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Differential requirements for DNA repair proteins in immortalized cell lines using alternative lengthening of telomere mechanisms

Alaina R. Martinez^{1,2}, Zeenia Kaul², Jeffrey D. Parvin^{1,*}, Joanna Groden^{2,*}

¹Department of Biomedical Informatics, The Ohio State University College of Medicine, Columbus, OH

²Department of Cancer Biology and Genetics, The Ohio State University College of Medicine, Columbus, OH

Abstract

Cancer cells require telomere maintenance to enable uncontrolled growth. Most often telomerase is activated, although a subset of human cancers are telomerase-negative and depend on recombination-based mechanisms known as ALT (Alternative Lengthening of Telomeres). ALT depends on proteins that are essential for homologous recombination, including BLM and the MRN complex, to extend telomeres. This study surveyed the requirement for requisite homologous recombination proteins, yet to be studied in human ALT cell lines, by protein depletion using RNA interference. Effects on ALT were evaluated by measuring C-circle abundance, a marker of ALT. Surprisingly, several proteins essential for homologous recombination, BARD1, BRCA2 and WRN, were dispensable for C-circle production, while PALB2 had varying effects on C-circles among ALT cell lines. Depletion of homologous recombination proteins BRCA1 and BLM, which have been previously studied in ALT, decreased C-circles in all ALT cell lines. Depletion of the non-homologous end joining proteins 53BP1 and LIG4 had no effect on C-circles in any ALT cell line. Proteins such as chromatin modifiers that recruit double-strand break proteins, RNF8 and RNF168, and other proteins loosely grouped into excision DNA repair processes, XPA, MSH2 and MPG, reduced C-circles in some ALT cell lines. MSH2 depletion also reduced recombination at telomeres as measured by intertelomeric exchanges. Collectively, the requirement for DNA repair proteins varied between the ALT cell lines compared. In sum, our study suggests that ALT proceeds by multiple mechanisms that differ between cell lines and that some of these depend on DNA repair proteins not associated with homologous recombination pathways.

INTRODUCTION

Telomeres are chromatin structures at chromosome ends that are critical for maintaining genomic integrity in normal somatic cells. Telomeres consist of TTAGGG DNA repeats and a protein complex known as shelterin that binds telomeric DNA to prevent recognition of the ends by the DNA repair machinery. Telomeres will shorten with each DNA replication cycle

*Correspondence: Joanna Groden; 986 Biomedical Research Tower, 460 West Twelfth Avenue, Columbus, Ohio 43210; Telephone: 614-292-0523; Joanna.Groden@osumc.edu; and Jeffrey D. Parvin; 904 Biomedical Research Tower, 460 West Twelfth Avenue, Columbus, Ohio 43210; Telephone: 614-685-4685; Jeffrey.Parvin@osumc.edu.

until telomeres become “too short” and trigger cellular senescence or apoptosis. In germ cells and somatic stem cells, the enzyme telomerase lengthens telomeres to counteract the loss of DNA sequences that occurs with DNA replication. In tumors, activation of telomere maintenance mechanisms confers immortality.

There are at least two broad categories of telomere maintenance mechanisms (TMM), one that uses the enzyme telomerase and a second category of recombination-based mechanisms known as alternative lengthening of telomeres (ALT) (Murnane *et al.*, 1994; Dunham *et al.*, 2000). Some tumors are composed of a mixture of cells using one or the other mechanism (Gocha *et al.*, 2013), while some mouse models of cancers suggest fluidity, with shifts between these telomere maintenance mechanisms (Chen *et al.*, 2010; Hu *et al.*, 2012). Expression of telomerase is associated with *de novo* synthesis of telomeres and is activated in the majority of tumors. Another subset of tumors maintains telomeres in the absence of telomerase and uses ALT. Cells in these tumors display distinct characteristics such as heterogeneous telomere lengths (Bryan *et al.*, 1995), ALT-associated PML bodies (APBs) (Yeager *et al.*, 1999), increased telomere-sister chromatid exchanges (T-SCEs) (Bechter *et al.*, 2004; Londoño-Vallejo *et al.*, 2004) and the abundance of linear and circular, single-stranded or double-stranded, extrachromosomal telomere repeat DNA (Ogino *et al.*, 1998; Cesare and Griffith, 2004). One type of extrachromosomal telomere repeat DNA, a C-circle, correlates precisely with ALT in 38 different immortalized human ALT-positive cells lines. C-circles are present when other ALT phenotypes disappear, no telomerase expression is measured and telomere length is maintained (Henson *et al.*, 2009).

Although ALT cells have distinct phenotypes, the exact mechanisms of ALT are not fully understood. Evidence suggests that recombination is the primary method for ALT telomere elongation (Draskovic and Londono Vallejo, 2013). A unique DNA sequence integrated into the telomeric region of a chromosome can be propagated, over time, to telomeric regions of other chromosomes which can result in an increased number of tagged telomeres per ALT cell (Dunham *et al.*, 2000). This could occur through intertelomeric exchanges and/or when an adjacent chromosome with a longer telomere is used as a template to extend a short telomere. Extrachromosomal circular DNAs could be used as substrates for rolling circle amplification (RCA) as described in yeast (Nosek *et al.*, 2005). RCA of C-circles could enable rapid increases in telomere length and would explain the heterogeneous telomere length phenotype in ALT cells. Intratelomeric elongation could occur by copying the telomere loop, using a sister chromatid as a template or by unequal T-SCE between a short and longer sister chromatid to allow the short sister chromatid to gain telomere length (Muntoni *et al.*, 2009).

Telomere elongation by ALT is likely facilitated by a network of DNA repair proteins. The MRE11/RAD50/NBS1 (MRN) complex senses and processes double-strand breaks (DSBs) to initiate repair and is required for ALT (Zhong *et al.*, 2007). Sequestration of MRN inhibits APB formation and facilitates telomere shortening (Jiang *et al.*, 2005; Zhong *et al.*, 2007). Other DNA repair proteins that may facilitate ALT, implicated by their presence at telomere synthesis sites in APBs, are RAD51, RAD52, RPA, BRCA1, RAP1, WRN and some of the Fanconi anemia proteins (Yeager *et al.*, 1999; Wu *et al.*, 2003; Fan *et al.*, 2009; Gocha *et al.*, 2014). Chromatin-associated proteins such as ATRX, DAXX and H3.3 (Heaphy *et al.*, 2011;

Schwartzentruber *et al.*, 2012), expression of non-coding telomeric repeats-containing RNAs (TERRA) (Arora *et al.*, 2014), nuclear receptors (Conomos *et al.*, 2012; Conomos *et al.*, 2014) and proteins involved in meiotic recombination pathways are also associated with ALT (Cho *et al.*, 2014).

Our previous publications have demonstrated that the RecQ-like DNA helicase BLM is required for ALT, in addition to MRN, and that the homologous recombination (HR) protein BRCA1 associates with BLM to promote ALT activities (Lillard-Wetherell *et al.*, 2004; Bhattacharyya *et al.*, 2009; Acharya *et al.*, 2014; Gocha *et al.*, 2014). In this study, we tested the requirement for BRCA1 in ALT cell lines using C-circle abundance as an ALT marker (Henson *et al.*, 2009) and expanded the survey to include twelve other DNA repair proteins. *siRNA* knockdowns were used to reduce protein expression in human ALT cell lines. BRCA1 knockdown reduced C-circles in five ALT cell lines, although we found that some of the other requisite HR proteins did not alter the C-circle phenotype. Mismatch repair (MMR), nucleotide excision repair (NER) and base excision repair (BER) proteins were evaluated and also were required for C-circle maintenance in some ALT cell lines. These results expand the scope of previous studies showing that ALT is not a single process and that some mechanisms of ALT depend on DNA repair proteins in excision DNA repair pathways.

MATERIALS AND METHODS

Cell lines

The ALT cell lines U-2 OS, Saos-2 and WI-38 VA-13 subline 2RA were obtained from ATCC as well as the telomerase-positive cell line, HeLa (Manassas, VA). G-292 and JFCF-6/T.1J/1–3C cell lines were kindly provided by Dr. Roger Reddel (Bryan *et al.*, 1997; Ng *et al.*, 2009). Saos-2, U-2 OS and G-292 are ALT cell lines derived from human osteosarcomas; WI-38 VA-13/2RA is an ALT cell line derived from human lung fibroblasts; JFCF-6/T.1J/1–3C is an ALT cell line derived from jejunal fibroblasts from a cystic fibrosis patient; HeLa is a telomerase-positive cell line derived from a human cervical adenocarcinoma. All cell lines were maintained in Dulbecco's Modified Eagles Medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA), with the exception of U-2 OS cells which were maintained in McCoy's 5A Medium (Gibco, Thermo Fisher Scientific), supplemented with 10% FBS (HyClone, GE Healthcare, Logan, UT) and grown at 37 degrees C, 5% CO₂.

siRNA knockdown

The list of *siRNAs* and the sources of the *siRNAs* used in these experiments are listed in Table 1. *siRNAs* were transfected into cultured cells in triplicate using *Dharmafect 1* transfection reagent (Dharmacon, GE Healthcare, Lafayette, CO) at a final concentration of 50 nM. Cells were treated with two rounds of *siRNA* transfection 48 h apart and DNA was collected for C-circle assays 72 h following the second transfection.

Western blots

Whole cell extracts were prepared from each cell line using NP-40-containing lysis buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1x mammalian protease inhibitor cocktail [Sigma-Aldrich, St Louis, MO]). The following primary antibodies were used for western blotting: anti-BRCA1 OSU246 (specific to 400–1,100 amino acid region) (Towler *et al.*, 2013); anti-BRCA1 (Ab-1) (Calbiochem, EMD Millipore, Darmstadt, Germany); anti-LAMIN B (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies include horseradish-peroxidase conjugated goat-anti-mouse, rabbit-anti-goat (Jackson ImmunoResearch Laboratories, West Grove, PA) and goat-anti-rabbit (Cell Signaling Technology, Danvers, MA).

qRT PCR assays

RNA extracts were collected using TRIzol reagent (Ambion, Thermo Fisher Scientific, Austin, TX) and RNA reverse-transcribed using RNase OUT at 40 U (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA), Oligo dT at 0.5 µg (Invitrogen, Thermo Fisher Scientific), *Superscript II* reverse transcriptase at 200 U (Invitrogen, Thermo Fisher Scientific) per 1 µg of total RNA. cDNA (20 ng) was analyzed by quantitative PCR with primers (900 nM) listed in Table 1 and 1x Power Sybr Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA). Most primers have been used in previous publications with demonstrated specificity (Table 1). PCR reactions were performed on Applied Biosystems StepOnePlus Real Time PCR machine. The quantitative C_T Method was used to analyze data.

C-circle assays

Total DNAs were extracted using the “DNeasy Blood & Tissue Kit” (Qiagen, Hilden, Germany) and treated with 0.5 µg/µl RNase A (Sigma-Aldrich). DNAs were analyzed for C-circles as previously described (Henson *et al.*, 2009). Briefly, 50 ng (Saos-2, U-2 OS), 100 ng (G-292 and JFCF-6/T.1J/1–3C), or 200 ng (WI-38 VA-13/2RA) of total DNAs were digested with *HinfI* and *RsaI* restriction enzymes followed by *ExoI/ExoV/λExo* nucleases (New England BioLabs, Ipswich, MA). Half of the digested DNAs were subjected to the C-circle assay; C96 synthetic C-circle served as a positive control; water and lambda DNA served as the negative controls. Digested DNA, 400 ng total DNA for input, and the C-circle amplified products were dot-blotted onto a nylon positively charged membrane (Amersham Hybond, GE Healthcare, Little Chalfont, United Kingdom) and crosslinked twice using a Strategene UV Stratalinker 2400. An end-labeled ^{32}P -(CCCTAA)₃ probe was hybridized to membrane in Ultrahyb-Oligo Hybridization Buffer (Ambion, Thermo Fisher Scientific) rotating at 42 degrees C. Membranes were washed with 2x SSC, 0.5 % SDS at 42 degrees C and exposed to a phosphor screen (GE) overnight and imaged on a Typhoon FLA 7000 imager. ImageQuant (GE) was used to quantitate signals from total DNA, digested DNA and C-circle DNA dots. Signal obtained from any leftover undigested DNA was subtracted from signal obtained from corresponding C-circle DNA; resulting number was divided by signal obtained from corresponding total DNA.

Telomere-sister chromatid exchange assays

Chromosome orientation FISH (CO-FISH) was performed as described previously to detect T-SCEs and intertelomeric exchanges with minor adaptations (Williams *et al.*, 2011). Transfected cells were treated with 3:1 BrdU/C at 10 μ M for 18 h (MP Biomedicals, Solon, OH) and treated with colcemid at 0.1 μ g/mL for 4 h (Roche, Basel, Switzerland). Cells were collected, treated with hypotonic solution (75 mM KCl) for 30 min at 37 degrees C and fixed in 3:1 methanol: acetic acid. Metaphase spreads were prepared and stained as previously described. Slides were hybridized with the fluorescent probe Alexa 488-labeled PNA (TTAGGG)₃ at 0.05 μ M (PNA Bio Inc., Newbury Park, CA) in a hybridization cocktail of 70% formamide/2x SCC for 2 h at 37 degrees C in a dark, moist chamber. Slides were washed once with 1x PBS, 0.1% Tween (Sigma-Aldrich) for 5 min followed by hybridization of Cy3-labeled PNA (CCCTAA)₃ probe at 0.05 μ M (PNA Bio Inc.) in hybridization cocktail for 3 h. Slides were washed two times in 1x PBS, 0.1% Tween at 55 degrees C for 10 min, shaking and four times in 2x SSC, 0.1% Tween for 5 min, shaking. Slides were then rinsed in deionized water, air-dried and mounted with VectaShield DAPI (Vector Laboratories, Burlingame, CA). Twenty metaphase spreads were analyzed per experiment, for a total of 60 metaphase spreads in three independent experiments, per treatment group. Samples were coded to blind the reader and analyzed using a Zeiss AxioVert 200 M with an attached AxioCam MRm camera (Oberkochen, Germany).

Homology-directed repair (HDR) assays

HDR assays were performed as previously described (Ransburgh *et al.*, 2010). Briefly, HeLa cells with stably-integrated HDR GFP substrate (HeLa-DR) were transfected with *siRNAs* on day 1 using Oligofectamine 2000 (Invitrogen, Thermo Fisher Scientific); cells were transfected on day 3 using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific) with *siRNAs* and a plasmid expressing the *I-SceI* endonuclease to initiate a DSB break. On day 5, flow cytometry was used to count the percent GFP-positive cells to correlate DNA repair competency.

Statistical analyses

Data were objectively compared between samples in the control group and samples in each experimental treatment group using a two-sided Welch's t-test (unpaired and unequal variance) to determine significance (*, ** and *** represent $P = 0.05$, $P = 0.01$, and $P = 0.001$, respectively). The variability between samples was analyzed and represented as the standard error of the mean.

RESULTS

BRCA1 depletion in five ALT cell lines decreases C-circles, a quantifiable marker of ALT

BRCA1 functions in the HR pathway (Table 2) and localizes to telomeres in ALT-positive cells, to APBs and with proteins implicated in ALT, including NBS1, RAD50 and BLM (Wu *et al.* 2003; Acharya *et al.* 2014; Conomos *et al.*, 2014; Gocha *et al.*, 2014). To determine if BRCA1 affects ALT, C-circle levels were evaluated in BRCA1-depleted ALT cells. C-circles are C-rich single-stranded circles of telomeric DNA with a (TTAGGG)_n primer; these C-

circles are specific and quantifiable markers of ALT activity that can be used as a substrate for extending telomeres (Henson *et al.*, 2009). Phage phi29 DNA polymerase, in the C-circle assay (CC assay), amplifies C-circles to a detectable level for quantification on a Southern blot or by qPCR (Henson *et al.*, 2009; Lau *et al.*, 2013). An advantage of using the CC assay is that C-circle half-life is short; thus changes in ALT activity can be detected within 24 h after transfection. Other ALT assays, such as determining changes in heterogeneous telomere lengths, requires numerous cell line passages; detecting changes in APBs and T-SCEs requires many steps for staining, imaging and counting cells. For these reasons, the CC assay was an optimal screening method for the discovery of ALT effectors.

Five ALT cell lines were used to test BRCA1 as an ALT effector: p53-null osteosarcoma tumor cell line, Saos-2; p53-positive osteosarcoma tumor cell line, U-2 OS; SV40-transformed lung fibroblast cell line, WI-38 VA-13 subline 2RA (herein named VA-13); jejunal fibroblast cell line from a cystic fibrosis patient, JFCF-6/T.1J/1–3C (referred to as JFCF-6/1–3C); mutant p53 osteosarcoma tumor cell line G-292. Successful depletion of BRCA1 protein in each ALT cell line was confirmed by comparing control *siRNA*-treated samples to the BRCA1-*siRNA* treated samples on a western blot (Figure 1A and Supporting Information Figure 2A). Although BRCA1, an essential HR protein, was successfully depleted in ALT cells, it was observed in culture that the cell growth was similar to that of control. The CC assay assessed changes in C-circle levels in BRCA1-depleted compared to control-treated ALT cells. Representative CC assay dot blots (Figure 1B) confirm that every ALT cell line has a different C-circle amount when comparing each control *siRNA*-treated sample to each other. A dot-blotted telomerase-positive HeLa sample demonstrates how telomerase-positive cell lines have low levels of C-circles, below the detection level by dot blots. Depletion of BRCA1 decreased C-circles in all ALT cell lines but had no detectable effect on C-circles in the HeLa telomerase-positive cell line (Figure 1B).

Quantitation of C-circle levels following BRCA1 depletion, in both osteosarcoma cell lines Saos-2 and U-2 OS, show C-circle levels fell approximately 2-fold below controls at a statistically significant change ($P = 0.010$, $P = 0.041$, respectively) (Figure 1C). In the ALT cell line with the lowest levels of C-circles, VA-13 (Figure 1B), there was an approximate 50-fold statistically significant ($P = 1.077 \times 10^{-4}$) reduction in C-circle abundance (Figure 1C). Supporting Information Figure 1 displays the raw data obtained from the BRCA1 depletion in VA-13 cells to demonstrate the observed variability between C-circle signals obtained from the three samples collected for each treatment group from three independent experiments. The signal from the input (total) DNA was consistent in all samples in control and BRCA1 siRNA depleted cells, but the recovery of C-circle DNA was reproducibly lower in the three BRCA1-depleted samples (Supporting Information Figure 1). A 3-fold and 1.75-fold reduction in C-circles were observed in a fibroblast ALT cell line JFCF-6/T.1J/1–3C and an additional osteosarcoma cell line, G-292, respectively (Supporting Information Figure 2B). Both reductions were statistically significant ($P = 0.047$ and $P = 0.003$, respectively). BRCA1 depletions using a second, non-overlapping *siRNA* were performed in Saos-2 and VA-13 cells (Supporting Information Figure 3A) and yielded similar results (Supporting Information Figure 3B). Our results suggest that BRCA1 is required for C-circle formation in five ALT cell lines and that it may be a required protein for ALT mechanisms.

Requirements for requisite homologous recombination proteins in telomere maintenance differ between ALT-positive cell lines

Proteins required for ALT may differ from those required for HR. We used the CC assay to ask whether other requisite HR proteins, BARD1, BRCA2 or PALB2, may have a role in ALT. Depletions were performed in three ALT cell lines by *siRNA*-mediated targeting. In Saos-2 cells, *siRNAs* for all three targets reduced mRNA expression 3.5-fold or more ($P < 0.05$) (Figure 2A). Surprisingly, BARD1 depletion, a protein that heterodimerizes with BRCA1 (Table 2) (Wu *et al.*, 1996), does not significantly affect C-circle levels in Saos-2 cells ($P = 0.069$) (Figure 2B). BRCA2 reduction in Saos-2 cells did not decrease C-circles significantly ($P = 0.081$). Depletion of PALB2 in Saos-2 cells resulted in a significant, 2-fold reduction of C-circles ($P = 0.001$) (Figure 2B). BRCA1 and BRCA2 interact via PALB2 to recruit the RAD51 recombinase to facilitate recombination in HR (Sy, *et al.*, 2009; Zhang *et al.*, 2009). *siRNAs* targeting these HR proteins in U-2 OS and VA-13 cells were also effective in significantly reducing expression levels by 2-fold or more ($P < 0.05$) (Figure 2C, E). Depletion of BARD1 or BRCA2 in U-2 OS and VA-13 did not significantly increase or decrease C-circle levels ($P = 0.429/P = 0.289$; $P = 0.280/P = 0.610$, respectively) (Figure 2D, F). In contrast to results observed in Saos-2, depletion of PALB2 in U-2 OS and VA-13 cells did not significantly affect C-circle levels ($P = 0.712$; $P = 0.069$, respectively) (Figure 2D, F). These results show that some HR proteins are required for ALT in some but not all ALT cell lines and that collectively BARD1 and BRCA2 may be dispensable for ALT activity. BRCA1 function in ALT seems to be independent of BARD1, striking in that these two proteins function interdependently in HR (Chiba and Parvin, 2001; Li and Yu, 2013; Lee *et al.*, 2015).

WRN, a RecQ-like DNA helicase similar in structure to BLM but with an exonuclease domain, is a protein that prevents aberrant HR (Table 2) and can be required for ALT processes (Chu and Hickson, 2009; Gocha *et al.*, 2014). Reduced expression of WRN in Saos-2, U-2 OS, and VA-13 cells (Figure 2A, C, E) was associated with a small but not significant increase in C-circles in Saos-2 and U-2 OS ($P = 0.116$; $P = 0.199$, respectively) (Figure 2B, D). C-circle levels were relatively similar to control in VA-13 cells ($P = 0.462$) (Figure 2F). RNF8 and RNF168 are E3 ubiquitin ligases that function in a signaling cascade to recruit DNA DSB repair proteins such as BRCA1 and 53BP1 to DNA lesions (Table 2) (Kolas *et al.*, 2007; Mailand *et al.*, 2007; Doil *et al.*, 2009). RNF8 and RNF168 were targeted by *siRNA* in three ALT cell lines (Saos-2, U-2 OS and VA-13). A 3.7-fold or more decrease in mRNA expression of RNF8 and RNF168 was achieved in Saos-2, U-2 OS and VA-13 cells ($P < 0.05$) (Figure 3A, C, E). Depletion of RNF8 or RNF168 resulted in a small increase in C-circles, in the Saos-2 and U-2 OS cells, which was not significant ($P = 0.381/P = 0.447$; $P = 0.250/P = 0.276$, respectively) (Figure 3B, D). In VA-13 cells, depletion of RNF8 or RNF168 decreased C-circle levels significantly by 6-fold and 3-fold ($P = 1.049 \times 10^{-3}$; $P = 0.015$), respectively (Figure 3F). These results suggest that the ubiquitin ligases RNF8 and RNF168 may function in ALT in some ALT cell types.

Non-homologous end joining proteins are not critical for ALT

Non-homologous end joining (NHEJ) is a second pathway by which DSBs can be repaired. Two NHEJ proteins, 53BP1 or LIG4, were knocked down in three ALT cell lines and C-

circles were evaluated to test whether NHEJ contributes to ALT. mRNA expression levels of 53BP1, a key NHEJ protein that interacts with BRCA1 to regulate DSB repair pathway choice, and LIG4, the DNA ligase needed for one of the final steps of NHEJ repair (Table 2) (Baumann and West, 1998; Bunting *et al.*, 2010), were reduced in three ALT cell lines, as detected by qPCR ($P < 0.05$) (Figure 3A, C, E). In Saos-2, U-2 OS or VA-13, depletion of 53BP1 or LIG4 resulted in a modest, but not statistically significant, increase in C-circle abundance, with the exception of LIG4 depletion in U-2 OS cells ($P = 0.519/P = 0.741$; $P = 0.438/P = 0.660$; $P = 0.084/P = 0.051$, respectively) (Figure 3B, D, F). These results suggest NHEJ proteins do not significantly affect the ALT process.

Nucleotide excision repair, DNA mismatch repair and base excision repair proteins contribute to ALT

Depletion of proteins in excision DNA repair pathways, defined as repair pathways that remove and replace damaged/mismatched DNA using the preserved complementary strand as a template, were also evaluated for ALT functions. *siRNA* transfection targeted three proteins activated at early steps in three different pathways: XPA in NER, MSH2 in MMR and DNA glycosylase N-methylpurine DNA glycosylase (MPG) in BER. In NER, XPA organizes the assembly of the repair complex at the incision site in both transcription-coupled (TC) NER and global-genome (GG) NER (Table 2) (Schärer, 2013). In MMR, MSH2 forms a complex with either MSH6 or MSH3 to scan DNA for a base mismatch or long insertions/deletions, respectively (Acharya *et al.* 1996). In BER, MPG cleaves N-glycosidic bonds to allow damaged bases to flip out and create an apyrimidinic/apurinic site (Roy *et al.*, 1996). All three *siRNAs* significantly reduced mRNA expression levels by 2-fold or more in Saos-2 cells ($P < 0.05$) (Figure 4A). Targeting XPA in Saos-2 cells had little effect on C-circle levels, as C-circle abundance was similar to that of the control cells; targeting MPG in Saos-2 increased C-circle abundance but not to a significant level ($P = 0.821$; $P = 0.429$, respectively) (Figure 4B). Targeting MSH2 significantly decreased C-circles 2.5-fold in Saos-2 cells ($P = 0.023$).

In U-2 OS and VA-13 ALT cell lines, depletion of each protein had different effects. XPA, MSH2 and MPG mRNA expression levels were significantly reduced by 5.5-fold or more upon treatment with *siRNAs* ($P < 0.05$) (Figure 4C, E). Depletion of XPA, MSH2 or MPG significantly reduced the abundance of C-circles in U-2 OS and VA-13 cell lines ($P = 0.031/P = 0.036/P = 0.012$; $P = 0.003/P = 0.001/P = 0.003$, respectively) (Figure 4D, F). This result suggests that multiple DNA repair pathways may be involved in ALT: HR, NER, MMR and BER. Surprisingly, the NER protein XPA or the BER protein MPG contribute to ALT activity in U-2 OS and VA-13 cells but are not critical for Saos-2. These data also suggest that different ALT mechanisms use unique combinations of DNA repair proteins and by extension that each ALT cell line may use different mechanisms.

MSH2 and MPG contribute to recombination rates

As DNA repair proteins unrelated to HR regulate C-circle levels in ALT cells, we asked whether telomeric recombination substrates other than C-circles may be affected by the depletion of excision DNA repair proteins. XPA, MSH2 or MPG were depleted in U-2 OS ALT cells (Figure 5A) followed by analysis of recombination by chromosome orientation

fluorescence in situ hybridization (CO-FISH). Changes in T-SCEs involving two sister chromatids and intertelomeric exchanges involving one sister chromatid can be visualized with this technique (Figure 5B) (Conomos *et al.*, 2014). Sixty metaphases were counted for each XPA, MSH2 or MPG depleted cell population. MSH2 depletion resulted in a 6% reduction in the average number of intertelomeric exchanges per metaphase ($P = 0.020$) (Figure 5B–D) and a small decrease in T-SCEs per metaphase compared to control (Figure 5B, C, E). Depletion of the NER protein XPA or the BER protein MPG had no effect on intertelomeric exchanges or T-SCEs (Figure 5D, E), similar to the observations of Hagelstrom *et al.* following XPF reduction (2010).

siRNA-mediated depletions of XPA, MSH2 or MPG (Figure 6A) were carried out in a HeLa-derived cell line, “HeLa-DR”, which contains a genome-integrated recombination substrate (GFP) to permit measurement of homology-directed repair (HDR) (Ransburgh *et al.*, 2010). Depletion of BRCA1 as a positive control, resulted in a 15-fold decrease in HDR compared to control ($P = 1.471 \times 10^{-4}$) (Figure 6B). Depletion of XPA had little effect on HDR compared to control ($P = 0.511$). MSH2 or MPG depletion generated 2-fold and 3-fold decreases in HDR, respectively, compared to controls ($P = 1.405 \times 10^{-3}$; $P = 0.008$, respectively) (Figure 6B), suggesting a role for these proteins in HDR.

DISCUSSION

ALT relies upon HR and some DNA repair proteins to maintain telomere length (Murnane *et al.*, 1994; Dunham *et al.*, 2000; Stavropoulos *et al.*, 2002; Wu *et al.*, 2003; Lillard-Wetherell *et al.*, 2004; Muntoni and Reddel, 2005; Bhattacharyya *et al.*, 2009). In this study, we asked whether this process depends on both requisite HR proteins and non-HR DNA repair proteins. CC assays measured C-circles as responsive and specific markers of ALT activity (Henson *et al.*, 2009). Our results demonstrate that: 1) many requisite HR proteins are not required for ALT; 2) the MMR protein MSH2 contributes to two types of telomere recombination events that could occur in ALT, recombination with C-circles and intertelomeric exchanges; 3) DNA repair proteins that function in NER, MMR or BER are required to promote C-circles in some ALT cell lines; and 4) ALT cell lines differ in their requirement for specific DNA repair proteins.

This study also evaluated BRCA1 in ALT. BRCA1 functions in homology-directed DSB repair (Scully *et al.*, 1996; Towler *et al.*, 2013). In telomerase-positive cells, BRCA1 inhibits telomerase activity in transfection studies, while loss of BRCA1 induces telomerase via multiple mechanisms (Xiong *et al.*, 2003; Ballal *et al.*, 2009). In ALT cells, BRCA1 localizes to telomeres and APBs, and its depletion reduces T-SCEs (Wu *et al.*, 2003; Acharya *et al.*, 2014; Cho *et al.*, 2014; Gocha *et al.*, 2014). In this study, BRCA1 depletion in five ALT cell lines decreases C-circles. Together, these data support a role for BRCA1 in ALT mechanisms.

Some requisite HR proteins such as BLM, BRCA1 and RAD51 have been evaluated in ALT, while others have yet to be characterized (Yeager *et al.*, 1999; Potts and Yu, 2007; Henson *et al.*, 2009; Cho *et al.*, 2014). Our data show that depletion of BARD1, the heterodimeric protein partner of BRCA1, has no effect on C-circles, even though depletion of BARD1

strongly impacts HDR (Westermarck *et al.*, 2003; Laufer *et al.*, 2007; Lee *et al.*, 2015). The HR protein BRCA2, a known binding partner of RAD51 (Moynahan *et al.*, 2001; Liu *et al.*, 2010), also had no effect on C-circles. In contrast, PALB2, a protein that bridges BRCA1 and BRCA2 (Sy *et al.*, 2009; Zhang *et al.*, 2009), significantly decreases C-circles in Saos-2 cells but not in the two other cell lines tested. These data suggest that PALB2 has an activity in ALT that is BRCA2-independent. Overall, HR pathways used in ALT diverge considerably from the characteristic HR pathway.

BLM was used as a positive control in our experiments as it is required for ALT in all cell lines examined to date (Bhattacharyya *et al.*, 2009; Bhattacharyya *et al.*, 2010; Mendez-Bermudez *et al.*, 2012). BLM depletion decreases C-circles in all ALT cell lines tested. Depletion of 53BP1 and LIG4, two NHEJ proteins, had no significant effect on C-circles in the ALT cell lines tested. Previous studies showed 53BP1 is not necessary for APB formation (Jiang *et al.*, 2007), although it localizes to telomere dysfunction-induced foci (TIFs) in ALT cells (Cesare *et al.*, 2009). Depletion of the E3 ubiquitin ligases RNF8 and RNF168, which mark chromatin sites of DNA DSBs and locate to TIFs in normal cells (Al-Hakim *et al.*, 2010; Jacobs, 2012), did not affect C-circles in Saos-2 or U-2 OS cells but were important in VA-13 cells. These results suggest that RNF8 and RNF168 may have a role in some ALT mechanisms.

In all the cell lines tested here, depletion of MSH2 had an effect on ALT characteristics. MMR protein alterations that increase microsatellite instability (MSI) may be incompatible with ALT due to disruption of the MRN complex (Giannini *et al.*, 2002; Muntoni and Reddel, 2005). However, alterations of MMR can initiate ALT and are correlated with ALT in some tumor types (Bechter *et al.*, 2004; Omori *et al.*, 2009). We were also surprised to observe an effect on ALT by depletion of the NER or BER proteins, XPA and MPG, respectively, in U-2 OS and VA-13 cells. Depletion did not alter T-SCEs or intertelomeric exchanges in the U-2 OS cell line, suggesting that these proteins may facilitate the use of one substrate such as C-circles, in preference to another, such as an adjacent chromosome, to elongate telomeres. Overall, our results suggest that proteins in DNA repair pathways other than HR can contribute to ALT mechanisms.

Lastly, the use of more than one ALT cell line in these studies illuminates the complex requirements for DNA repair proteins in ALT. PALB2 is uniquely required for ALT in Saos-2 cells; RNF8 and RNF168 are uniquely required for ALT in VA-13 cells; NER and BER proteins XPA and MPG, respectively, were required for ALT in VA-13 and U-2 OS. These results are summarized in Table 2. The results here highlight the complexity of ALT mechanisms and imply the flexibility with which cells can employ recombination-associated telomere elongation mechanisms. Consequently, approaching ALT therapeutically may warrant combinatorial therapies that are mechanism-specific. As in BRCA1-deficient breast cancers that utilize poorly-defined alternative pathways to become resistant to PARP inhibitors (Jaspers *et al.*, 2013), ALT cancers may be able to do the same following some therapeutic regimens.

Supplementary Material

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FIGURE 1.

Depletion of BRCA1 reduces C-circles in three ALT cell lines but not in a telomerase-dependent cell line. (A) Following two rounds of *siRNA* transfection targeting either the BRCA1 mRNA or luciferase (control), protein extracts were analyzed by immunoblotting with antibodies specific for BRCA1 (top) and LAMIN B as a loading control (bottom). (B) Representative dot blots of C-circle assay (CC assay) products. Total DNA extracted from control *siRNA* or *BRCA1 siRNA*-treated samples from 3 ALT cell lines and 1 telomerase-positive cell line (HeLa) were subjected to restriction and exonuclease digestion and DNA (25 ng) was subjected to the CC assay and dot blotting (bottom left). Total DNA (400 ng) was blotted to demonstrate equal input for the assay (top left). Controls included in the CC assay were dot-blotted: positive control C-96 synthetic C-circle (C96) and negative controls, no DNA (dash) and lambda DNA (λDNA) (bottom right). (C) Phosphorimager quantitation of C-circle abundance in 3 ALT cell lines. The results from three independent experiments

are presented as a fold-change in C-circle signal relative to the signal obtained in the control. Black bars, control *siRNA* average; Gray bars, *BRCA1 siRNA* average; error bars, standard error of the mean; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, determined by Welch's t-test.

FIGURE 2.

Depletion of homologous recombination proteins BARD1, BRCA2, PALB2 or WRN in ALT cells do not alter C-circle abundance. Following two rounds of *siRNA* transfection, cells were collected and analyzed for effectiveness of *siRNA* targeting by measuring mRNA abundance. (A) (C) (E) Changes in mRNA expression of targeted genes were determined by quantitative PCR (qPCR) shown as the average relative to the control $\pm$ standard error of the mean (SEM) from three or more independent experiments. All knockdowns significantly reduce expression ($P < 0.05$) as compared to control, determined by Welch's t-test. (B) (D) (F) Quantitation of C-circle levels after knockdown of each homologous recombination gene product. The averages from three independent experiments are presented normalized to the signal obtained by the control. Gray bars, averages of three or more experiments for each homologous recombination product knockdown; error bars, standard error of the mean; * P 0.05, ** P 0.01, *** P 0.001, determined by Welch's t-test.

FIGURE 3.

Depletion of RNF8 or RNF168 lowers C-circle levels in VA-13 cells and depletion of NHEJ proteins do not alter C-circle abundance in Saos-2, U-2 OS or VA-13 cells. Depletions and analyses were done as in Figure 2. **(A)** **(C)** **(E)** The change in mRNA expression of ubiquitin ligase and NHEJ products were analyzed by qPCR; averages in mRNA expression relative to the control $\pm$ standard error of the mean from three or more independent experiments. All knockdowns were significant ($P < 0.05$) compared to control, determined by Welch's t-test. **(B)** **(D)** **(F)** Quantitation of C-circle levels after knockdown of each ubiquitin ligase or NHEJ product. Results from three independent experiments are presented normalized to the signal obtained in the control transfection. Gray bars, averages of three or more experiments for each ubiquitin ligase or NHEJ product knockdown; error bars, standard error of the mean; * $P < 0.05$, ** $P < 0.01$, determined by Welch's t-test.

FIGURE 4.

Depletion of XPA (nucleotide excision repair), MSH2 (mismatch repair) or MPG (base excision repair) lowers C-circle levels in U-2 OS and VA-13 cells. Depletions and analyses were done as in Figure 2. **(A)** **(C)** **(E)** The change in mRNA expression of XPA (NER), MSH2 (MMR) and MPG (BER) gene products were determined by qPCR from three or more independent experiments. All knockdowns were significant ($P < 0.05$) compared to control, determined by Welch's t-test. **(B)** **(D)** **(F)** Quantitation of C-circle levels after knockdown of XPA (NER), MSH2 (MMR) or MPG (BER) gene products. Results from three independent experiments are presented relative to the signal obtained by the control. Gray bars, averages of three or more experiments for each XPA, MSH2 or MPG product knockdown; error bars, standard error of the mean; * P 0.05, ** P 0.01, *** P 0.001, determined by Welch's t-test.

FIGURE 5.

Depletion of MSH2 lowers intertelomeric exchanges in U-2 OS cells. Cells were transfected with two rounds of the appropriate *siRNA*, as done in Figure 2. (A) Quantitation by qPCR of targeted mRNAs in each transfection. All knockdowns were significant ($P < 0.05$) compared to control, determined by Welch's t-test. (B) Representative images of an intertelomeric exchange (one yellow signal) and telomere-sister chromatid exchange (T-SCE) event (two yellow signals). (C) Representative metaphase spreads of control and MSH2; arrows indicate intertelomeric exchanges, arrow heads indicate T-SCEs. (D) Quantitation of intertelomeric exchanges in U-2 OS cells, displayed as a density plot with each circle representing a metaphase, is plotted along the y-axis to indicate the % intertelomeric exchanges within that cell. A total of 60 metaphase spreads were counted for each treatment group, 20 metaphases were counted per independent experiment in which 3 independent experiments were performed. The red bar indicates the mean % intertelomeric exchanges

among the 60 metaphases counted for each treatment group; P -values determined by Welch's t-test. (E) Quantitation of T-SCEs in U-2 OS cells displayed as a density plot, with each circle representing a metaphase, is plotted along the y-axis to indicate the % T-SCEs within that cell. A total of 60 metaphase spreads were counted for each treatment group, 20 metaphases were counted per independent experiment in which 3 independent experiments were performed. The red bar indicates the mean % T-SCEs among the 60 metaphases counted for each treatment group; P -values determined by Welch's t-test.

FIGURE 6.

Depletion of MSH2 (MMR) or MPG (BER) proteins decrease homology-directed repair (HDR). HeLa-DR cells were transfected with the appropriate *siRNA* for luciferase (control), BRCA1, XPA, MSH2 or MPG. (A) Quantitation by qPCR of targeted mRNAs in each transfection. All knockdowns were significant ($P < 0.05$) compared to control, determined by Welch's t-test. (B) Measurement of GFP-positive cells determined by flow-cytometry and normalized relative to the control transfected cells. Gray bars represent the average HDR in the indicated experimental group relative to control, from three independent experiments; error bars, standard error of the mean; ** $P = 0.01$, *** $P = 0.001$, determined by Welch's t-test.

TABLE 1.Sequence of *siRNAs* and quantitative PCR primers

<i>siRNA</i> sequence or source	Quantitative PCR primers	
	Forward primer	Reverse primer
GL2: 5' CGUACGCGGAUACUUCGA3' (Hu <i>et al.</i> , 2014)	18S rRNA F: 5' AATCAGGGTTCGATTCCGGA3' (Hashimoto <i>et al.</i> , 2004)	18S rRNA R: 5' CCAAGATCCAACCTACGAGCT3' (Hashimoto <i>et al.</i> , 2004)
BRCA1 5787: 5' GCUCCUCACUCUUCAGU3' (Ransburgh <i>et al.</i> , 2010) BRCA1 2616: 5' GGUUCAAAGCGCCAGUCA3' (Sankaran <i>et al.</i> , 2005)	BRCA1 exon 11/12 F: 5' AAGAGGAACGGGCTTGAA3' (Fustier <i>et al.</i> , 2003)	BRCA1 exon 11/12 R: 5' CACACCCAGATGCTGCTTCA3' (Fustier <i>et al.</i> , 2003)
BARD1: 5' AGCUGAAUUAUACCAGA3' (Lee <i>et al.</i> , 2015)	BARD1 exon 3/4 F: 5' CAATGAGCTGTCAGATTTGAAAGAA3' (Y.Q. Zhang <i>et al.</i> , 2012)	BARD1 exon 3/4 R: 5' CGAGGGCTAAACACATTTTAATT3' (Y.Q. Zhang <i>et al.</i> , 2012)
BRCA2-2: 5' GGAGGACUCCUUAUGUCCAAAUUA3' (Ransburgh <i>et al.</i> , 2010)	BRCA2 exon 12/13 F: 5' GAAAATCAAGAAAAATCCTTAAAGGCT3' (Fustier <i>et al.</i> , 2003)	BRCA2 exon 12/13 R: 5' GTAATCGGCTCTAAAGAAACATGATG3' (Fustier <i>et al.</i> , 2003)
PALB2 169: 5' CUGAUCUUGGAUGUACAUAU3' ("this work")	PALB2 exon 12/13 F: 5' TGGGTGTGATGCTGTACTGT3' ("this work")	PALB2 exon 12/13 R: 5' CCAGCCAGCAAATGAGAGTC3' ("this work")
BLM: Silencer Pre-designed Human BLM <i>siRNA</i> (AM16704; Ambion)	BLM F exon 3/4 F: 5' GAATGGTTAAGCAGCGATG3' (De Luca <i>et al.</i> , 2013)	BLM R exon 3/4 R: 5' TCAATACATGGAACCTTCTCAG3' (De Luca <i>et al.</i> , 2013)
WRN: ON-TARGETplus Human WRN <i>siRNA</i> - SMARTpool (L-010378; Dharmacon)	WRN exon 34/35 F: 5' GACAGCGGACTTCAACCTTC3' (Yee <i>et al.</i> , 2012)	WRN exon 34/35 R: 5' TTGGCAAACACACAGGTAA3' (Yee <i>et al.</i> , 2012)
RNF8: 5' GGACAAUUAUGGACAACAA3' (Hu <i>et al.</i> , 2014; F. Zhang <i>et al.</i> 2012)	RNF8 exon 1/2 F: 5' GGTGCGAGGTGACTGTAGGAC3' (Bonanno <i>et al.</i> , 2013)	RNF8 exon 1/2 R: 5' GGGCAGATTTTGTATACCAGTTG3' (Bonanno <i>et al.</i> , 2013)
RNF168: 5' GGCGAAGAGCGAUGGAGGA3' (Galanty <i>et al.</i> , 2009; Hu <i>et al.</i> , 2014)	RNF168 exon 2/3 F: 5' TCAGCCAGTTCTGCTGCTCAGT3' (Gatti <i>et al.</i> , 2015)	RNF168 exon 2/3 R: 5' TCTTCTTCTCGCTGGCCCGT3' (Gatti <i>et al.</i> , 2015)
53BP1: 5' GAAGGACGGAGUACUAAUA3' (Galanty <i>et al.</i> , 2009; Hu <i>et al.</i> , 2014)	53BP1 exon 4/5 F: 5' GTCAGGTGCTTATGAGCAGTTACCTC3' (Bonanno <i>et al.</i> , 2013)	53BP1 exon 4/5 R: 5' TCCTCCACAGCAGGAGCAG3' (Bonanno <i>et al.</i> , 2013)
LIG4: 5' AGGAAGUAUUCUCAGGAAUUA3' (Galanty <i>et al.</i> , 2009; Hu <i>et al.</i> , 2014)	LIG4 F: 5' CACCTTGCGTTTCCACGAA3' (Liang <i>et al.</i> , 2008)	LIG4 R: 5' CAGATGCCTTCCCCCTAAGTTG 3' (Liang <i>et al.</i> , 2008)
XPA: ON-TARGETplus XPA <i>siRNA</i> SMARTpool (L-005067; Dharmacon)	XPA exon 4/5 F: 5' CACAATGGGGTGATATGAACTCTACT3' (Nymoen <i>et al.</i> , 2015)	XPA exon 4/5 R: 5' CCTTGCTTCTTCTAATGCTTCTTGACT3' (Nymoen <i>et al.</i> , 2015)
MSH2: ON-TARGETplus Human MSH2 <i>siRNA</i> SMARTpool (L-00399; Dharmacon)	MSH2 exon 13/14 F: 5' GGACTGCATCTTAGCCCGAGTAGG3' (Ahmadi <i>et al.</i> , 2015)	MSH2 exon 13/14 R: 5' GCCCATGCTAACCCAAATCCATCG3' (Ahmadi <i>et al.</i> , 2015)
MPG: 5' AAGAAGCAGCGACCAGCUAGA3' (Ström <i>et al.</i> , 2011)	MPG exon 4/5 F: 5' CTTCTGCATGAACATCTCCAGC3' (Song <i>et al.</i> , 2012)	MPG exon 4/5 R: 5' AGGGTGCTGCGAAGCTGACGC3' (Song <i>et al.</i> , 2012)

TABLE 2.

Summary of data from DNA repair depletions and C-circle measurements in ALT cell lines

DNA repair pathway	siRNA target	Function in pathway	Cell line	C-circle fold-change $\pm$ SEM	p-value
	Control (GL2)		Saos-2	1.00	N/A
			U-2 OS	1.00	
			VA-13	1.00	
HR	BRCA1	RAD51 recruitment, DSB repair, end resection (Scully <i>et al.</i> , 1997; Moynahan <i>et al.</i> , 1999; Schlegel <i>et al.</i> , 2006)	Saos-2	0.46 $\pm$ 0.08	0.0196
			U-2 OS	0.38 $\pm$ 0.11	0.0285
			VA-13	0.26 $\pm$ 0.08	0.0128
	BARD1	Forms E3 Ub ligase with BRCA1 (Wu <i>et al.</i> , 1996; Hashizume <i>et al.</i> , 2001; Lee <i>et al.</i> , 2015)	Saos-2	0.74 $\pm$ 0.07	0.0690
			U-2 OS	0.91 $\pm$ 0.10	0.4288
			VA-13	1.65 $\pm$ 0.49	0.2796
	BRCA2	RAD51 recruitment, DSB repair (Chen <i>et al.</i> , 1998; Moynahan <i>et al.</i> , 2001)	Saos-2	0.55 $\pm$ 0.14	0.0806
			U-2 OS	1.44 $\pm$ 0.31	0.2887
			VA-13	0.82 $\pm$ 0.32	0.6096
	PALB2	Mediates interaction between BRCA1 and BRCA2 for RAD51 recruitment (Sy <i>et al.</i> , 2009; Zhang <i>et al.</i> , 2009)	Saos-2	0.42 $\pm$ 0.04	0.0008
			U-2 OS	0.84 $\pm$ 0.38	0.7115
			VA-13	0.31 $\pm$ 0.19	0.0691
	BLM (+ control)	End resection, branch migration and dissolution of Holliday junctions; absence results in aberrant HR (Chu and Hickson, 2009)	Saos-2	0.29 $\pm$ 0.15	0.0399
			U-2 OS	0.13 $\pm$ 0.03	0.0012
			VA-13	0.17 $\pm$ 0.06	0.0007
	WRN	Promotes branch migration of Holliday junctions; absence results in aberrant HR (Chu and Hickson, 2009)	Saos-2	1.50 $\pm$ 0.19	0.1162
			U-2 OS	2.57 $\pm$ 0.83	0.1992
			VA-13	0.80 $\pm$ 0.24	0.4624
HR & NHEJ	RNF8	E3 Ub ligase, initiates signaling cascade for DSB repair proteins; recruits 53BP1 and BRCA1 (Kolas <i>et al.</i> , 2007; Mailand <i>et al.</i> , 2007)	Saos-2	1.42 $\pm$ 0.38	0.3811
			U-2 OS	2.70 $\pm$ 1.06	0.2495
			VA-13	0.16 $\pm$ 0.03	0.0010
	RNF168	E3 Ub ligase, initiates signaling cascade for DSB repair proteins; recruits 53BP1 (Doil <i>et al.</i> , 2009)	Saos-2	1.45 $\pm$ 0.48	0.4472
			U-2 OS	2.06 $\pm$ 0.71	0.2757
			VA-13	0.34 $\pm$ 0.08	0.0149
NHEJ	53BP1	Binds DSB ends and prevents extensive end resection (Iwabuchi <i>et al.</i> , 2003; Bunting <i>et al.</i> , 2010)	Saos-2	1.69 $\pm$ 0.89	0.5193
			U-2 OS	4.00 $\pm$ 3.13	0.4382
			VA-13	1.38 $\pm$ 0.12	0.0839
	LIG4	Performs final step of ligation to repair DSBs (Baumann and West, 1998)	Saos-2	1.21 $\pm$ 0.56	0.7411
			U-2 OS	0.88 $\pm$ 0.16	0.6597
			VA-13	2.08 $\pm$ 0.42	0.0507
NER	XPA	Recognizes and recruits NER proteins to damage site; involved in both GG & TC-NER (Schärer, 2013)	Saos-2	0.93 $\pm$ 0.27	0.8205
			U-2 OS	0.44 $\pm$ 0.14	0.0314
			VA-13	0.32 $\pm$ 0.08	0.0029

DNA repair pathway	siRNA target	Function in pathway	Cell line	C-circle fold-change $\pm$ SEM	p-value
MMR	MSH2	Heterodimerizes with MSH6 and MSH3, binds to DNA mismatch and initiates downstream MMR events (Acharya <i>et al.</i> , 1996)	Saos-2	0.38 $\pm$ 0.15	0.0234
			U-2 OS	0.36 $\pm$ 0.18	0.0359
			VA-13	0.35 $\pm$ 0.07	0.0006
BER	MPG	Methyl purine DNA glycosylase; severs N-glycosidic bond of damaged methylated purine (Roy <i>et al.</i> , 1996)	Saos-2	1.81 $\pm$ 0.82	0.4290
			U-2 OS	0.33 $\pm$ 0.12	0.0124
			VA-13	0.57 $\pm$ 0.07	0.0031

$\pm$, standard error of the mean; bolded *P*-values indicate statistical significance ($P < 0.05$)