New proto-oncogene Zbtb7c, a molecular functional switch of tumor suppressor p53

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Zbtb7c is a ubiquitously expressed BTB/POZ-domain class transcription factor. We investigated whether Zbtb7c has an effect on cell cycle progression and oncogenesis through regulated gene expression of key cell cycle regulatory genes in the Arf-Mdm2-p53-p21$^{Waf/Cip1}$ pathway. Zbtb7c repressed transcription of *p21*$^{Waf/Cip1}$ and *p19*^{Arf}, which requires p53. Zbtb7c interacts with p53 bound on the *p21*$^{Waf/Cip1}$ distal promoter via its zinc-finger domain and also interacts with transcriptional corepressors, such as NCoR and BCoR, causing the deacetylation of histones at the *p21*$^{Waf/Cip1}$ proximal promoter. Adenoviral delivery of the *Zbtb7c* gene potently promotes tumor growth in

mice transplanted with U343 cancer cells. In contrast, RNA that interfered with Zbtb7c mRNA abolished tumor growth. The molecular interactions among p53, Zbtb7c, and corepressors at the distal p53 binding element are important in the transcriptional repression of *p21*$^{Waf/Cip1}$, oncogenic cellular transformation, cell proliferation, and tumor growth. Zbtb7c is one of the key control genes of the Arf-Mdm2-p53-p21$^{Waf/Cip1}$ pathway as it acts at both transcription and protein levels. Our study also suggests that p53 can act as an oncogene in the presence of Zbtb7c.

Key words : BTB/POZ domain, zinc-finger domain , Zbtb7c, transcription factor, cell cycle, oncogene, Arf-Mdm2-p53-p21$^{Waf/Cip1}$, corepressors (NCoR, BCoR), histone modification

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I. Introduction

The BTB/POZ domain, originally found in Drosophila melanogaster bric-à-brac, tramtrack and broad complex transcription regulators and in pox virus zinc finger proteins, is an evolutionarily conserved protein-protein interaction domain with about 1,000 distinct BTB/POZ entries in available sequence databases[1] (http://smart.embl-heidelberg.de; http://btb.uhnres.utoronto.ca). The BTB/POZ domain regulatory proteins have various cellular regulatory functions. In particuar, some of the POK proteins with BTB/POZ domain and Kruppel like zinc finger are major determinant in differentiation, development, and oncogenesis. Promyelocytic leukemia zinc finger (PLZF)–null mice

display severe defects in limb development and germ stem cell maintenance[2,3]. Th-POK (T-helper-inducing POZ/Krüppel-like factor, also known as cKrox) has been recently reported as a master regulator of T-cell lineage commitment[4]. B-cell lymphoma 6 (Bcl-6) and PLZF, and hypermethylated in cancer-1 (HIC1) have been implicated in non-Hodgkin's lymphoma, acute promyelocytic leukemia, and spontaneous malignant tumors, respectively[5-8]. Recently, a ubiquitous transcription factor FBI-1 (also called Zbtb7a/Pokemon) was shown to act as a proto-oncogene by repressing transcription of Arf and thus down regulating p53, and promotes oncogenic cellular transformation. FBI-1 was suggested as a master regulator of cellular transformation and oncogenesis[11].

The cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ (hereinafter indicated as p21) is a major player involved in the negative regulation of cell cycle progression in mammalian cells and is the downstream cell-cycle regulator of Arf-MDM2-p53-p21 pathway[12,13]. *p21* gene is mainly regulated at the transcriptional level and is a transcriptional target of tumor suppressor p53 and plays a crucial role in mediating growth arrest when cells are exposed to DNA damaging agents. It has been shown that over expression of p21 results in G_1-, G_2-, or S-phase arrest. Whereas induction of p21 predominantly leads to cell cycle arrest, repression of p21 may have a variety of outcomes

depending on the cellular context. Apart from p53, a variety of other factors including Sp1/Sp3, Smads, AP2, STAT, BRCA1, E2F-1/E2F-3, and C/EBPα and ß are known to activate p21 gene transcription[14].

From the analysis of amino acid sequences of all available human POZ-domain proteins, we identified a novel Zbtb7c POK protein with POZ-domain and unique zinc-finger domain. We investigated whether Zbtb7c could regulate any components of the Arf-Mdm2-p53-p21 pathway, and, if it modulates the pathway, what is the physiological consequence of Zbtb7c action. We found that Zbtb7c is a master controller of Arf-Mdm2-p53-p21 pathway and represses transcription of p21 and Arf genes. Our data shows that the transcription repression of p21 by Zbtb7c involved p53, corepressors, and histone modifications at the promoter. Our data suggest that Zbtb7c acts as a protooncogene in the presence of p53, and induces cellular transformation, and promotes cell proliferation, and tumor growth.

II. Materials and Methods

1. Plasmids, antibodies, and reagents

$p21^{Waf/cip1}$-Luc plasmid was kindly provided by Dr. Yoshihiro Sowa of Kyoto Perpetual University of Medicine (Kyoto, Japan). pRB-Luc plasmid was a generous gift from Masayuki Sekimata (Fukushima Medical University, Fukushima, Japan). Various pGL2-p21-Luc, pGL2-p53-Luc, and pGL2-Arf-Luc reporter plasmids were prepared by cloning human promoter DNA fragments into pGL2-basic vector (Promega, WI). pcDNA3.1-Zbtb7c, pcDNA3.1-Zbtb7cΔPOZ, pcDNA3.1-p53 and various pcDNA3.1-p53 deletion constructs plasmids were prepared by cloning mouse brain cDNA or human cDNA fragments into pcDNA3.1 (Invitrogen, CA). GAL4-Zbtb7cPOZ plasmid was prepared by cloning mouse brain cDNA into pBIND. pTrex-Zbtb7c plasmid was cloned into pcDNA5.0/FRT/TO© (Invitrogen, CA) to prepare doxycyclin inducible Zbtb7c stable cells. pcDNA3.0-FBI-1, corepressors and VP16-corepressors was reported elsewhere[9,10]. To prepare recombinant GST-POZZbtb7c and GST-ZFZbtb7c proteins, cDNA fragments encoding POZ-domain (a.a 1-132) and zinc fingers (a.a 365-468) were cloned into pGEX4T1 or pGEX4T3 (Amersham Biosciences, NJ). All plasmid constructs were verified through sequencing.

Antibodies against p21, p53, GAPDH, His-Tag, Myc-Tag, FLAG-Tag, Ac-H3, Ac-H4, Gal4 and VP16 were purchased from Upstate (Charlottesville, VA), Chemicon (Temecula, CA), Calbiochem (San Diego, CA), and SantaCruz Biotech (SantaCruz, CA), respectively. Most of the chemical reagents were purchased from Sigma (St. Louis, MO).

2. Cell cultures/stable cell lines and animals

HeLa, 293A, CV-1, U343, NIH/3T3 and MB352 MEF cells were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco-BRL, MD) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, MD). Saos-2

cells were cultured in McCoy's 5A medium supplemented with 15% FBS. Stable 293Trex-Zbtb7c cells inducible by Doxycyclin were prepared by transfection of mammalian Flp-In™ T-REx™ host 293T cells (Invitrogen, CA) with a 9:1 ratio of pOG44, pcDNA5/FRT/TO©-Zbtb7c plasmid using Lipofectamin 2000 (Invitrogen, CA). Flp-In™ T-REx™ Zbtb7c stable cells overexpressing Zbtb7c were selected by culturing the transfected cells in a medium containing hygromycin (300 ㎍/ml) and blasticidin (15 ㎍/ml). To express the Zbtb7c protein, stable cells were cultured in a medium containing 1 ㎍/ml doxycyclin for the intended period of time.

Stable NIH/3T3 cells overexpressing Zbtb7c were prepared by infection of NIH/3T3 cells with a recombinant Lenti virus (LentiM1.4-Zbtb7c tagged with His and Myc peptide, Vectorcorea, Korea). After 2~3 days of incubation, stable cells were selected with puromycin (Sigma, MO).

For the *in vivo* tumour study, female BALB/c mice (six to seven weeks of age) were kept in a laminar air-flow cabinet maintained at 24 ± 2°C with 40% to 70% humidity under a 12 hrs light/dark cycle with a specific pathogen-free condition. All facilities were approved by the Association for Assessment and Accreditation of Laboratory Animal Care, and all animal experiments were conducted under the institutional guidelines established for the Animal Core Facility at the Yonsei University School of Medicine (Seoul, Korea).

3. Immunostaining of Zbtb7c

For immunostaining, CV-1 cells grown on coverslips (Sunshine Works, Seoul, Korea) were transfected with pcDNA3.1-Zbtb7c plasmid. Cells were fixed, permeabilized in 0.2% Triton X-100, blocked with 5% normal horse serum, incubated with mouse anti-His primary antibody, and finally incubated with FITC-conjugated anti-mouse IgG secondary antibody. After cells were washed with PBS, they were examined with an LSM 510 confocal laser scanning microscope (Carl Zeiss, Germany).

4. Transcriptional analysis of Arf, p53, $p21^{Waf/cip1}$, and Rb gene promoters

pGL2-Arf-Luc, pGL2-p53-Luc, pGL2-p21-Luc, and pGL2-Rb-Luc promoter reporter fusion plasmids, as well as pcDNA3.0-FBI-1, pcDNA3.1-Zbtb7c, pcDNA3.1-Zbtb7CΔPOZ, pcDNA3.1-p53, and pCMV-β-gal in various combinations were transiently transfected into HeLa, 293A and MB352 cells using Lipofectamine Plus reagent (Invitrogen, CA). After 24 hrs of incubation, cells were harvested and analyzed for luciferase activity. Reporter activity was normalized with contransfected β-galactosidase activity or protein concentration for transfection efficiency.

5. Western blot analysis

Cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl pH8.0, 1% NP-40, 0.25% sodium deoxycholic acid, 150 mM NaCl, 1 mM EGTA, Complete Mini-Protease cocktail). Cell extracts (50 μg) were separated using 12% SDS-PAGE gel electrophoresis, transferred onto Immun-Blot™ PVDF Membrane (Bio-Rad, CA), and blocked with 5% skim milk (BD Biosciences, MD). Blotted membranes were incubated with antibodies against FLAG-tag (Abcam, Cambridge, UK), GAPDH (Chemicon, CA), p21 (Upstate, NY), p53, His-Tag, Myc-Tag, and Gal4 (SantaCruz Biotech, CA) and then incubated with HRP conjugated mouse, rabbit, or goat IgG (Vector Laboratory, CA). Protein bands were visualized with ECL solution (PerkinElmer, CA).

6. Knock-down of Zbtb7c mRNA by siRNA

Three siRNA against Zbtb7c mRNA were designed and purchased from Ambion Inc. (Austin, TX): siZbtb7c-1, 5'-UCCAGUGCAUCGUGAAUGUtt-3', 5'-ACAUUCACGAUGCACUGGAtt-3'; siZbtb7C-2, 5'-CCAAGUUCGUGCACAACUAtt-3', 5'-UAGUUGUGCACGAACUUGGtt-3'; siZbtb7c-3, 5'-CAAUGCGAGUUCUGCUACAtt-3', 5'-UGUAGCAGAACUCGCAUUGtt-3'. The siRNA (150 pmoles) was transfected into NIH/3T3 cells by using Lipofectamine 2000 (Invitrogen, CA). After transfection, the cells were

harvested, total RNA was prepared, and RT-PCR analysis of mRNA was performed as described above.

7. Chromatin immunoprecipitation (ChIP) assays

The molecular interaction between Zbtb7c and p53 on the *p21*distal promoter as and the histone modification at the $p21^{Waf/cip1}$ proximal promoter *in vivo* were analyzed by ChIP assays. 293Trex-Zbtb7C cells were incubated with Doxycyclin. HeLa and Saos-2 cells were transfected with pcDNA3.1, pcDNA3.1-Zbtb7c, or pcDNA3.1-p53 using Lipofectamine Plus. Cells were fixed with formaldehyde (final 1%) to cross-link Zbtb7c, p53, and histones protein onto the *p21* promoter. The remaining ChIP procedures are reported elsewhere[9]. PCR reactions of immunoprecipitated DNA were carried out using oligonucleotide primer sets designed to amplify the upstream regulatory regions and the proximal promoter region of the *p21* gene. ChIP 1 primers (from bp -2307~-1947), forward, 5'-TGCTTGGGCAGCAGGCTGTG-3', reverse, 5'-GCAACCATGCACTTGAATGT-3'; ChIP 2 primers (bp, -1887~-1507), forward, 5'-AAAAAAGCCAGATTTGTGGC-3'; reverse, 5'-TGCTCACACCTCAGCTGGCG-3'; ChIP 3 primers (bp -1462 ~-1128), forward, 5'-TGTCCTCCCACCCCTACCTGG-3', reverse, 5'-AGAAATGAG TGATGTGTC-3'; ChIP 4 primers (bp -261~+39), forward, 5'-GGCTCACTT CGTGGGGAAAT-3', reverse, 5'-CACAAGGAACTGACTTCGGC-3'. To analyze H3 and H4 histone modification at the proximal promoter (from -133 bp to +100 bp), forward 5'-GCGCTGGGCAGCCAGGAGCC-3' and reverse 5'-CGCTCTCTCACCTCCTCT-3' oligonucleotides were used for ChIP assays.

8. Immunoprecipitation assays

Cells were washed, pelleted, and resuspended in a lysis buffer supplemented with protease inhibitors (20 mM Tris-HCl, pH7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100). Cell lysate was precleared, and the

supernatant was incubated overnight with anti-VP16 and anti-FLAG antibodies on a rotating platform at 4℃, followed by incubation with protein A-Sepharose Fast Flow beads. Beads were collected, washed, and resuspended in equal volumes of 5x SDS loading buffer. Immunoprecipitated proteins were separated with 12% SDS-PAGE. Western blot assay was performed as described above.

9. Mammalian two hybrid assays

CV1 cells were cotransfected with pG5-Luc, pGal4-POZZbtb7c, pVP16-corepressors, and pCMV-LacZ. After 24 hrs of transfection with Lipofectamin Plus (Invitrogen, CA), the CV-1 cells were harvested and assayed for luciferase activity. Luciferase activity was then normalized with β-galactosidase activity or protein concentration.

10. GST fusion protein purification, in vitro transcription and translation of corepressors or p53 and pull-down assays

Recombinant GST, GST-POZZbtb7c, and GST-ZFZbtb7c fusion proteins were prepared from *E. coli* BL21 (DE3) grown overnight at 18℃ in a medium containing 0.2 mM IPTG. The *E. coli* were lysed and purified using glutathione-agarose 4 bead affinity chromatography (Peptron, Taejeon, Korea). The purified proteins were then resolved with 12% SDS-PAGE to quantitate and assess purity. Corepressor and p53 polypeptides were prepared by incubating 1 μg of pcDNA3.0-corepressor expression plasmid and pcDNA3.1-p53 expression plasmid with TNT Quick-coupled Transcription/Translation Extract (Promega, WI), containing 40 ㎕ of TNT Quick Master Mix and 2 μl of [^{35}S]-methionine (1175.0 Ci/mol), (PerkinElmer Life Sciences, MA) at 30°C for 90 min. Polypeptide expression levels were then analyzed by running 1 ㎕ of the total mixture through 12% SDS-PAGE and autoradiography.

For GST-fusion protein pull-down assays, GST-fusion protein-agarose bead complexes were incubated with 10 ㎕ of *in vitro* translated [^{35}S]-

methione–labeled corepressors or p53 polypeptides at 4°C for 4 hrs in the HEMG buffer. The reaction mixtures were centrifuged, pellets were rinsed, and the bound proteins were separated using 12% SDS-PAGE. They were then exposed to X-ray film using an image-intensifying screen (Kodak, NY).

11. Foci formation assay

HeLa cells were cultured in six-well plates and then transfected with 0.5 ㎍/㎕ pcDNA3.0, pcDNA3.0-FBI-1, or pcDNA3.1-Zbtb7c using Lipofectamine plus reagent. Following transfection, cells were maintained in a medium supplemented with 10% FBS for two weeks in the presence of G418 (800㎍/ml). Colonies resistant to G418 were stained with crystal violet (0.5% crystal violet in 20% EtOH).

12. Fluorescence activated cell sorter (FACS) analysis

Cells were collected from six-well plates, washed, and fixed with cold 70% ethanol. Cells were then washed with PBS containing 1% horse serum, and cellular DNA was stained with propidium iodide (100 ㎍/ml) at 37℃ for 30 min. A cell cycle profile and forward scatter (FSC) were determined using a Becton Dickinson FACS caliber and the data were analyzed using ModFit LT 2.0 (Verity Software House, Inc., ME) and WindMDI 2.8 (Created by Joseph Trotter, Scripps Research Institute, CA).

13. BrdU incorporation

Cells were grown in DMEM containing 0.02 mM of BrdU for 12 hrs. The cells were fixed in 3.7% formaldehyde for 10 min, rinsed with PBS, and incubated for 10 min in 2N HCl. Fixed cells were then washed, blocked, incubated with anti-BruU monoclonal antibody, and finally incubated with goat anti-mouse-Cy2-conjugated secondary antibody. Cells were treated with DAPI solution (1 ㎍/ml), washed with PBS, and mounted for photography with a Radiance 2000 multi-photon (MP) imaging system (Bio-Rad, U.K.).

14. Preparation of recombinant adenovirus overexpressing Zbtb7c or siRNA against Zbtb7c mRNA

To prepare a recombinant adenovirus expressing Zbtb7c, the Zbtb7c cDNA was excised from pcDNA3.1-Zbtb7c plasmid with XhoI-AflII and cloned into the adenovirus E1 shuttle vector pCA14 (Microbix; Ontario, Canada) digested with XhoI-EcoRV, to generate pCA14-Zbtb7c. Also to prepare recombinant adenovirus expressing siRNA against Zbtb7c, annealed shRNA (sense: 5'-GATCC-CTCCAGTGCATCGTGAATGTTT-TTCAAGAG A(loop)-ACATTCACGATGCACTGGATT-TTTTTGGAA(loop)-A-3'), antisense: 5'-AGCTT-TTCCAAAAA(loop)-AATCCAGTGCATCGTGAATG T-TCTCTTGAA(loop)-AAACATTCACGATGCACTGGAG-G-3') were cloned into pSilencer 2.0-U6 (Ambion, Austin, TX) digested with BamHI-HindIII and subcloned into pΔE1sp1A vector digested with EcoRI-HindIII. The pCA14-Zbtb7c shuttle vector or pΔE1sp1A-U6-sh Zbtb7C vector was linearized by XmnI digestion, and the adenovirus vector vmdl324Bst (from Dr. Verca at the University of Fribourgh, Switzerland) containing the Ad5 genome deleted in the E1 and E3 region was linearized with BstBI digestion. The linearized pCA14-Zbtb7c or pΔE1sp1A-U6-sh Zbtb7c was cotransformed into *E. coli* BJ518 with the vmdl324Bst digested with BstBI for homologous recombination. To verify the respective homologous recombinant, the plasmid DNA was digested with HindIII, and the digestion pattern was analyzed. Proper homologous recombinant adenoviral plasmid was digested with *Pac*I and tranfected into 293 cells to generate the adenovirus expressing Zbtb7c (dl324-Zbtb7c) or siRNA against Zbtb7c (dl324-siZbtb7c). The virus was propagated in 293 cells and purified according to standard methods. PCR amplification and DNA sequencing using primers specific to the Zbtb7c or siRNA against Zbtb7c confirmed the genotype of the adenovirus.

The titer multiplicity of infection (MOI) used in this study was determined by the absorbency of the dissociated virus at 260 nm, where one

absorbency unit is equivalent to 1.1×10^{12} viral particles/ml. Particles: infectious unit (IU) ratio was 100:1.

15. MTT assays

To investigate the effect of Zbtb7c on cellular proliferation, brain glyoma cancer U343 cells were plated onto 24 well dishes at 30-80% confluency, and infected with 50 MOI of dl324 or dl324-Zbtb7c adenovirus. NIH/3T3-Zbtb7c cells were also plated and then infected with 50 MOI of dl324 or dl324-shRNA Zbtb7c adenovirus. The effect of recombinant viruses on U343 or NIH/3T3 cell proliferation was determined by measuring the conversion of the tetrazolium salt MTT to formazan. After 2, 4, 6, and 8 days of cell culture at 37^{o}C, 20 μl of MTT in PBS (2 mg/ml) was added to each well (Sigma, MO). After incubating for four hrs at 37^{o}C, the supernatant was discarded and the precipitate was dissolved with 1 ml of DMSO. Plates were then read on a microplate reader at 540 nm (Molecular Devices Corp., Sunnyvale, CA).

16. Analysis of Zbtb7C action on tumor growth in vivo

Two million U343 tumor cells were implanted under the skin of the abdomen in each anesthetized female BALB/c mouse. Once tumors had reached 100-120 mm^3 in volume, mice were injected intra-tumorly three times at two day intervals with 1×10^9 PFU of the adenovirus, either the control dl324 or dl324-Zbtb7c. For the dl324-siRNA Zbtb7c virus injections, viruses were injected five times at two day intervals with 2×10^8 PFU of the adenovirus. Tumor growth was monitored by measuring the length and width of the tumor three times a week using a caliper. Tumor volume was estimated on the basis of the following formula: volume = $0.523Lw^2$, where L refers to length (mm) and w refers to width (mm). Data are reported as the mean ± SEM. The significant differences were determined by Student's t-test (**). $P < 0.05$ was considered statistically significant.

17. Supplementary Experimental Procedures

(1) RT-PCR of Zbtb7c mRNA expression in FVB mouse or cells

Total RNA was isolated from brain, heart, liver, muscle, kidney, spleen, brown adipose tissues (BAT), and white adipose tissues (WAT) using TRIzol reagent (Invitrogen, CA). cDNAs were synthesized using 5 μg total RNA, random hexamer (10 pmol), and Superscript reverse transcriptase Ⅱ (200 units) in 20 $\mu \ell$ using the reverse transcription kit (Invitrogen, CA). Oligonucleotide primers used were as follows; Zbtb7c primer (forward, 5'-ACCATGGCCAATGACATTGATGA-3'; reverse, 5'-GTTGTTGGCTTCAG ACATGG-3'). β-actin (forward, 5'-ATGGATGACGATATCGCTGC-3', reverse, 5'- CACACTGTGCCCATCTACGA-3').

(2) EMSA (electro-mobility shift assay)

To identify the DNA binding consensus sequence for the Zbtb7c zinc fingers, we first prepared the [α-^{32}P]-dATP labeled probes by PCR using random oligonucleotides (5'-GAAGCTTAGACGGATCCATTGCA$(N)_{20}$CTG TAGGAATCCGGAGAATT-3') and two PCR primers (5'-GAAGCTTAGAC GGATCCA-3' 5'-AATTCTCCGGATTCCTAC-3') as a template. ^{32}P-labeled random oligonucleotide probes were purified using Sephadex^{TM} G-50 column chromatography (Amersham Biosciences, NJ). Each EMSA binding reaction was carried out for 30 min in 20 $\mu \ell$ of pH 7.9 binding buffer containing 10 mM HEPES, 60 mM KCl, 0.005 mM $ZnCl_2$, 1 mM dithiothreitol, 1% BSA, 7% glycerol, 2 μg recombinant FBI-1 zinc finger DNA binding domain, and Zbtb7c zinc-finger domain. The protein-DNA complexes were resolved with 4% non-denaturing polyacrylamide gel electrophoresis at room temperature in the 0.5X TBE (89mM TBE pH8.3) at 150 V for 2 hrs. The gels were exposed to X-ray film with a Kodak intensifying screen.

III. Results

1. Zbtb7c is a nuclear BTB/POZ-domain Krüppel-like zinc finger (POK) protein.

Zbtb7c conains a POZ-domain and four Krüppel-like zinc fingers and is simiar to the recently characterized protooncogene, FBI-1, in two key functional domains: the POZ-domain (81%) and the four Krüppel-like zinc fingers (88%) (Supplementary Fig. 1A, B). Zbtb7c, however, is quite different from FBI-1 in the overall amino acid sequence and in particular in the C2 half of each zinc finger, which probably differentiate it from FBI-1 in DNA binding activity and protein-protein interactions (Supplementary Figs. 1C, 2, 4). Indeed, Zbtb7c does not show DNA binding activity upon EMSA. Zbtb7c has a putative nuclear localization sequence (a.a., 470-486) juxtaposed to the fourth zinc finger. Immunohistochemical staining of Zbtb7c showed that it is located primarily in the nucleus (Fig. 1F), suggesting it has a role as a transcription factor.

2. Zbtb7c transcriptional repression of *p21*$^{Waf/Cip1}$ and *Arf* genes.

We investigated whether or not Zbtb7c also influences gene expression of the Arf-Mdm2-p53-p21 pathway, which are important in the regulation of cell cycle regulation. Transient cotransfection of the Zbtb7c expression vector and the promoter-Luc fusion reporter constructs in HeLa (also 293A cells) revealed that Zbtb7c potently repressed transcription of both the *Arf* and *p21* genes by 65% and 50%, respectively (Fig. 1a). Zbtb7c, however, did not repress transcription of the *Rb* gene. In stable 293Trex cells with the Doxycyclin inducible Zbtb7c expression system integrated, Zbtb7c induction repressed *p21* gene expression, as revealed by both RT-PCR and Western blot analyses (Fig. 1b). Even more clearly, in stable NIH/3T3-Zbtb7c established by infection with recombinant lentivirus expressing Zbtb7c, transcription of *p21* is potently repressed at both mRNA and protein levels (Fig. 1c). RNAi interference with Zbtb7c expression in NIH/3T3 by three different siRNA resulted in the derepression of *p21* mRNA expression (Fig. 1d). Knock-down of Zbtb7c through infection with the adenovirus expressing shRNA specific to Zbtb7c mRNA, also promoted potent derepression of *p21* gene transcription (Fig. 1e). Together, the data demonstrated that *p21*, a key regulator of the cell cycle and end regulator of the Arf-Mdm2-p53-p21 pathway, is potently repressed by Zbtb7c.

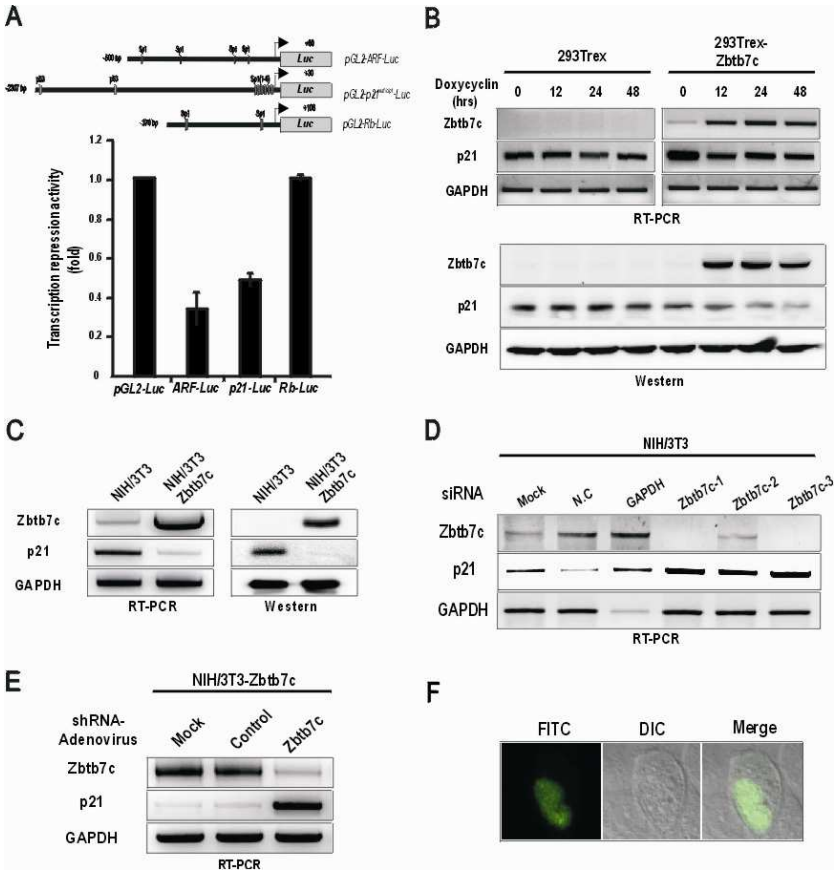


Figure 1. Zbtb7c potently represses transcription of the *p21*$^{Waf/cip1}$ and *Arf* genes. ***(A)*** Structures of the pGL2-p21$^{Waf/Cip1}$-Luc, pGL2-Arf-Luc, and pGL2-Rb-Luc constructs tested. Plasmid expressing Zbtb7c and reporter plasmid were transiently co-transfected into HeLa or 293A and luciferase activity was measured. Zbtb7c potently represses transcription of both the *p21*and *Arf* gene promoters by more than 50-65%, but it does not potently repress the *Rb* gene promoter. ***(B)*** RT-PCR and western blot analysis of the 293Trex and 293Trex-Zbtb7c cells after Doxycyclin induction. Upon induction of Zbtb7c expression, mRNA and protein expression of p21 decreased based on time elapsed. ***(C)***

RT-PCR and Western blot analysis of the NIH/3T3 and stable NIH/3T3-Zbtb7c cells infected with lentivirus. Zbtb7c overexpression decreased p21 mRNA and protein expression. ***(D, E)*** Knock-down of Zbtb7c expression, either by transfection with siRNA against Zbtb7c or by infection with the recombinant adenovirus expressing shRNA, increased p21 mRNA expression. RT-PCR analysis of the cDNAs prepared from NIH/3T3 or NIH/3T3-Zbtb7c cells transfected with three different siRNA against Zbtb7C mRNA. ***(F)*** Nuclear localization of Zbtb7c. The CV-1 cells transfected with the expression vector of 6x His-tagged ZbtbB7c were analyzed with immuno-histochemical staining using mouse anti-His antibody and FITC conjugated anti-mouse secondary antibody. Zbtb7c is primarily located in the nucleus.

3. Transcriptional repression of the p21$^{Waf/Cip1}$ promoter by Zbtb7c requires tumor suppressor p53.

Zbtb7c has features including its role as transcription factor, nuclear localization, zinc fingers, transcriptional repression, and molecular interaction with co-repressors. Therefore, we attempted to identify a DNA recognition sequence by CAST (cyclic amplification of sequence target) assays using a recombinant GST zinc-finger domain protein[25]. Our repeated EMSA attempts with randomly labeled oligonucleotides showed no detectable shifts in the bands, whereas the GST-FBI-1 zinc finger DNA binding domain provided retarded shifts in bands (Supplementary Fig. 3). Although Zbtb7c did not demonstrate DNA binding activity, Zbtb7c repressed transcription of the *p21* and *Arf* genes significantly. Accordingly, we tried to map the cis-regulatory element of the *p21* promoter responsible for transcriptional repression by Zbtb7c. Interestingly, Zbtb7c repressed transcription of the promoter with a -2.3 kb upstream sequence containing distal p53 binding sites (Fig. 2A). The data suggest transcriptional repression by Zbtb7c may require p53 bound at the distal regulatory element. We tested this possibility by cotransfecting the pGL2-p21-Luc (-2.3 kb) reporter plasmid with Zbtb7c expression plasmid into MB352 cells lacking p53. Zbtb7c did not repress transcription but transcriptional repression of the *p21* gene was restored through ectopic

expression of p53, suggesting Zbtb7c requires p53 for transcriptional repression (Fig. 2B). In HeLa cells with endogenous p53, when cells are treated with DNA damaging agent, etoposide, the *p21* gene is activated with induced p53. This p53 activation is again repressed by Zbtb7c (Fig. 2C). Cotransfection experiments with pGL2-p53 promoter-Luc and Zbtb7c expression plasmids in HeLa and 293A cells showed that Zbtb7c does not affect transcription of the p53 gene (Fig. 2D). Overall, these data suggest that Zbtb7c requires p53 bound at the distal p53 binding element to repress transcription of the *p21* gene, and Zbtb7c may be recruited to the distal promoter region through interaction with p53.

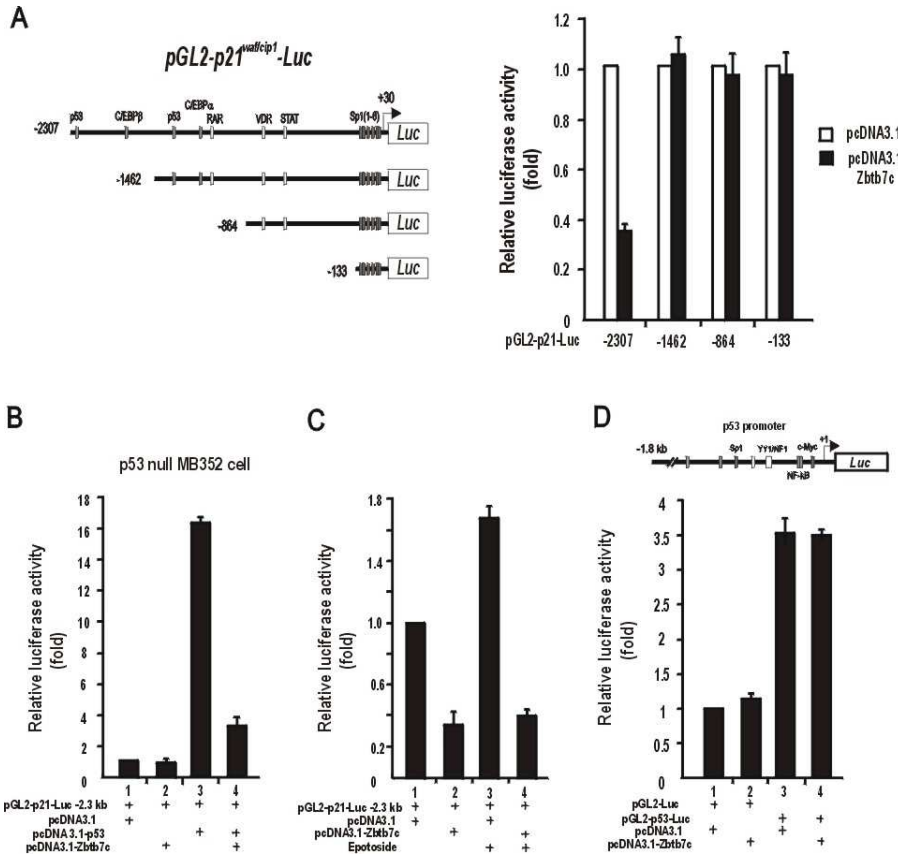


Figure 2. Transcriptional repression of the *p21*$^{Waf/Cip1}$ promoter by Zbtb7c requires tumor suppressor p53. ***(A)*** Structure of the *p21* promoter constructs tested. Plasmid expressing Zbtb7c and reporter plasmids were transiently co-transfected and analyzed for luciferase activity. Zbtb7c potently represses transcription of the p21$^{Waf/Cip1}$ promoter with -2.3 kb upstream regulatory sequence. ***(B, C)*** Plasmids expressing Zbtb7c, p53, and pGL2-p21-Luc were transiently co-transfected into MB352 MEF cells lacking endogenous p53. Zbtb7c potently represses transcription of *p21* promoter by 75% only when in the presence of co-transfected p53. When p53 expression is induced by etoposide, Zbtb7c strongly represses transcription of the *p21* promoter. ***(D)*** To

analyze whether or not Zbtb7c impacts transcription of the *p53* promoter, Zbtb7c expressing plasmid and pGL2-p53-Luc were transiently co-transfected and analyzed for luciferase activity. Zbtb7c does not repress transcription of the *p53* promoter.

4. Zbtb7c binding to the distal p53 binding region requires p53. Zbtb7c interacts directly with p53 via its zinc fingers.

Because Zbtb7c represses transcription of p21, we investigated whether or not Zbtb7c is targeted to the *p21* promoter and also which part of the promoter is involved in Zbtb7c binding via chromatin immunoprecipitation assays (ChIP). ChIP of the Zbtb7c overexpression 293Trex cells induced with Doxycyclin showed that Zbtb7c is specifically targeted to the distal p53 binding region (bp -2307 ~ -1947), but not targeted to other regions such as the C/EBPβ binding region (bp -1887 ~ -1507), the other p53 binding region (bp -1462 ~ -1128), and the proximal promoter (bp -261 ~ +39) (Fig. 3A).

Because transcriptional repression by Zbtb7c is only possible in the presence of p53, we tested by ChIP assays whether or not Zbtb7c binding to the distal p53 binding region relies on p53 in Saos-2 cells lacking p53. Saos-2 cells were transiently transfected with Zbtb7c-His expression vector in the presence or absence of the p53 expression vector. Ectopic p53 binds to the region regardless of the presence of Zbtb7c, but Zbtb7c binds to the region only in the presence of p53 (Fig. 3B).

The ChIP assays suggest but do not prove that Zbtb7c may be targeted to the *p21* distal promoter region by direct molecular interaction with p53. We tested this possibility by co-immunoprecipitation and GST-fusion protein pull-

down assays. Cell extracts prepared from control or stable 293Trex cells overexpressing FLAG-Zbtb7c were immunoprecipitated using M2 anti-FLAG antibody and analyzed with Western blot assays using antibody against p53. p53 and Zbtb7c interact with each other *in vivo* (Fig. 3C). We also investigated whether or not the molecular interaction between Zbtb7c and p53 is direct by *in vitro* GST-fusion protein pull-down assays. Recombinant GST-POZZbtb7c and GST-ZF of Zbtb7c were allowed to interact with *in vitro* translated [S^{35}]-methionine labeled p53. Only the GST-ZF domain, not the POZ-domain, interacts with the p53 polypeptide directly containing DNA binding and the oligomerization domain. The data indicated that Zbtb7c could be tethered to the *p21* distal promoter region by interacting directly with p53 using its zinc-finger domain (Fig. 3D, E).

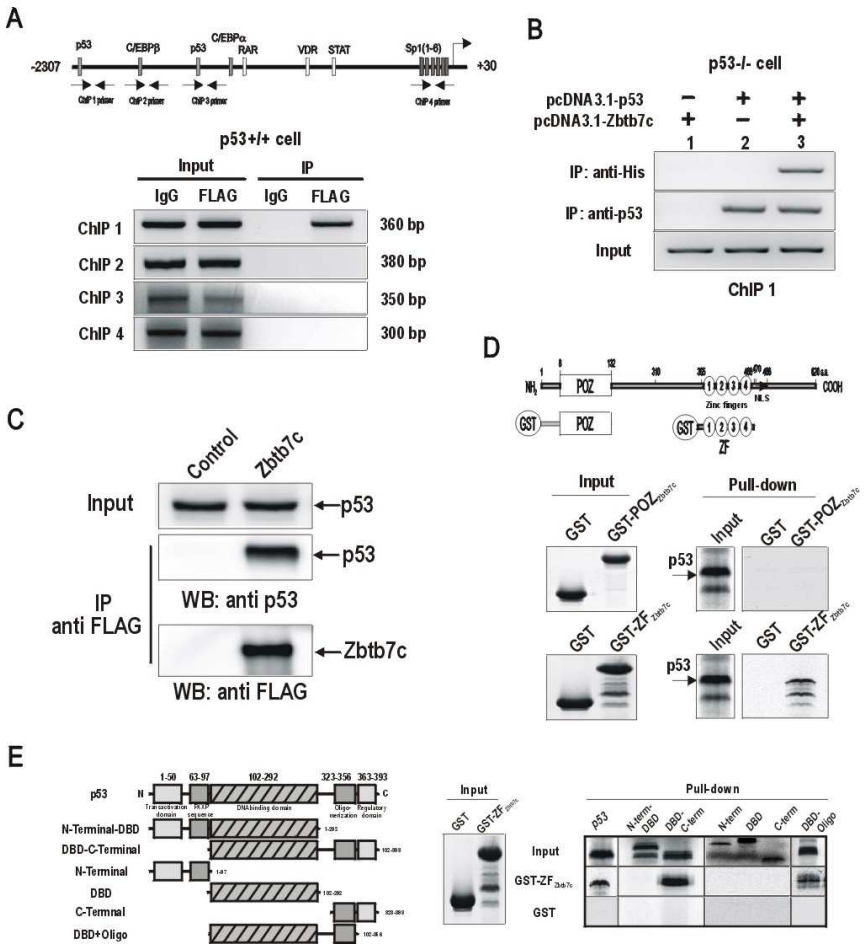


Figure 3. The ZF domain of Zbtb7c interacts with p53 *in vivo* and *in vitro*. ***(A)*** ChIP assay of the Zbtb7c binding site on the $p21^{Waf/Cip1}$ promoter in stable 293Trex-Zbtb7c cells with edogenous p53. Cells were induced with doxycyclin to express Zbtb7c-FLAG and immunoprecipitated with the antibodies indicated. Zbtb7c only binds to the distal p53 binding ChIP 1 region. ***(B)*** ChIP assay of Zbtb7c binding site on the $p21^{Waf/Cip1}$ promoter in Saos-2 cells lacking p53. Saos-2 cells lacking p53 were transfected with Zbtb7c and/or p53 expression plasmid, and immunoprecipitated with the antibodies indicated. Although p53 binds to the ChIP 1 region in the absence

or presence of Zbtb7c, Zbtb7c binds to the region only in the presence of p53. ***(C)*** Co-immunoprecipitation of Zbtb7c and p53. Lysates prepared from 293Trex cells and 293Trex-Zbtb7c cells were immunoprecipitated using the anti-FLAG antibody and analyzed using Western blotting with the anti-p53 antibody. ***(D, E)*** *In vitro* GST-fusion protein pull-down assays. Recombinant GST protein, GST-POZZbtb7c, or GST-ZFZbtb7c was incubated with [^{35}S]-methionine labeled p53 and its deletion mutants were pulled down and resolved with 15% SDS-PAGE. The gel was then exposed to X-ray film. Input, 10% of the p53 mutants added in the binding reactions. The zinc-finger domain of Zbtb7c interacts with the p53 fragment, including the DNA binding domain and oligomerization.

5. The POZ domain of Zbtb7c interacts with corepressors *in vivo* and *in vitro*.

Once targeted to the distal promoter region, Zbtb7c repressed transcription of *p21*, but mutant Zbtb7c△POZ did not. The data suggest that the POZ-domain is essential in transcriptional repression (Fig. 4A). Transcriptional repressors often repress transcription through interaction with corepressors such as mSin3A, SMRT, NCoR, and BCoR[8,11,17,20]. Mammalian two-hybrid assays in CV-1 cells using pG5-Luc, pGal4-POZZbtb7c, and pVP16-Corepressor fusion expression plasmids demonstrated that the POZ-domain interacts with NCoR and BCoR, but not with SMRT and mSin3A. The POZ-domain had particularly strong interaction with NCoR (Fig. 4B). Also, coimmunoprecipitation of the CV-1 cell extracts cotransfected with pGal4-POZZbtb7c and pVP16-corepressor fusion protein expression vectors showed that the POZ-domain of Zbtb7c interacts with NCoR or BCoR *in vivo* (Fig. 4C). GST-fusion protein pull-down assays using the recombinant GST-POZZbtb7c protein and *in vitro* translated [S^{35}]-Methionine labeled corepressor polypeptides also showed that POZZbtb7c can interact directly with NCoR or BCoR, but not with SMRT and mSin3A (Fig. 4D).

Often, corepressor complexes recruited by transcriptional repressors contain HDAC (histone deacetylase) proteins. These HDACs deacetylate the

histones of the nearby nucleosomes. Accordingly, we investigated whether or not HDAC inhibitor treatment of the CV-1 or HeLa cells cotransfected with pG5-Luc and pGal4-POZZbtb7c or pGL2-p21-Luc and pcDNA3.1-Zbtb7c affected the transcription of the reporter gene. HDAC inhibitor TSA significantly affected transcriptional repression by Zbtb7c, leading to an increase in transcription. This suggests the involvement of HDACs in transcriptional repression by Zbtb7c (Fig. 4E).

The proximal promoter of *p21* contains six GC-boxes recognized by Sp1-family transcription factors. These GC-boxes are critical in transcriptional regulation. Sp1, bound at the proximal promoter, was shown to communicate with p53 bound to the distal promoter via a looping mechanism that activates transcription[13]. Accordingly, we investigated whether or not the p53-Zbtb7c-Corepressor-HDAC complex could deacetylate H3 and H4 histones bound at the proximal promoter region. The ChIP assay showed that Zbtb7c significantly decreased acetylated histones 3 and 4 at the proximal promoter. The data suggest that histone deacetylation at the proximal promoter is probably responsible for transcriptional repression by the complex containing p53-Zbtb7c-corepressor-HDAC (Fig. 4F).

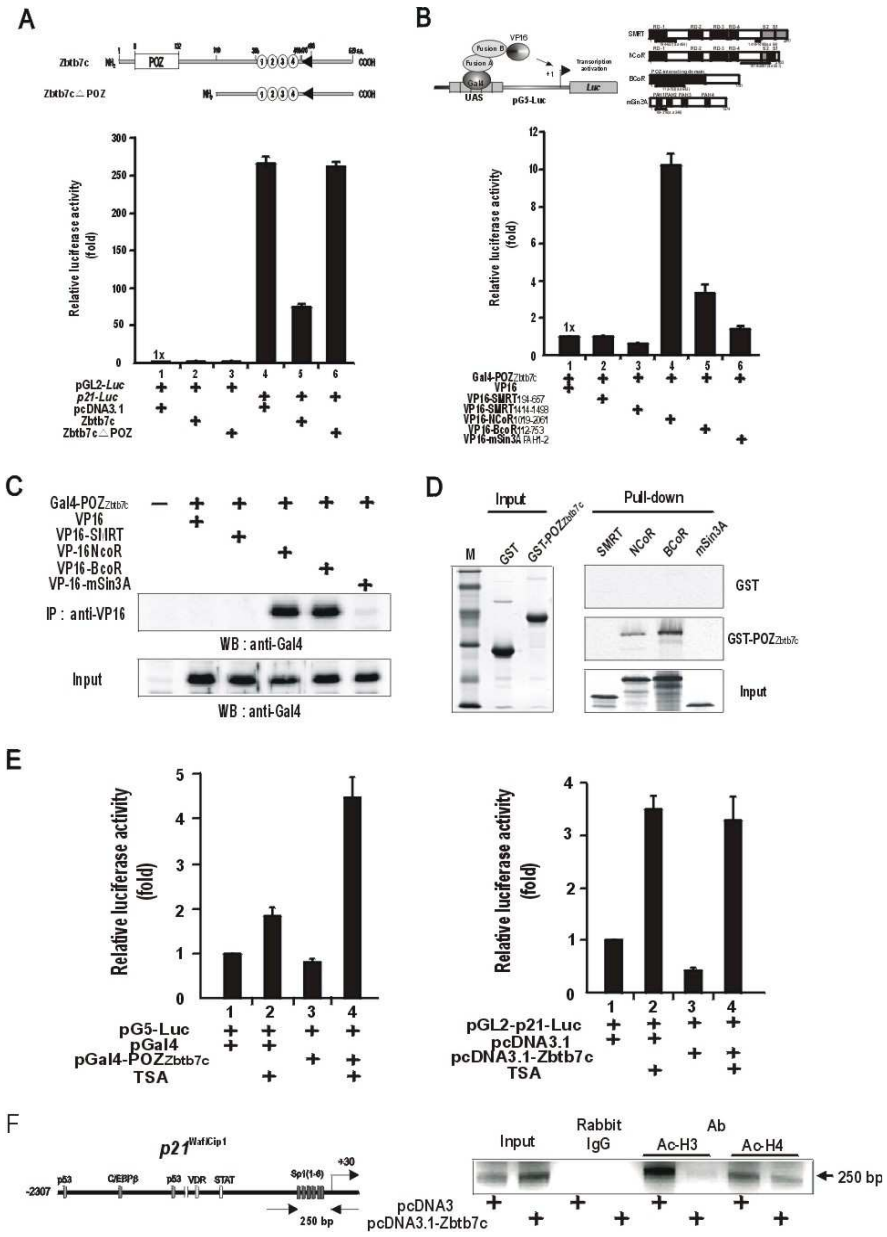


Figure 4. The POZ domain of Zbtb7c interacts directly with the corepressores *in vitro* and *in vivo*. *(A)* Diagram of Zbtb7c and Zbtb7c△POZ

lacking POZ-domain. The reporter pGL2-p21$^{Waf/Cip1}$-Luc and Zbtb7c, or its deletion mutant expression plasmids, were transiently co-transfected and luciferase activities were measured. The POZ-domain deletion mutant does not repress transcription of the p21 promoter. ***(B)*** Mammalian two-hybrid assays of protein-protein interaction between the POZ-domain and corepressor proteins. CV-1 cells were transfected with pG5-Luc, pGal4-POZZbtb7c, and pVP16-Corepressor expression plasmids. ***(C)*** Co-immunoprecipitation of Zbtb7c and corepressors, NCoR and BCoR. Cell extracts prepared from CV-1 cells co-transfected with pGal4-POZZbtb7c and pVP16-corepressor expression plasmids were immunoprecipitated with the anti-VP16 antibody and precipitates were analyzed using Western blot analysis with the anti-Gal4 antibody. ***(D)*** GST-fusion protein pull-down assays. Recombinant GST protein, or GST-POZZbtb7c, was incubated with *in vitro* synthesized [^{35}S]-methionine-labeled corepressors, pulled down, and resolved with 12% SDS-PAGE. The resulting gel was exposed to X-ray film. Input, 10% of the corepressors added in the binding reactions. ***(E)*** Plasmid mixtures of pG5-Luc and the pGal4-POZZbtb7c expression vector in CV-1 cells or pGL2-p21-Luc and the Zbtb7c expression vector were transiently co-transfected into HeLa cells. TSA treatment potently derepressed transcription of reporter genes, suggesting HDACs involvement in transcriptional repression via the POZ-domain of the Zbtb7c-corepressor. ***(F)*** ChIP assays of histone modification at the proximal promoter of the p21$^{Waf/Cip1}$ gene using antibodies against Ac-H3 and Ac-H4. HeLa cells were transfected with Zbtb7c and immunoprecipitated with the antibodies indicated. FBI-1 deacetylates histone Ac-H3 and Ac-H4 at the p21 proximal promoter region.

6. Zbtb7c induces cellular transformation and stimulates cell proliferation of NIH/3T3 and HeLa cells.

Because p21, a major negative regulator of cell cycle progression, is potently repressed by Zbtb7c at the transcriptional level, Zbtb7c may cause cellular transformation and promote cell cycle progression. We tested whether or not Zbtb7c could form foci of transformation in HeLa cells transfected with the Zbtb7c expression vector. The transfected cells formed a substantial number of foci, suggesting that Zbtb7c causes cellular transformation (Fig. 5A).

To investigate the effect of Zbtb7c on cellular proliferation, doxycyclin inducible stable 293Trex-Zbtb7c cells overexpressing Zbtb7c were analyzed by FACS. The percentage of cells in S phase increased from 29.7% to 50.3% through Zbtb7c following induction with doxycyclin. Cells in the G2-M phase decreased from 8.6% to 0.5% following induction. Alternatively, stable NIH/3T3-Zbtb7c cells expressing Zbtb7c constitutively were treated with an adenovirus expressing shRNA against Zbtb7c and analyzed using FACS. The percentage of cells in S phase decreased from 52.3% to 28.7% and cells in the G2-M phase decreased from 7.6% to 9.2% (Fig. 5B). Additionally, the effect of Zbtb7c overexpression on cellular proliferation was examined using BrdUrd incorporation as an index of cell proliferation. In NIH/3T3 cells, the

percentage of cells incorporating BrdUrd significantly increased from 2% to 37% in the cells stably expresssing Zbtb7c. Alternatively, knock-down of Zbtb7c expression by RNA interference of stable NIH3T3-Zbtb7c cells, yielded a significant decrease in the number of BrdU positive cells. Again, the data suggest that Zbtb7c promotes cell proliferation (Fig. 5C).

We also investigated whether or not Zbtb7c affects growth of brain glyoma U343 tumor cells via MTT assay. We infected U343 tumor cells with either the control adenovirus dl324 or the recombinant adenovirus overexpressing Zbtb7c, Ad-dl324-Zbtb7c. The MTT assay revealed that the U343 cells infected with Ad-dl324-Zbtb7c virus proliferated five times faster by day 4 compared to the control cells transfected with Ad-dl324 (Fig. 5D). We also observed, using the MTT assay, that RNA interference of Zbtb7c expression through infection with a recombinant adenovirus expressing shRNA against Zbtb7c mRNA in NIH/3T3-Zbtb7c cells inhibited cell proliferation (Fig. 5E). The data discussed above are in line with microscopic observations showing drastically increased cell proliferation of the stable NIH/3T3-Zbtb7c cells compared to control NIH/3T3 cells, both cultured for 72 hrs (Supplementary Fig. 4A, B).

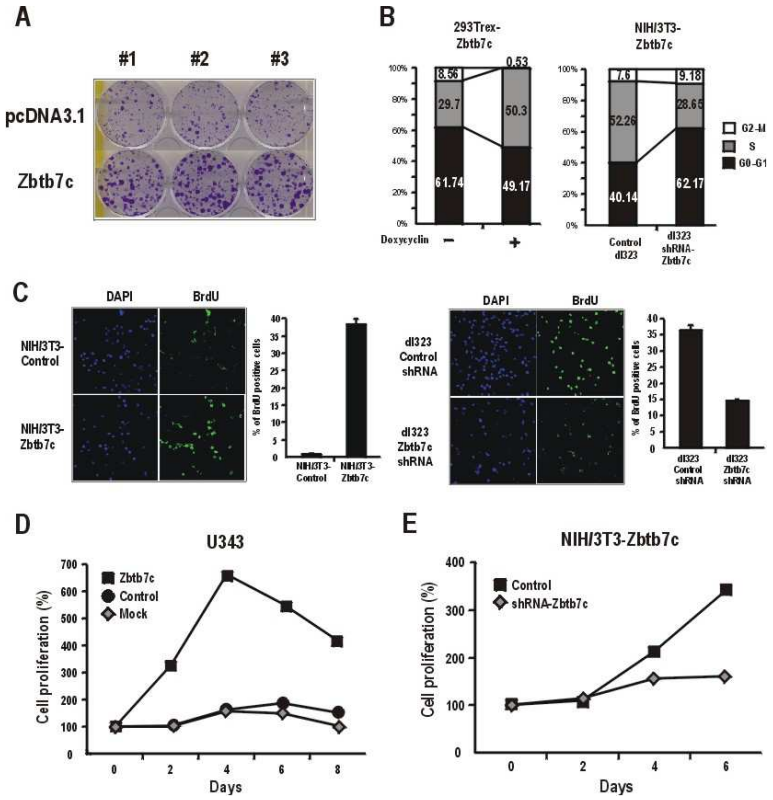


Figure 5. Zbtb7c causes cellular transformation and promotes cell cycle progression and proliferation. ***(A)*** Foci-formation assays. HeLa cells transfected with the Zbtb7C expression vector were selected by culturing the cells in a medium containing G418 for two weeks. Cells were fixed with 4% formaldehyde and stained with 0.1% crystal violet. The foci of the cells transfected with Zbtb7C expression vector were larger than those of the control cells. ***(B)*** FACS analysis. Stable 293Trex-Zbtb7c cells grown in the absence or presence of Doxycyclin and stable NIH/3T3-Zbtb7c cells infected with the recombinant adenovirus dl324 control (dl324-shRNA-Zbtb7c) were stained with propidium iodide and subjected to FACS analysis. When Zbtb7c expression increased, the number of cells in S-phase significantly increased and the number of cells in G2-M phase decreased. Alternatively, knock-down

of Zbtb7c expression yielded a significant decrease in the number of cells in S-phase. ***(C)*** BrdU incorporation assay. NIH/3T3 and NIH/3T3-Zbtb7C cells were grown in DMEM media containing BrdU. The cells were fixed in 3.7% formaldehyde, rinsed, and treated with 2N HCl. Cells were then incubated with anti-BrdU antibody, and further incubated with goat Cy2-conjugated anti-mouse secondary antibody. The cells were treated with DAPI, and those cells that had incorporated BrdU were counted. NIH/3T3-Zbtb7c cells demonstrated incorporation levels that were 19 times higher than NIH/3T3 control cells. Alternatively, knock-down of Zbtb7c expression yielded a significant decrease in the number of BrdU positive cells. ***(D)*** Brain glyoma cancer U343cells were infected with either 50 MOI of dl324 or dl324-Zbtb7c. Cell proliferation was determined by measuring the conversion of tetrazolium salt MTT to formazan, as described above. ***(E)*** MTT assay. Effect of Zbtb7c on cell proliferation of NIH/3T3-Zbtb7c. NIH/3T3-Zbtb7c cells were infected with either 50 MOI of dl324 or dl324-shRNA Zbtb7c. Cell proliferation was determined by measuring the conversion of tetrazolium salt MTT to formazan. After incubation at 37℃, 20 μl of MTT was added to each well. After 4 hrs incubation at 37℃, plates were read on a microplate reader at 540 nm.

7. Zbtb7c promotes potent tumor growth.

We also examined whether or not adenovirus Ad-dl324-Zbtb7c can promote tumor growth when administered intra-tumorally. After U343 tumor cells were implanted under the skin of female BALB/c mice, recombinant Ad-dl324-Zbtb7c or control, Ad-dl324, was injected into the tumor. Ad-dl324-Zbtb7c substantially promoted growth of the U343 tumors, while the control, Ad-dl324, did not promote growth. By day 60 of treatment, tumors treated with Ad-dl324-Zbtb7c reached an average volume of 1190.5 mm^3, and those treated with control Ad-dl324 reached only 390.4 mm^3, suggesting that Zbtb7c promotes significant tumor growth. Alternatively, we investigated whether tumor growth can be inhibited through knock-down of Zbtb7c expression by infecting it with a recombinant adenovirus expressing shRNA against Zbtb7c mRNA. RNA interference potently inhibited tumor growth, while the control virus gave no indication that inhibition of tumor growth occurred (Fig. 6). Overall, the two lines of data above suggest that Zbtb7c significantly promotes tumor growth by promoting cell proliferation.

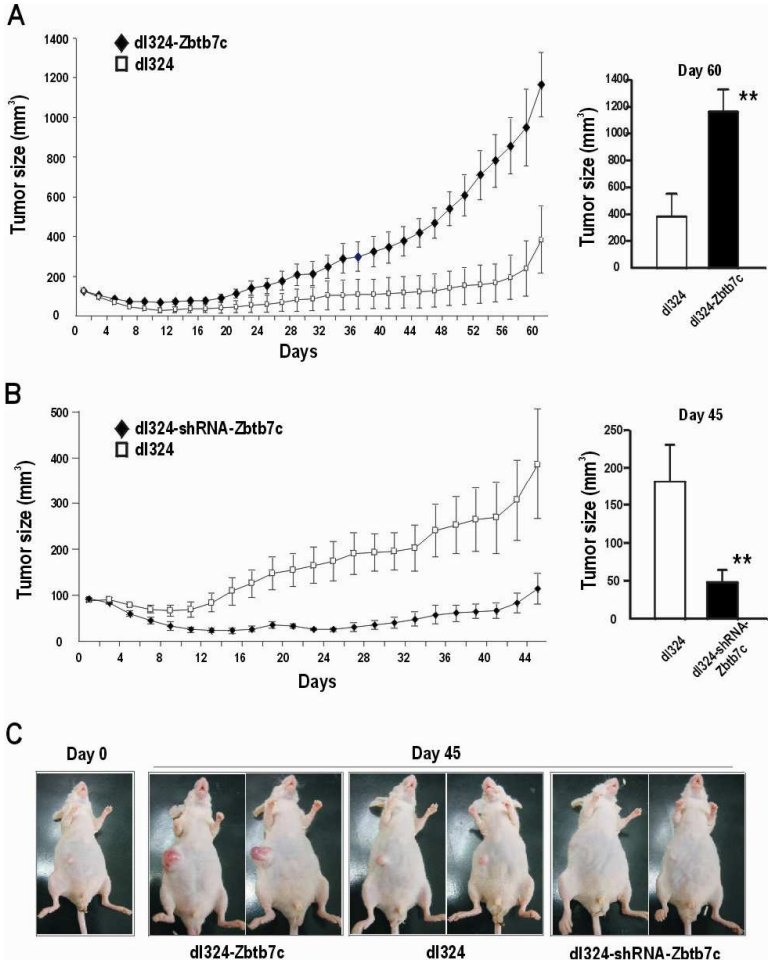


Figure 6. Zbtb7C promotes tumor growth and the knock-down of Zbtb7c inhibits tumor growth. ***(A)*** Brain glyoma U343 cells were implanted under the skin of the abdomen of anesthetized mice. Once the volume of the tumor volume reached 100-120 mm^3, mice were injected three times with either the control dl324 or dl324-Zbtb7C adenovirus and tumor volume was measured every other day. The tumor volume of dl324-Zbtb7c mice increased to about 3.3 times the size of the dl324 control. ***(B)*** Brain glyoma U343 cells were implanted in mice. When tumor volume in the mice reached 100-120 mm^3, they were injected five times with either the control dl324 or the dl324-

shRNA Zbtb7c adenovirus. Tumor volume markedly decreased with the dl324-shRNA Zbtb7c adenoviruses. ***(C)*** Pictures of mice injected with the control dl324 adenovirus, dl324/Zbtb7c, and dl324-shRNA Zbtb7c. Zbtb7c overexpression increased tumor growth and knock-down of Zbtb7c decreased tumor growth. Data are reported as the mean ± SEM. **P< 0.05 versus control dl324.

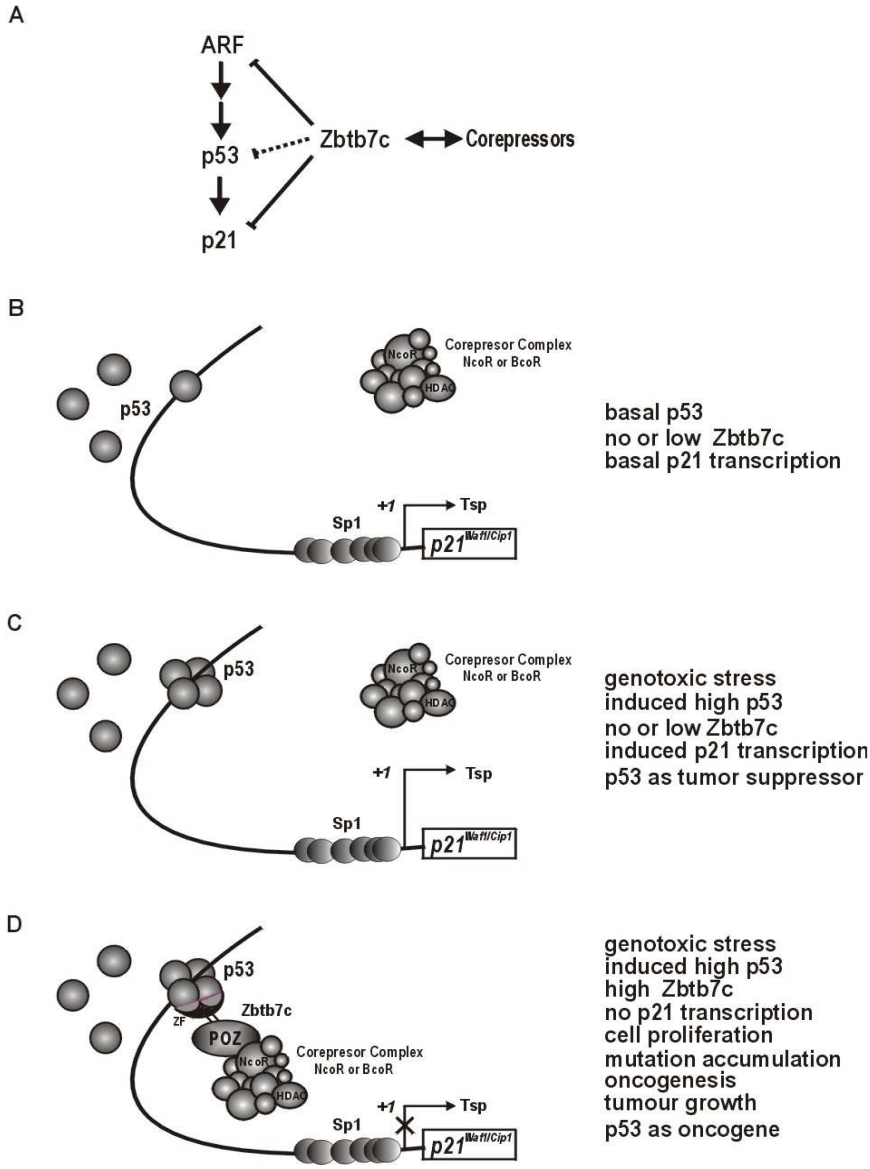
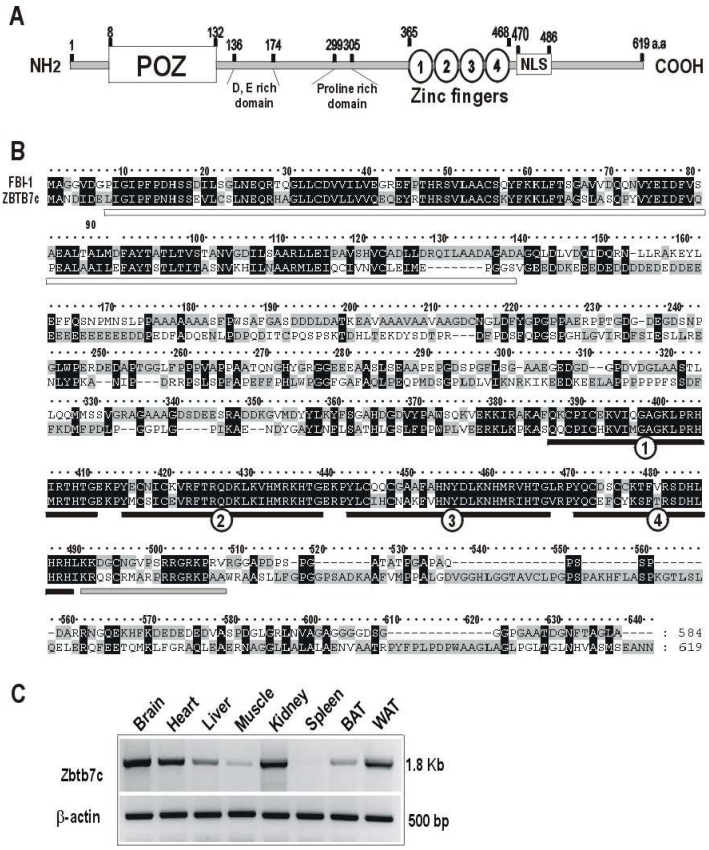
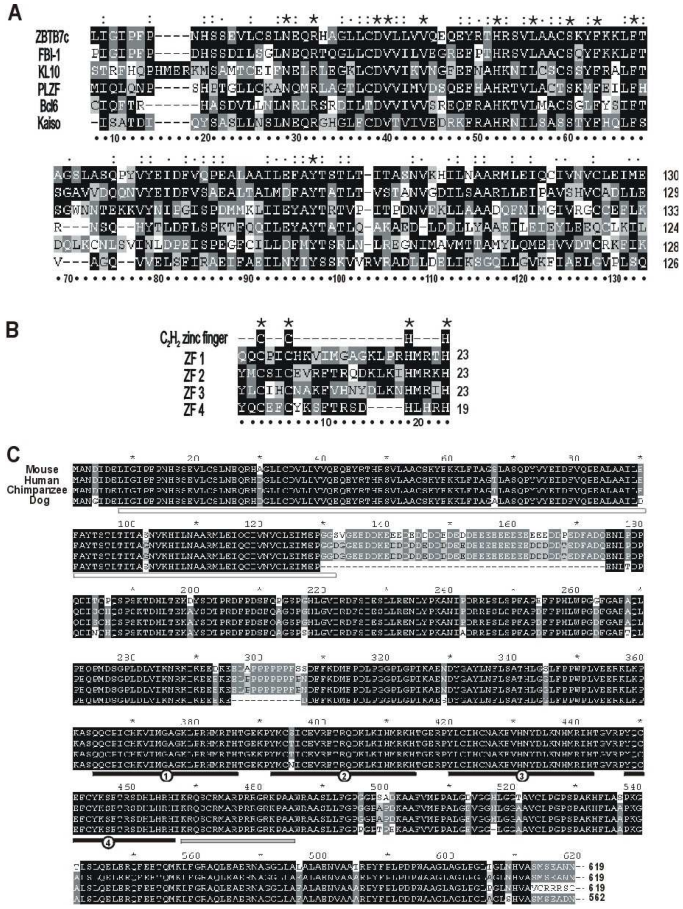


Figure 7. Model of transcriptional regulation of the Arf-p53-p21 pathway, particularly *p21*$^{Waf/Cip1}$, by Zbtb7c. ***(A)*** Diagram of the regulatory role of Zbtb7c on the Arf-p53-p21 pathway. Zbtb7c represses transcription of the Arf and p21 genes. The solid arrow line refers to transcriptional repression. The

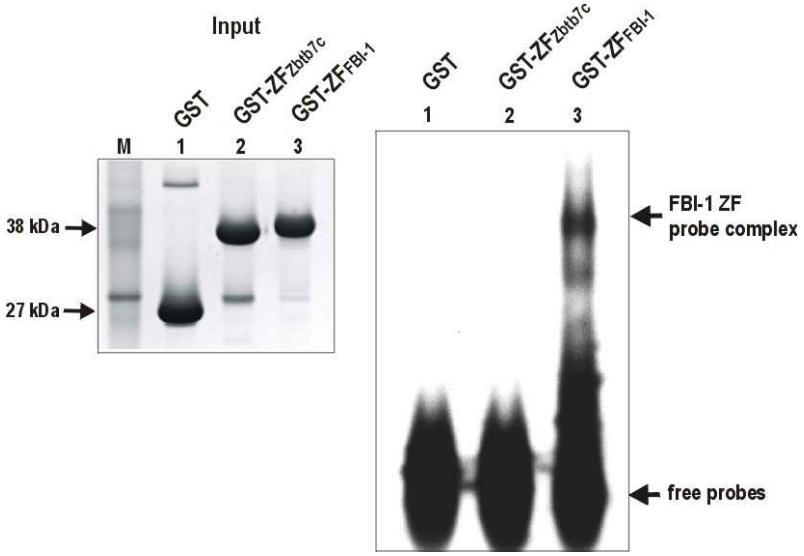
dotted line refers to protein interaction and inhibition of p53 dependent transcription activation. The double arrow line refers to molecular interaction. ***(B)*** Transcriptional regulation of the p21 gene in three different physiological states. In the normal state, where p53 and Zbtb7c expression are low, transcription of the p21 gene is controlled mainly by Sp1 family transcription factors. ***(C)*** When cells are challenged by stress (i.e. genotoxic stress) and the expression of Zbtb7c is low, p53 binds to the upstream regulatory element and communicates with proximal promoter bound Sp1. This potently induces p21 gene expression by arresting cell cycles and allowing time to repair DNA damage. ***(D)*** When expression of both p53 and Zbtb7c is high, as in cancer cells, Zbtb7c is recruited to the distal p53 binding element through interaction with p53 via its zinc fingers. The p53-Zbtb7c complex, in turn, recruits the corepressor-HDAC complex to deacetylated Ac-H3 and AcH4 histones at the proximal promoter where they potently repress transcription. Zbtb7c inhibits the p53-dependent transcriptional activation of p21. As a result, the role of p53 is dramatically changed from tumor suppressor to oncogenic factor because of Zbtb7c. Tsp, transcription start point.



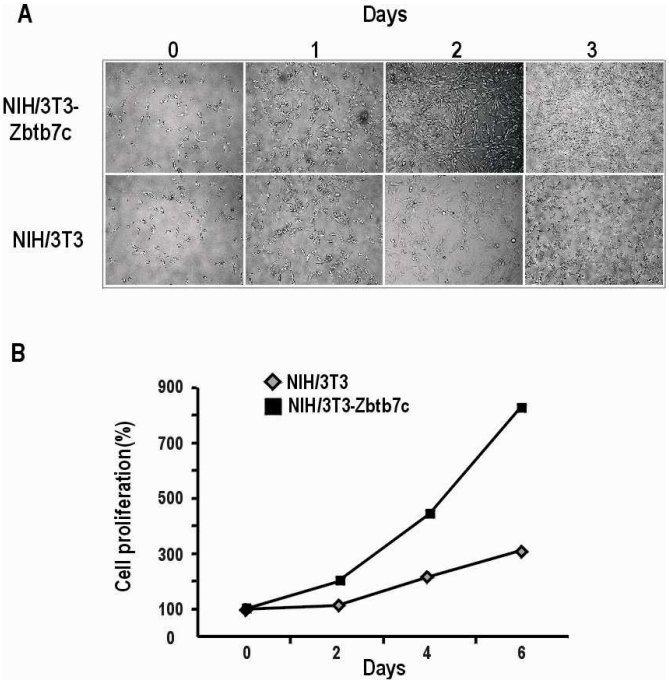
Supplement Figure 1. Zbtb7c is a ubiquitous POZ-domain Krüppel-like zinc finger protein. ***(A)*** Structure of Zbtb7c protein. Zbtb7c contains a POZ domain, D and E rich domain, Proline track, four zinc fingers, and a nuclear localization sequence. homologous to FBI-1 only in two domains, the POZ-domain and the zinc-finger domain. POZ domain, open box; Circles, zinc fingers; NLS, nuclear localization sequence. ***(B)*** Amino acid sequence comparison of Zbtb7c and FBI-1 using ClustalW software. Conserved amino acids are shaded black. The unfilled white bar is a POZ-domain and the solid black line with numbers refers to the C_2H_2 type zinc fingers. ***(C)*** Tissue distributions of Zbtb7c mRNA expression. RT-PCR analysis of cDNA prepared from total RNA of FVB mouse tissues.



Supplement Figure 2. Amino acid sequence comparison of mammalian Zbtb7c. ***(A)*** Amino acid sequence comparison of the POZ-domain of Zbtb7c with other mammalian POZ domains, generated by ClustalW software. The most highly conserved amino acids are shaded. Asterisks indicate amino acids that are perfectly conserved among all of the sequences compared. Dots indicate amino acids that are functionally similar. ***(B)*** Amino acid sequence comparison of Zbtb7C zinc fingers one to four with the C_2H_2 Krüppel-like zinc finger motif. ***(C)*** Amino acid sequence comparisons of four mammalian Zbtb7C types using Clustal W software. The open bar indicates the POZ domain, the solid black circles indicate zinc fingers, and the gray bar indicates the nuclear localization sequence (NLS).



Supplement Figure 3. EMSA of recombinant GST-Zbtb7c and FBI-1 zinc finger DNA binding domains. Zbtb7c does not bind to random oligonucleotide DNA probes. ***(A)*** SDS-PAGE of the recombinant GST or GST-fusion proteins used in EMSA. ***(B)*** EMSA. The [α-^{32}P]-dATP labeled random oligonucleotide probe mixture was incubated with the recombinant GST-Zbtb7c or the FBI-1 zinc finger DNA binding domain (2 μg) according to standard EMSA protocol. The protein-DNA complexes were separated using 4% non-denaturing polyacrylamide gel eletrophoresis at room temperature. The gels were then exposed to X-ray film with the Kodak intensifying screen (Kodak, NY). Probes bound to the zinc fingers of FBI-1 but not to zinc fingers of Zbtb7c or GST.



Supplement Figure 4. Cell proliferation of control NIH/3T3 and stable NIH/3T3-Zbtb7c cells. ***(A)*** NIH/3T3 and NIH/3T3-Zbtb7c cells (5x 10^5) were plated on a 6-well dish and cell proliferation was photographed daily. NIH/3T3-Zbtb7c cells grew much faster than NIH/3T3 cells. ***(B)*** MTT assay. To evaluate Zbtb7c cell proliferation effects, the cells were plated on 96-well plates. The proliferation of NIH/3T3 or NIH/3T3-Zbtb7c cells was determined by measuring the conversion of tetrazolium salt MTT to formazan. After cells were incubated at 37℃, 20 μl of MTT was added to each well. After 2 hrs, plates were read on a microplate reader at 570 nm. Again, NIH/3T3-Zbtb7c cells grew much faster than NIH/3T3 cells.

IV. Discussion

We investigated whether or not a novel member of the POK family Zbtb7c could regulate genes of the Arf-Mdm2-p53-p21 pathway, in order to illustrate some of the key biological functions carried out by Zbtb7c. We showed that Zbtb7c potently repressed Arf, p53, and p21 at transcriptional or protein levels and acts as a protooncogene in the presence of p53, causing cellular transformation and promoting cell cycle progression, cell proliferation, and tumor growth.

p53 tumour suppressor is a key transcription activator of *p21* gene and plays a crucial role in mediating growth arrest when cells are exposed to DNA damaging agents[6,7]. In this report, we have shown that, in a sence, p53 can act as transcription represssor in the presence of Zbtb7c or help the repressor activity of Zbtb7c. Previous report reports by others and microarray experiments clearly showed that p53 can act as transcription activator or repressor, as with many other transcription factors although less is known for the repressor side of p53 action[9,14,26]. The ability of p53 to repress transcription at various viral and cellular promoters has been known for some times[9]. p53 was shown to repress transcription by displacing or by interacting with transcription activators such as Sp1, glucocorticoid receptors, thyroid

hormone receptors, AP1, C/EBP, and hepatocyte nuclear factor (HNF4α1) or basal transcription factors such as TBP[22]. p53 bound onto the promoter interacted with mSin3A to repress transcription through recruitment of histone deacetylate and chromatin remodeling as revealed by ChIP assays[19].

The transcription repression of *p21* gene by Zbtb7c and p53 is unique and involves direct molecular interaction between p53 bound onto the distal promoter and Zbtb7c. In this case, p53 acts as an anchoring molecular dock for Zbtb7c to sit on the *p21* gene distal promoter. Subsequent recruitment of corepressor-HDAC complex to Zbtb7c-p53 complex bound onto distal promoter resulted in deacetylation of H3 and H4 histones at the proximal promoter of *p21* gene.

Although, the fundamental function of tumor suppressor p53 is to initiate various cellular defense mechanisms to counteract the DNA damage, the induction of p53 by DNA damaging agents can be detrimental in the presence of overexpressed Zbtb7c because p53 acts as an oncogenic factor instead. Molecular interaction between the Zbtb7c and p53 can block cell cycle regulators of Arf-Mdm2-p53-p21 pathway because Zbtb7c, and p53 wt or mutant form of p53 are often overexpressed in cancers. Zbtb7c is a potent protooncogene that can act in oncogenic transformation through repression of the Arf-Mdm2-p53-p21 pathway.

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국문요약

종양 억제 유전자 p53의 분자적인 기능을 조절하는 새로운 proto-oncogene Zbtb7c

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전 부 남

우리 연구팀은 POZ domain 계열 FBI-1의 세포 기능 조절에 대한 연구를 진행하고 있다. FBI-1과 밀접한 관계의 새로운 BTB/POZ domain protein에 관한 연구를 진행하기 위해 GeneBank database를 기초로 사람의 모든 BTB/POZ domain protein을 검색하여 BTB/POZ domain protein간의 amino acid sequence의 similarity를 분석하여 BTB/POZ polypeptides를 31개의 주요 그룹으로 분류해 phylogenic tree를 완성했다. 우리는 FBI-1과 가장 높은 homology를 가지고 있는 Zbtb7c를 발견하게 되었고 POZ domain 및 zinc finger motif만을 비교했을 때 각각 90%이상의 homology를 가지고 있는 것을 확인했다. Zbtb7c는 총 619개의 amino acid로 이루어져 있으며 구조적으로 FBI-1과 거의 유사하게 N-terminus에 POZ domain, C-terminus에 4개의

Kruppel-like zinc finger motif, 그리고 nuclear localization signal을 가지고 있었다.

Zbtb7c 는 human papillomavirus (HPV)68 의 E6 와 E7 gene 이 숙주세포의 chromosome 18q21 에 해당되는 곳에 integration 된 자궁 암세포에서 처음 분리되었고, 그 발현의 조직분포가 제한적인 것으로 생각되었지만, 우리는 RT-PCR 을 통해 mouse 에서 spleen 을 제외한 거의 모든 tissue 에서 Zbtb7c 가 발현되고 있음을 확인하게 되었다. 최근 FBI-1 이 세포주기를 촉진시킨다는 보고로 proto-oncogene 으로서 주목받고 있는데 우리는 아마도 Zbtb7c 역시 세포주기에 관계되는 Arf-Mdm2-p53-$p21^{Waf/Cip1}$ 과 RB 의 promoter 에 영향을 끼칠 것으로 예측하고, luciferase assay 를 실시하였다. 흥미롭게도, FBI-1 이 거의 모든 promoter 의 전사를 억제하는 것에 반해 Zbtb7c 는 오직 Arf 와 p21 의 전사만을 억제하는 것을 관찰하였다.

특히 우리는 p21 이 세포주기조절에 있어 최종적으로 기여하는 바가 크다고 판단하여 p21 의 전사를 억제하는 Zbtb7c 의 정확한 작용기전을 연구하고자 하였다. 그래서 promoter reporter assay, EMSA, ChIP, GST-pull down, 그리고 Immunoprecipitation assay 등을 실시한 결과 Zbtb7c 는 그것의 zinc finger 를 통해 p21 promoter 상의 distal 부분에 결합하는 p53 과 상호작용하는 것으로 나타났다. 마찬가지 위와 같은 실험방법을 통해 Zbtb7c 는 BTB/POZ domain 을 통해 NCoR 나

BCoR 와 같은 corepressor 와의 상호작용을 하는 것으로 나타났고 이를 통해 최종적으로 p21 proximal 부분의 Histone 을 deacetylation 시킴으로써 전사를 억제시킨다는 것을 알게 되었다. 뿐만 아니라, 이러한 전사적 억제효과가 현상적으로 잘 드러나는지를 관찰하기 위해 foci formation, FACS, BrdUrd, 그리고 MTT assay 를 실시한 결과 Zbtb7c 가 과발현 하게되면 세포주기중 S phase 의 양이 급격히 증가함으로써 세포주기가 급속히 촉진되는 것을 확인할 수 있었다.

최종적으로 이런 현상이 mouse 의 종양성장에 어떤 영향을 미치는지 알아보고자 인위적으로 mouse 에 종양을 형성시키고 Zbtb7c 를 과발현 시킬 수 있는 virus 를 infection 시켰을 때 그 종양의 크기가 대조군에 비해 현저하게 커진 것을 관찰할 수 있었으며 반대로 Zbtb7c 의 발현을 억제시키는 virus 를 infection 시켰을 때는 종양의 크기가 감소하는 것을 관찰할 수 있었다.

이렇듯 위와 같은 Zbtb7c 에 관한 본 연구는 세포주기조절에 관한 작용점의 파악과 발암 과정의 이해증진, 그리고 새로운 치료 방법의 도출에 필요한 중요한 연구 자료가 될 것으로 생각된다.

핵심 단어 : BTB/POZ domain, zinc-finger domain, Zbtb7c, 전사인자, 세포주기, 암유전자, Arf-Mdm2-p53-p21$^{Waf/Cip1}$, 유전자 전사억제자 (NCoR, BCoR), histone 조절