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DNA double strand break repair pathway choice in somatic mammalian cells

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Abstract

The major pathways of DNA double strand break (DSB) repair have key roles in suppressing genomic instability. However, if deployed in an inappropriate cellular context, these same repair functions can mediate chromosome rearrangements that underlie various human diseases, ranging from developmental disorders to cancer. Two major mechanisms of DSB repair predominate in mammalian cells, namely homologous recombination and non-homologous end joining. In this Review, we outline a 'decision tree' of DSB repair pathway choice in somatic mammalian cells, and consider how DSB repair dysfunction can lead to genomic instability. Stalled or broken replication forks present a distinctive challenge to the DSB repair system. Emerging evidence suggests that the 'rules' governing stalled fork repair pathway choice differ from those that operate at a conventional DSB.

Introduction

DNA double strand breaks (DSBs) and solitary DNA ends—whether products of chromosome breakage, replication fork stalling or telomere deprotection — pose an immediate threat to the stability of the genome, potentially provoking chromosome rearrangements and disrupting gene structure and function. Indeed, germline mutations in DSB repair genes cause genomic instability in numerous hereditary human disease syndromes that are associated with cancer predisposition, developmental disorders and premature aging[1]. Genetic disruption of any one of the major pathways of DSB repair causes genomic instability in mammalian primary cells, suggesting that the different DSB repair pathways normally work in harmony to minimize genome errors. However, not all breaks are created equal. A series of control mechanisms have evolved to ensure that the DSB repair pathway that is engaged is matched to the cellular context — including cell cycle phase and the local chromatin environment. This Review focuses on how these control mechanisms operate in normal cells and how their dysfunction can promote genomic

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instability. We first outline the pathways that are available for the repair of a conventional two-ended DSB and discuss the special challenge to the DSB repair system posed by one-ended breaks. We then consider the critical points at which commitment to each pathway occurs, and outline a 'decision tree' of DSB repair. Lastly, we address our emerging understanding of the 'rules' governing repair at stalled forks. Recent work shows that these rules differ substantially from those that operate at a conventional DSB. We suggest that at least one DSB repair pathway that has traditionally been considered error-prone, single strand annealing, may have a conservative function at stalled forks by suppressing tandem duplications at sites of aberrant replication fork restart.

Overview of DSB repair pathways

Two major pathways are predominantly involved in the repair of a two-ended DSB: non-homologous end joining (NHEJ) and homologous recombination (HR)[2–8] (Figure 1). 'Classical' NHEJ (cNHEJ)—so called to distinguish it from alternative end-joining (aEJ), the rejoining of DNA ends in the absence of cNHEJ genes—is a rapid, high capacity pathway in mammalian cells that joins two DNA ends with minimal reference to DNA sequence. cNHEJ can, however, accommodate very limited base-pairing between the two processed DNA ends, potentially forming repair joints with up to 4 base pairs of 'microhomology'[7]. By contrast, homologous recombination requires extensive sequence homology between the broken DNA and a donor DNA molecule, and entails templated DNA synthesis as a key step in the repair process.

Classical non-homologous end joining

cNHEJ is initiated by the binding of the Ku70–Ku80 (also known as XRCC6–XRCC5) heterodimer to DSB ends. Although several molecules of Ku can be loaded onto a DNA end *in vitro*, direct imaging of Ku at DSBs in living mammalian cells suggests that one dimer of Ku normally binds to each DNA end of a chromosomal DSB[9]. Ku nucleates the recruitment of other cNHEJ factors including the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), DNA ligase IV (LIG4) and the associated scaffolding factors XRCC4, XRCC4-like factor (XLF) and paralogue of XRCC4 and XLF (PAXX)[10–14]. XRCC4 is essential for LIG4 stability and function, while XLF and PAXX have partially redundant scaffolding roles, as revealed by studies of the cNHEJ-mediated process of V(D)J recombination in lymphocyte development[15,16]. Single molecule imaging of the cNHEJ reaction has revealed a two-stage mechanism of synapsis of the two ends of the DSB[17]. First, Ku70–Ku80 and DNA-PKcs establish long-range synapsis; second, the two ends become closely aligned in a process requiring XLF, non-catalytic functions of XRCC4–LIG4 and DNA-PKcs kinase activity[18]. A synaptic reaction can alternate between long-range and short-range states, suggesting that sampling of DNA end-binding partners is a dynamic process that is reversible until the process of ligation is completed. End processing by the nuclease Artemis, by specialized DNA polymerases λ and μ and other enzymes ensures compatibility of the ligated ends and is restricted to the short-range synaptic complex[19]. A number of accessory factors, some of which likely remain undiscovered, support or otherwise regulate C-NHEJ. These include the multifunctional Mre11/Rad50/Nbs1 (MRN) end recognition complex, which may assist in end bridging[20–22] and apratxin

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and PNK-like factor (APLF), which interacts with Ku80 and with poly(ADP ribose)-modified proteins in the vicinity of the DSB[23–25]. Several additional positive and negative regulators of Ku70–Ku80 have been identified[26–28]. For a 'Ku-binding motif" in a number of Ku70–Ku80-interacting proteins is thought to mediate their cNHEJ-regulatory functions[27].

Ku70–Ku80 is an abundant nuclear complex, which has high affinity for DNA ends that are either blunt or possess limited single-stranded DNA (ssDNA) overhangs. Long ssDNA tails have reduced affinity for Ku70–Ku80 and are channeled towards cNHEJ less efficiently[29]. Nucleolytic processing of DNA overhangs or chemically modified ends by the cNHEJ nuclease Artemis can re-establish Ku70–Ku80 access to the DNA end[30]. The reversible nature of cNHEJ synapsis prior to ligation suggests that later steps of the pathway may also be subject to regulation. Indeed, DNA-PKcs autophosphorylation is an important regulator of cNHEJ, affecting both Ku70–Ku80 binding and complex disassembly during ligation[18,31]. Further, interspecies variations in the efficiency of cNHEJ may reflect differences in the abundance or regulation of DNA-PKcs.

Homologous recombination

The second major pathway of DSB repair, homologous recombination, is a multi-step process that differs from cNHEJ in key aspects. Unlike cNHEJ, which operates throughout the vertebrate cell cycle to repair DSBs, HR is largely restricted to the S and G2 phases of the cell cycle[32]. The major conservative HR pathway in somatic cells involves recombination between sister chromatids — the identical copies of a post-replicative chromosome[32,33]. Sequence identity, alignment and physical cohesion of the two sister chromatids are thought to favour sister chromatid recombination over other potential recombination partners. HR entails the loading of the recombinase RAD51 (the eukaryotic homologue of RecA) onto ssDNA, either at DNA ends that have undergone DNA end resection to generate extended 3´ ssDNA tails, or at post-replicative ssDNA gaps, where no DSB is present[2,34]. Eukaryotic DNA end resection is initiated by the MRE11–RAD50–NBS1 (MRN) complex (Mre11–Rad50–Xrs2 in *Saccharomyces cerevisiae*), which also serves as a scaffold for activation of the multi-functional DNA damage signalling kinase ATM[35–37]. Mre11 endonuclease activity nicks the DNA strand of the DSB that possesses a free 5´ terminus, up to 300 nt internal to the DNA end, and Mre11 3´–5´ exonuclease activity extends the nick towards the DNA end. Efficient initiation of 'short-range' resection by MRE11 endonuclease activity requires interaction with CtBP-interacting protein (CtIP; also known as RBBP8; Sae2 in *S. cerevisiae* and Ctp1 in *Schizosaccharomyces pombe*) and is stimulated by protein blocks at the DNA end, such as Ku70–Ku80, replication protein A (RPA) or nucleosomes[38–45]. This initial processing step is thought to displace Ku70–Ku80 from DNA ends (Figure 1) and also provides an entry point for 'long range' resection, in which Exonuclease 1 (EXO1), in parallel with the helicase Bloom syndrome protein (BLM; Sgs1 in *S. cerevisiae*) and the endonuclease DNA2, mediate unwinding and/or nucleolytic digestion of the 5´ strand of the DNA end to form a long 3´ ssDNA tail[46–49]. Several additional DNA end resection regulators, both positive and negative, have been described. BRCA1, the product of one of the two major hereditary breast and ovarian cancer predisposition genes, in complex with its heterodimeric partner BARD1, interacts with CtIP

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and MRN and has been implicated in DNA end resection as well as in later stages of HR (discussed below).

Single-stranded DNA rapidly becomes coated with the abundant ssDNA binding heterotrimeric complex replication protein A (RPA). ssDNA bound to RPA cannot pair with other ssDNA sequences. Thus, RPA ‘melts’ secondary structure in ssDNA and limits spurious interactions with ssDNA intermediates of other nuclear processes. RPA also forms a barrier to the loading of the RAD51 recombinase and must be displaced by recombination mediators if HR is to proceed[34] (Figure 1). In budding yeast, Rad52 (Rad22 in *S. pombe*) is the key recombination mediator, while BRCA2, the product of the second major hereditary breast and ovarian cancer predisposition gene, serves that function in vertebrates and in some fungal species[50–52]. BRCA2, constitutively bound to the proteasomal component DSS1, interacts with both ssDNA and RAD51 monomers, as well as with BRCA1–BARD1 via the PALB2 protein[5]. BRCA2–DSS1 can act as a recombination mediator *in vitro*. The extent to which BRCA1–BARD1–PALB2 modulates this activity remains to be determined. The association of BRCA1 with proteins involved in DNA end resection and RAD51 loading suggests that BRCA1 might couple these two HR steps, perhaps analogous to how DNA end resection is coupled to RecA filament formation in *Escherichia coli*[53]. In *E. coli*, direct interactions between the RecB subunit of the DNA end resection complex RecBCD and RecA ensure timely loading of RecA at χ recombination hotspot sequences[54].

The RAD51 filament is a dynamic structure, subject to competing activities that promote its stability or disassembly. In *S. cerevisiae*, Rad51 paralogues promote the stability of the Rad51 filament and restrain its disassembly by the antirecombinase Srs2[55–57]. Loss of Srs2 alone promotes unrestrained, ‘toxic’ recombination and genomic instability[58]. These relationships indicate that RAD51 filament stability in normal physiology is regulated to optimize the efficiency of HR and, at the same time, to restrict RAD51 function to appropriate DNA substrates. RAD51-bound ssDNA mediates the homology search that defines HR, by invading duplex DNA molecules and facilitating base-pairing with complementary homologous DNA sequences in the invaded molecule. BRCA1–BARD1 has recently been implicated in facilitating RAD51-mediated homologous pairing, indicating that BRCA1 promotes multiple HR steps[59]. RecA or RAD51 nucleoprotein filaments form synaptic complexes containing a three-stranded DNA helix that supports heteroduplex formation — base-pairing between the invading strand and the complementary strand of the invaded molecule[60]. If a match of sufficient homology is made, the synapse is stabilized and the non-base-paired strand of the invaded molecule is displaced to form a displacement loop (D-loop)—a process driven by RAD51-mediated ATP hydrolysis and RAD51 filament disassembly[61]. The free 3′ end of the invading strand engages a DNA polymerase, extending the invading (nascent) strand using the invaded donor DNA molecule as a template for gene conversion (Figure 1). DNA polymerase δ (Pol δ) plays a major role in nascent strand synthesis, but translesion DNA polymerases have also been implicated in competition with Pol δ[62–64]. Gene conversion in yeasts and flies can entail multiple rounds of RAD51-mediated invasion, nascent strand extension, displacement and reinvasion[65,66]. The same process might also occur in vertebrates. Various motor proteins, including FANCM (Mph1 in *S. cerevisiae* and Fml1 in *S. pombe*), BLM and RTEL1 can disassemble D-loops,

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implicating these proteins in displacement of the nascent strand to limit the extent of gene conversion[44,67–70].

Subpathways of HR—The major pathway of somatic HR, synthesis-dependent strand annealing (SDSA), invokes RAD51-mediated invasion by only one end of the two-ended DSB, whereas the second end is resected but remains passive[2]. How this asymmetry is established is not well understood. Interestingly, a capacity for asymmetric resection of ionizing radiation-induced two-ended breaks was revealed in *S. cerevisiae* mutants lacking MRX or Sae2 function, suggesting that MRX or MRN may control this process[71]. For a two-ended DSB, the non-invading second end of the break enables HR termination by annealing with the displaced nascent strand. Because it does not involve formation of a Holliday junction, SDSA is a non-crossover pathway (Figure 1). By contrast, the classical HR pathway, DSB repair (DSBR), which is prominent in meiotic recombination, entails formation of a double Holliday junction (dHJ)[2]. Depending on the polarity of dHJ resolution, this can result in crossing over between the recombining molecules, detected in somatic cells as a sister chromatid exchange[72] (Figure 1). BLM, in complex with TopIIIα/RMI1/RMI2 (the BTR complex), promotes an alternative non-crossover dHJ dissolution mechanism[73]. Replicative responses following strand invasion, of which break-induced replication (BIR) is a prominent example, are discussed below.

Single strand annealing

Several additional pathways of DSB repair are recognized. Single strand annealing (SSA, Figure 2A) is a Rad51-independent mechanism that enables two homologous 3´-ssDNA ends (for example, at tandem repeats) to be joined by annealing, at the cost of a deletion between the repeats[2]. In repair of a conventional DSB, SSA is therefore an error-prone pathway. SSA requires extensive DNA end resection and RPA displacement to reveal complementary homologous sequences. Rad52 has a key role in RPA displacement and Rad51 loading in yeast, while its role in this process in mammalian cells appears less critical.

Alternative end joining

A related rejoining mechanism that operates on 3´-ssDNA ends is alternative end joining (aEJ, Figure 2B)—defined as non-homologous end joining that does not use the cNHEJ pathway[74]). Use of microhomology at the breakpoint is a prominent feature of aEJ and the term ‘microhomology-mediated end joining’ (MMEJ) is sometimes used synonymously with aEJ. However, this elision can be confusing, since limited microhomology use is also associated with cNHEJ. We will therefore use the term ‘MMEJ’ as a descriptive term, to note the presence of microhomology at a breakpoint, whether mediated by cNHEJ or aEJ. In metazoans, Pol θ (encoded by the *POLQ* gene) has been implicated in aEJ[75,76]. A Pol θ-associated helicase function can displace RPA from ssDNA, revealing internal microhomologies on ssDNA, while its polymerase function can stabilize the joint between the two DNA ends[77,78]. Additional DNA polymerases may be required to complete fill-in synthesis during aEJ[62]. *Polq* null mice reveal spontaneous genomic instability, implicating Pol θ (and possibly aEJ) in physiological functions[79]. These functions are probably executed at sites of stalled replication; in *Caenorhabditis elegans*, *pol-q1* (encoding Pol θ) suppresses

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large chromosomal deletions at sites of fork stalling, at the expense of allowing small deletions to form[80,81]. Combined deletion of *Ku70* and *Polq* in primary mouse cells induces a severe growth defect, while HR-defective cells reveal elevated expression of *POLQ*[82–84]. Taken together, these data suggest that Pol θ evolved to repair certain replication-associated DNA lesions that are poor substrates for cNHEJ. Pol θ has also been implicated as a mediator of pathological chromosome rearrangements[84].

Recent work suggests that not all mammalian aEJ is mediated by Pol θ. Class switch recombination (CSR) is an end joining process that is involved in the rearrangement of immunoglobulin heavy chain (*IgH*) loci in cytokine-stimulated B cells, in which both cNHEJ and aEJ pathways repair DSBs induced at CSR 'switch' regions, triggered by activation-induced cytidine deaminase (AID)-mediated cytosine deamination[74,85]. *Polq*$^{-/-}$ mouse B cells have normal CSR frequencies and normal spectra of microhomology use at CSR breakpoints, indicating that POLQ is not required for MMEJ during CSR[86,87]. However, *Polq* is required for formation of CSR junctions that contain nucleotide insertions[86]. A recent study reported that *Rad52*$^{-/-}$ mouse B cells have increased CSR frequencies in comparison to wild type cells, but fail to form CSR products with microhomology >4 bp[87]. Since C-NHEJ is not associated with microhomology >4 bp, this finding raises the interesting possibility that RAD52 contributes to mammalian aEJ during CSR and may compete with cNHEJ in this context. RAD52 has not been implicated in MMEJ in other settings[88]. It remains to be determined what specific features of CSR might enable RAD52 to contribute to aEJ.

Repair responses to one-ended breaks

A distinctive challenge for the DSB repair system arises at sites of broken or collapsed replication forks, since solitary DNA ends or one-ended breaks can arise in this context[89–92]. In this case, there is no immediate partner for end joining, and the absence of a second DNA end denies the possibility of engaging error-free SDSA.

Break-induced replication

In *S. cerevisiae*, 'break-induced replication' (BIR, Figure 1), a product of *RAD51*-dependent gene conversion, extends the nascent strand of a one-ended invasion to the end of the chromosome, frequently copying more than 100 kb from the donor chromosome, unless a disruptive event such as a collision with a replication fork prematurely terminates the process[91,93]. BIR that engages a heterologous chromosome donor results in a non-reciprocal translocation[94,95]. In *S. pombe*, a Rad22^{Rad52}-mediated form of BIR can occur at stalled forks[93]. BIR entails conservative DNA synthesis *via* a migrating bubble mechanism, generating extensive ssDNA tracts that are vulnerable to mutation and secondary chromosome rearrangements[58,96–99]. As a result, BIR is highly mutagenic. BIR in *S. cerevisiae* requires the gene *PIF1*, encoding the Pif1 helicase and *POL32*, encoding a non-essential subunit of Pol δ, whereas neither of these genes are required for conventional short tract SDSA[96,100,101]. However, SDSA-mediated long tract gene conversions (LTGCs) of only a few kilobases also require *POL32*[102]. BIR can be preceded by repeated rounds of

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LTGC and homologous template switching during the first ~10 kb of gene conversion[66]. These findings evince both similarities and distinctions between LTGC and BIR.

Other than the presence or absence of a second DNA end, the stability of D-loop/migrating bubble structures formed during nascent strand synthesis influences copying mechanism 'choice' during HR. Activities that mediate D-loop disassembly/nascent strand displacement channel HR towards SDSA, while factors that stabilize the D-loop favor LTGC or BIR and crossover outcomes in *S. cerevisiae*[103]. This interplay is supported by data implicating several motor proteins in D-loop metabolism. A recent study in *S. cerevisiae*, in which D-loop formation was directly quantified in response to a site-specific DSB, identified two parallel pathways of D-loop disruption, mediated by Mph1 and the Sgs1/Top3/Rmi1 (STR) complex or, in parallel, by the Srs2 helicase[104]. Similarly, the mammalian homologues of Mph1 (FANCM) and STR (the BLM/TopIIIα/RMI1/RMI2 complex), as well as several candidate homologues of Srs2, can disrupt D-loops *in vitro*[103], and both BLM and FANCM suppress LTGC during mammalian HR[92]. Interestingly, *SGS1* and *MPH1* impose a delay in the onset of BIR at a one-ended break[102,105,106]. These data suggest that the fate of the D-loop is intimately related to the balance between conservative and error-prone outcomes of HR.

In mammalian cells, the longest DSB-induced Rad51-mediated gene conversions reported to date are LTGC products of <10 kb, substantially less than the >100kb BIR tracts observed in yeast. Cells lacking BRCA1, CtIP, BRCA2, or paralogues of RAD51 reveal a bias in favor of LTGC[107–110]. This bias could reflect a failure to engage the second end of the break during SDSA termination or a specific bias in favour of BIR-type copying mechanisms[111]. An emerging literature suggests that some BIR-like processes in mammalian cells are RAD51-independent. First, RAD51-independent mitotic DNA synthesis ('MiDAS') occurs at common fragile sites (regions of the genome that exit S phase with incompletely replicated DNA)[112,113]. MiDAS is mediated by RAD52, DNA polymerase δ subunit 3 (POLD3; the mammalian homologue of *S. cerevisiae* Pol32) and the structure-specific nuclease MUS81–EME1 — possibly implicating the processing of stalled replication forks as an initiating event. Second, RAD51-independent and Pol δ-mediated BIR tracts of up to ~70 kb are provoked by DSBs on telomeres maintained by the recombination-mediated 'alternative lengthening of telomeres' (ALT) pathway[114]. Third, LTGC triggered at stalled mammalian replication forks is RAD51-independent[110].

Microhomology-mediated template switching

A distinct replicative response associated with a solitary 3´-ssDNA end is microhomology-mediated template switching (Figure 2C). This process entails microhomology-mediated synapsis of a free 3´-ssDNA tail with ssDNA donor sequences (possibly daughter strand gaps in postreplicative chromatin), followed by limited DNA synthesis of up to a few hundred base pairs, and completed by strand displacement. In *S. cerevisiae*, translesional DNA polymerases are implicated in the synthesis step[99]. Unlike BIR, the end product of microhomology-mediated template switching is not a full-blown chromosome translocation but the liberation of a 3´-ssDNA tail derived from the displaced nascent strand, similar in structure to the initiating 3´-ssDNA tail. Thus, microhomology-mediated template switching

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does not resolve the problem of the one-ended break; instead, it 'kicks the can down the road'. Microhomology-mediated template switching has been invoked to explain complex chromosome rearrangement breakpoints in cancer and other diseases, in which multiple short (i.e., a few hundred bp) ectopic sequences derived from distinct remote chromosomal loci are present within the breakpoint[115–117]. Such complex breakpoints may be products of 'futile cycles' of repeated microhomology-mediated template switching between different donor loci. Work in *E. coli* and *S. cerevisiae* has emphasized an association of microhomology-mediated template switching with BIR ("microhomology-mediated BIR")[118–121]. Multiple RAD51-mediated strand invasions can also generate complex breakpoints in yeast[122]. In contrast, experimental models of microhomology-mediated template switching in mammalian cells indicate an association with end joining[92,116,123,124]. Perhaps these species differences reflect the relative efficiency of end joining mechanisms in mammalian cells (where they have a major role) and yeast (where their role is minimal). In summary, the phenomenon of microhomology-mediated template switching suggests that solitary 3′-ssDNA tails are highly reactive DNA lesions that, if not channeled into a conservative repair pathway, can interact avidly with neighboring DNA molecules in uncontrolled and dangerous ways.

The chromatin response to DSBs

DSBs provoke an extensive chromatin response that has an important role in DSB repair. The PIKK-family DNA damage response kinases ATM (activated by interactions with MRN at the break[36]), ATR (activated by ssDNA and RPA sensors AtrIP and ETAA1[125–129]) and DNA-PKcs (activated by Ku70–Ku80[18]) have each been implicated in the phosphorylation of serine 139 in the carboxy-terminal tail of the histone variant H2A.X, forming "γ-H2A.X" chromatin domains[130]. The H2A.X signalling chromatin response is conserved from yeasts to mammals[130]. In vertebrates, γ-H2A.X directly recruits the adaptor protein MDC1, forming a specialized chromatin structure that can extend hundreds of kilobases away from the DSB[131]. γ-H2A.X/MDC1 decorated chromatin is multifunctional, supporting class switch recombination in activated B cells (an end-joining process), HR between sister chromatids and ATM signal amplification (mediated by interaction of MDC1 with the MRN complex), and also suppressing spurious end resection during V(D)J recombination in lymphocytes[130,132–135]. The MDC1-binding E3 ubiquitin ligase RNF8 catalyzes K63-linked polyubiquitylation of histone H2A, recruiting BRCA1–Abraxas–Rap80-containing complexes and a second E3 ubiquitin ligase, RNF168[136,137]. The BRCA1–Rap80 complex contains deubiquitylating enzymes, further editing the ubiquitin landscape of chromatin near the DSB, and has a role in antagonizing DNA end resection[138–141]. In parallel, 53BP1 — a homologue of the *S. cerevisiae* Rad9 and *S. pombe* Crb2 damage response proteins—is recruited to chromatin by binding to dual chromatin marks of histone H4 monomethylated on lysine 20 (H4K20me1) or H4K20me2, which are constitutive marks in mature chromatin and histone H2A monoubiquitylated on lysine 15 (H2AK15Ub; a target of RNF168)[142]. Both *H2A.X*-dependent and *H2A.X*-independent pathways of 53BP1 and BRCA1 recruitment to DSB sites have been described[143]. These complexes execute distinct functions in DSB repair, conforming quite well to a 'histone code' of DSB repair[144]. In chicken DT40 cells, *53BP1* null reveals epistasis with null alleles of cNHEJ genes and

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53BP1 similarly regulates cNHEJ in mammalian cells, primarily in an *H2A.X*-independent manner[144,145]. Numerous additional biochemical modifications of chromatin occur in the vicinity of the break, including recruitment of complexes that remodel chromatin and execute histone replacement[146–150]. An emerging literature points to a role for RNA in the DSB response[151,152]. These modifications may contribute to repair, signalling and epigenetic reprogramming in the vicinity of the break.

A 'decision tree' of DSB repair

Given such a bewildering array of DSB repair pathways, how does the cell select the pathway most appropriate for each DSB? In principle, all DSB repair pathways might compete for access to all free DNA ends. However, for a conventional two-ended DSB, the two major conservative DSB repair pathways, cNHEJ and HR, are dominant. In contrast, error prone pathways (such as SSA, aEJ, microhomology-mediated template switching and BIR) may act more opportunistically, scavenging on the products of aborted or incomplete cNHEJ or HR, or on problematic lesions such as one-ended breaks. In this way, the DSB repair system could be understood as a decision tree (Figure 3, 4), the branch-points ('nodes') of which represent points of commitment to cNHEJ or HR, points of physiological sub-pathway divergence (for example, during late stages of HR), or points at which repair intermediates are vulnerable to hijack by error-prone repair pathways. Presumably, in a well-regulated cell, each decision node is tuned to maximize the probability of conservative repair and minimize error-prone outcomes. Pathological conditions perturb this regulatory balance by disrupting DSB repair regulatory genes, by flooding the cell with levels of DNA damage that exceed the capacity of physiological pathways, or by allowing the formation of complex DNA lesions for which there are no good outcomes. For example, some of the recently identified examples of catastrophic chromosome rearrangement—chromothripsis and chromoplexy—reflect the action of end joining on an overwhelming burden of simultaneously arising DSBs[116,117].

"The wand chooses the wizard"

An important determinant of DSB repair pathway choice is the initiating DNA lesion itself: "The wand chooses the wizard"[153]. As discussed above, attempts to repair a one-ended break or solitary DNA end are necessarily error-prone. For a two-ended break, whether the DNA ends contain single stranded tails can affect repair pathway choice, since Ku70–Ku80 binds weakly to long ssDNA tails[29]. Similarly, a DNA end that is chemically blocked or that forms within compacted chromatin may require processing or extensive chromatin remodeling as an accompaniment to DSB repair. Complex patterns of single stranded gaps close to the free DNA end might also affect DSB repair because of intense activation of poly(ADP-ribose) polymerase (PARP) at ssDNA gaps. The spatial relationship between DSBs that form at heterologous loci affects the probability of their interaction; more closely positioned DSBs are more likely to interact, increasing the likelihood of rearrangement between the spatially proximate but genomically remote loci[154,155].

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The time factor

A second important determinant of DSB repair pathway choice is the time factor. In mammalian cells, the bulk of radiation-induced DSBs (which form in a genome-wide fashion across all cell cycle phases) are repaired with rapid kinetics, with a half-life in the order of a few minutes[156]. This rapid phase of repair requires cNHEJ genes, while HR and contributes to slower phases of repair[17,157,158]. A slower phase of Artemis-dependent cNHEJ has also been described, likely reflecting the involvement of DNA end processing prior to ligation[159]. In yeast mating-type switching, the interval between RAD51 loading at a DSB and its association with the intrachromosomal homologous donor is ~15 minutes, while the initiation of nascent strand synthesis requires an additional ~15 minutes[160,161]. Imaging of RAD51-mediated synapsis during mammalian interchromosomal HR suggests similar kinetics[162]. Although HR between sister chromatids might occur more rapidly than this, the kinetics of even the most efficient forms of HR are likely delayed in comparison to cNHEJ. The kinetics of error-prone mammalian repair pathways such as aEJ, microhomology-mediated template switching and BIR—arguably, repair pathways of last resort—are unknown. However, it is reasonable to assume that these processes are executed more slowly than cNHEJ.

The efficient nature of mammalian cNHEJ, combined with the role of Ku70-Ku80 as an 'early responder' at DNA ends lacking an extensive ssDNA tail, suggests that cNHEJ is a default repair pathway[163]. Consistent with this idea, mammalian cNHEJ competes with HR for repair of a site-specific chromosomal DSB[164]. Similarly, at an HO endonuclease-induced DSB in *S. cerevisiae*, cNHEJ acts on unresected DNA ends in precedence to and without reference to the status of the HR system[165].

Pathway choice and DNA end resection

At a molecular level, override of cNHEJ requires displacement of the Ku70-Ku80 complex from the DNA end. This may be accomplished by several mechanisms, including targeted degradation of Ku70-Ku80 by specific E3 ubiquitin ligases[166]. A major evolutionarily conserved mechanism for displacement of Ku70-Ku80 is the process of DNA end resection itself (Figure 1). Indeed, the engagement of DNA end resection is a key commitment step in HR, and its regulation through the cell cycle in part explains how HR is restricted to the S and G2 phases of the cell cycle.

Regulation of DNA end resection is one of the most important determinants of DSB repair pathway choice. Cell cycle-dependent kinase (CDK) activity, which increases as cells enter S phase, provides activating signals to the resection machinery and also to proteins that act later in HR[167–172]. Phosphorylation of CtIP on threonine 847 or *S. cerevisiae* Sae2 on serine 267 is essential for efficient activation of the MRE11 nuclease[170,173]. Thus, CtIP both senses cell cycle phase and transduces this information to initiate DNA end resection. A second significant connection made by CtIP in vertebrates is its binding to BRCA1 — an interaction that is regulated by phosphorylation of serine 327 on CtIP[174]. A role for BRCA1 in regulating DNA end resection during HR was suggested by the finding that BRCA1, like MRN and CtIP, is required for both HR-mediated and SSA-mediated repair of a site-specific

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DSB[175]. CtIP functions in HR, at least in part, independently of BRCA1; studies of the effect of BRCA1 loss on bulk DNA end resection have yielded variable results[107,176–178].

Remarkably, deletion of the DNA damage response gene *53BP1* suppresses the severe genomic instability of *BRCA1* mutants, as well as the sensitivity of some *BRCA1* mutant cells to PARP inhibitors[179]. This phenotypic suppression is especially prominent in those *BRCA1* hypomorphs for which the expressed *BRCA1* gene product retains the ability to bind to PALB2–BRCA2–Rad51 and hence, presumably, retains RAD51-loading mediator functions[180–182]. How does loss of 53BP1 lead to this striking phenotypic reversal? In the repair of a conventional DSB, 53BP1 mediates cNHEJ and also suppresses DNA end resection[144,179,183]. The latter activity led to the proposal that *53BP1* deletion suppresses genomic instability in *BRCA1* mutants by reversing a defect in DNA end resection. 53BP1 effectors include RIF1, PTIP and Rev7 (also called MAD2L2 — a subunit of the translesion DNA polymerase Pol ζ)[184–190]. Recent studies identified CTC-534A2.2, FAM35A and C20ORF19 as components of a Rev7-interacting 'shieldin' complex that functions downstream of 53BP1–RIF1–Rev7.[191–195]. Like 53BP1, the shieldin complex mediates cNHEJ, suppresses DNA end resection and mediates sensitivity of *BRCA1*-deficient cells to PARP inhibitors. FAM35A (also called SHLD2) contains ssDNA-binding oligonucleotide/oligosaccharide-binding (OB) fold domains, which are required for its antagonism of BRCA1. Interestingly, the results of a recent study suggested that shieldin promotes fill-in synthesis on ssDNA, potentially helping to blunt ssDNA tails[194]. The insight that shieldin antagonizes BRCA1 *via* interactions with ssDNA broadens the potential scope of 53BP1-shieldin complex function and raises some tantalizing questions: what are the key ssDNA structures over which BRCA1 and 53BP1 compete? Does 53BP1–shieldin execute functions on ssDNA additional to its known functions of resection suppression and cNHEJ? Which function of 53BP1–shieldin explains its role in conferring PARP inhibitor sensitivity to *BRCA1* mutants?

The competition between BRCA1 and 53BP1 can also be visualized in the context of the γ-H2A.X chromatin domain[130,196]. The balance between BRCA1 and 53BP1 on chromatin is affected by TIP60-mediated acetylation of residues close to H4K20, which can disrupt 53BP1 binding to H4K20me1 or H4K20me2 marks[197,198]. An E3 ubiquitin ligase activity of BRCA1–BARD1 can ubiquitylate histone H2A on lysine 27, recruiting the chromatin remodeller SMARCAD1 and facilitating 53BP1 repositioning[199]. 53BP1 is also subject to direct regulation by TIRR, a protein that blocks the H4K20me-binding domain of 53BP1[200].

Several mechanisms in addition to CDK-mediated phosphorylation of HR targets communicate cell cycle status to the DSB repair machinery. HR gene expression is up-regulated as cells transition from G1 into S phase. In mammalian cells in G1, DNA end resection is suppressed by the negative regulator HELB, which is inactivated as cells enter S phase[201]. The assembly of the BRCA1–PALB2–BRCA2–Rad51 recombinase complex is suppressed in G1 by proteasome-mediated degradation of PALB2, following its ubiquitylation by the E3 ubiquitin ligase cullin-3 (CUL3)–RBX1 and the adaptor protein Kelch-like ECH-associated protein 1 KEAP1[202]. A study of postreplicative chromatin provides an intriguing example of how S phase chromatin structure can favor HR. The TONSL–MMS22L heterodimer assists RAD51 loading and activity at stalled replication

forks[203–206]. The ankyrin repeat domain of TONSL was shown to bind to unmethylated lysine 20 of histone H4 (H4K20me0), an unmodified state that is restricted to newly incorporated histones[207]. By this mechanism, immature postreplicative chromatin provides a docking site for TONSL–MMS22L, while the dearth of H4K20me1 and H4K20me2 marks might also deny 53BP1's anti-BRCA1 activity stable access to chromatin to exert its anti-BRCA1 activity.

Entry into mitosis requires chromatin condensation and presents a unique challenge to DSB repair. Between late G2 and mid prophase the cell commits to mitotic entry even in the presence of DNA damage[208]. This transition is accompanied by an attenuation of DSB signalling. Although MRN is recruited to breaks in mitotic cells and ATM is activated, the chromatin response is restricted to γ-H2A.X modification and MDC1 recruitment, without activation of RNF8 and RNF168 E3 ubiquitin ligases or accumulation of BRCA1 or 53BP1 on chromatin[209]. This restraint of the DNA damage response is mediated by inhibitory phosphorylation of 53BP1 and RNF8 by mitotic kinases[210,211]. Indeed, unregulated reactivation of 53BP1 in mitosis provokes chromosome rearrangement and telomere fusions, reflecting inappropriate activation of cNHEJ.

Pathway choice and chromatin context

The chromatin context in which a DSB arises may have a broad influence on repair pathway choice. A study that measured the time-course of γ-H2A.X focus resolution (as a surrogate for DSB repair) in irradiated cells in G2 phase suggested that DSBs in heterochromatin are preferentially repaired by an HR mechanism requiring ATM[212]. Another study used chromatin-immunoprecipitation (ChIP) to evaluate the accumulation of HR and cNHEJ proteins at defined site-specific DSBs induced by the rare-cutting restriction endonuclease AsiSI[213]. In this setting, XRCC4 consistently accumulated in close proximity to each AsiSI target site, whereas RAD51 accumulation was more widely distributed around the break site, was maximal in G2 and showed a high level of variation in signal intensity between different AsiSI target sites. Of note, AsiSI target sites that revealed high levels of RAD51 accumulation were enriched in transcriptionally active genes, marked by the chromatin modification H3K36me3. The H3K36me3-binding factor LEDGF was implicated in RAD51 accumulation at transcriptionally active genes, consistent with previous work that linked this protein to CtIP function[214]. Interestingly, a ChIP study in undamaged cells revealed preferential accumulation of BRCA1 and PALB2 within the bodies of transcribed genes, suggesting that the accumulation of these proteins may be a scheduled accompaniment of transcription of some genes[215]. The mechanisms underlying these gene body-specific localization patterns are unclear. Possibly, collisions between replication and transcription, including the genome destabilizing properties of RNA-DNA hybrids (R-loops), might concentrate these factors at transcribed genes[216–218]. Alternatively, specific interactions with transcription complexes or epigenetic marks might be involved[150].

In yeast, DSBs are mobilized to the nuclear periphery as part of a nucleus-wide choreography of repair[219]. The question of whether DSBs are mobilized within the mammalian nucleus has been addressed by a number of studies. Mammalian DSBs do not seem to undergo mobilization that is precisely equivalent to that observed in yeast[220].

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However, γ-H2A.X foci were found to coalesce following DSB induction by alpha particles, which led to the suggestion that DSBs might undergo clustering during repair[221]. 53BP1 promotes mobility of deprotected telomeres, thereby facilitating long range rejoining[222,223]. Furthermore, endonuclease-induced DSBs that form in transcribed genes were observed to coalesce in G1, pending repair by HR at later stages of the cell cycle[213]. This observation has intriguing parallels with a cancer-associated chromosome rearrangement termed 'chromoplexy', in which chains of linked translocations between multiple transcribed loci mediate chromosome rearrangements in prostate cancer[224]. In both flies and mammalian cells, DSBs arising in constitutive heterochromatin are relocated to the periphery of heterchromatin before RAD51 becomes associated with the break sites[225–227]. Collectively, these studies suggest that regulated mobility of DNA ends occurs in specific chromatin contexts, contributing to a higher order level of DSB repair regulation.

Repair at stalled replication forks

Stalled replication forks differ from conventional DSBs in several important ways. The presence of branched DNA replication intermediates, ssDNA 'daughter strand gaps' (DSGs) and unresolved hemicatenanes in immature postreplicative chromatin, together with scaffolding by PCNA and other replisome components creates a unique environment for repair[228–232]. Attempted replication across a ssDNA nick in the parental template will convert the nick to a one-ended DSB, with the potential for misrepair of the broken fork, as discussed above. Forks that are stalled but not broken trigger a cascade of cellular responses that, when properly coordinated, are thought to minimize the risk of chromosome rearrangement at the site of stalling (Figure 5). These responses include: the activation of DNA damage signalling, controlled primarily by the Atr signaling kinase[233,234]; replisome disassembly (also termed 'fork collapse'), in which the CMG replicative helicase is extracted by the valosin-containing protein (VCP)/p97 ATPase following ubiquitylation of MCM helicase subunits[235]; remodeling of DNA structure ('fork remodeling')[90]; and the activation of repair responses, of which HR is a major component[236–238]. Reinitiation of replication ('replication restart') can also occur at the site of stalling, which can be an error-prone process[92,93]. Knowledge of the different fork remodeling steps in mammalian cells is almost certainly incomplete, as is the ability to quantify each step satisfactorily. Currently, fork remodeling is recognized to include resection of nascent lagging strands[239], fork reversal[240] and endonucleolytic processing of the stalled and reversed fork[241,242]. In vertebrates, timely, organized cleavage of stalled forks is a key step of conservative repair by HR[241,242]. In contrast, pathological states allow unscheduled processing of the stalled fork, leading to misrepair and genomic instability.

Replisome disassembly exposes the fork to topological stresses and remodelling activities that promote fork reversal[90,231,243] (Figure 5). Fork reversal generates a cruciate 'chicken foot' structure with a solitary DNA end that is formed by the annealing of the leading and lagging nascent strands. Several key mediators of fork reversal in mammalian cells have been identified, including RAD51, PARP, the translocase HLTF and the annealing helicases SMARCAL1 and ZRANB3[89,244–246]. The activity of RAD51 in fork reversal is counteracted by negative regulators such as PARI and RADX, which presumably restrict fork reversal to appropriate contexts[247,248]. In addition to its role in fork reversal, RAD51, in

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a Fanconi anaemia/BRCA pathway-dependent process, protects nascent daughter strands at stalled and reversed forks from degradation by MRE11[249–253].

Conservative repair at stalled forks

Major insights into the steps of stalled fork processing and repair in vertebrates have come from *in vitro* studies of replication-coupled interstrand DNA cross-link (ICL) repair in *Xenopus laevis* egg extracts[239,254]. An ICL covalently links the two parental DNA strands and an absolute block to replication, unless it can be 'traversed' with the assistance of FANCM[255] or directly 'unhooked' by the NEIL3 glycosylase[256]. In *X. laevis*, ICL repair is initiated following bidirectional replication fork stalling at the ICL[242]. The arrival of both opposing forks is required for replisome disassembly, asymmetric fork reversal and subsequent nucleolytic processing of the stalled fork for HR[240,242,257]. Nucleases regulated by the FANCD2–FANCI heterodimer introduce dual incisions in the stalled leading and lagging strands of one sister chromatid. This mechanism ensures that forks stalled at an ICL are processed to two-ended DSBs, thereby favouring conservative SDSA over LTGC or BIR. In contrast to the *X. laevis* ICL repair system, HR products at the replication termination switch (RTS1) replication fork barrier (RFB) in *S. pombe* occur without the formation of a DSB intermediate. Similarly, in *S. cerevisiae*, a site-specific RFB, derived from the *E. coli* replication termination system, comprising DNA replication terminus site-binding protein (Tus) bound to an array of seven *ter* sites, provokes no detectable incisions of the arrested fork[258]. Rearrangements at Tus–*ter* in *S. cerevisiae* arise from the processing of post-replicative ssDNA gaps[259]. Of note, the Fanconi anaemia pathway in yeasts is limited to orthologs of SLX4 (also known as FANCP) and FANCM; the stalled fork endonuclease-enabling FANCD2–FANCI heterodimer and its activator, the Fanconi core complex, are absent[68,260]. The presence of these later evolutionarily additions to the Fanconi anaemia pathway in higher eukaryotes might explain why scheduled incisions of the stalled fork have more prominent roles in stalled fork repair in vertebrates than in yeasts.

Rad51 is an 'early responder'—In mammalian cells, HR responses to site-specific fork stalling have been studied in replicating ICL-containing episomal plasmids and by use of a chromosomal Tus–*ter* RFB[92,110,261]. In contrast to the polar fork arrest observed in *E. coli* and *S. cerevisiae*, a Tus–*ter* RFB composed of six *ter* repeats mediates bidirectional fork stalling in mammalian cells[110]. HR induced at a Tus–*ter* RFB in wild type mammalian cells is a non-crossover pathway, generating predominantly STGC products of two-ended recombination, mediated by the canonical Fanconi anaemia–BRCA–RAD51 pathway[92,110]. These properties suggest that conservative HR at stalled forks is mediated by SDSA. The two DNA ends that participate in Tus–*ter* RFB-induced HR are presumably derived from the two opposing forks arrested at the Tus–*ter* RFB (Figure 5). However, in contrast to HR triggered by a conventional DSB, where cNHEJ avidly competes with HR, Tus–*ter* RFB-induced HR is unaffected by the status of the cNHEJ genes *Ku70* or *XRCC4*[237]. This suggests that the mechanism of SDSA at Tus/*Ter* RFBs differs significantly from SDSA at a conventional DSB. To understand this difference, it is helpful to review the known interactions between HR and cNHEJ at stalled forks.

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Studies of genetic interactions between HR and cNHEJ have provided important insights into the mechanisms of stalled fork repair. In *S. cerevisiae*, the Ku70–Ku80 complex, acting independently of Lig4, mediates lethality of *mre11* and *sae2* resection mutants exposed to the topoisomerase I inhibitor camptothecin (CPT)—a drug that generates one-ended breaks at broken forks[262,263]. This suggests that Mre11/Sae2-mediated resection at CPT-induced breaks is required to overcome the barrier formed by Ku70–Ku80 binding to the DNA end. Similarly, in human cells, Ku70–Ku80 (inferred by associated DNA-PKcs activity) accumulates transiently at CPT lesions and is rapidly displaced by MRE11 and CtIP, whereas delayed resection at CPT lesions allows misrepair by cNHEJ[264,265]. A recent study showed that HR induced by a DNA nicking enzyme ("nickase") is not affected by deletion of *Ku70* or *Xrcc4*[266]. One possible explanation for this finding is that the absence of a second DNA end at sites of nickase-induced fork breakage denies cNHEJ a productive outcome, although this hypothesis remains to be tested.

Given that Tus–*ter* RFB-induced HR is a product of two-ended recombination, why is cNHEJ denied access to these HR intermediates?[237]. A clue as to the underlying mechanism came from ChIP analysis of RAD51 recruitment to the Tus–*ter* RFB. In contrast to the DSB response, in which the Rad51 ChIP signal extends for several kilobases on either side of the DSB[213], RAD51 recruited to Tus–*ter* RFBs is localized to within 1 kb of the stall site and the ChIP signal is both more intense and more sustained than in the DSB response[237]. This distinctive pattern suggests that the principal DNA structures that recruit Rad51 to the stalled fork are not conventional DSBs — a conclusion corroborated by work in the *X. laevis* model of ICL repair[236]. Of note, lagging strand gaps normally arise, albeit transiently, during replication. Fork stalling renders these daughter strand gaps (DSGs) abnormally persistent, and nascent lagging strand resection at the site of fork stalling would further extend the size of the DSG[239]. Lagging strand DSGs could thus provide a platform for RAD51 recruitment as a very early step of stalled fork processing, prior to the formation of either a DNA end or a DSB[236,245]. An abundance of BRCA1 and BRCA2 at the stalled fork would ensure efficient RAD51 loading. In this model, RAD51 is an 'early responder' at stalled forks[267], acting as a sentinel repair factor in the stalled fork response analogous to Ku's pivotal role at a DSB (Figure 5). If leading and lagging strand synthesis were uncoupled at a leading strand DNA lesion, RAD51 might be loaded onto the resulting leading strand DSG prior to fork reversal[267].

Early and sustained RAD51 recruitment to the stalled fork could explain the exclusion of cNHEJ during stalled fork HR. If stalled fork HR in mammalian cells is initiated by RAD51-mediated fork reversal[240,245], the length of the 3′ ssDNA tail formed by fork reversal would be a reflection of the size of its precursor lesion, the lagging strand DSG (Figure 5). The first DNA end generated during stalled fork repair — an extended 3′ ssDNA tail produced by fork reversal — might therefore be incapable of binding Ku70-Ku80. In this way, the initial steps of stalled fork HR would remain 'invisible' to the cNHEJ pathway. Subsequent processing steps—more extensive fork reversal and nucleolytic incision of the reversed fork—could mobilize the RAD51-loaded DNA ends for SDSA. This model of stalled fork HR invokes two consecutive and distinct RAD51 loading steps: the first onto the lagging strand DSG, as a prelude to fork reversal; the second onto the 3′ ssDNA tail of the reversed fork, as a pre-requisite for SDSA.

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There is still much to be learned about the asymmetries associated with stalled fork processing for SDSA. How does the Fanconi anaemia pathway select one sister chromatid for incision, while leaving the other intact? During asymmetrical fork reversal, how is one fork selected to undergo reversal, and how does this asymmetry relate to the asymmetrical processing of DNA ends that is innate to SDSA?

Error-prone fork repair and restart

Error-prone fork repair may involve several distinct types of fork processing errors, including: pathological fork processing by opportunistic nucleases; aberrant interactions of solitary DNA ends formed by fork reversal; and aberrant fork restart. In our study of rearrangements at a chromosomally targeted Tus–*ter* RFB, we used high throughput genome-wide translocation sequencing (HTGTS) to identify DNA ends at Tus–*ter* that form translocations[92,268]. The major translocation-competent DNA lesions detected at Tus–*ter* by HTGTS were solitary DNA ends/one-ended breaks. This finding appears paradoxical, given the two-ended model of stalled fork HR discussed above, and in light of other methods that have revealed DNA ends of both polarities at stalled forks[269,270]. A possible explanation of this discrepancy is that two-ended intermediates of conservative repair are protected from translocation, whereas solitary DNA ends produced by aberrant fork processing are relatively translocation-prone.

The time factor influences whether stalled forks are processed in a conservative or error-prone manner. In *S. pombe*, HR at the RTS1 RFB is detectable within ~10 minutes of a fork stalling event, whereas aberrant fork restart is initiated only after ~60 minutes[93]. In mammalian cells, RAD51 supports the restart of hydroxyurea (HU)-stalled forks at which fork collapse has not yet occurred[271]. Presumably, in the context of transient HU-mediated nucleotide pool depletion, RAD51 protects stalled fork structures for a limited time period, allowing the replisome to be reactivated once the nucleotide pool is restored. However, more extensive exposure to HU leads to localized DNA damage responses and possibly fork breakage[271]. Pathological MRE11-mediated degradation of nascent strands in *BRCA* mutants is first detectable after ~30 minutes' incubation in HU, but is fully manifest only after ~5 hours[249]. Limited as the data is on this topic, it appears that pathological stalled fork repair pathways become operative only if physiological systems fail.

Replication restart and cancer—In bacteria, RecA-mediated invasion of the sister chromatid by a one-ended break at a broken fork is coupled to reassembly of a normal replisome by PriA, with resumption of conventional semi-conservative DNA synthesis[272]. To date, a PriA-like replisome re-loading activity has not been identified in eukaryotes. Consequently, once the replisome has been disassembled, fork restart in eukaryotes may be obligatorily error-prone. In *S. pombe*, forks stalled at an ectopically located RTS1 RFB engage both conservative and error-prone HR, including RAD51-dependent and RAD51-independent pathways of fork restart[273–275]. Stalled forks in *S. pombe* can be restarted by a $Rad22^{Rad52}$-mediated mechanism, but the restarted fork is unstable, being prone to rearrangement up to 75 kb downstream of the RFB[93,276]. The restarted fork in this setting is probably extended by BIR. In both *S. pombe* and *S. cerevisiae*, the mutagenic impact of BIR at stalled or broken forks is limited by the arrival of an opposing normal fork derived from

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the neighboring replicon[91,93]. A role for RAD52 in restarting collapsed mammalian replication forks has also been proposed[277].

RAD51-independent fork restart can occur at a mammalian Tus–*ter* RFB during the formation of tandem duplications in *BRCA1* mutant cells, recapitulating a highly specific ~10 kb TD 'rearrangement signature' that is observed in human *BRCA1*-linked breast and ovarian cancers[92,110,278,279]. BRCA1, BARD1 and CtIP suppress tandem duplications, whereas BRCA2, Rad51 and other HR mediators have no effect on tandem duplication formation. Tandem duplication formation in *BRCA1* mutants is seen in the stalled fork response but not in response to a conventional DSB[92]. This shows that aberrant stalled fork processing, not defective DSB repair, is the crucial trigger of tandem duplication formation in *BRCA1*-linked tumorigenesis. These findings directly implicate aberrant stalled fork restart in the formation of human cancer-associated chromosome rearrangements. Interestingly, FANCM and BLM — the same motor proteins that impose a delay on the onset of BIR in *S. cerevisiae*[102,105,106] — specifically suppress the formation of tandem duplications in *BRCA1* mutant cells[92]. These observations suggest that fork restart during tandem duplication formation may be mediated by BIR (Figure 6), and invite comparisons with aberrant fork restart in *S. pombe*[93,276].

The final step of tandem duplication formation in *BRCA1* mutant cells is mediated by cNHEJ, with formation of a non-homologous tandem duplication breakpoint (Figure 6)[92]. It is interesting to consider the possibility that this end joining step might be in competition with the error-prone SSA mechanism of DSB repair (Figure 2A). If the two DNA ends of the tandem duplication were repaired by SSA instead of cNHEJ, the duplicated segments would be collapsed to their original single copy status (Figure 6). In this setting, SSA, which is a *BRCA1*-dependent process, would perform a conservative function at stalled forks by counteracting the tendency of aberrantly restarted forks to form tandem duplications. Thus, a repair pathway that is considered to be error-prone in conventional DSB repair might mediate error-free repair at stalled forks.

Conclusion

Our understanding of the decision tree of mammalian DSB repair has reached quite sophisticated levels, enabling researchers to focus attention on higher order cellular processes that affect pathway choice, such as cell cycle status and the local chromatin environment. In contrast, some of the key 'rules' of repair pathway choice at stalled mammalian replication forks are only beginning to become clear — for example, the role of fork reversal and the exclusion of cNHEJ during conservative HR. The mechanisms that regulate remodelling of the stalled fork remain to be fully revealed and quantified in mammalian cells. We expect that ongoing research into repair pathway choice at stalled forks will yield additional insights into the origins of cancer and will reveal new therapeutic targets in diseases that are characterized by genomic instability.

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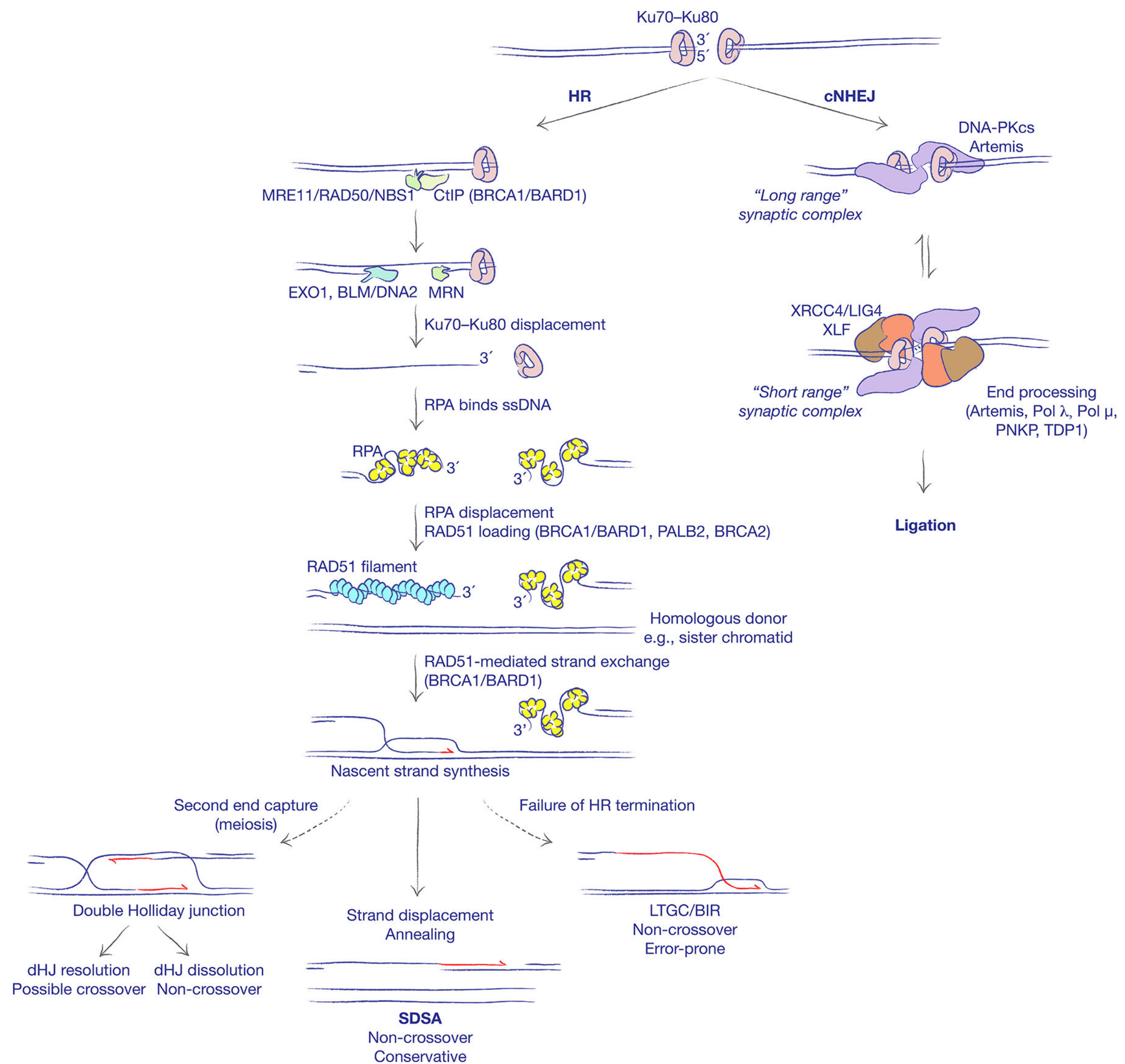

Figure 1. Two major pathways of DNA double strand break repair.

The binding of the Ku70–Ku80 heterodimer to DNA ends schedules repair of DNA double strand breaks (DSBs) by classical non-homologous end joining (cNHEJ). cNHEJ entails formation of a 'long range' synaptic complex, which is in equilibrium with a 'short range' synaptic complex. End processing by cNHEJ enzymes (as shown) and ligation are restricted to the short range complex. PNKP: Polynucleotide kinase-phosphatase. TDP1: Tyrosyl-DNA phosphodiesterase 1. The default engagement of cNHEJ can be disrupted by DNA end resection. The nuclease activity of MRE11 converts the blunt end into a 3′ single-stranded DNA (ssDNA) tail, displacing Ku70–Ku80 from the DNA end and establishing the possibility of repair by homologous recombination (HR). The replication protein A (RPA) complex avidly binds to ssDNA and must be displaced by recombination mediators to enable the formation of a RAD51 nucleoprotein filament. BRCA2 is the major recombination mediator in mammalian cells, likely acting in concert with PALB2 and the BRCA1–BARD1 heterodimer. Interactions between the two DNA ends at the recombination synapse, and operations on the D-loop formed following synapsis, influence which HR sub-

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pathway is engaged. The non-crossover synthesis-dependent strand annealing (SDSA) pathway is the predominant repair pathway in somatic cells. In meiotic cells, formation of a double Holliday junction (dHJ) intermediate can lead to crossing over. A failure to engage the second end of the break, or failure to displace the nascent strand leads to aberrant replicative HR responses of long tract gene conversion (LTGC) and break-induced replication (BIR). Established roles for *BRCA* gene products in HR are indicated in parentheses.

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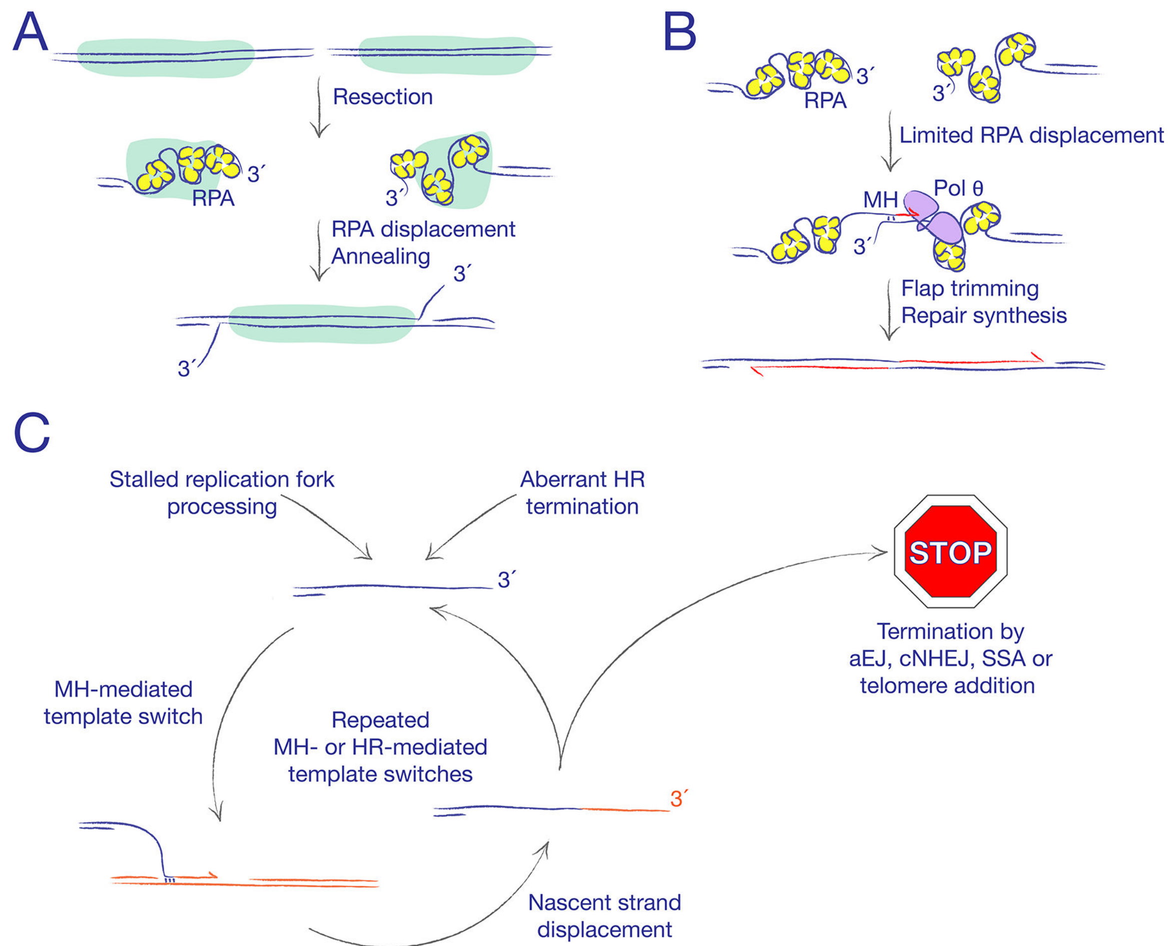

Figure 2. Alternative DSB repair pathways.

A. Single strand annealing (SSA) converts homologous repeats (marked in green) to a single copy, by annealing complementary single-stranded DNA (ssDNA) ends within each repeat. Replication protein A (RPA) must be displaced to expose complementary ssDNA for annealing. **B.** Alternative end joining (aEJ) rejoins DNA ends without use of classical non-homologous end joining (cNHEJ) proteins. MH: microhomology. Frequent use of microhomology-mediated end joining (MMEJ) is typical but not a defining feature of aEJ. The figure depicts the action of DNA polymerase θ (Pol θ), an important aEJ mediator in mammalian cells. **C.** Microhomology-mediated template switching can arise when a free 3′ ssDNA end lacks an immediately available partner for recombination or rejoining. The persistent ssDNA end is thought to interact with ssDNA gaps in neighbouring DNA molecules, leading to the synthesis of a few hundred base pairs templated on the ectopic donor strand. Multiple rounds of microhomology-mediated or HR-mediated template switching can give rise to complex breakpoints in cancer and in developmental disorders.

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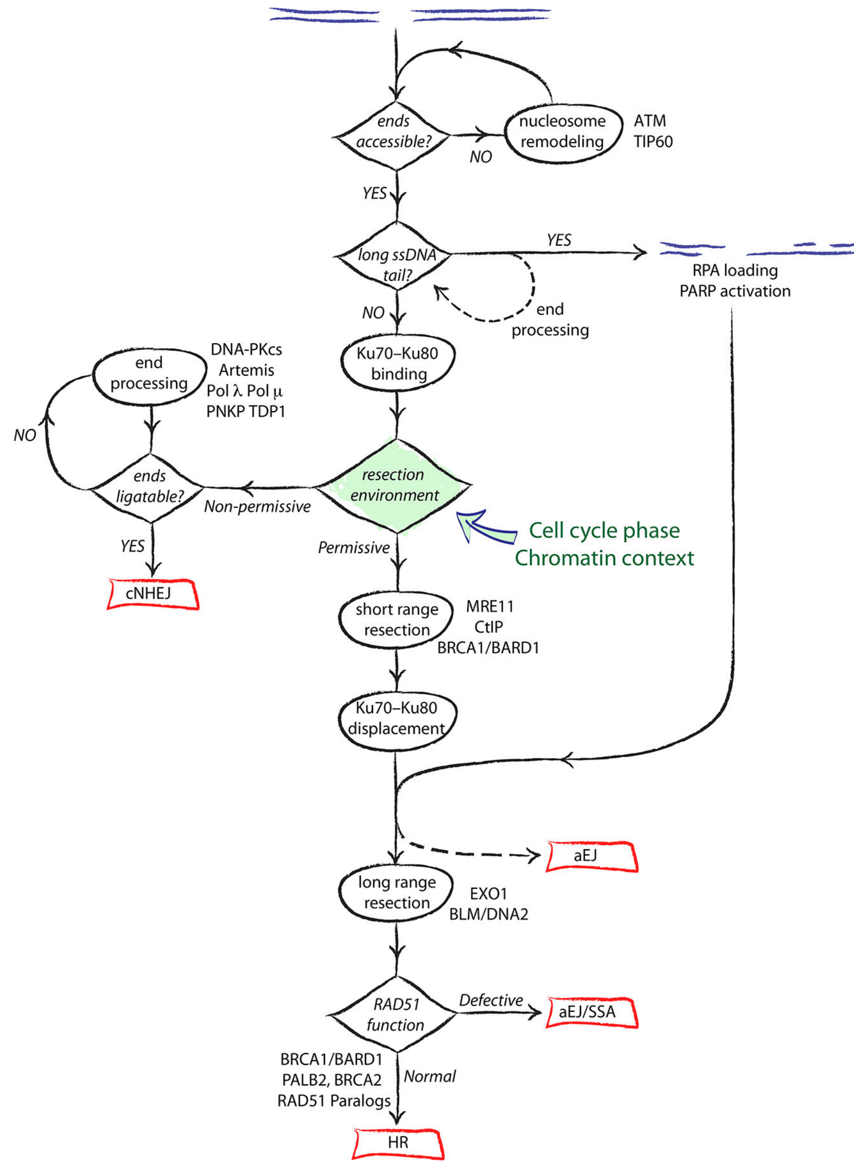

Figure 3. A decision tree of DNA double strand break repair.

DNA end resection has a crucial role in determining repair pathway choice. Cellular environments that disfavor resection enable Ku70–Ku80 retention at the DNA end, leading to classical non-homologous end joining (cNHEJ). A pro-resection environment favours Ku70–Ku80 displacement and the engagement of homologous recombination (HR). Error-prone pathways such as alternative end joining (aEJ) and single strand annealing (SSA) can act opportunistically on complex DNA ends or on recombination intermediates, hijacking the conservative HR process and leading to chromosome rearrangements.

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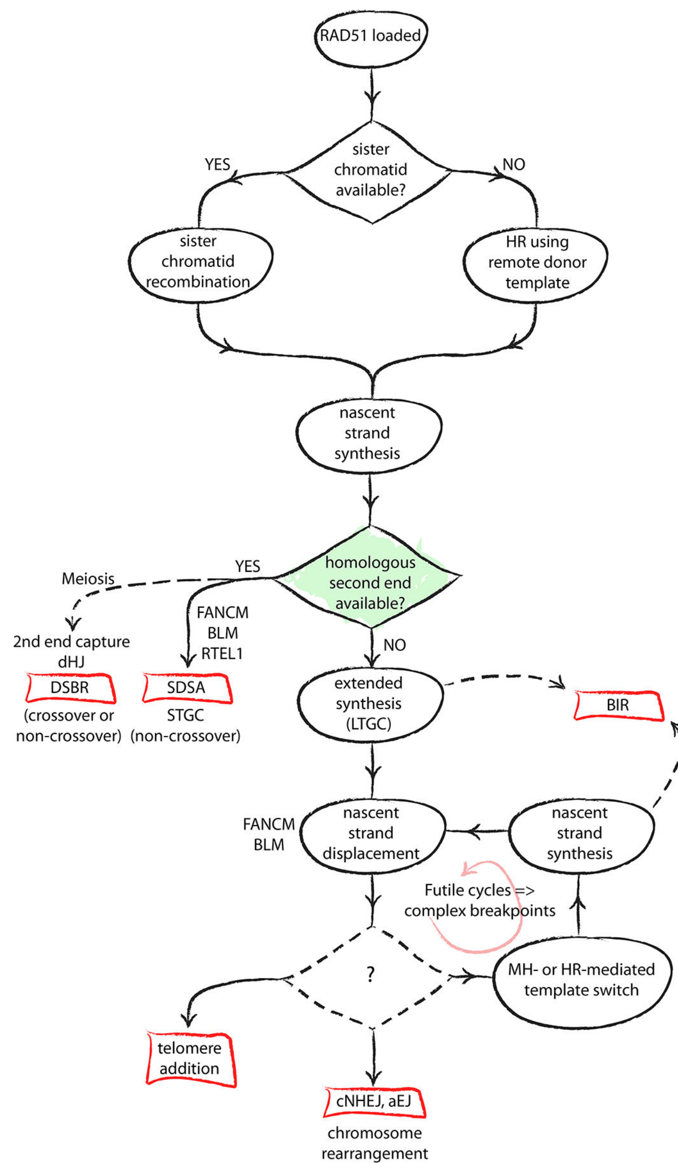

Figure 4. A decision tree of homologous recombination.

The schematic depicts the key role of the second end of the DNA double-stranded break (DSB) in determining the outcome of the homologous recombination (HR) process. Conservative outcomes are possible only if the second end is engaged for HR termination. The absence of a second end, or a failure to engage it in a timely fashion, leads to error-prone replicative HR outcomes — namely, long tract gene conversion (LTGC) and break-induced replication (BIR). Displacement of the nascent strand following LTGC places the solitary 3′ single-stranded DNA (ssDNA) end at risk of template switching and other spurious interactions, leading to complex breakpoints and chromosome rearrangements. The mechanisms that govern pathway selection for the displaced one-ended ssDNA end are unknown.

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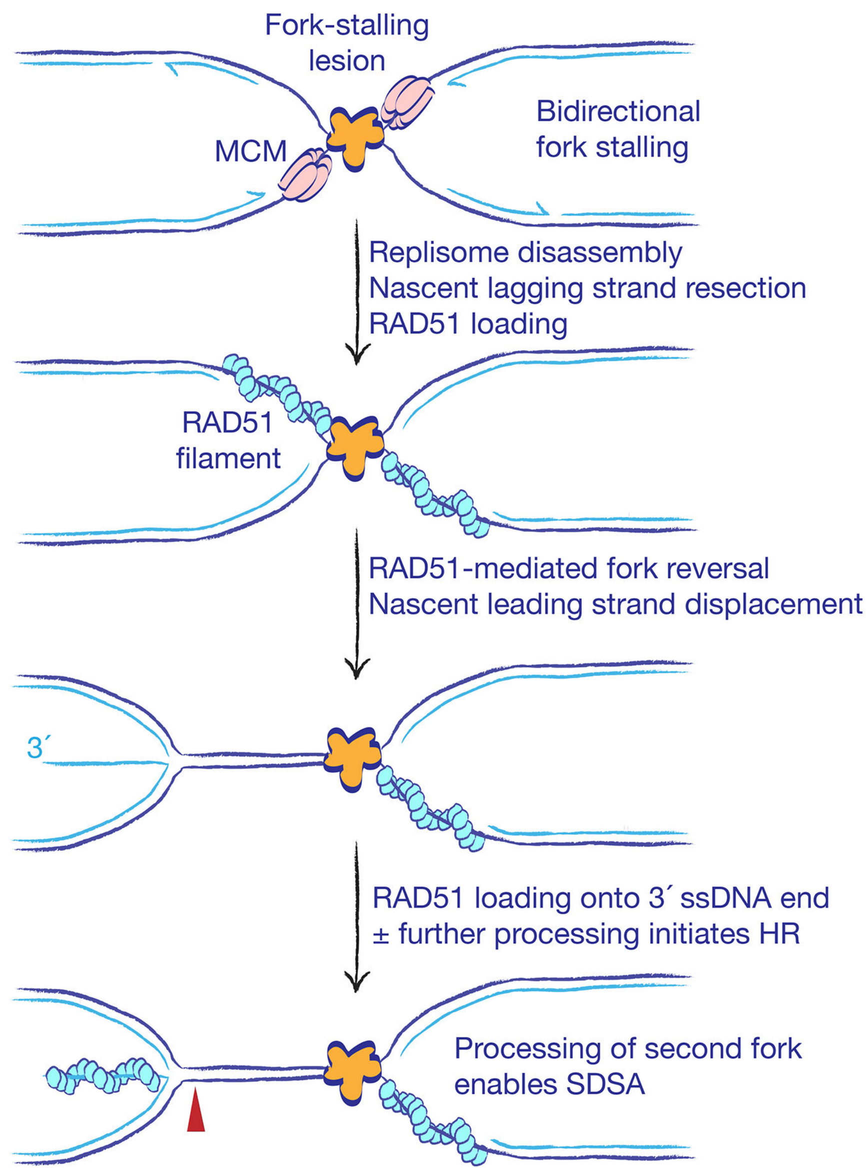

Figure 5. Rad51 is an 'early responder' at stalled forks.

The early steps of stalled fork processing for conservative homologous recombination (HR) entail bidirectional fork stalling, nascent lagging strand resection, replisome disassembly (also termed fork collapse) and asymmetric fork reversal. RAD51 acts early in stalled fork processing to facilitate fork reversal, which remodels lagging strand 'daughter strand gaps' into long 3´ single-stranded DNA (ssDNA) tails formed from the displaced leading daughter strand. The combination of ssDNA structural intermediates and avid BRCA-mediated RAD51-loading activity block Ku70–Ku80 access to DNA ends at stalled forks, making HR the default repair pathway in this context. Red arrowhead indicates possible site of nuclease-mediated cleavage that could liberate a RAD51-coated DNA end for HR. Processing of the opposing fork arrested at the site of stalling generates a second DNA end and enables conservative repair by SDSA.

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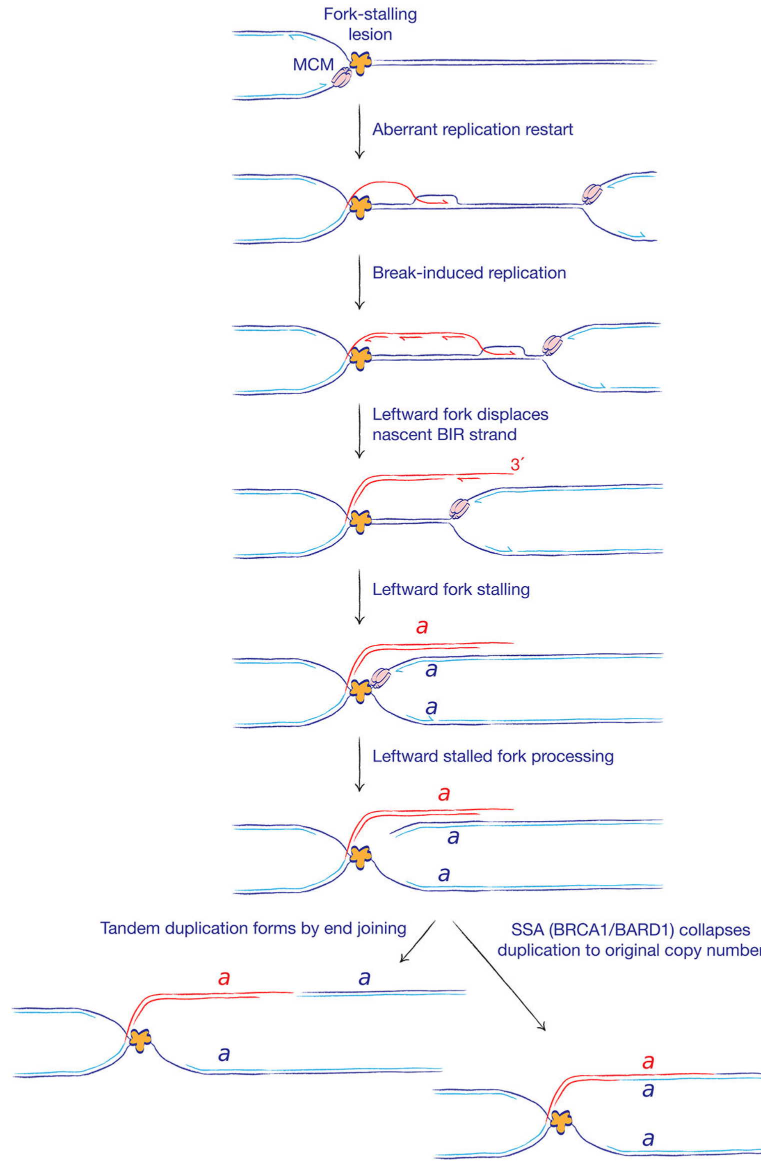

Figure 6. Single strand annealing may be a conservative repair pathway at stalled replication forks.

A solitary stalled fork may undergo aberrant fork restart, with the engagement of 'break-induced replication' (BIR)-type copying (red). Of note, BIR in this context might not entail a DNA break intermediate. Displacement of the BIR nascent strand by the converging opposing fork results in duplication of genomic segment *a*, bounded, at one end, by the site of fork stalling and, at the other end, by the site at which the BIR nascent strand was displaced. A non-homologous tandem duplication forms if these two DNA ends are repaired by end joining. By contrast, repair by single strand annealing (SSA; promoted by BRCA1–BARD1) would collapse the two copies of segment *a* back to a single copy, thereby suppressing tandem duplication formation and maintaining normal chromosome structure.