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Mechanisms of double-strand break repair in somatic mammalian cells

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Abstract

DNA chromosomal DSBs (double-strand breaks) are potentially hazardous DNA lesions, and their accurate repair is essential for the successful maintenance and propagation of genetic information. Two major pathways have evolved to repair DSBs: HR (homologous recombination) and NHEJ (non-homologous end-joining). Depending on the context in which the break is encountered, HR and NHEJ may either compete or co-operate to fix DSBs in eukaryotic cells. Defects in either pathway are strongly associated with human disease, including immunodeficiency and cancer predisposition. Here we review the current knowledge of how NHEJ and HR are controlled in somatic mammalian cells, and discuss the role of the chromatin context in regulating each pathway. We also review evidence for both co-operation and competition between the two pathways.

Keywords

double-strand break (DSB); double-strand break repair (DSB repair); homologous recombination; mammalian DNA repair; non-homologous end-joining (NHEJ)

INTRODUCTION

Chromosomal DSBs (double-strand breaks) can result from either endogenous or exogenous sources. Naturally occurring DSBs are generated spontaneously during DNA synthesis when the replication fork encounters a damaged template and during certain specialized cellular processes, including V(D)J recombination, class-switch recombination at the immunoglobulin heavy chain (IgH) locus and meiosis. In addition, exposure of cells to ionizing radiation (X-rays and gamma rays), UV light, topoisomerase poisons or radiomimetic drugs can produce DSBs and other types of DNA damage [1]. The ends of a DSB may contain additional chemical modifications, potentially requiring processing prior to the engagement of canonical DSB repair enzymes.

Failure to repair DSBs, or their misrepair, may result in cell death or chromosomal rearrangements, including deletions and translocations. This chromosomal instability can promote carcinogenesis and accelerate aging. Two major pathways have evolved to repair DSBs and thereby suppress genomic instability. Repair by HR (homologous recombination) can be error-free, but requires the presence of a homologous template, such as a sister chromatid

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(reviewed in [2,3]). The NHEJ (non-homologous end-joining) pathway joins the two ends of a DSB through a process largely independent of homology. Depending on the specific sequences and chemical modifications generated at the DSB, NHEJ may be precise or mutagenic (reviewed in [4]). Inherited defects in NHEJ account for approx. 15% of human severe combined immunodeficiency [4], whereas inherited defects in HR contribute to a variety of human cancers [5] (Table 1).

NON-HOMOLOGOUS END-JOINING

NHEJ is an efficient DSB repair pathway in multicellular eukaryotes such as mice and humans. NHEJ provides a mechanism for the repair of DSBs throughout the cell cycle, but is of particular importance during G_0 -, G_1 - and early S-phase of mitotic cells [6,7]. Briefly, the Ku70/Ku80 (Ku) protein binds with high affinity to DNA termini in a structure-specific manner and can promote end alignment of the two DNA ends [8,9]. The DNA-bound Ku heterodimer recruits DNA-PK_{cs} (DNA-dependent protein kinase catalytic subunit), and activates its kinase function [10]. Together with the Artemis protein, DNA-PK_{cs} can stimulate processing of the DNA ends [11]. Finally, the XRCC4 (X-ray repair complementing defective repair in Chinese hamster cells 4)–DNA ligase IV complex, which does not form a stable complex with DNA but interacts stably with the Ku–DNA complex, carries out the ligation step to complete repair [12] (Figure 1). A great deal is known about the process of NHEJ; however, due to space constraints we can only cover a small portion of the relevant literature.

Recognition

NHEJ is initiated by the binding of a heterodimeric complex composed of Ku70 and Ku80 to both ends of the broken DNA molecule. Ku interacts with many proteins *in vitro*, including DNA-PK_{cs} [13] and the XRCC4–DNA ligase IV complex [14]. Association of Ku with DNA ends may serve as a scaffold for the assembly of the NHEJ synapse. The Ku–DNA complex recruits DNA-PK_{cs}, a 460 kDa member of the PIKKs (phosphoinositide 3-kinase-like family of protein kinases) [13]. Ku then moves inward on the DNA, allowing DNA-PK_{cs} to contact DNA [15]. The association of DNA-PK_{cs} with both DNA and Ku leads to activation of the serine/threonine kinase activity of DNA-PK_{cs} [10]. Inward translocation of Ku also allows two DNA-PK_{cs} molecules to interact across the DSB, forming a molecular 'bridge' or synapse between the two DNA ends [16].

In yeast, the MRX (MRE11-RAD50-XRS2) complex participates in both NHEJ and HR, and is one of the first complexes to interact with a DSB [17]. The orthologous MRN [Mre11– Rad50–Nbs1 (Nijmegen breakage syndrome 1)] complex in vertebrates has an established role in HR [18,19]. Recent evidence suggests that mammalian MRN is also involved in NHEJ [20,20a–20c]. The Rad50 protein contains a high-affinity DNA-binding domain and a two-cysteine Zn^{2+} -binding hook that may assist synapsis [21]. In yeast, this is in fact the case, as DSB ends remain associated after break induction only when the MRX complex is intact [22,23].

The Mre11 polypeptide exhibits an *in vitro* nuclease function, cleaning hairpin structures and 3' single-strand overhangs at the ss (single-stranded)/ds (double-stranded) DNA junction as well as harbouring a 3'-to-5' exonuclease activity [24,25]. This nucleolytic function of Mre11 is not essential in yeast, and the major function of the MRX complex in yeast NHEJ appears to be to tether DNA ends and recruit the ligase complex [24]. Interestingly, the nuclease function of Mre11 is essential in mammals, perhaps reflecting a critical role in HR [26]. Vertebrate DNA-PK_{cs} may function in parallel with MRN as a DNA end-bridging factor for NHEJ [16,23]. This partial redundancy may explain why, in yeast, where no orthologues of DNA-PK_{cs} have been identified, NHEJ is relatively inefficient.

DNA-PK_{cs} can phosphorylate a number of substrates *in vitro*, including Ku70, Ku80, XRCC4, XLF, Artemis and DNA ligase IV [27–31]. However, it is not clear to what extent these phosphorylation events are required for NHEJ *in vivo*. Indeed, the best candidate substrate for DNA-PK is DNA-PK_{cs} itself; a number of autophosphorylation sites in DNA-PK_{cs} have been identified and *in vivo* phosphorylation of DNA-PK_{cs} occurs in response to DNA damage [32–35]. The phosphorylation status of DNA-PK_{cs} is known to influence its conformation and dynamics, probably serving to relieve the blockage of the ends by DNA-PK_{cs}, thus allowing further processing of the DNA [36].

Processing

Since DSBs can occur with a variety of different ends, a number of processing enzymes may be required to repair breaks. Ends must be transformed to 5'-phosphorylated ligatable ends in order for repair to be completed. One key end-processing enzyme in mammalian NHEJ is Artemis, a member of the metallo- β -lactamase superfamily of enzymes, which may be recruited to DSBs through its ability to interact with DNA-PK_{cs} [11,37]. Artemis possesses both a DNA-PK_{cs}-independent 5'-to-3' exonuclease activity and a DNA-PK_{cs}-dependent endonuclease activity towards DNA-containing ds–ssDNA transitions and DNA hairpins, each of which might be important for processing of DNA termini during NHEJ [11,38]. Inactivation of Artemis results in radiation sensitivity; however, cells lacking Artemis do not have major defects in DSB repair, suggesting that only a subset of DNA lesions are repaired in an Artemis-dependent *in vivo* [39].

Processing of complex lesions might lead to the creation of DNA gaps that must be filled in by DNA polymerases to enable break repair. The DNA polymerase X family of polymerases, including polymerase μ , polymerase λ and terminal deoxyribonuc-leotidyltransferase, have been implicated in fulfilling this role during NHEJ (reviewed in [40]).

Resolution

NHEJ is completed by ligation of the DNA ends, a step that is carried out by X4–L4 (a complex containing XRCC4, DNA ligase IV and XLF) [12]. XRCC4 has no known enzymatic activity, but is required for both NHEJ and V(D)J recombination [41]. It forms a homodimer that acts as a scaffold, interacting with Ku [14] and DNA [42]. The ligase activity of DNA ligase IV is stimulated by both XRCC4 [43] and XLF [44]. DNA ligase IV can ligate blunt DNA ends as well as those with compatible overhangs [12]. X4–L4 has the unique ability to ligate one DNA strand independent of the other, which might allow processing enzymes to act concurrently with the ligation machinery [45]. In addition to this ability, the complex is able to ligate across gaps of several nucleotides and can ligate some incompatible DNA ends with short overhangs [46,47]. NHEJ occurs even in the absence of X4-L4, suggesting that another ligase can partially substitute for DNA ligase IV [48].

ALTERNATIVE PATHWAYS OF NHEJ

A loosely defined alternative end-joining pathway operates in the absence of classical NHEJ factors such as Ku, XRCC4 or DNA ligase IV. These repair events frequently involve small deletions and entail short stretches of homology at the break point [48–52]. This MMEJ (microhomology-mediated end-joining) pathway dominates during alternative end-joining. However, MMEJ and alternative end-joining are not synonymous, since error-free ligation can occur at low frequency in the absence of X4-L4 [51]. Furthermore, MMEJ accounts for a proportion of V(D)J recombination events in wild-type cells [53,54]. Notably, yeast lacking MRX reveal reduced repair by MMEJ, but the complete set of genes that participate in alternative NHEJ in mammalian cells is not yet known [49,52,55].

Analysis of immune development in mice lacking X4-L4 has shown that alternative end-joining is fairly robust [48,56,57]. MMEJ also appears to contribute to genomic instability in cancer. Translocation breakpoints in human cancers very often reveal microhomology, suggesting a role for MMEJ in translocation [58]. MMEJ may also facilitate chemotherapy resistance by genetic reversion in cells lacking wild-type *BRCA2* (breast-cancer susceptibility gene 2) [59]. In these cases, in-frame microhomologous deletions flanking the original mutation occurred in the resistant cells. The genetic requirements for MMEJ in cancer remain unclear.

SPECIALIZED FUNCTIONS OF NHEJ

The vertebrate immune system is characterized by intrinsic DSB production and repair as a mechanism of diversification of the B- and T-lymphocyte repertoire (reviewed in [60]). Core members of the NHEJ pathway perform direct roles in V(D)J recombination. For example, Ku-deficient cells [61,62] and DNA ligase IV-deficient cells [63] are defective in both coding and signal joint formation. Cells harbouring mutations in DNA-PK_{cs} are severely impaired in their ability to form coding joints, but show little or no defect in signal joint formation [64–66]. Artemis is also implicated in the formation of coding joints, but not signal joints [67,68].

In contrast with V(D)J recombination, multiple DNA repair pathways are likely to be involved in CSR (class switch recombination), including base excision repair, mismatch repair and NHEJ [69]. DNA sequences located between S (switch) regions can be detected in the form of excised circularized DNA, consistent with the involvement of DSB intermediates in CSR [70,71]. Sequences from CSR junctions show little or no sequence homology between donor and acceptor S regions, and often contain short deletions or insertions of nucleotides, all of which are consistent with DSB repair by NHEJ [72]. Further evidence from knockout mice also suggests a role for NHEJ in CSR. DNA-PK_{cs}-deficient mice have significantly reduced levels of serum Ig isotypes, and the only detectable isotype in Ku-deficient mice containing rearranged *IgH* and *IgL* genes is IgM [73–75].

NHEJ also plays a role in telomere biology (reviewed in [76]). The formation of dicentric chromosomes as a consequence of DNA end-joining is a hallmark of telomere dysfunction. NHEJ appears to play a central role in the formation of dicentric chromosomes in cells with telomere dysfunction, since fusion of uncapped telomeres is strictly DNA ligase IV-dependent [77]. In addition, Ku, DNA-PK_{cs} and the MRN complex participate in multiple facets of normal telomere biology. All three components of the MRN complex bind telomeres, and disruption of the MRN complex leads to telomere length deregulation [78,79]. The Ku heterodimer and DNA-PK_{cs} also play roles in the regulation of normal telomere length [78,80–83].

CHROMATIN RESPONSE IN NHEJ

Chromosomal DSBs in eukaryotes provoke a rapid and extensive response in chromatin flanking the break, highlighted by phosphorylation of histone H2AX in mammalian cells (γ H2AX), on C-terminal Ser¹³⁹. γ H2AX facilitates repair of the break by either HR or NHEJ [84–88]. Phosphorylated H2AX is detected within 1 min of damage [89,90]. The H2AX phosphorylation site, Ser¹³⁹, is a common recognition site for the PIKKs, and in principle, all three major PIKK members, ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia mutated- and Rad3-related) and DNA-PK_{cs}, have the potential to phosphorylate H2AX. There is evidence that each of these kinases can carry out this phosphorylation when the others are compromised, but ATM seems to be the main kinase associated with γ H2AX formation under normal physiological conditions [91–93].

The γ H2AX mark around a DSB may extend more than 1 Mb from the break [89,90,93]. In *Saccharomyces cerevisiae*, γ H2AX is present in a 40–50 kb region around an unrepairable DSB and the greatest enrichment of γ H2AX occurred 3–5 kb on either side of the break, with

 γ H2AX absent in sequences 1–2 kb on both sides of the DSB [94]. In mammalian cells, γ H2AX is bound by MDC1 (mediator of DNA damage checkpoint 1), which interacts constitutively with the MRN complex and thereby activates ATM [95–97]. The interaction of MDC1 and γ H2AX has therefore been proposed to amplify the γ H2AX signal [95,97,98]. However, recent chromatin immunoprecipitation analysis suggests a more nuanced picture, whereby MDC1 may reinforce an existing γ H2AX signal, but the extent of spread of the signal is not dependent upon MDC1 [93]. This recent work raises the possibility that the signal that generates the γ H2AX mark is diffusable.

A number of DNA damage response proteins, such as MDC1, the MRN complex, ATM, 53BP1 (p53-binding protein 1) and BRCA1/BARD1 [BRCA1-associated RING (really interesting new gene) domain 1], accumulate on γ H2AX-containing chro-matin. MDC1 is a critical adaptor protein that directly interacts with γ H2AX [95]. 53BP1, BRCA1 and MRN/ATM can also associate with DSBs in H2AX^{-/-} cells, suggesting *H2AX*-independent roles at the DSB [99]. The recruitment of 53BP1 and BRCA1 to γ H2AX chromatin is indirect, requiring the activity of the E3 ubiquitin ligases RNF8 (RING finger protein 8) [100–103] and RNF168 [104,105].

 γ H2AX accumulates in an AID (activation-induced cytidine deaminase)-dependent manner at the IgH locus in cells undergoing switching [87], and B-cells from *H2AX* null mice reveal defects in CSR [85,86]. H2AX is not required for switch targeting, initial lesion formation or end-processing during CSR, suggesting that γ H2AX affects the efficiency of repair itself [86, 106]. However, V(D)J recombination appears to be unaffected by *H2AX* deletion [85].

Studies of CSR at the IgH locus and of the fusion of dysfunctional telomeres have revealed quantitative roles for H2AX, MDC1 and 53BP1 in 'long-range' NHEJ [107,108]. These defects are more severe in 53BP1-null mice than in H2AX- or MDC1-null mice, but less severe than that observed in cells lacking classical NHEJ [109]. 53BP1 localizes rapidly to DSBs and colocalizes with IR (ionizing radiation)-induced γ H2AX nuclear foci, but can also accumulate in the absence of H2AX [99,110]. 53BP1-deficient mice are immunodeficient, predisposed to T-cell lymphomas, and reveal severely diminished CSR but normal V(D)J recombination [109,111–113]. 53BP1 has also been implicated in*XRCC4*-dependent NHEJ of a conventional DSB [114]. 53BP1 accumulation on γ H2AX-containing chromatin is mediated by interaction of the 53BP1 tandem Tudor domain with the exposed constitutive chromatin mark, H4K20me2 (histone H4 dimethylated at Lys²⁰) [115]. It is not yet clear whether the same 53BP1–H4K20me2 interaction mediates the H2AX-independent functions of 53BP1.

HOMOLOGOUS RECOMBINATION

Several distinct mechanisms of 'homology-directed repair' have been identified. In yeast, these include HR, SSA (single-strand annealing) and BIR (break-induced replication; reviewed in [2]). Whereas HR potentially results in conservative repair of a DSB, both SSA and BIR are mutagenic pathways. Early steps in HR are the resection of the DNA ends to yield 3'-ssDNA overhangs (Figure 2, pathway A), followed by Rad51-mediated homologous DNA pairing and strand exchange (Figure 2, pathway B). In SSA, a DSB in or near one of two direct repeats leads to the annealing of complementary strands from each repeated sequence, yielding a homologous deletion (Figure 2, pathway F). In contrast with HR, BIR in yeast requires lagging-strand synthesis and appears to be mediated by formation of a replication fork (Figure 2, pathway G) [116]. Consequently, BIR can involve extensive copying from the donor, leading to non-reciprocal translocations and other types of genomic instability [117,118]. Although BIR has been invoked to explain some examples of genomic instability in mammalian cells, direct evidence for a mammalian BIR pathway is lacking.

Recognition

The MRN complex plays a critical role in the early DSB response. MRN complexes on adjacent DNA ends are thought to associate via Rad50 homodimerization to connect the DNA ends prior to repair [21,23,119]. In post-replicative repair, the broken ends may also be kept in close proximity with the neighbouring sister chromatid. Genetic studies in yeast link the cohesin complex (SMC1/3) and the related SMC5/6 complex to HR by maintaining close association of sister chromatids (reviewed in [120,121]). In addition to its role in tethering DNA ends, the MRN complex also recruits and activates the catalytic function of the ATM protein kinase through direct interaction of ATM and Nbs1 [122,123]. ATM phosphorylates numerous substrates in the DNA damage response, including histone H2AX, making H2AX phosphorylation an early marker in chromatin of DSB formation [93,124].

Processing

HR requires processing of the DSB to yield ssDNA containing a 3'-hydroxyl overhang. Genetic evidence in yeast suggests that this end processing involves the MRX complex, as deletions of *MRE11*, *RAD50* and *XRS2* slow down the rate of 5'-to-3' exonuclease activity *in vivo* [125–127]. However, Mre11 possesses a 3'-to-5' ATP-independent exonuclease activity, rather than the 5'-to-3' exonuclease activity required for generation of ssDNA with a 3'-hydroxyl end [25,124a,124b].

Recent evidence in yeast paints a more complex picture of 5'-end resection. In *S. cerevisiae*, Sae2 interacts with the MRX complex, and these proteins collaborate to trim the DNA ends to an intermediate form [124c]. The DSB is then processed more extensively by either the 5'-to-3' exonuclease activity of Exo1 or by the Sgs1 helicase in conjunction with an as-yet unidentified single-strand specific nuclease [124d,125]. Sae2, Exo1 and Sgs1 each have orthologues in mammalian cells {CtIP [CTBP (C-terminus-binding protein of adenovirus E1A)-interacting protein], Exo1 and BLM (Bloom's syndrome protein) respectively}, suggesting a general mechanism for DSB processing in eukaryotes. Indeed, mammalian CtIP, in association with BRCA1, has been implicated in DSB end-processing [133,134].

ssDNA is rapidly bound by the ssDNA-binding protein RPA (replication protein A), which melts the DNA's secondary structure [3]. However, the DNA strand invasion and homology search steps of HR require formation of a nucleoprotein filament composed of multimers of the Rad51 recombinase bound to ssDNA. Since RPA binds more avidly to ssDNA than Rad51, additional activities are required to load Rad51 on to RPA-coated ssDNA and to displace RPA. In mammalian cells, a critical mediator complex appears to include BRCA1/BARD1 and BRCA2(FANCD1)/DSS1, probably bridged by the PALB2 (partner and localizer of BRCA2) (FANCN) polypeptide [135,136]. Although each of these protein complexes is required for the formation of IR-induced Rad51 nuclear foci, the direct Rad51-loading function is provided by BRCA2, which interacts directly with Rad51 [137–139]. Studies on the *Ustilago maydis* BRCA2 orthologue Brh2, and a polypeptide harbouring critical functional domains of the human BRCA2 [140,141].

Many proteins involved in Rad51 function are products of hereditary cancer predisposition genes (Table 1), implying that failure to adequately regulate HR, and the consequent genomic instability, plays a causal role in cancer. The critical role of HR in suppressing genomic instability is reflected in the early embryonic lethality of mice lacking *Rad51*, *BRCA1* or *BRCA2*. The abundance of chromatid-type errors observed in such mutant cells suggests that the major function of these genes is to control SCR (sister chromatid recombination) [142, 143].

Resolution

The Rad51 nucleoprotein filament then captures duplex DNA and searches for homology. Studies using bacterial RecA indicate that the homology search probably occurs by way of random collisions between the nucleoprotein filament and the duplex DNA, thereby testing segments of the dsDNA in an iterative fashion until homology is found [144]. Following synapsis, the invading strand sets up a D-loop intermediate, whereby the 3'-end primes DNA synthesis using the duplex DNA as a template. It is presently unclear which polymerase(s) mediate D-loop extension *in vivo*, but Pol η (DNA polymerase η) can perform this function *in vitro* [145].

If the DSBs have occurred during the S- or G₂-phases of the cell cycle, the homology search may capture the neighbouring intact sister chromatid as a repair template for potentially error-free repair [146,147]. Most somatic HR events in either yeast or mammalian cells entail copying only a short tract from the donor DNA molecule (STGC; 'short tract' gene conversion). A small proportion of HR events in mammalian cells entail LTGC ('long tract' gene conversion), in which several kilobases are copied from the donor [147,148]. Mutation of the Rad51 paralogues, *XRCC3*, *Rad51C* or *XRCC2*, skews HR in favour of LTGC [149–151]. However, it is not clear whether STGC and LTGC represent different outcomes of a common HR mechanism, or whether LTGC is the product of a distinct mechanism, such as BIR. Thus far, the longest gene conversions identified in mammalian cells are <10 kb [152]; much less than the hundreds of kilobases that can be copied during BIR in yeast [117].

Strand invasion into a homologous sequence forms a D-loop intermediate and the 3'-end of the invading strand is extended by a polymerase. If the D-loop captures the second end of the break, the HJs (Holliday junctions) formed could yield crossover or non-crossover products (Figure 2, pathway E). However, crossing over is rare during somatic HR [147,150,153]. The SDSA (synthesis-dependent strand annealing) model was advanced to explain this fact. One SDSA model proposes that, following extension by 'bubble migration' (i.e. a minimal migrating D-loop), the invading strand is displaced and pairs (i.e. anneals) with the processed second end of the break (Figure 2, pathway C). In contrast, the 'double-strand break repair' model posits an extended D-loop, which captures the second end of the break, leading to the formation of double HJs (Figure 2, pathway D). Although it seems likely that SDSA is the major HR mechanism in somatic mammalian cells, double HJs probably arise during other recombination processes, such as daughter strand gap repair. HJ resolution is therefore relevant to somatic HR and genomic instability.

Once a HJ has been formed, it is able to undergo branch migration along DNA, generating increasing or decreasing lengths of heteroduplex DNA depending on the direction of junction travel (reviewed in [154]). Specialized enzymes in prokaryotes promote branch migration, and human Rad54 shows a strong preference for binding to branched substrates that resemble one end of a D-loop and can promote branch migration in either the 3' -to-5' or 5'-to-3' direction in an ATP-dependent manner [155]. Mammalian homologues of the *Escherichia coli* RecQ helicase, namely WRN (Werner's syndrome protein), BLM and RECQ5β, can catalyse branch migration, but disrupt HJs and show anti-recombinogenic characteristics *in vitro* [156].

The resolution of a HJ is probably executed by several distinct enzyme complexes. The product of the Bloom's syndrome gene, BLM, in complex with topoisomerase IIIa can dissolve double HJs to form non-crossover products [157]. Alternatively, the MUS81-EME1 complex may cleave HJs to produce crossovers with an exchange of flanking markers [158,159]. Recently, another HJ resolvase was identified in human cells, GEN1, which promotes junction resolution by a symmetrical cleavage mechanism that would be expected to give rise to crossovers and non-crossovers with equal efficiency [160]. There may also be other HJ resolvase activities

yet to be identified. In this regard, four recent papers have demonstrated a role for SLX4 in HJ resolution in higher eukaryotes [124a–124d].

SPECIALIZED FUNCTIONS OF HR IN SOMATIC CELLS

During DNA replication, a lesion encountered on one of the parental strands may cause the DNA polymerase complex to stall, potentially collapsing the replication fork. Arrested forks may be processed to form a DSB or replication may be reinitiated downstream of the lesion, leaving a ssDNA lesion that cannot be filled in due to the presence of the blocking lesion. These so-called DSGs (daughter strand gaps) could be repaired via sister chromatid recombination in an error-free manner (reviewed in [161]). Studies in the fission yeast *Schizosaccharomyces pombe* revealed that a replication fork barrier is capable of promoting recombination and chromosomal rearrangements at that locus [162]. Treatment of cells with HU (hydroxyurea) induces replication fork arrest, generating tracts of ssDNA on the lagging strand, but generating few DSBs in normal cells [163]. Structurally, HU-induced ssDNA may resemble DSGs and the accumulation of BRCA1, BRCA2 and Rad51 at sites of replication arrest in HU-treated cells suggests a probable role for these proteins in mediating repair of DSGs at stalled replication forks [164,165].

Cells maintain telomeric DNA repeats at a critical length that allows the assembly of 'T-loop' structures that protect the chromosome ends. Telomeric capping sequesters the 3' telomeric tail away from DNA damage sensors and processing activities within the cell (reviewed in [166]). Maintenance of telomere length normally requires telomerase, but this protein is non-essential in cells, indicating the existence of an alternative mechanism for telomere length maintenance. *S. cerevisiae* cells lacking telomerase gradually lose their telomeres and die, but rare survivors maintain telomeres through Rad52-dependent HR [167,168]. In these cases, telomere elongation can occur through BIR or gene conversion in which one telomere serves as a template for elongation of another [169,170].

In mammalian cells, the existence of an ALT (alternative lengthening of telomeres) pathway has been shown in cells lacking telomerase (reviewed in [171]). These cells exhibit sub-nuclear compartments containing telomeric DNA, telomere-binding proteins, recombination proteins such as Rad51 and Rad52, the MRN complex, RPA, and the WRN and BLM helicases [172]. In ALT cells, telomeric DNA is copied to other telomeres by means of HR and copy switching [173]. In support of this, Rad51D and Rad54 were reported to act at telomeres [174,175].

CHROMATIN RESPONSE IN HR

H2AX contributes to HR and SCR in a manner dependent upon the ability of H2AX to be phosphorylated on Ser¹³⁹, and upon the ability of γ H2AX to interact with MDC1 [88,95, 114]. Consistent with its role as a critical γ H2AX adaptor, MDC1 mediates *H2AX*-dependent HR, but the mechanisms responsible for this are not known. Possible mediators include RNF8 [100–102], MRN and BRCA1/BARD1 [96,98,176]; however, genetic analysis revealed that a *MDC1* mutant lacking the domain required for recruitment of MRN, RNF8, BRCA1 or 53BP1 to chromatin nonetheless retains HR function [114]. This separation of function is underscored by the fact that the major HR function of BRCA1 is independent of *H2AX* and, indeed, independent of the E3 ubiquitin ligase activity of BRCA1 itself [88,177].

BRCA1 exists in a number of distinct complexes in mammalian cells. The BRCA1–BARD1– Abraxas–MERIT40– Rap80 complex is recruited to IR-induced foci in a manner dependent upon the UIM (ubiquitin-interacting motif) of Rap80 [103,178–180]. Lys⁶³-linked polyubiquitin chains appear to be involved in DNA damage signalling, and recent studies have identified RNF8, a RING domain-containing E3 ubiquitin ligase as a key enzyme for this modification at DSBs [100–103]. RNF8 is also a direct binding partner of the MDC1 SQ-rich

domain and mediator of both 53BP1 and BRCA1 recruitment to chromatin [100–103]. RNF8 probably ubiquitinates H2A via a second E3 ubiquitin ligase, RNF168, to reinforce BRCA1 recruitment via Rap80 and 53BP1 through an uncharacterized mechanism [104,105].

COMPETITION BETWEEN NHEJ AND HR

Cells lacking classical NHEJ genes reveal a DSB repair bias in favour of HR, suggesting that these two major pathways normally compete to repair DSBs [181]. During V(D)J recombination, the RAG proteins play a role in specifying the preference for repair by NHEJ in these cells [182]. However, other rules must apply for unscheduled DSBs. The balance between NHEJ and HR shifts during the cell cycle, presumably reflecting the availability of a sister chromatid synthesized during S-phase [146,147]. A study in chicken DT40 cells deficient in NHEJ or HR factors revealed that NHEJ mutants were highly sensitive to IR in the G₁- and early S-phase of the cell cycle, whereas HR mutants were sensitive primarily in the S-/G₂- phase [6]. Similar studies in mammalian cells demonstrated that NHEJ-deficient cells have reduced repair at all cell cycle stages, whereas HR-deficient cells have a minor defect in G₁, but a greater impairment in S-/G₂-/M-phase [183,184].

Recent evidence suggests that the shift from NHEJ to HR as the cell cycle progresses is actively regulated. Analysis of end resection at an HO-induced DSB at *MAT* in yeast revealed that G_1 -arrested cells failed to initiate efficient end resection, which prevented loading of RPA and Rad51, and blocked Mec1/ATR activation [185]. This effect correlated with low levels of activity of the major cyclin-dependent kinase, CDK1 (cyclin-dependent kinase 1)/Cdc28 and, critically, inhibition of CDK1 activity in G_2 -phase prevented end resection and checkpoint activation. Under these conditions, Mre11 persists at the DSB site, consistent with the idea that processing of the break has stalled. This suggests that CDK1 controls Mre11-associated nuclease function at a DSB, but not the recruitment of Mre11 to DNA ends. Cdk activity also regulates HR in fission yeast [186].

Sae2 controls the initiation of DNA end resection in both meiotic and mitotic yeast and is itself a DNA endonuclease [130,187]. Recently, Sae2-mediated control of DSB resection in yeast was shown to depend on its CDK phosphorylation status [188]. Mutation of Sae2 Ser²⁶⁷ to a non-phosphorylatable residue (S267A) causes an end-processing phenotype comparable with deletion of Sae2 [188]. In contrast, a S267E mutant that mimics constitutive phosphorylation complements these phenotypes and overcomes the need for CDK activity in DSB end resection. The Sae2-null and S267A mutants show delayed HR and enhanced NHEJ, whereas the S267E mutant showed slightly enhanced recombination and a decrease in NHEJ efficiency. Thus CDK1/Cdc28-mediated phosphorylation of Sae2 modulates the balance between NHEJ and HR during the cell cycle. These results support a model in which the commitment to DSB end resection is regulated to ensure that a cell engages the most appropriate DSB repair pathway to optimize genome stability (Figure 3A). The motif encompassing Ser²⁶⁷ of Sae2 is highly conserved amongst orthologues in higher eukaryotes, and mutation of the analogous residue in human CtIP also produces hypersensitivity to camptothecin [188]. These results suggest that similar CDK control of DNA end resection operates in other organisms. CtIP is one of several proteins that interact with the BRCA1 C-terminal tandem BRCT repeat, and this interaction is important for efficient end resection. This, in turn, suggests that the BRCA1-CtIP interaction influences the balance between HR and NHEJ [134].

CO-OPERATION BETWEEN NHEJ AND HR

At the organismal level, HR and NHEJ clearly collaborate to suppress genomic instability. However, HR and NHEJ may also collaborate more directly to repair a subset of mammalian DSBs. This was revealed in work on a subpathway of HR termed LTGC. The majority of recombination events resolve after only a few hundred base pairs have been copied from the

donor. However, a small proportion of HR events involve more extensive copying from the donor, generating a LTGC spanning several kilobases [147–149,150,189]. Some of these LTGC events terminate without homology, presumably by use of NHEJ [147] (Figure 4). In a study of interchromosomal recombination, direct sequencing of the joints demonstrated that repair had occurred by NHEJ, with microhomology observed at approximately half of the junctions [189]. The genes involved in this coupled mechanism remain to be identified. Work from our laboratory suggests that both *XRCC4*-dependent and *XRCC4*-independent NHEJ pathways are capable of terminating LTGC in mammalian cells (A.J. Hartlerode and R. Scully, unpublished work). Therefore, both classical NHEJ and MMEJ may participate in resolving LTGCs.

Coupling of HR and NHEJ has also been observed in a less direct manner during other forms of recombination. In a study of ectopic recombination in *S. cerevisiae* between two unlinked homologous loci, a novel class of gene conversion events was observed that included extensive lengths of non-homologous sequence [190]. Co-operation between HR and NHEJ has also been deduced in some gene targeting events [191–193]. In *Drosophila melanogaster*, examples of incomplete LTGC events have been identified where repair is completed by an end-joining pathway that is independent of DNA ligase IV [194].

CONCLUSIONS

Significant progress in understanding the regulation of DSB repair in mammalian cells has been made in recent years. However, it is clear that much remains to be understood about these repair pathways and the complex interactions between them. Discoveries made in yeast have greatly advanced the understanding of both HR and NHEJ; however, the relationship between DSB repair pathways in yeast and higher eukaryotes is not always clearcut. Many protein complexes involved in DSB repair appear to function in more than one pathway. This highlights the need for more sophisticated tools to simultaneously examine HR and NHEJ in mammalian cells. Elucidation of these important disease-associated DSB repair functions may reveal new therapeutic targets in cancer and other disease states.

Abbreviations used

53BP1	p53-binding protein 1				
ALT	alternative lengthening of telomeres				
ATM	ataxia telangiectasia mutated				
ATR	ataxia telangiectasia mutated- and Rad3-related				
BARD1	BRCA1-associated RING domain 1				
BIR	break-induced replication				
BRCA	breast-cancer susceptibility gene				
CDK	cyclin-dependent kinase				
CSR	class switch recombination				
CtIP	CTBP (C-terminus-binding protein of adenovirus E1A)-interacting protein				
DNA-PK _{cs}	DNA-dependent protein kinase catalytic subunit				
DSB	double-strand break				
dsDNA	double-stranded DNA				

DSG	daughter strand gap			
HJ	Holliday junction			
HR	homologous recombination			
HU	hydroxyurea			
IR	ionizing radiation			
Ku	Ku70/Ku80			
LTGC	'long tract' gene conversion			
MDC1	mediator of DNA damage checkpoint 1			
MMEJ	microhomology-mediated end-joining			
MRN complex	Mre11–Rad50–NBS1 complex			
MRX complex	MRE11–RAD50–XRS2 complex			
NBS1	Nijmegen breakage syndrome 1			
NHEJ	non-homologous end-joining			
PALB2	partner and localizer of BRCA2			
PIKK	phosphoinositide 3-kinase-like family of protein kinase			
RING	really interesting new gene			
RNF	RING finger protein			
RPA	replication protein A			
S region	switch region			
SCR	sister chromatid recombination			
SDSA	synthesis-dependent strand annealing			
SSA	single-strand annealing			
ssDNA	single-stranded DNA			
STGC	'short tract' gene conversion			
X4-L4	complex containing XRCC4, DNA ligase IV and XLF			
XRCC	X-ray repair complementing defective repair in Chinese hamster cells			

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Figure 1. NHEJ in mammalian cells

Induction of a DSB forms DNA ends that are bound by the Ku heterodimer. Ku translocates inwards, allowing recruitment of DNA-PK_{cs} to the DNA termini. The two DNA-PK_{cs} molecules can then interact to tether the DSB ends together. Synapsis of DNA-PK_{cs} triggers phosphorylation of DNA-PK_{cs} [including autophosphorylation (autophos.)], altering the conformation and dynamics of DNA-PK_{cs}. Phosphorylation of DNA-PK_{cs} allows for recruitment of Artemis and other end-processing factors such as Pol X (DNA polymerare X) family members to generate the proper DNA ends required for ligation. Once the ends are processed, the X4-L4 complex, along with XLF, ligates the ends, repairing the break.



Figure 2. Homology-directed repair in eukaryotic cells

(A) Induction of a DSB is recognized by the MRN complex, which tethers the DNA ends together and participates in end processing. The CtIP–BRCA1–BARD1 complex co-operates with the MRN complex to aid in end resection. ssDNA is initially bound by the ssDNA-binding protein RPA to keep the ssDNA from forming secondary structures. BRCA1/BARD1 promotes accumulation of BRCA2 via PALB2. (B) BRCA2 catalyses the nucleation of Rad51 on to the free 5' end of a dsDNA–ssDNA junction. Once the Rad51 filament is assembled it captures duplex DNA and searches for homology. (C) The SDSA model predicts that a migrating D loop fails to capture the second DNA end and, following extension, the invading strand is displaced and anneals with the resected second end. (D) The DSB repair model predicts that

the second DNA end is captured by annealing to the extended D loop, forming two HJs. (E) The double HJ structure is then resolved to yield either crossover or non-crossover products. (F) The SSA pathway: a break near one of two direct repeat sequences leads to annealing of complementary strands from each repeated sequence. The product of this repair event contains a single copy of the repeat with a deletion of the intervening sequences. (G) BIR occurs when the 3' end of the invading strand leads to the formation of a replication fork, potentially copying long tracts from the donor DNA molecule. Dotted arrows indicate new DNA synthesis.



Figure 3. Relationships between HR and NHEJ in mammalian cells

(A) One of the early 'choices' in DSB repair is the extent to which the DNA ends are processed. In classical NHEJ, end resection may be minimal or absent. Should the ends be processed to yield a 3' overhang, repair can occur through either HR, SSA or MMEJ. (B) Defects in NHEJ skew DSB repair in favour of HR.



Figure 4. Model for termination of LTGC

In many LTGC events, gene conversion is thought to be terminated by homologous pairing. However, in a proportion of events, gene conversion is terminated without the use of homology, by NHEJ/MMEJ. This type of termination might result in chromosomal translocations.

Table 1 Disease and cancer incidence associated with impaired DSB repair

A summary of some human hereditary cancer predisposition syndromes that are known to be associated with germ-line mutation of individual DSB repair genes. The chromosome location is the location of the human gene on the chromosome. AD, autosomal dominant; AR, autosomal recessive; EBV, Epstein–Barr virus.

Gene	Inheritance	Chromosome location	Disease association	Cancer predisposition
Ku70	AR	22q11-13		T-cell lymphomas
Ku80	AR	2q35		Pro-B cell lymphomas
Artemis	AR	10p	Severe combined immunodeficiency	EBV-associated lymphomas
DNA ligase IV	AR	13q22-24	Ligase IV (LIG4) syndrome	Lymphoid malignancies
Cernunnos (XLF)	AR	2q35	Severe combined immunodeficiency	
BRCA1	AD	17q21	Hereditary breast cancer	Breast and ovarian cancer
BRCA2 (FANCD1)	AD (AR)	13q12.3	Hereditary breast cancer (Fanconi anaemia)	Breast and ovarian cancer
Mre11	AR	11q21	Ataxia telangiectasia-like disorder	
Rad50	AR	5q31		Slightly elevated breast cancer risk
Nbs1	AR	8q21	Nijmegen breakage syndrome	Haematologic malignancies
ATM	AR	11q22.3	Ataxia telangiectasia	Haematologic malignancies
ATR	AR	3q22-24	Seckel syndrome	
FANCD2	AR	3p25.3	Fanconi anaemia	Acute myeloid leukaemia, squamous cell carcinoma
BLM	AR	15q26.1	Bloom's syndrome	Broad spectrum (leukaemia, lymphoma, carcinoma)
PALB2 (FANCN)	AR	16p12	Fanconi anaemia	Breast and ovarian cancer
BRIP1 (BACH1/FANCJ)	AR	17q22	Fanconi anaemia	Breast cancer
CHEK2	AR	22q12.1	Li-Fraumeni syndrome 2	Breast cancer, sarcomas, brain tumours
WRN	AR	8p12-11.2	Werner's syndrome	Sarcomas