REV1 and polymerase ζ facilitate homologous recombination repair

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ABSTRACT

REV1 and DNA Polymerase ζ (REV3 and REV7) play important roles in translesion DNA synthesis (TLS) in which DNA replication bypasses blocking lesions. REV1 and Polζ have also been implicated in promoting repair of DNA double-stranded breaks (DSBs). However, the mechanism by which these two TLS polymerases increase tolerance to DSBs is poorly understood. Here we demonstrate that full-length human REV1, REV3 and REV7 interact *in vivo* (as determined by co-immunoprecipitation studies) and together, promote homologous recombination repair. Cells lacking REV3 were hypersensitive to agents that cause DSBs including the PARP inhibitor, olaparib. REV1, REV3 or REV7-depleted cells displayed increased chromosomal aberrations, residual DSBs and sites of HR repair following exposure to ionizing radiation. Notably, cells depleted of DNA polymerase η (Polη) or the E3 ubiquitin ligase RAD18 were proficient in DSB repair following exposure to IR indicating that Polη-dependent lesion bypass or RAD18-dependent monoubiquitination of PCNA are not necessary to promote REV1 and Polζ-dependent DNA repair. Thus, the REV1/Polζ complex maintains genomic stability by directly participating in DSB repair in addition to the canonical TLS pathway.

INTRODUCTION

Homologous recombination (HR) is a key pathway in mammalian cells for the repair of complex lesions including collapsed replication forks, interstrand DNA crosslinks and DSBs. During HR repair, the RAD51 protein forms nucleofilaments on resected 3' single-stranded DNA (ssDNA) formed at a DSB and promotes strand invasion into a homologous stretch of DNA, often the sister chromatid present during late S and G2 phases of the cell cycle. The invaded strand serves as a primer for DNA synthesis resulting in the generation of two restored duplex DNAs that are ultimately resolved by Holliday junction processing enzymes or through a DNA strand displacement and annealing mechanism referred to as 'synthesis-dependent strand annealing' (1,2). Cells deficient in a factor known to regulate or carry out HR repair typically display characteristic phenotypes indicative of genomic instability. This includes the accumulation of chromosomal aberrations and hypersensitivities to agents that directly or indirectly create DSBs. Although many of the proteins that participate in the early and late steps of HR have been fairly well characterized, the identity of the DNA polymerases involved in duplicating the sister chromatid sequence during HR repair have remained elusive. Genetic studies in yeast have identified roles for both DNA polymerases delta and epsilon (3–7). Among the TLS polymerases, Polη has been implicated in participating in HR repair based on both biochemical analyses and genetic studies performed in chicken DT40 cells (8,9). The observations that inherited truncating mutations in Polη are primarily associated with photosensitivity and skin cancer, and cell lines derived from such patients are not abnormally sensitive to ionizing radiation (IR), suggest that alternative DNA polymerases are important for HR repair in humans (10,11).

Polζ (polymerase zeta) is a leading candidate for facilitating HR repair since cellular deficiencies in this TLS polymerase are associated with radiosensitivity, embryonic lethality in mice, and high frequencies of chromosomal aberrations, phenotypes similar to those exhibited by HR repair deficient cells (12–17). In yeast and vertebrates, the Y-family polymerase REV1 is thought to promote Polζ-dependent TLS with the latter performing

an essential role in TLS by acting as an extension poly-merase following the insertion of a nucleotide opposite a wide variety of DNA lesions (18,19). Based on these ob-servations, we tested the hypothesis that both REV1 and Polζ are important for HR repair in human cells. Specifically, we examined whether full-length human REV1, REV3 and REV7 associate with one another in intact cells via co-immunoprecipitation studies and determined the importance of each gene product in facilitating HR repair via gene conversion. Our studies show that depletion of human REV1, REV3 or REV7 leads to very similar defects in DNA repair after IR or a site-specific DSB and demonstrate that REV1 and Polζ protect against IR-induced genomic instability. The fact that cells deficient in the RAD18 E3 ligase, the primary regulator of TLS, failed to exhibit similar deficiencies in our model system suggests that REV1 and Polζ operate in a DSB repair pathway separate from the canonical translesion DNA synthesis pathway.

MATERIALS AND METHODS

Cell culture, siRNA, flow cytometry and viability assays

HeLa and 293T/17 cells were obtained from the American Tissue Culture Collection (ATCC) and cultured in DMEM supplemented with 10% fetal bovine serum. U2OS and SV40-immortalized human fibroblasts contain-ing the DR-GFP reporter were obtained from Maria Jasin and cultured in DMEM supplemented with 10% fetal bovine serum. The human BL2 Burkitt's lymphoma cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (20). All siRNA duplexes were purchased from Qiagen and transfected into HeLa cells using X-tremeGENE reagent (Roche) as described (21). The sequences of siRNA targeting REV1, REV3, REV7, Polη, RAD18, FANCD2 and RAD51 are described and characterized with respect to knockdown efficiency (Supplementary Data; Supplementary Figures S1 and S2). Clonogenic survival assays and chromosomal aberration analyses were performed as described (21). Cells were irradiated at room temperature at a dose rate of 3 Gy/min using a Pantak DXT300 orthovoltage unit. Neocarzinostatin, etoposide, camptothecin and 30% H_2O_2 were purchased from Sigma. Olaparib was purchased from LC Laboratories. For cell cycle analysis, siRNA transfected HeLa cells were fixed in 70% ethanol, stained with propidium iodide in the presence of RNase A, and then analyzed on a FACSCaliber flow cytometer (Becton Dickinson). Viability was determined by measuring the percentage of BL2 cells excluding propidium iodide using an Accuri C6 flow cytometer.

Antibodies

Rabbit polyclonal anti-Polη (H-300), anti-53BP1 (H-300) and anti-REV1 (H-300) antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-ATM (Ser 1981, Rockland), anti-Rad51 (clone 14B4, GeneTex), and anti-RPA p34 (Ab-1, Lab Vision/NeoMarkers) were used for immunoflourescence. Anti-MAD2L2 (REV7) and anti-RAD18 polyclonal antibodies were obtained through Proteintech Group. Anti-Flag M2 monoclonal antibody and M2 antibody-conjugated agarose were purchased from Sigma-Aldrich. S1981P-ATM and 53BP1 immunofluorescence were performed as described (21). For immunofluorescent staining of RAD51 foci, cells were fixed in 3.7% para-formaldehyde supplemented with 0.5% triton-X-100, and then stained with monoclonal antibody followed by goat anti-mouse Alexa Fluor-488 (Invitrogen). For immunofluorescent staining of RPA p34 foci, cells were incubated in a detergent-containing buffer (0.5% triton X-100, 20 mM HEPES pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl and 300 mM sucrose) for 5 min at 4°C in order to reveal detergent extraction-resistant RPA p34 protein. Cells were then fixed in 3.7% para-formaldehyde supplemented with 0.5% triton-X-100 and stained as described above.

Co-immunoprecipitation assay

The plasmids used in these studies are described in the 'Materials and Methods' in Supplementary Data. 293T cells (2×10^6 cells/dish) were transfected with a total of 12 μg plasmid DNA (consisting of REV3-Flag alone or in combination with GFP-REV1 or GFP-REV7 at a 8.5:1.5 ratio) using the calcium phosphate method or X-tremeGENE siRNA Transfection Reagent to deliver plasmid DNA and/or siRNA into the cells. Cells were harvested 48 h later and lysed in 100-CSK buffer (10 mM PIPES [pH 6.8], 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA and 1 mM EDTA) containing 0.5% TX-100, 1X protease inhibitor cocktail (Roche), 1X phosphatase inhibitor cocktails 1 and 2 (Sigma Aldrich) and 1000 U of Benzonase (Sigma Aldrich) for 45 min on ice. The NaCl concentration was then adjusted to 300 mM NaCl and the samples were further incubated for 30 min on ice. After centrifugation (15 000 rpm for 20 min), the supernatant was diluted to 150 mM NaCl before immunoprecipitation. Cell extracts (equivalent to lysates obtained from eight 60 mm dishes) were pre-cleared with protein G plus/Protein A agarose (Calbiochem) prior to incubation with anti-FLAG M2 agarose or anti-GFP monoclonal (Roche) plus Protein G plus/Protein A for 2 h at 4°C. The beads were then washed 3× with 0.1% TX-150 mM CSK buffer. The immunoprecipitates were denatured and separated on SDS–PAGE gels. The proteins were transferred onto a nitrocellulose membrane and probed with the appropriate primary antibodies followed by secondary horseradish peroxidase (HRP) conjugated goat anti-rabbit or mouse antibodies (Thermo Scientific). Proteins were visualized using SuperSignal West Pico Chemiluminiscent Substrate (Thermo Scientific).

Homologous recombination assay

HeLa cells containing an integrated copy of the DR-GFP reporter substrate were constructed as described (22). DR-GFP-containing HeLa, U2OS or SV40-immortalized human fibroblasts were transfected twice with siRNA and then transduced with AdNGUS24i adenovirus encoding I-*SceI*. Two days later, the recombination frequency was

determined as the percentage of cells expressing GFP protein following flow cytometry analysis on an Accuri C6 flow cytometer.

RESULTS

REV1, REV3 and REV7 form a complex

Genetic evidence in yeast demonstrate that REV1, REV3 and REV7 are epistatic when performing lesion bypass suggesting they operate in a linear pathway or together as a complex (23). Consistent with this concept, yeast Rev1 and Polζ have been shown to physically interact; however, attempts to determine interactions of human REV3 with REV1 or REV7 have been limited to the analysis of small protein fragments due to the difficulty in purifying full-length REV3 (24–27). REV3 protein is expressed at very low levels in human cell lines and our attempts to detect endogenous REV3 with available antibody reagents were not successful. We therefore constructed an epitope-tagged version of REV3 and found that we can express Flag-tagged REV3 in 293T cells at detectable levels following immunoprecipitation and immunoblotting. To determine whether full length REV3 associates with REV1 and REV7 in human cells, we overexpressed Flag-tagged REV3 in 293T cells along with either GFP-REV1 or GFP-REV7 and immunopurified REV3-Flag via Flag-conjugated agarose. Although we had difficulty detecting REV3-Flag in the input lanes, we did observe that REV3-Flag associates with GFP-REV1 or GFP-REV7 when co-expressed after Flag immunoprecipitation (Figure 1A). Furthermore, endogenous REV1 or REV7 were co-purified with REV3-Flag immunoprecipitates when co-expressed with GFP-REV7 or GFP-REV1, respectively. We were also able to detect REV3-Flag associated with either GFP-REV1 or GFP-REV7 protein immunoprecipitated with GFP antibody in the reverse experiment (Figure 1B).

To further examine whether endogenous REV1 and REV7 proteins associate with REV3, we co-transfected REV3-Flag with siRNA specific for REV1 or REV7. As shown in Figure 1C, we observed association of endogenous REV1 and REV7 with REV3-Flag in anti-Flag immunoprecipitates, which demonstrates that REV1 and REV7 likely associate with REV3 independently of one another. Depletion of REV1 or REV7 does not affect the ability of the other to associate with REV3. Recently, Hara *et al.* (27) proposed that the interaction of REV3 with REV7 occurs first and this interaction produces a change in REV7 conformation such that a REV1 binding site is revealed. Our results indicate that full-length REV3 may harbor an additional binding site for REV1 based on the fact that REV7 does not appear to be necessary for REV3-Flag and REV1 to co-immunoprecipitate. Together, these results suggest that full length REV3 associates with both REV1 and REV7 in cells forming a ternary complex, consistent with the model that these proteins directly interact and cooperate together to perform lesion bypass and DSB repair (24,25).

REV1 and Polζ promote radioresistance and prevent IR-induced chromosomal instability

To investigate whether the REV1/Polζ complex is involved in promoting HR in human cells, we used siRNA to deplete REV1, REV3 or REV7 in HeLa cells as demonstrated in Supplementary Figure S1 (21). We validated our system by comparing the effects depleting REV1 or Polζ have on the cellular response to UV-C. It is well established that lesion bypass of UV-C-induced DNA damage requires Polη and/or REV1 and Polζ for efficient TLS (18,28–32). We therefore confirmed the effectiveness of depleting REV1, REV3, REV7 or Polη by showing that HeLa cells depleted of each individual protein arrested in S phase and accumulated γ-H2AX as an indication of extensive replication fork stalling following exposure to UV-C irradiation (Supplementary Data, Supplementary Figure S2). We next examined whether HeLa cells deficient in REV1 or Polζ exhibit altered responses to IR. HeLa cells are deficient in the G1 cell cycle checkpoint but are proficient in establishing the G2 cell cycle checkpoint ~12 h after IR. By 24 h, cells recover from growth arrest in G2 and return to a normal cell cycle distribution (data not shown). HeLa cells transfected with REV1, REV3 or REV7 siRNA were capable of generating an IR-induced G2 arrest but failed to recover and re-enter the cell cycle after 24 h suggesting a deficiency in DSB repair and presence of residual DNA damage (Figure 2A). The IR-induced prolonged G2 cell cycle checkpoint was not observed in HeLa cells transfected with either non-specific siRNA (Control) or siRNA targeting the TLS polymerase Polη. The specificity of this effect was further confirmed by using more than one siRNA sequence targeting each gene product. Consistent with a role in DSB repair, depletion of REV1, REV3 or REV7 resulted in enhanced radiosensitivity and the accumulation of chromosomal gaps and breaks (predominately the chromatid type) 24 h following exposure to IR (Figure 2B and C, respectively). Representative images of mitotic spreads isolated from irradiated HeLa cells are shown in Supplementary Data, Supplementary Figure S3.

To gain additional evidence that REV1 and Polζ are important for repairing IR-induced DSBs, we measured the induction and resolution of foci consisting of phosphorylated ATM (S1981P-ATM) and the checkpoint mediator protein, 53BP1. Both of these proteins localize to sites of DSBs as visible foci following immunofluorescent staining and are considered sensitive surrogate markers of DSBs (33,34). Irradiation of HeLa cells with 2 Gy resulted in extensive foci formation within 15 min, which resolved by 24 h consistent with completion of DSB repair, and the release from G2 cell cycle checkpoint control (Figure 2A; Supplementary Data and Supplementary Figure S4). In contrast to HeLa cells transfected with Control siRNA, cells transfected with siRNA specific for REV1, REV3 or REV7 failed to resolve S1981-phosphorylated ATM foci colocalized with 53BP1 within 24 h (Figure 2D and E; Supplementary Data, Supplementary Figure S5).
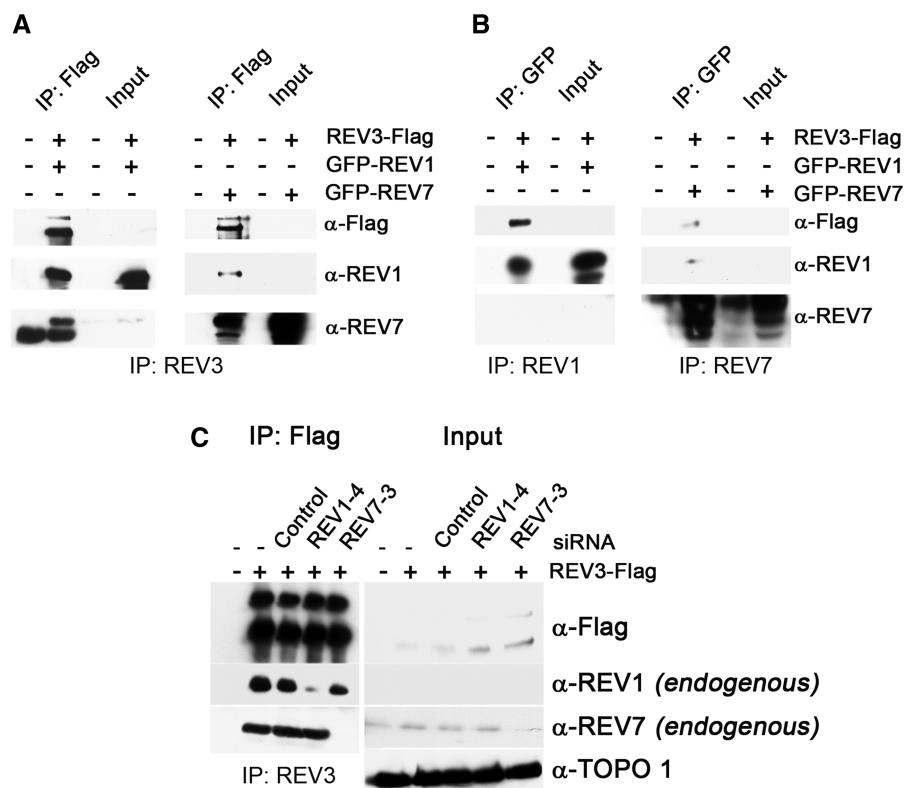
Figure 1. REV1, REV3, and REV7 form a ternary complex. 293T cells were transfected with a plasmid encoding REV3-Flag and a control plasmid or combinations of REV3-Flag with GFP-REV1 or GFP-REV7 encoding plasmids. Cell lysates were treated with benzonase, pre-cleared, and then subjected to immunoprecipitation and immunoblotting with the indicated antibodies. (**A**) REV3-Flag immunoprecipitates contain GFP-REV1 and endogenous REV7 (left) or GFP-REV7 and endogenous REV1 (right). (**B**) GFP-REV1 immunoprecipitates contain REV3-Flag (left). GFP-REV7 immunoprecipites contain REV3-Flag and endogenous REV1 (right). (**C**) REV3-Flag immunoprecipitates contain both endogenous REV1 and REV7. Specificity of REV1 and REV7 antibodies are demonstrated by the depletion of each protein with gene-specific siRNA co-transfected with REV3-Flag expression vector.

REV1 and Polζ are required for efficient homologous recombination repair

We next tested whether HeLa cells depleted of REV1, REV3 or REV7 were deficient in HR repair. A clonal population of HeLa cells were established containing an integrated DR-GFP reporter commonly used to measure gene conversion events induced by a site-specific DSB (22) (Figure 3A). Depleting cells of RAD51 essentially eliminated the ability of HeLa cells to efficiently carry out gene conversion as expected (Figure 3B; Supplementary Data and Supplementary Figure S6). Reduction of the principle regulator of TLS, RAD18 or Polη via siRNA resulted in very small decreases in HR repair induced by the I-*SceI* endonuclease (Figure 3B; Supplementary Data and Supplementary Figure S6). Importantly, we observed a significant reduction in gene conversion frequency in HeLa cells transfected with several different siRNAs targeting each individual REV protein using the DR-GFP reporter system, levels similar to those observed when HeLa cells are depleted of components of the Fanconi anemia pathway such as the FANCD2 protein (35,36) (Figure 3B). We extended this analysis to other cell lines such as U2OS osteosarcoma cells and human SV40-immortalized fibroblasts. Again, we found little impact on gene conversion efficiencies

when cells were deficient in either RAD18 or Polη (Figure 3C and D). Depletion of REV3 consistently interfered with HR repair efficiency in all three cell lines. Additionally, in the Hela DR-GFP cell line, co-depletion of REV1 and REV3, REV1 and REV7 or REV3 and REV7 did not produce additive effects on reducing gene conversion frequencies suggesting that these proteins participate in the same pathway to promote HR repair (Figure 3B). Consistent with an inability to complete HR repair of IR-induced DSBs, REV1, REV3 or REV7 siRNA transfected HeLa cells accumulated foci positive for the RAD51 protein (Figure 3E) or replication protein A, the latter marking resected DNA (Figure 3F). Overall, our results demonstrate that REV1 and Polζ contribute to HR repair of a defined DSB and provide a mechanistic explanation for the elevated numbers of DSBs (marked by S1981P-ATM and 53BP1) observed in irradiated cells deficient in these two TLS polymerases. Furthermore, the data suggest that REV1 and Polζ participate in a late step in HR, after the association of RPA and RAD51 at sites of DSBs, which is consistent with the REV1/Polζ polymerase complex participating in HR repair synthesis.

Next, we wanted to differentiate whether the apparent lack of DSB resolution observed in REV1, REV3
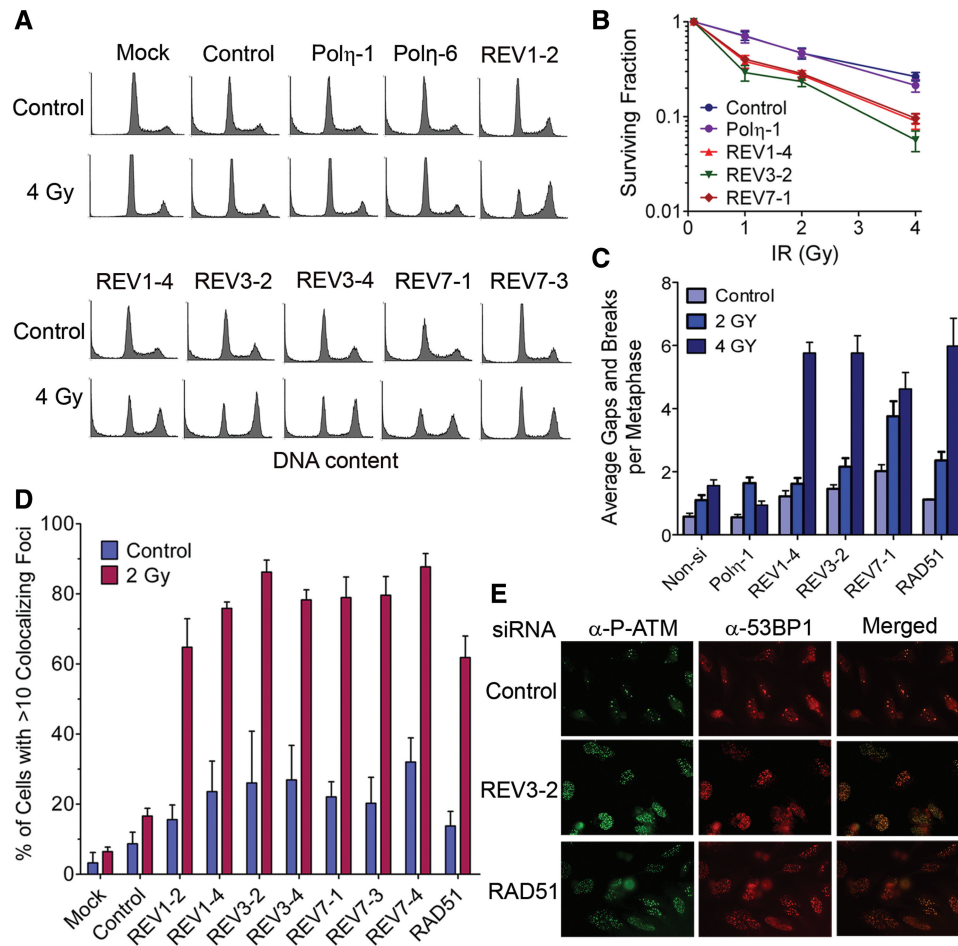
Figure 2. REV1 or Polζ (REV3 and REV7) depletion leads to hypersensitivity to ionizing radiation and a defect in DSB repair. HeLa cells were transfected with control siRNA (Control) or the indicated siRNAs targeting a specific DNA polymerase gene and assessed for their response to IR. (**A**) Depletion of REV1, REV3, or REV7 is associated with a prolonged IR-induced G2 cell cycle checkpoint. Cells were exposed to 0 or 4 Gy IR and subjected to cell cycle analysis 24 h later by flow cytometry. (**B**) Sensitivity to IR was measured using a standard clonogenic survival assay. Data are presented as the mean ± S.E.M from three independent experiments. (**C**) siRNA transfected HeLa cells were examined for the presence of chromosomal aberrations (gaps and breaks) 24 h after 2 or 4 Gy IR. Data from a representative experiment is expressed as the average number of gaps and breaks per metaphase ± SEM ($n = 50$). (**D**) HeLa cells transfected with the indicated siRNAs were exposed to 2 Gy IR and then fixed 24 h later. Cells were immunostained with S1981P-ATM and 53BP1 to mark sites of DSBs. The percentage of cells displaying 10 or more colocalized foci is shown as the mean ± SEM from three independent experiments. Representative images are shown in (**E**) and additional quantification data are shown in Supplementary Figure S5. HeLa cells transfected with RAD51-specific siRNA (HR-repair defective) are shown for comparative purposes in (C–E).

or REV7-depleted cells was due to a true deficiency in DSB repair, as opposed to the accumulation of replication-associated DSBs caused indirectly by the absence of TLS in irradiated cells. RAD18 is well characterized for its role in regulating TLS and post-replication repair in yeast and vertebrates by promoting the monoubiquitination of PCNA at stalled replication forks (37). Given the role of RAD18 in regulating REV1 and Polζ-dependent TLS, we tested whether RAD18-dependent PCNA monoubiquitination was important for the response of HeLa cells to IR. Depleting HeLa cells of RAD18 induced a pronounced cell cycle arrest in S phase and intense staining with the γ-H2AX antibody 24 h after exposure to UV-C irradiation demonstrating efficient knock down and diminished TLS activity promoted by RAD18 (Figure 4A and C). However, HeLa cells depleted of RAD18 did not exhibit

a prolonged G2 cell cycle checkpoint following IR (Figure 4B). RAD18-depleted HeLa cells efficiently resolved IR-induced foci consisting of S1981-phosphorylated ATM and 53BP1 suggesting that the lack of monoubiquitinated PCNA-regulated TLS per se does not lead to the accumulation of DSBs in irradiated cells progressing through S phase (Figure 4D and E).

Polζ-deficient cells are hypersensitive to agents that cause DSBs

To further investigate if Polζ is involved in HR repair, we tested whether human BL2 lymphoma cells deleted of the *REV3L* gene were hypersensitive to stresses that cause DSBs. Two different clones of *REV3L* knockout BL2 cells were examined in comparison to wild-type, Polη$^{-/-}$ and Polι$^{-/-}$ (polymerase iota) knockout cells. Both REV3$^{-/-}$ clones displayed a significant decrease in
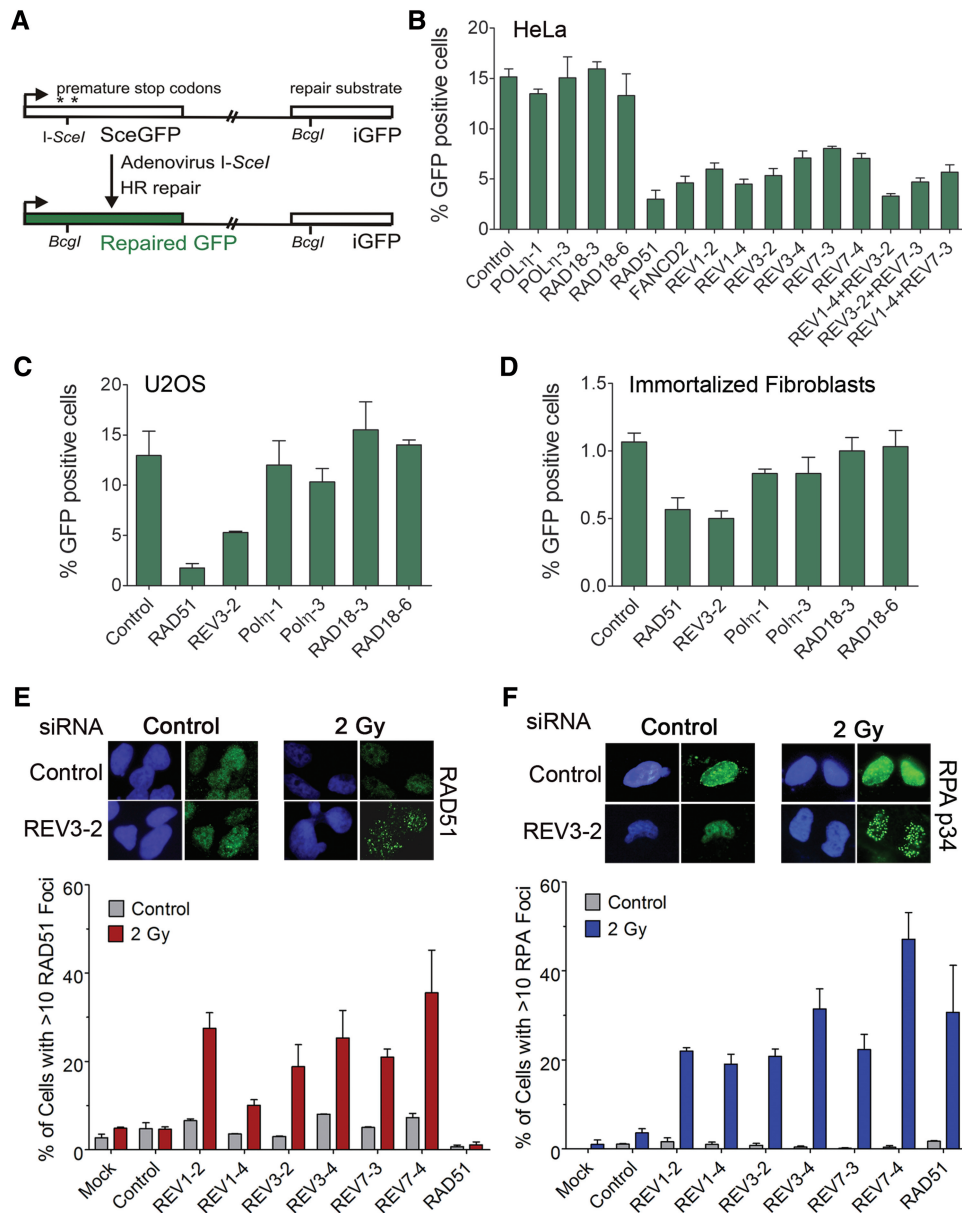
Figure 3. REV1 and Polζ promote HR repair. HeLa cells containing the DR-GFP reporter construct (**A**) integrated into the genome were transfected twice with the indicated siRNAs. The next day, cells were infected with adenovirus AdNGUS24i expressing the I-*SceI* enzyme. (**B**) Two days later, cells were assessed for GFP expression by flow cytometry. Control adenovirus did not promote detectable expression of GFP protein (data not shown). (**C** and **D**) U2OS or SV40-imortalized human fibroblasts containing the DR-GFP reporter were transfected twice with the inidicated siRNAs and analyzed as in (B). (**E** and **F**) REV1, REV3 or REV7-depleted HeLa cells display elevated numbers of RAD51 or RPA/p34 foci 24 h after exposure to 2 Gy IR. The percentage of cells exhibiting 10 or more RAD51 foci (E) or RPA/p34 (F) is shown for each siRNA. Representative images of cells scoring positive (REV3-2 transfected cells treated with 2 Gy IR) are shown. (B through F) Data are the mean ± SEM of at least three independent experiments.

viability following exposure to IR as expected. The Polη and Polι knockout BL2 cells were relatively radioresistant, similar to wild-type BL2 cells (Figure 5). This same trend in hypersensitivity to DNA damaging agents was observed when REV3$^{-/-}$ cells were challenged with neocarzinostatin, a radiomimetic drug that induces DNA damage similar to IR (Figure 5). The absence of REV3 did not confer increased sensitivity to H$_2$O$_2$, a chemical which through oxygen radical formation generates predominately base lesions and ssDNA breaks but very few DSBs (Figure 5). In contrast, BL2 cells deficient in REV3 were

hypersensitive to etoposide, a topoisomerase II poison that induces protein-associated DSBs, and mildly sensitive to camptothecin, a topoisomerase I directed-drug which causes replication-associated DSBs (Figure 5). Together these results suggest that REV3-deficient cells are particularly sensitive to treatments that cause DSBs rather than base lesions or ssDNA breaks. The fact that REV3L-deleted cells were hypersensitive to olaparib, an inhibitor of poly(ADP-ribose) polymerases, is consistent with these cells having a defect in HR repair (Figure 5) (38–40).
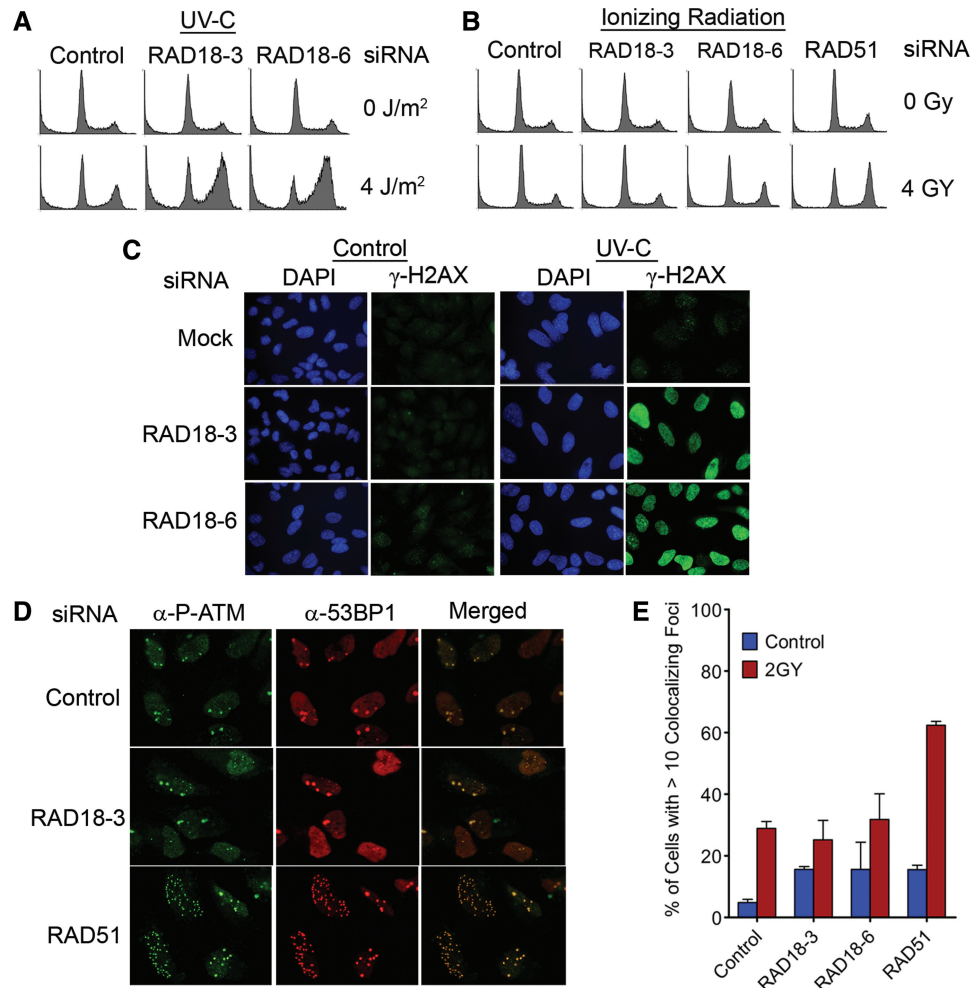
Figure 4. REV1 and Polζ promote radioresistance independently of RAD18. (**A** and **B**) HeLa cells transfected with siRNAs targeting RAD18 or RAD51 were exposed to 4 J/m² UV-C light or 4 Gy IR as indicated. Twenty-four hours later, cells were subjected to cell cycle analysis by flow cytometry to measure DNA content per cell. (**C**) HeLa cells depleted of RAD18 stain intensely for γ-H2AX 24 h after exposure to UV-C irradiation indicative of strong DNA damage response due to replication fork stalling and efficient protein knockdown. (**D**) RAD18-depleted HeLa cells are proficient in resolving foci containing S1981P-ATM and 53BP1 within 24 h. Cells transfected with RAD51 siRNA were analyzed as a positive control for HR repair deficiency. The mean percentage of cells exhibiting 10 or more co-localized foci is shown in (**E**). Data are the mean ± SEM of three independent experiments.

DISCUSSION

Although yeast or chicken DT40 cells lacking Polζ are proficient in gene conversion induced by a defined DSB (3,12,41), our data suggest that the REV1/Polζ complex participates in this specific repair pathway in human cells. When considering DSBs induced by less specific means, such as exogenous or endogenous chemicals, REV1 and Polζ may play an important role in replicating damaged ssDNA formed after resection. DNA damage induced by IR is complex and consists of single strand breaks, base lesions, sugar damage and apurinic/apyrimidinic sites (AP sites) (42). IR is also capable of creating clustered DNA damage in which two or more lesions are generated within close proximity of each other, and DSBs when lesions occur on opposite DNA strands (43). Thus, IR-induced DSBs are likely accompanied by additional localized base damage. In yeast, deletion of Rev1 or Rev3 results in a reduction of global IR-induced mutagenesis, as well as

mutagenesis associated with ssDNA formed during DSB repair (41,44–46). We found that human cells depleted of REV1, REV3 or REV7 are deficient in resolving DSBs and chromosomal aberrations induced by IR. We therefore postulate that the mammalian REV1/Polζ complex may be necessary to bypass lesions or aberrant structures formed in ssDNA as a product of strand resection and HR. In this scenario, REV1/Polζ-dependent DNA synthesis may be necessary to alleviate replication stalling during HR repair synthesis, including resected DNA formed subsequent to a defined DSB. Interestingly, our results suggest that REV1 and Polζ perform this function independently of RAD18 and associated PCNA monoubiquitination.

REV3⁻/⁻ BL2 cells are hypersensitive to a variety of agents that mechanistically create DSBs in the absence of base lesions suggesting that Polζ plays a direct role in promoting HR repair. These results are consistent with
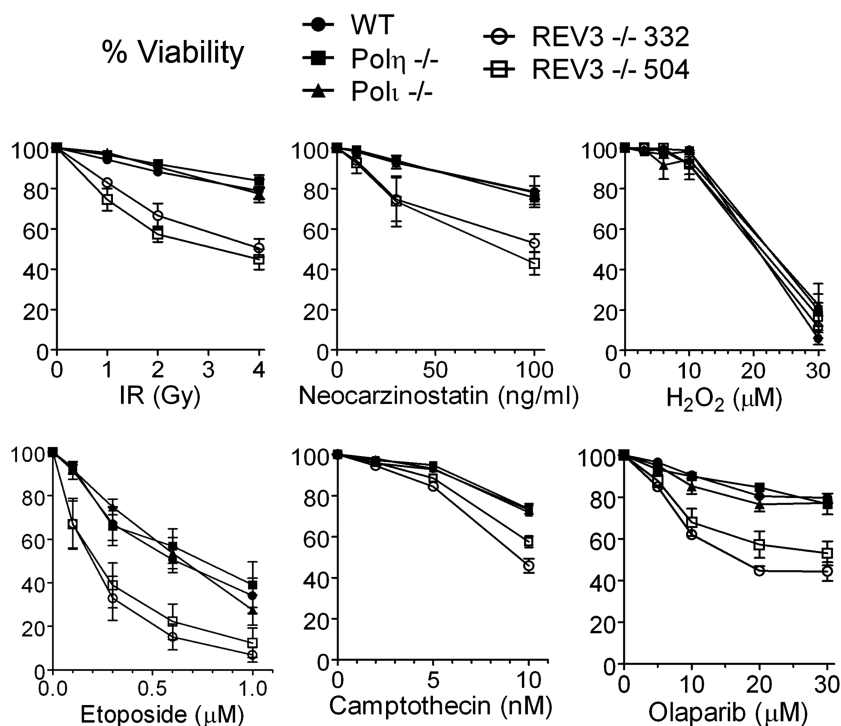
Figure 5. Deletion of the REV3L gene leads to hypersensitivity to agents that induce DSBs. Parental BL2 cells (WT) and BL2 cells deleted of the indicated genes were treated with various doses of DNA damaging agent and harvested 48 (IR, Neocarzinostatin, H_2O_2) or 72 h (etoposide, camptothecin, olaparib) later. Viability was determined by measuring the percentage of cells excluding propidium iodide by flow cytometry. Data represent the mean ± SEM of at least three independent experiments.

those observed in the model system—*Drosophila melanogaster*, where HR repair is also impaired in the absence of Polζ by ~50% (D. Kane and M. McVey, personal communication). Polζ displays the unique property of being able to extend mismatched primer DNA, a property thought to contribute to overall mutagenesis in yeast (25,47). In this model, REV1 and Polζ may directly participate in initial HR-repair synthesis, especially when the invading strand within a displacement loop (D-loop) contains a 3′ mismatch that would otherwise impede extension by other DNA polymerases.

Mammalian *REV3L* encodes a protein twice the size of yeast Rev3 and is essential for embryonic development (23). The recent observation that conditional loss of *REV3L* expression accelerates tumorigenesis in p53-null mice is consistent with Polζ playing an important role in preserving genomic stability by facilitating DSB repair (48). Persistence of DSBs due to REV3 deficiency can greatly affect genomic stability and contribute to cellular transformation. Although Polζ was originally associated with mutagenesis in yeast, these new observations made in mice and the results reported here indicate that mammalian Polζ acquired additional functions serving to protect genomic integrity in higher eukaryotes by directly facilitating HR repair.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures S1–S7, Supplementary methods.

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