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KR-POK Interacts with p53 and Represses Its Ability to Activate Transcription of p21WAF1/CDKN1A

Bu-Nam Jeon[1], Min-Kyeong Kim[1], Won-Il Choi[1], Dong-In Koh[1], Sung-Yi Hong[3], Kyung-Sup Kim[1], Minjung Kim[2], Chae-Ok Yun[2], Juyong Yoon[4], Kang-Yell Choi[4], Kyung-Ryul Lee[5], Kenneth P. Nephew[6], and Man-Wook Hur[1]

Abstract

Transcriptional regulation by p53 is thought to play a role in its ability to suppress tumorigenesis. However, there remain gaps in understanding about how p53 regulates transcription and how disrupting this function may promote cancer. Here we report a role in these processes for the kidney cancer–related gene *KR-POK* (ZBTB7C), a POZ domain and Krüppel-like zinc finger transcription factor that we found to physically interact with p53. Murine embryonic fibroblasts isolated from genetically deficient mice (*Kr-pok*$^{-/-}$ MEFs) exhibited a proliferative defect relative to wild-type mouse embryonic fibroblasts (MEF). The zinc finger domain of Kr-pok interacted directly with the DNA binding and oligomerization domains of p53. This interaction was essential for Kr-pok to bind the distal promoter region of the *CDKN1A* gene, an important p53 target gene encoding the cell-cycle regulator p21WAF1, and to inhibit p53-mediated transcriptional activation of *CDKN1A*. Kr-pok also interacted with the transcriptional corepressors NCoR and BCoR, acting to repress histone H3 and H4 deacetylation at the proximal promoter region of the *CDKN1A* gene. Importantly, *Kr-pok*$^{-/-}$ MEFs displayed an enhancement in *CDKN1A* transactivation by p53 during the DNA damage response, without any parallel changes in transcription of either the p53 or *Kr-pok* genes themselves. Furthermore, Kr-pok promoted cell proliferation *in vitro* and *in vivo*, and its expression was increased in more than 50% of the malignant human kidney cancer cases analyzed. Together, our findings define KR-POK as a transcriptional repressor with a pro-oncogenic role that relies upon binding to p53 and inhibition of its transactivation function. *Cancer Res; 72(5); 1137–48. ©2012 AACR.*

Introduction

The BTB/POZ domain–containing proteins play various cellular regulatory functions by interacting with proteins or by controlling transcription through binding to target gene promoters (1, 2). In particular, interactions between some of the POK family proteins and coregulators (e.g., BCoR, NCoR, SMRT, p300, and p120ctn) are major determinants in differentiation, development, hematopoiesis, tumor suppression, and oncogenesis (3–8). For example, promyelocytic leukemia zinc finger (PLZF) controls the development of invariant natural killer T-cell effector function and the maintenance of spermatogonial stem cells. T-helper–inducing POK (Th-POK, also known as cKrox) regulates the ratio of CD4 to CD8 cells through T-cell lineage commitment (9–12). Moreover, the aberrant expression of POK family proteins such as B-cell lymphoma 6 (BCL-6), FBI-1 (also called Pokemon), hypermethylated in cancer-1 (HIC-1), and PLZF fusion protein (PLZF-RARalpha) have been implicated in cancers such as lymphoma, spontaneous malignant tumors, and acute promyelocytic leukemia (13–19). Recently, the functions of these and other POK transcription factors have been characterized by several research groups, including ours (20–25). However, with the exception of the POK family proteins mentioned above, the functions of many POK family proteins remain uncharacterized, and the relationship between POK expression and their role in differentiation, development, tumor suppression, and oncogenesis needs to be investigated (3).

The activation of cyclin/cyclin-dependent kinase (CDK) complexes regulates cell proliferation through cell-cycle control (26). CDK inhibitors usually inhibit the activity of cyclin/CDK complexes and regulate cell-cycle arrest (27). The CDK inhibitor 1A (*CDKN1A* or p21) is a major player in cell-cycle control and is a major transcriptional target of p53 (28, 29). *CDKN1A* is mainly regulated at the transcriptional level by

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various oncogenes, tumor suppressors, and cellular regulators and is regulated posttranslationally at the level of degradation (30). The ability of p21 to inhibit proliferation may contribute to its tumor suppressor function (26, 27), and a number of oncogenes repress *CDKN1A* to promote cell growth and tumorigenesis (31). Whereas the induction of *CDKN1A* predominantly leads to cell-cycle arrest in G_1, G_2, or S-phase, repression of *CDKN1A* may have a variety of outcomes depending on the cellular context (31, 32). p21 is a key component in cell-cycle control and apoptosis that directs an antiapoptotic response following DNA damage (33). p53-dependent regulation of p21 expression likely contributes to how p53 influences cell survival or death upon DNA damage (32–35).

The tumor suppressor p53 protects the integrity of the genome. The inactivation of p53 plays a central role in tumorigenesis (36, 37). The p53 tetramer binds to its response element, in which it can recruit various transcriptional coregulators to regulate RNA polymerase II–mediated transcription at p53 target gene promoters (36, 37). Normally, the p53 protein is short-lived and present at low levels. However, in response to a variety of cellular stresses, p53 is stabilized and activated via a phosphorylation and acetylation cascade (38). Active p53 controls the expression of a set of genes required for the induction of cell-cycle arrest, senescence, DNA repair, and apoptosis (39–41). Regardless of the type of stress, the final outcome of p53 activation is either cell survival through cell-cycle arrest and DNA repair or cell death, but the mechanism leading to the choice between these outcomes has not yet been elucidated (39–41).

We characterized the molecular and cellular functions of a kidney cancer–related POK family transcription factor, KR-POK, as a proto-oncoprotein that controls cell proliferation. KR-POK is a potent negative regulator of *CDKN1A*, and the molecular interaction between p53 and KR-POK is critical for this regulation.

Materials and Methods

Plasmids, antibodies, and reagents

pcDNA3.0-FLAG-Kr-pok, pcDNA3.1-Kr-pok-Myc-His, and pcDNA3.1-Kr-pokΔPOZ constructs were prepared by cloning cDNA fragments into pcDNA3.0 or pcDNA3.1 (Invitrogen). GST-$POZ_{Kr\text{-}pok}$ and GST-$ZF_{Kr\text{-}pok}$ proteins and cDNA fragments encoding the POZ domain (a.a 1–132) and zinc fingers (a.a 365–468) were cloned into pGEX4T3 (Amersham Biosciences). All plasmid constructs were verified by sequencing. Antibodies and chemicals used are described in Supplementary Information.

Generation of *Kr-pok*$^{-/-}$ knockout mice and MEF preparation

Two sets of oligonucleotide primers (described in Supplementary Information) were used to amplify a *Kr-pok* gene NotI–XhoI DNA fragment (7.1 kb) and a BamHI–KpnI DNA fragment (4.5 kb) by the PCR amplification of mouse 129/SvJ mouse J1 genomic DNA. We incorporated the long and short arms into the targeting vector pPNT by ligation. The targeting vector was designed to replace the entire exon 4 of the *Kr-pok* gene (~1,330 bp) with a positive selection marker, the *PGK* promoter fused with a neomycin resistance gene. The targeting vector was linearized by digestion with NotI and introduced into 129/SvJ mouse J1 embryonic stem cells by electroporation. The ES cell clones resistant to G418 and ganciclovir were selected and analyzed for homologous recombination by Southern blotting. Two ES cell clones were injected into C57BL/6J blastocysts. *Kr-pok*$^{-/-}$ mice on a C57BL/6 genetic background were generated by standard laboratory animal procedures. *Kr-pok*$^{+/+}$ and *Kr-pok*$^{-/-}$ mouse embryonic fibroblasts (MEF) were prepared by standard protocol.

Cell cultures, stable cell lines, and animals

Cells were cultured in the media recommended by American Type Culture Collection. Stable NIH/3T3 cells overexpressing Kr-pok were prepared by infection with a recombinant lentivirus (LentiM1.4-Kr-pok tagged with His and Myc) and selected with puromycin.

For the *in vivo* tumor study, female nude mice (BALB/c-nu, 6–7 weeks of age) were kept in a laminar air flow cabinet maintained at 24 ± 2°C with 40% to 70% humidity under a 12-hour light/dark cycle and specific pathogen-free conditions. All facilities were approved by the Association for Assessment and Accreditation of Laboratory Animal Care, and all animal experiments were conducted in accordance with the institutional guidelines of the Animal Core Facility of the Yonsei University School of Medicine (Seoul, Korea).

Fluorescence-activated cell sorting analysis

Cells were fixed with 70% ethanol and washed with PBS containing 1% horse serum, and cellular DNA was stained with propidium iodide (100 μg/mL). A cell-cycle profile and forward scatter were determined using a Becton Dickinson FACSCalibur, and the data were analyzed using ModFit LT 2.0 (Verity Software House, Inc.).

BrdUrd incorporation

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 0.02 mmol/L of bromodeoxyuridine (BrdUrd) for 12 hours, fixed in 3.7% formaldehyde, incubated with anti-BrdUrd monoclonal antibody, and finally incubated with goat anti-mouse Cy2-conjugated secondary antibody. Cells were soaked in a 4′,6-diamidino-2-phenylindole (DAPI) solution and mounted for photography with a Radiance 2000 multi-photon (MP) imaging system (Bio-Rad).

MTT assays

To investigate the effect of Kr-pok on cellular proliferation, cells were plated onto 24-well dishes at 30% to 50% confluency and incubated. The cell growth of each sample was determined by measuring the conversion of the tetrazolium salt MTT to formazan.

GST-fusion protein purification, *in vitro* transcription, and translation of corepressors or p53 and pull-down assays

Recombinant GST, GST-$POZ_{Kr\text{-}pok}$, and GST-$ZF_{Kr\text{-}pok}$ fusion proteins were prepared from *E. coli* BL21 (DE3) by glutathione-agarose 4 bead affinity chromatography (Peptron).

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Corepressor and p53 polypeptides were prepared using TNT extracts in the presence of [^{35}S]-methionine (Promega). GST-fusion protein–agarose bead complexes were incubated with *in vitro* translated [^{35}S]-methionine–labeled corepressors or p53 polypeptides at 4°C for 4 hours in HEMG buffer. The reaction mixtures were centrifuged, pellets were rinsed, and the bound proteins were separated using 12% SDS-PAGE. The gels were exposed to X-ray film (Kodak).

Immunoprecipitation assays

Cells were washed, pelleted, and resuspended in a lysis buffer supplemented with protease inhibitors. The cell lysate was precleared, and the supernatant was incubated overnight with antibodies at 4°C, followed by incubation with protein A/G agarose beads. Beads were collected, washed, and resuspended in equal volumes of 5× SDS loading buffer. Immunoprecipitated proteins were separated with 12% SDS-PAGE. A Western blot analysis was done according to standard protocols.

Chromatin immunoprecipitation assays

Cells were fixed with formaldehyde (final 1%) to cross-link Kr-pok, p53, and histones onto the *CDKN1A* promoter. The remaining chromatin immunoprecipitation (ChIP) procedures have been reported elsewhere (21). Oligonucleotide primer sets used in ChIP are described in Supplementary Information.

Preparation of recombinant adenovirus overexpressing Kr-pok and shRNA against Kr-pok mRNA

The Kr-pok cDNA was cloned into the adenovirus E1 shuttle vector pCA14 (Microbix) to generate pCA14-Kr-pok. To prepare recombinant adenovirus expressing short hairpin RNA (shRNA) against Kr-pok, annealed shRNA DNA sequences [sense: 5′-GATCCCTCCAGTGCATCGTGAATGTTTTTCAAGAGA(loop)-ACATTCACGATGCACTGGATTTTTTTGGAA(loop)-A-3′, antisense: 5′-AGCTTTTCCAAAAA(loop)-AATCCAGTGCATCGTG-AATGTTCTCTTGAA(loop)-AAACATTCACGATGCACTGGAG-G-3′) were cloned into pSilencer 2.0-U6 (Ambion) and subcloned into the pΔE1sp1A vector. The remaining procedures have been described elsewhere and also in Supplementary Information (21).

Analysis of Kr-pok action on tumor growth *in vivo* in a xenograft assay

U343 tumor cells were implanted under the abdominal skin of female BALB/c-nu mice. Once tumors reached 100 to 120 mm^3 in volume, mice were injected intratumorally 3 times at 2-day intervals with either control dl324 or dl324-Kr-pok adenovirus (1×10^9 PFU). For the dl324-shKr-pok virus injections, viruses (2×10^8 PFU) were injected 5 times with 2-day intervals. To the study the synergistic antitumor effect of dl324-shKr-pok virus injection and γ-irradiation treatment, viruses (2×10^8 PFU) were injected 3 times at 2-day intervals, and mice were treated with 5 Gy γ-irradiation 2 days later (Gamma Cell 3000; Nordion Inc.). Tumor growth was monitored by measuring the length and width of the tumor 3 times a week using a caliper. Tumor volume was calculated as $0.523Lw^2$, in which L is the length and w is the width in mm.

Histology and immunohistochemistry

Histologic analyses of mice were conducted by following standard protocols. The 4-μm formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin reagent for counterstaining. 3,3′-Diaminobenzidine immunohistochemical staining was done according to the manufacturer's instructions (R.T.U. VECTASTAIN ABC Kit; Vector Laboratories, Inc.) after incubation with primary antibody.

Statistical analysis

Student t test was used for the statistical analyses.

Results

Kr-pok promotes cell proliferation

KR-POK, which is encoded by the *ZBTB7C* gene, contains a POZ domain (a.a. 8–132) and 4 Krüppel-like zinc fingers (a.a. 365–468). Murine KR-POK (Kr-pok) is primarily located in the nucleus and is expressed ubiquitously in mouse tissues (Supplementary Fig. S2). To study the function of the Kr-pok protein, we generated *Kr-pok* knockout mice by homologous recombination of mouse embryonic stem cells (Fig. 1A). The targeting vector was designed to replace the entire exon 4 of the *Kr-pok* gene with a positive selection marker, the *PGK* promoter fused with a neomycin resistance gene.

To investigate the effect of Kr-pok on cell proliferation, a flow cytometry [fluorescence-activated cell sorting (FACS)] analysis of *Kr-pok*$^{+/+}$ MEF and *Kr-pok*$^{-/-}$ MEF cells was done. The analyses revealed that the proportion of cells in the S-phase decreased from 35.2% to 20.1% with a loss of Kr-pok. In NIH/3T3 cells, Kr-pok increased the number of cells in the S-phase from 23.7% to 46.6%, and the number of cells in G_2-M phase decreased from 23.1% to 2.7% with the ectopic expression of Kr-pok (Fig. 1B). MTT assays *Kr-pok*$^{+/+}$ MEF, *Kr-pok*$^{-/-}$ MEF, and NIH/3T3 cells showed that Kr-pok promotes cell proliferation. Furthermore, BrdUrd incorporation assays indicated that Kr-pok promotes a transition from the G_0–G_1 to S-phase, indicating the proliferative effect of Kr-pok (Fig. 1C and D).

Kr-pok represses *CDKN1A* gene expression

Because Kr-pok has a significant effect on cell proliferation, we investigated whether Kr-pok regulates the expression of genes important in the cell cycle such as *CDKN1A*, which encodes p21. Transient cotransfection of the Kr-pok expression vector and the promoter Luciferase fusion constructs into HEK293 cells followed by transcription assays revealed that Kr-pok repressed the transcription of both the *ARF* and *CDKN1A* genes by 65% and 50%, respectively (Fig. 2A). p21 expression in *Kr-pok*$^{-/-}$ MEF cells was higher than that of *Kr-pok*$^{+/+}$ MEF cells at both the mRNA and protein levels. NIH/3T3 cells with ectopic expression of Kr-pok had lower p21 expression levels (Fig. 2B and C).

Transcriptional repression of the *CDKN1A* promoter by Kr-pok involves inhibition of p53 activity in transcription

Mapping of the upstream cis-regulatory element of the *CDKN1A* promoter that is responsible for transcriptional

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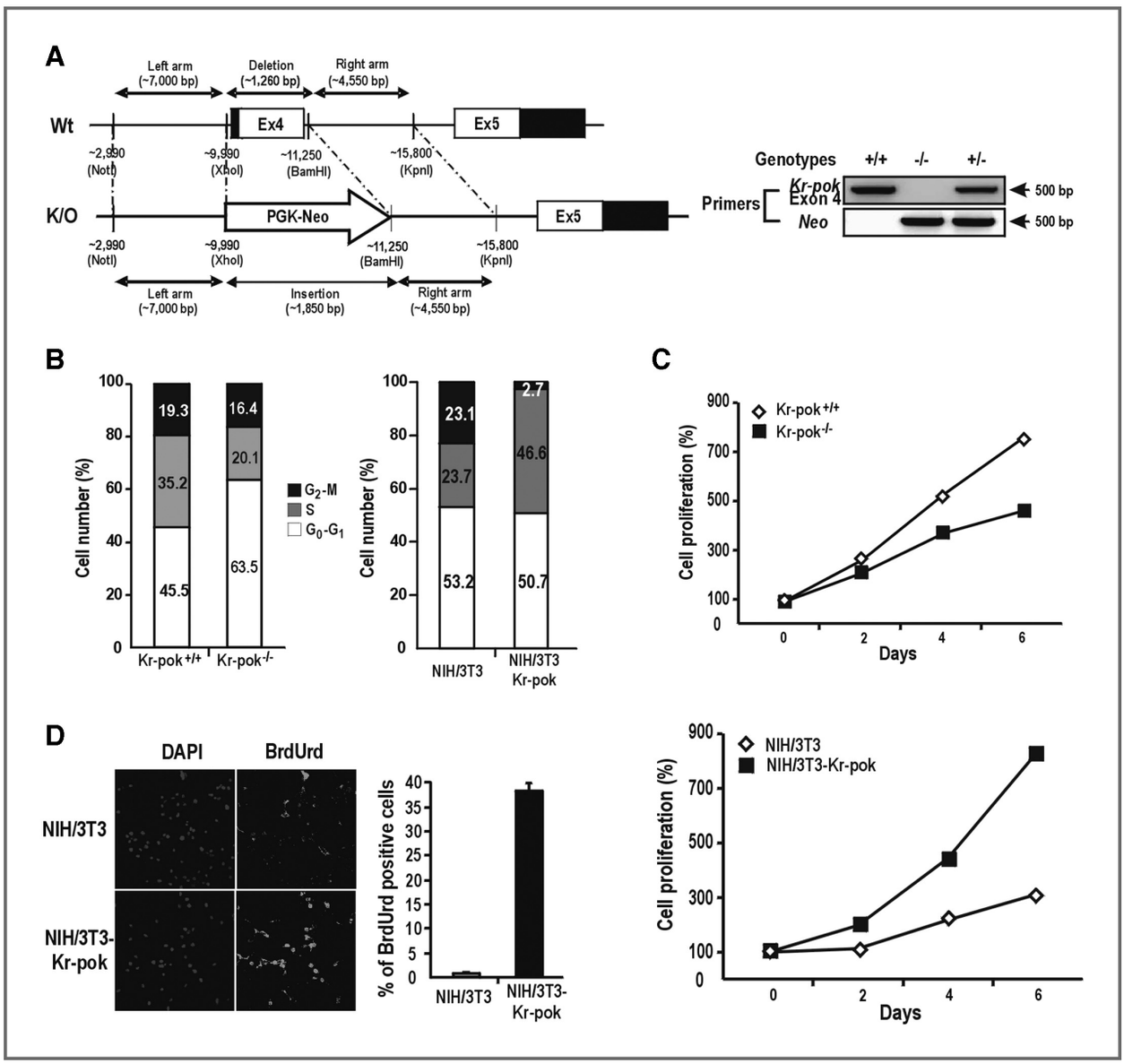

Figure 1. Kr-pok promotes cell proliferation. A, gene targeting approach to produce *Kr-pok*$^{-/-}$ mice by homologous recombination of mouse embryonic stem cells and genotyping of *Kr-pok*$^{-/-}$ mice by PCR. B, FACS analysis of *Kr-pok*$^{-/-}$ MEF cells and stable NIH/3T3-Kr-pok cells. C, MTT assay of cell proliferation. *Kr-pok*$^{+/+}$ and *Kr-pok*$^{-/-}$ MEF cells were cultured for 0 to 6 days, and analyzed for the MTT-to-formazan conversion by colorimetry at 540 to 600 nm. Alternatively, NIH3T3 and stable NIH/3T3-Kr-pok cell were grown 0 to 6 days and analyzed as above. D, BrdUrd incorporation assay. NIH/3T3 and NIH/3T3-Kr-pok cells were grown in DMEM containing BrdUrd. The cells were fixed, incubated with anti-BrdUrd antibody, and soaked with DAPI. Cells with incorporated BrdUrd were counted.

repression by Kr-pok showed that Kr-pok most potently repressed the transcription of the promoter with a 2.3-kb upstream sequence containing the distal p53 binding element (Fig. 3A). In p53-null MB352 cells cotransfected with pGL2-*CDKN1A*-Luc (-2.3 kb) and the Kr-pok expression vector, ectopic p53 potently activated the transcription of *CDKN1A*, an effect that was significantly inhibited by Kr-pok (Supplementary Fig. S3A). In HEK293 cells (wild-type p53) treated with the DNA-damaging agent etoposide, *CDKN1A* transcription was activated by induced p53, which was repressed by Kr-pok (Supplementary Fig. S3B). Transient cotransfection and transcription assays with pGL2-*TP53* promoter-Luc and Kr-pok expression plasmids of the same cells, revealed that Kr-pok did not affect the transcription of the *p53* gene (Supplementary Fig. S3C).

Furthermore, we investigated whether ectopic Kr-pok expression could repress endogenous *CDKN1A* gene expression in HCT116 p53$^{+/+}$ and p53$^{-/-}$ cells. Kr-pok repressed transcription in HCT116 p53$^{+/+}$ cells but only very weakly in HCT116 p53$^{-/-}$ cells, suggesting that Kr-pok inhibits

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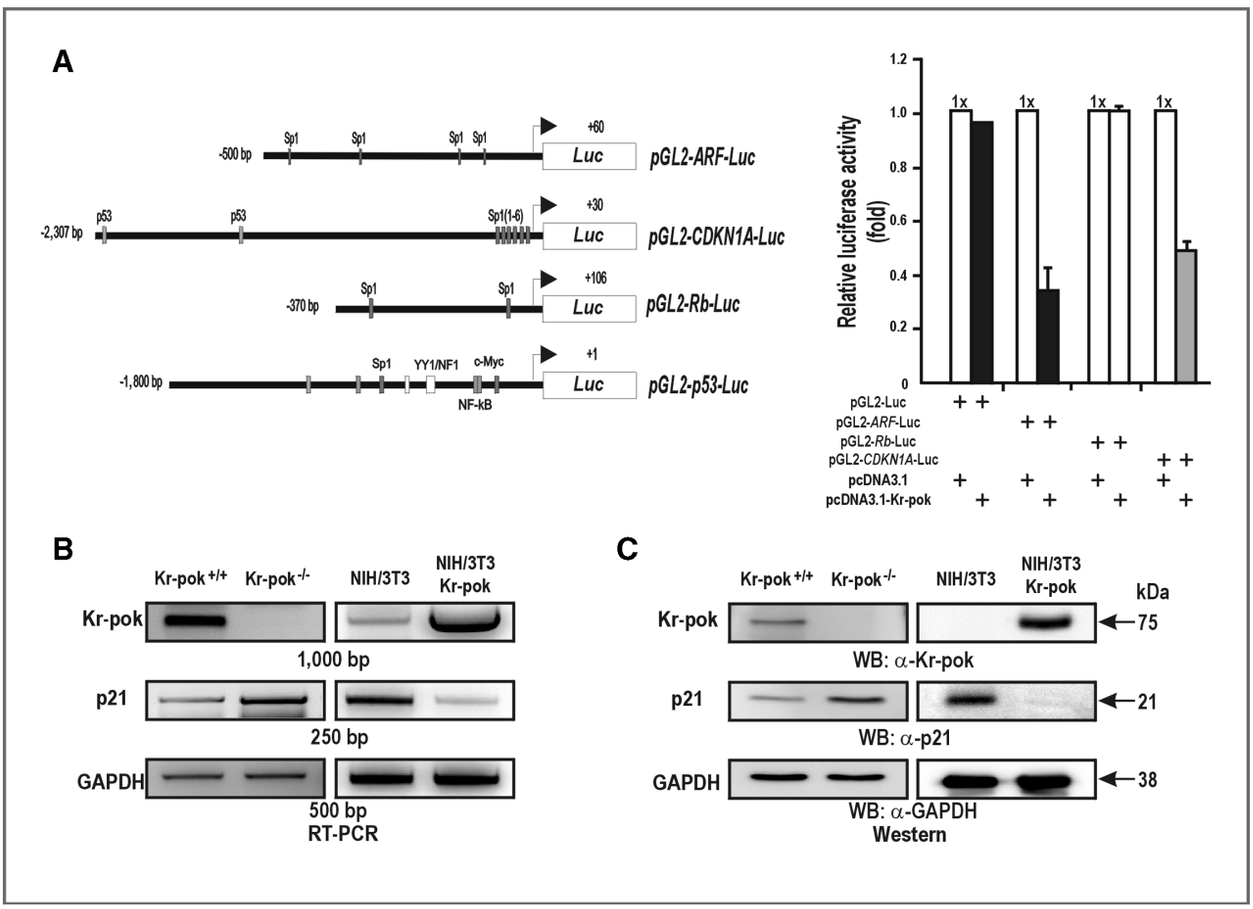

Figure 2. Kr-pok represses *CDKN1A* gene expression. A, transient transcription assays. The plasmid expressing Kr-pok and reporter plasmids were transiently cotransfected into HEK293 cells, and luciferase activity was measured. Shown left are the schematic structures of reporter plasmids. B, RT-PCR analysis of p21 mRNA isolated from the *Kr-pok*$^{+/+}$ and *Kr-pok*$^{-/-}$ MEF cells. Also examined is the p21 mRNA in the NIH/3T3 and stable NIH/3T3-Kr-pok cells infected with recombinant lentivirus. C, Western blot analysis of p21 expression in the *Kr-pok*$^{+/+}$ MEF, *Kr-pok*$^{-/-}$ MEF, NIH/3T3, and NIH/3T3-Kr-pok cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WB, Western blot.

endogenous p53 activity, which is important in the transcription of *CDKN1A* (Supplementary Fig. S3D).

Kr-pok interacts with p53, and the complex binds to the distal p53 binding element

Because Kr-pok inhibits the transcriptional activation of *CDKN1A* by p53, we investigated whether Kr-pok interacts with p53. Coimmunoprecipitation of total *Kr-pok*$^{+/+}$ MEF and *Kr-pok*$^{-/-}$ MEF cell lysates using anti–Kr-pok antibody and GST-fusion protein pull-down assays showed that p53 and Kr-pok interacted with each other directly by an interaction between the GST-$ZF_{Kr\text{-}pok}$ and the DNA binding oligomerization domain of p53 (Fig. 3B; Supplementary Fig. S4A and S4B). We next investigated whether Kr-pok binds to the *CDKN1A* promoter and the functional significance of the protein interaction between Kr-pok and p53 by ChIP assays. In p53 wild-type HEK293 cells, Kr-pok specifically binds to the distal p53 binding region (bp −2,307 to −1,947) but not to other regions tested (Fig. 3C and D).

We also investigated whether Kr-pok interacts with the p53 mutants (p53Mt) that are found frequently in human cancers and whether the *in vivo* binding requires the wild-type conformation of the DBD of p53. Kr-pok interacts with the p53Mt with mutations at the DNA binding domain or oligomerization domain (R175H, G245S, R248W, R273H, and K320R; Supplementary Fig. S4C and S4D). However, ChIP assays revealed that p53 with mutations in the DNA binding domain neither bind to the p53 binding element nor recruit Kr-pok. In contrast, p53K320R, which has a mutation in the oligomerization domain (a.a. 320), but retains DNA binding capability, recruited Kr-pok to the distal p53 binding element *in vivo* (Fig. 3D). The conformation of the p53 DNA binding domain and actual binding of p53 seems to be important in Kr-pok binding. These data indicate that Kr-pok can be tethered to the *CDKN1A* distal promoter region by directly interacting with p53 using its zinc-finger domain to repress the transcription of the *CDKN1A*.

We investigated whether the protein interaction between p53 and Kr-pok in transcriptional repression is limited to the

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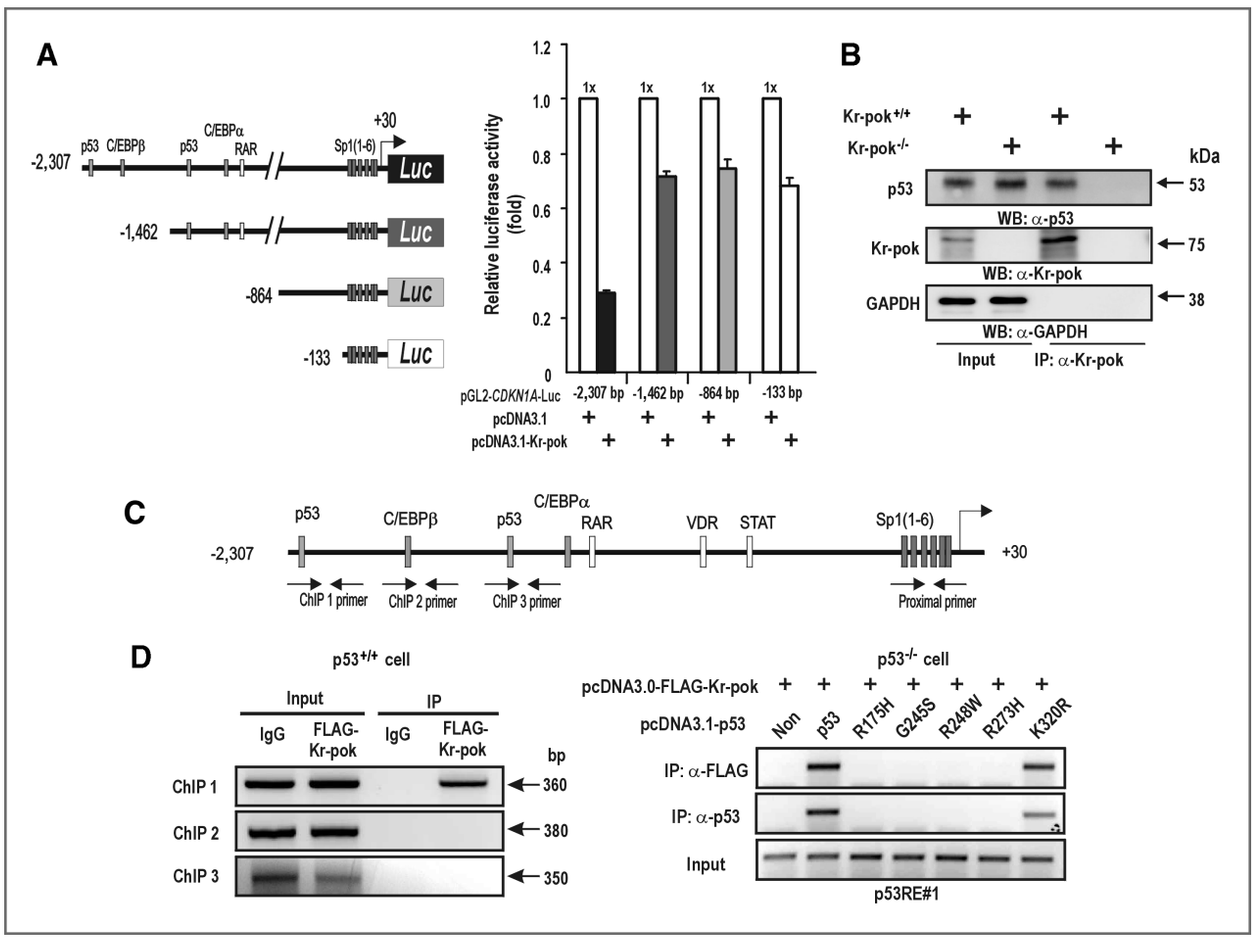

Figure 3. Kr-pok interacts with p53 and the complex binds to the distal p53 binding element. Kr-pok directly interacts with p53 via its zinc fingers. A, transient transcription assays. Plasmids expressing Kr-pok and the pGL2-*CDKN1A*-Luc fusion promoter constructs were transiently cotransfected into HEK293 cells and analyzed for luciferase activity. B, coimmunoprecipitation of endogenous Kr-pok and p53. Lysates prepared from the *Kr-pok*$^{+/+}$ and *Kr-pok*$^{-/-}$ MEF cells were immunoprecipitated using the anti-Kr-pok antibody and analyzed using Western blotting with anti-p53 antibody. C, structures of human *CDKN1A* gene promoter. Arrows at the promoter regions indicate the locations of ChIP PCR primer sets. D, left: ChIP assay of Kr-pok binding to the endogenous *CDKN1A* promoter in HEK293 cells. Cells were transfected with pcDNA3.0-FLAG-Kr-pok, and chromatin was immunoprecipitated with the antibodies indicated. Arrows indicate the primers used in ChIP. Right: ChIP assay of Kr-pok binding to the endogenous *CDKN1A* promoter in HCT116 p53$^{-/-}$ cells. HCT116 p53$^{-/-}$ cells were transfected with FLAG-Kr-pok and/or p53 wild-type and mutant expression plasmids and immunoprecipitated with the antibodies indicated. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IP, immunoprecipitation; WB, Western blot.

regulation of *CDKN1A* or whether the interaction is also significant in the other genes that are controlled by p53. Quantitative reverse transcriptase PCR (qRT-PCR) and Western blot analyses showed that *Bax*, *Noxa*, and *Survivin* expression was derepressed in *Kr-pok*$^{-/-}$ MEF cells but that Kr-pok has no effect on the *Puma* gene (Supplementary Fig. S5A and S5B). Interestingly, the antiapoptotic Survivin was derepressed in *Kr-pok*$^{-/-}$ MEF cells even though this gene is repressed by p53. p53 and Kr-pok may interact to repress the transcription of the *Bax*, *Noxa*, and *Survivin* gene promoters by a mechanism similar to that for *CDKN1A*. Although *Survivin* expression is increased in *Kr-pok*$^{-/-}$ MEF cells, cells still undergo cell-cycle arrest or apoptosis, probably because Kr-pok regulates other genes that are important in cell cycle or apoptosis.

KR-pok represses the transcriptional activation of the *CDKN1A* promoter via γ-irradiation–induced p53

Because Kr-pok represses *CDKN1A* gene transcription, we examined whether Kr-pok and γ-irradiation affect the expression of p53 and p21 in *Kr-pok*$^{+/+}$ MEF and *Kr-pok*$^{-/-}$ MEF cells. Endogenous p21 mRNA levels were higher in *Kr-pok*$^{-/-}$ MEF cells compared with that of *Kr-pok*$^{+/+}$ MEF cells, suggesting a repressive role of Kr-pok on endogenous *CDKN1A* gene transcription. Kr-pok mRNA expression in *Kr-pok*$^{+/+}$ MEF cells was not altered by γ-irradiation. p53 mRNA expression induced by γ-irradiation was similar regardless of *Kr-pok* genetic backgrounds. γ-Irradiation increased the transcription of the *CDKN1A* gene 2.6-fold in *Kr-pok*$^{-/-}$ MEF cells and 2-fold in *Kr-pok*$^{+/+}$ MEF cells, suggesting an inhibitory role of endogenous Kr-pok on

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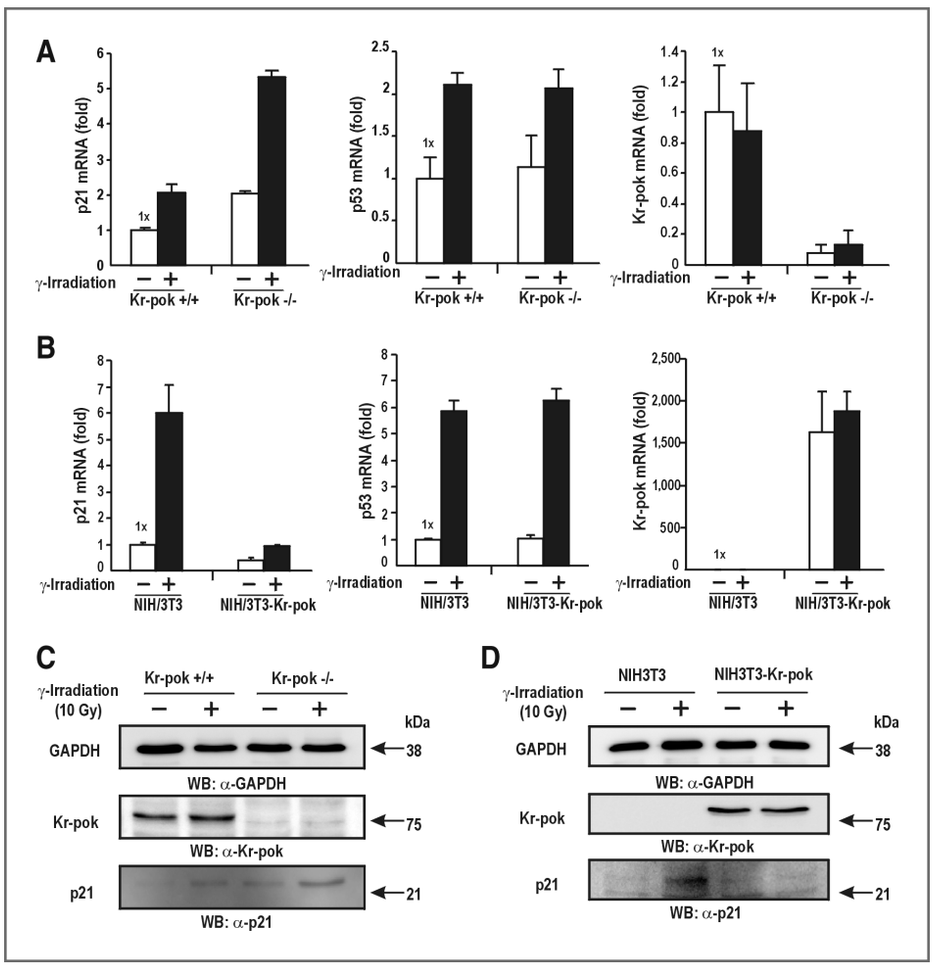

Figure 4. Kr-pok represses transcriptional activation of the *CDKN1A* promoter by γ-irradiation–induced p53. A, qRT-PCR analysis of p21 mRNA from *Kr-pok*$^{+/+}$ and *Kr-pok*$^{-/-}$ MEF cells exposed to γ-irradiation. B, qRT-PCR analysis of p21 mRNA from NIH/3T3 and stable NIH/3T3-Kr-pok cells exposed to γ-irradiation. C, Western blot analysis of p21 expression from *Kr-pok*$^{+/+}$ and *Kr-pok*$^{-/-}$ MEF cells exposed to γ-irradiation. D, Western blot analysis of p21 expression from NIH/3T3 and stable NIH/3T3-Kr-pok cells exposed to γ-irradiation. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WB, Western blot.

p53-dependent *CDKN1A* gene transcription. The Western blot analysis of p21 protein expression in *Kr-pok*$^{+/+}$ MEF and *Kr-pok*$^{-/-}$ MEF cell lysates showed similar results to the p21 mRNA analysis (Fig. 4A and C). The role of Kr-pok as a transcriptional repressor in response to DNA damage is also supported by a gain-of-function study in NIH/3T3 cells. γ-Irradiation increased *CDKN1A* gene transcription 6-fold in the NIH/3T3 cells, but the transcriptional activation was significantly decreased by ectopic Kr-pok and was increased only 2-fold by γ-irradiation. Kr-pok does not affect *p53* gene transcription. The Western blot analysis of p21 protein expression in NIH/3T3 cell lysates showed similar results to the p21 mRNA analysis (Fig. 4B and D).

Kr-pok interacts with transcriptional corepressors to repress transcription and deacetylate histones H3 and H4 at the proximal promoter via its POZ domain

Kr-pok binds to the p53 binding element with p53, and Kr-pok, thereby inhibits the transcriptional activation of *CDKN1A* by p53. Mutant Kr-pokΔPOZ, which lacks the POZ domain, did not inhibit transcription, suggesting that the POZ domain is important in transcriptional repression (Fig. 5A). Transcriptional repressors often inhibit transcription through an interaction with corepressors such as SMRT, NCoR, and BCoR. Mammalian 2-hybrid assays using pG5-Luc, pGal4-POZ, and pVP16-corepressor fusion expression plasmids have previously shown that the POZ domain interacts with NCoR and BCoR but not with SMRT (4, 7, 8). Our studies showed that the POZ domain strongly interacts with NCoR (Supplementary Fig. S6A). Corepressor complexes recruited by transcriptional repressors often contain histone deacetylase (HDAC) proteins. We found that the HDAC inhibitor TSA significantly decreased transcriptional repression by Kr-pok. This finding suggests a role for HDACs in transcriptional repression by Kr-pok (Supplementary Fig. S6B).

GST-fusion protein pull-down assays also showed that the POZ domain directly interacted with NCoR and BCoR (Fig. 5B). Coimmunoprecipitation of *Kr-pok*$^{+/+}$ and *Kr-pok*$^{-/-}$ MEF cell extracts showed that Kr-pok interacts with NCoR and HDAC3 (Fig. 5C; Supplementary Fig. S6C). The ChIP assay showed that Kr-pok decreased the amount of acetylated histones H3 and H4 at the proximal promoter (Fig. 5D). These data suggest that histone deacetylation at the *CDKN1A* gene proximal promoter is responsible for transcriptional repression by the p53–Kr-

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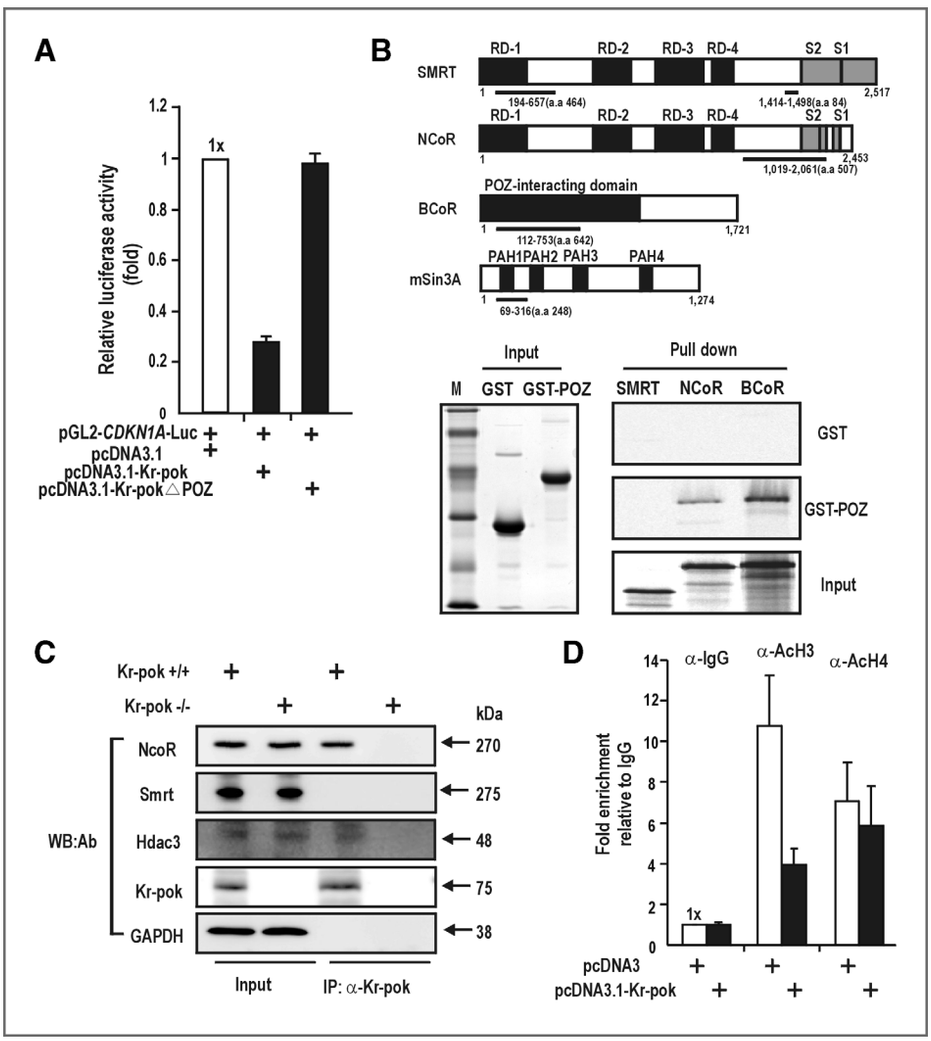

Figure 5. Kr-pok interacts with transcriptional corepressors to repress transcription and deacetylate histones H3 and H4 at the proximal promoter via its POZ domain. A, the pGL2-*CDKN1A*-Luc and expression vector of Kr-pok or Kr-pokΔPOZ were transiently cotransfected into HEK293 cells, and luciferase activities were measured. B, GST-fusion protein pull-down assays. Recombinant GST or GST-POZ was incubated with *in vitro* synthesized [^{35}S]-methionine–labeled corepressors, pulled down, and resolved by 12% SDS-PAGE. The gel was exposed to X-ray film. C, coimmunoprecipitation of endogenous Kr-pok, NCoR, SMRT, and HDAC3. Lysates prepared from the *Kr-pok*$^{+/+}$ and *Kr-pok*$^{-/-}$ MEF cells were immunoprecipitated with the anti-Kr-pok antibody and analyzed by Western blotting with the antibodies indicated. D, ChIP assays of the proximal promoter of endogenous *p21* using antibodies against acetylated H3 and H4.

pok-corepressor–HDAC3 complex on the distal p53-responsive element.

Kr-pok promotes tumor growth in a mouse xenograft model

The adenovirus Ad-dl324-Kr-pok, overexpressing Kr-pok, promoted the tumor growth after infecting of U343 MG tumor cells implanted under the skin of female nude mice (BALB/c-nu). U343 cells express p53WT and KR-POK, and form tumors easily. By day 60 of infection, tumors infected with Ad-dl324-Kr-pok reached an average volume of 1,190.5 mm^3, but those treated with Ad-dl324 (control) reached only 390.4 mm^3. Alternatively, the knockdown of Kr-pok expression by an infection with sh-Kr-pok adenovirus inhibited tumor growth by 71% (Fig. 6A, $n = 6$, $P < 0.05$). Finally, to study the feasibility of knocking down KR-POK to treat cancer, we examined whether the recombinant adenovirus, Ad-dl324-shKr-pok, plus γ-irradiation could significantly reduce tumor size. U343 MG tumor cells were implanted under the skin of female BALB/c-nu mice, recombinant Ad-dl324-shKr-pok, or control Ad-dl324 was injected into the tumor, and subsequently tumors were exposed to γ-irradiation (5 Gy). Whereas either Ad-dl324-shKr-pok or γ-irradiation alone reduced the tumor volume, the 2 agents combined induced potent and synergistic tumor volume reduction (Fig. 6B, $n = 6$, $P < 0.05$).

KR-POK expression is high in human kidney cancer tissues

An immunohistochemical analysis of human tissue microarrays of paired normal and cancer tissues using an antibody against KR-POK showed that KR-POK expression was significantly higher in kidney, cervix, parotid gland, soft tissue, and thymus cancer tissues compared with paired normal tissues (Fig. 7A and B). In particular, KR-POK expression was significantly higher than normal in 47 of 69 (68.1%) malignant kidney cancer cases tested, but in the other 22 malignant cases, the percentage of KR-POK–positive cells was similar to that of normal kidney tissues. The mean percentage of KR-POK–positive cells in normal kidney tissues was 1.71%, and that of malignant kidney

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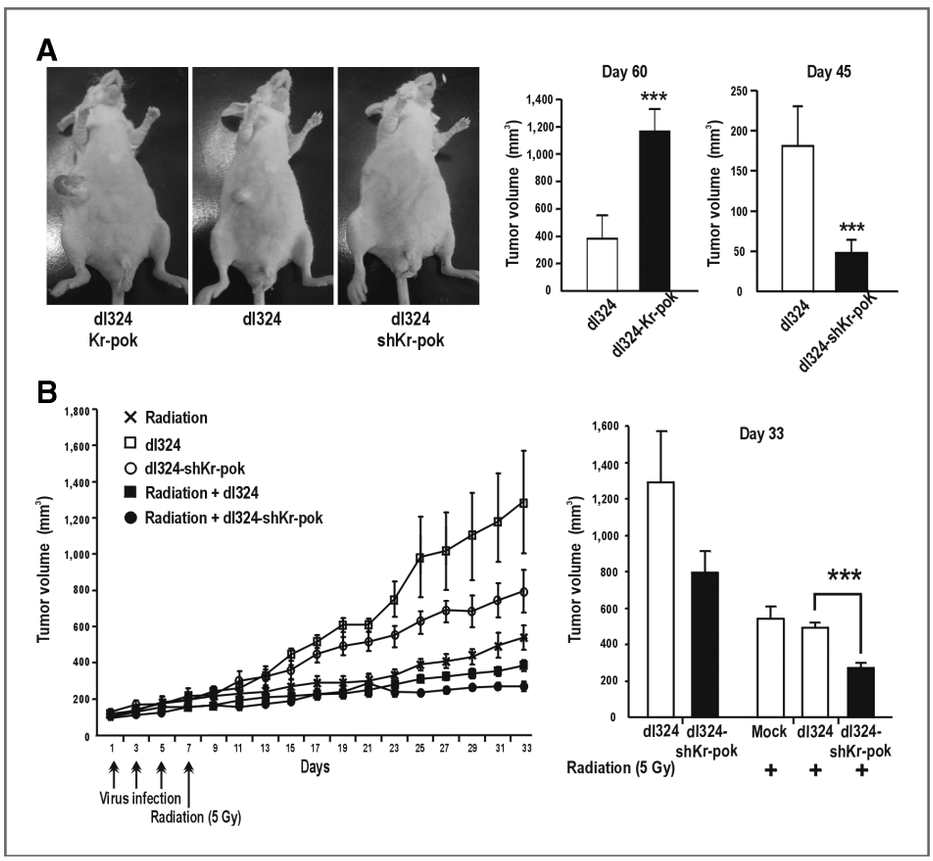

Figure 6. Kr-pok promotes tumor growth in a mouse xenograft model. A, tumor xenograft assay with brain glioma U343 cells. The tumor tissues were injected with the indicated recombinant adenoviruses, and tumor volume was measured every 2 days. Mice were injected with the control adenovirus dl324, dl324/Kr-pok, or dl324-shKr-pok. B, xenograft assay of tumor (brain glioma U343 cell derived) growth after combined treatment with γ-irradiation and adenovirus dl324-shKr-pok. The tumor tissues were injected with the control adenovirus dl324 or dl324-shKr-pok and subsequently exposed to γ-irradiation (5 Gy). Tumor volume was measured every other day. Tumor volumes are indicated as the mean ± SEM. ***, $P < 0.05$ versus control dl324.

cancer tissues with high KR-POK expression was 10.1% ($P < 0.05$), suggesting a high correlation between KR-POK expression and kidney cancer development (Fig. 7A, $P < 0.05$). Accordingly, we refer to this kidney cancer–related POK family transcription factor as KR-POK. High KR-POK expression in cancer tissue seems to increase tumor growth by stimulating cancer cell proliferation.

Discussion

POK family proteins with a BTB/POZ domain and a Krüppel-like zinc finger are major regulators of differentiation, development, hematopoiesis, oncogenesis, and tumor suppression (3–8). We characterized the biochemical and cellular functions of a proto-oncogenic POK family transcription factor, KR-POK, the expression of which is high in kidney cancer tissues [47 of 69 (68%) malignant kidney cancer patients]. This high expression suggests that a strong correlation exists between KR-POK expression level and kidney cancer development. Our investigation revealed that Kr-pok represses the transcription of *ARF* and the cell-cycle arrest gene *CDKN1A.* And p53-responsive *CDKN1A* gene is the direct target gene of Kr-pok. The transcriptional repression of *ARF* can down regulate p53 expression, and Kr-pok inhibits the transcriptional activity of p53, which results in the repression of *CDKN1A* gene expression. These data may explain why Kr-pok potently stimulates cell proliferation and tumor growth.

Our mechanistic study on the transcriptional repression of *CDKN1A* by Kr-pok provides information on the functions of protein interactions between Kr-pok, p53, and corepressors in *CDKN1A* gene transcription and perhaps in cell proliferation. From on our findings, we propose a hypothetical model of the transcriptional regulation of the *CDKN1A* gene by Kr-pok (Supplementary Fig. S7). In the absence of or with low levels of Kr-pok, when p53 expression is at basal levels or is induced by DNA damage, the transcription of the *CDKN1A* gene is activated by p53 and coactivator interaction, which acetylates histones at the proximal promoter. In cancer cells with high Kr-pok expression such as kidney cancer tissues, Kr-pok and p53 complex binding to the distal p53-responsive element represses transcription of the *CDKN1A* gene. The recruitment of a corepressor complex to the Kr-pok–p53 complex at the distal promoter results in the deacetylation of histones at the proximal promoter of *CDKN1A* (Supplementary Fig. S7). The conformation of the p53 DNA binding domain and actual binding of p53 seems to be important in Kr-pok binding and transcriptional repression by the p53 and Kr-pok complex. In addition, we found that the function of protein interaction between p53 and Kr-pok in transcriptional repression is not limited to *CDKN1A* but that the interaction

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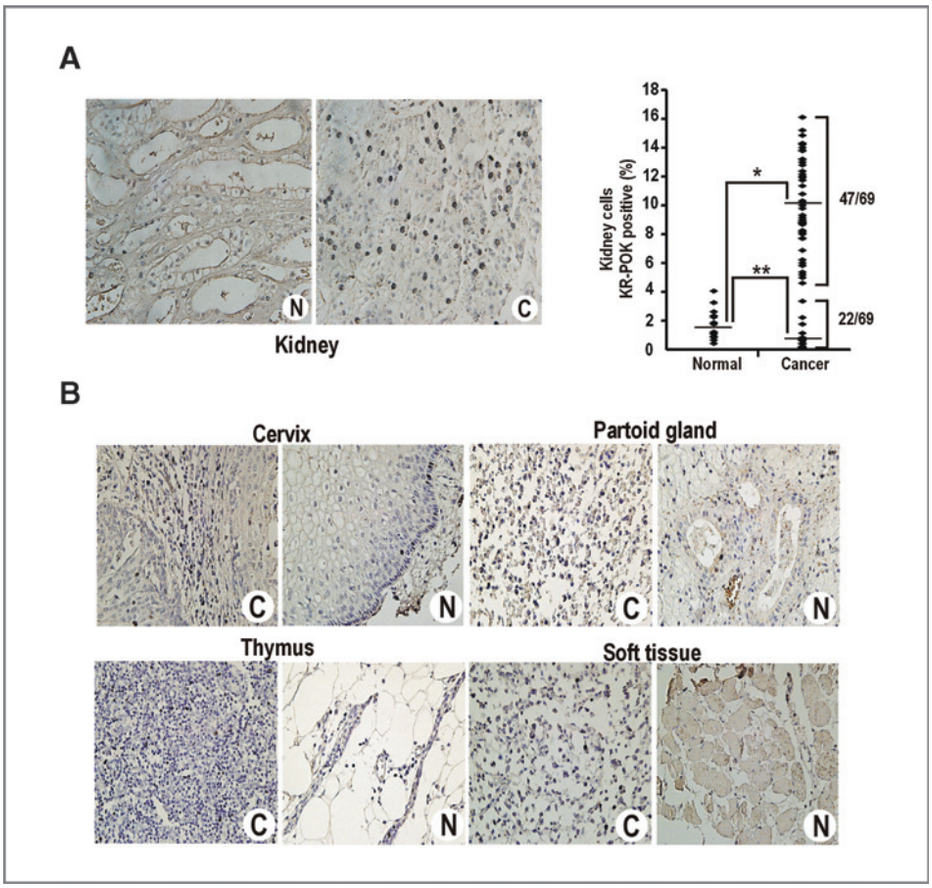

Figure 7. KR-POK expression is high in human kidney cancer tissues. A, immunohistochemical analysis of KR-POK expression in paired normal (N) and cancerous (C) kidney tissues. The microscopy images were analyzed by the ImmunoRatio Program (http://imtmicroscope.uta.fi/immunoratio; *, $P < 0.05$; **, No statistical significance, *t* test). ◆, percentage of KR-POK–positive cells in each normal and tumor kidney tissue; —, mean percentage of total KR-POK–positive cells (%). B, immunohistochemistry of paired normal and cancer tissues of the cervix, parotid gland, thymus, and soft tissues. The KR-POK expression was analyzed as above, revealing that KR-POK levels are high in these cancer tissues.

is also significant in the regulation of other genes that are reported to be controlled by p53, including *Bax*, *Noxa*, and *Survivin*, but not *Puma*.

KR-POK (APM-1, ZBTB7C) was originally isolated as an aberrant fusion transcript with papillomavirus oncogenes in cervical carcinoma cell lines. APM-1 was shown to inhibit foci formation in HeLa and CaSki cells and was proposed to be a tumor suppressor (42). However, our thorough investigation of the function of KR-POK in *Kr-pok*$^{+/+}$, *Kr-pok*$^{-/-}$ MEF, NIH/3T3, and HEK293 cells reveals that KR-POK is in fact a proto-oncoprotein candidate.

The tumor suppressor p53 is a DNA damage inducible transcriptional activator of *CDKN1A*, and it plays a crucial role in growth arrest, DNA repair, and apoptosis. Our data show that Kr-pok inhibits the transcriptional activation of *CDKN1A* by interacting with p53 and thereby inhibits cell-cycle arrest under DNA damage or normal physiologic conditions. p53 is an important transcriptional activator of many genes that regulate cell cycle, apoptosis, metabolism, development, differentiation, and forth. Moreover, p53 was found to act as a transcriptional repressor of many genes, such as dUTPase, Cdc20, and Pdk1, by an interaction with corepressors and other regulatory proteins (43–45). In particular, protein interactions among p53, mSin3A, and HDAC1 were found to be important in the regulation of *Map4* and *stathmin* gene expression. In other studies, p53 was found to interact with HDAC and Polycomb on the promoter of the tumor suppressor *Arf* (46). Kr-pok contains sequences for specific transcription factor features and acts as a transcriptional corepressor to recruit NCoR-HDAC complexes (47). In this respect, it is similar to FBI-1, which acts as an AR corepressor by recruiting NCoR and SMRT in prostate cancer cells (48).

Recently, our group published a paper on the regulation of the *CDKN1A* gene by MIZ-1 and Kr-pok at the proximal promoter region, where binding competition between MIZ-1 and Kr-pok is important. We were able to show novel actions of MIZ-1 and Kr-pok and the potential involvement of p53 and Sp1 in the process. Although we were able to dissect the molecular mechanism in great detail at the proximal promoter, the molecular events on the distal p53 binding elements were unknown. This article describes the molecular events of the protein interaction between p53 and Kr-pok on the distal p53 binding element, and we attempted to illustrate the functional significance of this unique protein interaction. p53 stimulates cell proliferation by interacting with Kr-pok, and this interaction is potentially important in oncogenesis or proliferation of cancer cells with high p53 and Kr-pok expression.

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Our findings on p53 and KR-POK have clinical implications that are relevant to genotoxic ionizing radiation therapy or chemotherapy. Many cancer treatment strategies, such as ionizing radiation and chemotherapy, take advantage of the p53 functions of inducing cell-cycle arrest and apoptosis in response to severe DNA damage. Our study suggests that for cancer tissue expressing a high level of KR-POK, additional therapeutic methods that can reduce KR-POK expression may be needed for successful cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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