Supporting Information

de Feraudy et al. 10.1073/pnas.1002175107

SI Materials and Methods

Cells. Cells were cultured in Eagle's minimal essential media with Earle's balanced salt solution supplemented with 10% FBS, 10 μg/mL streptomycin, 10 units/mL penicillin, 0.292 g/L glutamine, and 25 μg/mL blasticidin. Cells were maintained between 50% and 90% confluency, passaged 24–48 h before use, and grown at 37 °C in 5% CO_2. Cells were grown to 75% confluency and used in the exponential growth phase.

Irradiation and Drug Treatment. Cells were exposed either to ionizing radiation from a ^{137}Cs source or UV-C light (254 nm at a fluency of 2.65 J/m^{-2} per s). Cells were treated with 50 μM wortmannin, 100 μM SP600125, 50 μM KU55933, 2 mM caffeine, and 1 μM carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (ZVAD) immediately after irradiation and during subsequent incubations.

Western Blot. Cells were washed with PBS and lysed using radioimmunoprecipitation (RIPA) buffer [150 mM NaCl, 1% (vol/vol) Nonidet P-40, 0.5% (wt/vol) sodium deoxycholate, and 0.1% (wt/vol) SDS in 50 mM Tris-HCl (pH 8.0)] supplemented with protease (1836153; Roche) and phosphatase inhibitors (78420; Pierce) according to the manufacturer's protocol. Protein concentrations were determined using the BCA Protein Assay Kit (23225; Pierce). Total protein in 1× Laemmli buffer with 10% 2-mercaptoethanol was separated by SDS/PAGE, transferred overnight to a PVDF membrane (IPVH00010; Millipore) by electroblotting with 20% (vol/vol) methanol for low molecular weight and 5% (vol/vol) for high molecular weight proteins, and blocked for 1 h in 5% (wt/vol) dry milk/Tris buffered saline/0.1%(vol/vol) Tween-20. Membranes were incubated overnight at 4 °C with primary antiserum followed by incubation with a horseradish peroxidase-conjugated secondary antiserum for 1 h, and they were developed using enhanced chemiluminescence (32106; Pierce or 64–201BP; Millipore). JNK (9252), phospho-JNK (9251), ATM (2873), and phospho-H2AX Ser139 (2577) antibodies were purchased from Cell Signaling, beta-actin (A5441) was from Sigma, and phospho-ATM Ser1981 (05-740) was from Upstate. The xeroderma pigmentosum complementation group C protein (XPC) antibody was courtesy of Wim Vermeulen and Jan Hoeijmakers (Rotterdam, The Netherlands).

Flow Cytometry. Detection of cellular histone variant H2AX phosphorylated on serine 139 (γH2AX) was carried out using the H2AX phosphorylation assay kit for flow cytometry (Upstate Biotechnology). The assay was carried out according to the manufacturer's instructions with one modification: the FITC-labeled antibody was incubated overnight at 4 °C instead of 20 min on ice. Cells were suspended in flow buffer (Ca^{2+}- and Mg^{2+}-free 1× PBS with 1% BSA containing 10 μg/mL PI and 100 μg/mL RNaseA) and analyzed using a FACS Caliber Flow Cytometer (Becton Dickinson) equipped with CELL QUEST. Data were analyzed using both CELL QUEST and FLOJO software (Tree Star).

Immunofluorescence Microscopy. Cells were grown on dual-chambered slides (Nalge Nunc), fixed with 4% paraformaldehyde for 10 min, and permeabilized with 0.2% Triton X-100 for 5 min. Cells used for 53BP1 IF were fixed with 100% methanol for 20 min. Slides were either directly processed or air-dried for 5 min and stored at −80 °C. After storage/methanol fixation, slides were repermeabilized with −20 °C 50:50 acetone:methanol for 20 min, blocked in PBS, 10% FBS, and 1% BSA for 1 h at 37 °C, and incubated overnight at 4 °C with the following primary antibodies: mouse monoclonal γH2AX (Ser-139), 1:1,000 (Upstate Biotechnology); rabbit polyclonal γH2Ax (Ser-139), 1:1,000 (Novus Biologicals); rabbit polyclonal 53BP1, 1:300 (#4937; Cell Signaling); anti-phospho-Chk2 (Thr68; #2661; Cell Signaling); and anti-proliferating cell nuclear antigen, 1:200 (FL-261; SC7907; Santa Cruz). Secondary antibodies labeled with Alexa 488 or Alexa 555 (Molecular Probes) were added at 1:1,000, and slides were incubated at 37 °C for 1 h. Slides were mounted with ProLong Gold Antifade reagent containing DAPI (Molecular Probes). Images were acquired at room temperature with a Zeiss Axioplan 2 microscope equipped with a 100× Zeiss Plan-NEOFLUAR 1.3 Oil objective lens and a Zeiss Axiocam color camera under the control of AXIOVISION 4.2 software. Image processing with PHOTOSHOP 6.0 (Adobe Systems) was applied to whole images only. Images used for comparison between different treatments and/or cell lines were acquired with the same instrument settings and exposure times, and they were processed equally.

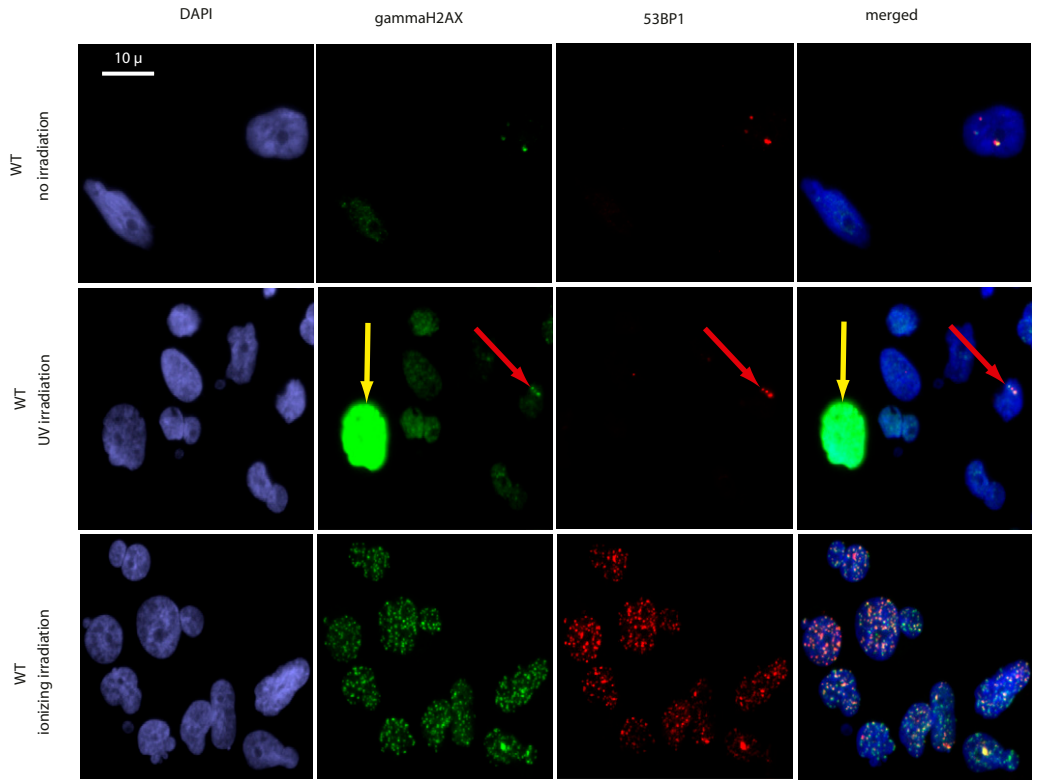

Fig. S1. Immunofluorescence analysis (IF) of GM637 cells stained for γH2AX and 53BP1 after UV and ionizing irradiation. Wild-type (WT) cells were SV40-transformed human fibroblasts (GM637). Cells were stained 30 min after 2 Gy and 4 h after 20 J/m^2. Data are representative of at least three independent experiments. Yellow arrow, UV-induced high levels of pan-nucleus γH2AX that did not colocalize with 53BP1; red arrow, γH2Ax foci colocalized with 53BP1.

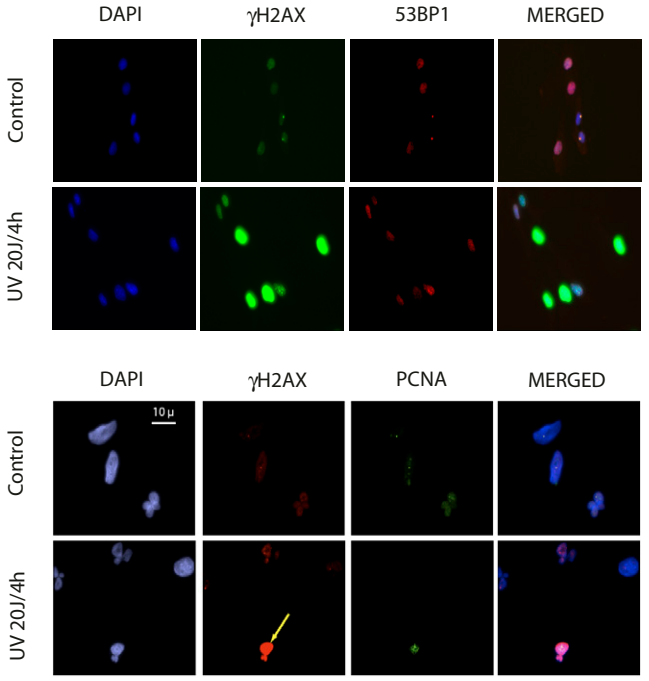

Fig. S2. High levels of pan-nucleus γH2AX were induced by UV damage in the absence of the marker of DSB 53BP1 foci. IF analysis of WT human fibroblasts (GM5659 human telomerase reverse transcriptase) stained for H2AX and 53BP1 after UV irradiation and IF analysis of WT human fibroblasts (GM5659 hTERT) stained for γH2AX and PCNA after UV irradiation are shown. Cells were stained (4 h at 20 J/m^2). Data are representative of at least three independent experiments. Yellow arrow, UV-induced high levels of pan-nucleus γH2AX.

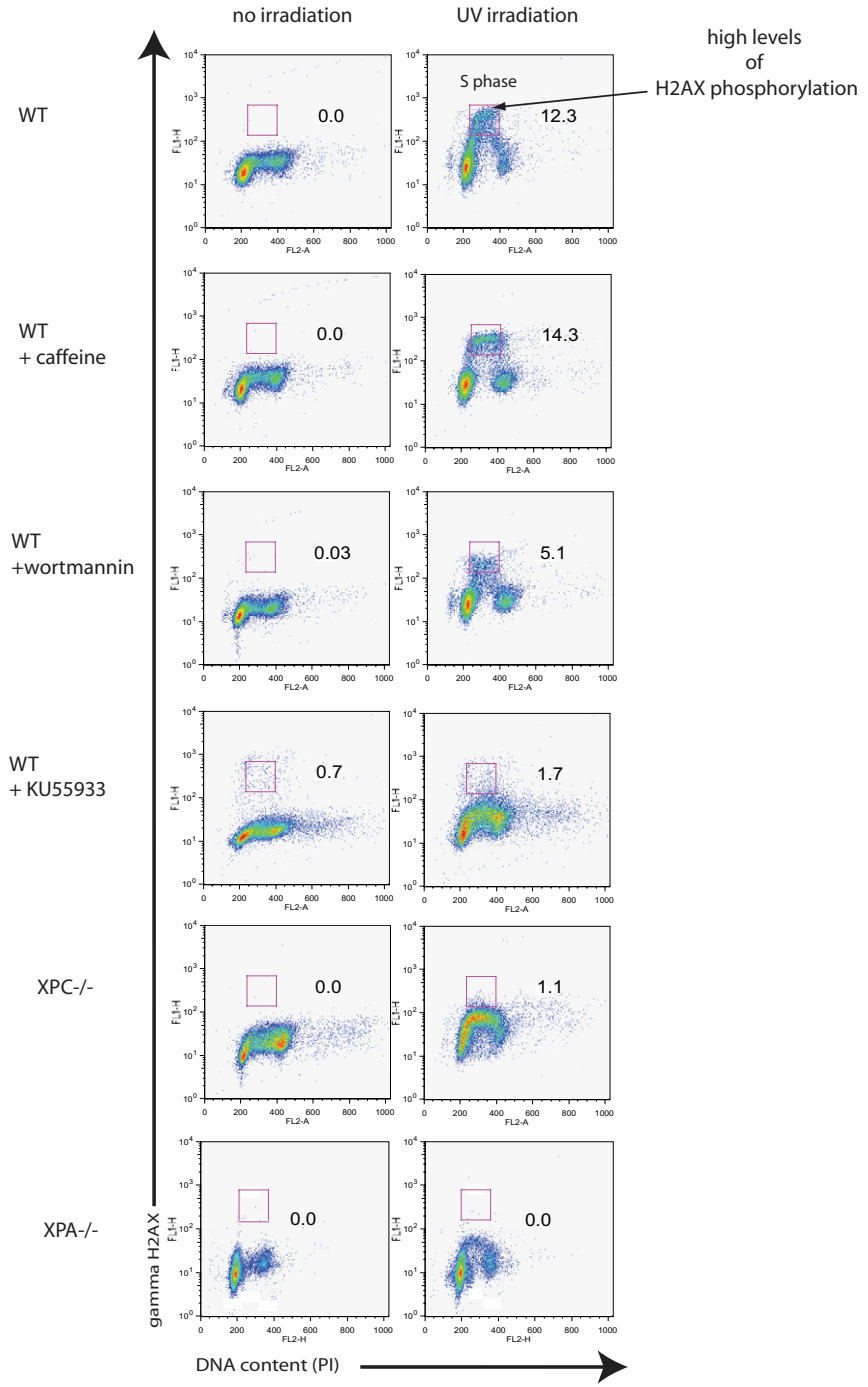

Fig. S3. Effect of caffeine, ataxia-telangiectasia mutated kinase (ATM) inhibition, and loss of xeroderma pigmentosum complementation group A or C proteins (XPA or XPC) on UV-induced high levels of pan-nucleus γH2AX during S phase. Flow cytometry profiles of hTERT-transformed fibroblasts stained for γH2AX and propidium iodide (PI) were used to measure DNA content and identify the G1, S, and G2 phases of the cell cycle. The high levels of γH2AX in S phase were quantified by measuring the fraction of cells within an S phase box at the same γH2AX levels in each profile (Fig. S3). WT, hTERT-transformed WT human fibroblasts (GM05659hTERT); XPC$^{-/-}$, hTERT-transformed XPC deficient cells (GM02993hTERT); XPA$^{-/-}$, hTERT-transformed XPA deficient cells (XP200SF hTERT). Large boldface numbers represent percentages of high levels of γH2AX in S phase. Data are representative of at least three independent experiments.

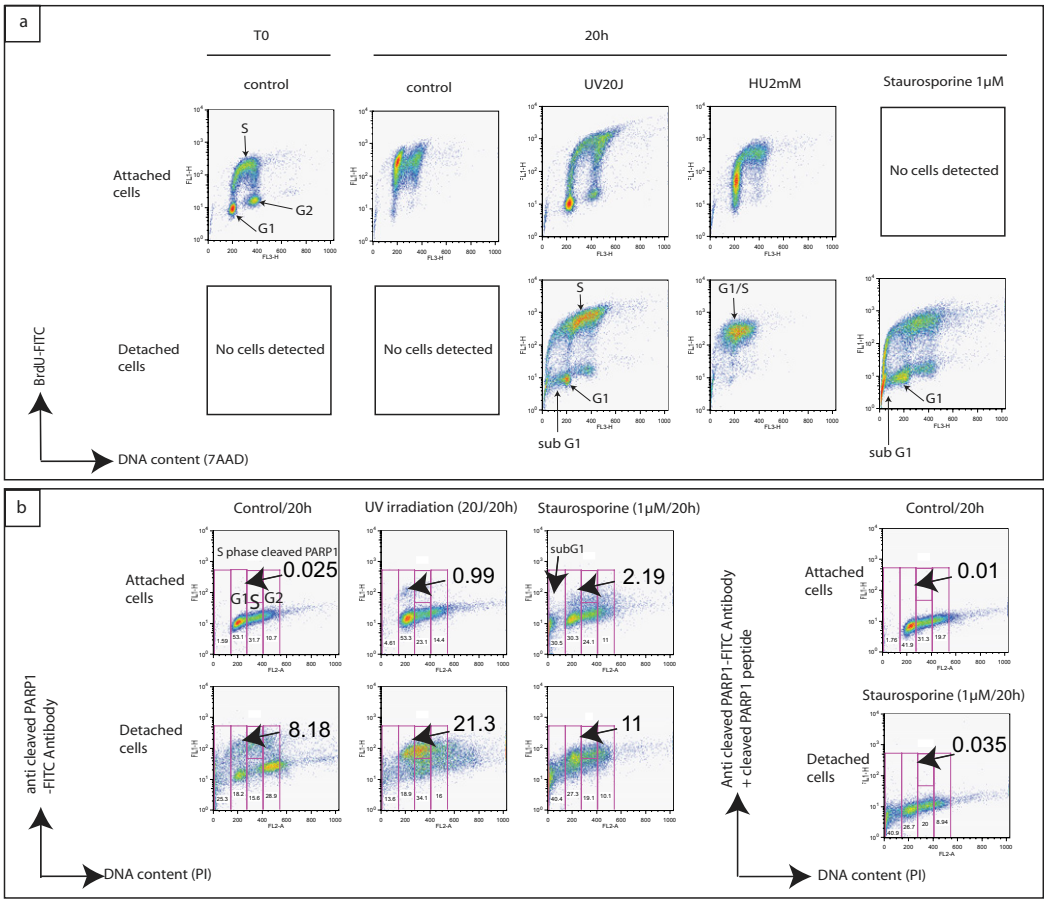

Fig. S4. Measure of apoptosis during S phase after UV irradiation. (*A*) To measure UV-induced apoptosis during S phase, we used an assay based on a property of SV40-transformed cells. SV40-transformed cells have high levels of p53, which predisposes them to an apoptotic response that involves detachment of S phase cells from their growth surface; this can be assessed by BrdU pulse labeling. S phase detached cells express high levels of the apoptotic marker cleaved p85-PARP1 and accumulate DNA degradation products. In contrast, cells that remain attached show none of these features. Treatment of the cells with ZVAD, which inhibits caspase-3 activation (an important step of apoptosis), inhibits their detachment from the growth surface. The percentage of apoptotic cells released from the substrate is calculated as the number of detached cells divided by the total number of attached plus detached cells. UV irradiation induced a rapid detachment of S phase SV40-transformed human fibroblasts from their growth surface. Flow cytometry profiles of human fibroblasts stained with bromodeoxyuridine. Cells attached to their growth surface were pulse labeled with BrdU for 15 min before or right after UV irradiation. BrdU incorporation was measured in 20,000 detached and attached cells by flow cytometry. Cells were simultaneously stained with 7AAD to identify the G1, S, and G2 phases of the cell cycle. Cells that were not harvested immediately after pulse labeling but were rinsed two times with PBS, irradiated with UV light (20 J), or incubated in hydroxyurea (HU; 2 mM) that induces cell death at the G1/S phase checkpoint or staurosporine (1 μM), a strong apoptosis inducer. HU and staurosporine were used as positive controls. BrdU incorporation was measured in attached and detached cells immediately after BrdU treatment (T0) and 20 h after BrdU treatment. Note that BrdU incorporation was slightly higher 20 h after pulse labeling because of the persistence of an intracellular pool of BrdU that was not washed away when the cells were rinsed and allowed to grow back into normal medium. Immediately after pulse labeling, attached S phase cells had incorporated detectable levels of BrdU. Staurosporine triggered a massive detachment of cells in all phases of the cell cycle (including S phase cells labeled with BrdU) and DNA degradation (sub-G1 population). In comparison, cells incubated in HU were arrested at G1/S phase checkpoint, consistent with the reduction of the intracellular nucleotides pool induced by this drug. Most detached cells incubated in HU were arrested in G1/S, which was expected; 20 h after UV irradiation, most detached cells were in S phase and had incorporated BrdU, indicating that they were already in S phase at time of irradiation. Thus, immediately after pulse labeling, S phase cells had incorporated detectable levels of BrdU, and UV irradiation caused these cells to later detach from the growth surface. Therefore, after UV damage, the main apoptotic response occurs within the S phase population at the time of irradiation. After HU or staurosporine, apoptosis occurred at different times according to their unique mechanisms of cell death. (*B*) After UV irradiation, detached cells were entering apoptosis, as assessed by their expression of high levels of apoptotic marker cleaved PARP-1 and accumulation of DNA degradation products. Flow cytometry profiles of WT human fibroblasts (GM637) stained for the early apoptotic marker cleaved protein poly(ADP ribose) polymerase (PARP-1) and PI (DNA content) to identify the G1, S, and G2 phases of the cell cycle. Cleavage of PARP-1 to generate a p85 fragment is a marker of caspase-dependent apoptosis. PARP-1 cleavage was measured in attached and detached cells 20 h after UV irradiation (UV = 20 J per 20 h) or treatment with staurosporine (staurosporine = 1 μM per 20 h) and in control cells (control = 20 h). The specificity of the anti-PARP-1 antibody was tested using a PARP-1 peptide mixed with the diluted antibody before adjunction of the mix to cells treated with UV or staurosporine (lowest row). After UV irradiation (20 J per 20 h), detached cells expressed the highest levels of cleaved PARP-1. This signal was the highest in S phase (21.3%). In contrast, cleaved PARP-1 expression in attached cells remained negligible (less than 2% in G1 and 0.99% in S phase). Only cells that detached from the growth surface were entering apoptosis 20 h after UV irradiation, and most of these apoptotic cells were in S phase. In parallel, detached cells accumulated DNA degradation products. These results confirm that apoptosis in UV-damaged SV40 transformed cells occurs during S phase through detachment from the growth surface followed by PARP-1 cleavage and DNA degradation. This UV-induced S phase apoptosis is mediated by caspases and can be inhibited by ZVAD (Fig. 4).

Table S1. Cell types, genotypes, and molecular characteristics

Designation	Genotype	Molecular characteristic
GM637*	WT	Normal fibroblasts†
GM15983*	XPC deficient	Lack DNA damage binding protein XPC†
GM16247 and GM16248*	XPC deficient + XPC cDNA	GM15983 corrected by a cDNA vector expressing the XPC WT protein†
XP200SF hTERT	XPA deficient	Lack the DNA damage-binding protein XPA‡
GM05659 hTERT*	WT	Normal fibroblasts‡
GM02993* hTERT	XPC deficient	Lack the DNA damage-binding protein XPC‡
A-T hTERT*	ATM deficient	A-T fibroblast lacks functional ATM‡
XP30RO	Pol η deficient	Lack bypass polymerase Pol η†

WT, wild type; A-T, ataxia-telangiectasia.
*Cells purchased from Corriell Cell.
†SV40 transformed.
‡Immortalized by hTERT in house.