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ORIGINAL ARTICLE

Ribosomal protein S27L is a direct p53 target that regulates apoptosis

H He and Y Sun

Division of Cancer Biology, Department of Radiation Oncology, University of Michigan Comprehensive Cancer Center, Ann Arbor, MI, USA

Ribosomal proteins were recently shown to regulate p53 activity by abrogating Mdm2-induced p53 degradation (L23, L11, L5) or by enhancing p53 translation (L26). Here, we report that a novel ribosomal protein, RPS27L (S27-like protein), is a direct p53 target. RPS27L, but not its family member RPS27, was identified as a p53 inducible gene in a genome-wide chip-profiling study. Further characterization revealed a p53-dependent induction of RPS27L in multiple cancer cell models. Indeed, a consensus p53-binding site was identified in the first intron of the RPS27L gene and a direct binding of p53 to this site was demonstrated both *in vitro* and *in vivo*. Characterization of a luciferase reporter driven by the RPS27L intron fragment revealed a p53-binding site-dependent transaction by wild-type p53, but not by several transactivating-deficient p53 mutants. This transactivation was enhanced by etoposide, a DNA damaging agent that activates p53 and was completely blocked by a dominant-negative p53 mutant. Functionally, overexpression of RPS27L within the physiological inducible levels promoted, whereas siRNA silencing of RPS27L inhibited, apoptosis induced by etoposide. This is the first report, to our knowledge, that p53 directly induces the expression of a ribosomal protein, RPS27L, which in turn promotes apoptosis.
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Keywords: RPS27L; p53; apoptosis; ribosomal protein; gene expression and siRNA silencing

Introduction

It is well established that p53 tumor suppressor plays a pivotal role in suppression of tumorigenesis. The importance of p53 in cancer biology is further supported by the facts that p53 is the most frequently mutated tumor suppressor gene in human cancers and cancer cells with p53 mutation are in general more resistant to current cancer therapies (Sun, 2006). As a transcription factor, p53 specifically binds to a consensus DNA-binding sequence, consisting of repeats of the 10 bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, located in the promoter or introns of the downstream target genes and transactivates the expression of these genes (el Deiry *et al*., 1992). p53 also transcriptionally represses many genes although its mechanism of action is less well defined (Ho and Benchimol, 2003). As a tumor suppressor, p53, upon activation by a variety of stresses, induces growth arrest that allows cells to repair the damaged DNA or apoptosis when the damage is too severe to repair (Vogelstein *et al*., 2000). This is achieved through transcription dependent and independent mechanisms of p53 action. For transcriptional dependent mechanism, p53 transcriptionally activates *p21* to induce G1 arrest and *14-3-3σ* to induce G2 arrest. On the other hand, p53 induces apoptosis through transactivation as well as transrepression of its downstream target genes. Activating genes include those involved in two major apoptotic pathways: the mitochondrial pathway, such as *BAX*, *NOXA*, *PUMA*, *p53AIP*, *PIGs* and *APAF-1*, and the death receptor pathway, such as *KILLER*/*DR5*, *FAS*, *PIDD*. p53 repressed cell survival genes include *IGFR*, *BCL-2* or *survivi*n (Levine, 1997; Vogelstein *et al*., 2000; Sun, 2006). A number of recent studies have also shown that cytoplasmic p53 can regulate apoptosis in a transcriptionally independent manner by binding to mitochondria and modulating BH3 family pro-apoptotic proteins such as BAX (Mihara *et al*., 2003; Chipuk *et al*., 2004).

As a key regulator of cell growth and cell death, p53 level is precisely regulated in cells. In unstressed normal cells, p53 is kept very low due to its binding to and being degraded by Mdm2. In response to many environmental stimuli, including DNA damaging agents, p53 is activated through phosphorylation and acetylation which separate it from Mdm2 binding (Giaccia and Kastan, 1998). Recent studies have shown that several ribosomal proteins can modulate p53 activity through modulation of Hdm2. Ribosomal protein L11 was found to bind to HDM2 and inhibit HDM2 function, leading to p53 stabilization and activation (Lohrum *et al*., 2003; Zhang *et al*., 2003; Bhat *et al*., 2004). Similarly, L23 as well as L5, two additional ribosomal proteins also bind to HDM2 and inhibits HDM2-induced p53 polyubiquitination and degradation, thus activating p53 (Dai and Lu, 2004; Dai *et al*., 2004; Jin

Correspondence: Professor Y Sun, Department of Radiation Oncology, University of Michigan Comprehensive Cancer Center, 4304 CCGC, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0936, USA.
E-mail: sunyi@umich.edu
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et al., 2004). Furthermore, p53 translation can be enhanced by ribosomal protein L26 (Takagi *et al*., 2005). Finally, a mitochondrial ribosomal protein L41 can directly bind to p53 and enhance translocation of p53 to the mitochondria, thus contributing to p53-induced apoptosis (Yoo *et al*., 2005). Our effort, through the chip profiling of entire human transcriptome for genes responsive to p53-induced growth arrest and apoptosis, led to identification of a novel ribosomal protein, RPS27L as a putative p53-inducible gene (Robinson *et al*., 2003; Sun, 2006). Here, we report that RPS27L is a direct p53 target. p53 directly binds to its consensus-binding site in the first intron of the RPS27L gene and transactivates its expression. Upon induction, RPS27L promotes, whereas upon silencing, RPS27L inhibits, apoptosis induced by etoposide. Thus, RPS27L could contribute to p53-induced apoptosis.

Results

The RPS27L *as a novel p53 inducible gene*

The *RPS27L*, a gene encoding a novel ribosomal protein S27-like, was identified as a p53-inducible gene through the chip profiling of entire human transcriptome using a p53-temperature sensitive H1299-p53A138V human lung cancer cell model (Robinson *et al*., 2003; Sun, 2006). The same analysis revealed that its family member, RPS27 was not subjected to p53 regulation (data not shown). To confirm the chip finding, H1299-p53A138V cells were cultured in 37°C for 24 h before shifting to 39°C (where p53 adapts a mutant conformation) or 32°C (where p53 adapts a wild-type conformation) for 6, 16 and 24 h. Cells were then harvested and subjected to Northern and Western blotting analyses. An anti-RPS27L antibody that specifically detected RPS27L, but not its family member RPS27 (see later in Figure 4a), was generated and used. As shown in Figure 1a, the levels of RPS27L mRNA and protein were low but detectable in cells grown at 39°C for various time points (lanes 1–3). Shifting the culture temperature for a wild-type p53 conformation caused a rapid induction of RPS27L at 6 h and the elevated levels remained up to 24 h (lanes 4–6). The results confirmed the chip-profiling data and showed that RPS27L can be induced by a functional p53. To further confirm p53 inducibility of RPS27L, we extended this study to two additional human cancer cell models. The first pair of cancer cell model used was human osteogenic sarcoma cell lines U2-OS (wild-type p53) and Saos-2 (p53-null). We used a DNA damaging agent, etoposide to induce p53 in U2-OS cells (Li *et al*., 2005). As shown in Figure 1b, treatment of U2-OS cells with etoposide caused a significant induction of RPS27L mRNA at 6 h, reaching a peak induction at 16 h and the elevated level remained at 24 h. The protein induction followed mRNA induction pattern, which started to occur at 16 h and reached the peak at 24 h. In contrast, the same etoposide treatment in p53-null Saos-2 cells did not cause any change of RPS27L mRNA and protein. The

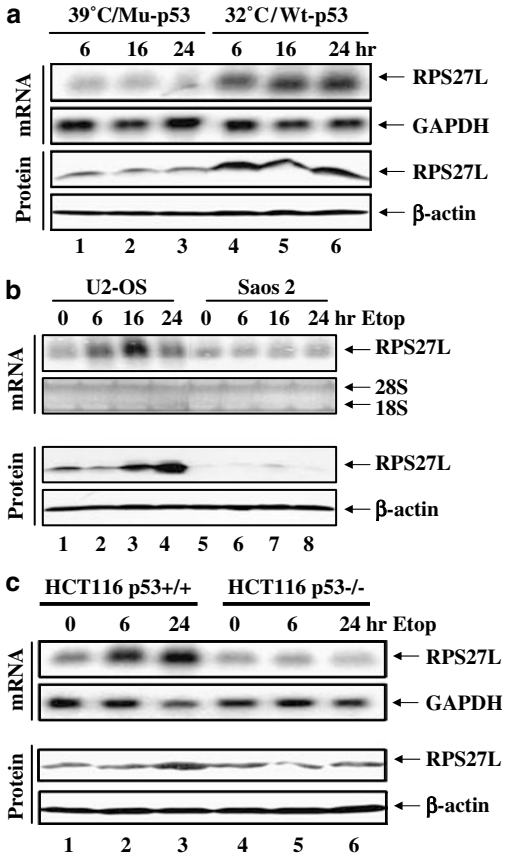

Figure 1 Wild-type p53-dependent induction of RPS27L mRNA and protein: (**a**) Human lung cancer cells: H1299-p53V138A cells were subcultured at 37°C and switched to either 39 or 32°C culture condition for a period of 6, 16 or 24 h. (**b**) Human osteogenic sarcoma cells: U2-OS (wild-type p53) and Saos-2 (p53-null) cells were treated with etoposide (25 μM) for 6, 16 and 24 h. (**c**). Human colon cancer cells HCT116 (p53+/+) and HCT116 (p53−/−) cells were treated with etoposide (50 μM) for 6 and 24 h. All samples were subjected to Northern and Western blotting analyses.

second pair of cancer cell model used was HCT116 cells harboring a wild-type p53 and its isogenic line with p53 gene deleted. After exposure to etoposide for 6 or 24 h RPS27L induction at both mRNA and protein levels occurs only in p53+/+ cells, but not in p53−/− HCT116 cells (Figure 1c). As expected, the same treatment induced p53 and its known target p21 in p53+/+, but not p53−/− cells (data not shown). Taken together, the results strongly suggested a direct role of p53 in induction of RPS27L expression.

p53 directly binds to its consensus-binding site in the first intron of the RPS27L *gene*

RPS27L has a LocusID:51065 under the NCBI locus-Link program. The NCBI Evidence Viewer revealed that human *RPS27L* gene contains four exons and three introns. The p53 consensus-binding site (5′-PuPuPu C(A/T)(A/T)GPyPyPyPuPuPuC(A/T)(A/T)GPyPyPy-3′) was searched for within the 2.2 kb promoter region and the 878 bp intron 1 region. A single putative-binding site with three mismatches (5′-GGGCATGTAGT

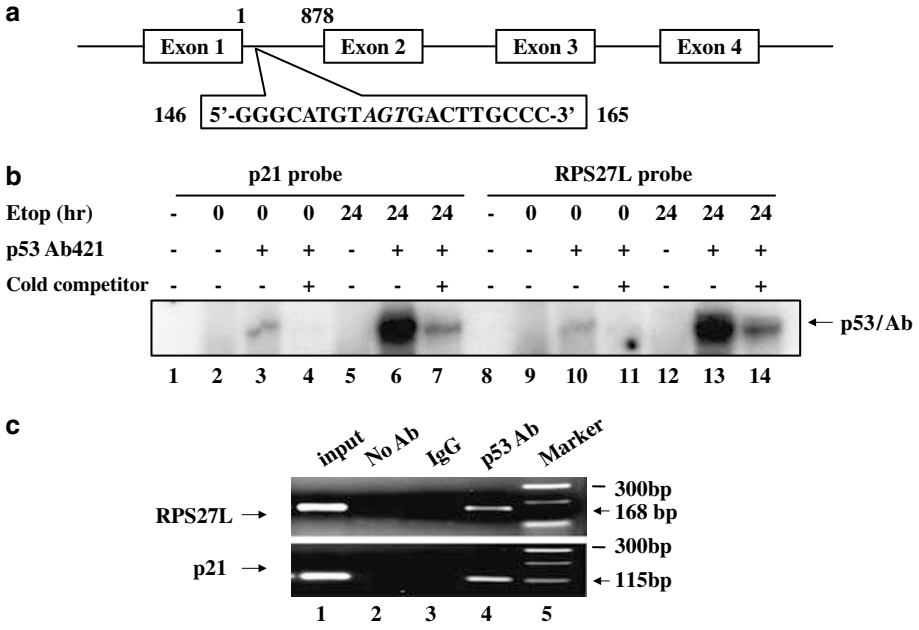

Figure 2 p53 directly binds to its consensus-binding site found in the first intron of RPS27L gene: (**a**). Genomic structure of the RPS27L gene. The RPS27L gene consists of four exons and three introns. The intron 1 sequence was labeled as nucleotides 1–878. A p53-binding site was identified at nts. 146–165 as indicated with three mismatched nucleotides italicized. Graph was not drawn in proportion. (**b**) *In vitro* p53 binding: U2-OS cells were either left untreated (0 h) or subjected to etoposide (25 μM) treatment for 24 h. Nuclear proteins were isolated and used in gel retardation assay using ^{32}P-labelled p53-binding site found in the promoter of p21 gene (p21 probe) or in the first intron of RPS27L gene (RPS27L probe). (**c**). *In vivo* p53 binding: U2-OS were treated with etoposide (25 μM) for 24 h and subjected to ChIP analysis. Input samples or samples precipitated with no Ab, IgG or p53 antibody was PCR amplified using primers specific for RPS27L (top panel) or p21 (bottom panel), respectively.

GACTTGCCC-3′, with mismatches underlined) was identified within the first intron and was designated as RPS27L probe. The RPS27L gene structure and p53-binding site location at the intron 1 is presented in Figure 2a. This 878 bp intron sequence was submitted GenBank with an accession number DQ785795. We next examined whether p53 binds to this putative consensus-binding site. Nuclear extracts were prepared from U2-OS cells either left untreated or treated with etoposide (25 μM to induce p53) for 24 h and subjected to gel retardation assay, using p53 consensus-binding site found in p21 promoter (p21 probe) as a positive control. As shown in Figure 2b, in untreated cells, the low level of p53 failed to bind to p21 or RPS27L probe in the absence of p53-Ab (lanes 2 and 9). A weakly binding was detected in the presence of p53 antibody which is known to enhance the binding (Bian and Sun, 1997b) (lanes 3 and 10). The binding is specific since it was completely blocked by the cold competitor (lanes 4 and 11). The p53 binding to p21 or RPS27L probe was significantly enhanced upon etoposide treatment (lanes 6 and 13). Again, the binding was detected only in the presence of p53 antibody and can be largely blocked by the cold competitor (lanes 5 and 12, 7 and 14). Thus, like to the p21 promoter, p53 directly bound to its consensus sequence in the first intron of the RPS27L gene.

We further confirmed this p53 direct binding using an *in vivo* chromatin immunoprecipitation assay (ChIP). U2-OS cells were treated with etoposide for 24 hr to activate p53 and subjected to ChIP analysis. As shown in Figure 2c (top panel), a 168 bp PCR-amplified fragment, corresponding to a portion of the RPS27L intron 1 where p53 bound to, was detected in the input sample (lane 1) or in the samples immunoprecipitated with p53 antibody (lane 4), but not in two control samples either without antibody added (lane 2) or with IgG added for immunoprecipitation (lane 3). Used as a positive control, the p53 binding to its consensus sequence in the p21 promoter was readily detected as a 115 bp fragment (Figure 2c bottom panel). Thus, we conclude from these results that p53 indeed directly binds to its consensus sequence in the intron of the RPS27L gene.

Wild-type p53- and p53-binding site-dependent transactivation of RPS27L luciferase reporter: blockage by a dominant negative p53 mutant

We next examined whether a direct p53 binding would lead to transcriptional activation. Two luciferase reporters driven by a 733 bp intron sequence (nts 146–878) containing p53-binding site (S27L-w/p53) or by a 713 bp intron sequence (nts 166–878) with p53-binding site deleted (S27L-w/o p53) were constructed and tested. Both constructs, along with the empty vector, pGL3 control were transiently co-transfected with Renillar luciferase (to control transfection efficiency) into H1299-p53A138V temperature-sensitive cells. Cells were then cultured either at 39 or 32°C and subjected to luciferase activity assay. The results were expressed as fold-activation of luciferase activity measured from cells grown at 32 vs 39°C after normalization with pGL3

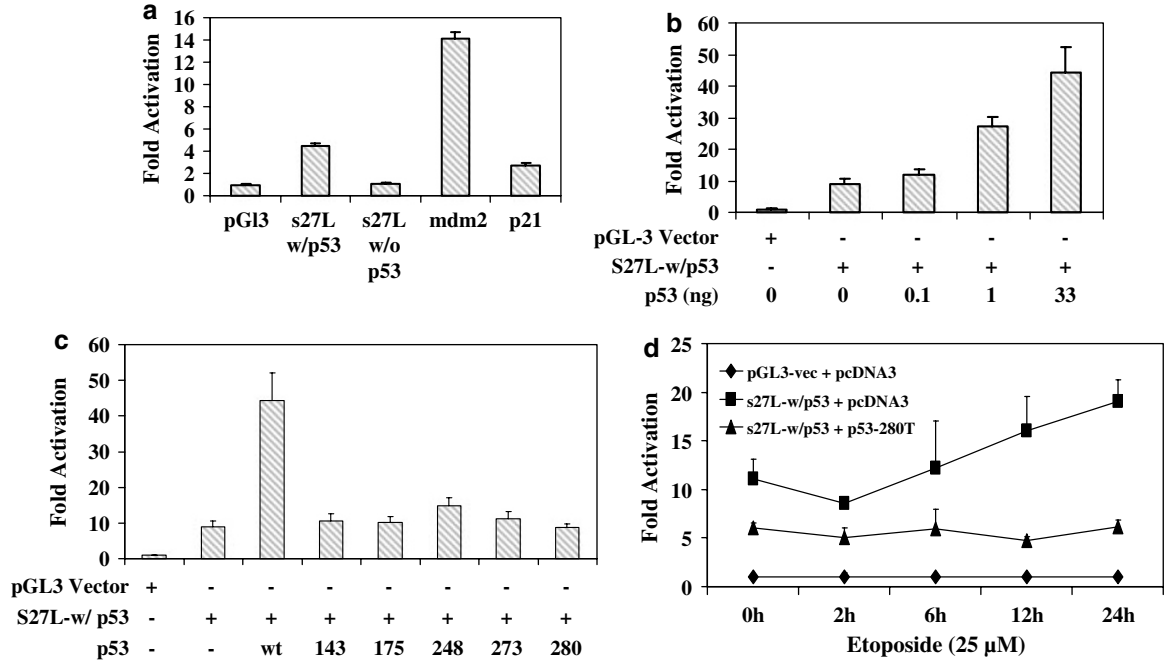

Figure 3 Wild-type p53-dependent transactivation of luciferase reporter driven by RPS27L intron sequence: (**a**) p53-binding site dependent: H1299-p53A138V cells were transiently transfected with the empty luciferase reporter (pGL3), and luciferase reporter driven by RPS27L intron sequence with p53-binding site (S27L-w/p53) or with the p53-binding site deleted (S27L-w/o-p53) or by Mdm2 intron sequence (Mdm2) or p21 promoter sequence (p21), along with Renillar luciferase reporter for the control of transfection efficiency. After transfection, cells were grown for 38 h either in 39 or 32°C before being subjected to luciferase activity. The results from three independent experiments in quadruplicate were presented as the fold induction (32 vs 39°C) after normalization with transfection efficiency. (**b**) p53 dose-dependent: p53-null H1299 cells were transiently transfected with the empty luciferase reporter (pGL3), and luciferase reporter driven by RPS27L intron sequence with p53-binding site and increasing amounts of p53 expressing plasmid (0, 0.3, 1, 33 ng per well in 96-well plate), along with Renillar luciferase reporter for the control of transfection efficiency. (**c**) Wild-type p53 dependent: p53-null H1299 cells were transiently transfected with the empty luciferase reporter (pGL3), and luciferase reporter driven by RPS27L intron sequence with p53-binding site and the plasmids expressing either wild-type p53 or different p53 mutants, along with Renillar luciferase reporter for the control of transfection efficiency. At 38 h post-transfection, luciferase activity was measured and the results from three independent experiments in quadruplicate were presented as the fold induction after normalization with transfection efficiency. (**d**) Activation by etoposide and blockage by a dominant negative p53 mutant: U2-OS cells were transiently transfected with pGL3 vector control and S27L-w/p53 in combination with pcDNA3 vector control or a plasmid expressing p53-280T, a dominant negative p53 mutant, along with Renillar luciferase reporter. At 24 h post-transfection, cells were treated with 25 μM etoposide for 2, 6, 12 and 24 h. Cells were harvested after treatment and subjected to luciferase activity assay. The results from three independent experiments in quadruplicate were presented as the fold induction after normalization with transfection efficiency.

vector controls and Renillar luciferase for transfection efficiency. As shown in Figure 3a, a 4.5-fold activation of luciferase activity was achieved in the S27L-w/p53 luciferase reporter. No difference was detected in S27L-w/o-p53 luciferase reporter, indicating that wild-type p53-mediated transactivation is dependent of this p53-binding site. Two positive controls, Mdm2-luciferase reporter (Mdm2) and p21-luciferase reporter (p21) were included in the assay and showed a 14- or 2.7-fold activation by wild-type p53, respectively.

We next determined whether the activation is p53 dose dependent. The p53-null H1299 cells were transiently transfected with S27L-w/p53 construct, along with different amounts of p53-expressing vector. As shown in Figure 3b, this 733-bp intron sequence caused a 10-fold induction of luciferase activity, compared to pGL3 vector control, indicating a promoter activity. The luciferase activity was further increased up to 45-fold as the amount of p53 expressing plasmid increased, clearly demonstrating a p53-dose dependency.

We further determined whether this *S27L* intron sequence was also subjected to transactivation by various p53 mutants. Plasmid DNAs expressing p53 mutants were individually co-transfected into p53-null H1299 cells with S27L-w/p53 luciferase reporter. The p53 mutants used were the p53-143A, p53-175H, p53-248W, p53-273H, four p53 mutants most commonly found in human cancers (Hollstein *et al*., 1991) and p53-280T, a dominant negative p53 mutant found in nasopharyngeal carcinomas (Sun *et al*., 1993a; Wallingford *et al*., 1997). As shown in Figure 3c, in contrast to wild-type p53, all the p53 mutants failed to induce any significant transactivation of the luciferase reporter. The results indicated that all tested p53 mutants have lost the activity to transcriptionally activate the *RPS27L* gene.

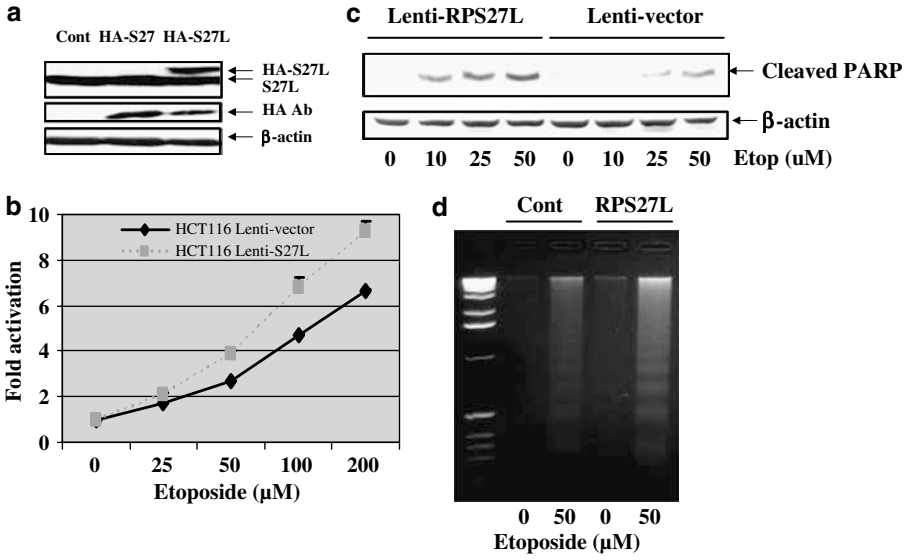

Figure 4 Expression of RPS27L promotes apoptosis induced by etoposide: (**a**) Anti-RPS27L antibody detects RPS27L, but not RPS27: HCT116 cells were infected with empty lenti-vector (Cont) or lenti-virus expressing HA-tagged RPS27 (HA-S27) or HA-tagged RPS27L (HA-S27L). Cells were subjected to Western blotting analysis using antibodies against RPS27L (top panel), HA (middle panel) and β-actin (bottom panel as loading control). (**b**) Expression of RPS27L enhances caspase-3 activation: HCT116 cells infected with the vector control and RPS27L were subjected to etoposide treatment at various drug concentrations. At 24 h post-treatment, cells were harvested and subjected to caspase-3 activity assay. The results of X $\pm$ s.e.m. from three independent experiments were expressed as fold-activation using caspase-3 activity value of the untreated control as 1. (**c**) Expression of RPS27L enhances PARP cleavage: HCT116 cells infected with the vector control and RPS27L were subjected to etoposide treatment at various drug concentrations for 24 h. Cells were then harvested and subjected to Western blotting analysis using antibodies against PARP (top panel) or β-actin (bottom panel as loading control). (**d**) Expression of RPS27L enhances DNA fragmentation: HCT116 cells infected with the vector control and RPS27L were subjected to etoposide treatment (50 μM) for 24 h. Cells were then harvested and subjected to DNA fragmentation analysis.

Finally, we determined whether endogenous p53, upon activation by a DNA damaging agent, would transactivate S27L-w/p53 luciferase reporter. The wild-type p53 containing U2-OS cells were transiently transfected with the reporter and subjected to etoposide treatment. As shown in Figure 3d, etoposide increased luciferase activity steadily in an incubation time-dependent manner reaching a twofold peak at 24 h. To further confirm that the etoposide-induced transactivation is p53-dependent, we transfected p53-280T, a known dominant-negative p53 mutant (Sun *et al*., 1993a; Wallingford *et al*., 1997), into U2-OS cells, followed by etoposide treatment and luciferase assay. As a control, the empty vector was used. Again as shown in Figure 3d, etoposide-induced activation is completely blocked by p53-280T. Taken together, all these results clearly demonstrated that *RPS27L* is subjected to p53 transactivation and is a novel p53 target gene.

Expression of RPS27L promotes apoptosis induced by etoposide

We next determined the biological significance of p53-induced *RPS27L* expression. The lenti-virus expressing HA-tagged RPS27L and its family member, HA-tagged RPS27 were constructed and infected into HCT116 colon cancer cells to make stable RPS27L and RPS27 expressing lines, along with the vector control line. As *RPS27L* encoding mRNA was extremely unstable after being cloned into various mammalian expressing vectors (unpublished data), we made this HA-tagged RPS27L construct using RPS27 encoding mRNA as template and mutated three nucleotides to the RPS27L encoding sequence. We first determined the specificity of a rabbit anti-RPS27L antibody raised against a 14 amino-acid peptide at the N-terminus of the protein, which differs in two amino acids from the corresponding peptide of RPS27. As shown in Figure 4a, this antibody detected both endogenous and HA-tagged RPS27L, but not HA-tagged RPS27 (top panel), whereas anti-HA tag antibody detected both HA-tagged RPS27L and HA-tagged RPS27 (middle panel), indicating that the antibody did not cross-react with RPS27 and was specific for RPS27L. As the level of exogenous expressed HA-RPS27L is comparable to the endogenous protein level, the lenti-virus infection led to a twofold increase of RPS27L level, which is within the physiological range because a twofold induction of endogenous RPS27L was observed after exposure of HCT116 cells to etoposide (Figure 1c). We then determined the effect of this forced expression of RPS27L on etoposide-induced apoptosis. We first measured caspase-3 activity in the vector- and RPS27L-infected cells upon exposure to different concentrations of etoposide for 24 h. As shown in Figure 4b, in both cell lines, the caspase-3 activity increased as the dose of etoposide increased. However, compared to the vector-infected cells, RPS27L-infected cells showed a 1.5-fold higher caspase-3 activity, particularly under higher drug concentrations, suggesting that RPS27L would promote etoposide-induced

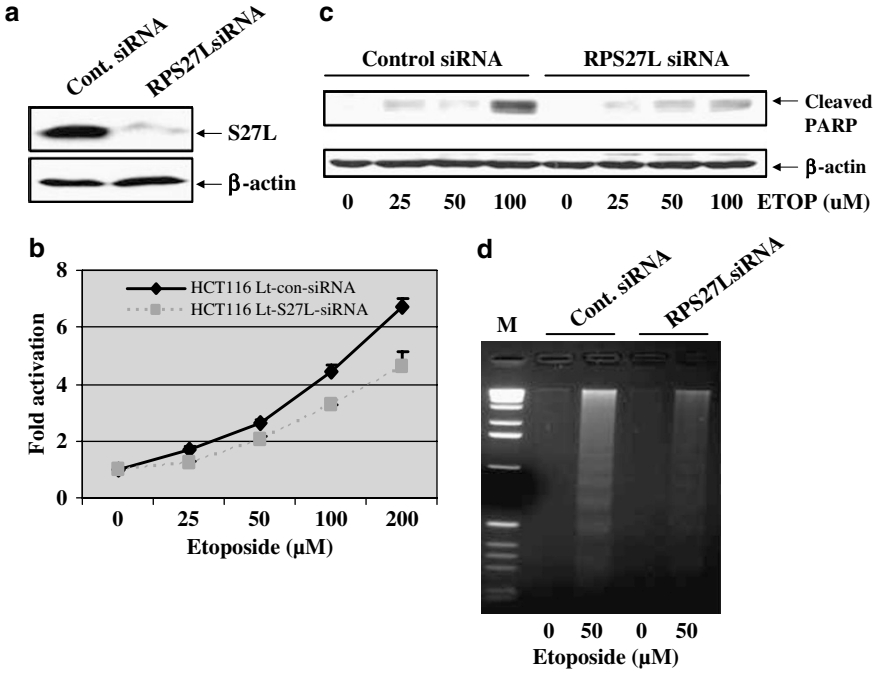

Figure 5 siRNA silencing of RPS27L inhibits apoptosis induced by etoposide: (**a**) siRNA silencing reduces the endogenous level of RPS27L: HCT116 cells were infected with lenti-virus expressing a scamble siRNA (Cont) or lenti-virus expressing siRNA specific for RPS27L (siRNA). Cells were subjected to Western blotting analysis using antibodies against RPS27L (top panel), and β-actin (bottom panel as loading control). (**b**) RPS27L silencing inhibits caspase-3 activation: HCT116 cells infected with lenti-virus expressing the control siRNA (con-siRNA) or RPS27L siRNA were subjected to etoposide treatment at various drug concentrations for 24 h, followed by caspase-3 activity assay. The results of X$\pm$s.e.m. from three independent experiments were expressed as fold-activation using caspase-3 activity value of the untreated control as 1. (**c**) Silencing of RPS27L inhibits PARP cleavage: HCT116 cells infected with lentivirus expressing control siRNA and RPS27L siRNA were subjected to etoposide treatment at various drug concentrations for 24 h. Cells were then harvested and subjected to Western blotting analysis using antibodies against PARP (top panel) or β-actin (bottom panel as loading control). (**d**) Silencing of RPS27L inhibits DNA fragmentation: HCT116 cells infected with lenti-virus expressing control siRNA and RPS27L siRNA were subjected to etoposide treatment (50 μM) for 24 h. Cells were then harvested and subjected to DNA fragmentation analysis.

caspase-3 activation. We next determined whether increased caspase-3 activity led to an increased PARP cleavage, a biochemical index for caspase-3 activation and apoptosis. Indeed, the PARP cleavage product was hardly detectable in un-treated cells and was detected in RPS27L-infected, but not in vector-infected cells after exposure to 10 μM etoposide. The level of PARP cleavage product was further increased as the dose of etoposide increased with a higher level seen in RPS27L-infected cells than that of the vector-infected cells (Figure 4c). Finally, we measured DNA fragmentation, a hall-marker of apoptosis. Treatment of the vector-infected cells with 50 μM etoposide induced a detectable level of DNA fragmentation, which is significantly increased upon RPS27L overexpression (Figure 4d). Taken together, these results demonstrated that expression of RPS27L would promote apoptosis induced by etoposide, suggesting that upon induction by p53, RPS27L could contribute to p53-induced apoptosis.

siRNA silencing of RPS27L inhibits apoptosis induced by etoposide

Finally, we determined whether RPS27L silencing would inhibit apoptosis-induced by etoposide, as one would expect, if RPS27L is a proapoptotic protein. The lenti-virus-based siRNA constructs were made and infected into HCT116 cells, along with the scrambled control siRNA. Indeed, infection of lenti-RPS27L siRNA, but not the control siRNA caused a significant reduction of endogenous RPS27L as shown in Figure 5a. As a consequence, etoposide-induced caspase-3 activation (Figure 5b), PARP cleavage (Figure 5c) and DNA fragmentation (Figure 5d) was all significantly reduced, particularly at high drug concentration, indicating an inhibition of etoposide-induced apoptosis by RPS27L silencing.

Discussion

Although previous studies have shown that various ribosomal proteins can regulate p53 and apoptosis through several mechanisms, including inhibition of Mdm2, enhancing p53 translation and enhancing p53 translocation to mitochondria (Lohrum *et al*., 2003; Zhang *et al*., 2003; Bhat *et al*., 2004; Dai and Lu, 2004; Dai *et al*., 2004; Jin *et al*., 2004; Takagi *et al*., 2005; Yoo *et al*., 2005), none of ribosomal proteins has been

previously identified, to the best of our knowledge, as a direct target of p53 that could contribute to p53-induced apoptosis upon induction by p53. Several lines of evidence presented in this study strongly indicate that RPS27L, a previously unknown protein, is a novel p53 target. (1) The expression of RPS27L is induced in a wild-type p53-dependent manner in multiple human cancer cell models. (2) A consensus p53-binding site was found in the first intron of RPS27L gene and p53 directly binds to the site. (3) Wild-type p53, but not its mutants, transactivates the luciferase reporter driven by this intron sequence in a p53-binding site-dependent manner. And finally (4) wild-type p53-mediated transactivation can be completely blocked by a dominant-negative p53 mutant. Thus, RPS27L joins the list of p53 direct targets in response to stress-induced p53 activation.

RPS27L is an uncharacterized novel protein that differs with only three amino acids (R5K, L12P, K17R) at the N terminus from its family member RPS27. Thus, RPS27L and RPS27 share a 96.3% identity at the protein level. However, at the mRNA level, they are quite diversified and only share a 73% identity within the coding region. The observations that the coding region of RPS27L mRNA is extremly unstable when being cloned into various expressing vectors (unpublished data) and that RPS27L mRNA is detectable in both human tissues and cancer cell lines (see Supplementary Material, Supplementary Figure 1S) indicated that either the 5′-end or 3′-end UTR region or both contribute to its mRNA stability.

Although no previous study has been conducted to understand the function of RPS27L, a number of studies have revealed a cancer relevance of its family member, RPS27. RPS27, also called metallopanstimulin-1 (MPS-1), was first identified as a growth factor inducible gene which encodes a 9.5 kDa protein of 84 amino acids with a zinc-finger-like motif (Chan *et al*., 1993; Fernandez-Pol *et al*., 1993; Wong *et al*., 1993). RPS27/MPS-1 is a nuclear protein and overexpressed in large quantities in various proliferating tissues (Fernandez-Pol *et al*., 1994) and in multiple human cancers, including carcinomas of colon (Ganger *et al*., 1997; Wong *et al*., 1993), prostate (Fernandez-Pol *et al*., 1997), breast (Atsuta *et al*., 2002), liver (Ganger *et al*., 2001), and head and neck (Lee *et al*., 2004). When overexpressed in cancer tissues, RPS27/MPS-1 is released by secretion or gradient diffusion into the extracellular space which allows for its measurement in serum (Lee *et al*., 2004). Upon releasing, RPS27/MPS-1 binds to albumin in serum and can be detected by radio-immuno assay or Western blotting as a 75 kDa band (Fernandez-Pol, 1996; Lee *et al*., 2004). The level of RPS27/MPS-1 was found to be elevated in sera of patients with various type of common cancers (Fernandez-Pol, 1996; Lee *et al*., 2004; Stack *et al*., 2004). It was, therefore, proposed that RPS27/MPS-1 can serve as a promising novel tumor marker, particularly for head and neck squamous cell carcinoma (Fernandez-Pol, 1996; Lee *et al*., 2004; Stack *et al*., 2004). Thus, RPS27 appears to be an oncogene involved in cell proliferation. Unfortunately, the studies on RPS27 were mostly descriptive in nature and limited to cancer biomarker identification. The biological function of this ribosomal protein is yet to be elucidated. Thus, it will be of interest to determine whether the increased level of RPS27 in human cancer is the cause or consequence of carcinogenesis and to understand the mechanism by which RPS27 increases as a result of increased transcription or translation or merely reflecting a mutant protein with a longer protein half-life, in analogs to p53.

Following the lead that RPS27L is a novel p53 target, we characterized its potential function in apoptosis regulation. Our results showed that indeed RPS27L can modulate apoptosis. Upon induction by p53, RPS27L appears to promote apoptosis induced by etoposide, whereas silencing of its expression with siRNA leads to inhibition of etoposide-induced apoptosis. Future study is directed to understand its mechanism of action by determining whether its activity is mediated through the Mdm2 binding, as shown by other ribosomal proteins (Lohrum *et al*., 2003; Zhang *et al*., 2003; Bhat *et al*., 2004; Dai and Lu, 2004; Dai *et al*., 2004; Jin *et al*., 2004), whether it acts in a p53-dependent manner or through other mechanisms.

In summary, we have characterized a novel p53 target, RPS27L that belongs to an uncharacterized ribosomal protein family. Our study presented here suggested that RPS27L could contribute to p53-induced apoptosis upon induction by p53.

Materials and methods

Cell cultures

All human cancer cell lines, except HCT116 cells, used in this study were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HCT116 cells were grown in McCoy's 5A, supplemented with 10% FBS. To change p53 conformation, the culture temperature for H1299-p53V138A cells was either 39°C (non-permissive for wild-type p53) or 32°C (permissive for wild-type). For drug treatment, subconfluent cells were incubated with different concentrations of etoposide (Sigma, St Louis, MO, USA) for various periods of time up to 24 h. The control cells were treated with dimethyl sulphoxide (DMSO).

Northern analysis

The analysis was performed as described (Sun *et al*., 1992) and detailed in Supplementary Materials.

Gel retardation assay

The assay was performed as described (Bian and Sun, 1997a, b). The probe sequence is RPS27L-I01:5′-GGGCATG TAGTGACTTGCCC-3′, consisting of the putative p53-binding site found in the first intron of the RPS27L gene. The positive control used is p21-p01: 5′-GAACATGTCC CAACATGTTG-3′

Chromatin immunoprecipitation assay

The assay was performed according to the protocol of Upstate Biotechnology Inc. (Lake Placid, NY, USA), as described (Li *et al*., 2005). The primer sequences for RPS27L are S27L-Ch01 5′-GAGTCATATGGGACGGATGAG-3′ and S27L-Ch02 5′-CAGCCACCGCCTCTGAATTGCA-3′ to generate a 168 bp

fragment. For p21, the primer sequences are p21-Ch01 5′-GTGGCTCTGATTGGCTTTCT-3′, and p21-Ch02 5′-CTG AAAACAGGCAGCCCAAGG-3′ to generate a 115 bp fragment. The IP antibody used was p53 Ab421 and normal IgG was used as negative controls. One tenth original DNA lysates was used as the input control.

Luciferase reporter constructions

Two luciferase reporter constructs driven by the RPS27L intron 1 sequence was made as follows: (a) RPS27L-w/p53: a 733 bp DNA fragment of the RPS27L intron 1 containing the p53-binding site was generated by PCR amplification of human placenta DNA (Oncor, Gaithersburg, MD, USA) with primers RPS27L-I01 (5′-GGGCATGTAGTGACTTGCCC-3′) and RPS27L-I04 (5′-CTGAACAAAGAAGCATATTATT AC-3′); (b) RPS27L-w/o p53: a 713 bp DNA fragment without p53-binding site was generated with primers RPS27L-I03 (5′-GGTCACCGCGGCGCGGCTGG-3′) and RPS27L-I04. The PCR fragments were gel-purified, first subcloned into a TA cloning vector (In Vitrogen, Carlsbad, CA, USA), and then to pGL-Basic-3 luciferase reporter (Promega, Madison, WI, USA) at *Kpn*I/*Xho*I sites. The resulting constructs were confirmed by sequencing.

DNA transfection and luciferase assay

Dispersed cells were seeded into 96-well plates at a cell concentration of 7.5×10^3 per well for H1299-p53V138A cells, 1×10^4 per well for H1299 cells, or 1.5×10^4 per well for U2-OS cells 16–24 h before transfection using TR01 reagent (America Pharma Source, Gaithersburg, MD, USA). The luciferase reporters, along with the control plasmids, were co-transfected with a Renillar construct in the presence or absence of constructs expressing wild-type or mutant p53 proteins. The luciferase assay was performed 38 h after transfection using the Dual-Glo Luciferase Assay System (Promega). The results from three independent experiments in quadruplicates were presented as the fold activation after normalization with transfection efficiency.

Antibody generation and affinity purification

A polyclonal antibody against RPS27L protein was generated using standard methods by Zymed Laboratories Inc. (South San Francisco, CA, USA). The antigen used is a 14-amino-acid peptide (RL-RPS27L: MPLARDLLHPSLEE) located in the N terminus of RPS27L protein. Two runs of affinity purification were performed using non-specific peptide derived from RPS27 with two amino acids difference (underlined) (KP-RPS27: MPLAKDLLHPSPEE) and RPS27L peptide used as the antigen.

Western blotting analysis

The assay was performed as described (Duan *et al*., 1999) with various antibodies against PARP (Cell Signaling Technology, Beverly, MA, USA), p53 (Ab-6, Calbiochem, San Diego, CA, USA), HA and β-actin (Sigma, St Louis, MO, USA), and RPS27L.

Lenti-virus-based RPS27L overexpression in HCT116 cells

The reverse transcriptase–polymerase chain reaction (RT–PCR) method (Sun *et al*., 1993b) was used to clone a cDNA fragment encoding the entire open reading frame of RPS27L with a HA-tag at the 5′-end. The primers used are S27L-HA-*Bam*HI-01: 5′-GGATCCGTTAACGCCACCATGTATCCA TATGATGTTCCAGATTATGCTCCTTTGGCTAGAGAT TTACTA-3; and S27L-*Not*I-02: 5′-GCGGCCGCTCATTA GTGTTGCTTTCTTCT-3′. The cDNA fragment, flanking the entire open reading frame of RPS27 with HA- tag sequence at the 5′-end was similarly generated by RT–PCR. The primers used are S27-HA-*Bam*I-01: 5′-GGATCCGTTAACGCCACC ATGTATCCATATGATGTTCCAGATTATGCT CCTCTC GCAAAGGATCTC-3; and S27-*Not*I-02: 5′-GCGGCCGCTT AGTGCTGCTTCCTCCTGA-3′. The PCR fragment was cloned into TA vector at first, and subcloned into FG9-EF1α. The resultant clones were sequenced to confirm the orientation and freedom of RT–PCR-generated mutation. As RPS27L lenti-virus construct made from the open reading frame of RPS27L mRNA did not express RPS27L protein due to a highly unstable mRNA with the coding region only (data not shown), we constructed a modified RPS27L construct, which expressed RPS27L protein from the open reading frame of RPS27 mRNA. We took advantage that RPS27L differs from RPS27 in only three amino acids at the N terminus and converted the coding nucleotides of these amino acids from RPS27 to RPS27L by introduction of corresponding mutations at the PCR primers. To generate this modified RPS27L lenti-virus construct, two-step PCR method was used with the following primers (note that the mutant nucleotides were in low case). S27Lm-HA-*Bam*HI-A: 5′-GGATCCGTTAACGC CACCATGTATCCAT ATGATGTTCCAGATTATGCTCC TCTCGCAAgGGATC TC-3′, S27Lm-B: 5′-GTGTTTCtTC TTCTCCTCTTCTaGA GAGGG-3′, S27Lm-C: 5′-CCCTCT CtAGAAGAGGAGAA GAaGAAACAC-3′, S27Lm-Flag-*Not*I-D: 5′-GCGGCCGCTTACTTGTCATCGTCGTCCTT GT AGTCGTGCTGCTTCCTCCTGA-3′. Two PCR fragments were amplified from Lenti-RPS27 with primer pairs of S27Lm-HA-*Bam*HI-A and S27Lm-B or S27Lm-C and S27Lm-Flag-*Not*I-D. The resulting two PCR fragments were then mixed and used as new PCR template with primer pair of S27Lm-HA-*Bam*HI-A and S27Lm-Flag-*Not*I-D. The final PCR products were first cloned into TA vector, then subcloned into Lenti-virus vector. Following DNA sequencing confirmation, the HA-RPS27L-Flag construct, along with the empty vector control and HA-RPS27 construct, was transiently co-transfected into 293 cells with plasmids expressing gag, env and polymerase. Virus-containing supernatants were collected and used to infect HCT116 cells. Expression of HA-RPS27, HA-RPS27L-Flag and endogenous RPS27L was determined by Western blotting using antibody against RPS27L or HA tag.

Lenti-virus-based siRNA silencing of RPS27L in HCT116 cells

A lenti-virus-based siRNA construct was used to make a LT-RPS27L-siRNA. Two siRNAs were made corresponding to amino acids from 1 to 7 or from 36 to 42, respectively. The sequences of these of RPS27L siRNA oligonucleotides are LT-s27L01-01: 5′-CATGCCTTTGGCTAGAGATTTCA AGAGAATCTCTAGCCAAAGGCATGTTTTTTGT-3′ and LT-s27L01-02: 5′-CTAGACAAAAAACATGCCTTTGGCT AGAGATTCTCTTGAAATCTCTAGCCAAAGGCATG-3′; LT-s27L02-01: 5′-TGTCCAGGTTGCTACAAGATTCAAG AGATCTTGTAGCAACCTGGACATTTTTTGT-3′ and LT-s27 L02-02: 5′-CTAGACAAAAAATGTCCAGGTTGCTACAA GATCTCTTGAATCTTGTAGCAACCTGGACA-3′. The control siRNA sequences are LT-Control-01: 5′-ATTGTATGC GATCGCAGACTTTTCAAGAGAAAGTCTGCG ATCGC ATACAATTTTTTGT-3′ and LT-Control-02: 5′-CTAGAC AAAAAATTGTATGCGATCGCAGACTTTCT CTTGAA AAGTCTGCGATCGCATACAAT-3′. These oligonucleotides were annealed to each other and ligated into H1 lentivirus vector, followed by DNA sequence confirmation. These vectors were then co-transfected into 293 cells, along with gag- and env-expressing plasmids. The supernatants

containing viable LT-RPS27L-siRNA virus (combination of two siRNAs) or LT-Cont-siRNA virus were collected and used to infect HCT116 cells. The change of the endogenous RPS27L was determined by Western blotting using anti-RPS27L antibody.

Caspase-3 activity assay

The activity of caspase 3 was analysed using a fluorogenic caspase 3 assay with Ac-DEVD-AFC as a substrate (Biomol Plymouth Meeting, PA, USA), as described by Bockbrader *et al*. (2005).

DNA fragmentation gel analysis

HCT116 cells infected with Lenti-S27L, LT-S27L-siRNA and their corresponding control vectors were cultured in 100 mm dishes and treated with etoposide (50 μM) for 24 h. Both detached and attached cells were collected, lysed, and subjected to DNA fragmentation assay as described (Sun *et al*., 1997).

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