DNA DAMAGE

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 are crucial for maintaining genomic stability. 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. The two major mechanisms of DSB repair in mammalian cells are non-homologous end joining (NHEJ) and homologous recombination. In this Review, we consider DSB repair-pathway choice in somatic mammalian cells as a series of 'decision trees', and explore how defective pathway choice can lead to genomic instability. Stalled, collapsed or broken DNA replication forks present a distinctive challenge to the DSB repair system. Emerging evidence suggests that the 'rules' governing repair-pathway choice at stalled replication forks differ from those at replication-independent DSBs.

One-ended breaks
Solitary DNA ends that lack an immediate second DNA end for rejoining or annealing.

Stalled replication forks
Replication forks that have been arrested at DNA damage sites or because of other causes.

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Two-ended DNA double-strand breaks (DSBs) and one-ended breaks — whether the result of chromosome breakage, dysfunctional replication fork processing or telomere deprotection — pose an immediate threat to the stability of the genome by provoking chromosome rearrangements, thereby disrupting gene structure and function. Indeed, germline mutations in DSB repair genes cause genomic instability in numerous hereditary human diseases, especially those associated with cancer predisposition, developmental disorders and premature ageing[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 genomic damage. 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 suited to the cellular context, including cell-cycle phase and the local chromatin environment.

In this Review we discuss how DSB repair control mechanisms operate in somatic cells and how their dysfunction can promote genomic instability. We first describe the pathways that repair 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 repair pathway occurs, and outline a 'decision tree' for DSB repair-pathway choice. Lastly, we discuss the emerging understanding of the regulation of repair at stalled DNA replication forks. Recent work shows that regulation of repair at stalled replication forks differs substantially from that of a conventional DSB. We suggest that at least one DSB repair pathway that has traditionally been considered error-prone — single-strand annealing (SSA) — mediates error-free repair at stalled forks by suppressing tandem duplications at sites of aberrant replication fork restart.

Overview of DSB repair pathways

Two pathways dominate the repair of two-ended DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR)[2–8] (FIG. 1). In mammalian cells, 'classical' NHEJ (cNHEJ) — so called to distinguish it from alternative end joining (aEJ), which functions in the absence of cNHEJ proteins — is a rapid, high-capacity pathway 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, thereby potentially forming repair joints with up to 4 bp of 'microhomology'[7]. By contrast, HR 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 NHEJ

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]. Ku70–Ku80 nucleates the recruitment of other cNHEJ

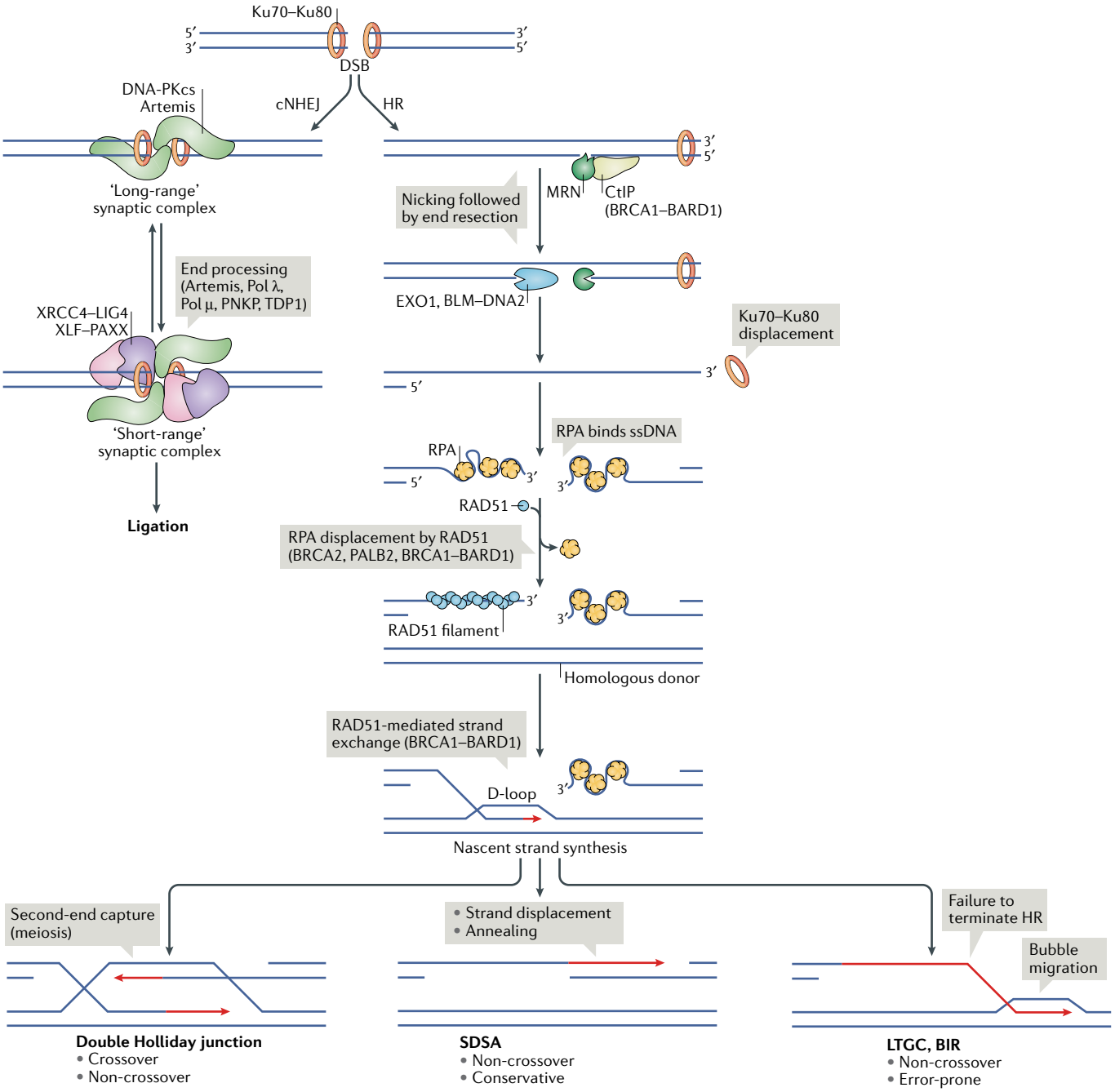

Fig. 1 | **The two major pathways of DNA double-strand break repair.** The binding of the Ku70–Ku80 heterodimer to DNA ends schedules repair of DNA double-strand breaks (DSBs) by classical non-homologous end joining (cNHEJ). cNHEJ entails formation of a 'long-range' synaptic complex, which can reversibly form a 'short-range' synaptic complex. DNA end ligation and processing by cNHEJ enzymes are restricted to the short-range complex. The default engagement of cNHEJ can be disrupted by DNA end resection, which facilitates repair by homologous recombination (HR). Resection is enabled by the endonuclease activity of the MRE11–RAD50–NBS1 (MRN) complex and the 5′–3′ strand resection activities of exonuclease 1 (EXO1) and the DNA2–Bloom syndrome protein (BLM) heterodimer, which together convert the blunt DSB end into a 3′ single-stranded DNA (ssDNA) tail. MRN 3′–5′ exonuclease activity displaces Ku70–Ku80 from the DNA end. The replication protein A (RPA) complex avidly binds to ssDNA and must be displaced by recombination 'mediators' to allow the formation of a RAD51 nucleoprotein filament. Breast cancer type 2 susceptibility protein (BRCA2) is the major recombination mediator in mammalian cells, likely acting in concert with partner and localizer of BRCA2 (PALB2) and the BRCA1–BRCA1-associated RING domain protein 1 (BARD1) heterodimer. Interactions between the two DNA ends at the recombination synapse and activities at the D-loop that is formed following synapsis influence which HR subpathway is engaged. The conservative, 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 intermediate can lead to crossing over. Failure to engage the second end of the break or failure to displace the nascent strand favours error-prone replicative HR responses of long-tract gene conversion (LTGC) and break-induced replication (BIR). Known roles of BRCA proteins in HR are indicated in parentheses; red arrows denote newly synthesized DNA strands. CtIP, CtBP-interacting protein; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; LIG4, DNA ligase IV; PAXX, paralogue of XRCC4 and XLF; PNKP, polynucleotide kinase-phosphatase; Pol, DNA polymerase; TDP1, tyrosyl-DNA phosphodiesterase 1; XLF, XRCC4-like factor.

Synapse
A DNA and protein complex in which two DNA molecules are brought into close proximity with the assistance of their associated proteins.

PARP inhibitors
Inhibitors of poly(ADP-ribose) polymerase (PARP) (especially PARP1) induce synthetic lethality in homologous recombination mutant cells through an unresolved mechanism that involves trapping of PARP1 on DNA.

factors, including 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] (FIG. 1). XRCC4 is essential for LIG4 stability and function, whereas 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 cNHEJ has revealed the existence of a two-stage mechanism of synapsis of the two ends of the DSB[17]. First, Ku70–Ku80 and DNA-PKcs establish a long-range synapse; second, the two DNA ends become closely aligned in a process requiring XLF, non-catalytic functions of XRCC4–LIG4 and the kinase activity of DNA-PKcs[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 ligation is completed. End processing by the nuclease Artemis, by the specialized DNA polymerases λ and μ and by other enzymes is restricted to the short-range synaptic complex, and ensures compatibility of the ligated ends[19]. A number of accessory factors, some of which likely remain unknown, support or otherwise regulate cNHEJ. These include the multifunctional MRE11–RAD50–NBS1 (MRN) (Mre11–Rad50–Xrs2 (MRX) in *Saccharomyces cerevisiae*) end recognition complex, which may assist in bridging the ends[20–22] and aprataxin 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]. 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, and has high affinity for DNA ends that are either blunt or possess short single-stranded DNA (ssDNA) overhangs. Long ssDNA tails have reduced affinity for Ku70–Ku80 and are channelled 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 before ligation suggests that steps in the pathway that follow Ku70–Ku80 binding may also be regulated. Indeed, DNA-PKcs phosphorylation by itself and by the multifunctional DNA damage response (DDR) kinase ATM is an important regulator of cNHEJ, affecting both Ku70–Ku80 binding and its disassembly following ligation[18,31,32].

Homologous recombination

The second major pathway of DSB repair, HR, is a multistep process that is intimately connected to human cancer risk, especially through the involvement of two major hereditary breast and ovarian cancer predisposition genes, *BRCA1* and *BRCA2*. Defects in HR confer exquisite sensitivity to inhibitors of the ssDNA-binding protein poly(ADP-ribose) polymerase (PARP) — a synthetic lethal interaction that has led to the use of PARP inhibitors as promising new cancer therapeutics in *BRCA*-linked cancer[33]. HR differs from cNHEJ in several key aspects. Unlike cNHEJ, which operates throughout the vertebrate cell cycle, HR is largely restricted to S phase and G2 phase of the cell cycle[34]. The major conservative, potentially error-free HR pathway in somatic cells carries out recombination between sister chromatids[34,35]. Sequence identity, spatial alignment and physical cohesion of the two sister chromatids are thought to favour recombination between sister chromatids over other potential recombination partners. HR entails the loading of the recombinase RAD51 (the eukaryotic homologue of bacterial RecA) onto ssDNA to form a nucleoprotein filament, either at DNA ends that have undergone DNA end resection to generate extended 3′ ssDNA tails (FIG. 1) or at post-replicative ssDNA gaps[2,36]. In eukaryotes, DNA end resection is initiated by the MRN complex, which also serves as a scaffold for activation of ATM[37–39]. The endonuclease activity of MRE11 nicks the strand that ends at a DSB with a free 5′ terminus, up to 300 nucleotides away from the break point, and the 3′–5′ exonuclease activity of MRE11 extends the nick towards the DNA end. Efficient initiation of such 'short-range' resection by the MRE11 endonuclease activity requires interaction with CtBP-interacting protein (CtIP; also known as RBBP8) and is stimulated by protein blocking the DNA end, such as Ku70–Ku80, replication protein A (RPA) or nucleosomes[40–47]. This initial processing step is thought to displace Ku70–Ku80 from the DNA ends and also provides an entry point for factors that carry out 'long-range' resection. This latter step is mediated by exonuclease 1, the endonuclease DNA2 and the Bloom syndrome helicase (BLM; Sgs1 in *S. cerevisiae*), which mediate the unwinding and nucleolytic digestion of the 5′ strand of the DNA end to form a long 3′ ssDNA tail[48–51] (FIG. 1). Other DNA end resection regulators, both positive and negative, have been described. For example, breast cancer type 1 susceptibility protein (BRCA1), in complex with BRCA1-associated RING domain protein 1 (BARD1), interacts with CtIP and MRN and is implicated in DNA end resection, as well as in later stages of HR (see below).

The emergent ssDNA is rapidly coated with the abundant RPA complex, which includes RPA1, RPA2 and RPA3. ssDNA bound by RPA cannot pair with other ssDNA. Thus, RPA opens secondary structures in ssDNA and limits spurious interactions with ssDNA intermediates of other nuclear processes. RPA also forms a barrier to the formation of a RAD51 nucleoprotein filament. RPA must therefore be displaced by recombination 'mediators' if HR is to proceed[36] (FIG. 1). In budding yeast, Rad52 is the key recombination mediator, whereas in vertebrates and in some fungal species, BRCA2 serves that function[52–54]. BRCA2, which is constitutively bound to the 26S proteasome complex subunit DSS1 (also known as SEM1), interacts with ssDNA, with RAD51 monomers and with BRCA1–BARD1 through partner and localizer of BRCA2 (PALB2)[5]. BRCA2 is thought to compete with RPA for ssDNA binding, thereby facilitating RPA displacement. The extent to which the recombination mediator function of BRCA2–DSS1 is modified by BRCA1–BARD1–PALB2 binding remains to be determined. The association of BRCA1 with proteins involved in both DNA end resection and RAD51 loading suggests that BRCA1 couples

χ sequences
Short sequences in bacterial genomes that serve as 'hotspots' for recombination; no equivalent has been identified in vertebrates.

Gene conversion
The transfer of genetic material from a donor sequence to a homologous acceptor during homologous recombination.

Translesion DNA polymerases
Specialized DNA polymerases that can traverse a damaged and unreadable DNA template.

Holliday junction
A four-way branched DNA structure that can mediate reciprocal exchanges between two homologous DNA molecules.

Non-crossover
A repair pathway that does not result in crossing over.

Crossing over
The exchange of genetic material between two homologous chromosomes.

these two HR steps, perhaps analogous to how DNA end resection is coupled to RecA filament formation in *Escherichia coli*[55]. In *E. coli*, direct interactions between RecA and RecB, which is a subunit of the DNA end resection complex RecBCD, ensure timely loading of RecA at recombination hotspot χ sequences[56].

The RAD51–ssDNA nucleoprotein filament is a dynamic structure that is subjected 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 helicase Srs2 (REFS[57–59]). Loss of Srs2 alone promotes unrestrained, 'toxic' recombination and genomic instability[60]. These relationships indicate that the stability of the RAD51 filament in normal physiological conditions is regulated to optimize the efficiency of HR and, at the same time, to restrict RAD51 function to appropriate DNA substrates. The RAD51–ssDNA nucleoprotein filament mediates the homology search that defines HR by invading duplex DNA molecules and facilitating base-pairing with complementary sequences. BRCA1–BARD1 facilitates RAD51-mediated homologous pairing, again indicating that BRCA1 promotes multiple HR steps[61]. RecA or RAD51 nucleoprotein filaments form synaptic complexes that contain a three-stranded DNA helix intermediate, which supports the formation of heteroduplex DNA composed of the invading strand and the complementary strand of the invaded molecule[62]. If sufficient base-paring takes place, 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[63]. The free 3′ end of the invading strand engages a DNA polymerase, which extends the invading (nascent) strand using the invaded donor DNA molecule as a template for gene conversion (FIG. 1). DNA polymerase δ (Pol δ) has a major role in nascent strand synthesis, but translesion DNA polymerases have been implicated in competing with Pol δ[64–66]. Gene conversion in yeasts and flies can entail multiple rounds of RAD51-mediated invasion, nascent strand extension, displacement and reinvasion[67,68]. The same process might also occur in vertebrates. Certain motor proteins, including Fanconi anaemia group M protein (FANCM; Mph1 in *S. cerevisiae*), BLM and regulator of telomere elongation helicase 1 (RTEL1) can disassemble D-loops, thereby potentially limiting the extent of gene conversion[46,69–72]. These functions are discussed in more detail later.

HR pathways. Several distinct pathways of HR are recognized, corresponding to distinct fates of the RAD51-mediated synapse (FIG. 1). The major pathway of somatic HR, 'synthesis-dependent strand annealing' (SDSA), invokes RAD51-mediated invasion by only one end of the two-ended DSB, while the second end is resected but remains passive[2]. The non-invading second end of the break facilitates HR termination by annealing with the displaced nascent strand. How this asymmetry between the two DNA ends is established is not well understood. *S. cerevisiae* mutants lacking MRX or Sae2 (the *S. cerevisiae* CtIP homologue) reveal asymmetric resection of ionizing radiation-induced DSBs, suggesting that MRX or MRN may control interactions between the invading and non-invading end during SDSA[73]. Because it does not involve formation of a Holliday junction, SDSA is a non-crossover pathway (FIG. 1). By contrast, the classical DSB repair HR pathway, which is prominent in meiotic recombination, entails the formation of a double Holliday junction (dHJ), with potential for crossing over during dHJ 'resolution'[2]. BLM, in complex with DNA topoisomerase 3α (TOP3α), RecQ-mediated genome instability protein 1 (RMI1) and RMI2, promotes an alternative non-crossover dHJ 'dissolution' mechanism[74]. Replicative responses following strand invasion — long-tract gene conversion (LTGC) and break-induced replication (BIR; FIG. 1) — are discussed later.

Single-strand annealing

SSA is a RAD51-independent DSB repair pathway that joins two homologous 3′ ssDNA ends (for example, at tandem repeats) through annealing, at the cost of deletion of the intervening sequence between the repeats[2] (FIG. 2a). SSA is therefore considered to be an obligatorily error-prone pathway. SSA requires extensive DNA end resection and RPA displacement to reveal complementary homologous sequences. In yeast, *RAD52* and its paralogue *RAD59* are required for SSA[4]. RAD52 also promotes SSA in mammalian cells, but there appear to be additional redundant annealing factors in mammals[75–77].

Alternative end joining

Another rejoining mechanism that operates on 3′ ssDNA ends is aEJ (FIG. 2b), which is defined as NHEJ without the use of cNHEJ factors[78]. Reliance on microhomology at the breakpoint is a prominent feature of aEJ, and the term microhomology-mediated end joining is sometimes used synonymously with aEJ; however, this use of the term microhomology-mediated end joining can be confusing because cNHEJ is also associated with a limited use of microhomology. We will therefore use the term microhomology-mediated end joining as a descriptive term to note the presence of microhomology at a breakpoint, whether repair is mediated by cNHEJ or by aEJ. In metazoans, Pol θ, which is encoded by the *POLQ* gene, has been implicated in aEJ[79,80]. A Pol θ-associated helicase function can displace RPA from ssDNA, thereby revealing internal microhomologies on the ssDNA ends, while its polymerase function can stabilize the joint between the two DNA ends[81,82]. Additional DNA polymerases may be required to complete fill-in synthesis during aEJ[64]. *Polq*-null mice exhibit spontaneous genomic instability, thereby implicating Pol θ (and possibly aEJ) in genome maintenance[83]. These functions are probably performed at sites of stalled replication; in *Caenorhabditis elegans*, Pol θ suppresses large chromosomal deletions at sites of fork stalling, at the expense of allowing small deletions to form[84,85]. Combined deletion of *Ku70* (also known as *Xrcc6*) and *Polq* in primary mouse cells induces a severe growth defect, and in HR-defective cells, *POLQ* expression is elevated[86–88]. 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[88].

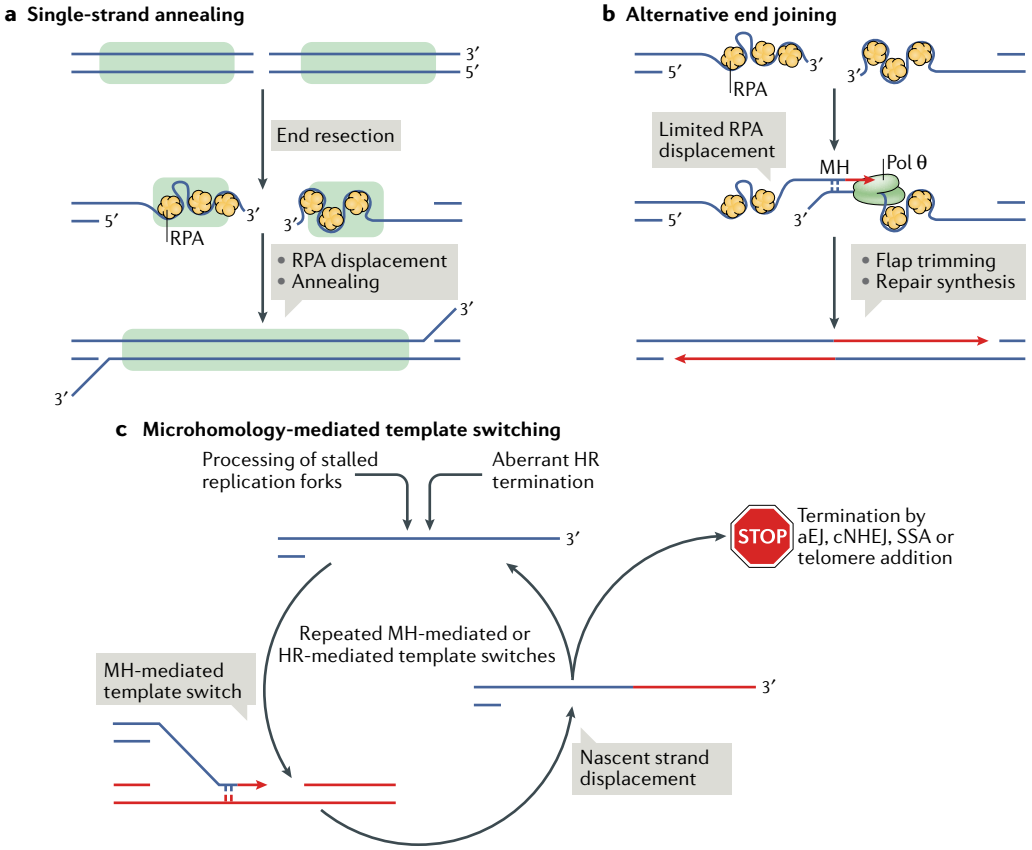

Fig. 2 | **Alternative DNA double-strand break repair pathways. a** | Single-strand annealing (SSA) converts sequence repeats (green boxes) into a single copy of the repeats by annealing complementary single-stranded DNA (ssDNA) ends from different repeats. Replication protein A (RPA) must be displaced to expose complementary ssDNA for annealing. **b** | Alternative end joining (aEJ) rejoins DNA ends without use of classical non-homologous end joining (cNHEJ) proteins. aEJ involves limited displacement of RPA, which reveals microhomology (MH) between strands and facilitates repair. In mammalian cells, DNA polymerase θ (Pol θ)-associated helicase activity can displace RPA from ssDNA, while its polymerase activity can stabilize the joint between the two DNA ends. **c** | Microhomology-mediated template switching can happen 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 regions of neighbouring DNA molecules, leading to the synthesis of up to a few hundred base pairs, templated by the ectopic donor strand. Multiple rounds of microhomology-mediated template switching or homologous recombination (HR)-mediated template switching can give rise to complex breakpoints in cancer and in developmental disorders. Termination of these futile cycles by aEJ, cNHEJ or SSA requires a second DNA end. Alternatively, de novo telomere addition could occur in the absence of a second end. Red arrows denote newly synthesized DNA strands.

Not all aEJ in mammalian cells is mediated by Pol θ. Class switch recombination (CSR) is an end joining process that is involved in the rearrangement of immunoglobulin heavy chain loci in cytokine-stimulated B cells, in which cNHEJ and aEJ pathways both repair DSBs induced at CSR regions[78,89]. *Polq*$^{-/-}$ mouse B cells have normal CSR frequencies and normal spectra of microhomology use at CSR breakpoints, indicating that Pol θ is not required for aEJ during CSR[90,91]. However, Pol θ is required for the formation of CSR junctions that contain nucleotide insertions[90]. A recent study reported that *Rad52*$^{-/-}$ mouse B cells have increased CSR frequencies in comparison with wild-type cells, but fail to form CSR products with breakpoints with microhomology of more than 4 bp (REF.[91]). Since cNHEJ is not associated with microhomology of more than 4 bp, this finding raises the interesting possibility that RAD52 contributes to mammalian aEJ during CSR and may compete with cNHEJ in CSR. RAD52 has not been implicated in microhomology-mediated end joining in other settings[75] and it remains to be determined what specific features of CSR might enable RAD52 to contribute to aEJ.

Repair responses to one-ended DSBs

A distinctive challenge for the DSB repair system arises at sites of broken replication forks or collapsed replication forks, since one-ended breaks or solitary DNA ends can arise in these contexts[92–95]. In this case, there is no immediate partner for end joining, and the absence of a second DNA end does not allow the possibility of engaging error-free SDSA.

Break-induced replication

In *S. cerevisiae*, BIR is an outcome of one-ended *RAD51*-dependent strand invasion (FIG. 1), in which the nascent strand is extended to the end of the chromosome. BIR can copy more than 100 kb from the donor chromosome, unless a disruptive event such as a collision with

Broken replication forks
Stalled replication forks that have lost their branched DNA structure due to interruption of both DNA strands of at least one sister chromatid.

Collapsed replication forks
Stalled replication forks that have lost the capacity to perform DNA synthesis due to disassembly of the replisome.

Replication restart
Resumption of DNA synthesis at a stalled fork; may be mediated by conventional semiconservative DNA synthesis or by error-prone mechanisms.

Migrating bubble
DNA synthesis mechanism of long-tract gene conversion and break-induced replication.

Daughter strand gaps
(DSGs). Post-replicative DNA single-strand gaps caused by interruption of the synthesis of the nascent daughter strand.

a replication fork prematurely terminates the process[94,96]. BIR that engages a heterologous chromosome donor results in a non-reciprocal translocation[97,98]. In *Schizosaccharomyces pombe*, a form of BIR mediated by Rad22 (orthologue of Rad52) can drive aberrant replication restart of the stalled fork[96]. BIR entails conservative DNA synthesis through a migrating bubble mechanism (FIG. 1), which generates extensive ssDNA tracts that are vulnerable to mutation and rearrangement[60,99–102]. As a result, BIR is highly mutagenic. BIR in *S. cerevisiae* requires the gene *PIF1*, which encodes the helicase Pif1, and *POL32*, which encodes a non-essential subunit of Pol δ; neither of these genes is required for conventional, short-tract SDSA[99,103,104]. However, LTGCs of only a few kilobases— which do not fit the classical definition of BIR in *S. cerevisiae* — also require *POL32* (REF.[105]). This suggests that there is significant mechanistic overlap between LTGC and BIR. Notably, BIR in *S. cerevisiae* can be preceded by repeated rounds of LTGC and homologous template switching during the first ~10 kb of gene conversion[68]. This finding suggests that the bubble migration mechanisms underlying LTGC and BIR differ in their processivity. In *S. cerevisiae*, activities that mediate D-loop disassembly or nascent strand displacement channel HR towards SDSA, whereas factors that stabilize the D-loop favour LTGC or BIR and crossover outcomes[106]. A recent study that directly quantified D-loop formation in response to a site-specific DSB identified two parallel pathways of D-loop disruption, mediated by Mph1 and the Sgs1 (the BLM orthologue)–Top3–Rmi1 complex or, in parallel, by the helicase Srs2 (REF.[107]). Furthermore, *SGS1* and *MPH1* impose a delay in the onset of BIR[105,108,109]. These findings suggest that the fate of the D-loop is intimately related to the balance between conservative (SDSA) and error-prone (LTGC, BIR) outcomes of HR.

In mammals, FANCM (mammalian homologue of Mph1), the BLM–TOP3α–RMI1–RMI2 complex and several candidate mammalian homologues of Srs2 can disrupt D-loops in vitro[106]. Furthermore, both BLM and FANCM suppress LTGC during HR in mammalian cells[95]. However, the longest DSB-induced RAD51-mediated gene conversions reported to date are LTGC products of less than 10 kb, which are substantially shorter than the more than 100 kb BIR tracts observed in yeast. Cells lacking BRCA1, CtIP, BRCA2 or paralogues of RAD51 reveal a bias in favour of LTGC[110–113]. 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 bubble migration copying mechanisms[114]. 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)[115,116]. MiDAS is mediated by RAD52, DNA Pol δ subunit 3 (the mammalian homologue of *S. cerevisiae* Pol32) and the structure-specific nuclease MUS81–EME1, thereby possibly implicating the processing of stalled replication forks in the initiation of MiDAS. Second, RAD51-independent and Pol δ-mediated BIR tracts of up to ~70 kb are provoked by DSBs at telomeres that are maintained by the recombination-mediated alternative lengthening of telomeres pathway[117]. Third, LTGC triggered at stalled replication forks is RAD51 independent[113].

Microhomology-mediated template switching

A distinct replicative response associated with a solitary 3′ ssDNA end is microhomology-mediated template switching (FIG. 2c). This process entails microhomology-mediated synapsis of a free 3′ ssDNA tail with ssDNA donor sequences (possibly daughter strand gaps (DSGs) in post-replicative chromatin), which is followed by limited DNA synthesis of up to a few hundred base pairs and is completed by nascent-strand displacement. In *S. cerevisiae*, translesion DNA polymerases are implicated in the synthesis step[102]. 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, which is similar in structure to the initiating 3′ ssDNA tail. Thus, microhomology-mediated template switching 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 breakpoints of chromosome rearrangement in cancer and other diseases, in which multiple short (a few hundred base pair) sequences derived from different loci are present within the breakpoint[118–120]. Such complex breakpoints may be products of futile cycles of repeated microhomology-mediated template switching between different donor loci. Incorporation of ectopic DNA fragments by end joining can also contribute to complex breakpoints[121]. Work in *E. coli* and *S. cerevisiae* has associated microhomology-mediated template switching with replication fork stalling ('fork stalling and template switching') and BIR ('microhomology-mediated BIR')[122–125]. Multiple RAD51-mediated strand invasions can also generate complex breakpoints in yeast[126]. Experimental models in mammalian cells of microhomology-mediated template switching associate it primarily with end joining[95,119,127,128], although the presence of hypermutation in some structural variations in human disease may suggest an underlying BIR mechanism[129]. Perhaps the species differences noted here reflect the greater importance in mammalian cells than in yeast of end joining pathways in genome maintenance. In summary, the phenomenon of microhomology-mediated template switching suggests that solitary 3′ ssDNA tails are highly reactive DNA lesions that can interact with remote chromosomal loci in uncontrolled and dangerous ways.

A decision tree for DSB repair

Given the array of available DSB repair pathways, how is the pathway most appropriate for the repair of any DSB selected? In principle, all DSB repair pathways might compete for access to all free DNA ends. However, the two major conservative DSB repair pathways, cNHEJ and HR, are dominant in the repair of a conventional two-ended DSB. By contrast, error-prone pathways such as SSA, aEJ, microhomology-mediated template switching and BIR may function more opportunistically, by

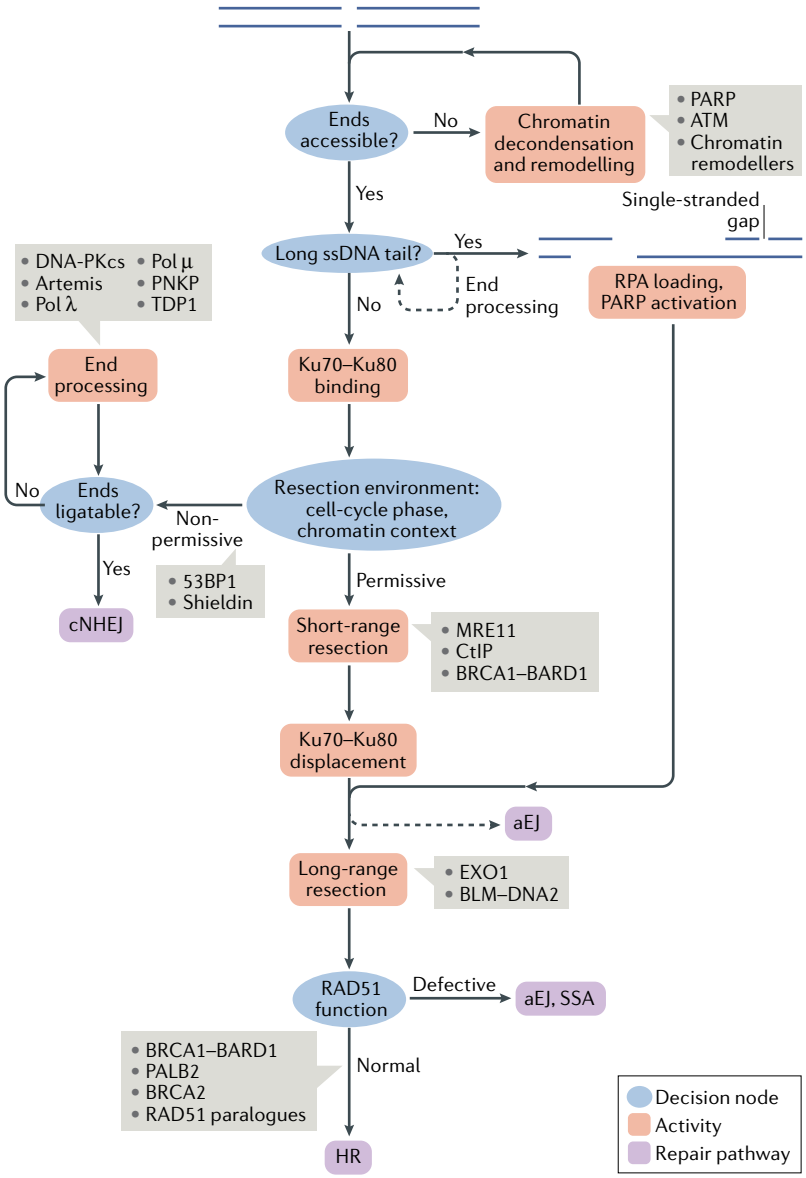

Fig. 3 | **A decision tree for DNA double-strand break repair.** Poly(ADP-ribose) polymerase (PARP) activation and ataxia telangiectasia mutated protein (ATM) signalling are part of an early response to the double-strand break (DSB) that may facilitate chromatin decondensation, chromatin remodelling and DNA end recognition. Initial DSB repair-pathway choice depends on the structure of the DNA ends. Blunt or minimally recessed DNA ends bind the classical non-homologous end joining (cNHEJ) factor Ku70–Ku80. Single-stranded DNA (ssDNA) or gapped DNA ends directly activate PARP and may evade end recognition by Ku70–Ku80 unless additional end processing occurs (dashed arrow). DNA end resection has a crucial role in determining the choice between cNHEJ and homologous recombination (HR). Cellular environments that disfavour end resection make possible Ku70–Ku80 retention at the DNA end, leading to cNHEJ. Additional end processing within the cNHEJ synapse facilitates ligation. Cellular environments that favour DNA end resection support the displacement of Ku70–Ku80 and the engagement of long-range resection required for HR. Error-prone pathways such as alternative end joining (aEJ; dashed arrow) and single-strand annealing (SSA) can function opportunistically on ssDNA ends or on recombination intermediates by hijacking the conservative HR process, leading to chromosome rearrangements. BARD1, BRCA1-associated RING domain protein 1; BLM, Bloom syndrome protein; 53BP1, p53-binding protein 1; BRCA1, breast cancer type 1 susceptibility protein; BRCA2, breast cancer type 2 susceptibility protein; CtIP, CtBP-interacting protein; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; EXO1, exonuclease 1; PALB2, partner and localizer of BRCA2; PNKP, polynucleotide kinase-phosphatase; Pol, DNA polymerase; TDP1, tyrosyl-DNA phosphodiesterase 1.

scavenging for the products of aborted or incomplete cNHEJ or HR, or at problematic lesions such as one-ended breaks. Thus, the DSB repair system could be depicted as a decision tree (FIGS 3,4), the branch-points ('nodes') of which represent points of commitment to cNHEJ or HR, points where physiological subpathways are selected (for example, during late stages of HR; FIG. 4) or points at which repair intermediates are vulnerable to hijacking 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 such as cancer perturb this regulatory balance by disrupting DSB-repair regulatory genes, by overwhelming the cell with DNA-damage levels that exceed its physiological repair capacity or by making possible the formation of complex DNA lesions, for which there are no good repair outcomes. For example, catastrophic chromosome rearrangements such as chromothripsis and chromoplexy reflect end-joining activity in conditions of an overwhelming DSB burden[119,120].

DNA end structure and pathway choice

An important determinant of DSB repair-pathway choice is the initiating DNA lesion itself: 'The wand chooses the wizard'[130]. As discussed earlier, attempts to repair a one-ended break are necessarily error-prone. At two-ended breaks, whether the DNA ends contain single-stranded tails can affect repair-pathway choice, since Ku70–Ku80 binds weakly to long ssDNA tails[29] (FIG. 3). Similarly, a DNA end that is chemically blocked or that forms within compact chromatin may require processing or extensive chromatin remodelling for DSB repair. Complex patterns of single-stranded gaps close to the free DNA end might also affect DSB repair because of intense activation of PARP (predominantly PARP1) 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, thereby increasing the likelihood of rearrangement between the spatially proximate but genomically remote loci[131,132].

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 rapidly repaired with a half-life of around a few minutes[133]. This rapid phase of repair requires cNHEJ genes, while HR contributes to slower phases of repair[17,134,135]. A slower phase of Artemis-dependent cNHEJ has also been described, likely reflecting the involvement of DNA end processing before ligation[136]. 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[137,138]. Imaging of RAD51-mediated synapsis during mammalian interchromosomal HR suggests similar kinetics[139]. Although HR between sister chromatids might occur more rapidly than this, the kinetics of even the most efficient forms of HR

Chromothripsis
Localized chromosome shattering and repair that occurs as a one-off catastrophe, generating an alternating copy number profile at the affected locus.

Chromoplexy
A closed chain of linked translocations, with little or no copy number alteration, observed in some cancers.

are likely slow in comparison with the kinetics of 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[140]. Consistent with this idea, mammalian cNHEJ competes with HR for repair of a site-specific chromosomal DSB[141]. Similarly, at an 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[142].

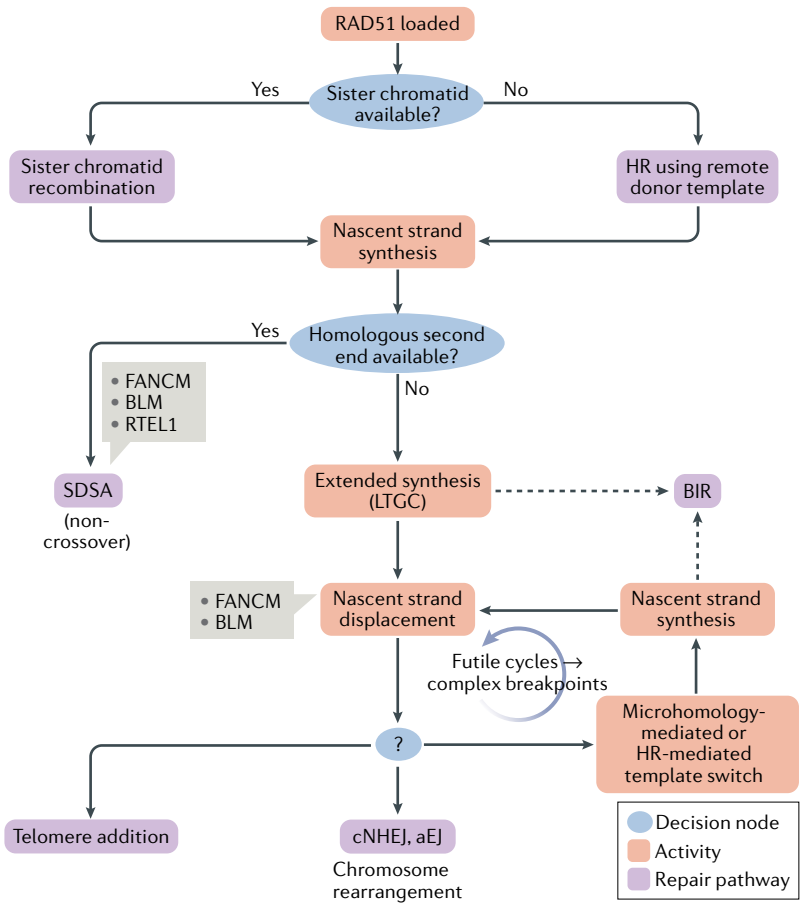

Fig. 4 | **A decision tree for homologous recombination in somatic cells.** Following formation of the RAD51 nucleoprotein filament, the availability of a sister chromatid favours conservative repair by sister chromatid recombination. In the absence of a sister chromatid, RAD51-mediated strand invasion of an ectopic homologous donor may occur by default. Once nascent strand synthesis has been initiated, the presence or absence of a second end of the DNA double-stranded break determines the outcome of the homologous recombination (HR) process. The conservative HR outcome of synthesis-dependent strand annealing (SDSA) is possible only if the second end is engaged for terminating HR. 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). The transition between LTGC and BIR (dashed arrows) is not well understood. Displacement of the nascent strand following LTGC places the solitary single-stranded DNA 3′ end at risk of spurious interactions, including the formation of chromosome rearrangements through classical non-homologous end joining (cNHEJ) or alternative end joining (aEJ), ectopic telomere addition or futile cycles of repeated template switching, which can lead to the formation of complex breakpoints. The mechanisms that govern pathway choice for the displaced one-ended single-stranded DNA end are unknown. BLM, Bloom syndrome protein; FANCM, Fanconi anaemia group M protein; RTEL1, regulator of telomere elongation helicase 1.

DNA end resection and pathway choice

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[143]. A major, evolutionarily conserved mechanism for displacement of Ku70-Ku80 is the process of DNA end resection itself (FIG. 1). Indeed, the engagement of DNA end resection is one of the most important determinants of DSB repair-pathway choice and a key commitment step in HR. Its regulation through the cell cycle in part explains how HR is restricted to S phase and G2 phase of the cell cycle. 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[144–149]. Phosphorylation of human CtIP at Thr847 or *S. cerevisiae* Sae2 at Ser267 is essential for efficient activation of the nuclease MRE11 (REFS[147,150]). Thus, CtIP both senses the cell-cycle phase (as a CDK phosphorylation target) and transduces this information to initiate DNA end resection. Another important regulatory role of CtIP in vertebrates is its binding to BRCA1 — an interaction that is regulated by phosphorylation of CtIP Ser327 (REF.[151]). BRCA1, such as MRN and CtIP, is required for both HR-mediated and SSA-mediated repair of a site-specific DSB, suggesting a role for BRCA1 in regulating DNA end resection[152]. CtIP functions, at least in part, independently of BRCA1, and studies of the effect of BRCA1 loss on bulk DNA end resection have yielded variable results[110,153–155].

Deletion of the DDR gene *53BP1* (also known as *TP53BP1*) suppresses the severe genomic instability of *BRCA1* mutants and the sensitivity of *BRCA1*-mutant cells to PARP inhibitors[156]. This suppression is especially prominent in *BRCA1* hypomorphs in which BRCA1 retains its ability to bind to PALB2–BRCA2–RAD51 and hence, presumably, RAD51-loading functions[157–159]. How does loss of 53BP1 lead to this striking phenotypic reversal? In the repair of a conventional DSB, 53BP1 both suppresses DNA end resection and promotes cNHEJ, although it is not a 'core' cNHEJ factor[156,160,161] (FIG. 3). 53BP1 effectors include RIF1, PTIP, REV7 (also called MAD2L2 — a subunit of the translesion DNA polymerase Pol ζ) and DYNLL1 (REFS[162–170]). Recent studies identified FAM35A (also called SHLD2) and other components of a 'shieldin' complex as key mediators of 53BP1 repair functions[171–175]. Like 53BP1, the shieldin complex suppresses DNA end resection and supports cNHEJ. SHLD2 contains ssDNA-binding oligonucleotide/oligosaccharide-binding fold domains, which are required for its repair functions. A recent study suggested that shieldin may promote fill-in synthesis of ssDNA, thereby converting ssDNA tails into blunt ends[174]. The insight that shieldin antagonizes BRCA1 through interactions with ssDNA broadens the potential function of the 53BP1–shieldin complex and raises some tantalizing questions. What are the

Box 1 | **The chromatin response in double-strand break repair**

DNA double-strand breaks (DSBs) provoke extensive changes in chromatin, which have an important role in DSB repair[253,254]. The DNA damage response kinases ATM (activated by MRE11–RAD50–NBS1 (MRN) at the break[38]), ATR (activated by single-stranded DNA and replication protein A sensors[255–259]) and DNA-dependent protein kinase catalytic subunit (activated by Ku70–Ku80 (REF.[18])) phosphorylate Ser139 of the histone variant H2AX (encoded by the gene *H2AFX*), thereby forming γ-H2AX chromatin domains[260]. In vertebrates, γ-H2AX recruits the adaptor MDC1 to form a specialized chromatin structure that can extend hundreds of kilobases away from the DSB[261]. γ-H2AX–MDC1-bound chromatin is multifunctional: it supports class switch recombination in activated B cells (which is an end-joining process), homologous recombination between sister chromatids and ATM signal amplification through an MDC1–MRN interaction, and also suppresses spurious end resection during V(D)J recombination in lymphocytes[260,262–265].

The MDC1-binding E3 ubiquitin ligase RNF8 catalyses Lys63 (K63)-linked polyubiquitylation of histone H2A, thereby recruiting BRCA1-containing complexes and a second E3 ubiquitin ligase, RNF168 (REFS[266,267]). A complex of BRCA1 and the K63-linked polyubiquitin binding protein RAP80 includes deubiquitylating enzymes, which further modulate the ubiquitylation of chromatin near the DSB. The RAP80 complex has a role in antagonizing DNA end resection[268–271]. In parallel, p53-binding protein 1 (53BP1) is recruited to chromatin by binding histone H4 monomethylated at Lys20 (H4K20me1) or H4K20me2 and histone H2A monoubiquitylated at Lys15 (a target of RNF168)[272]. 53BP1 regulates classical non-homologous end joining in vertebrate cell, in a manner independent of *H2AFX*[160,273]. 53BP1 also antagonizes the DNA end resection functions of BRCA1/BARD1. Thus, distinct histone post-translational modifications mediate distinct DSB repair functions, supporting the existence of a 'histone code' of DSB repair[160]. Numerous other histone post-translational modifications occur in the vicinity of DSBs, many of which have as yet undefined roles in repair[194,254,274–276].

The functional competition between BRCA1 and 53BP1 can also be visualized in the context of γ-H2AX chromatin domains[260,277]. The balance between BRCA1 and 53BP1 on chromatin is affected by TIP60-mediated acetylation of histone residues close to H4K20, which can disrupt 53BP1 binding to H4K20me1 or H4K20me2 (REFS[278,279]). Furthermore, BRCA1–BARD1 can ubiquitylate histone H2A at Lys27, thereby recruiting the chromatin remodeller SMARCAD1 and facilitating 53BP1 repositioning away from the DSB[280]. The activity of 53BP1 is also directly inhibited by TIRR, which blocks the H4K20me-binding domain of 53BP1 (REF.[281]).

key ssDNA structures over which BRCA1 and 53BP1 compete? Does the 53BP1–shieldin complex have ssDNA-related functions in addition to the functions of suppression of resection and promotion of cNHEJ? Which function of the 53BP1–shieldin complex explains its role in conferring PARP inhibitor sensitivity to *BRCA1* mutants?

In addition to CDK-mediated phosphorylation of HR targets, several other mechanisms communicate cell-cycle status to the DSB repair machinery. The expression of HR genes is upregulated as cells transition from G1 phase into S phase. In mammalian cells in G1 phase, DNA end resection is suppressed by DNA helicase B, which is inactivated as cells enter S phase[176]. The assembly of the BRCA1–PALB2–BRCA2–RAD51 recombinase complex is suppressed in G1 phase by proteasome-mediated degradation of PALB2, following its ubiquitylation by the E3 ubiquitin ligase cullin-3–RBX1 and the adaptor protein KEAP1 (REF.[177]). A study of post-replicative histone modification provided an intriguing example of how the features of chromatin in S phase can favour HR. The heterodimer TONSL–MMS22L supports RAD51 loading and activity at stalled replication forks[178–181]. The ankyrin repeat domain of TONSL can bind to unmethylated histone H4 (H4) K20, which is an unmodified-histone state that is restricted to histones newly incorporated into chromatin[182]. Thus, immature post-replicative chromatin provides a docking site for TONSL–MMS22L and eventually for RAD51, and the scarcity of monomethylated H4K20- and dimethylated H4K20-containing chromatin might also deny 53BP1 access to chromatin to exert its anti-BRCA1 activity (BOX 1).

Entry into mitosis involves chromatin condensation and presents a unique challenge to DSB repair. Between late G2 phase and mid prophase, the cell commits to mitosis even in the presence of DNA damage[183]. This transition is accompanied by an attenuation of the DDR. Although MRN is recruited to breaks in mitotic cells and ATM is activated, the chromatin-based response (BOX 1) is restricted to histone H2AX phosphorylation and MDC1 recruitment, without activation of RNF8 and RNF168 or accumulation of BRCA1 or 53BP1 on chromatin[184]. This attenuation of the DDR is mediated by inhibitory phosphorylation of 53BP1 and RNF8 by mitotic kinases[185,186]. Indeed, unregulated reactivation of 53BP1 in mitosis provokes chromosome rearrangement and telomere fusions, which reflect inappropriate activation of cNHEJ.

The chromatin context of pathway choice

The chromatin context in which a DSB arises may broadly influence repair-pathway choice (BOX 1). Measuring the time course of γ-H2AX foci resolution (a surrogate for DSB repair) suggested that DSBs in heterochromatin are repaired by an HR mechanism that requires ATM[187]. Chromatin-immunoprecipitation (ChIP) analysis of proteins at site-specific DSBs induced by the rare-cutting restriction endonuclease AsiSI revealed that the cNHEJ factor XRCC4 consistently accumulates in close proximity to each AsiSI-induced DSB, whereas accumulation of the HR factor RAD51 is more widely distributed around the break site and is highly variable between different AsiSI-induced DSBs[188]. Of note, AsiSI target sites with high levels of RAD51 were enriched in transcribed genes that were marked by trimethylated histone H3 K36 (H3K36me3). The H3K36me3-binding factor LEDGF was implicated in RAD51 accumulation at these sites, which is consistent with previous work that linked LEDGF to CtIP function[189]. ChIP analysis in undamaged cells revealed preferential accumulation of BRCA1 and PALB2 at transcribed genes[190]. Collisions between replication and transcription and the genome destabilizing properties of their accompanying RNA–DNA hybrids might concentrate these repair factors at transcribed genes[191–193]. Alternatively, specific interactions with transcription complexes or epigenetic modifications might be involved in this enrichment[194].

In yeast, DSBs are mobilized to the nuclear periphery as part of a nucleus-wide choreography of repair[195]. Mammalian DSBs do not appear to undergo mobilization to the nuclear periphery[196], yet γ-H2AX foci were found to coalesce following DSB induction, suggesting that DSBs might cluster during repair[197]. Similarly, 53BP1 promotes mobility of deprotected telomeres (exposed chromosome ends), thereby facilitating long-range telomere rejoining[198,199]. Furthermore, endonuclease-induced DSBs coalesce in cells in G1 phase at transcribed genes, pending repair by HR at later stages of

Prophase
The first stage of mitosis, during which chromosomes begin to condense.

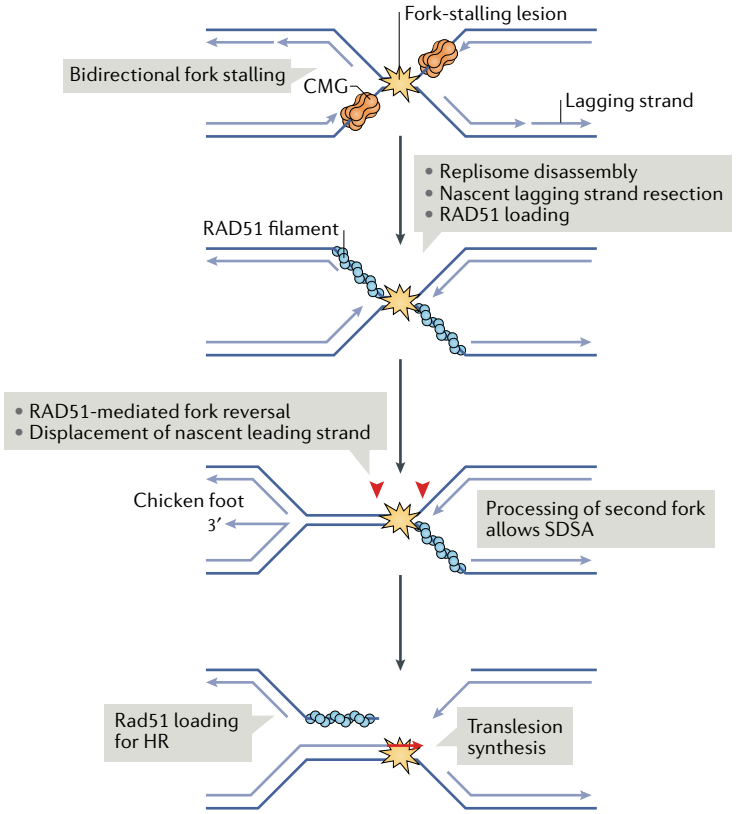

Fig. 5 | **RAD51 is an early responder at stalled replication forks.** The early steps of processing stalled replication forks for conservative homologous recombination (HR) entail bidirectional fork stalling, resection of the nascent lagging strands, replisome disassembly (also termed fork collapse) and reversal of one of the two collapsed replication forks. RAD51 functions early in the processing of stalled forks, before the formation of a double-strand break, to facilitate fork reversal, which remodels lagging strand 'daughter strand gaps' into a 'chicken foot' structure containing a long 3′ single-stranded DNA tail formed from the displaced leading daughter strand. The combination of structured single-stranded DNA, an avid pro-resection environment and efficient BRCA-mediated RAD51-loading may make DNA ends at stalled forks poor substrates for recognition by Ku70–Ku80, thereby channelling repair to HR. Endonucleolytic attack of each of the two opposing stalled forks liberates a two-ended double-strand break and allows conservative HR by synthesis-dependent strand annealing (SDSA; not shown). Translesion synthesis (red arrow) may bypass the residual fork-stalling lesion on the unbroken sister chromatid. The red arrowheads indicate possible sites of endonuclease-mediated cleavage of the stalled forks; other possible nuclease target sites are not shown. A second RAD51 loading step primes the released DNA ends for HR. CMG, CDC45–MCM2–7–GINS replicative helicase.

Hemicatenanes
Topological entanglements of two double-stranded DNA molecules, in which one strand of each duplex passes between the two strands of the other duplex.

Fork reversal
A stalled and collapsed replication fork in which reannealing of the parental strands has moved the branch-point of the fork backwards, extruding the annealed nascent strands to form a 'chicken foot' four-way DNA junction.

the cell cycle[188]. Finally, in both flies and mammalian cells, DSBs in constitutive heterochromatin that are not yet associated with RAD51 become relocated to the periphery of heterochromatin[200–202]. Collectively, these studies suggest that regulated mobility of DNA ends occurs in specific, higher-order chromatin contexts and contributes to DSB repair.

Repair at stalled replication forks

Stalled replication forks differ from conventional DSBs in several important ways. The presence of branched DNA replication intermediates, DSGs and unresolved hemicatenanes in immature post-replicative chromatin together with scaffolding of DDR factors by the DNA polymerase clamp PCNA and other replisome components creates a unique environment for repair[203–207]. Attempted replication across a (ssDNA) nick in the parental template will break the fork, converting the nick into a one-ended DSB. Forks that are stalled but not broken trigger a cascade of cellular processes that, when properly coordinated, are thought to minimize the risk of chromosome rearrangement at the site of stalling (FIG. 5). These processes include the activation of the DDR, which is primarily controlled by ATR[208,209]; replisome disassembly (also termed fork collapse), in which the CDC45–MCM2–7–GINS (CMG) replicative helicase is extracted by the ATPase valosin-containing protein (also known as p97 ATPase) following ubiquitylation of its MCM helicase subunits[210]; remodelling of DNA structure ('fork remodelling')[93]; and the activation of repair processes, of which HR is a major component[211–213]. Reinitiation of replication (replication restart) can occur at the collapsed fork and can be an error-prone process[95,96]. Currently, fork remodelling is known to include resection of nascent lagging strands[214], fork reversal[215] and endonucleolytic processing of the stalled and reversed fork[216,217]. In vertebrates, timely, organized cleavage of stalled forks is a key step of conservative repair by HR[216,217]. By contrast, pathological conditions, such as mitotic entry before the completion of DNA replication, allow premature fork breakage, leading to misrepair and genomic instability[218].

Replisome disassembly exposes the fork to topological stresses and remodelling activities that promote fork reversal[93,206,219]. Fork reversal generates a cruciate 'chicken foot' structure with a solitary DNA end that is formed by the reannealing of the parental leading and lagging strands (FIG. 5). RAD51 is a key mediator of fork reversal in mammalian cells, and is counteracted by negative regulators that presumably restrict fork reversal to appropriate contexts[220,221]. In addition to its role in fork reversal, RAD51 protects nascent daughter strands at stalled and reversed forks from degradation by MRE11 in a Fanconi anaemia–BRCA pathway-dependent manner[222–226].

Conservative repair at stalled forks

Studies of replication-coupled DNA interstrand crosslink (ICL) repair in *Xenopus laevis* egg extracts have provided considerable insight into the processing and repair of stalled replication forks in vertebrates[214,227]. An ICL covalently links the two DNA strands and constitutes an absolute block to replication, unless it can be 'traversed' with the assistance of FANCM[228] or directly severed by the glycosylase endonuclease 8-like 3 (REF.[229]). In *X. laevis*, ICL repair is initiated following bidirectional replication fork stalling at the ICL[217]. The arrival of both opposing forks is required for replisome disassembly, reversal of one of the two stalled forks and subsequent nucleolytic processing of both of the stalled forks for HR[215,217,230]. Nucleases regulated by the FANCD2–FANCI heterodimer and SLX4 (also known as FANCP) introduce dual incisions in the leading and lagging parental strands of one sister chromatid[216], which ensures that forks stalled at an ICL are processed into two-ended DSBs, thereby favouring conservative SDSA over LTGC or BIR. In contrast to *X. laevis* ICL repair, stalled fork HR in *S. pombe* occurs without the formation of a DSB

intermediate[231]. Of note, the stalled-fork-endonuclease-enabling FANCD2–FANCI heterodimer and its activator, the Fanconi core complex, are absent in yeasts[70,232]. 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 to replication fork stalling. In mammalian cells, HR responses to site-specific fork stalling have been studied in replicating ICL-containing episomal plasmids and by use of a replication fork barrier (RFB) comprising the DNA replication terminus (*ter*) site-binding protein (Tus) bound to an array of six chromosomally inserted *ter* sites[95,113,233]. In this setting, the Tus–*ter* RFB mediates bidirectional fork stalling[113]. HR induced at a Tus–*ter* RFB in wild-type mammalian cells is a non-crossover pathway, generating predominantly short-tract gene conversions of two-ended recombination, mediated by the canonical Fanconi anaemia–BRCA–RAD51 pathway[95,113]. These properties suggest that conservative HR at stalled mammalian 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 (FIG. 5). However, in contrast to HR triggered by a conventional DSB, in which cNHEJ avidly competes with HR, Tus–*ter* RFB-induced HR is unaffected by the status of the cNHEJ genes *Ku70* and *Xrcc4* (REF.[212]). This suggests that the mechanism of SDSA at Tus–*ter* RFBs differs considerably from SDSA at a conventional DSB. To understand this difference, it is helpful to discuss the known interactions between HR and cNHEJ at stalled forks.

Studies of genetic interactions between HR and cNHEJ have provided important insights into the mechanisms of repair of stalled forks. In *S. cerevisiae*, the activity of the Ku70–Ku80 complex independently of Lig4 is lethal in *mre11* or *sae2* resection mutants exposed to the topoisomerase I inhibitor camptothecin, which may generate either stalled or broken forks[234,235]. This suggests that DNA end resection is required to overcome the barrier to HR formed by Ku70–Ku80 binding to DNA ends at camptothecin-induced lesions. Similarly, in human cells, Ku70–Ku80 (inferred by associated DNA-PKcs activity) transiently accumulates at camptothecin lesions and is rapidly displaced by MRE11 and CtIP, whereas delayed resection at camptothecin lesions allows misrepair by cNHEJ[236,237]. A recent study in mouse cells showed that HR induced by a DNA nicking enzyme ('nickase') is not affected by deletion of *Ku70* or *Xrcc4* (REF.[238]). A possible explanation for this finding is that the absence of a second DNA end at sites of nickase-induced fork breakage does not allow a productive cNHEJ outcome, although this hypothesis remains to be tested.

Given that Tus–*ter* RFB-induced HR in mammalian cells is a product of two-ended recombination, why is cNHEJ denied access to these HR intermediates[212]? A clue 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[188], RAD51 recruited to Tus–*ter* RFBs is localized to within 1 kb of the stalling site and the ChIP signal is both more intense and longer sustained than in the DSB response[212]. 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 on ICL repair in *X. laevis* egg extracts[211] and on a Fanconi anaemia-associated dominant negative allele of *RAD51* that confers ICL sensitivity on cells without disrupting DSB-induced HR[239].

Lagging strand gaps normally arise, albeit transiently, during replication. Fork stalling renders these DSGs abnormally persistent, and nascent lagging strand resection at the site of fork stalling would further extend the size of DSGs[214]. Lagging strand DSGs could thus provide a platform for RAD51 recruitment as a very early step of stalled fork processing, before the formation of either a DNA end or a DSB[211,240]. If leading and lagging strand synthesis were to become uncoupled at a leading strand DNA lesion, RAD51 might be loaded onto the resulting leading strand DSG before fork reversal[241]. 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[241] and functions (downstream of RPA) as a sentinel repair factor, analogously to the pivotal role of Ku at DSBs (FIG. 5).

Early and sustained RAD51 recruitment to stalled forks could explain the exclusion of cNHEJ during stalled fork HR. If stalled fork HR in mammalian cells were initiated by RAD51-mediated fork reversal[215,240], the length of the 3′ ssDNA tail formed by fork reversal would reflect the span of its precursor lesion, the RAD51-coated lagging strand DSG (FIG. 5). The first DNA end generated during the repair of the stalled fork — an extended 3′ ssDNA tail produced by fork reversal — might therefore be incapable of binding Ku70–Ku80, and the initial steps of stalled fork HR would remain 'invisible' to the cNHEJ pathway. Subsequent processing steps, such as more extensive fork reversal and nucleolytic incision of the reversed fork could mobilize 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 processed fork, as a prerequisite for SDSA (FIG. 5).

There is still much to be learned about the asymmetries associated with processing of stalled forks 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 preferentially 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 deregulated fork processing by opportunistic nucleases, aberrant interactions of solitary DNA ends formed by fork reversal and aberrant fork restart (in which the restarted fork is abnormal). In a study of rearrangements at a

chromosomally inserted Tus–*ter* RFB, high-throughput genome-wide translocation sequencing was used to identify DNA ends at Tus–*ter* that form translocations[95,242]. The major translocation-competent DNA lesions detected at Tus–*ter* by high-throughput genome-wide translocation sequencing were solitary DNA ends. This finding appears paradoxical, given the two-ended SDSA model of stalled fork HR discussed earlier, and the findings obtained with direct DNA end-sequencing methods, which revealed DNA ends of both polarities at stalled forks[243,244]. A possible explanation for this discrepancy is that two-ended intermediates of SDSA at stalled forks are protected from translocation, whereas solitary DNA ends produced by aberrant fork processing are relatively translocation-prone.

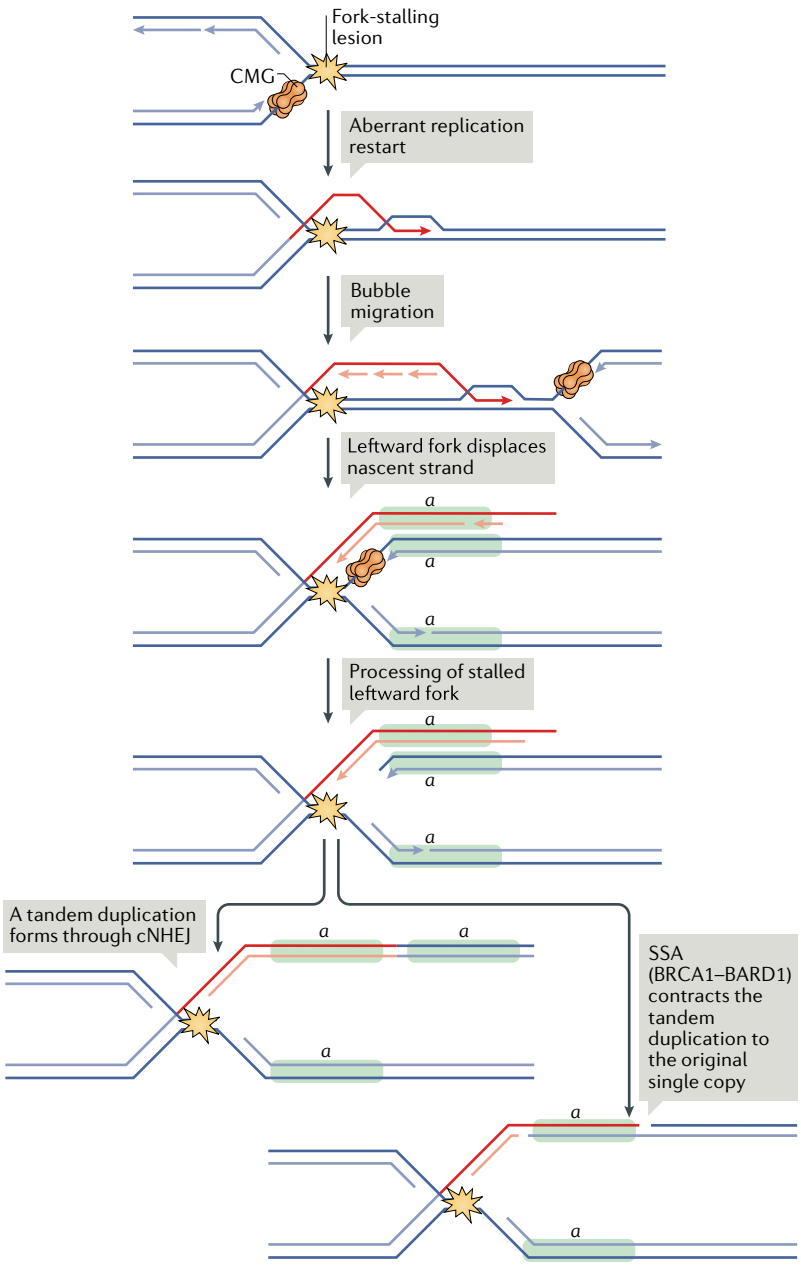

Fig. 6 | **Single-strand annealing may function as a conservative repair pathway at stalled replication forks.** A solitary stalled fork (represented by the CDC45–MCM2–7–GINS (CMG) replicative helicase) may undergo aberrant fork restart through a break-induced replication-type bubble migration mechanism (shown in red). Displacement of the nascent strand of the restarted fork by the converging opposing fork results in duplication of genomic segment '*a*' (green box), bounded, at one end, by the site of fork stalling and, at the other end, by the site at which the nascent strand was displaced. A tandem duplication forms if the two DNA ends are repaired by classical non-homologous end joining (cNHEJ). By contrast, repair by single-strand annealing (SSA), which is promoted by BRCA1–BARD1, would contract the two copies of segment '*a*' back to a single copy, thereby suppressing the formation of a tandem duplication and maintaining normal chromosome structure. BARD1, BRCA1-associated RING domain protein 1; BRCA1, breast cancer type 1 susceptibility protein.

The time factor influences whether stalled forks are processed in a conservative or error-prone manner. In *S. pombe*, HR at the replication termination switch (RTS1) RFB is detectable within ~10 minutes of fork stalling, whereas aberrant fork restart is initiated only after ~60 minutes[96]. In mammalian cells, RAD51 supports the restart of forks that have stalled (but not yet collapsed) following hydroxyurea-induced nucleotide pool depletion[245]. Presumably, RAD51 protects stalled fork structures for a limited time, thereby allowing replisome reactivation (restart of a normal fork) once the nucleotide pool is restored. More prolonged exposure to hydroxyurea leads to localized DDRs suggestive of fork breakage[245]. Deregulated MRE11-mediated degradation of nascent strands in *BRCA* mutants is first detected after ~30 minutes of hydroxyurea treatment, but is fully manifested only after ~5 hours in hydroxyurea[222]. Although these data are still limited, it appears that pathological repair of stalled replication forks occurs only when physiological repair fails.

Replication fork restart and cancer

In bacteria, RecA-mediated invasion of the sister chromatid by a one-ended break at a broken replication fork is coupled to reassembly of a normal replisome by PriA and resumption of conventional semiconservative DNA synthesis[246]. To date, a PriA-like replisome reloading activity has not been identified in eukaryotes. Consequently, in eukaryotes, once the replisome has been disassembled, restart of the collapsed or broken fork may be obligatorily error-prone. In *S. pombe*, forks stalled at an ectopic RTS1 RFB engage both conservative and error-prone HR, including RAD51-dependent and RAD51-independent pathways of fork restart[231,247,248]. A Rad22-mediated mechanism can restart stalled forks, but the restarted fork is extended by BIR, and is unstable and prone to rearrangement up to 75 kb downstream of the RFB[96,249]. A role for RAD52 in restarting collapsed mammalian replication forks in mammals has also been proposed[250]. In yeasts, the mutagenic impact of BIR at stalled or broken forks is limited by the arrival of the opposing, unhindered fork from the neighbouring replicon[94,96].

Aberrant, RAD51-independent fork restart can occur at a mammalian Tus–*ter* RFB, leading to the formation of tandem duplications in *BRCA1*-mutant cells[95]. This model system recapitulates highly specific, ~10-kb-long tandem duplications that are commonly observed in human *BRCA1*-linked, but not *BRCA2*-linked, breast and ovarian cancers[95,113,251,252]. In the Tus–*ter* RFB model, BRCA1, BARD1 and CtIP, but not BRCA2 or RAD51, suppress tandem duplications, suggesting the

involvement of a DNA end resection step in the suppression mechanism. In *BRCA1*-mutant cells, the duplications occur in response to fork stalling, but not in response to conventional DSBs. These findings directly implicate aberrant restart of stalled forks in the formation of human cancer-associated chromosome rearrangements. FANCM and BLM — the motor proteins that impose a delay on the onset of BIR in *S. cerevisiae*[105,108,109] — specifically suppress the formation of tandem duplications in *BRCA1*-mutant cells[95], suggesting that fork restart that causes a tandem duplication is mediated by BIR-type bubble migration (FIG. 6).

The formation of tandem duplications in *BRCA1*-mutant cells is completed by cNHEJ, which joins in tandem the DNA segment synthesized following fork restart and the DNA replicated by the converging opposing replication fork[95] (FIG. 6). We propose that in normal, BRCA1-proficient cells, this end joining step is in competition with SSA. SSA requires BRCA1, BARD1 and CtIP, but not BRCA2 or RAD51 — a pattern of dependencies that matches the requirements for suppression of tandem duplications. If the two DNA ends of the duplicated segment were repaired by SSA instead of cNHEJ, the duplication would be converted back into the original single copy (FIG. 6). Therefore, unlike the contribution of SSA to repair of replication-independent DSBs, where it is obligatorily error-prone, SSA at stalled replication forks may perform a conservative repair function by counteracting the tendency of aberrantly restarted forks to form tandem duplications. Thus, a repair pathway that is considered to be error-prone during conventional DSB repair might mediate error-free repair at stalled forks.

Conclusion

Our knowledge of the decision trees for mammalian DSB repair is well established, enabling researchers to focus their attention on higher-order cellular processes that affect pathway choice, such as the cell cycle and the local chromatin environment. By contrast, some of the key determinants 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 in favour of conservative HR. The mechanisms that regulate the remodelling of stalled forks remain to be fully understood and quantified in mammalian cells. We expect that ongoing research into repair-pathway choice at stalled replication forks will yield additional insights into the process of tumorigenesis and will reveal new therapeutic targets in diseases characterized by genomic instability.

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Competing interests
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