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Defects in transcription coupled repair interfere with expression of p90^{MDM2} in response to ultraviolet light

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Ultraviolet (UV) irradiation transiently stabilizes p53 through a mechanism that may require a decrease in the activity of the ubiquitin ligase, p90^{MDM2}. Conversely, the recovery of low levels of p53 following UV exposure may depend on an increase in p90^{MDM2}. The level of p90^{MDM2} is increased by UV light following the p53-dependent induction of an internal *mdm2* promoter, P2. If this induction of *mdm2* were critical for the recovery of low levels of p53 following UV exposure, defects in *mdm2*'s transcription would result in a prolonged increase in p53. Cells defective in transcription coupled repair (TCR) maintain high levels of p53 for a prolonged period following UV exposure. Such cells also have defects in general transcription after UV irradiation. We investigated whether TCR-deficient cells express diminished levels of *mdm2* mRNA and p90^{MDM2} following UV exposure. We found that transcription of *mdm2* was reduced in TCR-deficient cells. The uninducible *mdm2* promoter, P1, was more sensitive to the inhibitory effects of UV irradiation than the P2 promoter. The decrease in transcription from the P1 promoter was sufficient to reduce the level of p90^{MDM2} and correlated with a prolonged increase in p53. Thus, p53-independent transcription of *mdm2* appears critical to p53's regulation. *Oncogene* (2001) **20,** 5856–5864.

Keywords: p53; MDM2; ultraviolet; transcription

Introduction

The p53 tumor suppressor is an important mediator of the response of mammalian cells to ultraviolet (UV) irradiation. In UV-treated cells, p53 is transiently stabilized, resulting in induction of a battery of p53-responsive genes encoding proteins involved in apoptosis, growth arrest, and DNA repair (Maltzman and Czyzyk, 1984; Ziegler *et al*., 1994; Geyer *et al*., 2000; Hwang *et al*., 1999). p53's apoptotic function is thought to protect against UV-induced skin tumorigenesis because mice lacking p53 have an increased incidence of UV-induced tumors (Li *et al*., 1995; Jiang *et al*., 1999).

The mechanisms controlling the stability of p53 in response to UV light are unclear. Initially, the half-life of p53 is lengthened from approximately 20 min to several hours, allowing p53 to accumulate (Maltzman and Czyzyk, 1984). Ubiquitination of p53 is reduced within 1 h of UV-exposure, suggesting that proteosomal degradation of p53 is diminished (Maki and Howley, 1997). After a period of time that correlates with the dose of UV light, the half-life of p53 becomes shortened (Abrahams *et al*., 1995). This period is lengthened in cells deficient in the repair of UV-induced lesions, suggesting that either DNA damage or the repair machinery influences the kinetics of p53's recovery to its low basal level (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996; Dumaz *et al*., 1997).

The changes in p53's stability in response to UV-exposure may be determined by the activity of the ubiquitin ligase, p90^{MDM2}, which controls the basal level of p53 in untreated, diploid human fibroblasts (Haupt *et al*., 1997; Kubbutat *et al*., 1997; Blaydes and Wynford-Thomas, 1998). Multiple mechanisms have been proposed to reduce p90^{MDM2}'s ability to ubiquitinate p53 following UV exposure, but none have been shown to be important for p53's stabilization. For example, UV exposure results in changes in p53's phosphorylation status that potentially disrupt the p90^{MDM2}/p53 interaction (Shieh *et al*., 1997). Although disruption of this interaction by neutralizing antibodies stabilizes p53, it is not clear that the interaction between p53 and p90^{MDM2} is blocked in the UV response (Blaydes and Wynford-Thomas, 1998). In fact, Geyer *et al*. (2000) found no decrease in the amount of the p90^{MDM2}/p53 complex in UV-treated human fibroblasts under conditions in which p53 was stable.

A second model proposes that a decrease in the specific activity of p90^{MDM2}'s ubiquitin ligase is critical for p53's stabilization. For example, direct modification of p90^{MDM2} by the small protein SUMO-1 has been shown to reduce p90^{MDM2}'s ability to ubiquitinate p53 (Buschmann *et al*., 2000). Sumoylation has been reported to retard the migration of p90^{MDM2} on polyacrylamide gels from 75 kDa to 90 kDa (Buschmann *et al*., 2000). The 90 kDa form of p90^{MDM2} is selectively reduced in human fibroblasts exposed to a high dose (45 J/m^2) of UV light, leading to the

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suggestion that decreased sumoylation of p90^{MDM2} allows p53 to accumulate (Buschmann *et al*., 2000).

Third, a decrease in synthesis of p90^{MDM2} protein could also stabilize p53 (Wu and Levine, 1997). Indeed, transcription of *mdm2* is initially repressed by UV light in rat embryo fibroblasts and in rat epithelial cells, leading to the hypothesis that it is a decrease in the level of p90^{MDM2} that allows p53 to become stable (Wu and Levine, 1997; Blaydes *et al*., 1997). However, the decreases in *mdm2* mRNA occurred only in response to high doses of UV light and may have been the result of genotoxicity rather than a specific regulatory mechanism.

Later in the damage response, the short half-life and low level of p53 are recovered (Abrahams *et al*., 1995). The switch in p53's half-life may depend on an increase in either the specific activity or level of p90^{MDM2}. The level of p90^{MDM2} rises in response to UV light through the p53-dependent induction of an internal *mdm2* promoter, P2 (Saucedo *et al*., 1998). The resulting high level of p90^{MDM2} has been proposed to facilitate the recovery of p53's short half-life in a feedback loop (Prives, 1998). If an increased level of p90^{MDM2} were critical for the recovery of p53's short half-life, a diminished capacity to transcribe *mdm2* would be expected to lead to a prolonged accumulation of p53. For example, the increase in p90^{MDM2} may be abrogated in human fibroblasts defective in the TCR aspect of nucleotide excision repair (NER). Following UV exposure, TCR-deficient cells fail to recover normal rates of transcription and maintain high levels of p53 (Mayne and Lehmann, 1982; Dumaz *et al*., 1997). Moreover, in the absence of TCR, p53 is stabilized in response to lower doses of UV light than in repair-proficient fibroblasts (Yamaizumi and Sugano, 1994). If the induction of *mdm2* were diminished in TCR-deficient cells, p90^{MDM2} might not reach levels sufficient to destabilize p53.

The observation that cells capable of TCR but deficient in global genome NER stabilize p53 at doses of UV light similar to those required by repair-proficient cells led to two models for p53's stabilization in response to UV light (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996). Both models were based on the interpretation that persistent lesions in actively transcribed genes block transcription and mediate p53's stabilization. In one model, a signal for p53's stabilization emanates from the stalled transcription complex (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996). In the other model, unrepaired lesions interfere with transcription of a gene encoding a short-lived protein involved in p53's degradation (Ljungman *et al*., 1999). The *mdm2* gene fits this description since p90^{MDM2} is short-lived and its level is regulated through transcription (Juven *et al*., 1993; Wu *et al*., 1993; Saucedo *et al*., 1998, 1999). A transcription block that decreased the level of p90^{MDM2} could prolong p53's accumulation in TCR-deficient cells.

In addition to forming transcription-blocking lesions in the template, UV light reduces transcription by directly inhibiting the function of the transcription machinery (Rockx *et al*., 2000). Nuclear extracts from UV-irradiated cells have a reduced capacity to transcribe undamaged templates (Rockx *et al*., 2000). While repair-proficient cells recover the ability to transcribe, TCR-deficient cells do not (Rockx *et al*., 2000). The inhibition of transcription correlates with a decrease in the hypophosphorylated form of RNA polymerase II which is exacerbated in TCR-deficient cells (Rockx *et al*., 2000). In TCR-deficient cells, the defective transcriptional machinery may be incapable of inducing *mdm2* to levels sufficient to lead to p53's destabilization.

Here we tested the hypothesis that UV light regulates the level of p53 by modulating the level of p90^{MDM2} through changes in transcription of *mdm2*. First, we asked whether repression of *mdm2*'s transcription reduces the level of p90^{MDM2} and allows the initial stabilization of p53 in response to UV light. Second, we tested whether the induction of *mdm2*'s transcription is diminished in TCR-deficient fibroblasts that maintain high levels of p53. We found that, prior to the stabilization of p53, transcription of *mdm2* was slightly inhibited in repair-proficient cells. However, this decrease in *mdm2* mRNA was not sufficient to decrease the level of either the 75 or 90 kDa form of p90^{MDM2}. Thus, a reduction in the level of p90^{MDM2} is not required for the accumulation of p53 in response to UV light. In contrast, the level of p90^{MDM2} appears to be important for the recovery of low, basal levels of p53. In TCR-deficient fibroblasts, transcription from the *mdm2* P1 promoter is inhibited, leading to a diminished accumulation of p90^{MDM2}. These data suggest that stabilization of p53 does not depend on a reduction in the level of p90^{MDM2} but that recovery of p53's short half-life may require a sufficient increase in p90^{MDM2}.

Results

Transcription of mdm2 *is inhibited slightly by UV light in fibroblasts proficient in nucleotide excision repair*

To determine whether repression of *mdm2* contributes to the stabilization of p53 by UV light in repair-proficient human fibroblasts, we measured the levels of both *mdm2* mRNAs following exposure to UV light. In untreated human fibroblasts, *mdm2* mRNA from the P1 promoter is approximately 10 times more abundant than mRNA from the P2 promoter (Seavey *et al*., 1999). However, mRNA from the P2 promoter may contribute equally to the basal level of p90^{MDM2} since it is translated approximately 10 times more efficiently in some cell types (Landers *et al*., 1997; Brown *et al*., 1999). Therefore, although the P2 promoter of *mdm2* has been shown to be specifically induced by UV light (Seavey *et al*., 1999), repression of either promoter could significantly affect the level of p90^{MDM2}. The levels of *mdm2* mRNAs were measured before and after exposure to UV light using a quantitative S1 nuclease protection assay that differentiates between mRNAs synthesized from the uninducible P1 promoter and the UV-inducible P2 promoter (Seavey *et al*., 1999).

We treated two strains of repair-proficient fibroblasts with a dose of 10 J/m² of UV light which is known to stabilize p53 in this cell type (Seavey *et al*., 1999). In both strains of fibroblasts, the amount of mRNA from the P1 promoter was maintained for 2 h following UV exposure but declined 22% by 4 h (Figure 1). In contrast, the level of mRNA from the *mdm2* P2 promoter was reduced 28% by 1 h and rose threefold by 8 h post-treatment. Thus, a dose of 10 J/m² of UV light causes a small decrease in the level of each *mdm2* mRNA that could potentially contribute to stabilization of p53 by reducing the level of the ubiquitin ligase $p90^{MDM2}$.

To determine whether the slight reduction in *mdm2* mRNA was sufficient to reduce the level of $p90^{MDM2}$, we performed Western analyses of $p90^{MDM2}$ in lysates from UV-irradiated fibroblasts. By 2 h, there was an increase above the basal level of both the 75 kDa and 90 kDa forms of $p90^{MDM2}$ (Figure 2), demonstrating that the decrease in *mdm2* mRNAs was not sufficient to inhibit expression of $p90^{MDM2}$. Moreover, the increase in $p90^{MDM2}$ may result from a post-translational modification of $p90^{MDM2}$ because it preceded the induction of the P2 promoter (Figure 1). Although sumoylation of $p90^{MDM2}$ has been shown to increase its stability (Buschmann *et al*., 2000), this modification cannot account for the increase in both forms of $p90^{MDM2}$ because sumoylation converts the 75 kDa form of $p90^{MDM2}$ to the p90 kDa form (Buschmann *et al*., 2000).

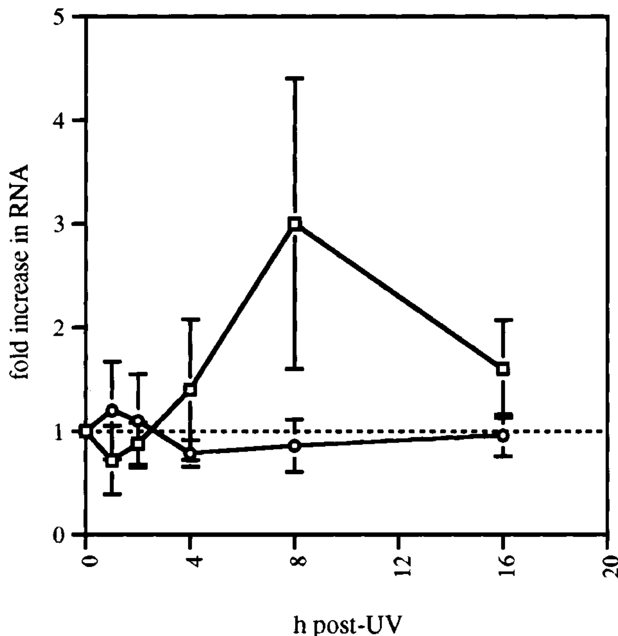

Figure 1 Levels of *mdm2* mRNAs in repair-proficient fibroblasts following treatment with UV light. Normal human fibroblasts were treated with 10 J/m² of UV light and the amounts of *mdm2* mRNAs from the P1 and P2 promoters were measured by S1 nuclease protection. The graph represents the means from two strains of repair-proficient fibroblasts (GM00037 and GM00011), plus and minus standard deviation ($n=4$). Fold change in the level of mRNA from the P1 promoter in response to UV light (circles). Fold change in the level of mRNA from the P2 promoter in response to UV light (squares)

We investigated whether the apparent increase in $p90^{MDM2}$ is specific to a change in the epitope for 2A10, which is known to be sensitive to phosphorylation (Khosravi *et al*., 1999). A similar increase in $p90^{MDM2}$ was seen with an antibody that recognizes a different epitope in $p90^{MDM2}$, suggesting that there is a bona fide increase in the amount of $p90^{MDM2}$ protein (Figure 3, compare a and b). Despite this increase in $p90^{MDM2}$, the level of p53 protein also increased by 1–2 h post-UV exposure (Figure 3c). Thus, stabilization of p53 by UV light does not require a reduction in the level of $p90^{MDM2}$. In contrast, the decrease in p53 at 8 h post-UV exposure was concurrent with the peak level of $p90^{MDM2}$ as well as with the peak increase in *mdm2* mRNA (Figure 1). This result supports the hypothesis that induction of *mdm2* causes an increase in $p90^{MDM2}$ that contributes to the destabilization of p53.

Transcription of mdm2 *is inhibited by UV light in fibroblasts deficient in nucleotide excision repair*

Although a decrease in the level of $p90^{MDM2}$ is not necessary for p53 to accumulate, an increase in $p90^{MDM2}$ may be critical for the recovery of low levels of p53. A failure to induce *mdm2* would allow p53 levels to

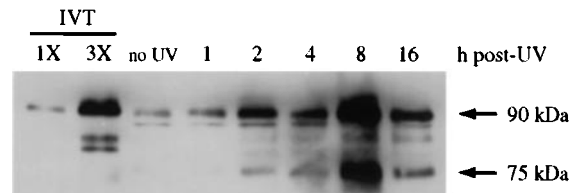

Figure 2 Western analysis of $p90^{MDM2}$ in UV-treated repair-proficient fibroblasts (GM00011) following treatment with a dose of 10 J/m² UV light. An *in vitro* translation reaction expressing *mdm2* was used as a positive control (IVT). The membrane was incubated with anti-MDM2 monoclonal 2A10

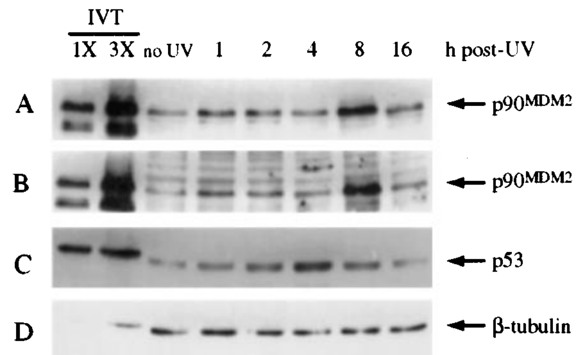

Figure 3 Western analysis of $p90^{MDM2}$ and p53 in UV-treated repair-proficient fibroblasts (GM00011) following treatment with a dose of 10 J/m² UV light. *In vitro* translation reactions expressing *p53* and *mdm2* were used as positive controls (IVT). Duplicate membranes were cut and the top halves were incubated with either anti-MDM2 monoclonal 2A10 (**a**) or anti-MDM2 monoclonal IF-2 (**b**). The bottom half of the membrane in **a** was incubated with anti-p53 antibody DO-1 (**c**) and the bottom half of the membrane in **b** was incubated with anti-β-tubulin as a loading and transfer control (**d**)

remain high as they do in NER-deficient fibroblasts (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996). We hypothesized that transcription of *mdm2* would be inhibited in NER-deficient fibroblasts which are defective in transcription following UV exposure (Mayne and Lehmann, 1982). We measured the levels of *mdm2* mRNAs in cells from two patients in complementation group A of xeroderma pigmentosum (XPA) which are defective in both TCR and global genome NER (Sugasawa *et al*., 1998). In both XPA strains, the levels of both *mdm2* mRNAs were reduced following exposure to 10 J/m^2 of UV light (Figure 4). The level of *mdm2* mRNA transcribed from the P1 promoter decreased 24% 4 h following treatment with UV light, similar to that in normal cells. However, the amount of this mRNA continued to decline in XPA cells, whereas it recovered in repair-proficient cells (Figure 4). Moreover, in neither XPA strain was the level of mRNA transcribed from the P2 promoter of *mdm2* increased as it was in repair-proficient cells. Instead, it declined to 42% of the basal level by 16 h (Figure 4). These results demonstrate that NER is essential for the normal regulation of *mdm2* expression in the UV response.

The decrease in mRNAs from both *mdm2* promoters was predicted to result in a decrease in p90^{MDM2}. Instead, there was an increase in the level of p90^{MDM2} that peaked at 2 h (Figure 5), consistent with the interpretation that the initial increase in p90^{MDM2} is independent of the induction of the *mdm2* P2 promoter. No further increase in the level of p90^{MDM2} was seen at 8 h. The level of p53 increased by 2 h, supporting the conclusion that a decrease in p90^{MDM2} is not required for stabilization of p53. p53 continued to accumulate in XPA cells, as shown previously (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996). Thus, the prolonged accumulation of p53 in NER-deficient fibroblasts may be due to diminished induction of *mdm2* and p90^{MDM2}.

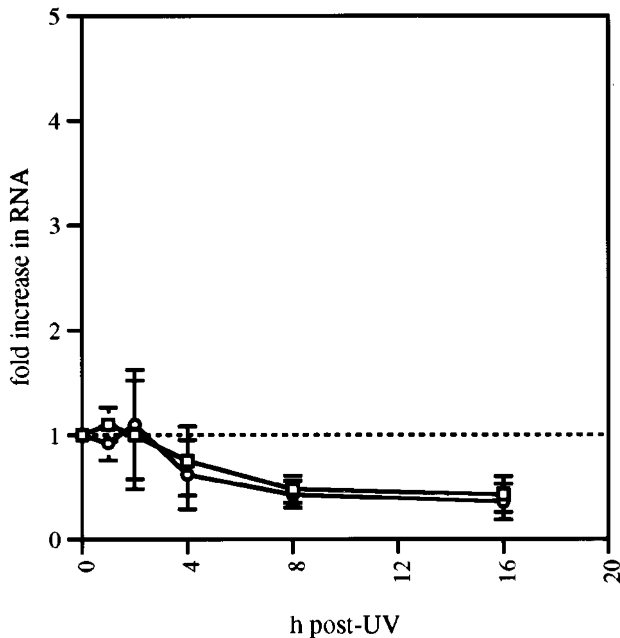

Figure 4 Decrease in *mdm2* mRNAs in cells deficient in both TCR and global genome NER following treatment with UV light. XPA cells were treated with 10 J/m^2 of UV light and the amounts of *mdm2* mRNAs from the P1 and P2 promoters were measured by S1 nuclease protection. The graph represents the means from two strains of repair-deficient fibroblasts (XPA strains GM02990 and GM05509), plus and minus standard deviation ($n = 3$). Fold change in the level of mRNA from the P1 promoter in response to UV light (circles). Fold change in the level of mRNA from the P2 promoter in response to UV light (squares)

The P1 promoter can be inhibited by defects in either TCR or global genome NER

Like XPA cells, fibroblasts defective in TCR but capable of global genome NER maintain high levels of p53 for a prolonged period following UV exposure (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996). If induction of *mdm2* is necessary for the recovery of low levels of p53, then the prolonged accumulation of p53 in TCR deficient cells may be due to an inability of these cells to induce *mdm2*. We hypothesized that transcription of *mdm2* would be reduced in fibroblasts from patients with Cockayne syndrome (CS) which lack the ability to perform TCR but perform global genome repair (Venema *et al*., 1990). Like XPA cells, CS cells are defective in recovering the rate of transcription following UV exposure (Mayne and Lehmann, 1982; Rockx *et al*., 2000). We analysed the levels of *mdm2* mRNAs in cells from two patients in CS complementation group B (CSB) (Troelstra *et al*., 1992). In both CSB strains, there was a 60% decrease in the level of *mdm2* mRNA from the P1 promoter by 8 h following exposure to 10 J/m^2 of UV light (Figure 6). However, in contrast to XPA cells, CSB cells showed a threefold induction of

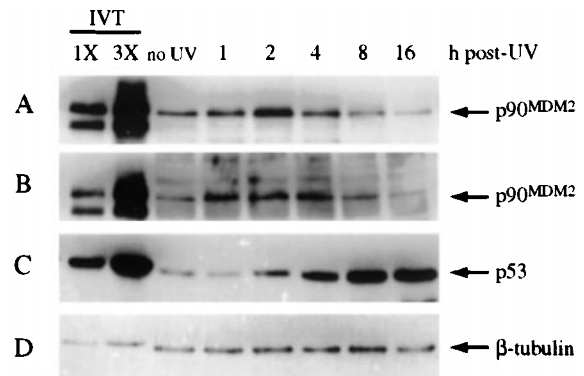

Figure 5 Western analysis of p90^{MDM2} and p53 in XPA strain GM02990, deficient in both TCR and global genome NER, following treatment with a dose of 10 J/m^2 UV light. *In vitro* translation reactions expressing *p53* and *mdm2* were used as positive controls (IVT). Duplicate membranes were cut and the top halves were incubated with either anti-MDM2 monoclonal 2A10 (**a**) or anti-MDM2 monoclonal IF-2 (**b**). The bottom half of the membrane in **a** was incubated with anti-p53 antibody DO-1 (**c**) and the bottom half of the membrane in **b** was incubated with anti-β-tubulin as a loading and transfer control (**d**)

transcription from the *mdm2* P2 promoter by 8 h following treatment (Figure 6). Thus, a defect in TCR appears to inhibit basal transcription from the P1 promoter but allow induction of the P2 promoter.

The decrease in mRNA from the P1 promoter at 8 h was sufficient to dampen the increase in p90^{MDM2}. In CSB cells, the level of p90^{MDM2} peaked at 2 h, as it did in repair-deficient XPA cells (Figure 7). At 8 h, the level of p90^{MDM2} was not increased above the level at 2 h, even though the P2 promoter was induced. Since transcription from the P1 promoter was reduced at this time, this result suggests that the level of mRNA from the P1 promoter is an important determinant of the peak level of p90^{MDM2} in the UV response. The level of p53 increased by 2 h following UV exposure and remained high at 16 h, suggesting that the level of p90^{MDM2} may be insufficient to decrease p53 to basal levels.

Cells capable of TCR but defective in global genome NER recover low levels of p53 with kinetics similar to those of repair-proficient cells (Yamaizumi and Sugano, 1994). To determine whether the induction of *mdm2*'s transcription and the increase in p90^{MDM2} were normal in cells proficient in TCR but defective in global genome NER, we analysed fibroblasts from XP complementation group C (Venema *et al*., 1991). XPC cells recover the rate of RNA synthesis with kinetics similar to those of normal cells (Yamaizumi and Sugano, 1994). In one strain (GM03176), there was no inhibition of expression from either *mdm2* promoter following exposure to 10 J/m^2 of UV light (Figure 8a). In this strain, the P2 promoter was induced to a higher level than in repair-proficient cells (Figure 8a). In a second strain of XPC cells (GM02992), mRNAs from both promoters were reduced 55% initially. By 8 h, transcription from the P1 promoter had recovered and transcription from the P2 promoter was induced to a higher level than in repair-proficient cells (Figure 8b). In this strain, the induction of the P2 promoter was prolonged to 16 h. Although the two XPC strains differed in their response to UV exposure, by 8 h both expressed the basal level of mRNA from the P1 promoter and an induced level from the P2 promoter.

In both strains of XPC cells, p90^{MDM2} increased within 1 h of UV exposure, and peaked at 8 h, as in repair-proficient cells (Figure 9). This peak in p90^{MDM2} correlated with the peak in p53; the level of p53 was highest at 8 h but diminished by 16 h. These data support the hypothesis that an increase in p90^{MDM2} is necessary to return p53 to basal levels. This increase in p90^{MDM2} requires TCR but not global genome repair and appears to depend on both basal expression from the P1 promoter and induction of the P2 promoter.

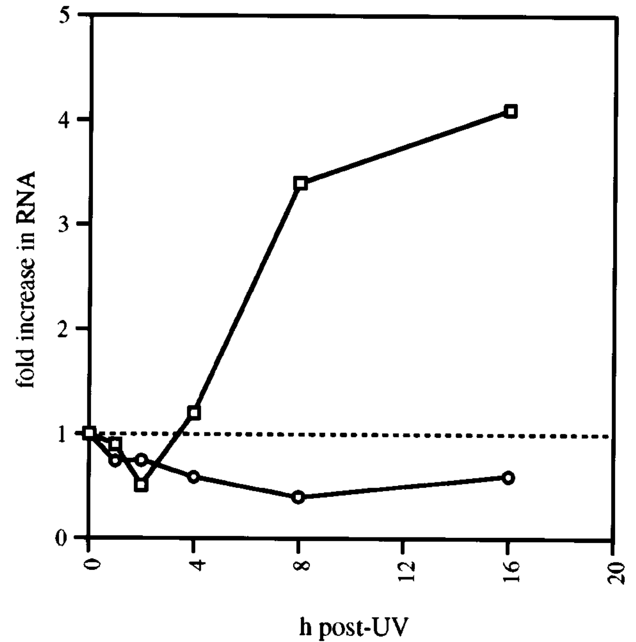

Figure 6 Prolonged induction of transcription from the *mdm2* P2 promoter in fibroblasts proficient in global genome NER, but not TCR, following treatment with UV light. Fibroblasts from two patients with Cockayne syndrome B were treated with 10 J/m^2 of UV light and the amounts of *mdm2* mRNAs from the P1 and P2 promoters were measured by S1 nuclease protection. The graph represents the means from two strains of CSB fibroblasts (GM01629 and GM00739) ($n=2$). Fold change in level of mRNA from the P1 promoter in response to UV light (circles). Fold change in level of mRNA from the P2 promoter in response to UV light (squares)

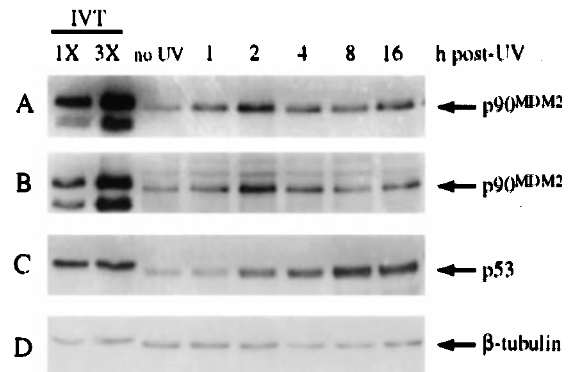

Figure 7 Western analysis of p90^{MDM2} and p53 in fibroblasts proficient in global genome NER, but not TCR (CSB strain GM01629) following treatment with a dose of 10 J/m^2 of UV light. *In vitro* translation reactions expressing *p53* and *mdm2* were used as positive controls (IVT). Duplicate membranes were cut and the top halves were incubated with either anti-MDM2 monoclonal 2A10 (**a**) or anti-MDM2 monoclonal IF-2 (**b**). The bottom half of the membrane in **a** was incubated with anti-p53 antibody DO-1 (**c**) and the bottom half of the membrane in **b** was incubated with anti-β-tubulin as a loading and transfer control (**d**)

Discussion

Transient stabilization of p53 appears to be critical for the cellular response to DNA damage (Prives, 1998). Although the mechanisms regulating p53's stability in response to UV light are unclear, several models invoke an initial decrease in the level of p90^{MDM2} that allows p53 to become stable (Wu and Levine, 1997;

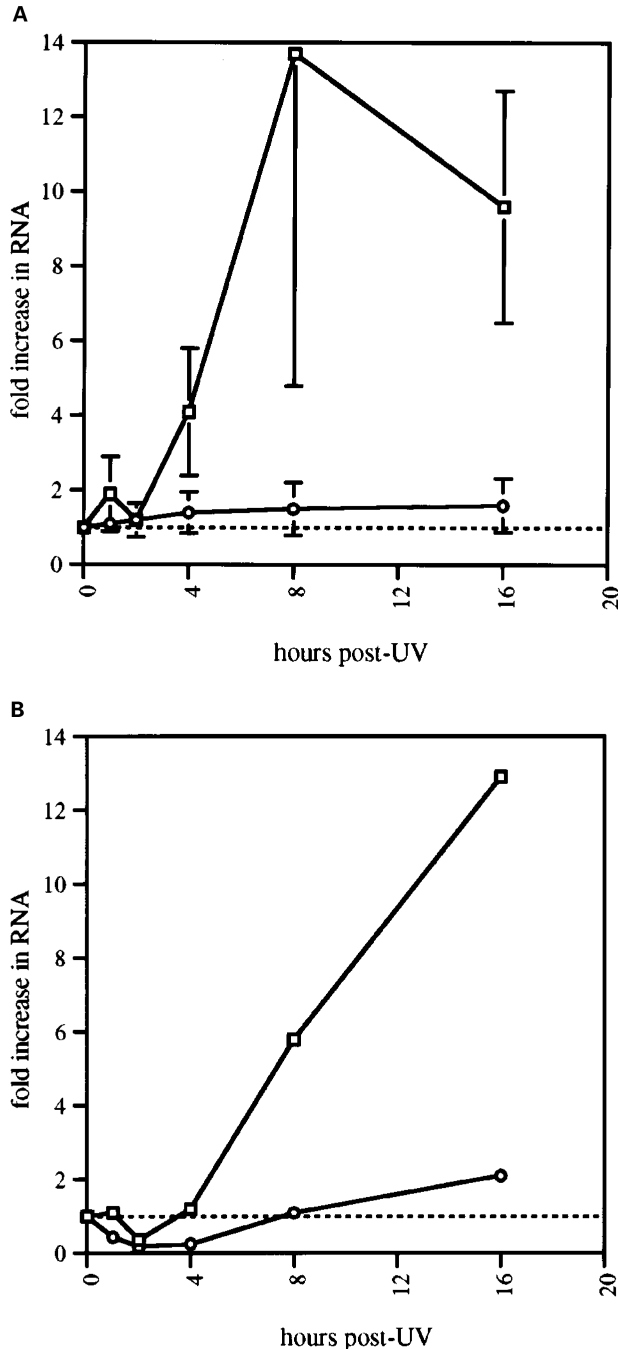

Figure 8 Induction of *mdm2*'s transcription in fibroblasts deficient in global genome NER, but not TCR, following treatment with UV light. Two strains of XPC fibroblasts were treated with 10 J/m^2 of UV light and the amounts of *mdm2* mRNAs from the P1 and P2 promoters were measured by S1 nuclease protection. The results from each strain are shown separately because expression from the P1 promoter differed reproducibly between strains. (**a**) Strain GM03176 ($n=3$). (**b**) Strain GM02992 ($n=2$). Fold change in the level of mRNA from the P1 promoter in response to UV light (circles). Fold change in the level of mRNA from the P2 promoter in response to UV light (squares)

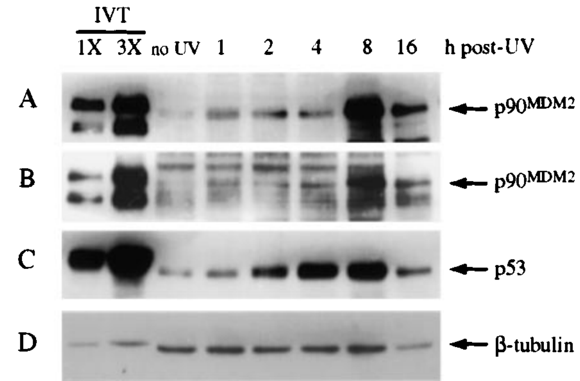

Figure 9 Western analysis of p90^{MDM2} and p53 in fibroblasts proficient in TCR, but not global genome NER, (XPC strain GM03176) following treatment with 10 J/m^2 of UV light. *In vitro* translation reactions expressing *p53* and *mdm2* were used as positive controls (IVT). Duplicate membranes were cut and the top halves were incubated with either anti-MDM2 monoclonal 2A10 (**a**) or anti-MDM2 monoclonal IF-2 (**b**). The bottom half of the membrane in **a** was incubated with anti-p53 antibody DO-1 (**c**) and the bottom half of the membrane in **b** was incubated with anti-β-tubulin as a loading and transfer control (**d**)

Blaydes *et al*., 1997, Buschmann *et al*., 2000). In contrast, the subsequent destabilization of p53 has been proposed to depend on an increase in p90^{MDM2} mediated by the p53-dependent induction of the *mdm2* P2 promoter. Here we investigated whether changes in *mdm2*'s transcription control the levels of p90^{MDM2} and, indirectly, p53. We find that the level of p90^{MDM2} is not reduced prior to p53's stabilization. Instead, there was an early, transcription-independent rise in the level of p90^{MDM2}. A second increase in p90^{MDM2} followed induction of *mdm2*'s transcription and preceded p53's destabilization. Thus, transcription-mediated changes in the level of p90^{MDM2} do not regulate p53's stabilization in response to UV exposure. Rather, they appear important for the later destabilization of p53.

No reduction in the level of p90^{MDM2} occurred in repair-proficient fibroblasts exposed to a dose of UV light known to elevate p53's level and transcriptional activation function in this cell type (Seavey *et al*., 1999). We reasoned that a decrease in p90^{MDM2} would be evident at this dose if it were critical for the stabilization and activation of p53 by UV light. In addition, we had shown that this dose kills 90% of human fibroblasts as measured by their ability to form colonies when plated 24 h after exposure (Seavey *et al*., 1999). By these criteria, 10 J/m^2 is a high dose of UV light. Our results differ from reports of significant UV-dependent decreases in both *mdm2* mRNA and p90^{MDM2} protein in repair-proficient cells (Wu and Levine, 1997; Blaydes *et al*., 1997; Buschmann *et al*., 2000). A reasonable explanation is that the higher doses of UV light used in these other studies led to a nonspecific inhibition of transcription or translation. Indeed, one study reported that transcription of *mdm2* in rat embryo fibroblasts was inhibited by a high dose (20 J/m^2) but not by a low dose (4 J/m^2) of UV light (Wu and Levine, 1997). Here we have shown that a decrease in p90^{MDM2} mediated by repression of *mdm2* transcription is not required for stabilizing p53 in repair-proficient human fibroblasts.

In all fibroblast strains studied, UV-exposure elicited an initial increase in the level of $p90^{MDM2}$ without a concomitant increase in transcription of *mdm2*. This result was most evident in XPA cells which fail to induce *mdm2*, suggesting that a post-transcriptional mechanism contributes to the increase in $p90^{MDM2}$ in UV-treated human fibroblasts. A modification of $p90^{MDM2}$ that stabilizes the protein would be expected to increase its steady-state level. One such modification is sumoylation which converts $p90^{MDM2}$ from a 75 kDa to a more stable 90 kDa form (Buschmann *et al*., 2000). However, increased sumoylation of $p90^{MDM2}$ cannot account for the increases seen here because both the 75 kDa and 90 kDa forms of $p90^{MDM2}$ are increased. Phosphorylation is another modification that could mediate the increase in $p90^{MDM2}$. Although we saw no evidence for phosphorylation changes such as a shift in mobility, $p90^{MDM2}$ is known to be highly phosphorylated and a subtle change may not be evident under the conditions used here (Momand *et al*., 1992). The level of $p90^{MDM2}$ would also rise with an increase in translational efficiency. Although the translational efficiency of *mdm2* is not known to be enhanced by DNA damage, ionizing irradiation has been shown to increase the translational efficiency of *p53* (Fu and Benchimol, 1997).

Oscillations in the level of $p90^{MDM2}$ have been proposed to allow fluctuations in p53's level such that DNA repair has a chance to proceed before p53 stimulates apoptosis (Bar-Or *et al*., 2000). Our results invite a modification of this model. Here, there was a biphasic increase in the level of $p90^{MDM2}$. The initial increase appears to be post-transcriptional, peaking at 2 h after exposure in cells of all repair genotypes. In contrast, the second peak correlated with transcription from both *mdm2* promoters and was abrogated in TCR-deficient cells. The first peak did not prevent p53's accumulation whereas the second peak correlated with p53's destabilization. $p90^{MDM2}$ can block p53's interaction with the transcriptional machinery without stimulating p53's ubiquitination (Kubbutat *et al*., 1999). Perhaps the initial rise in $p90^{MDM2}$ delays p53's function while allowing the protein to accumulate. Indeed, p53's transcriptional activation function did not increase for at least 2 h after p53 began to accumulate. In this revised model, $p90^{MDM2}$'s ubiquitin ligase function would be selectively inhibited following UV exposure. This model is consistent with data from Geyer *et al*. (2000) which showed that p53 accumulated after UV exposure even though $p90^{MDM2}$ and p53 remained bound.

The p53-dependent induction of *mdm2* (Seavey *et al*., 1999) has been proposed to allow $p90^{MDM2}$ to accumulate to levels sufficient to return p53 to its basal level (Prives, 1998). Our data support the idea that an increase in $p90^{MDM2}$ promotes the recovery of low levels of p53, however we found that both *mdm2* promoters contribute substantially to the second peak in $p90^{MDM2}$. For example, repair-proficient and CSB cells induce the P2 promoter equivalently, but the second peak in $p90^{MDM2}$ was diminished in CSB cells in which mRNA from the P1 promoter was reduced. Transcription from the P2 promoter is also critical for the increase in $p90^{MDM2}$ because XPA cells, which showed a progressive decrease in mRNAs from both promoters, did not have a second increase in $p90^{MDM2}$. The failure of CSB and XPA cells to accrue $p90^{MDM2}$ correlates with prolonged accumulation of p53, supporting the idea that a high level of $p90^{MDM2}$ is required to destabilize p53. This increase in $p90^{MDM2}$ depends on the level of transcription from both *mdm2* promoters.

Transcription from the *mdm2* P1 promoter appears more sensitive to the inhibitory effects of UV exposure than transcription from the P2 promoter. Although mRNAs from both promoters were decreased initially in CSB cells, mRNA from the P2 promoter rose while mRNA from the P1 promoter remained reduced. UV light inhibits transcription through both cis and trans mechanisms. In cis, lesions in the template block elongation (Donahue *et al*., 1994). In trans, modifications of the transcriptional machinery inhibit transcription even of templates that are free of lesions (Rockx *et al*., 2000). The inhibition correlates with a decrease in the level of the hypophosphorylated form of RNA polymerase II which is important for transcriptional initiation (Rockx *et al*., 2000). Over time, repair-proficient cells recover the ability to transcribe whereas TCR-deficient fibroblasts fail to recover (Mayne and Lehmann, 1982; Rockx *et al*., 2000). However, the recovery of transcription does not correlate with the repair capacity of the cells (Mayne and Lehmann, 1982), indicating that the defects in transcription and repair are separable (Ganesan *et al*., 1999; Van Hoffen *et al*., 1999).

The inhibition of *mdm2*'s transcription was most severe in XPA cells which cannot repair UV-induced lesions. Whereas XPA cells show no induction of the P2 promoter, CSB cells, which can repair by the global genome NER pathway, recover normal levels of P2 mRNA. Although this observation appears to suggest that repair facilitates the induction of the P2 promoter, repair would be expected to improve elongation through the larger P1 template which remains reduced in CSB cells. Thus, the repair capacity of the cell does not completely explain the differential effects of UV light on the two *mdm2* promoters. An alternative explanation is provided by the observation that, while both XPA and CSB cells lack the hypophosphorylated form of RNA polymerase II following UV irradiation (Rockx *et al*., 2000), the transcription defect is more severe in XPA cells (Mayne and Lehmann, 1982; Rockx *et al*., 2000). Together these observations suggest that the P1 promoter may be more sensitive than the P2 promoter to defects in the transcription machinery such as a lack of hypophosphorylated RNA polymerase II. We propose that defects in repair and transcription combine to inhibit *mdm2*'s expression in XPA cells.

In human fibroblasts, induction of the P2 promoter by UV light requires p53 (Seavey *et al*., 1999). Thus, p53 appears able to stimulate the P2 promoter even

when transcription from the P1 promoter is inhibited. Zhu *et al*. (2000) previously showed that p53 can stimulate transcription when general transcription is inhibited by UV light.

Thus, p53 may be able to stimulate transcriptional initiation in the absence of abundant levels of hypophosphorylated RNA polymerase II (Rockx *et al*., 2000). In addition, p53 is known to stimulate NER (Ford and Hanawalt, 1995). Indeed, Huang *et al*. (1998) showed UV-irradiated reporter plasmids containing p53-response elements were transcribed following transfection into UV-irradiated cells while plasmids containing other binding sites were not. Although the authors concluded that p53 stimulated repair of the plasmids, thereby allowing transcription, repair was not measured. Thus, it is possible that Huang *et al*. (1998) detected an ability of p53 to stimulate transcription through damaged templates. The ability of p53 to stimulate transcription when general transcription is inhibited may ensure that p53 can exert its tumor suppressive function under conditions of severe DNA damage (Jimenez *et al*., 2000). However, it does not ensure that the feedback from *mdm2* is sufficient to get p53 back under control.

Materials and methods

Cell culture

Diploid human skin fibroblast strains were obtained from the NIGMS Human Genetic Mutant Cell Repository at the Coriell Institute for Medical Research and used between passages 19 and 25. Two strains from normal, repair-proficient, individuals were used (GM00011 and GM00037). Four strains from patients with xeroderma pigmentosum (XP) were analysed. Two XP strains were in complementation group A (XPA, GM02990 and GM05509) and two were in complementation group C (XPC, GM03176 and GM02992). We used two strains from individuals with Cockayne syndrome in complementation group B (GM01629 and GM00739) (Troelstra *et al*., 1992). Fibroblasts were grown in Dulbecco's modification of Eagle's medium with 20% fetal calf serum (Summit), supplemented with nonessential amino acids, essential amino acids and vitamins. Before exposure of cells to UVC light (wavelength of 254 nm), the media was removed and cells were rinsed with phosphate buffered saline. The distance between the plates and the lamp was adjusted so that the fluence of the UVC light source (Glo-Mark Systems, Inc.) was 1 $J/m^2/s$ as measured by a Blak-Ray J-225 UV meter (UV photoproducts, San Gabriel, CA, USA).

S1 nuclease digestion

RNA was isolated using Tri reagent (Molecular Research Center, Inc.). The probe was a 488 basepair *Xba*I-*Apa*LI fragment from pTA-L-mdm2 containing all of exon 1, none of exon 2, all of exon 3 and 44 bp of exon 4 (Landers *et al*., 1997). Following end-labeling with $^{32}PO_4$ using T4 kinase (New England Biolabs), the probe was denatured and hybridized overnight to 30 μg of total RNA. The hybridization products were incubated with S1 nuclease (Gibco) and products were separated on polyacrylamide gels. A positive control, corresponding to mRNA transcribed from the P1 promoter of human *mdm2* (containing exon 1 spliced to exon 3) was synthesized *in vitro* from pHDM (Chen *et al*., 1993). A 25 bp ladder was obtained from Gibco and end-labeled with $^{32}PO_4$ using T4 kinase. Products were quantified using a Molecular Dynamics PhosphorImager.

Western analysis

Whole cell lysates were generated as described (Seavey *et al*., 1999) and protein concentrations were determined by the method of Lowry, using the Biorad DC Protein Assay. Forty micrograms of total cell protein were separated on duplicate polyacrylamide gels and transferred to nitrocellulose. Equal loading and transfer was confirmed using Ponceau S. The membranes were cut and the top portions were incubated with either hybridoma supernatant containing the anti-MDM2 2A10 antibody (1 : 50) or monoclonal antibody IF-2 (1 : 100) (Ab-1, Oncogene Science). The bottom portions were incubated with DO-1 monoclonal antibody (Ab-1, Oncogene Science) (1 : 1000) to detect p53 or with anti-β-tubulin (clone KMX-1, Boehringer Mannheim) (1 μg/ml) as a control for loading and transfer. As a positive control, two amounts of *in vitro* translation reactions containing p53 and MDM2 were included. All four monoclonal antibodies were revealed using goat anti-mouse Ig conjugated to horseradish peroxidase (American Qualex) and Enhanced Chemiluminescence (ECL, Amersham).

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References

Abrahams PJ, Schouten R, van Laar T, Houweling A, Terleth C and van der Eb AJ. (1995). *Mutat. Res.,* **336,** 169 – 180.

Bar-Or RL, Maya R, Segel LA, Alon U, Levine AJ and Oren M. (2000). *Proc. Natl. Acad. Sci. USA,* **97,** 11250 – 11255.

Blaydes JP, Gire V, Rowson JM and Wynford-Thomas D. (1997). *Oncogene,* **14,** 1859 – 1868.

Blaydes JP and Wynford-Thomas D. (1998). *Oncogene,* **16,** 3317 – 3322.

Brown CY, Mize GJ, Pineda M, George DL and Morris DR. (1999). *Oncogene,* **18,** 5631 – 5637.

Buschmann T, Fuchs SY, Lee C-G, Pan Z-Q and Ronai Z. (2000). *Cell,* **101,** 753 – 762.

Chen J, Marachel V and Levine AJ. (1993). *Mol. Cell. Biol.,* **13,** 4107 – 4114.
Donahue B, Yin S, Taylor J-S, Reines D and Hanawalt PC. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 8502 – 8506.
Dumaz N, Duthu A, Ehrhart J-C, Drougard C, Appella E, Anderson CW, May P, Sarasin A and Daya-Grosjean L. (1997). *Mol. Carcinol.,* **20,** 340 – 347.
Ford JM and Hanawalt PC. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 8876 – 8880.
Fu L and Benchimol S. (1997). *EMBO J.,* **16,** 4117 – 4125.
Ganesan AK, Hunt J and Hanawalt PC. (1999). *Mutation Res.,* **433,** 117 – 126.
Geyer RK, Nagasawa H, Little JB and Maki CG. (2000). *Cell Growth Differ.,* **11,** 149 – 156.
Haupt Y, Maya R, Kazaz A and Oren M. (1997). *Nature,* **387,** 296 – 299.
Huang J, Logsdon N, Schmieg F and Simmons DT. (1998). *Oncogene,* **17,** 401 – 411.
Hwang BJ, Ford JM, Hanawalt PC and Chu G. (1999). *Proc. Natl. Acad. Sci. USA,* **96,** 424 – 428.
Jiang W, Ananthaswamy HN, Muller HK and Kripke ML. (1999). *Oncogene,* **18,** 4247 – 4253.
Jimenez G, Nister M, Stommel JM, Beeche M, Barcarse EA, Zhang X-Q, O'Gorman S and Wahl GM. (2000). *Nature Genet.,* **26,** 37 – 43.
Juven T, Barak Y, Zauberman A, George DL and Oren M. (1993). *Oncogene,* **8,** 3411 – 3416.
Khosravi R, Maya RR, Gottlieb T, Oren M, Shiloh Y and Shkedy D. (1999). *Proc. Natl. Acad. Sci. USA,* **96,** 14973 – 14977.
Kubbutat MHG, Jones SN and Vousden K. (1997). *Nature,* **387,** 299 – 303.
Kubbutat MHG, Ludwig RL, Levine AJ and Vousden K. (1999). *Cell Growth Differ.,* **10,** 87 – 92.
Landers JE, Cassel SL and George DL. (1997). *Cancer Res.,* **57,** 3562 – 3568.
Li G, Ho VC, Berean K and Tron VA. (1995). *Cancer Res.,* **55,** 2070 – 2074.
Ljungman M and Zhang F. (1996). *Oncogene,* **13,** 823 – 831.
Ljungman M, Zhang F, Chen F, Rainbow AJ and McKay BC. (1999). *Oncogene,* **18,** 583 – 592.
Maki CG and Howley PM. (1997). *Mol. Cell. Biol.,* **17,** 355 – 363.
Maltzman W and Czyzyk L. (1984). *Mol. Cell. Biol.,* **4,** 1689 – 1694.
Mayne LV and Lehmann AR. (1982). *Cancer Res.,* **42,** 1473 – 1478.
Momand J, Zambetti G, Olson DC, George D and Levine AJ. (1992). *Cell,* **69,** 1237 – 1245.
Prives C. (1998). *Cell,* **95,** 5 – 8.
Rockx DAP, Mason R, van Hoffen A, Barton MC, Citterio E, Bregman DB, van Zeeland AA, Vrieling H and Mullenders LHF. (2000). *Proc. Natl. Acad. Sci. USA,* **97,** 10503 – 10508.
Saucedo LJ, Carstens BP, Seavey SE, Albee LD and Perry ME. (1998). *Cell Growth Differ.,* **9,** 119 – 130.
Saucedo LJ, Myers CD and Perry ME. (1999). *J. Biol. Chem.,* **274,** 8161 – 8168.
Seavey SE, Holubar M, Saucedo LJ and Perry ME. (1999). *J. Virol.,* **73,** 7590 – 7598.
Shieh S-Y, Ikeda M, Taya Y and Prives C. (1997). *Cell,* **91,** 325 – 334.
Sugasawa K, Ng JMY, Masutani C, Iwai S, van der Spek PJ, Eker APM, Hanaoka F, Bootsma D and Hoeijmakers JHJ. (1998). *Mol. Cell,* **2,** 223 – 232.
Troelstra C, van Gool A, de Wit J, Vermeulen W, Bootsma D and Hoeijmakers JH. (1992). *Cell,* **71,** 939 – 953.
Van Hoffen A, Kalle WHJ, de Jong-Versteeg A, Lehmann AR, van Zeeland AA and Mullenders LHF. (1999). *Nucleic Acids Res.,* **27,** 2898 – 2904.
Venema J, Mullenders LHF, Natarajan AT, van Zeeland AA and Mayne LV. (1990). *Proc. Natl. Acad. Sci. USA,* **87,** 4707 – 4711.
Venema J, van Hoffen A, Karcagi V, Natarajan AT, van Zeeland AA and Mullenders LHF. (1991). *Mol. Cell. Biol.,* **11,** 4128 – 4134.
Wu L and Levine AJ. (1997). *Mol. Med.,* **3,** 441 – 451.
Wu X, Bayle JH, Olson D and Levine AJ. (1993). *Genes Dev.,* **7,** 1126 – 1132.
Yamaizumi M and Sugano T. (1994). *Oncogene,* **9,** 2775 – 2784.
Zhu Q, Wani MA, El-Mahdy M, Wani G and Wani AA. (2000). *Mol. Carcinol.,* **28,** 215 – 224.
Ziegler A, Jonason AS, Leffell DJ, Simon JA, Sharma HW, Kimmelman J, Remington L, Jacks T and Brash DE. (1994). *Nature,* **372,** 773 – 776.