

# p53 and p21 Regulate Error-Prone DNA Repair to Yield a Lower Mutation Load

## Short Article

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### Summary

Regulation of mutation rates is critical for maintaining genome stability and controlling cancer risk. A special challenge to this regulation is the presence of multiple mutagenic DNA polymerases in mammals. These polymerases function in translesion DNA synthesis (TLS), an error-prone DNA repair process that involves DNA synthesis across DNA lesions. We found that in mammalian cells TLS is controlled by the tumor suppressor p53, and by the cell cycle inhibitor p21 via its PCNA-interacting domain, to maintain a low mutagenic load at the price of reduced repair efficiency. This regulation may be mediated by binding of p21 to PCNA and via DNA damage-induced ubiquitination of PCNA, which is stimulated by p53 and p21. Loss of this regulation by inactivation of p53 or p21 causes an out of control lesion-bypass activity, which increases the mutational load and might therefore play a role in pathogenic processes caused by genetic instability.

### Introduction

Despite the presence of multiple error-free DNA repair mechanisms (Friedberg et al., 1995), a significant number of DNA lesions escape repair. During DNA replication these lesions are bypassed by specialized, low-fidelity DNA polymerases in a process termed translesion DNA synthesis (TLS), translesion replication (TLR), or error-prone repair (Goodman, 2000; Livneh, 2001; Prakash and Prakash, 2002). The biological significance of TLS is indicated by the hereditary disease xeroderma pigmentosum variant (XP-V), where the absence of an active TLS polymerase, DNA polymerase  $\eta$  (pol $\eta$ ), causes sunlight sensitivity and predisposition to skin cancer (Johnson et al., 1999; Masutani et al., 1999; McCulloch et al., 2004; Washington et al., 2001). The multiplicity of TLS polymerases and their highly mutagenic activity pose a potential threat to the cell and must be therefore tightly regulated to prevent an escalation in the muta-

tional load and control cancer risk. The molecular mechanism of the regulation of TLS in mammals is largely unknown. However, ubiquitination of PCNA, the DNA sliding clamp that interacts with TLS polymerases (Goodman, 2000; Livneh, 2001; Prakash and Prakash, 2002), appears to be involved in the process, most likely by recruiting TLS polymerases (Hoeye et al., 2002; Kanouche et al., 2004; Stelter and Ulrich, 2003; Watanabe et al., 2004). Here, we report that p53, a major tumor suppressor and a key regulator of cell cycle arrest and apoptosis (Oren, 2003; Vogelstein and Kinzler, 2004), regulates the efficiency and mutagenicity of TLS. This activity involves p21 via its PCNA binding domain and may involve stimulation of PCNA monoubiquitination.

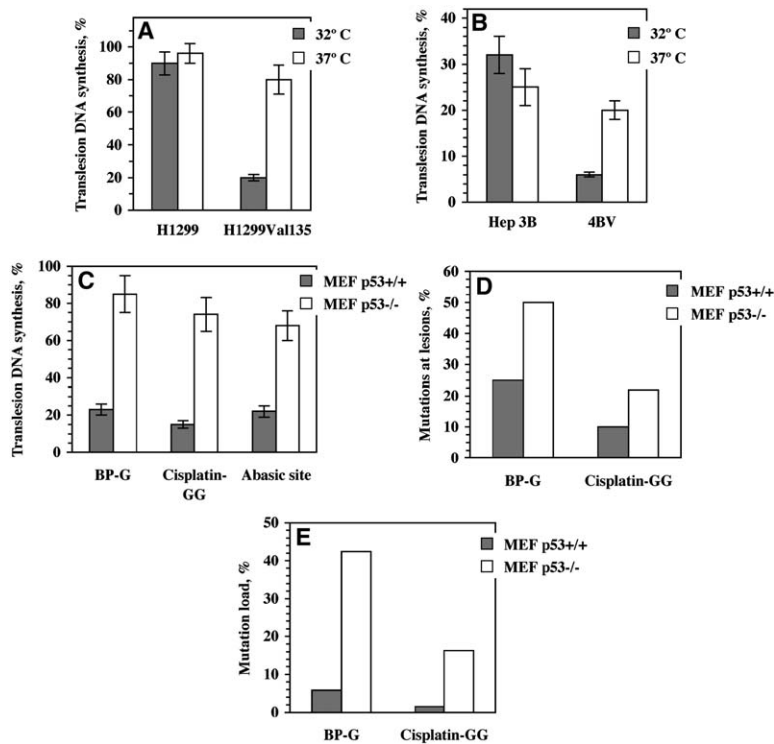
### Results

#### The p53 Protein Suppresses TLS

To study the effect of p53 on TLS, we used a quantitative model TLS assay system that was developed in our laboratory (Avkin et al., 2002, 2004; Avkin and Livneh, 2002). The assay (Figures S1 and S2 available in the Supplemental Data with this article online) consists of transient transfection of cultured cells with a gapped plasmid (Figure S1) carrying a site-specific lesion in the ssDNA region. Plasmids that were fully filled in by TLS are isolated and transformed into an indicator *E. coli* strain for analysis of the extent and mutability of the TLS reaction (Figure S2). This system was previously shown to be specific for TLS and responsive to the type of DNA damage and the DNA polymerase composition of the cell (Avkin et al., 2002, 2004; Avkin and Livneh, 2002). The effect of p53 on TLS was assayed by using the p53 null H1299 lung cancer cell line and a derivative thereof, H1299Val135, stably expressing a temperature-sensitive mouse p53 mutant. The p53Val135 mutant has wild-type p53 activity at 32°C, but not at 37°C (Michalovitz et al., 1990). Figure 1A shows the results of TLS across a synthetic abasic site, a common replication-blocking lesion (Friedberg et al., 1995), assayed in H1299Val135 cells. When p53 was inactive (37°C), TLS across the abasic site was 80%. Remarkably, p53 activation (32°C) led to a 4.2-fold decrease in TLS, down to 19%. This was not a mere temperature effect, as indicated by the similar TLS obtained in parental H1299 cells at 32°C and 37°C (Figure 1A). To examine whether the reduced TLS observed at 32°C is an indirect outcome of the cell cycle arrest imposed by p53, we assayed TLS in 4BV cells, derived from the p53 null Hep-3B hepatocarcinoma cell line by stable transfection with p53Val135 (Friedman et al., 1997). Despite p53 activation and p21 induction, 4BV cells fail to undergo growth arrest at 32°C, probably due to a nonfunctional retinoblastoma protein (Friedman et al., 1997). As seen in Figure 1B, activation of p53 in 4BV cells (32°C) caused a 3.2-fold decrease in TLS compared to 37°C, arguing that the reduction in TLS is due to p53 activity rather than to a general growth arrest.

To rule out the possibility that the effect of p53 on TLS was due to p53 overexpression in transfected cell lines, we performed the assay in p53<sup>+/+</sup> and p53<sup>-/-</sup> mouse

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tion obtained by multiplying the percentage of TLS (from [C]) by the corresponding percentage of mutagenic bypass (from [D]). White columns,  $p53^{-/-}$  MEFs; gray columns,  $p53^{+/+}$  MEFs. Details of the assay, the numeric results, and the calculations are presented in the [Experimental Procedures](#) and in [Tables S1–S5](#).

embryonic fibroblasts (MEFs). As can be seen in [Figure 1C](#), TLS across an abasic site in  $p53^{-/-}$  MEFs was 68%, 3.1-fold higher than in  $p53^{+/+}$  MEFs (22%), similar to the effect obtained with the human cell lines. Bypass across abasic sites in mammalian cells involves an aphidicolin-sensitive DNA polymerase of unclear identity ([Avkin et al., 2002](#)). Based on the *S. cerevisiae* paradigm,  $\text{pol}\delta$  and  $\text{pol}\zeta$  may be implicated ([Haracska et al., 2001](#)). To examine the generality of the p53 effect, we determined TLS across two additional types of DNA damage, which are bypassed by different DNA polymerases: benzo[a]pyrene-guanine (BP-G) and cisplatin-GG, bypassed primarily by  $\text{pol}\kappa$  ([Avkin et al., 2004](#); [Ogi et al., 2002](#)) and  $\text{pol}\eta$  ([Johnson et al., 1999](#); [Masutani et al., 1999](#)), respectively. As seen in [Figure 1C](#), bypass across the BP-G adduct in the  $p53^{-/-}$  MEFs was 85%, 3.7-fold higher than in  $p53^{+/+}$  cells (23%). Bypass across cisplatin-GG was 74% in  $p53^{-/-}$  cells, 4.9-fold higher than in  $p53^{+/+}$  cells (15%) ([Figure 1C](#)). Thus, the effect of p53 on TLS is general.

### The p53 Protein Increases the Fidelity of TLS

A critical property of TLS is its fidelity, which determines to a large extent the mutagenic outcome of unrepaired DNA lesions. We examined the mutagenicity of TLS by DNA sequence analysis of plasmids that were filled in by TLS in cultured cells. Mutagenicity was quantified by determining the percent of incorrect nucleotides inserted opposite the lesion. Fifty percent of TLS events across BP-G were found to be mutagenic in  $p53^{-/-}$  MEFs, in contrast to only 25% in  $p53^{+/+}$  MEFs ([Figure 1D](#) and [Table S4](#);  $p = 0.02$ ). As for cisplatin-GG, 23% of TLS

**Figure 1. The p53 Protein Suppresses the Efficiency of TLS but Increases Its Fidelity**

(A) The TLS assay was performed with H1299Val135 cells expressing a temperature-sensitive p53 protein. The cells were transfected with the gap-lesion plasmid GP21, which carries a synthetic abasic site in the ssDNA region, along with the control and carrier plasmids. After transfection, cells were incubated in parallel at 32°C (gray columns) or 37°C (white columns). As a control, H1299 cells were assayed at the two temperatures.

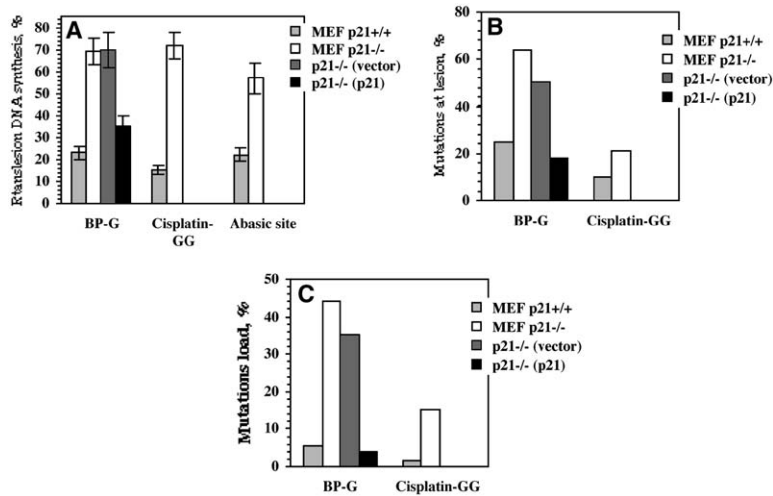
(B) The experiments were performed as in (A), except that the Hep3B cell line and its derivative 4BV expressing the p53Val135 mutant were used.

(C–E) TLS in  $p53^{-/-}$  and  $p53^{+/+}$  mouse embryo fibroblasts assayed with gap-lesion plasmids carrying either an abasic site, a benzo[a]pyrene-G (BP-G) adduct, or a cisplatin-GG adduct. (C) Efficiency of TLS. (D) Mutagenicity of the TLS reaction, namely the percentage of incorrect nucleotides inserted opposite the lesion (sequences other than C for BP-G, and sequences other than CC for cisplatin-GG). The statistical significance of the differences in mutagenesis between the two cell types was calculated by the chi-square test, yielding  $p$  values of 0.02 and 0.009 for BP-G and cisplatin-GG, respectively. (E) The mutational load of the TLS reactively.

events were mutagenic in  $p53^{-/-}$  MEFs, whereas only 10% were mutagenic in  $p53^{+/+}$  MEFs ([Figure 1D](#) and [Table S5](#);  $p = 0.009$ ). In the absence of coding information in an abasic site, accurate bypass cannot be defined, and therefore differences cannot be measured for this lesion. This 2-fold lower mutagenicity observed in  $p53^{+/+}$  cells, when combined with the lower TLS efficiency, causes a much greater decrease in the mutational load. Because not all gap-lesion structures give rise to a mutation via TLS (some are bypassed accurately, and some do not survive), we define the mutation load as the fraction of gap-lesion structures that give rise to mutations. In our system, the mutation load is obtained by multiplying the efficiency of TLS by the fraction of incorrect insertions opposite the lesion. In the absence of p53, the mutational loads of cisplatin-GG and BP-G were 10.9- and 7.3-fold higher than in the presence of p53, respectively ([Figure 1E](#)).

### The p53-Regulated p21 Protein Suppresses TLS and Increases Its Fidelity

The effect of p53 may be direct, via involvement in TLS, or indirect, via its activity as a transcription factor. Of the many known proteins induced by p53, we focused on p21 because it interacts with PCNA and inhibits PCNA-dependent DNA replication by  $\text{pol}\delta$  ([Fotedar et al., 2004](#); [Waga et al., 1994](#)). In addition, as described above, ubiquitination of PCNA was proposed to recruit  $\text{pol}\eta$  to sites of DNA damage. TLS across three DNA lesion types was therefore assayed in  $p21^{+/+}$  and  $p21^{-/-}$  MEFs. For all lesions, TLS was higher in the  $p21^{-/-}$  MEFs compared to  $p21^{+/+}$  MEFs: 3-fold for BP-G, 4.8-fold for



(C) The mutational load of the TLS reaction obtained by multiplying the percentage of TLS (from [A]) by the corresponding percentage of mutagenic bypass (from [B]). Gray columns, *p21*<sup>+/+</sup> MEFs; white columns, *p21*<sup>-/-</sup> MEFs; dark gray columns, *p21*<sup>-/-</sup> MEFs transfected with an empty vector; and black columns, *p21*<sup>-/-</sup> MEFs transfected with a plasmid expressing p21. Details of the assay, detailed numeric values of the results, and the calculations are presented in the [Experimental Procedures](#) and in [Tables S3–S5](#) and [S7](#).

cisplatin-GG, and 2.6-fold for the synthetic abasic site (Figure 2A). Transfection of *p21*<sup>-/-</sup> MEFs with a plasmid expressing p21 suppressed the TLS efficiency across BP-G down to a level similar to that observed in *p21*<sup>+/+</sup> MEFs (Figure 2A), indicating that it is indeed p21 that is affecting TLS. When the fidelity of TLS was examined, p21 was found to exert an effect similar to p53. TLS across BP-G and cisplatin-GG was 2.6-fold (Figure 2B and Table S4; *p* = 0.006) and 2.1-fold (Figure 2B and Table S5; *p* = 0.056), respectively, more mutagenic in *p21*<sup>-/-</sup> MEFs compared to *p21*<sup>+/+</sup> MEFs. Reintroduction of p21 suppressed the mutagenicity of TLS across BP-G in *p21*<sup>-/-</sup> MEFs (Figure 2B and Table S4; *p* = 0.015). Taking into account the higher extent of TLS in the *p21*<sup>-/-</sup> MEFs, the overall mutational load of BP-G and cisplatin-GG in these cells was 7.8- and 10.1-fold higher than in *p21*<sup>+/+</sup> MEFs, respectively (Figure 2C). We found no effect of UV irradiation of the cells on TLS in our system (Table S6). This may be because the transfection itself is a stress treatment to cells and is known to induce p53 and cell cycle arrest (Renzing and Lane, 1995). In addition, UV radiation is not needed for DNA gap formation, as gaps are provided in our system by the gap-lesion plasmid. These two conditions combined appear to be sufficient to elicit specific TLS without treating the cell with a DNA damaging agent.

### The p21 Protein Affects TLS via Its PCNA-Interacting Domain and Partially Complements the Effect of p53 Deficiency

The C-terminal domain of p21 was shown to interact with PCNA (Gulbis et al., 1996), whereas its N terminus interacts with cyclin-dependent kinases (Rousseau et al., 1999). In order to examine whether the PCNA-interacting portion of p21 is sufficient to suppress TLS, we used *p21*<sup>-/-</sup> MEFs transfected with a plasmid encoding the C-terminal portion of p21 (amino acids 114–159). As seen in Figure 3, plasmids expressing the intact p21 protein (Figure 3C, lane 2) or its C-terminal portion (Figure 3D, lane 3) suppressed TLS across a BP-G lesion to a similar extent (Figure 3A). In contrast, expressing

### Figure 2. The p21 Protein Suppresses the Efficiency of TLS but Increases Its Fidelity in MEFs

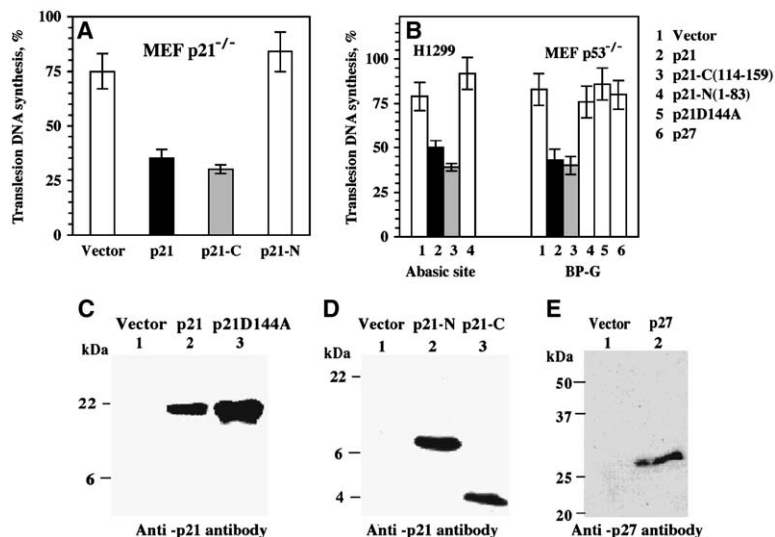
The TLS assays were each performed with a different gap-lesion plasmid carrying either an abasic site, a BP-G adduct, or a cisplatin-GG adduct.

#### (A) Efficiency of TLS.

(B) Mutagenicity of the TLS reaction, namely the percentage of incorrect nucleotides inserted opposite the lesion (sequences other than C for BP-G, and sequences other than CC for cisplatin-GG). The statistical significance of the differences in mutagenesis between the *p21*<sup>+/+</sup> and *p21*<sup>-/-</sup> MEFs was calculated by the chi-square test, yielding *p* values of 0.006 and 0.056 for BP-G and cisplatin-GG, respectively. The *p* value was 0.015 for the results of the complementation experiment (*p21*<sup>-/-</sup> cells transfected with the p21-expressing plasmid versus the empty vector).

the N-terminal portion of p21 (amino acids 1–83; Figure 3D, lane 2) had no effect on TLS (Figure 3A).

The experiments presented so far suggest that p53 and p21 exert similar effects on TLS. To examine whether the entire effect of p53 can be attributed to the induction of p21, we measured TLS in p53 null H1299 cells transfected with p21 expression plasmids. Expression of p21 caused a decrease in TLS across an abasic site, and a similar effect was caused by the C-terminal fragment of p21 (amino acids 114–159), but not its N-terminal fragment (amino acids 1–83) (Figure 3B). A similar pattern of suppression of TLS by p21 was observed with another lesion (BP-G) in *p53*<sup>-/-</sup> MEFs (Figure 3B). No effect was observed with a plasmid expressing the p27 protein (Figure 3B, column 6, and Figure 3E, lane 2), indicating that the effect of p21 cannot be mediated by another cell cycle inhibitor. To further examine whether the effect of p21 is mediated via its PCNA-interacting domain, we expressed the p21D144A mutant protein (Figure 3C, lane 3), which carries a single amino acid substitution in the PCNA binding site and is defective in PCNA binding (equivalent to p21D149A in humans [Kontopidis et al., 2005; Zheleva et al., 2000]). As can be seen in Figure 3B (column 5), in contrast to wt p21, this p21D144A mutant protein had no effect on TLS. Taken together, these results suggest that the inhibition of TLS by p21 is mediated via its interaction with PCNA. The effects obtained in this series of experiments were approximately 2-fold, less than the 3.7- to 4.9-fold TLS suppression attributed to active p53 (see Figure 1), suggesting partial complementation in *p53*<sup>-/-</sup> MEFs by p21. Thus, although the effect of p53 on TLS is mediated to a large extent via p21, it may also have an additional component. We examined whether this component may involve the induction of TLS polymerases by p53. To that end, we measured by immunoblotting the level of four TLS DNA polymerases, pol $\eta$ , pol $\kappa$ , pol $\iota$ , and hREV1, in H1299 and H1299Val135 cells grown under either permissive or restrictive temperatures. We found no significant effect of p53 on the expression of these DNA polymerases (Figure S3).



**Figure 3. Suppression of TLS by p21 Is Mediated via Its C-Terminal PCNA-Interacting Region and Partially Restores Suppression of TLS to  $p53^{-/-}$  MEFs**

(A) Efficiency of TLS across an abasic site in  $p21^{-/-}$  MEFs expressing p21, its C-terminal, or its N-terminal portion.

(B) Efficiency of TLS in cells lacking p53, which express p21 mutants. TLS across an abasic site was assayed in H1299 cells and across BP-G in  $p53^{-/-}$  MEFs. White columns (also numbered 1 in [B]), the empty vector pCDNA3; black columns (also numbered 2 in [B]), cells expressing the intact p21 gene; gray columns (also numbered 3 in panel B), cells expressing p21-C(114-159); white columns (also numbered 4 in panel B), cells expressing p21-N(1-83); white column numbered 5, cells expressing p21D144A; and white column numbered 6, cells expressing p27.

(C) Immunoblot showing expression of p21 and p21D144A.

(D) Immunoblot showing expression of p21-N (amino acids 1-83) and p21-C (amino acids 114-159).

(E) Immunoblot showing expression of p27. Details of the assay, detailed numeric values of the results, and the calculations are presented in the [Experimental Procedures](#) and in [Tables S7 and S8](#).

### UV-Induced Monoubiquitination of PCNA Is Stimulated by p53 and p21

A possible pathway by which p53 and p21 may exert their effect on TLS is via PCNA monoubiquitination, which was implicated to be involved in TLS as described above. To examine this possibility, we measured the effect of p53 on UV-induced PCNA ubiquitination in two cell systems: H1299Val135 cells (ts p53 mutant active at 32°C) compared to H1299 cells (p53 null), and U2OS cells expressing wild-type p53, in which p53 was stably knocked down by shRNA, compared to control U2OS cells stably transfected with *lacZ* shRNA (Figures 4A and 4B). The triton-insoluble chromatin bound PCNA was observed under all conditions, being lowest in unirradiated H1299Val135 at 32°C, possibly a result of the cell cycle arrest caused by activated p53 (Figure 4A, lane 2; the band is seen only at higher exposure of the gel). Interestingly, under these conditions, UV caused a 5-fold increase in the fraction of chromatin bound PCNA (Table S9, column 5 rows 3 and 4). Essentially no ubiquitination of PCNA was observed in unirradiated cells (Figure 4A, lanes 1-4); however, upon UV irradiation, a fraction of the chromatin bound PCNA became monoubiquitinated (Figures 4A and 4B, lanes 6 and 8). Strikingly, in UV-irradiated H1299Val135 cells grown at 32°C, where both active p53 and p21 were expressed (Figure 4A, lanes 5 and 6; Table S9), 45.5% ± 5% of the chromatin bound PCNA was ubiquitinated (Figure 4A, lane 6), 5.3-fold higher than in the parental H1299 cells lacking p53 grown at the same temperature (8.6%; Figure 4A, lane 8; Table S9). Interestingly, UV irradiation led also to the retention of p21 in the chromatin bound fraction (Figure 4A, compare lanes 2 and 6). A similar effect of p53 on PCNA ubiquitination was observed in U2OS cells. In UV-irradiated U2OS cells expressing p53 (Figure 4B, lane 6) 16% of the chromatin

bound PCNA was monoubiquitinated, 8-fold higher than in U2OS cells in which p53 expression was suppressed by p53 shRNA (2%; Figure 4B, lane 8). Thus, the presence of active p53 stimulates the UV-induced monoubiquitination of chromatin bound PCNA.

To examine the involvement of p21 in this effect, we assayed UV-induced PCNA monoubiquitination in U2OS cells in which p21 expression was knocked down by using a p21-specific siRNA oligonucleotide. As expected, the expression of p21 was reduced in cells transfected with the p21-specific synthetic siRNA compared to cells transfected with the control oligonucleotide (Figure 4C, even-numbered lanes and odd-numbered lanes, respectively). The amount of triton-insoluble p21 was considerably lower than the amount of soluble p21 (Figure 4C, bottom boxes), and UV irradiation diminished the amount of the soluble p21 (Figure 4C, lanes 7, 9, and 11), but not the chromatin bound insoluble p21 (Figure 4C, lanes 1, 3, and 5). Again, monoubiquitination of PCNA was not observed in unirradiated cells (Figure 4C, lanes 1, 2, 7, and 8) and was restricted to chromatin bound (triton-insoluble) proteins in UV-irradiated cells (Figure 4C, lanes 3-6). Remarkably, UV-induced monoubiquitination of PCNA was suppressed by 7-fold (at 10 J/m<sup>2</sup>) and 3-fold (at 20 J/m<sup>2</sup>) in cells transfected with the p21-specific siRNA compared to the control cells (Figure 4C, lanes 3-6). Thus, like p53, p21 is needed for efficient UV-induced PCNA monoubiquitination.

### Discussion

The presence of multiple mutagenic TLS DNA polymerases in mammalian cells poses a special challenge to genome stability, as their unrestrained action may lead to a high mutational burden, which may cause severe malfunction. Our study suggests that p53 and p21

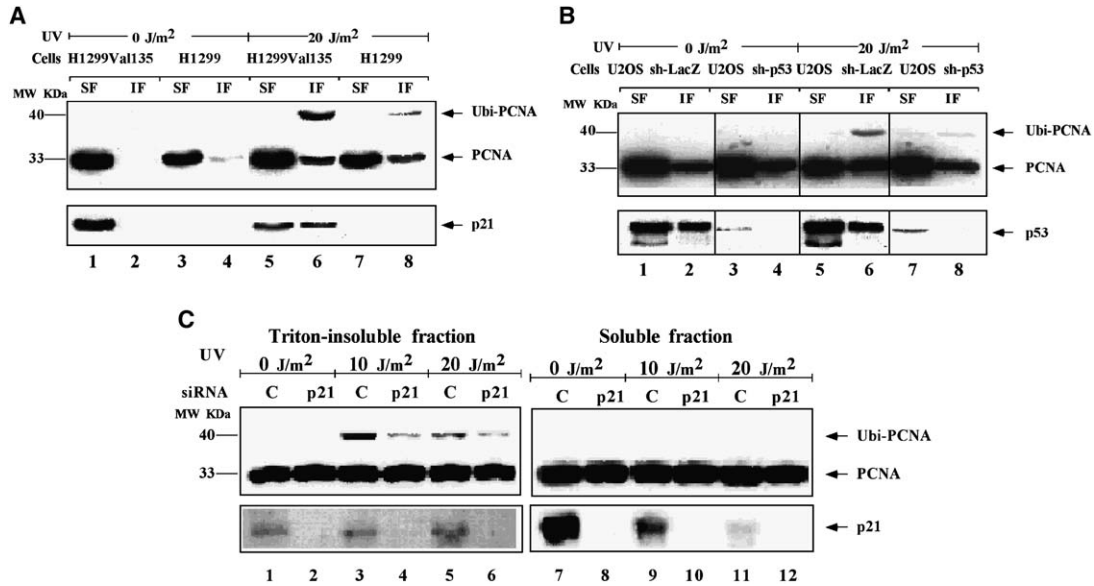


Figure 4. The p53 Protein Stimulates UV-Induced Ubiquitination of PCNA in Human Cells

(A) H1299 cells (p53 null) and their derivative H1299Val135 cells expressing a ts p53 protein were incubated at 32°C (the permissive temperature) and then UV irradiated (20 J/m<sup>2</sup>) and incubated for 6 hr at the same temperature. Equal amounts (14 μg) of total protein from Triton-soluble (SF) and Triton-insoluble (IF) fractions were fractionated by electrophoresis in a 12% SDS-PAGE gel and immunoblotted with anti-PCNA (top box) or anti-p21 antibodies (bottom box).

(B) A similar experiment was performed with U2OS cells stably expressing shRNA to *lacZ* or p53 at 37°C. Ten micrograms of total protein was loaded in each lane, and the gel was immunoblotted with anti-PCNA (top box) or anti-p53 (bottom box) antibodies. The order of the lanes of the original gel image was changed for clarity, but they all are from the same gel and the same exposure. Quantification is shown in Table S9.

(C) A similar experiment was performed with U2OS cells transfected with p21-specific siRNA or control siRNA at the indicated UV doses. Triton-insoluble (lanes 1–6) and triton-soluble (lanes 7–12) proteins from these cells were immunoblotted with anti-PCNA (top box) or anti-p21 (bottom box) antibodies. The images on the right and left sides are from different gels. The amount of triton-insoluble p21 was considerably lower than the amount of soluble p21, and therefore, a longer exposure time was needed for the former, leading to a higher background in the gel image. “C” stands for the control siRNA.

have a key function in this regulation by restraining the mutational burden caused by TLS. This may represent a fidelity-for-efficiency tradeoff, which acts to assure a tolerable rate of mutagenesis. According to this model, in cells where p53 or p21 is inactivated, TLS is grossly deregulated, leading to unrestrained high and mutagenic bypass of DNA lesions. This provides new insight regarding p53 and p21 and may be part of the “guardian of the genome” activity of p53 (Lane, 1992).

Our results, obtained with a model system, predict that chromosomal mutagen-induced mutagenesis, which is a manifestation of chromosomal TLS, will be suppressed by p53. Indeed, UV-induced HPRT mutation frequency was reported to be lower in a SAOS-2 cell line derivative stably expressing wild-type p53, compared to the parental p53 null SAOS-2 line (Yagi et al., 1998), and similar results were reported in other studies (Havre et al., 1995; Yamagishi et al., 1997; Yuan et al., 1995). Thus, the plasmid assay system seems to share at least some regulatory elements with chromosomal TLS.

The mechanism by which p53 regulates TLS is not fully understood, but it does not seem to involve increased expression of TLS polymerases in human cells (Figure S3), consistent with a previous report (Velasco-Miguel et al., 2003). We propose that regulation of TLS by p53 may be mediated, at least in part, via binding of p21 to PCNA and PCNA monoubiquitination. The p21 binding site on PCNA, which overlaps the polδ binding site (Gulbis et al., 1996; Zhang et al., 1998), is in the vicin-

ity of K164, which is the site of PCNA ubiquitination. Thus, binding of p21 to PCNA stalled at a lesion might fulfill a dual function: (1) stimulation of PCNA ubiquitination, e.g., by causing dissociation of the large polδ (125 kDa) thereby increasing the accessibility of K164 and (2) a general inhibition of binding of “nonlicensed” polymerases to PCNA. Under this situation, the ubiquitination may enable binding of the “correct” TLS polymerase (e.g., polη), whereas the bound p21 may inhibit binding of other polymerases, leading to higher fidelity of bypass. Consistently, we have observed retention of p21 in the chromatin bound fraction of extracts from UV-irradiated p53 proficient cells (Figure 4A, lanes 5 and 6; Figure 4C, lanes 3 and 5), although we do not know yet whether this p21 is associated with the PCNA. In the absence of p21, and with less ubiquitinated PCNA, other less accurate TLS polymerases may gain access to DNA, leading to higher though less accurate TLS. This model is consistent with our observation that UV-induced ubiquitination is stimulated by p53 and p21. It was recently reported that p21 inhibits UV-induced PCNA ubiquitination in a pathway that is not dependent on its interaction with PCNA (Soria et al., 2006). However, those results were obtained with overexpressed p21, which might have masked some of its normal regulatory effects.

In summary, our results suggest an unexpected layer of regulation of TLS in which p53 and p21 are key players and might act, at least in part, via binding of p21 to PCNA

and via stimulation of PCNA monoubiquitination by p53 and p21. The results presented justify an in-depth examination of the significance of the p53 and p21 regulation of TLS and mutagenesis in pathogenic processes such as carcinogenesis.

#### Experimental Procedures

##### Construction of Plasmids

The gapped plasmids used are shown in Figure S1. The construction of gapped plasmids containing a site-specific synthetic abasic site (GP21), or a (+)-trans-BPDE-N2-dG adduct (GP-BPG1) in the ssDNA region, and the control gapped plasmid GP20-cm was previously described (Avkin et al., 2002, 2004; Reuven et al., 1998). A gapped plasmid carrying a site-specific cisplatin-GG adduct (GP-cisPtGG1) was prepared by a similar procedure, (see Supplemental Data). The construction of plasmids expressing intact or mutant p21 proteins, and p27 is described in the Supplemental Data.

##### TLS Assay in Mammalian Cells

The quantitative TLS assay was previously described (Avkin et al., 2004). Further details are presented in the Supplemental Data.

##### Analysis of UV-Induced Ubiquitination of PCNA

H1299 and H1299Val135 cells were incubated at 32°C (at which p53Val135 is active) for 14 hr, after which they were UV irradiated at 20 J/m<sup>2</sup> and returned to 32°C for 6 hr. Extraction of Triton-soluble and insoluble fractions was done as described (Kannouche et al., 2004). Protein samples (14 µg) were fractionated by electrophoresis in a 12% SDS-PAGE gel, followed by immunoblotting and probing with anti-PCNA antibody (PC-10, Sigma) or anti-p21 antibody (F5 or C19, Santa Cruz Biotechnology). The monoubiquitinated PCNA band was identified based on its reactivity with anti-PCNA antibodies, on its apparent size (approximately 40 kDa), and on its comigration with HA-ubiquitinated PCNA immunoprecipitated with anti-PCNA antibodies and detected with anti-HA antibodies. The same experiment was conducted with U2OS cells (which contain a wild-type p53) stably expressing shRNA to *lacZ* or p53 (Brummelkamp et al., 2002), except that cells were incubated at 37°C throughout the experiment, and blots were probed in parallel with an anti-PCNA antibody (PC-10) or anti-p53 antibody (a mixture of monoclonal antibodies PAb1801 and DO-1). The effect of p21 on PCNA ubiquitination was similarly examined with U2OS cells transfected with p21-specific synthetic siRNA (Qiagen, Hilden, Germany) or the commercially supplied control siRNA at 10 nM. At 72 hr after transfection, the cells were irradiated with UV doses of 0, 10, or 20 J/m<sup>2</sup>, and 6 hr later, PCNA ubiquitination and p21 amounts were examined by immunoblotting.

##### Statistical Analysis

The statistical significance of the differences in mutation frequency obtained with pairs of different cell types or different expression plasmids was calculated by the chi-square test using the SAS software (SAS Institute, Cary, NC).

##### Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, three figures, and nine tables and can be found with this article online at <http://www.molecule.org/cgi/content/full/22/3/407/DC1/>.

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