Published in final edited form as:

DNA Repair (Amst). 2013 July ; 12(7): 480–487. doi:10.1016/j.dnarep.2013.04.024.

Repair of Chromosomal Double-Strand Breaks by Precise Ligation in Human Cells

William Y. Lin, John H. Wilson, and Yunfu Lin*

Verna and Marrs McLean Department of Biochemistry and Molecular Biology Baylor College of Medicine Houston, TX 77030, USA

Abstract

Double-strand breaks (DSBs), a common type of DNA lesion, occur daily in human cells as a result of both endogenous and exogenous damaging agents. DSBs are repaired in two general ways: by the homology-dependent, error-free pathways of homologous recombination (HR) and by the homology-independent, error-prone pathways of nonhomologous end-joining (NHEJ), with NHEJ predominating in most cells. DSBs with compatible ends can be re-joined in vitro with DNA ligase alone, which raises the question of whether such DSBs require the more elaborate machinery of NHEJ to be repaired in cells. Here we report that chromosomal DSBs with compatible ends introduced by the rare-cutting endonuclease, IScel, are repaired by precise ligation nearly 100% of the time in human cells. Precise ligation depends on the classical NHEJ components Ku70, XRCC4, and DNA ligase IV, since siRNA knockdowns of these factors significantly reduced the efficiency of precise ligation. Interestingly, knockdown of the tumor suppressors p53 or BRCA1 showed similar effects as the knockdowns of NHEJ factors. In contrast, knockdown of components involved in alternative NHEJ, mismatch repair, nucleotide excision repair, and single-strand break repair did not reduce precise ligation. In summary, our results demonstrate that DSBs in human cells are efficiently repaired by precise ligation, which requires classical NHEJ components and is enhanced by p53 and BRCA1.

INTRODUCTION

Double strand breaks (DSBs) on chromosomes are potentially damaging to the cell. DSBs can result from either endogenous or exogenous sources of DNA damage [1]. DSBs occur naturally during DNA replication when the replication fork encounters a nick on the template strand; they can happen as a result of damage by the reactive oxygen species generated during electron transport; and they can also arise as programmed events during certain cellular processes such as V(D)J recombination, class-switch recombination, and meiotic recombination. Exogenous agents that generate DSBs include ionizing radiation, UV light, and chemicals such as topoisomerase inhibitors and chemotherapy drugs. DSBs can be highly detrimental to mammalian cells, causing cell death if unrepaired [2]. Thus, it is critical for mammalian cells to repair DSBs efficiently and appropriately. Here, we examined the ability of human cells to repair chromosomal DSBs that have compatible, complementary DNA ends.

© 2013 Elsevier B.V. All rights reserved.

*Correspondence: yunful@bcm.edu, Phone: (713) 798-5477, Fax: (713) 796-9438.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Mammalian cells possess two main pathways of DSB repair: homologous recombination (HR), which typically uses the identical sequence on the sister chromatid as the template, and non-homologous end-joining (NHEJ), which joins broken ends without the need for a homologous donor [3–5]. In contrast to HR, which restores the original sequence, NHEJ often generates mutations at the site of the repaired break [6,7]. In various mammalian systems, the most common mutations are small deletions of one to a few nucleotides, although larger deletions and some insertions also arise [8–11]. In the classical pathway for NHEJ (c-NHEJ), the key initial step is the binding of the Ku70/Ku80 complex to the DNA ends, which serves to recruit other c-NHEJ factors possessing endonuclease, polymerase and ligase activities [4], although not all these enzymatic activities are required for every c-NHEJ event. Binding of Ku at both ends of a DSB is thought to tether the ends and enable repair via c-NHEJ. The binding of Ku to DNA ends facilitates its subsequent binding to DNA-PKcs (DNA-dependent protein kinase catalytic subunit) [12]. Recruitment of DNA-PKcs activates its kinase activity, which phosphorylates itself, as well as other c-NHEJ factors, including Ku70, Ku80, XRCC4, XLF, Artemis, and LIG4 [3]. Depending on their structure, DNA ends might need to be processed prior to their final ligation by the XLF/XRCC4/LIG4 complex, which has been shown to be capable of ligating both compatible and blunt DNA ends in vitro [13].

DSBs with compatible ends could, in principle, be directly ligated by DNA ligase in the cell, without the need for additional components, much as compatible restriction ends can be joined in vitro. Direct ligation—precise ligation—of a chromosomal DSB was first showed by Lin et al, who used the rare-cutting endonuclease ISceI, to introduce DSBs with compatible ends [14]. The frequency of precise ligation ranged from 30–70% in four independent cell lines. However, the product of precise ligation was susceptible to further cutting by ISceI until the recognition site was mutated or the enzyme was lost; thus, the measured efficiencies of precise ligation were likely underestimated. In addition, this previous study did not investigate the repair pathway that was used to accomplish precise ligation.

In this report, we used a novel substrate design that eliminated the problem of repetitive ISceI cutting and also allowed us to probe the cellular requirements for precise ligation. Using this new strategy, we found that DSBs are repaired almost exclusively by precise ligation in proliferating human cells. Moreover, we showed that depletion of the c-NHEJ components—Ku70, XRCC4, or LIG4—or of the tumor suppressors p53 or BRCA1 significantly reduced the efficiency of precise ligation, identifying precise ligation as a subpathway of c-NHEJ.

MATERIALS AND METHODS

ISceI recognition site analysis

Plasmid pUC19 was digested with *Bam*H I and *Hind* III and ligated to annealed pairs of DNA oligos with compatible ends contained ISceI sites that differed from the wild-type ISceI recognition site, TAGGGATAACAGGGTAAT, at one position. After ligation, the mixture was transformed into *E. coli* competent cells, three clones were picked for each mutated ISceI site, and individual clones were tested for ISceI cutting by incubating 0.5 µg of plasmid DNA with one unit of ISceI (Biolabs) at 37°C for 30 minutes. We introduced all possible single nucleotide substitutions at the first five positions and the last four positions in the ISceI site.

Cell lines and cell culture

Plasmid pTNeo99-7, which was described previously [15], contains the herpes simplex virus type 1 thymidine kinase (*tk*) gene fused to the coding region of the *neo* gene. The *tk-neo* gene in pTNeo99-7 is disrupted by a 22-bp oligonucleotide that contains the 18-bp recognition site for *ISceI*, flanked by *SacI* sites. pTNeo99-7 was digested with *SacI* to remove the insert and ligated with DNA oligos with mutant *ISceI* sites—

cTACGGATAACAGGGTAATCTAGCTAACTAGTAGGGATAACAGGGTAAGgagct and cCTTACCCTGTTATCCCTACTAGTTAGCTAGATCATCCTGTTATCCGTAagct—to generate plasmid pHPL. The underlined sequences are the recognition sites for *ISceI* and the bold letters indicate the specific nucleotide changes in mutants 711 and 205. To make HPL cell lines, 1 µg of pHPL was linearized with *AloI* and electroporated into human HT1080 cells. Cells at density of 500,000 cells/10 cm dish were then selected for hygromycin (300 µg/mL) for 2 weeks. Individual colonies were picked and the number of copies of the *tk-neo* gene was determined by Southern blotting. Cell lines HPL9, HPL16 and HPL19 contained a single copy of *tk-neo* gene and were used for further study. All cells were grown at 37°C with 5% CO₂ in DMEM/F-12 medium supplemented with 10% fetal bovine serum and 1% MEM nonessential amino acids.

DSB introduction and cell selection

Plasmid pCMV-3xnl*s* *ISceI* encodes an *ISceI* endonuclease with three copies of a nuclear localization sequence. To introduce a DSB in the *tk-neo* gene, 2 µg of pCMV-3xnl*s* *ISceI* were transfected into about 1 million HPL cells in each well of a 6-well dish, using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were plated in 10 cm dishes at a density of 100,000 cells per dish in the presence of 600 µg/mL G418 (Sigma) for two weeks.

siRNA treatment and qRT-PCR

The siRNAs (Dharmacon) used in this study are listed in Table 1. For siRNA treatments, about 100,000 HPL9 cells were plated in each well of a 6-well plate on day 3. On day 2, we transfected cells with siRNAs at a final concentration of 200 nM, using oligofectamine (Invitrogen). Transfections with 200 nM vimentin-siRNA served as controls; this siRNA has no detectable effect on cells. On day 0, the cells were again transfected with siRNA. On day 1, the cells were transfected with pCMV-3xnl*s* *ISceI*. On day 2, cells were plated in the presence of G418 as shown above. Knockdown efficiency was determined by measuring the mRNA levels of target genes using RNA isolated on day 1. All siRNAs used in this study reduced the mRNA levels of their target gene by 70% to 90%. The potential effects of siRNA transfection on expression of *ISceI* from pCMV-3xnl*s* were determined by measuring *ISceI* mRNA levels in total RNA isolated on day 2, using primers Sce-1: GGTCCGAAGTCTAACTGCTG and Sce-2: CCACTGATCGTACAGCAGAC. For all qRT-PCR reactions, the levels of β-actin gene mRNA were also measured and used to normalize for the amount of total RNA used in individual PCR reactions.

Characterization of G418-resistant clones and nested PCR

G418-resistant clones were picked and grown for genomic DNA preparation. About 400 ng of genomic DNA were used for each genomic PCR using primers: PL5-ACGTACGGCGTTGCGC and PL4- TAGCCAGGTCAAGCCGCTC; or PL9-GGTCCAGGTCCACTTCGC and PL8-ACATGCATGGGACGGAGGC. The PCR products were analyzed for the efficiency of cutting by *ISceI* (Biolabs). PCR products that were not cleaved by *ISceI* were sequenced using primers PLSeq1-CCGAGCCGATGACTTACTGGC or PLSeq2-TGCCATAAGGTATCGCGCG. For nested PCR, genomic DNA was amplified using primers PL1:

CGCCTTGTAGAAGCGCGTATGG and PL2: CCTGCAGATACCGCACCG. The PCR product of PL1 and PL2 was amplified using internal primers PL3: ACGCGTCTGCGTTCGAC and PL4.

Statistics

Statistical analyses of significance were conducted using Student's *t*-test to compare the means and standard deviations derived from multiple experiments.

RESULTS

Novel selection assay for studying precise ligation

ISceI, a yeast mitochondrial intron-encoded double stranded DNA endonuclease, is a unique rare-cutting enzyme with a core recognition site of 18 bp: TAGGGATAACAGGGTAAT [16]. Because this long recognition sequence does not exist in most genomes, it can be inserted into chromosomes to serve as a specific target of ISceI, a process that has proven useful for studying the repair of site-specific DSBs in cells [17–21]. Unlike the more common recognition sites for restriction enzymes, not every base in the ISceI site is absolutely required for ISceI activity; some nucleotide substitutions minimally affect the efficiency of cutting [22]. We chose to exploit the inherent ambiguity of the ISceI recognition sequence to eliminate the problem of re-cutting after precise ligation [14,23]. Our goal was to design a tandem pair of ISceI sites, both of which could be cleaved, but constructed so that the hybrid site created by rejoining would not be cuttable (Figure 1). To find appropriately mutated recognition sites, we tested all possible single nucleotide substitutions at positions 1 to 3 and 14 to 18 for their effects on the efficiency of cleavage by ISceI. We found a G to C substitution at the third nucleotide and eight substitutions in the last five nucleotides that minimally affected ISceI cutting (Figures 1A and 1B). We then tested all eight combinations of substitutions from the left and the right halves to find ones that prevented ISceI cleavage; we found two double substitutions that dramatically reduced cutting (data not shown).

We chose to use one set of mutant recognition sites: mutant 711, which contains a G to C substitution at position 3, and mutant 205, which contains a T to G substitution at position 18. Cleavage of mutants 711 and 205, followed by the rejoining of their mutant halves, generates the hybrid site, mutant 916, which has both mutations (Figure 1D). The cleavage properties of the individual mutant ISceI sites are shown in Figure 1C.

To generate a selection assay for precise ligation, we inserted the tandem pair of mutant 711 and 205 ISceI sites into the *tk* portion of a *tk*-neo gene, and integrated the construct into the genome of HT1080 human cells (Figure 1D). Between the two mutant ISceI sites, we included 12 extra nucleotides containing stop codons in all three reading frames (Figure 1D). These stop codons prevent synthesis of the portion of the gene encoding neomycin resistance, rendering cells sensitive to the antibiotic G418. Cleavage of the tandem ISceI sites followed by precise ligation removes the stop codons, allowing the cells to grow in the presence of G418. Thus, ISceI treatment of cells containing the modified *tk*-neo construct and selection for neomycin-resistant colonies provides a selection assay for precise ligation.

It is instructive to consider what types of event—other than precise ligation—might give rise to neomycin-resistant colonies. One event might be cleavage at one ISceI site followed by NHEJ, which typically involves deletions or insertions of one or a few nucleotides [8–11]. These potentially common events are not expected to produce neomycin-resistant colonies because the stop codons between the sites will prevent expression of the *tk*-neo fusion product. However, large deletions that eliminate the adjacent stop codons and leave the reading frame intact would allow expression of the TK-neo fusion protein. Similarly, NHEJ

events that occur after cleavage of both ISceI sites and remove or add a multiple of 3 nucleotides without generating new stop codons at the junctions would also generate colonies that survive neomycin selection. As documented below, these alternative events occur at low frequency.

DSBs repair by precise ligation

To assay for DSB repair by precise ligation, we tested three independently derived human HT1080-derived cell lines, HPL9, HPL16 and HPL19, each of which contains a single copy of the randomly integrated *tk*-neo gene containing the tandem pair of mutant ISceI sites (Figure 1D). We introduced DSBs into the integrated *tk*-neo genes in the cells by transfecting plasmid pCMV3xnlS ISceI, which expresses ISceI. DSB repair events were monitored by colony formation in the presence of G418, which selects for cells that express the *tk*-neo fusion gene. Neomycin-resistant cells arose at frequencies ranging from 3.4 to 7.8×10^{-3} (Table 2). By contrast, the frequency of G418-resistant colonies in untreated cells was less than 10^{-6} (data not shown). For each cell line, 48 G418-resistant colonies were further characterized by ISceI cutting and DNA sequencing. We found that 143 of the 144 colonies arose by precise ligation between the two ISceI sites (Table 2). The remaining colony contained a deletion that encompassed both ISceI sites (data not shown). In summary, these results show that this selection assay detects DSB repair events that arise primarily by precise ligation.

Precise ligation versus c-NHEJ

Although the selection assay preferentially detects precise ligation, it is blind to c-NHEJ events that remove a number of nucleotides that is not a multiple of 3. Since the majority of c-NHEJ events are associated with changes of a few nucleotides, with one-nucleotide deletions being the most common [8,9,11], it was possible that c-NHEJ events actually outnumbered those that arose by precise ligation. To test that possibility, we transfected HPL9 cells with plasmid pCMV3xnlS ISceI and analyzed the re-joining events in a nonselective way. To eliminate DNA that had not been cleaved at both ISceI sites and re-joined—by precise ligation or c-NHEJ—we digested the genomic DNA from treated cells with *Spe* I, which cleaves between the mutant 711 and 205 sites, preventing amplification of genomes that had not been cleaved by ISceI (Figure 1D). The DNA was then amplified by PCR, the PCR products were digested with *Spe* I, and the intact molecules were amplified again by PCR using nested primers. The final PCR products were then cloned and individual clones were analyzed by DNA sequencing. Among the 56 clones that were analyzed, 53 (95%) resulted from precise ligation events and 3 (5%) contained small deletions characteristic of c-NHEJ. These results showed that DSBs with compatible ends are much more efficiently repaired by precise ligation than by events that remove nucleotides in the joining process, as is typical for c-NHEJ.

Precise ligation depends on c-NHEJ components

The key question is whether precise ligation represents a distinct process from c-NHEJ or is a subpathway of c-NHEJ that requires no processing of the ends. To distinguish these possibilities, we individually knocked down the c-NHEJ factors Ku70, XRCC4, and LIG4 in HPL9 cells. As shown in Figure 2A, siRNA-mediated depletion of Ku70, XRCC4, and LIG4 significantly reduced the frequency of precise ligation in ISceI-treated cells, as judged by the frequency of G418-resistant colonies.

To test the possibility that the decrease in the frequency of G418 resistance might be due to reduced expression of ISceI in siRNA transfected cells, we measured ISceI mRNA in cells treated with control siRNA or siRNAs targeted to Ku70, XRCC4, or LIG4. After transfection of plasmid pCMV3xnlS ISceI, we found that the levels of ISceI mRNA after

siRNA treatment for Ku70, XRCC4 or LIG4 ranged from 83% to 124% of the amount in the control (vimentin siRNA) transfection, which was defined as 100%. Thus, knockdown of the expression of these genes has no significant influence on ISceI expression, eliminating the possibility that the reduced frequency of G418-resistant clones after knockdown of c-NHEJ components was caused by decreased expression of ISceI.

For each siRNA treatment, we analyzed 48 clones to determine the nature of the repair events. More than 98% of the clones from each of the siRNA-treated cells arose by precise ligation (Table 3). The reduced frequency, but same type of event—precise ligation—suggests that knockdown of c-NHEJ components partially compromises the c-NHEJ pathway, with the result that the fewer ends are joined, but they are still joined by precise ligation. These results indicate that Ku70, XRCC4, and LIG4 are essential for efficient precise ligation, suggesting that precise ligation is a subpathway of c-NHEJ that depends on key c-NHEJ components.

p53 and BRCA1 modulate precise ligation

Genomic instability, a hallmark of tumorigenesis, is enhanced by the loss of function of certain tumor suppressors, including p53 and BRCA1, which affect DSB repair in various ways [1,24,25]. To test their role, if any, in precise ligation, we knocked down expression of p53 and BRCA1 in HPL9 cells. As shown in Figure 2A, knockdown of either p53 or BRCA1 significantly reduced the repair of ISceI-induced DSBs, as assessed by selection for neomycin resistance. As shown above for knockdown of c-NHEJ components, siRNA knockdown of p53 or BRCA1 did not affect the expression levels of ISceI (data not shown). Isolation of individual repair products and analysis of their end-joining junctions revealed that most DSB repair occurred by precise ligation; however, the knockdown of BRCA1 raised the proportion of deletions to 10–15%, a significant increase relative to knockdown of p53 or NHEJ components (Table 3). This result indicates that knockdown of BRCA1 not only partially compromises the c-NHEJ pathway, but also diverts some substrates away from end joining by precise ligation. As expected the deletions were all multiples of 3 nucleotides and ranged from 9 to a few hundred base pairs (Figure 3). These results indicate that both p53 and BRCA1 play a role in precise ligation, suggesting a potential link to the genomic instability in tumor cells with mutations in these genes.

Effects of alt-NHEJ and other DNA repair pathways

In mammalian cells, NHEJ consists of two subpathways: the classical pathway, c-NHEJ, and an alternative pathway, alt-NHEJ. The alt-NHEJ pathway typically involves DNA end resection and uses short lengths of homology to join DNA ends. The alt-NHEJ pathway depends on XRCC1 and LIG3, which are used in the joining reaction, and on PARP1, which is required to direct broken ends toward repair by alt-NHEJ or by homologous recombination (HR) [26]. To test whether alt-NHEJ is used for precise ligation, we individually knocked down XRCC1, LIG3, and PARP1, and measured the efficiency of repair by precise ligation in HPL9 cells. As shown in Figure 2B, siRNA knockdown of XRCC1 or LIG3 did not affect the frequency of G418 resistant colonies, indicating that the alt-NHEJ pathway is not involved in precise ligation. Surprisingly, however, siRNA knockdown of PARP1 stimulated precise ligation more than 2 fold, suggesting that its normal action removes substrates from the c-NHEJ pathway.

To test the roles of other DNA repair pathways in precise ligation of DSB, we chose key factors in three DNA repair pathways: nucleotide excision repair (NER) component XPA (xeroderma pigmentosum type A) [27], mismatch repair (MMR) factor MSH2 [28], and single-strand break (SSB) repair factor TDP1 (tyrosyl DNA phosphodiesterase) [29]. As shown in Figure 2B, knockdown of XPA, MSH2, or TDP1 did not affect the efficiency of

DSB repair. The pattern of repair events was also not affected (data not shown). These results suggest that NER, MMR, and SSB repair do not participate in repairing DSBs via precise ligation.

DISCUSSION

In this report, we describe a novel selection assay for the repair of chromosomal DSBs by precise ligation of the ends. The assay includes three key features. First, the target for ISceI cleavage is a tandem pair of mutant ISceI sites that are individually cleavable, but whose rejoined halves are not. This arrangement eliminates the recutting of precise ligation products that was noted as a potential problem in previous studies [14,23]. Second, between the mutant ISceI sites we inserted a spacer sequence that carries stop codons in all three reading frames. These termination codons ensure that repair events at either single ISceI site will not be detected in the assay. Finally, the *tk-neo* fusion gene serves as a reporter for the in-frame removal of the stop codons between the mutant ISceI sites, which were positioned in the TK portion of the fusion gene. Removal of the stop codons allows the neomycin resistance gene to be expressed. Using this assay, we show that 99% of the G418-resistant colonies that arise after treatment with ISceI are due to precise ligation. Thus, this assay represents a substantial improvement over previous assays, which included up to 70% mutant NHEJ junctions among the selected products [14,23,30].

Because our selection assay is blind to most NHEJ events, which commonly remove one or a few nucleotides [8,9,11], we used a nonselective analysis to examine all the ways the two ISceI-cut ends were rejoined. Using a restriction enzyme that cleaves in the spacer between the two ISceI sites, we eliminated uncut parental molecules and those cleaved at a single ISceI site, allowing preferential amplification of molecules that had been cleaved at both ISceI sites and joined by either precise ligation or mutagenic NHEJ. Analysis of the PCR products showed that 95% had been joined by precise ligation, with 5% possessing the mutant junctions typical of NHEJ. Thus, cells presented with a chromosomal DSB that has compatible, complementary ends, preferentially rejoin the ends by precise ligation. These results validate the concerns expressed in earlier studies that their measured frequencies of precise ligation of ISceI-induced DSBs (as low as 30%) could have resulted from the re-cleavage of rejoined, wild type sites until ISceI-resistant sites were generated [14,23].

A clean assay for precise ligation permitted us to define precise ligation as a subpathway of c-NHEJ. Although precise ligation is commonly accomplished in the test tube using DNA ligase alone, in the cell it requires the c-NHEJ machinery, including Ku70, XRCC4, and DNA ligase IV, since siRNA-mediated depletion of these factors significantly reduced the efficiency of repair by precise ligation. This may not be surprising since DNA ends are tightly bound by Ku70/Ku80 [31], which is thought to tether DNA ends to one another and serve as a docking site for other c-NHEJ proteins, including the XRCC4/LIG4 complex, which rejoins DNA ends during DSB repair. Moreover, in cell-free assays, the Ku complex has been shown to stimulate ligation of compatible DNA ends by DNA ligase IV [32]. Although c-NHEJ typically involves end processing, DNA synthesis, and ligation [4], we show that cells prefer to join ends precisely when presented with compatible ends. It should be noted that the requirements for Ku and XRCC4/LIG4 in precise ligation match a previous conjecture on how compatible ends would be handled by cells [33] and confirm the involvement of Ku suggested by previous studies in Ku80-negative CHO cells [23].

Knockdown of key components involved in alt-NHEJ did not reduce the frequency of precise ligation, indicating the alt-NHEJ does not play a role in the precise joining of ends. Knockdown of XRCC1 and DNA ligase III, which are required for the final ligation step during alt-NHEJ [26], had no effect on the frequency of precise ligation; thus, they do not

normally ligate compatible ends. By contrast, knockdown of PARP1, whose binding to DNA ends directs repair away from the c-NHEJ pathway by preventing the binding of the Ku complex [26], increased precise ligation more than twofold. This result suggests that roughly equal numbers of the ISceI-cleaved substrates are normally processed by c-NHEJ and by a PARP1-dependent pathway—either alt-NHEJ or HR [26]—and when PARP1 activity is compromised, all the substrates are directed to the c-NHEJ pathway instead. The PARP1-dependent flow is unlikely to be down the alt-NHEJ pathway because even in the absence of selection for precise ligation events, only 5% of substrates were joined imprecisely, as might have resulted from alt-NHEJ. Even if all these substrates were re-directed to the c-NHEJ pathway, they could not account for the twofold increase in precise ligation events. Thus, it seems likely that the PARP1-dependent flow is down the HR pathway, leading either to the repair of the cleaved substrate off an uncut molecule or to its ultimate loss, if no uncut parental molecules are present.

It is significant that mammalian cells seem to be poised to join DSBs in a way that preserves genomic integrity whenever possible; when DNA ends are compatible, cells overwhelmingly prefer to join them by precise ligation using the machinery of c-NHEJ. The loss of this predisposition toward maintaining genomic integrity is a hallmark of the early events in tumor development, which are often accompanied by mutations in the tumor suppressor genes, p53 and BRCA1 [34,35]. As we show here, the knockdown of either p53 or BRCA1 decreases the efficiency of precise ligation, indicating that these tumor suppressors normally function to promote precise ligation, in agreement with several previous studies [36–40]. Given the complicated effects of both p53 and BRCA1 on DSB repair, it is not certain how they normally bias a cell toward precise ligation. Nevertheless, it seems likely that BRCA1 exerts its effects by direct action on proteins involved in end-joining; for example, BRCA1 inhibits the exonuclease activity of the MRN complex, which may help to preserve the nucleotides at the broken end [40–42]. The significant number of deletions associated with BRCA1 knockdown, for example, could arise by diversion of some molecules destined for the HR pathway into the alt-NHEJ pathway. By contrast, although p53 has been reported to bind to DNA ends [43,44], its effect on precise ligation could be due to its activity as a transcriptional regulator, one of the several ways p53 interacts with DNA repair pathways [45]. It has been proposed, for example, that p53 promotes end-joining fidelity by enhancing expression of hnRNP G, which was found associated with DNA ends and may protect them from nuclease activity [37]. Whatever the mechanistic link between end joining and p53 and BRCA1, our studies suggest that the high rate of mutagenesis in tumor cells may be partially caused by the decrease in precise ligation during DSB repair.

In summary, we have developed a novel assay for precise ligation, and we have demonstrated that human cells choose to repair chromosomal DSBs with compatible ends almost exclusively by precise ligation. We have also shown that precise ligation requires key factors in the c-NHEJ pathway of repair and is sensitive to the status of two important tumor suppressors, p53 and BRCA1. This assay should be applicable to the search for proteins that modulate precise ligation and for chemicals that interfere with their function. The ability to impede precise ligation could potentially be useful in combination with radiotherapy and chemotherapy, by enhancing the rate of mutagenesis after treatments that introduce DSBs, thereby, increasing the cell's sensitivity [46,47].

Acknowledgments

This work was supported by a grant from the NIH (GM38219) to J.H.W.

References

1. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet.* 2001; 27:247–254. [PubMed: 11242102]
2. Rich T, Allen RL, Wyllie AH. Defying death after DNA damage. *Nature.* 2000; 407:777–783. [PubMed: 11048728]
3. Hartlerode AJ, Scully R. Mechanisms of double-strand break repair in somatic mammalian cells. *Biochem J.* 2009; 423:157–168. [PubMed: 19772495]
4. Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu Rev Biochem.* 2010; 79:181–211. [PubMed: 20192759]
5. Shrivastav M, De Haro LP, Nickoloff JA. Regulation of DNA double-strand break repair pathway choice. *Cell Res.* 2008; 18:134–147. [PubMed: 18157161]
6. Pardo B, Gomez-Gonzalez B, Aguilera A. DNA repair in mammalian cells: DNA double-strand break repair: how to fix a broken relationship. *Cell Mol Life Sci.* 2009; 66:1039–1056. [PubMed: 19153654]
7. Burma S, Chen BP, Chen DJ. Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. *DNA Repair (Amst).* 2006; 5:1042–1048. [PubMed: 16822724]
8. Lin Y, Waldman AS. Capture of DNA sequences at double-strand breaks in mammalian chromosomes. *Genetics.* 2001; 158:1665–1674. [PubMed: 11514454]
9. Lin Y, Waldman AS. Promiscuous patching of broken chromosomes in mammalian cells with extrachromosomal DNA. *Nucleic Acids Res.* 2001; 29:3975–3981. [PubMed: 11574679]
10. Roth DB, Porter TN, Wilson JH. Mechanisms of nonhomologous recombination in mammalian cells. *Mol Cell Biol.* 1985; 5:2599–2607. [PubMed: 3016509]
11. Roth DB, Wilson JH. Nonhomologous recombination in mammalian cells: role for short sequence homologies in the joining reaction. *Mol Cell Biol.* 1986; 6:4295–4304. [PubMed: 3025650]
12. Nick McElhinny SA, Snowden CM, McCarville J, Ramsden DA. Ku recruits the XRCC4-ligase IV complex to DNA ends. *Mol Cell Biol.* 2000; 20:2996–3003. [PubMed: 10757784]
13. Grawunder U, Wilm M, Wu X, Kulesza P, Wilson TE, Mann M, Lieber MR. Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells. *Nature.* 1997; 388:492–495. [PubMed: 9242410]
14. Lin Y, Lukacsovich T, Waldman AS. Multiple pathways for repair of DNA double-strand breaks in mammalian chromosomes. *Mol Cell Biol.* 1999; 19:8353–8360. [PubMed: 10567560]
15. Smith JA, Waldman BC, Waldman AS. A role for DNA mismatch repair protein Msh2 in error-prone double-strand-break repair in mammalian chromosomes. *Genetics.* 2005; 170:355–363. [PubMed: 15781695]
16. Colleaux L, d'Auriol L, Betermier M, Cottarel G, Jacquier A, Galibert F, Dujon B. Universal code equivalent of a yeast mitochondrial intron reading frame is expressed into *E. coli* as a specific double strand endonuclease. *Cell.* 1986; 44:521–533. [PubMed: 3004738]
17. Rouet P, Smih F, Jasin M. Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proc Natl Acad Sci U S A.* 1994; 91:6064–6068. [PubMed: 8016116]
18. Brenneman M, Gimble FS, Wilson JH. Stimulation of intrachromosomal homologous recombination in human cells by electroporation with site-specific endonucleases. *Proc Natl Acad Sci U S A.* 1996; 93:3608–3612. [PubMed: 8622983]
19. Sargent RG, Brenneman MA, Wilson JH. Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. *Mol Cell Biol.* 1997; 17:267–277. [PubMed: 8972207]
20. Lukacsovich T, Yang D, Waldman AS. Repair of a specific double-strand break generated within a mammalian chromosome by yeast endonuclease I-SceI. *Nucleic Acids Res.* 1994; 22:5649–5657. [PubMed: 7838718]
21. Choulika A, Perrin A, Dujon B, Nicolas JF. Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of *Saccharomyces cerevisiae*. *Mol Cell Biol.* 1995; 15:1968–1973. [PubMed: 7891691]

22. Colleaux L, D'Auriol L, Galibert F, Dujon B. Recognition and cleavage site of the intron-encoded omega transposase. *Proc Natl Acad Sci U S A*. 1988; 85:6022–6026. [PubMed: 2842757]
23. Guirouilh-Barbat J, Huck S, Bertrand P, Pirzio L, Desmaze C, Sabatier L, Lopez BS. Impact of the KU80 pathway on NHEJ-induced genome rearrangements in mammalian cells. *Mol Cell*. 2004; 14:611–623. [PubMed: 15175156]
24. Moynahan ME, Jasin M. Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. *Nat Rev Mol Cell Biol*. 2010; 11:196–207. [PubMed: 20177395]
25. Negrini S, Gorgoulis VG, Halazonetis TD. Genomic instability--an evolving hallmark of cancer. *Nat Rev Mol Cell Biol*. 2010; 11:220–228. [PubMed: 20177397]
26. Ciccia A, Elledge SJ. The DNA damage response: making it safe to play with knives. *Mol Cell*. 2010; 40:179–204. [PubMed: 20965415]
27. Hanawalt PC, Spivak G. Transcription-coupled DNA repair: two decades of progress and surprises. *Nat Rev Mol Cell Biol*. 2008; 9:958–970. [PubMed: 19023283]
28. Jiricny J. The multifaceted mismatch-repair system. *Nat Rev Mol Cell Biol*. 2006; 7:335–346. [PubMed: 16612326]
29. Caldecott KW. Single-strand break repair and genetic disease. *Nat Rev Genet*. 2008; 9:619–631. [PubMed: 18626472]
30. Dahm-Daphi J, Hubbe P, Horvath F, El-Awady RA, Bouffard KE, Powell SN, Willers H. Nonhomologous end-joining of site-specific but not of radiation-induced DNA double-strand breaks is reduced in the presence of wild-type p53. *Oncogene*. 2005; 24:1663–1672. [PubMed: 15688024]
31. Blier PR, Griffith AJ, Craft J, Hardin JA. Binding of Ku protein to DNA. Measurement of affinity for ends and demonstration of binding to nicks. *J Biol Chem*. 1993; 268:7594–7601. [PubMed: 8463290]
32. Gu J, Lu H, Tippin B, Shimazaki N, Goodman MF, Lieber MR. XRCC4:DNA ligase IV can ligate incompatible DNA ends and can ligate across gaps. *EMBO J*. 2007; 26:1010–1023. [PubMed: 17290226]
33. Lieber MR, Ma Y, Pannicke U, Schwarz K. The mechanism of vertebrate nonhomologous DNA end joining and its role in V(D)J recombination. *DNA Repair (Amst)*. 2004; 3:817–826. [PubMed: 15279766]
34. Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell*. 1997; 88:323–331. [PubMed: 9039259]
35. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011; 144:646–674. [PubMed: 21376230]
36. Lin Y, Waldman BC, Waldman AS. Suppression of high-fidelity double-strand break repair in mammalian chromosomes by pifithrin-alpha, a chemical inhibitor of p53. *DNA Repair (Amst)*. 2003; 2:1–11. [PubMed: 12509264]
37. Shin KH, Kim RH, Kang MK, Kim SG, Lim PK, Yochim JM, Baluda MA, Park NH. p53 promotes the fidelity of DNA end-joining activity by, in part, enhancing the expression of heterogeneous nuclear ribonucleoprotein G. *DNA Repair (Amst)*. 2007; 6:830–840. [PubMed: 17387044]
38. Wang HC, Chou WC, Shieh SY, Shen CY. Ataxia telangiectasia mutated and checkpoint kinase 2 regulate BRCA1 to promote the fidelity of DNA end-joining. *Cancer Res*. 2006; 66:1391–1400. [PubMed: 16452194]
39. Zhuang J, Zhang J, Willers H, Wang H, Chung JH, van Gent DC, Hallahan DE, Powell SN, Xia F. Checkpoint kinase 2-mediated phosphorylation of BRCA1 regulates the fidelity of nonhomologous end-joining. *Cancer Res*. 2006; 66:1401–1408. [PubMed: 16452195]
40. Thompson EG, Fares H, Dixon K. BRCA1 requirement for the fidelity of plasmid DNA double-strand break repair in cultured breast epithelial cells. *Environ Mol Mutagen*. 2012; 53:32–43. [PubMed: 22170030]
41. Durant ST, Nickoloff JA. Good timing in the cell cycle for precise DNA repair by BRCA1. *Cell Cycle*. 2005; 4:1216–1222. [PubMed: 16103751]
42. Paull TT, Cortez D, Bowers B, Elledge SJ, Gellert M. Direct DNA binding by Brca1. *Proc Natl Acad Sci U S A*. 2001; 98:6086–6091. [PubMed: 11353843]

43. Bakalkin G, Selivanova G, Yakovleva T, Kiseleva E, Kashuba E, Magnusson KP, Szekely L, Klein G, Terenius L, Wiman KG. p53 binds single-stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain. *Nucleic Acids Res.* 1995; 23:362–369. [PubMed: 7885831]
44. Lee S, Elenbaas B, Levine A, Griffith J. p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. *Cell.* 1995; 81:1013–1020. [PubMed: 7600570]
45. Gatz SA, Wiesmuller L. p53 in recombination and repair. *Cell Death Differ.* 2006; 13:1003–1016. [PubMed: 16543940]
46. Thoms J, Bristow RG. DNA repair targeting and radiotherapy: a focus on the therapeutic ratio. *Semin Radiat Oncol.* 2010; 20:217–222. [PubMed: 20832013]
47. Gullotta F, De Marinis E, Ascenzi P, di Masi A. Targeting the DNA double strand breaks repair for cancer therapy. *Curr Med Chem.* 2010; 17:2017–2048. [PubMed: 20423312]
48. Martinez JJ, Seveau S, Veiga E, Matsuyama S, Cossart P. Ku70, a component of DNA-dependent protein kinase, is a mammalian receptor for Rickettsia conorii. *Cell.* 2005; 123:1013–1023. [PubMed: 16360032]
49. Com E, Lagadec C, Page A, El Yazidi-Belkoura I, Slomianny C, Spencer A, Hammache D, Rudkin BB, Hondermarck H. Nerve growth factor receptor TrkA signaling in breast cancer cells involves Ku70 to prevent apoptosis. *Mol Cell Proteomics.* 2007; 6:1842–1854. [PubMed: 17617666]
50. Muylaert I, Elias P. Knockdown of DNA ligase IV/XRCC4 by RNA interference inhibits herpes simplex virus type I DNA replication. *J Biol Chem.* 2007; 282:10865–10872. [PubMed: 17296606]
51. Ahnesorg P, Smith P, Jackson SP. XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining. *Cell.* 2006; 124:301–313. [PubMed: 16439205]
52. Hubert L Jr, Lin Y, Dion V, Wilson JH. Topoisomerase 1 and single-strand break repair modulate transcription-induced CAG repeat contraction in human cells. *Mol Cell Biol.* 2011; 31:3105–3112. [PubMed: 21628532]
53. Liang L, Deng L, Nguyen SC, Zhao X, Maulion CD, Shao C, Tischfield JA. Human DNA ligases I and III, but not ligase IV, are required for microhomology-mediated end joining of DNA double-strand breaks. *Nucleic Acids Res.* 2008; 36:3297–3310. [PubMed: 18440984]
54. Lin Y, Leng M, Wan M, Wilson JH. Convergent transcription through a long CAG tract destabilizes repeats and induces apoptosis. *Mol Cell Biol.* 2010; 30:4435–4451. [PubMed: 20647539]
55. Quinn JE, Kennedy RD, Mullan PB, Gilmore PM, Carty M, Johnston PG, Harkin DP. BRCA1 functions as a differential modulator of chemotherapy-induced apoptosis. *Cancer Res.* 2003; 63:6221–6228. [PubMed: 14559807]
56. Lin Y, Dion V, Wilson JH. Transcription promotes contraction of CAG repeat tracts in human cells. *Nat Struct Mol Biol.* 2006; 13:179–180. [PubMed: 16388310]
57. Lin Y, Wilson JH. Transcription-induced CAG repeat contraction in human cells is mediated in part by transcription-coupled nucleotide excision repair. *Mol Cell Biol.* 2007; 27:6209–6217. [PubMed: 17591697]

- Chromosomal DSBs at multiple loci are almost exclusively repaired by precise ligation.
- Repair of DSBs by precise ligation requires classical NHEJ components.
- The tumor suppressors p53 and BRCA1 increase the efficiency of precise ligation.

Figure 1.

An assay for precise ligation. A. Effects of single nucleotide substitution on ISceI cleavage. Sixteen examples of results with mutant ISceI recognition sites are shown. Three samples are marked to indicate their susceptibility to cleavage: resistant to cleavage, “-”; partially cut “+”; well cut “++”. The lane marked WT is the wild-type ISceI recognition site. M indicates the marker DNA ladder. B. Summary of the effects of single nucleotide substitutions on ISceI cleavage efficiency. C. Efficiency of ISceI cleavage of the mutant recognition sites 711, 205, and 916. D. Modified *tk-neo* gene. A 54-bp DNA fragment was inserted at the *Sac*I site of the HSV1-*tk* gene. The insert includes two mutated ISceI recognition sites: mutant 711, which contains a G to C substitution at position 3 of the ISceI site, and mutant 205, which contains a T to G substitution at position 18. Between the two

ISceI sites, we included three stop codons, designed to terminate translation for all three reading frames, and a *Spe* I site (ACTAGT). Cleavage and rejoining of mutants 711 and 205 generates the hybrid site, mutant 916, and eliminates the *Spe* I site.

Figure 2.

Effects of the knockdown of DNA repair proteins on precise ligation. A. NHEJ pathway. B. Alt-NHEJ and other DNA repair pathways. In each case, the relative frequencies of G418-resistant colonies formed after transfection of plasmid pCMV3xnlS ISceI into HPL9 cells are shown. The frequency after vimentin siRNA treatment was defined as 100%. For each siRNA treatment, the data are the average frequency from at least 6 assays. Statistical significance is indicated: * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

Figure 3. Deletions among the G418-resistant colonies that arose after BRCA1 knockdown. The extents of deletion of material from each side are represented schematically. The sequences across the junctions are shown with microhomology indicated by capital letters. The total size of each deletion is indicated in base pairs.

Table 1

Sequences of siRNAs used in this study.

siRNA	Sequences ^a
Ku70-1	GGAAGAGAUAGUUUGAUUU ^[48]
Ku70-2	GAUGCCCUUACUGAAAAA ^[49]
XRCC4-1	AAUCUUGGGACAGAACCUGAAA ^[50]
XRCC4-2	AUAUGUUGGUGAACUGAGA ^[51]
LIG4-1	AAGCCAGACAAAAGAGGUGAA ^[50]
XRCC1	GGAAGAUCUUCAGGGUGUGGUAGU ^[52]
PARP-1	GUGGCGAAGAAGAAAUCUA ^[52]
LIG3-1	CGACCUUUUAGACUCAAUU ^[53]
p53-1	GCUUCGAGAUUUCCGAGAGCUGAA ^[54]
p53-2	CCGGACGAUUAUGAACAAGGUUCA ^[54]
BRCA1-1	AACCTGTCTCCACAAAGTGTG ^[55]
BRCA1-2	GACUUCUACAGAGUGAACCC ^[55]
XPA-1	GCUACUGGAGGCAUGGCUA ^[56]
MSH2-1	UCUGCAGAGUGUUGUGCUU ^[57]
TDP1-1	GGGAGCCGAUGAAUCAAGUGGUUA ^[52]

^a Numbers in parentheses indicate references describing the siRNAs used in this study

Table 2

Analysis of DSB repair in three cell lines.

Cell line	G418 ^R frequency ($\times 10^{-3}$) ^a	Total analyzed	Indel ^b	Precise ligation (%)
HPL9	7.8 ± 0.8	48	0	48 (100)
HPL16	3.7 ± 0.8	48	0	48 (100)
HPL19	3.4 ± 0.6	48	1	47 (98)

^aFrequencies were calculated as the number of G418-resistant colonies divided by the number of viable cells plated. Each frequency is the average of 6 experiments.

^bIndel stands for insertions or deletions. This assay only detects indels that remove or add a number of nucleotides that is a multiple of 3.

Table 3

Analysis of DSB repair events after knockdown of DNA repair factors.

siRNA	Total analyzed	Indel ^a	Precise ligation (%)
vimentin	48	1	47 (98)
Ku70-1	48	1	47 (98)
Ku70-2	48	0	48 (100)
XRCC4-1	48	1	47 (98)
XRCC4-2	48	0	48 (100)
LIG4-1	48	1	47 (98)
p53-1	48	0	48 (100)
p53-2	48	0	48 (100)
BRCA1-1	48	6	42 (88)
BRCA1-2	48	7	41 (85)

^aIndel stands for insertions or deletions. This assay only detects indels that remove or add a number of nucleotides that is a multiple of 3.