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Exploiting the Microhomology-Mediated End-Joining Pathway in Cancer Therapy

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Abstract

Repair of DNA double strand breaks (DSB) is performed by two major pathways: homology-dependent repair and classical non-homologous end joining. Recent studies have identified a third pathway: microhomology-mediated end-joining (MMEJ). MMEJ has similarities to homology-dependent repair in that repair is initiated with end resection, leading to single-stranded 3' ends which require microhomology upstream and downstream of the DSB. Importantly, the MMEJ pathway is commonly upregulated in cancers, especially in homologous recombination-deficient cancers which display a distinctive mutational signature. Here we review the molecular process of MMEJ as well as new targets and approaches exploiting the MMEJ pathway in cancer therapy.

Keywords

MMEJ; DNA repair; cancer; POLQ

INTRODUCTION

DNA Damage and Double-Stranded Breaks

Maintenance of genome integrity is of upmost importance for cellular survival (1). Loss of genomic integrity results in permanent changes to DNA sequence and is the source of many human diseases, notably cancer (2–7). Genome integrity is achieved by DNA repair pathways, collectively known as the DNA damage response (DDR) (8). Double-stranded breaks (DSBs) are the most deleterious form of DNA damage (1). DSBs are generated either exogenously, for example, from exposure to ionizing radiation, or endogenously, for example, from reactive oxygen species secondary to aerobic metabolism or from errors during DNA replication and meiosis (9–11). DSBs were traditionally thought to be repaired

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CONFLICT OF INTEREST DISCLOSURE

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by either of two distinct pathways, distinguished by the presence or absence of homology: homologous recombination repair (HR) and classical non-homologous end-joining (NHEJ), respectively (Figures 1A–B).

Microhomology-Mediated End-Joining as a Third Pathway for DSB Repair

Evidence for a third pathway that could repair DSBs first emerged following the observation that NHEJ-deficient yeast and hamster cells retained some degree of end-joining activity (12,13). Because this pathway was first reported in NHEJ-deficient cells, it was initially termed alternative end-joining and viewed as a backup mechanism if HR and NHEJ were unavailable. Subsequent work, especially in mammalian cells, has suggested a more substantial role for this pathway, now more commonly referred to as microhomology-mediated end-joining (MMEJ) (14,15).

CURRENT UNDERSTANDING OF DSB REPAIR BY MMEJ

The MMEJ pathway is a stepwise pathway (Figure 1C). It is initiated by end-resection near the DSB exposing short regions of complementary sequences ranging from 2 to 20 nucleotides (microhomologies). These microhomologies are used to align the DNA ends with the occurrence of end bridging. Next, the resultant 5' flaps, created following alignment, are processed. A polymerase then fills in any gaps, followed ultimately by ligation (16).

End-Resection

The MMEJ pathway is initiated by end-resection. Following a DSB, poly (ADP-ribose) polymerase-1 (PARP-1) competes with Ku70-Ku80 for binding to DSBs ends, facilitating, though not essential for, recruitment of end-resection factors (17,18). As in HR, the initial end-resection in MMEJ is performed by the MRN complex (MRE11, RAD50, and NBS1) stimulated by C-terminal binding protein interacting protein (CtIP), with the initial creation of a 3' single-strand region (19–21). If the microhomologies are distant, studies have suggested that further resection is performed by Bloom helicase-DNA2 helicase/nuclease and exonuclease 1 (EXO1); however, their roles have not been genetically confirmed. Furthermore, opposing data suggests that long range resection by EXO1 decreases MMEJ (14,19,20,22–27).

Normally, single-stranded DNA (ssDNA) is bound by Replication protein A (RPA) to stabilize the secondary structure. In yeast, RPA functions as a negative regulator of MMEJ. MMEJ is dramatically increased following end resection while HR remains relatively stable when a hypomorphic mutant allele of one of components of yeast RPA is expressed (28). Recently, Shukla et al. demonstrated that the 5-hydroxymethylcytosine binding, ESC-specific protein (HMCES), previously shown to protect stalled replication forks, can bind ssDNA and promote MMEJ repair (29,30). Furthermore, HMCES binds class switch regions and protects ssDNA overhangs to promote MMEJ, and mice deficient in HMCES have significant defects in class switch recombination, similar to mice deficient in the specialized DNA polymerase of the MMEJ pathway, polymerase theta (POLQ) (30,31). Taken together,

these studies suggest that RPA is the critical ssDNA binding protein in HR repair, while HMCES is the critical ssDNA binding protein in MMEJ (Figures 1B–C).

End-Bridging and Alignment

End-bridging and alignment of microhomology sequences are next accomplished through the activities of PARP-1, the MRN complex, and POLQ. Recent *in vitro* studies suggest that PARP-1 may compete with Ku70–80 for binding to DSBs and may exhibit end-bridging activity through an unknown mechanism (32). PARP-1 also appears to have a role in the recruitment of POLQ to sites of DNA damage. Regarding the MRN complex, *in vitro* studies have shown that cohesive ends inhibit MRE11-mediated degradation. If a region of microhomology is revealed during resection, MRE11 stalls, thereby stabilizing the junction site (33,34). In addition, scanning force microscopy revealed a complex of MRE11 and RAD50 which can tether linear DNA molecules (35). These findings suggest that the MRN complex, while performing nucleolytic degradation, can facilitate DNA end-bridging and microhomology alignment. POLQ contains a N-terminal helicase domain, a long, unstructured central region, and a C-terminal polymerase domain (36). The crystal structure of the POLQ helicase domain revealed a tetrameric organization, suggesting a role of microhomology alignment and strand annealing in preparing a substrate for the polymerase domain (37). Indeed, both domains are required for MMEJ, and mutation of a conserved residue in the helicase domain reduces MMEJ (38,39).

Tail and Flap Processing

The processes of DNA end-bridging and alignment result in the generation of nonhomologous 3' tails. These tails must be removed in order to complete the repair process. In some DNA repair processes, such as single-strand annealing, nonhomologous 3' tails are removed by the heterodimeric structure-specific endonuclease ERCC1/XPF (Excision repair cross-complementing group 1 / Xeroderma pigmentosum, complementation group F) (40). ERCC1/XPF is also employed in nucleotide excision repair, in the Fanconi Anemia interstrand crosslink repair, and in base excision repair (41–43). Consequently, it was originally hypothesized that ERCC1/XPF was the endonuclease complex that removes nonhomologous 3' flaps in MMEJ. However, murine ERCC1 −/− cells demonstrate only a minor MMEJ defect (44). These studies suggest that another functionally-distinct flap endonuclease, such as flap endonuclease 1 (FEN1), might be involved in the removal of nonhomologous 3' tails in the MMEJ pathway.

Gap filling in MMEJ is performed by POLQ, through the process of strand displacement and DNA synthesis, and this process creates displaced 5' ssDNA flaps requiring subsequent removal (45). FEN1 is a structure-specific endonuclease that recognizes and cleaves these 5' ssDNA flaps (46). Using a cell-free system created from rat testes and thymus to study proteins involved in MMEJ, Sharma et al. demonstrated that siRNA-mediated depletion of FEN1 strongly reduces MMEJ activity (47). More recently, genetic screens to identify genes required for the upregulation of MMEJ and the survival of BRCA2-deficient cells identified FEN1, and specifically the 5' flap endonuclease activity of FEN1 (48). Furthermore, siRNA-mediated depletion of FEN1 resulted in a defect in MMEJ repair in a cell-based MMEJ

reporter assay, confirming that FEN1 is an essential enzyme in MMEJ, and suggesting that FEN1 may be the critical endonuclease which cleaves 5' flaps created by POLQ.

Polymerase Theta (POLQ) Gap Filling

Once the DNA microhomology regions are aligned and the 3' nonhomologous tails have been removed, there will be gaps between the duplex DNA. The specialized DNA polymerase involved in MMEJ, POLQ, was first identified from studies in *Drosophila* by using DSBs induced by P-element transposition or sequence-specific endonuclease (38,49). Subsequent studies have shown that POLQ is essential for MMEJ in all mammalian species examined to date (50). POLQ is a 290 kDA A-family DNA polymerase with a unique structure containing a N-terminal helicase domain, a long, unstructured central region, and a C-terminal polymerase domain (36).

The N-terminal domain of POLQ contains a superfamily 2 Hel308-typeS helicase domain. The helicase domain has both ATPase and DNA unwinding activities, which can act independently of each other. The ATPase activity is required for both the suppression of HR by binding RAD51 and inhibiting its assembly along ssDNA, and the survival of HR deficient cells (36,51). Expression of POLQ mutated at ATPase catalytic residues did not reduce RAD51 foci formation, nor decrease recombination frequency (51). The ATPase activity also facilitates removal of RPA from ssDNA to allow MMEJ and inhibit HR (52). Conceivably, with removal of RPA from ssDNA, HMCES could bind, protecting ssDNA overhangs and promoting MMEJ (30). *In vitro,* the POLQ helicase domain can unwind DNA, thereby facilitating displacement synthesis by the POLQ polymerase domain. Further studies are required to understand the coordination of these domains *in vivo* (53).

The long, unstructured central region of POLQ contains a RAD51 interaction motif. Protein interaction studies of POLQ demonstrated that this site directly interacts with RAD51 via three distinct motifs, though only one region (amino acids 847 to 894) was both necessary and sufficient for RAD51 binding. Interaction of the central domain of POLQ with RAD51 inhibited RAD51-ssDNA nucleoprotein filament assembly. In this way, POLQ acts as an anti-recombinase capable of inhibiting HR and promoting MMEJ (51). This process appears to mirror that of the POLQ helicase domain which can displace RPA, inhibit HR, and promote MMEJ (52). These interactions of the POLQ helicase domain with RPA and the POLQ central domain with RAD51, respectively, demonstrate the competitive nature between MMEJ and HR, occurring at the level of ssDNA binding. In addition, the central domain of POLQ regulates POLQ multimerization (54).

The C-terminal domain of POLQ contains an A-family DNA polymerase domain that performs gap filling. *In vitro* and cellular experiments have elucidated POLQ's gap filling mechanism. POLQ anneals to short regions of ssDNA microhomology of 2 to 6 base-pairs (37,55,56). Because POLQ can uniquely prime DNA synthesis from non-optimal base-pairing leading to the introduction of insertions at the break sites, with the 3' flaps acting as a template, it is highly error-prone (45,57,58). In addition, the POLQ polymerase is proofreading deficient and quite promiscuous because of its robust terminal transferase activity (59). Biochemically, which domains of POLQ are necessary for DNA synthesis depend on the substrate (54). Purified POLQ polymerase domain alone is active on short

ssDNA and short 3' overhangs. Longer ssDNA substrates require both the POLQ N-terminal helicase domain and the C-terminal polymerase domain, and it is the helicase domain's DNA annealing ability that is required rather than its helicase activity. Further, extension of long ssDNA substrates requires multimers of POLQ, and multimerization requires the central region (54).

End Ligation

DSB repair by MMEJ is completed upon ligation of the DNA ends. In mammalian cells, three DNA ligases are encoded: DNA ligase I, DNA ligase III, and DNA ligase IV (60). Ligase IV functions in NHEJ and its deficiency does not impact MMEJ (61). Substantial literature has demonstrated that Ligase III is the major ligase in MMEJ. First, *in vitro* biochemical and plasmid-rejoining studies identified Ligase III (32,62). Second, these studies were confirmed *in vivo,* where mouse cells deficient in Ligase III demonstrated reduced frequency of chromosomal translocations. In this case, the few remaining translocations lacked areas of microhomology commonly seen in MMEJ (63). Third, sequential depletion of Ligase III followed by Ligase I depletion was additive, suggesting the latter is a backup ligase (63). Ligase III forms a stable complex with the scaffold protein, X-ray repair cross-complementing protein 1 (XRCC1), and both proteins in the complex interact with PARP-1 (64,65). This interaction with PARP-1 is not required for recruitment of Ligase III/XRCC1 to DSBs as there is no reduction in recruitment in the presence of PARP inhibition (15). Rather, it appears that the MRN complex might recruit Ligase III/XRCC1 to DSBs during repair by MMEJ (66).

MMEJ'S ROLE IN HUMAN DISEASE

Because of the mutagenic nature of POLQ, it is likely that POLQ and hence MMEJ are only activated under rare circumstances. Indeed, POLQ protein expression is highly limited in normal tissue (51,67). These rare circumstances might include the following. First, as POLQ expression greatly increases in HR-deficient cancers, the presence of active HR could inhibit expression of POLQ to prevent a more mutagenic DSB repair (51,68). Perhaps the BRCA1 protein, which has some transcriptional functions, reduces the transcription of the POLQ gene (69). Second, embryonic stem cells and somatic cells rely on different DSB repair pathways (70). MMEJ might play a greater role during gametogenesis, embryogenesis, or in hematopoietic stem cells as expression of POLQ is greatest in the testis, human placental tissue, and hematopoietic stem cells (36,71). Third, POLQ appears to have a prominent role in the repair of four-stranded guanine rich structures (G-quadruplex) (72–74). These structures can have both beneficial and deleterious functions. On the one hand, G-quadruplexes can protect telomeres, regulate transcription, and promote immunoglobulin gene recombination. On the other hand, these structures are prone to genomic instability and can inhibit DNA replication through fork stalling (75). Indeed, mice deficient in POLQ have increased genomic instability (76). Finally, POLQ might play a preferred role in the repair of repetitive DNA, as HR could be a deleterious option given the possibility of recombination between repetitive elements.

MMEJ and Cancer

One of the hallmarks of cancer is genomic instability (77). The repair of DSBs by MMEJ is an intrinsically mutagenic pathway. The creation of templated insertions at DSB junction sites is characteristic of MMEJ (38,51,78,79). Templated insertions are small insertions, typically, 3 to 30 bps, synthesized from DNA flanking the DSB junction into sites of microhomology deletions (38). Templated insertions, are abundant in the ClinVar database, raising the question of MMEJ's contribution to malignancy (80). Microhomology signatures have been observed at sites of oncogenic chromosomal translocation breakpoints in primary human cancer cells, raising the possibility that MMEJ could be the causative mechanism of these translocation (23,26). Further, review of The Cancer Genome Atlas (TCGA) demonstrates that POLQ is overexpressed in breast, lung, bladder, colorectal, gastric, glioblastoma, pancreatic, prostate, melanoma, and uterine cancers, correlating with poor prognosis (67,81). The elevated POLQ expression in these tumors may result, at least in part, from an underlying HR deficiency.

Cancers deficient in HR up-regulate the expression of POLQ as a survival strategy. Aberrant use of MMEJ could create more mutations in cancers, promoting both cancer growth and resistance (82). These cancers become addicted to POLQ expression, routing the repair of DSBs to MMEJ, as evidenced by the accumulation of a distinct pattern of MMEJ-mediated templated insertions (51,68,83). This mutation signature, initially termed mutation signature 3, was identified by whole genome sequence analysis of HR-deficient tumor and thought to typify tumors that were potentially HR-deficient (84). In actuality, this signature better represents up-regulation and addiction to POLQ rather than a specific signature for HR deficiency (85). Indeed, in a recent revision of mutational signatures in cancers, signature 3 has been further resolved. The MMEJ specific signature, now defined as insertion-deletion signature 6, is characterized predominantly by greater than 5bp deletions with overlapping microhomology at deletion boundaries and is correlated with single-base substitution signature 3 (86).

THERAPEUTIC APPLICATION OF MMEJ FOR CANCER TREATMENT

Loss of DNA repair pathways is a common feature of cancers and likely provides a growth advantage (87). Concomitantly, there is increased dependence on the remaining DNA repair pathways. This dependence renders the cancer vulnerable to inhibition of the remaining DNA repair pathways and is the basis of the synthetic lethality. This concept of synthetic lethality has been successfully exploited by the use of PARP inhibitors in breast and ovarian cancers harboring inactivating mutations in BRCA1 or BRCA2, and could similarly be employed with MMEJ inhibition (88,89). Because PARP-1 is also a component of MMEJ (see section 2), PARP inhibitors also inhibit MMEJ (15). Indeed, inhibition of MMEJ appears to be a major mechanism of synthetic lethality of PARP inhibitors. Loss of expression of POLQ or other MMEJ genes in HR-deficient cells is synthetically lethal (51,68). Some cancers also have mutations in NHEJ genes, and these cancers exhibit synthetically lethality from depletion of POLQ (58,90). Importantly, some HR-deficient tumors acquire PARP inhibitor resistance by downregulating NHEJ. This offers the opportunity for using a POLQ inhibitor, since NHEJ-deficient cells are also dependent on

MMEJ. Taken together these observations provide a strong rationale for targeting MMEJ in the treatment of cancers, including PARP inhibitor resistant cancers (Figure 2) (88,89).

Synthetic Lethality: Targeting MMEJ and HR

POLQ has limited protein expression in normal tissue but is overexpressed in wide variety of cancers (51,67,81). This overexpression is increased with concomitant p53 loss (91). These cancers may be addicted to POLQ expression because of the need to repair DSBs (51,68,83). Furthermore, POLQ, via its ATPase activity and RAD51 binding ability, can prevent the accumulation of toxic RAD51 on resected ssDNA, thereby enhancing cancer cell survival. Loss of POLQ in these tumors results in cell death by impairing MMEJ-mediated of DSBs and promoting the buildup of toxic RAD51 (51,68). Thus, POLQ is an attractive target for drug development in HR-deficient cancers (Figure 2A) (92).

The POLQ protein has three domains: a N-terminal helicase domain, a long, unstructured central region, and a C-terminal polymerase domain (36). POLQ inhibitors could be designed to target either the N-terminal helicase domain or the C-terminal polymerase domain. Targeting the polymerase domain would inhibit repair of resected DSBs at the gap-filling step in HR-deficient cancers. Targeting the helicase domain, however, might be a better option, as MMEJ repair of DSBs would be inhibited and toxic RAD51 foci would accumulate (51). POLQ inhibitors are currently undergoing active pre-clinical development. Indeed, Zhou et al. have recently identified the antibiotic Novobiocin as a specific POLQ helicase domain inhibitor that selectively kills HR-deficient cancer cells *in vitro* and *in vivo* (93).

In addition to POLQ, FEN1 is an essential enzyme in MMEJ, required at least for removal of the 5' flap and potentially the 3' flap (47,48). Depletion of FEN1 results in a defect in MMEJ repair and is also synthetically lethal with HR deficiency, recapitulating the synthetic lethality observed with POLQ inhibition (48,51,68). Hydroxyurea based FEN1 inhibitors have been developed that induce synthetic lethality and did not induce toxicity as a monotherapy (94). Importantly, FEN1 is not exclusive to the MMEJ pathway. It is also involved in long-patch base excision repair, thereby adding to its value as a target for anti-cancer drug development.

Synthetic Lethality: Targeting More Than HR-Deficiency

Synthetic lethality occurs not only in HR-deficient cells but also in cells deficient in NHEJ, where depletion of POLQ decreases cell survival (58). There are only a few cancers with underlying defects in NHEJ (90). However, DNA-dependent protein kinase (DNA-PK) is a key component of the NHEJ pathway, and DNA-PK inhibitors are currently under development because they block NHEJ and enhance the activity of DNA-damaging agents (95,96). DNA-PK inhibition results in a significant increase in MMEJ-mediated templated insertions and, coupled with genetic downregulation of POLQ, results in significantly reduced viability (91). These results provide evidence for the application of POLQ inhibition beyond HR-deficient cancers and for combination treatment strategies (Figures 2B–C).

Cell cycle DNA damage checkpoints allow time for repair by the DDR. The protein kinases, ataxia-telangiectasia-mutated (ATM) and ataxia-telangiectasia and Rad3-related protein

(ATR), play central roles in these DNA damage checkpoints, creating an opportunity for synthetic lethality (97). ATM is activated by DSBs and initiates a DNA damage response via a signaling cascade of phosphorylation with activation of checkpoint kinase 2 (CHK2) and ultimately p53, as well as activation of the HR pathway. ATR, in contrast, is activated by replication stress. Replication stress is common in oncogene-driven cancer cells, but not in normal tissue (98). Through its kinase activity, ATR activates checkpoint kinase 1 (CHK1) and ultimately Wee1-like protein kinase (WEE1), leading to prolongation of the S/G2 cell cycle phase (99).

Replication stress activates the ATR pathway to prevent replication fork collapse and allow DNA replication to continue (99). By creating single-strand breaks at replication forks, using either a Cas9 nickase or a topoisomerase inhibitor, Wang and colleagues demonstrated synthetic lethality with POLQ deficiency, demonstrating a role for MMEJ in replication stress tolerance (100). Inhibition of the ATR-CHK1-WEE1 signaling cascade is likely to prove useful in combination with POLQ inhibition (Figure 2B). Inhibitors of ATR, CHK1, or WEE1 are currently under investigation in clinical trials and have activity through their ability to promote replication stress. Gemcitabine is another agent which can promote replication stress. A randomized phase II clinical trial of the ATR inhibitor M6620 plus gemcitabine demonstrated efficacy through the induction of replication stress in cancer cells (101). Thus, combination treatment strategies involving induction of replication stress with gemcitabine coupled with POLQ inhibition provides a strong rationale for future trials. Finally, a targeted CRISPR screen of 309 murine genes known to be involved in DDR uncovered 140 genes synthetically lethal with POLQ deficiency with the majority of these identified genes being previously unknown. It is likely that whole genome wide CRISPR screening to identify genetic vulnerabilities that interact with POLQ will reveal new combination therapy targets.

Targeting MMEJ to Overcome Therapy Resistance

The success of PARP inhibitors (PARPi) in the treatment of breast and ovarian cancers harboring inactivating mutations in BRCA1 or BRCA2 has demonstrated the power of synthetic lethality (88,89). Unfortunately, these cancers ultimately acquire PARPi resistance, with the primary mechanisms of resistance being either restoration of HR repair or replication fork stabilization (102). Reactivation of HR in PARPi resistant cancers would be predicted to alleviate dependence on MMEJ for DSB repair. However, POLQ via its RAD51 binding ability, prevents accumulation of toxic RAD51 secondary to abortive HR. Though HR is often reactivated in PARPi resistant cancers, through somatic reversion of BRCA1/2 mutations or via downregulation of NHEJ, inhibition of POLQ could still result in accumulation of toxic RAD51, resulting in cellular toxicity (51). Furthermore, POLQ and the MMEJ are required for coping with replication stress (100). Inhibition of POLQ in PARPi resistant cancers would still result in increased replication stress and cellular toxicity. Indeed, PARPi resistant tumors are sensitive to the specific POLQ inhibitor, Novobiocin, both *in vitro* and *in vivo* (93). Targeting MMEJ by inhibition of POLQ might help prevent the development of PARPi resistance or re-sensitize PARP inhibitor resistant cancers (Figure 2C).

Cancer cells often lose expression of p53 or express a mutant version (103). Loss of p53 function in cancer cells increases resistance to ionizing radiation, a common treatment modality for cancer (104,105). Given POLQ is overexpressed in p53-deficient cancers, this raises the question of whether POLQ inhibition might re-sensitize p53-deficient cancers to ionizing radiation or if POLQ inhibition could be used as an adjuvant with radiation treatment (91). Indeed, treatment of bone marrow stromal cells derived from POLQ deficient mice demonstrated increased sensitivity to ionizing radiation (106).

Biomarker-Guided Development of MMEJ Inhibitors

The development of predictive biomarkers to guide the application of MMEJ inhibition in cancers is crucial. There are at least four potential MMEJ/POLQ biomarkers. First, since POLQ is overexpressed in HR-deficient cancers or in p53-deficient cancers, dependence on POLQ activity would be predicted by upregulation of POLQ protein expression (51,68,83,91). Assessment of POLQ expression in cancers, via immunohistochemistry or RT-PCR, could serve as a predictive biomarker for response to POLQ inhibition. Second, depletion of POLQ in HR-deficient cancers leads to RAD51 accumulation (51). Assessing RAD51 accumulation could serve as a pharmacodynamic biomarker for POLQ inhibition. Third, the MMEJ pathway is required for alleviation of replication stress and is synthetically lethal with ATR inhibition (100). Replication stress activates the ATR pathway (99). Phosphorylation of RPA is downstream of ATR activation and is an immunohistochemical marker of replication stress (107). Thus, the assessment of phosphorylated-RPA could be a biomarker of the effectiveness of an MMEJ inhibitor. Finally, templated insertions are a biomarker of ongoing (or previous) MMEJ activity in the tumor cells and are a potential indicator of POLQ inhibitor efficacy (84,85). The development of biomarkers will be extremely useful in future clinical trials of POLQ inhibitors.

SUMMARY

DNA is under constant threat from both endogenous and exogenous damage, and the maintenance of genomic integrity is critical for cell survival (1). The most deleterious type of DNA damage is DSBs. Mammalian cells have three main pathways to repair DSBs: NHEJ, HR, and MMEJ. Initially, NHEJ and HR were the best studied DSB repair pathways and were thought to be the dominant pathways, with MMEJ as a weaker alternative pathway. However, evidence has since emerged to support MMEJ as a more substantial third pathway given the functional relevance of the pathway to both DSB repair and human disease. Indeed, the prevalence of a distinctive MMEJ mutational signature of templated insertions in up to 20% of all human cancers provides evidence of the widespread relevance of this pathway. Given the success of targeting DDR dependencies as a therapeutic approach to cancers, for example with PARP inhibitors in HR-deficient ovarian and breast cancers, targeting MMEJ is especially enticing. Furthermore, targeting MMEJ could be helpful in the setting of acquired drug resistance to prior therapies.

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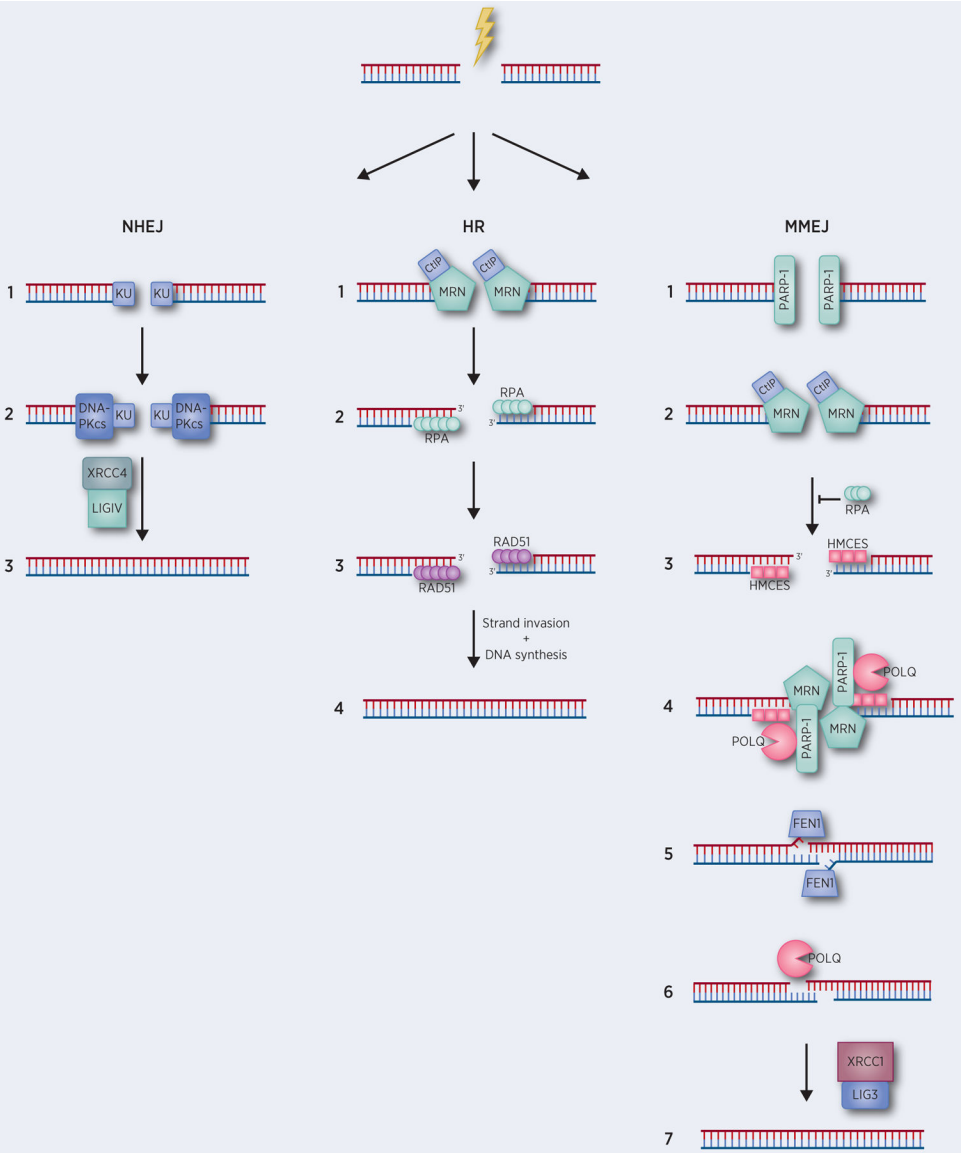

FIGURE 1: DSB Repair Pathways.

Repair of DSBs is a stepwise pathway. **(A)** NHEJ. (1) Ku70-Ku80 (Ku) heterodimer recognizes DSB end. (2) Recruitment of DNA-PKcs to Ku70-Ku80 heterodimer-DSB ends. If incompatible ends, further processing is required. (3) Ligation of DNA ends by XRCC4-DNA ligase IV. **(B)** HR. (1) MRN, stimulated by CtIP performs end-resection. (2) ssDNA is bound by RPA. (3) RAD51 replaces RPA, forming a RAD51-ssDNA nucleoprotein filament. (4) RAD51-ssDNA nucleoprotein filament performs strand invasion of the sister chromatid, leading to template-dependent strand extension followed by strand annealing. **(C)** MMEJ. (1) PARP-1 competes with Ku70–80 to bind DSBs ends, recruiting MRN and CtIP. (2) End-resection is initiated by MRN, stimulated by CtIP, in a stepwise manner first with the creation of a 3' single-strand region endonucleolytically, subsequently followed by 3' to 5' exonucleolytic digestion. (3) Competition for binding of single-stranded DNA by RPA or HMCES with HMCES-ssDNA coating favored by MMEJ. (4) End-bridging and

microhomology alignment. End-bridging is mediated by a combination of PARP-1, MRN, and POLQ. (5) Flap processing. Non-homologous 3' tails are removed by ERCC1/XPF, FEN1, and likely as-yet identified endonucleases. Non-homologous 5' tails are removed by FEN1. (6) POLQ gap filling. POLQ can uniquely prime DNA synthesis from non-optimal base-pairing leading distinctive templated insertions. (7) Ligation of ends by LIG3/XRCC1. See text for more details.

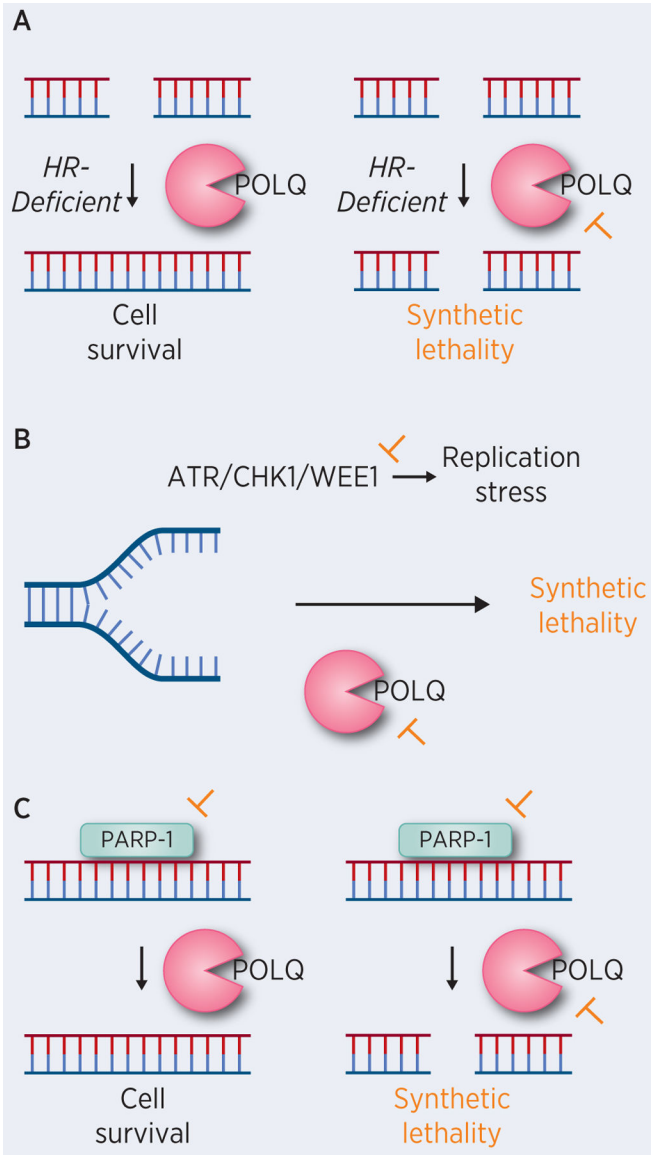

FIGURE 2: Therapeutic Application of MMEJ for Cancer Treatment.

(A) HR-deficient cancers require MMEJ to repair DSBs. Inhibition of POLQ results in synthetic lethality in HR-deficient cancers. (B) POLQ deficiency is synthetically lethal with ATR inhibition. Inhibition of the ATR-CHK1-WEE1 signaling cascade leading to replication stress in combination with POLQ inhibition would result in synthetic lethality. (C) PARP inhibition results in DSBs which can be repaired by MMEJ. Inhibition of both PARP and POLQ (MMEJ) induces synthetic lethality.