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# The *Saccharomyces cerevisiae* Dna2 can function as a sole nuclease in the processing of Okazaki fragments in DNA replication

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## ABSTRACT

**During DNA replication, synthesis of the lagging strand occurs in stretches termed Okazaki fragments. Before adjacent fragments are ligated, any flaps resulting from the displacement of the 5′ DNA end of the Okazaki fragment must be cleaved. Previously, Dna2 was implicated to function upstream of flap endonuclease 1 (Fen1 or Rad27) in the processing of long flaps bound by the replication protein A (RPA). Here we show that Dna2 efficiently cleaves long DNA flaps exactly at or directly adjacent to the base. A fraction of the flaps cleaved by Dna2 can be immediately ligated. When coupled with DNA replication, the flap processing activity of Dna2 leads to a nearly complete Okazaki fragment maturation at subnanomolar Dna2 concentrations. Our results indicate that a subsequent nucleolytic activity of Fen1 is not required in most cases. In contrast Dna2 is completely incapable to cleave short flaps. We show that also Dna2, like Fen1, interacts with proliferating cell nuclear antigen (PCNA). We propose a model where Dna2 alone is responsible for cleaving of RPA-bound long flaps, while Fen1 or exonuclease 1 (Exo1) cleave short flaps. Our results argue that Dna2 can function in a separate, rather than in a Fen1-dependent pathway.**

## INTRODUCTION

All cells must replicate their DNA before each cell division. While leading strand DNA synthesis occurs continuously in a 5′ to 3′ direction, the lagging strand is synthesized in short stretches termed Okazaki fragments due to the 5′ to 3′ polarity of DNA polymerases. First, the DNA polymerase α-primase complex (pol α) synthesizes a ∼30 nucleotides (nt) long RNA–DNA primer. The replication factor C (RFC) then binds the junction between the RNA–DNA primer and the parental DNA strand and initiates the loading of PCNA. Recruitment of PCNA mediates a switch from pol α to polymerase δ (pol δ), which extends the newly synthesized DNA strand to up to ∼200 nt (1,2). RNAse H1 is primarily responsible for the removal of RNA from DNA (3–6). PCNA binds pol δ (7,8) and enhances its processivity (9), and also serves as a binding platform for further replicative factors including Fen1 and DNA ligase I (Lig1 or Cdc9) (8,10–14). After pol δ reaches the 5′ end of the downstream Okazaki fragment it may continue DNA synthesis leading to the displacement of the RNA–DNA primer. This creates 5′-terminated flaps of various lengths that must be cleaved before ligation by Lig1 can occur. This process is also important for the maintenance of genome stability as it contributes to the removal of RNA as well as DNA from the initial primer synthesized by error-prone pol α (15,16).

Dna2 is an essential protein that was found to be necessary for DNA replication *in vivo* (17–19). While it is not necessary for bulk DNA synthesis (20), newly replicated DNA in *dna2–1* cells contained low molecular weight fragments, showing that Dna2 is required for sealing nicks in newly replicated DNA, reminiscent of cells lacking Lig1 (18,21). Dna2 has both DNA nuclease and helicase activities (22–25). While a loss of its nuclease activity is lethal, helicase-deficient mutants are viable under some growth conditions (19,24). This suggested that specifically the nuclease activity of Dna2 is essential for DNA replication. The Pif1 helicase was shown to stimulate the displacement activity of pol δ, leading thus to long flap formation and providing requirement for Dna2 (26–28). In accord, *pif1*Δ mutation rescues the lethality of *dna2*Δ cells (29). Additional deletion of *pol32* (pol δ subunit responsible for DNA strand displacement activity) further suppresses the growth defects of *pif1*Δ *dna2*Δ cells (29,30). At the same time, the lethality of *dna2*Δ cells can be also rescued by a mutation of *rad9*, leading to inactivation of DNA damage checkpoint (31). Nevertheless, these data collectively suggest that Dna2 *in vivo* is required for the processing of long flaps in *Saccharomyces cerevisiae* DNA replication.

Short flaps are primarily processed by Fen1 that is a component of the Okazaki fragment maturation complex composed of pol δ, PCNA and Lig1 (7,8,10–14,32). It has been demonstrated that Fen1 becomes incapable of cleaving flaps

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that are long enough to bind RPA. Thus, RPA mediates the nuclease switch between Fen1 and Dna2 (33). While inhibiting DNA cleavage by Fen1 (26,33), RPA promotes the nuclease of Dna2 (22,33,34). However, recombinant Dna2 was shown to only shorten long flaps to ∼5–8 nt, even when used at very high concentrations. DNA cleavage at these positions did not support ligation and therefore a second nuclease activity was needed (22,33,35). As Dna2 was found to be part of a complex with Fen1 *in vivo* (36), it was proposed that Fen1 must act downstream of Dna2 (32,33,35). Indeed, a combination of recombinant Dna2 and Fen1 allowed Okazaki fragment processing *in vitro*, leading to a two-step Okazaki fragment processing model (33). In contrast to *dna2*Δ cells, *rad27*Δ mutants are viable, albeit grow slowly and display elevated recombination and mutation rates (37,38). The viability of *rad27*Δ cells seems to contradict the model where Fen1 acts downstream of Dna2 (32,33,35). To this point, Fen1 activity was proposed to be redundant with that of Exo1 (33). Indeed, overexpression of Exo1 could suppress some of the phenotypic defects of *rad27*Δ cells and *exo1*Δ *rad27*Δ mutant is lethal (39). However, there is no evidence to date suggesting that Dna2 physically interacts with Exo1, nor that Exo1 acts downstream of Dna2. On the contrary, Dna2 and Exo1 nucleases function in strictly separate pathways during DNA end resection (40). Furthermore, the direct interaction between Dna2 and Fen1 could not be confirmed in a later study (41). Also, overexpression of Dna2 suppressed the growth defects associated with deletion of *rad27*, while overexpression of Fen1 suppressed the lethality of *dna2*Δ cells (36). Also this supports the notion that Dna2 and Fen1 can function in separate pathways. Despite that, available *in vitro* data with recombinant Dna2 were in contrast with such an explanation.

Here we show that Dna2 cleaves DNA flaps near their base, and is thus able to support complete Okazaki fragment maturation without the requirement of Fen1 during DNA replication *in vitro*. This finding provides a possible explanation of the diverse phenotypes of *dna2*Δ and *rad27*Δ cells. It strongly suggests that Dna2 can function as the sole nuclease in the processing of at least a fraction of long DNA flaps in DNA replication.

## MATERIALS AND METHODS

### DNA substrates

The oligonucleotides used to prepare the flapped substrates were as follows: 'bottom' X12–4NC and 'top' Flap 19 X12–4C were annealed with a variety of 'top flap' oligonucleotides to prepare flapped substrates of 30 (oligonucleotide 292), 8 (293) and 4 nt in length (294): X12–4NC, 5′-GCGATAGTCTCTAGACAGCATGTC CTAGCAAGCCAGAATTCGGCAGGCTA-3′; Flap 19 X12–4C, 5′-TAGCCTGCCGAATTCTGGC-3′; 292, 5′-GGTACTCAAGTGACGTCATAGACGATTACATT GCTAGGACATGCTGTCTAGAGACTATCGC-3′; 293, 5′-GGATTACATTGCTAGGACATGCTGTCTAGAGA CTATCGC-3′; 294, 5′-GACATTGCTAGGACATGCTG TCTAGAGACTATCGC-3′.

For 8 nt double flap substrate, oligonucleotides X12–4NC, Flap 20 X12–4C and 293 were annealed. The sequence of Flap 20 X12–4C is: 5′-TAGCCTGCCGAATT CTGGCA-3′.

The oligonucleotides were $^{32}$P-labeled at the 3′ end with [alpha-$^{32}$P] cordycepin-5′-triphosphate and terminal deoxynucleotidyl transferase (New England Biolabs) according to manufacturer instructions. Unincorporated nucleotides were removed using MicroSpin G25 columns (GE Healthcare).

The circular 3.197 kb-long ssDNA used in the replication assays was pGEM-3Zf(−) that was prepared as described previously (42). This DNA was annealed with either pR_T12flap 5′-TTTTTTTT TTTTACCATCTGGCCCCAGTGCTGCAATG-3′ (primer with a 12 nt-flap), pR_T30flap 5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCA TCTGGCCCCAGTGCTGCAATG-3′ (primer with a 30 nt-flap), or pR_20 5′-GCGATCTGTCTATTTCGTTC-3′ (fully complementary primer without a flap).

### Recombinant proteins

RPA protein was prepared as described (43). Wild-type Dna2 as well as nuclease-dead E675A, helicase-dead K1080E and the E675A/K1080E double mutant were expressed and purified as described previously (25). We note that care must be taken during cell lysis to prevent a loss of activity. Also, Dna2 is particularly sensitive to oxidation, so reducing agents must be included throughout the procedure. Yeast *LIG1* (*CDC9*) gene was amplified from yeast genomic DNA by polymerase chain reaction (PCR) using primers Lig1_for 5′- ACGCATTAGCTA GCGGATCCCTGGAAGTTCTGTTCCAGGGGCCC ATGCGCAGATTACTGACCGGTTG-3′ and Lig1_rev 5′-ACGCATTACTCGAGATTTTGCATGTGGGATTG GT-3′. The PCR product was digested by NheI and XhoI restriction endonucleases (both New England Biolabs) and cloned into corresponding sites in pFB-MBP-Sgs1-his vector (44), creating pFB-MBP-Lig1-his. Lig1 was expressed in insect *Sf*9 cells and purified by affinity chromatography as described previously for Sgs1 (44).

Yeast *FEN1* (*RAD27*) was amplified from yeast genomic DNA by PCR using primers Fen1_for: 5′- ACGCATTA GCTAGCGAATTCCTGGAAGTTCTGTTCCAGGG GCCCATGGGTATTAAAGGTTTGAATGC-3′ and Fen1_rev: 5′- ACGCATTACTCGAGTCTTCTTCCC TTTGTGACTT-3′. The PCR product was digested by NheI and XhoI restriction endonucleases (both New England Biolabs) and cloned into corresponding sites in pFB-MBP-Sgs1-his vector (44), creating pFB-MBP-Fen1-his. Maltose-binding protein (MBP) tag was excised from this vector by digestion with the restriction endonuclease BamHI (New England Biolabs) followed by self-ligation of pFB-Fen1-his. Then, a fragment coding for PP-MBP (PP is PreScission protease cleavage site) was amplified by PCR from the pFB-MBP-Sgs1-his vector (44) using primers XhoI_PP2G_MBP_for 5′-ACGCATTACTCGAGCTGGAAGTTCTGTTCCAG GGGCCCGGTGGTATGAAAATCGAAGAAGGTAA-3′ and MBP_XhoI_rev 5′-ACGCATTACTCGAGCCCG AGGTTGTTGTTATTGT-3′. The PCR product was digested by XhoI restriction endonuclease (New England

Biolabs) and cloned into pFB-Fen1-his directly after Fen1, creating pFB-Fen1-MBP-his. Fen1 was expressed in insect *Sf*9 cells. Cells were lysed and MBP-tagged Fen1 was first incubated with amylose resin as described previously for Sgs1 (44). Eluates from amylose resin were applied on Ni-NTA agarose column and extensively washed with wash buffer (50 mM Tris–HCl pH 7.5, 2 mM β-mercaptoethanol, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1 M NaCl and 20 mM imidazole), followed by a wash with the same buffer, but containing 0.3 M NaCl and 60 mM imidazole. Protein was eluted with wash buffer containing 0.3 M NaCl and 0.3 M imidazole. MBP and His tags were cleaved by PreScission protease and the eluate was incubated with glutathione and amylose resins. The sample was centrifuged (2000 *g*, 5 min) and supernatant containing Fen1 was dialyzed into 50 mM Tris–HCl pH 7.5, 5 mM β-mercaptoethanol, 150 mM NaCl, 10% glycerol and frozen in small aliquots.

Yeast three subunit pol δ and pol δ exonuclease-deficient variant Pol3-D520V (3' exo- Pol3-DV, mutation introduced by site-directed mutagenesis) were expressed in the yeast strain WDH668 as described previously (45) and purified according to existing protocols (27). PCNA and RFC were expressed and purified from *Escherichia coli* by minor modifications of previously established procedures (46,47).

### Nuclease assays

Nuclease assays were performed in a 15 μl volume in 25 mM Tris-acetate pH 8.5, 10 mM magnesium acetate (unless indicated otherwise), 1 mM adenosine triphosphate (ATP), 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin (BSA, New England Biolabs), 1 mM phosphoenolpyruvate, 80 U/ml pyruvate kinase, 1 nM DNA substrate (in molecules) and recombinant proteins as indicated. Where indicated, RPA was present at 27 nM, which is sufficient to fully cover the entire DNA in the reactions assuming all DNA was single-stranded. Samples were incubated at 30°C for 30 min, reaction was stopped by adding an equal amount of formamide dye (95% (v/v) formamide, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.01% bromophenol blue), samples were heated at 95°C for 4 min and separated on 20% denaturing polyacrylamide gels (ratio acrylamide:bisacrylamide 19:1, Biorad). After fixing in a solution containing 40% methanol, 10% acetic acid and 5% glycerol for 30 min, the gels were dried on DE81 chromatography paper (Whatman), and exposed to storage phosphor screens (GE Healthcare). The screens were scanned by Typhoon phosphor imager (GE Healthcare).

### Replication assays

Replication assays with plasmid-based substrates were performed similarly as described previously (32) in a 15 μl volume in 25 mM Tris-acetate pH 8.5, 10 mM magnesium acetate, 125 mM NaCl, 1 mM ATP, 1 mM dithiothreitol, 0.1 mg/ml BSA (New England Biolabs), 1 mM phosphoenolpyruvate, 80 U/ml pyruvate kinase, 100 μM dNTPs (each) and 6.4 nM (molecules, 100 ng) ssDNA substrate. PCNA (20 nM), RFC (20 nM) and RPA (1 μM, concentration saturating 100% of DNA) were added to the reaction and preincubated for 1 min at 30°C. Pol δ (5 nM), Dna2 and/or yeast Fen1 (concentrations as indicated in figures or figure legends) and Lig1 (20 nM) were then added and the reactions were incubated, if not indicated otherwise, at 30°C for 60 min. The reactions were stopped by adding 5 μl of 2% stop solution (150 mM EDTA, 2% sodium dodecyl sulphate, 30% glycerol, bromophenol blue) and 1 μl proteinase K (20.3 mg/ml, Roche) for 10 min at 30°C and separated on 1% agarose gels containing GelRed (1:10,000 v/v, Biotinum). Gels were analyzed by an AlphaImager gel imaging system.

### Pulldown assays

To test for interactions between Dna2 and RPA or PCNA, 2 μg of recombinant Dna2 was diluted in Tris-buffered saline (TBS, 50 mM Tris–HCl pH 7.5, 150 mM NaCl), bound to anti-HA resin (25 μl, Pierce) and washed with TBS-T (TBS containing 0.05% Tween 20). Then, recombinant RPA (1.33 μg) or PCNA (0.34 μg) were added and the resin was incubated at 4°C for 1 h. The resin was again washed with TBS-T and proteins were eluted with 0.1 M glycine pH 2.5, according to manufacturer's instructions. The proteins in the eluate were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis stained with silver.

## RESULTS

### Dna2 cleaves DNA flaps near their base

Previously, we were able to purify recombinant *S. cerevisiae* Dna2 with high levels of DNA helicase and nuclease activities (25,34). Here we tested the behavior of Dna2 and mutant variants (Supplementary Figure S1A) on flapped substrates that mimic structures arising upon displacement synthesis during Okazaki fragment processing (Figure 1A). We first used a substrate with a 30 nt-long 5′ ssDNA flap and examined the exact cleavage position by *S. cerevisiae* Dna2 in the presence of RPA. Cleavage at exactly the base of the flap (position 0) would produce a fragment of 32 nt in length. It is known that Dna2 must load onto the 5′ ssDNA end and translocate along the ssDNA flap before cleavage occurs (23,35). We show that wild-type Dna2 protein efficiently cleaved the flap at −1, 0 or +1 positions in most cases (Figure 1B, cleavage at −1 position leaves behind a flap of 1 nt in length, cleavage adjacent to flap base within dsDNA corresponds to +1 position). In contrast, nuclease-dead (E675A) and double-dead (nuclease- and helicase-dead, E675A/K1080E) Dna2 variants did not show any activity, demonstrating that the cleavage is inherent to the nuclease of Dna2 and not a product of a contamination (Figure 1B). These results differ from those published previously (33,35), which reported that Dna2 only shortens flaps up to the length of ∼5–8 nt in a vast majority of cases. The position of cleavage was unchanged in magnesium concentrations between 2 and 10 mM, indicating that DNA melting near the flap base cannot explain the observed position of cleavage (compare Figure 1B with Supplementary Figure S1C). A comparison of DNA flap cleavage by Dna2 and Fen1 is shown in Supplementary Figure S1D; Fen1 cleaves past the flap just within the dsDNA. Furthermore, Dna2 cleaved the long flap sequentially, with the

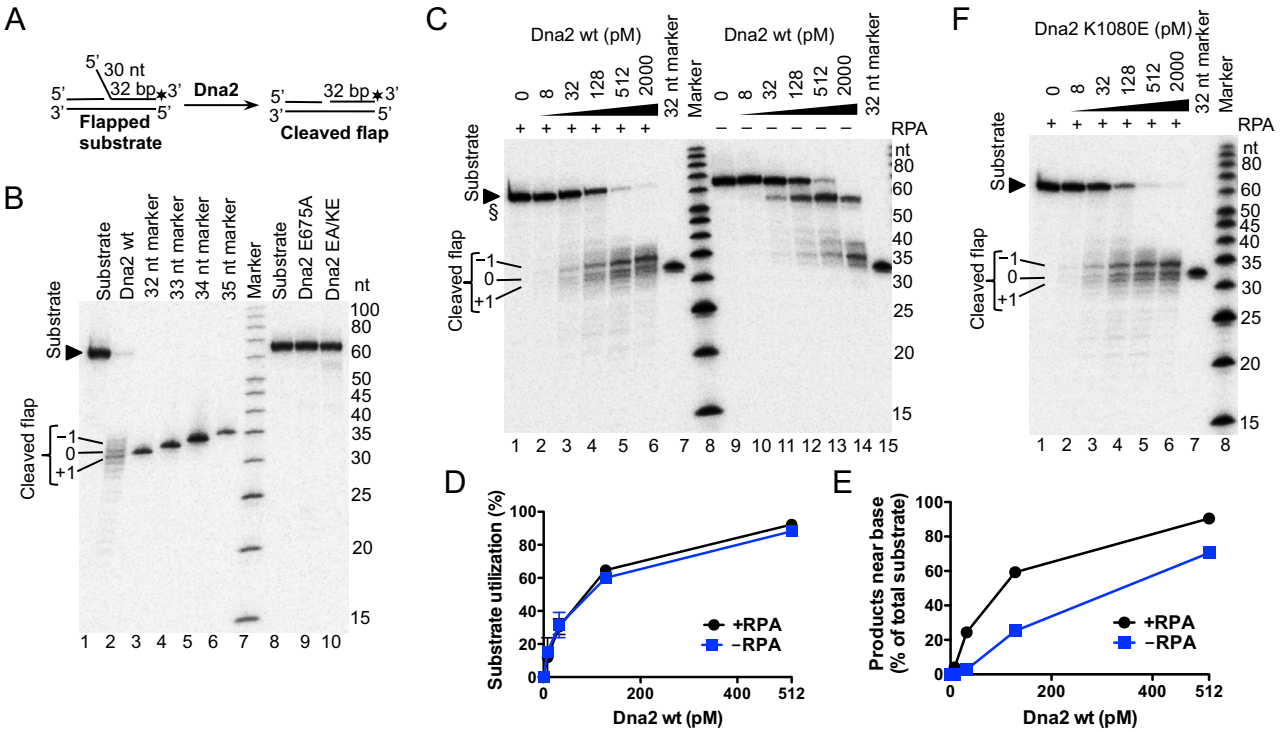


**Figure 1.** Dna2 cleaves DNA near a base of a flap. (**A**) Nuclease assay. *, radioactive label. (**B**) Wild-type (wt), nuclease-dead (E675A) or double-dead (E675A/K1080E, EA/KE) Dna2 variants (all 2 nM) were incubated with a DNA substrate containing a 30 nt-long flap, in the presence of RPA in a buffer containing 2 mM magnesium acetate. The reaction products were separated on 20% polyacrylamide denaturing urea gel. Cleavage at the base of the flap produces a fragment of 32 nt in length (position 0). (**C**) Increasing concentrations of Dna2 were incubated with a substrate containing a 30 nt-long flap as in (B), but in 10 mM magnesium acetate buffer, with or without RPA, as indicated. §, substrate cleaved by Dna2 in the absence of RPA. (**D**) Quantification of experiments such as in (C). Averages shown, $n = 2$; error bars, s.e.m. (**E**) Quantitation of products cleaved within 5 nt of flap base from (C). Averages shown, $n = 2$; error bars, s.e.m. (**F**) Experiment as in (C), but with helicase-dead Dna2 K1080E variant in the presence of RPA.

first cut being ∼5–10 nt away from the flap end (Supplementary Figure S1E). This is in agreement with previous observations that Dna2 must load on a free flap end and translocate along DNA before cleavage occurs (35). RPA did not stimulate the overall efficiency of DNA cleavage by Dna2, but promoted cutting at positions near the flap base (Figure 1C-E). Without RPA, Dna2 often cleaved at −1 or 0 positions, while no cleavage at +1 position was observed (Figure 1C, right part). Furthermore, without RPA, a large fraction of the flap was only cleaved ∼20 nt away from the flap base (Figure 1C, right part), likely due to secondary structure in the flap that prevents Dna2 translocation along the ssDNA, which is in full agreement with previous data (48). With RPA, Dna2 cleaved majority of the substrate in the vicinity of the flap base (Figure 1C, left part, Figure 1E). We also show that Dna2 was able to cleave an 8 nt-long flap in the same manner as the 30 nt-long flap, but could not cleave a 4 nt-long flap (Supplementary Figure S2A and B), which is instead as expected a good substrate for Fen1 (Supplementary Figure S2C–E). RPA inhibited the cleavage of the 30 nt-long flaps by Fen1, while it had no effect on the processing of the short flaps (Supplementary Figure S2C–G), in agreement with previous data (33,35,49–54). The helicase activity of Dna2 did not significantly affect the position of cleavage (Figure 1F and Supplementary Figure S3A and B). In summary, these data suggest that Dna2 is able to cleave flaps of at least 8 nt in length at or very near their base.

## The product of Dna2 can be directly ligated

The capacity of Dna2 to cut at or near the flap base prompted us to investigate whether a fraction of the cleaved flaps can be ligated by the cognate Lig1. Previously, it was shown not to be the case as Dna2 was leaving a ∼5–8 nt flap behind that prevented ligation in a vast majority of cases (33). This gave rise to the two-step model where a second nucleolytic activity was needed (33). We show in Figure 2A–C that ∼10–15% of the cleaved flap structures could be directly ligated by Lig1, which corresponded to the flaps cleaved exactly at the base (position '0' in Figure 2B). RPA moderately increased ligation efficiency (Figure 2C, primary data for reactions without RPA not shown), while the helicase of Dna2 had no effect (Figure 2D; Supplementary Figure S3C). Furthermore, ∼15–25% of 8 nt-long flap structures cleaved by either wild-type or helicase-dead Dna2 were ligated by Lig1 as well (Supplementary Figure S4A–D, primary data for reactions without RPA not shown). In contrast, the nuclease activity of Fen1 resulted in almost undetectable ligation efficiency on 4 nt or longer flaps (∼1% product, Supplementary Figure S5A and B). This is most likely due to the fact that Fen1 predominantly cleaves DNA not exactly at the flap base, but just inside dsDNA (+1 po-

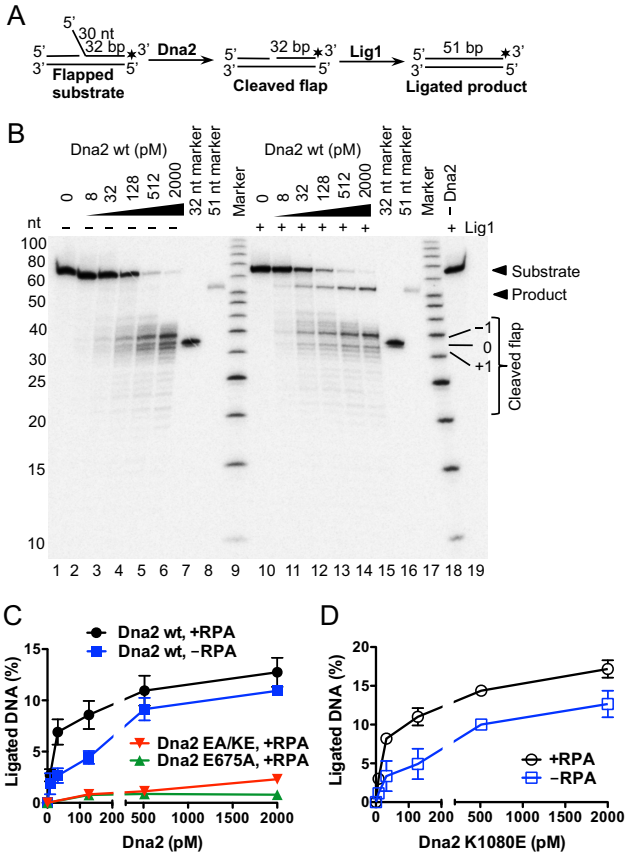


**Figure 2.** The product of Dna2 can be directly ligated. (**A**) Assay. *, radioactive label. (**B**) Increasing concentrations of wild-type Dna2 were incubated with a substrate containing a 30 nt-long flap with or without Lig1, in the presence of RPA. The reaction products were separated on a 20% polyacrylamide denaturing urea gel. Cleavage at the base of the flap produces a fragment of 32 nt in length. Ligation of the cleaved intermediate results in a final product of 51 nt in length. (**C**) Quantitation of data such as in (B), with wild-type or mutants of Dna2 (nuclease-dead, E675A; helicase and nuclease-dead, EA/KE). The $^{32}$P label was placed either at the 3′ terminus of the flapped oligonucleotide or at the 5′ terminus of the upstream primer. Averages shown, $n = 2$; error bars, s.e.m. (**D**) Quantitation of experiments such as in (B), but with helicase-dead Dna2 K1080E. The $^{32}$P label was placed either at the 3′ terminus of the flapped oligonucleotide or at the 5′ terminus of the upstream primer. Averages shown, $n = 2$; error bars, s.e.m.

sition, Supplementary Figure S1D) and this cleavage position does not produce a substrate for Lig1. In accord, Fen1 is known to prefer a substrate containing an additional 1 nt 3′ flap (55). Taken together, these data indicate that a fraction of flap structures cleaved by Dna2 exactly at their base (∼15–20%) can be directly ligated by Lig1. The nuclease but not the helicase of Dna2 is essential for this process.

### Dna2 is highly efficient in flap processing during replication

In replication, the processing of flap structures occurs coupled with DNA synthesis by pol δ (1). It was shown that pol δ can accommodate for inaccurate cleavage (9,32,56,57). Hence, flap cleavage at the +1 position (in dsDNA), can be coupled with 1 nt synthesis by pol δ, which creates a ligatable substrate (9). Likewise, cleavage at the −1 position (leaving behind a 1 nt flap) can be accommodated by the proofreading 3′-5′ exonuclease activity of pol δ. By going 1 nt backward, the exonuclease of pol δ likewise leads to a ligatable substrate (57). We expressed and purified the three-subunit pol δ, PCNA and RFC to test whether Dna2 on its own can mediate efficient flap processing in the context of ongoing DNA replication. Previously, it was shown that Dna2 was required for cleavage of exclusively long flaps (e.g. 30 nt) that are bound by RPA and efficient maturation was only achieved in conjunction with Fen1 (32,33). We used a plasmid based ssDNA substrate with a primer containing a 30 nt-long ssDNA flap (Figure 3A; B, lane 2). Pol δ in conjunction with RFC, PCNA, RPA and Lig1 efficiently synthesized DNA (Figure 3B, lane 3, open circular DNA, ocDNA), but no covalently closed supercoiled DNA (scDNA) was detected, showing that the flap structure prevented ligation. As shown in Figure 3B, lanes 4–8, supplementing the reactions further with Dna2 resulted in nearly complete Okazaki fragment maturation already at sub-nanomolar Dna2 concentrations (see also Figure 3C). DNA synthesis was fully dependent on the presence of pol δ, PCNA, RFC and RPA but did not require Lig1, while generation of scDNA required additionally both Dna2 and Lig1 (Figure 3D). The same results were obtained when we used a substrate with a 12 nt-long flap (Supplementary Figure S6A–D). Nuclease- and helicase-dead Dna2 was not able to support the reactions (Figure 3E, Supplementary Figure S6E). When using a substrate without a flap, the maturation was fully independent of Dna2 (Figure 3F), as expected, but still required all other components (Figure 3G). Next, we tested to which extent the Okazaki fragment maturation activity of Dna2 is dependent on the proofreading exonuclease of pol δ. To this point, we substituted wild-type pol δ with 3′ exo- pol δ variant, Pol3-DV, in the replication assays (Figure 4A). The generation of scDNA was strongly inhibited in the absence of the pol δ exonuclease (Figure 4B, lane 14 and Figure 4C), showing that the proofreading activity of pol δ is very important for flap processing during DNA replication in conjunction with Dna2, in accord with a previous study (57).

Fen1, in contrast, was unable to process the 30 nt-long flap in the presence of RPA (Supplementary Figure S7A and B), while it very efficiently supported the reactions with a 4 nt-long flapped substrate (Supplementary Figure S7C-E) and to a lesser degree reactions with a 12 nt-long flaps (Supplementary Figure S7F–H) as expected (58). Furthermore, Fen1 was fully incapable to process the 4 nt-long flaps without the 3′ exonuclease of pol δ (Supplementary Figure S7J and K), most likely due to the increased strand displacement activity that was described for the Pol3-DV mutant (57). Genetic studies showed that *rad27Δ pol3-DV* cells grow very slowly and an additional deletion of *rad51* is lethal (56,57). However, the lethality of the *rad27Δ pol3-DV rad51Δ* triple mutant can be rescued by overexpression of Dna2, suggesting that Dna2 can process these flaps in the absence of Fen1 (57). Our experiments demonstrate that Dna2 in concert with DNA replication is highly efficient in flap processing as a single nuclease, thus suggesting that Dna2 may function in Okazaki fragment maturation as the sole flap processing enzyme.

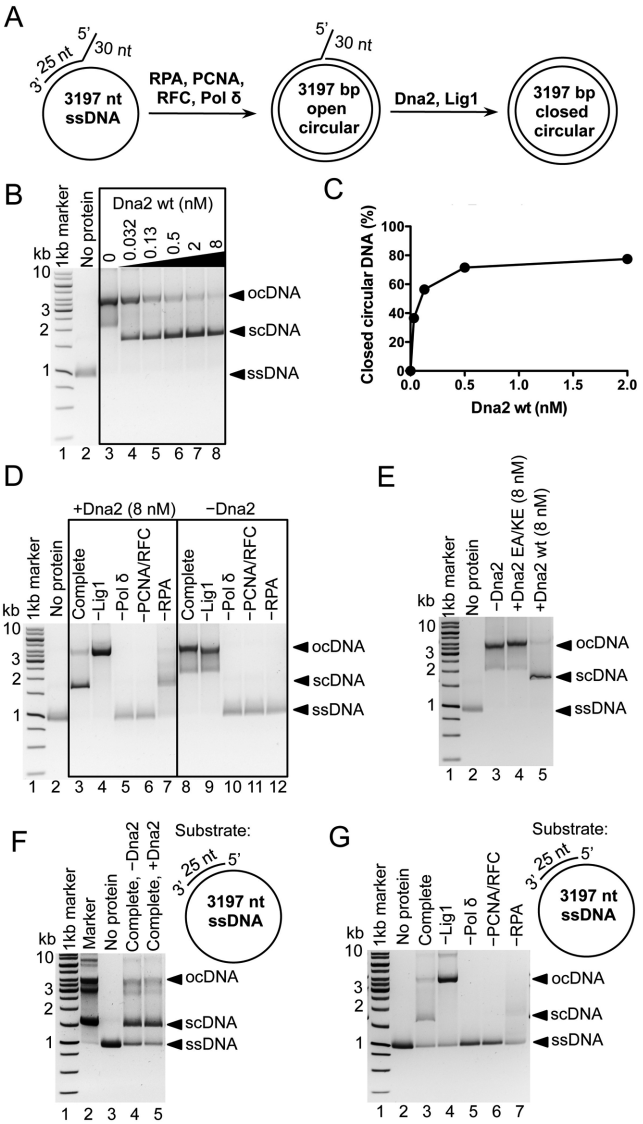


**Figure 3.** Dna2 is highly efficient in flap processing during replication. (**A**) Replication assay. See 'Materials and Methods' section for details. (**B**) Dna2 is required for the completion of replication of a substrate containing a 5′ ssDNA flap of 30 nt in length. Reactions contained pol δ, PCNA, RFC, RPA, Lig1 and Dna2 as indicated. Positions of substrate ssDNA, open circular intermediate (ocDNA) and closed circular supercoiled final product (scDNA) are indicated. Final product appears in a Dna2 concentration dependent manner. (**C**) Quantitation of data such as in (**B**). Averages shown, $n = 3$; error bars, s.e.m. (**D**) Pol δ, RFC, PCNA and RPA are required for DNA synthesis, Dna2 and Lig1 for the formation of scDNA product. A 'complete' reaction contained all components as described in (B) without Dna2. Proteins were omitted from the reactions as indicated. (**E**) Nuclease activity of Dna2 is required for flap processing. Nuclease- and helicase-dead Dna2 E675A/K1080E variant was used where indicated. (**F**) Same assay as in (**E**), but using a substrate without a flap. Dna2 was not required for the processing of the flap-less substrate. A 'complete' reaction contained all components as described in (B) without Dna2. Marker, a sample containing DNA species corresponding to scDNA, linear DNA and ocDNA. (**G**) Replication assay with a flap-less substrate. Protein components were omitted from the reactions where indicated. A 'complete' reaction contained all components as described in (B) without Dna2.

## Dna2 does not function with Fen1 in a concerted manner

Our data so far indicated that the Dna2 nuclease was remarkably capable of promoting flap processing during DNA replication. To test whether Fen1 can further stimulate the ligation efficiency in conjunction with Dna2, we combined the two nucleases together with RPA and Lig1 on the 30 nt-long flap oligonucleotide-based substrate (Figure 5A). As shown in Figure 5B and C, the presence of Fen1 lowered the ligation efficiency from ∼15 to ∼5% in a Fen1 concentration-dependent manner, in accord with the observed cleavage position of Fen1 that precludes ligation (Supplementary Figure S1D).

We next analyzed the activity of Fen1 and Dna2 in replication-coupled assays on plasmid-based DNA substrates. As Fen1 was incapable to process the 30 nt-long flapped substrates (Supplementary Figure S7A and B), we tested whether it could promote the production of covalently closed DNA in concert with Dna2. Using a suboptimal Dna2 concentration (0.13 nM), we show in kinetic assays that an equimolar concentration of Fen1 had no effect on the ligation efficiency (Figure 5D, E and G). When a 10-fold higher Fen1 concentration was used, a moderate stimulation of the reaction was observed (Figure 5F and G). Therefore, Fen1 was able to complete the flap processing downstream of Dna2 in some cases. We showed above that Dna2 cleaves the flap sequentially; thus, we believe that Fen1 could process the flaps that were previously shortened by Dna2. The fact that the stimulation occurred at Fen1 concentrations that exceeded those of Dna2 argues against the notion that both enzymes function in a coordinated manner. However, this assumes that both protein preparations contain an identical proportion of an active enzyme. To study the effect of Fen1 on flap processing by Dna2 in more detail, we next performed experiments with an even lower Dna2 concentration (32 pM) and titrated Fen1 into the reactions. We observed that Fen1 promoted the formation of covalently closed DNA at concentrations equal or higher than 128 pM (Supplementary Figure S8A–C). However, very similar Fen1 concentrations promoted the 4 nt-long flap processing without Dna2 (Supplementary Figure S7C–E). In case of a concerted reaction, we would expect Fen1 being more efficient in reactions with Dna2 rather then on its own, which was not the case. We conclude that Dna2 is sufficient for Okazaki fragment processing in most cases on its own without Fen1. However, we do not exclude that Fen1 can function downstream of Dna2 in a small number of cases when either Dna2 does not cleave near the base of the flap or when pol δ displaces the annealed 5′ end upon Dna2 cleavage before ligation occurs. In accord, we observed that Fen1 but not Dna2 could promote processing of a substrate without a flap (Supplementary Figure S9A–E). These results collectively argue against the requirement for a cooperation between Dna2 and Fen1 in flap processing and rather suggest that their action is not concerted in most cases.

PCNA has a central function in lagging strand DNA replication as it interacts with RFC, pol δ, Lig1 and Fen1 (7,8,10,11,13,14). PCNA also stimulates Fen1 activity (54). PCNA thus not only promotes DNA synthesis by pol δ as a processivity factor (9), but also serves as a docking platform

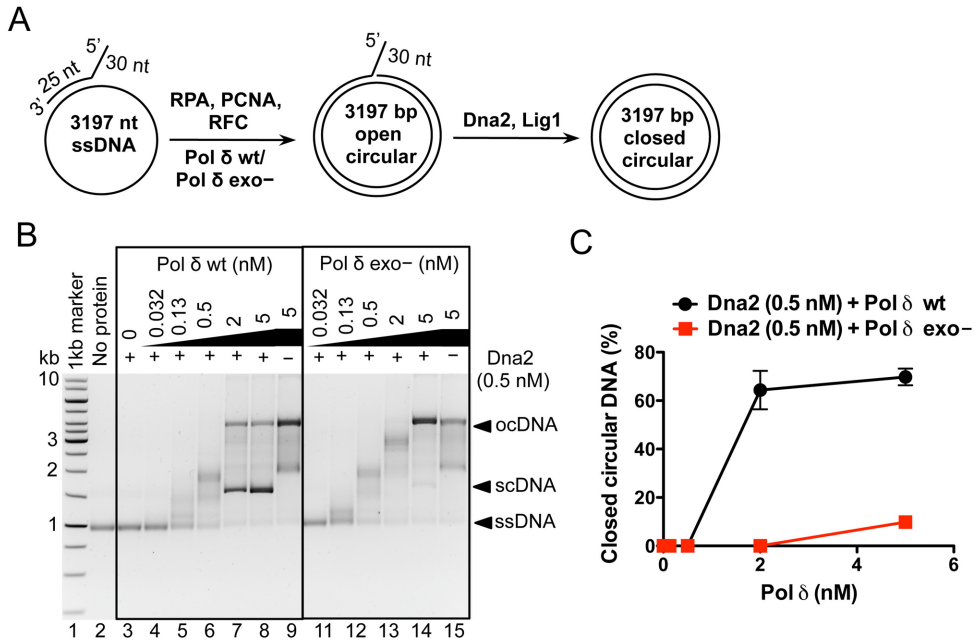


**Figure 4.** Effect of pol δ exonuclease activity on Okazaki fragment processing by Dna2 and Fen1. (**A**) Replication assay with a 30 nt-long flapped primer. (**B**) Reactions contained RFC, PCNA, RPA, Lig1 and Dna2, where indicated. Pol δ wild-type or the exonuclease-deficient mutant (pol δ exo−) were titrated into the reactions. Positions of substrate ssDNA, open circular intermediate (ocDNA) and closed circular supercoiled final product (scDNA) are indicated. In the presence of pol δ exo−, Dna2 stimulates the completion of replication only to a minor extent. (**C**) Quantitation of data such as in (B). Averages shown, $n = 2$; error bars, s.e.m.

for factors required for Okazaki fragment maturation (59). We show in Supplementary Figure S10 that Dna2 can also directly interact with PCNA under physiological salt concentrations. In addition, as demonstrated previously (60), we confirm that Dna2 interacts with RPA (Supplementary Figure S10). The observation that Dna2 binds PCNA is in accord with human DNA2, which was found to be in complex with replication component And-1 *in vivo* (61). Thus, our data in conjunction with previous work suggest a model (Figure 6) where Dna2 is primarily responsible for the processing of long DNA flaps coated with RPA. This is facilitated by the direct interaction between Dna2 and RPA, and the capacity of Dna2 to degrade RPA-coated ssDNA more rapidly than naked DNA (10,12,34,35,60,62). Fen1 is primarily responsible for short flaps and is recruited to those *via* its structure specific DNA-binding capacity (32,35,50). The polymerase and 3′-5′ exonuclease of pol δ is then required in most cases downstream of both Dna2 and Fen1 before adjacent fragments can be sealed by Lig1 (9,57).

## DISCUSSION

Synthesis of the lagging DNA strand is discontinuous and occurs in short fragments of ∼200 nt in length. In order to complete DNA replication, the adjacent Okazaki fragments must be ligated to achieve integrity of the nascent DNA. Direct ligation is often not possible due the displacement synthesis of the lagging DNA strand by pol δ, which leads to flap structures of various lengths (1,2). It has been established that Fen1 cleaves short flaps that are not bound by RPA (33). The strand displacement activity of pol δ, in concert with the Pif1 helicase, can lead to longer flaps that become a substrate for RPA (26). Binding of ssDNA by RPA inhibits the cleavage by Fen1 (33). It has been proposed that the nuclease of Dna2 is specifically involved in the processing of these RPA-bound long flaps (32,33). Importantly, it has been presented that Dna2 only shortens long flaps to ∼5–8 nt. In previous preparations of Dna2, only a very small proportion of flaps was cleaved at the base, which was attributed to the dsDNA melting capacity of RPA at low magnesium concentrations, which creates a substrate that Dna2 can cut (63). Therefore, Fen1 was proposed to function downstream of Dna2 (33,35). This hypothesis was however in contrast with the viability of *rad27*Δ but lethality of *dna2*Δ mutants (19,36–38). Previously, we characterized recombinant Dna2 that exhibited vigorous nuclease and helicase activities (25). Here we show that recombinant Dna2 cleaves efficiently DNA directly at or on either side of the flap base (Figure 1). The cleavage of DNA by Dna2 is unchanged in magnesium concentrations up to 10 mM and requires the nuclease but not the helicase activity of Dna2. A fraction of flaps that are cleaved precisely at their base can be directly ligated by Lig1 (Figure 2). When coupled with DNA replication, Dna2 was remarkably effective in flap processing by allowing a nearly complete Okazaki fragment maturation at sub-nanomolar concentrations (Figure 3), supported by the polymerase and 3′-5′ exonuclease activities of pol δ (Figure 4) (9,57). This showed that in most cases the activity of Fen1 downstream of Dna2 is not required, arguing against the two-nuclease model. We showed that adding Fen1 to Dna2 reactions only led to an increase in ligation efficiency when Fen1 concentration exceeded that of Dna2, and we failed to obtain evidence for cooperativity between Fen1 and Dna2 (Figure 5). Our results thus demonstrate that Fen1 does not promote

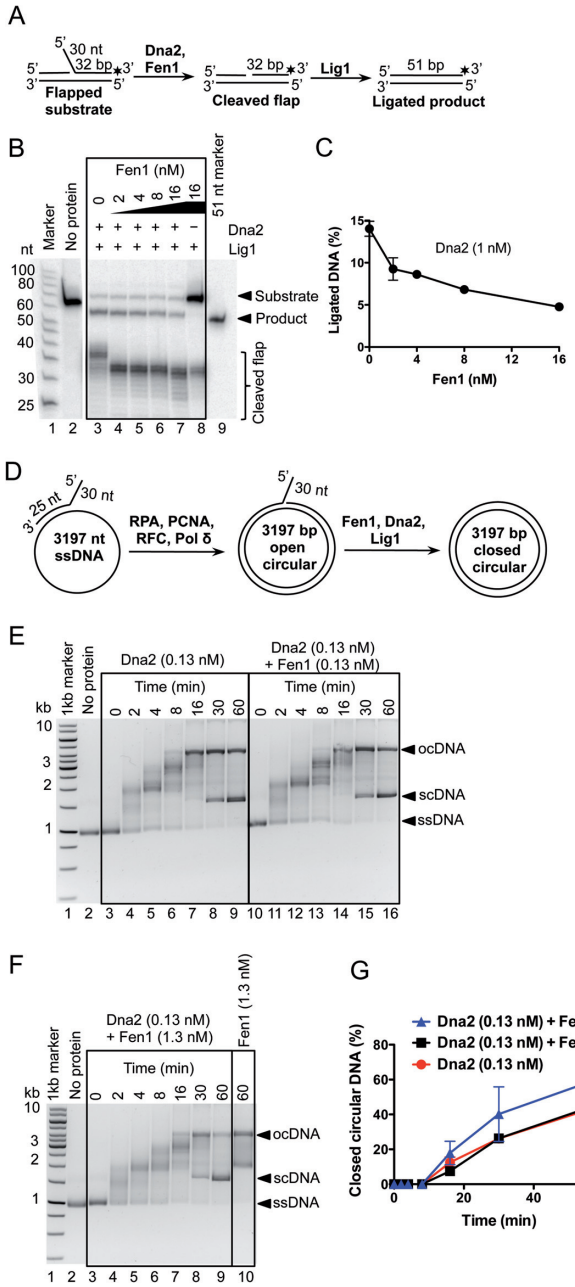


**Figure 5.** Effect of Fen1 on the flap processing by Dna2. (**A**) Assay. *, radioactive label. (**B**) Increasing concentrations of Fen1 were used to supplement reactions containing RPA, and Dna2 or Lig1 as indicated. The presence of Fen1 led to a decrease of the final ligated reaction product. (**C**) Quantitation of data such as in (B). Averages shown, $n = 2$; error bars, s.e.m. (**D**) Replication assay. (**E**) Kinetic replication reactions contained pol δ, PCNA, RFC, RPA, Lig1 and either 0.13 nM Dna2 (left part) or 0.13 nM Dna2 and 0.13 nM Fen1 (right part). Positions of substrate ssDNA, open circular intermediate (ocDNA) and closed circular supercoiled final product (scDNA) are indicated on the right. Samples were terminated at various time points, as indicated. (**F**) Same assay as in (E) but with 0.13 nM Dna2 and 1.3 nM Fen1. (**G**) Quantitation of experiments such as in (E and F). Averages shown, $n = 3$; error bars, s.e.m.

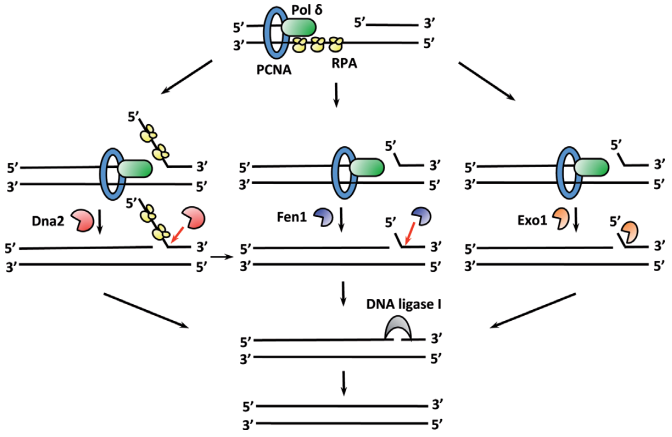


**Figure 6.** Model of Okazaki fragment processing in eukaryotes. In cases when flaps formed upon the strand displacement activity of pol δ are long enough to bind RPA, Dna2 alone is primarily responsible for their processing in most cases (left). Short flaps are primarily processed by Fen1, Exo1 or possibly other nucleases (middle and right). See text for details.

the processing of a large fraction of long flaps by Dna2 and suggest that Dna2 can function in Okazaki fragment processing as the sole nuclease. However, we believe that Fen1 can still function downstream of Dna2 in cases when Dna2 cleaves the flap inaccurately or when 5′ strand displacement occurs again upon Dna2 cleavage before the fragments are ligated.

Dna2 in multiple organisms was proposed to be part of the replication complex. It has been shown that *Xenopus laevis* Dna2 is forming a complex with Mcm10 (minichromosome maintenance complex component 10) and human Dna2 was found to be in complex with And-1 during G1/S transition (61,64). Here we report that *S. cerevisiae* Dna2 interacts with PCNA (Supplementary Figure S10), similarly to Fen1 (10,12). This, together with previously published data, might suggest that Dna2 and Fen1 travel with the DNA replication machinery as components of the Okazaki fragment maturation complex. Alternatively, Dna2 can be recruited to gapped DNA near unprocessed flaps *via* its interaction with PCNA post-replicatively. This is supported by our observation that the levels of Dna2 are low in early S and increase significantly in late S/G2 phase of the cell cycle (Levikova, M. and Cejka, P., unpublished results). In contrast, human FEN1 is highly expressed in the G1 and S phases of the cell cycle and gets rapidly degraded in late S/G2 (65). This would suggest that Dna2 functions rather late in DNA replication to cleave flaps that are refractory to Fen1 and/or Exo1. In accord, yeast *dna2* mutants are proficient in bulk DNA synthesis, but arrest in G2/M phase of the cell cycle (21). Similarly, replication fork progression is not affected in human cells upon DNA2 downregulation; these cells also accumulate in late S/G2 (66). In contrast *fen1* cells accumulate in S phase due to a block in DNA replication at non-permissive temperature (67). Collectively, we show that Dna2 can function in flap processing independently of Fen1. These results are in agreement with the lethality of *dna2*Δ mutation (36), viability of *rad27*Δ or *exo1*Δ cells (37–39), as well as the lethality of *rad27*Δ *exo1*Δ double mutants (39). The flap processing activity of Dna2

described here may also play a role in other processes of DNA metabolism not limited to DNA replication.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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