

Robust chromosomal DNA repair via alternative end-joining in the absence of X-ray repair cross-complementing protein 1 (XRCC1)

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Classical nonhomologous DNA end-joining (C-NHEJ), which is a major DNA double-strand break (DSB) repair pathway in mammalian cells, plays a dominant role in joining DSBs during Ig heavy chain (IgH) class switch recombination (CSR) in activated B lymphocytes. However, in B cells deficient for one or more requisite C-NHEJ factors, such as DNA ligase 4 (Lig4) or XRCC4, end-joining during CSR occurs by a distinct alternative end-joining (A-EJ) pathway. A-EJ also has been implicated in joining DSBs found in oncogenic chromosomal translocations. DNA ligase 3 (Lig3) and its cofactor XRCC1 are widely considered to be requisite A-EJ factors, based on biochemical studies or extrachromosomal substrate end-joining studies. However, potential roles for these factors in A-EJ of endogenous chromosomal DSBs have not been tested. Here, we report that *Xrcc1* inactivation via conditional gene-targeted deletion in WT or XRCC4-deficient primary B cells does not have an impact on either CSR or *IgH/c-myc* translocations in activated B lymphocytes. Indeed, homozygous deletion of *Xrcc1* does not impair A-EJ of I-SceI-induced DSBs in XRCC4-deficient pro-B-cell lines. Correspondingly, substantial depletion of Lig3 in Lig4-deficient primary B cells or B-cell lines does not impair A-EJ of CSR-mediated DSBs or formation of *IgH/c-myc* translocations. Our findings firmly demonstrate that XRCC1 is not a requisite factor for A-EJ of chromosomal DSBs and raise the possibility that DNA ligase 1 (Lig1) may contribute more to A-EJ than previously considered.

Double-strand breaks (DSBs) can be caused by environmental factors (e.g., ionizing radiation, UV exposure), metabolic byproducts (free radicals), replication stress, and programmed gene rearrangements in developing lymphocytes (1, 2). In vertebrates, there are two major DSB repair pathways, namely, homologous recombination (HR) and classical nonhomologous DNA end-joining (C-NHEJ) (1). HR requires a long, intact DNA template to initiate repair (2). In contrast, C-NHEJ directly joins DNA ends without overlapping nucleotides as well as ends with very short stretches of complementary nucleotides, referred to as microhomologies (MHs) (1–3). During DSB repair by C-NHEJ, DSB recognition is provided by the Ku70/Ku80 complex and joining is mediated by the XRCC4/ligase 4 (Lig4) ligation complex (1). These four factors are evolutionarily conserved in their roles in C-NHEJ and considered to be core C-NHEJ factors. In the absence of core C-NHEJ factors, DSBs still can be repaired at reduced efficiencies by an alternative end-joining (A-EJ) process (2, 4). A-EJ was initially identified based on experiments that showed linear extrachromosomal plasmid substrates could recircularize in C-NHEJ-deficient cells (5, 6). Subsequently, A-EJ was implicated in generating recurrent oncogenic chromosomal translocations found in progenitor B-cell tumors from mice doubly deficient for XRCC4 or Lig4 and the p53 tumor suppressor (7, 8).

Ig heavy chain (IgH) class switch recombination (CSR) in activated B cells replaces the C_μ constant region exons with one of a series of sets of downstream exons (“C_H genes”) that encode different IgH constant regions to carry out switching from IgM to a different IgH isotype, such as IgG1 or IgA. During CSR, DSBs in the donor switch region upstream of C_μ (S_μ) are joined to DSBs in a downstream acceptor S region, followed by the end-joining of the two broken S regions (9). In WT activated B cells, CSR DSBs are joined largely by C-NHEJ via either direct or short MH-mediated joins (10). However, in cells deficient in one or more of the core C-NHEJ factors, CSR breaks are joined at reduced levels, but still robustly, by an A-EJ process that is heavily biased toward MH-mediated joins (10, 11). A-EJ also repairs yeast endonuclease I-SceI-generated DSBs within chromosomally integrated substrates in Ku80- or XRCC4-deficient cell lines (12, 13). Although joins formed by A-EJ tend to be more biased toward MHs than those of C-NHEJ, both in frequency and in length of MHs (14), MH use is not an absolute criterion for A-EJ (2). For example, direct joins can comprise up to 20–50% of the total joins of CSR-associated or I-SceI-mediated chromosomal DSBs in Ku-deficient B cells or CHO cells, respectively (11–13).

The precise nature of A-EJ has been enigmatic. There may be more than one A-EJ pathway, and A-EJ pathways may potentially vary in the absence of particular C-NHEJ factors, with some even representing variant C-NHEJ pathways (1, 11). However, A-EJ operates even in the combined absence of C-NHEJ recognition (Ku70) and joining (Lig4) components, clearly demonstrating independence from C-NHEJ (11, 15). Thus, a working definition of A-EJ has been suggested to be any form of end-joining occurring in the absence of a core C-NHEJ factor (2). Factors that have been reported to function in chromosomal A-EJ in the context of CSR, V(D)J recombination, I-SceI substrates, and/or

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chromosomal translocations include Nbs1 (16), Mre11 (17–19), and CtIP (20, 21). These factors are thought to influence the choice between C-NHEJ and A-EJ pathways by mediating DNA end resection to uncover MHs and promote A-EJ. In addition, recent studies have indicated that Lig3 and, to lesser extent, Lig1 can mediate A-EJ associated with particular chromosomal translocations (22). Lig4 and its requisite cofactor XRCC4 are absolutely required for C-NHEJ during V(D)J recombination (2, 23). Therefore, A-EJ in the absence of Lig4 or XRCC4 might be expected to use one of the other cellular ligases, namely, Lig1 or Lig3 (2).

Lig1 is the replicative ligase and is involved in joining Okazaki fragments during lagging strand synthesis (24). Lig1 has also been implicated in long-patch base excision repair (BER) and nucleotide excision repair (25). The XRCC1/Lig3 complex operates in general short-patch BER and in single-strand break repair (26). Lig3 has both nuclear and mitochondrial isoforms (27), with the mitochondrial isoform, but not the nuclear isoform, operating independent of XRCC1 and being required for cell viability via maintenance of mtDNA integrity (28, 29). Both Lig3 and Lig1 have been implicated in A-EJ based on extra-chromosomal plasmid reporter assays, biochemical studies (30–33), and assays using chromosomal endonuclease sites (22); all these studies concluded that Lig3 is a key ligase for A-EJ. The XRCC1 BER factor serves as a scaffold to recruit other BER factors, including nuclear Lig3, which it also stabilizes (25, 34). XRCC1 was described as a mammalian A-EJ factor, based on biochemical experiments (30). Whereas XRCC1 might be hypothesized to function in A-EJ via stabilizing Lig3, the XRCC1 plant ortholog has been proposed to be involved in A-EJ, although plants lack Lig3 (35). In this regard, XRCC1 also has nonoverlapping roles with Lig3 in nuclear DNA repair, because XRCC1, but not Lig3, depletion leads to sensitivity to DNA-alkylating agents (28, 29). Recently, studies of XRCC1 haploinsufficient B cells led to the conclusion that XRCC1 functions in A-EJ during CSR and formation of *IgH/c-myc* translocations (36).

To define potential roles of XRCC1 and Lig3 in A-EJ further, we have now assayed A-EJ in the context of CSR, joining of I-SceI DSBs, or chromosomal translocations in primary B cells and B-cell lines in which the functional *Xrcc1* gene was eliminated by

gene-targeted mutation or in which Lig3 protein was reduced to nearly undetectable levels by Lig3-specific shRNA.

Results

Conditional Inactivation of *Xrcc1* in Activated B Cells Causes Genotoxic Stress Sensitivity. To investigate a potential role of XRCC1 in CSR in mice, we conditionally inactivated *Xrcc1* alone or together with *Xrcc4* specifically in mature B cells. Mice that conditionally delete *Xrcc1* in B cells (“CX1 mice”) were generated by crossing mice carrying either two copies of a *loxP*-flanked (“floxed”) *Xrcc1* allele (37) or one copy of a floxed *Xrcc1* allele and one copy of an *Xrcc1* null allele with CD21-Cre transgenic mice (38). For combined deletion of both *Xrcc1* and *Xrcc4* (“CX1X4”) in B cells, CX1 mice were crossed with conditional *Xrcc4* KO mice (“CX4”; mice either carrying 2 floxed *Xrcc4* alleles or 1 floxed *Xrcc4* allele and 1 copy of an *Xrcc4* null allele) (10). In comparison to controls (Ctrls; either WT mice or mice with floxed alleles without CD21-Cre, because they give indistinguishable results), CX1 and CX1X4 mice had similar fractions of splenic B cells (Fig. 1A). XRCC1 protein levels were generally below detection levels in mature CX1 B cells and reduced to low levels in CX1X4 B cells (Fig. 1B and Fig. S1A and B). XRCC4 levels were dramatically reduced in both CX4 and CX1X4 activated B cells. Consistent with B cell-specific deletion, protein levels of these factors were normal in mutant and double-mutant thymocytes (Fig. 1B and Fig. S1A). XRCC1 is required for the stability of nuclear Lig3 (37). Conditional deletion of *Xrcc1* caused a substantial reduction in Lig3 protein levels in CX1 and CX1X4 B cells; however, consistent with less complete XRCC1 inactivation, residual Lig3 levels were higher in the CX1X4 B-cell population (Fig. S1A). Lig1 protein levels were normal in both CX1 and CX1X4 B cells (Fig. S1B).

Cellular sensitivity to the alkylating agent methane methyl sulfonate (MMS) is a hallmark of XRCC1 deficiency (34, 37). To test whether CX1 or CX1X4 B cells are functionally deficient for XRCC1, we examined MMS sensitivity. Isolated mature B cells from either CX1 or CX1X4 spleens were stimulated with α CD40 plus IL-4 (α CD40/IL-4) for 1, 2, or 3 days, and MMS was added at the indicated concentrations for 6 h (Fig. 1C and Fig. S1C). *Xrcc1* deletion rendered CX1 B cells highly sensitive to MMS (Fig. 1C and Fig. S1C), indicating that these cells are functionally XRCC1-

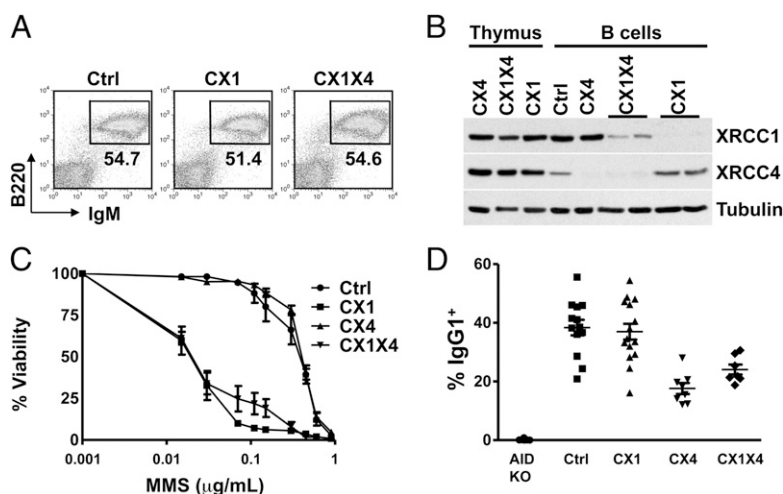


Fig. 1. XRCC1 is dispensable for normal and A-EJ-mediated CSR to IgG1. (A) Splenic B cells from Ctrl, CX1, or CX1X4 mice were stained with IgM and B220 antibodies and analyzed by flow cytometry. (B) Deletion levels in CX1 and CX1X4 splenic B cells. Extracts from purified activated B cells or thymocytes from CX1 ($n = 2$), CX1X4 ($n = 2$), or Ctrl mice were immunoblotted with the indicated antibodies. (C) Elevated genotoxic stress sensitivity of mature CX1 and CX1X4 B cells activated with α CD40/IL-4 for 3 d. B cells from at least three mice per genotype were used for each MMS concentration. (D) Surface IgG1 expression of B cells stimulated with α CD40/IL-4 for 4 d. A total of 13 Ctrl, 15 CX1, 8 CX4, and 7 CX1X4 mice were analyzed in independent experiments. *AID*^{-/-} (AID KO) mice were included as negative Ctrl. The results of IgG1 time course experiments are presented in Fig. S1D. Data are means \pm SEM.

deficient. In contrast, *Xrcc4* deletion (CX4) did not render cells sensitive to MMS (Fig. 1C and Fig. S1C). In comparison to both Ctrl and CX4 B cells, combined *Xrcc1* and *Xrcc4* deletion (CX1X4) also rendered a large portion of the B cells sensitive to MMS (Fig. 1C and Fig. S1C). However, especially at early time points, CX1X4 B cells displayed a biphasic MMS sensitivity curve (Fig. 1C and Fig. S1C), consistent with the presence of B cells with either complete or incomplete *Xrcc1* deletion. Analyses of the MMS curves suggested that 50–80% of the CX1X4 cells were fully MMS-sensitive at day 1, with the number increasing to 80% and 90%, respectively, at days 2 and 3 (Fig. 1C and Fig. S1C). We conclude that CD21-Cre-mediated *Xrcc1* deletion is more efficient in B cells carrying only floxed *Xrcc1* alleles than in B cells carrying both floxed *Xrcc1* and *Xrcc4* alleles. However, most CX1X4 B cells are functionally XRCC1-deficient by day 2 or day 3 of *in vitro* stimulation when CSR occurs.

Conditional *Xrcc1* Inactivation in B Cells Does Not Impair C-NHEJ- or A-EJ-Mediated CSR or *IgH/c-myc* Translocation Formation. To test the effect of *Xrcc1* inactivation on end-joining in the context of CSR, we assayed CX1 or CX1X4 splenic B cells for surface IgG1 expression after *in vitro* stimulation with α CD40/IL-4. B cells from CX1, CX1X4, CX4, and Ctrl mice were analyzed in independent time course experiments at days 2, 3, and 4 of stimulation (Fig. 1D and Fig. S1D). Despite XRCC1 deficiency (Fig. 1B) and functional inactivation (Fig. 1C), CX1 B cells did not display significant IgG1 CSR defects at early or late time points (Fig. 1D and Fig. S1D). In contrast, CX4 B cells showed reduced IgG1 CSR levels (Fig. 1D and Fig. S1D), as previously demonstrated (10). CX1X4 B cells underwent CSR to IgG1 at levels similar to CX4 cells, at both early and late time points (Fig. 1D and Fig. S1D), indicating that *Xrcc1* inactivation in a large fraction of the B-cell population did not affect A-EJ-mediated CSR. We also stimulated CX1 and CX1X4 B cells with bacterial LPS plus α IgD-dextran to induce CSR to IgG3 and obtained similar results as for switching to IgG1 (Fig. S1E).

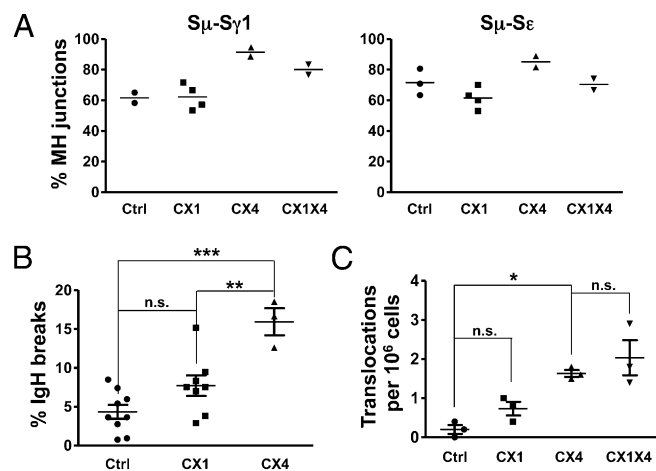


Fig. 2. XRCC1 deficiency does not affect switch region junctions, *IgH* genomic instability, or *IgH/c-myc* translocations. (A) Levels of MH-mediated S_{μ} - $S_{\gamma 1}$ joins (Left) and S_{μ} - S_{ϵ} joins (Right) in Ctrl, CX1, CX4, and CX1X4 activated B cells. S region junction data are shown in detail in Table S1. (B) CX1 B cells do not display altered levels of *IgH* locus breaks. The percentage of unrepaired *IgH* locus breaks in activated Ctrl, CX1, and CX4 B cells is shown. Data are means \pm SEM. A summary of all FISH data is included in Table S2. (C) XRCC1 is dispensable for *IgH/c-myc* translocations in CX1 and CX1X4 B cells. *IgH/c-myc* translocations were amplified from B cells cultured with α CD40/IL-4 for 4 d. Data are means \pm SEM. One-way ANOVA analysis with a Tukey posttest was used for calculation of statistical significance. n.s., not significant; * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

To address a potential role of XRCC1 in CSR and A-EJ further, we analyzed CSR junction sequences. In WT B cells, up to half of the S region joins are direct and the rest are mediated by short, ≤ 4 -nt MHs (10). In CX4 B cells, nearly 90% of junctions were MH-mediated as described (10). On the other hand, S_{μ} - $S_{\gamma 1}$ and S_{μ} - S_{ϵ} junctions from α CD40/IL-4-activated CX1 B cells showed similar levels of direct and MH-mediated junctions as Ctrl cells (Fig. 2A and Table S1). CX1X4 S_{μ} - $S_{\gamma 1}$ and S_{μ} - S_{ϵ} junctions also had increased MHs compared with Ctrl or CX1 junctions but not to the extent of CX4 junctions (Fig. 2A and Table S1). The latter finding is consistent with the presence of a small population of B cells in CX1X4 mice that did not undergo Cre-mediated deletion of *Xrcc4*.

Absence of DNA repair factors, such as Artemis, causes activation-induced cytidine deaminase (AID)-dependent *IgH* locus breaks in B cells activated for CSR without markedly diminishing overall CSR levels (39). Thus, we tested whether B cell-specific *Xrcc1* deletion leads to unrepaired AID-mediated *IgH* breaks or translocations. For this purpose, we used two sensitive DSB repair defect assays, namely, metaphase FISH (39) and PCR-mediated amplification of *IgH/c-myc* translocations (40). Compared with Ctrl, CX1 B cells did not show significant differences in frequency of *IgH* breaks (Fig. 2B and Table S2), in general translocations involving the *IgH* locus (Table S2), or in *IgH/c-myc* translocations (Fig. 2C). In contrast, CX4 B cells, as expected (10, 41), had elevated *IgH* breaks, *IgH* translocations, and *IgH/c-myc* translocations (Fig. 2B and C and Table S2). Although FISH data were not available for CX1X4 B cells, these cells showed a similar frequency of *IgH/c-myc* translocations in comparison to CX4 cells (Fig. 2C). Because the majority of CX1X4 B cells are functionally inactivated for XRCC1 by day 2 of activation (Fig. S1C), the finding that CX1X4 B cells have similar *IgH/c-myc* translocation levels as CX4 B cells indicates that XRCC1 is not required for A-EJ in this context.

***Xrcc1* Deletion Does Not Have an Impact on Joining of I-SceI Chromosomal DSB Substrates.** Our studies of CX1 and CX1X4 B cells indicate that XRCC1-independent A-EJ mechanisms can mediate CSR and chromosomal translocations. However, because residual XRCC1 protein remains in a subset of CX1X4 B cells (Fig. 1B and Fig. S1A and B), we sought to confirm this observation by a different approach. For this purpose, we generated Abelson murine leukemia virus (v-Abl)-transformed pro-B-cell lines (hereafter referred to as v-Abl-transformed pro-B lines) from progenitor B cells derived from bone marrow of mice carrying conditional *Xrcc4* and *Xrcc1* KO alleles ($X4^{c/c}X1^{c/c}$) and introduced a single retroviral end-joining reporter construct containing two I-SceI target sites. Joining of the two I-SceI sites eliminates an out-of-frame translation start site and results in expression of GFP (19). Following retroviral transduction, clones with unique genomic integration sites of the reporter substrate were identified by Southern blotting and three different $X4^{c/c}X1^{c/c}$ reporter lines (clones 26, 27, and 150), each with a unique single-copy substrate integration, were treated with recombinant Tat-Cre protein to generate subclones that were either null for XRCC4 ($X4^{-/-}$), or double-deficient for both XRCC1 and XRCC4 ($X4^{-/-}X1^{-/-}$). Absence of XRCC4 protein or of both XRCC1 and XRCC4 proteins in the respective lines was confirmed by Western blot analysis (Fig. 3A and Fig. S2A). Ablation of XRCC1 protein also resulted in a $>80\%$ reduction in total cellular Lig3 protein in $X4^{-/-}X1^{-/-}$ cells (Fig. 3A and Fig. S2B), consistent with prior findings showing that XRCC1 stabilizes Lig3 (25, 34).

To test DSB repair in $X4^{-/-}X1^{-/-}$ v-Abl-transformed pro-B lines, we transduced them with retroviral vectors encoding an I-SceI-glucocorticoid receptor (GR) fusion protein and subsequently induced nuclear translocation of the I-SceI-GR fusion protein by treatment of cells with triamcinolone acetonide (42). Average joining levels, based on the GFP reporter, were $20.6 \pm 2.2\%$ for the $X4^{c/c}X1^{c/c}$ clone 26 line, whereas in $X4^{-/-}$ and $X4^{-/-}$

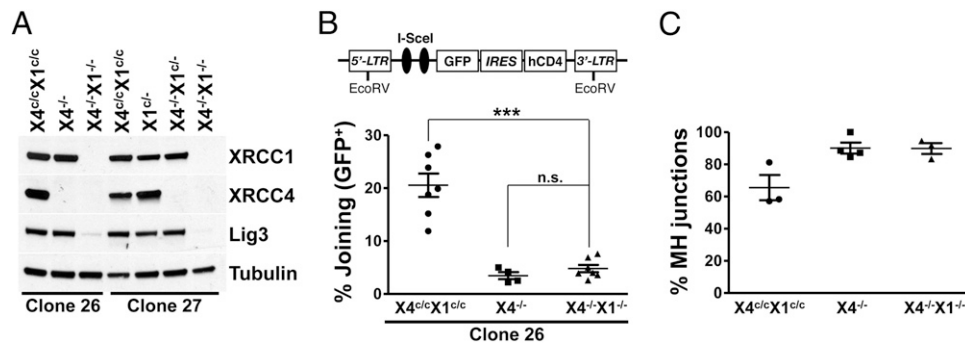


Fig. 3. *Xrcc4*^{-/-}*Xrcc1*^{-/-}-transformed pro-B lines do not display XRCC1-dependent end-joining defects. (A) Western blot analysis revealed complete absence of XRCC1 and XRCC4 proteins in subclones from two independent v-Abl-transformed pro-B lines. Lig3 levels are dramatically reduced in XRCC1 null cells. (B) (Upper) Illustration of I-SceI chromosomal reporter. (Lower) Quantification of I-SceI chromosomal joining for X4^{c/c}X1^{c/c} (*n* = 7), X4^{-/-} (*n* = 4), and X4^{-/-}X1^{-/-} (*n* = 7) v-Abl clonal lines with single-copy I-SceI chromosomal substrates integrated in the same location (clone 26). Data are means ± SEM. One-way ANOVA analysis with a Tukey posttest was used for calculation of statistical significance. n.s., not significant; ****P* < 0.0001. Tables S3 and S4 list details on I-SceI joining experiments. (C) *Xrcc1* deletion does not affect the nature of I-SceI junctions in XRCC4 null cells. I-SceI junctions were amplified from v-Abl cells of the indicated genotypes. Data are means ± SEM.

X1^{-/-} clone 26 sublines, joining levels decreased to 3.5 ± 0.7% and 4.8 ± 0.7%, respectively, which were not significantly different (Fig. 3B, Fig. S3, and Table S3). We obtained similar results for two additional lines with independent integration sites (Table S4). Therefore, abrogation of XRCC1 in XRCC4-deficient lines did not have a measurable impact on A-EJ activity.

To test whether XRCC1 loss affects the use of MH-mediated vs. direct joins in XRCC4 null cells, we PCR-amplified I-SceI junctions from X4^{-/-}X1^{-/-}, X4^{-/-}, and X4^{c/c}X1^{c/c} v-Abl transformants. We used primers flanking the I-SceI sites (located 650 bp apart) to allow for amplification of joins with relatively large resections. Because the two I-SceI sites in the reporter construct are in opposite orientations, joining involves DNA ends with non-complementary 4-nt overhangs. We analyzed 237 independent X4^{c/c}X1^{c/c} junctions, excluding junctions with short (<10 bp) insertions, which could be mediated by terminal deoxynucleotidyl transferase (43), and found ~34% direct junctions vs. 66% MH-mediated junctions (Fig. 3C). For X4^{-/-} cells, we observed that the frequency of MH-mediated joins increased to ~90%, with only about 10% of the junctions being direct (Fig. 3C). We analyzed 119 junctions from X4^{-/-}X1^{-/-} cells and found levels of MH-mediated vs. direct joins (~90% vs. 10%, respectively) similar to those of X4^{-/-} cells (Fig. 3C). These findings indicate that XRCC1 is not required to achieve the MH-mediated A-EJ repair of DSBs observed in XRCC4-deficient B cells.

Lig3 Inactivation in WT or Lig4-Deficient B-Lineage Cells Does Not Have a Measurable Impact on CSR or Translocations. XRCC1 and Lig3 are often considered to function together to mediate A-EJ (23, 31, 44–47). Conditional *Lig3* inactivation in primary B cells gave results very similar to those we described above for conditional *Xrcc1* inactivation (Fig. S4 and Table S1), but we had no functional assay for *Lig3* ablation. We also tried to inactivate *Lig3* conditionally alone or in combination with *Lig4* in v-Abl-transformed pro-B lines carrying conditional *Lig3* KO alleles (*Lig3*^{c/c}) or both conditional *Lig3* and *Lig4* KO alleles (*Lig3*^{c/c}*Lig4*^{c/c}) by Tat-Cre treatment. Screening of 412 Tat-Cre-treated *Lig3*^{c/c} subclones and ~600 *Lig3*^{c/c}*Lig4*^{c/c} subclones did not yield any *Lig3* null subclones, indicating that total *Lig3* inactivation is cell-lethal. Thus, to address a potential role of Lig3 in chromosomal A-EJ further, we assayed IgA switching in WT and *Lig4*^{-/-} CH12F3 cells transduced with lentiviruses expressing either Lig3-specific or scrambled Ctrl shRNAs. After 3 d of stimulation with αCD40/IL-4/TGF-β, ~60% of WT (transduced with a scrambled Ctrl shRNA) and Lig3-depleted WT CH12F3 cells had switched to IgA as evidenced by surface-staining flow

cytometry (Fig. 4B and C), despite depletion of cellular Lig3 in both by more than 90% (Fig. 4A and Fig. S5A). Like Lig4- or XRCC4-deficient primary B cells, 3-d stimulated *Lig4*^{-/-} CH12F3 cells infected with a Ctrl shRNA undergo CSR via A-EJ at about 30–50% of WT levels (10, 48) (Fig. 4B and C). Notably, *Lig4*^{-/-} CH12F3 cells expressing Lig3 shRNA also still underwent CSR at similar levels to the Ctrl shRNA-expressing *Lig4*^{-/-} CH12F3 cells based on accumulation of IgA⁺ cells (Fig. 4B and C), despite depletion of Lig3 levels by more than 90% (Fig. 4A and Fig. S5A). We did note, however, ~40% lower median fluorescence intensity of the IgA⁺ fraction of *Lig4*^{-/-} shLig3 cells in comparison to that of *Lig4*^{-/-} shCtrl cells (Fig. 4B). In this regard, we found similar relative levels of IgA-switched Ctrl and Lig3-depleted *Lig4*^{-/-} CH12F3 cells at days 2 and 3 of stimulation, indicating the absence of a kinetic delay in switching in the latter (Fig. 4C and Fig. S5B). Thus, the decreased surface IgA expression most likely reflects general adverse effects of Lig3 depletion in *Lig4*^{-/-} CH12F3 cells, as also reflected by slightly lower cell viability of Lig3-depleted vs. Ctrl *Lig4*^{-/-} CH12F3 cells [mean ± SEM (%): 34.2 ± 3.8 vs. 45.8 ± 2.3; *P* = 0.025, unpaired two-tailed *t* test].

Lig4^{-/-} CH12F3 cells transduced with either Ctrl or Lig3 shRNA showed similar levels of direct and MH-mediated Sμ-Sα junctions (Table S5). Together, these findings suggest that substantial Lig3 depletion does not measurably influence the level or quality of A-EJ-mediated CSR in CH12F3 cells. To assess a potential role of Lig3 in chromosomal end-joining further, we analyzed levels of *IgH/c-myc* translocations in stimulated WT or *Lig4*^{-/-} CH12F3 cells stably expressing either scrambled Ctrl or Lig3 shRNA (Fig. 4D). Deletion of *Lig4* significantly increased the frequency of *IgH/c-myc* translocations in CH12F3 cells (Fig. 4D), consistent with findings in primary B cells (11, 15). In contrast, Lig3 knockdown did not detectably alter *IgH/c-myc* translocation frequency in WT CH12F3 cells (Fig. 4D). Moreover, Lig3 depletion did not reduce the frequency of *IgH/c-myc* translocations in the absence of Lig4 (Fig. 4D).

Discussion

In mammalian cells, A-EJ has been shown to mediate both intrachromosomal joining for CSR (10) and interchromosomal translocation junctions (8, 22). XRCC1, a Lig3-stabilizing cofactor, and Lig3 itself have been very widely assumed to provide major end-ligation functions in A-EJ (22, 23, 31, 36, 44–47). We have now unequivocally established that XRCC1 is not required for A-EJ repair of I-SceI DSBs in XRCC4-deficient pro-B lines. We also found that conditional depletion of XRCC1 in XRCC4-

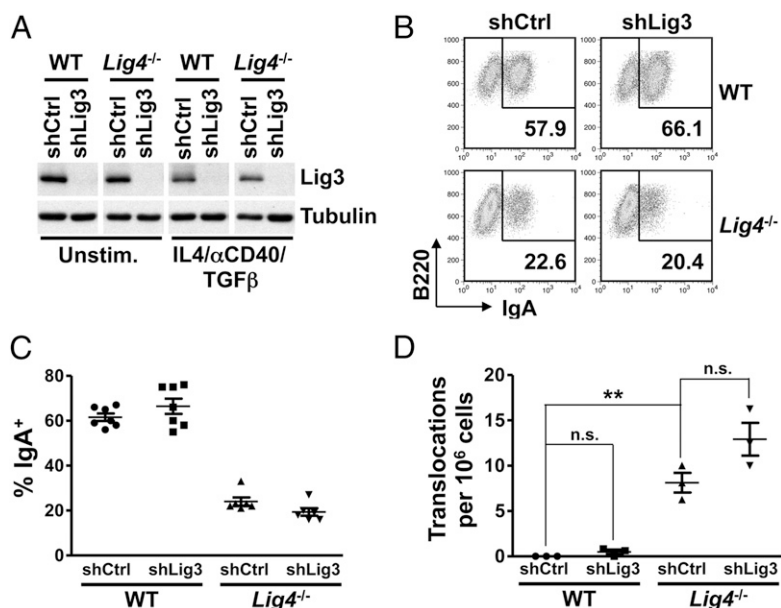


Fig. 4. shRNA-mediated depletion of Lig3 in B-cell lines does not affect CSR or frequency of *IgH/c-myc* translocations. (A) Lig3 protein levels in CH12F3 cells of the indicated genotypes expressing either Ctrl or Lig3 shRNA. Blots were stripped and probed for tubulin as a loading Ctrl. Unstim., unstimulated. (B) Representative example of flow cytometry results for IgA expression of WT or *Lig4*^{-/-} CH12F3 cells stably expressing either Ctrl or Lig3 shRNA on day 3 of stimulation with IL-4/ α CD40/TGF- β . Cultures propagated without cytokines and analyzed in parallel contained <1% IgA⁺ cells. (C) Summary of CSR experiments in CH12F3 B-cell lines. Means \pm SEM from seven (WT shCtrl and WT shLig3) or six (*Lig4*^{-/-} shCtrl and *Lig4*^{-/-} shLig3) independent experiments are shown. (D) Lig3 depletion does not affect the frequency of *IgH/c-myc* translocations. Translocations were analyzed in CH12F3 cells stimulated for 3 d with IL-4/ α CD40/TGF- β . Results from three independent knockdown experiments using WT shCtrl, WT shLig3, *Lig4*^{-/-} shCtrl, or *Lig4*^{-/-} shLig3 CH12F3 cells are shown. One-way ANOVA analysis with a Tukey posttest was used to assess statistical significance. Data are means \pm SEM. n.s., not significant; ***P* < 0.01.

deficient primary B cells, of which the vast majority were functionally XRCC1-deficient based on MMS sensitivity, led to no measurable impact on the formation of CSR junctions by A-EJ and did not alter the abundant use of MHs for their formation. Conditional depletion of XRCC1 in XRCC4-deficient primary B cells also had no apparent effect on the frequency of *IgH/c-Myc* translocations. We conclude that XRCC1 is not a requisite factor for major pathways of chromosomal A-EJ.

XRCC1 was previously implicated in A-EJ based on plasmid assays and biochemical experiments (30, 31, 33). Our current finding of no requisite role for XRCC1 in A-EJ might be rationalized with these earlier results if joining of DSBs in transient DNA substrates has different requirements than joining of chromosomal DSBs. In this context, cells lacking the C-NHEJ factor XLF have a V(D)J defect in extrachromosomal V(D)J recombination substrate assays but not for chromosomally integrated V(D)J recombination substrates or for V(D)J recombination of endogenous Ig and T-cell receptor loci (49, 50). A recent study found normal CSR in LPS-stimulated *Xrcc1*^{+/-} splenic B cells but noted a reduction in MH length at $\Sigma\mu$ - $\Sigma\gamma$ 3 switch junctions and also found reduced levels of *IgH/c-myc* translocations in *Xrcc1*^{+/-} B cells, leading to the conclusion that XRCC1 plays a role in A-EJ in both processes (36). However, although we did not analyze $\Sigma\mu$ - $\Sigma\gamma$ 3 junctions, we did not find altered $\Sigma\mu$ - $\Sigma\gamma$ 1 or $\Sigma\mu$ - $\Sigma\epsilon$ junctions in XRCC1-deficient B cells, where XRCC1 protein levels were reduced to almost undetectable levels. Likewise, we found no reduction in translocations upon conditional *Xrcc1* inactivation in XRCC4-proficient or -deficient B cells. Another recent study reported that nuclear Lig3 is the primary ligase for translocation formation in the context of zinc finger endonuclease-mediated chromosomal DSBs in mouse ES cells (22). In our experiments, depletion of Lig3 to extremely low levels did not affect CSR, CSR junctions, or the frequency of *IgH/c-myc* translocations. However, because complete *Lig3* ablation appears cell-lethal, we cannot unequiv-

ocally rule out the possibility that very low residual Lig3 levels contribute to the A-EJ we observe in *Lig4*-deficient cells.

Overall, our findings demonstrate that XRCC1 is not required for major known forms of A-EJ, and thus cannot be considered a chromosomal A-EJ factor at this time. Our results also rule out a requisite role for the XRCC1/Lig3 complex in A-EJ and raise the further possibility that there may be significant A-EJ mechanisms that function independent of both Lig3 and Lig4. Because eukaryotic cells contain only three known enzymes that ligate DNA ends (Lig1, Lig3, and Lig4) (25), the major candidate for this role would be Lig1, which has already been suggested to play a role in A-EJ, albeit a more modest role than Lig3 (22, 51). Future experiments will need to address a direct role for Lig1 in A-EJ in *Lig3/4*-deficient B lymphocytes, as well as the relative contributions of Lig1 and Lig3 to A-EJ in XRCC4/*Lig4*-deficient and WT B cells.

Materials and Methods

Mice. All experiments involving mice were performed according to protocols approved by the Institutional Animal Care Facility of Children's Hospital Boston. *Xrcc1*^{oc}, *Lig3*^{oc}, *Xrcc4*^{oc}, and *CD21CreLig4*^{oc} (CL4) mice were reported (15, 28, 37).

B-Cell Purification and Culture. Splenic B cells were isolated and stimulated as described elsewhere (52).

Generation of v-Abl-Transformed Pro-B Lines. The v-Abl kinase-transformed pro-B cells were generated (53) and used as described in *SI Materials and Methods*.

CH12F3 Cell Culture and shRNA-Mediated Knockdown. Details on WT and *Lig4*^{-/-} (48) CH12F3 cell culture and shRNA experiments are provided in *SI Materials and Methods*.

Switch Region Junction Analysis. $\Sigma\mu$ - $\Sigma\gamma$ 1 and $\Sigma\mu$ - $\Sigma\epsilon$ junctions were analyzed as described previously (10). Conditions for $\Sigma\mu$ - $\Sigma\alpha$ junction analysis in CH12F3 cells are described in *SI Materials and Methods*.

Two-Color IgH FISH and IgH/c-myc Translocations. Metaphases were prepared and processed as described elsewhere (39). All FISH samples were analyzed in a blinded manner. IgH/c-myc translocation analysis in primary B cells was performed as described elsewhere (40). Details on IgH/c-myc translocation analysis in CH12F3 cells are provided in *SI Materials and Methods*.

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