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ZBTB2, a Novel Master Regulator of the p53 Pathway

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We found that ZBTB2, a POK family transcription factor, is a potent repressor of the ARF-HDM2-p53-p21 pathway important in cell cycle regulation. ZBTB2 repressed transcription of the ARF, p53, and p21 genes, but activated the HDM2 gene. In particular, ZBTB2 repressed transcription of the p21 gene by acting on the two distal p53 binding elements and the proximal Sp1 binding GC-box 5/6 elements. ZBTB2 directly interacted with Sp1 via its POZ domain and zinc fingers, which was important in the repression of transcription activation by Sp1. ZBTB2 and Sp1 competed with each other in binding to the GC-box 5/6 elements and the two p53 binding elements. ZBTB2 directly interacted with p53 via its zinc fingers, inhibiting p53 binding and repressing transcription activation by p53. The POZ domain, required for transcription repression, interacted with corepressors such as BCoR, NCoR, and SMRT. The interactions deacetylated histones Ac-H3 and Ac-H4 at the proximal promoter. Although ectopic ZBTB2 stimulated cell proliferation, knockdown of ZBTB2 expression decreased cell proliferation and DNA synthesis. Overall, our data suggest that ZBTB2 is a potent proto-oncogenic master control gene of the p53 pathway and, in particular, is a potent transcription repressor of the cell cycle arrest gene p21 by inhibiting p53 and Sp1.

The POZ domain is an evolutionarily conserved protein-protein interaction motif found in many cellular regulatory proteins (1, 2). POZ domain genes, first identified in Drosophila and poxvirus, have since been found in organisms ranging from yeast to humans (3, 4). As many as 184 known human proteins, 96 Drosophila proteins, and 137 Caenorhabditis elegans proteins are estimated to contain the POZ domain.

POZ domain proteins are involved in many critical cellular processes such as apoptosis (5), development (6, 7), ion channel activity (4), oncogenesis (8–10), and transcription (10–16). In particular, some of the POZ domain Krüppel-like zinc finger (POK) proteins are the major determinants of development, differentiation, and oncogenesis. PLZF-null mice display severe defects in limb development and germ stem cell maintenance (7, 17). T helper-inducing POZ/Krüppel-like factor (Th-POK/eKrox) has been recently reported as a master regulator of T-cell lineage commitment (18). BCL-6, PLZF, and HIC1 have been implicated in non-Hodgkin lymphoma, acute promyelocytic leukemia, and spontaneous malignant tumors, respectively (8, 9, 19). Recently, FBI-1 (also called Pokemon) has been shown to act as a proto-oncogene by repressing transcription of the act gene, causing down-regulation of p53 and promoting oncogenic cellular transformation (10).

The most striking property of some POZ domain transcription factors is their ability to repress transcription via their POZ domains (10–16, 20), although a few POZ domain transcription factors activate transcription (21, 22). This characteristic probably underlies many biological processes controlled by these factors. The ability of the domain to interact with key regulatory proteins such as corepressor proteins and other transcription factors appears to be important for repression. In particular, the POZ domains of human BCL-6, FBI-1, HIC-1, and PLZF interact with BCoR, histone deacetylase, mSin3A, and SMRT/N-CoR (12–16, 20, 23).

The cyclin-dependent kinase inhibitor p21 is a major player in cell cycle arrest in mammalian cells and the downstream cell cycle regulator of the ARF-HDM2-p53-p21 pathway (Refs. 24 and 25 and references therein). The p21 gene, mainly regulated at the transcriptional level, 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 (Refs. 24–26 and references therein). Overexpression of p21 results in G1-, G2-, or S-phase arrest upon exposure to DNA-damaging agents (27–29). Whereas induction of p21 predominantly leads to cell cycle arrest, repression of p21 may have a variety of

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The abbreviations used are: POK, POZ domain Krüppel-like zinc finger; ARF, alternative reading frame gene; BCL-6, B-cell lymphoma-6; BCoR, BCL-6 interacting corepressor; BTB, bric-a-brac tramtrack broad complex; POZ, poxvirus and zinc finger; ChIP, chromatin immunoprecipitation; CV-1, African green monkey kidney cell, EMSA, electromobility shift assay; FACS, fluorescence-activated cell sorter; FBI-1, factor that binds to the inducer of short transcripts of human immunodeficiency virus-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; HDM2, human analogue of mouse double minute oncogene; LacZ, β-galactosidase gene; Luc, luciferase; NCoR, nuclear receptor corepressor; PLZF, promyelocytic leukemia zinc finger protein; Rb, retinoblastoma; SMRT, silencing mediator for retinoid and thyroid receptors; Sp1, specificity protein 1; RT, reverse transcription; siRNA, small interfering RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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outcomes depending on the cellular context (Ref. 26 and references therein, and Ref. 27). Aside from p53, a variety of other factors, including Smads, AP2, STAT, BRCA1, E2F-1/E2F-3, and C/EBPα and -β, activate the transcription of p21. In addition to its role in responding to DNA damage, p21 has also been implicated in terminal differentiation, replicative senescence, and protection from p53-dependent and -independent apoptosis (Ref. 26 and references therein).

Sp1 family transcription factors that bind at the proximal promoter (bp −120 to −50) of the p21 gene represent another group of major regulators that affect p21 gene expression (Ref. 26 and references therein). Sp1 is one of the best characterized transcription factors that bind to GC-rich DNA sequences in numerous cellular and viral genes (Refs. 30 and 31 and references therein). The six Sp1 binding GC-boxes of the p21 gene proximal promoter have been shown to be important; mutation of the sites not only significantly affects transcription but also disrupts synergistic transcription activation by Sp1, p53, and other signals that regulate p21 gene transcription (26, 32). Among the six GC-boxes, GC-box 3 mediates p21 induction by various agents such as transforming growth factor-β, butyrate, histone deacetylase inhibitor trichostatin A, lovastatin, and Ca2+. In contrast, GC-boxes 1 and 2 mediate transcriptional activation by phorbol esters and okadaic acid, tumor suppressor protein BRCA1, and gut-enriched Krüppel-like factor (GKLF, KLF4). To date, no specific role has been attributed to the most proximal and overlapping GC-boxes 5 and 6 (Ref. 26 and references therein). Together, these observations suggest that the specificity of utilizing different proximal GC-boxes under different p21 gene regulation conditions is important.

In this article, we investigated whether a novel POK family protein, ZBTB2, could regulate any components of the ARF-HDM2-p53-p21 pathway, and examined the mechanisms and physiological consequences of ZBTB2 action. ZBTB2 repressed transcription of the ARF, p53, and p21 genes, and potently activated the HDM2 gene, which overall down-regulates the p53 pathway significantly. ZBTB2 increased cell proliferation significantly. Our data suggest that ZBTB2 may be a master regulator of the p53 pathway and may play a critical role in important biological processes controlled by p21 and other genes of the p53 pathway.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Reagents—The p21-Luc plasmid was kindly provided by Dr. Yoshihiro Sowa of the Kyoto Perpetual University of Medicine (Kyoto, Japan). The various pGL2-p21-Luc, pGL2-p53-Luc, pGL2-ARF-Luc, pGL2-HDM2-Luc, pcDNA3.1-p53, pcDNA3.1-POZ, pcDNA3.1-Sp1, and pCMV-LacZ plasmids were prepared by cloning the cDNA fragments into pcDNA3.1-ZBTB2 plasmid was prepared by cloning the cDNA fragment encoding the POZ domain (amino acids 24–117) into pBIND (Promega). To prepare recombinant GST-POZZBTB2 and GST-ZFZBTB2 proteins, cDNA fragments encoding the POZ domain (amino acids 24–117) and zinc fingers (amino acids 254–468) were cloned into pGEX4T3 (Amersham Biosciences). The pPac-PL-ZBTB2 plasmid was prepared by cloning the cDNA fragments into pPac-PL. All plasmid constructs were verified by sequencing.

Antibodies against p21, p53, Sp1, GAPDH, Myc tag, FLAG tag, Ac-H3, Ac-H4, and HDAC3 were purchased from Upstate (Charlottesville, VA), Chemicon (Temecula, CA), Calbiochem, and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Most of the chemical reagents were purchased from Sigma.

Cell Cultures—HEK293A, HeLa, MB352, and CV-1 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Saos-2 cells were cultured in McCoy’s 5A medium (Invitrogen) supplemented with 15% fetal bovine serum. Drosophila SL2 cells were cultured in Schneider’s Drosophila medium (Invitrogen) supplemented with 10% fetal bovine serum.

Transcriptional Analysis of ARF, HDM2, p53, and p21 Genes—The pGL2-ARF-Luc, pGL2-HDM2-Luc, pGL2-p53-Luc, and various pGL2-p21-Luc promoter reporter fusion plasmids as well as pcDNA3.1-ZBTB2, pcDNA3.1-ZBTB2ΔPOZ, pcDNA3.1-p53, pcDNA3.1-Sp1, and pCMV-LacZ in various combinations were transiently transfected into several cell lines such as HEK293A, HeLa, MB352, Saos-2, and CV-1 using Lipofectamine Plus reagent (Invitrogen). After 24–36 h of incubation, cells were harvested and analyzed for luciferase activity. Reporter activity was normalized with cotransfected β-galactosidase activity for transfection efficiency.

RT-PCR of ZBTB2 mRNA Expression in FVB Mice or Cells—Total RNA was isolated from brain, heart, liver, muscle, kidney, spleen, brown adipose tissues, and white adipose tissues or cells using TRIzol reagent (Invitrogen). Synthesis of cDNA was done using 5 μg of total RNA, random hexamer (10 pmol), and Superscript reverse transcriptase II (200 units) in 20 μl using a reverse transcription kit (Invitrogen). The following oligonucleotide PCR primers were used: murine Zbtb2 (forward, 5'-CCAACCATGAGCATTATCTCTA-3'; reverse, 5'-TTCTACCTCCTGGATCTGGTG-3'), β-actin (forward, 5'-ATGAGTGACGATATCGCTGC-3', reverse, 5'-CACACTGTGCCCATCTAGA-3'), Human ZBTB2 (forward, 5'-GATCGGTACCCATTGCGAAAAGGTCTCCTGG-3', reverse, 5'-GATCCCTGCGAGGAGGAAAGGC-3'), and GAPDH (forward, 5'-ACACAGTGCCATGCTACAC-3', reverse, 5'-TCCAACCCCTGTGCTGTA-3').

Western Blot Analysis—Cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.25% sodium deoxycholic acid, 150 mM NaCl, 1 mM EGTA, complete Mini-Protease mixture). Cell extracts (40 μg) were separated using 12% SDS-PAGE gel electrophoresis, transferred onto Immun-Blot polyvinylidene difluoride membranes (Bio-Rad), and blocked with 5% skim milk (BD Biosciences). Blotted membranes were incubated with antibodies against FLAG tag (Sigma), GAPDH (Chemicon), p21, p53, Sp1, Myc tag, (Santa Cruz Biotechnology) and then incubated with horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibody (Vector Laboratory). Protein bands were visualized using ECL solution (PerkinElmer Life Sciences).
Knock-down of ZBTB2 mRNA by siRNA—Four siRNA against ZBTB2 mRNA were designed and purchased from Dharmacon (Lafayette, CO): siZBTB2-1, 5'-GAUCAUCAGUGAGACAAGUU-3', 5'-PCUUGUUCAAACUGAUCCUU-3'; siZBTB2-2, 5'-CAGUGAAUCCGAAGAAAUUU-3', 5'-PUAUUUUGCCAUUCACCUGU-3'; siZBTB2-3, 5'-CGACCCGUUGAUCAGAUAUU3', 5'-PUCUUAAGCAGAUCGCUU-3'; and siZBTB2-4, 5'-AGACGA-

AGGGCAGAU CCCAUUU-3', 5'-'PAAUGGAUCGCCUCUGUCUU-3'. The siRNA (200 pmol) were transfected into HEK293A cells using Lipofectamine 2000 (Invitrogen). After transfection, cells were harvested, and total RNA was prepared. RT-PCR analysis of mRNA was performed as described above.

Chromatin Immunoprecipitation (ChIP) Assays—The molecular interaction between ZBTB2 and p53 or Sp1 on the p21 gene promoter and histone modification at the p21 proximal promoter in HEK293A, Saos-2, and Drosophila SL2 cells were analyzed by following the standard ChIP assay protocol, as described elsewhere (20, 22).

PCR of chromatin immunoprecipitated DNA was carried out using oligonucleotide primer sets designed to amplify the upstream regulatory regions and proximal promoter region of the p21 gene. p53 RE-1 binding primers (bp, −2307–1947), forward, 5'-GCTTGGGCAGCAGGTGTG-3', reverse, 5'-GCAACCATGCACTTGAATGT-3'; p53 RE-2 binding primers (bp, −1462–1128), forward, 5'-TGCTCTCCACCCCTACCTGG-3', reverse, 5'-AGAATGAGTGTGTGC-3'; proximal GC-boxes ChIP primers (bp, −261 to approximately +39), forward, 5'-GCTCATTGCGGGAAT3', reverse, 5'-CACAAGGAACTGACT-3'.

Immunoprecipitation Assays—Cells were washed, pelleted, and resuspended in a lysis buffer supplemented with protease inhibitors (20 mm Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100). Cell lysate was precleared, and the supernatant was incubated overnight with anti-FLAG antibody on a rotating platform at 4 °C, 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 2SDS-PAGE. Western blot assay was performed as described above.
Mammalian Two-hybrid Assays—CV-1 cells were co-transfected with pG5-Luc, pGal4-POZZBTB2, pVP16-corepressors, and pCMV-LacZ. After 24 h of transfection with Lipofectamine Plus (Invitrogen), CV-1 cells were harvested and assayed for luciferase activity. Luciferase activity was then normalized with cotransfected β-galactosidase activity.

GST Fusion Protein Purification, in Vitro Transcription and Translation of Corepressors, p53, or Sp1, and Pull-down Assays—Recombinant GST, GST-POZZBTB2, and GST-ZFZBTB2 fusion proteins were prepared from Escherichia coli BL21 (DE3) grown for 5 h at 37 °C in a medium containing 1 mM isopropyl 1-thio-β-D-galactopyranoside. 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, p53, and Sp1 polypeptides were prepared by incubating 1 μg of pcDNA3.0-corepressor, pcDNA3.1-p53, and pcDNA3.1-Sp1 expression plasmid with Tnt Quick-coupled Transcription/Translation Extract (Promega) containing 40 μl of Tnt Quick Master Mix and 2 μl of [35S]methionine (1175.0 Ci/mol) (PerkinElmer Life Sciences) at 30 °C for 90 min. Polypeptide expression levels were then analyzed by running 1 μl 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 μl of in vitro translated [35S]methionine-labeled corepressors, p53, and Sp1 polypeptides at 4 °C for 4 h in HEMG buffer. The reaction mixtures were centrifuged, pellets were rinsed, and the bound proteins were separated using 12% SDS-PAGE. Gels were then exposed to x-ray film using an image-intensifying screen (Kodak).

Preparation of Recombinant Adenovirus Overexpressing ZBTB2—ZBTB2 cDNA was cloned into the adenovirus E1 shuttle vector pCA14 (Microbix, Ontario, Canada), to generate pCA14-ZBTB2. The pCA14-ZBTB2 shuttle vector was linearized by XmnI digestion, and the adenovirus vector vmdl324Bst (from Dr. Verca at the University of Fribourg, Switzerland) containing the Ad5 genome deleted in the E1 and E3 regions was also linearized with BstBI digestion. The linearized pCA14-ZBTB2 and vmdl324Bst
digested with BstBI were co-transformed into E. coli BL518 for homologous recombination. Proper homologous recombinant adenoviral plasmid was digested with Pael and transfected into HEK293 cells to generate the adenovirus expressing ZBTB2 (dl324-ZBTB2). Propagation and titration of the recombinant virus were carried out by standard methods. PCR amplification and DNA sequencing using primers specific to ZBTB2 confirmed the adenovirus genotype.

Electromobility Shift Assays (EMSA) — EMSAs were carried out as described previously (20, 22, 33). The probe sequences of Sp1 response elements on the p21 gene proximal promoter or the sequences of p53 response elements on the p21 gene distal promoter used in EMSA were as follows (only top strands are shown): GC-box 1, 5′-GATCCGAGCGCGGGTCCCGCCTC-3′; GC-box 2, 5′-GATCTCCCGGCGCGCGGCGC-3′; GC-box 3, 5′-GATCCGAGCGGGGTCCCGCCTC-3′; GC-box 4, 5′-GATCCTTGGGCGGGCGGCGG-3′; GC-box 5/6, 5′-GATCCGCGGC-3′. Oligonucleotide Pull-down Assays — HEK293A cells were lysed in HKMG buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl2, 10% glycerol, 1 mM dithiothreitol, and 0.5% Nonidet P-40). Cellular extracts were incubated with 1 μg of biotinylated double-stranded oligonucleotides (p53 RE-1, p53 RE-2, and Sp1 GC-box 5/6) for 16 h. The sequences of the oligonucleotides are as follows (only top strands are shown): Sp1 GC-box 5/6, 5′-CCTTAGGGCGCCGGCGGGCGGGTGTATATCGGC-3′; p53 RE-1, 5′-GATCCGTTAGAGGAAAGACTGGGATCTGTCTG3′; and p53 RE-2, 5′-GATCCATCAGGAACATGTTGAGCTC-3′. To collect DNA-bound proteins, the mixtures were incubated with streptavidin-agarose beads and precipitated by centrifugation. The precipitate was analyzed by Western blot assay as described above.

FACS Analyses — HEK293A cells were transfected with pcDNA3.1-ZBTB2 expression vector or siZBTB2 RNA in the presence or absence of p53 expression vector. Cells were transfected with pcDNA-FLAG-ZBTB2 expression vector. Cells (1.5 × 104 cells) were transfected with pGL2-p21-Luc Wt, pPac PL-Sp1, and increasing amounts of pPac PL-FLAG-ZBTB2. Cells were washed, fixed with methanol, and stained with 50 μg/ml of ribonuclease A for 30 min at 37 °C in the dark. DNA content, cell cycle profiles, and forward scatter were analyzed by FACS Calibur (BD Biosciences) with emission detection at 488 nm (excitation) and 575 nm (peak emission). Data were analyzed using ModFit LT 2.0 (Verity Software House, Inc., ME) and WindMDI 2.8 (Joseph Trotter, The Scripps Research Institute).

RESULTS

ZBTB2, a Novel POK Protein, Represses Transcription of ARF, p53, and p21 Genes of the p53 Pathway but Activates Transcription of HDM2 — ZBTB2 cDNA encodes a protein composed of 514 amino acids (supplemental Fig. S1). ZBTB2 contains an N-terminal POZ domain at amino acids 24 to 117 and four zinc fingers at the C terminus. ZBTB2 mRNA was expressed in all male FVB mouse tissues examined, and was particularly high in the spleen (supplemental Fig. S2D). SAGE analysis by the Cancer Genomic Anatomy Project (CGAP) showed that ZBTB2 mRNA expression is higher in cancers derived from the retina, thyroid, liver, peritoneum, ovary, and muscle compared with normal tissues (nci.nih.gov). Immunocytochemistry in HEK293A cells detected ZBTB2 in both the cytoplasm and nuclear speckles of human HEK293A cells transfected with pcDNA3.1-ZBTB2 overexpression vector. The precipitate was analyzed by Western blot assay as described above.

FACS Analyses — HEK293A cells were transfected with pcDNA3.1-ZBTB2 expression vector or siZBTB2 RNA in the presence or absence of p53 expression vector. Cells were transfected with pcDNA-FLAG-ZBTB2 expression vector. Cells (1.5 × 104 cells) were transfected with pGL2-p21-Luc Wt, pPac PL-Sp1, and increasing amounts of pPac PL-FLAG-ZBTB2. Cells were washed, fixed with methanol, and stained with 50 μg/ml of ribonuclease A for 30 min at 37 °C in the dark. DNA content, cell cycle profiles, and forward scatter were analyzed by FACS Calibur (BD Biosciences) with emission detection at 488 nm (excitation) and 575 nm (peak emission). Data were analyzed using ModFit LT 2.0 (Verity Software House, Inc., ME) and WindMDI 2.8 (Joseph Trotter, The Scripps Research Institute).

MTT Assays — Confluent HEK293A cells grown on 10-cm culture dishes were transfected with pcDNA3.1-ZBTB2 or siZBTB2 RNA in the presence or absence of p53 expression vector. Cells (1 × 105 cells) were transfected to 6-well culture dishes and grown for 0–6 days. At 0, 2, 4, and 6 days, cells were incubated for 1 h at 37 °C with 500 μl/well MTT (2 mg/ml). Precipitates were dissolved with 1 ml of dimethyl sulfoxide. Cellular proliferation was determined from the conversion of MTT to formazan using a SpectraMax 250 (Molecular Device Co.) at 570 nm. p value was calculated using the statistical analysis program SPSS (Statistical Package for the Social Sciences) (Chicago, IL).

Preparation of Anti-ZBTB2 Antibody — To obtain a rabbit polyclonal antibody against ZBTB2 protein, one white rabbit was immunized subcutaneously with a synthetic peptide (amino acids 500–514 of ZBTB2; VLASIKKEQETVL) three times at 3-week intervals. Blood was collected, incubated at 37 °C for 90 min, and centrifuged. The supernatant was incubated with the protein A/G-agarose beads (Santa Cruz Biotechnology). The beads were collected and washed, and the antibody was eluted. The titer of the antibody was tested by Western blot assay of the HEK293A cells transfected with the pcDNA3.1-ZBTB2 overexpression vector.

FIGURE 2. ZBTB2 represses transcription activation by Sp1 on the minimal p21 promoter and pG5-5x(GC)-Luc. ZBTB2 competes with Sp1 to bind to the proximal GC-box 5/6. ZBTB2 interacts with Sp1 to weakly inhibit Sp1 binding. A and B, Sp1 activated transcription of pGL2-p21-Luc (∼131 bp) and pG5-5x(GC)-Luc, and ZBTB2 repressed transcriptional activation by Sp1. C, EMSA. The 32P-labeled Sp1 binding GC-box probes were incubated with GST-ZF-ZBTB2 (0.5 μg) and separated by 4% non-denaturing PAGE. D, ChIP assay in Drosophila SL2 cells transfected with pGL2-p21-Luc Mt, pPac PL-Sp1, and/or pPac PL-FLAG-ZBTB2. ZBTB2 could not bind to the mutated proximal promoter. X, mutated GC-box 5/6. E, ChiP assay of ZBTB2 binding on the p21 Wt promoter in Drosophila SL2 cells. Cells were transfected with pGL2-p21-Luc Wt, pPac PL-Sp1, and increasing amounts of pPac PL-FLAG-ZBTB2. F, ChiP assay of the endogenous p21 gene in human HEK293A cells transfected with pcDNA3-FLAG-ZBTB2. ZBTB2 competed with Sp1 to bind to the proximal promoter and inhibited Sp1 binding, but not in the 3′-untranslated region. Bottom, histogram of ChIP assays. G, co-immunoprecipitation of ZBTB2 and Sp1. Cell lysates prepared from HEK293A cells transfected with pcDNA3-FLAG-ZBTB2 expression vector were co-immunoprecipitated using anti-FLAG antibody (Ab) and analyzed by Western blotting using anti-Sp1 antibody. H, in vitro GST fusion protein pull-down assays. Recombinant GST, GST-POZZBTB2, or GST-ZF-ZBTB2 was incubated with [35S]methionine-labeled Sp1, pulled down, and resolved by a 10% SDS-PAGE. Input, 10% of the Sp1 added in the binding reactions. I, ChiP assay of endogenous ZBTB2 binding to the proximal GC-box 5/6 elements of endogenous p21 in HEK293A cells. ChiP PCR primer sets are as in Fig. 2, oligonucleotide pull-down assay of ZBTB2 binding to the proximal GC-box 5/6 elements of the p21 gene promoter. HEK293A cell extracts were incubated with biotinylated double-stranded oligonucleotides. The mixtures were further incubated with streptavidin-agarose beads and precipitated by centrifugation. The precipitate was analyzed by Western blot (WB) assay using antibody against ZBTB2. IP, immunoprecipitation.
band, suggesting the possibility of post-translational modification of the protein.

Recently, several reports have implicated POZ domain proteins such as FBI-1, BCL-6, and Miz-1 in cell cycle regulation (10, 21). We investigated whether ZBTB2 regulates expression of the genes (ARF, HDM2, p53, and p21) of the p53 pathway, which is important in cell cycle regulation. In human HEK293A cells, ZBTB2 repressed transcription of three genes (ARF, p53, and p21) of the p53 pathway by more than 60–80%, and repression was particularly effective in the p21 gene. In contrast, ZBTB2 increased transcription of HDM2, which could induce degradation of p53 (Fig. 1A) (33).

Because all of the transcriptional regulatory effects of ZBTB2 on the p53 pathway converged onto expression of the p21 gene, an important negative regulator of cell cycle progression, we investigated the molecular mechanisms of transcriptional regulation of the p21 gene in detail. RT-PCR and Western blot analysis of HEK293A cells infected with either control adenovirus or recombinant virus overexpressing ZBTB2 showed that ZBTB2 repressed p21 gene expression (Fig. 1B). Knock-down of ZBTB mRNA by RNA interference with four different siRNAs also resulted in derepression of p21 gene transcription, particularly with ZBTB2 number 2 siRNA (Fig. 1, C–E). Taken together, these data suggest that ZBTB2 is a major negative transcriptional regulator of the p53 pathway, particularly of the p21 gene. Furthermore, we mapped the cis-regulatory elements of the p21 gene promoter responsible for transcriptional repression by ZBTB2. Transcription assays indicate that ZBTB2 can repress transcription by acting on the small proximal regulatory element concentrated with Sp1 binding GC-boxes (bp, −133 to approximately +30) (Ref. 26 and references therein). We observed more potent repression with the longer promoter constructs containing the distal p53 binding elements (Fig. 1, F and G).

ZBTB2 Competes with Sp1 to Bind to the Proximal Promoter GC-box 5/6 of the p21 Gene and ZBTB2 Interacts with Sp1—The above data suggested that Sp1 and the GC-boxes could be involved in repression by ZBTB2. Accordingly, we tested whether ZBTB2 could repress transcriptional activation by Sp1 on the short p21 proximal promoter and artificial pG5-5x(GC)-Luc with the well characterized Sp1 binding GC-box (34) in HEK293A cells. Sp1 activated transcription of

![Graph A](image1.png)  
![Graph B](image2.png)  
![Graph C](image3.png)  

**Graph A**: Relative luciferase activity (fold) for pGL2-p21-Luc (2.3 kb) and various constructs. 
**Graph B**: Similar to A, showing effects of pcDNA3-ZBTB2 or pcDNA3-p53 on transcription. 
**Graph C**: Effect of ZBTB2 knock-down on p21 transcription. 

**Figure 1**: Schematic representation of the p21 promoter and ZBTB2 binding sites. 

**Figure 2**: Electrophoretic mobility shift assay (EMSA) showing ZBTB2 binding to the p21 proximal promoter GC-box.
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the HEK293A cellular extract transfected with the FLAG-ZBTB2 expression vector showed that Sp1 and ZBTB2 interacted with each other in vivo (Fig. 2G). GST fusion protein pull-down assays also demonstrated that both the POZ and ZF domains of ZBTB2 interact directly with Sp1 (Fig. 2H). ZBTB2 not only competed with Sp1 to bind to the proximal GC-box 5/6 elements, but also interacted with Sp1 to inhibit Sp1 binding, repressing transcription of the p21 gene.

We also examined whether endogenous ZBTB2 binds the proximal GC-box 5/6 of the endogenous p21 gene by ChIP and oligonucleotide pull-down assays using antibody against ZBTB2. Apparently, endogenous ZBTB2 binds to the proximal promoter GC-box 5/6 of the endogenous p21 gene (Fig. 2I and J).

ZBTB2 Represses Transcription of p21 through Binding Competition between ZBTB2 and p53 on the Distal p53 Binding Elements and ZBTB2 Interacts with p53—Because more robust transcription repression was observed with pGL2-p21-Luc −2.3-kb and −1.5-kb constructs compared with pGL2-p21-Luc −131 bp (Fig. 1, F and G), we suspected that other repression mechanisms involving p53 and/or distal p53 binding elements might be possible. ZBTB2 repressed transcription of pGL2-p21-Luc by 50% in MB352 cells lacking endogenous p53. Ectopic p53 increased p21 gene expression, which was repressed by ZBTB2 (Fig. 3A). In HEK293A cells, treatment with the DNA damaging agent etoposide increased p21 gene expression by inducing p53, which was again repressed by ZBTB2 (Fig. 3B). Additional transcriptional analysis of pGL2−6x(p53RE)−Luc with five copies of the p53 binding elements of the p21 gene in the proximal promoter showed that ZBTB2 blocked transcription activation by p53 (Fig. 3C). These data suggest that ZBTB2 may inhibit transcription of the p21 gene by directly acting on the distal p53 binding elements.

EMSA showed that the zinc finger DNA binding domain of ZBTB2 could bind to p53 RE-1 and 2, suggesting a potential binding competition between p53 and ZBTB2 (Fig. 3D). We investigated whether the two proteins competed with each other in vivo. Co-immunoprecipitation and Western blot assays of ZBTB2 and p53 binding on the distal p53 binding elements of the endogenous p21 gene in HEK293A cells. Western blot; IP, immunoprecipitation; Ab, antibody.

FIGURE 3. ZBTB2 represses transcription of the p21 gene by binding competition with p53 on the distal p53 binding elements in vivo. A, transcription analysis. MB352 cells lacking p53 were transiently co-transfected with expression vector of p53 and/or ZBTB2 and pGL2-p21-Luc WT (−2.3 kb), and luciferase activity was measured. B, transcription analysis in human HEK293A cells. Etoposide treatment of cells increased p21 gene transcription, which was repressed by ZBTB2. C, transcriptional activation of pGL2−6x(p53RE)−Luc by ectopic p53 was repressed by ZBTB2 in Saos-2 cells lacking p53, p53RE, distal p53-binding elements of the p21 gene promoter. D, EMSA. Two 32P-labeled p53 binding element probes of the p21 gene were incubated with GST-ZFZBTB2 (0.5 μg) and separated by 4% nondenaturing PAGE. E, ChIP assay of ZBTB2 binding on the distal p53 binding elements of the endogenous p21 gene in HEK293A cells. Cells were transfected with increasing amounts of FLAG-ZBTB2 expression vector. Right, histograms of ZBTB2 and p53 binding on p53 binding elements 1 and 2. G, ChIP assay of endogenous ZBTB2 binding to the distal p53 binding elements of the endogenous p21 gene in HEK293A cells. H, oligonucleotide pull-down assay of endogenous ZBTB2 binding to the distal p53 binding elements of the p21 gene. WB, Western blot; IP, immunoprecipitation; Ab, antibody.

FIGURE 4. ZBTB2 interacts directly with p53 and inhibits p53 binding by protein-protein interactions. A, ChIP assay of ZBTB2 binding on the proximal promoter of pG13-Luc in Saos-2 cells. Although ZBTB2 itself could not bind to this p53 binding element (−17941 bp), its presence still decreased p53 binding. B, co-immunoprecipitation of ZBTB2 and p53. HEK293A cells lysates prepared from cells transfected with FLAG-ZBTB2 expression vector were immunoprecipitated (IP) using anti-FLAG antibody and analyzed by Western blotting (WB) using anti-p53 antibody. C, in vitro GST fusion protein pull-down assay. Recombinant GST, GST-PDZ-ZBTB2, or GST-ZF-ZBTB2 was incubated with 32P-labeled p53, pulled down, and resolved by 10% SDS-PAGE. The gel was then exposed to x-ray film. Input, 10% of the p53 added in the binding reactions. Ab, antibody.
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A

Relative luciferase activity (fold)

B

Relative luciferase activity (fold)

C

ZBTB2

D

pcDNA3-FLAG-ZBTB2

E

Relative luciferase activity (fold)

F

Relative luciferase activity (fold)

G

Fold enrichment relative to IgG

p53

Sp1(5/6) Tsp (±)

-2307 bp

p21

ChIP primer

Ab

pcDNA3 pcDNA3-ZBTB2

Input IgG Ac-H3 Ac-H4

pcDNA3 pcDNA3-ZBTB2

233 bp

pcDNA3.0 ZBTB2

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other for the sites of the endogenous p21 gene using ChIP assays. Expression vectors of ZBTB2 and p53 were transiently co-transfected in Saos2 cells lacking p53. ZBTB2 bound to p53 RE-1 by competing with p53 in a dose-dependent manner, but binding competition between ZBTB2 and p53 at p53 RE-2 was less pronounced (Fig. 3E). We found a similar binding competition between ZBTB2 and p53 on the endogenous p21 gene promoter in HEK293A cells, and again the binding competition on p53 RE-2 by ZBTB2 was also less effective (Fig. 3F).

Using newly prepared antibody against ZBTB2, we also investigated whether endogenous ZBTB2 could bind to the distal p53 binding elements of the endogenous p21 gene using ChIP and oligonucleotide pull-down assays. ZBTB2 binds specifically to p53 RE-1 and p53 RE-2 (Fig. 3, G and H).

We then used ChIP to determine whether ZBTB2 and p53 competed with each other on the well characterized pG13-Luc, which has 13 copies of the p53 binding element (5′-CCAG-GCAAGTCCAGGCGAG-3′) in the proximal promoter (24). Expression vectors of ZBTB2, p53, and pG13-Luc reporter plasmids were transiently co-transfected in p53-null Saos2 cells, and chromatin was immunoprecipitated using the indicated antibodies. Contrary to our expectations, ZBTB2 did not bind to the pG13-Luc reporter gene, possibly reflecting the binding specificity of ZBTB2 to only some p53 binding sequences (such as those of the p21 gene). ZBTB2 did, however, decrease p53 binding to the pG13-Luc reporter gene quite effectively (Fig. 4A). The data suggest that ZBTB2 may repress transcription of the p21 gene by directly interacting with p53, and thus interfering with p53 binding on the proximal promoter of pG13-Luc. Co-immunoprecipitation and Western blot assays of HEK293A cells transfected with the FLAG-ZBTB2 expression vector revealed that ZBTB2 and p53 interact with each other in vitro (Fig. 4B). GST fusion protein pull-down assay also showed that the GST-ZFZBTB2 domain, not GST-POZZBTB2, interacted with p53 in vitro, suggesting that p53 and ZBTB2 interact directly with p53 via its zinc fingers of ZBTB2 (Fig. 4C).

The POZ Domain of ZBTB2 Interacts with the Corepressor-HDAC Complex to Deacetylate Histones Ac-H3 and Ac-H4 at the Proximal Promoter—ZBTB2 repressed transcription by direct binding competition with transcription activators such as Sp1 and p53, but it was not clear how it repressed transcription once bound to the proximal and/or distal regulatory elements. To map the domain of ZBTB2 important in transcription repression, we prepared a mutated ZBTB2 construct with the POZ domain deleted (ZBTB2ΔPOZ). ZBTB2 repressed transcription of the p21 gene but the mutant ZBTB2ΔPOZ did not, indicating that the POZ domain of ZBTB2 is required in transcriptional repression (Fig. 5A).

Transcriptional repressors, including some POZ domain proteins such as PLZF and BCL-6, often repress transcription through interaction with corepressors such as SMRT, NCoR, BCoR, and mSin3A. Mammalian two-hybrid assays in CV-1 cells using pG5-Luc, pGal4-POZZBTB2 and pVP16-corepressor fusion protein expression vectors demonstrated that the POZ domain of ZBTB2 interacts with SMRT, NCoR, and BCoR (Fig. 5B). In addition, GST fusion protein pull-down assays showed that POZZBTB2 can interact directly with SMRT, NCoR, and BCoR (Fig. 5C). Corepressor complexes recruited by transcriptional repressors often contain HDAC proteins. These HDACs deacetylate the histones of nearby nucleosomes to repress transcription. Co-immunoprecipitation and Western blot analysis of HEK293A cell extracts transfected with the FLAG-ZBTB2 expression vector using anti-SMRT and anti-HDAC3 antibodies revealed that ZBTB2 and SMRT-HDAC interacted with each other in vivo (Fig. 5D), indicating that ZBTB2 may repress transcription of the p21 gene by interacting directly with the corepressor-HDAC complex via its POZ domain.

HDAC inhibitor trichostatin A treatment of CV-1 or HEK293A cells co-transfected with pG5-Luc and pGal4-POZZBTB2 or pGL2-p21-Luc and ZBTB2 expression vectors significantly affected transcriptional repression by ZBTB2 or the POZ domain of ZBTB2 on both pG5-Luc and pGL2-p21-Luc, resulting in a significant increase in transcription (Fig. 5, F and G). These data implicate the involvement of HDACs in transcriptional repression by ZBTB2.

Corepressor-HDACs recruited by the repressors may deacetylate the histones of nearby nucleosomes around the proximal promoter. Accordingly, we used ChIP to examine whether the acetylation status of histones H3 and H4 at the proximal promoter of the endogenous p21 gene was altered by ZBTB2-corepressor-HDAC complexes in HEK293A cells transfected with the FLAG-ZBTB2 expression vector. The complex significantly decreased acetylated histones H3 and H4 at proximal promoter 4 (Fig. 5H).

ZBTB2 Stimulates HeLa Cell Proliferation and Increases the Percentage of Cells in S-phase—A major regulator of cell cycle arrest, p21, was potently repressed by ZBTB2 at the transcriptional level. HeLa cells stably expressing ZBTB2 showed a significant increase in cell proliferation (Fig. 6A). MTT assays revealed that whereas ZBTB2 overexpression significantly increased cell proliferation, ZBTB2 knock-down by siRNA decreased cell proliferation in the presence or absence of p53 (Fig. 6, B–F). FACs analysis also showed that ZBTB2 stimulated cell cycle progression and
increased the number of HEK293A cells in S-phase (38.3% in control versus 48.5% in HEK293A-ZBTB2). Furthermore, the number of cells in the G2-M phase decreased from 5.7 to 0% (Fig. 6G). Knock-down of endogenous ZBTB2 mRNA resulted in a decrease in the number of cells in S-phase and a concomitant increase in the number of cells in G0-G1 and G2-M phases (Fig. 6H). We also observed similar changes in cell cycle progression in the presence of p53, and ZBTB2 reversed the cell cycle arrest effect of p53 as in the MTT assays (Fig. 6I).

**DISCUSSION**

Our investigation revealed that ZBTB2 represses transcription of ARF, p53, and p21 and activates transcription of HDM2. Potent repression of ARF and activation of the HDM2 gene can
result in an overall increase in \textit{HDM2} gene expression, which can decrease p53 activity or stability. The mechanism of repression of the p53 pathway by ZBTB2 is directed toward the inhibition of p53 activity or stability. In addition to potently modulating expression of \textit{ARF} and \textit{HDM2} and thereby eventually affecting p21 expression by down-regulating p53 expression, ZBTB2 directly repressed transcription of the p21 gene (Fig. 7A). Although details of the transcription regulation of \textit{ARF}, p53, and \textit{HDM2} by ZBTB2 remains largely unexplored and requires further investigation, ZBTB2 may be a master control gene of the p53 pathway and thus of cell proliferation, cell cycle progression, and oncogenesis.

Our investigation of regulation of the cell cycle arrest gene p21 revealed that p21 is the direct target of ZBTB2, with a complex molecular mechanism of transcription repression that involves Sp1, p53, proximal GC-box 5/6, and two distal p53 binding elements of p21. ZBTB2 competes with Sp1 to bind to the proximal Sp1 binding GC-box 5/6, which is a direct target of regulation by Sp1 and Sp family members. Intriguingly, ZBTB2 binds to this particular site to repress transcription activation by Sp1.

Our data also showed that ZBTB2 binds to distal p53 binding elements by competing with p53, and that ZBTB2 also interacts with p53 directly to inhibit p53 binding and to repress the contribution of p53 to transcription. The site was shown to mediate the induction of p21 by genotoxic stresses. Accordingly, DNA damaging signals that result in p53-mediated induction of p21 can be blocked by ZBTB2. Overall, these molecular features of ZBTB2 may explain how ZBTB2 acts as a master control gene of cell proliferation by potently blocking the p53 pathway.

Based on our finding, we propose a hypothetical model of transcriptional regulation of the p21 gene by ZBTB2 (Fig. 7). Under normal cellular conditions where p53 is expressed at low basal levels and ZBTB2 is not present or low compared with Sp1, the p21 gene is expressed at low basal levels driven by Sp1, and cells proliferate normally. Challenge with a genotoxic stress induces production of tumor suppressor p53, which binds to the distal p53 response elements and activates transcription of p21 by interacting with the Sp1 bound at the proximal GC-box. The induced p21 arrests cell cycle progression, allowing cells to repair DNA damage. In cells without DNA damage where ZBTB2 expression is high, ZBTB2 represses transcription...
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directly by binding to both the proximal GC-box 5/6 and distal p53 binding elements. ZBTB2 bound to the regulatory elements recruits the corepressor-HDAC complex, causing deacetylation of histones Ac-H3 and -H4 around the proximal promoter and repressing transcription. When cells are under genotoxic stress and ZBTB2 expression is high or in cancerous tissues that have high levels of p53 and ZBTB2, ZBTB2 represses transcription directly by binding to both the proximal GC-box 5/6 and distal p53 binding elements. Although p53 expression is also highly induced under these conditions, p53 has to compete with ZBTB2 to bind to the distal p53 binding elements and is also affected by molecular interactions between p53 and ZBTB2 that further impede binding. Although p53 is present, transcription of the p21 gene is potently repressed by ZBTB2. Cells proliferate without cell cycle arrest, mutations accumulate, and cells are likely to undergo oncogenic transformation.

ZBTB2 has characteristics of Sp1-family Krüppel-like zinc finger proteins and binds to some of the GC-boxes that are similar to the GC-boxes recognized by Sp1. This finding suggests that GC-boxes recognized by Sp1 may be transcriptional repression targets of ZBTB2, and that Sp1-ZBTB2 binding competition may be a general mechanism of transcriptional repression of some ZBTB2 target genes. Molecular interactions occurring both in the proximal and distal promoter of the p21 gene are unique and may be also relevant to the transcription regulation of other genes of the Arf-HDM2-p53-p21 pathway. How ZBTB2 potently activates (as much as 80-fold under our assay conditions) transcription of the HDM2 gene (which has a p53 binding element in the P2 promoter and is activated by p53) remains unclear and needs further investigation. The strong induction of the HDM2 gene may induce rapid degradation of p53, significantly blocking the protective effect of p53 in the cellular response to DNA damage. Taken together, our findings suggest that ZBTB2 may play a critical role in regulating important biological processes such as DNA repair, cell growth, differentiation, and apoptosis by regulating the transcription of p21 and other genes of the p53 pathway.

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