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Ei24, a Novel E2F Target Gene, Affects *p53*-independent Cell Death upon Ultraviolet C Irradiation*

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Background: *Rb* loss deregulates E2F and thus induces the expressions of E2F target genes.
Results: *Ei24* is an E2F target up-regulated in $Rb^{-/-}$ MEFs and affects susceptibility of *p53*-deficient MEFs against UVC.
Conclusion: E2F1 can provide a p53-independent modulation of cellular sensitivity against UVC via *Ei24*.
Significance: EI24 may be a potential therapeutic target for treating *p53*-deficient tumors.

The deficiency of retinoblastoma (*Rb*) gene deregulates E2F transcription factors and thus induces E2F target genes directly or p53 target genes indirectly via mouse $p19^{Arf}$ (or $p14^{ARF}$ in humans), an E2F target gene. Here, we identified that etoposide-induced 2.4 mRNA (*Ei24*)/p53-induced gene 8 (*Pig8*), a p53 target gene involved in apoptosis and autophagy, was up-regulated in $Rb^{-/-}$ mouse embryonic fibroblasts (MEFs). The *Ei24* promoter was activated by E2F1 via multiple E2F-responsive elements, independently of the previously reported p53-responsive element. Chromatin immunoprecipitation assays revealed that E2F1 directly acts on the mouse *Ei24* promoter. We observed that Ei24 expression was suppressed in $p53^{-/-}$ MEFs upon UVC irradiation, which was exacerbated in $p53^{-/-}$ $E2f1^{-/-}$ MEFs, supporting the positive role of E2F1 on *Ei24* transcription. Furthermore, *Ei24* knockdown sensitized $p53^{-/-}$ MEFs against UVC irradiation. Together, our data indicate that *Ei24* is a novel E2F target gene contributing to the survival of *p53*-deficient cells upon UVC irradiation and thus may have a potential significance as a therapeutic target of certain chemotherapy for treating *p53*-deficient tumors.

The retinoblastoma gene (*Rb*)[4] was the first tumor suppressor gene to be identified in humans, and its loss of function is implicated in a wide variety of human cancers, including retinoblastomas (1). The *Rb* gene product (pRb) is a critical component of the cell cycle because it regulates the activity of E2F transcription factors, which induce the expression of genes required for cell cycle progression, including cyclins, cyclin-dependent kinases (CDKs), and proliferating cell nuclear antigen (2). When E2F is activated or deregulated, Rb-induced cell cycle arrest is compromised (3). Furthermore, deregulated E2F transcription factors can enforce quiescent cells to re-enter the cell cycle (4).

pRb/E2F is controlled by p53, which is a critical tumor suppressor known as the guardian of the genome (5). More than 50% of human cancers contain a mutation of p53 (6), and p53-mediated tumor suppression is mainly dependent on its activity as a transcription factor. The mutations occur most frequently in the DNA-binding domain of the p53 protein (7). In fact, p53 can activate a plethora of genes that regulate cell cycle progress, apoptosis, and DNA repair under stressful conditions (8). $p21^{Waf1}$ is a well known p53 target gene that is involved in the regulation of the Rb/E2F pathway. $p21^{Waf1}$ can directly bind to and inhibit all cyclin-CDK complexes, whereas $p16^{Ink4a}$ specifically inhibits cyclin D-associated complexes of CDK4 and CDK6 (9). The resulting hypophosphorylation of pRb blocks the activation of E2F transcription factors.

In addition to their roles in cell cycle progression, *E2F* genes are important for apoptosis. In fact, diverse proapoptotic genes are directly regulated by E2F transcription factors (2), whose physiological significance has been demonstrated using mouse models deficient for E2F transcription factors. *E2f1*-deficient mice exhibit defects in apoptosis and are rather prone to tumorigenesis (10, 11). E2F2 and E2F3 are required for S phase entry induced by c-Myc (12). These phenotypes are supported by E2F-mediated regulation of proapoptotic genes including *Bax/Bad*, *Apaf1*, *caspase-3*, and *caspase-9* (2).

Etoposide-induced 2.4 mRNA (*Ei24*), also known as p53-induced gene 8 (*Pig8*), was first identified as an up-regulated transcript in etoposide-treated NIH3T3 cells (13) and was later characterized as a p53 target gene (14). *Ei24* was also identified as a prerequisite gene for executing autophagy (15). Because its ectopic expression induces cell death and suppresses cellular growth, *Ei24* has been regarded as a tumor suppressor gene with a proapoptotic function (14). In support of this characterization, *Ei24* is located on chromosome 11q24, which is fre-

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[4] The abbreviations used are: Rb, retinoblastoma; Apaf1, apoptosis protease-activating factor 1; CDK, cyclin-dependent kinase; CHX, cycloheximide; *Ei24*, etoposide-induced 2.4 mRNA; ER, estrogen receptor; MEF, mouse embryonic fibroblast; 4-OHT, 4-hydroxytamoxifen; *Pig8*, p53-induced gene 8; pRb, retinoblastoma gene product; RE, responsive element.

quently lost in neoplastic lesions, including breast cancers (16). Furthermore, because Ei24 is a Bcl2-binding protein localized on the endoplasmic reticulum with potential roles in preventing the spreading of tumors (17), it is believed to be a critical downstream regulator of p53 in tumor suppression. We recently reported that Ei24 stabilizes protein kinase Cα (PKCα) and that its deficiency attenuates PKCα signaling and skin carcinogenesis in mice (18), suggesting that the physiological role of EI24 is complex and may manifest differently according to tissue- and/or in a stress-specific manner.

Even though a large number of genes have been identified as E2F target genes, the role of pRb/E2F has not been fully elucidated. Recently, we analyzed the alteration in the gene expression pattern induced by *Rb* deficiency (19). Among the deregulated genes, the induction of *Ei24* was observed in $Rb^{-/-}$ mouse embryonic fibroblasts (MEFs). In the present study, we characterized *Ei24* as an E2F target gene, suggesting that Ei24 may act as a key mediator in the downstream pathways of both pRb/E2F and p53.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Reporter Gene Assay—MEFs deficient for *Rb*, *p53*, and/or *E2f1* were prepared as described previously (20). In addition to MEFs, NIH3T3 fibroblasts and H1299 cells were grown in DMEM containing 10% fetal bovine serum (Sigma). Reporter gene assays were conducted using NIH3T3 and H1299 cells. Cells were transiently transfected using WelFect-EX plus reagent (WelGENE) or Lipofectamine 2000 reagent (Invitrogen). Firefly and *Renilla* luciferase activities were measured 24 h after transfection using Dual-Luciferase Reporter Assay System (Promega) and a luminometer (Turner Designs).

Constructs—pBabe-HA-ER and pBabe-HA-ER-E2F1 were generous gifts from Dr. Kristian Helin (Biotech Research and Innovation Center, University of Copenhagen). pSG5L-HA-E2F1 (Addgene plasmid 10736) was routinely used as an effector for the luciferase and chromatin immunoprecipitation (ChIP) assays. pcDNA3-HA-E2F1, -E2F2, and -E2F3 were generous gifts from Dr. Joseph R. Nevins (Duke University) and were used to evaluate the effect of individual E2F members on the activation of the *Ei24* promoter. pcDNA3-FLAG-p53 was a generous gift from Dr. Jaewhan Song (Yonsei University) and was used to validate the activation of the human *EI24* promoter. pRL-SV40 (Promega) was used as an internal control for the Dual-Luciferase Reporter Assay System (Promega). The mouse *Ei24* promoter was cloned from 129 mouse genome and subcloned into pGL3 Basic vector (Promega). Site-directed mutagenesis was done using the following primers: 5′-GTGTTATAAGCCCCGGCTCTCC-3′ for BS1, 5′-GGTATATGAGCAGGCGAACCGAG-3′ for BS2, and 5′-CCGCGGCGGTAGGGTGTATTGGTAGGTGCAGGCCGC-3′ for BS3. The human *EI24* promoter region (−2093/+477) was amplified by PCR from human *EI24* BAC clone (clone ID: RP11-57M13) and was subcloned into pGL3 Basic vector. The reporter gene constructs were verified by direct sequencing. Lentiviral constructs encoding mouse Ei24-specific shRNAs were purchased from Sigma.

Stable Cell Lines and Treatments—Retroviruses were generated with pBabe-HA-ER and pBabe-HA-ER-E2F1, as described previously, and were used for the infection (21). After selection with puromycin (Sigma), infected NIH3T3 cells were cloned and used after the expressions of HA-ER and HA-ER-E2F1 were confirmed. Cells were pretreated with cycloheximide (10 μg/ml, Sigma) for 1 h and then co-treated with 30 nM, 300 nM, or 3 μM 4-hydroxytamoxifen (4-OHT, Sigma) dissolved in ethanol for 24 h. Infectious lentiviral particles were produced as described previously (18) and were used to infect $p53^{-/-}$ MEFs. Infected cells were selected by treatment with puromycin (Sigma), and polyclonal cell populations were used for the following experiments. UVC irradiation and the following cell death assays, done by measuring fluorescein isothiocyanate (FITC)-labeled annexin V (annexin V-FITC) staining and propidium iodide permeability were conducted as described previously (20).

Northern Blot Analysis and Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNAs were separated and transferred onto a nylon membrane (Amersham Bioscience). Prehybridization and hybridization were carried out as described previously (19). cDNA was synthesized using SuperScript III System (Invitrogen). RT-PCR analyses were conducted using the following primer pairs: 5′-CCTTTCTGCAGGACCTTGGCA-3′ and 5′-CTCTTGCTTCCGCTCTACACTCTG-3′ for *Ei24* (NM_007915), 5′-GGTGAAAGAACTGGAGAAGCG-3′ and 5′-GTGCTTATCCTTCTCTCGC-3′ for *Stathmin* (NM_019641) (22), and 5′-ATCACTGCCACCCAGAAGAC-3′ and 5′-CACCACCTTCTTGATGTCATC-3′ for *Gapdh* (NM_008084).

ChIP Assays—ChIP assays were done using the E2F1-specific antibody (sc-251x) and following the protocol from Santa Cruz Biotechnology. PCR amplification was performed using the following primers: 5′-CTAGCAAAATAGGCTGTCCC-3′ (a forward primer specific for pGL3 Basic), 5′-AGGTCCAGCCAACTACAATT-3′ (−401/−382 of *Ei24* promoter, used for detecting endogenous *Ei24* promoter), and 5′-CCAGGCTGCGATTTGCAA-3′ (−88/−105, used for detecting endogenous *Ei24* promoter and the *Ei24* reporter gene construct).

RESULTS

Up-regulation of Ei24 in $Rb^{-/-}$ MEFs—To identify novel *Rb/E2f*-regulated genes, we previously analyzed the gene expression profile of $Rb^{-/-}$ MEFs (19). Among the deregulated genes, we observed the highly up-regulated expression of *Ei24*, which has been identified as a p53 target gene (14). Northern blot and quantitative RT-PCR analyses confirmed the significant increase of *Ei24* mRNA in $Rb^{-/-}$ MEFs (Fig. 1, *A* and *B*). Consistent with this finding, known E2F target genes including apoptosis protease-activating factor 1 (*Apaf1*) (23) and *Stathmin* (22) were also up-regulated in $Rb^{-/-}$ MEFs (Fig. 1*B*). These data indicate that *Ei24* expression can be regulated by Rb/E2F.

Because deregulated E2F1 directly up-regulates $p19^{Arf}$, *Rb* deficiency alters the expressions of diverse p53 target genes (24). To examine whether *Ei24* transcription is directly induced by E2F1, stable cell lines expressing the hemagglutinin (HA)-tagged ligand-binding domain of the estrogen receptor (ER) and estrogen receptor-fused E2F1 (ER-E2F1) were established

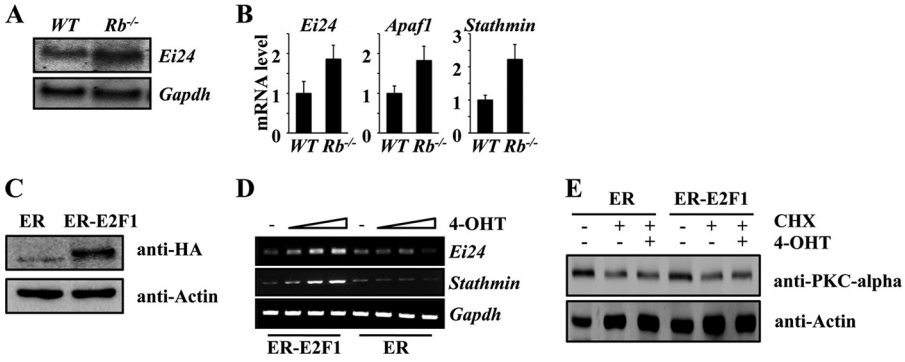

FIGURE 1. *A*, Northern blot analysis of *Ei24* expression in wild-type (WT) and $Rb^{-/-}$ MEFs. *B*, quantitative RT-PCR analyses of *Ei24*, *Apaf1*, and *Stathmin* expressions in WT and $Rb^{-/-}$ MEFs. *C*, Western blot analysis of NIH3T3 stable cell lines expressing the HA-tagged ER-E2F1. *D*, effect of 4-OHT treatment on the expression of *Ei24* and *Stathmin* in NIH3T3 cells stably expressing ER or ER-E2F1. Cells were pretreated with CHX to prevent *de novo* protein synthesis before treating cells with increasing doses of 4-OHT. Gene expressions were monitored by semiquantitative RT-PCR. *E*, effect of CHX treatment in NIH3T3 cells stably expressing ER or ER-E2F1. *De novo* synthesis was effectively blocked and confirmed by PKCα protein as a control.

using *Ink4a/Arf*-deficient NIH3T3 as a parental cell line (Fig. 1*C*). Because 4-OHT binding causes the translocation of the ER into the nucleus, the activity of ER-E2F1 can be conditionally activated by 4-OHT (25). These cell lines were pretreated with cycloheximide (CHX) to prevent *de novo* protein synthesis and then co-treated with 4-OHT for 24 h to activate the transcriptional activity of ER-E2F1 (Fig. 1, *D* and *E*). The data revealed that the *Ei24* transcript level was considerably up-regulated in cells expressing ER-E2F1 but not in cells expressing ER only (Fig. 1*D*). Consistently, *Stathmin*, a known E2F target gene, showed a comparable expression pattern (Fig. 1*D*). In Fig. 1*E*, the effect of CHX treatment was confirmed by PKCα protein levels as a control on Western blot assays (18). Because *de novo* protein synthesis was blocked, these results indicate that *Ei24* should be transcriptionally regulated by E2F1.

Ei24 Is a Novel E2F Target Gene—To identify potential cis-elements responsive to E2F transcription factors, the proximal region of the Ei24 promoter was analyzed with the TFBIND software (26). Three putative E2F-responsive elements (RE1, 2, and 3) and two cryptic REs with moderate similarity to the consensus were identified in the proximal region of mouse and human *EI24* promoters (Fig. 3*A* and data not shown). Based on this analysis, the corresponding regions of the *Ei24* promoters were cloned from human and mouse genomes, and a series of reporter genes was constructed. Similar to mouse *Ei24* (14), human *EI24* promoter was strongly activated by p53 (Fig. 2*A*). Because *Rb* deficiency up-regulated Ei24 expression in MEFs (Fig. 1*A*), and as pRb mainly regulates E2F1–3 (2), we examined the effect of these E2F transcription factors on the activation of the *Ei24* promoter. Although E2F1–3 comparably activated the synthetic E2F reporter construct, they had variable effects on the activation of the *Ei24* promoter (Figs. 2*B* and 3*B*). E2F1 showed an ~4-fold higher luciferase activity compared with basal activity, whereas E2F2 and E2F3 increased the activity by ~2-fold (Figs. 2*B* and 3*B*). These results suggest that, among the different E2Fs, deregulation of E2F1 has a major effect on the transcriptional activation of *Ei24* in $Rb^{-/-}$ MEFs.

To discriminate the relative potential of the putative E2F-responsive elements on the *Ei24* promoter, they were deleted or mutated. The longest promoter (−396 ~ +453) was strongly activated by E2F1 in NIH3T3 cells (Fig. 3*C*). The deletion of two

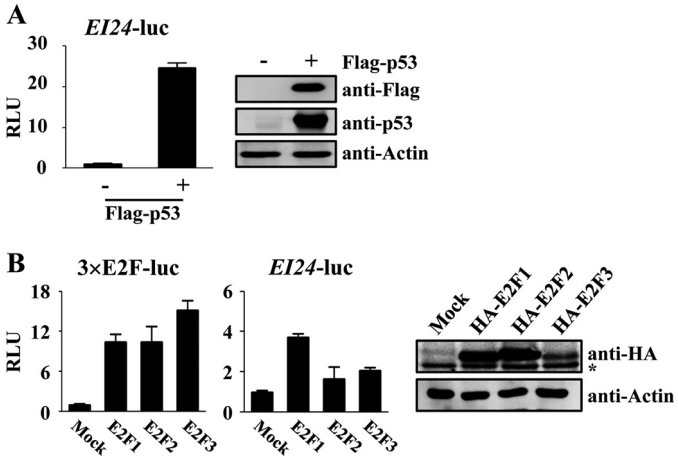

FIGURE 2. *A*, effect of p53 on the human *EI24* promoter (−2093/+477). The human *EI24* promoter was strongly activated by p53. *B*, differential effects of E2F transcription factors on the human *EI24* promoter. A synthetic E2F-responsive reporter gene (3×E2F-Luc) was used to confirm the activity of individual E2F transcription factors. *, nonspecific signal.

cryptic REs had no effect on the E2F1-mediated activation of reporter genes, but the responsiveness of the *Ei24* promoter-luciferase reporter to E2F1 was almost completely blocked by deleting RE1, 2, and 3 (Fig. 3*C*). In addition to the deletion, site-directed mutations in RE1 and RE2 significantly decreased E2F1-induced reporter gene activity but did not completely abolish the reporter gene activation by E2F1 (Fig. 3*D*, *upper panel*). An RE3 mutation had minimal effect when RE1 and 2 were intact (Fig. 3*D*, *lower panel*). However, the combined mutations of RE1–3 effectively suppressed the reporter gene activation by E2F1 (Fig. 3*D*, *lower panel*). These results imply that RE1, 2, and 3 are important for E2F-mediated activation of the *Ei24* promoter.

Based on the E2F1-dependent activation of the *Ei24* promoter, we examined whether E2F1 binds directly to the *Ei24* promoter. An *Ei24* reporter gene containing wild-type E2F-responsive elements was transiently transfected into H1299 cells with or without an E2F1 expression construct, and ChIP assays were conducted with an E2F1-specific antibody (Fig. 3*E*, *left panel*). Although the signals from the input were similar, a more prominent signal was detected in the cells transfected with the E2F1-expressing construct (Fig. 3*E*, *left panel*). As

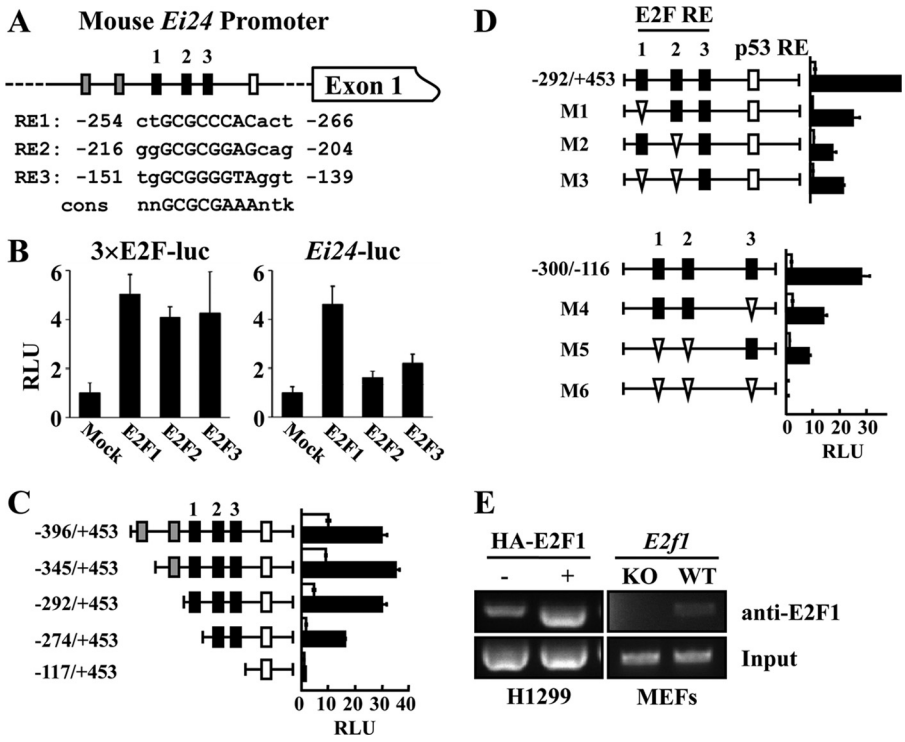

FIGURE 3. *A*, schematic representation of the proximal *Ei24* promoter. The sequences for putative E2F-responsive elements (RE1, 2, and 3) and a consensus sequence for E2F-responsive elements (*cons*) are denoted. *B*, differential effects of E2F transcription factors on the *Ei24* promoter (−292/+453). The *Ei24* reporter gene (*Ei24-Luc*) containing RE1–3 and p53 RE without the cryptic E2F REs was used. *C*, effect of *Ei24* promoter deletions on their responsiveness to E2F1. *D*, effect of site-directed mutagenesis on E2F1-dependent activation of the reporter genes. *Upper panel*, promoter region of *Ei24*, −292/+453. Each mutant construct is denoted as M1, M2, and M3 for the site-directed mutation of RE1, RE2, and both, respectively. *Lower panel*, the promoter region of *Ei24* encompassing −300/−116. It does not contain p53 RE. M4, M5, and M6 indicate the mutant constructs with site-directed mutation on RE3 only, commonly on RE1 and RE2, and on all three REs, respectively. ▽, site-directed mutation ruining the E2F consensus sequence. *White bars*, basal promoter activity; *black bars*, E2F-activated reporter gene activity. *White boxes*, previously reported p53-responsive elements; *gray boxes*, cryptic E2F-responsive elements; *black boxes*, putative E2F-responsive elements. The average of a triplicate experiment ± S.D. (*error bars*) is shown. *E*, ChIP analysis showing the association of the *Ei24* promoter with E2F1. The *Ei24* reporter gene containing wild-type RE1–3 (−300/−116) were transiently transfected into *p53*-deficient H1299 cells with or without E2F1 expression construct, and ChIP analysis was done (*left panel*) or endogenous interaction of E2f1 with the *Ei24* promoter was monitored using wild-type and *E2f1*$^{-/-}$ MEFs (*right panel*). E2F1-associated chromatin fractions were immunoprecipitated with an E2F1-specific antibody detecting both human and mouse E2F1 proteins.

E2F1 is functional in H1299 cells (27), a relatively weak signal may also be detected without E2F1 overexpression (Fig. 3*E*, *left panel*). To verify this result, the ChIP assay was done in an endogenous condition using wild-type and *E2f1*$^{-/-}$ MEFs with the same E2F1-specific antibody. The promoter region of the *Ei24* promoter containing RE1–3 was specifically immunoprecipitated from the wild-type MEFs, but no signal was detected from the *E2f1*$^{-/-}$ MEFs (Fig. 3*E*, *right panel*), suggesting the direct binding of E2F1 on the *Ei24* promoter. Taken together, these results imply that *Ei24* is a novel transcriptional target of E2F1.

Ei24 Expression and Its Physiological Effect upon UVC Irradiation—Because E2F1 is important in the response to DNA damage (28), the expression of *Ei24* might be altered by genotoxic stresses independent of p53. To test this possibility, we employed *p53*$^{-/-}$ and *p53*$^{-/-}$ *E2f1*$^{-/-}$ MEFs to test the p53-independent role of E2F1 in regulating *Ei24* expression upon genotoxic stresses. Adriamycin, etoposide, and mitomycin C did not induce significant expressional changes between these genotypes (data not shown), but UVC irradiation significantly reduced *Ei24* expression in both genotypes (*e.g.* mock *versus* 10 J/m^2, $p < 0.005$ for *p53*$^{-/-}$ and $p < 0.05$ for *p53*$^{-/-}$ *E2f1*$^{-/-}$) (Fig. 4). Notably, the reduction in expression was more severe in the *p53*$^{-/-}$*E2f1*$^{-/-}$ MEFs than in the *p53*$^{-/-}$ MEFs (Fig. 4). Consistently, *Stathmin*, an E2F target gene, showed a similar pattern of a reduction in expression in a time-dependent experiment (Fig. 4*B*). These results indicate that UVC irradiation suppresses *Ei24* expression upon *p53* deficiency and that E2F1 mitigates the negative effect of UVC irradiation on *Ei24* expression in MEFs.

Because UVC irradiation is a potent inducer of both p53-dependent and p53-independent cell death, we examined whether Ei24 knockdown might affect UVC-induced cell death in p53$^{-/-}$ MEFs. *Ei24* expression was suppressed with lentiviruses encoding *Ei24*-specific short hairpin RNAs (shRNAs) in *p53*$^{-/-}$ MEFs (Fig. 5*A*). When these MEFs were irradiated with 20 J/m^2 UVC, annexin V-FITC- and/or propidium iodide-stained cells were prominently detected at 24 and 48 h (Fig. 5*B*). Notably, *Ei24* knockdown significantly increased UVC-induced cell death (Fig. 5, *B* and *C*). In contrast to previous reports showing the proapoptotic function of *Ei24*, these results provide evidence that EI24 may have a protective role against UVC-induced cell death in a *p53*-deficient genetic background.

DISCUSSION

In the present study, we identified *Ei24*, a p53 target gene, as an up-regulated gene in *Rb*$^{-/-}$ MEFs, and we subsequently characterized it as an E2F1 target gene. p53 and pRb/E2F are

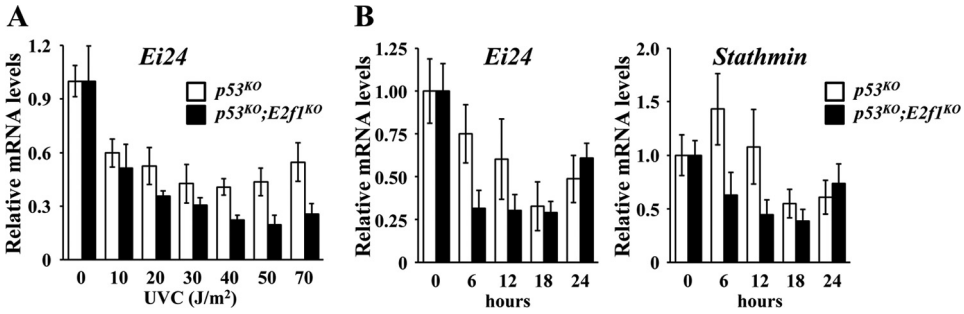

FIGURE 4. *A*, quantitative RT-PCR analysis showing the dose-dependent expression pattern of *Ei24* at 6 h after UVC irradiation in $p53^{-/-}$ and $p53^{-/-}E2f1^{-/-}$ MEFs. *B*, time-dependent expression of *Ei24* and *Stathmin* after 20 J/m^2 UVC irradiation in $p53^{-/-}$ and $p53^{-/-}E2f1^{-/-}$ MEFs. The data are represented as relative values to the mock.

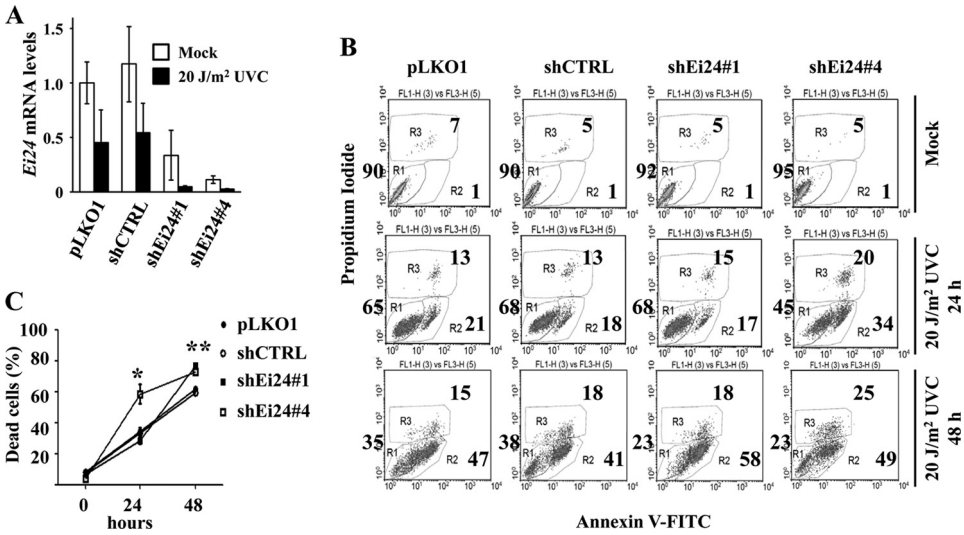

FIGURE 5. *A*, *Ei24* expression in $p53^{-/-}$ MEFs infected with lentiviruses encoding an empty vector (pLKO1), a control shRNA (shCTRL), and two independent shRNAs targeting *Ei24* (shEi24#1 and #4). UVC irradiated cells were harvested at 6 h after treatment. *B*, UVC-induced cell death at 24 and 48 h after 20 J/m^2 UVC irradiation. Note that the analytic areas of shEi24#4 were shifted to adjust focus of dead cells at 48 h because of the highly severe UVC-induced cell death of shEi24#4 cells at that time point. Experiments were conducted in triplicate. *C*, statistical analysis of dead cells observed in *B*. *, Student's *t* test, $p < 0.05$; **, $p < 0.005$.

closely related in modulating diverse cellular physiologies. When *Rb* is deficient, deregulated E2F up-regulates the expression of $p19^{Arf}$, an important tumor suppressor gene alternatively encoded by *Ink4a/Arf* locus (24, 29, 30). $p19^{Arf}$ then executes a critical role in regulating the p53 activity by compromising the MDM2-mediated degradation of p53 (31–33). This molecular interaction allows diverse p53 and E2F target genes to be simultaneously induced in $Rb^{-/-}$ MEFs. Besides the *Ink4a/Arf* locus, cross-talk between p53 and pRb/E2F is also achieved by sharing target genes or by regulating distinct genes of a common physiological process. For example, *Apaf1* is directly up-regulated by both p53 and E2F1 (23). *Bax*, a p53 target gene, and *Apaf1*, a common target of E2F and p53, are critical components for transducing intrinsic apoptotic signals and cooperate with E2F target genes including *caspases-3* and *7–9*, to properly execute apoptosis (34). Along these lines, considering its expressional regulation, EI24 may participate in cellular processes that are commonly governed by both p53 and E2F.

The potential significance of *Ei24* has been highlighted as a p53 target gene for apoptosis and growth suppression (14) and as an autophagy gene (15). As a lethal stress, UVC irradiation actively induces cell death through both p53-dependent and independent pathways (35, 36). Our results indicate that *Ei24* expression is suppressed by UVC irradiation (Fig. 3), presumably by the activation of pRb and/or its homologues, p107 and p130 (37). However, because this phenomenon was accelerated by *E2f1* deficiency, E2F1 should have a positive role in regulating Ei24 expression upon UVC irradiation. Previous studies have described *Ei24* as a proapoptotic gene, and thus its putative role as a tumor suppressor is expected (14, 17). In fact, etoposide induces *Ei24* expression (13), and *Ei24* knockdown confers cellular resistance to etoposide-induced cell death in NIH3T3 cells (38). Although these lines of evidence suggest the possible role of EI24 as a tumor suppressor, we recently reported that *Ei24*-heterozygous knock-out mice manifested attenuated skin tumorigenesis phenotypes upon 7,12-dimethylbenz[α]anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment (18). Consistently, *Ei24* knockdown sensitizes $p53^{-/-}$ MEFs to UVC irradiation (Fig. 5), raising the possibility that EI24 might exert a protective effect under a certain stress independently of p53. These results also suggest that the physiological role of EI24 in cell death is complicated and may be context- and/or environment-dependent. Meanwhile, its physiological role can be deduced from the function of EI24 as an autophagy gene (15).

Autophagy is also regulated by both p53 and E2F1 (39). Because UVC irradiation seriously damages cells, the damaged organelles, including mitochondria, should be removed by autophagy. If they are not properly eliminated by autophagy, lethal materials produced and released by the damaged mitochondria (*e.g.* reactive oxygen species or proapoptotic molecules) may make *Ei24*-silenced cells susceptible to UVC irradiation.

As *Ei24* knockdown increased the sensitivity of $p53^{-/-}$ MEFs against UVC irradiation (Fig. 5), EI24 might be critical for determining cellular survival under a certain stress independently of p53. In other words, it is highly possible that EI24 is a critical factor modulating the survival of p53-deficient cells in human diseases, *e.g.* cancers. Therefore, EI24 can be considered as a potential therapeutic target in *p53*-deficient tumors.

Taken together, these results imply that EI24 is a critical mediator/effector required for the crosstalk between p53 and pRb/E2F pathways dedicated to tumor suppression. Therefore, understanding and identifying the specific conditions that require EI24 may provide important clues for the prevention and treatment of neoplastic diseases in humans, and it may be helpful to scrutinize the molecular mechanisms of EI24 action and to evaluate the extent of mutations and expression of *Ei24* gene in diverse human cancers for proper cancer treatments.

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