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REVIEW ARTICLE

Dna2 on the road to Okazaki fragment processing and genome stability in eukaryotes

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Abstract

DNA replication is a primary mechanism for maintaining genome integrity, but it serves this purpose best by cooperating with other proteins involved in DNA repair and recombination. Unlike leading strand synthesis, lagging strand synthesis has a greater risk of faulty replication for several reasons: First, a significant part of DNA is synthesized by polymerase α, which lacks a proofreading function. Second, a great number of Okazaki fragments are synthesized, processed and ligated per cell division. Third, the principal mechanism of Okazaki fragment processing is via generation of flaps, which have the potential to form a variety of structures in their sequence context. Finally, many proteins for the lagging strand interact with factors involved in repair and recombination. Thus, lagging strand DNA synthesis could be the best example of a converging place of both replication and repair proteins. To achieve the risky task with extraordinary fidelity, Okazaki fragment processing may depend on multiple layers of redundant, but connected pathways. An essential Dna2 endonuclease/helicase plays a pivotal role in processing common structural intermediates that occur during diverse DNA metabolisms (e.g. lagging strand synthesis and telomere maintenance). Many roles of Dna2 suggest that the preemptive removal of long or structured flaps ultimately contributes to genome maintenance in eukaryotes. In this review, we describe the function of Dna2 in Okazaki fragment processing, and discuss its role in the maintenance of genome integrity with an emphasis on its functional interactions with other factors required for genome maintenance.

Keywords: *Dna2; Fen1; DNA replication; lagging strand synthesis; Okazaki fragment processing; DNA repair; DNA recombination; genome instability*

Introduction

Cell-free replication of simian virus (SV) 40 DNA, developed by Li and Kelly (1984), made a great contribution to discoveries of many cellular factors for eukaryotic replication machinery. Replication of SV40 DNA is initiated by the large tumor antigen (T ag) encoded in SV40 viral DNA. SV40 T ag acts as both initiator and replicative helicase for SV40 origin containing DNA. It recognizes and unwinds SV40 origin DNA to establish replication forks. All subsequent replication events are carried out by cellular factors (Li and Kelly, 1984; Dodson *et al.*, 1987). Extensive studies of this system by many researchers led to identification of replication protein A (RPA, the eukaryotic single-stranded DNA binding protein), replication factor C (RFC, the clamp loader), proliferating cell nuclear antigen (PCNA, a polymerase clamp), pol α-primase, pol δ, Fen1, DNA ligase I, and topoisomerases (Murakami *et al.*, 1986; Prelich *et al.*, 1987a; 1987b; Wobbe *et al.*, 1987; Yang *et al.*, 1987; Fairman and Stillman, 1988; Ishimi *et al.*, 1988; Wold and Kelly, 1988; Lee, S.H. *et al.*, 1989; Tsurimoto and Stillman, 1989; Weinberg and Kelly, 1989; Wold *et al.*, 1989). In addition, genetic analyses of human homologs in budding and fission yeasts and the *in vitro* replication system combined with immuno-depletion technique in *Xenopus* egg extracts confirmed the observations obtained from the SV40 studies. In addition, they contributed greatly to the understanding of other important aspects of eukaryotic DNA replication such as cell-cycle regulated

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initiation and checkpoint control of chromosomal DNA replication (Johnson and O'Donnell, 2005; Sclafani and Holzen, 2007). This review will concentrate on roles of Dna2 in eukaryotic Okazaki fragment processing and how it works together with other protein factors for reliable and complete processing of Okazaki fragments. In addition to its relevance as a fundamental biological process, dysfunction of Okazaki fragment processing could be the basis of genome instability in yeast and in other eukaryotes. We will also speculate how genome instability is instigated by dysfunction of this process. Recent advances in mechanisms and factors for DNA replication, recombination, and repair have been well described in many excellent reviews by others (Kornberg and Baker, 1992; Pâques and Haber, 1999; Alberts, 2003; Cahill *et al.*, 2006; Iyer *et al.*, 2006; Burgers and Seo, 2006; Fortini and Dogliotti, 2007; Shrivastav *et al.*, 2008; Shuck *et al.*, 2008; Budzowska and Kanaar, 2009; Hübscher, 2009).

Once replication origins are activated to establish replication forks, nascent DNA is synthesized both continuously (in the leading strand) and discontinuously (in the lagging strand) at each replication fork because of the anti-parallel nature of two template strands and the ability of DNA polymerases to synthesize DNA in the 5′ to 3′ direction only. Lagging strand DNA replication begins with RNA–DNA primers made by the four-subunit pol α-primase complex, which are further extended by pol δ to form discrete DNA fragments called "Okazaki fragments" (~150 nucleotides) (reviewed in Hübscher and Seo, 2001; MacNeill, 2001; Rossi *et al.*, 2006). Synthesis of RNA and DNA in the hybrid primers are catalytically coupled in the pol α-primase complex; synthesis of RNA (~10 nt) is followed by a short extension of ~20-nt DNA (Bullock *et al.*, 1991), catalyzed by Pri1 and Pol1 subunit, respectively (reviewed in Garg and Burgers, 2005; Burgers, 2008). The other two subunits, Pri2 and Pol2, are also essential factors and required to stabilize and regulate the catalytic subunits. Two catalytic cores are connected through interactions between Pol1 and Pri2 subunits. It should be noted that pol α is devoid of proofreading function to correct misincorporated nucleotides. The five-subunit RFC complex recognizes primer RNA–DNA ends; the binding of RFC to these ends dissociates pol α-primase from the primers and loads PCNA (Maga *et al.*, 2000), which eventually recruits pol δ to the primer–template junction (a process called "polymerase switching"). PCNA is a ring-structured homotrimeric complex which tethers pol δ onto a primer-template junction (Burgers, 1991). Each subunit of RFC belongs to AAA+ family proteins that possess characteristic ATP-binding/hydrolysis motifs (Cullmann *et al.*, 1995). ATP binding to RFC ultimately facilitates ring opening of PCNA, resulting in PCNA loading onto primers (Chen, S. *et al.*, 2009). The inner diameter of the PCNA ring is large enough to encircle double-stranded (ds) DNA. The DNA binding of RFC-PCNA promotes ATP hydrolysis of RFC, which induces closure of PCNA ring and subsequent dissociation of RFC from template DNA. Pol δ is composed of three subunits in *Saccharomyces cerevisiae* (four in other eukaryotes) (Garg and Burgers, 2005; Burgers, 2009). The largest Pol3 subunit contains both polymerase and 3′ to 5′ exonuclease (proofreading) activities, and the other two (in *S. cerevisiae*) subunits are required for PCNA binding and stable interactions between subunits. PCNA loaded onto primers binds pol δ, increasing the processivity of pol δ that allows more efficient DNA synthesis. The RNA moiety in a RNA–DNA primer is present at the 5′ end of every nascent Okazaki fragment and should be removed prior to the joining of Okazaki fragments to form a linear duplex DNA. This process is called "Okazaki fragment processing."

Some initial problems with Okazaki fragment processing in eukaryotes

At least the RNA portions of RNA–DNA primers of Okazaki fragments are processed prior to their joining to produce complete duplex DNA in lagging strand through a series of complex enzymatic reactions that require a number of enzymes (Bambara *et al.*, 1997; Waga and Stillman, 1998). However, none of the eukaryotic polymerases possess intrinsic 5′ to 3′ exonuclease activity for Okazaki fragment processing, unlike the well-characterized prokaryotic polymerase, *Escherichia coli* DNA pol I (Kornberg and Baker, 1992). In the previous model for Okazaki fragment maturation in eukaryotes, Fen1 provides the 5′ to 3′ exonuclease activity, and with the assistance of RNase HI removes the RNA segments on Okazaki fragments (Ishimi *et al.*, 1988; Goulian *et al.*, 1990; Waga and Stillman, 1994). RNase HI hydrolyzes the initiator RNA of the primer DNA leaving a single ribonucleotide at the RNA–DNA junction, which is subsequently removed by the 5′ to 3′ exonuclease activity of Fen1. This view is reminiscent of a prokaryotic model for Okazaki fragment processing (Waga and Stillman, 1994).

Two findings with regard to mammalian Fen1 and RNase HI argued against this view: (i) Fen1 is a structure-specific endonuclease that cleaves the 5′-unannealed single-stranded (ss) DNA or RNA at the duplex junction (Harrington and Lieber, 1994; Murante *et al.*, 1994; Bambara *et al.*, 1997; Lieber, 1997); and (ii) in addition, mammalian RNase HI can cleave 5′ of the last ribonucleotide of ssRNA–DNA hybrid molecules (Murante *et al.*,. 1998). These findings suggested that Okazaki fragment maturation could occur through a more complex process than inferred previously. For example, Okazaki fragment processing proceeds via formation of a 5′-ss "flap" prior to the action of Fen1 and/or RNase HI, a process that is poorly understood previously because an enzyme(s)

responsible for the formation of this "flap" structure was not clearly identified. The candidate enzyme could be a DNA helicase translocating ahead of DNA pol δ. Alternatively, pol δ itself could contribute to formation of flap structure by catalyzing displacement DNA synthesis. However, the ability of pol δ to catalyze displacement DNA synthesis was controversial.

Although the role of Fen1 and RNase HI in Okazaki fragment maturation has been well established *in vitro* in SV40 DNA replication, deletion of *RAD27* encoding yeast Fen1 or *RNH201* (formerly called *RNH35*) ending the catalytic subunit of RNase HII (a yeast homolog of human RNase HI) revealed that *RAD27* and *RNH35* are dispensable (Frank *et al.*, 1998b; Qiu *et al.*, 1999) in *S. cerevisiae* unlike other genes whose products are essential for DNA replication. Yeast strains carrying a deletion of *RAD27* are viable at 30°C, but displayed severe growth defects at 37°C, producing cells with a terminally arrested phenotype, an indication of a defect in DNA replication (Sommers *et al.*, 1995; Reagan *et al.*, 1995). Human RNase HI was known to have two subunits (Frank *et al.*, 1998a), and the deletion of the catalytic subunit did not affect cell viability in yeast (Frank *et al.*, 1998b). Therefore, it is puzzling that the *RAD27*- and/or *RNH35*-deleted yeast mutant strains are viable if Fen1/RNase HII (RNase HI in humans) is assumed to be the only enzymes that generate ligatable nicks in processing of Okazaki fragments. This is especially true considering that DNA ligase I, which acts upon nicks produced by combined action of Fen1/RNase HII, is essential in yeast (Nasmyth, 1977). Thus, the viability of yeast cells lacking Fen1 argues strongly for the existence of a more critical (or a redundant) pathway for the *in vivo* processing of Okazaki fragments.

Discovery of Dna2

Dna2 is a well-conserved essential endonuclease/helicase, and its biochemical activities are well-suited to process Okazaki fragments. The *DNA2* gene was originally identified in a systematic screening of genes involved in DNA replication using permeabilized yeast nuclei. This screening method, first developed by Hereford and Hartwell (1971), was based on the inability of permeabilized nuclei of replication-defective mutants to incorporate radio-labeled nucleotides into DNA. This method led to identification of *dna154* from several temperature-sensitive (ts) DNA replication mutants of *S. cerevisiae* (Kuo *et al.*, 1983). The *dna154* allele was later renamed *dna2-1* (Budd and Campbell, 1995). The S-phase nuclear extracts of ts *dna2-1* cells supported semi-conservative replication of plasmid DNA only at permissive temperature, suggesting a function of Dna2 in S-phase DNA replication (Braguglia *et al.*, 1998). By complementation analysis, the *DNA2* gene was cloned and found to encode an essential 172 kDa protein with conserved helicase motifs in its C-terminal region. Originally, Dna2 was reported as a potential replicative DNA helicase with a 3′ to 5′ directionality (Budd and Campbell 1995). Later, it was confirmed that Dna2 translocated in the 5′ to 3′ direction (Bae and Seo, 2000). It was observed that radiolabeled DNA substrates used for helicase assays were partly degraded with Dna2 preparations. The degradation of the helicase substrates was at first attributed to the presence of a nuclease(s) associated tightly with Dna2 (Budd and Campbell, 1995). This observation led to the finding that Dna2 interacted physically with Rad27 *in vivo*. Moreover, overexpression of *DNA2* suppressed temperature-sensitive growth defects of *rad27Δ*. Conversely, multicopy expression of *RAD27* also suppressed *dna2-1* mutant (Budd and Campbell, 1997). Subsequently, it was shown that Dna2 possessed potent intrinsic endonuclease activity that is highly specific to ssDNA (Bae *et al.*, 1998). This was confirmed by mutational analyses of amino acid residues critical for endonuclease activity (Budd *et al.*, 2000; Lee, K.H. *et al.*, 2000). It should be noted that the helicase activity of Dna2 is dispensable for certain growth conditions (Bae *et al.*, 2002), whereas the endonuclease activity of Dna2 is not, indicating that Dna2 endonuclease plays a more fundamental function *in vivo* than its helicase activity.

In parallel with the discovery of *DNA2* gene in budding yeast, its homolog in fission yeast was also discovered. Ten new ts cell-division-cycle (*cdc*) mutants were isolated from *Schizosaccharomyces pombe* (Nasmyth and Nurse, 1981). One of the replication defective mutants, *cdc24*, could synthesize bulk DNA at 36°C, but the DNA synthesized were not complete. This mutant arrested at non-permissive temperature in late S or G2 phase and suffered from chromosome breakage. The *cdc24*$^+$ gene was cloned during the study of *cdc24-G1*, an independently isolated *cdc24* allele (Gould *et al.*, 1998). A genetic suppressor of *cdc24-G1* (and *cdc24-M38*) mutants was isolated, which was identified as a homolog (*dna2*$^+$) of budding yeast *DNA2*. Genetic experiments with *dna2-C2* ts mutant revealed that *dna2*$^+$ was not required for bulk DNA synthesis and arrested at late S-phase at restrictive temperature (Kang *et al.*, 2000). In addition, the chromosomes of mutant cells at restrictive temperature underwent extensive breakage, similarly to *cdc17* (DNA ligase I) mutant, raising the possibility that nicks might not be sealed in *dna2* mutation. Furthermore, *dna2*$^+$ interacted genetically or physically with a number of replication factors required for lagging strand elongation and maturation (*cdc24*$^+$, *rad2*$^+$, *cdc1*$^+$, *cdc27*$^+$, and *cdc17*$^+$). Similarly, *S. cerevisiae dna2* ts mutants (*dna2-1* and *dna2-22*) could synthesize bulk DNA at non-permissive temperature based on FACS analyses. Besides, newly synthesized DNAs in *dna2-1* mutant cells at non-permissive temperature were low molecular weight fragments (Budd and Campbell, 1995; Fiorentino and Crabtree, 1997).

DNA2 was also identified in the screening performed for isolating synthetic lethal genes with *CTF4* which interacts with pol α, in keeping with *DNA2* involvement in lagging strand DNA replication (Formosa and Nittis, 1999). Taken together, the genetic observations from budding and fission yeasts and biochemical activities of *S. cerevisiae* Dna2 strongly suggested that *DNA2* plays an essential and direct function in processing of Okazaki fragment during lagging strand DNA synthesis. The interaction of Dna2 with Fen1 further supports this notion because it was demonstrated that human Fen1 plays a role in removing RNA primers for joining adjacent Okazaki fragments (Ishimi *et al.*, 1988; Goulian *et al.*, 1990). Many excellent reviews are available of the roles of Fen1 in DNA replication as well as in recombination and repair (Henneke *et al.*, 2003; Lieber 1997; Liu *et al.*, 2004a; Shen *et al.*, 2005).

In higher eukaryotes, a human homolog of yeast *DNA2* was first identified from a sequence database. Subsequently *Caenorhabditis elegans*, *Xenopus laevis*, and *Arabidopsis thaliana DNA2* were found (Eki *et al.*, 1996; Liu *et al.*, 2000). In *Xenopus* egg extracts, bulk DNA replication was inhibited by depletion of Xenopus Dna2 (XDna2) (Eki *et al.*, 1996). However, the addition of recombinant XDna2 did not restore chromosomal DNA replication, raising the possibility that other replication factors were depleted along with XDna2. Alternatively, recombinant XDna2 from insect cells was not active to support DNA replication in *Xenopus* egg extracts. The phenotype of homozygous *DNA2* deletion mutant was examined in *C. elegans* (Lee, K.H. *et al.*, 2003). This mutant showed reduced brood size, and the embryonic lethality observed depended on growth temperature. The helicase and endonuclease activities of *C. elegans* Dna2 were also characterized biochemically (Kim, D.H. *et al.*, 2005).

Dna2, a RecB-like endonuclease, is modulated by ATP

Initial characterization of *S. cerevisiae* recombinant Dna2 purified from insect cells revealed that it possessed multiple intrinsic biochemical activities. First, it possesses ssDNA-specific endonuclease activity. Dna2 did not cleave dsDNA, ssRNA, or dsRNA. The endonuclease activity of Dna2 requires ssDNA ends for efficient cleavage. With partial duplex DNA, it cleaved both 5′ and 3′ ssDNA overhang, but not dsDNA region in the absence of ATP (Bae *et al.*, 1998). The ssDNA flanked by duplex DNA was resistant to Dna2 cleavage. Moreover, RPA strongly protected the internal ssDNA region from cleavage by Dna2 (Bae and Seo, 2000). Although Dna2 cleaved ssDNA near the duplex DNA region, it could not cleave at the junction between duplex and ssDNA, and thus left short (~6 nt) ssDNA even with very high levels of Dna2 (Bae *et al.*, 1998; 2001a). Thus, Dna2 is not able to create nicks that can be sealed by DNA ligase I. Second, it has ssDNA-dependent ATPase (Bae *et al.*, 1998; Budd *et al.*, 1995). Like its endonuclease specificity, only ssDNA supports the hydrolysis of ATP or dATP (Bae *et al.*, 1998; 2002). Third, it contains weak helicase activity that translocates in the 5′ to 3′ direction. For the helicase reaction, only ATP and dATP support DNA unwinding activity of Dna2. This is in accordance with the ability of Dna2 to hydrolyze only ATP and dATP. The unwinding activity of wild-type Dna2 was observed when its endonuclease activity was suppressed by decreasing Mg^{2+} concentrations (Bae *et al.*, 1998). Purified Dna2 enzymes from *X. laevis* and *S. pombe* lacked detectable helicase activity *in vitro* despite the presence of well conserved helicase motifs, in contrast to those from other organisms such as *S. cerevisiae, C. elegans,* and *Pyrococcus horikoshii* (Budd *et al.*, 1995; Bae and Seo, 2000; Liu *et al.*, 2000; Higashibata *et al.*, 2003; Kim, D.H. *et al.*, 2005). The helicase activity of human Dna2 is controversial (Kim, J.H. *et al.*, 2006; Masuda-Sasa *et al.*, 2006a). It appears that human Dna2 helicase activity was so low that it was detectable only with very high levels of enzyme (Masuda-Sasa *et al.*, 2006a). Another possibility is that there may be an additional protein to activate low helicase activity of human Dna2 *in vivo*. Alternatively, it may act upon specific types of substrate. Fourth, ATP hydrolysis enables Dna2 to degrade duplexed DNA from 3′ ends. When nuclease assays were performed with ΦX174 single-stranded circular (ssc) DNA annealed with an oligonucleotide containing a 5′non-complementary tail, both yeast and *Xenopus* Dna2 cleaved 5′ ssDNA tail in the absence of ATP (Bae *et al.*, 1998; Liu *et al.*, 2000). In the presence of ATP (or dATP), however, Dna2 is able to degrade duplexed DNA from the 3′ ends. Dna2 unwinds partially the 3′ end of oligonucleotide annealed to ΦX174 sscDNA due to its 5′ to 3′ helicase activity, generating a short stretch of 3′ ssDNA tail, which is susceptible to Dna2 engaged in unwinding or free Dna2 in the solution. This was also observed with a mixture of helicase-negative and endonuclease-negative mutant Dna2 enzymes, indicating that unwinding and cleavage is not necessarily coupled. This observation suggested that ATP-dependent translocation of Dna2 along ssDNA could influence the cleavage pattern of 5′ ssDNA tail as well. In keeping with this, with simple Y-fork substrates, Dna2 could cleave DNA within duplex region past the ss/dsDNA junction (Bae and Seo, 2000). In addition, ATPase/helicase-deficient Dna2K1080E protein was not affected at all by the presence of ATP. These results raise the possibility that the way that endonuclease activity of Dna2 is harnessed *in vivo* can be modulated by its own helicase activity that depends on ATP hydrolysis.

Dna2 enzymes from various organisms share endonuclease activity in common with varying levels of ATP/helicase activity. For example, *Xenopus* Dna2 lacked detectable ATPase/helicase activity, while *C. elegans*

Dna2 contained both activities comparable to yeast Dna2. This is consistent with the notion that the endonuclease activity of Dna2 is essentially required for its physiological function. Amino acid residues critical for essential endonuclease activity of Dna2 were identified by mutational analyses (Budd *et al.*, 2000; Lee, K.H. *et al.*, 2000). In one study (Budd *et al.*, 2000), amino acid residues that are likely responsible to bind Mg^{2+} were mutated based on the sequences conserved among *E. coli* RecB nuclease family members (Aravind *et al.*, 1999). Overexpression of these mutated *dna2* genes inhibited the growth of *dna2-1* cells at permissive temperature. Subsequent analyses of the purified mutant proteins confirmed that they possessed significantly reduced endonuclease activity *in vitro*. Thus, endonuclease-negative mutant Dna2 acted in a dominant-negative fashion, suggesting that Dna2 acts jointly *in vivo* with other proteins and this joint action could be critical for its cellular function. In the other study, eight charged amino acids, which are highly conserved in the central domain of Dna2 homologs, were mutated and examined the ability of the mutant genes to complement *dna2* null mutant. Recombinant proteins from the mutant genes that failed to complement even when their expression was driven by a GAL4 promoter did not display any detectable endonuclease activity. In contrast, those mutated genes that complemented *dna2Δ* with increased levels of their expression showed partial endonuclease activity. These results established a correlation between the extent of complementation *in vivo* and the level of endonuclease activity *in vitro*. Weak nuclease mutant complemented *dna2Δ* only when it was overexpressed, whereas overexpression of nuclease-negative mutant did not (Lee, K.H. *et al.*, 2000). This result demonstrated that the intrinsic endonuclease activity of Dna2 carries out an essential function *in vivo*. The AddAB complex, a bacterial helicase-nuclease complex contains 4 Cys residues near the conserved RecB domain which is required to bind Fe^{2+} (Yeeles *et al.*, 2009). This Fe-S cluster is critical for AddAB in binding to and processing dsDNA ends. Eukaryotic Dna2 also contains a Fe-S cluster, and thus by analogy it could be involved in processing of double-strand breaks (see below).

Tripartite structure of yeast Dna2

All eukaryotic Dna2 proteins share two catalytic domains for helicase and endonuclease and their sizes range from 120 kDa (1077 amino acids, *X. laevis*) to 172 kDa (1522 amino acids, *S. cerevisiae*). The size difference among Dna2 proteins from various organisms can be attributed to varying lengths of non-conserved N-terminal domains. The 300–400 amino acid N-terminal regions present in *S. cerevisiae* and *S. pombe* are not found in metazoan homologs. In *S. cerevisiae* Dna2, the endonuclease activity is encoded in the central domain of the protein, and the helicase domain in its C-terminal domain. Proteolytic digestion of yeast Dna2 with subtilisin (nonspecific protease) rapidly degraded the N-terminal domain, liberating a ~120-kDa fragment that contained catalytic domains for ATPase and endonuclease activities (Bae *et al.*, 2001b). Consistent with this, a recombinant Dna2 derivative lacking the N-terminal 405 amino acids (Dna2Δ405N) also retained both activities. It was observed initially that the subtilisin-treated Dna2 or the recombinant Dna2Δ405N enzyme displayed elevated levels of ATPase and endonuclease activities compared to the wild-type enzyme. This result confirmed that the N-terminal region of Dna2 is dispensable for catalytic activities, but raised an interesting possibility that it has a regulatory role for the catalytic activities. In support of this, intramolecular interactions were detected between the N-terminal 405 amino acid domain and the 693–853 amino acid region located between the ATPase and endonuclease domains. These findings led to a hypothesis that there might be a protein factor(s) *in vivo* that regulates these interactions, thereby regulating the catalytic activities of Dna2. Despite intact catalytic activities of Dna2Δ405N, yeast cells expressing Dna2Δ405N displayed temperature-sensitive growth defects, suggesting that the N-terminal 405 amino acid domain is important for a cellular function. Subsequent analyses revealed that the N-terminal region of Dna2 is the motif required for interaction with RPA (see below). Hydrodynamic analyses of Dna2 revealed that the wild-type Dna2 protein is monomeric with a prolate shape, while Dna2Δ405N is spherical (Bae *et al.*, 2001b).

The elevated level of catalytic activities of Dna2Δ405N was observed only when ΦX174 (or M13 ssDNA)-based partial duplex DNA (as substrates for endonuclease assays or cofactor DNA for ATPase assays) were used, but not with substrates prepared with oligonucleotides (unpublished results, Y.-S. Seo). When oligonucleotide-based DNA substrates were used, levels of both ATPase and endonuclease activities of Dna2 and Dna2Δ405N were indistinguishable. Dna2Δ405N appeared to have increased stability during incubation compared to wild-type Dna2 (unpublished results, C.-H. Lee and Y.-S. Seo). Thus, the way by which Dna2 interacts with substrate or cofactor DNA is likely to be affected by secondary structures present in the bulk ΦX174 or M13 sscDNA. Later, it turned out that the N-terminal domain has intrinsic DNA-binding activity which is specific for hairpin structure (see below for its potential function).

S. cerevisiae Dna2 is a nuclear protein with three classic NLS (nuclear localization signal) motifs based on sequence analysis. However, none of them were found in vertebrate homologs including chicken, mouse, rat, cattle, and human (Choe *et al.*, 2002; Zheng *et al.*, 2008). In *S. cerevisiae* Dna2, two overlapping bipartite NLS (nuclear localization signal) sequences are located within the first 48 amino acids in the N-terminus (Kosugi *et al.*, 2009), while the third bipartite NLS is found in

the C-terminus. It appears that the third NLS alone is sufficient for nuclear translocation since cells with the *dna2Δ405N* allele devoid of the first two NLS grow as well as wild type at 25°C, although they do not at 37°C (Bae *et al.*, 2001a; 2001b; unpublished observation).

Human Dna2 does not have the N-terminal domain that is present in yeasts, and lacks the C-terminal portion corresponding to the third bipartite NLS of yeast Dna2. Thus, it appears that human Dna2 lacks all three NLS, suggesting that it may not have a nuclear function unlike yeast Dna2. This is supported by the finding that human Dna2 is exclusively present in mitochondria rather than nucleus (Zheng *et al.*, 2008). However, it was shown that Dna2 is still important for a nuclear function because knockdown of human Dna2 resulted in the formation of internuclei chromatin bridge (Duxin *et al.*, 2009), which is indicative of defective resolution of replicated DNA. Recently, it was shown that yeast Dna2 is localized in cytoplasm in G1 and imported to nucleus in S-M phases, which is in contrast to the previous observation that Dna2 was localized in nucleus even in G1 (Choe *et al.*, 2002). The translocation appears to be a regulated process since the translocation of Dna2 between cytoplasm and nucleus was abrogated by a mutation in predicted CDK1 phosphorylation site of Dna2 (Kosugi *et al.*, 2009).

Dna2 is a flap-processing enzyme that requires a concerted action of helicase and endonuclease activities

The mechanistic model of how Dna2 participates in Okazaki fragment maturation was first proposed, based on the *in vitro* results obtained with flap-structured substrates that mimic the *in vivo* intermediate DNA structure generated during Okazaki fragment maturation (Bae and Seo, 2000). *In vivo* evidence accumulated from budding and fission yeasts supports the notion that Dna2 is involved in some stage of Okazaki fragment processing. In addition, the fact that Dna2 could remove the chimeric RNA–DNA flap more efficiently than a DNA-only flap is in keeping with its physiological role as a flap-processing nuclease that is well suited to remove the initiator RNA (Bae and Seo, 2000). However, the *in vitro* finding that Dna2 degraded flap rapidly, but not completely, suggests that Dna2 should jointly act to produce ligatable nicks with an additional nuclease such as Fen1 (Bae *et al.*, 2001a). An *in vitro* reconstitution of lagging strand DNA synthesis performed with RPA, RFC, PCNA, pol δ, and Dna2 on double-primed ΦX174 sscDNA demonstrated that flaps can be generated by displacement DNA synthesis by pol δ, and that the flaps generated this way can be directly and efficiently cleaved by Dna2 endonuclease activity (Bae and Seo, 2000).

Dna2 required a free 5′ end for the cleavage of flap DNA, since it was not able to cleave the flap substrates that contains bulk obstacle at the 5′ end such as biotin-streptavidin. Blocking of 5′ ends of ssDNA also inhibited translocation of Dna2 along ssDNA in the presence of ATP (Bae and Seo, 2000). Duplex DNA in the 5′ end of flaps also inhibited Dna2-catalyzed cleavage of flap (Kao *et al.*, 2004a). This indicates that Dna2 binds the 5′ end of ssDNA and slides along the single-stranded region to the point of cleavage, which is similar to a "tracking mechanism" proposed for Fen1 action (Murante *et al.*, 1995). Unlike Fen1, however, Dna2 appears to use threading-like mechanism in which a hole or cleft in Dna2 allows passage of single-stranded region but not larger structure. This idea is supported by the observation that tracking of Dna2 was blocked by a branched structure in the 5′-flap region, which was not observed with Fen1 (Kao *et al.*, 2004a).

The requirement of Dna2 helicase activity for yeast viability supports the notion that DNA unwinding activity is normally necessary for its physiological function *in vivo*. The unwinding and endonuclease activities of Dna2 were greatly affected by the ratio of ATP to Mg^{2+} (Bae and Seo, 2000; Bae *et al.*, 2002; Higashibata *et al.*, 2003; Kim, D.H. *et al.*, 2005). When $[ATP]/[Mg^{2+}]$ is high, the endonuclease activity of wild-type Dna2 was suppressed, while its helicase activity was enhanced. Both endonuclease and helicase activities of Dna2 acted in a distributive manner (Bae *et al.*, 2002). The presence of two different catalytic activities in one polypeptide of Dna2 implies that both activities act in a collaborative manner. The addition of ATP, which activates helicase activity, altered the cleavage pattern of flap DNA by Dna2. The average size of flaps cleaved in the absence of ATP became shorter than that observed in the presence of ATP (Bae *et al.*, 2002). Furthermore, the addition of ATP allowed Dna2 to cleave secondary-structured flap via its combined action of helicase and nuclease activities. Mixing of Dna2K1080E helicase-negative and Dna2D657A nuclease-negative mutant failed to do so, indicating that these two essential activities should be coupled for removing secondary-structured flap. In accordance with this, simultaneous expression of both mutant proteins in *dna2Δ* cell did not allow cells to grow, emphasizing the importance of the concerted action of two activities of Dna2. Recently, it was reported that Dna2 is capable of unwinding G-quadruplex DNA structures, another critical role of Dna2 in resolving the structural intermediates arising during DNA metabolisms (Masuda-Sasa *et al.*, 2008).

Okazaki fragment processing in eukaryotes

Important features of the current model

The fact that Dna2 is essential *in vivo* (Budd *et al.*, 1995; Lee *et al.*, 2000), whereas Fen1 is dispensable

under a certain growth condition (Reagan *et al.*, 1995) predicts that processing of long flaps depends on a non-redundant essential pathway, while the removal of short flaps (thus formation of ligatable nicks) occurs through multiple redundant pathways. The current model for Okazaki fragment processing in eukaryotes is based primarily upon the one first proposed by Bae *et al.* (2001a) and others thereafter (Figure 1). The unique feature of this model is that the 5′-end region (containing the primer RNA) of Okazaki fragments is converted into a flap and then processed by several nucleases, which allows a number of different strategies to remove primer RNA and generate ligatable nicks. This model comprises several fundamental steps: (i) synthesis of primer RNA–DNA by pol α-primase; (ii) elongation of the primer RNA–DNA – after polymerase switching (pol α to pol δ with help of RFC and PCNA), the primer RNA–DNA is further extended by pol δ to generate full-length Okazaki fragments; (iii) generation of flaps by displacement DNA synthesis by pol δ – the size of flaps determines how they are processed, with short flaps being directly processed by Fen1, and long flaps processed by a sequential action of Dna2 and Fen1 which is governed by RPA; and (iv) sealing of nicks – continuous dsDNA in the lagging strand is produced from short pieces of Okazaki fragments by sealing the nicks.

Involvement of Dna2 in the cleavage of long flaps

Involvement of Dna2 in the cleavage of long flaps was supported by many elaborate genetic experiments. First, *dna2-1* was lethal in combination with a mutation in pol δ (*pol3-01*) which increased strand displacement synthesis of pol δ. Meanwhile, deletions of Pol32 subunit, which limits strand displacement activity of pol δ *in vitro*, suppressed growth defects and methyl-methanesulfonate sensitivity of *dna2-1* and *dna2-2* (Burgers and Gerik, 1998; Garg *et al.*, 2004; Johansson *et al.*, 2004; Budd *et al.*, 2005). Similar results were also obtained in *S. pombe*.

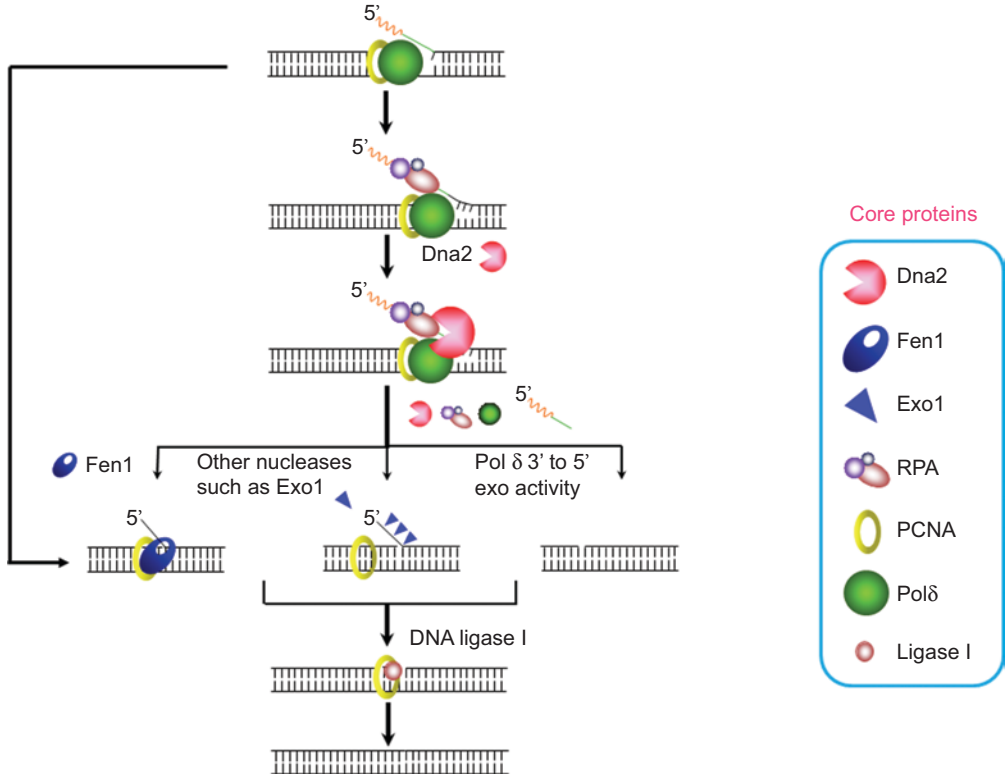

Figure 1. A role of Dna2 in processing of Okazaki fragments in eukaryotes. (1) The RNA containing the 5′ terminus of an Okazaki fragment is rendered single-stranded by displacement DNA synthesis catalyzed by pol δ. (2) RPA rapidly forms an initial complex with the nascent flap structure and (3) then recruits Dna2 to form a ternary complex. This leads to the initial cleavage of RNA-containing segments by Dna2. (4) The remaining short flap DNA can be processed further either by Fen1, which is loaded onto the DNA through protein–protein interactions with PCNA (Fen1-dependent) or alternatively by other nucleases, possibly Exo1 or Dna2 itself (Fen1-independent). (5) Finally, the resulting nick is sealed by DNA ligase I. For simplicity, the "idling and nick translation" mechanism was not shown (refer to the text for details). Nicks generated by this mechanism are directly channeled into step (5).

The C-terminal region of Cdc27, the subunit of *S. pombe* pol δ, is essential for its *in vivo* function, and a mutant *cdc27-R22* allele (a frameshift mutation of Cdc27 lacking the PCNA binding motif) markedly reduced processivity of pol δ *in vitro* (Reynolds *et al.*, 2000; Zuo *et al.*, 2000). The ts growth defects of *dna2-C2* were suppressed when it was combined with either *cdc27-R22* or *pol3-R18* (Tanaka *et al.*, 2004). All of these results suggest that Dna2 becomes critical in a situation under which long flaps are generated. Results of elegant genetic studies using *rad27* mutants further support this notion (Jin *et al.*, 2003). When *rad27-p* (impaired interactions with PCNA) was combined with *pol3-5DV* (a mutant allele of a pol δ subunit, defective in 3′ to 5′ exonuclease and increased in displacement DNA synthesis), the double mutant cells were lethal in the absence of *RAD51* that is essential for double-strand break (DSB) repair. This supports the notion that DSBs occur in the absence of Dna2 and can be repaired by *RAD51*-dependent DSB repair pathway (see below). The lethal phenotype of *rad27-p pol3-5DV rad51Δ* was suppressed by overexpression of Dna2, suggesting that increased levels of long flaps resulting from mutant pol δ were processed by Dna2 (Jin *et al.*, 2003). The suppression of *dna2* mutant cells by RPA also supports the notion that Dna2 is involved in processing of ssDNA long enough to bind RPA (Bae *et al.*, 2001a). Consistent with this, RPA-bound flaps were more readily cleaved than naked flaps (Bae *et al.*, 2001b), which was further confirmed by others (Ayyagari *et al.*, 2003; Jin *et al.*, 2003; Kao *et al.*, 2004b). Because the binding of RPA to long flaps does not allow Fen1 to access the substrate, creation of ligatable nicks from long flaps is blocked in the absence of Dna2 and thus cells are not viable. In the absence of Dna2, the large number of RPA-bound flaps could induce chronic cell cycle arrest, leading to apoptotic cell death. *In vivo*, the cooperation between Dna2 and Fen1 can be more demanding to generate ligatable nicks from long-flap substrates due to abundance of RPA. Thus, the primary role of Dna2 during Okazaki fragment processing in eukaryotes appears to maintain flap length as short as possible during replication. Long flaps, once formed, could impose formidable burdens to cells most likely due to their tendency to bind proteins nonspecifically or to form hairpin or higher-ordered structure that are difficult to be processed.

RPA as a critical nuclease switch

The findings that RPA also plays a role in Okazaki fragment processing *in vivo* are supported by several independent observations. First, a mutation in *DNA2* was identified during a synthetic lethal screen with *rfa1Y29H*, a ts mutant allele of *RFA1* (S. Brill, personal communication), indicating a functional interaction between Dna2 and RPA. Furthermore, Dna2 and Rpa1 (a large subunit of RPA encoded by *RFA1*) physically interacted each other both *in vivo* and *in vitro* (Bae, K.H. *et al.*, 2003). Second, the 32 kDa subunit of RPA was crosslinked to RNA–DNA primers in the lagging strand of replicating SV40 chromosomes (Mass *et al.*, 1998). This crosslinking was observed only with early RNA–DNA primer intermediates and was not detected with mature lagging strand products. The genetic interaction between RPA and Dna2 was discovered from screening of suppressors that rescued ts growth defects of *dna2Δ405N* mutant when expressed in a multicopy plasmid (Bae *et al.*, 2001a). *RFA2*, the middle subunit of RPA, was found as one of the multicopy suppressors and subsequent studies showed that other two subunits, *RFA1* and *RFA3*, also acted as suppressors.

RPA is able to bind efficiently ssDNA > 20-nt and to physically interact with Dna2 as mentioned above. Consistent with this, RPA affected the way in which endonuclease activities of both Dna2 and Fen1 act upon ssDNA flap *in vitro*. Dna2-catalyzed cleavage of 5′ flap DNA was dramatically stimulated by RPA at physiological salt concentration. In contrast, RPA markedly inhibited Fen1-catalyzed cleavage of 5′ flap. This inhibition was readily relieved by the addition of Dna2 (Bae *et al.*, 2001a). The 5′ flaps longer than the site size of RPA first bind RPA, forming rapidly a ternary complex with Dna2. Dna2 within the complex then cleaves the RPA-bound flap, leaving shortened flaps (mostly ~6-nt). This releases RPA from the flap, and thus the remaining flaps are no longer resistant to and can be completely removed by Fen1 to produce ligatable nicks. Therefore, RPA acts as a molecular switch between Dna2 and Fen1, which governs the sequential action, Dna2 followed by Fen1, of the two endonucleases (Bae *et al.*, 2001a). Two different mechanistic models have been suggested for recycling of RPA from long flaps. The first model requires the catalytic activity of Dna2; the cleavage of RPA-bound flap by Dna2 releases RPA, Dna2, and flap DNA (Bae *et al.*, 2001a). The ternary complex formation of Dna2–RPA–DNA was readily disassembled by the addition of Mg^{2+} that activates Dna2 endonuclease activity, while Dna2D657A (endonuclease-deficient, but capable of forming a complex) failed to do so. The other model invokes the fact that Dna2 itself acts as a factor that causes dissociation of RPA from the substrate regardless of its nuclease activity based on the results obtained with endonuclease-deficient Dna2E675A (Stewart *et al.*, 2008). The dissociation of RPA was not dependent on the helicase activity or tracking of Dna2 along the flap that is believed to dismantle RPA from flaps (Stewart *et al.*, 2008). It was also proposed that Dna2 is not passively dissociated from flaps after flap cleavage; Fen1 is still required to actively disengage Dna2 (Stewart

et al., 2006; Stewart *et al.*, 2009). We believe that action of Dna2 endonuclease is primarily responsible for removing all bound proteins, RPA and Dna2, from the ternary complex (Bae *et al.*, 2001a).

RPA can help Dna2 indirectly through its dsDNA-destabilizing activity. RPA can melt dsDNA in a Mg^{2+} or ATP-independent manner, in contrast to *bona fide* helicase activity (Georgaki and Hübscher, 1992; Georgaki *et al.*, 1993; Treuner *et al.*, 1996). In experiments performed with secondary-structured flap, RPA alone allowed Dna2 or Dna2K1080E (helicase-negative mutant) to cleave a flap with a short hairpin (10-bp) in the middle. This result demonstrated that double-stranded regions, if short, were first melted by RPA, and then became susceptible to Dna2 cleavage (Bae *et al.*, 2002). In support of this, multicopy expression of RPA suppressed the lethal phenotype of *dna2K1080E* mutant strain. Thus, the dsDNA-destabilizing activity of RPA could be at least partially substituted for the helicase activity of Dna2. The presence of RPA allowed Dna2 (or Dna2K1080E) to cleave the flap past the junction of ssDNA and dsDNA in the absence of ATP, most likely due to the ability of RPA to melt the duplex region of the flap-containing strand (Stewart *et al.*, 2008). The helicase activity of Dna2 was also stimulated by RPA (Bae *et al.*, 2002). The stimulation of endonuclease and helicase activities of Dna2 by RPA was species-specific since *E. coli* SSB failed to do so. Consistent with this, both helicase and endonuclease activities of *C. elegans* Dna2 were stimulated only by *C. elegans* RPA, neither by human nor yeast RPA (Bae *et al.*, 2002; Kim, D.H. *et al.*, 2005).

Multiple ways to remove primer RNAs

The primer RNAs can be removed in the following ways. (i) Primer RNAs remain duplexed with template before pol δ arrives. However, they can be degraded by the endonucleolytic activity of RNase HI (RNase HII in yeast), as demonstrated with *in vitro* experiments with mammalian proteins (Huang *et al.*, 1994; Turchi *et al.*, 1994). The terminal ribonucleotide can be removed by 5′ to 3′ exonuclease activity of Fen1, completing removal of the RNA-initiated primer. (ii) If primer RNA is converted into RNA-containing flaps, primer RNA can be efficiently removed by Dna2 along with some part of primer DNA (Bae and Seo, 2000). (iii) Mammalian RNase HI is capable of cleaving RNA from the chimeric RNA–DNA present in the 5′ flap, and thus named junction RNase activity (Murante *et al.*, 1998). (iv) Primer RNAs can be removed, gradually as they are displaced by pol δ, by Fen1 via a "nick translation" mechanism (Garg *et al.*, 2004). The diverse ways to remove primer RNAs are in keeping with the fact that the *RNH35* gene encoding the catalytic subunit of RNase HII complex in *S. cerevisiae* is not essential for viability (Frank *et al.*, 1998b).

Multiple ways to create ligatable nicks

Once Dna2 removes major part of long flaps, nicks can be created by a variety of ways from the remaining short flaps. The short flap DNA can be processed further by Fen1 (Fen1-dependent) to create nicks or by other nucleases such as Exo1 or 3′ to 5′ exonuclease of pol δ (Fen1-independent) to create nicks. Exo1, an exonuclease, is thought to be a backup protein with an overlapping function with Fen1. In the absence of Fen1, *EXO1* becomes essential (Tishkoff *et al.*, 1997). Consistent with the notion that Exo1 is involved in processing of Okazaki fragments, overexpression of Exo1 suppressed growth defect of *dna2-1* strain (Budd *et al.*, 2005). In keeping with the idea of multiple ways of nick creation, mouse nuclear extracts containing mutant Fen1 enzymes that did not interact with PCNA displayed a reduced rate (~50% compared to wild type) of Okazaki fragment maturation, but still higher (~20–30%) than Fen1-depleted extracts (Zheng *et al.*, 2007). This result also indicates that both Fen1 and other nuclease(s) contributed to processing of flaps. A single pathway to process long flaps but multiple pathways to create nicks can account for why Dna2 is essential, but Fen1 is dispensable at least in yeast. However, it should be noted that simultaneous inactivation of any two ways of nick creation (e.g., *rad27Δ exo1Δ* or *rad27Δ pol3-5DV rad51Δ*) results in lethality. Thus, creation of nicks is just as essential as ligation of nicks. It appears that the extent of Fen1 dependence for nick creation varies among eukaryotic organisms; for example, lower eukaryotes such as fission and budding yeasts are less dependent on Fen1 for nick creation, and are thus viable in its absence, whereas Fen1 is indispensable in multicellular eukaryotes such as *C. elegans* and mice, indicating that a great fraction of nicks are generated by Fen1 in higher eukaryotes (Liu *et al.*, 2004a; Parrish *et al.*, 2003).

Pif1 and production of long flaps

Eukaryotic pol δ was shown to have displacement DNA synthesis activity (Klungland and Lindahl, 1997; Mossi *et al.*, 1998; Podust *et al.*, 1995). For example, calf pol δ was shown to displace duplex DNA up to 274 bp (Mossi *et al.*, 1998). Thus, extents of displacement DNA synthesis could determine the length of flaps. Recently, Pif1 was shown to affect the length of flaps.

Pif1 is a 5′ to 3′ helicase involved in telomere homeostasis and mitochondrial DNA repair and recombination (Lahaye *et al.*, 1991; 1993; Schulz and Zakian, 1994; Zhou *et al.*, 2000; Boulé *et al.*, 2005). The helicase activity of Pif1 inhibits the *de novo* addition of telomeric repeats

by dissociating telomerase, thereby preventing telomeres from growing excessively long (Schulz and Zakian, 1994; Boulé *et al.*, 2005). Evidence that Pif1 is involved in Okazaki fragment processing was first obtained from genetic studies with *S. pombe*. The *S. pombe pfh1*$^+$ gene, a homolog of *S. cerevisiae PIF1*, was shown to interact genetically with *cdc24*$^+$ (Tanaka *et al.*, 2002). Mutations in *pfh1*$^+$ in one of the conserved motifs among Pfh1, Pif1, and Rrm3 were found to suppress the ts phenotype of *cdc24-M38* mutant. Curiously, expression of wild-type *pfh1*$^+$ interfered with the ability of the two mutant *pfh1* alleles (*pfh1-R20 and pfh1-R23*) to suppress ts phenotype of *cdc24-M38*, indicating that simultaneous inactivation of both *pfh1*$^+$ and *cdc24*$^+$ was important for the suppression observed. In support of this, the purified Pfh1-R20 protein exhibited significantly reduced levels of ATPase and helicase activities compared to wild type (Ryu *et al.*, 2004). The genetic interactions between *cdc24*$^+$ and *pfh1*$^+$ prompted investigations with regard to the relationship between *dna2*$^+$ and *pfh1*$^+$ since *cdc24*$^+$ was known to interact genetically and physically with *dna2*$^+$ (Gould *et al.*, 1998; Tanaka *et al.*, 2004). Like *cdc24-M38* mutant, expression of catalytically inactive *pfh1-R20* suppressed ts growth defects of *dna2-C2* mutant (Ryu *et al.*, 2004). Based on these *in vivo* and *in vitro* results, it was proposed that the role of Pfh1 in Okazaki fragment processing is to help displacement DNA synthesis by pol δ, contributing to the formation of long flap structure. The fact that long flaps are primarily processed by Dna2 could explain how the inactive Pfh1-R20 enzyme suppresses *dna2-C2* mutant. The abrogation of a pathway that leads to long flaps would make cells viable in the absence of Dna2, by rendering them less dependent on Dna2 function. This is confirmed in *S. cerevisiae*. Selective inactivation of nuclear Pif1 function suppressed the lethal phenotype of *dna2Δ* strains (Budd *et al.*, 2006). However, the *dna2Δ pif1Δ* strain was viable at 30°C, but not at 37°C. This suggests that *PIF1* deletion alone is not enough to completely bypass Dna2 requirement. Further deletion of *POL32* made *dna2Δ pif1Δ* strain viable even at 37°C (Stith *et al.*, 2008), thus bypassing the requirement of Dna2. Furthermore, deletion of *PIF1* or *POL32* suppressed growth defects of *rad27Δ pol3-D520V* (exonuclease defective pol δ) mutant which is believed to produce longer flaps (Stith *et al.*, 2008). The function of mitochondrial Pif1 was unaffected by a mutation that affected only nuclear Pif1 (Budd *et al.*, 2006). These results further strengthen the notion that Dna2 is responsible for processing of long flaps. In the absence of Pif1, wild-type yeast pol δ has a very limited ability of displacement DNA synthesis, generating mostly short flaps. The short flaps were rapidly processed via "idling" and "nick translation" of pol δ in a cooperative manner with Fen1 (Garg *et al.*, 2004). If Fen1 does not act after displacement synthesis, pol δ moves back and forth by degrading and resynthesizing DNA ("idling") with its 3′ to 5′ exonuclease and polymerase activities until the short flaps are cleaved by Fen1. Flaps that escaped early cleavage by Fen1 could become longer by two interrelated mechanisms: (1) strand displacement synthesis influenced by processivity of pol δ and (2) elongation of flaps catalyzed by Pif1 helicase.

The cleavage of dynamic equilibrating flaps by Fen1 created by pol δ was less inhibited by RPA than fixed flap-structured substrate (Rossi *et al.*, 2008). This is most likely because double flaps are shorter on average than 5′ single flap, and they are thus less likely to bind RPA than 5′ single flap. The addition of Pif1 in this reaction increased flap displacement and increased RPA binding, leading to inhibition of Fen1 cleavage. This supports the idea that the combined action of pol δ and Pif1 favors the formation of 5′ long flaps. In keeping with this, it was shown recently that Pif1 helicase directly stimulated the displacement DNA synthesis catalyzed by pol δ *in vitro* (Pike *et al.*, 2009). In addition, Pif1 was shown to stimulate Dna2-catalyzed cleavage of short flaps with ATP-dependent manner, which indicates that flaps become long enough to be cleaved by Dna2 in the presence of Pif1. The involvement of Pif1 helicase strongly argues for the possibility that flaps could be generated long *in vivo*.

Equilibrating flaps and Dna2

If the long flaps are not processed in time by Dna2 and Fen1, how could they be processed? They can anneal with the template DNA strand in a competitive manner with the 3′ end region of the newly synthesized upstream Okazaki fragment, like what happens at a Holliday junction. This produces many intermediates (depending of the initial sizes of 5′ flaps) with varying length of both 5′ and 3′ flaps (termed "equilibrating flaps"). Among these, Fen1 is able to cleave the double flaps with one-nt 3′ flap only, which is regarded as a physiological substrate for Fen1. With this, Fen1 cleaves endonucleolytically the 5′ flap strand at one nucleotide into the duplex region from the base of the double flaps. Thus, once a 5′ flap is cleaved, the one-nt 3′ flap anneals back to the template, generating a nick that can be readily ligated. Since only short flaps could be readily converted into an active form (via equilibration process) for Fen1, Fen1 alone would be sufficient to cleave short flaps. However, long flaps would be kinetically slow in the conversion of equilibrating flaps into a physiological substrate form for Fen1. Besides, long flaps have increased probability to form secondary structure. This probability would increase in proportion to the flap length. The secondary structure interferes with not only equilibration process but also Fen1 tracking into the base of flap (Liu *et al.*, 2004b). In addition, long flaps (> 25 nt) tend to bind RPA readily,

which inhibits Fen1-catalyzed cleavage. Dna2 is well suited to remove long double flaps since the 3′ flap stimulates cleavage of the 5′ flap by Dna2 and *vice versa* (Kim, J.H. *et al.*, 2006). The mutual stimulation renders the two flaps very short, so that they can be readily processed by Fen1.

Differences between human and yeast Dna2 enzymes

Both human and *C. elegans* Dna2 were purified and extensively characterized (Kim, D.H. *et al.*, 2005; Kim, J.H. *et al.*, 2006; Masuda-Sasa *et al.*, 2006a). *C. elegans* Dna2 is quite similar to yeast Dna2, but human Dna2 is not. Human Dna2 cleaves 3′ ssDNA flaps more efficiently than 5′ flaps in contrast to yeast Dna2 (Bae *et al.*, 1998; Kim, J.H. *et al.*, 2006). The other differences observed between yeast and human Dna2 include: human Dna2 lacks robust helicase activity and the N-terminal domain required for binding and resolving secondary structured flap; and the endonuclease activity of human Dna2 is inhibited by RPA (Kim, J.H. *et al.*, 2006). These marked differences in structure and biochemical properties indicate that human Dna2 may require other proteins for their function in processing of Okazaki fragments or may not carry out the same function *in vivo* as determined for *S. cerevisiae* Dna2. It was found that human Dna2 was able to cleave both 5′ flaps and 3′ flaps efficiently in equilibrating flaps by virtue of the mutual stimulatory effect of one flap on the cleavage of the other by human Dna2. This property would be useful when equilibrating flaps are formed, since it would ensure rapid processing of double flaps so that they are readily converted to a physiological form of substrate that is susceptible to Fen1 (Kim, J.H. *et al.*, 2006). In addition to this, two new activities of Dna2 were discovered, revealing new insight into mechanisms with regard to Okazaki fragment processing in higher eukaryotes. It was shown that Dna2 contains single-strand annealing and ATP-independent (rather inhibited by ATP) strand exchange activities both in *S. cerevisiae* and human Dna2 enzymes (Masuda-Sasa *et al.*, 2006b). It was proposed that the annealing and strand-exchange activities of Dna2 could contribute to rapid generation of a form of substrate (from equilibrating flaps) suitable for cleavage by Dna2 itself or Fen1 (Masuda-Sasa *et al.*, 2006b).

Some points to ponder; a means to replace the editing function of pol α

Although the ratio of long to short flaps *in vivo* has not been determined, the proposed mechanism by which long flaps are removed appears to have a significant advantage if all flaps are processed in a manner that requires Dna2. This model permits eukaryotic cells to remove the DNA beyond the RNA–DNA junction in the primers. In eukaryotes, the DNA in the primers is synthesized by error-prone pol α, whereas the entire genome of prokaryotes is synthesized by polymerases with a proofreading function (Garcia-Diaz and Bebenek, 2007; O'Donnell, 2006). Therefore, it would be beneficial if eukaryotic cells remove the potentially mutagenic DNA in the primers. The removal of the entire RNA–DNA primer synthesized by the pol α–primase complex may abrogate the need to correct any errors inserted by pol α. The current model shows that flaps with the size (~ 35 nt) comparable to the entire RNA–DNA primer can be removed most efficiently by the combined action of Dna2, RPA and Fen1.

Involvement of Dna2 in other DNA metabolisms

Role of DNA2 in telomere maintenance

Telomeres are specialized structures at chromosomal ends that consist of repeat sequences, playing a crucial role in telomere maintenance. They prevent chromosomes from degradation by nuclease and ensure complete DNA replication without loss of genetic information in conjunction with telomerase. Involvement of Dna2 in telomere metabolisms was first discovered from a screening that identified 10 DOT genes (disruptor of telomeric silencing); high-copy expression of the DOT genes led to expression of the normally silent genes near the telomeres (Singer *et al.*, 1998). The N-terminal Dna2 fragment (1-337 aa) identified from the screening suppressed silencing not only at telomere but also at the two HM loci (*HML* and *HMR*) and tandemly duplicated repeats of rDNA (ribosomal DNA). This result implies that Dna2 might be somehow involved in the regulation of gene silencing. Alternatively, overexpression of truncated Dna2 lacking the catalytic domain exerted dominant positive or negative effects on DNA replication, thereby indirectly affecting chromatin assembly thus relieving gene silencing near telomeres (Laman *et al.*, 1995; Singer *et al.*, 1998). Another piece of evidence that Dna2 is involved in telomere maintenance came from studies of telomeric repeat destabilization in *FEN1* deletion mutant (Parenteau and Wellinger, 1996). Lagging strand DNA synthesis copes with the so called "end-replication problem" at telomeric ends due to lack of space for the last piece of Okazaki fragment to be synthesized, resulting in gradual loss of chromosomal ends. To prevent loss of DNA from the end by following rounds of replication, the unreplicated 3′ overhang (G-rich strand) at the end of telomere is extended by telomerase to provide template DNA for further priming to synthesize new Okazaki fragments. In the *rad27Δ* strain, the G-rich

3′ ssDNA overhang was accumulated at the restrictive temperature. The results above suggest that compromised lagging strand DNA synthesis due to deletion of *FEN1* left the G-rich strand unused as template, accumulating G-rich 3′ overhang. This ssDNA arises in a manner dependent on DNA replication even in the absence of telomerase (Dionne and Wellinger, 1996; 1998). A similar result was obtained when Dna2 was overexpressed in wild-type cells, suggesting that both Dna2 and Fen1 are involved in telomeric DNA replication (Parenteau and Wellinger, 1999). In addition, overexpression of wild-type Dna2 resulted in reduction of telomere length, whereas 17 newly isolated *dna2* mutant alleles caused lengthening of telomere (Formosa and Nittis, 1999). In contrast, a *dna2-2* mutant displayed reduced levels of telomeric addition in *rad52Δ* cells lacking recombination-dependent addition of telomeres (Choe *et al.*, 2002). It was shown that Dna2 is localized at telomeres by immunofluorescence experiments (Choe *et al.*, 2002). Dna2 was colocalized with telomeres during G1 phase in a manner dependent on its interaction with Sir3, a component of silent chromatin, and became dispersed in S phase. This implies that Dna2 has separable roles in a cell-cycle dependent manner, the function for telomere maintenance in G1 phase and DNA replication in S phase. Despite some discrepancies in the influence of Dna2 on telomere length, all these findings indicate that Dna2 is involved in telomere maintenance. Est2 and Est1 are a catalytic subunit and a RNA binding protein, respectively, of the telomerase complex. Telomeres become shorter in the absence of either of the two proteins, leading to rapid senescence of the mutant cells. The two double mutants, *dna2-2 est2Δ* and *dna2-2 est1Δ*, entered senescence more rapidly than *est2Δ* or *est1Δ* single mutant. The Rad50-dependent, but Rad51-independent Type II survivors appeared earlier from *dna2-2 est1Δ* than from *est1Δ* single mutant, suggesting that inactivation of Dna2 led to telomerase-independent telomere elongation (Choe *et al.*, 2002). Type II survivors also required *RAD52*, *RAD59*, *SGS1* and *POL32*, suggesting that in the absence of *DNA2* an alternative recombination pathway is activated that leads to elongation of telomere (McEachern and Haber, 2006; Lydeard *et al.*, 2007).

The G-rich 3′ overhang can be formed by resection of replicated telomeres. After synthesizing nascent DNA up to the very end of the leading strand, the C-rich template strand is degraded, leaving a G-rich 3′ overhang. The influence of Dna2 on telomere length in *S. pombe* was investigated in an attempt to discover a nuclease required to produce a G-rich 3′ overhang. The Rad50–Rad32–Nbs1 complex was originally believed to resect C-rich strands. However, the formation of a G-rich 3′ overhang did not depend on the nuclease activity of Rad32 (Tomita *et al.*, 2003). Deletion of putative nuclease *exo1*$^+$ or *rad2*$^+$ in the *taz1*-deleted strain did not affect the amount of G-rich overhang. However, the G-rich overhang was hardly detected in *dna2-C2* mutant cell even at a semi-permissive temperature (30°C), suggesting that Dna2 is the nuclease required for C-rich strand resection (Tomita *et al.*, 2004). It was shown that Dna2-C2 mutant protein binds telomere at 25°C *in vivo*, although binding was decreased at 30°C. In keeping with this, Trt1, the catalytic subunit of telomerase, was not able to bind to telomere in *dna2-C2* mutant at 30°C. These results suggest that Dna2 may regulate telomere length by directly binding to telomere and controlling telomerase activity.

Role of DNA2 in double-strand break repair

Flap structures can occur not only in Okazaki fragments, but also during DNA repair such as base excision repair (BER), nucleotide excision repair (NER), and recombination processes. Fen1 is required for virtually all DNA transactions that involve formation of ssDNA flap structure. Thus it is conceivable that Dna2 is likely to play roles in all DNA transactions, provided that flaps are long. Like Fen1, therefore, inactivation of Dna2 is likely to affect many DNA metabolisms.

The ts *dna2-22* mutant displayed increase in recombination rate and chromosome loss rate at non-permissive temperature (Fiorentino and Crabtree, 1997). Consistent with this, the *dna2-2* mutant cells showed hyper-recombination of rDNA, causing reduced lifespan of *S. cerevisiae* (Hoopes *et al.*, 2002). The *rad27Δ rad52Δ and dna2-1 rad27Δ* double mutants are synthetic lethal. Cells with *dna2-1 rad52Δ* and *dna2-2 rad52Δ* double mutations exhibited synergistic growth defects. This is also supported by *S. pombe* data that functions of *rhp51*$^+$ (recombination gene *RAD51* homolog) were required for viability of *dna2* mutants (Tsutsui *et al.*, 2005). These results support the notion that incomplete replication by defective lagging strand synthesis can be repaired by Rad52-dependent homologous recombination (HR) pathway (Reagan *et al.*, 1995; Tishkoff *et al.*, 1997; Budd and Campbell, 2000). Thus, it is likely that dysfunction of Dna2 and/or Fen1 in Okazaki fragment processing causes DSB that can be repaired by Rad52-dependent HR. In *S. pombe*, *dna2-C2* mutant cells displayed extensive chromosomal fragmentation such as *cdc9* (DNA ligase I) mutation. Previously, it was also proposed that Dna2 is involved in DSB repair, based on results obtained from increased sensitivity of *dna2* mutants to DNA damaging agents (Budd and Campbell, 2000). The *dna2* mutant strains were sensitive to X-ray, and it was hypothesized that lethal X-ray damages in DNA are repaired by DSB-initiated HR. In keeping with this, Dna2 initially localized at telomeres became diffused throughout nuclei after treatment of DSB-inducing bleomycin (Choe *et al.*, 2002).

In *E. coli,* the RecBCD nuclease catalyzes the formation of 3′ overhang from DSB ends to initiate homologous recombination (reviewed in Spies and Kowalczykowski, 2005). However, in eukaryotes, a nuclease responsible for this resection remains elusive. The MRX (Mre11/Rad50/Xrs2) complex and Sae2 have been known to initiates 5′ to 3′ resection from DSB ends, producing the 3′ ssDNA overhang. The mechanism for this had not been clearly understood because the Mre11 nuclease in this complex degrades one strand from duplex ends in the 3′ to 5′ direction, which would then generate 5′ overhang ssDNA. Moreover, the expression of *mre11-H125N*, the nuclease-negative mutant, still maintained nearly wild-type level of resection activity (Lee, S.E. *et al.*, 2002; Llorente and Symington, 2004). Extensive efforts to find an enzyme(s) responsible for long-range resection of DSB ends revealed that Sgs1 cooperates with Dna2 for the resection in a redundant fashion with Exo1 (Mimitou and Symington, 2008; Zhu *et al.*, 2008). Deletion of *EXO1* resulted in significant reduction in the 5′ to 3′ long-range resection of HO-induced DSB ends, and additional deletion of *SGS1* resulted in nearly complete loss of resection (Zhu *et al.*, 2008). Double mutant *sgs1Δ exo1Δ* showed synergistic growth defect in DSB-inducing agent phleomycin (a DSB inducing agent). Subsequently, Dna2 was identified as an interacting partner of Sgs1 in this resection. Both helicase activity of Sgs1 and nuclease activity of Dna2 were essential for this resection, whereas the helicase activity of Dna2 was dispensable. The latter result was obtained using the mutant *dna2-2* enzyme that is assumed helicase-negative based on its mutation in one of the conserved helicase motifs. Therefore, Sgs1, complexed with Top3 and Rmi1, would unwind DSB ends to produce a structure suitable to cleavage by Dna2 endonuclease. Only the 5′ ssDNA tail of the two ssDNA tails was then cleaved by Dna2 (Mimitou and Symington, 2009a; 2009b; Zhu *et al.*, 2008). At present, it is unclear how Dna2 cleaves preferentially one of the two ssDNA overhang during DSB resection. To assess the contribution of Dna2-dependent resection in DSB repair, single-strand annealing (SSA) events (which occur independently of Rad51) were measured. The result was that the Sgs1-Dna2 pathway is primarily responsible for DSB repair by SSA in the absence of Rad51 (Zhu *et al.*, 2008).

Recently, it was shown that the requirement of Dna2 for DSB repair entirely depends on genetic interactions between Mre11, Dna2, Pif1 and Sgs1 in the presence of ionizing radiation (IR) (Budd and Campbell, 2009). The double *dna2Δ pif1Δ* mutant cells are resistant to X-ray, suggesting Dna2 is dispensable in the absence of Pif1 not only for DNA replication but also for DNA repair. However, the double mutants showed synergistic growth defects in combination with a nuclease-defective *mre11-D56N* or *mre11-H125N* when irradiated with X-rays, indicating that the nuclease activity of Mre11 is critical for resistance to X-ray. Inability of nuclease-negative MRE11 to complement X-ray sensitivity of *dna2Δ pif1Δ mre11Δ* implies that the nuclease activity of Mre11 is required for a Dna2-independent pathway. Interestingly, expression of the nuclease-negative Dna2 (helicase-positive) mutant protein was toxic to the growth of *dna2Δ pif1Δ* double mutant cells at 30°C (semi-permissive temperature) regardless of X-ray irradiation and at 23°C (permissive temperature) with X-ray irradiation. However, expression of Dna2 lacking both activities did not affect the growth of the double mutant cells at both temperatures even irradiated with X-rays, emphasizing the importance of coupling of the two activities in DNA repair as well as replication (Bae *et al.*, 2002; Budd and Campbell, 2009). Based on the results above, it seems that Dna2, in conjunction with Sgs1 helicase activity, is a major player in DSB end resection for SSA or possibly Rad51-dependent HR. This is consistent with the results obtained from *in vitro* experiments that XDna2 is a major nuclease in *Xenopus* for the 5′ to 3′ strand-specific processing of DNA ends as well as for SSA (Liao *et al.*, 2008).

Role of human DNA2 in mitochondrial DNA replication and repair

Recently, human Dna2 has been implicated in mitochondrial DNA replication and repair based on the observations that it is localized predominantly in mitochondria (Duxin *et al.*, 2009; Zheng *et al.*, 2008). Although it was shown that human Dna2 was exclusively detected in mitochondria (Zheng *et al.*, 2008), a small fraction of Dna2 was also detected in nuclei (Duxin *et al.*, 2009). Pol γ, required for mitochondrial DNA replication and BER, was shown to physically interact with human Dna2 (Zheng *et al.*, 2008). In addition, the primer-extension in D-loop substrates by pol γ-catalyzed displacement DNA synthesis was markedly stimulated specifically by Dna2, not by Fen1 (Zheng *et al.*, 2008). These authors proposed that the helicase activity of human Dna2 might contribute to this stimulation by unwinding downstream fork structure, thus providing template for pol γ. However, it should be noted that human Dna2 helicase activity is feeble or undetectable (Kim, J.H. *et al.*, 2006; Masuda-Sasa *et al.*, 2006a). If this were the case, direct stimulation of pol γ by human Dna2 could have led to robust primer extension in the D-loop. It was also shown that synergistic action of human Dna2 and Fen1 on flap substrates cooperated with pol γ-driven displacement synthesis and resulted in the formation of ligated products. The fact that pol γ is responsible for replication of mitochondrial DNA and it interacts functionally with Dna2 indicates that human Dna2 is involved in mitochondrial DNA replication and/or repair (Zheng *et al.*, 2008). The combined action

of Dna2, Fen1, and pol γ was very efficient for processing abasic nicked or flapped long-patch BER (LP-BER) intermediates. This property appears to have a critical physiological relevance to the repair of oxidative DNA damages in mitochondria; mitochondrial DNA is constantly at risk of oxidative DNA damages that require BER for their repair owing to abundant reactive oxygen species (Harman, 1972; Sohal and Weundruch, 1996; Zheng *et al.*, 2008; Duxin *et al.*, 2009). The importance of LP-BER and single-nucleotide BER (SN-BER) in mitochondrial DNA repair and the role of Dna2 in this context are well described elsewhere (Copeland and Longley, 2008).

Dna2 and genome instability

DNA replication proteins serve better for maintaining genome integrity during DNA synthesis by cooperating with other proteins involved in DNA repair and/or recombination. Damaged DNA is known to have propensity for recombination (Hartwell and Weinert, 1989), and now it is well established that recombination plays a crucial role in correcting damaged DNA produced endogeneously or exogenously. DNA bases could be spontaneously modified by metabolic intermediates, and single- or double-strand DNA can undergo breakage, resulting in aberrant DNA structures in DNA. If they are not repaired properly, replication would cease or lead to permanent changes or loss of genetic information. Incomplete DNA replication or unrepaired damaged DNA causes cell cycle arrest by activation of checkpoint pathways (Shrivastav *et al.*, 2008; Reinhardt and Yaffe, 2009). DNA replication, repair, and recombination are intricately networked at the functional level to maintain genome integrity. For example, mutations in one of replication or repair genes often lead to a marked increase in the rate of recombination and chromosome loss (Hartwell and Smith, 1985).

Okazaki fragment processing as a converging point for DNA replication and genome maintenance factors

A number of *DNA2*-interacting genes were identified with a synthetic genetic array technique using two conditional mutant alleles of *DNA2*, and clustering analysis of the network profiles of identified genes revealed most, if not all, of the pathways in which Dna2 could participate (Budd *et al.*, 2005). These include Okazaki fragment processing, DNA repair, DNA recombination, and chromatin dynamics. It appears that the genes in the network together safeguard the DNA replication process in redundant or partially overlapping manners to protect the fidelity of genome inheritance. In keeping with this, recent findings from subsequent studies revealed that Dna2 is involved in telomere maintenance, resection of DSBs, and mitochondrial DNA replication/repair, highlighting the diverse roles of Dna2 in many DNA transactions. The genes that showed any genetic interaction with *DNA2* are summarized in table 1.

In eukaryotes, DNA portions in the primer RNA–DNAs are synthesized by error-prone pol α and thus are potentially mutagenic. Fen1 has been implicated in removing mismatches present in this region; mismatch-containing DNA primers are thermodynamically unstable, readily forming a micro-flap structure sensitive to Fen1 cleavage (Rumbaugh *et al.*, 1999). Another mechanism is via an exonuclease that interacts with and confers a proofreading function on pol α-primase complex. The first candidate nuclease was the wild-type form of p53 that contained intrinsic 3′ to 5′ exonuclease (Mummenbrauer *et al.*, 1996; Melle and Nasheuer, 2002). The second one was a human 3′ to 5′ exonuclease (exoN) (Brown *et al.*, 2002). The properties of both nucleases suggested they may function as a proofreader for the exonuclease-deficient replicative DNA pol α. The amount of DNA synthesized by pol α is not negligible, accounting for ~10% of total genomic DNA. Besides, the small size (~ 150 nt) of Okazaki fragments in eukaryotes requires a great number (2×10^7/cell division in humans) of Okazaki fragments synthesized, processed, and ligated per cell division. Therefore, correct processing of all Okazaki fragments requires apparently extraordinary fidelity. For this reason, lagging strand synthesis could be a major source from which genome instability is initiated. Extensive generation of flaps could also contribute to high incidence of genome instability. Flaps consist of ssDNA that can form a variety of structures in sequence context. It is obvious that additional activities such as for resolution of these structures are essential prior to cleavage of structured flaps by Dna2.

Multilayers of redundant pathways to mistake-free processing of Okazaki fragments

We have been carrying out screening of multicopy suppressors and synthetic lethal genes using *dna2K1080E* (helicase-negative), *dna2Δ405N* (N-terminal deletion), and *dna2D657A* (endonuclease-negative) mutant alleles, which resulted in identification of a number of genes such as *MGS1, MPH1,* and *VTS1* (Kang *et al.*, 2009b; Kim, J.H. *et al.*, 2005; Lee, C.H. *et al.*, 2009). The genes identified in the screening can be tentatively categorized into four groups according to their mode of suppression; the first group included *PIF1* and *POL32*. They are likely to suppress *dna2* by suppressing long flap formation as discussed before. The second group included *MGS1*, *MPH1*, and *VTS1*. They stimulated endonuclease activities of Dna2 and/or Fen1. The third group included recombination/repair proteins such as *RAD52* and *MUS81-MMS4*. They are likely to repair damages caused by inadequate function of Dna2. The fourth group includes *SML1* and

Table 1. Genes genetically interacting with yeast *DNA2* (*S. cerevisiae* and *S. pombe*). The 'Cellular Function' in the first column represents the most well-defined function of a given gene or a predicted function when there is not yet a clearly defined function. It may not be the function directly related to *DNA2* because many proteins are multifunctional. Some genes are listed twice when they are synthetic sick or lethal with *dna2* mutant and act as multicopy suppressors. The 'Gene' in the second column was either deleted or overexpressed in a multicopy plasmid, if not specified, to observe the phenotypic relationship (type of Interaction) with *dna2* mutation as shown in the third column. Other mutant alleles used are indicated in parenthesis. Refer to the reference for the mutant *dna2* strain used in each experiment. Genes that have synthetic defects with *DNA2* are based on results obtained from double mutants. Triple or quadruple mutants are not included in this list. Some triple interactions are described in the text. *S. pombe* genes were denoted in italic with superscript "+"; for example, "*dna2*$^{+}$".

Cellular Function	Gene	Type of interaction	Reference
DNA replication initiation	*CDC6* (*cdc6-1*)	Synthetic sick	Formosa and Nittis (1999)
	MCM10 (*mcm10-1*)	Synthetic lethal	Araki *et al.*, (2003)
	CDC7/DBF4	Multicopy suppression	Kim and Seo (unpublished)
	RAD27	Synthetic lethal	Budd and Campbell (1997)
	CTF4	Synthetic lethal	Formosa and Nittis (1999)
	CDC9 (*cdc9-1*)	Synthetic lethal	Ireland *et al.,* (2000)
	RPA1 (*rfa1-Y29H*)	Synthetic lethal	Bae, K.H. *et al.*, (2003)
	EXO1	Synthetic lethal	Budd *et al.*, (2005)
	RNH201	Synthetic lethal	Budd *et al.*, (2005)
	RNH202	Synthetic lethal	Budd *et al.*, (2005)
	POL1 (*pol1-1*)	Synthetic lethal	Budd *et al.*, (2005)
	PRI1 (*pri-m4*)	Synthetic lethal	Budd *et al.*, (2005)
	POL3 (*pol3-01*)	Synthetic lethal	Budd *et al.*, (2005)
	cdc17^{+} (*cdc17-K42*)	Synthetic lethal	Kang *et al.*, (2000)
	rad2^{+}	Synthetic lethal	Kang *et al.*, (2000)
	cdc24^{+}(*cdc24-M38*)	Synthetic lethal	Kang *et al.*, (2000)
	RAD27	Multicopy suppression	Budd and Campbell (1997)
	RPA1	Multicopy suppression	Bae *et al.*, (2001a)
	RPA2	Multicopy suppression	Bae *et al.*, (2001a)
	RPA3	Multicopy suppression	Bae *et al.*, (2001a)
	EXO1	Multicopy suppression	Budd *et al.,* (2005)
	CDC9	Multicopy suppression	Lee and Seo (unpublished)
	cdc24$^{+\,a}$ (*cdc24-M38 and -G1*)	Multicopy suppression	Gould *et al.,* (1998)
	cdc27^{+}	Multicopy suppression	Kang *et al.*, (2000)
	cdc17^{+}	Multicopy suppression	Kang *et al.*, (2000)
	rad2^{+}	Multicopy suppression	Kang *et al.*, (2000)
	cdc1^{+}	Multicopy suppression	Kang *et al.*, (2000)
	POL32	Synthetic rescue	Budd *et al.*, (2006)
	PIF1	Synthetic rescue	Budd *et al.*, (2006)
	pfh1^{+}(*pfh1-R20*)	Synthetic rescue	Ryu *et al.*, (2004)
	pol3^{+}(*pol3-R18*)	Synthetic rescue	Tanaka *et al.,* (2004)
	cdc27^{+}(*cdc27-R22*)	Synthetic rescue	Tanaka *et al.,* (2004)
Ribosomal DNA replication	*RRM3*	Synthetic lethal	Weitao *et al.,* (2003b)
	FOB1^{b}	Variable	Hoopes *et al.,* (2002)/ Budd et al., (2005)-
DNA repair or recombination	*RAD52*	Synthetic sick	Budd and Campbell (2000)
	SGS1	Synthetic lethal	Weitao *et al.,* (2003a)
	SRS2	Synthetic lethal	Weitao *et al.,* (2003a)
	MRE11	Synthetic lethal	Budd *et al.*, (2005)
	RAD50	Synthetic lethal	Budd *et al.*, (2005)
	XRS2	Synthetic lethal	Budd *et al.*, (2005)
	SAE2	Synthetic lethal	Budd *et al.*, (2005)
	TOP3	Synthetic sick	Budd *et al.*, (2005)
	MMS1	Synthetic lethal	Budd *et al.*, (2005)
	MMS22	Synthetic lethal	Budd *et al.*, (2005)
	SLX5	Synthetic lethal	Budd *et al.*, (2005)
	SLX8	Synthetic lethal	Budd *et al.*, (2005)
	RAD6	Synthetic sick	Budd *et al.*, (2005)

Table 1. Continued on next page.

Table 1. Continued.

Cellular Function	Gene	Type of interaction	Reference
	EXO1	Synthetic lethal	Budd *et al.*, (2005)
	YEN1	Synthetic sick	Budd *et al.*, (2005)
	ELG1	Synthetic lethal	Budd *et al.*, (2005)
	rad50[+]	Synthetic sick	Tomita *et al.*, (2004)
	MGS1	Multicopy suppression	Kim, J. H. *et al.*, (2005)
	MPH1	Multicopy suppression	Kang *et al.*, (2009b)
	MUS81/MMS4	Multicopy suppression	Kang *et al.*, (2009a)
	ELG1	Multicopy suppression	Kang and Seo (unpublished)
	PSO2	Multicopy suppression	Lee and Seo (unpublished)
	RAD52	Multicopy suppression	Lee and Seo (unpublished)
Replication fork-pausing complex and replication checkpoint	*MRC1*	Synthetic lethal	Budd *et al.*, (2005)
	CSM3	Synthetic lethal	Budd *et al.*, (2005)
	TOF1	Synthetic lethal	Budd *et al.*, (2005)
Regulation of dNTP production	*SML1*	Multicopy suppression	Lee and Seo (unpublished)
Sister chromatid cohesion	*CTF4*	Synthetic lethal	Formosa and Nittis (1999)
	CTF18	Synthetic lethal	Formosa and Nittis (1999)
	CTF18	Multicopy suppression	Kang and Seo (unpublished)
Cell cycle regulation	*CDC4* (*cdc4-1*)	Synthetic sick	Formosa and Nittis (1999)
	CDC15 (*cdc15-2*)	Synthetic sick	Formosa and Nittis (1999)
Checkpoint	*RAD9*[c]	Variable	Fiorentino and Crabtree (1997)
	MEC1[d]	Synthetic rescue	Budd *et al.*, (2005)
	TEL1[d]	Synthetic sick	Budd *et al.*, (2005)
Chromatin assembly/	*SPT16* (*spt16-16*)	Synthetic lethal	Formosa *et al.*, (2001)
disassembly,remodeling,	*POB3* (*pop3-21*)	Synthetic lethal	Schlesinger and Formosa (2000)
and nucleosome	*RAD6*	Synthetic sick	Budd *et al.*, (2005)
modification	*BRE1*	Synthetic sick	Budd *et al.*, (2005)
	SWD1	Synthetic sick	Budd *et al.*, (2005)
	SWD3	Synthetic sick	Budd *et al.*, (2005)
	HST3	Synthetic lethal	Budd *et al.*, (2005)
	RPD3	Synthetic lethal	Budd *et al.*, (2005)
	PHO23	Synthetic lethal	Budd *et al.*, (2005)
	RTF1	Synthetic sick	Budd *et al.*, (2005)
	ASF1	Synthetic sick	Budd *et al.*, (2005)
Oxidative stress response	*LYS7*	Synthetic lethal	Budd *et al.*, (2005)
	SOD1	Synthetic lethal	Budd *et al.*, (2005)
Osmotic stress response	*HOG1*	Synthetic lethal	Budd *et al.*, (2005)
Ubiquitination andprotein degradation	*CDC16* (*cdc16-1*)	Synthetic sick	Formosa and Nittis (1999)
	UBC4	Synthetic sick	Budd *et al.*, (2005)
	RAD6	Synthetic sick	Budd *et al.*, (2005)
Cell growth control	*TOR1*	Multicopy suppression	Fiorentino and Crabtree (1997)
	TOR2	Multicopy suppression	Fiorentino and Crabtree (1997)
Cell wall remodeling	*PKC1*	Multicopy suppression	Kang and Seo (unpublished)
Polarized cell growth	*CLA4*	Synthetic sick	Budd *et al.*, (2005)
RNA interacting proteins	*CAF20*	Synthetic lethal	Budd *et al.*, (2005)
mRNA processing	*RTT103*	Synthetic sick	Budd *et al.*, (2005)
Nuclear import and export	*MSN5*	Multicopy suppression	Lee and Seo (unpublished)
Transcription regulation	*VTS1*	Multicopy supperssion	Lee, C.H. *et al.*, (2009)

[a] In this case, overexpression of *dna2*[+] suppressed ts phenotype of *cdc24-M38 and cdc24-G1 mutant.*

[b] Deletion of *FOB1* suppressed to a limited extent the reduced life span of *dna2-2* strain (Hoopes *et al.*, 2002) and synthetic lethality of *dna2-2 ctf4* (Budd *et al.*, 2005).

[c] Effects of *RAD9* deletion on *dna2* mutants varied from synthetic rescue to no effect, depending on mutant alleles used (Budd *et al.*, 2005; Fiorentino and Crabtree, 1997; Formosa and Nittis, 1999).

[d] Deletion of *MEC1* or *TEL1* was accompanied with deletion of *SML1*.

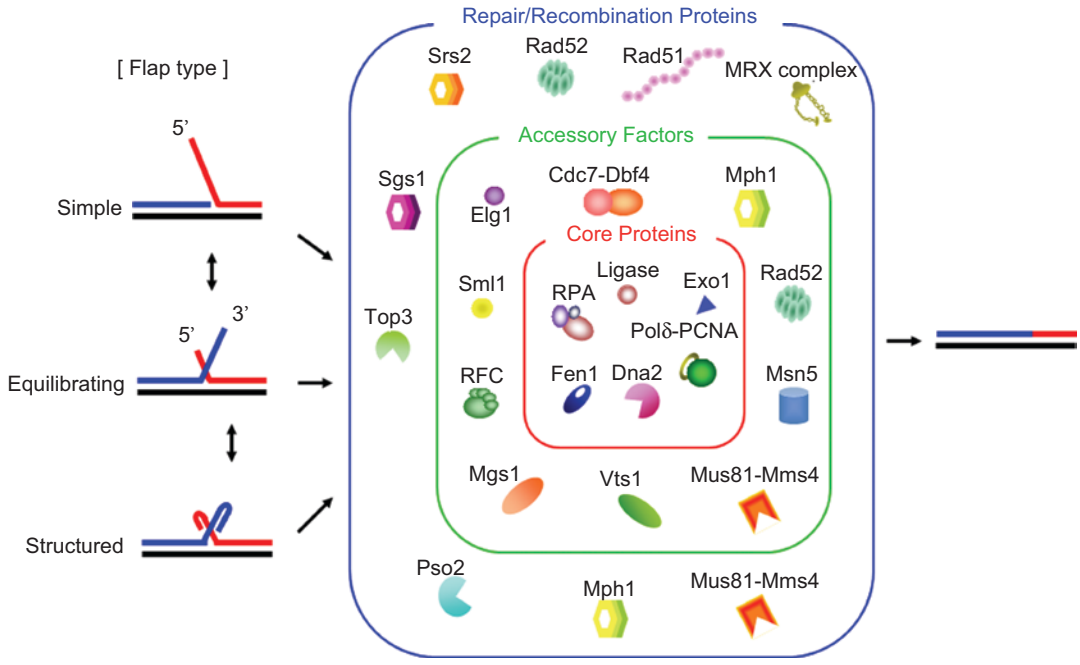

Figure 2. Multiple layers of redundant pathways of Okazaki fragment processing. Various flap structures, exemplified by three types only, can be generated during lagging strand synthesis. In most cases, it is believed that they can be processed by the combined action of "core proteins" in the first layer (indicated as the red box), the basic machinery for Okazaki fragment synthesis. "Accessory factors" that constitute the second layer (indicated as the green box) function mostly to potentiate enzymatic activities of Dna2 or Fen1 or both. When core proteins fail to function, unprocessed flaps left can be removed by proteins in the third layer (indicated as the blue box) that contains factors for DNA repair and recombination (see text for details). Msn5 or Sml1 may not be directly related to Dna2 or Fen1 and thus need to be tested in this regard. Note that some proteins can belong to several layers.

MSN5, whose immediate relationship to Okazaki fragment processing is not clear presently. Based upon their functional categories, we propose that mistake-free processing of Okazaki fragments relies on multilayers of redundant pathways as illustrated in Figure 2. The first layer consists of "core proteins", the basic machinery for Okazaki fragment synthesis and processing as described above. The second layer contains protein factors, termed "accessory factors" that can directly stimulate endonuclease activity of either Dna2 or Fen1 or both. Some proteins such as Msn5 or Sml1 may not be directly related to Dna2 or Fen1 and thus need to be tested in this regard. The third layer includes factors working for DNA repair and recombination. Note that some proteins belong to several layers. Among these, we describe some suppressors that supplement the impaired function of Dna2 since it was feasible to analyze interplay of each suppressor with Dna2 and Fen1 at functional levels. We also include others' findings if they fit in this regard. This design of Okazaki fragment processing seems to serve a single purpose, that is, the creation of ligatable nicks from all possible types of flaps (Figure 2).

Functional interactions of Dna2 with genome maintenance factors

MGS1 (Maintenance of genome stability 1) was identified as a multicopy suppressor of *dna2Δ405N* ts mutant (Kim, J.H. *et al.*, 2005). It encoded an RFC-related protein that contains DNA-dependent ATPase and DNA annealing activities (Hishida *et al.*, 2001). The *mgs1Δ* mutant cells displayed an increased rate of HR and required the *RAD6* epistatsis group of genes for viability. This group of genes is involved in post-replicational repair (Hishida *et al.*, 2002), indicating that DNA damages, accumulated in the absence of *MGS1* during replication, are repaired by the *RAD6*-dependent pathway. The suppression of *dna2Δ405N* by *MGS1* depended on a functional copy of *RAD27*, and Mgs1 stimulated the endonuclease activity of Fen1 *in vitro* (Kim, J.H. *et al.*, 2005). Consistent with this, multicopy expression of *RAD27* suppressed the ts phenotype of *dna2Δ405N*. Mph1 (Mutator phenotype 1) also acted as a suppressor specific for the helicase-negative *dna2K1080E* and *dna2Δ405N* mutants, and stimulated nuclease activities of both Fen1 and Dna2 (Kang *et al.*, 2009b). Mph1, a 3′ to 5′ helicase, is implicated in the error-free DNA damage bypass pathway for faulty DNA replication as well as in controlling of HR in favor of non-crossover events (Prakash *et al.*, 2005; 2009; Schürer *et al.*, 2004). In contrast, the suppression of the *dna2* helicase-negative mutant did not require the ATPase/helicase activity of Mph1, suggesting that Mph1 plays a variety of roles in DNA metabolism (Kang *et al.*, 2009b).

Human Bloom and Werner helicases (BLM and WRN, respectively) that belong to the human RecQ-family were also tested for their potential roles in

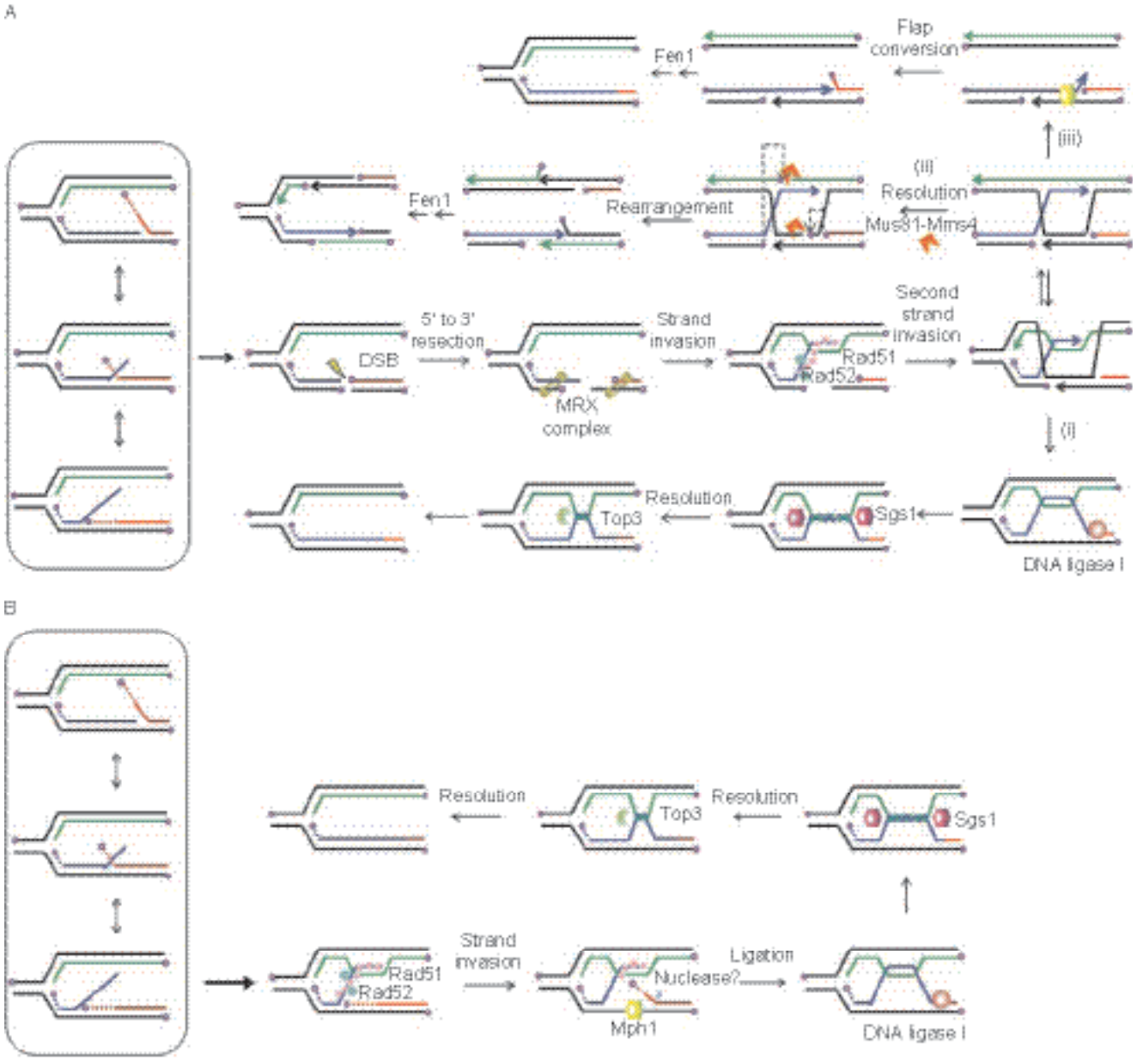

Figure 3. Possible repair pathways for unprocessed flaps that require Mus81-Mms4, Sgs1-Top3, and Mph1. The unprocessed flap can be repaired via either DSB-dependent (A) or DSB-independent (B) pathways. (A) In the DSB-dependent pathway, replicated lagging strand containing unprocessed flap undergoes double-strand break, which is resected by the MRX complex. The resulting 3′ overhang starts homologous recombination by invading homologous leading strand DNA. The resulting recombination intermediate can enter three different subpathways. (i) When nicks in the intermediate are sealed, it is resolved by the Sgs1-Top3 pathway. If nicks are available, the recombination intermediate is subjected to either (ii) nick-directed cleavage by Mus81-Mms4 or (iii) branch migration by Mph1 helicase. (B) If DSB is not involved, the 3′ flap resulting from a 5′ unprocessed flap via "equilibration" can initiate recombination by invading homologous leading strand DNA. The intermediate can be converted into substrates for the Sgs1-Top3 pathway by forming pseudo double Holliday junctions. Resolution of the intact Holliday junction could produce intermediates that require Mus81-Mms4 for further resolution (not shown for simplicity). The circle at the end of DNA strands denotes the 5′ end. Some 3′ ends are indicated by arrow heads to denote DNA extension by DNA pol δ. See text for details.

budding yeast. Both human genes, when their expressions were highly induced, were able to suppress drug sensitivity and growth defects of *dna2-1* mutant at the non-permissive temperature (Imamura and Campbell, 2003; Sharma *et al.*, 2004a). It was shown that both BLM and WRN proteins stimulated endonuclease activity of Fen1 *in vitro* (Brosh *et al.*, 2001; Sharma *et al.*, 2004a; 2004b). However, it appears that the mechanism for *in vivo* suppression is different between the two helicases, because helicase activity was essential for suppression of *dna2-1* with BLM, but not with WRN. The C-terminal $WRN_{940-1432}$ fragment (lacking the catalytic domain) was

sufficient for suppression of *dna2-1* and stimulation of the Fen1 endonuclease activity. The C-terminal $BLM_{966-1417}$ fragment devoid of the catalytic domain was able to stimulate Fen1 endonuclease activity with non-structured flaps, but not with the secondary-structured flap substrate. Thus, the helicase activity of BLM is required to resolve secondary structure in the flaps, thereby facilitating Fen1-catalyzed cleavage. This activity was not determined with WRN (Sharma *et al.*, 2004a; Wang and Bambara, 2005). BLM could resolve the secondary structure in the 5′ flap or remove a blocking primer annealed to the 5′ flap by translocating from the base of the flap in the 3′ to 5′ direction. This may account for the suppression of *dna2-1* which showed significantly reduced helicase activity (Budd *et al.*, 2000; Wang and Bambara, 2005). These results suggest that WRN might suppress *dna2-1* simply by stimulating Fen1 like Mgs1 and Mph1. In case of BLM, however, it probably would act jointly with Dna2 because the resolution of the secondary structure would most likely produce long flaps during DNA metabolism, including Okazaki fragment maturation. It should be noted that human Dna2 is devoid of or lacks DNA helicase activity unlike yeast Dna2, and thus it may depend on other helicase such as BLM (Kim, J.H. *et al.*, 2006; Masuda-Sasa *et al.*, 2006a) in humans.

The common feature of *MGS1*, *MPH1*, WRN, and BLM is that they all display genome instability, which is regarded relatively mild compared to Fen1. It was found that they all are capable of stimulating Fen1. Can we explain their relatively weak mutator phenotype in relation to Dna2 and Fen1 function? The stimulation of Fen1 by these enzymes may lead to a reduced size of flaps from the beginning via "idling and nick translation" as described above. It is conceivable that when formation of short flaps is favored, cells would be less dependent on the function of Dna2. Thus, overexpression of one of these enzymes could suppress *dna2* mutation. The reduced frequency of long flap formation would put chromosomes at reduced risk of genome instability, since long flaps are more difficult to process. In addition, cells lacking these enzymes would be in a state with a reduced Fen1 function compared to the wild type. Despite the normal protein level of Rad27, lack of a stimulator is likely to lead to low levels of Rad27 activity *in vivo* than required for optimal function. Thus, the inadequate activity of Fen1 could be one reason that may account for moderate genome instability observed in the absence of these genes despite the presence of wild-type Fen1. Besides, the reduced level of Fen1 may lead to faulty Okazaki fragment processing and the accumulation of damaged lesions that should be repaired. It should be noted that the mutator phenotype of *MPH1* was dependent on ATPase/helicase activity, but the stimulation of Fen1 (and thus suppression of *dna2* mutants) did not require its enzymatic activity (Scheller *et al.*, 2000; Kang *et al.*, 2009b). This result indicates that each gene above could contribute to the genome stability in a unique way other than via its interaction with Dna2 and Fen1. In summary, it appears that accurate and reliable processing of all Okazaki fragments depend critically on the interaction of Dna2 or Fen1 with protein factors required for maintenance of genome integrity, while genome integrity could be severely threatened if the interaction fails somehow.

Repair of faulty processing of Okazaki fragments

Recently, we found that defects of several mutant *dna2* alleles including *dna2K1080E* were rescued by overexpression of Mus81-Mms4 (Kang *et al.*, 2009a), indicating a role of Mus81-Mms4 in Okazaki fragment processing. Mus81-Mms4 is a structure-specific endonuclease that can cleave nicked Holliday junctions, D-loop, replication forks, and 3′-flaps (Boddy *et al.*, 2001; Kaliraman *et al.*, 2001; Bastin-Shanower *et al.*, 2003; Ciccia *et al.*, 2003; Whitby *et al.*, 2003). The common structural feature of Mus81-Mms4 substrates is the presence of three- or four-way junctions containing a 5′ end at the junction, which can serve to direct the cleavage reaction (Boddy *et al.*, 2001; Chen, X.B. *et al.*, 2001; Kaliraman *et al.*, 2001; Hollingsworth and Brill, 2004; Ciccia *et al.*, 2008). When replication forks encounter damaged DNA and stall, Mus81-Mms4 produces collapsed replication forks to re-establish replication forks (Doe *et al.*, 2002; Whitby *et al.*, 2003; Hanada *et al.*, 2007). Cells attempt to repair collapsed replication forks (produced by Mus81-Mms4 action) by synthesis-dependent strand annealing initiated by the double-strand break.

The purified Mus81-Mms4 complex stimulated endonuclease activities of both Dna2 and Fen1 (Kang *et al.*, 2009a). In addition, Fen1 stimulated the endonuclease activity of Mus81-Mms4. Consistent with this *in vitro* finding, overexpression of Rad27 rescued the growth defects caused by mutations of *mus81* combined with *sgs1Δ*. These results support the notion that Mus81-Mms4 is also a player in repair of unprocessed flaps because of its genetic and functional interaction with *DNA2* and *RAD27*. In addition, mutations in *SGS1* and *TOP3* are synthetically lethal or sick with *dna2* mutant. Based on these genetic and biochemical interactions between *DNA2*/*RAD27* and *MUS81-MMS4*/*SGS1-TOP3*, we propose models how they collaborate in repairing unprocessed flaps as shown in Figure 3.

Flaps may not be processed for a variety of reasons; for example, flaps that are too long and form a structure cannot be cleaved by Dna2 and Fen1, the two core processing enzymes. Unprocessed 5′ flaps can be corrected by protein factors involved in DNA repair/recombination via either DSB-dependent or DSB-independent pathways. Both processes would produce the same recombination intermediates which are normally toxic to cells if not removed. They can be substrates for Sgs1-Top3 or

Mus81-Mms4 pathways that resolve the toxic recombination intermediates. It appears that the choice between the two pathways is governed by the availability of nicks in the recombination intermediates. In DSB-dependent pathway, replicated lagging strand undergoes DSB, which is resected by the MRX complex to generate a 3′ ssDNA overhang prior to strand invasion. The 3′ overhang starts homologous recombination as shown Figure 3A. The recombinant intermediate then can be channeled into three different sub-pathways. (i) When nicks present in the intermediate are sealed, it is resolved by the Sgs1-Top3 pathway. If nicks are available, there are two conceivable pathways to resolve the recombination structure: (ii) nick-directed cleavage of the intermediate DNA by Mus81-Mms4; and (iii) branch migration by Mph1 helicase activity prior to cleavage by Mus81-Mms4. The resolution of recombination intermediates by Mus81-Mms4 produces crossover recombinant DNAs that possess both a 5′ flap and a gap. The 5′ flaps and gaps can be repaired by the same proteins required for lagging strand synthesis. The branch migration by Mph1 helicase activity (Prakash *et al.*, 2009) produces the 3′ flap structure, which can be converted into a 5′ flap via "equilibration" process for Fen1 cleavage. Note that the flaps newly formed are not the same as the original unprocessed flap in their positions, lengths, and nucleotide sequences. Due to these differences, the new flaps might not have the problems as the original flaps had. If DSB is not involved, the 3′ flap converted from the 5′ unprocessed flap via "equilibration" could initiate recombination. The intermediate can be converted into pseudo double Holliday junctions, a structure that requires Sgs1-Top3 for resolution. Cleavage of the intact Holliday junction could produce intermediates that require Mus81-Mms4 for further resolution as shown in Figure 3A.

The models proposed above are far from being complete and need to be tested rigorously in many aspects in the future. In this model, however, the 5′ flaps generated directly by Mus81-Mms4 cleavage or indirectly by Mph1 can be subsequently removed by Fen1. The mutual stimulatory effects of Fen1 and Mus81-Mms4 suggest more effective means to repair unprocessed flap. Under this situation, mutual stimulation could constitute a feed-forward and feedback stimulatory mechanism that could increase greatly the overall rate of both the resolution of toxic recombination intermediates and the removal of 5′ flap (Figure 3). In this way, the two endonucleases, although they differ in their structural substrate specificity, act jointly to repair unprocessed flap with a remarkable efficiency. This process can be further facilitated by other enzymes such as BLM (Zhang *et al.*, 2005) or Mph1 (Kang *et al.*, 2009b) that could not only stimulate Fen1 but also migrate Holliday junction. In addition, Rad54 was found to strongly stimulate Mus81-Mms4 in an ATP-dependent fashion in humans and yeasts (Matulova *et al.*, 2009; Mazina and Mazin, 2008). The involvement of Mus81-Mms4 and its multiple interactions with other protein factors in repair of Okazaki fragment processing is one example to show the complexity of dynamic design to cope with a variety of problems intrinsically associated with Okazaki fragment processing.

Repeat instability and Dna2

The first evidence that Dna2 is important to maintain repeat stability came from studies of minisatellite DNA stability in *dna2* mutant cells. It was shown that the *dna2-1* destabilized the CEB1 minisatellite DNA in vegetatively growing cells, although with a reduced efficiency compared to *rad27Δ* mutant (Cederberg and Rannug, 2006; Lopes *et al.*, 2002). It was proposed that processing of minisatellite-containing flaps by Dna2 is critical to preserve the minisatellite DNA.

Instability of trinucleotide repeats (TNR) is associated with human genetic diseases such as fragile X syndrome (CGG), Huntington's disease (CAG), myotonic dystrophy (CTG), Friedreich's ataxia (GAA), etc. (reviewed in Orr and Zoghbi, 2007). The underlying cause of these diseases is attributed to instability, typically expansion, of TNR as shown in the parentheses above (O'Donovan *et al.*, 2003). Two major models for repeat expansion have been proposed based on the requirement of replication or recombination for expansion to occur (Usdin and Grabczyk, 2000). The replication model can be categorized into two distinct pathways; one model based on DNA slippage interpreted from the results obtained using post-replication repair (PRR) mutants including Srs2 (Bhattacharyya and Lahue, 2004; 2005; Daee *et al.*, 2007). The other model is based on incomplete processing of Okazaki fragments due to secondary structure present in the flaps (Freudenreich *et al.*, 1998; Schweitzer and Livingston, 1998; White *et al.*, 1999; Spiro and McMurray, 2003; Yang and Freudenreich, 2007). Biochemical experiments with regard to the latter model have been feasible and thus were performed extensively (Lee, S. and Park, 2002; Veeraraghavan *et al.*, 2003; Liu and Bambara, 2003; Liu *et al.*, 2004b; Ruggiero and Topal, 2004). TNR-containing flaps formed hairpin structure spontaneously in solution, and were resistant to Fen1-catalyzed cleavage as predicted (Spiro *et al.*, 1999). If this hairpin flap is ligated prior to being removed, the next round of DNA replication would result in lengthening of TNR. Consistent with the role of Fen1 in creation of ligatable nicks, deletion of Fen1 increased markedly the rate of expansion. In the absence of Fen1, flaps tend to grow long, increasing the probability of expansion. Although genetic and biochemical data in *S. cerevisiae* are well consistent, however, the role of Fen1 in TNR stability in higher eukaryotes is unclear at present. TNR instability can be partially attributable to Fen1-deficiency in

mice. In *Drosophila* and humans, this relationship has not yet been clearly demonstrated (Otto *et al.*, 2001; van den Broek *et al.*, 2006; Moe *et al.*, 2008).

Compared to Fen1, yeast Dna2 is better suited to remove hairpin flaps using DNA helicase and endonuclease activities in a concerted manner (Bae *et al.*, 2002; Kao *et al.*, 2004b). The N-terminal domain of Dna2 possesses hairpin binding activity, and it functions to target Dna2 to secondary-structured flaps (unpublished observation, Y.-S. Seo). The addition of ATP (thereby activating helicase of Dna2) helped the endonuclease activity resolving the secondary-structure flaps (Bae *et al.*, 2002). These biochemical properties have rendered Dna2 the prime candidate that safeguards integrity of repeat DNA such as trinucleotide repeats that could form secondary structures in ssDNA flaps; the abilities to bind to hairpin flaps, to unwind the hairpin, and to subsequently cleave the unwound flap reside in a single polypeptide of Dna2. In our preliminary observation, however, the increased rates of CTG repeat expansion and contraction were observed in *dna2Δ405N* mutant strain when 84 repeats of CTG reside in a multicopy plasmid. This result was in keeping with our *in vitro* data. However, this observation was not repeated with TNR repeats inserted into a chromosome (unpublished observation, Y.-S. Seo). Consistent with this, a *dna2-1* mutant, which was shown to retain reduced levels of helicase/endonuclease activities, did not display significant changes in TNR expansion frequency (Callahan *et al.*, 2003; Ireland *et al.*, 2000). This observation suggests that Dna2 may not contribute significantly to the maintenance of TNR repeat despite its biochemical properties ideally suited to this task. Alternatively, the frequency of long flaps formed *in vivo* during replication is not so great that Dna2 does not play a significant role in this regard. Another explanation for this is that there is a redundant pathway that could act conjointly or in parallel with Dna2 in keeping the TNR repeat intact. We prefer the possibility that TNR in a multicopy showed instability in a manner dependent on functional Dna2. In order to clarify the role of Dna2 in maintenance of TNR, more genetic and biochemical analyses should be carried out to find factors that work together with Dna2.

Summary and perspectives

We have reviewed the evidence for the dynamic design of Okazaki fragment processing and the roles of Dna2 and its interacting partners in carrying out this design. In addition, protein factors for DNA replication, repair, and recombination can merge at lagging strand synthesis for reliable and complete accomplishment of Okazaki fragment processing, which is to produce perfect continuous duplex DNA. Core proteins can process most Okazaki fragments to create ligatable nicks. When structured flaps are generated, additional activities are required. To maintain genome integrity, it appears crucial to remove branched ssDNA from duplex DNA, since they are sites of discontinuity and are constantly subjected to DNA modifying enzymes including various polymerases, helicases, and nucleases. Correct modifications could give rise to a desirable result such as formation of perfect stable duplex, whereas faulty modifications could lead to changes in DNA sequence or chromosomal structure. Since the branched ssDNA, exemplified by flaps, can take very diverse structures in context of the DNA sequence, there exist many additional enzymatic activities to cope with this structural problem. They are likely to constitute redundant or backup systems for a core pathway. To date, many results with regard to Dna2 functions have been obtained from studies using yeasts as model organisms. However, recent findings with human Dna2 suggest that there are some variations in the primary function of Dna2 among eukaryotes. For example, knockdown of human Dna2 resulted in the formation of an internuclei chromatin bridge, indicating a novel role of Dna2 in resolution of chromosome directly or indirectly (Duxin *et al.*, 2009). Despite some observed functional variations, the essential properties of Dna2 seem to remain unchanged: single-strand specific endonuclease activity and its ability to collaborate with a number of other proteins to maintain genome integrity. Further studies of Dna2 in mammals will not only uncover the precise mechanisms by which Dna2 participates in a given process, but will also shed light on how the genome becomes altered, thereby causing human genetic disease.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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