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Pierce1, a Novel p53 Target Gene Contributing to the Ultraviolet-Induced DNA Damage Response

Young Hoon Sung¹, Hye Jin Kim¹, Sushil Devkota¹, Jusik Roh¹, Jaehoon Lee^{1,2}, Kunsoo Rhee³, Young Yil Bahk^{1,4}, and Han-Woong Lee¹

Abstract

Retinoblastoma (Rb) and *p53* genes are mutated or inactivated in most human cancers and mutually regulate each other. Recently, we reported that expression of diverse genes was altered in *Rb*-deficient mouse embryonic fibroblasts (MEF). In this study, we found that *Pierce1*, a novel transcript upregulated in *Rb*-deficient MEFs, is a transcriptional target of p53. Although *Pierce1* promoter did not respond to the ectopic expression of E2F1, it was strongly activated by p53 via 2 cis-elements. Consistently, the expression of *Pierce1* was induced by genotoxic stresses that activate p53 but was not detected in *p53*-deficient MEFs. *Pierce1* was posttranslationally stabilized by ultraviolet C (UVC) irradiation, and UVC-activated ATR (ataxia telangiectasia-mutated and Rad3-related) signaling suppressed proteosomal degradation of *Pierce1* protein. Furthermore, knockdown of *Pierce1* compromised the checkpoint response of wild-type MEFs to UVC irradiation, accompanying the diminished expression of p53 target genes. Together, our data suggest that *Pierce1* is an important p53 target gene contributing to normal DNA damage response and may play crucial roles in maintaining genomic integrity against genotoxic stresses, including UVC irradiation. *Cancer Res*; 70(24); 10454–63. ©2010 AACR.

Introduction

The retinoblastoma protein (pRb) is an important tumor suppressor that is regulated by p16^{INK4a}, p14^{ARF}, p21^{WAF1}, and p53, which are the core components of the signaling network central to tumor suppression (1). The INK4a/ARF locus, which is one of the most frequently mutated loci in human cancers, encodes both p14^{ARF} (p19^{Arf} in mice) and p16^{INK4a} proteins (2). p16^{INK4a} directly inhibits the cyclin D-dependent kinases CDK4 and CDK6, thereby maintaining pRb in its hypophosphorylated, antiproliferative state (3). On the other hand, p19^{Arf} inhibits the action of an E3 ubiquitin ligase, Mdm2, and thus stabilizes p53 (4, 5). Because p19^{Arf} is upregulated in retinoblastoma (*Rb*)-deficient mouse embryonic fibroblasts (MEF) and is also a direct transcriptional target of the E2F transcription factor (6, 7), dysregulation

of E2Fs by *Rb* deficiency contributes to p53 activation (8–10).

As a guardian of the genome, p53 is an essential tumor suppressor gene responsible for maintaining the integrity of the genome (11) and is mutated or lost in approximately 50% of all human cancers (12). Genetic deletion of p53 equivalently predisposes mice to various types of cancers (13). In addition, the incidence of tumors in p53 heterozygous knockout mice is higher than that in wild-type mice, with a frequent p53 loss of heterozygosity (14). p53 induces expression of numerous downstream target genes in response to genotoxic, oxidative, and oncogenic stresses. Most mutations in p53 gene are found in the DNA binding region, indicating that transcriptional activity is critical for its tumor suppressor function (15). The consensus sequence of a p53-responsive element is composed of 2 copies of a 10-bp degenerate sequence, 5'-RRRC-WWGY-3', with or without a nonspecific spacer between them (16). p53 binds to this consensus sequence as a tetramer (17) and modulates the expression of genes necessary for various physiologic processes (18).

The DNA damage response is a signal transduction event evoked by genotoxic and endogenous replication stresses, which monitors and ensures the genomic integrity and thus acts as a critical barrier to tumorigenesis (19). p53 is activated as an essential effector by preceding signal transducers of the DNA damage response (19), which is exclusively elicited by 2 phosphoinositide 3-kinase-related protein kinases—ATM (ataxia telangiectasia-mutated) and ATR (ATM and Rad3-related; ref. 20). ATR is highly homologous to ATM; however, its activation and target specificity are divergent from those of ATM in response to different forms of genotoxic stress (21). It

Authors' Affiliations: ¹Department of Biochemistry, College of Life Science and Biotechnology, and Laboratory Animal Research Center, Yonsei University, Seoul, Korea; ²Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon, Korea; ³Department of Biological Sciences and Research Center for Functional Cellulomics, College of Natural Sciences, Seoul National University, Seoul, Korea; and ⁴Department of Integrated OMICS for Biomedical Sciences, Graduate School, Yonsei University, Seoul, Korea

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Corresponding Author: Han-Woong Lee, Department of Biochemistry, College of Life Science and Biotechnology, and Laboratory Animal Research Center, Yonsei University, Seoul 120-749, Korea. Phone: 82-2-2123-5698; Fax: 82-2-2123-8107. E-mail: hwl@yonsei.ac.kr

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responds to rarely occurring double-strand breaks (DSBs), whereas ATR plays important roles in a wide range of DNA damage responses induced both by endogenous replication stresses in proliferating cells and by exogenous genotoxic stresses such as ultraviolet (UV)-induced DNA damages and DSBs (20). Both ATR and ATM activate DNA damage responses by directly and indirectly phosphorylating downstream targets, including p53 (20).

To date, the physiologic significance and molecular mechanisms of p53 action in the DNA damage response, as well as its abnormalities in various neoplastic diseases, have not been completely elucidated. Recently, we identified a novel transcript, RbEst47, that was upregulated in Rb-deficient MEFs (22). Interestingly, this gene was found to be transcriptionally activated by p53 but not by E2F and thus was designated as p53-induced expression 1 in Rb^{-/-} cells (Pierce1). Pierce1 (Entrez Gene ID: 69327) is located on mouse chromosome 2, which is syntenic with human chromosome 9q34.3. In this study, we confirmed that Pierce1 is indeed a novel p53 target gene and evaluated its function in p53-mediated DNA damage responses. Our data suggest that Pierce1 is important for the physiologic response against UVC irradiation and could be crucial to maintain genomic integrity upon UVC irradiation.

Materials and Methods

Mouse embryo fibroblasts

MEFs were prepared and cultured as previously described (22). E2F1 (23), Rb (24), and p53 (14) knockout mice were purchased from Jackson Laboratory, and p21^{Waf1} knockout mice (25) were a generous gift from Dr. P. Leder (Harvard Medical School). All mice were managed as described previously (22). Genotypes were determined using 3-primer PCR reactions, and detailed genotyping protocols will be provided upon request.

Reporter gene constructs and luciferase assays

Reporter genes were constructed by subcloning the genomic DNA fragment containing Pierce1 promoter region and a part of its exon 1 from the 129SvJ strain into pGL3 Basic vector (Promega). p53-responsive elements were predicted using TFBIND software (26) and by directly comparing DNA sequences with the consensus sequence (16). PCR mutagenesis was conducted using mutant primers (5'-CAGGACGCAT-TACCGCAGCAGGCACAGAATTAATCTGGGC-3' for BS1; 5'-ccatGGCAGATACgtaaacgacagacttGTAACCTCTCaggc-3' for BS3) and was verified by sequencing. Reporter genes and effector constructs including pcDNA3-p53, pCMV-E2F1, pcDNA3-p21^{Waf1}, pcDNA3-p16^{Ink4a}, and pcDNA3-p19^{Arf} were transfected into NIH3T3 (22) and H1299 cells (27) with Lipofectamine PLUS reagent (Invitrogen). Luciferase assays were conducted as previously described (27).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were conducted using a p53-specific antibody (sc-6243x) and protocols from Santa Cruz Biotechnology. PCR amplification was carried out using the following primers: 5'-CTAGCAAAATAGGCTGTC-CC-3' (pGL3-specific) and 5'-tagccaccagcctctctat-3' (p21^{WAF1}-

specific) for internal control; 5'-CTAGCCAA-AGCAACCTACAA-3' (Pierce1-specific) and 5'-CTTTATGTTTTTGGCGTCTTCCA-3' (pGL3-specific) for BS1; 5'-CTAGCAAAATAGGCTGTCCC-3' (pGL3-specific) and 5'-GCAGATCCTGGACGAATGAA-3' (Pierce1-specific, primary PCR) or 5'-TGGGATTTCCACGAC-CAATA-3' (Pierce1-specific, nested PCR) for BS3. 5'-CGTCCAG-GATCTGCCTCCTA-3' and 5'-AGAATCTTCCCAGGGCGGAA-3' for endogenous mouse Pierce1 promoter flanked by BS1 and BS3; 5'-CCAGAGGATACCTTGCAAGGC-3' and 5'-TCTCTGTC-TCCATTCATGCTCCTCC-3' for endogenous mouse p21^{Waf1} promoter.

RNA isolation, Northern blot analysis, and real-time quantitative reverse transcription-PCR

Northern blot analysis was conducted as described previously (22). MEFs were treated for 12 hours with etoposide (Sigma), cisplatin (Sigma), adriamycin (Sigma), or γ -ray irradiation (¹³⁷Cs). For UVC irradiation, UV cross-linker (Stratagene) or germicidal UV lamp of a clean bench was used. UVC doses were determined using UVX Radiometer with UVX-25 detector (Ultra-Violet Products). cDNA was synthesized using SuperScript III System (Invitrogen), and quantitative PCR was conducted using SYBR Green (Bio-Rad) and IQ5 Multicolor real-time PCR Detection System (Bio-Rad). Experiments were conducted in triplicate and signals were normalized to mouse Gapdh and human actin (primers from Origene). All other primers are listed in Supplementary Table S1.

Pierce1 expression constructs and stable cell lines

A mouse cDNA encoding full-length Pierce1 protein (22) was subcloned in-frame into pcDNA3-HA (Invitrogen) for N-terminal tagging (HA-Pierce1). To produce retroviruses, HA-Pierce1 cDNA was subcloned into the pLPCX (Clontech). Ecotropic retroviruses were produced and used to transduce NIH3T3 cells as described previously (28). Transduced cells were pooled after puromycin selection to avoid variability between clones.

Pierce1-specific antibody

The GST-Pierce1 fusion protein was produced and purified using pGEX4T1 according to the manufacturer's instruction (Amersham). Antisera were obtained using rabbits as described previously (29). Pierce1-specific antibody was affinity purified with a GST-Pierce1-transferred nitrocellulose membrane strip and eluted with 100 mmol/L of glycine, pH 2.5. Alternatively, ImmunoPure IgG Purification Kit (Pierce) was used according to the manufacturer's instructions.

Western blot analysis

Western blot analyses were conducted using antibodies against Pierce1, HA (Santa Cruz), p53 (Santa Cruz), phospho-p53 (Ser-15; Cell Signaling), Apaf1 (Alpha Diagnostic Intl.), Pten (Cell Signaling), and Gapdh (Santa Cruz) as described previously (22).

BrdU incorporation assays

MEFs were incubated in culture media containing 10 μ mol/L of 5-bromo-2'-deoxyuridine (BrdU; Sigma) for 2 hours prior

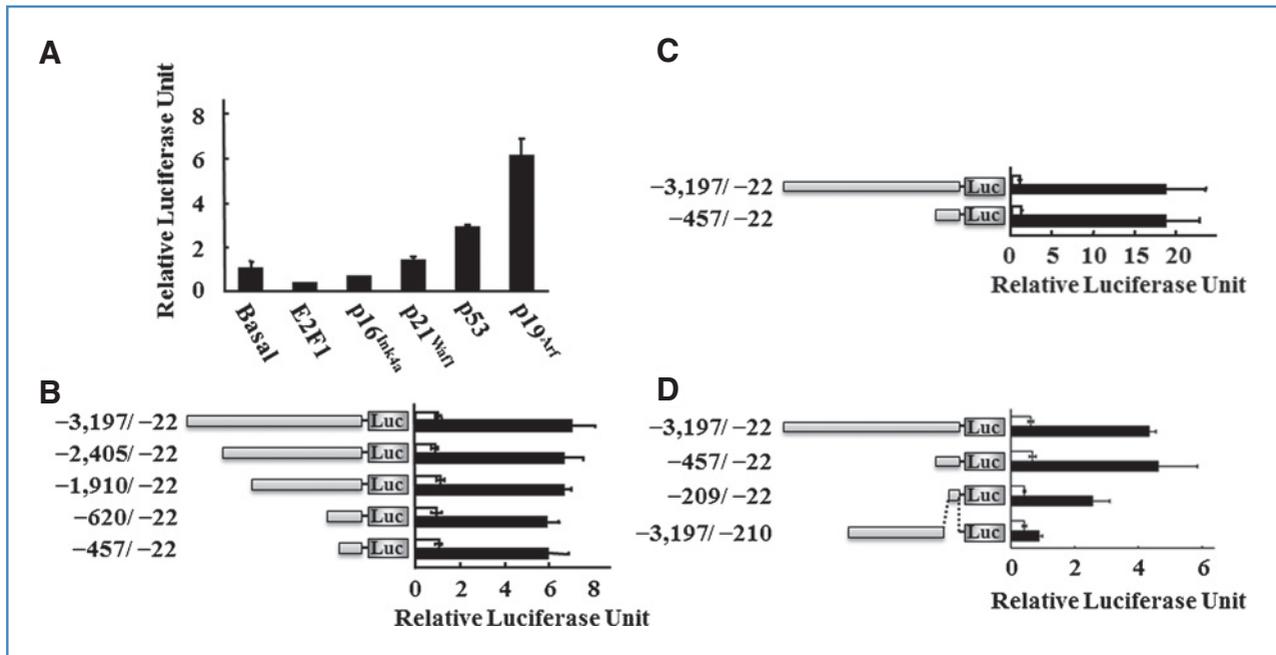


Figure 1. Reporter gene assays showing p53-dependent activation of the Pierce1 promoter. A, luciferase reporter gene was constructed using the upstream region (–3,197 to –22) of the mouse Pierce1 locus, which contains the full-length promoter region. Constructs expressing E2F1, p16^{Ink4a}, p19^{Arf}, p21^{Waf1}, or p53 were cotransfected with the reporter gene construct into NIH3T3 cells. B and C, serially deleted luciferase reporters were constructed, and their p53-dependent activity was measured in NIH3T3 cells containing wild-type p53 (B) and in p53-deficient H1299 cells (C). D, proximal promoter region was further deleted, and the p53-dependent promoter activation was evaluated in NIH3T3 cells. B to D, open bars: empty vector; solid bars: p53 expression construct.

to harvest. Cells were fixed and stained using fluorescein-conjugated anti-BrdU antibody according to the manufacturer's instructions (PharMingen). Cell populations were analyzed using a FacsCalibur (BD Biosciences).

Clonogenic assays

Stable cells conditionally overexpressing HA-Pierce1 were plated into 6-well plates (1×10^3 cells/well). After cells attached, the medium was replaced with the fresh medium with or without doxycycline. Cells were irradiated with indicated doses of UVC 12 hours after media change ($n = 6$). The medium was changed every other day for 12 days, and formalin-fixed cells were stained with crystal violet (Sigma). After photography with Gel Documentation System (Bio-Rad), the clonal growth was densitometrically analyzed using Quantity One software (Bio-Rad).

Results

Pierce1 is directly regulated by p53

pRb directly inhibits E2Fs and is indirectly regulated by CDK inhibitors p16^{Ink4a} and p21^{Waf1} (1). Because Pierce1 was discovered as an upregulated transcript in Rb^{-/-} MEFs (22), the effect of pRb pathway on its promoter activity was evaluated in Ink4a/Arf-deficient NIH3T3 cells by overexpressing E2F1, p16^{Ink4a}, p19^{Arf}, p21^{Waf1}, and p53 (Fig. 1A). When a luciferase reporter gene containing Pierce1 promoter and a

part of its exon 1 (–3,197 to –22) was cotransfected with constructs overexpressing E2F1, p16^{Ink4a}, p19^{Arf}, p21^{Waf1}, and p53 into Ink4a/Arf-deficient NIH3T3 cells, p53 and its activator p19^{Arf} induced the reporter gene activity more than 2.5- and 6-fold, respectively, whereas E2F1, p16^{Ink4a}, or p21^{Waf1} did not (Fig. 1A). These results indicate that the Pierce1 promoter can be directly regulated by p53 but not by pRb and E2F1.

To define the p53-responsive region, we analyzed p53-dependent activation of serially deleted Pierce1 promoters (Fig. 1B and C). No significant difference was observed by deleting from –3,197 to –458 (Fig. 1B). In addition, both basal activity and p53 responsiveness of the proximal region (–457 to –22) were similar to those of the full-length construct in both p53-proficient NIH3T3 cells and p53-deficient H1299 cells (Fig. 1B and C, respectively). Further deletions showed that this region is essential for p53 to fully activate the Pierce1 promoter and also revealed that at least 2 p53-responsive elements exist in this region (Fig. 1D). Its sequence analysis revealed 3 putative p53-responsive elements (BS1–3) highly homologous to p53 consensus sequence (75%–90%; Fig. 2A). Consistent with Figure 1D, even though BS2 and BS3 deleted, the reporter construct containing only BS1 was significantly activated by p53 (Fig. 2B). This result was confirmed by site-directed mutagenesis of BS1 (Fig. 2B). Similar to Figure 1D, BS1 deletion considerably attenuated its p53 responsiveness, which made it frequently difficult to distinguish its p53-induced reporter activity. However, increasing doses of p53

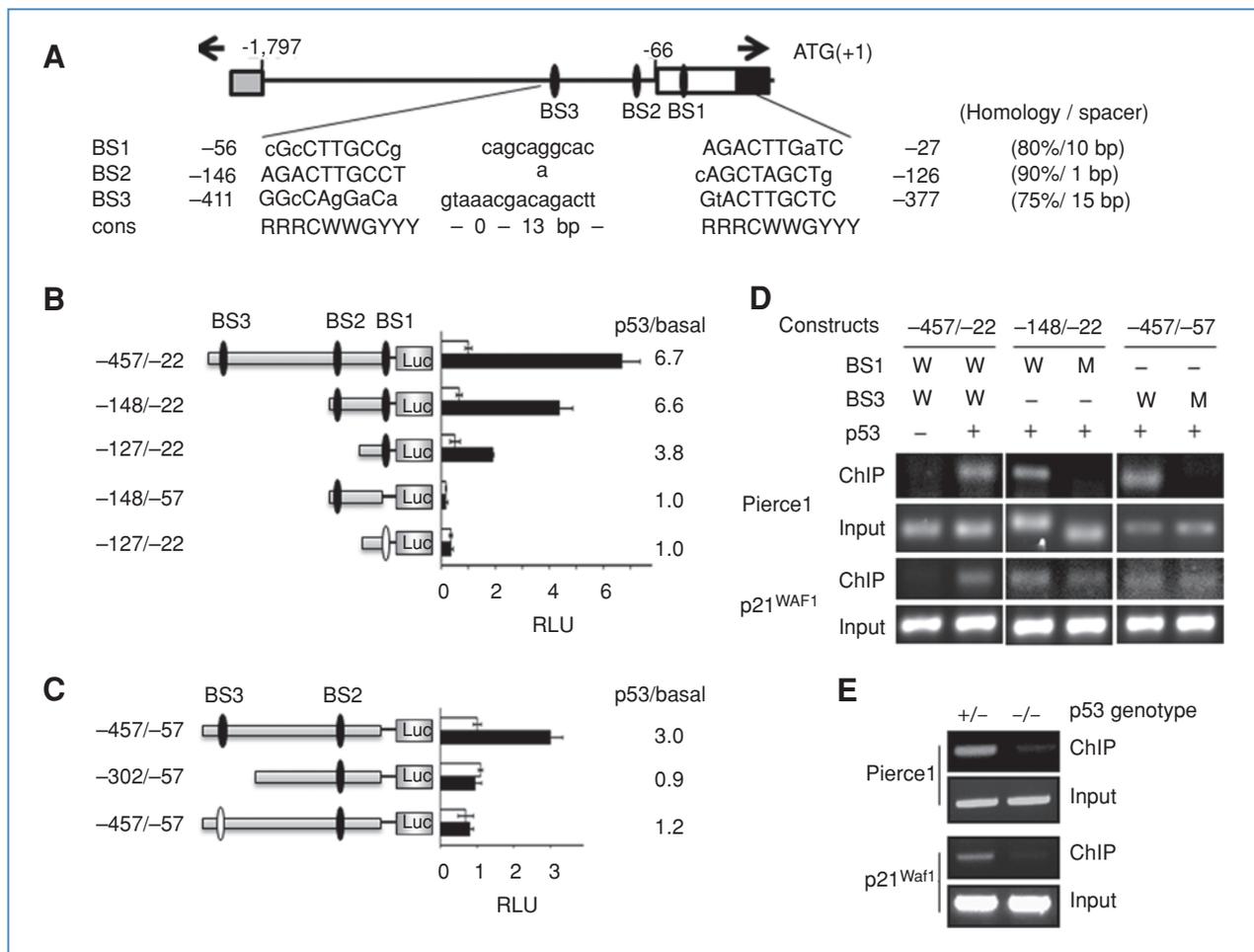


Figure 2. Direct activation of the Pierce1 promoter by p53. A, gene structure and putative p53-responsive elements (REs) on the mouse Pierce1 locus. Mismatches with the p53 consensus sequence in lowercase. R, purine; Y, pyrimidine; W, adenine, or thymine. B and C, p53 responsiveness of BS1 (B) and BS3 (C) in NIH3T3 cells. Solid ovals, wild-type p53-REs; empty ovals, mutated p53-REs. Open bars, empty vector; solid bars, p53 expression construct. Results are expressed as an average of triplicate experiments, and error bars indicate standard deviation. D, ChIP of the Pierce1 promoter by anti-p53 antibody. Denoted reporter genes were transfected into p53-deficient H1299 cells with (+) or without (-) p53 expression constructs. p21^{WAF1} promoter-luciferase construct was transfected as an internal control. W, wild-type; M, mutant. Chromatin fractions immunoprecipitated with p53 were used as template for PCR reactions. Results for BS3 are from nested PCR reactions. E, endogenous interaction between p53 and mouse Pierce1 promoter. p53-bound chromatin fractions were immunoprecipitated from p53^{+/-} and p53^{-/-} primary MEFs, using an endogenous p53-specific antibody. The interaction of p53 with Pierce1 promoter was monitored by amplifying the central part (162 bp) of the promoter region flanked by BS1 and BS3. Endogenous p53-dependent ChIP of endogenous p21^{Waf1} promoter was used as a positive control.

clearly activated it and 300 ng of p53 expression construct was enough to elicit the maximal reporter activity (Supplementary Fig. S1; Fig. 2C). As BS2 did not respond to p53 (Fig. 2B and C) and mutation of BS3 abrogated p53-dependent reporter activation (Fig. 2C), BS1 and BS3 are responsible for p53-dependent activation of Pierce1 promoter.

To confirm the direct interaction of p53 with BS1 and BS3, ChIP assays were conducted using a p53-specific antibody after transfecting Pierce1 reporter gene constructs with or without p53 into p53-deficient H1299 cells (Fig. 2D). The p53-responsive p21^{WAF1} reporter gene was cotransfected as an internal positive control (30). As expected, p21^{WAF1} reporter gene was immunoprecipitated with a p53-specific antibody in cells overexpressing p53 (Fig. 2D, bottom). Pierce1 reporter gene containing both BS1 and BS3 was specifically immuno-

precipitated under the same conditions (Fig. 2D, left). The reporter gene harboring only wild-type BS1 was also immunoprecipitated with p53-specific antibody, whereas its mutations abrogated it (Fig. 2D, middle). The size difference in inputs reflects the differential use of multiple cloning sites on the pGL3 basic vector for wild-type and mutant BS1-containing reporter genes (Fig. 2D, middle). BS3 signals were not detected by primary PCR reactions (data not shown). However, nested PCR reactions using the primary PCR products as template successfully amplified p53-precipitated DNAs containing wild-type BS3 but not those of mutated BS3 (Fig. 2D, right). Consistent with reporter gene assays (Supplementary Fig. S1), these data reflect the weak p53 responsiveness of BS3 compared with that of BS1. To show that this observation is compatible with the endogenous control of Pierce1 promoter

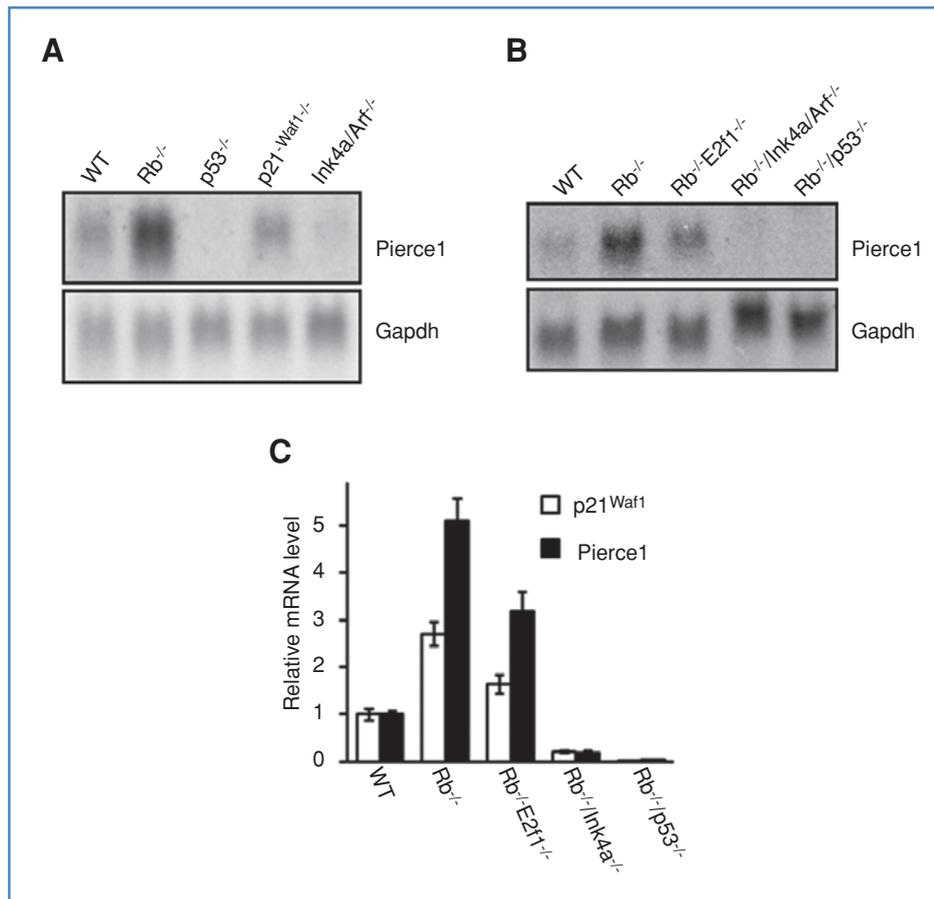


Figure 3. The essential role of p53 in Pierce1 transcription. A, Northern blot analysis of Pierce1 expression in MEFs with indicated genotypes. B and C, MEFs of compound genetic backgrounds were used for Northern blot analyses of Pierce1 (B) and real-time quantitative PCR analyses of Pierce1 and p21^{Waf1} (C).

by p53, we monitored the association of endogenous p53 with endogenous Pierce1 promoter using p53^{+/-} and p53^{-/-} MEFs (Fig. 2E). The Pierce1 promoter region, -308/-147, between BS1 and BS3 was specifically immunoprecipitated by endogenous p53 from p53^{+/-} MEFs, but not from p53^{-/-} MEFs (Fig. 2E), showing the endogenous interaction of p53 with BS1 and BS3 on the Pierce1 promoter. Taken together, these results confirm that p53 directly binds to the Pierce1 promoter through BS1 and BS3.

p53-dependent expression of Pierce1 in Rb^{-/-} MEFs

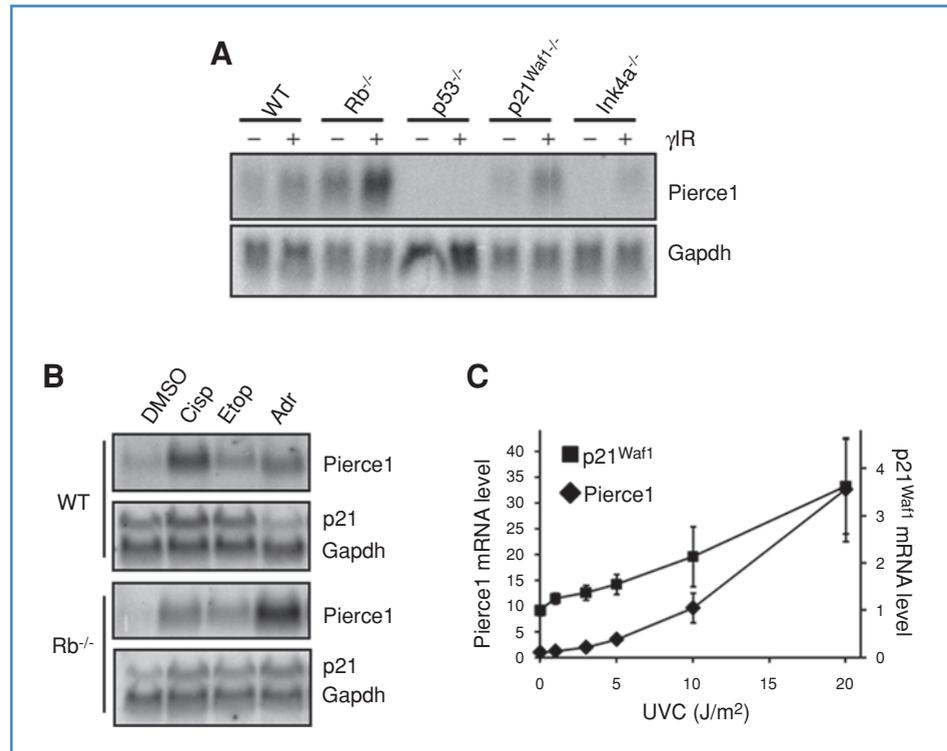
It is well known that Rb deficiency upregulates the E2F target gene p19^{Arf} and thus increases p53 levels by interfering with Mdm2 (31). As the Pierce1 promoter was strongly activated by p19^{Arf} (Fig. 1A), we genetically investigated the role of Rb/E2F1-Ink4a/Arf-p53 pathway in regulating Pierce1 expression (Fig. 3). As compared with wild-type MEFs, the level of Pierce1 transcript was considerably lowered in Ink4a/Arf-deficient MEFs (32) and was undetectable in p53^{-/-} MEFs (Fig. 3A). Although p21^{Waf1} is a critical inhibitor of CDK2, which phosphorylates and thus inactivates pRb (33), p21^{Waf1} deficiency does not significantly alter the expression level of p19^{Arf} (6) and had little effect on Pierce1 expression (Fig. 3A). Interestingly, Pierce1 expression was significantly attenuated

in MEFs deficient for both Rb and E2F1 as compared with Rb^{-/-} MEFs (Fig. 3B and C). Furthermore, Pierce1 expression was severely downregulated in Rb/Ink4a/Arf-deficient MEFs, and was not detected in Rb/p53 double-knockout MEFs (Fig. 3B and C). This expression pattern was coincident with that of p21^{Waf1}, a well-known p53 target gene (Fig. 3C). Therefore, these results indicate that the strengthened E2F-p19^{Arf}/Mdm2-p53 pathway is likely a primary cause of Pierce1 induction in Rb^{-/-} MEFs.

Pierce1 expression is induced by genotoxic stresses

Genotoxic stresses can activate p53 and should therefore increase p53-dependent transcription of Pierce1 as well. As shown in Figure 4A, Pierce1 expression was elevated in response to γ -irradiation in all genotypes, except for p53-deficient MEFs. γ -irradiation induced robust expression of Pierce1 in Rb^{-/-} MEFs, and the amplitude of Pierce1 induction was smaller in Ink4a/Arf^{-/-} MEFs than in wild-type MEFs (Fig. 4A), reminiscent of the positive role of p19^{Arf} in p53 activity (31). As anticipated, both basal and γ -ray-induced expression of Pierce1 was not affected by p21^{Waf1} deficiency (Fig. 4A). Cisplatin, etoposide, and adriamycin consistently upregulated Pierce1 expression in both wild-type and Rb^{-/-} MEFs (Fig. 4B). The extent to which it was induced varied

Figure 4. Pierce1 induction by genotoxic stresses. A, Northern blot analysis of Pierce1 in γ -irradiated MEFs with indicated genotypes. B, Northern blot analysis of wild-type (WT) or $Rb^{-/-}$ MEFs treated for 12 hours with cisplatin (Cisp, 25 μ mol/L), etoposide (Etop, 2 μ g/mL), or adriamycin (Adr, 400 ng/mL). C, real-time quantitative PCR analysis of Pierce1 and $p21^{Waf1}$ in wild-type MEFs 6 hours after UVC irradiation.



according to *Rb* genotype and different genotoxic stresses (Fig. 4B). Although both etoposide and adriamycin are topoisomerase II inhibitors (34), Pierce1 expression was more strongly induced by adriamycin than by etoposide in wild-type MEFs (Fig. 4B). It was most highly induced by cisplatin in wild-type MEFs, but the maximal Pierce1 expression was triggered by adriamycin in $Rb^{-/-}$ MEFs (Fig. 4B). In addition, it was strongly activated by UVC irradiation in a dose-dependent manner (Fig. 4C). PIERCE1 induction was also observed in primary human cell lines, albeit to a lesser extent than in MEFs (Supplementary Fig. S2). Taken together, these results indicate that p53-activating genotoxic stresses can induce Pierce1 transcription, thus emphasizing the positive role of p53 in regulating Pierce1 expression.

Pierce1 protein is stabilized by UVC independently of its transcriptional activation

As the expression of Pierce1 was regulated at the level of transcription by UVC (Fig. 4C), we tested whether its protein level was proportional to the transcript level upon UVC irradiation, using Pierce1-specific polyclonal antibody (Supplementary Fig. S3). Compared with p53-deficient MEFs (Fig. 5A, lane 1), a weak Pierce1 signal was detected in wild-type MEFs under the normal condition (Fig. 5A, lane 2), but was considerably upregulated upon UVC irradiation (Fig. 5A, lane 3). This confirms that expression of both Pierce1 transcript and protein is increased by UVC irradiation.

For the biochemical studies, we retrovirally established NIH3T3 stable cells, designated as NP1, that overexpress HA-tagged mouse Pierce1 (HA-Pierce1) under the control of

the human cytomegalovirus (CMV) immediate early (IE) promoter, as opposed to the Pierce1 promoter, and the control cells, NX1, that harbor the empty vector. UVC irradiation dramatically elevated the level of HA-Pierce1 protein (Fig. 5B). It has recently been suggested that the CMV-IE promoter can be activated by UVC irradiation independently of p53 (35). However, the transcript level of HA-Pierce1 was not increased in NP1 cells 6 hours after UVC irradiation (Supplementary Fig. S4), the time point at which the accumulation of HA-Pierce1 protein was observed (Fig. 5B). These results indicate the stabilization of HA-Pierce1 by UVC irradiation. It was confirmed using an NIH3T3 stable cell line conditionally expressing HA-Pierce1 (Supplementary Fig. S5). As Pierce1 protein contains putative PEST signals (22), which may induce the rapid turnover of Pierce1 protein, we considered that UVC irradiation may inhibit proteasome-mediated degradation of Pierce1 protein. After HA-Pierce1 expression was turned on by doxycycline, the conditional cells were incubated with the proteasome inhibitors MG132, zLLnV, and LLnL (Fig. 5C). We also monitored the protein levels of $p21^{Waf1}$ and p53 as positive controls (Fig. 5C). These drugs stabilized HA-Pierce1 protein to varying degrees (Fig. 5C). p53 and $p21^{Waf1}$ were also stabilized under these conditions (Fig. 5C). These data suggest that Pierce1 protein is easily degraded via proteasomes and that this process can be inhibited or suppressed by UVC irradiation.

UVC irradiation activates the ATR-mediated DNA damage response (36) and its kinase activity is inhibited by caffeine (37). As UVC irradiation stabilized the Pierce1 protein, the ATR-mediated DNA damage response may be involved in

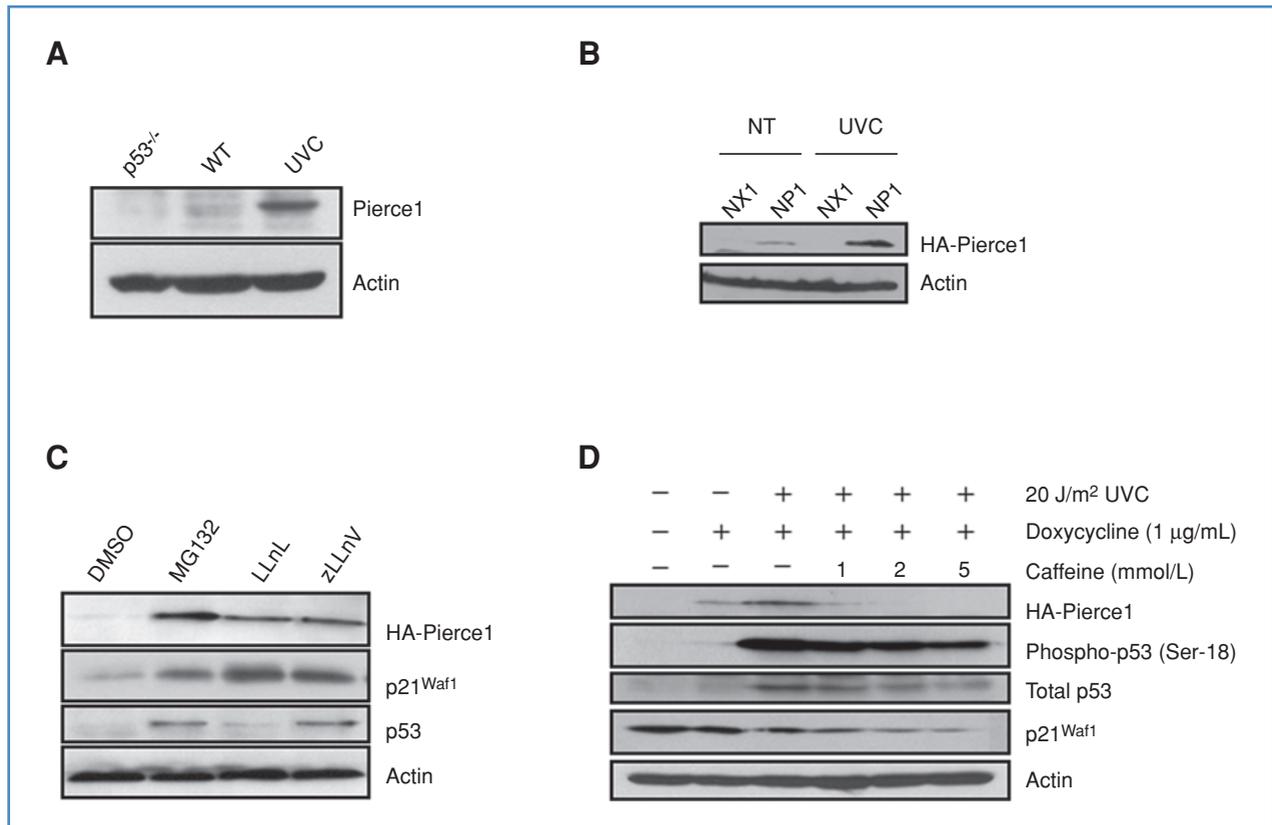


Figure 5. Posttranslational regulation of Pierce1 by UVC irradiation and proteasome-mediated degradation. A, Western blot analysis of endogenous Pierce1 in p53^{-/-}, wild-type (WT), and UVC-irradiated wild-type MEFs (10 J/m² UVC, 8 hours). B, Western blot analysis of HA-Pierce1 in normal and UVC-irradiated (20 J/m²) stable cell lines. NX1, control cells; NP1, HA-mouse Pierce1-expressing cells. C, Western blot analysis of HA-Pierce1, p53, and p21^{Waf1} in a conditional NIH3T3 cell line (Supplementary Fig. S5). The expression of HA-Pierce1 was induced by treating doxycycline for 24 hours, followed by treatment with MG132 (20 μmol/L), LLnL (20 μmol/L), or zLLnV (50 μmol/L) for 4 hours. D, Western blot analysis showing the effect of caffeine on UVC-induced HA-Pierce1 stabilization. After incubation with doxycycline for 16 hours, cells were pretreated with the indicated concentrations of caffeine for 1 hour prior to UVC irradiation. Cells were harvested 6 hours after UVC irradiation.

the posttranslational regulation of Pierce1. The half-maximal inhibition concentration (IC₅₀) of ATR by caffeine is approximately 1.1 mmol/L (37). Pretreatment of the conditional cells with caffeine abolished UVC-induced stabilization of HA-Pierce1 protein (Fig. 5D). As expected, caffeine also decreased UVC-induced Ser-18 phosphorylation of mouse p53 (homologous to human Ser-15) and the protein accumulation of p53 and p21^{Waf1} (Fig. 5D). These results indicate that Pierce1 is posttranslationally regulated by the ATR-mediated DNA damage response.

Pierce1 is important for eliciting UVC-induced checkpoint responses

As a p53 target gene, it is feasible that Pierce1 knockdown could alter the UVC-induced DNA damage response triggering cell-cycle arrest and apoptosis. Treatment with a Pierce1-specific small interfering RNA (siRNA; siPierce1 #2) efficiently depleted Pierce1 expression up to approximately 80% in wild-type MEFs (Supplementary Fig. S6). The extent of UVC-induced apoptosis was measured by flow cytometric analysis of annexin V-FITC-positive and propidium iodide (PI)-positive cell popu-

lations but was not altered by Pierce1 knockdown (data not shown). In addition, Pierce1 knockdown had little effect on BrdU incorporation under the normal condition (siCTRL 22.7 ± 0.5% vs. siPierce1 22.5 ± 0.6%; Fig. 6A; Table 1). Although cell-cycle arrest was clear upon UVC irradiation (20 J/m²), BrdU incorporation was slightly higher in Pierce1-silenced MEFs (4.6 ± 0.5%) than that in control siRNA (siCTRL)-treated MEFs (3.0 ± 0.7%) at 9 hours ($P < 0.05$, Fig. 6A; Table 1). G₁ and G₂/M cell populations were not significantly changed (Table 1). Although UVC-induced S-phase arrest was mostly Pierce1-independent, these data indicate that UVC-induced S-phase arrest was mildly affected by Pierce1 knockdown at that time point. Clonogenic assays were also conducted using the conditional cell line to observe the long-term effect of HA-Pierce1 overexpression (Fig. 6B). Although the cell-cycle profile was not changed (flow cytometric analysis of anti-BrdU-FITC/PI-stained cells, data not shown), the clonogenic survival were mildly diminished by HA-Pierce1 overexpression without UVC irradiation (Fig. 6B). Furthermore, the overexpression of HA-Pierce1 affected the clonogenic survival upon UVC irradiation (Fig. 6B). Therefore, these data suggest that Pierce1 has moderate effects on the DNA

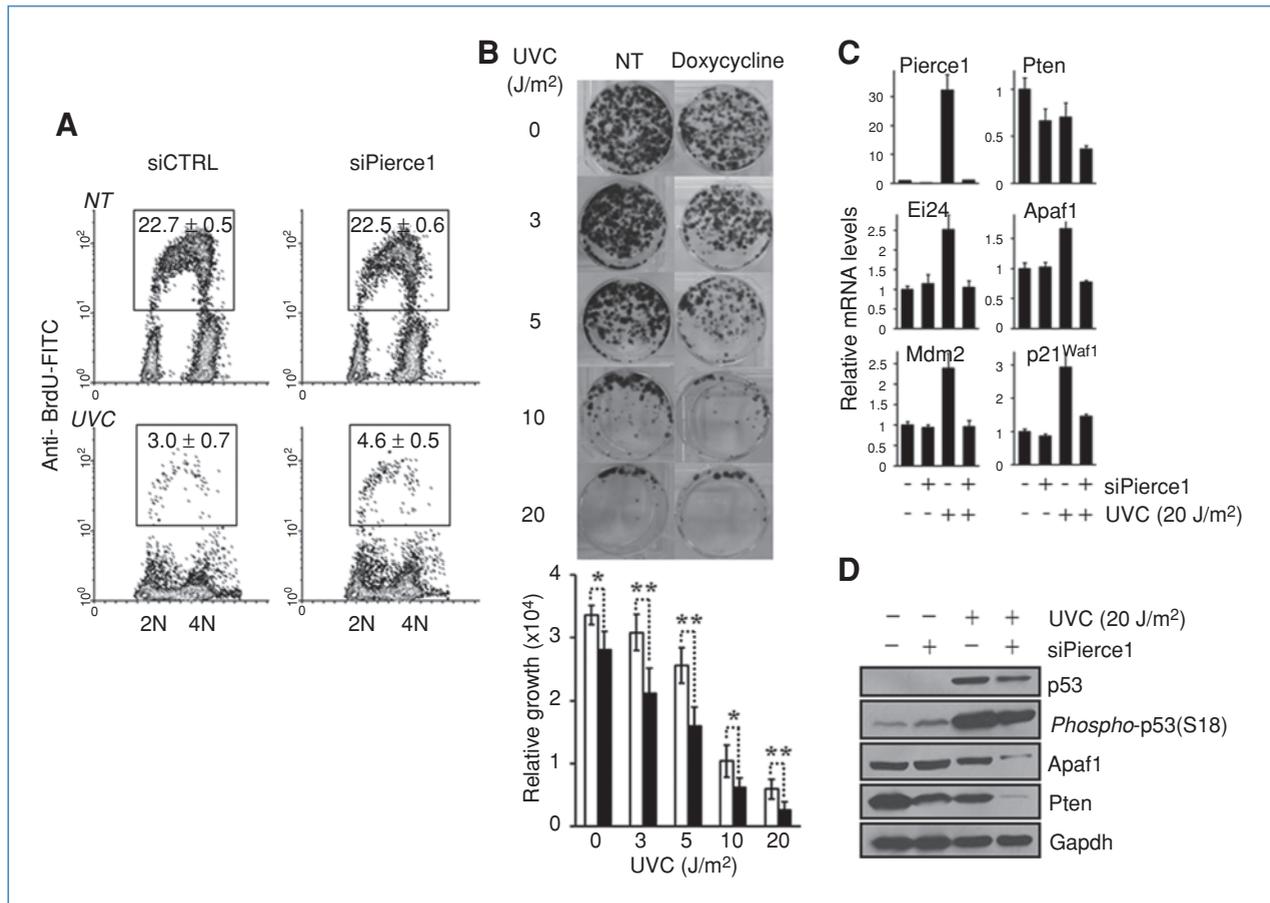


Figure 6. Effect of Pierce1 expression on clonal growth and checkpoint responses to UVC irradiation. A, flow cytometric analysis of BrdU incorporation. Wild-type MEFs were treated with the indicated siRNA for 48 hours and then irradiated with 20 J/m² of UVC. MEFs were harvested 9 hours after UVC irradiation. BrdU was treated for the final 2 hours. B, clonogenic assay ($n = 6$). HA-Pierce1 was conditionally induced using doxycycline treatment, and their clonogenic survival was compared with those of untreated controls after irradiation of indicated UVC doses. Top, representative photographs; bottom, statistical analysis. *, $P < 0.05$; **, $P < 0.005$. C, real-time quantitative PCR analyses of p53 target genes in MEFs 18 hours after UVC irradiation. D, Western blot analyses. Protein samples were prepared from an aliquot of MEFs used in C.

damage response, contributing to both cell-cycle arrest and clonogenic survival against UVC irradiation.

We also examined the effect of Pierce1 knockdown on the expression of p53 target genes, including Apaf1, etoposide-induced 24 (Ei24), Mdm2, Pten, and p21^{Waf1} (38). When the expression of Pierce1 was efficiently depleted, the expressional change was not clear under the normal condition (Fig. 6C). However, expressions of Apaf1, Ei24, Mdm2, and p21^{Waf1} were obviously abrogated in Pierce1-silenced MEFs at 18 hours after UVC irradiation (Fig. 6C). Although Pten is a p53 target gene (39), it was reported to be repressed by UVB irradiation (40). Notably, Pten was severely downregulated by UVC irradiation upon Pierce1 knockdown (Fig. 6C). Likewise, the levels of total p53 and its UVC-induced phosphorylation (Ser-18) were diminished by Pierce1 knockdown (Fig. 6D). Consistent with Figure 6C, the protein levels of Apaf1 and Pten were significantly decreased by Pierce1 knockdown upon UVC irradiation (Fig. 6D). These results suggest that Pierce1 may be important for properly activating the expression of p53 target genes upon UVC irradiation.

In conclusion, this study identifies Pierce1 as a novel p53 target gene and provides molecular and physiologic evidence that it may be an important mediator of the DNA damage response induced by UVC irradiation.

Discussion

We identified Pierce1 as a novel p53 target gene from Rb^{-/-} MEFs (Figs. 1–3), emphasizing the important cross talk between pRb and p53 (1). Although p53 activity is upregulated in Rb^{-/-} MEFs, DNA damage responses against diverse genotoxic stresses are severely abrogated. Rb^{-/-} MEFs have a defect in G₁/S-phase arrest upon γ -irradiation (41). Topoisomerase II inhibitors, such as adriamycin and etoposide, normally induce cell-cycle arrest in all phases, and cisplatin primarily causes S-phase arrest in wild-type MEFs (42). However, considerable fractions of Rb^{-/-} MEFs are still proliferative upon treatment with these genotoxic stresses (41–45). Notably, although distinctive genotoxic stresses comparably induced p21^{Waf1} in wild-type and Rb^{-/-} MEFs, the induction of Pierce1 severely

Table 1. Quantitation of cell populations observed in Figure 6A

	siRNA	G1 ^a	S ^a	G2/M ^a
NT ^b	siCTRL	47.0 ± 0.6	22.7 ± 0.5	30.4 ± 1.0
	siPierce1	44.5 ± 1.4	22.5 ± 0.6	33.0 ± 0.9
	<i>P</i> ^c	0.04	0.72	0.03
UVC (20 J/m ²)	siCTRL	54.3 ± 0.8	3.0 ± 0.7	42.7 ± 0.9
	siPierce1	52.4 ± 0.2	4.6 ± 0.5	42.9 ± 0.3
	<i>P</i> ^c	0.02	0.03	0.68

^aExpressed as an average of triplicate experiments ± SD.

^bNot treated.

^cStudent's *t* test of triplicate experiments.

fluctuated (Fig. 4B). The expression of Pierce1 may be more sensitive to the type of genotoxic stress, and the expressional changes of Pierce1 may reflect unknown molecular or physiologic changes upon Rb deficiency.

In addition to transcriptional control, Pierce1 protein can be posttranslationally regulated by UVC irradiation (Fig. 5). It is well documented that the UVC-induced DNA damage response is primarily activated through ATR (20, 36), which can be inhibited by caffeine (37). Extensive studies have shown that DNA damage responses are accompanied by diverse posttranslational modifications contributing to the protein stability (46). For example, although the turnover of p53 protein is very rapid under normal conditions, ATR stabilizes and activates p53 through direct and indirect phosphorylation via checkpoint kinase 1 upon UVC irradiation (20). On the basis of the presence of potential PEST signatures in Pierce1 homologues (22) and the relatively low expression level of HA-Pierce1 protein in our stable cell lines (Fig. 5), Pierce1 protein would be unstable under normal conditions. Because proteasome inhibitors dramatically increased the level of HA-Pierce1 protein (Fig. 5C), UVC irradiation may trigger specific signaling events to inhibit proteasome-mediated degradation of Pierce1 proteins. Finally, as caffeine, an ATR inhibitor, compromised UVC-induced stabilization of HA-Pierce1 protein (Fig. 5D), the ATR pathway may be critical for posttranslational regulation of Pierce1.

ATR is the prime initiator for UVC-induced DNA damage responses (20). Thus, Pierce1 may transiently play a role under the stressful conditions such as UVC irradiation. In fact, the effect of Pierce1 knockdown on selected p53 target genes was effective only upon UVC irradiation (Fig. 6C). As these observations were made at 18 hours after UVC irradiation and it was lesser at an earlier time point (data not shown), these effects may become progressively severe. Similar situations are involved in regulating mismatch repair (MMR) proteins that maintain genomic stability by correcting DNA errors and regulating cellular responses to DNA damage (47). Human MutL proteins, a subset of MMR proteins, are posttranslationally stabilized by ATM and contribute to augmented p53 activation during the DNA damage response (48). These data support the idea that Pierce1, as a potential downstream molecule of ATR, can modulate the UVC-induced DNA damage response.

The effects of Pierce1 knockdown on p53 target genes were physiologically conflicting. Pierce1 knockdown induced downregulation of proapoptotic genes including Ei24, Apaf1, and Pten (Fig. 6C), suggesting that Pierce1 is proapoptotic. On the other hand, Pierce1 knockdown was accompanied by the downregulation of p21^{Waf1} (Fig. 6C). As p21^{Waf1} deficiency can alter the cellular response to genotoxic stresses from cell-cycle arrest to apoptosis (49), Pierce1 may be important for cellular survival after UVC irradiation. Finally, although Mdm2 expression, which is essential for p53 degradation, was diminished, p53 protein levels and its overall activity were decreased by Pierce1 knockdown upon UVC irradiation (Fig. 6C and D). Although the molecular mechanisms remain to be elucidated, the physiologic output of UVC irradiation was moderately affected by Pierce1 (Fig. 6A and B; Table 1). Notably, as the expressional changes of p53 target genes were preceded by the physiologic abnormality, Pierce1 may be involved in processes required for an earlier response of wild-type MEFs upon UVC irradiation such as detecting genotoxic lesions and transducing DNA damage responses.

In conclusion, our data suggest that Pierce1 is indeed a novel p53 target gene and may participate in important function(s) required for eliciting appropriate DNA damage responses. Further studies revealing the molecular and physiologic aspects of Pierce1 will be helpful for in-depth understanding of p53 pathways and the design of new cancer therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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