

Temporal and differential regulation of KAISO-controlled transcription by phosphorylated and acetylated p53 highlights a crucial regulatory role of apoptosis

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Transcriptional regulator KAISO plays a critical role in cell cycle arrest and apoptosis through modulation of p53 acetylation by histone acetyltransferase p300. KAISO potently stimulates apoptosis in cells expressing WT p53, but not in p53-mutant or p53-null cells. Here, we investigated how KAISO transcription is regulated by p53, finding four potential p53-binding sites (p53-responsive DNA elements; p53REs) located in a distal 5'-upstream regulatory element, intron 1, exon 2 coding sequence, and a 3'-UTR region. Transient transcription assays of pG5-p53RE-Luc constructs with various p53REs revealed that p53 activates *KAISO* (*ZBTB33*) transcription by acting on p53RE1 (−4326 to −4227) of the 5'-upstream region and on p53RE3 (+2929 to +2959) of the exon 2 coding region during early DNA damage responses (DDRs). ChIP and oligonucleotide pulldown assays further disclosed that p53 binds to the p53RE1 and p53RE3 sites. Moreover, ataxia telangiectasia mutated (ATM) or ATM-Rad3-related (ATR) kinase-mediated p53 phosphorylation at Ser-15 or Ser-37 residues activated *KAISO* transcription by binding its p53RE1 or p53RE3 sites during early DDR. p53RE1 uniquely contained three p53-binding half-sites, a structural feature important for transcriptional activation by phosphorylated p53 Ser-15-Ser-37. During the later DDR phase, a KAISO-mediated acetylated p53 form (represented by a p53QRQ acetyl-mimic) robustly activated transcription by acting on p53RE1 in which this structural feature is not significant, but it provided sufficient KAISO levels to confer a p53 “apoptotic code.” These results suggest that the critical apoptosis regulator *KAISO* is a p53 target gene that is differently regulated by phosphorylated p53 or acetylated p53, depending on DDR stage.

KAISO was originally isolated as a protein interacting with p120ctn, a structural analogue of β -catenin, a member of the

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This article contains Figs. S1 and S2.

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Wnt signaling pathway (1). KAISO exhibits DNA-binding activity to KAISO-binding sites (CTGCNA) and methylated CpG dinucleotides of target genes associated with cancer and/or embryonic development (2–4). In that regard, KAISO interacts with transcriptional corepressors, such as NCoR1, Sin3A, and Groucho, to mediate DNA methylation-dependent transcriptional repression (5).

Previously, we demonstrated that KAISO is induced by DNA damage in p53-expressing cells and interacts with a p53/p300 complex to increase acetylation of p53 Lys-320 and Lys-382 residues while decreasing Lys-381 acetylation. Moreover, the p53 with this particular acetylation pattern potently induces cell cycle arrest and apoptosis by activating transcription of *CDKN1A* (cyclin-dependent kinase inhibitor 1) and various apoptosis effector genes. KAISO is a critical regulator of p53-mediated apoptosis, under genotoxic stress conditions, in mammalian cells (6). We also showed that *APAF1*, encoding the core molecule of the apoptosome, is transcriptionally activated by KAISO (7).

p53, normally short-lived and present at low levels, is a key mediator of cellular responses to a broad range of genotoxic stresses (8). In response to DNA damage and other cellular stresses, both the protein level and activities of p53 are greatly increased. The crucial regulators of the genotoxic stress response, ataxia telangiectasia mutated (ATM)² and ATM-Rad3-related (ATR) protein kinases, phosphorylate many cellular substrates, including p53 (9–12). p53-dependent transcriptional outcomes can be determined by the presence of p53-responsive DNA elements (p53REs), levels of induced p53, p53 post-translational modifications, its interacting proteins, and the epigenetic landscape of p53 target gene promoters. In particular, post-translational modifications, including phosphorylation and acetylation, were shown to regulate p53 stability, site-specific DNA-binding activity, and transactivation potential. Protein kinases, including ATR, ATM, and DNA-PK, were shown to phosphorylate several serine and/or threonine residues of the N terminus of p53, effectively regulating its function. Phosphorylation of p53 Ser-15, *in vivo*, is stimulated

² The abbreviations used are: ATM, ataxia telangiectasia mutated; ATR, ATM-Rad3-related; qPCR, quantitative real-time PCR; DNA-PK, DNA-dependent protein kinase; CBP, CREB-binding protein; PCAF, p300/CBP-associated factor; DDR, DNA damage response; p53RE, p53-responsive DNA elements; PTM, post-translational modification; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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by DNA-damaging agents, including ionizing radiation and UV (13). Ser-15 phosphorylation might stabilize p53 by preventing p53 nuclear export (14). In addition, phosphorylation of human p53 on serines 15 and 37 *in vitro* by DNA-PK was reported to inhibit p53 interaction with its repressor, Mdm2 (15). Phosphorylation at Ser-37 is also important for the transcriptional activity of p53 (16).

Another important modification of p53, acetylation, also plays important roles in response to various types of DNA damage. Acetylation has been shown to increase p53 sequence-specific DNA-binding capacity, through the recruitment of coactivators, and to enhance its stabilization by inhibiting ubiquitination of p53 by MDM2 (17–20). Specifically, p53 is acetylated at Lys-370, Lys-372, Lys-381, and Lys-382 by p300/CBP and at Lys-320 by p300/CREB-binding protein–associated factor (PCAF) (21–24).

Phosphorylation at the p53 N terminus enhances its interaction with the acetyltransferase p300/CBP and acetylates p53. Phosphorylation at N-terminal serines, such as Ser-15, -33, and -37, has been reported to recruit p300/CBP and PCAF to induce p53 acetylation in response to DNA damage (25, 26). Phosphorylation of p53 at Ser-46 by UV-activated HIPK2 facilitates CBP-mediated acetylation of p53 at Lys-382 to promote p53-dependent gene expression (27). These reports suggest that phosphorylation and acetylation of p53 may selectively induce p53 target gene expression and exert p53 functions, such as apoptosis.

The gene *TP53* is absent or mutated in a high proportion of human cancers (28, 29), often leading to expression of a full-length protein that is deficient in certain functions, such as specific DNA binding. Many p53-mutant forms exert a dominant-negative effect, serving to abrogate the ability of WT p53 to inhibit cellular transformation or mediate DNA repair (30). Disruption of p53 functions promotes checkpoint defects, cellular immortalization, genomic instability, and inappropriate survival, allowing the continued proliferation of abnormal cells (31, 32). Activated p53 binds to specific DNA sequences of its target genes after DNA damage, functioning as a homotetramer and binding to p53-response elements; however, mutants of p53, including each of the four “hot spots” frequently altered in human cancers, fail to bind to the p53-binding consensus dimer (33).

The consensus-binding site of p53 contains two copies of a 10-bp motif (5'-PuPuPuC(A/T)(T/A)GPyPyPy-3'), separated by a 0–21-bp spacer. One copy of the motif was insufficient for site-specific DNA binding (34). Genes activated by p53 include a negative cell cycle regulator, *CDKN1A* (*p21*), and pro-apoptotic genes, such as *Bax* and *Puma*, which trigger cell cycle arrest, DNA damage repair (DDR), cellular senescence, apoptosis, and other responses, to determine cell fate following genotoxic stress (35–37).

In this study, we found that *KAIISO* expression is dramatically increased by DNA damage only in cells with WT p53 and not in cells with p53 hot spot mutations. Consequently, we investigated how *KAIISO* gene expression is controlled by p53 through p53REs in its 5'-upstream regulatory and coding regions. During early phases of DDR, p53 phosphorylated at Ser-15 and Ser-37 up-regulates *KAIISO* and early DDR genes to activate

apoptosis during later phases of DDR. Phosphorylated p53 Ser-15-Ser-37 and “acetylation-coded” (“cell death code”) (6) p53 differentially regulate *KAIISO* expression via time differences of their expression and differential reading of its structural features by p53RE1.

Results

KAIISO gene expression is increased only in cells expressing WT p53 and not in the cells with hot spot p53 mutants or lacking p53

Previously, we showed that temporal expression patterns of *KAIISO* and p53 are similar in cells treated with etoposide (6). We further investigated expression patterns of *KAIISO* and p53 in various cell types expressing no p53, WT p53, or hot spot p53 mutations upon treatment with the DNA-damaging agent etoposide. *KAIISO* and *p53* mRNA levels were increased by 7–16-fold at 3 h of etoposide treatment in HCT116 colorectal cancer p53^{+/+} cells. However, in HCT116 p53^{-/-} cells or in p53-mutated cancer cells, such as SNU61, Colo320DM, LS1034, and HT-29, neither *KAIISO* nor *TP53* mRNA levels were significantly increased by etoposide (Fig. 1A). We observed similar *KAIISO* and p53 expression pattern at the protein level (Fig. 1B). In addition, ectopic p53 increased transcription of endogenous *KAIISO* in H1299 lung cancer cells lacking endogenous p53 (Fig. 1C).

We also tested whether other types of DNA-damaging agents (cisplatin, an alkylating reagent, and 5-fluorouracil, a pyrimidine analog) also induce *KAIISO* transcription, via p53. Those assays showed that cisplatin induced *KAIISO* transcription only in HCT116 cells expressing p53, in a fashion similar to that of etoposide (a topoisomerase inhibitor). In contrast, 5-fluorouracil induced *KAIISO* transcription weakly and slowly in p53^{+/+} cells (Fig. S1). These results suggest that WT p53 is required for *KAIISO* expression in cells treated with DNA-damaging agents.

p53 activates *KAIISO* transcription by acting on its p53RE1 (bp –4326 to –4227) of 5'-upstream regulatory regions and p53RE3 (bp +2929 to +2959) in its exon 2 coding sequence region

Because *KAIISO* expression increased only in cells with WT p53, we investigated how p53 activates transcription of the *KAIISO* gene. Computational analysis of the *KAIISO* gene transcription unit (*i.e.* 5'-upstream regulatory region, promoter, exon, intron, and 3'-UTR) was conducted by MAC vector (version 7.2.3). This assessment revealed four potential p53REs: 5'-upstream regulatory region (bp –4326 to –4227), intron 1 (bp +2449 to +2522), exon 2 coding sequence region (bp +2929 to +2959), and 3'-UTR (bp +5129 to +5151) (Fig. 2A). We next prepared pG5-(p53RE)-Luc reporter constructs with the p53REs (one copy of all four distinct *KAIISO* promoter p53REs) placed at the proximal promoter and tested which p53RE(s) is important for transcriptional activation by WT p53. In H1299 cells, ectopic p53 increased luciferase expression with p53RE1 in the *KAIISO* 5'-upstream regulatory region and p53RE3 of the exon 2 coding sequence region, by 4.4- and 1.4-fold, respectively. These data suggest that p53RE1 may be more important for transcriptional activation of *KAIISO* by p53 (Fig.

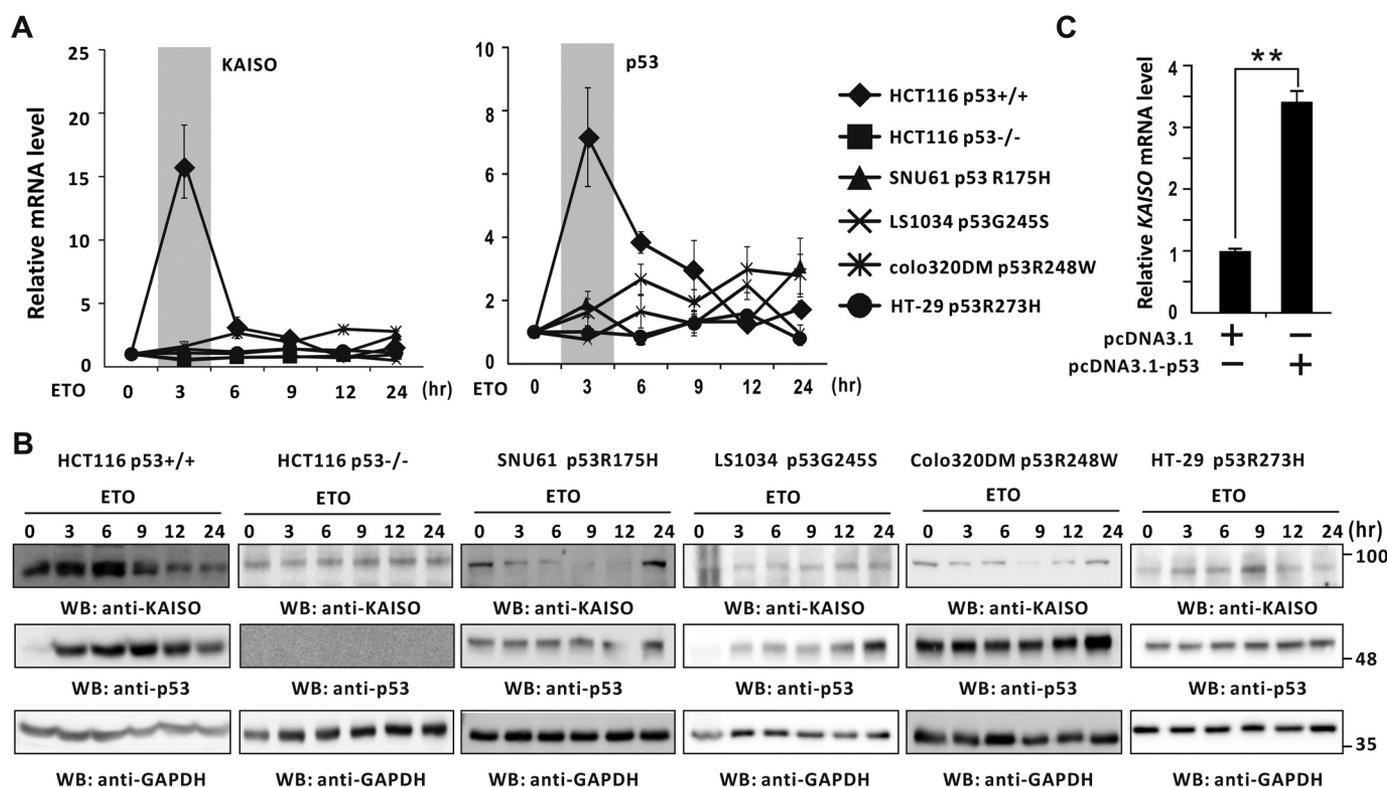


Figure 1. Induction of *KAISO* expression by DNA damage is p53-dependent. A and B, RT-qPCR and Western blot analysis (WB) of *KAISO* and p53 expression. HCT116 p53^{+/+}, HCT116 p53^{-/-}, SNU61, Colo320DM, LS1034, and HT-29 cells were treated with etoposide (60 μ M), harvested at the indicated times, and analyzed for mRNA by RT-qPCR and protein expression by Western blot analysis, respectively. GAPDH was used as control. C, RT-qPCR analysis. H1299 p53-null cells were transfected with p53 expression or control vector and analyzed for *KAISO* mRNA levels by RT-qPCR.

2B). In HCT116 p53^{+/+} cells transfected with pGL2-*KAISO*-Luc-4.6 kb construct with p53RE1, etoposide treatment increased reporter expression by 1.8-fold (Fig. 2C).

As described above, we successfully identified *KAISO* gene p53-response elements mediating transcriptional activation by WT p53 and depleted *KAISO* expression in cells expressing hot spot p53 mutants. Consequently, we next investigated whether hot spot p53 mutants could activate transcription of the reporter constructs with p53RE1 or -3 at the proximal promoter (pG5-2x(p53RE1)-Luc or pG5-4x(p53RE3)-Luc). Transient transcription assays in p53-null H1299 cells showed that WT p53 significantly increased reporter expression by acting on p53RE1 or p53RE3. However, hot spot p53 mutants could not increase reporter expression (Fig. 2D).

p53 directly binds p53RE1 and 3 of the *KAISO* gene

Because we showed that WT p53 enhances transcriptional activation of *KAISO* by acting on its p53-response elements of its 5'-upstream regulatory region and its exon 2 coding region, we investigated whether p53 actually binds to two *KAISO* p53-binding elements by oligonucleotide pulldown assays. Interestingly, p53RE of the 5'-upstream regulatory region contains three copies of the 10-bp p53-binding motif (5'-PuPuPuC(A/T)(T/A)GPyPyPy-3'). Consequently, we designed three probes, #1, #2, and #3, to cover two consensus p53 half-sites, with probe #3 containing three p53-binding half sites. Extracts from p53-null H1299 cells transfected with a p53 expression vector were incubated with the probes conjugated to agarose beads, showing that p53 bound to probes #1, #2, and #3 of p53RE1 and

probe #4 of p53RE3. Interestingly, p53 bound more strongly to probe #3 (Fig. 3A). To further test whether p53 bound to p53REs of the *KAISO* gene, *in vivo* ChIP assays showed that p53 bound to p53RE1 and p53RE3 of *KAISO* but not to its intron 1 p53RE2 (Fig. 3B).

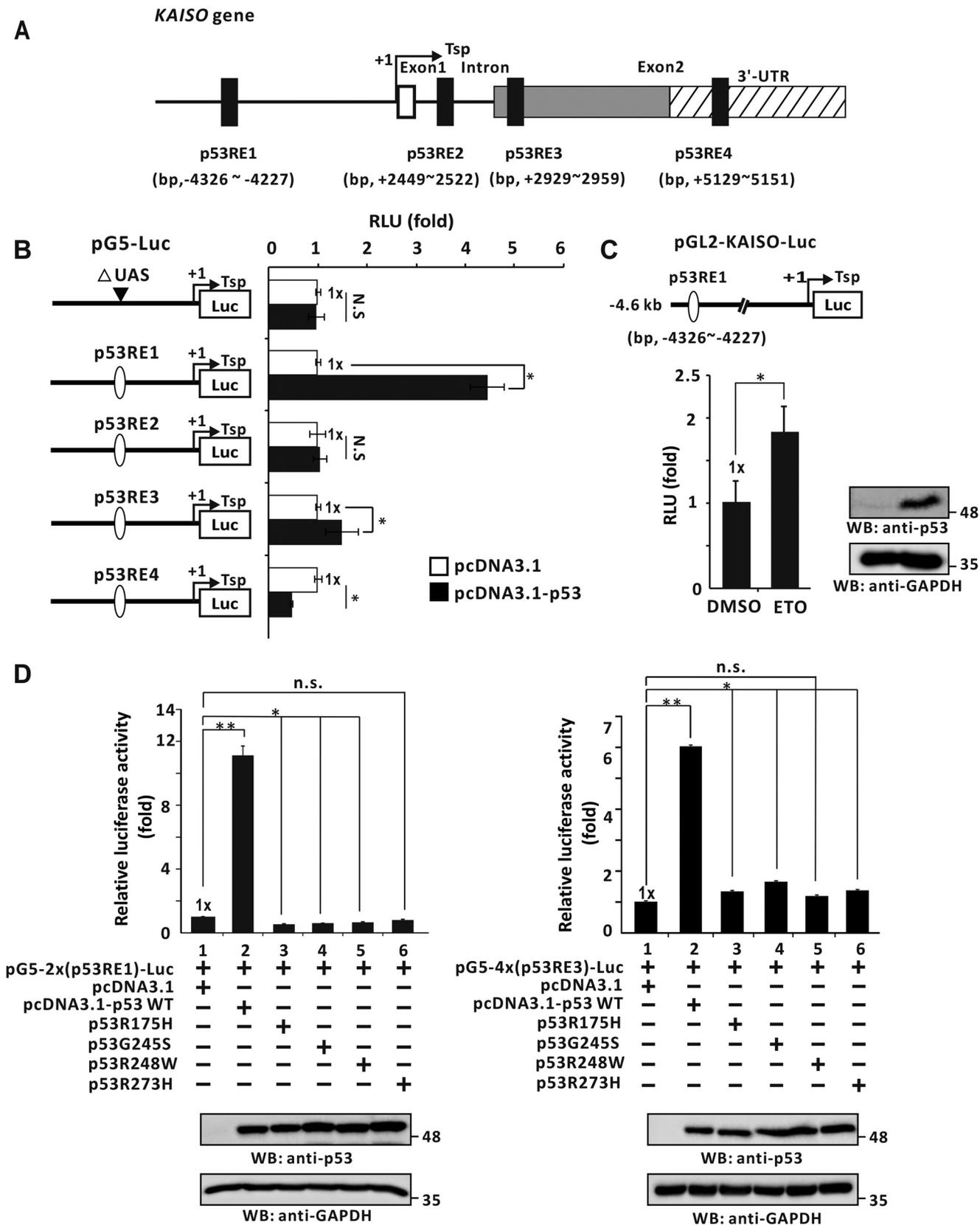
Temporal expression of mRNA and protein of *KAISO*, p53, phosphorylated p53, acetylated p53, and p53 target genes

In humans, a number of serine residues at the p53 N terminus, comprising its transactivation domain, become phosphorylated by various kinases, including ATM and ATR, during early DNA damage responses (9–16). Because *KAISO* is an early response p53 target gene induced by DNA damage and is a critical regulator of apoptosis, we suspected that phosphorylated p53 may play a role in *KAISO* transcription activation, and a particular combination of phosphorylation at serine or threonine residues of p53 may be important in specific *KAISO* promoter binding and subsequent transcription activation. First, we investigated the expression patterns of *KAISO*, p53, p53 target genes (regulating cell cycle arrest and apoptosis (*p21*, *Bax*, *Puma*, and *Noxa*)), and p53 post-transcriptional modifications (PTMs) in HCT116 p53^{+/+} cells treated with etoposide. Upon etoposide treatment, *KAISO* mRNA levels gradually increased up to 6 h, reactivated at 9 h, and peaked at 24 h. In contrast, p53 mRNA levels increased by 3 h, reactivated at 9 h, peaked at 24 h, and decreased thereafter. Moreover, expression of the p53 target genes *p21*, *BAX*, and *PUMA* increased by 3 h of etoposide treatment and increased steadily until 6 h. Among these, *p21* expression decreased after peaking at 24 h, similar to

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the expression pattern of *KAISO*. Conversely, *BAX* and *PUMA* expression levels increased by 6 or 9 h and decreased thereafter, whereas *NOXA* transcription increased by 3 h and reactivated at 9 h, resembling the expression pattern of *p21* (Fig. 4A).

We also analyzed protein expression levels of *KAISO*, p53, p53 target genes, and p53 PTMs to explore their regulatory relationships, using HCT116 p53^{+/+} cells. Upon etoposide treatment, *KAISO* expression increased steadily from 3 to 36 h,



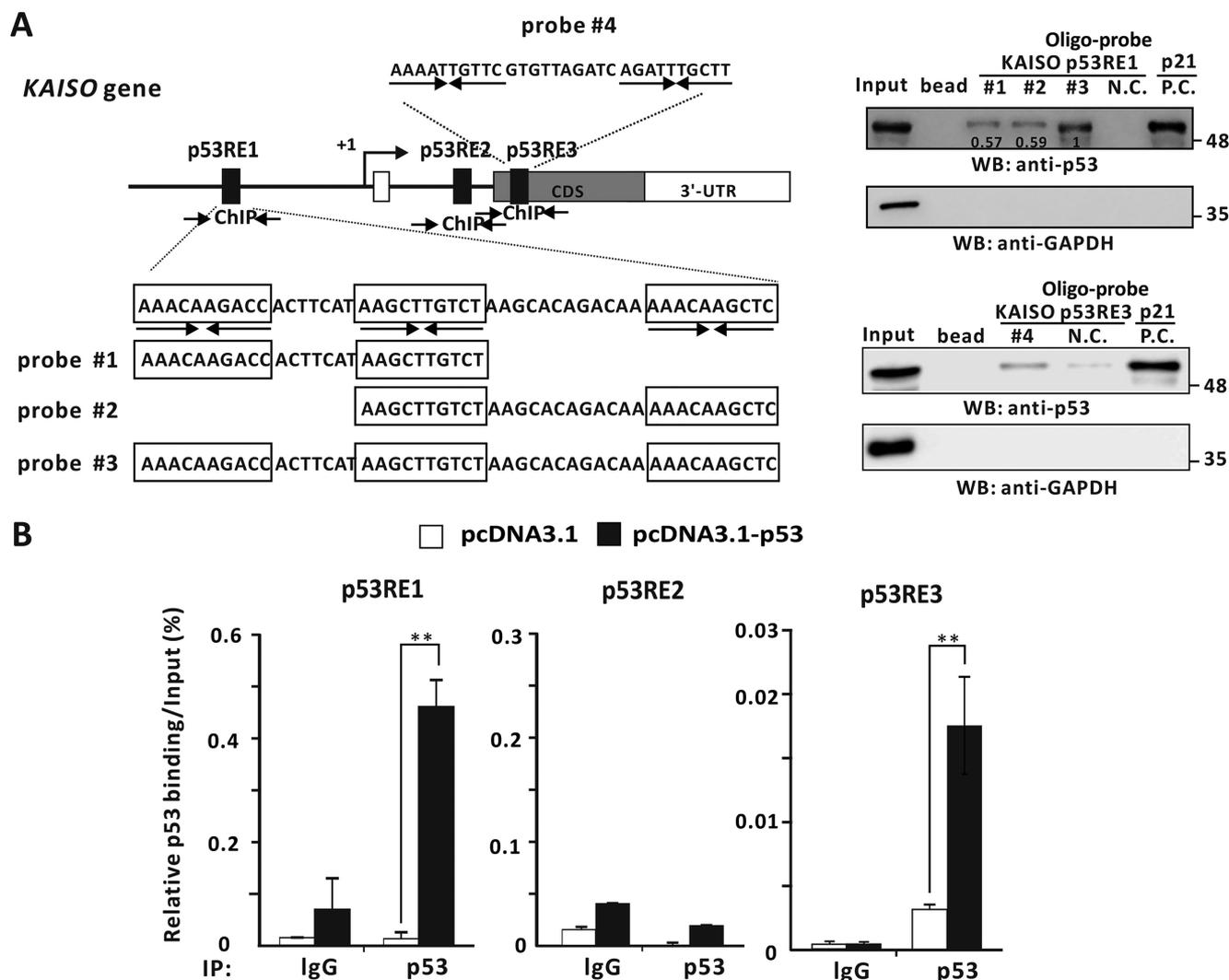


Figure 3. p53 binds to the p53REs of the 5'-upstream regulatory region and coding region of KAISO. A, structure and oligonucleotide pull-down/Western blotting assay of p53REs of the KAISO gene. Endogenous p53REs of KAISO 5'-upstream regulatory region (ChIP#1, bp -4354 to -4254), intron 1 (ChIP#2, bp +2426 to +2556), and exon-coding sequence region (ChIP#3, bp +2885 to +2982) are shown above. Arrows, locations of the ChIP oligonucleotide primer sets spanning the p53-responsive elements (p53REs). Probe #1, #2, #3, and #4 represent the p53REs used for oligonucleotide pull-down assays. Shown is an oligonucleotide pull-down/Western blotting assay of p53 binding to the p53REs of KAISO 5'-upstream regulatory region and exon 2 coding sequence region. H1299 p53-null cell extracts with ectopic p53 were incubated with biotinylated double-stranded oligonucleotides. The mixtures were further incubated with streptavidin-agarose beads and precipitated by centrifugation. The precipitate was analyzed by a Western blotting assay (WB) using antibodies against p53 or GAPDH. B, ChIP assays of p53 binding to a potential p53RE of the endogenous KAISO 5'-upstream regulatory region and coding region. H1299 p53-null cells were transfected with a p53 expression vector and immunoprecipitated (IP) with anti-p53 antibody, followed by PCR amplification of the region flanking p53RE1, p53RE2, and p53RE3. **, $p < 0.01$. Error bars, S.D.

whereas p53 expression increased from 3 to 24 h, peaking at 24 h and decreasing thereafter. Specifically, phosphorylation of p53 Ser-15 and Ser-37 increased at 3 h of etoposide treatment, and peaked at 9 or 24 h and then decreased thereafter, reminiscent of the expression pattern of p53. In contrast, acetylation of p53 showed different patterns, depending on the location of the acetylated lysine. For example, lysine 320

acetylation was low and remained relatively unchanged, whereas lysine 381 acetylation remained unchanged but decreased weakly after 36 h. Moreover, acetylation of p53 lysine 382 showed a pattern similar to that of phosphorylation of p53 Ser-15 or Ser-37. This pattern of p53 modifications may be involved in the regulation of p53 target gene expression (Fig. 4B).

Figure 2. p53 activates KAISO gene transcription by acting on the p53RE1 located in the 5'-upstream regulatory region. A, four potential p53REs identified in the KAISO gene: 5'-upstream regulatory region, intron 1, exon 2 coding sequence, and 3'-UTR. B, structure of the four pG5-Luc reporter plasmid constructs with the KAISO p53REs and transient transcription assays. The upstream activation sequence (UAS) (bp +1 to +97) was removed from pG5-Luc reporter plasmid. H1299 p53-null cells were transiently co-transfected with pG5-1x(p53RE)-Luc reporter plasmid and WT p53 expression vector. All assays were performed in triplicate. Error bars, S.D. C, HCT116 p53^{+/+} cells were transfected with pGL2-KAISO-Luc-4.6 kb containing the KAISO promoter (bp -4441 to +165). After treatment with etoposide for 24 h, luciferase activity was measured and normalized to total cellular protein. Data presented are the average of three independent assays. Error bars, S.D. *, $p < 0.05$. D, transient transcription analysis of the p53REs of the KAISO gene fusion reporter plasmid constructs. pG5-2x(p53RE1)-Luc or pG5-4x(p53RE3)-Luc and WT p53 expression vector or four hot spot p53 mutant expression vectors were transiently co-transfected in H1299 p53-null cells, and luciferase activities were measured. Luciferase activities were normalized to co-expressed β -gal activity, and data are presented as the average of three independent assays. Error bars, S.D. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant. WB, Western blotting.

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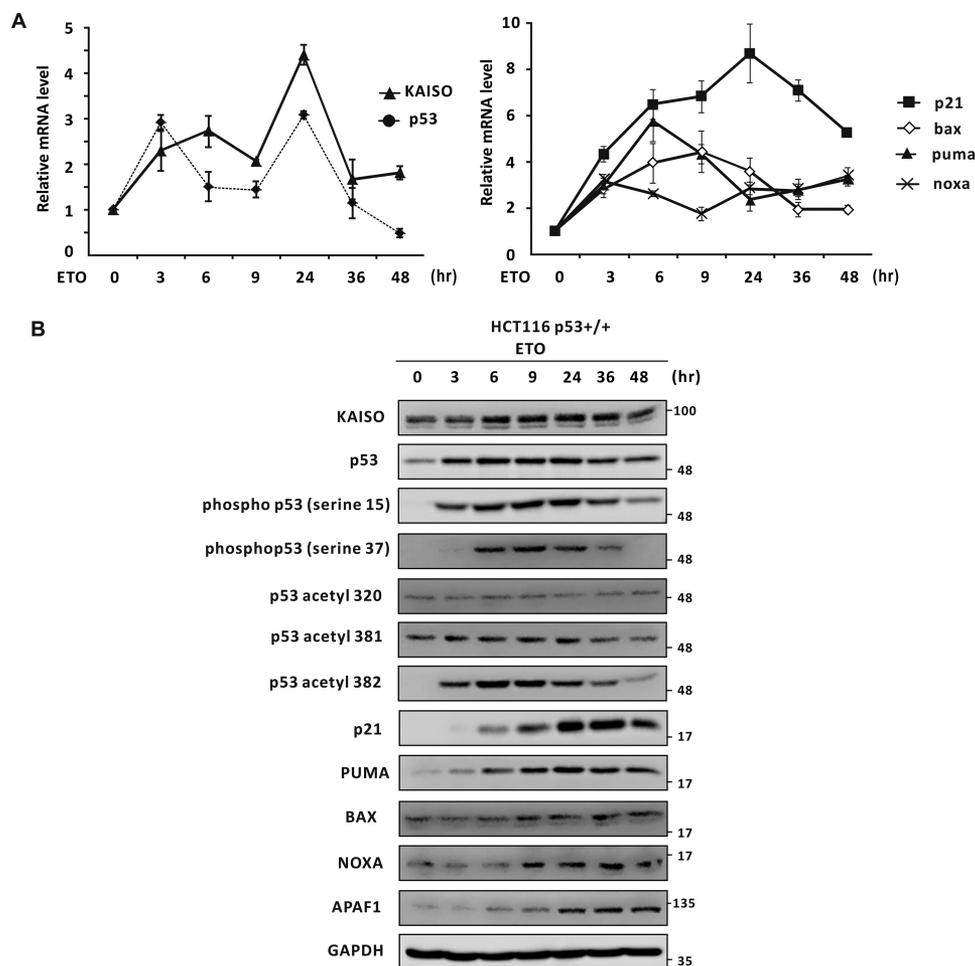


Figure 4. Temporal expression of mRNA and protein of *KAISO*, p53, p53 phosphorylated at Ser-15 or Ser-37, p53 acetylated at Lys-320, Lys-381, or Lys-382, and a p53 target gene. A and B, temporal expression of mRNA and protein of *KAISO*, p53, p53 phosphorylated at Ser-15 or Ser-37, p53 acetylated at Lys-320, Lys-381, or Lys-382, and p53 target genes in HCT116 p53^{+/+} cells treated with etoposide (ETO). The HCT116 p53^{+/+} cells treated with etoposide (60 μ M) were harvested at the indicated times and analyzed for mRNA by RT-qPCR and protein expression by Western blot analysis, respectively. GAPDH was used as control. Error bars, S.D.

It was intriguing that this pattern (“code”) of p53 Ser-15/Ser-37 phosphorylation and Lys-381/382 acetylation preceded induction of p21, PUMA, BAX, NOXA, and APAF1 (key factors regulating cell cycle arrest and apoptosis), suggesting that these particular modifications are important for expression of these genes. Overall, the protein expression patterns of *KAISO* and p53 were similar (Fig. 4B), suggesting that this p53 modification code may be important for *KAISO* expression during DNA damage responses.

p53 phosphorylated at Ser-15 and/or Ser-37 activates *KAISO* gene transcription

We next investigated whether p53 phosphorylation is important for *KAISO* transcriptional activation and which of five p53 serine residues, known to be phosphorylated upon treatment with etoposide, were critical for *KAISO* gene transcription (Fig. 5A). Consequently, we transfected WT p53 or phosphomimic p53 mutants (p53-S6D, S15D, S20D, S37D, and S46D) into p53-null H1299 cells, with or without etoposide treatment, and analyzed endogenous *KAISO* mRNA expression. Those results showed that the p53-S15D and -S37D mutants up-regulated

KAISO, similar to WT p53. However, whereas transcriptional activation of *KAISO* by WT p53, p53-S15D, and p53-S37D was slightly higher in cells treated with etoposide, the difference was statistically insignificant, suggesting that Ser-15 or Ser-37 phosphorylation is sufficient for *KAISO* transcription activation during early DNA damage responses in p53-null, etoposide-treated H1299 cells (Fig. 5B). Furthermore, we prepared additional p53 expression constructs, including nonphosphorylatable p53 mutants (p53-S15A, p53-S37A, and p53-S15A-S37A) and dual p53 phosphomimic (p53-S15D-S37D) (Fig. 5C). We then transfected these plasmid expression vectors into p53-null H1299 cells and analyzed *KAISO* gene expression without etoposide treatment. *KAISO* expression is induced by WT p53, but nonphosphorylatable p53 mutants (p53-S15A, S37A, and S15A-S37A) gave relatively weaker transcription activation than WT p53. Considering that p53-S15A, S37A, and S15A-S37A similarly activated *KAISO* transcription, single phosphorylation at either Ser-15 or Ser-37 appeared to be sufficient for transactivation (Fig. 5D). Moreover, transcriptional activation potential of phosphorylation of p53 Ser-15 was not affected by phosphorylation of Ser-37, and vice versa.

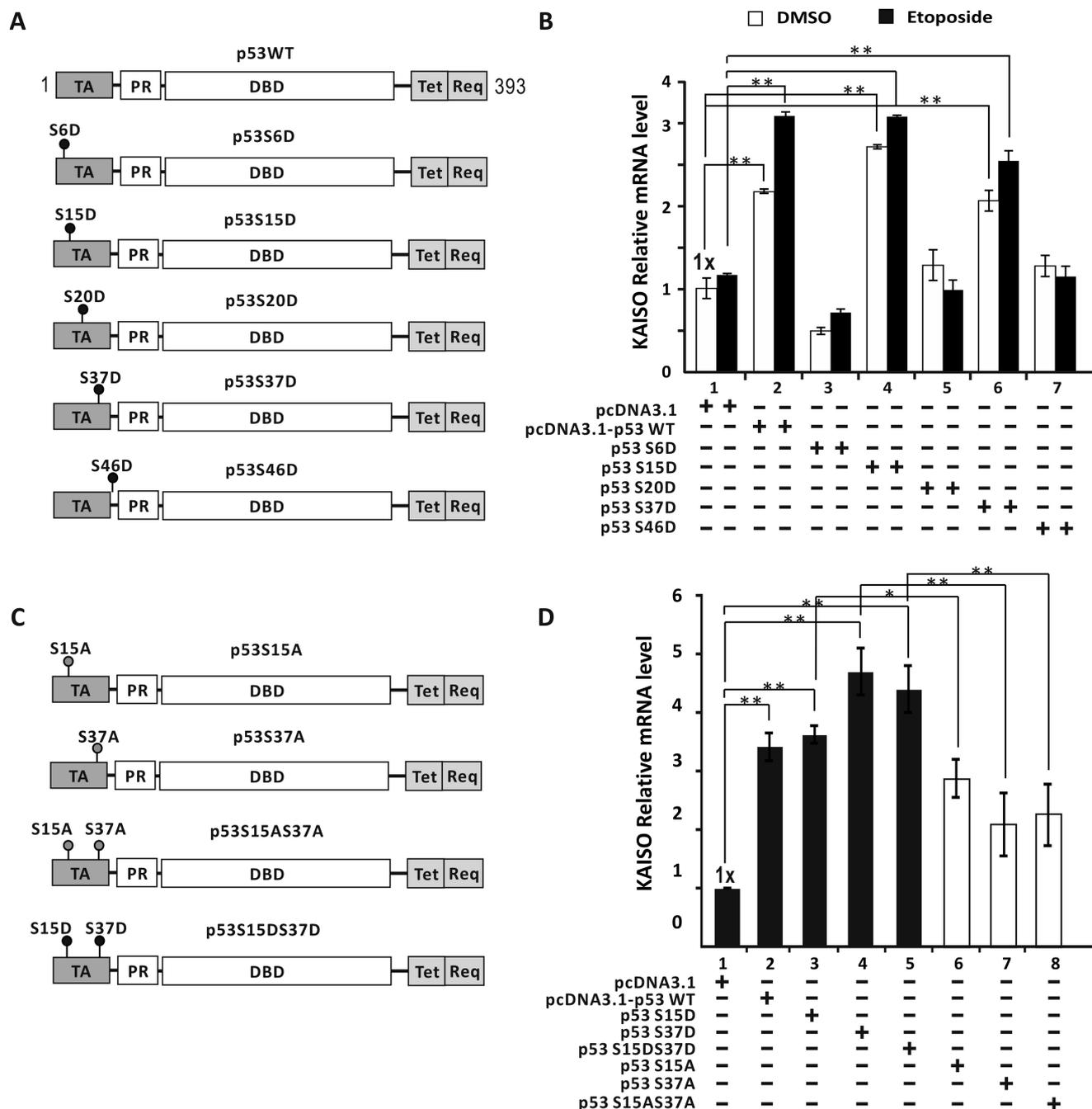


Figure 5. Phosphorylated p53 at Ser-15 and/or Ser-37 residues activates *KAISO* transcription. A, WT p53 and mimics of phosphorylated p53 at serine residues. Filled circles, phosphomimics, p53 serine residue X; TA, transactivation domain (residues 1–42); PR, proline-rich domain (residues 40–92); DBD, DNA-binding domain (residues 101–306); Tet, tetramerization domain (residues 307–355); Reg, regulatory domain (residues 356–393). B, RT-qPCR analysis of *KAISO* mRNA expression. H1299 p53-null cells were transfected with WT p53 expression or p53 phosphomimic mutant expression vector and analyzed for *KAISO* mRNA, with or without etoposide (60 μ M) treatment for 3 h. C, structures of p53, p53-S15D and/or -S37D, and p53-S15A and/or -S37A. p53-S15A/S37A is a dominant-negative form of p53-S15D/S37D. Filled circles, phosphorylation target p53 serine residue. Filled red circles, nonphosphorylatable p53 serine residue. D, RT-qPCR analysis. H1299 p53-null cells were transfected with WT p53, p53 phosphomimic mutant (Ser \rightarrow Asp), or p53 nonphosphorylatable Ser-15/37 \rightarrow Ala-15/37 mutant expression vector and analyzed for *KAISO* mRNA for 3 h. Error bars, S.D.; *, $p < 0.05$; **, $p < 0.01$.

Alternatively, we tested whether the p53 phosphomimics (p53-S15D, p53-S37D, and p53-S15D-S37D) could activate *KAISO* gene transcription. All three p53 mutants activated *KAISO* transcription similarly, suggesting that p53 Ser-15 and/or Ser-37 residues may be phosphorylated, because p53 S15D and/or S37D could up-regulate *KAISO* (Fig. 5D). As described above (Fig. 5B), simultaneous modification of Ser-15 and Ser-37 did not provide additional transcriptional activation.

Identification of unique structural features of the p53RE1 of *KAISO* involved in transcription activation by phosphorylated p53 Ser-15 and/or Ser-37

Because p53-S15D and/or -S37D could up-regulate *KAISO* mRNA expression, we tested whether *KAISO* protein expression could be rescued by p53-S15D-S37D in p53-null H1299 cells by Western blotting. We transfected H1299 cells with a

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p53 phosphomimic expression vector (p53-S15D·S37D) or a nonphosphorylatable p53 mutant (p53-S15A·S37A) and analyzed expression patterns of *KAIISO*, p53, and p53 target gene (*p21*, *PUMA*) proteins. Expression levels of the p53 mutants were comparable. *KAIISO* expression was increased by the p53 phosphomimic p53-S15D·S37D but not noticeably increased by the nonphosphorylatable p53-S15A·S37A, which only weakly induced the p53 target genes *p21* and *PUMA*. Together, these data demonstrate that functional rescue of *KAIISO* expression is only possible with WT p53 (phosphorylated p53 Ser-15 and Ser-37) in p53-null H1299 cells (Fig. 6A).

As shown in Fig. 2, WT p53 activated reporter gene transcription by acting on the *KAIISO* promoter p53REs-1 and/or -3. We also investigated how p53-phospho-S15D·S37D activates *KAIISO* transcription, showing that ectopic p53-S15D·S37D increased expression of p53RE1 and -RE3 luciferase fusion vectors by 13.6- and 3.5-fold, respectively, in p53-null H1299 cells. Transcription activation by p53-S15D·S37D was 3 times stronger than that by WT p53 (Fig. 6B).

Interestingly, unlike many other p53-response elements with two half binding elements (33), p53RE1 contains three half p53-binding elements. Consequently, we investigated whether this unique structural feature was recognized by WT p53 or the p53 dual phosphomimic p53-S15D·S37D and was functional in transcriptional regulation.

To investigate which two copies of p53 half-sites could mediate transcriptional activation, we prepared four *KAIISO* promoter-luciferase gene reporter constructs: pG5-1x(p53RE1)-Luc WT, pG5-1x(p53RE1)-Luc MT1, pG5-1x(p53RE1)-Luc MT3, and pG5-1x(p53RE1)-Luc MT1, -2, and -3 with mutations introduced at p53-binding half-sites (Fig. 6C). Transcription assays showed that ectopic WT p53 activated transcription of the pG5-1x(p53RE1)-Luc WT reporter construct by 1.8-fold. Mutations of the p53RE1 half-site at MT1 or MT3 weakly decreased p53 reporter expression, and mutation of all three p53-binding sites effectively nullified reporter expression (Fig. 6D, lanes 6–10).

Interestingly, the p53 dual phosphomimic, p53-S15D·S37D, strongly activated *KAIISO* expression by 4.7-fold, whereas the p53RE1-MT1 or MT3 mutations down-regulated *KAIISO* transcription by 50%, and mutation of all three sites completely abolished *KAIISO* transcriptional activation by p53 S15D·S37D (Fig. 6D, lanes 1–5). These results suggest that the unique structural feature of p53RE1 is functional and important for *KAIISO* transcriptional activation by p53-S15D·S37D or possibly by p53 endogenously phosphorylated during early DNA damage responses.

We next investigated whether phosphorylated p53 Ser-15 and/or Ser-37 could activate transcription of the reporter constructs with p53RE1 or -3 within the *KAIISO* proximal promoter (pG5-1x(p53RE1)-Luc or pG5-1x(p53RE3)-Luc). Transient transcription assays in p53-null H1299 cells showed that the p53 phosphomimic mutants, p53-S37D and p53 S15D·S37D, increased reporter expression by acting on p53RE1 or p53RE3, whereas nonphosphorylatable p53-S37A and p53 S15A·S37A could not activate reporter expression. However, the p53 phosphomimic, p53-S15D, increased reporter expression by acting on p53RE1 or p53RE3, whereas nonphosphorylatable

p53-S15A did not decrease reporter expression significantly. These findings indicate that phosphorylated p53-Ser-37-Ser-15 and/or Ser-37 alone are necessary for *KAIISO* gene expression via p53RE1 and/or p53RE3 (Fig. 6E).

We then wished to determine whether the p53 phosphomimic p53-S15D·S37D physically interacts with *KAIISO* p53RE1, using oligonucleotide pulldown assays. To that end, extracts from p53-null H1299 cells transfected with a p53-S15D·S37D expression vector were incubated with agarose beads conjugated to the above-described probes #1, #2, and #3 of p53RE1. The p53-S15D·S37D bound more strongly to the probe #3, in accord with the above data (Fig. 7A). We further tested whether this particular form of p53 bound to p53-binding elements in the endogenous *KAIISO* gene promoter, via ChIP. Those assays clearly showed that p53-S15D·S37D strongly bound p53RE1 and -RE3 (Fig. 7B).

We next examined whether phosphorylated p53-Ser15 or Ser-37 binds to the p53 REs of *KAIISO* and influences *KAIISO* transcription at 6 and 24 h post-etoposide treatment (coinciding with up-regulation of *p53* and *KAIISO* genes; Fig. 4A). ChIP assays, using an antibody specific to phosphorylated p53 Ser-15 or Ser-37 showed that the phosphorylated p53 bound well to p53RE1 at 6 and 24 h. Interestingly, phosphorylated p53 Ser-15 or Ser-37 bound well to p53RE3 at 6 h but decreased significantly by 24 h. These interesting binding patterns, revealed by ChIP, revealed differential DNA binding patterns of phosphorylated p53 Ser-15 and/or Ser-37, providing information on how *KAIISO* may be regulated.

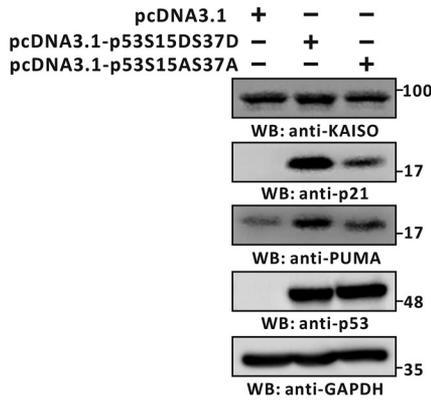
The revealed DNA-p53 binding interaction patterns matched very well with the Western blotting and RT-qPCR results. For example, p53 Ser-15 phosphorylation increased over 3–24 h, whereas p53 Ser-37 phosphorylation increased over 6–9 h and decreased by 24 h. These expression patterns were also reflected in DNA-p53 binding at p53RE1 and -RE3. Overall, our data showed that p53 RE1 is the primary element interacting with phosphorylated and acetylated p53 and that p53RE3 is only mediating transcriptional activation by phosphorylated p53 (Fig. 7C).

***p53QRQ*, a mimic of *KAIISO*-mediated acetylated p53, activates *KAIISO* transcription via p53RE1**

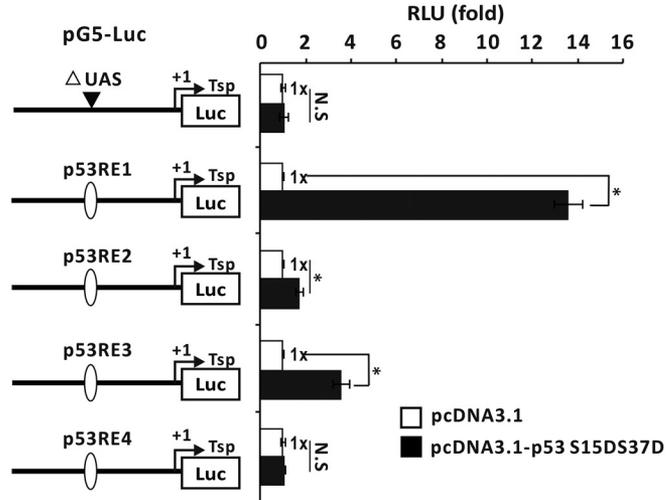
As noted above, *KAIISO* expression is induced during early DDRs and up-regulated again, at later time periods, when cells are exposed to etoposide. Moreover, a *KAIISO*-p53-p300 complex formation increases acetylation of p53 Lys-320 and Lys-381 residues but decreases acetylation of Lys-381 by p300 (5), and these events are important to increase p53 DNA binding and transcriptional activation of p53 target apoptotic genes at later time periods. Moreover, p53QRQ (K320Q, K381R, and K382Q), a p53 triacetyl mimic, represents a *KAIISO*-mediated p53 acetylation pattern or code that might regulate *KAIISO* transcription. Therefore, we tested which p53RE(s) might be important for transcriptional activation of *KAIISO* by the phosphomimic p53QRQ, which may be functionally important in later stages of DDRs.

Further assessments showed that in p53-null H1299 cells, ectopic p53QRQ increased luciferase expression of reporter constructs only via p53RE1 in the *KAIISO* 5'-upstream regulatory

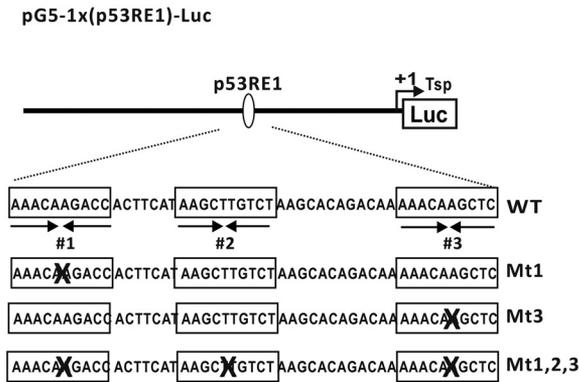
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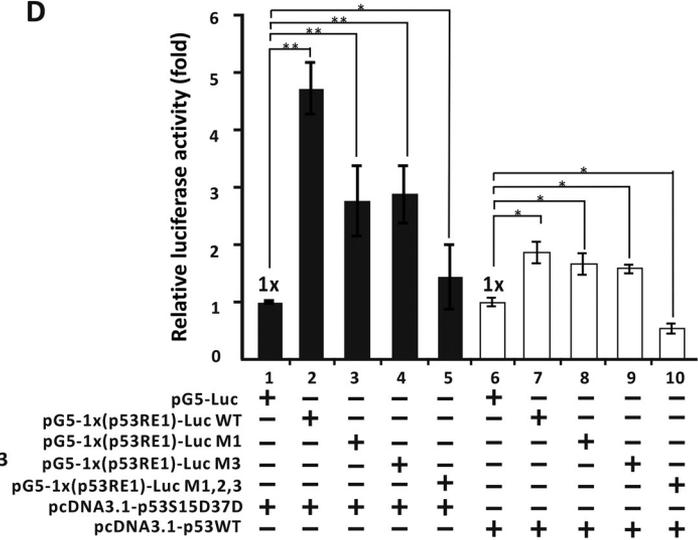
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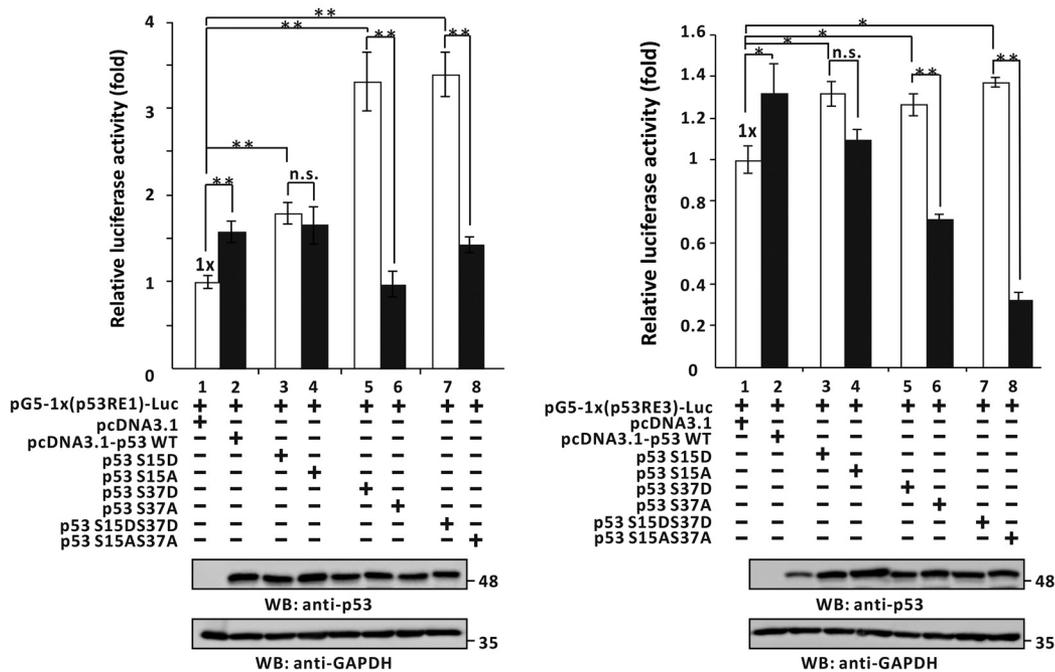
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D



E



Transcriptional regulation of *KAISO* gene

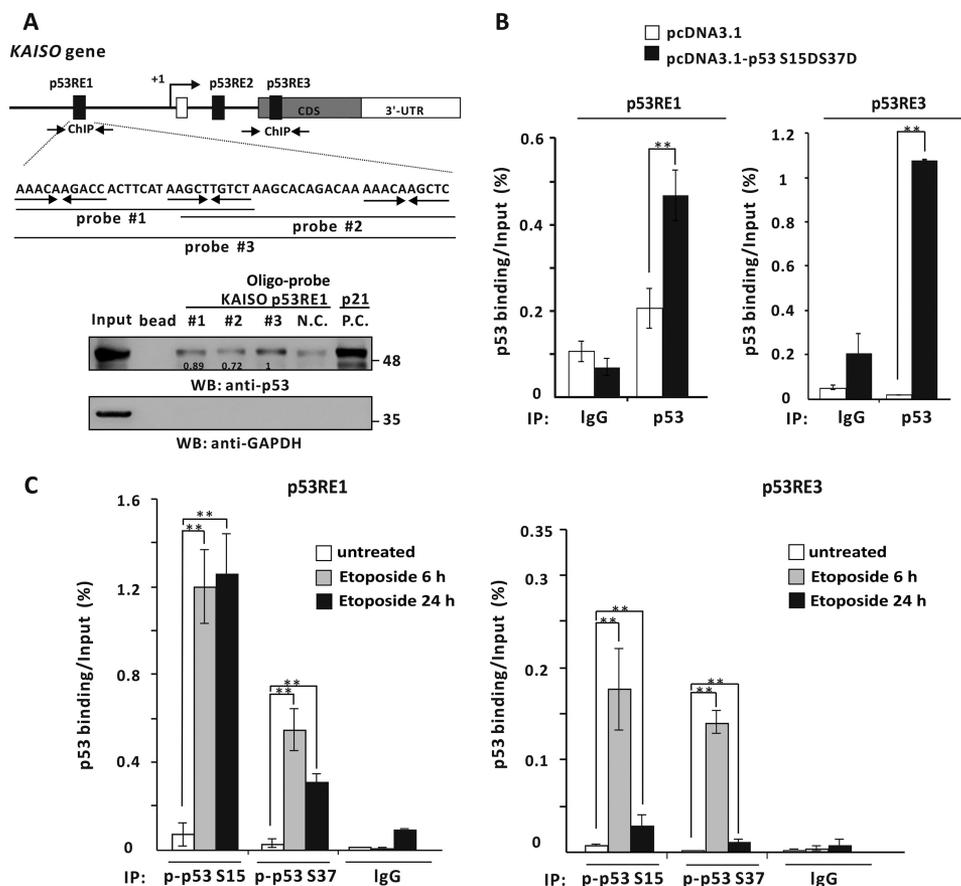
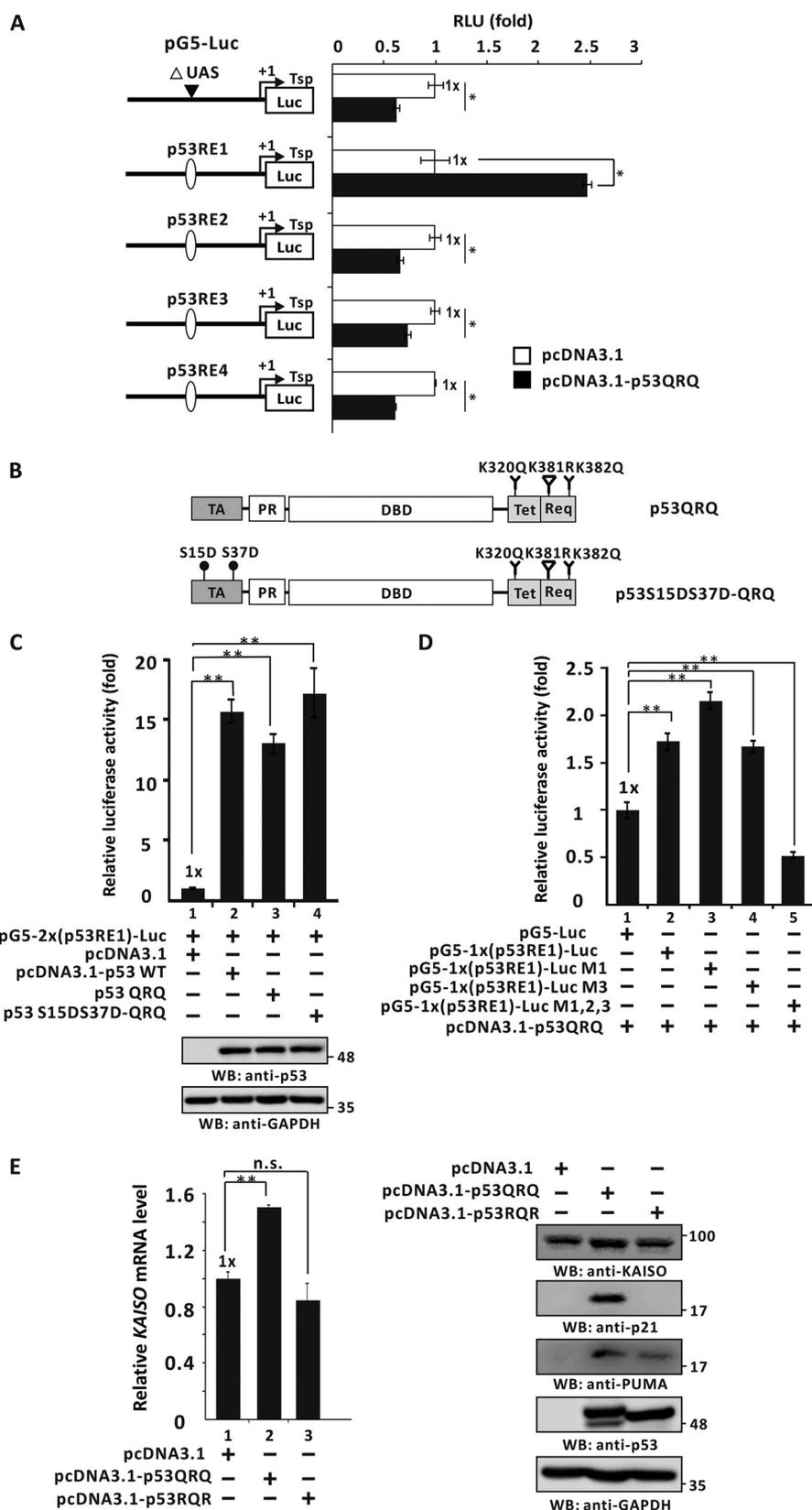


Figure 7. The endogenous phosphorylated p53 at Ser-15 or Ser-37 and p53-S15D-S37D, a mimic of phosphorylated p53 Ser-15 and Ser-37, bind to the p53RE1 and -RE3 of the *KAISO* gene. *A*, oligonucleotide pulldown/Western blotting assay of p53 binding to the p53RE1 of *KAISO*. Mimics of phosphorylated p53-S15D-S37D bind to the p53RE1 *in vitro*. Shown is the structure of p53RE1 and three probes used in oligonucleotide pulldown assays of p53 binding. H1299 p53-null cell extracts with ectopic p53-S15D-S37D expression were incubated with biotinylated double-stranded oligonucleotides, precipitated, and analyzed by a Western blotting assay (WB) using antibodies against p53 or GAPDH. *B*, ChIP assays of p53-S15D-S37D binding to the p53RE1 and -RE3 of endogenous *KAISO*. H1299 p53-null cells were transfected with a p53-S15D-S37D expression vector and immunoprecipitated (IP) with an anti-p53 antibody, followed by PCR amplification of the region flanking p53RE1 and -RE3. Shown is the average of three independent assays. Error bars, S.D. **, $p < 0.05$. *C*, ChIP assays of endogenously phosphorylated p53 Ser-15 or Ser-37 binding to p53RE1 and -RE3 of the endogenous *KAISO* gene. HCT116 p53^{+/+} cells treated with etoposide (60 μ M) were harvested at 6 or 24 h, immunoprecipitated with a specific anti-phosphorylated p53 Ser-15 or Ser-37 antibody, and amplified by PCR of the region flanking p53RE1 and -RE3. Shown is the average of three independent assays. Error bars, S.D. **, $p < 0.05$.

region and not via p53RE3 of the exon 2 coding sequence region and was clearly different from p53-S15A and/or p53-S37A (Fig. 8A). To investigate whether p53 PTMs (*i.e.* phosphorylated p53 form, p53QRQ, and potentially transient phosphorylated p53QRQ form) affect p53's transcriptional activation of *KAISO* via p53RE1, p53-null H1299 cells were transfected with the pG5-2x(p53RE1)-Luc construct and expression vectors for p53, p53QRQ, and p53-S15D-S37D-QRQ, showing that transcription of the *KAISO* reporter construct was increased by WT p53 and similarly by p53-S15D-S37D-QRQ and p53QRQ (Fig. 8, B and C).

The p53 phosphomimic p53-S15D and/or -S37D can activate early transcription of *KAISO*, a transcriptional regulator that is critical for activation of apoptotic gene expression. The actual player in apoptosis is a post-translationally modified p53 form having a particular functional code, as mimicked by p53QRQ. This particular form of p53 selectively binds apoptotic gene promoters to activate their transcription and later activates *KAISO* gene transcription to provide *KAISO* required for the generation of sufficiently PTM-coded p53 for apoptosis. Transient transcription assays showed that p53QRQ activated the *KAISO* promoter by 1.8-fold, whereas mutations of p53-

Figure 6. Identification of p53-response element and transcriptional regulation of *KAISO* by p53 and a p53-S15D-S37D, a mimic of phosphorylated p53 Ser-15 and Ser-37. *A*, Western blot analysis (WB) of *KAISO*, p53 target genes (*p21*, *PUMA*), and p53 expression of the cell extracts prepared from the H1299 p53-null cells transfected with control vector, p53-S15D-S37D, or p53-S15A-S37A. N.S., not significant. *B*, structure of the four pG5-Luc reporter plasmid constructs with the *KAISO* p53REs and transient transcription assays. H1299 p53-null cells were transiently co-transfected with pG5-1x(p53RE1)-Luc reporter plasmid and p53-S15D-S37D expression vector. All assays were performed in triplicate. Error bars, S.D. *C* and *D*, structure of the pG5-1x(p53RE1)-Luc reporter plasmid constructs with WT p53RE1 and mutations at p53RE half-sites (Mt1, Mt2, Mt1,2,3) and transient transcription assays. H1299 p53-null cells were transfected with reporter plasmids, WT p53, and a mimic of phosphorylated p53-S15D-S37D expression vector and were analyzed for luciferase activity. Reporter activities were normalized to co-expressed β -gal activity. Data presented are the average of three independent assays. Error bars, S.D. *, $p < 0.05$; **, $p < 0.01$. *E*, pG5-Luc reporter plasmid constructs with p53RE1 or p53RE3 of the *KAISO* gene and transient transcription assays. H1299 p53-null cells were transfected with reporter plasmids and WT p53 or a phosphomimic mutant p53-S15D and/or S37D or p53 nonphosphorylatable p53-S15A or S37A expression vector and were analyzed for luciferase activity. Reporter activities were normalized to co-expressed β -gal activity. Data presented are the average of three independent assays. Error bars, S.D. *, $p < 0.05$; **, $p < 0.01$.



Transcriptional regulation of *KAIISO* gene

binding half-site 1 or 3 showed little effect on transcriptional activation, although mutations of all three sites abolished transcriptional activation by p53QRQ (Fig. 8D). This mode of regulation is quite different from those of p53-S15D and/or -S37D (with regard to -fold activation and binding behavior to the p53RE1 and/or -RE3), suggesting a specific role of phosphorylated p53 during early phases of DDR transient and p53 with its *KAIISO*-mediated “acetylation code” in late phases of DDR. Furthermore, we investigated whether ectopic p53QRQ and/or p53 functionally negative forms of transient p53QRQ, such as p53RQR (K320R, K381Q, and K382R), can modulate endogenous *KAIISO* gene expression after 24 h of H1299 cell culture. Those assays showed that p53QRQ activated *KAIISO* gene expression at both the mRNA and protein levels, but p53RQR could not. Likewise, p53 target gene expression was activated by p53QRQ, but only very weakly by p53RQR, although p53QRQ and -RQR were comparably expressed (Fig. 8E).

p53QRQ binds to the p53RE1 of the KAIISO gene, and its binding is enhanced at a late stage of DNA damage responses

We also investigated whether transcriptional regulatory behaviors, like DNA binding, were different for p53QRQ. Oligonucleotide pulldown assays showed that p53QRQ binds to the p53RE1 of the *KAIISO* 5'-upstream regulatory region, and ChIP assays showed that p53 binds to *KAIISO*'s p53RE1. However, unlike the phosphomimic p53-S15D and/or -S37D, p53QRQ showed similar binding activity to all three probes (e.g. p53QRQ bound to probes #1 and #2 similarly). However, the presence of all three intact elements did not increase DNA-binding activity of p53QRQ (Fig. 9, A and B).

We next examined whether acetylated p53 Lys-382 binds to the p53REs of *KAIISO* and was involved in *KAIISO* transcription at the time points of 6 and 24 h post-etoposide treatment, times at which p53 and *KAIISO* were both activated. Interestingly, acetylated p53 lysine 382 bound well to p53RE1 at 6 h, and intriguingly, binding was increased further at 24 h. These binding patterns, revealed by ChIP, are interesting and show the temporal differential DNA-binding patterns of acetylated p53 Lys-382 (representing p53 acetylated Lys-320 and Lys-382 and nonacetylated Lys-381 or p53QRQ) (Fig. 9C).

We also investigated the effect of p53 phosphorylation on p53 acetylation and binding to p53RE1 and, thereby, *KAIISO* expression. ChIP assays showed that whereas WT p53 bound well to p53RE1, neither p53-S15A and/or pS37A nor p53RQR (two dominant negative forms of p53-S15D and/or p53-S37D and p53QRQ) bound p53RE1, suggesting again the importance

of phosphorylation or acetylation at Ser-15, Ser-37, Lys-320, Lys-381, and Lys-382 in binding to the p53RE1 during the induction of *KAIISO* in early or late DNA damage responses (Fig. 9D).

The results of our studies highlight the importance of phosphorylated p53 (particularly phosphorylation at Ser-15 and Ser-37) in the initial early-phase expression of *KAIISO* and acetylated p53 (p53 Ac-K281, -K320, and -K382) in the induction of *KAIISO* for apoptotic gene expression. Although p53RE1 located in the 5'-upstream regulatory element plays a major role, p53RE3 is also important for up-regulation of initial early-phase *KAIISO* induction, and structural features of p53RE1 are critical for promoter element recognition by phosphorylated p53 Ser-15 and/or Ser-37. Moreover, this structural feature is not recognized by p53, with *KAIISO*-mediated acetylation code for late-period *KAIISO* induction, in the DNA damage response (Fig. 10).

Discussion

KAIISO, a critical regulator of apoptosis, is induced by etoposide and inhibits cell proliferation by inducing apoptosis in p53-expressing cells (6, 7). However, little is known of how *KAIISO* transcription is regulated. Here, we demonstrate that *KAIISO* is a target gene of p53, which can activate *KAIISO* expression via p53RE1 (bp -4326 to -4227) and p53RE3 (bp +2929 to +2959) elements in colon cancer cells (Figs. 1 and 2B).

p53 is a multifunctional tumor suppressor protein and is considered the “guardian of the genome” (38). Stress-induced p53 activation involves p53 post-translational modifications on multiple sites by phosphorylation, acetylation, and other modifications (22, 40–42). Phosphorylation has been studied most intensively and has been proposed to play a critical role in the stabilization and activation of p53. We proposed that an early PTM of p53, phosphorylation at Ser-15 and Ser-37 residues, increased *KAIISO* expression during early cellular DNA damage responses. Multiple serine/threonine kinases have been implicated in p53 phosphorylation. Saito *et al.* (43) suggested that ATM mediates phosphorylation of p53 at multiple sites in response to ionizing radiation. In particular, Ser-15 phosphorylation has been shown to enhance the interaction of p53 with the transcriptional co-activators CBP and PCAF, which subsequently acetylates and activates transactivational activity by p53. ATM mainly phosphorylates Ser-15, in response to ionizing radiation and chemotherapeutic drugs, whereas ATR phosphorylates both Ser-15 and Ser-37 when cells are exposed to UV and inhibitors of replication (10, 11, 21, 44).

Figure 8. p53QRQ, a mimic of p53 with *KAIISO*-mediated apoptotic code (acetylation at 320 and 382, no acetylation at p53 Lys-381) activates the transcription of a *KAIISO* reporter via the p53RE1 of the 5'-upstream regulatory region of *KAIISO* gene. A, structure of the four pG5-Luc reporter plasmid constructs with the p53REs of the *KAIISO* gene and transient transcription assays. Identification of the p53RE critical for transcriptional activation of *KAIISO* by p53QRQ. H1299 p53-null cells transfected with pG5-Luc reporter plasmid with p53RE and p53QRQ expression vectors were analyzed for luciferase activity, normalized to co-expressed β -gal activity. Data presented are the average of three independent assays. Error bars, S.D. *, $p < 0.05$; **, $p < 0.01$. B and C, structures of p53QRQ and p53-S15D-S37D-QRQ, and transient transcription assays. Shown below are the structures of p53QRQ and p53-S15D-S37D-QRQ. Transient transcription analysis of the p53REs of *KAIISO* gene fusion reporter plasmid constructs is shown. pG5-2x(p53RE1)-Luc and WT p53 or p53QRQ or p53-S15D-S37D-QRQ expression vectors were transiently co-transfected in H1299 p53-null cells, and luciferase activities were measured. Luciferase activities were normalized to co-expressed β -gal activity, and data presented are the average of three independent assays. D, transient transcription assays. Mutations were introduced to test the function of each p53-binding half-sites in transcriptional activation of the reporter gene by p53QRQ. H1299 p53-null cells were transiently co-transfected with pG5-1x(p53RE1)-Luc reporter plasmid and p53QRQ expression vector. All assays were performed in triplicate. Error bars, S.D. E, RT-qPCR analysis and Western blot analysis (WB). H1299 p53-null cells were transfected with control vector, p53QRQ, or p53RQR expression and analyzed for *KAIISO*, p53 target genes (*p21*, *PUMA*), and p53 expression. Data presented are the average of three independent assays. Error bars, S.D.; *, $p < 0.05$; **, $p < 0.01$; n.s., not significant.

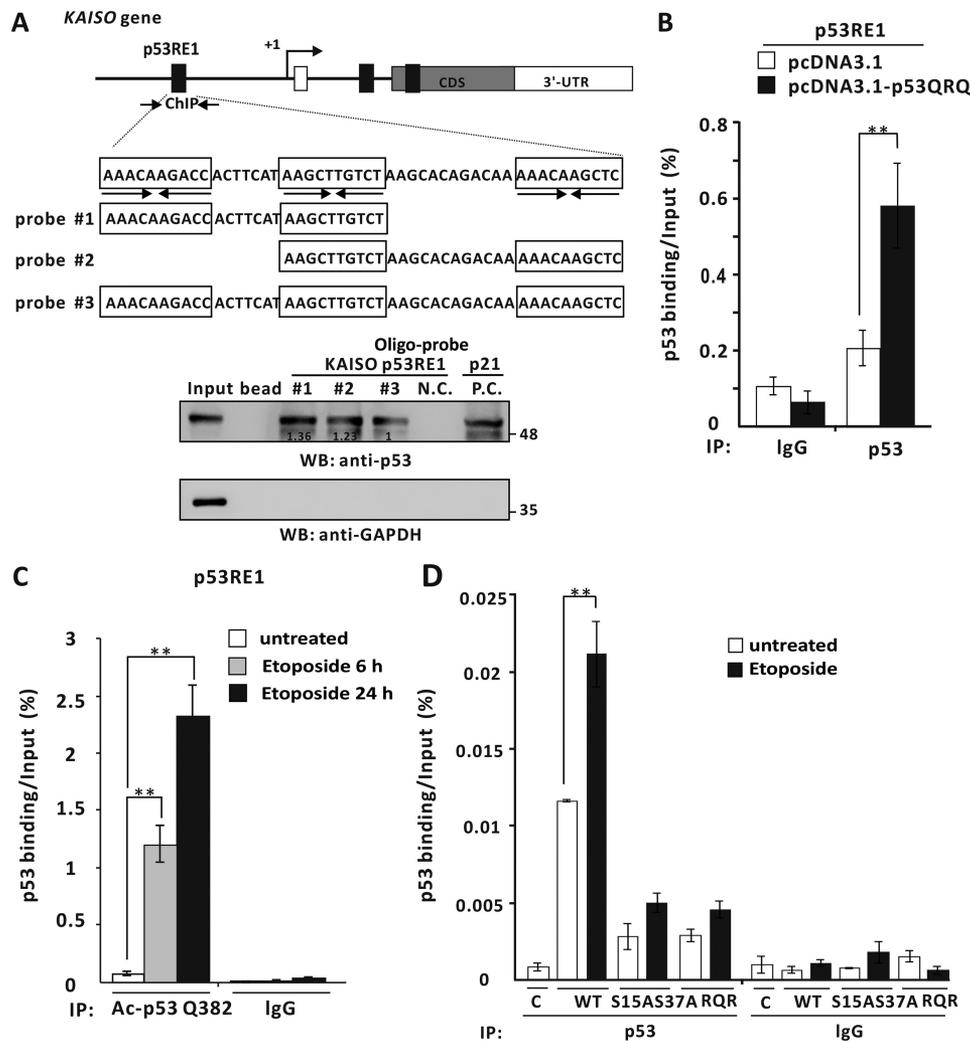


Figure 9. The mimic of a *KAISO*-mediated acetylated p53, p53QRQ, binds to the p53RE1 of the 5'-upstream regulatory region of *KAISO*. **A**, structure and oligonucleotide pulldown/Western blotting assay (WB) of p53RE1 of the *KAISO* gene. p53RE1 contains three p53-binding half-sites. The locations of oligonucleotide primer-binding sites of the ChIP assay of p53QRQ binding are indicated by arrows. Probes #1 to #3 used in oligonucleotide pulldown assays are indicated below. Oligonucleotide pulldown and Western blotting assays of p53QRQ binding to p53RE1 probes are shown. H1299 p53-null cells extracts with ectopic p53QRQ were incubated with biotinylated double-stranded oligonucleotides. The mixtures were further incubated with streptavidin-agarose beads and precipitated by centrifugation. The precipitate was analyzed by a Western blotting assay using antibodies against p53 (DO-1) or GAPDH. p53QRQ bound similarly to all three probes. 3'-UTR was used as negative control. p53RE1 of the *p21/CDKN1A* gene was positive control. **B**, ChIP assay of p53QRQ binding to the p53RE1 of the endogenous *KAISO* gene in the cells transfected with pcDNA3.1-p53QRQ. IP, immunoprecipitation. **C**, ChIP assays of endogenously acetylated p53-K382 binding to p53RE1 of endogenous *KAISO*. HCT116 p53^{+/+} cells treated with etoposide (60 μ M) were harvested at 6 or 24 h, immunoprecipitated with a specific anti-acetylated p53 Lys-382 antibody, and amplified by PCR of the region flanking p53RE1. Shown is the average of three independent assays. Error bars, S.D. **, $p < 0.05$. **D**, PTMs of p53, including phosphorylation of Ser-15 and Ser-37 and acetylation of Lys-382, PTMs critical for binding the *KAISO* p53RE1. Dominant-negative forms of phosphorylated p53 at Ser-15 and/or Ser-37 (p53-S15A-S37A) or the *KAISO*-mediated acetylated form (p53RQR) were expressed in H1299 cells treated with etoposide (60 μ M) and analyzed for p53 binding. The mutations strongly attenuated p53 binding.

Our study revealed that p53 is phosphorylated at Ser-15 and Ser-37 residues during early-phase DNA damage responses, a time point at which *KAISO* is also induced, in HCT116 cells expressing WT p53 (Fig. 4B). Our data further suggest that p53 phosphorylated at Ser-15 and Ser-37 may play a role in *KAISO* gene transcription by acting on p53RE1 and p53RE3 (Fig. 6, B–E).

p53 acetylation also plays an important role in transcriptional activation of p53 target genes. Previous studies have shown that p53 acetylation enhances its ability to bind DNA and promote transcription of downstream targets after DNA damage. p53 can be acetylated by various histone acetyltransferases (e.g. p300/CBP, Tip60, and PCAF) and deacetylated by histone deacetylases (HDAC1, -2, -5, and -6 and sirtuin

(SIRT)-1, -2, and -3), respectively (17, 45–50). Phosphorylation of p53 at Ser-15, Ser-33, and Ser-37 was reported to recruit p300/CBP and PCAF to acetylate p53 in response to DNA damage (24, 25). Moreover, Wang *et al.* (39) showed that p53 phosphorylation at Ser-15 leads to acetylation of p53 at Lys-320 and Lys-373/Lys-382 by p300 upon treatment with the DNA-hypomethylating agent 5-aza-2'-deoxycytidine. Upon treatment of the cells with etoposide, *KAISO* forms a complex with p53 and p300 to modulate acetylation at three key lysine residues of p53 by acetyltransferase p300, increasing acetylation at Lys-320 and Lys-382 while decreasing acetylation of Lys-381. Through such p53 post-translational modifications, *KAISO* up-regulates various apoptotic genes to induce cell death (6).

Transcriptional regulation of *KAISO* gene

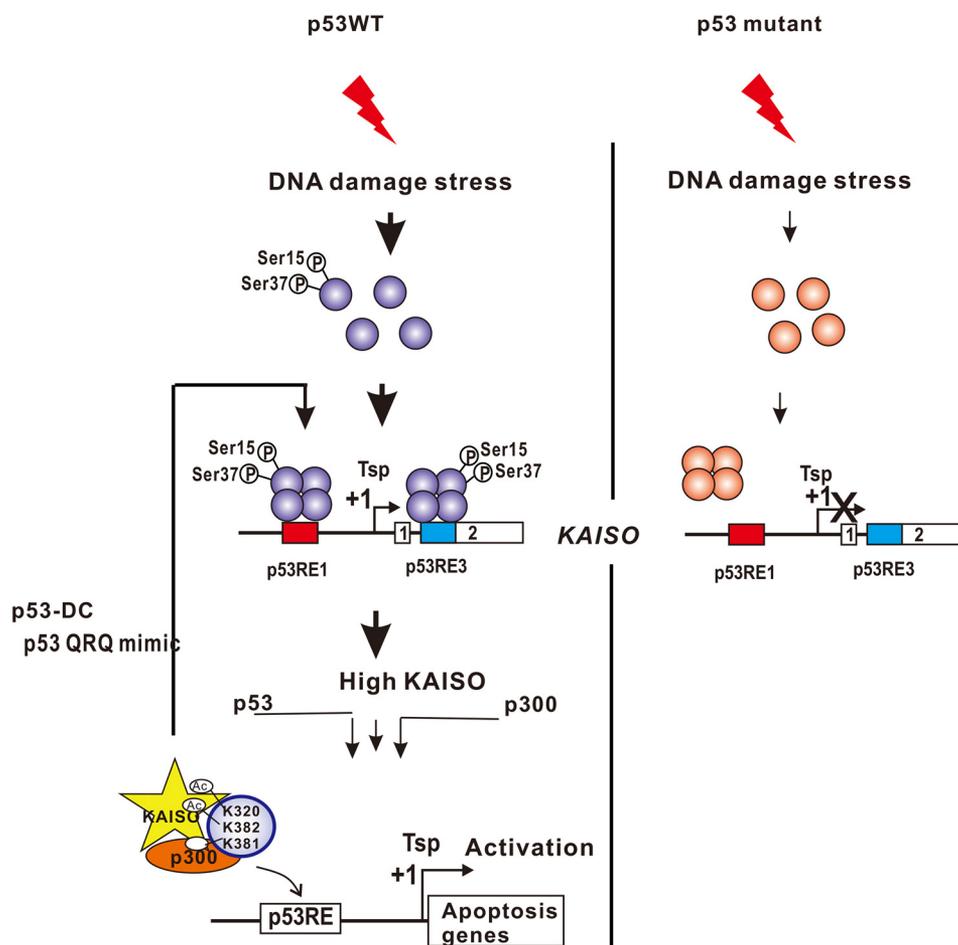


Figure 10. Hypothetical model of *KAISO* gene transcriptional activation by p53 during DNA damage responses. In cells undergoing DNA damage responses (e.g. by treatment with etoposide, cisplatin, etc.), *KAISO* is transcribed as an early DNA damage response gene, activated by p53, and robustly activated again at later periods of the DNA damage response, which is critical for apoptosis. *KAISO* gene and post-translational modification of p53, either by phosphorylation or acetylation, and *KAISO* promoter structure (particularly p53RE1 and p53RE3) play important roles. DNA-damaging stress induces phosphorylation of Ser-15 and/or Ser-37 of p53 by ATM or ATR. This particular form of p53 binds the two elements but preferentially to the p53RE1, with three unique p53-binding half-sites. The initial pulse of *KAISO* expression is critical in the generation of a *KAISO*-mediated acetylated form of p53 (Ac-K320, deacetylated Lys-381, mimicked by p53QRQ). In turn, acetylated p53 (called the p53 “death code,” p53DC) robustly activates *KAISO* gene transcription by acting on p53RE1, thus generating sufficient *KAISO* and thereby apoptotic p53 required for the induction of apoptotic gene expression.

We also observed that *KAISO* transcription is activated twice upon treatment with etoposide, both in the early phase and later phase of the DDR process. Therefore, we hypothesized that phosphorylated p53 activates transcription of *KAISO* in early DDR, and acetylated p53 activates *KAISO* in late DDR. We found that the phosphorylated p53 at Ser-15 and/or Ser-37 can activate early-phase *KAISO*, required to invoke the p53 “death code,” which is required to activate *KAISO* robustly and to induce transcription of apoptotic genes. Intriguingly, both phosphorylated and acetylated p53 showed differences in selection of p53-binding response elements (p53REs) and in the magnitude of transcriptional activation. Specifically, p53 phosphorylated at Ser-15 and/or Ser-37 selectively bound p53RE1 and -RE3 but preferred p53RE1 with the unique arrangement of three p53-binding half-sites. In contrast, acetylated p53, generated by interaction with *KAISO* and p300, is a potent transactivator of *KAISO*, acting only on p53RE1, but not particularly affected by the unique structure of p53RE1. Our study indicates that the mode of *KAISO* transcriptional activation by acetylated p53 during late DDR may be different from *KAISO* activation medi-

ated by phosphorylated p53 Ser-15 and Ser-37 in early DDR time periods.

Both phosphorylated p53 and acetylated p53 may be expressed and coexist, and they are thought to regulate different sets of genes, depending on p53 PTM codes. For example, *KAISO* expression is regulated by mixed PTMs of p53, with transcriptional activation by phosphorylated p53 as well as acetylated p53 and the *KAISO*-mediated acetylation code (i.e. p53QRQ) during DDR responses. In that event, p53QRQ continuously increases p53 target genes, such as *p21*, *PUMA*, and *BAX*, as well as *KAISO*. In HCT116 p53^{+/+} cells, *KAISO* mRNA levels peaked at 6 h and then increased again to peak at 24 h (Fig. 4A). *KAISO* can also be regulated by the different modes. As described above, our studies highlight the importance of post-translationally modified forms of p53 in the activation of *KAISO* gene transcription and apoptosis during early and late stages of genotoxic stress. Our studies also implicate that mutation in any of the p53 codons for Ser-15, Ser-37, Lys-320, Lys-381, and Lys-382 likely causes defective *KAISO* and apoptotic gene induction.

We also tested whether *KAIISO* can induce apoptosis in WT p53 or hot spot mutant-expressing cells by flow cytometry. The SNU61, Colo320DM, LS1034, and HT-29 cells expressing endogenous p53 hot spot mutant were transfected with *KAIISO* expression vector and were analyzed for apoptosis. Ectopic *KAIISO* expression in these cells did not stimulate apoptosis. In the H1299-null cells transfected with *KAIISO*, WT p53, p53-R175H, p53-G245S, p53-R248W, and p53-R273H expression vector, only the cells transfected with WT p53 and *KAIISO* showed strong apoptosis (Fig. S2), suggesting the critical roles of functional p53 and *KAIISO* apoptotic cell death.

In conclusion, we herein investigated the molecular mechanism of *KAIISO* gene transcription by post-translationally modified p53 during DNA damage responses, successfully identifying two p53-responsive DNA regulatory elements (p53RE1 and p53RE3) critical for the activation of the *KAIISO* promoter. Moreover, p53 phosphorylation at Ser-15 and/or Ser-37 and p53 with the *KAIISO*-mediated acetylation code differentially recognize p53RE1 or p53RE3. p53RE1 has unique structural features preferably recognized by phosphorylated p53-Ser-15-Ser-37, which are important in early induction of *KAIISO* gene transcription. Complex formation of p53, *KAIISO*, and p300 resulted in p53 coded with a particular acetylation pattern that only recognizes p53RE1 may contribute to *KAIISO* gene transcription during late DNA damage responses. Such findings have implications for transactivational activity by p53 and other tumor-suppressing signal pathways.

Experimental procedures

Plasmids, antibodies, and reagents

To prepare *KAIISO* promoter-Luc gene fusion reporter plasmids, the *KAIISO* promoter was PCR-amplified from genomic DNA isolated from HEK293 cells and cloned into pGL2-Luc vector to generate a reporter plasmid (pGL2-*KAIISO*-Luc-4.6 kb). The following oligonucleotide PCR primers were used for pGL2-*KAIISO*-Luc-4.6 kb plasmid. The primers were designed to amplify a 1606-bp DNA fragment (bp -4441 to -2835) and 3000-bp DNA fragment (bp -2835 to +165), respectively. The following primer pairs were used: for the 1606-bp DNA fragment region (bp -4441 to -2835), 5'-GATCGGTACCGAT-ATCCACAACTGACCTCGC-3' (forward) and 5'-GATCC-TCGAGCTCACGGTTCCAGAATATGCC-3' (reverse); for the 3000-bp DNA fragment region (bp -2835 to +165), 5'-GATCCTCGAGCAGTTTGACACTATGCATAGTT-3' (forward) and 5'-GATCAAGCTTCAAGAAGAAGCCGCACTC-CCCA-3' (reverse).

The pG5-(p53RE)-Luc reporter plasmid was prepared by cloning a p53RE double-stranded oligonucleotide into pG5-Luc. Short oligonucleotides were heated at 95 °C for 5 min and cooled slowly to room temperature. Oligonucleotides were phosphorylated at their 5'-ends by T4 polynucleotide kinase (Takara, CA) and Klenow enzyme (Roche, Mannheim, Germany) by incubating at 37 °C for 30 min. Oligonucleotide sequences of the p53REs of the *KAIISO* gene of the four pG5-Luc reporter plasmid constructs were as follows: p53RE1 (55-mer) of the 5'-upstream regulatory region, 5'-CTAGCAAACAAG-ACCACTTCATAAGCTTGTCTAAGCACAGACAAAAC-

AAGCTCC-3' (forward) and 5'-TCGAGGAGCTTGT- TGTCTGTGCTTAGACAAGCTTATGAAGTGGTCTTGT- TTG-3' (reverse); intron region p53RE2 (79-mer), 5'-CTA- GCAAACCTTGCTCAACTCAGGAGCAAGCCATGAAATT- GGACACTTGTCCAAAAGCCAACCTGTATGAACAATT- TCC-3' (forward) and 5'-TCGAGGAAATTGTTTCATACAG- GTTGCTTTTGGAAACAAGTGTCCAATTTTCATGGCTT- GCTCCTGAGTTGAGCAAGTTT-3' (reverse); p53RE3 of the exon 2 coding region (36-mer), 5'-CTAGCAAATTGTT- CGTGTTAGATCAGATTTGCTTC-3' (forward) and 5'- TCGAGAAGCAAATCTGATCTAACACGAACAATTTTG-3' (reverse); p53RE4 in 3'-UTR (28-mer), 5'-CTAGCAAATAAG- TTTTAGAGTTGTTCTC-3' (forward) and 5'-TCGAGAGA- ACAACTCTAAACTTATTTG-3' (reverse). Oligonucleotide primers were purchased from Macrogen (Seoul, Korea). Preparation of pcDNA3.1, pcDNA3.1-*KAIISO*, pcDNA3.1-p53, pcDNA3.1-p53QRQ, pcDNA3.1-p53-R175H, pcDNA3.1-p53-G245S, pcDNA3.1-p53-R248W, and pcDNA3.1-p53-R273H plasmids used was reported elsewhere (24). All plasmid constructs were verified by sequencing.

Antibodies against p53 (DO1; sc-126), GAPDH (6C5; sc-32233), p21 (H-51; sc-397), BAX (B-9; sc-7480), and NOXA (FL-54; sc-30209) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). Antibody against PUMA (NBP1-76639) was purchased from Novus Biologicals (Centennial, CO). Antibodies against p53 Ser-15 (9284S), p53 Ser-37 (9289S), p53 (9282S), and APAF1 (8969S) were purchased from Cell Signaling Technology (Boston, MA). Antibody against p53 acetyl-Lys-381 (ab61241), p53 acetyl-Lys-382 (ab75754), and *KAIISO* (ab12723) were purchased from Abcam (Cambridge, UK). Antibody against p53 acetyl-Lys-320 (02-1283) was purchased from Sigma-Aldrich. To obtain a polyclonal antibody against *KAIISO*, a white rabbit was immunized subcutaneously with recombinant HIS-*KAIISO*(Δ ZF) (amino acids 1-499) protein six times at 2-week intervals. Blood was collected, incubated at 37 °C for 90 min, and centrifuged. Following centrifugation, the supernatant was incubated with Affi-Gel 10 gel beads (Bio-Rad) cross-linked to a recombinant *KAIISO*(Δ ZF) protein. The precipitated beads were then collected and washed with PBS, and the antibody was eluted in 1 M Tris (pH 7.6). Most of the chemical reagents (etoposide, DMSO) were purchased from Sigma.

Cell cultures

HEK293A, HCT116 p53^{+/+}, HCT116 p53^{-/-}, and HT-29 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL), and H1299, SNU61, Colo320DM, and LS1034 cells were cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum (Gibco-BRL) and 1% penicillin-streptomycin. Cultures were maintained in humidified incubators at 37 °C in an atmosphere of 5% CO₂ and 95% air. SNU61, Colo320DM, and LS-1034 cells were obtained from the Korean Cell Line Bank (Seoul, Korea).

qPCR analysis of *KAIISO*, p53, and GAPDH mRNA expression in cells

Total RNA was isolated from the HCT116 p53^{+/+}, HCT116 p53^{-/-}, H1299, SNU61, Colo320DM, LS1034, and HT-29 cells using TRIzol reagent (Invitrogen). cDNAs were synthesized

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using 2 μg of total RNA, oligo(dT) primer, and Superscript reverse transcriptase II (200 units/ μl) (Invitrogen). RT-qPCR was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Reactions were subjected to quantification using the ABI PRISM 7300 RT-PCR system (Applied Biosystems). All reactions were performed in triplicate. GAPDH mRNA or 18S RNA was used as control. The oligonucleotide primers used for the qPCR assays are as follows: *KAISO*, 5'-CCGAGATTCTGCCACAAA-3' (forward) and 5'-GGGCGAGTTATTGCTAGCACTAG-3' (reverse); 18S RNA, 5'-AGTCCCTGCCCTTTGTACACA-3' (forward) and 5'-GATCCGAGGGCCTCACTAAAC-3' (reverse); p53, 5'-CCTGAGGTTGGCTCTGACTGTA-3' (forward) and 5'-AAAGCTGTTCCGTCCCAGTAGA-3' (reverse); GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse). All qPCR primers were purchased from Macrogen.

Site-directed mutagenesis

The p53 mutants were prepared by site-directed mutagenesis using the pcDNA3.1-p53 expression plasmid as a template. Conditions of PCR were as follows: denaturation step at 95 °C for 5 min, followed by 18 cycles at 95 °C for 30 s, 55 °C for 1 min, and an elongation step at 68 °C for 10 min. The oligonucleotides used for generation of the mutants were as follows: p53-R175H, 5'-GACGGAGGTTGTGAGGCACTGCCCCACC-ATGAGC-3' (forward) and p53-R175H 5'-GCTCATGGTG-GGGGCAGTGCCTCACAACCTCCGTC-3' (reverse); p53-G245S, 5'-CAGTTCCTGCATGGGCAGCATGAACCGGAGGCCA-3' (forward) and p53-G245S 5'-TGGGCCTCCGTTTCATGCTGCCATGCAGGAACTG-3' (reverse); p53-R248W, 5'-CATGGGCGGCATGAACTGGAGGCCCATCC-TCACCA-3' (forward) and 5'-TGTTGAGGATGGGCCTCCAGTTCATGCCGCCATG-3' (reverse); p53-R273H, 5'-GAACAGCTTTGAGGTGCATGTTTGTGCCTGTCCTG-3' (forward) and 5'-CAGGACAGGCACAAACATGCACCTCAAAGCTGTTTC-3' (reverse); p53-S15A, 5'-CGTCGAGCC-CCCTCTGGCTCAGGAAACATTTTCAG-3' (forward) and 5'-CTGAAAATGTTTCCTGAGCCAGAGGGGGCTCGAC-3' (reverse); p53-S15D, 5'-CGTCGAGCCCCCTCTGGAT-CAGGAAACATTTTCAG-3' (forward) and 5'-CTGAAAATGTTTCCTGATCCAGAGGGGGCTCGACG-3' (reverse); p53-S37A, 5'-CTGTCCCCCTTGCCGGCCCAAGCAATGG-ATGATT-3' (forward) and 5'-AATCATCCATTGCTTGGG-CCGGCAAGGGGGACAG-3' (reverse); p53-S37D, 5'-CTGT-CCCCCTTGCCGGACCAAGCAATGGATGATT-3' (forward) and 5'-AATCATCCATTGCTTGGTCCGGCAAGGGGGACAG-3' (reverse).

Transcriptional analysis of reporter plasmid with p53-responsive element of *KAISO* gene

Various pG5-(p53RE)s-Luc reporter plasmids and pcDNA3.1-p53, pcDNA3.1-p53-S15D, pcDNA3.1-p53-S15A, pcDNA3.1-p53-S37D, pcDNA3.1-p53-S37A, pcDNA3.1-p53-S15D-S37D, pcDNA3.1-p53-S15A-S37A, pcDNA3.1-p53QRQ (K320Q, K381R, K382Q), or pcDNA3.1-p53-S15D-S37D-QRQ expression plasmids were transiently transfected into H1299 cells using Lipofectamine Plus (Invitrogen). After 24–36 h of

culture, the cells were harvested and analyzed for luciferase activity using a Microplate LB 96V luminometer (EG&G Berthold, Gaithersburg, MD). Reporter activity was normalized to the activity of co-expressed β -gal to normalize the transfection efficiency and is shown as the average of three independent assays.

Western blot analysis of protein expression

Transfected cells were harvested and lysed in radioimmune precipitation buffer (50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.25% sodium deoxycholic acid, 150 mM NaCl, 1 mM EGTA, and complete Mini-Protease mixture). The cell lysates (50 μg) were separated using 8 or 10% SDS-polyacrylamide gels. The proteins were transferred onto Immun-BlotTM PVD membranes (Bio-Rad), and the membranes were blocked with 5% skim milk (BD Biosciences) or 3% BSA. Membrane blots were then incubated with antibodies followed by incubation with anti-mouse or rabbit secondary antibodies conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA). Protein bands were visualized with ECL solution (PerkinElmer Life Sciences).

FACS analysis

H1299, SNU61, Colo320DM, LS1034, and HT-29 cells were transfected with the *KAISO* expression vector or control vectors and washed, stained with propidium diiodide (1 $\mu\text{g}/\text{ml}$; BD Biosciences) and annexin V-FITC (1:25; BD Biosciences) in annexin V binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM CaCl_2), and incubated for 15 min at room temperature in the dark. Apoptotic cells were analyzed using a FAC-SCalibur flow cytometer (BD Biosciences) at detection wavelengths of 488 nm (excitation) and 575 nm (peak emission). The obtained data were analyzed using ModFit LT 2.0 (Verity Software House, Inc.) and Wind MDI 2.8 (Joseph Trotter, Scripps Research Institute). For FACS analysis of apoptosis, cells were transfected with the *KAISO*, WT p53, or mutant p53 expression vectors and stained using propidium iodide and an annexin V apoptosis detection kit (BD Biosciences).

Oligonucleotide pulldown assays

Cells were lysed in HKMG buffer (10 mM HEPES (pH 7.9), 100 mM KCl, 5 mM MgCl_2 , 10% glycerol, 1 mM DTT, and 0.5% Nonidet P-40). Oligonucleotide probes were heated at 95 °C for 5 min and cooled slowly to room temperature to allow annealing. The cellular extracts were incubated with 1- μg biotinylated, double-stranded oligonucleotides of various potential p53-response elements of the *KAISO* gene (p53RE#1, p53RE#2, p53RE#3, and p53RE#4) for 16 h. To collect the DNA-bound proteins, the mixtures were incubated with streptavidin-agarose beads for 4 h, washed five times with HKMG buffer, and precipitated by centrifugation. The precipitates were analyzed by Western blotting using antibodies against *KAISO* and p53, as described above. Oligonucleotide sequences were as follows: *KAISO* 5'-upstream regulatory region p53RE#1 (37-mer), 5'-AGACAAAACAAGACCCTTCATAAGCTTGTCTAAGCA-3' (forward) and 5'-TGCTTAGACAAGCTTATGAAGTGGTCTTGTCTTGTCT-3' (reverse); p53RE#2 (42-mer), 5'-TCATAAGCTTGTCTAAG CACAGACAAAACAAGCTC-

ACTGT-3' (forward) and 5'-ACAGTGAGCTTGT'TTTTGTCTGTGCTTAGACAAGCTTATGAA-3' (reverse); p53RE#3 (59-mer), 5'-AGACAAAACAAGACCACTTCATAAGCTTGTCTAAGCACAGACAAAAACAAGCTCACTGT-3' (forward) and 5'-ACAGTGAGCTTGT'TTTTGTCTGTGCTTAGACAAGCTTATGAAGTGGTCTTGT'TTTTGTCT-3' (reverse); exon 2 *KAISO*-coding region p53RE#4, 5'-GTTCTAAAATTGTTTCGTGTTAGATCAGATTTGCTTGATGA-3' (forward) and 5'-TCATCAAGCAAATCTGATCTAACACGAACAATTTTAGAAC-3' (reverse); *KAISO* 3'-UTR p53RE4 (negative control), 5'-GGCTACCACATAGTAGAGAATGGAATGAAG-3' (forward) and 5'-CTTCATTCCATTCTCTACTATGTGGTAGCC-3' (reverse); *p21* promoter p53RE (positive control), 5'-GTCAGGAACATGTCCCAACATGTTGAGCTC-3' (forward) and 5'-GAGCTCAACATGTTGGGACATGTTCCTGAC-3' (reverse).

Quantitative ChIP and qPCR analysis (ChIP-qPCR)

To test the interaction between p53 and the *KAISO* promoter elements, H1299 cells were transfected with 5 μ g of expression vector of pcDNA3.1-p53, pcDNA3.1-p53QRQ, pcDNA3.1-p53RQR, pcDNA3.1-p53-S15D-S37D, or pcDNA3.1-p53-S15A-S37A. HCT116 p53^{+/+} cells were treated with etoposide for 6 h or 24 h. Cells were fixed with 1% formaldehyde final concentration, for 20 min at room temperature, to cross-link protein–DNA complexes. Cells were harvested, washed three times in 1 \times PBS, and lysed in a 1% SDS, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA. The lysate was sonicated to shear genomic DNA into fragments ranging from 500 to 1000 bp. The sonicated supernatant was then 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 precleared with salmon sperm DNA/protein A–agarose-50% slurry for 1 h at 4 $^{\circ}$ C. Precleared supernatant was incubated with antibody overnight at 4 $^{\circ}$ C 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 h at 4 $^{\circ}$ C, with rotation, and then pelleted by centrifugation. Beads were washed three times in 1 \times PBS, including a protease inhibitor, incubated with 300 μ l of elution buffer (1% SDS, 0.1 M Na₂CO₃), and rotated for 15 min to remove excess agarose. Eluted supernatants, as well as input DNA samples, were then decross-linked by incubating at 65 $^{\circ}$ C for 4 h. The supernatant was extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol to recover DNA, and incubated with oligonucleotide primer sets designed to amplify regions flanking the p53-binding sites of the *KAISO* gene. Primer sets are as follows: p53RE#1 of *KAISO* 5'-upstream regulatory region, 5'-ATCACTGGGAGCCTGATCAAG-3' (forward) and 5'-TGGTGT-TTCTGGGTGTCACA-3' (reverse); p53RE#2 of the *KAISO* first intron region, 5'-GTTCAAATCTCTGTAACAATG-3' (forward) and 5'-AACCAAAGTTCAGCATAATTT-3' (reverse); p53RE#3 of the *KAISO* exon 2 coding region, 5'-CTGGGCAAGTTGTTGAACTG-3' (forward) and 5'-CTAA-TAACTGCCCTGATTAA-3' (reverse); p53RE#4 of the *KAISO* 3'-UTR, 5'-GTGTGTTTTGTCTTGTATATG-3' (forward) and 5'-AGTACTGACTTATTCTGACAT-3' (reverse).

Statistical analysis

Student's *t* test was used for the statistical analyses. *p* values of <0.05 were considered statistically significant.

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