Supporting Information

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SI Text

Cell Culture and Transfections. V79 CHO cells were cultured in F-12 Nutrient Mixture (Gibco), supplemented with 10% FCS, penicillin G (100 units/mL) and streptomycin (100 μg/mL). The cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. One day before transfection, 400,000 cells were seeded into six-well plates containing glass coverslips. At a confluence of 70–80%, the cells were transfected with 1 μg XPD-pEGFP (or the mutated construct) using 4 μL FuGENE HD reagent (Roche) and incubated for another 18 h.

Induction of UV Foci. After removal of the culture medium, the cells were rinsed with PBS, covered by a polycarbonate filter (Millipore) with 5-μm pores and irradiated using a UV-C source (254 nm, 150 $J \cdot m^{-2}$). Subsequently, the filter was removed and the cells were returned to complete medium for 30 min at 37 °C before paraformaldehyde fixation.

Immunocytochemistry. Cells were washed with PBS and fixed for 15 min at room temperature using 4% (vol/vol) paraformaldehyde. The cells were then permeabilized twice with PBS containing 0.1% (vol/vol) Tween 20 for 10 min and DNA was denatured with 0.07 M NaOH for 8 min. Next, the samples were washed three times with 0.1% Tween 20 and incubated (30 min, 37 °C) with 20% FCS in PBS to inhibit unspecific binding. The samples were incubated (1 h at 37 °C in 5% FCS) with primary antibodies (MBL International Corp.) directed against cyclobutane pyrimidine dimers (CPDs) (catalog number TDM-2; dilution 1:1,000). The samples were then washed with 0.1% Tween 20, blocked twice for 10 min with 20% FCS, and treated with Alexa Fluor 594 dye-conjugated secondary antibodies (Invitrogen; dilution 1:400) for 30 min at 37 °C. After washing with 0.1% Tween 20 in PBS, the nuclei were stained for 10 min with Hoechst dye 33258 (200 ng/mL). Finally, the samples were washed three times and analyzed using an oil immersion objective.

Image Analysis. Fluorescence measurements were carried out through a 63x oil immersion objective lens with a numerical aperture of 1.4 (EC-Plan-Neo-Fluar, Zeiss) using an Ar^+ source (488 nm). The average fluorescence intensities were measured in the area of accumulation and normalized to the background signal in a neighboring area of identical size. The background-corrected values are finally expressed as the percentage of wild-type xeroderma pigmentosum group D (XPD)-GFP controls. Total protein expression levels were assessed by measuring the overall green fluorescence of nuclei whose foci were subjected to quantitative determination. Only cells containing comparably low amounts of GFP constructs were included in these quantitative comparisons.

Fluorescence Recovery After Photobleaching on Local Damage Analysis. This method was applied to probe the stability by which human XPD interacts with damaged sites in living cells. The dynamics of XPD-GFP fusions bound to UV foci was analyzed at high time resolution using a Leica TCS SP5 confocal microscope equipped with an Ar^+ laser (488 nm) and a 40x oil immersion lens (numerical aperture of 1.4). The assays were performed in a controlled environment at 37 °C and a CO_2 supply of 5%. In CHO cells transfected with GFP constructs, regions of interest (ROI) corresponding to the sites of XPD accumulation were defined 30–45 min after the induction of repair foci by UV-C irradiation (254 nm, 150 $J \cdot m^{-2}$) through polycarbonate filters. These ROI were photobleached at 100% laser intensity until the fluorescence reached a level equivalent to that of the nuclei outside the UV foci. Fluorescence redistributions to each ROI were monitored 10 times using 700-ms intervals followed by 10 frames of 5 s and 6 frames of 20 s. Simultaneously, a reference ROI of the same size was measured for each time point to correct for overall bleaching (1). The values were used to calculate ratios between the damaged area in the foci and the corresponding intensity before bleaching. In the data display, the first fluorescence measurement after photobleaching is set to zero, while all following data points are normalized to the highest plateau level.

DNA Helicase Substrates. Forked substrates were constructed on the basis of 30-mer oligonucleotides (5′-GCCTGCAGTCAGCGTCGACTCGAA**TT**CCCG-3′) or 51-mers (5′-CATGATTACGGCCATATCGAGCGGGAA**TT**CGAGTCGACGCTGACTGCAGGC-3′) containing a single CPD at the position of the underlined thymines. These oligonucleotides (or their undamaged counterparts) were annealed with a partially complementary 125-mer sequence and a flanking oligonucleotide to obtain a duplex of 81 base pairs with overhangs of 44 residues (Fig. S1). Briefly, the 30-mers and 51-mers (100 pmol) were 5′ end-labeled with [γ^{32}P]ATP (6,000 Ci/mmol; Perkin Elmer) and mixed with 200 pmol each of the other two oligonucleotides. Alternatively, 5′ end-labeled partial duplexes were obtained by ^{32}P labeling of the flanking 95-mer or the complementary 125-mer sequence. Hybridizations were performed in a 100-μL volume containing 50 mM Tris · HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. Subsequently, the annealed oligonucleotides were ligated for 18 h at 16 °C in the presence of 5 units T4 DNA ligase (Fermentas). The full-length 125-mer strands were purified on denaturing 8% (wt/vol) polyacrylamide gels, visualized by autoradiography, recovered by electroelution (Bio-Rad) and reannealed to yield partial duplex helicase substrates.

1. Oksenych V, de Jesus BB, Zhovmer A, Egly J-M, Coin F (2009) Molecular insights into the recruitment of TFIIH to sites of DNA damage. *EMBO J* 28:2971–2980.

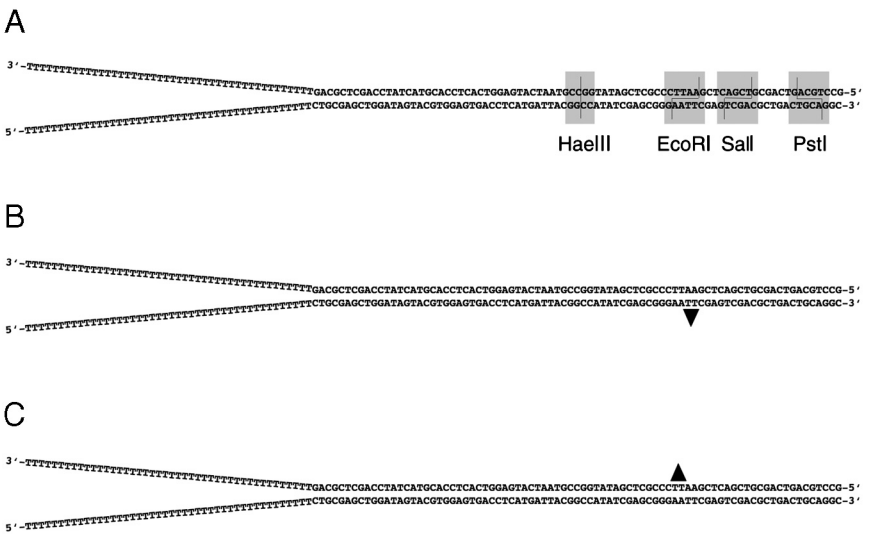

Fig. S1. Forked DNA substrates used for helicase assays. (*A*) Undamaged partial duplex indicating the position of the different restriction sites. (*B*) Forked substrate containing a single CPD in the EcoRI site along the 5′ → 3′ translocated strand. (*C*) Forked substrate with a CPD in the EcoRI site along the 3′ → 5′ displaced strand.

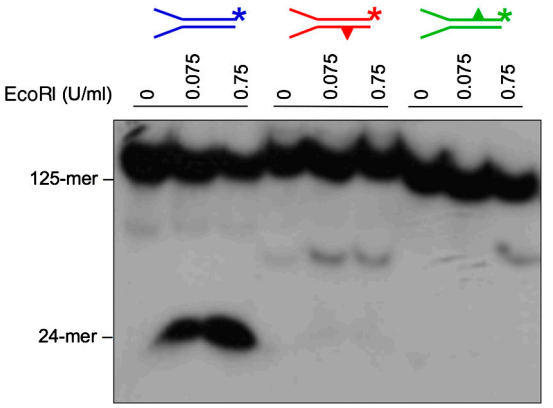

Fig. S2. Inhibition of EcoRI digestion by a CPD located either in the 5′ → 3′ strand or the opposing 3′ → 5′ strand. The specific cleavage of the undamaged forked DNA yields a short radiolabeled fragment of 22 nucleotides in length.

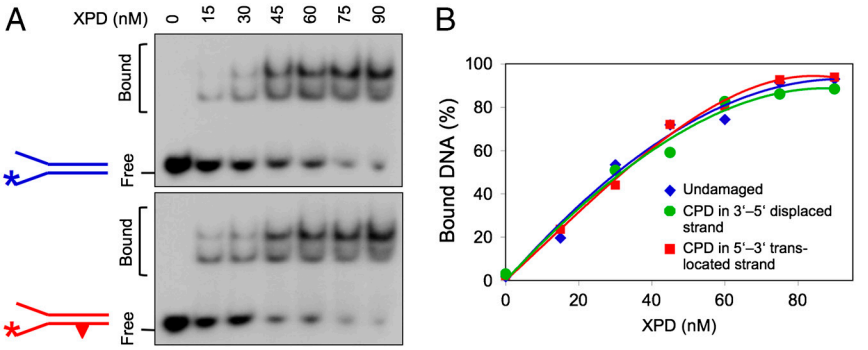

Fig. S3. Binding of XPD protein to forked DNA substrates in the absence of ATP. (*A*) Electrophoretic mobility shift assays showing the equivalent binding of FaXPD (15–90 nM) to undamaged (*Upper*) or damaged (*Lower*) substrates (5 nM). (*B*) Quantification of triplicate binding assays with each DNA helicase substrate.

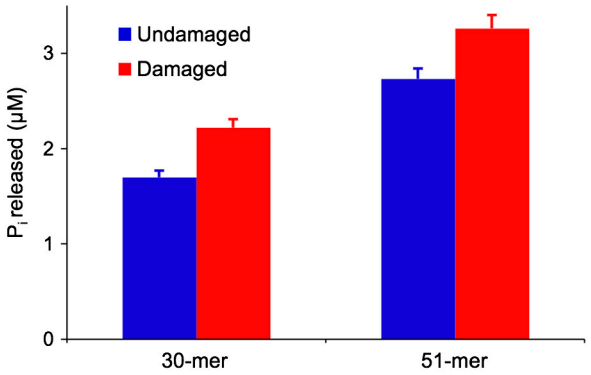

Fig. S4. Stimulation of ATPase activity by a CPD lesion in short oligonucleotides. FaXPD protein (60 nM) was incubated with 30-mer or 51-mer oligonucleotides (5 nM) and 3 mM ATP. The P_i release was determined after incubations of 30 min at 25 °C. The nucleotide sequence and the position of single CPDs in these damaged oligomers are shown in *Materials and Methods*.

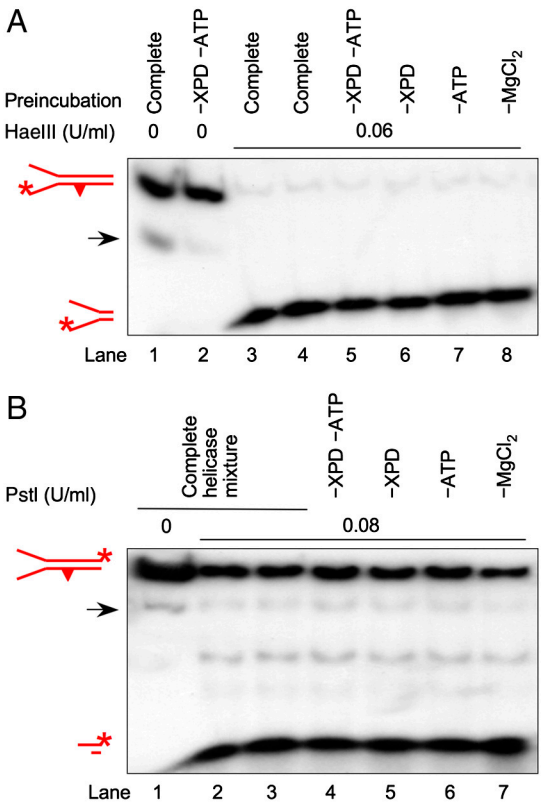

Fig. S5. Protection assays with HaeIII and PstI, whose restriction sites are located in the forked substrates 15–16 nucleotides away from the CPD. (*A*) Probing of helicase reaction products with HaeIII. The FaXPD helicase (60 nM) was preincubated (15 min) with partial duplexes (5 nM) and ATP (3 mM), followed by treatment with HaeIII (0.03 unit/mL). The samples (20 μL) were supplemented with 5 μL loading buffer and analyzed on native 5% (wt/vol) polyacrylamide gels. HaeIII restriction generates a radiolabeled product of 85 residues. Lanes: 1, helicase reaction without subsequent addition of HaeIII; 2, control sample incubated without FaXPD, ATP, and HaeIII; 5–8, control reactions with incomplete helicase mixtures. The arrow indicates the position of the displaced strand. (*B*) Probing of helicase reaction products with PstI. The FaXPD helicase (60 nM) was preincubated (15 min) with partial duplexes (5 nM) and ATP (3 mM), followed by treatment with 0.08 unit/mL of PstI, which generates a radiolabeled product of seven residues. In this case, samples were analyzed on a native 5% (wt/vol) polyacrylamide gel. Lanes: 1, helicase reaction without subsequent addition of PstI; 4–7, control reactions with incomplete helicase mixtures. The arrow indicates the position of the displaced strand.

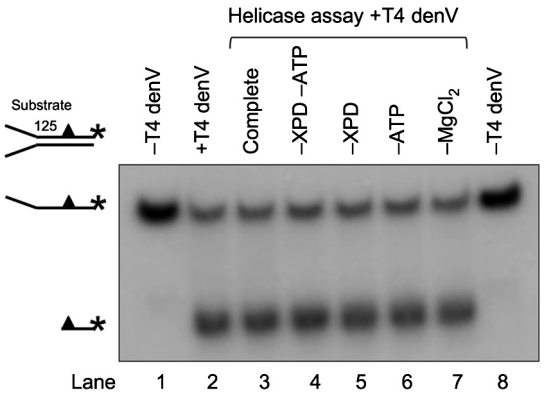

Fig. S6. The XPD helicase fails to protect a CPD located in the 3′–5′ displaced strand of forked duplexes. A glycosylase protection assay was performed after preincubation (15 min) of FaXPD protein (60 nM) with forked substrate (5 nM) containing a CPD in the displaced strand. These helicase reaction products were probed by incubation with T4 denV, as indicated, and resolved on a denaturing polyacrylamide gel. Lanes: 1 and 8, control samples without subsequent addition of T4 denV; 4–7, control reactions with incomplete helicase mixtures.