

A novel role of the Dna2 translocase function in DNA break resection

Adam S. Miller,^{1,4} James M. Daley,^{1,4} Nhung Tuyet Pham,² Hengyao Niu,³ Xiaoyu Xue,¹ Grzegorz Ira,² and Patrick Sung¹

¹Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06520, USA; ²Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030, USA; ³Molecular and Cellular Biochemistry Department, Indiana University, Bloomington, Indiana 47405, USA

DNA double-strand break repair by homologous recombination entails nucleolytic resection of the 5' strand at break ends. Dna2, a flap endonuclease with 5'–3' helicase activity, is involved in the resection process. The Dna2 helicase activity has been implicated in Okazaki fragment processing during DNA replication but is thought to be dispensable for DNA end resection. Unexpectedly, we found a requirement for the helicase function of Dna2 in end resection in budding yeast cells lacking exonuclease 1. Biochemical analysis reveals that ATP hydrolysis-fueled translocation of Dna2 on ssDNA facilitates 5' flap cleavage near a single-strand–double strand junction while attenuating 3' flap incision. Accordingly, the ATP hydrolysis-defective dna2-K1080E mutant is less able to generate long products in a reconstituted resection system. Our study thus reveals a previously unrecognized role of the Dna2 translocase activity in DNA break end resection and in the imposition of the 5' strand specificity of end resection.

[*Keywords:* DNA repair; double-strand break; end resection; homologous recombination]

Supplemental material is available for this article.

Received December 26, 2016; revised version accepted February 17, 2017.

DNA double-strand breaks (DSBs) are highly cytotoxic and can cause gross chromosomal rearrangements, including translocations and deletions, which can in turn lead to oncogenesis (Stephens et al. 2009, 2011; Liu et al. 2012; Jeggo and Lobrich 2015). Thus, ensuring that DSBs are repaired accurately is of critical importance to the maintenance of genome integrity and cancer avoidance. Eukaryotic cells possess two major DSB repair pathways. Nonhomologous end-joining (NHEJ) entails religation of the break ends, whereas homologous recombination (HR) requires a homologous DNA template, usually the sister chromatid, to guide accurate repair (San Filippo et al. 2008; Chiruvella et al. 2013; Mehta and Haber 2014). HR provides a major tool for DSB and DNA replication fork repair in the S and G2 phases of the cell cycle when the sister chromatid is available. NHEJ is the default DSB repair mechanism in G1 cells and is also operational during the other cell cycle phases (Karathanasis and Wilson 2002).

In HR, the 5' strands of the DSB ends are nucleolytically resected, leading to the formation of 3' ssDNA tails (Supplemental Fig. S1A; Daley et al. 2015; Symington 2016). These DNA tails become coated by the ssDNA-binding protein replication protein A (RPA), which is subsequently replaced by the Rad51 recombinase to form a he-

lical polymer known as the presynaptic filament, a process that is enhanced by HR mediator proteins such as yeast Rad52 and human BRCA2 (Prakash et al. 2015). The presynaptic filament then functions together with other accessory factors—e.g., the DNA motor protein Rad54—to mediate a search for DNA homology in the sister chromatid. DNA strand invasion occurs upon the location of DNA homology to yield a DNA joint called the displacement loop (D loop) (Sung et al. 2003). This is followed by DNA synthesis within the nascent D loop and resolution of the resulting DNA intermediates via one of several mechanistically distinct pathways to yield noncrossover or crossover recombinants (San Filippo et al. 2008).

Aside from being an integral step in HR, DNA end resection helps determine repair pathway choice commitment, as resected DNA ends are not readily amenable to NHEJ (Daley et al. 2005). Extensive studies in the past several years have shown that DNA end resection is subject to intricate regulation that occurs in a cell cycle-dependent manner (Daley et al. 2015; Symington 2016). As shown initially in yeast, several nucleases function in end resection. The Mre11–Rad50–Xrs2 (MRX) complex initiates the resection process together with Sae2 by

⁴These authors contributed equally to this work.

Corresponding authors: patrick.sung@yale.edu, james.daley@yale.edu, gira@bcm.edu

Article published online ahead of print. Article and publication date are online at <http://www.genesdev.org/cgi/doi/10.1101/gad.295659.116>.

© 2017 Miller et al. This article is distributed exclusively by Cold Spring Harbor Laboratory Press for the first six months after the full-issue publication date (see <http://genesdev.cshlp.org/site/misc/terms.xhtml>). After six months, it is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at <http://creativecommons.org/licenses/by-nc/4.0/>.

incising the 5' DNA strand near the DSB end via the Mre11 endonuclease activity (Supplemental Fig. S1A; Huertas et al. 2008; Garcia et al. 2011; Cannavo and Cejka 2014). Mre11 also possesses 3'-to-5' exonuclease activity, allowing it to convert the DNA nick into a gap (Paull and Gellert 1998; Cannavo and Cejka 2014), which creates the entry point for two partially redundant nucleases, exonuclease 1 (Exo1) and Dna2, to mediate long-range resection (Supplemental Fig. S1A; Gravel et al. 2008; Mimitou and Symington 2008; Zhu et al. 2008). Exo1 is a 5'-to-3' exonuclease, whereas Dna2 is an endonuclease that acts on the 5' flap generated as a result of DNA unwinding by the Sgs1 helicase (Bae et al. 1998; Budd et al. 2000; Cejka et al. 2010; Niu et al. 2010).

Dna2, a member of the SF I family of helicases, possesses ATP hydrolysis-dependent 5'-to-3' DNA helicase activity. Dna2 was initially identified as a helicase involved in DNA replication (Kuo et al. 1983; Budd and Campbell 1995), and genetic and biochemical analysis suggests that its main function in replication relates to cleavage of 5' DNA flaps during Okazaki fragment maturation in parallel with Rad27/FEN-1 (Bae et al. 1998, 2002; Bae and Seo 2000; Budd et al. 2000; Lee et al. 2000; Cannavo et al. 2013; Pinto et al. 2016). Mutation of the conserved Walker A lysine residue K1080, which is needed for ATP binding, to glutamate (K1080E mutation) abolishes ATPase and helicase activities (Budd et al. 1995). Cells harboring the *dna2-K1080E* mutation are inviable unless the DNA helicase Pif1 is also absent (Bae et al. 2002; Budd et al. 2006). Based on this observation, it has been suggested that Dna2 cleaves the DNA flap generated as a result of Okazaki fragment unwinding by Pif1 (Ryu et al. 2004; Budd et al. 2006). The helicase activity of Dna2 has been implicated in the processing of Okazaki fragment-derived DNA flaps that contain secondary structure (Bae et al. 2002) and in unwinding G4 quadruplex DNA present at telomeres (Masuda-Sasa et al. 2008). However, existing evidence suggests that the Dna2 helicase activity is not needed for DSB end resection. Specifically, yeast cells harboring the *dna2-R1253Q* helicase mutation can resect DSB ends at the normal rate (Zhu et al. 2008). In apparent concordance with this observation, the helicase activity of Dna2 appears to be dispensable for resection in reconstituted systems consisting of purified proteins (Cejka et al. 2010; Niu et al. 2010).

Here, we present new evidence that ATP hydrolysis by Dna2 is in fact germane for DSB end resection. First, we show that the *dna2-K1080E* mutation causes a major resection defect in cells lacking Exo1, which rely on the Sgs1–Dna2 pathway for long-range resection. Importantly, biochemical experiments show that ATP alters the DNA cleavage pattern in a manner indicative of ATP hydrolysis-driven Dna2 translocation in the 5'-to-3' direction prior to strand incision. In our reconstituted resection system containing Sgs1 and RPA, while Dna2 generates a wide range of cleavage products between 12 and 100 nucleotides (nt) in length, the K1080E mutant produces only short products of ≤ 12 nt. Based on these findings, we present a model in which ATP hydrolysis fuels the translocation of Dna2 on ssDNA flaps in the 5'-to-3' direction. On a 5'

flap, Dna2 moves toward the ss–dsDNA junction and incises DNA when it pauses near the junction. This same activity induces the enzyme to translocate off the end of a 3' flap, which is expected to help impose the 5'-to-3' polarity of end resection in cells. Our results thus reveal novel roles of the DNA translocase activity of Dna2 in enhancing the efficiency and enforcing the polarity of DNA break end resection in eukaryotic cells.

Results

The dna2-K1080E mutation engenders a DNA end resection defect in exo1 cells

In the Dna2-dependent pathway of long-range DNA end resection, the Sgs1 helicase separates the strands in duplex DNA to create a forked “Y” structure. The 5' strand in the fork is cleaved endonucleolytically by Dna2, yielding a 3' ssDNA tail that becomes coated by RPA. Whereas the helicase activity of Sgs1 is essential for strand unwinding, the helicase-dead *dna2-K1080E* mutant is capable of efficient resection in vitro (Cejka et al. 2010; Niu et al. 2010). Another helicase-defective mutant, *dna2-R1253Q*, has been shown to resect an HO-induced DSB in vivo at nearly the wild-type level (Zhu et al. 2008). These results have led to the view that the helicase activity of Dna2 is important for Okazaki fragment processing but dispensable for DSB end resection. We sought to re-examine this issue by probing for differences in resection in the absence of Dna2 ATPase activity using both genetic and biochemical approaches.

We first asked whether a resection defect would be revealed in the *dna2-K1080E* mutant in the *exo1* background. In the absence of Exo1, cells must rely solely on the Sgs1–Dna2 pathway for long-range resection. Since *dna2* mutations, including *dna2-K1080E*, are lethal in yeast (Bae et al. 2002), these experiments were performed in the *pif1-m2* mutant background that suppresses lethality of *dna2-K1080E* (Budd et al. 2006). Long-range resection was monitored 10 kb away from the HO endonuclease-induced DSB site. Consistent with previous observations, we saw negligible delay at 10 kb in the *dna2-K1080E* mutant (Fig. 1A), while the deletion of *EXO1* led to a significant resection defect (Zhu et al. 2008). Importantly, the long-range resection deficit in *exo1* cells became greatly exacerbated by the *dna2-K1080E* mutation (Fig. 1A). Moreover, combining the *exo1* and *dna2-K1080E* mutations resulted in a synergistic increase in cellular sensitivity to the topoisomerase I inhibitor camptothecin, which induces DSBs that are reliant on HR for repair, and to hydroxyurea, which induces stalled replication forks that require HR for timely restart (Fig. 1B). These results thus reveal a previously unrecognized requirement for the Dna2 ATPase activity in resection.

Bimodal regulation of Dna2-mediated flap cleavage by ATP

Early biochemical studies of the *dna2-K1080E* mutant found that it possesses the wild-type level of nuclease

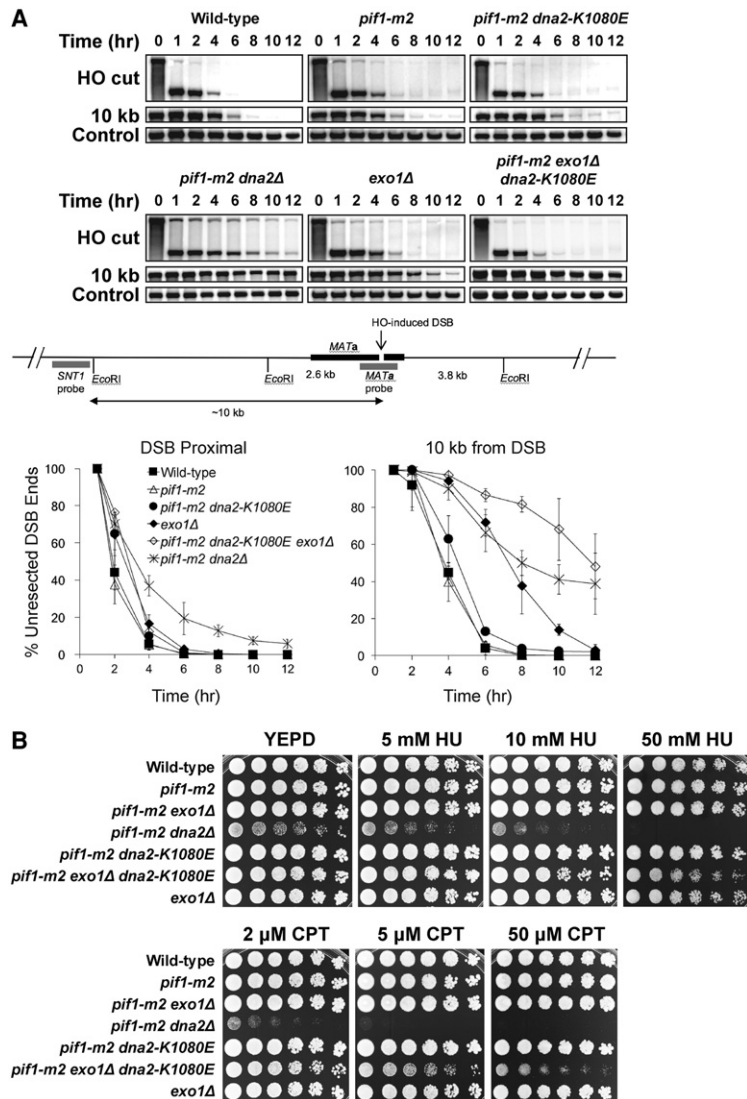


Figure 1. DNA end resection defect and drug sensitivity of *dna2-K1080E exo1* cells. (A) Resection in cells of the indicated genotype was monitored by Southern blotting (Zhu et al. 2008) at the HO cut site and 10 kb away. Quantitation showing the extent of resection, with the signal being normalized against that of the *TRA1* control locus. Experiments were done in triplicate, and error bars represent one standard deviation. (B) The *dna2-K1080E* mutation causes sensitivity to hydroxyurea and camptothecin in the *exo1* background. Cells of the indicated genotypes were tested.

activity (Budd et al. 1995). Results from our laboratory corroborate this conclusion within the context of our reconstituted system of DNA end resection (Niu et al. 2010). Given the resection defect revealed in the above genetic experiments, however, we sought to re-examine whether and how the *dna2-K1080E* mutation impacts DNA end resection biochemically. We purified wild-type Dna2 and the K1080E mutant protein as reported previously (Niu et al. 2010) and analyzed their activity on DNA substrates that model resection intermediates. We specifically assessed early time points in these experiments to minimize secondary cleavage of primary nuclease products. We first used a Y-shaped substrate with 44-nt flaps and a 31-base-pair duplex region, which mimics a DSB end that has undergone DNA strand separation (Fig. 2A). To assess DNA cleavage, the 5' end of the 5' flap strand was labeled with ^{32}P . In the absence of ATP, Dna2 efficiently cleaved the labeled flap strand, generating products in the range of 2–13 nt (Fig. 2A). When ATP was included in the reaction, novel cleavage prod-

ucts in the range of ~35–40 nt were generated in addition to the shorter products seen without ATP (Fig. 2A). Notably, increasing the 5' flap length to 59 nt led to even longer products in the range of ~50 nt (Fig. 2C). Thus, a major cleavage site in a 5' flap strand of 44 or 59 nt is ~9 nt from the ss-dsDNA junction.

Long 5' flap cleavage products were seen upon substitution of ATP with dATP but not when ADP, other nucleotides, and nonhydrolyzable ATP analogs were used (Supplemental Fig. S2A). Labeling of the 3' terminus of the duplex region revealed that Dna2 also acts on sites in the dsDNA region in the presence of ATP, although the majority of the incision sites remains in the 5' ssDNA flap region (Supplemental Fig. S2B). These data suggest that Dna2 is capable of catalyzing strand separation in the duplex region before cleavage occurs. We also found that a 3' flap strand is not necessary for the ATP-dependent shift in 5' flap incision sites, as Dna2 displayed a similar incision pattern on a partial duplex that harbors a 5' overhang only (Supplemental Fig. S2C).

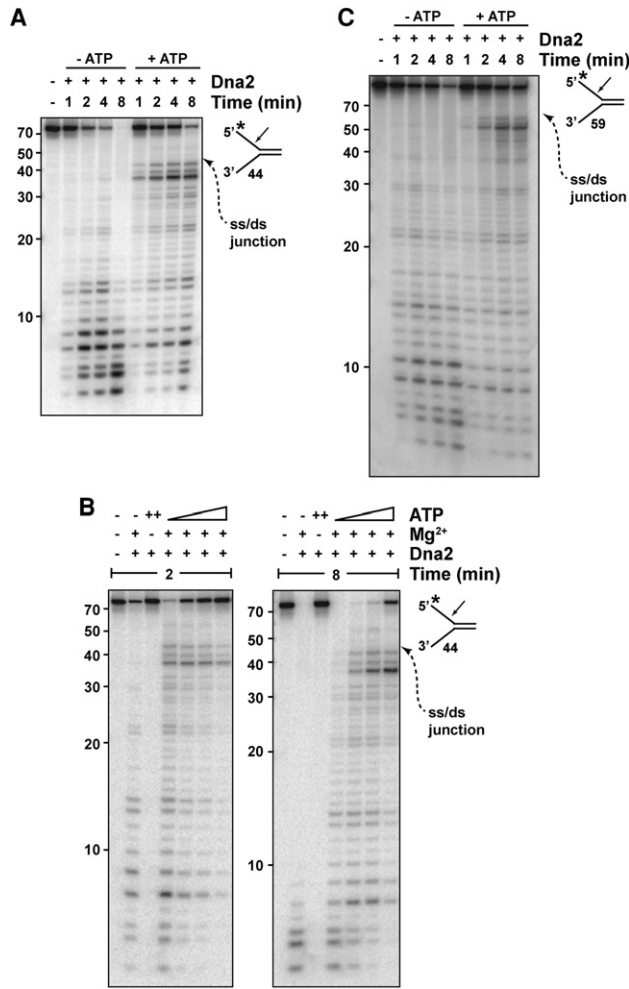


Figure 2. Regulation of Dna2 activity by ATP. (A) Dna2 (10 nM) was incubated with 5 nM Y substrate containing 44-nt overhangs with or without 1 mM ATP for the indicated times. The asterisk denotes the location of the radiolabel, and the arrow marks the position where a 44-nt ssDNA product would be located. (B) Dna2 activity was monitored as in A but with 0.5, 1, 2, or 4 mM ATP. The reactions were incubated for 2 min (left panel) and 8 min (right panel). (C) Same as in A, except that the Y substrate harbored 59-nt overhangs. See also Supplemental Figure S2.

The above observations are consistent with Dna2 translocating in the 5'-to-3' direction in an ATP hydrolysis-dependent manner until it reaches the vicinity of the ssDNA-dsDNA junction, where Dna2 pauses, creating a time window for DNA flap cleavage to occur. In the absence of ATP, Dna2 can still engage the 5' flap but does not translocate, and repeated cycles of DNA engagement and cleavage lead to the formation of the short products observed in Figure 2A. This model makes the prediction that the *dna2-K1080E* mutant protein, which cannot hydrolyze ATP (Supplemental Fig. S3A; Budd et al. 1995), would not generate the large cleavage products seen with the wild-type enzyme. Indeed, *dna2-K1080E* produced only short cleavage products on the 44-nt 5' flap substrate with or without ATP being present (Fig. 3A).

In cells, the 5' strand of a DSB end is resected rapidly, but the 3' strand remains remarkably stable. This is surprising in view of the fact that Dna2 can cleave both 5' and 3' flaps *in vitro* (Bae et al. 1998; Cejka et al. 2010; Niu et al. 2010). Previous studies have shown that the ssDNA-binding protein RPA helps impose the 5' polarity of DNA end resection by enhancing 5' flap incision while attenuating 3' flap cleavage (Bae et al. 2003; Stewart et al. 2008; Cejka et al. 2010; Niu et al. 2010). We considered the possibility that ATP hydrolysis by Dna2 would attenuate 3' flap cleavage by fueling the translocation of Dna2 to facilitate its dissociation from the 3' flap terminus. To test this idea, we assessed the action of Dna2 on a Y structure with 44-nt flaps in which the 3' end of the 3' flap strand was labeled with ^{32}P (Fig. 3B). Dna2 was able to cleave the 3' flap efficiently in the absence of ATP, producing mainly 3- to 6-nt products (Fig. 3B). However, 3' flap cleavage became strongly attenuated upon ATP addition (Fig. 3B). Similar results were obtained when the radiolabel was moved to the 5' terminus of the duplex region of the Y structure (Supplemental Fig. S3B). Thus, ATP-fueled translocation of Dna2 on ssDNA minimizes the incision of 3' flaps and is therefore expected to help enforce the 5' strand specificity of DSB end resection in cells.

Next, we asked how RPA might influence flap cleavage by Dna2 in the presence of ATP. Importantly, ATP still induced the formation of long cleavage products from the Y structure with flap strands of 44 nt in a reaction that contained RPA (Fig. 3C). Consistent with published data (Cejka et al. 2010; Niu et al. 2010), RPA enhanced 5' flap cleavage, leading to the accelerated disappearance of the substrate (Fig. 3C). Notably, the ~35- to 40-nt cleavage products were less stable with RPA being present, which very likely stemmed from the stimulation of secondary cleavage events by RPA (Fig. 3C). These results indicate that RPA does not interfere with the translocation of Dna2 on DNA significantly. This is consistent with the deduction that Dna2-RPA interaction facilitates reciprocal exchange of the two factors during resection (Stewart et al. 2008; Zhou et al. 2015).

Deficiency of dna2-K1080E in a reconstituted resection system

Finally, we ascertained the importance of the Dna2 translocase function in resecting 5' ssDNA created by Sgs1-mediated unwinding in a reconstituted system. This reaction context is more physiologically relevant because (1) the ssDNA strands generated by Sgs1 are homologous and thus have the ability to reanneal, unlike our synthetic oligonucleotide structures that harbor noncomplementary flap strands, and (2) formation of long DNA flaps is possible, as would be expected in the cellular setting. We used a randomly radiolabeled 2-kb dsDNA fragment as substrate in this endeavor. Because Sgs1 requires ATP hydrolysis to unwind dsDNA, we could not simply omit ATP as we did with analyses of the oligonucleotide-based DNA substrates. Instead, we monitored the size of nuclease products generated by Dna2 and the *dna2-K1080E* mutant in conjunction with Sgs1 and RPA. Importantly, while

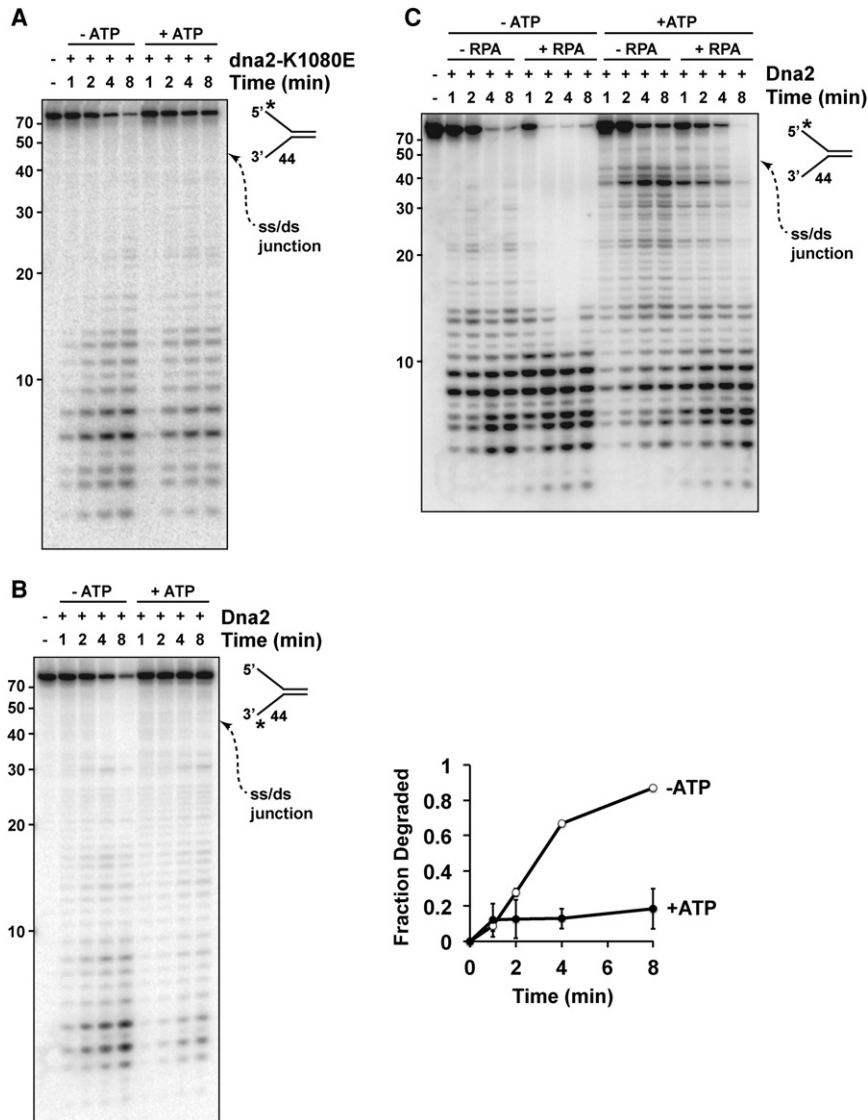


Figure 3. Analyses of *dna2*-K1080E and RPA in DNA flap cleavage. (A) Unlike Dna2, 5' flap cleavage by *dna2*-K1080E is not affected by ATP. The reaction was conducted as in Figure 2A. The arrow indicates the position on the gel where a 44-nt ssDNA product would be located. (B) ATP inhibits 3' flap cleavage by Dna2. The reaction was conducted as in Figure 2A, except that the ^{32}P label was placed at the 3' terminus of the duplex region as denoted by the asterisk. Experiments were done in triplicate, and error bars represent one standard deviation. (C) ATP induces long 5' DNA cleavage products when RPA is present. The reaction was conducted as in Figure 2A with the inclusion of 20 nM RPA. See also Supplemental Figure S3.

Dna2 produced products ranging from 2 nt to >100 nt, *dna2*-K1080E generated products of <13 nt, with the bulk of them being ≤ 10 nt (Fig. 4A). Quantitation of the data revealed a clear deficit of *dna2*-K1080E in product formation in the 12- to 100-nt range (Fig. 4A, right panel). The results thus suggest that the DNA translocase activity of Dna2 endows it with the ability to incise flaps generated by Sgs1-RPA-mediated unwinding of dsDNA to form long resection products.

Discussion

The role of the DNA-dependent ATPase function of Dna2 in DSB end resection has remained enigmatic since the protein was first implicated in HR in yeast genetic studies (Zhu et al. 2008). Here, we documented an unexpected role for this Dna2 attribute in the resection process both in vitro and in cells. Specifically, a resection defect for the *dna2*-K1080E mutant is revealed in the *exo1* back-

ground, where the Sgs1-Dna2 pathway becomes solely responsible for long-range resection. We observed this effect by physically monitoring resection at a DSB created by the HO endonuclease and examining the sensitivity of cells to the clastogenic agents camptothecin and hydroxyurea. Both of these agents induce lesions that require Dna2- or Exo1-dependent resection for initiation of recombination-based repair (DSBs with topoisomerase-linked ends for the former and stalled replication forks for the latter).

Biochemically, ATP alters the Dna2 cleavage specificity on 5' flaps and inhibits 3' flap cleavage, but the *dna2*-K1080E mutant is unresponsive to ATP. In a reconstituted resection system containing Sgs1 and RPA, wild-type Dna2 produces short as well as long cleavage products, whereas *dna2*-K1080E yields only short fragments.

The findings reported here provide the basis for a model of Dna2 action in DNA break end resection. In this model, Dna2 hydrolyzes ATP to fuel its translocation to the base of a 5' DNA flap generated by the Sgs1 helicase in conjunction with RPA, where DNA incision occurs near the

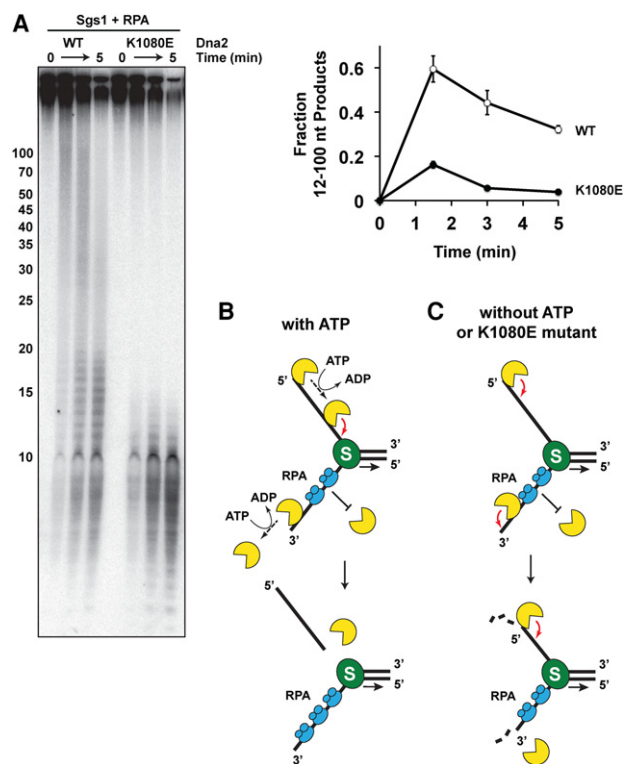


Figure 4. Analysis of Dna2 and dna2-K1080E in a reconstituted DNA end resection system. (A) Dna2 (wild type [WT]; 5 nM) or 5 nM dna2-K1080E (K1080E) was incubated with 20 nM Sgs1, 200 nM RPA, and 0.25 nM 2-kb randomly labeled dsDNA substrate for the indicated times. Quantitation shows the fraction of products in the 12- to 100-nt range relative to the total sum of all nuclease products. The experiment was done in triplicate, and error bars represent one standard deviation. (B) Model for ATP hydrolysis-fueled Dna2 translocation in end resection. Dna2 is shown in yellow, Sgs1 is in green, and RPA is in blue. The dotted arrow indicates the direction of Dna2 translocation, and the red arrow denotes the Dna2 cleavage site. At the expense of ATP hydrolysis, Dna2 translocates to the base of the 5' flap, where DNA incision occurs. RPA can block Dna2 from accessing the 3' flap. When Dna2 engages the 3' flap, ATP hydrolysis causes it to translocate on the DNA and dissociate from the end. (C) Model for how Dna2 functions in the absence of ATP or when the K1080E mutation prevents ATP hydrolysis. In this case, Dna2 fails to translocate and instead incises DNA near the 5' terminus of a DNA flap. Moreover, a 3' ssDNA end that is not protected by RPA becomes susceptible to Dna2 nuclease action.

duplex junction (Fig. 4B). In the absence of ATP or in the case of the K1080E mutant, Dna2 is unable to move and instead cleaves a small DNA section from the end of the 5' flap (Fig. 4C). This mechanism of action is consistent with previous biochemical data addressing the role of Dna2 in Okazaki fragment processing (Bae and Seo 2000). On substrates that resemble intermediates formed during Okazaki fragment processing, ATP has been reported to induce a shift in the Dna2 cleavage pattern similar to what we observed in end resection (Bae and Seo 2000). In this regard, the helicase activity of Dna2 has been suggested to power its traversal over the RNA primer

associated with Okazaki fragments (Balakrishnan et al. 2010). Together, the available data suggest that ATP hydrolysis promotes 5'-to-3' Dna2 translocation on ssDNA flaps in a similar manner in both end resection and Okazaki fragment maturation.

RPA has been shown to protect the 3' flap DNA from cleavage by Dna2 (Bae et al. 2003; Stewart et al. 2008; Cejka et al. 2010; Niu et al. 2010). However, during the resection process, the 3' flap strand may remain RPA-free for a short time upon its generation or become momentarily exposed when a bound RPA undergoes exchange with another protein factor; e.g., a free RPA molecule, Dna2, or the recombinase Rad51 (Gibb et al. 2014; Zhou et al. 2015). Under these circumstances, the DNA translocase activity of Dna2 is expected to prompt its disengagement from the 3' flap strand (Fig. 4B), thus imposing an additional layer of resection polarity control.

Materials and methods

Yeast DSB end resection assay and drug sensitivity tests

All yeast strains used to study resection at the HO-induced DSB and sensitivity to damaging agents were derivatives of the haploid yeast strain JKM139 (*MATa ho hml::ADE1 hmr::ADE1 ade1-100 leu2-3,112 lys5 trp1::hisG ura3-52 lys5 ade3::GAL10::HO*). The *dna2-K1080E* mutant was constructed within a *pif2-m2* strain using in vivo site-directed mutagenesis (Stuckey and Storici 2013), and the mutation was confirmed by sequencing. The kinetics of resection was measured as described previously (Niu et al. 2010). For hydroxyurea and camptothecin sensitivity tests, cultures grown in YEPD were diluted to 1×10^7 cells per milliliter and then further serially diluted by 10-fold before being spotted on YEPD plates containing the indicated concentration of drug. The plates were photographed after a 3-d incubation at 30°C.

Protein expression and purification

Affinity epitope-tagged forms of Sgs1, RPA, Dna2, and dna2-K1080E were purified as described previously (Niu et al. 2010).

DNA substrates

The oligonucleotide-based substrates used in this study have been described previously (Daley et al. 2014). The indicated oligonucleotides were either 5' end-labeled with γ - 32 P-ATP (Perkin-Elmer) and T4 polynucleotide kinase (New England Biolabs) or 3' end-labeled with α - 32 P-dATP (Perkin-Elmer) and terminal transferase (New England Biolabs). After annealing of complementary oligonucleotides, substrates were purified by electrophoresis in native 10% polyacrylamide gels run in TBE buffer (90 mM Tris, 90 mM boric acid, 0.5 mM EDTA at pH 8.0) at 4°C and electroelution into the same buffer before being concentrated in an Amicon Ultra-4 centrifugal concentrator.

Nuclease assays

Nuclease assays were conducted for the indicated time at 30°C with the indicated radiolabeled DNA substrate (5 nM for the oligonucleotide-based substrates or 0.25 nM for the 2-kb substrate used in Fig. 4) in 10 μ L of nuclease reaction buffer (25 mM Tris-HCl at pH 7.5, 1 mM DTT, 100 μ g/mL BSA). All of the reactions contained 2 mM MgCl₂ unless otherwise indicated and were supplemented

with the indicated amount of ATP. The nucleotides used in Supplemental Figure S2A were dATP, dGTP, dCTP, dTTP (Denville Scientific), ADP, AMP-PNP, and ATP γ S (Roche) at a final concentration of 1 mM. The reaction was terminated by adding 0.25 mg/mL proteinase K, 0.08% Orange G dye, 10% glycerol, and 90% formamide and was analyzed in a 20% urea-polyacrylamide TBE sequencing gel. After fixing with 50% methanol and 20% poly(ethylene glycol)-400 (Sigma-Aldrich) for 10 min, gels were dried onto 3-mm Whatman paper before being analyzed in a Personal Molecular Imager FX phosphorimager (Bio-Rad).

ATPase assay

ATPase assay was performed at 30°C as described previously (Niu et al. 2010). Briefly, 20 nM Dna2 or mutant was incubated with 33 μ M ssDNA nucleotides in 20 μ L of buffer [20 mM HEPES-KOH at pH 7.5, 5 mM Mg(C₂H₃O₂)₂, 1 mM DTT, 0.2 mM ATP, 0.4 Ci γ -³²P-ATP] for the indicated time at 30°C. The reaction was terminated by adding 2 μ L of 0.1 M EDTA. Next, 1 μ L of the reaction mixtures was spotted onto a PEI cellulose TLC plate (Select Scientific), which was developed in with 0.15 M formic acid/0.15 M LiCl. The TLC plates were air-dried and then subjected to phosphorimaging analysis.

Acknowledgments

This study was supported by National Institutes of Health grants ES007016, CA220188, CA220123, ES027154, ES021441, and GM080600 and Cancer Prevention Research Institute of Texas grant RP140456.

References

- Bae SH, Seo YS. 2000. Characterization of the enzymatic properties of the yeast dna2 helicase/endonuclease suggests a new model for Okazaki fragment processing. *J Biol Chem* **275**: 38022–38031.
- Bae SH, Choi E, Lee KH, Park JS, Lee SH, Seo YS. 1998. Dna2 of *Saccharomyces cerevisiae* possesses a single-stranded DNA-specific endonuclease activity that is able to act on double-stranded DNA in the presence of ATP. *J Biol Chem* **273**: 26880–26890.
- Bae SH, Kim DW, Kim J, Kim JH, Kim DH, Kim HD, Kang HY, Seo YS. 2002. Coupling of DNA helicase and endonuclease activities of yeast Dna2 facilitates Okazaki fragment processing. *J Biol Chem* **277**: 26632–26641.
- Bae KH, Kim HS, Bae SH, Kang HY, Brill S, Seo YS. 2003. Bimodal interaction between replication-protein A and Dna2 is critical for Dna2 function both in vivo and in vitro. *Nucleic Acids Res* **31**: 3006–3015.
- Balakrishnan L, Polaczek P, Pokharel S, Campbell JL, Bambara RA. 2010. Dna2 exhibits a unique strand end-dependent helicase function. *J Biol Chem* **285**: 38861–38868.
- Budd ME, Campbell JL. 1995. A yeast gene required for DNA replication encodes a protein with homology to DNA helicases. *Proc Natl Acad Sci* **92**: 7642–7646.
- Budd ME, Choe WC, Campbell JL. 1995. DNA2 encodes a DNA helicase essential for replication of eukaryotic chromosomes. *J Biol Chem* **270**: 26766–26769.
- Budd ME, Choe W, Campbell JL. 2000. The nuclease activity of the yeast DNA2 protein, which is related to the RecB-like nucleases, is essential in vivo. *J Biol Chem* **275**: 16518–16529.
- Budd ME, Reis CC, Smith S, Myung K, Campbell JL. 2006. Evidence suggesting that Pif1 helicase functions in DNA replication with the Dna2 helicase/nuclease and DNA polymerase δ . *Mol Cell Biol* **26**: 2490–2500.
- Cannavo E, Cejka P. 2014. Sae2 promotes dsDNA endonuclease activity within Mre11–Rad50–Xrs2 to resect DNA breaks. *Nature* **514**: 122–125.
- Cannavo E, Cejka P, Kowalczykowski SC. 2013. Relationship of DNA degradation by *Saccharomyces cerevisiae* exonuclease 1 and its stimulation by RPA and Mre11–Rad50–Xrs2 to DNA end resection. *Proc Natl Acad Sci* **110**: E1661–E1668.
- Cejka P, Cannavo E, Polaczek P, Masuda-Sasa T, Pokharel S, Campbell JL, Kowalczykowski SC. 2010. DNA end resection by Dna2–Sgs1–RPA and its stimulation by Top3–Rmi1 and Mre11–Rad50–Xrs2. *Nature* **467**: 112–116.
- Chiruvella KK, Liang Z, Wilson TE. 2013. Repair of double-strand breaks by end joining. *Cold Spring Harb Perspect Biol* **5**: a012757.
- Daley JM, Laan RL, Suresh A, Wilson TE. 2005. DNA joint dependence of pol X family polymerase action in nonhomologous end joining. *J Biol Chem* **280**: 29030–29037.
- Daley JM, Chiba T, Xue X, Niu H, Sung P. 2014. Multifaceted role of the Topo IIIa–RMI1–RMI2 complex and DNA2 in the BLM-dependent pathway of DNA break end resection. *Nucleic Acids Res* **42**: 11083–11091.
- Daley JM, Niu H, Miller AS, Sung P. 2015. Biochemical mechanism of DSB end resection and its regulation. *DNA Repair (Amst)* **32**: 66–74.
- Garcia V, Phelps SE, Gray S, Neale MJ. 2011. Bidirectional resection of DNA double-strand breaks by Mre11 and Exo1. *Nature* **479**: 241–244.
- Gibb B, Ye LF, Kwon Y, Niu H, Sung P, Greene EC. 2014. Protein dynamics during presynaptic-complex assembly on individual single-stranded DNA molecules. *Nat Struct Mol Biol* **21**: 893–900.
- Gravel S, Chapman JR, Magill C, Jackson SP. 2008. DNA helicases Sgs1 and BLM promote DNA double-strand break resection. *Genes Dev* **22**: 2767–2772.
- Huertas P, Cortes-Ledesma F, Sartori AA, Aguilera A, Jackson SP. 2008. CDK targets Sae2 to control DNA-end resection and homologous recombination. *Nature* **455**: 689–692.
- Jeggo PA, Lobrich M. 2015. How cancer cells hijack DNA double-strand break repair pathways to gain genomic instability. *Biochem J* **471**: 1–11.
- Karathanasis E, Wilson TE. 2002. Enhancement of *Saccharomyces cerevisiae* end-joining efficiency by cell growth stage but not by impairment of recombination. *Genetics* **161**: 1015–1027.
- Kuo C, Nuang H, Campbell JL. 1983. Isolation of yeast DNA replication mutants in permeabilized cells. *Proc Natl Acad Sci* **80**: 6465–6469.
- Lee KH, Kim DW, Bae SH, Kim JA, Ryu GH, Kwon YN, Kim KA, Koo HS, Seo YS. 2000. The endonuclease activity of the yeast Dna2 enzyme is essential in vivo. *Nucleic Acids Res* **28**: 2873–2881.
- Liu P, Carvalho CM, Hastings PJ, Lupski JR. 2012. Mechanisms for recurrent and complex human genomic rearrangements. *Curr Opin Genet Dev* **22**: 211–220.
- Masuda-Sasa T, Polaczek P, Peng XP, Chen L, Campbell JL. 2008. Processing of G4 DNA by Dna2 helicase/nuclease and replication protein A (RPA) provides insights into the mechanism of Dna2/RPA substrate recognition. *J Biol Chem* **283**: 24359–24373.

- Mehta A, Haber JE. 2014. Sources of DNA double-strand breaks and models of recombinational DNA repair. *Cold Spring Harb Perspect Biol* **6**: a016428.
- Mimitou EP, Symington LS. 2008. Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. *Nature* **455**: 770–774.
- Niu H, Chung WH, Zhu Z, Kwon Y, Zhao W, Chi P, Prakash R, Seong C, Liu D, Lu L, et al. 2010. Mechanism of the ATP-dependent DNA end-resection machinery from *Saccharomyces cerevisiae*. *Nature* **467**: 108–111.
- Paull TT, Gellert M. 1998. The 3' to 5' exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks. *Mol Cell* **1**: 969–979.
- Pinto C, Kasaciunaite K, Seidel R, Cejka P. 2016. Human DNA2 possesses a cryptic DNA unwinding activity that functionally integrates with BLM or WRN helicases. *Elife* **5**: e18574.
- Prakash R, Zhang Y, Feng W, Jasin M. 2015. Homologous recombination and human health: the roles of BRCA1, BRCA2, and associated proteins. *Cold Spring Harb Perspect Biol* **7**: a016600.
- Ryu GH, Tanaka H, Kim DH, Kim JH, Bae SH, Kwon YN, Rhee JS, MacNeill SA, Seo YS. 2004. Genetic and biochemical analyses of Pfh1 DNA helicase function in fission yeast. *Nucleic Acids Res* **32**: 4205–4216.
- San Filippo J, Sung P, Klein H. 2008. Mechanism of eukaryotic homologous recombination. *Annu Rev Biochem* **77**: 229–257.
- Stephens PJ, McBride DJ, Lin ML, Varela I, Pleasance ED, Simpson JT, Stebbings LA, Leroy C, Edkins S, Mudie LJ, et al. 2009. Complex landscapes of somatic rearrangement in human breast cancer genomes. *Nature* **462**: 1005–1010.
- Stephens PJ, Greenman CD, Fu B, Yang F, Bignell GR, Mudie LJ, Pleasance ED, Lau KW, Beare D, Stebbings LA, et al. 2011. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. *Cell* **144**: 27–40.
- Stewart JA, Miller AS, Campbell JL, Bambara RA. 2008. Dynamic removal of replication protein A by Dna2 facilitates primer cleavage during Okazaki fragment processing in *Saccharomyces cerevisiae*. *J Biol Chem* **283**: 31356–31365.
- Stuckey S, Storici F. 2013. Gene knockouts, in vivo site-directed mutagenesis and other modifications using the delitto perfetto system in *Saccharomyces cerevisiae*. *Methods Enzymol* **533**: 103–131.
- Sung P, Krejci L, Van Komen S, Sehorn MG. 2003. Rad51 recombinase and recombination mediators. *J Biol Chem* **278**: 42729–42732.
- Symington LS. 2016. Mechanism and regulation of DNA end resection in eukaryotes. *Crit Rev Biochem Mol Biol* **51**: 195–212.
- Zhou C, Pourmal S, Pavletich NP. 2015. Dna2 nuclease–helicase structure, mechanism and regulation by Rpa. *Elife* **4**: e09832.
- Zhu Z, Chung WH, Shim EY, Lee SE, Ira G. 2008. Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. *Cell* **134**: 981–994.