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Mechanisms of eukaryotic DNA double strand break repair

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TABLE OF CONTENTS

1. ABSTRACT

For all cells, a DNA double strand break (DSB) is a dangerous lesion that can have profound consequences for the genome. If a DSB is encountered during mitosis, chromosomal separation may be adversely affected. Alternatively, during S phase a DSB may cause replication fork stalling or collapse. Improperly repaired DSBs can result in chromosomal rearrangements, senescence or activation of apoptotic pathways. Cells have developed sophisticated recombination pathways to metabolize and repair DSBs quickly as well as the capacity to differentiate physiologically occurring breaks from life threatening lesions. The two major pathways of recombination repair are known as non-homologous end-joining (NHEJ) and homologous recombination (HR). In this review, we will discuss the detection, response, and repair of DSBs in eukaryotes.

2. INTRODUCTION

Maintaining genomic stability is essential for cellular survival. Throughout the lifetime of a cell, DNA is continually exposed to a variety of damaging factors. Double strand breaks (DSBs) are among the most serious and difficult to repair DNA lesions due to the lack of an undamaged complementary strand. DSBs that are undetected or misrepaired can cause chromosomal aberrations and threaten cellular survival. Mutations in many of the genes involved in DSB detection and repair manifest severe phenotypes such as radiosensitivity, immunodeficiency, cancer predisposition and premature aging. Two highly conserved recombination pathways have evolved to repair DSBs, homologous recombination (HR) and non-homologous end-joining (NHEJ). HR is an error-free mechanism of repair that uses a homologous sequence as a template. The homologous sequence generally used is

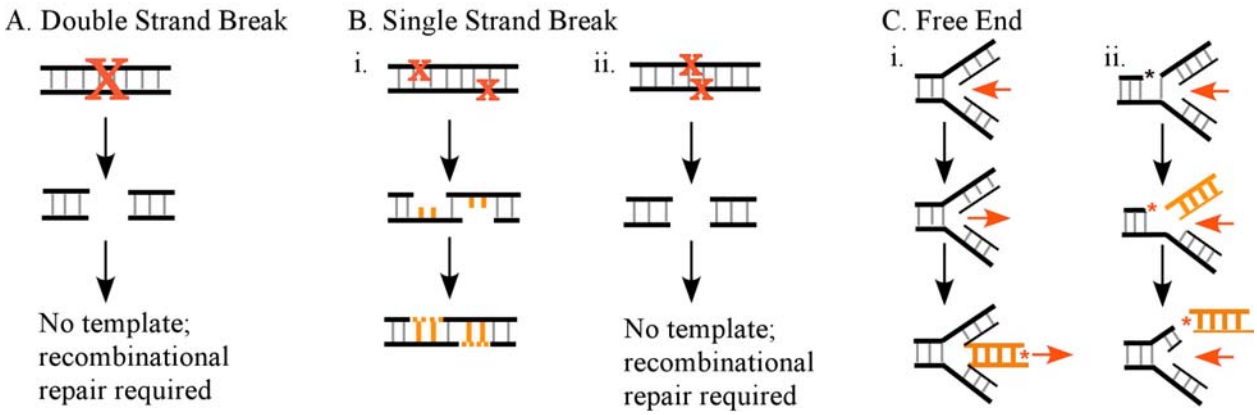

Figure 1. Defining Double Stranded Breaks. A. DSBs are created when both strands have been broken resulting in the absence of a template for repair. The large red X indicates a break on both strands. B. Each red x indicates a ssb found on separate strands. (i). SSBs can occur far enough apart, such that a template (orange) can be used to prime DNA synthesis. (ii). SSBs on separate strands that occur close enough will result in a DSB. C. A free end is defined as one half of a DSB (orange DNA with asterisk). The red arrow represents the direction of the replication fork. (i). Free ends arise during replication fork reversal. (ii). Free ends can also arise if replication proceeds through an unrepaired SSB which is represented by the black asterisk.

the sister chromatid, linking this repair pathway to replication. For the most part, HR is limited to replicating cells in the S, G2, or M phase of the cell cycle. Consequently, differentiated cells in higher vertebrates rarely use HR repair. In contrast to HR, NHEJ is an error prone mechanism. But despite this, NHEJ plays a significant role in mammalian DSB repair. The high conservation of these pathways in all organisms and the preference of an error prone mechanism over no repair indicate the potential threat of these lesions and the significance of efficient repair mechanisms. In this review we will focus on how the cells detect and respond to DNA DSBs in mammalian cells.

3. DEFINING DOUBLE STRAND BREAKS

Cellular DNA is constantly exposed to internal and external damaging agents. These agents can attack not only the nitrogenous bases, but also the sugar phosphate backbone (1). Breaks that occur on only one DNA strand (known as a single strand break, SSB) or base damage, which include individual and multiple bases, can be rapidly repaired. In these repair pathways, the intact strand opposite the damage can act as a template to prime DNA synthesis (reviewed in (1). A double strand break requires a break in the sugar phosphate backbone of each of the two strands in duplex DNA (Figure 1A). If the breaks in each strand are sufficiently far apart so that the strands remain attached through base pairing, they will be repaired as separate single stranded breaks (2) (Figure 1Bi). However, if the single stranded breaks in the complementary strand are close together, the ends will dissociate from one another, resulting in a DSB (2) (Figure 1Bii). A free DNA end can also be considered as a DSB. Free ends can develop during S phase as a result of replication fork reversal or progression through a nick in the DNA (3, 4) (Figure 1Ci and 1Cii, respectively). Because the DNA ends can become physically detached, a DSB represents a very serious threat to genomic integrity. Recombinational repair is necessary to resolve these genome destabilizing structures.

4. THE CAUSE AND EFFECTS OF DOUBLE STRAND BREAKS

Just one DSB can ultimately lead to cell death or carcinogenesis if improperly repaired (5). And yet, these events arise frequently. These lesions can arise from common environmental sources and natural cellular processes (Figure 2). Exogenous sources of DSBs include ionizing radiation (IR) and chemical toxins. The major sources of IR exposure come from medical x-rays and the decay of radon gas found in the soil (6-9). Ionizing radiation damages DNA directly and indirectly. Radiation can directly ionize DNA molecules, such that DNA is the initial target. Indirect affects are a result from the ionization of molecules in close proximity to DNA. In particular, the hydrolysis of water produces highly reactive free radicals that interact with the DNA molecule. These free radicals can assault the double helix causing multiple base and strand damage (10-12). Some of the chemical toxins that create DSBs include radiomimetic drugs that are commonly used to treat cancer, for example, the antibiotic, bleomycin (8, 13). All of these DNA damaging agents are harmful to the cell due to the generation of highly reactive oxidative free radicals. High enough doses can create enough radicals that produce a cluster of damage within a few base pairs along the DNA (10-12). If these multiple lesions are within a few helical turns of DNA, the clusters of damage that result from ionizing radiation can result in a DSB (2, 14). Topoisomerase inhibitors, such as camptothecin, can also create DSBs. Rather than generating reactive oxidative species, these compounds trap an enzyme-DNA intermediate and inhibit religation of the DNA. By inhibiting the religation event, a single strand break is generated (15). In non-dividing cells, the single strand breaks can quickly be repaired, however, in replicating cells, the progression of the replicating fork

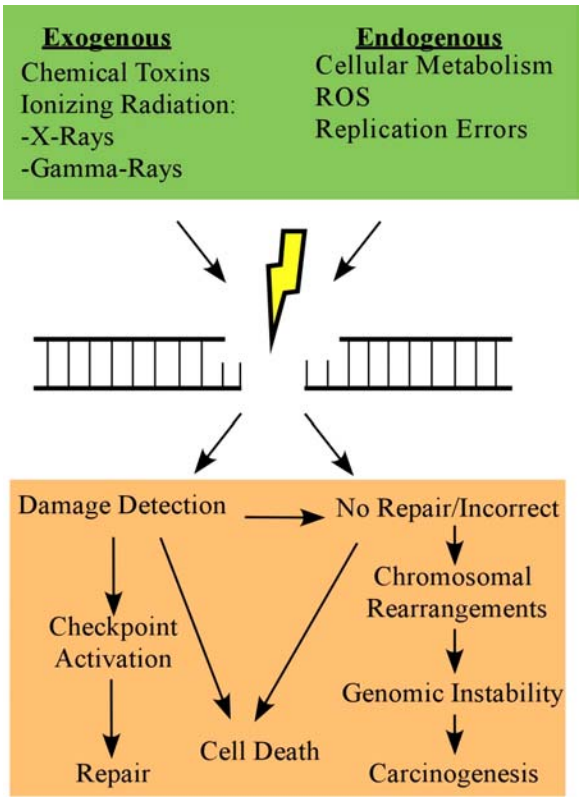

Figure 2. Cause and Effects of Double Strand Breaks. The factors involved in creating DSBs are found in the green boxes. The downstream events that occur as a result of DSBs are found in the orange box.

through the nick generates a DSB (Figure 1Cii). Because the induction of these DSBs is dependent upon replication, drugs that inhibit topoisomerase activity are also used to treat cancer.

DSBs can also arise from endogenous factors such as oxidative metabolism as well as errors in DNA replication (1, 4, 16, 17). Similar to IR, products of cellular metabolism include reactive oxygen species that are capable of assaulting the DNA to generate nicks and DSBs (18, 19). During chromosomal replication, fork progression through an unrepaired nick can potentially generate DSBs (Figure 1Cii) (3, 4, 16). Similarly, if the replication machinery encounters a bulky adduct it may cause reversal of the replication fork, generating a branched intermediate that must be cleaved and repaired by recombination proteins to reset a new replication fork (Figure 1Ci) (17).

Consequently, cells have evolved several protective responses to these potentially devastating lesions (Figure 2). Upon damage detection, dividing cells can activate cell cycle checkpoints to arrest cell growth until the damage is repaired. Details of cell cycle checkpoints have been recently reviewed in (20, 21). Additionally, detection of these breaks activates and recruits repair proteins (21-23). Once the repair pathways have restored the integrity of the DNA, the cell can resume replication. If the extent of damage outweighs the success of repair, the cell can induce apoptosis or a state of replicative death known as senescence (5).

Persistent DSBs or inaccurate repair can have profound effects. The incorrect rejoining of broken ends can produce aberrant chromosomal rearrangements resulting in cell death or chromosomal instability that can lead to carcinogenesis. These rearrangements include reciprocal translocations, insertions, duplications, and deletions (1, 24). Large chromosomal deletions can lead to the loss of tumor suppressor genes or to the activation of proto-oncogenes, resulting in carcinogenesis (24, 25).

Paradoxically, some breaks are intentionally generated and are required intermediates for cellular activities. These activities include meiotic recombination and lymphocyte development. An important aspect of meiosis is DNA strand exchange to generate genetic diversity with DSBs being required to initiate this recombination (26). DSBs are also required during the development of T and B lymphocytes to generate antigen receptor and immunoglobulin, respectively, diversities necessary for vertebrate immune system function (reviewed in (27).

5. DSB DETECTION AND RESPONSE

The harmful effects of just one DSB emphasize the importance of a sensitive damage detection system and rapid response. There are significant obstacles that need to be addressed for efficient repair. First, sensing the break must be both selective and extremely sensitive. Not only must the cells be able to detect a single DSB, but they must also differentiate between a threatening lesion and the common intermediates of normal DNA metabolism (i.e. replication intermediates, meiotic recombination, V(D)J recombination, and telomeres). Upon sensing the break, chromatin modification is also important. DNA is normally packaged with histones that may block the numerous proteins that are found to associate at the sites of damage (28). Lastly, it is essential that the signal that detects the break can be rapidly amplified in order to induce the global cellular functions that are involved in the response. Some of these cellular functions include cell cycle control, transcriptional regulation and/or post-translational modification of repair proteins, and apoptosis, if necessary.

We have only just begun to gain insights into how these breaks are initially sensed. Recently, an elegant experiment using marked DSBs and fluorescently labeled repair proteins, allowed the authors to visualize the cellular events that take place in a live cell in response to IR (29). The evidence indicated that the Mre11 complex was detected first at the sites of DSBs. The multi-subunit Mre11 complex composed of Mre11, Rad50, and Nbs1 in higher organisms or Xrs2 in *Saccharomyces cerevisiae*, (MRN or MRX, respectively) has been implicated in playing a role in numerous cellular responses to DSBs. Some of these responses include detection, activation of the

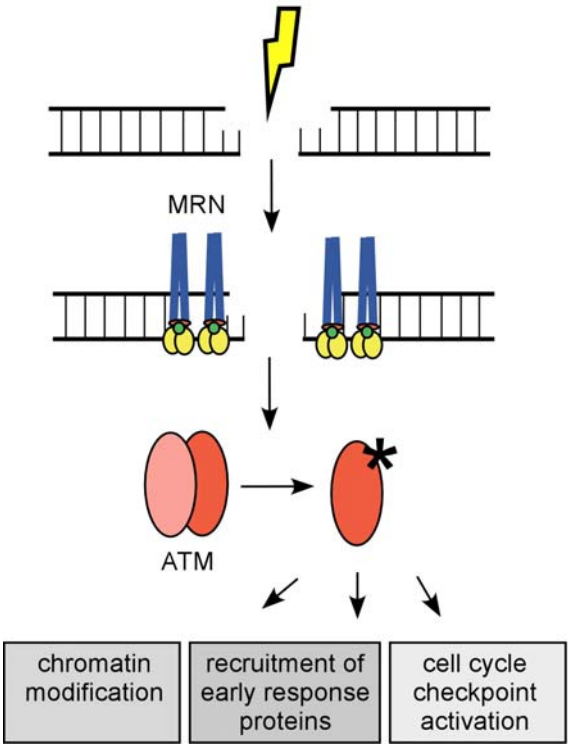

Figure 3. ATM Activation is Critical for DNA Damage Response. The initial sensing of the break may be first detected by the Mre11 complex found at sites of the break (MRN, shown in blue and yellow). MRN is required for ATM activation. Upon DSB detection, the inactive ATM dimer is dissociated and becomes activated (inactive dimer, red ovals). The activated, phosphorylated ATM (red oval with asterisk) can phosphorylate its numerous substrates to activate and recruit proteins involved in the damage response system (grey boxes).

ataxia telangiectasia mutated (ATM) kinase, end processing, repair, telomere maintenance and DSB-induced cell cycle checkpoint activation (Figure 3) (reviewed in (30-32). Rad50 and Mre11 are highly conserved throughout all kingdoms and the disruption of the MRN complex in mice results in embryonic lethality (33, 34). The significant role of this complex and its involvement at an early step on DSBR make it a candidate for being the DNA damage detector, but the exact mechanism remains to be elucidated.

Chromatin modification is another crucial aspect of DSB detection and response. Eukaryotic DNA is packaged such that DNA is wrapped around nucleosomes made of a histone octomer. This structure may make the DNA inaccessible to the replication, repair, and transcriptional machinery (35, 36). Following damage, chromatin is modified to allow access of the repair proteins to the damage site. The covalent modifications usually occur on the exposed tails of the histone octomer. These modifications which can include acetylation, phosphorylation and methylation, act as surface markers that can be recognized by nonhistone proteins that can further alter the state of chromatin leading to a variety of downstream events (36). One example is the INO80 complex, an ATP-dependent chromatin remodeling complex. Through chromatin immunoprecipitation, INO80 has been shown to be recruited to DSBs within 30-60 minutes of the induction of a single DSB (37, 38). This recruitment is dependent upon the damage induced phosphorylation of histone, H2AX (37, 38). It is speculated that the recruitment of the nucleosome remodeling complex, INO80, to DSBs allows repair proteins to gain access to chromatin.

Chromatin modification also plays a role in recruiting and localizing repair factors to the break. The phosphorylation of the histone H2A variant, H2AX, has been extensively studied. This variant has been shown to be rapidly phosphorylated in response to DSBs (39, 40). Phosphorylated H2AX (gamma-H2AX) results in the formation of gamma-H2AX foci found along the chromatin regions that are flanking the damaged sites (39, 40). Many of the proteins that are involved in the early response to breaks have been found to co localize with these foci. These factors include Brca1, MRN, and 53BP1 (41-43). The generation of H2AX deficient mice and cell lines has provided insights into the role in cellular responses to DSBs (44-47). Although H2AX deficiency is not required for survival, it does cause increased IR sensitivity and genomic instability (45, 47). In gamma-H2AX deficient cells, IR-induced foci are not observed, however, Nbs1, Brca1, and 53BP1 are still able to localize to sites of damage. Additionally, IR-induced cell cycle checkpoints are still activated (44, 45, 47). On the basis of these results, it seems that essential DNA damage detectors and checkpoint activators, including ATM, are rapidly recruited to the break independent of H2AX. The activation of ATM results in the phosphorylation of the multiple H2AX molecules flanking the damaged region. Gamma-H2AX may act as a signal or scaffolding protein to help recruit and concentrate other repair factors to the damaged site, including the chromatin remodeling complex, INO80. Phosphorylation of the multiple H2AX molecules may also act to amplify the signal and damage response. Gamma-H2AX and additional histone modifications may also play a role in blocking transcription in the damaged region until the break has been repaired.

Methylation is another type of histone modification that has recently been shown to recruit the repair protein, 53BP1. Specifically, Huyen et al (48) have shown that the tudor domain of 53BP1 interacts with histone H3 methylated at lysine 79 (H3-K79). This interaction is required to recruit 53BP1 to sites of damage. Interestingly, H3-K79 is methylated throughout the entire genome. The authors suggested that a break may result in chromatin relaxation at the site of damage which may expose the methylated H3-K79 tail which may act as a signal to recruit the damage sensor protein, 53BP1 (48, 49).

Another critical factor for efficient detection and repair is the rapid amplification of the signal in order to induce the global cellular functions that are involved in the damage response system (50). Members of the

phosphatidyl inositol 3-kinase (PI3-K) family which include ATM, ATR, and DNA-PK have been implicated as essential transducers in this response pathway (51). The kinase that is primarily involved in IR-induced DSB detection is the ataxia telangiectasia mutated (ATM) kinase. Upon DSB detection, the inactive ATM dimer is autophosphorylated and dissociated to become activated (52). Once activated, phosphorylated ATM moves to sites of damage where it can phosphorylate its numerous substrates (52, 53). Many of the substrates are involved in cell cycle checkpoints, including p53, Mdm2, Chk2, Nbs1, Brca1, FancD2 and SMC1 (54). To protect the genome, specific cell cycle checkpoints can be activated during G_1, S, G_2, or mitosis and at the G_1/S and G_2/M boundaries (20). The activation of ATM is crucial to many of these checkpoint activations (Figure 3).

The mechanism of ATM activation is a topic that has recently attracted much attention. Evidence indicates that ATM activation is a result of intermolecular autophosphorylation resulting in ATM dimer dissociation (52). ATM autophosphorylation is thought to occur by a chromatin modification event that resulted from the damage (52). Additionally, the Mre11 complex has been shown to interact with fragmented DNA and ATM (55-57). Biochemical evidence reveals that the MRN complex is capable of activating ATM kinase activity (58, 59). Whether chromatin modification induces ATM autophosphorylation for activation or the MRN complex is required for ATM activation is currently unclear. It will be important to understand the initiation of this critical signal transducer for developments in cancer therapy.

6. DSB REPAIR PATHWAYS AND HUMAN DISEASES ASSOCIATED WITH REPAIR DEFICIENCY

Once the damage is detected, repair can take place. The cell has evolved two distinct mechanisms to repair the damaged chromosome, homologous recombination (HR) and non-homologous end-joining (NHEJ) (Figure 4). During HR repair, a homologous sequence is used as a template resulting in an essential error-free repair mechanism. On the other hand, NHEJ rejoins the broken strands regardless of sequence. HR and NHEJ are essential in preserving genomic integrity and avoiding the devastating consequences of an unrepaired DSB (1, 25, 60). The analysis of the prokaryotic HR pathway has helped tremendously in our understanding of the more complex eukaryotic HR pathway. Until recently, the NHEJ pathway had only been described in eukaryotes, even though prokaryotic cells had been shown to display DNA end-joining activity (61, 62). Within the last few years, researchers have begun to identify bacterial homologues of the NHEJ pathway (63, 64). This conservation from bacteria to man emphasizes the importance of each DSB repair pathway.

Interestingly, the significance of each pathway differs amongst each organism. Yeast generally rely on HR as the major repair pathway in response to IR, whereas mammals tend to use NHEJ as the major repair pathway (65-67). It is important to note that the pathways are not mutually exclusive. Cellular conditions play an important role in determining which pathway is used to repair the DSB. These conditions include cell cycle and embryonic developmental stage. HR is most efficient in late S and G2 phase because of the availability of the sister chromatid (67). HR is also predominant in early embryonic developmental stages. This may be explained by the rapidly dividing cells at this stage and/or the potential threat error-prone repair may have at this early stage of development.

The mechanisms and factors involved in each pathway vary. However, mutations in some of the key proteins involved in damage response and repair result in chromosomal instability syndromes that vary on the clinical level, but have remarkably similar cellular phenotypes. Some of the common disorders associated with defective DSB detection and repair include LIG4 syndrome, radiosensitive severe combined immunodeficiency (RS-SCID), ataxia-telangiectasia (AT), AT-like disorder (ATLD), and Nijmegen breakage syndrome (NBS). LIG4 syndrome and RS-SCID are caused by mutations in DNA Ligase IV and Artemis, respectively, each of which has a role in the NHEJ pathway (68, 69). LIG4 syndrome is associated with developmental and growth retardation, characteristic facial features, immunodeficiency, and cancer predisposition (68). RS-SCID, on the other hand, is not associated with any obvious developmental abnormalities, but patients display severe immunodeficiency and a predisposition to cancer (70). The other commonly studied diseases that are associated with defective DSBR are AT, ATLD, and NBS, which are a result of mutations in ATM, Mre11, and Nbs1, respectively (71-76). These three proteins are thought to play a key role in DNA damage detection and response. As the names imply, AT and ATLD patients display similar clinical features to one another, including progressive cellular ataxia and immunodeficiency. Predisposition to cancer is observed in AT patients, however, due to the limited number of ATLD patients, it is still under investigation as to whether they have increased cancer risks (71, 72, 76). NBS patients display similar features to LigIV patients, including the characteristic facial features, cancer predisposition, mild mental and growth retardation, but are less immunodeficient than LIG4 patients (74, 75, 77, 78). The range of clinical factors observed in these syndromes may be as a result of their significance to each repair pathway. For instance, Mre11, Nbs1, and LigIV play essential roles in HR or NHEJ repair, while Artemis appears to be necessary for V(D)J recombination..

At the cellular level, these disorders exhibit similar phenotypes (77, 79, 80). As a result of being defective in DSB repair, the cells are sensitive to IR and other DSB inducing agents. Patients that have genomic instability syndromes are extremely sensitive to certain chemotherapy, making proper diagnosis and treatment essential. In addition to being IR sensitive, there is also an increased rate of spontaneous and IR-induced chromosomal breaks and rearrangements that lead to chromosomal instability and may explain the increase risk for developing cancer (77, 79, 80). Mutations in proteins that are involved in activating cell cycle checkpoints such as those found in

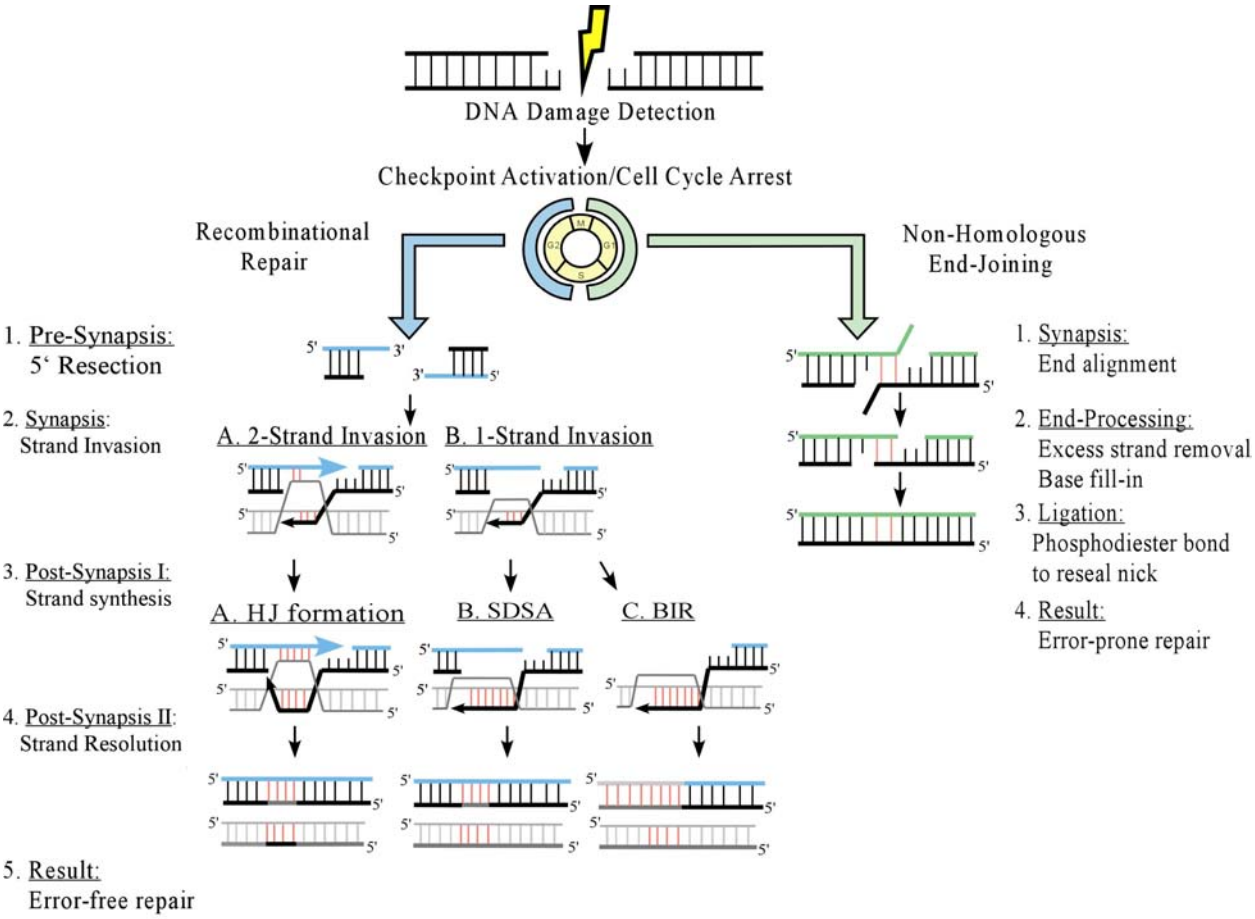

Figure 4. Double Strand Break Repair Pathways. Upon DSB detection, cell cycle checkpoints are activated causing the arrest of cell growth. The stage of the cell cycle will determine the repair pathway. Nonhomologous end-joining is depicted on the right in green. Briefly, the ends are joined together during synapsis, leading to the removal of unpaired ends during end processing. Next, the ends are ligated together resulting in a rejoined sequence containing possible deletions. Homologous recombination is depicted on the left in blue. HR consists of three stages pre-synapsis, synapsis, and post-synapsis. During pre-synapsis the 5′ ends of the DSB are resected to create long 3′ tails. A strand exchange protein is loaded onto the tails and the homologous duplex is invaded. Synapsis ends with the alignment of the homologous sequences. A 3′ branch displacement loop (D-loop) is formed during strand invasion and is converted into a 4 branched structure called a Holliday junction (HJ) in post-synapsis. Resolution of the HJ may result in genetic recombination (crossover) or noncrossover products. Two alternative mechanisms of HR include Synthesis Dependent Strand Annealing (SDSA) and break-induced replication (BIR). Pre-synapsis and synapsis take place during SDSA. This pathway of HR can bypass HJ formation and any novel genetic rearrangements resulting from HJ resolution. During SDSA the invading strand is simply replicated for a long distance past the break and then displaced from the homologous duplex and realigned with the other broken end. Now the broken end has a template that can be primed for DNA synthesis. BIR usually occurs during S phase and often involves a free end. In BIR, the invading strand is replicated until the end of the chromosome. Similar to SDSA, recombinant crossover products are avoided.

NBS, AT, and ATLD cell lines, also display radio-resistant DNA synthesis (73, 81, 82). While there is no gross defect in DSB repair in NBS, AT or ATLD cells, clearly the response to this damage is aberrant. Comparison of the clinical and cellular phenotypes of the chromosomal instability syndromes has provided helpful insights into the function of the individual components, as well as the significance of each pathway.

7. NON-HOMOLOGOUS END JOINING

The basic mechanism of NHEJ is the rejoining of two broken ends directly, regardless of sequence (Figure 5). This imperfect process is the predominant DSB repair pathway in multicellular eukaryotes. As a result of damage, the broken ends can be very diverse and are usually incompatible. It is critical that the two ends are maintained in close proximity and processed with as minimal modifications as possible. The overall process involves the DNA-dependent protein kinase, DNA-PK, which is comprised of the Ku70/80 heterodimer and the catalytic subunit, DNA-PKcs (83, 84). Upon activation of DNA-PK, nucleases and polymerases specific for end processing are recruited to the ends to fill in the gaps and remove flaps prior to rejoining. After the ends have been processed, the XRCC4/ligase IV complex completes the

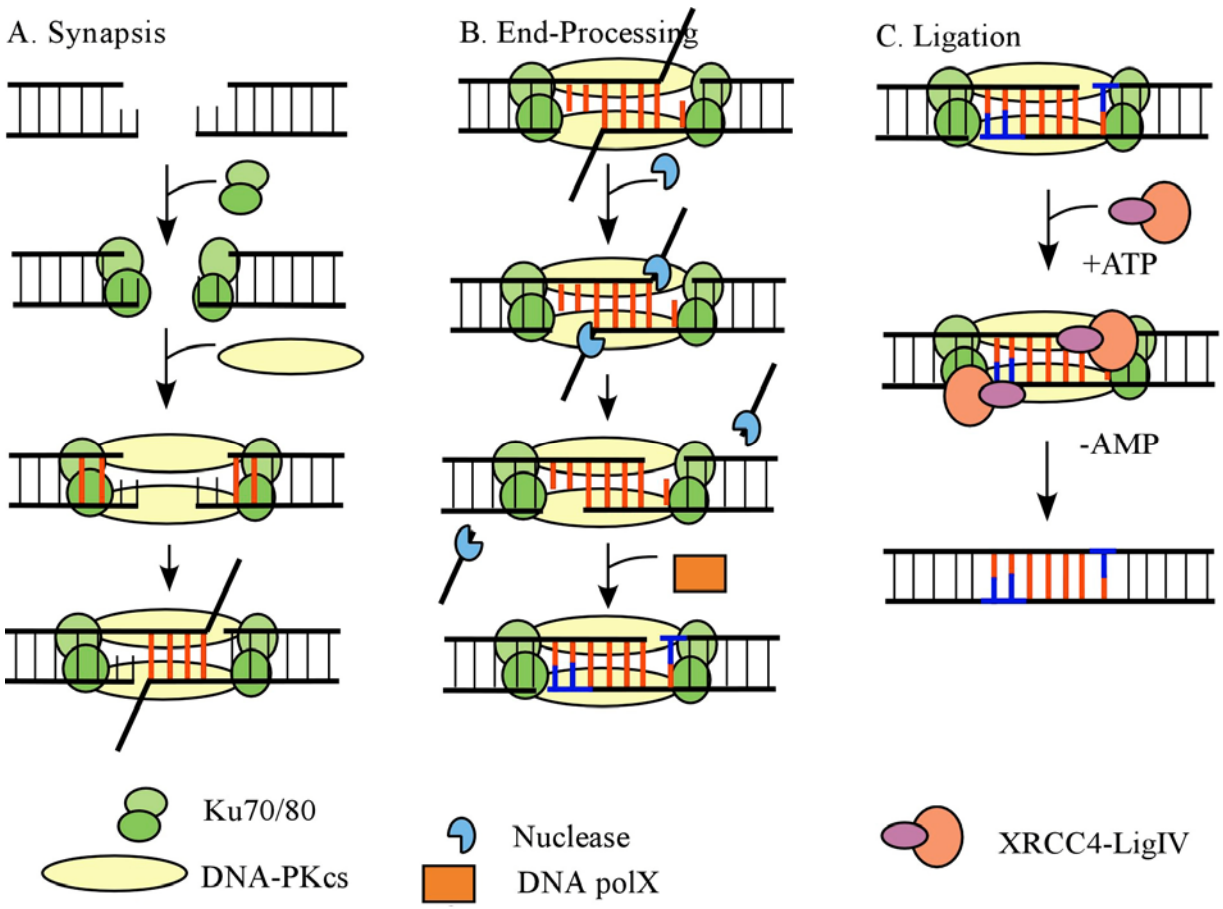

Figure 5. Nonhomologous End-joining. A. During synapsis, the Ku 70/80 heterodimer (green) is recruited to the free ends of the break. This allows for the recruitment of the catalytic subunit, DNA PKcs (yellow). The ends are aligned (shown in red) usually generating displaced non-complimentary strands. DNA PK kinase is activated, allowing for bridging and recruitment of other repair factors found in the subsequent steps. B. During end processing, nucleolytic activity is required to remove the ends generated during synapsis (blue). Possible nucleases include FEN-1, WRN and Artemis. Once the ends are removed, the gaps are filled in with DNA synthesis factors that my include members of the DNA Pol X family (orange square). C. The final step is ligation. DNA PK recruits XRCC4-LigIV complex (purple/red). This complex requires ATP to complete the DNA joining reaction.

final ligation step (85, 86). The biochemical characteristics of each protein will be discussed in terms of their functions in the three phases of NHEJ: synapsis, end processing and ligation.

7.1. Synapsis

Synapsis is the physical bridging of two DNA ends to ensure ligation. There are several salient features during the synaptic phase of end joining (Figure 5A). Primarily, the broken ends must be held in close proximity so that they can be aligned. Next, it is important that the ends are protected from unwanted nucleases but yet available to recruit other end joining factors to allow the subsequent repair steps to proceed (87). Additionally, most breaks will require processing either by a nuclease or polymerase to allow for completion of repair. The alignment, protection, and recruitment of repair factors are achieved through the DNA-dependent protein kinase, DNA-PK (88-90). The exact roles of the DNA-PK holoenzyme have yet to be determined. Structural and biochemical evidence suggest that DNA-PK can not only help align and bridge the two DNA strands together, but similar to ATM, it can amplify the signal, activate, and recruit the downstream factors specific to NHEJ.

The Ku heterodimer, made up of Ku70 (69 kDa) and Ku80 (83 kDa), binds duplex DNA in a structure specific manner with a distinct preference for DNA ends. The substrates include blunt ends, hairpin ends, and 5' or 3' overhangs (91). The crystal structure of Ku70/80 reveals a ring-like structure that can thread onto the duplex DNA with minimal DNA contact (92). Minimal DNA contact may explain the multiple substrates recognized by Ku70/80. Ku70/80 forms a cradle-like structure suggesting it can protect a DNA end while recruiting the other end joining factors (92). Thus, DNA bound Ku70/80 facilitates the recruitment of the catalytic subunit of DNA-PK, DNA-PKcs (89).

DNA-PKcs is a 470 kDa kinase which shares sequence homology to other members of the PI3-K family (91, 93). The recruitment of DNA-PKcs to the Ku-bound DNA complex induces an inward translocation of Ku, providing room for DNA-PKcs to bind to the DNA termini (94). The exact function of DNA-PK remains to be elucidated. Based on the size and shape, DNA-PK may be important for not only bridging the DNA ends together, but may also act as a shield to protect the DNA from unwanted nucleases (95). In addition to its possible structural role in NHEJ, DNA-PK also displays kinase activity that is essential for DNA end joining (96). DNA-PK kinase activity has also been shown to be severely reduced in S phase cells when compared to cells in the G1 phase (97). Cell cycle dependent kinase activation may be one possible mechanism that the cell uses to influence repair pathway use.

Similar to ATM, the activation of DNA-PK kinase may result in a signaling cascade promoting the NHEJ pathway, rather than cell cycle checkpoints. There are many potential substrates that have been shown to be phosphorylated by DNA-PK, *in vitro*. The substrates include XRCC4, WRN helicase, Artemis, gamma-H2AX and p53 (98-102). However, only DNA-PK autophosphorylation has been confirmed to be a physiological event (97, 103). Interestingly, the catalytic subunit is found only in higher eukaryotes. The role of DNA-PK may be replaced with other proteins that are involved in DNA bridging activity. One possible candidate is the Mre11 complex. Scanning force microscopy images reveal the Mre11 complex can hold linear DNA strands together (104, 105). In yeast, this complex may take the place of DNA-PK and provide a role as a bridging factor. However, the precise role of the Mre11 complex in NHEJ in higher eukaryotes is still being investigated.

7.2. End Processing

In the simplest type of DSB, two complimentary ends can reanneal directly once they have been brought together by DNA-PK. However, this is usually not the case, as the two broken ends are rarely compatible. Frequently, there are damaged bases as a result of the damage (91). End processing is required for the removal of excess DNA and filling in of gaps that are generated by end alignment (Figure 5B). During synapsis, the sequences can align at regions of microhomology. These are regions that share similar sequences of 1-4 nucleotides (106). However, these microhomologous regions are not essential for alignment (87, 107). The various substrates generated by the damage may require an assortment of enzymes that are specific to the break. These enzymes may include helicases for DNA unwinding, nucleases to remove excess strands, polymerases to fill in gaps, or phosphatases and/or kinases to prime the 5′ or 3′ DNA ends. To date, we have numerous candidates that may be involved in end processing.

Possible nucleases involved in the removal or resection of single strands include FEN-1, WRN, and Artemis. FEN-1 is an endonuclease that is capable of cleaving 5′ flaps. FEN-1 is a prime candidate for 5′ flap removal due to its ability to interact with Pol4 and Dnl4 (ligase IV homolog), which are components of NHEJ in *S. cerevisiae* (108, 109). Another candidate for nucleolytic processing is the WRN protein, which is defective in the premature aging disease, Werner syndrome. In addition to having both helicase and 3′ to 5′ exonuclease activity, WRN has been found to be regulated and phosphorylated by DNA-PK. Ku70/80 and the Mre11 complex have been shown to interact and stimulate the nuclease and helicase activity of WRN, respectively (110, 111). By recruiting exonucleases that are regulated by DNA-PK, the extent of degradation may be limited thus preventing extensive deletions and increasing the fidelity of repair (101). Another important candidate involved in end processing is Artemis. Artemis possesses a range of nuclease activities including 5′ to 3′ exonucleolytic activity on single stranded DNA, endonucleolytic activity on 5′ and 3′ overhangs and hairpin opening. The opening of hairpins is stimulated by its interaction with DNA-PKcs (99). Mutations in Artemis result in severe combined immunodeficiency due to a defect in V(D)J recombination (69, 70). Recent evidence has shown that Artemis is a downstream target of ATM indicating its role in the damage checkpoint response (112, 113). The possibility that there may be more than one protein for each activity with overlapping functions makes it much more difficult to determine the functional contribution of each in repair.

In addition to the nucleolytic removal of excess DNA, DNA polymerases are required to fill in the gaps that arise during end-processing. Yeast Pol4, a member of the Pol X family, has been implicated in NHEJ end processing (114). Pol4 has also been shown to interact with yeast Dnl4/Lif1 (Ligase4/XRCC4) and Fen-1 suggesting this to be the main polymerase involved in NHEJ (108, 115). Pol mu, Pol lambda, and terminal deoxyribonucleoidyl transferase are members of the mammalian Pol X family that are thought to be involved in NHEJ gap filling. All three of these proteins have been implicated in DNA end joining reactions and interact with XRCC4/LigaseIV (116-118). Similar to the nucleases, their exact role in end processing has yet to be determined. Because DNA ends are aligned using whatever homology is available, end-processing may be unique to each break. It is likely that multiple factors have overlapping roles in the processing of the DNA ends during NHEJ.

7.3. Ligation

The third step in NHEJ is ligation (Figure 5C). Before ligation can occur, the ends must be properly aligned, flaps must be removed, and the gaps filled in. The ligation process is dependent on the LigIV/XRCC4 complex. Ligase IV (LigIV) shares homology with ligases I and III in the N-terminal region which includes the ligase active site. However, LigIV differs from the other mammalian ligases in that it has a long C- terminal extension containing two BRCT motifs. The reaction mechanisms and chemistry of eukaryotic ligases are similar to prokaryotic ligase (119, 120). Briefly, ligases utilize ATP for the DNA joining reaction. In the first stage, ATP is used to adenylate the enzyme in its active site. Next, the AMP group is transferred from the ligase, to the 5′ side of

the nicked DNA. Lastly, the nick is sealed with the elimination of AMP. This ultimately results in the formation of a phosphodiester bond. Ligase IV or XRCC4 knock-out mice are embryonic lethal indicating the essential role for this complex (121, 122). Alternatively, LigIV and/or Xrcc4 may be involved in an additional undefined function that is necessary for survival through development. Interestingly, Ku and DNA PK mouse knockouts are viable suggesting redundancy in the initial steps of NHEJ or that the unrepaired lesions are channeled into the HR pathway. In addition to the embryonic lethality of LigIV knockout mice, they also display massive neuronal apoptosis and defective lymphocyte development (123). Inactivation of ATM could restore neuronal development but not lymphocyte development (123). This suggests ATM and LigIV work together to remove or possibly repair endogenously produced DSBs during neuronal development.

LigIV is stabilized and activated by interaction with XRCC4 (85, 86, 124). XRCC4 strongly associates with LigIV at the extended C terminus in between the BRCT motifs (85, 124, 125). The crystal structures of XRCC4 alone and bound to the interacting peptide of LigIV indicate that the C terminus of LigIV can interact with an XRCC4 dimer (126, 127). The N terminal globular head domain of XRCC4 may interact with DNA, while the helical C terminal domain forms a coiled coil complex with another XRCC4 molecule that can interact with a single LigIV molecule (126, 127).

In addition to stabilizing LigIV, XRCC4 may play a role in recruiting the ligase to the damaged DNA ends that are held together by DNA-PK. Biochemical evidence suggests that the XRCC4-LigIV complex is recruited to DNA ends by Ku via protein-protein interactions (128, 129). Direct interaction between LigIV and Ku, as well as between XRCC4 and DNA-PKcs has been shown. These specific interactions may ensure the correct orientation of the ligase molecule (130). XRCC4 can be phosphorylated by DNA-PKcs, *in vitro* (98). However, complementation of XRCC4-deficient cells with XRCC4 alleles mutated in the DNA-PK phosphorylation sites could still repair IR induced DSBs similarly to the cells complemented with wild type XRCC4 (131). This suggests that the phosphorylation of XRCC4 may not be critical for ligase activity, but possibly required for other cellular functions.

8. HOMOLOGOUS RECOMBINATION

As an error free mechanism of recombination, HR is dependent on the sequence identity or homology of recombinant DNA substrates (65). The requirements for sequence identity is typically met by the use of the replicated sister chromatid and thus limits the use of HR to the S and G2 phases of the cell cycle (132). Conversely, in meiosis the association of the homologues contributes to recombination that leads to enhancement of genetic diversity. HR contributes to the repair of DSBs in organisms from all three kingdoms of life and the conservation among the key protein components involved underscores the conservation of mechanism. While this mechanism is more complex than NHEJ, studies in *E. coli* have revealed a detailed picture of HR. As expected the eukaryotic pathway has additional complexity but the overall mechanism is similar to the prokaryotic (reviewed in (133). During HR, the cell must be able to search and locate a homologous sequence, promote a DNA strand exchange resulting in a joint molecule between the damaged DNA end and its homologous dsDNA template, and finally, resolve the duplex molecules.

A primary method for identification of genes involved in the HR pathways of eukaryotes is the utilization of mutagenesis followed by genetic screens in *S. cerevisiae* for mutants sensitive to IR and other damaging agents (133). Interestingly, many of the proteins found to be involved in eukaryotic HR do not share sequence homology to prokaryotes, but rather biochemical and functional homology. There are many proteins involved in this pathway and will be further discussed in terms of their functions in the three phases of HR: pre-synapsis, synapsis and post-synapsis (Figure 6).

8.1. Pre-synapsis

Presynapsis is the first stage of HR, in which the damaged strand is processed so that synapsis can take place. The initial processing generates a single-stranded 3′ overhang that forms a functional nucleoprotein filament for strand invasion (Figure 6A). A 3′ overhang is generated through nuclease resection, and then is loaded with the strand exchange protein, Rad51, and other proteins. The processing and formation of the DNA filament is a complex task that requires many factors. A nuclease is required for 5′-end resection followed by the single-stranded DNA binding protein (RPA) that stabilizes the ssDNA along with a number of mediators involved in the promotion or stabilization of the Rad51 nucleoprotein filament.

The eukaryotic nuclease involved in end processing has yet to be determined. The Mre11 complex is considered a likely candidate. However, the exonuclease activity of the Mre11 protein has a polarity of 3′ to 5′ that would be unable to generate the 3′ overhangs that have been observed. In addition, mutations that disrupt nuclease activity without affecting MRN complex formation do not show severe IR sensitivity nor do they significantly reduce DSB processing (134-136). This suggests that the Mre11 exonuclease may not resect the 5′ ends. One model is that Mre11 recruits another nuclease to the DSB to carry out the resection. Another possibility is that the nuclease activity of Mre11 is only required for aberrant structures formed at sites of DSBs. Evidence in support of this has been published by Lobachev and colleagues (137) where the presence of a palindrome at the DSB site requires the nuclease activity of the Mre11 protein for efficient repair. Another nuclease candidate responsible for end resection is Exo1 which displays 5′ to 3′ exonuclease activity (138, 139). A double yeast mutant of Exo1 and a nuclease deficient Mre11 display an increase in IR sensitivity but can still repair breaks (139). However, the activation of the Mec1 signaling pathway after blocked replication damage

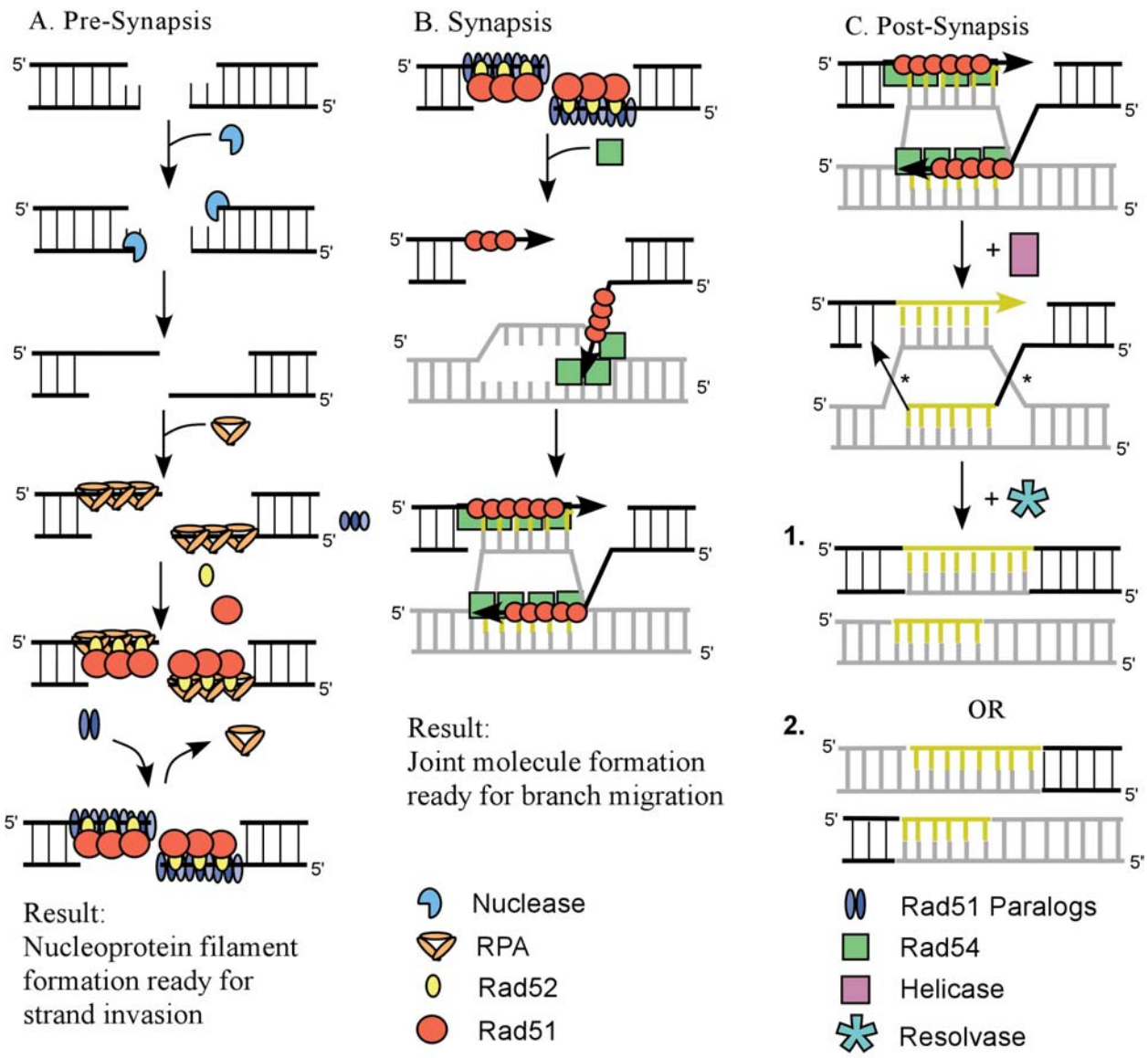

Figure 6. Homologous Recombination. A. Presynapsis is the first stage of HR, in which the damaged strand is processed so that synapsis can take place. The overall process is to generate a nucleoprotein filament that is ready for strand invasion. Nuclease activity is required to generate 3′ overhangs. Possible candidates includes the Mre11 of the MRN complex or Exo1 (blue). Single strand binding protein, RPA (orange) facilitates the assembly of the strand exchange protein, Rad51 (red). There are numerous stabilizing factors involved in Rad51 recruitment and RPA displacement. These include Rad52 and the Rad51 paralogs shown in yellow and dark blue, respectively. B. During synapsis, the nucleoprotein filament invades the homologous sister chromatid (grey) in search for a homologous sequence. Binding of Rad54 (green) to DNA induces topological changes known as a D-loop. Rad54 may facilitate the alignment of the Rad51 nucleoprotein filament (broken DNA strand) with the homologous sequence (grey) resulting in the formation of a joint molecule. C. In post-synapsis the homologous sequences are aligned and can now be used as a template for repair. As a result of the strand invasion the DNA strands are crossed and form structures known as Holliday junctions (asterisk). DNA helicases (purple) and endonucleases (blue star) are required to resolve the HJ. Two different products may result from cleavage, a noncrossover (1) or a crossover (2). The crossover is a truly recombinant duplex with new genetic sequences flanking the site of cleavage, while a noncrossover retains the parental flanking sequences with a small portion of heteroduplex DNA.

was inhibited in the Exo1 and Mre11 double mutants (140). Yeast Mec1 (ATR, in mammals) is the kinase largely responsible for checkpoint responses following UV damage, stalled replication forks, and occasionally DSBs (141). Mre11 and Exo1 are thought to cooperate in the generation of long ssDNA regions that can recruit Mec1 (ATR) to sites of damage (140). Thus, it may be that Mre11 and ExoI are both capable of contributing to the

processing of DSBs to facilitate HR. Currently it is unclear if these proteins cooperate in this function or if they operate independently of each other.

Once the 3′ overhang has been generated, RPA is recruited for strand protection. The eukaryotic single-stranded DNA binding protein, RPA, is a heterotrimer with three subunits of 70, 34 and 14 kDa (142). The 34 kDa subunit is phosphorylated in a cell cycle dependent manner and may alter the binding of RPA to various DNA and proteins. RPA is necessary for HR; however it can inhibit Rad51 activity in pre-synapsis by competing for the ssDNA binding sites in the resected 3′ overhangs (143-145). RPA stimulates HR by inhibiting premature annealing and shielding ssDNA from disruptive secondary structures that prevent formation of the Rad51 nucleoprotein filament (146). For *in vitro* strand exchange reactions, Rad51 must be added first to avoid competition with RPA (145, 147, 148). *In vivo*, RPA probably binds first to the 3′ tail (145).

A number of accessory proteins are involved in the loading of Rad51 onto the 3′ overhangs in order to overcome the inhibitory effects of RPA. These mediators include Rad52 and a group of proteins known as the Rad51 paralogs (147, 149-151). In *S. cerevisiae,* Rad52 is an essential component to HR, but this has not been observed in mammals (133). The Rad52 protein promotes annealing and binds both single-stranded and double-stranded DNA with a preference for single-stranded ends (152). The Rad52 protein interacts with itself, forming heptameric ring-like oligomers with a central pore that form higher order structures on DNA (153-155). Cellular studies of green florescence protein tagged Rad52 constructs show that the protein localizes to centers of DNA repair and recombination in S phase (156, 157). Rad52 interacts with both the 34 kDa subunit of RPA and Rad51 through a central domain and the carboxyl terminus, respectively (158, 159). In rad52 mutants, damage induced Rad51 foci are not observed (160). Alone, Rad52 can not displace RPA directly from DNA, but it may play a role in recruiting Rad51 to ssDNA and assist Rad51 in displacing RPA (148). Additionally, Rad52 has been shown to remain at the damaged site after Rad51 has dissociated, suggesting Rad52 may also play a role in downstream events (160, 161).

The Rad51 paralogs also assist with the loading of Rad51 onto the single strand. In vertebrates there are five Rad51 paralogs: Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3 (162-167). These proteins can form various pairwise complexes and a larger quaternary complex consisting of Rad51B, Rad51C, Rad51D, and XRCC2 (151, 168-175). Genetic deletion of each Rad51 paralog results in genetic instability and increased IR sensitivity indicating a role in DSB repair (176-179). Biochemical analysis of the paralogs reveal strand annealing activity and preferential binding to ssDNA and branched DNA strands (151, 169). In addition to assisting Rad51 binding, the Rad51C protein may play a role in Holliday junction processing in mammals (180).

The strand exchange protein, Rad51, is highly conserved in all eukaryotes. Rad51 has a polymerization interface that facilitates nucleation and formation of the protein filament on single-stranded DNA. Rad51 homologues bind both single-stranded and double-stranded DNA with equal affinity, with a preference for duplex with a single stranded tail (181-183). Genetic analysis of RAD51 knockouts in *S. cerevisiae* demonstrates viability in spite of IR sensitivity and meiotic defects. A RAD51 deletion in vertebrate embryonic stem cells is lethal (184, 185). Chromosomal breakage, G2/M arrest and cell death occur when an inducible RAD51 is turned off in Chicken DT40 cells (186). Immunofluorescence studies have revealed Rad51 forms nuclear foci during S phase in the absence of exogenous DNA damage and in response to the induction of DSBs (187, 188).

8.2. Synapsis

Upon Rad51 loading onto the 3′ tail to create the nucleoprotein filament, strand invasion occurs into the sister chromatid resulting in the formation of a joint molecule (Figure 6B). During the strand invasion event there is a reciprocal strand invasion that prompts a search for homology in the duplex DNA with the broken single-strand end and aligns the homologous DNA sequences when found. The synaptic nucleoprotein filament of Rad51 forms a three-branched displacement loop (D-loop) with the homologous duplex DNA. Interestingly, even though the strand exchange is a major hallmark of this pathway, the mechanistic details remain unknown.

The Rad54 protein is member of the chromatin remodeling SWI12/SNF2 family that translocates along DNA. Rad54 has vigorous dsDNA dependent ATPase activity and can directly interact with Rad51 (189, 190). Like other SWI12/SNF2 family proteins, Rad54 functions as a DNA translocating motor that possesses chromatin remodeling activity (191, 192). Binding of Rad54 to DNA induces topological changes and as it tracks along DNA it is able to disrupt nucleosomes (191, 192). This may facilitate homologous DNA alignment by Rad51, allowing the broken DNA to pair with the duplex (193, 194). The melting of secondary DNA structure by Rad54 may also initiate D loop formation (192). In addition to disrupting nucleosomes, Rad54 translocation might disrupt other proteins bound to DNA, including the nucleoprotein filament of the invading strand. Rad54 has been shown to be required for the disassembly of Rad51 and Rad52 (189). By displacing DNA bound proteins, the exposure of short regions of single stranded DNA may facilitate the alignment and annealing of two complimentary sequences.

8.3. Postsynapsis

Strand invasion results in a joint molecule intermediate known as the Holliday junctions (HJ). HJ are DNA structures consisting of two duplex crossed strands, resulting in a four strand cross (195). This heteroduplex DNA molecule is a dynamic structure that can undergo branch migration. In mammalian cells, this branch migration has been measured to reach up to lengths of 2700 base pairs (196). Followed by branch migration, the junctions are cut in order to separate and resolve the strands (Figure 6C). Two different products may result from strand resolution, a crossover or noncrossover. The crossover is a

truly recombinant duplex with new genetic sequences flanking the site of cleavage, while a noncrossover retains the parental flanking sequences with a small portion of heteroduplex DNA. Although most of the data on strand resolution has come from what we have learned about in prokaryotes, mammalian cells have been shown to have the similar activities of branch migration and HJ resolution (196).

The precise mechanism and all required proteins involved in postsynapsis have not been determined completely. Generally speaking, in order for branch migration and HJ strand nicking to take place, helicase activities and endonucleolytic activities, respectively, are required. Fractionated mammalian cell extracts have revealed ATP-dependent branch migration activity (197). The proteins responsible have yet to be identified. Members of the RecQ helicase family may be good candidates (reviewed in (198)). The substrates of the RecQ helicase family are notable because they include D-loops, HJs, duplex DNA and duplex containing a replication bubble (199, 200). In addition to DNA unwinding, in vitro analysis indicates members of this helicase family are capable of catalyzing branch migration (199, 201, 202). Mutations in three out of the five known human RecQ helicases are responsible for the genome instability disorders Werner syndrome, Bloom syndrome and Rothmund-Thomson syndrome (203-205). The corresponding helicases are referred to as WRN, BLM and RECQ4. Mutations in the BLM gene can result in increased sister chromatid exchanges (hyper-recombination) in addition to chromosomal breakage and cancer predisposition (206, 207). BLM forms a complex with topoisomerase III alpha (BLM-TOPOIIIα) that limits recombinant crossover events (208). The loss of this activity in BLM cells may explain the hyper-recombination events and chromosomal aberrations observed.

In order to resolve the strands, a HJ structure-specific endonuclease, resolvase, is required. One possible candidate is Mus81, a flap/fork endonuclease, that displays weak HJ cleavage activity in vitro (201, 209, 210). However, depletion of Mus81 from fractionated cell extracts still displays HJ resolution activity (180). Interestingly, depletion of Rad51C or XRCC3 from fractionated cell extracts result in a loss in HJ resolution activity (180). These results suggest Rad51C and XRCC3 may be required for HJ resolution.

An important aspect to consider during HJ resolution is the alternative outcomes that can arise. In meiotic cells, a cross over event is likely to be favored during meiotic recombination in order to generate genetic diversity. In contrast, unwanted DSBs that occur in mitotic cells should favor a non-crossover resolution that may prevent possible mutations. The factors involved in resolution may be activated and/or recruited based on the type of cell, cell cycle, or extent of damage. Additionally, there are alternative mechanisms that bypass HJ formation to prevent recombinant crossover products. Two of these mechanisms are known as synthesis dependent strand annealing (SDSA) and break-induced replication (BIR) and have been reviewed (211, 212) (Figure 4). Briefly, both mechanisms contain a single strand invasion, rather than two. During SDSA the invading strand is simply replicated for a long distance past the break, followed by its displacement from the homologous duplex and realignment with the other broken end. The broken end now has a template that can be primed for DNA synthesis. BIR usually involves a single strand invasion using a free end that is replicated until the end of the chromosome. In both cases, HJ formation is avoided thus preventing a recombinant crossover event. Similarly to HJ resolution, there may be multiple factors involved.

9. CONCLUSIONS AND PERSPECTIVES

DSBs are among the most serious and difficult to repair DNA lesions. Detection and repair of these breaks is essential for genomic stability. Ironically, these insults are frequent events that occur as a result of common sources and are deliberately initiated in physiological conditions to maintain genetic diversity. How the cell is able to detect these harmful breaks and initiate the damage response pathways is an ongoing investigation. Understanding the precise role of the MRN complex may reveal this process. The activation of ATM is also essential in our understanding of detection and response. The role of ATM as a damage sensor and its critical role in checkpoint activation make it another critical player involved in maintaining genomic stability. Furthermore, the role of chromatin modification, which has been shown to play a major role in transcriptional regulation, is just starting to emerge in the DNA repair field. Chromatin modification has been linked to DSB detection, however, the factors involved and mechanisms employed remain to be elucidated. Understanding the mechanisms and components of DSB repair will also provide insights into treatment for patients that have genetic instability syndromes, as well as other mutations that can lead to defective DSB repair. A common theme in both the HR and NHEJ repair pathways is the overlapping functions of many of the proteins, in particular the strand processing enzymes. The significance of having these multiple processing enzymes may be to use backup systems in case a mutation occurs in one protein or pathway, or they may be necessary for the extent of damage that has occurred. Unfortunately, it has been a struggle to determine their precise role in repair due to the overlapping functions and lethality of knocking out many of the key factors involved.

The characterization of the clinical symptoms of DSB repair defective patients and their cellular phenotypes has revealed increased sensitivity to IR as well as chromosomal rearrangements and checkpoint deficiencies. Given the damage sensitivity of DSB repair deficient cells, these pathways are an attractive target for development of therapeutics to inhibit these pathways in tumors. One of the ongoing goals of DSB repair research is to elucidate the pathways underlying chromosomal instability in order to develop better anti-cancer drug regimens that can be offered in conjunction with current therapies. Ultimately, the study of DSB repair will likely provide important clues to understanding complex cellular phenotypes such as senescence, aging, and tumorigenesis.

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Abbreviations: DSB: double strand break, DSBR: double strand break repair, NHEJ: non-homologous end joining, HR: homologous recombination, SSB: single strand break, IR: ionizing radiation, MRN: Mre11, Rad50, Nbs1 or Mre11 complex, ATM: ataxia telangiectasia mutated kinase, DNA-PKcs: DNA-Protein Kinase catalytic subunit, HJ: Holliday junction

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