Transcriptional regulation of *APAF1* by KAISO and p53

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Transcriptional regulation of *APAF1* by KAISO and p53

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ABSTRACT

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KAISO, a POK family protein, has been conflictingly characterized as both a tumor suppressor and an oncoprotein. Here, KAISO was induced by the DNA-damaging agent etoposide to enhance apoptosis in a p53-dependent manner via upregulation of APAF1, the core molecule of the apoptosome. Previously, we found that p53 interacts with KAISO, which in turn modulates p300 acetylation of p53 lysine residues. Moreover, p53 activates *APAF1* transcription, and p53 binding is further enhanced by KAISO at the *APAF1* promoter distal p53 response element (p53RE#1, bp, -765 ~ -739). Interestingly, an NF-κB response element, located close to p53RE#1, bound NF-κB to transcriptionally repress *APAF1* by disrupting interactions between

KAISO and p53 within a p53-KAISO-p300 activating complex, formed upon exposure to genotoxic stress. Subcellular fractionation also revealed that ectopic expression of the NF-κB family member RelA/p65 led to depletion of KAISO in the nucleus and predominant KAISO localization to the cytoplasm. Thus, while KAISO enhances p53-dependent apoptosis by activating or enhancing *APAF1* gene expression, RelA/p65 decreases apoptosis by blocking interaction between KAISO and p53.

Key words: KAISO, p53, APAF1, apoptosis, transcription factor, p65, BTB/POZ protein

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I. INTRODUCTION

Apoptosis is the process of programmed cell death that occurs universally in multicellular organisms[1]. The biochemical events of apoptosis are characterized by changes in cell morphology that include blebbing, cell shrinkage, nuclear fragmentation, and chromatin condensation, in addition to chromosomal DNA fragmentation[2, 3]. Cell death signals also induce various apoptosis-regulatory proteins before activation of pro-apoptotic enzymes. These proteins often play a critical role in regulating apoptosis by affecting p53 expression or activity. Two widely known, major regulatory cell death cascades are the intrinsic and extrinsic apoptosis pathways. The intrinsic pathway is regulated by p53 and pro-apoptotic proteins of the B-cell

lymphoma 2 (BCL-2) family such as p53-upregulated modulator of apoptosis (PUMA), Bcl-2-associated X protein (BAX), and BH3-interacting domain death agonist (BID). PUMA, a major player in apoptosis, seems to be exclusively controlled by transcription, and p53 is indispensable for its transcriptional activation[16, 17]. The extrinsic pathway is induced by death ligands binding to death receptors such as FAS (CD95 or apoptosis antigen 1 [APO-1]), death receptor 5 (DR5), and p53 apoptosis effector related to PMP-22 (PERP)[18, 19]. Activation of the intrinsic apoptosis pathway ultimately results in release of cytochrome C from the mitochondria, and activation of caspase 9 and its downstream caspases 3, 6, and 7[20, 21]. Expression of death receptor genes and apoptotic protease-activating factor 1 (APAF 1, which binds caspase 9) is also controlled by p53[22, 23].

Dysregulated apoptosis can contribute to many human diseases such as cancer, autoimmunity, heart disease, immunodeficiencies, and neurodegenerative disorders[4], often as a consequence of inappropriate caspase activation. Caspases are a group of cysteine proteases with specificity for aspartic acid residues that are expressed as inactive pro-enzymes and proteolytically activated to form active tetramers during apoptosis. Many caspases participate in signaling and execution of apoptosis[5].

APAF1 is the core molecule of the apoptosome, the “executioner” of mitochondria-dependent apoptosis. The apoptosome is a wheel-like particle containing seven APAF1 monomers that, like seven spokes, radiate from a

central hub. APAF1 possesses an amino-terminal caspase-recruitment domain (CARD) that interacts with the CARD domain of procaspase-9, a central CED-4-like domain, and a long carboxy-terminal domain extremely rich in WD-40 repeats. The CARD domain is located in the central hub. Cytochrome c interacts with the WD40 domain of the apoptosome, which forms the distal part of the spoke[6]. Following its release from mitochondria, cytochrome c displaces the APAF1 CARD domain from the WD40 domain to take its place and bind dATP/ATP. This multiprotein complex undergoes a conformational change in which APAF1 has a more extended conformation; this is required for efficient assembly of the apoptosome and APAF1 CARD domain binding to procaspase-9, which is then cleaved to release its mature, activated form, caspase 9. Caspase-9 then recruits and activates other caspases such as caspase-3 and caspase-7[7]. Deficiency of Apaf1 in mouse leads to embryonic lethality, thus demonstrating the importance of apoptosis in proper development. Moreover, mutation of APAF1 is often found in human melanoma, and its deletion contributes to malignant transformation in various mouse models of cancer[24].

In addition to mutation, the *APAF1* gene has also been identified as a DNA methylation target during the development of malignant melanoma[8]. These tumors are particularly difficult to treat as they are resistant to drug-induced cell death, despite the fact that they generally retain expression of wild-type p53. Methylation of CpG dinucleotides in the *APAF1* promoter, and

subsequent recruitment of histone deacetylases, results in the transcriptional silencing of *APAF1*. In the absence of APAF1 expression, proteolytic activation of procaspase-9 is blocked, and cells are rendered less susceptible to drug-induced apoptosis. These findings suggest that APAF1 is a key factor in chemotherapy resistance.

APAF1 expression is controlled both at the transcriptional level and via several post-translational mechanisms. At the protein level, the activity of APAF1 may be modulated by proteolytic cleavage regulated by protein chaperones, such as heat shock protein family members[9]. APAF1 expression may also be translationally controlled by internal ribosome entry in certain cell types, serving to maintain APAF1 levels during the later stages of apoptosis, when cap-dependent translation may be inhibited[10, 11]. Transcriptional regulation of *APAF1* has also been implicated in several important biological processes, notably in tumorigenesis and development of the mammalian central nervous system. The *APAF1* promoter contains a number of sites for known transcription factors, and oligonucleotide microarray analysis identified *APAF1* as a transcriptional target of E2F1 and p53, both of which are critical for regulation of cell cycle progression and apoptosis[12, 13]. Through its activation by E2F1, APAF1 has been linked to disruption of the retinoblastoma (RB) pathway, an event that occurs in the development of most cancers. In normal cells, RB family proteins (pRBs) negatively regulate the activity of E2F1 until the cells enter into DNA

replication. However, when E2F1 is overexpressed or the function of pRB is otherwise compromised, cells undergo both hyperproliferation and apoptosis[12]. Thus, transcriptional activation of *APAF1* by E2F1 provides one mechanism by which deregulation of the RB pathway might lead to p53-independent apoptosis.

p53 is the most widely studied tumor suppressor, and acts in response to various cellular stresses to mediate a number of anti-proliferative processes. p53 can be activated by DNA damage, hypoxia, or irregular oncogene expression to promote cell-cycle checkpoints, DNA repair, cellular senescence, and apoptosis[13-15]. The human p53 protein is composed of 393 amino acids and is a known transcriptional regulator consisting of five functional domains[14]. The N-terminal transcriptional activation domain of p53 is required for activating p53 target genes. The DNA-binding domain mediates DNA sequence-specific binding of p53 to p53 response elements. The tetramerization domain forms the interaction of p53 monomers to dimers, and the interaction of these dimers to form tetramers. Tetramerization is particularly important to for p53 positive regulation of gene expression[25]. These three domains contribute to transcriptional activation of various p53 targets, including *p21/CDKN1A* and the proapoptotic genes *BAX*, *PUMA*, and *NOXA*[13].

The p53 tumor suppressor, which is normally maintained at low levels in the nucleus, is stabilized and activated following exposure to DNA damaging

agents and other stimuli that could cause genetic instability. p53 induces either cell cycle arrest or apoptosis through the activation or repression of target genes[26]. Turnover of p53 is predominantly regulated by MDM2-mediated ubiquitination and degradation. Genotoxic stress triggers a series of post-translational modifications of p53 that contribute to its stabilization, nuclear accumulation, and biochemical activation[15, 16]. The rapid activation of p53 by ionizing radiation is mediated by its phosphorylation by the ataxia-telangiectasia-mutated (ATM) protein[27]. Upon activation, p53 and its downstream effectors (*e.g.*, p21, BAX) regulate different responses, including cell cycle arrest and apoptosis. Although p53 induces a number of pro-apoptotic genes besides APAF1, expression of APAF1 has been shown to be essential for p53-dependent apoptosis[12].

POK family proteins, a subfamily of the human BTB/POZ-domain regulatory proteins, have been implicated in many biological processes, including embryonic development, cell differentiation, inflammation, apoptosis, and oncogenesis[17]. Several members of this family are well-known transcription factors. Recently, it was reported that some of the POK family proteins function not only as tumor suppressors but also as oncoproteins, depending on the cellular context. POK proteins have an N-terminal POZ domain and Krüppel-type (C_2H_2) zinc finger domains in their C-termini. The POZ domain mediates either homo- or hetero-dimerization with other proteins such as corepressors, histone deacetylases, and other transcriptional regulators.

The C-terminal zinc fingers, by contrast, bind specific DNA sequences and also interact with other regulatory proteins.

Another POK protein, KAISO (also known as ZBTB33), was originally isolated in a yeast-two-hybrid screen as an interaction protein for p120ctn[21]. KAISO is composed of 671 amino acids, with a molecular weight of 100 kDa. KAISO has a POZ domain important in protein-protein interactions and three C_2H_2 zinc fingers at its carboxy terminus[21]. Among the POK protein family, KAISO is a unique transcription factor having bimodal DNA-binding properties. Following KAISO binding to methylated CpG dunucleotides and a sequence specific KAISO-binding site, it then recruits co-repressor components such as NCoR1, SIN3A, and Groucho[18]. KAISO expression is increased in murine intestinal cancer and is also expressed in human colon cancer. When crossed with tumor-susceptible ApcMin/+ mice, KAISO-null mice had delayed onset of intestinal tumorigenesis[19], suggesting that KAISO plays a role as an oncogene in intestinal cancer. On the other hand, KAISO has been suggested as a potential tumor suppressor that can block cancer progression by cell cycle regulation[20, 21]. Thus, the role of KAISO as either a tumor suppressor or oncogene remains uncertain.

Recently, we found that KAISO is induced by DNA-damage with an expression level similar to that of p53. Ectopic p53 induced potent apoptosis, and KAISO interacted with p53 and p300 to alter acetylation of three p53 lysine residues. These data suggest that KAISO is upregulated by DNA

damage prior to p53 expression, and that KAISO interacts with a p53-p300 complex to increase acetylation of K320 and K382 lysine residues of p53, while preventing acetylation of K381. KAISO may also regulate cell cycle arrest and apoptosis by acetylation of p53 through p53-p300-KAISO complex formation (our unpublished data).

Like p53, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a stress-inducible transcription factor important for cell proliferation and apoptosis[23]. NF-κB is a multiple protein complex that regulates DNA transcription through specific NF-kB binding sites (5'-GGGPuNNPyPyCC-3') in the promoters or enhancers of its target genes[28]. NF-κB is expressed in almost all animal cell types and tissues and plays an important role in mediating responses to stimuli such as genotoxic stress, inflammation, free radicals, infection, ultraviolet irradiation, and antigen presentation[29]. In mammals, the NF-κB family consists of five proteins, RelA/p65, RelB, c-Rel, p105/p50 (NF-κB1), and p100/52 (NF-κB2) that form functionally active homo- and hetero-dimers with each other. The N-terminus of NF-κB proteins has a Rel homology domain in common, and the C-terminus of RelA, RelB, and c-Rel includes a transactivation domain. NF-κB1 and NF-κB2 undergo processing to generate p50 and p52, respectively[30]. Transcription factor NF-κB complexes are located in the cytoplasm and remain in a transcriptionally inactive form by interaction with IκB, until a cell receives stimuli. Following proteasome-dependent degradation of IκB,

released RelA/p65 accumulates in the nucleus to transcriptionally activate specific genes involved in immune and inflammatory responses and cell growth[31].

NF-κB activation can suppress cell death and is important to protect cells from the apoptotic caspase cascade induced by bacterial lipopolysaccharide, the cytokines TNF and IL-1, and other stimuli[32]. NF-κB impedes apoptosis by transcriptional activation of target genes suppressing apoptosis, whose encoded proteins act to inhibit the apoptotic caspase cascade. Dysregulation of NF-κB contributes to cancer, autoimmunity, inflammatory diseases, improper immune development, viral infection, and septic shock[33]. NF-κB activity is exquisitely regulated at multiple levels, thus influencing the expression of its various downstream genes. Furthermore, NF-κB signalling is constitutively activated in cancer cells, and this activation can interrupt chemotherapy responses that induce cell death[34].

Considering a crucial role for p53 and NF-κB in tumor progression, their considerable interplay is not surprising. Activation of NF-κB suppresses cell death and is needed to defend against apoptosis induced by noxious cellular stress stimuli, inducing transcription of the negative regulator of p53, MDM2[35]. Analogously, p53 inhibits NF-κB activity by competitively binding to NF-κB response elements and antagonizing NF-κB binding to its co-regulators (*e.g.*, p300) on target gene promoters[36]. Likewise, NF-κB can antagonize p53 function by cross-competing with its transcriptional co-

regulators[37]. In contrast, p53 and NF-κB can work together in transactivating genes having both p53 and NF-κB response elements in their promoters (*e.g.*, *SKP2*), under specific conditions[38] in which p53 needs NF-κB to induce apoptosis. Thus, depending upon the situation, the relationship between p53 and NF-κB can be either antagonistic or cooperative. Furthermore, we believe this is the first study showing that (1) KAISO can increase transcriptional activation of *APAF1* by interacting with p53; and (2) RelA/p65 can impede the activation of *APAF1* by reducing the interaction between p53 and KAISO. This study reveals possible mechanisms of transcriptional regulation of *APAF1* via molecular interactions between the *APAF1* promoter, p53, KAISO, and RelA/p65.

II. MATERIALS AND METHODS

1. Plasmids, antibodies, and Reagents

We searched the human *APAF1* promoter sequence in the NCBI database. To prepare APAF1 promoter-Luc fused reporter plasmids, the putative APAF1 promoter was amplified by polymerase chain reaction (PCR) from HeLa cell genomic DNA and cloned into pGL2-Basic vector to generate a reporter plasmid (pGL2-APAF1-Luc plasmid). The following oligonucleotide PCR primers were used for pGL2-APAF1-mutant NF-κB binding site: forward, 5'-GGGTTTCTTTTCGCCC-3', reverse, 5'-GGGCGGGCCGACCCCC-3'. Primers were purchased from MacroGen. Preparation of pcDNA3, pcDNA3-p53, pcDNA3-KAISO, and pcDNA3-RelA/p65 plasmids used in this study has been reported elsewhere (46, 54). All plasmid constructs were verified by sequencing.

Antibodies against GAPDH, p53, p65, APAF1, p300, HDAC1, and (Ac)-Lysine were purchased from Millipore (Billerica, MA, USA), Calbiochem (San Diego, CA, USA), Cell Signaling Technology (Beverly, MA, USA), and Santa Cruz Biotechnology (Santa Cruz, CA, USA). To obtain a polyclonal antibody against KAISO, a white rabbit was immunized subcutaneously with a mixture of HIS-KAISO(ΔZF) (a.a., 1~499) recombinant polypeptides, 6 times, at 2-week intervals. Blood was collected, incubated at 37℃ for 90 min, and centrifuged. Following centrifugation, the supernatant was incubated with

Affi-Gel 10 Gel beads cross-linked to a recombinant KAISO(ΔZF) (Bio-Rad, U.K.). The precipitated beads were then collected and washed with PBS, and the antibody was eluted (1 M Tris pH 7.6). Most chemical reagents were purchased from Sigma (St. Louis, MO, USA).

2. Quantitative Real-time PCR (qPCR) Analysis of APAF1, p53, KAISO, and GAPDH mRNA expression in cells

Total RNA was isolated from the HEK293 cells using TRIzol reagent (Invitrogen, CA). cDNAs were synthesized using 2 μg of total RNA, oligo(dT) primer, and Superscript reverse transcriptase II (200 units/μl) (Invitrogen, CA). The qRT-PCR was performed suing SYBR Green Master Mix (Applied Biosystems, Foster City, CA) 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 mRNA was used as control. The following oligonucleotide PCR primers were used for qRT-PCR: APAF1 forward, 5'-AAAAGGGGATAGAACCAGAGG-3', reverse, 5'-TGCGGCACCTCAAGTCTTC-3', KAISO forward, 5'-CCGAGATTCTGCCCACAAA-3', reverse, 5'-GGGCGAGTTATTGCTAGCACTAG-3', p53 forward, 5'-CCTGAGGTTGGCTCT GACTGTA-3'; reverse, 5'-AAAGCTGTTCCGTCCCAGTACA-3', GAPDH forward, 5'-ACCACAGTCCATGCCATCAC-3'; reverse, 5'-TCCACCACCCTGT

TGCTGTA-3'. Primers were purchased from MacroGen.

3. Cell cultures

HEK293, HeLa, H1299, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's medium (DMEM) and RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS), 100 g/ml streptomycin and 100 units/ml penicillin, and grown at 37℃ in a humidified, 5% CO2 incubator. All cell culture media and supplements were from Gibco-BRL (Grand Island, NY, USA).

4. Transcription analysis of p53 and NF-κB responsive elements of the *APAF1* promoter

To analyze transcriptional regulation, the human *APAF1* promoter-reporter fusion plasmid, p53 or/and KAISO expression vectors in various combinations were transiently transfected into H1299 cells using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA, USA) and analyzed for luciferase activity as reported elsewhere. The pGL2- *APAF1* full length promoter, pGL2-*APAF1* deletion constructs, pTK-LUC-*APAF1*-NF-κB region construct reporter fusion plasmids, pcDNA3.1-p53 wild-type, pcDNA3.1-Kaiso and pcDNA3-p65 in various combination were transfected. After 24 to 36 h of incubation, transfected cells were harvested and analyzed for

luciferase activity using a Microplate LB 96V luminometer (EG&G Berthold, Gaithersburg, MD, USA). All reactions were performed in triplicate. Reporter activity was normalized to co-transfected β-galactosidase activity or total cellular protein to determine transfection efficiencies.

5. Western Blotting

Cells were harvested and lysed in RIPA buffer. Cell extracts (30 g) were separated using 10% SDS-PAGE gel electrophoresis, transferred onto Immun-BlotTM PVDF membranes (Bio-Rad, Hercules, CA, USA), and blocked with 5% skim milk (BD Biosciences) or BSA. Membrane blots were then incubated with antibodies against GAPDH, APAF1, p53, KAISO, RelA/p65, p300, and (Ac)-Lysine, followed by incubation with anti-mouse or rabbit secondary antibodies conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA). Protein bands were visualized with ECL solution (PerkinElmer Life Sciences, Waltham, MA, USA).

6. Fluorescence-activated cell sorting (FACS) Analysis

HEK293, HCT116 p53+/+, and p53-/- cells were transfected with KAISO expression or control vectors. Cells were washed, stained with propidium iodide (1μg/ml, BD Biosciences, San Jose, CA, USA) and annexin V-FITC (1:25, BD Biosciences) in annexin V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM CaCl2) and incubated for 15 min at room

temperature in the dark. Apoptotic cells were detected using a BD FACSCalibur (BD Biosciences), and the data were analyzed using FlowJo (Ashland, OR, USA). All assays were performed in triplicate.

7. Cell Growth Assays

To investigate the effect of KAISO on cell growth, cells were grown in 24-well dishes to 60% confluency after cells were transfected with KAISO expression vector, and further incubated for 0-3 days. The cell growth of each sample was measured by counting the number of cells using a hematocytometer.

8. Immunoprecipitation Assays

HEK293 cells (transfected with expression or control vector, if necessary) were washed, pelleted, and resuspended in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and complete Mini-Protease cocktail. Cell lysates were precleared, and the supernatant was incubated overnight with anti-p53, KAISO, antibody on a rotating platform at 4 ℃, followed by incubation with protein A-Sepharose Fast Flow beads (Santa Cruz, CA, USA). The beads were collected, washed, and resuspended in 5X SDS loading buffer (1 M Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, 2-mercaptoethanol, 1% bromophenol blue), as reported previously (46). Immunoprecipitated proteins were separated with 10% SDS-

PAGE. Western blot analysis was performed as described above using the appropriate antibodies.

9. Electrophoretic Mobility Shift Assays(EMSA)

The EMSAs were performed as described previously. The oligonucleotide probes were annealed by heating at 95℃ for 10 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 ℃. Labeled double-stranded oligonucleotides were purified using Sephadex™ G-50 (Amersham Biosciences, Uppsala, Sweden) columns. Binding reactions were carried out in 20 μl of binding buffer containing 10 mM HEPES, pH 7.9, 100 mM KCl, 20 mM $MgCl_2$, 40% glycerol, 0.1 μg of recombinant p53 or KAISO, and 10,000 cpm of probe at room temperature for 20 min. 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℃ with a Kodak intensifying screen. The probe sequences of p53 response elements on the *APAF1* promoter or on the *p21* distal promoter used in EMSA are as follows (only top strands are shown): p53RE#1, 5’-GATCCACTTAC CAGGCCAGGCCCAGGCACGTCCCCAGCGACA

GCAGGCTCAGGCACGTTCGGGGTCTGCCCAGCCCCCGCC-3’; p53RE#2, 5’-

GATCCCTTGGGCCCCGACTTCTTCCGGCTCTTCACCTCAGACATGTCTGGAG ACCCTAGGACGACAAGCCCAGGGCAGCTTCTTCACCAGG-3'; p53RE#3, 5'-GATCCGTCCGCGGGGTAGGCGGGCACTTCTACGCGCGCGGGCATGAGCCGTG GCAGGAGTGCGCGGCGGCAGCGGTGGC-3'; p53RE on *p21* distal promoter, 5'-GATCCGTTAGAGGAAGAAGACTGGGCATGTCTG-3'. Primers were purchased from MacroGen.

10. DNase I Footprinting Assays

Recombinant proteins were incubated in binding buffer with 20,000 cpm of ^{32}P-labeled DNA and 1 μg of poly (dI-dC) at 0 ℃ for 30 min in total volume of 50 μl. The digestions were performed at this same temperature after adding a solution containing 10 mM HEPES, pH 7.6, 60 mM KCl, 25 mM $MgCl_2$, 5 mM $CaCl_2$, 7% glycerol and 0.2-2 units DNase I. The amount of enzyme and the time of digestion were determined each time a new probe was prepared. After the incubation, the reaction was stopped by the addition of 320 μl of stop solution (20 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 250 mM NaCl, 0.5% SDS). This mixture was extracted with 400 μl phenol / chloroform / isoamyl alcohol (25 : 24 : 1), vortexed briefly, and centrifuged for 10 min at 4 ℃. The DNA was precipitated at -20 ℃ by adding 3 M sodium acetate and 1 ml of ethanol. The precipitate was collected by centrifugation for 10 min, washed once 70% ethanol, and dried. The pellets

were redissolved in 4 μl of formamide-containing loading dye, boiled for 5 min, and cooled on ice immediately prior to loading onto a DNA sequencing gel of 6% polyacrylamide and 7 M urea. Pre-electrophoresis and electrophoresis were carried out in 0.5X TBE at 2000 V. To identify the regions protected from DNase digestion, the labeled fragment was subjected to the base-specific chemical modification and cleavage reaction as described by Maxam and Gilbert and analyzed on parallel lanes.

11. *In vitro* protein acetylation assays

Recombinant His-KAISO (a.a. 1-672, full-length) and His-p53 (a.a. 1-393, full-length) proteins were expressed in E. coli DH5 and purified by affinity chromatography. Full-length p300 was purchased from Active motif (Carlsbad, CA). KAISO protein (5 μg) was incubated with p300 (200 ng) and His-p53 (6 μg) in acetylation assay buffer (100 mM Tris-HCl (pH 8), 20% glycerol, 2 mM DTT, 0.1 M NaCl, 20 mM butylic acid, 10 mM acetyl CoA) for 2 hrs at 37 ℃, and the mixture was resolved by SDS-PAGE.

12. Oligonucleotide Pull-down Assays

Cells were lysed in HKMG buffer and the extracts incubated overnight with 1 g biotinylated double-stranded oligonucleotides. Oligonucleotide probes were heated at 95℃ for 5 min, cooled slowly to room temperature to allow annealing, and pull-down procedures performed as reported elsewhere

(46). Oligonucleotides sequences were (only top strands are shown): APAF1 promoter p53RE1, 5’-GGCCCAGGCACGTCCCCAGCGACAGCAGGCTCAG-3’; p53RE2, 5’-TCAGACATGTCTGGAGACCCTAGGACGACAAGCCCAG-3’; p53RE3, 5’-CACTTCTACGCGCGCGGGCATGAGCCGTGGCAGGAGTGCGC-3’; and a p53RE in the CDKN1A distal promoter, 5’-GATCCGTTAGAGGAAGAAGACTGGGCATGTCTG-3’. Oligonucleotides were purchased from MacroGen.

13. Quantitative Chromatin Immunoprecipitation (ChIP)-qPCR Assays

To test the interaction between KAISO, p53 and the *APAF1* promoter, H1299 cells were transiently transfected with 5 μg of pcDNA3.1-Kaiso and/or pcDNA3.1-p53. After, cells were treated with final concentration 1% of formaldehyde for 20 min at room temperature in other to crosslink protein-DNA complexes. Cells were harvested, washed three times in 1X PBS, and lysed in a 1% SDS, 50 mM Tris-HCl (pH 8.0), 10.0 mM EDTA. 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 8.0), 167 mM NaCl, 1.2 mM EDTA) and pre-cleared with salmon sperm DNA/protein A agarose-50% slurry for 1 hr at 4 ℃. Pre-cleared supernatant was incubated with antibody overnight at 4 ℃ with rotation. To collect DNA-protein-antibody complex, salmon sperm

DNA/protein A agarose-50% slurry was added to the mixture. The mixture was incubated for 2 hrs at 4 ℃ with rotation and pelleted by centrifugation. Beads were washed three times in 1X PBS including protease inhibitor, and incubated with 300 μl of elution buffer (1% SDS, 0.1 M Na_2CO_3) and rotated for 15 min to remove excess agarose. Eluted supernatants as well as input DNA samples were then decrosslinked by incubating at 65 ℃ for 4 hrs. After decrosslinking, the supernatant was extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol to recover DNA. PCR reactions were performed using the DNA form the immunoprecipitated reactions and oligonucleotide primer sets designed to amplify the promoter region.

III. RESULTS

1. KAISO expression is induced by etoposide treatment and inhibits cell proliferation by inducing apoptosis

Recent studies show that POK family proteins function not only as tumor suppressors but also as oncoproteins, depending on the cellular context (39-54). Some of these, like BTBD4, ZNF238, FAZF, HIC2, and ZBTB8, are induced by DNA damage in HCT116 $p53^{+/+}$ cells (our unpublished data). KAISO, one POK family protein, is induced at both mRNA and protein levels by etoposide treatment of HEK293 cells (Fig. 1A and B). KAISO mRNA expression was weakly activated at 1 hour of etoposide treatment, reached its highest peak at 6 hours, and declined thereafter. For p53, transcription was activated at 2 hours, kept the same transcription level from 2 to 6 hours, and then was reactivated after 6 hours (Fig. 1A). However, p53 and KAISO protein expression profiles differed somewhat from mRNA expression profiles, as KAISO protein expression increased after 2 hours and further increased after 6 hours, while p53 protein expression increased at 2-3 hours and remained constant afterward (Fig. 1B). Because KAISO protein expression correlated with p53 expression, we next tested whether KAISO can also regulate various p53-modulated cellular processes, such as cell cycle arrest and apoptosis, in HEK293 cells. Direct cell counting assays revealed

that KAISO expression vector-transfected HEK293 cells showed little cell growth for 3 days (Fig. 1C), while flow cytometry analysis showed that ectopic KAISO significantly induced apoptosis (Fig. 1D). These results suggest that KAISO is induced by etoposide to inhibit cell proliferation by inducing apoptosis.

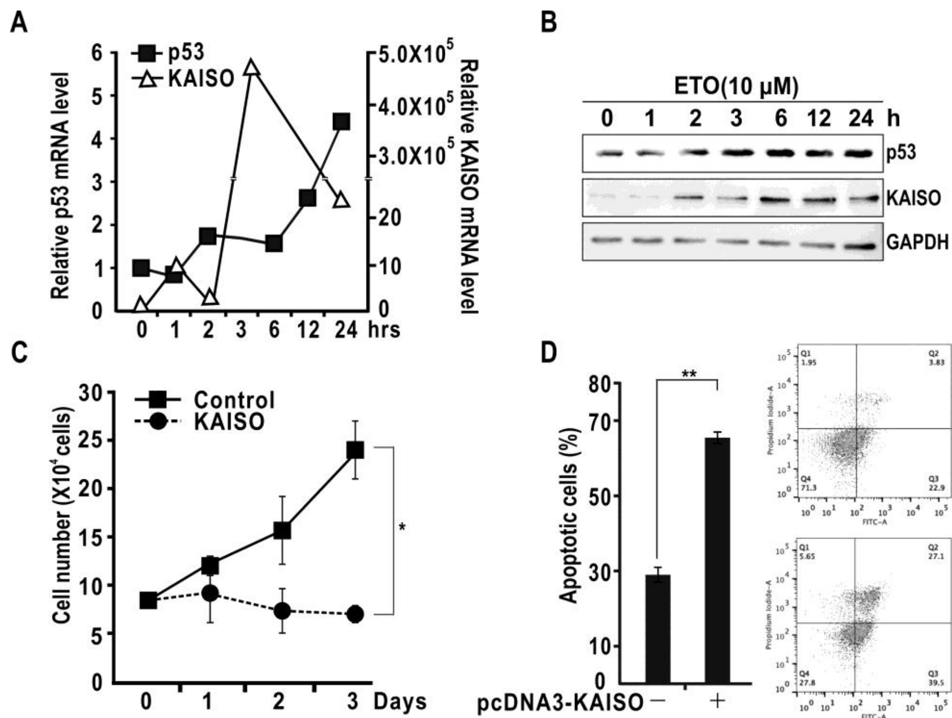


Figure 1. KAISO is induced by etoposide, and ectopic KAISO induces apoptosis. (A, B) RT-qPCR and western blot analysis of KAISO and p53. HEK293 cells treated with etoposide (10 μM) were harvested at the indicated times. GAPDH was used as a control. (C) Growth curve analysis. HEK293 cells transfected with KAISO expression or control vector were counted daily for 3 days. Mean values of three independent experiments are shown. *, $p < 0.05$. Error bars represent S.D. D, Flow cytometry analysis. HEK293 cells transfected with KAISO expression or control vector were stained with

propidium iodide and annexin V, using an apoptosis detection kit (BD Biosciences). **, $p < 0.005$.

2. KAISO inhibits cell proliferation by inducing apoptosis only in p53-positive cells

p53 plays a critical role in cell cycle arrest and apoptosis (55-58). KAISO is induced by etoposide and ectopic KAISO inhibits cell proliferation by inducing apoptosis (Fig. 1). As KAISO may exert these biological activities in a p53-dependent or -independent manner, we investigated KAISO activity in HCT116 $p53^{+/+}$ or $p53^{-/-}$ cells. Direct cell counting assays revealed that HCT116 $p53^{+/+}$ cells transfected with KAISO expression vector showed little cell growth for 3 days, while KAISO-transfected HCT116 $p53^{-/-}$ cells showed no difference in cell growth compared to control cells (Fig. 2A). Flow cytometry analysis further showed that ectopic KAISO induced apoptosis significantly only in HCT116 $p53^{+/+}$ cells (Fig. 2B). These results indicate that inhibition of cell proliferation and apoptosis induction by KAISO requires p53.

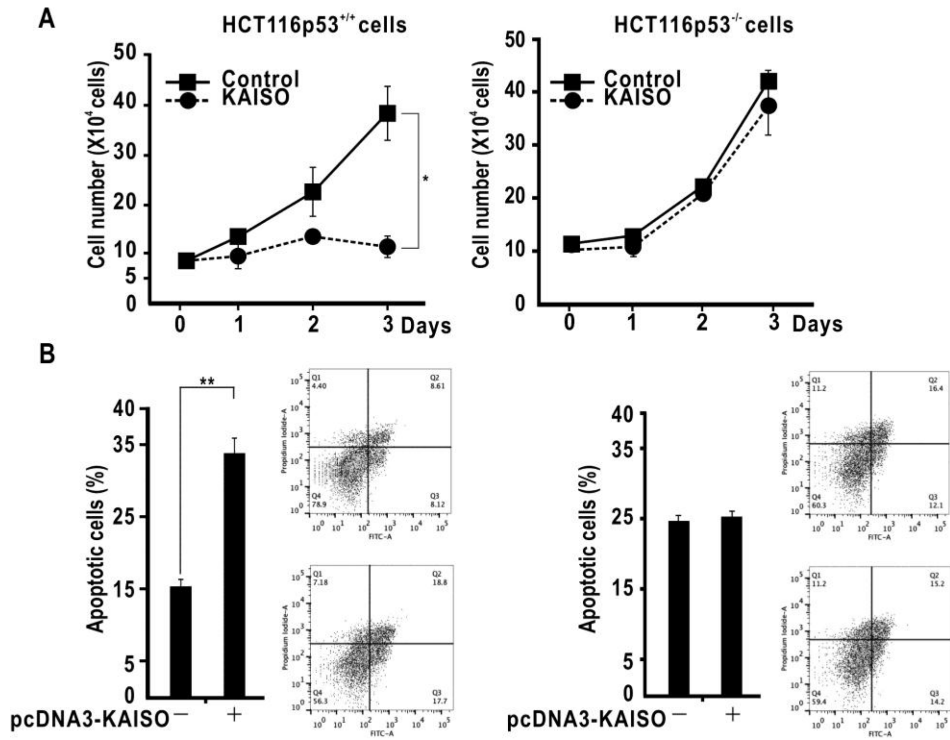


Figure 2. KAISO induces apoptosis in HCT116 $p53^{+/+}$, but not HCT116 $p53^{-/-}$, cells. (A) Growth curve analysis. HCT116 $p53^{+/+}$ (left panel) or $p53^{-/-}$ (right panel) cells transfected with KAISO expression or control vector were counted daily for 3 days. Mean values of three independent experiments are shown. *, $p < 0.05$. Error bars represent S.D. (B) Flow cytometry analysis. HCT116 $p53^{+/+}$ (left panel) and $p53^{-/-}$ (right panel) cells transfected with KAISO expression or control vector were stained with propidium iodide and annexin V, using an apoptosis detection kit (BD Biosciences). Mean values of three independent experiments are shown.**, $p < 0.03$.

3. KAISO activates APAF1 expression by interacting with p53

APAF1 is a critical component of p53-dependent apoptosis, and the *APAF1* gene promoter is activated by p53 via two p53 response elements (p53REs)[59, 60]. Because KAISO induced apoptosis in p53-positive cells (Fig. 2B), we examined whether KAISO might induce *APAF1* in concert with p53. We found that ectopic KAISO activated endogenous *APAF1* transcription in HCT116 $p53^{+/+}$, but not HCT116 $p53^{-/-}$, cells (Fig. 3A). We further noticed that ectopic KAISO increased p53 transcription approximately 2-fold, and Western blot analysis showed that ectopic KAISO increased APAF1 expression only in p53-positive cells (Fig. 3B). Unlike p53 gene transcription, ectopic KAISO did not change p53 protein expression levels significantly different than the control (Fig. 3B). These results indicate that ectopic KAISO requires the presence of p53 to induce *APAF1*.

p53 is an important transcriptional activator of *AFAF1*[60], and KAISO expression is induced similarly to p53 (Fig. 1). Activity of p53 is often regulated by post-translational modifiers and/or factors that interact with p53. For example, HAT proteins (e.g., p300/CBP or PCAF) interact with and acetylate p53, leading to its full activation[61-64]. Since KAISO likely affects transcriptional activation of *APAF1* via p53, we tested whether KAISO interacts with p53. Co-immunoprecipitation and western blot analysis of HEK293 cell lysates with ectopic KAISO expression showed that KAISO and

p53 directly interact with each other (Fig. 3C). This interaction may be important in modulating p53 transcriptional activation of *APAF1*.

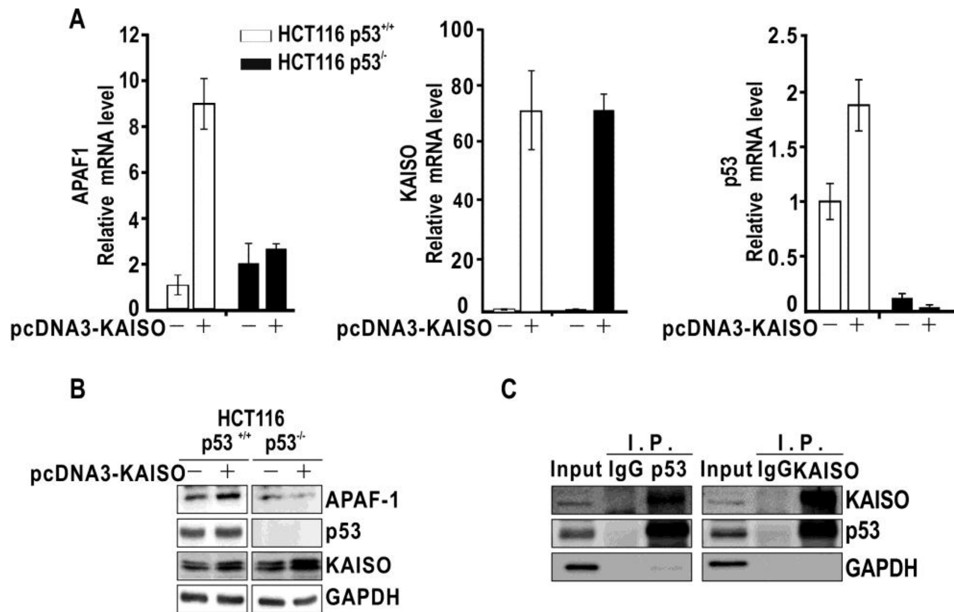


Figure 3. KAISO interacts with p53 to increase APAF1 expression. (A, B) RT-qPCR and western blot analysis. HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were transfected with KAISO expression or control vector and analyzed for APAF1, KAISO, and p53 mRNA (A) or protein (B). GAPDH was used as a control. (C) Co-immunoprecipitation of KAISO and p53. HEK293 cells transfected with KAISO expression vector were immunoprecipitated.

4. KAISO enhances transcriptional activation of the human *APAF1* promoter by acting on its most distal p53 response element (bp, -765 ~ -739)

We showed above that KAISO activates *APAF1* transcription only in the presence of p53. Subsequently, we investigated the molecular mechanism of why KAISO requires p53 and how KAISO and p53 cooperatively activate transcription of *APAF1*. Since it has been reported that p53 regulates *APAF1* at the mRNA level[60], we hypothesized that KAISO enhances transcriptional activation of *APAF1* in concert with specific promoter elements that bind p53. To determine response elements jointly affected by p53 and KAISO, we cloned three deletion constructs of the *APAF1* promoter into pGL2-Luc reporter plasmids possessing various p53 response elements (p53REs). Ectopic p53 induced luciferase activity of pGL2-*APAF1* 1.25kb construct in H1299 $p53^{-/-}$ cells, which further increased more than 2-fold when the cells were co-transfected with p53 and KAISO (Fig. 4B). With the other two constructs, pGL2-*APAF1* 0.6kb and -0.3kb, ectopic p53 or KAISO conferred only weak or no activation (Fig. 4B), suggesting that KAISO may affect transcriptional activation of *APAF1* by p53 via a far distal promoter element, p53RE#1 (bp, -765 ~ -739), but not p53RE#2 (bp, -604 ~ -572). These results suggest that KAISO affects the transcription of *APAF1* by p53 via p53RE#1,

and this transcriptional activation may be mediated by interaction between KAISO and p53.

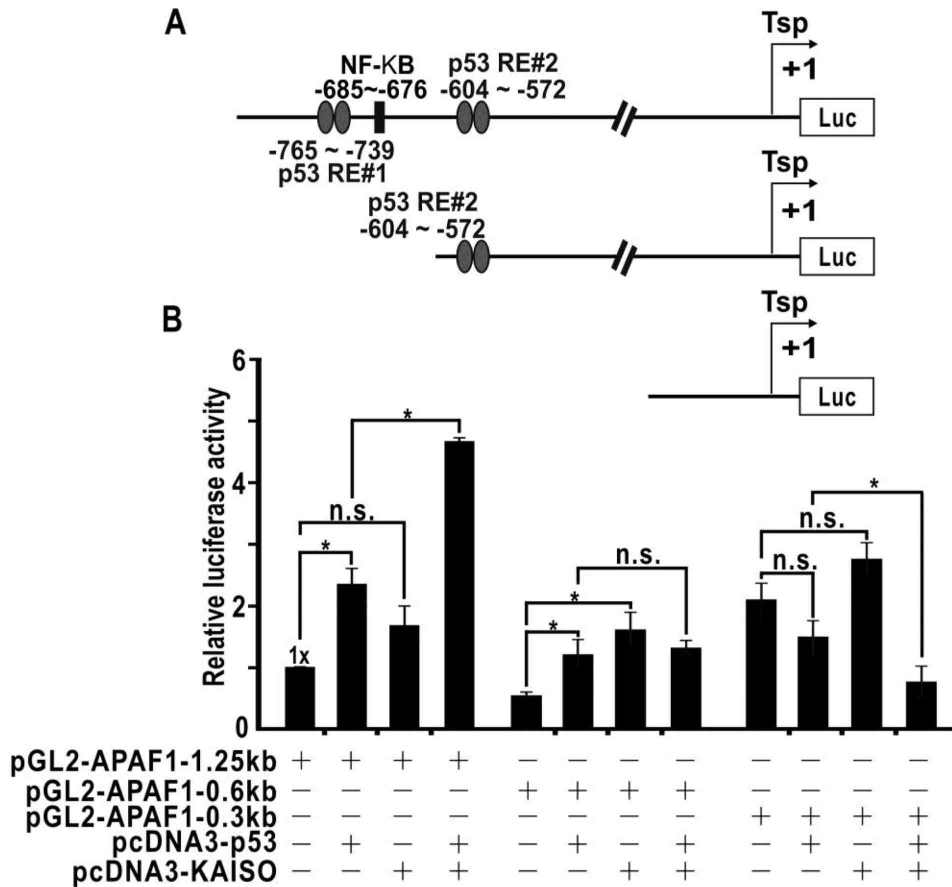


Figure 4. KAISO enhances p53 transcriptional activation of *APAF1* exclusively through its most distal p53RE, #1 (bp, -765 ~ -739). A, Structures of three *APAF1* promoter-luciferase gene fusion reporter plasmid constructs. 5' upstream deletion constructs were designed to include or exclude specific p53REs. B, *In vitro APAF1* reporter assay. H1299 p53-null cells were transfected with the *APAF1* promoter-reporter fusion plasmids shown in (A) and KAISO and/or p53 expression vectors, and analyzed for

luciferase activity. Luciferase activities were normalized to cotransfected β-galactosidase activity. Data presented are the average of three independent assays. Error bars represent S.D. *, $p < 0.05$. Error bars represent S.D.

5. p53 directly binds the p53REs of the *APAF1* promoter, and KAISO enhances p53 binding only to p53RE#1

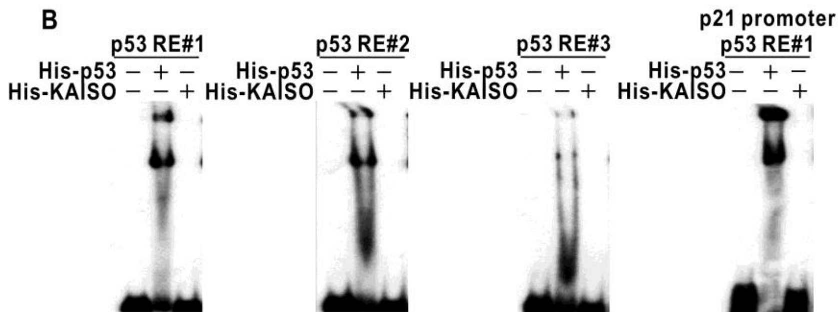
Figure 4 showed that transcriptional activation of *APAF1* by p53 is enhanced by ectopic KAISO, suggesting a possible direct interaction between the two proteins. To further investigate possible KAISO/p53 interaction at p53REs, we used an electrophoretic mobility shift assay (EMSA). EMSA showed that recombinant p53 protein bound strongly to p53RE#1 and p53RE#2 and weakly to p53RE#3, as predicted by the strength of the p53 response elements determined by Genomatix software, while recombinant KAISO protein lacked any ability to bind the p53REs by itself (Fig. 5B). As a positive control, we showed that recombinant p53 bound strongly to the p53RE#1 of the *p21WAF/CDKN1A* promoter.

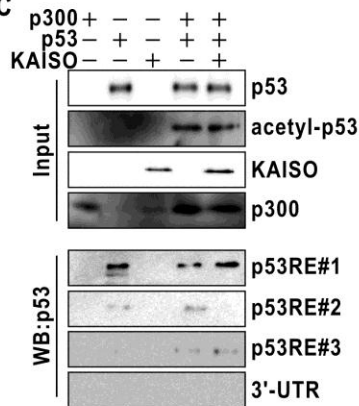
p300/CBP-mediated p53 acetylation is commonly modulated by p53-activating agents and p53 interaction partners such as MDM2, KAISO, *etc*. Recently, we found that KAISO interacts with p53 and p300 to increase acetylation of p53 lysines K320 and K382, while decreasing acetylation of K381. Thus, KAISO may regulate cell cycle arrest and apoptosis by altering the acetylation of p53 through a p53-p300-KAISO complex (our unpublished data).

Oligonucleotide pull-down assays further showed that p53 by itself binds strongly to p53RE#1 and weakly to p53RE#2 and p53RE#3, while a

p53/p300 mixture bound fairly weakly to all three p53REs (Fig. 5C). Interestingly, the binding of a p53/p300/KAISO mixture increased only in p53RE#1 (Fig. 5C). We also investigated whether lysates from HCT116 p53$^{+/+}$ cells transfected with KAISO expression vector could give similar results, showing that p53 binding (in the presence of KAISO) was enhanced only at p53RE#1 and was weak at p53RE#2, with KAISO binding only at p53RE#1 (Fig. 5D). We also investigated whether p53 actually bound to the *APAF1* promoter p53REs by ChIP-reChIP. Those assays revealed that p53 bound to p53RE#1 and p53RE#2, and that KAISO increased p53 binding at p53RE#1 but slightly decreased p53 binding at p53RE#2 (Fig. 5E). These data agreed with our in vitro oligonucleotide pull-down assays showing KAISO enhancement of p53 binding to p53RE#1. Thus, by molecular interaction, KAISO specifically increases DNA binding ability of p53 to p53RE#1 of the *APAF1* promoter.

A
Tsp
+1
p53 RE#2
-604 ~ -572
-765 ~ -739
p53 RE#1
-141~-123
p53 RE#3


B
p53 RE#1
p53 RE#2
p53 RE#3
p21 promoter
p53 RE#1
His-p53
His-KAISO


C
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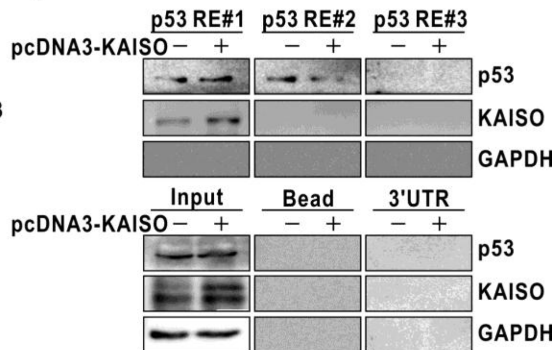
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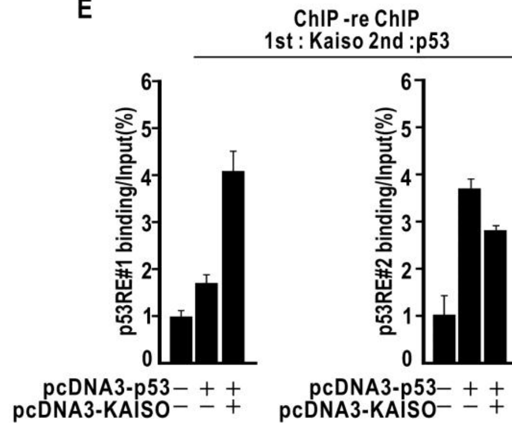
E
ChIP -re ChIP
1st : Kaiso 2nd :p53
p53RE#1 binding/Input(%)
p53RE#2 binding/Input(%)
pcDNA3-p53
pcDNA3-KAISO

Figure 5. p53 directly binds p53REs #1 and #2 of the *APAF1* promoter, but KAISO enhances p53 binding only to p53RE#1. (A) Locations of the three p53 response elements of *APAF1* promoter. (B) EMSA. Three ^{32}P-[α-dATP]-labeled p53 binding probes were incubated with His-p53 and His-KAISO and separated by 4% nondenaturing PAGE. A p53RE in the *p21WAF/CDKN1A* promoter was used as a positive control. (C) *In vitro* acetylation assay and oligonucleotide pull-down assay. The *in vitro* acetylation mixtures were incubated with probes/agarose beads. The precipitate was analyzed by western blot using antibody against p53. 3'-UTR, 3'-untranslated region; IP, immunoprecipitation. (D) Oligonucleotide pull-down assays. HCT116 p53$^{+/+}$ cell extracts transfected with KAISO expression or control vector were incubated with probes-agarose beads and analyzed as described above. (E) ChIP-reChIP analysis of p53 or KAISO binding to the *APAF1* promoter. Chromatin from H1299 cells transfected with p53 and KAISO expression vectors was immunoprecipitated with anti-p53 or KAISO antibodies, followed by PCR amplification of p53REs #1 or #2. DNA binding activity of p53 to the *APAF1* promoter was increased by KAISO, and p53 and KAISO bound as a complex (p53RE#1, left).

6. RelA/p65 represses transcription of *APAF1* by trapping KAISO in the cytoplasm

A putative NF-κB response element within the *APAF1* promoter (bp, -685~-676) is located close to the most distal p53 response element (p53RE#1) that binds p53 and KAISO (Fig. 5A). To investigate whether RelA/p65 affects *APAF1* transcriptional activation by interaction of p53 and KAISO, H1299 $p53^{-/-}$ cells were transfected with a pGL2-luc-*APAF1* 1.25kb construct and expression vectors of p53, KAISO and p65 in various combinations. Reporter activity of the construct in the presence of KAISO was slightly decreased from that with p53 alone, while the addition of p53 reversed RelA/p65 transcriptional repression. However, the addition of RelA/p65 decreased KAISO/p53 additive transcriptional activation > 2-fold (Fig. 6B). To further investigate whether RelA/p65 affects transcriptional activation of *APAF1* by p53 and KAISO, lysates from HEK293 cells were immunoprecipitated with anti-RelA/p65 or anti-KAISO antibodies and analyzed for KAISO or RelA/p65 by western blot, revealing that KAISO interacts with p65 endogenously (Fig. 6C). Because ectopic RelA/p65 localizes to the cytoplasm, we predicted that this interaction may affect nuclear localization of KAISO and thereby repress its transcriptional activation of *APAF1*. Consequently, we performed western blot analysis following subcellular fractionation of H1299 cells transfected with RelA/p65 or control vector. Those assays showed that

endogenous KAISO localized to both the nucleus and the cytoplasm under normal conditions, while ectopic RelA/p65 constrained KAISO to the cytoplasm (Fig. 6D). RelA/p65 binds to and inhibits KAISO activity by cytoplasmic sequestration because ectopic RelA/p65 is localized to the cytoplasm. Ectopic RelA/p65 disrupts interaction between p53 and KAISO (Fig. 6E). Thus, RelA/p65 can repress *APAF1* by trapping KAISO in the cytoplasm to inhibit its nuclear interaction with p53 at the *APAF1* promoter.

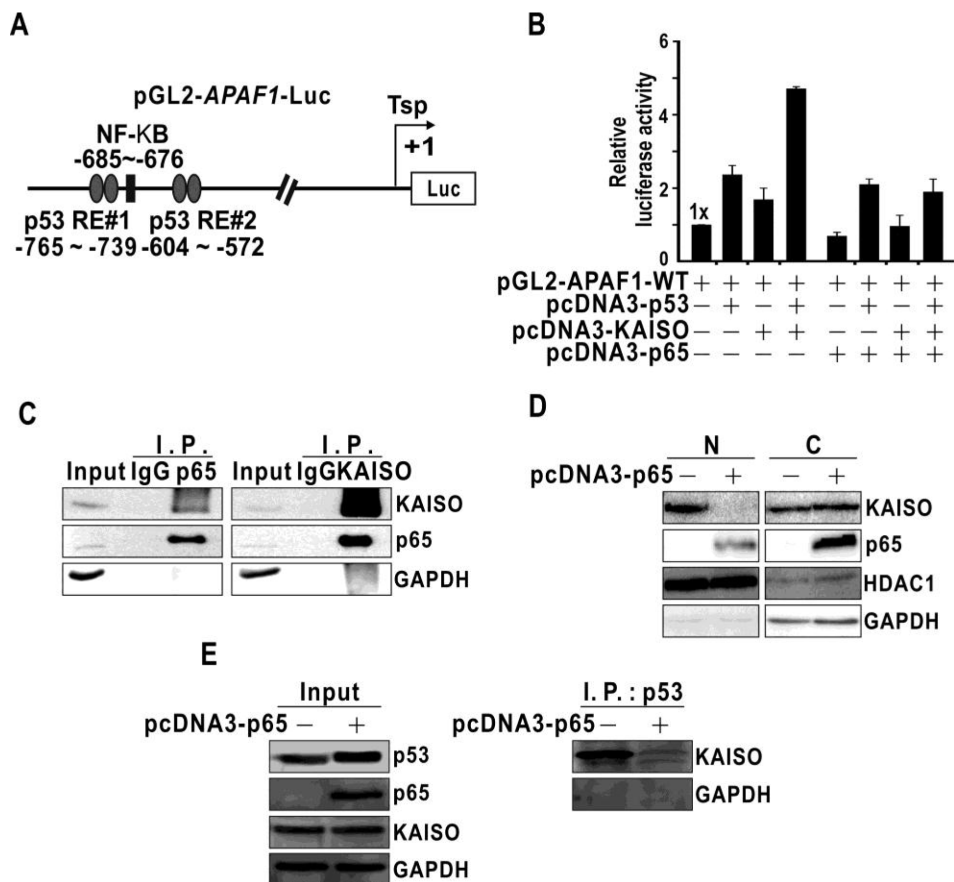

Figure 6. RelA/p65 represses transcription activation of *APAF1* by p53 and KAISO by trapping KAISO in the cytoplasm. (A) Structure of the *APAF1* promoter-luciferase gene fusion reporter plasmid constructs. Binding sites for p53 or NF-κB are indicated as filled ovals or box. (B) Transient transcription assays. H1299 p53-null cells co-transfected with various mixtures of *APAF1* promoter-reporter gene fusion plasmid, p53, p65 and/or KAISO expression vectors were analyzed for luciferase activity. Luciferase

activities were normalized to co-expressed β-galactosidase activity, and data presented are the average of three independent assays. Error bars represent standard deviations, S.D. (C) Co-immunoprecipitation of KAISO and p65. HEK293 cell lysates were immunoprecipitated using anti-KAISO antibody and analyzed by western blot using anti-p65 antibody. Conversely, the lysates were also immunoprecipitated by anti-p65 antibody and analyzed by western blotting using anti-KAISO antibody. IgG, negative control. (D) Nuclear and cytoplasmic cell extracts from H1299 cells transfected with RelA/p65 expression or control vector were immunoblotted for KAISO, p65, HDAC1 (nuclear marker) and GAPDH (cytoplasmic marker). N, nuclear fraction; C, cytoplasmic fraction. (E) Co-immunoprecipitation of p53, KAISO and p65. HEK293 cell lysates were immunoprecipitated using anti-p53 antibody and analyzed by western blotting using anti-p53, anti-p65 and anti-KAISO antibody. IgG, negative control.

IV. DISCUSSION

POK family proteins, including PLZF, BCL6, and FBI-1, have been reported to play critical roles in various biological processes, including embryonic development, cell differentiation, inflammation, proliferation, and apoptosis[16]. Recently, it was also reported that one POK protein, KAISO, can be considered either a potential tumor suppressor or oncoprotein. Here, we found that KAISO is induced by the DNA-damaging agent etoposide in p53-positive cells (e.g., HCT116 $p53^{+/+}$ and HEK293 cells) in which it activates apoptosis (Fig. 1). Thus, KAISO may act as a tumor suppressor in a p53-dependent manner.

Our unpublished data show that KAISO induced by DNA-damage forms a complex with p53 and p300, and that KAISO can induce de novo acetylation of p53 at K320, inhibit acetylation at K381, and enhance acetylation of K382. Thus, KAISO-mediated acetylation of p53 alters its DNA binding to p53 response elements (p53REs), and potently induces apoptosis by activation of *APAF1* and other genes.

p53 interacts with cofactors including HATs (*e.g*., p300/CBP, Tip60, MOZ, MOF and PCAF) and HDACs (HDACs 1, 2, 5, and 6; and SIRTs 1, 2, and 3) and can be acetylated or deacetylated, drastically affecting its DNA binding activity and ability to transcriptionally activate or repress a group of genes determining cell fate[65, 66]. For example, p53 activated by severe

genotoxic stresses acquires enhanced DNA binding ability, via acetylation, to activate transcription of apoptotic and cell cycle regulators, including death receptors, *p21/CDKN1A, PUMA, BAX, APAF1*, *etc*[67-72].

Accordingly, KAISO-associated acetylation of p53 may allow it to interact with p53RE#1 and activate transcription of *APAF1*. Although p53RE#2 also moderately contributes to *APAF1* transcriptional activation, it would be interesting to investigate which post-translationally modified form of p53 binds that element and the functional significance of such an event. We investigated whether p53 modification generated by interaction with KAISO and p300/CBP affects apoptosis. p53QRQ (K320Q K381R K382Q) mimics interaction of p53-KAISO-p300/CBP, while p53RQR (K320R K381Q K382R) is a functionally negative form generated by site-directed mutagenesis. Transient transcription assays in H1299 cells indicated that while wild-type p53 and KAISO could activate transcription of *APAF1*, while p53QRQ, p53RQR lacked such ability. Indeed, *APAF1* transcriptional activation by p53QRQ was greater than that by p53 and KAISO. These results suggest that KAISO may function as a tumor suppressor by regulating acetylation of p53 and promoting apoptosis by activating transcription of *APAF1*.

The *APAF1* promoter contains a number of sites for known transcription factors, including p53, E2F1, and Sp1, that are involved in the activation of apoptosis and cell cycle control. Consistent with this, we identified an NF-κB response element located -685~-676 bp upstream of the *APAF1* transcription

start point, close to p53RE#1. NF-κB is involved in various cellular processes such as cell proliferation, apoptosis, development, and immune responses, and NF-κB and p53 often collaborate or counteract to maintain homeostasis[72]. Considering these critical physiological roles of p53 and NF-κB, their reciprocal relationship in the transcriptional regulation of *APAF1* is worth investigation. In particular, we found that RelA/p65, a subunit of NF-κB, repressed *APAF1* transcription by itself, and also when p53 and KAISO were co-present. Moreover, our data strongly supports RelA/p65 binding to and inhibiting KAISO activity by cytoplasmic sequestration (as ectopic RelA/p65 is localized to the cytoplasm). Thus, RelA/p65 can repress *APAF1* by trapping KAISO in the cytoplasm to inhibit its nuclear interaction with p53 at the *APAF1* promoter. In summary, NF-κB may antagonize p53 upregulation of *APAF1* by disrupting p53/KAISO/p300 complexes.

V. CONCLUSION

Recently, some of the POK family proteins are reported to function as either tumor suppressors or oncoproteins. I found that the expression of KAISO is induced by DNA-damage agent etoposide treatment in HEK293 cells in the pattern similar to the expression of p53 (Fig. 1A and B). I found that KAISO induced by etoposide induces potent apoptosis. p53 plays critical roles in determining cell fate including apoptosis following cellular stresses. KAISO induces apoptosis in the HCT116 $p53^{+/+}$ cells but not HCT116 $p53^{-/-}$ cells, suggesting the possibility that KAISO might induce apoptosis by modulating p53 activity.

APAF1 is the key regulator of apoptosis and was reported as a transcriptional target of p53. I found that KAISO activates transcription of *APAF1* only in the cells expressing p53. Transient transcription assays showed that KAISO enhances transcription activation of *APAF1* by p53 only through the far distal p53RE#1 (bp, -765 ~ -739) in H1299 cells. KAISO does not influence transcription activation of *APAF1* by p53 on the p53RE#2 or -#3. Functional significance of moderate or weak APAF1 activation by p53 through the p53RE#2 or -#3 and nature of p53 bound at these two elements remains to be established.

Post-translational modifications of p53 have important roles in its stability and function. Co-immunoprecipitation revealed that KAISO and p53

interacts each other. This interaction may be important in the regulation of p53 activity, particularly in the regulation of *APAF1* transcription. Koh *et al*. recently found that KAISO affects acetylation of p53 by p300 and confers p53 a death code (ac-K320, non-Ac-381, and Ac-K382). p53QRQ, a mimic of p53 with death code, potently activates APAF1 expression only with the distal p53RE#1, again confirming the importance the particular element in DNA damage response by KAISO. Interestingly, KAISO enhances the DNA binding activity of p53 on only p53RE#1 of the *APAF1* promoter. KAISO lacks the binding activity by itself and requires p53 to bind to the p53RE#1 of the *APAF1* promoter.

And interestingly, a putative NF-κB binding site is located close to the distal p53RE#1 responding to the p53 and KAISO. I found that RelA/p65 can repress transcription activation of *APAF1* by p53 and KAISO. RelA/p65 appears to trap KAISO in cytoplasm by interacting with KAISO. RelA/p65 may suppress apoptosis via transcription repression of *APAF1* by molecular interaction with p53 and KAISO.

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ABSTRACT (IN KOREA)

KAISO와 p53에 의한 APAF1의 발현 활성화의 분자생물학적 작용 기전

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안해민

POK 계열 단백질들은 아포토시스(apoptosis, 예정 세포사), 분화, 발달, 종양 억제와 종양 형성에 중요하다. 일부 POK 계열 단백질들은 종양 억제 단백질 또는 전발암 단백질로 보고 되었다. KAISO의 발현은 HEK293 세포가 DNA-손상 물질에 노출시 유도되며, p53의 발현 양상과 유사하다. 아포토시스는 다세포 생물에서 나타나는 매우 보존된 메커니즘 중에 하나이다. KAISO는 p53 존재시에 아포토시스를 활성화함으로써 세포 증식을 억제한다. *APAF1*은 사이토크롬 C와 dATP와 결합하여 저중합체의 아포토좀 (apoptosome)을 형성하며, 케스페이즈 케스케이드를 가동시킨다. *APAF1* promoter에서의 전사조절은 E2F1과 p53에 의해 조절된다는 것이 이미 알려

져 있다. KAISO가 p53 존재 시 *APAF1*의 발현을 mRNA와 단백질 수준에서 증가시킴을 발견하였다. p53과 KAISO와 마찬가지로, *APAF1* 역시 etoposide에 의해 유도된다. Transient transcription 분석기법을 이용하여 KAISO는 p53RE#1 (bp, -765 ~ -739)에 작용하는 p53에 의해 이루어지는 전사활성화를 증가시킨다. 흥미로운 점은 KAISO는 p53RE#2나 -#3에 작용하는 p53에 의한 *APAF1*의 약한 전사활성화에 영향을 미치지 않는다. Co-immunoprecipitation/western blot 분석기법을 통해 KAISO와 p53이 체내에서 결합함을 보았다. KAISO와 p53은 상호작용을 통해, *APAF1* promoter에서 p53RE#1에 결합하는 p53을 증가시킨다는 것을 EMSA, oligonucleotide pull-down, ChIP 분석기법을 통해 발견하였다.

전사인자인 NF-κB는 세포주기, 아포토시스, 분화, 면역과 염증반응에 대한 유전자의 중요한 조절자이다. *APAF1* promoter에서 NF-κB의 결합부위는 p53과 KAISO에 의해 작용하는 p53RE#1과 가까이에 위치한다. NF-κB에 의한 APAF1의 조절이 세포사멸에 중요할 것으로 생각되어 NF-κB가 p53과 KAISO에 의한 *APAF1*의 전사 활성화에 영향을 미치는지 조사하였다. RelA/p65는 p53과 KAISO에 의한 *APAF1*의 전사적 활성을 크게 억제하였다. RelA/p65는 세포질에서 KAISO와 결합하여 KAISO의 핵안으로 이동을 차단함으로써 KAISO

에 의해 유발된 p53의 아세틸화에 영향을 준다. 따라서, KAISO는 *APAF1* 유전자 발현을 활성화 또는 향상시킴으로써 p53 의존적 아포토시스를 향상시키는 반면, RelA/p65는 KAISO와 p53의 상호작용을 막음으로서 아포토시스를 감소시킨다. RelA/p65은 p53/KAISO와 *APAF1*의 발현과 아포토시스에서 상반적인 역할을 나타낸다.

핵심되는 말 : KAISO, p53, APAF1, 아포토시스, 전사 인자, p65, BTB/POZ 단백질