Classical and Alternative End-Joining Pathways for Repair of Lymphocyte-Specific and General DNA Double-Strand Breaks

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Contents

Abstract

Classical nonhomologous end joining (C-NHEJ) is one of the two major known pathways for the repair of DNA double-strand breaks (DSBs) in mammalian cells. Our understanding of C-NHEJ has been derived, in significant part, through studies of

programmed physiologic DNA DSBs formed during V(D)J recombination in the developing immune system. Studies of immunoglobulin heavy-chain (IgH) class-switch recombination (CSR) also have revealed that there is an "alternative" end-joining process (A-EJ) that can function, relatively robustly, in the repair of DSBs in activated mature B lymphocytes. This A-EJ process has also been implicated in the formation of oncogenic translocations found in lymphoid tumors. In this review, we discuss our current understanding of C-NHEJ and A-EJ in the context of V(D)J recombination, CSR, and the formation of chromosomal translocations.

1. INTRODUCTION

We live under the constant assault of DNA-damaging factors; at any given moment, our cells encounter harmful agents and must react in order to preserve genomic stability. DNA damage caused either by environmental factors such as various forms of radiation (i.e., UV or ionizing radiation) or by-products of cellular metabolism such as reactive oxygen species is one of the most harmful events an organism can encounter. If unrepaired, DNA damage can lead to the activation of cell-cycle checkpoints and cell-cycle arrest or cell death. Improper repair can cause chromosomal aberrations that interfere with DNA replication or gene expression (reviewed by Bassing & Alt, 2004; Franco, Alt, & Manis, 2006; Gostissa, Alt, & Chiarle, 2011; Zhang et al., 2010). Of the various kinds of DNA lesions, the complete disruption of both DNA strands—a double-strand break (DSB)—is particularly harmful. However, programmed physiologic DSBs are generated in the course of normal cellular development during V(D)J recombination in developing B and T lymphocytes and during immunoglobulin heavy-chain (IgH) class-switch recombination (CSR) in the context of B cell-mediated immune responses (reviewed by Chaudhuri et al., 2007; Dudley, Chaudhuri, Bassing, & Alt, 2005; Lieber, 2010). Due to their potential danger, the repair of such physiological DSBs, like that of general DSBs, is tightly linked to endogenous DNA repair pathways. Thus, studies of DSB repair processes involved in V(D)J recombination and CSR in lymphocytes have yielded many key insights into general principles of DNA DSB repair.

The two major pathways known to recognize and repair DSBs in eukaryotic cells are homologous recombination (HR) and "classical" non-homologous end joining (C-NHEJ) (reviewed by Bassing & Alt, 2004; Franco, Alt, et al., 2006; Heyer, Ehmsen, & Liu, 2010; Lieber, 2010;

Weinstock, Richardson, Elliott, & Jasin, 2006; Fig. 1.1). DSB repair via HR requires a separate template sequence with a long stretch of homology to the sequence containing the DSB. Such templates are usually provided by a sister chromatid or homologous chromosome (reviewed by Heyer et al., 2010; Weinstock et al., 2006). As such, HR operates after DNA replication in the S and G2 phases of the cell cycle. Because it uses a homologous template to repair damage, HR fully restores the genetic information (reviewed by Heyer et al., 2010). Another form of homology-directed repair, single-strand annealing (SSA), mediates joining between interspersed nucleotide repeats in the genome (Elliott, Richardson, & Jasin, 2005; Sugawara, Ira, & Haber, 2000; reviewed by Heyer et al., 2010). Because the intervening sequence is deleted in the repaired product, this form of repair results in the loss of genetic information (Elliott et al., 2005; Sugawara et al., 2000; reviewed by Heyer et al., 2010). C–NHEJ rejoins DSBs and, in contrast to HR, does not rely on sequence homology to do so (reviewed by Lieber, 2010). In this regard, C–NHEJ can operate in all phases of the cell cycle (reviewed by Lieber, 2010; Mills, Ferguson, & Alt, 2003). The fast kinetics of C–NHEJ in rejoining DSBs promote genomic stability by preventing removal of larger segments of DNA around DSBs via resection and by suppressing chromosomal translocations, at least for the majority of repair events. However, C–NHEJ can be mutagenic as it introduces small deletions and insertions during the repair process (reviewed by Lieber, 2010). As discussed in Sections 4.1 and 4.2 below,
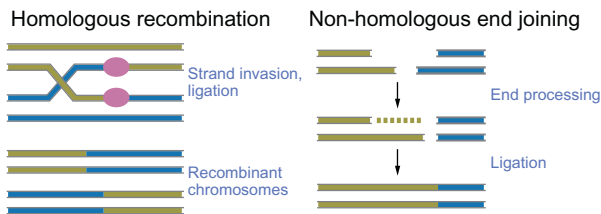
Figure 1.1 Homologous recombination versus C-NHEJ. HR repairs DNA breaks using a long homologous DNA template, which is not generally available before DNA replication. HR is active in cycling cells, in the S and G2 phases of the cell cycle, and fully preserves genetic information. C-NHEJ can join DNA ends without sequence homology and therefore can operate in nondividing cells and in the G1 phase of the cell cycle. C-NHEJ can process DNA ends before ligation, through end resection or nucleotide insertions, which allows ligation of ends with complex modifications but can cause loss of genetic information.

C–NHEJ is the only repair pathway employed for V(D)J recombination and it also predominates in CSR.

In recent years, evidence for an end-joining pathway (or pathways) in addition to C–NHEJ has emerged. This "alternative" end-joining (A-EJ) process for DNA DSB repair has been discovered in various contexts, but the details of A-EJ have remained rather elusive. A-EJ does not normally participate in V(D)J recombination, even in the absence of C–NHEJ (Taccioli et al., 1993; reviewed by Schatz & Swanson, 2011), due to active exclusion from this reaction (Corneo et al., 2007; Cui & Meek, 2007). However, A-EJ can mediate up to 50% of wild-type (WT) CSR levels in C–NHEJ-deficient B cells, although its role in CSR in normal B cells remains to be definitively characterized (Yan et al., 2007). A-EJ DSB repair has been suggested to pose a particular threat to genomic integrity due to its apparent predisposition to participate in the joining of DSBs between heterologous chromosomes to generate translocations (Simsek & Jasin, 2010; Zhang & Jasin, 2011; reviewed by Zhang et al., 2010). The mechanistic nature and potential physiologic and nonphysiologic activities of A-EJ have been a subject of intense study by the DNA repair field.

In this review, we will first briefly introduce the V(D)J recombination and CSR reactions, as required for the discussion of end-joining repair pathways in the context of these processes. We will then provide an in-depth discussion of what we know about C–NHEJ and, particularly, A-EJ and will conclude with a discussion of unresolved issues and future directions.

2. V(D)J RECOMBINATION

The ability of B cells to mount an effective immune response against a vast array of pathogens relies on the generation of a diverse population of B cells that each expresses a unique B cell receptor (BCR), the secreted form of which is an antibody. BCRs comprise two identical Ig heavy (IgH) and two identical Ig light (IgL) chains, with each IgH and IgL chain containing N-terminal variable and C-terminal constant regions. The exons encoding the variable regions of BCRs are assembled in developing B cells through a process of genetic rearrangements termed V(D)J recombination (reviewed by Tonegawa, 1983; Tonegawa, Brack, Hozumi, & Pirrotta, 1978). The murine *IgH* locus is located at the telomeric end of chromosome 12 and contains a large number of V (variable), D (diversity), and J (joining) gene segments, spread over three megabases (reviewed by Schatz & Swanson, 2011; Fig. 1.2). Variable region exon assembly in the Ig loci
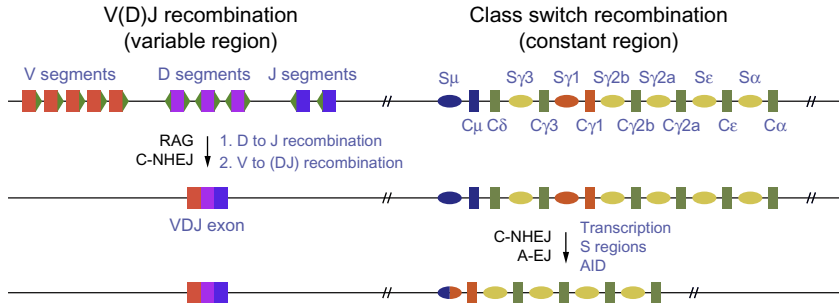
Figure 1.2 Genomic rearrangements in the *IgH* locus. The murine *IgH* locus is located at the telomeric end of chromosome 12. Left, V(D)J recombination; developing B cells in the bone marrow rearrange the variable regions of their immunoglobulin loci in a stepwise fashion. Rearrangement of *IgH* begins with D–J recombination, followed by V to (DJ). The DNA breaks between V, D, and J gene segments (squares) and adjacent RSSs (triangles) are introduced by the RAG1/2 complex. RAG1/2 also channels these breaks into repair by C-NHEJ. Right, CSR; mature B cells in peripheral lymphoid organs acquire additional effector functions by expressing new classes of antibodies. At the level of DNA, CSR involves breaks in S regions, which are long and repetitive intronic regions upstream of constant region genes (C). S-region breaks are generated by AID, which is targeted to S regions by RNAPII-mediated transcription. The final step in CSR is a DNA joining event between two different S regions, which leads to the expression of the downstream constant region gene. End joining in the context of CSR is performed by both C-NHEJ and A-EJ.

occurs in an ordered manner, with D_H to J_H rearrangement taking place before V_H to $D_H J_H$ joining (Alt et al., 1984). Productive assembly of an *IgH* $V_H D J_H$ leads to expression of an IgH chain that signals the assembly of *IgL* variable region exon via the joining of V_L and J_L segments (reviewed by Perlot & Alt, 2008). Expression of IgH and IgL chains together in the form of a surface BCR leads to the generation of mature naive B cells that migrate to peripheral lymphoid organs, where upon interaction of cognate antigens with their BCR, they can differentiate into antibody–producing cells that secrete the complete Ig molecule as an antibody (reviewed by Chaudhuri et al., 2007).

The "V(D)J recombinase" involved in the assembly of variable region exons employs the lymphocyte-specific *recombination activating gene 1* (RAG1) and RAG2 components to generate specific DSBs (Oettinger, Schatz, Gorka, & Baltimore, 1990; Schatz, Oettinger, & Baltimore, 1989), the lymphocyte–specific *terminal deoxynucleotidyl transferase* (TdT) to diversify junctional regions (Alt & Baltimore, 1982), and the C–NHEJ pathway to process and join broken V, D, and J segments (Taccioli et al., 1993).

RAG1 and RAG2 together form the RAG endonuclease, which is expressed specifically in the G1 cell–cycle phase in developing B and T lymphocytes (reviewed by Schatz & Swanson, 2011). The RAG endonuclease initiates the assembly of both *IgH* and *IgL* variable region exons in developing B lymphocytes and the assembly of related T cell receptor variable region exons in developing T lymphocytes. Developmental stage and lineage specificity of V(D)J recombination is further ensured by controlled accessibility of antigen receptor gene segments to RAG activity (Yancopoulos & Alt, 1985). RAG generates DSBs between specific recombination signal sequences (RSSs) and the coding sequence of the V, D, and J segments (reviewed by Jung, Giallourakis, Mostoslavsky, & Alt, 2006; Schatz & Swanson, 2011). RSSs consist of a conserved heptamer followed by a 12- or 23-base pair (bp) nonconserved spacer and a conserved AT-rich nonamer (reviewed by Schatz & Swanson, 2011; Tonegawa, 1983). RAG binds and cleaves a pair of RSSs predominantly according to the 12/23 rule, that is, RAG cleavage will generally only occur if one of the participating gene segments is flanked by an RSS with a 12-bp spacer, while the other one has a 23-bp spacer (reviewed by Tonegawa, 1983). RAG generates a single-strand nick between the V, D, or J gene segment and the adjacent RSS, followed by a nucleophilic attack of the 3′-OH on the phosphate group located opposite on the complementary 5′-strand (McBlane et al., 1995). This reaction is carried out in the context of a synaptic complex and yields two DSBs with distinct ends: hairpin-sealed V, D, or J coding ends (CEs) and blunt 5′-phosphorylated signal ends (SEs) (reviewed by Schatz & Swanson, 2011). CEs and SEs are retained in a postcleavage synaptic complex until they are joined.

CEs must be opened and processed before joining to yield coding joins, whereas the blunt SEs are joined directly to form signal joins (reviewed by Schatz & Swanson, 2011). The processing of CEs prior to joining frequently leads to the addition or removal of nucleotides through the action of Pol X polymerases (TdT, pol μ, pol λ) or exonucleases. TdT is a lymphocyte-specific-V(D)J recombination component which appears to have evolved to expand the antigen receptor repertoire by diversification of V(D)J junctions (Alt & Baltimore, 1982; Gilfillan, Dierich, Lemeur, Benoist, & Mathis, 1993; Komori, Okada, Stewart, & Alt, 1993). The end processing and joining components of the V(D)J recombinase comprise the ubiquitously expressed C-NHEJ factors (Taccioli et al., 1993; reviewed by Lieber, 2010; Schatz & Swanson, 2011). In this regard, the RAG2 component of

the RAG endonuclease directly excludes both the HR and the A-EJ processes from V(D)J repair in WT cells (Corneo et al., 2007; Cui & Meek, 2007). Thus, A-EJ can mediate repair of RAG-dependent DSBs in cells expressing a particular mutant of the RAG2 protein (Corneo et al., 2007). HR may be also excluded from the reaction based on the fact that V(D)J recombination is initiated and completed in the G1 cell-cycle phase when HR is not active (Lee, Neiditch, Salus, & Roth, 2004). As discussed in more detail in Sections 4.3 and 4.4, V(D)J recombination also employs components of the *ataxia telangiectasia-mutated kinase* (ATM)-dependent DNA DSB response which appear, at least in part, to functionally overlap with certain C-NHEJ functions (Liu et al., 2012; Oksenych et al., 2012; Zha, Guo, et al., 2011).

3. IgH CLASS-SWITCH RECOMBINATION

Antigen-dependent activation of peripheral B cells can lead to two additional somatic alterations of antibody gene structure, namely, variable region exon somatic hypermutation, which allows selection of B cells that produce higher affinity antibodies, and CSR, which changes the expressed antibody constant region and allows generation of antibodies with different effector functions. In mice, there are eight different sets of C_H exons (referred to as "C_H genes") within a 200-kb region downstream of the assembled V(D)J exon (reviewed by Chaudhuri et al., 2007). CSR is a DSB-dependent recombination/deletion process that results in the replacement of the exons encoding the initially expressed IgH constant region ($C\mu$) with one of a set of downstream C_H exons ($C\gamma1$, $C\gamma2b$, $C\gamma3$, $C\gamma2a$, $C\epsilon$, or $C\alpha$), resulting in a change in the antibody class from IgM to IgG1, IgG2b, IgG3, IgG2a, IgE, or IgA, respectively (reviewed by Chaudhuri et al., 2007). With the exception of $C\delta$, each C_H gene is preceded by a long (1–10 kb), repetitive switch (S) region (reviewed by Chaudhuri & Alt, 2004; Honjo, Alt, & Neuberger, 2004; Fig. 1.2), which are the targets of the CSR-specific DSBs. $S\mu$, $S\epsilon$, and $S\alpha$ have short, 5-bp repeats and are more homologous in repeat structure to each other; $S\gamma$ repeats are longer (up to 49 bp) and are less homologous to $S\mu$ (reviewed by Chaudhuri et al., 2007).

Both CSR and SHM are initiated by the *activation-induced cytidine deaminase* (AID) enzyme (Muramatsu et al., 2000), which is a single-stranded (ss) DNA-specific cytidine deaminase (reviewed by Chaudhuri et al., 2007). AID is targeted to single-stranded S-region DNA by transcription, where it deaminates deoxycytidine to deoxyuridine (reviewed by Chaudhuri

et al., 2007). Transcription through S regions generates an ssDNA substrate for AID via R-loop formation and/or by RPA-dependent mechanisms (Basu et al., 2005; Chaudhuri et al., 2003; Tian & Alt, 2000; Yu, Chedin, Hsieh, Wilson, & Lieber, 2003). AID targeting in part may involve association with the RNA polymerase (RNAP) II complex via the Spt5 factor in association with stalled RNAPII (Pavri et al., 2010). Access of AID to the template DNA strand, which may be blocked by association of this strand with RNA, appears to be promoted via actions of the RNA exosome complex (Basu et al., 2011). AID deamination lesions in S regions are processed into DSBs (Catalan et al., 2003; Petersen et al., 2001) through co-opted activities of the base excision repair (BER) and mismatch repair pathways (reviewed by Di Noia & Neuberger, 2007). To complete CSR, DSBs in the donor Sμ region and a downstream acceptor S region are joined via an end-joining event (reviewed by Chaudhuri et al., 2007). In the context of this joining reaction, the intervening region between the two S-region DSBs, including Cμ, is deleted out in the form of an excision circle, while the other two ends are joined, resulting in the juxtaposition of the downstream C_H gene to the V(D)J exon and expression of a new constant region under the control of the V(D)J exon promoter (reviewed by Chaudhuri et al., 2007; Fig. 1.2).

Once DSBs are generated in two participating S regions, they must be brought together (synapsed) over 100–200-kb distances for joining. How this synapsis event occurs is still being investigated. However, experimental evidence supports the notion that chromosomal DSBs separated over distances of 100 kb or more may be joined at relatively high frequency by general DNA repair mechanisms that maintain chromosomal integrity and suppress translocations (Zarrin et al., 2007). In this context, normal CSR requires both the ATM-dependent DSB response and C-NHEJ. In the absence of either, many CSR DSBs remain unjoined, leading to chromosomal *IgH* locus breaks and translocations (Franco et al., 2008; Franco, Gostissa, et al., 2006; Ramiro et al., 2006; Yan et al., 2007). As discussed in more detail in Section 5, in the absence of C-NHEJ, CSR also can be carried out by A-EJ at up to 50% of WT levels (Yan et al., 2007). A proposed obligatory factor for achieving requisite levels of long-range CSR joins via general repair mechanisms is the introduction of multiple DSBs into a given set of S regions in a given activated B cell. Frequent DSB introduction into participating S regions is proposed to allow some DSBs to be joined between S regions to carry out CSR, even though most are rejoined or joined over shorter distances
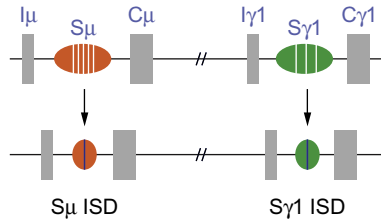
Figure 1.3 Internal switch deletions—AID-dependent recombination/deletion events within a given S region. AID-generated lesions can lead to ISDs in mature B cells undergoing CSR. Sμ contains more frequent ISDs than downstream S regions, such as Sγ1. ISDs may result from end resection of a single AID lesion followed by joining or from the joining of two independent AID-dependent S-region breaks within the same S region. In WT cells, ISD junctions are either direct or MH mediated, suggesting that both C-NHEJ and A-EJ are involved during ISD formation. Homology-directed pathways such as SSA could potentially be involved as some S regions contain relatively long repeats.

within a given S region (Zarrin et al., 2007). In this context, joining of two AID-initiated DSBs within a given S region can also cause internal switch deletions (ISDs) (Alt, Rosenberg, Casanova, Thomas, & Baltimore, 1982; Dudley et al., 2002; Gu, Zou, & Rajewsky, 1993; Iwasato, Shimizu, Honjo, & Yamagishi, 1990; Fig. 1.3); ISDs also might be caused by resection of the ends of a single DSB within an S region region before joining (Boboila, Jankovic, et al., 2010). Even though sequences within a given S region tend to be repetitive in nature, characterized ISD junctions also appear to result predominantly from end joining rather than a mechanism involving HR (Boboila, Jankovic, et al., 2010; Dunnick, Hertz, Scappino, & Gritzmacher, 1993; Yancopoulos et al., 1986).

4. CLASSICAL NONHOMOLOGOUS DNA END JOINING

In mammalian cells, C-NHEJ is the major mechanism for DSB repair utilized when an intact DNA template is not available to initiate HR. C-NHEJ predominates in G0 and G1 but can operate in all phases of the cell cycle (reviewed by Lieber, 2010; Mills et al., 2003). Therefore, C-NHEJ is a predominant DSB repair pathway in both dividing and nondividing cells (reviewed by Rooney, Chaudhuri, & Alt, 2004). The C-NHEJ joining reaction can utilize DSB ends without homologous nucleotides, as well as ends with short (1–4) nucleotide homologies (reviewed by Lieber, 2010). Joining of blunt DNA ends or ligation of DNA ends that become blunt after end processing via resection or fill-in
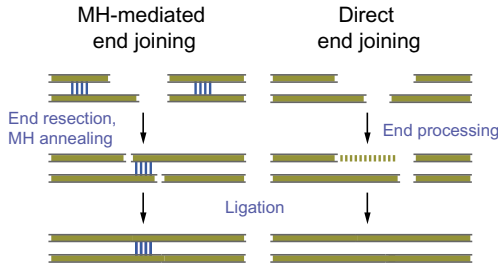
Figure 1.4 Microhomology-mediated versus direct DNA end joining. MH-mediated joining is facilitated by base-pairing interactions between MHs present in single-strand overhangs. C-NHEJ joins both MH and blunt (direct) ends, whereas A-EJ shows a strong preference for MH-mediated joining.

by DNA polymerases is referred to as "direct" joining. Joining of ends with short homologies is generally referred to as "microhomology (MH)–mediated" end joining (Fig. 1.4).

4.1. C-NHEJ during V(D)J recombination

C–NHEJ repairs both general DSBs and programmed DSBs generated during V(D)J recombination and CSR (reviewed by Rooney, Chaudhuri, et al., 2004). Indeed, findings that formed the basis for the elucidation of C–NHEJ, and much of what we know about the pathway from later work, came from studies of end joining during V(D)J recombination, a reaction that is absolutely dependent on C-NHEJ (Taccioli et al., 1993). Seven C–NHEJ pathway members have been identified, including Ku70, Ku80 (also known as Ku86), *X-ray repair cross-complementing protein 4* (XRCC4), *DNA ligase 4* (Lig4), *DNA-dependent protein kinase, catalytic subunit* (DNA–PKcs), Artemis, and *XRCC4-like factor* (XLF; also known as NHEJ1 or Cernunnos) (reviewed by Lieber, 2010; Fig. 1.5). Ku70, Ku80, XRCC4, and Lig4 are evolutionarily conserved, as they have homologs in yeast; these proteins are considered "core" C–NHEJ factors that are absolutely required for all C–NHEJ reactions including the joining of both hair pinned CEs and blunt RS ends during V(D)J recombination (reviewed by Lieber, 2010; Rooney, Chaudhuri, et al., 2004). DNA–PKcs and Artemis are only found in vertebrates and are required for joining a subset of DNA ends that must be further processed before end joining (reviewed by Lieber, 2010; Rooney, Chaudhuri, et al., 2004).

Artemis and XLF were discovered based on being mutated in human immunodeficient patients (Buck, Malivert, et al., 2006; Moshous et al., 2001).
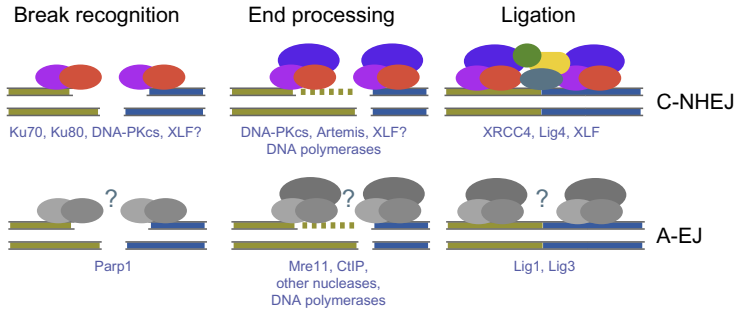
Break recognition **End processing** **Ligation**

C-NHEJ

Ku70, Ku80, DNA-PKcs, XLF?   DNA-PKcs, Artemis, XLF?   XRCC4, Lig4, XLF
                              DNA polymerases

A-EJ

Parp1   Mre11, CtIP,   Lig1, Lig3
               other nucleases,
               DNA polymerases

Figure 1.5 DNA end joining repair. Upper, C-NHEJ; DSB ends are recognized by the Ku70/Ku80 heterodimer, which recruits DNA-PKcs to form the DNA-PK holoenzyme. DNA-PK phosphorylates and recruits Artemis, which further processes DSB ends. End ligation is performed by the XRCC4/Lig4 complex. Ku70, Ku80, XRCC4, and Lig4 are evolutionarily conserved "core" C-NHEJ factors that are required for all C-NHEJ reactions. DNA-PKcs and Artemis are accessory factors found in vertebrates and are required for a subset of DNA ends that need further processing, such as CEs in V(D)J recombination. XLF interacts with XRCC4, but its precise function in NHEJ is unknown; because of its functional overlap with DSBR factors, it could be potentially involved in any of the repair stages. Lower, A-EJ; no factors with roles exclusive to A-EJ have yet been identified. Several proteins have been implicated in A-EJ, and each is known to function in other DNA repair pathways. Parp1 may sense the DNA break and recruit XRCC1/Lig3 for end ligation. Mre11 and CtIP are involved in end resection and may have additional functions in the context of the DSB response complex. In addition, DNA polymerases and other nucleases may process ends before ligation. The identity of the ligation complex is not clear yet and may be context dependent.

The end processing activity of Artemis is activated by DNA–PKcs; in this regard, both are required for processing and joining hair pinned V(D)J CEs but are largely dispensable for joining blunt RS ends (Blackwell et al., 1989; Lieber et al., 1988; Ma, Pannicke, Schwarz, & Lieber, 2002; Malynn et al., 1988; Rooney et al., 2002). Although the precise function of XLF is unknown, it was considered a C-NHEJ factor based on the radiosensitivity and impaired V(D)J recombination phenotype of cells from humans with XLF mutations (Buck, Malivert, et al., 2006) and also based on its direct interaction with XRCC4/Lig4 (Ahnesorg, Smith, & Jackson, 2006; Callebaut et al., 2006), which separately led to its identification (Ahnesorg et al., 2006). XLF promotes the ligation activity of Lig4 (Ahnesorg et al., 2006) and, in biochemical assays, aids in the joining of DSBs with incompatible or blunt ends (Gu, Lu, Tsai, Schwarz, & Lieber, 2007; Tsai, Kim, & Chu, 2007). However, in contrast to other C–NHEJ factors, XLF appears largely dispensable for chromosomal V(D)J

recombination in developing mouse lymphocytes (Li et al., 2008; Zha, Alt, Cheng, Brush, & Li, 2007). However, the critical role of XLF in C–NHEJ is much more apparent in the context of deficiency of ATM or its downstream DNA DSB response factors (Liu et al., 2012; Oksenych et al., 2012; Zha, Guo, et al., 2011), as discussed below.

During C–NHEJ, Ku, which consists of the Ku70/Ku80 heterodimer, binds and protects DSB ends and promotes end ligation carried out by the XRCC4/Lig4 complex (reviewed by Lieber, 2010; Rooney, Chaudhuri, et al., 2004). XRCC4 functions as a scaffolding protein to stabilize Lig4 and stimulates its readenylation and may also help to align DSB ends before ligation (reviewed by Lieber, 2010). For DNA ends that require processing before ligation, for example, the hairpin-sealed CEs formed during V(D)J recombination, the Ku complex recruits DNA-PKcs to form the DNA–PK holoenzyme (reviewed by Lieber, 2010). DNA-PKcs phosphorylates and activates Artemis, which processes CEs through its endonuclease activity (Ma et al., 2002; reviewed by Lieber, 2010). DNA-PKcs also functions in direct joining of DSBs that do not require processing, as evidenced by the requirement for DNA-PKcs for fully normal rejoining of blunt RS ends during V(D)J recombination (Errami et al., 1998; Fukumura et al., 1998, 2000). The precise function of DNA-PKcs in this context is unknown but may involve serving to tether DSB ends or via phosphorylation of other proteins (reviewed by Lieber, 2010; Weterings & Chen, 2007). Notably, the ATM DSB response factor serves a functionally redundant role with DNA-PKcs in RS joining as deficiency for either of these factors alone has, at most, relatively modest effects on this direct joining reaction while deficiency for both severely impairs RS joining (Gapud et al., 2011; Zha, Jiang, et al., 2011; see Section 4.3). Conceivably, DNA-PKcs may have functions in DSB repair that are independent of its kinase activity, potentially involving tethering of DSB ends. Finally, DNA-PKcs binds to XRCC4, Lig4, and Artemis, suggesting that it may have functions in the recruitment of C–NHEJ factors to DSB ends (reviewed by Lieber, 2010; Neal & Meek, 2011).

V(D)J recombination does not occur in B cells deficient in Ku70, Ku80, XRCC4, or Lig4 (Frank et al., 1998; Gao, Sun, et al., 1998; Gu et al., 1997; Li et al., 1995; Nussenzweig et al., 1996; Taccioli, Gottlieb, et al., 1994; Taccioli et al., 1993; Zhu, Bogue, Lim, Hasty, & Roth, 1996) because of a nearly absolute requirement for C–NHEJ to join ends generated in the context of V(D)J recombination (Taccioli, Cheng, Varghese, Whitmore,

& Alt, 1994; Taccioli et al., 1993). As mentioned earlier, this restriction appears to reflect the ability of RAG, through unknown mechanisms, to channel the repair reaction specifically into C-NHEJ (Corneo et al., 2007; Cui & Meek, 2007). In addition, this restriction may be augmented by the restriction of V(D)J recombination to the G1 cell-cycle phase. Due to their absolute requirement for V(D)J recombination, mice lacking any of the core C-NHEJ factors exhibit a complete lack of mature B and T cells, referred to as severe combined immunodeficiency (SCID) (Frank et al., 1998; Gao, Sun, et al., 1998; Gu et al., 1997; Li et al., 1995; Nussenzweig et al., 1996). Deficiency for DNA-PKcs or Artemis in mice also causes a SCID phenotype (Bosma, Custer, & Bosma, 1983; Gao, Chaudhuri, et al., 1998; Kurimasa, Ouyang, et al., 1999; Lieber et al., 1988; Rooney et al., 2003, 2002; Taccioli et al., 1998), which for DNA-PKcs deficiency is often "leaky," due to low residual levels of V(D)J recombination, which over time give rise to some mature B and T cells (reviewed by Bosma, 1992). Mice deficient for core C-NHEJ factors also have marked growth defects and radiosensitivity associated with their DSB repair defects. In the case of XRCC4 or Lig4 deficiency, they die late in embryonic development in association with severe neuronal apoptosis in addition to their SCID phenotype (Frank et al., 1998; Gao, Sun, et al., 1998). The neuronal apoptosis and embryonic lethality, but not SCID phenotype, of XRCC4- or Lig4-deficient mice can be rescued by deficiency for the p53 tumor suppressor, which normally leads to cell-cycle arrest or death in response to G1 DSBs (Frank et al., 2000; Gao et al., 2000). Consistent with a role for DNA-PKcs and Artemis in processing only a subset of DSBs, DNA-PKcs- or Artemis-deficient mice have at most mild radiosensitivity, lack overt neuronal apoptosis, and do not display growth defects (Gao, Chaudhuri, et al., 1998; Rooney et al., 2003, 2002; Taccioli et al., 1998).

Humans with mutations in XLF display an immunodeficiency phenotype consistent with defects in the repair of RAG-mediated DSBs (Buck, Malivert, et al., 2006). In contrast, XLF-deficient mice are not immunodeficient and developing lymphocytes in these mice are not substantially impaired for V(D)J recombination (Li et al., 2008). Surprisingly, although V(D)J recombination of extrachromosomal substrates occurs in XLF-deficient lymphocytes, XLF-deficient nonlymphoid cells, such as embryonic stem cells or embryonic fibroblasts, are defective in these assays (Li et al., 2008; Zha et al., 2007), suggesting that XLF deficiency might be compensated by another factor, or several factors, during the repair of

RAG-initiated DSBs in lymphocytes (Li et al., 2008). Indeed, subsequent studies demonstrated that XLF has functionally overlapping roles with ATM-dependent DNA DSB response (DSBR) factors (Liu et al., 2012; Oksenych et al., 2012; Zha, Guo, et al., 2011; see below).

4.2. C-NHEJ during IgH CSR

Unlike V(D)J recombination, there is no evidence that AID or any other factor specifically directs CSR into a particular repair pathway. Indeed, it has been argued that CSR may operate mainly via the same factors that are involved in the repair of general chromosomal DSBs (Zarrin et al., 2007; reviewed by Chaudhuri et al., 2007). Yet, C-NHEJ has been shown to play a major role in CSR DSB repair. While S regions are repetitive, the level of homology between S regions is not sufficient to provide a substrate for HR (reviewed by Chaudhuri et al., 2007). In this regard, nearly all characterized CSR junctions are generated by end joining. Most CSR junctions in WT cells are characteristic of C-NHEJ because they tend to display direct joins or joins that involve very short MHs (Boboila, Yan, et al., 2010; Dunnick et al., 1993; Yan et al., 2007) and/or short insertions (Yan et al., 2007; reviewed by Stavnezer, Bjorkman, Du, Cagigi, & Pan-Hammarstrom, 2010). Correspondingly, deficiency for either XRCC4 or Lig4 in activated B cells results in a major reduction in the levels of CSR along with the accumulation of substantial levels of AID-dependent *IgH* locus chromosomal breaks (Yan et al., 2007). These latter findings clearly demonstrated a critical role for C-NHEJ in CSR; however, unlike V(D)J recombination, CSR still occurred in C-NHEJ-deficient cells via end joining at up to 50% of WT levels, implicating a role for A-EJ (Yan et al., 2007; see Sections 5.1 and 5.2).

Early experiments with Ku70- or Ku80-deficient mature B cells suggested a nearly complete deficiency for CSR, as well as severe proliferation defects (Casellas et al., 1998; Manis et al., 1998). In the latter context, it was noted that impaired proliferation in the absence of Ku might have contributed to lack of CSR in these Ku-deficient cells (Manis et al., 1998; reviewed by Chaudhuri & Alt, 2004). Indeed, subsequent experiments that employed more stringent B cell purification and robust stimulation protocols (Cheng et al., 2009; Yan et al., 2007) demonstrated that Ku70- or Ku80-deficient B cells are definitively impaired for CSR but that, again, CSR can occur at up to 50% WT levels in their absence via A-EJ (Boboila, Yan, et al., 2010). The role of the other C-NHEJ factors in CSR has also been studied in detail in mice. DNA-PKcs-deficient B cells undergo variable levels of CSR, which

for some IgH isotypes were only moderately reduced (Bosma et al., 2002; Callen et al., 2009; Franco et al., 2008; Kiefer et al., 2007; Manis, Dudley, Kaylor, & Alt, 2002), whereas Artemis-deficient B cells undergo CSR at relatively normal levels (Rivera-Munoz et al., 2009; Rooney, Alt, Sekiguchi, & Manis, 2005). However, sensitive fluorescence *in situ* hybridization assays for *IgH* locus chromosomal breaks revealed clear CSR end-joining defects for both DNA-PKcs-deficient and, to a lesser extent, Artemis-deficient B cells (Franco et al., 2008). In this regard, DNA-PKcs and Artemis appear to be required for joining a fraction of CSR DSBs, perhaps primarily those that require further processing (Franco et al., 2008; Rivera-Munoz et al., 2009). In comparison to WT, XLF-deficient B cells display slightly lower CSR levels, increased *IgH* breaks, and a slight increase in MH-mediated joins, indicating a more prominent role, in normal cells, for XLF in repair of CSR DSBs than V(D)J DSBs (Li et al., 2008; Zha, Guo, et al., 2011)

4.3. Roles of the ATM-dependent DSB response in C-NHEJ

The ATM-dependent DSB response (DSBR) recognizes DNA DSBs and generates chromatin-associated complexes around DSBs that promote DSB repair via C-NHEJ (reviewed by Downs, Nussenzweig, & Nussenzweig, 2007; Franco, Alt, et al., 2006; Zha, Boboila, & Alt, 2009). DSBs generated during both V(D)J recombination and IgH CSR lead to a DSBR (reviewed by Bassing & Alt, 2004; Downs et al., 2007). Initial DSB detection appears to involve the *meiotic recombination 11 homolog A* (Mre11)/Rad50/*Nijmegen breakage syndrome 1* (Nbs1) (MRN) complex (reviewed by van den Bosch, Bree, & Lowndes, 2003), which is required for activation of several phosphatidylinositol 3-kinase-like serine/threonine kinases, including DNA-PKcs and ATM (reviewed by Shiloh, 2003). Activated ATM phosphorylates a multitude of factors, including the histone variant H2AX (to form γH2AX), Nbs1, *mediator of DNA-damage checkpoint protein 1* (MDC1), and 53BP1, which was initially identified as a p53-binding protein (reviewed by Bassing & Alt, 2004; Downs et al., 2007). This ATM-dependent DSB response leads to the assembly of macromolecular foci over large (two megabases or more) chromatin regions flanking the DSB (Rogakou, Boon, Redon, & Bonner, 1999; Rogakou, Pilch, Orr, Ivanova, & Bonner, 1998; reviewed by Bassing & Alt, 2004; Downs et al., 2007; Franco, Alt, et al., 2006). These foci are thought to serve as scaffolds to stabilize DSB ends and facilitate the

recruitment of additional repair and checkpoint signaling factors (reviewed by Bassing & Alt, 2004; Downs et al., 2007). ATM also directly activates cell-cycle checkpoints in response to DSBs, such as the p53-dependent G1/S checkpoint that allows for DSB repair and prevents replication of damaged DNA and the associated risk of chromosomal translocations (reviewed by Bassing & Alt, 2004; Franco, Alt, et al., 2006). Failure to repair DSBs in the context of such checkpoints can induce cell-cycle arrest or apoptosis to eliminate cells that contain damaged DNA in a cell type-specific manner (reviewed by Bassing & Alt, 2004; Downs et al., 2007). Deficiencies for DSBR components lead to genomic instability, unrepaired chromosomal breaks, and translocations, as well as defects in V(D)J recombination and CSR, albeit to varying degrees (Bassing et al., 2002, 2003; Bredemeyer et al., 2006; Callén et al., 2007; Celeste et al., 2002; Difilippantonio et al., 2008; Franco, Gostissa, et al., 2006; Zha, Guo, et al., 2011).

ATM deficiency in mice leads only to minor defects in V(D)J recombination and lymphocyte development (Barlow et al., 1996; Borghesani et al., 2000; Lumsden et al., 2004; Reina-San-Martin, Chen, Nussenzweig, & Nussenzweig, 2004; Xu et al., 1996). In this context, humans with mutations in ATM (reviewed by Lavin, 2008) and ATM-deficient mice develop T cell lymphomas with translocations involving the T cell receptor loci owing to defects in V(D)J recombination during T cell development (Zha et al., 2010). H2AX deficiency does not overtly affect V(D)J recombination, indicating that H2AX, like ATM, is not absolutely required for the repair of RAG-mediated DSBs (Bassing et al., 2002; Celeste et al., 2002). However, dual deficiency for H2AX and p53 in mice leads to T and B cell lymphomas, with some of the latter carrying oncogenic translocations consistent with aberrant V(D)J recombination (Bassing et al., 2003; Celeste et al., 2003). In this context, H2AX protects V(D)J recombination-generated DNA ends from resection by CTBP-interacting protein (CtIP) in G1-arrested B cells and, thereby, influences C-NHEJ and prevents aberrant repair of V(D)J DSBs that could cause genomic instability (Helmink et al., 2011). 53BP1-deficient mice are immunodeficient, radiation sensitive, and prone to developing lymphomas (Manis et al., 2004; Morales et al., 2006; Ward, Minn, van Deursen, & Chen, 2003; Ward et al., 2004). MDC1-deficient mice are radiation sensitive and display genomic instability and defects in CSR (Lou et al., 2006). However, neither MDC1 nor 53BP1 deficiency have any apparent effect on V(D)J recombination (Lou et al., 2006; Manis et al., 2004; Ward et al., 2004; but see below).

Deficiencies for ATM, H2AX, or MDC1 lead to reduced CSR ranging from 30% to 50% of WT levels (Celeste et al., 2002; Lou et al., 2006; Lumsden et al., 2004; Manis et al., 2004; Petersen et al., 2001; Reina-San-Martin et al., 2004, 2003; Ward et al., 2004), and activated B cells deficient for these factors accumulate substantial levels of AID-dependent *IgH* locus breaks, indicating a role for these DSBR factors in end joining during CSR (Bassing et al., 2003; Celeste et al., 2002; Franco, Gostissa, et al., 2006; Lumsden et al., 2004; Petersen et al., 2001; Reina–San-Martin et al., 2004). In contrast, 53BP1 deficiency very severely impairs CSR (Manis et al., 2004; Ward et al., 2004), even though it leads to more modest general genomic instability (Franco, Gostissa, et al., 2006; Liu et al., 2012; Oksenych et al., 2012). Various functions have been proposed for 53BP1 that might contribute to its prominent role in CSR, including roles in C-NHEJ, regulation of DSB resection, long-range end synapsis, and checkpoint activation (Boboila et al., 2012; Bothmer et al., 2010; Bunting et al., 2010; Difilippantonio et al., 2008; Xie et al., 2007). Notably, despite the much greater impact of 53BP1-deficiency on CSR, the frequency of *IgH* breaks is not higher in 53BP1-deficient B cells activated for CSR than B in cells lacking either H2AX or ATM (Franco, Gostissa, et al., 2006). Overall, it appears that 53BP1 plays a unique functional role in CSR beyond any it plays in the context of the normal DSB response, a role that is still under intense investigation.

The increased frequency of *IgH* locus breaks and translocations in B cells deficient for H2AX, ATM, MDC1, or 53BP1 demonstrated that these factors participate in end joining during CSR (Franco, Gostissa, et al., 2006). In this regard, functional overlap between DSBR and C-NHEJ factors has been revealed in several contexts. For example, DNA-PKcs-deficient cells require ATM for the repair of RS ends, indicating that DNA-PKcs and ATM have overlapping roles in the context of V(D)J recombination (Gapud et al., 2011; Zha, Jiang, et al., 2011). Similarly, in the context of CSR, broken DNA ends in ATM-deficient cells require DNA-PKcs for normal repair, as combined deficiency for both proteins results in large insertions, genomic instability, and an additive reduction in CSR levels (Callen et al., 2009). The overlapping kinase activities of ATM and DNA-PKcs, and potentially a shared spectrum of substrate proteins, may mediate the functional overlap of these factors. In this context, both factors are known to phosphorylate several factors implicated in the DSB response, such as H2AX, *structural maintenance of chromosomes 1* (SMC1), and *Krüppel-associated box (KRAB) zinc finger protein (ZFP) protein-associated protein 1* (KAP1) (Callen et al., 2009; Kitagawa,

Bakkenist, McKinnon, & Kastan, 2004; Tomimatsu, Mukherjee, & Burma, 2009; Ziv et al., 2006), as well as *bona fide* C-NHEJ factors (Ku, XRCC4, Lig4, Artemis) (reviewed by Lieber, 2010). In addition, DNA-PKcs and ATM might have other types of functionally overlapping activities such as tethering ends of DSBs. As described in more detail in Section 4.4, a major functional overlap in C-NHEJ has been found for XLF and various DSBR factors.

4.4. Overlapping roles of DSBR factors and XLF in C-NHEJ

As mentioned above, ATM and H2AX were found to have, at most, only modest roles in C-NHEJ during V(D)J recombination. However, recent work revealed that, under certain conditions, these two factors can indeed play key roles in the repair of RAG-mediated DSB ends during V(D)J recombination (Zha, Guo, et al., 2011). This important functional role for these factors in C-NHEJ was previously missed because of the unexpected functional redundancies between the ATM DSBR factors and XLF (Liu et al., 2012; Oksenych et al., 2012; Zha, Guo, et al., 2011). Thus, in contrast to individual deficiencies, combined loss of XLF/ATM mimics C-NHEJ deficiency: it causes a nearly complete block in the repair of RAG-dependent DSBs in developing lymphocytes and reduces CSR in mature B cells to the same extent as deficiencies for core C-NHEJ factors (Zha, Guo, et al., 2011). These findings established that XLF-deficient B and T lineage cells require ATM and ATM-deficient cells require XLF to perform C-NHEJ (Zha, Guo, et al., 2011). Notably, combined XLF/ATM deficiency does not appear to markedly affect A-EJ during CSR, consistent with the notion that XLF is a C-NHEJ factor and not an A-EJ factor (Zha, Guo, et al., 2011).

To test possible mechanisms for the ATM and XLF functional redundancy, several studies tested the hypothesis that the redundancy may be mediated in the context of the overall ATM-dependent DSBR and, therefore, extend to additional DSBR factors beyond ATM. In this regard, combined deficiency of XLF and H2AX reduced V(D)J recombination, albeit to a lesser degree than combined XLF/ATM deficiency (Zha, Guo, et al., 2011) and with, at least in part, a different outcome in the form of increased resection of the unjoined V(D)J DSBs (Zha, Guo, et al., 2011). In the latter context, H2AX limits excessive ATM-dependent resection of unrepaired RAG-dependent DSB ends in Artemis-deficient pro-B cells by neutralizing the activity of CtIP (Helmink et al., 2011). Indeed, combined absence of H2AX and XLF similarly led to ATM-dependent resection of unrepaired RAG breaks, potentially due to a combined end-joining defect in the absence of XLF and H2AX and the lack

of H2AX to protect the persistent broken DSB ends (Zha, Guo, et al., 2011). A very similar functional overlap also was found for XLF and 53BP1 during V(D)J recombination (Liu et al., 2012; Oksenych et al., 2012). Thus, in comparison to individual deficiencies, combined XLF/53BP1 deficiency in mice results in increased general genomic instability and a severe block in early lymphocyte development associated with severely impaired V(D)J recombination (Liu et al., 2012; Oksenych et al., 2012). As for combined H2AX and XLF deficiency, unjoined V(D)J ends in the context of dual XLF and 53BP1 deficiency also undergo extensive resection (Liu et al., 2012; Oksenych et al., 2012), consistent with a role for 53BP1 in protecting ends from resection (Bothmer et al., 2011). Notably, combined XLF/53BP1 deficiency also appears to potentially predispose to thymic lymphomas in mice (Liu et al., 2012), pointing to an overlapping function of XLF and 53BP1 in tumor suppression.

Overall, current studies indicate that XLF is functionally redundant with multiple ATM-dependent DSBR factors and implicate XLF as a key factor in facilitating C–NHEJ in the context of chromatin. The precise mechanistic nature of these overlapping activities of DSBR factors with XLF is currently unclear; however, there are several possibilities (Liu et al., 2012; Oksenych et al., 2012; Zha, Guo, et al., 2011). One possibility is that DSBR factors and XLF are involved in the same processes. Alternatively, DSBR factors might function in separate processes from those that involve XLF that do not lead to marked defects in end-joining repair unless both are impaired. As one example, XLF might help recruit repair factors to increase reaction kinetics (Ahnesorg et al., 2006; Gu, Lu, Tsai, et al., 2007; Tsai et al., 2007), while the DSBR may tether and protect ends or activate checkpoints that allow ends to persist together long enough for joining (reviewed by Bassing & Alt, 2004; Downs et al., 2007). In this example, loss of one of these activities might be countered by the other, but loss of both could lead to severe joining defects (Zha, Guo, et al., 2011). Going forward, it will also be informative to examine whether additional ATM substrates, such as MDC1 or other factors implicated in the repair of DSBs, including certain C–NHEJ factors such as DNA–PKcs, have any functionally overlapping roles with XLF in mediating efficient chromosomal C–NHEJ.

4.5. C-NHEJ suppresses genomic instability

The DSBR and C–NHEJ mechanisms discussed in Sections 4.1–4.4 above ensure genomic integrity by rapidly repairing DSBs. In turn, a hallmark of cells deficient in C–NHEJ factors is the presence of widespread unrepaired

chromosomal breaks (Boboila, Jankovic, et al., 2010; Ferguson et al., 2000; Franco et al., 2008; Ramiro et al., 2004; Yan et al., 2007; reviewed by Ferguson & Alt, 2001; Rooney, Chaudhuri, et al., 2004; Zha et al., 2009). Moreover, a significant fraction of the broken chromosomal ends in C–NHEJ–deficient cells are involved in illegitimate repair that joins different chromosomes together to generate chromosomal translocations (Boboila, Jankovic, et al., 2010; Wang, Gostissa, et al., 2009; Yan et al., 2007; Zhu et al., 2002). Notably, C–NHEJ–deficient cells appear to have significantly more chromosome than chromatid breaks, suggesting that most breaks occur in the G1 phase of the cell cycle and are replicated during S phase (Franco, Gostissa, et al., 2006). ATM or 53BP1 deficiency also lead primarily to chromosome breaks, again indicating a major role in G1 (Franco, Gostissa, et al., 2006). However, H2AX deficiency causes both chromatid breaks (post–replication) and chromosome breaks (pre–replication), suggesting prominent roles for this factor in DSB repair in both S and G1 phases of the cell cycle (Franco, Gostissa, et al., 2006; Zha, Sekiguchi, Brush, Bassing, & Alt, 2008). Most primary cells with genomic instability do not undergo cell division and are eliminated by cell–cycle checkpoints (Ferguson et al., 2000). Rarely, however, cells with chromosomal breaks can persist and even differentiate further, such as in the case of ATM–deficient B cells carrying unrepaired V(D)J breaks (Callén et al., 2007).

C–NHEJ deficiencies in mice compatible with survival of the animal post-natally (Ku70-, Ku80-, XLF-, DNA-PKcs-, or Artemis deficiency) do not generally promote tumors, despite leading to chromosomal breaks and trans-locations (reviewed by Gostissa et al., 2011). Mice in which *Xrcc4* or *Lig4* are conditionally deleted in mature B cells (Boboila, Jankovic, et al., 2010; Wang et al., 2008) are not tumor prone either. In some cases, complete inactivation of *Ku70* (Gu et al., 1997) or *DNA-PKcs* (Kurimasa, Kumano, et al., 1999), or a point mutation that affects the function of DNA-PKcs (reviewed by Custer, Bosma, & Bosma, 1985) or Lig4 (Rucci et al., 2010), gave rise to thymic tumors with variable frequency. Because these mutant mice exhibit "leaky" T cell development, these tumors are thought to arise during T cell development and rearrangement of the TCR loci. Environmental factors and differences in genetic backgrounds may also affect the variable incidence of these tumors (Rucci et al., 2010).

Mice lacking both C–NHEJ factors and the G1/S checkpoint protein p53 display rapid and aggressive tumor onset. Notably, these mice develop both medulloblastomas (XLF, XRCC4, or Lig4 deficiency) (Li et al., 2008; Zhu et al., 2002) and pro–B cell tumors (Ku80, XRCC4, Lig4, or Artemis deficiency) with the latter tending to dominate (Difilippantonio et al., 2000;

Frank et al., 2000; Gao et al., 2000; Rooney, Sekiguchi, et al., 2004). Pro-B cell tumors in Ku80/p53–, XRCC4/p53–, or Lig4/p53 double–deficient mice show RAG–dependent translocations between the J_H region of the *IgH* locus on chromosome 12 and *c-myc* on chromosome 15 (Difilippantonio et al., 2002; Zhu et al., 2002). In contrast, Artemis/p53 tumors carry mostly *IgH/n-myc* intrachromosome 12 translocations (Rooney, Sekiguchi, et al., 2004). Why the Artemis/p53 mice have *n-myc* as a translocation target is a very interesting unanswered question. The translocations promote tumorigenesis via gene amplification and overexpression of the *c-myc* and *n-myc* loci (Rooney, Sekiguchi, et al., 2004). Gene amplification is a classical mechanism for oncogene activation in human and mouse neural and other solid tumors (e.g., Kohl et al., 1983; Yan et al., 2006) but is only more rarely observed in human lymphoid tumors (Martin–Subero et al., 2005; reviewed by Kuehl & Bergsagel, 2002). Mature B cells conditionally deleted for *Xrcc4* and *p53* generate tumors harboring translocations that join CSR–induced *IgH* S–region breaks to *c-myc* (Wang et al., 2008), a classical mechanism of oncogene activation in various human mature B cell lymphomas (reviewed by Gostissa et al., 2011; Küppers & Dalla–Favera, 2001). This class of translocations results in deregulated *c-myc* expression that is driven over hundreds of kilobases by regulatory elements in the 3′–end of the *IgH* locus (Gostissa, Ranganath, Bianco, & Alt, 2009; Wang et al., 2008). Overall, mouse studies have established C–NHEJ as a major mechanism for suppressing genomic instability, including chromosomal translocations that can lead to oncogenic transformation of murine lymphoid cells. To date, however, there have only been a limited number of examples of C–NHEJ deficiencies associated with human malignancies (Buck, Moshous, et al., 2006; Du et al., 2012; Hill et al., 2006; Moshous et al., 2003).

5. ALTERNATIVE END JOINING

5.1. Overview

Early evidence for A–EJ came from studies showing that C–NHEJ–deficient yeast cells (Boulton & Jackson, 1996) and mammalian cell lines (Kabotyanski, Gomelsky, Han, Stamato, & Roth, 1998) efficiently repair linear plasmid substrates. In such "transient" assays, and in biochemical studies, end–joining activity in cells lacking C–NHEJ factors was found to depend on the *X-ray repair cross-complementing protein 1* (XRCC1)/*DNA ligase 3* (Lig3) complex (Audebert, Salles, & Calsou, 2004; Wang et al., 2005). Additionally, *poly-ADP-ribose polymerase 1* (Parp1), a factor with

known roles in single-strand break DNA repair, also was found to play a role in end joining of nonchromatinized DSBs in the absence of Ku70 or DNA-PKcs (Audebert et al., 2004; Wang et al., 2006). Assays relying on extrachromosomal plasmid substrates have been very useful for elucidating A–EJ and revealing certain aspects of the process. However, not all findings of such studies are necessarily directly applicable to the repair of endogenous chromosomal DSBs because repair of transiently transfected plasmids does not occur in the context of native chromatin. On the other hand, V(D)J recombination on transiently introduced substrates was found to be completely abrogated in the absence of C-NHEJ factors (Taccioli, Gottlieb, et al., 1994; Taccioli et al., 1993). Correspondingly, V(D)J recombination was also found to be essentially abrogated in Ku–, XRCC4–, Lig4–, or DNA-PKcs-deficient mice (Frank et al., 1998; Gao, Chaudhuri, et al., 1998; Gao, Sun, et al., 1998; Gu et al., 1997; Li et al., 1995; Nussenzweig et al., 1996; Taccioli, Gottlieb, et al., 1994; Taccioli et al., 1993; Taccioli et al., 1998; Zhu et al., 1996). Together, these V(D)J recombination findings led to the notion that C-NHEJ may be the only robust pathway for joining chromosomal DSBs and that A–EJ represents a "backup" or "error-prone" repair pathway, unable to function on chromatinized substrates (Verkaik et al., 2002; Wang et al., 2003; Wang et al., 2005). Subsequent studies, however, demonstrated that A–EJ indeed can repair endogenous chromosomal DSBs, in some cases, relatively robustly, in various biological contexts.

One of the first clear indications of A–EJ functioning in the context of chromosomal substrates came from studies of translocation junctions in pro-B cell lymphomas derived from mice deficient for the C-NHEJ core factors XRCC4 or Lig4 and lacking the tumor suppressor p53 (Zhu et al., 2002). Notably, more than a dozen such A–EJ oncogenic translocation junctions were isolated and were all found to have occurred by end joining and to have involved MH–mediated joining (Zhu et al., 2002). These findings clearly demonstrated that A–EJ can fuse RAG-initiated *IgH* DSBs to DSBs around the *c-myc* gene to generate oncogenic *IgH/c-myc* translocations associated with pro-B cell lymphomagenesis (Zhu et al., 2002). Additional studies showed that Ku80-deficient or XRCC4-deficient cells can repair DSBs generated by the yeast meganuclease I-SceI in chromosomally integrated reporter substrates via A–EJ (Guirouilh–Barbat, Rass, Plo, Bertrand, & Lopez, 2007; Guirouilh–Barbat et al., 2004). Evidence that A–EJ can function at relatively robust levels in a true physiological context came from studies that demonstrated that B cells deficient for XRCC4 or Lig 4 could undergo CSR at up to 50% of WT levels via an

A–EJ pathway that used MH for nearly all CSR joins (Yan et al., 2007). Similar findings were made by studies of CSR in a Lig4-deficient B cell lymphoma line (Han & Yu, 2008). In addition, more recent studies extended these findings to CSR in B cells deficient for Ku or both Ku and Lig4 (Boboila, Yan, et al., 2010; see Section 5.2).

The almost complete reliance on MH for end joining of DSBs in yeast lacking the Ku70 (Yku70p) or Lig4 (Dnl4p) homologs indicated that end joining in the absence of C–NHEJ in *Saccharomyces cerevisiae* requires MHs, leading to the A–EJ joining pathway being referred to as "MH-mediated end joining" (MMEJ) (Ma, Kim, Haber, & Lee, 2003; reviewed by Haber, 2008). The findings that A-EJ-catalyzed translocation junctions and CSR junctions in XRCC4- or Lig4–deficient B cells were nearly all MH mediated (in contrast, e.g., to WT CSR junctions which have approximately 30–60% direct joins; Yan et al., 2007) were consistent with these yeast studies and the plasmid–based mammalian cell studies that found A–EJ junctions display a strong bias toward MH joining (Kabotyanski et al., 1998; Verkaik et al., 2002; Wang et al., 2003). Thus, even A–EJ in mammalian cells has been referred to as MMEJ (reviewed by McVey & Lee, 2008). However, the requirement for MH for A–EJ joining in mammalian cells is not absolute; thus, I–SceI- or RAG-mediated DSBs in XRCC4–deficient murine ES cells and Chinese hamster ovary (CHO) cells can utilize direct joining (Corneo et al., 2007; Guirouilh-Barbat et al., 2007; Simsek & Jasin, 2010). Further, junctions of repaired DSBs within I–SceI reporter substrates in Ku80–deficient CHO cells (Guirouilh–Barbat et al., 2004), translocation junctions in Ku70–deficient ES cells (Simsek & Jasin, 2010), and S-region junctions in Ku70–deficient or Ku70/Lig4 double–deficient mature B cells (Boboila, Jankovic, et al., 2010; Boboila, Yan, et al., 2010) all show a substantial proportion of direct joins, clearly indicating that MH is not a strict requirement of A–EJ. Whether or not MH–mediated or direct A–EJ represent the same or different pathways is unknown. However, until this question is resolved, it has been proposed that it may be more accurate to define mammalian A–EJ as any DSB repair process occurring in the absence of "core" C–NHEJ factors (reviewed by Zha et al., 2009; Zhang et al., 2010).

5.2. Distinct forms of A-EJ in B cells

A–EJ is often discussed as a single pathway, but it is possible that observed A–EJ activities represent multiple pathways using different components. Moreover, it is still unclear whether DSB repair in XRCC4- or Lig4–deficient cells represents an A–EJ pathway completely distinct from C–NHEJ or a subpathway

of C-NHEJ, as both factors function only in the final ligation step and the upstream C-NHEJ components remain intact (reviewed by Lieber, 2010; Lieber, Lu, Gu, & Schwarz, 2008). However, in the context of CSR, A-EJ operates relatively robustly in the absence of either the DSB recognition component (Ku70 or Ku80) or the specific ligation component (Lig4) of C-NHEJ and even in the absence of both (Boboila, Yan, et al., 2010). In this context, there appear to be at least two distinct forms of A-EJ that can mediate CSR (Boboila, Yan, et al., 2010). One form operates in the absence of Lig4 or Xrcc4 and relies on Ku and either Lig1 or Lig3 and is strongly biased toward longer MHs (Boboila, Yan, et al., 2010; Yan et al., 2007). The second form of A-EJ occurs in the absence of Ku or the combined absence of Ku and Lig4 and preferentially uses MH but also frequently uses direct joining (Boboila, Yan, et al., 2010). This Ku- and Lig4-independent form of A-EJ clearly established the existence of an A-EJ pathway or pathways that are totally distinct from C-NHEJ, as, again, both the DSB recognition and the DSB ligation components of C-NHEJ are absent in the double-deficient cells, which would obviate the C-NHEJ pathway (Boboila, Yan, et al., 2010).

The finding that there are substantial numbers of direct CSR joins in Ku-deficient and Ku/Lig4 double-deficient B cells, but not in Xrcc4- or Lig4-defcient B cells, is intriguing but mechanistically unclear. Direct joins in Ku70/Lig4 double-deficient B cells might be promoted by DNA polymerases such as TdT, pol μ, or pol λ that gain access to DNA ends and insert random nucleotides to generate end homologies (Gu, Lu, Tippin, et al., 2007; Komori, Pricop, Hatakeyama, Bona, & Alt, 1996; reviewed by Lieber, 2010), which could then be used for MH-mediated joining which would include junctions that were MH mediated but in which the MH was not obvious (occult) in the final junctions (Boboila, Yan, et al., 2010). Another explanation for the observed direct joins in Lig4/Ku70-deficient cells, but not in Lig4/XRCC4-deficient cells, would be that they occur via a different ligase that can access the joins in the absence of Ku (Boboila, Yan, et al., 2010). Elucidation of potential roles for the various polymerases and other end-modifying factors in Ku-deficient cells and the exact role of Ku in end processing and recruitment of factors influencing joining pathways and MH usage should provide important insights into mechanisms that promote A-EJ.

Overall, it seems reasonable to approach A-EJ mechanistically as a repair process or processes that involve several phases, namely, (a) DSB recognition and tethering, (b) end processing, and (c) ligation and dissociation of the repair complex. Whether these steps necessarily occur sequentially for each strand or simultaneously on both strands is unknown. While knowledge of the A-EJ pathway is still rather rudimentary, some recent studies have

implicated roles for a number of known factors involved in DSB repair and these will be discussed in the following sections.

5.3. Factors implicated in DSB end recognition and tethering during A-EJ

DSBs must be recognized and the ends must be held together (tethered) for sufficient time to allow joining. In terms of DSB recognition and tethering, the relative utilization of MH versus forming direct joins during CSR via A–EJ is influenced by Ku, as outlined above (Boboila, Jankovic, et al., 2010; Boboila, Yan, et al., 2010), although the mechanistic function of Ku in this context is still unknown. Another putative factor proposed to be involved in early steps of A–EJ is Parp1, a member of an 18–protein family of nuclear PARP enzymes that mark histones, transcription factors, and other target proteins by adding poly–ADP–ribose moieties onto amino acid residues (Robert, Dantzer, & Reina-San-Martin, 2009; Shockett & Stavnezer, 1993; reviewed by Caldecott, 2003; Huber, Bai, de Murcia, & de Murcia, 2004). Parp1 and Parp2 are known to function as DNA-damage sensors and to recruit other proteins in response to single-strand breaks (reviewed by Huber et al., 2004). Involvement of Parp1 in A–EJ was initially based on biochemical experiments (Audebert et al., 2004) and plasmid assays in Ku-deficient cells (Wang et al., 2006). However, a role for Parp1 in CSR has been suggested based on the finding that S-region junctions in Parp1 null cells displayed an apparent decrease in MH-mediated joins and an increase in junctional insertions (Robert et al., 2009). However, because both C-NHEJ and A-EJ can utilize MHs, Parp1 theoretically might influence either pathway. Notably, siRNA-mediated Parp1 depletion resulted in a severe defect in end joining of chromosomal I–SceI DSBs in Ku–deficient, but not WT CHO cells, indicating that Parp1 may promote chromosomal A–EJ (Mansour, Rhein, & Dahm–Daphi, 2010). Going forward, it would be useful to examine whether Parp1/XRCC4 double-deficient cells show lower CSR levels than XRCC4–deficient B cells, which would be expected if Parp1 were required for A–EJ. However, negative results in such an experiment would be difficult to interpret due to the presence of Parp2, which might substitute for Parp1 activity. In this regard, Parp1 and Parp2 double deficiency is embryonically lethal, whereas either Parp1– or Parp2–null mice survive and have only modest DNA repair defects (Menissier de Murcia et al., 2003). Such potential functional redundancies between Parp1 and Parp2 might also extend to A–EJ in particular contexts. Overall, there is much more to be done with respect

to DSB recognition and tethering during A–EJ. In this regard, as outlined above for C–NHEJ, the ATM-dependent DSBR has been implicated in recognition and tethering of DSBs. However, potential roles for DSBR factors in A–EJ DSB recognition or tethering largely remain to be investigated; although Mre11 has been studied in the context of DSB processing during A–EJ, as discussed in Section 5.4.

5.4. Factors implicated in DSB processing during A-EJ

Many ends joined by A–EJ likely require DSB end processing, including resection. A–EJ frequently uses short stretches of complementary nucleotides, situated either at the ends of the DNA break (terminal MH) or embedded into the ends and requiring end resection to be functionally revealed. Indeed, in *S. cerevisiae*, Mre11 facilitates MH joining in Yku70p-deficient strains, probably by promoting end resection (Ma et al., 2003). By extension, possible roles for mammalian Mre11 in MH–mediated joining have been proposed (Nussenzweig & Nussenzweig, 2007). Mre11 is a component of the conserved MRN complex, which activates specific DNA-damage responses and chromatin changes around the DSB site (reviewed by Stracker & Petrini, 2011). Mre11 can process DNA ends through its endo- and exonuclease activities (Hopkins & Paull, 2008; Paull & Gellert, 1998, 2000) and has been implicated in HR (Limbo et al., 2007; Takeda, Nakamura, Taniguchi, & Paull, 2007). Reduction of Mre11 protein levels or chemical inhibition of Mre11 decreased chromosomal end joining in WT as well as C–NHEJ-deficient backgrounds, potentially implicating Mre11 in both C–NHEJ and A–EJ (Dinkelmann et al., 2009; Xie, Kwok, & Scully, 2009). In the context of A–EJ, Mre11 seems to be involved in end processing, as Mre11 depletion in XRCC4-deficient cells limits end resection (Xie et al., 2009). B cells in which *Mre11* is conditionally deleted show elevated genomic instability, indicating a potential role for Mre11 in end joining during CSR (Dinkelmann et al., 2009). However, because loss of either Mre11 or only its nuclease domain causes B cell proliferation defects, some of the reduction in CSR may be due to impaired cell survival (reviewed by Zha et al., 2009). Elucidation of the precise roles of Mre11 and its domains in CSR and their relevance for A–EJ and general DSB repair will require further studies.

Another factor proposed to be involved in DSB end processing during A–EJ is CtIP. CtIP participates in several DNA repair pathways, including SSA, HR, and A–EJ (Bennardo, Cheng, Huang, & Stark, 2008;

Limbo et al., 2007; Sartori et al., 2007; Yun & Hiom, 2009). CtIP has been proposed to modulate repair pathway choice between A-EJ and HR in chicken DT40 cells (Yun & Hiom, 2009). Not much yet is known about the biological role of CtIP in lymphocytes. What is known is that CtIP promotes hairpin opening and end resection of RAG-mediated DSBs in H2AX-deficient cells (Helmink et al., 2011). In these cells, DSBs processed by CtIP cannot be efficiently joined by C-NHEJ and the remaining joints show deletions and MH leading to the suggestion that A-EJ may potentially join these ends (Helmink et al., 2011; reviewed by Helmink & Sleckman, 2012). Additional evidence for a putative role of CtIP in A-EJ in lymphocytes comes from studies of CtIP in CH12F3 B cell lines (Lee-Theilen, Matthews, Kelly, Zheng, & Chaudhuri, 2011). In these cells, CtIP depletion reduced CSR levels and caused a shift toward a higher proportion of direct CSR joins (Lee-Theilen et al., 2011). In comparison to Ku70 depletion alone, combined reduction of CtIP and Ku70 levels in CH12F3 B cells appeared to cause an additive reduction in CSR and resulted in CSR joins that are more frequently direct or contain shorter MHs (1–4 nucleotides) (Lee-Theilen et al., 2011). Based on these findings, CtIP was proposed to promote MH-mediated joining in the context of CSR, both in WT and C-NHEJ-deficient cells. Further clarification of the role of CtIP in A-EJ versus C-NHEJ awaits further studies in primary B cells. CtIP has also been implicated in the formation of translocations originating from I-SceI-induced DSBs in mouse embryonic stem cells (Zhang & Jasin, 2011). In this system, CtIP depletion reduces translocation frequency in both WT and Ku-deficient cells, thus potentially implicating CtIP in A-EJ-mediated translocation formation (Zhang & Jasin, 2011). Alternatively, the reduced translocation frequency in CtIP-depleted cells could be due to cell viability defects caused by impaired HR-mediated repair. Experiments addressing translocation formation in G1-arrested cells should help dissect the roles of CtIP in HR versus A-EJ.

5.5. Factors implicated in DSB joining during A-EJ

Several studies have addressed the ligation step of A-EJ. Lig4 is not considered to be an A-EJ factor due to its requisite role for C-NHEJ during V(D)J recombination. However, Lig4 theoretically might still function in some form of A-EJ, for example, in Ku-deficient cells. Beyond Lig4, mammalian cells contain only two other known DNA ligases: Lig1 and Lig3 (reviewed by Tomkinson, Vijayakumar, Pascal, & Ellenberger, 2006). Lig1 is required

for joining of Okazaki fragments during DNA replication and functions in long-patch BER (reviewed by Timson, Singleton, & Wigley, 2000). Lig3 is implicated in short-patch BER and single-strand break repair and is stabilized by its XRCC1 cofactor (Caldecott, McKeown, Tucker, Ljungquist, & Thompson, 1994, reviewed by Caldecott, 2003). Results from plasmid joining assays and biochemical experiments implicated Lig3 and XRCC1 in A-EJ (Audebert et al., 2004; Wang et al., 2005, 2006). Consequently, these factors have been very widely considered to be major chromosomal A-EJ factors (Yan et al., 2007; reviewed by Ciccia & Elledge, 2010; Mladenov & Iliakis, 2011).

Both Lig1 and Lig3 deficiency cause cell lethality (Puebla-Osorio, Lacey, Alt, & Zhu, 2006). Lig3 has both nuclear and mitochondrial isoforms (Lakshmipathy & Campbell, 1999), and the nuclear, but not the mitochondrial isoform, associates with XRCC1 (Lakshmipathy & Campbell, 2000). Notably, mitochondrial Lig3, but not the nuclear Lig3, is critical for cell survival (Gao et al., 2011; Simsek, Furda, et al., 2011). Thus, cellular complementation with mitochondrially targeted Lig1 (mtLig1) or Lig3 (mtLig3) allows subsequent deletion of the endogenous *Lig3* gene in mouse ES cells (Simsek, Furda, et al., 2011). Surprisingly, Lig3-deficient cells generated by this approach (Simsek, Furda, et al., 2011), or via conditional *Lig3* inactivation by tissue-specific Cre expression (Gao et al., 2011), are not IR sensitive, indicating that Lig3 deletion does not cause substantial defects in nuclear DNA repair. Yet, mtLig1- or mtLig3-complemented Lig3-deficient mouse ES cells showed reduced translocations between two zinc finger endonuclease-induced chromosomal DSBs (Simsek, Brunet, et al., 2011), consistent with a Lig3 role in end joining leading to these translocations, although other interpretations are possible. However, very substantial depletion of Lig3 in either WT or Lig4-deficient primary B cells or CH12F3 B cell lines did not cause measurable CSR defects, alter MH usage in CSR junctions, or markedly affect generation of *IgH/c-myc* translocations (Boboila et al., 2012). There are several potential interpretations consistent with the apparently negative findings on the role of Lig3 in A-EJ based on its depletion in B-lineage cells, including (a) there is redundancy between Lig1 and Lig3 in A-EJ, (b) low Lig3 levels are sufficient to support normal A-EJ, or (c) Lig3 does not participate in A-EJ during CSR in activated B cells. In any case, although these findings on Lig3-depleted activated B cells do not rule out a role for Lig3 in A-EJ, they are consistent with a major role for Lig1 as an A-EJ ligase in an endogenous context

(Boboila et al., 2012). Further studies using approaches other than gene ablation will be required to evaluate relative contributions of Lig1 and Lig3 to A-EJ.

XRCC1 is a scaffolding protein that stabilizes Lig3; correspondingly, Lig3 protein levels are drastically reduced in XRCC1-deficient cells (Boboila et al., 2012; Lee et al., 2009; Tebbs et al., 1999). XRCC1 has been implicated in A-EJ based on biochemical experiments in Ku70- or XRCC4-depleted extracts (Audebert et al., 2004) and its known interactions with Lig3. Another line of evidence implicating XRCC1 in A-EJ is its role in Ku-independent repair in *Arabidopsis thaliana* (Charbonnel, Gallego, & White, 2010). However, whether this function is relevant for mammalian A-EJ is unclear because plants lack a Lig3 homolog. Thus, unlike mammalian XRCC1, which is associated with nuclear Lig3, *Arabidopsis* XRCC1 may function by recruiting other repair complexes to carry out DSB repair in the absence of C-NHEJ. Inactivation of a single copy of XRCC1 in primary mouse B cells was reported to alter MH usage in CSR junctions and to reduce *IgH/c-myc* translocation levels, leading to the conclusion that XRCC1 is an A-EJ factor for CSR (Saribasak et al., 2011). Unlike Lig3 deficiency, loss of XRCC1 is compatible with cell survival (Tebbs et al., 1999), and XRCC1-deficient and XRCC1/XRCC4 double-deficient murine pro-B cell lines have been generated (Boboila et al., 2012). Surprisingly, XRCC1/XRCC4 double-deficient lines showed no differences from XRCC4-deficient lines with respect to the repair of I-SceI-induced chromosomal DSBs, definitively showing that XRCC1 is not necessary for A-EJ in this context (Boboila et al., 2012). In addition, conditional *Xrcc1* inactivation in mature primary WT or XRCC4-deficient B cells did not impair A-EJ-mediated Sμ–Sγ1 or Sμ–Sε switch junction formation or alter their frequency of *IgH/c-myc* translocations (Boboila et al., 2012). Similarly, genetic ablation of *Xrcc1* in Lig4 null CH12F3 B cell lines did not impact their ability to undergo CSR (Han, Mao, & Yu, 2012). Thus, current findings reveal that XRCC1 is not required for normal A-EJ of various types of chromosomal DSBs in B-lineage cells and thus cannot be considered a requisite A-EJ factor, at least in these contexts (Boboila et al., 2012).

Lig1 also has been implicated in A-EJ (Boboila et al., 2012; Simsek, Brunet, et al., 2011). However, like *Lig3* inactivation, *Lig1* inactivation causes embryonic and cellular lethality (Petrini, Xiao, & Weaver, 1995), making direct genetic studies in mice impossible. Truncations that leave

the Lig1 N-terminus intact also result in embryonic lethality but are compatible with cell survival (Bentley et al., 2002, 1996). Cells with such Lig1 truncations display genomic instability and impaired DNA replication, due to defects in the joining of Okazaki fragments (Bentley et al., 2002). Point mutations that impair Lig1 function have been described in humans with Bloom syndrome, which have severely reduced lymphocyte numbers and suffer from immunodeficiency; however, these mutations do not affect V(D)J recombination, supporting, but not proving, the notion that Lig1 does not play a critical role in C-NHEJ (Petrini, Donovan, Dimare, & Weaver, 1994). As outlined above, studies of Lig3-deficient cells suggest that Lig1 contributes to A-EJ-mediated translocation formation when Lig3 is absent (Simsek, Brunet, et al., 2011). Also, as mentioned above, findings that XRCC1/Lig3 depletion does not impair A-EJ-mediated CSR or *IgH/c-myc* translocation formation in XRCC4- or Lig4-deficient B-lineage cells are consistent with a major role for Lig1 in A-EJ (Boboila et al., 2012).

5.6. Potential physiologic roles of A-EJ

A major open question is whether A-EJ operates in the presence of intact C-NHEJ and what its physiologic role may be. Several lines of evidence argue that A-EJ may function in the presence of C-NHEJ on substrates that are repetitive in nature and therefore provide frequent opportunity for MHs, such as S regions. First, ISD junctions isolated from WT S regions display frequent MH usage (Boboila, Jankovic, et al., 2010; Dunnick et al., 1993; Yancopoulos et al., 1986). Second, in the absence of C-NHEJ, A-EJ in primary cells catalyzes significant levels of CSR, and although the absolute levels are reduced to about 50% of those of WT cells, the kinetics of join accumulation appear to be similar (Yan et al., 2007). This result led to the suggestion that A-EJ is relatively robust in ability to join a subset of CSR ends, likely those that provide sufficient MH (Yan et al., 2007). Third, Sμ–Sα junctions in human B cells (Pan-Hammarstrom et al., 2005) and Sμ–Sε junctions in mouse B cells (Boboila, Yan, et al., 2010; Yan et al., 2007) use MHs longer than four nucleotides, which may be more characteristic of A-EJ than of C-NHEJ (Daley & Wilson, 2005; Komori et al., 1993; reviewed by Chaudhuri et al., 2007). These latter findings are consistent with the possibility that A-EJ may participate more robustly in the generation of CSR junctions between DSBs in S regions that are more homologous (Sμ, Sε, Sα) and, therefore, may

provide more potential MHs to use as joining substrates, as well as longer potential MHs (Yan et al., 2007). Overall, there is still much to be done to elucidate any potential major role for A–EJ in physiological processes.

5.7. ISD may provide a model for studying MH-mediated A-EJ

Because of the repetitive nature of S regions, ISD breaks may be repaired preferentially by mechanisms utilizing MHs (Boboila, Jankovic, et al., 2010; Yancopoulos et al., 1986). Notably, B cells deficient in one or more C–NHEJ core factor(s) display considerably more ISDs than WT, both in Sμ and downstream S regions, such as Sγ1 (Boboila, Jankovic, et al., 2010). ISD junctions in C–NHEJ-deficient cells are likely mediated by A-EJ because the limited number analyzed occurred via end joining and contained either MHs or insertions (Boboila, Jankovic, et al., 2010). Therefore, A–EJ appears to mediate ISD joins preferentially over joining DSBs in different S regions to carry out CSR. Whether or not ISD is actually increased in C–NHEJ-deficient B cells remains to be determined. Thus, the relative level of ISD may be underestimated in WT cells, as these cells often undergo CSR on both alleles, which would mask many potential ISD events. Attempts to circumvent this problem by analyzing WT B cells that did not undergo CSR are potentially limited by the possibility that such cells are not adequately stimulated for AID DSB-inducing activity which would also lower their ISD levels.

Keeping the above caveats in mind, there are several potential mechanisms that might lead to increased ISD and decreased CSR in C–NHEJ-deficient activated B cells that would have implications for A-EJ. One possibility would be that A-EJ might join DNA ends within the same S region more efficiently than joining ends from different S regions because the repetitive internal nature of individual S regions provides many more opportunities for MH annealing than would be found in two different S regions (Boboila, Jankovic, et al., 2010). Indeed, if degree of homology influences the choice of DNA repair pathway, joins within the same S region might be repaired preferentially by an MH-biased A-EJ pathway even in WT cells (Boboila, Jankovic, et al., 2010; Yan et al., 2007). It has also been noted that ISDs could result from either joining two DSBs within a given S region or via resection and joining of a single DSB within an S region (Boboila, Jankovic, et al., 2010). Thus, a related explanation for the frequent ISD events in C–NHEJ-deficient B cells would be that such ISDs reflect high levels of DSBs that persist

long enough to be extensively resected (Boboila, Jankovic, et al., 2010). In this scenario, the increase in ISD observed in C-NHEJ-deficient cells might reflect rejoining of single DSBs following extensive end resections and not an actual increase in joining DSBs within an S region. In WT cells, such DSB rejoining within an S region would also occur but be largely invisible, as current ISD assays only detect large deletions via Southern blotting. On the other hand, extensive resection could help reveal internal S-region MHs and further promote MH-mediated A-EJ (Bothmer et al., 2010). Finally, as efficient long-range end joining of DSBs between S regions is promoted by the ATM-dependent DSBR (Bothmer et al., 2011; Franco, Gostissa, et al., 2006; Reina-San-Martin, Chen, Nussenzweig, & Nussenzweig, 2007; Reina-San-Martin et al., 2003), a preference for ISD versus long-range CSR joining in the absence of C-NHEJ might occur if A-EJ was not efficiently recruited by the DSBR, a possibility that could also explain the putative predisposition of this pathway to catalyze translocations (Boboila, Jankovic, et al., 2010).

5.8. The role of A-EJ in the formation of chromosomal translocations

The notion that A-EJ mediates the majority of chromosomal translocations, potentially even in the presence of C-NHEJ, has received much attention. Translocation junctions in XRCC4-deficient pro-B cell tumors (Zhu et al., 2002), mature B cell tumors (Wang et al., 2008), splenic B cells (Wang, Gostissa, et al., 2009), or murine ES cells (Simsek & Jasin, 2010) predominantly (but not exclusively) display short MHs, suggesting that they are all mediated by MH-biased end-joining pathways. In addition, chromosomal translocation junctions in Ku70- or Ku70/Lig4-deficient mature B cells, Ku80-deficient CHO cells, and Ku70-deficient murine ES cells are also mediated through end joining and show a bias toward MH joining (Boboila, Jankovic, et al., 2010; Guirouilh-Barbat et al., 2004; Zhang & Jasin, 2011). Importantly, the finding of frequent chromosomal translocations in cells deficient for both Ku70 and Lig4 definitively demonstrates that an A-EJ pathway that is independent of C-NHEJ can catalyze translocations in primary cells (Boboila, Jankovic, et al., 2010). Translocation junctions isolated from a subset of human tumors provide evidence for extensive end processing, deletions, insertions, and frequent MH usage (Kitada & Yamasaki, 2007; Mattarucchi et al., 2008; reviewed by Zhang & Rowley, 2006). In addition, cellular extracts from late-stage, aggressive

bladder tumors and urothelial carcinoma cell lines (Bentley, Diggle, Harnden, Knowles, & Kiltie, 2004; Windhofer, Krause, Hader, Schulz, & Florl, 2008) repair DSBs predominantly through MH, suggesting that tumor cells might extensively utilize A–EJ for DSB repair, although the caveat remains that C–NHEJ can also use MH. The observation that I-SceI-mediated translocation junctions in WT cells have more MH than predicted by chance (Simsek & Jasin, 2010; Zhang & Jasin, 2011) is consistent with the notion that an MH-prone A–EJ pathway can mediate translocations in the presence of C–NHEJ, at least in some contexts (Simsek & Jasin, 2010). Recently, a high-throughput method was developed that allowed the identification and analysis of extremely large numbers of translocation junctions (tens of thousands) from activated primary B cells and revealed that a large fraction (75–90%) resulted from end-joining events that contained short MHs at the junctions (Chiarle et al., 2011). Extension of this approach to different DSB repair-deficient or DSBR-deficient backgrounds should give insight into potential roles for MHs in the context of different end-joining pathways on a genome-wide scale.

6. PERSPECTIVE

Over the past several years, significant advances have been made in elucidating DSB repair by C–NHEJ and A–EJ pathways. Thus far, however, experimental approaches used to study A–EJ have mostly relied on experimental reduction of candidate factors in C–NHEJ-deficient cells and the notion that any observed additional defects in DNA repair indicate a role of the candidate factor in A–EJ. However, such approaches are often hampered by the unavailability of cells deficient for both C–NHEJ and candidate factor or because single or combined deficiency causes proliferation defects or cell lethality. In this context, A–EJ candidate factors have also been knocked down or knocked out in C–NHEJ-proficient cells and differences in the nature of DSB repair junctions (i.e., decreases in MH usage or length, size of deletions, etc.) were used to argue for involvement of the candidate factor in A–EJ pathways. This particular approach can also be problematic because both C–NHEJ and A–EJ can utilize MH for joining, which makes it difficult to unequivocally determine the involvement of a given factor in A–EJ versus C–NHEJ. Going forward, additional approaches to fully elucidate A–EJ pathways might include defining what factors are found at DSBs in various types of C–NHEJ-deficient cells.

A key for elucidating the nature of A–EJ will be to elucidate the factors that recognize DSBs in preparation for joining by this pathway. Ku is considered a primary DSB recognition factor for DSBs in the context of C–NHEJ, and it also appears to influence how DSBs are joined in Lig4-deficient cells, indicating that it could function in some types of A–EJ. However, how are DSBs recognized for A–EJ in Ku-deficient cells? In the absence of Ku or other C–NHEJ factors, the ATM DSBR still generates foci at DSBs (Mills et al., 2004; Riballo et al., 2004; Wang, Ghosh, & Hendrickson, 2009). Repair foci formed by H2AX and other DSBR factors augment repair by attracting repair factors and by holding DSB ends in proximity, but much about the role of these repair foci and, in particular, how they carry out their functions in end joining is still unknown. Important questions remain regarding the interaction of the DSBR machinery with C–NHEJ factors, both at the genetic and the biochemical level, and whether the DSBR machinery could also be directly involved in A–EJ. An unanswered question is whether DSBR foci could contribute to holding DSB ends together during A–EJ in the combined absence of Ku and Lig4.

Another important question is how DSBs engage particular repair pathways. Are there factors that bind all DSBs, examine the nature of the DSB and type of damage, and then select the appropriate repair mechanisms by recruiting specific repair factors (e.g., Bothmer et al., 2010; Yun & Hiom, 2009)? It has been proposed that Ku and the MRN complex bind to DSBs and then recruit factors to carry out C–NHEJ, HR, or A–EJ (reviewed by Ciccia & Elledge, 2010; Neal & Meek, 2011). In this context, the choice of repair pathway would likely be affected by cell-cycle phase and the complement of available repair factors in a given cell type. For example, the cell lineage-specific phenotypes of XLF deficiency in the context of the repair of RAG-dependent DSBs illustrates that repair outcomes can be determined in a cell type-specific manner (Li et al., 2008). Moreover, whereas mature B cells can efficiently utilize A–EJ for CSR, developing lymphocytes cannot use A–EJ for V(D)J recombination because of RAG excluding A–EJ, and possibly other contributing factors such as cell-cycle restrictions, if, for example, A–EJ employed certain HR factors not expressed in G1. One interpretation of the finding that Lig4 or XRCC4 deficiency during neural development causes neuronal cell death is that A–EJ may not be available to contribute significantly to DSB repair in this cell lineage or at this developmental stage. However, it is also possible that newly generated neurons simply

have a very strong apoptosis-inducing checkpoint in response to persisting DSBs (Gao, Sun, et al., 1998). The specific role of Lig4/XRCC4 and the role of DNA repair during neuronal development clearly is an important unresolved question that warrants further investigation. In general, studies of DSB repair processes in various developmental stages and cell lineages may yield important novel insights into the various repair pathways.

One particularly intriguing issue is the apparently opposite role of A–EJ and C–NHEJ in the context of translocation formation. The ability of C–NHEJ to suppress translocations may involve its speculated recruitment by the DSBR complex, which is thought to stabilize the ends of spatially separated DSBs on the same chromosome to facilitate end joining (Boboila, Jankovic, et al., 2010; Zarrin et al., 2004; Zhang et al., 2012; reviewed by Bassing & Alt, 2004). In contrast, A–EJ may indeed be an error-prone pathway whose mutagenic potential and an intrinsic ability to potentiate interchromosomal over intrachromosomal joining only becomes unleashed in the absence of C–NHEJ. Thus, if certain types of cancer cells became more dependent on A–EJ (e.g., Tobin, Robert, Nagaria, et al., 2012; Tobin, Robert, Rapoport, et al., 2012), this pathway could potentially be an attractive target for cancer therapy. However, before such approaches can be considered, further studies of the mechanistic role of A–EJ in translocation formation are required, and ideally, factors with exclusive functions in A–EJ would need to be identified. It also should be noted that the increased frequency of unrepaired DSBs in the absence of a more robust C–NHEJ pathway would provide a much higher level of substrates for translocation formation by A–EJ (Boboila, Jankovic, et al., 2010; reviewed by Gostissa et al., 2011; Zhang et al., 2010). Thus, beyond any potential increase in the ability of A–EJ versus C–NHEJ to join DSBs interchromosomally, the overall increase in translocations in C–NHEJ-deficient cells could also reflect both the level of consequence of slower or less robust local repair and the persistence of DSB translocation substrates (Yan et al., 2007; reviewed by Gostissa et al., 2011).

A major question regarding A–EJ is the potential physiologic role of this type of repair if it has any. What is the purpose of A–EJ and why would it be needed in addition to C–NHEJ? Moreover, although several factors have been proposed to function in A–EJ, these are all factors already known to be involved in other DNA repair pathways such as HR and BER. Could this indicate that repair factors from different repair contexts work together in A–EJ and imply that there are no exclusive A–EJ factors? The identification

of putative A–EJ-specific factors, if they exist, would be the best way to begin to elucidate potential functions of A–EJ. It will be quite important to investigate in which cell-cycle phase A–EJ operates, as this could give additional insights into the nature of this repair process. Further characterization of A–EJ mechanisms and factors may also offer insights into the biology of CSR. In the context of CSR, increased IgE production is an important component in the pathogenesis of allergic diseases and asthma, and the notion that A–EJ may mediate some or many Sμ–Sε junctions in normal B cells is intriguing and could potentially provide new opportunities for allergy treatment. Finally, as deficiencies for C-NHEJ have not been frequently associated with human cancers, further elucidation of whether A–EJ functions robustly in C-NHEJ proficient cells will help elucidate whether this pathway is involved in the generation of translocations that can contribute to oncogenic transformation of C-NHEJ-proficient lymphoid and other types of cells.

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