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DNA Polymerase theta (Polθ) – an error-prone polymerase necessary for genome stability

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Abstract

Mammalian cells have evolved multiple pathways to repair DNA double strand breaks (DSBs) and ensure genome stability. In addition to non-homologous end-joining (NHEJ) and homologous recombination (HR), cells evolved an error-prone repair pathway termed microhomology-mediated end joining (MMEJ). The mutagenic outcome of MMEJ derives from the activity of DNA polymerase theta (Polθ) – a multidomain enzyme that is minimally expressed in normal tissue but overexpressed in tumors. It has become evident that Polθ expression is critical for the proliferation of HR deficient cancer cells. As a result, this mutagenic repair emerged as an attractive target for cancer therapy, and inhibitors are currently in pre-clinical development. Here we review the multifunctionality of this enigmatic polymerase, focusing on its role during DSB repair in mammalian cells and its impact on cancer genomes.

Keywords

DNA damage; DNA Polymerase theta; Polθ dysfunctional telomeres; DSB; MMEJ; alt-EJ

DNA double-strand break repair in mammalian cells

DNA double-strand breaks (DSBs) are highly deleterious lesions that arise due to exogenous agents, including ionizing radiation and chemotherapeutic drugs. In addition, DSBs accumulate as a result of DNA replication, meiosis, and the assembly and diversification of antigen receptor genes by Class Switch and V(D)J recombination[1]. The ends of linear chromosomes may also be recognized as DSBs when telomeres are rendered dysfunctional following telomerase deficiency or upon the removal of the protective Shelterin complex[2]. Different sources of DSBs lead to diverse chemistry at DNA ends that can be resolved using various pathways to ensure genome stability.

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Declaration of interests

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The most studied DSB repair mechanisms include non-homologous end-joining (NHEJ) that uses little or no homology to seal DNA ends, and homologous recombination (HR) that employs the sister chromatid as template to promote error-free repair (Figure 1). NHEJ is the main DSB repair pathway in mammalian cells. This canonical end-joining pathway is initiated when the Ku70/80 (Ku) heterodimer recognizes broken DNA ends and recruits DNA-dependent protein kinase catalytic subunit (DNA-PKcs). The DNA-PK complex then phosphorylates numerous factors to promote end-joining by Ligase 4 (Lig4)[3]. NHEJ employs additional end-processing enzymes that are essential for the joining of chemically incompatible ends, including Artemis, polynucleotide kinase 3' phosphatase (PNKPT1), and two family X-Polymerase – Polλ and Polμ[3]. Although NHEJ can act throughout the cell cycle, its activity is predominant in G1. On the other hand, the activity of HR is restricted to S and G2 phases of the cell cycle[4]. HR requires DNA resection, where nucleolytic degradation of a DSB generates a 3' single-stranded (ss) DNA overhang. This process is initiated by the MRE11-RAD50-NBS1 complex (MRN) in conjunction with CtIP. Extended resection is then catalyzed redundantly by EXO1 and DNA2-BLM[5]. Following resection, the 3' ssDNA tail is bound by RPA which is subsequently exchanged for RAD51 through the action of BRCA2 and PALB2[6]. RAD51 nucleofilaments mediate strand invasion and homology search on the sister chromatid. Ultimately, missing nucleotides are filled-in by copying the undamaged chromatid and repair is completed with minimal alterations to the original sequence[6].

Microhomology-mediated end joining, an intrinsically mutagenic repair pathway

In addition to the well-characterized NHEJ and HR, mammalian cells employ a mechanistically distinct, yet less-understood pathway termed Microhomology-Mediated End Joining (MMEJ). MMEJ is part of a broader and error-prone mechanism of end joining, known as alternative end joining (alt-EJ) (Figure 1). Repair by MMEJ is driven by the annealing of micro-homologous sequences flanking the DNA ends, and its outcome is mutagenic due to deletions and insertions that scar break sites. MMEJ activity has been detected in all kingdoms of life and studied in bacteria[7,8], yeast[9], flies[10], worms[11], plants[12], fish[13] and mammals[14,15]. Its activity was first described in NHEJ defective *S. cerevisiae* mutants[9] and found to be dependent on the presence microhomology[9]. Early evidence for MMEJ in mammalian cells emerged from the analysis of Class Switch Recombination (CSR) in NHEJ-deficient B cells[14]. MMEJ was initially viewed as a back-up pathway for canonical repair. Consistent with substrates being rerouted to MMEJ when HR and NHEJ are compromised, cells lacking BRCA1/2, Ku, and Lig4 rely on MMEJ for survival[16–18]. However, it has become evident that in certain contexts, MMEJ operates even when NHEJ and HR are proficient. For example, MMEJ was found to be essential to repair DSBs in the developing zebrafish embryos[13]. Furthermore, MMEJ activity was detected during V(D)J recombination in NHEJ-proficient B cells that carry mutations in the RAG recombination genes[15]. Accordingly, MMEJ is no longer viewed as a back-up repair mechanism. Nevertheless, it remains unclear as to when MMEJ prevails over other repair pathways and what prevents it from accessing substrates that are typically repaired by HR and NHEJ.

Evidence for MMEJ activity at deprotected telomeres emerged from the analysis of telomerase-deficient mice, where chromosome end-to-end fusions persisted in the absence of Lig4 and DNA-PKcs[19]. Subsequently, sequence analysis of telomere fusions in cells derived from chronic lymphocytic leukemia (CLL) and ataxia-telangiectasia-like disorder (ATLD) patients highlighted an MMEJ signature comprising frequent microhomology, deletions, and insertions[20–22]. Furthermore, depletion of MMEJ factors compromised the ability of cancer cells to escape telomere crisis[20], indicating that this mutagenic repair can foster genome instability in the early stage of tumorigenesis to promote cancer progression. Mechanistic insight into how mammalian telomeres suppress MMEJ was obtained through genetic experiments in mouse cells [23,24] which revealed that mutagenic repair is fully unleashed upon the depletion of all subunits of the Shelterin complex and in the absence of Ku. Interestingly, the function of MMEJ at telomeres is not confined to the very tip of chromosomes. DSBs internal to telomeric repeats are also repaired by MMEJ as opposed to canonical NHEJ, potentially implicating MMEJ during the repair of specialized loci such as telomere repeats[25].

Mechanistic basis of microhomology-mediated end joining

Our current understanding of the mechanistic basis of MMEJ derives primarily from genetic studies in model organisms and based on different sources of DSBs. The first step in MMEJ is shared with HR and involves MRE11/CtIP dependent resection. This exposes flanking microhomology and allows base pairing of ssDNA ends[26]. Evidences point to a role for PARP1 in facilitating annealing of resected ends[27,28]. Following synapsis, DNA polymerase theta (Polθ) fills-in the flanking ssDNA regions. This then stabilizes paired intermediates and prevents long-range resection that would otherwise promote HR. MMEJ is completed when Ligase 3 seals DNA ends[29]. Annealing can also be driven by internal microhomology which then leads to 3’ ssDNA flaps that must be removed prior to fill-in synthesis and ligation. Recent work implicated the flap endonuclease, FEN1 during this processing step. Specifically, FEN1 was identified in a genome-wide CRISPR-Cas9 screen for genes that are synthetic lethal with mutant BRCA genes and found to promote MMEJ using a reporter system[30].

Only a handful of MMEJ factors have been characterized so far, and efforts are currently underway to unveil the full genetic make-up of this pathway. A better understanding of factors upstream of Polθ, including ones that shape the chromatin landscape of DSBs, will provide insight into when and how cells opt for mutagenic MMEJ over HR and NHEJ. A recent study identified HMCES (5-Hydroxymethylcytosine binding, embryonic stem cell-specific protein) as a novel MMEJ factor[31] that promotes efficient CSR in mature B cells. *In vitro* assays detected HMCES binding at resected DNA substrates, suggesting that it could act prior to the annealing step. However, the precise function of HMCES during MMEJ and whether it is active beyond CSR remains to be determined.

MMEJ mutagenicity is attributed to the promiscuous activity of Polθ

A distinguishing feature of MMEJ in higher eukaryotes is the presence of nucleotide insertions at break sites. Insertions, which are highly mutagenic, have been attributed to the

activity of Polθ that is encoded by the *POLQ* gene. Polθ was identified in *D. melanogaster* through the analysis of mus308 mutants that displayed hypersensitivity to interstrand cross-links inducing agents[32]. Thereafter, Polθ activity was linked to MMEJ during the repair of DSBs induced by P-element transposition in flies[33]. Since then, the role of Polθ in MMEJ has been characterized in several multicellular organisms including worms, fish, mammals, and plants, whereas its homologs are lacking in yeast and other fungi[34]. In mammals, inhibition of Polθ sensitizes cells to DSB inducing agents, including bleomycin, etoposide, camptothecin and irradiation[35,36]. Its function in MMEJ-mediated repair has been established based on the analysis of endonuclease-cleavage reporter constructs, CRISPR-Cas9 induced breaks in human and mouse cells[16,17,36–39], and chromosomal translocation in mouse cells[7] (Figure 2). In all cases, Polθ activity scarred repair junctions by means of nucleotide insertions at break sites. Notably, Polθ-driven MMEJ activity was also found to be critical during the random integration of foreign DNA into host genomes in plants and mammals[12,37,40]. Work from our laboratory established the role of Polθ during the processing of dysfunctional telomeres[17]. Sequence analysis of telomere fusions in cells lacking NHEJ revealed breakpoints with non-telomeric nucleotide insertions that were diminished upon Polθ inhibition[17]. Taken together, these studies underscore templated insertions by Polθ as an evolutionary conserved feature of MMEJ. This raises the question of why would cells retain a mutagenic repair mechanism when more accurate pathways such as HR and NHEJ are in place. Emerging evidence from *C. elegans* suggests that repair by MMEJ could promote genome diversification. Specifically, insertions and small deletions indicative of Polθ activity in lab strains are also evident in the genomes of wild isolates[41]. It would be interesting to investigate whether Polθ is a source of genome diversity in higher eukaryotes including human germ cells.

DNA polymerase Polθ as a multifunctional enzyme

Polθ is a unique enzyme, as it is the only DNA polymerase that contains a helicase-like domain at its N-terminus (Figure 3), and the modalities are separated by a large unstructured central domain. Structural analysis of the different enzymatic domains confirmed that they form dimers and possibly multimers in solution. It has been proposed that dimerization helps Polθ tether DNA ends together and stabilize synapsed intermediates. Whether Polθ dimerization occurs *in vivo* is yet to be established[42–44].

Polθ-polymerase domain belongs to the A-family DNA polymerases but lacks proofreading activity[45]. Polθ-polymerase activity has been investigated biochemically using a range of DSB substrates and tested genetically in several model systems. *In vitro* characterizations of human Polθ-polymerase identified template-dependent as well as template-independent DNA synthesis[46,47]. Templated nucleotides are copied from regions flanking the break sites *in trans* and *in cis*. The latter employs a unique “snap-back” reaction that self-copies the ssDNA end[47]. On the other hand, non-templated insertions have been mainly characterized *in vitro* using full-length protein as well as the polymerase domain and are attributed to a terminal transferase activity that is stimulated by Mn^{++}[48]. However, template-independent activity is abolished in the presence of Mg^{++} and when ssDNA substrates that cannot self-anneal were used[49]. Of note, Polθ-dependent nucleotide insertions not matching flanking DNA sequences were detected at chromosomal translocations as well as telomere fusions in

mouse cells[47]. These nucleotides could either be inserted by a template-independent activity or through iterative cycles of templated copying, slippage, and re-priming. The identification of a separation of function mutant would be critical to ascertain the relevance of a terminal transferase Polθ activity *in vivo.*

Polθ-helicase belongs to the SF2 family of helicases that includes Hel308 and RecQ[50]. Inhibition of Polθ-helicase manifested in reduced microhomology at repair junctions in flies[10] and impaired chromosomal translocation in mouse cells[51]. *In vitro* assays uncovered a strong ATPase activity that is stimulated by ssDNA[43,50]. Recent work from our laboratory revealed that Polθ-helicase exhibits an ATP-dependent DNA annealing activity that facilitate base-pairing of ssDNA, even when pre-bound by RPA. In effect, we showed that Polθ employs its ATPase activity to counteract RPA binding and promote the annealing of resected DNA substrates. This would ultimately favor repair by MMEJ while preventing HR[51]. Consistent with a role for Polθ-helicase in suppressing HR, Ceccaldi and colleagues found that inhibition of Polθ-helicase increases RAD51 loading and HR-mediated repair of I-SceI-based reporters[16]. Furthermore, inhibition of Polθ-helicase in mouse cells enhanced the efficiency of HR-mediated CRISPR-Cas9 gene targeting[51].

The function of Polθ in other repair pathways

In addition to its critical role during MMEJ, Polθ has overlapping functions with other repair pathways, including translesion synthesis, base-excision repair (BER), and replication repair (Figure 2). Polθ catalyzes DNA synthesis across abasic sites and possesses a mild 5'-dRP lyase activity[45,50,52], both relevant for BER. Genetic data suggest that Polθ could serve as a back-up for Polβ during BER. Specifically, DT40 cells lacking both Polθ and Polβ are sensitive to MMS due to reduced BER activity[53]. Furthermore, a CRISPR-Cas9 genome-wide screen in *Polq* deficient mouse cells identified multiple genes involved in the BER pathway as synthetic lethal interactors with Polθ[54]. Biochemical studies have determined that Polθ is unable to bypass pyrimidine dimers, a type of UV photoproduct. However, the polymerase domain is capable of extending mismatched DNA termini and could therefore indirectly contribute to repairing UV-induced lesions. In support of this hypothesis, $Polq^{-/-}$ mice have an increased risk of developing UV-induced skin cancers, and the incidence raises dramatically when combined with *Polh* deficiency[55].

MMEJ activity is maximal in S phase and it is therefore not surprising that Polθ has been implicated in the repair of replication forks. The first evidence for Polθ function in replication repair came upon examining G4 DNA stability in *C. elegans*. Worms lacking FANCJ, which ensures replication across G4 sites, rely on Polθ to repair collapsed forks and prevent the accumulation of large deletions[11]. Furthermore, Polθ depletion in human cells reduces replication speed and compromises replication fork restart in the presence of hydroxyurea[16]. Direct evidence for Polθ role at collapsed forks is based on a recent study using Xenopus egg extracts. Specifically, supplementing frog extracts with mitotic kinases to trigger replisome disassembly led to fork breakage and Polθ dependent rearrangement[56]. The observation that Polθ mediated repair is not inhibited by mitotic kinases raises the interesting possibility that MMEJ is the predominant DSB repair pathway in mitosis when NHEJ and HR are known to be greatly repressed[4].

Polθ inhibition during cancer therapy

Expression of Polθ is tightly regulated; the polymerase is largely repressed in somatic cells and upregulated in several human cancers, including lung, gastric, and colorectal[57]. In addition, Polθ levels are especially high in HR deficient breast[58] and ovarian cancers[16] and associated with poor clinical outcomes. The underlying mechanism that regulates Polθ expression remains unknown. Nevertheless, its expression pattern and the reliance of BRCA mutated cells on MMEJ for survival[16,17] renders this unique polymerase an appealing target for cancer treatment[59]. In fact, efforts are currently underway to develop Polθ inhibitors for the treatment of HR defective tumors.

Exploiting synthetic lethality in DNA repair to eliminate cancer cells is best exemplified by the PARP1-BRCA genetic interaction. The profound impact of PARP1 inhibition on the survival of a sizeable fraction HR defective tumors led to the development of five PARP inhibitors that are currently in clinical trials. Despite the clinical benefits achieved in several *BRCA*-mutated cancers, resistance to therapy is a common theme among all PARP inhibitors. Resistance mechanisms are not fully understood, but evidence suggests that at least a subset of these tumors involve mutations in NHEJ factors that would respond to Polθ inhibition[60,61].

While Polθ synthetic lethality manifests in the context of HR and NHEJ defective tumors, rearrangements consistent with MMEJ activity have been detected in wide range of cancers[62]. Furthermore, MMEJ footprint is found at telomere fusions in CLL[21], translocations that drive lymphomas[63], and during chromothripsis[64]. Mutagenic repair by Polθ could drive genome plasticity that fuels cancer progression and therefore, its inhibition could limit the mutational rate that renders tumors resistant to cytotoxic therapies. In conclusion, targeting Polθ has substantial clinical potential and this can only be realized upon a complete understanding of the mechanistic basis of MMEJ.

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Biography

Schimmel *et al.*, 2017. This study reveals that in mouse embryonic stem cells Polθ acts in parallel and redundantly with NHEJ to repair DSBs and that virtually all DSBs are repaired through these two pathways. The author induced a blunt DSB with CRISPR-Cas9 in a reporter gene and analyzed the repair products revealing the mutation spectra of Polθ and NHEJ factors. The study supports the notion that Polθ repair relies on microhomology. Surprisingly, in the case of long 3’ or 5’ ssDNA overhangs, NHEJ dominates over MMEJ.

Deng *et al.*, 2019. This is the first study that provides evidence of Polθ-mediated MMEJ events during mitosis. The authors showed that in *X. laevis* extracts the replisome disassembly during mitosis causes replication fork breakage that form rearrangements driven Polθ and characterized by microhomology.

Balagere *et al.*, 2019. This study shows that HMCES, a protein reported to protect stalled replication forks, plays an additional role in the MMEJ pathway during class switch recombination in murine B cells. The author demonstrates the ability of HMCES to interact with DNA overhangs of different lengths.

Black *et al.*, 2019. The authors used a biochemical approach to characterize MMEJ at the molecular level using full-length human Polθ. This study shows that Polθ-helicase domain is essential for MMEJ. In addition, its central domain was proposed to regulate of Polθ multimerization and DNA substrates requirements during MMEJ.

Feng *et al.*, 2019. This study expands the synthetic lethality interactions of Polθ. Through a CRISPR-Cas9 screen in mouse cells, the authors identified 140 genes that are required for the survival of Polθ deficient cells. These genes belong to several pathways, including DNA repair, DNA damage signaling, and chromatin structure. Breast cancers with alterations in many of these genes had increased Polθ and had an MMEJ genomic footprint.

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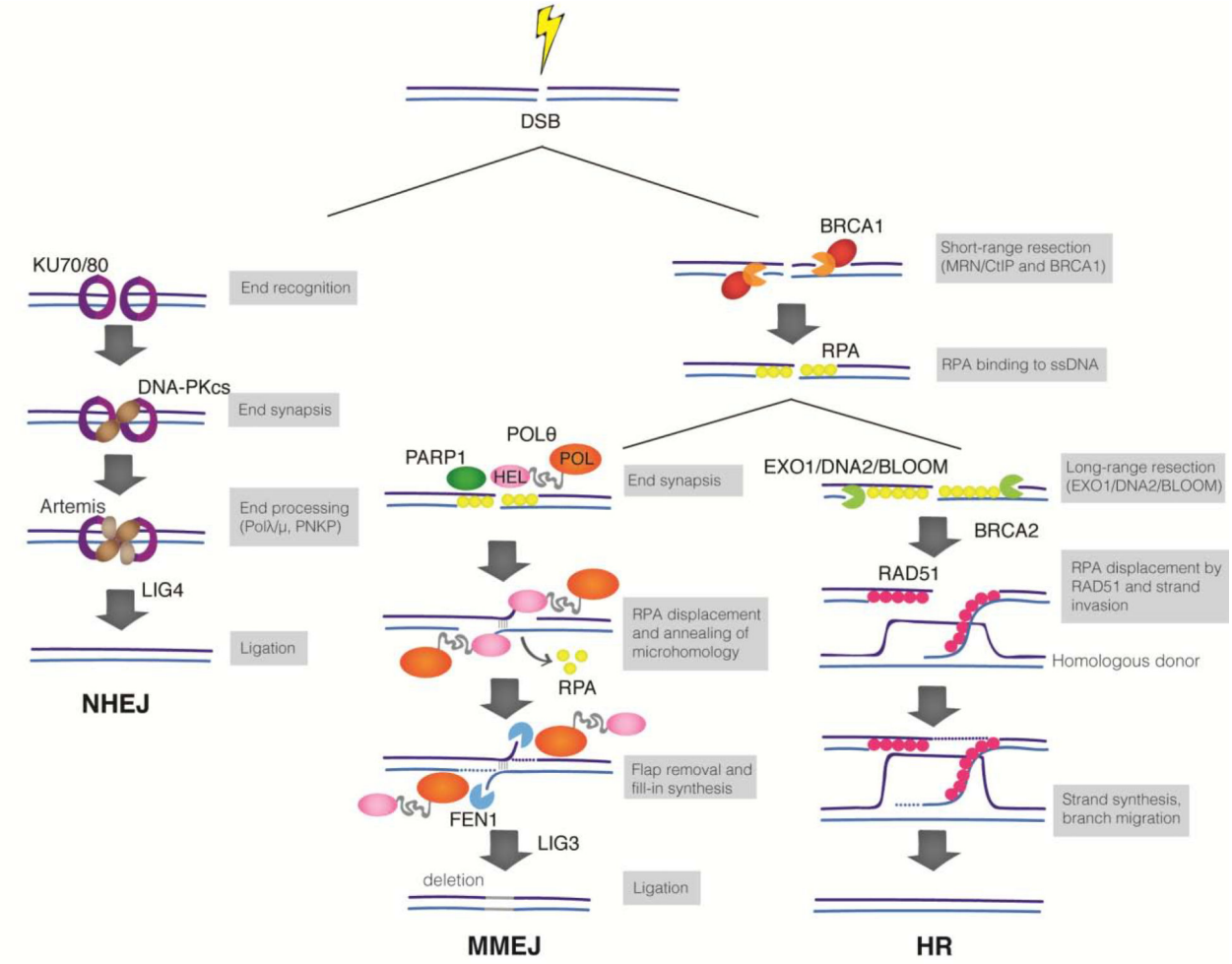

Figure 1. Major DSB repair pathways in mammalian cells.

DSBs in G1 are primarily repaired by NHEJ. The Ku heterodimer recognizes broken DNA and recruits DNA-PKcs to orchestrate end-joining by Lig4. During NHEJ, minimal processing of DNA ends leads to repair with minimal alteration to the original sequence. DSBs in S/G2 phases of the cell cycle are subjected to end-resection by MRN/CtIP, leading to short ssDNA that is rapidly coated by RPA. Resected DSBs are substrates for MMEJ and HR, and the choice between these pathways is poorly understood. Polθ-helicase displaces RPA to promotes the synapsis of the opposing ends. If annealing occurs using internal microhomology, flaps are generated and are processed by FEN1. Polθ fills-in the gapped DNA and hands over the substrate to Lig3 to seal the end. When resected DNA is subject to long end-resection by EXO1/DNA2 and BLM, RPA1 is exchanged for Rad51 to promote strand invasion and copying from the sister chromatid.

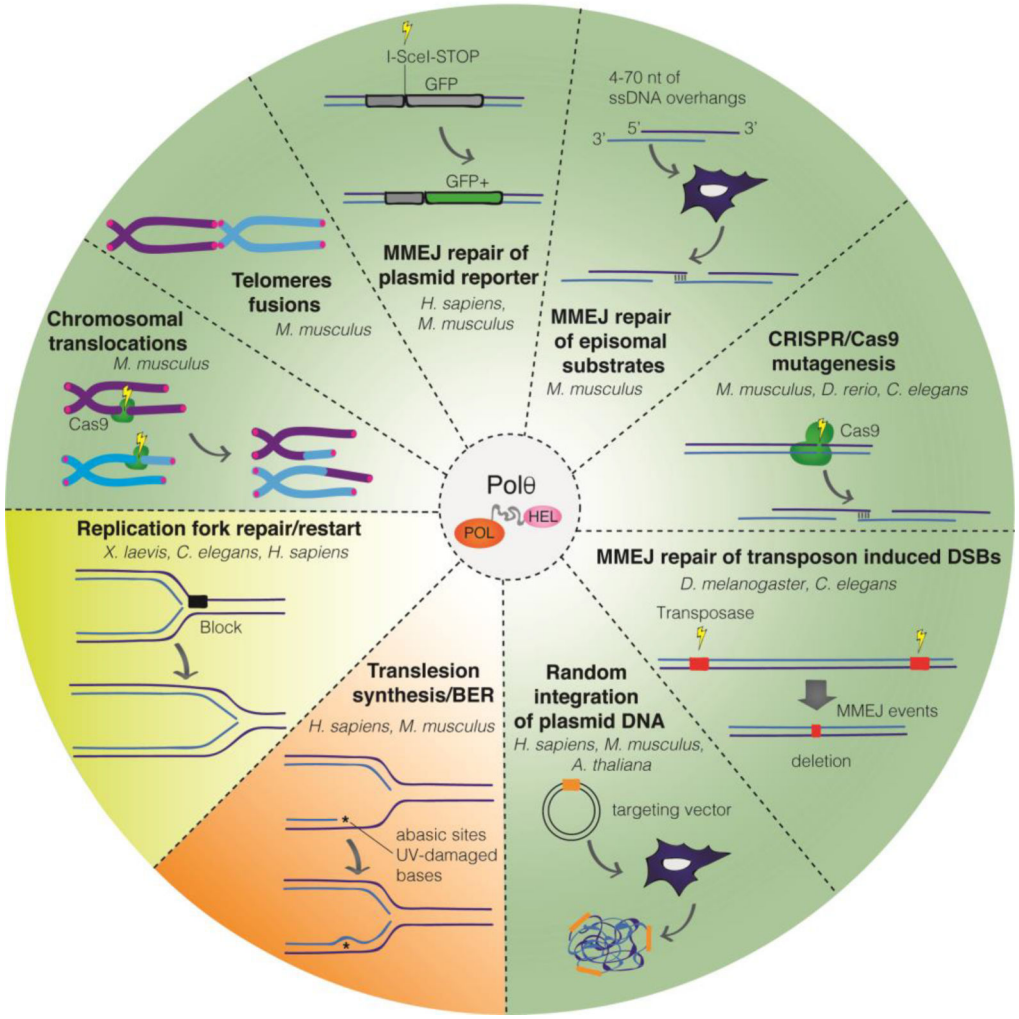

Figure 2. Overview of all reported Polθ activities.

Representation of the various activities that are carried out by Polθ. The major and well-established Polθ function is during DSB repair by MMEJ and has been studied using different substrates and in various model systems (green). Polθ has also been linked to the repair of breaks associated with replication forks (yellow). Lastly, Polθ employs its translesion synthesis activity to bypass UV-damaged bases and abasic sites (orange).

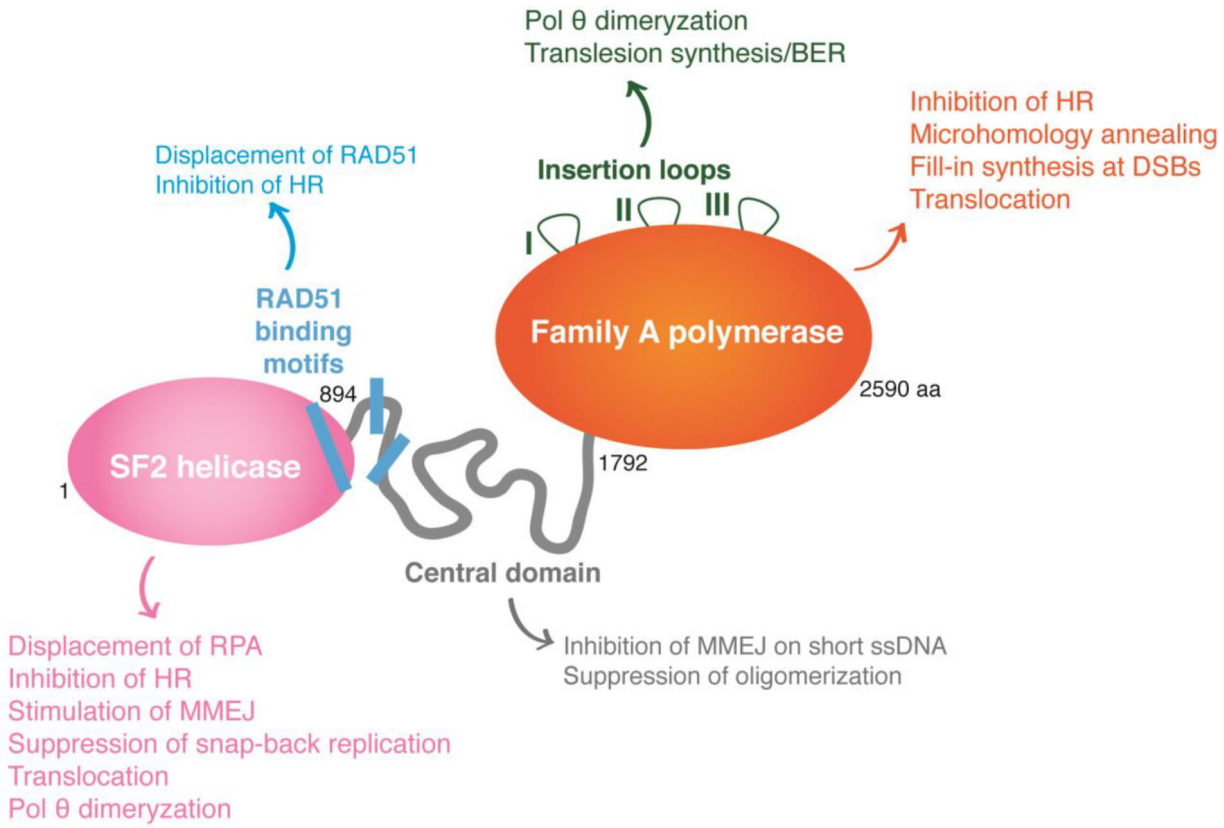

Figure 3. Polθ is a unique multidomain enzyme.

Schematic representation of the different domains of human Polθ, depicting the structure and function of its helicase domain (pink), the unstructured central domain (grey) and the family-A polymerase domain (orange). The helicase domain drives MMEJ activity during chromosomal translocations, promotes Polθ dimerization, and suppresses HR and snap-back replication. The central domain contains three predicted RAD51 binding motifs that are not conserved in mouse. These motifs are implicated in suppression of HR through the inhibition of RAD51 nucleofilament formation. The primary and unequivocal function of the polymerase domain is to perform fill-in synthesis during MMEJ. The polymerase domain has also been reported to participate in tethering DNA ends. The polymerase domain comprised three insertion loops that are essential for Polθ to bypass bulky lesions and abasic sites, and were proposed to promote dimerization.