Polθ promotes the repair of 5'-DNA-protein crosslinks by microhomology-mediated end-joining

Gurushankar Chandramouly
Thomas Jefferson University

Shuren Liao
Fox Chase Cancer Center

Timur Rusanov
Washington University School of Medicine in St. Louis

Nikita Borisonnik
Thomas Jefferson University

Marissa L Calbert
Thomas Jefferson University

See next page for additional authors

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Authors
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Graphical Abstract

Highlights

- Polθ confers resistance to DNA-protein crosslink (DPC) agents
- Polθ promotes MMEJ repair of 5’-DPCs in Xenopus egg extracts
- Polθ promotes MMEJ repair of 5’-DPCs in mammalian cells
- Polθ acts independently of NHEJ and HR to repair 5’-DPCs

Authors

Gurushankar Chandramouly, Shuren Liao, Timur Rusanov, ..., Tomasz Skorski, Hong Yan, Richard T. Pomerantz

Correspondence

richard.pomerantz@jefferson.edu

In brief

Chandramouly et al. find that Polθ protects cells from DNA-protein crosslink (DPC) agents and promotes microhomology-mediated end-joining (MMEJ) repair of DPCs occurring at double-strand breaks (DSBs) in Xenopus egg extracts as well as mammalian cells. Polθ-mediated repair of DPCs occurring at DSBs is independent of non-homologous end-joining (NHEJ) and homologous recombination (HR).
Pol(θ) promotes the repair of 5’-DNA-protein crosslinks by microhomology-mediated end-joining

Gurushankar Chandramouly,1,6 Shuren Liao,4,5 Timur Rusanov,2 Nikita Borisonnik,1 Marissa L. Calbert,1,3 Tatiana Kent,1 Katherine Sullivan-Reed,3 Umeshkumar Vekariya,2 Ekaterina Kashkina,3 Tomasz Skorski,3 Hong Yan,4 and Richard T. Pomerantz1,6,*

1Thomas Jefferson University, Sidney Kimmel Cancer Center, Department of Biochemistry and Molecular Biology, Philadelphia, PA 19107, USA
2Washington University School of Medicine, Department of Pathology & Immunology, St. Louis, MO 63110, USA
3Fels Cancer Institute for Personalized Medicine, Temple University Lewis Katz School of Medicine, Philadelphia, PA 19140, USA
4Fox Chase Cancer Center, Philadelphia, PA 19111, USA
5These authors contributed equally
6Lead contact
*Correspondence: richard.pomerantz@jefferson.edu
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SUMMARY

DNA polymerase θ (Pol(θ)) confers resistance to chemotherapy agents that cause DNA-protein crosslinks (DPCs) at double-strand breaks (DSBs), such as topoisomerase inhibitors. This suggests Pol(θ) might facilitate DPC repair by microhomology-mediated end-joining (MMEJ). Here, we investigate Pol(θ) repairing DPCs by monitoring MMEJ in Xenopus egg extracts. MMEJ in extracts is dependent on Pol(θ), exhibits the MMEJ repair signature, and efficiently repairs 5’ terminal DPCs independently of non-homologous end-joining and the replisome. We demonstrate that Pol(θ) promotes the repair of 5’ terminal DPCs in mammalian cells by using an MMEJ reporter and find that Pol(θ) confers resistance to formaldehyde in addition to topoisomerase inhibitors. Dual deficiency in Pol(θ) and tyrosyl-DNA phosphodiesterase 2 (TDP2) causes severe cellular sensitivity to etoposide, which demonstrates MMEJ as an independent DPC repair pathway. These studies recapitulate MMEJ in vitro and elucidate how Pol(θ) confers resistance to etoposide.

INTRODUCTION

Elucidating the mechanisms of double-strand break (DSB) repair can inform strategies for reducing cellular resistance to genotoxic chemotherapeutics, such as those that promote DNA-protein crosslinks (DPCs). A relatively newly discovered DSB repair pathway referred to as alternative end-joining or microhomology-mediated end-joining (MMEJ) acts independently of non-homologous end-joining (NHEJ) and homologous recombination (HR) (Kent et al., 2015; Mateos-Gomez et al., 2015; Yousefzadeh et al., 2014). The early phase of MMEJ likely requires poly-ADP ribose polymerase 1 (PARP1) that facilitates recruitment of the essential MMEJ factor DNA polymerase θ (Pol(θ)) to sites of DNA damage (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015). MMEJ functions during S/G2 cell cycle phases and acts on 3’ single-stranded DNA (ssDNA) overhangs like HR (Truong et al., 2013). Mre11 and CtIP are essential for MMEJ and initiate the resection process, resulting in 3’ ssDNA overhangs (Black et al., 2016; Sfeir and Symington, 2015). Pol(θ) facilitates synopsis of the 3’ ssDNA termini by using minimal base pairing (≥2 bp) within microhomologous sequence tracts and then extends each overhang, resulting in stabilization of the DNA synapse (Black et al., 2016, 2019; Kent et al., 2015). Following additional end processing, DNA ligase 3 (Lig3) or ligase 1 seals the DNA (Lu et al., 2016; Sfeir and Symington, 2015).

Pol(θ) is important for DSB repair in HR-deficient cancer cells and is therefore synthetic lethal with HR factors such as BRCA1/2 (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015). Pol(θ) also confers resistance to multiple chemotherapy agents (Higgins et al., 2010; Wang et al., 2019; Yousefzadeh et al., 2014). For example, Pol(θ) promotes resistance to chemotherapy agents that cause DPCs at DSBs, such as topoisomerase inhibitors etoposide and camptothecin and ionizing radiation (IR) (Wang et al., 2019; Yousefzadeh et al., 2014). This suggests that Pol(θ) plays a role in repairing toxic DPCs by the MMEJ pathway.

Previously characterized DPC repair processes include tyrosyl-DNA phosphodiesterase 1 and 2 (TDP1/2) that cleave 3’ and 5’ tyrosyl-DNA crosslinks, respectively; protease-mediated (i.e., SPRTN) pathways that act at stalled replication forks; and HR that uses MRN-CtIP to initiate endonucleolytic cleavage of DPCs at DSBs (Deshpande et al., 2016; Juarez et al., 2018; Nakano et al., 2009; Stingele et al., 2017). NHEJ also promotes DPC repair downstream of tyrosyl-DNA phosphodiesterase 2 (TDP2) (Gómez-Herreros et al., 2013). Whether Pol(θ) contributes to these DPC repair mechanisms or acts by MMEJ to confer resistance to DNA-protein crosslinking agents has remained unknown. Here, we demonstrate that Pol(θ) acts independently of TDP2 to confer resistance to etoposide and show that Pol(θ) promotes MMEJ repair of DSBs harboring 5’-terminal DPCs using Xenopus egg.
Figure 1. Polq confers resistance to DNA-protein crosslinking agents
(A) Bar plots showing percentage of colonies relative to control after treatment with the indicated topoisomerase inhibitors in Polq+/+ and Polq−/− mESCs. Data represent mean. n = 3 ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Statistical significance from two-sample t test: p = 0.004 for camptothecin and p = 0.001 for etoposide.

(B) Plot showing percentage of colonies relative to control after treatment with the indicated concentrations of etoposide in Polq+/+ and Polq−/− mouse bone marrow cells. Data represent mean. n = 3 ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. Statistical significance from two sample t test: p = 0.00017 for 20 nM, p = 0.0001 for 50 nM, p = 0.00003 for 100 nM.

(C) Plot showing percentage of colonies relative to control after treatment with indicated concentrations of formaldehyde in Polq+/+ and Polq−/− mESCs (left) and bone marrow cells (right). Data represent mean (n = 3) ± SEM (left), SD (right). *p < 0.05, **p < 0.01, ***p < 0.001. Statistical significance from two-sample t test: p = 0.001 for 200 nM formaldehyde and p = 0.00001 for 400 nM formaldehyde (left). Statistical significance from two-sample t test: p = 0.0002 for 400 nM (right).

(D) Plot showing percentage of colonies relative to control after treatment with indicated concentrations of etoposide in HCT116 cells with indicated gene knockouts. *p < 0.05, **p < 0.01, ***p < 0.001. Statistical significance from two-sample t test: p < 0.00001 for 3.8 nM.

(legend continued on next page)
extracts and a cellular MMEJ reporter assay. Together, these data explain how Polq promotes resistance to DPC agents such as etoposide.

RESULTS

Polq promotes resistance to DNA-protein crosslinking agents

One of the mechanisms by which DPCs are repaired is through the MRN-CtIP complex (Stingele et al., 2017). MRN acts with CtIP to initiate 5′-3′ DNA resection during S phase and G2 that is required for HR (Syed and Tainer, 2018). The nuclease activities of Mre11 and CtIP confer resistance to etoposide and camptothecin that covalently link Top2 and Top1, respectively, onto 5′ and 3′ DNA ends (Hoa et al., 2016; Makharashvili et al., 2014; Stingele et al., 2017). MRN-CtIP and its yeast homolog complex (Mre11-Rad50-Xrs2 along with Sae2) perform endonucleolytic cleavage of DNA just upstream of DSB ends that is simulated by 5′ biotin-streptavidin conjugation that models DPCs (Aparicio et al., 2016; Cannavo and Cejka, 2014; Deshpande et al., 2016). Additional studies have shown that BRCA1 collaborates with MRN-CtIP to facilitate DPC repair, suggesting that the HR pathway acts on DSBs containing protein adducts (Aparicio et al., 2016; Nakamura et al., 2010). Indeed, multiple studies demonstrate that HR-deficient cells are sensitive to topoisomerase inhibitors (Al Abo et al., 2014; Gómez-Herreros et al., 2013; Nakamura et al., 2010; Treszczamsky et al., 2007). Intriguingly, HR and MMEJ are thought to share the same DNA resection mechanism involving MRN and CtIP (Truong et al., 2013), which suggests that HR and MMEJ process identical DSB ends. Based on this idea, we hypothesized that Polq-dependent MMEJ facilitates the repair of DSBs containing DPCs.

Polq promotes cellular resistance to topoisomerase crosslinking agents etoposide and camptothecin (Wang et al., 2019; Yousefzadeh et al., 2014). To confirm this and to begin to explore Polq involvement in DPC repair, we first tested the sensitivity of isogenic Polq+/+ and Polq−/− mouse induced pluripotent cells (iPSCs) to etoposide and camptothecin. Consistent with prior reports, Polq null cells were significantly more sensitive to these topoisomerase-DNA crosslinking agents, as revealed by clonogenic survival (Figure 1A), Polq−/− Lin− Sca1+c-Kit+(LSK) murine stem/early progenitor bone marrow cells were also highly sensitive to etoposide, as expected (Figure 1B). To explore a more general role for Polq in DPC repair we tested the susceptibility of Polq−/− iPSCs and Polq−/− LSK murine bone marrow cells to formaldehyde that acts as a non-specific DNA-protein crosslinking agent. Consistent with a general role for Polq in DPC repair, we observed a significant reduction in the survival of Polq−/− versus Polq+/+ cells following formaldehyde treatment (Figure 1C). These data show that Polq confers resistance to formaldehyde.

Importantly, Polq-dependent MMEJ occurs independently of NHEJ. This infers that Polq promotes resistance to DNA-protein crosslinking agents independently of NHEJ. TDP2 acts upstream of NHEJ to cleave 5′ DPCs, such as 5′-phosphotyrosine adducts, by its 5′-tyrosyl DNA phosphodiesterase activity (Pommier et al., 2014; Stingele et al., 2017). Thus, we envisaged that Polq acts independently of TDP2 to confer resistance to etoposide. As a control, we found that TDP2−/− human HCT116 cells are highly sensitive to etoposide as expected (Figure 1D). POLQ−/− HCT116 cells were also significantly more sensitive to etoposide than WT HCT116 cells (Figure 1D). Dual deficiency in Polq and TDP2, however, further increased cellular sensitivity to etoposide (Figure 1D). Hence, these genetic data demonstrate that Polq plays a major role in 5′ DPC repair that is independent of TDP2-mediated NHEJ. To gain insight into whether other MMEJ factors contribute to etoposide resistance, we compared the effects of etoposide in mouse bone marrow cells null for Polq versus those null for Parp1, which also promotes MMEJ and facilitates Polq recruitment to DNA breaks (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015). The results demonstrate a similar sensitivity to etoposide in Polq−/− and Parp1−/− cells, and no significant increase in sensitivity is observed in Polq−/− Parp1−/− double-knockout cells (Figure 1E). These data indicate that Polq and PARP1 act within the same pathway (e.g., MMEJ) to confer etoposide resistance.

Polq promotes DPC repair in Xenopus egg extracts

To directly examine Polq DPC repair, we recapitulated MMEJ by using membrane-free extracts from unfertilized interphase Xenopus eggs that are incapable of initiating replication (Liao et al., 2016). Because MRN-CtIP cleaves 5′ DNA-streptavidin linkages by Mre11 endonuclease activity (Deshpande et al., 2016) and MRN-CtIP is essential for the resection initiation step of MMEJ (Truong et al., 2013; Zhang and Jasim, 2011), we used a similar model system that uses 5′ avidin-biotin linkages (Figure 2A, top right). Mre11 and CtIP are essential for the 5′–3′ resection of DNA containing 5′-biotin-avidin adducts in Xenopus egg extracts (Liao et al., 2016). Mre11 is also essential for resecting DNA possessing 5′-phosphotyrosine adducts that model 5′ DNA-Top2 covalent lesions following etoposide treatment (Liao et al., 2016). To assess whether MMEJ is active in Xenopus egg extracts and capable of repairing DSBs with 5′ DPCs, uniformly 3P-labeled 5′ avidin-conjugated DNA 5.7 kb in length was incubated with extracts during a time course in HEPES buffer containing ATP and a ATP regenerating system as in previous studies (Liao et al., 2016). Reactions were terminated by the addition of EDTA and proteinase K that degrades protein but leaves DNA intact. Radiolabeled DNA was then resolved in native agarose gels and visualized by phosphorimager. Consistent with previous studies (Liao et al., 2016), we observed the formation of high-molecular-weight products that were due to intermolecular DSB repair and thus concatemer formation (Figure 2A, right). The repair junction sequencing analysis below confirmed intermolecular DSB repair (Figure 3). Dimers were also formed that were subsequently converted to concatemers during the time course (Figure 2A, right). Minor degradation of the DNA was also detected...
during the earlier time points, which has been shown to be due to 5′–3′ resection in prior studies (Liao et al., 2016). In the absence of 5′ DNA-protein adducts (clean ends; 5′ phosphate), the majority of products resembled circular supercoiled DNA monomers that have previously been shown to be due to NHEJ and occur independently of Mre11 (Figure 2A, left; Di Virgilio and Gautier, 2005; Labhart, 1999). Concatemer products were still formed in the absence of 5′-avidin, albeit to a lesser extent (Figure 2A, left).

To determine whether PolI promotes repair of the DSBs containing 5′-avidin-biotin linkages, the polymerase was depleted from extracts by using a polyclonal antibody generated against the polymerase domain of Xenopus PolI (495 C-terminal residues). Our data demonstrate efficient (>90%) depletion of PolI from extracts (Figure 2B). The DSB repair time course assay was repeated using PolI-depleted versus mock-depleted extracts. PolI-depleted extracts caused a dramatic reduction in the formation of high-molecular-weight concatemer DSB repair products versus mock-depleted extracts even after 3 h, indicating that PolI promotes 5′ DPC repair during DNA end-joining (Figure 2D, left). The majority of 5′-avidin conjugated DNA in PolI-depleted extracts were resected, and a smaller fraction...
was converted into dimers, likely due to residual Polq (Figure 2D, left, lanes 6–9). To validate Polq involvement in this process, we purified full-length human Polq by using previously described methods (Figure 2C; Black et al., 2019) and then tested whether adding back recombinant Polq to the depleted extracts rescues concatemer formation. Our recent biochemical studies demonstrate that full-length Polq is fully active in MMEJ even at low 1- to 3-nM concentrations (Black et al., 2019). Consistent with this result, adding even small amounts of recombinant Polq to the Polq-depleted extracts resulted in partial rescue of concatemer DSB repair products (Figure 2D, right). We note that we were unable to further concentrate full-length recombinant Polq due to precipitation issues. Therefore, only a modest rescue of concatemer formation was observed due to a limited concentration of the large 290-kDa enzyme. Nevertheless, our data clearly demonstrate that depletion and replenishment of Polq abolishes and rescues, respectively, DSB repair of DNA containing 50 DPCs.

Because MMEJ is known to act independently of NHEJ, the observed 50 DPC repair process is expected to occur independently of essential NHEJ factors such as Ku70/80. Indeed, we found that depletion of Ku70 had no significant effect on 50 DPC repair, as indicated by concatemer formation (Figure 2D, right). We note that we were unable to further concentrate full-length recombinant Polq due to precipitation issues. Therefore, only a modest rescue of concatemer formation was observed due to a limited concentration of the large 290-kDa enzyme. Nevertheless, our data clearly demonstrate that depletion and replenishment of Polq abolishes and rescues, respectively, DSB repair of DNA containing 50 DPCs.

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Polq promotes DPC repair in mammalian cells

To confirm the ability of Polq to promote DPC repair in mammalian cells, we developed a reporter assay that detects MMEJ of a split green fluorescent protein (GFP) expression construct conjugated with 5’-terminal DPCs (Figures 4A and 4B). Here, the upstream and downstream portions of a GFP expression vector were synthesized by PCR with a non-specific AT-rich sequence adjacent to a 6-bp overlapping sequence tract as microhomology. The DNA constructs were prepared with either 5’-biotin-streptavidin linkages on both ends (Figure 4A) or with 5’-phosphotyrosine adducts on the ends proximal to the microhomology tracts along with 5’-biotin-streptavidin linkages on the opposite ends (Figure 4B). The 5’-phosphotyrosine adducts are formed following partial proteolytic degradation of Top2 after etoposide induces covalent crosslinking of a Top2 active site tyrosine residue to the 5’-phosphate at DNA ends (Gao et al., 2012; Shi et al., 2012). Controls showed highly efficient conjugation of the 5’-biotinylated DNA constructs with streptavidin prior to transfection (Figure S1B). MMEJ of the left and right 5’-adducted DNA constructs in cells following co-transfection is expected to use the 6-bp microhomology tract and thus activate GFP expression (Figures 4A and 4B). This is based on the fact that MMEJ typically results in relatively large deletions flanked by microhomology. For example, recent studies demonstrate that microhomology tracts (≥3bp) are efficiently used to repair CRISPR-Cas9-induced DSBs in human cells by MMEJ, resulting in predictable microhomology-mediated deletions (Grajcarek et al., 2019).

We co-transfected the left and right DNA constructs containing either 5’-streptavidin or 5’-phosphotyrosine adducts proximal to the microhomology tracts, along with a mCherry expression vector as an internal transfection control, into Polq+/+ and Polq−/− mouse embryonic stem cells (mESCs) (Matesos-Gomez et al., 2017) and POLQ+/+ and POLQ−/− HEK293T cells generated by CRISPR-Cas9 engineering (Figure S1C). At 72-h post-transfection, %GFP and %mCherry were measured by fluorescence activated cell sorting. Transfection efficiency was measured using mCherry. GFP+ frequencies were normalized to both transfection efficiency and parallel control samples. Representative fluorescence-activated cell sorting (FACS) plots demonstrate activation of GFP following co-transfection of the left and right GFP DNA reporter constructs with 5’-streptavidin and 5’-phosphotyrosine adducts, indicating MMEJ (Figure 4C). We note that the left and right PCR DNA constructs were treated with DpnI prior to their purification in order to degrade the CMV-GFP plasmid PCR template and thus prevent any possible background GFP expression. The absence of Polq in both mESCs and 293T cells resulted in significantly lower GFP following transfection of DNA constructs with 5’-streptavidin and 5’-phosphotyrosine (Figures 4D and 4E; Figures S2A and S2B). Hence, Polq promotes the repair of DSBs carrying 5’-streptavidin and 5’-phosphotyrosine adducts, which requires deletion of the DNA termini and use of the 6-bp microhomology. As a control, we found that a similar reduction in GFP was observed following siRNA suppression of Polq (Figure 4F; Figures S2C and S2F). siRNA knockdown of the MMEJ factor Lig3 also significantly reduced MMEJ of both 5’-adducted DNA constructs, whereas knockdown of BRCA1 had no effect, as expected (Figure 4F;
Figures S2C and S2F). Taken together, these data confirm that both 5'-adducted GFP reporters are repaired by MMEJ. Additionally, we found that expression of Polq WT, but not a polymerase mutant, rescues MMEJ of the 5'-adducted DNA substrates (Figures S2D and S2E). The results of these MMEJ reporter assays are consistent with those obtained from Xenopus egg extracts in which similar DNA substrates with 5' protein (avidin) adducts are repaired by Polq-dependent MMEJ. Furthermore, because replication initiation requires specific replication origin protein loading and strict cell-cycle control, and exogenous DNA is not replicated in mammalian cells in the absence of large T-antigen and its replication origin, our GFP assay reports on MMEJ events that occur independently of the replication fork, similar to the Xenopus MMEJ system. Hence, our data indicate that Polq-dependent MMEJ is capable of promoting 5' DPC repair independently of the replisome.

DISCUSSION

Here, we discover the ability of Polq-dependent MMEJ to repair DSBs possessing 5' DPCs. Consistent with these findings, we discover that Polq confers cellular resistance to formaldehyde and confirm Polq’s ability to promote cellular tolerance to etoposide and camptothecin. We also find that Polq acts independently of TDP2 to promote resistance to etoposide, which indicates MMEJ as an independent mechanism of 5' DPC repair.

Separate studies have shown that MRN along with CtIP promotes endonucleolytic cleavage of DSBs carrying 5' DPCs (Deshpande et al., 2016; Liao et al., 2016; Figure 4G). In fact, 5'-streptavidin stimulates endonucleolytic activity of MRN-CtIP and the yeast ortholog MRX-Sae2 complex (Cannavo and Cejka, 2014; Deshpande et al., 2016). Because MRN-CtIP promotes MMEJ, and MMEJ and HR share the same resection initiation process (Truong et al., 2013), we propose a model whereby MRN-CtIP endonucleolytic activity cleaves DPCs at DSB ends (Figure 4G). This model is consistent with the requirement for Mre11 and CtIP in resection of DSBs containing 5' DPCs in Xenopus egg extracts (Liao et al., 2016) and biochemical studies of MRN-CtIP and MRX-Sae2 (Cannavo and Cejka, 2014; Deshpande et al., 2016). Once the DNA-protein adduct is cleaved, the resection machinery can complete its process along with additional nucleases and helicases (i.e., Dna2, BLM, and Exo1) that ultimately leads to MMEJ or HR (Figure 4G).

Previous studies demonstrated that the BRCA1-CtIP interaction is needed for MRN-CtIP repair of 5'-Top2 DNA adducts in replication-competent Xenopus egg extracts (Aparicio and Gautier, 2016). MMEJ repair of 5' DPCs in the replication-incompetent extracts used here, however, shows no dependency on BRCA1 (Figure S3). We reconcile the difference in the requirement for BRCA1 in 5' DPC repair in the prior report due to replication fork coupling. For example, replication fork coupling with Top2-DNA crosslinks was implicated in triggering BRCA1-dependent MRN-CtIP 5' DPC repair in replication-competent Xenopus extracts (Aparicio and Gautier, 2016). Thus, in the context of the replication fork, BRCA1 likely plays a primary role in facilitating DPC repair by HR, whereas Polq MMEJ probably acts as a backup mechanism. This is consistent with the synthetic lethal relationship between BRCA1 and Polq. Despite the ability of Polq to promote DPC repair independently from the replication fork in our assays, MMEJ is likely to be activated following replication-DPC collisions because Polq confers resistance to topoisomerase inhibitors. Future studies will be needed to elucidate the upstream signaling mechanisms responsible for coordinating Polq MMEJ repair of DPCs.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.108820.

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AUTHOR CONTRIBUTIONS


### STAR METHODS

#### KEY RESOURCES TABLE

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact: Richard T. Pomerantz (Richard.Pomerantz@jefferson.edu).

Materials availability
Cell lines generated in this study are available upon request from the lead contact.

Data and code availability
This study did not generate any unique datasets or code. The datasets supporting this study have not been deposited in a public repository.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Lin-cKIT+ primary cells, Polq+/+, Polq−/− mESCs and POLQ−/− HEK293T cells were cultured in appropriate media (more information are provided in Method details) and incubated at 37°C with 5% CO2.

METHOD DETAILS

Cell lines
The following oligonucleotides (IDT, IA) were used for constructing plasmids that express PolQ or TDP2 gRNAs (PAM sequences are in parenthesis and not part of oligos): TTCTATAGGATCTATCA(TGG)/TGATGAACTCTATGAA (PolQ); ATATACTGTAGC TGACTC(TGG)/GAGTCAGCTACAGTTATAT (TDP2). Mutant cells were isolated by the HPRT co-targeting method as previously described (Liao et al., 2015). PolQ status was determined by amplifying the target region from wild-type and mutant cells and then Sanger sequencing (Genewiz, NJ). TPD2 status was determined by western blot with an anti-TDP2 antibody (Bethyl Laboratories, TX) against samples prepared from wild-type and mutant cells. Primary murine bone marrow cells were isolated from mice. Polq+/+ and Polq−/− mice were obtained by breeding Polq−/− mice (JAX #006194), Parp1−/− mice were provided by Roberto Caricchio (Temple University). They were crossed with Polq−/− mice to make the Polq−/−Parp1−/− mice. Lin-cKIT+ primary cells were isolated by magnetic sorting using the EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit (StemCell) followed by EasySep Mouse CD117 (cKIT) Positive Selection Kit (StemCell), and were subsequently cultured in IMDM + 10% FBS supplemented with a cocktail of growth factors (3 ng/mL IL3, 3 ng/mL IL6, 5 ng/mL SCF). Polq+/+, Polq−/− mESCs and iPSCs were generated in prior studies as described (Mateos-Gomez et al., 2015, 2017).

Clonogenic survival assays
Polq+/+ and Polq−/− iPSCs from a stock culture were plated on six well plates at 500 cells/well. Cells were treated with the indicated concentrations of camptothecin, etoposide or formaldehyde 24 h after plating. Medium was replaced every three days with the same media until the colonies were ready for staining in 8-10 days. Medium was removed from plates, cells were rinsed with PBS. Fixation was carried out with Acetic acid/methanol (1:7) for 30 minutes followed by staining of colonies with 0.5% crystal violet for 2 hours at room temp. Dishes were rinsed with water and left for drying overnight at room temp. Counting of colonies was performed with ImageJ software. Polq+/+, Polq−/−, Parp1−/− and Polq−/−Parp1−/− Lin−Sca1−c-Kit+ (LSK) murine stem/early progenitor bone marrow cells were plated at 10^4 cells per well in a 96 well plate and treated with etoposide or formaldehyde at the indicated concentrations. After 48 h, cells were counted via trypan blue exclusion and immediately plated in MethoCult (StemCell) containing threshold level (1/10X) of growth factors. Colonies were counted after approximately 7 days. HCT116 WT and mutant cells were grown in DMEM supplemented with 10% fetal bovine serum, 2mM L-glutamine, non-essential amino acids, and penicillin/streptomycin at 37°C in a humidified incubator containing 5% CO2. Wild-type HCT116 cells and the indicated mutant HCT116 cells were seeded in 6 well plates at 500 cells/well. After two days, etoposide was added to each well at the indicated concentrations. Plates were incubated for 9 more days and then stained with crystal violet to visualize colonies. Colonies were counted and the averages and standard deviations of surviving colonies as percentages of no drug controls were calculated and plotted. For comparisons of averages, a one-tailed t test was conducted at 95% confidence interval (C.I.).
Preparation of Xenopus egg extracts and immunodepletion

Membrane-free extracts derived from unfertilized interphase Xenopus eggs were prepared as described (Liao et al., 2016). For immunodepletions, extracts (40 μl + 20 μl ELB (10 mM HEPES (pH 7.5), 250 mM sucrose, 2.5 mM MgCl₂, 50 mM KCl, 1 mM DTT)) were incubated with 20 μl Protein A Sepharose beads pre-coated with 80 μl of the rabbit serum or no serum at 4°C for 1.5 h. After two rounds of depletion, extracts were saved as 5 μl aliquots at −80°C. Rabbit antibodies against Xenopus Ku and Pol Ḷ were raised against the Ku70 subunit and the C-terminal 495 amino acids of Pol Ḷ. Recombinant Xenopus Ku and Pol Ḷ (C-terminal 495 residues) were over-expressed as GST-fusion proteins in E. coli, purified as inclusion bodies, and injected into rabbits following standard procedures.

DSB repair of 5'-avridin conjugated DNA in Xenopus egg extracts

The DNA substrates were prepared by amplifying a 5.7 kb plasmid using Pfu DNA polymerase (Promega, WI) and oligonucleotides carrying 5'-biotin (Midland, TX) in the presence of 32P-α-dATP (Perkin Elmer). PCR products were purified by gel-filtration with Sepharose CL-2B beads (Sigma-Aldrich, MO). Peak fractions were pooled and concentrated to 37.5 ng/μl. 5'-avridin DNA was prepared by pre-incubating 5'-biotin DNA (20 ng/μl) with Neutravidin (4 μg/μl) (Pierce/ThermoScientific, IL) for 10 minutes. A typical repair assay contained 5 μl non-depleted extracts, mock depleted extracts, or extracts depleted of Pol Ḷ, Ku70 or BRCA1. 0.5 μl 10x ATP mix (20 mM ATP/200 mM phosphocreatine/0.5 mg/ml creatine kinase/50 mM DTT), 1.5 ng/μl DNA, and ELB buffer (total volume = 7.5 μl). Reactions were incubated at room temp and samples taken at the indicated times were mixed with an equal volume of 2% SDS/25 mM EDTA. At the end, samples were brought up to 10 μl with H₂O and treated with 1 μl proteinase K (10 mg/ml) at room temp for 2 hours. Products were separated on 1% TAE/agarose gels by electrophoresis and gels were dried and exposed to X-ray films. For analysis of repair junctions, the 2 kb fragment bordering the junction was amplified by PCR, subcloned into a pUC vector, and sequenced by the Sanger method (Genewiz, NJ).

Construction of mutant cells

The following oligonucleotides (IDT, IA) were used for constructing plasmids that express PolQ or TDP2 gRNAs (PAM sequences are in parenthesis and not part of oligos): TTCATATAGGAGTTCATCA(TGG)/TGATGAACTCCTATATGAA(PolQ); ATATAACTGTAGCT(TGG)/GAGTCAGCTACAGTTATAT (TDP2).

Mutant cells were isolated by the HPRT co-targeting method as previously described (Liao et al., 2015). PolQ status was determined by amplifying the target region from wild-type and mutant cells and then Sanger sequencing (Genewiz, NJ). TDP2 status was determined by western blot with an anti-TDP2 antibody (Bethyl Laboratories, TX) against samples prepared from wild-type mice (JAX #006194). Lin-cKIT+ primary cells were isolated by magnetic sorting using the EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit (StemCell) followed by EasySep Mouse CD117 (cKIT) Positive Selection Kit (StemCell), and were subsequently cultured in IMDM + 10% FBS supplemented with a cocktail of growth factors (3 ng/mL IL3, 3 ng/mL IL6, 5 ng/mL SCF). PolQ+/+ and PolQ−/− mESCs and iPSCs were generated in prior studies as described (Mateos-Gomez et al., 2015, 2017).

Synthesis of DNA substrates for cellular MMEJ reporter assay

PCR preparation followed recommended conditions for the Phusion® High-Fidelity DNA Polymerase (New England BioLabs®) with 10ng of the 3kb pCMV-GFP plasmid in 1x Phusion HF Buffer. PCR for the left-flank DNA with 6bp of internal microhomology (PCR1.6I.SA2.) was performed with forward primer RP500B and RP506B. PCR for the right-flank DNA with 6bp of internal microhomology (PCR2.6I.SA2.) was performed with primers RP507B and RP503B. Following PCR, left or right flank DNA constructs were pooled together and digested with DpnI (New England Biolabs®) in 1X CutSmart® buffer and then purified via QIAGEN® QIAquick PCR Purification Kit. Purified PCR was then conjugated to Streptavidin (Sigma) at 110 ng/μl of PCR and 0.8 μg/μl Streptavidin in 10 mM Tris–HCl 7.5, 100 mM NaCl at 37°C for 1 hr. Conjugation was confirmed by resolution in a 0.8% agarose gel stained with ethidium bromide. To synthesize DNA substrates with 6 bp microhomology and a 5'-phosphotyrosine, this procedure was repeated using different left-flank DNA and right-flank DNA for the PCR. PCR for the left-flank DNA was performed with forward primer RP500B and reverse primer RP506-5'-phosphotyrosine (The Midland Certified Reagent Co.). PCR for the right-flank DNA was performed with forward primer RP507-5'-phosphotyrosine (The Midland Certified Reagent Co.) and reverse primer RP503B.

Cellular MMEJ reporter assay

2 x 10⁵ mouse embryonic stem cells were transfected with 0.25 μg each of left and the right flank of GFP in suspension along with 100 ng of pCAGGS-mcherry (https://www.addgene.org/41583/) using Lipofectamine 2000 (Invitrogen). As a negative control, buffer that was used to re-suspend DNA in experimental wells was used for transfection in control wells. For HEK293T, 1 x 10⁵ cells were plated and 24 hours later, 0.25 μg each of left and the right flank of GFP in along with 100 ng of pCAGGS-mcherry was transfected using Lipofectamine 2000. For overexpression of POLOWT (https://www.addgene.org/64875/) as well as POLO-DY2230AA polymerase mutant (https://www.addgene.org/64878/), 1 x 10⁶ cells HEK293T cells were plated and after 24 hours later, 200 ng of either POLOWT or POLO-DY2230AA polymerase mutant was transfected using lipofectamine 2000. 24 hours after transfection of the POLO plasmids, 0.25 μg each of left and the right flank of GFP along with 100 ng of pCAGGS-mcherry was transfected using Lipofectamine 2000. For siRNA experiments, cells were transfected with 20 pmol siRNA along with 0.25 ug each of left and right flank of GFP, 100 ng of mCherry. Positive GFP and RFP cell frequencies were measured 3 days post transfection by flow cytometry using
GUAVA easycyte 5-HT (Luminex corp) in independent replicates. For all the MMEJ reporter assays, the frequency of repair events was normalized using percentage of red fluorescence signal generated by simultaneous transfection with mcherry expression vector (pCAGGS-mcherry). For comparisons Polq+/+ versus Polq−/−, each repair value normalized to transfection efficiency is expressed relative to Polq+/+ (Polq+/+ = 1). In the case of siRNA experiments, each repair value normalized to transfection efficiency is expressed relative to non-targeting siRNA (siControl = 1). Data is represented as the mean and standard error of the mean of two independent experiments, with triplicates per condition per experiment. Statistical analysis was by two sample t test.

RT-qPCR
A portion of mES cells from MMEJ reporter assays performed with siRNA was used for RNA extraction. RNA was extracted using High Capacity cDNA Reverse Transcription Kit (Thermofisher Scientific Catalog # 4368814). Analysis of first-strand cDNA was by Power SYBR Green PCR master mix (Applied Biosystems Catalog # 4367659). An Applied Biosystem StepOne plus PCR System was used for RT-qPCR. We used conventional SYBR green RT-qPCR assays of Gapdh and the siRNA-targeted gene. Primers used for RT–qPCR:

- Mouse BRCA1—sense: CGAGGAAATGGCAACTTGCCTAG;
- Mouse BRCA1—antisense: TCACTCTGCGAGCAGTCTTCAG;
- Mouse POLQ—sense: GTCGAGAGGAGCTTGTTTGC;
- Mouse POLQ—antisense: CGCTTGTTTGTTCCTGTCCC;
- Mouse LIG3—sense: AAG GCA GAC TTT GCT GTG GT;
- Mouse LIG3—antisense: AAT GCT TTG GAA TCG GTT TG;
- Mouse GAPDH—sense: CATCACTGCCACCCAGAAGACTG;
- Mouse GAPDH—antisense: ATGCCAGTGAGCTTCCCGTTCAG.

mRNA was measured in triplicates with a standard curve generated for each gene using cDNA obtained from each sample. The expression level of target genes was normalized to internal Gapdh.

Immunoblotting
A portion of 293T cells from the MMEJ reporter assays performed after overexpression of POLQWT or POLQ-DY2230AA polymerase mutant was used for western blotting analysis. Cells were resuspended in IP lysis buffer (Cat. No: 87787, Thermo scientific, USA) and laemmli buffer was used to make whole-cell protein extracts. Equal amounts (20 μg) whole-cell protein lysates were separated on 4–20% bis tris gels (GenScript) by electrophoresis then transferred onto Protran BA85 nitrocellulose membrane (Whatman, Germany) and immunoblotted with antibodies against Actin (MA-5-11869,1:20000, Invitrogen) or POLQ (PA5-69577,1:500, Invitrogen) overnight followed by secondary antibodies IRDye 800CW (926-32210, 1:10000) or IRDye 680CW (926-68073, 1:10000). Blots were scanned using ODYSSEY software.

QUANTIFICATION AND STATISTICAL ANALYSIS
Two sample t test was used in Figures 1, 4, and S2. Mean, SEM and SD values are shown, p < 0.05 was considered statistically significant. Statistically significant p values and number of replicates are indicated in the Figure legends.
Supplemental information

Polθ promotes the repair of 5′-DNA-protein crosslinks by microhomology-mediated end-joining

Gurushankar Chandramouly, Shuren Liao, Timur Rusanov, Nikita Borisonnik, Marissa L. Calbert, Tatiana Kent, Katherine Sullivan-Reed, Umeshkumar Vekariya, Ekaterina Kashkina, Tomasz Skorski, Hong Yan, and Richard T. Pomerantz
Figure S1

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Ku Marker

| Ku86 - Ku70 | -170 | -116 | -86 | -68 | -56 | -36 | -32 |

B

Left DNA    | Right DNA
---------    |   
~ t         |   

Streptavidin

30' 60' 120' 180'

C

SF2 helicase domain
Central domain
A-family Pol domain

Inactive exonuclease domain

GAGCGATTGATCAGACCATTGG IN EXON 4
CTAACAGATCCATCTTTATCTCTATGAGCGATTGATCAGACCATTGGCTCTCT
CAATTGTGAGACTGAAATATGAAGAATGCTCTTGATGGAGA

HEK 293T POLQ -/- CLONE #4: -1/-1 bp, 1 bp deletion in both alleles:
CAGTCTGCAACATTGAGAGGCAATG--TCTGATCAATCGCCTGATAGAGGAAA
Figure S1. Controls for testing the structural and genetic requirements for MMEJ of DSBs.

(A) Controls showing Ku depletion has no effect on intermolecular DSB repair in Xenopus egg extracts. Western blot showing depletion of Ku (-Ku) and positive control for anti-body and presence of Ku via Western blot of control extracts (Control; % extracts)(top left). SDS protein gel showing purified recombinant Ku proteins (bottom left). Gel showing a time course of DNA end-joining products formed in Xenopus egg extracts from the $^{32}$P-internally labeled DNA substrate indicated at top (right). Supercoiled monomer and relaxed monomer products require the presence of Ku (compare right two panels). Multimers which are due to Polq-dependent MMEJ occur more efficiently in the absence of Ku (second panel from right), indicating competition between NHEJ and MMEJ. -control, mock depleted extracts; -control +Ku, mock depleted extracts with recombinant Ku proteins added; -Ku, Ku depleted extracts; -Ku +Ku, Ku depleted extracts with recombinant Ku added back.

(B) Non-denaturing gel showing left and right DNA MMEJ reporter constructs with and without streptavidin conjugation. Slower migration of DNA demonstrates streptavidin conjugation.

(C) gRNA sequence used to generate POLQ-/- HEK293T cells via CRISPR-Cas9 engineering. Schematic representation of human Polq with protein domains indicated. Approximate location of the gRNA sequence (red) designed from Exon 4 is indicated. The genome sequence flanking the gRNA sequence (red) is shown in grey. POLQ-/- clone #4 was generated by CRISPR-Cas9 engineering and carries a 1 bp deletion in both alleles. Sequence of the region harboring the 1 bp deletion is indicated in blue.
Figure S2. Controls for Pol-dependent MMEJ repair of 5’ DPCs in cells.

(A) Bar plot showing relative GFP following co-transfection of left and right MMEJ reporter DNA constructs conjugated with streptavidin (left) and following co-transfection of left and right MMEJ reporter DNA constructs conjugated with phosphotyrosine (right) in Polq+/+ and Polq-/- mESCs. GFP+ frequencies are normalized to transfection efficiency. Raw data pooled from two separate experiments performed in triplicate for each condition. +/- s.e.m. * P < 0.05, **P<0.01, ***P<0.001. * = statistical significance from two sample t-test between Polq+/+ vs Polq-/-.

(B) Same in POLQ+/+ and POLQ-/- HEK293T cells. GFP+ frequencies are normalized to transfection efficiency. Raw data pooled from two separate experiments performed in triplicate for each condition. +/- s.e.m. * P < 0.05, **P<0.01, ***P<0.001. * = statistical significance from two sample t-test between POLQ+/+ vs POLQ-/-.

(C) Bar plot showing relative GFP following co-transfection of left and right MMEJ reporter DNA constructs conjugated with streptavidin (left), following co-transfection of left and right MMEJ reporter DNA constructs conjugated with phosphotyrosine (right) in Polq+/+ mESCs and following co-transfection of siRNA control, siRNA against LIG3, siRNA against BRCA1 and siRNA against Pol in Polq+/+ mESCs. GFP+ frequencies are normalized to transfection efficiency. Raw data pooled from two separate experiments performed in triplicate for each condition. +/- s.e.m. * P < 0.05, **P<0.01, ***P<0.001. * = statistical significance from two sample t-test between si control vs si Lig3, P = 0.048; si control vs siPolq, P = 0.009 (left), between si control vs si Lig3, P = 0.01; si control vs siPolq, P = 0.02 (right).

(D) Bar plot showing relative GFP following overexpression of indicated plasmids and co-transfection of left and right MMEJ reporter DNA constructs conjugated with streptavidin (left) and following co-transfection of left and right MMEJ reporter DNA constructs conjugated with phosphotyrosine (right) in HEK293T cells. GFP+ frequencies are normalized to transfection efficiency. Raw data pooled from one experiment performed in triplicate for each condition. +/- s.e.m. * P < 0.05, **P<0.01, ***P<0.001. * = statistical significance from two sample t-test between Empty vector vs wtPOLQ. P = 0.002 (left), P = 0.001 (right).

(E) Immunoblot with whole cell extracts of POLQ-/HEK293T cells that were overexpressed with indicated plasmids and used for assay in S2C. Immunoblotting was performed against POLQ antibody (top) and actin (bottom, loading control).

(F) RT qPCR analysis of Lig3, Brca1 and Polq expression. mRNA levels were corrected with internal control for GAPDH in siRNA-treated cells used in Figures 4F and S2C as well as normalized to non-targeting siRNA (siControl = 1). Data represent mean. n = 3 +/- s.e.m. * P < 0.05, **P<0.01, ***P<0.001. Statistical significance was determined from two sample t-test. P values are as follows: siControl vs siLig3 = 0.005; siControl vs siBrca1 = 0.002; siControl vs siPolq = 0.003.
Figure S3  MMEJ repair of 5' DPCs occurs in the absence of BRCA1

(A) Western blots showing the presence and absence of BRCA1 in mock depleted (control) and BRCA1 depleted *Xenopus* egg extracts. % extracts loaded indicated at right.

(B) Non-denaturing gels showing a time course of DSB repair of the indicated DNA substrate in mock depleted (control; left) and BRCA1 depleted (right) *Xenopus* egg extracts.
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