

Harnessing the p53-PUMA Axis to Overcome DNA Damage Resistance in Renal Cell Carcinoma¹

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Abstract

Resistance to DNA damage–induced apoptosis is a hallmark of cancer and a major cause of treatment failure and lethal disease outcome. A tumor entity that is largely resistant to DNA-damaging therapies including chemo- or radiotherapy is renal cell carcinoma (RCC). This study was designed to explore the underlying molecular mechanisms of DNA damage resistance in RCC to develop strategies to resensitize tumor cells to DNA damage–induced apoptosis. Here, we show that apoptosis-resistant RCC cells have a disconnect between activation of p53 and upregulation of the downstream proapoptotic protein p53 upregulated modulator of apoptosis (PUMA). We demonstrate that this disconnect is not caused by gene-specific repression through CCCTC-binding factor (CTCF) but instead by aberrant chromatin compaction. Treatment with an HDAC inhibitor was found to effectively reactivate PUMA expression on the mRNA and protein level and to revert resistance to DNA damage–induced cell death. Ectopic expression of PUMA was found to resensitize a panel of RCC cell lines to four different DNA-damaging agents tested. Remarkably, all RCC cell lines analyzed were wild-type for p53, and a knockdown was likewise able to sensitize RCC cells to acute genotoxic stress. Taken together, our results indicate that DNA damage resistance in RCC is reversible, involves the p53-PUMA axis, and is potentially targetable to improve the oncological outcomes of RCC patients.

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Introduction

Patients with metastatic renal cell carcinoma (RCC) have, with a few exceptions, no curative options. Tyrosine kinase inhibitors have successfully been introduced into the clinical care of patients with advanced clear cell RCC, but most patients experience a progression-free survival benefit of only 12 to 14 months. Additional treatment approaches are hence urgently needed [1]. RCCs are commonly resistant to conventional anticancer therapies such as radiotherapy and chemotherapy. The underlying molecular mechanisms are, with

some exceptions such as expression of multidrug resistance transporters [2], poorly understood.

On the molecular level, chemo- or radiotherapy is believed to function mainly through the induction of DNA damage. In particular, DNA double-strand breaks (DSBs) are highly toxic and trigger an acute cellular response. After induction of a DNA DSB, a cascade of events is initiated to halt cell cycle progression and activate DNA repair mechanisms, which, in the case of a DSB, involve nonhomologous end joining or homology-directed repair. If the damage is too severe to be repaired, cells either enter premature senescence or undergo apoptosis [3]. Activation of p53 has long been shown to be a key event in this scenario, and a number of critical downstream mechanisms involved in different branches of the p53-mediated response to DNA damage have been identified. Whereas the cell cycle arrest involves, among others, the transcriptional upregulation of the cyclin-dependent kinase (CDK) inhibitor p21^{Cip1}, p53-dependent apoptosis involves genes such as *PUMA*, *NOXA*, and *BAX*, which participate in the mitochondrial pathway of apoptosis [4].

The BH3-only protein PUMA has been identified as a key regulator of p53-dependent and -independent apoptosis following genotoxic insults [5–7]. PUMA binds and inhibits antiapoptotic BCL-2 proteins, thereby relieving the inhibition of the proapoptotic proteins BAK/BAX and inducing mitochondrial outer membrane permeabilization [8]. Members of the BCL-2 network are frequently disrupted in primary RCCs, and furthermore, functional studies have shown that the profound DNA damage resistance of this tumor type involves defects in p53-mediated proapoptotic signaling [9–12].

Here, we show that resistance to DNA damage-induced apoptosis in RCC cells involves a disconnect between p53 activation and upregulation of the proapoptotic protein PUMA that can be reverted by histone deacetylase (HDAC) inhibition. Our results underscore that the DNA damage resistance in RCC is reversible and may hence translate into novel therapeutic concepts to improve patient outcome.

Material and Methods

Cells Culture and Transfections

Human embryonic kidney (HEK) 293 as well as Caki-1, Caki-2, and A498 RCC cell lines were obtained commercially and maintained as recommended by the distributor (CLS). The media were supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 mg/ml streptomycin (PAA). Plasmids used for transfections were pCMV-p53 and pHA-PUMA (both from Bert Vogelstein through Addgene) or empty control vectors. The target sequences for siRNA-mediated knockdown of CCCTC-binding factor (CTCF) was 5'-ACCAGTGTGATTACGCTTGTA-3' (Qiagen). For knockdown of PUMA, pooled siRNA duplexes (Trilencer-27, OriGene, SR308982) were used. For knockdown of p53, shRNA constructs (TR320558; OriGene) were tested for maximum efficiency, and two were selected for further experiments. Knockdown of protein expression was confirmed by immunoblot analyses. For transient transfections, 0.5×10^6 were transfected with 2 μ g of plasmid DNA or 15 μ l of 20 μ M annealed RNA duplexes (Neon Transfection System, Life Technologies).

Antibodies and Reagents

Antibodies used were directed against ATM (MAT3-4G10/8; Sigma), phospho-ATM S1981 (Rockland), CTCF (D31H2; Cell

Signaling), cleaved caspase-3 (5A1E; Cell Signaling), cyclin A (6E6; Leica Biosystems), PARP-1 (Ab-2; Calbiochem), PUMA (Cell Signaling or Millipore), p53 (DO-1; Santa Cruz), β -actin (AC-15, Sigma), or GAPDH (FL-335; Santa Cruz). Neocarzinostatin (NCS), cisplatin, gemcitabine, daunorubicin, and trichostatin A (TSA) were obtained commercially (Sigma).

Flow Cytometry

After 72-hour treatment with NCS (200 ng/ml), 1×10^6 cells were washed twice with PBS (1% BSA) and fixed with 70% ethanol, then treated with 10 μ g/ml RNase A in PBS, and incubated at 37°C for 30 minutes before staining with 50 μ g/ml propidium iodide for 30 minutes at 37°C. The cells were then analyzed using a FACSCalibur flow cytometer and the ModFit software.

Immunofluorescence Microscopy

Cells were grown on coverslips and processed for immunofluorescence analysis as previously described. An antibody against PUMA (Cell Signaling) was used at a 1:50 dilution followed by an Alexa Fluor 488-conjugated donkey secondary antibody (Life Technologies). Cells were analyzed using a DM5000B Leica fluorescence microscope.

Quantitative Polymerase Chain Reaction

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis was performed as previously described. Primers to p53 (forward: 5'-GAGGTTGGCTCTGACTGTACC-3', reverse: 5'-TCCGTCCCAGTAGATTACCAC-3'), PUMA (forward: 5'-ATGGCGGACGACCTCAAC-3', reverse: 5'-AGTCCCATGAAGAGATTGTACATGAC-3'), p21^{Cip1} (forward: 5'-TGTCCGTCAGAACCCATGC-3', reverse: 5'-AAAGTCGAAGTCCATCGCTC-3'), CTCF (forward: 5'-ACCAACCAGCCCAAACAGAAC-3', reverse: 5'-GTATTCTGGTCTTCAACCTGAATGATA-3'), and GAPDH (forward: 5'-ACAACCTTTGGTATCGTGGAAGG-3', reverse: 5'-GCCATCACGCCACAGTTTC-3') were obtained from Integrated DNA Technologies. GAPDH served as the reference gene for relative quantification.

Cell Viability Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltriazolium bromide (MTT) assay (Molecular Probes) was used according to the manufacturer's protocol. The absorbance at 570 nm was measured using a Glomax Multi luminometer (Promega).

Cell Treatment and Immunoblotting

Cells were cultured in the presence of NCS (200 ng/ml), cisplatin (10 μ M), gemcitabine (10 μ M), daunorubicin (0.5 μ M), and/or TSA (1 μ M or 0.5 μ M) for the time intervals indicated. After treatment, adherent and floating cells were harvested, washed, and processed for immunoblot analysis. Cells were lysed in NP-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40) containing protease inhibitors (10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 μ M phenylmethylsulfonyl fluoride, and 2 μ M vanadate). Thirty or 50 μ g of protein was separated by 10% SDS-PAGE electrophoresis and blotted onto a nitrocellulose membrane. Membranes were incubated with primary antibody at 4°C overnight, washed with 0.1% Tween 20 in PBS thrice, and incubated with horseradish peroxidase-conjugated secondary antibody (Life Technologies) for 2 hours at room temperature. Then, proteins were detected with an ECL detection system

(Thermo Scientific). All Western blot experiments were performed at least thrice.

Statistical Analysis

Student's *t* test for independent samples was used to compare experimental groups. *P* values $\leq .05$ were considered significant, and all tests were performed two-sided. Data analysis was performed using the SPSS software package (SPSS) or GraphPad Prism (GraphPad).

Results

DNA Damage–Induced Apoptosis, But Not Cell Cycle Arrest, Is Impaired in Renal Cancer Cells

To explore cell fate decisions in response to DNA damage in benign and malignant renal cells, we treated HEK293 and p53 wild-type RCC cell lines Caki-2, Caki-1, and A498 with the radiomimetic, DNA breakage–inducing compound NCS. NCS induced apoptosis in HEK293 cells after 72 hours (30.5% vs 0.1% sub-G1 cells in controls, Figure 1A). In contrast, Caki-2 cells failed to undergo apoptosis and instead arrested mainly in the G2/M phase of the cell

cycle (37.9% vs 10.1% in controls, Figure 1A). We also analyzed Caki-1 and A-498 cells, but these RCC cell lines showed a mixed response with an increase of both apoptotic as well as cell cycle–arrested cells (Figure 1A). Because of the virtually complete absence of apoptosis, we decided to interrogate the defect in DNA damage–induced apoptosis in Caki-2 cells in greater detail.

In immunoblot analyses, both cell lines, HEK293 and Caki-2, showed an increase of phospho-ATM S1981, confirming an induction of DNA damage by NCS (Figure 1B). HEK293 cells are known to harbor high levels of constitutively active p53 [13], and we did not detect any additional changes in the abundance of p53 in HEK293 cells. Immunoblot analysis of PARP cleavage confirmed an onset of apoptosis at 72 hours in HEK293 cells (Figure 1B). Why cyclin A levels remained largely unchanged in these cells remains to be determined, but cyclin A has been proposed to participate in apoptosis in other cell types [14].

Apoptosis-resistant Caki-2 cells showed an increase of p53 expression, a decrease of cyclin A expression, but no PARP cleavage, which confirms the flow cytometric results.

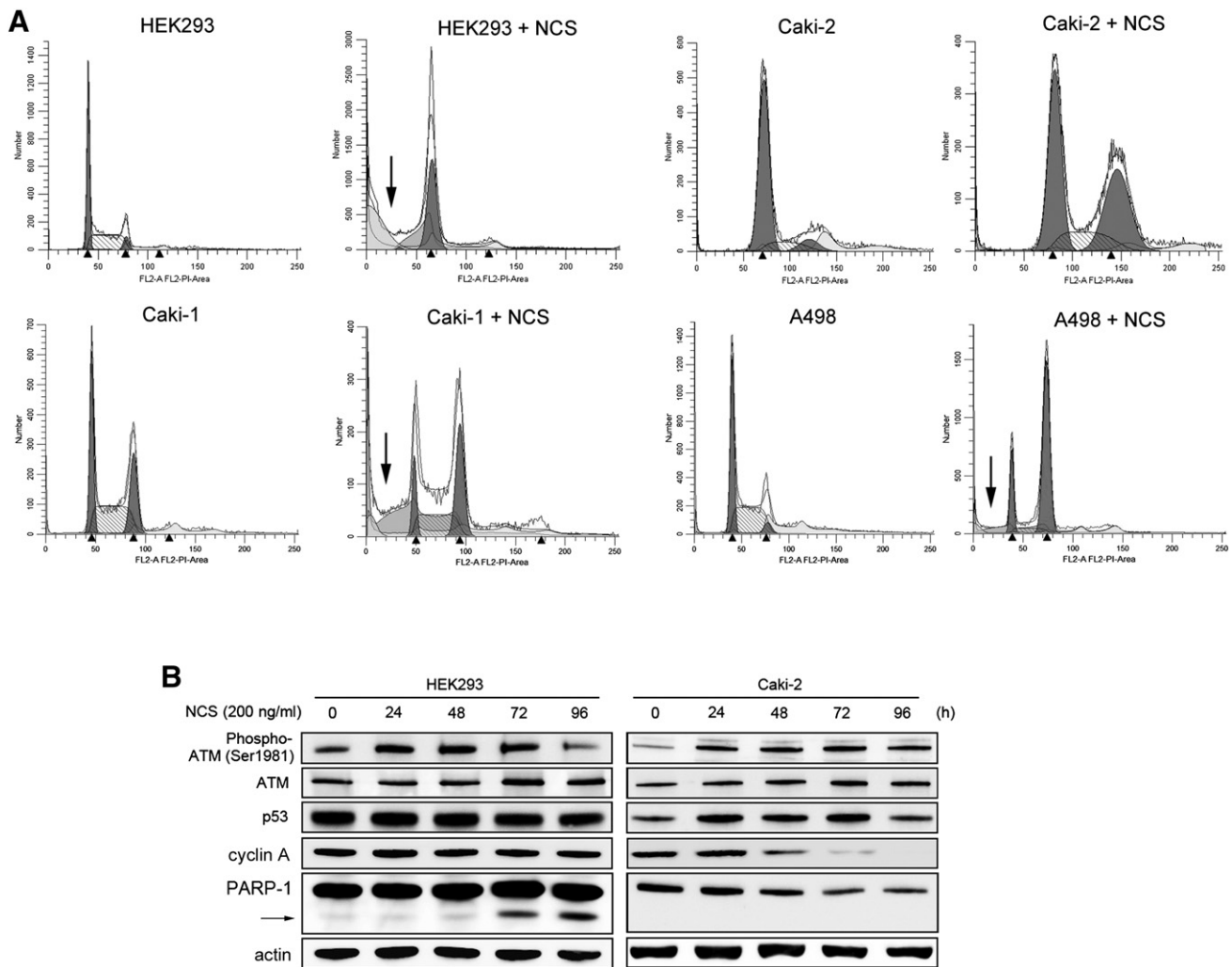


Figure 1. Caki-2 RCC cells are resistant to DNA damage–induced apoptosis. (A) Representative flow cytometric analyses of HEK293 cells in comparison to Caki-2, Caki-1, and A498 RCC cell lines after 72-hour treatment with 200 ng/ml NCS or left untreated. Note the increase of apoptotic cells as evidenced by a sub-G1 population (arrows) in HEK293, Caki-1, and A498 cells but not in Caki-2 cells, which undergo a G2/M arrest instead. (B) Immunoblot analysis of HEK293 and Caki-2 cells either untreated (0 hour) or treated with NCS for the time intervals indicated. Note the induction of apoptosis versus cell cycle arrest as evidenced by PARP cleavage (arrow) and reduction of cyclin A expression, respectively.

Taken together, these results highlight the profound differences in the response to DNA-damaging agents between noncancerous and malignant renal cells.

DNA Damage–Induced Upregulation of PUMA Is Defective in Apoptosis-Resistant RCC Cells

We next asked whether the differences in the response to genotoxic stress between HEK293 cells and Caki-2 cells involve a differential activation of p53 downstream targets including the CDK inhibitor p21^{Cip1} and the proapoptotic BH3-only protein PUMA.

Using qRT-PCR, we found that HEK293 showed an increase of p21^{Cip1} and PUMA mRNA expression, whereas Caki-2 cells upregulated only p21^{Cip1} but not PUMA mRNA (Figure 2A), which is in line with both the cell cycle arrest phenotype and the absence of DNA damage–induced apoptosis in the latter cell type.

To further corroborate the notion that Caki-2 cells are defective in PUMA upregulation in response to DNA damage, we performed an

immunofluorescence microscopic analysis. We found an increase of PUMA expression in NCS-treated HEK293 cells, whereas PUMA expression remained below the detection level in NCS-treated Caki-2 cells (Figure 2B).

Immunoblot analyses showed that HEK293 cells upregulated PUMA in response to NCS, whereas the PUMA expression in Caki-2 cells remained at a significantly lower level despite a robust increase of p53 expression (Figure 2C).

Taken together, these results show that the defect in DNA damage–induced apoptosis in Caki-2 is associated with a defect to upregulate PUMA expression to a proapoptotic level on the mRNA and protein level despite p53 stabilization.

We next analyzed the effects of ectopically expressed PUMA in Caki-2 cells to determine whether these cells are still responsive to PUMA-induced cytotoxicity. Ectopically expressed PUMA, but not p53, was found to significantly reduce cell viability at 48 hours posttransfection (Figure 2D). These results suggest an impairment of

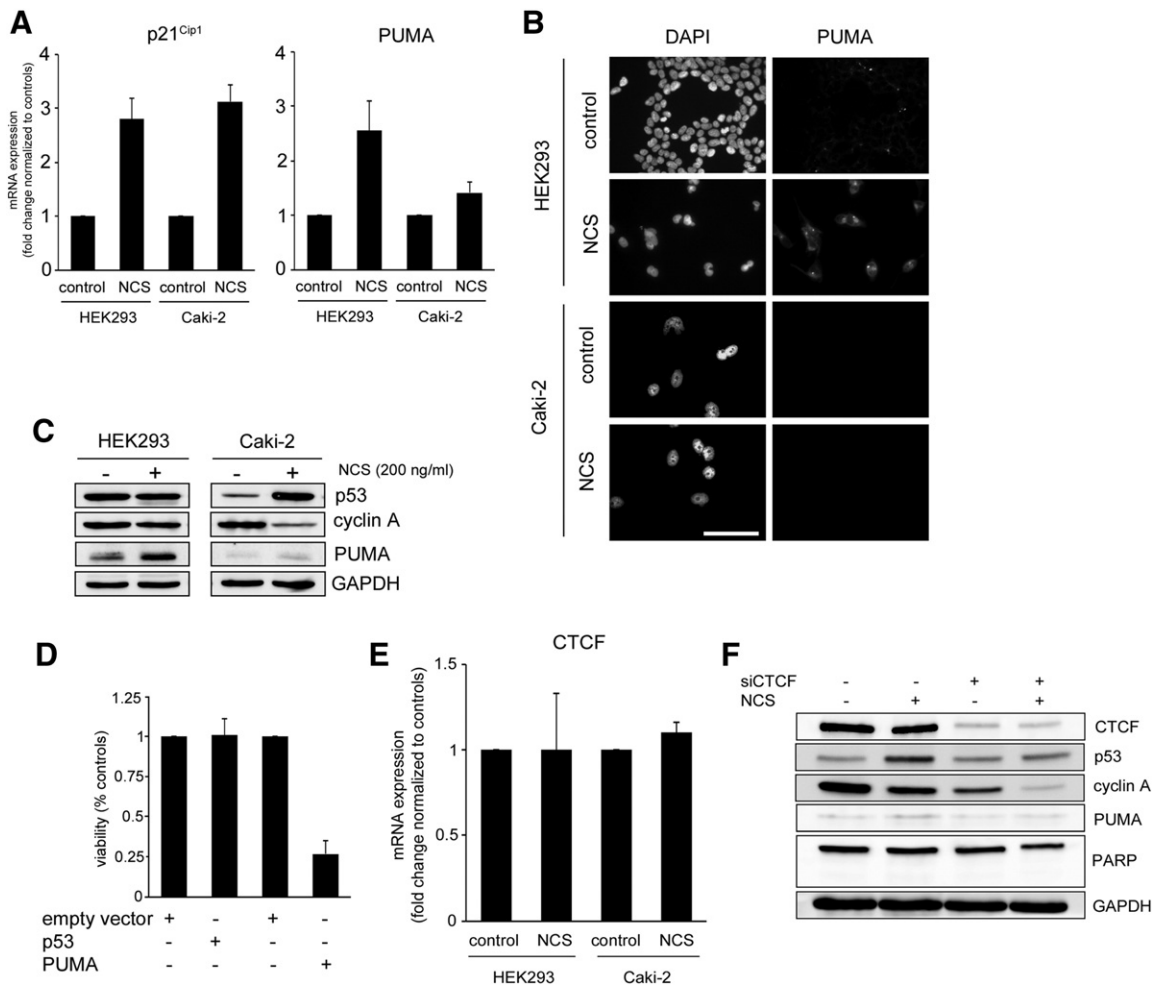


Figure 2. Resistance to DNA damage–induced apoptosis is caused by defective PUMA upregulation. (A) qRT-PCR analysis of HEK293 and Caki-2 cells for p21^{Cip1} and PUMA mRNA expression after NCS treatment with 200 ng/ml for 72 hours or left untreated (control) and normalization to the housekeeping gene GAPDH. Mean and standard errors of three independent experiments are shown. (B) Immunofluorescence microscopic analysis of PUMA expression in cells after treatment with 200 ng/ml NCS or left untreated. Scale bar = 50 μm. (C) Immunoblot analysis of HEK293 and Caki-2 cells treated with NCS 72 hours or left untreated. (D) Cell viability of Caki-2 cells normalized to controls following transient overexpression (48 hours) of p53 or PUMA. Each bar represents mean and standard error of three experiments. (E) qRT-PCR analysis of HEK293 and Caki-2 cells for CTCF mRNA expression after NCS treatment with 200 ng/ml for 72 hours or left untreated (control) and normalization to the housekeeping gene GAPDH. Mean and standard errors of three independent experiments are shown. (F) Immunoblot analysis of Caki-2 cells transfected with siRNA duplexes targeting CTCF or control duplexes for 24 hours and treated with 200 ng/ml NCS for 72 hours or left untreated.

PUMA transcription and not a general defect in the execution of apoptosis as cause of the resistance of Caki-2 cells to DNA damage–induced cell death.

We therefore asked whether the insulator protein and gene-specific PUMA repressor CTCF [15] may be involved in the defect to upregulate PUMA in response to DNA damage. CTCF mRNA expression levels were unchanged in both cell types following DNA damage (Figure 2E). Knockdown of CTCF did not lead to increased PUMA expression or enhanced apoptosis (Figure 2F), which makes a role of CTCF in PUMA repression in the cell system used here unlikely.

PUMA-Induced Apoptosis Can Be Reactivated by HDAC Inhibition

We next tested whether PUMA repression may be a consequence of general chromatin compaction and analyzed the effect of HDAC inhibition on PUMA expression using the HDAC class I and II inhibitor TSA. We first used TSA as monosubstance at a 1- μ M concentration and found enhanced spontaneous apoptosis of Caki-2 cells beginning at 24 hours (Figure 3A) together with an increase of PUMA mRNA (Figure 3B) and protein expression (Figure 3C). The induction of apoptosis was confirmed by PARP cleavage (Figure 3C).

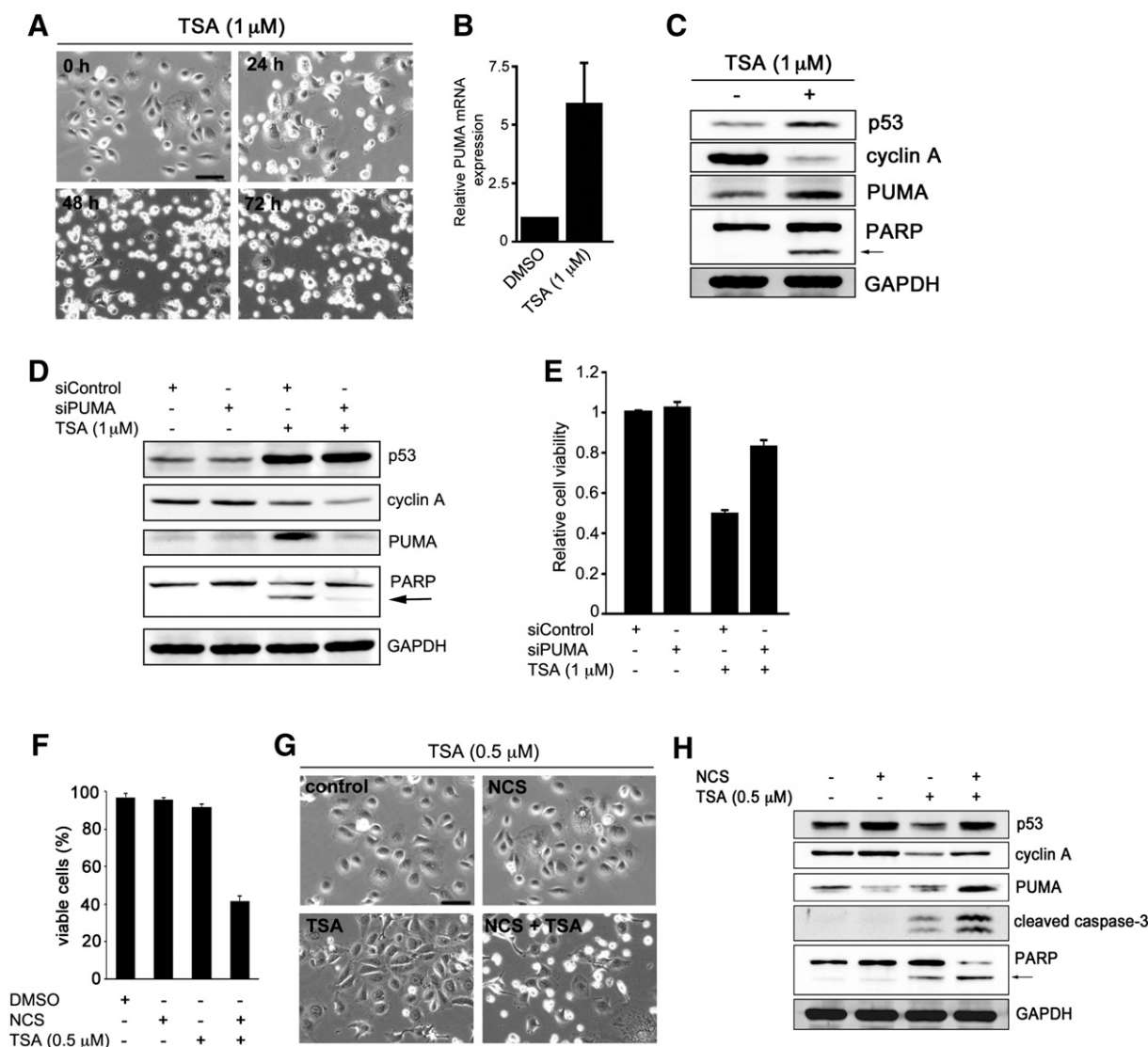


Figure 3. HDAC inhibition restores PUMA expression and DNA damage–induced apoptosis in Caki-2 cells. (A) Brightfield microscopic analysis of Caki-2 cells treated with 1 μ M TSA for the indicated time intervals. Note appearance of cells with apoptotic morphology at 24 hours. Scale bar = 20 μ m. (B) qRT-PCR analysis of PUMA mRNA expression following treatment with 1 μ M TSA or DMSO used as control for 24 hours. (C) Immunoblot analysis of Caki-2 cells treated with 1 μ M TSA for 24 hours or left untreated. Note the reexpression of PUMA and induction of PARP cleavage following TSA treatment. (D) Immunoblot analysis of Caki-2 cells for p53, cyclin A, PUMA, PARP cleavage (arrow), and GAPDH after transfection with siRNA duplexes targeting PUMA or control siRNA (24 hours) followed by treatment with 1 μ M TSA for 36 hours or left untreated. Note the suppression of PUMA upregulation and apoptosis by TSA in cells transfected with PUMA siRNA. (E) Relative cell viability (MTT) analysis of Caki-2 cells after transfection with siRNA duplexes targeting PUMA (24 hours) followed by treatment with 1 μ M TSA for 36 hours. (F) Trypan blue dye exclusion assay of Caki-2 cells treated with 200 ng/ml NCS and/or a sublethal concentration of TSA (0.5 μ M) in comparison to DMSO-treated controls. (G) Brightfield microscopic analysis of Caki-2 cells treated for 24 hours with either DMSO, 200 ng/ml NCS, 0.5 μ M TSA, or a combination of 200 ng/ml NCS and 0.5 μ M TSA. Scale bar = 20 μ m. (H) Immunoblot analysis of Caki-2 cells treated with 200 ng/ml NCS and/or 0.5 μ M TSA for 24 hours or left untreated. Note reexpression of PUMA and induction of apoptosis as evidenced by PARP cleavage and expression of cleaved caspase-3.

To directly prove that the upregulation of PUMA is the cause of TSA-induced cell death, we used siRNA and found that depletion of PUMA led to a reduced induction of PARP cleavage (Figure 3D) and a reduction in the loss of viability associated with TSA treatment at a 1- μ M concentration (Figure 3E).

To explore synergistic effects between HDAC inhibition and acute DNA damage, we decided to use a sublethal concentration of TSA (0.5 μ M, Figure 3F). Combination treatment of Caki-2 cells with NCS and sublethal TSA concentration resulted in significant cytotoxicity (Figure 3G). At the same time, PUMA expression was increased together with an apoptotic response as evidenced by enhanced PARP cleavage and cleaved caspase-3 expression (Figure 3H).

Collectively, these results show that PUMA expression and DNA damage-induced apoptosis can be effectively reactivated in RCC cells by HDAC inhibition.

PUMA and p53 Expression Determines the Sensitivity to Acute DNA Damage in RCC Cells

We next asked whether the sensitivity of RCC cells to other DNA-damaging agents than NCS could likewise be modulated by ectopic expression of PUMA and what the role of p53 in this scenario would be. In this context, it is important to emphasize that all three RCC cell lines tested (Figure 1) are wild-type for p53 and that p53 was not completely inactive but rather shifting cells toward a cell cycle arrest by p21^{Cip1} induction (Figure 2A). Using transient overexpression of

PUMA and two independent siRNAs targeting p53, we analyzed the consequences of these changes in PUMA or p53 expression in Caki-2, Caki-2, or A498 RCC cells treated with either NCS or the chemotherapeutic agents cisplatin, gemcitabine, or daunorubicin (Figure 4). Under all conditions tested, ectopic expression of PUMA was found to be associated with a significant increase of drug-induced cytotoxicity (Figure 4). Likewise, knockdown of p53 by either one or both siRNA duplexes was found to be associated with enhanced cytotoxic effects. These results underscore the importance of the PUMA-p53 axis in the global DNA damage sensitivity of RCC cells.

Discussion

RCC cells have long been known to be resistant to DNA damage-induced apoptosis, but the underlying molecular mechanisms are not fully understood. Here, we extend the spectrum of mechanisms involved in RCC DNA damage resistance by demonstrating a disconnect between p53 and upregulation of its downstream target and key proapoptotic protein PUMA. We show that the deficiency in PUMA upregulation is due to aberrant chromatin compaction and that inhibition of HDACs can restore PUMA expression and apoptosis in response to DNA damage. In addition, we found that depletion of p53 can sensitize RCC cells to acute DNA damage-induced cell death.

Almost all members of pro- and antiapoptotic members of the BCL-2 family of proteins have been shown to be altered in RCC [9–11,16,17].

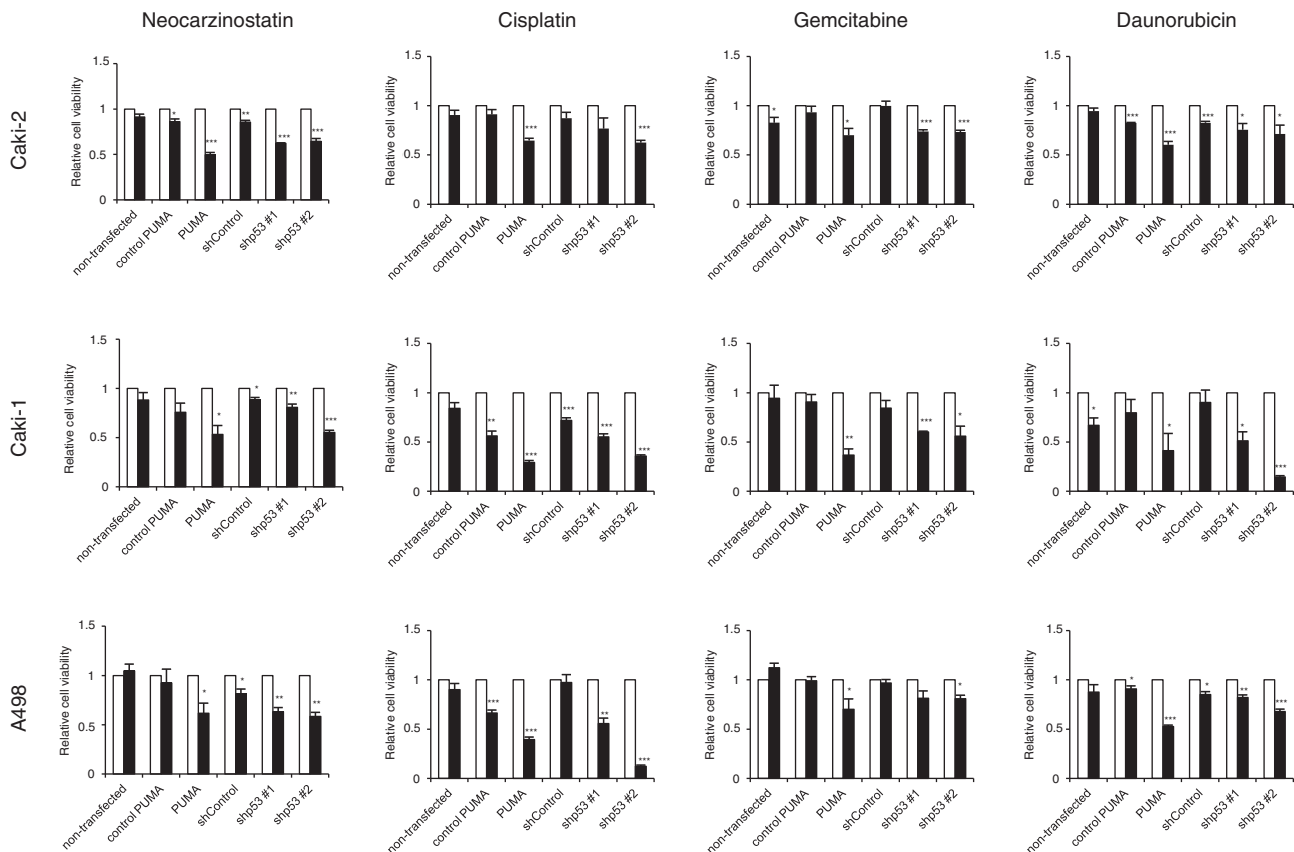


Figure 4. PUMA and p53 expression modulates sensitivity of RCC cells to various DNA-damaging agents. Relative cell viability (MTT) analysis of Caki-2, Caki-1, and A498 RCC cells following treatment with either NCS (200 ng/ml), cisplatin (10 μ M), gemcitabine (10 μ M), or daunorubicin (0.5 μ M) for 24 hours after transient transfection with PUMA (48 hours) or control plasmid, or two shRNAs to knockdown p53 or control shRNA (48 hours). Nontransfected cells are shown as control. Asterisks indicate statistically significant differences (* $P \leq .05$, ** $P \leq .005$, *** $P \leq .0005$; Student's t test for independent samples, two-tailed).

The present report extends this knowledge by showing that the disconnect between p53 and PUMA can be reverted by HDAC inhibition. This finding has potential clinical relevance for the use of HDAC inhibitors as sensitizing agents to chemo- or radiotherapy in RCC. This notion is underscored by the finding that RCCs frequently overexpress HDACs [18]. The role of altered histone modifications other than acetylation as well as aberrant DNA methylation patterns, frequent findings in RCC [19], in disrupting the p53-PUMA axis requires further analyses. Another factor that could influence the evolution of DNA damage resistance in RCC is the extensive intratumoral heterogeneity of RCC [20]. Acute and relatively severe exogenous DNA damage is likely to be an infrequent event during malignant progression and rather associated with chemo- or radiotherapy. How endogenous and low-intensity DNA damage, for example, caused by DNA replication stress shapes RCC genomes and affects the p53-PUMA axis remains to be determined. A limitation of our study is that only one RCC line has been interrogated in detail for the role of PUMA in apoptotic defects. Additional experiments in particular with tumor-derived primary cell cultures and cell lines are hence needed. Importantly, HDAC inhibitors have previously been successfully used as apoptosis-sensitizing agents in RCC in the context of other proapoptotic stimuli [21–24].

An initially unexpected finding was that depletion of p53 likewise sensitized RCC cells to DNA-damaging agents. All cell lines tested were wild-type for p53 and have an intact ability to undergo a cell cycle arrest (Figure 1). Because results presented in the present study underscore the dichotomy between cell cycle arrest and apoptosis in RCC cells, it is possible that an impaired ability to halt cell cycle progression in an acute and p53-dependent manner is an intrinsically DNA damage-sensitizing event. Support for this notion stems from a number of findings. First, targeted deletion of p53 has been shown to sensitize cells to DNA strand break-induced cell death, which was mimicked by p21^{Cip1} deficiency, suggesting that the inability to upregulate this CDK inhibitor was responsible for enhanced apoptosis [25]. Second, cells in which p53 stability and function were compromised due to viral oncogene expression were likewise sensitized to DNA damage-induced apoptosis, although p53-independent mechanisms are likely to contribute [26]. Third, p53 has been implicated in cell fate decisions [27], and the inability to arrest the cell cycle due to p53 deficiency may trigger proapoptotic pathways for example through mitotic catastrophe [28].

Collectively, our results underscore that DNA damage resistance in RCC is, in principle, reversible and may hence be targetable as part of novel combination therapies with the goal to improve the oncological outcomes of RCC patients.

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