Emerging insights into alternative end-joining: Mechanisms, genome instability and therapeutic opportunities in cancer (Review)

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Received June 18, 2025; Accepted September 2, 2025

DOI: 10.3892/ijo.2025.5809

Abstract. Genome instability is a central hallmark of cancer, driven by aberrant DNA damage responses that facilitate tumor evolution and resistance to therapy. Although canonical non-homologous end joining and homologous recombination are well-characterized pathways for repairing DNA double-strand breaks (DSBs), recent advances have revealed that cancer cells increasingly depend on alternative end-joining (alt-EJ) to survive persistent DNA damage that arises from intrinsic stresses or external therapies. Alt-EJ, characterized by its reliance on microhomologous sequences at DSB sites, promotes mutation accumulation and chromosomal rearrangements, thereby driving genomic instability and tumor progression. Despite its pivotal role in cancer biology, the molecular regulation, contextual determinants and dualistic role of alt-EJ in maintaining genome integrity compared with promoting instability remain incompletely understood. The present review integrated the latest mechanistic insights into alt-EJ, elucidated its regulatory networks and interactions with canonical DSB repair pathways and discussed its consequences for cancer genome integrity and evolution. Furthermore, it highlighted the emerging potential of alt-EJ as a therapeutic vulnerability for cancer, underscoring the urgent need to translate these discoveries into innovative treatment strategies aimed at overcoming therapy resistance and improving patient outcomes.

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Key words: alternative end-joining, DNA damage response, genome instability, cancer therapeutic targets, DNA double strand break repair

Contents

1. Introduction

The maintenance of genome integrity is fundamental to cellular homeostasis and organismal health, yet the genome is continually challenged by both endogenous and exogenous genotoxic stresses. DNA damage response (DDR) pathways orchestrate detection, signaling and repair of DNA lesions to preserve genomic stability. Among various types of DNA damage, DNA double-strand breaks (DSBs) represent the most lethal and deleterious lesions, with the potential to compromise chromosome integrity and cellular viability if unrepaired or misrepaired (1). Inefficient or erroneous DDR leads to genome instability, a hallmark of cancer that fuels tumor initiation, progression and therapeutic resistance (2,3). Germline mutations in key DDR genes, such as breast cancer gene (BRCA) 1/2 and ataxia-telangiectasia mutated (ATM), are known to predispose individuals to cancer by impairing efficient DSB repair, underscoring the crucial role of DDR in tumor suppression (4-7).

Cells incur tens of DSBs per day under physiological conditions, resulting from replication stress, oxidative damage and metabolic byproducts (8). In cancer cells, rapid proliferation and oncogene-induced replication stress exacerbate DSB accumulation, while therapeutic interventions such as ionizing radiation (IR) and chemotherapeutics further increase the burden of DSBs (9-11). Successfully evolving or surviving

cancer cells often exhibit enhanced or altered DNA repair competencies, which contributes to therapeutic resistance and disease relapse (12).

Classical DSB repair in human cells primarily involves non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ quickly rejoins broken DNA ends with minimal processing, while HR uses a homologous template for error-free repair, predominantly during S/G_2 phases (13,14). However, a third repair pathway, alternative end-joining (alt-EJ), has emerged as a distinct mechanism with a unique biological footprint, which is also referred to as microhomology-mediated end joining (MMEJ) or alternative NHEJ (alt-NHEJ) or theta mediated end joining (TMEJ) (15-19). Alt-EJ leverages microhomologous DNA sequence at DSB ends for annealing, frequently resulting in deletions, insertions or complex chromosomal rearrangements (1,20,21).

Alt-EJ was initially considered a backup or error-prone compensatory pathway used only when NHEJ or HR fails. However, a growing body of evidence in recent years has revealed that alt-EJ is actively engaged and tightly regulated in various contexts within cancer cells in particular (14,22,23). The increased reliance on alt-EJ by cancer cells is likely multi-factorial, reflecting intrinsic DDR alterations, replication stress adaptation and selective pressures from DNA-damaging therapies. Importantly, the inherent mutagenic potential of alt-EJ contributes not only to genome destabilization but also to tumor heterogeneity and evolution (15,22,24,25). Furthermore, a recent study has discovered that alt-EJ plays an important role in maintenance of extrachromosomal DNA in tumor cells (15).

Beyond oncogenic contexts, alt-EJ also operates in normal physiology. During immunoglobulin class switch recombination (CSR) in activated B cells, cytidine deaminase (AID) is induced and creates lesions in donor and acceptor switch (S) regions that are processed into DSBs by UNG and APE1 (26). While canonical NHEJ (c-NHEJ) mediates most joins, restriction of c-NHEJ factors or enhanced end resection diverts repair to alt-EJ (27,28). Alt-EJ mediated CSR exhibits a distinctive mutational signature: Increased microhomology at junctions with longer resection tracts, larger deletions within S regions, templated insertions consistent with POLQ mediated end joining and an elevated rate of inter chromosomal translocations (29-32). These features illustrate that alt-EJ is intrinsically mutagenic even in a programmed developmental setting.

Despite significant advances having been made in recent years, critical aspects of the molecular regulation of alt-EJ, the contextual cues that determine its engagement over canonical pathways and its precise effect on cancer genome landscapes remain incompletely defined. This gap hampers full exploitation of alt-EJ as a therapeutic target, although promising evidence has shown that inhibition of alt-EJ components sensitizes resistant tumors and enhances treatment efficacy (22,33,34).

The present review aimed to consolidate current knowledge of alt-EJ mechanisms, including the key factors and regulatory networks that control its activity; to dissect its dual roles in maintaining genome integrity compared with driving instability; and to evaluate its emerging contributions to cancer initiation, progression and therapeutic response. Furthermore, it highlight the therapeutic potential of targeting alt-EJ vis-à-vis recent advances in inhibitors and biomarkers that could transform cancer treatment paradigms.

2. Alt-EJ in DSB Repair

DSB repair mechanisms. DSBs are primarily repaired via three major pathways in cancer cells: NHEJ, HR and alt-EJ, with single-strand annealing (SSA) also used in certain circumstances (Fig. 1). These pathways differ in mechanism, repair fidelity and cell cycle regulation, collectively maintaining genome stability while balancing repair efficiency and accuracy (21,35-38).

NHEJ is the predominant DSB repair pathway throughout the cell cycle and is especially active during G_1 phase. It involves recognition of broken DNA ends by the Ku70/80 heterodimer, which recruits and activates the kinase of DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and Artemis to process DSB ends, followed by ligation with XRCC4-LIG4-XLF complex (39-41). NHEJ typically re-ligates breaks with minimal processing, which occasionally causes small insertions or deletions.

HR offers high-fidelity repair by using a homologous template, primarily in S/G_2 phases. The MRN complex (MRE11-RAD50-NBS1) recognizes DSBs, which activates ATM kinase, initiates MRE11-mediated resection and produces 3'single-stranded DNA (ssDNA) overhangs coated by RPA, facilitating replacement by RAD51 through BRCA1/2 mediation (13). This mediates strand invasion and template-directed repair via gene conversion or break-induced replication. HR deficiency (HRD) tumors usually show hypersensitivity poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi) (42-44).

SSA, although less common, uses extensive homology between repeat sequences flanking a DSB, which contributes to genome instability when dysregulated (38). Initiation of SSA requires extensive 5'-3' end resection producing long 3' (ssDNA) tails bound by RPA, followed by RAD52-mediated annealing of complementary repeats (45,46).

Molecular mechanisms of alt-EJ. Alt-EJ was first discovered in NHEJ-deficient cells and considered an alternate repair route for rejoining DSBs in the presence of compromised NHEJ and HR (47,48). At present, alt-EJ has gained prominence as a distinct, regulated pathway that contributes to both physiological repair processes and pathological genome instability, particularly in cancer. Alt-EJ utilizes short microhomologous sequences, typically 2-20 base pairs, near DSB ends to facilitate alignment and repair. Unlike NHEJ, which ligates DNA ends with minimal processing, or HR, which uses templates for error-free repair, alt-EJ requires DNA end resection and often results in deletions, insertions and chromosomal rearrangements, thereby contributing markedly to genomic instability in cancer (49,50).

The Alt-EJ process is initiated by rapid detection of DSBs and stabilization of broken DNA ends, predominantly mediated by PARP1, which recruits repair factors and modulates chromatin at break sites (51,52). The MRN complex, in concert with CtIP, catalyzes 5'-3' resection of the DNA ends, generating 3' ssDNA overhangs required for microhomology exposure (1,53). When microhomology sites are spaced farther

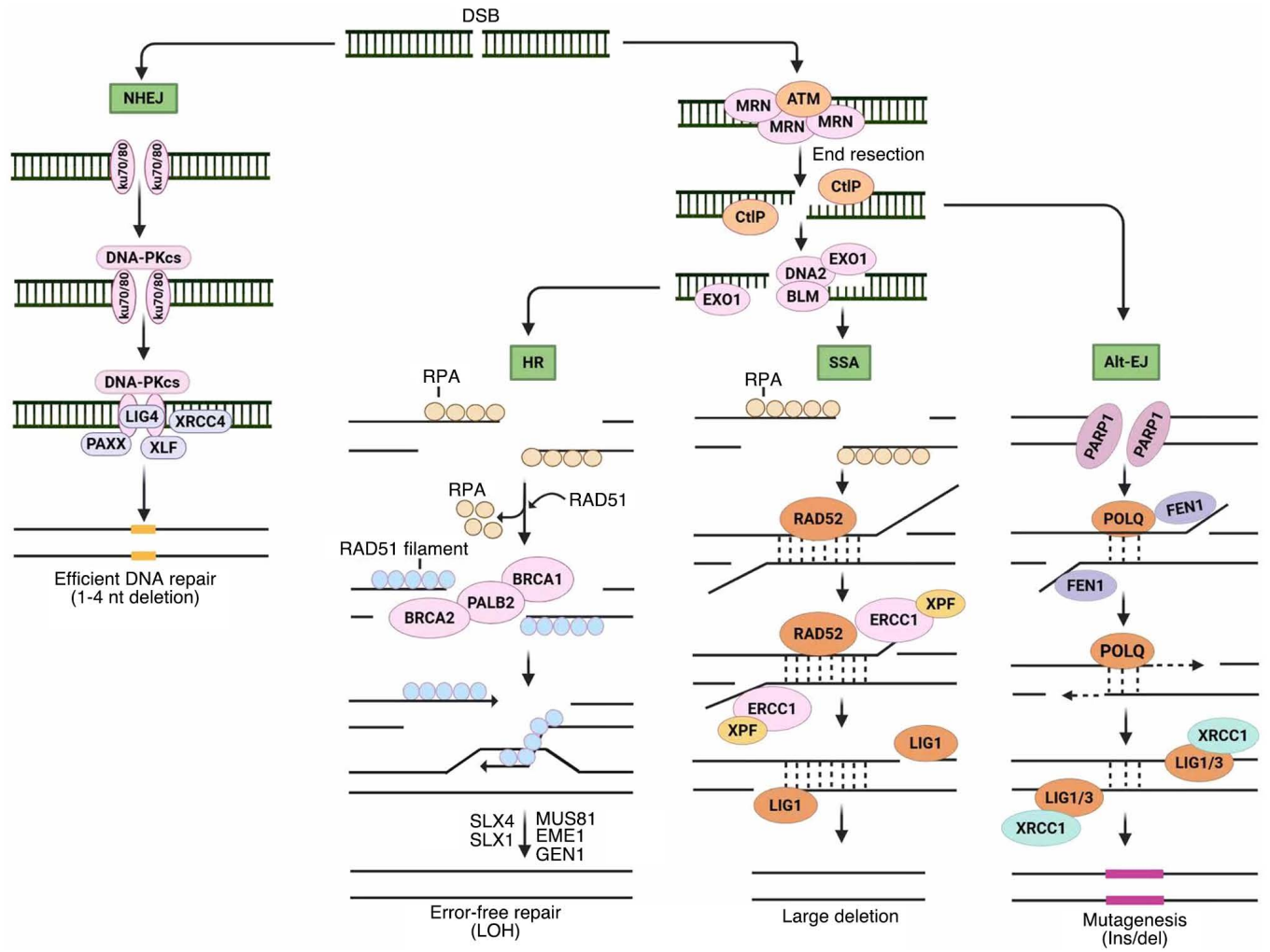

Figure 1. Major pathways for DSB repair. NHEJ begins with Ku70-Ku80 hetero-dimer attaching to DNA ends. DNA-PKcs recruitment and auto-phosphorylation bring the DNA ends together and allow ligation by XRCC4-LIG4 and XLF or PAXX. Resection by MRN complex and CtIP promotes homology-directed repair. Long-range resection creates RPA-coated ssDNA overhangs using BLM-DNA2 helicase-nuclease or EXO1 nucleases. HR occurs when BRCA1, PALB2 and BRCA2 facilitate loading RAD51 onto ssDNA and displace RPA. RAD51 nucleoprotein filaments invade the DNA-synthesis template sister chromatids. Alternatively, substantial resection generates a substrate for SSA, where RAD52 promotes homologous sequence annealing on each DNA end. ERCC1 and XPF handle 3'single-stranded flaps for LIG1-mediated DNA ligation. DSB resection also activates alt-EJ via PARP1, where POLQ anneals short homologous sequences, synthesizes DNA and re-ligates DNA ends using LIG1 or LIG3. DSB, DNA double-strand break; NHEJ, non-homologous end joining; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; ssDNA, single-stranded DNA; HR, homologous recombination; alt-EJ, alternative end-joining; PARP, poly (ADP-ribose) polymerase; XRCC4, X-ray repair cross-complementing protein 4; LIG4, DNA ligase 4; XLF, XRCC4-like factor; PAXX, paralogue of XRCC4 and XLF; CtIP, c-terminal-binding protein-interacting protein; RPA, replication protein A; BLM, Bloom syndrome helicase; DNA2, DNA replication helicase/nuclease 2; EXO1, exonuclease 1; BRCA1/2, breast cancer gene 1/2; PALB2, partner and localizer of BRCA2; SSA, single strand annealing; ERCC1, excision repair cross complementation group 1; XPF, xeroderma pigmentosum group F; POLQ, DNA polymerase theta; LIG1/3, DNA ligase1/3; Ins/del, insertion or deletion; LOH, loss of heterogeneity; nt, nucleotide.

apart, exonuclease 1 (EXO1) and Bloom syndrome helicase (BLM) extend this resection, producing longer ssDNA tracts (21,53,54). This extensive resection sharply distinguishes alt-EJ from NHEJ, which repairs minimally processed ends.

Alt-EJ exploits short stretches of microhomology exposed on the complementary 3' ssDNA overhangs for annealing. This step is inherently mutagenic, frequently generating nucleotide deletions or insertions at repair junctions (21,49). DNA Pol θ, encoded by POLQ, is the key mediator of this process, performing DNA synthesis that extends and stabilizes annealed microhomologies, thereby bridging the broken ends (55,56).

POLQ is unique in its domain architecture and consists of an N-terminal helicase-like domain and a C-terminal A-family polymerase domain linked by a central region. The domains both contribute to the end-joining activity of POLQ by stabilizing DNA synapses and catalyzing synthesis across partially paired or mismatched templates (18). POLQ's low-fidelity polymerase lacks 3'-5' proofreading and tolerates base mispairing, enabling it to efficiently extend from short microhomologies but often introducing mutations or templated insertions that constitute the signatures of alt-EJ repair (57,58). The final ligation step in alt-EJ is mediated by LIG1 or LIG3, in complex with XRCC1 (59-61).

Beyond POLQ, DNA polymerase λ (Polλ), an X-family polymerase typically involved in NHEJ, has been implicated in an alternative alt-EJ mechanism. Polλ promotes alt-EJ independently of canonical NHEJ factors by stabilizing DNA end

synapses with minimal base pairing and inserting nucleotides at gaps flanked by microhomologies of 4-6 base pairs. This polymerase acts on a range of substrates, including 5-12 nucleotide single-stranded overhangs and small gaps, expanding the diversity of polymerases supporting alt-EJ (62).

Following gap-filling synthesis by POLQ or Polλ, displaced 3' ssDNA flaps must be removed to generate proper DNA ends for ligation. The apurinic/apyrimidinic endonuclease APE2 has recently emerged as a critical nuclease in this process. APE2 exhibits 3'-5' exonuclease and flap endonuclease activities, enabling it to cleave 3' flaps generated during alt-EJ and facilitate end processing necessary for subsequent repair (63-65). Intriguingly, APE2 depletion sensitizes homologous recombination-deficient cells, highlighting its complementary role and synthetic lethality relationship to POLQ (63). This synergy underscores the importance of APE2 in maintaining genome stability by enabling POLQ-driven alt-EJ in contexts where NHEJ or HR is defective.

PARP1 recruitment is essential, particularly when NHEJ and HR are compromised, as it promotes assembly of alt-EJ factors at damage sites (23). POLQ is a central determinant of alt-EJ, not only for carrying out annealing and extension at microhomologies but also for antagonizing HR by preventing RAD51 filament formation (66-68). The 9-1-1 (RAD9A-RAD1-HUS1)/Rad9-Hus1-Rad1-interacting nuclear orphan (RHINO) complex also directs POLQ to DSBs during mitosis, highlighting the cell cycle-specific regulation of alt-EJ (69).

Although microhomology usage is often considered a defining feature of alt-EJ, numerous repair junctions formed by this pathway lack clear microhomologous sequences. For example, studies quantifying chromosomal aberrations suggest that up to 50% of events attributed to alt-EJ occur without detectable microhomology, even when they require POLQ or other essential alt-EJ components (18,70). This may reflect variability in DNA end resection, the influence of local sequence context, or the capacity of alt-EJ polymerases such as POLQ to facilitate end joining through template-independent synthesis (56,71). These findings indicate that alt-EJ is a mechanistically flexible and heterogeneous pathway and that reliance solely on microhomology as a marker may underestimate its role in genome instability and cancer.

Factors that affecting Alt-EJ. The efficiency and reliance on alt-EJ in cells, especially cancer cells, are shaped by a complex interplay between intrinsic genomic factors, regulatory molecules, cell cycle status and external signals (Fig. 2). Alt-EJ activity is governed by the degree of 5'-3' resection at DSB ends, initiated by the MRN complex with CtIP and further extended by nucleases/helicases such as EXO1 and BLM (53,72). This resection exposes microhomologies, with the length of ssDNA determining whether short (2-6 bp) or longer (>10 bp) microhomologies are used (73). Factors such as 53BP1 restrain resection, favoring classical NHEJ and loss or suppression of 53BP1 skews repair towards alt-EJ (74). Deficiencies in other factors, such as the ssDNA-binding protein RPA, can also potentiate alt-EJ by increasing the use of longer microhomologies and promoting chromosomal rearrangements (73,75). Moreover, components mutated in Fanconi Anemia (FA), a disorder characterized by defective interstrand crosslink (ICL) repair, have been implicated in modulating alt-EJ activity, suggesting cross-talk between replication stress responses and alt-EJ (23,76,77).

Alt-EJ is also subject to regulation by extracellular signals, notably TGFβ. Upon activation in the tissue microenvironment, TGFβ ligands bind to heteromeric complexes of serine/threonine kinase receptors, TGFβ receptor type I and type II, initiating downstream phosphorylation cascades primarily mediated by receptor-regulated suppressor of mother against decapentaplegic (Smad) proteins (Smad2, Smad3 and Smad4) that translocate to the nucleus, or by non-canonical TGFβ pathways (Fig. 2). These TGFβ signaling transduction pathways orchestrate a broad array of biological functions that govern cell cycle, differentiation, apoptosis and importantly, DDR pathways (25,78,79). Active TGFβ signaling supports ATM-dependent DDR and suppresses alt-EJ gene expression (80). Conversely, TGFβ pathway inhibition, frequently observed in the tumor microenvironment or certain viral infections, upregulates alt-EJ components (POLQ, PARP1 and LIG1) and increases reliance on this mutagenic pathway, leading to more frequent chromosomal aberrations (25,81). Mechanistically, this effect is partly mediated by the microRNA miR-182, which is upregulated upon TGFβ inhibition and acts to repress key HR and NHEJ repair effectors, thereby shifting repair pathway choice towards alt-EJ (24).

Oncogenic viruses such as human papillomavirus (HPV) further hijack the alt-EJ machinery. A critical step in HPV-mediated oncogenesis is the integration of viral DNA into the host genome, which not only ensures persistent viral replication but also promotes genomic instability and malignant transformation. Parfenov *et al* (82) performed comprehensive genomic profiling of 279 head and neck squamous cell carcinoma (HNSCC) specimens using next-generation sequencing techniques and found that ~60% of HPV integration events occurred within regions characterized by microhomology at the junction sites. Since microhomology usage is a hallmark of alt-EJ, the authors' study suggests that HPV exploits this error-prone repair pathway to facilitate viral insertion and tumorigenesis.

This hypothesis is supported by several mechanistic studies demonstrating elevated alt-EJ activity in HPV-positive cancers (Fig. 2). For example, Liu *et al* (24) reported markedly higher expression of key alt-EJ factors in HPV-positive HNSCC compared with HPV-negative tumors. Functional assays in that study showed that HPV-positive tumor cells preferentially rely on PARP1-dependent alt-EJ for repairing DSBs, highlighting a shift in DSB repair pathway choice induced by HPV infection. Furthermore, Liu *et al* (25) analyzed transcriptomic data from The Cancer Genome Atlas (TCGA) that comprised 243 HPV-negative and 36 HPV-positive HNSCC samples, where the authors found unsupervised clustering based on alt-EJ gene signatures distinctly segregated HPV-positive tumors into a cluster characterized by high expression of alt-EJ-associated genes. Similarly, Guix *et al* (79) validated this signature in patient-derived xenografts and primary tumor tissues via NanoString assays, finding that HPV-positive samples exhibited an upregulated alt-EJ signature. Independent validation by Leeman *et al* (83) also demonstrated that HPV infection increases the frequency of DNA deletions flanked by microhomology by alt-EJ. The authors' mechanistic dissection

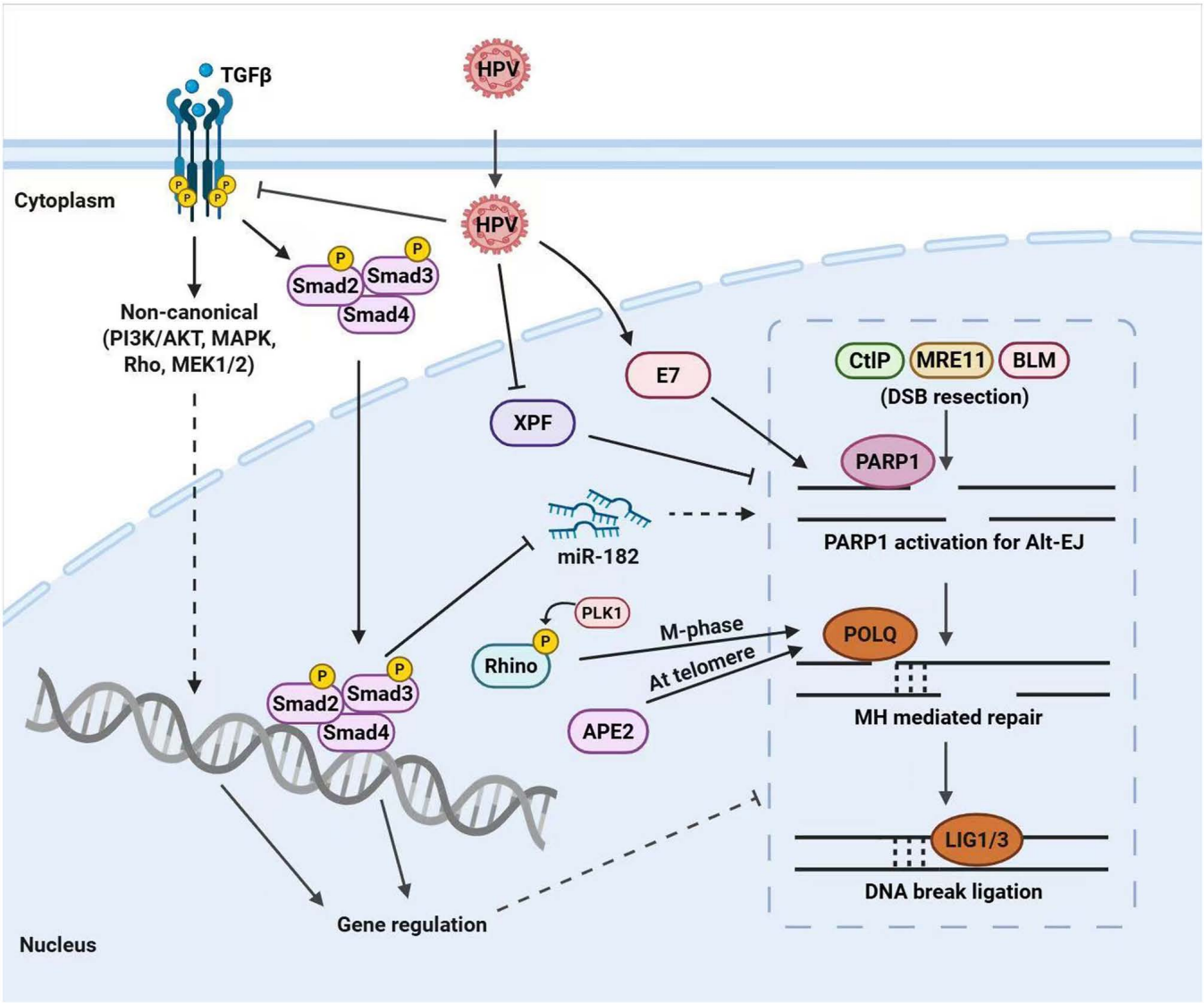

Figure 2. Schematic illustration of alt-EJ effectors. The alt-EJ pathway is influenced by several factors. TGFβ signaling begins in the cytoplasm and activates Smad proteins and non-canonical pathways to regulate DDR through miR-182 and other mechanisms (24,25,78). Loss of TGFβ signaling, such as with the effects induced by HPV, causes enhanced alt-EJ activity (25,79). The HPV oncoprotein E7 directs DNA repair to alt-EJ (83). PLK1 facilitate RHINO to accumulate in the M phase recruit POLQ to the break site (69). APE2 interacts with POLQ in an epistatic way for alt-EJ (63). CtIP, Mre11 and BLM proteins regulate DNA end resection, which subsequently facilitates the process of strand annealing and the coupling of fragmented DNA ends through the annealing of microhomologies. alt-EJ, alternative end-joining; DDR, DNA damage response; TGFβ, transforming growth factor-beta; Smad, suppressor of mothers against decapentaplegic; miR, microRNA; HPV, human papillomavirus; PLK1, polo-like kinase 1; APE2, apurinic/apyrimidinic endodeoxyribonuclease 2; RHINO, Rad9-Hus1-Rad1-interacting nuclear orphan; POLQ, DNA polymerase θ; CtIP, c-terminal-binding protein-interacting protein; MRE11, Meiotic recombination 11; BLM, Bloom syndrome helicase; PARP, poly (ADP-ribose) polymerase.

revealed that the HPV oncoprotein E7 promotes a shift in DSB repair pathway choice toward alt-EJ. The aforementioned evidence suggests the existence of a strategy wherein a virus hijacks alt-EJ in its host for its oncogenic agenda.

Crosstalk between alt-EJ and other DSB repair pathways. The interplay between alt-EJ and the canonical DSB repair pathways, HR and NHEJ, reflects the remarkable plasticity of the cellular DNA damage response. Disruption or inhibition of HR or NHEJ frequently redirects DSB repair toward alt-EJ, suggesting that alt-EJ is a flexible and adaptable mechanism, which may be exploited by cancer cells to maintain survival under genotoxic stress (24,84). Studies have revealed that the regulation of alt-EJ is a dynamic and multifaceted process involving complex coordination between numerous signaling pathways and repair proteins, including TGFβ, PARP1 and POLQ and factors involved in DNA end resection (22,62,85). Such regulation is critical because dysregulated repair pathway choice can profoundly affect genome stability, with significant implications for tumorigenesis and therapeutic resistance.

Although the alt-EJ mechanism was originally described as a backup pathway to NHEJ and HR, emerging evidence now positions alt-EJ as an independent and potentially competitive repair mechanism that operates even in the presence of intact HR and NHEJ pathways (1,86). Alt-EJ is preferentially engaged during the S and G_2 phases of the cell cycle when limited DNA end resection has occurred, contributing 10-20% of DSB repair activity in various mammalian cell contexts (48). This

is particularly salient in conditions of replication stress, where alt-EJ facilitates rapid repair of collapsed replication forks, balancing between the cytotoxic risk of error-prone repair and the cellular imperative to maintain genomic integrity.

A key nexus of crosstalk exists between HR and alt-EJ centers on POLQ, a multifunctional enzyme with both DNA polymerase and N-terminal helicase domains endowed with ATPase and DNA unwinding activities (50,57,87,88). POLQ's helicase domain mediates critical molecular antagonism of HR by binding RAD51 and thereby preventing RAD51 nucleoprotein filament assembly on RPA-coated ssDNA, a prerequisite for HR-mediated strand invasion. This inhibitory interaction facilitates the suppression of HR and promotes alt-EJ, effectively channeling repair towards a more error-prone pathway (68,88). The interaction is structurally mediated by a disordered central domain within POLQ (residues 847-894) that is essential for RAD51 binding and displacement, thus highlighting a direct molecular mechanism for pathway choice regulation (68,89). However, the question of whether POLQ's ATPase activity regulates the stability and dynamics of RAD51 filaments remains to be elucidated.

The synthetic lethal relationship between HRD and alt-EJ dependency further exemplifies their functional interplay. HR-deficient tumors rely heavily on alt-EJ for repairing complex DSBs, especially those that arise from replication fork collapse, events characterized by single-ended DSBs that cannot be repaired by classical NHEJ due to the absence of a second DSB end (90). Therefore, POLQ-mediated alt-EJ is potentially vital in bridging ssDNA gaps formed during replication stress and fork collapse, particularly important in HRD contexts or following treatment with PARPi (85,91,92).

Single-ended DSBs typically arise when an unrepaired single-strand break is converted into a DSB, leading to replication fork collapse. It is also important to appreciate that single-ended DSBs, the predominant form of DSB in unperturbed cells due to unrepaired single-strand breaks converted into DSBs during replication, are primarily repaired by HR (93,94). Alt-EJ repair of single-ended DSBs tends to be detrimental, often causing chromosomal abnormalities and increased genome instability and is thus suppressed under normal conditions to preserve genomic integrity (94). However, alt-EJ can act as a salvage pathway in HR-compromised scenarios, albeit at the cost of increased mutagenesis and chromosomal rearrangements (90). This delicate balance illustrates an intricate regulatory network where repair pathway choice is tightly controlled to limit deleterious outcomes while facilitating survival.

3. Alt-EJ effects in genome instability

Gene mutations and genomic scars. Alt-EJ often introduces insertions and deletions (indels) at DSB repair sites. This pathway not only fosters genetic alterations within coding regions but also leaves distinctive 'genomic scars'. These scars frequently involve microhomology regions that facilitate end alignment prior to repair, leading to characteristic deletions, rearrangements and templated or non-templated insertions, which can be detected as a genomic signature for alt-EJ activity.

In the context of cancer, the mutagenic potential of alt-EJ is especially significant. For example, loss of TGFβ signaling increases alt-EJ in pan-cancer, which lead to increased genomic alterations (25). Notably, a subset of types of cancer with upregulated alt-EJ has shown a specific indel mutation signature, characterized by >5 base pair deletions and overlapping microhomology at deletion boundaries (25). Furthermore, in BRCA-mutant tumors, increased reliance on alt-EJ not only drives tumor progression but also engenders specific mutational signatures that can be exploited for diagnosis or therapeutic targeting. These mutational signatures include recurrent deletions featuring microhomology at breakpoint junctions and complex rearrangements that impact genome stability. Such patterns serve as genomic scars indicative of defective HR repair and are associated with increased sensitivity to PARPi (95-98).

POLQ facilitates annealing of microhomologous regions and contributes to the characteristic junctional mutations. In a *C. elegans* study, the results analyzing ~7,000 deletion breakpoints demonstrated that POLQ-dependent alt-EJ accounts for a significant portion of mutations induced by alkylating agents such as ethyl methane sulfonate and UV/TMP treatments (95). These breakpoints often include small deletions due to microhomology and show inserted DNA sequences, either copied from close (templated) or newly added (non-templated) region.

The frequent insertion of short sequences at breakpoints can be attributed to POLQ's terminal transferase activity and its propensity for template switching processes that generate diverse repair signatures, including partial ssDNA intermediates (99). This accumulation of ssDNA renders cells more susceptible to further damage or mutations, especially under conditions of external genotoxic stress or endogenous replicative stress. Alt-EJ leads to exposure of ssDNA regions and increased opportunities for nucleotide misincorporation or further damage. Notably, hypermutagenesis tends to extend over a defined physical distance (~7-9 kb) from the breakpoints, indicating that error-prone repair is not confined solely to immediate junctions but can propagate along adjacent DNA regions (100). Notably, this hypermutability appears to be an intrinsic feature of alt-EJ and not solely dependent on POLQ activity, suggesting that the pathway's inherent mechanics predispose to extensive mutagenic outcomes.

Chromosomal aberrations and translocations. Alt-EJ often results in chromosomal aberrations and translocations. In *Saccharomyces cerevisiae* (budding yeast), the presence of multiple simultaneous DSBs increases the likelihood of promiscuous end joining via alt-EJ, generating chromosomal translocations and complex rearrangements (101). This finding highlights the potential for alt-EJ to misrepair disparate DNA ends. Consistently, a high frequency of microhomology at translocation junctions has been detected in human tumor cells, supporting a mechanistic link between alt-EJ and chromosomal rearrangements in cancer (102,103). This microhomology enrichment at breakpoints contrasts with the more blunt-end ligation characteristic of NHEJ, underscoring the distinct repair signature of alt-EJ. Furthermore, loss of TGFβ signaling increases IR-induced chromosome aberrations in HNSCC cells, but knock-down of POLQ suppresses the effects, suggesting a critical role for alt-EJ (24). Cisplatin

treatment also induces chromosomal aberrations, especially in human papillomavirus (HPV)-positive HNSCC with preference for alt-EJ (76).

To investigate the direct role of alt-EJ in chromosomal translocation formation, studies induced targeted DSBs on distinct chromosomes using site-specific nucleases such as CRISPR-Cas9 or I-SceI endonuclease (104,105). Experiments performed in both murine and human cells deficient in NHEJ factors demonstrated that loss of key NHEJ components, notably XRCC4, substantially elevated the frequency of reciprocal translocations up to fivefold compared with wild-type controls (104,105). Furthermore, microhomologies were observed at ~60% of these breakpoint junctions, strongly implicating alt-EJ in the formation of the chromosomal aberrations. Beyond XRCC4, other proteins critical to alt-EJ function, such as CtIP, which initiates DNA end resection, and LIG3, which catalyzes the final ligation, have been shown to influence chromosomal rearrangement frequencies. For example, mouse cells depleted of CtIP or LIG3 exhibit reduced alt-EJ activity and corresponding decreases in chromosomal aberrations, indicating that a functional alt-EJ machinery is required for these instability phenotypes (70,106).

Telomere fusions. Telomeres, the specialized nucleoprotein structures capping chromosome ends, are essential for preserving genomic integrity by preventing chromosome ends from being recognized as DSBs. However, critically short or dysfunctional telomeres that result from replicative attrition or loss of protective factors become 'uncapped', exposing chromosome ends as DNA breaks vulnerable to erroneous repair pathways. In these contexts, alt-EJ plays a significant role in driving telomere fusions, which contribute to profound genome instability. Indeed, telomere fusion events mediated by alt-EJ have been observed in aggressive cancers including glioblastomas, where they correlate with poor prognosis and increased genomic chaos (107-109).

Recent investigations have provided critical mechanistic insights into alt-EJ mediators in telomere fusion. For example, one study that used mouse embryonic fibroblasts lacking TRF2 and Ku demonstrated robust NHEJ-independent telomere fusions that are highly dependent on POLQ and this POLQ-dependent fusion process is sensitive to inhibition by PARPi, highlighting a potential therapeutic vulnerability in tumors reliant on alt-EJ for telomere maintenance (69). Importantly, the same study revealed that APE2 acts as an epistatic partner of POLQ in executing these fusion events, as simultaneous depletion of POLQ and APE2 did not further reduce telomere fusions compared with single depletions. These findings not only strengthen evidence that POLQ is a central player in alt-EJ-mediated telomere fusion but also identify APE2 as a novel cooperating factor in this pathway.

Consistent with the pronounced effects of alt-EJ in genome instability, recent discoveries have also increasingly linked aberrant activation of alt-EJ to cancer development, progression and therapeutic resistance. In HRD tumors, such as those harboring BRCA1/2 mutations, reliance on alt-EJ fosters the accumulation of microhomology-associated deletions and templated insertions, generating genomic scars that drive tumor evolution and heterogeneity (25,55,95,98). These genomic features are associated with poor clinical prognosis and have been proposed as predictive biomarkers for response to genotoxic therapies and PARPi. Large-scale pan-cancer transcriptomic analyses have further revealed that tumors with elevated alt-EJ activity frequently exhibit high expression of POLQ and PARP1, correlating with increased chromosomal instability and resistance to standard treatments (22,25,68,98). Together, these findings support the emerging view that alt-EJ is an active contributor to malignant transformation. Understanding these oncogenic consequences of alt-EJ and factors that affect alt-EJ activity are critical for harnessing its components as potential diagnostic markers and novel therapeutic targets in cancer.

4. Potential cancer targets in Alt-EJ

DDR contributes to cancer cell resistance to genotoxic therapies. Numerous cancers exhibit an abnormal dependence on alt-EJ, creating unique therapeutic vulnerabilities that can be exploited by targeting DDR components involved in alt-EJ (Table I) (110,111). Among alt-EJ factors, PARP1 stands out as a well-validated and promising target in oncology. Multiple PARPi have been FDA-approved or are currently in clinical trials for diverse types of cancer, particularly tumors harboring HRD (112-114). PARPi exemplify synthetic lethality, where cancer cells defective in HR, commonly due to BRCA1/2 mutations in breast and ovarian cancers, become selectively vulnerable to PARP1 inhibition. Beyond BRCA mutations, emerging evidence reveals that defects in multiple DDR components, including ATM and SMAD4, also confer hypersensitivity to PARPi (115,116).

In HNSCC, functional deficiencies in key DSB repair genes such as DNA-PKcs and BRCA2, particularly in HPV-positive tumors, sensitize cells to PARPi. Weaver *et al* (117) demonstrated that PARP inhibition with veliparib impaired cell survival and delayed tumor growth by exploiting these DDR deficiencies. Furthermore, metastatic castration-resistant prostate cancer with frequent HR pathway alterations has been increasingly targeted with PARPi in clinical studies (118).

Due to the importance of PARP1 in DSB repair, especially for alt-EJ, PARPi are expected to have synergistic effects with IR. In addition to DSBs, IR also generates other types of DNA damage, such as single strand break (SSB) or base lesions. PARPi are able to convert these DNA lesions to DSBs, thus increasing the DSB load and producing synergistic effects with radiotherapy (119). Indeed, PARPi-enhanced therapeutic efficacy has been detected across multiple solid tumors (116,118,120,121). These synergistic effects are detected not only in tumors treated with low linear energy transfer (LET) IR such as X-rays, but also in those treated with high-LET particle radiation. It is well established that high-LET IR induces complex clustered DNA damage comprising both SSBs and DSBs and alt-EJ may play an important role in the repair of these complex DNA damages (122). PARPi enhances the radiosensitivity of cancer cells exposed to particle irradiation in various studies (123-126). Combinatorial PARP1-targeting strategies are also quickly advancing. For example, co-targeting PARP1 and cell cycle checkpoint kinases such as CHK1 or Wee1 displays differential radiosensitization related to HPV status: PARP1 plus CHK1 inhibition enhances radiosensitivity in

Table I. Inhibition effects of alt-EJ targets in cancer.

First author/s, year	Target	Inhibitor	Cancer type	Therapeutic effects	(Refs.)
Jagsi *et al*, 2018	PARP1	Veliparib	Breast Cancer	Increases radiosensitivity in breast cancer patients	(121)
de Bono *et al*, 2020	PARP1	Olaparib	Prostate cancer	Improve progression-free survival in patients with metastatic castration-resistant prostate cancer	(147)
Liu *et al*, 2018	PARP1	Olaparib	Bladder cancer	Increases radiosensitivity in cancer cell line	(116)
De Haan *et al*, 2019	PARP1	Olaparib	Breast cancer, NSCLC, HNSCC	Enhances radiosensitivity in phase1clinical trial of the patients	(120)
Weaver *et al*, 2015	PARP1	Veliparib	HNSCC	Reduces cell viability and mouse xenograft tumor growth *in vitro*	(117)
Wang *et al*, 2020	PARP1	Niraparib	HNSCC	Increases radiosensitivity in cancer cell lines and increased proton vs. photon RBE	(148)
Zhou *et al*, 2021	POLQ	Novobiocin	Breast cancer, ovarian cancer	Enhances chemosensitivity to PARPi in cancer cell lines and mouse xenograft and patient derived xenograft model	(131)
Zatreanu *et al*, 2021	POLQ	ART558	Breast cancer	Enhances synthetic lethality and the effect of PARPi in BRCA1-mutant cell *in vivo* and *in vitro*	(132)
Rodriguez-Berriguete *et al*, 2023	POLQ	ART558 ART899	Colorectal cancer, NSCLC, bladder cancer	Increases the radiosensitivity of cancer cell lines and mouse xenograft model	(130)
Fried *et al*, 2024	POLQ	RTx-161	Breast cancer	Induces synthetic lethality and enhances the effect of PARPi in HRD cells	(133)
Hossain *et al*, 2021	APE2	Celastrol	Pancreatic cancer	Enhances the effect of chemotherapy drugs	(145)
Chen *et al*, 2008	LIG1/3	L67, L82, L189	Breast cancer, colon cancer	Enhances radiosensitivity and cytotoxicity to DNA damaging agent	(140)
Tobin *et al*, 2013	LIG1/3 PARP1	L67 NU1025	CML	Enhances combine sensitivity of DNA repair inhibitors	(143)
Liu *et al*, 2018	TGFBRI PARP1	LY2157299 Olaparib	HNSCC	Increases radiosensitivity of a mouse tumor model	(24)
Hintelmann *et al*, 2021	PARP1 Weel	Olaparib Adavosertib	HNSCC	Increases radiosensitivity to cancer cell	(149)
Zuo *et al*, 2023	XPF PARP1	F06 Olaparib	HNSCC	Induces chemosensitivity in a mouse tumor model	(76)
Molkentine *et al*, 2021	PARP1 Chk1 Weel	Niraparib MK-8776, MK-1775	HNSCC	Increases radiosensitivity to cancer cell and xenograft tumor model	(127)

alt-EJ, alternative end-joining; PARP, poly (ADP-ribose) polymerase; POLQ, DNA polymerase θ; APE2, apurinic/apyrimidinic endonuclease 2; XPF, xeroderma pigmentosum complementation group F; Chk1, checkpoint kinase 1; NSCLC, non-small cell lung cancer; CML, chronic myeloid leukemia; HNSCC, head and neck squamous cell carcinoma; RBE, relative biological effectiveness; HRD, homologous recombination deficiency.

HPV-positive cells, whereas PARP1 plus Wee1 inhibition is more effective in HPV-negative tumors (127). Zuo *et al* (76) found that, due to the increased reliance of XPF-deficient HNSCC on alt-EJ to repair ICLs, inhibiting both PARP1 and the endonuclease XPF enhanced the effects of cisplatin on HPV-negative HNSCC *in vitro* and *in vivo*. Another recent study has revealed that polymerase α (POLA1) inhibition, in combination with PARP inhibition, synergizes to increase replication stress and DSB accumulation in BRCA1-deficient backgrounds, sensitizing cancer cells to PARPi treatment (128). This suggests that disruption of replication processes can further compromise genome stability, amplifying alt-EJ dependency. Such findings underscore the importance of personalized strategies that account for tumor genotype and DDR pathway context, for which alt-EJ addiction is an important factor.

The clinical success of PARPi has firmly established the potential of targeting alt-EJ in cancer therapy. Beyond PARP1, POLQ has emerged as a critical, targetable player central to the alt-EJ mechanism. Results from preclinical studies have demonstrated that POLQ inhibitors have significant anti-tumor effects in DDR-deficient contexts or under DNA damage overload during genotoxic treatments (129,130). One breakthrough in POLQ-targeted therapy came with the identification of novobiocin, an antibiotic repurposed as a selective POLQ inhibitor through high-throughput small-molecule library screening. Novobiocin directly binds the ATPase domain of POLQ, blocking its recruitment to DNA damage sites and inhibiting alt-EJ repair (131). Functionally, novobiocin preferentially induces the death of BRCA-deficient cells and synergizes with PARPi to exacerbate DNA repair defects. Notably, novobiocin suppresses tumorigenesis in BRCA1-deleted triple-negative breast cancer mouse models and in patient-derived xenografts that have developed resistance to PARPi therapy as a consequence of 53BP1 loss, highlighting that POLQ inhibition may overcome the resistance mechanisms.

Similarly to novobiocin, the investigational compound ART558 demonstrates nanomolar affinity for POLQ and selectively inhibits proliferation in BRCA2-deficient cancer cells *in vitro* (132). ART558 also effectively targets PARPi-resistant BRCA1-null cells and organoids, a trait shared with the next-generation POLQ inhibitor ART812, which has offered improved bioavailability and pharmacokinetics in animal models. ART812 markedly suppresses growth of PARP inhibitor-resistant BRCA1-knock-out xenografts, reinforcing the clinical translation potential of POLQ inhibitors for overcoming therapeutic resistance.

More recent advances include a new class of POLQ polymerase-specific inhibitors, represented by RTx-161 and RTx-152, which exhibit highly potent inhibitory activity (IC_{50} values of 4-6 nM) and demonstrate broad efficacy in eliminating HRD tumors, as well as overcoming PARPi resistance in genetically diverse backgrounds, including some HR-proficient tumors (133). These inhibitors differ mechanistically by targeting the polymerase function rather than the ATPase activity, effectively trapping POLQ on DNA and preventing repair completion.

Beyond monotherapy, POLQ inhibition shows considerable promise as a radiosensitizing strategy. Preclinical evidence indicates that POLQ inhibitors such as ART558 and its derivative ART899 potentiate the cytotoxic effects of IR across diverse tumor models, especially under hypoxic or S-phase-enriched conditions common in solid tumors, where conventional therapies often falter or resistance emerges (134). Encouragingly, a Phase I clinical trial (NCT04991480) is currently underway to evaluate safety and efficacy of combining POLQ inhibition with radiotherapy, which may open new avenues for more precise and less toxic cancer treatments (130).

Synthetic-lethal interactions between POLQ and key DDR genes (for example, BRCA1, BRCA2 and ATM) have been detected in *in vitro* and *in vivo* models, emphasizing the therapeutic potential of targeting POLQ in genetically defined cancer populations (56,68,135). Despite these advances, further investigations are imperative to deepen our mechanistic understanding of POLQ-mediated alt-EJ, optimize inhibitor potency and refine biomarkers to predict treatment response. Such efforts will ultimately bridge molecular insights with clinical oncology, thereby enhancing personalized medicine approaches that exploit alt-EJ dependence for cancer therapy.

Although PARP1 and POLQ remain the most extensively studied therapeutic targets within the alt-EJ pathway, several other factors integral to alt-EJ-mediated DSB repair have emerged as promising candidates for cancer therapy. Flap endonuclease 1 (FEN1) is an essential nuclease that processes 5'-flap structures generated during DNA end resection and strand displacement synthesis within alt-EJ (136,137). FEN1's activity is crucial for the removal of displaced DNA flaps that otherwise hinder efficient alt-EJ. Loss of FEN1 function critically impairs alt-EJ efficiency, paralleling the loss of POLQ in HRD cells and resulting in synthetic lethality (56,68,136). Importantly, novel FEN1 inhibitors, structurally related to hydroxyurea derivatives, have demonstrated selective cytotoxicity against HRD cancer cells without substantial toxicity to normal cells, underscoring their therapeutic potential (56,68,136).

FANCD2, a central player in the FA pathway, have been identified in synthetic lethal interactions with POLQ function. FANCD2 also promotes POLQ recruitment to DNA breaks, facilitating alt-EJ activation (138). Preclinical studies have demonstrated that dual depletion of POLQ and FANCD2 markedly sensitizes cancer cells to cisplatin and PARP inhibitors, particularly in lung and ovarian cancer models. For example, short interfering RNA-mediated co-suppression of POLQ and FANCD2 resulted in markedly increased cell death and tumor volume reduction in xenograft models, highlighting the synthetic lethal potential of targeting this axis (68,139).

DNA ligases, particularly LIG1 and LIG3, are critical components of the alt-EJ pathway. Some years ago, Chen *et al* (140) reported that structure-based drug design led to the development of small molecules that inhibit human DNA ligases by targeting their DNA-binding domains. For instance, compounds such as L82 selectively inhibit LIG1; L67 inhibits both LIG1 and LIG3; and L189 targets LIG1, LIG3 and LIG4. These inhibitors have demonstrated efficacy *in vitro* by impairing DNA repair processes. In cell-based studies, L67 and L189 exhibited cytotoxicity and synergized with DNA-damaging agents, particularly in cancer cell lines, highlighting their potential to sensitize tumors to chemotherapy.

LIG1 has been identified as a synthetic lethal target in cancers harboring BRCA1 mutations. Using CRISPR/Cas9 screening and validation assays, researchers have shown that BRCA1-mutant cells depend heavily on LIG1 activity for survival, whereas BRCA1/2 wild-type cells are less affected. Notably, LIG1's catalytic function is essential for this dependency, suggesting that its inhibition disrupts sealing of single-strand DNA nicks during alt-EJ. Depletion of LIG1 leads to accumulation of unrepaired DNA nicks and results in tumor stasis in xenograft models. These findings demonstrate that targeting LIG1 can exploit the synthetic lethality associated with BRCA1 deficiency, making it a promising therapeutic avenue in such malignancies (141).

In addition, resistance to tyrosine kinase inhibitors such as imatinib in chronic myeloid leukemia, as well as resistance to endocrine therapies in certain breast cancers, have been linked to reliance on the alt-EJ pathway. Resistant cells often exhibit elevated levels of PARP1 along with DNA ligases,

especially LIG3. Combined inhibition of PARP1 and LIG3 impairs alt-EJ-mediated repair, markedly reducing the survival of these therapy-resistant cancer cells. Importantly, the degree of sensitivity to this combination correlates with the expression levels of PARP1 and ligases in both cell lines and patient-derived samples, supporting their potential as biomarkers and therapeutic targets (142,143).

Another player recently implicated in alt-EJ is APE2. APE2 facilitates alt-EJ by processing damaged DNA ends and modulating telomere fusions (63), which positions APE2 as a promising therapeutic target (63,136,144). The first APE2 inhibitor identified, Celastrol, originally characterized for its anti-inflammatory properties, has been shown to inhibit APE2's ssDNA binding and 3'-5' exonuclease activity, thereby attenuating ATR checkpoint activation in pancreatic cancer models (145). Given APE2's role in processing the 3'-DNA-protein adducts generated by PARPi, APE2 inhibition may augment PARPi efficacy to overcome resistance in both BRCA-deficient and proficient tumors.

Alessandra Brambati *et al* (69) recently described RHINO as an M-phase accumulated protein that facilitates POLQ recruitment to DSBs, thereby promoting alt-EJ-mediated repair. This finding uncovers a temporal regulation of alt-EJ repair activity during the cell cycle and suggests that RHINO could be a novel candidate target for cancer therapy. Modulation of RHINO function may provide new avenues for targeted intervention, particularly in cancers addicted to alt-EJ due to HRD or other reasons.

5. Conclusion and future prospective

Alt-EJ plays a critical and complex role in genome instability and cancer biology. Mounting evidence highlights alt-EJ not merely as a backup repair pathway but as a pivotal contributor to both tumor development and therapeutic response. Alt-EJ operates with inherently lower fidelity, leading to mutational signatures characterized by deletions, insertions and chromosomal rearrangements, which drive genome instability and can contribute to therapeutic resistance in cancer.

The present review emphasized that alt-EJ is regulated by intricate molecular mechanisms influenced by both intrinsic cellular components and extrinsic factors, underscoring its role as a highly coordinated and context-dependent repair pathway rather than a simple fail-safe mechanism. Recognizing these complexities presents unique challenges and opportunities: Future research should therefore focus on deciphering the precise molecular basis of alt-EJ-mediated mutagenesis, which will be crucial for optimizing therapeutic strategies aimed at mitigating its deleterious effects while exploiting its vulnerabilities to enhance cancer cell destruction.

Mutations in DDR genes prevalent in numerous types of cancer often increases alt-EJ reliance (14,146). This dependency offers a significant therapeutic window. Specifically targeting alt-EJ components such as POLQ, PARP1 and emerging factors such as FEN1 and APE2 can selectively control HRD tumors. Recent advances in understanding alt-EJ mechanisms, coupled with novel therapeutic approaches including combination regimens with radiotherapy or immunotherapy, hold great promise for improving outcomes in these clinically challenging cancers.

Despite recent advances, substantial gaps remain about the molecular determinants of tumor reliance on alt-EJ and the heterogeneity of responses to DDR-targeting therapies. Coordinated, systematic studies are needed to define how alt-EJ interacts with other repair pathways, translate this biology into predictive and pharmacodynamic biomarkers and uncover the routes by which tumors evade therapy. This foundation will enable context specific treatment strategies that exploit alt-EJ inhibition to maximize antitumor efficacy while minimizing toxicity.

Translating these insights into clinical benefit requires clinically deployable assays that quantify alt-EJ dependence and confirm on target inhibition, alongside a rigorous understanding of resistance to alt-EJ-directed agents, including POLQ and PARP1 inhibitors. Anticipated resistance mechanisms include on target alterations, pathway rewiring toward NHEJ or HR, adaptation to replication stress and pharmacologic tolerance; sensitive early indicators should guide timely countermeasures and adaptive trial design. Clarifying how alt-EJ-driven lesions interface with immune checkpoint signaling and with chromatin and epigenetic regulation of end resection will inform combinations with immunotherapy and epigenetic agents. Equally important is optimization of dose, fractionation and sequencing for pairings with radiotherapy and other DDR regulators, supported by standardized analytic pipelines to measure alt-EJ activity across platforms and over time. Addressing these priorities will enable biomarker guided patient selection, anticipate therapeutic resistance and deliver precise, durable exploitation of alt-EJ vulnerabilities in the clinic.

In conclusion, alt-EJ represents a promising and expanding frontier for cancer therapy. By deepening our mechanistic understanding and integrating emerging molecular insights with innovative drug development, the field is poised to translate alt-EJ targeting into effective, personalized interventions that improve both prognosis and quality of life for patients with alt-EJ-dependent malignancies.

Acknowledgements

The authors thank Dr Lei Wang from the International Cancer Center, Shenzhen University (China), for his support and advices.

Funding

The present study was supported by National Natural Science Foundation of China (grant nos. 82373212 and 82073007 to QL; grant no. 82203967 to LM); Guangdong Basic and Applied Basic Research Foundation (grant no. 2023A1515011945 to LM); Shenzhen Science and Technology Program (grant no. JCYJ20240813142112017 to LM). HaiYa Young Scientist Foundation of Shenzhen University General Hospital (grant no. 2025-HY006 to XL).

Availability of data and materials

Not applicable.

Authors' contributions

Conceptualization was by QL, investigation was by QL and NA. Writing and original draft preparation was by NA, LM

and QL. Writing, reviewing and editing was by QL, NA, LM and XL. Visualization was by NA and QL. Supervision was by QL, LM and XL. Funding acquisition was by QL, XL and LM.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work the authors used AI tools in order to improve language and readability and subsequently the authors reviewed and edited the content as needed and take full responsibility for the content of the present manuscript.

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