Supplemental Information

Supplemental Material and methods

Cell culture and establishment of cell lines

MRC5 (wild-type) and XP30RO (XP-V, deficient for Poln) are SV-40 transformed fibroblasts (previously published in (Quinet et al., 2014) and were cultured as described in the main article. The expression of Rev1 and Rev3L was stably knocked down (KD) from XP4PA cells using lentiviral vectors carrying specific short-hairpin RNA (shRNA) from the Mission shRNA Library (Sigma-Aldrich, Saint Louis, MO, USA) with the following shRNA sequences: for Rev1, CCGGGATGTTGACATGGAGTCAATACTCGAGTATTGACCTCCATGCAACATCTTTTTTGT (NM_016316.2-425s21c1), for Rev3L CCGGCGGAGCCATAATGAATAAATTCTCGAGAATTTATTCATTATGGCTCCGTTTTTG (NM_002912.1-1014s1c1). A non-target shRNA sequence (shC02, Sigma-Aldrich) was used as a control (shCT). Briefly, lentivirus production was achieved by transfecting HEK293FT with Mission Lentiviral Packaging mix (Sigma-Aldrich) and the plasmid carrying the shRNA sequence of interest using linear PEI (Polyethylenimine, Polyscience, Warrington, PA, USA) as a transfection agent. Cell supernatants were collected 72 h after transfection, filtered-sterilized and concentrated by ultracentrifugation. Transduced cells were selected with 0.3 µg/ml of puromycin (Sigma-Aldrich).

For experiment with MRC5 cells depleted for XPC, previously published esiRNA against XPC (HU-03344-1) and esiRNA for GFP (Sigma-Aldrich) as a control (Berra et al., 2013) were used as described in the main article.

Quantitative RT- qPCR

Total RNA was extracted with an Ambion RNA extraction kit (Life Technologies) then treated with DNase (Promega). A High-Capacity cDNA Reverse Transcription Kit (Life Technologies) was used to synthesize cDNA. Quantitative PCR was performed using SYBR Green PCR Master Mix (Life Technologies) by the 7500 Real Time PCR System (Life Technologies). Primers used were: Rev1 forward: CCCAGACATCAGAGCTGTATAAT, Rev1 reverse: CTTCCCTGTGCTCTGTACTT; Rev3L forward: TGCAAAGCGGAGCCATAAT, Rev3L reverse: ATGAAGAGCTGTAGGAGGTAGG; XPV forward: GGTGAGTCCACAGGTCTTT, XPV reverse: GACAGAGGCCCCCTAGCTTTT. GAPDH was used as an endogenous
control (forward: ACCCACTCCTCCACCTTTGA, reverse: CTGTTGCTGTAGCCAAATTCGT). The results were calculated according to the 2^\(-\Delta\Delta C_t\) methodology.

**Cell viability**

Cell viability was assessed using the Cell Proliferation Kit II (XTT, Roche). 2x10^4 cells were plated in a 12-well plate the day prior to UV irradiation, and the cell proliferation assay was performed 72 h after treatment. Following the manufacturer’s instructions, 300 µl of XTT labeling mixture were added to each well and incubated for approximately 1.5 h at 37°C. The absorbance was measured at 492 and 650 nm, and the difference between the two values corresponds to the final result. Cell viability is expressed as a percentage of the corresponding control.

**Cell Cycle and SubG1 analyses**

Both procedures were performed independently. For cell cycle analysis, at the indicated times, cells were incubated with 10 µM bromodeoxyuridine (BrdU, Sigma-Aldrich) for 20 min at 37°C, washed twice with PBS, then fixed with chilled 75% ethanol and stored at -20 °C for at least 2 h. The samples were treated with pepsin (14 µM, 250 U/mg, Sigma-Aldrich) for 20 min at 37°C, then with 2 M HCl for 20 min at room temperature (RT). The cells were then blocked and permeabilized with Bu buffer (0.5% FBS, 0.5% Tween-20, 20 mM Heps in PBS) before incubation with anti-BrdU antibody (mouse, Dako, Glostrup, Denmark) followed by anti-mouse FITC antibody (Sigma-Aldrich). DNA content was stained with propidium iodide (PI) solution (20 µg/ml PI, 200 µg/ml RNase A (Invitrogen, Life Technologies), 0.1% Triton X-100) for 30 min at RT. For experiments with nocodazole, 100 ng/ml nocodazole (Sigma-Aldrich) was added to the medium right after UV exposure for 24 h. For SubG1 analyses, for each sample, the supernatant with detached cells was collected and mixed with attached cells before fixation with 70% ethanol and storage at -20°C. The staining of DNA content was then performed with PI solution as described above.

**Single-stranded DNA detection by modified neutral comet assay**

10^5 cells were seeded in 35-mm dishes the day before UVC irradiation. Twenty-four hours after treatment, the cells were resuspended in 0.5% of low melting point agarose and spread onto microscope slides pre-coated with 1.5% agarose. The cells were lysed overnight at 4°C in chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% dimethyl sulfoxide, pH 10). The slides were then washed three times in S1 nuclease buffer (50 mM NaCl, 30 mM sodium acetate, pH 4.6 and 5%
glycerol) before the addition of S1 nuclease (Invitrogen, Life Technologies) at 20 U/ml in 1X S1 nuclease buffer and 50 mM NaCl (both supplied by the manufacturer) to half the slide for 30 min at 37°C. As a control, the other half of each slide was incubated with the same solution, but without the nuclease. The slides were then washed with chilled neutral electrophoresis solution (300 mM sodium acetate, 100 mM Tris, acetic acid, pH 8.5) before being placed horizontally in an electrophoresis chamber and allowed to equilibrate in this solution for 1 h. Electrophoresis was performed for 1 h at 14 V and 20 mA. Slides were subsequently neutralized with neutralization buffer (0.4 M Tris, pH 7.5), and then fixed with ice-cold ethanol. Comets were stained with ethidium bromide, imaged with a fluorescence microscope (Olympus BX51, Olympus, Center Valley, PA, USA), and at least 50 comets were scored for each condition (with and without S1 nuclease) per slide with Kinetic Imaging Komet 6.0 (Andor ™ Technology, Belfast, UK).

**DNA fiber assay and detection of ssDNA gaps on ongoing forks with S1 nuclease**

The progression of replication forks upon UV exposure was evaluated by a DNA fiber assay, with a 20 min pulse of chlorodeoxyuridine (CldU, Sigma-Aldrich) before UVC irradiation and a 60 min pulse of iododeoxyuridine (IdU) afterwards (Quinet et al., 2014). Briefly, 3x10^5 cells were seeded in 35-mm plates the day before treatment. The cells were incubated with 20 µM chlorodeoxyuridine (CldU, Sigma-Aldrich) in complete culture medium for 20 min at 37°C, washed twice with PBS and exposed to UVC. Immediately after irradiation, the cells were incubated with 200 µM iododeoxyuridine (IdU) in complete culture medium for 60 min at 37°C. For experiments with cells expressing photolyases, upon UV irradiation, the cells were incubated with 200 µM IdU in PBS supplemented with 5% FBS for 60 min at RT on the photoreactivation apparatus. The cells were then trypsinized, and 2,500 cells were lysed for 5 min with lysis solution (0.5% SDS, 200 mM Tris-HCl pH 7.4 and 50 mM EDTA) on a glass slide. For experiments with the ssDNA-specific endonuclease S1, after an IdU pulse, the cells were treated with CSK100 buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM MOPS, 0.5% Triton X-100) for 10 min at RT, then incubated with S1 nuclease buffer (50 mM NaCl, 30 mM sodium acetate pH 4.6, 10 mM zinc acetate and 5% glycerol) with or without S1 nuclease (Invitrogen, Life Technologies) at 20 U/ml for 30 min at 37°C. The cells were then scraped in PBS, centrifuged for 5 min at 7000 rpm and lysed for 10 min with lysis solution (described above). The slides were then tilted to allow DNA to spread along the slide. The samples were then fixed in methanol-acetic acid (3:1) and stored in 70% ethanol at 4°C overnight. DNA was denatured with 2.5 M HCl for 1 h then blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich). IdU was
stained with mouse-anti-BrdU antibody (BD Biosciences) followed by anti-mouse Alexa Fluor 594 secondary antibody (Invitrogen, Life Technologies). CldU incorporation was detected by rat anti-BrdU antibody (Accurate Chemicals) and anti-rat Alexa Fluor 488 antibody (Invitrogen, Life Technologies). The slides were mounted using Fluoroshield (Sigma-Aldrich), and DNA fibers were imaged using a fluorescent microscope (Axiovert 200, Zeiss, Jena, Germany) at a magnification of 1,000X. Analyses were performed using Zeiss LSM Browser Software. All experiments were performed twice independently, and at least 80 fibers were counted for each slide.

**Immunofluorescence** was performed as described elsewhere (Quinet et al., 2014). Briefly, cells were fixed immediately after photoreactivation with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and blocked with 5% BSA 0.05% Tween-20. For detection of DNA damage, DNA was unwind with 2 M HCl at 37°C prior to blocking. Antibodies used were: anti-Cyclin A (Santa Cruz 1/50), anti-BrdU (BD Biosciences 1/50), anti-6-4PP and anti-CPD (CosmoBio 1/200) as primaries antibodies and anti-mouse Alexa Fluor 594 or 488 secondary antibodies (Invitrogen, Life Technologies, 1/1000).

**References**

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Supplemental Data

Figure S1. (A) Averages and s.e.m. of percentages of cells in each phase of the cell cycle from three independent experiments, 24 and 72 h after the indicated UVC doses. Statistical significances between UV-exposed cells and non-irradiated cells were assessed for S and G2 phases by two-way ANOVA followed by Bonferroni test. (B) Cell cycle distribution of MRC5 cells depleted for XPC with siRNA (siXPC) or treated with a control siRNA (siCT) upon the indicated conditions. (C) Representative cell cycle distributions from at least two experiments of XP-C cells depleted with shRNA 24 h after 0 or 5 J/m² with or without nocodazole.
Figure S2. (A) Gene expression in isolated clones (cl.) from XP-C shRev1 and XP-C shRev3L populations (pop) by RT-qPCR expressed as relative expression (means ± s.e.m) of correspondent population (B) Cell viability 72 h after 1, 2 or 3 J/m² UVC, expressed as means (± s.e.m.) of percentages of non-irradiated control of two independent experiments. (C) Cell cycle distribution of XP-C shRev1 pop, XP-C shRev1 cl. 3, XP-C shRev3L pop and XP-C shRev3L cl.7 at 24 and 72 h after 2 J/m² or 24 h after 5 J/m², with or without nocodazole (noco).
**Figure S3.** CldU track (thymidine analog pulse prior to UVC irradiation) length averages in micrometers from 2 independent experiments from both UV-irradiated and non-irradiated cells in XP-C cells depleted for TLS Pols with shRNA (left) or siRNA (right). Statistical significances were assessed by one-way ANOVA followed by Bonferroni test (* \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \)).

**Figure S4. Detection of ssDNA gaps.** Neutral comet assay with or without ssDNA-specific S1 endonuclease 24 h after the indicated UVC doses in the presence of absence of nocodazole in XP-C shRev3L cells (A), XP-C shCT (B) and XP-C shRev1 clone 3 (C). Results from two independent experiments are shown and the significance of differences was assessed by one-way ANOVA followed by Bonferroni test.
**Figure S5.** (A) Representative cell cycle distributions from at least two experiments of TLS Pols-depleted XP-C cells (using shRNA) 24 h after 0 or 20 J/m², with or without nocodazole. (B) Representative immunofluorescence images of XP-C cells depleted for TLS Pols 24 h after 0 or 20 J/m² with nocodazole. BrdU was added to the medium 20 min prior to harvesting to allow staining of S-phase cells and Cyclin A-positive cells are in late S/G2 phase of the cell cycle.
Figure S6. PRR tracts density in XP-C cells exposed to the indicated UVC doses. 20 fibers were analyzed for 0 J/m² (medium: 0.005 Kb-1), 16 fibers for 20 J/m² (0.016 Kb-1) and 25 fibers were scored for 30 J/m² (medium: 0.021 Kb-1). * P < 0.05 between 20 and 30 J/m² assessed by unpaired t-test.
Figure S7. Immunodetection of 6-4PP and CPD in AdCPDphr, Ad6-4phr or mock-transduced cells in photorepair conditions (light) or without photoreactivation (dark). In XP-C shCT cells, the magnification used was 400X (A) and in XP-C shCT, XP-C shPolη, XP-C shRev1 or XP-C shRev3L, the magnification used was of 1,000X for 6-4PP and 400X for CPD (B).
Figure S8. Cell viability 72 h after irradiation with low doses of UVC, with or without caffeine (CAF), in AdCPDphr, Ad6-4phr or mock-transduced cells. Cells were photoreactivated (left panels) or maintained in the dark for photoreactivation control (right panels). All results are expressed as percentage of the correspondent non-irradiated control. Experiments were performed twice independently, and the significance of differences was assessed by two-way ANOVA followed by Bonferroni test (ns, non significant, * $P < 0.005$, ** $P < 0.01$, *** $P < 0.001$).
Figure S9. CldU/IdU ratios from XP-C cells depleted for TLS Pols upon 0 or 50 J/m². At least 150 fibers were scored per experiment and experiments were performed twice independently. Statistical significances were assessed by one-way ANOVA followed by Bonferroni test (ns, non significant; ** P < 0.01, *** P < 0.001).

Figure S10. (A) CldU/IdU ratios from XP-C cells transduced with Ad6-4phr, AdCPDphr or mock-treated exposed to 0 (NI) or 50 J/m² from three independent experiments (≥150 fibers each). (B) CldU/IdU ratios from XP-C shRev3L cells exposed to 0 (-) or 50 J/m² (+) and treated with S1 (+) or not (-) from two independent experiments (≥80 fibers each). (+). Statistical significances were determined by one-way ANOVA followed by Bonferroni test (ns, non significant; * P < 0.05, ** P < 0.01, *** P < 0.001).