Correction

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Correction for "KAISO, a critical regulator of p53-mediated transcription of *CDKN1A* and apoptotic genes," by Dong-In Koh, Dohyun Han, Hoon Ryu, Won-Il Choi, Bu-Nam Jeon, Min-Kyeong Kim, Youngsoo Kim, Jin Young Kim, Lee Parry, Alan R. Clarke, Albert B. Reynolds, and Man-Wook Hur, which was first published October 6, 2014; 10.1073/pnas.1318780111 (*Proc. Natl. Acad. Sci. U.S.A.* **111**, 15078–15083).

The authors note that Fig. 6 and Fig. S3 in the *SI Appendix* appeared incorrectly due to errors in figure preparation. The corrected Fig. 6 and its legend appear below. The *SI Appendix* has been corrected online.

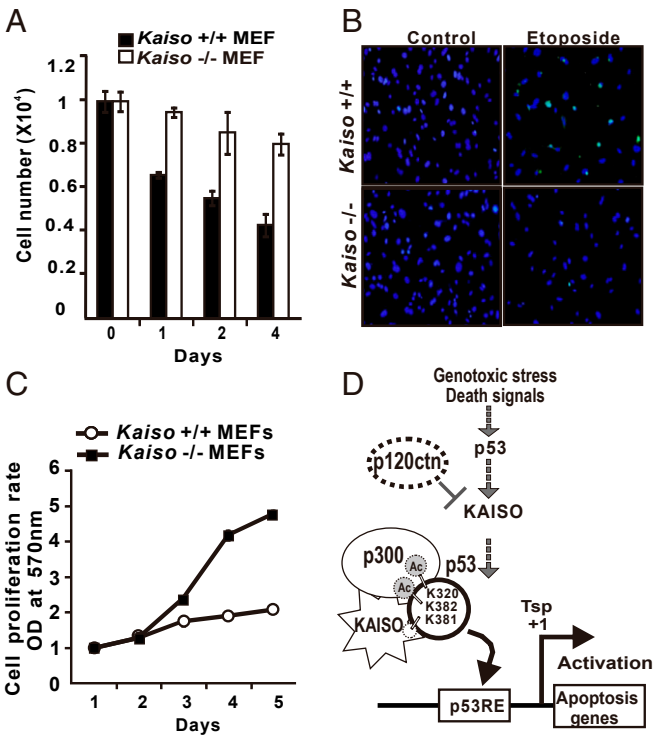

Fig. 6. Kaiso inhibits proliferation of MEFs and plays a critical role in apoptosis. (*A*) MTT assays. MEFs were treated with etoposide and surviving cells were counted. (*B*) TUNEL assays of the MEFs treated with etoposide. DNA strand breaks, green; nuclei, DAPI; apoptotic cells, cyan. (*C*) MTT assay. MEF cells were seeded and grown for 0–5 d. Cell growth was determined by measuring the conversion of the tetrazolium salt MTT to formazan. (*D*) Molecular mechanism of KAISO action in transcription activation of *CDKN1A* and apoptotic genes.

Published June 28, 2021.

www.pnas.org/cgi/doi/10.1073/pnas.2109163118

KAISO, a critical regulator of p53-mediated transcription of *CDKN1A* and apoptotic genes

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Edited by Carol Prives, Columbia University, New York, NY, and approved September 15, 2014 (received for review October 8, 2013)

An unresolved issue in genotoxic stress response is identification of induced regulatory proteins and how these activate tumor suppressor p53 to determine appropriate cell responses. Transcription factor KAISO was previously described to repress transcription following binding to methylated DNA. In this study, we show that *KAISO* is induced by DNA damage in p53-expressing cells and then interacts with the p53–p300 complex to increase acetylation of p53 K320 and K382 residues, although decreasing K381 acetylation. Moreover, the p53 with this particular acetylation pattern shows increased DNA binding and potently induces cell cycle arrest and apoptosis by activating transcription of *CDKN1A* (cyclin-dependent kinase inhibitor 1) and various apoptotic genes. Analogously, in *Kaiso* KO mouse embryonic fibroblast cells, p53-to-promoter binding and up-regulation of *p21* and apoptosis gene expression is significantly compromised. KAISO may therefore be a critical regulator of p53-mediated cell cycle arrest and apoptosis in response to various genotoxic stresses in mammalian cells.

KAISO | p53 | cell cycle arrest | apoptosis | p300

The POZ/BTB family protein KAISO was first isolated based on its interaction with the cell adhesion catenin, p120ctn, a protein with structural similarity to the beta-catenin of the Wnt signaling pathway (1, 2). KAISO has been described as a methyl CpG-binding domain (MBD) protein, and binds to both a sequence-specific KAISO binding site and methylated CpG dinucleotides within target gene promoters (3–5). KAISO has been shown to interact with the nuclear receptor corepressor (NCoR) and mediate DNA methylation-dependent transcriptional repression (4, 6, 7). It has also been suggested that KAISO is a transcription factor that regulates the cell cycle and is a potential tumor suppressor able to block cancer progression (2, 8). However, when crossed with intestinal and mammary tumor-susceptible *ApcMin*/+ mice, *Kaiso*-null mice showed a delayed onset of intestinal tumorigenesis, suggesting that Kaiso deficiency decreases tumor size and initiation (9). Likewise, Kaiso has been shown to be up-regulated in murine intestinal tumors and is expressed in human colon cancers (9), suggesting that Kaiso may be an oncogene that induces intestinal cancer. Thus, the role of KAISO as either a tumor suppressor or an oncogene remains ambiguous.

The tumor suppressor p53 is a key mediator of cellular responses to a variety of stresses (10). It is activated in response to DNA damage and mediates cell cycle arrest, DNA repair, senescence, and apoptosis (10, 11). How p53 is able to discriminate between different stresses and elicit specific cellular response is important and intriguing, but remains largely elusive. When cells are exposed to stresses such as DNA damage, transcriptional outcomes can be determined by the sequence of p53-responsive DNA element (p53RE), levels of induced p53, posttranslational modifications of p53, p53-interacting proteins, and the epigenetic landscape of p53 target gene promoter. Posttranslational acetylation of p53 by acetyltransferases has been found to be important in cell cycle arrest and/or apoptosis by increasing p53 DNA binding and transcription activation potential (12–14). More specifically, how p53 is activated by a particular signal and how it reads a specific p53RE to regulate transcription, of distinct target genes, remains largely unresolved. These questions are important, as the transcriptional output of p53 target genes regulates cell fate decisions in response to a specific genotoxic stress.

Apoptosis, an essential process in mammalian growth and development and cancer, can be induced by cancer therapeutic agents, hypoxia, mitotic abnormalities, and ionizing irradiation. In mammals, there are two major apoptosis pathways (15). The intrinsic apoptosis pathway is regulated by p53 and proapoptotic proteins of the B-cell lymphoma 2 (BCL-2) family of proteins, including p53 up-regulated modulator of apoptosis (PUMA), phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), 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 apoptosis pathway is induced by "death ligands" that bind to death receptors, including FAS (CD95 or apoptosis antigen 1 [APO-1]), death receptor 5 (DR5), and p53 apoptosis effector related to PMP-22 (PERP). Activation of the apoptosis pathway releases cytochrome C from the mitochondria and activates caspase 9 and the effector caspases 3, 6, and 7 that finalize the apoptosis process (15, 18). Expression of death receptor genes and apoptotic protease-activating factor 1 (APAF-1) is also controlled by p53 (19, 20).

In this study, we found that KAISO expression is increased by etoposide treatment in a p53-dependent manner and forms a complex with p53 and the acetyltransferase p300. Subsequently, we

Significance

Transcription factor KAISO (POZ/BTB family protein, ZBTB33) expression is induced by genotoxic stress in a tumor suppressor p53-dependent manner. KAISO then interacts with p53 and the acetyltransferase p300 to modulate p300 acetylation of p53 and imposing upon p53 a "code," i.e., acetylation at K320 and K382, and inhibition of acetylation at K381. This coded p53 shows increased DNA binding to p53 response elements in the promoters of *CDKN1A* (cyclin-dependent kinase inhibitor 1) and apoptosis genes, subsequently inducing cell cycle arrest and potent apoptosis. KAISO is a critical regulator of DNA damage responses in multiple cell types and carries out this function by regulating p53-mediated cell cycle arrest and apoptosis.

Author contributions: D.-I.K., H.R., and M.-W.H. designed research; D.-I.K., D.H., W.-I.C., B.-N.J., and M.-K.K. performed research; D.H., H.R., Y.K., J.Y.K., L.P., A.R.C., and A.B.R. contributed new reagents/analytic tools; D.-I.K., D.H., Y.K., J.Y.K., and M.-W.H. analyzed data; and D.-I.K. and M.-W.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1318780111/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1318780111

comprehensively investigated the functions of KAISO in response to DNA damage and the molecular mechanisms underpinning cell cycle arrest and apoptosis induced by a KAISO–p53–p300 interaction.

Results

KAISO Induces Cell Cycle Arrest and Apoptosis and Forms a Protein Complex with p53 and p300. Recently, we and others found that some BTB/POZ-domain proteins with Krüppel-like zinc-fingers (POK family) function either as tumor suppressors or as oncoproteins (21). We investigated which POK transcription factors are induced in response to DNA damage and could serve as tumor suppressors. Human HEK293 cells were exposed to the DNA-damaging agent etoposide and the expression of various POK transcription factors analyzed. We found that KAISO, p21, p53, BAX, and PUMA were increased both at the mRNA and protein levels (*SI Appendix*, Fig. S1*A*). We further explored whether KAISO could regulate the p53-controlled cell cycle arrest and apoptosis. Analysis of cell proliferation by direct cell number counting or MTT cell viability assay and flow cytometry showed that ectopic KAISO inhibited cell proliferation and increased the number of cells undergoing apoptosis (Fig. 1*A* and *SI Appendix*, Fig. S1 *B–D*). In contrast, a KAISO K8 mutant, lacking the POZ domain and zinc-finger functional domains, showed attenuated apoptotic activity and an inability to induce apoptosis genes (*SI Appendix*, Fig. S2). Activation and posttranslational modification of p53 by acetyltransferases (p300, PCAF, TIP60) are known to be key regulators of p53-mediated apoptosis (12, 22–24). Consequently, we investigated whether p53 or acetyltransferases interact with endogenous KAISO under normal and etoposide-treated conditions. We found that p53 and KAISO interact with each other under both conditions; more protein complex, however, was formed following etoposide treatment. KAISO also interacted with p300 and TIP60, but not PCAF, under normal condition. Whereas the level of the complex of KAISO and TIP60 was not altered, KAISO/p300 complex levels increased following etoposide treatment (Fig. 1*B*).

We also investigated whether these proteins interact directly with each other and mapped their interacting domains. Coimmunoprecipitation and GST-fusion pull-down assays showed that KAISO, p53, and p300 interact with each other to form a heterotrimeric complex (Fig. 1*C* and *SI Appendix*, Fig. S3). KAISO interacted with the C-terminal DNA-binding domain of p53 via its POZ domain and zinc-finger DNA-binding domain (ZFDBD). The POZ domain interacted directly with the DUF906 domain (no. 3) of p300, encompassing its acetyltransferase enzymatic region. These data suggest that KAISO may regulate cell cycle arrest and apoptosis by affecting acetylation of p53 by p300 through p53–p300–KAISO complex formation.

KAISO Influences the Ability of p300 to Acetylate the Three Lysine Residues of p53 and Confers p53 a KAISO-Specific Acetylation Code. We next investigated possible influence of KAISO on p53 acetylation by p300. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of an in vitro p53 acetylation reaction mixture (absent KAISO) revealed that lysine residues of p53 reported to be acetylated by p300 (K164, K305, K370, K372, K373, K381, K382, and K386) could be acetylated (*SI Appendix*, Fig. S4 *I–L*). Interestingly, addition of KAISO to the above reaction mixture affected acetylation of three lysine residues of p53 as follows: increased acetylation of K382, acetylation of K320 (previously reported not to be acetylated by p300), and inhibition of acetylation of K381 (Fig. 2 and *SI Appendix*, Fig. S4 *B–H*). For K320, although we were unable to obtain a clean extracted ion chromatogram (XIC), we observed a mass spectrum corresponding to an acetylated p53 polypeptide fragment (amino acids 320–333, K(Ac)KPLDGEYFTLQIR) only in the p53–KAISO–p300 reaction mixture (Fig. 2*A*). K381, a residue previously known as one of the target sites of acetylation, ubiquitination, and p53 degradation, was blocked from acetylation following its interaction with KAISO (Fig. 2 *B* and *C*). Western blot assays of HEK293 cell lysates with ectopic or knocked-down KAISO, using anti–Ac-K320-, anti–Ac-K381-, and anti–Ac-K382-specific antibodies, revealed that KAISO promoted acetylation of p53 at K320 and K382, but inhibited acetylation of K381 (*SI Appendix*, Fig. S4 *M* and *N*). These results are consistent with our LC-MS/MS data described above.

Molecular Interactions Between KAISO, p53, and p300 Increase p53 DNA Binding to p53REs in *CDKN1A* and Apoptotic Genes. Because KAISO induces cell cycle arrest and apoptosis, we investigated whether and how KAISO might regulate expression of *CDKN1A*, *PUMA*, *BAX*, and *p53*. p21 and PUMA, which represent the major regulators of cell cycle arrest and apoptosis, are directly regulated at the transcriptional level by p53. Whereas ectopic KAISO increased p21, PUMA, and BAX expression, KAISO knockdown decreased their expression in HEK293 cells (Fig. 3*A*). KAISO did not affect p53 expression. Thus, KAISO may directly bind p53 response elements or otherwise affect p53 activity to activate expression of p53 target genes. Oligonucleotide pull-down assays revealed that KAISO alone (absent p53) lacks the ability to bind the p53RE of *CDKN1A* and *PUMA* (compare binding patterns in $p53^{+/+}$ vs. $p53^{-/-}$ HCT116 human colon cancer cells transfected with KAISO expression vector (Fig. 3*B*).

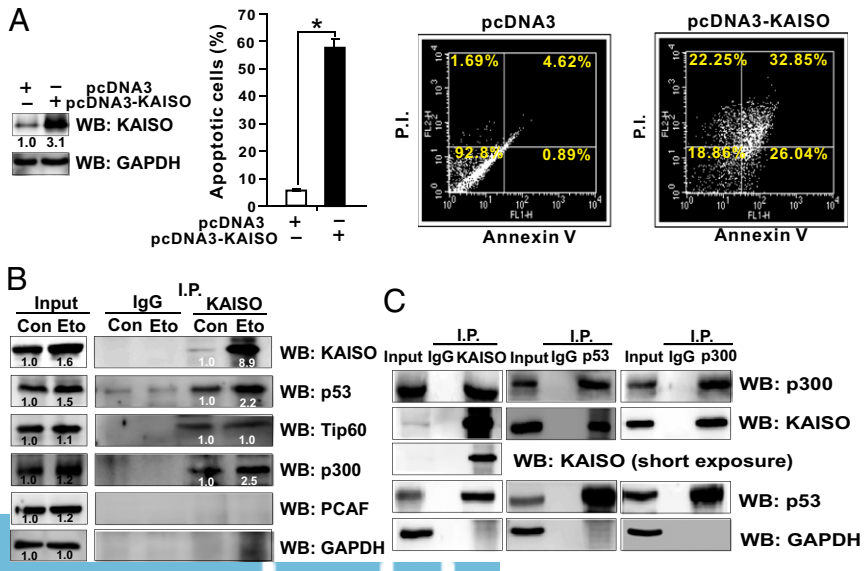

Fig. 1. KAISO induces apoptosis and interacts with the p53–p300 complex. (*A*) Western blot of KAISO overexpression in HEK293 cells transfected with KAISO vector (*Left*). Bar graph showing quantification of flow cytometry analysis of apoptosis of HEK293 cells transfected with KAISO expression vector (scatterplots shown on *Right*). $*P < 0.005$. (*B*) Coimmunoprecipitation and Western blots of endogenous KAISO, p53, and histone acetyltransferase proteins. HEK293 cells were treated with vehicle or etoposide and the cell lysates prepared 24 h posttreatment. (*C*) Coimmunoprecipitation and Western blots showing the various interactions among p53, KAISO, and p300. HEK293 cells were transfected with a KAISO expression vector and cell lysates immunoprecipitated with anti-p300, anti-p53, or anti-KAISO antibodies.

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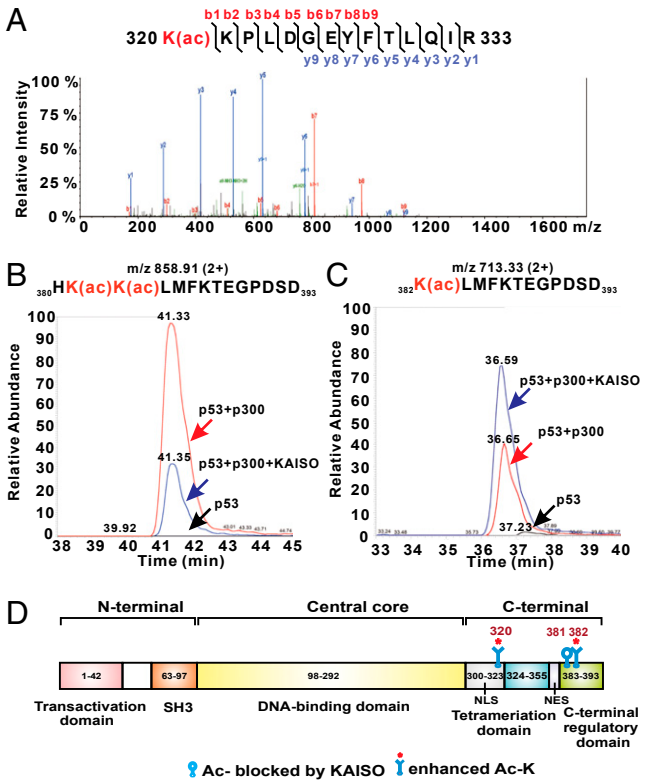

Fig. 2. Acetylation of p53 residues K320, K381, and K382 by p300 is modulated by KAISO. (*A*) Mass spectrometry (MS) analysis of a p53 peptide acetylated at K320 by KAISO–p300. The acetylated lysine residue is shown as K(ac). (*B* and *C*) An extracted ion chromatogram (XIC) of the acetylated peptide 380-HK(Ac)K(Ac)LMFKTEGPDSD-393 [mass to charge ratio (*m/z*): 858.91, charge 2+] and peptide 382-K(Ac)LMFKTEGPDSD-393 (*m/z*: 713.33, charge 2+). Acetylation levels of K381 and K382 were quantified by comparing XICs of the acetylated peptides, including the acetylation sites K381 and K382 in the three different in vitro reactions (p53 only, p53 + p300, and p53 + p300 + KAISO). An XIC of the precursor peptides was plotted in 10-ppm mass windows using the Xcalibur program. *X* axis, retention time; *y* axis, relative signal intensity. (*D*) Summary of the LC-MS/MS analysis of the p53 in vitro acetylation by p300 + KAISO.

Oligonucleotide pull-down assays of the in vitro p53 acetylation reaction mixture p53 + p300 ± KAISO showed that p53 binding to p53RE1 of *CDKN1A* and *PUMA* was significantly increased by KAISO (*SI Appendix*, Fig. S5). ChIP assays showed that KAISO increased DNA binding of p300 and p53 to the p53REs of the *CDKN1A* and *PUMA* promoters (Fig. 3*C*). ChIP-ReChIP assays also showed that ectopic p53WT and KAISO bind together to the p53REs of the promoter of *CDKN1A*, *PUMA*, and other apoptosis genes, in H1299 p53-null human lung cancer cells (Fig. 3*D* and *SI Appendix*, Fig. S6 *C* and *D*). Additional ChIP assays of p53 binding in *Kaiso* WT and KO mouse embryonic fibroblasts (MEFs) treated with etoposide showed that p53 binding to *Cdkn1a* and apoptosis gene promoter regions flanking the p53REs increased only in the presence of Kaiso (Fig. 3*E* and *SI Appendix*, Fig. S6*F*). We also found that KAISO binding to *CDKN1A* and apoptosis gene promoters required p53 in the HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells treated with etoposide (Fig. 3*F* and *SI Appendix*, Fig. S6*G*). These data suggest that the in vivo molecular interactions between KAISO, p53, and p300 regulate the combinatorial acetylation of the three abovementioned lysine residues of p53, and that these events are important in increasing p53 DNA binding and transcriptional activation of p53 target genes such as *CDKN1A*, *PUMA*, and other apoptotic genes.

Intriguingly, the p53REs of CDKN1A and apoptotic genes regulated by KAISO showed a bipartite symmetrical arrangement of their half sites (RRRCWWGYYY). These two half sites face each other with a 0- or 1-nucleotide spacer between them (→← spacer →←). In contrast, KAISO did not enhance p53-mediated repression of the antiapoptosis gene *Survivin* or activation of the *DDA3* gene at the transcriptional level (*SI Appendix*, Fig. S6*C*). The latter two genes have two half sites in a similar orientation separated by 3- and 9-nucleotide spacers, respectively (*SI Appendix*, Table S2). Introduction of an extra 5-nucleotide spacer between the two half sites of p53REs of *CDKN1A* and *PUMA* significantly decreased p53 binding in a p53 + p300 + KAISO mixture, but not in a p53 + p300 (lacking KAISO) mixture or p53 alone (*SI Appendix*, Fig. S5). These results suggest that combinatorial modification of the three p53 lysine residues may induce a conformational change such that it can more efficiently bind two p53RE half sites with 0- or 1-nucleotide spacers.

The Acetylation Pattern of the Three p53 Lysines Affected by KAISO/p300 Is Critical for Transcriptional Activation of p53 Target Genes, *CDKN1A*, and Apoptotic Genes. To study possible functions of KAISO/p300-associated p53 acetylation in apoptosis, we used site-directed mutagenesis to prepare p53 mutants representing the above-described p53 code: an acetyl mimetic, p53QRQ (K320Q, K381R, K382Q), and a functionally negative form of p53QRQ, p53RQR (K320R, K381Q, K382R). Transient transcription assays in H1299 cells showed that ectopic p53 could activate transcription of endogenous *CDKN1A* and apoptotic genes. Additionally, when KAISO and p53 were coexpressed, they further stimulated the expression of apoptosis genes. Similarly, the p53QRQ mutant potently activated transcription of the same genes, in contrast to the functionally negative form, p53RQR (*SI Appendix*, Fig. S7*A*). Under these test conditions, the expression levels of p53WT, p53QRQ, and p53RQR were all similar, at both the mRNA and protein levels (*SI Appendix*, Fig. S7 *C* and *D*).

We further used flow cytometry to demonstrate that p53QRQ could induce apoptosis in H1299 cells, comparable to that seen following coexpression of KAISO and p53 (Fig. 4). In contrast, the functionally negative p53RQR mutant showed no significant effect on apoptosis. Moreover, p53 could not induce apoptosis when KAISO expression was knocked down by siRNA, suggesting that KAISO is essential for this p53 function (Fig. 4 and *SI Appendix*, Fig. S7 *B* and *E*). These data collectively suggest that the combinatorial acetylation of three p53 lysine residues by p300 (imposed by KAISO) is important and sufficient for transcriptional activation of a group of p53 target genes that regulate apoptosis.

KAISO Plays an Indispensable Role in *Cdkn1a* and Apoptotic Gene Expression in MEF Cells. We also investigated the role of Kaiso in cell cycle arrest and apoptosis using a loss-of-function approach. The level of p53 expression was nearly identical in MEF cells with both *Kaiso* WT and KO phenotypes, but the expression of the p53 target genes *Cdkn1a*, *Bax*, *Puma*, death receptor genes (*Fas* and *Dr5*) of the extrinsic pathway (9, 25, 26), and caspase genes was low or not detected in *Kaiso* KO MEF cells (Fig. 5*A* and *SI Appendix*, Fig. S8 *C* and *D*). Whereas etoposide and the death receptor ligands FasL and Trail induced expression of *Kaiso*, *p53* and other genes important in cell cycle arrest and apoptosis (*Cdkn1a*, *Bax*, *Puma*, *Caspase 8*, *Caspase 3*, *Dr5*, and *Fas*) in Kaiso WT MEF cells, they did not induce expression of these genes in *Kaiso* KO MEF cells, even when p53 was induced (Fig. 5*B* and *SI Appendix*, Fig. S8 *C* and *D*). Interestingly, expression of two critical players in apoptosis, *Caspase 8* and *Caspase 3* was also significantly decreased in the absence of Kaiso, which may also reflect a decrease in the expression of death receptors and apoptotic protease-activating factor 1 (Apaf-1) (*SI Appendix*, Fig. S8 *C* and *D*). RT-qPCR analysis indicated that mRNA expression of *Cdkn1a* and apoptosis pathway genes was very low or not detectable in *Kaiso* KO MEF cells. Even when *Kaiso* KO MEF cells were treated with etoposide, all of the genes were not induced, although p53 mRNA induction was similar to that in WT MEF cells (Fig. 5*C*). We also noticed that death receptor ligands could activate KAISO expression (*SI Appendix*, Fig. S8 *C* and *D*), and that KAISO

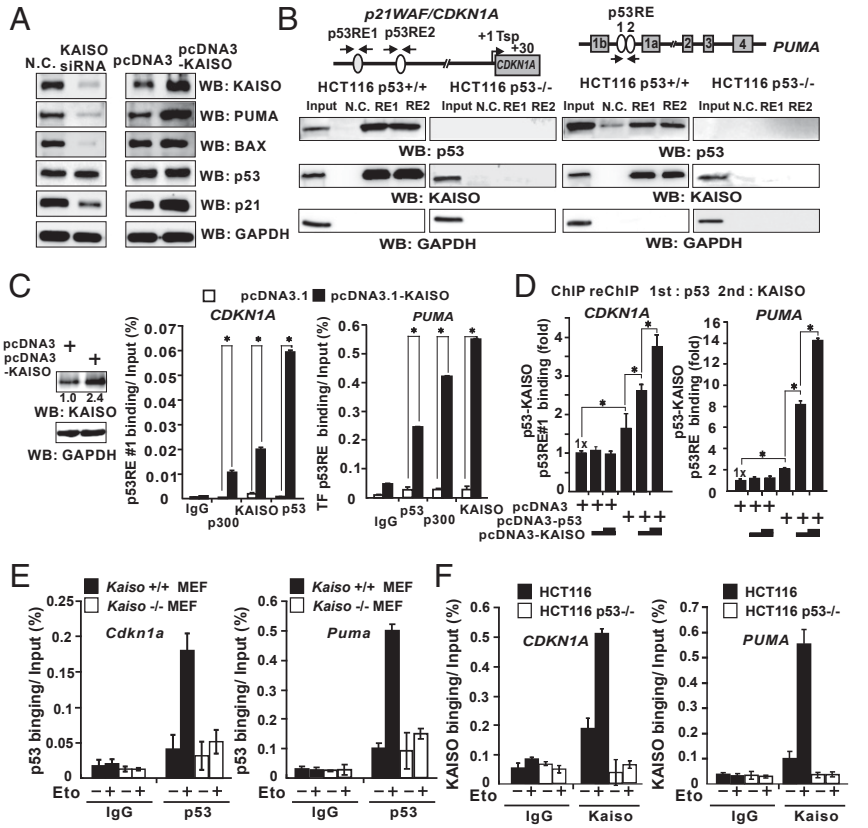

Fig. 3. KAISO increases expression of p53 target *CDKN1A* and *PUMA* genes. KAISO increases p53 binding to the p53REs of CDKN1A and PUMA and binds as a KAISO–p53–p300 complex. (*A*) Western blot analysis of endogenous p53, BAX, and PUMA expression in HEK293 cells transfected with either KAISO expression vector or KAISO siRNA. (*B*) Oligonucleotide pull-down assays of the p53REs of the *CDKN1A* and *PUMA* promoter. Whole cell lysates of HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells transfected with KAISO expression vector were used. (*C*) ChIP assays of KAISO, p53, and p300 binding to the p53REs of *CDKN1A* and *PUMA* in HEK293 cells. IgG, ChIP control. The ChIP enrichment of transcription factor binding was normalized to the input DNA. $*P < 0.01$. (*D*) ChIP-ReChIP analysis. H1299 cells were transfected with p53WT and/or KAISO expression vectors. $*P < 0.01$. First ChIP antibody, anti-p53; second ChIP antibody, anti-KAISO. (*E*) ChIP assays of p53 binding in Kaiso WT and KO MEFs treated with etoposide (40 μM) for 24 h. (*F*) ChIP assays of Kaiso binding in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells treated with etoposide (100 μM) for 24 h. IgG, ChIP control. Error bars, SDs.

mRNA knockdown attenuated apoptosis of HCT116 cells by the death receptor ligand Trail (*SI Appendix*, Fig. S8 *A* and *B*).

We also investigated whether RNA polymerase II (Pol II) binding during transcription of apoptotic genes, *Kaiso*, and transformation-related protein 53 (*Trp53*) was affected by the presence or absence of Kaiso in MEFs exposed to etoposide. ChIP assays of RNA polymerase II binding to exonic regions (thus detecting elongating RNA Pol II) showed that RNA Pol II binding to *Kaiso* and apoptotic genes significantly decreased following etoposide treatment in *Kaiso* KO MEFs. In contrast, RNA Pol II binding to the downstream exons of *Trp53* was increased by etoposide in both *Kaiso* WT and KO MEFs. Furthermore, ChIP assays of RNA Pol II involved in transcriptional initiation, elongation, and termination, using RNA Pol II antibodies (CTD4H8, Ser2Ph, and Ser5Ph), showed that expression of Kaiso and/or p53 increases RNA Pol II in elongation and termination stages and transcription of *PUMA* and *BAX* (Fig. 5*D* and *SI Appendix*, Fig. S9 *A–D*). These results suggest that Kaiso plays an important role in the transcriptional activation of p53 target apoptosis genes.

Kaiso Inhibits Proliferation of MEFs and Plays a Critical Role in Apoptosis. We showed that chemotherapeutic agents, ionizing radiation, and UV radiation, known stressors that induce apoptosis via p53 expression, could activate Kaiso expression (*SI Appendix*, Fig. S10 *A* and *B*), which would eventually affect activity of p53 and the expression of apoptosis genes. Furthermore, we analyzed cell growth rates of *Kaiso* WT and KO MEFs after pretreatment with etoposide. *Kaiso* WT MEFs underwent a severe decreases in cell number at 1–4 d postetoposide treatment, whereas *Kaiso* KO MEFs showed mild decrease in cell number and were relatively etoposide resistant (Fig. 6*A*). Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assays revealed fewer apoptotic cells among *Kaiso* KO MEFs compared with *Kaiso* WT MEFs. The ratios of apoptotic cells to the total number of 4′, 6-diamidino-2-phenylindole (DAPI)-positive cells were 44% (SD ± 2.6%) for

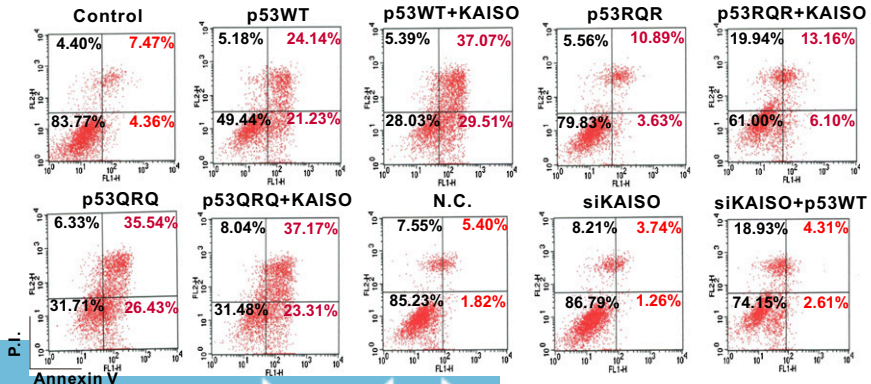

Fig. 4. The p53 mimic (p53QRQ), representing the combinatorial acetylation of p53 imposed by KAISO, induces potent apoptosis. Flow cytometry analysis of apoptosis of H1299 cells transfected with various combinations of KAISO siRNA and expression vectors of p53WT and/or KAISO, p53RQR, and p53QRQ.

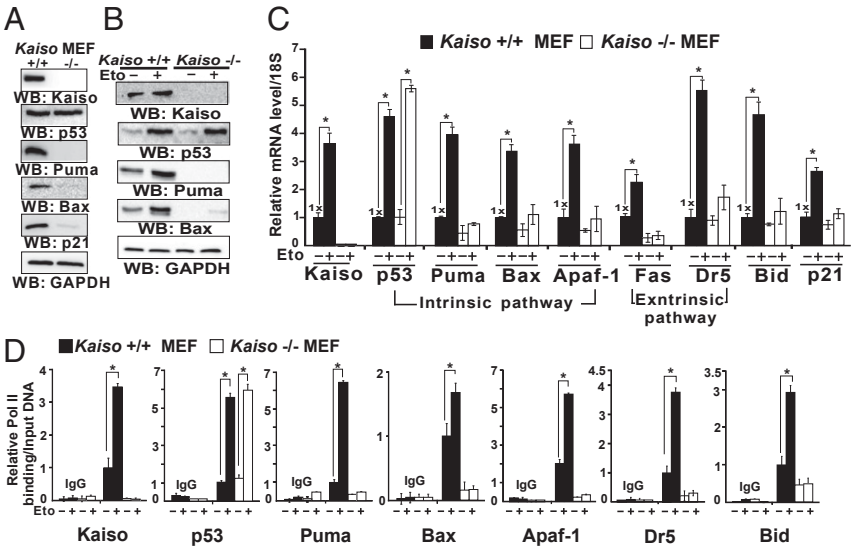

Fig. 5. Kaiso is a critical regulator of *Cdkn1a* and apoptotic gene transcription. (*A*) Western blots of Kaiso, p21, Puma, and p53 expressed in *Kaiso* KO MEF cells. GAPDH, control. (*B*) Western blots of Bax, Puma, and p53 expressed in *Kaiso* WT and KO MEF cells treated with etoposide. (*C*) RT-qPCR assays of mRNA levels of p21 and apoptotic genes in *Kaiso* WT and KO MEF cells treated with etoposide. The mRNA levels were normalized to 18S RNA. $*P < 0.05$. (*D*) ChIP assays of RNA polymerase II (elongating stage) bound at the downstream exons of *Kaiso*, *p53*, and apoptosis pathway genes. *Kaiso* WT and KO MEFs were treated with etoposide and immunoprecipitated using the antibody indicated. IgG, ChIP control antibody. $*P < 0.05$.

Kaiso WT and 2.24% (SD ± 1.0%) for Kaiso KO MEFs, respectively (Fig. 6*B*). KAISO thus induces potent apoptosis in the WT MEF cells exposed to etposide.

Microarray analyses of spleen and testis tissues of *Kaiso* WT and KO mice demonstrated that *Puma*, *Noxa*, *Dr5*, and *FasL* mRNA were significantly decreased in KO mouse tissues (*SI Appendix*, Table S1). In the brain, kidney, and testis tissues of C57BL/6J *Kaiso* KO mice (9), p53 expression levels were nearly identical to those in *Kaiso* WT mice; however, the expression levels of *Puma* were markedly decreased at both the mRNA and protein levels (*SI Appendix*, Fig. S10 *C* and *D*). Immunohistochemical (IHC) analysis of testis tissues showed that the expression of *Bax* decreased significantly in the absence of Kaiso (*SI Appendix*, Fig. S10*E*). The number of cells positive for proliferating cell nuclear antigen (Pcna) in *Kaiso* knockout mouse tissues was high, compared with wild-type controls. Also, MEF KO cells prepared from the *Kaiso* KO mouse embryos were morphologically similar to MEF WT cells but grew 2.5 times faster after 5 d (Fig. 6*C*). These results show that KAISO inhibits cell proliferation and plays a role in apoptosis.

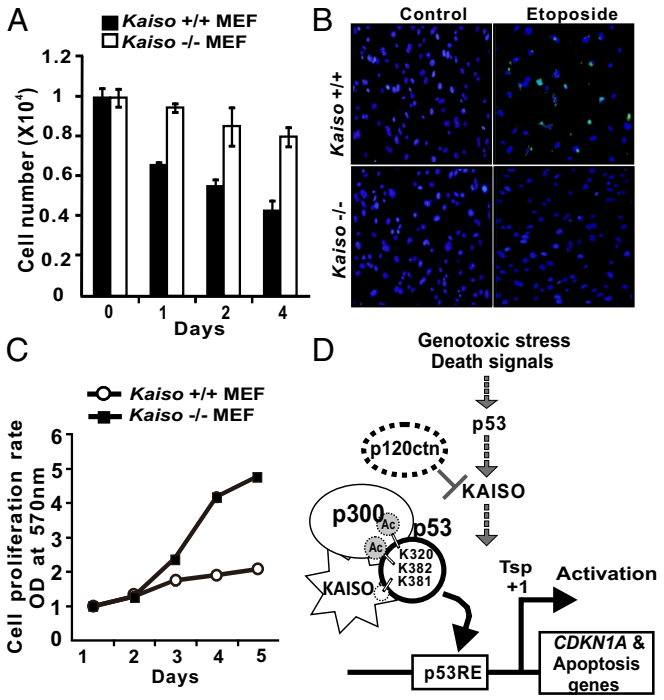

Fig. 6. Kaiso inhibits proliferation of MEFs and plays a critical role in apoptosis. (*A*) MTT assays. MEFs were treated with etoposide and surviving cells were counted. (*B*) TUNEL assays of the MEFs treated with etoposide. DNA strand breaks, green; nuclei, DAPI; apoptotic cells, cyan. (*C*) MTT assay. MEF cells were seeded and grown for 0–5 d. Cell growth was determined by measuring the conversion of the tetrazolium salt MTT to formazan. (*D*) Molecular mechanism of KAISO action in transcription activation of *CDKN1A* and apoptotic genes.

Discussion

One of the interesting questions surrounding p53-mediated transcription is how p53 specifically recognizes only certain subsets of promoters and ignores numerous other promoters in response to different stimuli (27). p53-binding site sequences, p53 posttranslational modifications (23, 28), and p53-interacting proteins are important determinants of the diversity of p53-mediated transcriptional responses. For example, increased DNA binding and stability have been demonstrated following acetylation of p53 lysine residues K120, K164, and K320 by Tip60/hMOF, p300/CBP, and PCAF, respectively, and p300/CBP-mediated acetylation of lysine residues K370, K372, K373, K381, K382, and K386 in the p53 C terminus (13, 25, 26, 28–32). Each of these findings highlights the importance of modification of specific lysine residues, but the combined effect of multiple p53 modifications on gene expression is largely unknown.

The p53 protein interacts with coactivators or corepressors and can be acetylated or deacetylated to respectively activate or repress its transcriptional activity. Proteins interacting with p53 can modulate acetylation levels by altering the acetyltransferase or deacetylase activity of coregulators, thus affecting promoter DNA binding by p53 and transcription activation potential (23, 28–32).We demonstrated that expression of KAISO is induced by etoposide in the cells expressing p53 and forms a heterotrimeric complex with p53 and p300. KAISO also affected p300 acetylation of three key lysine residues of p53, increasing acetylation at K320 and K382, while inhibiting acetylation of K381. We also showed that p53QRQ, a mutant that mimics the p53 acetylation pattern following its interaction with KAISO–p300, could similarly activate transcription of *CDKN1A* and apoptotic genes and thereby induce cell cycle arrest and apoptosis (refer to model shown in Fig. 6*D*). Levine and Oren (27) and Levine and coworkers (33) discussed the influence of p53 posttranslational modifications, p53RE arrangements, and spacer lengths, and p53RE nucleotide sequences on the activation of p53 and its

downstream target genes. How does KAISO activate transcription of a group of genes that plays roles in cell cycle arrest and apoptosis? The p53REs of *CDKN1A* and apoptosis genes regulated by KAISO showed a bipartite symmetrical arrangement of p53RE half sites (RRRCWWGYYY) with a 0- or 1-nucleotide spacer. In contrast, KAISO did not enhance transcriptional regulation of genes having the p53RE with 3- or 9-nucleotide spacers (*SI Appendix*, Table S2 and Fig. S6*C*). The combined effect of modification of the three p53 lysine residues may be induction of a conformational change that can more efficiently bind the two half sites of the p53REs separated by 0- or 1-nucleotide spacers.

Kaiso KO C57BL/6J mice maintained in the heterogeneous genetic backgrounds Ola120 and C3H were viable and fertile, and they exhibited no detectable abnormalities of development or gene expression (9). *Kaiso*-deficient mice, in the absence of the tumor suppressor APC, were susceptible to intestinal cancer, suggesting that *Kaiso* might be an oncogene (9). By contrast, KAISO has also been suggested to be a potential tumor suppressor repressing transcription of *MMP7*, *Cyclin D1*, and *Wnt11*, genes involved in oncogenesis and metastasis (1, 2, 34). However, *Kaiso* KO mice maintained in a C57BL/6J genetic background for three generations showed several distinct phenotypes such as splenomegaly, testis atrophy, and increased body weight and size. The failure to observe overt phenotypes in previous studies and in our *Kaiso* KO G1 and G2 generation mice may indicate a role for Kaiso as a methylcytosine-binding domain (MBD) family protein involved in the epigenetic regulation of target genes. Such regulation could be maintained for three generations after *Kaiso* knockout or may be due to differences in the genetic backgrounds in which *Kaiso* gene function was tested. Microarray analysis of differentially expressed mRNA in mouse tissues showed massive changes in mRNA expression of as many as 4,000 genes. Our investigation consistently revealed that KAISO plays an important role in cell cycle arrest and apoptosis in HEK293, H1299, and MEF cells and *Kaiso* KO mice. Expression of *Cdkn1a*, *Puma*, and *Bax* genes was significantly decreased in *Kaiso*-null MEFs and in various tissues of *Kaiso* KO mice. KAISO may be a potential tumor suppressor inducing cell cycle arrest and apoptosis. Interestingly, HCT116 cells transfected with KAISO siRNA showed an increase in apoptosis when treated with etoposide (7), suggesting that KAISO is antiapoptotic. In contrast, we found that KAISO was induced by etoposide robustly enough to overcome or rescue knocked down KAISO expression in both HCT116 and HEK293 cells (*SI Appendix*, Fig. S10 *F* and *G*). Our similar experiment carried out with *Kaiso* KO MEFs clearly showed that etoposide treatment induced little apoptosis (Fig. 6*A*), further suggesting that KAISO is proapoptotic.

We also found that other cellular stresses, including UV radiation, γ-irradiation, chemotherapeutic anticancer drugs, and death receptor ligands also induced KAISO expression. These agents may induce apoptosis through KAISO, similar to etoposide. In summary, our study exemplifies the importance of KAISO to p53-mediated transcription of *CDKN1A* and apoptotic genes.

Materials and Methods

***Kaiso* KO Mice.** Animal experiments were approved by the Committee on Animal Investigations of Yonsei University.

LC-MS/MS. Mass spectrometry, in-gel tryptic digestion, and on-line nano LC-MS/MS were performed as previously reported (35, 36).

ChIP/Re-ChIP Analysis. ChIP/Re-ChIP assays were performed to assess KAISO, p53, and p300 binding to the promoters of apoptotic genes in vivo, and the sequences of the oligonucleotide primers used in these assays are listed in *SI Appendix, SI Materials and Methods*.

In Vitro Protein Acetylation Assays. KAISO protein was incubated with p300 and His-p53 in in vitro p53 acetylation assay buffer for 1 h at 37 °C, and the mixture was then resolved by SDS/PAGE, stained using Coomassie Blue, and gel regions containing p53 were excised and analyzed by mass spectrometry.

The full methods are available in *SI Appendix, SI Materials and Methods*.

ACKNOWLEDGMENTS. This work was supported by Do-Yak Research Grant 2011-0028817 (to M.-W.H.) and Medical Research Center Grant 2011-0030086 (to M.-W.H.) from the National Research Foundation of Korea (NRF) of the Korean Government (MSIP).

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