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Lagging Strand Synthesis and Genomic Stability

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1. Introduction

In eukaryotic cells, DNA replication starts at many origins in each chromosome during S phase of cell cycle. Each origin is activated at different time points in S phase, which takes place once and only once per cell cycle. In yeast and most likely higher eukaryotes, the origin-recognition complex (ORC) and several other initiation factors play a pivotal role in activation and regulation of replication origins. Briefly, the ORC-bound origins are sequentially activated and deactivated along the progression of cell cycle. The prereplicative complex (pre-RC) is formed by loading the replicative helicase MCM complex onto the ORC-bound origins with the aid of Cdc6 and Cdt1. This complex is activated by S-phase cyclin dependent kinases (Cdks) when cells enter S phase. The elevated levels of Cdk activities lead to removal of some initiation proteins such as Cdc6 by proteolysis, allowing the pre-RC to be further activated for subsequent DNA synthesis. The irreversible removal of initiation factors is a major mechanism to ensure DNA to be replicated once and only once per cell cycle. The assembly of replication initiation complex and its activation are well reviewed in many literatures (Sclafani & Holzen, 2007; Remus & Diffley, 2009; Araki, 2010). Activation of origins leads to the establishment of bidirectional replication forks for the DNA synthesis of leading and lagging strands.

2. Overview of lagging strand synthesis

Leading strand synthesis, once initiated, occurs in a highly processive and continuous manner by a proofreading DNA polymerase. Unlike leading strands, lagging strands are synthesized as discrete short DNA fragments, termed 'Okazaki fragments' which are later joined to form continuous duplex DNA. Synthesis of an Okazaki fragment begins with a primer RNA-DNA made by polymerase (Pol) α-primase. The synthesis of RNA portion (~ 10 to 15 ribonucleotides) and subsequent extension of short (~20 to 30 nucleotides, nt) DNA are coupled. The recognition of a primer RNA-DNA by the Replication-Factor C (RFC) complex leads to dissociation of Pol α-primase and loading of proliferating cell nuclear antigen (PCNA), resulting in recruitment of Pol δ to the primer-template junction, a process called 'polymerase switching.' Then the primer RNA-DNA is elongated by Pol δ. When Pol δ encounters a downstream Okazaki fragment, it displaces the 5′ end region of the Okazaki fragment, generating a single-stranded (ss) nucleic acid flap. The flaps formed can be efficiently processed by the combined action of Flap endonuclease 1 (Fen1) and Dna2 to

eventually create nicks. The nicks are finally sealed by DNA ligase 1 to complete Okazaki fragment processing. The current model is summarized in Fig. 1.

3. Potential risks associated with lagging strand synthesis in eukaryotes

Lagging strand maturation appears to be intrinsically at high risks of suffering DNA alterations for several reasons. First, a substantial part (up to 20%) of short Okazaki fragments (~150-nt in average) is synthesized by Pol α which does not contain a proofreading function (Conaway and Lehman, 1982; Bullock et al., 1991). Thus, the high-incidence errors in Okazaki fragments, if not effectively removed, could become a source of genome instability. Second, the *modus operandi* of Okazaki fragment processing could put eukaryotic chromosomes at risks of DNA alteration. It involves the formation and subsequent removal of a flap structure (Bae & Seo, 2000; Bae et al., 2001a); flaps could be a source of a potential risk because they can take a variety of structures according to their sizes and sequences. Third, since the size of Okazaki fragments is very small, cells require a great number (for example, 2 x 10^7 in humans) of Okazaki fragments to be synthesized, processed, and ligated per cell cycle. This bewilderingly great number of events would make infallible processing of all Okazaki fragments dependent on multiple back-up or redundant pathways. Forth, lagging strand synthesis is mechanistically more complicated than leading strand synthesis, implying that the sophisticated machinery for this process may come across accidents in many different ways. Therefore, failsafe synthesis of lagging strand is highly challenging by virtue of the complex multi-step process and the sophisticated machinery for Okazaki fragment processing.

4. 'Core' factors for synthesis and maturation of lagging strands

The protein factors required for synthesis of lagging strands include Pol α-primase, Pol δ, PCNA, RFC, RPA, Fen1 (5′ to 3′ exonuclease or MF1, maturation factor 1), RNase H, and DNA ligase 1. In essence, a combined action of these factors was sufficient and necessary for completion of lagging strand synthesis in vitro in simian virus 40 DNA replication (Ishimi et al., 1988; Waga & Stillman, 1994). Among them, the two nucleases Fen1 and RNase H were shown to have roles in the removal of primer RNA of Okazaki fragments. In yeasts, however, the deletion of genes encoding Fen1 (RAD27) or RNase H (RNH35) was not lethal, indicating the presence of redundant pathways in eukaryotes (Tishkoff et al., 1997a; Qui et al, 1999). In addition, Dna2, which was originally reported as a helicase (Budd & Campbell 1995; Budd et al., 1995), was shown to play a critical role in the processing of Okazaki fragments using its endonuclease activity (Bae et al., 1998; Bae & Seo, 2000; Bae et al., 2001a; MacNeill, 2001; Kang et al., 2010). Displacement DNA synthesis by Pol δ generates flap structures, which can be substrates for Dna2 and Fen1 endonuclease activities (Bae & Seo, 2000). For the convenience sake, all enzymes (Pol δ, PCNA, RFC, RPA, Fen1, RNase H, Dna2, and DNA ligase 1) described early from yeast and human studies are referred to as 'core' factors for synthesis of lagging strands in this chapter. We refer to all the others as 'auxiliary' factors which may not be needed normally, but become critical under specific circumstances (Fig. 1 and see also Fig. 3). These factors have been screened for their abilities to suppress the crippled function of Dna2 or Fen1. It is believed that (i) the 'auxiliary' factors come to assist the 'core' machinery that does not function appropriately, (ii) they provide additional enzymatic activities to resolve hairpin or higher-ordered structures in flaps, or

(iii) they are needed to resolve toxic recombination intermediates arising during lagging strand metabolism. Thus, it is the multiplicity of 'auxiliary' factors that allows the 'core' machinery to be fine-tuned in response to diverse situations with regard to Okazaki fragment processing.

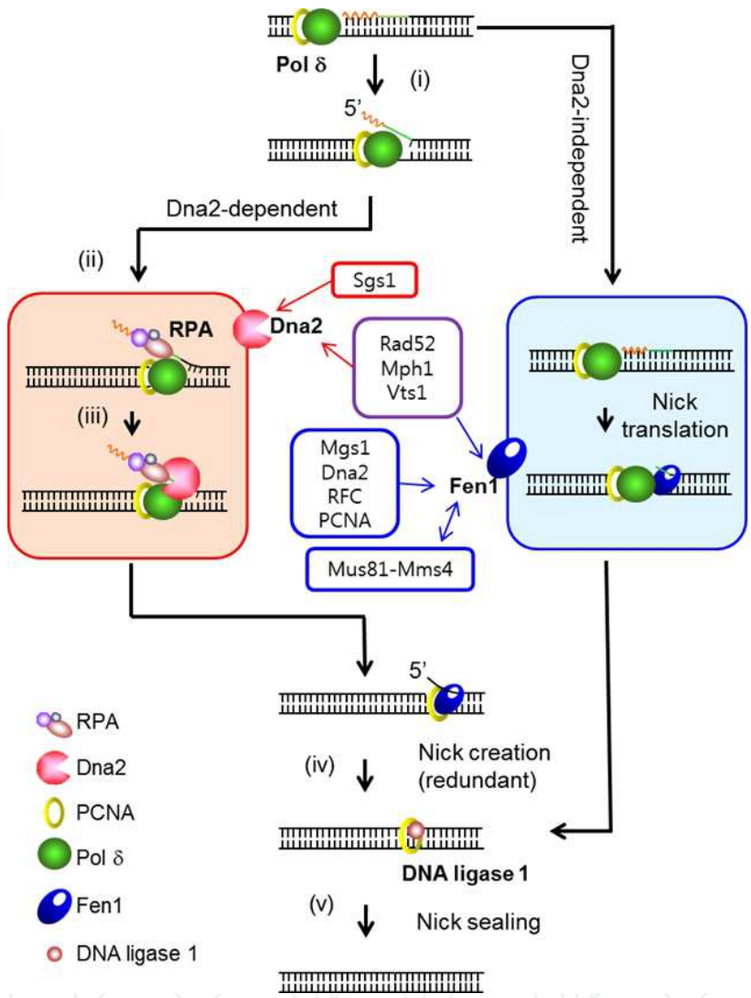

Fig. 1. A current model for processing of Okazaki fragments in eukaryotes. Dna2-dependent pathway includes: (i) The 5' terminus of an Okazaki fragment containing the primer RNA-DNA is rendered single-stranded by displacement DNA synthesis catalyzed by Pol δ. (ii) RPA rapidly forms an initial complex with the nascent flap structure and (iii) then recruits Dna2 to form a ternary complex. This leads to the initial cleavage of RNA-containing segments by Dna2, (iv) leaving a short flap DNA that can be further processed either by Fen1 (Fen1-dependent) or by other nucleases, possibly Exo1 or 3' exonuclease of Pol δ (Fen1-independent) (not shown; see the text for details). (v) Finally, the resulting nick is sealed by DNA ligase 1. Short flaps can be processed directly by Fen1 (Dna2-independent pathway) that involves the 'idling' (not shown) or 'nick translation' (see the text for details). Nicks generated by this mechanism are directly channelled into the nick sealing step. 'Auxiliary' factors that stimulate Dna2 or Fen1 or both are boxed and their targets are indicated by arrowheads. A double arrowhead indicates mutual stimulation.

4.1 Multiple pathways in parallel with Fen1

Fen1 is a major, but not the only enzyme that can create ligatable nicks directly from flap structures (Harrington & Lieber, 1994; Murante et al., 1995; Liu et al., 2004; Garg & Burgers 2005). In vivo studies demonstrated that double-strand break(DSB)-induced DNA repair, which requires replication of both leading and lagging strands, still occurred 50% in Fen1-deficient strains compared to wild type (Holmes & Haber, 1999), indicating that the 50% of the repair events were carried out with nicks created by nuclease(s) other than Fen1. The ability of Pol δ to switch from displacement DNA synthesis to its 3′ exonuclease could constitute a pathway to create nicks; the retrograde 3′ exonucleolytic degradation of a newly elongated end, followed by annealing of the displaced flap to the lagging strand template, can be a mechanism for nick formation (Jin et al., 2001). The overexpression of Exo1 in rad27Δ restored growth of the mutant cells at the nonpermissive temperature (Tishkoff et al., 1997b). Single mutant cells with either rad27Δ or exo1Δ were viable, whereas rad27Δ exo1Δ double mutants were not (Budd et al., 2000; Tishkoff et al., 1997b). Yeast Exo1 has 5′ exonuclease activity acting on double stranded (ds) DNA and an associated 5′-flap endonuclease activity (Tran et al., 2001). In addition, yeast rad27Δ cells (lacking yeast Fen1) were not lethal, but temperature-sensitive (ts) in growth, consistent with existence of multiple pathways for nick generation in yeasts. It was shown that Pol δ has a unique ability to maintain dynamically the nick position in conjunction with Fen1, via a process called 'idling'. In addition, Pol δ cooperates with Fen1 and PCNA to carry out 'nick translation' to progressively remove primer RNA-DNAs (Garg et al., 2004). The endonuclease activity of Fen1 can keep cleaving a flap while it is being displaced by Pol δ, allowing nicks to be changed in their positions along with Pol δ movement.

4.2 Structured flaps are special types of DNA damage that could cause genome instability

Failure to create nicks by Fen1 in a timely manner could cause genome instability. The importance of Fen1 in this regard was clearly demonstrated by the dramatic increase of small (5- to 108-bp) duplications flanked by 3- to 12-bp repeats in rad27Δ mutants (Tishkoff et al., 1997a). This unusual type of duplication mutations is in keeping with the current model of Okazaki fragment processing; unprocessed flaps, rapidly accumulated in the absence of Fen1, are ligated with the 3′-end of the downstream Okazaki fragment, resulting in duplication mutations. In the absence of Fen1, many types of repeat DNA sequences in eukaryotic chromosomes are not stably maintained. These include dinucleotide, trinucleotide, micro- or mini-satellite DNA, and telomeric DNA (Johnson et al., 1995; Kokoska et al., 1998; Xie et al., 2001; Freudenreich et al., 1998; Spiro et al., 1999; White et al., 1999; Maleki et al., 2002; Lopes et al., 2002; Lopes et al., 2006). Most notably, expansion of trinucleotide repeats such as CTG/CAG or CGG/CCG has been extensively studied using yeasts as model system (Schweitzer & Livingston, 1998; Freudenreich et al., 1998; Shen et al., 2005), because of their clinical relevance to many human neurodegenerative diseases such as Fragile X Syndrome, Huntington's Disease, and Myotonic Dystrophy (Pearson et al., 2005; Kovtun & McMurray, 2008). All of the disease-causing trinucleotide repeats are able to form secondary or higher-ordered structures in solution, such as hairpins (CAG, CTG, CGG, and CCG repeats), G quartets (CGG repeats), and triplexes (GAA and CTT) (Fig. 2).

Trinucleotide repeats, once displaced by Pol δ, could reanneal to the template in a misaligned manner. If they are joined to the 3′ end of the new Okazaki fragment, followed by a subsequent round of DNA replication, the repeats could be expanded. In yeast, stability

of trinucleotide repeats is greatly affected by their orientation with respect to nearby replication origins (Freudenreich et al., 1997; Miret et al., 1998). The orientation-dependent and sequence-specific instability of trinucleotide repeats support the model that expansions of CTG and CAG tracts result from aberrant DNA replication via hairpin-containing Okazaki fragments. In addition, telomere repeats are not stably maintained in the absence of functional Fen1 in yeasts (Parenteau & Wellinger, 1999 and 2002). Although Fen1 is critical for repeat stability in yeasts, it remains unclear in mice or humans (Spiro & McMurray, 2003; Moe et al., 2008; van den Broek et al., 2006). One explanation is that unlike yeasts, mammals may have more diverse pathways to remove or prevent formation of long flaps, since instability of the trinucleotide repeats occurs through formation of long flaps. Alternatively, Fen1 is responsible for formation of most nicks in mammals because deletion of Fen1 caused embryonic lethality in mice (Kucherlapati et al., 2002). The human minisatellite DNA became unstable in rad27 or dna2 mutant cells when it was inserted into one of the yeast chromosomes (Lopes et al., 2002; Cederberg & Rannug, 2006). These data also are in keeping with the idea that improperly processed 5′ flap instigates minisatellite destabilization. DNA instability associated with secondary or higher-ordered structures in the flap indicates that structures formed during DNA metabolisms can be regarded as special forms of DNA damage that need to be immediately removed (Fig. 2). The role of Fen1 in safeguarding the genome integrity has qualified Fen1 as a tumor suppressor in mammals and its physiological importance was recently reviewed with an emphasis on studies of human mutations and mouse models (Zheng et al., 2011).

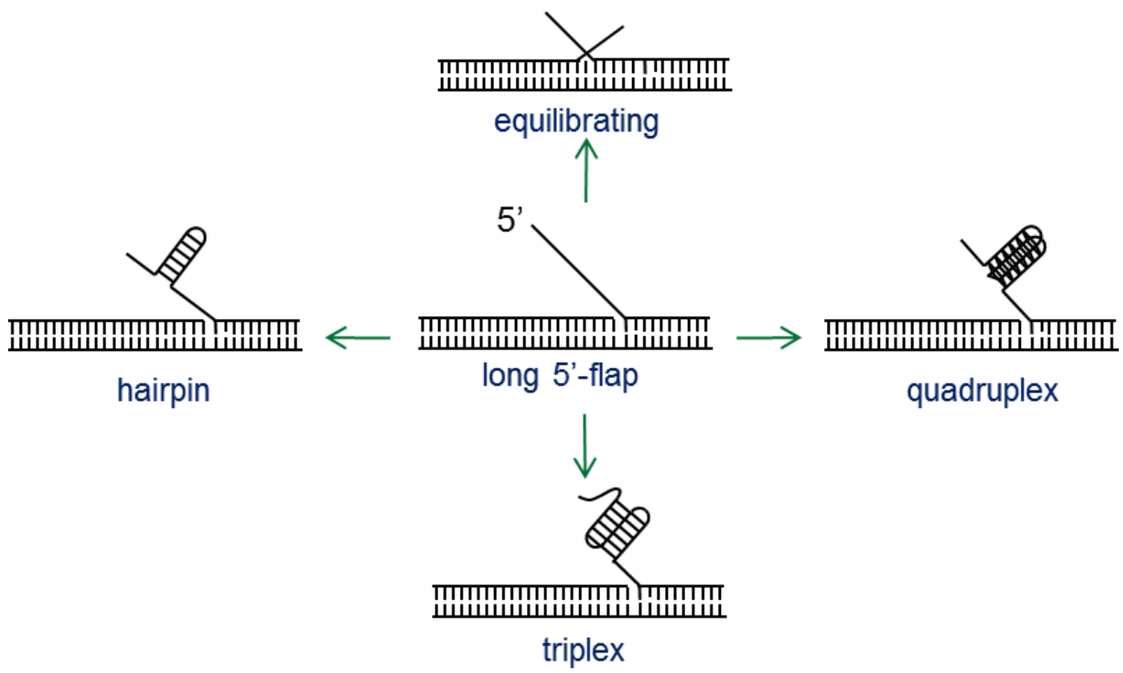

Fig. 2. A variety of structures are possible in unprocessed 5′-ssDNA flaps. If an excessively long 5′ flap is not processed in a timely manner, the flap can reanneal back to the template DNA, generating an 'equilibrating' flap which is more difficult to process by Fen1 alone. Alternatively, it could form hairpin or higher-order structures such as triplex or quadruplex according to the sequence context.

4.3 Dna2 as a preemptive means to prevent formation of long flaps

4.3.1 Long flaps are in vivo substrates preferred by Dna2

Dna2 is highly conserved throughout eukaryotes and contains at least two catalytic domains for helicase and endonuclease activities (Budd & Campbell, 1995; Budd et al., 1995; Bae et al., 1998; Bae et al., 2001b). Genetic data from fission and budding yeasts indicate that the endonuclease activity of Dna2 is essential, playing an essential role in vivo in Okazaki fragment processing (Kang et al., 2000; Lee et al., 2000; Budd et al., 2000; Kang et al., 2010). There are several lines of evidence that long flaps can be formed in vivo that need the action of Dna2. 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 is difficult to be processed. In this sense, any structural intermediates formed in flaps can be regarded as a special type of DNA damage. The requirement of Dna2 endonuclease and helicase activities for a complete removal of long or hairpin flaps supports the idea that the major role of Dna2 is to prevent formation of excessively long flaps by cleaving them into shorter ones as soon as they occur. The flaps shortened by Dna2 are not able to form secondary or higher-ordered structure. Thus, Dna2 functions to maintain flaps as short as possible during replication. The marked increase of unusual duplications or trinucleotide expansions in the absence of Fen1 (Tishkoff et al., 1997a) provide strong evidence that long flaps are produced in vivo. It was shown that calf thymus Pol δ was able to displace downstream duplex DNA longer than 200 bps in vitro, revealing its intrinsic ability to form extensive flaps (Podust & Hubscher, 1993; Podust et al., 1995; Maga et al., 2001). In vitro reconstitution experiments using yeast enzymes showed that a portion of flaps grows long up to 20- to 30-nt, although flaps formed in vitro are primarily short, up to 8-nt in length (Rossi & Bambara, 2006). The frequency of long flaps can be affected by sequence in the lagging strand template or by interactions of Pol δ/Dna2 with other proteins. For example, Pol δ lacking PCNA-interaction tends to preferentially generate short flaps (Jin et al., 2003; Garg et al., 2004; Tanaka et al., 2004). In contrast, Pif1 helps to create long flaps through its helicase activity in vitro (Rossi et al., 2008) and in vivo (Ryu et al., 2004). Several other elaborate genetic experiments are in keeping with involvement of Dna2 in the cleavage of long flaps. First, dna2-1 was lethal in combination with a mutation in Pol δ (pol3-01) which increased strand displacement synthesis. Meanwhile, deletions of Pol32 subunit, which reduces strand displacement activity of Pol δ in vitro, suppressed the growth defects of dna2-1 and dna2-2 (Burgers & Gerik, 1998; Garg et al., 2004; Johansson et al., 2004). Similar results were also obtained in S. pombe (Reynolds et al., 2000; Zuo et al., 2000; Tanaka et al., 2004). The observation that overexpression of RPA alleviates the requirement of Dna2 helicase activity (Bae et al., 2002) is also consistent with formation of long flaps in vivo. In order for dsDNA-destabilizing activity of RPA to substitute for the helicase activity of Dna2, flaps should be at least long enough to form hairpin structure.

4.3.2 RPA acts as a molecular switch between Dna2 and Fen1

Several independent observations indicate that RPA plays a critical role in Okazaki fragment processing in conjunction with Dna2; (i) a mutation in DNA2 was identified during a synthetic lethal screen with rfa1Y29H, a ts mutant allele of RFA1. Furthermore, Dna2 and Rpa1 (a large subunit of RPA encoded by RFA1) physically interacted with each other both in vivo and in vitro (Bae et al., 2003). (ii) The 32 kDa subunit of RPA was crosslinked to primer RNA–DNA in the lagging strand of replicating SV40 chromosomes (Mass et al., 1998). (iii) 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). The fact that RPA binds most efficiently ssDNA longer than 20-nt and interacts genetically with Dna2 is consistent with the idea that the in vivo substrates of Dna2 are long ssDNA flaps. In vitro, RPA markedly stimulated Dna2-catalyzed cleavage of 5′ flap at physiological salt concentration (Bae et al., 2001a), which was further confirmed by others (Ayyagari et al., 2003; Kao et al., 2004). However, RPA inhibited Fen1-catalyzed cleavage of 5′ flaps. This inhibition was readily relieved by the addition of Dna2 (Bae et al., 2001a). Thus, a 5′ flap longer than 20-nt first binds RPA, and then rapidly recruits Dna2 to form a ternary complex. Dna2-catalyzed cleavage of the flap releases free RPA-bound ssDNA and a shortened flap (mostly 6-nt). The short flap produced is 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 for the sequential action in cleavage of long flaps, Dna2 followed by Fen1, of the two endonucleases (Bae et al., 2001a).

4.3.3 A concerted action of helicase and endonuclease activities for removal of hairpin flaps

The presence of both endonuclease and helicase activities in one polypeptide of Dna2 implies that both activities act in a collaborative manner. The lethality of dna2 mutation lacking helicase activity (Budd et al., 1995) suggests that DNA unwinding activity is critical for its physiological function in vivo. The addition of ATP not only activates helicase activity, but also alters the cleavage pattern of flap DNA by Dna2. The average size of cleaved flaps is expanded in the presence of ATP (Bae et al., 2002). Furthermore, the addition of ATP allowed wild type Dna2, not helicase-negative Dna2K1080E mutant, to cleave secondary-structured flap via its combined action of helicase and nuclease activities (Bae et al., 2002). The mixture of helicase-negative Dna2K1080E and nuclease-negative Dna2D657A mutant enzymes failed to recover wild type action on these structured flaps. Therefore, it is critical essential that these two essential activities should be concerted. In keeping with this, simultaneous expression of both mutant proteins in dna2Δ cell did not allow cells to grow. Dna2 is also capable of unwinding G-quadruplex DNA structures, suggesting another critical role of Dna2 helicase in resolving the structural intermediates arising during DNA metabolisms (Masuda-Sasa et al., 2008). It was also shown that concerted action of exonuclease and gap-dependent endonuclease activities of Fen1 could contribute to the resolution of trinucleotide-derived secondary structures formed during maturation of Okazaki fragments (Singh et al., 2007).

4.3.4 Dna2 as an alternative means to remove mismatches

Since the Pol α-synthesized DNA in Okazaki fragments is highly mutagenic, eukaryotic cells need to eliminate this mutagenic DNA to prevent accumulation of errors. Recently, it was shown that in yeast Pol α incorporates ribonucleotides more frequently than Pol δ or Pol ε (Nick McElhinny et al., 2010b). The unrepaired ribonucleotides in DNA could inflict a potential problem on DNA replication because Pol ε has difficulty bypassing a single ribonucleotide present within a DNA template in yeasts. This again emphasizes that processing of Okazaki fragments is associated with high risks of DNA alterations. It has been puzzling that eukaryotic cells maintain a low mutation rate, despite the fact that a substantial portion (~10%) of total DNA is synthesized by Pol α, a flawed DNA polymerase. To account for this enigma, it was proposed that in mammals Pol α is associated with a 3′

exonuclease that may confer a proofreading function on Pol α (Bialec and Grosse, 1993). In yeasts, an intermolecular proofreading mechanism was proposed in which Pol δ could play a role in proofreading errors made by Pol α during initiation of Okazaki fragments (Pavlov et al., 2006). Mismatch repair (MMR) can correct mismatches in the Pol α-synthesized DNA (Modrich & Lahue 1996; Kolodner & Marsischky,1999; Kunkel & Erie, 2005). One unsolved fundamental problem in eukaryotic MMR, however, is the strand discrimination signal, although a strand-specific nick is generally believed to be the signal (Holmes et al., 1990; Thomas et al., 1991; Modrich, 1997). Equally possible is that the presence of flaps, which may be as abundant as nicks in lagging strand, could act as the strand discrimination signal. At any rate, the accuracy of MMR would depend on the rate at which nicks or flaps (the strand discrimination signals) are being removed. Thus, MMR could be unreliable if MMR is kinetically slower than sealing nicks. The ability of Dna2 to efficiently remove the RPA-bound flap containing the whole RNA-DNA primer could offer an alternative mechanism to remove mismatches present in the primer DNA of Okazaki fragments.

5. Multi-factorial interplays as a means to ensure high-fidelity replication of lagging strand

If one of the 'core' factors is crippled, a redundant factor(s) that works in parallel can reveal itself. In our laboratory, we have focused on isolating genetic suppressors that can rescue dna2 mutations in order to identify redundant pathways for Okazaki fragment processing. Most suppressors isolated turned out to have roles in maintenance of genome integrity, in keeping with the notion that faulty processing of Okazaki fragment could lead to genome instability. The in vivo and in vitro interactions of the suppressors with Dna2 or Fen1 suggest that Okazaki fragment processing is a converging place for DNA replication, repair, and recombination proteins to ensure removal of flaps in an accurate and timely manner in eukaryotes.

5.1 RNase H2 as an enzyme to clean up ribonucleotides in lagging strands

Both type I and type II RNase H play a role in the removal of ribonucleotides present in duplex DNA (Ohtani et al., 1999; Cerritelli & Crouch, 2009). The S. cerevisiae RNase H2 enzyme is active as a heterotrimeric complex that consists of Rnh201, Rnh202, and Rnh203, which are encoded by RNH201 (formerly known as RNH35), RNH202, and RNH203, respectively (Jeong et al., 2004). Expression analyses and other results suggest that RNase H2 plays roles in DNA replication and/or repair (Frank et al., 1998; Qiu et al., 1999; Arudchandran et al., 2000). Since rnh201Δ and rnh202Δ displayed synthetic lethal interactions with dna2-1 and rad27Δ, yeast RNase H2 has been implicated in Okazaki fragment processing (Budd et al., 2005). The unique ability of eukaryotic RNase H2 (type II) to cleave the 5′ side of a single ribonucleotide embedded within duplex DNA suggests an additional role, that is, the removal of ribonucleotides misincorporated into DNA (Rydberg & Game, 2002). The catalytic activity of RNase H2 was critical for a pathway requiring the function of RAD27 since all rnh201 mutant alleles failed to complement the growth defect of rad27Δrnh201Δ. Moreover, the addition of 20 mM hydroxyurea to growth media rescued the ts phenotype of dna2Δ405N, but failed to suppress the double mutants, dna2Δ405N rnh201Δ, dna2Δ405N rnh202Δ and dna2Δ405N rnh203Δ (Nguyen et al., 2011). Thus, the suppression of dna2 mutation also depends on a functional RNase H2, suggesting that RNase H2 plays a critical role in the removal of primer RNAs if cells have impaired Dna2.

An alternative explanation, which is not mutually exclusive from the above possibility, is that the addition of 20 mM HU might have led to a decreased ratio of deoxyribonucleotides to ribonucleotides, causing a dramatic increase in ribonucleotide incorporation. This might render cells more dependent on the clean-up function of RNase H2 to remove misincorporated ribonucleotides present in newly synthesized DNA strands by replicative polymerases (Nick McElhinny et al., 2010a). The fact that Pol α misincorporates ribonucleotides more frequently than Pol δ or Pol ε is consistent with a more critical role of RNase H2 in lagging strand synthesis than in leading strand (Nick McElhinny et al., 2010b). It was shown that in humans, Rnh202-PCNA interaction is important to recruit RNase H2 to replication foci (Bubeck et al., 2011). Since the biochemical activity of RNase H2 is dedicated to the removal of ribonucleotide incorporated into DNA, the interaction between PCNA and RNase H2 may function to recruit RNase H2 to lagging strands for Okazaki fragment processing. It was also shown that elevated levels of misincorporated ribonucleotides during DNA replication cause genomic instability (Nick McElhinny et al., 2010a). Mutations in the human homologs of the three yeast RNase H2 subunits are related to the development of Aicardi-Goutieres syndrome (Crow et al., 2006).

5.2 Many stimulators of Dna2 and Fen1 to prevent formation of structural intermediates

5.2.1 Mgs1

MGS1 (Maintenance of Genome Stability 1) of S. cerevisiae was found to act as a multicopy suppressor of the ts growth defect of dna2Δ405N mutation (Kim et al., 2005). Mgs1 stimulated the structure-specific nuclease activity of yeast Fen1 in an ATP-dependent manner. ATP binding but not hydrolysis was sufficient for the stimulatory effect of Mgs1. Suppression of dna2Δ405N required the presence of a functional copy of RAD27. MGS1 is a highly conserved enzyme containing both DNA-dependent ATPase and DNA annealing activities, playing a role in post-replicational repair processes (Hishida et al., 2001 and 2002).

5.2.2 Vts1

VTS1 (*vti*1–2 *s*uppressor) of S. cerevisiae was originally identified as a multicopy (and lowcopy) suppressor of vti1-2 mutant cells that displayed defects in growth and vacuole transport (Dilcher et al., 2001). The Vts1 protein is also highly conserved in eukaryotes and encodes a sequence- and structure-specific RNA binding protein that has a role in posttranscriptional regulation of a specific set of mRNAs with cognate binding sites at their 3′-untranslated region (Aviv et al., 2003). VTS1 was identified as a multi-copy suppressor of helicase-negative dna2K1080E. The suppression was allele-specific since overexpression of Vts1 did not suppress the ts growth defects of dna2Δ405N (Lee et al., 2010). Purified recombinant Vts1 stimulated the endonuclease activity of wild type Dna2, but not of Dna2Δ405N devoid of the N-terminal domain, indicating that the activation requires the N-terminal domain of Dna2. Stimulation of Dna2 endonuclease activity by Vts1 appeared to be the direct cause of suppression, although it also stimulated Fen1 activity.

5.2.3 PCNA and RFC

RFC and PCNA are processivity factors for Pol δ and Pol ε. RFC, a clamp loader of PCNA, consists of five subunits (Rfc1 to 5) which share significant homology in seven regions referred to as RFC boxes (box II-VIII) (Cullman et al., 1995; Majka & Burgers, 2004). Although PCNA has been well known for its ability to stimulate Fen1 (Li et al., 1995; Tom et

al., 2000; Frank et al., 2001; Gary et al., 1999; Gomes & Burgers, 2000), human RFC complex was recently found to markedly stimulate Fen1 activity via multiple stimulatory motifs per molecule (Cho et al., 2009). Fen1 stimulation by RFC is a separable function from ATP-dependent PCNA loading to primer ends. Analysis of stimulatory domain of RFC4 revealed that only a small part ($RFC4_{170-194}$; subscripts indicate positions of amino acids) of it was sufficient to stimulate Fen1 activity and among them, the four amino acid residues were critical for Fen1 stimulation (Cho et al., 2009). The multiple stimulatory motifs present in the RFC complex could contribute to more rapid formation of ligatable nicks as an integral part of replication machinery while it moves along with replication forks (Masuda et al., 2007).

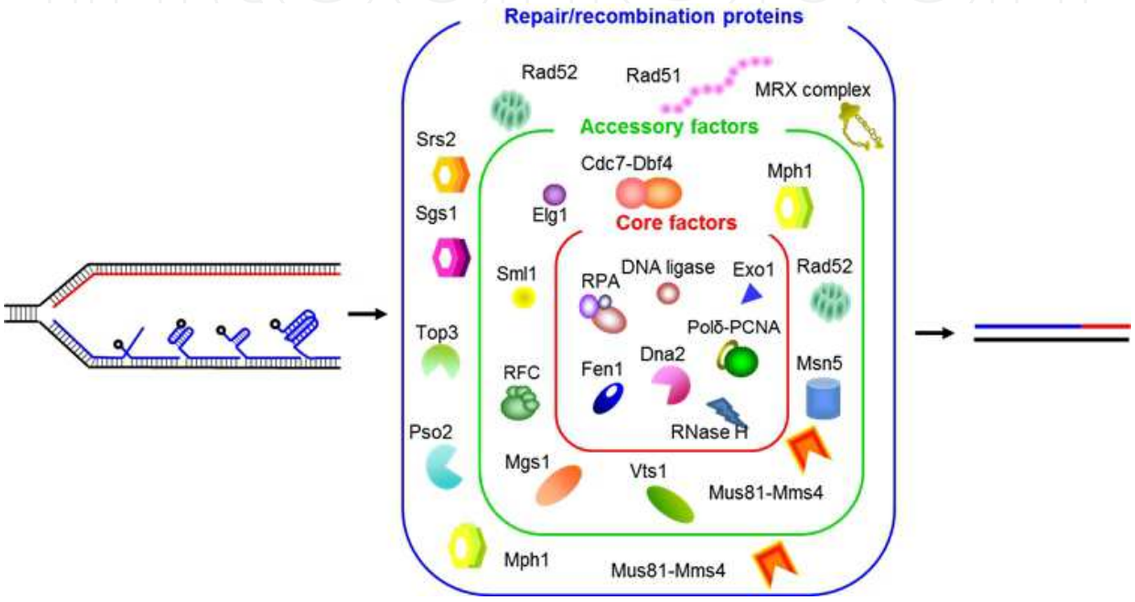

Fig. 3. Multiple layers of redundant pathways for failsafe processing of Okazaki fragments. Various flap structures, exemplified by four types only, can be generated during lagging strand synthesis. In most cases, it is believed that they can be processed by the combined action of 'core' factors in the first layer (indicated in the red box), the basic machinery for Okazaki fragment synthesis. 'Accessory factors' that constitute the second layer (indicated in the green box) function mostly to strengthen enzymatic activities of Dna2 and/or Fen1. When the 'core' proteins fail to function, unprocessed flaps can be removed by proteins in the third layer (indicated in 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 more than one layer. Pol α-primase is not shown for simplicity.

5.2.4 Mus81-Mms4

Mus81-Mms4 is a structure-specific endonuclease that can cleave nicked Holliday junctions, D-loop, replication forks, and 3'-flaps that could arise in vivo during the repair of damaged replication forks (Boddy et al., 2001; Kaliraman et al., 2001; Bastin-Shanower et al., 2003; Ciccia et al., 2003; Whitby et al., 2003). Overexpression of Mus81 suppressed the lethality of helicase-negative dna2K1080E (Kang et al., 2010) as well as dna2-2 and dna2-4, the two other dna2 mutant alleles isolated by others (Formosa & Nittis, 1999). In addition, Mus81-Mms4

and Fen1 stimulated each other in a manner requiring a specific protein-protein interaction. This indicates that the three endonucleases, Rad27, Mus81-Mms4, and Dna2, collaborate to remove a variety of structural intermediates in vivo.

5.2.5 Mph1 and Rad52

MPH1 was first identified as a mutator phenotype 1 gene (Entian et al., 1999), and the mph1Δ mutant displayed increased mutation rates and sensitivity to a variety of DNA damaging agents (Scheller et al., 2000). Based on this and other genetic studies, MPH1 is proposed to function in an error-free DNA damage bypass pathway that requires homologous recombination (Schürer et al., 2004). It was shown that Mph1 has DNA-dependent ATPase and 3′ to 5′ helicase activities (Prakash et al., 2005). Overexpression of Mph1 increased gross chromosomal rearrangements (GCR) by partially inhibiting homologous recombination through its interaction with RPA (Banerjee et al., 2008). These data suggest that Mph1 is important in maintaining the integrity of genome. MPH1 was isolated as a multicopy suppressor of dna2Δ405N and dna2K1080E. Purified Mph1 markedly stimulated the endonuclease activities of both Dna2 and Fen1 in vitro in an ATP-independent manner (Kang et al., 2009). Stimulation depends on the specific protein-protein interaction between the N-terminal domain of Dna2 and Mph1. Since overexpression of Mph1 also suppressed the dna2Δ405N mutant, the suppression of the Dna2 defect by Mph1 is due to the stimulation of Fen1 activity, and not of Dna2. Rad52 that mediates exchanging RPA with Rad51 in ssDNA is a multi-copy suppressor of dna2K1080E. Purified Rad52 is able to stimulate both Fen1 and Dna2 in vitro (Lee et al., 2011). The stimulation is independent of the recombination activity of Rad52.

5.3 Speculations on the presence of numerous stimulators of Dna2 and Fen1

In addition to the proteins mentioned above, the list of proteins that stimulate Fen1 and Dna2 is growing, which are most likely involved in maintenance of genome integrity. In humans, WRN, BLM, and RecQ5, the human homologues of yeast RecQ are an example of Fen1 stimulator (Brosh et al., 2001; Wang et al., 2005; Speina et al., 2010). Recently, it was shown that Dna2 and Pif1 can contribute to rapid nick formation by stimulating FEN1 (Henry et al., 2008). In addition, low levels of RPA also stimulated Fen1 activity particularly when short flaps were used as substrates. The acquisition of the ability of Fen1 or Dna2 to be stimulated by many proteins that work in close proximity may have conferred evolutionary benefits, because such an ability may permit faster generation and sealing of DNA nicks. Rapid generation and sealing of ligatable nicks may be more favorable in the preservation of genome integrity by converting unstable nicked DNA into stable duplex DNA.

5.4 Repair of faulty processing of Okazaki fragments

5.4.1 Homologous recombination as a last resort to repair faulty Okazaki fragments

When rad27-p (impaired interactions with PCNA) was combined with pol3-5DV (a mutant allele of a Pol δ subunit, defective in 3′ exonuclease and increased in displacement DNA synthesis), the double mutant cells were lethal in the absence of RAD51 that is essential for DSB repair (Jin et al., 2003). 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 required elevated levels of Dna2 for appropriate processing. In addition, the result above raises the possibility that excess levels of long flaps produced in

rad27-p pol3-5DV cells could undergo DSB that can be harmlessly repaired by RAD51-dependent repair pathway. This idea is further supported by a number of genetic data. First, dna2-C2 mutant cells displayed extensive chromosomal fragmentation like cdc9 (DNA ligase 1) mutation in S. pombe (Kang et al., 2000). Second, rad27Δ rad52Δ, dna2-1 rad27Δ, dna2-1 rad52Δ, dna2-2 rad52Δ double mutants are synthetic lethal (Jin et al., 2003; Budd et al., 2005). Third, ts dna2-22 mutant displayed increase in the rates of recombination and chromosome loss at non-permissive temperature (Fiorentino and Crabtree, 1997). Forth, the dna2-2 mutant cells showed hyperrecombination of rDNA, causing reduced life span of S. cerevisiae (Hoopes et al., 2002). In S. pombe, it was shown that functions of rhp51$^+$ (recombination gene RAD51 homolog) were required for viability of dna2 mutants (Tsutsui et al., 2005). Moreover, Rad52 was isolated as a multi-copy suppressor of helicase-negative dna2K1080E. Rad52 plays a role in the formation of Rad51-ssDNA filament by exchanging RPA with Rad51 (Song and Sung, 2000). Thus, the mediator function of Rad52 is crucial to initiate strand invasion. The rad52-QDDD-308-311-AAAA (rad52-QDDD/AAAA) mutant cells failed to form MMS-induced DNA repair foci and were not able to repair MMS-induced damage (Plate et al., 2008). Moreover, the mutant Rad52-QDDD/AAAA protein barely interacted with RPA and showed inefficient recombination mediator activity while retaining wild type levels of DNA binding activity (Plate et al., 2008). The suppression of dna2 mutation by Rad52 required the mediator activity of Rad52; rad52QDDD/AAAA mutant was not able to suppress dna2K1080E (Lee et al., 2011). This suggests that faulty Okazaki fragment could lead to elevated levels of homologous recombination. In support of this, we discovered that dna2Δ405N showed increases in the rates of inter- and intra-chromosomal recombination and unequal sister chromatid recombination (Lee et al., 2011). Our results suggest that incomplete replication of lagging strand synthesis due to faulty processing of Okazaki fragments could be efficiently repaired via Rad52-dependent homologous recombination pathway (Fig. 4) (Reagan et al., 1995; Tishkoff et al., 1997b; Budd and Campbell, 2000). Recently, it was found that Dna2 itself is a critical player in DSB repair by directly participating in long-range resection of DSB ends in cooperation with Sgs1 in a redundant fashion with Exo1 (Mimitou and Symington, 2008; Zhu et al., 2008). Both helicase activity of Sgs1 and nuclease activity of Dna2 were essential for this resection, whereas the helicase activity of Dna2 was dispensable (Mimitou and Symington, 2008 and 2009; Zhu et al., 2008; Niu et al., 2010; Shim et al., 2010).

5.4.2 Roles of Mph1 and Mus81-Mms4 as structure managers

The involvement of Mph1, Mus81-Mms4, and Rad52 in Okazaki fragment processing is particularly interesting, not only because of their abilities to stimulate the endonuclease activity of Dna2 and/or Fen1, but also because of their roles in recombinational repair of lagging strand replication defect as suggested previously (Ii & Brill, 2008). We found that Mph1 is a multipurpose helicase that can unwind a variety of DNA structures such as junction structures containing three or four DNA strands. Mph1 is able to unwind fixed double-flap DNA (an intermediate form of equilibrating flaps) in such a way that among the two flaps the displacement of 5′ flap occurs first (Kang et al., 2011). Thus, the helicase activity of Mph1 could contribute to Okazaki fragment processing by facilitating conversion of equilibrating flaps into 5′ flaps, which are readily cleaved by Fen1. In addition, Mph1 was able to efficiently displace hairpin-containing oligonucleotides, as long as short (~5-nt) ssDNA regions were present at the ssDNA/dsDNA junction. The ability of Mph1 to

displace 5′ secondary-structure flaps may allow cells to strip off the chronically problematic Okazaki fragments from the template, resulting in a gap equivalent in size to an Okazaki fragment, which can be filled in by Pol δ. Fen1 and Mus81-Mms4 appear to function in two separate processes because of their different substrate specificity (5′- and 3′-flap specific, respectively), the mutual stimulation observed in yeasts suggests a more direct inter-functional role between the two structure-specific endonucleases. The joint role of Fen1 and Mus81-Mms4 could come into effect via the interconversion between the substrates specific for each endonuclease. The 5′ or 3′ flap can be converted into a 3′ or 5′ flap, respectively, in a manner similar to that seen in Holliday junction migration. The equilibrating flaps (see Fig. 1. for structure) could be processed more rapidly if 5′ and 3′ flap specific enzymes could stimulate each other's activity.

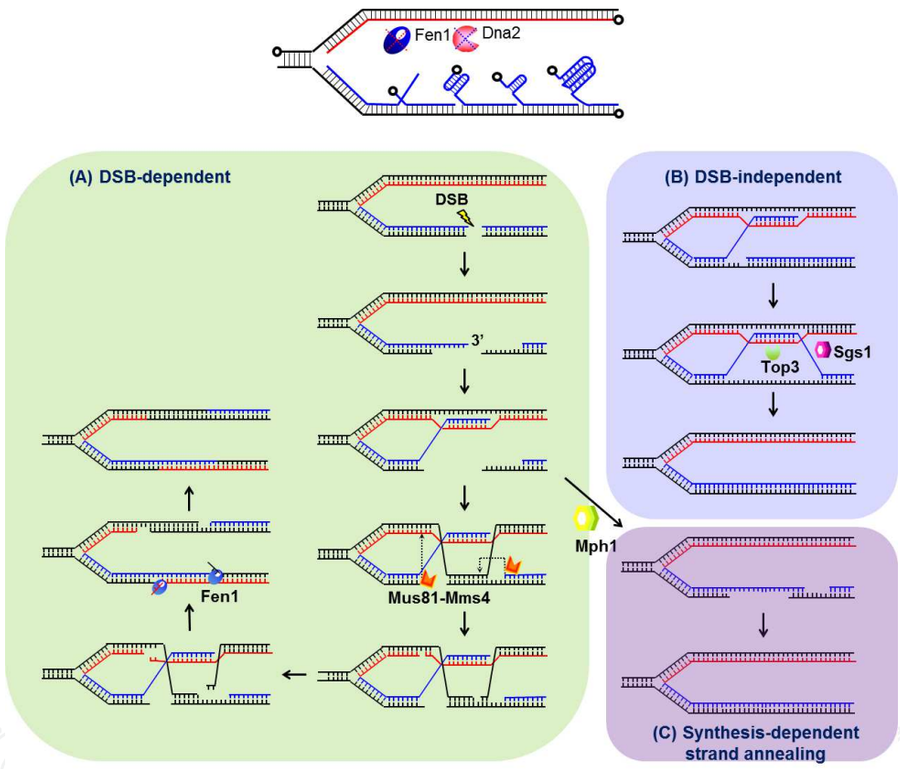

Fig. 4. Possible repair pathways for unprocessed flaps due to malfunction of Fen1 and/or Dna2. The unprocessed flap can be repaired via either DSB-dependent or -independent pathway. (A) In DSB-dependent pathway, replicated lagging strand containing unprocessed flap undergoes a DSB, followed by resection by the MRX complex (not shown). The resulting 3′ overhang starts homologous recombination by invading leading strand DNA. (B) If DSB is not involved, the 3′ flap, which could result from a 5′ unprocessed flap via 'equilibration,' can initiate recombination by invading leading strand DNA. If nicks are available, the resulting recombination intermediate can be resolved by Mus81-Mms4 catalyzed nick-directed cleavage (not shown in *B*). Alternatively, the intermediate can be converted into substrates for the Sgs1-Top3 pathway by forming pseudo double Holliday junctions (not shown in *A*). (C) Mph1 can remove the D-loop formed, facilitating synthesis-dependent strand annealing.

A helicase such as Mph1 could facilitate the interconversion process by virtue of its ability to displace the downstream strand. The product formed by this reaction would contain either 5′ or 3′ ssDNA flap depending on the polarity of the helicase involved, generating the structures suitable for cleavage by either Mus81-Mms4 or Fen1. Most likely candidates for such a function would include the helicases with branch migration activities such as WRN, RecQ1, and Mph1 (Prakash et al., 2009; Opresko et al., 2009; Burgreev et al., 2008). The human BLM helicase was shown to stimulate nuclease activity of the Mus81-Eme1 complex (Zhang et al., 2005). In addition, Rad54 was found to strongly stimulate Mus81-Mms4 in an ATP-independent manner in humans and yeasts (Matulova et al., 2009). Alternatively, a nuclease(s) that can simultaneously process both 5′ and 3′ double flaps could reduce the length of both flaps. This could more rapidly generate a DNA substrate that can be processed by either Fen1 or Mus81-Mms4. It was shown that endonuclease activity of human Dna2 is stimulated in the presence of double flaps (Kim et al., 2006).

6. Concluding remarks

Processing of Okazaki fragments is a complicated process at high risks of various types of DNA alterations such as base change, repeat expansion, and small duplications due to the involvement of anomalous structural DNA– a special type of DNA damages which, if left unrepaired, can promote genome instability. Examples of anomalous structure include nicks, unprocessed flaps, DSBs, and recombination intermediates. Formation of anomalous structures can be prevented by preemptive actions of Dna2 and/or by numerous 'auxiliary' factors that enhance endonuclease activities of Fen1 or Dna2. Alternatively, anomalous structures can be repaired by first forming DSBs, a key event that activates recombination. DSB-mediated recombination is regarded as the basis of genetic instability in eukaryotes since it can be a source of illegitimate recombination in higher organisms. A diverse array of auxiliary factors identified up to date may be a mirror image of a variety of structural intermediates present in vivo. The highly dynamic and capricious nature of structural intermediates renders correct processing of Okazaki fragments a formidable task which has to rely on a number of factors important for genome maintenance. Thus, Okazaki fragment processing is a platform where a number of proteins with roles in DNA replication and repair/recombination act together to minimize the hazardous outcome associated with its mechanisms in eukaryotes. In the future, the biggest challenge would be complete understanding of how each of the factors involved is regulated to fit into the complicated and dynamic network of protein-protein interactions required for failsafe processing of Okazaki fragments.

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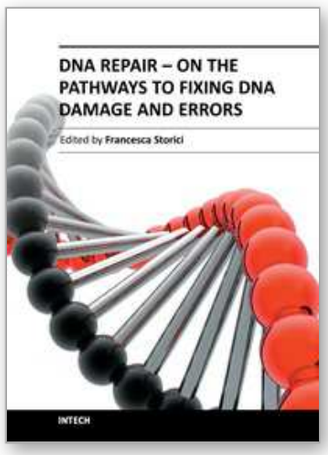

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DNA repair is fundamental to all cell types to maintain genomic stability. A collection of cutting-edge reviews, DNA Repair - On the pathways to fixing DNA damage and errors covers major aspects of the DNA repair processes in a large variety of organisms, emphasizing foremost developments, questions to be solved and new directions in this rapidly evolving area of modern biology. Written by researchers at the vanguard of the DNA repair field, the chapters highlight the importance of the DNA repair mechanisms and their linkage to DNA replication, cell-cycle progression and DNA recombination. Major topics include: base excision repair, nucleotide excision repair, mismatch repair, double-strand break repair, with focus on specific inhibitors and key players of DNA repair such as nucleases, ubiquitin-proteasome enzymes, poly ADP-ribose polymerase and factors relevant for DNA repair in mitochondria and embryonic stem cells. This book is a journey into the cosmos of DNA repair and its frontiers.

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