Polymerase θ-helicase efficiently unwinds DNA and RNA-DNA hybrids

Ahmet Y. Ozdemir¹, Timur Rusanov¹, Tatiana Kent¹, Labiba A. Siddique¹, and Richard T. Pomerantz*¹

From the ¹Fels Institute for Cancer Research, Department of Medical Genetics and Molecular Biochemistry, Temple University Lewis Katz School of Medicine, Philadelphia, Pennsylvania, 19140, USA

Running title: Polθ-helicase unwinds DNA

*To whom correspondence should be addressed: Richard T. Pomerantz: Fels Institute for Cancer Research, Department of Medical Genetics and Molecular Biochemistry, Temple University Lewis Katz School of Medicine, Philadelphia, Pennsylvania, 19140, USA; richard.pomerantz@temple.edu; Tel. (215) 707-7623.

Keywords: DNA repair, DNA replication, microhomology-mediated end-joining, alternative end-joining, helicase, polymerase

ABSTRACT

POLQ is a unique multifunctional replication and repair gene that encodes for a N-terminal superfamily 2 helicase and a C-terminal A-family polymerase. Although the function of the polymerase domain has been investigated, little is understood regarding the helicase domain. Multiple studies have reported that Polθ-helicase is unable to unwind DNA. However, it exhibits ATPase activity that is stimulated by single-strand DNA which presents a biochemical conundrum. In contrast to previous reports, we demonstrate that Polθ-helicase (residues 1-894) efficiently unwinds DNA with 3’-5’ polarity, including DNA with 3’ or 5’ overhangs, blunt-ended DNA, and replication forks. Polθ-helicase also efficiently unwinds RNA-DNA hybrids, and exhibits a preference for unwinding the lagging strand at replication forks, similar to related HELQ helicase. Finally, we find that Polθ-helicase can facilitate strand displacement synthesis by Polθ-polymerase, suggesting a plausible function for the helicase domain. Taken together, these findings indicate nucleic acid unwinding as a relevant activity for Polθ in replication repair.

POLQ is a unique gene in higher eukaryotes that encodes for a N-terminal superfamily 2 (SF2) helicase and a C-terminal A-family polymerase with a large central domain that lacks any known enzymatic domain (Fig. 1a)(1-3). Understanding the biochemical activities and cellular functions of Polθ have become a priority because it has been found to be essential for the error-prone double-strand break (DSB) repair pathway known as microhomology-mediated end-joining (MMEJ) or alternative end-joining (alt-EJ)(3-9). Remarkably, Polθ expression has also been shown to be important for the proliferation of cells deficient in the homologous recombination (HR) pathway, such as due to mutations in BRCA1 or BRCA2(4,10). Recent studies additionally demonstrate that Polθ is responsible for random DNA integration into the genomes of mammalian cells, and for T-DNA integration into plant genomes(11-13). In addition to these functions, Polθ was shown to be essential for DSB repair in zebra fish embryos and is involved in replication timing and potentially replication fork repair(10,14,15). Thus, the recent expansion of Polθ studies has revealed multiple essential and important functions for this enigmatic protein in DNA repair and cancer proliferation.

Although multiple studies have begun to elucidate the functions of the polymerase domain (Polθ-polymerase), very little is understood about the helicase domain (Polθ-helicase) which is a SF2 helicase member (Fig. 1a). For example, a seminal report investigating Polθ activities found that the helicase exhibits ATPase activity as predicted from its conserved helicase motifs (i.e. nucleotide
binding, ssDNA binding, and core helicase motifs; Fig. 1a)(16). However, although Polθ-helicase exhibits robust ATPase activity, the study failed to identify any DNA unwinding activity by the enzyme(16). Consistent with this, a more recent study reported that Polθ-helicase is unable to unwind DNA(17). Interestingly, Ceccaldi, et al. reported that Polθ-helicase interacts with RAD51 via specific binding motifs and exhibits anti-recombinase activity due to its ability to counter RAD51 activity(10). Despite these initial findings, the biochemical and cellular functions of Polθ-helicase have yet to be fully elucidated.

Because Polθ-helicase is most closely related to Hel308/HELQ-type and RecQ-type helicases, it likely shares activities with these widely studied groups of motor proteins(1,18). For example, many RecQ helicases exhibit both DNA unwinding and annealing activities(19). Because these mechanisms can compete with one another, they can also mask each other in biochemical assays. For example, in recent studies we found that Polθ-helicase exhibits DNA annealing activity, similar to RecQ-type helicases(20). Specifically, Polθ-helicase promotes ssDNA annealing in an ATP-independent manner in the absence of the ssDNA binding protein RPA(20). However, when RPA is pre-bound to ssDNA Polθ-helicase requires ATP hydrolysis to promote ssDNA annealing(20). These studies link the ATP-dependent annealing activity of the helicase to ALT-EJ by showing that it counteracts RPA to promote end-joining(20)(reviewed in(21)).

Because Polθ-helicase promotes DNA annealing, we envisaged that this activity likely opposes its unwinding function, and if so this would explain why DNA unwinding by the helicase has been difficult to detect. Indeed, here we demonstrate that by masking ssDNA annealing, we observe that Polθ-helicase efficiently unwinds several different types of DNA substrates with 3’-5’ polarity, including replication forks, blunt-ended DNA and DNA with 3’ or 5’ overhangs. We further demonstrate that Polθ-helicase efficiently unwinds RNA-DNA hybrids and preferentially displaces the lagging strand from model replication forks, similar to the related HELQ/Hel308 helicase. These findings suggest Polθ-helicase DNA unwinding contributes to the many activities of Polθ in genome maintenance, and highlight a new activity for this enigmatic multi-functional enzyme.

Results
Polθ-helicase unwinds DNA in an ATP and dATP dependent manner
Considering that Polθ-helicase exhibits annealing activity like related RECO-type helicases(20), it can conceivably rewind DNA after unwinding it which would prevent detection of its unwinding function. We therefore developed an assay that would mask the annealing activity immediately following DNA unwinding by the helicase. Polθ-helicase (residues 1-894) was expressed and purified from E. coli using a N-terminal tandem hexahistidine-SUMO-tag which was subsequently cleaved (Fig. 1b)(20). The purified helicase was incubated with a radio-labeled DNA substrate containing a 3’ ssDNA overhang, referred to as partial ssDNA (pssDNA), in standard buffer conditions in the presence of MgCl$_2$ (Fig. 1c). Next, the ATPase activity of the helicase was initiated by adding ATP along with excess ssDNA trap that is identical to the short strand within the pssDNA substrate. Here, if the helicase unwinds the DNA duplex then the excess unlabeled ssDNA trap will preferentially anneal to the complementary long strand within the pssDNA substrate. Consistent with this, we detected helicase dependent unwinding in the presence of the ssDNA trap (Fig. 1c), and show that excess sequence-specific ssDNA trap is essential for detection of Polθ-helicase unwinding (Supplementary Fig. 1a). To our knowledge, these data are the first to document Polθ-helicase unwinding.

Next, we utilized the optimized unwinding assay to further characterize the enzyme’s unwinding activity on various substrates. Unexpectedly, we observed that the helicase is able to unwind substrates containing 3’ and 5’ overhangs with similar efficiency (compare Fig. 1c and Fig. 1d). Although related SF2 enzymes such as HELQ, also known as Hel308, translocate along ssDNA with a 3’-5’ polarity(18), our data presented insofar fail to reveal a particular polarity exhibited by Polθ-helicase. Nevertheless, we proceeded to determine which nucleotide cofactors support the enzyme’s unwinding activity. The results show that the helicase exclusively utilizes nucleotides containing adenine, but more efficiently unwind DNA in the presence of ATP compared to deoxyribonucleoside triphosphate (dATP)(Fig. 1c). We further find that the Polθ-
Polθ-helicase unwinds DNA

Polθ-helicase preferentially unwinds DNA with 3' overhangs

Although Polθ-helicase demonstrated a similar ability to unwind DNA containing a 3' or 5' ssDNA overhang, it would be unprecedented for such an enzyme to actively translocate along ssDNA in both directions. Thus, an alternative interpretation of the data presented in Figure 1c and 1d is that Polθ-helicase actively translocates along ssDNA with a single polarity, but is capable of initiating unwinding at blunt or 3' recessed ends. To further investigate the enzyme's ATP-dependent directional movement, we assayed unwinding on 3' and 5' overhang substrates that contain longer DNA duplexes in order to increase the energy barrier to unwinding (Fig. 2a). For example, the substrates used in Figure 1 include a duplex region 15 base pairs (bp) in length, whereas the substrates used in the current figure contain 23 bp of double-strand DNA. Importantly, the 23 bp duplex sequence on the 3’ and 5’ overhang substrates is identical to prevent differences in melting temperature, and thus the amount of energy required for unwinding. The results demonstrate that Polθ-helicase unwinds the 3’ overhang substrate, but not the 5’ overhang substrate, indicating a 3’-5’ polarity, similar to related HELQ/Hel308 (Fig. 2a)(18).

The rate of unwinding by the helicase was next examined on multiple substrates to potentially identify its preference for a particular substrate. We utilized identical conditions to assay the enzyme on pssDNA containing 3’ or 5’ overhangs, duplex DNA, and a replication fork (Fig. 2b-2e). Here, again we employed substrates with the same double-strand DNA sequence and thus identical melting temperature. The results show that although the helicase unwinds each substrate under identical conditions, it exhibits the highest rate of unwinding on pssDNA harboring a 3’ overhang, which is consistent with 3’-5’ directional movement along ssDNA (Fig. 2f). We presume the enzyme unwinds the replication fork at a slower rate due to a second enzyme acting on the 5’ overhang that can conceivably impede helicase translocation on the 3’ overhang. Taken together, the results presented insofar in Figure 2 demonstrate that Polθ-helicase preferentially unwinds DNA containing 3’ overhangs, but is also capable of unwinding double-strand DNA, DNA with 5’ overhangs, and replications forks. We note that although the enzyme can unwind blunt-ended DNA substrates, it fails to do so on longer substrates even at relatively high concentrations (Supplementary Fig. 1b). This suggests that multiple Polθ-helicase molecules are unable to act cooperatively to unwind long substrates, as indicated for SF1-type helicase UvrD(22). Because many helicases function with and are stimulated by the ssDNA binding protein RPA, we assessed whether RPA promotes Polθ-helicase unwinding activity in Figure 2g. Here, we determined the efficiency of unwinding the 23 bp duplex substrate by relatively low amounts of either Polθ-helicase, RPA, or both proteins combined. The results show that the addition of both proteins results in synergistic activity which is indicated by a significantly higher yield of unwound DNA (Fig. 2g). Future studies will be required to determine whether RPA stimulation of Polθ-helicase occurs by a specific protein-protein interaction.

Several lines of evidence indicate the involvement of RNA-DNA structures in contributing to both genome instability and DNA repair. For example, R-loops have long been associated with replicative stress and genome instability, whereas more recent work indicates that RNA-DNA hybrids can also promote DNA repair by mechanisms that remain to be elucidated(23-27). Considering the importance of RNA-DNA structures in DNA repair and genome instability, we proceeded to examine whether Polθ-helicase unwinds RNA-DNA duplexes with similar efficiency. Indeed, using identical substrate sequences, our results show that RNA-DNA substrates are also efficiently unwound by Polθ-helicase (Fig. 3a,b). Here again, the enzyme more rapidly unwinds the substrate containing a 3’ overhang (Fig. 3a). We note that the helicase...
Polθ-helicase unwinds DNA shows substantially lower efficiency of unwinding a blunt ended RNA-DNA duplex (Fig. 3c). This is consistent with inefficient unwinding of a blunt ended DNA-DNA duplex (see Fig. 2d). Failure of Polθ-helicase to unwind a RNA-DNA substrate containing a 3' RNA overhang indicates that this enzyme exclusively translocates along ssDNA (Fig. 3d). The helicase also fails to unwind a RNA-RNA substrate which further demonstrates its inability to translocate along RNA (Fig. 3e).

Polθ-helicase efficiently unwinds substrates modeled after stalled replication forks

A previous report demonstrates that mammalian Polθ acts in response to replication stress and promotes replication fork progression or fork stability(10). For example, Polθ was shown to form cellular foci in response to ultraviolet light and confer cellular resistance to hydroxyurea (HU) treatment(10). Furthermore, Polθ was demonstrated to promote replication fork progression in the absence of exogenous DNA damaging agents, and cells deficient in Polθ exhibit a prolonged S phase delay and a significant increase in stalled or collapsed forks following HU treatment(10). Thus, although Polθ has an essential role in alt-EJ, additional lines of evidence suggest it might exhibit separate functions in response to replicative stress, such as replication fork restart(10).

We further examined Polθ-helicase activity on different types of replication forks to provide insight into its potential functions during replication. Time courses of Polθ-helicase unwinding were performed on replication forks containing leading or lagging strands, leading and lagging strands, or a fork lacking leading and lagging strands (Fig. 4a). The results clearly show that the helicase preferentially unwinds the fork containing the lagging strand but lacking the leading strand (Fork B; Fig. 4a). These data suggest a possible function for Polθ-helicase in replication fork repair. For example, following arrest of the leading strand polymerase, such as due to an encounter with a lesion, the replicative helicase is known to continue to unwind the fork resulting in a large leading strand gap. In contrast, the lagging strand polymerase can continue to act on its respective template, generating Okazaki fragments(29). Hence, fork collapse is often modeled as Fork B which specifically lacks a leading strand. We next investigated whether Polθ-helicase more efficiently unwinds the lagging strand which is common among Hel308 type enzymes. Indeed, similar to HELQ/Hel308 activity, we find that Polθ-helicase preferentially unwinds the lagging strand at a replication fork which further supports a potential role in response to replication stress like HELQ/Hel308 (Fig. 4b).

Polθ-helicase promotes strand displacement synthesis by Polθ-polymerase

A unique feature of POLQ is that it is the only known gene in multicellular organisms to encode for a helicase and polymerase. Other known helicase-polymerase fusion proteins are more common in bacteria, archaea, and viruses, and are involved in replication and repair(30). A conceivable function for Polθ-helicase unwinding activity is to facilitate strand displacement synthesis by the Polθ-polymerase domain. For example, although some polymerases exhibit proficient strand displacement activity, which enables DNA unwinding downstream of the 3' primer terminus during replication, many polymerases such as those involved in chromosomal replication require the unwinding activity of auxiliary helicases to perform replication of double-strand DNA. We tested whether Polθ-polymerase exhibits strand displacement activity on a replication fork containing a leading strand in Figure 4c. The results show that the polymerase possesses limited strand displacement activity in the presence of all four dNTPs and ATP as indicated by its inability to fully extend the leading strand primer (Fig. 4c, lane 2). Given that Polθ-helicase exhibits 3'-5' polarity, we evaluated whether it promotes strand displacement activity by the polymerase domain. Indeed, addition of the helicase under identical conditions with ATP facilitates Polθ-polymerase primer extension through the downstream DNA duplex, as indicated by a 4-fold increase in run-off product (Fig. 4c, lane 3). Hence, these data suggest a plausible function for the helicase domain in facilitating Polθ-polymerase strand displacement synthesis during replication repair.

Discussion

Polθ has multiple documented activities in DNA replication and repair, including alt-EJ, replication repair, translesion synthesis, and replication initiation(4-8,10,15,31,32). Although the activities
and cellular functions of Polθ-polymerase have been investigated, little is understood regarding the enzymatic activities of Polθ-helicase. For example, although studies have shown that the helicase exhibits ATPase activity that is stimulated by ssDNA, multiple reports failed to detect a DNA unwinding function which is common among DNA helicases sharing sequence homology to Polθ, such as HELQ/Hel308 and RecQ-type helicases(10,16-18,28,33-35). In this report, we demonstrate that Polθ-helicase exhibits robust DNA unwinding activity with 3′-5′ directionality. The helicase preferentially unwinds DNA substrates containing 3′ ssDNA overhangs, but additionally unwinds substrates with 5′ overhangs, blunt-ended DNA, RNA-DNA hybrids and replication forks. Because Polθ-helicase also performs ssDNA annealing(20), this function counters its unwinding which likely explains why Polθ unwinding has been difficult to detect in previous studies(16,17).

Several lines of evidence have supported a role for Polθ in replication fork repair. For example, a recent report demonstrated that suppression of Polθ significantly slows the velocity of replication forks even in the absence of exogenous DNA damaging agents(10). This report also show that knockdown of Polθ expression impairs fork progression and halts cells in S-phase following HU treatment(10). These data indicate that Polθ either promotes replication fork stability or replication fork repair. Considering that several SF2 helicases, such as HELQ/Hel308 and RecQ subclasses, are involved in replication fork repair, it is not unreasonable to assume a similar function for Polθ-helicase which is closest in relation to HELQ/Hel308(18,28,36-38). For example, prior studies showed that mammalian HELQ/Hel308 is recruited to stalled replication forks and is involved in repairing interstrand crosslinks which arrest replication forks(28,37,38). Similar to HELQ/Hel308, Polθ-helicase unwinds DNA with 3′-5′ polarity and exhibits a preference for unwinding substrates modeled after collapsed replication forks, such as those lacking a leading strand(28,35). We also find that Polθ-helicase is unable to unwind long substrates and thus exhibits non-processive unwinding activity like HELQ/Hel308(18,35). Hence, our studies confirm similar biochemical activities between Polθ-helicase and HELQ/Hel308 which suggests these enzymes perform similar replication repair functions.

Structural and sequence comparisons between Polθ-helicase and Hel308 type enzymes provide further evidence for shared mechanisms of helicase activity (Fig. 5). For example, superposition of Polθ-helicase and the co-crystal structure of Hel308 in complex with partially unwound DNA reveals a similar orientation of the β-hairpin motif, previously shown to act as a wedge to facilitate duplex unwinding by Hel308 (Fig. 5b)(39). Although the sequence of this motif is not closely conserved between Polθ-helicase and HELQ/Hel308 type enzymes (Fig. 5a), superposition of Polθ-helicase and Hel308 suggests the slightly smaller β-hairpin in Polθ facilitates DNA duplex separation by a similar mechanism (Fig. 5b). Another interesting structural similarity between these enzymes is the previously reported auto-inhibitory helix-loop-helix domain 5 which contains a highly conserved Arg-Ala-Arg (RAR) motif (Fig. 5c)(35,39). For instance, a prior report demonstrated that domain 5 within Hel308 suppresses its unwinding activity(35). Specifically, deletion of this domain or mutation of a conserved arginine (R662) in this region, which was shown to interact with extruded ssDNA in the co-crystal structure of Hel308 in complex with partially unwound DNA, resulted in a dramatic increase in helicase activity(35,39). These types of helicase autoinhibitory domains found in both SF1 and SF2 members may be modulated by interacting proteins or specific nucleic acid structures(35). Thus, Polθ-helicase activity may be substantially stimulated by protein or DNA interactions that change the orientation of the autoinhibitory domain. We speculate that the structurally and sequence conserved domain 5 in Polθ-helicase exhibits an autoinhibitory mechanism like Hel308 (Fig. 5c).

Despite the similar unwinding activities between Polθ and HELQ/Hel308, DNA unwinding is countered by the annealing function of Polθ-helicase. For instance, detection of DNA unwinding by Polθ-helicase requires masking the opposite annealing activity by addition of excess ssDNA trap. In contrast, HELQ/Hel308 has been shown to unwind DNA in the absence of a ssDNA trap and therefore does not likely exhibit strong annealing activity like Polθ-helicase(28). Interestingly, other SF2 helicases such as those from the RecQ subclass exhibit ssDNA annealing,
however, in many cases this activity is suppressed by ATP(19). In contrast, the respective annealing activities of Polθ and RECQL5 helicases are not suppressed by ATP, and these enzymes share ~18% sequence homology (1,20). RECQL5 also unwinds DNA with low processivity like Polθ-helicase(40). Despite these similarities, we were unable to detect strand exchange activity by Polθ-helicase which RECQL5 has been shown to exhibit (Supplementary Fig. 1c). Another similar function between Polθ-helicase and RECQL5 is their ability to interact with RAD51 and counteract its activity. For example, both Polθ-helicase and RECQL5 promote dissociation of RAD51-mediated D-loops in vitro, and these enzymes suppresses homologous recombination in cells(10,41). Taken together, Polθ-helicase shares similar characteristics with RECQL5 and HELQ/Hel308.

Because Polθ is known to promote the proliferation of BRCA-deficient cancer cells and is considered a promising oncology drug target, it will be important to determine whether the unwinding function of the helicase domain contributes to cancer cell survival(4,10). For example, although the helicase domain was recently shown to promote alt-EJ via annealing and counteracting RPA, its unwinding activity may also contribute to this pathway(20). For example, Polθ-helicase unwinding may enable microhomology annealing or strand displacement synthesis by Polθ-polymerase during alt-EJ (Fig. 6a). Since Polθ-polymerase exhibits poor strand displacement synthesis, DNA unwinding ahead of the polymerase would be a plausible function for the helicase during alt-EJ (Fig. 6a). Considering that RNA-DNA hybrids have recently been shown to form at DNA breaks, Polθ-helicase dissociation of these structures may also contribute to DSB repair(27).

Importantly, it remains unclear whether alt-EJ independent roles for Polθ-helicase exist and enable the proliferation of BRCA-deficient cells. For example, although a previous report suggested Polθ-helicase suppresses HR via its RAD51 interaction motif(10), a more recent study was unable to confirm this mechanism in cells(20). Because Polθ-helicase exhibits robust unwinding of replication forks, it can conceivably play a compensatory role in BRCA-deficient cells during replication repair. For instance, lagging strand unwinding can contribute to fork reversal and replication restart (Fig. 6b, left). Alternatively, the helicase can potentially promote replication by facilitating strand displacement synthesis by the polymerase domain (Fig. 6b, right). This activity could conceivably aid in replication restart by extending nascent primers. Future studies will be required to further characterize the molecular basis of Polθ-helicase unwinding and determine whether this activity contributes to the many functions of Polθ in replication and repair.

Experimental Methods

Nucleic acid unwinding.

5 nM 5’-32P radiolabeled DNA, RNA-DNA or RNA templates were preincubated at room temperature in buffer (25 mM Heps- NaOH, pH 7.0, 2 mM DTT, 0.01% NP-40, 40 mM KCl, 5% glycerol, 1 mM MgCl2 ) then mixed with the indicated amounts of Polθ-helicase for 5 min. This was followed by the addition of 2 mM ATP and 200 nM ssDNA trap for the indicated times at 30º C in a total volume of 20 µl. Reactions were terminated by the addition of 4 µl of non-denaturing stop buffer (0.2 M Tris-HCl, pH 7.5, 10 mg/ml proteinase K, 100 mM EDTA, and 0.5 % SDS) then resolved in non-denaturing 12% polyacrylamide gels and visualized by phosphorimager (Fujifilm FLA 7000) or autoradiography. For RPA stimulation experiments, the indicated amounts of RPA were pre-incubated with DNA for 5 min, then the indicated amounts of Polθ-helicase were added for an additional 5 min. Reactions were then initiated as above. Unwinding experiments utilizing substrates with a 23 bp duplex were incubated at 37º C.

Sequence alignment.

The indicated amino acid sequences of the helicase domain of Homo sapiens Polθ and other indicated SF2/Ski2 family helicases were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/; European Bioinformatics Institute) default settings. Location and numbers of β-sheets and α-helices are indicated for Polθ-helicase domain based on previous structural analysis(17).

Superposition of Polθ-helicase and Hel308 structures.

The Ca bound form of Hel308 (PDB code 2p6r) was used as reference onto which the Polθ-
Polθ-helicase unwinds DNA

The Polθ-helicase domain (PDB code 5aga) was superimposed using Swiss-PDBViewer(42). Using least-squares fitting option, 1,432 matching atoms were found to superimpose with an RMSD of 1.55 Å. Images were generated with PyMOL software(43).

Polθ strand displacement synthesis.
10 nM 5'-32P radiolabeled DNA pre-incubated at room temperature in 25 mM Tris-HCl, pH 8.8, 1 mM DTT, 0.01% NP-40, 0.1 mg/ml BSA, 10% glycerol, 10 mM MgCl₂ was mixed with or without 50 nM Polθ-helicase. Next, 2 mM ATP and 20 µM dNTPs were added along with 400 nM of unlabeled ssDNA trap for 30 min at 30º C. Polθ-polymerase was added for an additional 20 min in a total volume of 20 µl. Reactions were terminated by the addition of 20 µl of 2x denaturing stop buffer (90% formamide and 50 mM EDTA) then resolved in denaturing urea polyacrylamide gels and visualized by phosphorimager (Fujifilm FLA 7000).

DNA and RNA. Templates are as follows. Figure 1c,e,f,h and Figure 2b, RP469D/RP470D. Figure 1d and Figure 2c, RP469D/RP470D. Figure 2a, AO8/AO1, AO8/AO10. Figure 2g, AO8/AO1. Figure 2d, RP469D/RP469DC. Figure 3a, RP469R/RP470D. Figure 3b, RP469R/RP470R. Figure 2e, RP469D/RP469DC. Figure 3c, RP469R/RP470R. Figure 3d, RP469D/RP469DC. Figure 3e, RP469R/RP470R. Figure 4a, Fork A: RP470D/AO12/RP485, Fork B: RP470D/RP485/AO13, Fork C: RP470D/AO12/RP485/AO13, Fork D: RP470D/RP485. Figure 4b, Leading strand fork: RP470D/AO12/AO18, Lagging strand fork: RP470D/AO12/AO17/AO19. Figure 4c, RP470D/AO12/RP485. DNA and RNA were 32P-5'-radiolabeled with T4 polynucleotide kinase (NEB) and [γ-32P] ATP (Perkin Elmer). Substrates were annealed by mixing a ratio of 1:2 of short to long strands then boiling and slow cooling to room temperature. DNA and RNA oligonucleotides (Integrated DNA Technologies) are as follows (5'-3'). RP469D, CTGGTCGAGCTGAGTACGAGT; RP469R, CUGUCCUGCAUGAUG; RP469DC, CATACA TGCGAGCAGC; RP470D, CATCATGCAGG ACAGTCGAGATGCAGTCAG; RP470R, CAUCAUGCAGCACAGUCGGAUGCAG; RP484, TCGGATCGAGTCAGATCGAGCAG; AO8, CATGCTGTCTAG; AO10, TTTCGAGCTTCTTGCCTCTGTCTGATGCTAG; AO12, CTGACTGACGT; AO13, AGCAAAAGGCGAAG; AO17, TGCTGAGCTTCTTGCCTCTGTCTGATGCTAG; AO18, TGATTGCGATTACTGTCATGATG; AO19, AGCCCTCTAGATGCA.

Proteins. Polθ-helicase, Polθ-polymerase, and RPA were purified as described(20). Ambion RNase inhibitor was purchased from ThermoFisher Scientific.

Acknowledgements: This work was supported by National Institutes of Health grant 1R01GM115472-01 to R.T.P.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: A.Y.O. and T.R. performed experiments and interpreted data. L.A.S. quantitated data and contributed to figure production. T.K. purified proteins and performed structure and sequence analysis. R.T.P. designed experiments, interpreted data and wrote the manuscript.
Polθ-helicase unwinds DNA

References

1. Black, S. J., Kashkina, E., Kent, T., and Pomerantz, R. T. (2016) DNA Polymerase theta: A Unique Multifunctional End-Joining Machine. Genes (Basel) 7
2. Wood, R. D., and Doublie, S. (2016) DNA polymerase theta (POLQ), double-strand break repair, and cancer. DNA repair
3. Sfeir, A., and Symington, L. S. (2015) Microhomology-Mediated End Joining: A Back-up Survival Mechanism or Dedicated Pathway? Trends in biochemical sciences 40, 701-714
4. Mateos-Gomez, P. A., Gong, F., Nair, N., Miller, K. M., Lazzerini-Denchi, E., and Sfeir, A. (2015) Mammalian polymerase theta promotes alternative NHEJ and suppresses recombination. Nature 518, 254-257
5. Yousefzadeh, M. J., Wyatt, D. W., Takata, K., Mu, Y., Hensley, S. C., Tomida, J., Bylund, G. O., Doublie, S., Johansson, E., Ramsden, D. A., McBride, K. M., and Wood, R. D. (2014) Mechanism of suppression of chromosomal instability by DNA polymerase POLQ. PLoS Genet 10, e1004654
6. Chan, S. H., Yu, A. M., and McVey, M. (2010) Dual roles for DNA polymerase theta in alternative end-joining repair of double-strand breaks in Drosophila. PLoS Genet 6, e1001005
7. Kent, T., Chandramouly, G., McDevitt, S. M., Ozdemir, A. Y., and Pomerantz, R. T. (2015) Mechanism of microhomology-mediated end-joining promoted by human DNA polymerase theta. Nature structural & molecular biology 22, 230-237
8. Koole, W., van Schendel, R., Karambelas, A. E., van Heteren, J. T., Okihara, K. L., and Tijsterman, M. (2014) A Polymerase Theta-dependent repair pathway suppresses extensive genomic instability at endogenous G4 DNA sites. Nat Commun 5, 3216
9. Wyatt, D. W., Feng, W., Conlin, M. P., Yousefzadeh, M. J., Roberts, S. A., Mieczkowski, P., Wood, R. D., Gupta, G. P., and Ramsden, D. A. (2016) Essential Roles for Polymerase theta-Mediated End Joining in the Repair of Chromosome Breaks. Molecular cell
10. Ceccaldi, R. L., J.C.; Amunugama, R.; Hajdu, I.; Primack, B.; Petalcorin, M.I.R.;; O’Connor, K. W. K., P.A.; Elledge, S.J.; Boulton, S.J.; Yusufzai, Y.T.; and & D’Andrea, A. D. (2015) Homologous-recombination-deficient tumours are dependent on Polθ-mediated repair. Nature 517
11. Zelensky, A. N., Schimmel, J., Kool, H., Kanaar, R., and Tijsterman, M. (2017) Inactivation of Pol theta and C-NHEJ eliminates off-target integration of exogenous DNA. Nat Commun 8, 66
12. van Kregten, M., de Pater, S., Romeijn, R., van Schendel, R., Hooykaas, P. J., and Tijsterman, M. (2016) T-DNA integration in plants results from polymerase-theta-mediated DNA repair. Nat Plants 2, 16164
13. Saito, S., Maeda, R., and Adachi, N. (2017) Dual loss of human POLQ and LIG4 abolishes random integration. Nat Commun 8, 16112
14. Thyme, S. B., and Schier, A. F. (2016) Polq-Mediated End Joining Is Essential for Surviving DNA Double-Strand Breaks during Early Zebrafish Development. Cell Rep
15. Fernandez-Vidal, A., Guitton-Sert, L., Cadoret, J. C., Drac, M., Schwob, E., Baldacci, G., Cazaux, C., and Hoffmann, J. S. (2014) A role for DNA polymerase theta in the timing of DNA replication. Nat Commun 5, 4285
16. Seki, M., Marini, F., and Wood, R. D. (2003) POLQ (Pol theta), a DNA polymerase and DNA-dependent ATPase in human cells. Nucleic acids research 31, 6117-6126
17. Newman, J. A., Cooper, C. D., Aitkenhead, H., and Gileadi, O. (2015) Structure of the Helicase Domain of DNA Polymerase Theta Reveals a Possible Role in the Microhomology-Mediated End-Joining Pathway. Structure 23, 2319-2330
18. Marini, F., and Wood, R. D. (2002) A human DNA helicase homologous to the DNA cross-link sensitivity protein Mus308. The Journal of biological chemistry 277, 8716-8723
19. Khadka, P., Croteau, D. L., and Bohr, V. A. (2016) RECQL5 has unique strand annealing properties relative to the other human RecQ helicase proteins. DNA repair 37, 53-66
Polθ-helicase unwinds DNA

20. Mateos-Gomez, P. A., Kent, T., Deng, S. K., McDevitt, S., Kashkina, E., Hoang, T. M., Pomerantz, R. T., and Sfeir, A. (2017) The helicase domain of Polθthata counteracts RPA to promote alt-NHEJ. Nature structural & molecular biology 24, 1116-1123

21. Campbell, J. L., and Li, H. (2017) Polθthata helicase: drive or reverse. Nature structural & molecular biology 24, 1007-1008

22. Runyon, G. T., and Lohman, T. M. (1989) Escherichia coli helicase II (uvrD) protein can completely unwind fully duplex linear and nicked circular DNA. The Journal of biological chemistry 264, 17502-17512

23. Sollier, J., Stork, C. T., Garcia-Rubio, M. L., Paulsen, R. D., Aguilera, A., and Cimprich, K. A. (2014) Transcription-coupled nucleotide excision repair factors promote R-loop-induced genome instability. Molecular cell 56, 777-785

24. Keskin, H., Shen, Y., Huang, F., Patel, M., Yang, T., Ashley, K., Mazin, A. V., and Storici, F. (2014) Transcript-RNA-templated DNA recombination and repair. Nature 515, 436-439

25. Aguilera, A., and Garcia-Muse, T. (2012) R loops: from transcription byproducts to threats to genome stability. Molecular cell 46, 115-124

26. Plosky, B. S. (2016) The Good and Bad of RNA:DNA Hybrids in Double-Strand Break Repair. Molecular cell 64, 643-644

27. Ohle, C., Tesorero, R., Schermann, G., Dobrev, N., Sinning, I., and Fischer, T. (2016) Transient RNA-DNA Hybrids Are Required for Efficient Double-Strand Break Repair. Cell 167, 1001-1013 e1007

28. Tafel, A. A., Wu, L., and McHugh, P. J. (2011) Human HEL308 localizes to damaged replication forks and unwinds lagging strand structures. The Journal of biological chemistry 286, 15832-15840

29. Pages, V., and Fuchs, R. P. (2003) Uncoupling of leading- and lagging-strand DNA replication during lesion bypass in vivo. Science 300, 1300-1303

30. Guilliam, T. A., Keen, B. A., Brissett, N. C., and Doherty, A. J. (2015) Primase-polymerases are a functionally diverse superfamily of replication and repair enzymes. Nucleic acids research 43, 6651-6664

31. Hogg, M., Seki, M., Wood, R. D., Doublie, S., and Wallace, S. S. (2011) Lesion bypass activity of DNA polymerase theta (POLQ) is an intrinsic property of the pol domain and depends on unique sequence inserts. J Mol Biol 405, 642-652

32. Yoon, J. H., Roy Choudhury, J., Park, J., Prakash, S., and Prakash, L. (2014) A role for DNA polymerase theta in promoting replication through oxidative DNA lesion, thymine glycol, in human cells. The Journal of biological chemistry 289, 13177-13185

33. Fairman-Williams, M. E., Guenther, U. P., and Jankowsky, E. (2010) SF1 and SF2 helicases: family matters. Curr Opin Struct Biol 20, 313-324

34. Zhang, L., Xu, T., Maeder, C., Bud, L. O., Shanks, J., Nix, J., Guthrie, C., Pleiss, J. A., and Zhao, R. (2009) Structural evidence for consecutive Hel308-like modules in the spliceosomal ATPase Brr2. Nature structural & molecular biology 16, 731-739

35. Richards, J. D., Johnson, K. A., Liu, H., McRobbie, A. M., McMahon, S., Oke, M., Carter, L., Naismith, J. H., and White, M. F. (2008) Structure of the DNA repair helicase hel308 reveals DNA binding and autoinhibitory domains. The Journal of biological chemistry 283, 5118-5126

36. Croteau, D. L., Popuri, V., Opresko, P. L., and Bohr, V. A. (2014) Human RecQ helicases in DNA repair, recombination, and replication. Annual review of biochemistry 83, 519-552

37. Takata, K., Reh, S., Tomida, J., Person, M. D., and Wood, R. D. (2013) Human DNA helicase HELQ participates in DNA interstrand crosslink tolerance with ATR and RAD51 paralogs. Nat Commun 4, 2338

38. Adelman, C. A., Lolo, R. L., Birkbak, N. J., Murina, O., Matsuzaki, K., Horejsi, Z., Parmar, K., Borel, V., Skehel, J. M., Stamp, G., D’Andrea, A., Sartori, A. A., Swanton, C., and Boulton, S. J. (2013) HELQ promotes RAD51 parologue-dependent repair to avert germ cell loss and tumorigenesis. Nature 502, 381-384
39. Buttner, K., Nehring, S., and Hopfner, K. P. (2007) Structural basis for DNA duplex separation by a superfamily-2 helicase. *Nature structural & molecular biology* **14**, 647-652

40. Popuri, V., Huang, J., Ramamoorthy, M., Tadokoro, T., Croteau, D. L., and Bohr, V. A. (2013) RECQL5 plays co-operative and complementary roles with WRN syndrome helicase. *Nucleic acids research* **41**, 881-899

41. Hu, Y., Raynard, S., Sehorn, M. G., Lu, X., Bussen, W., Zheng, L., Stark, J. M., Barnes, E. L., Chi, P., Janscak, P., Jasins, M., Vogel, H., Sung, P., and Luo, G. (2007) RECQL5/Recql5 helicase regulates homologous recombination and suppresses tumor formation via disruption of Rad51 presynaptic filaments. *Genes & development* **21**, 3073-3084

42. Guex, N., and Peitsch, M. C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **18**, 2714-2723

43. DeLano, W. L. (2002) Unraveling hot spots in binding interfaces: progress and challenges. *Curr Opin Struct Biol* **12**, 14-20

**Figure legends**

**Figure 1** Polθ-helicase unwinds DNA in an ATP and dATP dependent manner

A. Schematic of Polθ. B. Denaturing SDS gel of purified Polθ-helicase. C. Schematic of unwinding assay (left). Non-denaturing gel showing Polθ-helicase unwinding of the indicated DNA substrate with a 3’ ssDNA overhang (right). D. Non-denaturing gel showing Polθ-helicase unwinding of the indicated DNA substrate with a 5’ ssDNA overhang. E,F Non-denaturing gels showing Polθ-helicase DNA unwinding in the presence of the indicated nucleotides. % unwinding is indicated. G. SDS gel of purified Polq-helicase K121M (left). Non-denaturing gel showing the lack of Polθ-helicase K121M DNA unwinding (right). *, 32P.

**Figure 2** Polθ-helicase preferentially unwinds DNA with 3’ overhangs

A. Non-denaturing gels showing Polθ-helicase unwinding of DNA substrates containing 3’ (left) and 5’ (right) ssDNA overhangs. B-E. Non-denaturing gel showing a time course of Polθ-helicase unwinding of the indicated DNA substrates. F. Plot showing rate of Polθ-helicase unwinding of the DNA substrates from panels B-E. Data represent mean, n = 3 ±s.d. G. Non-denaturing gel showing RPA stimulation of Polθ-helicase unwinding (left). Bar chart showing % unwinding by indicated proteins (right). n = 3 ±s.d. *** = < 0.001 p value , student’s unpaired t-test. % unwinding is indicated. *, 32P.

**Figure 3** Polθ-helicase unwinds RNA-DNA hybrids

A-E. Non-denaturing gels showing a time course of Polθ-helicase unwinding of the indicated RNA-DNA and RNA-RNA substrates. Gray line, RNA oligonucleotide. Black line, DNA oligonucleotide. % unwinding is indicated. *, 32P.

**Figure 4** Polθ-helicase unwinding activity at replication forks

A. Non-denaturing gel showing a time course of Polθ-helicase unwinding of the indicated DNA replication fork substrates. B. Non-denaturing gel showing a time course of Polθ-helicase unwinding of the indicated DNA replication fork substrates containing leading (left) or lagging (right) strands. % unwinding is indicated. Plot of % unwinding data from left and middle panels (right). C. Denaturing gel showing Polθ-polymerase leading strand extension in the presence (lane 3) and absence (lane 2) of Polθ-helicase. % run-off product is indicated. *, 32P.
**Figure 5** Sequence and structural comparison of Polθ-helicase and HELQ/Hel308 enzymes

A. Sequence alignment of motifs IVa-VI of Polθ/Hel308/Ski2 related SF2 helicases. β-hairpin, motifs IV-VI, and secondary structures are indicated. * = identical residues, : = residues sharing very similar properties, . = residues sharing some properties, red = small and hydrophobic, blue = acidic, magenta = basic, green = hydroxyl, sulphhydryl, amine. Sequences were aligned using clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) default settings. B. Superposition of Polθ-helicase (Green; PDB 5AGA) and Hel308 in complex with DNA (Blue; PDB 2p6r) highlighting the β-hairpin motif. C. Superposition of Polθ-helicase (Green; PDB 5AGA) and Hel308 (Blue; PDB 2va8) highlighting the conserved Ala-Arg-Ala (RAR) motif in domain 5. Conserved arginines are represented as sticks.

**Figure 6** Models of Polθ-helicase activity during replication and repair

A. Model of Polθ-helicase activity during alt-EJ. Polθ-helicase dissociates RPA in an ATP-dependent manner, then facilitates DNA synapse/annealing and Polθ-polymerase strand displacement synthesis. Polθ-helicase unwinding may also enable microhomology annealing. B. Models of Polθ-helicase activity during replication fork repair. Polθ-helicase performs lagging strand unwinding at collapsed replication forks (left). Polθ-helicase DNA unwinding enables strand displacement synthesis by Polθ-polymerase during replication restart (right).
Fig. 2

Panel A: Gel electrophoresis showing unwinding of DNA by Polθ helicase at different concentrations. The graph shows % unwinding over time.

Panel B: Similar experiment as panel A but with 15 bp DNA.

Panel C: Experiment as in panel A but with 15 bp DNA.

Panel D: Experiment as in panel A but with 15 bp DNA.

Panel E: Experiment as in panel A but with 15 bp DNA.

Panel F: Graph showing time course of unwinding with different initial distances.

Panel G: Experiment showing % unwinding with Polθ-helicase and hRPA at different concentrations.
Fig. 4

A

Fork A

\[3' \rightarrow 5'\]

15 bp

Fork B

\[3' \rightarrow 5'\]

15 bp

Fork C

\[3' \rightarrow 5'\]

15 bp

Fork D

\[3' \rightarrow 5'\]

15 bp

B

Leading strand fork

\[3' \rightarrow 5'\]

15 bp

Lagging strand fork

\[3' \rightarrow 5'\]

15 bp

C

Pol\(\beta\)-Helicase

Pol\(\beta\)-Polymerase

Run-off product

Primer

5.7 22.3 \% Run-off
Fig. 5

A

HsPolγ 414 AFHACVEEEQDI12GAFHQLLVLAGDASAARHRL175QFDYAGTILTYGQ+GAGGTVYSELICK 497
DmHus308 545 AFHACVEEEQDI12GAFHQLLVLAGDASAARHRL175QFDYAGTILTYGQ+GAGGTVYSELICTCN 628
HsHel308 641 AYHAKGLLEKRERAYTVCLFLCCPLLAAGDVRLALAILAFLAPF+DVEFSFPAQYLGACAGTSELILLQ 724
AfHel308 301 AFHAGLIGNQADTVAAPYKIN13VPVAVGETALAGTVKVKLAVLAFSYYGTYREDVYQRGQGECHEGAAVIVG 387
Pa0592 298 AFHACVEEQLVEQGFVLKLDLCFLCQK13RATTVVTVQGK13RATTVVTVQGK13RATTVVTVQGK 385
yMrR4 473 GILHGLPL13KTV13GVESGLASYARTTVTVMGQQFFWGGXYG13GAGGGLDKIVMD1R13 560
hMrR4 453 GILHGLPL13KTV13GVESGLASYARTTVTVMGQQFFWGGXYG13GAGGGLDKIVMD1R13 540
ySK12 693 AAVHFLP13KTV13GVESGLASYARTTVTVMGQQFFWGGXYG13GAGGGLDKIVMD1R13 780
hSK12 633 GILHGLPL13KTV13GVESGLASYARTTVTVMGQQFFWGGXYG13GAGGGLDKIVMD1R13 720

B

Hel308

DNA duplex

Polθ-helicase

C

Hel308

Polθ-helicase
Fig. 6

A

RPA
Polθ-polymerase
Polθ-helicase

RPA dissociation, DNA synapse formation

DNA unwinding, polymerase extension of paired overhang

Flap cleavage, ligation

Alt-EJ

B

Lagging strand unwinding

Fork progression

Polθ-polymerase
Polθ-helicase
**Polymerase θ–helicase efficiently unwinds DNA and RNA-DNA hybrids**
Ahmet Yûnus Ozdemir, Timur Rusanov, Tatiana Kent, Labiba A Siddique and Richard T Pomerantz

*J. Biol. Chem.* published online February 14, 2018

Access the most updated version of this article at doi: [10.1074/jbc.RA117.000565](https://doi.org/10.1074/jbc.RA117.000565)

**Alerts:**
- When this article is cited
- When a correction for this article is posted

[Click here](https://www.jbc.org/Alerts) to choose from all of JBC’s e-mail alerts