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Fen1 is induced p53 dependently and involved in the recovery from UV-light-induced replication inhibition

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Mouse embryonic fibroblasts (MEFs) that lack p53 are hypersensitive to the cytotoxic and genotoxic effect of ultraviolet (UV-C) light. They also display a defect in the recovery from UV-C-induced DNA replication inhibition. An enzyme involved in processing stalled DNA replication forks is flap endonuclease 1 (Fen1). Gene expression profiling of UV-C-irradiated MEFs revealed *fen1* to be upregulated, which was confirmed by RT–PCR and Western blot experiments. Increased Fen1 levels upon UV-C exposure are due to transcriptional activation, as revealed by inhibitor studies. Fen1 induction was dose- and time-dependent; it occurred on protein level already 3 h after irradiation. Induction of Fen1 by UV-C requires p53 since it was observed in p53 wild-type (wt) but not in p53 null (p53−/−) fibroblasts. Fen1 upregulation paralleled the increase in p53 protein level in replicating wt cells, whereas in nonreplicating cells both Fen1 and p53 were not induced by UV-C. The mouse *fen1* promoter was cloned and shown to harbor a p53 consensus sequence to which p53 binds. In cotransfection experiments, p53 stimulated the expression of a *fen1* promoter–reporter construct. Transgenic expression of Fen1 in p53 null cells attenuated UV-C light-induced DNA replication inhibition, supporting the hypothesis that Fen1 induction is involved in the recovery of cells from DNA damage.
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Introduction

The cellular genome is permanently stressed by a multitude of endogenous and exogenous DNA damaging insults. To maintain genomic integrity, cellular defense mechanisms, including DNA repair, have evolved (Christmann *et al*., 2003). One of the cellular defense mechanisms includes the tumor suppressor protein p53, which is paradigmatic of a genotoxic stress response. p53 is a sequence-specific transcription factor (el-Deiry *et al*., 1992). It also acts as a binding partner for different proteins that play a central role in cell cycle control (Janus *et al*., 1999), DNA replication and DNA repair (Smith *et al*., 2000). Upon exposure to UV-C light p53 is stabilized (Maltzman and Czyzyk, 1984), which is due to its own phosphorylation (Chehab *et al*., 1999) and downregulation of its binding partner Mdm2 (Khosravi *et al*., 1999). This results in increased nuclear translocation of p53, enhanced DNA binding and transcriptional activity of target genes (Lakin and Jackson, 1999). Induction of p53 has been reported to result in cell cycle arrest, which occurs either in the G1 or the G2/M phase (Kastan *et al*., 1991), increasing the time available for repair of damaged DNA prior to replication or the entry of cells into mitosis. In contrast, the G1/S transition and the intra-S phase checkpoint is p53-independent (Bartek *et al*., 2004).

Immortalized embryonic fibroblasts from mice (MEFs) deficient for p53 (p53−/−) are hypersensitive to the cytotoxic, apoptosis-inducing and clastogenic effect of UV-C light as compared to the corresponding wild-type (wt) cells, which was shown in two independent studies (Lackinger and Kaina, 2000; Smith *et al*., 2000; Lackinger *et al*., 2001). Hypersensitivity of p53-deficient cells was explained by inefficient removal and/or processing of UV-C-induced DNA damage. The major cytotoxic UV-C light-induced photolesions are (6–4) photoproducts and cyclobutane pyrimidine dimers (CPDs), which are repaired by nucleotide excision repair (NER). If not repaired, they cause DNA replication inhibition and S phase arrest (Chan *et al*., 1985). The mechanism of DNA replication inhibition upon the induction of DNA damage is not fully understood. It is believed to be the direct consequence of inhibition of DNA polymerases that encounter the lesion, or a regulatory phenomenon that involves checkpoint proteins, or both.

In an attempt to elucidate the cause of the hypersensitivity of p53 knockout mouse fibroblasts, we studied their S phase response and DNA repair capacity upon treatment with UV-C light. We show that p53 null cells are unable to remove UV-C-induced pyrimidine dimers (CPDs) from DNA and, at the same time, exhibit a sustained DNA replication blockade as compared to isogenic wt cells. This suggests that a p53-regulated function is involved in the cellular recovery from DNA replication inhibition.

A candidate gene involved in the regulation of the S phase arrest is Fen1. Fen1 has pleiotropic functions,

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playing a critical role not only in DNA repair but also in DNA replication. It is indispensable for long-patch base excision repair (Klungland and Lindahl, 1997) stimulating strand displacement and repair synthesis by DNA polymerase β (Prasad *et al*., 2000). It exhibits $5'\rightarrow3'$ exonuclease activity and a structure-specific endonuclease activity by which DNA flap structures are cleaved (Harrington and Lieber, 1994). During DNA replication, Fen1 is responsible for the removal of Okazaki fragments (Bae *et al*., 2001). It also suppresses triplet repeat expansion generated by DNA slippage (Ruggiero and Topal, 2004) and facilitates the removal of mismatches that occur during synthesis of Okazaki fragments (Rumbaugh *et al*., 1999). Recently, it has been reported that Fen1 exhibits gap endonuclease activity, which is critical in resolving stalled DNA replication forks. Expression of human Fen1 in yeast Fen-1-deficient cells mediates resistance to UV-C light (Zheng *et al*., 2005).

To elucidate whether Fen1 is involved in the cellular UV response, we analysed Fen1 expression in wt and p53 null cells. Our results revealed that in MEFs *fen1* is induced p53 dependently by UV-C light. We also show that over-expression of *fen1* in p53 null cells attenuates the UV-C-induced S phase arrest. The available data suggest that *fen1* is a novel p53-dependent genotoxic stress inducible gene that plays a role in the recovery of cells from replication arrest following UV-C treatment.

Results

Cytotoxic response of wt and p53-deficient cells

To investigate the p53 response following UV-C treatment, we used isogenic MEFs that are wt and knockout (p53−/−) for p53. Since MEFs tend to lose functional p53 during cultivation, the expression of p53 in wt cells was carefully checked before each experimental series. As shown in Figure 1a, wt cells display p53 mRNA (which was not upregulated by UV-C light) and p53 protein, which was significantly enhanced in nuclear extracts upon UV-C treatment as expected. In p53−/− cells neither the mRNA nor the protein was present. Moreover, nuclear extracts from wt but not p53−/− cells exhibited UV-C light-induced p53 binding to a p21 promoter oligonucleotide harboring the p53-binding site (Figure 1b). MEFs deficient in p53 are hypersensitive to UV-C light, displaying a lower viability (Figure 1c) and a higher frequency of apoptosis (Figure 1d).

Replication blockage and DNA repair upon UV-C treatment

Inhibition of DNA replication is an immediate–early event in cells treated with UV-C. In order to study the impact of p53 on DNA replication inhibition after UV-C treatment, DNA replication of wt and p53−/− MEFs was examined by BrdU incorporation at different times after exposure to a dose of 7.5 J/m^2. As shown in

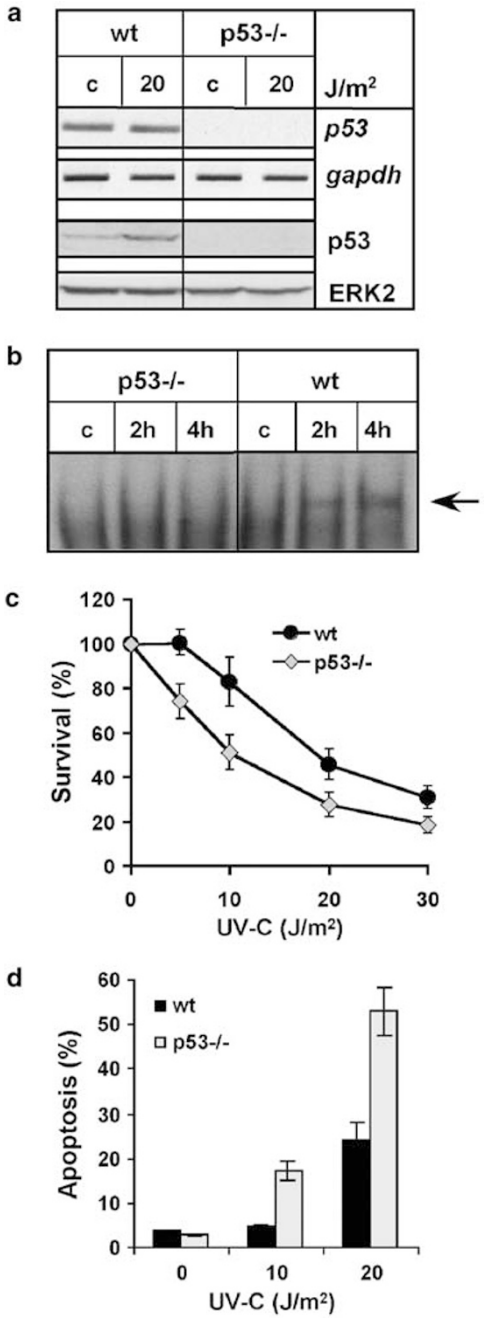

Figure 1 Phenotype of wt and p53−/− mouse fibroblasts. (**a**) Expression of p53 mRNA (upper panel) and protein (lower panel) as determined by RT–PCR and Western blot analysis, respectively. Wt and p53−/− cells were exposed to 20 J/m^2 UV-C. After 4 h nuclear protein extracts and total RNA were prepared. RNA was subjected to RT–PCR using *p53* or, for control, *gapdh*-specific primers. For Western blot analysis, the filter was incubated with anti-p53 or, for loading control, anti-ERK2 antibody. (**b**) EMSA: wt and p53−/− cells were exposed to 7.5 J/m^2 UV-C. After 2 and 4 h, nuclear protein was prepared and subjected to EMSA using oligonucleotides containing the p53 binding site of p21 (for sequence see Figure 7b). (**c**) Cytotoxicity assay: wt and p53−/− cells were treated with different UV-C doses. After 48 h, survival was measured by the WST-1 assay. (**d**) Frequency of apoptosis: wt and p53−/− cells were exposed to different doses of UV-C and 72 h later apoptosis was measured by annexinV-FITC/PI staining. Data points are the mean of three independent experiments

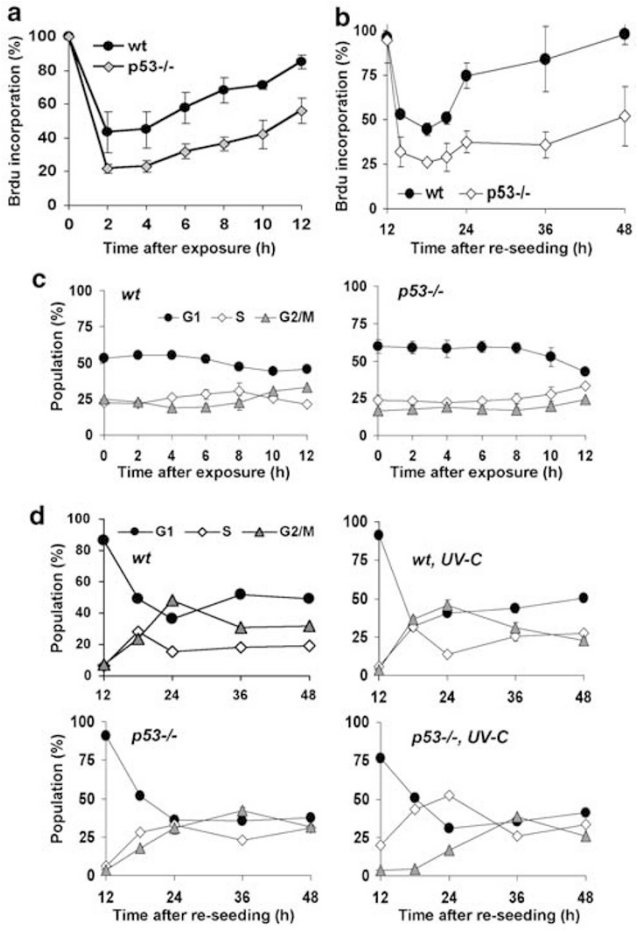

Figure 2 UV-C-induced replication blockage and cell cycle distribution. (**a**) DNA replication in wt and p53−/− fibroblasts as a function of time after UV-C treatment (7.5 J/m^2 UV-C), as measured by BrdU incorporation. All measure points are the mean of at least three independent experiments. (**b**) DNA replication in cells irradiated with UV-C (7.5 J/m^2) 12 h after reseeding of confluent cells. BrdU incorporation was measured by pulse labelling for 30 min at the indicated times after reseeding. (**c**) Cell cycle distribution of exponentially growing wt and p53−/− cells as a function of time after irradiation with 7.5 J/m^2 UV-C, as measured by flow cytometry. (**d**) Cell cycle distribution of reseeded wt and p53−/− cells (upon 3 days of confluence) not irradiated (left panels) and irradiated with 7.5 J/m^2 UV-C, which occurred 12 h after reseeding

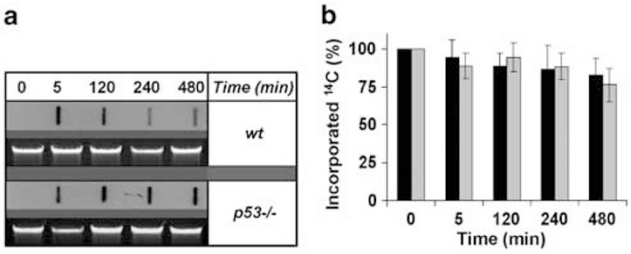

Figure 3 Repair of UV-C-induced DNA damage. (**a**) Induction and repair of UV-C lesions in wt and p53−/− mouse fibroblasts as determined by Southwestern blot analysis. Cells were exposed to 7.5 J/m^2 UV-C. At different times later, genomic DNA was isolated, equal amounts of DNA were blotted and subjected to incubation with anti-CPD antibodies. For loading control, the same amount of DNA was run on a parallel agarose gel (lower panel). (**b**) Dilution of CPDs due to DNA replication was analysed by labelling of genomic DNA with [^{14}C]thymidine 12 h before irradiation and measurement of remaining activity at different time points after irradiation in a scintillation counter. Data points are the mean of three independent experiments. Black and gray bars represent wt and p53−/− cells, respectively

Figure 2a, DNA synthesis in wt cells was reduced to 40% of control level 2 h after treatment and recovered almost completely 12 h later. In contrast, p53−/− cells displayed a stronger initial block of DNA replication (reduction to 20% of control level after 2 h) and an incomplete recovery within the 12 h postexposure period.

Upon irradiation with UV-C, cells activate cell cycle checkpoints via p53. To exclude the possibility that the observed block to replication results from G1/S inhibition and therefore a lower fraction of cells in S phase, we analysed in a parallel experiment the cell cycle distribution. Neither in the wt nor in the p53 null population a clear change in the cell cycle distribution profile was observed upon low dose of UV-C (7.5 J/m^2), as measured up to 12 h after exposure (Figure 2c). This indicates that the reduced BrdU incorporation upon UV-C is indeed due to inhibition of DNA replication itself. To further substantiate the finding of S phase blockage in p53 null cells, we treated synchronized cells with UV-C upon their entrance into S phase. In parallel experiments we monitored BrdU incorporation and cell cycle distribution. As shown in Figure 2b, BrdU incorporation was maximally reduced 6 h after irradiation (i.e., 18 h after reseeding) in cells that have re-entered S phase, with a stronger effect in p53−/− cells. The cell cycle distribution at different times after re-seeding is shown in Figure 2d. Neither wt nor p53−/− cells are blocked from re-entering S phase upon 7.5 J/m^2. Overall, the data suggest that the sustained decline in BrdU incorporation in p53-deficient cells is due to DNA replication inhibition.

UV-C light induces the formation of cyclobutan pyrimidine dimers (CPDs), which are known to block DNA replication (Chan *et al*., 1985). Thus, one might speculate that the observed replication blockage in p53-deficient cells is a consequence of impaired removal of CPDs from the DNA. We therefore determined the induction and repair of CPDs by immunoblot analysis. As shown by a representative blot in Figure 3a, in UV-C unexposed cells CPDs were not detectable, whereas they were significantly formed 5 min after treatment with 7.5 J/m^2 UV-C. This occurred to a similar level in wt and p53−/− cells (Figure 3a). Within the 8 h postincubation period, CPDs were clearly removed from DNA in wt cells, whereas in p53−/− cells no removal was observed (Figure 3a). The observed loss of CPDs from DNA in wt cells within the 8 h postincubation period is not due to dilution by DNA replication, which was found to be negligible in control experiments in which the decrease of [^{14}C]thymidine in the DNA of prelabelled cells was determined (Figure 3b).

Expression of Fen1 upon UV-C exposure

To elucidate the effect of p53 on the regulation of Fen1 upon UV-C treatment of MEFs, the expression of *fen1* mRNA was examined by real-time RT–PCR and, for confirmation, by semiquantitative RT-PCR. As shown in Figure 4a, exposure with a dose of 7.5 J/m^2 UV-C gave rise to a significant increase in the level of *fen1* mRNA in wt, but not p53−/− cells. The increase in the mRNA level was a fast response, already detectable 3 h after irradiation with a further increase in the 6 h postincubation period. Comparable results were obtained by semiquantitative RT–PCR, which was performed to verify the specificity of the product that was quantified by real-time RT–PCR. Also in this experiment, the level of *fen1* mRNA increased upon UV-C irradiation only in wt cells in a dose- and time-dependent manner (Figure 4b).

To clarify whether the induction of *fen1* mRNA and protein is the result of mRNA stabilization or promoter activation, cells were treated with the transcription-blocking agent actinomycin D. As demonstrated in Figure 4c, treatment of wt cells with the inhibitor only slightly reduced the amount of *fen1* mRNA, indicating its high stability. Treatment with actinomycin D completely prevented the increase in fen1 mRNA by UV-C, suggesting that the observed accumulation of *fen1* mRNA is dependent on RNA *de novo* synthesis.

Fen1 protein expression was further studied by Western blot analysis. In wt cells the level of Fen1

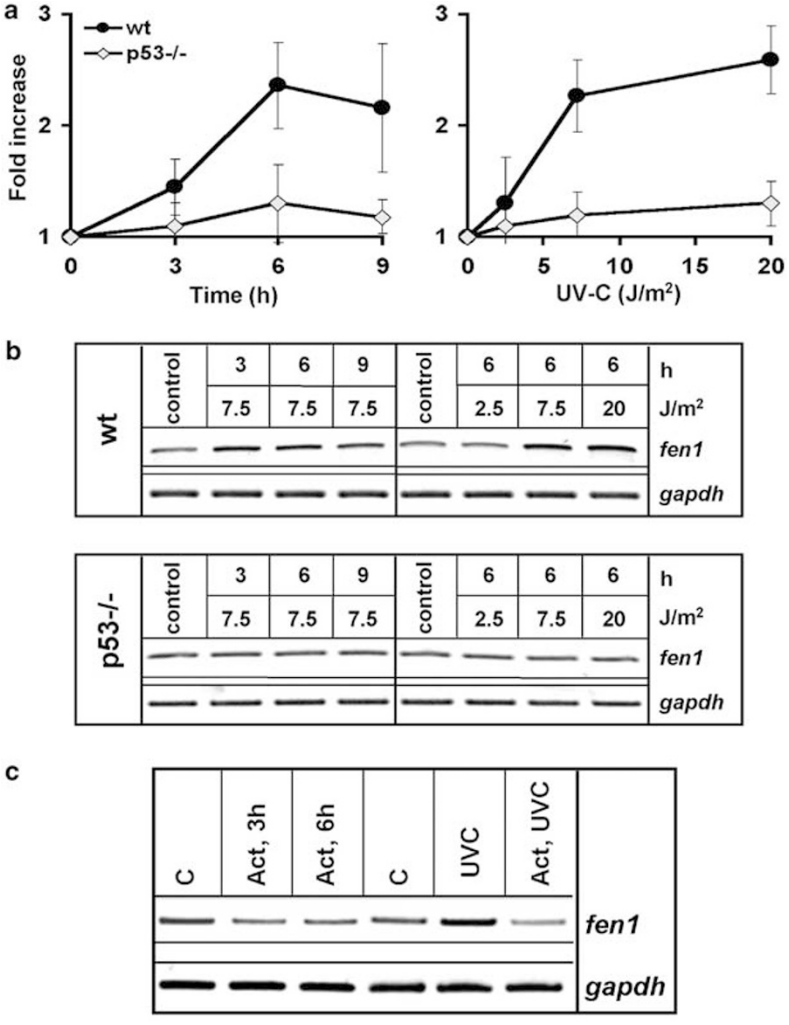

Figure 4 Induction of *fen1* mRNA by UV-C light. (**a**,**b**) *Fen1* mRNA level in UV-C-treated cells. (**a**) Exponentially growing fibroblasts were exposed to 7.5 J/m^2 UV-C for different time periods (left panel) or to different doses of UV-C and postincubated for 6 h until harvest (right panel). Total RNA was isolated and real-time RT–PCR was performed using *fen1* or, as positive control, *gapdh*-specific primers. For quantification, the expression was normalized with *gapdh* and the untreated control was set to one. Data are the mean of three independent experiments ± s.d. (**b**) In a different set of experiments, mRNA of control and treated cells was used to perform semiquantitative RT–PCR in order to confirm the specificity of the amplified RT–PCR product. The blots show one representative experiment performed with wt or p53−/− cells. (**c**) Induction of *fen1* is blocked by the transcriptional inhibitor. Wt cells were exposed to 0 or 7.5 UV-C in the presence or absence of 1 μM actinomycin D for 3 or 6 h. Total RNA was isolated and RT–PCR was performed using *fen1* or, as positive control, *gapdh*-specific primers

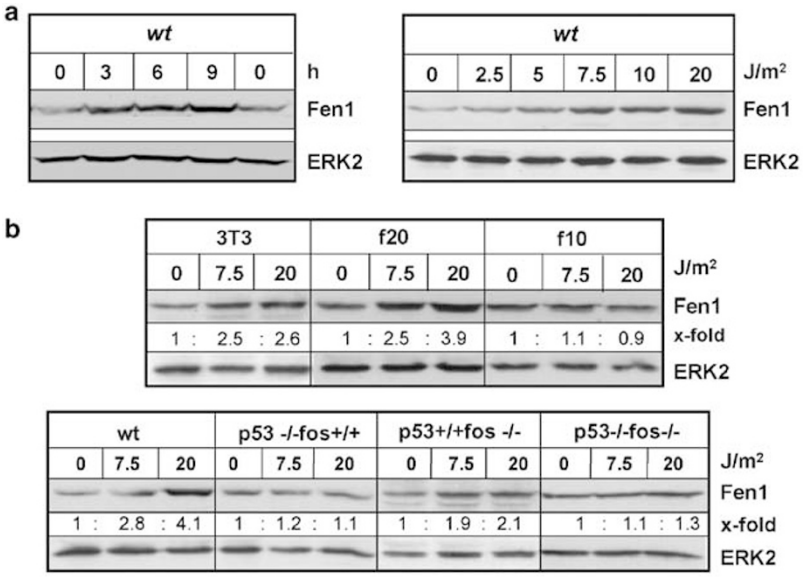

Figure 5 Induction of Fen1 protein by UV-C light. (**a**) Dose- and time-dependence of Fen1 protein level in UV-C treated cells. Exponentially growing fibroblasts were exposed to 7.5 J/m^2 UV-C and harvested after different times indicated in the figure, or to different doses, and post-incubated for 8 h until harvest (right panel). Cell extracts were prepared and subjected to Western blot analysis. (**b**) Induction of Fen1 in different cell lines exposed to 0, 7.5 or 20 J/m^2 UV-C and recovered for 8 h. Mouse 3T3 and f20 cells are p53wt, the line f10 is p53 mt and fos−/− (upper panel). In the lower panel, other lines are shown that are phenotypically wt, p53−/−, fos−/−, and double knockout (p53−/−fos−/−). The filters were incubated with anti-Fen1 and, for loading control, anti-ERK2 antibody

increased as a function of UV-C dose (Figure 5a, right panel) and time (Figure 5a, left panel). The increase in Fen1 was a long-lasting effect; it occurred between 3 and 6 h after irradiation (Figure 5a) and was still detectable 18 h later (data not shown).

To see whether Fen1 is also inducible in other rodent cell lines, we assayed 3T3 and f20 mouse fibroblasts: both are p53 proficient (data not shown). A line isogenic to f20 is f10, which expresses mutated p53 (Haas and Kaina, 1995, and data not shown). In the lines expressing wt p53, Fen1 increased after UV-C light exposure, whereas in f10 cells no increase was observed (Figure 5b, upper panel). f10 cells are not only mutated in p53 but also deficient for c-Fos. Previously we reported that *c-fos* knockout cells (both established and primary *fos*−/− cell lines) are hypersensitive to UV-C light (Haas and Kaina, 1995; Lackinger and Kaina, 2000; Lackinger *et al*., 2001). Therefore, we were interested to learn whether c-Fos deficiency impacts Fen1 induction. Data shown in Figure 5b (lower panel) revealed that Fen1 was induced in wt and *p53*+/+*fos*−/− cells but not in *p53*−/−*fos*+/+ as well as in the double knockout *p53*−/−*fos*−/− cell line. This shows that p53, but not c-Fos, is critically involved in the upregulation of Fen1 by UV-C light.

Proliferation dependence of Fen1 induction

To elucidate whether the inducible expression of Fen1 is dependent on proliferation, we compared resting (confluent) cells and cells that were stimulated to divide by reseeding. As shown in Figure 6a, confluent cells express Fen1 protein at a very low level, confirming previous reports (Kim, 1998; Warbrick *et al*., 1998). In confluent cells, Fen1 was not induced by UV-C. At 12 h after reseeding, the basal level of Fen1 was significantly enhanced, but Fen1 was still not induced by UV-C (as measured 8 h later). Induction of Fen1 by UV-C was observed at later times, i.e. 18, 24 and 36 h after reseeding (Figure 6a, upper panel). The data clearly suggest that induction of Fen1 by UV-C requires cell proliferation; it does not occur in nonreplicating cells. Parallel to Fen1 we investigated the expression of p53 under the same experimental conditions (Figure 6a, lower panel). Nuclear p53 protein did not increase upon UV-C irradiation in confluent cultures or in cells irradiated 12 h after reseeding (and measured 8 h later). At later times after reseeding ($\geqslant$18 h), however, p53 was induced by UV-C similar to the time course of Fen1 induction.

To further establish cell cycle-independent Fen1 induction, we exposed synchronized wt cells at the G1/S transition (12 h after reseeding) to 7.5 J/m^2 UV-C and measured the accumulation of Fen1 at various time points after exposure. As shown in Figure 6b (upper panel), Fen1 accumulation can be seen clearly 12–24 h after irradiation. Interestingly, under the same conditions accumulation of nuclear p53 was observed already 6 h after UV-C. p53 accumulation therefore precedes the induction of Fen1 (Figure 6b, lower panel). In non-irradiated controls, nuclear accumulation of p53 and increase in Fen1 were not observed. The data support the conclusion that UV-C-mediated induction of Fen1 is replication dependent and involves p53.

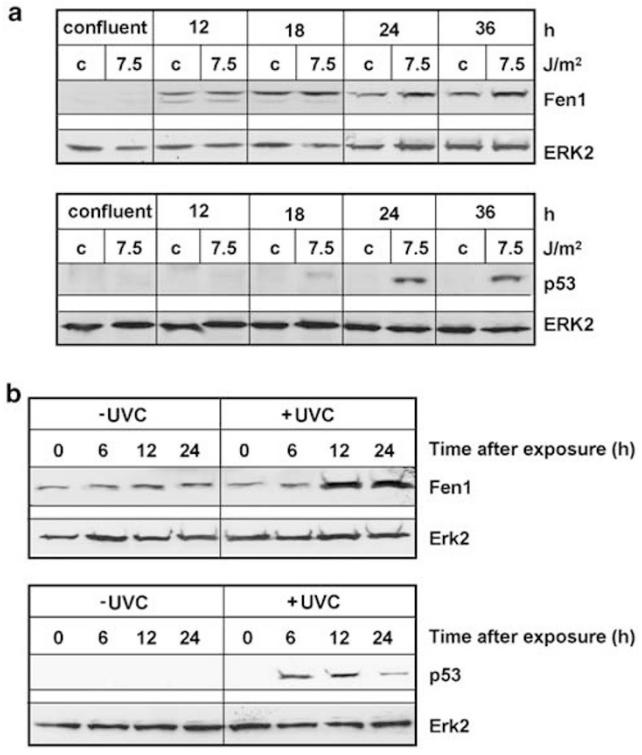

Figure 6 Fen1 induction is related to cell proliferation. (**a**) Replication-arrested confluent cells and cells stimulated to proliferation were not exposed (control, c) or were exposed to 7.5 J/m² UV-C at different time points upon reseeding. Cells were harvested 8 h after the time points indicated. Fen1 expression was detected by Western blotting in whole cell extracts (upper panel) and p53 expression was detected in nuclear extracts (lower panel). ERK2 served as loading control. (**b**) Replication-arrested confluent cells were reseeded and 12 h later exposed (+ UVC) or not exposed (− UVC) to 7.5 J/m² UV-C. Thereafter cells were harvested at the time points indicated. Fen1 expression was detected in whole-cell extracts (upper panel) and p53 in nuclear extracts (lower panel). ERK2 served as loading control

fen1 *promoter activation requires p53*

To analyse the role of p53 in the regulation of Fen1 expression on gene level, a 1.5 kb genomic fragment 5′ of the ATG codon of *fen1* was cloned from DNA of wt MEFs. The sequence is outlined in Figure 7a. The activity of the *fen1* promoter was confirmed in transfection experiments. As shown in Figure 7c, transfection of the *fen1*-*β*-gal fragment exhibited promoter activity, which was significantly enhanced if the plasmid was cotransfected with a p53 expression plasmid (4-fold increased promoter activity as compared to cotransfection with pcDNA3 alone or the corresponding p53 antisense construct). This indicates that the *fen1* promoter is positively regulated by p53. Support for this was obtained by p53 binding studies. Screening for putative transcription factor binding sequences revealed the presence of two potential p53 binding sites in the mouse **fen-1** promoter (labelled in bold in Figure 7a and b). The nucleotides differing between the potential binding sites and the p53 consensus sequence (el-Deiry *et al*., 1992) are marked by asteriks (Figure 7b). Electromobility shift assays (EMSA) were performed using radioactively labelled oligonucleotides harboring either one of the p53 binding sites of the *fen1* promoter or, for control, the p53 binding site of the **p21** promoter (see Figure 7b). The oligonucleotides were incubated with nuclear extracts obtained from MEFs that were wt or null for p53 (both were preirradiated with UV-C light in order to increase the nuclear p53 level). As shown in Figure 7d, there was only one band detectable upon incubation of the oligonucleotides using the wt but not the p53 null extract. Competition experiments with nonradioactively labelled oligonucleotides containing the p53 consensus sequence of the *p21* or the *fen1* promoter or, for control, the AP-1 consensus sequence demonstrated competition only by the p53 sequence, revealing the specificity of the binding complex (Figure 7e).

Transfection of fen1 *attenuates DNA replication blockage*

As shown above (see Figure 2), p53-deficient MEFs are characterized by an impaired recovery from the UV light-induced block of DNA replication. To see whether increased expression of Fen1 has impact on the extent of DNA replication inhibition, p53−/− cells were transfected with a *fen1* expression plasmid (*pcDNA3fen1*), irradiated with UV-C light and checked for BrdU incorporation at various times thereafter. As shown in Figure 8, p53−/− cells transfected with *pcDNA3fen1* clearly exhibited a better recovery from UV-C induced replication inhibition than mock-transfected (vector only) cells. Although the recovery was not as high as in wt cells (which is likely due to the expression level of Fen1) the data support the hypothesis that p53 driven-Fen1 expression plays a supporting role in the recovery of cells from UV-C light-induced DNA replication inhibition.

Discussion

MEFs deficient for p53 are hypersensitive to the cytotoxic, apoptosis-inducing and clastogenic effect of UV-C light (Lackinger and Kaina, 2000; Smith *et al*., 2000; Lackinger *et al*., 2001). Here, we show that p53-deficient MEFs display a significantly reduced recovery from the block of DNA replication induced by UV-C light, as compared to isogenic wt cells. This was found both in primary (data not shown) and established MEFs, which shows that the defect is not cell line specific but attributed to the p53 deficiency itself. The observed block of DNA replication did not result from G1/S inhibition and, therefore, a lower fraction of cells in S phase, since with the dose of UV-C used no change in the cell cycle distribution between wt and p53 null fibroblasts was observed. In previous reports p53-inactivated cells were shown to exhibit delayed progression through S phase upon cisplatin treatment (Hawkins *et al*., 1996). DNA replication was also significantly inhibited in p53-inactivated cells after UV-C exposure, which was associated with enhanced sensitivity to

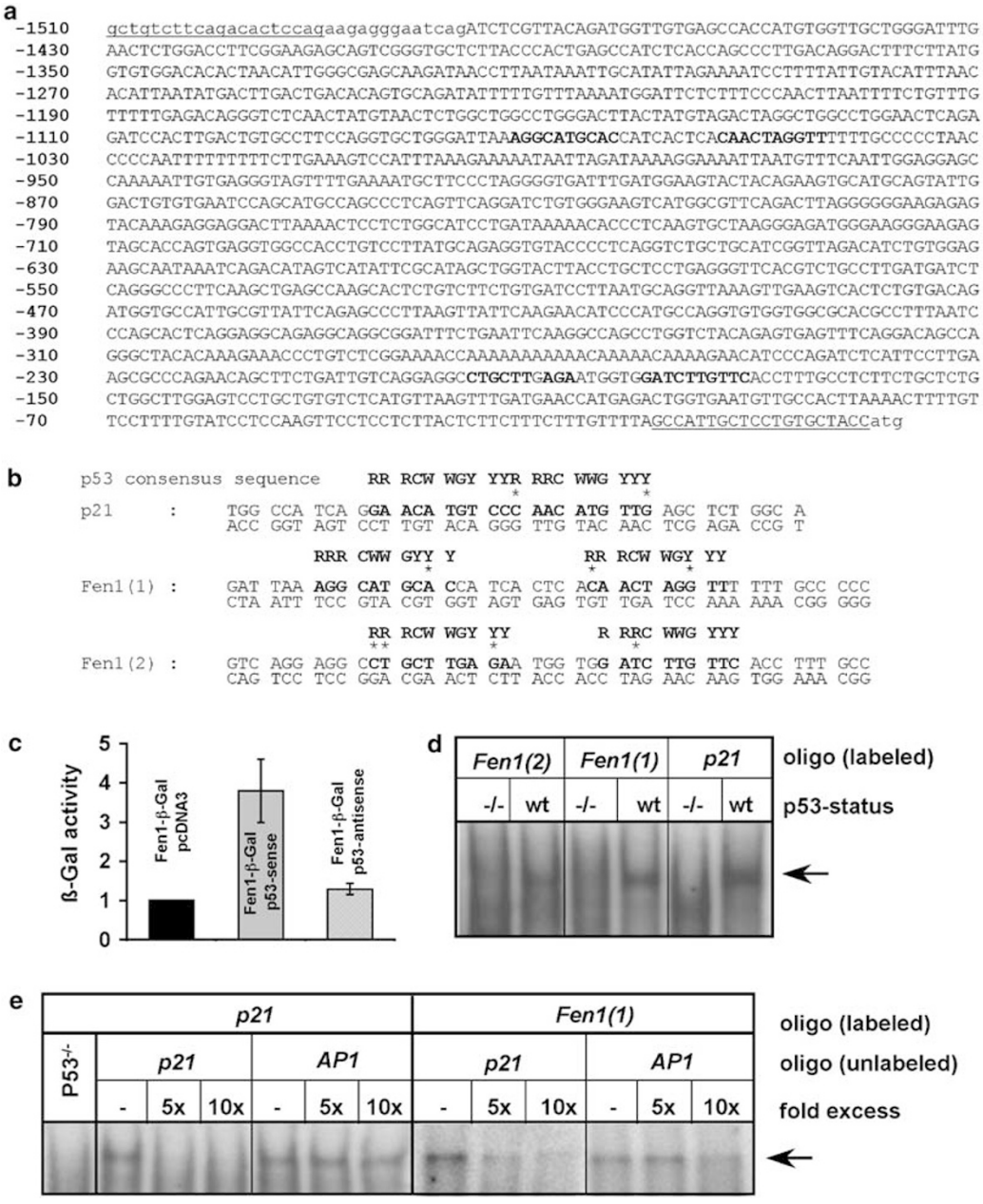

Figure 7 p53-dependent regulation of the *fen1* promoter. (**a**) Nucleotide sequence of a 1.5 kb *fen1* promoter fragment. Primers used for cloning are underlined and potential p53 binding sites are printed in bold. Additional sequences obtained from the database are shown in small letters. (**b**) Sequences of oligonuceotides harboring the p53 response element of the *p21* gene, and potential p53 response elements in two *fen1* promoter fragments used for EMSA. Differences in the p53 consensus sequence are indicated by asteriks. (**c**) Promoter activity as measured by *β*-galactosidase assay. The activity of the plasmid *pTOPOblue*(*fen1-prom*) was measured 48 h after transfection of CHO cells. Fen1-*β*-Gal pcDNA3 designates co-transfection with *pTOPOblue*(*fen1-prom*) and *pcDNA3*. Fen1-*β*-Gal p53-sense designates co-transfection with *pTOPOblue*(*fen1-prom*) and human *p53* cDNA expression vector. Fen1-*β*-Gal p53-antisense designates co-transfection with *pTOPOblue*(*fen1-prom*) and human *p53* cDNA antisense expression vector. (**d**) Binding of p53 to promoter fragments as determined by EMSA. Oligonucleotides (shown in **b**) were incubated with nuclear extracts from p53−/− and wt cells (both were pre-exposed to 20 J/m^2 UV-C light 4 h before harvest) and subjected to EMSA. (**e**) EMSA competition assay. For checking the specificity of the reaction, a 5- or 10-fold excess of unlabelled oligonucleotides of either p21 (with p53 binding sequence) or AP-1 promoter (without p53 binding sequence) was added to the assay

UV-C light (Cistulli and Kaufmann, 1998). The pronounced replication blockage in p53-deficient cells might be related to an impaired removal of UV-C-induced lesions from DNA. Thus, we show that under the treatment conditions used (7.5 J/m^2) p53 knockout MEFs are unable to remove CPDs from DNA, whereas the corresponding wt cells are able to do so. This is in line with a previous report with mouse fibroblasts (Smith *et al*., 2000) and mouse hepatocytes (Prost *et al*., 1998).

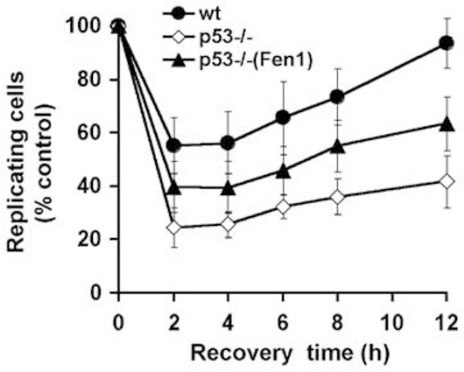

Figure 8 Transgenic Fen1 expression attenuates UV-C-induced DNA replication inhibition. DNA replication upon UV-C was measured by BrdU-ELISA. p53 null cells were transfected with *pcDNA3-fen1* or, for control, with *pcDNA3*. Cells were reseeded and 24 h later treated with 7.5 J/m^2 UV-C. DNA replication was measured at the times indicated by incorporation of BrdU added to the medium 1 h before harvest. Data are the mean of three independent experiments ± s.d. Transfection efficiency of p53 null cells was 40% as determined in a parallel experiment with a GFP expression vector

To analyse whether Fen1 is involved in the recovery from UV-C light-induced replication blockage, we analysed the Fen1 expression in our cell lines. The results indicate that Fen1 expression is induced by UV-C light which is dependent on the presence of p53. We demonstrate that induction of *fen1* by UV-C occurs in p53 wt MEFs on the level of mRNA, protein and the promoter. Fen1 induction was clearly dose- and time-dependent. Increased levels of Fen1 were seen after 3 h with a maximum 6–9 h after irradiation. Fen1 mRNA and protein accumulation was found only in p53 wt but not in p53 null cells, indicating that it is a p53-specific response. It appears not to be c-Fos dependent since *c-fos* knockout cells expressing p53 wt responded in the same way as the isogenic wt control. The time course of Fen1 accumulation in cells that were stimulated to divide coincided with the increase of p53 level after UV-C irradiation. Interestingly, Fen1 induction upon UV-C was not observed in nonproliferating cells, which was also true for p53 stabilization. The level of both proteins increased only in exponentially growing cells upon irradiation. We therefore conclude that replication-related events (presumably stalled replication forks) trigger p53 upregulation upon UV-C light, which in turn stimulates Fen1 expression. We should note that blockage of DNA synthesis by hydroxyurea (HU) was reported to lead to nuclear accumulation of p53 without inducing p53 target genes (Gottifredi *et al*., 2001). HU did not stimulate Fen1 expression (data not shown). Therefore, it seems that replication blockage *per se* is not sufficient for the induction of Fen1. It rather appears that additional signals are required that fully activate p53 transcriptional activity upon genotoxic damage.

The involvement of p53 in the regulation of *fen1* was further established by cotransfection experiments using the *fen1* promoter and a p53 expression plasmid. Cotransfection of p53 wt resulted in a significant increase in *fen1* promoter activity, whereas transfection of p53(antisense) did not stimulate the promoter. A search for binding sites revealed in the *fen1* promoter of mouse the presence of two putative binding sites exhibiting the p53 consensus sequence. Electromobility shift assays showed that one of these binding sites is recognized by p53. Induction of *fen1* mRNA was prevented by actinomycin D. Collectively, the data show that in MEFs, *fen1* is a p53-dependent UV-C light inducible gene whose upregulation results in increased Fen1 protein expression.

In view of these data, it was pertinent to hypothesize that the observed induction of Fen1 in MEFs plays a role in the DNA damage response. Thus, an attractive hypothesis would be that Fen1 upregulation prompts cells to recover from the UV-C-induced block of DNA replication. Maximal DNA replication inhibition in MEFs was observed 2 h after irradiation, and recovery started about 4 h later. Increased Fen1 levels were seen as early as 3 h after irradiation. It further increased with time and paralleled the recovery of the cells. The hypothesis that Fen1 is involved in DNA replication recovery is further supported by the finding that transfection of Fen1 in p53 null cells significantly attenuated the UV-C-induced DNA replication arrest. This is in line with a very recent report showing that human Fen1 is critical in resolving stalled DNA replication forks (Zheng *et al*., 2005). A prolonged S phase delay in response to treatment with UV-C light was also reported for human cells expressing non-functional Fen1 (Shibata and Nakamura, 2002). In line with this is the finding that transfection of cells with a Fen1 antisense construct resulted in delayed S phase progression (Shi *et al*., 2001). Also, homozygote inactivation of *fen1* is embryonic lethal because of the inability of blastocyst cells to perform DNA synthesis (Larsen *et al*., 2003). The exact role of Fen1 in replication fork movement upon DNA damage is not yet known. A reasonable model describes Fen1 to play a role in the conversion of stalled replication forks into recombination substrates, which are involved in repairing collapsed replication forks (Henneke *et al*., 2003; Sharma *et al*., 2004). Irrespective of the exact mechanism of cellular recovery from DNA replication blockage, the inability of MEFs lacking p53 to upregulate Fen1 might cause accumulation of arrested replication forks, nuclease attack at collapsed forks and the formation of lethal DNA double-strand breaks. This process could explain the hypersensitivity of p53 knockout MEFs to UV-C light-induced cell death, which is due to apoptosis (Lackinger and Kaina, 2000). It also explains that p53-deficient MEFs exhibit a higher chromosomal aberration frequency upon UV-C (Lackinger and Kaina, 2000), which is indicative of nonrepaired replication-mediated DNA breaks. Interestingly, expression of human Fen1 in yeast Fen1 deficient cells provoked resistance to UV-C light, supporting the model of an involvement of Fen1 in protection against UV-C (Zheng *et al*., 2005).

To our knowledge, this is the first report describing induction of Fen1 on transcriptional level by genotoxic

stress. Fen1 appears to be a novel player in the complex p53-dependent cellular genotoxic stress response, contributing to cellular protection against UV-C light and presumably other genotoxic stresses. It will be an interesting issue of future work to elucidate in more detail the signalling mechanism leading to the upregulation of various DNA repair genes and their coregulation with other cellular defence functions upon exposure of cells to genotoxic stress.

Material and methods

Cell lines

The cell lines used (fos+/+1-98M = wt, fos−/−7-98M = fos−/−, p53−/−2-98M = p53−/−, fosp53−/−BK10 = fosp53−/−, Swiss albino 3T3, f20 and f10) were described previously (Haas and Kaina, 1995; Lackinger and Kaina, 2000; Lackinger *et al*., 2001). The cells were grown in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS), in 7% CO_2 at 37°C. CHO cells were grown under the same conditions with 5% FBS.

UV-C treatment

Growth medium was removed and cells were irradiated with UV-C at a dose rate of 1 J/m^2 per second with a radium NSE 11-270 low pressure UV-C lamp (Philips). Thereafter, the removed medium was returned and cells were incubated at 37°C for the appropriate time periods.

Preparation of cell extracts and Western blot analysis

Whole-cell extracts were prepared as described previously (Christmann and Kaina, 2000). Samples of 25 μg protein extract were separated by 10% SDS–PAGE and electroblotted onto nitrocellulose membranes, which were then incubated with antibodies as described (Christmann *et al*., 2002). Monoclonal anti-Fen1 antibody (BD Pharmingen Laboratories) was diluted 1 : 500 in 5% nonfat dry milk, 0.2% Tween/PBS and incubated overnight at 4°C. A polyclonal anti-ERK2 antibody (Santa Cruz Biotechnology) was diluted 1 : 3000 and incubated for 2 h at room temperature. The protein–antibody complexes were visualized by ECL reagent (GE Health care, formerly known as Amersham). Quantification of signal intensity was performed using the Multianalyst Software and the Gel Doc 1000 system from Biorad. Signal intensity of ERK2, which is not regulated (background data), served as loading control. Fen1 intensity in relation to ERK1 in the untreated control was set to 1.

Preparation of nuclear extracts and EMSA

Nuclear cell extracts were prepared and subjected to EMSA as described previously (Christmann and Kaina, 2000). The sequence of the oligonucleotides specific for the p53-binding site in p21 and the two potential p53-binding sites in Fen1 is shown in Figure 7b.

Preparation of RNA, RT–PCR and real-time RT–PCR

Total RNA was isolated using the RNA II Isolation Kit from Machery and Nagel. A volume of 2 μg RNA were transcribed into cDNA by Superscript II (Invitrogen) in a volume of 40 μl, and 3 μl was subjected to RT–PCR. RT–PCR was performed using specific primers (MWG Biotechnology) and Red-Taq Ready Mix (Sigma-Aldrich). The PCR program used was: 1.5 min at 94°C, ((denaturation: 45 s, 94°C; annealing: 1 min 56–62°C; elongation: 1 min, 72°C) 25 cycles) and 10 min at 72°C. Real-time PCR was performed using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics) and the light cycler of Roche Diagnostics.

BrdU incorporation

Cells were cultured in DMEM (10% FBS) and, after exposure to UV-C, the thymidine analog BrdU (10 μM) was added to the medium. After 1 h of incubation, the incorporation was analysed by the BrdU Incorporation Kit (Roche) in a microplate reader.

Cloning of the fen1 cDNA and promoter

The *fen1* promoter of mouse was cloned by RT–PCR amplification of a genomic DNA fragment (1510 bp in size) 5′ to the coding region of the gene. It was cloned into the *pBlue-Topo* vector (Invitrogen) resulting in the *fen1* reporter plasmid designated as *pTopo(fen1-prom)*. The Fen-1 expression plasmid was constructed as follows: *fen1* cDNA was amplified by RT–PCR from mouse mRNA and cloned into the *Xho*I/*Hind*III site of the expression vector *pcDNA3* (Invitrogen) resulting in the *pcDNA3-fen1* expression plasmid.

Transfection

For transient transfection, 10^6 wt, p53−/− or CHO cells were seeded per 10-cm dish. One day later, cells were transfected with 2 μg of *pcDNA3-p53sense*, *pcDNA3-p53antisense* (Grombacher *et al*., 1998), *pTopo*(*fen1-prom*), or *pcDNA3-fen1* vector, using Effectene reagent as described by the manufacturer (Qiagen).

[^{14}C]thymidine incorporation

In all, 10^6 cells were seeded per 5-cm dish. The next day, 0.005 μCi [^{14}C]thymidine was added to the medium. After 24 h, cells were exposed to UV-C and genomic DNA was isolated after the indicated time points. DNA (1 μg) was mixed with 4 ml scintillation cocktail and counted in a liquid scintillation counter. The incorporated radioactivity of the non-UV-C exposed probe was set to 100%.

β-*Galactosidase assay*

CHO cells were cotransfected with pcDNA3 and **pTopo (fen1-prom)** (mock control), **pcDNA-p53sense** and **pTopo (fen1-prom)**, **pcDNA3-p53antisense** and **pTopo(fen1-prom)**, or **pTopo(fen1-prom)** alone. At 2 days after transfection, cell extracts were prepared by several freeze/thaw cycles and the β-galactosidase activity was determined by the β-Gal assay kit from Invitrogen.

Southwestern slot-blot analysis

Genomic DNA was isolated from subconfluently growing cells using the QIA(amp) blood mini kit (Qiagen). DNA (0.5 μg) was transferred to a positively charged nylon membrane (Hybond plus, GE Health care, formerly known as Amersham) by vacuum slot-blotting, denaturated with 0.3 M NaOH, neutralized with 5 × SSC and fixed by baking the membrane for 2 h at 80°C. Monoclonal antibodies specific for thymine dimers (Kamiya Biomedical Company) were used at a dilution of 1 : 100. Additional Western blot procedure and detection were performed as described above.

Flow cytometry

Cell cycle analysis was performed as described (Tomicic *et al*., 2003).

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