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Supporting Information for

A disease-associated XPA allele interferes with TFIIH binding and primarily affects transcription-coupled nucleotide excision repair.

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Other supporting materials for this manuscript include the following:

Datasets / spreadsheet S1

Supporting Text: Extended Methods

Cell lines. All cell lines are listed in Table S1. Human U2OS-Flp-In/T-REx cells, RPE1-iCas9 cells and primary fibroblasts were cultured at 37°C in an atmosphere of 5% CO_2 in DMEM, supplemented with antibiotics, 10% fetal calf serum (FCS) and glutaMAX (Gibco).

Plasmids. All plasmids are listed in Table S2. A plasmid expressing DDB2-mCherry was described previously (1). pX458 encoding Cas9 was purchased from Addgene. pOG44 encoding the Flp recombinase was purchased from Thermo Fisher. pU6-sgXPA-PGK-puro-2A-tagBFP was obtained from the Sigma-Aldrich library, available in the LUMC (Table S2). The neomycin resistance gene in pcDNA5/FRT/TO-Neo (Addgene) was replaced with a Puromycin resistance gene. A PCR fragments containing EGFP-C1 (Clontech) including the multiple cloning site was inserted into pcDNA5/FRT/TO-puro. PCR fragments containing EGFP-NLS or EGFP-XPA were inserted into pcDNA5/FRT/TO-Puro. Overlap PCR was used to generate EGFP-XPA with amino acid substitution H244R (EGFP-XPA^{H244R}) or C108F (EGFP-XPA^{C108F}). Overlap primers are provided in Table S3. A region spanning the PGK promoter was amplified by PCR and used to replace the CMV promoter in pEGFP-C1-IRES-PURO (2). The XPA^{WT} and XPA^{H244R} genes were inserted into pPGK-EGFP-C1-IRES-PURO. All sequences were verified by Sanger sequencing.

Generation of U2OS knockout cells. U2OS-Flp-In/T-Rex cells were co-transfected with 3 µg pU6-sgXPA-PGK-puro-2A-tagBFP (Sigma-Aldrich library from the LUMC) and 3 µg pX458 (Addgene) encoding Cas9 in 3.6 ml optiMEM glutaMAX with 10% FCS (Thermo Fisher) and 6 µl lipofectamine 2000 (Thermo Fisher). Starting 24 - 36h after transfection, cells were selected with puromycin (1 µg/ml) for 3 days and seeded at low density without puromycin. Individual clones were isolated and screened for loss of protein-of-interest expression and absence of stable Cas9 expression by western blot analysis and / or sanger sequencing.

Generation of RPE1-hTERT knockout cells. Parental RPE1-hTERT cells stably expressing inducible Cas9 (iCas9) that are also knockout for *TP53* and the puromycin-N-acetyltransferase *PAC1* gene were described previously (referred to as RPE1-iCas9) (3). RPE1-iCas9 cells were transfected with Cas9-2A-EGFP (pX458; Addgene #48138) containing a CSB guide RNA from the TKOv3 library (Addgene #125517) or co-transfected with Cas9-2A-EGFP (pX458; Addgene #48138) together with two pU6-sgXPC-PGK-puro-2A-tagBFP plasmids each encoding different XPC guide RNAs from the LUMC/Sigma-Aldrich sgRNA library using lipofectamine 2000 (Invitrogen). The used sgRNAs are listed in Table S3 and plasmids in Table S2. Cells were FACS sorted on BFP/EGFP and plated at low density after which individual clones were isolated, expanded and verified by western blot analysis and/or Sanger sequencing. To generate double knockout (dKO) cells, the resulting RPE1-iCas9 CSB-KO (clone 15) or XPC-KO (clone 5) cells were co-transfected with 3 µg pU6-sgXPA-PGK-puro-2A-tagBFP (Sigma-Aldrich library from the LUMC) and 3 µg pX458 (Addgene) encoding Cas9 in 3.6 ml optiMEM glutaMAX with 10% FCS (Thermo Fisher) and 6 µl lipofectamine 2000 (Thermo Fisher). Cells were FACS sorted on BFP/EGFP and plated at low density after which individual clones were isolated, expanded and verified by western blot analysis and/or Sanger sequencing.

Stable integration of EGFP-tagged XPA proteins by Flp-In. U2OS-Flp-In/T-Rex cells knockout for XPA were used to stably express inducible EGFP-XPA^{WT}, EGFP-XPA^{H244R}, or EGFP-XPA^{C108F} by co-transfecting the pCDNA5/FRT/TO-Puro plasmid encoding EGFP-tagged fusion proteins (5 µg), together with pOG44 plasmid encoding the Flp recombinase (0.5 µg) in 6ml optiMEM glutaMAX with 10% FCS (Thermo Fisher) and 10 µl lipofectamine 2000 (Thermo Fisher). After selection on 1 µg/mL puromycin, single clones were isolated and expanded. In addition, wild type U2OS Flp-In/T-REx cells were used to stably express EGFP-NLS. Stable U2OS-Flp-In/T-REx clones were incubated with 2 µg/mL doxycycline to induce expression of EGFP-tagged proteins.

Stable integration of EGFP-tagged XPA proteins by random integration. RPE1-iCas9 cells double knockout for either CSB/XPA (clone 14) or XPC/XPA (clone 18) were transfected with either pPGK-EGFP-XPA^{WT}-IRES-PURO or pPGK-EGFP-XPA^{H244R}-IRES-PURO. Cells were selected with 1 µg/mL puromycin to generate a polyclonal stable cell-line. Homogenous expression of EGFP-XPA proteins was verified by western blot analysis and fluorescence microscopy.

Transient expression of DDB2-mCherry. U2OS(FRT) cells stably expressing EGFP-XPA variants were transfected with 0.2 µg plasmid encoding DDB2-mCherry in 1 ml optiMEM glutaMAX with 10% FCS (Thermo Fisher) and 2 µl lipofectamine 2000 (Thermo Fisher) in 12-well plates. At 24 hours after transfection, cells were subjected to UV-C laser irradiation and followed by live-cell imaging as described below.

Immunoprecipitation for Co-IP. Cell pellets of ~50x10^6 cells were lysed in EBC-150 buffer I (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, 2 mM $MgCl_2$ supplemented with protease and phosphatase inhibitor cocktails (Roche)) and 500 U/mL Benzonase® Nuclease HC (Novagen) for 1 hour at 4°C under rotation. The lysates were cleared from insoluble chromatin and were subjected to immunoprecipitation with EGFP Trap beads (Chromotek) for 1.5 hours at 4°C. The beads were then washed 4-6 times with EBC-150 buffer II (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA) and boiled in Laemmli sample buffer. Bound proteins were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. All antibodies are listed in Table S4.

Western blotting. Cell extracts were generated by cell lysis and boiled in Laemmli sample buffer. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4-12% bis-tris Criterion Xt pre-cast gels (Biorad) in NuPAGE™ MOPS SDS running buffer (Thermo Fisher) and transferred to 0.45 µm PVDF membranes (Immobilon). Protein expression was analyzed by immunoblotting with the indicated antibodies in commercial blocking buffer (Rockland) (Table S4), followed by detection using the Odyssey infrared imaging scanning system (LI-COR biosciences, Lincoln, Nebraska USA).

Clonogenic Survival assays. Cells were plated at low density in culture dishes and allowed to attach. When required cells were directly plated in medium containing 2 µg/mL doxycycline to induce EGFP-XPA expression for 24h prior to further treatment and doxycycline was supplemented to the culture medium for the full length of the experiment. Cells were either irradiated with increasing doses of UV-C light, or treated with increasing doses of Illudin S (for 72 hours), or treated with increasing doses of trabectedin for the remainder of the experiment. Cells were allowed to form clones for 7-10 days. To visualize clones, cells were subjected to methylene blue fixation and staining. Cell survival was defined as the percentage of cells able to form clones, relative to the untreated condition.

Unscheduled DNA synthesis. Primary fibroblasts or U2OS cells were used for general UDS. RPE1 cells deficient in TC-NER (CSB-KO cells) were used to specifically measure UDS by GG-NER (GGR-UDS). RPE1 cells deficient in GG-NER (XPC-KO cells) were used to specifically measure UDS by TC-NER (TCR-UDS). Cells were plated in DMEM supplemented with 10% FCS. Subsequently, cells were placed in DMEM supplemented with 0-1% FCS for at least 24 hours prior to UV irradiation to reduce the excess of available deoxy-thymidine in the culture medium. When required, expression of EGFP-tagged XPA proteins was induced by treatment with 2 µg/mL doxycycline in U2OS cells. Cells were locally UV irradiated through 5 µm pore filters (Millipore) with 30 J/m^2 (general UDS or GGR-UDS) or 100 J/m^2 (TCR-UDS) and immediately pulse-labelled with 20 µM 5-ethynyl-deoxy-uridine (EdU; VWR) and 1 µM FuDR (Sigma Aldrich) for 1 hour (general UDS or GGR-UDS) or 4 hours (TCR-UDS). After medium-chase with DMEM containing 10 µM thymidine for 30 min, cells were fixed with 3.7% formaldehyde in PBS for 10-15 min at room temperature and stored in PBS. Next, cells were permeabilized for 20 min in PBS with 0.5% Triton-X100, washed 2 times with PBS + 3% BSA (Thermo Fisher) and incubated in PBS with 1.5% BSA for 10 minutes. The incorporated EdU was visualized by click-it chemistry, labelling the cells for 1 hour with a mix of 60 µM atto azide-Alexa647 (Atto Tec), 4 mM copper sulphate (Sigma) and 10 mM ascorbic acid (Sigma) in a 50 mM Tris-buffer (pH8). After this, the cells were post-fixed with

2% PFA for 10 min and blocked with 100 mM Glyine. Cells were washed extensively with PBS, DNA was denatured with 0.5 M NaOH for 5 minutes, blocked with 10% BSA (Thermo Fisher) in PBS for 15 minutes and equilibrated in 0.5% BSA and 0.05% Tween20 in PBS (WB buffer). Sites of local UV damage were visualized by labelling the cells for 2 hours at room temperature, or overnight at 4 °C, with mouse anti-CPD (Cosmo Bio; 1:1000 in WB buffer). After primary antibody incubation, cells were washed extensively with WB buffer, stained with goat anti-rabbit IgG-Alexa555 (1:1,000 in WB buffer) for 1 hour, again washed extensively with WB buffer, stained with 0.1 µg/mL DAPI, washed extensively with PBS and mounted in Polymount (Brunschwig). Antibody detail are provided in Table S4.

Quantification of ERCC1 and EGFP-XPA levels at local damage. Cells were plated in DMEM supplemented with 10% FCS. When required, expression of EGFP-tagged proteins was induced by treatment with 2 µg/mL doxycycline for at least 24 hours. Subsequently, cells were locally UV irradiated through 5 µm pore filters (Milipore) with 30 J/m^2 (primary fibroblasts) or 100 J/m^2 (U2OS(FRT) cells). At 15-20 min after UV irradiation, cells were fixed with 3.7% formaldehyde in PBS for 10-15 min at room temperature and stored in PBS. Next, cells were permeabilized by incubation with 0.5% TritonX100 in PBS for 5 minutes. Cells were subsequently treated with with 100 mM Glycine in PBS for 10 minutes to block unreacted aldehyde groups, washed extensively with PBS and blocked with 0.5% BSA and 0.05% Tween20 in PBS (WB buffer) for 10-15 minutes. The TFIIH subunit p89/XPB (used as damage marker) and ERCC1 were visualized by labeling the cells 2 hours at room temperature, or overnight at 4 °C, with mouse anti-ERCC1 and rabbit anti-p89/XPB, both 1:100 in WB buffer. After primary antibody incubation, cells were washed extensively with WB buffer, stained with goat anti-rabbit or anti-mouse IgG-Alexa 488, 555 and/or 647 (1:1,000 in WB buffer) for 1 hour, again washed extensively with WB buffer, counterstained with 0.1 µg/mL DAPI, washed extensively with PBS and mounted in Polymount (Brunschwig). Antibody detail are provided in Table S4.

Nascent transcript level measurements. Cells were plated in DMEM supplemented with 10% FCS. Subsequently, cells were placed in DMEM supplemented with 1% FCS for at least 24 hours prior to UV irradiation or Illudin S treatment to reduce the excess of available uridine in the culture medium. When required, expression of EGFP-tagged proteins was induced by treatment with 2 µg/mL doxycycline for at least 24 hours. Cells were UV irradiated (6 J/m^2 for U2OS cell lines or 9 J/m^2 for primary fibroblasts) or exposed to 30 ng/ml Illudin S for 3 hrs, allowed to recover for the indicated time periods, and pulse-labelled with 400 µM 5-ethynyl-uridine (EU; Axxora) for 1 hour. After medium-chase with DMEM without supplements for 15 min, cells were fixed with 3.7% formaldehyde in PBS for 15 min and stored in PBS. Nascent RNA was visualized by click-it chemistry, labeling the cells for 1 hour with a mix of 60 µM atto azide-Alexa594 (Atto Tec), 4 mM copper sulfate (Sigma), 10 mM ascorbic acid (Sigma) and 0.1 µg/mL DAPI in a 50 mM Tris-buffer (pH8). Cells were washed extensively with PBS and mounted in Polymount (Brunschwig).

Microscopic analysis of fixed cells. Images of fixed samples were acquired on a Zeiss AxioImager M2 or D2 widefield fluorescence microscope equipped with 63x PLAN APO (1.4 NA) oil-immersion objectives (Zeiss) and an HXP 120 metal-halide lamp used for excitation. Fluorescent probes were detected using the following filters: DAPI (excitation filter: 350/50 nm, dichroic mirror: 400 nm, emission filter: 460/50 nm), Alexa 555 (excitation filter: 545/25 nm, dichroic mirror: 565 nm, emission filter: 605/70 nm), Alexa 647 (excitation filter: 640/30 nm, dichroic mirror: 660 nm, emission filter: 690/50 nm). Images were recorded using ZEN 2012 (blue edition v1.1.0.0) software and analyzed in Image J (1.48v).

UV-C laser micro-irradiation. Cells were grown on 18-mm Quartz coverslips and placed in a Chamlide CMB magnetic chamber in which growth medium was replaced by CO_2-independent Leibovitz's L15 medium. Laser tracks were made by a diode-pumped solid state 266 nm Yttrium Aluminum Garnet laser (Average power 5 mW, repetition rate up to 10 kHz, pulse length 1 ns) in a UGA-42-Caliburn/2L Spot Illumination system (Rapp OptoElectronic) with laser power set to 2.5% as described previously (4). This was combined with live cell imaging in an environmental chamber set to 37°C on an all-quartz widefield fluorescence Zeiss Axio Observer 7 microscope, using a 100x

(1.2 NA) ultrafluar glycerol-immersion objective. The laser system is coupled to the microscope via a triggerbox and a neutral density (ND-1) filter blocks 90 % of the laser light. A HXP 120 V metal-halide lamp was used for excitation. Images were acquired in Zeiss ZEN and quantified in Image J.

Generation of mass spectrometry samples. All mass spectrometry was performed in quadruplicate. Cells were plated in DMEM supplemented with 10% FCS. Expression of EGFP-tagged proteins was induced by treatment with 2 µg/mL doxycycline for at least 24h. Cells were either kept untreated or exposed to 20 J/m^2 UV-C light and allowed to recover for 1 hour. Individual mass spectrometry samples, were subsequently subjected to co-immunoprecipitation using EGFP Trap beads as described above. After pulldown, the beads were washed 2 times with EBC-150 buffer II and 2 times with 50 mM NH_4HCO_3 followed by overnight digestion using 2.5 µg trypsin at 37°C under constant shaking. All samples were desalted using a Sep-Pak tC18 cartridge by washing with 0.1 % formic acid and concentrated on STAGE-tips as described previously (5). Finally, peptides were eluted with 0.1 % formic acid / 60 % acetonitrile and lyophilized using a SpeedVac RC10.10 (Jouan, France) and dissolved in 10 µl 0.1% formic acid.

Mass spectrometry data acquisition. Mass spectrometry was performed essentially as previously described (6). Samples were analyzed on a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher, Germany) coupled to an EASY-nanoLC 1000 system (Proxeon, Odense, Denmark). Trypsin-digested peptides were separated using a 20 cm fused silica capillary (ID: 75 µm, OD: 375 µm, Polymicro Technologies, California, US) in-house packed with 1.9 µm C18-AQ beads (Reprospher-DE, Pur, Dr. Maisch, Ammerburch-Entringen, Germany). Peptides were separated by liquid chromatography using a gradient from 2% to 30% acetonitrile with 0.1% formic acid at a flow rate of 200 nl/min for 40 min followed by 25 minutes of column re-conditioning. The mass spectrometer was operated in positive-ion mode at 2.8 kV with the capillary heated to 250°C. Data-dependent acquisition (DDA) mode was used to automatically switch between full scan MS and MS/MS scans, employing a top 7 method. Full scan MS spectra were obtained with a resolution of 70,000, a target value of $3x10^6$ and a scan range from 300 to 1,600 m/z. Higher-Collisional Dissociation (HCD) tandem mass spectra (MS/MS) were recorded with a resolution of 35,000, a Acquisition Gain Charge (AGC) target value of $1x10^5$ and a normalized collision energy of 25%. Maximum injection times for MS and MS/MS were 250ms and 120ms, respectively. The minimum AGC target for performing a DDA scan was set to $1x10^4$. For all samples, the precursor ion masses selected for MS/MS analysis were subsequently dynamically excluded from MS/MS analysis for 20 sec. Precursor ions with a charge state of 1 or greater than 6 were excluded from triggering MS/MS events.

Mass spectrometry data analysis. Raw mass spectrometry data were further analysed in MaxQuant v 1.6.7.0 according to (7) using standard settings with the following modifications. Maximum missed cleavages by trypsin was set to 4. Label-free quantification was activated, not enabling Fast LFQ. Searches were performed against an *in silico* digested database from the human proteome including isoforms and canonical proteins (Uniprot, 27th May 2019). Carbamidomethyl (C) was disabled as fixed modification. The match between runs feature was activated and iBAQ quantification was also enabled with Log fit. Analysis was further carried out in the Perseus Computational Platform v1.6.7.0 according to (8). LFQ intensity values were log2 transformed and potential contaminants and proteins identified by site only or reverse peptide were removed. Samples were grouped in experimental categories and proteins not identified in 4 out of 4 replicates in at least one group were also removed. Missing values were imputed using normally distributed values with a 1.8 downshift (log2) and a randomized 0.3 width (log2) considering whole matrix values. Two sample t-tests were performed to compare groups. Analyzed data were exported from Perseus and further processed in Microsoft Excel 2016 for comprehensive visualization.

Expression and purification of XPA^{WT} and XPA^{H244R}. Wild-type or mutant full-length XPA with an N-terminal His_6 tag was expressed in *Escherichia coli* Rosetta pLysS cells. Cells were grown in LB medium containing 50 µg/ml kanamycin to OD_{600} = 0.6 at 37 °C, then OD_{600} = 1.2 at 18 °C,

followed by induction with 1 mM isopropyl β-D-thiogalactoside at 18 °C overnight. Cells were collected by centrifugation at 6,500 rpm for 20 min and all subsequent purification steps were performed at 4 °C. The cell pellet from 1 L of culture was resuspended in 20 mL of lysis buffer (100 mM Tris-HCl at pH 8.0, 500 mM NaCl, 20 mM imidazole, 5 mM 2-mercaptoethanol, 10 μM $ZnCl_2$, 200 μg/ml lysozyme, 0.5 mM PMSF, 1 mM benzamidine, and 10% glycerol, and protease inhibitor (Roche)). The suspended pellets were Dounce homogenized 20 times and sonicated with an amplitude of 30 (5 sec on/10 sec off) for 2 min. The lysate was clarified by centrifugation at 20,000 rpm for 40 min and filtration of the supernatant through a 0.45 mm syringe filter. The supernatant was incubated for 90 min at 4 °C with 2 mL of Ni-resin pre-equilibrated with Ni-loading buffer (100 mM Tris-HCl at pH 8.0, 500 mM NaCl, 10 mM imidazole, 5 mM 2-mercaptoethanol, 10 μM $ZnCl_2$, and 10% glycerol). The resin was washed twice with Ni-wash buffer (100 mM Tris-HCl at pH 8.0, 500 mM NaCl, 30 mM imidazole, 5 mM 2-mercaptoethanol, 10 μM $ZnCl_2$, and 10% glycerol) and eluted with Ni-elution buffer (100 mM Tris-HCl at pH 8.0, 500 mM NaCl, 250 mM imidazole, 5 mM 2-mercaptoethanol, 10 μM $ZnCl_2$, and 10% glycerol). The eluent was applied to a 1 mL Hi-trap heparin column equilibrated with Hep-buffer (20 mM Tris-HCl at pH 8.0, 50 mM NaCl, 5 mM 2-mercaptoethanol, 10 μM $ZnCl_2$, and 10% glycerol) and eluted using a linear gradient with Hep/NaCl-buffer (20 mM Tris-HCl at pH 8.0, 1.5 M NaCl, 5 mM 2-mercaptoethanol, 10 μM $ZnCl_2$, and 10% glycerol) over 5 column volumes. The XPA protein eluted at approximately 800 mM NaCl. The proteins were further purified on a HiLoad 16/600 Superdex 75pg column with buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1 mM dithiothreitol, 10 μM $ZnCl_2$, and 10% glycerol), where they eluted at 50-60 ml. The proteins were obtained at a concentration of ~0.05 mg/mL, yielding a total of 1 mg per liter of cell culture. The XPA H244R protein was analyzed by mass spectrometry after excising band from SDS-PAGE gel by digestion with trypsin. The tryptic digests were separated by online reversed-phase chromatography using a Thermo Scientific Eazy nano LC 1200 UHPLC equipped with an autosampler using a reversed-phase peptide trap Acclaim PepMapTM 100 and a reversed-phase analytical PepMapTM RSLC C18 column and analyzed on an Orbitrap Fusion Lumos mass spectrometer. The MS samples were analyzed using Sequest (XCorr Only, Thermo Fisher Scientific, San Jose, CA, USA; version IseNode in Proteome Discoverer 2.2.0.388).

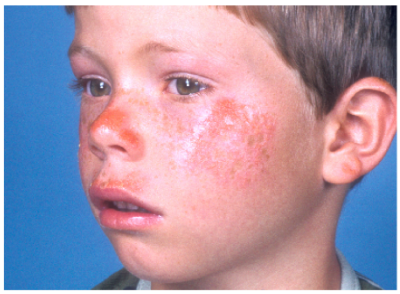
Electrophoretic mobility shift assay (EMSA). EMSA was conducted using three-way junction substrate as described in our previous paper (9). The annealed three-way junction oligonucleotides (100nM) with fluorescein-label were incubated with wild-type or mutant XPA (0, 20, 40, 60, 80 nM) in a 10 μL mixture containing 25 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mg/ml BSA, 5% glycerol, and 1 mM EDTA at 25 °C for 30 min. The reaction mixture was loaded onto 8% native polyacrylamide gel and run at 4 °C for 2 hr at 20 mA with 0.5% TBE buffer. Gels were scanned using an Amersham Typhoon RGB imager.

In vitro NER activity assay with purified proteins. A plasmid containing a site-specific acetylaminofluorene (AAF) lesion was incubated with purified NER protein (5 nM of XPC-RAD23B, 10 nM of TFIIH, 20 nM of XPA, 41.6 nM of RPA, 27 nM of XPG, and 13.3 nM of XPF-ERCC1) was used. All proteins were >95% pure and produced as previously described: XPC-RAD23B (10), TFIIH (11), RPA (12), XPG (13), ERCC1-XPF (14). The reactions were carried out in repair buffer containing 45 mM HEPES-KOH pH 7.8, 5 mM MgCl2, 0.3 mM EDTA, 40 mM phosphocreatine (di-Tris salt, Sigma), 2 mM ATP, 1 mM DTT, 2.5 μg BSA, 0.5 μg creatine phosphokinase (Sigma), and NaCl (to a final concentration of 70 mM) and 50 nM of purified wild-type or mutant XPA in a total volume of 9 μl were pre-warmed at 30 °C for 10 min. 1 μl of plasmid containing AAF (25 ng/μl) was added to reaction mixture and incubated at 30 °C for different incubation times (0, 5, 10, 20, 45, and 90 min). 0.5 μl of 1 μM of a 3′-phophorylated oligonucleotide for product labeling was added and the mixture heated at 95 °C for 5 min. The mixture was allowed to cool down to room temperature for 15 min. 1 μl of a Sequenase/[α-^{32}P-dCTP mix (0.25 units of Sequenase and 2.5 μCi of [α-^{32}P]-dCTP per reaction) was added and the mixture incubated at 37 °C for 3 min. Then 1.2 μl of dNTP mix (100 μM of each dATP, dTTP, dGTP; 50 μM dCTP) added to mixture and incubated for another 12 min. The reactions were stopped by adding 12 μl of loading dye (80% formamide/10 mM EDTA) and heating at 95 °C for 5 min. 6 μl of sample was loaded on 14% sequencing gel (7 M urea, 0.5x TBE) at 45 W for 2,5 hours. The reaction products were visualized using a PhosphorImager (Amersham Typhoon RGB, GE Healthcare Bio-Sciences). Two

independent repetitions were performed. The NER products were quantified using ImageQuant TL and normalized to the amount of NER product formed with XPA^{WT} at 90 min.

Supplementary Figure 1

a



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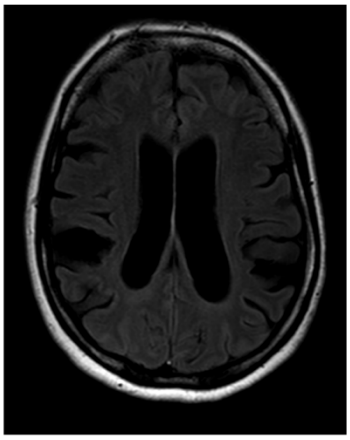

Clinical features of the two Dutch XP-A patients. (**a**) 1979, 8-year-old male patient (XP3LD) showing UV sensitivity. (**b**) 2017, female patient (XP2LD) at age 41 years, MRI brain showing diffuse atrophy with widening of the ventricles and sulci. Both patients gave informed consent for publication of photographs and medical data.

Supplementary Figure 2

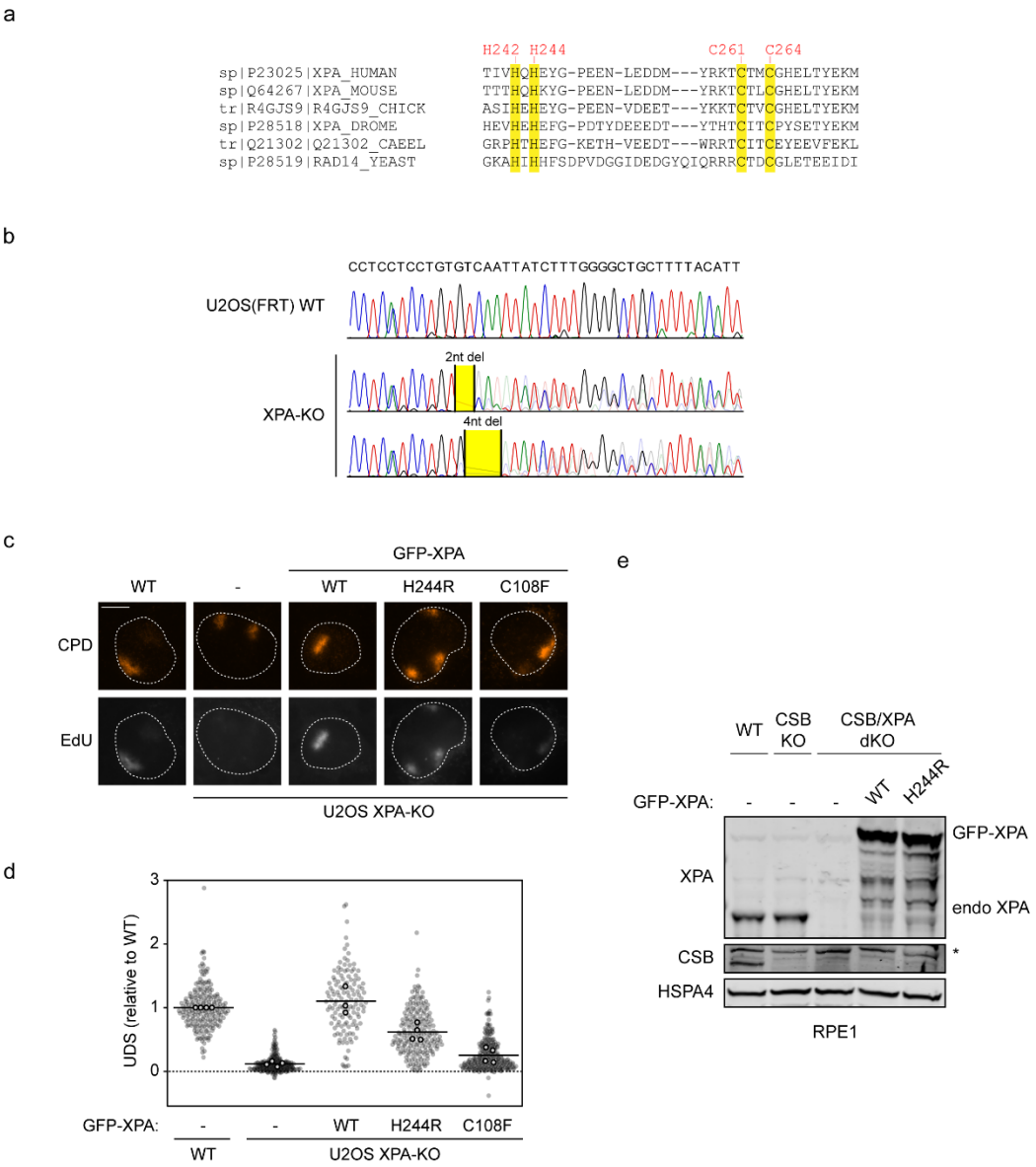

Figure S2. Sequencing of XPA knockout and profile of purified XPA^{H244R} protein. (**a**) Alignment of the C-terminal region of XPA from human (*Homo sapiens*), mice (*Mus Musculus*), chicken (*Gallus gallus*), flies (*Drosophila melanogaster*), worms (*Caenorhabditis elegans*) and yeast (*Saccharomyces cerevisiae*). The highly conserved H242, H244, C261 and C264 residues are indicated. The alignment was generated using the CLUSTAL O (1.2.4) multiple sequence alignment tool. (**b**) Sequence confirmation of the XPA-KO in U2OS(FRT) cells compared to WT cells. XPA-KO cells show a 2 nt and 4 nt deletion at genomic DNA. (**c**) Representative images and (**d**) quantification of EdU incorporation in the indicated U2OS(FRT) cells after 30 J/m^2 UV-C irradiation through 5 µm pore membranes. Sites of local damage are identified by CPD staining. The EdU signal has been normalized for CPD levels at sites of local damage. All cells are depicted as individual data points with the bar representing the mean of all data points. The individual means of 4 biological replicates are depicted as points with black circles. (**e**) Expression levels of CSB and XPA in RPE1 WT, CSB-KO, CSB/XPA-dKO cells without or with constitutive expression of GFP-XPA^{WT} or GFP-XPA^{H244R}. HSPA4 is used as a loading control.

Supplementary Figure 3

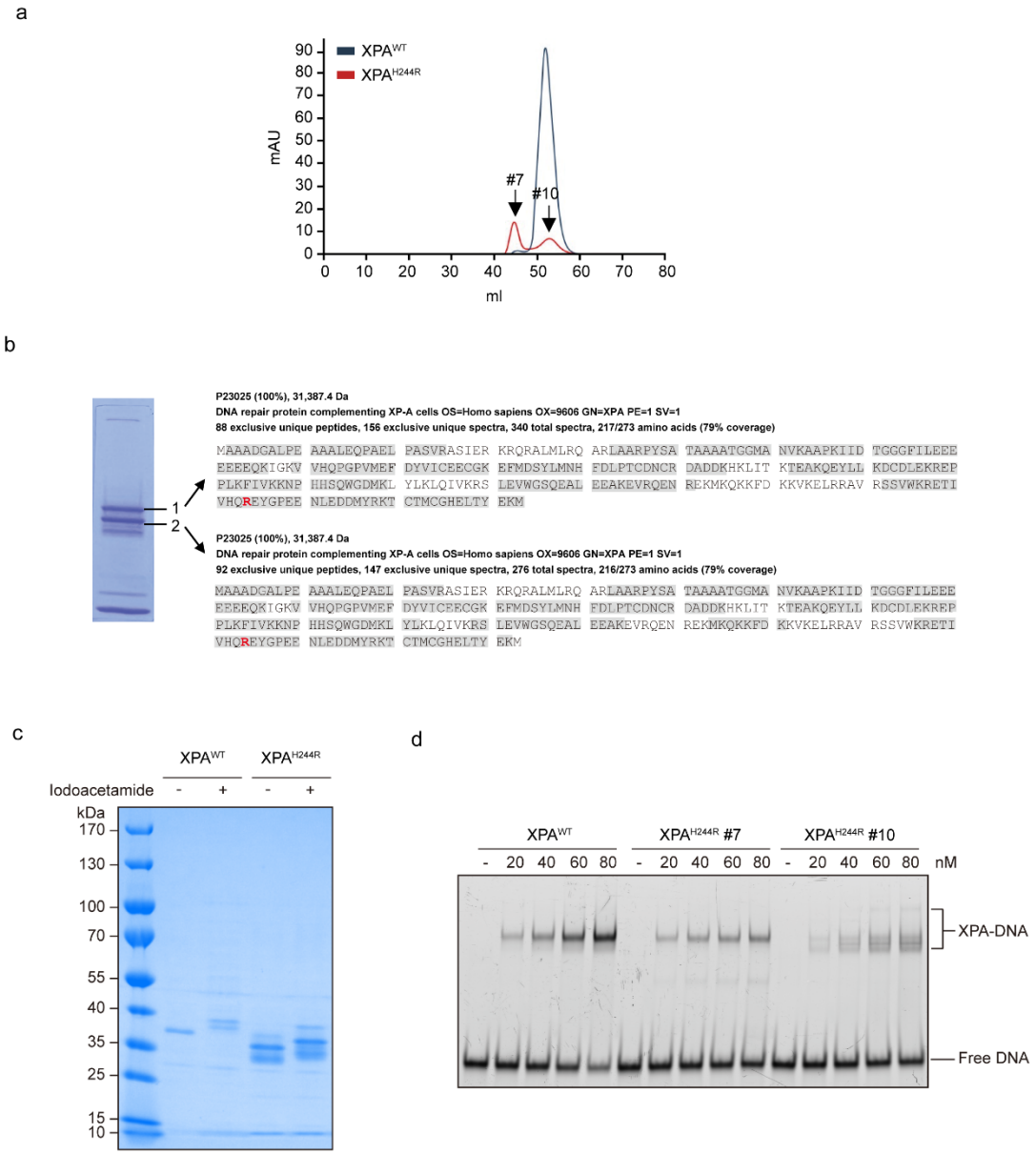

Figure S3. The H244R mutation in XPA reduces in vitro NER activity. (**a**) Profile of gel-filtration purification for XPA^{H244R}. #7 was eluted from the first peak and #10 was eluted from the second peak. Purified XPA^{WT} was eluted from the second peak. (**b**) The two main bands on the SDS gel from the XPA^{H244R} protein was analyzed with mass spectrometry (MS) analysis. The location of band 1 is expected location for XPA^{H244R}, whereas location of band 2 is lower than expected. Both band 1 and 2 were sequenced with MS analysis and both of them were full length XPA^{H244R} protein. (**c**) XPA proteins were incubated for 10 min at 50°C and treated with Iodoacetamide (25 mM) for 10 min at RT in the dark. Dithiothreitol (DTT; 100 mM) was added along with loading buffer and the proteins analyzed on an 8-16% SDS-PAGE gel. All bands were shifted to lower mobility upon Iodoacetamide treatment, but the relative mobility of the bands of the H244R bands were not altered. (**d**) EMSA analysis of XPA^{H244R} proteins with 5'-FAM labeled three-way DNA junction (100 nM). Protein concentration and position of DNA is indicated on the gel.

Supplementary Figure 4

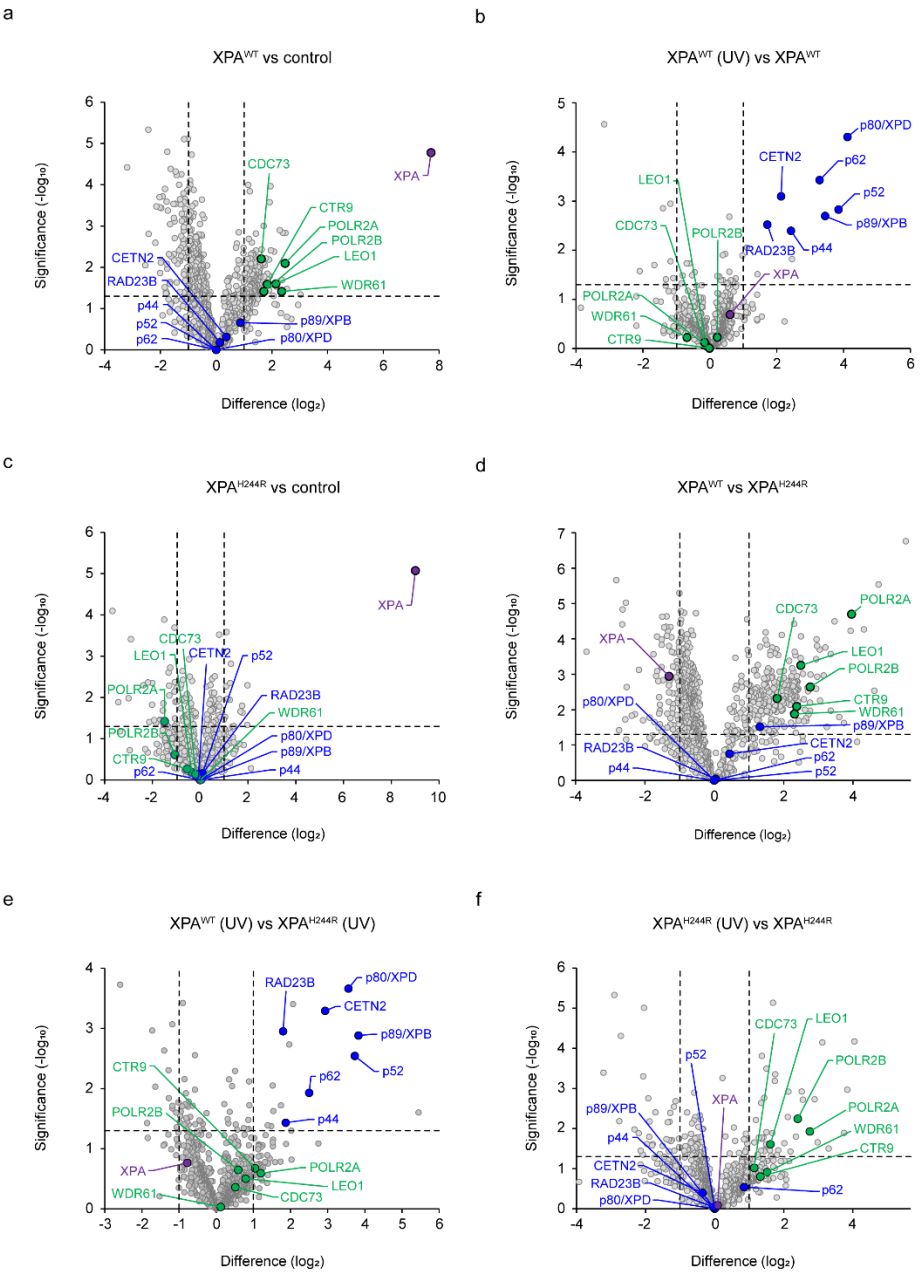

Figure S4. Volcano plots of XPA^{WT} and XPA^{H244R} interactomes. (**a**) Volcano plot depicting the enrichment of proteins after pull-down of EGFP-XPA^{WT} versus EGFP-NLS analyzed by label-free MS. The enrichment (log_2) is plotted on the x-axis and the significance (2-sided t-test -log_{10} p-value) is plotted on the y-axis. Highlighted are XPA (purple), subunits of RNAPII and PAF1C (green) or NER proteins (blue). (**b**) As in a, but for pull-down of EGFP-XPA^{WT} at 1h after irradiation with 20 J/m^2 UV-C compared to unirradiated, mock-treated EGFP-XPA^{WT}. (**c**) As in a, but for pull-down of EGFP-XPA^{H244R} compared to EGFP-NLS. (**d**) As in a, but for pull-down of EGFP-XPA^{WT} compared to EGFP-XPA^{H244R}. (**e**) As in a, but for pull-down of EGFP-XPA^{WT} at 1h after irradiation with 20 J/m^2 UV-C compared to EGFP-XPA^{H244R} at 1h after irradiation with 20 J/m^2. (**f**) As in a, but for pull-down of EGFP-XPA^{H244R} at 1h after irradiation with 20 J/m^2 UV-C compared to unirradiated, mock-treated EGFP-XPA^{H244R}.

Supplementary Figure 5

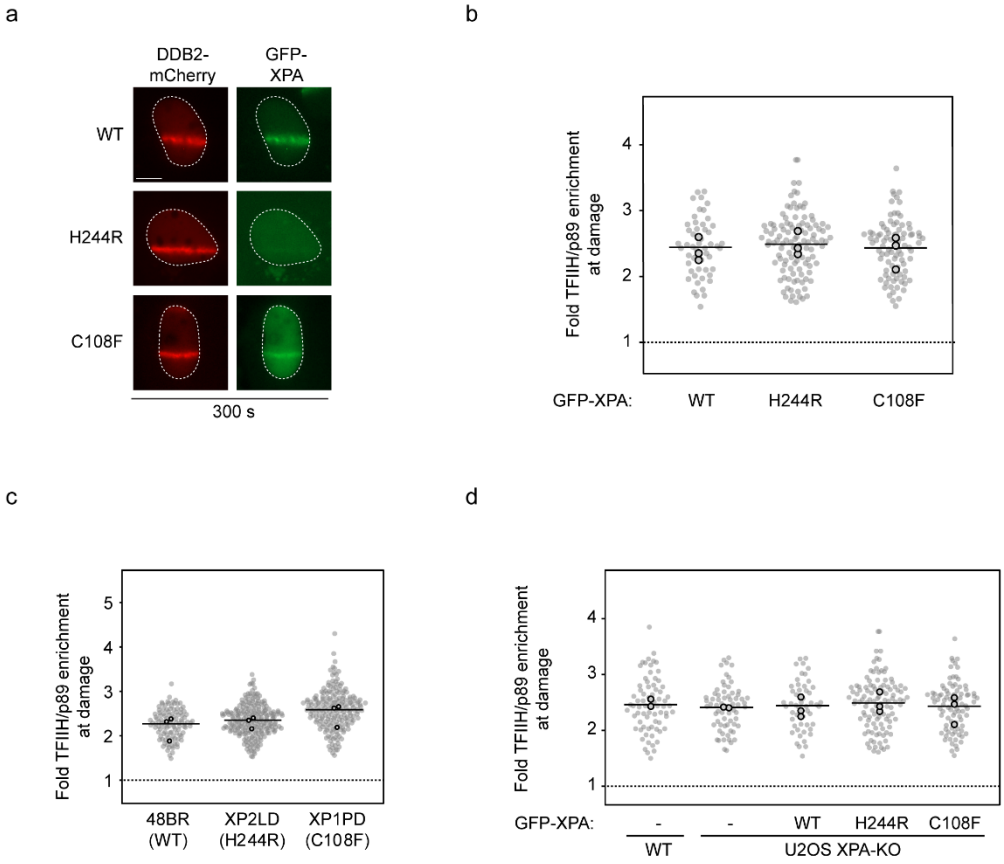

Figure S5. Recruitment control experiments. (**a**) Representative images of the recruitment of indicated EGFP-tagged XPA variants to UV-C laser tracks, after the transient expression of mCherry-DDB2, measured by live cell imaging. mCherry-DDB2 recruitment to laser tracks was used to confirm damage induction, even in the absence of GFP-XPA recruitment. (**b**) Quantification of the recruitment of TFIIH/p89 to sites of local UV-C irradiation, confirming equal damage induction, in the experiment shown in **Figure 4d**. (**c**, **d**) Quantification of the recruitment of TFIIH/p89 to sites of local UV-C irradiation, confirming equal damage induction, in the experiments presented in **Figure 4f, h.** All cells are depicted as individual data points with the bar representing the mean of all data points. The individual means of 3 biological replicates are depicted as colored points with black circles.

Supplementary Figure 6

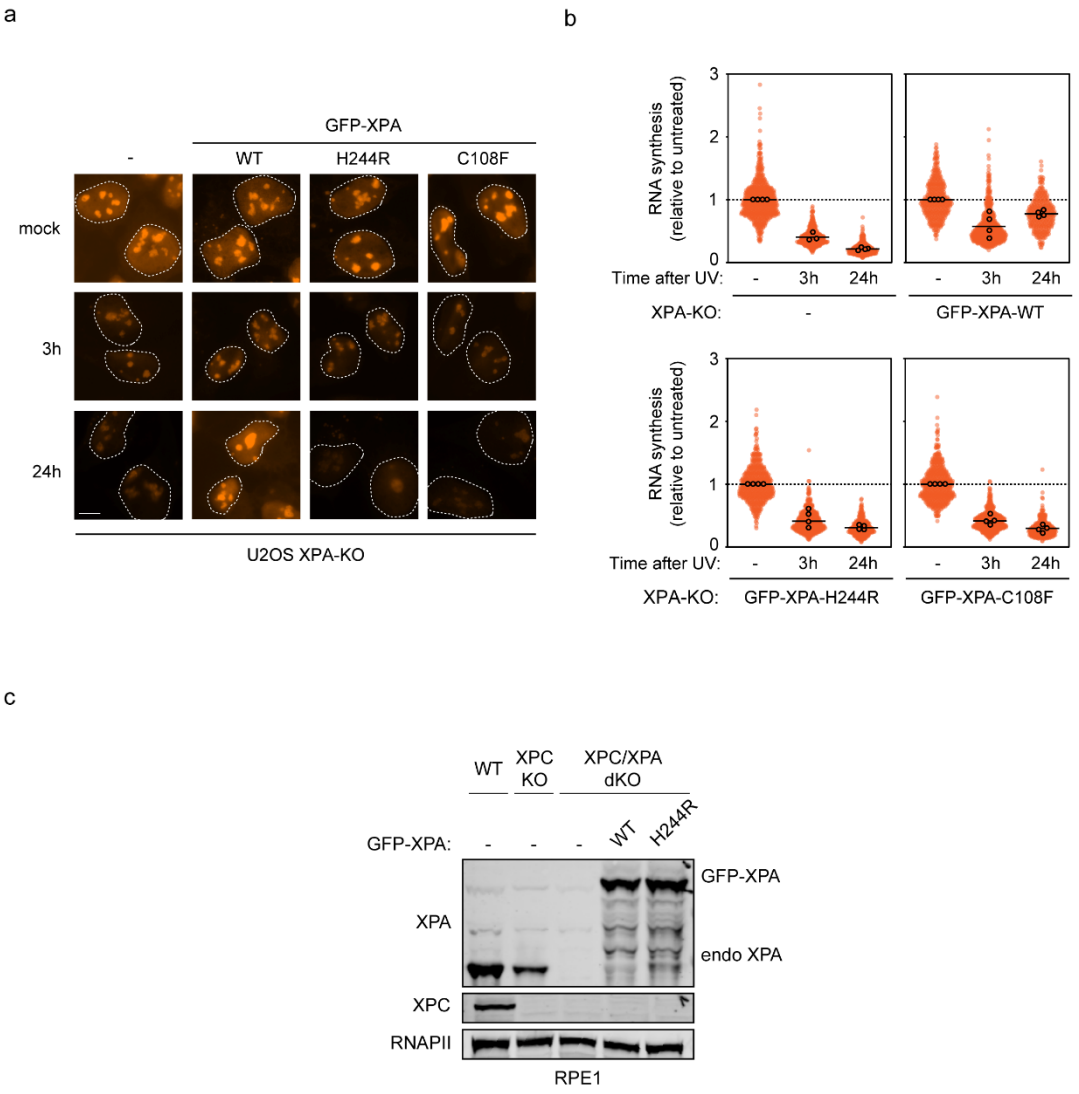

Figure S6. Rescue cells expressing XPA^{H244R} are defective in TC-NER. (**a**) Representative images of U2OS(FRT) XPA-KO cells reconstituted with the indicated GFP-XPA cDNAs and pulse-labeled with 5-ethynyl-uridine (5-EU). Cells were either mock-treated or irradiated with 6 J/m^2 UV-C and 5-EU pulse-labeled at indicated timepoints after UV. (**b**) Quantification of RNA synthesis from (**a**), relative to the mock condition per cell line. All cells are depicted as individual data points with the bar representing the mean of all data points. The individual means of 3-4 biological replicates are depicted as colored points with black circles. (**c**) Quantification of EdU incorporation in the indicated RPE1 cells deficient in GG-NER (XPC-KO cells) to specifically measure UDS by TC-NER after 100 J/m^2 UV-C irradiation through 5 µm pore membranes. Sites of local damage are identified by CPD staining. The EdU signal has been normalized for CPD levels at sites of local damage. All cells are depicted as individual data points with the bar representing the mean of all data points. The individual means of 4 biological replicates are depicted as grey points with black circles.

Table S1. Cell lines

Primary fibroblasts			
Cell lines	XPA variant (nucleotide seq)	XPA variant (amino acid seq)	Origin
48BR/B primary fibroblast with XPA^{WT}	WT	WT	Alan Lehmann (University of Sussex)
XP1PD primary fibroblast with XPA^{C108F}	Allele 1) del C349–T353 Allele 2) G323T	Allele 1) frameshift Allele 2) C108F	Coriell Institute for Medical Research
XP2LD primary fibroblast with XPA^{H244R}	Homozygous A731G	Homozygous H244R	Remco van Doorn (LUMC)
U2OS(FRT)			
Cell lines	Description		Origin
U2OS(FRT)	WT		(15)
U2OS(FRT) XPA-KO (clone 2-8)	Allele 1) 2 nt deletion Allele 2) 4 nt deletion		(15)
U2OS(FRT) EGFP-XPA-WT (clone 8)	XPA-KO+DOX-inducible expression of $EGFP\text{-}XPA^{WT}$		This study
U2OS(FRT) EGFP-XPA-H244R (clone 12)	XPA-KO+DOX-inducible expression of $EGFP\text{-}XPA^{H244R}$		This study
U2OS(FRT) EGFP-XPA-C108F (clone 8)	XPA-KO+DOX-inducible expression of $EGFP\text{-}XPA^{C108F}$		This study
U2OS(FRT) EGFP-NLS	WT cells expressing DOX inducible EGFP tagged to a NLS		(16)
RPE1-hTERT-iCas9-PuroS-TP53-KO (abbreviated to RPE1-iCas9)			
Cell lines	Description		Origin
RPE1-iCas9	RPE1-hTERT cells expressing inducible Cas9 (iCas9) knockout for TP53 and PuroS		(3)
RPE1-iCas9 CSB-KO (clone 15)	CSB knockout in the RPE1-iCas9 background		(3)
RPE1-iCas9 CSB/XPA-dKO (clone 14)	XPA knockout generated in the RPE1-iCas9 CSB-KO background		This study
RPE1-iCas9 CSB/XPA-dKO + $EGFP\text{-}XPA^{WT}$	CSB/XPA double KO stably expressing $EGFP\text{-}XPA^{WT}$		This study
RPE1-iCas9 CSB/XPA-dKO + $EGFP\text{-}XPA^{H244R}$	CSB/XPA double KO stably expressing $EGFP\text{-}XPA^{H244R}$		This study
RPE1-iCas9 XPC-KO (clone 5)	XPC knockout in the RPE1-iCas9 background		(3)
RPE1-iCas9 XPC/XPA-dKO (clone 18)	XPA knockout generated in the RPE1-iCas9 XPC-KO background		This study
RPE1-iCas9 XPC/XPA-dKO + $EGFP\text{-}XPA^{WT}$	XPC/XPA double KO stably expressing $EGFP\text{-}XPA^{WT}$		This study
RPE1-iCas9 XPC/XPA-dKO + $EGFP\text{-}XPA^{H244R}$	XPC/XPA double KO stably expressing $EGFP\text{-}XPA^{H244R}$		This study
RPE1-iCas9 XPC/CSB-KO	CSB knockout generated in the RPE1-iCas9 XPC-KO background		This study

Table S2. Plasmids

Plasmids	Description	Origin
pCDNA5/FRT/TO-Puro-EGFP-C1	Adjusted from pCDNA5/FRT/TO-NEO and combined with EGFP-C1 (from Clontech)	This study
pCDNA5/FRT/TO-Puro-EGFP-NLS	Based on pCDNA5/FRT/TO-Puro-EGFP-C1; Replaced EGFP-C1 with EGFP-NLS	(16)
pCDNA5/FRT/TO-Puro-EGFP-XPA^{C108F}	EGFP-XPA^{C108F} coding sequence	This study
pCDNA5/FRT/TO-Puro-EGFP-XPA^{H244R}	EGFP-XPA^{H244R} coding sequence	This study
pCDNA5/FRT/TO-Puro-EGFP-XPA^{WT}	EGFP-XPA^{WT} coding sequence	This study
pDDB2-mCherry	Encodes DDB2-mCherry (in a Clontech C1 backbone)	(1)
pEGFP-C1-IRES-PURO	Adjusted from pEGFP-C1 to insert IRES-Puro	(2)
pOG44	Encodes the Flp recombinase	Thermo Fisher
pPGK-EGFP-C1-IRES-PURO	Replaced the CMV promoter for a PGK promoter	(3)
pPGK-EGFP-XPA^{H244R}-IRES-PURO	EGFP-XPA^{H244R} coding sequence	This study
pPGK-EGFP-XPA^{WT}-IRES-PURO	EGFP-XPA^{WT} coding sequence	This study
pU6-sgXPA-PGK-puro-2A-tagBFP	Targets XPA exon 2: 5-GCCCCAAAGATAATTGACACAGG-3	Sigma-Aldrich library Available at LUMC
pU6-sgXPC-1-PGK-puro-2A-tagBFP	Targets XPC exon 5: 5-CCGAAGATATGTCTCAAACTCCA-3	Sigma-Aldrich library Available at LUMC
pU6-sgXPC-2-PGK-puro-2A-tagBFP	Targets XPC exon 2 : 5-TGGGGGTTTCTCATCTTCAAAGG-3	Sigma-Aldrich library Available at LUMC
pX458	Encodes Cas9	Addgene

Table S3. Primers

Primers to generate mutated XPA constructs		
XPA-C108F-Fw	oML#118	5-GTAATATGCGAAGAATTTGGGAAAGAATTTATGG-3
XPA-C108F-Rv	oML#119	5-CCATAAATTCTTTCCCAAATTCTTCGCATATTAC-3
XPA-H244R-Fw	oML#122	5-CGATTGTTCATCAACGTGAGTATGGACCAG-3
XPA-H244R-Rv	oML#123	5-CTGGTCCATACTCACGTTGATGAACAATCG-3
Primers to check EGFP-XPA expressing U2OS(FRT) cells		
XPA_nt192-216_Fw	oML#443	5-CCCAAAGATAATTGACACAGGAGGA-3
XPA_nt766-792_Rv	oML#442	5-GCCACACATAGTACAAGTCTTACGGT-3
Primers to check primary cell lines		
Primary XPA_C108F_Fw	oML#476	5-CTTGATTTTAGCCTGCTGAGACCCATG-3
Primary XPA_C108F_Rv	oML#477	5-TGGCAGAACCATCGGCATCCTTC-3
Primary XPA_H244R_Fw	oML#480	5-TGTGTCCTATTGTGTTACTCAACTCACTTCTG-3
Primary XPA_H244R_Rv	oML#481	5-CTACCACTTCTGCACCTACTCTAGCAC-3
Primers to check XPA-KO		
XPA-sgRNA2_F2	oML#218	5-GTTAGACTAGCTGGGACCTTCA-3
XPA-sgRNA2_R2	oML#219	5-ACCCCTAAGTACATCAGCCACA-3
sgRNA sequences		
XPA		5-GCCCCAAAGATAATTGACACAGG-3
CSB		5-AGACAGAATGATCCGATGAG-3
XPC		5-CCGAAGATATGTCTCAAACTCCA-3 and 5-TGGGGGTTTCTCATCTTCAAAGG-3

Table S4. Antibodies

Antibodies	Host	Manufactorer (Cat.nr; clone)	WB	IF	
Anti-Mouse A488	Goat	Thermo Fisher Scientific (A-11029)		1:1,000	aML#013
Anti-Mouse A555	Goat	Thermo Fisher Scientific (A-21424)		1:1,000	aML#015
Anti-Mouse A647	Goat	Thermo Fisher Scientific (A-21235)		1:1,000	aML#017
Anti-mouse CF770	Goat	VWR (#20077)	1:10,000		aML#009
Anti-Rabbit A488	Goat	Thermo Fisher Scientific (A-11304)		1:1,000	aML#012
Anti-Rabbit A555	Goat	Thermo Fisher Scientific (A-21429)		1:1,000	aML#014
Anti-Rabbit A647	Goat	Thermo Fisher Scientific (A-21245)		1:1,000	aML#016
Anti-rabbit CF680	Goat	VWR (#20067)	1:10,000		aML#010
Cas9	Mouse	Cell signalling (7A9-3A3 #14697)	1:2,000		aML#031
CPD	Mouse	Cosmo Bio (CAC-NM-DND-001)		1:500	aML#020
CSA	Rabbit	Abcam (ab137033)	1:750		aML#028
CSB	Rabbit	Santa Cruz (sc25370; H-300)	1:200		aML#003
EGFP	Mouse	Roche (11814460001)	1:1,000		aML#011
ERCC1	Mouse	Santa Cruz (sc-17809 D-10)	1:500	1:100	aML#066
HSPA4	Rabbit	Novus Biologicals, NBP1-81696	1:1000		aML#114
RNAPII / RPB1-S2	Rabbit	Abcam (ab5095)	1:1,000		aML#024
TFIIH – p44	Mouse	Gift J-M Egly	1:2,000		aML#075
TFIIH – p62	Mouse	Santa Cruz (sc-48431 (g-10))	1:1,000		aML#038
TFIIH – p80 (XPD)	Mouse	Abcam (ab54676)	1:1,000		aML#029
TFIIH – p89 (XPB)	Rabbit	Santa Cruz (sc-293; S-19)	1:1,000	1:100	aML#040
TFIIH – p89 (XPB)	Mouse	Gift of J-M Egly	1:1,000		aML#073
Tubulin	Mouse	Sigma (T6199)	1:1,000		aML#008
XPA	Rabbit	gift of R. Wood (CJ1)	1:10,000	1:1,000	aML#079
XPC	Rabbit	Novus Biologicals (NB100-58801)	1:2,000		aML#077
XPC	Mouse	Sigma (WH0007508M1-100UG)	1:1,000		aML#067

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