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Main steps in DNA double-strand break repair: an introduction to homologous recombination and related processes

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Abstract

DNA double-strand breaks arise accidentally upon exposure of DNA to radiation, chemicals or result from faulty DNA metabolic processes. DNA breaks can also be introduced in a programmed manner, such as during the maturation of the immune system, meiosis or cancer chemo- or radiotherapy. Cells have developed a variety of repair pathways, which are fine-tuned to the specific needs of a cell. Accordingly, vegetative cells employ mechanisms that restore the integrity of broken DNA with the highest efficiency at the lowest cost of mutagenesis. In contrast, meiotic cells or developing lymphocytes exploit DNA breakage to generate diversity. Here, we review the main pathways of eukaryotic DNA double-strand break repair with the focus on homologous recombination and its various sub-pathways. We highlight the differences between homologous recombination and end-joining mechanisms including non-homologous end-joining and microhomology-mediated end-joining, and offer insights into how these pathways are regulated. Finally, we introduce non-canonical functions of the recombination proteins, in particular during DNA replication stress.

1 Overview of DNA double-strand break repair pathways

1.1 Formation and types of DNA breaks

Our DNA is under a constant threat of damage from radiation, chemicals or aberrant DNA metabolic processes (Jackson and Bartek 2009; Ciccia and Elledge 2010; Tubbs and Nussenzweig 2017). A large fraction of DNA lesions involve modifications of DNA bases, such as oxidation, ultraviolet light-induced pyrimidine dimers, methylation or creation of abasic sites, which leave the phosphodiester backbone intact. Other abnormalities result in the disruption of the phosphodiester backbone. The most common of these are DNA single-strand breaks (SSBs), where only one DNA strand is interrupted (Fig. 1a). SSBs normally do not compromise the integrity of double-stranded DNA (dsDNA). However, if an SSB is left unrepaired and the lesion is encountered by DNA machinery that separates the DNA duplex into two component single-strands (ssDNA), such as during DNA replication, an SSB can be converted into a one-ended DNA double-strand break (DSB), see Fig. 1b. Both SSBs and DSBs can arise as a result of ionizing radiation (IR), which may occur either directly or indirectly *via* generation of reactive oxygen species (Ward 1988). As a result, radiation-induced DNA damage results in complex lesions, where both SSBs and DSBs are accompanied by oxidative DNA damage (Olive et al. 1990; Olive 1998). The most common source of accidental IR exposure is the radioactive radon gas that accumulates in certain locations in the basements of old homes (Jackson and Bartek 2009). IR remains one of the most effective treatments during anti-cancer therapy, as it preferentially affects rapidly dividing cancer cells (Jackson and Bartek 2009; Baskar et al. 2014). SSBs and DSBs also arise during aberrant DNA topoisomerase reactions, which can occur spontaneously or upon exposure to specific inhibitors that are often used as anti-cancer chemotherapeutics (Holm et al. 1989; Canela et al. 2017). Finally, DNA breaks under certain conditions result from a nuclease attacking diverse DNA intermediates, including stalled DNA replication forks. As will be introduced in chapter 7, DSB-like structure may also arise during a process termed replication fork reversal without template DNA breakage.

Depending on the mechanism of break formation, a DSB can either be one-ended or two-ended (Fig. 1b,c). Most one-ended DSBs arise when DNA replication encounters an SSB and the replication fork falls apart, or when a DNA replication fork stalls and one of the arms is cleaved by a nuclease. Two-ended DSBs typically form when both strands of linear dsDNA are broken simultaneously, or when two ssDNA breaks form in an immediate proximity; in this case, the broken end would contain a short stretch of overhanging ssDNA. Additionally, depending on the mechanism of the DSB formation,

DNA ends may be either chemically "clean" or "dirty". The so-called clean DNA breaks, apart from the broken phosphodiester backbone, bear normal DNA chemistry. Dirty DNA ends instead contain additional adducts that may include anything from small chemical groups to covalently attached proteins. DNA breaks induced by DNA nucleases are generally clean, but many DSBs induced by ionizing radiation are dirty (Olive et al. 1990). Likewise, DSBs induced by aberrant DNA topoisomerase reactions result in a covalently attached DNA topoisomerase at the break end (Tse et al. 1980). As will be described below, the mechanism of DSB repair depends largely on whether the break is one- or two-ended, chemically clean or dirty, as well as the cell cycle stage.

Our cells have developed numerous mechanisms for repairing DSBs with a minimal loss of genetic information. However, if a DSB is repaired incorrectly, it can lead to mutations and chromosomal rearrangements, resulting in aberrant regulation of cellular growth and cancer development or even cell death (Jackson and Bartek 2009). Therefore, accurate recognition and repair of DSBs is essential to maintain genomic integrity and prevent tumorigenesis.

1.2 End-joining and homologous recombination mechanisms repair DNA double-strand breaks

Eukaryotic cells use two main processes for DSB repair, end-joining and homologous recombination (HR), see Fig. 2a. The end-joining pathways can be further divided into canonical non-homologous end-joining (NHEJ) and alternative non-homologous end-joining (altNHEJ), also termed microhomology-mediated end-joining (MMEJ). The MMEJ abbreviation will be used hereafter in this review. As the name indicates, NHEJ and MMEJ involve the direct ligation of two DSB ends with little or no sequence homology required, see below for details (Chang et al. 2017). Therefore, a key feature of end-joining is that a repair template, such as the sister chromatid, is not required, so it can occur during any phase of the cell cycle. Both NHEJ and MMEJ processes typically lead to a limited loss of genetic information resulting in short deletions at the DSB site. Additionally, because NHEJ and MMEJ mechanisms are template-independent, ligation of the incorrect ends, if multiple DSBs are present, can generate large deletions or chromosomal rearrangements (Chang et al. 2017). The end-joining pathways can only repair two-ended DSBs, and abnormal structures at the break sites ("dirty" ends, especially protein blocks) may inhibit this type of repair. In summary, end-joining pathways represent fast, but potentially mutagenic DSB repair processes (Fig. 2a).

In contrast, HR requires a homologous sequence as a template for repair (Kowalczykowski 2015). This allows the recombination machinery to restore any missing genetic information in the vicinity of the break site, and, as a result, HR is largely accurate. In most cases in vegetatively growing cells, the sister chromatid is used as the repair template. This restricts recombination to cell cycle stages when the sister chromatid is available, which includes the S and G2 phases, and thus necessitates a strict control mechanism (Orthwein et al. 2015). HR is capable of repairing both one- and two-ended DSBs and can also repair dirty DNA breaks; in particular those with covalently attached proteins. In contrast to end-joining, HR is mechanistically more complicated, involves a larger number of enzymes, and is thus comparatively slower but more accurate (Kowalczykowski 2015; Chang et al. 2017).

Recent years brought breakthroughs in genome editing technologies, which were spearheaded by the development of engineered nucleases such as zinc finger nucleases (ZFNs) or transcription-activator like effector nucleases (TALENs) (Lombardo et al. 2007; Bedell et al. 2012). The majority of genome editing applications now exploit the bacterial clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system (Jinek et al. 2012; Mali et al. 2013). The common denominator of these approaches is the capacity to induce a site-specific DSB. The choice of the DSB repair pathway then dictates the result of editing (Fig. 2b). Imprecise repair by NHEJ or MMEJ gives rise to "indel" mutations (insertion or deletions, although deletions are much more common) at the break site, which may disrupt the reading frame of the targeted gene and thus result in a loss of function. Conversely, if a DNA template is provided, the recombination machinery may get involved, which can mediate precise alteration of the DNA sequence, including introduction of DNA segments or correction of a pathogenic mutation (Fig. 2b). The advance of these genome editing technologies brought renewed interest in understanding the balance between the DSB repair pathways, as the inhibition of MMEJ and NHEJ repair promotes HR-based precise genome editing (Chu et al. 2015; Mateos-Gomez et al. 2017; Schimmel et al. 2017; Zelensky et al. 2017).

The key process that stands at the crossroads between end-joining and HR is the initial processing of the DNA break (Cejka 2015). NHEJ and MMEJ require little DNA end processing (see chapter 2 for more details). In contrast HR (including all its sub-pathways) is initiated by DNA resection at the break site that exposes long tracts of ssDNA. This ssDNA is then used in a search for a homologous dsDNA sequence (such as the sister chromatid) that serves as a template for the largely accurate repair of the DSB by the recombination pathway. At the same time, extended DNA end resection makes

the DNA break generally non-ligatable and inhibits end-joining. Therefore, extensive DNA end resection commits DSB repair to the HR-mediated pathway and accordingly, the initiation of DNA end resection is strictly controlled (Symington and Gautier 2011; Chapman et al. 2012; Shibata 2017). This control mechanism allows HR to be initiated only when a repair template is available (S/G2 phase), and thus limits the potential for illegitimate recombination (i.e., recombination between not fully homologous DNA sequences). This is elegantly achieved through the activation of key resection factors by cyclin-dependent kinase (CDK)-catalyzed phosphorylation (Ira et al. 2004; Huertas et al. 2008). It should be pointed out that this simple model has been challenged, and there is evidence that limited or even extended DNA end resection, occurring in the G1 phase, is involved in the canonical NHEJ pathway (Biehs et al. 2017). Elucidating the details and the regulation of these processes will be an exciting direction of future research. Since misregulation of these DSB repair pathways is believed to result in genome rearrangements that are typical in many cancer types, understanding these processes is highly relevant for human health.

1.3 End-joining and recombination processes involve several sub pathways

Both HR and end-joining processes are not simple linear pathways. Both can be divided into several sub-pathways, which significantly differ in terms of repair mechanisms and enzyme requirements. Here, we will introduce the basic principles of these repair processes; a more detailed description that includes the key enzymatic players will be provided in subsequent chapters.

With regard to the end-joining mechanisms, canonical NHEJ significantly differs from MMEJ (Fig. 3). Whereas canonical NHEJ requires no or very limited homology (less than 4 nt) between the broken DNA molecules, MMEJ was found as a DNA end-joining event that occurs independently of the key NHEJ factors and usually involves short stretches of microhomology (2-20 nt) between the two broken DNA ends to mediate repair (Seol et al. 2017). Therefore, MMEJ is sometimes considered a separate process that stands between the NHEJ and HR pathways.

Recombination processes, in a broad sense, can be divided into single-strand annealing (SSA), synthesis-dependent strand annealing (SDSA), break induced replication (BIR) and canonical HR (also called canonical DNA double-strand break repair, DSBR) (Kowalczykowski 2015). The main conceptual differences between the mechanisms of these sub-pathways are schematically illustrated in Fig. 4. Depending on whether the flanking sequences of the recombining DNA molecules are exchanged or

not, recombination leads to crossover or non-crossover recombination products. A crossover is defined as an event where the distal arm of the broken DNA is swapped with the distal arm of the template DNA molecule. As schematically depicted in Fig. 4d, a crossover results in the "blue" DNA molecule ultimately joined with the "red" one. Crossovers that occur between two homologous loci of sister chromatids give rise to "equal" sister chromatid exchanges, which are mutagenically silent (Fig. 4). In contrast, crossovers that occur between two ectopic loci (non-homologous loci, such as in repetitive sequences) of sister chromatids give rise to "unequal" sister chromatid exchanges. Likewise, crossovers resulting from recombination between non-sister (homologous) chromosomes also lead to gross genome rearrangements. Furthermore, events when genetic information from one DNA molecule gets unidirectionally transferred into another DNA molecule are referred to as gene conversions. This occurs when a DNA sequence is copied from a donor template to the broken DNA molecule during BIR, SDSA or canonical HR. As above, gene conversion can be mutagenic when an ectopic site of a sister chromatid or non-sister chromosome is used as a template. Mutations arising in the course of DSB repair by either NHEJ or HR may give rise to a loss of heterozygosity, which is a key driver of tumorigenesis (Fearon and Vogelstein 1990).

SSA involves DNA end resection to reveal repetitive DNA sequences, which are subsequently annealed. The resulting DNA flaps are cleaved and the strands are ligated. SSA leads to the deletion of the DNA sequence between the two repeats, and is thus a very mutagenic repair process (Fig. 4a). SSA is restricted to situations when two repeats flank the break site, and can successfully restore the integrity of DSBs within repetitive DNA sequences. Although SSA is conceptually similar to MMEJ, it is usually grouped together with recombination mechanisms because of enzyme requirements (see below). Additionally, unlike MMEJ, SSA requires extensive DNA end resection, which is shared among all recombination sub-pathways (Bhargava et al. 2016).

In most cases, DNA end resection is followed by the invasion of the resected DNA into the dsDNA template, forming a joint molecule intermediate (Kowalczykowski 2015). This initiates DNA synthesis, which restores missing genetic information at the break site. In SDSA, the joint molecule intermediate is destabilized and the nascent DNA is annealed to the other end of the broken DNA molecule (Fig. 4b). SDSA is one of the least mutagenic recombination sub-pathways, resulting in a non-crossover repair product. In BIR, DNA synthesis proceeds all the way to the end of the template DNA, copying the sequence of the entire chromosome arm (Fig. 4c). BIR is thus a unique

pathway for repair of one-ended DSBs resulting from collapsed DNA replication forks (Sakofsky and Malkova 2017). Additionally, BIR allows telomere lengthening in the absence of telomerase (Sakofsky and Malkova 2017). The genetic result of BIR is a non-reciprocal crossover.

In the canonical recombinational DSBR pathway, the joint molecule (D-loop) intermediate is processed into double Holliday junctions (dHJs), which may be processed into non-crossovers or crossovers, depending on the dHJ processing pathway utilized (see chapter 5) (Fig. 4d) (Szostak et al. 1983). Although the various repair events described above share some processing steps, they have different degrees of mutagenic potential. To date, many of the key repair factors for each pathway have been identified; however, how a cell determines which pathway to use for DSB repair is still poorly understood. The initial processing of broken DNA ends seems to be the key step that determines which pathway is used to repair a DSB, and will be described in the next section.

2 Processing of DNA breaks for repair

DNA end resection involves the degradation of the 5'-terminated DNA strand in the 5' to 3' direction from the break site to generate a 3' ssDNA overhang. Generation of this 3'-terminated ssDNA is essential to allow for the usage of homologous DNA sequences for repair. The homology may be either between the two resected ends of the broken DNA molecule, such as in case of MMEJ or SSA, or between the resected broken DNA molecule and an intact dsDNA template, such as in case of BIR, SDSA and canonical recombinational DSBR. Nucleolytic resection of DNA ends typically inhibits canonical NHEJ.

2.1 Processing of DNA ends for canonical NHEJ

Many canonical NHEJ events involve little or no processing of the broken DNA ends. The initial step of NHEJ involves the binding of the DNA ends by the Ku70-80 heterodimer, which forms a ring that encircles the duplex DNA (Gottlieb and Jackson 1993; Ramsden and Gellert 1998). This protects DNA ends from degradation, and recruits additional NHEJ components (Fig. 3a). Next, the Ku70-80-bound ends are tethered by DNA-dependent protein kinase catalytic subunit (DNA-PKcs), followed by ligation of the broken DNA ends by the XRCC4-XLF complex and DNA Ligase IV. The yeast Mre11-Rad50-Xrs2 (MRX) complex has a structural role to promote ligation, while the function

of the human MRE11-RAD50-NBS1 (MRN) complex in NHEJ is less apparent (Chen et al. 2001; Huang and Dynan 2002; Zhang et al. 2007; Rass et al. 2009; Xie et al. 2009).

In the case of DNA ends that are not directly ligatable, which may include those with DNA overhangs, gaps or blocking chemical groups, limited DNA end processing may be required. This involves a nucleolytic removal of overhangs or chemical groups by the human Artemis nuclease, which cleaves at the junctions of single and double-stranded DNA, and is activated by DNA-PKcs (Ma et al. 2002; Chang et al. 2017; Lobrich and Jeggo 2017). Artemis may not be the only NHEJ nuclease; other proteins, including APLF, Werner's syndrome helicase (WRN), the MRN complex, FEN1 and EXO1 may also play a role in some cases (Chang et al. 2017). Alternatively, the filling of DNA gaps at breaks may facilitate ligation, which is carried out by DNA polymerases μ and λ (Bebenek et al. 2014; Moon et al. 2014). Additionally, polynucleotide kinase (PNK) may remove 3' phosphate groups and phosphorylate 5' OH groups, which may be necessary for ligation (Chappell et al. 2002). Finally, one recent study found that extended DNA end resection can occur prior to NHEJ in G1 (Biehs et al. 2017), but further mechanistic analysis is required to fully understand this process.

2.2 Processing of DNA end for homologous recombination

In contrast to NHEJ, extended DNA end resection is an obligate step that initiates all recombination pathways. DNA end resection of the 5'-terminated DNA strand occurs in two main steps (Mimitou and Symington 2008; Zhu et al. 2008). The first step is catalyzed by the MRN complex and CtIP in human cells, and MRX and Sae2 in *S. cerevisiae* (Johzuka and Ogawa 1995; Keeney and Kleckner 1995; Paull and Gellert 1998; Sartori et al. 2007). The nucleolytic processing by these proteins is limited to the vicinity of the DNA end (generally up to 300 nt in yeast), and is thus referred to as short-range DNA end resection (Zhu et al. 2008). The most likely mechanism for the short range-resection by MRX/N is illustrated in Fig. 5. Resection is initiated by the endonucleolytic cleavage of the 5'-terminated DNA strand away from the DNA end, followed by 3'→5' exonuclease that proceeds back toward the DNA end.

Both exonuclease and endonuclease activities during the first resection step are likely catalyzed by MRE11/Mre11 in human and yeast cells (Neale et al. 2005; Garcia et al. 2011; Cannavo and Cejka 2014; Shibata et al. 2014). MRE11/Mre11 first endonucleolytically cleaves 5' terminated DNA in the vicinity of the DNA end. This endonucleolytic cleavage requires the ATPase activity of RAD50/Rad50 as well as CtIP/Sae2 and NBS1 (but not strictly Xrs2 in yeast) as co-factors (Cannavo and Cejka

2014; Anand et al. 2016; Deshpande et al. 2016; Oh et al. 2016; Kim et al. 2017). Importantly, the capacity of Sae2 and CtIP to promote MRE11/Mre11 depends on phosphorylation of key residues in CtIP/Sae2, at least some of which are under cyclin-dependent kinase CDK control (Huertas et al. 2008; Huertas and Jackson 2009; Cannavo and Cejka 2014; Anand et al. 2016; Deshpande et al. 2016). Cell-cycle dependent phosphorylation of CtIP/Sae2 represents one of the key control mechanisms that allow resection (and hence recombination) to initiate only in S and G2 phases of the cell cycle when a sister chromatid is available as a template for repair (Orthwein et al. 2015). Downstream of the endonuclease cut, MRE11/Mre11 subsequently uses its 3'→5' exonuclease activity to proceed back toward the DNA end, generating a 3' ssDNA overhang. Additionally, another nuclease, EXD2, may function alongside MRE11 exonuclease in human cells (Broderick et al. 2016). It has been also proposed that CtIP/Sae2 are nucleases (Lengsfeld et al. 2007; Makharashvili et al. 2014; Wang et al. 2014), but their potential catalytic functions in resection remain undefined.

The initial endonucleolytic cleavage away from the DNA end allows the resection machinery to bypass end-binding factors or non-canonical structures that may be present at the break end. This includes protein blocks, such as Spo11 in meiosis, stalled topoisomerases or Ku (Keeney and Kleckner 1995; Keeney et al. 1997; Neale et al. 2005; Bonetti et al. 2010; Mimitou and Symington 2010; Langerak et al. 2011; Chanut et al. 2016). Indeed, the efficiency of 5' DNA end cleavage *in vitro* by MRN-CtIP or MRX-Sae2 is stimulated by the presence of protein blocks at DNA ends (Cannavo and Cejka 2014; Anand et al. 2016; Deshpande et al. 2016). The short-range DNA end resection pathway is absolutely required for the processing of protein-blocked DNA ends, but may be dispensable for the resection of clean DNA ends in yeast (Neale et al. 2005; Zhu et al. 2008; Mimitou and Symington 2010). Instead, both MRE11 and CtIP are required for all resection events in human cells, although it remains to be defined whether resection always depends on the nuclease of MRE11 (Sartori et al. 2007).

The initial endonucleolytic cleavage by MRE11/Mre11 creates entry sites for the long-range resection enzymes. These subsequently catalyze resection in the 5'→3' direction away from the DNA end to generate extended ssDNA overhangs (up to several kilobases in length) and represent the second step of the resection process. The 3'→5' exonucleolytic DNA degradation by MRE11/Mre11 and the 5'→3' degradation by the long-range enzymes downstream of the endonucleolytic cut has been termed "bi-directional" resection. In addition to the nuclease function, the MRN/MRX complex has non-catalytic (i.e. structural) roles to recruit the long-range resection enzymes (Cejka et

al. 2010a; Nicolette et al. 2010; Niu et al. 2010; Nimonkar et al. 2011). The long-range resection factors include either of two nucleases, EXO1/Exo1 or DNA2/Dna2, which are well conserved between human and yeast cells (Gravel et al. 2008; Mimitou and Symington 2008; Zhu et al. 2008). EXO1/Exo1 is a dsDNA specific exonuclease (Tran et al. 2002), which specifically degrades the 5'-terminated DNA strand within dsDNA, generating 3' ssDNA overhangs. In contrast DNA2/Dna2 is an ssDNA specific 5'→3' nuclease that cannot process dsDNA on its own, and requires a cognate RecQ family helicase partner (Bae et al. 1998; Zhu et al. 2008; Levikova et al. 2013). This includes Sgs1 in yeast and either Bloom syndrome helicase (BLM) or WRN in human cells (Sturzenegger et al. 2014; Pinto et al. 2016; Levikova et al. 2017). Sgs1/BLM/WRN unwinds dsDNA to generate ssDNA, which becomes rapidly coated by replication protein A (RPA). RPA-coated ssDNA is subject to degradation by DNA2/Dna2 (Cejka et al. 2010a; Niu et al. 2010). RPA was found to promote 5' DNA end degradation by DNA2/Dna2, while at the same time inhibiting 3' end degradation. RPA, which physically interacts with the RecQ family helicase and DNA2/Dna2, is thus a critical factor that enforces the correct DNA polarity of DNA end resection by DNA2/Dna2 (Cejka et al. 2010a; Niu et al. 2010). Both human DNA2 and yeast Dna2, in addition to their essential nuclease activity, contain a helicase domain, which likely functions as an ssDNA translocase to facilitate the degradation of 5'-overhanged DNA by the DNA2/Dna2 nuclease (Levikova et al. 2017; Miller et al. 2017). The individual subunits of the Sgs1-Dna2-RPA, BLM-DNA2-RPA and WRN-DNA2-RPA complexes stimulate the activities of their partners to form integrated molecular resection machines (Cejka et al. 2010a; Niu et al. 2010; Pinto et al. 2016).

Multiple DNA end resection mechanisms described above lead to the formation of 3’-tailed ssDNA coated by RPA. The key function of RPA is to protect ssDNA from the action of nucleases and prevent the formation of secondary structures that might arise by self-annealing of ssDNA (Wold 1997). As will be described in the section below, in SDSA, BIR and canonical recombinational DSBR pathways, RPA must be replaced with the strand exchange protein RAD51. In contrast, SSA shares the initial DNA end resection with other recombination sub-pathways, but is RAD51-independent. Instead of using intact dsDNA as a template, SSA functions by annealing the two resected strands of DNA, using large stretches of sequence homology to form a stable complex between the two resected broken DNA molecules (Fig. 4a). This is dependent on RAD52/Rad52, which has a capacity to anneal RPA-coated ssDNA. After annealing, the non-complementary sequences are cleaved by XPF-ERCC1 (Rad1-Rad10 in yeast) and

any remaining gaps are filled and ligated to complete the repair of the DSB (Bardwell et al. 1994; Ivanov et al. 1996; Mortensen et al. 1996; Shinohara et al. 1998).

Broken DNA molecules signal the presence of DNA damage to the cellular checkpoint machinery. Generally, DSBs activate the ATM (Tel1 in yeast) kinase. In both systems, MRN/MRX plays a structural role to activate ATM/Tel1 (Carson et al. 2003; Uziel et al. 2003; Lee and Paull 2004; Lee and Paull 2005). Upon resection, RPA-coated ssDNA then activates the ATR-ATRIP (Mec1-Ddc2 in yeast) pathway (Zou and Elledge 2003). ATM/Tel1 and ATR/Mec1 sensors phosphorylate hundreds of protein targets at SQ/TQ motifs, which activate the proper response to DNA damage. This includes regulation of DNA repair components and checkpoint proteins, leading to cell cycle arrest and thus providing time for repair (Jackson and Bartek 2009). Additionally, unsuccessful repair and prolonged cell cycle arrest lead to the activation of apoptosis in higher eukaryotes (Jackson and Bartek 2009).

2.3 Processing of DNA ends for Alt-EJ (MMEJ)

In the absence of the key canonical NHEJ factors, including Ku70-80, DNA-PKcs, XRCC4, XLF and DNA Ligase IV, cells can still repair DSBs through an end-joining event referred to as MMEJ (Simsek and Jasin 2010; Chang et al. 2017) (Fig. 3b). The frequency of MMEJ in DSB repair is not very clear yet (Sfeir and Symington 2015), but it has been established that the process is more common in human cells compared to yeast. One of the hallmarks of MMEJ is the use of shared sequence microhomology between the break points, which is generally limited to ∼2-20 nt in length (Chang et al. 2017). Overlapping homologies were observed in junction points upon translocations in human cancers (Stephens et al. 2009), suggesting that they may arise due to MMEJ. Currently, only a few factors have been implicated in mediating human MMEJ, including FANCA, PARP1, DNA Ligase III, CtIP, DNA2 and Pol θ (Pol theta, also known as PolQ), but none of these appear to be absolutely essential for MMEJ (Audebert et al. 2004; Bennardo et al. 2008; Simsek et al. 2011; Howard et al. 2015; Mateos-Gomez et al. 2015; Mateos-Gomez et al. 2017). The use of microhomology for repair indicates that MMEJ and recombination-based mechanisms may share elements of DNA end resection. This is also supported by observations that Ku70-80 inhibits MMEJ, and Ku is known to be inhibitory for DNA end resection (Mimitou and Symington 2010). The end resection factors CtIP and DNA2 in human cells have been identified as being important to promote MMEJ events but it is still unclear if this is due to a direct role in DNA end resection (Zhang and Jasin 2011; Howard et al. 2015). Likewise, the genetic requirements for MMEJ remain to be defined

in yeast. Interestingly, MMEJ was increased in *mre11*, *sae2* and *sgs1 exo1* mutants, indicating that the absence of the canonical DNA end resection factors is not limiting for MMEJ (Wang et al. 2006; Deng et al. 2014). Whether another yet unidentified nuclease is essential for MMEJ is not clear. RPA is clearly a strong inhibitor of MMEJ in both yeast and human cells (Deng et al. 2014; Mateos-Gomez et al. 2017). This shows that the more extensive resection creating ssDNA of lengths that strongly associate with RPA channels repair toward HR. In accord, the motor activity of human Pol θ was found to displace RPA to promote MMEJ, showing that the balance between HR and MMEJ is controlled by the opposing activities of Pol θ and RPA (Mateos-Gomez et al. 2017).

3 Formation of RAD51-ssDNA filament on resected DNA

Resection of the 5'-terminated DNA strand at DSBs leads to the formation of 3'-overhanged ssDNA, which is initially coated by RPA. In a subsequent step, RPA is replaced by the key recombination protein RAD51 (Rad51 in yeast) (Fig. 6). RAD51 and ssDNA form a nucleoprotein filament, also called a pre-synaptic filament. This catalyzes the signature step of the recombination pathway, which includes homology search, pairing with the intact donor (also called template) dsDNA and strand invasion (Benson et al. 1994; Sugiyama et al. 1997). The transient interaction of the RAD51 nucleoprotein filament with the template dsDNA is referred to as a synaptic complex. This ultimately leads to the displacement of one of the template DNA strands forming a displacement loop (D-loop, also called joint molecule) intermediate (Fig. 6), which represents the post-synaptic stage. All these steps are controlled (both positively and negatively) by a number of recombination regulators, which often physically interact with RAD51 and allow recombination to occur only in the proper context.

To understand mechanisms underlying the regulation of RAD51, it is important to understand that RAD51 has a capacity to bind both single- and double-stranded DNA, but only ssDNA binding is thought to promote recombination, while dsDNA binding by RAD51 is generally inhibitory (Zaitseva et al. 1999). Furthermore, RAD51 binds and hydrolyzes ATP. ATP binding, but not ATP hydrolysis, by RAD51 is required for stable RAD51-DNA binding; in contrast, ATP hydrolysis by RAD51 is required for the steps downstream of DNA invasion (Sung 1994; Baumann et al. 1996; van Mameren et al. 2009). RAD51 can also hydrolyze ATP non-productively (i.e., without catalyzing strand invasion); also in this case, ATP hydrolysis leads to a reduction in its capacity to bind DNA. Recombination can be stimulated on several levels by affecting these RAD51 activities. This includes RAD51 loading on RPA-coated ssDNA (i.e. exchange of RPA with RAD51), reduction of RAD51's capacity to bind dsDNA, stabilization of the nascent

nucleoprotein filament by inhibiting its disassembly, as well as remodeling of the RAD51 nucleoprotein filament into a conformation that is optimally permissive for DNA strand exchange. These control mechanisms are in place to prevent aberrant recombination (Heyer 2015). This includes recombination between non-homologous sequences, which may lead to DNA translocations and genome rearrangements, or recombination during physiological processes of DNA metabolism when ssDNA is present, such as during unperturbed DNA replication or transcription. The proper interplay of both positive and negative recombination regulators ascertains that recombination occurs only when it is needed to optimally maintain genome stability (Heyer 2015).

The first of the control mechanisms is the replacement of RPA with RAD51 on resected ssDNA. Due to the higher affinity toward ssDNA, RPA initially outcompetes RAD51 for ssDNA binding. To overcome this apparent inhibitory effect of RPA, cells have various recombination mediator proteins that help load RAD51 onto ssDNA, displacing RPA in the process. In yeast, the key recombination mediator that displaces RPA from ssDNA is Rad52. The interaction of Rad51 with Rad52 is required for this process (Benson et al. 1998; Song and Sung 2000). Human RAD52, in contrast, possesses no recombination mediator activity, and its contribution to recombination in human cells is much more subtle than in yeast, while *Caenorhabditis elegans* and *Drosophila melanogaster* lack RAD52. The main mediator protein in higher eukaryotes, including worms, flies and humans, is the product of the breast cancer susceptibility gene 2 (BRCA2) and its homologues (Yang et al. 2005; Petalcorin et al. 2007; Jensen et al. 2010). Depletion of BRCA2 in cells treated with ionizing radiation (IR) leads to persistent RPA and decreased RAD51 foci formation on broken DNA, clearly indicating its role in RAD51 loading (Yuan et al. 1999). BRCA2 contains eight BRC repeats (conserved motifs of about 35 amino acids), which can all independently interact with RAD51, although only four to five repeats associate with RAD51 at a given time (Carreira et al. 2009; Jensen et al. 2010). The repeats 1-4 bind RAD51 with a high affinity and promote RAD51 nucleation on ssDNA; repeats 5-8 show a lower affinity to free RAD51 and rather stimulate the growth of the nascent RAD51 filament (Jensen et al. 2010; Liu et al. 2010; Thorslund et al. 2010). Mechanistically, the BRC repeats 1-4 of BRCA2 promote ssDNA binding of RAD51 by inhibiting its ATPase activity, which stabilizes ssDNA binding of RAD51. Additionally, BRCA2 promotes recombination by inhibiting RAD51's dsDNA binding activity (Jensen et al. 2010). The displacement of RPA from ssDNA by BRCA2 is further facilitated by DSS1, a direct interaction partner of BRCA2 (Yang et al. 2002; Zhao et al. 2015). BRCA2 also interacts with PALB2, which was shown to promote RAD51-mediated DNA strand exchange on its own (Buisson et al. 2010). In cells, PALB2

mediates BRCA2's recruitment to DNA damage and bridges BRCA2's interaction with BRCA1 (Sy et al. 2009). Cellular assays established that these interactions are critical for recombination, although the mechanisms how BRCA1 and PALB2 proteins affect BRCA2's recombination mediator activity remain to be defined. RAD51 nucleoprotein filament assembly is also stimulated by RAD54, independently of RAD54's ATPase activity (Wolner and Peterson 2005). Additional proteins including the MMS22L-TONSL complex may promote the RAD51 nucleoprotein filament assembly during perturbed DNA replication (Piwko et al. 2016).

The activity of RAD51 is also promoted by a group of proteins termed RAD51 paralogs. The respective genes likely arose during evolution through a duplication of the RAD51 gene, and share around 20 to 30 % of sequence homology with RAD51. The paralogs are represented by five polypeptides in human cells: RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3, which form two major complexes: RAD51B-RAD51C-RAD51D-XRCC2 (BCDX2) and RAD51C-XRCC3 (CX3) (Masson et al. 2001). Neither of the paralog proteins nor complexes exhibits DNA strand exchange activity on their own, and all likely function *via* regulating RAD51. Phenotypically, depletion of these complexes generally results in fewer RAD51 foci in response to ionizing radiation. This resembles, although to a lesser extent, the depletion of BRCA2. The paralogs likely function in the same pathway as BRCA2 as indicated by epistatic interactions (Qing et al. 2011). The first mechanistic report indicated that the RAD51B-RAD51C proteins, similarly to BRCA2, have a recombination mediator activity to facilitate loading of RAD51 on RPA-coated ssDNA, displacing RPA in the process (Sigurdsson et al. 2001). In yeast, the RAD51 paralogs include Rad55 and Rad57 (Sung 1997; Liu et al. 2011a). Rad55-Rad57 physically interact with the Shu complex, comprising of Csm2, Psy3, Shu1 and Shu2 polypeptides, and Rad52. The subunits of the supercomplex synergize in their capacity to displace RPA and facilitate Rad51-ssDNA nucleoprotein filament formation (Gaines et al. 2015). The human ortholog of Shu2 is SWS1, which forms a complex with SWSAP1 (Martin et al. 2006; Liu et al. 2011b). The heterodimer binds DNA and interacts with RAD51, RAD51D and XRCC2. Depletion of SWS1 results in a reduction of spontaneous and radiation-induced RAD51 foci. This suggests that SWS1 in humans also likely functions in conjunction with the RAD51 paralogs, but their interplay, as well as their relationship to BRCA2's function, remain undefined. Evidence from the *C. elegans* model system instead suggests that the RAD51 paralogs, represented by RFS-1 and RIP-1, function downstream of BRCA2, specifically in the stabilization of the RAD51 nucleoprotein filament and its remodeling into a species that is optimally capable to

invade template dsDNA (Taylor et al. 2015; Taylor et al. 2016). RAD51 filaments are also stabilized by the SWI5-SFR1 complex (Akamatsu and Jasin 2010; Tsai et al. 2012).

It is well established that there is a balance between factors that promote the formation and disrupt RAD51/Rad51 nucleoprotein filaments (Heyer 2015). In yeast, Srs2 can dismantle Rad51 filaments due to its ATP-powered DNA translocase activity, and additionally *via* stimulating the ATP hydrolysis of Rad51 through a direct physical interaction, which destabilizes the nucleoprotein filament (Krejci et al. 2003; Veaute et al. 2003; Liu et al. 2011a). In humans, no direct homolog of Srs2 has been identified, yet the most probable candidates for this function are RECQ5, FBH1, PARI or BLM helicases (Bugreev et al. 2007b; Hu et al. 2007; Fugger et al. 2009; Schwendener et al. 2010; Moldovan et al. 2012; Patel et al. 2017). To elucidate the functional interplay of the various RAD51 regulators represents a challenge for future research. The proper balance between pro- and anti-recombination factors is required to execute recombination only when it is needed and thus prevent illegitimate recombination and genome rearrangements.

4 Homology search and DNA strand exchange

Once the pre-synaptic filament is formed and stabilized, it begins the search for a homologous sequence. In most cases in vegetative cells, the sister chromatid is used as a repair template. A recent report demonstrated that the human BRCA1 protein, which is similarly as BRCA2 a tumor suppressor that is frequently mutated in familiar breast and ovarian cancers, directly promotes DNA invasion. This likely occurs through promoting the assembly of the synaptic complex or promoting the homology search (Zhao et al. 2017). Despite the complex nuclear environment, the course of homology search – the process that occurs immediately before the synaptic phase – is relatively fast. In yeast, it has been demonstrated that the mobility of a cut chromosome is increased, which allows the Rad51 filaments to explore a larger nuclear volume (Dion et al. 2012; Mine-Hattab and Rothstein 2012). Additionally, broken DNA relocalizes to the nuclear periphery (Oza et al. 2009; Chiolo et al. 2011; Horigome et al. 2014; Ryu et al. 2015). Although the exact mechanism of homology search is still undefined, it is suggested that the pre-synaptic filament randomly probes the genome by making multiple temporary contacts with different DNA duplexes (Forget and Kowalczykowski 2012; Renkawitz et al. 2014; Qi et al. 2015). Contacts with very short microhomologies are unstable; instead, contacts with more than 7 nt of homology are more stable, which allows the presynaptic complex to probe flanking sequences for additional homology. Once

homology is identified, the pre-synaptic filament invades the duplex DNA, displaces the original strand and binds its complementary sequence by Watson-Crick pairing. It has been estimated that the efficiency of repair in yeast was decreased to ~14% when template sequence diverged by about one in every eight nucleotides (Anand et al. 2017). Following strand invasion, the displaced ssDNA within the D-loop structure is stabilized by RPA, which prevents reversal of the D-loop formation (Lavery and Kowalczykowski 1992; Eggler et al. 2002). Additionally, RAD51 is removed from the heteroduplex dsDNA of the D-loop structure. This function is catalyzed by RAD54, a dsDNA translocase that uses its ATPase-powered motor activity to displace RAD51 from dsDNA (Solinger et al. 2002; Mason et al. 2015)(Fig. 6). RAD54 thus promotes D-loop stability, which allows DNA synthesis and facilitates canonical HR (Ceballos and Heyer 2011; Wright and Heyer 2014).

The 3'-terminated strands within D-loop structures that resist disassembly can prime DNA synthesis. Although translesion polymerases have been implicated in HR (Kawamoto et al. 2005; McIlwraith et al. 2005), most of DNA synthesis during recombination is likely catalyzed by either polymerase δ or polymerase ε (Li et al. 2009; Hicks et al. 2010; Wilson et al. 2013). However, the DNA synthesis during recombination is about three orders of magnitude more error-prone than in DNA replication, most likely due to limited activity of DNA mismatch repair during recombination-dependent repair DNA synthesis (Hicks et al. 2010). DNA synthesis extends the length of the paired duplex, which further stabilizes the joint molecule, leading to the recovery of the missing genetic information in the broken DNA molecule by using the invaded molecule as a template. Downstream of strand invasion and DNA synthesis, recombination proceeds into either of three recombination sub-pathways: BIR, SDSA and canonical HR (see chapter 1.3 for general introduction to these pathways and Fig. 4). In BIR, the invaded DNA molecule is stabilized, and DNA synthesis proceeds along the whole length of the template DNA *via* a bubble-like structure to the chromosome end (Llorente et al. 2008; Sakofsky and Malkova 2017). The extended DNA synthesis is likely catalyzed by polymerase δ, in conjunction with the Pif1 helicase, which promotes the strand displacement activity of polymerase δ (Wilson et al. 2013). Interestingly, DNA synthesis during BIR occurs conservatively, and thus dramatically differs from canonical DNA replication (Donnianni and Symington 2013). How exactly the synthesis of the complementary strand is achieved remains undefined. The BIR pathway occurs at the cost of elevated mutagenesis, and is thus primarily utilized when no alternative is available, such as in the absence of the second DNA end.

In SDSA, the extended invaded DNA strand separates from the template DNA, and anneals to the second end of the broken DNA molecule. DNA synthesis and ligation then complete the repair process. In canonical HR instead, through a process termed second DNA end capture, the second broken DNA end anneals to the displaced ssDNA strand of the D-loop structure (Fig. 4). The strand annealing employs the Rad52 protein in yeast (Nimonkar et al. 2009). Although human RAD52 also possesses the strand annealing activity (McIlwraith and West 2008; Jensen et al. 2010), the effects of RAD52-deficiency are modest and are particularly revealed only in the absence of BRCA2 (Feng et al. 2011). This raises questions whether the second end capture in human cells is mediated by annealing or a second DNA strand invasion event (Kowalczykowski 2015).

The balance between SDSA and canonical recombinational DSBR is regulated by activities that either disrupt or promote stability of the D-loop structure. In most cases, the stability of D-loops is dictated by motor proteins such as DNA helicases that act by moving the junctions. Mph1 in yeast cells and BLM, RECQ1 and RTEL1 in human cells have been implicated in D-loop dissociation and promotion of SDSA (Bugreev et al. 2007b; Barber et al. 2008; Bugreev et al. 2008; Prakash et al. 2009; Daley et al. 2013; Mitchel et al. 2013). In yeast, D-loops can also be disrupted or "dissolved" by an alternative mechanism that involves topoisomerase III (Top3), which is also thought to promote SDSA (Fasching et al. 2015). Whether this process also functions in human cells is not yet clear. Additionally, RAD54's branch migration activity has been implicated to disrupt D-loops downstream of RAD51-mediated strand invasion *in vitro* (Bugreev et al. 2007a), but the biological significance of this function may be restricted to limiting recombination with non-homologous DNA (Ceballos and Heyer 2011; Wright and Heyer 2014). Regulating the balance between SDSA and HR is important, as it affects the genetic outcome of recombination. Whereas SDSA only leads to non-crossover products, canonical HR is a pathway that can potentially produce crossovers, as will be described in the next chapter.

5 Processing recombination intermediates

D-loop stability determines the pathway choice between SDSA and canonical recombinational DSBR. In SDSA, the D-loop is disrupted, whereas in recombinational DSBR, the D-loop is stabilized and becomes a substrate for annealing with the second resected dsDNA end (Fig. 4). This gives rise to a "double" or "complement-stabilized" D-loop. This structure forms as a result of annealing activity catalyzed by Rad52 in yeast, and does not represent a second strand invasion step (Nimonkar et al. 2009). Rad52

was shown to promote annealing of ssDNA to D-loops generated by cognate Rad51 and Rad54 in the presence of RPA, indicating that the process likely requires direct protein-protein interactions. This function of Rad52 is apparent in unicellular eukaryotes, while RAD52 in human cells has a much less defined function (Feng et al. 2011). It is possible that in high eukaryotes other proteins such as BRCA2 might be involved in second end capture in addition to RAD52. It remains to be established whether second end capture in human cells employs annealing and/or strand invasion mechanisms.

Following second end capture, DNA synthesis and ligation gives rise to a central intermediate of canonical HR, termed a double Holliday junction (dHJ)(Duckett et al. 1988). As both DNA molecules are physically linked at the junction points, HJs need to be processed prior to separation of both DNA molecules (Fig. 7). A failure to process HJs leads to chromosome segregation defects, and may be one of the mechanisms responsible for genome instability (Wechsler et al. 2011). Due to the homology between the recombining DNA molecules, a key feature of endogenous HJs is their mobility: the junction points can move in either direction to a limited extent spontaneously, or more extensively in ATP-hydrolysis driven reactions catalyzed by molecular machines, such as DNA helicases or translocases. Double HJs can be processed by resolution or dissolution-based mechanisms. As will be described below, these processes are enzymatically distinct and lead to diverse genetic outcomes. Whereas dissolution leads to non-crossover products, resolution gives rise to both crossovers and non-crossovers (Fig. 4 and Fig. 7).

5.1 Dissolution of double Holliday junctions

Dissolution separates the recombining DNA molecules without exchanging the flanking sequences. As somatic cells employ mechanisms to maximally preserve genome integrity, dissolution is the default mode of dHJ processing. In yeast, dissolution is carried out by the Sgs1-Top3-Rmi1 (STR) complex (Cejka et al. 2010b), while in human cells the "dissolvasome" consists of BLM, topoisomerase IIIα and RMI1-RMI2, forming the BTRR complex (Wu and Hickson 2003; Singh et al. 2008; Xue et al. 2013). As Sgs1, BLM can unwind various DNA structures and branch migrate HJs *in vitro* (Wu and Hickson 2003). The dissolution reaction involves migration of the two Holliday junctions toward each other (i.e. convergent branch migration) by the combined activity of the RecQ family BLM/Sgs1 helicase and the strand passage activity of the TOPOIIIα/Top3 type IA topoisomerase (Fig. 7a). As both Holliday junctions link two DNA molecules of long lengths, the ends are not free to rotate, and the dHJ structure is

topologically constrained. Therefore, a branch migration activity of Sgs1/BLM is not sufficient to migrate endogenous HJs (Cejka et al. 2010b; Chen et al. 2014). It has been demonstrated that the strand passage activity of TOPOIIIα/Top3 is required to relieve positive supercoiling that would otherwise form ahead of (between) the convergently migrating junctions and prevent further movement (Chen et al. 2014). Mechanistically, Top3/TOPOIIIα creates a transient nick in ssDNA that allows the other ssDNA strand to pass through it, thereby allowing the relaxation of the torsional stress forming between the junctions during convergent branch migration. RMI1/Rmi1 does not significantly affect the initial DNA branch migration step, but seems to specifically promote a late step just prior to dissolution (Cejka et al. 2010b; Bocquet et al. 2014). The last predicted intermediate of convergent branch migration is a hemicatenane: this structure represents a junction between two dsDNA molecules, where one strand of one duplex is wrapped around another strand from the second DNA duplex (Fig. 7a). It has been proposed that RMI1/Rmi1 specifically promotes the processing of a hemicatenane (Cejka et al. 2012). RMI2, which is only present in high eukaryotes, likely has only a minor function in dHJ dissolution, and may have other roles such as to target BLM to blocked DNA replication forks (Singh et al. 2008). Finally RPA, which physically interacts with BLM/Sgs1 and RMI1/Rmi1 subunits of the STR/BTRR complexes, also stimulates dissolution (Cejka et al. 2010b; Xue et al. 2013). This likely stems from RPA's capacity to promote the helicase of BLM/Sgs1 and strand passage of TOPOIIIα-RMI1/Top3-Rmi1, likely through its ssDNA binding activity to prevent re-annealing, as well as because of the direct physical interaction of RPA with RMI1/Rmi1 (Brosh et al. 2000; Plank et al. 2006; Cejka et al. 2012; Xue et al. 2013). The unique mechanism of dHJ processing by dissolution exclusively results in non-crossover events, which maintains genome stability in vegetative cells (Fig. 7a). BLM-deficient cells are characterized by elevated levels of sister chromatid exchanges, which are a hallmark of genome rearrangements and may contribute to cancer predisposition of Bloom syndrome patients (Chaganti et al. 1974). These rearrangements result from elevated usage of pathways that are alternative to dissolution and that are dependent on the structure-specific nucleases described in the next chapter.

5.2 Resolution of double Holliday junctions

Double HJs that evade dissolution are resolved by structure specific nucleases later in the cell cycle to give rise to both crossover and non-crossover recombination products. Nucleolytic cleavage also represents the only option for the processing of single HJs.

These may form during DSB repair where only one strand invades template DNA, or when one of the D-loop arms has been cleaved prior to second end capture (Wechsler et al. 2011; Shah Punatar et al. 2017). To date, three structure-specific nucleases capable of processing HJs or similar structures have been identified in eukaryotic cells, including human MUS81-EME1 and yeast Mus81-Mms4, human SLX1-SLX4 and yeast Slx1-Slx4 as well as human GEN1 and yeast Yen1 (Dehe and Gaillard 2017). The Mus81-Mms4 complex in yeast was found to be essential for viability in cells lacking Sgs1-Top3, indicating its role in the processing of recombination intermediates that would have otherwise been processed by the Sgs1-Top3 complex (Hickson and Mankouri 2011). Mus81 belongs to the XPF family of nucleases and represents the catalytic subunit of the heterodimer. Both yeast and human Mus81-Mms4/MUS81-EME1 complexes prefer to cleave replication forks, 3' flaps and nicked HJs (Gaillard et al. 2003; Ehmsen and Heyer 2008). MUS81-EME1/Mus81-Mms4 cleave intact HJs inefficiently in an asymmetric manner by introducing a nick a few nucleotides away from the junction, producing gapped and flapped DNA products, which are unsuitable for ligation and necessitate further processing (Wyatt et al. 2013). In human cells, MUS81 forms another complex with EME2, which has a broader substrate specificity than MUS81-EME1, and has roles in replication fork restart (Pepe and West 2014b). SLX1/Slx1 belongs to the GYI-YIG family of nucleases and is the catalytic subunit of the SLX1-SLX4 heterodimer. The SLX1-SLX4/Slx1-Slx4 complex can cleave branched structures with a preference toward Y-structures, 5' flaps and replication forks. While it can cleave intact HJs, the cleavage is inefficient, asymmetrical and creates poorly ligatable products (Fricke and Brill 2003; Fekairi et al. 2009; Svendsen et al. 2009; Wyatt et al. 2013). Both MUS81 and SLX1 complexes are thus non-canonical HJ resolvases. The only *bona-fide* eukaryotic HJ resolvase is GEN1/Yen1, which belongs to the RAD2/XPG family of nucleases. As the *Escherichia coli* RuvC, GEN1/Yen1 dimerizes on HJs, and cleaves HJs by a dual incision mechanism that produces two ligatable products (Dunderdale et al. 1991; Ip et al. 2008; Rass et al. 2010). However, both GEN1 and Yen1 are capable of cleaving other branched DNA structures as well. The random nature of HJ cleavage by the various nuclease complexes gives rise to both crossover and non-crossover recombination products (Shah Punatar et al. 2017) (Fig. 7).

In human cells, both the SLX1-SLX4 and MUS81-EME1 dimers associate to form the SLX1-SLX4-MUS81-EME1 tetramer (SM complex). The formation of the SM complex is mediated by the interaction between MUS81 and SLX4 polypeptides (Wyatt et al. 2013). Biochemical studies demonstrated that the SM complex exhibits higher HJ resolution activity compared to its individual subunits. Mechanistically, SLX1-SLX4 makes the first

nick in the HJ creating a substrate for MUS81-EME1, which then makes a second counter-nick in the opposing strand, allowing for efficient HJ resolution (Castor et al. 2013; Wyatt et al. 2013). This activity is further enhanced by interaction with a third nuclease complex, the XPF-ERCC1 dimer, which has a structural (i.e. non-catalytic) role to promote HJ cleavage (Wyatt et al. 2017)(Fig. 7b). The catalytic subunit within the XPF–ERCC1 dimer is XPF, which has established functions in nucleotide excision repair. It should be pointed out that the functions of MUS81-EME1/Mus81-Mms4 and SLX1-SLX4/Slx1-Slx4 are not specific to processing recombination products, and both enzyme complexes may cleave structures arising during other DNA repair processes, as well as during replication or telomere maintenance. Likewise, the SLX1-SLX4-MUS81-EME1-XPF-ERCC1 (SMX) trinuclease complex has a broad substrate specificity that is not restricted to HJ cleavage and likely functions in multiple DNA metabolic pathways (Wyatt et al. 2017). The formation of the nuclease complexes appears specific to higher eukaryotes, as no such collaborative action of nucleases has been observed in yeast.

5.3 Regulation of Holliday junction processing

To maximally preserve genome stability, vegetative cells evolved mechanisms that facilitate dissolution pathway usage over resolution, which limits crossover formation. Additionally, the activity of the nucleases capable of cleaving branched DNA structures must be carefully controlled to avoid promiscuous DNA cleavage. The preferential employment of dissolution over resolution by structure-specific nucleases is governed by tight spatial and temporal control (Matos and West 2014). The STR/BTRR complex is likely active in any phase of the cell cycle, and thus processes the majority of dHJs from S-phase to mitosis in both yeast and human cells. The activity of yeast Mus81-Mms4 is instead low in S-phase, and becomes elevated at the onset of mitosis by phosphorylation of the Mms4 subunit by CDK and Cdc5 (Matos et al. 2011; Matos et al. 2013). In human cells, the phosphorylation of EME1 does not activate MUS81 directly, but rather promotes the formation of the SLX1-SLX4-MUS81-EME1 (SM) complex with increased activity toward HJs (Wyatt et al. 2013). Yeast Yen1 is inactive in its phosphorylated state in S phase, and becomes activated by Cdc14-mediated dephosphorylation late in mitosis. Interestingly, phosphorylation of Yen1 not only inhibits its catalytic activity by limiting its association with DNA, but also prevents nuclear import (Kosugi et al. 2009; Blanco et al. 2014). Nuclear exclusion appears to be the primary mechanism that restricts human GEN1 activity (Chan and West 2014). The nuclear export sequence within GEN1 ascertains that GEN1 can process recombination intermediates only when the nuclear

envelope breaks down during mitosis. These regulatory mechanisms collectively ascertain that the structure-specific nucleases are only activated late in the cell cycle to remove any residual junctions that were not processed by the dissolvasome complex. Although potentially mutagenic, efficient processing of all joint molecules by resolution mechanisms is essential to prevent chromosome missegregation in mitosis (Wechsler et al. 2011).

6 Specialized roles of DSB repair pathways

Cells evolved mechanisms to repair accidental DNA breaks to achieve maximal efficiency and accuracy in the maintenance of genome integrity. However, not all DSBs are pathological, and there are several cases when DSBs are introduced deliberately, which serves specific physiological purposes. The best examples are processes occurring during lymphocyte development and in meiosis. As will be seen below, during these events cells make use of the DSB repair pathways to instead generate diversity. Both processes represent fascinating examples of the plasticity of the DSB repair systems.

6.1 DSB repair pathways in the immune system

V(D)J recombination is a process that occurs during B and T lymphocyte development and involves a random rearrangement of the variable (V), diversity (D) and joining (J) segments of immunoglobulin genes (Arya and Bassing 2017) (Fig. 8a). The random assembly of these VDJ segments allows producing a wide variety of antigen receptors from a limited number of gene segments. Despite its name, V(D)J recombination is facilitated by canonical NHEJ factors, and does not involve homologous recombination. Disruption of key NHEJ factors results in severe immune disorders in humans and mice, indicating their essential role in V(D)J recombination. The MRN complex was found to have a function in V(D)J recombination, but this appears to be dependent on its capacity to promote checkpoint signaling *via* activation of the ATM kinase, and not a role in HR (Uziel et al. 2003; Helmink et al. 2009).

V(D)J recombination is initiated by the recombination-activating genes 1 and 2 (RAG1 and RAG2), which are expressed in developing lymphocytes and therefore restrict this process to these cells (McBlane et al. 1995; Schatz and Swanson 2011). RAGs bind to the recombination signal sequences (RSS) flanking the V, D, and J gene segments and first create an ssDNA nick at the junction between the coding region and the RSS. There are two types of RS sequences, which differ with respect to the length of a spacer region between the two identical sequences. The spacers are of 12 or 23 bps in

length, giving rise to RS12 and RS23 sequences (Fig. 8a). Next, RAGs catalyze the formation of a paired complex, where the RS12 and RS23 sequences associate with the same RAG complex. The free hydroxyl group of the nicked strand then invades the phosphodiester bond of the intact strand, generating a DSB with different DNA ends. The coding end of the DSB contains a hairpin loop, whereas the signal end is blunt. Next, the blunt signal ends are ligated together to form a circular piece of DNA that is lost during subsequent cell divisions. The hairpin coding ends are processed and joined by the NHEJ machinery to fuse the respective gene segments. First, Ku70-80 and DNA-PKcs bind to the coding ends and recruit other additional NHEJ factors, including Artemis, XRCC4-XLF and DNA ligase IV (Fig. 8a). Next, the Artemis nuclease is activated upon autophosphorylation of DNA-PKcs and opens the hairpin loop of the coding ends, which can then be ligated to the other coding ends by the XRCC4-XLF and DNA ligase IV complex (Li et al. 1995; Casellas et al. 1998; Ma et al. 2002). The creation of DSBs with different ends and the requirement for association of the 12- and 23-RS sequences in human B and T cells is essential for regulated processing to ensure that coding sequences of V, D and J, but not homotypic gene segments are joined. The final product of V(D)J recombination is a DNA coding sequence consisting of randomly rearranged V, D and J gene segments (Schatz and Swanson 2011).

Class switch recombination (CSR) and somatic hypermutation (SHM) only occur in activated germinal center B cells. CSR leads to the change of antibody isotype and thus its effector function, while SHM affects the variable regions of the immunoglobulin genes to promote diversity of antibodies. Both CSR and SHM are independent of RAGs and instead are triggered by activation-induced cytidine deaminase (AID) (Muramatsu et al. 2000; Arakawa et al. 2002; Petersen-Mahrt et al. 2002)(Fig. 8b). AID specifically deaminates cytosines into uracils, resulting in the formation of U:G mismatches. During CSR, the Cμ exon is exchanged by Cγ/ε/α exons, resulting in the change of the antibody isotype from IgM to IgG, IgE or IgA (Methot and Di Noia 2017). Unlike RAGs that are targeted to very specific sequences, AID in CSR is active on large regions (1-3 kb) of repetitive DNA sequences upstream of C_H genes (coding for constant regions of immunoglobulin heavy chains), called the switch (S) regions. The U:G mismatches are mainly processed by the base excision repair machinery (BER), which creates ssDNA breaks as intermediates of the repair process (Rada et al. 2002; Imai et al. 2003; Schrader et al. 2005). Closely spaced ssDNA breaks then give rise to staggered DSBs (DSBs with ssDNA overhangs)(Fig. 8b). Additionally, a smaller fraction of DNA breaks may form as a result of a non-canonical function of the DNA mismatch repair machinery, which involves the exonuclease activity of Exo1 (Ehrenstein and Neuberger 1999;

Schrader et al. 2007; Bregenhorn et al. 2016). The exonucleolytic processing of one DNA strand then collides with a nick in the opposite DNA strand, leading to DSBs (Fig. 8b). Irrespectively of the exact mechanism, the resulting DSBs often contain ssDNA overhangs, which need to be either filled or cleaved prior to joining by the canonical NHEJ machinery. DSBs with overhangs bearing microhomologies may also be joined by the MMEJ pathway (Lee-Theilen et al. 2011). As with V(D)J recombination, CSR is also dependent on end-joining mechanisms, and it is thus not a homologous recombination process. Interestingly, MRN was found important for CSR, where it likely has a structural role to promote both canonical NHEJ and MMEJ pathways (Dinkelmann et al. 2009).

Somatic hypermutation is, as CSR, dependent on AID (Arakawa et al. 2002; Pham et al. 2003). In contrast to CSR, SHM leads to antibody diversification through mutagenesis in the V regions of light and heavy chains of immunoglobulins. It has been estimated that the mutation rate in the V regions during SHM is about 6 orders of magnitude higher than in the rest of the genome. The majority of mutations are single base substitutions, with a small fraction of short insertions or deletions. This suggests that the formation of a DSB is not an obligate step in SHM, and in fact NHEJ-deficient cells do not show significant defects in SHM. However, some DSBs form during SHM (Papavasiliou and Schatz 2000). To this point, it has been demonstrated that DSBs during SHM can be repaired by homologous recombination (Papavasiliou and Schatz 2000; Zan et al. 2003); furthermore, HR has been implicated as a safeguard against off target AID activity (Hasham et al. 2010; Zahn et al. 2014). Interestingly, MRN was found to promote SHM, but the underlying mechanisms remain unclear (Yabuki et al. 2005).

Finally, the insertion of DNA fragments of various sizes has been recently reported to occur in the switch region of immunoglobulin genes (Tan et al. 2016; Pieper et al. 2017). The DNA sequences originate from another part of the genome and the transfer occurs *via* a copy-paste rather than a cut-paste process. This may represent a novel mechanism for antibody diversification, although the molecular pathways required for these transactions are unknown. Interestingly, insertions of fragments from the collagen receptor LAIR1 into immunoglobulin genes lead to antibodies that are broadly reactive to malaria (Tan et al. 2016; Pieper et al. 2017). It is important to point out that during B cell maturation, clones that produce high affinity antibodies are positively selected while low affinity clones are eliminated. Due to the selection process, even infrequent events may thus become physiologically relevant (Pieper et al. 2017).

6.2 Homologous recombination in meiosis

Meiosis is a specialized cell division that is required for genome haploidization (Keeney et al. 2014). This is essential to form spores or gametes in sexually reproducing unicellular or multicellular eukaryotes, and makes sure that ploidy is maintained with each successive generation (Fig. 9). DSBs are introduced during meiosis, and become substrates for the homologous recombination machinery (Sun et al. 1989; Sun et al. 1991). The function of meiotic recombination in most organisms including yeast and vertebrates (but not *Drosophila* and *Caenorhabditis*) is to make physical connections between homologous chromosomes to facilitate their proper alignment and subsequent segregation. Additionally, recombination creates genetic diversity in the population by exchanging DNA regions between maternal and paternal chromosomes. The key mechanistic differences between HR in vegetative cells and during meiosis are (a) the formation of DNA breaks, which are induced in a programmed manner during meiosis; (b) the preferential usage of the homologous chromosome as a template for repair and (c) a bias for preferential resolution of recombination intermediates into crossovers that are non-randomly spaced (Fig. 9) (Hunter 2015).

DSBs are introduced during the first meiotic division by the SPO11/Spo11 transesterase, which is evolutionarily conserved from yeast to mammals (Keeney and Kleckner 1995; Keeney et al. 1997; Mahadevaiah et al. 2001; Robert et al. 2016). SPO11 is a topoisomerase-like protein that cleaves both strands of dsDNA, but remains covalently attached to the 5'-terminated DNA strand upon cleavage. In yeast, at least 9 other proteins are required for Spo11 to cleave DNA, including the MRX complex (Keeney 2008). Interestingly, the nuclease activity of MRE11 is dispensable for DNA cleavage by Spo11 in yeast, but it is instead essential to initiate the processing of Spo11-bound DNA breaks (Borde et al. 2004; Lam and Keeney 2014). As with other protein-blocked DNA ends, the first resection step requires the nuclease activity of the MRN/MRX complex with its co-factor CtIP/Sae2 to remove SPO11/Spo11 from the break ends (Keeney and Kleckner 1995; Neale et al. 2005). Resection by MRX-Sae2 can proceed up to several hundred of nucleotides in length. This is followed by long-range resection, which appears to be exclusively carried out by the Exo1 branch in yeast meiotic cells (Zakharyevich et al. 2010; Mimitou et al. 2017). In mice, the average resection length is 0.9 kb (Lange et al. 2016), which is similar to the value obtained in yeast (Zakharyevich et al. 2010).

The resected 3’ DNA tail is bound by RPA and successively replaced by DMC1 (a meiosis specific strand exchange protein) together with RAD51 (Bishop et al. 1992; Cloud et al. 2012). Whereas RAD51 is the only strand exchange protein in vegetative cells, meiotic cells employ both RAD51 and DMC1. In addition to the recombination

mediators such as yeast Rad52 and human BRCA2 that function during recombination in both vegetative cells and in meiosis, meiotic cells make use of an additional regulator, the HOP2-MND1 complex (Leu et al. 1998; Petukhova et al. 2005). The heterodimer stabilizes the RAD51/DMC1 complex on ssDNA, reduces the affinity of RAD51 to dsDNA and modulates the conformation of the nucleoprotein filament to promote DNA strand exchange (Chi et al. 2007; Pezza et al. 2007).

The second key dissimilarity between meiotic and mitotic HR is the template choice for repair. Meiotic cells preferentially use the homologous chromosome instead of the sister chromatid in order to fulfill the requirement to generate genetic variability (Schwacha and Kleckner 1994; Baudat et al. 2000; Peoples et al. 2002). How this is achieved is not yet fully understood. The template bias is regulated by cohesion (Kim et al. 2010), depends in part on the role of DMC1 to promote strand exchange between homologs, and requires the DNA damage response cascade involving Mec1, Tel1 and meiosis-specific components such as Mek1 (Schwacha and Kleckner 1997; Niu et al. 2005; Carballo et al. 2008).

The third key characteristic of meiotic HR is a more frequent resolution of joint molecule intermediates such as dHJs or their precursors into crossovers. Most meiotic non-crossover products appear early and result from the processing of unstable intermediates by SDSA (Allers and Lichten 2001; Bishop and Zickler 2004). The joint molecules that can be readily observed are termed single-end invasions (containing presumably D-loop structures), which later mature into dHJs (Hunter and Kleckner 2001). Most of these joint molecules are resolved synchronously later, which is triggered by the phosphorylation of yet undefined targets by the Cdc5 kinase (Allers and Lichten 2001; Sourirajan and Lichten 2008). Although the molecular mechanism remains unclear, the preferential processing of joint molecule intermediates into crossovers is achieved by the usage of a meiosis-specific pro-crossover pathway, which is responsible for up to ~80% of meiotic crossovers in *S. cerevisiae* (Zakharyevich et al. 2012). This pathway ensures that every chromosome receives at least one crossover (positive crossover interference), thereby assuring the exchange of genetic information between the homologous chromosomes. At the same time, the formation of one crossover suppresses the formation of additional crossovers in its vicinity (negative crossover interference), making sure that the crossovers are evenly spaced, as two crossovers in immediate vicinity limit the exchange of genetic information between the homologous chromosomes. The proper number of crossovers and their distribution is thus balanced to enable efficient chromosome segregation and genetic exchange (Hunter 2015).

The exact mechanism of crossover interference remains to be defined. It is apparent that it is regulated by the components of the synaptonemal complex that juxtaposes homologs along their entire length (Sym and Roeder 1994; Hunter 2015). The interference signaling involves Tel1 (Garcia et al. 2015). Crossover interference also requires topoisomerase II, suggesting that interference may be regulated by mechanical or topological stress along the meiotic chromosome axes (Zhang et al. 2014). The meiotic crossover-specific pathway involves the ZMM group of proteins, which includes Zip1, Zip2, Zip3, Zip4, Spo16, Mer3 and Msh4-Msh5 in *S. cerevisiae* (Lynn et al. 2007; Shinohara et al. 2008). These proteins form or facilitate the formation of the synaptonemal complex, or function more downstream to stabilize D-loop structures in a way that protects their disassembly by motor proteins (Mazina et al. 2004; Snowden et al. 2004). Similarly in mice, the putative SUMO-conjugating factor RNF212 was found to stabilize meiotic recombination factors including MSH4-MSH5 at designated recombination sites, which is essential for crossing over. This is likely achieved via sumoylation of selected targets along the chromosome axes, which functionally interacts with HEI10-dependent ubiquitination and proteasomal degradation (Wei et al. 2003; Reynolds et al. 2013; Rao et al. 2017).

The crossover-specific resolution in budding yeast, plants and mammals depends on the nuclease activity of Mlh3, which is a part of the Mlh1-Mlh3 heterodimer (Lipkin et al. 2002; Nishant et al. 2008; Zakharyevich et al. 2012; Ranjha et al. 2014; Rogacheva et al. 2014). Unexpectedly, Exo1 was found to have a non-catalytic function in crossover resolution together with Mlh1-Mlh3 (Zakharyevich et al. 2010). How the ZMM proteins and Mlh1-Mlh3 achieve the crossover-specific resolution of dHJs or their precursors remains to be described (Fig. 7c). Interestingly, only crossovers from the ZMM-Mlh1-Mlh3 pathway – termed Class I crossovers – display interference. Joint molecules that are processed by the structure-specific nucleases (Mus81-Mms4, Slx1-Slx4 and Yen1 in yeast) result in both crossovers and non-crossovers. Crossovers resulting from these nucleases are interference independent and account for ∼20% of crossovers in *S. cerevisiae* (de los Santos et al. 2003; Zakharyevich et al. 2012). In contrast, Mus81-Eme1 generates most meiotic crossovers in the budding yeast *Schizosaccharomyces pombe*, while the MEI-9, an XPF-like protein, resolves meiotic joint molecules in *Drosophila* (Boddy et al. 2001; Yildiz et al. 2002).

7 Role of recombination proteins in promoting the stability of DNA replication forks

In addition to repairing DSBs, recombination proteins have critical functions in other pathways of DNA metabolism. Specifically, recombination is one of the pathways that contribute to the repair of DNA crosslinks, which has been covered elsewhere (Hinz 2010). Furthermore, recombination in multiple ways promotes the stability of replicating DNA (Branzei and Szakal 2017). Abnormalities such as protein blocks, chemical modifications, abnormal secondary structures or collisions of replication forks with the transcription machinery can impede DNA replication. Depending on the nature of the replication stress, cells utilize various mechanisms that help them deal with these situations (Fig. 10a-d). This includes re-priming of DNA replication and/or translesion DNA synthesis, which lead to lesion bypass. Postreplicative repair can fill any gaps left behind by the replication machinery (Bianchi et al. 2013; Garcia-Gomez et al. 2013; Mouron et al. 2013; Branzei and Szakal 2016; Guilliam and Doherty 2017; Vaisman and Woodgate 2017). In some cases the replication fork can fall apart, resulting in a single-ended DSB, which is repaired as described in chapter 1.3. Here, we will focus on two related functions of recombination proteins that were uncovered only in recent years, namely in the replication fork reversal and the protection of DNA from nucleolytic degradation at stalled replication forks.

Replication fork reversal is the conversion of a stalled DNA replication fork into a four-way junction (Fig. 10b). Fork reversal is achieved by the annealing of the two nascent DNA strands and the re-annealing of the two parental strands, which resembles a DNA double-strand break. Fork reversal can thus lead to DSB formation in the absence of template breakage (Fig. 10b). Although arising through a very different mechanism, the resulting four-way junction is structurally identical to a Holliday junction that forms during DNA recombination (Fig. 10b). The concept of replication fork reversal has been proposed a long time ago (Higgins et al. 1976). In bacteria, it has been established as a way that contributes to the restart of stalled replication forks. In *E. coli*, the actions of RecA (a homologue of RAD51), followed by branch migration by RuvAB, or the motor activity of RecG, can drive replication fork reversal (Seigneur et al. 1998; McGlynn and Lloyd 2001; Gupta et al. 2014). For a long time, this process was believed to represent only a pathological reaction in eukaryotic cells (Sogo et al. 2002; Hu et al. 2012). Specifically, fork reversal in yeast was described to occur in checkpoint-defective cells (Lopes et al. 2001). Only recently, it has been demonstrated that replication fork reversal can be beneficial to prevent DNA template breakage in human cells, and that it is a global response to DNA replication stress (Ray Chaudhuri et al. 2012; Neelsen and Lopes 2015; Zellweger et al. 2015). Electron microscopy revealed that the regressed

arms of the replication forks can reach and occasionally exceed 1 kb in length, implicating that they form in the course of an active and enzyme-driven process.

One of the first proteins shown to promote replication fork reversal *in vivo* was RAD51 (Zellweger et al. 2015). In accord, RAD51, to a limited extent, can drive migration of Holliday junctions via polymerization on DNA (Rossi et al. 2011). However, RAD51 *per se* has no motor activity, so it is unlikely that RAD51 can drive fork reversal on its own. The best candidates for this function are motor proteins such as DNA helicases or translocases. To this point, the motor protein RAD54 was shown to catalyze fork reversal *in vitro* in a way that was stimulated by RAD51 (Bugreev et al. 2011). Other proteins, including SMARCAL1, ZRANB3, HLTF, FBH1, RECQ5 and FANCM were proposed to function in fork reversal *in vitro* or *in vivo* (Kanagaraj et al. 2006; Gari et al. 2008; Betous et al. 2012; Ciccia et al. 2012; Bhat et al. 2015; Fugger et al. 2015; Kile et al. 2015; Kolinjivadi et al. 2017; Taglialatela et al. 2017; Vujanovic et al. 2017). However, the interplay of these factors with RAD51 is not defined, and it is not clear to which extent these motor proteins can function in a redundant manner. Interestingly, fork reversal likely does not require BRCA2, showing that, unlike in DSB repair, the function of RAD51 in promoting fork reversal is BRCA2 independent (Prakash et al. 2015; Kolinjivadi et al. 2017; Mijic et al. 2017). Fork reversal allows time for DNA repair ahead of the fork, or may result in DNA damage bypass (i.e. damage tolerance, in case when one reversed arm templates DNA synthesis of the complementary strand). Once this is completed, DNA replication must resume. Several mechanisms have been described that promote the restoration of reversed replication forks, which involve motor proteins such as RECQ1, RAD54 or the nucleolytic degradation of the reversed arms by MRE11, EXO1, the DNA2-WRN nuclease-helicase pair or by the MUS81-dependent cleavage, with different implication for genome stability (Bugreev et al. 2011; Berti et al. 2013; Pepe and West 2014a; Thangavel et al. 2015; Lemacon et al. 2017). While replication fork reversal may be pathologic in some cases, it has been demonstrated that inhibition of fork reversal results in increased DNA breakage and genome instability, underpinning the role of this process in preventing genome rearrangements (Neelsen and Lopes 2015; Mijic et al. 2017).

As described in chapter 3, RAD51 is the main DNA strand exchange protein in eukaryotes. It remains to be defined whether RAD51 has a structural or a catalytic function in replication fork reversal. There is, however, one key function of RAD51 that is DNA repair and recombination independent. It has been demonstrated that BRCA1, BRCA2 and RAD51 function together to protect stalled – and possibly reversed – DNA replication forks from degradation by the MRE11 nuclease (Hashimoto et al. 2010;

Schlacher et al. 2011; Schlacher et al. 2012; Mijic et al. 2017). It has been shown that a stable RAD51 nucleoprotein filament is required to protect DNA from MRE11 (Kolinjivadi et al. 2017; Zadorozhny et al. 2017). The BRCA1 and BRCA2 factors likely act directly or indirectly to stabilize the RAD51 nucleoprotein filament. It is being discussed whether the essential function of BRCA2 is to prevent DNA degradation at stalled forks by stabilizing RAD51, or to promote canonical DSB repair (Ray Chaudhuri et al. 2016; Feng and Jasin 2017). At the same time, unrestricted RAD51 activity at stalled forks is equally detrimental: the RADX factor was recently identified as a negative regulator of RAD51 at stalled forks (Dungrawala et al. 2017). These experiments collectively demonstrated that in DNA replication, the RAD51 protein, in addition to performing canonical "recombination" functions has other, previously unrecognized roles in replication fork reversal and protection from nucleolytic degradation. To elucidate the underlying molecular mechanisms of these processes represents a challenge to researchers in the coming years.

Figure legends

Fig. 1 An overview of DNA breaks. **a** A single-stranded DNA (ssDNA) break arises when only one strand of double-stranded DNA is interrupted. **b** If an ssDNA break is encountered by DNA replication, it gives rise to a one-ended double-stranded DNA (dsDNA) break. **c** A two-ended dsDNA break forms when dsDNA is broken into two pieces.

Fig. 2 An overview of the two main pathways for DNA double-strand break repair in human cells. **a** Main differences between end-joining and homologous recombination pathways. **b** DNA double-strand break repair pathway usage gives rise to different outcomes during genome editing with CRISPR-Cas9. Whereas end-joining often results in random mutations in the vicinity of the break site that may disrupt the reading frame of the targeted gene, homologous recombination may mediate the precise replacement of genetic information.

Fig. 3 An overview of DNA end-joining repair mechanisms. **a** Overview and main factors of non-homologous end-joining. **b** Overview and main factors of microhomology-mediated end-joining.

Fig. 4 An overview of homologous recombination pathways. A schematic representation of **a** single-strand annealing, **b** synthesis-dependent strand annealing, **c** break-induced replication and **d** canonical DNA double-strand break repair pathway that involves generation of a double Holliday junction, which can be processed by either topologic dissolution (d1) or nucleolytic resolution (d2). The various pathways differ in terms of mutagenic potential and whether they lead to crossover or non-crossover products, as indicated. The green triangles indicate DNA replication sites. Newly synthesized DNA is illustrated using dashed lines.

Fig. 5 An overview of DNA end resection in human cells. The first (short-range) resection step involves the MRE11 nuclease and the second (long-range) step either EXO1 or DNA2 nuclease. DNA end resection leads to the generation of 3' overhanged DNA at DNA double-strand break sites. The dashed blue lines indicate degraded DNA.

Fig. 6 An overview of the RAD51 filament formation (presynaptic phase) and the invasion of template dsDNA (postsynaptic phase) in human cells. Both positive and negative regulators of the process are indicated.

Fig. 7 An overview of double Holliday junction processing mechanisms in the canonical recombinational DSBR pathway. In vegetative cells, double Holliday junctions can be processed by either dissolution (**a**) that involves helicase-coupled topoisomerase or by resolution (**b**) that involves nucleolytic cleavage of the junctions. Meiotic cells in most organisms preferentially use a dedicated crossover pathway (**c**), and to a smaller degree nucleases that are common to both meiotic and mitotic cells (**b**). For simplicity, human nomenclature is used. The involvement of nucleases listed in panels b and c in human (or mouse) meiotic cells generally remains to be defined. Yeast *S. cerevisiae* cells use a crossover-specific pathway that involves the Exo1-Mlh1-Mlh3 complex and the structure-specific nucleases Mus81-Mms4, Slx1-Slx4 and Yen1. See text for details.

Fig. 8 An overview of specialized roles of the DSB repair pathways in human lymphocytes. **a** Mechanism of V(D)J recombination, where DNA breaks are introduced by RAG1-RAG2. **b** Mechanism of class switch recombination, where DNA breaks are indirectly caused by the action of AID. In both cases end-joining, but not homologous recombination pathways, are responsible for DNA break repair.

Fig. 9 An overview of specialized roles of the homologous recombination pathway in meiosis. The main differences between recombination in meiotic and vegetative cells are indicated.

Fig. 10 An overview of pathways that relieve DNA replication stress in human cells. **a** When a DNA replication fork encounters an obstacle, it may be bypassed by translesion synthesis. **b** A DNA replication fork can also reverse, which may temporarily protect DNA from breaking. Replication fork reversal can also lead to lesion bypass. Factors that may regulate the various steps of replication fork reversal and restart are indicated. RAD51 likely mediates replication fork reversal and protects reversed replication forks from degradation by the MRE11 nuclease. **c** Replication repriming downstream of a lesion is another mechanism of DNA damage tolerance. **d** Replication forks can also break, and become a substrate for homologous recombination as indicated in Fig. 4.

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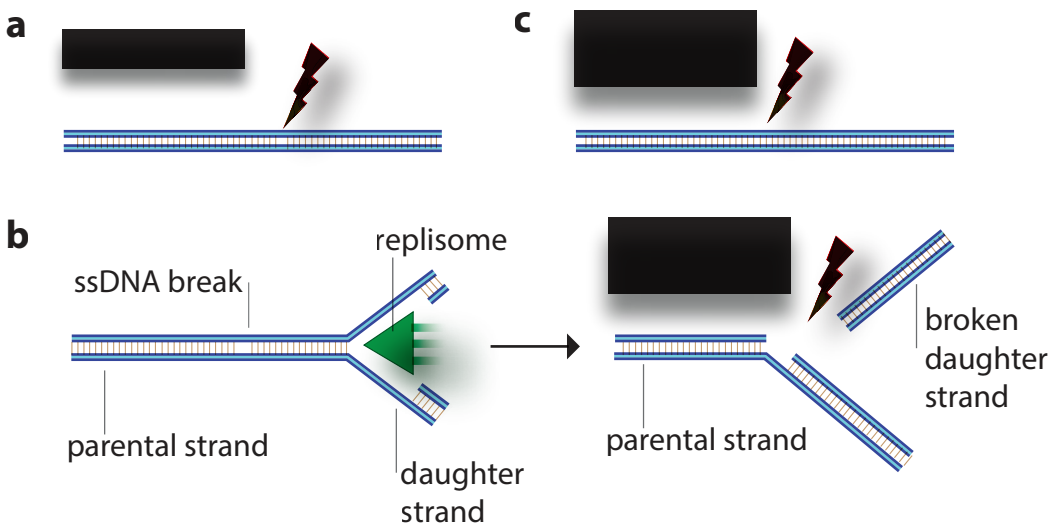
Figure 1


a
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strand

Figure 2

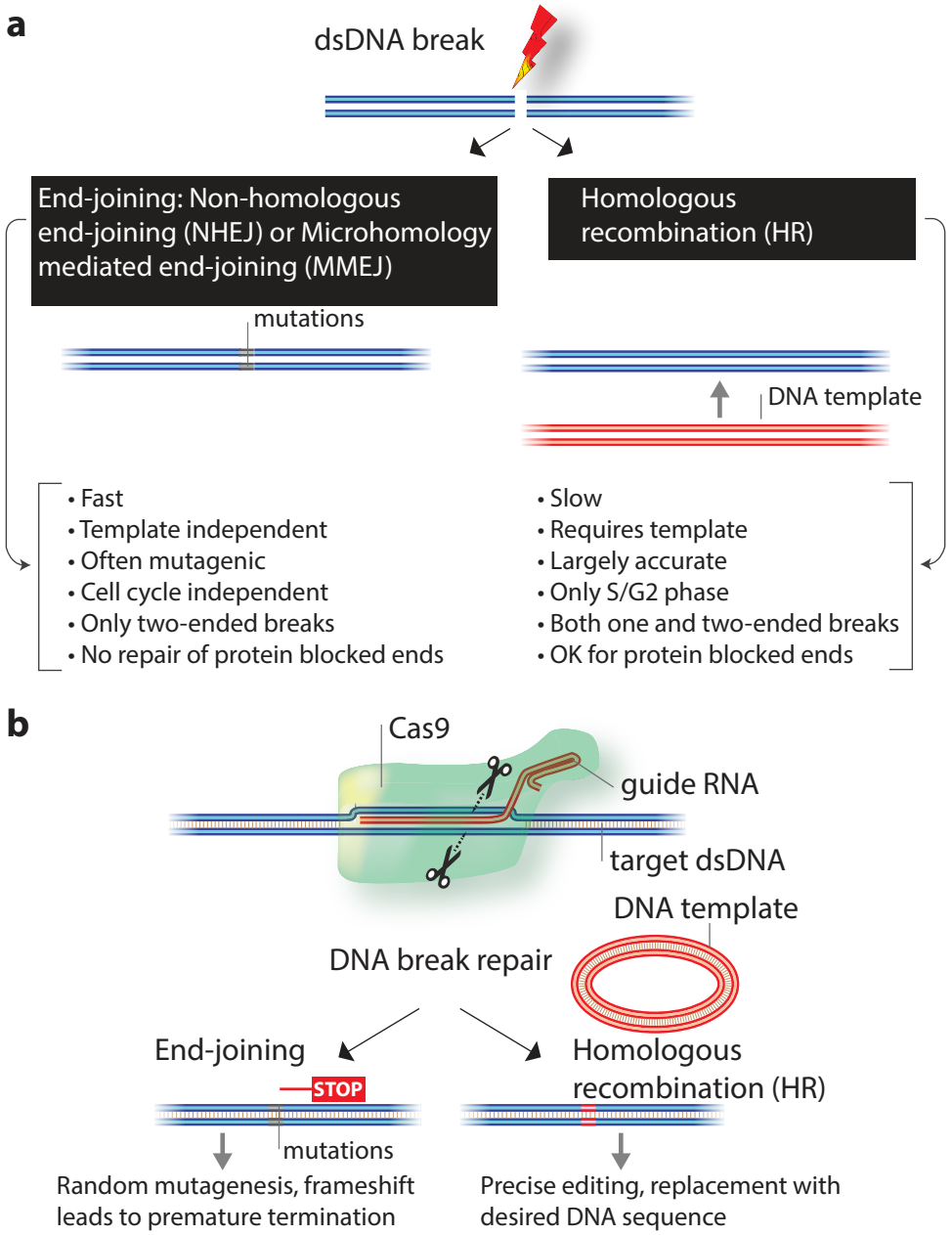

a
dsDNA break
End-joining: Non-homologous end-joining (NHEJ) or Microhomology mediated end-joining (MMEJ)
Homologous recombination (HR)
mutations
DNA template
• Fast
• Template independent
• Often mutagenic
• Cell cycle independent
• Only two-ended breaks
• No repair of protein blocked ends
• Slow
• Requires template
• Largely accurate
• Only S/G2 phase
• Both one and two-ended breaks
• OK for protein blocked ends
b
Cas9
guide RNA
target dsDNA
DNA template
DNA break repair
End-joining
STOP
mutations
Homologous recombination (HR)
Random mutagenesis, frameshift leads to premature termination
Precise editing, replacement with desired DNA sequence

Figure 3

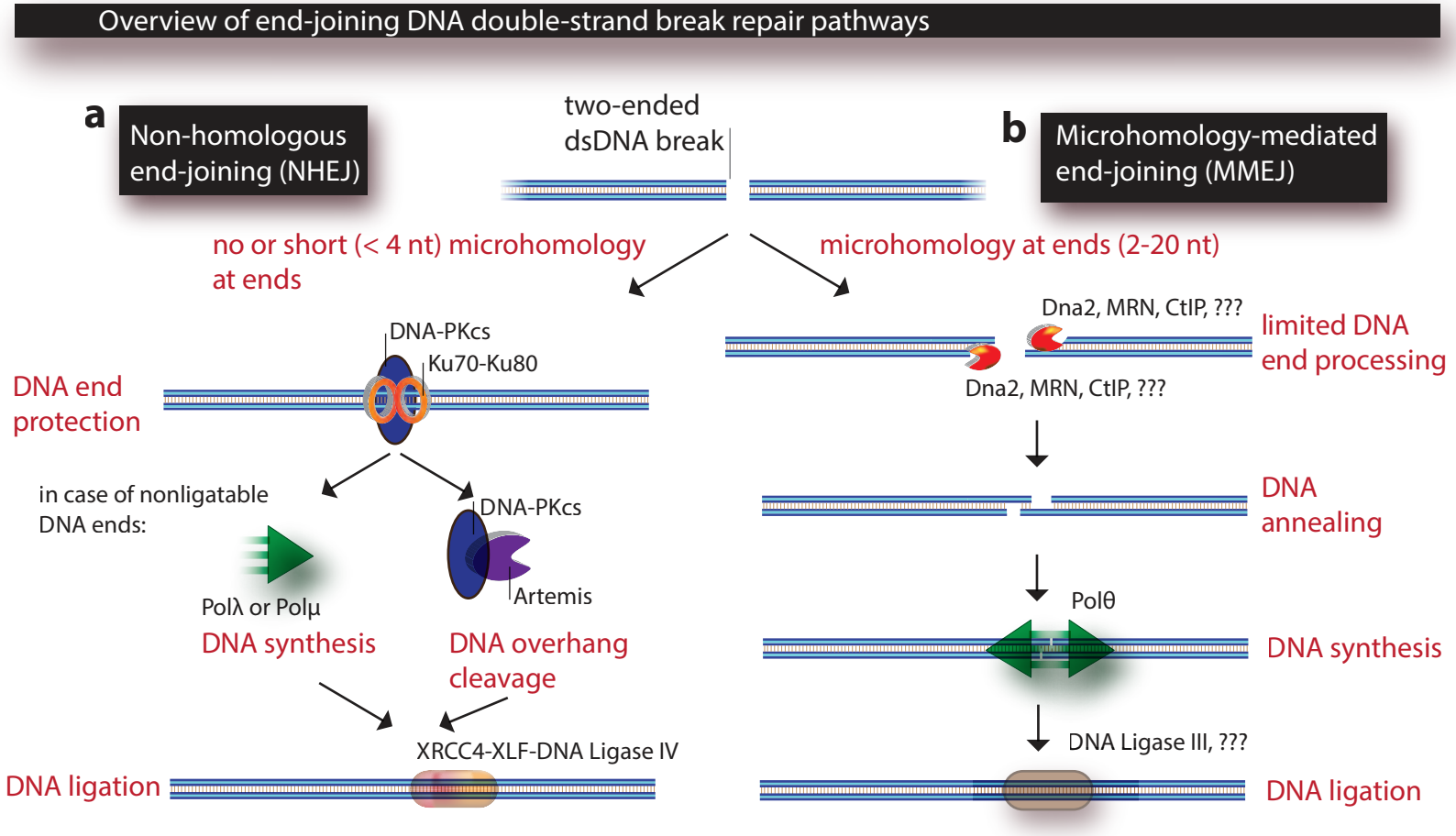

Overview of end-joining DNA double-strand break repair pathways
a
Non-homologous end-joining (NHEJ)
two-ended dsDNA break
b
Microhomology-mediated end-joining (MMEJ)
no or short (< 4 nt) microhomology at ends
microhomology at ends (2-20 nt)
DNA-PKcs
Ku70-Ku80
DNA end protection
Dna2, MRN, CtIP, ???
limited DNA end processing
Dna2, MRN, CtIP, ???
in case of nonligatable DNA ends:
DNA-PKcs
Artemis
DNA annealing
Polλ or Polμ
DNA synthesis
DNA overhang cleavage
Polθ
DNA synthesis
XRCC4-XLF-DNA Ligase IV
DNA Ligase III, ???
DNA ligation
DNA ligation

Figure 4

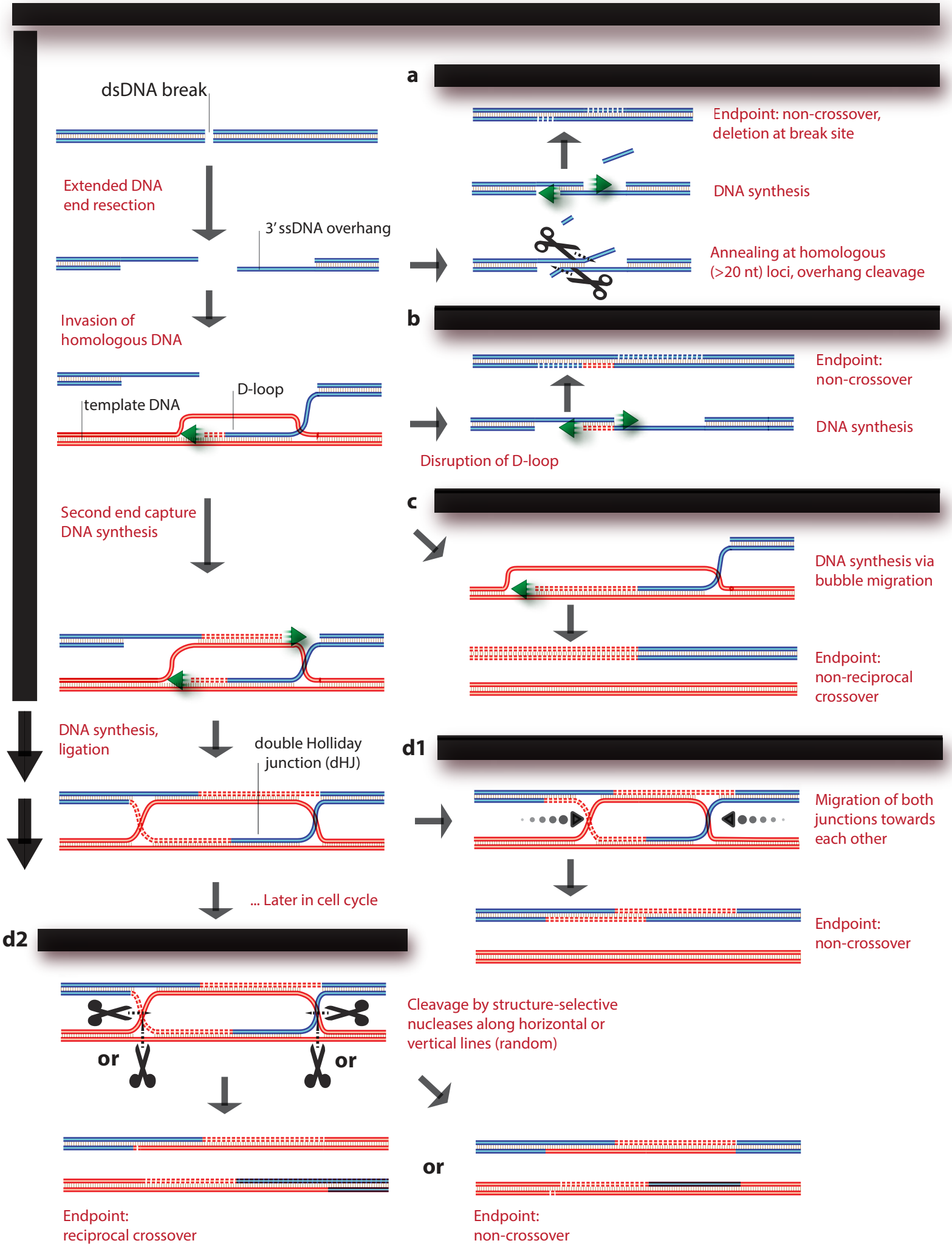

dsDNA break
Extended DNA end resection
3′ ssDNA overhang
Invasion of homologous DNA
template DNA
D-loop
Second end capture DNA synthesis
DNA synthesis, ligation
double Holliday junction (dHJ)
... Later in cell cycle
d2
or
or
Endpoint: reciprocal crossover
a
Endpoint: non-crossover, deletion at break site
DNA synthesis
Annealing at homologous (>20 nt) loci, overhang cleavage
b
Endpoint: non-crossover
DNA synthesis
Disruption of D-loop
c
DNA synthesis via bubble migration
Endpoint: non-reciprocal crossover
d1
Migration of both junctions towards each other
Endpoint: non-crossover
Cleavage by structure-selective nucleases along horizontal or vertical lines (random)
or
Endpoint: non-crossover

Figure 5

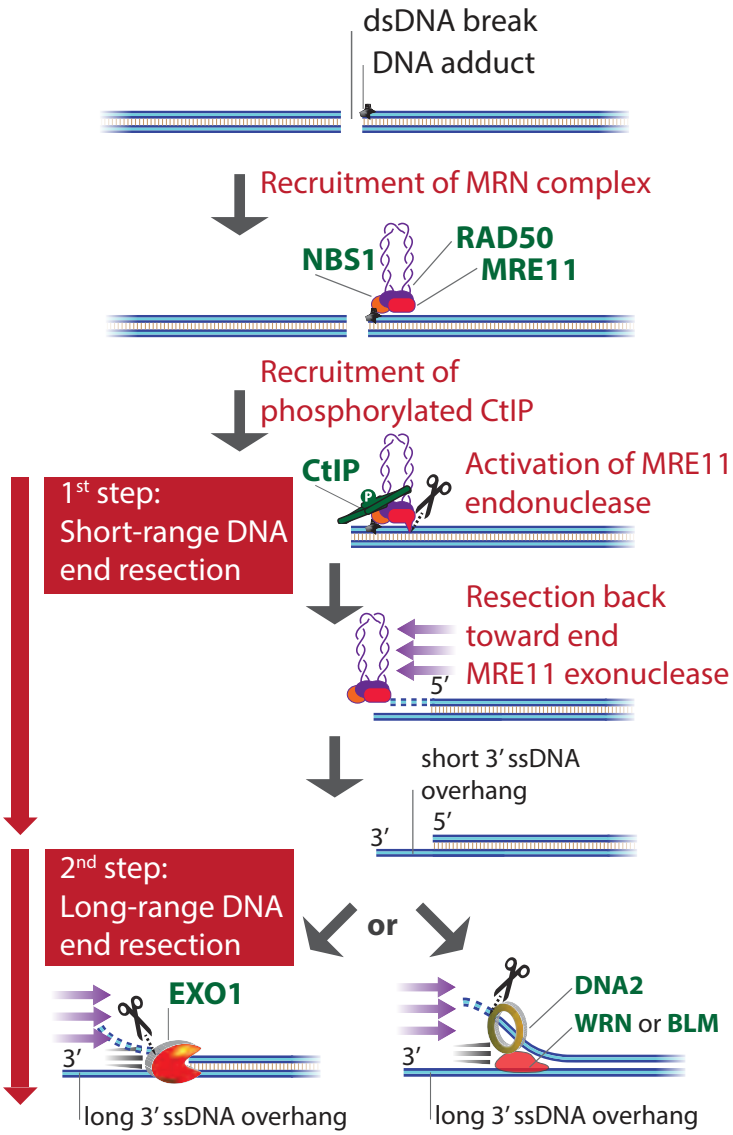
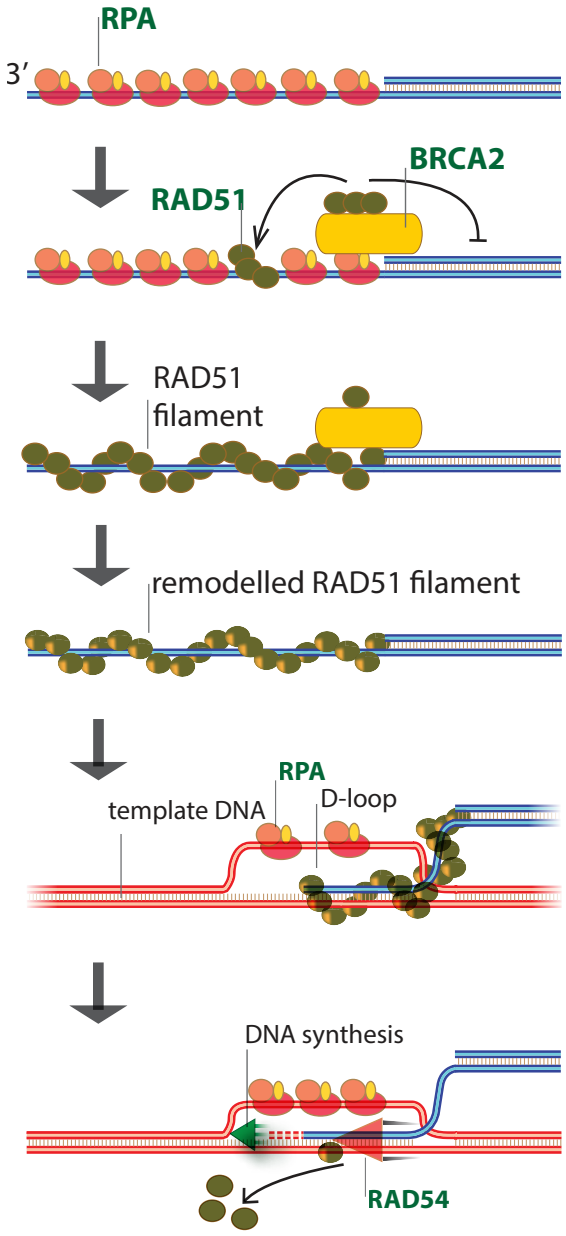

dsDNA break
DNA adduct
Recruitment of MRN complex
NBS1
RAD50
MRE11
Recruitment of phosphorylated CtIP
CtIP
Activation of MRE11 endonuclease
1st step: Short-range DNA end resection
Resection back toward end MRE11 exonuclease
5′
short 3′ ssDNA overhang
3′
5′
2nd step: Long-range DNA end resection
or
EXO1
3′
long 3′ ssDNA overhang
DNA2
WRN or BLM
3′
long 3′ ssDNA overhang

Figure 6



Resected RPA-coated 3′ ssDNA overhang

BRCA2 exchanges RPA for RAD51 on overhanged ssDNA, blocks dsDNA binding of RAD51

RAD51 paralogs
DSS1
SWS1 ?

RAD51 nucleoprotein filament

RAD51 paralogs
RAD54
RAD52
PALB2

RECQ5
FBH1
PARI
BLM

Active RAD51 filament

RAD51 paralogs

BRCA1

Invasion of template dsDNA, RPA stabilizes D-loop by binding to displaced ssDNA

RAD54

RTEL1
BLM
RECQ1
RAD54

Removal of RAD51 from dsDNA by RAD54, DNA synthesis by Polδ or Polε

Figure 7

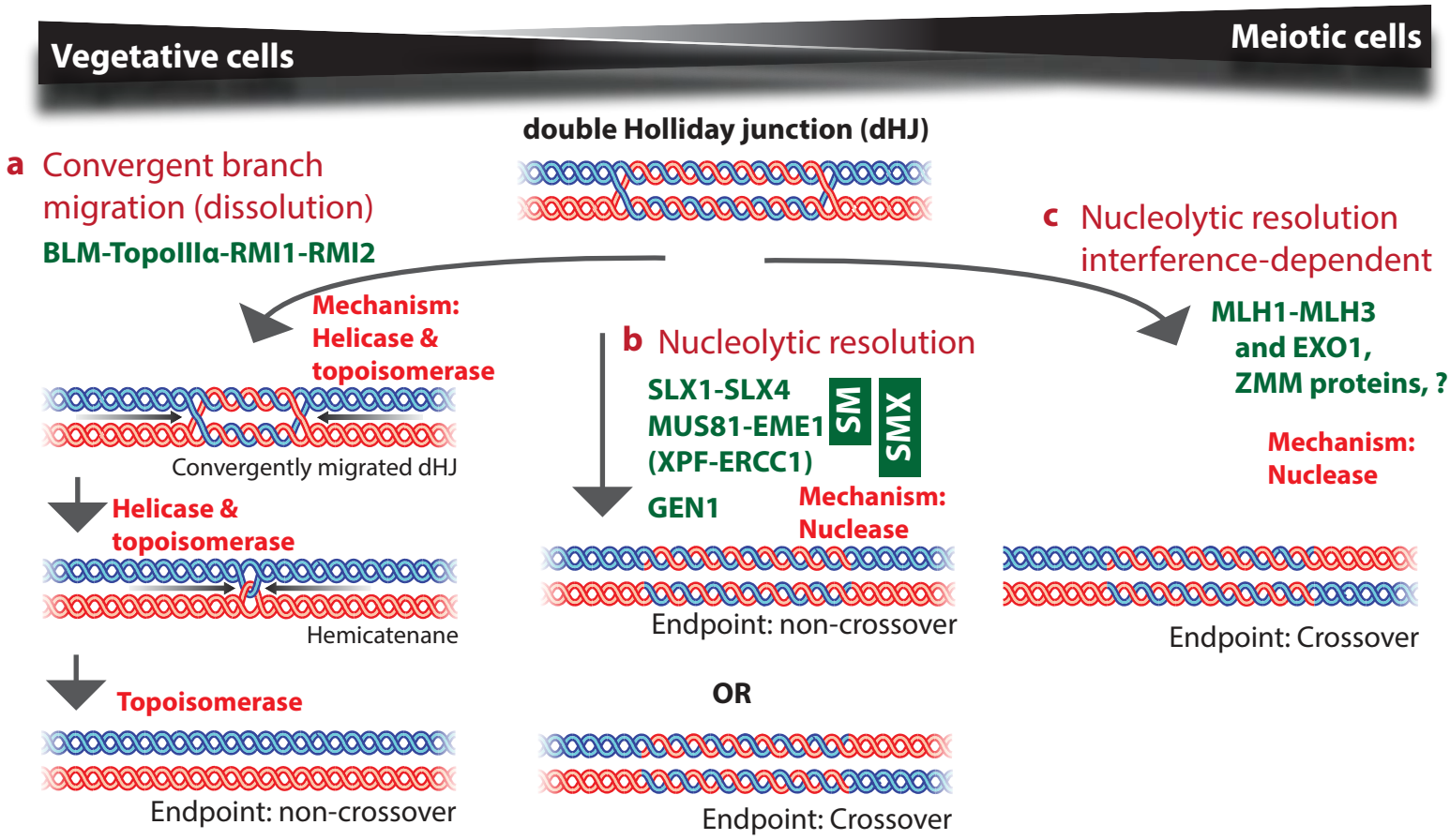

Vegetative cells
Meiotic cells
double Holliday junction (dHJ)
a Convergent branch migration (dissolution)
BLM-TopoIIIα-RMI1-RMI2
Mechanism: Helicase & topoisomerase
Convergently migrated dHJ
Helicase & topoisomerase
Hemicatenane
Topoisomerase
Endpoint: non-crossover
b Nucleolytic resolution
SLX1-SLX4
MUS81-EME1
(XPF-ERCC1)
SM
SMX
GEN1
Mechanism: Nuclease
Endpoint: non-crossover
OR
Endpoint: Crossover
c Nucleolytic resolution interference-dependent
MLH1-MLH3
and EXO1,
ZMM proteins, ?
Mechanism: Nuclease
Endpoint: Crossover

Figure 8

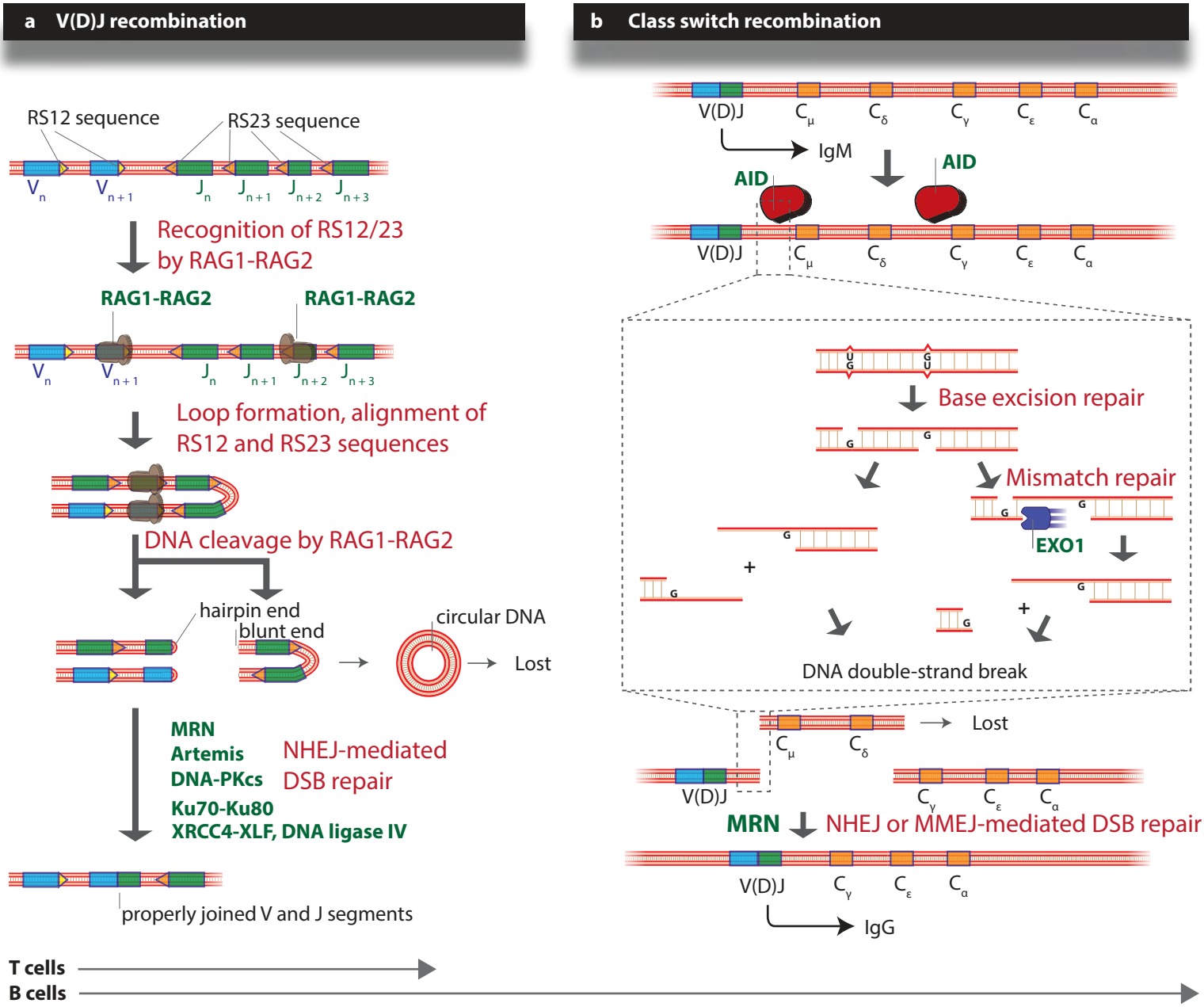

a V(D)J recombination
RS12 sequence
RS23 sequence
Recognition of RS12/23 by RAG1-RAG2
RAG1-RAG2
RAG1-RAG2
Loop formation, alignment of RS12 and RS23 sequences
DNA cleavage by RAG1-RAG2
hairpin end
blunt end
circular DNA
Lost
MRN
Artemis
DNA-PKcs
Ku70-Ku80
XRCC4-XLF, DNA ligase IV
NHEJ-mediated DSB repair
properly joined V and J segments
b Class switch recombination
V(D)J
IgM
AID
AID
Base excision repair
Mismatch repair
EXO1
DNA double-strand break
Lost
V(D)J
MRN
NHEJ or MMEJ-mediated DSB repair
V(D)J
IgG
T cells
B cells

Figure 9

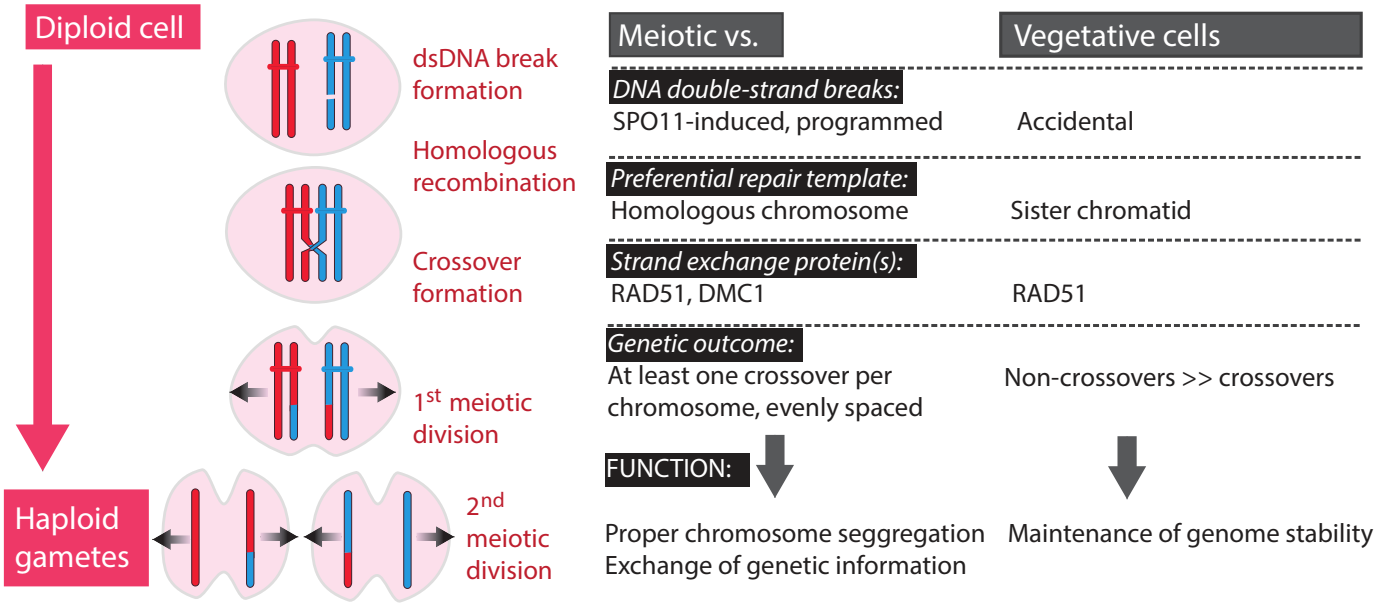
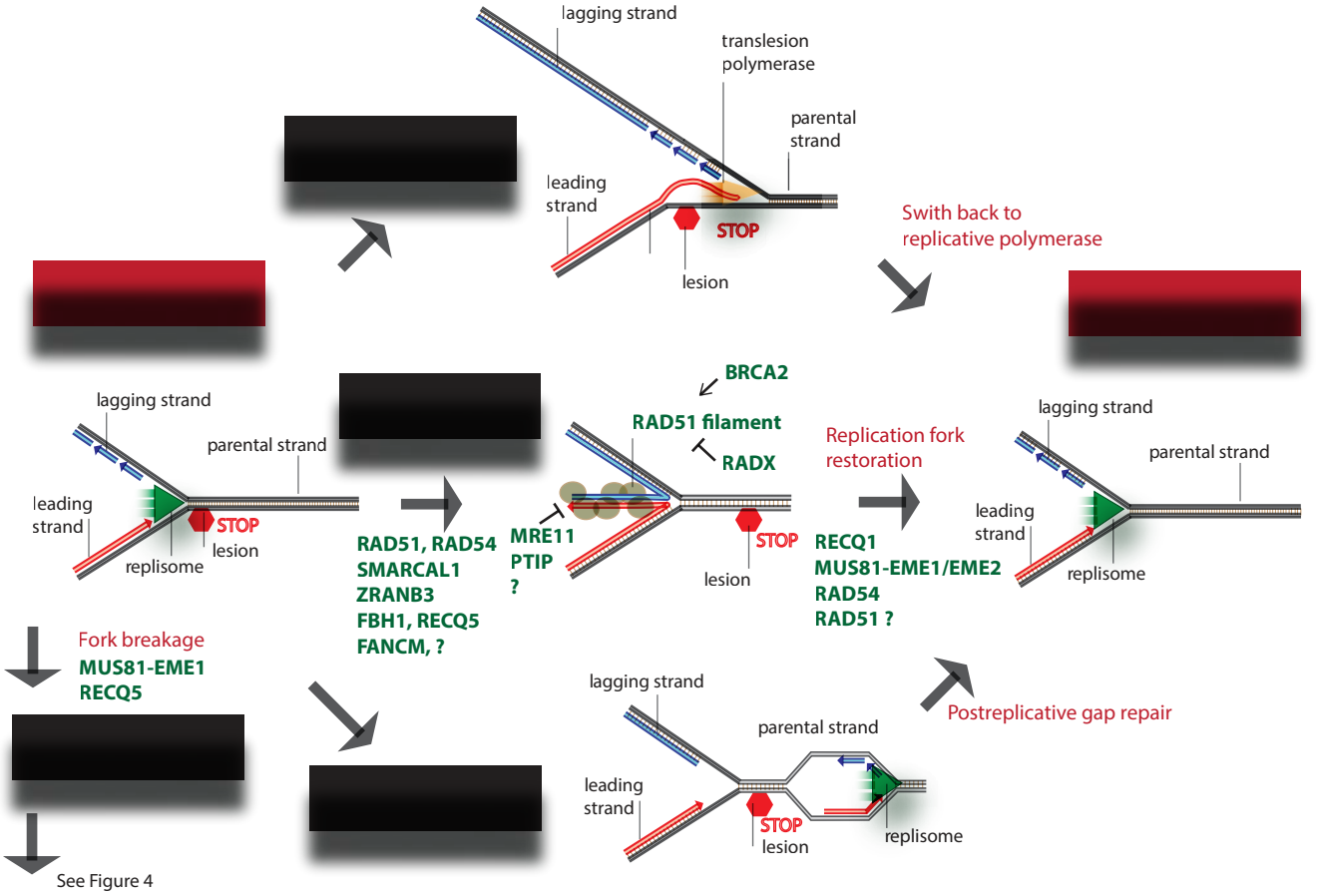

Diploid cell
dsDNA break formation
Homologous recombination
Crossover formation
1st meiotic division
Haploid gametes
2nd meiotic division
Meiotic vs.
Vegetative cells
DNA double-strand breaks:
SPO11-induced, programmed
Accidental
Preferential repair template:
Homologous chromosome
Sister chromatid
Strand exchange protein(s):
RAD51, DMC1
RAD51
Genetic outcome:
At least one crossover per chromosome, evenly spaced
Non-crossovers >> crossovers
FUNCTION:
Proper chromosome seggregation
Exchange of genetic information
Maintenance of genome stability

Figure 10


lagging strand
translesion polymerase
parental strand
leading strand
STOP
lesion
Swith back to replicative polymerase
lagging strand
parental strand
leading strand
STOP
lesion
replisome
RAD51, RAD54
SMARCAL1
ZRANB3
FBH1, RECQ5
FANCM, ?
BRCA2
RAD51 filament
RADX
MRE11
PTIP
?
STOP
lesion
Replication fork restoration
RECQ1
MUS81-EME1/EME2
RAD54
RAD51 ?
lagging strand
parental strand
leading strand
replisome
Fork breakage
MUS81-EME1
RECQ5
See Figure 4
lagging strand
parental strand
leading strand
STOP
lesion
replisome
Postreplicative gap repair