Deletion of the Nucleotide Excision Repair Gene *Ercc1* Reduces Immunoglobulin Class Switching and Alters Mutations Near Switch Recombination Junctions

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Abstract

The structure-specific endonuclease ERCC1-XPF is an essential component of the nucleotide excision DNA repair pathway. ERCC1-XPF nicks double-stranded DNA immediately adjacent to 3′ single-strand regions. Substrates include DNA bubbles and flaps. Furthermore, ERCC1 interacts with Msh2, a mismatch repair (MMR) protein involved in class switch recombination (CSR). Therefore, ERCC1-XPF has abilities that might be useful for antibody CSR. We tested whether ERCC1 is involved in CSR and found that *Ercc1*$^{-/-}$ splenic B cells show moderately reduced CSR in vitro, demonstrating that ERCC1-XPF participates in, but is not required for, CSR. To investigate the role of ERCC1 in CSR, the nucleotide sequences of switch (S) regions were determined. The mutation frequency in germline Sμ segments and recombined Sμ-Sγ3 segments cloned from *Ercc1*$^{-/-}$ splenic B cells induced to switch in culture was identical to that of wild-type (WT) littermates. However, *Ercc1*$^{-/-}$ cells show increased targeting of the mutations to G:C bp in RGYW/WRCY hotspots and mutations occur at sites more distant from the S–S junctions compared with WT mice. The results indicate that ERCC1 is not epistatic with MMR and suggest that ERCC1 might be involved in processing or repair of DNA lesions in S regions during CSR.

Key words: ERCC1-XPF • antibody • heavy chain • DNA repair • mouse B cells

Introduction

Activation of B lymphocytes by antigen and costimulatory signals, usually from T lymphocytes, initiates two processes of antibody diversification. Somatic hypermutation (SHM) introduces mutations in the variable region genes, which, in conjunction with antigen selection, generates antibodies with increased affinity. The second process, class switch recombination (CSR), enables B cells to diversify the constant region and thereby the effector function of the antibody molecule, while maintaining the same antigen-binding domain. These two mechanisms have several shared features but both are poorly understood.

CSR occurs by an intrachromosomal deletional recombination between switch (S) region sequences located upstream of the constant region genes. S region sequences consist of tandem repeats of short (20–80 bp) consensus elements, extending from 2 to 10 kb in length, and recombination can occur at any site within the S regions. The process is thought to be initiated by the creation of double-strand breaks within the S regions (1, 2), consistent with the ability to detect the deleted DNA as a circle (3). Although the different S regions have short sequence elements in common (e.g., GGGGT, GGGCT, or GAGCT), they differ too much to undergo homologous recombination and CSR is thought to occur by a type of nonhomologous end joining (NHEJ; ref-

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Abbreviations used in this paper: AID, activation-induced cytidine deaminase; BER, base excision repair; CFSE, carboxyfluorescein diacetate succinimidyl ester; CSR, class switch recombination; GL, germline; MMR, mismatch repair; NER, nucleotide excision repair; NHEJ, nonhomologous end joining; S, switch; SHM, somatic hypermutation; UNG, uracil DNA glycosylase.

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erences 4 and 5). This is supported by the occurrence of very few bp of microhomology at the S–S junctions, which is typical of NHEJ (6), and by the demonstration that the NHEJ proteins Ku70 and Ku80 are required for CSR (7–9). Although DNA-PKcs is required for switching to all isotypes except IgG1 (10), interestingly, its kinase activity is not (11).

Activation-induced cytidine deaminase (AID) is essential for both SHM and CSR (12, 13), and the data indicate its role is to convert dC to dU residues within variable genes and S regions (14–16). Resolution of the dU residues could introduce mutations that are characteristic both of SHM and of segments surrounding S–S junctions (17, 18). Furthermore, removal of the dU residues by the base excision repair (BER) pathway could introduce the DNA breaks necessary to initiate CSR. This has not been demonstrated; however, CSR is 90% reduced in B cells from mice deficient in the BER enzyme uracil DNA glycosylase (UNG) that excises dU residues (19), and even more severely reduced in some patients with hyper IgM syndrome caused by deleterious mutations in UNG (20).

Mismatch repair (MMR) proteins are also involved in both CSR and SHM, although their roles are not yet clear. B cells from mice deficient in Msh2, Msh6, Mlh1, or Pms2 and combinations thereof have reduced abilities (two- to sevenfold) to undergo CSR and have altered S–S recombination junctions (21–27). Additionally, mutations in segments surrounding the CSR junctions are increased in MMR-deficient mice (25, 28), consistent with the known role for Msh2, Msh6, Mlh1, and Pms2 in identification of DNA mismatches and recruitment of repair factors during MMR.

Msh2 and Msh3 participate in homologous recombination in *Saccharomyces cerevisiae* (29, 30), removing nonhomologous DNA ends in double-strand break repair (31). *Rad1* and *rad10* mutants have phenotypes similar to *msh2* and *msh3* mutants with regard to homologous recombination (32, 33) and DNA end processing (31, 34, 35). Rad1 and Rad10 form a complex with endonuclease activity (36), and Rad10 and Msh2 interact in two-hybrid and coimmunoprecipitation assays (37, 38). Thus, it has been proposed that Msh2-Msh3 recognize recombination and repair intermediates, and then recruit the endonuclease Rad10-Rad1 for incision (34). Consistent with its role in yeast, the available evidence suggests that one of the roles of Msh2 in CSR is to perform end processing (21, 24, 39). However, current evidence indicates that Msh3 has little or no role in CSR (26, 27).

ERCC1-XPF, the mammalian homologue of Rad10-Rad1, is a structure-specific endonuclease that nicks double-stranded DNA immediately adjacent to a 3′ single-stranded tail (40). This activity allows it to remove nonhomologous 3′ DNA ends, facilitating homologous recombination (41–44). In addition, ERCC1-XPF is an essential component of the nucleotide excision repair (NER) pathway. This pathway removes helix-distorting DNA lesions by incising the damaged strand on either side of the lesion to remove the damaged patch followed by resynthesis to fill the gap (45).

S regions must be transcribed to undergo CSR, and the transcript has been shown to form an RNA–DNA hybrid (R loop) with S region sequences (46, 47). This structure results in the formation of single-stranded DNA, which can be a target for AID (15, 48–51). ERCC1-XPF has been shown in vitro to cleave R loop structures at the site of the double-strand DNA–R loop transition (52). However, the absolute requirement for AID in CSR makes it highly unlikely that ERCC1-XPF is required to initiate CSR by recognition of R loops before cytidine deamination by AID.

To investigate a potential role for ERCC1-XPF, we analyzed CSR in splenic B cells isolated from mice deficient in ERCC1. Our data show that ERCC1-XPF is not essential for CSR, as has been recently reported by others (53, 54). However, we find that switching efficiency is reduced in ERCC1-deficient B cells and that the location and specificity of mutations introduced into S regions during CSR are altered, indicating that ERCC1-XPF participates in the DNA repair required for CSR.

Materials and Methods

Mice. *Ercc1*$^{-/-}$ mice and littermate controls were generated by heterozygote crosses in a mixed C57Bl/6:FVBn genetic background. Animals were killed by CO_2 asphyxiation 19–21 d postpartum and their spleens were removed. Genotyping was performed by PCR as described previously (55). Mice were typed for the *pol* ι gene as described previously (56) and the *Ercc1*$^{-/-}$ mice and their WT littermates were *pol* ι$^{+/+}$.

B Cell Isolation and Cultures. Splenic B cells were isolated by T cell depletion with antibody and complement, and cultured as described previously (24). To induce switch recombination to IgG3, 50 μg/ml LPS (Sigma-Aldrich) and 0.3 ng/ml anti–δ dextran (provided by C. Snapper, Uniformed Services University of the Health Sciences, Bethesda, MD) were added at the initiation of culture. To induce switch recombination to IgG1, LPS and IL-4 (800 U/ml; provided by W. Paul, National Institutes of Health, Bethesda, MD) were added. LPS and IFN-γ (10 U/ml) were used to induce IgG2a switching, and LPS and dextran sulfate (30 μg/ml; Amersham Biosciences) were used to induce IgG2b switching. LPS, 2 ng/ml TGF-β1, IL-4, 150 U/ml IL-5 (BD Biosciences), and anti–δ dextran were used to induce IgA CSR. To induce IgE CSR, cells were cultured at a lower density (12.5×10^4/ml) and treated with LPS and IL-4 (50,000 units/ml) for 5 d (57). Some cultures also included 100 ng/ml BLyS (Human Genome Sciences). BLyS did not alter the relative amount of CSR obtained in WT and *Ercc1*$^{-/-}$ B cell cultures. To detect IgE expressed on B cell surfaces, it is necessary to remove any IgE bound to CD23 (low affinity IgE receptor) due to IgE secretion into the cultures by the few cells that have switched to IgE expression. We tested both anti-CD23 blocking antibody (57) or treatment of fixed cells with acid (50 mM NaOAc, 85 mM NaCl, 5 mM KCl, 1% FCS, pH 4.0) and found that acid treatment alone worked as well as both procedures combined (see Fig S1, available at http://www.jem.org/cgi/content/full/jem.20040052/DC1, and unpublished data). Without this treatment, we found artifactual staining with anti-IgE antibody, in which 70–90% of the cells were stained.

For carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, cells were washed in serum-free HBSS (GIBCO BRL) and resuspended at 40×10^6/ml. An equal volume of 2.4 μM

CFSE was added and cells were incubated at 37°C for 12 min, and then washed with medium containing 10% FCS. Cells were then diluted and cultured as described above.

PCR Amplification and Cloning of Sμ–Sγ3 Junctions and Germline (GL) Sμ and Sγ3 Segments. Genomic DNA was isolated from purified splenic B cells ex vivo or after culture as indicated (24). Sμ–Sγ3 junctions E8–E86 were amplified from genomic DNA by PCR using the Expand Long Template Taq and Pfu polymerase mix (Roche) and the primers μ3-H3 and g3-2 (24). Sμ–Sγ3 junctions E188–E245 were amplified using primers Smu1 and Sg3-4 (25). These primers hybridize 168 bp 5′ to and 40 bp 3′ to the first primer set. Different primers were used for GL 5′ Sμ sequences, 5μ3 and 3μ2 (28, 58). Sequences located at the 5′ end of this segment were analyzed. The primers used to amplify the GL Sγ3 fragment were γ3-1 and g3-2 (28).

PCR products were cloned into the vector pCR4-TOPO (Invitrogen). For measurement of the mutation frequency and spectrum within recombined Sμ–Sγ3 segments, only clones whose Sμ or Sγ3 segments were completely sequenced were included. The PCR error frequency in our experiments was previously determined by sequencing several independent amplifications of the single Sμ–Sγ3 junction from the plasmacytoma TIB114, using procedures identical to ones used for splenic B cell junctions (28).

Online Supplemental Material. The following supplemental figures are available at http://www.jem.org/cgi/content/full/jem.20040052/DC1: Fig. S1, surface IgE expression (FACS® data); Fig. S2, surface IgG or IgA expression in total *Ercc1*$^{-/-}$ B cell populations relative to WT cells; Fig. S3, nucleotide mutations in GL Sμ segments; Fig. S4, sequences of Sμ–Sγ3 junctions

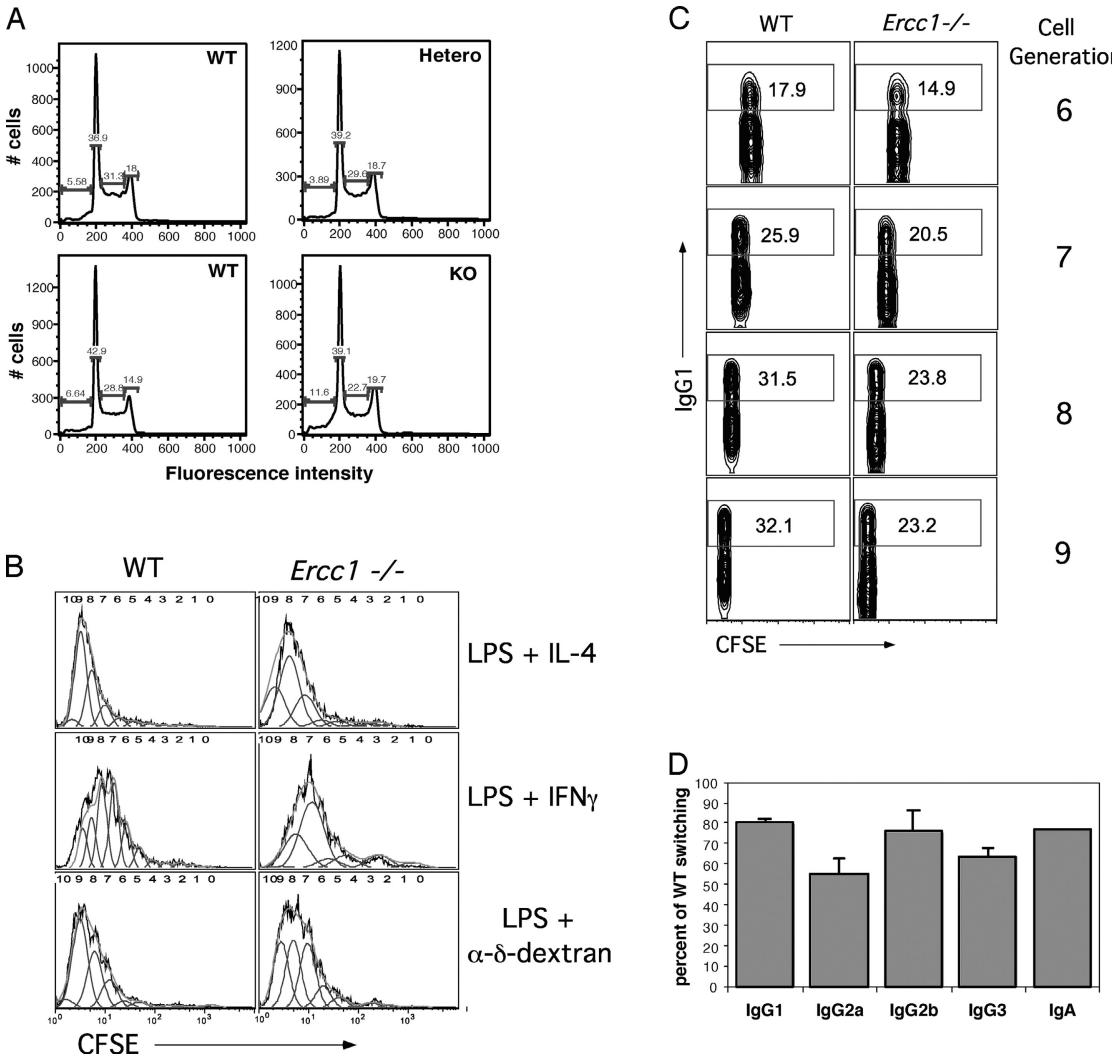

Figure 1. Reduced switching in *Ercc1*$^{-/-}$ splenic B cells is not due to a reduction in cell proliferation. (A) The percent of *Ercc1*$^{-/-}$ B cells in S phase, as determined by flow cytometry of propidium iodide–stained cells, after stimulation with LPS plus anti–δ dextran for 2 d is slightly reduced. One heterozygous and two WT littermates are shown for comparison. (B) CFSE-stained B cells cultured for 4 d with the indicated inducers lose CFSE staining by 50% with each cell division. The dark line represents the fluorescence tracing and the peaks within the fluorescence tracing are integrations representing individual cell generations that are enumerated above the graph. (C) Cells from the indicated generations were gated on and analyzed for IgG1 expression (other isotypes not shown). (D) Surface IgG or IgA expression by *Ercc1*$^{-/-}$ cells is shown as a percent of expression of WT cells (+ SEM) after 4 d in culture with appropriate CSR inducers. Only cells that divided six or more times were analyzed and the percent switching to each isotype relative to WT is shown (+ SEM). $n = 3$ for all isotypes, except for IgA, where $n = 1$. The differences between WT and *Ercc1*$^{-/-}$ switching for all isotypes are highly significant: $P < 0.001$ as determined by a paired *t* test.

Table I. *In Vitro Ercc1$^{-/-}$ Class Switching in Successive Cell Generations as a Percent of WT Switching*

Cell generation	IgG1	IgG2a	IgG3
6	83.2	82.5	52.3
7	79.1	70.4	61.4
8	75.6	74.6	56.4
9	72.3	77.7	53.2

from *Ercc1*$^{-/-}$ cells; Fig. S5, sequences of recombined Sμ segments (using μ3-H3 and g3-2 primers) from *Ercc1*$^{-/-}$ cells; Fig. S6, sequences of recombined Sγ3 segments (using μ3-H3 and g3-2 primers) from *Ercc1*$^{-/-}$ cells; Fig. S7, sequences of recombined Sμ segments (using Smu1 and Sg3-4 primers) from *Ercc1*$^{-/-}$ cells; and Fig. S8, sequences of recombined Sγ3 segments (using Smu1 and Sg3-4 primers) from *Ercc1*$^{-/-}$ cells.

Results

ERCC1-deficient mice have a severe growth defect and die ~3 wk after birth due to liver failure (59–61). Spleens harvested from 19–21-d-old *Ercc1*$^{-/-}$ mice had 10-fold fewer cells than their WT littermates, disproportionate compared with their total body weight (~40% of WT). To determine whether these mice have mature B cell populations, we stained spleen cells with anti-B220, anti-CD23, and anti-CD21, and analyzed them by flow cytometry. Despite their reduced cellularity, *Ercc1*$^{-/-}$ spleens contain populations of marginal zone follicular and immature B cells that are proportional to those of their WT littermates (unpublished data).

Next, we examined splenic B cells from *Ercc1*$^{-/-}$ mice in an in vitro isotype switching assay to determine if class switching is affected by ERCC1 deficiency. Spleens were harvested from KO and WT littermates at 19–21 d postnatum, and B cells were cultured for 4 d with LPS and cytokines to induce CSR to IgG3, IgG1, IgG2b, IgG2a, and IgA as described previously (23). We found a moderate reduction (20–55%) in class switching to all of these isotypes in the *Ercc1*$^{-/-}$ cells, which was highly reproducible and significant ($P < 0.001$; Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20040052/DC1). We also attempted to measure IgE CSR in cells cultured for 5 d, but were unable to detect significant levels of IgE surface–expressing cells in WT or *Ercc1*$^{-/-}$ cultures (Fig. S1; refer to Materials and Methods).

Although mature B cell populations appeared to be normal, the serious growth defects in *Ercc1*$^{-/-}$ mice led us to ask whether a decrease in the rate of cell division may underlie the decrease in class switching. Indeed, in both [^{3}H]thymidine uptake (not depicted) and propidium iodide cell cycle analysis (Fig. 1 A), mutant B cells did lag slightly behind WT B cells in the rate of cell division (76% of control cells). To control for the rate of cell division, we stained cells with the vital dye CFSE to follow cell division in culture. After 4 d, we further stained the cells with PE-coupled anti-Ig isotype antibodies to assay class switching. By day 4, most of the viable cells in both *Ercc1*$^{-/-}$ and WT cultures had divided five or more times, with *Ercc1*$^{-/-}$ cells lagging slightly behind WT cells (Fig. 1 B). We then analyzed class switching in cells that had divided six to nine times. Although the frequency of switched cells increased with cell division number in both *Ercc1*$^{-/-}$ and WT cells (Fig. 1 C and not depicted), switching was still reduced in

Table II. *The Mutation Frequency in Switch Region Segments from Ercc1*$^{-/-}$ *Splenic B Cells Induced to Undergo CSR in Culture with LPS Plus Anti–δ Dextran Does Not Differ from WT Littermates*

Row		Mutation frequency ($\times 10^{-4}$)	Total nucleotides of sequenced	Number of mutations	p-value[b]
WT B cells[a]					
1	GL 5′ Sμ	15.1	33,743	51	0.041
2	Recombined Sμ	35.0	8,003	28	<0.001
3	Recombined Sγ3	30.3	12,890	39	<0.001
Ercc1$^{-/-}$ B cells					
4	GL 5′ Sμ	17.9	24,536	44	0.012
5	Recombined Sμ	41.2	8,254	34	<0.001
6	GL Sγ3 (5′ + 3′)[c]	8.4	18,537	18	0.560
7	Recombined Sγ3	31.5	9,534	30	<0.001
8	PCR error frequency[d]	7.4	12,182	9	

[a]WT (+/+) 3-wk-old *ercc1* littermates.
[b]Significance of difference from PCR error frequency (Fisher's exact *t* test).
[c]GL Sγ3 segments cloned from mature WT mice from a different background (*pms2* and *msh2*) had a mutation frequency of 8.0×10^{-4} mutations per bp (reference 28).
[d]Determined from eight independent PCR amplifications of a recombined Sμ–Sγ3 segment from the Sμ segment, the TIB114 myeloma cell line (reference 28).

Table III. *Sequence Specificity of Mutations in Sμ and Sγ3 Segments from Activated[a] Ercc1$^{-/-}$ and WT Splenic B Cells Differ*

	Recombined Sμ[b]	p-value[d]	Recombined Sγ3[b]	p-value[d]	GL 5′ Sμ[c]	p-value[d]
Percent of mutations at G:C nucleotides:						
WT littermate	78.5% (28 mutations)	0.048	51% (39 mutations)	0.244	39% (56 mutations)	0.407
Ercc1$^{-/-}$[e]	71% (34 mutations)	0.212	68% (31 mutations)	0.575	51% (45 mutations)	0.352
DNA sequence:	*59% G/C*		*61% G/C*		*43% G/C*	
Percent of mutations at G:C within RGYW/WRCY Hotspots[e]:						
WT littermate	68% (28 mutations)	<0.001	23% (39 mutations)	0.683	22% (51 mutations)	0.095
Ercc1$^{-/-}$	68% (34 mutations)	<0.001	45% (31 mutations)	0.002	40% (45 mutations)	<0.001
DNA sequence:	*25% G:C bp in hotspots*		*20% G:C bp in hotspots*		*13.2% G:C bp in hotspots*	

[a]Activated for 4 d with LPS or with LPS plus anti–δ dextran.
[b]Segments amplified using the μ3H3 and g3-2 or the Smu1 and Sg3-4 primers.
[c]Segments amplified using the 5μ3 and 3μ2 primers.
[d]Difference from random DNA sequence base composition by Fisher's exact test.
[e]Only includes mutations at central two G:C bp in RGYW/WRCY sequence.

all *Ercc1*$^{-/-}$ cell populations relative to WT cells (Fig. 1 C and Table I). We analyzed expression of switched isotypes in cells that had divided six or more times in three independent experiments and found that class switching was reduced in the *Ercc1*$^{-/-}$ B cells relative to WT cells ($P < 0.001$ for all isotypes shown; Fig. 1 D). From this we conclude that the moderate but consistent reduction in class switching in *Ercc1*$^{-/-}$ B cells is not due to reduced cell division, and that ERCC1 may therefore have a role in CSR.

Altered Mutation Spectrum in the Unrecombined Sμ Alleles from Ercc1$^{-/-}$ *B Cells.* Unrecombined or GL Sμ alleles in B cells that have been activated to undergo CSR accumulate nucleotide substitutions, presumably due to aborted switch events (9, 28, 58, 62). To begin to determine whether ERCC1 might be involved directly in CSR, we examined the mutation frequency and spectrum in the GL 5′ Sμ segments from activated *Ercc1*$^{-/-}$ B cells compared with their 3-wk-old WT littermates (Fig. S3 shows the mutations identified in both genotypes). Although the mutation frequency was not significantly different between these two genotypes (Table II, rows 1 and 4), the mutation spectra differed (Table III). Especially striking is the finding that mutations in GL Sμ segments from *Ercc1*$^{-/-}$ cells focused to the two central G:C bp within RGYW/WRCY hotspots ($P < 0.001$), whereas GL Sμ mutations in WT littermates were not significantly targeted to hotspots. These data suggest that ERCC1-XPF participates in the error-prone mechanisms that repair DNA lesions initiated in the Sμ segment by AID, reducing the fraction of mutations at AID hotspots. However, because the frequency of mutation is not increased in the absence of ERCC1, other repair mechanisms appear to compensate for this function of ERCC1-XPF. The increased targeting of GL Sμ mutations to G:C bp in hotspots differs from our previous findings in *msh2*$^{-/-}$ cells.

Switch Recombination Junctions Are Not Altered in Ercc1$^{-/-}$ *Cells.* Because ERCC1-XPF is required for end processing during double-strand break repair in yeast and possibly in mammals (34, 41, 63), we asked whether Sμ–Sγ3 recombination junctions are altered in *Ercc1*$^{-/-}$ B cells. We PCR amplified, cloned, and sequenced 38 Sμ–Sγ3 junctions from *Ercc1*$^{-/-}$ B cells stimulated with LPS plus anti–δ dextran for 4 d. Similarly, 46 Sμ–Sγ3 junctions were amplified from 3-wk-old WT (*Ercc1*$^{+/+}$) littermates. No difference in the lengths of junctional microhomology or frequency of insertions at Sμ–Sγ3 junctions was observed between mutant and WT littermates (Table IV). As we have previously found that Msh2 deficiency results in decreased junctional microhomology and increased insertions at junctions, these data provide a further distinction be-

Table IV. *Sμ–Sγ3 Junctions in Ercc1*$^{-/-}$ *B Cells Are Similar to Junctions from WT Littermates*

	Percent of junctions with indicated length of microhomology						
Mouse	0–1 bp	≥2 bp	≥5 bp	≥8 bp	≥10 bp	Number of sequences	Percent with inserts
WT littermates	70	30	11	9	4	46	17
Ercc1$^{-/-}$	63	37	13	8	8	38	16

tween the roles of ERCC1-XPF and Msh2. However, there was an increase in length of microhomology patches and insertions in junctions cloned from both the WT and *Ercc1*$^{-/-}$ cells in these experiments compared with previously analyzed junctions from 3-mo-old WT mice (24). This difference might be due to age-related changes or differences in genetic backgrounds, but demonstrates the importance of determining the sequences of S junctions from WT littermates in each experiment.

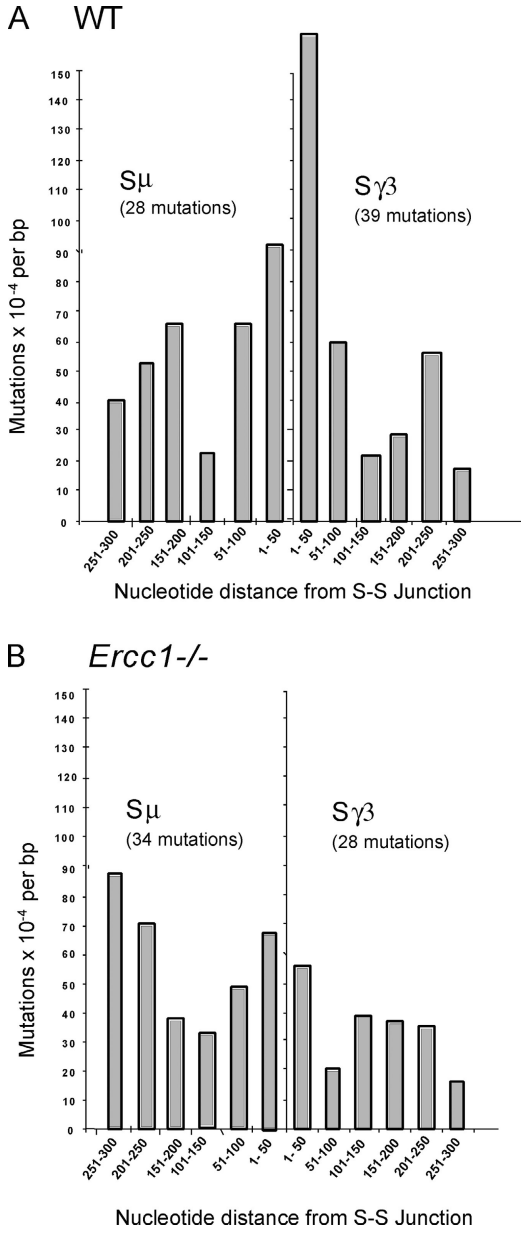

Figure 2. Mutations are more clustered at the Sμ–Sγ3 junctions in (A) WT B cells than in (B) *Ercc1*$^{-/-}$ B cells. The mutation density per 50-bp segment is plotted. Only segments in which the entire sequence was available are included. The significance of the differences in mutation distribution on both the Sμ and Sγ3 sides, and also in toto, are all $P < 0.001$; two-tailed Mann-Whitney test.

Mutations Extended Further from the Sμ–Sγ3 Junctions in Ercc1$^{-/-}$ *Cells and Increased Targeting to G:C bp in Hotspots on the Sγ3 Side.* Mutations are also frequently observed in the nucleotides surrounding switch recombination junctions, extending ~200 bp on either side of the junction (6, 28, 64, 65). Because the S segments are not within exons, the mutations do not affect antibody function and are readily tolerated, but they do have implications as to the mechanism of CSR. To determine whether ERCC1 might be involved in repair of S–S recombination junctions, we examined the mutations surrounding Sμ–Sγ3 junctions from *Ercc1*$^{-/-}$ and WT littermates. The sequences from *Ercc1*$^{-/-}$ cells are shown in Figs. S5–S8. There was no change in mutation frequency in DNA segments surrounding these junctions in *Ercc1*$^{-/-}$ compared with WT littermate cells (Table II, rows 2 and 3. vs. 5 and 7). However, the positions of the mutations relative to the Sμ–Sγ3 junctions were significantly different ($P < 0.001$; Fig. 2). WT cells have the highest density of mutations within 50 bp of the switch junction (Fig. 2 A), whereas mutations were spread much more distally in *Ercc1*$^{-/-}$ cells (Fig. 2 B). In fact, on the Sμ side, the mutation frequency did not decrease with increasing distance from the junction in *Ercc1*$^{-/-}$ cells. Because most of the recombined Sμ segments sequenced were 300 bp or less, we could not determine if the mutations extended even further from the junction. This pattern of mutations is strikingly different from the pattern observed in Msh2-deficient cells ($P < 0.001$), in which we found that mutations were even more focused to the S–S junctions than in the WT cells (28). Although the mutations did not extend further from the junction on the Sγ3 side in *Ercc1*$^{-/-}$ cells as they do on the Sμ side, there were significantly fewer mutations within 50 bp of the junction compared with WT cells.

Similar to the *Ercc1*$^{-/-}$ GL Sμ segments, mutations in the recombined Sγ3 segments in *Ercc1*$^{-/-}$ cells also preferentially occurred at G:C bp in hotspots ($P = 0.002$), unlike mutations in WT Sγ3 segments (Table III). Mutations in recombined Sμ segments in WT cells strongly favor G:C bp in hotspots ($P < 0.001$), and there was no further increase in targeting of the mutations to G:C bp hotspots in *Ercc1*$^{-/-}$ B cells (Table III). These data suggest that similar to the GL Sμ segment, the mutation spectrum of switched segments is altered in the absence of ERCC1, suggesting a role for ERCC1-XPF in processing CSR intermediates.

Discussion

ERCC1-XPF Participates in, but Is Not Essential for, CSR. Data presented in this work demonstrate that B cells deficient in ERCC1 have a mildly reduced ability to undergo CSR that is not due to reduced cell proliferation. Sequence analysis of the S region segments in cells induced to switch with LPS plus anti–δ dextran demonstrated a clear difference from WT littermates in the targeting of mutations found within GL Sμ segments and recombined Sγ3 segments. Furthermore, the mutations in recombined

Sμ segments from *Ercc1*$^{-/-}$ B cells did not cluster near the S–S junctions, unlike those in WT cells and in *msh2*$^{-/-}$ cells (28). These data demonstrate that ERCC1-XPF participates in, but is not required for, CSR. Specifically, the endonuclease appears to function during the repair of Sμ segments that initiate CSR but fail to recombine, and also during successful CSR.

Our data appear to conflict with previous conclusions that ERCC1-XPF is not involved in CSR. Winter et al. (53) reported that splenic B cells from *Ercc1*$^{-/-}$ mice switch in culture to IgG1 and IgE as efficiently as WT cells. Although the *Ercc1*$^{-/-}$ mouse line used in their study was independently derived from ours (59), these mice are exact phenocopies. To explain this discrepancy, we note that IgG1 is the isotype least affected in our assays, and that IgG1 switching is less sensitive than other Ig isotypes to various DNA repair deficiencies, including DNA-PKcs (10, 11) and MMR (21, 23). In addition, Winter et al. (53) measured IgE CSR by assaying surface-bound IgE, which can be inaccurate due to the presence of low affinity IgE receptors (CD23) on B cells (57, 66, 67). In fact, when we did not acid wash the B cells before staining with anti-IgE antibody, we found that up to 80% of the B cells in the cultures bound anti-IgE antibody (Fig. S1). More recently, it was found that B cells deficient in XPF, the catalytic subunit of the ERCC1-XPF endonuclease (68), also appeared to switch normally in culture (54). In this work the levels of IgG1, IgG2b, and IgG3 secreted by activated B cells from WT and *xpf*$^{-/-}$ B cells were found to be similar. However, neither of the above mentioned studies compared the specificity of mutations in the S regions from activated mutant and WT B cells. The fact that ERCC1 deficiency has such a modest effect on the level of CSR might be due to redundancy amongst DNA repair systems.

ERCC1 Is Not Epistatic with Msh2 in CSR. Our findings do not support the model that ERCC1-XPF is recruited by Msh2 to perform end processing during CSR (21, 23). Sμ–Sγ3 junctions from Msh2-deficient cells show a reduction in the length of microhomology between the Sμ and Sγ3 sequences compared with junctions from WT cells (24). In addition, there is an increased occurrence of inserted nucleotides at these junctions. B cells lacking the Sμ tandem repeats are absolutely dependent upon Msh2 to undergo CSR (39), whereas mice lacking Sμ repeats, but with WT Msh2, show only two- to fivefold reduced switching (69). These findings suggest that Msh2 is involved in processing DNA ends during recombination (21, 23, 24, 39). Although Msh2 may indeed be involved in processing DNA ends, it does not appear to do so by recruiting the endonuclease ERCC1-XPF, unlike the yeast system in which the homologues Rad1–Rad10 are epistatic with *msh2* and *msh3* mutants (35). Sμ–Sγ3 junctions in *Ercc1*$^{-/-}$ cells differ strikingly from *msh2*$^{-/-}$ junctions, as does the pattern of mutations surrounding the junctions. Our results demonstrate that ERCC1-XPF does not function with Msh2 in CSR. However, ERCC1-XPF may still be involved in 3′ end processing in CSR, as it could be recruited to junctions by other proteins.

ERCC1-XPF Deficiency Affects the Specificity of Mutations in GL Sμ and in Recombined Sγ3 Segments. Mutations in the GL Sμ segments in LPS + anti–δ dextran-activated WT splenic B cells do not commonly occur at G:C bp within the known AID target (RGYW/WRCY) sequences (Table III). AID is required for introduction of mutations into GL Sμ segments in activated B cells, and it is hypothesized that these mutations are caused by error-prone repair of AID-initiated lesions that do not result in CSR (58, 62). Abasic sites, which would be produced by AID and subsequent UNG activity, are normally targets for the BER enzyme AP endonuclease, which incises the DNA backbone (70, 71). Although repair is error free when DNA polymerase β inserts the correct nucleotide at this break (72–74), it is likely that in B cells error-prone polymerases (e.g., pol ι, η, and ζ) are recruited to the break, resulting in insertion of any nucleotide at the dC bp targeted by AID, and also introduction of mutations in flanking sequences (18, 56, 75–78). The increased incidence of mutations at G:C bp within AID target sequences and decreased incidence at other sites in *Ercc1*$^{-/-}$ B cells indicates a role for ERCC1-XPF in AID-initiated mutagenesis.

NER can also recognize and repair abasic sites in vitro (79) and might compete with BER to repair AID-initiated lesions. Additionally, NER is specialized for correcting mutations that distort the DNA helix, e.g., G:A mispairs (44, 79), which might be generated from AID-initiated lesions. Although NER is also typically error free, this repair machinery excises a patch of 24 to 32 nucleotides surrounding a lesion site (44, 80), and if abasic residues created by AID and UNG were clustered, this could instigate error-prone repair in the ~30 bp flanking the lesion(s). NER cannot occur in the absence of ERCC1-XPF, which could explain the focusing of mutations at G:C bp in *Ercc1*$^{-/-}$ B cells.

ERCC1-XPF Suppresses Mutations at Sites Distal to the Sμ–Sγ3 Junctions. The observation that in *Ercc1*$^{-/-}$ B cells mutations are spread more distally from S–S junctions suggests that in WT cells, ERCC1-XPF is required for the prevention and/or repair of the more distal mutations. The observation that the mutations extend further from the junction on the Sμ side than on the Sγ3 side might be due to a greater rate of transcription on the Sμ side before CSR (unpublished data). To account for the role of ERCC1-XPF in suppressing distal mutations, it is possible that a large Sμ segment (hundreds of nucleotides in length) may undergo end processing during CSR and if 3′ flaps are not removed in the absence of ERCC1, mutations would be observed over the entire region.

Conclusions. Our data, taken together with previous reports, suggest that components of three DNA repair systems, BER, MMR, and NER, are involved in repairing the lesions initiated by AID during the process of CSR. Although it is unclear how ERCC1-XPF contributes to CSR, it is possible that it is involved in repairing lesions instigated by AID and that it also contributes to creation of DNA ends suitable for CSR.

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