

# Ribosomal Protein S27-like, a p53-Inducible Modulator of Cell Fate in Response to Genotoxic Stress

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## Abstract

**Activation of the p53 tumor suppressor upon DNA damage elicits either cell cycle arrest or apoptosis, and the precise mechanism governing cell fate after p53 response has not been well defined. Through genomic analysis, we have identified the ribosomal protein S27-like (RPS27L) as a novel p53 transcriptional target gene. Although RPS27L mRNA levels were consistently induced after diverse p53 activating signals, its change in protein level was stimuli-dependent: it was up-regulated when cells were arrested in response to DNA-damaging agents Adriamycin or VP16 but was down-regulated when cells underwent apoptosis in response to antimetabolite agent 5-fluorouracil. RPS27L is a nuclear protein that forms nuclear foci upon DNA damage. Depletion of RPS27L resulted in deficiency in DNA damage checkpoints, leading to conversion of DNA damage-induced p53 response from cell cycle arrest to apoptosis. We further show that RPS27L positively regulates p21 protein expression. Through this mechanism, RPS27L induction by p53 facilitates p21-mediated cell cycle arrest and protects against DNA damage-induced apoptosis. Thus, RPS27L modulates DNA damage response and functions as a part of the control switch to determine cell fate to DNA damage-p53 response.** [Cancer Res 2007;67(23):11317–26]

## Introduction

In response to DNA damage, mammalian cells activate a protection system to enable repair to continue normal life cycle or they may activate the apoptotic machinery in the face of excessive and irreparable damage (1). The tumor suppressor p53 is believed to play important roles in DNA damage response. As a transcription factor with DNA-binding activity, p53 binds to many target genes and actively regulates their transcription (2–5). Major consequences of p53 activation after DNA damage include the induction of cell cycle arrest, senescence, or apoptosis (6–8). It is well known that p53-dependent cell cycle arrest is primarily mediated through transcriptional induction of the cyclin-dependent kinase inhibitor p21 (9, 10). The mechanism for p53-induced apoptosis, however, is less clear. p53 might induce apoptosis

through transcriptional activation of apoptotic target genes, such as *PUMA*, *BAX*, *NOXA*, *BID*, *PIG3*, *CD95*, *DR5*, or *p53AIP1* (6, 7, 11), or through a transcription-independent mechanism involving direct Bax/Bak activation in the mitochondria (12–15).

The cellular response to p53 activation after DNA damage varies by cell type and stimuli. The response could be the initiation of DNA repair and the damage checkpoint, leading to cell cycle arrest or apoptosis as a result of defective DNA repair. For example, activation of p53 by the DNA-damaging agent Adriamycin resulted in p53-dependent cell cycle arrest in HCT116 cells, whereas in the same cells p53 activation by the DNA analogue 5-fluorouracil (5-FU) gave rise to apoptosis (16). The signals governing cell fate after p53 response to different genotoxic stresses are yet to be understood. Nevertheless, increasing evidence suggests that p53-dependent transcription often elicits an antiapoptotic response in human cancer cells, and p21 is believed to be the key molecule that mediates this antiapoptotic response (17–19). In addition to the well-defined p21 function as a cyclin-dependent kinase inhibitor, p21 also acts to inhibit proapoptotic effectors, such as procaspase-3, caspase 8, and ASK1 (19, 20). Depletion of p21 in cancer cells led to enhanced apoptosis in response to DNA damage both *in vitro* and *in vivo* (16, 21–24). Inhibition of p21 transcription through overexpression of Myc (25) or inhibition of p21 protein translation by the mTOR inhibitor RAD001 (26, 27) can lead to the conversion of DNA damage-induced p53 response from growth arrest to apoptosis. Thus, manipulation of p21 level seems to be a feasible approach for modulating chemotherapeutic response (18).

In this study, we identify ribosomal protein S27-like (RPS27L) as a novel p53 target that participates in DNA damage response. Functional analysis indicates that RPS27L functions as an important molecular determinant of cell fate after DNA damage-induced p53 activation.

## Materials and Methods

**Cell culture and drugs.** The human colorectal cancer HCT116 cells and the p53 knockout HCT116 cells were kindly provided by Dr. Bert Vogelstein. The human osteosarcoma cell lines U2OS and Saos-2 were purchased from American Type Culture Collection. Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Invitrogen). Adriamycin, VP16, and 5-FU were purchased from Sigma-Aldrich.

**Microarray hybridization and data analysis.** Total RNA was extracted with the use of Trizol reagent (Invitrogen) and Qiagen RNeasy kit (Qiagen) according to manufacturer's instructions. The methods for probe labeling reaction and microarray hybridization were described previously (2). For all experiments, universal human reference RNA (Stratagene) was used to generate a reference probe for drug-treated and untreated samples. Total RNA (30 µg) from experimental samples or equal amounts of universal human reference RNA were labeled with Cy5 and Cy3, respectively, by using Superscript II reverse transcriptase (Invitrogen). The log<sup>2</sup> ratios of each time

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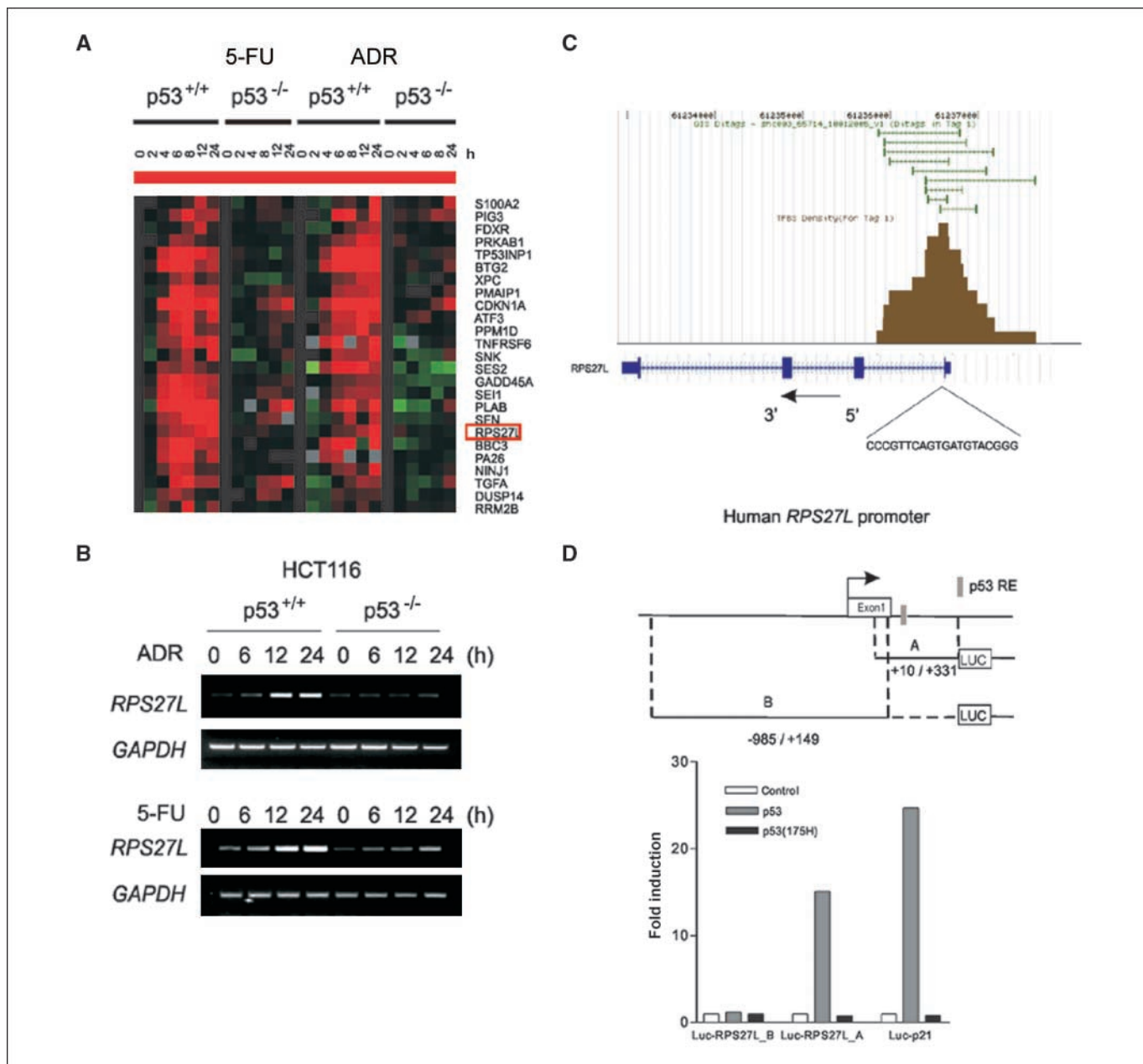
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point were then normalized for each gene to that of untreated cells (time 0) to obtain the relative expression pattern. The genes that showed substantial differences after drug treatment were selected based on a 2-fold change in expression values for at least two time points across all experimental conditions. Genes that met the criteria were further analyzed using clustering and display programs (rana.stanford.edu/software) developed by Eisen et al. (28).

**Reverse transcription-PCR analysis.** Total RNA was extracted using RNeasy kit (Qiagen). All reverse transcription-PCR (RT-PCR) was done

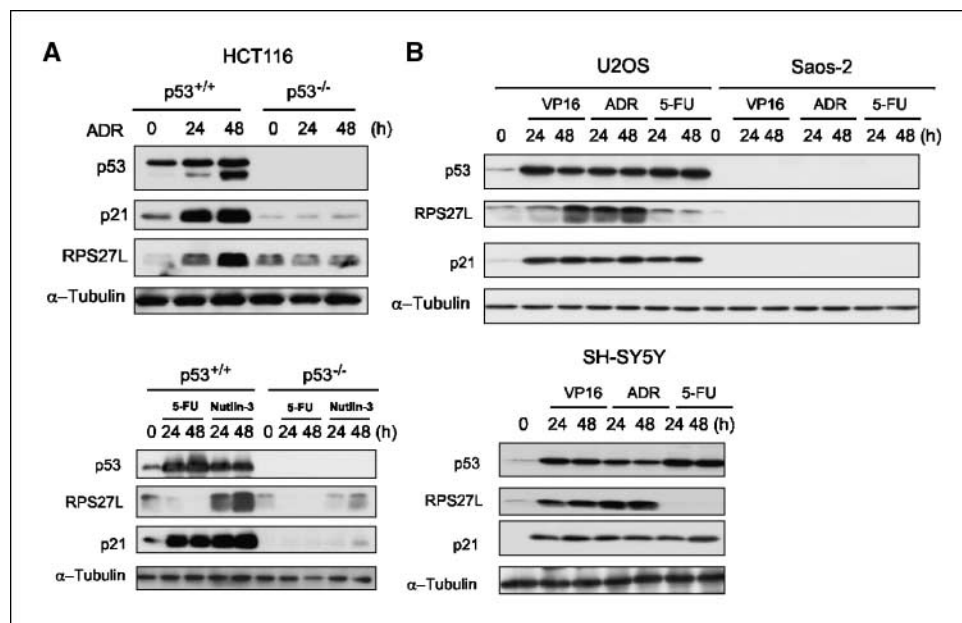
using the Titanium One Step RT-PCR kit (BD Clontech). Primer sequences are as follows: p21, forward 5'-ATGTCAGAACCGGCTGGGGA-3'/reverse 5'-ATCACAGTCGGGCTCAGCT-3'; RPS27L, forward 5'-GTGACGACCTACGCACACGA-3'/reverse 5'-GTGCTGCTCTCCTCCTGAAGG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-CAAAGTTGTCATGGATGACC-3'/reverse 5'-CCATGGAGAAGGCTGGGG-3'.

**Flow cytometry.** Cell cycle analysis was done by DNA content quantification. The cells were fixed with 70% ethanol and stained with propidium iodide (50 µg/mL) staining. The stained cells were analyzed by



**Figure 1.** *RPS27L* is a direct transcriptional target of p53. **A**, cluster analysis of microarray data showing genes up-regulated by the genotoxic agents 5-FU and Adriamycin in a p53-dependent manner. **B**, RT-PCR analysis of *RPS27L* mRNA expression in p53<sup>+/+</sup> and p53<sup>-/-</sup> HCT116 cells treated with Adriamycin (1 µmol/L) or 5-FU (375 µmol/L) for the indicated times. *GAPDH* was used as the loading control. **C**, p53 binds to the first intron of the *RPS27L* gene. Genome-wide p53-binding targets in HCT116 cells have previously been performed using ChIP-PET technology (30). Nine PETs binding to the first intron of the *RPS27L* gene in HCT116 cells treated with 5-FU. The overlapped region contains a consensus p53-binding motif. **D**, p53 activates the *RPS27L* gene promoter containing the p53 binding site. *Top*, schematic structure of the *RPS27L* gene promoter. Two luciferase reporter constructs containing the ChIP-validated p53 binding site (fragment A) in the first intron and the 1.1-kb *RPS27L* promoter region (fragment B) were constructed. *p53 RE*, p53 response elements. *Bottom*, the above constructs were cotransfected with wild-type p53 and the DNA-binding mutant p53 (175H) and luciferase activity was measured. A reporter construct containing the p21 promoter was used as a positive control.

**Figure 2.** RPS27 protein is differentially expressed in response to distinct stress signals. **A**, p53<sup>+/+</sup> and p53<sup>-/-</sup> HCT116 cells were treated with Adriamycin (ADR; 1  $\mu$ mol/L; *top*), 5-FU (375  $\mu$ mol/L), and Nutlin-3 (10  $\mu$ mol/L; *bottom*) for the indicated times. p53, RPS27L, and p21 protein levels were determined by Western blot analysis.  $\alpha$ -Tubulin was examined as a loading control. **B**, U2OS, Saos-2, and SH-SY5Y cells were treated with VP16 (10  $\mu$ mol/L), Adriamycin (1  $\mu$ mol/L), or 5-FU (375  $\mu$ mol/L) for the indicated times. p53, p21, and RPS27L protein levels were determined by Western blot analysis.  $\alpha$ -Tubulin was examined as a loading control.



FACScalibur (BD Bioscience). For BrdUrd incorporation assay and mitochondrial membrane potential detection, the BrdUrd flow kit and JC-1 staining kit (both from BD Bioscience) were used, respectively, following the instruction manual. Stained cells were analyzed by FACScalibur (BD Bioscience) and quantified by using CellQuest software (BD Bioscience).

**Gene silencing by RNA interference.** The small interfering RNA (siRNA) oligo targeting RPS27L (sequence, GGTGCTACAAGATTACTA) and negative control siRNA (sequence, TTCTCCGAACGTGTCACGT) was purchased from Sigma-Proligo, and transfection was conducted using Lipofectamine 2000 (Invitrogen). To generate stable knockdown cell lines, the siRNA sequences were cloned into the pSIREN-RetroQ retroviral expression vector (BD Bioscience) according to the manufacturer's instruction. Virally infected cells were selected in a medium containing 2  $\mu$ g/mL puromycin for 2 weeks and individual drug-resistant clones were collected and expanded. Two additional siRNAs against RPS27L were also used: 5'-CATGCCTTTGGCTAGAGATT-3' and 5'-TGTCACAGTTGCTACAAGATT-3'.

**Protein analysis and generation of anti-RPS27L antibody.** Cells were harvested and lysed with radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and proteinase inhibitors]. Protein concentrations were determined with the Bradford protein assay kit (Bio-Rad). Protein samples (20–50  $\mu$ g) were separated by SDS-PAGE, transferred onto an Immobilon membrane (Millipore), and blotted with anti-p53 and anti-p21 antibodies (Santa Cruz) and anti-MDM2 and anti-PUMA antibodies (Merck). The rabbit polyclonal antibody to RPS27L was raised against a 14-amino acid peptide from human RPS27L (LHPSLE-EKKKKHKK).

**Luciferase report assay.** The promoter elements of RPS27L were cloned into the pGL3-luciferase vector (Promega). HCT116 p53<sup>-/-</sup> cells were plated in 24-well cell culture plates and cotransfected with p53 expression vectors and RPS27L promoter plasmids. Twenty-four hours after transfection, the luciferase activities were measured using the dual luciferase system (Promega) as previously described (2).

**Immunofluorescence staining and confocal microscopy.** The cells were seeded in four-well or eight-well culture slides. After treatment, cells were fixed with 3.7% paraformaldehyde in PBS and permeabilized with 0.2% Triton-X100. Cells were sequentially incubated with 10% FBS in PBS for blocking primary antibodies in 1:100 dilution (RPS27L as indicated above, c-Myc from Roche,  $\gamma$ H2AX from Upstate, and phosphorylated ATM from

Cell Signaling) and Alexa Fluor 488 or Alexa Fluor 546-conjugated secondary antibodies (Invitrogen) in 1:1,000 dilution for 1 h each and mounted in Fluorsave (Merck) mounting medium. DRAQ5 (Biostats) was diluted 1:1,000 in mounting medium for nuclear staining. The stained cells were examined by Zeiss LSM510 confocal microscopy.

**Plasmids.** pcDNA4/RPS27L-Myc was generated by RT-PCR using normal colon tissue total RNA (Ambion), PowerScript reverse transcriptase (Clontech), and Platinum PCR SuperMix High Fidelity (Invitrogen) with the primers 5'-GGTACCATGCCTTTGGCTAGAGATT-3' (forward) and 5'-GAATTCCTAGTGTGCTTCTCTAAATGA-3' (reverse). The PCR product and empty vector were digested with *Kpn*I and *Eco*RI (New England Biolab) and ligated with T4 ligase (New England Biolab), followed by transformation and selection.

**Cytokinesis blocked micronucleus assay.** After treatment with Adriamycin, cells were incubated with cytochalasin B (5  $\mu$ g/mL; Sigma) for an additional 22 h. The cells were then trypsinized and subsequently fixed using a combination of both Carnoy's fixative (acetic acid/methanol, 1:3) and three to four drops of formaldehyde (to fix the cytoplasm). Fixed cells were dropped onto clean slides and stained with 3  $\mu$ g/mL acridine orange (which differentially stains cytoplasm and nucleus). One thousand binucleated cells were scored for each sample.

**Alkaline single-cell gel electrophoresis (comet) assay.** Cells were treated with Adriamycin with the doses mentioned above. The treated cells were subjected to a single-cell gel electrophoresis (comet) assay as described earlier (29) and stained with SYBR green dye. The tail moment of the comets was generated using the Metasystems (Germany) analysis software comet imager version 1.2. Fifty randomly chosen comets were analyzed per sample. The extent of DNA damage observed was expressed as tail moment, which corresponded to the fraction of the DNA in the tail of the comet.

## Results

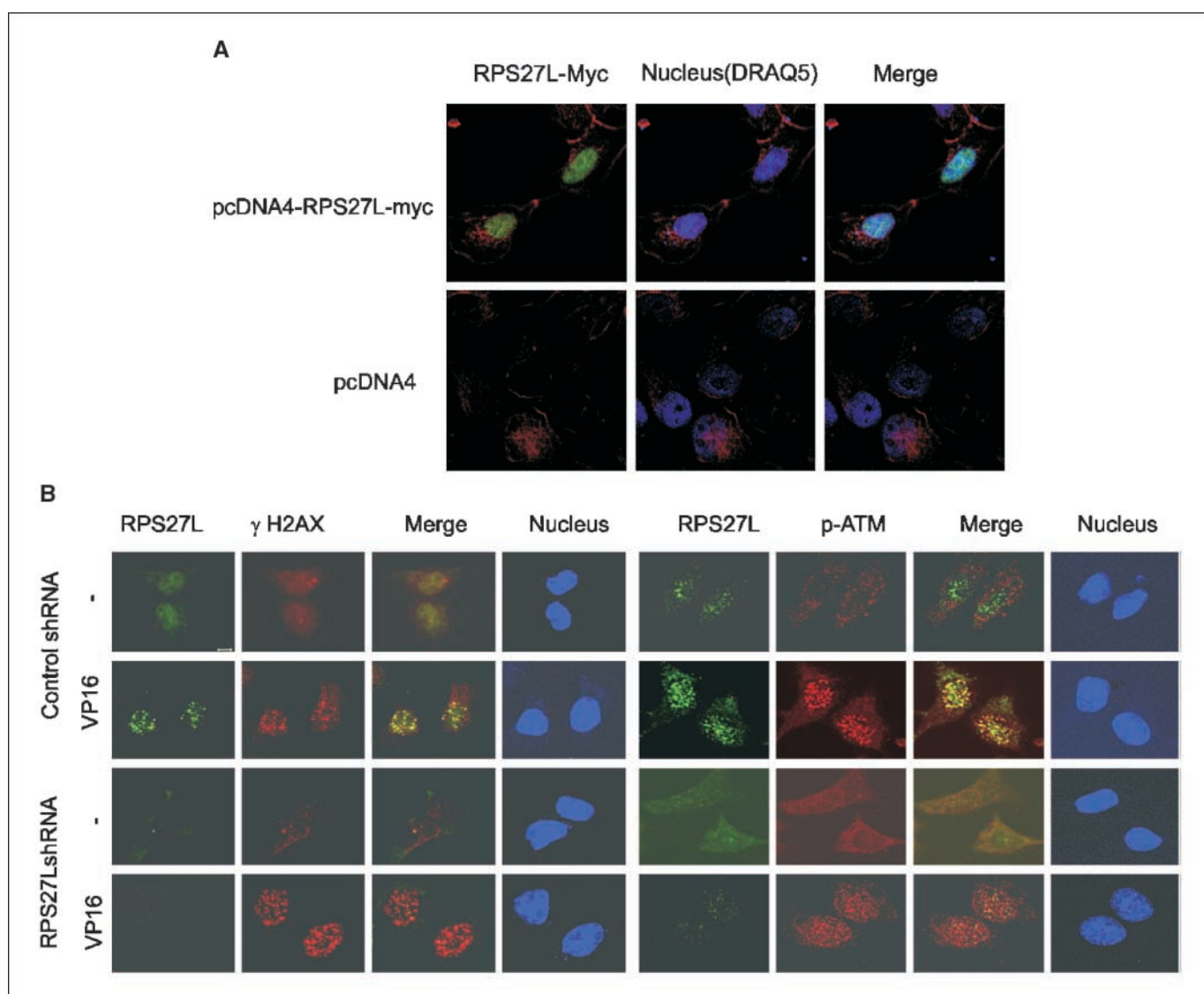
**Genomic analysis identifies RPS27L as a direct transcriptional target of p53.** p53 exerts its tumor suppressor function predominately through transcriptional regulation on downstream targets (6, 7, 30). In our previous efforts to identify additional p53 targets through large-scale gene expression analysis (2), we had observed that *RPS27L*, which encodes RPS27L with a previously unknown function, was strongly regulated by p53. Figure 1A is the



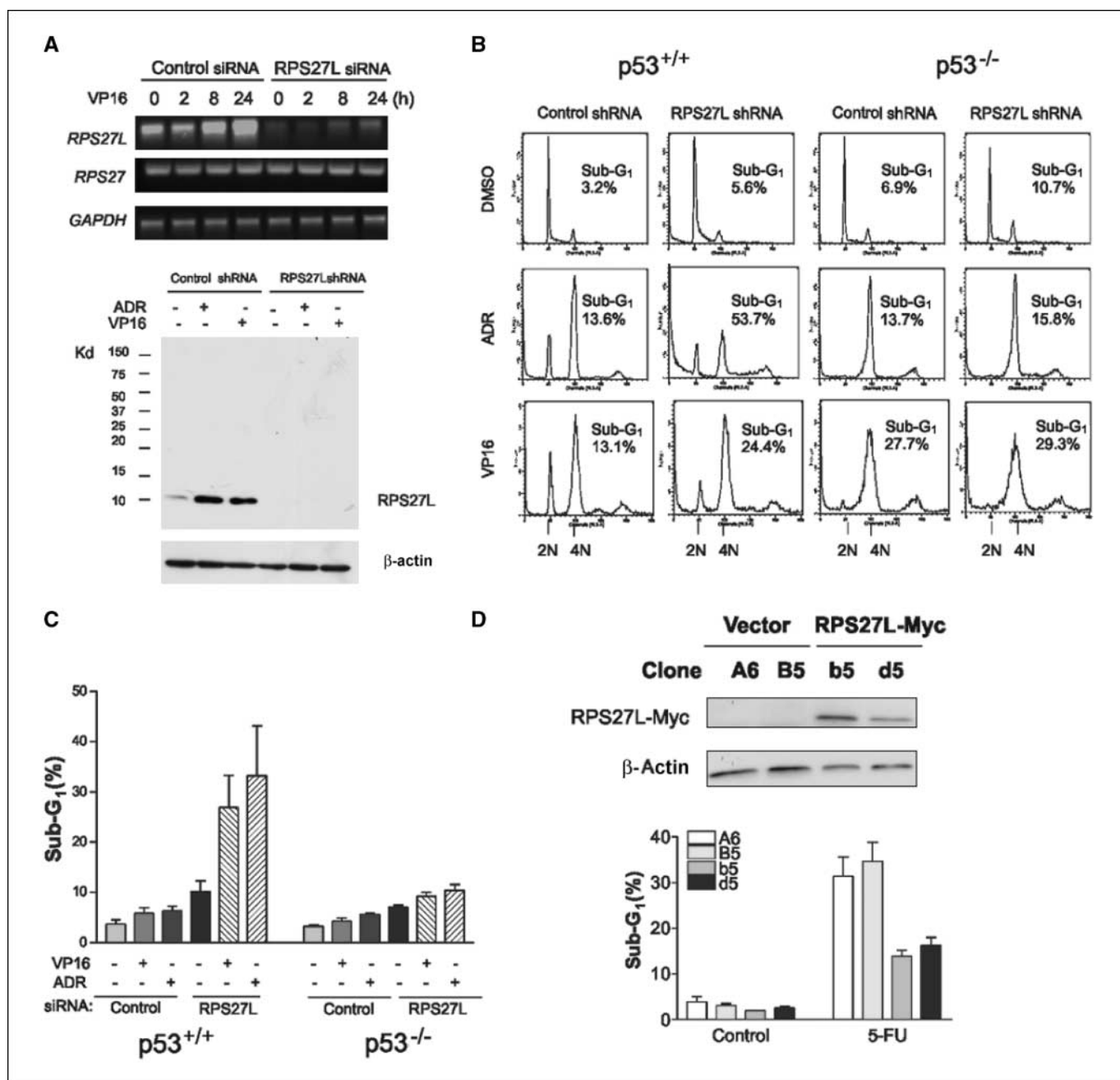
microarray analysis showing a set of genes whose expression was induced by the DNA-damaging agents Adriamycin and 5-FU in p53 wild type but not in p53 knockout HCT116 cells. In both drug treatments, *RPS27L*, together with *bona fide* p53 targets, such as *cdkn1a* (encodes p21), *Pmaid* (encodes Noxa), and *ccd3* (encodes Puma), was up-regulated in a p53-dependent manner. To validate the microarray data, we did RT-PCR analysis. Figure 1B shows that *RPS27L* mRNA was strongly induced after Adriamycin or 5-FU treatment in p53 wild-type, but not in p53 knockout, HCT116 cells. Of note, 5-FU also slightly induced *RPS27L* in p53<sup>-/-</sup> cells, indicating that 5-FU can also induce *RPS27L* through other mechanisms. Furthermore, we have previously identified up to 500 p53-binding targets in HCT116 cells treated with 5-FU by using chromatin immunoprecipitation (ChIP)-positron emission tomography (PET) technology (31). Among these p53-binding targets, *RPS27L* was found to be strongly bound by p53 through a p53-

binding motif located in the first intron (Fig. 1C). Thus, *RPS27L* expression seems to be up-regulated by p53 through direct DNA binding.

To assess whether the ChIP-identified p53 binding is functional, we cloned a genomic DNA fragment containing the p53-binding site in the first *intron* (Luc-RPS27L-A), as well as a fragment spanning a 1.3-kb promoter region (Luc-RPS27L-B) into a luciferase reporter plasmid. When cotransfected into p53 knockout HCT116 cells together with a p53 expression plasmid, only the reporter containing the intronic sequences was activated by p53 wild-type but not by a DNA binding-deficient p53 mutant (R175H; Fig. 1D). As a positive control, wild-type but not the mutant p53 also activated a reporter containing the p21 promoter. These results confirm that the p53 binding located in the first intron, as determined by ChIP analysis, is functional and confers the p53 responsiveness.



**Figure 3.** RPS27L is a nuclear protein that forms DNA damage foci upon DNA damage. *A*, HCT116 cells were transfected with a Myc-tagged RPS27L expression vector. The transfected cells were fixed and stained with anti-Myc antibody and FITC-conjugated antimouse immunoglobulin (green), followed by confocal microscopy examination. Nuclei were stained with DRAQ5 (blue). Rodamine-phalloidin was used for counterstaining of the actin cytoskeleton (red). *B*, HCT116 control and RPS27L-depleted cells were treated with VP16 (20 μmol/L) for 16 h. Colocalization of RPS27L with γ-H2AX or phosphorylated ATM was detected by immunofluorescence staining with anti-RPS27L (green) and anti-γ-H2AX or anti-phosphorylated ATM (1981; red). Nuclei were stained by DRAQ5 (blue).



**Figure 4.** RPS27L expression modulates p53-dependent apoptosis. *A*, RPS27L, RPS27, and GAPDH mRNA levels or RPS27L protein expression were determined by RT-PCR in control and RPS27L shRNA cells treated with VP16 at indicated times. RPS27L protein expression was examined by Western blot in control and RPS27L shRNA cells. *B*, HCT116 p53<sup>+/+</sup> and p53<sup>-/-</sup> cells stably expressing the RPS27L shRNA or a control shRNA were treated with Adriamycin (1 μmol/L) or VP16 (10 μmol/L) for 48 h. Cell death was measured by cells with sub-G<sub>1</sub> DNA content. *C*, HCT116 p53<sup>+/+</sup> and p53<sup>-/-</sup> cells were transfected with RPS27L siRNA, followed by VP16 (10 μmol/L) or Adriamycin (1 μmol/L) treatment for 24 h. Cell death was measured by cells with sub-G<sub>1</sub> DNA content. Columns, mean of three independent experiments; bars, SD. *D*, RPS27L-myc expression in HCT116 clones expressing empty vector or RPS27L-myc (top). The control clones (A6 and B5) and RPS27L-myc expressing clones (b5 and d5) were treated with 5-FU (375 μmol/L) for 24 h, and cell death was measured by FACS analysis of sub-G<sub>1</sub> population (bottom).

**p53-dependent RPS27L protein level induction is stimuli dependent.** To investigate whether the p53-induced increase in RPS27L mRNA also gives rise to the protein induction, we performed immunoblot analysis of p53 wild-type and p53 knockout HCT116 cells treated with Adriamycin, 5-FU, or Nutlin-3, a small molecule MDM2 antagonist that directly activates p53 (32). As anticipated, Adriamycin or Nutlin-3 treatment resulted in the accumulation of p53 and p21 in p53 wild-type HCT116 cells.

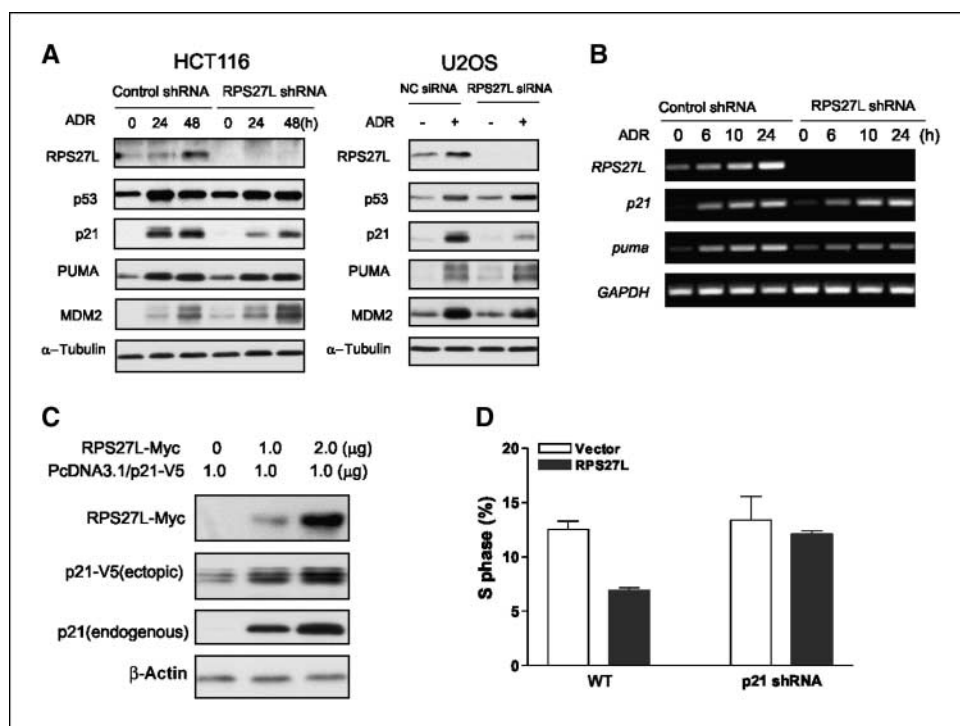
RPS27L protein induction, as detected by an antibody raised against a RPS27L peptide, was also detected after Adriamycin or Nutlin-3 treatment in these cells (Fig. 2A). Surprisingly, 5-FU-induced up-regulation of RPS27L mRNA did not result in increased protein expression as expected. Rather, it was even down-regulated. To extend our observations to additional cell lines or stimuli, we treated U2OS and SH-SY5Y cells (p53 wild type) as well as Saos-2 cells (p53 deficient) with Adriamycin, VP16, and 5-FU (Fig. 2B).

RPS27L protein levels were markedly induced, together with p53 and p21, in Adriamycin-treated or VP16-treated U2OS and SH-SY5Y cells, but not in Saos-2 cells. Again, RPS27L protein levels were either unchanged or decreased in response to 5-FU. Collectively, these results indicate that, despite the consistent induction of *RPS27L* mRNA after various p53-activating signals, its change in protein level depends on the type of genotoxic stress.

**RPS27L is a nuclear protein that forms nuclear foci upon DNA damage.** To further characterize the involvement of RPS27L in DNA damage response, we evaluated the cellular localization of RPS27L. We overexpressed c-Myc-tagged RPS27L in HCT116 cells and detected its expression by immunofluorescence staining with an anti-c-Myc antibody. The results indicate that ectopically expressed RPS27L localizes to the nucleus (Fig. 3A). To determine the cellular location of the endogenous RPS27L and assess its participation in DNA damage response, we performed immunofluorescence studies using the anti-RPS27L antibody in both HCT116 wild-type cells and HCT116 cells depleted of RPS27L (see below). Because of the low expression of RPS27L in untreated cells, we obtained low nuclear staining in these cells (data not shown). Upon DNA damage by VP16, we detected a nuclear foci-like staining pattern in HCT116 cells with anti-RPS27L (Fig. 3B). Moreover, RPS27L staining partially colocalized with phosphorylated histone H2AX ( $\gamma$ -HA2X), a hallmark of DNA double-strand breaks (33) or phosphorylated ATM upon VP16 treatment. Depletion of RPS27L expression abolished these colocalizations, although it had no effect on  $\gamma$ -HA2X or phosphorylated ATM foci formation. These data suggest that RPS27L is a nuclear protein that participates in DNA damage response and is recruited to a subset of DNA double-strand breaks.

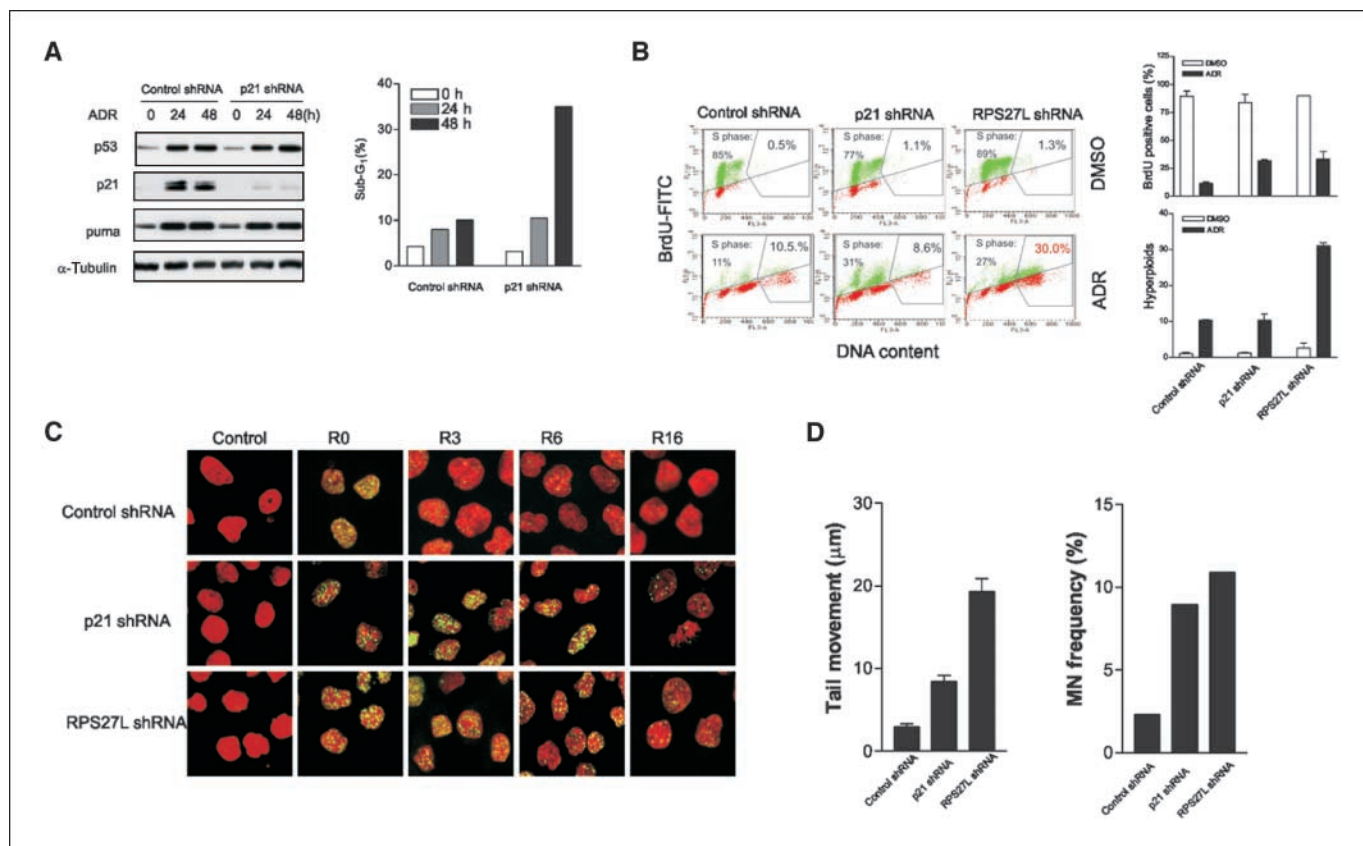
**RPS27L regulates apoptotic response to genotoxic stress.** In response to DNA damage, p53 induces cell cycle arrest, senescence, or apoptosis, depending on the cellular context or type of stress. In HCT116 cells, it is known that the DNA damaging agent, such as

Adriamycin, induces a p53-dependent cell cycle arrest, whereas DNA analogue 5-FU treatment triggers a p53-dependent apoptosis (Supplementary Fig. S1; refs. 16, 34). Because RPS27L protein expression was induced in Adriamycin-treated HCT116 cells but decreased in response to 5-FU treatment, we next set out to determine the functional role of RPS27L in these cellular responses. First, we investigated whether silencing of RPS27L would convert Adriamycin-induced cell cycle arrest to apoptosis in HCT116 cells. To achieve this aim, we generated cell lines derived from HCT116 that constitutively expressed a short hairpin RNA (shRNA) targeting RPS27L (RPS27L shRNA) or a nonspecific control shRNA (control shRNA) in both p53 wild-type and p53 knockout backgrounds. The targeted sequence for gene silencing was efficient and specific, as it almost completely ablated RPS27L expression and prevented its induction after DNA damage while having no effect on closely related RPS27 (Fig. 4A). In p53 wild-type HCT116 cells expressing the control shRNA, VP16 or Adriamycin treatment for 48 h primarily resulted in a cell cycle arrest response in both G<sub>1</sub> and G<sub>2</sub> phase with minimal cell death, whereas cells expressing RPS27L shRNA exhibited marked loss of G<sub>1</sub> arrest but an increase in cell death upon Adriamycin or VP16 treatment (Fig. 4B). This effect seemed to be p53 dependent, as RPS27L depletion in p53 knockout HCT116 cells did not give the same results. Knockdown of RPS27L through transient siRNA transfection also resulted in a marked increase in cell death upon Adriamycin or VP16 treatment only in p53 wild-type cells (Fig. 4C). Similar effects were also seen in p53 normal lung carcinoma A549 and osteosarcoma U2OS cells but not in p53-deficient Saos-2 cells (data not shown), indicating that the increased cell death response to DNA-damaging agents is a general feature of RPS27L depletion. Importantly, targeting RPS27L by using two additional siRNA against RPS27L also resulted in evident loss of G<sub>1</sub> arrest and significant induction of cell death upon Adriamycin and VP16 treatment (see Supplementary Fig. S2). This indicates that the



**Figure 5.** RPS27L depletion impairs p21 accumulation upon DNA damage. *A*, HCT116 control and RPS27L-depleted cells were treated with Adriamycin (1  $\mu$ mol/L) for 24 and 48 h. The expression levels of p53 and its target genes p21, Puma, and MDM2 were determined by Western blot analysis.  $\alpha$ -Tubulin was used as a loading control. U2OS cells were transfected with RPS27L siRNA or the control siRNA. The transfected cells were treated with Adriamycin (1  $\mu$ mol/L) for 24 h. Expression levels of p53 and its target genes. *B*, RT-PCR analysis of RPS27L and p21 mRNA levels in HCT116 control and RPS27L-depleted cells treated with Adriamycin as indicated. *C*, HCT116 cells were transfected with pcDNA-p21-V5 and increasing amounts of pcDNA-RPS27L-Myc. p21-V5 and RPS27L-Myc protein expression was examined by anti-V5 and anti-Myc, respectively. The endogenous p21 was examined by using anti-p21 antibody.  $\beta$ -Actin was used a loading control. *D*, HCT116 and HCT116 p21 shRNA cells were cotransfected with RPS27L expressing vector or control vector, together with the EGFP-expressing vector. After 24 h, cells positive for EGFP were analyzed for cells in S phase.





**Figure 6.** RPS27L deficiency results in defective cell cycle checkpoint and DNA repair. *A*, Western blot analysis of p53, p21, and Puma in HCT116 control and p21-depleted cells treated with Adriamycin for 24 h. Cell death was evaluated by propidium iodide staining and FACS analysis (*right*). *B*, HCT116 control, RPS27L-depleted, and p21-depleted cells were treated with Adriamycin (1  $\mu\text{mol/L}$ ) for 24 h. Cells were labeled with BrdUrd for 30 min and stained with FITC-conjugated anti-BrdUrd and 7-amino-actinomycin D. Incorporation of BrdUrd (*y* axis) and total DNA content (*x* axis) were analyzed by flow cytometry. The representative histogram indicates the percentages of cells in S phase and in hyperploids (>4N DNA content), respectively. *Columns*, mean of three independent experiments; *bars*, SD. *C*, HCT116 control, RPS27L-depleted, and p21-depleted cells were treated with VP16 (20  $\mu\text{mol/L}$ ) for 3 h. Afterwards, VP16 was removed by replacing with fresh medium, and cells were harvested at 0, 3, 6, and 16 h for anti- $\gamma\text{H2AX}$  staining (*green*). Stained cells were examined by confocal microscopy. Nuclei were stained with DRAQ5 (*red*). *D*, DNA damage as measured by the comet assay in HCT116 control, RPS27L-depleted, and p21-depleted cells after 24 h of Adriamycin treatment (*left*). The damage distribution, measured as tail moment (product of tail length and fraction of DNA), differed between the two cell lines. Tail moment (in microns) after treatment is given. The number of micronuclei (in percentage) was measured by the cytokinesis blocked micronucleus assay after a 24-h Adriamycin treatment (*right*). Data for Adriamycin treatment (1  $\mu\text{mol/L}$ ) of HCT116 control, RPS27L-depleted, and p21-depleted cells in comparison with the untreated samples. A total of 1,000 binucleated cells were scored. *Columns*, percentage of micronuclei in 1,000 counted cells.

observed effect upon RPS27L depletion is not due to off-target of RPS27L siRNA.

To further define the nature of this cell death response, we used another assay, namely flow cytometric detection of cells stained with JC-1. The JC-1 staining identifies cell death events associated with the loss of mitochondria membrane potential ( $\Delta\Psi_m$ ). Treatment of HCT116 RPS27L shRNA cells with Adriamycin resulted in a marked decrease in  $\Delta\Psi_m$  compared with the control cells (33.5% versus 13.5%), indicating an apoptotic cell death involving mitochondrial dysfunction (Supplementary Fig. S3). Taken together, these results suggest that RPS27L mediates an antiapoptotic response upon DNA damage.

As RPS27L protein level was reduced in apoptotic response to 5-FU, we next sought to determine whether overexpression of RPS27L would inhibit 5-FU-induced apoptosis. To this end, we had established HCT116 cells stably expressing exogenous RPS27L-myc (Fig. 4D). Although the stable clones were unable to express high levels of exogenous RPS27L that was also down-regulated after 5-FU treatment (data not shown), the two clones we generated did exhibit reduced apoptotic response to 5-FU compared with the control clones that express an empty vector (Fig. 4D). This result

further supports the idea that RPS27L functions as an antiapoptotic molecule and its decrease upon 5-FU treatment might contribute to 5-FU-induced apoptosis.

**RPS27L regulates p21 protein accumulation upon DNA damage.** We next sought to delineate the pathway via which RPS27L functions in the DNA damage response. Because only p53 wild-type cells were sensitized to DNA damage upon loss of RPS27L, RPS27L should genetically function within this pathway. We examined the effects of RPS27L silencing on p53 and its downstream targets p21, PUMA, and MDM2 by immunoblot analysis. Silencing of RPS27L did not seem to affect p53 induction by Adriamycin (Fig. 5A). However, p21 induction was markedly compromised in RPS27L-depleted cells compared with the control cells, whereas the induction of PUMA or MDM2 was unaffected by RPS27L depletion. This indicates that depletion of RPS27L impairs the p21 induction by DNA damage, and this effect seems to be selective. A similar result was also seen in U2OS cells, indicating that the observed effect on p21 upon loss of RPS27L is not cell type specific.

We further found that the reduced p21 protein level was not due to inhibited p21 transcription because p21 mRNA levels were not

significantly changed in RPS27L-depleted cells compared with the control cells (Fig. 5B). To obtain direct evidence that RPS27L has the ability to regulate p21 protein expression, we cotransfected a p21-expressing vector with increasing amounts of an RPS27L-expressing vector into HCT116 cells, and the result showed that overexpression of RPS27L up-regulated p21 protein expression in a dose-dependent manner (Fig. 5C). Furthermore, cells expressing RPS27L show reduced cells in S phase but not in cells lacking of p21 (Fig. 5D). Collectively, these findings suggest that RPS27L positively regulates p21 protein expression and functions as antiproliferation molecule. Loss of RPS27L results in attenuated p21 protein induction after DNA damage.

To determine whether p21 protein down-regulation seen in RPS27L shRNA cells arises from decreased mRNA stability or increased protein degradation, we evaluated the p21 mRNA and protein levels in the presence of actinomycin D or cycloheximide that inhibits transcription and protein synthesis, respectively. The result indicated that the stabilities of p21 mRNA and protein levels in RPS27L shRNA cells were similar to that in control cells (Supplementary Fig. S4A and B). These results suggest that the down-regulation of p21 upon loss of RPS27L was not due to changes in p21 mRNA or protein stability. Furthermore, we did not find that p21 physically interacts with RPS27L by coimmunoprecipitation assay (data not shown).

**RPS27L regulates cell cycle checkpoint, DNA repair, and genome stability in DNA damage response.** Cells lacking p21 are defective in cell cycle arrest and are more sensitive to DNA damage (21, 23, 35–37). Because RPS27L positively regulates p21 protein level, we reasoned that the cells depleted of RPS27L would have associated phenotypes similar to cells lacking p21. To facilitate this comparison, we created a HCT116 cell line stably expressing p21 shRNA. In these cells, p21 expression and its induction after Adriamycin treatment were confirmed to be markedly inhibited and, as anticipated, exhibited increased sensitivity to Adriamycin (Fig. 6A). Moreover, knockdown of RPS27L in p21 shRNA cells did not result in further increase in apoptosis upon DNA damage, further supporting that RPS27L, at least in part, mediates through p21 for its biological function (Supplementary Fig. S5).

To further characterize the effect of RPS27L knockdown on cell cycle progression upon DNA damage, we performed fluorescence-activated cell sorting (FACS) analysis with 7-amino-actinomycin D staining (for total DNA content) and BrdUrd labeling (for DNA synthesis) and compared the results with that of p21 shRNA cells. HCT116 cells lacking RPS27L or p21 exhibited similar levels of DNA synthesis in untreated cells. However, after Adriamycin treatment for 24 h, parental HCT116 cells underwent a dramatic decrease in DNA synthesis (from 85% to 11%), whereas in HCT116 cells lacking p21 or RPS27L this decrease in DNA synthesis was partially rescued (from 77% to 31% and from 86% to 27%, respectively; Fig. 6B). This result indicates that cells lacking RPS27L or p21 did prematurely leak through the G<sub>1</sub> checkpoint entering S phase and signifies that RPS27L, like p21, is required for efficient DNA damage cell cycle checkpoint in G<sub>1</sub> phase. In addition, RPS27L-depleted HCT116 cells exhibited a substantial accumulation of cells with a hyperploid DNA content (>4N) compared with the control cells (30% versus 10.5%). This feature, however, was not observed in p21-depleted cells (Fig. 6B).

A deficient DNA damage checkpoint is expected to increase DNA damage.  $\gamma$ -H2AX foci are considered to be sensitive indicators of double-strand breaks, and in p21-deficient cells, DNA damage-induced H2AX foci remains elevated after DNA-damaging agents

have been removed (38). We next looked at  $\gamma$ -H2AX focus formation after VP16 treatment and monitored the reversibility post-VP16 washout in RPS27L or p21 shRNA cells. Figure 6C shows that 1-h treatment with VP16 induced prominent double-strand break foci, as evidenced by strong  $\gamma$ -H2AX staining. In control cells,  $\gamma$ -H2AX staining was decreased over time, and by 16 h after VP16 removal, the  $\gamma$ -H2AX staining was almost undetectable, suggesting a proficient DNA repair process in these cells. In contrast, both p21-depleted or RPS27L-depleted HCT116 cells displayed a sustained  $\gamma$ -H2AX staining up to 16 h after VP16 removal, pointing to the increased DNA damage or delayed repair in these cells. These results further support our hypothesis of a protective role for RPS27L in DNA damage response.

To examine DNA damage directly, we next used the comet assay. The comet assay is a single-cell gel electrophoresis assay, in which damaged DNA migrates to form a “comet tail” that is proportional to the amount of DNA damage (39). HCT116 control, p21 shRNA, and RPS27L shRNA cells were incubated with Adriamycin for 24 h, and cells were harvested and processed for the comet assay. As shown in Fig. 6D, Adriamycin-induced DNA damage was significantly enhanced in RPS27L-depleted cells and, to a lesser extent, in p21-deficient cells. Moreover, we observed increased micronucleus frequency in both RPS27L-depleted and p21-depleted cells upon Adriamycin treatment, as evaluated by micronuclei assay, which proves to be a reliable indicator of chromosomal damage and genomic instability (Fig. 6D; refs. 29, 40). Overall, these experiments support that the loss of RPS27L leads to increased DNA damage upon Adriamycin treatment. We propose that the induction of RPS27L by p53 facilitates cell cycle checkpoint and chromosome stability at least in part through promoting p21 accumulation. Collectively, these functions effectively protect against the DNA damage response.

## Discussion

In this study, we identify the RPS27L protein as a novel p53 transcriptional target participating in DNA damage response. Importantly, we showed its functional role in DNA damage as protective and it antagonizes the p53-mediated apoptotic response. Depletion of RPS27L-sensitized cells to DNA-damaging chemotherapeutic agents by converting p53 response from growth arrest to apoptosis. Interestingly, p53-dependent RPS27L protein level induction depended on the type of genotoxic stress. Whereas its induction in Adriamycin-treated cells was associated with the cell cycle arrest response, its decrease upon 5-FU treatment correlated with a strong apoptosis. RPS27L therefore might function as a control switch to determine cell fate after p53 activation. The reason for RPS27L protein down-regulation after 5-FU treatment is not clear, but it must occur at the posttranscriptional levels because its mRNA level is up-regulated after 5-FU treatment. The detailed mechanism for this regulation needs to be further investigated.

While this manuscript was in preparation, He et al. reported a similar finding indicating RPS27L as a novel p53 target that regulates apoptosis upon DNA damage (41). However, the data contrast with our findings, showing that RPS27L function as a proapoptotic molecule that mediates p53-induced apoptosis in etoposide (VP16)-treated HCT116 cells. In He and Sun's study, high doses of VP16 (50–200  $\mu$ mol/L) was used to induce apoptosis in HCT116 cells. It is well known that DNA-damaging agents, such as Adriamycin and VP16, at low doses would induce p53-dependent



cell cycle arrest rather than apoptosis in HCT116 cells, p53<sup>-/-</sup> cells, due to lack of p53-p21 induction for G<sub>1</sub> cell cycle checkpoint, are more sensitive to DNA-damaging response (16). When given in high doses, VP16 would induce apoptosis in HCT116 cells, but apoptosis induced under this condition does not depend on p53, as similar levels of apoptosis were induced in both p53 wild-type and p53<sup>-/-</sup> HCT116 cells (Supplementary Fig. S2). Although these authors found that RPS27L knockdown reduced the apoptosis rate, despite modestly, in HCT116 cells treated with high doses of VP16, they did not show the p53 dependence of this effect. Therefore, the discordant findings between the two studies might be due to the different doses of VP16 used in the study. In fact, we do not agree that the phenotype change under the high doses of VP16 in HCT116 cells is relevant to p53. Nevertheless, our studies by integrating with different aspects of analysis using valid p53-dependent cellular models, combined with mechanistic insights, clearly argue the functional role of RPS27L as an antiapoptotic molecule in DNA damage response.

p21 has been suggested to be critical in determining cellular sensitivity to DNA-damaging agents, as p21-deficient cells are defective in the cell cycle checkpoint and are highly sensitive to DNA damage (18). In addition to transcriptional regulation by p53, p21 expression can also be regulated through posttranscriptional mechanisms, either through protein translation or protein stability. Inhibiting p21 transcription through Myc overexpression leads to conversion of DNA damage p53 response from growth arrest to apoptosis (25). The mTOR inhibitor RAD001 has been shown to sensitize cells to DNA damage-induced apoptosis through interference with p21 protein translation (26). The same effect can also be achieved by inhibiting p21 protein stability through knockdown of the p21-binding protein WISP39 (27). The concurrent induction of both RPS27L and p21 by p53 seems to serve as a novel mechanism to sustain p21 protein increase and to maintain an effective defensive mechanism against the DNA damage response. Although the underlying mechanism by which RPS27L regulates p21 protein expression remains to be determined, our preliminary data suggests that this regulation is not through direct interaction with p21 and might be involved in other mechanisms.

p53 is a multifunctional transcriptional factor which induces many target genes. Depending on the cellular context, p53

activation can induce DNA repair (e.g., by induction of XPC and p53R2), cell cycle arrest (e.g., by induction by p21), or apoptosis (e.g., by induction of Bax and Puma). In addition to the role of RPS27L in regulating cell cycle checkpoint through p21 as we presented in this study, RPS27L also forms damage foci and colocalizes with H2A $\gamma$  or ATM upon DNA damage. This feature distinguishes RPS27L from p21 and indicates that RPS27L might have additional functions in regulating DNA damage response. In addition, RPS27L induction by p53 seemed to be required to prevent tetraploidy upon DNA damage. Loss of the tetraploidy checkpoint due to a missing cell cycle arrest has been believed to contribute to the genomic instability induced by p53 inactivation (42, 43). Thus, it raises the possibility that RPS27L might also have a role in maintaining genome stability upon DNA damage. This feature on tetraploidy regulation seemed to be also independent of its effect on p21, as p21-deficient cells seemed to have no such effect. These functions of RPS27L, along with its role in cell cycle control through regulation of p21, may suggest a role for the protein in a highly effective DNA repair mechanism in cell cycle-arrested cells, protecting these cells against DNA damage-induced apoptosis and placing RPS27L in the p53 pathway, which determines cell fate in response to DNA damage.

In conclusion, our findings identify a novel p53 target that participates in DNA damage response and plays an important role in regulating cell cycle checkpoints, DNA repair, and chromosome stability. In particular, it points to a novel mechanism in regulation of p21. Given that DNA damage induces apoptosis rather than cell cycle arrest in the absence of RPS27L, RPS27L seems to be a critical determinant of the cellular outcome of p53 activation. Therefore, RPS27L could be a promising pharmacologic target for modulating sensitivity to DNA-damaging chemotherapeutic agents.

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