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Sustained activation of p53 in confluent nucleotide excision repair-deficient cells resistant to ultraviolet-induced apoptosis

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ABSTRACT

p53 activation is one of the main signals after DNA damage, controlling cell cycle arrest, DNA repair and apoptosis. We have previously shown that confluent nucleotide excision repair (NER)-deficient cells are more resistant to apoptosis induced by ultraviolet irradiation (UV). Here, we further investigated the effect of cell confluence on UV-induced apoptosis in normal and NER-deficient (XP-A and XP-C) cells, as well as the effects of treatments with the ATM/ATR inhibitor caffeine, and the patterns of p53 activation. Strong p53 activation was observed in either proliferating or confluent cells. Caffeine increased apoptosis levels and inhibited p53 activation in proliferating cells, suggesting a protective role for p53. However, in confluent NER-deficient cells no effect of caffeine was observed. Transcription recovery measurements showed decreased recovery in proliferating XPA-deficient cells, but no recovery was observed in confluent cells. The levels of the cyclin/Cdk inhibitor, $p21^{Waf1/Cip1}$, correlated well with p53 activation in proliferating cells. Surprisingly, confluent cells also showed similar activation of $p21^{Waf1/Cip1}$. These results indicate that reduced apoptosis in confluent cells is associated with the deficiency in DNA damage removal, since this effect is not clearly observed in NER-proficient cells. Moreover, the strong activation of p53 in confluent cells, which barely respond to apoptosis, suggests that this protein, under these conditions, is not linked to UV-induced cell death signaling.

1. Introduction

DNA damage is the initial trigger for mutagenesis and carcinogenesis, if lesions remain unrepaired, and the cell does not undergo apoptosis. Ultraviolet (UV) irradiation is an important source of DNA damage, especially on sun-exposed areas of the body, mainly generating cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts ((6-4) PPs). These lesions are normally removed by an efficient DNA repair system known as nucleotide excision repair (NER) [1,2]. The NER pathway involves several proteins with distinct activities, such as XP (XPA through XPG) and CS proteins (CSA and CSB). Mutations

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Abbreviations: CS, Cockayne's syndrome; CPD, cyclobutane pyrimidine dimers; GGR, global genome repair; TCR, transcription-coupled repair; TTD, trichothiodystrophy; XP, xeroderma pigmentosum.

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on XP proteins are found in xeroderma pigmentosum, a rare disease characterized by photosensitivity, high incidence of skin cancer, and eventually associated to neurological symptoms [3]. CS proteins are mutated on Cockayne's syndrome, which is characterized by growth and mental retardation, retinal abnormalities and severe photosensitivity [4]. Mutations in XP genes can also result in trichothiodystrophy (TTD), a disease characterized by brittle hair and nails due to deficiency in sulfur-rich protein synthesis [5]. NER works in two sub-pathways: one that repairs exclusively the transcribed strand of active genes (transcription-coupled repair – TCR), and another that repairs the genome overall (global-genome repair – GGR) [1,2]. Cells from XP patients are deficient for both TCR and GGR, with the exception of the XP-C and XP-E complementation groups, which are exclusively impaired for GGR. On the other hand, CS cells show exclusive impairment for TCR [4].

Along with the repairing role of the NER system, the tumor suppressor protein p53 is normally activated after DNA damage causing cell cycle arrest, thereby providing more time for proper DNA repair [6]. The role of p53 in cell cycle control is mainly exerted through cell cycle checkpoints. After DNA damage, activation of the so-called damage sensor kinases, ATM, ATR and DNA-PK, is the primary known signal for cell cycle checkpoints. p53 is phosphorylated and activated by ATM in response to ionizing radiation (IR), and also by ATR in response to UV irradiation [6]. p53 activation then leads to transcriptional activation of the cyclin-dependent kinases inhibitor $p21^{Waf1/Cip1}$, that binds and inactivates cyclin-Cdk complexes responsible for mediating G1/S or G2/M phases transition, thus leading to cell cycle arrest [7]. Besides its role in cell cycle arrest, it is noteworthy that p53 has also been shown to play a direct role in the NER pathway, preferentially affecting GGR. Cells from Li-Fraumeni patients, homozygous for mutations in p53, have shown decreased GGR but not TCR [8]. The role of p53 in GGR has also been shown in human mammary cells induced to degrade p53 by expression of HPV-16 E6 [9]. It was later shown that the effect of p53 on GGR is probably due to its transcriptional control of the NER proteins XPC [10] and p48/DDB2 (XPE) [11], as well as GADD45 [12].

If the cell is unable to repair DNA damage, it triggers apoptosis in order not to pass on mutations. Apoptosis is a controlled mode of cell death, characterized by cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation in a characteristic pattern [13,14]. p53 has a crucial role in apoptosis acting as a transcriptional factor and controlling the expression of genes necessary for cell death induction. These genes include pro-apoptotic members of the Bcl-2 family of proteins such as Bax, Bid, Noxa and PUMA, besides death-related receptors such as FasR and DR5 [15].

The role of p53 in apoptosis, as well as in other cellular processes, is regulated by modulation of p53 activity [16]. In this process, the E3 ubiquitin ligase MDM2 plays a central role, as it targets p53 for degradation by 26S proteasome. Phosphorylation of p53 disrupts the binding of MDM2 and stabilizes the protein. Several kinases have been discovered to phosphorylate p53, such as the UV-activated ATR and the IR-activated ATM, besides the checkpoint kinases Chk1 and Chk2 [6], as well as other stress-activated kinases, such as ERK, p38 and JNK [17].

The fact that cells from our skin, which correspond to the sun-exposed areas of our body, have an *in vivo* quiescent metabolism, a lower turnover and are found in intimate association with other cells, much similar to what is observed in confluent cells in culture, prompted us to investigate the effect of cell confluence on the apoptotic response of NER-deficient cells after UV irradiation [18]. Primary confluent cells are more resistant to UV-induced apoptosis, and this effect was more pronounced in XPC-deficient cells, impaired only in GGR. This result suggests that, by being more resistant to apoptosis induced by UV irradiation, NER-deficient cells from our skin may be more susceptible to DNA damage-induced malignant transformation, as defective removal of the DNA lesions may lead the cells to perpetuate a genomic mutation.

In this study, we examined the status of p53 activity and the p53-regulated cell cycle inhibitor $p21^{Waf1/Cip1}$ in order to identify possible differences between proliferating and confluent cells. The effect of the radiosensitizing agent caffeine, a potent inhibitor of DNA damage sensor kinases [19–21], was also tested in cells irradiated with UV. Here we show activation of p53 and p21 in all cell lines and conditions tested. Caffeine increased the sensitivity of proliferating normal and DNA repair-deficient cells to UV irradiation, while it inhibited p53 activation. However, confluent XP-A and XP-C cells treated with caffeine did not show increased sensitivity to UV. Moreover, no significant differences were observed in the levels of p53 activation between proliferating and confluent cells, and similar results were observed for the p53-regulated cell cycle inhibitor $p21^{Waf1/Cip1}$. Results with caffeine suggest a role for p53 in cell protection in proliferating cells, possibly independent of DNA repair. However, p53 activation in UV-irradiated confluent cells does not seem to be crucial for cell protection, or related to cell death induction.

2. Materials and methods

2.1. Cell lines and culture

Primary fibroblasts were derived from skin biopsies from xeroderma pigmentosum patients (complementation groups A and C – XP-A (XP456VI and XP21VI) and XP-C cells (XP17VI and XP148VI), and normal cells (198VI)). These cells were kindly provided by Dr. Alain Sarasin (IGR, Villejuif, France). Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY), supplemented with 10% fetal calf serum (FCS, Cultilab, Campinas, SP, Brazil), 100 U/mL penicillin G sodium, 100 μg/mL streptomycin and 0.25 mg/mL amphotericin B. Cells were grown at 37 °C, in a humidified, 5% CO_2 atmosphere.

2.2. Cell irradiation

Proliferating cells (approx. 1×10^6 cells, 60% confluence) cultured in 35 or 100 mm dishes, were irradiated with UV in PBS, using a germicidal UV lamp (UVC, max. emission 254 nm). The UV dose was monitored by a VLX 3W radiometer (Vilber Lourmat, Torcy, France). Confluent cells were grown until confluence and maintained for 3–4 days after reaching confluence in media containing 10% FCS, with frequent changes in culture

medium. Culture medium was exchanged to a medium containing low serum (0.1% FCS) 1 day before the irradiation, after which fresh low serum medium was added to the cells. Cells were further cultivated during different periods of time, before harvesting for apoptosis assays or Western blot analysis.

2.3. Determination of [^{3}H]-thymidine incorporation

Proliferating and confluent cells were compared for [^{3}H]-thymidine incorporation, a measure of DNA synthesis [22]. Confluent cells were maintained in a quiescent state during 5 days, with periodic medium changes, to assure DNA synthesis inhibition. Cells were then incubated with ^{3}H-thymidine (4 μCi/mL, GE Healthcare, Piscataway, NJ) for 6 h. The medium was discarded, and the cells were washed twice with PBS. 15% TCA was added for 5 min, and the cells were washed twice with ethanol for 5 min. 1 mL 0.3 M NaOH was added for 10 min and the lysate was scraped from culture dishes. 200 μL of this alkaline solution was applied to filter paper, and the remainder was used for DNA quantification by measurement of absorbance at 260 nm. Filter paper was sequentially washed with 15% TCA, ethanol and acetone for 5 min each. [^{3}H]-thymidine incorporation was measured by liquid scintillation and standardized according to the amount of DNA.

2.4. Determination of RNA synthesis

Cells were irradiated as mentioned before, and maintained in a culture medium for increasing times. The medium was changed for one containing 3% dialyzed FCS and cells were pulse-labeled for 1.5 h with 2 μCi/mL ^{3}H-uridine (GE Healthcare) [23]. After this time, cells were tripsinized and lysed with a solution containing 300 mM NaCl, 20 mM Tris–HCl pH 8.0, 2 mM EDTA, 1% SDS and 200 μg/mL proteinase K. Samples were applied to filter paper, fixed with 15% TCA and washed with ethanol. ^{3}H-Uridine incorporation was then measured by liquid scintillation, and standardized according to the amount of DNA.

2.5. Apoptosis detection

Apoptosis was measured by quantification of sub-diploid nuclei (sub-G_1 events) after cell cycle analysis of propidium iodide stained cells [18]. Briefly, adherent cells were harvested by tripsinization, together with floating cells, resuspended in 70% ethanol/PBS, and stored at 4 °C. Cells were then stained with 50 μg/mL propidium iodide for 1 h in the presence of 40 μg/mL of RNase A (DNase-free). Measurements were carried out in a FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). 10,000 events were analysed for each sample. Results were analyzed with the CellQuest Software (BD Biosciences).

2.6. Western blots

Cells lysates (40–50 μg protein/lane) were subject to electrophoresis in 10–12% SDS-polyacrylamide gels, and electroblotted onto polyvinylidene difluoride membranes (Hybond-P, GE Healthcare). Monoclonal antibody against p53 was from Dako (Cambridgeshire, UK). Polyclonal antibody against p21$^{Waf1/Cip1}$ was from BD Biosciences (San Jose, CA). Polyclonal antibody against β-tubulin was from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were from Sigma Chemical Co. (St. Louis, MO). Membranes were exposed to a Biomax Light film (Kodak, Rochester, NY) after chemiluminescence detection using ECL Plus (GE Healthcare).

3. Results

3.1. Inhibition of DNA synthesis in confluent cells

Confluent cells undergo cell cycle arrest due to contact inhibition, causing a reduction in the number of cells in the S phase of the cell cycle, which means inhibition of DNA synthesis [24]. In order to confirm DNA synthesis inhibition in confluent cells, [^{3}H]-thymidine incorporation was measured. All the confluent cells tested (198VI/normal cells, XP456VI/XP-A and XP17VI/XP-C) showed strong DNA synthesis inhibition (approximately 90%) when compared to proliferating cells (Fig. 1). These results confirmed that confluent cells were arrested in the cell cycle due to contact inhibition.

3.2. Effect of cell confluence on apoptosis in NER-deficient cells

We had previously found that confluent NER-deficient cells, especially XP-C cells, were much more resistant to apoptosis when compared to proliferating ones [18]. In the present work, those results were confirmed using a wider range of UV doses. Apoptosis measurements were carried out by sub-G1 quantification. Different UV doses were used for each cell line, in order to work with similar apoptosis levels in each case. For XP-C cells, apoptosis levels were assessed at two different

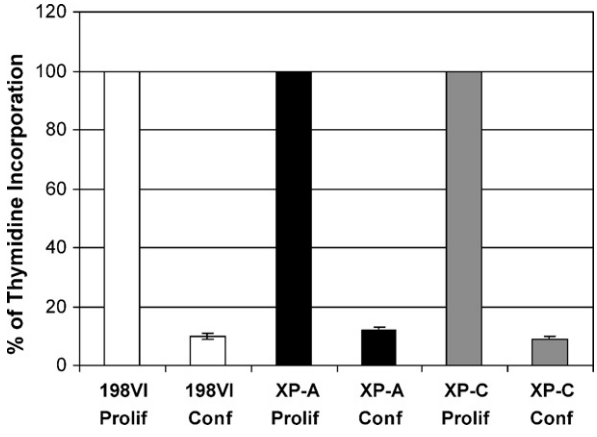

Fig. 1 – DNA synthesis measurement in proliferating or confluent NER-deficient cells. Proliferating or confluent 198VI (normal), XP456VI (XP-A) and XP17VI (XP-C) cells were labeled with [^{3}H]-thymidine (4 μCi/mL) for 6 h. Cells were trypsinized, lysed, and samples were applied to filter paper, fixed with 15% TCA and washed with ethanol. [^{3}H]-thymidine incorporation was measured by liquid scintillation and standardized according to the amount of DNA. Error bars represent the mean of 2 determinations ± S.E.M.

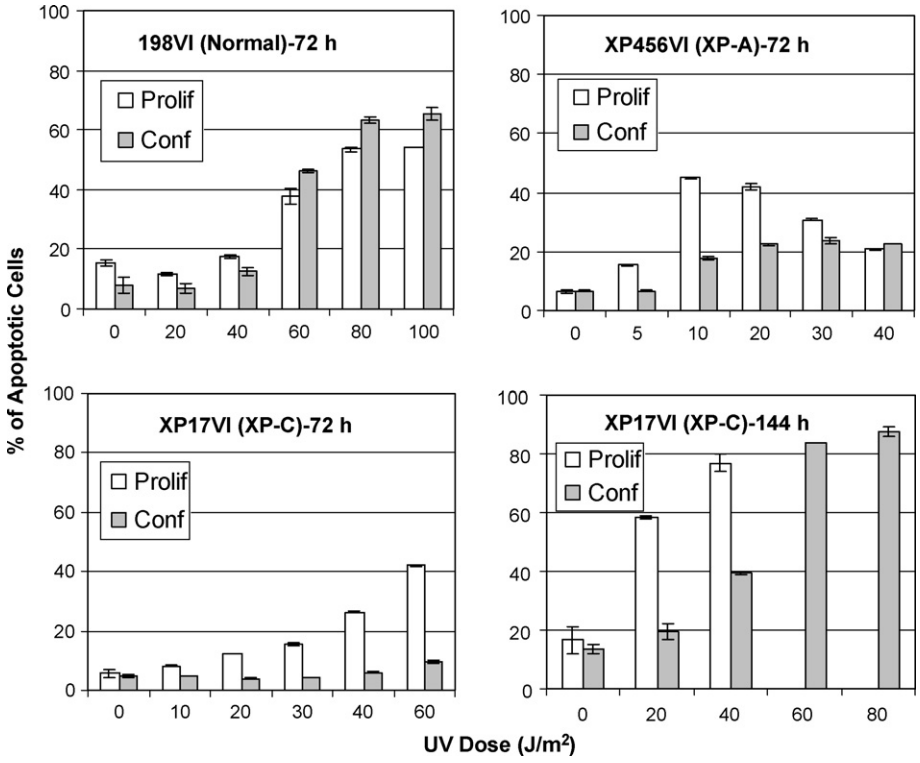

Fig. 2 – Effect of cell confluence on the induction of apoptosis by UV irradiation in NER-deficient cells. Proliferating or confluent 198VI (normal), XP456VI (XP-A) and XP17VI (XP-C) cells were irradiated with the indicated UV doses, and harvested after 72 or 144 h, as indicated, for the quantification of sub-G_1 cells by FACS. Error bars represent the mean of 2 determinations ± S.E.M.

times (72 and 144 h) after irradiation with UV doses ranging from 10 to 80 J/m^2. The results are shown in Fig. 2. Increased resistance to apoptosis in confluent cells was observed in XP-A and XP-C cells, although this effect was more evident for XP-C cells. Considering 72 h after UV, very low levels of apoptosis were observed for confluent XP-C cells, even at high UV doses, while for proliferating cells there was a consistent increase in the levels of apoptosis. These XP-C cells were more resistant to UV irradiation even at later times (144 h) after irradiation. Even though they show high levels of apoptosis at the higher UV doses, they are still more resistant than proliferating cells which could not even be detected at those time points after UV (only debris were observed in the cell culture medium).

Proliferating XP-A cells, which were irradiated with doses ranging from 5 to 40 J/m^2, showed the highest level of apoptosis induction at 10 J/m^2, with decreasing levels at higher UV doses. Confluent XP-A cells were less susceptible to apoptosis, especially at lower UV doses, but showed only a small increase in apoptosis levels at higher UV doses (≥10 J/m^2). As a result, at the high UV dose of 40 J/m^2 there seems to be an inversion in the tendency of confluent cells of being less susceptible to apoptosis. As the effect of confluence is only observed at lower UV doses for these cells, this partially explains why in our previous work, where higher UV doses were used, we did not observe decreased levels of apoptosis in confluent XP-A cells [18]. This inversion in UV susceptibility also appears to occur in normal cells (198VI), although less evident. When irradiated with 20–40 J/m^2, proliferating 198VI cells were more susceptible to apoptosis compared to confluent ones, while at UV doses ranging 60–100 J/m^2, confluent cells were more susceptible to apoptosis.

3.3. *Effect of caffeine on apoptosis in confluent and proliferating NER-deficient cells*

Caffeine has long been known for enhancing the cytotoxic effects of UV and γ irradiation, and chemotherapeutic agents. Thus, we decided to check for the effect of caffeine on UV-induced apoptosis in confluent and proliferating cells, in order to test whether this treatment could sensitize confluent cells to apoptosis. In parallel, we also used other cell lines with deficiencies in the same NER proteins in order to test whether the effect of confluence was due to the repair deficiency and not to cell variability. Only lower UV-doses were tested in this case. Both XPA-deficient (XP456VI and XP21VI) and XPC-deficient cells (XP17VI and XP148VI) were more resistant to apoptosis when in confluence, strengthening a connection between the confluence effect and DNA repair deficiency (Fig. 3). Proliferating XP-A as well as XP-C cells, when treated with caffeine, presented a consistent increase in the percentage of apoptotic cells. However, confluent XP-A and XP-C cells were refractory to the caffeine radiosensitizing effect. On the other hand, cells proficient in DNA repair (198VI) showed increased levels of apoptosis when treated with caffeine, regardless of the confluence status. These results suggest that the absence of an effect of caffeine

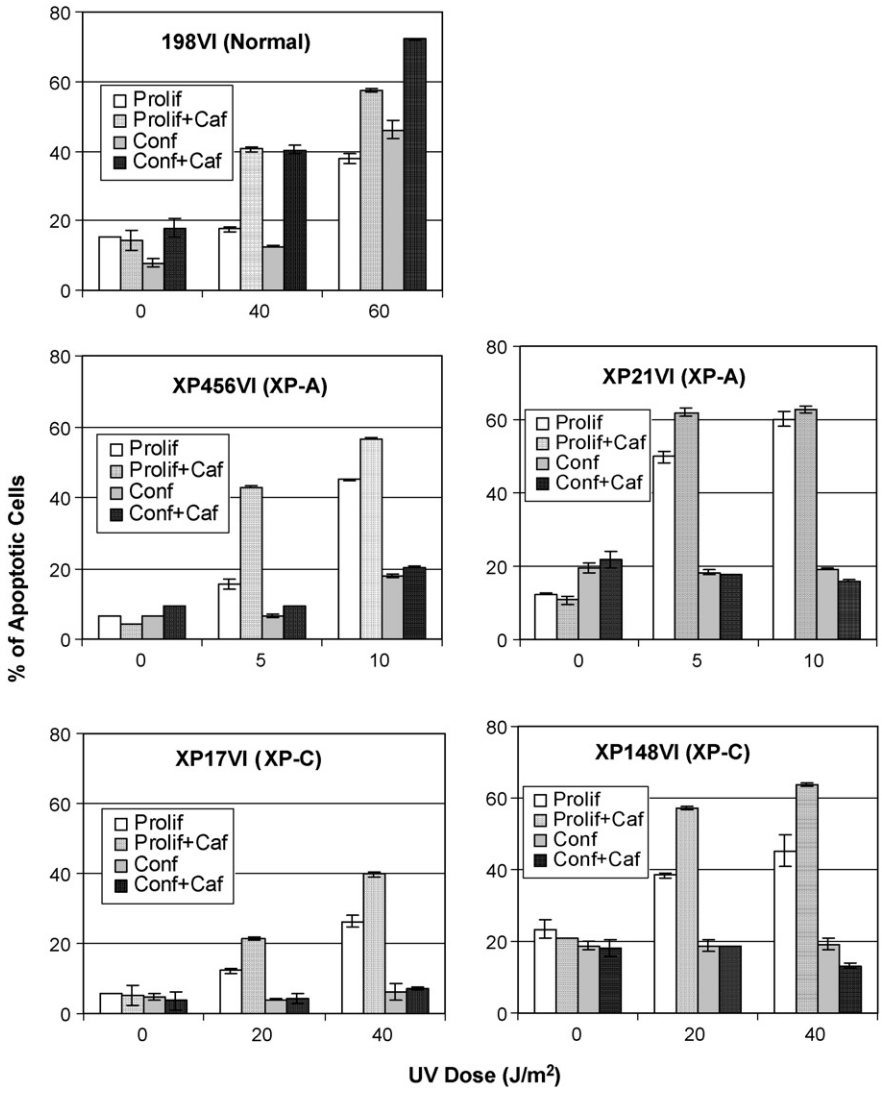

Fig. 3 – Effect of caffeine on the induction of apoptosis by UV in proliferating or confluent cells. Proliferating or confluent 198VI (normal), XP456VI and XP21VI (XP-A), and XP17VI and XP148VI (XP-C) cells were irradiated with the indicated UV doses, treated with 6 mM of caffeine, and harvested after 72 h for the quantification of sub-G_1 cells by FACS. Error bars represent the mean of 3 determinations ± S.E.M.

in confluent cells may be related to their DNA repair deficiency, since only DNA repair-deficient cells were insensitive to caffeine.

3.4. *Differential transcription recovery in confluent and proliferating cells*

Transcription inhibition has long been known as a hallmark of DNA damage [25]. This is due to the physical hindrance of RNA polymerase II progression during transcription. Impairment of RNA polymerase movement is mainly caused by CPDs, as they are removed more slowly, when compared to (6-4) PPs [26]. After DNA repair, transcription is normally restored. However, TCR-deficient cells are characterized by a pronounced impairment of transcription recovery when compared to normal cells [25,27], whereas persistence of these lesions in the transcribed strand of active genes is believed to be the main signal to apoptotic cell death due to the accumulation of transcription complexes at the sites of lesions [28,29]. The recovery from transcription inhibition was therefore evaluated in proliferating and confluent cells. Proliferating XP-A, XP-C and normal cells showed decreased transcription 3 h after UV irradiation. Transcription inhibition was followed by progressive recovery that started after 6 h, and was almost complete after 24 h for normal and XP-C cells (Fig. 4). XP-A cells showed almost no transcription recovery, as expected, since these cells are impaired in both TCR and GGR, while XP-C cells are deficient only in GGR. On the other hand, confluent XP-A and XP-C cells showed a delay in transcription inhibition, with minimum values 6 h after irradiation and no transcription recovery being observed even 24 h later. A similar behavior was observed for transcription inhibition in confluent normal cells. Thus, when compared with proliferating cells, confluent cells had a delay in transcription inhibition, and neither TCR-proficient

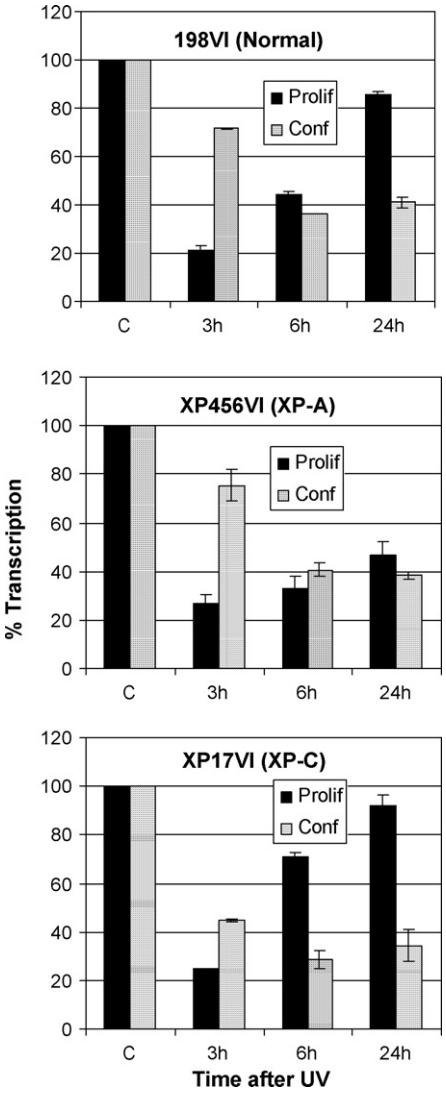

Fig. 4 – Effect of UV irradiation in the transcription rate of proliferating or confluent NER-deficient cells. Proliferating or confluent 198VI (normal), XP456VI (XP-A) and XP17VI (XP-C) cells were irradiated with 10 J/m^2 (XP-A), 40 J/m^2 (XP-C) or 60 J/m^2 (normal cells). Cells were maintained in culture for the indicated times. Nascent RNA was then pulse-labeled for 1.5 h by incubation of the cells with 2 μCi/mL of ^{3}H-uridine. Cells were trypsinized, lysed, and samples were applied to filter paper, fixed with 15% TCA and washed with ethanol. Uridine incorporation was then measured by liquid scintillation. Error bars represent the mean of 3 determinations ± S.E.M.

nor TCR-deficient cells displayed transcription recovery 24 h after UV irradiation.

3.5. *Reduced apoptosis in confluent cells is not related to differences in p53 activation*

In order to check for possible differences in p53 activation between proliferating and confluent cells, p53 total levels were assessed by Western blot. p53 activation was observed after UV irradiation in all cell types tested (Fig. 5). p53 activation peaked 24 h after irradiation for proliferating XP-A and XP-C cells, and later on (48 h) for cells maintained in confluence. Interestingly, basal p53 levels were higher in confluent cells, probably indicating stabilization of the protein in cell cycle-arrested cells. A comparison of p53 activation in proliferating and confluent NER-deficient cells, however, showed no significant differences in the maximum levels of total p53 protein under these conditions. Interestingly, when only proliferating cells were analyzed, activation of p53 was considerably lower in cells normal for DNA repair when compared with NER-deficient cells.

The effect of treatments with the radiosensitizing agent caffeine on p53 activation after UV irradiation was also evaluated. Fig. 5 shows p53 activation was highly reduced in proliferating or confluent XP-A and XP-C cells treated with caffeine. This suggests caffeine prevents phosphorylation and consequent stabilization of p53, probably through inhibition of activation of ATM and ATR kinases [19]. The effect of caffeine on p53 activation was also observed in normal cells (198VI), although less pronounced.

3.6. *p53 activation correlates with increased levels of p21$^{Waf1/Cip1}$*

p21$^{Waf1/Cip1}$ acts as a cyclin-dependent kinase inhibitor causing cell cycle arrest after DNA damage [7]. p21$^{Waf1/Cip1}$ is also transcriptionally activated by p53. In this study, p21$^{Waf1/Cip1}$ levels correlated well with p53 activation kinetics, suggesting that p53 transactivates p21$^{Waf1/Cip1}$, as expected (Fig. 5). While in proliferating cells p53 levels remained high even 48 h after irradiation, p21$^{Waf1/Cip1}$ levels decreased after 24 h. In confluent 198VI and XP-A cells, however, high levels of p21$^{Waf1/Cip1}$ were still observed 48 h after irradiation, since maximum p53 levels were reached at this time, while XP-C cells displayed a decrease in p21$^{Waf1/Cip1}$ levels after 24 h. Induction of p21$^{Waf1/Cip1}$ is associated with cell cycle arrest, which is assumed to allow cells more time for DNA damage removal. This makes sense in proliferating cells, but in the case of confluent cells, which are growth arrested due to contact inhibition, p21$^{Waf1/Cip1}$ induction would not be necessary for this purpose.

4. Discussion

We have previously shown that confluent NER-deficient cells have an increased resistance to apoptosis [18]. The present report further investigates the mechanisms involved in the resistance of confluent cells to DNA damage. One of the approaches consisted in treating the cells with the radiosensitizing agent caffeine immediately after UV irradiation. Caffeine is a known inhibitor of cell cycle checkpoints, acting on ATM, ATR and DNA-PK kinases [19,20]. These kinases are responsible for phosphorylation and activation of checkpoint kinases Chk1 and Chk2, besides the tumor suppressor protein p53, among other substrates. p53 activation triggers cell cycle arrest, in order to allow more time for DNA repair. Checkpoint inhibition by caffeine inhibits p53 phosphorylation and activation. Increased levels of apoptosis were observed in 198VI cells,

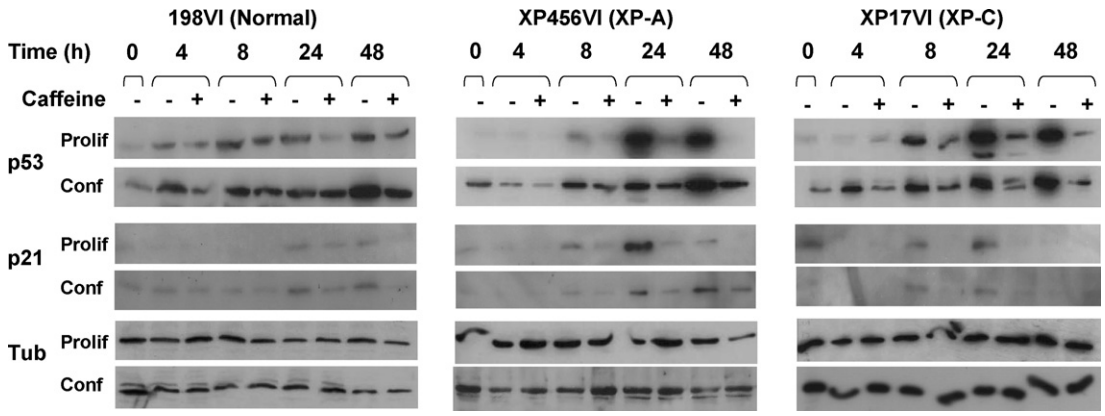

Fig. 5 – Activation of p53 and levels of p21 in confluent or proliferating NER-deficient cells irradiated with UV. Proliferating or confluent 198VI (normal), XP456VI (XPA) and XP17VI (XP-C) cells were irradiated with 10 J/m^2 (XP-A), 40 J/m^2 (XP-C) or 60 J/m^2 (normal cells), and treated or not with 6 mM caffeine. Cells were harvested after the times mentioned in the figure, lysed and subjected to SDS-PAGE and "Western blot" using antibodies against p53 and p21. Tubulin was used as loading control.

normal for DNA repair, when treated with caffeine, regardless of the state of confluence. Proliferating NER-deficient XP-A and XP-C cells showed a similar effect of sensitization to UV when treated with caffeine. However, when in confluence, XP-A and especially XP-C cells were more resistant to apoptosis, and did not show any increase in apoptosis levels when treated with caffeine. Increased resistance to apoptosis in confluent cells has been observed in other systems, such as human diploid fibroblasts subject to oxidative stress [30] or primary keratinocytes irradiated with UVB [31,32]. However, in these reports, only normal cells were used. Earlier studies from Kantor [33,34] reported decreased survival rates of quiescent human dermal fibroblasts deficient in NER when irradiated with UV, compared to normal cells. But the authors did not compare quiescent and proliferating cells. Although not working with confluent cells, other authors have previously described the effect of cell cycle arrest on apoptosis induction in DNA repair-deficient cells. Using serum starvation to induce cell cycle arrest, McKay et al. [35] observed that induction of apoptosis in XP-A and XP-B cells depends on S phase entry, that is, DNA synthesis. Dunkern and Kaina [36] showed that induction of apoptosis in DNA repair-deficient Chinese hamster cells, mutated for the helicase XPB and the endonuclease XPF, depends on DNA synthesis and cell cycle progression. Our previous work [18] described decreased susceptibility to UV-induced apoptosis in confluent NER-deficient XP-C and XP-D cells, compared to proliferating cells. However, the decreased susceptibility to UV was not as clear for DNA repair proficient (normal) cells, especially at high UV-doses. In that work, the results indicated that confluent XP-A cells, irradiated at higher UV doses, displayed increased apoptotic levels, when compared to proliferating cells. The results shown here, using two different cell lines, indicate, however, that cells defective for XPA protein, irradiated at lower UV doses, also present a protective response when maintained in a quiescent state by confluency. Strikingly, for XP-C cells, the decreased apoptotic response in confluent cells was, however, also observed even at high UV irradiation doses. This suggests that, at least in the case of primary dermal fibroblasts, reduced UV-induced apoptosis in confluent cells could be due to a certain difference between normal cells and DNA repair-deficient cells in the signaling pathways leading to apoptosis. One intriguing possibility is that the excision repair itself could signal to apoptosis. In fact, DNA damage removal results in a single strand gap, which must be filled in by DNA synthesis, a process that could introduce a fragile DNA region, which could in turn sensitize DNA repair-proficient cells to cell death. This explanation is speculative at this point, but the data with caffeine (increased UV-induced apoptosis observed in confluent cells only when DNA repair is not defective) reinforces the need for effective repair for cell death signaling after UV-irradiation of quiescent cells. Indeed, Matsumoto et al. recently found that histone γH2AX phosphorylation in response to UV irradiation is replication-dependent in proliferating cells, and replication-independent in quiescent growth-arrested cells [37]. γH2AX phosphorylation in quiescent cells showed to be mediated by ATR and to be dependent on NER. The authors propose that an inefficient gap-filling, due to limited amounts of replication factors, would increase the half-life of ssDNA-gap intermediates formed in the last step of NER, resulting in γH2AX phosphorylation in UV-irradiated quiescent cells. These results correlate well with our findings and may explain the differential susceptibility to apoptosis. Besides its role in repair factor recruitment, γH2AX phosphorylation has been shown to be involved in apoptosis signaling [38]. In confluent cells, inefficient NER could reduce γH2AX phosphorylation and result in lower levels of apoptosis. Apoptosis induction in proliferating cells, on the other hand, could rely on other apoptotic pathways, including replication-dependent activation of ATR [39].

The results from transcription recovery experiments showed lower recovery levels for proliferating XP-A cells, deficient in TCR and GGR, when compared to XP-C cells, deficient only in GGR, or normal cells proficient in TCR and GGR, as expected. However, confluent cells showed a delay in transcription inhibition and no recovery 24 h after irradiation. This was surprising, since the persistence of DNA lesions in the transcribed strand of active genes is argued to be one

of the main triggers for apoptosis induction [25]. Thus, the results indicate that, at least in confluent NER-deficient cells, transcription inhibition does not seem to significantly contribute to UV-induced apoptosis signaling. In this situation, the decreased levels of DNA replication in confluent cells might explain why confluent NER-deficient cells are more resistant to apoptosis, in agreement with previous works, which indicated that DNA replication is necessary for apoptosis in UV-irradiated cells [35,36,40]. Similar results using TCR-deficient cell lines confirmed that transcription inhibition is not related to UV-induced apoptosis, at least in confluent cells [41].

We also checked for differences in the activation patterns of the tumor suppressor protein p53 in proliferating versus confluent cells. Surprisingly, no significant differences were observed at the maximum levels of p53 activation between proliferating and confluent NER-deficient cells. The activation of p53 in quiescent cells in response to DNA damage (including by UV irradiation), thus independent of DNA replication, has also been shown recently by Derheimer et al. [42]. The authors correlate this activation to an RNA transcription-based stress response involving RPA, ATR, and p53, as a DNA damage sensor mechanism. However, in our work this mechanism would not explain the differences in cell death activation observed between proliferating and confluent cells.

Confluent cells also activated p53 slightly earlier than proliferating cells. p53 activation was abrogated by caffeine treatment in proliferating and confluent cells, suggesting that ATR/ATM kinases are involved in the phosphorylation of p53, and in the accumulation of this protein after UV-irradiation of cells under both conditions. Inhibition of p53 activation by caffeine in proliferating cells was accompanied by increased levels of apoptosis, suggesting that activation of p53 has a protective role in this process regardless of the NER status. This corroborates previous results from McKay et al. [43], who observed increased levels of UV-induced apoptosis in normal and XP-C cells after p53 inactivation by transfection with HPV-E6. However, in the opposite direction to what we observed, no increase in apoptosis was noted in XP-A cells with inactivated p53. This may be due to differences in the method used to inhibit p53. Transfection with HPV-E6 targets total p53 to E6 binding and consequent degradation by the ubiquitin/proteasome pathway [44], while treatment with caffeine inhibits p53 phosphorylation by ATM/ATR.

Since p53 activation in NER-deficient cells did not show a direct correlation to the induction of apoptosis, we assessed the levels of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$. The kinetics of increase in $p21^{Waf1/Cip1}$ levels paralleled the kinetics for p53 activation levels, suggesting $p21^{Waf1/Cip1}$ was one of the p53 targets in these cells after UV irradiation. Caffeine also abrogated the increase in $p21^{Waf1/Cip1}$ levels, further confirming that its activation is controlled by p53. In proliferating cells, $p21^{Waf1/Cip1}$ activation results in cell cycle arrest in order to allow more time for DNA repair. This is carried out by the binding of $p21^{Waf1/Cip1}$ and inactivation of cyclin-Cdk complexes that mediate G1/S or G2/M phase progression [7]. However, in confluent cells, the cell cycle is already arrested and there is no need for an increase in $p21^{Waf1/Cip1}$ levels to stop the cell cycle. The role for activated $p21^{Waf1/Cip1}$ in confluent cells is still unknown and it could be just fortuitous, as the cells are programmed to do so after DNA is damaged. As the reduction of $p21^{Waf1/Cip1}$ activation by caffeine does not affect the low apoptotic levels in confluent cells, this protein does no seem to be essential for cell protection. This will thus require further studies.

In our observations, the accumulation of p53 does not correlate directly with the levels of UV-induced apoptosis, although p53 activation is probably contributing to induce cell cycle arrest and DNA repair, thus increasing cell survival, especially in proliferating cells. Studies with cells from Li–Fraumeni patients, with mutated p53, demonstrated the direct role of this protein in the GGR of the NER pathway [8], due to transcriptional control of the NER proteins XPC [10] and p48/DDB2 (XPE) [11], and of GADD45 [12]. However, a role in DNA repair would not explain any protective effect of p53 in NER-deficient cells. Moreover, the absence of a caffeine effect in sensitizing confluent NER-deficient cells, despite the suppression of p53 activation, may suggest that p53 is not the only factor determining cell fate, when in confluence. The participation of p53 as a direct mediator of UV-induced apoptosis was also challenged in a recent work with XP-G cells [45]. Although this protein accumulated after UV, the use of siRNA to knockdown its expression showed p53 is not required for cell death.

In conclusion, decreased apoptosis levels in confluent NER-deficient cells suggest that, at least in the case of primary dermal fibroblasts, this could be due to differences between normal cells and DNA repair-deficient cells in the signaling pathways leading to apoptosis. The results shown here reveal that important differences in cell responses to DNA damage may occur depending on the cell cycle status. The relevance of such data is that most of the cells in the organism, including skin fibroblasts and keratinocytes, are found, in fact, in a non-proliferating, quiescent condition, which may present similarities to that found in confluent cells. The fact that the confluence effect on prevention of apoptosis is more pronounced in NER-deficient cells, particularly XP-C cells, is striking and raises the possibility that the resistance to apoptosis may also contribute to the increased cancer susceptibility in XP patients. The recent finding that bladder cancer cells show low expression of XPC protein and are more resistant to apoptosis [46] reinforces the importance of this idea. Therefore, a more complete investigation of apoptotic pathways and/or the protective mechanisms of NER-deficient cells in confluence may help to understand the consequences of DNA damage in XP patients, as well as in the normal population.

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REFERENCES

[1] P.C. Hanawalt, Subpathways of nucleotide excision repair and their regulation, Oncogene 21 (2002) 8949–8956.
[2] R.M. Costa, V. Chigancas, R.S. Galhardo, H. Carvalho, C.F. Menck, The eukaryotic nucleotide excision repair pathway, Biochimie 85 (2003) 1083–1099.
[3] A. Stary, A. Sarasin, The genetics of the hereditary xeroderma pigmentosum syndrome, Biochimie 84 (2002) 49–60.
[4] P.C. Hanawalt, DNA repair: the bases for Cockayne syndrome, Nature 405 (2000) 415–416.
[5] P.H. Itin, A. Sarasin, M.R. Pittelkow, Trichothiodystrophy: update on the sulfur-deficient brittle hair syndromes, J. Am. Acad. Dermatol. 44 (2001) 891–920.
[6] Z.A. Stewart, J.A. Pietenpol, p53 signaling and cell cycle checkpoints, Chem. Res. Toxicol. 14 (2001) 243–263.
[7] G.P. Dotto, $p21^{WAF1/Cip1}$: more than a break to the cell cycle? Biochim. Biophys. Acta 1471 (2000) M43–M56.
[8] J.M. Ford, P.C. Hanawalt, Li-Fraumeni syndrome fibroblasts homozygous for p53 mutations are deficient in global DNA repair but exhibit normal transcription-coupled repair and enhanced UV resistance, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 8876–8880.
[9] M.A. El-Mahdy, F.M. Hamada, M.A. Wani, Q. Zhu, A.A. Wani, p53-degradation by HPV-16 E6 preferentially affects the removal of cyclobutane pyrimidine dimers from non-transcribed strand and sensitizes mammary epithelial cells to UV-irradiation, Mutat. Res. 459 (2000) 135–145.
[10] S. Adimoolam, J.M. Ford, p53 and DNA damage-inducible expression of the xeroderma pigmentosum group C gene, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 12985–12990.
[11] B.J. Hwang, J.M. Ford, P.C. Hanawalt, G. Chu, Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 424–428.
[12] M.B. Kastan, Q. Zhan, W.S. El-Deiry, F. Carrier, T. Jacks, W.V. Walsh, B.S. Plunkett, B. Vogelstein, A.J. Fornace Jr., A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxiatelangiectasia, Cell 71 (1992) 587–597.
[13] T. Dragovich, C.M. Rudin, C.B. Thompson, Signal transduction pathways that regulate cell survival and cell death, Oncogene 17 (1998) 3207–3213.
[14] G.P. Amarante-Mendes, D.R. Green, The regulation of apoptotic cell death, Braz. J. Med. Biol. Res. 32 (1999) 1053–1061.
[15] J. Yu, L. Zhang, The transcriptional targets of p53 in apoptosis control, Biochem. Biophys. Res. Commun. 331 (2005) 851–858.
[16] G. Liu, X. Chen, Regulation of the p53 transcriptional activity, J. Cell Biochem. 97 (2006) 448–458.
[17] Q.B. She, N. Chen, Z. Dong, ERKs and p38 kinase phosphorylate p53 protein at serine 15 in response to UV radiation, J. Biol. Chem. 275 (2000) 20444–20449.
[18] H. Carvalho, R.M.A. Costa, V. Chiganças, R. Weinlich, G. Brumatti, G.P. Amarante-Mendes, C.F. Menck, Effect of cell confluence on ultraviolet light apoptotic responses in DNA repair-deficient cells, Mutat. Res. 544 (2003) 159–166.
[19] J.N. Sarkaria, E.C. Busby, R.S. Tibbetts, P. Roos, Y. Taya, L.M. Karnitz, R.T. Abraham, Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine, Cancer Res. 59 (1999) 4375–4382.
[20] A.M. Bode, Z. Dong, The enigmatic effects of caffeine in cell cycle and cancer, Cancer Lett. 247 (2007) 26–39.
[21] R.S. Tibbetts, K.M. Brumbaugh, J.M. Williams, J.N. Sarkaria, W.A. Cliby, S.Y. Shieh, Y. Taya, C. Prives, R.T. Abraham, A role for ATR in the DNA damage-induced phosphorylation of p53, Genes Dev. 13 (1999) 152–157.
[22] J. Sambrook, D.W. Russel, Molecular Cloning: A Laboratory Manual, third ed., Cold Spring Harbor Laboratory Press, New York, 2001.
[23] M. Yamaizumi, T. Sugano, UV-induced accumulation of p53 is evolked through DNA damage of actively transcribed genes independently of the cell cycle, Oncogene 9 (1994) 2775–2784.
[24] S.D. Rhode, K.A. Ellem, Control of nucleic acid synthesis in human diploid cells undergoing contact inhibition, Exp. Cell Res. 53 (1968) 184–204.
[25] M. Ljungman, F. Zhang, Blockage of RNA polymerase as a possible trigger for UV light-induced apoptosis, Oncogene 13 (1996) 823–831.
[26] B.A. Donahue, S. Yin, J.S. Taylor, D. Reines, P.C. Hanawalt, Transcript cleavage by RNA polymerase II by a cyclobutane pyrimidine dimer in the DNA template, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 8502–8506.
[27] L. Mayne, A. Lehman, Failure of RNA synthesis to recover after UV irradiation: an early defect in cells from individuals with Cockayne's syndrome and xeroderma pigmentosum, Cancer Res. 42 (1982) 1473–1478.
[28] V. Chiganças, L.F. Batista, G. Brumatti, G.P. Amarante-Mendes, A. Yasui, C.F. Menck, Photorepair of RNA polymerase arrest and apoptosis after ultraviolet irradiation in normal and XPB-deficient rodent cells, Cell Death Differ. 9 (2002) 1099–1107.
[29] V. Chiganças, E.N. Miyaji, A.R. Muotri, J.F. Jacsyn, G.P. Amarante-Mendes, A. Yasui, C.F. Menck, Photorepair prevents ultraviolet-induced apoptosis in human cells expressing the marsupial photolyase gene, Cancer Res. 60 (2000) 2458–2463.
[30] J. Naderi, M. Hung, S. Pandey, Oxidative stress-induced apoptosis in dividing fibroblasts involves activation of p38 MAP kinase and over-expression of Bax: Resistance of quiescent cells to oxidative stress, Apoptosis 8 (2003) 91–100.
[31] V. Chaturvedi, J.Z. Qin, M.F. Denning, D. Choubey, M.O. Diaz, B.J. Nickoloff, Apoptosis in proliferating, senescent, and immortalized keratinocytes, J. Biol. Chem. 274 (1999) 23358–23367.
[32] V. Chaturvedi, J.Z. Qin, L. Stennett, D. Choubey, B.J. Nickoloff, Resistance to UV-induced apoptosis in human keratinocytes during accelerated senescence is associated with functional inactivation of p53, J. Cell Physiol. 198 (2004) 100–109.
[33] G.J. Kantor, Effects of UV, sunlight and X-ray radiation on quiescent human cells in culture, Photochem. Photobiol. 44 (1986) 371–378.
[34] G.J. Kantor, C. Warner, D.R. Hull, The effect of ultraviolet light on arrested human diploid cell populations, Photochem. Photobiol. 25 (1977) 483–489.
[35] B.C. McKay, C. Becerril, J. Spronck, M. Ljungman, Ultraviolet light-induced apoptosis is associated with S-phase in primary human fibroblasts, DNA Repair 1 (2002) 811–820.
[36] T.R. Dunkern, B. Kaina, Cell proliferation and DNA breaks are involved in ultraviolet light-induced apoptosis in nucleotide excision repair-deficient chinese hamster cells, Mol. Biol. Cell. 13 (2002) 348–361.
[37] M. Matsumoto, K. Yaginuma, A. Igarashi, M. Imura, M. Hasegawa, K. Iwabuchi, T. Date, T. Mori, K. Ishizaki, K. Yamashita, M. Inobe, T. Matsunaga, Perturbed gap-filling synthesis in nucleotide excision repair causes histone H2AX phosphorylation in human quiescent cells, J. Cell Sci. 120 (2007) 1104–1112.
[38] C. Lu, F. Zhu, Y.Y. Cho, F. Tang, T. Zykova, W.Y. Ma, A.M. Bode, Z. Dong, Cell apoptosis: requirement of H2AX in DNA ladder

formation, but not for the activation of caspase-3, Mol. Cell 23 (2006) 121–132.
[39] D. Shechter, V. Costanzo, J. Gautier, Regulation of DNA replication by ATR: signaling in response to DNA intermediates, DNA Repair 3 (2004) 901–908.
[40] L.F. Batista, V. Chigancas, G. Brumatti, G.P. Amarante-Mendes, C.F. Menck, Involvement of DNA replication in ultraviolet-induced apoptosis of mammalian cells, Apoptosis 11 (2006) 1139–1148.
[41] R.M. Costa, C. Quayle, J.F. Jacysyn, G.P. Amarante-Mendes, A. Sarasin, C.F. Menck, Resistance to ultraviolet-induced apoptosis in DNA repair deficient growth arrested human fibroblasts is not related to recovery from RNA transcription blockage, Mutat. Res. 640 (2008) 1–7.
[42] F.A. Derheimer, H.M. O'Hagan, H.M. Krueger, S. Hanasoge, M.T. Paulsen, M. Ljungman, RPA and ATR link transcriptional stress to p53, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 12778–12783.
[43] B.C. McKay, C. Becerril, M. Ljungman, p53 plays a protective role against UV- and cisplatin-induced apoptosis in transcription-coupled repair proficient fibroblasts, Oncogene 20 (2001) 6805–6808.
[44] M. Scheffner, J.M. Huibregtse, R.D. Vierstra, P.M. Howley, The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53, Cell 75 (1993) 495–505.
[45] V. Clement, I. Dunand-Sauthier, M. Wiznerowicz, S.G. Clarkson, UV-induced apoptosis in XPG-deficient fibroblasts involves activation of CD95 and caspases but not p53, DNA Repair 6 (2007) 602–614.
[46] Z. Chen, J. Yang, G. Wang, B. Song, J. Li, Z. Xu, Attenuated expression of xeroderma pigmentosum group C is associated with critical events in human bladder cancer carcinogenesis and progression, Cancer Res. 67 (2007) 4578–4585.