Molecular basis for processing of topoisomerase 1-triggered DNA damage by Apn2/APE2

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SUMMARY

Topoisomerase 1 (Top1) incises DNA containing ribonucleotides to generate complex DNA lesions that are resolved by APE2 (Apn2 in yeast). How Apn2 engages and processes this DNA damage is unclear. Here, we report X-ray crystal structures and biochemical analysis of Apn2-DNA complexes to demonstrate how Apn2 frays and cleaves 3′ DNA termini via a wedging mechanism that facilitates 1–6 nucleotide endonucleolytic cleavages. APN2 deletion and DNA-wedge mutant Saccharomyces cerevisiae strains display mutator phenotypes, cell growth defects, and sensitivity to genotoxic stress in a ribonucleotide excision repair (RER)-defective background harboring a high density of Top1-incised ribonucleotides. Our data implicate a wedge-and-cut mechanism underpinning the broad-specificity Apn2 nuclease activity that mitigates mutagenic and genome instability phenotypes caused by Top1 incision at genomic ribonucleotides incorporated by DNA polymerase epsilon.

In brief

Apn2/APE2 nucleases resolve chemically diverse DNA damage, including endogenous DNA lesions created by topoisomerase 1 (Top1). Williams et al. report the structure and functional characterization of Apn2-DNA complexes showing how the enzyme wedges and frays DNA ends and how the inactivation of Apn2 function causes mutagenesis in budding yeast.

Graphical Abstract

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AUTHOR CONTRIBUTIONS

Conceptualization and methodology, J.S.W. and R.S.W.; investigation, J.S.W., J.L.W., D.C.A., B.D.W., and E.W.; formal analysis, J.S.W., J.L.W., J.K., and R.S.W.; writing – original draft, J.S.W., J.L.W., and R.S.W.; writing – review & editing, J.S.W., J.L.W., D.C.A., R.S.W., and T.A.K.; resources and supervision, R.S.W. and T.A.K.

SUPPLEMENTAL INFORMATION

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DECLARATION OF INTERESTS

The authors declare no competing interests.
INTRODUCTION

Nuclear genome replication is performed with high fidelity, in which the correctly paired nucleotide is incorporated into the growing DNA chain to avoid mutations and other forms of genome instability (Burgers and Kunkel, 2017). However, recent evidence from studies performed using multiple organisms implicates ribonucleotides stably incorporated into DNA as a common source of DNA damage (Clark et al., 2011; Klein, 2017; Nick McElhinny et al., 2010; Reijns et al., 2012; Tumbale et al., 2014). In addition to rendering the DNA backbone several orders of magnitude more susceptible to spontaneous hydrolysis (Li and Breaker, 1999), enzymatic processing of unrepaired single ribonucleotides in genomic DNA causes a significant threat to DNA integrity (reviewed by Williams et al., 2016; Wallace and Williams, 2014). This is due to the action of topoisomerase 1 (Top1), an enzyme critical for the removal of supercoils that arise during replication and transcription (Pommier et al., 2016). Top1-dependent processing of unrepaired ribonucleotides produces unligatable 5’-OH and 2’,3’-cyclic phosphate DNA ends that can proceed through error-free or mutagenic repair pathways (Huang et al., 2015; Sparks and Burgers, 2015). In ribonucleotide excision repair (RER)-defective cells, various types of DNA instability, which include DNA double-strand breaks and recombination, have been reported (Allen-Soltero et al., 2014; Conover et al., 2015; Huang et al., 2017; O’Connell et al., 2015; Potenski et al., 2014), and Top1-cleavage at unrepaired ribonucleotides is associated with several cellular phenotypes that include replication stress and checkpoint activation (Williams et al., 2013).
When a Top1-induced DNA nick occurs within a tandemly repeated DNA sequence, it can be converted into a short deletion (Kim et al., 2011). One mechanism of avoiding deletions that occur in the absence of RER is mediated by the unwinding of DNA from the 5’ side of a nick by the Srs2 helicase, which, together with the Exo1 nuclease, generates a DNA gap that can be repaired via gap filling (Potenski et al., 2014). An additional mechanism by which Top1-induced RNA-DNA damage can be repaired is via processing by the apurinic/apyrimidinic (AP) endonuclease 2 (Apn2) (Li et al., 2019). Apn2 resolves the 3’ DNA-end damage produced when Top1 cleaves at a ribonucleotide by also creating a gap that can be filled and ligated in an error-free manner. However, the molecular mechanism of Apn2 action and the mutagenic consequences of loss of Apn2 in an RER-defective strain beyond an effect on short deletion mutagenesis have not been explored.

Apn2 is historically known for its role in DNA base excision repair (BER), in which a lesion is first removed by a damage-specific monofunctional glycosylase to produce an AP site. The DNA backbone at this AP site is then cleaved by an AP endonuclease, with Apn1 identified as the primary AP endonuclease and Apn2 serving a minor and/or backup role in cells (Unk et al., 2000). However, Apn2 and its vertebrate APE2 homolog are exonuclease-endonuclease-phosphatase (EEP) family proteins that execute a number of catalytic activities, including 3’−5’ exonuclease, 3’ resection, AP-endonuclease, and phosphodiesterase functions (Alvarez-Quilon et al., 2020; Andres et al., 2015; Unk et al., 2001; Wallace et al., 2017), indicating that the other DNA lesions generating 3’ DNA-end damage require Apn2 processing and resolution. In addition, the unique Apn2 domain architecture couples proliferating cell nuclear antigen PCNA binding via a PCNA-interacting peptide (PIP) to a C-terminal zinc finger (Zf)-glycine-arginine-phenylalanine (GRF) DNA-binding domain required for processive DNA-end resection (Wallace et al., 2017). Other EEP domain-containing family members include tyrosyl-DNA phosphodiesterase 2 (TDP2) (Schellenberg et al., 2012) and AP endonuclease 1 (APE1) (Whitaker and Freudenthal, 2018). The human homolog of Apn2, APE2, was recently demonstrated to be capable of removing endogenous blocked 3’ DNA ends, including those that arise following Top1 incision at a genomic ribonucleotide (Alvarez-Quilon et al., 2020). The processing of genomic ribonucleotides produces DNA strand breaks with damaged 3’ termini in the form of 2’−3’ cyclic phosphate ends (Alvarez-Quilon et al., 2020; Huang et al., 2017; Wallace and Williams, 2014). Loss of APE2 is lethal in BRCA1 or BRCA2-mutated cells that are deficient in homologous recombination (HR) repair, and APE2 is therefore a target for personalized medicine approaches in breast cancer (Alvarez-Quilon et al., 2020; Mengwasser et al., 2019). APE2 synthetic lethality is likely linked to the inability of APE2 to repair endogenous 3’ DNA damage, including DNA damage generated by Top1 processing of ribonucleotides in DNA (Alvarez-Quilon et al., 2020).

It remains unclear how Apn2 homologs perform their functions, especially in the context of 3’ DNA-end damage generated by Top1-cleavage at unrepaired ribonucleotides. Integrated structural, biochemical, and genetic results demonstrate that the 3’ cyclic phosphate DNA ends produced by Top1 cleavage at a ribonucleotide can be processed by Apn2 by using a DNA-end-fraying mechanism. Structure-guided Apn2 DNA-binding mutants undermine Apn2 functions and result in growth defects and mutator phenotypes in yeast. Our results show how Apn2 is able to recognize, accommodate, and cleave diverse forms of 3’ DNA-
end damage, and describe the mutagenic and genome instability consequences of failure of Apn2 to repair 3’ DNA damage produced when Top1 processes one of the most abundant DNA lesions, a genomic ribonucleotide incorporated during DNA synthesis.

RESULTS

X-ray structural analysis of apo Apn2

To understand the Apn2 DNA-processing molecular mechanism, we crystallized and determined the X-ray structure of the Saccharomyces cerevisiae Apn2 catalytic core domain (amino acids 1–407) in both DNA-free and DNA-bound states (see STAR Methods; Table S1). Overall, the Apn2 catalytic domain structure resembles human APE1 and TDP2 EEP fold enzymes that have a central 10 stranded β sandwich flanked with several helical elements that dictate the diverse substrate specificities (Freudenthal et al., 2015; Schellenberg et al., 2012, 2016). In the DNA-free crystal form, Apn2 crystallized as an interlocked domain-swapped dimer (Figures S1A–S1C). Domain swapping and inter-dimer interactions are mediated by the C-terminal helical region of the EEP domain and a β hairpin insertion element specific to Apn2 (Figures S1A and S1C). Like TDP2 (Schellenberg et al., 2012, 2016), APE1 (Freudenthal et al., 2015) and the DNA-free structure of Xenopus laevis APE2 (Alvarez-Quilon et al., 2020), the Apn2 active site contains a single coordinated magnesium ion, suggesting a common single metal-mediated hydrolytic mechanism for these enzymes (Figure S1A).

Structure of an Apn2-DNA complex

We conducted extensive co-crystallization trials of Apn2 in complex with a variety of blunt-ended DNAs, nicked SSB substrates, or Abasic-site mimic (furan) containing substrates. Initial efforts to crystallize the native enzyme with DNA failed, likely because Apn2 degrades its substrate during the crystallization experiment. To block the Apn2 nuclease activity, we used an active site variant targeting a Mg$^{2+}$-binding motif (E59Q) and the proposed catalytic base (D222N). To further stunt DNA substrate hydrolysis, we modified the five 3’ terminal bases of the oligonucleotide co-crystallization substrates with a phosphorothioate backbone. Together, these protein and substrate modifications facilitated crystallization and structure determination of an Apn2-DNA complex to 2.73 Å resolution bound to a 13-bp duplex substrate (Figure 1; Table S1). The asymmetric unit contains four Apn2 molecules bound to the ends of two double-stranded DNA (dsDNA) molecules (Figure S1D).

In the structures captured, the 3’ termini are bound approximately 1–2 nt short of the active center (Figure 1D). Unexpectedly, two different DNA complexes in the crystal were co-crystallized from the same set of 13-nt oligonucleotides (Figures 1D and S1D). For the first bound DNA, one strand of DNA anneals to itself via a 12-bp duplex region and has 1-nt 5’ overhangs. A second bound blunt-ended DNA duplex forms from the annealing of two 13-nt DNA strands (duplex 2, Figure S1D). For the two bound duplex molecules, each of the four Apn2 catalytic domains binds to either two duplexes (Apn2 chains A and C, Figure S1E) or to a single duplex (Apn2 chains B and D, Figure S1F) through two DNA-binding surfaces mapping to the active site face and/or a backside face of the molecule (Figure S1G).
Compared to two other EEP fold enzymes, the APE1-DNA (Freudenthal et al., 2015), and TDP2-DNA complexes (Schellenberg et al., 2012), the Apn2 DNA binding mode is distinct (Figure S2). Apn2 DNA-binding regions on the active site face localize to the α1-α2 loop and a DNA “wedge loop” (WL) comprising the α12-β12 connector (Figures 1B–1D, S2A, and S2B). This DNA WL and residue Y33 in the α1-α2 loop form a contiguous surface that interrogates the DNA substrate minor groove, frays the DNA duplex end, and directs the 3′ strand toward the active site (Figures 2A, 2C–2E and S2C). A secondary DNA duplex binding site is formed by residues K201, R205, R208 (helix α6), and R273 (helix α10) on the back side of the Apn2 EEP domain (Figure 2B). Near the active site, Y33 stabilizes the DNA-bound WL via van der Waals interactions with the DNA backbone and a hydrogen bonding to the side chain of N317 of the wedge (Figure 2A). WL residues W312 and R319 anchor the loop to the EEP core, while residues K316, N317, and L318 penetrate into the DNA minor groove (Figures 2C and 2D). In the DNA-bound state, the unpaired 5′ terminal deoxy thymidine (dT) of duplex 1 base stacks with Y323 at the frayed DNA end. Altogether, these DNA interactions contribute to DNA-end binding and separation of the DNA strands, and they direct the 3′ terminus toward the active site.

Apn2 DNA binding induces several structural rearrangements, including remodeling of the C-terminal domain-swapped dimer helix and DNA binding-dependent conformational changes of the DNA wedge (Figures 2C–2F). In the apo state, the WL is compacted and adopts partial helical structure (Figure 2F). By comparison, all of the DNA-bound molecules undergo loop decompaction resembling the blooming of flower petals (Figure 2F). Intriguingly, structural remodeling of an analogous DNA binding element is also observed for mouse TDP2 (Figures S2C–S2E; Schellenberg et al., 2016).

**Apn2 DNA-binding site mutants affect its 3′ exonuclease activity**

We hypothesized that the DNA minor groove wedging observed in the X-ray structure facilitates DNA strand separation and 3′ nucleolytic processing of diverse 3′ modified substrates reported for yeast Apn2 (Li et al., 2019). Consistent with published data, we find that the 3′ exonuclease of recombinant Apn2 is active on correctly paired (Figure 3A, substrate 3′-OH, lane 4), mismatched (Figure 3A, substrate 3′ GA mismatch, lane 11), ribonucleotide (3′ ribonucleotide, Figure 3A, lane 18), and cyclic 2′−3′ phosphate (2′−3′cycPO₄; Figure 3A, lane 25) DNA ends. While Apn2 alone processes 1–5 nucleotides of a 3′ recessed DNA substrate, its activity on all of the substrates is markedly stimulated by the addition of equimolar PCNA (Figure 3A, lanes 5–7, 12–14, 19–21, and 26–28).

To test roles for Apn2 active site motifs and DNA-binding elements in 3′ DNA-end processing, we analyzed mutant Apn2 exonuclease activities. Compared to WT Apn2 (Figure 3B, lanes 4–7), the relative activities of a α1-α2 loop mutant (Y33E) showed reduced PCNA-stimulated exonuclease activity compared to wild type (WT) (Figure 3B, lanes 9–12). However, the backside-binding site mutants (R205E and R208E) had no impact (R205E, Figure 3B, lanes 14–17) or a very limited impact (R208E, Figure 3B, lanes 19–22) on PCNA-stimulated exonuclease activity (Figure 3D). A deletion mutation within the DNA WL (ΔWL, Figures 3C–3E) resulted in impaired PCNA-independent resection (Figure 3C, lanes 1–3) and salt-sensitive attenuation of Apn2 3′ resection activity in the presence of
PCNA (Figure 3D, lanes 8–10 and 18–20). By comparison, the double active site mutant (D222N/E59Q) used for crystallization is completely inactive (Figure 3C, lanes 11–13 and 21–23). That none of the DNA-binding mutations completely blocked activity is consistent with contributions of the α1-α2 loop and wedge Apn2 DNA-binding regions to a complex multipoint DNA interaction interface that also includes the PCNA-DNA interaction and DNA binding via the Apn2 C-terminal Zf-GRF domain (Wallace et al., 2017). Consistent with this hypothesis, combination of the Y33E and ΔWL mutations significantly impaired Apn2 catalytic activity, even at high enzyme concentrations (1 μM) (Figures S3A and S3B). Our combined results indicate that both Y33E and WL are important for Apn2 DNA-end processing function, consistent with our structural observations.

The WL defines 3′ endonucleolytic cleavage product lengths

To probe the nature of Apn2 DNA cleavages and to map nucleolytic cleavage sites, we assessed Apn2 activity on 3′ FAM modified DNA substrates (Figure 4A). Control DNA cleavage product ladders were generated using 5′−3′ T7 and lambda exonucleases, or by synthesis of a 3′ FAM-labeled 5-nt DNA ladder (Figure 4A, lanes 3–5). On the 3′ FAM-labeled substrate, WT Apn2-PCNA exhibited 3′ processing activity that directly removes the 3′ FAM label and also performed an array of DNA substrate cleavages that produce 1-, 2-, 5-, and 6-nt endonucleolytic products (Figure 4A, lanes 7–9). Intriguingly, the ΔWL mutant alters Apn2 cleavage specificity, as the cleavage distribution showed 1- and 6-nt products increased relative to the WT enzyme (Figures 4A, lanes 10–12, and S3C).

To confirm the positions of the cleavage sites, we modified 1, 2, and 3 terminal nucleotide steps of the substrate with a cleavage-resistant phosphorothioate backbone. Addition of a single phosphorothioate at the 3′ terminus (substrate 1PT; Figure 4A) specifically slowed accumulation of the 1-nt cleavage product by WT and ΔWL Apn2, but enhanced cleavages that produced a 2-nt product (Figures 4A, lanes 14–19, and S3C). For the 1PT substrate, the ΔWL Apn2 also yielded a 4-nt product that is not observed for WT Apn2. Two phosphorothioates limited accumulation of the 1- and 2-nt products, as predicted. However, Apn2-PCNA digestion of the 2PT substrate also produced 3-nt cleavage products that were not observed on an unmodified phosphodiester backbone (Figure 4A, lanes 23–28). Three 3′ terminal phosphorothioate backbone substitutions attenuated 1- to 3-nt cleavages, with observable 4- to 6-nt products produced (Figures 4A, lanes 30–35, and S3C). Overall, these data point to dynamic 3′ DNA-end binding by Apn2-PCNA, which can fray DNA ends and catalyze endonucleolytic incisions ranging from 1- to 6-nt. Furthermore, the WL regulates the Apn2 endonuclease, and WL mutations alter overall enzymatic activity and salt sensitivity (Figures 3B–3D), as well as the distribution of DNA cut sites. The data point to a “wedge and cut” mechanism that helps to explain the accommodation of variable 3′ modified substrates by Apn2 (Figure 4B).

Apn2 is important for preventing ribonucleotide-triggered genome instability in yeast

Based on our structural and biochemical data supporting a critical role for Apn2 in processing damaged 3′ DNA ends, we tested the requirement for Apn2 in yeast strains deficient in RER. Unrepaired ribonucleotides can be cleaved by Top1 to generate 5′-OH and 2′−3′ cyclic phosphate DNA ends, the latter of which has been demonstrated to be
a substrate for Apn2 (Li et al., 2019). The failure of RER to remove ribonucleotides incorporated into DNA during replication causes various forms of genome instability, and many of them, including 2- to 5-bp deletions in repeat DNA sequences, are the result of processing by Top1 (Huang et al., 2015; Kim et al., 2011; Sparks and Burgers, 2015; Williams et al., 2013, 2016). We therefore tested whether the loss of APN2 affects the deletion rate in an RNase H2-deficient strain using a URA3 forward mutation reporter gene placed adjacent to the ARS306 origin of replication in one of two opposite orientations (OR1 versus OR2). Spontaneous mutation rates were calculated by measuring the frequency of 5-fluoroorotic acid (5-FOA) resistance caused by the mutation of URA3 (Table S4), and the ura3 mutants were sequenced in order to interrogate the spectrum of mutations (Figure S4; Table S4). Consistent with a previous study using a reversion assay (Li et al., 2019), we observed an approximate 2-fold increase in the rate of 2- to 5-bp deletions (Figure 5A) when comparing apn2Δ rnh201Δ to rnh201Δ. This is consistent with a contribution of 3’ DNA-end processing by Apn2 to preventing short deletion mutations.

A M644G Pol ε mutant has increased propensity for the incorporation of ribonucleotides into DNA during leading strand synthesis (Nick McElhinny et al., 2010). Interestingly, in the study conducted by Li et al. (2019), the authors were unable to construct a pol2-M644G rnh201Δ apn2Δ haploid strain as it was inviable in their strain background. Because of this, their analysis of Apn2 in modulating TOP1 mutagenesis was limited to the use of a reversion assay that monitored one specific mutagenic event, 2-bp deletions. However, in the strain background used here, the pol2-M644G rnh201Δ apn2Δ haploid strain is viable, albeit extremely sick and slow growing. Our ability to propagate these strains facilitates a robust and more comprehensive analysis of Apn2 functions. We thus extended this genetic analysis to include a pol2-M644G rnh201Δ strain containing a higher density of unrepaired genomic ribonucleotides than does an rnh201Δ mutant in which WT POL2 is expressed (Lujan et al., 2012; Williams et al., 2013, 2015). We deleted APN2 in the rnh201Δ, pol2-M644G, and pol2-M644G rnh201Δ diploid strains. Following sporulation and dissection, we found that only in the presence of high ribonucleotide incorporation and impaired RER (pol2-M644G rnh201Δ) did deletion of APN2 result in extremely small colonies (Figure 5B) and confer an increase in doubling time compared to the other mutants (Figure S5A). This suggests that the processing of unrepaired ribonucleotides incorporated into the nascent leading strand by the M644G Pol ε mutant generates a substrate that requires Apn2 for processing and repair. Consistent with this growth impairment being due to the loss of Apn2-dependent processing of the damaged 3’ DNA ends produced following Top1 cleavage at genomic ribonucleotides, deletion of TOP1 alleviated the small colony size and slow growth of the pol2-M644G rnh201Δ apn2Δ strain (Figures 5B and S5A). To confirm that the impaired growth of this strain was due to unrepaired single ribonucleotides and not RNA-DNA hybrids, whose structures are also processed by RNase H2, we deleted APN2 in the Rnh201-RED (ribonucleotide excision defective) mutant that is specifically unable to process single ribonucleotides but retains activity on RNA-DNA hybrids (Chon et al., 2013). The pol2-M644G rnh201-RED apn2Δ haploid strain formed small spore colonies (Figure S5B) and had an elevated doubling time similar to that of the pol2-M644G rnh201Δ apn2Δ mutant (Figure S5A).
Growth of the pol2-M644G rnh201Δ/RED strain is impaired when the cells are grown in the presence of the replication inhibitor hydroxyurea (HU), which causes deoxynucleoside triphosphate (dNTP) pool depletion and replication fork stalling (Lopes et al., 2001; Reichard, 1988; Williams et al., 2013, 2017). Growth is improved upon the deletion of TOP1, suggesting that Top1 cleavage at unrepaired ribonucleotides causes genome instability that sensitizes the cells to replication stress. To investigate whether the role of Apn2 in processing ribonucleotide-triggered DNA damage is important for resistance to HU, we performed a spot dilution assay of the pol2-M644G ± rnh201/RED apn2Δ mutants on rich medium ± HU. Loss of APN2 in the pol2-M644G rnh201Δ strain impaired growth and resulted in hypersensitivity to HU, a phenotype also observed for the pol2-M644G rnh201-RED apn2Δ strain (Figure 5C). Consistent with this hypersensitivity to replication stress being due to the failure of Apn2 to repair 3′ DNA damage produced when Top1 cleaves at a ribonucleotide, deletion of TOP1 improved growth and reduced sensitivity to HU.

**Apn2 is important for preventing ribonucleotide-triggered mutations**

Spontaneous mutation rates and specificity were determined for the pol2-M644G rnh201Δ apn2Δ mutant to determine the contribution of Apn2 to mutagenesis in a strain with a high density of unrepaired ribonucleotides in the leading strand (Figures 5D, 6A, and S4B; Tables S5 and S6). In terms of deletion mutagenesis, the loss of APN2 did not have a significant effect on the rate of Δ2–5 bp in the pol2-M644G rnh201Δ strain (Figure 6B). In this case, there was an approximate 2-fold (16 versus 11 3 10^{-8}) decrease in the rate of Top1-dependent Δ2–5 bp when compared to the APN2+ strain (Table S6), likely due to the increase in base pair substitutions (discussed below).

Interestingly, the deletion of APN2 resulted in an increased rate of A to T transversions at base pair positions 279 and 686 in the URA3-OR1 reporter gene in the pol2-M644G rnh201Δ strain. This rate decreases upon deletion of TOP1 (Figure 6C). These mutations were originally identified as polymerase-specific error signatures of the M644G Pol ε variant enzyme, likely due to the incorporation of a deoxythymidine monophosphate (dTMP) mismatch opposite a template T (Pursell et al., 2007). The A279T and A686T mutations are also a signature of loss of proofreading by Pol ε (St Charles et al., 2015; Williams et al., 2012), and they are largely refractory to DNA mismatch repair (Lujan et al., 2012). The rate of transversions at these URA3 hotspots is highest (fold increase of 4.5-fold for A279T and 4.7-fold for A686T) in the pol2-M644G rnh201Δ apn2Δ strain as compared to the RNase H2-proficient pol2-M644G apn2Δ mutant. Combined with the propensity of M644G Pol ε for ribonucleotide incorporation (Nick McElhinny et al., 2010), this suggests that it may be Pol ε incorporation of a ribouridine 5′-phosphate (rUMP) mismatch opposite a template T that is the initiating lesion. Loss of APN2 did not affect spore colony size (Figure S5C) or the rate of A279T and A686T mutations in the proofreading-deficient pol2-4 rnh201Δ mutant (Figure S4C; Table S7), consistent with the demonstration that Pol ε proofreading of ribonucleotides is weak (Williams et al., 2012). Incision at a misincorporated ribouridine (rU) by Top1 may create a damaged 3′ DNA substrate for Apn2, as deletion of TOP1 suppresses the elevated rates of A279T and A686T in the pol2-M644G rnh201Δ apn2Δ mutant (Figure 6C; Table S7).
**Analysis of Apn2 DNA-binding mutants in vivo**

We evaluated the contribution of Apn2 DNA processing to genome maintenance by constructing yeast strains expressing Apn2 mutants with specific defects in DNA binding and 3′-end processing. As defined structurally and biochemically, the Apn2 Y33 residue (part of the α1-α2 loop) and the WL together form a DNA interaction surface, fray the duplex DNA end, and direct the 3′ strand toward the enzyme active site (Figures 2A and 4B). We constructed both an Apn2-Y33E mutant and a WL deletion strain by replacing amino acids 312–323 with 2 glycine residues (Apn2-ΔWL) to test the in vivo importance of these specific disruptions to DNA binding and fraying, and measured cell growth and sensitivity to replication stress in a pol2-M644G mutant background deficient in RER. The RNase H2-deficient apn2-ΔWL and -Y33E mutants had increased doubling times that were reduced when TOP1 was deleted (Figures S6A–S6C). The pol2-M644G apn2-ΔWL/Y33E rnh201Δ strains were also hypersensitive to HU, and again, the growth of each was improved upon the loss of TOP1 (Figures S6A and S6B). These results are consistent with important roles for Apn2 DNA binding and 3′ end processing in promoting cell growth and genotoxin resistance in the presence of Top1-induced DNA damage.

We next measured spontaneous mutation rates and specificity in RNase H2-deficient apn2-ΔWL and -Y33E mutant strains expressing the pol2-M644G variant ± TOP1 and compared our results to what we observed in the complete absence of APN2 (Figures S7A–S7D; Table S6). Consistent with the biochemical demonstration of partial loss of nuclease activity in these mutants (Figure 3D), both the overall mutation rate (Table S6) and A to T transversion rates at positions 279 and 686 were elevated relative to an APN2+ strain, but less than what was observed in the apn2Δ mutant (Figure 6D). The partial loss of function in the WL mutant may also be affected by an alteration in cleavage specificity (Figure 4A), which may in turn affect base-base mutagenesis resulting from aberrant processing of Top1-incised ribonucleotides. The rate of 2- to 5-bp deletions in the RNase H2-deficient Apn2 mutant strains was similar to that observed in the rnh201Δ apn2Δ strain (Figure S6D).

**DISCUSSION**

Our results are consistent with a model in which the Apn2 DNA WL and the α1-α2 loop engage and splay the DNA duplex apart, directing the damaged 3′ strand toward its active site. Accessibility of the active site pocket and the ability of Apn2 to catalyze variable endonucleolytic incisions ranging from 1 to 6 nt affords Apn2 a broad specificity through a “wedge and cut” mechanism that is also highly stimulated by its interaction with PCNA. While the present structures do not capture the active center bound conformation of the enzyme, we hypothesize that basic residues and the active site catalytic metal stabilize the 3′ strand for cleavage initiated by the catalytic water that is hydrogen bonded to D222. The backside patch of basic residues on helices α6 and α10 could provide a potential second site of DNA interaction that we speculate accommodates the DNA duplex upstream of the damage and allows the intact DNA strand opposite the damage to wrap around the enzyme (Figure 4B). More work is needed to decipher precisely how Apn2 engages DNA nicks and gaps.
The broad substrate specificity of Apn2 gives the enzyme the ability to accommodate a range of biologically relevant 3′ DNA damage. Our work further corroborates a critical role for Apn2 in the processing of the products of Top1 incision at ribonucleotides incorporated by Pol ε during leading strand synthesis. Apn2 DNA-end binding is facilitated by a dynamic DNA binding wedge that rearranges upon substrate engagement (Figure 2F). Interestingly, these rearrangements occur in topologically similar DNA binding loops that are dynamic in other EEP fold enzymes such as TDP2 (Schellenberg et al., 2016). For TDP2, the active site DNA binding loop adopts variable and extended protease-accessible conformations in the absence of DNA, but compacts upon DNA binding to complete active site assembly (Schellenberg et al., 2016) (Figures S2B–S2E). Overall, our results show how structural variability in the DNA binding modes differentiate Apn2-DNA interactions from the TDP2-DNA and APE1-DNA complexes (Figures S2B–S2E). The structures underscore how differentially evolved substrate binding interfaces have been built on a common EEP catalytic domain scaffold.

Overall, our genetic and mutagenesis data are consistent with an important role for Apn2 in preventing ribonucleotide triggered genomic instability and mutagenesis (Figure 7). Together, observations of impaired growth rates, genotoxin sensitivity, and elevated mutation rate in the pol2-M644G rnh201Δ apn2Δ mutant are consistent with a model in which Top1-generated DNA damage is a substrate for Apn2 activity. Observed elevated rates of A to T transition mutations at URA3 positions 279 and 686 implies that DNA ends produced by Top1 cleavage at unrepaired ribonucleotides require Apn2 for processing and resolution. These observations also suggest that Apn2 may process a Top1cc-triggered lesion resulting from ribonucleotide incision to promote short deletion mutagenesis. However, this hypothesis for short deletion formation is disfavored based on the observations that (1) genetic interactions support a role for Apn2 in Top1cc repair, but probably not Top1cc removal, in the absence of Tdp1 (Liu et al., 2002), and (2) an apn2Δ rnh201Δ strain is associated with a modest effect on the rate of 2- to 5-bp deletions when compared to the rnh201Δ single mutant (Li et al., 2019; Figure 5A), and therefore is not a strong contributor to direct resolution of Top1cc intermediates that lead to ribonucleotide-dependent deletions in budding yeast. In fact, in a pol2-M644G rnh201Δ mutant, APN2 deletion causes a small decrease (1.5-fold) in the rate of 2- to 5-bp deletions (Figure 6B), suggesting that Apn2 may make a minor contribution to gap production following Top1 action at ribonucleotides in a strain containing a high density of unrepaired ribonucleotides. Our data are supportive of a wedge-and-cut mechanism facilitating broad-specificity Apn2 nuclease activity. These activities help defray the mutagenic and genome instability phenotypes linked to Top1 incision of genome-embedded ribonucleotides incorporated by DNA polymerase ε.

**Limitations of the study**

Our structural analysis captured a DNA-end bound state of Apn2. A limitation of the study stems from the fact that additional DNA contacts proximal to the active site, and to downstream DNA on nicked or 3′ recessed DNA ends likely contribute to the Apn2-DNA substrate complex. One possible explanation that DNA is not bound in the active center here is that ablation of the metal binding site in the Apn2 crystallization construct used precludes stable binding of nucleic acid in the active site. Additional work will be required to capture...
Apn2 homologs bound to nicked or gapped DNA substrates to understand how the protein engages these structures in the context of Top1-generated DNA damage.

**STAR★METHODS**

**RESOURCE AVAILABILITY**

**Lead contact**—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, R. Scott Williams (williamsrs@niehs.nih.gov).

**Materials availability**—All materials generated by this study are listed in the Key Resources Table and are available from the lead contact without restriction. Coordinates and structure factors for Apn2 (RCSB 7N3Z) and the Apn2-DNA complex (RCSB 7N3Y) are deposited in the RCSB protein data bank.

**Data and code availability**

- The data presented in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Bacterial strain**—BL21-AI cells (Life Technologies) were transformed with the constructs for protein expression overnight at 15°C in Terrific Broth. Induction of expression was carried out by the primary addition of 0.1% (w/v) final concentration L-Arabinose (GoldBio).

**Yeast strains**—Saccharomyces cerevisiae strains are isogenic derivatives of strain Δ(−2)~7B-YUNI300 (MATa CAN1 his7–2 leu2Δ:kanMX ura3Δ trp1–289 ade2–1 lys2ΔGG2909–2909). Please see Table S3 for derivative strains. Strains were grown in YPDA medium (1% yeast extract, 2% bacto-peptone, 250 mg/L adenine, 2% dextrose, 2% agar for plates) at 30°C.

**METHOD DETAILS**

**Apn2 and PCNA protein purification**—The Apn2FL (full length) and Apn2Cat (residues 1–407) were PCR amplified and subcloned into the pET MBP His6 LIC cloning vector (2Cc-T), containing a TEV-cleavable C-terminal MBP fusion tag (Addgene). Apn2Cat(D222N/E59Q) was cloned by site-directed mutagenesis of the Apn2Cat construct. Apn2FL mutants were cloned by site-directed mutagenesis of the Apn2FL construct. The wedge loop deletion construct was made by replacing Apn2 residues W312 through Y323 with two glycine residues. BL21-AI cells (Life Technologies) were transformed with the constructs for protein expression overnight at 15°C in Terrific Broth. Induction of expression was carried out by the primary addition of 0.1% (w/v) final concentration L-Arabinose (GoldBio). Following Amylose affinity chromatography, MBP fusion proteins were diluted.
in ddH2O and loaded onto 5-mL ion exchange columns for gradient elution (GE Healthcare; HiTrap Heparin). For crystallography, proteins were subjected to overnight TEV protease cleavage for MBP tag removal prior to ion-exchange chromatography. Purest fractions were pooled and purified by Superdex 200 (GE Healthcare) gel filtration for final polishing. Final purity was assessed by SDS-PAGE and fractions pooled and concentrated for subsequent experiments. The yeast PCNA protein was a kind gift from Andrea Kaminski.

**Apn2\textsuperscript{Cat} and Apn2\textsuperscript{Cat(D222N/E59Q)}-DNA crystallization—** Apn2\textsuperscript{Cat} protein was buffer exchanged into 10 mM Tris pH 8.0, 100 mM NaCl, 1 mM MgCl\textsubscript{2}, and 1 mM TCEP and concentrated to 10 mg/mL. Crystallization trials with commercially available screens (Qiagen) were utilized for initial hit discovery using the sitting drop vapor diffusion method. Initial crystals of the Apn2\textsuperscript{Cat} domain were optimized to grow in 25% PEG 6000, 0.1 M Tris pH 8.5 at 20°C. 10 mg/mL Apn2\textsuperscript{Cat(D222N/E59Q)} protein in 10 mM Tris pH 8.0, 100 mM NaCl, 1 mM CaCl\textsubscript{2}, and 1 mM TCEP with 1.2-fold molar excess DNA was used for crystallization trials with commercially available screens (Qiagen) using the sitting drop vapor diffusion method. Apn2\textsuperscript{Cat(D222N/E59Q)} was crystallized with two different duplex DNA substrates in the same crystal made by performing annealing in the presence of 13-mer strands A (5’ TCCGAAATTTCGG) and B (5’ CCGAAATTTCGGA), each with five phosphorothioate bonds at the 3’ ends to inhibit Apn2 nuclease activity. One DNA substrate has a blunt-end resulting from A annealing to B, where the second substrate has a 1 nucleotide overhang resulting from strand A self-annealing. Crystals of the Apn2\textsuperscript{Cat(D222N/E59Q)}-DNA complex were optimized to grow in 0.2 M trisodium citrate, 20% PEG 3350 at 20°C. Crystals were cryoprotected with mother liquor supplemented with 26% ethylene glycol and flash-frozen in liquid nitrogen for X-ray data collection.

**Data collection, structure solution and refinement for Apn2\textsuperscript{Cat} and Apn2\textsuperscript{Cat(D222N/E59Q)}-DNA—** X-ray data was collected at 105K on beamline 22-ID of the Advanced Photon Source at a wavelength of 1.000 Å. Data reduction and scaling was performed with the HKL2000 suite (Otwinowski and Minor, 1997). The Apn2\textsuperscript{Cat} structure was solved by molecular replacement with PDB 3g0r (Lakomek et al., 2010) as a model, performed by the PHENIX software suite (Adams et al., 2010). A combination of density modification with Autobuild and manual refinement in COOT (Emsley and Cowtan, 2004) produced a model that was refined to 1.994 Å in PHENIX (see Table S1). The Apn2\textsuperscript{Cat(D222N/E59Q)}-DNA structure was solved by molecular replacement with PDB 3g0r (Lakomek et al., 2010) as a model, performed by the PHENIX software suite (Adams et al., 2010). A combination of density modification with Autobuild and manual refinement in COOT (Emsley and Cowtan, 2004) produced a model that was refined to 2.728 Å in PHENIX (see Table S1). NCS restraints were not used in the refinement. The structures and associated experimental data were deposited in the RCSB PDB as 7N3Z and 7N3Y for Apn2\textsuperscript{Cat} and Apn2\textsuperscript{Cat(D222N/E59Q)}-DNA, respectively.

**Preparation of oligonucleotide substrates—** The construction and sequence of the 3’ recessed substrates for the exonuclease cleavage assays are detailed in Table S2.
Exonuclease cleavage assays—For nuclease assays involving 6-FAM labeled DNA substrates, purified Apn2FL or Apn2FL mutants in storage buffer (10 mM Tris pH 8.0, 75 mM NaCl, 1 MgCl2, 1 mM TCEP, 50% glycerol) were diluted in 1X reaction buffer (10 mM Tris pH 8.0, 1 MgCl2, 1 mM TCEP) and combined with PCNA, for a final concentration of 250 nM each. To begin reaction, 50 nM DNA substrate, in 1X reaction buffer, was added to the Apn2FL/PCNA complex in a final reaction volume of 30 μL. Samples were incubated at 37°C for an hour and 10 μL aliquots were removed at indicated time points. For testing effect of salt on Apn2FL activity, 1X reaction buffers were prepared containing additional 10 mM NaCl, and reactions were carried out as described above. For the 3′-phosphorothioate experiments, BSA (Sigma) was added to the 1X reaction buffer to a final concentration of 0.1 mg/mL. All reactions were quenched with 2-fold gel loading buffer (98% formamide, 10 mM EDTA), denatured at 95°C for 10 min, and cooled at 4°C for 2 min prior to sample loading. Samples were loaded and resolved on a 20% TBE-Urea gel. Gels were imaged using a Typhoon 9500 imager (GE Healthcare) and viewed using ImageJ.

Yeast strain construction and growth analysis—Saccharomyces cerevisiae strains are isogenic derivatives of strain Δ(−2)|−7B-YUNI300 (MATa CAN1 his7−2 leu2Δ:kanMX ura3Δ trpl−289 ade2−1 lys2Δ:GG2899−2900) (Pavlov et al., 2001), and relevant genotypes are listed in Table S3. The pol2-M644G, rnh201Δ and top1Δ mutants have been described previously (Williams et al., 2013), and the Rnh201-RED mutant is from (Huang et al., 2017). The Apn2 Y33E and wedge loop deletion (ΔWL; replacement of amino acids 312–323 with 2 glycine residues) mutants were generated using a plasmid containing Apn2 C-terminally tagged with 5x-FLAG and flanked by 600 bp of upstream and downstream sequences. Mutagenesis was performed using the Quikchage II site-directed mutagenesis kit (Agilent). Construction of diploids heterozygous for APN2/apn2::natMX4 was performed by deletion replacement of one copy of APN2. Transformants were verified by marker selection and PCR analysis.

Doubling time was measured for logarithmically growing cultures using between 4 and 10 replicates for each experiment. Data is displayed as the mean D_t +/- standard deviation (SD).

Spot dilution assays—Spot assays were performed by spotting 10-fold dilutions of exponentially growing cells onto YPDA agar in the absence or presence of varying concentrations of hydroxyurea (HU; Sigma H8627). Plates were photographed following growth at 30°C for 3 days.

Spontaneous mutation rate and sequencing analysis—Mutation rate analysis was performed in strains containing the URA3 reporter gene placed in one of two opposing orientations adjacent to an efficient, early-firing replication origin, ARS306. Mutation rates and specificity were determined by measuring fluctuation analysis as described (Shcherbakova and Kunkel, 1999). The ura3 gene from single, independent 5-FOA-resistant colonies was PCR-amplified and sequenced. Specific mutation rates were calculated by multiplying the fraction of that mutation type by the total mutation rate for each strain.
QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analysis was performed using GraphPad Prism 8. p-value determination for doubling time measurements was performed using the unpaired Students t-test. Statistical analysis of overall mutation rate comparisons was performed using a one-sided nonparametric Mann Whitney test. Statistical details can be found in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Apn2 frays DNA ends to facilitate its broadly specific 3’ DNA-end healing activity
- Apn2 binds duplex DNA differently than other EEP family proteins TDP2 and APE1
- Products of Top1 incision at ribonucleotides are biologically important Apn2 substrates
- Top1-dependent mutagenesis is uncovered in yeast strains deficient in APN2 and RER
Figure 1. X-ray crystal structure of the Apn2-DNA complex
(A) Domain architecture of Apn2.
(B) Crystal structure of the N-terminal EEP domain of *S. cerevisiae* Apn2 bound to DNA with 1 of the 4 Apn2 protomers in the asymmetric unit of the DNA complex crystal represented.
(C) An orthogonal view of the catalytic domain structure bound to DNA.
(D) A surface representation of the Apn2-DNA complex with the electron density map of the DNA overlaid (final 2Fo-Fc, 1.0σ at 2.73 Å).
Figure 2. Apn2 DNA interactions

(A) Surface representations of the DNA, the WL, and Y33 reveal tight shape complementarity. The 180° rotation shows how Y33, together with the WL, interrogates the minor groove of incoming DNA, making van der Waals contacts with the DNA backbone and a charge interaction with the side chain of N317, stabilizing both the DNA and the WL.

(B) Basic surface comprised residues K201, R205, R208, and R273 (green surfaces and sticks) create a groove that recognizes the backbone of the second DNA molecule in the crystal.
(C) The WL changes conformation upon DNA binding. The conformational change of the WL allows for new side chain contacts to recognize the DNA substrate, essentially splaying open the strands to make available the damaged 3’ end for cleavage. The WL appears to “bloom,” with conserved residues W312 and R319 serving as the central anchor point out from which the other residues unfurl. The WL tilts down with the DNA interaction to allow N317 to begin to intercalate between the strands and to position K316 for direct interaction with the 3’ DNA termini to be processed.

(D) Simplified illustrated model of a fully “bloomed” WL.

(E) Orthogonal views of (C).

(F) Overlay of ribbon representations of DNA-free and DNA-bound WLs in all Apn2 protomers from both the DNA-free and DNA-present crystal structures.
Figure 3. Apn2 nuclease processes a variety of 3′ DNA termini

(A) Denaturing gel electrophoresis of dsDNA oligonucleotide substrates carrying a 3′ hydroxyl, 3′ G:A mismatch, 3′ ribouridine (rU), or terminal 2′,3′-cyclic phosphate (see Table S2 for substrates) incubated with recombinant S. cerevisiae maltose binding protein (MBP)-Apn2, PCNA, or MBP as controls. Yellow, position of the FAM label; numbers, oligonucleotide lengths. Gels shown are representative of 3 technical replicates.
(B) Denaturing gel electrophoresis exonuclease assays of WT, Y33E, R205E, and R208E mutant Apn2 in the presence and absence of PCNA with the 3′ hydroxyl substrate (see Table S2 for substrates). Gels shown are representative of 3 technical replicates.

(C) Denaturing gel electrophoresis of salt-sensitivity exonuclease time course assays of WT and WL deletion mutants in the absence of PCNA. Gels shown are representative of 3 technical replicates.

(D) Denaturing gel electrophoresis of salt-sensitivity exonuclease time course assays of WT, WL deletion, and catalytically dead Apn2 in the presence of PCNA with a 3′ hydroxyl substrate (3′OH) (Table S2). Gels shown are representative of 3 technical replicates.

(E) Quantification of the band intensities observed in the 10 mM NaCl, 60 min lanes in (C) using Fiji. Experiments are representative example of gels run 3 times.

(F) Sequence alignment of the WL with anchor residues highlighted in red and pink circles denoting DNA interaction. The deletion mutation used is indicated.
Figure 4. The WL guides endonucleolytic cleavage events

(A) A 3’ FAM-labeled substrate with increasing, non-hydrolyzable phosphorothioate bonds at the 3’ end demonstrate a difference in PCNA-stimulated endonucleolytic cleavage pattern between WT Apn2 and the WL deletion mutant. DNA ladders were produced by T7 (lane 3) or lambda exonuclease (lane 4) digestion of substrate, and by direct synthesis 5-nt 3’-FAM-labeled DNA ladder (lanes 5, 20, 21, and 36). Gels shown are representative of 3 technical replicates.

(B) Model of Apn2 recognizing and processing DNA substrate.
Figure 5. Apn2 is important for suppressing mutagenesis and promoting normal cell growth and genotoxin resistance in the presence of a high density of Top1-incised nascent leading strand ribonucleotides
(A) Δ2 to 5-bp mutation rates for the URA3-OR1 (orientation 1) and URA3-OR2 reporter genes in the WT, rnh201Δ, and rnh201Δ apn2Δ mutants were calculated as the proportion of each type of event among the total mutants sequenced, multiplied by the overall mutation rate (Table S4).

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(B) Tetrad analysis of an _APN2/apn2::natMX_ diploid in a homozygous _POL2_ or _pol2-M644G_ strain ± _RNH201_ and _TOP1_. Plates were photographed after 3 days of growth at 30°C.

(C) Deleting _APN2_ in the _pol2-M644G_ RNase H2-defective strains impairs growth and causes genotoxin sensitivity that is reduced upon the deletion of _TOP1_. Serial (10-fold) dilutions of cells were plated on rich medium ± HU and photographed after 3 days’ growth at 30°C.

(D) Overall spontaneous mutation rates for yeast strains expressing _pol2-M644G_ ± _RNH201_, _APN2_, and _TOP1_ were determined by fluctuation analysis using a _URA3_ reporter assay. The rate of mutation conferring resistance to 5-FOA was determined as _ura3_ mutants are resistant to 5-FOA. The median rate ± the 95% confidence interval is displayed. *p = 0.0028. N represents the number of mutation rate replicates and is as follows: _pol2-M644G_: 57, _pol2-M644G apn2Δ_: 36, _pol2-M644G rnh201Δ_: 24, _pol2-M644G rnh201Δ apn2Δ_: 46, and _pol2-M644G rnh201Δ apn2Δ top1Δ_: 32.
Figure 6. Apn2 prevents base-base mismatch mutagenesis caused by Top1 cleavage at ribonucleotides

(A) The coding strand of the 804-bp URA3 gene is shown (OR1). Sequence changes observed in independent ura3 mutants are depicted in red above the coding sequence for the pol2-M644G rnh201Δ strain (n = 126; Nick McElhinny et al., 2010) and in blue beneath the coding sequence for the pol2-M644G rnh201Δ apn2Δ strain (n = 191). Letters indicate single-base substitutions, closed triangles indicate single-base additions, open triangles indicate single-base deletions, and short lines indicate 2–5 bp deletions.
(B) Δ2- to 5-bp mutation rates in the pol2-M644G ± RNH201, APN2, and TOP1 were calculated as the proportion of each type of event among the total mutants sequenced, multiplied by the overall mutation rate (Table S6).

(C) As in (B), but for the A286T and A686T transversion hotspots in the pol2-M644G strain ± RNH201, APN2, and TOP1.

(D) As in (C), but for the Apn2 DNA-binding mutants.
A model depicting the consequences of Top1 cleavage at unrepaired ribonucleotides incorporated by Pol ε during DNA replication. Processing of a Top1-induced nick can be repaired via mutagenic or error-free repair pathways. Results in yeast demonstrate the importance of Apn2 for promoting cell growth and HU resistance and preventing single-base mismatch mutagenesis in a strain with a high density of unrepaired ribonucleotides (pol2-M644G RNase H2-defective). As these phenotypes are dependent on the presence of Top1, this suggests that the “dirty” 3’ DNA end produced by Top1 incision at a ribonucleotide

Figure 7. Overall model of Apn2 processing of 3’ DNA ends produced when Top1 cleaves at genomic ribonucleotides
requires Apn2 for processing and resolution. This may involve multiple enzymatic functions possessed by Apn2, including 3’ exonucleolytic, unwinding, and endonuclease activities.
### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** |       |            |
| BL21-Al E. coli     | Invitrogen | Cat# C607003 |
| **Chemicals, peptides, and recombinant proteins** |       |            |
| Recombinant *S. cerevisiae* Apn2<sup>FL</sup> (full-length) | This paper | N/A |
| Recombinant *S. cerevisiae* Apn2<sup>ΔWL</sup>, Y<sup>33E</sup>, R<sup>205E</sup>, R<sup>208E</sup>, D<sup>222N/E<sup>59Q</sup></sup> | This paper | N/A |
| Recombinant *S. cerevisiae* Apn2<sup>Cat</sup> (residues 1–407) | This paper | N/A |
| Recombinant *S. cerevisiae* Apn2<sup>Cat(D222N/E59Q)</sup> | This paper | N/A |
| Recombinant *S. cerevisiae* PCNA | Andrea Kaminski | N/A |
| Hydroxyurea (HU) | Sigma | Cat# H8627 |
| 5-Fluoroorotic acid (5-FOA) | US Biological | Cat# F21270 |
| Yeast extract | ThermoFisher Scientific | Cat# 211677 |
| Bacto-peptone | ThermoFisher Scientific | Cat# 214010 |
| Agar | Sigma | Cat# A3159 |
| Adenine | Dextrose | ThermoFisher Scientific | Cat# D16 |
| **Critical commercial assays** |       |            |
| Quikchange II site-directed mutagenesis kit | Agilent | Cat# 200524 |
| **Deposited data** |       |            |
| Coordinate of *S. cerevisiae* Apn2<sup>Cat</sup> | This paper | PDB: 7N3Z |
| Coordinate of *S. cerevisiae* Apn2<sup>Cat(D222N/E59Q)</sup>-DNA | This paper | PDB: 7N3Y |
| **Experimental models: Organisms/strains** |       |            |
| Saccharomyces cerevisiae: Δ(|−2|)-7B-YUNI300 (parent) | Pavlov et al., 2001 | N/A |
| **Oligonucleotides** |       |            |
| Substrate oligonucleotides for *in vitro* reactions See Table S2 | This paper | N/A |
| Crystallization oligonucleotide A 5′-TCCGAAAT*T*T*C*G*G (+ denotes phosphorothioate bond) | This paper | N/A |
| Crystallization oligonucleotide B 5′-CCGAAATT*T*C*G*G*A (+ denotes phosphorothioate bond) | This paper | N/A |
| Yeast mutagenesis primers:Apn2-Y33E-for 5′-AGAAA CCTTTTTTCAGGCAACATTTTCTCAA | This paper | N/A |
| Apn2-Y33E-rev 5′-TGGAAATGTTGCTCATGAAAAAGGTTTCT | This paper | N/A |
| Apn2-ΔWL-for 5′-CTTTAAAAGTATACAGTCGGCGG CGGCTACCGG ATAGATTTT | This paper | N/A |
| Apn2-ΔWL-rev 5′-AAAATCTATCCGTAGGCCGCCGACTGTACATTTTAAG | This paper | N/A |
| **Recombinant DNA** |       |            |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| 2Cc-T ScApn2FL       | This paper | N/A        |
| 2Cc-T ScApn2FL mutants (DWL, Y33E, R205E, R208E, D222N/E59Q) | This paper | N/A        |
| 2Cc-T ScApn2Cat      | This paper | N/A        |
| 2Cc-T ScApn2Cat D222N/E59Q | This paper | N/A        |

**Software and algorithms**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| PHENIX               | Adams et al., 2010 | N/A        |
| Coot                 | Emsley and Cowtan, 2004 | N/A        |
| HKL2000              | HKL Research Inc. | N/A        |
| PyMOL                | Schrödinger | N/A        |
| ImageJ               | Fiji | N/A        |
| Prism 8.0            | GraphPad | N/A        |
| SeqMan Pro           | DNA star Lasergene 15 | N/A        |