Repair of Strand Breaks by Homologous Recombination

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In this review, we discuss the repair of DNA double-strand breaks (DSBs) using a homologous DNA sequence (i.e., homologous recombination [HR]), focusing mainly on yeast and mammals. We provide a historical context for the current view of HR and describe how DSBs are processed during HR as well as interactions with other DSB repair pathways. We discuss the enzymology of the process, followed by studies on DSB repair in living cells. Whenever possible, we cite both original articles and reviews to aid the reader for further studies.

It was long recognized by radiation biologists that γ-irradiation was capable of causing DNA strand breaks. Geneticists posited that radiation-induced broken ends were treated differently from the ends of chromosomes (see Webb et al. 2013). Much of our early view of how HR is used in yeast to repair strand breaks emerged from studies of γ-irradiation-induced breaks and targeted integration during the 1970s and early 1980s. A model for the repair of γ-irradiation-induced breaks was proposed by Resnick and involved pairing of the broken ends with the homolog (Resnick 1976).

This model foreshadowed the repair of broken plasmid DNA (Orr-Weaver et al. 1981). Plasmid molecules containing yeast genes were linearized with restriction enzymes that cut once within a yeast gene and were introduced into yeast cells via transformation (Fig. 1A). By cutting these plasmids, the number of transformants compared to uncut circular DNA was increased 10- to 1000-fold. In addition, most of the transformants resulted from plasmid integration into the yeast genome. In plasmids that contained two yeast genes, the integration event was directed to the chromosomal copy of the gene that was cut on the plasmid. Gaps in the plasmid created by removing sequences from the yeast gene also directed integration into the homologous gene in the genome and were filled-in using the chromosomal sequence as a template (Orr-Weaver et al. 1981). The addition of yeast origins of replication to the plasmids allowed the detection of those events that restored the plasmid sequences unassociated with an integration event (noncrossover [NCO]) versus integration events associated with gap repair (crossover [CO]) (Fig. 1A) (Orr-Weaver and Szostak 1983). These observations showed that gene conversion of the gapped sequences oc-

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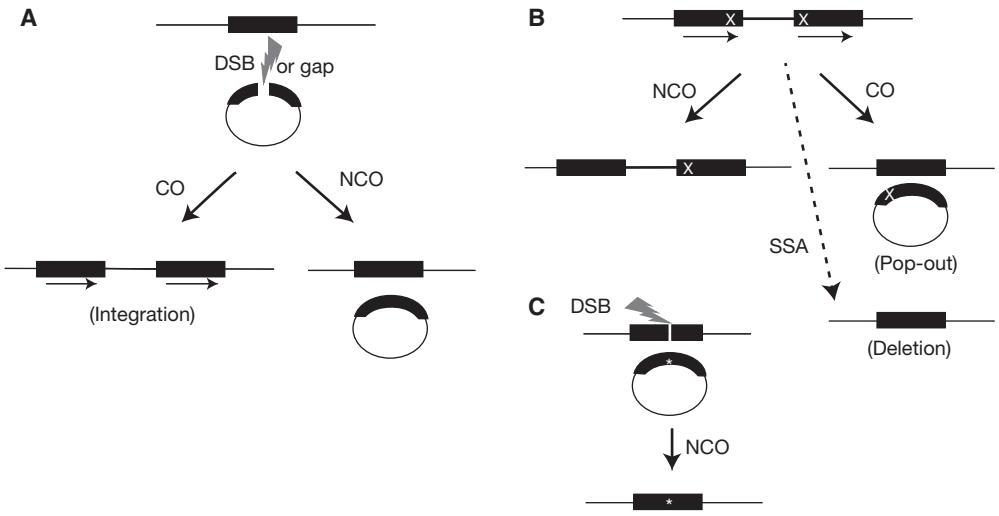

Figure 1. HR events. (*A*) Plasmid-by-chromosome HR initiated by a DSB or gap (lightning bolt) in the plasmid. The black bars represent the homology between the chromosome and plasmid. In this case, the chromosomal sequence is used as the donor template to repair the gap in the recipient plasmid. Crossovers (COs) lead to plasmid integration and the formation of direct repeats (indicated by arrows). Noncrossovers (NCOs) are also detected if the plasmid contains an origin of replication. (*B*) Recombination between direct repeats is frequently used to assay HR. Different mutations (X) are present in each repeat. HR associated with gene conversion of one mutation leads to restoration of one intact repeat (solid black bars), which is wild type. NCOs maintain the direct repeat configuration, whereas a CO leads to a plasmid "pop-out" event. Whereas bona fide HR events (i.e., those involving gene conversion) maintain both repeats, SSA (for details, see Fig. 2) leads to deletion of one repeat and the segment between the repeats. (*C*) DSB-induced gene targeting. Because nonhomologous integration of plasmids in mammalian cells is so efficient, creation of a site-specific DSB in the chromosome is used to induce HR. For the purposes of genome modification (*), the repair of this DSB can be from an incoming plasmid donor sequence, which can be circular (shown) or linear (not shown).

curred, which lead directly to the DSB repair model for HR (Szostak et al. 1983), which in its simplest form postulated a double Holliday junction (dHJ) recombination intermediate flanking the gap. Holliday junctions are named for the cross-strand exchange structure in Robin Holliday's model for genetic recombination (Holliday 1964).

Discovery of a role for HR in DSB repair in mammalian cells lagged significantly behind that of yeast and other model organisms. In fact, cell culture and mouse studies had originally pointed investigators away from considering HR as having a vital role in mammalian cells. Analysis of the fate of integrated DNA following transfection or injection in cells and in transgenic mice showed nonhomologous sites of integration (Lacy et al. 1983; Smithies et al. 1985), unlike the uniform homologous integration of transformed DNA in yeast. More direct studies of DSBs in plasmid DNA showed efficient nonhomologous repair (i.e., nonhomologous end-joining [NHEJ]) (Wake et al. 1984; Chiruvella et al. 2013). Subsequently, antigen receptor rearrangement was determined to involve DSB repair by NHEJ (Roth et al. 1992), which piqued the interest of investigators in NHEJ, but dissuaded them from considering that HR played a significant role in DSB repair in mammalian cells. Furthermore, when sequence repeats (homology) were provided in the plasmids, repair was consistent with annealing of complementary strands (single-strand annealing [SSA]) (Lin et al. 1984) rather than the invasion of one homologous strand into the other, the defining step of HR (Fig. 2). These observations were important for understanding the deletions that occur during direct repeat recombination in

fungi and mammalian cells (Rudin and Haber 1988; Liang et al. 1996).

The publication of DSB repair studies in yeast (Orr-Weaver and Szostak 1983) and the proposal of the DSB repair model (Orr-Weaver et al. 1981; Szostak et al. 1983) prompted direct investigation into a role for HR in mammalian cells. Initial studies were based on plasmid-by-chromosome recombination (Fig. 1A), in which a double-strand gap was introduced into the plasmid that could be repaired from the chromosome, leading to the production of intact virus (Jasin et al. 1985). These experiments showed a high frequency of DSB repair by HR. As much as 25% of the successfully transfected cells produced wild-type virus soon after plasmid transfection and about 10% of the initially gapped plasmids appeared to have undergone HR. Although designed for a virus readout, these experiments provided clear proof of DSB repair by HR in mammalian cells.

The product of these plasmid-by-chromosome HR experiments was essentially an NCO. An alternative outcome predicted by the original DSB repair model is a CO, which could not be detected in the viral system. Thus, the approach was revised to target the same locus but with a promoterless selectable marker gene substituting for viral sequences (Jasin and Berg 1988). DSB repair leading to plasmid integration (CO) was observed (Fig. 1A), further showing the recombinogenic nature of DSBs in mammalian cells, and led to efficient selection for the marker, now a frequently used approach. The enrichment of homologous integration events with a DSB was estimated to be 100-fold. Despite the large effect, however, these CO events were notably significantly less frequent than NCO

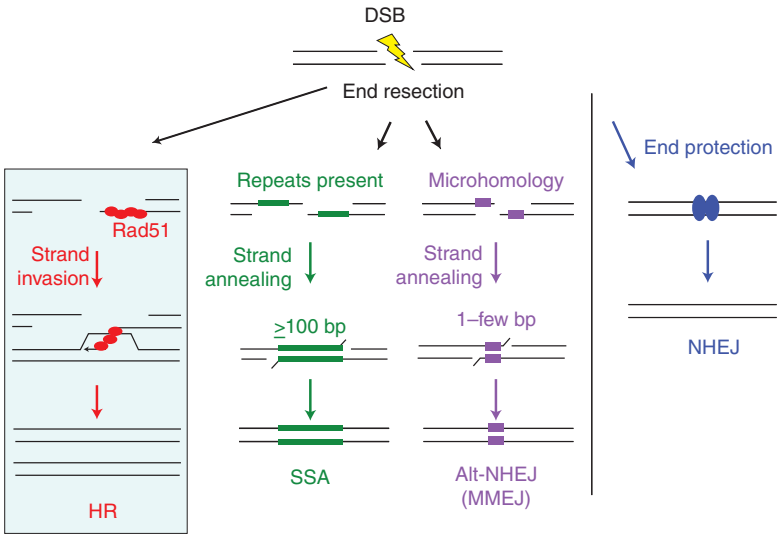

Figure 2. HR is only one pathway of DSB repair and can collaborate with and compete with other pathways to repair DSBs. An early determinant of DSB repair pathway choice is DNA end resection—the processing of DNA ends to generate 3′ single-strands, which is required for HR but inhibits canonical NHEJ in which DNA ends are protected with minimal processing before joining. The defining step of HR is strand invasion by Rad51 or a related recombinase (red balls). Rad51 forms a nucleoprotein filament on single-stranded DNA after end resection. End resection also provides an intermediate in nonconservative single-strand annealing (SSA) and alternative-NHEJ (alt-NHEJ) involving microhomology, as it gives rise to single strands, which can anneal at long (SSA) or short (alt-NHEJ) complementary sequences (green and purple bars, respectively). HR and SSA require more extensive end resection than alt-NHEJ, because minimal end resection will uncover microhomologies present near DNA ends. Alt-NHEJ involving microhomology is also termed microhomology-mediated NHEJ (MMEJ).

events. The choice of CO and NCO outcomes in HR will be discussed below.

DIRECT REPEAT RECOMBINATION

In addition to plasmid repair studies, direct repeat recombination assays were developed in yeast, taking advantage of plasmids integrated at their homologous chromosomal site (Jackson and Fink 1981; Klein and Petes 1981). The integrations were often designed to create direct repeats with two different mutations (Fig. 1B). Selecting for recombination between the direct repeats leads to prototrophs that maintain the plasmid and these are called gene convertants. Prototrophs associated with plasmid loss are called "pop-outs." Depending on the configuration of the alleles, triplications can also arise. Variations on these assays have formed the basis of many recombination studies in fungi and mammalian cells (Klein 1995; Liang et al. 1996; Lambert et al. 1999) including the now widely used GFP repeats (Pierce et al. 1999).

SITE-SPECIFIC DSBs

Molecular insights into DSB repair required the development of systems to introduce site-specific DSBs in the genome. Expression of the HO and I-*Sce*I endonucleases have been used to create site-specific DSBs in yeast (Rudin and Haber 1988) and mammalian cells (Rouet et al. 1994b) and later in other species including flies (Bellaiche et al. 1999; Rong et al. 2002), plants (Puchta et al. 1993, 1996), and bacteria (Ponder et al. 2005). The HO endonuclease was discovered studying mating type switching in budding yeast, which is initiated by a DSB (Strathern et al. 1982; Kostriken et al. 1983; Haber 2012). The HO endonuclease targets the active mating locus, creates a site-specific DSB, and then one of the two silent copies elsewhere on the chromosome is used as a template to switch mating type information. Studies of this highly regulated process led to the notion that the processed ends of a DSB could undergo "ectopic" recombination. The model of plasmid gap repair was used to explain the gene conversion of the silent locus into the *MAT* cassette (Orr-Weaver et al. 1981). Subsequent experiments in *Schizosaccharomyces pombe* (Arcangioli and de Lahondes 2000), where mating type is initiated by a nick that is converted to a DSB, and later in *Saccharomyces cerevisiae* (Ira et al. 2006) showed that the DNA synthesis during mating type switching is conservative.

Studies of mitochondrial genetics in budding yeast led to the discovery of an endonuclease-initiated HR event that involves cleavage of an 18-base-pair recognition site (Colleaux et al. 1988). The site is present only in mitochondrial genomes that do not contain the ORF encoding the endonuclease, because the ORF interrupts the recognition site (Dujon 1989). When mitochondria, which contain the ORF, fuse with ones that do not, the endonuclease cleaves the site in the ORF-minus genomes, initiating DSB gap repair. The ORF is subsequently copied into the broken site, formally a gene conversion event, which renders the site no longer sensitive to the endonuclease. The endonuclease responsible for this site-specific DSB is I-*Sce*I and the ORF encoding it was codon optimized for protein expression in *E. coli* (Colleaux et al. 1986), allowing expression vectors to be developed for a variety of cell types, including yeast (Plessis et al. 1992) and mammalian cells (Rouet et al. 1994a).

Many studies of DSB repair have centered on site-specific breaks introduced by the HO and I-*Sce*I endonucleases. Various assays have been designed to assess how different cell types and different mutations affect the process of DSB repair. Direct repeat assays with the DSB cut site inserted in different places are used to measure HR (here defined as a gene-conversion event), SSA, and/or NHEJ. Importantly, the efficiency of cutting by HO and I-*Sce*I is high enough in yeast cells to physically monitor DSB repair, leading to mechanistic insights into this process and the genetic control of discrete steps.

GENOME ENGINEERING BY REPAIR OF CHROMOSOME DSBs

DSB-induced plasmid recombination experiments were performed contemporaneously with the burgeoning field of gene targeting in mammalian cells for the purposes of modifying

genes at their endogenous loci (Capecchi 1989). The finding that DSBs in plasmids induced recombination with the chromosome suggested that homologous DSB repair could be co-opted in mammalian cells for genome engineering. However, two limitations were immediately noted: the relatively low frequency of CO events (Jasin and Berg 1988), and that formally the plasmid, not the chromosome, is the one modified because it carries the DSB (Jasin et al. 1985). Thus, what was required to efficiently modify the genome by HR was a DSB in the chromosome, something that was unnecessary in yeast because homologous integration of plasmids was already much more efficient than nonhomologous events. This requirement could be met by expressing I-*Sce*I (Rouet et al. 1994a) and introducing its target site into the mammalian genome (Rouet et al. 1994b). I-*Sce*I was used for this purpose because its target site was well defined and long enough so that its expression was not expected to be toxic to cells even with complex genomes (Rouet et al. 1994a). DSBs in the mammalian genome are repaired by HR using this system (Fig. 1C), such that homologous gene targeting was increased several orders of magnitude (Rouet et al. 1994b). DSBs were also efficiently repaired by NHEJ in this system, with an estimate of approximately twice the level of NHEJ as HR (Rouet et al. 1994b). However, any such estimates comparing HR and NHEJ frequencies should be used with caution, given that the most common HR and NHEJ events (precise repair from the sister chromatid and religation of the site, respectively) are not scored in assays that require modification of the DSB site to be detected.

DSB repair studies with the I-*Sce*I endonuclease form the basis of current genome engineering approaches in mammalian cells. Critical for the success of these approaches is an endonuclease directed to cleave the locus to be modified. Nucleases using an array of zinc finger DNA-binding domains fused to a *Fok*I endonuclease domain were the first designed to target an endogenous mammalian locus (Urnov et al. 2005), similar to an approach developed for *Drosophila* (Bibikova et al. 2003). Nuclease design was greatly facilitated by the discovery of the simple DNA recognition code of TAL effector proteins from pathogenic bacteria, which invade plants (Boch et al. 2009; Moscou and Bogdanove 2009) and even more so recently by the discovery of an RNA-guided nuclease in bacterial adaptive immunity termed CRISPR/Cas9 (Gasiunas et al. 2012; Jinek et al. 2012). These "designer" nucleases are being used for genome engineering in a variety of organisms in addition to mammalian cells, including zebrafish (Hwang et al. 2013) and livestock (Carlson et al. 2012).

SINGLE-STRAND BREAKS AND HR

Single-strand breaks and gaps, like DSBs, can also be repaired by HR, as first described in *E. coli* (Michel et al. 2007). In the fission yeast *Schizosaccharomyces pombe*, the mating type switching system is likely induced from a site-specific nick or gap left on one chromosome, which in the next round of DNA synthesis becomes a DSB that is used to initiate the switch (Arcangioli and de Lahondes 2000). Interestingly, this DSB repair is conservative, likely to avoid COs that would result in deletion of the silent cassettes. The mechanism responsible for generating the nick at the fission yeast mating type locus is still unknown. Evidence that single-strand nicks or gaps are likewise repaired by HR in mammalian cells comes not only from studies with drugs like camptothecin but also from the use of site-specific nickases, such as meganucleases (Davis and Maizels 2011), zinc finger nucleases (Kim et al. 2012; Ramirez et al. 2012; Wang et al. 2012), CRISPR/Cas9 (Cong et al. 2013; Mali et al. 2013), and corrupted Rag proteins (Lee et al. 2004). For purposes of genome engineering, nickases have the advantage that single-strand breaks are not repaired by error-prone NHEJ, although the induction of HR is usually much less than that from DSBs.

MEIOSIS

The connection between mitotic HR repair and meiosis was made clear when DNA DSBs were identified as initiators of meiotic recombination (Sun et al. 1989). The search for the enzyme responsible for creating meiotic DSBs revealed

a conserved gene, *SPO11* (Bergerat et al. 1997; Keeney et al. 1997). *SPO11*, first identified as being defective in budding yeast sporulation (Esposito and Esposito 1969), which is the end product of successful meiosis, is homologous to the archael type VIA topoisomerase subunit with functional homologs identified in many eukaryotes (Keeney 2008), including mouse (Baudat et al. 2000; Romanienko and Camerini-Otero 2000). When introducing a DSB, Spo11 protein remains covalently bound to the ends before it is removed during endonucleolytic processing (Neale et al. 2005; Garcia et al. 2011). In organisms such as yeast and mouse, HR promotes pairing of chromosome homologs in addition to providing the physical connections between chromosome homologs (COs) critical for proper disjunction during meiosis I (Davis et al. 2001; Tesse et al. 2003; Henderson and Keeney 2004; Kauppi et al. 2013).

STEPS OF DSB REPAIR BY HR

The defining step of HR is the invasion of 3′ single-stranded DNA into a homologous duplex (Figs. 2 and 3). Single-stranded DNA generated by resection of the ends of a DSB provides a substrate for assembly of the Rad51 filaments needed for strand invasion; moreover, the invading 3′ end provides a primer for repair synthesis templated by the intact duplex. The strand invasion intermediate (D loop) can be resolved in a number of different ways, ultimately leading to an NCO or CO.

Initiating Homologous Recombination: End Resection and the Control of Pathway Choice

As a key early step in HR reactions, the generation of 3′ single-stranded DNA by resection of the 5′ end acts as a control point for DSB repair pathway choice (Kass and Jasin 2010; Symington and Gautier 2011). End resection was observed in budding yeast by physical analysis of the fate of DSBs in vivo (Pâques and Haber 1999). HO endonuclease-generated DSBs undergo resection at their 5′ ends while maintaining relatively stable 3′ ends. Identifying the role of factors involved in resection proved challenging largely because of redundancy, but a breakthrough came from two laboratories in 2008 (Mimitou and Symington 2008; Zhu et al. 2008). Initial processing of the ends involves Mre11/Rad50/Xrs2, termed MRX (MRN in mammals), and the Sae2 protein. In mammalian cells, the functional counterpart of Sae2 is CtIP, as evidenced by the dependence on CtIP for recruitment of RPA to nuclear foci and for ATR activation (Sartori et al. 2007). Of note, the breast cancer suppressor BRCA1 interacts with MRN (Zhong et al. 1999) and CtIP (Yu et al. 1998) and importantly promotes both HR and SSA (Moynahan et al. 1999; Stark et al. 2004), as does CtIP (Sartori et al. 2007; Bennardo et al. 2008), suggesting that BRCA1 may also be involved in the initial step of end resection (Stark et al. 2004). More extensive resection involves the 5′-3′ exonuclease Exo1 or the combined helicase/nuclease activities of Sgs1/Dna2 (Mimitou and Symington 2008; Zhu et al. 2008). Roles for the mammalian counterparts EXO1 and BLM, respectively, is supported by in vitro end resection assays (Nimonkar et al. 2011).

Although programmed DSBs are channeled into specific repair pathways (e.g., meiotic DSBs into HR and immune system DSBs into NHEJ), DSBs from nucleases or radiation can be repaired by either HR or NHEJ pathways (Rouet et al. 1994b; Liang et al. 1998; Rothkamm et al. 2003). HR itself is regulated and is promoted during the S/G_2 phase of the cell cycle (Ira et al. 2004; Escribano-Diaz et al. 2013). It is important to note that HR occurs within a cellular milieu in which NHEJ is also active, especially in mammalian cells where NHEJ is a prominent pathway throughout the cell cycle (Rothkamm et al. 2003). As such, end resection is a major determinant of whether canonical NHEJ or HR is used at DSBs. As shown by direct molecular analysis, resection is increased in yeast mutated in the canonical NHEJ proteins Ku and Dnl4/Lif1 (LIG4/XRCC4 in mammalian cells), with a greater effect in the Ku mutant (Lee et al. 1998; Zhang et al. 2007). Because Ku binds DNA ends, it presumably physically blocks access of the end resection machinery, whereas Dnl4/Lif1 may act to stabilize Ku binding to DNA ends. In cases in which the HR machinery successfully

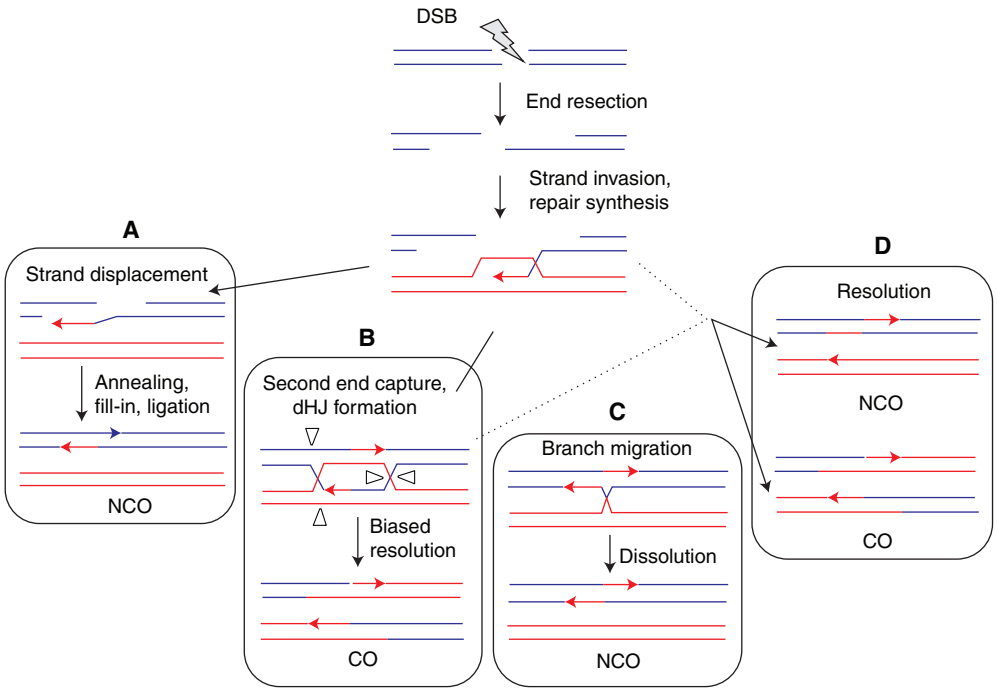

Figure 3. HR of a resected DSB after strand invasion and repair synthesis showing independent and interrelated steps for resolution. (*A*) Synthesis-dependent strand annealing involves displacement by DNA helicase(s) of the newly synthesized strand. The two ends can anneal by their shared complementarity. Synthesis, nuclease, and ligase reactions finish the event. (*B*) Double Holliday junctions (dHJs) are formed when the D loop captures the second end. In meiotic cells, dHJ resolution is biased to give COs. (*C*) dHJs can be dissolved without crossover by the action of the BLM(Sgs1)/TOP3α/RMI1 complex. (*D*) Resolution of intermediates that escape the BLM(Sgs1) complex may occur from the action of different resolvases (e.g., MUS81/EME1[Mms4], GEN1[Yen1], etc.).

competes with Ku, MRX (MRN) and Sae2 (CtIP) may clip Ku from DNA ends, similar to its action to remove Spo11 from 5′ ends during meiotic recombination (Garcia et al. 2011).

Consistent with an increase in a resected intermediate, mutation of Ku or LIG4/XRCC4 in mammalian cells increases HR, with mutation of Ku showing the more profound effect (Pierce et al. 2001). Mutation of DNA-PKcs, which does not have a yeast homolog, also increases HR, again less profoundly than Ku (Pierce et al. 2001; Allen et al. 2002). Interestingly, an increase in HR in NHEJ mutants occurs whether the DSB is introduced by I-*Sce*I endonuclease, which generates a 3′ overhang, or the RAG recombinase, which generates a hairpin-capped end (Pierce et al. 2001; Weinstock and Jasin 2006). Like HR, SSA is increased with loss of Ku or LIG4/XRCC4 (Stark et al. 2004; Bennardo et al. 2009), in agreement with increased end resection in these mutants.

In addition to HR and SSA, a third DSB repair pathway initiates with end resection—that of microhomology-mediated NHEJ (MMEJ), also termed alternative NHEJ (alt-NHEJ) to distinguish it from canonical NHEJ (Fig. 2). Like HR and SSA, alt-NHEJ at microhomology is suppressed by canonical NHEJ components like Ku and LIG4/XRCC4, but it can also operate in the presence of the canonical pathway (Weinstock et al. 2007; Simsek and Jasin 2010; Simsek et al. 2011; Escribano-Diaz et al. 2013). On the other hand, end resection factors like CtIP promote alt-NHEJ, as well as HR and SSA (Bennardo et al. 2008; Simsek et al. 2011; Zhang and Jasin 2011).

Besides canonical NHEJ proteins like Ku and LIG4, the DNA damage response protein 53BP1 has a role in NHEJ (Nakamura et al. 2006; Stavnezer et al. 2008), and as with canonical NHEJ factors, 53BP1 disruption is associated with increased HR (Nakamura et al. 2006; Xie et al. 2007). In 2009, a surprising discovery was made showing that *53bp1* mutation rescues the lethality associated with *Brca1* mutation in mice (Cao et al. 2009). Disruption of canonical NHEJ factors like Ku, however, does not similarly rescue (Bunting et al. 2012). Evidence indicates that *53bp1* disruption restores HR in BRCA1-deficient cells, likely by relieving 53BP1 inhibition of end resection at DSBs (Bouwman et al. 2010; Bunting et al. 2010). The yeast 53BP1 ortholog, Rad9, also regulates DNA end resection (Lazzaro et al. 2008). Thus, 53BP1 and BRCA1 may act antagonistically on end resection to control DSB repair pathway choice.

Likely a number of other proteins will be identified that regulate end resection, given the central role of end resection in DSB repair pathway choice. Several laboratories have recently described the RIF1 protein as an effector of DNA end resection control by 53BP1 (Chapman et al. 2013; Di Virgilio et al. 2013; Escribano-Diaz et al. 2013; Feng et al. 2013; Zimmermann et al. 2013). Thus, like disruption of 53BP1, loss of RIF1 restores end resection in BRCA1-depleted cells, as measured by RPA accumulation on chromatin and Rad51 focus formation. Genetic assays support this interpretation because HR, SSA, and alt-NHEJ are all increased by RIF1 depletion (Gunn and Stark 2012; Escribano-Diaz et al. 2013).

Rad51 Assembly and Strand Invasion: Another Decision Point

Rad51, a DNA-dependent ATPase that forms nucleoprotein filaments with DNA, is a homolog of the bacterial RecA protein (Shinohara et al. 1992, 1993; Cox 2007). The mechanism of strand exchange was illuminated by crystal structures of RecA filaments in complex with single- and double-stranded DNA (Chen et al. 2008), and is likely relevant to how Rad51 proteins function. Single-stranded DNA within the RecA filament has a repeating unit of three nucleotides, which maintains a B-form structure, while between every triplet the DNA is significantly stretched. The triplets in single-stranded DNA can pair through canonical Watson-Crick hydrogen bonds with complementary triplets in homologous duplex DNA, which is crucial for a stable interaction, as RecA itself has few contacts with the complementary DNA to stabilize the interaction. ATP hydrolysis promotes dissociation of the newly formed heteroduplex DNA and the displaced single strand.

Replication protein A (RPA) binds avidly to single-stranded DNA and effectively competes with Rad51, such that a number of proteins termed mediators are necessary to displace RPA to promote Rad51 binding (San Filippo et al. 2008). Critical mediators are Rad52 in yeast and BRCA2 in mammalian cells, as Rad51 recruitment to DSBs, and hence HR, are substantially impaired when these proteins are disrupted (Sugawara et al. 2003; Lisby et al. 2004; San Filippo et al. 2008; Moynahan and Jasin 2010). Formation of a Rad51 filament directs repair into an HR pathway and suppresses SSA. Thus, disruption of either BRCA2 or Rad51 leads to increased SSA while reducing HR (Moynahan et al. 2001; Tutt et al. 2001; Stark et al. 2004). The opposing effects on HR and SSA with Rad51 or BRCA2 disruption contrast with BRCA1 or CtIP disruption, in which both pathways are reduced, and RIF1 disruption, in which both pathways are increased. Where checked, alt-NHEJ is affected similarly to SSA. Overall, these results are consistent with an upstream role in HR for BRCA1/CtIP in promoting end resection, which is suppressed by 53BP1/RIF1, but a downstream role in HR for Rad51/BRCA2 in strand invasion. Importantly, Rad51 filament formation suppresses the potentially mutagenic pathways of SSA and alt-NHEJ, while promoting more precise HR.

Completing HR: Many Avenues

The extended strand invasion intermediate has many potential fates, ultimately leading to either an NCO or CO (Fig. 3) (Klein and Symington 2012). This "choice" of fate can have critical

consequences. In meiotic cells, at least one DSB per chromosome pair must be resolved as a CO to ensure proper segregation of homologous chromosomes at the first meiotic division (Baker et al. 1976). However, in mitotic cells, COs between homologous chromosomes can lead to loss of heterozygosity of the segment of chromosome distal to the CO, an important mechanism for initiation of some types of tumors, whereas COs between repeats can lead to copy number variation, such as from duplications or deletions (Moynahan and Jasin 2010). Nonetheless, both COs and NCOs are observed in mitotic and meiotic cells, even if the balance is weighted differently for each (Cole et al. 2010; LaRocque et al. 2011).

In a primary pathway in mitotic cells to generate NCOs, the newly synthesized DNA strand dissociates from the D loop to anneal to the other DNA end, which has been termed synthesis-dependent strand annealing (Fig. 3A) (Nassif et al. 1994; Ferguson and Holloman 1996). In contrast, a key pathway in meiotic cells involves "capture" of the other DNA end by the D loop to form a dHJ; although originally postulated to be resolved as either a CO or NCO (Orr-Weaver and Szostak 1983), dHJ resolution appears to be biased to the formation of COs (Allers and Lichten 2001) by the combined activity of a MutL complex and Exo1 (Fig. 3B) (Zakharyevich et al. 2012). However, dHJs that form in mitotic cells can also be "dissolved" by the branch migration and topoisomerase activity of the BLM (Sgs1)/TOP3α/RMI1 complex (Fig. 3C) (Wu and Hickson 2003). Intermediates that escape the action of the BLM complex can potentially be resolved by several different resolvases, including MUS81/EME1(Mms4), GEN1(Yen1), and SLX1/SLX4 (Ho et al. 2010; Wechsler et al. 2011; De Muyt et al. 2012; Zakharyevich et al. 2012), the choice of which may be cell-cycle regulated (Fig. 3D) (Matos et al. 2011). Further complicating this already complex picture, Sgs1 regulates the formation of both COs and NCOs in meiosis (De Muyt et al. 2012; Klein and Symington 2012; Zakharyevich et al. 2012). Whether BLM performs a similarly complex role in mammals is still an open question.

CELL BIOLOGY OF DSB REPAIR IN LIVING CELLS

Immunohistochemistry provides a view of where the repair and recombination proteins are localized in the cell after DNA damage. For example, the colocalization of RAD51 and the breast cancer susceptibility genes, BRCA1 and BRCA2, in nuclear foci was important for linking them in a common pathway (Scully et al. 1997; Chen et al. 1998). As a result of the development of fluorescent tags, it has been shown that many repair proteins involved in HR form parts of dynamic giga-Dalton assemblies that contain many hundreds to thousands of copies of the different proteins (Lisby and Rothstein 2004). The proteins flow into and out of these complexes as shown by fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) analyses in mammalian cells (Essers et al. 2002). In addition, cells constantly assemble and disassemble these complexes as part of a quality control mechanism designed to monitor appropriate sites for HR activity (Kanaar et al. 2008). At this time, the completion of recombination events cannot be detected in individual cells and therefore one must be cautious in the interpretation of what the appearance of a focus represents—an attempt to recombine or a successful event. In addition, not all HR events may lead to detectable foci (Lisby and Rothstein 2004).

Choreography of the DNA Damage Response

DNA damage can be introduced into chromosomes via γ-irradiation, clastogens, laser microirradiation (in mammalian cells), or by using site-specific endonucleases. Precise tagging of chromosomal regions can be achieved by inserting multiple tandem arrays of bacterial operators into the chromosome and expression of fluorescently tagged repressor proteins allows the visualization of the specific chromosomal sites (Robinett et al. 1996). Addition of a meganuclease cut site adjacent to the array allows the monitoring of the proteins that are recruited to a site-specific DSB (Lisby et al. 2004). By combining tagged proteins and sites with mu-

tants in the repair process and using time-lapse photography after induction of the break, many aspects of the choreography of the DNA damage response have been revealed (Lisby et al. 2004).

In budding yeast, repair centers are formed after DSBs, which are accompanied by the recruitment of checkpoint and repair proteins (Lisby and Rothstein 2004). One of the first proteins to form a focus after induction of DNA damage by γ-irradiation is the Mre11 protein, part of the Mre11/Rad50/Xrs2 (MRX) complex. It is also the first protein recruited to a site-specific DSB (Lisby et al. 2004). Resection of endonuclease-induced DSBs only occurs in S or G_2 and is controlled by cyclin-dependent kinases (Ira et al. 2004). On the other hand, DSBs induced by γ-irradiation can be resected at any stage of the cell cycle (Barlow et al. 2008). The absence of the Mre11 protein blocks the appearance of Tel1 (the yeast ATM homolog) in foci, but interestingly does not block the appearance of many other downstream proteins including Rpa1, members of the yeast 9-1-1 complex (Ddc1, Rad17, and Mec3), Rad53 (a yeast CHK2 homolog), and the recombination protein, Rad52 (Lisby et al. 2004). Recruitment of budding yeast Rad9 (a 53BP1 homolog) and Rad53 depend on both Tel1 and Mec1 (the ATR homolog), whereas the downstream recombination protein, Rad52, does not (Lisby et al. 2004). The entire cascade of recombination proteins in yeast depends first on the recruitment of RPA to single-stranded DNA, which in turn recruits Rad52, which is necessary to recruit the Rad51 recombinase (Lisby et al. 2004).

In mammalian cells, like in yeast, recombination proteins are recruited to DSBs and many of these steps are conserved (Wyman and Kanaar 2006). For example, the mammalian Mre11/Rad50/Nbs1 (MRN) complex is one of the first sensors of DSBs followed by the downstream checkpoint proteins of the 9-1-1 complex (Rad9, Rad1, and Hus1) (Petrini and Stracker 2003). Other steps use slightly different players. For example, Rad52 in mammalian cells does not have as strong a recombination phenotype as in yeast (Essers et al. 2002; Stark et al. 2004) and it is the BRCA2 protein that is necessary to recruit Rad51 to IR-induced foci for HR (Yuan et al. 1999; Moynahan et al. 2001). However, knocking down both Rad52 and BRCA2 leads to synthetic lethality pointing to a role for Rad52 in mammalian cells (Feng et al. 2011). In addition, the Rad51 paralogues, RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3, are also required to recruit Rad51 to foci (Bishop et al. 1998; Takata et al. 2000; Rodrigue et al. 2006), whereas in budding yeast, the Rad51 paralogues, Rad55 and Rad57 are not (Lisby et al. 2004), although Rad51 foci are dimmer in a *rad57* mutant (Fung et al. 2009). The Rad54 protein in mammalian cells plays a role both early and late in the recruitment of Rad51 to foci, the early role not being ATPase dependent (Agarwal et al. 2011).

In mammalian cells there are many more proteins found at laser-induced DSBs (Bekker-Jensen and Mailand 2010). In addition, the studies in mammalian cells have defined microcompartments with single-stranded DNA (RPA localization) as well as surrounding chromatin flanking the DSB after irradiation (Bekker-Jensen et al. 2006). Some recombination proteins, like the MRN complex and BRCA1, are in both compartments (Bekker-Jensen et al. 2006). However, there is much protein recruitment activity in the adjacent surrounding chromatin (Polo and Jackson 2011). For example, after a DSB, PARP activity is detected by the appearance of poly(ADP ribose) at a DSB (D'Amours et al. 1999; Luijsterburg and van Attikum 2012). Its accumulation and H2AX phosphorylation (γH2AX) signal the recruitment of MDC1 (Stucki et al. 2005), which in turn recruits 53BP1 (Luijsterburg and van Attikum 2012) and then the E3 ubiquitin ligases, RNF8, RNF168, and BRCA1 (Huen et al. 2007; Kolas et al. 2007; Mailand et al. 2007; Wang and Elledge 2007; Doil et al. 2009; Stewart et al. 2009). These later proteins are necessary for the recruitment of the SUMO ligases PIAS1 and PIAS4, which help stabilize some of the ubiquitin conjugants (Galanty et al. 2009; Morris et al. 2009). It is thought that all of these processes aid in both the assembly and disassembly of recombination foci. Understanding their regulation will help us gain insights into their various functions during DSB repair.

INCREASED CHROMOSOME MOBILITY AFTER DNA DAMAGE

Recently, studies have focused on the movement of chromosomal loci before and after DNA damage. Many support the notion that DSBs cause increased mobility within the nucleus. For example, in yeast, cells that contain two double-strand breaks often form only a single repair focus, suggesting that the broken chromosomes move to a repair center (Lisby et al. 2003b). Furthermore, DSBs that are unable to be repaired move to the yeast nuclear periphery (Nagai et al. 2008; Oza et al. 2009). On the other hand, chromosome mobility in mammalian cells does not increase in G_1 cells after induction of a single DSB, however mobility does increase slightly in the absence of *Ku80* (Soutoglou et al. 2007). Different results were seen after creating DSBs in HeLa cells with α-particles (Jakob et al. 2009). On the other hand, γH2AX foci are more mobile favoring the view that distant DSBs can be juxtaposed (Aten et al. 2004). Increased chromosome movement of uncapped telomeres in mouse cells has recently been associated with the 53BP1 repair protein (Dimitrova et al. 2008). In addition, 53BP1 plays a role in long-range VDJ joining reactions, underlining the importance of properly regulating chromosome mobility (Difilippantonio et al. 2008). All of these results highlight the role of the DNA damage response and of repair proteins in the higher mobility of chromosomal loci.

High-resolution 4D tracking studies in the mid-1990s showed that chromosomal loci in budding yeast show confined diffusion exploring approximately 3% of the nuclear volume (Marshall et al. 1997). Recent work in haploid and diploid yeast cells has shown that after DNA damage, there is an increase in chromosome mobility (Hajjoul et al. 2009; Dion et al. 2012; Mine-Hattab and Rothstein 2012). Because the confinement radius of the broken chromosome more than doubles from that seen in the absence of DSBs, it means that it explores a more than 10 times larger nuclear volume. The increased mobility is general, as the dynamics of the unbroken chromosomes also increase depending on the number of DSBs as revealed by studies with γ-irradiation at doses delivering from between ~4 DSBs and ~20 DSBs per nucleus (Mine-Hattab and Rothstein 2012). In diploid cells, the pairing of the homologous chromosomes can be observed in real time, and once started it takes approximately 20 min before the loci separate again (Mine-Hattab and Rothstein 2012). This separation is associated with the disassembly of the repair center. Importantly, the single repair center associated with a DSB necessarily has the two ends held together and this tethering is at least partially dependent on MRX and Sae2 (Lisby et al. 2003a; Kaye et al. 2004; Lobachev et al. 2004; Clerici et al. 2005). Thus, the two ends of a DSB most often form a single Mre11 or Rad52 focus, suggesting that the homology search that occurs after a break has both ends ready for strand invasion at the homologous chromosomal locus. In summary, all of these studies suggest that the increase in chromosomal mobility likely facilitates the search for homology.

Combining genetics and cell biological studies have revealed some of the key players in regulating increased chromosome mobility. For example, in both haploid and diploid yeast cells, increased DNA dynamics depend on the Rad51 recombinase, which is similar to the observation that Rad51 is important for the movement of an unrepaired DSB to the nuclear periphery in haploid yeast cells (Oza et al. 2009; Dion et al. 2012; Mine-Hattab and Rothstein 2012). Similarly, in *Drosophila,* Rad51 is important for the increased mobility of heterochromatic DNA after induction of DSBs (Chiolo et al. 2011). Increased chromosome mobility in haploid yeast also depends on another recombination protein, Rad54, as well as on the checkpoint proteins, Rad9 and Mec1 (Dion et al. 2012). In addition, the pairing between homologous *MAT* and *HML* loci on chromosome III in haploid yeast cells seen after an HO-induced DSB at the *MAT* locus depends on two recombination and repair helicase proteins, Sgs1 and Srs2 (Houston and Broach 2006). Finally, delaying the appearance of single-stranded DNA in the absence of Sae2, a protein involved very early in the processing of DNA ends, also delays increased chromosome mobility (Mine-Hattab and Rothstein 2012).

All of these studies point to the regulation of chromosome movements in response to DNA damage. It is interesting to note that increased mobility is not associated with a higher coefficient of diffusion, suggesting that global changes in the properties of chromatin after damage (Kruhlak et al. 2006; Ziv et al. 2006) may affect the movement of chromosomal loci.

PERSPECTIVES

Over the years, much progress has been made concerning the repair of DSBs; however, many questions remain in the field. How does the cell "know" what to do when confronted with broken DNA? What is the mechanism governing increased chromosome mobility? To what is the circuitry responding when it triggers the repair event? Clearly the state of the chromatin is used to both interpret and act as the scaffold to build repair centers. What are all of the proteins doing in the gigadalton complexes that form around broken DNA? Why do so many mediator proteins seem to be required to load Rad51 onto single-stranded DNA? How is the chromosomal end that is bound by Rad51 organized in this complex to find its partner? It is likely that concentrating the proteins in centers help ensure that biochemical reactions proceed rapidly and efficiently. They also play an important role in coordinating the reactions at the ends.

Finally, it is notable that HR proteins act as tumor suppressors (Walsh and King 2007; Turnbull and Rahman 2008). Furthermore, agents that cause strand breaks are tumorigenic. Thus, understanding DSB repair processes and the factors involved in them continue to be a priority. Still, the relationship between HR and tumor suppression remains to be elucidated. It is unclear whether genomewide instability is sufficient to promote tumorigenesis or whether a subset of loci is particularly vulnerable to lead to transformation. Synthetic lethality approaches are being exploited for therapy of HR-deficient tumors (Brough et al. 2011). Presumably, synthetic lethal screens will continue to be fruitful for developing therapeutic approaches and also to provide insight into the relationship of HR with other cellular pathways.

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