Mechanism of DNA resection during intrachromosomal recombination and immunoglobulin class switching

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DNA double–strand breaks (DSBs) are byproducts of normal cellular metabolism and obligate intermediates in antigen receptor diversification reactions. These lesions are potentially dangerous because they can lead to deletion of genetic material or chromosome translocation. The chromatin-binding protein 53BP1 and the histone variant H2AX are required for efficient class switch (CSR) and V(D)J recombination in part because they protect DNA ends from resection and thereby favor nonhomologous end joining (NHEJ). Here, we examine the mechanism of DNA end resection in primary B cells. We find that resection depends on both CtBP-interacting protein (CtIP, Rbbp8) and exonuclease 1 (Exo1). Inhibition of CtIP partially rescues the CSR defect in 53BP1– and H2AX–deficient lymphocytes, as does interference with the RecQ helicases Bloom (Blm) and Werner (Wrn). We conclude that CtIP, Exo1, and RecQ helicases contribute to the metabolism of DNA ends during DSB repair in B lymphocytes and that minimizing resection favors efficient CSR.

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Abbreviations used: AID, activation-induced cytidine deaminase; A-NHEJ, alternative nonhomologous end joining; ATM, ataxia-telangiectasia mutated; C-NHEJ, classical NHEJ; CSR, class switch recombination; CtIP, CtBP-interacting protein; DSB, DNA double-strand break; ssDNA, single-stranded DNA.

DNA double-strand breaks (DSBs) can be repaired locally or recombined to produce chromosome rearrangements. These events are common to lymphomas, leukemias, and sarcomas, and can also be found in prostate, lung, and other solid cancers (Futreal et al., 2004; Mitelman et al., 2007; Nussenzweig and Nussenzweig, 2010; Robbiani and Nussenzweig, 2012). The mechanisms that govern chromosomal rearrangements are only partially understood, but paired DSBs are sufficient to initiate translocation, whereas chromosome deletion can result from joining of two paired intrachromosomal DSBs or from resection of a single break. A shared feature of these reactions is the loss of genetic information, ranging from a few nucleotides to several megabases (Robbiani et al., 2008; Bothmer et al., 2010, 2011).

Lymphocytes are particularly prone to transformation by chromosome rearrangements, in part because they undergo programmed DNA damage during *Ig* and *TCR* gene diversification reactions (Nussenzweig and Nussenzweig, 2010;

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Gostissa et al., 2011). In mature B cells, DSBs are requisite intermediates in class switch recombination (CSR), which is a deletional recombination reaction that alters the effector function of antibody molecules. During CSR, the enzyme activation–induced cytidine deaminase (AID) converts cytosines into uracils at switch regions on the *Ig heavy chain gene* (*Igh*). Excision of uracils from both DNA strands leads to staggered DNA breaks at donor and acceptor switch regions. These lesions are normally detected as DSBs by the Mre11–Rad50–NBS1 (MRN) complex, which initiates a cascade of events that leads to phosphorylation of H2AX, recruitment of 53BP1, and, eventually, end joining by classical or alternative nonhomologous end-joining pathways (C- or A-NHEJ; Petersen et al., 2001; Reina-San-Martin et al., 2004; Neuberger et al., 2005; O'Driscoll and Jeggo, 2006; Stavnezer et al., 2008; Noon and Goodarzi, 2011; Gostissa et al., 2011). However, *Igh* breaks can also be aberrantly joined to AID-mediated DSBs in non-*Ig* genes, including oncogenes that are commonly translocated to the *Ig* loci in lymphomas (Robbiani et al., 2008; Chiarle et al., 2011; Klein et al., 2011; Hakim et al., 2012; Zhang et al., 2012).

In addition to their role in initiating the DNA damage response, 53BP1 and H2AX are essential for CSR (Petersen et al., 2001; Manis et al., 2004; Ward et al., 2004). These proteins are believed to contribute to the switch reaction by facilitating synapsis and by protecting DNA ends from resection. In their absence, broken DNA is resected to produce single-stranded DNA (ssDNA), which serves as a substrate for repair by alternative pathways characterized by increased junctional microhomology (Bothmer et al., 2010; Bunting et al., 2010; Gostissa et al., 2011; Helmink et al., 2011). It has been suggested that alternative repair favors abortive open and shut joining within a switch region (intra-switch recombination) at the expense of productive joining between different switch regions (inter-switch recombination; Bothmer et al., 2010). However, the precise role of resection and the factors that mediate this process during CSR have not been defined.

Genetic studies in yeast and work in human cells have implicated endonucleases, exonucleases, and helicases in DNA resection. Specifically, yeast Sae2 (CtBP-interacting protein [CtIP]) has been implicated together with the MRX (MRN) complex in the initial trimming phase of resection, whereas Exo1 and the RecQ helicase Sgs1 have been shown to promote the subsequent extension of resected tracts (Mimitou and Symington, 2008; Zhu et al., 2008; Symington and Gautier, 2011). Less is known about the effectors of DNA resection in mammalian cells, especially in the context of physiological DNA damage in primary cells. Knockdown of CtIP protects DNA ends from being resected during V(D)J recombination in the absence of H2AX (Helmink et al., 2011), and in lymphocytes undergoing CSR, inhibition of ataxia–telangiectasia mutated (ATM) suppresses the increased end resection that occurs in the absence of 53BP1 (Bothmer et al., 2010). However, ATM is a kinase, not a nuclease, and it acts on a multitude of targets during the DNA damage response (Matsuoka et al., 2007; Bensimon et al., 2010).

Here, we show that both CtIP and Exo1 are in part responsible for the processing of DNA ends during intrachromosomal joining of tandem DSBs. In addition, we show that preventing resection promotes CSR in 53BP1- and H2AX-deficient lymphocytes.

RESULTS

Resection by CtIP and Exo1

In the absence of 53BP1 or H2AX, DNA ends are resected and repaired more frequently via junctional microhomology characteristic of alternative nonhomologous end joining (A-NHEJ), a phenotype that is reverted by ATM inhibition (Bothmer et al., 2010, 2011; Bunting et al., 2010). To identify the enzymes responsible for resection, we analyzed joining products between two I-SceI sites spaced by 96 kb at the *Igh* locus on mouse chromosome 12 (Fig. 1 A, IgH$^{I\text{-}96k}$; Bothmer et al., 2010). The analysis was performed in AID$^{-/-}$ B lymphocytes, to exclude potentially confounding effects of AID breaks.

Experiments in yeast have shown that MRX (MRN) together with Sae2 (CtIP) can resect DSBs to produce a few hundred nucleotides of ssDNA at the break (Symington and Gautier, 2011). To determine whether CtIP mediates DNA end resection at DSBs, we used short hairpin RNAs (sh-CtIP1 and sh-CtIP2) to deplete this protein from activated B cells (Fig. 1 B). CtIP-depleted IgH$^{I\text{-}96k/+}$53BP1$^{-/-}$AID$^{-/-}$ B lymphocytes expressing I-SceI were isolated by cell sorting, and recombination joins were analyzed by PCR. Loss of CtIP decreased the frequency of joins displaying extensive resection (Fig. 1 C, 47.8 to 33.8%; P = 0.001) and the mean number of resected nucleotides (Fig. 1 C, 124.1 to 63.1 nt; P = 0.006). Moreover, diminished end processing was also observed in 53BP1-proficient B cells (Fig. 1 D, 37.1 to 25.7%; P = 0.024). We conclude that CtIP is in part responsible for processing intrachromosomal DSBs in the presence or absence of 53BP1.

Exo1 possesses 5′ to 3′ exonuclease and 5′ flap endonuclease activities, which in yeast promote the extension of resected tracts up to 2–5 kb (Symington and Gautier, 2011). To determine the role of Exo1 in resection, we produced IgH$^{I\text{-}96k/+}$53BP1$^{-/-}$AID$^{-/-}$Exo1$^{-/-}$ mice by breeding, and then assayed resection of DNA breaks created by I-SceI. Similar to mice expressing a mutant form of Exo1 (Bardwell et al., 2004; Wei et al., 2003), B lymphocyte development was not altered in the absence of Exo1 (unpublished data). Compared with IgH$^{I\text{-}96k/+}$53BP1$^{-/-}$AID$^{-/-}$Exo1$^{+/+}$ controls, junctions with extensive resection were reduced (Fig. 1 E, 51.1 to 27.3%; P < 0.0001), as was the mean number of resected nucleotides (Fig. 1 E, 96.3 to 53.3 nt; P = 0.020). Lack of Exo1 also resulted in decreased DNA-end processing in 53BP1-sufficient B cells (Fig. 1 F, 37.1 to 28.1%; P = 0.016). We conclude that Exo1 resects intrachromosomal DSBs.
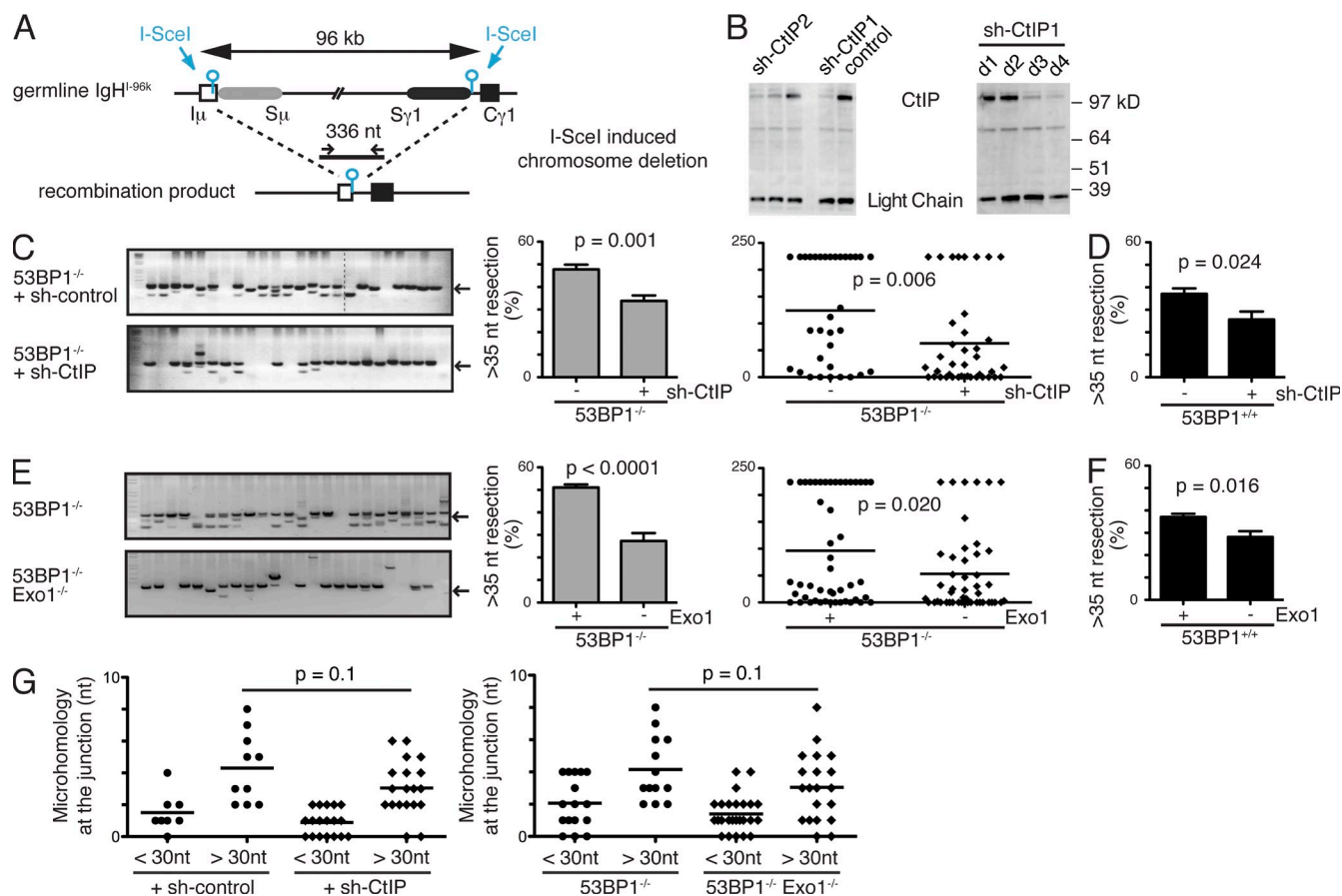
Figure 1. CtIP and Exo1 promote DNA resection during the joining of intrachromosomal I–SceI–induced DSBs. (A) Schematic representation of the IgH[I-96k] allele before (top) and after (bottom) recombination by I-SceI. Blue circles indicate the I-SceI sites. Black arrows represent the position of the PCR primers for amplifying the recombination products. The perfect (nonresected) join is 336 nt. Sμ and Sγ1 are Switch μ and Switch γ1 regions, respectively. (B, left) Identification of sh-RNAs against CtIP. Western Blot analysis of protein lysates from activated B cells infected with candidate shRNAs and analyzed after 96 h in culture. The two best shRNAs were selected for the experiments (sh-CtIP1 and sh-CtIP2). (right) Time course of knock down by sh-CtIP1 (d = days since beginning of the culture). Two and one experiment, respectively. (C, left) Representative ethidium bromide–stained agarose gels with PCR products obtained after I-SceI recombination in IgH[I-96k]53BP1^{−/−}AID^{−/−} B cells infected with I-SceI and sh-RNAs against CtIP (sh-CtIP1) or lacZ (sh-control), followed by sorting of the double-infected population. Arrows point to the 300 nt size, under which bands are scored as extensively resected. Vertical dotted line indicates merge of two adjacent pictures of the same gel. (middle) Histogram with the frequency of I-SceI–induced recombination products with extensive resection (>35 nt). (right) Resection dot plot, with each dot representing one sequence. On the Y axis is the number of resected nucleotides. (D) Same as in Fig. 1 C (middle), but for IgH[I-96k]AID^{−/−} B cells (53BP1 proficient). (E) As in Fig. 1 C for IgH[I-96k]53BP1^{−/−}AID^{−/−}Exo1^{−/−} B cells and Exo1-proficient control. (F) Same as in Fig. 1 C (middle), but for IgH[I-96k]AID^{−/−}Exo1^{−/−} and IgH[I-96k]AID^{−/−}Exo^{+/+} B cells (53BP1 proficient). (G) Dot plots showing the number of nucleotides microhomology at the junctions from recombination products of I-SceI–infected IgH[I-96k]53BP1^{−/−}AID^{−/−} B cells from the indicated experimental conditions. Sequences were grouped according to the extent of resection (more or less than 30 nt). Error bars in histograms indicate standard deviations. Horizontal lines in dot plots indicate the means. P-values were calculated using the unpaired two-tailed Student's t test. All graphs represent data from at least two separate PCR reactions for each of two independent experiments.

Chromosome translocation breakpoints are often characterized by the presence of microhomology (Gostissa et al., 2011). To determine if the partial decrease in DNA end resection in the absence of CtIP or Exo1 was accompanied by changes in junctional microhomology, we sequenced recombination products (Figs. 1, C and E) and measured the extent of microhomology. We found that the remaining resected joins are still characterized by extensive junctional microhomology (Fig. 1 G). This indicates that neither of the two nucleases alone is required for DNA repair by A-NHEJ.

Mre11, Wrn, and Blm

Mre11, a component of the MRN complex, has been implicated in DNA end resection both in yeast and mammalian cells (Symington and Gautier, 2011). To test the role of Mre11 in resection in B cells, we treated I-SceI–expressing IgH[I-96k/+]53BP1^{−/−}AID^{−/−} cultures with the small molecule inhibitor Mirin at the biologically active concentration of 10 μM (Dupré et al., 2008; and unpublished data). No measurable effect on resection was observed after Mre11 inhibition (Fig. 2 A). In addition, we found no difference in the microhomology at the junctions (unpublished data).
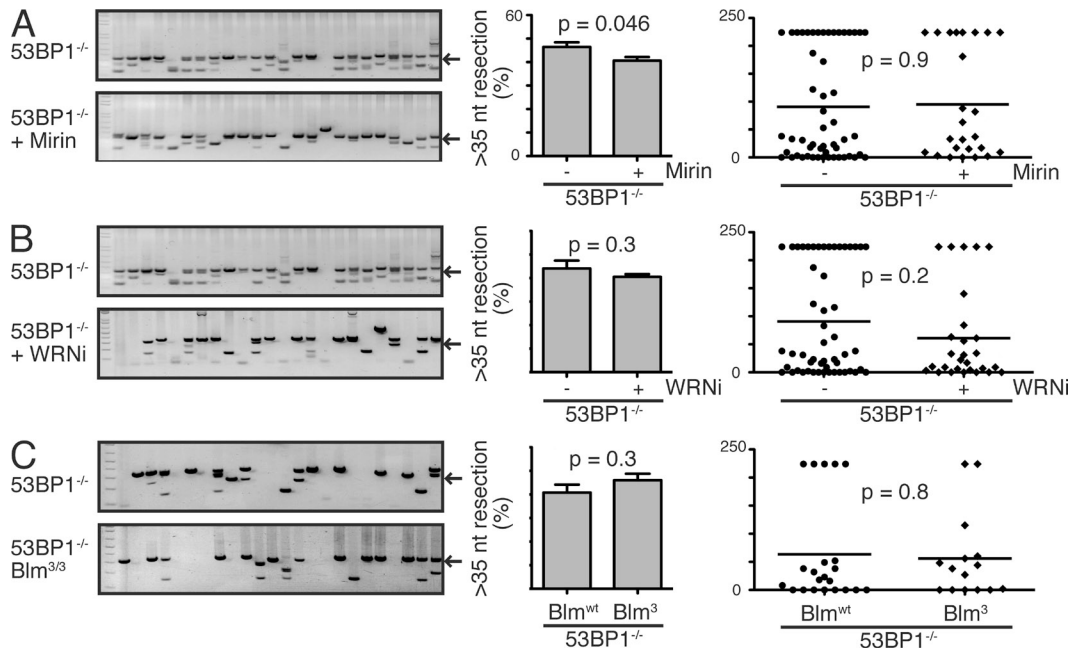
Figure 2. Mre11, Wrn, and Blm do not alter DNA resection during I–SceI recombination. (A) IgH$^{I-96k/+}$53BP1$^{-/-}$AID$^{-/-}$ B cells cultured in the presence or absence of the Mre11 inhibitor (Mirin) as in Fig. 1 C. The frequency of joins with extensive resection was 46.5% for untreated and 40.6% for Mirin treated. The mean numbers of resected nucleotides were 90.7 and 95.2 nt, respectively. (B) IgH$^{I-96k/+}$53BP1$^{-/-}$AID$^{-/-}$ B cells cultured in the presence or absence of the Wrn inhibitor (WRNi) as in Fig. 1 C. The frequency of joins with extensive resection was 44.1% for untreated and 40.5% for WRNi treated. Nucleotide resection was 90.7 and 61 nt, respectively. (C) B cells from IgH$^{I-96k/+}$53BP1$^{-/-}$AID$^{-/-}$Blm$^{3/3}$ and Blm proficient control as in Fig. 1 C. The frequency of joins with extensive resection was 40.8% for Blm-proficient and 46.1% for Blm^3. Nucleotide resection was 63.4 and 53.3 nt, respectively. All graphs represent data from at least two separate PCR reactions for each of two independent experiments.

We conclude that, in contrast to CtIP, inhibition of Mre11 does not alter DNA end resection in 53BP1-deficient B cells.

Sgs1, a RecQ DNA helicase, has been implicated together with Exo1 in the extension of DNA end resection in yeast (Symington and Gautier, 2011). Five RecQ proteins exist in mammals (Wrn, Blm, RecQ1, RecQ4, and RecQ5). Although Blm is viewed as the closest orthologue of Sgs1, Wrn is the only family member that possesses both DNA helicase and 3′ to 5′ exonuclease activities (Brosh and Bohr, 2007). To investigate the role of Wrn in resection, we treated I–SceI–infected IgH$^{I-96k/+}$53BP1$^{-/-}$AID$^{-/-}$ lymphocytes with the small molecule inhibitor NSC19630 (WRNi), which specifically blocks Wrn helicase activity (Aggarwal et al., 2011). At the biologically active concentrations of 1.5–2 µM (Aggarwal et al., 2011; and see below), WRNi did not significantly alter resection or junctional microhomology (Fig. 2 B and not depicted). To determine the role of the Blm helicase, we bred the functionally null Blm^3 allele (Luo et al., 2000) to produce IgH$^{I-96k/+}$53BP1$^{-/-}$AID$^{-/-}$Blm$^{3/3}$ mice. Compared with IgH$^{I-96k/+}$53BP1$^{-/-}$AID$^{-/-}$Blm$^{+/+}$ controls, neither resection, nor junctional microhomology was significantly altered (Fig. 2 C and not depicted). We conclude that neither Wrn helicase activity nor Blm deficiency significantly alters processing of intrachromosomal DSBs in the absence of 53BP1.

Inhibition of resection promotes *Ig* class switching

During CSR, B lymphocytes replace *Igµ* by a downstream constant region, such as *Igγ1* (Fig. 3 A). 53BP1 and H2AX are essential for this reaction, in part because they protect DNA ends from resection (Petersen et al., 2001; Manis et al., 2004; Ward et al., 2004). Interfering with ATM promotes CSR in the resection-prone 53BP1$^{-/-}$ and H2AX$^{-/-}$ backgrounds, and this is associated with inhibition of DSB resection (Bothmer et al., 2010, 2011). To determine whether DNA end resection contributes to this class switch defect, we analyzed CSR in 53BP1$^{-/-}$ and H2AX$^{-/-}$ B cells under conditions that impair DNA resection.

First, we tested the role of CtIP by transducing activated B cells with retroviruses expressing anti-CtIP shRNA. CtIP depletion partially rescued class switching in 53BP1$^{-/-}$ and H2AX$^{-/-}$ B cells (Fig. 3 B). The rescue of switching was not associated with significant changes in cell proliferation (unpublished data). Importantly, CtIP knock down did not significantly alter wild-type switching (Fig. 3 C), AID expression, or switch region transcription (Fig. 3 D). We conclude that DSB resection by CtIP is in part responsible for the switching defect in 53BP1 and H2AX deficiency.

The absence of a CSR defect in CtIP-depleted wild-type cells is in contrast to a report that silencing CtIP reduced AID levels and CSR in the CH12 B cell line (Lee-Theilen et al., 2011). To exclude possible off-target effects of the hairpins

used for CtIP depletion, we next measured class switching in B cells that were genetically deficient for CtIP. Because CtIP-null mice are embryonically lethal (Chen et al., 2005), we intercrossed the previously reported CtIP-null allele (CtIP$^-$; Chen et al., 2005) to a new CtIP conditional allele (CtIP^{co}), in which exon 2 is flanked by *loxP* sites (unpublished data; and see Materials and methods). Absence of CtIP was verified by Western Blot (Fig. 4 A). In agreement with

the shRNA results, class switching was not significantly altered by the absence of CtIP (Fig. 4 B). Moreover, in contrast to the reported effect of CtIP depletion on the extent of microhomology at switch junctions of CH12 cells (Lee-Theilen et al., 2011), our analysis of Sμ–Sγ1 junctions of CD19$^{cre/+}$CtIP$^{co/-}$ B cells shows a similar pattern to control (Figs. 4, C and D). We conclude that the absence of CtIP does not alter class switching or microhomology at Sμ–Sγ1 junctions in primary B cells.

We next explored the role of helicases. WRNi treatment did not affect wild-type switching (unpublished data). However, CSR was enhanced in both 53BP1$^{-/-}$ and H2AX$^{-/-}$ B cells treated with this helicase inhibitor (Fig. 5 A). The effect of WRNi was notably more pronounced than ATM inhibitor Ku55933 (ATMi; Bothmer et al., 2010; Fig. 5 A), despite a decrease in AID and switch germline transcripts (Fig. 5 B). Blm deficiency negatively impacts class switching in wild-type B cells (Babbe et al., 2009). To test the role of Blm in a resection-prone background, we generated CD19$^{cre/+}$Blm$^{lox/lox}$ 53BP1$^{-/-}$ mice. Switching was increased but only to a small extent by loss of Blm compared with 53BP1$^{-/-}$ control (Fig. 5 C), and this was accompanied by higher switch μ germline transcription (Fig. 5 D). We conclude that 53BP1 and H2AX contribute to *Ig* class switching in part by preventing the action of Wrn and Blm helicases on DSBs at switch regions.

DISCUSSION

DSBs predispose to genomic rearrangements, which are common in cancer and are often accompanied by the loss of genetic information (Futreal et al., 2004; Mitelman et al., 2007; Nussenzweig and Nussenzweig, 2010; Robbiani and Nussenzweig, 2012). To investigate the molecular mechanism responsible for the loss of genomic sequence during DNA recombination reactions, we took advantage of a previously established assay to measure the DNA end resection during joining of paired intrachromosomal DSBs in the *Igh* locus separated by 96 kb (Bothmer et al., 2010).

CtIP has been implicated in the initial trimming phase of DNA end resection (Symington and Gautier, 2011). Although CtIP deletion is lethal (Chen et al., 2005), knockdown experiments in human cells show that it binds to the MRN complex and promotes accumulation of replication protein A (RPA) at sites of laser-induced DNA damage, suggesting that it produces ssDNA (Sartori et al., 2007). Moreover, although CtIP does not appear to have any direct nuclease activity, enzymatic assays in vitro have demonstrated that it enhances the nuclease activity of the MRN (Sartori et al., 2007). Exo1 has been involved in the extension phase of resection (Symington and Gautier, 2011). In mammalian cells, Exo1 rapidly accumulates at sites of DSBs and is required for proper DNA repair, as its absence leads to chromosome instability and defects in RPA accumulation upon ionizing radiation (Bolderson et al., 2010). In addition, biochemical DNA end resection assays with purified human proteins have shown that Exo1 activity is stimulated by the presence of the MRN, RPA, and BLM (Nimonkar et al., 2011).
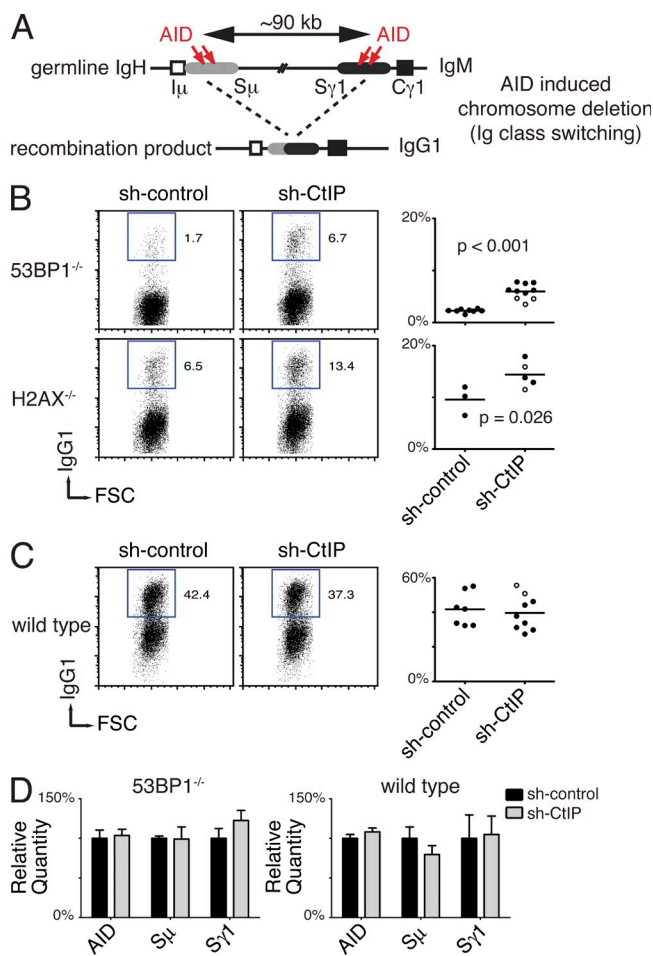
Figure 3. CtIP contributes to the Ig class switch defect of 53BP1$^{-/-}$ and H2AX$^{-/-}$ B cells. (A) Schematic representation of the IgH allele prior (top) and after (bottom) AID-induced recombination, which results in Ig class switching from IgM to IgG1. (B, left) Representative flow cytometry plots measuring the percentage of CSR to IgG1 upon infection of 53BP1$^{-/-}$ or H2AX$^{-/-}$ B cells with sh-RNAs against CtIP or lacZ control (gated on the GFP$^+$ infected population). Mean CSR values for sh-control and sh-CtIP, respectively: 2.3 and 6.0% in 53BP1$^{-/-}$; 9.6 and 14.4% in H2AX$^{-/-}$. FSC is forward scatter for cell size. (right) Summary dot plot of class switching results. Filled and empty circles represent results with sh-CtIP1 and shCtIP-2, respectively. Horizontal lines in dot plots indicate the means. P-values were calculated using a paired two-tailed Student's *t* test. Each dot represents an independent culture. (C) Wild-type cells infected with shRNAs against CtIP, as in B. (D) Q-PCR analysis of AID, and germline switch μ and switch γ1 transcription upon culture of sh-CtIP1–infected 53BP1$^{-/-}$ or wild-type cells. Data are expressed as relative quantity of control (black bars). Three independent samples were used.
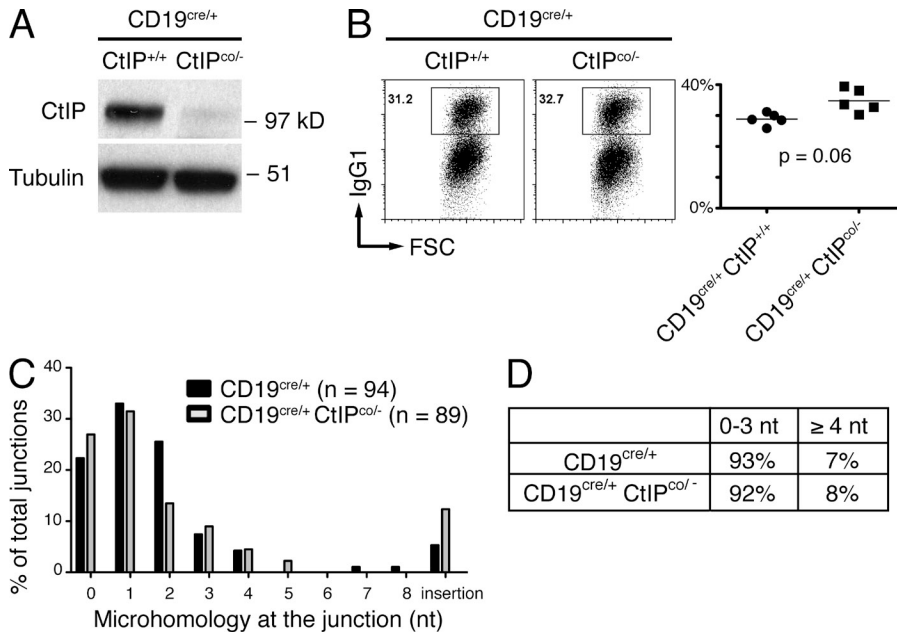
Figure 4. CtIP–deficient B cells have normal class switching and microhomology at switch junctions. (A) Western blot for CtIP on B cells activated in vitro for 2 d. Representative of two experiments. (B, left) Representative flow cytometry plots measuring the percentage of CSR to IgG1 in B cells of the indicated genotypes. Mean CSR values are 28.9% for CD19$^{cre/+}$CtIP$^{+/+}$ and 34.8% for CD19$^{cre/+}$CtIP$^{co/-}$. FSC is forward scatter for cell size. (right) Summary dot plot of class switching results. Horizontal lines in dot plots indicate the means. Each dot represents an independent culture. (C) Nucleotide (nt) microhomology at Sμ-Sγ1 junctions of CD19$^{cre/+}$CtIP$^{co/-}$ and control. Sequences from two independent experiments. (D) Summary of junctions with 0–3 nt microhomology versus extensive microhomology (≥ 4 nt) for the same sequences as in C.

	0-3 nt	≥ 4 nt
CD19$^{cre/+}$	93%	7%
CD19$^{cre/+}$ CtIP$^{co/-}$	92%	8%

The contribution of CtIP or Exo1 to resection of DSBs that occur during DNA recombination reactions is largely unknown, as are the players responsible for resection in the absence of 53BP1. ATM regulates CtIP and Exo1 by phosphorylation, which controls their accumulation at DSBs (Li et al., 2000; You et al., 2009; Bolderson et al., 2010). Our experiments show that interfering with CtIP or Exo1 significantly reduced DSB resection in 53BP1$^{-/-}$ and wild-type B cells. These results are consistent with and likely account for our finding that inhibiting ATM interferes with DNA end resection (Bothmer et al., 2010). However, neither the knockdown

of CtIP nor the knockout of Exo1 abrogates resection entirely, and therefore these two factors are either partially redundant or there are additional, yet to be identified, mechanisms that mediate DNA end resection. Our findings in activated B cells that CtIP promotes resection are consistent with CtIP promoting resection of DNA ends during V(D)J recombination in H2AX-deficient pre–B cells (Helmink et al., 2011) and supporting DNA deletion during translocation (Zhang and Jasin, 2011).

Microhomologies are frequent at breakpoints of chromosome translocations (Robbiani et al., 2008; Gostissa et al.,
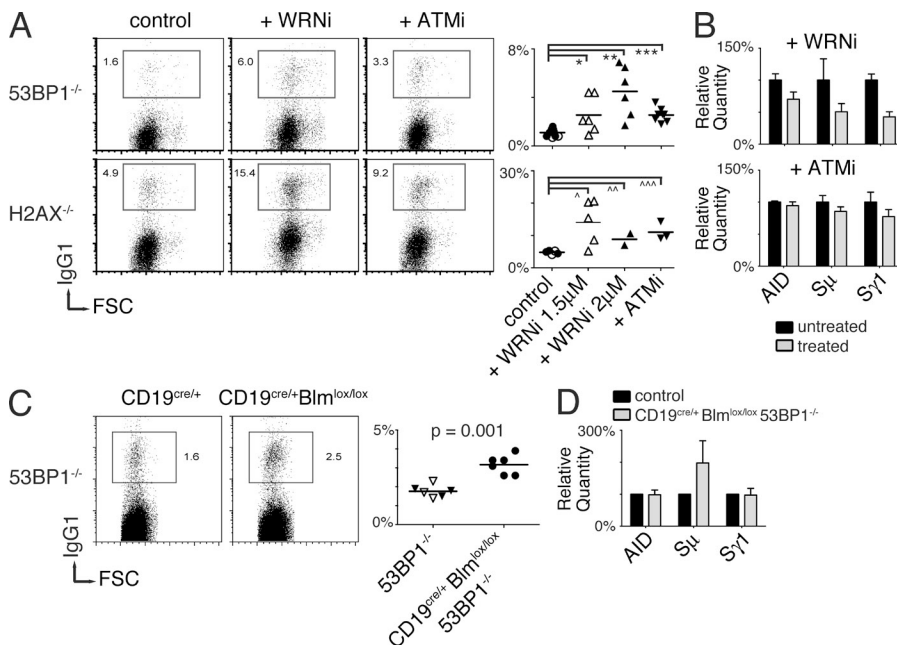
Figure 5. Helicases are in part responsible for the switching defect of 53BP1– and H2AX–deficient B cells. (A) Cells treated with WRNi, ATMi, or controls, as in Fig. 3 B. Filled and empty circles represent results with untreated and DMSO only controls, respectively. Mean CSR values (control, +WRNi 1.5 μM, +WRNi 2 μM, and +ATMi, respectively) are as follows: 1.1, 2.6, 4.5, and 2.6% for 53BP1$^{-/-}$; 4.8, 14.0, 8.8, and 11.0% for H2AX$^{-/-}$. P-values for 53BP1$^{-/-}$ are as follows: *, 0.08; **, 0.02; ***, 0.0005; for H2AX$^{-/-}$: ^, 0.04; ^^, 0.26; ^^^, 0.05. Each dot represents an independent culture. (B) 53BP1$^{-/-}$ cells treated with WRNi or ATMi, as in Fig. 3 D. Three independent samples. (C) CD19$^{cre/+}$Blm$^{lox/lox}$53BP1$^{-/-}$ and control cells (filled triangles are 53BP1$^{-/-}$, empty triangles are CD19$^{cre/+}$53BP1$^{-/-}$), as in Fig. 3 B. Mean CSR values are 1.8% in control and 3.2% in CD19$^{cre/+}$Blm$^{lox/lox}$53BP1$^{-/-}$. Each dot represents an independent culture. (D) As in Fig. 3 D for CD19$^{cre/+}$Blm$^{lox/lox}$53BP1$^{-/-}$ cells and control. Six independent samples.

2011). Although classical NHEJ (C-NHEJ) can produce joins with microhomology, these are favored by the A-NHEJ pathway, which joins DSBs when the core C-NHEJ factors (Ku70, Ku80, XRCC4, or Lig4) are absent (Boboila et al., 2010; Gostissa et al., 2011). Thus, the A-NHEJ pathway is particularly active under conditions when DNA ends are poorly protected, such as loss of 53BP1 (Bothmer et al., 2010). Loss of CtIP or Exo1 in 53BP1-deficient cells results in a partial decrease in DNA end resection, but the remaining resected joins still show extensive junctional microhomology. Thus, neither factor alone is essential for DNA repair by A-NHEJ.

Together with CtIP, Mre11 is also involved in the initial trimming of broken DNA ends (Symington and Gautier, 2011). Although chemical inhibition of Mre11 with Mirin did not decrease resection, this treatment also inhibits ATM activation (Dupré et al., 2008), and ATM activity is needed for resection (Bothmer et al., 2010). Thus, it is possible that Mirin also causes defects in joining that interfere with the formation of joins being scored in the assay.

CSR in lymphocytes is profoundly impaired by the absence of 53BP1 or H2AX (Petersen et al., 2001; Manis et al., 2004; Ward et al., 2004; Franco et al., 2006). We have proposed that 53BP1 and H2AX protect broken DNA ends and inhibit resection-associated A-NHEJ while promoting C-NHEJ repair. Because A-NHEJ favors microhomology-driven intra-switch recombination within the repetitive switch regions, inhibiting resection and A-NHEJ would support successful inter-switch recombination (Bothmer et al., 2010, 2011). In agreement with this idea, loss of CtIP in 53BP1-deficient cells results in a partial rescue of CSR, and has no significant effect on primary wild-type B cells. Our results are consistent with the observation that CtIP is responsible for resection during V(D)J recombination (Helmink et al., 2011), and contrasts a report that silencing CtIP reduced AID levels and CSR in the CH12 B cell line (Lee-Theilen et al., 2011).

The RecQ family of DNA helicases plays an important role in maintaining genomic stability, and some members have been implicated in the processing of broken DNA ends (Brosh and Bohr, 2007; Gravel et al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008; Nimonkar et al., 2011; Symington and Gautier, 2011). The increase in CSR upon interference with Wrn or Blm helicases contrasts with their lack of effect on resection during recombination between intrachromosomal I-SceI sites. We speculate that the difference in the two assays might arise if Wrn and Blm are not engaged in the repair of paired intrachromosomal I-SceI breaks, but participate in the repair of AID-mediated DNA lesions that occur within the repetitive, G-rich and highly transcribed switch regions (Stavnezer et al., 2008). Genetic experiments will be necessary to clarify the role of Wrn during DNA processing and class switching.

In summary, CSR in lymphocytes is profoundly impaired by the absence of 53BP1 or H2AX and this is associated with increased resection, which favors microhomology- or homology-driven abortive intra-switch recombination within the repetitive

switch regions (Petersen et al., 2001; Manis et al., 2004; Ward et al., 2004; Bothmer et al., 2010, 2011). Our experiments reveal that CtIP and Exo1 mediate resection and that preventing nucleolytic processing of broken DNA ends promotes *Ig* class switching.

MATERIALS AND METHODS

Mice. IgH[I-96k] (Bothmer et al., 2010), AID$^{-/-}$ (Muramatsu et al., 2000), 53BP1$^{-/-}$ (Ward et al., 2004), Blm^3 (Blm[tm3Brd], NCI mouse repository; Luo et al., 2000), Blm[lox/lox] (Blm[tm4Ches]; P. Leder, Harvard University, Boston, MA Chester et al., 2006), CD19[Cre] (Rickert et al., 1997), CtIP$^{+/-}$ (Chen et al., 2005), and H2AX$^{-/-}$ (Petersen et al., 2001; Celeste et al., 2002) mice were previously described. Exo1$^{-/-}$ mice were generated by homologous recombination in WW6 embryonic stem cells. In the conditional CtIP allele (CtIP[co]), exon 2 (encoding the N-terminal 36 aa, including the initiator methionine) is flanked by a pair of *loxP* recombination signals. It was generated using a conditional CtIP targeting vector, which consisted of a 7.7-kb genomic fragment containing exons 1 and 2. One *loxP* site was inserted into a unique *Nhe*I site in intron 1, while a second *loxP* site together with an *FRT*-flanked *PGK-neo* cassette (with a transcriptional orientation opposite to that of CtIP) was cloned into a unique *EcoR*V site in intron 2. A HSV-thymidine kinase (HSV-TK) gene cassette was included in the construct as a negative selection marker against random integration. Gene targeting in 129Sv ES cells and blastocyst injections were performed after standard techniques. The *FRT*-flanked *PGK-neo* cassette was removed by mating heterozygous CtIP[coneo/+] mice with FLPeR mice (The Jackson Laboratory). Unless otherwise specified, mice were homozygous for the indicated alleles. All experiments were in accordance with protocols approved by the Rockefeller University Institutional Animal Care and Use Committee.

B cell culture and retroviral infection. For I-SceI recombination and CSR assays, CD43$^-$ resting B cells were isolated from spleens, stimulated with LPS and interleukin 4 (IL-4), and infected as previously described (Bothmer et al., 2011). The pMX-IRES-GFP based retrovirus encoding for I-SceI was previously described (Robbiani et al., 2008). For double-infection experiments, I-SceI was cloned into pMX-IRES-mCherry. The short hairpins were as follows: sh-control (against lacZ), 5′-TGCTGTTGACA-GTGAGCGAGGCCTGTATGTGGTGGATGAATAGTGAAGCCACA-GATGTATTCATCCACCACATACAGGCCGTGCCTACTGCCTC-GGA-3′; sh-CtIP1, 5′-TGCTGTTGACAGTGAGCGAGCTCTCTATG-TACAAATGAATTAGTGAAGCCACAGATGTAATTCATTTGTA-CATAGAGAGCGTGCCTACTGCCTCGGA-3′; and sh-CtIP2, 5′-TGCTGTTGACAGTGAGCGCGTAAATTCTCTCCAGACCAAATAG-TGAAGCCACAGATGTATTTGGTCTGGAGAGAATTTACTTG-CCTACTGCCTCGGA-3′. Sh-RNAs were cloned into a pMX-IRES-GFP backbone upon amplification with the following primers: fwd, 5′-CA-GAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG-3′; rev, 5′-CTAAAGTAGCCCCTTGAATTCCGAGGCAGTAGGCA-3′. Cultures were analyzed at 96 h.

PCR assay for measuring resection. The PCR assay for measuring resection at recombination joins was performed as detailed previously (Bothmer et al., 2010). In brief, B cells were infected in vitro and analyzed after 4 d. Nested PCR reactions with primers flanking the I-SceI recognition sites (see Fig. 1 A) were performed on genomic DNA that was diluted for detection of single recombination events. PCR products were run on agarose gels and scored by visual inspection for resection of >35 nt and extracted for direct sequencing.

Small molecule inhibitors. Mirin (TOCRIS Bioscience; Dupré et al., 2008) was used at 10 μM; ATMi (Ku55933; TOCRIS Bioscience) was used at 2.5 μM; and WRNi (NSC19630, NCI Diversity Set; Aggarwal et al., 2011) was used at 1.5 μM or 2 μM, as indicated.

Flow cytometry and cell sorting. B cell cultures co-infected with I-SceI (mCherry[+]) and sh-RNA (GFP[+]) were sorted for double-positivity on a FACSAria (BD). For Ig class switch assays, cell suspensions were stained with fluorochrome-conjugated anti-IgG1 antibodies (BD). For cell division analysis, cells were labeled at 37°C for 10 min in 2.5 µM CFSE. Samples were acquired on a FACSCalibur (BD) and analyzed with FlowJo (Tree Star).

Western Blot. In Fig. 1 B CtIP was detected in whole-cell extracts with the antibody A300-488A (Bethyl Laboratories), followed by secondary anti–light chain. In Fig. 4 A, antibody 14–1 was used (Yu and Baer, 2000).

Q-PCR for AID and germline transcripts. RNA from stimulated B cells (96 h) was extracted from TRIzol (Invitrogen), followed by cDNA preparation with Superscript II Reverse transcription (Invitrogen) according to the manufacturer's protocol. Q-PCR was performed using the Brilliant III SYBR Green Q-PCR Master Mix (Agilent). Reactions were performed in triplicates for each of at least three mice of each genotype, and analyzed using MX3005P Q-PCR software (Stratagene). Values were normalized to GAPDH. The following primers were used: GAPDH-F, 5′-TGAAGCAG-GCATCTGAGGG-3′; GAPDH-R, 5′-CGAAGGTGGAAGAGT-GGGAG-3′; AID-F, 5′-GAAAGTCACGCTGGAGACCG-3′; AID-R, 5′-TCTCATGCCGTCCCTTGG-3′; µGLT-F, 5′-TAGTAAGCGAG-GCTCTAAAAAGCAT-3′; µGLT-R, 5′-AGAACAGTCCAGTGTAG-GCAGTAGA-3′; γ1GLT-F, 5′-GGCCCTTCCAGATCTTTGAG-3′; γ1GLT-R, 5′-GGATCCAGAGTTCCAGGTCACT-3′.

Analysis of microhomology at switch junctions. DNA was harvested from cultured B cells and amplified using nested reactions. First reaction: 5′-AATGGATACCTCAGTGGTTTTTAATGGTGGGTT-3 and 5-CAATTAGCTCCTGCTCTTCTGTGG-3′. Nested reaction: 5′-GAC-CCAGGCTAAGAAGGCAATCC-3 and 5-GACTGGGTTCCCACA-CTCTTACCTGCC-3′. After cloning in TOPO vector and sequencing, microhomology was determined by manual alignment.

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