

Homologous recombination mediates S-phase-dependent radioresistance in cells deficient in DNA polymerase η

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DNA polymerase η (pol η) is the only DNA polymerase causally linked to carcinogenesis in humans. Inherited deficiency of pol η in the variant form of xeroderma pigmentosum (XPV) predisposes to UV-light-induced skin cancer. Pol η -deficient cells demonstrate increased sensitivity to cisplatin and oxaliplatin chemotherapy. We have found that XP30RO fibroblasts derived from a patient with XPV are more resistant to cell kill by ionising radiation (IR) than the same cells complemented with wild-type pol η . This phenomenon has been confirmed in Burkitt's lymphoma cells, which either expressed wild-type pol η or harboured a pol η deletion. Pol η deficiency was associated with accumulation of cells in S-phase, which persisted after IR. Cells deficient in pol η demonstrated increased homologous recombination (HR)-directed repair of double strand breaks created by IR. Depletion of the HR protein, X-ray repair cross-complementing protein 3 (XRCC3), abrogated the radioresistance observed in pol η -deficient cells as compared with pol η -complemented cells. These findings suggest that HR mediates S-phase-dependent radioresistance associated with pol η deficiency. We propose that pol η protein levels in tumours may potentially be used to identify patients who require treatment with chemo-radiotherapy rather than radiotherapy alone for adequate tumour control.

Introduction

DNA polymerase η (pol η) was first discovered as the enzyme lacking in the variant form of xeroderma pigmentosum (XPV) (1,2). XPV patients suffer from a greatly increased sensitivity to sunlight; deficiency of pol η predisposes to the development of UV-induced skin cancers, often on multiple body sites (3,4). Whereas the seven other complementation groups of XPV are characterized by defects in different components of the nucleotide excision repair pathway and lack the ability to repair damage caused by various DNA-damaging agents, cells from patients suffering from XPV are unable to tolerate these forms of damage during S-phase to complete DNA replication (5). As part of the cellular translesion synthesis system, pol η has the ability to replicate past certain types of DNA damage, such as cyclobutane pyrimidine dimers created by UV radiation (6). To date, pol η is the only DNA polymerase that has been causally linked to the development of cancer (7).

Radiotherapy is one of the most efficacious treatments for cancer. Ionising radiation (IR) exerts its effects by inducing DNA damage, either by depositing its energy directly within cells or by acting indirectly through ionization of cellular water and the creation of reactive

radicals (8). Various forms of DNA damage have been described as a consequence of IR treatment, such as damage to DNA bases or the sugar backbone. DNA double strand breaks (DSBs) are considered to be the main toxic lesion caused by IR. Cells employ two main pathways to repair DSBs, homologous recombination (HR) and non-homologous end-joining (9). Since HR requires a sister chromatid as a template for accurate repair, it can only take place after replication; therefore the majority of DSBs are repaired by non-homologous end joining (10,11).

Whereas the role of pol η in the repair or bypass of UV radiation-induced DNA lesions has been well characterised, its involvement in cellular responses to IR is less clear. A published study investigating the radiosensitivity of a panel of cell lines derived from patients with XPV and similar inherited syndromes did not report increased radiosensitivity for XPV cells as compared with cell lines representative of the general population, but pol η protein levels were not presented and isogenic pol η -deficient and pol η -expressing cells were not available for comparison (12). In a separate study, chicken DT40 cells deficient in pol η were not found to be more sensitive to IR than wild-type cells (data not shown); an effect attributed by the investigators to loss of functional p53 (13).

In this study, we examined the role of pol η in mediating clonogenic survival after exposure to IR and we investigated the mechanisms related to DNA damage repair by which pol η may influence radiosensitivity. Using three model systems, we show that pol η -deficient cells are more resistant to cell kill by IR and that HR mediates S-phase-dependent radioresistance in the context of pol η deficiency.

Materials and methods

Cell lines and cultures

XP30RO cells, simian virus 40-transformed fibroblasts derived from a patient with XPV, harbour a 13 bp deletion leading to the expression of a truncated and non-functional pol η fragment. XP30RO/pol η cells contain a complementary DNA vector expressing wild-type pol η . Both cell lines were a gift from Dr. Alan Lehmann (University of Sussex, Brighton, UK). The pol η wild-type type I Burkitt's lymphoma BL-2 cell line and the corresponding cell lines in which pol η had been knocked out by HR (BL-2 POLH^{-/-}) have previously been described (15). The HCT116 colorectal cancer cell line and the SQ20B laryngeal cancer cell line were purchased from ATCC (Teddington, Middlesex, UK). XP30RO, XP30RO/pol η , HCT116 and SQ20B cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and 1% penicillin/streptomycin (all from Invitrogen). Cells containing the pol η vector were cultured in the presence of 100 μ g/mL zeocin (Invitrogen). BL-2 and BL-2 POLH^{-/-} cells were grown in RPMI medium containing 10% foetal calf serum and 1% penicillin/streptomycin. All cells were grown in a humidified atmosphere containing 5% CO₂ at 37°C.

siRNA knockdown

Cells were reverse-transfected with four small interfering RNAs (siRNAs) against four different sequences of POLH mRNA at a total concentration of 10 nM (siRNA 1 sense: 5'-AAACUGGCCUGUGGACUAA-3'; anti-sense: 5'-UUA GUC CAC AGG CCA GUU U-3'; siRNA 2 sense: 5'-GAA GUU AUG UCC AGA UCU U-3'; anti-sense: 5'-AAG AUC UGG ACA UAA CUU C-3'; siRNA 3 sense: 5'-GCA CUU ACA UUG AAG GGU U-3'; anti-sense: 5'-AAC CCU UCA AUG UAA GUG C-3'; siRNA 4 sense: 5'-GCA AUU AGC CCA GGA ACU A-3'; anti-sense: 5'-UAG UUC CUG GGC UAA UUG C-3'; purchased from Eurogentec). X-ray repair cross-complementing protein 3 (XRCC3) was knocked down by 10 nM siRNA (sense: 5'-GGA CCU GAA UCC CAG AAU U-3'; anti-sense: 5'-AAU UCU GGG AUU CAG GUC C-3'; obtained from Dharmacon). For all experiments using transfections, a control with non-targeting siRNA (AllStars negative control siRNA; Qiagen) and a mock-transfection control were included. Transfections were carried out using Dharmafect-1 according to the manufacturer's instructions. Knockdown efficiency was confirmed by western blot analysis.

Abbreviations: DSB, double strand break; HR, homologous recombination; IR, ionising radiation; PBS, phosphate-buffered saline; pol η , polymerase η ; siRNA, small interfering RNA; XPV, xeroderma pigmentosum; XRCC3, X-ray repair cross-complementing protein 3.

Clonogenic survival assays

Clonogenic survival assays were performed in 10 cm plastic dishes; cells were plated and allowed to adhere before treatment. Plates were irradiated using a ¹³⁷Cs irradiator (dose rate 1.81 Gy/min) at room temperature. For knock-down experiments, transfections were performed as described above, and cells were re-plated to 10 cm dishes and allowed to adhere before irradiation 48 h after transfection. Remaining cells after plating were used to test for knock-down efficiency using western blot analysis. Colonies were stained with 4% methylene blue 10–14 days after irradiation and only colonies containing more than 50 cells were counted. All clonogenic assays were performed in triplicate, and survival curves represent at least three independent experiments. The surviving fraction was calculated with the following formula: $(\# \text{colonies} / \# \text{plated cells})_{\text{irradiated}} / (\# \text{colonies} / \# \text{plated cells})_{\text{untreated}}$.

Cell viability assays

The BL-2 cell line and its pol η -deficient clones (BL-2 POLH^{-/-} cl82 and cl123) were plated in serial dilutions on 96-well plates (40 000 to 312 cells per well) and treated with IR using a ¹³⁷Cs irradiator at room temperature. Cells were cultured for 5 days following treatment, then media was supplemented by adding resazurin (Sigma) to a concentration of 10 μ g/mL. Resazurin reduction after 3 h was measured colorimetrically on a Perkin Elmer Envision 2103 plate reader (560–590 nm). To obtain a measure for relative proliferation, fluorescence data from each treatment dose were plotted against cell number using a minimum of four samples per data point. The cell number plated to obtain 50% resazurin conversion was interpolated from the graphs. The ratio of untreated cells plated to treated cells plated, at 50% resazurin conversion, was plotted on semi-logarithmic scale to produce relative proliferation curves for each cell line. All curves presented represent five independent experiments.

Western blot analyses

Cells were harvested and washed in phosphate-buffered saline (PBS), and protein was extracted in lysis buffer. Protein content was measured using a NanoDrop spectrophotometer (Thermo Scientific). For each sample, 40 μ g of total protein from whole-cell lysates was run on a sodium dodecyl sulphate–polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were probed with rabbit polyclonal antibodies against pol η (Atlas Antibodies, dilution 1:1000), P21 (Abcam, dilution 1:200), phosphorylation of p53 at Ser15 (Cell Signaling, dilution 1:1000) or XRCC3 (Novus Biologicals, dilution 1:2000). Equal loading of the gels was confirmed by immunoblotting with a mouse monoclonal antibody against α -actin (Abcam). Blots were visualized and quantified using the Odyssey image analysis system (Li-Cor Biosciences).

Fluorescence-activated cell sorting

To assess cell cycle profiles, cells were harvested and washed in PBS twice before fixation with ice-cold 70% ethanol. Cell pellets were again washed with PBS and incubated with 40 μ g/mL propidium iodide solution containing 250 μ g/mL RNase A for 30 min at 37°C. A FACSCalibur system (Becton-Dickinson) was used for the analysis. Ten thousand events were recorded, and cell cycle profiles were analysed and modelled using the Modfit LT software. Experiments were repeated at least three times, and statistical significance of independent experiments was examined by the Student's *t*-test.

Analysis of nuclear foci

Cells were plated in 96-well plates and allowed to attach before being irradiated with 5 Gy. Fixation with 4% paraformaldehyde was performed at different time points after treatment as indicated in the Results section. Cells were permeabilised with 0.3% Triton X-100 in PBS, and unspecific binding was blocked with PBS solution containing 3% bovine serum albumin. Cells were incubated with the primary antibodies against γ H2AX (Millipore) or RAD51 (Santa Cruz Biotechnology) overnight at 4°C. After three washing steps with PBS, secondary anti-mouse and anti-rabbit antibodies coupled with Alexa Fluor 488 were incubated for 1 h. Nuclei were stained with 4',6-diamidino-2-phenylindole, and images were taken and analysed using the IN CellAnalyzer system (GE Healthcare). For the analysis, different filters were used to exclude irregularly shaped cells and ultra-bright 4',6-diamidino-2-phenylindole staining. Cells with more than nine γ H2AX foci or more than five RAD51 foci were counted as positive. Statistical analysis was performed using the two-sided Student's *t*-test.

Results

Pol η determines cellular sensitivity to IR

XP30RO cells are derived from a patient with the variant form of XPV and contain a homozygous deletion in the *POLH* gene leading to the expression of a non-functional 42-amino acid peptide. These

cells have previously been shown to exhibit increased sensitivity to cisplatin and UV radiation, which is reversed in cells containing a pol η -expressing vector (14).

We examined the response of XP30RO and XP30RO/pol η cells to IR using clonogenic assays. The XP30RO cells showed significantly increased clonogenic survival as compared with their pol η -rescued counterparts ($P < 0.01$, paired Student's *t*-test; Figure 1A). To rule out a cell line-specific phenomenon or an indirect effect caused by the POLH vector, we tested a different pair of isogenic cell lines that differed in the expression of pol η protein. In the BL-2 cell model system, Burkitt's lymphoma cells either expressed wild-type pol η or harboured a pol η deletion caused by HR-mediated knockout (15). Since lymphoma cells grow in suspension, resazurin-based viability assays were performed in pol η wild-type BL-2 cells and two pol η -knockout clones (cl-82 and cl-123). The resazurin-based assay also allowed analysis of survival data obtained with lower radiation doses (up to 6 Gy) than those used for the clonogenic survival experiments performed with XP30RO fibroblasts (Figure 1A). As shown in Figure 1C, both pol η -deficient BL-2 clones exhibited higher viability when measured 5 days after IR treatment ($P < 0.01$, paired Student's *t*-test).

Interestingly, despite differences between the model systems used and radiation doses applied, the radiation enhancement ratios for the effect of pol η expression were similar for both model systems (Figure 1A as compared with Figure 1C). Although the cell lines used have been considered “functionally” deficient in p53 on account of binding of the large T antigen to p53 or due to over-expression of MDM2 (16,17), we demonstrated induction of total p21 and phosphorylation of p53 protein in pol η -expressing and pol η -deficient clones of both XP30RO and BL-2 cell lines by IR (Supplementary Figure 1 is available at *Carcinogenesis* Online). Consistent with the presence of functional p53, cell cycle analysis of the BL2 cell lines 6 h after IR showed reduced entry into S-phase, indicative of the p53-activated G1/S checkpoint (Supplementary Figure 2 is available at *Carcinogenesis* Online).

To confirm the effects of pol η on cellular survival of cancer cells after exposure to IR, we used siRNA against pol η to knock down protein expression in two cancer cell lines of different histological origins. HCT116 adenocarcinoma colorectal and SQ20B squamous cell laryngeal cancer cell lines were reverse-transfected with siRNA against pol η or non-targeting siRNA; knockdown efficiency was quantified by western blotting. Knockdown of more than 75% was achieved in both cell lines after 48 h and pol η protein levels remained suppressed for the following 24 h (Figure 2B and 2D). For clonogenic survival experiments, cells were irradiated at 48 h after transfection. As shown in Figure 2, pol η knockdown in both cell lines led to a significant increase in clonogenic survival ($P < 0.05$ for HCT116 cells, $P < 0.01$ for SQ20B cells, statistical analysis performed by paired Student's *t*-test).

Taken together, the experiments shown in Figures 1 and 2 demonstrate that the expression of pol η protein accounts for differences in survival following IR treatment.

Pol η -deficiency is associated with S-phase accumulation

A principal role of pol η is the bypass of lesions to prevent stalling of replication forks during the S-phase of the cell cycle. It is also known that cells in S-phase exhibit a relative increase in radioresistance as compared with cells in other phases of the cell cycle (18,19). In order to study the effect of pol η -deficiency on cell cycle profiles, we analysed pol η -deficient cells in comparison to their respective pol η -expressing counterparts, without and with treatment with IR. In un-irradiated cells, BL-2 POLH^{-/-} cells had a significantly higher proportion of cells in S-phase as compared with the parental BL-2 cells (Figure 3A). This increased proportion of S-phase cells in BL-2 POLH^{-/-} cells as compared with BL-2 cells was maintained following treatment with IR. Although treatment with IR did not significantly increase the proportion of total cells in S-phase, it was associated with a reduction of cells in early S-phase, and accumulation of cells in mid-S-phase, known to be a particularly radioresistant phase of

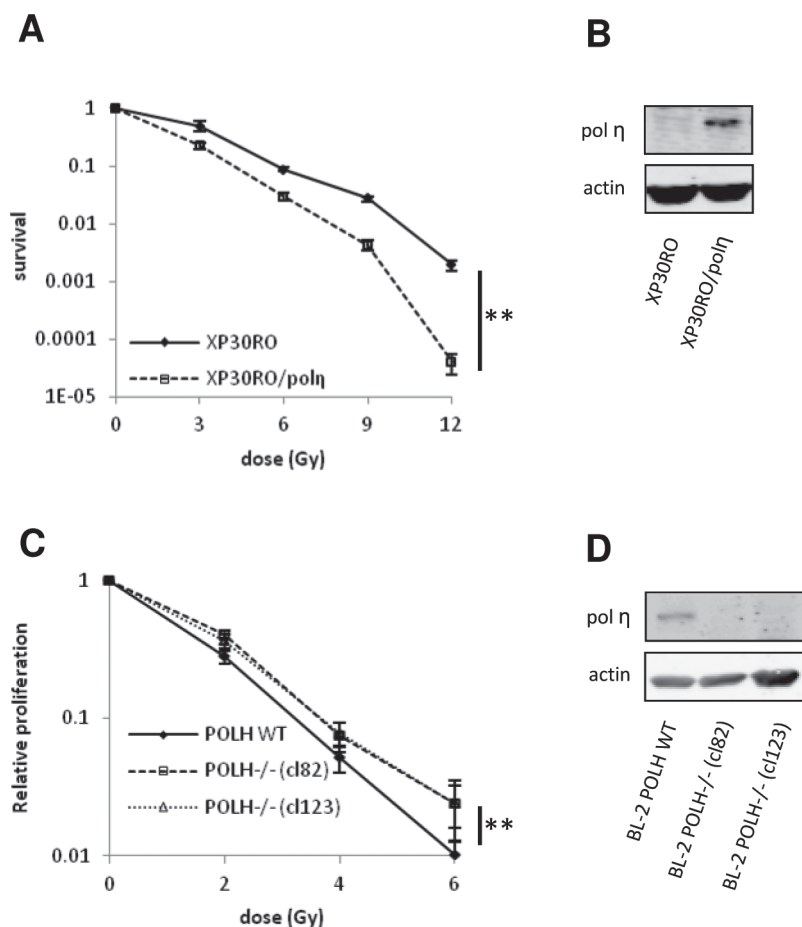


Fig. 1. Pol η -deficiency is associated with decreased sensitivity to ionizing radiation. (A) Clonogenic survival assay showing increased survival of XP30RO human fibroblasts compared to XP30RO/pol η cells after treatment with IR. Each data point represents three independent experiments. Error bars show standard error of the mean. Statistical analysis performed by paired Student's *t*-test; $**P < 0.01$. (B) Western blot showing levels of pol η protein in XP30RO cell lines. (C) Viability assay in pol η -proficient BL-2 and pol η -knockout (cl-82 and cl-123) cell lines after IR. Each data point represents five independent experiments. Error bars show standard error of the mean. Statistical analysis performed by paired Student's *t*-test; $**P < 0.01$. (D) Western blot showing pol η protein levels in POLH wild-type and two POLH knockout BL-2 cell clones.

the cell cycle (Figure 3B and Supplementary Figure 2 is available at *Carcinogenesis* Online). To confirm that pol η deficiency results in a greater S-phase proportion in untreated cells, cell cycle profiles of XP30RO cells were compared with XP30RO/pol η cells (Figure 3C) and knockdown of pol η was performed by siRNA in HCT116 cells, which demonstrated a significant difference in the proportion of S-phase cells as compared with treatment with non-targeting siRNA (Figure 3D). In all three model systems, pol η deficiency was associated with a significant increase of cells in the S-phase of the cell cycle, presumably due to delay of progression through replication in pol η defective cells.

Pol η deficiency alters the kinetics of the repair of DSBs

To study whether pol η -mediated alterations in cell cycle profiles influence DNA damage repair kinetics, we performed immunofluorescence analysis of γ H2AX foci as markers of DSBs in living cells. Untreated XP30RO cells had an approximately 2-fold higher proportion of cells with more than nine γ H2AX foci than the XP30RO/pol η cell line (5.9% versus 3.2%; $P < 0.001$ by Student's *t*-test), suggesting that pol η -deficient cells have a higher baseline DNA damage response to endogenously created lesions. As shown in Figure 4A, exposure of XP30RO and XP30RO/pol η cell lines to 5 Gy of IR led to an increase in the proportion of cells that were γ H2AX foci-positive, measured by the number of foci above a threshold level of 9 foci per cell. As compared with the pol η -deficient cells, the XP30RO/pol η cells had a higher percentage of

γ H2AX-positive cells from 6 h post-IR onwards and exhibited persistence of unrepaired DSBs. Significantly, at 24 h after radiation, the pol η -expressing cells still had about 50% more cells positive for γ H2AX foci ($P < 0.01$ by Student's *t*-test) than the pol η -deficient counterparts (Figure 4A and 4B).

It has been suggested that S-phase-dependent radioresistance can be mediated by increased homology-directed DNA repair (20,21). We assessed the levels of HR activity in pol η -deficient cells after IR via quantification of RAD51 foci. After irradiation with 5 Gy, there was a significantly higher proportion of RAD51-positive cells in the XP30RO cell line as compared with the corresponding pol η -expressing cell line ($P < 0.001$ at 6 h, statistical analysis performed by Student's *t*-test; Figure 4C and 4D). This finding was consistent with the faster resolution of γ H2AX foci in pol η -deficient cells and supported the hypothesis that increases in clonogenic survival of pol η -deficient cells following IR are due to increased homology-directed DNA damage repair.

XRCC3 knockdown abrogates the survival benefit of pol η deficiency after IR

To further test the hypothesis that increased HR activity accounts for survival differences between pol η -expressing and pol η -deficient cells following IR, we knocked down a key HR protein, XRCC3, in the context of treatment with IR. Clonogenic survival assays were performed after reverse transfection with XRCC3 siRNA for 48 h. Knockdown efficiency was above 70% for XP30RO and XP30RO/pol η cells;

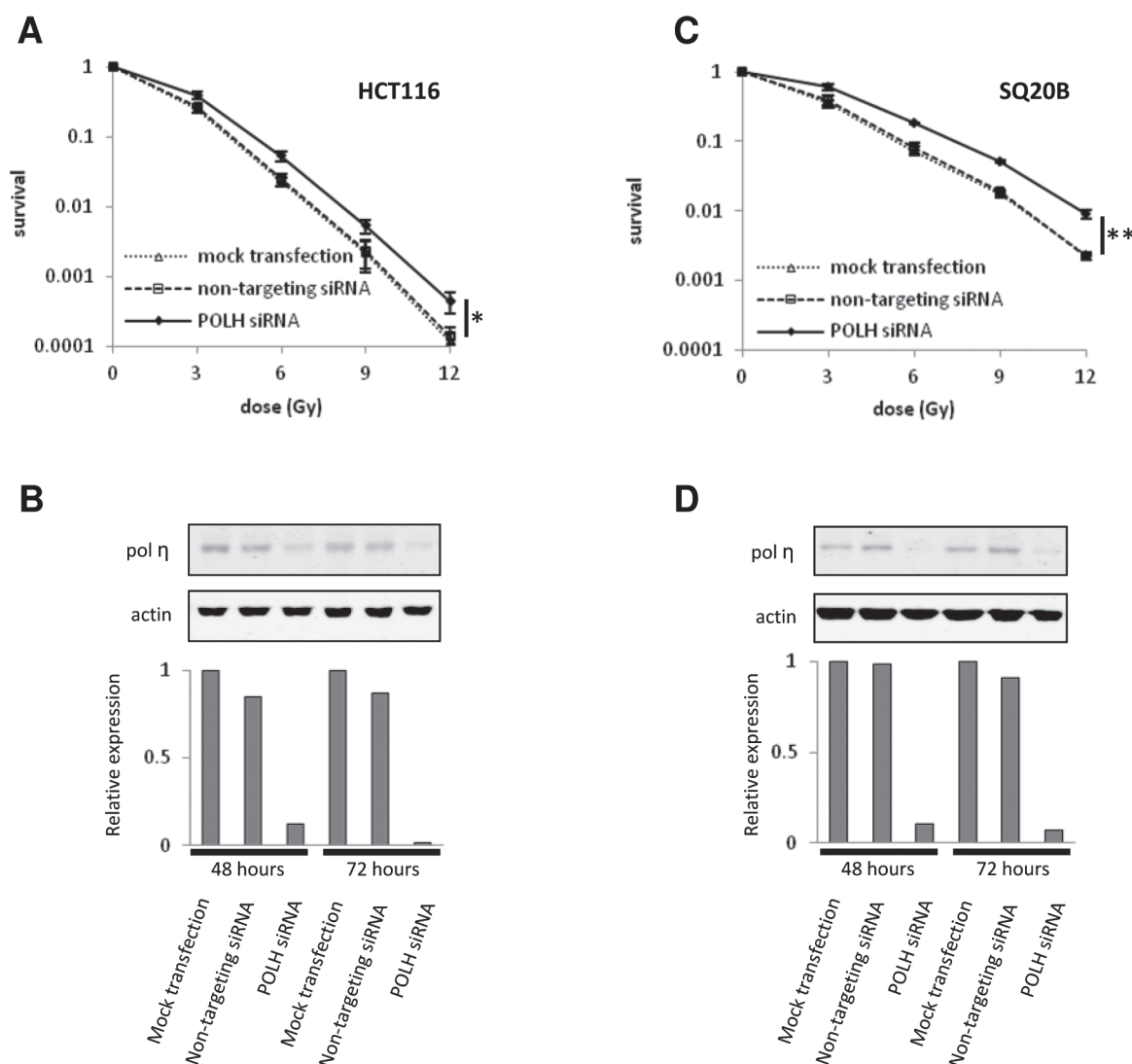


Fig. 2. Pol η knockdown increases resistance of cancer cells to IR. Clonogenic survival in (A) HCT116 and (C) SQ20B cells after pol η knockdown. Cells were either transfected with non-targeting siRNA or siRNA against pol η for 48 h before irradiation. Each data point represents three independent experiments. Error bars show standard error of the mean. Statistical analysis performed by Student's *t*-test; * $P < 0.05$, ** $P < 0.01$. B, D. Western blot analyses showing efficiency of pol η protein knockdown after 48 and 72 h. Knockdown efficiency was above 75% for both HCT116 and SQ20B cells (bar diagrams).

XRCC3 knockdown reduced the survival of XP30RO cells to the levels of XP30RO/pol η cells (Figure 5A and 5B). This finding was consistent with the hypothesis that the increased radioresistance of XP30RO cells was mediated by an HR-dependent mechanism. Additionally, depletion of XRCC3 decreased survival significantly ($P < 0.01$, Student's paired *t*-test) in XP30RO cells but not in XP30RO/pol η cells (Figure 5A), suggesting that cells which express pol η are less dependent on HR to repair IR-induced DNA damage than pol η -deficient cells.

Discussion

We report for the first time the interesting observation that loss of pol η results in increased resistance to IR. This finding initially seems counter-intuitive, since usually loss of a DNA repair protein results in sensitivity to DNA damaging agents. However, in recent times, there has been considerable recognition of competition or redundancy in DNA repair pathways, and that in some circumstances loss of a repair pathway can have paradoxical results.

Unlike UV irradiation that leads to well-defined DNA lesions that can be repaired with high fidelity by enzymes such as pol η during the S-phase of the cell cycle (6), exposure to IR creates a variety of DNA

lesions such as base lesions, single strand breaks and DSBs. DSBs, the major toxic lesions by which IR exerts its effects, are repaired by two major pathways, non-homologous end-joining and HR. Aberrations in DNA repair pathways such as HR can contribute to radioresistance and may be useful as biomarkers to select patients for or against radiotherapy treatment or as targets to improve the efficacy of radiation treatment, for example by the addition of chemotherapy (22,23).

In this study, we have shown for the first time that pol η status influences cellular survival after treatment with IR. Previously published studies performed *in vitro* have suggested that pol η has a role in the bypass of oxidative DNA lesions (24–30) and that it can determine sensitivity to cisplatin chemotherapy (31,32). In contrast to studies in which pol η deficiency resulted in *greater* sensitivity to damaging agents, our data suggest that lack of pol η expression results in *lower* sensitivity to treatment with IR. It should be noted that IR induces phosphorylation of p53 protein in the cell lines used in the study reported here.

To explain the phenomenon of radioresistance, we have found that pol η -deficiency has a major effect on cell cycle profiles, notably increasing the S-phase population as compared with pol η -expressing

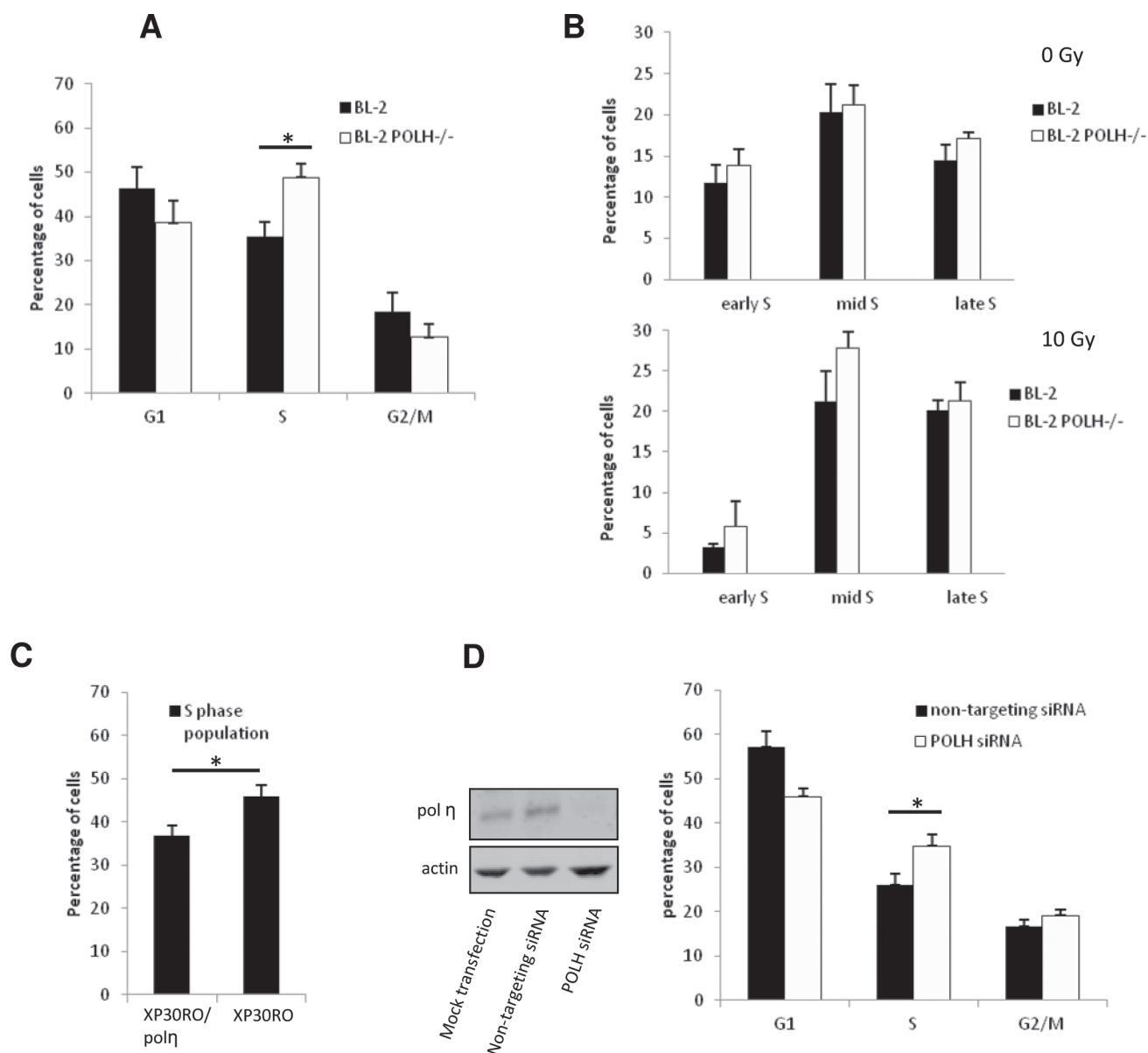


Fig. 3. Pol η deficiency is associated with accumulation of cells in S-phase. (A) Cell cycle profiles of un-irradiated POLH wild-type and POLH^{-/-} BL-2 (cl-82) cells. (B) POLH wild-type and POLH^{-/-} BL-2 (cl-82) were treated with 10 μ M BrdU 5.5 h after IR (10 Gy) and cells were collected 30 min later. As compared with the profile in mock-irradiated cells (upper panel), POLH^{-/-} BL-2 cells had a higher proportion of cells in mid-S-phase cells post-IR than the POLH wild-type cells (lower panel). (C) FACS analysis showing S-phase population in untreated XP30RO and XP30RO/pol η fibroblast cells. (D) Cell cycle profile of HCT116 either transfected with 10 nM non-targeting siRNA or POLH siRNA. Knockdown efficiency was confirmed by western blot analysis. Data from the original cell cycle histogram data were pooled and depicted as bar diagrams. Error bars show the standard of the mean of at least five independent experiments. Statistical analysis was performed by Student's *t*-test; **P* < 0.05.

cells. Pol η has previously been linked to the maintenance of genomic stability in the absence of exogenous DNA lesions, and alterations of cell cycle profiles have been described in undamaged pol η -knockdown cells (33). Consistent with the hypothesis that S-phase cells are relatively radioresistant as compared with G1- and G2/M-phase cells (18,19), we found consistently higher levels of S-phase in pol η -deficient cell lines. This finding is consistent with previously published studies in which cells deficient in pol η accumulated in S-phase in response to DNA damage induced by chemical agents (34,35).

In order to link radioresistance with the repair kinetics of DNA DSBs, we measured γ H2AX foci post-IR. Our finding that lack of pol η expression results in lower sensitivity to treatment with IR suggested that pol η -deficient cells should have greater proficiency in

repairing IR-induced damage. The measurement of residual γ H2AX foci that are not resolved within 24 h of IR has previously been linked with cellular radiosensitivity (36,37). We demonstrated a 50% higher level of residual γ H2AX foci at 24 h in pol η -expressing cells as compared with pol η -knockout counterparts.

Recently, the importance of HR repair has been implicated in determining S-phase-dependent radioresistance (21,38,39). Cells deficient in components of the HR pathway demonstrate different degrees of radioresistance in S-phase (20). HR requires an intact sister chromatid or homologous chromosome as a template for accurate repair and can therefore only be carried out during S or G2 phases of the cell cycle (40). We observed an increase in RAD51 foci in pol η -deficient cells after IR, suggesting a dependency on HR, and an abrogation of the survival benefit of pol η deficiency after knockdown of the

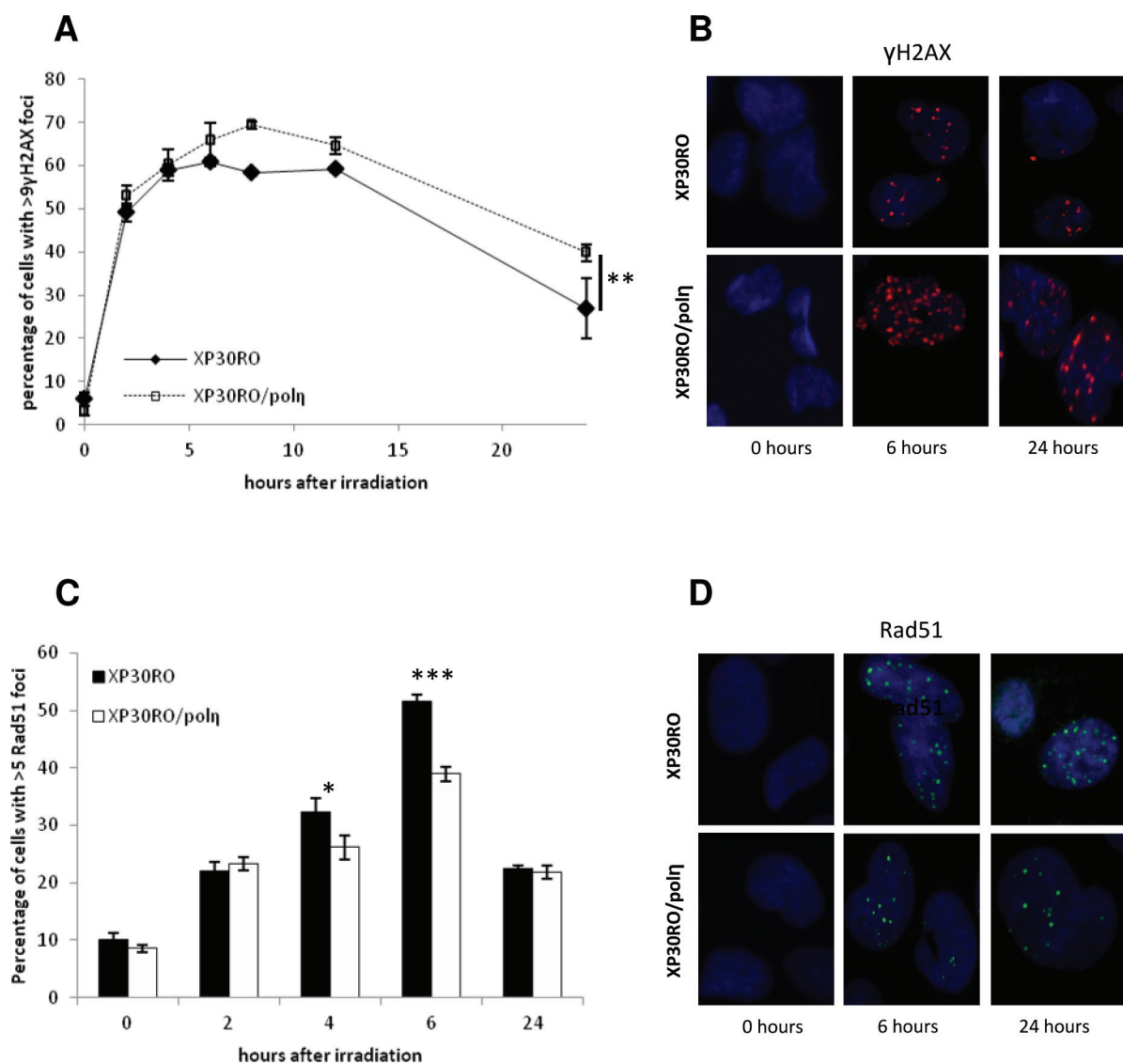


Fig. 4. DNA repair protein foci formation in XP30RO cells after irradiation. (A, B) Pol η -deficient XP30RO cells and the same cells transfected with pol η vector were irradiated with 5 Gy and fixed at the time points shown. γ H2AX foci were visualized by immunostaining; analysis was performed using the automated IN Cell Analyzer system. Cells with more than 9 γ H2AX foci were counted as positive, and the percentage of positive cells is plotted against time after IR. Data points represent three independent experiments. (C, D) For RAD51 foci analysis, cells were treated with 5 Gy irradiation dose and fixed and stained at the time points shown. Cells with more than 5 RAD51 foci were counted as positive. Bars represent three independent experiments. Statistical analysis was performed by two-sided Student's *t*-test; **P* < 0.05, ****P* < 0.001. Sample pictures were prepared using ImageJ software.

HR protein, XRCC3. Both findings suggest that pol η -deficient cells depend on the HR pathway more than pol η -expressing cells to repair IR-induced DNA damage. An increase in HR events has previously been described for pol η -deficient cells after exposure to UV radiation (41,42).

Conversely, pol η does appear to have a role in HR. Pol η has been reported to be able to perform D-loop extension during HR (13,43). These findings were made using oligonucleotide constructs and reporter assays. The biological relevance of pol η -mediated strand extension in live cells is not currently known. Indeed, based on current levels of knowledge, pol η expression does not appear to be essential for HR to occur in human cells. Based on the data presented above, we propose a model in which pol η deficiency requires cells to employ increased HR activity to repair endogenous and exogenous DNA damage and therefore delay progression from S-phase (Figure 6). The biological consequence of an increased S-phase population is relative radioresistance of pol η -deficient cells by enabling more rapid repair of IR-induced DSBs by HR.

The clinical implications of our findings are potentially wide-ranging. Some of the largest improvements observed over the past three decades in clinical outcomes for patients with solid malignancies have been from combined treatment with chemotherapy and radiotherapy (concurrent chemo-radiation). An example of this clinical benefit is locally advanced stage cervix cancer, for which the administration of cisplatin-based chemotherapy concurrent with radiotherapy reduced the risk of death by 30–50% as compared with radiotherapy alone in a meta-analysis of five randomised controlled trials (44). Similarly, improvements in local control and survival have been demonstrated by the addition of platinum-based chemotherapy to radiotherapy for cancer of the head and neck, lung cancer, rectal cancer and oesophageal cancer (45). It has been shown previously that pol η mRNA and protein levels can vary significantly in gastric cancer and lung cancer tissues from patients and that pol η levels may predict clinical response to cisplatin or oxaliplatin chemotherapy (46,47). In the context of our data presented here and the variation in pol η protein levels that appear to exist within tissues (46),

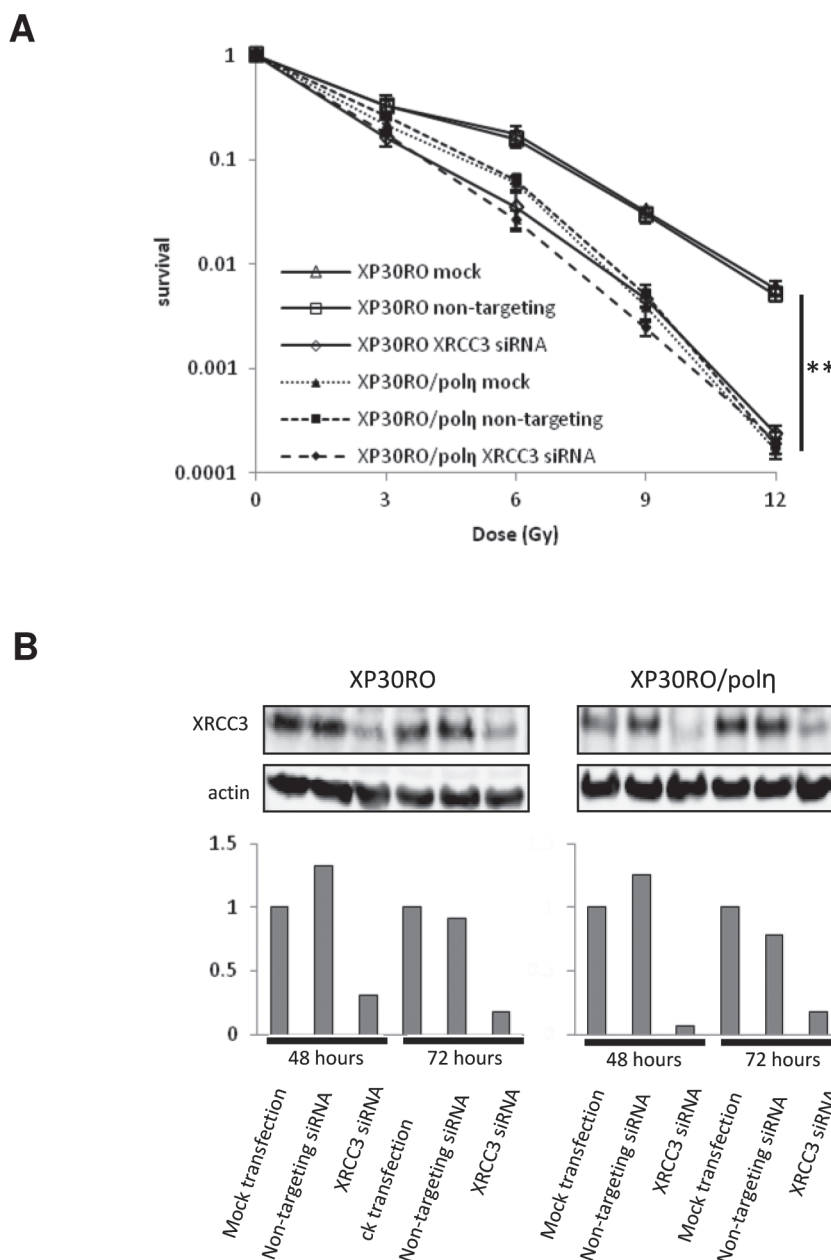


Fig. 5. Abrogation of radioresistance of pol η -deficiency by knockdown of the HR protein, XRCC3. (A) Clonogenic survival assays were performed in XP30RO and XP30RO/pol η cells after XRCC3 knockdown for 48 h and then treatment with IR. Controls used mock transfection and non-targeting siRNA. Each data point represents three independent experiments; error bars show the standard error of the mean. Statistical analysis performed using Student's *t*-test; ***P* < 0.01. (B) Western blot analysis showing knockdown efficiency of XRCC3 siRNA after 48 and 72 h. Quantification shows more than 70% knockdown efficiency for both cell lines.

we propose that cells within a tumour with high levels of pol η may be more radiosensitive, yet may be more resistant to platinum chemotherapy, and that cells within the same tumour with low levels of pol η protein may be more sensitive to chemotherapy yet may be more radioresistant. Based on this translational hypothesis, we suggest the need for clinical studies to quantify the heterogeneity of expression of pol η within human cancer tissue to determine its potential utility as a predictive biomarker for platinum chemotherapy and radiotherapy and when both treatment modalities are necessary for effective treatment.

Supplementary material

Supplementary Figures 1 and 2 can be found at <http://carcin.oxford-journals.org/>

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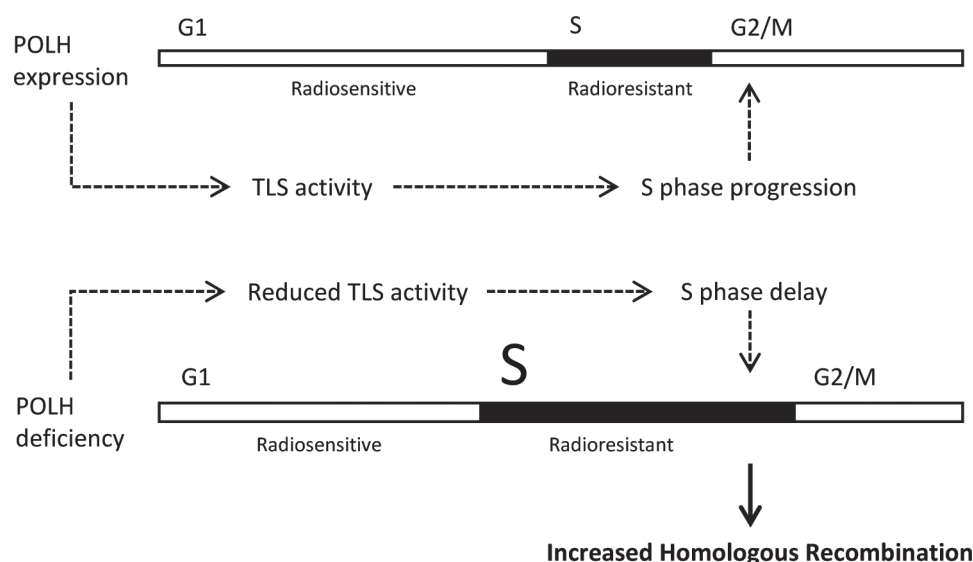


Fig. 6. Hypothetical model for the increase in radioresistance mediated by pol η deficiency. Cells proficient in pol η -mediated translesion synthesis progress through S-phase and have a higher proportion of cells in radiosensitive phases of the cell cycle. In contrast, cells lacking pol η are delayed in S-phase, presumably since they need to employ other S-phase-specific DNA repair mechanisms to deal with damage to DNA by endogenous and exogenous factors. Redistribution of pol η -deficient cells towards the relatively radioresistant stages of S-phase decreases overall radiosensitivity, probably mediated via a dependence on homologous recombination repair.

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