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EXO1 suppresses double-strand break induced homologous recombination between diverged sequences in mammalian cells

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Abstract

DNA double-strand breaks (DSBs) can be repaired through several mechanisms, including homologous recombination (HR). While HR between identical sequences is robust in mammalian cells, HR between diverged sequences is suppressed by DNA mismatch-repair (MMR) components such as MSH2. Exonuclease I (EXO1) interacts with the MMR machinery and has been proposed to act downstream of the mismatch recognition proteins in mismatch correction. EXO1 has also been shown to participate in extensive DSB end resection, an initial step in the HR pathway. To assess the contribution of EXO1 to HR in mammalian cells, DSB-inducible reporters were introduced into *Exo1*^{-/-} mouse embryonic stem cells, including a novel GFP reporter containing several silent polymorphisms to monitor HR between diverged sequences. Compared to HR between identical sequences which was not clearly affected, HR between diverged sequences was substantially increased in *Exo1*^{-/-} cells although to a lesser extent than seen in *Msh2*^{-/-} cells. Thus, like canonical MMR proteins, EXO1 can restrain aberrant HR events between diverged sequence elements in the genome.

Keywords

homologous recombination; EXO1; DNA mismatch repair; diverged sequences

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1. Introduction

To preclude genome instability, cells must efficiently repair DNA damage in as accurate a manner as possible. Failure to do so can lead to cell death or transformation. A particularly toxic DNA lesion is the double-strand break (DSB). Cells repair DSBs through several mechanisms, including homologous recombination (HR) in which the broken DNA utilizes an unbroken homologous sequence as a template for repair [1]. An alternative pathway, single-strand annealing (SSA), can be used if the DSB occurs between or within two homologous sequences, such as direct repeats. During SSA, DNA ends are extensively resected to uncover complementary strands, which can anneal to result in loss of the intervening sequence. Alternatively, a DSB can be repaired by non-homologous end joining (NHEJ), where ends that cannot be directly ligated may be processed by nucleases, leading to loss of genetic information at the site of the DSB. Both canonical and alternative NHEJ mechanisms have been reported, the latter of which may involve limited resection to reveal microhomologies [2].

HR is considered an error-free repair pathway because unlike other DSB repair pathways, it can restore genetic information lost at the site of the DSB. The identical sister chromatid is preferred as a template for HR compared to the homolog in mammalian cells [3]. HR between the many repetitive sequences present throughout the genome has the potential to lead to deletions, duplications, and other genome rearrangements, but it is highly suppressed by sequence divergence [4]. The DNA mismatch repair machinery (MMR) has been shown to suppress recombination between diverged sequences in several organisms [5–10], including mammals, where loss of MSH2 results in similar frequencies of HR between identical and diverged sequences [11,12]. Recombination between diverged sequences can result in heteroduplex DNA within the D-loop structures. In vitro studies suggest that a conformational change of the MSH2-MSH6 complex at D-loops may facilitate the later rejection steps of these recombination intermediates [13]. The Sgs1 helicase in yeast has also been reported to suppress HR between diverged sequences, potentially as a helicase that unwinds the heteroduplex DNA-containing D-loops, although its homolog in mammals, BLM, does not appear to perform a similar role [12].

Exonuclease I (EXO1) is a 5' to 3' exonuclease which interacts with the MMR machinery [14–17]. It has been proposed that EXO1 acts in the excision step of MMR, after mismatch recognition in prokaryotes and eukaryotes [18–21]. EXO1 is also involved in the 5' to 3' end resection at DSB ends to initiate HR in both yeast and mammalian systems [22]. These dual functions may be affected by both the catalytic and structural functions of EXO1 [23–25].

Because of its contribution to both MMR and end resection, we test here the role of EXO1 in HR using *Exo1*^{-/-} mouse embryonic stem (ES) cells [26]. To examine HR between diverged sequences, a novel reporter was developed from the widely-used DR-GFP reporter in which recombinants are identified on the basis of GFP expression [27]. In this new reporter, DR-GFP-11 mu, silent polymorphisms have been introduced along the length of the GFP gene. Using this reporter in a plasmid assay as well as an established reporter in a chromosomal assay, we find that overall HR between diverged sequences is substantially increased in

Exo1^{-/-} cells, although HR between identical sequences, as well as SSA, is not clearly affected, in line with the existence of redundant resection pathways. Molecular analysis of NHEJ events, however, shows a small decrease in the size of deletions at the junctions in the mutant cells. In summary, while EXO1 is not essential for HR between identical sequences, it plays a major role in suppressing HR between diverged sequences.

2. Materials and Methods

2.1. Plasmid constructs and DNA manipulations

DR-GFP was previously described [27]. DR-GFP-11mu was derived from hprtDR-GFP [28]. iGFP (794 bp) was subcloned into the HindIII site of pBlueScript.KS-. QuikChange II Site-Directed Mutagenesis was used to introduce 11 silent polymorphisms into iGFP, as per manufacturer's instructions (Agilent Technologies, Santa Clara, CA). This results in iGFP-11mu, with an increase of 1.4% divergence, relative to iGFP. The T to C point mutation resulting in an SfaNI site loss also includes loss of the endogenous BcgI cleavage site of iGFP [27]. iGFP-11mu was then cloned back into hprtDR-GFP, replacing iGFP with iGFP-11mu and resulting in DR-GFP-11mu. Each mutation and location is summarized in Supplementary Table 1.

For complementation assays, mouse *Exo1* cDNA was expressed using a pCAGGS expression vector [29]. pCAGGS.Exo1 was cloned by inserting NotI/KpnI digested fragments of mouse *Exo1* cDNA from pCMV.Exo1 into a modified pCAGGS vector containing NotI and KpnI. Mouse *Exo1*^{D173A} cDNA [30] was generated by a three-way SLiCE method as previously described [31]. Briefly, full-length mouse *Exo1* cDNA carrying the D173A mutation was PCR amplified from pCAGGS.Exo1 in two reactions using pCAGGS SLF (5' GTCTCATCATTTTGGCAAAG) with Exo1 DA R (5' CCAAATGCGAGGAGGgCAGAGTCCTCTGTG) and Exo1 DA F (5' CACAGAGGACTCTGcCCTCCTCGCATTGG) with pCAGGS SLR (5' TGAGGAGTGAATTCCTCGAA), respectively. Approximately 30 bp of end homologies and the Exo1 D173A mutation were introduced by these reactions. The wild-type mouse *Exo1* cDNA was removed from pCAGGS.Exo1 by NotI/EcoRV digestion and substituted with the two PCR fragments by SLiCE, resulting in pCAGGS.Exo1^{D173A} cDNA expression vector. Correct incorporation of the D173A mutation was confirmed by Sanger sequencing.

2.2. Cell lines and integration of repair substrates

Wild-type, *Msh2*^{-/-} [32], and *Exo1*^{-/-} (*Exo1*^{null/null} [26]) male mouse ES cells were cultured on gelatin-coated dishes in standard medium supplemented with 833 U/ml of ESGRO leukemia inhibitory factor (Millipore, Netherlands), as previously described [33]. *Msh2*^{-/-} cells and their wild-type counterpart were derived from E14 ES cells [32], whereas *Exo1*^{-/-} cells and their wild-type counterpart were derived from mouse blastocysts from *Exo1*^{+/-} matings. *Exo1*^{-/-} DR-GFP cells were generated by targeting the DR-GFP reporter constructs as previously described [34]. Briefly, 1×10^7 *Exo1*^{-/-} cells were electroporated (250V; 950 µF) with 75 µg of XhoI-digested p59xDR-GFP plasmid. The cells were selected in 110 µg/ml hygromycin and 1 µg/ml puromycin 48 hours following electroporation. After

selection, the individual clones were confirmed by Southern analysis for single integration at the *Pim1* locus. Two targeted clones were used for each genotype.

Wild-type and *Exo1*^{-/-} H-DR-WT and H-DR-8mu cell lines were generated by targeting the H-DR-WT and H-DR-8mu reporter constructs as previously described [11,12]. Briefly, wild-type and *Exo1*^{-/-} cells were cultured in HAT+ medium for 14 days prior to electroporation with 20 µg of SacI-XhoI-digested H-DR-WT and H-DR-8mu substrates. After selection, cell lines were confirmed by Southern blot analysis for single integration at the *Hprt* locus. Two targeted clones were used for each genotype (clones 1.3 and 1.7 for wild-type and clones 10 and 12 for *Exo1*^{-/-}). The *Exo1* genotype was confirmed in each cell line by PCR amplification. A 280 bp wild-type allele fragment is specifically amplified using primers A (5' CTCTTGCTCTGGGCTGATATGC) and B (5' ATGGCGTGCGTGATGTTGATA) and a 300 bp *Exo1*⁻ allele fragment is specifically amplified using primers B and C (5' AGGAGTAGAAGTGGCGCAAGG), using following cycling conditions: 94°C for 10 min, 40 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min; 72°C for 5 min.

Wild-type and *Exo1*^{-/-} SA-GFP lines were generated from E14 mouse embryonic stem cells by a CRISPR/Cas9-mediated genome editing approach. The SA-GFP substrate used in this study was described previously [35], except that the *puro*^R sequence between the two tandem *GFP* repeats is replaced with human *KU70* intronic sequence and that the substrate is targeted to a different genomic locus, *Rosa26*. Single-copy integration of the SA-GFP substrate to *Rosa26* was confirmed by PCR and Southern blot analysis. *Exo1* targeting was carried out by co-introducing a CRISPR/Cas9-mediated DSB in exon 4 of the *Exo1* gene and a promoterless *neo* resistant gene flanked by homology arms as the repair template (Fig. S1A) [36]. After 8 days of G418 selection (200ng/ml), resistant clones were isolated and expanded, and subjected to genomic DNA extraction and genotyping [36]. The *Exo1* genotype was determined by PCR amplification (Fig. S1B). Universal primers: mExo1-LA-in-F, CTTCCTGGCTACCATGTGTCC; mExo1-RA-in-R, GTATCCTATGGCCTATGGCACC. 5' confirmation primers: mExo1-5out-F, TGTCAAATCCCTTGGGTGC; Neointernals, CCCGCTTCAGTGACAACG. 3' confirmation primers: Neo-internal-F2, CGATCAGGATGATCTGGACG; mExo1-3out-R, GAAGCTGCTTCCCTTTAAGAAGG. OneTaq polymerase mix (New England Biolabs, Ipswich, MA) was applied in all genotyping PCR reactions as per manufacturer's instructions: denature at 95 °C for 2 min, followed by 32 cycles of 95 °C for 30 s, 60 °C for 1 min, and 68 °C for 2 min. A clone was chosen which was presumed to be biallelically targeted, as it demonstrated the correct targeting event by PCR and no evidence for a second mutation.

EXO1 expression in wild-type and *Exo1*^{-/-} SA-GFP lines was confirmed by Western blot (Fig. S1C). Cells were harvested and lysed on ice for 30 min in 10 mM Tris, pH 8, 1 mM EDTA, 10% glycerol, 0.5% NP-40, and 400 mM NaCl with freshly added 1 mM DTT and 1X protease/phosphatase inhibitor cocktails (Pierce). Lysates were centrifuged at 13000 g for 20 min and the supernatant was collected. Proteins were separated on a 10% gel (Bio-Rad) and transferred to a PVDF membrane at 100V for 80 min. Blocking was performed in 5% milk/PBST. Primary and secondary antibodies were incubated at 4°C overnight or at

room temperature for 1 hour, respectively. Each incubation was followed by three 10-min washes in PBST. The membrane was developed using Enhanced ECL (PerkinElmer). Antibodies were: anti-EXO1 (Bethyl Laboratories; A302-640A) and anti- α -tubulin (Sigma; T9026).

2.3. HR assays

For plasmid HR assays, 4×10^6 ES cells were co-transfected with 20 μ g of either DR-GFP or DR-GFP-11mu plasmid DNA and 20 μ g of either the I-SceI expression vector (pCBASce) [37] or an empty vector (pCAGGS) (250V; 950 μ F) and plated onto 60 mm plates. For EXO1 complementation assays, an additional 20 μ g of either empty (pCAGGS) or full-length *Exo1* cDNA (pCAGGS.Exo1) was electroporated with the above plasmids. For complementation with *Exo1*^{D173A} cDNA, 3.4 to 4×10^6 ES cells were cotransfected (225V; 950 μ F) with 16 μ g of each plasmid as described above. Cells were additionally transfected with 16 μ g of empty vector (pCAGGS), or full-length *Exo1* cDNA (pCAGGS.Exo1), or *Exo1*^{D173A} cDNA (pCAGGS.Exo1^{D173A}).

Complementation with *Exo1* cDNA was confirmed by Western Blot (Figure S3). 5×10^6 *Exo1*^{-/-} DR-GFP ES cells were electroporated as above with 20 μ g of I-SceI expression vector (pCBASce) and/or full length *Exo1* cDNA (pCAGGS.Exo1), harvested 24 h and/or 48 h after electroporation, and lysed on ice for 30 min in 10 mM Tris, pH 8, 1 mM EDTA, 10% glycerol, 0.5% NP-40, and 400 mM NaCl with freshly added 1 mM DTT and 1X protease/phosphatase inhibitor cocktails (Pierce). Lysates were centrifuged at 13000 g for 20 min and the supernatant was collected. Proteins were separated on a 4–15% gel (Bio-Rad) and transferred to a PVDF membrane at 22V overnight. Blocking was performed in 5% milk/PBST. Primary and secondary antibodies were incubated at 4°C overnight or at room temperature for 1 hour, respectively. Each incubation was followed by three 10-min washes in PBST. The membrane was developed using Enhanced ECL (PerkinElmer). Antibodies were: anti-EXO1 (Bethyl Laboratories; A302-640A) and anti- α -tubulin (Sigma; T9026) and anti-HA (Covance; MMS 101-P) to detect HA-tagged I-SceI (HA-I-SceI). Wild-type J1 DR-GFP ES cells and *Exo1*^{-/-} DR-GFP ES cells that were not electroporated served as controls for *Exo1* expression and were processed as above. For MSH2 Western blot, wild-type J1 DR-GFP and *Exo1*^{-/-} DR-GFP ES cell lysates were used as described above, with the exception that protein transfer occurred for 1 hour at 100V and secondary antibodies were incubated for 20 minutes. Antibodies were: anti-MSH2 (EMD Millipore; NA27) and anti-GAPDH (Cell Signaling Technology; 5174). Band intensities were determined using ImageJ software (NIH), normalized to GAPDH, and compared relative to wild-type control.

For plasmid HR assays, flow cytometric analysis was performed on a Beckton Dickinson FACScan to determine the frequency of HR by measuring the percentage of GFP-positive cells using FlowJo software. The percentage of GFP+ in the absence of I-SceI expression were extremely low, as frequencies from transfection of the empty vector (pCAGGS) ranged from 0.002–0.04%. Relative HR frequencies of DR-GFP and DR-GFP-11mu were determined by normalizing the absolute HR frequencies to DR-GFP. Statistical analyses comparing the absolute HR frequencies between DR-GFP and DR-GFP-11mu in the same cell line was determined by a paired student *t* test. Statistical analyses comparing the

absolute and relative HR frequencies between different cell lines were determined for each experiment by either paired or unpaired student *t* test, where applicable. Statistical analyses comparing absolute and relative HR frequencies between complemented and uncomplemented *Exo1*^{-/-} cells were determined by paired student *t* test.

For intrachromosomal DR-GFP HR assays, 2.5×10^6 ES cells were electroporated (250V; 950 μ F) with 30 μ g of the I-SceI expression vector (pCBASce) [37] or empty vector (pCAGGS) and plated onto 60 mm dishes. Flow cytometric analysis was performed on a Beckton Dickinson FACScan to determine the frequency of HR by measuring the percentage of GFP-positive cells at 24- and 48-hour time points. The percentage of GFP+ cells in the absence of I-SceI expression were extremely low, as frequencies from transfection of the empty vector (pCAGGS) were less than 0.02%. Statistical analysis comparing the absolute HR frequencies between wild-type (J1) clones and *Exo1*^{-/-} clones was determined by an unpaired student *t* test.

For intrachromosomal H-DR assays, 2×10^7 cells were electroporated with 25 μ g of pCBASce (400V, 250 μ F). 9/10 of electroporated cells were plated on 9×15 cm plates; G418 was added 24 hr later to select for *neo*⁺ colonies. The remaining electroporated cells were plated in serial dilutions (1/1000, 1/2000, 1/10000) without selection to calculate the total number of cells that survived electroporation. Recombination frequencies were calculated by the ratio of G418-resistant colonies to the total number of cells surviving electroporation. Statistical analyses comparing the absolute and relative HR frequencies between different cell lines were determined for each experiment by either paired or unpaired student *t* test, where applicable.

For intrachromosomal SSA assays, 1×10^6 wild-type or *Exo1*^{-/-} cells were nucleofected (Amaza nucleofector II, program A-024) with 4 μ g of pCBASce [36]. Flow cytometric analysis was performed on a Beckton Dickinson FACScan to determine the frequency of SSA by measuring the percentage of GFP-positive cells at 48-hour post transfection. Statistical analysis comparing the absolute SSA frequencies between wild type (E14) and *Exo1*^{-/-} was determined by an unpaired student *t* test.

2.4. Gene conversion tract analysis

Genomic DNA was isolated by phenol/chloroform extraction and amplified from H-DR-8mu *neo*⁺ colonies with primers Neo1 (5'GCCAATATGGATCGGCCATTGAACAA) and Neo3 (5'CCTCAGAAGAACTCGTCAAGA). PCR product was directly digested with appropriate restriction enzymes and resolved on 1.5% agarose gel. Mean gene conversion tract lengths ($\pm$ standard error of the mean) were determined by the last polymorphism converted. Statistical analyses comparing mean gene conversion tract lengths between different cell lines were determined by unpaired student *t* test.

2.5. Analysis of NHEJ DSB repair events

H-DR-WT cell lines were electroporated with 25 μ g of pCBASce. Colonies were allowed to grow with no selection for 9–12 days. DNA from pooled cells was isolated by phenol/chloroform extraction. Pooled DNA was digested with I-SceI to eliminate *S2neo* sequences that were not cleaved by I-SceI in vivo. I-SceI digested genomic DNA was precipitated,

resuspended in Tris-EDTA (TE) buffer, amplified with primers Neo1 and Neo3, and subcloned into pCR2.1 vector using TA Cloning Kit as per manufacturer's instructions (Invitrogen, Carlsbad, CA). Plasmid DNA from individual clones was isolated (Qiagen, Valencia, CA) and digested with NcoI and I-SceI to identify NHEJ repair events. NcoI⁻/I-SceI⁻ clones were sequenced across the DSB junction.

3. Results

3.1. EXO1 is not essential for HR or SSA between identical sequences

EXO1 is involved in DNA end resection to generate single-stranded DNA for strand invasion, the first step in HR, but other resection pathways exist [38,39]. To test the requirement for EXO1 in HR, we targeted the DR-GFP reporter [27] (Fig. 1A) to the *Pim1* locus in *Exo1*^{-/-} ES cells [40]. In DR-GFP, two non-functional copies of the GFP gene are separated by 2.5 kb. The first copy, SceGFP, is nonfunctional due to the replacement of 11 bp within the GFP gene to create a premature stop codon and an I-SceI endonuclease site within the GFP coding sequence. The second copy, iGFP, is truncated at both the 5' and 3' ends, but is identical to SceGFP except for the 11-bp substitution at the I-SceI site. Expression of I-SceI endonuclease results in a DSB within SceGFP; HR repair using iGFP as a template results in restoration of a wild-type *GFP* gene and GFP fluorescence. Two different clones of each genotype were transfected with the I-SceI expression vector. Little difference in HR levels was observed 24 hours after transfection in different clones (wild type: $1.85 \pm 0.16\%$ SEM, *Exo1*^{-/-}: $1.44 \pm 0.16\%$; $p = 0.10$; Fig. 1B); at 48 hours after transfection, the percent GFP-positive cells was somewhat higher in the *Exo1*^{-/-} cells (wild type: $3.60 \pm 0.26\%$, *Exo1*^{-/-}: $4.29 \pm 0.3\%$; $p = 0.11$), but this was likely due to clonal variation.

SSA repair of a DSB between direct repeats requires sufficient DNA end resection to uncover complementary sequences that can anneal [41]. To determine if EXO1 has an essential role in SSA repair requiring long range resection, *Exo1* was disrupted in ES cells containing the SA-GFP reporter [35] (Fig. S1A–C). After I-SceI cleavage, 3.0 kb must be resected to reveal 300 bp of complementary sequence that can anneal, resulting in a 2.7 kb deletion and restoration of a functional *GFP* gene that can be quantitatively measured by FACS (Fig. S1D). No statistically significant difference in SSA frequencies was found between wild-type and *Exo1*^{-/-} cells ($1.0 \pm 0.15\%$ GFP-positive cells for wild type vs. $0.88 \pm 0.07\%$ GFP-positive cells for *Exo1*^{-/-}; $p = 0.52$). Thus, EXO1 is not required for either HR or SSA, consistent with the presence of redundant resection pathways in mammalian cells.

3.2. EXO1 suppresses plasmid-based HR between diverged sequences

Sequence divergence ranging from 1.0 to 1.5% substantially suppresses HR in wild-type mouse ES cells but not in *Msh2*^{-/-} cells [11,12]. To simplify testing which proteins participate in this suppression, including EXO1, we modified DR-GFP to create DR-GFP-11mu in which 11 silent polymorphisms are present along the length of iGFP (Fig. 1A, Supplementary Table S1). The polymorphisms result in 11 new restriction sites and 1.4% sequence divergence between iGFP and iGFP-11mu.

To compare HR between homologous and diverged sequences, DR-GFP or DR-GFP-11mu were co-transfected with an I-SceI expression vector into wild-type ES cells, and GFP+ cells were quantified 48 hours later. DSB-induced HR between the diverged GFP sequences was reduced nearly 8-fold compared to HR between the nearly identical sequences in wild-type cells ($4.9 \pm 0.4\%$ SEM for DR-GFP vs. $0.65 \pm 0.11\%$ for DR-GFP-11mu; $p < 0.01$; Fig. 1C). The sequence divergence in the DR-GFP-11mu substrate is clearly recognized in wild-type cells by the MMR machinery, as HR frequencies of DR-GFP-11mu were reduced by only 20% compared to DR-GFP in MSH2-deficient ES cells, which was not statistically significant (5.6 ± 0.6 for DR-GFP vs. $4.5 \pm 0.2\%$ for DR-GFP-11mu; $p = 0.08$; Fig. 1C). These relative levels of HR between identical and diverged sequences in both wild-type and *Msh2*^{-/-} cell lines are similar to those previously reported using a long-term colony assay with a drug selection marker [12].

Given its interaction with the MMR machinery [15–17], we examined whether EXO1 contributes to the suppression of HR between diverged sequences. DR-GFP and DR-GFP-11mu were co-transfected with the I-SceI expression vector in *Exo1*^{-/-} ES cells [26] and GFP-positive cells were analyzed 48 hours later. Compared to the 8-fold reduction in wild-type cells, *Exo1*^{-/-} cells suppressed HR between diverged sequences to a significantly lower extent, such that HR was reduced by only 48% ($2.3 \pm 0.5\%$ for DR-GFP vs. $1.2 \pm 0.3\%$ for DR-GFP-11mu; $p < 0.01$; Fig. 1C). However, the failure to suppress recombination by *Exo1*^{-/-} cells was not as extensive compared to *Msh2*^{-/-} cells, which resulted in a reduction in HR by only 20% (Fig. 1C). Given that EXO1 interacts with MSH2, we determined whether this observation was due to a deficiency in MSH2 expression, rather than a direct role of EXO1. Western blot analysis demonstrated that MSH2 expression in *Exo1*^{-/-} cells was not reduced compared to wild-type cells (Fig. S2).

To confirm that the impaired suppression of HR was due to loss of EXO1, mouse *Exo1* was transiently co-transfected together with the I-SceI expression vector (Fig. S3A). The expression of full-length *Exo1* cDNA reduced the percent of GFP-positive cells by ~6-fold with DR-GFP-11mu compared to DR-GFP, whereas without *Exo1* expression, it was reduced by only 23% (Fig. 1D). To determine if this suppression of recombination with diverged sequence was due to the catalytic function of EXO1, an *Exo1*^{D173A} cDNA was constructed. While the EXO1 D173A mutant has defective catalytic activity, it nevertheless maintains DNA binding activity in the contexts of both the yeast [30] and human proteins [42]. *Exo1*^{-/-} cells were co-transfected with the *Exo1*^{D173A} and I-SceI expression vectors and either DR-GFP or DR-GFP-11mu (Fig. S3B). Cells expressing EXO1^{D173A} showed only a mild suppression of recombination between diverged sequences similar to cells transfected with an empty vector (~40%), much less than the ~6-fold decrease with wild-type EXO1 (Fig. 1E). These results support a contribution of EXO1 catalytic activity in suppression of HR between non-identical sequences.

3.3. EXO1 suppresses intrachromosomal HR between diverged sequences

To further investigate a role for EXO1 in suppressing HR between diverged sequences, we tested HR in the context of a chromosomal DSB. For this, the H-DR-WT and H-DR-8mu reporters were used in which individual recombinants could be selected using a neomycin

resistance gene (*neo*) (previously described in [11]. These reporters contain two non-functional copies of the *neo* gene (Fig. 2A). The first copy, *S2neo*, contains a full-length *neo* gene disrupted by the 18-bp I-SceI site inserted at the original NcoI site of *neo*, introducing a stop codon. The downstream copy of *neo*, either *pneo-WT* (H-DR-WT) or *pneo-8mu* (H-DR-8mu), is truncated at both ends but contains a wild-type sequence at the NcoI site; *pneo-8mu* also contains 8 silent polymorphisms, for 1.2% sequence divergence with *pneo-WT*. Expression of I-SceI followed by HR using the *pneo-WT* or *pneo-8mu* donor restores the NcoI site and results in a *neo+* clone. Both H-DR-WT and H-DR-8mu were targeted to the *Hprt* locus of wild-type and *Exo1*^{-/-} cells. Two targeted cell lines of each genotype were used for subsequent analysis.

Similar to what was observed with the GFP assays (Fig. 1B), *Exo1*^{-/-} cells had robust levels of HR using the *neo* reporter. In wild-type ES cells, 1.2% heterology reduced intrachromosomal HR >7-fold, from $18.7 \pm 2.3 \times 10^{-3}$ with H-DR-WT to $2.6 \pm 0.4 \times 10^{-3}$ with H-DR-8mu ($p < 0.01$; Fig. 2B). By contrast, this level of heterology had much less of an impact in *Exo1*^{-/-} cells: HR was reduced only ~2-fold compared to homologous sequences, from $32.3 \pm 0.3 \times 10^{-3}$ with H-DR-WT to $16.2 \pm 1.0 \times 10^{-3}$ with H-DR-8mu ($p < 0.01$; Fig. 2B). However, similar to what was observed with the plasmid HR assay, the failure to suppress recombination by *Exo1*^{-/-} cells was not as extensive compared to *Msh2*^{-/-} cells, which resulted in a reduction in HR with H-DR-8mu by only 16% [11]. Overall, these results further support the notion that EXO1 plays a role in suppressing HR between diverged sequences, regardless as to whether HR occurs between episomal or chromosomal sequences.

3.4. Gene conversion tracts in *Exo1*^{-/-} cells

The 8 silent polymorphisms in the H-DR-8mu reporter were used to compare gene conversion tracts in the wild-type and *Exo1*^{-/-} cells. The *neo+* gene was PCR amplified from individual *neo+* colonies and digested with restriction enzymes relevant to each of the silent polymorphisms. In both wild-type and *Exo1*^{-/-} cells, the majority of gene conversion tracts only involved conversion at the DSB or additionally the closest polymorphisms flanking the DSB on either side (NsiI and NruI polymorphisms located 8 and 46 bp to the right and left side of the NcoI site, respectively; Fig. 2C). As such, the median gene conversion tract length was 13 bp for both genotypes. In most conversion tracts, polymorphisms were fully converted, indicating mismatch correction of heteroduplex DNA or conversion of sequences in a double-strand gap. However, a fraction of tracts in both wild-type and *Exo1*^{-/-} cells contained both genotypes at one or a few polymorphisms. The fraction of these mixed tracts was higher in *Exo1*^{-/-} cells than wild-type cells (24% and 13%, respectively) consistent with a potentially relevant biological role for EXO1 in mismatch correction of heteroduplex DNA, although the level did not reach statistical significance ($p = 0.186$; Fig. 2C).

3.5 EXO1 processes larger deletions during NHEJ repair

NHEJ repair of a DSB can result in nucleolytic processing of the DNA ends. Due to EXO1's exonuclease activity, the role of EXO1 in class switch recombination and somatic hypermutation [26,43], and the role of yeast Exo1 in the processing of DNA ends during

NHEJ [44], we isolated and investigated the processed junctions that resulted from NHEJ in *Exo1*^{-/-} cells. The I-SceI expression vector was electroporated into wild-type H-DR-WT and *Exo1*^{-/-} H-DR-WT cell lines, and genomic DNA was isolated from unselected cell pools 9 to 12 days later. The *S2neo* sequence was PCR amplified using primers Neo1 and Neo3 and subcloned into the pCR2.1 vector using the TA Cloning Kit (Invitrogen). DNA from individual clones was subjected to restriction digestion with both NcoI and I-SceI (Fig. 3A). Failure to cleave by either NcoI or I-SceI suggests repair by NHEJ with end processing. To determine the sequence at the DSB repair junction, NcoI⁻/I-SceI⁻ clones were sequenced.

The analysis of repair junctions did not reveal any differences between wild-type and *Exo1*^{-/-} cells in blunt end joining (i.e., no microhomologies), in the frequency of deletion events involving microhomologies (Figure 3B), in the size of microhomologies used during NHEJ repair, or in the insertion/deletion ratio. In addition, the analysis of deletion events did not show a statistically significant difference in the distribution of the size of deletions ($p = 0.11$, Mann-Whitney; Fig. 3B) between wild-type and *Exo1*^{-/-} cells, and one repair junction dominated in both cell types. However, in NHEJ repair events in wild-type cells, a significantly greater proportion contained 10 or more nucleotide deletions (33.3% in wild-type cells, vs. 13.2% in *Exo1*^{-/-} cells; $p = 0.0059$). This data suggests that EXO1 is involved in the processing of longer (> 10 nt) excision tracts at DSB ends during NHEJ.

4. Discussion

DNA damage repair requires many components, some of which are also involved in multiple repair pathways to maintain genome integrity. One of these multifunctional repair components is EXO1, which has been reported to have dual roles in both end resection during HR [22] as well as the excision step during MMR [15,20,45], perhaps by separable catalytic and structural functions [23–25]. While the end resection and MMR excision functions most likely requires the 5' to 3' exonuclease activity of EXO1, the outcome of this activity differs: its role in end resection could promote recombination between identical sequences, while its role in MMR could suppress recombination between diverged sequences. Here we directly tested the role of EXO1 in recombination between both identical and diverged sequences in mammalian cells. We found that EXO1 is not essential for HR but plays a significant role in suppressing HR between diverged sequences, similar to other proteins involved in MMR [11,12]. Our results provide evidence that the catalytic function of EXO1 is responsible for this suppression. Like MSH2, EXO1 also participates in the repair of mismatches present in heteroduplex DNA during HR, as evidenced by a two-fold increase in the number of gene conversion tracts in which one or more polymorphisms were of both genotypes in mutant cells. However, the frequency of mixed tracts was not statistically different in *Exo1*^{-/-} cells compared to wild-type cells, suggesting that EXO1 does not play as significant role in mismatch repair in heteroduplex DNA as MSH2.

The increase in HR between diverged sequences in *Exo1*^{-/-} cells was observed in both the DR-GFP-11mu plasmid assay and the H-DR-8mu intrachromosomal assay. Interestingly, the relative HR frequencies between diverged sequences compared to homologous sequences are somewhat lower than those observed in *Msh2*^{-/-} ES cells using both the DR-GFP-11mu

assay (48% vs 81%) and H-DR-8mu assay (50% vs 84%) (Fig. 1C, and [11,12]), indicating that this core MMR factor (MSH2) may have a more important role in suppressing HR between diverged sequences. However, it should be noted that there can be some variability between assays (i.e., the plasmid complementation assay in Fig. 1D shows a greater recovery of events between diverged sequences, 77%).

Nicholson and colleagues analyzed the contribution of the components of the *S. cerevisiae* MMR machinery and found recombination between inverted repeats with ~1.1% sequence divergence was dependent on Msh2, but, interestingly, not Exo1 [46]. Similarly, heteroduplex rejection during SSA, which is facilitated by unwinding, rather than by nuclease degradation, was also independent of Exo1 [47]. However, double mutants of *exo1* / with other MMR components (Rad1 and Pms1) suggest an additive role for suppressing HR involving sequence divergence (SSA was not analyzed). These data suggest that EXO1 in mammalian cells may have a more significant role in suppressing recombination between diverged sequences than yeast Exo1, perhaps due to the proposed role of mammalian EXO1 in heteroduplex rejection at branched D-loops [13].

Overall, HR between nearly identical sequences was not clearly affected in *Exo1*^{-/-} ES cells. While this outcome is similar to *Msh2*^{-/-} ES cells [11], a role for EXO1 may have been anticipated given its role in end resection. Like HR, SSA was also not clearly affected, similar to results in MEFs [48]. These results are in keeping with extensive long-range resection involving two parallel pathways in eukaryotes: one that includes the BLM-TOPIIIα-RMI complex with DNA2 and another that includes EXO1 [22,39]. It is also possible that differences in the significance of EXO1 for HR exist in cell types from different tissues or species. In human cells depleted of EXO1, HR frequencies were reduced by 70% relative to HR frequencies in control siRNA knockdown cells, using an MCF7 cell line containing the DR-GFP reporter [49]. Overexpression of nuclease-dead EXO1 also resulted in reduced HR (~50%) relative to HR frequencies in cells transfected with full-length EXO1, suggesting that the end resection activity of EXO1 may play more of a role in HR in human cells. In mouse ES cells, it is likely that in the absence of the EXO1-dependent end resection pathway, DSB repair events are directed into the BLM-TOPIIIα-RMI1/DNA2 end resection pathway. It should be noted that BLM is not absolutely required for HR either, although small decreases in HR have been noted in BLM mutant cells [12]. We did observe a small decrease in NHEJ events with deletions > 10 bp, indicating a role for EXO1 in the generation of larger excision tracts at DSBs, which may be due to its exonuclease activity and processing of DSB ends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- EXO1 suppresses double-strand break induced homologous recombination between diverged sequences in mammalian cells.
- The contribution of mammalian EXO1 to homologous recombination (HR) was investigated.
- A novel GFP reporter to monitor HR between diverged sequences was developed.
- EXO1 was not essential for HR between identical sequences.
- HR between diverged sequences was substantially increased in *Exo1*^{-/-} cells.
- EXO1 catalytic activity is required for the increase in HR between diverged sequences.

Fig. 1. EXO1 is not required for HR between homologous sequences, but suppresses HR between diverged sequences

A. GFP recombination substrates. DR-GFP and DR-GFP-11mu were used to measure HR between homologous [27] and diverged sequences, respectively. Each contains two non-functional copies of GFP separated by 2.5 kb of sequence. SceGFP (white) contains a disrupting I-SceI recognition sequence inserted at the wild-type Bcgl sequence. iGFP (grey) is truncated at the 3' and 5' ends (overlap of SceGFP and iGFP shown in black horizontal lines). Repair of an I-SceI-induced DSB by HR leads to GFP expression (green). In DR-GFP, SceGFP and iGFP are identical except at the I-SceI site. In DR-GFP-11mu, 11 silent polymorphisms have been introduced along the length of iGFP (vertical lines), resulting in a gain or loss of restriction sites. X, XhoI; Av, AvrII; S, SacI; A, AatI; Nd, NdeI; N, NcoI; K, KpnI; Sm, SmaI.

B. Intrachromosomal HR frequencies using DR-GFP reporter 24 hours and 48 hours after I-SceI transfection are given. Data represent average % GFP-positive cells from 4 independent experiments and S.E.M. using two clones of each wild-type J1 and *Exo1*^{-/-} ES cells.

C. Mouse ES cells of the indicated genotypes were co-transfected with an I-SceI expression vector and either DR-GFP or DR-GFP-11mu. HR was determined by % GFP-positive cells measured by flow cytometry 48 hours later. HR frequencies are relative to those obtained with the DR-GFP substrate. Error bars represent the S.E.M. The number of paired (e.g., DR-GFP and DR-GFP.11mu) experiments is 7 for wild type, 4 for *Msh2*^{-/-}, and 6 for *Exo1*^{-/-}.

D. *Exo1*^{-/-} cells were co-transfected with a mouse *Exo1* cDNA expression vector (*Exo1*^{WT}) or an empty expression vector (-) along with the I-SceI expression vector and the indicated HR reporter. HR frequencies relative to DR-GFP are plotted (with S.E.M.). Average HR frequencies (with S.E.M.) from five independent experiments are presented below the graph.

E. *Exo1*^{-/-} cells were co-transfected with a full length *Exo1* cDNA expression vector (*Exo1*^{WT}), an empty expression vector (-), or catalytically dead *Exo1* cDNA expression vector (*Exo1*^{D173A}) along with the I-SceI expression vector and the indicated HR reporter. HR frequencies relative to DR-GFP are plotted (with S.E.M.). Average HR frequencies (with S.E.M.) from three independent experiments are presented below the graph.

Fig. 2. Intrachromosomal homologous recombination, recombination between diverged sequences, and gene conversion tract lengths are increased in Exo1^{-/-} cells

A. Intrachromosomal recombination substrates measure homologous recombination (H-DR-WT) and recombination between diverged sequences (H-DR-8mu). S2neo is nonfunctional due to an insertion of the I-SceI recognition sequence at the endogenous NcoI site. pneo-WT is truncated at both the 5' and 3' ends; pneo-8mu is similar but contains 8 silent restriction site polymorphisms that increase the divergence with S2neo to ~1.2%. After I-SceI cleavage, noncrossover gene conversion between the two repeats results in neo+ recombinants with the

I-SceI converted to the NcoI site (striped). For H-DR-8mu, gene conversion may also result in the incorporation of the polymorphisms. A, ApaI; L, ApaLI; P, PstI; B, BamHI; X, XbaI; Nr, NruI; Ns, NsiI; Pm, PmlI.

B. EXO1 suppresses recombination between both homologous and diverged sequences. The H-DR substrates were targeted to the Hprt locus in Exo1^{+/+} and Exo1^{-/-} ES cells. After I-SceI expression, recombination frequency was determined by dividing the total number of neo⁺ colonies by the number of cells surviving electroporation. Frequencies of recombination relative to H-DR-WT are given below. Data represent the mean and S.E.M. of 3 or 4 independent experiments.

C. Gene conversion tract lengths in wild-type and Exo1^{-/-} cells. neo⁺ clones derived from H-DR-8mu cell lines transfected with I-SceI expression vector were analyzed to determine the minimal extent of DSB-induced gene conversion. Distance between each polymorphism is indicated (in bp). Shown are the minimal lengths of the conversion tracts. Polymorphisms that were partially converted, suggestive of hDNA (mixed, grey bar) are given. Number of clones in each class for each cell line are indicated.

Fig. 3. EXO1 may play a role in processing large deletions associated with NHEJ
A. Molecular analysis distinguishes between DSB repair mechanisms at the I-SceI site in H-DR-WT. Cells are transfected with I-SceI, grown under non-selective conditions, and pooled genomic DNA is isolated. Genomic DNA is digested with I-SceI to eliminate non-informative uncut events and then amplified with Neo1 and Neo3.
B. Deletion sizes of NHEJ events were determined by cloning Neo1 and Neo3 PCR products (from A) and then sequenced to determine processing at the DSB. All deletion events are presented, and average deletions size ($\pm$ S.E.M) is given. While deletion size distribution is

not significant ($p = 0.11$, Mann-Whitney), the proportion of deletion events greater than 10 bp was larger in wild-type cells ($p = 0.0059$). Number of NHEJ repair events with microhomologies is indicated.

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