# The Fen1 extrahelical 3′-flap pocket is conserved from archaea to human and regulates DNA substrate specificity

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## ABSTRACT

**Fen1 is a key enzyme for the maintenance of genetic stability in archaea and eukaryotes and is classified as a tumor suppressor. Very recent structural data obtained from *Archaeoglobus fulgidus* Fen1 suggest that an extrahelical 3′-flap pocket is responsible for substrate specificity, by binding to the unpaired 3′-flap and by opening and kinking the DNA. Since the extrahelical 3′-flap pocket in archaeal Fen1 contains seven amino acids that are conserved to a great extent in human Fen1, we have mutated the four conserved or all seven amino acids in the human Fen1 extrahelical 3′-flap pocket to alanine. Our data suggest that the human extrahelical 3′-flap pocket mutants have lost substrate specificity to the double-flap DNA. Moreover, loss of high affinity for the unpaired 3′-flap suggests that the extrahelical 3′-flap pocket is essential for recognition and processing of the 'physiological' template. Human PCNA could stimulate the human Fen1 extrahelical 3′-flap pocket mutants but not restore their specificity. Thus the substrate specificity of Fen1 has been functionally conserved over a billion years from archaea to human.**

## INTRODUCTION

Flap endonuclease 1 (Fen1) belongs to a family of structure-specific nucleases that are evolutionarily conserved between Archaea and Eukarya (reviewed in 1–4). Its flap endonuclease activity as well as its 5′→3′ exonuclease activity allows Fen1 to remove the RNA primers during lagging strand synthesis and damaged DNA fragments in various DNA repair pathways (reviewed in 4). Based on protein sequence comparison and biochemical assays, two major conserved motifs, the N (N-terminal) and I (intermediate) motifs, were found to be essential for the nuclease activities of Fen1 (3,5). A third motif at the C-terminal end is involved in the interaction with proliferating cell nuclear antigen (PCNA) (3,6,7). All crystal structures of Fen1 homologs solved to date (8–11) showed a conserved helical arch located above the globular domain that contains the active site. This flexible loop, in addition to the catalytic site, was shown to be essential for flap cleavage (12) and it has been proposed that the 5′-end of the DNA flap could thread through the hole of the loop tracking the length of the 5′-tail (13).

The cleavage rate on a single-5′-flap substrate, which is usually used to measure Fen1 endonuclease activity, is significantly increased by the presence of a 1 bp overlap between the upstream and downstream duplex regions. This stimulatory effect has been reported previously for Fen1 homologs from Archaea to Eukarya (13–19). When the cleavage specificity of Fen1 nucleases was analyzed on a single-5′-flap substrate, the heterogeneity in cleavage position encountered (either at the junction or 1 nt into the annealed region) seemed to be inconsistent with the proposed roles of Fen1 in DNA replication and repair. In the case of the substrate with an overlap between the upstream and downstream duplexes (also called the double-flap substrate), the site of cleavage was found to be exclusively 1 nt into the annealed region (16,18,19), allowing DNA ligase I to seal the resulting nicks (16,18). In contrast, a portion of the products from the 'traditional' single-5′-flap substrate was not ligated. This led to the proposition that there is a region or pocket in the enzyme that specifically recognizes that 3′-flap nucleotide (16). A study of the T5 5′-nuclease also suggested that there could be room for an additional base at the primer terminus (10), and analysis of the interaction between the 5′-nuclease domain of bacterial DNA polymerase I and different 3′-tailed substrates also supported the binding of 1–2 nt at the 3′-terminus into an enzyme binding pocket (20).

Two additional findings suggest that a double-flap is formed and cleaved during eukaryotic DNA replication *in vivo*. Firstly, a rad27p G240D mutant (rad27p is the *Saccharomyces cerevisiae* homolog of Fen1) exhibited very low endo- and exonuclease activities on a conventional single-flap substrate, however, it efficiently cleaved a double-flap substrate (18). The mutant showed a mutator phenotype but it could grow with almost equal efficiency as the wild-type. Secondly, a dual mode of binding was shown for human Fen1 to a double-flap substrate at a 1:1 molar ratio (19), whereas a 100-fold excess of Fen1 to a single-flap substrate was required to obtain complex formation.

Very recently, the structure of the archaeon *Archaeoglobus fulgidus* Fen1 bound to a duplex DNA was solved, and FRET

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experiments and modeling suggest that *A.fulgidus* Fen1 binds to the unpaired DNA 3′-end (3′-flap), opens and kinks the DNA and promotes conformational closing of the flexible helical clamp to facilitate 5′ cleavage specificity (11). This led us to analyze whether the extrahelical 3′-flap pocket is also conserved in human Fen1 and to study the effect of replacing the seven amino acids, which comprise this extrahelical 3′-flap pocket, by alanine.

## MATERIALS AND METHODS

### Nucleic acid substrates

Oligonucleotides used to prepare the substrates for Fen1 nuclease assays were purchased from Microsynth GmbH (Balgach, Switzerland) and their sequences are listed in Table 1. They were labeled at the 5′-end in a buffer containing 70 mM Tris–HCl (pH 7.6), 10 mM MgCl$_2$, 5 mM DTT, an equimolar amount of [$\gamma$-$^{32}$P]ATP to the 5′-end to be labeled and T4 polynucleotide kinase (New England Biolabs) for 45 min at 37°C. T4 polynucleotide kinase was heat-inactivated at 80°C for 15 min and free ATP was removed on Microspin™ G-25 columns. To generate the substrates for the nuclease assays, the appropriate oligonucleotides were mixed in a 1:1 molar ratio in a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, heated to 75°C and slowly cooled to room temperature. The substrates for the electromobility shift assays were prepared according to the same protocol.

### Enzymes and proteins

Human Fen1 cDNA was cloned into the pET 23d vector (Novagen) (6). The ∆P mutant was purified as described previously (6). Wild-type and mutant proteins were over-expressed in *Escherichia coli* strain BL21(DE3)pLysS as histidine-tagged proteins and purified to near homogeneity using nickel charged metal chelating resin (HiTrap; Amersham Pharmacia Biotech) and SP Sepharose using fast protein liquid chromatography. Human PCNA was produced in *E.coli* using the plasmid pT7/hPCNA and purified to homogeneity as described (21).

### Generation of two extrahelical 3′-flap pocket mutant Fen1 enzymes

Mutagenic PCR was performed using DyNAzyme™ (Finnzymes) and the primers listed in Table 2. Following digestion of the parental, non-mutated pET23d vector template with DpnI (New England Biolabs), mutated plasmids were transformed into *E.coli* and plasmid DNA was isolated. To introduce all the desired mutations, this protocol was repeated several times. Finally, DNA sequencing reactions were performed to verify introduction of the desired mutations and absence of additional mutations.

### Fen1 nuclease assay

Assay conditions were identical to those described previously (19). The enzyme titration assays were performed in a final volume of 12.5 µl containing the following ingredients: 40 mM Tris–HCl (pH 7.5), 10 mM MgCl$_2$, 1 mM dithiothreitol, 200 µg/ml bovine serum albumin and 50 fmol DNA substrate. After addition of wild-type (wt) and mutant Fen1, reactions were incubated for 15 min at 30°C and stopped with 2.5× stop buffer (95% formamide, 20 mM EDTA, 0.05% each bromophenol blue and xylene cyanol). Products were separated on 19% denaturing polyacrylamide gels and visualized by autoradiography. For all experiments, the product size was determined by using appropriate radioactively labeled oligonucleotides (nt) of identical sequence to the template. For PCNA stimulation of Fen1, reactions were performed as

**Table 1.** Oligonucleotides used to create the Fen1 substrates used in this study

| Oligonucleotide | Size | Sequence (5′→3′) |
|---|---|---|
| Upstream primers | | |
| Up1 | 30mer | TCGAGGTCGACGGTATCGATAAGCTTGATA |
| Up2 | 31mer | TCGAGGTCGACGGTATCGATAAGCTTGATA**C** |
| Up3 | 31mer | TCGAGGTCGACGGTATCGATAAGCTTGATAT |
| Up4 | 32mer | TCGAGGTCGACGGTATCGATAAGCTTGATA**CG** |
| Downstream primers | | |
| Dn1 | 19mer | TCGAATTCCTGCAGCCCGG |
| Dn2 | 39mer | **GTCATGATAGATCTGATCGC**TCGAATTCCTGCAGCCCGG |
| Template | | |
| T | 49mer | CCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGA |

Unannealed nucleotides are in bold.

**Table 2.** Primers used to create the Fen1 mutants used in this study

| Mutant | Amino acid | Sequence (5′→3′) |
|---|---|---|
| LTFR | L53 | GGGTGGGGATGTG**GC**GCAGAATGAGGAGGG |
| | T61 | GGAGGGTGAGACC**GCT**AGCCACCTGATGGG |
| | F316 R320 | GTGTGGTGAAAAGCAG**GCC**TCTGAGGAG**GC**AATCCGCAGTGGG |
| LQTKFSR | L53 Q54 | CGCCAGGGTGGGGATGTG**GCCGC**GAATGAGGAGGGTGAGACC |
| | T61 | GGAGGGTGAGACC**GCT**AGCCACCTGATGGG |
| | K314 F316 S317 R320 | GTTCATGTGTGGTGAA**GC**GCAG**GCCGC**GGAGGAG**GC**AATCCGCAGTGGGG |

Mutated nucleotides are in bold.

described above except that the buffer was replaced by 50 mM Bis–Tris (pH 6.5) and 100 mM NaCl was added (22). For

calculation of PCNA stimulation, the gels were quantified on a PhosphorImager using the ImageQuant software (Molecular Dynamics). The SEM was calculated from the results of three independent experiments.
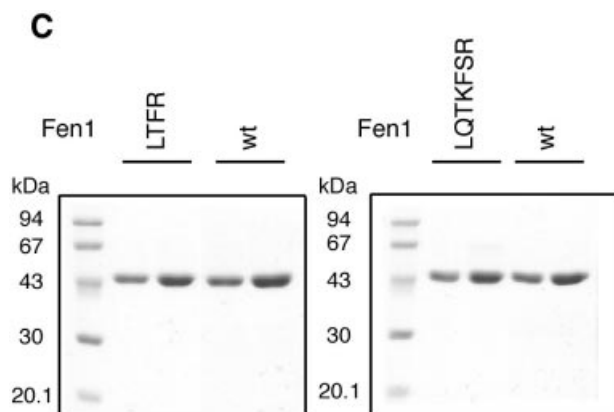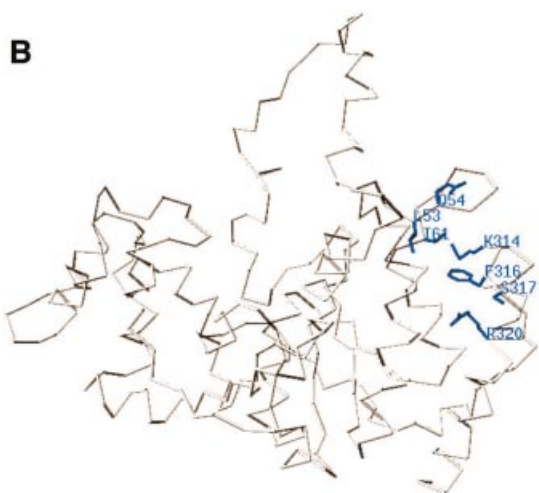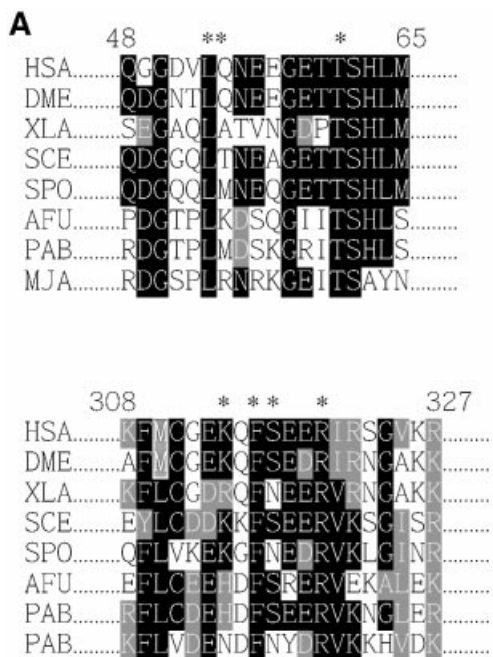
### Electrophoresis mobility shift assay (EMSA) for Fen1

Assay conditions were the same as described previously (19). The binding reactions were carried out in a final volume of 20 μl containing 50 mM Tris–HCl (pH 8.0), 10 mM NaCl, 5 mM EDTA, 100 μg bovine serum albumin, 4% Ficoll, 50 fmol labeled substrate and the indicated amounts of wt or mutant Fen1. After incubation for 10 min at room temperature, reactions were loaded on 6% polyacrylamide gels containing 0.5× TBE and run first at 50 V for 45 min and then at 100 V for 90 min. Finally, the bands were visualized by autoradiography.

### Pull down assay with $His_6$–Fen1

Aliquots of 8 μg Fen1 wt or mutant proteins were bound on $Ni^{2+}$ beads. To prevent non-specific binding, the beads were pretreated with 10 μg Sf9 cell extract prior to the addition of 460 ng PCNA in 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.025% NP-40 and incubated for 2 h at 4°C. The beads were then washed five times with the same buffer, but in addition 60 mM imidazole was included. Bound proteins were eluted by boiling in SDS–PAGE loading buffer and resolved on a 12% SDS–PAGE gel. PCNA was detected by western blot analysis using the PC10 antibody (Santa Cruz Biotechnology).

## RESULTS

### The amino acids in the extrahelical 3′-flap pocket of human Fen1 are conserved from archaea to human

Modeling of the extrahelical 3′-flap pocket from the structure of the archaeon *A.fulgidus* Fen1 (11) suggests that this region might be very similar in human Fen1, since seven amino acids within this extrahelical 3′-flap pocket are conserved in Fen1 from Archaea (*Methanococcus janaschii*, *Pyrococcus abyssi* and *A.fulgidus*), to two yeasts (*Saccharomyces cerevisiae* and *Schizosaccaromyces pombe*), to a fly (*Drosophila melanogaster*), to a frog (*Xenopus laevis*) and to human (*Homo sapiens*) (Fig. 1A and B). The human amino acids L53, Q54, T61, K314, F316, S317 and R320 are all in the extrahelical 3′-flap



**Figure 1.** The amino acids in the extrahelical 3′-flap pocket of human Fen1. (**A**) Amino acid alignments of different Fen1 proteins. HSA, *Homo sapiens*; DME, *Drosophila melanogaster*; XLA, *Xenopus laevis*; SCE, *Saccharomyces cerevisiae*; SPO, *Schizosaccaromyces pombe*; AFU, *Archaeoglobus fulgidus*; PAB, *Pyrococcus abyssi*; MJA, *Methanococcus janaschii*. Identical amino acids are shaded black, similar amino acids grey. The asterisks indicate the seven amino acids in the extrahelical 3′-flap pocket of human Fen1: L53, Q54, T61, K314, F316, S317 and R320. (**B**) The 3-dimensional structure of human Fen1 was modeled with the Swiss-Pdb viewer according to the structure of *M.janaschii* Fen1 (9). (**C**) Two mutant human Fen1 proteins were designed: Fen1 (LTFR), where the four conserved amino acids (L53, T61, F316 and R320) were mutated to alanine; Fen1 (LQTKFSR) where the seven amino acids L53, Q54, T61, K314, F316, S317 and R320 were mutated to alanine. Mutagenesis, recombinant production and purification were carried out as described in Materials and Methods. (Left) Fen1 (LTFR) and Fen1 wt, 5 and 10 μg each; (right) Fen1 (LQTKFSR) and Fen1 wt, 5 and 10 μg each were analyzed by 10% SDS–PAGE.

pocket (Fig. 1B) and correspond to the amino acids L47, K48, T55, H308, F310, S311 and R314 of *A.fulgidus* (see fig. 1C in 11). We have constructed two mutants of human Fen1. First, the four conserved amino acids L53, T61, F316 and R320 and, second, the seven amino acids L53, Q54, T61, K314, F316, S317 and R320 (Fig. 1A) were mutated to alanine, since these amino acids are important for *A.fulgidus* Fen1 binding to the unpaired DNA 3′-end. The two mutants carrying a His tag, called Fen1 (LTFR) and Fen1 (LQTKFSR), respectively, were produced in bacteria and purified to apparent homogeneity (Fig. 1C). The preparations were free of contaminating nucleases, as tested on linear and supercoiled plasmid DNA (data not shown).

### Human Fen1 extrahelical 3′-flap pocket mutants have reduced exonuclease activities and a less stringent cleavage pattern

First we tested exonuclease activities on a nicked DNA substrate and on a nicked substrate containing an unpaired 3′-end. From Figure 2 it is evident that both mutants had a greatly reduced activity compared to Fen1 wt (compare lanes 4–6 to 7–9 and 10–12, respectively). In addition, both mutants synthesized a two base product compared to the one base product of Fen1 wt. Furthermore, the activity of Fen1 (LQTKFSR) is considerably lower than the four amino acid mutant Fen1 (LTFR). This result is surprising since we would expect no effect on a substrate lacking a 3′-flap when the 3′-flap pocket is mutated. This might be explained by a tendency of Fen1 wt to bind to the 3′-end of the nick and to induce a 3′-flap. A similar activity profile was found with the unpaired DNA 3′-end substrate (Fig. 2, lanes 13–22) and the previously observed stimulation of Fen1 wt by the unpaired 3′-flap (19) is lost in both mutants. In summary, the two extrahelical 3′-flap pocket mutants have reduced exonuclease activities, which are coupled with a loss of product specificity. The effect was more pronounced with Fen1 (LQTKFSR), suggesting that the extrahelical 3′-flap pocket is important for the exonuclease activity of human Fen1.

### Human Fen1 extrahelical 3′-flap pocket mutants have reduced endonuclease activities and a less stringent cleavage pattern

It has been found that Fen1 has high cleavage specificity for double-flap DNA substrates and this specificity suggests that Fen1 contains a region or pocket that can specifically bind to the 3′-flap (16). This has been confirmed at the structural level for archaeal Fen1, where this binding induces a kinking of the DNA substrate (11). We therefore next tested different flap DNA substrates and compared the two extrahelical 3′-flap Fen1 mutants with their wild-type counterpart. Figure 3A shows that Fen1 wt has a clear preference for double-flap substrates (compare lanes 4–6 with lanes 8–10 and 12–14 in Fig. 3A). A 2 nt non-complementary flap mainly resulted in a 1 nt longer product (22 nt), likely due to the transition to the optimal one nt 3′-flap (lanes 16–18). When the four amino acid extrahelical 3′-flap pocket mutant Fen1 (LTFR) was tested with four DNA substrates we found that, first, >100 times more molecules of Fen1 were required for cutting, second, two bands appeared with all four substrates tested and, third, the previously observed stimulation by the unpaired 3′-flap (19) is lost (compare lanes 4 and 8 in Fig. 3B with lanes 4 and 8 in
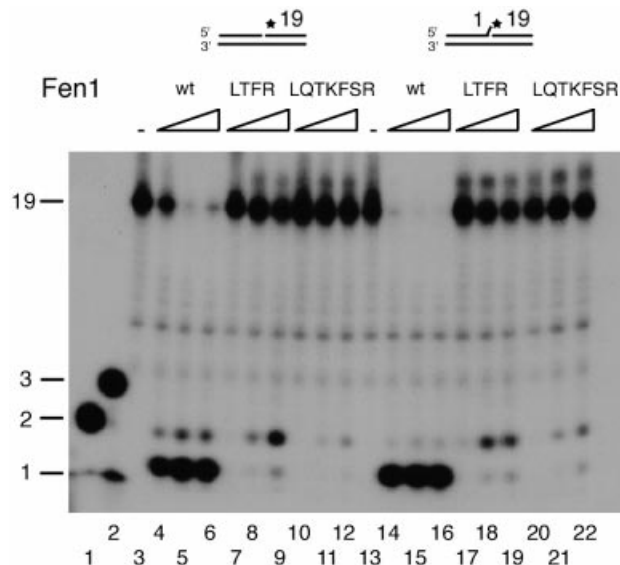


**Figure 2.** Both human Fen1 extrahelical 3′-flap pocket mutants have reduced exonuclease activities and a less stringent cleavage pattern. Fen1 exonuclease activities were determined as described in Materials and Methods on a nicked (lanes 3–12) and on a 3′-overhang nicked (lanes 13–22) DNA substrate. Lanes 1 and 2, oligonucleotide (nt) markers of 1, 2 and 3 nt; lanes 3 and 13, no enzyme control; lanes 4–6 and 14–16, 5, 50 and 500 ng Fen1 wt; lanes 7–9 and 17–19, 5, 50 and 500 ng Fen1 (LTFR); lanes 10–12 and 20–22, 5, 50 and 500 ng Fen1 (LQTKFSR). DNA substrate and product size (in nt) are indicated on the left of the figure.

Fig. 3A). With the 'physiological' complementary 3′-flap substrate the product was mainly 1 nt longer (22 nt) than with Fen1 wt and only a small proportion of the correct 21 nt product was made (compare lanes 12–14 of Fig. 3A and B). For the single-flap and non-complementary 3′-flap substrates two products appeared (21 and 22 nt), whereas the wild-type showed a clear preference for the 21 nt product (compare lanes 4–6 and 8–10 of Fig. 3B with lanes 4–6 and 8–10 of Fig. 3A). This suggests that substrate specificity was severely hampered in this mutant. Finally, the seven amino acid extrahelical 3′-flap pocket mutant Fen1 (LQTKFSR) showed a similar altered cleavage pattern to the Fen1 (LTFR) mutant and with the 'physiological' complementary 3′-flap substrate the product was exclusively 1 nt longer (22 nt) than with Fen1 wt (lanes 12–14 of Fig. 3C). Only the 2 nt non-complementary 3′-flap substrate showed the expected product of 22 nt (lanes 16–18 in Fig. 3C). These data suggest that replacement of only the four conserved amino acids L53, T61, F316 and R320 resulted in a severe loss of substrate specificity, while replacement of all seven conserved amino acids L53, Q54, T61, K314, F316, S317 and R320 resulted in complete loss of the 3′-flap specificity.

### Human Fen1 extrahelical 3′-flap pocket mutants have lost the specific binding to double-flap DNA

EMSA recently showed two binding modes of human Fen1, but only with the double-flap DNA structure containing a 3′-flap (19). Using gel filtration experiments we could show that the faster migrating complex had a molecular weight which corresponded to a 1:1 complex between Fen1 and the double-flap DNA (data not shown). Again as expected, Fen1 wt
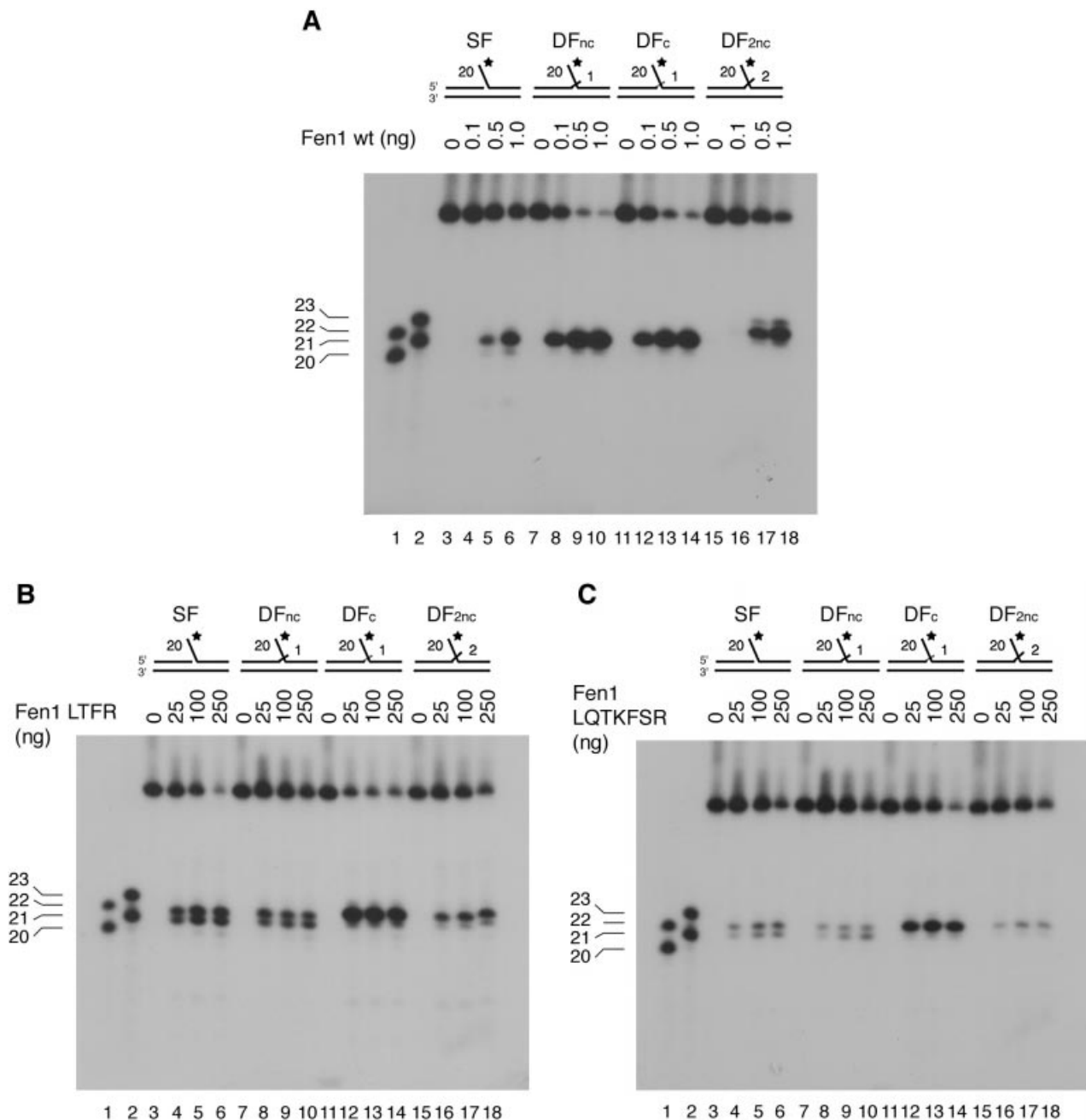
**Figure 3.** Both human Fen1 extrahelical 3′-flap pocket mutants have reduced endonuclease activities and a less stringent cleavage pattern. (**A**) The amounts of Fen1 wt indicated at the top of the autoradiogram were tested on a flap (SF) (lanes 3–6), on a non-complementary double-flap (DF$_{nc}$) (lanes 7–10), on a complementary double-flap (DF$_c$) (lanes 11–14) and on a two 3′-nucleotide overhang (DF$_{2nc}$) (lanes 15–18) DNA substrate as outlined in Materials and Methods. Lanes 1 and 2 contain oligonucleotide (nt) markers of 20 and 22 nt and 21 and 23 nt, respectively. (**B**) The amounts of Fen1 (LTFR) indicated at the top of the autoradiogram were tested on a flap (SF) (lanes 3–6), on a non-complementary double-flap (DF$_{nc}$) (lanes 7–10), on a complementary double-flap (DF$_c$) (lanes 11–14) and on a two 3′-nucleotide overhang (DF$_{2nc}$) (lanes 15–18) DNA substrate as outlined in Materials and Methods. Lanes 1 and 2 contain oligonucleotide (nt) markers of 20 and 22 nt and 21 and 23 nt, respectively. (**C**) Shown on the top of the autoradiogram, the indicated amounts of Fen1 (LQTKFSR) were tested on a flap (SF) (lanes 3–6), on a non-complementary double-flap (DF$_{nc}$) (lanes 7–10), on a complementary double-flap (DF$_c$) (lanes 11–14) and on a two 3′-nucleotide overhang (DF$_{2nc}$) (lanes 15–18) DNA substrate as outlined in Materials and Methods. Lanes 1 and 2 contain oligonucleotide (nt) markers of 20 nt and 22 nt, or 21 nt and 23 nt, respectively. Product size (in nt) is indicated on the left of the figure.

revealed two binding modes on both double-flap substrates (Fig. 4A, lanes 7–10 and 12–15). This dual binding mode was lost with both extrahelical 3′-flap pocket mutants (Fig. 4B and C). In contrast to a Fen1 mutant lacking the C-terminal 20

amino acids, which is completely unable to bind DNA (6), these two mutants have not lost the property of general DNA binding, but rather DNA-specific binding. Although the slightly weaker binding of the Fen1 (LTQKFSR) mutant to
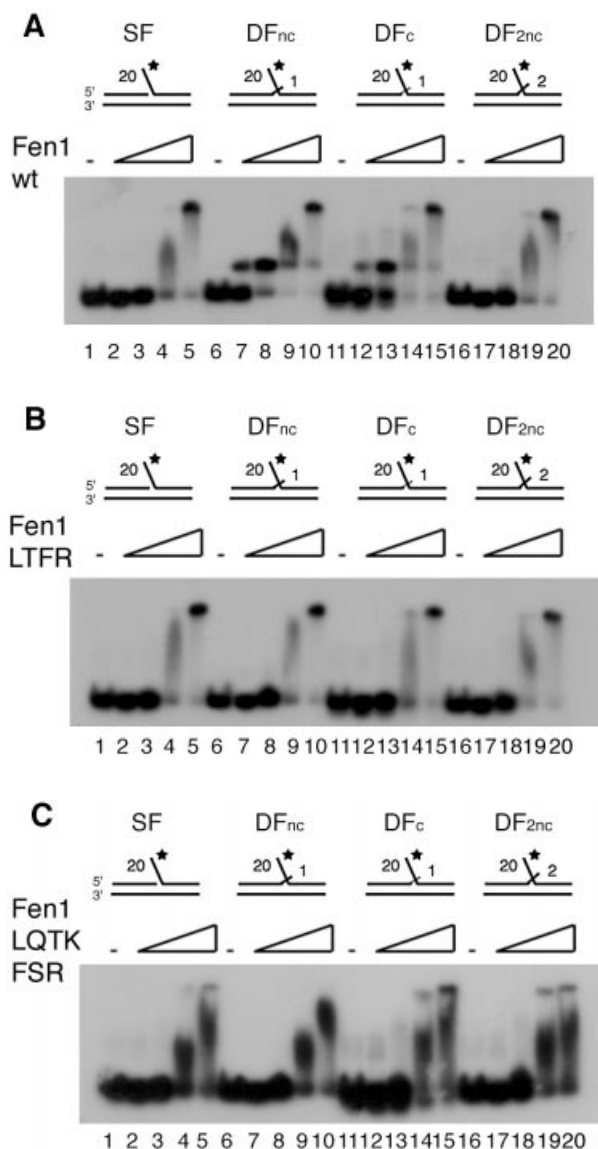
**Figure 4.** Both human Fen1 extrahelical 3′-flap pocket mutants have lost specific binding to double-flap DNA. The EMSA assays were carried out as outlined in Materials and Methods. (**A**) Aliquots of 0, 5, 50, 200 and 400 ng Fen1 wt were tested on a flap (SF) (lanes 1–5), on a non-complementary double-flap (DF$_{nc}$) (lanes 6–10), on a complementary double-flap (DF$_{c}$) (lanes 11–15) and on a two 3′-nucleotide overhang (DF$_{2nc}$) (lanes 16–20) DNA substrate. (**B**) Aliquots of 0, 5, 50, 200 and 400 ng Fen1 (LTFR) were tested on a flap (SF) (lanes 1–5), on a non-complementary double-flap (DF$_{nc}$) (lanes 6–10), on a complementary double-flap (DF$_{c}$) (lanes 11–15) and on a two 3′-nucleotide overhang (DF$_{2nc}$) (lanes 16–20) DNA substrate. (**C**) Aliquots of 0, 5, 50, 200 and 400 ng Fen1 (LQTKFSR) were tested on a flap (SF) (lanes 1–5), on a non-complementary double-flap (DF$_{nc}$) (lanes 6–10), on a complementary double-flap (DF$_{c}$) (lanes 11–15) and on a two 3′-nucleotide overhang (DF$_{2nc}$) (lanes 16–20) DNA substrate.

the different DNA flap substrates may indicate a perturbation of its structure by the seven amino acid replacements, our data in Figures 2 and 3 show that this mutant is still enzymatically active. Therefore, we conclude that the Fen1 (LQTKFSR) mutant has not lost its general DNA binding ability. These

data suggest that the mutant Fen1 proteins could still bind to DNA but were no longer able to properly accommodate the 3′-flap and kink the DNA substrate. Fluorescent resonance energy transfer (FRET) experiments with *A.fulgidus* Fen1 are in agreement with these observations (11). In conclusion, the extrahelical 3′-flap pocket mutant Fen1 enzymes were no longer able to distinguish the 'physiological' substrate from other ones.

### Human Fen1 extrahelical 3′-flap pocket mutants can interact and are stimulated by PCNA, but the normal cleavage pattern cannot be restored by PCNA

PCNA has been shown to bind to Fen1 and to stimulate its activity by >50 times under physiological salt conditions (22–25). We therefore tested the two extrahelical 3′-flap pocket mutants for their capacity to bind PCNA. Both mutants bound PCNA, the four amino acid mutant Fen1 (LTFR) as strongly as Fen1 wt, while the binding of the seven amino acid mutant Fen1 (LQTKFSR) to PCNA was reduced by 50% (Fig. 5A). These binding experiments were nicely confirmed in a PCNA stimulation assay (Fig. 5B–D). On a single-flap DNA substrate, Fen1 wt was stimulated by PCNA in a dose-dependent manner and this stimulation was specific, since BSA showed no stimulatory effect (Fig. 5B, compare lanes 5–9 with lanes 10–14). Both extrahelical 3′-flap pocket mutants were stimulated by PCNA (Fig. 5C), although 100 times more Fen1 (LTFR) or 250 times more Fen1 (LQTKFSR) had to be included to see a similar effect (compare Fig. 5C with B). Quantification of the stimulation showed that the four amino acid mutant Fen1 (LTFR) was stimulated by PCNA to the same extent as Fen1 wt, whereas the seven amino acid mutant Fen1 (LQTKFSR) was stimulated to a lesser extent (Fig. 5D), probably due to its reduced interaction with PCNA (Fig. 5A). Finally, we analyzed whether PCNA could restore the substrate specificity of the mutant Fen1 enzymes. As seen from Figure 5B, Fen1 wt mainly synthesized a product of 21 nt, while the two mutants produced about equal amounts of the 21 and 22 nt cleavage products (Fig. 5C). These data are in agreement with those seen in Figure 3. In summary, the extrahelical 3′-flap pocket mutant Fen1 enzymes could be stimulated by PCNA but the substrate specificity could not be restored.

## DISCUSSION

### The substrate specificity of Fen1 is evolutionarily conserved

The data presented in this work clearly indicate that the substrate specificity of Fen1 is conserved through evolution in an extraordinary way and that this conservation is seen in the structural similarity of the hydrophobic wedge in general and the extrahelical 3′-flap pocket in particular. The biochemical and functional data presented in this paper suggest that the extrahelical 3′-flap pocket, as structurally identified in the archaeon *A.fulgidus* Fen1 (11), appears to be almost identical to human Fen1. The observation that a 1 nt double-flap structure is the optimal substrate for human Fen1 and that with this substrate a DNA–protein complex could be observed at a 1:1 molar ratio (19 and data not shown) suggested that a double-flap substrate containing a 1 nt 3′-tail is the *in vivo*
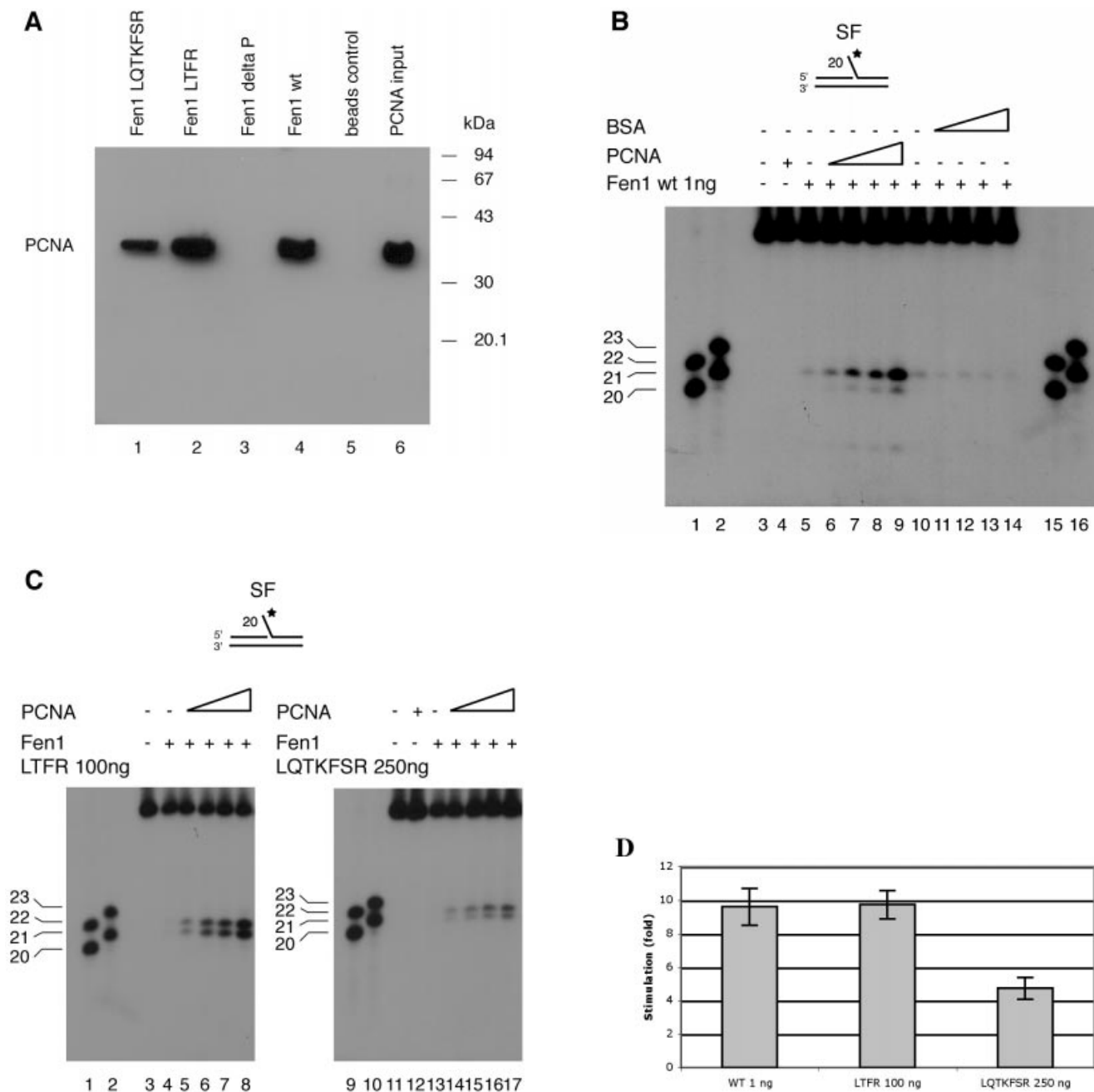
**Figure 5.** Both human Fen1 extrahelical 3′-flap pocket mutants can interact and be stimulated by PCNA, but the normal cleavage pattern cannot be restored. (**A**) Fen1 wt and mutant proteins (8 μg) were incubated with PCNA (460 ng) and pull-down experiments performed as outlined in Materials and Methods. PCNA was detected by western blot analysis. Lane1, Fen1 (LQTKFSR) and PCNA; lane 2, Fen1 (LTFR) and PCNA; lane 3, Fen1 ΔP (Fen1 lacking the PCNA binding domain) and PCNA, negative control; lane 4, Fen1 wt and PCNA, positive control; lane 5, binding of PCNA to the beads, non-specific control; lane 6, 46 ng PCNA. (**B**) Stimulation of Fen1 wt by PCNA and BSA in the presence of the single-flap DNA substrate was performed as outlined in Materials and Methods. Lanes 1 and 2, oligonucleotide (nt) markers of 20 and 22 nt and 21 and 23 nt, respectively; lane 3, no enzyme control; lane 4, PCNA control; lanes 5–9, 0, 25, 50, 100 and 250 ng PCNA tested with 1 ng Fen1 wt; lanes 10–14, 0, 25, 50, 100 and 250 ng BSA tested with 1 ng Fen1 wt, negative control; lanes 15 and 16, oligonucleotide (nt) markers of 20 and 22 nt and 21 and 23 nt, respectively. (**C**) Cleavage specificity of the two extrahelical 3′-flap pocket mutants in the presence of PCNA. Reactions were performed as outlined in Materials and Methods. Lanes 1 and 2, oligonucleotide (nt) markers of 20 and 22 nt and 21 and 23 nt, respectively; lane 3 and 11, no enzyme control; lanes 4–8, 0, 25, 50, 100 and 250 ng PCNA tested with 100 ng Fen1 (LTFR); lane 12, PCNA control; lanes 13–17, 0, 25, 50, 100 and 250 ng PNA tested with 250 ng Fen1 (LQTKFSR); lanes 9 and 10, oligonucleotide (nt) markers of 20 and 22 nt and 21 and 23 nt. Product size in nucleotides is indicated on the left of each gel. (**D**) Stimulation of Fen1 wt and the two extrahelical 3′-flap mutants by PCNA. The bar graph documents the maximal stimulation ± SEM of Fen1 wt (1 ng), Fen1 (LTFR) (100 ng) and Fen1 (LQTKFSR) (250 ng) endonuclease activity by saturating amounts of PCNA. The stimulation was calculated as the quotient between the activity in the presence and absence of PCNA.

substrate for human Fen1. This has been described previously for the Fen1 homolog in yeast, rad27p (18). This double-flap

substrate could also reflect an *in vivo* intermediate between two conformations that would be interchangeable from a

double-flap to a single-flap containing a +1 5′-flap, provided the single 3′-flap is complementary. The 3′-terminal nucleotide is recognized by a region or hydrophobic pocket in the Fen1 protein (11,16) that could accommodate a mononucleotide. Our results suggest that, as proposed recently for the archaeal *A.fulgidus* Fen1, binding to the 3′-flap anchors the DNA in a defined orientation and positions the scissile phosphate near the active site. The kink promotes conformational closing of the flexible helical clamp and thus can facilitate the cleavage specificity at the 5′-flap to be cleaved.

## Implications of a double-flap structure for DNA transactions carried out by Fen1 *in vivo*

A double-flap DNA structure ensures specific recognition of an unpaired 3′ nucleotide at a junction in DNA replication. Due to the universal directionality of any DNA polymerase, replication at the lagging strand is discontinuous (reviewed in 26). This occurs every 200 bases, thus resulting in more than $10^7$ Okazaki fragment maturation events per replication of the human genome. During their maturation, the DNA polymerase δ holoenzyme performs strand displacement synthesis and the Okazaki fragment is processed by the concerted action of replication protein A, PCNA, Fen1 and subsequent ligation by DNA ligase I (27,28). 'Freezing' of the double-flap structure for Fen1 is likely guaranteed by the concerted action of these proteins, but how they are positioned around PCNA is not known. In *Sulfolobus solfataricus*, where PCNA is a heterotrimer, one subunit binds Fen1, the second DNA polymerase and the third DNA ligase (29). This suggests that kinking the DNA at the physiological double-flap DNA would guarantee concomitant binding of Fen1 endonuclease, DNA polymerase δ and DNA ligase I to precisely coordinate DNA synthesis, flap cutting and DNA ligation. A frozen double-flap structure would be the optimal contribution from the DNA side to precisely perform the many millions of Okazaki fragment processing events.

In long patch base excision repair it has been proposed that DNA ligase I can act as a patch size mediator (30) that can obviously determine proper Fen1 positioning for 5′-flap cleavage in the presence of the DNA polymerase δ holoenzyme (DNA polymerase δ, PCNA and replication factor C) (31). Again, 'freezing' of the double-flap DNA would guarantee proper ligation, regardless of the size of the base excision repair patch.

Uncovering how cleavage specificity is guaranteed by means of the extrahelical 3′-flap pocket which positions the DNA to allow precise cleavage may explain how Fen1 deals with its paramount role in DNA replication and in preventing a situation in the genome that leads to unwanted genetic exchanges and eventually to a cancerous phenotype.

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