The nuclelease activity of DNA2 promotes exonuclease 1–independent mismatch repair

Lyudmila Y. Kadyrova1, Basanta K. Dahal1, Vaibhavi Gujjar1, James M. Daley1, Patrick Sung1, and Farid A. Kadyrov1,2

From the 1Department of Biochemistry and Molecular Biology, Southern Illinois University School of Medicine, Carbondale, Illinois, USA; 2Department of Biochemistry and Structural Biology, University of Texas Health Science Center, San Antonio, Texas, USA

Received for publication, December 8, 2021, and in revised form, March 9, 2022. Published, Papers in Press, March 15, 2022, https://doi.org/10.1016/j.jbc.2022.101831

© 2022 THE AUTHORS. Published by Elsevier Inc on behalf of American Society for Biochemistry and Molecular Biology. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

For correspondence: Farid A. Kadyrov, fkadyrov@siumed.edu.

Present address for Basanta K. Dahal: Finless Foods, 6460 Hollis St, Emeryville, CA 94608, USA.

Present address for James M. Daley: Department of Biological Chemistry, University of Michigan School of Medicine, Ann Arbor, MI, 48109, USA.

The DNA mismatch repair (MMR) system is a major DNA repair system that corrects DNA replication errors. In eukaryotes, the MMR system functions via mechanisms both dependent on and independent of exonuclease 1 (EXO1), an enzyme that has multiple roles in DNA metabolism. Although the mechanism of EXO1-dependent MMR is well understood, less is known about EXO1-independent MMR. Here, we provide genetic and biochemical evidence that the DNA2 nuclease/helicase has a role in EXO1-independent MMR. Biochemical reactions reconstituted with purified human proteins demonstrated that the nuclease activity of DNA2 promotes an EXO1-independent MMR reaction via a mismatch excision-independent mechanism that involves DNA polymerase δ. We show that DNA polymerase ε is not able to replace DNA polymerase δ in the DNA2-promoted MMR reaction. Unlike its nuclease activity, the helicase activity of DNA2 is dispensable for the ability of the protein to enhance the MMR reaction. Further examination established that DNA2 acts in the EXO1-independent MMR reaction by increasing the strand-displacement activity of DNA polymerase δ. These data reveal a mechanism for EXO1-independent mismatch repair.

The mismatch repair (MMR) system has been conserved from bacteria to humans (1, 2). It promotes genome stability by suppressing spontaneous and DNA damage-induced mutations (1, 3–11). The key function of the MMR system is the correction of DNA replication errors that escape the proof-reading activities of replicative DNA polymerases (1, 4–10, 12). In addition, the MMR system removes mismatches formed during strand exchange in homologous recombination, suppresses homologous recombination, initiates apoptosis in response to irreparable DNA damage caused by several anticancer drugs, and contributes to instability of triplet repeats and alternative DNA structures (1, 4, 5, 7–11, 13–18). The principal components of the eukaryotic MMR system are MutSα (MSH2-MSH6 heterodimer), MutLα (MLH1-PMS2 heterodimer in humans and Mlh1-Pms1 heterodimer in yeast), MutSβ (MSH2-MSH3 heterodimer), proliferating cell nuclear antigen (PCNA), replication factor C (RFC), exonuclease 1 (EXO1), RPA, and DNA polymerase δ (Pol δ). Loss-of-function mutations in the MSH2, MLH1, MSH6, and PMS2 genes of the human MMR system cause Lynch and Turcot syndromes, and hypermethylation of the MLH1 promoter is responsible for ∼15% of sporadic cancers in several organs (19, 20). MMR deficiency leads to cancer initiation and progression via a multistage process that involves the inactivation of tumor suppressor genes and action of oncogenes (21).

MMR occurs behind the replication fork (22, 23) and is a major determinant of the replication fidelity (24). The correction of DNA replication errors by the MMR system increases the replication fidelity by ∼100 fold (25). Strand breaks in leading and lagging strands as well as ribonucleotides in leading strands serve as signals that direct the eukaryotic MMR system to remove DNA replication errors (26–30). MMR is more efficient on the lagging than the leading strand (31). The substrates for MMR are all six base–base mismatches and 1 to 13-nt insertion/deletion loops (25, 32–34). Eukaryotic MMR commences with recognition of the mismatch by MutSα or MutSβ (32, 34–36). MutSα is the primary mismatch-recognition factor that recognizes both base–base mismatches and small insertion/deletion loops whereas MutSβ recognizes small insertion/deletion loops (32, 34–37). After recognizing the mismatch, MutSα or MutSβ cooperates with RFC-loaded PCNA to activate MutLα endonuclease (38–43). The activated MutLα endonuclease incises the discontinuous daughter strand 5′ and 3′ to the mismatch. A 5′ strand break formed by MutLα endonuclease is utilized by EXO1 to enter the DNA and excise a discontinuous strand portion encompassing the mismatch in a 5′→3′ excision re-action stimulated by MutSα/MutSβ (38, 44, 45). The generated gap is filled in by the Pol δ holoenzyme, and the nick is ligated by a DNA ligase (44, 46, 47). DNA polymerase ε (Pol ε) can substitute for Pol δ in the EXO1-dependent MMR reaction, but its activity in this reaction is much lower than that of Pol δ (48). Although MutLα endonuclease is essential for MMR in vivo, 5′ nick-dependent MMR reactions reconstituted in the presence of EXO1 are MutLα-independent (44, 47, 49).
DNA2 and MMR

EXO1 deficiency in humans does not seem to cause significant cancer predisposition (19). Nevertheless, it is known that Exo1−/− mice are susceptible to the development of lymphomas (50). Genetic studies in yeast and mice demonstrated that EXO1 inactivation causes only a modest defect in MMR (50–53). In agreement with these genetic studies, a defined human EXO1-independent MMR reaction that depends on the strand-displacement DNA synthesis activity of Pol δ holoenzyme to remove the mismatch was reconstituted (54). Furthermore, an EXO1-independent MMR reaction that occurred in a mammalian cell extract system without the formation of a gapped excision intermediate was observed (54). Together, these findings implicated the strand-displacement activity of Pol δ holoenzyme in EXO1-independent MMR.

In this study, we investigated DNA2 in the context of MMR. DNA2 is an essential multifunctional protein that has nuclease, ATPase, and 3′→5′ helicase activities (55–57). Previous research ascertained that DNA2 removes long flaps during Okazaki fragment maturation (58–60), participates in the resection step of double-strand break repair (61–63), initiates the replication checkpoint (64), and suppresses the expansions of GAA repeats (65). We have found in vivo and in vitro evidence that DNA2 promotes EXO1-independent MMR. Our data have indicated that the nuclease activity of DNA2 enhances the strand-displacement activity of Pol δ holoenzyme in an EXO1-independent MMR reaction.

Results

A dna2 allele causes a defect in Exo1-independent MMR

We started this work to investigate whether DNA2 has a role in MMR. Our initial genetic analysis in Saccharomyces cerevisiae demonstrated that introduction of a temperature-sensitive dna2 allele (55), dna2-P504S, into a WT strain caused a 3-fold increase in the CAN1 mutation rate (Fig. 1). We then used the CAN1 mutation assay to examine how dna2-P504S interacted with exo1Δ and msh6Δ, two alleles that cause MMR defects. The data showed that there was a synergistic relationship between dna2-P504S and exo1Δ, but not between dna2-P504S and msh6Δ (Fig. 1). Next, we determined can1 mutation spectra in the WT, dna2-P504S, exo1Δ, dna2-P504S exo1Δ, msh2Δ, and msh2Δ dna2-P504S exo1Δ strains (Table 1). The can1 spectrum in the dna2-P504S strain was dominated by base–base substitutions, but some 1-nt deletions and mutational events that caused CAN1 loss were also observed. Comparison of the mutation spectra in the dna2-P504S, exo1Δ, and dna2-P504S exo1Δ showed that there was a synergistic interaction between dna2-P504S and exo1Δ for base substitutions and 1-nt deletions. Further analysis revealed that msh2Δ was epistatic to dna2-P504S exo1Δ for base substitutions. The observations that (i) msh2Δ was epistatic to dna2-P504S exo1Δ for base substitutions and that (ii) there was a synergistic relationship between dna2-P504S and exo1Δ for base substitutions supported the hypothesis that DNA2 is involved in an Msh2-dependent pathway that repairs base–base mismatches in an Exo1-independent manner. We also observed that there was a weak synergistic relationship between msh2Δ and dna2-P504S exo1Δ for 1-nt deletions. This is likely a result of participation of DNA2 in another genetic stabilization pathway.

A physical interaction between MutLa and PCNA is essential for MMR (66). A mutation, pms1-Q723A, that results in an amino acid change in the PCNA-binding motif of MutLa causes a strong defect in EXO1-independent MMR (66). We investigated how pms1-Q723A and dna2-P504S interacted with each other in the CAN1 mutation assay. Measurements of the mutation rates and analysis of the mutation spectra revealed that pms1-Q723A was epistatic to dna2-P504S for base substitutions in CAN1 (Table 1). This finding provided additional genetic evidence for a role of DNA2 in EXO1-independent MMR.

DNA2 promotes an EXO1-independent MMR reaction in a reconstituted system via an excision-independent mechanism

We next utilized biochemical approaches to investigate DNA2 in the context of MMR reactions. A previous study described a defined EXO1-independent human MMR reaction that occurs on heteroduplex DNAs in the presence of MutSa, MutLa, PCNA, RFC, RPA, and Pol δ (54). In this EXO1-independent MMR reaction, MutLa incises a heteroduplex DNA 5′ to the mismatch in a MutSa+, PCNA+, and RFC-dependent manner, and Pol δ holoenzyme utilizes the 5′ strand break to perform a strand-displacement DNA synthesis that removes the mismatch. We examined whether DNA2 affected the EXO1-independent MMR reaction on a 3′ heteroduplex DNA. Human DNA2 for these and following experiments was produced in and purified from insect Sf9 cells (57) (Fig. S1). The data revealed that the purified DNA2 increased the level of MMR in the EXO1-independent reaction by ~3 fold (Fig. 2A, lanes 2–3 and graph). In the next series of experiments, we analyzed whether the presence of MutSa, MutLa, or Pol δ was necessary for the DNA2-promoted MMR reaction. As expected, these experiments showed that the omission of MutSa, MutLa, or Pol δ abolished the DNA2-promoted MMR reaction (Fig. 2A, lanes 4–6 and graph). Thus, we concluded that the DNA2-promoted MMR reaction on a 3′ heteroduplex occurred in a MutSa+, MutLa+, and Pol δ-dependent manner.

**Figure 1.** A synergistic interaction between dna2-P504S and exo1Δ for can1 mutations. Spontaneous CAN1 mutation rates were measured as described under Experimental procedures. The data are presented as medians with 95% confidence intervals.
EXO1 excises the DNA mismatch in the mismatch excision reaction (45, 47, 67). To better understand the mechanism of DNA2-promoted MMR, we performed experiments to analyze whether the addition of DNA2 to a reaction mixture containing MutSα, MutLα, PCNA, RFC, RPA, and a 3′ heteroduplex led to a mismatch excision (Fig. 2B). In agreement with a previous study (38), a control experiment showed that a small level of mismatch excision took place in the reaction mixture containing MutSα, MutLα, PCNA, RFC, and RPA (Fig. 2B, lanes 1–2). This excision was a result of the activation of MutLα endonuclease by MutSα, PCNA, RFC, and the mismatch (38).

However, we found that the supplementation of the five-protein system with DNA2 did not trigger an increase in the level of mismatch excision (Fig. 2B, lanes 2–3). This finding indicated that the mechanism of the DNA2-promoted MMR reaction is different from that of the EXO1-dependent MMR reaction.

Protein–protein interactions are involved in MMR reactions (4). We studied whether DNA2 physically interacted with the MMR factors MutSα and MutLα in a pull-down assay. The data showed that MutSα-containing agarose beads pulled down the purified DNA2 protein (Fig. 2C), but agarose beads containing MutLα did not (Fig. S2). Thus, these experiments

---

Table 1

| Genotype | Base–base substitutions | 1-nt deletions | 1-nt insertions | MMR loss | Other mutations | Total |
|----------|-------------------------|----------------|----------------|----------|----------------|-------|
| WT (n=50) | 20                      | 1.5            | 1.5            | <0.5     | 1.5            | 24 (21–34) |
| dna2-P504S (n=50) | 42                      | 8              | <2             | 8        | 11             | 70 (50–90) |
| exo1Δ (n=50) | 110                     | 14             | <2.8           | <2.8    | 14             | 140 (130–190) |
| dna2-P504S exo1Δ (n=80) | 280                   | 68             | 29             | 23       | 63             | 460 (300–510) |
| msh2Δ (n=82) | 435                    | 390            | 90             | <11      | <11            | 910 (900–1200) |
| msh2Δ dna2-P504S exo1Δ (n=79) | 425                 | 655            | 61             | 61       | <15            | 1200 (1100–1700) |
| msh2Δ dna2-P504S pms1-Q723A (n=51) | 85                   | 36             | 11             | 3        | 3              | 140 (110–180) |
| dna2-P504S pms1-Q723A (n=50) | 80                     | 110            | 35             | 5        | 20             | 250 (230–320) |

n, a number of can1 mutants sequenced. The mutations were identified by DNA sequencing. 95% confidence intervals are in parentheses.
revealed that DNA2 physically interacts with the mismatch recognition factor MutSα.

**DNA2 involves its nuclease activity to promote an EXO1-independent MMR reaction**

Human DNA2 has both helicase and nuclease activities (56, 57, 68). The DNA2-D277A variant lacks the nuclease activity and the DNA2-K654R mutant does not have the helicase activity (57). We investigated whether the DNA2-D277A and DNA2-K654R variants affected the EXO1-independent MMR reaction on a 3’ heteroduplex DNA. We determined that the nuclease-deficient DNA2-D277A variant did not promote the 3’ gap-directed EXO1-independent MMR reaction (Fig. 3A and graph) but the helicase-deficient DNA2-K654R mutant protein did (Fig. 3B, lane 6 and graph). In agreement with the former result, we established the double mutant DNA2-D277A-K654R variant was not able to enhance the 3’ gap-directed EXO1-independent MMR reaction. These findings indicated that DNA2 relies on its nuclease activity to promote the EXO1-independent MMR reaction on a 3’ heteroduplex DNA.

**DNA2 increases the strand-displacement DNA synthesis activity of the Pol δ holoenzyme**

In addition to 3’ heteroduplexes, the MMR system corrects mismatches on 5’ heteroduplexes. We therefore studied if DNA2 and its variants affected mismatch removal on a 5’ heteroduplex in the presence of MutSα, MutLα, PCNA, RFC, and RPA. In line with our earlier data obtained using a 3’ heteroduplex DNA substrate (Fig. 3), we found that DNA2 and DNA2-K654R promoted mismatch removal on a 5’ heteroduplex in the presence of MutSα, MutLα, PCNA, RFC, and RPA, but DNA2-D277A and DNA2-D277A-K654R did not (Fig. 4B, lanes 2–6, and graph). We next asked whether the DNA2-promoted mismatch removal reaction on the 5’ heteroduplex could occur in the absence of the mismatch recognition factor MutSα or MutLα endonuclease. The data showed that the omission of MutSα or MutLα from the reaction mixture did not abolish the DNA2-promoted mismatch removal reaction on a 5’ heteroduplex (Fig. 4B, lanes 7–8, and graph). This suggested that DNA2 enhanced the MutSα-and MutLα-independent mismatch correction reaction by increasing the strand-displacement activity of Pol δ holoenzyme. Further support for this idea came from an experiment in which we ascertained that DNA2 strongly increased the strand displacement–based mismatch removal on the 5’ heteroduplex by a four-protein system consisting of Pol δ, PCNA, RFC, and RPA (Fig. 5A). In addition, we determined that human Pol ε, an enzyme that does not have a significant strand-displacement activity (48, 69), was not able to replace Pol δ in the DNA2-promoted mismatch correction reaction on the 5’ heteroduplex (Fig. 5B).

We also utilized a Southern hybridization with a 32P-labeled probe to visualize the DNA2-promoted strand-displacement DNA synthesis products that were separated on denaturing agarose gels. The experiments showed that DNA2 enhanced the strand-displacement DNA synthesis activity of Pol δ.

![Figure 3. The nuclease activity of DNA2 facilitates EXO1-independent MMR on a 3’ heteroduplex.](image-url)

**DNA2 and MMR**

A. The effects of different concentrations of DNA2 and the nuclease-deficient DNA2-D277A on EXO1-independent MMR on a 3’ heteroduplex. B. The effects of the helicase-deficient DNA2-K654R and the nuclease- and helicase-deficient DNA2-D277A-K654R on EXO1-independent MMR that occurred on a 3’ heteroduplex. The data in the graphs are averages ±1 S.D. (n > 3). MMR, DNA mismatch pair.
holoenzyme on the 5' heteroduplex and 5' homoduplex with a similar efficiency (Fig. 5C, lanes 3 and 8), and that MutSα did not affect the DNA2-promoted strand-displacement DNA synthesis on either DNA (Fig. 5C, lanes 4 and 9).

We next performed experiments to determine the size of DNA products formed by DNA2 in reconstituted strand-displacement DNA synthesis reactions that occurred in the presence or absence of MutSα. The substrate in these reactions was a 6.4-kb circular ssDNA that was annealed with 13 oligonucleotides, one of which had a base mismatch and was labeled at its 5' end with 32P. The results showed that DNA2 formed 8 to 14 nt products in the strand-displacement DNA synthesis reactions in a MutSα-independent manner (Fig. S3). This finding suggests that when the Pol δ generated an 8 to 14 nt flap during the strand-displacement reaction, it was removed by DNA2.

Discussion

EXO1 is the only exonuclease that has been shown to excise the mismatch in eukaryotic MMR (45, 50, 52, 53, 70). EXO1 preferentially acts on DNA replication errors formed by Pol α (71), but it is also involved in the correction of mismatches produced by Pol δ and Pol ε (71, 72). Unlike the loss of MutSα or MutLα, the loss of EXO1 does not confer a strong mutator phenotype on yeast and mice (50, 52, 53). This finding indicates that EXO1-independent MMR removes the majority of DNA replication errors when EXO1 is not available. Although significant progress has been made in understanding EXO1-independent MMR (54, 73–78), this MMR pathway has remained enigmatic.

Prior research revealed that the strand-displacement activity of Pol δ plays a role in EXO1-independent MMR (54, 73, 74). Furthermore, the nucleases FEN1, Rad27, and FAN1 have been implicated in EXO1-independent MMR (75–78). To address the question of whether there is another player in EXO1-independent MMR (54, 73–78), this MMR pathway has remained enigmatic.

Figure 4. The nuclease activity of DNA2 enhances EXO1-independent MMR on a 5' heteroduplex. MMR reactions were conducted as described in Fig. 2 except that the DNA substrate was a 5' heteroduplex (a 5' G-T DNA), which carried a nick 128 bp 5’ to a G-T mispair. To score MMR, the reaction products were cleaved with HindIII and Clal. A, a graphical representation of the 5' MMR assay. B, MMR products that were generated in the presence of indicated proteins. The data in the graph were obtained by quantification of gel images including the one shown and are averages ±1 S.D. (n > 3). MMR, DNA mismatch pair.
**DNA2 and MMR**

**Figure 5. DNA2 increases the strand-displacement activity of Pol δ holoenzyme.** MMR reactions on a 5′-G-T DNA and control reactions on a 5′-A-T DNA were performed as described in Fig. 4 except that reactions in A and C were carried out for 10 min. A, the effect of DNA2 on the strand-displacement activity of Pol δ holoenzyme on a 5′-G-T DNA. B, the effect of replacement of Pol δ with Pol ε on the DNA2-promoted MMR on a 5′-G-T DNA. The data in A and B are averages ± 1 S.D. (n > 3). C, a Southern hybridization analysis of strand-displacement products that were formed on 5′-A-T and 5′-G-T DNAs in the presence of indicated proteins. Recovered products of the MMR reactions were cleaved with AccI, separated in denaturing agarose gels, transferred onto nylon membranes, and hybridized with a 32P-labeled probe (5′-ACCTCAGGCATGACCTGATAGCC-3′) that is complementary to the discontinuous strand of the 5′-A-T and 5′-G-T DNAs. The indirectly labeled products were visualized using a Typhoon phosphorimager. The sketches outline the 5′-A-T and 5′-G-T DNAs and indicate relative positions of the 32P-labeled probe. The arrow marks a location of smallest strand-displacement products that removed the G-T mismatch from the DNA. MMR, DNA mismatch pair; Pol δ, DNA polymerase; Pol ε, DNA polymerase ε.

Our biochemical experiments have shown that DNA2 promotes a defined EXO1-independent MMR reaction that relies on the strand-displacement activity of Pol δ holoenzyme (Figs. 2–4). Earlier research revealed that DNA2 harbors both nuclease and helicase activities (55, 57) and that the helicase activity of DNA2 remains silent in the presence of the DNA2 nuclease activity (57). Our analysis of the helicase- and nuclease-deficient variants of DNA2 has established that it is the nuclease activity of DNA2 that enhances the EXO1-independent MMR reaction (Fig. 3). Subsequent experiments indicated that the DNA2 nuclease activity increases the efficiency of the EXO1-independent MMR reaction by enhancing strand displacement by the Pol δ holoenzyme (Fig. 5). This finding suggests that removal of ssDNA tails by DNA2 increases the strand-displacement DNA synthesis by Pol δ holoenzyme. Unlike Pol δ, Pol ε does not have a significant strand-displacement activity (48, 69). Our observation that the replacement of Pol δ with Pol ε inactivates the DNA2-promoted MMR reaction (Fig. 5B) supports the conclusion that the strand-displacement activity of Pol δ holoenzyme drives mismatch removal in the DNA2-promoted MMR reaction.

MutSα activates mismatch excision by EXO1 (45). The functional MutSα–EXO1 interaction is likely to be driven by the physical contact between the two proteins (45, 52). Although DNA2 and MutSα physically interact with each other (Fig. 2C), we have been unable to detect that MutSα increases the DNA2-promoted strand-displacement activity of Pol δ holoenzyme on a mismatch-containing DNA (Fig. 5, A and C). It might be that our system lacks a factor or a protein modification that enables MutSα to stimulate DNA2 in a mismatch-dependent manner.

A basic feature of the eukaryotic MMR mechanism is that MutLα endonuclease incises the discontinuous daughter strand in a MutSα-, PCNA-, and RFC-dependent manner to afford mismatch correction (38, 39). The endonuclease function of MutLα provides a downstream factor with a window of opportunity to enter the DNA via a MutLα-generated strand break to remove the mismatch. Previous research uncovered a role for the strand-displacement activity of the Pol δ holoenzyme in mismatch removal in the absence of EXO1 (48, 54, 73, 74). We have now shown that DNA2 contributes to EXO1-independent mismatch removal by enhancing the strand-displacement activity of Pol δ.

Of importance is an observation that like DNA2, the other two nucleases, Rad27/FEN1 and FAN1, that contribute to EXO1-independent MMR (75–78) have 5′ flap endonuclease activities (79–83), and one of them (Rad27) enhances the strand-displacement DNA synthesis by a Pol δ holoenzyme (78). This observation reinforces the view that the strand-displacement activity of the Pol δ holoenzyme plays a key role in EXO1-independent MMR. It will be important to perform quantitative analyses to determine the effects of EXO1, FEN1, Rad27, and FAN1 on the reconstituted DNA2-promoted MMR reaction to better understand the impact of DNA2 on MMR.

**Experimental procedures**

*S. cerevisiae strains and measurements of the mutation rates*

Yeast strains used in this work were the haploid WT strain E134 (MATa ade5-1 lys2-InsE-A14 trp1-289 his7-2 leu2-3,112 ura3-52) (53) and its mutant derivatives. The gene knockouts were generated utilizing PCR-amplified disruption cassettes (84) and the lithium/PEG-based transformation method (85). All gene disruptions were confirmed by PCR. The replacement of DNA2 with dna2-P504S allele was performed using the integration-excision method. The presence of the dna2-P504S mutation was confirmed by DNA sequencing.
Spontaneous CAN1 mutation rates were measured using fluctuation tests that were carried out according to a previously described method (86). Briefly, single colonies obtained from 2 to 3 independent isolates of the same yeast genotype were used to start 12 to 24 cultures each in 3-ml YPD medium (1% yeast extract, 2% bactopeptone, 2% dextrose), supplemented with 60 mg/l adenine and 60 mg/l uracil that were grown at 23 °C. Dilutions of the cultures were plated on a synthetic complete medium to score the total number of cells and on a drop-out medium that lacked arginine and contained 60 mg/l L-canavanine to score the total number of can1 mutants. Colony counts were utilized to calculate the spontaneous CAN1 mutation rates with the Drake formula (87, 88). Mutation rates are presented as median values with 95% confidence intervals.

**Human proteins**

Human MutLα, MutSα, PCNA, Pol δ, Pol ε, RFC, and RPA were isolated in near-homogeneous forms as previously described (48, 54). Human DNA2, DNA2-D277A, DNA2-K654R, and DNA2-D277A-K654R that were flag-tagged at the C termini were each purified from insect Sf9 cells by chromatographies on α-Flag M2 beads (Sigma) and a MonoS column (GE HealthCare). Baculoviruses that carried the codon-optimized DNA2, DNA2-D277A, DNA2-K654R, and DNA2-D277A-K654R genes (57) were used for production and purification of DNA2 and its variants.

**MMR and mismatch excision reactions**

MMR reactions were carried out at 37 °C in 40-μl mixtures that contained 20 mM Hapes–NaOH (pH 7.4), 5 mM MgCl2, 3 mM ATP, 110 mM KCl, 25 μM dGTP, 25 μM dATP, 25 μM dTTP, 25 μM dCTP, 4 mM DTT, 0.2 mg/ml bovine serum albumin, 0.3 μg DNA (a 3′-gapped heteroduplex, a 5′-nicked heteroduplex, or a 5′-nicked homoduplex), and indicated human proteins. When human MutSα, MutLα, PCNA, RFC, RPA, Pol δ, and Pol ε were present in the reaction mixture, their concentrations were 25, 10, 30, 10, 10, and 20 nM, respectively. Human DNA2 and its variants were included in the reaction mixtures at 0 to 30 nM as indicated. The 3′-gapped heteroduplex DNA (7.54 kb) contained a 21-nt gap that was 304 bp 3′ to an A-C mispair, the 5′-nicked heteroduplex (6.44 kb) carried a nick that was 128 bp 5′ to a G-T mismatch (70), and the 5′-nicked homoduplex DNA was identical to the 5′-nicked heteroduplex except that it lacked a mismatch. Unless noted otherwise, MMR reactions were carried out for 20 min. Mismatch excision reactions were performed exactly as the MMR reactions except that the reaction mixtures lacked the four dNTPs. MMR and mismatch excision reactions were each terminated by the addition of a 30-μl mixture containing 0.31% SDS, 0.36 M NaCl, 12 mM EDTA, 0.3 μg/μl proteinase K, and 1.8 μg/μl glycogen, followed by incubation of the mixtures at 50 °C for 15 min. The mixtures were extracted with phenol/chloroform, and the DNAs from the supernatants were precipitated with isopropanol. MMR on the 3′-gapped heteroduplex was scored by cleavage of the recovered reaction products with BspEI and AlwNI. To score MMR on the 5′-nicked heteroduplex, the recovered reaction products were cleaved with HindIII and ClaI. To determine the level of mismatch excision on the 3′-gapped heteroduplex, the recovered reaction products were digested with HindIII and AlwNI. After cleavage with restriction endonucleases, the recovered reaction products were separated in 1% agarose gels in 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) and stained with ethidium bromide. The images were obtained with a cooled charge-coupled device camera (Fotodyne) and the DNA species were quantified using an ImageJ software.

**Coimmunoprecipitation assays**

Coimmunoprecipitation assays were performed as previously described (48). Antibodies against human MSH2 (sc-376384, Santa Cruz Biotechnology), DNA2 (sc-393323, Santa Cruz Biotechnology), and MLH1 (sc-271978, Santa Cruz Biotechnology) were used in coimmunoprecipitation assays.

**Data availability**

All data are contained within the article.

**Supporting information**—This article contains supporting information (67).

**Acknowledgments**—We are grateful to Petr Cejka for a gift of the human codon-optimized DNA2 constructs and Tim Formosa for providing a dna2-I plasmid.

**Author contributions**—L. Y. K., P. S., and F. A. K. methodology; L. Y. K., B. K. D., V. G., and F. A. K. investigation; L. Y. K., J. M. D., P. S., and F. A. K. resources; L. Y. K., B. K. D., V. G., and F. A. K. formal analysis; and L. Y. K. and F. A. K. wrote the writing—original draft.

**Funding and additional information**—Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R01GM132128 (to F. A. K.) and by the National Cancer Institute of the National Institutes of Health under Award Number R35CA241801 (to P. S.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

**Conflicts of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: EXO1, exonuclease 1; MMR, DNA mismatch repair; Pol δ, DNA polymerase δ; Pol ε, DNA polymerase ε; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; RPA, replication protein A.

**References**

1. Modrich, P., and Lahue, R. (1996) Mismatch repair in replication fidelity, genetic recombination, and cancer biology. Annu. Rev. Biochem. 65, 101–133
2. Kunkel, T. A., and Eri, D. A. (2005) DNA mismatch repair. Annu. Rev. Biochem. 74, 681–710
DNA2 and MMR

3. Strand, M., Prolla, T. A., Liskay, R. M., and Petes, T. D. (1993) Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. *Nature* **365**, 274–276

4. Iyer, R. R., Pluciennik, A., Burdett, V., and Modrich, P. L. (2006) DNA mismatch repair: Functions and mechanisms. *Chem. Rev.* **106**, 302–323

5. Li, G. M. (2008) Mechanisms and functions of DNA mismatch repair. *Cell Res.* **18**, 85–98

6. Hsieh, P., and Yamane, K. (2008) DNA mismatch repair: Molecular mechanism, cancer, and ageing. *Mech. Ageing Dev.* **129**, 391–407

7. Boiteux, S., and Jinks-Robertson, S. (2013) DNA repair mechanisms and the bypass of DNA damage in Saccharomyces cerevisiae. *Genetics* **193**, 1025–1064

8. Jiricny, J. (2013) Postreplicative mismatch repair. *Cold Spring Harb. Perspect. Biol.* **5**, a012633

9. Kunkel, T. A., and Erie, D. A. (2015) Eukaryotic mismatch repair in relation to DNA replication. *Annu. Rev. Genet.* **49**, 291–313

10. Kadyrova, L. Y., and Kadyrov, F. A. (2016) Endonuclease activities of MutLalpha and its homologs in DNA mismatch repair. *DNA Repair (Amst)* **38**, 42–49

11. Chakraborty, U., and Alani, E. (2016) Understanding how mismatch repair proteins participate in the repair/anti-recombination decision. *FEBS Lett.* **580**, 2071

12. Pannafino, G., and Alani, E. (2021) Coordinated and independent roles for MLH subunits in DNA repair. *Cells* **10**, 948

13. Manhart, C. M., and Alani, E. (2016) Roles for mismatch repair family proteins in promoting meiotic crossing over. *DNA Repair (Amst)* **38**, 84–93

14. Khristich, A. N., and Mirkin, S. M. (2020) On the wrong DNA track: DNA mismatch repair: Functions and mechanisms. *FEMS Yeast Res.* **18**, fow071

15. Manhart, C. M., and Alani, E. (2016) Distinct DNA repair pathways cause genomic instability at alternative DNA structures. *Nat. Commun.* **11**, 236

16. Zhao, X., Kumari, D., Miller, C. J., Kim, G. Y., Hayward, B., Vitolio, A. G., Pinto, R. M., and Usdin, K. (2021) Modifiers of somatic expansion in huntington’s disease and other triplet repeat diseases: DNA repair turns to the dark side. *J. Biol. Chem.* **286**, 1092–1099

17. McKinney, J. A., Wang, G., Mukherjee, A., Christensen, L., Subramanian, S. H. S., Zhao, J., and Vasquez, K. M. (2020) Distinct DNA repair pathways cause genomic instability at alternative DNA structures. *Nat. Commun.* **11**, 236

18. Intervention of hMutSb and hMutSa. *Cell Res.* **6**, 1912–1914

19. Zhao, X., Kumari, D., Miller, C. J., Kim, G. Y., Hayward, B., Vitolio, A. G., Pinto, R. M., and Usdin, K. (2021) Modifiers of somatic expansion in huntington’s disease. *J. Huntington’s Dis.* **10**, 149–163

20. Lujan, S. A., Williams, J. S., Clausen, A. R., Clark, A. B., and Modrich, P. T. (2007) Saccharomyces cerevisiae MutLalpha and its homologs in DNA mismatch repair. *DNA Repair (Amst)* **6**, 1751–1764

21. Holmes, J., Clark, S., and Modrich, P. (1990) Strand-specific mismatch correction in nuclear extracts of human and *Drosophila melanogaster* cell lines. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 5837–5841

22. Thomas, D. C., Roberts, J. D., and Kunkel, T. A. (1991) Heteroduplex repair in extracts of human HeLa cells. *J. Biol. Chem.* **266**, 3744–3751

23. Lujan, S. A., Williams, J. S., Clausen, A. R., Clark, A. B., and Kunkel, T. A. (2013) Ribonucleotides are signals for mismatch repair of leading-strand replication errors. *Mol. Cell* **50**, 437–443

24. Ghodgaonkar, M. M., Lazzaro, F., Olivera-Pimentel, M., Artola-Boran, M., Cojka, P., Reijns, M. A., Jackson, A. P., Plevani, P., Muzi-Falconi, M., and Jiricny, J. (2013) Ribonucleotides misincorporated into DNA act as strand-discrimination signals in eukaryotic mismatch repair. *Mol. Cell* **50**, 323–332

25. Reyes, G. X., Kolodziejczyk, A., Devakumar, L., Kubota, T., Kolodner, R. D., Putnam, C. D., and Hombauer, H. (2021) Ligation of newly replicated DNA controls the timing of DNA mismatch repair. *Curr. Biol.* **31**, 1268–1276.e1266

26. Pavlov, Y. I., Mian, I. M., and Kunkel, T. A. (2003) Evidence for preferential mismatch repair of lagging strand DNA replication errors in yeast. *Curr. Biol.* **13**, 744–748

27. Drummond, J. T., Li, G.-M., Longley, M. J., and Modrich, P. (1995) Isolation of an hMSH2–p160 heterodimer that restores mismatch repair to tumor cells. *Science* **268**, 1909–1912

28. Sigel, J., Littman, S. J., Drummond, J. T., and Modrich, P. (1998) Isolation of hMutSb from human cells and comparison of the mismatch repair specificities of hMutSb and hMutSa. *J. Biol. Chem.* **273**, 19895–19901

29. Palombo, F., Gallinari, P., Iaccarino, I., Lettieri, T., Hughes, M., D’Arrigo, A., Truong, O., Hsuan, J. I., and Jiricny, J. (1995) GTBP, a 160-kilodalton protein essential for mismatch-binding activity in human cells. *Science* **268**, 1912–1914

30. Palombo, F., Iaccarino, I., Nakajima, E., Ikejima, M., Shimada, T., and Jiricny, J. (1996) hMutSb, a heterodimer of hMSH2 and hMSH3, binds to insertion/deletion loops in DNA. *Curr. Biol.* **6**, 1181–1184

31. Stone, J. E., and Pets, T. D. (2006) Analysis of the proteins involved in the in vivo repair of base-base mismatches and four-base loops formed during meiotic recombination in the yeast Saccharomyces cerevisiae. *Genetics* **173**, 1223–1239

32. Kadyrova, F. A., Dzantiev, L., Constantin, N., and Modrich, P. (2006) Endonucleolytic function of MutLalpha in human mismatch repair. *Cell* **126**, 297–308

33. Kadyrova, F. A., Holmes, S. F., Arana, M. E., Lukianova, O. A., O’Donnell, M., Kunkel, T. A., and Modrich, P. (2007) Saccharomyces cerevisiae MutLalpha is a mismatch repair endonuclease. *J. Biol. Chem.* **282**, 37181–37190

34. Pluciennik, A., Dzantiev, L., Iyer, R. R., Constantin, N., Kadyrov, F. A., and Modrich, P. (2010) PCNA function in the activation and strand direction of MutLalpha endonuclease in mismatch repair. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 16066–16071

35. Iyer, R. R., Pluciennik, A., Genschel, J., Tsai, M. S., Reese, L. S., and Modrich, P. (2010) MutLalpha and proliferating cell nuclear antigen share binding sites on MutSbeta. *J. Biol. Chem.* **285**, 11730–11739

36. Smith, C. E., Bowen, N., Grahame, W. J., Goellner, E. M., Srivatsan, A., and Kollodner, R. D. (2015) Activation of Saccharomyces cerevisiae mlh1pms1 endonuclease in a reconstituted mismatch repair system. *J. Biol. Chem.* **290**, 21580–21590

37. Ortega, J., Lee, G. S., Gu, L., Yang, W., and Li, G. M. (2021) Mispair-bound human MutS-MutL complex triggers DNA incisions and activates mismatch repair. *Cell Res.* **31**, 542–553

38. Zhang, Y., Yuan, F., Presnell, S. R., Tian, K., Gao, Y., Tomkinson, A. E., and Arrigo, A. (2022) 298(4) 101831

39. Li, G. M. (2008) Mechanisms and functions of DNA mismatch repair. *J. Biol. Chem.* **283**, 39752–39761
DNA2 and MMR

48. Rodriges Blanko, E., Kadyrova, L. Y., and Kadyrov, F. A. (2016) DNA mismatch repair interacts with CAF-1- and ASF1A-H3-H4-dependent histone (H3-H4)2 tetramer deposition. J. Biol. Chem. 291, 9203–9217

49. Modrich, P. (2006) Mechanisms in eukaryotic mismatch repair. J. Biol. Chem. 281, 30305–30309

50. Wei, K., Clark, A. B., Wong, E., Kane, M. F., Mazur, D. J., Parris, T., Kolas, N. K., Russel, R., Hou, H., Jr., Knertz, B., Yang, G., Kunkel, T. A., Kolodner, R. D., Cohen, P. E., and Edelmann, W. (2003) Inactivation of Exonuclease 1 in mice results in DNA mismatch repair defects, increased cancer susceptibility, and male and female sterility. Genes Dev. 17, 603–614

51. Szankasi, P., and Smith, G. R. (1995) A role for Exonuclease 1 from M. cerevisiae in mutation avoidance and mismatch correction. Science 267, 1166–1169

52. Tishkoff, D. X., Boeger, A. L., Bertrand, P., Filosi, N., Gaida, G. M., Kane, M. F., and Kolodner, R. D. (1997) Identification and characterization of Saccharomyces cerevisiae EXO1, a gene encoding an exonuclease that interacts with MSH2. Proc. Natl. Acad. Sci. U. S. A. 94, 7487–7492

53. Tran, H. T., Gordenin, D. A., and Resnick, M. A. (1999) The 3′→5′ exonucleases of DNA polymerases delta and epsilon and the 5′→3′ exonuclease Exo1 have major roles in postreplication mutation avoidance in Saccharomyces cerevisiae. Mol. Cell. Biol. 19, 2000–2007

54. Kadyrova, F. A., Genschel, J., Fang, Y., Penland, E., Edelmann, W., and Modrich, P. (2009) A possible mechanism for exonuclease 1-independent eukaryotic mismatch repair. Proc. Natl. Acad. Sci. U. S. A. 106, 8495–8500

55. Budd, M. E., and Campbell, J. L. (1997) A yeast gene required for DNA replication encodes a protein with homology to DNA helicases. Proc. Natl. Acad. Sci. U. S. A. 94, 7642–7646

56. Budd, M. E., Cho, W., and Campbell, J. L. (2000) The nuclelease activity of the yeast DNA2 protein, which is related to the RecB-like nucleases, is essential in vivo. J. Biol. Chem. 275, 16518–16529

57. Pinto, C., Kasacuinaite, K., Seidel, R., and Cejka, P. (2016) Human DNA2 possesses a cryptic DNA unwinding activity that functionally integrates with BLM or WRN helicases. Elife 5, e18574

58. Budd, M. E., and Campbell, J. L. (1997) A yeast replicative helicase, DNA2 helicase, interacts with yeast FEN-1 nuclease in carrying out its essential function. Mol. Cell Biol. 17, 2136–2142

59. Burgers, P. M. (2009) Polymerase dynamics at the eukaryotic DNA replication fork. J. Biol. Chem. 284, 4041–4045

60. Jin, Y. H., Ayyagari, R., Resnick, M. A., Gordenin, D. A., and Burgers, P. M. (2003) Okazaki fragment maturation in yeast. II. Cooperation between the polymerase and 3′-5′-exonuclease activities of Pol delta in the creation of a ligatable nick. J. Biol. Chem. 278, 1626–1633

61. Zhu, Z., Chung, W. H., Shim, E. Y., Lee, S. E., and Ira, G. (2008) Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. Cell 134, 981–994

62. Ni, H., Chung, W. H., Zhu, Z., Kwon, Y., Zhao, W., Chi, P., Prakash, R., Seong, C., Liu, D., Lu, L., Ira, G., and Sung, P. (2010) Mechanism of the ATP-dependent DNA end-resection machinery from Saccharomyces cerevisiae. Nature 467, 108–111

63. Ciepła, P., Cannavo, E., Polaczek, P., Masuda-Sasa, T., Pokharel, S., Campbell, J. L., and Kowalczykowski, S. C. (2010) DNA end resection by Dna2-Sgs1-RPA and its stimulation by Top3-3Rml and Mre11-Rad50-Xrs2. Nature 467, 112–116

64. Kumar, S., and Burgers, P. M. (2013) Lagging strand maturation factor Dna2 is a component of the replication checkpoint initiation machinery. Genes Dev. 27, 313–321

65. Zhang, Y., Shishkin, A. A., Nishida, Y., Marcinkowski-Desmond, D., Saini, N., Volkov, K. V., Mirkin, S. M., and Lobachev, K. S. (2012) Genome-wide screen identifies pathways that govern GAA/TTT repeat fragility and expansions in dividing and nondividing yeast cells. Mol. Cell 48, 254–265

66. Genschel, J., Kadyrova, L. Y., Iyer, R. R., Dahal, B. K., Kadyrov, F. A., and Modrich, P. (2017) Interaction of proliferating cell nuclear antigen with PM2S is required for MutLα activation and function in mismatch repair. Proc. Natl. Acad. Sci. U. S. A. 114, 4930–4935

67. Dantzig, L., Constantin, N., Genschel, J., Iyer, R. R., Burgers, P. M., and Modrich, P. (2004) A defined human system that supports bidirectional mismatch-provoked excision. Mol. Cell 15, 31–41

68. Budd, M. E., Cho, W. C., and Campbell, J. L. (1995) DNA2 encodes a DNA helicase essential for replication of eukaryotic chromosomes. J. Biol. Chem. 270, 26766–26769

69. Ganai, R. A., Zhang, X. P., Heyer, W. D., and Johansson, E. (2016) Strand displacement synthesis by yeast DNA polymerase epsilon. Nucleic Acids Res. 44, 8229–8240

70. Genschel, J., Bazemore, L. R., and Modrich, P. (2002) Human exonuclease 1 is required for 5′ and 3′ mismatch repair. J. Biol. Chem. 277, 13302–13311

71. Libertí, S. E., Larrea, A. A., and Kunkel, T. A. (2013) Exonuclease 1 preferentially repairs mismatches generated by DNA polymerase alpha. DNA Repair (Amst) 12, 92–96

72. Hombauer, H., Campbell, C. S., Smith, C. E., Desai, A., and Kolodner, R. D. (2011) Visualization of eukaryotic DNA mismatch repair reveals distinct recognition and repair intermediates. Cell 147, 1040–1053

73. Amin, N. S., Nguyen, M. N., Oh, S., and Kolodner, R. D. (2001) exon1-Dependent mutator mutations: model system for studying functional interactions in mismatch repair. Mol. Cell Biol. 21, 5142–5155

74. Stith, C. M., Sterling, J., Resnick, M. A., Gordenin, D. A., and Burgers, P. M. (2008) Flexibility of eukaryotic Okazaki fragment maturation through regulated strand displacement synthesis. J. Biol. Chem. 283, 34129–34140

75. Johnson, R. E., Kovvali, G. K., Prakash, L., and Prakash, S. (1995) Requirement of the yeast RTH1 5′ to 3′ exonuclease for the stability of single repetitive DNA. Science 269, 238–240

76. Liu, S., Lu, G., Ali, S., Liu, W., Zheng, L., Dai, H., Li, H., Xu, H., Hua, Y., Zhou, Y., Ortega, J., Li, G. M., Kunkel, T. A., and Shen, B. (2015) Okazaki fragment maturation involves alpha-segment error editing by the mammalian FEN1/MutSα functional complex. EMBO J. 34, 1829–1843

77. Kratz, K., Artola-Boran, M., Kobayashi-Era, S., Koh, G., Oliveira, G., Kobayashi, S., Oliveira, A., Zou, X., Richter, J., Tsuda, M., Sasanuma, H., Takeda, S., Loizou, J. I., Sartori, A. A., Nik-Zainal, S., et al. (2021) FANCD2-associated nucleosome 1 partially compensates for the lack of Exonuclease 1 in mismatch repair. Mol. Cell Biol. 41, e003021

78. Calil, F. A., Li, B. Z., Torres, K. A., Nguyen, K., Bowen, N., Putnam, C. D., Calil, F. A., Li, B. Z., Torres, K. A., Nguyen, K., Bowen, N., Putnam, C. D., and Kunkel, T. A. (2013) Identi...
DNA2 and MMR

84. Gueldener, U., Heinisch, J., Koehler, G. J., Voss, D., and Hegemann, J. H. (2002) A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res.* **30**, e23

85. Gietz, R. D., and Woods, R. A. (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol.* **350**, 87–96

86. Kadyrova, L. Y., Mertz, T. M., Zhang, Y., Northam, M. R., Sheng, Z., Lobachev, K. S., Shcherbakova, P. V., and Kadyrov, F. A. (2013) A reversible histone H3 acetylation cooperates with mismatch repair and replicative polymerases in maintaining genome stability. *PLoS Genet.* **9**, e1003899

87. Drake, J. W. (1991) A constant rate of spontaneous mutation in DNA-based microbes. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 7160–7164

88. Shcherbakova, P. V., and Kunkel, T. A. (1999) Mutator phenotypes conferred by MLH1 overexpression and by heterozygosity for mlh1 mutations. *Mol. Cell Biol.* **19**, 3177–3183