Molecular and Functional Characterization of the ZNF509 in the Regulation of Cell Proliferation

Bu-Nam Jeon

Department of Medical Science

The Graduate School, Yonsei University

Molecular and Functional Characterization of the ZNF509 in the Regulation of Cell Proliferation

Directed by Professor Man-Wook Hur

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Bu-Nam Jeon is approved.

Thesis Supervisor : Man-Wook Hur

Thesis Committee Member #1 : Hoguen Kim

Thesis Committee Member #2 : Kang-Yell Choi

Thesis Committee Member #3 : Sahng Wook Park

Thesis Committee Member #4 : Chae-Ok Yun

The Graduate School

Yonsei University

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Cell proliferation is a process which is precisely controlled by interaction between a number of regulatory proteins. In cancer cells, the aberrant expression of oncogenes and/or tumor suppressor genes causes dramatic changes in regulatory programs controlling cell proliferation. Recently, the POK family of transcription factors was characterized as important oncogenes or tumor suppressors that regulate the expression of cell cycle control genes.

In this study, ZNF509, a novel POK family of transcription factors that is induced by p53, was characterized to be a transcriptional activator of the

CDKN1A cell cycle arrest gene. It was shown that ZNF509 directly bound to the *CDKN1A* proximal promoter by EMSA, Oligonucleotide pull-down, and ChIP assays. Interaction of ZNF509 and MIZ-1 activated the transcription of *CDKN1A* synergistically. This interaction between ZNF509 and MIZ-1 recruited the co-activator p300, and the ZNF509-MIZ-1-p300 complex increased histone Ac-H3 and -H4 levels at the *CDKN1A* proximal promoter. Knockdown of endogenous ZNF509 expression caused decrease in *CDKN1A* transcription, and resulted in increasing cell proliferation.

ZNF509 not only arrested cell cycle progression but also inhibited apoptosis when cells were exposed to DNA damaging agents. Expression of p53 was increased by etoposide treatment regardless of ZNF509 expression and, in turn, p53 increases *PUMA* gene expression. Expression of *PUMA* gene decreased moderately only in the presence of ZNF509. IP, GST pull-down, Oligonucleotide pull-down, and ChIP assays showed that ZNF509 directly interacted with p53 via its zinc-fingers, resulting in inhibition p53 binding to regulatory elements of the *PUMA* promoter and suppression of *PUMA* transcription.

These results suggest that ZNF509 serves important functions in cell fate decision directly toward cell survival. First, ZNF509 potently activates transcription of *CDKN1A* through interaction with MIZ-1. Second, ZNF509

inhibits apoptosis by repressing the *PUMA* gene expression by inhibiting DNA binding of p53 to the *PUMA* promoter.

Key words: POK, transcription, ZNF509, MIZ-1, p300, p53, CDKN1A, cell cycle arrest, PUMA, apoptosis

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I. INTRODUCTION

The BTB transcription factors were so named because of a distinct and unique N-terminal domain that was first identified in the *Drosophila* proteins Broad complex, Tramtrack, and Bric a brac[1,2]. These factors are also known as poxvirus and zinc finger (POZ) proteins. The human POZ domain has since been found in over 200 proteins, which are classified according to the presence of an N-terminal POZ domain used for interactions with other proteins and kelch or zinc-finger C-terminal domains for DNA interactions with the promoters of target genes. The BTB/POZ domain forms homo- and hetero-dimers and controls transcription through interactions with transcriptional co-factors[3]. In most cases, the BTB/POZ and zinc-finger (POK) proteins regulate transcription of target genes through chromatin

modification.

Proteins containing BTB/POZ domains are known to have various cellular regulatory functions[4,5]. In particular, interactions between certain POK family proteins and co-regulators (e.g., BCoR, NCoR, SMRT, p300 and p120ctn) are the major determinants in cell differentiation, development, hematopoiesis, tumor suppression, and oncogenesis[1,2,4-8]. For example, promyelocytic leukemia zinc-finger (PLZF) protein controls the development of invariant natural killer T cell effector function and the maintenance of spermatogonial stem cells. T-helper-inducing POK (Th-POK, also known as cKrox) regulates the ratio of CD4 to CD8 expression through T-cell lineage commitment[9-12]. Also, aberrant expression of the POK family proteins such as B-cell lymphoma 6 (BCL-6), FBI-1 (also called Pokemon), hypermethylated in cancer-1 (HIC-1) and PLZF fusion protein (PLZF-RARalpha) has been implicated in cancers such as lymphoma, spontaneous malignant tumors, and acute promyelocytic leukemia[13-19]. With the exception of the POK family proteins described above, the functions of many other POK family proteins remain largely uncharacterized and the relationship between POK expression and their roles in differentiation, development, tumor suppression, and oncogenesis need to be investigated[1,2].

Cell growth and division are divided into phases with growth occurring in

the G1 phase and the DNA replication during the S phase. During the G2 phase, cells continue to grow and prepare for mitosis and cell division during M phase[20]. Three principal checkpoints control the cell cycle; the G1 checkpoint makes the key decision as to whether the cell should divide, delay division, or enter a resting stage. The G2 checkpoint assesses the success of DNA replication and promotes the start of mitosis and the accuracy of mitosis is assessed at the M checkpoint. The M checkpoint occurs during metaphase, and promotes the exit from mitosis and the beginning of G1[21]. At each checkpoint, cyclin-dependent kinases (CDKs) phosphorylate histones and other proteins that carry the cell cycle past the checkpoint. The cell cycle is controlled by interactions between the cyclins and CDKs. During G1 phase, the cell gradually accumulates cyclin, which binds to CDK to form a complex. When the level of the cyclin/CDK complex exceeds a certain limit, the G1 phase ends and DNA replication begins[22,23]. Cell division is normally turned off in cells by proteins, called tumor suppressor proteins, which prevent cyclins from binding to CDKs. The most widely studied tumor suppressor protein is p53. p53 functions at the G1 phase checkpoint by sensing DNA integrity and activates the DNA repair system if the DNA is damaged. p53 stops the cell cycle at the G1 phase checkpoint to allow time for DNA repair by inducing the transcription of *CDKN1A*, whose protein product binds to

CDK/cyclins to prevent them from interacting. Without the CDK/cyclin complex, the cell cycle does not proceed to S phase and DNA replication cannot occur[20-23].

The tumor suppressor p53 protects the integrity of the genome. The p53 tetramer binds to its target gene promoter response elements where it recruits various transcriptional co-regulators to regulate RNA polymerase II–mediated transcription[24,25]. Normally, p53 is short-lived and present at low levels; however, in response to a variety of cellular stresses p53 is stabilized by ATM/ATR-mediated phosphorylation and an acetylation cascade by interacting with the general transcriptional co-factors[26]. Active p53 controls the expression of a set of genes required for the induction of cell cycle arrest, senescence, DNA repair, and apoptosis. Regardless of the type of stress, the final outcome of p53 activation is either cell survival through cell cycle arrest and DNA repair or cell death; however, the mechanism leading to the choice between these fates has not yet been elucidated[27-29]. A variety of genotoxic and non-genotoxic stresses lead to stabilization and activation of p53 that are mediated by multiple post-translational modifications. More than 36 different amino acids within p53 are modified in various biochemical and cell culture studies. The N-terminus of p53 is heavily phosphorylated whereas the C-terminus may be phosphorylated, acetylated, methylated, neddylated,

ubiquitinated, or sumoylated[30].

Mutation or inactivation of p53 plays a central role in tumorigenesis. Mutation or functional inactivation of the tumor suppressor p53 is an almost universal feature of cancer[31]. The clinical significance of p53 protein accumulation remains unresolved. Mouse models bearing knock-in mutations of p53 have established that mutant p53 proteins can promote tumor formation and invasion and metastasis through dominant negative inhibition of wild-type p53 or through a gain of function that can inhibit or activate the function of other proteins[32,33].

Activation of cyclin/CDK complexes regulates cell proliferation through cell cycle control[34]. CDK inhibitors usually inhibit the activity of cyclin/CDK complexes and regulate cell cycle arrest[35]. The CDK inhibitor 1A (*CDKN1A*) gene product p21 is a negative regulator of the cell cycle through G1, S and M phase checkpoints and is a major transcriptional target of p53[36,37]. The *CDKN1A* gene is mainly regulated at the transcriptional level by various oncogenes, tumor suppressors, and cellular regulators[38]. The ability of p21 to inhibit proliferation may contribute to its tumor suppressor function, and a number of oncogenes repress *CDKN1A* to promote cell growth and tumorigenesis[39]. Whereas induction of *CDKN1A* predominantly leads to cell cycle arrest in G1, G2, or S phase, repression of *CDKN1A* may have a variety

of outcomes depending on the cellular context, such as cell proliferation[39,40]. p21 is a key component in cell cycle control and apoptosis, directing an anti-apoptotic response following DNA damage[41]. It is likely that p53-dependent regulation of *CDKN1A* expression and subsequent p21 production contributes to how p53 influences cell survival or death upon DNA damage[40-43]. Apart from p53, a variety of other factors including Sp1/Sp3, Smads, AP2, STAT, BRCA1, E2F-1/E2F-3, and C/EBPα and β are known to activate *CDKN1A* transcription.

Apoptosis is a normal component in the development of multicellular organisms and is critically important for their survival by removing damaged or infected cells that may interfere with normal function[44]. The extrinsic and intrinsic pathways represent the two major well-studied apoptotic processes. The extrinsic pathway is mediated by a subgroup of the tumor necrosis factor receptor superfamily that includes TNFR, Fas, and TRAIL. Activation of these death receptors leads to the recruitment and activation of initiator caspases, including caspases 8 and 10, that involves the formation and activation of complexes such as the death inducing signaling complex (DISC). This leads to the activation of an effector caspase, typically caspase 3, which is responsible for the cleavage of a number of death substrates that lead to the well known characteristic marks of an apoptotic cell[45,46].

The intrinsic pathway is largely centered around and is regulated by the mitochondria[44]. The major player of this pathway is p53, which induces pro-apoptotic gene expression. The most widely studied form of intrinsic apoptosis is initiated by the stress-mediated release of cytochrome c from the mitochondria that results in the formation of the apoptosome. The apoptosome then activates initiator caspase, typically caspase 9, which leads to the activation of caspase 3. In response to apoptotic stimuli, pro-apoptotic members of the Bcl-2 protein family become activated and act on the mitochondria to induce the release of cytochrome c. Other pro-apoptotic proteins are also released by the mitochondria including Smac/Diablo (Second mitochondrial derived activator of caspase/Direct IAP-binding protein with a low pI), the serine protease Omi/HtrA2, endonuclease G, and apoptosis inducing factor. Activation of the mitochondrial pathway can also occur following activation of the extrinsic pathway. This has been shown to occur via caspase 8 cleavage of the pro-apoptotic Bcl-2 member Bid to its activated tBid form[47-49].

The release of mitochondrial apoptotic factors is regulated by the pro- and anti-apoptotic Bcl-2 family proteins, which either induce or prevent the permeabilization of the outer mitochondrial membrane[50,51]. It was shown that some pro-apoptotic family members, including *Bax*, *Noxa*, and *PUMA*, are

transcriptional targets of p53. Elucidation of the p53-dependent pathway, resulting in mitochondrial outer membrane permeabilization through pro-apoptotic Bcl-2 family proteins, is key to understanding the mechanism of stress-induced apoptosis[50-52].

PUMA (p53 upregulated modulator of apoptosis) is a Bcl-2 homology 3 (BH3)-only Bcl-2 family member and a critical mediator of p53-dependent and -independent apoptosis[53,54]. *PUMA* is normally expressed at a very low level in cells and tissues, but is rapidly induced in response to a wide range of stresses, including genotoxic stress, growth factor and cytokine withdrawal, and infection[54]. Several p53 binding sites are located in the intron 1 region of the *PUMA* gene in humans and mice. Among the transcription factors that activate *PUMA*, the function of p53 is best understood. By DNA damage, p53 is recruited to two p53-responsive elements in the *PUMA* promoter and the binding of p53 facilitates modification of the core histone by acetylation of histones H3 and H4, which activates transcription[55,56]. The proapoptotic activity of PUMA protein requires its interaction with other Bcl-2 family members and mitochondria localization[53,54]. PUMA induces apoptosis by activating the multidomain proapoptotic protein Bax and/or Bak through its interaction with anti-apoptotic Bcl-2 family members, thereby triggering mitochondrial dysfunction and caspase activation. PUMA ablation or

inhibition leads to apoptosis deficiency, underlying increased risks for cancer development and therapeutic resistance[55-57].

In this study, the molecular and regulatory function of ZNF509, a novel BTB/POZ domain protein, in cell proliferation and apoptosis. I was found that ZNF509 activates transcription of *CDKN1A* and represses transcription of *PUMA*, which favors cell survival over apoptosis upon exposure to genotoxic stress.

II. MATERIALS AND METHODS

1. Plasmids, antibodies and reagents

The pGL2-*ARF*-Luciferase (Luc), pGL2-*HDM2*-Luc, pGL2-*p53*-Luc, various pGL2-*CDKN1A*-Luc constructs, pG13-Luc, and the pcDNA3.1-p53 plasmid used in this study have been reported elsewhere[60]. Dr. Kyung-Sup Kim, Yonsei University of Medicine, kindly provided the HA-p300 plasmid. The pcDNA3.0-ZNF509, pcDNA3.1-MIZ-1, and various p300 deletion plasmids were prepared by cloning cDNA fragments into pcDNA3.0 or pcDNA3.1 vectors (Invitrogen, CA). To prepare recombinant GST-POZ_{ZNF509} and GST-ZF_{ZNF509} proteins, cDNA fragments encoding the POZ domain (amino acids (a.a.) 25 to 121) and zinc-finger domains (a.a. 395 to 585) were cloned into pGEX4T3 (Amersham Biosciences, NJ). All plasmid constructs were verified by sequencing.

Antibodies against GAPDH, p53, p21, MIZ-1, p300, Myc-tag, FLAG-tag, acetylated (Ac)-H3, and Ac-H4 were purchased from Upstate (Charlottesville, VA), Chemicon (Temecula, CA), Calbiochem (San Diego, CA) or Santa Cruz Biotechnology (Santa Cruz, CA). To obtain rabbit polyclonal antibody against ZNF509 protein, a white rabbit was immunized subcutaneously with a mixture of GST-POZ_{ZNF509} (a.a. 25 to 121) and GST-ZF_{ZNF509} (a.a. 395 to 585)

recombinant polypeptides 6 times at 2-week intervals. Blood was collected, incubated at 37 ºC for 90 min, and centrifuged. The supernatant was incubated with protein A/G agarose beads (Santa Cruz Biotechnology). The beads were collected and washed, and the antibody was eluted. Chemical reagents were purchased from Sigma (St. Louis, MO).

2. Cell culture and transient transfection assay

HEK293A, HCT116 $p53^{+/+}$, and HCT116 $p53^{-/-}$ cells were maintained at 37 ºC in a humidified incubator with 5% CO_2 air in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100.0 μg/ml streptomycin and 100 units/ml penicillin. Cell culture medium and supplements were from Invitrogen (Gibco-BRL, MD).

The pGL2-*ARF*-Luc, pGL2-*HDM2*-Luc, pGL2-*p53*-Luc, pG13-Luc, various pGL2-*CDKN1A*-Luc promoter reporter fusion plasmids, pcDNA3.0-ZNF509, pcDNA3.1-MIZ-1, HA-p300, pcDNA3.1-p53 and pCMV-LacZ in various combinations were transiently transfected into HEK293A cells using Lipofectamine Plus reagent (Invitrogen, CA). After 24 to 36 h of incubation, the cells were harvested and analyzed for luciferase activity using a Microplate LB 96V luminometer (EG&G Berthold, MD). All the reactions were performed in triplicate. Reporter activity was normalized with co-

transfected β-galactosidase activity or protein concentration for transfection efficiency.

3. Immunocytochemistry

HEK293A cells were transfected with pcDNA3.0-FLAG-ZNF509 plasmid, washed, and fixed with cold methanol and formaldehyde. The cells were permeabilized, washed, blocked with horse serum, and incubated with mouse anti-FLAG primary antibody. After thorough washing, cells were further incubated with FITC-conjugated anti-mouse IgG secondary antibody (Jackson Immuno Research Laboratories, ME). Finally, the cells were soaked with 4, 6-diamidino-2-phenylindole (1.0 mg/ml). The cells were mounted and examined using a Carl Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Germany).

4. Total RNA isolation and tissue distribution of *Zfp509* mRNA Expression in FVB mice

Total RNA was isolated from brain, liver, heart, spleen, white adipose tissue (WAT), brown adipose tissue (BAT), muscle, and kidney tissues and from various cell lines the using TRIzol reagent (Invitrogen). Synthesis of cDNA was conducted using 5.0 μg total RNA, random hexamers (10.0 pmol),

and Superscript reverse transcriptase II in a total volume of 20 μl using a reverse transcription kit (Invitrogen). PCR reactions were performed using the following amplification conditions: initial 94 ºC denaturation for 3 min, 30 cycles of amplification at 94 ºC for 30 sec, 55 ºC for 30 sec, 72 ºC for 30 sec, and a final extension reaction at 72 ºC for 5 min. The following oligonucleotide PCR primers were used: murine Zfp509 (forward, 5'-TTGCTGTTCACAGCTGCCAC-3'; reverse, 5'-TTAGCCTGCGGGCCTTCCAC-3'), GAPDH (forward, 5'-ACCACAGTCCATGCCATCAC-3'; reverse, 5'-TCCACCACCCTGTTGCTGTA-3').

5. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was conducted using the SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers. Reactions were subjected to qRT-PCR quantification using the ABI PRISM 7300 RT-PCR System (Applied Biosystems). All reactions were performed in triplicate. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was measured as an invariant control. The following qRT-PCR oligonucleotide primers sets were used: ZNF509 (forward, 5'-AGATGGCAGCTGCACAGAACTGCCTTT-3'; reverse, 5'-GGGACCAGCTGCGTGTTTAT-3'), MIZ-1 (forward, 5'-AGACCCACGACACGGACAA-3’; reverse, 5’-CCGTCAGCGATGTGGATCT-3’),

p53 (forward, 5'-CCTGAGGTTGGCTCTGACTGTA-3'; reverse, 5'-AAAGCTGTTCCGTCCCAGTAGA-3'), p21 (forward, 5'-AGGGGACAGCAGAGGAAG-3'; reverse, 5'-GCGTTTGGAGTGGTAGAAATCTG-3'), BAX (forward, 5'-TGGAGCTGCAGAGGATGATTG-3'; reverse, 5'-GCTGCCACTCGGAAAAAGAC-3'), PUMA (forward, 5'-CCTGGAGGGTCCTGTACAATCT-3'; reverse, 5'-TCTGTGGCCCCTGGGTAAG-3'), GAPDH (forward, 5'-CCCCTTCATTGACCTCAACTAC-3'; reverse, 5'-TCTCGCTCCTGGAAGATGG-3').

6. Purification of GST fusion protein and GST pull-down assays: *in vitro* transcription and translation of p300 and p53

Recombinant GST, GST-POZ_{ZNF509}, and GST-ZF_{ZNF509} fusion proteins were prepared from *E. coli* BL21 (DE3) grown for 5 h at 37 ºC in medium containing 1.0 mM IPTG. *E. coli* cells were lysed and recombinant proteins were purified using glutathione-agarose 4-bead affinity chromatography (Peptron, Daejeon, Korea). Purified proteins were resolved with 12% SDS-PAGE to quantitate and assess purity.

p300 or p53 polypeptides were prepared by incubating 1.0 μg p300 or p53

expression plasmids with TNT Quick Coupled Transcription/Translation Extract (Promega, WI) containing 30.0 μl TNT Quick Master Mix and 1.0 μl [^{35}S]-methionine (1175.0 Ci/mol) (PerkinElmer Life Sciences, MA) at 30 ºC for 90 min. Polypeptide expression levels were then analyzed by resolving 1.0 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 *in vitro* translated [^{35}S]-methionine labeled p300 or p53 polypeptides at 4 ºC for 4 h in HEMG buffer (40.0 mM HEPES pH 7.9, 100.0 mM KCl, 0.2 mM EDTA, 5.0 mM $MgCl_2$, 0.1% NP40, 10% Glycerol, 1.5 mM DTT and complete mini-protease cocktail). 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, NY).

7. Western blot analysis

Cells were harvested and lysed in RIPA buffer (50.0 mM Tris-HCl pH 8.0, 1% NP-40, 0.25% sodium deoxycholic acid, 150.0 mM NaCl, 1.0 mM EGTA and complete mini-protease cocktail). Cell extracts (30.0 μg) were separated using 12% SDS-PAGE gel electrophoresis, transferred onto Immun-Blot^{TM} PVDF membranes (Bio-Rad, CA), and blocked with 5% skim milk (BD

Biosciences, MD). Blotted membranes were incubated with antibodies against GAPDH, FLAG, c-Myc, p53, p21, PUMA, ZNF509, MIZ-1 and p300, and then incubated with anti-mouse or rabbit secondary antibody conjugated with HRP (Vector Laboratory, CA). Protein bands were visualized with ECL solution (PerkinElmer Life Sciences).

8. Co-immunoprecipitation

Cells were washed, pelleted, and resuspended in a lysis buffer (20.0 mM Tris-HCl, pH 7.5, 150.0 mM NaCl, 10% glycerol, 1% Triton X-100 and complete mini-protease cocktail). The cell lysates were pre-cleared, and the supernatant was incubated overnight with anti-FLAG or anti-Myc antibody on a rotating platform at 4 ºC, followed by incubation with protein A-Sepharose Fast Flow beads. The beads were collected, washed, and resuspended in equal volumes of 5x SDS loading buffer. Immunoprecipitated proteins were separated with 12% SDS-PAGE. Western blot analysis was performed as described above using the appropriate antibodies.

9. Oligonucleotide pull-down assay

Cells were lysed in HKMG buffer (10.0 mM HEPES pH 7.9, 100.0 mM KCl, 5.0 mM $MgCl_2$, 10% glycerol, 1.0 mM DTT, 0.5% NP40 and complete

mini-protease cocktail). Cellular extracts were incubated with 1.0 μg biotinylated double stranded oligonucleotides for 16 h. To collect DNA-bound proteins, the mixtures were incubated with NeutrAvidin-agarose beads (Thermo Fisher Scientific Inc., USA) for 2 h, washed with HKMG buffer, and precipitated by centrifugation. The precipitate was analyzed by Western blot analysis using the appropriate antibodies. The sequences of the oligonucleotides are as follows (only top strands are shown): *CDKN1A* promoter GC-box#1, 5'-GATCGGGAGGGCGGTCCCG-3'; GC-box#2, 5'-GATCTCCCGGGCGGCGCG-3'; GC-box#3, 5'-GATCCGAGCGCGGGTCCCGCCTC-3'; GC-box#4, 5'-GATCCTTGAGGCGGGCCCG-3'; GC-box#5/6, 5'-GATCGGGCGGGGCGGTTGTATATCA-3'; p53RE1, 5'-GATCCGTTAGAGGAAGAAGACTGGGCATGTCTG-3'; p53RE2, 5'-GATCCATCAGGAACATGTCCCAACATGTTGAGCTC-3'. *PUMA* promoter p53RE1, 5'-CCCGGTGTCTGGGCGATCTCCCC-3'; p53RE2, 5'-CAGGGAAACCCCCGGCGCGGAG-3'. *ZNF509* promoter p53RE, 5'-CAAGTTCCCTGATAACATGCCCTTAGGTTTGGTT-3'. Oligonucleotide probes were annealed by heating at 95 ºC for 5 min and cooling slowly to room temperature.

10. Electromobility shift assays (EMSA)

The oligonucleotide probes were annealed by heating at 95 ºC for 5 min and slowly cooled to room temperature. Annealed oligonucleotides were labeled with [α-^{32}P]-ATP and Klenow enzyme (Roche, Mannheim, Germany) by incubating for 30 min at 37 ºC. Labeled double-stranded oligonucleotides were purified using Sephadex^{TM} G-50 (Amersham Biosciences, Uppsala, Sweden) columns. Binding reactions were conducted in 20.0 μl binding buffer containing 10.0 mM HEPES (pH 7.9), 60.0 mM KCl, 1.0 mM dithiothreitol, 1% BSA, 7% glycerol, and purified recombinant GST-ZF_{ZNF509} (200.0 ng). The protein-DNA complexes were resolved from free probe using 4% non-denaturing PAGE at room temperature in 0.5x TBE (89.0 mM Tris-Borate, 2.0 mM EDTA, pH 8.3) buffer. Dried gels were exposed to X-ray film at -70 ºC with a Kodak intensifying screen. The probe sequence on the *CDKN1A* proximal promoter used in EMSA was as follows (only the top strand is shown): GC-box#1, 5'-TTGGGAGGGCGGTCCCG-3'; GC-box#3, 5'-TTCGAGCGCGGGTCCCGCCTC-3'; GC-box#5/6, 5'-TTGGGCGGGGCGGTTGTATATCA-3'.

11. Chromatin immunoprecipitation (ChIP) assay

Cells were treated with formaldehyde (final 1%) to cross-link proteins to

the DNA promoter sequence. Cells were washed and lysed with SDS lysis buffer (1% SDS, 10.0 mM EDTA, 50.0 mM Tris-HCl, pH 8.0). The lysate was sonicated to shear DNA into fragments ranging from 500 to 1000 bp. The sonicated supernatant was diluted 10-fold with ChIP dilution buffer (1% SDS, 1% Triton X-100, 16.7 mM Tris-HCl, pH 8.1, 167.0 mM NaCl, 1.2 mM EDTA) and incubated with antibody overnight at 4 °C with rotation. To collect DNA-protein-antibody complex, protein A/G-agarose slurry was added to the mixture. The mixture was incubated for 2 h at 4 °C with rotation and pelleted by brief centrifugation. After extensive washing of the pellet with washing buffers (low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer, and TE buffer) as recommended by the manufacturer, the pellet was dissolved with 500.0 μl elution buffer and spun to remove excess agarose. The supernatant was treated with 20.0 μl 5.0 M NaCl and heated to 65 °C for 2 h to reverse the protein-DNA cross-link. After treatment with EDTA and proteinase K, the supernatant was extracted with phenol/chloroform and precipitated with ethanol to recover DNA. PCR reactions using the immunoprecipitated DNA were conducted using oligonucleotide primer sets designed to amplify the promoter region. *CDKN1A* distal promoter (forward, 5'-TGCTTGGGCAGCAGGCTGTG-3'; reverse, 5'-GCAACCATGCACTTGAATGT-3'), proximal promoter (forward,

5’-GCGCTGGGCAGCCAGGAGCC-3’; reverse, 5’-CGCTCTCTCACCTCCTCT-3’), 3’-UTR (forward, 5'-GTCACCCTGCCCAACCTTAG-3'; reverse, 5'-TCCTTCCCATCGCTGTCACA-3'), *ZNF509* promoter (forward, 5’-AGCTTCATCCCTGCCCAATTGTA-3’; reverse, 5’-TTGAATAAGTAATACAAAAATAT-3’), 3’-UTR (forward, 5’-TGCCTTCTAACTAGCCAGAGAAT-3’; reverse, 5’-AAATGCTACAAATTTCAATCTTG-3’), *PUMA* promoter (forward, 5’-TCGCTGGGGGTGTGGATCTGT-3’; reverse, 5’-GGCCCGCTCCAAAGCCGCCCC-3’), 3’-UTR (forward, 5’-CCCCAGCCCAGCCTGGGGTGC-3’; reverse, 5’-CACGGGCCCCCTCCCAGGAGG-3’).

12. Quantitative chromatin immunoprecipitation (qChIP)

Intracellular molecular interactions among the ZNF509, MIZ-1, and p300 proteins on the endogenous *CDKN1A* proximal promoter, and the modification of histones H3 and H4 at the *CDKN1A* proximal promoter, were analyzed by the standard ChIP assay protocol as described above. The SYBR Green PCR Master Mix (Applied Biosystems) was used for ChIP quantification using gene-specific primers and the ABI PRISM 7300 RT-PCR System (Applied Biosystems). All reactions were performed in triplicate. IgG

was used as a negative control for the qChIP assays. The following qChIP oligonucleotide primers sets were used: *CDKN1A* proximal promoter (forward, 5'-GATCGCTACCGCGCTGGGCAGCCAGGAGCCT-3'; reverse, 5'-TCGTCACCCGCGCACTTAGA-3'), 3'-UTR (forward, 5'-GTCACCCTGCCCAACCTTAG-3'; reverse, 5'-TCCTTCCCATCGCTGTCACA-3').

13. Knock-down of *ZNF509* mRNA expression by siRNA

Two siRNAs against *ZNF509* mRNA were designed and purchased from Bioneer (Daejeon, Korea): siZNF509-1, 5'-AUGCAAAGGAAGUUAGUAA(dTdT)-3', 5'-UUACUAACUUCCUUUGCAU(dTdT)-3'; siZNF509-2, 5'-AGAAAUACGCAGAGCAAGU(dTdT)-3', 5'-ACUUGCUCUGCGUAUUUCU(dTdT)-3'; Negative siRNA, 5'-CCUACGCCACCAAUUUCGU(dTdT)-3', 5'-ACGAAAUUGGUGGCGUAGG(dTdT)-3'. The siRNA (200.0 pmol) was transfected into cells using Lipofectamine 2000 (Invitrogen). Efficiency of *ZNF509* knockdown was confirmed by qRT-PCR.

14. Foci formation assay

HEK293A cells were cultured in 6-well culture plates and then transfected with 0.5 μg pcDNA3.0 and pcDNA3.0-ZNF509 using Lipofectamine plus reagent. Following transfection, cells were maintained in a medium supplemented with 10% FBS for one week in the presence of G418 (800.0 μg/ml). Colonies resistant to G418 were stained with crystal violet (0.5% crystal violet in 20% EtOH).

15. MTT assay

Confluent HEK293A cells grown on 10.0 cm culture dishes were transfected with pcDNA3.0-ZNF509 and/or the pcDNA3.1-MIZ-1 expression plasmid or siZNF509 RNA. The cells (1.5x 10^5 cells) were transferred to 6-well culture dishes and grown for 0 to 3 days. The cells were incubated for 1 h at 37 ºC with 500.0 μl/well MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (2.0 mg/ml)]. Precipitates were dissolved with 1.0 ml DMSO. Cellular proliferation was determined from the conversion of MTT to formazan using a SpectraMAX 250 at 570 nm (Molecular Device Co.).

16. Fluorescence-activated cell sorting (FACS) analysis

Confluent HEK293A cells grown in 10.0 cm culture dishes were

transfected with pcDNA3.0-ZNF509 or siZNF509 RNA. The cells were washed, fixed with methanol, and stained with 50.0 μg/ml propidium iodide in 100.0 μg/ml ribonuclease A for 30 min at 37 ºC in the dark. DNA content, cell cycle profiles, and forward scatter were analyzed using a FACSCalibur (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).

III. RESULTS

1. ZNF509 is a POK family protein.

The human genomic sequence encoding the predicted ZNF509 protein maps to chromosome 4p16.3. *ZNF509* cDNA (Gene ID: 166793) encodes a protein composed of 765 amino acids (GenBank Accession No. NP_660334.3, Fig. 1A). ZNF509 contains an N-terminal POZ domain from amino acids 25 to 121 and seven Krüppel-like C_2H_2 type zinc-fingers at the C-terminus (Fig. 1B). Immunocytochemistry of HEK293A cells using anti-FLAG-tag antibody showed ZNF509 expression in both the cytoplasm and nucleus (Fig. 1C). *Zfp509* mRNA was expressed in all male FVB mouse tissues examined with high mRNA expression levels in white adipose tissues (WAT) and kidney (Fig. 1D).

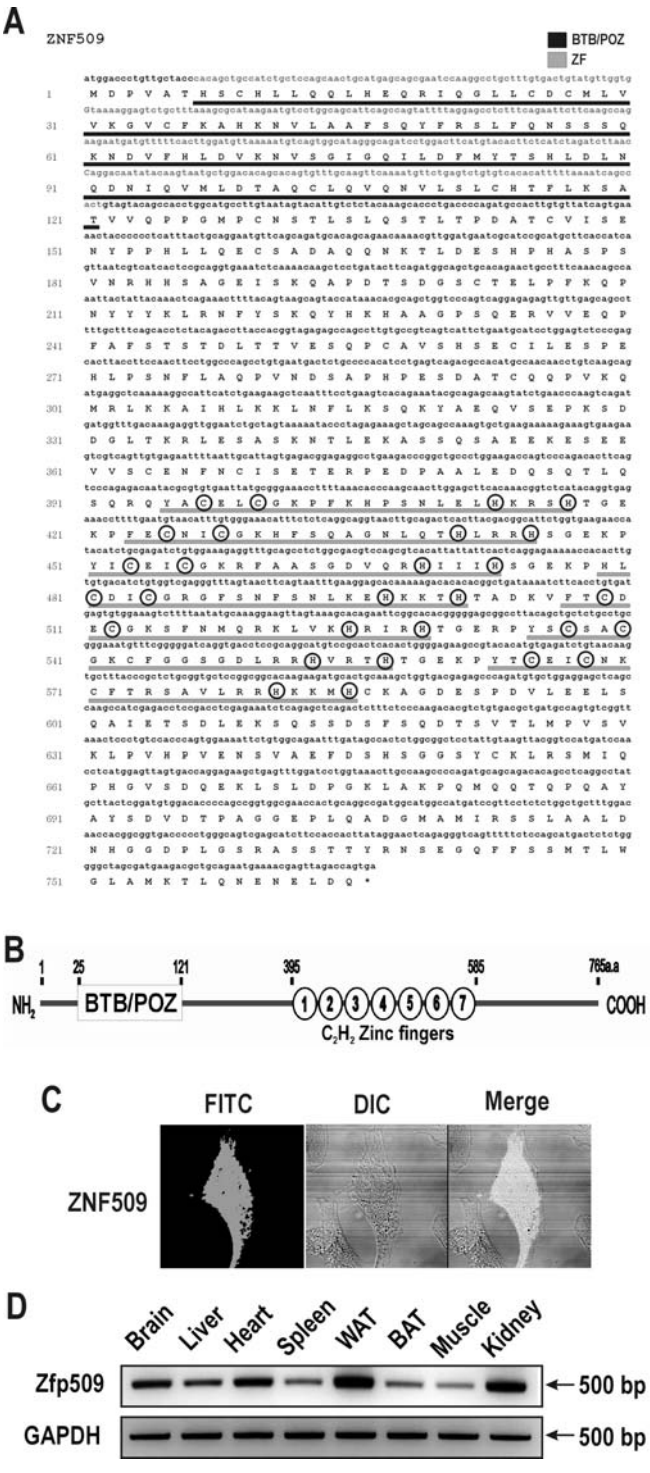

A
ZNF509
BTB/POZ
ZF
B
1
25
121
395
585
765a.a
NH2
BTB/POZ
1 2 3 4 5 6 7
COOH
C2H2 Zinc fingers
C
FITC
DIC
Merge
ZNF509
D
Brain
Liver
Heart
Spleen
WAT
BAT
Muscle
Kidney
Zfp509
500 bp
GAPDH
500 bp

Figure 1. Characterization of a novel BTB/POZ domain and a Krüppel-like zinc-finger (POK) protein, ZNF509. (A) Nucleotide and amino acid sequence of ZNF509. (B) Structure of ZNF509. ZNF509 is composed of 765 amino acids and contains an N-terminal BTB/POZ-domain and seven C_2H_2-type zinc-fingers. (C) Immonocytochemistry of ZNF509 expression in HEK293A cells that were transfected with a FLAG-ZNF509 expression plasmid. (D) Tissue distribution of mouse *Zfp509* mRNA expression. Semi-quantitative (RT-PCR) analysis of cDNA prepared from total RNA of male FVB mouse tissues. GAPDH was measured as a control.

2. ZNF509 is a downstream target gene of p53.

p53 is a well known transcription factor that is closely connected with transcriptional expression of cell cycle regulators. Results from semi-quantitative (RT-PCR) analysis of cDNA in several cancer cells and immortalized cells showed that ZNF509 expression is regulated according to p53 expression. To definitively test whether p53 regulates *ZNF509* expression, HEK293A cells were treated with etoposide, a DNA damaging agent that increases endogenous p53 expression in a time or dose dependent manner. *ZNF509* mRNA and protein expression of the cells treated with etoposide were increased (Figs. 2A and B). Also, *ZNF509* mRNA and protein expression were compared in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells treated with etoposide. Etoposide was found to increase *ZNF509* mRNA and protein expression in HCT116 $p53^{+/+}$ cells, but not in HCT116 $p53^{-/-}$ cells (Fig. 2C, D), suggesting that p53 regulates *ZNF509* expression.

Two potential p53 binding elements were identified in the *ZNF509* promoter region using the MacVector program. Chromatin immunoprecipitation (ChIP) assays with p53 antibody were conducted to test whether p53 binds to these p53 binding sites in HEK293A cells. Endogenous p53 was found to bind to the potential p53 binding sites within the *ZNF509* promoter (Fig. 3A). Furthermore, ZNF509 was shown to bind to the proximal

region of the *CDKN1A* promoter by oligonucleotide pull-down assays (Fig. 3B). qRT-PCR and Western blot analysis in HCT116 $p53^{-/-}$ cells transfected with the exogenous p53 expression plasmid showed that p53 activates *ZNF509* gene transcription (Fig. 3C, D). These results indicate that p53 directly binds to the *ZNF509* promoter to activate transcription of the gene.

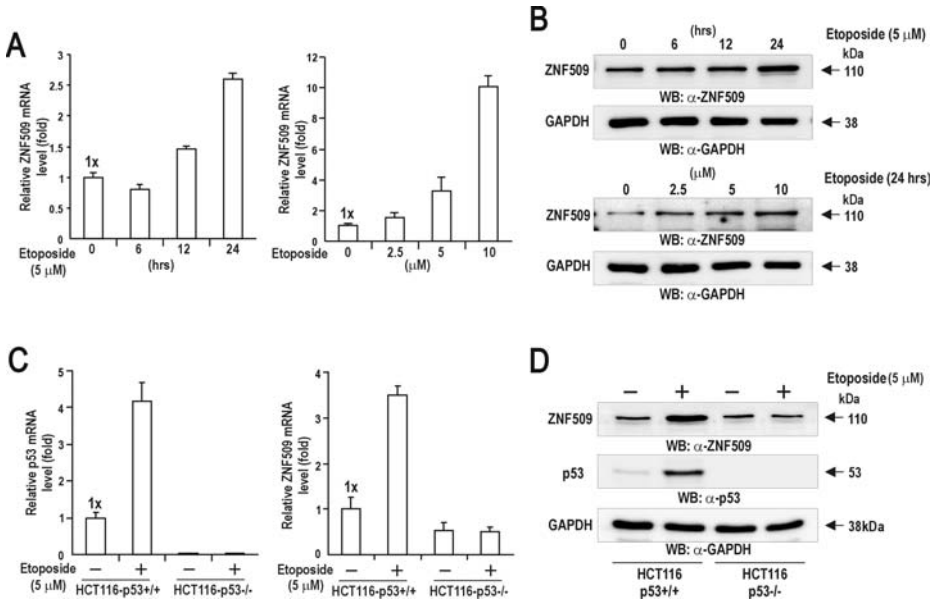


Figure 2. p53 activates transcription of *ZNF509* by binding to p53 binding elements. HEK293A cells were treated with etoposide in either a time- or dose-dependent manner. *ZNF509* mRNA (A) and protein expression (B) level in cell lysates were analyzed by qRT-PCR and Western blots. Endogenous *ZNF509* mRNA and protein expression of HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells treated with etoposide (5.0 μM) were analyzed by qRT-PCR and Western blots. GAPDH was measured as a control.

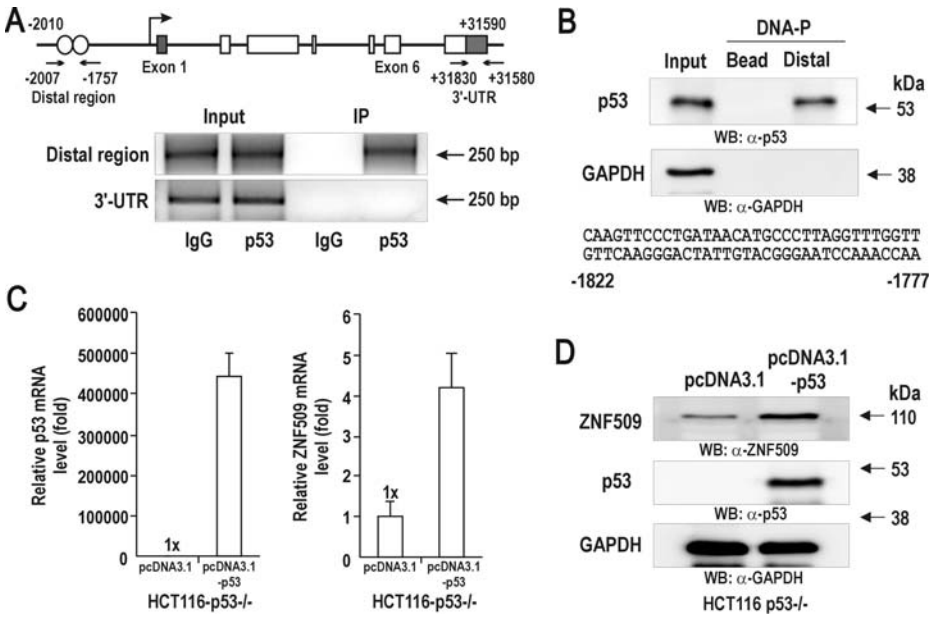


Figure 3. p53 activates transcription of *ZNF509* by binding to p53 binding elements. (A) ChIP assay of p53 binding to the endogenous *ZNF509* promoter at -2007 bp to -1757 bp. HEK293A cell lysates were fixed with formaldehyde and immunoprecipitated with a p53 antibody. (B) Oligonucleotide pull-down assay / Western blot assay of p53 binding to the distal *ZNF509* promoter region (-1822 bp to -1777 bp). p53 activates transcription of the *ZNF509* gene. HCT116 $p53^{-/-}$ cells were transfected with a p53 expression plasmid. *ZNF509* mRNA (C) and protein expression (D) levels in cell lysates were analyzed by qRT-PCR and Western blots. GAPDH, control; 3'-UTR, 3'-untranslated region; IP, immunoprecipitation; DNA-P, oligonucleotide pull-down assay.

PART I

3. ZNF509 arrests the cell cycle at the G2-M phase in HEK293A cells.

Serial analysis of gene expression (SAGE) analysis by the Cancer Genomic Anatomy Project (CGAP) showed that ZNF509 expression is lower in cancer tissues derived from the breast, pancreas, ovary, prostate, and skin compared with normal tissues (http://cgap.nci.nih.gov/SAGE). Based on this data, I investigated whether ZNF509 regulates the cell cycle. HEK293A cells stably transfected with a ZNF509 expression plasmid showed very slow cell growth and formed a few foci (Fig. 4A). MTT assays also showed that ectopic ZNF509 expression significantly inhibited cell growth while knockdown of ZNF509 expression stimulated cell proliferation (Fig. 4B). FACS analysis revealed that ectopic ZNF509 expression arrested the cell cycle as the population of cells in the G2-M phase overexpressing ZNF509 increased from 16.5% to 67.5%. The number of cells in S phase decreased from 29.8% to 5%. Knockdown of ZNF509 expression resulted in the opposite effect (Fig. 4C).

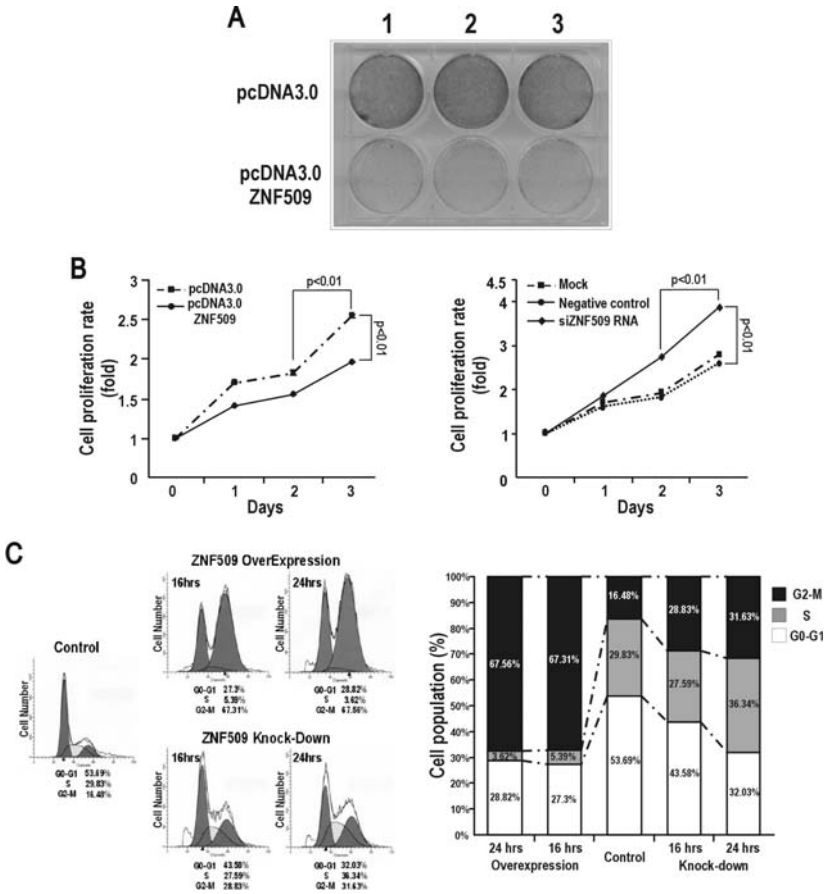


Figure 4. ZNF509 arrests the cell cycle in HEK293A cells. (A) Foci formation assay. Cells transfected with a ZNF509 expression plasmid were cultured in medium containing G418 and stained with 0.1% crystal violet. (B) MTT assay of cells grown for 0, 1, 2, or 3 days. Cells were transfected with either the control vector or ZNF509 expression plasmid and analyzed for cell growth. Alternatively, cells were transfected with either a negative control siRNA or ZNF509 siRNA. *p* values are <0.01. (C) FACS analysis. Cells were transfected with ZNF509 expression plasmid or ZNF509 siRNA for 16 or 24 h, stained with propidium iodide, and analyzed by FACS.

4. ZNF509 activates transcription of the *CDKN1A* gene in HEK293A cells.

Whether ZNF509 regulates expression of *ARF*, *HDM2*, *TP53,* and *CDKN1A* genes, which are important in cell cycle regulation, was investigated using promoter-Luc gene fusion plasmids. ZNF509 was shown to activate transcription of both *TP53* and *CDKN1A* transcriptional fusions in HEK293A cells (Fig. 5A); however, ZNF509 did not activate transcription of *TP53* when analyzed by qRT-PCR and Western blot (Supplementary Fig. 1). Knockdown of ZNF509 mRNA by RNA interference was confirmed by transcription assays with two different siRNAs that repressed p21 (Fig. 5B). Also, qRT-PCR and Western blot analysis of the HEK293A cells transfected with the ZNF509 expression plasmid showed that ZNF509 can increase p21 expression by acting on the proximal promoter of *CDKN1A* (Fig. 5D, E). Knockdown of *ZNF509* expression resulted in a decrease in p21 transcription (Fig. 5F, G). Taken together, these data suggest that ZNF509 activates transcription of *CDKN1A* to arrest cell cycle progression.

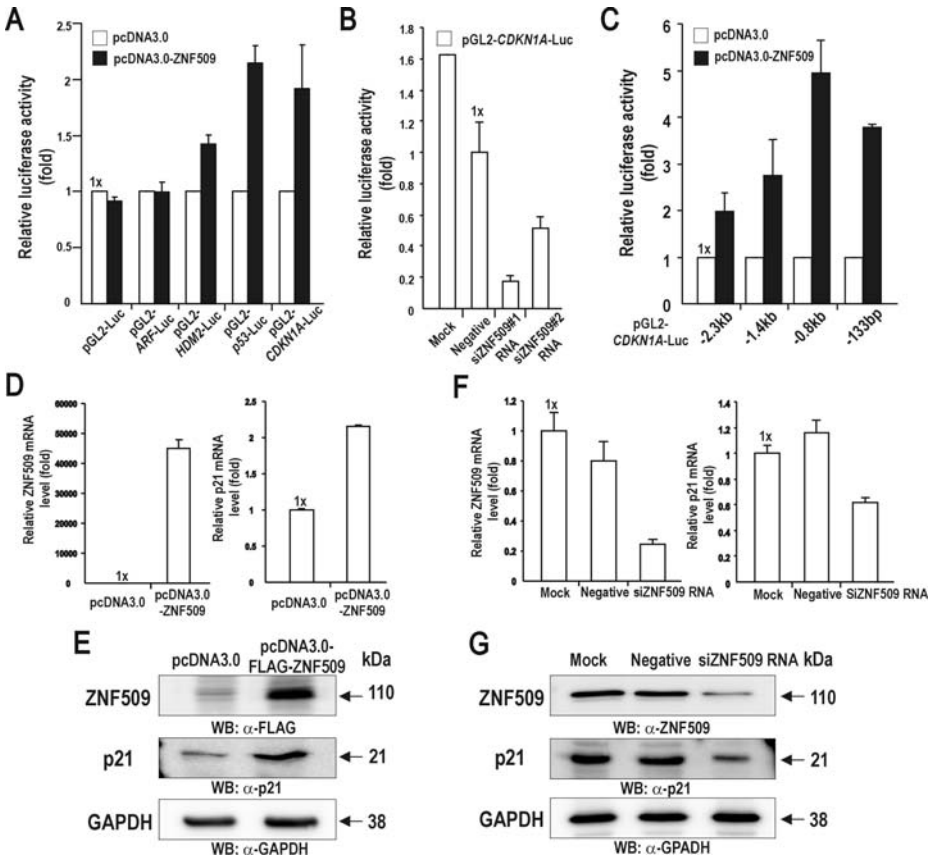


Figure 5. ZNF509 activates transcription of *CDKN1A* in HEK293A cells. (A, B, C) Transient transcription analysis of *ARF*, *HDM2*, *TP53* and various *CDKN1A* promoters fused with the luciferase gene. ZNF509 expression plasmids or ZNF509 siRNA and reporter plasmids were transiently co-transfected and luciferase activity was measured. Error bars represent standard deviations. (D, E, F, G) qRT-PCR and Western blot analysis of endogenous ZNF509 expression. cDNA for qRT-PCR and protein for Western blots were prepared from the cell lysates transfected with a ZNF509 expression plasmid or ZNF509 siRNA. GAPDH, control; Mock, no siRNA; Negative, scrambled siRNA.

5. ZNF509 zinc-fingers bind to the GC-box#3 and #5/6 of the *CDKN1A* proximal promoter.

Since ZNF509 was shown to activate transcription of the *CDKN1A* gene, the *cis*-regulatory elements of the *CDKN1A* gene promoter responsible for transcriptional activation by ZNF509 were mapped. Transcription assays using four different promoter-Luc fusion reporter plasmids indicated that ZNF509 activates transcription by acting on a proximal promoter element (Fig. 6A). I investigated whether ZNF509 was targeted to the proximal promoter by ChIP assays. HEK293A cells were transfected with a FLAG-ZNF509 expression plasmid and ChIP assays using anti-ZNF509 antibody showed that ZNF509 binds to the proximal region and not to any other region of the promoter, such as the distal p53 binding region or the 3'-UTR of the *CDKN1A* gene (Fig. 6B).

Oligonucleotide pull-down assays verified that ZNF509 binds to the proximal promoter region (Fig. 6C) and also identified specific target sequences for ZNF509 binding within the *CDKN1A* proximal promoter. ZNF509 was shown to preferentially bind to the GC-box#3 and #5/6 probes over the other GC-box probes tested (Fig. 6D). In addition, EMSA showed that the ZNF509 zinc-fingers directly interacted with the GC-box#1, #3, and #5/6 (Fig. 6E). These data suggest that the GC-box#3 and #5/6 of the proximal promoter is very important for transcriptional activation of the *CDKN1A* gene by ZNF509.

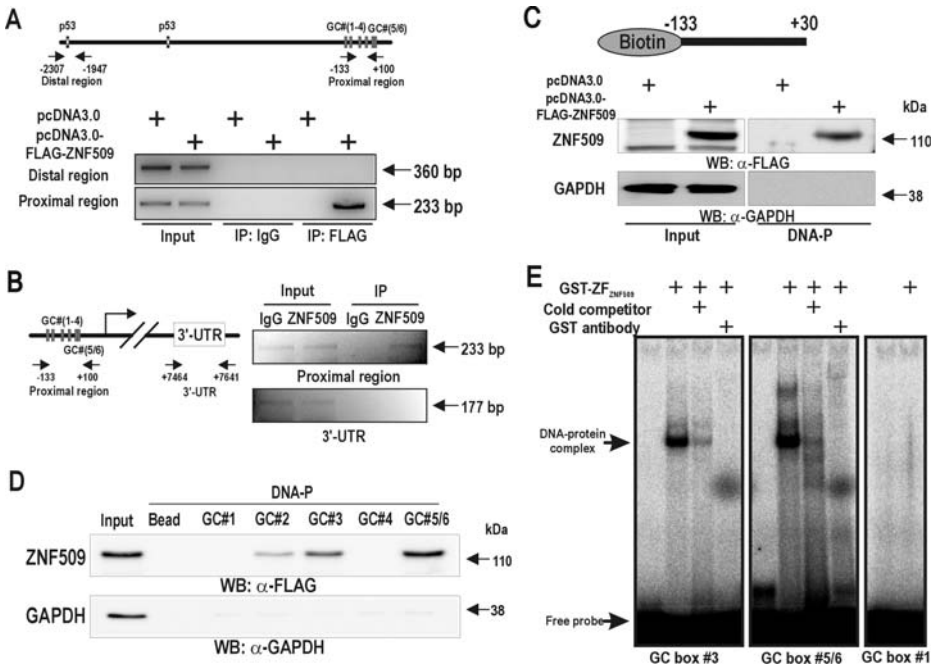


Figure 6. ZNF509 zinc-fingers bind to the proximal GC-box#3 and #5/6 of the *CDKN1A* proximal promoter. (A, B) ChIP assay of ZNF509 binding on the *CDKN1A* promoter. HEK293A cells were transfected with a FLAG-ZNF509 expression plasmid and immunoprecipitated with anti-FLAG (A), or ZNF509 (B) antibody. (C, D) Oligonucleotide pull-down assays of ZNF509 binding to the proximal promoter elements of *CDKN1A*. HEK293A cell extracts that were transfected with a FLAG-ZNF509 expression plasmid were incubated with biotinylated double-stranded oligonucleotides. The mixtures were further incubated with NeutrAvidin-agarose beads and precipitated by centrifugation. The precipitate was analyzed by Western blot using antibody against FLAG. (E) EMSA. The ^{32}P-labeled GC-box probes were incubated with GST-ZF$_{ZNF509}$ and separated by 4% non-denaturing PAGE. GAPDH, control; 3'-UTR, 3'-untranslated region; IP, immunoprecipitation; DNA-P, oligonucleotide pull-down assay.

6. The POZ domain of ZNF509 is important in transcription activation and interacts directly with p300. ZNF509-p300 complex acetylates histone H3 and H4.

Transcriptional activators often activate transcription by interaction with co-activators such as p300/CBP, PCAF, and SRC-1, to name a few[61-64]. Therefore, it was investigated whether ZNF509 and/or p300 co-regulate expression of the *CDKN1A* gene. ZNF509 and p300 synergistically activated transcription of the *CDKN1A* gene in HEK293A cells that were co-transfected with the respective expression plasmids (Fig. 7A). Co-immunoprecipitation and Western blots of HEK293A cell extracts transfected with the FLAG-ZNF509 expression plasmid with anti-p300 antibody showed that the ZNF509 and p300 proteins interact with each other (Fig. 7B). In addition, GST-fusion protein pull-down assays showed that the POZ domain of ZNF509 interacts directly with the DUF906 domain of p300 (Fig. 7C).

Furthermore, I investigated whether p300 associated with the *CDKN1A* promoter in a ZNF509 dependent manner. HEK293A cells were transfected with a ZNF509 expression plasmid or siZNF509 RNA. ChIP assays showed that binding of p300 to the *CDKN1A* proximal promoter increased in the presence of ZNF509; in the absence of ZNF509 expression, p300 binding was weak (Fig. 7D). Co-activators recruited by transcription activators acetylate the histones of nearby nucleosomes around the proximal promoter; ChIP assays showed that the ZNF509-p300 complex significantly increased acetylated histones H3 and H4 at the *CDKN1A* promoter in HEK293A cells (Fig. 7E).

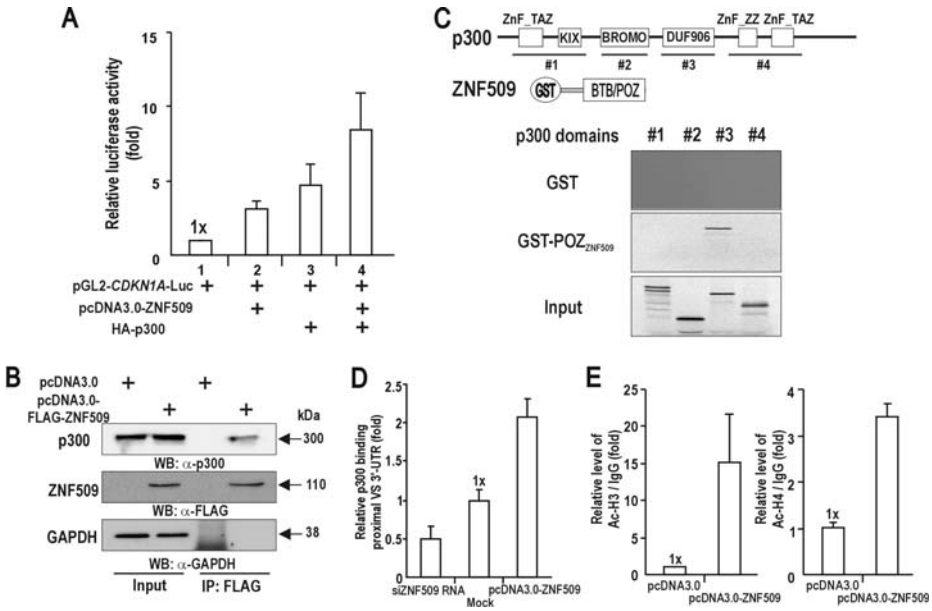


Figure 7. The ZNF509 POZ domain interacts directly with p300. The ZNF509-p300 complex acetylates histones H3 and H4. (A) Transcriptional regulation of the *CDKN1A* promoter by ZNF509 and p300. Expression plasmids containing ZNF509 or/and p300 and CDKN1A promoter-Luc fusion reporter plasmids were transiently co-transfected into HEK293A cells and luciferase activity was measured. Error bars represent standard deviations. (B) Co-immunoprecipitation of ZNF509 and p300. Cell lysates prepared from HEK293A cells transfected with a FLAG-ZNF509 expression plasmid were immunoprecipitated using anti-FLAG antibody and analyzed by Western blot using anti-p300 antibody. (C) *In vitro* GST-fusion protein pull-down assay. Recombinant GST or GST-POZ$_{ZNF509}$ was incubated with [^{35}S]-methionine-labeled p300 polypeptide fragments, pulled down, and resolved by 15% SDS-PAGE. The gel was then exposed to X-ray film. Input was 10% of the p300 fragments added in the binding reactions. (D, E) p300 by interaction with

ZNF509 via its POZ domain increases the transcription of *CDKN1A* by ZNF509-directed acetylation of H3 and H4. ChIP assays of p300 binding and histone modifications at the proximal promoter of *CDKN1A* using antibodies against p300, Ac-H3, and Ac-H4. HEK293A cells were transfected with FLAG-ZNF509 or siZNF509 RNA and immunoprecipitated using the antibodies indicated. GAPDH, control; 3'-UTR, 3'-untranslated region; IP, immunoprecipitation.

7. ZNF509 interacts with the transcriptional activator MIZ-1.

The results described above suggested that ZNF509 activates transcription of the *CDKN1A* gene. Therefore, I investigated additional mechanisms for how ZNF509 activates transcription at the *CDKN1A* promoter. Based on previous studies on the BTB/POZ protein interaction network, I found that the POZ domain of ZNF509 interacts with MIZ-1 (Fig. 8A), characterizing MIZ-1 as a transcriptional activator of the *CDKN1A* gene. Co-immunoprecipitation and Western blot analysis of HEK293A cells transfected with FLAG-ZNF509 or Myc-MIZ-1 expression plasmids revealed that ZNF509 and MIZ-1 interact with each other (Fig. 8B). Oligonucleotide pull-down assays and ChIP assays showed that ZNF509 and MIZ-1 bind to the *CDKN1A* proximal promoter. However, these two proteins bind more strongly to the *CDKN1A* promoter in the presence of ZNF509 and MIZ-1 expression plasmids (Fig. 8C, D).

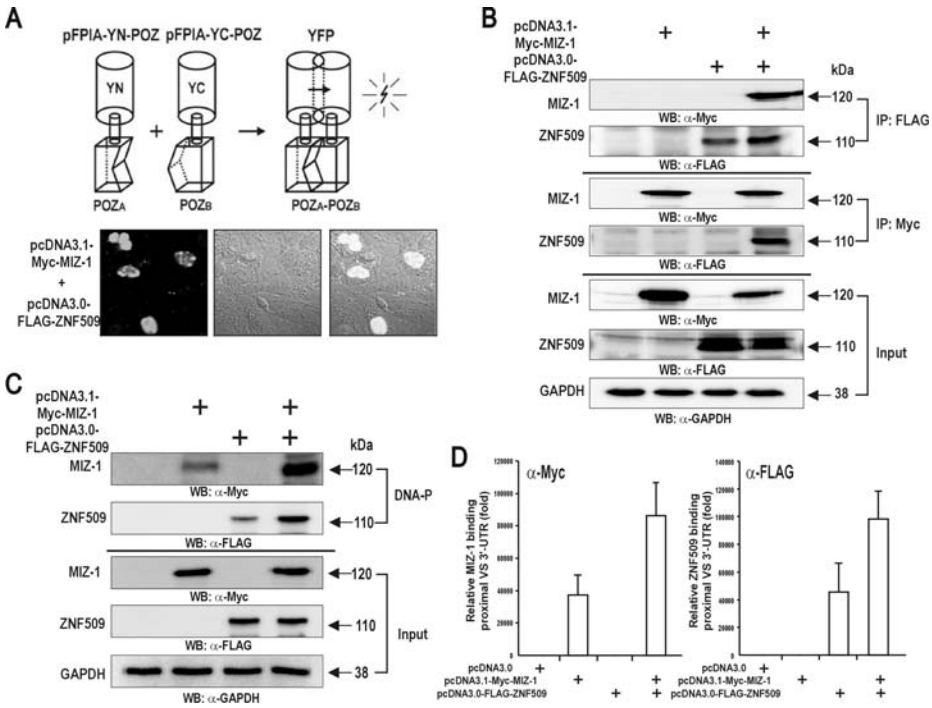


Figure 8. ZNF509 interacts with the transcription factor, MIZ-1. (A) BiFC/FRET assays. CV1 cells were transfected with YN-tagged ZNF509 POZ and YC-tagged MIZ-1 POZ domain pFPIAA expression plasmids and analyzed with confocal microscopy. (B) Cell lysates prepared from HEK293A cells transfected with FLAG-ZNF509 and/or Myc-MIZ-1 expression plasmids were immunoprecipitated using anti-FLAG or anti-Myc antibody and analyzed by Western blot using the antibodies indicated. (C) Oligonucleotide pull-down assays of ZNF509 and MIZ-1 binding to the proximal region of the *CDKN1A* promoter. HEK293A cell extracts transfected with FLAG-ZNF509 and/or Myc-MIZ-1 expression plasmids were incubated with biotinylated double-stranded oligonucleotides. The mixtures were further incubated with NeutrAvidin-agarose beads and precipitated by centrifugation. The precipitate

was analyzed by Western blot using antibody against FLAG or Myc. (D) ChIP assay of ZNF509 or MIZ-1 binding on the *CDKN1A* promoter. HEK293A cells were transfected with FLAG-ZNF509 and/or Myc-MIZ-1 expression plasmids and immunoprecipitated with FLAG or Myc antibody. GAPDH, control; 3'-UTR, 3'-untranslated region; IP, immunoprecipitation; DNA-P, oligonucleotide pull-down assay.

8. ZNF509 and MIZ-1 synergistically activate *CDKN1A* transcription.

I investigated the functional significance of the molecular interaction between ZNF509 and MIZ-1 on *CDKN1A* transcription. ZNF509 and MIZ-1 were shown to activate transcription independently and once co-transfected, the two transcription factors synergistically activated transcription (Fig. 9A). Also, qRT-PCR and Western blot analysis verified the results of the reporter gene assay (Fig. 9B, C). MTT assays showed that cells co-transfected with ZNF509 and MIZ-1 had a slower growth rate than cells transfected with ZNF509 or MIZ-1 expression plasmids alone (Fig. 9D). These results indicate that ZNF509 and MIZ-1 interact to activate transcription of *CDKN1A* and the transcriptional activation involves recruitment of p300 to ZNF509 and MIZ-1 complex (Fig. 9E).

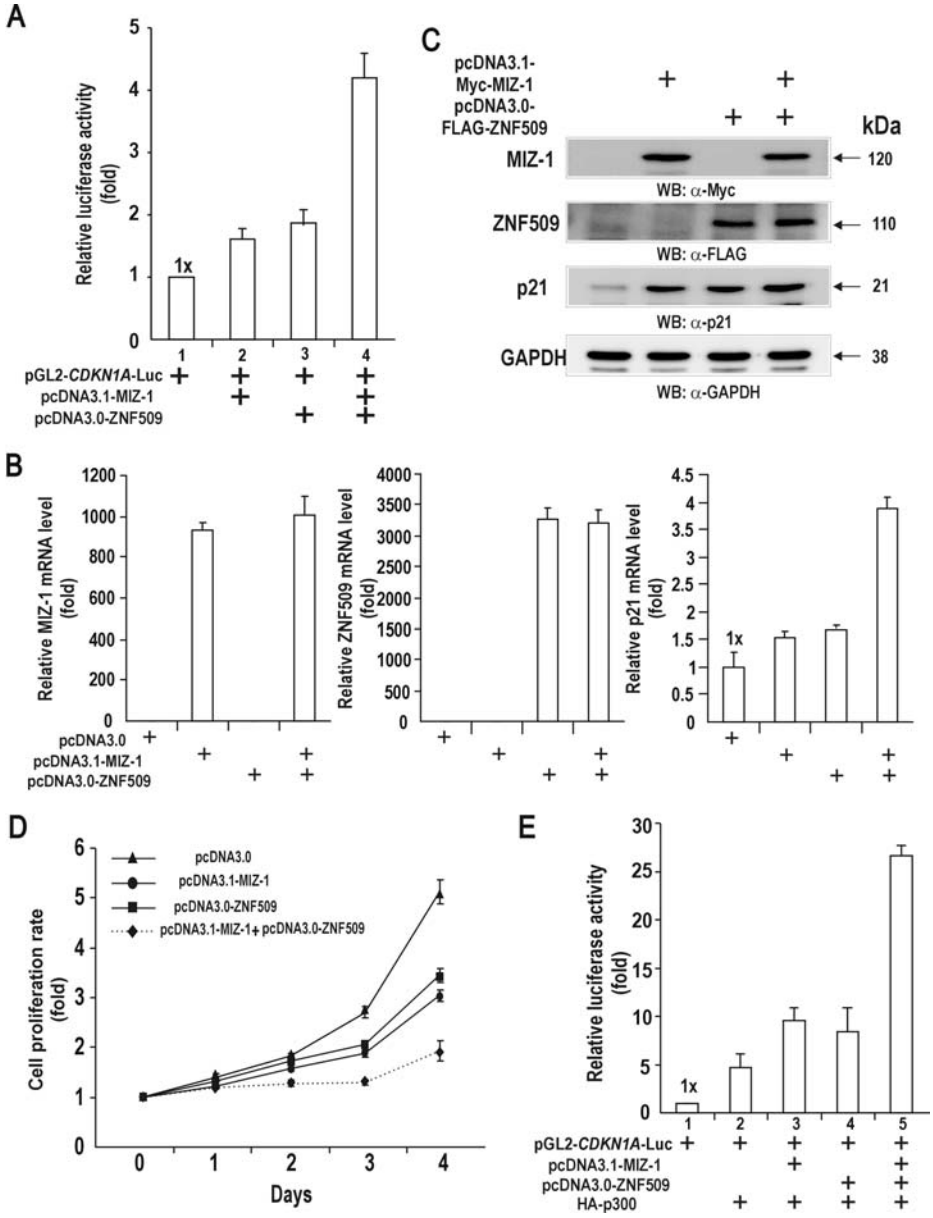


Figure 9. ZNF509 and MIZ-1 synergistically activate transcription of *CDKN1A*. (A) Transcriptional regulation of the *CDKN1A* promoter by

ZNF509 and MIZ-1. Expression plasmids with ZNF509 and/or MIZ-1 and reporter plasmids were transiently co-transfected into HEK293A cells and luciferase activity was measured. Error bars represent standard deviations. (B, C) qRT-PCR and Western blot analysis of p21 expression by ZNF509 and/or MIZ-1. cDNA for qRT-PCR and proteins for Western blot were prepared from HEK392A cell lysates transfected with ZNF509 and/or MIZ-1 expression plasmids. (D) MTT assay of cells grown for 0, 1, 2, 3 or 4 days. Cells were transfected with either control vector or ZNF509 expression plasmid in the absence or presence of MIZ-1 expression plasmid and analyzed for cell growth. (E) Transcriptional regulation of the *CDKN1A* promoter by ZNF509, MIZ-1 and p300. ZNF509, MIZ-1, and p300 expression plasmids and reporter plasmids were transiently co-transfected into HEK293A cells and luciferase activity was measured. Error bars represent standard deviations. GAPDH, control.

PART II

1. ZNF509 decreases etoposide-induced apoptosis in HCT116 p53$^{+/+}$ cells.

I have shown that ZNF509 activates *CDKN1A* expression and inhibits cell proliferation particularly in the presence of MIZ-1. However, some cell cycle regulators such as oncogenes and tumor suppressors have more than one function when the cells were exposed to various stimuli[65-67]. So, it was investigated whether ZNF509 increased apoptosis when cells were treated with the DNA damaging agent, etoposide. Unexpectedly, ZNF509 decreased the number of cells undergoing apoptosis in HCT116 p53$^{+/+}$ cells treated with etoposide (Fig. 10).

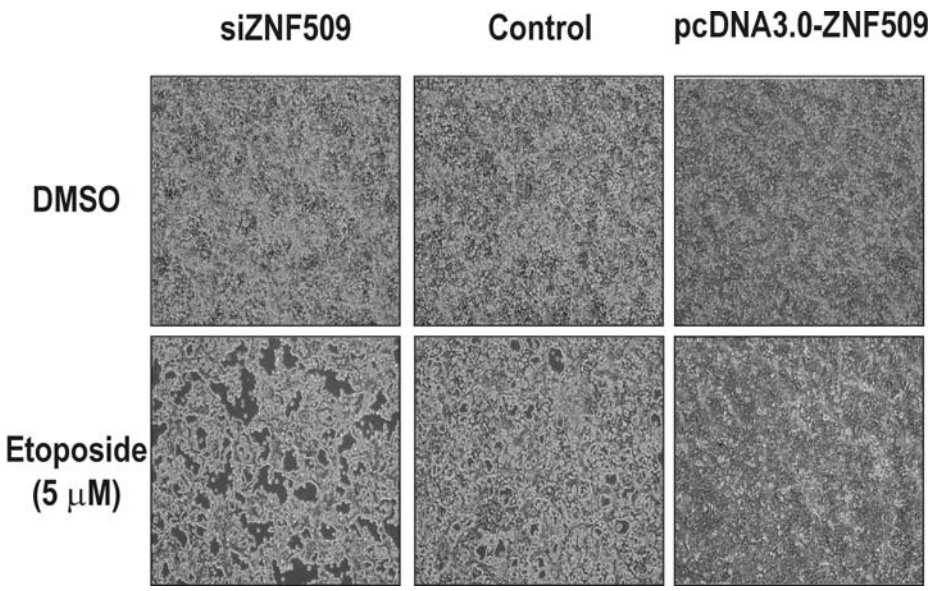


Figure 10. ZNF509 decrease etoposide-induced apoptosis in HCT116 p53$^{+/+}$ cells. HCT116 p53$^{+/+}$ cells were transfected with ZNF509 expression plasmid and siZNF509 RNA. Control cells were co-transfected with pcDNA3.0 vector and negative siRNA. After 2 days, the cells were treated with etoposide (5.0 μM) and analyzed for cell growth.

2. ZNF509 decreases *PUMA* gene expression upon etoposide treatment.

When cells are exposed to DNA damage agents, p53 is induced to direct cells to undergo apoptosis by mobilizing p53 downstream genes. Therefore, it was investigated whether ZNF509 regulates expression of apoptosis related genes that contain p53 response elements in HCT116 $p53^{+/+}$ cells when exposed to DNA damage. Transcriptional analysis of a pG13-Luc reporter plasmid (an artificial p53 response element reporter plasmid) showed that ZNF509 could inhibit the transcriptional activity of p53 (Fig. 11A). Also, qRT-PCR and Western blot analysis of HCT116 $p53^{+/+}$ cells transfected with the ZNF509 expression plasmid showed that ZNF509 could repress *PUMA* gene expression when the cells were treated with etoposide. Also, knockdown of ZNF509 expression resulted in the activation of *PUMA* gene transcription (Fig. 11B, C). These data suggest that ZNF509 is a transcriptional repressor that represses the activity of p53 in response to DNA damage.

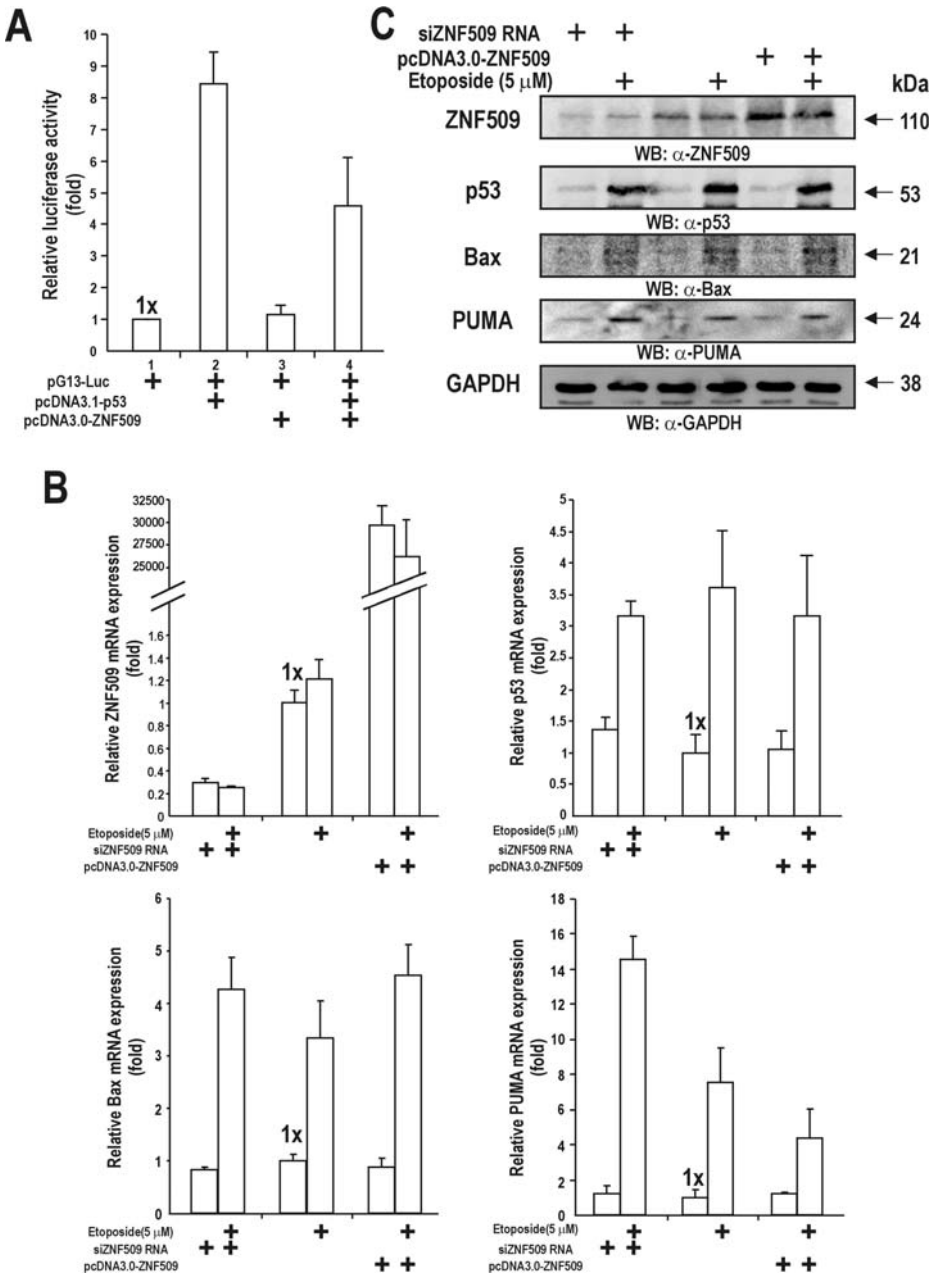


Figure 11. ZNF509 represses *PUMA* gene expression induced by etoposide

treatment. (A) Transcriptional regulation of pG13-Luc (with 13x p53RE at the proximal promoter) by p53 and ZNF509. p53 and/or ZNF509 expression and reporter plasmids were transiently co-transfected into HCT116 $p53^{+/+}$ cells and luciferase activity was measured. Error bars represent standard deviations. (B) qRT-PCR. HCT116 $p53^{+/+}$ cells transfected with ZNF509 expression plasmid or siZNF509 RNA were treated with etoposide and analyzed for *ZNF509*, *p53*, *Bax*, and *PUMA* mRNA expression by qRT-PCR. (C) Western blot analysis. HCT116 $p53^{+/+}$ cells treated as above were analyzed for ZNF509, p53, Bax and PUMA expression. GAPDH, control.

3. ZNF509 interacts with the tumor suppressor p53.

Because ZNF509 represses transcriptional activation of *PUMA* and pG13-Luc by p53, it was suspected that ZNF509 might effect p53 action by protein-protein interactions. It was investigated whether ZNF509 interacts with p53 by co-immunoprecipitation and *in vitro* GST-fusion protein pull-down assays. These assays demonstrated that ZNF509 and p53 interact with each other directly by molecular interaction between the GST-ZF domain of ZNF509 and the polypeptide containing the DNA binding domain of p53 (Fig. 12A, B). Oligonucleotide pull-down assays were conducted to test if ZNF509 could bind to the p53 binding elements of the *PUMA* gene. Although ZNF509 was not shown to directly bind, ZNF509 inhibited the binding of p53 to the p53 RE-1 and p53 RE-2 regions of the *PUMA* promoter (Fig. 12C).

Furthermore, I investigated whether ZNF509 is targeted to the PUMA promoter by ChIP assays in the presence or absence of DNA damage. ChIP was performed on either HCT116 $p53^{+/+}$ cells or HCT116 $p53^{+/+}$-FLAG-ZNF509 stably transfected cells treated with etoposide. ZNF509 inhibited DNA binding of p53 to the *PUMA* promoter when the cells were exposed to the DNA damaging agent (Fig. 12D), indicating that ZNF509 inhibits p53-mediated activation of *PUMA* transcription and that ZNF509 has anti-apoptotic function in response to DNA damage.

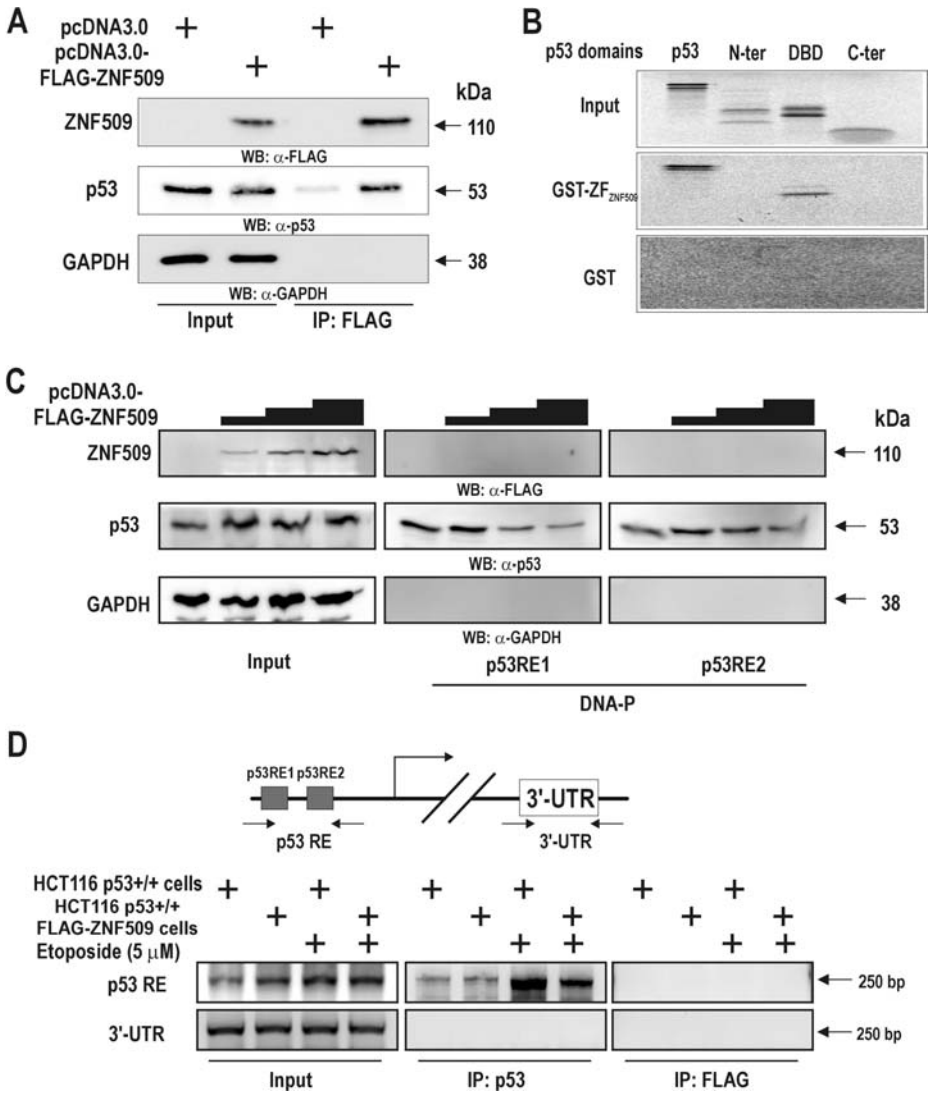


Figure 12. ZNF509 interacts with tumor suppressor p53. (A) Co-immunoprecipitation of ZNF509 and p53. Cell lysates prepared from HEK293A cells transfected with FLAG-ZNF509 expression plasmid were immunoprecipitated using anti-FLAG antibody and analyzed by Western blot using the indicated antibodies. (B) *In vitro* GST-fusion protein pull-down

assays. Recombinant GST or GST-ZF_{ZNF509} was incubated with [^{35}S]-methionine-labeled p53 polypeptide fragments, pulled down, and resolved by 15% SDS-PAGE. The gel was then exposed to X-ray film. Input was 10% of the p53 mutants added in the binding reactions. (C) Oligonucleotide pull-down assay of ZNF509 and p53 binding to two p53 binding elements of the *PUMA* promoter. HEK293A cell extracts transfected with FLAG-ZNF509 expression plasmid were incubated with biotinylated double-stranded oligonucleotides. The mixtures were further incubated with NeutrAvidin-agarose beads and precipitated by centrifugation. The precipitate was analyzed by Western blot using antibody against FLAG or p53. (D) ChIP assay of ZNF509 or p53 binding to the *PUMA* promoter. HCT116 $p53^{+/+}$ cells and HCT116 $p53^{+/+}$ - FLAG-ZNF509 cells were treated with etoposide (5.0 μM) and immunoprecipitated with anti-FLAG or p53 antibody. GAPDH, control; 3'-UTR, 3'-untranslated region; IP, immunoprecipitation; DNA-P, oligonucleotide pull-down assay.

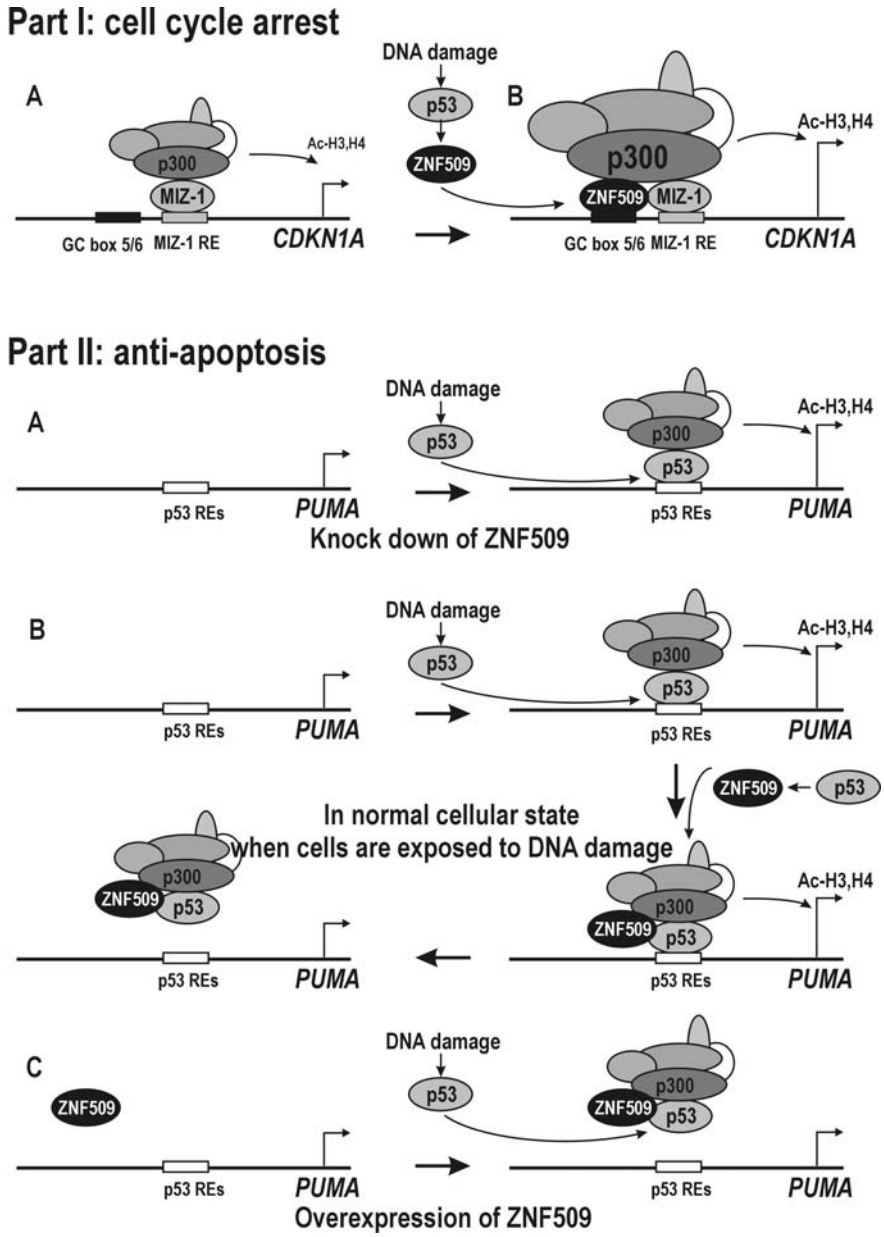


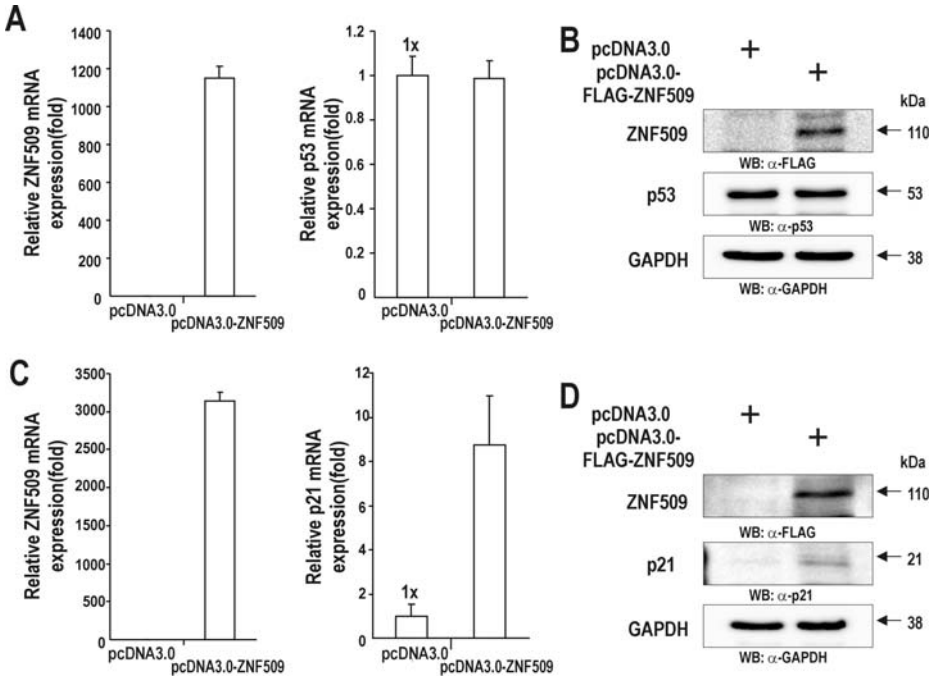
Figure 13. Hypothetical model for the transcriptional regulation of *CDKN1A* and *PUMA* genes by ZNF509.

Part I

ZNF509 arrests cell cycle proliferation. (A, B) In the normal cellular state, MIZ-1 interacts with p300 to acetylate nearby histones and activates transcription. Under conditions when genotoxic agents induce p53, p53 activates ZNF509 expression. Then ZNF509 interacts both with p300 and MIZ-1, which synergistically increase binding to the promoter and activate transcription of the *CDKN1A* gene.

Part II

Inhibition of apoptosis by ZNF509. Depending on cellular conditions, apoptosis is induced. (A) Under knockdown of ZNF509 expression and when genotoxic agents induce p53, p53 increases binding to the promoter to activate transcription of the *PUMA* gene. (B) The normal cellular state. After (A), ZNF509 is induced by p53; ZNF509 inhibits activation of *PUMA* gene transcription through decreased binding of p53 to the promoter. (C) Under high levels of ZNF509 expression and when genotoxic agents induce p53, ZNF509 inhibits p53 binding to the promoter to repress *PUMA* expression through interactions with both p300 and p53.



Supplementary Figure 1. ZNF509 does not regulate *TP53* transcription. (A, B) qRT-PCR and Western blot analysis of p53 expression in HEK293A cells transfected with ZNF509 expression plasmid. (C, D) qRT-PCR and Western blot analysis of p21 expression in HCT116 $p53^{-/-}$ cells transfected with ZNF509 expression plasmid. GAPDH, control.

IV. DISCUSSION

The POK family proteins have been implicated in many biological processes, including embryonic development, cell differentiation, inflammation, apoptosis, and oncogenesis. Several members of this family are well-characterized transcriptional regulators. In this study, I investigated a novel POK transcription factor, ZNF509, with a highly conserved N-terminal BTB/POZ domain and seven zinc-finger motifs at the C-terminus of the protein. ZNF509 was shown to localize in both the nucleus and cytoplasm of HEK293A cells. *ZNF509* mRNA was detected in almost all tissues of the adult male FVB mouse including the brain, liver, heart, spleen, kidney, WAT, BAT, and muscle (Fig. 1). Based on its characteristics, it was hypothesized that ZNF509 may function as transcription regulator of genes important in key biological processes in various tissues.

I identified the *CDKN1A* gene, which encodes p21, an inhibitor of the cyclin-dependent kinase Cdk2, as a transcriptional activation target of ZNF509. ZNF509 was shown to bind to the proximal GC-box#3 and #5/6 elements, and ZNF509 synergistically activated transcription of *CDKN1A* by interacting with MIZ-1. Also, when bound at the proximal promoter elements, ZNF509 and MIZ-1 recruited higher levels of p300, causing the robust

acetylation of histones H3 and H4 around the *CDKN1A* proximal promoter region to activate transcription of *CDKN1A*. Results presented here also show that *ZNF509* expression is induced by tumor suppressor p53, and the promoter region of *ZNF509*, which is bound by p53, has two GC-rich p53 binding elements. Overall, these molecular features of *ZNF509* indicate how ZNF509 can control the cell cycle by potent transcriptional activation of the *CDKN1A* gene.

Based on these findings, I propose a hypothetical model for the transcriptional regulation of *CDKN1A* by ZNF509 (Fig. 13 Part I). In the normal cellular state, MIZ-1 interacts with p300 to acetylate nearby histones to activate *CDKN1A* transcription. However, under conditions when genotoxic agents induce p53, p53 activates *ZNF509* expression. Then, ZNF509 interacts both with p300 and MIZ-1, which synergistically increase binding to the promoter and activate transcription of the *CDKN1A* gene.

ZNF509 interaction with the GC-box#3 and #5/6 elements and MIZ-1 is important for the synergistic activation of *CDKN1A* transcription. Although ZNF509 can directly bind to the GC-box#3 and #5/6 elements, it can recognize the GC-box#3 and #5/6 elements with enhanced affinity through interactions with MIZ-1. In addition, the GC-box#3 and #5/6 elements are the target sites for not only non-POK oncogenes but also several other POK

family proteins, which were reported to be oncogenes. ZNF509 can protect transcriptional repression of *CDKN1A* from other oncogenes through competitive binding on the GC-box#3 and #5/6 elements. Moreover, MIZ-1 is a target protein which offers promoter binding ability to the oncogenes such as c-Myc and Gfi-1 without directly binding DNA[68,69]. ZNF509 can inhibit protein-protein interactions of MIZ-1 with these oncogenes.

Under normal cellular conditions where p53 is expressed at low basal levels, *CDKN1A* and *PUMA* genes are expressed at low basal levels driven by Sp1, leading to normal cell proliferation. Challenge with a genotoxic stress induces production of tumor suppressor p53, which plays a central role in the decision of whether the outcome of DNA damage will be growth arrest or apoptosis through activating transcription of *CDKN1A* and/or *PUMA* genes. Here, I also identify *PUMA*, a pro-apoptotic gene, as a ZNF509 target. Although ZNF509 does not effect transcription of *PUMA* in the absence of DNA damage, ZNF509 inhibits DNA damage induced by p53 transcriptional activity. It was confirmed that ZNF509 and p53 directly interact with each other through molecular interactions between the ZF domain of ZNF509 and the DNA binding domain of p53. These molecular features of ZNF509 may explain how ZNF509 acts to control apoptosis by potently repressing the *PUMA* gene.

Based on these findings, I propose a hypothetical model for the transcriptional regulation of the *PUMA* gene by ZNF509 (Fig. 13 Part II). Under low levels of ZNF509 expression and when genotoxic agents induce p53, p53 increases binding to the promoter and activates transcription of *PUMA* gene. However, under high levels of ZNF509 expression and when genotoxic agents induce p53, ZNF509 inhibits p53 binding to the proximal promoter to repress *PUMA* expression through interactions with both p300 and p53.

I investigated whether ZNF509 is induced by p53 to arrest cell cycle proliferation through the transcriptional activation of *CDKN1A*. ZNF509 inhibits transcriptional activity of p53 through interactions with p53 when cells are under DNA-damaging conditions. This molecular mechanism is very similar to that of Mdm2, which can interact with p53 even though it is one of downstream target genes for p53. Unlike Mdm2, which induces ubiquitination and degradation of p53, the function of ZNF509 may be to inhibit p53 through DNA binding. However, ZNF509 does not change the expression of *PUMA* without DNA damage. Therefore, I believe that ZNF509 might have an effect on the acetylation or phosphorylation of p53 through DNA damage. Future research investigations will determine whether ZNF509 can modify the active form of p53.

ZNF509 functions in anti-apoptotic events and can arrest cell cycle, suggesting that ZNF509 may be a tumor suppressor. This type of dual function has also been reported for other proteins of the Bcl-2 family[50,51,65]. Bcl-2 was shown to have effects on proliferation (anti-apoptotic) by the observation that when deprived of growth factor, bone marrow cells expressing Bcl-2 were arrested in the G0-G1 phase and protected from apoptosis. These observations suggested that Bcl-2 has anti-apoptotic and cell cycle arrest functions. Other members of the Bcl-2 family, including Bcl-xL, Bcl-w, and E1B19K, also have dual functions in cell cycle regulation.

The significance of the data presented here is that ZNF509 is a new dual function protein with negative cell growth and anti-apoptotic functions. The dual function of ZNF509 may be beneficial in cells placed under certain conditions. DNA damage induces cell cycle arrest, DNA repair, and irreparable events such as senescence and apoptosis. The damaged cells decide which route to take on their own according to the severity of DNA damage. In particular, incomplete repair of damaged DNA prior to replication can result in the accumulation of genetic changes. Consequently, cells have to decide to repair DNA and must block the function of induced apoptosis-related proteins by DNA damage and arrest cell cycle progression until cells finish DNA repair.

These findings coincide with the dual function of ZNF509 in both cell cycle arrest and anti-apoptosis. For example, ZNF509 was transiently upregulated when cells were exposed to DNA damage. In this instance, upregulated ZNF509 may arrest cell cycle progression through its interaction with MIZ-1 and p300, thus activating transcription of the *CDKN1A* gene. At the same time, ZNF509 may exert an anti-apoptotic function by repressing transcription of *PUMA* through its interaction with p53 to ensure cell survival until DNA repair is completed. Thus, ZNF509 may control not only the activation of DNA repair enzymes and other anti-apoptotic factors but also the repression of pro-apoptotic factors. Because ZNF509 arrests cell cycle progression without apoptosis, ZNF509 might be important in the DNA repair processes. It will be exciting to study whether the ZNF509 regulates the expression and activity of genes related with DNA repair including *Ku*, *PCNA*, *XPC*, *ERCC1*, and *BRAC1*, to name a few.

V. CONCLUSION

I found that a novel POK family transcription factor, ZNF509, which is induced by p53, is a transcriptional activator of a cell cycle arrest gene, *CDKN1A*, with direct binding ability to the *CDKN1A* proximal promoter. Then ZNF509 interacts both with p300 and MIZ-1, which synergistically increase binding to the promoter and activate transcription of *CDKN1A* gene by increasing histone Ac-H3 and -H4 at the promoter.

ZNF509 not only arrests cell cycle progression but also inhibits apoptosis by when cells are exposed to DNA damaging agent. p53 expression is increased by etoposide treatment, and p53, in turn, increases *PUMA* gene expression. However, p53 increased the transcription of the *PUMA* gene only moderately in the presence of ZNF509. ZNF509 inhibits p53 binding to the proximal promoter of PUMA, to repress PUMA expression through protein interactions among ZNF509, p300 and p53.

My data suggest that ZNF509 has dual functions important in cell fate decision directing toward cell survival. First, ZNF509 potently activates transcription of *CDKN1A* to arrest cell cycle progression. Second, ZNF509 inhibits apoptosis by repressing *PUMA* gene expression.

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ABSTRACT (IN KOREAN)

새로운 ZNF509 단백질의 동정과 세포 증식 조절

<지도교수 허만욱>

연세대학교 의과대학원 의과학과

전부남

세포주기 조절은 여러 관련 단백질들간의 상호작용을 통해 조절된다. 암세포는 발암인자 (oncogene)나 종양억제인자 (tumor suppressor)들의 비정상적인 발현변화로 인해 세포증식 조절 과정의 변화에 의하여 발생될 수 있다. 최근에 POK family 전사인자들이 세포주기 조절 유전자들의 발현을 조절할 수 있는 발암인자나 종양억제인자로 보고가 되고 있다.

본 저자는 종양억제인자 p53에 의하여 발현이 유도되고 세포주기 억제 유전자인 *CDKN1A*의 전사를 활성화 시킬 수 있는 새로운 POK family 전사인자인 ZNF509를 발견하였다. 생화학 및

분자생물학적 연구방법을 이용하여 ZNF509가 *CDKN1A* 유전자의 근접 프로모터 부위 (proximal promoter)에 직접적으로 결합할 수 있고, 전사보조 조절자 (co-activator)인 p300과 단백질간 상호작용을 통하여 프로모터 주변으로 끌어와 p300의 히스톤 아세틸트랜스퍼레이즈 (HAT) 활성을 이용하여 *CDKN1A* 근접 프로모터 주변의 nucleosomes의 히스톤 단백질들을 아세틸화 시킴으로서 nucleosomes의 구조를 열어 전사를 활성화시킴을 관찰하였다.

또한, *CDKN1A* 유전자의 전사를 활성화 시킬 수 있는 다른 POK family 전사인자 MIZ-1과 단백질간 상호작용을 하여 두 단백질의 *CDKN1A* 근접 프로모터 부위 결합 능력을 크게 증가시키고 전사과정을 크게 활성화하였다. 이러한 p21의 발현 증가는 세포증식을 억제하게 된다

그 외에도 ZNF509는 세포가 DNA 손상물질에 노출되었을 때 세포의 사멸을 막는 기능도 수행한다. Etoposide와 같은 DNA 손상물질에 의해 종양억제인자인 p53의 발현이 증가하게 되면 p53은 세포사멸을 주도하는 유전자 PUMA의 전사를 활성화 시킨다. 그러나 이러한 p53의 작용은 ZNF509가 과발현되어 있을 때에는 두 단백질간 상호작용에 의하여 p53의 이러한 기능이 차단된다.

ZNF509는 자신의 zinc-finger domain과 p53의 DNA 결합 domain간 직접적인 상호작용을 통하여 p53이 *PUMA* 프로모터에 결합하지 못하게 함으로서 *PUMA* 유전자의 전사활성화를 억제하여 세포사멸을 억제한다.

본 저자의 연구결과는 ZNF509가 세포의 운명을 결정하는데 있어 일정 역할을 수행하며, 세포의 생존에 필요한 두 가지 기능을 가지고 있음을 보여주고 있다. 첫째, ZNF509는 MIZ-1, p300과의 상호작용을 통해 *CDKN1A*의 전사를 활성화, p21 발현의 증가를 시켜 세포 증식을 억제한다. 둘째, 유전체 손상 시 유도되는 종양억제자 p53에 의하여 발현이 유도되는 세포사멸 주도 유전자 *PUMA*의 전사를 p53과 ZNF509간의 단백질 상호작용을 통해 p53의 기능을 억제함으로써 세포사멸을 억제한다.

핵심 되는 단어: POK, 전사, ZNF509, MIZ-1, p300, p53, CDKN1A, 세포 주기 억제, PUMA, 세포 사멸