Eukaryotic Translation Initiator Protein 1A Isoform, CCS-3, Enhances the Transcriptional Repression of *p21CIP1* by Proto-oncogene FBI-1 (Pokemon/ZBTB7A)

Won-Il Choi^{1*}, Youngsoo Kim^{2*}, Yuri Kim¹, Mi-young Yu¹, Jungeun Park², Choong-Eun Lee³, Bu-Nam Jeon¹, Dong-In Koh¹ and Man-Wook Hur¹

¹Department of Biochemistry and Molecular Biology, Brain Korea 21 Project for Medical Science, Institute of Genetic Science, Yonsei University School of Medicine, Seoul, ²Cancer Research Institute and College of Medicine, Seoul National University, Seoul, ³Department of Biological Science, Sungkyunkwan University, Suwon, *The first two authors contributed equally

Key Words

eEF1A • CCS-3 • Transcription • Repression • FBI-1 • p21 • Pokemon • POZ domain • Co-repressors

Abstract

FBI-1, a member of the POK (POZ and Krüppel) family of transcription factors, plays a role in differentiation, oncogenesis, and adipogenesis. eEF1A is a eukaryotic translation elongation factor involved in several cellular processes including embryogenesis, oncogenic transformation, cell proliferation, and cytoskeletal organization. CCS-3, a potential cervical cancer suppressor, is an isoform of eEF1A. We found that eEF1A forms a complex with FBI-1 by co-immunoprecipitation, SDS-PAGE, and MALDI-TOF Mass analysis of the immunoprecipitate. GST fusion protein pull-downs showed that FBI-1 directly interacts with eEF1A and CCS-3 via the zinc finger and POZ-domain of FBI-1. FBI-1 co-localizes with either eEF1A or CCS-3 at the nuclear periplasm. CCS-3 enhances transcriptional repression of the *p21CIP1* gene (hereafter referred to as *p21*) by FBI-1. The POZ-domain of FBI-1 interacts with the co-repressors, SMRT and BCoR. We found that CCS-3 also interacts

with the co-repressors independently. The molecular interaction between the co-repressors and CCS-3 at the POZ-domain of FBI-1 appears to enhance FBI-1 mediated transcriptional repression. Our data suggest that CCS-3 may be important in cell differentiation, tumorigenesis, and oncogenesis by interacting with the proto-oncogene FBI-1 and transcriptional co-repressors.

Copyright © 2009 S. Karger AG, Basel

Introduction

FBI-1 (also called Pokemon or ZBTB7A) is a transcriptional repressor member of the BTB/POZ domain and Krüppel-like zinc finger family [1-2]. Originally, FBI-1 was isolated as a cellular factor that binds to the inducer of short transcripts of the human immunodeficiency virus-1 and the proximal GC-rich sequence of *ADH5/FDH* [3, 4]. Molecular cloning revealed that FBI-1 is a transcription factor with a POZ domain at the N-terminus and Krüppel-type four C₂H₂

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2009 S. Karger AG, Basel
1015-8987/09/0236-0359\$26.00/0

Accessible online at:
www.karger.com/cpb

Man-Wook Hur, Ph.D.
Department of Biochemistry and Molecular Biology,
Yonsei University School of Medicine,
134, ShinChon-Dong, SeoDaeMoon-Ku, Seoul (Korea)
Tel. +82-2-2228-1678, Fax +82-2-312-5041, E-Mail mwhur2@yuhs.ac

zinc fingers at the C-terminus [4, 5]. Western blot analysis showed that FBI-1 is a 75 kDa protein. FBI-1 has a variety of biological functions such as HIV-1 Tat trans-activation, enhancement of NF- κ B activity, adipogenesis, osteoclastogenesis, B-cell lymphoma, and repression of *ADH5/FDH* gene transcription [4, 6-10]. SAGE (serial analysis of gene expression) analysis shows that FBI-1 expression is increased in multiple cancers (available at www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Hs&CID=465623). Recent reports show that FBI-1 causes cancer in the thymus, liver, and spleen by repressing the tumor suppressor gene *ARF*, which in turn lowers the expression of another tumor suppressor gene, *p53* [11]. Also, LRF (the mouse homolog of FBI-1) regulates B-versus T-lymphoid lineage fate decision [12].

Eukaryotic translation elongation factor 1A (eEF1A) is a 50 kDa monomer that carries the aminoacyl-tRNA to the A-site of the ribosome during translation [13 and references therein]. eEF1A also plays a role in several other cellular processes including embryogenesis, senescence, oncogenic transformation, cell proliferation, apoptosis, and cytoskeletal organization [14-24]. Though mainly present in the cytoplasm [25 and references therein], a small amount of eEF1A has been previously located in the nucleus [26-31]. Even though eEF1A binds viral RNA and RNA polymerase [32-34], the involvement of eEF1A in nuclear processes largely remains to be established. CCS-3 protein (cervical cancer suppressor 3) was isolated through a yeast two-hybrid screen as a protein that interacts with the BTB/POZ-domain family transcriptional repressor, PLZF (promyelocytic leukemia zinc finger protein), and is proposed to be an isoform of eEF1A [35]. CCS-3 interacts directly with PLZF. CCS-3 acts as a transcriptional repressor of a PLZF responsive reporter construct and enhances transcriptional repression by PLZF. It has been suggested, though not proven, that CCS-3 interacts with transcriptional co-repressors [35]. These data suggest that the molecular interaction between CCS-3 and PLZF might be important in the biological functions of PLZF [35].

This study investigates biological functions carried out by the proto-oncogene FBI-1. Recently, we and others found that FBI-1/LRF potently represses the ARF-Mdm2-p53-p21 pathway, which is a major cell cycle control pathway [11, 36, 37]. FBI-1 might carry out key regulatory functions, such as cell cycle control, by interacting with other regulatory proteins. To understand the biological function of FBI-1, we isolated a nuclear protein complex by immunoprecipitation using an antibody specific to FBI-1. MALDI-TOF mass analysis showed that eEF1A, a

eukaryotic translation elongation factor, interacts with FBI-1. This study investigated and showed that CCS-3, an eEF1A isoform, enhances transcriptional repression by FBI-1 by interacting with both co-repressor proteins and the FBI-1 POZ-domain. These interactions may affect oncogenic transformation and cell proliferation by potently repressing *p21* gene transcription.

Materials and Methods

Plasmids, antibodies, and reagents

pGL2-p21-Luc-Wt (-2.3 kb) and pGL2-p21-Luc Wt (-133 bp) plasmids were kindly provided by Dr. Yoshihiro Sowa of the Kyoto Perpetual University of Medicine (Kyoto, Japan). pcDNA3-FBI-1, pcDNA3-eEF1A, and pcDNA3.1-CCS-3 plasmids were prepared by cloning the respective cDNA fragments into the pcDNA3 or pcDNA3.1 vector (Invitrogen, CA, USA). To prepare recombinant GST-fusion proteins, cDNA fragments encoding POZFBI-1 (a.a. 1-130) and ZFDBDFBI-1 (a.a. 382-490) were cloned into pGEX4T1 or pGEX4T3 (Amersham Biosciences, NJ, USA). The mammalian expression plasmids for Gal4-POZFBI-1 (a.a. 1-130) and Gal4-CCS-3 (a.a. 1-361) fusion proteins were constructed by subcloning amplified cDNA fragments into a pCMX-Gal4 fusion expression plasmid. The expression plasmids for VP16-corepressors, NCoR (a.a. 1709-2215), SMRT (a.a. 194-657), BCoR (a.a. 112-753), and mSin3A fusion proteins (pKH73/110EF-NCoR, pKH135EF-BCoR) were kindly provided by Drs. Ronald Evans (The Salk Institute, CA, USA), Vivian Bardwell (University of Minnesota, MN, USA), and Dominique Leprince (Institut Pasteur de Lille, France) [38]. The pTREx-FBI-1 plasmid was cloned into pcDNA5.0/FRT/TO[®] (Invitrogen, CA, USA) to prepare a stable cell line with doxycycline inducible FBI-1 expression. All plasmid constructs were verified by sequencing.

Antibodies against GAPDH, eEF1A, His-Tag, and Flag-Tag were purchased from Upstate (Charlottesville, VA, USA), Chemicon (Temecula, CA, USA), Calbiochem (San Diego, CA, USA), and SantaCruz (SantaCruz, CA, USA), respectively. Most chemical reagents were purchased from Sigma (St. Louis, MO, USA).

Cell culture and stable cell lines

HeLa, HEK293A, and CV-1 cells were cultured in Dulbecco's modified eagle medium (DMEM, Gibco-BRL, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, MD, USA).

Doxycycline inducible HEK293TREx-FBI-1 stable cells were prepared by transfecting mammalian Flp-In[™] T-REx[™] into HEK293T cells (Invitrogen, CA, USA) with a 9:1 ratio of pOG44: pcDNA5/FRT/TO[®]-FBI-1 plasmid using Lipofectamine 2000 (Invitrogen, CA, USA). Flp-In[™] T-REx[™] FBI-1 stable cells were selected by culturing the transfected cells in a medium containing hygromycin (300/ml) and blasticidin (15/ml). To induce FBI-1 expression, stable cells were cultured in medium containing 1/ml doxycycline for the intended time period.

Preparation of nuclear extracts

Nuclear extracts were prepared according to the method by Dignam et al. [39]. HEK293TREx-FBI-1 stable cells were harvested by centrifugation and suspended in two packed cell pellet volumes of buffer A [10 mM HEPES (pH 7.9 at 4°C), 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM DTT] and lysed by ten strokes in a glass Dounce homogenizer. The homogenate was centrifuged for 10 min at 2,000 rpm to collect the nuclear pellet. This pellet was subjected to a second centrifugation for 20 min at 25,000x g to remove residual cytoplasmic material. The resulting pellet was designated as crude nuclei. The nuclei were resuspended in buffer C [20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM DTT] with a glass Dounce homogenizer (tight type, Wheaton, NJ, USA). The resulting suspension was stirred gently with a magnetic stir bar for 30 min and centrifuged for 30 min at 25,000x g. The resulting clear supernatant was dialyzed against 50 volumes of buffer D [20 mM HEPES pH 7.9, 20% (v/v) glycerol, 0.15 M NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM DTT] for five hours. The dialysate was centrifuged at 25,000x g for 20 min, and the resulting precipitate discarded. In contrast, the supernatant, designated as the nuclear extract, was frozen as aliquots in liquid nitrogen and stored at -80°C.

Isolation of the nuclear protein complex containing FBI-1

To isolate the nuclear proteins containing FBI-1 from HEK293TREx-FBI-1 stable cells, nuclear extract was pre-cleared with anti-mouse IgG-agarose (Sigma, MO, USA) for 2 hrs and incubated with anti-Flag M2-agarose (Sigma, MO, USA) overnight at 4°C. The anti-Flag M2-agarose resin was washed first with wash buffer (20 mM HEPES pH 7.5, 1 mM EDTA, 10% glycerol, 200 mM KCl, 0.2% NP-40) and then TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl). The bound proteins were then eluted with 3x Flag Peptide (Sigma, MO, USA).

MALDI-TOF mass spectrometry

Gel spots were excised, destained by reduction using 30 mM potassium ferricyanide/100 mM sodium thiosulfate, and washed with water. The gel pieces were then incubated with 0.2 M NH₄HCO₃ for 20 min, dehydrated, shrunk twice with 100% acetonitrile, and dried by vacuum centrifugation. For "in gel" digestion with trypsin, gel pieces were rehydrated in digestion buffer containing 0.05 M NH₄HCO₃ and 10 ng/μl of modified porcine trypsin (Promega, Madison, USA) at 4°C for 30-45 min. Then the excess supernatant was removed and the gel pieces were covered with 30 μl of 0.05M NH₄HCO₃ buffer. Digestion was performed overnight at 37°C, and after the 'in-gel' tryptic digestion, tryptic peptides were extracted from the gel particles. Samples were desalted using a GELoader tip (Eppendorf AG, Germany) packed with POROS 20 R2 resin (Applied Biosystems Inc., CA, USA). Peptide binding and washing were carried out in 0.1% trifluoroacetic acid (TFA) in water. To produce the MALDI sample matrix, α-cyano-4-hydroxy cinnamic acid was dissolved in a solution containing 70% acetonitrile and 0.1% TFA at 5 g/l. The elution was performed with 1 μl of sample

matrix, and the eluted peptides were directly spotted on the target plate. Protein identification was carried out using peptide mass fingerprinting (PMF) using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager DE-PRO MALDI-TOF mass spectrometer, Applied Biosystems Inc., CA, USA). Mass spectra were registered in reflectron positive ion mode. The mass accuracy was set at 50 ppm for PMF analysis. Database searches for PMF were performed using the MASCOT search program, developed by Matrix Science Ltd. (access is available on <http://www.matrix.science.com>), at the NCBI database (<http://www.ncbi.nlm.nih.gov/entrez>) using the ExpASY Molecular Biology Server at the SWISSPROT database (<http://www.expasy.org>).

Immunoprecipitation and Western blot analysis

Stable HEK293TREx cells with a doxycycline inducible FBI-1-Flag expression system were washed, pelleted, and resuspended in lysis buffer supplemented with protease inhibitors (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100). Cell lysates were pre-cleared and the supernatant was incubated with anti-Flag M2 antibody (SantaCruz Biotechnology, CA, USA) on a rotating platform overnight at 4°C followed by incubation with protein G-Sepharose Fast Flow beads. The beads were collected, washed, and resuspended in an equal volume of 5x SDS loading buffer. The immunoprecipitated proteins were separated by 12% SDS-PAGE and transferred onto a Immobilon-P™ PVDF Membrane (Bio-Rad, CA, USA). The membranes were blocked with 5% skim milk (BD Biosciences, MD, USA) and then blotted by incubation with Flag-tag, GAPDH, eEF1A antibodies. The membranes were then incubated with either HRP conjugated mouse or rabbit IgG. Protein bands were visualized with ECL solution (PerkinElmer, CA, USA).

To investigate protein-protein interaction between the CCS-3 and FBI-1, HEK293A cells were transfected with the pcDNA3.1-Myc-CCS-3 mammalian expression vector. The cell lysate was immunoprecipitated with anti-Myc tag antibody. The precipitate was separated by SDS-PAGE and analyzed by Western blot using anti FBI-1 antibody (Abcam, Cambridge, UK).

GST fusion protein purification, in vitro transcription and translation of co-repressors and pull-down assays

GST, GST-POZFBI-1, or GST-ZFDBDFBI-1 proteins were prepared from *E. coli* BL21 (DE3) transformed with the GST, GST-POZFBI-1, or GST-ZFDBDFBI-1 protein expression plasmids. Protein expression was induced by treating the transformed *E. coli* with 0.5 mM isopropyl-1-thio-D-galactopyranoside (IPTG) for 4 hrs at 37°C. The bacterial pellets were lysed with lysis buffer containing 1x PBS, 1 mM PMSF, 2 mM EDTA, and 0.2 mg/ml lysozyme, then sonicated 3-5 times at a 0.5 cycle with 50% amplitude (Dr. Hielscher GmbH, Germany). The recombinant proteins were purified with glutathione-agarose 4 beads by affinity chromatography (Peptron, Daejeon, Korea). The purified proteins were resolved by 12% SDS-PAGE to quantitate and assess purity. The same aliquot amount of the protein-agarose bead

Fig. 1. eEF1A is a component of the HEK293T cell nuclear protein complex that contains the proto-oncogene FBI-1. (A) Isolation of a nuclear protein complex that interacts with FBI-1 from doxycycline-inducible stable HEK293-TREx cells over-expressing Flag-tagged FBI-1. FBI-1 expression was induced overnight, and the nuclei were separated by glycerol gradient centrifugation. Nuclear lysates were immunoprecipitated using anti-Flag M2-Agarose antibody. The immunoprecipitates were separated by 12% SDS-PAGE and stained with a silver staining solution. The bands were isolated and subjected to MALDI-TOF mass spectrometry analysis. (B) Peptide mass fingerprinting to identify protein by MALDI-TOF mass spectrometry. Eight polypeptides that showed significant homology to eEF1A upon amino acid sequencing are listed. The 50 kDa protein was identified as eEF1A. (C) MALDI mass spectrum of the 50 kDa protein band that was immunoprecipitated by Flag-FBI-1.

complex was used in GST-fusion protein pull down assays. The eEF1A and CCS-3 polypeptides were prepared by incubating 1 µg of pcDNA3.0 eEF1A and CCS-3 expression plasmids with TNT quick-coupled transcription/translation extract (Promega, WI, USA), containing 40 µl of TNT quick master mix and 2 µl of [³⁵S]-Methionine (1175.0 Ci/mol, PerkinElmer Life Sciences, Inc.) at 30°C for 90 min. Polypeptide expression levels were then analyzed by running 1 µl of the total mixture on a 12% SDS-PAGE and analyzing the gel by autoradiography. The purified GST fusion proteins (5 µg) were incubated with GSH-agarose (Sigma, MO, USA) for 1 hr in HEMG buffer (40 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 5 mM MgCl₂, 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, Germany)) at 4°C for 1 hr. After washing the agarose-GST protein complexes three times with 1 ml of cold HEMG buffer, 10 µl of *in vitro* translated eEF1A1 and CCS-3 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, the supernatants removed, and the pellets washed five times with cold HEMG buffer. The bound proteins were separated by 10% SDS-PAGE. Finally, the SDS-PAGE gel was dried and exposed to X-ray film using an image-intensifying screen (Kodak, NY, USA).

Immunocytochemical staining of FBI-1, eEF1A, and CCS-3

For immunostaining, HEK293A cells were grown on coverslips (Sunshine Works, Seoul, Korea) and transfected with pcDNA3.1-FBI-1 and either pcDNA3.0-eEF1A or pcDNA3.1-CCS-3 plasmids. Cells were fixed, permeabilized with 0.2% Triton X-100, blocked with 5% normal horse serum, incubated with mouse anti-Flag primary antibody, washed thoroughly, and then incubated with FITC-conjugated anti-mouse IgG secondary antibody. For double-staining, after washing, the cells were fixed with formaldehyde, blocked, and incubated with a different primary antibody (rabbit anti-His

Fig. 2. FBI-1 directly interacts with eEF1A and CCS-3 via its zinc finger DNA binding domain and the FBI-1 POZ-domain. (A) Structures of FBI-1, GST-POZFBI-1, GST-ZFDBDFBI-1, eEF1A and CCS-3. CCS-3 is an eEF1A isoform that lacks the N-terminus 100 a.a. GTP-binding domain. (B) Co-immunoprecipitation of eEF1A and FBI-1. Nuclear extracts prepared from stable HEK293-TREx-Flag-FBI-1 cells were immunoprecipitated with anti-Flag M2 agarose beads, and the precipitates were analyzed by Western blot using antibodies against eEF1A, GAPDH, and Flag-Tag. GAPDH, control. FBI-1 is a nuclear protein that interacts with eEF1A *in vivo*. (C) Co-immunoprecipitation of CCS-3 and FBI-1. HEK293A cells were transfected with a Myc-CCS-3 expression vector and immunoprecipitated with anti-Myc antibody. The precipitate was analyzed by Western blot using an antibody against FBI-1. (D) GST-fusion protein pull-down assays. GST, GST-POZFBI-1, and GST-ZFDBDFBI-1 were incubated with *in vitro* translated [³⁵S]-Methionine-labeled eEF1A and CCS-3, and pulled-down. The precipitates were resolved by 12% SDS-PAGE, and the gel was exposed to X-ray film. FBI-1 directly interacts with eEF1A or CCS-3.

antibody). Subsequently, the cells were incubated with secondary antibody (anti-rabbit antibody conjugated with Rhodamine) and washed. Finally, the cells were washed with a solution containing 4, 6-diamidino-2-phenylindole and mounted on glass with mounting medium. All samples were examined with a LSM 510 confocal laser-scanning microscope (Carl Zeiss, Germany).

Transcriptional analysis of pGL2-p21-Luc by FBI-1, eEF1A, and CCS-3

Various combinations of pGL2-p21-Luc, pcDNA3.1-FBI-1, pcDNA3.0-eEF1A, pcDNA3.1-CCS-3, and pCMV-LacZ were transiently transfected into HEK293A cells using Lipofectamine Plus reagent (Invitrogen, CA, USA). After 24 hrs of incubation, the transfected cells were harvested and analyzed for luciferase activity. Activity was normalized using co-transfected β -galactosidase activity or protein concentration. Analysis of variance was employed to calculate the p value using statistical analysis program, SPSS (Statistical Package for the Social Sciences) (Chicago, USA).

Mammalian two-hybrid assays

The CV-1 cells were co-transfected with various combinations of pG5-Luc (0.2 μ g), pGal4-POZFBI-1 (0.3 μ g), pGal4-CCS-3 (0.3 μ g), pVP16-corepressors (0.3 μ g), and pCMV-LacZ (0.2 μ g). After 24 hrs of transfection with Lipofectamine Plus, the transfected CV-1 cells were harvested and assayed for luciferase activity. Luciferase activity was normalized using co-transfected β -galactosidase activity or protein concentration.

MTT assay

Human HEK293A cell were plated to ~50% confluence in 6 well culture dishes, transfected with FBI-1 or/and CCS-3 expression vector (1 μ g) using Lipofectamine Plus reagent (Invitrogen, CA, USA). Transfected cells were incubated for 0, 2, 4, 6 days and incubated with 1 ml/well MTT (2 mg/ml). Precipitates were dissolved with 1 ml of dimethylsulfoxide. Cellular proliferation was determined from the conversion of MTT to formazan using a SpectraMAX 250 (Molecular Device Co., Sunnyvale, CA) at 570 nm.

Fig. 3. FBI-1 co-localized with eEF1A and CCS-3 in the nuclear periplasm. (A) Cellular localization of eEF1A and FBI-1. Arrow indicates the direction of expression scanning of eEF1A (red) and FBI-1 (green). (B) Cellular localization of CCS-3 and FBI-1. Arrow indicates the direction of expression scanning of CCS-3 (red) and FBI-1 (green). Expression vectors for Flag-FBI-1 (green) and His-tagged eEF1A or CCS-3 (red) were co-transfected into HEK293A cells. The cells were fixed, hybridized with anti-Flag and anti-His antibodies, and further incubated with secondary antibody conjugated to either Rhodamine or FITC. Confocal microscopy showed that FBI-1 is present mainly in the nuclear periplasm. eEF1A and CCS-3 are present mainly in the cytosol, but low levels of eEF1A and CCS-3 are detected in the nucleus as reported previously [20, 21]. FBI-1 co-localizes with eEF1A and CCS-3 in the nuclear periplasm.

Results

eEF1A is a component of the nuclear protein complex containing the proto-oncogene FBI-1

Biological functions of a protein can be understood by determining interacting protein(s). To understand the biological functions of the proto-oncogene FBI-1, we isolated a nuclear protein complex containing FBI-1 from stable HEK293-TREx-Flag-FBI-1 cells by immunoprecipitation using anti-Flag M2-agarose beads. The immunoprecipitates were separated by 12% SDS-PAGE and stained with a silver staining solution (Fig. 1A). A 50 kDa protein band was excised from the gel, digested with trypsin, and subjected to MALDI-TOF mass spectrometry analysis. The 50 kDa protein was identified as eEF1A (eukaryote translation elongation factor 1A), which carries the amino acyl-tRNA onto the ribosome

during translation and is also involved in several cellular processes, including oncogenic transformation and cell proliferation (Fig. 1).

The zinc finger DNA binding domain and the POZ-domain of FBI-1 directly interact with eEF1A and its isoform, CCS-3, in vivo and in vitro

Although we isolated eEF1A as a component of the nuclear protein complex containing Flag-FBI-1, we confirmed the molecular interaction by co-immunoprecipitation and GST-fusion pull-down assays. The nuclear extracts prepared from control and doxycycline inducible stable HEK293-TREx-FBI-1-Flag cells were immunoprecipitated using anti-Flag M2 agarose beads or control IgG conjugated agarose beads. The precipitates were analyzed by western blotting using

Fig. 4. CCS-3 enhances transcriptional repression of the cell cycle arrest gene, *p21*, by the proto-oncogene FBI-1 in HEK293A cells. FBI-1 represses transcription of the *p21* promoter by more than 70%. CCS-3 enhances the FBI-1 transcriptional repression by an additional 30–50%. (A) Structures of the pGL2-p21-Luc (-2.3 kb or -131 bp) plasmids. The *p21* promoter has two p53 binding sites at the distal promoter, as well as C/EBP α , β , STAT, RAR, VDR, and six Sp1 binding sites at the proximal promoter. FBI-1 represses transcription of the two promoter constructs by acting on the distal p53 binding elements and proximal promoter Sp1 binding GC-box 3 element. (B) CCS-3 enhances FBI-1-mediated transcriptional repression. The HEK293A cells were co-transfected with pGL2-p21-Luc (-2.3 kb or -131 bp), pcDNA3 FBI-1, and/or pcDNA3 CCS-3 expression plasmids. After 48 hrs, the cells were harvested and analyzed for luciferase activity. Luciferase activities were normalized with co-transfected β -galactosidase activity. The average of three independent experiments is shown. Bars, standard deviations (SD). Analysis of variance was employed to calculate the p value using SPSS program. A p value <0.05 was considered to be significant. (C) RT-PCR and Western blot analysis of *p21* gene repression by FBI-1 at the mRNA and protein levels in the transfected HEK293A cells. GAPDH and Tubulin, controls for RT-PCR and Western blot analysis, respectively.

antibodies against eEF1A, GAPDH, and Flag-Tag. The data show that FBI-1 interacts with eEF1A (Fig. 2B).

Additionally, we investigated whether CCS-3, eEF1A isoform, could interact with FBI-1 by co-immunoprecipitation. HEK293A cells were transfected with a mammalian expression vector, pcDNA3.1-Myc-CCS-3. The cell lysate was immunoprecipitated with anti-Myc tag antibody, and the precipitate was analyzed by western blot using anti-FBI-1 antibody. Clearly, FBI-1 interacts with CCS-3 (Fig. 2C).

We used GST-fusion protein pull-down assays to further investigate whether the molecular interaction between FBI-1 and eEF1A (or its isoform, CCS-3,) is direct. GST, GST-POZFBI-1, and GST-ZFFBI-1 were mixed with *in vitro* translated [35 S]-Methionine labeled eEF1A or CCS-3 polypeptides and pulled-down. Subsequent SDS-PAGE and autoradiography showed that both eEF1A and CCS-3 interacted via the zinc finger DNA binding domain and the POZ-domain of FBI-1 (Fig. 2D).

Because CCS-3 is an isoform of eEF1A that lacks the 100 a.a. N-terminal GTP-binding domain, eEF1A interacts with FBI-1 via the a.a.102-462 region, the region that overlaps with CCS-3 (a.a. 1-361) (Fig. 2D).

FBI-1 and eEF1A or CCS-3 co-localize in the nuclear periplasm

To investigate the cellular localization of FBI-1 and eEF1A or CCS-3, the expression vectors for Flag-tagged FBI-1 and His-tagged eEF1A or His-tagged CCS-3 were co-transfected into HEK293A cells. Cells were fixed, hybridized with anti-Flag and anti-His antibodies, and incubated with Rhodamine or FITC conjugated secondary antibodies. As reported previously [7], confocal microscopy showed that FBI-1 is present mainly in the nuclear periplasm, especially when FBI-1 is over-expressed. eEF1A and CCS-3 are preferentially localized in the cytosol. eEF1A was previously shown to be actively exported out of the nucleus by Exp5 [25], however, a low level of eEF1A and CCS-3 were previously

Fig. 5. FBI-1 interacts with co-repressors (SMRT, BCoR) via the POZ-domain, and CCS-3 also interacts with co-repressors (SMRT, BCoR). (A) Diagram of the mammalian two-hybrid assay to analyze the molecular interaction between FBI-1 or CCS-3 and co-repressors. CCS-3 increased luciferase expression by two-fold. CV-1 cells were co-transfected with various combinations of pG5-Luc, pGal4-POZFBI-1, pVP16-corepressor, and pcDNA3.1-CCS-3 expression plasmids. After 48 hrs in culture, the cells were harvested and analyzed for luciferase activity. Luciferase activities were normalized to co-transfected β -galactosidase activity. Histogram represents mean $\pm$ standard deviation (S.D.) of three independent experiments. P values are indicated for the observed differences. A p value <0.05 was considered to be significant. (B) Mammalian two-hybrid assay of molecular interaction between CCS-3 and co-repressors. CCS-3 interacts with the co-repressors. (C) Model of protein-protein interactions among the POZ-domain of FBI-1, CCS-3, and co-repressors. X, X', transcription co-repressors.

identified in the nucleus [26-31]. eEF1A was also detected in our purified nuclear extract, and our immunoprecipitate of the nuclear protein complex (Fig 1A). Confocal microscopic image analysis of stained cells in cross-sections apparently shows that FBI-1 and eEF1A or CCS-3 co-localize at the nuclear periplasm (Fig. 3A, B), though eEF1A was less pronounced.

CCS-3 enhances transcriptional repression of the cell cycle arrest p21 gene promoter by the proto-oncogene FBI-1

We analyzed the functional significance of FBI-1-eEF1A or FBI-1-CCS-3 interactions on the transcriptional regulation of target genes. Recently, we and others found that FBI-1 is a major regulator of ARF-Mdm2-p53-p21 pathway (p53 pathway) [11, 36, 37]. In particular, FBI-1 represses transcription of the *p21* promoter by more than 70~80% by acting on both the proximal Sp1 binding GC-box 3 element and the distal p53 responsive regulatory elements. The transcriptional repression involved

competitive binding with Sp1 and p53 on the proximal promoter and distal binding elements, respectively. Once FBI-1 succeeded in binding, interaction with the co-repressors played an important role in transcriptional repression by deacetylating histone H3 and H4 at the promoter [37].

We investigated whether CCS-3 or eEF1A could affect transcriptional repression of two *p21* promoter constructs by FBI-1. We co-transfected eEF1A or CCS-3 expression vectors with FBI-1 and the pGL2-p21-Luc reporter plasmid (-2.4 kb or -133 bp) in HEK293A cells (Fig. 4A). In the two promoter constructs tested, CCS-3 co-expression significantly enhances FBI-1-induced transcriptional repression by an additional 30~50%, depending on the promoter construct (Fig. 4B). Also, FBI-1 expression decreased the endogenous p21 protein and mRNA levels, and CCS3 co-expression further decreased p21 protein and mRNA expression (Fig. 4C). Ectopic eEF1A did not show a clear regulatory effect on FBI-1-mediated transcriptional repression of the *p21*

Fig. 6. The CCS-3 and FBI-1 increases cell proliferation, and molecular interaction between FBI-1 and CCS-3 further stimulates cell proliferation. MTT assays were performed in HEK293A cells transfected with FBI-1 or/and CCS-3 expression vector. HEK293A cells grown for 0, 2, 4, 6 days and analyzed for conversion of MTT to formazan. All assays were performed in triplicate. Error bars are included, but are too tight to see. Analysis of variance was employed to calculate the p value. A P value <0.05 was considered to be significant.

promoter (data not shown), perhaps due to rapid nuclear export of eEF1A. In contrast, CCS-3, which lacks the N-terminus (1-100 a.a.) of eEF1A, appears to be somewhat refractory to nuclear export, residing to a greater extent in the nuclear periplasm as revealed by immunocytochemistry (Fig. 3B), thereby explaining why CCS-3 was able to enhance transcriptional repression by FBI-1.

The FBI-1 POZ-1 domain and CCS-3 interact with co-repressors (SMRT, BCoR) independently

We recently found that, by mammalian two-hybrid assays and co-immunoprecipitation, the FBI-1 POZ-domain interacts with co-repressors (SMRT, BCoR) [37, 40] (Fig. 5A). Such molecular interactions are important in the transcriptional repression of FBI-1 target genes, such as *p21* and *Rb* [37, 40]. Additionally, the FBI-1 POZ-domain is important in transcriptional repression [4, 40]. In mammalian two-hybrid assays, luciferase reporter activities indicate the molecular interaction between the POZ-domain and the co-repressors. Accordingly, we investigated whether the molecular interaction between the POZ-domain and the co-repressors is affected by ectopic CCS-3 expression in CV-1 cells. Interestingly, ectopic CCS-3 increased the reporter activity (Fig. 5A).

Fig. 7. A hypothetical model of CCS-3 action on FBI-1-mediated transcriptional repression of the *p21* promoter. (A) Inhibition of *p21* of p53 pathway, by FBI-1 and CCS-3. Both FBI-1 and CCS-3 interact with corepressors, which potentiate transcriptional repression of *p21* by FBI-1. Arrow (→), transcriptional activation; solid line with ⊥, transcriptional repression; double arrow (↔) protein-protein interaction. (B) Model of transcriptional repression of *p21* by FBI-1 and CCS-3. The proto-oncogene FBI-1 attacks three critical elements (Sp1 binding GC-box 3, and two p53 responsive elements) in basal constitutive and inducible expression. CCS-3 is recruited to the *p21* proximal promoter and distal p53 binding regulatory elements by interacting with the POZ-domain of FBI-1. FBI-1 represses transcription of *p21*, which is further enhanced by molecular interaction between CCS-3 and additional transcriptional co-repressors. Tsp (+1), transcription start site; ZF, zinc-finger DNA binding domain.

CCS-3 may simply increase the interaction between co-repressor and FBI-1, however, the reporter activity increased by approximately two-fold. These data suggest that CCS-3 might interact with co-repressors while bound to the POZ-domain of FBI-1 bound at the proximal UAS (upstream activator binding sequence) of the test pG5-UAS-Luc promoter. Therefore, the corepressor-CCS3 interaction may be independent of corepressor-FBI-1

interaction. Accordingly, we investigated whether CCS-3 interacts with transcriptional co-repressors by mammalian two-hybrid assays. Indeed, CCS-3 interacts with SMRT and more strongly with BCoR (Fig. 5B). In contrast, the POZ-domain interacts with SMRT more strongly than BCoR. These data suggest that CCS-3 enhances the transcriptional repression potential of FBI-1 by interacting with the POZ-domain of FBI-1 and recruiting additional co-repressors (Fig. 5C).

The CCS-3 and FBI-1 increases cell proliferation, and molecular interaction between FBI-1 and CCS-3 further stimulates cell proliferation

We investigated the functional significance of the molecular interaction between FBI-1 and CCS-3 on cell proliferation by carrying out MTT assays in human HEK293A cells. The assays clearly showed that although CCS-3 or FBI-1 could increase cell proliferation independently, co-transfection of CCS-3 and FBI-1 significantly further stimulated cell proliferation. The data suggested that the molecular interaction is important in the repression of cell cycle arrest gene *p21* and accordingly in the stimulation of cell proliferation (Fig. 6).

Discussion

FBI-1 was originally isolated as a BTB/POZ class transcription factor that bound to HIV-1 LTR and *ADH5/FDH* gene regulatory elements [3-5]. Subsequent investigation revealed that FBI-1 carries out various cellular regulatory functions, such as modulating HIV-1 transcription, nuclear trapping of NF- κ B, adipocyte differentiation, osteoclastogenesis, and transcriptional repression of the *ADH5/FDH* gene [3-10]. FBI-1 expression is often increased in cancer tissues. Recently, Maeda *et al.* and we showed that FBI-1/LRF is a potent proto-oncogene that regulates *ARF* gene of the p53 pathway and *Rb* gene [11, 40]. LRF caused oncogenic cellular transformation, and transgenic mice over-expressing LRF showed multiple cancers [11].

Analyzing FBI-1 protein-protein interactions will help us to understand its various cellular regulatory functions. This study shows that FBI-1 interacts with eEF1A. Although eEF1A is principally involved with translation initiation (13 and references therein), several reports have suggested that eEF1A is also involved in oncogenic transformation and cell proliferation [14 and references therein; 15-21]. For example, an eEF1A isoform, PTI-1

(tumor-inducing gene 1), has an oncogenic nature, identified in prostate, breast, colon, and lung cancer cell lines [22, 23]. In addition, over-expressing eEF1A mRNA has been correlated with increased metastatic potential in mammary adenocarcinoma [24]. Recently, another eEF1A isoform, CCS-3, was isolated as a protein that interacts with the BTB/POZ-domain transcription factor, PLZF [35].

Static images from confocal microscopy show that eEF1A and CCS-3 are predominantly located in the cytoplasm, but this result does not suggest that eEF1A and CCS-3 are located only in the cytoplasm. Some reports suggest that both proteins are also detected in the nucleus. Interaction with other proteins can change the cellular localization of a particular protein, especially when this interaction is critical for cellular functions. For example, the zinc finger protein ZPR1 is present in the cytoplasm of quiescent mammalian cells. EGF stimulation of mammalian cells triggers the formation of ZPR1/eEF1A complexes and translocation of both ZPR1 and eEF1A from the cytoplasm to the nucleus [26]. Disrupting the binding between ZPR1 and eEF1A by mutational analysis caused cells to accumulate in the G2/M phase of the cell cycle, resulting in defective growth. Reconstituting the ZPR1/eEF1A interaction restored normal growth. Thus, the formation of nuclear ZPR1/eEF1A complexes is essential in the normal growth of proliferating cells [26].

Interestingly, Exp5 exports eEF1A from the nucleus via tRNA, thereby confining most eEF1A to the cytoplasm. This finding suggests that eEF1A must be necessary and present in the nucleus (25). Some reports have suggested that 10-15% of cellular translation occurs in the nucleus, indicating that some eEF1A should be present [27-35]. Cross sections of confocal microscopic images show that the FBI-1 and eEF1A staining patterns overlap in the nuclear periplasm. In addition, confocal cross sections also showed that CCS-3 and FBI-1 staining patterns overlap in a broader region of the nuclear periplasm. This may explain the significant transcriptional repression by CCS-3.

The member of the p53 pathway primarily regulated by FBI-1 is *p21* [37]. FBI-1 caused oncogenic transformation and potentially promoted cellular proliferation by inhibiting *p21* gene expression, which is important in cell cycle arrest [37, 41, 42]. FBI-1 repressed transcription by acting on both the proximal promoter and distal p53 responsive elements through binding competition with Sp1 and p53. Interestingly, CCS-3, an eEF1A isoform, enhances the transcription repression of *p21* by FBI-1. This repression is similar to enhancing

transcriptional repression of the PLZF target reporter promoter by PLZF [35]. As CCS-3 interacts with PLZF, it also interacts with the FBI-1 POZ-domain, which is important in the transcriptional repression of *p21* by FBI-1.

Intriguingly, we found that CCS-3 interacted with transcriptional co-repressors SMRT and BCoR, independent of FBI-1, and it could repress transcription of *p21*. CCS-3 further enhances transcriptional repression by FBI-1 by recruiting additional transcriptional co-repressor-HDAC complex when tethered to the FBI-1 POZ-domain. This molecular mechanism may also apply to the transcription repression by PLZF. CCS-3 interaction with transcriptional co-repressors might be important in FBI-1 oncogenic activity by recruiting more co-repressor-HDAC complex to the promoters of FBI-1 target genes important in cell growth and proliferation, e.g. *p21*.

It is generally accepted that over-expressing translation initiation factors is a significant event in oncogenic cell transformation and malignancy. The protein interaction between CCS-3 or eEF1A and the proto-oncogene FBI-1 might initiate a cellular regulatory mechanism that couples translation and cell proliferation.

Abbreviations

Ab (antibody); ADH5/FDH (alcohol dehydrogenase 5/formaldehyde dehydrogenase); BCoR (BCL-6 interacting co-repressor); bp (base pair); BTB/POZ (bric-à-brac, tramtrack, and broad complex and pox virus zinc finger); CCS-3 (cervical cancer suppressor 3);

CMV (cytomegalovirus); CV-1 (african green monkey kidney cell); DBD (DNA binding domain); DMEM (Dulbecco's modified eagle medium); DTT (1,4-dithiothreitol); FBI-1 (factor that binds to the inducer of short transcripts of human immunodeficiency virus-1); FBS (fetal bovine serum); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); GST (glutathione S-transferase); HIV (human immunodeficiency virus); IP (immunoprecipitation); IPTG (isopropyl-1-thio- β -D-galactopyranoside); *LacZ* (β -galactosidase gene); *Luc* (luciferase gene); NCoR (Nuclear receptor co-repressor); PCR (polymerase chain reaction); PLZF (promyelocytic leukemia zinc finger protein); Pokemon (POK erythroid myeloid ontogenic factor); POZ (poxvirus and zinc finger); PVDF (Poly vinylidene di-fluoride); Rb (retinoblastoma); RT-PCR (reverse transcription and polymerase chain reaction); SAGE (serial analysis of gene expression); SDS-PAGE (sodium-dodecyl sulfate-polyacrylamide gel); SMRT (silencing mediator for retinoid and thyroid receptors); Sp1 (specificity protein 1); Wt (wild type); ZFDBD (zinc finger DNA binding domain).

Acknowledgements

This work was supported by a National Research Laboratory research grant (ROA-2003-000-10318-0 to MWH), a Medical Research Center Grant (R13-2002-054-05002-0 to MWH) and also in part by a Nuclear Basic Research Grant (2008-01735) from the Korean Science and Engineering Foundation of the Korean Ministry of Science and Technology.

References

- Bardwell VJ, Treisman R: The POZ domain: a conserved protein-protein interaction motif. *Genes Dev* 1994;8:1664-1677.
- Kelly KF, Daniel JM: POZ for effect-POZ-ZF transcription factors in cancer and development. *Trends Cell Biol* 2006;16:578-587.
- Pessler F, Pendergrast PS, Hernandez N: Purification and characterization of FBI-1, a cellular factor that binds to the human immunodeficiency virus type 1 inducer of short transcripts. *Mol Cell Biol* 1997;17:3786-3798.
- Lee DK, Suh D, Edenberg HJ, Hur MW: POZ domain transcription factor, FBI-1, represses transcription of ADH5/FDH by interacting with the zinc finger and interfering with DNA binding activity of Sp1. *J Biol Chem* 2002;277:26761-26768.
- Morrison DJ, Pendergrast PS, Stavropoulos P, Colmenares SU, Kobayashi R, Hernandez N: FBI-1, a factor that binds to the HIV-1 inducer of short transcripts (IST), is a POZ domain protein. *Nucleic Acids Res* 1999;27:1251-1262.
- Pendergrast PS, Wang C, Hernandez N, Huang S: FBI-1 can stimulate HIV-1 Tat activity and is targeted to a novel subnuclear domain that includes the Tat-P-TEFb-containing nuclear speckles. *Mol Biol Cell* 2002;13:915-929.
- Lee DK, Kang JE, Park HJ, Kim MH, Yim TH, Kim JM, Heo MK, Kim KY, Kwon H J, Hur MW: FBI-1 activates transcription of NF-kB responsive E-selectin gene by nuclear localization of p65 subunit of NF-B. *J Biol Chem* 2005;280:27783-27791.

- 8 Laudes M, Christodoulides C, Sewter C, Rochford JJ, Considine RV, Sethi JK, Vidal-Puig A, O'Rahilly S: Role of the POZ zinc finger transcription factor FBI-1 in human and murine adipogenesis. *J Biol Chem* 2004;279:11711-11718.
- 9 Kukita A, Kukita T, Ouchida M, Maeda H, Yatsuki H, Kohashi O: Osteoclast-Derived Zinc Finger (OCZF) protein with POZ domain, a possible transcriptional repressor, is involved in osteoclastogenesis. *Blood* 1999;94:1987-1997.
- 10 Davies JM, Hawe N, Kabarowski J, Huang QH, Zhu J, Brand NJ, Leprince D, Dhordain P, Cook M, Morris-Kay G, Zelent A: Novel BTB/POZ domain zinc-finger protein, LRF, is a potential target of the LAZ-3/BCL-6 oncogene. *Oncogene* 1999;18:365-375.
- 11 Maeda T, Hobbs RM, Merghoub T, Guernah I, Zelent A, Cordon-Cardo C, Teruya-Feldstein J, Pandolfi PP: Role of the proto-oncogene Pokemon in cellular transformation and ARF repression. *Nature* 2005;433:278-285.
- 12 Maeda T, Merghoub T, Hobbs RM, Dong L, Maeda M, Zakrzewski J, van den Brink MR, Zelent A, Shigematsu H, Akashi K, Teruya-Feldstein J, Cattoretti G, Pandolfi PP: Regulation of B versus T lymphoid lineage fate decision by the proto-oncogene LRF. *Science* 2007;316:860-866.
- 13 Merrick WC, Nyborg J: The protein biosynthesis elongation cycle; in Sonenberg N, Hershey JWB, Mathews MB (eds): *Translational Control of Gene Expression*. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press, 2000, pp 89-125.
- 14 Lamberti A, Caraglia M, Longo O, Marra M, Abbruzzese A, Arcari P: The translation elongation factor 1A in tumorigenesis, signal transduction and apoptosis: review article. *Amino Acids* 2004;26:443-448.
- 15 Sonenberg N: Translation factors as effectors of cell growth and tumorigenesis. *Curr Opin Cell Biol* 1993;5:955-960.
- 16 Al-Maghrebi M, Anim JT, Olalu AA: Up-regulation of eukaryotic elongation factor-1 subunits in breast carcinoma. *Anticancer Res* 2005;25(3c):2573-2577.
- 17 Kato MV, Sato H, Nagayoshi M, Ikawa Y: Upregulation of the elongation factor-1alpha gene by p53 in association with death of an erythroleukemic cell line. *Blood* 1997;90:1373-1378.
- 18 Dapas B, Tell G, Scaloni A, Pines A, Ferrara L, Quadrifoglio F, Scaggiante B: Identification of different isoforms of eEF1A in the nuclear fraction of human T-lymphoblastic cancer cell line specifically binding to aptameric cytotoxic GT oligomers. *Eur J Biochem* 2003;270:3251-3262.
- 19 Krieg PA, Varnum SM, Wormington WM, Melton DA: The mRNA encoding elongation factor-1 (eEF-1) is a major transcript at the midblastula transition in *Xenopus*. *Dev Biol* 1989;133:93-100.
- 20 Shepherd JCW, Walldorf U, Hug P, Gehring WJ: Fruit flies with additional expression of the elongation factor eEF-1 live longer. *Proc Natl Acad Sci USA* 1989;86:7520-7521.
- 21 Tatsuka M, Mitsui H, Wada M, Nagata A, Nojima H, Okayama H: Elongation factor-1 gene determines susceptibility to transformation. *Nature* 1992;359:333-336.
- 22 Gopalkrishnan RV, Su ZZ, Goldstein NI, Fisher PB: Translational infidelity and human cancer: role of the PTI-1 oncogene. *Int J Biochem Cell Biol* 1999;31:151-162.
- 23 Su Z, Goldstein NI, Fisher PB: Antisense inhibition of the PTI-1 oncogene reverses cancer phenotypes. *Proc Natl Acad Sci USA* 1998;95:1764-1769.
- 24 Edmonds BT, Wyckoff J, Yeung YG, Wang Y, Stanley ER, Jones J, Segall J, Condeelis J: Elongation factor-1 alpha is an overexpressed actin binding protein in a metastatic rat mammary adenocarcinoma. *J Cell Sci* 1996;109:2705-2714.
- 25 Bohnsack MT, Regener K, Schwappach B, Saffrich R, Paraskeva E, Hartmann E, Görlich D: Exp5 exports eEF1A via tRNA from nuclei and synergizes with other transport pathways to confine translation to the cytoplasm. *EMBO J* 2002;21:6205-6215.
- 26 Gangwani L, Mikrut M, Galcheva-Gargova Z, Davis RJ: Interaction of ZPR1 with translation elongation factor-1 alpha in proliferating cells. *J Cell Biol* 1998;143:1471-1484.
- 27 Colling DA, Wastenays GO, Miyazaki M, Williamson RE: Elongation factor 1 is a component of the subcortical actin bundles of characean algae. *Cell Biol Int* 1994;18:1019-1024.
- 28 Janssen GMC, Van Damme HTF, Kriek J, Amons R, Moller W: The subunit structure of elongation factor 1 from *Artemia salina*; why two -chains in this complex. *J Biol Chem* 1994;269:1-8.
- 29 Barbarese E, Koppel DE, Deutscher MP, Smith CL, Ainger K, Morgan F, Carson JH: Protein translation components are colocalized in granules in oligodendrocytes. *J. Cell Sci* 1995;108:2781-2790.
- 30 Billaut-Mulot O, Fernandez-Gomez R, Loyens M, Ouaisi A: Trypanosoma cruzi elongation factor-1: nuclear localization in parasites undergoing apoptosis. *Gene* 1996;174:19-26.
- 31 Sanders J, Brandsma M, Janssen GMC, Dijk J, Moller W: Immunofluorescence studies of human fibroblasts demonstrate the presence of the complex of elongation factor-1 in the endoplasmic reticulum. *J Cell Sci* 1996;109:1113-1117.
- 32 Blackwell JL, Brinton MA: Translation elongation factor-1 alpha interacts with 3' stem-loop region of West Nile virus genomic RNA. *J Virol* 1997;71:6433-6444.
- 33 Blumenthal T, Carmichael GG: RNA replication: function and structure of Q-replicase. *Ann Rev Biochem* 1979;48:528-548.
- 34 Das T, Mathur M, Gupta AK, Janssen GMC, Banerjee AK: RNA polymerase of vesicular stomatitis virus specifically associates with translation elongation factor-1 for its activity. *Proc Natl Acad Sci USA* 1998;95:1449-1454.
- 35 Rho SB, Park YG, Park K, Lee SH, Lee JH: novel cervical cancer suppressor 3 (CCS-3) interacts with the BTB domain of PLZF and inhibits the cell growth by inducing apoptosis. *FEBS Lett* 2006;580:4073-4080.
- 36 Toledo F, Wahl GM: Regulating the p53 pathway: *in vitro* hypotheses, *in vivo* veritas. *Nat Rev Cancer* 2006;6: 909-923.
- 37 Choi WI, Jeon BN, Yun CO, Kim PH, Kim SE, Choi KY, Kim SH, and Hur MW: Proto-oncogene FBI-1 represses transcription of p21CIP1 by inhibition of transcription activation by p53 and Sp1. *J Biol Chem* 2009; in press.
- 38 Lee JA, Suh DC, Kang JE, Kim MH, Park H, Lee MN, Kim JM, Jeon BN, Roh HE, Yu MY, Choi KY, Kim KY, Hur MW: Transcriptional activity of Sp1 is regulated by molecular interactions between the zinc finger DNA binding domain and the inhibitory domain with corepressors, and this interaction is modulated by MEK. *J Biol Chem* 2005;280:28061-28071.
- 39 Dignam JD, Lebovitz RM, Roeder RG: Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 1983;11:1475-1489.
- 40 Jeon BN, Yoo JY, Choi WI, Lee CE, Yoon HG, Hur MW: Proto-oncogene FBI-1 (Pokemon/ZBTB7A) represses transcription of the tumor suppressor Rb gene via binding competition with Sp1 and recruitment of corepressors. *J Biol Chem* 2008;283:33199-33210.
- 41 El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993;75:817-825.
- 42 El-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y: WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res* 1994;54:1169-1174.