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Routing strand lesions during DNA replication: Coordination between single-strand break repair and double-strand break repair shapes lesion fate

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Abstract

DNA replication constantly faces obstacles that threaten genome stability. Faithful duplication requires replication forks to smoothly process template damage, chemical or topological blocks, and repair intermediates. Traditionally, replication-associated lesions are described by their initial structures (nicks, gaps, etc.). However, their S-phase identities are highly transitional: a template single-strand break (SSB) can become a one-ended double-strand break (DSB) upon fork arrival, a replication fork stalled by a template block can reverse, restart, or collapse, and a repriming event leaves a post-replicative single-stranded DNA (ssDNA) gap. The biological consequence of a lesion depends heavily on its structural transitions and how it is "routed" through distinct repair, bypass, or cleavage pathways. In this review, we shift our focus from static structural identities to dynamic lesion routing, emphasizing the coordination between SSB repair (SSBR) and DSB repair (DSBR). We first summarize the major sources of replication-associated strand lesions, and then discuss how strand lesions are managed across three processing windows: SSBR before fork encounter, the overlap zone in which gaps and stressed forks can be buffered without immediate DSB conversion, and broken-fork/DSBR-centered processing after DSB formation, including homologous recombination (HR)-mediated restart, break-induced replication (BIR)-like synthesis, or end joining. Depending on the routing network, lesion processing can result in accurate completion, delayed resolution or salvage, mutagenic completion, structural genome scars, or cytotoxic genome instability. Finally, we discuss how this dynamic framework refines our understanding of DNA damage response (DDR)-targeted cancer therapies, drug resistance, and treatment outcomes in different genetic or therapeutic contexts.

Keywords: DNA replication, single-strand break repair, double-strand break repair, single-stranded DNA gap, replication fork restart, strand lesion routing

1. Introduction

Both prokaryotic and eukaryotic cells undergo DNA replication to duplicate their genetic material before cell division. During DNA replication, the replication machinery unwinds the DNA double helix and synthesizes daughter strands using the parental strands as templates, resulting in two DNA molecules that inherit one parental strand and one newly synthesized strand, a process known as semiconservative replication. In eukaryotic cells, DNA replication occurs during the S phase of the cell cycle and requires the coordinated action of multiple proteins, including helicases, primases, DNA polymerases, sliding clamps, topoisomerases, nucleases, and ligases[1]. The whole process is tightly coordinated to ensure both accuracy and completion, as defective DNA replication compromises genome stability and contributes to developmental disorders, aging, and tumorigenesis[2].

However, replication templates are not always intact and clean. Replication forks frequently encounter obstacles such as endogenous DNA damage, DNA secondary structures, topological complexes, and incompletely repaired intermediates that can obstruct fork progression[3,4]. At the same time, replication itself generates transient strand discontinuities and replication-associated DNA intermediates that are normally resolved as part of the replication process, but may become pathological if they persist or are

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processed incorrectly[5,6]. Replication-associated strand lesions include pre-existing lesions encountered by the fork, intermediates generated directly during DNA replication, and secondary lesions or unresolved states produced through replication-dependent structural conversion[7,8].

A key characteristic of replication-associated strand lesions is that their identities are transitional, instead of being fixed. A template-strand single-strand break (SSB) can be converted into a one-ended double-strand break (DSB) when encountered by an advancing replication fork[9,10]; a nascent-strand discontinuity can persist as a post-replicative single-stranded DNA gap[11-13]; a stalled fork can be remodeled into a reversed fork or processed by nucleases[14,15]; and a collapsed fork-associated break can be turned into replication protein A (RPA)-coated single-stranded DNA during end resection of homologous recombination (HR)-mediated repair[16,17]. Here, "lesion routing" refers to the context-dependent progression of a lesion through structural lesion conversion, repair or tolerance pathway engagement, and biological outcome. These transitions mean that the biological consequence of a lesion is determined not only by its initial structure, but also by its timing, strand location, fork status, and how it is routed into distinct repair or tolerance pathways.

In this review, we discuss DNA lesion routing during replication, with an emphasis on replication-associated strand lesions and their structural transitions. We begin by introducing pre-existing template lesions, replication-generated intermediates, and secondary replication-dependent lesions or unresolved states. We then follow these lesions in the order in which they are handled: early SSB repair (SSBR)-centered repair before fork encounter, protective buffering in the overlap zone (the functional interval rather than an independent repair pathway) before DSB formation, and DSBR-centered restart or repair after fork collapse. These routes can lead to different final outcomes, including accurate completion, delayed resolution or salvage, mutagenic completion, structural genome scars, or cytotoxic genome instability. Finally, we discuss how this routing view may help improve DDR-targeted therapies: not only by supporting more dynamic therapeutic strategies, but also by helping predict drug resistance and treatment outcomes (Figure 1).

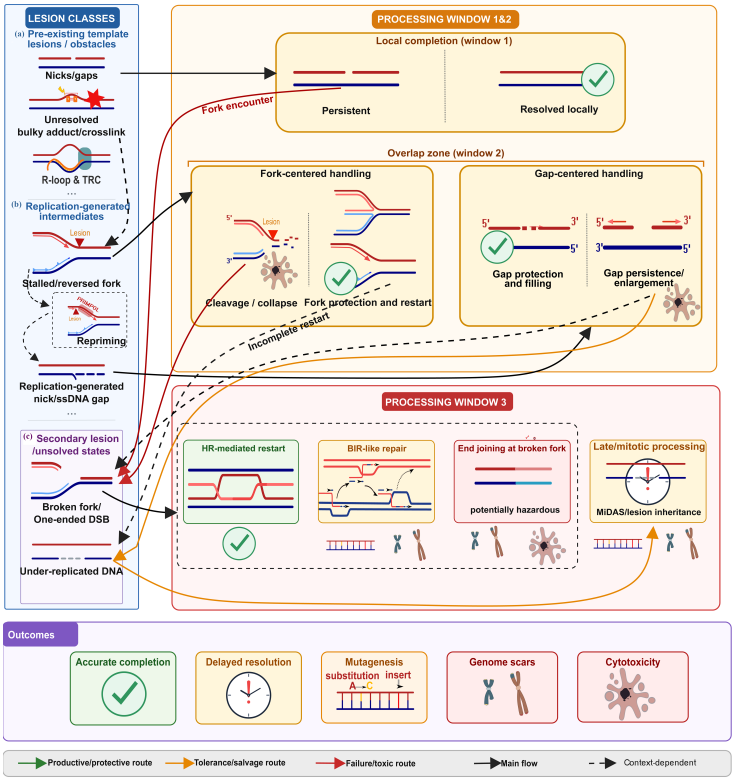


Figure 1. Overall routing of replication-associated strand lesions. Replication-associated lesions and intermediates are organized according to their origin and structural state into three classes: (a) Pre-existing template lesions or obstacles present before fork encounter; (b) Intermediates generated at or behind the replication fork; (c) Secondary lesions or unresolved states. During processing window 1, template lesions may be resolved locally before fork arrival, whereas post-replicative gaps and stalled or reversed forks enter gap-centered or fork-centered handling in the overlap zone of windows 2. Successful buffering includes gap filling or fork restart. Failure or delayed completion can generate broken forks or one-ended DSBs, or under-replicated DNA, for which processing window 3 shows representative downstream routes, including HR-mediated restart, BIR-like repair, end joining at broken forks, and late or mitotic processing. Under-replicated DNA may undergo MiDAS or persist as inherited lesions. Possible outcomes include accurate or delayed completion, as well as mutagenesis, genome scars, or cytotoxicity. Green, orange, and red arrows indicate productive/protective, tolerance/salvage, and failure/toxic routes, respectively; black arrows indicate the main structural flow, and dashed arrows indicate context-dependent transitions. TRC: transcription-replication conflict; SSB: single-strand break; DSB: double-strand break; HR: homologous recombination; BIR: break-induced replication; MiDAS: mitotic DNA synthesis; BER: base excision repair.

2. Main Body

2.1 Replication-associated strand lesions: Sources, identities, and transitions

Replication-associated strand lesions can be defined by their structures, origins, and especially their positions relative to the replication fork. Timing matters, as structurally similar lesions or strand discontinuities can have different outcomes depending on whether they are formed ahead of, at, or behind the replication fork. Here, we organize strand lesions according to when and where they arise relative to replication: The first group contains template lesions that already exist before an advancing fork approaches, including unrepaired base excision repair (BER)/SSBR intermediates, template SSBs, trapped topoisomerase cleavage complexes, bulky adducts, crosslinks, and other fork-blocking structures. The second group contains intermediates generated during ongoing DNA replication, including immature Okazaki-fragment intermediates, nascent post-replicative ssDNA gaps, and stalled or reversed forks. A third group consists of secondary replication-dependent lesions or unresolved states that emerge when initial lesions or replication intermediates persist or undergo further structural conversion, including broken forks, one-ended DSBs, and under-replicated DNA[18,19]. These categories describe lesion origin, timing, and structural state, whereas subsequent structural lesion conversion, repair pathway engagement, and biological outcomes depend on the specific replication context. During replication, one structural state can transition into another as the fork approaches, bypasses, stalls, reverses, or collapses[20]. These dynamic transitions reflect the continuous interplay between DNA synthesis, genome protection, and repair pathway choices[21-23]. In this section, we summarize the major lesion sources and structural transitions, while their detailed processing and repair mechanisms are discussed in Section 2.2, Section 2.3, and Section 2.4 (Table 1 and Figure 2).

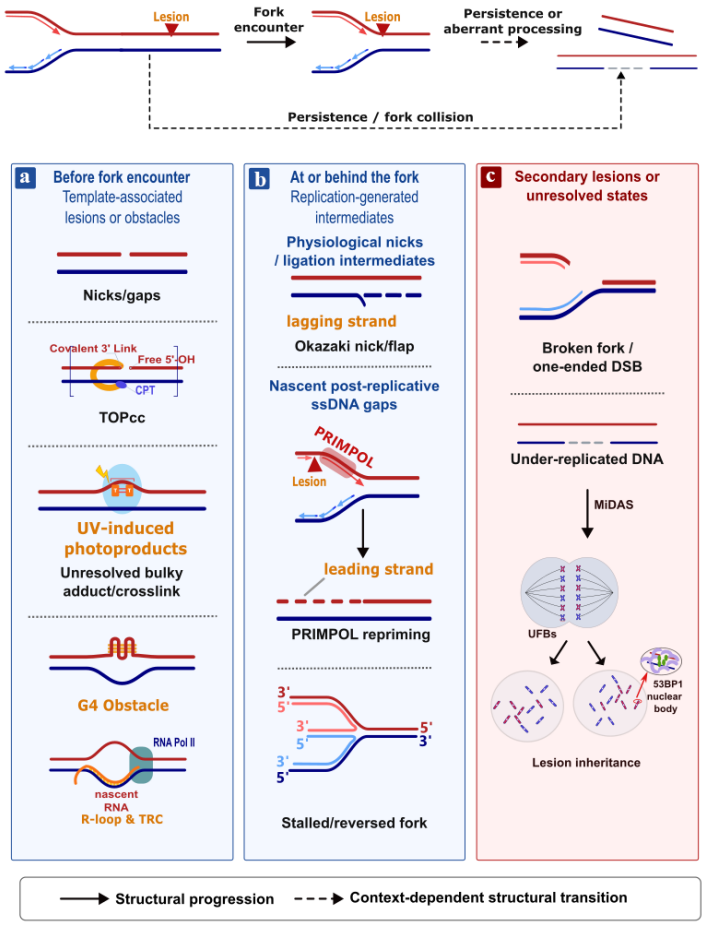


Figure 2. Lesion origins and structural transitions relative to replication. Lesions and intermediates are classified by their timing and position relative to the replication fork. (a) Template-associated lesions or obstacles present before fork encounter include nicks and gaps, trapped TOPccs, unresolved bulky adducts or crosslinks, G4 structures, and R-loops associated with TRCs; (b) Replication-generated intermediates arising at or behind the fork include physiological Okazaki-fragment nick/flap intermediates, nascent post-replicative ssDNA gaps generated by repriming, and stalled or reversed forks; (c) Persistence or aberrant processing can produce secondary lesions or unresolved states, including broken forks or one-ended DSBs and under-replicated DNA. Under-replicated DNA may undergo mitotic DNA synthesis or be inherited by daughter cells. Solid arrows indicate structural progression; dashed arrows indicate context-dependent structural transitions. CPT: camptothecin; G4: G-quadruplex; SSB: single-strand break; BER: base excision repair; TOPcc: topoisomerase cleavage complexes; TRC: transcription-replication conflict; ssDNA: single-stranded DNA; DSB: double-strand break; MiDAS: mitotic DNA synthesis; UV: ultraviolet.

Table 1. Replication-associated strand lesions: sources, transitions, and outcomes. Lesions and intermediates are grouped according to their origin and timing relative to replication, with representative transition risks and major outcomes.

	Lesion/intermediate	Source	Timing relative to replication	Transition risk	Major outcomes
A. Template-associated lesions present before fork encounter					
1	**Template SSB/BER-SSBR intermediate (nicks and gaps)**[9,10,24]	Oxidative/base damage; AP-site incision; direct SSBs; incomplete BER/SSBR;blocked or abortively ligated termini	Present before fork encounter	Persistence until fork encounter can convert a repairable single-strand lesion into a broken fork/one-ended DSB	Local repair; restored replication after broken-fork repair; cytotoxicity if unresolved
2	**TOPcc**[25-27]	Physiological TOP1/TOP2 cleavage intermediates trapped by endogenous stress or topoisomerase poisons	Present before fork encounter	Trapped TOPcc can obstruct fork progression; TOP1cc can stall or collapse an advancing fork, whereas persistent TOP2cc can generate protein-linked two-ended DSBs.	Resolution and restart; fork stress; replication-associated DSBs; genome instability or cell death
3	**Unresolved bulky adduct/crosslink**[19,28,29]	UV photoproducts; bulky chemical adducts; interstrand crosslinks	Present before fork encounter	Fork blockage can trigger repriming, reversal, incision or collapse, generating new replication intermediates	Lesion bypass/removal; post-replicative gaps; stalled/reversed forks; break formation if processing fails
4	**RNA-associated and non-B DNA obstacle**[30-33]	R-loops; G-quadruplexes; embedded ribonucleotides; RNA-DNA structures	Present before fork encounter	Persistent structures can impede fork progression or generate nicks/gaps during processing	Resolution without breakage; fork slowing/reversal; strand lesions or DSB formation in unresolved contexts
B. Replication-generated intermediates					
5	**Okazaki nick/flap**[34-37]	Lagging-strand synthesis and Okazaki-fragment maturation	Generated during replication, behind the progressing fork	Persistence after defective flap processing or ligation can leave post-replicative strand discontinuities	Normal maturation; persistent nick/gap; replication stress or breakage if unresolved
6	**Post-replicative nascent ssDNA gap**[38-41]	PRIMPOL-dependent repriming;incomplete lagging-strand synthesis; polymerase uncoupling; defective gap suppression/filling	Generated during replication, behind the progressing fork	Gap persistence or nuclease-mediated enlargement may increase ssDNA burden and, in some contexts, promote break formation	Gap completion; persistent ssDNA gaps; mutagenesis; break formation in selected contexts
7	**Stalled/reversed fork**[23,42-45]	Fork-blocking lesions, dNTP shortage, transcription-replication conflict, oncogenic	Generated during replication, at the replication fork	Failure of fork protection or inappropriate nuclease cleavage can produce fork	Fork restart; prolonged stalling/reversal; nascent-strand degradation; fork

		replication stress		degradation or fork breakage	collapse/break formation
C. Secondary replication-dependent lesions and unresolved intermediates					
8	**Broken fork/one-ended DSB**[9,10,46,47]	Fork collision with a template nick/TOP1cc; cleavage of stalled or reversed forks; collapse of vulnerable replication intermediates	Secondary lesion formed after fork encounter or fork collapse	May remain one-ended, undergo resection and template-directed restart, or acquire a second end through fork convergence/additional processing	Restored replication; mutagenic or rearranged repair products; loss of replication capacity or cell death
9	**Under-replicated DNA/mitotic replication intermediate**[48-51]	Incomplete replication at late-replicating regions or common fragile sites; unresolved replication stress	Persists from late S/G2 into mitosis or the next cell cycle	Mitotic processing, cleavage or incomplete resolution can generate chromosome breaks and inherited lesions	MiDAS-associated completion/salvage; chromosome bridges; micronuclei; structural genome instability; inherited lesions/53BP1 nuclear bodies

SSB: single-strand break; BER: base excision repair; SSBR: single-strand break repair; AP: apurinic/apyrimidinic; DSB: double-strand break; TOPcc: topoisomerase cleavage complex, including TOP1cc and TOP2cc; UV: ultraviolet; ssDNA: single-stranded DNA; PRIMPOL: primase-polymerase; dNTP: deoxyribonucleoside triphosphate; MiDAS: mitotic DNA synthesis; 53BP1: p53-binding protein 1.

2.1.1 Template-associated lesions or obstacles

2.1.1.1 BER/SSBR intermediates and template SSBs (nicks and gaps)

Oxidative metabolism generates base lesions and direct SSBs. BER processing itself generates transient nick or gap intermediates[52]. These lesions are normally filled and ligated locally, but blocked termini or incomplete repair can prolong their existence. Outside S phase, these lesions are canonical BER or SSBR substrates. In S phase, however, an unrepaired SSB or BER intermediate on the template strand can be converted into a replication-associated break when encountered by an advancing fork. A leading-strand template nick can generate a blunt one-ended DSB when the eukaryotic Cdc45-MCM-GINS (CMG) replicative helicase reaches and runs off the nicked template; a lagging-strand template nick can generate breaks with different structures and CMG unloading routes[9]. Therefore, in S phase, the key transition is from a repairable SSB/BER intermediate before fork arrival to a one-ended DSB after fork collision.

2.1.1.2 Topoisomerase-linked lesions

Topoisomerases are essential for replication: TOP1 introduces a transient single-strand nick to relieve positive supercoiling, forming a covalent 3'-phosphotyrosyl intermediate[53]; TOP2 passes one DNA duplex through a transient double-strand break via a 5'-phosphotyrosyl linkage[54]. These cleavage products are normally short-lived and rapidly resealed. However, chemotherapeutics (camptothecin or etoposide), oxidative damage, or topological stress like R-loops can convert them into trapped topoisomerase cleavage complexes (TOPcc), typical protein-linked lesions on the replication templates[25]. A trapped TOP1cc on the template strand can block replication fork progression, causing the fork to stall, reverse, or collapse into a one-ended DSB. Unresolved TOP2 cleavage complexes instead directly produce persistent protein-linked two-ended DSBs that need end processing and can drive translocations. Thus, TOPccs show how a transient enzymatic intermediate can become a replication-blocking lesion and subsequently a replication-associated strand break.

2.1.1.3 Unresolved crosslink- or adduct-induced fork obstacles

Template strands are not always "clean" during DNA replication. Replication forks encounter template obstacles arising from oxidative by-products, ultraviolet light, crosslinking chemotherapeutics, etc.[19]. DNA crosslinks and bulky adducts are generally not strand breaks when they first arise, but if not resolved, they become replication-associated lesion sources when encountered by a progressing fork. Interstrand crosslinks physically block helicase unwinding, and bulky intrastrand adducts mainly block replicative polymerases and may uncouple DNA unwinding from synthesis. Fork encounter can cause a stalled or reversed fork, a nascent ssDNA gap left by a repriming event downstream, a repair-associated nick or DSB generated by incision, or even a collapsed fork. The key transition here is from a chemically stable template obstacle to an unpredictable replication-associated strand lesion or fork intermediate.

2.1.1.4 RNA-associated and non-B DNA replication obstacles

RNA-associated structures and non-B DNA conformations can also act as template-associated replication obstacles. During active transcription, nascent RNA can hybridize back to the template DNA strand and form R-loops, leaving the non-template DNA strand as ssDNA, which can generate secondary structures or impede fork progression[55,56]. Embedded ribonucleotides in template DNA, on the other hand, can become strand discontinuities after incision[57]. In addition, G-quadruplexes and other non-B DNA structures can form at specific sequence regions and impede polymerase progression or fork movement, causing fork slowing, stalling, or remodeling[58]. These RNA-containing structures or non-B DNA structures are not necessarily strand breaks when they first arise, but incomplete removal or incorrect processing of them can generate nicks, gaps, or abnormal DNA ends; and persistent R-loops or non-B DNA structures can block fork progression, promote fork reversal, or contribute to fork collapse[30,59]. In this sense, RNA-associated obstacles link transcription, RNA metabolism, and DNA secondary structures to lesion routing during DNA replication.

2.1.2 Replication-generated intermediates

2.1.2.1 Physiological nicks and ligation intermediates

DNA replication generates transient strand discontinuities, especially on the lagging strand. Okazaki-fragment maturation transiently produces flaps and ligatable nicks that are normally processed and sealed before they persist as post-replicative discontinuities[34,35,60]. If flap cleavage is delayed or ligation fails or is aborted, nicks, gaps, flaps, or abortive-ligation intermediates can remain behind the progressing fork[61]. The transition is mainly temporal and conditional: a normal lagging-strand intermediate becomes a lesion when it persists beyond the Okazaki maturation window.

2.1.2.2 Nascent post-replicative ssDNA gaps

Nascent post-replicative ssDNA gaps, on the other hand, are generated behind the progressing fork. On the leading strand, nascent ssDNA gaps arise when PRIMPOL reprimes downstream of blocking lesions on the leading strand, leaving a post-replicative ssDNA gap behind the fork[38]. On the lagging strand, incomplete Okazaki fragment maturation or polymerase uncoupling can also generate gaps. In most cases, these gaps may subsequently be filled, persist, or undergo nuclease-mediated enlargement. In some contexts, prolonged or aberrantly processed gaps can contribute to break formation, but post-replicative gaps should not be regarded as obligate pre-DSB intermediates. Their defining feature is their location behind the fork as a replication-generated nascent-strand discontinuity. Unlike template-strand discontinuities present ahead of the fork, these nascent ssDNA gaps are generated behind the fork and can be routed toward gap filling, damage tolerance, persistence, or, in selected contexts, break formation.

2.1.2.3 Stalled and reversed forks

Other than being bypassed by repriming and leaving a nascent ssDNA gap, a template obstacle could simply block polymerase movement, helicase unwinding, or both, causing fork slowing or stalling. Replication forks stall at bulky DNA adducts, protein-DNA barriers, non-B DNA structures including G-quadruplexes and R-loops, or under conditions of dNTP shortage or oncogene-driven replication stress[31,62-65]. Stalled forks are not broken yet, but are vulnerable, as they can be remodeled into reversed forks, generating structurally different intermediates that are vulnerable to further processing. If fork stabilization and restart succeed, replication can resume without DSB formation; if the intermediate persists or is cleaved, it can instead be converted into a broken fork. Stalled and reversed forks are therefore replication-generated intermediates whose structural fate depends on subsequent protection, restart, or cleavage.

2.1.3 Secondary lesions or unresolved states

2.1.3.1 Broken fork or replication-generated one-ended DSBs

Broken forks and replication-associated one-ended DSBs are secondary lesions generated when an initial template lesion or replication intermediate undergoes fork collision, cleavage, or collapse. Collision with a leading-strand template nick can cause CMG run-off and generate a blunt one-ended DSB, whereas cleavage or processing of stalled, reversed, or otherwise vulnerable fork structures can generate breaks with different end structures[9,10]. Unlike a conventional two-ended DSB, a one-ended broken fork initially lacks a matched second DNA end at the same locus. But if a fork arriving from the opposite direction reaches the lesion before restart, the one-ended break can become a two-ended DSB[9,10,46]. In the view of routing, replication-generated one-ended DSBs are better viewed as broken forks awaiting restart, rather than DNA ends to be ligated.

2.1.3.2 Under-replicated DNA and mitotic processing

Incomplete replication can persist at late-replicating regions and difficult-to-replicate loci, including common fragile sites, leaving under-replicated DNA that extends into G2 or mitosis[48,66]. Such regions may undergo mitotic processing, including cleavage and mitotic DNA synthesis (MiDAS), or persist as inherited lesions into the next cell cycle[49,50,67]. Under-replicated DNA represents a late unresolved replication state rather than an ordinary DSB or a general form of gap repair[68,69]. Its subsequent salvage processing and potential genomic consequences are discussed in later sections.

2.2 SSBR-centered processing before fork encounter

In S phase, the SSBR pathway is not just a housekeeping pathway that fixes nicks and gaps. It also acts as an early guard against the conversion of template DNA nicks, short gaps, and chemically blocked termini into replication-generated DSBs, such that strand lesions that are detected, cleaned, filled, and ligated before fork arrival can be resolved as local repair events, without generating a replication-associated DSB. If the same lesion persists as an unrepaired nick or gap on the template strand until the fork approaches, collision may occur and generate a one-ended DSB at a broken fork, which subsequently requires replication-coupled restart rather than ordinary SSBR[9,10]. It is not simply the original state of the lesion, but also whether SSBR completes repair in time, that determines whether a strand lesion remains a local repair event or becomes a replication-coupled chromosome break.

The early PARP1/2-XRCC1 response functions as a coordinator of early SSBR rather than a simple SSBR initiator. PARP1 rapidly binds nicks and gaps through its DNA-binding zinc-finger domains and synthesizes poly(ADP-ribose) (PAR) on itself and nearby proteins[70]; PARP2 can also contribute with a preference for 5'-phosphorylated breaks or nicks. The PAR signal promotes local chromatin relaxation and provides docking sites for downstream factors, especially the scaffold protein XRCC1, which itself has little catalytic activity, but coordinates PNKP and APTX for end processing, POL β for gap filling, and LIG3 for nick sealing. In addition to LIG3, LIG1 also contributes, particularly in replication-associated contexts[36,71]. By contrast, PARG degrades PAR chains, and other ADP-ribosylhydrolases such as TARG1, MacroD1, and ARH3 remove the residual mono-ADP-ribose mark[72-74]. This turnover limits PAR signaling and helps recycle PARP1/2 and associated repair factors. Efficient assembly and timely disassembly keep the lesion transient and under control. In contrast, PARP inhibition may trap PARP1 on DNA lesions and prolong the persistence of unrepaired SSBs, which might be encountered by an advancing fork during replication, presenting an example of not just "failed SSB repair", but also failure of lesion resolution before fork encounter[75].

The chemistry of DNA ends also matters for successful SSBR. Only a minority of SSBs carry 3'-hydroxyl (3'-OH) and 5'-phosphate (5'-P) ends that can be directly ligated. Oxidative damage and BER intermediates often leave atypical termini, such as 3'-phosphoglycolate (3'-PG), 3'-phosphate (3'-P), 5'-hydroxyl (5'-OH), 5'-deoxyribose-5-phosphate (5'-dRP), and 3'-phospho-α,β-unsaturated aldehyde (3'-PUA) ends. Abortive ligation can produce 5'-AMP adducts, and trapped topoisomerase cleavage complexes can leave covalent 3'- or 5'-phosphotyrosyl protein adducts. Those ends of different chemical structures are handled by different enzymes: PNKP restores ligatable 3'-OH/5'-phosphate ends by removing 3'-phosphate and phosphorylating 5'-OH ends[76]; the dRPase activity of POL β excises the 5'-dRP during short-patch BER and fills the gap[52]; APTX deadenylates 5'-AMP[61]; TDP1 resolves TOP1-linked 3'-phosphotyrosyl ends and can also process some oxidative 3' blocks[77,78], whereas TDP2 is more relevant to 5'-phosphotyrosyl ends generated by TOP2 cleavage complexes[79]. XRCC1 helps concentrate several of these activities at SSBs, although exceptions exist; for example, TDP2 recruitment can be promoted by ZATT/ZNF451-SUMO-dependent mechanisms[80,81]. End cleaning can be a rate-limiting step in SSBR. If end cleaning is slow or incomplete, an SSB can persist until the fork arrives, increasing the risk of replication-dependent structural conversion.

By contrast, lagging-strand synthesis actually provides a physiological example of how strand discontinuities can be ligated without being converted into DSBs. During Okazaki fragment maturation, Pol α-primase synthesizes an RNA-DNA primer, Pol δ extends the fragment and displaces the 5' end of the downstream fragment into a short flap. Most short flaps are cleaved by FEN1, generating a ligatable nick. Longer flaps can be trimmed by DNA2 before FEN1 completes processing, with RNase H2 helping remove RNA primer in some settings. Throughout this process, PCNA coordinates the sequential handoffs by organizing Pol δ, FEN1, RNase H2, and LIG1 on the nascent strand, allowing newly generated nicks to remain locally protected until ligation[34-37]. LIG1 then seals the final nick, restoring an intact strand. These nascent-strand intermediates are conceptually distinct from the template-strand SSBR substrates discussed above. Okazaki maturation illustrates an important S-phase principle: nicks and flaps are not automatically chromosome breaks if they are processed in a timely manner within a protected, enzyme-accessible environment. It also implies that S-phase cells can keep strand discontinuities under control before they become chromosome breaks. If a template lesion is not resolved by SSBR before fork encounter, however, replication-specific buffering or tolerance mechanisms, distinct from SSBR itself, may still prevent DSB formation, setting up the next layer of routing.

2.3 The overlap zone: Pre-DSB buffering and tolerance during replication

The boundary between SSBR-centered processing and broken-fork/DSB-centered processing is not sharp. Many single-strand lesions are quickly resolved before cells enter S phase, but some nicks, unligated repair intermediates, base lesions and their repair intermediates, or protein-linked nicks persist until a replication fork approaches. At the same time, fork-blocking lesions, including trapped TOPcc, bulky adducts, crosslinks, R-loops, G-quadruplexes, protein-DNA barriers, nucleotide shortage, or oncogene-induced fork stress, can directly obstruct fork progression. Even at that point, DSB formation or engagement of DSB-centered repair is not inevitable. Repair can still be attempted on the damaged strand, the fork may still be restartable, or may be halted without collapsing into a break. We refer to this intermediate state as the overlap zone. It is not an independent pathway, but a functional interval in which SSBR-derived lesions and fork-blocking lesions are managed before structural conversion to a broken fork/DSB or entry into DSB-centered processing. Repriming, translesion synthesis (TLS), template switching (TS), fork reversal, fork protection, and restart are considered here as replication-specific buffering or tolerance mechanisms rather than SSBR or DSBR pathways themselves. The

central problem in this zone is therefore not simply how to repair a lesion but how to limit its conversion into a broken fork or DSB. In other words, cells use this interval to keep single-strand lesions and stressed replication intermediates in non-DSB states when possible[23,82] (Figure 3).

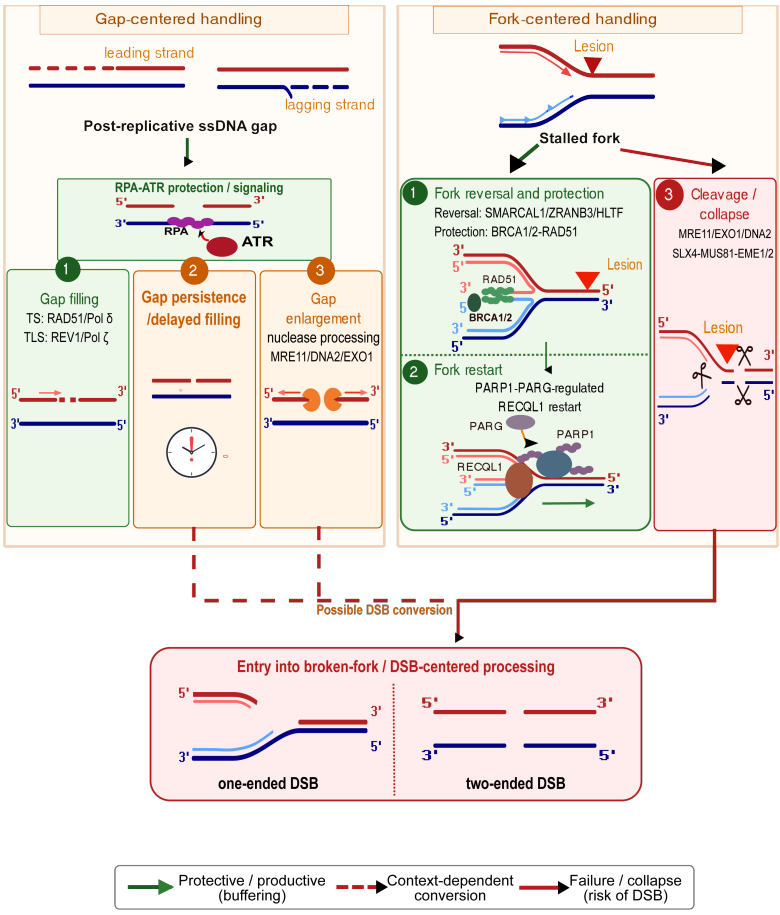


Figure 3. The overlap zone: pre-DSB buffering and tolerance. Replication-associated intermediates in the overlap zone are handled through gap-centered and fork-centered mechanisms before entry into DSB-centered processing. Post-replicative ssDNA gaps are protected by RPA-ATR signaling and may undergo filling through TS or TLS, persist for delayed filling, or undergo nucleolytic enlargement. Fork-centered responses include fork reversal and protection, PARP1-PARG-regulated RECQL1 restart, and nuclease-mediated cleavage or collapse. The lower panel distinguishes a replication-associated one-ended DSB or broken fork from a conventional two-ended DSB. Green arrows indicate productive or protective buffering, dashed red arrows indicate context-dependent DSB conversion, and solid red arrows indicate cleavage or collapse associated with DSB risk. DSB: double-strand break; ssDNA: single-stranded DNA; RPA: replication protein A; TS: template switching; TLS: translesion synthesis.

At the gap-centered branch, cells first try to keep the problem within a single-strand context. Here, we focus primarily on nascent ssDNA gaps generated during fork-level restart or bypass. These post-replicative gaps arise on nascent DNA behind the fork and are structurally distinct from template-strand nicks or gaps present ahead of the fork, as discussed in Section 2.2. ssDNA gaps can arise when replication is reinitiated downstream of a blocking lesion, either through PRIMPOL-mediated repriming on the leading strand or through discontinuous lagging-strand synthesis, leaving an unreplicated region to be filled after fork passage[38,83]. These gaps are not simple SSBs, and their fate depends on whether they are filled, protected, or allowed to worsen. Firstly, RPA coats ssDNA and activates ATR-CHK1 signaling to provide a temporary protective platform, buying more time for completion of repair. Next, gap filling may occur through TLS, RAD18-PCNA-dependent lesion bypass, or template switching with the newly replicated sister chromatid. These processes represent replication-specific gap filling or damage-tolerance mechanisms rather than canonical SSBR. At the same time, BRCA1/2 and RAD51 also contribute before DSB formation by limiting gap accumulation and protecting vulnerable ssDNA or nascent DNA from nuclease attack[84]. For example, BRCA2 can suppress PRIMPOL-dependent repriming and ssDNA gap formation through its interaction with MCM10, while BRCA1/BARD1-dependent PCNA ubiquitination helps suppress ssDNA gap formation during normal replication[85]. If these filling and protective mechanisms fail, the same gap can be enlarged by nucleases including MRE11, EXO1, DNA2, etc. Such processing products can amplify RPA-ATR signaling and create substrates for gap repair and also increase dependence on BRCA/RAD51-mediated protection. Thus, gap accumulation should be viewed as a replication-stress intermediate with context-dependent fates rather than as an obligate pre-DSB state: accurate completion, mutagenic filling, persistent ssDNA, and, in selected contexts, break formation all remain possible outcomes[39,86,87].

In parallel, primary fork-blocking lesions can enter a fork-centered branch, in which stalled forks can be managed through repriming, remodeling, protection, or restart to avoid immediate collapse. One option is repriming. PRIMPOL-mediated repriming allows DNA synthesis to restart downstream of a blocking lesion, allowing replication to continue at the cost of leaving a post-replicative ssDNA gap which is then handled by the gap-centered branch. Another option is fork reversal. Instead of bypassing the obstacle and leaving it behind, the fork is remodeled by factors such as SMARCAL1, ZRANB3, and HLTF into a reversed structure, temporarily moving the nascent strands away from the lesion and creating time for repair or controlled restart. But this strategy is protective only if the regressed arm of the reversed fork is protected. BRCA1, BRCA2, RAD51, and RAD51 paralogs stabilize these structures and limit MRE11- or EXO1-dependent degradation of nascent DNA. PARP/PARG-dependent PARylation dynamics and RECQ1-mediated branch migration further determine whether a reversed fork remains protected or restarts after the obstacle is removed. Repriming trades an immediate fork collapse for a delayed gap, whereas fork reversal delays the decision between restart and collapse. The roles of BRCA1/2 and RAD51 here are distinct from their canonical roles in DSB repair: by suppressing gap accumulation and protecting reversed forks, these factors participate in replication-specific buffering before DSB formation[42,88,89]. Failure of these buffering mechanisms can instead result in structural conversion to a broken fork or one-ended DSB, whose subsequent processing is discussed in Section 2.4.

2.4 Broken-fork processing and replication-coupled DSB repair

Replication-generated DSBs have multiple origins. They can arise when an advancing fork runs into a template-strand break, a trapped topoisomerase cleavage complex, or an aberrantly processed post-replicative gap or gap-associated structure[9,10,25,90]. Incompletely replicated regions that persist into mitosis represent a distinct late replication state and are discussed separately below in the context of MiDAS[48,66]. Replication-generated DSBs are special in the sense that, although two-ended DSBs can arise from TOP2 cleavage complexes, closely opposed strand breaks, or converging forks that process the same lesion from both sides, a large fraction of fork-collapse products are one-ended DSBs, generated when a fork collides with a leading-strand template nick, a TOP1 cleavage complex, or a cleaved fork structure[9,10]. These one-ended breaks remain embedded in a replication context: the lesion may retain or lose replisome components depending on the mode of fork collapse, and CMG fate can influence the structure presented for subsequent processing, so the repair substrate is shaped not only by the broken DNA end but also by the state of the disrupted replisome. Because a one-ended DSB lacks a matched second end at the same locus, simple ligation to an unrelated DNA end risks deletion, fusion, or translocation. A converging fork can subsequently alter this substrate architecture by generating a second end, creating a two-ended DSB context. The repair problem is then not only to seal a break, but to restore a template-directed replication structure.

The first determinative processing step for many one-ended DSBs is DNA end resection. The MRN (MRE11-RAD50-NBS1) complex and cyclin-dependent kinase (CDK)-phosphorylated CtIP initiate end processing in S/G2, after which longer-range resection is extended mainly by EXO1 or by the DNA2-BLM/WRN pathway[91-93]. This reaction converts a protein-bound blunt or near-blunt broken fork end into a 3' ssDNA overhang. RPA coats the exposed ssDNA to protect it from nucleases and activates ATR-CHK1 signaling, which promotes checkpoint activation, limits further origin firing, and helps stabilize the damaged fork environment[94-96]. Resection also suppresses end joining: BRCA1-BARD1 promotes resection and counteracts p53-binding protein 1 (53BP1)-RIF1-Shieldin-mediated end protection, while Ku prefers blunt ends and becomes less compatible with long 3' ssDNA overhangs as resection proceeds[97,98]. Resection functions as a pathway-choice determinant rather than simply HR initiation: it does not repair the fork by itself, but it prepares the broken end for recombination and reduces the chance that a one-ended break will be ligated to an inappropriate end.

After resection, the major template-directed route is BRCA-RAD51-dependent HR-mediated restart. In newly replicated chromatin, damage is signaled by H2A ubiquitination and nascent chromatin is marked by H4K20me0 enrichment. Both changes are recognized by BARD1 and recruit the BRCA1-BARD1 complex, helping bias repair pathways toward sister-chromatid-templated recombination[99,100]. BRCA1 then recruits PALB2, which then recruits BRCA2. BRCA2 replaces RPA with RAD51 and stabilizes the RAD51 filament on the resected 3' ssDNA[101,102]. With the help of RAD51 paralogs and RAD54, this filament invades the intact sister chromatid and forms a D-loop, using the broken 3' end to prime repair synthesis[103]. In this way, a functional replication structure can be regained through a homologous template, rather than by simply re-joining the broken end to another DNA end. Successful recombination uses the sister chromatid as template to restore chromosome integrity and allow replication to be completed, either by rebuilding a functional new fork or by extension from the invaded site until the region is replicated. This is a major replication-coupled repair pathway for restoring a broken fork after collapse.

If canonical BRCA-RAD51-mediated restart fails or is delayed, one-ended DSBs can be rescued by break-induced replication (BIR) or BIR-like synthesis. BIR is best studied in yeast as a recombination pathway in which one broken end invades a homologous template and drives extensive DNA synthesis through a migrating D-loop rather than through a normal bidirectional fork[17,104]. Mammalian cells also show BIR-like repair of collapsed forks, with factors such as RAD52, POLD3, PIF1, and MUS81 involved in some contexts[105,106]. This route can be useful because it allows a cell to finish replication in a template-directed way, preventing incorrect ligation to a distant end. However, it is not equivalent to canonical HR. Unlike replication fork restart or HR, in which the D-loop is locally stabilized and terminated, BIR involves long-tract D-loop-dependent migration, occasionally switches templates, and can

continue far beyond the lesion site. Long-tract synthesis and sometimes repeated template engagement increase the risks of template switching, mutation accumulation, loss of heterozygosity, copy-number alteration, and complex rearrangements[106]. Compared with fork restart, BIR-like repair is a salvage pathway that can carry substantial genomic costs: it can prevent some consequences of fork collapse, including inappropriate end-joining, but it does so by accepting a higher burden of genome instability.

End joining can be particularly hazardous for replication-generated one-ended DSBs. Canonical non-homologous end joining (c-NHEJ) can be appropriate in many conventional two-ended DSB contexts because, after the break, Ku70-Ku80 and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) can synapse two nearby ends immediately and hand them to LIG4-XRCC4 for ligation[107]. A one-ended DSB, however, has no correct partner end nearby. If c-NHEJ captures it, the available partner may be an unrelated broken fork, a non-sister chromatid, or a distant DNA end, producing deletions, translocations, dicentric chromosomes, or chromosome fusions[46]. Moreover, alternative end joining (alt-EJ), also referred to in different contexts as microhomology-mediated end joining (MMEJ) or POLθ-dependent end joining (TMEJ), provides another set of end-joining routes. The use of these terms varies with context, and they are not necessarily interchangeable, although many alt-EJ reactions involve resected DNA ends and exposed microhomologies, and MMEJ specifically depends on annealing of microhomologous sequences before ligation[108-110]. For one-ended DSBs, this adds another potentially mutagenic layer because alt-EJ may capture an inappropriate distal end, produce deletions, or cause rearrangements. Recent work further suggests that POLθ-dependent fork-MMEJ can act directly at broken replication forks and generate asymmetric deletions or templated insertions distinct from MMEJ operating at replication-independent two-ended DSBs, while ATR signaling appears to suppress this fork-MMEJ route while favoring resection-coupled BIR-like repair[47]. PARP1-dependent alternative end joining may also compete at some breaks, so PARP inhibition or PARP trapping can alter, rather than simply enhance, backup end-joining pathway usage[111]. For one-ended replication-generated DSBs, genome-preserving repair depends not only on activation of template-dependent recombination repair, but also on the efficient exclusion of end-joining pathways that can result in chromosomal rearrangement.

2.5 Lesion fate after different routing outcomes

The fate of replication-associated lesions and intermediates such as nicks, short gaps, BER/SSBR intermediates, stalled forks, and one-ended breaks is not determined by origin alone. As discussed above, the same lesion can be routed into different handling modes and repair pathways. The outcome depends on whether that routing restores the original DNA structure, delays resolution for later processing, repairs the lesion with tolerable scars, or induces cytotoxic genome instability when unresolved or toxic intermediates overwhelm the remaining repair and tolerance capacity.

2.5.1 Accurate completion

The most favorable outcome is the complete restoration of the original DNA structure. This can be achieved by correct processing of these lesions when the appropriate template and repair pathway are available. In this case, the original duplex is restored, the fork or chromosome structure is maintained, and little or no detectable sequence or structural alteration is transmitted to daughter cells. Checkpoint and DNA damage signals may appear during processing, but they remain transient and disappear once the intermediate is resolved[95,96].

2.5.2 Delayed resolution and salvage

Some lesions are not resolved during the S phase in which they arise. Instead, they are carried forward as protected gaps, stalled or reversed fork intermediates, under-replicated regions, or inherited lesions in daughter cells, often associated with 53BP1-positive nuclear bodies[112]. This outcome represents a controlled delay in lesion resolution. Such delayed intermediates can follow distinct later routes. Persistent or under-replicated DNA may undergo late or mitotic salvage processing, including MiDAS[48,51,66], whereas lesions transmitted into daughter cells can be protected or retained in 53BP1-positive nuclear bodies for processing in the next cell cycle[49,50,112]. These mechanisms are not equivalent to ordinary gap filling, and successful completion is not necessarily scarless. In particular, MiDAS can promote completion of under-replicated regions but may involve BIR-like or conservative synthesis and can be accompanied by structural alterations or genome instability[48,51,66].

2.5.3 Mutagenic completion

When accurate repair is unavailable or bypass is favored, the cell may still survive by accepting some local sequence errors. This fate includes lesion bypass or gap filling that leaves base substitutions, small indels, short conversion tracts, or local loss of heterozygosity (LOH). MMEJ and BIR repair products can also fall into this category because they often leave sequence-level alterations. In this case, repair is completed, but fidelity is sacrificed. Such processing may preserve cell viability while sacrificing sequence fidelity[113,114].

2.5.4 Structural genome scars

A broader-scale outcome is the formation of chromosome-scale scars. These arise when misrouted or unresolved intermediates are joined, copied, broken, or rearranged in ways that preserve cell viability but alter chromosome structure. The resulting scars include large deletions, duplications, copy-number variations (CNVs), inversions, translocations, chromosome bridges, micronuclei, and

chromothripsis-like rearrangements. Unlike local mutagenic completion, these outcomes involve larger-scale alterations of chromosome structure or copy number[115].

2.5.5 Cytotoxic genome instability

The final outcome occurs when the lesion burden or toxicity exceeds all remaining repair and tolerance capacity, compromising cell viability. This can occur when broken forks cannot be restarted, ssDNA accumulates beyond protection, trapped protein-DNA complexes block fork progression, degraded forks cannot be restored, or unresolved intermediates enter mitosis and lead to chromosome missegregation. At this point, routing no longer preserves genome integrity or viability. The consequences include replication catastrophe, mitotic catastrophe, apoptosis, senescence, or permanent proliferative arrest. Therapeutic killing by PARP inhibition, platinum treatment, or other replication-stressing agents takes advantage of these cytotoxic outcomes, illustrating that lesion fate depends not simply on the absence of repair but also on which processing pathways remain available and whether they preserve or compromise cell viability[116].

3. Therapeutic Implications and Perspectives

3.1 Therapeutic implications: Redirecting lesion routes

So far, DDR-targeted therapies have mainly been considered in terms of damage induction or repair inhibition. However, from a lesion-routing perspective, they may also impose routing pressure on replication-associated lesions. This idea is clearest for PARP inhibitors (PARPi), the most clinically established DDR-targeted drugs, which are used in BRCA-mutant or broader HR-deficient/platinum-sensitive ovarian, breast, pancreatic, and prostate cancers[117-119]. PARPi primarily inhibit PARP1 catalytic activity and may also alter PARP1/2 dissociation at DNA lesions, although the contribution and mechanism of PARP trapping remain debated; the resulting lesions may include persistent nicks, gaps, protein-DNA complexes, fork blocks and, in some contexts, transcription-replication conflicts[40,120]. Their cytotoxicity depends on whether cells can route these intermediates into fork protection, gap repair and HR, or instead accumulate toxic replication-associated DNA damage[121-124]. Along the same routing chain, PARPi resistance mechanisms can be distinguished by the level at which they act: at the downstream processing level, tumors may restore HR through reversion mutations[125], or lose 53BP1/Shieldin to release the block on DNA end resection[126,127], thereby restoring end resection and, in some BRCA1-deficient contexts, HR capacity; at the fork level, restored fork or gap protection reduces the conversion of lesions into toxic intermediates, while residual HR activity or incomplete HR deficiency also modulates drug sensitivity[124,128]; at the drug-target level, loss or mutation of PARP1 directly weakens drug engagement[129]. Together, these mechanisms suggest that resistance can reflect an adaptive rearrangement of lesion-routing pressure rather than a simple on-off switch of repair capacity.

A second class of strategies is still experimental or used mainly in combination therapy, which also targets replication-stress tolerance rather than the lesion itself. This category includes ATR, CHK1, and WEE1 inhibitors[123,130]. In normal S phase, ATR-CHK1 and WEE1 jointly restrain CDK activity and define the tolerance window for replication stress[131]. By shortening checkpoint-mediated delay, altering origin firing, and weakening replication-stress tolerance, inhibition of these kinases can convert lesions that might otherwise be stabilized, bypassed, or repaired into under-replicated DNA, fork breakage, or mitotic DNA damage. This tolerance window has a molecular boundary: once replication stress exceeds the coverage capacity of the RPA pool, global fork collapse can ensue[132]. More recently, PKMYT1, another CDK-restraining kinase beyond WEE1, has become a new target of this class: PKMYT1 inhibition is synthetic lethal with *CCNE1* amplification, forcing tumors under high replication stress into premature mitosis with unfinished DNA replication[133], and acts synergistically with ATR inhibition[134]. Whether directed at checkpoint kinases or CDK-restraining kinases, the shared logic of these strategies is to compress the tolerance window, shifting lesion routing from tolerance and repair toward collapse and mitotic catastrophe.

The third group, however, targets backup route dependence: when BRCA/RAD51-mediated HR or fork/gap protection is impaired, tumors may rely on POLQ/TMEJ, gap sealing and controlled nuclease processing. POLQ is among the best-validated of these dependencies: HR-deficient tumors depend on POLQ-mediated end joining for survival[135], and POLQ also seals post-replicative ssDNA gaps[110]; its inhibitors selectively kill BRCA-deficient and some PARPi-resistant tumors[136], and synthetic-lethal network analysis further suggests that a significant proportion of tumors may carry this dependency[137]. Beyond POLQ, RAD52, a backup homologous recombination/single-strand annealing factor, is synthetically lethal with BRCA2 deficiency, providing another targetable backup route for HR-deficient tumors[138]. The deubiquitinating enzyme USP1 represents a dependency at the fork/gap-protection level: BRCA1-deficient tumors rely on USP1 to maintain fork stability[139]; sensitivity to its inhibitors correlates directly with ssDNA gap accumulation[140]; and USP1 inhibition has overcome PARPi resistance in preclinical models[141] and entered early clinical trials[142]. At the nuclease level, the restraint of MRE11 activity by BRCA2 and PARP1 is a key element of fork protection[88], and MUS81 becomes essential for replication-stress tolerance in BRCA2-deficient cells[143]. Nuclease targeting remains largely preclinical[143] and requires careful toxicity and biomarker selection. Therapy response may also reflect routing bias rather than a simple repair-proficient or repair-deficient state. Defining which backup routes remain available in a given tumor may help guide biomarker selection, combination strategies, and approaches to overcome acquired resistance.

3.2 Conclusion and perspective

A key focus of the replication stress field still lies on lesion type and endpoint readouts. Many commonly used methods, including DSB accumulation, γH2AX formation, checkpoint activation, chromosome aberrations, and cell survival, are highly informative but are often endpoint measurements[144,145]. They can tell us whether replication-associated damage has accumulated or whether this damage affects cell survival, but they do not necessarily reveal how a lesion was routed into that outcome. Methodological innovations in recent years have already provided several new or improved tools that could help address this problem. SSB-seq can map single-strand nicks[146]; END-seq, breaks labeling *in situ* and sequencing (BLISS), and related methods can locate DSBs[147,148]; ssDNA gap-mapping strategies can detect post-replicative gaps[39]; DNA fiber and 5-ethynyl-2'-deoxyuridine (EdU) pulse-chase assays can follow fork progression and lesion bypass[149]; isolation of proteins on nascent DNA (iPOND) and chromatin-associated proteomics can identify proteins enriched at nascent DNA or stressed forks[150]; and live-cell reporters and inducible nickase or DSB-induction systems based on Cas9 or other endonucleases can add temporal and spatial control to defined lesions[10]. However, most of these approaches capture one state, one layer of interaction, or a limited time window. The next challenge is to develop approaches that combine structural, spatiotemporal, and functional information so that lesion transition, protection, bypass, repair, and their modulation can be monitored more directly. In this sense, "routing mapping" could help reveal how a nick, gap, stalled fork, or collapsed fork moves through alternative repair or tolerance pathways before it eventually resolves into a final local, genomic, or cell-level survival phenotype.

Unresolved questions still arise from this routing view. One central issue is what pushes a replication-associated strand lesion beyond the range of local SSBR and into a state that requires broken-fork/DSB-centered processing during replication. When a replication fork approaches, a template lesion may be repaired locally before fork encounter, whereas fork encounter may trigger bypass or repriming that leaves a post-replicative gap, induces fork remodeling, or cause structural conversion to a broken fork/DSB[40]. Of course, which of these outcomes occurs is likely influenced by lesion position, fork speed, chromatin context, cell-cycle stage, local protein availability and the balance between repair, protection and cleavage activities[28]. But is there any molecular "judge" for these route choices? Are these choices determined simply by competition, or sequentially attempted until one route works, or controlled by mechanisms that actively bias pathway choice? This question is especially important for separating the functions of BRCA1/2 and RAD51 in gap suppression or fork protection from their canonical roles in HR after DSB formation[84,151]. Similar distinctions are needed in PARP biology. PARP catalytic activity, PARP trapping, and interaction networks may all change the distribution of repair pathways, but they are unlikely to depend solely on lesion type[120]. A routing network may help explain why the same drug can produce different outcomes and which factors shape the final outcomes.

This perspective is also important for cancer biology and therapy. Tumors carrying different DDR deficiencies may differ in which routes they preferentially permit, restrict, or favor during lesion processing. Tumors with HR deficiencies, fork protection deficiencies, gap filling deficiencies, or different PARP activities may show distinct routing biases, even when they display similar endpoint markers of replication stress[152,153]. Understanding these biases could improve DDR-targeted drug selection and combination strategies: therapies may be designed to shift lesion processing away from tolerable routes toward more toxic ones. This strategy could also help to predict resistance that may arise later by restoring a blocked route or engaging an alternative tolerance pathway[154]. Thus, replication-associated strand lesions should be understood in a more dynamic way rather than as fixed entities. From this view, replication stress-related therapeutic strategies may eventually be monitored in a timely manner and adjusted as lesion routing shifts among different routes[155]. More broadly, the lesion-routing framework links structural lesion conversion, repair pathway engagement, and biological outcome across the course of DNA replication, providing a dynamic complement to conventional structure-based lesion classification.

Declarations

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Authors contribution

Yang Y: Investigation, writing-original draft, writing-review & editing.
Wen J: Investigation, visualization, writing-review & editing.
Wei S: Writing-review & editing.
Shao Z: Conceptualization, project administration, supervision, visualization, writing-review & editing.

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