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In vivo and *in vitro* studies of Mgs1 suggest a link between genome instability and Okazaki fragment processing

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ABSTRACT

The non-essential *MGS1* gene of *Saccharomyces cerevisiae* is highly conserved in eukaryotes and encodes an enzyme containing both DNA-dependent ATPase and DNA annealing activities. *MGS1* appears to function in post-replicational repair processes that contribute to genome stability. In this study, we identified *MGS1* as a multicopy suppressor of the temperature-sensitive *dna2Δ405N* mutation, a *DNA2* allele lacking the N-terminal 405 amino acid residues. Mgs1 stimulates the structure-specific nuclease activity of Rad27 (yeast Fen1 or yFen1) in an ATP-dependent manner. ATP binding but not hydrolysis was sufficient for the stimulatory effect of Mgs1, since non-hydrolyzable ATP analogs are as effective as ATP. Suppression of the temperature-sensitive growth defect of *dna2Δ405N* required the presence of a functional copy of *RAD27*, indicating that Mgs1 suppressed the *dna2Δ405N* mutation by increasing the activity of yFen1 (Rad27) *in vivo*. Our results provide *in vivo* and *in vitro* evidence that Mgs1 is involved in Okazaki fragment processing by modulating Fen1 activity. The data presented raise the possibility that the absence of *MGS1* may impair the processing of Okazaki fragments, leading to genomic instability.

INTRODUCTION

The completion of DNA synthesis at the lagging strand is a complicated process requiring the concerted action of at least 23 polypeptides in eukaryotes (1–6). Owing to their short length (100–200 nt) and the large size of eukaryotic genome, an enormous number of Okazaki fragments ($\sim 2 \times 10^7$ in humans) must be synthesized and processed during a single cell division cycle. Based on current models of Okazaki fragment synthesis and processing, polymerase (pol) α-primase synthesizes short RNA–DNA primers on the lagging DNA template (7). This is then followed by polymerase switching from the non-processive low-fidelity pol α-primase to the processive proofreading pol δ (8). The pol δ–PCNA complex then elongates the short RNA–DNA primers until it encounters the 5′ end of the previously synthesized Okazaki fragment, at which point displacement synthesis leads to the formation of an RNA–DNA flap structure (9).

Studies with purified proteins suggest that the displaced RNA–DNA flap structure is cleaved completely by the combined action of Dna2 endonuclease/helicase and Fen1 (10,11). The length of the RNA–DNA flap generated by pol δ appears to be governed by the binding of replication-protein A (RPA) to the displaced flap (12). When the length of the flap reaches 35 nt, RPA bound to the displaced flap recruits the Dna2 helicase–endonuclease (10), which inhibits any further displacement synthesis. Dna2 then cleaves the flap, most probably removing the entire RNA–DNA segment originally synthesized by the non-proofreading pol α–primase complex, leaving a shortened flap structure that is cleaved by the Fen1 nuclease. Finally, the nicked DNA produced is finally sealed by DNA ligase I. Although there is substantial evidence that both Dna2 and Fen1 are essential for Okazaki fragment processing (13–16), short DNA flaps can be processed only by Fen1 (15–17). These findings suggest that the role of Dna2 is probably related to the length of the flaps generated *in vivo* by the pol δ-catalyzed displacement reaction.

Exploring lagging-strand DNA synthesis in detail is crucial for understanding the genome instability that can occur during DNA replication. For example, mutations in Werner or Bloom helicases (and their homologs in other organisms) cause genome instability in humans and are implicated in Okazaki fragment processing by virtue of their functional interactions with

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Fen1. *In vitro*, both Werner and Bloom helicases markedly stimulate Fen1-mediated flap structure (18–20). *In vivo*, overexpression of the human Bloom syndrome gene suppresses replication and repair defects of yeast *dna2* mutants (21). The role of the Werner and Bloom helicases in Okazaki fragment processing, however, is not clear. The non-essential yeast *MGS1* gene is another example in this enigmatic category. It encodes a highly conserved protein related to RFC and *Escherichia coli* RuvB, and possesses DNA-dependent ATPase and DNA annealing activities (22). *mgs1Δ* mutant cells display an increased rate of homologous recombination and require the *RAD6* epistatsis group of genes for viability. This group of genes is involved in post-replicational repair (23), indicating that DNA damages, accumulated in the absence of *MGS1*, are repaired by the *RAD6*-dependent pathway.

The human homolog of yeast Mgs1 was identified as a WHIP (also called WRNIP1) by two-hybrid screening (24). Recently, WHIP was found to interact physically with DNA pol δ and stimulate its DNA synthetic activity by increasing its initiation frequency (25). Though many of the biochemical and genetic properties of Mgs1 have been defined, its role contributing to genome stability is unknown.

We have carried out experiments designed to identify novel factors involved in eukaryotic Okazaki fragment processing. These resulted in the identification of *MGS1* as a multicopy suppressor that rescues the temperature-sensitive growth defect of *dna2Δ405N* (a *dna2* mutant allele lacking the N-terminal 405 amino acid residues). To better define the role of Mgs1 in Okazaki fragment processing, we prepared recombinant Mgs1 and investigated its interaction with Dna2 and yFen1. We found that in the presence of ATP, the purified Mgs1 enzyme stimulates the nuclease activity of Fen1 and had no effect on the endonuclease activity of Dna2. The suppression of the *dna2Δ405N* phenotype by *MGS1* overexpression required the presence of a functional copy of *RAD27*. Our *in vivo* and *in vitro* results demonstrate that Mgs1 functions by stimulating Fen1 and suggest that a probable source of genomic instability in the absence of *MGS1* is faulty Okazaki fragment processing.

MATERIALS AND METHODS

Enzymes, nucleotides and antibodies

The oligonucleotides used in this study were commercially synthesized from Genotech (Daejeon, Korea) and their sequences are listed in Table 1. Nucleoside triphosphates were obtained from Sigma (St Louis, MO). [γ-^{32}P]ATP (>5000 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). Adenosine-5′-(γ-thio)-triphosphate (ATPγS) and adenosine-5′-(β,γ-imido) triphosphate (AppNp) were from Boehringer Mannheim (Germany). Restriction endonucleases and polynucleotide kinase were from KOSCO Inc. (Sungnam, Korea). M2 anti-flag monoclonal antibodies were purchased from Sigma. Secondary antibodies were from Amersham Biosciences. Yeast Fen1 and Dna2 were purified as described (9,26). Rabbit polyclonal anti-yFen1 antibodies were prepared as described (27).

Preparation of substrates

The oligonucleotide-based partial duplex substrates (except the equilibrating flap substrates; see below) were prepared as described previously (9) using the synthetic oligonucleotides listed in Table 1. Oligonucleotides used as substrates, the position of radioisotopic label in the substrates, and substrate structures are as described in each figure. The equilibrating flap substrates were prepared as described previously (28) with the following modifications. A downstream oligonucleotide was first labeled with [γ-^{32}P]ATP by polynucleotide kinase and the labeled oligonucleotide was then annealed to a template oligonucleotide along with an upstream oligonucleotide (molar ratio of 1:3:10). The resulting equilibrating flap substrate was gel purified prior to use as described (9).

Screening for multi-copy suppressors of dna2Δ405N temperature sensitivity

A *Saccharomyces cerevisiae* genomic DNA library in a pYEp13-based multicopy plasmid (ATCC27323) was transformed into yJA2 (*MAT**a** ade1 ura3 lys1 trp1 his7 leu2 GAL*$^{+}$

Table 1. Oligonucleotides used to construct DNA substrates in this study

No.	Nucleotide sequences (length in nt)
1	5′-AGGTCTCGACTACCACCCGTCCACCCGACGCCACCTCCTG-3′ (40)
2	5′-CAGGAGGTGGCGTCGGGTGGACGGGATTGAAATTTAGGCTGGCACGGTCG-3′ (50)
3	5′-CGACCGTGCCAGCCTAAATTTCAATC-3′ (26)
4	5′-TCGGACGCTCGACGCCATTAATAATGTTTTC-3′ (31)
5	5′-GAAAACATTATTAATGGCGTCGAGCGTCCGTAGGCACAAGGCGAACTGCTAACGG-3′ (55)
6	5′-CCGTTAGCAGTTCGCCTTGTGCCTAG-3′ (26)
7	5′-TTTTTTTTTTTTTCGGACGCTCGACGCCATTAATAATGTTTTC-3′ (43)
8	5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCGGACGCTCGACGCCATTAATAATGTTTTC-3′ (60)
9	5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCGGACGCTCGACGCCATTAATAATGTTTTC-3′ (90)
10	5′-TAGGTCTCGACGACTAACTCTAGTCGTTGTTCCACCCGTCCACCCGACGCCACCTCCTG-3′ (58)
11	5′-CAGGAGGTGGCGTCGGGTGGACGGGTGGAACAACGACTAGAGTTAGTCGTCGAGACCTATTGAAATTTAGGCTGGCACGGTCG-3′ (84)
12	5′-CGACCGTGCCAGCCTAAATTTCAATAGGTCTCGACGACT-3′ (39)
13[a]	5′-CGAACAAUUCAGCGGCTTTAACCGGACGCTCGACGCCATTAATAATGTTTTC-3′ (52)
14	5′-CGGACGCTCGACGCCATTATTATTGTTTTC-3′ (30)
15	5′-GAAAACATTATTAATGGCGTCGAGCTAGGCACAAGGCGAACTGCTAACGG-3′ (50)
16	5′-CGGTTAGCAGTTCGCCTTGTGCCTA-3′ (25)
17	5′-CCGTTAGCAGTTCGCCTTGTGCCT-3′ (24)
18[b]	5′-G[b]AAAACATTATTAATGGCGTCGAGCGTCCGTAGGCACAAGGCGAACTGCTAACGG-3′ (55)

[a]The underlined are ribonucleotides.
[b]The superscript b indicates a biotinylated nucleotide.

dna2Δ405N) (29). The transformants were grown at 25°C for one day and then shifted to 37°C for 4–5 days. Total DNA was isolated from ~20 colonies formed at 37°C and transformed into *E.coli* to recover plasmid DNAs. Insert DNAs in plasmids isolated from *E.coli* were analyzed by nucleotide sequencing. Among these, four clones were identified as *MGS1*. The genomic DNA containing a promoter and open reading frame (ORF) of wild-type *MGS1* was amplified by PCR and the amplified fragment was cloned into 2 μm origin pRS424 (Trp1 as a selection marker), which was transformed back into the yJA2 mutant strain to confirm suppression of *dna2Δ405N* mutation. Transformants were grown in liquid media and the cells were spotted in 10-fold dilutions (10^5, 10^4, 10^3, 10^2 and 10^1 cells) onto SD-Trp plates that were incubated for 3 days at 25 or 37°C as shown.

Construction of vectors and strains

A DNA fragment containing the entire *MGS1* ORF and upstream 574 bp regions was amplified by PCR using yeast genomic DNA as template and a pair of primers; 218W-1 primer (5′-CGG AAT TCG TTC CCT TAT AAT TGT GCT TC-3′) and 218W-2 primer (5′-GCC GAG CTC GTA TCT ATG CTC CTC AAG CC-3′). The amplified fragments were purified using QIA-quick gel extraction kit (Qiagen) and subcloned into pCR2.1 TOPO vector (Invitrogen) to produce pCR2.1 TOPO-MGS1. Nucleotide sequences of MGS1 were verified by DNA sequencing. The plasmid, pCR2.1 TOPO-mgs1K183E (a mutant Mgs1 with a lysine to glutamate change at position 183) was constructed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) using the primers; KE-1 primer (5′-CTC CAG GTG TAG GAG AGA CTT CAC TAG CTA G-3′) and KE-2 primer (5′-CTA GCT AGT GAA GTC TCT CCT ACA CCT GGA G-3′). The EcoR1–Sac1 fragments derived from pCR2.1 TOPO-MGS1 or pCR2.1 TOPO-mgs1K183E were ligated into pRS424 to obtain pRS424-MGS1 or pRS424-mgs1 KE, respectively. In order to produce pET28-MGS1-FLAG or pET28-mgs1KE (which expresses C-terminally FLAG-tagged wild-type or mutant enzyme, respectively), PCR was performed with MGS1–1 primer (5′-CGG CCA TGG CTA GTA ACA AGA GGA CAT CTG TAG AAC-3′) and MGS1-FLAG-2 primer (5′-CGC GAA TTC CTA CTT ATC GTC ATC ATC TTT ATA ATC CGC TAA AAC AGC ACC CTG ATA TGA CTG TCT CAA GTC-3′) using pCR2.1 TOPO-MGS1 or pCR2.1 TOPO-mgs1K183E as template DNAs. Amplified fragments were excised from an agarose gel, purified and subcloned into pET28 (Novagen).

To generate yHJ2 strain, the pRS326-RAD27 plasmid was transformed first into yJA2 and then chromosomal *RAD27* was deleted by transforming PCR products obtained with a pair of primers (5′-CAT ATA TGC CAA GGT GAA GGA CCA AAA GAA GAA AGT GGA AAA AGA ACC CCG CAT ATG ATC CGT CGA GTT C-3′ and 5′-GAA AAG CGT TGA CAG CAT ACA TTG GAA AGA AAT AGG AAA CGG ACA CCG GAC CAT AAT TCC GTT TTA AGA GC-3′) as described previously (30).

Purification of Mgs1

The pET28-Mgs1-FLAG plasmid was transformed into *E.coli* BL21 (DE3) and cells were induced with Isopropyl-β-D-thiogalactopyranoside (final concentration, 0.4 mM) for 3 h. Cells were collected by centrifugation, resuspended in buffer H_{50} [50 mM HEPES–KOH, pH 7.4, 50 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 0.5% Nonidet P-40 (NP-40), 1 mM phenylmethlysulfonyl fluoride, 0.1 mM benzamidine, 1.25 μg/ml leupeptin and 0.625 μg/ml pepstatin A; the subscript number indicates NaCl in mM] and sonicated. The supernatant obtained after centrifugation was loaded onto 20 ml SP-Sepharose column (Pharmacia) pre-equilibrated with H_{50}. This column was washed with 5-column volumes of H_{100} and eluted with H_{500}. The eluted proteins were pooled, mixed with 0.5 ml of anti-FLAG M2 agarose (Sigma) equilibrated with H_{500} and incubated at 4°C for 4 h. After extensive washing with H_{500}, bound proteins were eluted with 0.5 ml of H_{500} containing 3× FLAG peptide (0.5 mg/ml, Sigma) by incubation at 4°C overnight. The eluted material was subjected to glycerol gradient sedimentation and fractions were analyzed for proteins and ATPase activity as described (26). Activity peak fractions were stored at −80°C. The Mgs1K183E mutant enzyme was purified using the same procedure described above.

Nuclease and ATPase assays

Standard nuclease assays were performed in reaction mixtures (20 μl) containing 50 mM Tris–HCl, pH 7.8, 2 mM $MgCl_2$, 2 mM DTT, 0.25 mg/ml BSA, DNA substrate (15 fmol) and with or without 2 mM ATP. Reactions were incubated at 37°C for 15 min, followed by the addition of 20 μl of 2× stop solution (95% formamide, 20 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol). The nucleolytic products were boiled for 1 min and subjected to electrophoresis for 1.5 h at 35 W in 1× TBE (45 mM Tris, 45 mM boric acid and 1mM EDTA) through 15% denaturing gel containing 7 M urea as described previously (26). The gels were dried on DEAE-cellulose paper and autoradiographed. Labeled DNA products were quantified with the use of a PhosphorImager (Molecular Dynamics, Inc.). The background level detected in the absence of added nuclease enzymes was <2% of the input substrate, and this value was subtracted from the amount of cleaved products formed in the presence of yFen1.

Standard ATPase assays were carried out in reaction mixtures (20 μl) containing 25 mM Tris–HCl, pH 7.8, 2 mM DTT, 2 mM $MgCl_2$, 0.25 mg/ml BSA, 50 μM cold ATP, 20 nM [γ-^{32}P]ATP (>5000 Ci/mmol) and 100 ng of ΦX174 sscDNA or other polynucleotides as indicated. After incubation at 37°C for 20 min, aliquots (2 μl) were spotted onto a polyethyleneimine-cellulose plate (Baker,J.T. Inc.) and developed in 0.5 M LiCl/1.0 M formic acid. The products were analyzed using a PhosphorImager.

Gel mobility shift assay

Reaction mixtures (20 μl) containing 50 mM Tris–HCl, pH 7.8, 5% glycerol, 2 mM DTT, 0.25 mg/ml BSA, 15 fmol of substrate and indicated amounts of Mgs1 were incubated on ice for 20 min. Glycerol and bromophenol blue were added to 10 and 0.05%, respectively, and reactions were separated for 1.5 h on 6% acrylamide gel in 0.5× TBE in 4°C. The complexes formed were measured with PhosphorImager.

In vitro binding and immunoblotting

The interaction of Mgs1 and Fen1 was examined as follows; purified C-terminal FLAG-tagged Mgs1 (Mgs1-FLAG; 10 pmol) and Fen1 (10 pmol) were incubated on ice for 30 min in binding buffer (50 mM HEPES–KOH, pH 7.4, 0.2 mg/ml BSA, 125 mM NaCl and 0.05% NP-40), followed by the addition of anti-FLAG antibody conjugated agarose. After 2 h of rocking at 4°C, protein complexes formed were collected by centrifugation and were subjected to 10% SDS–PAGE, followed by western blotting using anti-FLAG or anti-yFen1 antibodies.

In order to examine whether Mgs1 stimulates complex formation of Fen1 with DNA substrate, Mgs1 (25 pmol) was pre-incubated at 4°C for 1 h with the biotinylated DNA substrate (4 pmol) in the above binding buffer, and the mixtures were then supplemented with purified yFen1 (2 pmol), followed by incubation for additional 1 h. Protein–substrate complexes were then precipitated with streptavidin agarose. After centrifugation and washing, the enzymes present in the complexes were analyzed as described above.

RESULTS

Multi-copy expression of MGS1 suppresses the temperature-sensitive growth defect of dna2Δ405N

In a continuing effort to identify novel proteins involved in Okazaki fragment maturation in association with Dna2 endonuclease/helicase, we isolated several multi-copy suppressors—including *MGS1*—that rescue the temperature-sensitive growth defect of *dna2Δ405N*, an allele that lacks the N-terminal 405 amino acid residues of Dna2 (Materials and Methods). By subcloning, we confirmed that the *MGS1* gene alone is sufficient to suppress the *dna2Δ405N* temperature-sensitive phenotype (Figure 1A).

To determine whether the DNA-dependent ATPase activity of Mgs1 is involved in the suppression of *dna2Δ405N*, we constructed a mutant *mgs1* allele (*mgs1K183E*) by substituting glutamate for lysine at amino acid position 183, which abolishes ATPase activity (22). The multicopy plasmid of pRS424-mgs1K183E failed to suppress the temperature-sensitive phenotype of *dna2Δ405N* cells (Figure 1A). As a positive control, we included a plasmid (pRS424-RAD27) that expresses yFen1 and is known to allow *dna2Δ405N* cells to

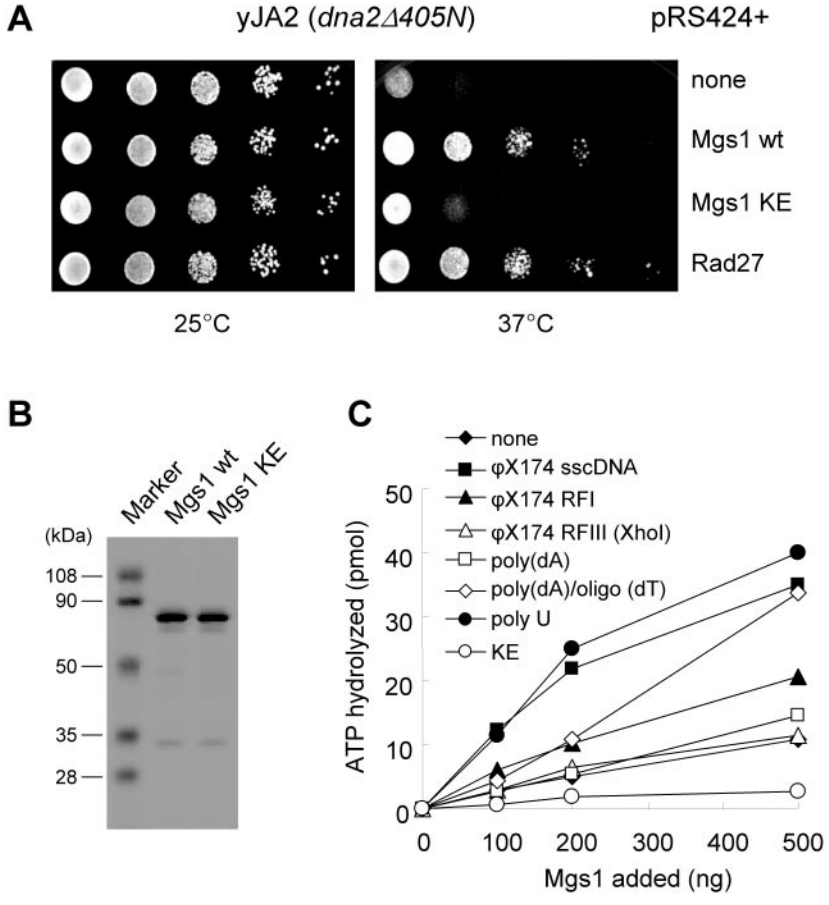


Figure 1. (**A**) Suppression of the temperature-sensitive growth defect of *dna2Δ405N* by overexpression of wild-type *MGS1*. pRS424 (none) and its derivatives expressing either wild-type Mgs1 (Mgs1 wt), ATPase-negative Mgs1K183E (Mgs1 KE) or yFen1 (Rad27) were transformed separately into yJA2, a *dna2Δ405N* strain. Transformants were grown in liquid media and the cells were spotted in 10-fold dilutions (10^5, 10^4, 10^3, 10^2 and 10^1 cells) onto SD-Trp plates. The plates were incubated for 3 days at 25 or 37°C as indicated. (**B**) Purified Mgs1 and Mgs1 KE proteins (0.5 μg each) were subjected to a 10% SDS–PAGE. The gel was Coomassie-stained. (**C**) Influence of DNA cofactors on the ATPase activity of Mgs1. ATP hydrolysis was measured as described in Materials and Methods. Reaction mixtures containing indicated amounts of Mgs1 and 100 ng of DNA cofactors as indicated were incubated for 20 min at 37°C. RFI, replication form I; RFIII (XhoI), replication form III generated by cleaving RFI with XhoI; poly$(dA)_{400}$/oligo$(dT)_{20}$, molar ratio = 1:20 (the superscript indicates the length of each polymer).

grow at 37°C (Figure 1A) (29). All the transformants were able to grow at the permissive temperature regardless of plasmids transformed (Figure 1A, left panel). These results indicate that the ATPase activity of Mgs1 is essential for the suppression of temperature-sensitive growth defect of *dna2Δ405N*.

Influence of cofactor DNAs on Mgs1 ATPase activity

It was shown previously that both yeast and human Mgs1 contain DNA-dependent ATPase activity (22,25). The hydrolysis of ATP by yeast Mgs1 was stimulated by the addition of either single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) with a preference for ssDNA. In contrast, ATP hydrolysis by WHIP1 (also called WRNIP1), the human homolog of yeast Mgs1 was stimulated only in the presence of duplex DNA with ends. Because of this discrepancy in the structures of the DNA cofactors, we examined a variety of DNA preparations for their ability to stimulate the ATPase activity of the yeast Mgs1.

For this purpose, we constructed plasmids that expressed FLAG-epitope tagged Mgs1 either at its C-terminus (Mgs1-FLAG) or at its N-terminus (FLAG-Mgs1). The overexpression of Mgs1-FLAG suppresses the temperature-sensitive growth defect of *dna2Δ405N*, indicating that it is functionally interchangeable with wild-type Mgs1. In contrast, the FLAG-Mgs1 failed to rescue the growth defect of *dna2Δ405N*, indicating that a modification at its N-terminus interferes with its biological function. For this reason, wild-type and mutant Mgs1K183E (both FLAG-tagged at their C-termini) were expressed in *E.coli* and purified to near homogeneity as described in Materials and Methods.

Glycerol gradient sedimentation of the wild-type Mgs1 yields a protein peak fraction that coincided with a single peak of ATPase activity (data not shown), demonstrating that the ATPase activity is intrinsic to Mgs1. An SDS–PAGE analysis of wild-type and mutant Mgs1 are shown in Figure 1B. As expected, Mgs1KE was devoid of detectable ATPase activity, whereas wild-type Mgs1-FLAG hydrolyzed ATP (Figure 1C). Mgs1 hydrolyzed ATP most efficiently in the presence of ΦX174 sscDNA with a turnover rate of ∼0.4 ATP/min/enzyme. In the absence of DNA, Mgs1 hydrolyzed ATP less efficiently (∼20% of the activity obtained with ΦX174 sscDNA) (Figure 1C). Poly(dA)/oligo(dT) stimulated the ATPase activity of Mgs1 weakly (∼2-fold), whereas poly(dA) failed to do so (Figure 1C). We found that the varying molar ratio of poly(dA) to oligo(dT) did not affect the efficiency of ATP hydrolysis by Mgs1 (data not shown). ΦX174 RFI DNA stimulated ATP hydrolysis ∼2-fold, but this stimulation was not observed when the RFI form was converted to RFIII with XhoI (Figure 1C), suggesting that ssDNA regions transiently present in the supercoiled DNA stimulated the ATPase activity of Mgs1. Collectively, these results suggest that DNA molecules containing both ssDNA and dsDNA structures are most effective in supporting the ATPase activity of Mgs1. Interestingly, we found that a ribonucleic acid such as poly(U) stimulates the ATPase activity as efficiently as ΦX174 sscDNA (Figure 1C).

Mgs1 stimulates the endonuclease activity of yeast Fen1

Suppression of the *dna2Δ405N* mutation is observed in the presence of multicopy of the *MGS1* gene as well as by overexpression of either the mutant *dna2Δ405N* allele itself or *RAD27* (29). Therefore, the simplest explanation is that the suppression might stem from the ability of Mgs1 to stimulate either Dna2Δ450N or yFen1 activity. Purified Mgs1 did not affect the helicase or nuclease activities of Dna2. We tested both wild-type and mutant Dna2 enzymes with substrates containing varying lengths (10–60 nt) of flaps, and failed to observe any stimulatory effect of Mgs1 on Dna2-catalyzed cleavage of such substrates (data not shown). However, it significantly stimulated yFen1 structure-specific nuclease activity (Figure 2). To examine the influence of Mgs1 on yFen1 activity, a double-flapped DNA substrate containing both 5′ and 3′ unannealed tails (15 and 1 nt long, respectively) was used as substrate, which when cleaved by yFen1 yields 16 nt long product.

In the absence of ATP, Mgs1 stimulates yFen1 weakly (∼2-fold) at the highest concentration (450 fmol, 30 ng) of Mgs1 added (Figure 2A, lane 6). The stimulation plateaued after the addition of 75 fmol (5 ng) of Mgs1 (Figure 2A, lanes 4–6). The addition of ATP inhibited yFen1 ∼3-fold (data not shown). For this reason, we used nearly three times more enzyme (0.2 fmol) in reactions containing ATP. The additional enzyme resulted in the same level of yFen1 activity (Figure 2A, complex lanes 2 and 9). In the presence of 2 mM ATP, Mgs1 stimulated the activity of yFen1 significantly more than its absence (Figure 2A, compare lanes 2–6 and 9–13). This stimulation increased in proportion to the level of Mgs1 added. Mgs1 alone contained no detectable nuclease activity (Figure 2A, lanes 7 and 14). Nor did Mgs1 alter size of the cleavage product formed by yFen1. We also examined influence of ATP on Mgs1 stimulation of yFen1 using the same level of Fen1 (0.07 fmol) as that used in Figure 2A (lanes 2–7). Under these conditions, the stimulatory effect of ATP was higher (∼10-fold) than that observed higher levels of yFen1 (Figure 2B).

Fen1-mediated suppression of *dna2Δ405N* growth defect

The finding that Mgs1 stimulates Fen1 activity in an ATP-dependent fashion is consistent with our *in vivo* observations that overexpression of wild-type Mgs1, but not ATPase-negative mutant Mgs1K183E, suppressed the *dna2Δ405N* growth defect. Since Mgs1 did not stimulate the activities of the dna2Δ405N mutant enzyme, it is probable that its *in vivo* suppressor activity is due to stimulation of yFen1 activity as shown above (Figure 2A and B). If this were the case, suppression of *dna2Δ405N* by Mgs1 should depend on the presence of a functional Fen1. For this reason, we examined whether multicopy *MGS1* could rescue the *rad27Δ dna2Δ405N*.

Because *rad27Δ dna2Δ405N* double-mutants are lethal (data not shown), we first introduced a plasmid (pRS326-RAD27, Ura3 as a selection marker) containing wild-type *RAD27* into yJA2 (*dna2Δ405N*) and then deleted the chromosomal *RAD27* to construct yHJ2 (*rad27Δ dna2Δ405N).* The strain grew normally at 37°C provided wild-type *RAD27* (in the plasmid pRS326-RAD27) was present (Figure 2C). The yHJ2 strain harboring wild-type *RAD27* was transformed with pRS424 (Trp1 as a selection marker) alone or pRS424 derivatives containing wild-type *MGS1*, mutant *mgs1KE* or *RAD27* and transformants were grown on a plate containing

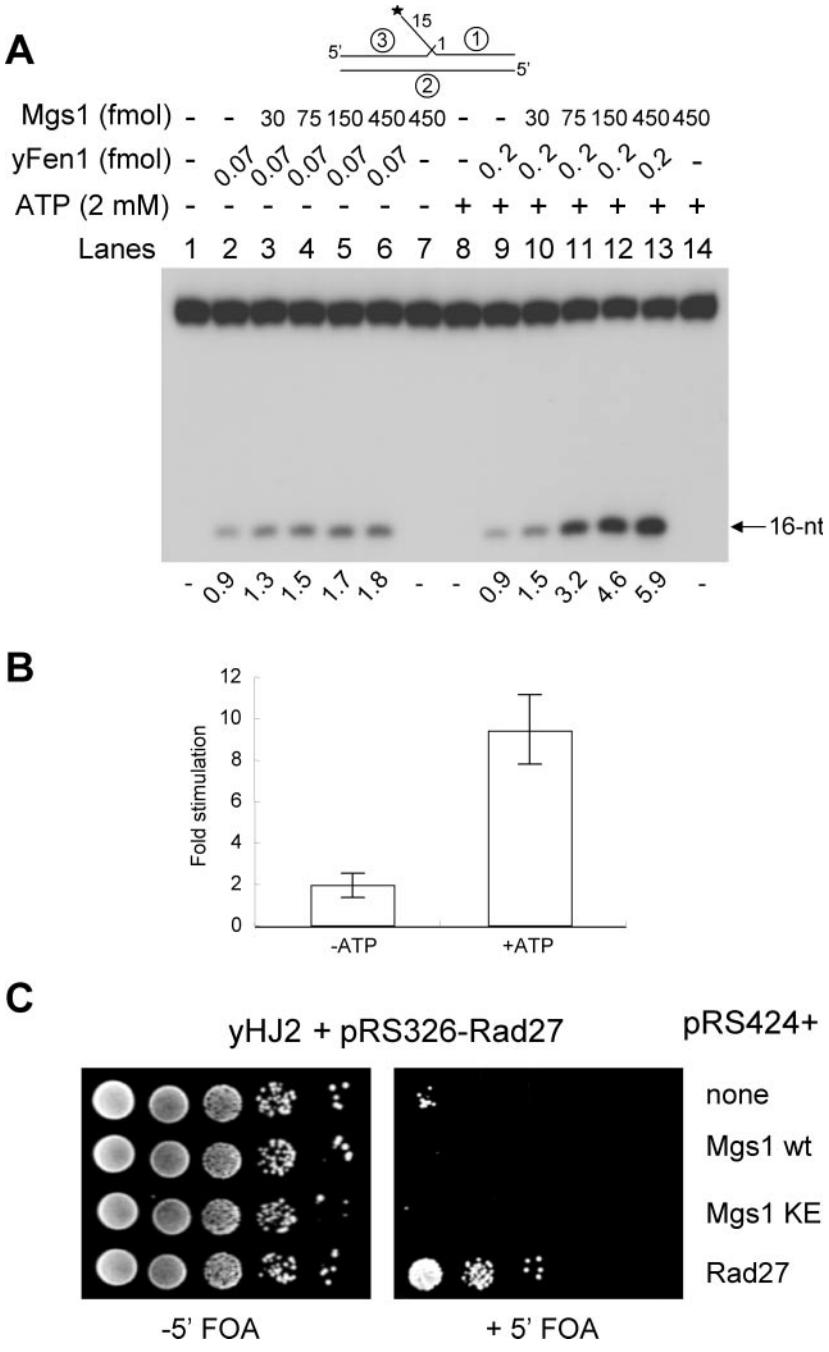


Figure 2. Mgs1 stimulates yFen1 activity in the presence of ATP. (**A**) Structure-specific endonuclease activity of yFen1 (0.07 fmol in the absence of ATP or 0.2 fmol in the presence of 2 mM ATP) was measured as described in Materials and Methods. Schematic structure of a double-flap substrate is illustrated at the top of the figure. The circled number indicates the oligonucleotide used as listed in Table 1. An asterisk in the substrate indicates the position of the 5′ ^{32}P-label. The double-flap substrate (15 fmol) was incubated at 37°C for 15 min with indicated amounts (in fmol) of yFen1 and increasing levels of Mgs1. The addition (plus) or omission (minus) of 2 mM ATP or enzymes is so indicated. The products were separated by electrophoresis on a 15% denaturing polyacrylamide gel. (**B**) Fold stimulation of yFen1 activity by Mgs1. The amounts of yFen1 and Mgs1 were 0.07 and 450 fmol, respectively. The result presented is the mean value obtained from four independent experiments carried out as described in (A) in the absence (−ATP) and presence (+ATP) of 2 mM ATP. Error bars are indicated. (**C**) Overexpression of wild-type *MGS1* does not suppress the growth defect of *rad27Δ dna2Δ405N* double-mutant. pRS424 (none, Trp1 as a selection marker) and its derivatives expressing wild-type Mgs1 (Mgs1 wt), ATPase-negative Mgs1K183E (Mgs1 KE) or yFen1 (Rad27) were separately introduced into the *rad27Δ dna2Δ405N* double-mutant strain harboring pRS326-RAD27 (Ura3 as a selection marker). Transformants were grown in liquid media and cells were spotted in 10-fold dilutions (10^5, 10^4, 10^3, 10^2 and 10^1 cells) onto a plate with 5′ FOA or without 5′ FOA. The plates were incubated for 4 days at 37°C.

5′ fluoroorotic acid (5′ FOA). We found that the control plasmid containing wild-type *RAD27* restored the growth defect efficiently, whereas wild-type *MGS1* or mutant *mgs1KE* did not (Figure 2C). This result supports that the suppressor activity of *MGS1* is due to its stimulation of yFen1 activity. Consistent with this, overexpression of *MGS1* did not suppress the growth defect of the *rad27Δ* mutant (data not shown).

ATP binding is important for stimulation of Fen by Mgs1

We examined whether ATP hydrolysis by Mgs1 was required for the stimulation of the enzymatic activity of Fen1. We found that ATPγS was as efficient as ATP in supporting the Mgs1 stimulation of yFen1 (data not shown). Because ATPγS can be hydrolyzed by a subset of ATPase enzymes, this experiment was repeated using another non-hydrolyzable ATP analog, AppNp. The rate of product accumulation by yFen1 in the presence of Mgs1 (450 fmol, 30 ng) was 4–5 times faster than its absence (Figure 3A, lanes 7–11). When ATP was replaced with AppNp, the rate of product formation was nearly the same as that observed with ATP (Figure 3A, lanes 13–17, and Figure 3B). These results indicate that the binding of ATP to Mgs1, rather than its hydrolysis, is required to stimulate Fen1 activity. In the presence of ADP, Mgs1 stimulated Fen1 as effectively as in the presence of ATPγS or AppNp (data not shown).

Mgs1 stimulation of Fen1 depends on flap length

The Dna2Δ405N enzyme cleaves long flap DNAs poorly, particularly those containing secondary structure (Y. H. Kang, B. C. Lee, J. H. Kim, D. H. Kim, G. H. Ryu,

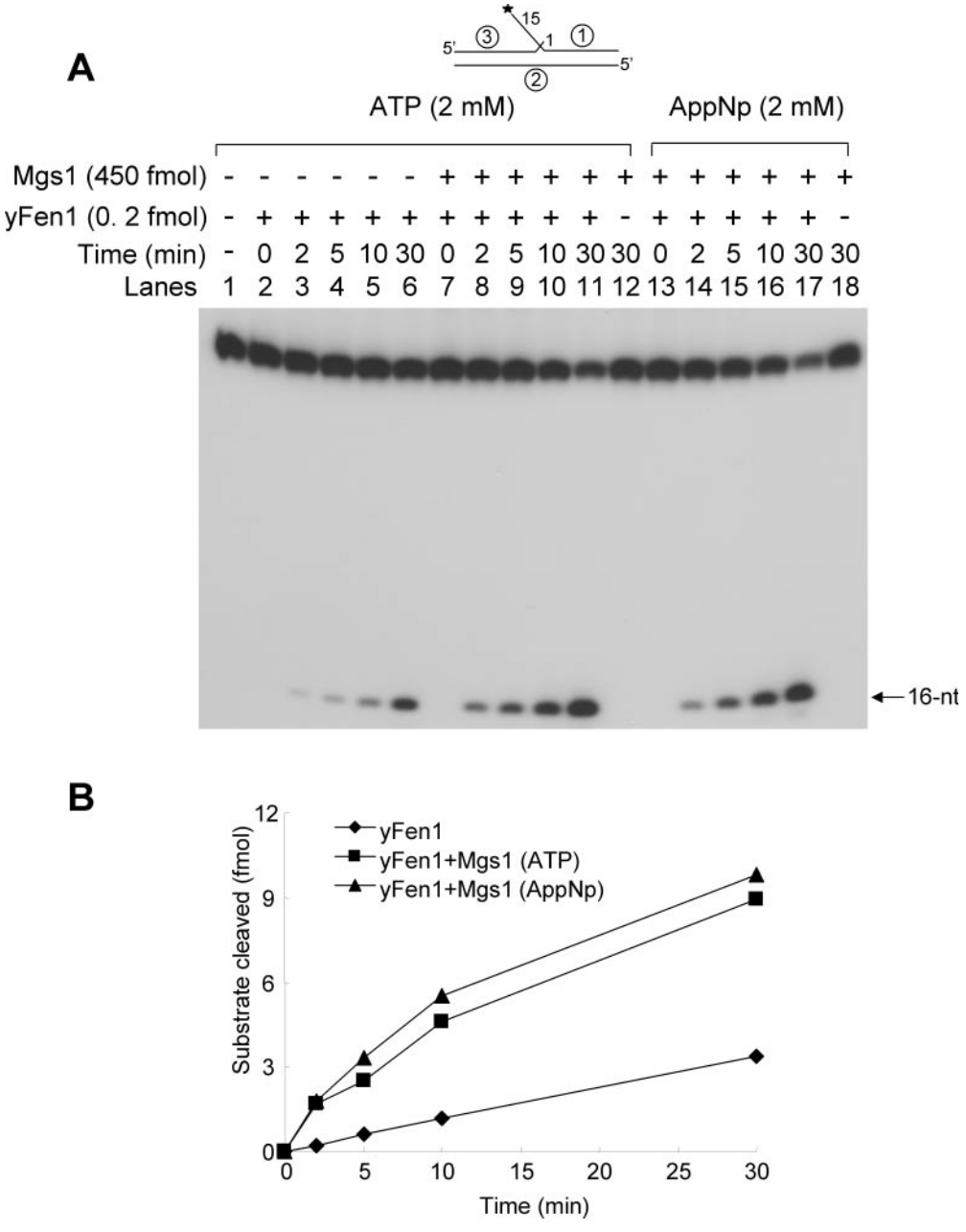
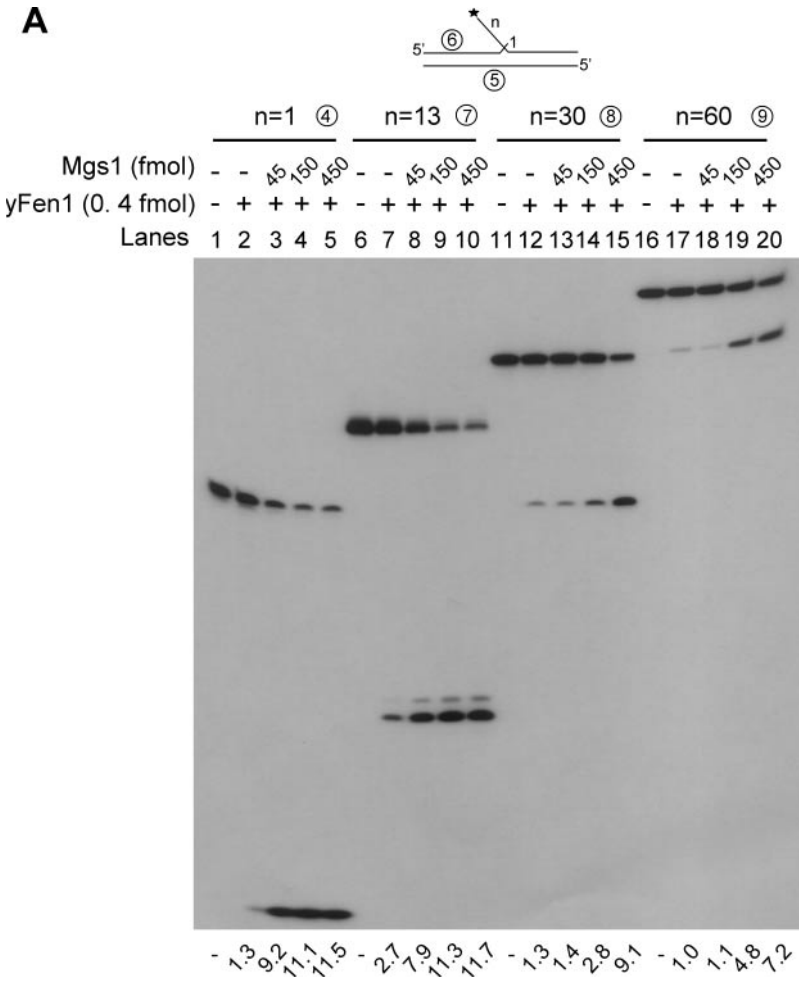


Figure 3. ATP hydrolysis is not required for stimulation of yFen1 by Mgs1. (**A**) The reaction mixtures containing indicated amounts of yFen1 and Mgs1 were incubated for increasing time periods (0, 2, 5, 10 and 30 min) in the presence of 2 mM ATP or 2 mM AppNp. The substrate used was the same as shown in Figure 2A. Products formed were analyzed as described in Figure 2A. (**B**) The amount (fmol) of product formed in reactions described in (A) was plotted against incubation period.


A
n=1 ④
n=13 ⑦
n=30 ⑧
n=60 ⑨
Mgs1 (fmol)
yFen1 (0. 4 fmol)
Lanes
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
- 1.3 9.2 11.1 11.5 - 2.7 7.9 11.3 11.7 - 1.3 1.4 2.8 9.1 - 1.0 1.1 4.8 7.2
B
Mgs1 (fmol)
yFen1 (0. 4 fmol)
Lanes
1 2 3 4 5 6 7 8 9 10 11 12
- 1.1 5.1 7.2 8.1 - - 0.8 2.5 4.8 6.6 -

K. Y. Moon, H. D. Kim, M. J. Kang, S. H. Bae and Y. S. Seo, manuscript submitted). Therefore, we investigated whether Mgs1 preferentially stimulated the cleavage of long flaps by Fen1 *in vitro* and thereby restores long flap cleavage in the *dna2Δ405N* mutant *in vivo*. For this purpose, four additional substrates with oligo(dT) flaps of varying lengths (1, 13, 30 and 60 nt) were constructed. Because ATP is required for the maximal stimulation of yFen1 by Mgs1, 2 mM ATP was added to all subsequent experiments. As shown in Figure 4A, low levels (45 fmol, 3 ng) of Mgs1 stimulated efficiently the Fen1 cleavage reaction with substrates containing 1 and 13 nt flaps. With 30 and 60 nt flap substrates, however, the stimulation showed a sigmoidal response to the amounts of Mgs1 added. In the presence of these two substrates, low levels (45 fmol) of Mgs1 hardly stimulated flap cleavage by yFen1. Only higher levels (450 fmol) of Mgs1 resulted in a significant stimulation of Fen1 activity (Figure 4A, lanes 15 and 20). This raises the possibility that excess ssDNA present in the longer flap substrates may need to be coated with Mgs1, thus decreasing the level of Mgs1 available to stimulate Fen1 activity.

We also examined the effect of Mgs1 on cleavage of 'equilibrating flap' substrates containing 5′ and 3′ flaps (31), both of which are complementary to the template oligonucleotide. In such substrate, both flaps can competitively reanneal either partially or completely to the template and form a variety of double-flap intermediates. As shown in Figure 4B, such equilibrating flap substrates were also cleaved more efficiently by yFen1 in the presence of increasing amounts of Mgs1 (Figure 4B, lanes 2–6). Poly(U) supported ATP hydrolysis by Mgs1 as efficiently as ΦX174 sscDNA, we investigated whether Mgs1 could stimulate the cleavage of flaps containing a 5′-terminal RNA segment. As shown in Figure 4B (lanes 8–11), Mgs1 stimulated the nucleolytic removal of the RNA–DNA chimeric flap with a terminal 12 nt RNA and 10 nt DNA segment. Thus, the presence of a terminal RNA segment in the flap did not abrogate the effect of Mgs1 on yFen1.

Structure-specific stimulation of Mgs1

We investigated whether the Mgs1 activation of Fen1 activity is restricted to flap DNA substrates. For this purpose, three structurally distinct substrates containing a nicked duplex, a fork-structure and a 1 nt gapped flap were used. The 5′–3′ exonuclease activity of Fen1 was marginally stimulated in the presence of a nicked duplex DNA (Figure 5, lanes 2–5). In contrast, the Fen1-dependent cleavage of both fork-structured and 1 nt gapped flap substrates was significantly stimulated by Mgs1 (Figure 5, compare lanes 8–11 and 14–17). It should be noted that cleavage of the nicked and fork-structured substrates required significantly more yFen1 (20 and 4 fmol, respectively, compared with 0.07–00.4 fmol for flap substrates). This result indicates that Mgs1 requires ssDNA for efficient stimulation of Fen1 in addition to ATP. In support of this, Mgs1 did not stimulate yFen1 efficiently when a hairpin structure was present in the flap substrate (data not shown).

Binding of Mgs1 to a flap DNA is important for stimulation

The results described above indicate that flap substrates cleaved more efficiently by yFen1 in the presence of Mgs1 should contain at least 1 nt of unannealed ssDNA. We postulate that ssDNA flaps may act as a binding site for Mgs1, which is required to stimulate Fen1. To test this possibility, we investigated the requirements of various DNA substrates for Mgs1 binding using electrophoretic gel mobility shift assays as described in Materials and Methods. We first examined the binding of Mgs1 to ssDNA containing substrates. These included double-flap, equilibrating-flap, 5′ overhang and 3′ overhang substrates. The former two substrates are efficiently cleaved by yFen1, whereas the latter two are not.

All four substrates formed a complex with Mgs1 with comparable efficiency although the 5′ overhang substrate was slightly less efficient (Figure 6A, lanes 7–9). The amount of Mgs1–substrate complex formed was proportional to the amount of Mgs1 added (Figure 6A). In the presence of 3 pmol of Mgs1, ∼40–80% of the substrate formed a complex with Mgs1 (Figure 6A, lanes 2, 5, 8 and 11). However, Mgs1 formed a complex inefficiently (<6%) under the same conditions with the nicked-duplex substrate (Figure 6B, lane 2). Cleavage of this substrate was poorly stimulated by Mgs1 (Figure 5). In contrast, the 1 nt double (Figure 6B, lanes 4–6) and single-flap (Figure 6B, lanes 7–9) substrate formed detectable complexes with Mgs1. The presence of the 1 nt long 5′ DNA in the flap appears critical for Mgs1 binding, since the 1 nt gapped substrate was hardly complexed by Mgs1 (Figure 6B, lanes 10–12). This finding suggests a strong correlation between binding of Mgs1 to the ssDNA flap and its ability to stimulate yFen1 activity.

Mgs1 does not form a stable complex with Fen1

We examined whether purified Mgs1 and yFen1 interacted using an *in vitro* binding assay. Mgs1-FLAG and yFen1 enzymes were mixed and then incubated with agarose-conjugated anti-FLAG antibodies. Analysis of the immunocomplexes formed did not reveal any physical association of Mgs1 with Fen1 in solution (Figure 7A).

We then examined whether Mgs1 stimulated the binding of Fen1 to substrate DNA. For this purpose, we prepared a substrate in which the template strand was biotinylated at its 5′-terminus as shown in Figure 7B. After 1 h incubation of the enzyme(s) and the biotinylated substrate at 4°C as indicated in Figure 7B, the biotinylated substrate was precipitated with streptavidin agarose beads. In the absence of the substrate, neither Mgs1 nor yFen1 was detected in the precipitated DNA (Figure 7B, lanes 3 and 4), whereas they were detected in the presence of the substrate (Figure 6B, lanes 5 and 6).

Figure 4. Influence of flap size on yFen1 stimulation by Mgs1. (**A**) The reaction mixtures containing 2 mM ATP were incubated at 37°C for 15 min with a fixed amount of yFen1 (0.4 fmol) and increasing levels of Mgs1 as indiated. Reaction products were analyzed in 15% denaturing PAGE. *n* denotes the number of nucleotides presented in the 5′ flap in the substrate. The amount (fmol) of substrate cleaved is presented at the bottom of figure. (**B**) Mgs1 stimulates yFen1-catalyzed cleavage of 'equilibrating' and RNA–DNA chimeric flaps. The schematic structures of the equilibrating and RNA–DNA chimeric flap substrate are shown at the top of the figure. Asterisks indicate the position of the 5′ ^{32}P-label in each substrate. Nuclease assays were carried out as described in Materials and Methods. Reaction mixtures containing 2 mM ATP were incubated at 37°C for 15 min with indicated amounts of yFen1 and Mgs1 and the products formed were analyzed as described in Figure 2A. The amount (fmol) of substrate cleaved is presented at the bottom of figure.

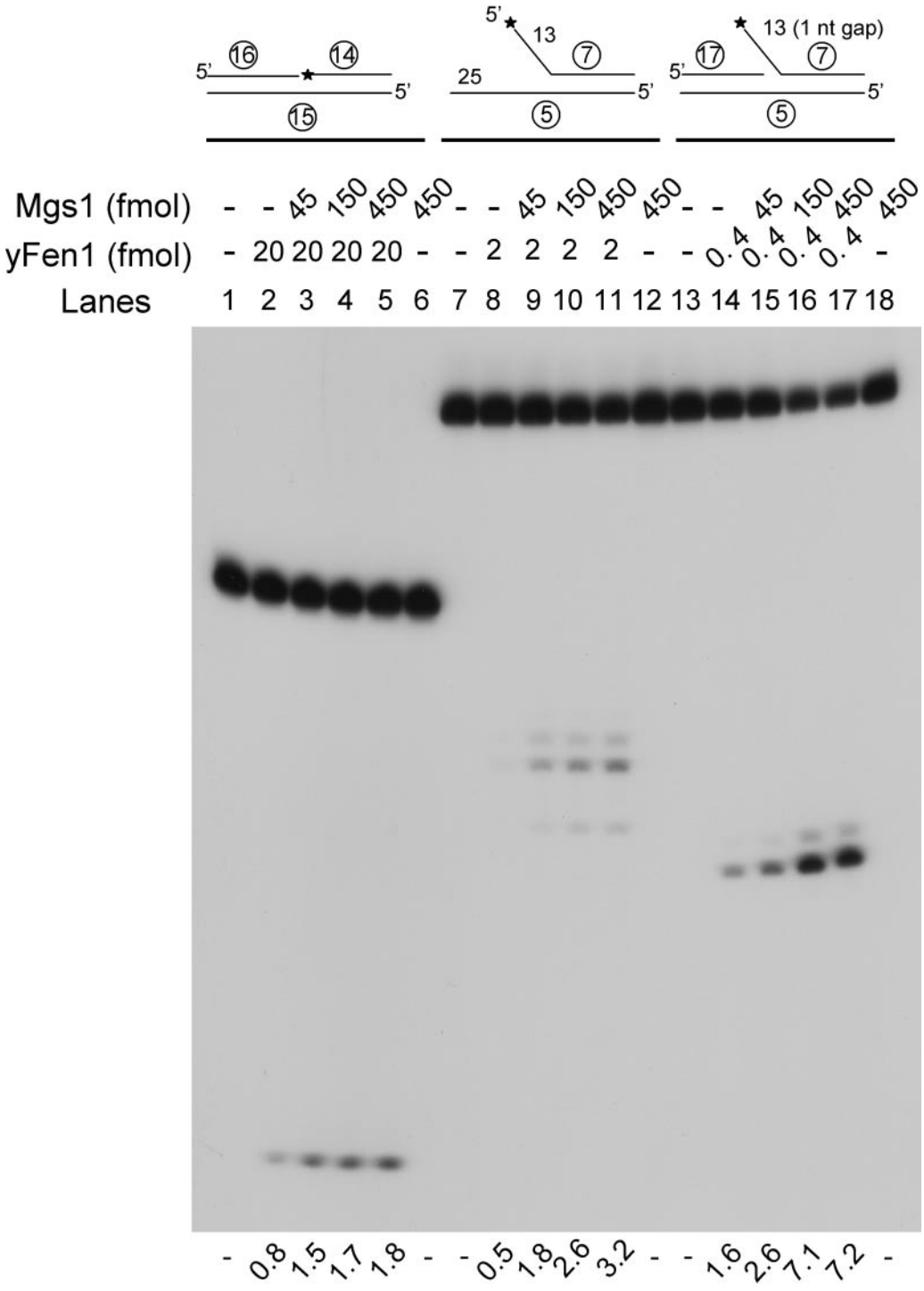


Figure 5. Stimulation of yFen1 by Mgs1 with fork-structured or flap substrates. The levels of yFen1 and Mgs1 added are as indicated. The schematic structures of nicked, forked and 1 nt gapped flap substrates are indicated at the top of the figure. Asterisks indicate positions of the 5′ ^{32}P-label in each substrate. Standard nuclease assays were as described in Materials and Methods. Reaction mixtures containing 2 mM ATP were incubated at 37°C for 15 min with indicated amounts of yFen1 and Mgs1 and products were analyzed as described in Figure 2A. The amount (fmol) of substrate cleaved is presented at the bottom of figure.

When both proteins were combined with the substrate (Figure 6B, lane 7), the amount of protein detected was the same as when each enzyme alone was used. We also found that addition of ATP had no effect on the binding of the two proteins to the substrate (Figure 7B, lanes 7 and 9). This observation indicates that there is either no physical interaction between Mgs1 and Fen1 or that their interaction is too weak to withstand the assays described here. The apparent

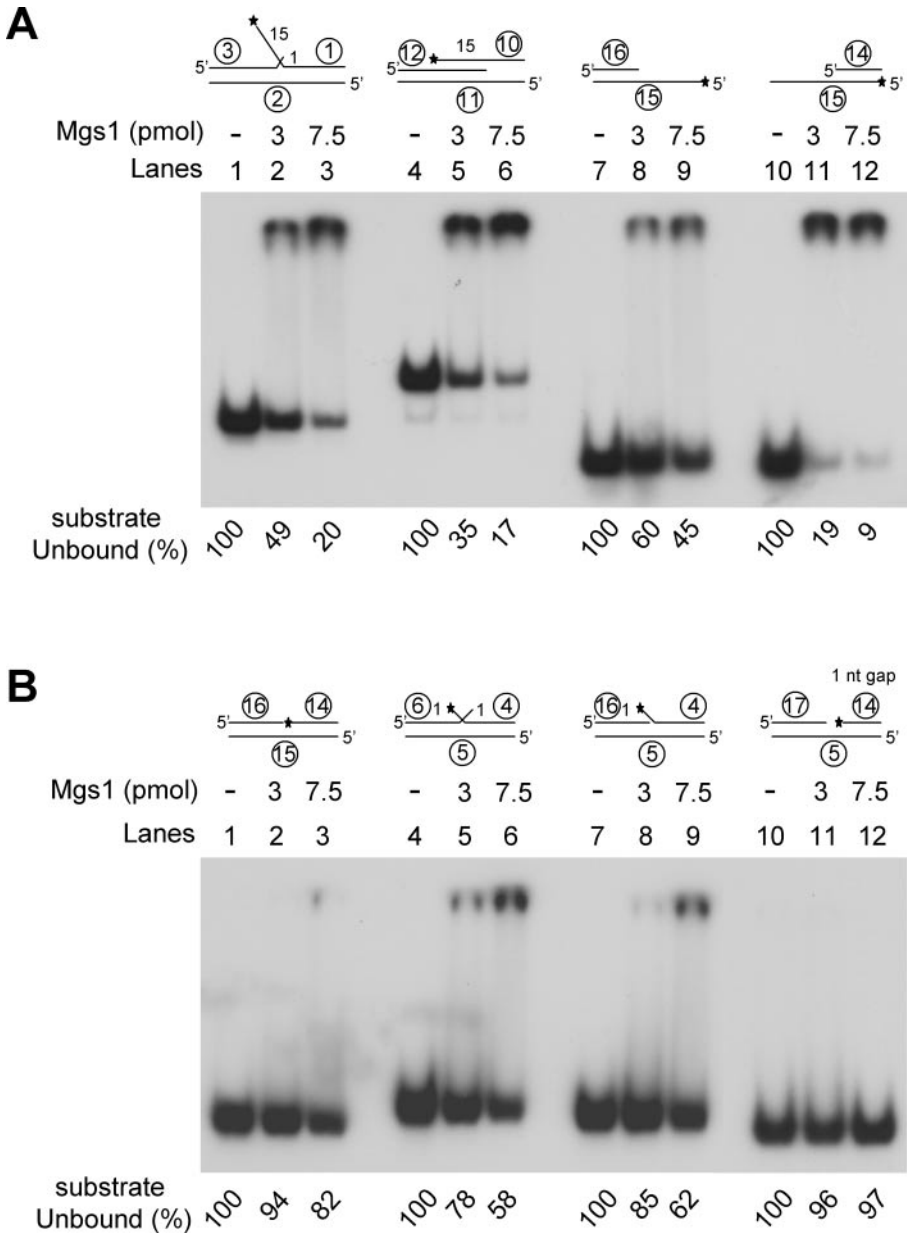


Figure 6. Analysis of DNA structures required to form an Mgs1–DNA complex. Requirements for Mgs1 to form a complex with substrate DNA. (**A**) Electrophoretic mobility shift assays were performed as described in Materials and Methods. Each substrate (15 fmol) shown at the top of the gel was incubated with increasing amounts (3 and 7.5 pmol) of Mgs1 at 4°C for 20 min. The Mgs1–substrate complexes formed were analyzed in 6% PAGE and autoradiography. The amount (%) of substrate unbound was measured and presented in the bottom of the gel. (**B**) The same experiment shown in (A) was repeated with nicked, 1 nt double-flap, 1 nt 5′ flap and 1 nt gapped duplex substrates.

stimulation of yFen1 activity by Mgs1 and our failure to detect any stable interaction between the two enzymes suggest that the physical interaction, if any, between Mgs1 and yFen1 is transient or intrinsically weak and the transient interaction may be critical for the stimulation of Fen1 activity by Mgs1.

DISCUSSION

In this report, we showed that Mgs1 stimulates yFen1 activity and that this effect is enhanced by ATP. Although *MGS1* was isolated as a multicopy suppressor of *dna2Δ405N*, we failed to detect any observable stimulation of endonuclease activity of Dna2Δ405N (a mutant enzyme devoid of N-terminal 405 amino acids) by Mgs1. Our results indicate that the suppression of *dna2Δ405N* by *MGS1* overexpression occurs indirectly via its stimulation of Fen1 activity. This view is in keeping with our previous genetic observation that overexpression of Fen1 suppresses the temperature-sensitive phenotype of the *dna2Δ405N* mutant (29). It was shown that overexpression of either wild-type *DNA2* (32) or mutant *dna2Δ405N* (J. H. Kim and Y. S. Seo, unpublished data) suppresses temperature-dependent growth defects of *rad27Δ* mutants. This mutual suppression supports the notion that

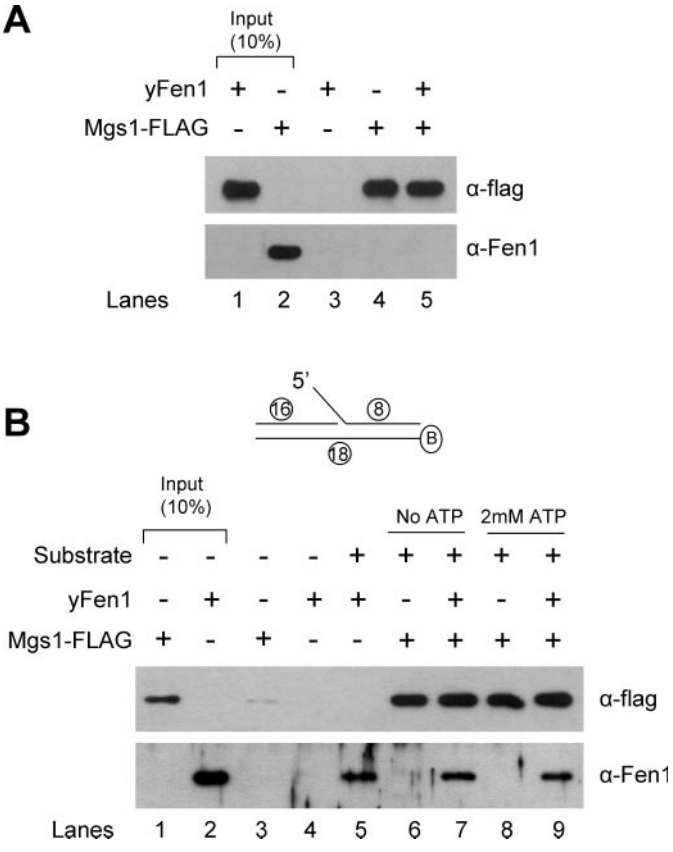


Figure 7. Examination of interaction between Mgs1 and Fen1. (**A**) Mgs1-FLAG (10 pmol) was mixed with or without Fen1 (10 pmol) in binding buffer at 4°C for 2 h. Anti-FLAG antibody conjugated agarose beads were added and the immunoprecipitated material after centrifugation and washing was analyzed by western blotting using anti-FLAG (α-FLAG) or anti-yFen1 (α-Fen1) antibodies as described in Materials and Methods. (**B**) Mgs1 (25 pmol) was preincubated at 4°C for 1 h with the biotinylated flap substrate (4 pmol) (shown at the top of the figure) and binding buffer in the presence and absence of 2 mM ATP. Mixtures were then supplemented with yFen1 (2 pmol), followed by an additional 1 h incubation. The substrate DNA was precipitated with streptavidin agarose beads and the bound proteins were analyzed as described in (B).

Dna2 and Fen1 share overlapping functions, although each has its own preferred substrate for cleavage (10,13,15,16). However, it is possible that stimulation of Fen1 activity by Mgs1 may not be required for the processing of Okazaki fragments in the presence of intact Dna2, considering that deletion of Mgs1 hardly affected growth of yeast cells.

We noted that the stimulation of Fen1 activity by Mgs1 required a high ratio of Mgs1 to the DNA substrate. In the presence of ATP, an excess amount (3- to 5-fold, 45 and 75 fmol) of Mgs1 was necessary for a significant stimulation of Fen1 in a reaction containing 15 fmol of DNA substrates (Figure 2A, lane 11, Figure 4A, lane 11, and Figure 4B, lane 11). The finding that WRNIP1, the human homolog of yeast Mgs1 exists in a homo-oligomeric complex, most probably an octamer (25) may account for why the excess amount of Mgs1 is required for a meaningful stimulation of Fen1 activity.

Although our finding indicates that ATP hydrolysis is not required for the stimulation of Fen1 by Mgs1, it is possible that ATP hydrolysis could be important for its other functions. It is conceivable that ATP hydrolysis is required for Mgs1 if it acts as a Holliday junction migration motor protein, possibly as suggested by its homology to *E.coli* RuvB and its genetic interaction with *TOP3* (22,23). This activity, along with the strand annealing activity of Mgs1 (22), may alter the equilibrating flap structures to facilitate the formation of preferred structures for Fen1 cleavage. For example, Fen1 may be loaded through the 5′ end flap to the duplex junction, but cleavage may not occur until the equilibrating flap junction migrates to form a 1 nt 3′ flap (31). Therefore, cleavage of this substrate may be increased by branch migration and annealing activities of Mgs1, which most probably would require ATP hydrolysis. However, we found that ATP hydrolysis was not required to stimulate Fen1 activity by Mgs1 with CTG-repeat equilibrating substrates (data not shown). ATP hydrolysis may be important with longer equilibrating flaps than that (27 nt) tested in this study.

Moreover, it appears that the DNA annealing activity of Mgs1 may not play a role in stimulating Fen1 nuclease activity for two reasons: first, its stimulation by Mgs1 was not altered significantly even with equilibrating flap substrates and second, ATP addition reduced the DNA annealing activity of Mgs1, while it stimulates Fen1 activity (22). Presently, the role of ATP hydrolysis in stimulation of Fen1 activity is not clear. Nevertheless, the specific rescue of the *dnaΔ405N* mutation and stimulation of Fen1 are consistent with the notion that Mgs1 plays a direct role in processing of Okazaki fragments.

Although Mgs1 stimulated Fen1 activity significantly, we failed to detect a direct protein–protein interaction between Mgs1 and Fen1. This contrasts to results obtained with Bloom or Werner helicase or hnRNP A1, all of which stimulate Fen1 activity by specific protein–protein interactions (18,19,33). The mode by which Mgs1 stimulates Fen1 also differs from that of other Fen1 stimulatory proteins such as PCNA (34) and the Rad9–Rad1–Hus1 complex (20). These proteins cease to stimulate Fen1 activity when their entry onto the ssDNA–dsDNA junction is blocked by a bulky complex at the duplex ends of the substrate. In contrast, Mgs1 stimulates Fen1 cleavage regardless of the presence of a bulky complex present at the DNA termini (data not shown). For this reason, we suggest that Mgs1 binds directly to ssDNA–dsDNA junctions, and the resulting Mgs1–substrate complex becomes more easily accessible to Fen1. This view is in keeping with our observation that Mgs1 did not stimulate cleavage of the nicked flap substrate significantly, to which Mgs1 bound poorly. Mgs1 bound to the flap may adopt a conformation in the presence of ATP that is more readily recognized by Fen1. Our failure to detect a stable complex between Mgs1 and Fen1 suggests that the interaction may be transient, which is important for rapid turnover of enzymes.

In *S.cerevisiae*, *mgs1Δ* mutant cells are viable and exhibit elevated levels (∼4- to 13-fold) of recombination relative to wild-type (22). Cells lacking *MGS1* display a synthetic lethal interaction with *rad6* and exhibit a synergistic growth defect with *rad18* and *rad5*, which are members of the *RAD6* epistasis group of post-replicational repair gene (23). Thus, Mgs1 becomes essential in the absence of post-replicational repair. This observation, together with our results described above, indicates that in the absence of a functional Mgs1, processing of Okazaki fragments is impaired and this

defect can be efficiently overcome by a post-replicational repair pathway that requires genes belonging to *RAD6* epistasis group.

Recently, it was shown that WHIP1, a mammalian homolog of Mgs1, interacts physically with DNA pol δ and increases the initiation frequency of pol δ (25). It was suggested that this interaction is required under particular conditions such as replication fork arrest. These findings suggest another mechanism by which Mgs1 suppresses the *dna2Δ405N* mutation. Namely, the interaction of Mgs1 with pol δ may lead to a reduced size of flaps by affecting pol δ-catalyzed displacement DNA synthesis. Processing of short flaps is less dependent on the function of Dna2 because they can be processed by Fen1 alone. Another possibility is that Mgs1 in association with pol δ may increase the recruitment of Fen1, which would cleave flaps before they grow to a length processed by Dna2. If this were the case, Mgs1 could stimulate flap removal as rapidly as they are generated by pol δ-catalyzed displacement reaction. Recently, it was shown that ligatable nicks are generated by two different mechanisms; (i) in the presence of Fen1, pol δ and Fen1 together go through multiple cycles of displacement synthesis and flap cutting and (ii) in the absence of Fen1, idling by pol δ predominates to maintain ligatable nicks (35). The biochemical role of Mgs1 in our study is not incompatible with this model, since the stimulation of Fen1 by Mgs1 can further facilitate nick maintenance carried out by both pol δ and Fen1. The absence of a functional Mgs1 in this regard is probably to result in faulty Okazaki fragment processing and the accumulation of damaged lesions that can be repaired by a *RAD6*-dependent post-replicational repair pathway.

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Conflict of interest statement. None declared.

REFERENCES

1. Stillman,B. (1994) Smart machines at the DNA replication fork. *Cell*, **78**, 725–728.
2. Bambara,R.A., Murante,R.S. and Henricksen,L.A. (1997) Enzymes and reactions at the eukaryotic DNA replication fork. *J. Biol. Chem.*, **272**, 4647–4650.
3. Waga,S. and Stillman,B. (1998) The DNA replication fork in eukaryotic cells. *Annu. Rev. Biochem.*, **67**, 721–751.
4. Hubscher,U. and Seo,Y.S. (2001) Replication of the lagging strand: a concert of at least 23 polypeptides. *Mol. Cells*, **12**, 149–157.
5. Kao,H.I. and Bambara,R.A. (2003) The protein components and mechanism of eukaryotic Okazaki fragment maturation. *Crit. Rev. Biochem. Mol. Biol.*, **38**, 433–452.
6. Liu,Y., Kao,H.I. and Bambara,R.A. (2004) Flap endonuclease 1: a central component of DNA metabolism. *Annu. Rev. Biochem.*, **73**, 589–615.
7. Conaway,R.C. and Lehman,I.R. (1982) A DNA primase activity associated with DNA polymerase alpha from *Drosophila melanogaster* embryos. *Proc. Natl Acad. Sci. USA*, **79**, 2523–2527.
8. Tsurimoto,T. and Stillman,B. (1991) Replication factors required for SV40 DNA replication *in vitro*. II. Switching of DNA polymerase alpha and delta during initiation of leading and lagging strand synthesis. *J. Biol. Chem.*, **266**, 1961–1968.
9. Bae,S.H. and Seo,Y.S. (2000) Characterization of the enzymatic properties of the yeast dna2 Helicase/endonuclease suggests a new model for Okazaki fragment processing. *J. Biol. Chem.*, **275**, 38022–38031.
10. Bae,S.H., Bae,K.H., Kim,J.A. and Seo,Y.S. (2001) RPA governs endonuclease switching during processing of Okazaki fragments in eukaryotes. *Nature*, **412**, 456–461.
11. MacNeill,S.A. (2001) DNA replication: partners in the Okazaki two-step. *Curr. Biol.*, **11**, R842–R844.
12. Maga,G., Villani,G., Tillement,V., Stucki,M., Locatelli,G.A., Frouin,I., Spadari,S. and Hubscher,U. (2001) Okazaki fragment processing: modulation of the strand displacement activity of DNA polymerase delta by the concerted action of replication protein A, proliferating cell nuclear antigen, and flap endonuclease-1. *Proc. Natl Acad. Sci. USA*, **98**, 14298–14303.
13. Kao,H.I., Veeraraghavan,J., Polaczek,P., Campbell,J.L. and Bambara,R.A. (2004) On the roles of *Saccharomyces cerevisiae* Dna2p and Flap endonuclease 1 in Okazaki fragment processing. *J. Biol. Chem.*, **279**, 15014–15024.
14. Kao,H.I., Campbell,J.L. and Bambara,R.A. (2004) Dna2p helicase/nuclease is a tracking protein, like FEN1, for flap cleavage during Okazaki fragment maturation. *J. Biol. Chem.*, **279**, 50840–50849.
15. Ayyagari,R., Gomes,X.V., Gordenin,D.A. and Burgers,P.M. (2003) Okazaki fragment maturation in yeast. I. Distribution of functions between FEN1 and DNA2. *J. Biol. Chem*, **278**, 1618–1625.
16. 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 δ in the creation of a ligatable nick. *J. Biol. Chem.*, **278**, 1626–1633.
17. Henneke,G., Friedrich-Heineken,E. and Hubscher,U. (2003) Flap endonuclease 1: a novel tumour suppresser protein. *Trends Biochem. Sci.*, **28**, 384–390.
18. Brosh,R.M.,Jr, von Kobbe,C., Sommers,J.A., Karmakar,P., Opresko,P.L., Piotrowski,J., Dianova,I., Dianov,G.L. and Bohr,V.A. (2001) Werner syndrome protein interacts with human flap endonuclease 1 and stimulates its cleavage activity. *EMBO J.*, **20**, 5791–5801.
19. Sharma,S., Sommers,J.A., Wu,L., Bohr,V.A., Hickson,I.D. and Brosh,R.M.,Jr (2004) Stimulation of flap endonuclease-1 by the Bloom's syndrome protein. *J. Biol. Chem.*, **279**, 9847–9856.
20. Wang,W. and Bambara,R.A. (2005) Human Bloom protein stimulates flap endonuclease 1 activity by resolving DNA secondary structure. *J. Biol. Chem.*, **280**, 5391–5399.
21. Imamura,O. and Campbell,J.L. (2003) The human Bloom syndrome gene suppresses the DNA replication and repair defects of yeast dna2 mutants. *Proc. Natl Acad. Sci. USA*, **100**, 8193–8198.
22. Hishida,T., Iwasaki,H., Ohno,T., Morishita,T. and Shinagawa,H. (2001) A yeast gene, MGS1, encoding a DNA-dependent AAA(+) ATPase is required to maintain genome stability. *Proc. Natl Acad. Sci. USA*, **98**, 8283–8289.
23. Hishida,T., Ohno,T., Iwasaki,H. and Shinagawa,H. (2002) *Saccharomyces cerevisiae* MGS1 is essential in strains deficient in the RAD6-dependent DNA damage tolerance pathway. *EMBO J.*, **21**, 2019–2029.
24. Branzei,D., Seki,M., Onoda,F. and Enomoto,T. (2002) The product of *Saccharomyces cerevisiae* WHIP/MGS1, a gene related to replication factor C genes, interacts functionally with DNA polymerase delta. *Mol. Genet. Genomics*, **268**, 371–386.
25. Tsurimoto,T., Shinozaki,A., Yano,M., Seki,M. and Enomoto,T. (2005) Human Werner helicase interacting protein 1 (WRNIP1) functions as a novel modulator for DNA polymerase delta. *Genes Cells*, **10**, 13–22.
26. Bae,S.H., Choi,E., Lee,K.H., Park,J.S., Lee,S.H. and Seo,Y.S. (1998) Dna2 of *Saccharomyces cerevisiae* possesses a single-stranded DNA-specific endonuclease activity that is able to act on double-stranded DNA in the presence of ATP. *J. Biol. Chem.*, **273**, 26880–26890.
27. Harlow,E. and Lane,D. (1988) *Antibodies: A Laboratory Manual*. Chapter 5. Immunizations, Cold Spring Harbor Laboratory Press, NY, pp. 53–138.
28. Kao,H.I., Henricksen,L.A., Liu,Y. and Bambara,R.A. (2002) Cleavage specificity of *Saccharomyces cerevisiae* flap endonuclease 1 suggests a double-flap structure as the cellular substrate. *J. Biol. Chem.*, **277**, 14379–14389.
29. Bae,S.H., Kim,J.A., Choi,E., Lee,K.H., Kang,H.Y., Kim,H.D., Kim,J.H., Bae,K.H., Cho,Y., Park,C. and Seo,Y.S. (2001) Tripartite structure of

Saccharomyces cerevisiae Dna2 helicase/endonuclease. *Nucleic Acids Res*., **29**, 3069–3079.
30. Baudin,A., Ozier-Kalogeropoulos,O., Denouel,A., Lacroute,F. and Cullin,C. (1993) A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res*., **21**, 3329–3330.
31. Liu,Y., Zhang,H., Veeraraghavan,J., Bambara,R.A. and Freudenreich,C.H. (2004) *Saccharomyces cerevisiae* flap endonuclease 1 uses flap equilibration to maintain triplet repeat stability. *Mol. Cell. Biol*., **24**, 4049–4064.
32. 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.
33. Chai,Q., Zheng,L., Zhou,M., Turchi,J.J. and Shen,B. (2003) Interaction and stimulation of human FEN-1 nuclease activities by heterogeneous nuclear ribonucleoprotein A1 in alpha-segment processing during Okazaki fragment maturation. *Biochemistry*, **42**, 15045–15052.
34. Tom,S., Henricksen,L.A. and Bambara,R.A. (2000) Mechanism whereby proliferating cell nuclear antigen stimulates flap endonuclease 1. *J. Biol. Chem*., **275**, 10498–10505.
35. Garg,P., Stith,C.M., Sabouri,N., Johansson,E. and Burgers,P.M. (2000) Idling by DNA polymerase d maintains a ligatable nick during lagging-strand DNA replication. *Genes Dev*., **18**, 2764–2773.