Chapter 6
Mechanism of Lagging-Strand DNA Replication in Eukaryotes

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Abstract This chapter focuses on the enzymes and mechanisms involved in lagging-strand DNA replication in eukaryotic cells. Recent structural and biochemical progress with DNA polymerase α-primase (Pol α) provides insights how each of the millions of Okazaki fragments in a mammalian cell is primed by the primase subunit and further extended by its polymerase subunit. Rapid kinetic studies of Okazaki fragment elongation by Pol δ illuminate events when the polymerase encounters the double-stranded RNA-DNA block of the preceding Okazaki fragment. This block acts as a progressive molecular break that provides both time and opportunity for the flap endonuclease 1 (FEN1) to access the nascent flap and cut it. The iterative action of Pol δ and FEN1 is coordinated by the replication clamp PCNA and produces a regulated degradation of the RNA primer, thereby preventing the formation of long-strand displacement flaps. Occasional long flaps are further processed by backup nucleases including Dna2.

Keywords DNA replication • Lagging strand • Okazaki fragment maturation • DNA polymerase α-primase • DNA polymerase δ • Flap endonuclease 1 • Dna2

6.1 Introduction

Three DNA polymerases are responsible for the bulk of genomic DNA replication, Pol α, Pol δ, and Pol ε. A preponderance of evidence supports the following division of labor at the replication fork: The Pol α-primase complex primes synthesis on both the leading and lagging strands, with Pol ε synthesizing the leading strand and Pol δ synthesizing the discontinuous Okazaki fragments that make up the lagging strand (Burgers 2009). This model has been supported by analysis of replication errors (Pursell et al. 2007; Nick McElhinny et al. 2008; Larrea et al. 2010), studies of

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polymerase localization on replication forks (Yu et al. 2014), and genomic rNMP incorporation studies (Nick McElhinny et al. 2010a; Miyabe et al. 2011; Reijns et al. 2015; Daigaku et al. 2015; Koh et al. 2015; Clausen et al. 2015). Biochemical studies have shown that Pols ε and δ replicate their respective strands spontaneously in the presence of purified CMG helicase (Cdc45-Mcm_{2-7}-GINS) complex (Georgescu et al. 2014a, 2015) and are excluded from the incorrect strand (Schauer and O'Donnell 2017). For these reasons, the model placing Pol ε on the leading strand and Pol δ on the lagging strand has become widely accepted.

A recent study has suggested an alternate arrangement of polymerases at the replication fork (Johnson et al. 2015), concluding that Pol δ replicates both strands of the replication fork. These conclusions have become a matter of debate in the field (Stillman 2015; Johnson et al. 2016; Burgers et al. 2016), and some very recent biochemical data support a very limited engagement of Pol δ during the initiation of leading-strand DNA replication (Yeeles et al. 2017). However, no study disputes the current model of lagging-strand DNA replication involving the synthetic activities of Pol α-primase and Pol δ, which will be the primary focus of this review.

6.2 Priming by Pol α-Primase

DNA synthesis on both strands of the fork is initiated by the synthesis of RNA primers by the Pol α-primase complex. Pol α and its associated primase each contain one accessory subunit, forming a hetero-tetrameric complex overall, often designated as the eukaryotic primosome. The polymerase catalytic and accessory subunits are Pol1 and Pol12, respectively, in budding yeast and p180 and p70 in human cells (Johansson and Dixon 2013). The catalytic subunit is one of the four, eukaryotic B-family polymerases, which comprises a conserved polymerase domain and a separate C-terminal domain that is connected to the polymerase domain by a flexible linker (Klinge et al. 2009; Suwa et al. 2015; Kilkenny et al. 2012; Baranovskiy et al. 2016a). Interactions between the catalytic and the accessory subunit are made through this C-terminal domain (denoted $p180_C$ below). Similarly, the primase contains a catalytic and an accessory subunit: Pri1 and Pri2, respectively, in yeast, and p49 and p58 in humans. Integral to the mechanism described below, the primase accessory subunit contains two domains (N-terminal and C-terminal, denoted $p58_N$ and $p58_C$ below) connected by a flexible linker (Baranovskiy et al. 2015, 2016b).

The primase initiates RNA synthesis de novo, synthesizing an 8–10-nucleotide primer that is transferred to the polymerase subunit of the Pol α-primase complex for extension with dNTPs (Baranovskiy et al. 2016b; Singh et al. 1986; Kuchta et al. 1990; Kuchta and Stengel 2010) and then creating an ~30-nucleotide hybrid primer that becomes the substrate for Pol δ (Bullock et al. 1991; Murakami and Hurwitz 1993). The mechanism by which Pol α-primase makes uniformly sized RNA primers has long been unclear. Recent structural and biochemical studies with the human and yeast primosome have contributed to the proposal of a new model for primer synthesis (Klinge et al. 2009; Baranovskiy et al. 2015; Vaithiyalingam et al. 2014;

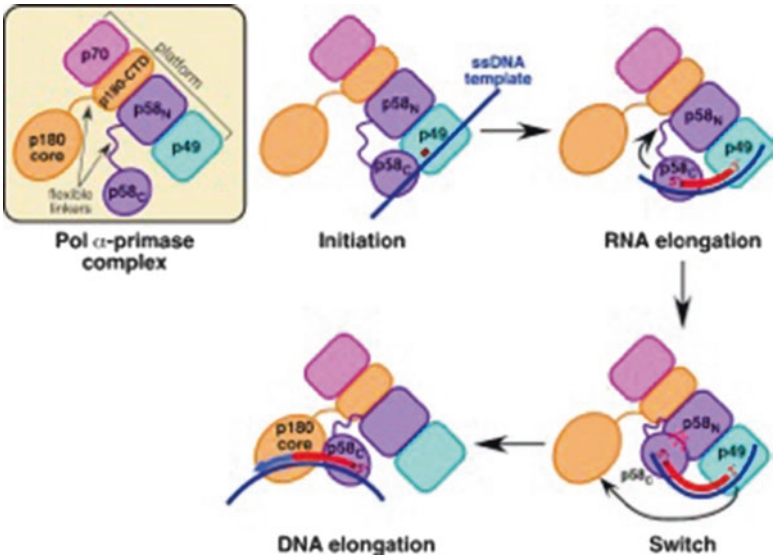

Fig. 6.1 Priming of DNA synthesis by Pol α-primase. The sequential steps in the initiation of RNA priming, the elongation of the RNA primer, and the switch to DNA synthesis are shown. The model of Pol α-primase is based on Baranovskiy et al. (2016a)

Agarkar et al. 2011; Nunez-Ramirez et al. 2011; Sauguet et al. 2010; Kilkenny et al. 2013; Perera et al. 2013). This model is outlined by Baranovskiy et al. and described below (Baranovskiy et al. 2016a).

The crystal structure of the apo form of the primosome (not bound to DNA) indicates that the entire complex is built upon a stable platform with a p49-p58$_N$-p180$_C$-p70 arrangement (human subunit designations). Flexible linkers connect the polymerase (p180$_{core}$) and the C-terminal half of the primase accessory subunit (p58$_C$) to this platform (Fig. 6.1). Large conformational changes of p180$_{core}$ and p58$_C$ with respect to the primosome platform enable the substrate exchanges necessary for priming and extension. De novo RNA synthesis occurs at the interface of the primase catalytic and accessory subunits (p49-p58$_C$ interface) (Zerbe and Kuchta 2002). As the new primer grows, p58$_C$ retains interactions with its 5′-terminus and rotates away from p49. Eventually, this rotation brings on steric clashes between p58$_C$ and p58$_N$ (Fig. 6.1). Molecular modeling predicts that these clashes would occur when the RNA primer had reached about ten nucleotides in length, providing an explanation for why RNA primers longer than ten nucleotides are rarely produced (Baranovskiy et al. 2016a).

After further RNA synthesis is inhibited, the DNA/RNA duplex is intramolecularly transferred from the primase to the polymerase subunit (Fig. 6.1). Since p58$_C$ makes extensive contacts with the duplex and p49 is only weakly bound, it is predicted that p58$_C$ delivers the primer terminus to the polymerase. Molecular modeling of the potential transfer complex predicts that the polymerase is only able to access the 3′-primer terminus when the primer is at least nine nucleotides in length,

consistent with biochemical data (Baranovskiy et al. 2016a). Pol α extends the RNA primers for an additional 20–30 nucleotides with dNTPs, yielding a 30–40-nucleotide-long primer. This estimate dates back to early in vitro SV40 replication studies (Bullock et al. 1991; Murakami and Hurwitz 1993).

6.3 Polymerase Exchanges at Pol α-Synthesized Primers

After primer synthesis, Pol α is exchanged for Pol ε or Pol δ for further high-fidelity DNA replication. It is still unclear how DNA synthesis by Pol α remains so precisely limited and how polymerase exchange occurs. Several mechanisms have been proposed. First, it has been hypothesized that the different helical characteristics of the RNA/DNA duplex and double-stranded DNA may be sensed by the Pol α active site. Pol α has been shown to bind more tightly to RNA/DNA duplexes, which adopt an A-form helix, than to the B-form DNA helix (Perera et al. 2013). As Pol α extends the RNA primer with dNTPs, the A-form helix initially present will be converted to a B-form helix. It has been proposed that the formation of the B-form structure inhibits further synthesis by Pol α (Perera et al. 2013). However, the biochemical experiments supporting this hypothesis were performed using poly(dT) templates, where formation of triplex structures (dT-dA-dT) after limited replication causes inhibition of DNA synthesis by most DNA polymerases, and not just Pol α (Mikhailov and Bogenhagen 1996; Zhang et al. 2016). As a result, the extent to which Pol α extended these homopolymeric templates was artificially low.

Alternately, it has been suggested that the switch from Pol α to Pol δ is mediated by loading of PCNA onto 3′-primer termini by the RFC complex (Schauer and O'Donnell 2017; Tsurimoto and Stillman 1991; Eki et al. 1992; Yuzhakov et al. 1999; Maga et al. 2000; Mossi et al. 2000). In the absence of PCNA, the RFC complex has been shown to inhibit Pol α activity when present at high concentrations (Yuzhakov et al. 1999; Maga et al. 2000). However, Pol α inhibition is greatly enhanced when both RFC and PCNA are both present, suggesting that clamp loading is integral to polymerase switching (Schauer and O'Donnell 2017; Tsurimoto and Stillman 1991). Polymerase switching has also been shown to be stimulated by the presence of the single-stranded binding protein RPA at the template-primer junction. RPA directly binds RFC, providing specificity of PCNA loading and the displacement of Pol α (Yuzhakov et al. 1999; Gomes and Burgers 2001). Regardless of the exact details of the mechanism of Pol α ejection, the preponderance of evidence points to PCNA loading by RFC as essential to the recruitment of Pol δ, which prevents rebinding of Pol α.

CMG helicase-dependent leading- and lagging-strand synthesis has recently been reconstituted in vitro using the budding yeast replication system (Georgescu et al. 2015, 2014b; Yeeles et al. 2017; Devbhandari et al. 2017). These studies have provided biochemical support for the current model of the eukaryotic replication fork, with Pol ε replicating the leading strand and Pol δ the lagging strand, and Pol

α priming synthesis on both strands. It appears from these studies that replicating a bidirectional fork in the presence of the CMG helicase complex enforces the division of labor of the replication machinery; i.e., Pol ε is suppressed on the lagging strand, and Pol δ is suppressed on the leading strand (Schauer and O'Donnell 2017). Interestingly, Diffley and coworkers found that leading-strand replication proceeded more efficiently if the initial elongation of the leading-strand primer was carried out by Pol δ, followed by a second exchange from Pol δ to Pol ε (Yeeles et al. 2017). Presumably, the latter polymerase exchange occurs when the elongating PCNA-Pol δ complex collides with the leading CMG complex ahead of it and GINS enforces the exchange to Pol ε.

6.4 Strand Displacement Synthesis and Nick Translation

Pol δ extends primers on the lagging strand until it reaches the 5′-end of the preceding Okazaki fragment. Before ligation, however, the initiator RNA at the 5′-terminus of the primer must be removed. Biochemical and genetic studies support a model in which the initiator RNA is predominantly removed through the joint action of Pol δ and flap endonuclease 1 (FEN1), a structure-specific nuclease (Grasby et al. 2012; Balakrishnan and Bambara 2013). When Pol δ collides with the previous Okazaki fragment, it continues replicating through limited displacement of the RNA primer, forming a short 5′-flap. This flap is the substrate for FEN1; repetition of strand displacement synthesis by Pol δ followed by FEN1 cleavage removes the initiator RNA (Garg et al. 2004; Rossi and Bambara 2006; Stodola and Burgers 2016). Most frequently, one- or two-nucleotide products are liberated by FEN1 (Stodola and Burgers 2016; Stith et al. 2008). After removal of the RNA through these iterative actions of Pol δ and FEN1, a process termed "nick translation," the nick can be sealed by DNA ligase. These basic steps are sufficient to process the vast majority of Okazaki fragments. On rare occasions, strand displacement synthesis may become decoupled from flap cutting, and flaps can grow to lengths that cannot be processed by FEN1 (Murante et al. 1995; Bae et al. 2001a). Backup mechanisms, described below, are required to cleave these flaps so that they do not lead to DNA damage.

Pol δ possesses two enzymatic activities, a DNA polymerase activity and a 3–5′-exonuclease activity. The exonuclease is required for proofreading of misincorporated nucleotides during DNA replication but also plays an important role in Okazaki fragment maturation (Jin et al. 2001). After Pol δ initiates strand displacement synthesis, the forward, flap-generating movement of the polymerase is countered by the exonucleolytic activity of the polymerase. After formation of a short flap, the exonuclease activity of Pol δ cuts out the nucleotides that the polymerase had inserted, with the release of dNMPs. Repeated short-flap formation followed by exonucleolytic cleavage back to the nick position has been termed "polymerase idling." This activity appears to be unique to the lagging-strand polymerase; Pol ε exhibits

very weak strand displacement and idling activities (Garg et al. 2004; Ganai et al. 2016). In most sequence contexts, idling is sufficient to restrict forward movement of Pol δ to within three nucleotides of the nick position, and most frequently, one- or two-nucleotide products are liberated by FEN1 (Garg et al. 2004; Stodola and Burgers 2016; Stith et al. 2008).

Polymerase idling is not the only restraint placed on strand displacement. As Pol δ initiates strand displacement, the rate of forward polymerase movement slows down progressively as the 5′-flap grows longer (Stodola and Burgers 2016), i.e., the growing flap inhibits further synthesis in a length-dependent manner. Thus, the nascent flap acts as a "molecular brake" on the polymerase. Idling and flap inhibition allow Pol δ to produce a substrate with a short 5′-flap for FEN1 while simultaneously limiting extensive strand displacement synthesis. This cooperation is necessary. If the rate of strand displacement remained constant irrespective of flap length, idling alone would be insufficient to constrain the polymerase near the nick.

Surprisingly, the ability of Pol δ to displace the duplex region of the preceding Okazaki fragment is not dependent on the nature of the block, i.e., RNA versus DNA, but solely on the stability of the duplex (Stodola and Burgers 2016; Stith et al. 2008). Extensive strand displacement synthesis is favored in sequence contexts with low duplex stability such as AT-rich sequences. Furthermore, when flaps reach a critical length, Pol δ continues strand displacement synthesis in a manner that is decoupled from its regulatory mechanisms, generating long flaps (Ayyagari et al. 2003). This "critical length" remains poorly defined. It is possible that the "molecular brake" exerted on Pol δ only applies in situations where flaps are very short, perhaps due to interactions between the polymerase and the 5′-end of the flap (Koc et al. 2015). Perhaps, the failure of the flap-controlling mechanisms could be caused by a failure of very long flaps to interact with the enzyme. Further investigation is required to more fully examine this phenomenon.

6.5 Short-Flap Processing by FEN1

The iterative action of Pol δ and FEN1 removes initiator RNA so that nick ligation can occur. In vitro, these enzymes together comprise an efficient maturation machine, rapidly degrading either RNA or DNA annealed downstream of Pol δ (Stodola and Burgers 2016; Stith et al. 2008; Lin et al. 2013). In the absence of DNA ligase, nick translation can continue indefinitely unless it is blocked by other DNA-binding proteins, as observed in yeast (Smith and Whitehouse 2012). Much effort has been dedicated to determine the structure of FEN1's optimal substrate. The consensus model is that FEN1 most efficiently cuts double-flap structures with a single-nucleotide 3′-flap and a variable length 5′-flap (Kao et al. 2002; Tsutakawa et al. 2011, 2014). Irrespective of the length of the 5′-flap, FEN1 cuts a single base into the 5′-duplex region, yielding a ligatable nick when the single-nt

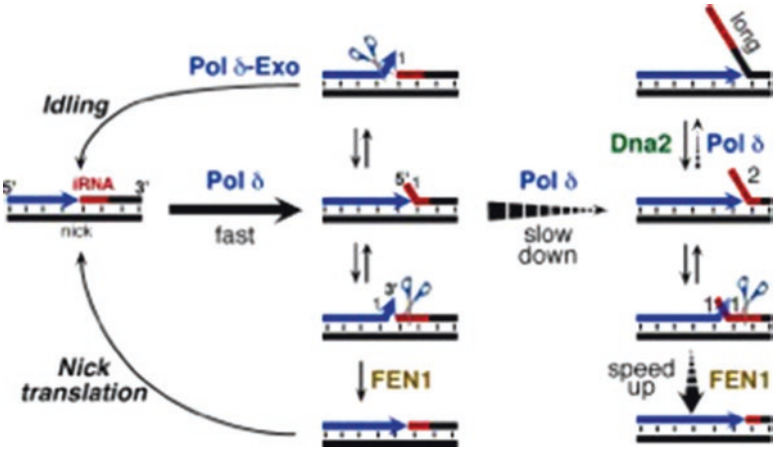

Fig. 6.2 Regulatory steps that limit strand displacement synthesis by Pol δ. The formation of long flaps is restricted by the 3′-exonuclease activity of Pol δ (*idling*), by a progressive slowdown of strand displacement synthesis as the flap grows, and by cutting of the nascent flap by FEN1. FEN1 cleavage may be accelerated when double-flap structures are formed as flaps grow in size, thereby further limiting their length. Long flaps that still do occur are trimmed by Dna2

3′-flap reanneals to the template (Fig. 6.2) (Kao et al. 2002; Kaiser et al. 1999; Xie et al. 2001).

Whether this optimal substrate, requiring at least two unpaired nucleotides, represents the substrate that is *most often* cut during nick translation has recently been addressed. Data suggest that the major FEN1 substrate in nick translation results from a single-nucleotide flap (Stodola and Burgers 2016). Since a 3′-flap is required for FEN1 activity, we hypothesize that the single-nucleotide 5′-flap formed by Pol δ strand displacement must re-equilibrate into a single-nucleotide 3′-flap and a fully base-paired 5′-junction before cutting (Fig. 6.2). Although shown to be efficiently cut by FEN1, this single-flap structure is not processed as avidly as double-flap structures. Thus, these data suggests that in most contexts, the major FEN1 substrate is not actually the optimal substrate (Stodola and Burgers 2016; Kao et al. 2002). These observations could be interpreted as a contradiction, but it may in fact represent another layer of regulation limiting the formation of long flaps. It is likely that during nick translation, FEN1 binds and cuts double-flap structures more avidly than single 3′-flap structures. The higher-affinity binding of FEN1 to these double-flap intermediates would aid in the preferential recruitment of FEN1 to longer flaps in the case that the enzyme was not associated with PCNA-Pol δ at the start of strand displacement synthesis. Such a mechanism would ensure that flaps longer than a single nucleotide are processed before Pol δ strand displacement extends too far.

6.6 Alternative and Long-Flap Processing

The occurrence of long flaps in the cell was initially inferred from genetic studies in *S. cerevisiae*. Deletion of *RAD27*, which encodes FEN1, is associated with a dramatic increase in the occurrence of duplications between direct repeats, up to ~100 nt in length, as it could result from slippage mispairing of long 5′-flaps (Tishkoff et al. 1997a). The related exonuclease 1 (Exo1) also shows flap processing activity and can process nascent flaps generated during strand displacement synthesis by Pol δ, although less efficiently than FEN1 (Tran et al. 2002; Sparks et al. 2012). However, the spectrum of mutations observed in an *exo1Δ* strain is most consistent with a defect in mismatch repair rather than in Okazaki fragment maturation (Tran et al. 2001). Because *rad27 exo1* double mutants are lethal, the model has been proposed that Exo1 serves as a backup nuclease for FEN1, and in the absence of both enzymes, the burden of long flaps overwhelms the ability of the cell to process them (Stith et al. 2008; Tishkoff et al. 1997b). Further genetic studies have highlighted Dna2 as the principal enzyme responsible for processing long flaps. For instance, the conditional lethality of *DNA2* mutations is suppressed by overexpression of *RAD27*, and the temperature sensitivity of *rad27Δ* is suppressed by *DNA2* overexpression (Budd and Campbell 1997).

Based on biochemical studies, FEN1 has been apportioned the task of processing short flaps and Dna2 that of long flaps (reviewed in Burgers 2009; Kang et al. 2010; Balakrishnan and Bambara 2010; Burgers and Kunkel 2017). Long flaps are operationally defined as those longer than ~20 nucleotides, the length at which RPA stably binds flaps (Kumaran et al. 2006). FEN1 itself can cleave long flaps in vitro, but when the 5′-flap is coated with RPA or assumes a secondary structure, FEN1 cutting is abrogated (Murante et al. 1995). In wild-type cells, long flaps could be formed in certain sequence environments, such as AT-rich sequences, where strand displacement synthesis by Pol δ is predicted to be very rapid (Stodola and Burgers 2016). Alternatively, Pol δ strand displacement could become decoupled from flap cutting for other reasons, e.g., if FEN1 and Exo1 were absent from the replisome. In addition, the generation of long flaps is enhanced by the action of Pif1 helicase (Budd et al. 2006; Rossi et al. 2008) or by a defect in the proofreading activity of Pol δ (Jin et al. 2003). Therefore, backup mechanisms are required to process long flaps and rescue replication forks (Stith et al. 2008; Jin et al. 2001, 2005).

S. cerevisiae Dna2 is a multifunctional enzyme with nuclease, helicase, and cell-cycle checkpoint activities (Lee et al. 2000; Budd et al. 2000; Bae et al. 2001b; Kumar and Burgers 2013). Of these activities, the nuclease is most critical to Okazaki fragment maturation. Dna2 nuclease threads onto the 5′-end of flaps, displacing RPA before cutting DNA (Stewart et al. 2010; Zhou et al. 2015). In several reports, Dna2 was observed to cleave flaps several nucleotides away from their base, leaving behind a ~5–8-nucleotide 5′-flap (Bae et al. 2001a, 1998; Kao et al. 2004). Additionally, in one report, cutting at the base of the flap was also observed (Levikova and Cejka 2015). However, regardless of the exact cleavage accuracy of Dna2, efficient Okazaki fragment maturation of long-flap intermediates requires additional

nucleolytic processing beyond that by Dna2. Either additional 5′-flap cutting by FEN1 or 3′-exonucleolytic processing by the proofreading activity of Pol δ is required to produce ligatable nicks with high efficiency. When in biochemical studies, Dna2 was the only 5′-nuclease provided, the maturation of long flaps carried out with a proofreading-defective form of Pol δ produced ligatable nicks very inefficiently (Jin et al. 2003; Levikova and Cejka 2015). Consistent with these biochemical results is the observation that yeast mutants defective for the Pol δ 3′-exonuclease activity are exquisitely sensitive to additional defects in FEN1 (Jin et al. 2001).

A recent electron-microscopic study of isolated fission yeast replication forks provides structural support for the existence of the long-flap pathway (Liu et al. 2017). In wild-type cells, 10% of the isolated forks had associated with it a 40–50-nt-long flap. Often, these long flaps were detected kilobases distant from the fork. Because the EM methodology cannot detect very short flaps and nicks that are normally generated during short-flap processing, one conclusion from these data is that long flaps are rare. The frequency of long flaps increased in *rad2* (*S. pombe* FEN1) mutants as well as in *dna2* mutants. These results are consistent with the model, because FEN1 defects are expected to generate more long flaps while Dna2 defects are expected to abrogate their resolution. Accordingly, quantification of the frequency of long flaps in the *dna2*$^{-}$ mutant should give a good estimate of their normal occurrence during Okazaki fragment maturation. In *dna2*$^{-}$ cells, 32% of the forks showed long flaps, and the average distance between long flaps was about 6.5 kb. If one assumes that an Okazaki fragment is ~150 nt in length, one can estimate that long flaps are generated at a frequency of 1–2%. With about 50,000 Okazaki fragments being generated per fission yeast cell cycle, this amounts to as many as 500–1000 long flaps, which makes it unsurprising that *dna2* is essential for cell growth in *S. pombe*, as it is in *S. cerevisiae* (Kang et al. 2000; Budd et al. 1995).

The same EM study also determined the role of fission yeast RNase H2 and Exo1 in Okazaki fragment maturation (Liu et al. 2017). Defects in RNase H2 (*rnh201Δ)* did not result in a significant increase in the frequency of long flaps, suggesting that this enzyme does not participate in the degradation of the RNA primers during Okazaki fragment maturation. However, an *exo1Δ* mutant showed a clear increase in the frequency of long flaps, suggesting that Exo1 participates in Okazaki fragment maturation in wild-type cells. When compared with the known phenotypes of *S. cerevisiae exo1Δ* (see above), it appears that in *S. pombe*, Exo1 plays a more prominent role in Okazaki fragment maturation.

While there is strong evidence in both yeasts for the processing of long flaps by Dna2, the situation is less clear in human cells. Human Dna2 has been shown to play a role in nuclear genome maintenance, specifically promoting the rescue of stalled replication forks (Thangavel et al. 2015). However, currently there is no strong evidence for a role for Dna2 in Okazaki fragment maturation analogous to its role in both yeasts (Duxin et al. 2009, 2012). It is unknown whether human Okazaki fragment maturation can be accomplished by just FEN1 and Exo1 or whether long flaps are processed by additional nucleases redundant with Dna2, or different nucleases.

6.7 DNA Ligation

Following the removal of initiator RNA, nicks are sealed by DNA ligase I (*cdc9* in budding yeast and *LIG1* in human cells) (Howes and Tomkinson 2012). The eukaryotic ligase contains a conserved PCNA-interacting protein motif that binds PCNA in the interdomain connection loop (Vijayakumar et al. 2007). This interaction is important for localizing ligase to replication foci and for completing Okazaki fragment maturation in mammalian cells (Montecucco et al. 1998; Levin et al. 2000). PCNA has also been shown to stimulate ligase activity on nicked DNA substrates (Tom et al. 2001). Despite these effects, it is unclear whether the ligase is actually a stable component of the PCNA-mediated maturation complex like Pol δ and FEN1. During in vitro Okazaki fragment maturation, yeast ligase acts distributively, with the position of ligation following RNA removal dependent on the ligase concentration (Ayyagari et al. 2003). The cause of this observation remains unclear, but it is possible that when Pol δ and FEN1 are bound to PCNA, ligase cannot gain access to the PCNA ring, resulting in distributive ligation.

6.8 Limits to Nick Translation and the Size of Okazaki Fragments

The transient nature of Okazaki fragments has made the study of their properties in vivo difficult. However, advances have been made in recent years in isolating and examining Okazaki fragments in vivo and also in reconstituting lagging-strand replication in vitro. Both approaches have yielded new insights into the controls placed on Okazaki fragment synthesis and maturation.

Recent in vitro replication studies showed that, when lagging-strand replication was coupled to leading-strand synthesis by CMG-Pol ε, Pol α spontaneously primed on the lagging strand (Georgescu et al. 2015; Yeeles et al. 2017). The distance between priming events decreased as the concentration of Pol α in the assay was raised, indicating that priming itself is stochastic (Yeeles et al. 2017). PCNA-Pol δ spontaneously extended these Pol α-synthesized primers, producing Okazaki fragments that ranged from 100 to 500 nucleotides (Georgescu et al. 2015; Yeeles et al. 2017). Chromatin structure further modulated the size distribution of Okazaki fragments (Devbhandari et al. 2017; Kurat et al. 2017).

Maturation of these synthesized Okazaki fragments minimally requires FEN1 and DNA ligase as well as PCNA-Pol δ. In the absence of ligase, Pol δ and FEN1 could perform nick translation indefinitely (Ayyagari et al. 2003), although this would represent a major inefficiency in lagging-strand DNA replication. There is strong evidence that the chromatin context of the cell places a limit on the amount of nick translation synthesis that can be performed by Pol δ FEN1 (Smith and Whitehouse 2012). By purifying Okazaki fragments from a budding yeast strain deficient for DNA ligase, the Whitehouse Group found that the size distribution of Okazaki fragments

was strongly influenced by the placement of nucleosomes, with Okazaki fragment termini preferentially located at nucleosome dyads (Smith and Whitehouse 2012). Thus, it appears that a bound nucleosome upstream of the nick translation machinery is enough to block its further movement. This phenomenon has been extended to other DNA-binding proteins that bind the double-stranded DNA downstream of the migrating nick; transcription factor binding sites have been shown to be correlated with Okazaki fragment termination sites (Reijns et al. 2015; Smith and Whitehouse 2012). The lagging-strand replication machinery has also been shown to be blocked by double-stranded DNA-binding proteins in vitro (Koc et al. 2016).

It is currently unknown whether nick translation regularly extends to where nucleosomes or protein blocks are positioned or whether DNA is ligated before PCNA-Pol δ and FEN1 reach these blocks. Since the observations discussed above were generated in a yeast strain deficient for ligase, this data may report more on the limits placed on the maturation machinery rather than representing true Okazaki fragments (Smith and Whitehouse 2012; Smith et al. 2015). Since ligase acts distributively, in some situations ligation could leave some Pol α-synthesized DNA in the mature genome, despite the fact that more extensive nick translation would replace the lower-fidelity DNA produced by Pol α with that of the higher-fidelity Pol δ (Kadyrov et al. 2009; Liu et al. 2015). Indeed, several studies have shown that a significant amount of Pol α-synthesized DNA remains in the mature yeast genome (Reijns et al. 2015; Nick McElhinny et al. 2010b; Lujan et al. 2014). It remains to be determined to what extent nucleosome positioning directly influences the retention of Pol α-synthesized DNA.

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