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Predominant role of DNA polymerase eta and p53-dependent translesion synthesis in the survival of ultraviolet-irradiated human cells

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ABSTRACT

Genome lesions trigger biological responses that help cells manage damaged DNA, improving cell survival. Pol eta is a translesion synthesis (TLS) polymerase that bypasses lesions that block replicative polymerases, avoiding continued stalling of replication forks, which could lead to cell death. p53 also plays an important role in preventing cell death after ultraviolet (UV) light exposure. Intriguingly, we show that p53 does so by favoring translesion DNA synthesis by pol eta. In fact, the p53-dependent induction of pol eta in normal and DNA repair-deficient XP-C human cells after UV exposure has a protective effect on cell survival after challenging UV exposures, which was absent in p53- and *Pol H*-silenced cells. Viability increase was associated with improved elongation of nascent DNA, indicating the protective effect was due to more efficient lesion bypass by pol eta. This protection was observed in cells proficient or deficient in nucleotide excision repair, suggesting that, from a cell survival perspective, proper bypass of DNA damage can be as relevant as removal. These results indicate p53 controls the induction of pol eta in DNA damaged human cells, resulting in improved TLS and enhancing cell tolerance to DNA damage, which parallels SOS responses in bacteria.

INTRODUCTION

Ultraviolet light (UV) damages DNA primarily through the formation of two bulky lesions, cyclobutane pyrimidine dimers (CPDs) and (6-4)-photoproducts (6-4PPs), which distort the DNA structure and cause the stalling of replicative polymerases. Cells have evolved different mechanisms to avoid the deleterious effects of DNA damage, including their removal by nucleotide excision repair (NER) (1). For unrepaired lesions, the replication of damaged DNA uses specialized translesion synthesis (TLS) polymerases that can bypass these lesions (2). In humans, a failure of these mechanisms leads to genetic syndromes, such as xeroderma pigmentosum (XP) and Cockayne syndrome (CS), which possess complex phenotypes of a high frequency of cancer and/or severe neurodegeneration and aging (1,2).

TLS is a conserved mechanism triggered by DNA polymerases, which have active sites capable of accommodating damaged templates. Thus, this mechanism of DNA damage tolerance helps the replisome when it encounters unrepaired lesions (3). However, the same structural adaptations that allow these polymerases to bypass lesions lead to a very low fidelity when copying undamaged DNA templates, which may ultimately increase the risk of mutagenesis (3). The main TLS polymerases belong to the Y-family, such as the eukaryotic pols eta, iota, kappa and REV1, and to the B-family, including pol zeta, which is formed by the subunits REV7 and REV3 (3).

Cells from XP-variant (XP-V) patients were originally described as proficient in lesion removal, but deficient in DNA

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synthesis after UV irradiation (4,5). Consistently, XP-V patients carry mutations in the DNA polymerase eta (pol eta)-encoding gene, *POLH* (6). While pol eta is an error-prone polymerase for some DNA lesions, the bypass of the most frequent UV lesions, TT-CPDs by pol eta is error free (7). In the absence of the accurate replication across DNA damage by pol eta (8) the mutation frequency by other error prone polymerases increases, accelerating the onset of skin cancer in XP-V patients (9). Pol eta is also important for the tolerance of other replication-blocking lesions, including thymine glycol (10), 8-oxoguanine (11) and those induced by some genotoxic chemotherapeutic agents (12). Although pol eta is mainly known for its role on TLS, it also participates on homologous recombination (13) and is involved in the replication of fragile sites (14).

The p53 tumor-suppressor protein is a key transcription factor that modulates many DNA damage responses and has thus been called the 'Guardian of the Genome'. The cell responses that are controlled by this protein affect checkpoint cell cycle arrest, DNA repair, cellular senescence and apoptosis (15,16). In addition to ensuring genomic integrity after genotoxic insults, p53 controls cell proliferation and differentiation. In general, the network of p53 target genes plays important roles in cancer prevention and aging (17). Part of the control of gene expression by this protein depends on the p53 response element (p53-RE), a regulatory region that is observed on the target genes and where p53 binds for transcriptional activation (18).

Genes that encode the NER recognition proteins *XPC* and *DDB2* possess canonical p53 REs (18) and are induced after UV treatment in human cells (19–21). In fact, early experiments with DNA repair-proficient and XP-C fibroblasts demonstrated that low UV doses increased the repair capacity of a transduced UV-damaged reporter gene and that this induced repair was p53 dependent (22). However, despite the utmost importance of p53 and NER, the actual mechanism that links both pathways during the processing of the damaged human genome, leading to increased cellular resistance to genotoxic damage, remains unclear. Similar to the NER genes mentioned above, pol eta possesses a p53-RE in its promoter region (23). Recently, this DNA polymerase was shown to be induced in several human cell lines after treatment with chemotherapeutic agents in a p53-dependent manner, and was linked to acquired resistance to chemotherapy (24,25).

The aim of this work was to explore the relevance of the p53-dependent induction of pol eta, in both normal and NER-deficient backgrounds. In fact, pol eta induction was observed in different cell lines that were treated with different genotoxic agents, including UV exposure. Remarkably, pol eta induction was entirely dependent on p53 as p53 depletion was sufficient to prevent pol eta upregulation. Surprisingly, p53 was also required for pol eta recruitment to the chromatin fraction after genotoxic stress. To assess the biological relevance of changes in pol eta expression, we designed experiments in which UV challenge was preceded by the delivery of a lower UV dose (UVC split-dose assay). This pre-treatment was sufficient to partially protect cells from death in a manner that dependend on an increase in cell proliferation and the attenuation of replication arrest after higher UVC exposure. Such a protective response was dependent on both p53 and pol eta, specifically at the level of a more rapid replication elongation of nascent DNA on damaged templates. Together, these results reveal that in human cells, TLS can be upregulated by p53 in response to the accumulation of UV-damaged DNA. Such p53-pol eta pathway protects both NER-proficient and -deficient cells from cell death suggesting that cell survival after UV can be modulated by the efficiency of DNA damage tolerance pathways.

MATERIALS AND METHODS

A more detailed description of Materials and Methods can be found in Supplemental Information S1.

Cell lines and gene silencing

Human diploid primary XP-C fibroblasts XP189VI carrying the homozygous frameshift mutation c1643_1644delTG (26) were used at passages <12–14. Human primary wild-type skin fibroblasts (NHF) were obtained from a control individual with no XP phenotype. XP-V primary fibroblasts were derived from a skin biopsy of an XP-V Brazilian patient, XP05MG and carried a point mutation at the intronic region of *POLH* (c.1249-1 G>A, unpublished data). Complemented XP-V cells (XP30RO-SV) were a kind gift of Dr Patricia Kannouche. Human melanoma cell lines SK-MEL-28 and SK-MEL-27 were purchased from ATCC. XP-C cell lines that were stably silenced for p53 and DNA polymerase eta were established using lentiviral vectors carrying a short hairpin sequence for the *TP53* and *POLH* genes. pLKO1-puro shRNA (Mission® shRNA, Sigma-Aldrich, Saint Louis, MO, USA), a cell line expressing a non-targeting shRNA, was generated as a control (pLKO.1-puro SHC002 non-target shRNA). Cells were grown in DMEM (LGC-Lab Trade, Cotia, SP, Brazil) that was supplemented with 15% FCS (Cultilab, Campinas, SP, Brazil) and 1% Penicillin/Streptomycin (Invitrogen, Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified 5% CO_2 atmosphere. For shRNA sequences, viral vector production and transduction please refer to SI 1.

Flow cytometry (cell cycle and SubG1 analyses)

Flow cytometry experiments were performed as previously described (27,28). For the cell cycle analysis by the concomitant PI-BrdU staining, a pulse of 10 μM bromodeoxyuridine (BrdU, Sigma-Aldrich) was performed for 20 min after the second UVC irradiation. After 24 h, the cells were harvested, washed with PBS and fixed with 80% ethanol for at least 24 h at −20°C.

DNA fiber assay

For the analysis of the progression of the replication fork after UVC irradiation, we performed DNA fiber experiments as previously described (29). Briefly, 3×10^5 cells were plated in 35-mm dishes and 16 h later received a pulse of 20 μM chlorodeoxyuridine (CldU, Sigma-Aldrich) for 20 min. The cells were washed twice with PBS, UVC-irradiated and then received the iododeoxyuridine pulse (Idu, 200 μM, Sigma-Aldrich) for 1 h.

Western blot and chromatin fractionation

For whole-cell extracts, protein samples were prepared as described in (28). Chromatin fractionations were performed as previously described (30). The antibodies that were used were mouse anti-POLH (B-7 sc-17770, Santa Cruz, Dallas, TX, USA) or rabbit anti-POLH (A301-231A, Bethyl, Montgomery, TX, USA), rabbit anti-p21 (C-19 sc-397, Santa Cruz), mouse anti-p53 (DO-7 M7001, Dako, Glostrup, Denmark), rabbit anti-XPC (H-300 sc-30156, Santa Cruz), rabbit anti-β-tubulin (H-235 sc-9104, Santa Cruz), mouse anti-Lamin B1 (ZL-5 ab20396, Abcam, Cambridge, UK) and mouse anti-GAPDH (6C5 sc-32233, Santa Cruz).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA), and the significance was determined using a one-way ANOVA followed by Tukey's Multiple Comparison Test.

RESULTS

The generation of bulky adducts triggers a p53-dependent accumulation and chromatin recruitment of DNA polymerase eta

To investigate the effects of genotoxic agents on the expression of pol eta, different cell lines were irradiated with UVC or UVB or treated with cisplatin. Protein expression was followed for different periods of time after treatment. We detected a 2- to 3-fold increase in the protein levels of DNA pol eta 24 h after UVC treatment both in NER-proficient wild-type (WT) and in NER-deficient (XP-C) primary fibroblasts (Figure 1A). Given that *POLH* has a p53-responsive element in its promoter region (23), we evaluated whether the upregulation of the pol eta protein levels after UVC irradiation was related to p53. Working in XP-C primary fibroblasts, the maximal induction of pol eta protein levels was detected between 20 and 28 h after treatment, when the levels of p53 also increased. The pol eta levels began to increase at approximately 16 h and lasted for approximately 36 h, when the protein levels began to decrease (Figure 1B). The addition of pifithrin-α to the cell media (inhibiting the transcriptional activity of p53 in the nucleus) impaired pol eta induction (Supplementary Figure S1A), similar to what is observed after silencing p53 expression with p53 shRNA (shp53 XPC cells, Figure 1C). The induction of pol eta 24 h after UVC and UVB irradiation was observed in human WT-p53 melanoma cells (SK-MEL-37, Supplementary Figures S1B and S1C), which demonstrates that this observation is not restricted to fibroblasts. Other genotoxic agents, such as cisplatin, also led to pol eta induction in p53-WT tumor cells (Supplementary Figure S1D). Reinforcing the requirement of p53 transcriptional activity, p53-mutated (L145R) SK-MEL-28 melanoma cells did not show increased pol eta levels after genome damage (Supplementary Figures S1C and S1D). Moreover, the transduction of these cells with retroviral vectors containing the wild-type cDNA sequence of p53 restored their ability to induce pol eta in response to genotoxic stress (Supplementary Figure S1E).

The recruitment of pol eta to the chromatin after genotoxic stress also depends on p53 because pol eta was not detected in the chromatin-enriched fraction of UVC-irradiated XP-C cells that were depleted of p53 (Supplementary Figure S1F), although it accumulated in the soluble cytoplasmic fraction (Supplementary Figure S1G). Pol eta-induced transcription was also observed, with a large increase in the *POLH* mRNA levels in both WT and XP-C cells after equitoxic UVC doses. This induction occurred in a p53-dependent manner because no significant induction was observed in shp53 XP-C cells 24 h after UVC irradiation (Figure 1D).

Cell death and replication stress are attenuated by the pre-irradiation of human primary fibroblasts

The simple increase in the protein levels of pol eta in the cells does not mean that this protein is functional for the cellular response to UV. To determine whether pol eta induction is biologically relevant, UVC split-dose experiments were performed, in which pre-irradiating the cells with low UVC doses led to increased levels of pol eta before challenging them with higher doses. The selected pre-doses were the lowest doses that led to detectable levels of pol eta induction by Western Blot in XP-C cells. The challenging doses were chosen to promote high levels of cell killing when delivered as single dose. We reasoned that such settings would facilitate the detection of protective effects of the split-doses in cell survival assays. As depicted in Figure 2A, NER-deficient XP-C cells received a pre-dose of 6 J/m^2 UVC, 16 h after plating, followed by a challenging dose of 20 or 40 J/m^2 UVC which was delivered 24 h after the pre-dose. As expected, the pre-dose leads to <5% of cell death (Figure 2B). The cell cycle and apoptosis (SubG1) (27,31) were monitored 24 and 72 h after the challenging doses, respectively. The cell viability was also quantified by a proliferation assay (30). The results indicate that UV pre-irradiation had a protective effect on the cells that received the challenging doses, which showed a significant reduction of the SubG1 population and better survival efficiency compared with the cells that received only the challenging doses (Figure 2B and C, and Supplementary Figure S2A). Such protective effects were clearly dependent on p53 because they were not observed in the p53-silenced cells (Figure 2B and C).

To evaluate the effects of pre-irradiation on the progression of the cell cycle, the cells were incubated with BrdU for 30 min immediately after the challenging dose. This incubation allowed us to evaluate the cell cycle progression and the ability to replicate the DNA of cells that were in S phase at the time of UVC irradiation (challenging dose). Samples were collected and fixed 24 h after the BrdU pulse. The samples that received the challenging doses alone were mostly arrested in G1 or had only a small proportion of cells progressing through S phase, as shown by the small amount of BrdU incorporation. In contrast, cells that had previously received a pre-dose were able to progress through the cycle, nearly doubling the proportion of cells in the late S and G2 phases (Figure 2D). The cells that received only a single dose of 46 J/m^2, which represented the sum of the pre-dose and the highest dose, were strongly arrested at G1

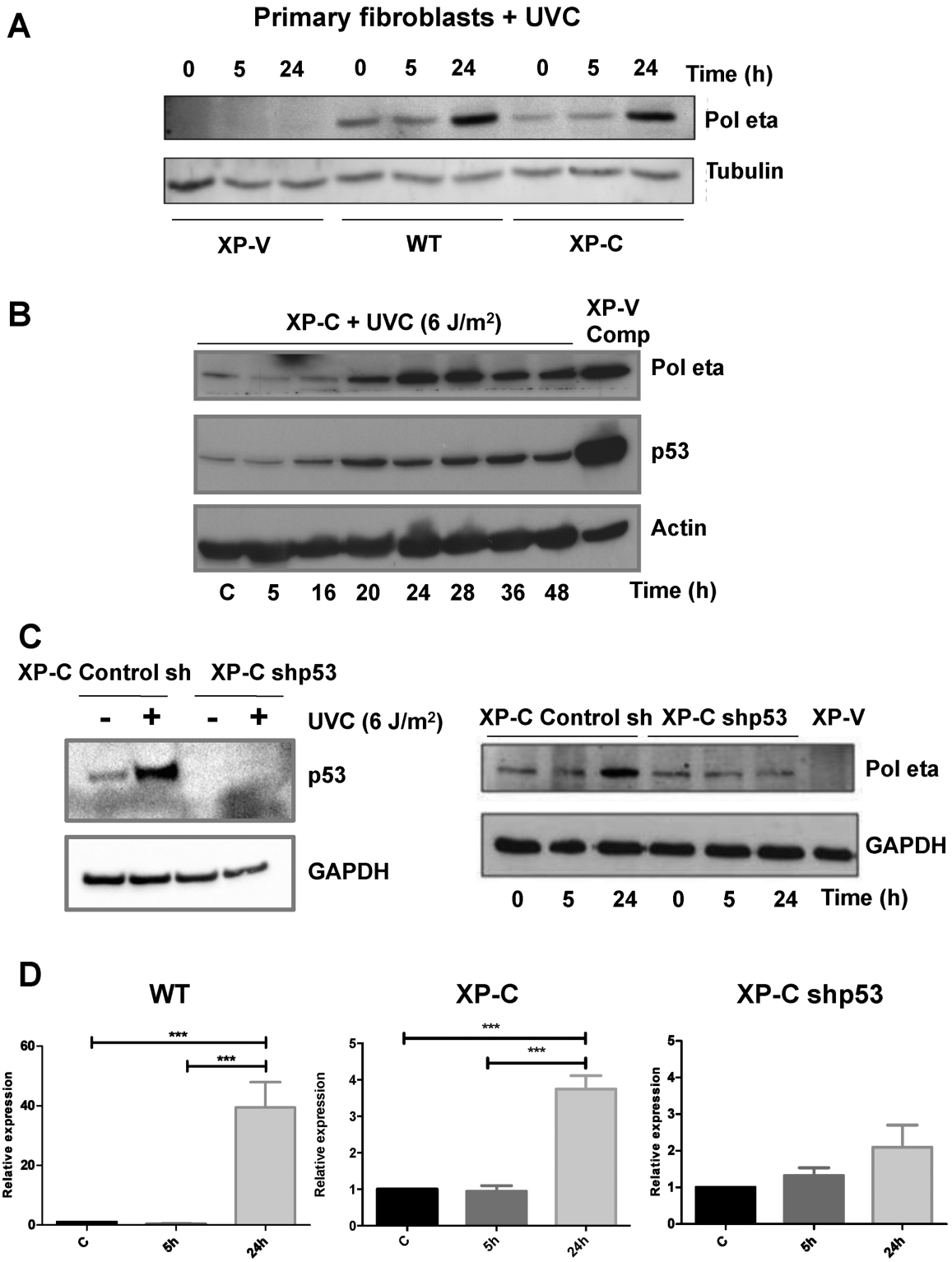

Figure 1. P53-dependent induction of DNA pol eta after the UV irradiation of human fibroblasts. (**A**) Pol eta is induced by UVC in primary fibroblasts. Pol eta was detected by western blot after irradiation with 6 J/m^2 (XP-C) and 40 J/m^2 (WT) UVC; the samples were collected 0, 5 and 24 h after treatment. The pol eta protein was undetectable in the XP-V cell line. (**B**) Kinetics of Pol eta induction. XP-C cells were treated with 6 J/m^2 and harvested at the indicated time points. (**C**) Left panel: Western blot analysis of the total protein content from XP-C cells that were silenced for p53 (shp53) with lentiviral vectors. The cells were treated with 6 J/m^2 and harvested 24 h later. Right panel: pol eta induction is absent in p53-silenced XP-C primary fibroblasts. The cells were treated with 6 J/m^2 and harvested at the indicated time points. (**D**) *POLH* mRNA transcription increased after UVC treatment. The cells were treated with 6 J/m^2 (XP-C and p53 shRNA XP-C) and 40 J/m^2 (WT) and collected at the indicated time points. mRNA transcription was quantified as the relative expression compared with that of the respective non-irradiated controls. The values represent the means (error bars indicate SD) of three independent experiments. *** $P < 0.001$.

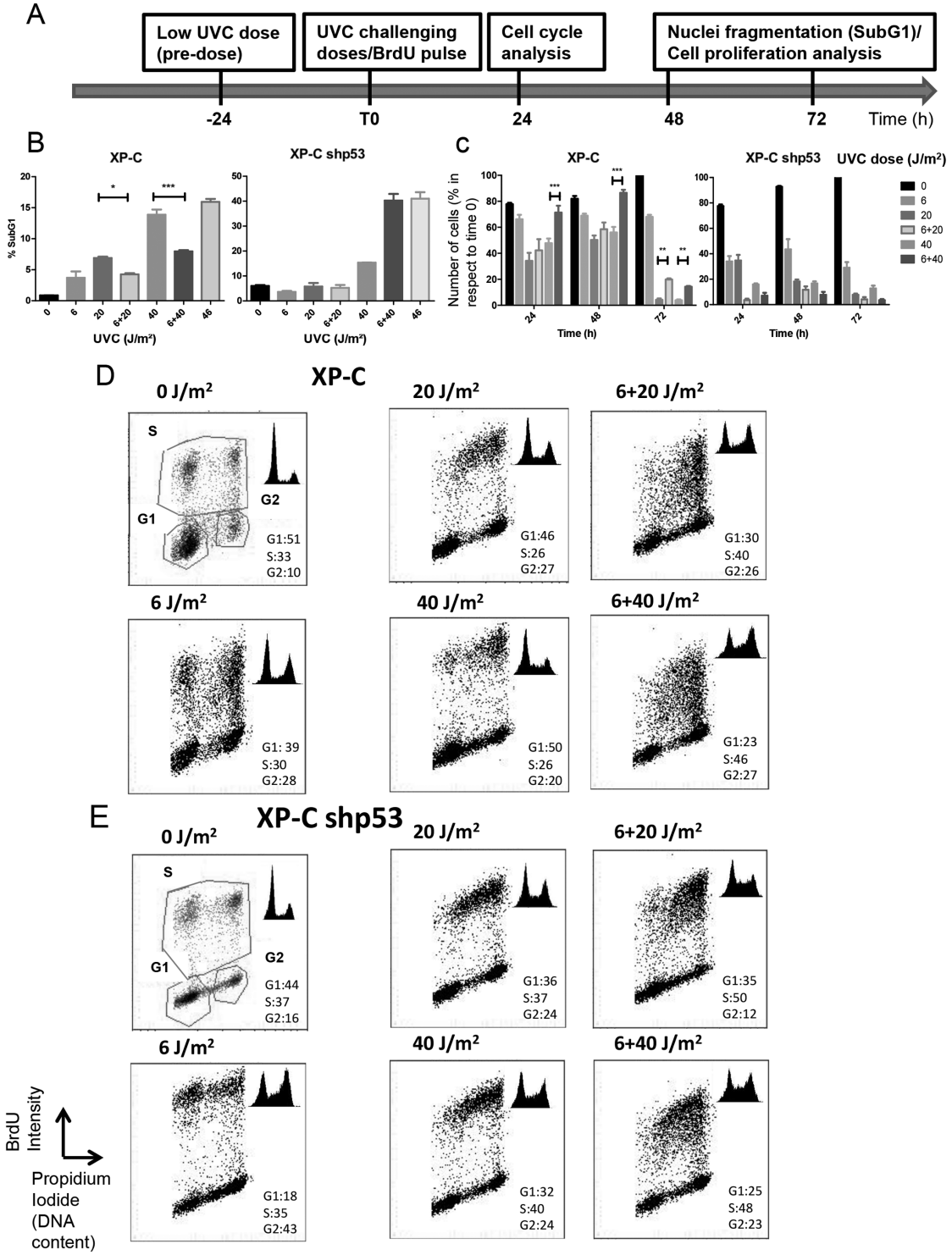

Figure 2. The delivery of UVC split doses increases cell survival and DNA replication in p53-WT cells. (**A**) Scheme of the experimental UV split-dose procedure. (**B**) UVC split doses decrease cell death after challenging UVC irradiation in p53-WT cells. Percentages of the hypodiploid cells (SubG1) fraction were detected by flow cytometry 72 h after the challenging dose. p53 was depleted in XP-C cells by the lentiviral delivery of short-hairpin RNA sequences. The values represent the means (error bars indicate SD) of three independent experiments. $*P < 0.05$ and $***P < 0.001$. (**C**) Cell survival 24, 48 and 72 h after the indicated single- or split-UVC doses (in J/m^2) as described in the Materials and Methods. ** $P < 0.01$ and ***$P < 0.001$. (**D**) DNA replication in cells that were submitted to UVC split doses increased after challenging UVC doses. The cell cycle phase distribution and S-phase cell progression of XP-C cells were evaluated after BrdU incorporation that immediately followed the challenging UV dose and PI staining. Samples were collected and fixed 24 h after the BrdU pulse. The upper left panel shows an example of the quantification of G1, S and G2-phase populations and the corresponding values in %. Representative plots of three independent experiments are shown, with the DNA content on the X-axis and the BrdU incorporation on the Y-axis. The corresponding cell-cycle distribution as determined by PI staining is shown in the top right corner of each graph. (**E**) shp53 XP-C cells did not show increased DNA replication after UVC split doses. The experimental procedure was as described in (**D**).

(similar to the cells that were irradiated to only 40 J/m^2), thus demonstrating that the effects that were observed in the split-dose group are not due to the total UV dose (Supplementary Figure S2B). It is important to note that the cells that were irradiated by a single dose of 6 J/m^2 presented a larger population of cells in S phase at the time of the challenging dose. However, the cells that received 6 J/m^2 and were followed up to 48 h (Supplementary Figure S2B) varied from the split-dose groups at 24 h (Figure 2D), which indicates that these observations are mainly due to the effects of the pre-dose in the ability to replicate more DNA. The split-dose effects on the cell cycle were also dependent on the presence of p53 because much smaller differences were observed with shp53 XP-C cells in the BrdU-incorporating S-phase cells compared with the split- and single-dose groups (Figure 2E). The control shRNA XP-C cell line resulted in a similar increase in the number of cells in the late S and G2 phases after the challenging doses in the cells that were pre-irradiated (Supplementary Figures S3A and S3B). Taken together, these results demonstrate that irradiation with low doses of UV irradiation induces p53-dependent responses that counteract the cell-cycle-arresting signals arising after challenging doses of UV irradiation.

Pre-irradiation minimizes replication fork arrest after challenging UVC doses in p53-WT cells

The increased capacity of pre-irradiated cells to progress through S-phase after a UV-challenge suggests that cells may better elongate DNA at active forks that encounter DNA lesions. To address this question, we performed DNA fiber assays in DNA-repair-proficient WT, XP-C and p53-silenced XP-C fibroblasts. Samples were incubated with CldU for 20 min before the challenging dose, followed by a second IdU pulse of 60 min immediately after the challenge (Figure 3A).

In non-irradiated cells, because the DNA replication speed is unaltered when comparing the first and second tracks, a CldU/IdU ratio of 0.33 was observed as expected (Figure 3A and B). A pre-dose of 6 J/m^2 followed by 20 or 40 J/m^2 delivered 24 h later was used for XP-C cells, while for WT fibroblasts, a pre-dose of 40 J/m^2 followed by challenging doses of 60 and 80 J/m^2 were used. Figure 3B, C and D shows the cumulative frequency distribution of the CldU/IdU ratio (expressed as the percentage of cumulative forks), the distribution of the CldU/IdU ratios and the average CldU/IdU ratios, respectively, for WT, XP-C and XP-C shp53 cells. Figure 3 shows that UV-irradiation caused an increase in the CIdU/IdU ratios, as the IdU track was shorter than in unirradiated controls. Such a shortening of the IdU track was previously reported (28,29) and it is expected to result from the encounter of elongating DNA with DNA lesions. Strikingly, the CldU/IdU ratios were reduced in both control and repair-deficient cells when the samples were pre-irradiated, which indicated an increased ability of these cells to elongate nascent DNA across UV-induced lesions (Figure 3B–D). In agreement with the results of the cell cycle, in p53-silenced cells, no difference in the CldU/IdU ratio was observed when comparing samples that were pre-irradiated or not. Such result indicated that p53 promotes the DNA elongation of nascent DNA across damaged templates in pretreated cells (Figure 3B–D and Supplementary Figure S3C). Importantly, the improved elongation of nascent DNA in pre-irradiated cells was observed in both WT and XP-C cells, thus indicating that at least during this time period (60 min post-UV), the removal of DNA lesions by NER may not be central for such an improvement in DNA replication. We inferred that such results could be linked to the induction of pol eta, which might avoid persistent stalling of the replication forks at DNA lesions.

Pol eta is necessary for the protective effects of split doses in GG-NER-deficient cells

To further investigate the role of pol eta induction in the split-dose effects, we used lentiviral vectors carrying shRNA against *POLH* to generate primary XP-C fibroblasts that were silenced for this polymerase (Figure 4A). As observed in Figure 4B (left panel), the XP-C cells that were silenced for pol eta were not protected by the pre-irradiation. Also, no decrease in the amount of apoptotic cells (revealed by the percentage of cells in SubG1) was observed in these cells when comparing the split- and single-dose groups. Moreover, *POLH*-silenced XP-C cells did not show increased cell survival in the pre-dosed groups, as observed in XP-C cells; in contrast, their proliferation was impaired by both protocols, an effect that was even more pronounced in the split-dose group (Figure 4B, right panel, and Supplementary Figure S2A, right panel). In fact, and in agreement with previously published results, a single dose of 40 J/m^2 was significantly more toxic for XP-C cells lacking pol eta (28). Figure 4C shows that after the split-dose treatment, pol eta-silenced XP-C cells fail to accumulate in G2 and are not protected from cell cycle arrest in S phase as are XP-C cells (Figure 2D). Moreover, similarly to the results obtained with the p53-depleted cells, when analyzing the elongation of nascent DNA, no difference in the cell cycle profile was noticed when comparing single and split-doses protocols in *POLH*-silenced cells (Figure 4D). Hence, pol eta is necessary for the acquired protective effect by the pre-treated cells. These results indicate that, by facilitating nascent DNA elongation across damaged-DNA templates, pol eta overcomes cell cycle and replication fork arrest in pre-treated samples.

We next asked whether the split doses would also have an important effect on XP-V cells, which are deficient in pol eta but proficient in NER. Similar split-dose experiments with a pre-dose of 10 J/m^2 were used for the XP-V cells 24 h before the challenging doses (40 and 60 J/m^2). Figure 4E shows that pre-treatment did not confer significant protection for the cells when assessing the SubG1 population. We conclude that pol eta is necessary for the enhancement of cell survival in pre-irradiated cells, even when GG-NER is fully active.

XP-C cells are proficient in transcription-coupled repair (TCR), meaning that transcription-blocking lesions in transcribed strand of active genes are being removed in such XP background. We therefore asked whether the protective mechanism conferred by pre-irradiation could also be observed in TCR-deficient XP-A cells. Contrary to results obtained in XP-C cells, in XP-A cells the pre-doses tested

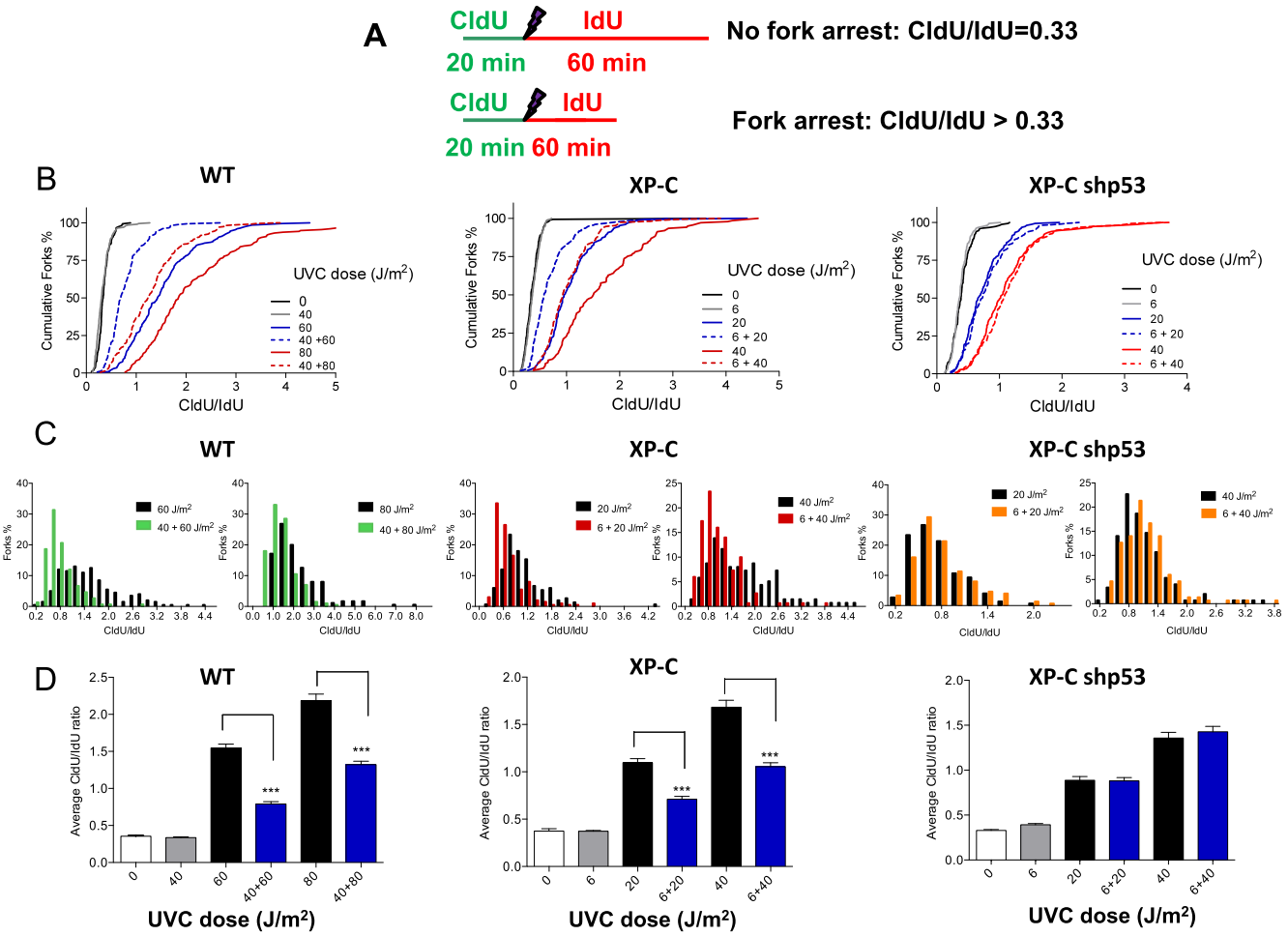

Figure 3. UVC pre-irradiation decreases replication fork arrest after challenging UVC doses in p53-WT cells. (**A**) Scheme of the DNA fiber experiment. The lightening represents the UVC irradiation. (**B**) Cumulative frequency distribution of the CldU/IdU ratio expressed as the percentage of cumulative forks; (**C**) distribution of the CldU/IdU ratios for WT, XP-C and XP-C shp53 cells; and (**D**) average CldU/IdU ratio. The length of the CldU track is the same for all of the cell groups. The values represent the means (error bars indicate SD) of two independent experiments. $***P < 0.001$.

(1 or 3 J/m^2) did not protect the cells from the challenging doses (Supplementary Figure S3D). Moreover, the split-dosed group was even less viable than the one that received a single challenging irradiation (Supplementary Figure S3D). These results showed that the cell death triggering capacity of persistent transcription-blocking lesions cannot be overcome by an improvement in the cell capacity to synthesize DNA across damaged-templates. This result reinforces the existence of a signaling pathway that strictly links the protective effects of pre-irradiation and the effectiveness of the TLS activation in cells. It follows that such an improvement in the TLS capacity of pre-irradiated cells can indirectly compensate a defect in NER, but can be evidenced only when TCR is fully functional.

Transcription levels of other TLS polymerases may also be induced by UV irradiation

To further investigate whether other TLS polymerases were also induced after UVC irradiation, we performed Real-Time PCR experiments to detect changes in the mRNA levels of different TLS polymerases after equitoxic UVC doses (leading to <5% of apoptosis). It is important to note that these experiments do not reflect the protein levels, and therefore do not reflect activity or protein modifications. The mRNA levels of both *XPC* and *DDB2* were evaluated as positive controls because they are bona-fide p53-responsive genes (19,20) that were upregulated under these experimental settings (Supplementary Figure S4). The levels of mRNA of at least one more specialized polymerase, REV1, increased after low doses of UVC irradiation in primary cell lines in a p53-dependent fashion, which indicated that a more general effect of the TLS machinery may also be part of the cellular responses to genotoxic stress, as orchestrated by p53 (Supplementary Figure S4). Two other TLS polymerases of the Y-family, pol kappa (POLK) and pol iota (POLI), were significantly induced in control fibroblasts after UVC irradiation (Supplementary Figure S4). Curiously, however, these two genes were not induced in XP-V and XP-C cells, which indicated that they are modulated under different control mechanisms (which may also involve the regulation of post-translational modifications of the polymerases). In contrast, the mRNA expression of pol theta (POLQ), a polymerase that is important for the firing of

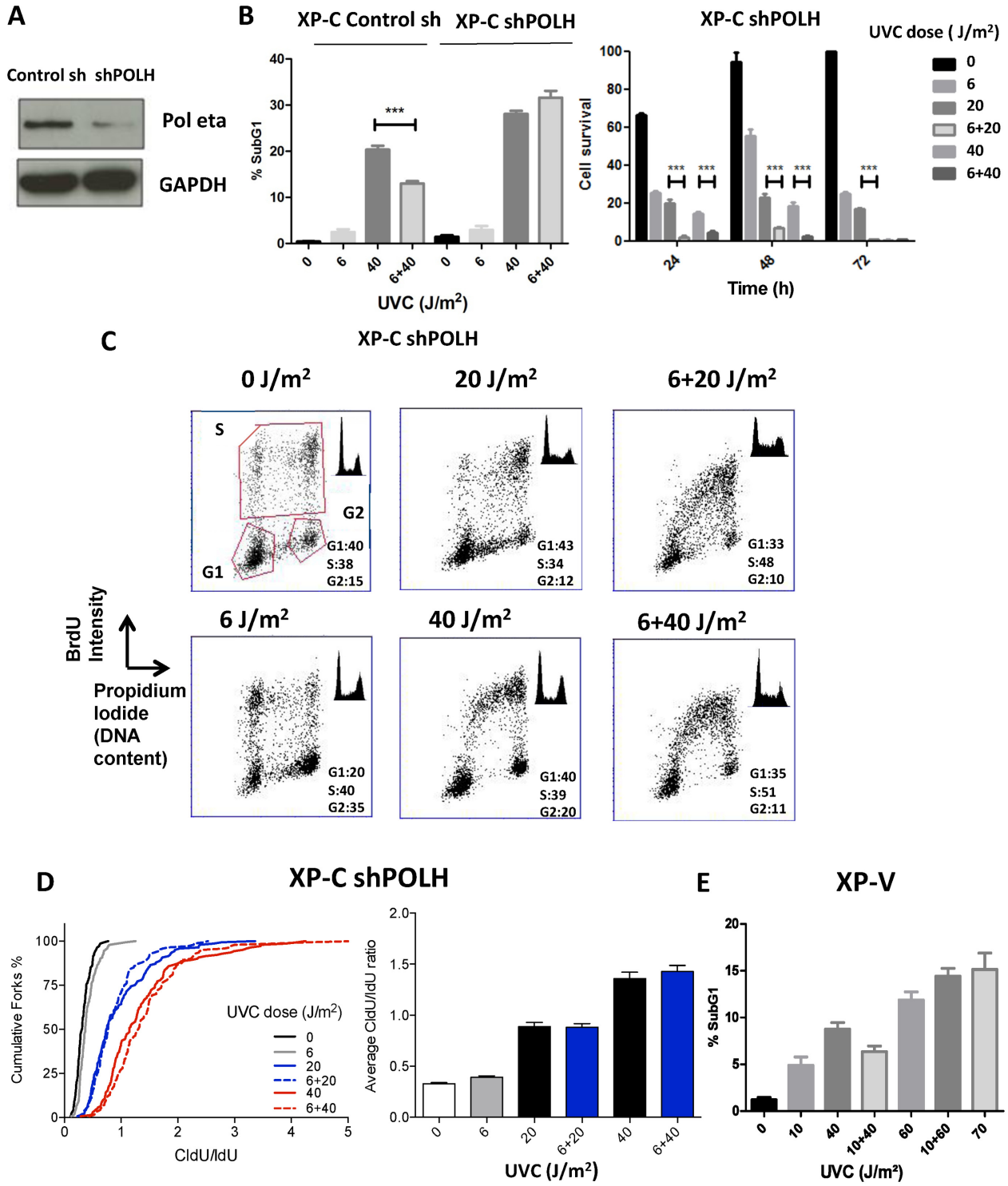

Figure 4. Pol eta is required to improve the cell survival of GG-NER-deficient cells after split-dose treatment. (**A**) Western blot analysis of the total protein content from XP-C cells that were silenced for *POLH* (shPOLH) with lentiviral vectors. (**B**) SubG1 population was detected 72 h after UVC treatment with the indicated doses in XP-C control sh and XP-C shPOLH cells. The values represent the means (error bars indicate SD) of three independent experiments. The cell proliferation of POLH-silenced XP-C cells was determined 24, 48 and 72 h after the indicated single of split-doses of UVC (in J/m^2). ***$P <$ 0.001. (**C**) UVC split-dose effects on the cell cycle and DNA replication depend on pol eta in XP-C cells. The cell cycle phase distribution and S-phase cell progression of XP-C cells were evaluated by PI staining and BrdU incorporation immediately after the challenging UV dose. (**D**) UVC pre-irradiation has no effect in replication fork arrest after challenging UVC doses in *POLH*-depleted XP-C cells. Cumulative frequency distribution of the CldU/IdU ratio expressed as the percentage of cumulative forks (left panel), and average CldU/IdU ratio (right panel) for XP-C shPOLH cells. The values represent the means (error bars indicate SD) of two independent experiments. (**E**) Split-dose protective effects are lost in XP-V cells, particularly at higher UVC doses. The SubG1 population was detected as described above. The values represent the means (error bars indicate SD) of three independent experiments.

new replication origins (32), is not significantly induced by UVC irradiation, although its expression decreases sharply in UV-irradiated p53-silenced XP-C cells (Supplementary Figure S4). While protein levels should be monitored to ultimately conclude on the regulation of the function of these proteins, together, these observations indicate that changes in the levels of TLS proteins might be central to determining the efficiency of cell responses following UV irradiation.

DISCUSSION

In the absence of DNA damage removal, the stalling of blocked replicative polymerases at replication forks may ultimately lead to cell death or genomic instability (33), and cells rely on tolerance mechanisms that allow replication through damage by recombination or translesion synthesis (TLS) (2).

In human cells, we found that pol eta (mRNA and protein) is sharply upregulated after genotoxic stress, in a p53 dependent way, consistent with the p53-RE found in the promoter region of DNA pol eta (23). As summarized in Figure 5, this pol eta upregulation, investigated by split dose experiments, results in higher amounts of cells progressing through the S-phase of cell cycle, compared with the cells that were irradiated with a single UVC dose or in p53-silenced cells. The pre-treated cells were also able to better cope with the stalled replication forks, showing higher DNA replication rates, and lower levels of cell death (Figure 5B). This cell-protection phenomenon was dependent on p53 as it was highly attenuated when p53 was silenced. In agreement with previous reports (34,35), we speculate that the increased DNA damage tolerance capacity in pre-treated samples would lead to less checkpoint activation (due to less ssDNA that could activate ATR), meaning that pol eta is key to maintain the homeostasis of the cell cycle progression (right number of elongation forks, origin firing and checkpoint activation) in the presence of DNA damage.

DNA fiber assays helped to directly evaluate the elongation of nascent DNA across damaged DNA. Previous studies (36) have predicted that pre-treatment would induce a mechanism which facilitates DNA replication across damaged templates, thus allowing the reactivation of arrested replication forks. In fact, DNA fibers were longer in cells that were subjected to a pre-treatment when compared with fibroblasts irradiated with a single challenging dose. Shorter DNA fibers may result from more stalling, more resection of nascent DNA or a less-efficient re-start of the replication fork. Consistently, DNA elongation is slower in XP-V cells after UVC irradiation compared with pol eta-expressing controls (37), indicating that pol eta is limiting for DNA elongation through UV-induced DNA damage. The better ability of replication through damaged DNA templates also depends on the action of p53, as shown by the shorter DNA fibers in p53-silenced XP-C cells both in the split- and single-dose groups. These results show that, even if TLS is very efficient, UV-induced DNA damage still limits DNA elongation growth in human cells, which is improved in cells that have p53-dependent induced levels of pol eta.

Experiments performed with cells that were silenced for the *POLH* gene demonstrated that pol eta is necessary to protect GG-NER-deficient XP-C fibroblasts after split-dose protocols. Moreover, NER-proficient XP-V cells were unable to activate the protective mechanism of a split-dose protocol, meaning that pol eta has a more relevant role in cell survival protection in a split-dose context than does DNA damage removal due to NER induction (XPC and DDB2). In contrast, the overexpression of pol eta alone by adenoviral transduction was not sufficient to support any increase in cell survival or DNA replication in NER-deficient XP-A SV40-transformed fibroblasts (38). Such result may indicate that arrested transcription complexes, generated in TCR-deficient cells, constitute a more problematic burden that can trigger cell death despite the fact that pol eta could be improving DNA replication at DNA lesions which are not encountered by transcription bubbles.

Other TLS polymerases might also be involved in the p53-mediated response to pretreatment in both pol eta-deficient and pol eta-proficient cells. We detected a p53-dependent increase in mRNA levels after UVC pre-irradiation of at least one other Y-family TLS polymerase, REV1. In fact, previous observations confirm that pol eta and REV1 are both necessary for the bypass of CPDs at the replication forks in human cells (39). The UV-induced upregulation of mRNA of two other Y-family TLS polymerases, pols kappa and iota, was also detected in WT cells, supporting the idea that increasing bypass abilities is one of the main mechanisms on which cells rely to address genotoxic damage. Indeed, several TLS polymerases are overexpressed in different human cancers (40) and are involved in acquired resistance to chemotherapy (24,25,40).

The similarities between the inducible TLS system in human cells and the SOS response in bacteria regarding their importance for damage tolerance should also be highlighted. Several similar SOS-like consequences have been described for mammalian cells. For example, the pre-treatment of cells with low doses of genotoxic agents greatly increases the survival of UV-irradiated viral particles (36). It was speculated that the blockage of DNA synthesis by genotoxic treatment would induce a mechanism that facilitates the replication of the damaged templates, thus enhancing virus reactivation (36). Collectively, as a consequence of the activation of inducible responses to DNA damage, both prokaryotic and eukaryotic cells improve their abilities to address the lethal effects of genotoxic agents (41–43).

This work clearly demonstrates that human cells activate late responses to DNA damage that improve DNA replication through damaged templates, which helps cells survive genotoxic stress. Such mechanism of DNA damage tolerance is dependent on both p53 and DNA polymerase eta, and is important even for the survival of cells that have optimal capacities to remove DNA damage (NER-proficient samples). This study also provides mechanistic data, which may explain the similarities between the general clinical symptoms in the GG-NER-deficient XP-C patients and the pol eta-deficient XP-V patients. In fact, this manuscript highlights the importance of the optimal tolerance of unrepaired UV lesions, even in cells that are actively removing these lesions throughout the genome.

Another important conclusion of this study is that the mechanism that promotes cell survival in pre-treated cells is directly linked to the promotion of nascent DNA elongation across damaged-DNA templates. Remarkably, the SOS

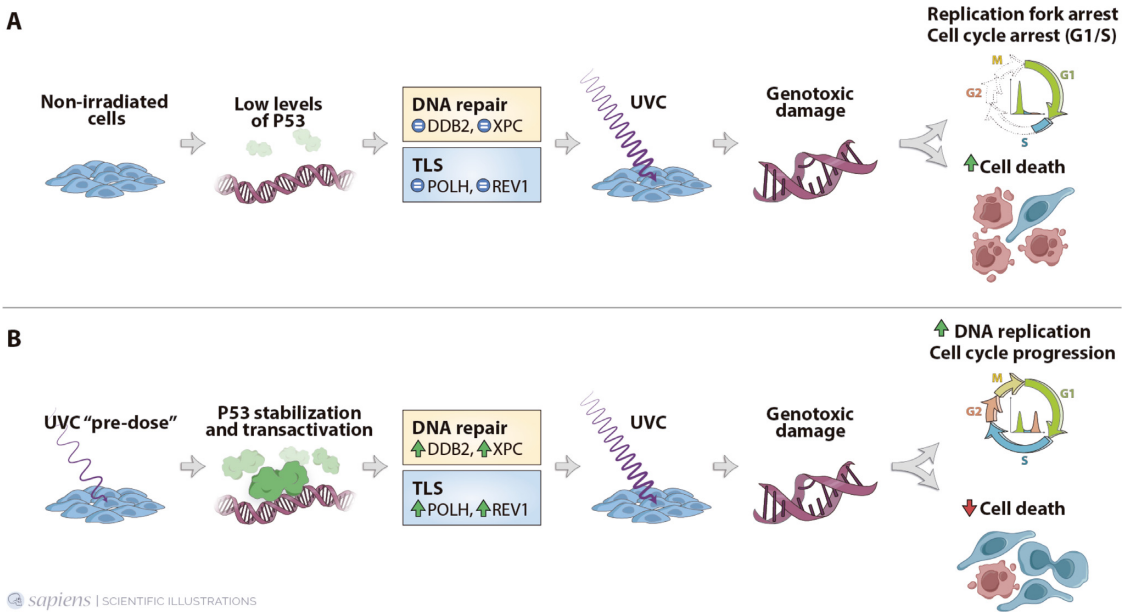

Figure 5. Proposed model for the cellular responses leading to improved DNA damage replication and cell tolerance after UVC split-dose. (**A**) After a high UVC dose, the genotoxic load leads to the arrest of the replication fork and the blockage of cells in G1 and early S-phase of the cell cycle; the persistence of the blocked replication fork ultimately causes cell death. (**B**) In cells that were previously treated with low UVC doses, DNA damage leads to the stabilization and transactivation of p53, the master regulator of the adaptive response, by activated kinases (not shown). p53 stabilization leads to changes in gene expression, including increased expression of repair proteins, such as XPC and DDB2, as well as TLS polymerases, such as pol eta and REV1. When confronted with the next higher UVC dose, cells are able to better address the genotoxic stress. Less replication fork arrest, due to increased ability to progress in the cell cycle, consequently leads to higher DNA replication rates, which has a protective effect on cell survival.

response in bacteria is highly mutagenic due to the induction of error-prone TLS polymerases (44). We were not able to evaluate whether this induction is mutagenic, although the main TLS polymerase that is involved (pol eta) is almost error-free in the context of UV-induced CPDs. Moreover, previous work has indicated that overexpression of this protein does not lead to increased mutagenesis in human cells (45). Moreover, the lack of pol eta (in XP-V cells) results in increased UV-induced mutagenesis, and consequently patients with defects in pol eta are cancer-prone. However, as many other DNA damage responses, an unfortunate aspect of the SOS-like responses in human cells might be the protection of tumor cells which may also rely on this response to acquire resistance to anticancer therapy.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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