models.[7] Consequently, the *Rb* gene functions are important to suppress tumorigenesis by inhibiting cell cycle progression.

So far, many studies of Rb function focused on various cell function regulated by the Rb/E2F complex at protein level. Although, studying Rb function at protein level is important, investigations on how transcription of *Rb* gene is regulated are also important in understanding the functions of Rb and cellular function carried out by the protein.[8] The *Rb* promoter is located upstream of the human *Rb* gene within an unmethylated CpG-rich DNA sequence and contains no CCAAT or TATA box, indicating that the transcription initiation complex is recruited to initiator-binding factors.[8, 9] A sequence within the promoter, often referred to as the essential regulatory region which contains the DNA binding motifs for Sp1, E4TF1, Fli-1, p53, ATF, E2F, and CREB1, has been shown to be important for both basal and regulated transcription.[10] The Sp1 and ATF sites within the essential regulatory region are thought to be essential activators of basal promoter activity.[11] Also, MyoD is associated with CREB and targeted to the *Rb* promoter CRE in a complex also containing the p300 transcriptional coactivator.[8, 12] The resulting multiprotein complex stimulates transcription from the *Rb* promoter, so that

MyoD is a key event in the process of skeletal muscle differentiation.[13] Also, GABP and E4TF1 activate transcription of *Rb* promoter by directly binding to RBF motif, upon induction of differentiation, the GABP cofactor HCF-1 is recruited to and coactivates the *Rb* promoter with GABP.[14, 15] In contrast, YY1, which exerts an inhibitory effect on *Rb* gene expression, is removed from the promoter as cells advance through myogenesis and translocates from the nucleus to the cytoplasm.[15-17] All these data suggests that multiple transcription factors act on the transcription of *Rb* genes and greatly affect the biological functions carried out by *Rb* gene.

The BTB/POZ (broad complex, Tramtrack, and bric-a-brac/poxvirus and zinc finger) domain is an evolutionarily conserved protein-protein interaction domain that is found at the N-terminus of various cellular and viral regulatory proteins.[18] The proteins containing the BTB/POZ domain have several C-terminal structures important in their biological functions, such as the zinc finger, actin-binding repeats, and ion channel motifs.[18, 19] The POZ-domains of PLZF (promyelocytic leukemia zinc finger) and Bcl-6 (B-cell lymphoma-6) have been shown to interact with BCoR (BCL-6 interacting corepressor), NCoR (nuclear receptor co-repressor), mSin3A (mammalian homologs of yeast repressor switch-independent), SMRT (silencing mediator for retinoid and thyroid hormone receptors), and histone deacetylases.[20-25] POZ-domain

proteins are strongly involved in many critical cellular processes such as development, oncogenesis, apoptosis, ion channel activity, and transcription, as shown in some transcription repressor such as PLZF, Bcl-6 and FBI-1. Also, GAGA, a transcription regulator of *Drosophila*, facilitates long-range activation by providing a protein bridge that mediates enhancer–promoter communication and thus stimulate transcription by linking an enhancer to its cognate promoter.[26-28] Strikingly, in addition to facilitating activation by a remote enhancer *in cis*, GAGA was also shown to direct activation of a promoter by an enhancer located on a separate DNA molecule. Enhancer function *in trans* is critically dependent on POZ-domain-mediated GAGA oligomerization, enabling GAGA to bind two DNA molecules (e.g., PRE and promoter) simultaneously.[26-28] Currently, there are 183 proteins with BTB/POZ domain. Among them 43 POK (POZ and Krüppel zinc finger) family proteins are important in embryogenesis, cell differentiation, and tumorigenesis.[18] In particular, PLZF and Bcl-6 were shown to be important in hematopoiesis, and in the development of lymphoma, and leukemia and other cancers. Interactions between POZ domains and corepressor proteins (BCoR, NCoR, mSin3A, and SMRT) and other transcription factors could regulate transcription of many genes. The transcription repression by the molecular interaction was proposed to be important in the oncogenisis.[29-31]

We have been investigating on the biological function of various BTB/POZ-domain proteins, especially FBI-1. FBI-1 (Factor that Binds to the Inducer of short transcripts of human immunodeficiency virus-1) was purified as a cellular factor that binds specifically to the wild-type IST (inducer of short transcripts) elements of human immunodeficiency virus-1 (HIV-1) long terminal repeats (LTR) and the proximal promoter of the *ADH5/FDH* gene, and its cDNA was cloned.[32-35] FBI-1 is a ubiquitous transcription factor that contains a BTB/POZ domain at its N terminus and Krüppel-like zinc fingers at its C terminus.[33] There have been several recent reports on the function of FBI-1. FBI-1 stimulates Tat (transactivator of transcription) activity of HIV-1 LTR and represses human *ADH5/FDH* gene expression by interacting with Sp1 zinc fingers.[19, 35] The mouse counterpart of FBI-1, LRF (leukemia/lymphoma-related factor), is co-immunoprecipitated and co-localized with Bcl-6.[19] The rat homolog of FBI-1, OCZF (osteoclast-derived zinc finger), is a transcription repressor and is involved in osteoclastogenesis.[30] SAGE (serial analysis of gene expression) analysis shows that the expression of FBI-1 is increased in cancer tissues (available at www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Hs&CID=104640).

Recent studies showed that FBI-1 could play important cellular functional roles by regulating gene expression by various protein-protein,

protein-DNA interactions. FBI-1 was shown to interact with Sp1 zinc finger DNA binding domain and thus regulates the transcription of ADH5/FDHgene and also was shown to enhance transcription mediated by NF-κB by the interaction between the POZ-domain and RHD of NF-κB. [19, 32] FBI-1could selectivly bind to active chromatin and increase the activation potential of Tat. More recently, FBI-1 was shown to repress transcription of several transcription factors, important in cell cycle control such as cyclin A, cdk2, E2F-4, p130, and p170 although the detailed mechanism remains obscure.[35-36]

Recently, FBI-1 or Pokemon was shown as a proto-oncogene to promote.[37] Transgenic mice overexpressing FBI-1 was shown to repress transcription of a tumor suppressor gene ARF by binding to the promoter region. $p14^{ARf}$ is the transcription activator of p53, another tumor suppressor. Accordingly, repression of ARF can eventually inhibit expression of p53, and promotes of oncogenesis in thymus, liver, spleen, and tumor infiltration into bone marrow. In FBI-1 knockout mice, overexpression of ARF increased expression of p53, induces senescence apoptosis, and eventually blocks cellular differentiation.[37-39] FBI-1 is overexpressed in solid tumors such as colon cancer and bladder cancer in which the normal function of the ARF/p53 pathway is frequently lost.[31, 38] Accordingly, it is likely that FBI-1 has multiple additional target genes by which it can exert its oncogenic activity. These data

suggest that FBI-1 would have multiple additional target genes related oncogenesis.

We suspected that FBI-1 might be involved in the transcriptional regulation of *Rb* genes important in cell cycle control and tumor suppression. We and others investigated potential binding sites for FBI-1 and generated a GC rich DNA binding consensus sequences, 5'-GDGGGYYYY-3' or RMGACCCCCCCCC.[37, 40] It is similar to Sp1 binding consensus sequences, 5'-KRGGMGKRRY-3'.[41-43] We found 10 several potential FBI-1 binding sites (FREs) in the proximal promoter region of *Rb* gene and found that FBI-1 could repressed transcription of Rb gene with 400 bp upstream regulatary sequence. We investigated the molecular mechanism of transcriptional regulation of *Rb* genes by proto-oncogene, FBI-1. FBI-1 represses transcription by interacting with corepressors. Also, FBI-1 competes with Sp1 in binding to some of the key regulating elements, GC-boxes and FREs.

FBI-1 represses Rb at the gene transcription levels, and it could drive cells into S phase. We suggest that FBI-1 overexpression, found in cancer cells, might be involved in altered checkpoint controls during oncogenesis.

II. Materials and Methods

1. Plasmid construction

pGL3-*Rb* gene promoter (bp -370 to +106) fusion plasmid was kindly provided by Dr. Masayuki Sekimata (Fufushima Medical University School of Medicine, Japan). pGL2-*Rb* gene promoter fusion plasmid was prepared by enzyme digestion of pGL3-*Rb* gene promoter into pGL2-Basic plasmid (Promega, WI). Various mutant *Rb* promoter and reporter gene fusion plasmids were prepared by site directed mutagenesis kit (Stratagene, CA). The expression plasmids for the VP16-corepressors, the BCoR (a.a. 112-753), NCoR (a.a. 1709-2215), mSin3A (a.a. 69-316), and SMRT (a.a. 194-657) fusion proteins (pKH135EF-BcoR, pKH73/110EF-NCoR, pCMX-mSin3A, and pCMX-SMRT), were kindly provided by Drs. Ronald Evans (The Salk Institute, CA), Vivian Bardwell (University of Minnesota, MN) and Dominique Leprince (Institut Pasteur de Lille, Lille, France), respectively.[38-40] pcDNA3-FBI-1, pcDNA3-FBI-1ΔPOZ, and pG5-*Luc* were reported elsewhere.[32] Also, to prepare pGL2-FRE cluster-*tk**-*Luc*, pGL2-6x (Sp1-1) -*tk**-*Luc*, and pGL2-3x (Sp1-2) -*tk**-*Luc*, the parent pGL2-*tk**-*Luc* was mutated at the two Sp1 binding sites of *tk* (thymidine kinase) minimal promoter. 5'-GCCCCCGCCC-3' and 5'-GGGGCGGCG-3' sequences of the

*tk** promoter were mutated into 5'-GCCCCCGAAC-3' and 5'-GGTTCGGCG-3' respectively. For site directed mutagenesis two oligonucloetides (only top strands are shown), 5'-GATCAGATCTGGATCCGGCCCCGCCCAGCG-3' and 5'-CGCAGATGCAGTCGGTTCGGCGCGGTCCGAGG-3' were used. To prepare pGL2-FRE cluster-*tk**-*Luc*, a promoter region with 4 FRE cluster (149 bp fragment ranging from -318 bp to -170 bp) was PCRed from *Rb* promoter using two oligonucleotides , 5'-GATCGGTACCGGATAGGGATGAGGC-3' and 5'-GATCCTCGAGGAAACCTGGCGTGGG-3', and cloned into pGL2-*tk**/*Kpn Ⅰ*-*Xho Ⅰ*. pGL2-6x (Sp1-1) -*tk**-*Luc* was prepared by cloning 6 copies of GC-box1 (top strand, 5'-GTGACGCCGCGGGCGGAAGTGACGTTTTCCCGCGGTTGGACGCG-3') and pGL2-3x (Sp1-2) -*tk**-*Luc* was prepared by cloning 3 copies of GC-box2 (top strand, 5'-AGTTGCCGGGCGGGGGAGGG-3') in front of *tk** promoter of pGL2-*tk**-*Luc*. To prepare, the GST-POZ_{FBI-1} fusion protein expression plasmid, the cDNA fragment encoding the POZ-domain of FBI-1 was subcloned into pGEX4T3 (Amersham Biosciences, NJ) and reported elsewhere.[32] The expression plasmids for Sp1ZDBD (a.a. 622-778) and FBI-1ZFDBD (a.a. 366-495; 5'-GATCGGATCCGCCTGGTCGCAGAAGGTGGAG-3'; 5-GATCGTCGACCGAGGGGACGCCGTTGCAGCCGTC-3') were prepared

by cloning PCRed cDNA fragments cloned into pGEX4T1. The mammalian expression plasmids of the corepressors were prepared by subcloning the cDNA fragments encoding for BCoR (a.a. 112-753), NCoR (a.a. 1709-2215), mSin3A (a.a. 69-316), and SMRT (a.a. 194-657) into pcDNA3.0 (Invitrogen, CA) and reported elsewhere.[21-23]

2. Cell culture/ stable cell line

HeLa cells were cultured in Dulbecco' modified eagle medium (DMEM, Gibco-BRL, MD) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, MD) at 37℃, 5% CO_2. Stable HeLa cells overexpressing FBI-1 were prepared by transfection of HeLa cells with a recombinant Lenti virus, LentiM1.4-FBI-1 tagged with Flag peptide. Briefly, 350 ㎕ of 4×10^7 TU/㎖ of LentiM1.4-FBI-1 (Vectorcorea, Korea) was used to infect HeLa cells plated on 12-well plate supplemented with 8 ㎍/㎖ of polybrene (Sigma, MO) to increase infection efficiency and incubated at 37°C, 5% CO_2 for 6~8 hrs and replaced with fresh culture medium. After 2~3 days of incubation, cells were transferred onto a 6-well plate and selected stable cells with 1 ㎍/㎖ of puromysin (Sigma, MO). Control stable cells were prepared by infection with LentiM1.4-LacZ (Vectorcorea, Korea).

3. Electrophoretic mobility shift assays (EMSAs)

The oligonucleotide probes (100 picomoles each in 83.3 mM Tris-HCl, pH 8.0, 16.7 mM $MgCl_2$, 166.7 mM NaCl) were annealed by heating at 93℃ for 5 min and cooling down slowly to room temperature. After diluting the solution containing the annealed oligonucleotides with water to 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl, 100 picomoles of annealed oligonucleotides for EMSAs were labeled with [α-^{32}P] dATP and Klenow enzyme (Roche, Mannheim, Germany) by incubating at 37℃ for 10 min. ^{32}P labeled, double-stranded oligonucleotides were purified with sephadex^{TM} G-50 (Amersham Biosciences, NJ). For competition assay, unlabeled oligonucleotides (200x cold competitor) were added to the reaction mixture. The sequences of FREs and Sp1 binding GC-box oligonucleotides are as follows: top strand sequences are shown. FRE1, 5'- GATCGGATGAGGCCC ACAGTCACC-3'; FRE2, 5'-GATCCCACAGTCACCCACCAGACT-3'; FRE3, 5'-GATCAGGGGGTGGTTCTGGGTAGA-3'; FRE4, 5'-GATCCGC CTGGACCCACGCCAGGT-3'; GC-box1, 5'-GATCACGCCGCGGGCGGA AGTGAC-3'; GC-box2, 5'-GATCAGTTGCCGGGCGGGGGAGGG-3'. Each binding reaction was carried out in 20 ㎕ of binding buffer containing 10 mM HEPES, pH 7.9, 60 mM KCl, 5 μM $ZnCl_2$, 1 mM dithiothreitol, 1% BSA, 7% glycerol, 0.1 ㎍ recombinant FBI-1ZFDBD (zinc finger DNA

binding domain) or Sp1ZFDBD, and 10,000 cpm probe at room temperature for 30 min. Where indicated, antibody against Tag, FBI-1 or Sp1 was added to EMSA binding reaction. The protein-DNA complexes were resolved from free probe by 4% non-denaturing polyacrylamide gel electrophoresis (PAGE) at room temperature in 0.5x TBE buffer (89 mM Tris-Borate, 2 mM EDTA, pH 8.3) at 150 V for 1.5 hrs. The dried gels were exposed to X-ray film at -70℃ with a Kodak intensifying screen (Kodak, NY).

4. GST Fusion Protein Purification, in Vitro Transcription and Translation of Corepressors

GST or GST fusion protein expression was prepared from *E. coli* BL21 (DE3) transformed with GST or GST fusion proteins expression plasmid. The *E. coli* were induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 hrs at 37°C. The cells were lysed by lysis buffer containing 1x PBS, 1 mM PMSF, 2 mM EDTA, and 0.2 mg/㎖ lysozyme, then sonicated 3 ~ 5 times to make lysates. The recombinant proteins were purified with glutathione-agarose 4 bead by affinity chromatography (Peptron, Daejeon, Korea). The purified proteins were resolved with 12% SDS-PAGE to quantitate and assess purity. The same amount of aliquot of the protein-agarose bead complex was used in GST-fusion protein pull down assays.

The corepressor polypeptides were prepared *in vitro* by incubating 1 μg of pcDNA3.0 corepressor expression plasmids with TNT Quick-coupled Transcription/Translation Extract (Promega, WI), containing 40 μl of TNT Quick Master Mix and 2 μl of [^{35}S] methionine (1175.0 Ci/mol, PerkinElmer Life Sciences, Inc.) at 30°C for 90 min. Polypeptide expression levels were then analyzed by running 3 μl of the total mixture on a 12% SDS-PAGE.

5. GST Fusion Protein Pull-down Assays

The purified GST fusion proteins (5 μg) were incubated with GSH-agarose (Sigma, MO) for 1 hr in HEMG buffer (40 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 10% glycerol, 1.5 mM dithiothreitol, and protease inhibitor mixture, 1 tablet/50 ml of a protease inhibitor mixture (Roche, Mannheim, Germany) at 4°C for 1 hr. After the agarose-GST protein complexes were washed three times with 1 ml of cold HEMG buffer, 10 μl of the *in vitro* translated corepressors were added and incubated in HEMG buffer at 4°C for 4 hrs. The reaction mixtures were centrifuged at 3,000x *g* at 4°C, and the supernatants were removed and the pellets were washed five times with cold HEMG buffer. The bound proteins were separated by a 12% SDS-PAGE. The SDS-PAGE gel was dried and exposed to X-ray film using image-intensifying screen (Kodak, NY).

6. Site-directed Mutagenesis of p*Rb-Luc*

To investigate the role of FBI-1 binding sites, mutations were introduced into the proximal promoter sequence of *Rb* gene using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). To introduce mutations, the following oligonucleotides were used (only top strands are shown): Mutations were introduced into the core binding sequences (5'-GDGGGYYYY-3' to 5'-GDAAAYYYY-3') of various FRE and GC-Box using the following oligonucleotides primers: mFRE1, 5'-CCCGGGATAGGGATGAAATTTACAGTCACCCACCAGA-3'; mFRE2, 5'-ATGAGGCCCACAGTCATTTACCAGACTCTTTGTAT-3'; mFRE3, 5'-CACCCCGGCCTGGAGGAAAAAATTCTGGGTAGAAGCAC-3'; mFRE4, 5'-CTGGAAGGCGCCTGGATTTACGCCAGGTTTCCCAG-3'; mGC-Box1,5'-ACGTGACGCCGCGGGCAAAAGTGACGTTTTCCCG-3'; mGC-Box2, 5'-CGCTCAGTTGCCGGGCAAGGGAGGGCGCGTCCGG-3'. PCR cycling conditions used in site directed mutagenesis were 18 cycles of amplication of following reaction: denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 68°C for 10 min. Amplified mixtures were treated with *Dpn I* (Stratagene, CA) at 37°C for 1 hr and aliquotes were used to transform competent *E. coli*. All the constructs were confirmed by DNA

sequencing using ABI automatic DNA sequencer (Ramlsey, MN).

7. Chromatin Immunoprecipitation (ChIP) Assays

To investigate whether *in vivo* molecular interaction between FBI-1 and the FRE elements on the *Rb* promoter is actually occurring, ChIP assay was performed with commercial kit (Upstate Inc., VA). Subconfluent HeLa cells on a 10 cm dish were transfected with 1 μg of pGL2-*Rb-Luc* plasmid and 3 μg of pcDNA3.0 or pcDNA3.0-FBI-1-Flag using Lipofectamine Plus (Invitrogen, CA) and grown for additional 48 hrs. HeLa cells were treated with formaldehyde (final 1 %) to cross-link FBI-1 protein to the *Rb* promoter. Cells were washed with cold phosphate-buffered saline and lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0). The lysate was sonicated to shear DNA into 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, pH 8.1, 167 mM NaCl, 1.2 mM EDTA) and incubated with antibodies against mouse M2-Flag antibody (Sigma, MO) or control mouse IgG overnight at 4°C with rotation. To collect DNA-FBI-1-antibody complex, a salmon sperm DNA/protein A-agarose slurry was added to the mixture. The mixture was incubated for 1 h at 4°C with rotation and pelleted in a DNA/protein A-agarose complex by brief centrifugation (4000

rpm) at 4°C. After extensive washing of the pellet with washing buffers (low salt immune complex wash buffer, high salt immune complex wash buffer, LiCi immune complex wash buffer, and TE buffer) recommended by the manufacturer, the pellet was dissolved with 500 ㎕ of elution buffer and spun to remove agarose. Supernatant was treated with 20 ㎕ of 5 M NaCl and heated to 65°C for 4 hrs to reverse protein-DNA cross-link. 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 the two sets of oligonucleotide primers designed to amplify the proximal and distal promoter regions of *Rb* promoter. Distal FRE cluster region (bp -370 to -147: forward primer: 5'-CACTAGCCAGATATTCCCTGCGGGG-3', reverse primer: 5'-TAAGTCATGAGGAATTAAACTGGGA-3'). Proximal FRE cluster region (bp -131 to +93: forward primer: 5'-CACCGACCAGCGCCCCAGTTCCCC A-3', reverse primer: 5'-GGGAGGACGCGCGCGCACGTCG-3').

8. Mammalian Two-hybrid Reporter Assays

African green monkey kidney cells (CV-1) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/㎖ of streptomycin, and 100 units/㎖ of penicillin

(Invitrogen, CA). The cells were inoculated on 6-well tissue culture plates at a density of 5x 10^5 cells/well in 2 ㎖ of DMEM. After the cells were grown for 16 hrs, they were transiently transfected with the POZ-domain of FBI-1 fused with Gal4 expression plasmid (pCMX-GAL4-POZ), the corepressors fused with VP16 expression plasmid (pCMX-VP16-BCoR, pCMX-VP16-NCoR, pCMX-VP16-mSin3A, and pCMX-VP16-SMRT), and the reporter plasmid (pG5-*Luc*) using Lipofectamine Plus reagent (Invitrogen, CA) in serum-free OptiMEM, according to the manufacturer's recommendations. After 3 hrs, the cells were supplied with fresh, complete DMEM, and allowed to grow for additional 36 hours. The cells were then harvested and lysed in 100 ㎕ of reporter lysis buffer (Promega, WI), vortexed for 1 min, and then centrifuged at 12,000 rpm for 15 min at 4°C. The luciferase reporter assays were performed with 5 ㎕ of cell extracts, using 50 ㎕ of luciferase assay reagent (Promega, WI) on a luminometer (Microplate Luminometer LB 96V, EG&G Berthold, MD). Luciferase activities were normalized with cotransfected β-galactosidase activity.

9. Transcription analysis of *Rb* gene promoter

Cell culture was carried out as described above. The 0.3 ㎍ of Wt or mutant of *Rb* promoter fused with *luc* plasmid and 0.5 ㎍ of pcDNA3.0,

pcDNA3.0-FBI-1 and pcDNA3.0-FBI-1ΔPOZ were transiently transfected into HeLa cells grown on 6-well culture vessel, using Lipopectamine PLUS reagent (Invitrogen, CA) according to manufacturer's suggestion. The HeLa cells were transfected as described in Mammalian Two-hybrid Reporter Assays section. After 36 hrs of incubation, cells were harvested and lysed in 100 ㎕ of reporter lysis buffer. Luciferase activities were normalized with protein concentration of the transfected cells.

10. RT-PCR (Reverse Transcriptase Polymerase Chain Reaction)

Total RNA was isolated from HeLa cells and stable HeLa cells expressing FBI-1 or LacZ using TRIzol®reagent (Invitrogen, CA). cDNAs were synthesized using 5 ㎍ total RNA, 10 pmol of random hexamer, and 200 units of superscript reverse transcriptase Ⅱ in 20 ㎕ using reverse transcription kit (Invitrogen, CA). PCR was performed by using the following amplification condition: 94°C denaturation 5 min, 23 cycles of amplication reaction cycling of at 94°C for 30 sec, 62°C or 55°C for 30 sec, and 72°C for 40 sec, and final extension reaction at 72°C for 7 min. The primers used for PCR FBI-1 cDNA were 5'-GGCCTGCTGTGCGACGTGGT-3' and 5'-CAGCAGGCGGGCGGCGCTGA-3'. The primers for *Rb* were 5'-AAAGAA AAAGGAACTGTGGG-3' and 5'-AACTGCTGGGTTGTGTCAAA-3'. The

primers for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. The PCR products were separated by 1% agarose gel electrophoresis and visualized with ethidium bromide staining.

11. Western Blot Analysis of Rb and FBI-1

HeLa cells and stable HeLa cells overexpressing FBI-1 or LacZ were harvested and lysed in TEN buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1 M NaCl). Cell extract (40 μg) were separated by a 10% SDS-PAGE. Proteins were transferred onto a Immun-Blot™ PVDF Membrane (Bio-Rad, CA) with TRANS-Blot® Semi-Dry Transfer –cell (Bio-Rad, CA) at 15 V for 1 hr by transfer buffer (25 mM Tris-base, 0.2 mM glycine, and 20% methanol, pH 8.5) and blocked with 5% skim milk (BD biosciences, NJ) in TBST (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, and 0.001% Tween 20) for 10 min. Blotted membranes were incubated with Ab-FBI-1 (Sigma, MO), Ab-Rb (BD biosciences, NJ), and Ab-α-tubulin (Calbiochem, SD) diluted 1:1000 ratio at 4°C for overnight. Membranes were washed three times with TBST for 10 min and incubated with a 1 : 2000 dilution of horseradish peroxidase-conjugated anti-mouse IgG (Vector, CA) or anti-goat IgG (Santa Cruz, CA) antibody at room temperature for 1 hr. Blots were washed with TBST more

than three times and developed with the ECL system (PerkinElmer, CA) according to the manufacturer's protocols.

12. Cell cycle analysis with FACS (Fluorescence Activated Cell Sorter)

HeLa cells and stably FBI-1 or LacZ overexperssing HeLa cells were harvested. The cells were gently washed with PBS and then fixed with ice-cold 70% methanol at -20°C for 1 hr. The fixed cells were washed with PBS, and stained with 50 ㎍/㎖ PI (Propidium Iodide) in the presence of 100 ㎍/㎖ ribonuclease A for 30 min at 37°C in the dark. DNA content was analyzed by flow cytometry analyzer FACSCalibur (BD biosciences, NJ). DNA content was assessed by cell treatment with PI and emission detection at the excitation wavelengths 488 nm and the peak emission 575 nm.

III. Results

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

Recently, FBI-1 was shown to have proto-oncogenic activity by repressing tumor suppressor ARF gene (mouse $p19^{Arf}$, human $p14^{Arf}$), which in turn lower expression of tumor suppressor gene, p53. While, over expressed FBI-1 caused oncogenesis in thymus, liver, spleen, and tumor infiltration into bone marrow, down-regulated FBI-1 caused cellular senescence, apoptosis, and blockage of differentiation.[37, 38] Considering that Pokemon is also overexpressed in solid tumors such as colon cancer and bladder cancer in which the normal function of the ARF/p53 pathway is frequently lost, it is likely that Pokemon has multiple additional target genes by which it can exert its oncogenic activity.[37, 38]

Pokemon consensus DNA binding sequences by CAST analysis selected a specific GC–rich sequence for Pokemon binding, which shows a certain similarity to the consensus sequence for the transcription factor Sp1.[37, 40] We suspected that the target gene controlled by FBI-1 is not limited to pARF and in fact many other genes with certain GC-box located in the promoter or enhancer could be regulated by FBI-1.

Accordingly, we investigated whether the other gene such as *Rb* which is

directly involved with cell cycle control and tumor suppression, is the target of Pokemon action. Using recently characterized consensus sequence and MacVector 7.0 program (Accelrys, San Diego, CA), we found four FBI-1 binding sites (FRE) and two GC-boxes on the Rb promoter. The four potential FREs have varying degree of sequence homology to the consensus sequence (5'-GDGGGYYYY-3') (Fig. 2A).[40]

We transiently cotransfected into HeLa cells with three FBI-1 expression plasmids (pcDNA3-FBI-1, pcDNA3-FBI-1ΔPOZ, and pcDNA3-POZ-NLS) with the *Rb-Luc* fusion gene reporter plasmid and analyzed reporter luciferase gene expression (Fig. 2B). FBI-1 repressed transcription of *Rb* promoter by more than 50%, compared to the control. FBI-1 with deleted POZ-domain showed much weaker transcription repression and repressed transcription only by 20%, suggesting that the POZ-domain is important in transcription repression. The POZ-domain polypeptide with NLS could not repress transcription of *Rb* promoter, but rather increase reporter gene expression by 20%. Since the POZ-domain-NLS polypeptide lacks zinc fingers DNA binding domain and cannot bind to the promoter, the binding to the promoter is important in transcription repression (Fig. 2C). These data suggest that *Rb* gene is a newly identified transcription target gene of FBI-1 and the POZ-domain of FBI-1 plays an important role in the transcription repression of *Rb*

gene.

To investigate whether FBI-1 could repress gene expression of *Rb* gene in HeLa cells, we prepared stable HeLa cells overexpressing FBI-1 established by recombinant Lentivirus transfection and selection. We investigated whether overexpession of FBI-1 could repress expressions of mRNA and protein of *Rb* gene by RT-PCR and western blot analysis, respectively. The ectopic FBI-1 decreased mRNA and protein level of *Rb* gene. The control stable cells established with LacZ Lentivirus or control HeLa cells with no virus transfection, did not show any significant change in expression of mRNA or protein of *Rb* gene (Fig. 2D, E). It is clear that *Rb* gene is the new target gene of FBI-1 action and the repression could be important in oncogenesis.

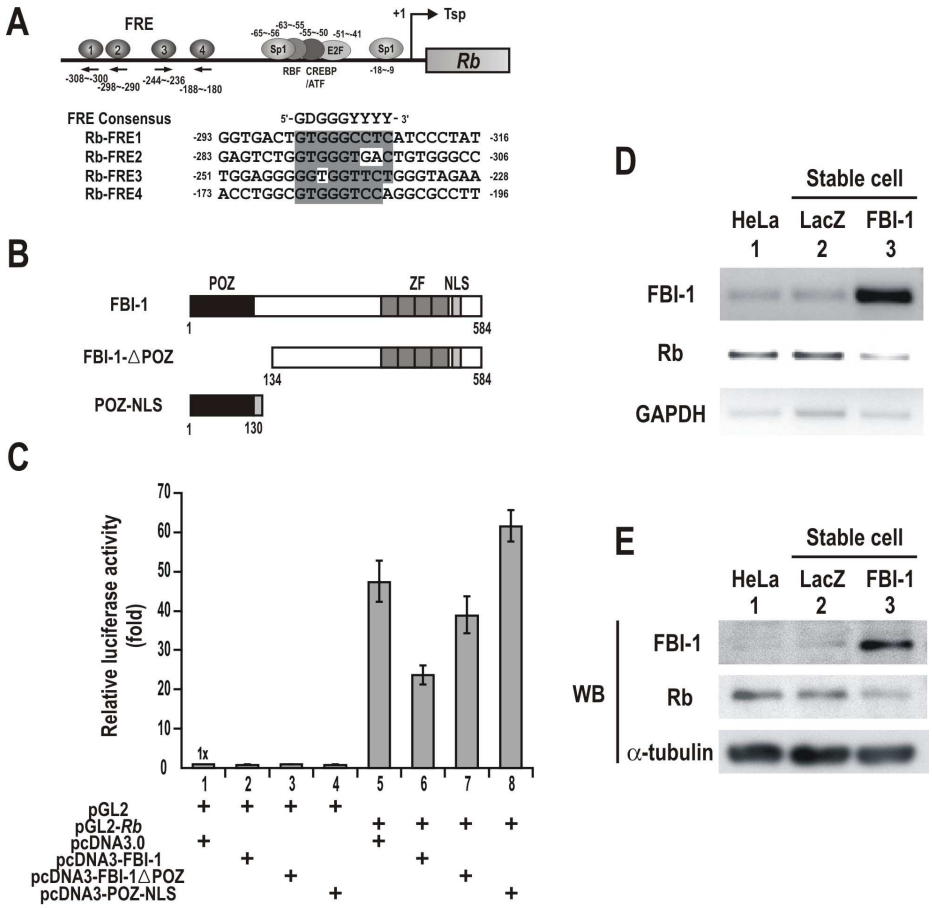

A
FRE
+1
Tsp
Sp1
E2F
Sp1
Rb
RBF CREBP /ATF
FRE Consensus 5'-GDGGGYYYY-3'
Rb-FRE1 GGTGACTGTGGGCCTCATCCCTAT
Rb-FRE2 GAGTCTGGTGGGTGACTGTGGGCC
Rb-FRE3 TGGAGGGGGTGGTTCTGGGTAGAA
Rb-FRE4 ACCTGGCGTGGGTCCAGGCGCCTT
B
POZ
ZF
NLS
FBI-1
FBI-1-△POZ
POZ-NLS
C
Relative luciferase activity (fold)
pGL2
pGL2-Rb
pcDNA3.0
pcDNA3-FBI-1
pcDNA3-FBI-1△POZ
pcDNA3-POZ-NLS
D
Stable cell
HeLa
LacZ
FBI-1
FBI-1
Rb
GAPDH
E
Stable cell
HeLa
LacZ
FBI-1
WB
FBI-1
Rb
α-tubulin

Figure 2. Repression of transcription activity of the *Rb* promoter by FBI-1 in HeLa cells. (A) Structure of *Rb* gene promoter and sequence comparison of putative four FBI-1 binding sites (FRE). Transcription factor binding sites are indicated by the shaded circles. Putative four FREs are located at bp -180 to -308 region. FBI-1, factor that binds to the inducer of short transcripts of human immunodeficiency virus-1; *Rb*, retinoblastoma gene, FRE; FBI-1 binding site (B) Schematic diagram of the FBI-1 and deletion mutant constructs. FBI-1 has a POZ-domain at N-terminus, four Krüppel-like zinc fingers, and nuclear localization signal (NLS) at C-terminus. POZ, poxvirus and zinc finger of FBI-1; ZF, Krüppel-like zinc fingers domain; NLS, nuclear localization signal (C) FBI-1 represses transcription of *Rb* gene promoter by more than 50%. Also FBI-1 with POZ-domain deleted show much weaker transcription repression. Interestingly, the POZ-domain with NLS attached activates transcription. (D), (E) FBI-1 represses expressions of mRNA and protein of *Rb* gene by RT-PCR (D) and western blot analysis (E), respectively. FBI-1, stable HeLa cells over expressing FBI-1; LacZ, stable HeLa cells over expressing LacZ; WB, western blot; GAPDH, control of RT-PCR; α-tubulin, control of western blot analysis.

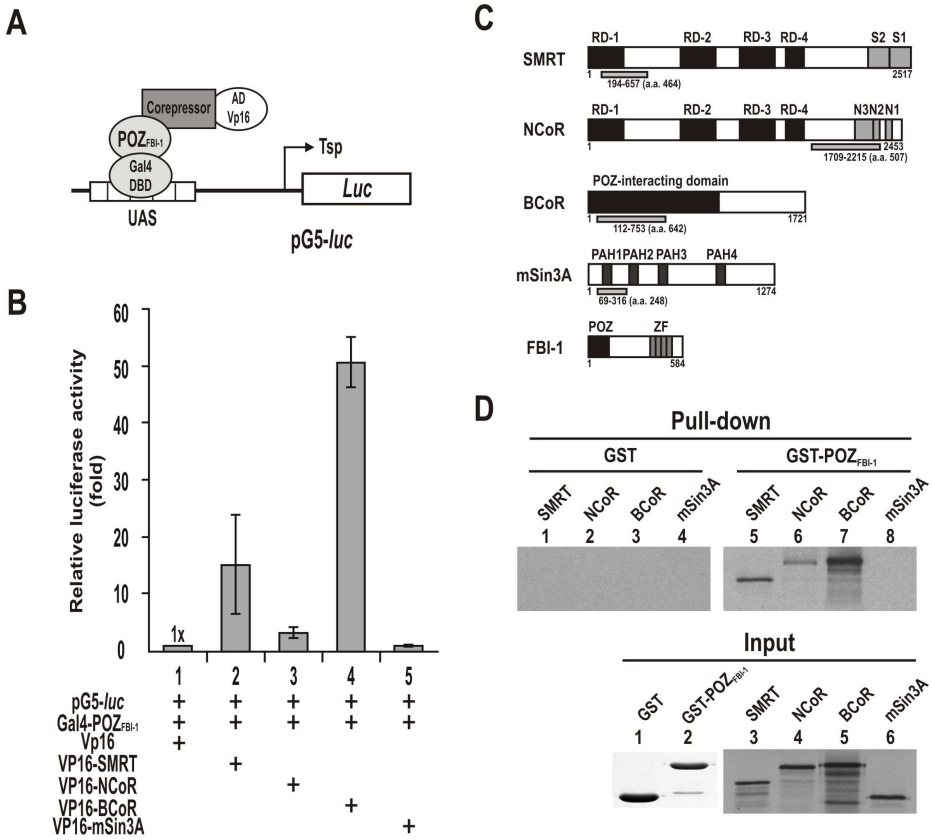
2. The POZ-domain of FBI-1 interacts with the corepressors *in vivo* and *in vitro.*

We found that FBI-1 could repress transcription of *Rb* gene, which might be important in oncogenesis. One of the possibilities is the molecular interaction between the functional POZ-domain of FBI-1 with the corepresssors and resulting histone modification, could repress transcription, as reported for PLZF and Bcl-6.[21-23] Accordingly, we investigated whether the POZ-domain of FBI-1 can interact with the corepressors *in vivo* using Gal4-UAS reporter mammalian two-hybrid assay system (Fig. 3A). We fused the POZ-domain of FBI-1 with the DBD of Gal4 transcription factor and targeted to the UAS (upstream activation sequence) of Gal4 mammalian two hybrid reporter gene construct (pG5-*Luc*) in the presence or absence of the VP16AD-corepressor (BCoR, NCoR, mSin3A, and SMRT) fusion protein constructs. We found that the POZ-domain of FBI-1 could interact with BCoR, NCoR, and SMRT, but not with mSin3A. Especially, the BCoR showed the most strong molecular interaction, which is followed by SMRT, and NCoR (Fig. 3B).

To investigate whether the molecular interaction between the POZ-domain and corepressors are direct, GST-fusion protein pull down assays were performed. We incubated the recombinant GST-POZ domain or GST protein

with *in vitro* synthesized [^{35}S] labeled corepressors (Fig. 3C), and pulled down. The assays showed that the GST-POZ of FBI-1 interacted strongly with BCoR, which is followed by SMRT, and NCoR. As in mammalian two hybrid assays, mSin3A did not interact with the POZ-domain (Fig. 3D).

Our data overall suggest that the POZ-domain of FBI-1 interacts with the corepressors directly *in vivo* and *in vitro*. Because the corepressors were shown to be important in transcription repression by recruiting histone modification enzymes such as HDACs, the molecular interaction between FBI-1 and corepressors might be important in the transcription repression of *Rb* gene by FBI-1.


A
Corepressor
AD
Vp16
POZFBI-1
Gal4
DBD
Tsp
Luc
UAS
pG5-luc
B
Relative luciferase activity (fold)
60
50
40
30
20
10
0
1x
1 2 3 4 5
pG5-luc + + + + +
Gal4-POZFBI-1 + + + + +
Vp16 +
VP16-SMRT +
VP16-NCoR +
VP16-BCoR +
VP16-mSin3A +
C
SMRT
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1 194-657 (a.a. 464) 2517
NCoR
RD-1 RD-2 RD-3 RD-4 N3N2N1
1 2453
1709-2215 (a.a. 507)
BCoR
POZ-interacting domain
1 112-753 (a.a. 642) 1721
mSin3A
PAH1PAH2 PAH3 PAH4
1 69-316 (a.a. 248) 1274
FBI-1
POZ ZF
1 584
D
Pull-down
GST
GST-POZFBI-1
SMRT NCoR BCoR mSin3A SMRT NCoR BCoR mSin3A
1 2 3 4 5 6 7 8
Input
GST GST-POZFBI-1 SMRT NCoR BCoR mSin3A
1 2 3 4 5 6

Figure 3. The POZ-domain of FBI-1 directly interacts with corepressors *in vitro and in vivo*. (A) Diagram of mammalian two-hybrid assay used for interaction study between the POZ-domain of FBI-1 and corepressors. (B) The POZ-domain of FBI-1 interacts with corepressors. It interacts most strongly with BCoR, followed by SMRT, and rather weakly interacts with NCoR. It did not interact with mSin3A. (C) Structure of the corepressors, SMRT, NCoR, BCoR, and mSin3A, and FBI-1. RD, repression domain; N3-1 and S1-2, domains involved in interaction with nuclear receptor; PAH, mediates protein-protein interactions. Domains of corepressors used *in vitro* pull-down assays are indicated below by light gray filled bars. ZF, zinc finger domain; POZ, POZ-domain of FBI-1 used in pull-down of [^{35}S] methionine-labeled corepressors polypeptides. (D) Recombinant GST, and GST-POZFBI-1 fusion proteins were incubated with the *in vitro* synthesized [^{35}S] methionine-labeled corepressors polypeptides, and then were pulled down. The precipitated samples were resolved by a 12% SDS-PAGE and exposed to X-ray film.

3. FBI-1 binds to the proximal promoter of *Rb* gene *in vivo* and *in vitro.*

We investigated whether FBI-1 could bind to the potential FBI-1 binding FRE elements located in the promoter region of *Rb* gene by EMSA assays. We prepared four ^{32}P labeled FRE oligonucleotide probes (bp -308 to -300, bp -298 to -290, bp -244 to -236, and bp -188 to -180) and allowed to interact with recombinant FBI-1ZFDBD (zinc finger DNA binding domain ; a.a. 366-495). FBI-1ZFDBD binds to the FRE2, 3, 4 probes quite well and it also binds to the FRE1 relatively weakly compared to FRE2, 3, and 4. Cold competitor competed well and antibody specific to GST caused binding inefficient or prevented the probe from binding to the FREs (Fig. 4A, B).

We further investigated whether FBI-1 binds to the *Rb* promoter *in vivo* by ChIP assays in HeLa cells. We cotransfected FLAG tagged FBI-1 expression palsmid and *Rb-Luc* fusion reporter plasmid into HeLa cells. ChIP assays showed that the antibody against the Flag Tag, precipitated the distal promoter region with four FRE elements, but it did not precipitated the proximal region. Control IgG antibody did not precipitate neither proximal nor distal region of *Rb* gene promoter (Fig. 4C). Our data indicated that FBI-1 could bind to the promoter region of *Rb* gene *in vitro* and *in vivo* directly.

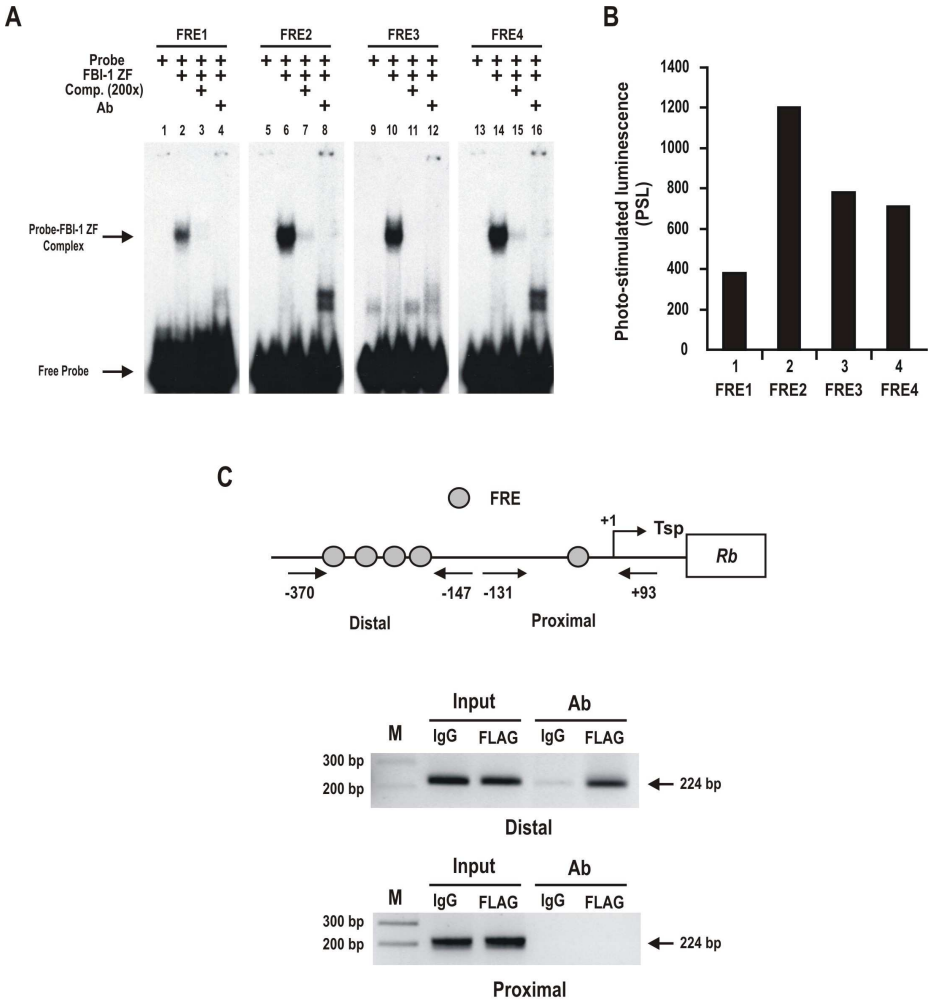

A
FRE1
FRE2
FRE3
FRE4
Probe
FBI-1 ZF
Comp. (200x)
Ab
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Probe-FBI-1 ZF Complex
Free Probe
B
Photo-stimulated luminescence (PSL)
1400
1200
1000
800
600
400
200
0
1 2 3 4
FRE1 FRE2 FRE3 FRE4
C
FRE
+1
Tsp
Rb
-370
-147 -131
+93
Distal
Proximal
Input
Ab
M
IgG FLAG IgG FLAG
300 bp
200 bp
224 bp
Distal
Input
Ab
M
IgG FLAG IgG FLAG
300 bp
200 bp
224 bp
Proximal

Figure 4. FBI-1 binds to the *Rb* promoter *in vitro* and *in vivo*. (A) EMSA. Four ^{32}P labeled FRE oligonucleotide probes (bp -308 to -300, bp -298 to -290, bp -244 to -236, bp -188 to -180) were incubated with recombinant zinc finger DNA binding domain of FBI-1 (a.a. 382-490) and separated by a 4% nondenaturating polyacrylamide gel. (B) Phosphoimager analysis of the EMSA-PAGE gel. The relative molecular interaction of probe-FBI-1 zinc finger complex retarded (lanes 2, 6, 10, and 14). (C) Schematic diagram of the *Rb* gene promoter and PCR primers used in ChIP assay. pcDNA3.0-FBI-1-flag and *Rb-Luc* plasmids were transfected into HeLa cells and chromatin was immunoprecipitation with anti-Flag antibody.

4. Not only FBI-1 binding to FREs but also Sp1 binding GC-box 2 are important in the transcription repression by FBI-1.

To investigate the role of the FBI-1 binding to FRE elements in the transcription repression of the *Rb* gene, we prepared the mutant FRE oligonucleotides for EMSA by substituting the core GGG with AAA (Fig. 5A). We also analyzed the molecular interaction among Sp1, FBI-1, and GC-boxes of the *Rb* proximal promoter, because we have found that some of the Sp1 binding GC boxes bind to FBI-1 fairly well (data unpublished). We mutated two GC-boxes 5'-CGGGCGGAAG-3' and 5'-CGGGCGGGGG-3' sequences of the *Rb* promoter into the 5'-CGGGCAAAAG-3' and 5'-CGGGCAAGGG-3', respectively, for EMSA assays. FBI-1 did bind well to all the Wt probes but not to any of mutant FREs, which indicates that the core GGG sequence is critical in FBI-1 binding (Fig. 5B).

Having shown that the FBI-1ZFDBD does not bind to mutant probes, we introduced the mutations into the promoter region of *Rb* gene by PCR-based site-directed mutagenesis. Also, we made mutant form of the Sp1 binding site on the *Rb* promoter by similar method. We transiently transfected the Wt or mutant *Rb-Luc* plasmids in HeLa cells to investigate *in vivo* function of FRE1-4 elements and Sp1 binding GC boxes 1, 2 (Fig. 5C). Mutations of FREs increased the transcription activity of *Rb* promoter,

compared to the Wt promoter by 20-130%. The data suggests that FBI-1 acts as transcription repressor by binding to the FREs of *Rb* promoter. First Sp1 binding site mutant at bp -59 and -60 showed drastically reduced luciferase gene expression compared to the wild type *Rb* promoter. This data indicate that first Sp1 binding site is important in transcription of *Rb* gene, as reported previously.[11] However, interestingly mutation of second Sp1 binding site at bp -12 and -13 showed increased luciferase activity compared to the wild type *Rb* promoter (Fig. 5D). The data potentially suggest that second Sp1 binging site is either occupied by repressor member of Sp1-superfamily or by FBI-1 as shown by EMSA with FBI-1ZFDBD. Mutation of element prevent binding by Sp1 repressor member or FBI-1, and resulted in increase transcription although it wasn't shown that FBI-1 binds to second Sp1 binding site of *Rb* promoter by ChIP assay.

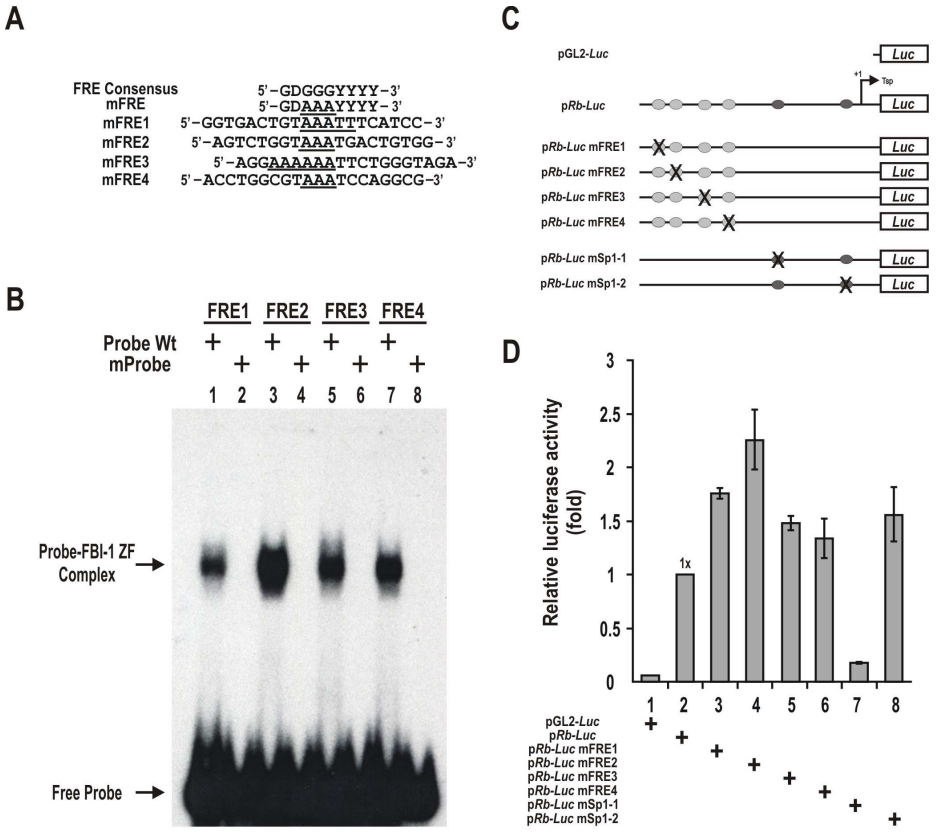

A
FRE Consensus 5'-GDGGGYYYY-3'
mFRE 5'-GDAAAYYYY-3'
mFRE1 5'-GGTGACTGTAAATTTCATCC-3'
mFRE2 5'-AGTCTGGTAAATGACTGTGG-3'
mFRE3 5'-AGGAAAAAATTCTGGGTAGA-3'
mFRE4 5'-ACCTGGCGTAAATCCAGGCG-3'
B
FRE1 FRE2 FRE3 FRE4
Probe Wt + + + +
mProbe + + + +
1 2 3 4 5 6 7 8
Probe-FBI-1 ZF Complex
Free Probe
C
pGL2-Luc
pRb-Luc
pRb-Luc mFRE1
pRb-Luc mFRE2
pRb-Luc mFRE3
pRb-Luc mFRE4
pRb-Luc mSp1-1
pRb-Luc mSp1-2
Luc
+1
Tsp
D
Relative luciferase activity (fold)
1x
0
0.5
1
1.5
2
2.5
3
1 2 3 4 5 6 7 8
pGL2-Luc
pRb-Luc
pRb-Luc mFRE1
pRb-Luc mFRE2
pRb-Luc mFRE3
pRb-Luc mFRE4
pRb-Luc mSp1-1
pRb-Luc mSp1-2

Figure 5. Mutation of core GGG sequence of FREs and binding by FBI-1. The core sequence (GGG) of FRE consensus sequence is important in binding by FBI-1. (A) Sequences comparisons of the consensus and mutant probes. The core GGG sequence of the 4 FREs in the *Rb* promoter was substituted with AAA. (B) EMSA. The zinc finger of FBI-1 does bind to the natural but not to the mutant probes. (C) Schematic diagram of a series of *Rb-Luc* Wt and mutant plasmids. (D) Mutations of FREs and Sp1-2 increased the transcription of *Rb* promoter, compared to the Wt promoter by 20-130 %. Sp1 binding site at GC-box 1 mutant showed drastically reduced luciferase gene expression compared to the wild type *Rb* promoter.

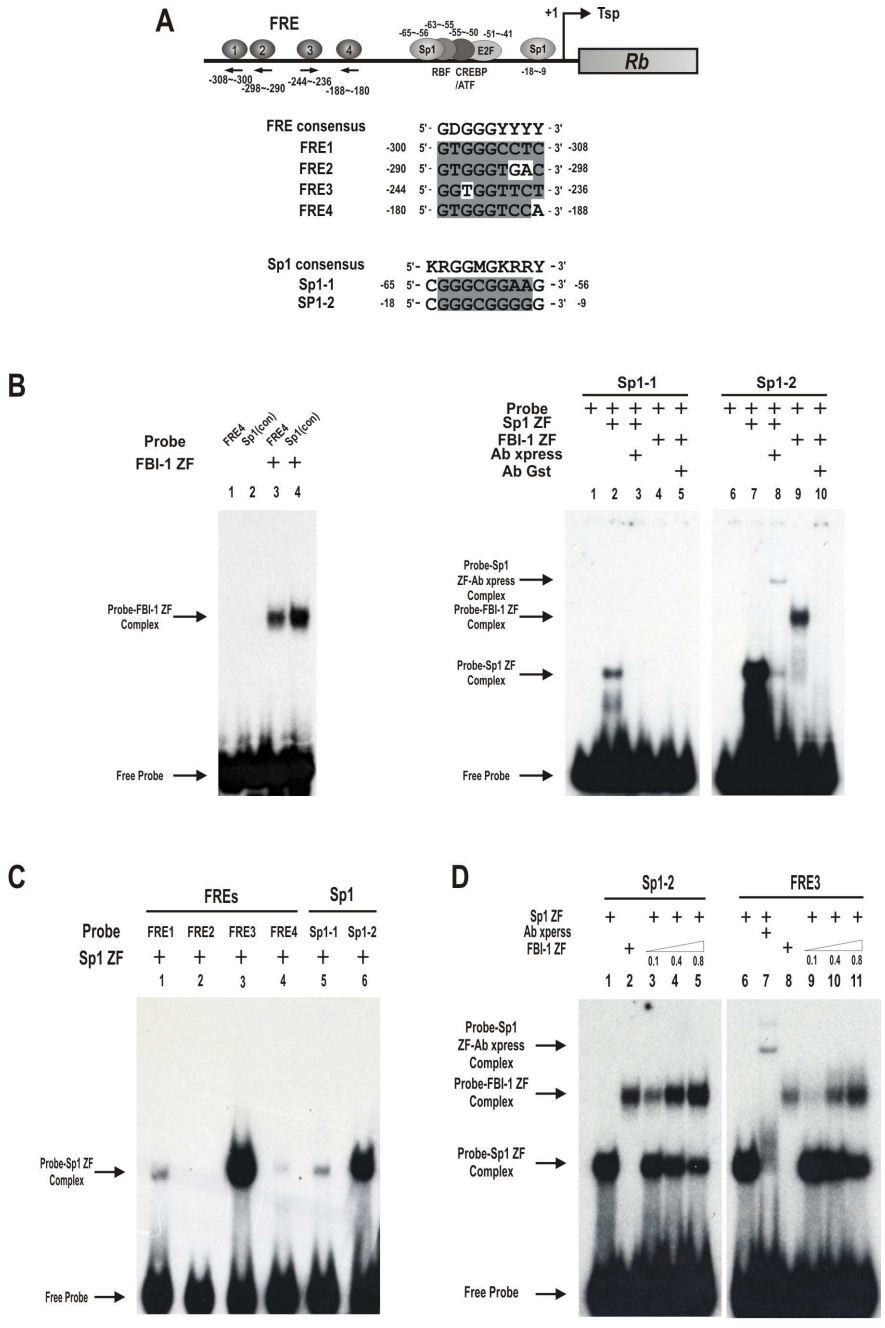
5. FBI-1 and Sp1 compete for some of the FREs and Sp1 binding GC-box

Although the binding of FBI-1 to the FREs and recruitment of corepressor-HDAC complex can explain the transcription repression of *Rb* promoter by FBI-1, we investigated alternative possibility of transcription repression by FBI-1. This is based on the our previous finding that FBI-1 and Sp1 both bind to the GC-rich sequence (consensus sequence of FBI-1, 5'-GDGGGYYYY-3'; consensus sequence of Sp1, 5'-KRGGMGKRRY-3') and some of the binding sites can be bound both by FBI-1 and Sp1.[37, 40-43] Firstly, we investigated whether FBI-1ZFDBD could bind to the typical Sp1 consensus probe (5'-GATCATTCGATCGGGGCGGGGCGAGC-3') by EMSA. FBI-1ZFDBD could bind to the typical Sp1 consensus probe quite well (Fig. 6B). Based on this finding, we carried out EMSA to see whether two GC-boxes of *Rb* promoter can be bound by FBI-1ZFDBD. FBI-1ZFDBD does not bind to GC-Box 1, while the some FBI-1ZFDBD binds to GC-box 2 quite well, indicating that FBI-1 can indeed bind to some of the GC-boxes but not all (Fig. 6B). Particularly, FBI-1 binds well to Sp1-2 site and Sp1 binds partially well to FRE3. Therefore, binding competition between Sp1 and FBI-1 can be important in the transcription repression of a gene with FBI-1 binding GC-box in their promoters.

Also we investigated whether Sp1 could bind to some of the FREs. Sp1 indeed bound to three FRE1, 3, 4 elements at varying degree of binding affinity, but it did not bind to the FRE2 (Fig. 6C). FRE2 probe is the one to which FBI-1 binds most strongly (Fig. 5B, lane 3). Accordingly, FRE2 is the *cis*-regulatory element that is bound only by FBI-1. Recruitment of corepressor-HDAC complex via the POZ-domain of FBI-1 bound onto FRE2 may be important in transcription repression of *Rb* gene. The three FREs (1, 3, and 4) and GC-box2 are the regulatory elements to which FBI-1 and Sp1 bind. And competition between FBI-1 and Sp1 may determine the level of transcription particularly on the FRE3 and Sp1-2 sites. Once FBI-1 occupies the sites, it may further repress transcription by above mentioned corepressor mechanism.

Above data showed that Sp1 binds to FRE3 most strongly and the binding was far stronger than to the two GC-Boxes. Therefore, we did binding competition assays for the two elements (FRE3 and Sp1-2). Sp1ZFDBD (100 ng) was added to the probes in the presence of increasing amount of FBI-1ZFDBD (100 ng, 400 ng, and 800 ng). Increasing amount of FBI-1 decreased the FRE3 probe-Sp1 or Sp1-2 probe-Sp1 interaction, clearly demonstrating the competition between FBI-1 and Sp1 toward the probes (Fig. 6D).

We found that, on the *Rb* promoter, proto-oncogene FBI-1 (or Pokemon) could repress transcription by competing with Sp1 for FRE3 and GC-box 2. This mechanism of repression is radically different from the previously proposed mechanism that FBI-1 POZ-domain interacts with Sp1ZFDBD and prevents Sp1 from binding to GC-box target sequence.[19] Our new finding is an important addition in our understanding on the role of POZ-domain in the oncogenic development involving tumor suppressor *Rb* gene.


A
FRE
+1
Tsp
Sp1
E2F
Sp1
Rb
RBF CREBP /ATF
-308~-300
-298~-290
-244~-236
-188~-180
-65~-56
-63~-55
-55~-50
-51~-41
-18~-9
FRE consensus 5'- GDGGGYYYY - 3'
FRE1 -300 5'- GTGGGCCTC - 3' -308
FRE2 -290 5'- GTGGGTGAC - 3' -298
FRE3 -244 5'- GGTGGTTCT - 3' -236
FRE4 -180 5'- GTGGGTCCA - 3' -188
Sp1 consensus 5'- KRGGMGKRRY - 3'
Sp1-1 -65 5'- CGGGCGGAAG - 3' -56
SP1-2 -18 5'- CGGGCGGGGG - 3' -9
B
Probe
FBI-1 ZF
FRE4
Sp1(con)
FRE4
Sp1(con)
1 2 3 4
Probe-FBI-1 ZF Complex
Free Probe
Sp1-1
Sp1-2
Probe
Sp1 ZF
FBI-1 ZF
Ab xpress
Ab Gst
1 2 3 4 5
6 7 8 9 10
Probe-Sp1 ZF-Ab xpress Complex
Probe-FBI-1 ZF Complex
Probe-Sp1 ZF Complex
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FREs
Sp1
Probe
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Probe-Sp1 ZF Complex
Free Probe
D
Sp1-2
FRE3
Sp1 ZF
Ab xperss
FBI-1 ZF
0.1 0.4 0.8
1 2 3 4 5
6 7 8 9 10 11
Probe-Sp1 ZF-Ab xpress Complex
Probe-FBI-1 ZF Complex
Probe-Sp1 ZF Complex
Free Probe

Figure 6. FBI-1 and Sp1 compete in binding to some of the FRE and GC-box. (A) Schematic diagram of the *Rb* gene promoter and sequence comparison of four potential FREs and two Sp1-binding sites. (B) EMSA. FBI-1 can bind to Sp-1 consensus sequence probe. *Rb*-Sp1 probes were used in EMSA with zinc finger of FBI-1. Only SP1-2 region binds to FBI-1. (C) Sp1 binds quite well to three FREs, Sp1-1 and Sp1-2. Sp1 binds most strongly to FRE3. (D) EMSA. Sp1 and FBI-1 compete with each other in binds to Sp1-2 site and FRE 3.

6. FBI-1 regulates transcription activity of fragments of *Rb* promoter

We have shown that FBI-1 repressed transcription of *Rb* gene. In order to investigate which part of *Rb* gene promoter is the target of FBI-1 repression, we divided the promoter into 3 segments: the FRE cluster located at -318~-170 bp, cluster containing Sp1-1, ATF2, E2F, CREB1 and so on binding sits located at -74~-31 bp, and the Sp1-2 GC-box (-24~-5 bp) located just in front of transcription start site (Fig. 7A). We prepared that *Rb* promoter segment-*Luc* fusion constructs that have 1 copy of FRE cluster, 6 copies of Sp1-1 contain region, and 3 copies of Sp1-2 inserted upstream of *tk** promoter (mentioned at page 14). We cotransfected FBI-1 expression plasmid with the *Rb* fragment-*Luc* gene fusion reporter plasmid into the HeLa cells and analyzed the reporter gene expression. Expression of pGL2-FRE cluster-*tk**-*Luc* was increased by overexpressed FBI-1 compared to the control. However, this increasing may not so significant, because luciferase activity was very low. Expression of pGL2-6x (Sp1-1) -*tk**-*Luc* was repressed by overexpressed FBI-1 by 45 %. We found that FBI-1 cannot bind to the GC-box 1 by EMSA assay (Fig. 6B). The 6 copies of Sp1-1 containing region have many binding sites of other transcription factors such as ATF2, E2F1, CREB1 and so on. So we can guess that FBI-1 may bind to those binding sites or interact with these transcription factors and represses transcription. And expression of pGL2-3x

(Sp1-2) -*tk**-*Luc* was repressed by overexpressed FBI-1 by 70 %. We suggest that overexpressed FBI-1 binds competitively with Sp1 at Sp1-2 region of *Rb* promoter and represses transcription (Fig. 7).

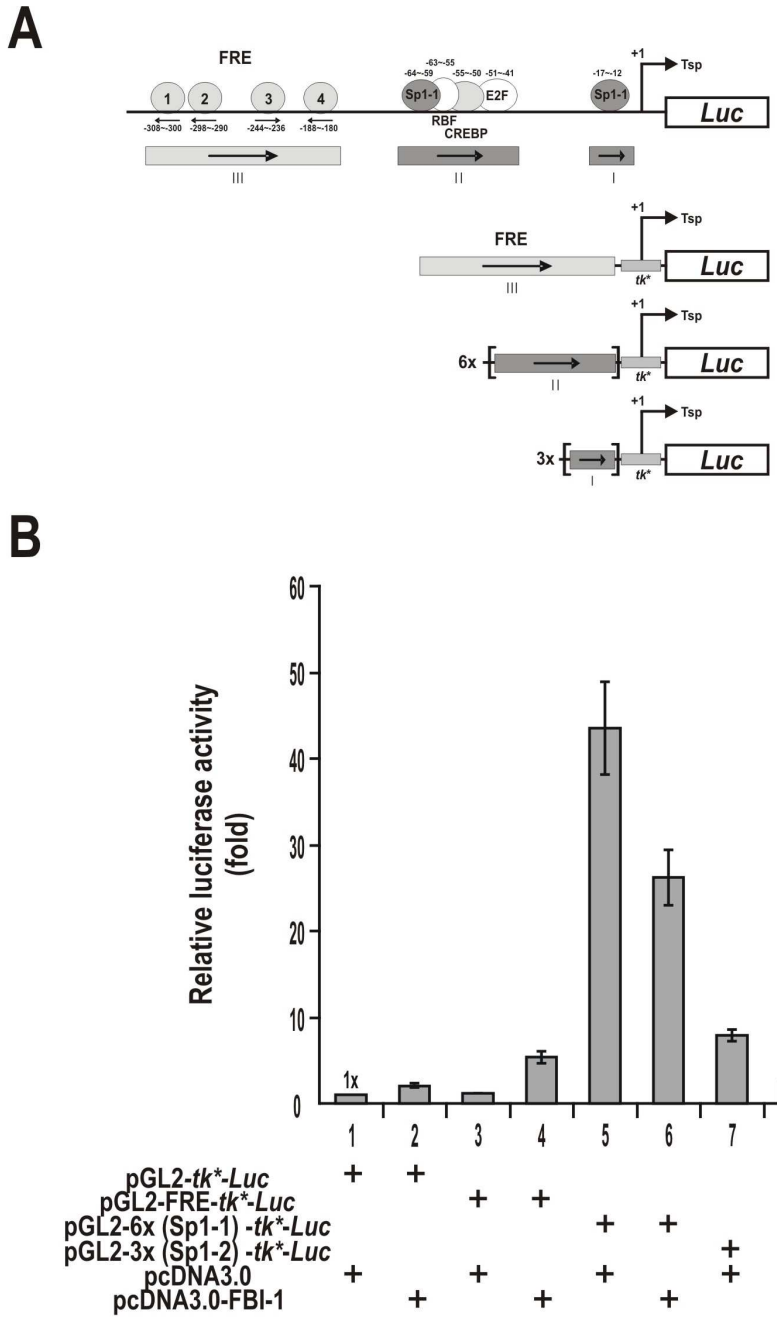

A
FRE
1
2
3
4
-308~-300
-298~-290
-244~-236
-188~-180
-63~-55
-64~-59
-55~-50
-51~-41
Sp1-1
E2F
RBF
CREBP
-17~-12
Sp1-1
+1
Tsp
Luc
III
II
I
FRE
tk*
6x
3x
B
Relative luciferase activity
(fold)
60
50
40
30
20
10
0
1x
1
2
3
4
5
6
7
8
pGL2-tk*-Luc
pGL2-FRE-tk*-Luc
pGL2-6x (Sp1-1) -tk*-Luc
pGL2-3x (Sp1-2) -tk*-Luc
pcDNA3.0
pcDNA3.0-FBI-1

Figure 7. FBI-1 regulates transcription of fragments of *Rb* promoter. (A) Schematic diagram of the *RB* promoter construct and FRE, 6 copies of Sp1-1, and 3 copies of Sp1-2 fragments of *Rb* gene inserted in *tk** promoter. (B) FBI-1 expression plasmid was cotransfected with the *Rb* fragment fused *tk**-*Luc* gene into HeLa cells. Expression of pGL2-FRE cluster-*tk**-*Luc* was increased by overexpressed FBI-1 compared to the control. Expression of pGL2-6x (Sp1-1) -*tk**-*Luc* was repressed by overexpressed FBI-1 by 45 %. And expression of pGL2-3x (Sp1-2) -*tk**-*Luc* was repressed by overexpressed FBI-1 by 70 %.

7. FBI-1 stable cells increase in S phase.

We investigated whether overexpression of FBI-1 in HeLa cells could increase cell number in S phase by promoting cell cycle progression by FACS analysis. Cells in S phase were increased in FBI-1 overexpressing HeLa cells by more than 34% compared to the LacZ stable HeLa cells (Fig. 8). This suggests that more cells in S phase existed because the cell cycle could not be arrest at the check point G1 by repression of *Rb* gene expression by FBI-1.

These data suggest that cell cycle progression by overexpressed of FBI-1 could affect biological processes such as cell differentiation, apoptosis, and tumorigenesis by repression of *Rb* gene.

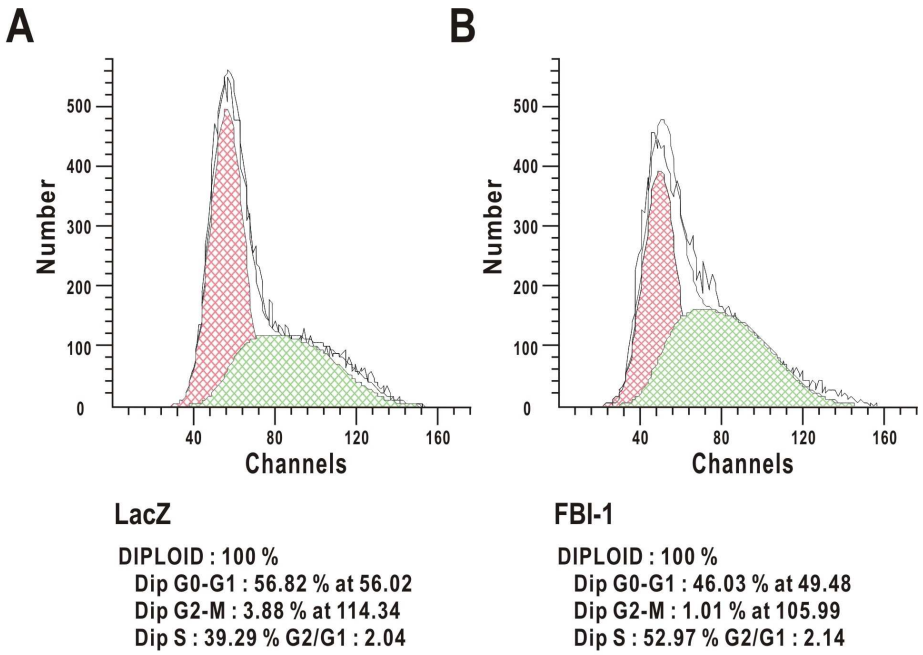


Figure 8. FBI-1 overexpressing HeLa cells increase in S phase. Flow cytometry analysis of cell cycles in LacZ stale HeLa cells (A) and FBI-1 overexpressing HeLa cells (B). FBI-1 overexpressing HeLa cells in S phase were increased 34% more compared to the LacZ stable HeLa cells

IV. Discussion

Recently, over expression of FBI-1 in mice caused cancer in thymus, liver, spleen, and also tumor infiltration into bone marrow. FBI-1 repressed *ARF* gene expression, which resulted in repression of tumor suppressor p53.[37, 38, 48] On the other hands, down-regulation of FBI-1 by knocking out FBI-1 gene caused cellular senescence, apoptosis, and blockage of differentiation. FBI-1 is overexpressed in solid tumors such as colon cancer and bladder cancer in which the normal function of the ARF/p53 pathway is frequently lost.[31, 38] Accordingly, it is likely that FBI-1 has multiple additional target genes by which it can exert its oncogenic activity. These data suggest that FBI-1 would have multiple additional target genes related oncogenesis. We suspected that FBI-1 might regulate genes that have more direct and critical role in oncogenesis such as *Rb* gene.

Rb is a tumor suppressor gene that regulate G1 check point of cell cycle by making a complex with transcriptor E2F.[44-46] Abnormalities in Rb cause Retinoblastoma, small cell lung carcinoma, osteosarcoma, breast carcinoma, head and neck tumors, and mantle cell lymphomas.[1, 2, 49] Most of studies about Rb function have investigated in Rb protein level. Investigations on how transcription of *Rb* gene is regulated are important in understanding the

cellular function of Rb.[10, 14] For this reason, we set out to investigate whether FBI-1 could regulate gene expression of *Rb* at transcription level. Firstly, we studied whether *Rb* gene promoter has potential FBI-1 binding sites by searching for the sequences that show certain degree of sequence homology with the FBI-1 consensus sequence (GDGGGYYYY) using MacVector7.0. Among those sequences, we found four potential FBI-1 binding sites which have more than 80 percentages identity with the FBI-1 consensus sequences and contain the core sequence (-GGG-) that we consider as important 'core'. Transcription analysis showed that FBI-1 repressed *Rb* gene transcription by more than 50% in HeLa cells. Mutant FBI-1 lacking POZ-domain was not able repress to transcription, suggesting that POZ-domain is important in transcription repression (Fig. 2C).

The highly conserved BTB/POZ domain is involved in protein-protein interaction. In particular, the interactions between POZ-domain and corepressors (BCoR, NCoR, mSin3A and SMRT) are important in the biological functions such as transcription repression, immunological function, oncogenesis, and etc carried out by the POZ class regulatory proteins.[30, 32-36] For example, GAGA, transcription regulator of *Drosophila*, facilitates long-range activation by providing a protein bridge that mediates enhancer–promoter communication and thus stimulate transcription by linking an

enhancer to its cognate promoter.[26-28] Strikingly, in addition to facilitating activation by a remote enhancer *in cis*, GAGA was also shown to direct activation of a promoter by an enhancer located on a separate DNA molecule. Enhancer function *in trans* is critically dependent on POZ-domain-mediated GAGA oligomerization, enabling GAGA to bind two DNA molecules (e.g., PRE and promoter) simultaneously.[26-28] PLZF, the most well characterized BTB/POZ-domain proteins, interacts with NCoR and mSin3A. Also, Bcl-6, oncogenic protein important in B-cell lymphoma, binds to BCoR, NCoR and SMRT.[20-24] Accordingly, we investigated whether the POZ-domain of FBI-1 interacts with corepressors such as BCoR, NCoR, mSin3A and SMRT. The POZ-domain of FBI-1 interacts with the corepressors except mSin3A. Interestingly, the POZ-domain of FBI-1 binds most strongly with BCoR, which is followed by SMRT, and NCoR (Fig. 3).

BCoR was identified as a noble Bcl-6-interacting protein. It is expressed ubiquitously in human tissues.[24] BCoR interacts with class Ⅰ (HDAC1 and HDAC3) and class Ⅱ (HDAC4 and HDAC5) HDACs, suggesting that, through BCoR, FBI-1 may recruit HDACs to repress transcription.[47, 51, 52] Therefore, class Ⅰ and class Ⅱ HDACs may be involved in the transcription repression of FBI-1 target genes.

We found that the zinc finger of FBI-1 could directly bind to the promoter of *Rb* gene by EMSA and ChIP assays (Fig. 4). Our mutagenesis of FREs and EMSA demonstrated that GGG of FRE consensus sequence is important in FBI-1 binding to DNA. To investigate *in vivo* function of each FRE, *Rb* promoter-*luc* reporter plasmids with or without mutation at FRE were transiently transfected into HeLa cells. Luciferase activity drived by these mutant *Rb* promoter was increased by 20~130% compared to Wt *Rb* promoter (Fig. 5D). These data suggest that FREs in *Rb* promoter are important in transcription repression by FBI-1.

The consensus sequences of FBI-1 (GDGGGYYYY) and Sp1 (KRGGMGKRRY) are very similar in that they are GC-rich (Fig. 6A). [37, 40-43] We investigated whether Sp1 could bind to any of the 4 FREs of *Rb* promoter. Sp1 can bind to three FREs, except FRE2 (Fig. 6C). The FRE2 site was bound by FBI-1 most strongly (Fig. 3A). Also *Rb* promoter with mutation at FRE2 showed 2.3 fold higher luciferase activity than Wt *Rb* promoter (Fig. 5D). FBI-1 appears to repress transcription of *Rb* gene by binding to FRE2, and recruitment with corepressors such as BCoR, NCoR, and SMRT. Although, the two GC-boxes could be bound by FBI-1, FBI-1 could only bind to GC-box 2 (Fig. 6B). We considered that GC-box 1 region is important in the transcription of *Rb* gene since the mutation in GC-box 1 significantly

decreased transcription compared to Wt *Rb* promoter (Fig.5B). However, FBI-1 cannot bind to the GC-box 1 (Fig. 6B), nevertheless, artificial promoter with 6 copies of Sp1-1 was repressed by ectopic FBI-1 (Fig. 7B). It is also possible that FBI-1 may also bind to the sites occupied by other transcription factors such as ATF2, E2F1, and CREB1, or interact with these transcription factors, to repress *Rb* gene expression.

The transcription of *Rb* gene may be regulated by binding competition between FBI-1 and Sp1 at FRE1, FRE3, FRE4, and GC-box 2 sites which were shown to bind Sp1 and FBI-1. FRE3 and GC-box 2 is the site where Sp1 binds quite strongly. So, we investigated whether FBI-1 could compete with Sp1 at FRE3 and GC-box 2 sites by EMSA assay and found that FBI-1 and Sp1 bind competitively to FRE3 and GC-box 2 (Fig. 6D). Our data suggest that competitive binding between Sp1 and FBI-1 onto FRE3 and GC-box 2, and recruitment of corepressors by FBI-1 may be a novel mechanism of transcription regulation of *Rb* gene by FBI-1.

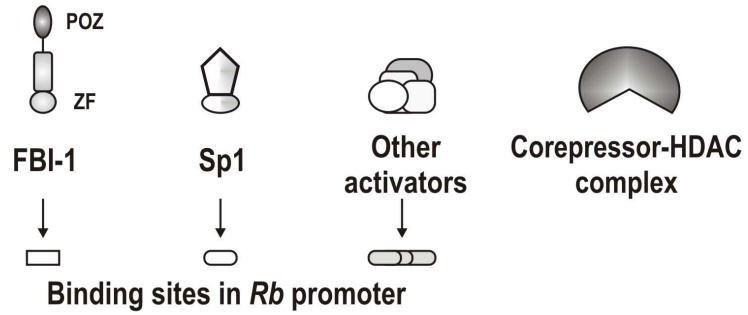
Base on our findings, we came up with the following mechanisms of transcriptional regulation of *Rb* gene by FBI-1. In case when FBI-1 is highly expressed as in cancer, FBI-1 could repress transcription by two mechanisms. Firstly, FBI-1 binds to FRE3 and Sp1-2 by competing with Sp1 and significantly lower transcription level. FBI-1 can also bind to FRE2 which

does not involved binding competition with Sp1. Once anchored to the FRE2, FRE3, and Sp1-2, the POZ-domain of FBI-1 could recruit HDAC-corepressors complexes and caused histone deacetylation, which repress transcription. Secondary, the POZ-domain of FBI-1 bound at FREs cluster located at bp -308 to -180 could exclude Sp1 binding at FRE3 by the closing of the site by POZ-domain-POZ-domain interaction bound at FRE1, 2 and 4. Also FBI-1 binding to GC-box 2 can be significant in make a loop structure of the *Rb* promoter via POZ-domain-POZ domain interaction that involves FBI-1 on the Sp1-2 and any of the FBI-1 bound at the FRE cluster located further upstream. The formation of this loop structure may effectively exclude the transcription activators such as Sp1, ATF2, CREB1, and E2F1 bound at the region bp -65 to -41 which is locate in between FRE clusters and Sp1-2 site. This exclusion can be important in the transcription repression of *Rb* gene by FBI-1 in the situation where FBI-1 expression is elevated.

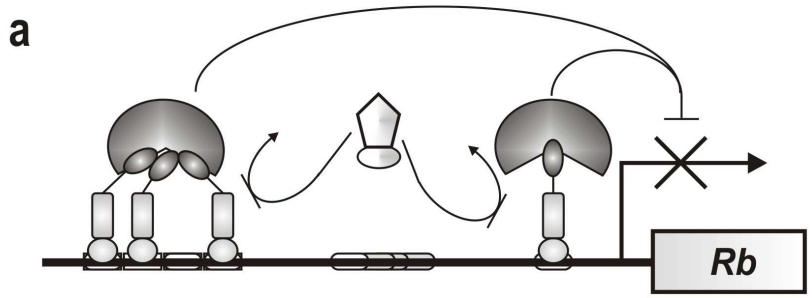
It is also intriguing to imagine how *Rb* gene might be regulated when FBI-1 expression level is sufficiently low, as in normal cells. When FBI-1 expression is low, it cannot compete with Sp1 to FRE3 and Sp1-2. And the FREs at the FRE cluster and Sp1-2 is not likely to be occupied by FBI-1. Accordingly, Sp1 binding FRE3 is likely opened for Sp1 binding and Sp1-2 is also more likely occupied by Sp1. In this case no looping is possible by the

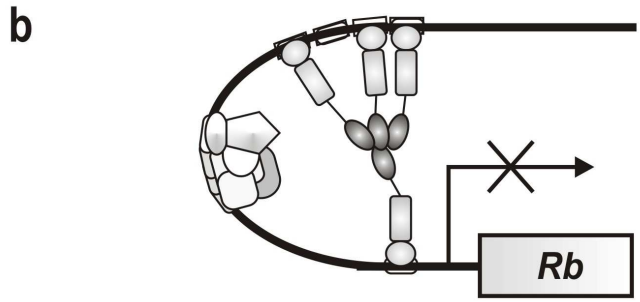
POZ-domain-POZ-domain interaction. With Sp1 bound at the FRE3, Sp1-2, and at the region bp -65 to -41, *Rb* gene is likely activated by Sp1 and maintain certain cellular level of transcription corresponding to the Sp1 expression level. Also, the transcription activators such as Sp1, ATF2, CREB1, and E2F1 bound at the region bp -65 to -41, may fully exert their transcription activation potential. FBI-1 is not able to exclude Sp1 binding at FRE3, and to make a loop structure of *Rb* promoter. Although the mechanism appears plausible, we need some more investigation to supporting current mechanism (Fig. 9).

Regulation of *Rb* gene expression can affect cellular functions.[53, 54] For example, MyoD and CREB regulates *Rb* promoter and the regulation is important in muscle cell differentiation.[13] Induction of *Rb* gene transcription by MyoD, via the CREB, is a key event in the process of muscle differentiation. Also, GABP and HCF-1 bind to the *Rb* promoter and activates transcription and induces muscle differentiation.[14, 15] In contrast, MIZF and YY1 were shown to repress transcription of *Rb* gene and inhibit muscle formation.[15-17] We investigated whether FBI-1 regulates any cellular functions by repressing *Rb* gene expression. One of the primary functions of Rb is inhibition of cell cycle progression.[45, 46, 50] Accordingly we investigated whether RB repression by FBI-1 has any effect on the cell cycle progression.



High FBI-1 level





Low FBI-1 level

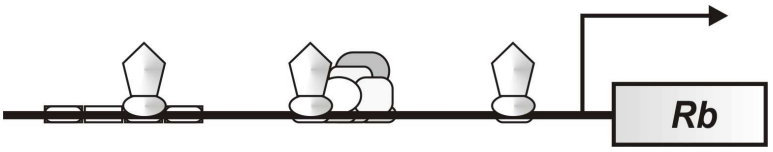


Figure 9. Hypothetical mechanisms of transcriptional regulation of *Rb* gene by FBI-1. Top part of this figure is schematics of FBI-1, Sp1, other activator such as Sp1, ATF2, CREB1, and E2F1, and corepressor-HDAC complex. Square box means FBI-1 binding site, FRE, elliptical box means Sp1 binding site, GC-box, and light gray elliptical box means another activator binding sites. In high expression of FBI-1: Firstly, FBI-1 binds to FREs and GC-box 2 on *Rb* promoter by competing with Sp1. After FBI-1 binding, chromatin modification by corepressor-HDAC complex recruited by the POZ-domain of FBI-1 may repress transcription of *Rb* gene (a). Secondary, *Rb* promoter is formed a loop structure by POZ-domain-POZ-domain interaction of FBI-1 bound at FREs cluster and GC-box 2 region. This loop structure may exclude the transcription activators such as Sp1, ATF2, CREB1, and E2F1. So *Rb* expression is down regulated (b). In low expression of FBI-1: In this case no looping is possible by the POZ-domain-POZ-domain interaction. Not only Sp1 but also another activator could bind to *Rb* promoter and activate transcription of *Rb* gene. Interestingly, Sp1 also could bind to three FREs, especially FRE3.

We prepared stable HeLa cells over expressing FBI-1 and LacZ gene and analyzed the cell cycle progression by FACS. Overexpressed FBI-1 increased number of cells in S phase by more than 34% compared to LacZ stable HeLa cells (Fig. 7). Rb forms complexes with proteins of E2F family and inhibits transcription by recruiting proteins involved in transcriptional repression.[3, 6, 55] And when Rb level is low caused by transcription repression by FBI-1, Rb no longer efficiently form complexes with E2Fs. The free E2F proteins, by forming complex with DP proteins, then activates expression of a number of genes that are likely to regulate or promote entry into S phase, including DNA polymerase α, thymidylate synthase, ribonucleotide reductase, cyclin E, and dihydrofolate reductase.[3] We suggest that FBI-1 functions are important to oncogenesis by promoting cell cycle progression through transcription repression of *Rb* gene expression.

Overall, we were able to demonstrate the noble mechanism of FBI-1 as a protoncogene regulating *Rb* gene expression. FBI-1 represses transcription of *Rb* gene by binding mainly onto FRE2, and also onto FRE3 and Sp1-2 site of *Rb* promoter by competition Sp1. And histone modification through recruited corepressor-HDAC complex formed on the POZ-domain of FBI-1 anchored onto FRE2, 3, and Sp1-2 site may be important in sustained transcription repression of *Rb* gene by FBI-1. The repression of *Rb* gene expression could

change the cellular function controlled by Rb in the cell cycle progression control and FBI-1 increased cell proliferation.

V. Conclusion

1. We investigated whether *Rb* is the target gene affected by FBI-1, during oncogenesis.
2. *Rb* proximal promoter (~-370 bp) has four FBI-1 binding sites (FRE; bp -308 to -300, bp -298 to -290, bp -244 to -236, and bp -188 to -180).
3. FBI-1 represses transcription of *Rb* gene by direct binding to FREs and GC-box 2 We confirmed binding of FBI-1 *in vitro* and *in vivo* by EMSA and ChIP assays, respectively.
4. The POZ-domain of FBI-1 interacts with the corepressors such as BCoR, NCoR, and SMRT, and the interaction may play important role in transcription repression of *Rb* gene through histone modification.
5. FBI-1 competes with Sp1 in binding to three FREs, especially FRE3, and GC-box 2 sites.
6. Overexpressed FBI-1 promotes cell cycle progression and thus increases cell numbers in S phase.
7. We found that *Rb* gene is targeted by proto-oncogene FBI-1.

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국문요약

Rb 유전자의 전사조절을 통한 FBI-1의 Rb 기능 조절

지도교수 **허만욱**

연세대학교 대학원 의과학과

유정윤

FBI-1 (Factor that Binds to the Inducer of short transcripts of human immunodeficiency virus-1) 은 N-말단에 POZ-domain과 C-말단에 4개의 Krüppel-like zinc finger를 갖는 단백질으로 여러 유전자들의 promoter에 작용하여 전사작용인자로써 지방조직의 분화, 발암과정에 있어서 중요한 역할을 한다. 최근 연구에 의해 FBI-1은 발암과정에 관여되고 있으며, 여러 암세포에서 FBI-1의 발현이 증가되어있다고 보고되었다. 우리는 FBI-1이 종양 억제 유전자 중의 하나인 *Retinoblastoma* (*Rb*) 유전자의 전사에 어떠한 영향을 주는지

알아보았다. Rb 단백질은 세포 주기의 조절, 세포사멸, 세포 분화 그리고 종양 억제에 중요한 역할을 하는 단백질이다. 우리는 FBI-1의 결합가능 서열로 도출된 서열 GDGGGYYYY 서열을 이용하여 *Rb* promoter에 여러 FBI-1 결합 부위 (FRE) 가 존재하고, 이를 통하여 FBI-1이 *Rb* 유전자의 전사를 조절할 수 있을 것으로 추측하였다. EMSA를 통해 4군데의 결합 가능 부위 (bp -308 to -300, bp -298 to -290, bp -244 to -236, bp -188 to -180) 를 발견하였다. 우리는 FBI-1이 *Rb* promoter에 직접적으로 결합함으로써, *Rb* 유전자의 전사를 억제시킨다는 것을 확인하였다. *Rb* 유전자는 Sp1, E4TF1, Fli-1, p53, ATF, E2F, CREB1, CDF-1와 같은 전사인자들에 의해 발현이 조절된다. *Rb* 유전자의 FRE 서열과 Sp1 결합 서열이 GC가 많이 존재한다는 점에서 유사하다. 우리는 EMSA를 통해 FRE1, FRE3, FRE4와 GC-box 2 지역이 FBI-1과 Sp1이 모두 결합이 가능하고, FRE3과 GC-box 2 부위에서 FBI-1이 Sp1과 경쟁적으로 결합할 수 있음을 발견하였다. FBI-1과 Sp1과의 경쟁적인 결합이 *Rb* 유전자의 전사를 억제하는 중요한 기전이라 생각된다. 또한, POZ-도메인 부위는 단백질-단백질 상호작용 기능을 가지는데, FBI-1의 POZ 부위가 BCoR, NCoR, SMRT와 결합함을 확인하였고, 이것이 *Rb* 유전자의 전사를 억제시키는데 중요하게 작용한다고 여겨진다.

FBI-1은 *Rb*의 발현을 억제함으로써 Rb가 수행하고 있는

세포기능에 영향을 줄 것이라 생각되어, FBI-1이 과발현된 HeLA 세포를 제조하고, FACS로 세포주기를 조사한 결과, DNA 합성단계 (S phase) 의 HeLA 세포수가 증가됨을 발견하였다.

결론적으로 FBI-1은 *Rb* 유전자의 발현을 조절하고, 이에 따라 세포 주기 진행을 촉진함으로써 발암과정에 있어서 중요한 역할을 하는 단백질이라 생각한다.

핵심되는 말 : Rb, *Rb* promoter, FBI-1, Pokemon, 전사 억제, Sp1, BCoR, NCoR, SMRT, 세포주기, 발암과정