Review

Okazaki fragment maturation: nucleases take centre stage

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Completion of lagging strand DNA synthesis requires processing of up to 50 million Okazaki fragments per cell cycle in mammalian cells. Even in yeast, the Okazaki fragment maturation happens approximately a million times during a single round of DNA replication. Therefore, efficient processing of Okazaki fragments is vital for DNA replication and cell proliferation. During this process, primase-synthesized RNA/DNA primers are removed, and Okazaki fragments are joined into an intact lagging strand DNA. The processing of RNA/DNA primers requires a group of structure-specific nucleases typified by flap endonuclease 1 (FEN1). Here, we summarize the distinct roles of these nucleases in different pathways for removal of RNA/DNA primers. Recent findings reveal that Okazaki fragment maturation is highly coordinated. The dynamic interactions of polymerase δ, FEN1 and DNA ligase I with proliferating cell nuclear antigen allow these enzymes to act sequentially during Okazaki fragment maturation. Such protein–protein interactions may be regulated by post-translational modifications. We also discuss studies using mutant mouse models that suggest two distinct cancer etiological mechanisms arising from defects in different steps of Okazaki fragment maturation. Mutations that affect the efficiency of RNA primer removal may result in accumulation of unligated nicks and DNA double-strand breaks. These DNA strand breaks can cause varying forms of chromosome aberrations, contributing to development of cancer that associates with aneuploidy and gross chromosomal rearrangement. On the other hand, mutations that impair editing out of polymerase α incorporation errors result in cancer displaying a strong mutator phenotype.

Keywords: Okazaki fragment maturation, FEN1, DNA2, PCNA, RPA, cancer

Introduction

Replication of double-stranded DNA is the central process in cell proliferation. In eukaryotic cells, DNA replication initiates at multiple origins on each chromosome and proceeds bi-directionally from each origin into the flanking DNA, forming a DNA replication fork (Figure 1). As the replication machinery moves along the DNA, the synthesis of both new strands occurs simultaneously. To achieve this, replication is semi-discontinuous: the leading strand is synthesized continuously by polymerase ε (Pol ε), from a single initiation event at the replication origin, whereas the lagging strand is initiated by the primase [a hetero-tetramer of RNA polymerase and DNA polymerase α (Pol α) that synthesizes the RNA primer and a short portion of DNA (termed the α-segment)] and is extended by polymerase δ (Pol δ) as a series of discrete Okazaki fragments (Pursell et al., 2007; Kunkel and Burgers, 2008; Nick McElhinny et al., 2008; Burgers, 2009). Primase synthesizes primers to initiate both leading and lagging strand synthesis. On the leading strand, Pol α is displaced by the combined action of replication factor C (RFC), proliferating cell nuclear antigen (PCNA) and Pol ε (Lovett, 2007; Kunkel and Burgers, 2008; Burgers, 2009). As a result, a highly processive polymerization complex is assembled, which carries out the leading strand DNA synthesis (Lovett, 2007; Kunkel and Burgers, 2008; Burgers, 2009). On the other hand, the DNA Pol α/primase complex places frequent RNA primers, 7–14 nucleotides (nt) in length, on the lagging strand and elongates them by addition of 10–20 deoxyribonucleotides. Subsequently, Pol α is displaced by Pol δ via an RFC/PCNA-dependent polymerase switching mechanism, which initiates a processive DNA synthesis of segments of nascent lagging strand DNA, ~200 nt in length (Waga and Stillman, 1994; Bambara et al., 1997; Burgers, 2009). The RNA primers in these nascent segments, or Okazaki fragments, must be removed, and the Okazaki fragments are joined into an intact lagging DNA strand. This process is dependent on the actions of nucleases. Over the last two decades, several nucleases, including RNase H, flap endonuclease 1 (FEN1) and Dna2, have been implicated as being involved in processing of Okazaki fragments (Turchi et al., 1994; Waga and Stillman, 1994; Bae et al., 2001; MacNeill, 2001). Here, we review recent advances in elucidating the roles of FEN1 and other nucleases in the Okazaki fragment maturation process. We also discuss insights into how nucleases and other enzymes are regulated in this highly ordered process and the biological consequences of impairing one of these steps.

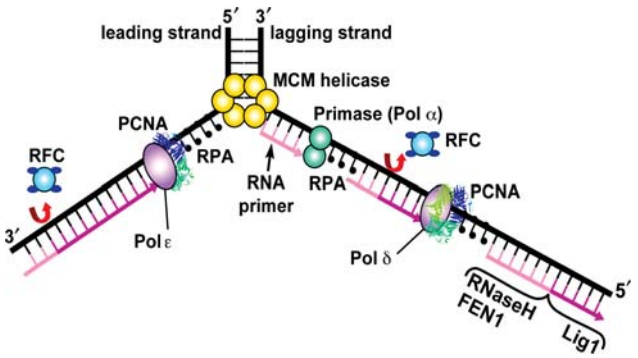

Figure 1 Enzymes and reactions in the DNA replication fork. Major proteins factors present in a typical replication fork include: (**i**) mini-chromosome maintenance (MCM) proteins (six homo-subunits), which are helicases for opening up the DNA duplex to initiate a DNA replication fork; (**ii**) RPA, a single-stranded DNA binding protein to protect the DNA template from nuclease cleavage; (**iii**) primase (a complex of RNA polymerase and Pol α), which synthesizes RNA primers and a short DNA fragment to initiate Okazaki fragments; (**iv**) Pol δ, the DNA polymerase responsible for synthesizing the major portion of Okazaki fragments; (**v**) Pol ε, the DNA polymerase responsible for leading strand DNA synthesis; (**vi**) PCNA, which is the DNA clamp for the processivity of DNA polymerase and coordination of Okazaki fragment maturation processes; (**vii**) RFC, which is the clamp loader for PCNA to load onto DNA duplex; (**viii**) nucleases, including RNase H, DNA2 and FEN1 for removal of RNA primers and (**ix**) DNA Lig I, which joins processed Okazaki fragments into an intact DNA lagging strand. Black lines represent the DNA template, while pink ones are the newly synthesized DNA and light pink ones are the RNA primers.

FEN1-dependent cleavage of the short primer flap

Three distinct pathways have been proposed to elucidate how RNA/DNA primers are removed by nucleases during Okazaki fragment maturation (Figure 2). RNase H, which degrades ribonucleotides on RNA–DNA hybrid duplex, was initially identified as an important enzyme for RNA primer removal (Turchi and Bambara, 1993; Turchi et al., 1994; Waga and Stillman, 1994; Waga et al., 1994). The biochemical property of RNase H makes it a perfect candidate for the nuclease involved in RNA primer removal. It has been hypothesized that RNase H cleaves all ribonucleotides of the RNA primer except the last one, which is removed by the 5′ exonuclease activity of FEN1 (Figure 2) (Turchi and Bambara, 1993; Turchi et al., 1994; Waga and Stillman, 1994; Waga et al., 1994). However, in yeast, deletion of individual genes encoding RNase H1 or RNase H2, or combinations of these null mutations, does not cause an obvious phenotype (Frank et al., 1998; Qiu et al., 1999), suggesting that this pathway is not the primary mechanism for RNA primer removal and other pathways may be responsible for processing of RNA/DNA primers.

It has been indicated that during lagging strand DNA synthesis, the replicating DNA polymerase/PCNA complex encounters and displaces the RNA primer of the previous Okazaki fragment, resulting in a 5′ single-stranded flap structure (Bambara et al., 1997; Liu et al., 2004; Burgers, 2009). There are two distinct mechanisms for processing the displaced RNA primer in eukaryotic cells, depending on the length of the RNA primer flap (Figure 2). In the first model, Pol δ displaces the RNA primer to create a predominantly short flap of 2–10 nt (Bambara et al., 1997; Liu et al., 2004). Cleavage of such short flap structures is mediated by FEN1, which recognizes branched nucleic acid structures that contain a single-stranded 5′ flap and threads through or slides down the single-stranded DNA and cleaves the DNA substrate at the junction between double-stranded and single-stranded DNA, precisely producing a ligatable DNA end (Figure 2) (Harrington and Lieber, 1994, 1995; Murante et al., 1994; Nolan et al., 1996; Bambara et al., 1997; Zhu et al., 1997; Hosfield et al., 1998; Shen et al., 2005). More recently, Burger's group has further proposed a model to elucidate the highly coordinated actions of Pol δ and FEN1 in removal of short RNA primer flaps (Garg et al., 2004; Stith et al., 2008). They revealed that in most cases the interaction between the 3′ exonuclease and the polymerase activities of Pol δ limits the forward movement of Pol δ, allowing the addition of only 1–2 nt in the strand displacement DNA synthesis. In addition, after gap-filling, Pol δ is replaced by

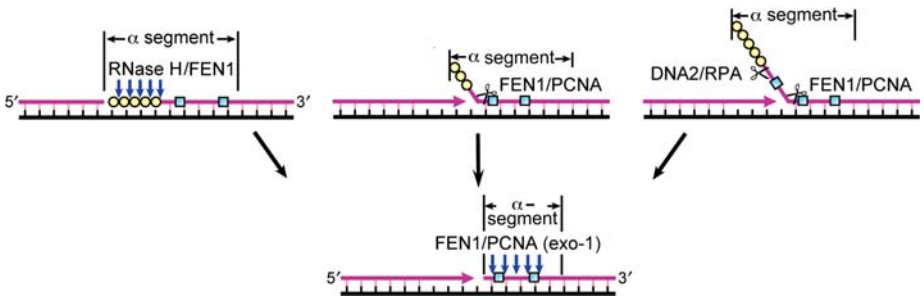

Figure 2 Distinct roles of nucleases in sequential processing of RNA/DNA primers (α-segment). Upper panels show three different pathways involved in processing RNA primers: (**i**) FEN1-mediated short flap cleavage (middle); (**ii**) long flap degradation by sequential actions of DNA2 and FEN1 (right) and (**iii**) RNA primer removal by RNase H/FEN1 exonuclease (left). The bottom panel indicates the action of FEN1 or exonuclease to edit out incorporation errors of Pol α. Yellow circles represent ribonucleotides and cyan squares represent mismatched deoxyribonucleotides. Black lines correspond to DNA templates and pink lines correspond to newly synthesized DNA. Blue arrows indicate cleavage by RNase H or the exonuclease activity of FEN1 or exo-1.

FEN1 via a hand-off mechanism to cleave the mono- or di-ribonucleotide flap. If the RNA primer remains present, Pol δ returns. The Pol δ-driven gap filling and FEN1-mediated short flap cleavage continue for several cycles until all ribonucleotides are removed, leading to DNA–DNA ends that are sealed by DNA ligase I (Lig I).

The FEN1-dependent short flap pathway has been shown to be the dominant pathway in the removal of RNA primers. This is supported by several *in vivo* studies using yeast and mouse models. FEN1 deficiency in mice causes defects in DNA replication, failure of cell proliferation and embryonic lethality (Kucherlapati et al., 2002; Larsen et al., 2003; Zheng et al., 2007a). In yeast, deletion of Rad27 (FEN1 yeast homolog) causes slow growth at the permissive temperature (30°C) and cell death at the stringent temperature (37°C). Furthermore, rad27△ mutant yeast cells display a unique duplication mutator phenotype, which is likely due to the ligation of an un-removed flap with the downstream Okazaki fragment. However, the observation that rad27△ mutations are, surprisingly, not lethal suggests that redundant nucleases are involved in RNA primer removal in yeast. One such nuclease is the 5′ exonuclease 1 (Exo1), which also displays 5′ flap endonuclease activity, albeit at a much lower level than FEN1 (Lee and Wilson, 1999). Deletion of both RAD27 and EXO1 in yeast causes synthetic lethality, suggesting a role for Exo1 in backing up the function of FEN1 in RNA primer removal and/or DNA recombination (Tishkoff et al., 1997a). DNA recombination provides an alternative pathway to process RNA primers when FEN1 is deficient (Tishkoff et al., 1997a). On the other hand, long flap structures may form in the absence of RAD27, and the RNA primers may be removed by the alternative long flap pathway.

Sequential actions of DNA2 and FEN1 in removal of long primer flap

The existence of the long flap pathway during Okazaki fragment maturation was revealed by studies of Dna2 mutant yeast strains, indicating that DNA2 is essential for DNA replication in yeast. Dna2 proteins, members of the nuclease/helicase family (Budd et al., 1995), are present in numerous organisms and have distinct nuclease, ATPase and helicase domains (Budd et al., 1995; Bae and Seo, 2000). Biochemical studies revealed that DNA2 nucleases preferentially recognized and cleaved 5′ long flap DNA structures (Bae et al., 1998; Bae and Seo, 2000; Bae et al., 2001; Kim et al., 2006; Masuda-Sasa et al., 2006; Zheng et al., 2008). However, unlike the typical FEN1, which cleaves single-stranded flap DNA substrates at the junction between single-stranded and double-stranded DNA, DNA2 nucleases remove a portion of DNA in the middle of the long single-stranded DNA flap, generating a short flap (Bae et al., 1998; Bae and Seo, 2000; Bae et al., 2001; Kim et al., 2006; Masuda-Sasa et al., 2006; Zheng et al., 2008). Single-stranded RNA/DNA flaps longer than 30 nt may attract the single-stranded DNA binding protein, replication protein A (RPA), which inhibits the flap endonuclease activity of FEN1 (Bae et al., 2001; Chai et al., 2003). Seo's group first proposed that the binding of RPA to the single-stranded RNA primer recruits Dna2 and stimulates its nuclease activity to cleave a large portion of the long RNA/DNA primer flap, as Dna2 can clamp onto the site (Figure 2) (Bae et al., 2001). This generates a short flap (∼5–7 nt) that resists binding by RPA and cleavage by Dna2. The remaining flap is removed by FEN1 to produce a substrate for ligation (Figure 2). In addition, in FEN1-deficient yeast, the short flap can be processed by the 3′ exonuclease activity of Pol δ via a 5′ and 3′ flap equilibrium mechanism, in which the 5′ flap is converted into a 3′ flap (Jin et al., 2001, 2003).

Yeast genetic studies have offered supporting evidence for the formation of the long flap structure *in vivo* and mechanistic insights into the long flap pathway. rad27△ mutant yeast cells contain duplication mutations that may be as long as 100 nt, indicating that the flap can be up to 100 nt long (Tishkoff et al., 1997b). In addition, compromising the 3′ exonuclease activity of Pol δ and FEN1 in yeast causes synthetic lethality; however, overexpression of Dna2 can rescue these double mutant cells (Jin et al., 2001, 2003). More recently, a study on Pif1, a DNA helicase important for DNA replication and telomere stability, suggested that the helicase promotes the generation of long flaps (Rossi et al., 2008; Pike et al., 2009). Supporting this hypothesis, deletion of Pif1 can suppress the lethal phenotype of the dna2△ mutant yeast strain, in which processing of the long flap fails (Rossi et al., 2008; Pike et al., 2009). Although Pol δ predominantly displaces the short DNA flap, long flaps may occur due to the presence of robust helicase activity such as Pif1 and/or dysfunction of Pol δ and FEN1.

Despite evidence suggesting its role in yeast, the alternative long flap pathway may not be important in mammalian Okazaki fragment maturation. In human cells, DNA2, unlike its yeast homolog, predominantly migrates into the mitochondria (Zheng et al., 2008) and the residual nuclear DNA2 is not associated with DNA replication foci (Zheng et al., 2008; Duxin et al., 2009), indicating that human DNA2 is not a primary component in human DNA replication machinery. This finding suggests that the DNA2-mediated long flap pathway is critical for RNA primer removal only in yeast and other lower eukaryotic organisms.

Editing out Pol α errors

At first glance, the processing of Okazaki fragments might appear to only remove the short RNA primers, replace them with DNA and join DNA fragments. However, primase lacks a proofreading function (Foiani et al., 1997; Pavlov et al., 2006); therefore, the initial deoxyribonucleotides in the Okazaki fragment contain mis-incorporated bases (Figure 2), which are highly mutagenic and must be removed. Otherwise, these initiation regions of Okazaki fragments would be mutation hotspots, leading to dramatic elevation of mutation rates. Three mechanisms may be involved in suppressing DNA mutations resulting from the incorporation errors of Pol α. First, the 3′ exonuclease activity of Pol δ may form a complex with Pol α and help to proofread Pol α errors during Pol α-driven RNA–DNA primer synthesis. Supporting this hypothesis, mutant yeast cells carrying the L868M Pol α mutation, which reduces its DNA polymerase fidelity, displayed a 6-fold increase in mutation rate and the proofreading-

defective Pol δ mutant yeast (exo-Pol δ) had a 7-fold increase in mutation rate. However, the L868M Pol α and exo-Pol δ double mutant displayed a 70-fold elevation of the mutation rate (Pavlov et al., 2006), suggesting the two polymerases may functionally interact with each other and the 3′ exonuclease of Pol δ may proofread mis-incorporation errors of Pol α. Second, if the mis-incorporation escapes proofreading, it may be edited out by FEN1 during RNA primer removal (Figure 2). The mis-paired nucleotide could be displaced in the 5′ flap and removed by FEN1 through the short flap pathway or by the concerted action of DNA2 and FEN1 in the long flap pathway (Figure 2). On the other hand, if the mis-paired nucleotide is not displaced into the flap, the 5′ exonuclease activity of FEN1 may remove a few nucleotides from the 5′ end of the nick, followed by Pol δ-driven gap-filling (nick-translation) to edit out the incorporation error (Figure 2). Consistent with this view, we observed that mouse embryonic fibroblasts or yeast cells carrying the FEN1 exonuclease-defective mutation E160D (or E158D in yeast) had a 25-fold increase in rates of base substitution (Zheng et al., 2007b). Last, the incorporation error may be repaired via the DNA mismatch repair pathway, in which the MSH2/MLH1 complex recognizes the mismatched base and recruits EXO1 to remove it from either the 5′–3′ or the 3′–5′ direction (Figure 2) (Kolodner and Marsischky, 1999; Sun et al., 2002; Wei et al., 2003). These multiple mechanisms ensure the accuracy of lagging strand DNA synthesis.

Regulation of Okazaki fragment maturation processes

Completion of lagging strand DNA synthesis requires processing of up to 50 million Okazaki fragments per cell cycle in mammalian cells. Even in yeast, the Okazaki fragment maturation happens $\sim 1 \times 10^6$ times during a single round of DNA replication. Therefore, efficient processing of Okazaki fragments is vital for DNA replication and cell proliferation. Because Okazaki fragment maturation involves multiple enzymatic reactions, including Pol δ-driven gap-filling, FEN1-mediated flap cleavage and Lig I-catalyzed DNA ligation, these enzymes must be effectively recruited to the replication site to execute their function. However, they also need to promptly dissociate from DNA substrates, allowing downstream enzymes to access DNA substrates. Thus, different enzymes will not compete for DNA substrates and ensure efficient completion of Okazaki fragment maturation processes. Recent studies from our and other groups have shed light onto how cells achieve highly ordered Okazaki fragment maturation processes (Chapados et al., 2004; Sporbert et al., 2005; Dore et al., 2006; Zheng et al., 2007a). PCNA has been implicated as a platform for recruiting different enzymes to the replication sites and coordinating the sequential actions of polymerase, FEN1 and DNA Lig I at DNA replication forks (Figure 3) (Chapados et al., 2004; Sporbert et al., 2005; Dore et al., 2006). Pol δ, FEN1 and Lig I share the same PCNA-binding motif, Qxx(L/I)xxF(F/Y) (Gary et al., 1997; Levin et al., 2000; Frank et al., 2001), and both the motif QRSIESFFK in Lig I and the motif QGRLDDFFK in FEN1 can bind to a subunit of PCNA.

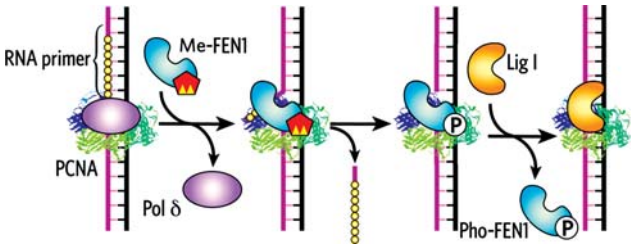

Figure 3 Model for post-translational modifications that mediate the interaction between FEN1 and PCNA, and thus regulate the dynamic actions of FEN1 in processing of Okazaki fragment maturation. The Pol δ/PCNA complex drives the gap filling and formation of the flap structure in Okazaki fragment mutation. Methylated FEN1 is recruited to the replication fork by interacting with PCNA, replacing Pol δ. Methylation of FEN1 ensures its interaction with and stimulation by PCNA to remove the flap structure. After DNA flap cleavage, FEN1 undergoes de-methylation and subsequent phosphorylation, leading to FEN1 dissociation from the DNA nick. DNA Lig I is then recruited by interaction with PCNA and seals the nicks between the two Okazaki fragments.

This physical interaction is critical for coordinating FEN1 and Lig I during DNA replication (Chapados et al., 2004; Refsland and Livingston, 2005; Sporbert et al., 2005; Subramanian et al., 2005; Dore et al., 2006). Two models have been proposed to elucidate how PCNA coordinates the actions of FEN1 and Lig I (Chapados et al., 2004; Refsland and Livingston, 2005; Subramanian et al., 2005; Dore et al., 2006). In the first model, rotary-handoff (Chapados et al., 2004; Dore et al., 2006), both FEN1 and Lig I recognize the PCNA-bound DNA substrate, which can rotate at the PCNA site. The rotation allows FEN1 and Lig I, which are bound to one of three binding sites on PCNA, to sequentially access intermediate DNA substrates. The second model proposes that the binding of PCNA by FEN1 and by Lig I is mutually exclusive. The competition between Lig I and FEN1 for PCNA binding is crucial for the sequential loading of FEN1 and Lig I onto the DNA replication fork (Subramanian et al., 2005). Supporting this model, the co-crystal of the human Lig I-DNA complex indicates that Lig I encircles the DNA substrate with a similar ring size and shape to that of PCNA, and that PCNA binding of FEN1 or Lig I excludes the other from interacting with PCNA (Pascal et al., 2004). In either situation, the interaction of PCNA with FEN1 and Lig I is critical for efficient transition from the FEN1 cleavage reaction to Lig I-mediated DNA ligation. In fact, a mutation in FEN1, which disrupts the physical interaction with PCNA, resulted in retarded processing of α-segments and severe pathological consequences (Zheng et al., 2007a).

One critical question is how FEN1 and other enzymes can efficiently bind to PCNA and DNA substrates, and then dissociate once the nuclease reaction is complete to avoid blocking the Lig1 reaction during DNA replication. Our recent discovery about FEN1 offers an exemplary case in solving this puzzle (Guo et al., 2010). We demonstrated that FEN1 was methylated at residue R192, which prevented FEN1 phosphorylation S187. In late S or G2 phase, the amount of methylated FEN1 decreased, which coincided with an increase in phosphorylated FEN1. Furthermore, we showed that methylated FEN1 effectively

interacted with PCNA (Guo et al., 2010), but phosphorylated FEN1 promptly dissociated from PCNA (Henneke et al., 2003; Guo et al., 2010). Thus, the interplay between FEN1 methylation and phosphorylation provides a mechanism for the nuclease to dynamically associate and dissociate with PCNA and thus the DNA substrate (Guo et al., 2010). Therefore, we propose the following model to illuminate how PCNA coordinates FEN1 and other enzymes during the processing of Okazaki fragments (Figure 3). In the early stage of Okazaki fragment maturation, FEN1 exists in a methylated form, which enables it to replace Pol δ and access PCNA and the flap structure. Upon cleavage of the RNA primer flap, FEN1 is demethylated, which allows the nuclease to be phosphorylated by cell cycle-dependent kinases. Phosphorylation of FEN1 causes the nuclease to fall off PCNA and the DNA nicks, leading to recruitment of Lig I and DNA ligation. In addition, we postulate that similar to methylation and phosphorylation of FEN1, post-translational modifications may occur in other Okazaki fragment maturation proteins and play important roles in regulating the actions of these proteins during processing of Okazaki fragments. Determining the dynamic modifications and corresponding protein–protein interactions of these enzymes at different stages of the Okazaki fragment maturation process will eventually provide a high-resolution picture of how different DNA replication enzymes and accessory proteins are able to process millions of Okazaki fragments within hours.

RNA primer processing in mitochondria

Each cell contains hundreds to thousands of mitochondria and each mitochondrion contains several copies of the mitochondrial DNA (mtDNA) genome (Shadel and Clayton, 1997). Mammalian mtDNA is a circular molecule of approximately 16 kb and is typically replicated via a strand-displacement mode (Shadel and Clayton, 1997). DNA synthesis is initiated at the replication origin of the H-strand (O_H) to replicate the single-stranded H-strand, which results in a D-loop structure. The expansion of the D-loop structure exposes the replication origin of the L-strand, leading to DNA synthesis of the L-strand in the opposite direction. Thus, unlike nuclear DNA replication, mtDNA replication by the strand displacement mode only involves leading strand DNA synthesis and requires only one primer for synthesis of one copy of mtDNA (Shadel and Clayton, 1997). Recently, using two-dimensional gel electrophoresis of the replication intermediates, Holt's group observed that mitochondrial replication forks were similar to those in nuclear DNA replication. This observation suggests that the strand-coupled DNA replication mechanism is also used to replicate mtDNA (Holt et al., 2000; Yang et al., 2002). Other studies showed that both mechanisms were employed to replicate mtDNA in eukaryotic cells (Fish et al., 2004).

It is generally accepted that RNA primers are removed during a maturation process in mtDNA replication, regardless of which replication mode is used (Shadel and Clayton, 1997; Bogenhagen and Clayton, 2003). However, the molecular mechanisms by which RNA primers are removed in mitochondria remain unclear. A study has shown that RNase H1 is critical for mitochondrial biosynthesis (Cerritelli et al., 2003). More recently, we and others have found that FEN1 and DNA2, which are involved in nuclear Okazaki fragment maturation, migrate into mitochondria (Copeland and Longley, 2008; Liu et al., 2008; Zheng et al., 2008; Duxin et al., 2009). Therefore, we hypothesize that all three mechanistic pathways for nuclear Okazaki fragment maturation may also be critical in mtDNA replication (Figure 2). (i) RNase H cleaves the RNA portion, and FEN1 then removes the last ribonucleotide and a portion of the deoxyribonucleotides, generating a ligatable DNA end. (ii) During DNA synthesis, the RNA primer can be displaced into a flap shorter than 10 nt, which is cleaved by FEN1. A nick-translation reaction coupling the nuclease-driven flap cleavage and polymerase-driven displacement gap-filling may efficiently remove the whole RNA primer. (iii) Extensive displacement may occur and result in a long flap structure, which is not an optimal substrate for FEN1. Mitochondrial single-stranded DNA binding protein binds to the single-stranded flap and further suppresses the flap endonuclease activity. In such a case, the DNA2 nuclease/helicase is required to cleave the long flap, producing a short flap for FEN1 to cut.

Defects in Okazaki fragment maturation and cancer

Failure in Okazaki fragment maturation in yeast has been linked to increases in DNA strand breaks, mutator phenotypes and other genome instabilities, which suggests that defects in Okazaki fragment maturation may contribute to cancer initiation and/or progression. Severe defects in Okazaki fragment maturation may halt DNA replication and induce cell death. Therefore, we postulate that mutations or deletions that eliminate the functions of the genes essential for this process are rare in human populations, but those causing subtle functional alterations in this process may exist. These subtle defects in Okazaki fragment maturation processes do not affect growth and development, but result in accumulation of varying forms of genome instabilities, leading to early onset of cancer.

Supporting this hypothesis, homozygous knockout mutant mice for genes such as FEN1 and Lig 1 exhibit failure of cell proliferation and early embryonic lethality (Bentley et al., 2002; Kucherlapati et al., 2002; Larsen et al., 2003). In addition, we have recently identified an FEN1 mutation F343A/F344A (FFAA), which disrupts the interaction between FEN1 and PCNA (Zheng et al., 2007a). Consequently, the FFAA FEN1 mutant proteins fail to localize to replication sites to execute FEN1's function in Okazaki fragment maturation. The FFAA/FFAA MEF cells grow slower than wild-type cells and mice homozygous for the FFAA FEN1 mutation die immediately after birth due to pancytopenia and pulmonary hypoplasia (Zheng et al., 2007a). Interestingly, the FFAA FEN1 mutation displayed partial defects in Okazaki fragment maturation, which allowed heterozygous mutant mice to survive although they accumulated unligated nicks in the genome. These single-stranded DNA breaks collapse DNA replication forks in the next round of DNA replication, resulting in

double-strand breaks, which subsequently cause chromosome breaks and/or gross chromosomal rearrangements (GCR). In addition, the mutant cells are more likely to become polyploid and aneuploid. These chromosomal aberrations, commonly observed in human cancers, have been thought to promote cancer development (Zheng et al., unpublished data).

Another group of genetic mutations may affect non-essential processes for editing out Pol α errors, thus increasing the mutation rate but not affecting cell proliferation. As a consequence, cells have an elevated chance of activating oncogenes or inactivating tumor suppressor genes due to somatic DNA mutations. A previous study showed a point mutation D400A at the 3′ exonuclease domain of Pol δ, which may be responsible for proofreading Pol α errors, increased the DNA mutation frequency and cancer incidence (Goldsby et al., 2001). More recently, we identified a group of exonuclease-defective FEN1 mutations in human cancer (Zheng et al., 2007b). We modeled these human FEN1 mutations in mice by introducing the E160D FEN1 mutation into the mouse genome. The E160D FEN1 mutation eliminates 90% of the exonuclease activity, which is likely important for editing out the incorporation errors of Pol α, but retains the flap endonuclease activity critical for RNA primer removal (Zheng et al., 2007b). Thus, E160D MEF cells have a DNA replication and proliferation rate similar to wild-type cells but display high rates of base substitution. Mutant mice bearing the E160D FEN1 mutation grow and develop normally like their wild-type littermates, but develop mutator phenotype-associated lung cancer at relatively early stages (Zheng et al., 2007b). These mouse studies provide convincing evidence supporting the concept that defects in Okazaki fragment maturation cause cancer. We further suggest that there are two distinct molecular mechanisms in cancer development due to defects in different processes during Okazaki fragment maturation. Mutations that affect the efficiency of RNA primer removal are likely to result in DNA strand breaks, which cause GCR and/or chromosome instabilities and subsequently cancer that associates with the chromosomal aberrations. On the other hand, mutations that affect the editing out of Pol α incorporation errors may lead to cancer displaying a strong mutator phenotype.

Concluding remarks

Recent studies have provided compelling evidence indicating the distinct roles of nucleases in Okazaki fragment maturation. In yeast, FEN1-mediated cleavage of short RNA primer flaps and degradation of long flaps by the sequential actions of DNA2/FEN1 are both critical for RNA primer removal and DNA replication. However, recent biochemical studies have suggested that DNA2 may not localize to the replication sites and play an important role in RNA primer removal in mammals. Studies using DNA2 knockout mutant mice will clarify this issue. Moreover, characterization of FEN1 and DNA2 mutant mouse models will provide high resolution pictures elucidating the role of these nucleases in mammalian cells.

Several lines of evidence have emerged to support the hypothesis that Okazaki fragment maturation is a highly ordered process and that PCNA plays a vital role in coordinating different enzymes in this process. An exciting observation is that the interplay between methylation and phosphorylation of FEN1 modulates its dynamic interactions with PCNA, providing insights into how a nuclease is able to access DNA substrates when needed and promptly dissociate from them when its job is done. However, many other questions remain, including: (i) how methylated FEN1 actively binds PCNA, while phosphorylated FEN1 separates from PCNA; (ii) what enzyme demethylates FEN1; (iii) whether post-translational modifications of Pol δ and Lig1, similar to those of FEN1, regulate the dynamic interactions with PCNA and DNA substrates; and (iv) whether genetic or environmental factors that change the post-translational modifications of these enzymes disrupt the highly ordered Okazaki fragment maturation processes and cause cancer. Future studies using a combination of biochemical, structural and mouse genetic approaches will fill these knowledge gaps.

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