Lagging Strand Replication Proteins in Genome Stability and DNA Repair

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1. Introduction

Because of the antiparallel structure of DNA, cells employ significantly different mechanisms to replicate the two DNA strands.[1] Leading strand replication is the continuous extension of one strand in the 5′ to 3′ direction toward the separating replication fork (Figure 1, I). Lagging strand replication is the creation and joining of a series of segments, designated Okazaki fragments (Figure 1). These small stretches of DNA are extended away from the separation point of the parental strands. In eukaryotes, Okazaki fragments are initiated by RNA primers and subsequently extended by DNA for a distance of about 100 nucleotides (nts) in yeast and higher eukaryotes[2] and up to 2000 nts in some bacteria.[3] In most eukaryotic cells, millions of these segments must be made and joined to complete replication of the genome. Cells have evolved an efficient group of proteins to carry out this task.[4] The significance of these proteins in maintaining the fidelity of the genome is twofold. First, the joining process offers the opportunity for aberrant intermediates to form that can lead to sequence duplications or deletions, double or single strand breaks, and other lesions. However, the lagging strand proteins have evolved to suppress aberrant joining.[2] These properties will be discussed in this review. Second, the synthesis and cleavage to remove the initiator RNA and the ligation of the Okazaki fragments are similar to the excision of damage and resynthesis reactions that must occur during repair of many types of lesions. Therefore, many of the lagging strand proteins have evolved dual roles in DNA replication and repair.[4] These roles and the mechanism by which cells distribute the lagging strand proteins into repair functions will also be discussed.

2. Lagging Strand DNA Replication

2.1. Pathways for Short Flap Processing

To understand the dual roles of lagging strand replication proteins in replication and repair and how they work to maintain genome stability, it is necessary to understand their activity during normal replication (Figure 1). On the lagging strand, replication is primed by RNA/DNA primers synthesized by the polymerase α/primase (pol α) complex. In

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eukaryotes, the primers contain about 10 nts of RNA followed by about 10−20 nts of DNA.[1] Then, in a process known as polymerase switching, pol α dissociates and DNA polymerase δ (pol δ) binds and extends the Okazaki fragments in the 5′ to 3′ direction to their full length. Processive synthesis by pol δ is facilitated by the sliding clamp proliferating cell nuclear antigen (PCNA), which tethers pol δ to the DNA, and the PCNA loading molecule replication factor C (RFC). Upon encountering the 5′ end of a downstream Okazaki fragment, the pol δ strand displaces the primer into a single stranded flap (Figure 1, II). The flap is processed by flap endonuclease 1 (FEN1) to form a nick (Figure 1, III).[1,5,6]

Using in vitro DNA replication as a model, the mechanism of FEN1 activity has been elucidated. On oligonucleotide substrates, it has been shown that FEN1 tracks from the 5′ end of the flap[7,8] and cleaves specifically at the base of 5′ flaps.[7,9,10] (Various flap intermediate structures are outlined in Figure 2 and will be referred to throughout the text.) The

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tracking mechanism is necessary for endonucleolytic cleavage as it has been shown that cleavage is inhibited by structures which block the 5′ end of flap substrates.[7,8] FEN1 prefers cleavage of a double flap substrate, a substrate containing a 5′ flap with a 1 nt 3′ flap overhang (Figure 2, II).[11,12] Such structures presumably form transiently during strand displacement synthesis. In addition, FEN1 is also a 5′−3′ exonuclease. Both its endonuclease and exonuclease activities are stimulated by PCNA. PCNA tethers FEN1 to its cleavage site at the base of flaps.[8] Ultimately, once a nick is generated by FEN1 endonucleolytic cleavage, it is sealed by DNA ligase I to yield the continuous double stranded DNA (Figure 1, IV).[1] An interaction between DNA ligase I and PCNA also stimulates ligase activity.[13] It has been proposed, then, that PCNA serves as a platform for the

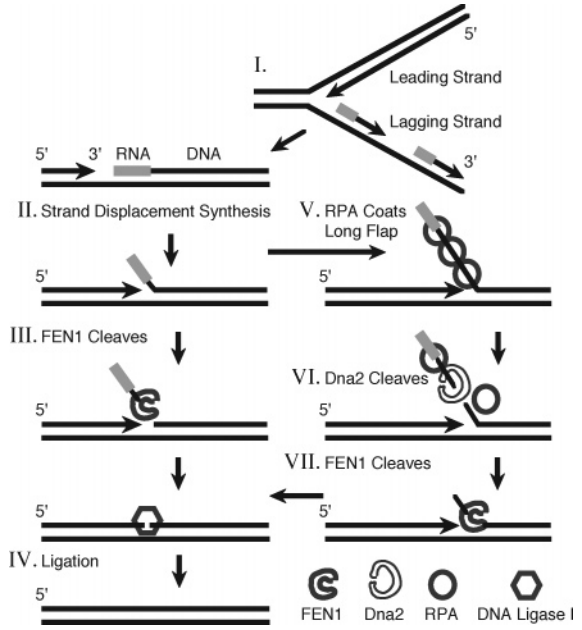

Figure 1. Eukaryotic lagging strand synthesis mechanisms. (I) Leading strand synthesis proceeds continuously 5′ to 3′ in the direction of the advancing replication fork, while lagging strand synthesis proceeds discontinuously via Okazaki fragments away from the separating fork. (II) Pol δ continues extension of the 3′ end of the upstream Okazaki fragment until it displaces the 5′ end of the downstream fragment into a single stranded flap in a process termed strand displacement synthesis. (III) Short single stranded flaps up to about 12 nts are readily cleaved by FEN1, resulting in formation of a nick. (IV) The nick is sealed by DNA ligase I to generate the double stranded DNA. (V) Long flaps, about 20−30 nts in length, that escape FEN1 cleavage are coated by RPA. (VI) RPA stimulates cleavage by Dna2, resulting in formation of a short flap. (VII) The short flap, no longer bound by RPA, is cleaved by FEN1, to generate the ligatable nick.

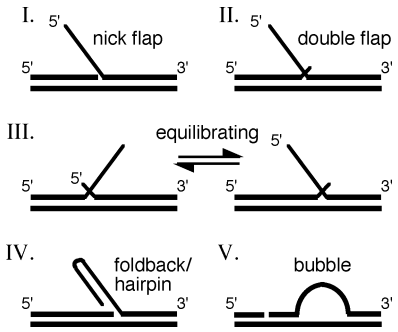

Figure 2. Flap intermediates. (I) A nick flap intermediate contains a 5′ flap, such that the junction between the upstream fragment and the downstream fragment forms a nick. (II) A double flap intermediate contains a 5′ flap with a 1 nt 3′ flap overhang from the upstream fragment. (III) An equilibrating intermediate occurs when a region of the 3′ end of the upstream fragment and a region of the 5′ end of the downstream fragment, which are complementary to the template, equilibrate into various flap structures. (IV) A foldback or hairpin intermediate may form due to complementarity in the sequence of the 5′ flap. (V) A bubble intermediate occurs when a downstream fragment is bound to a template such that a unique sequence between the 5′ and 3′ ends remains unbound.

recruitment of proteins to the lagging strand during replication and mediates the sequential protein "handoffs" in the process.[2,5,14]

The process of strand displacement synthesis and flap cleavage is necessary to remove the RNA/DNA primer that initiates the Okazaki fragments.[1] Current research suggests that if the length of the flap displaced by pol δ is short, up to about 12 nts in length, then FEN1 is able to readily cleave and create a nick.[15] In vitro studies of lagging strand processing in *Saccharomyces cerevisiae* (*S. cerevisiae*) indicate that the pol δ 3′−5′ exonuclease and Rad27 (*S. cerevisiae* homologue of FEN1) cooperate to maintain a short displacement length, since reconstitutions with 3′−5′ exonuclease deficient pol δ (pol δ−exo$^-$) show increased displacement synthesis.[16,17] A synthetic lethal interaction between *rad27* null and *pol* δ−exo$^-$ in double mutant *S. cerevisiae* strains further supports this hypothesis.[18−20]

2.2. Pathways for Long Flap Processing

If the displaced flap escapes cleavage by FEN1 and achieves a length of 20−30 nts, it will be bound by the single stranded DNA binding protein replication protein A (RPA) (Figure 1, V).[21−23] The presence of RPA inhibits cleavage by FEN1 yet stimulates cleavage by Dna2.[22,24] Dna2 is a multifunctional enzyme with ATPase, single stranded DNA-specific endonuclease, and helicase activities that is essential in yeasts, both *S. cerevisiae* and *Schizosaccharomyces pombe* (*S. pombe*).[25−27] Homologues of Dna2 have also been recently identified in *Xenopus laevis* (*X. laevis*)[28] and *Caenorhabditis elegans* (*C. elegans*).[29,30] Like FEN1, Dna2 also requires the unique tracking mechanism for cleavage.[31,32] However, unlike FEN1, the 5′ nuclease activity of Dna2 favors cleavage of longer flaps (>27 nts).[24] Dna2 cleaves at the single stranded region along flaps, but it does not cleave at the base of a 5′ flap to generate a nick.[24] Consequently, Dna2 cleavage results in a shorter flap that is no longer bound by RPA and can be cut by FEN1 to support the subsequent ligation reaction (Figure 1, VI and VII).[22,31] It is likely, then, that Dna2 and FEN1 act sequentially to process long flaps.[22] Copurification and genetic studies in *S. cerevisiae* reveal a synthetic lethal interaction between *dna2* and *rad27* mutants.[33] In addition, overexpression of Rad27 in *S. cerevisiae dna2* mutant or deletion backgrounds partially rescues the temperature sensitive growth phenotype.[33,34] Although both Dna2 and FEN1 are able to process long flaps,[15,22] it is possible that long flaps form structures, such as foldbacks or bubbles (Figure 2, IV and V), that are inhibitory to cleavage by both enzymes. Yet, the two enzymes may cooperate to process long flaps forming structures inhibitory to cleavage. Consistent with this possibility, the helicase activity of Dna2 has been shown to help resolve structure-containing flaps[24,35] and promote FEN1 cleavage.[24]

Additional proteins may also be necessary to resolve some flap structures into cleavable intermediates. It has been proposed that the RecQ helicases participate in resolving DNA structures inhibitory to replication.[36,37] Both Bloom syndrome protein (BLM) and Werner syndrome protein (WRN) are members of the RecQ family of DNA helicases, mutated in patients with Bloom syndrome and Werner syndrome, respectively. Bloom syndrome is characterized by growth deficiency and skin lesions. Cells cultured from Bloom syndrome patients display ultraviolet (UV) radiation sensitivity and genome instabilities consistent with defects in DNA replication. Similarly, Werner syndrome cells exhibit incomplete resolution of repair intermediates.[36]

Recent results demonstrate that both BLM and WRN stimulate FEN1 cleavage activity on oligonucleotide 5′ flap substrates.[38−42] In the absence of ATP, BLM moderately stimulates FEN1 cleavage on substrates containing a foldback on the 5′ flap and on bubble substrates (Figure 2, IV and V). Yet, in the presence of ATP, the stimulation of FEN1

activity by BLM increases, indicating that the ATP-dependent BLM helicase activity is allowing FEN1 access to the structured substrates.[42] Moreover, immunoprecipitation and fluorescence localization studies in HeLa cells show an interaction between FEN1 and BLM and FEN1 and WRN, respectively.[40,41] The C-terminal region of BLM, which shares homology with the FEN1 interaction domain of WRN, mediates the functional and physical interaction between BLM and FEN1.[40] Together these results suggest that the RecQ helicases, specifically BLM and WRN, are important in resolving replication and repair intermediates having foldback and bubble structures that inhibit normal replication.[40−42] In support of this connection is the synthetic lethal interaction between *sgs1*, the *S. cerevisiae* homologue of *BLM* and *WRN*, and *rad27* mutants.[43,44] In addition, the temperature sensitive growth defects and damage sensitivity of a *S. cerevisiae dna2* helicase mutant (*dna2-1*) were lessened by expression of human BLM,[45] perhaps due to BLM stimulation of Rad27. WRN has also been shown to rescue the *dna2-1* mutant phenotypes, via its C-terminal domain.[46]

2.3. Additional Genetic Connections between Lagging Strand Proteins and DNA Repair

Although *rad27* null is not lethal in *S. cerevisiae*, deletion mutants are temperature sensitive and display phenotypes consistent with defects in DNA replication and repair, mainly sensitivity to alkylating agents and UV radiation.[47,48] Also, a deletion of *FEN1* in chicken DT40 cells results in an increased sensitivity to methylating agents and peroxide.[49] However, in *C. elegans*, inactivation of *FEN1* expression by RNA interference (RNAi) results in embryonic lethality.[29] Furthermore, homozygous deletion of *FEN1* in mice causes growth failure, suggesting an embryonic lethal mutation.[50,51] Primary cells cultured from homozygous null mouse blastocysts failed to proliferate. The null cells underwent extensive apoptosis when treated with radiation, indicating that deletion of *FEN1* disrupts both replication and response to radiation.[51] These results suggest that FEN1 is important in both DNA replication and repair.

Additionally, synthetic lethal screens in *S. cerevisiae* have identified several proteins that possess essential interactions with *RAD27*. Some of these interactions are particularly relevant to the role of Rad27 in DNA repair. First, studies identified synthetic lethal interactions between *rad27* null and mutants in most of the *RAD52* group genes (*rad50*, *rad51*, *rad52*, *rad54*, *rad55*, *rad57*, *rad59*, *mre11*, and *xrs2*).[43,48] The *RAD52* group genes play roles in double strand break repair via homologous recombination,[52] demonstrating a significance of *RAD27* and *RAD52* genes in repair. Second, a synthetic lethal interaction[53] in the double mutant *DDC1* and *RAD27* was also identified. *DDC1* is the *S. cerevisiae* homologue of both the human and *S. pombe RAD9* gene, a PCNA analogue with a role in the DNA damage checkpoint (discussed in section 8).[54] The synthetic lethality between *ddc1* and *rad27* mutants highlights the role of both proteins in recognizing and/or processing DNA damage. Furthermore, additional proteins, including *S. cerevisiae* Rad17 and Rad24 damage response proteins (discussed in section 8), were identified to have synthetic sick interactions with mutant *RAD27*.[43,53] A majority of these interacting proteins are involved in DNA repair, once more highlighting the importance of FEN1 in repair.

Moreover, a synthetic lethal interaction between mutants in *FEN1* and exonuclease I (*EXO1*), a 5′−3′ exonuclease,[55,56] in some genetic backgrounds in yeast[57] suggests that these two enzymes have overlapping functions in the cell. Overexpression of *EXO1* in *rad27* null cells relieves the temperature sensitivity, suggesting that, in *rad27* mutants, EXO1 can serve as a backup exonuclease for Rad27 in removal of the RNA primer during Okazaki fragment maturation.[55,56,58] In support of this, recombinant human EXO1 displays riboexonuclease activity on synthetic oligonucleotide substrates.[56] Thus, the synthetic lethal interaction between *rad27* and *exo1* mutants is presumably a replication defect. However, *exo1* mutants do not have impaired growth or sensitivity to UV radiation, yet they display a mutator phenotype consistent with mismatch repair defects, indicating a cellular role of EXO1 in mismatch repair.[55,56] A physical interaction between EXO1 and Msh2,[55,56] a mismatch repair protein (discussed in section 3.3.3), supports this hypothesis. More recent studies reveal that EXO1 is recruited to stalled replication forks, indicating that it may also function in pathways for replication fork restart.[59]

Thus, the genetic connections between FEN1 and repair proteins, in addition to highlighting the proteins' roles in repair, also raise the possibility that unprocessed FEN1 substrates can accumulate and could be subsequently processed by DNA damage repair pathways. Furthermore, there is some evidence that Dna2 is involved in damage repair pathways because *dna2* mutants are sensitive to X-ray radiation and mildly sensitive to UV radiation, although the roles of Dna2 in repair processes are uncertain.[34]

3. Complexities of Replicating Repeating Sequences

3.1. Minisatellite Instability

Many unstable regions of the genome contain repeated sequences of various lengths that present problems when they are replicated. One class of repeat, the minisatellite repeats, contain regions of DNA with tandemly repeated units 11−100 nts long. Minisatellites are polymorphic in repeat number and are prone to sequence expansions and contractions. They are often characterized as fragile sites on chromosomes, meaning that unprocessed replication or repair intermediates can lead to single and double strand breaks.[60−62]

Repeat sequences exhibit instability during both mitosis and meiosis. In fact, meiotic instability events may arise more frequently than mitotic instability events.[63,64] This review will focus on mitotic instability because it is likely the result of aberrant lagging strand replication, whereas meiotic instability of repeat sequences may rely more on faulty repair of double strand breaks[63,65] or other processes peculiar to meiosis and gametogenesis. For a review of meiotic instability of minisatellites, see ref 66.

In 1997 Tishkoff et al.[67] reported that mutations in *RAD27* destabilize DNA sequences flanked by 3 to 12 nt direct repeats. In addition, they reported that double null mutants of *RAD27* together with either *RAD51* or *RAD52* are inviable. Given that Rad51 and Rad52 are involved in double strand break repair, it can be inferred that the replication defects of the *rad27* null mutants led directly or indirectly to double strand breaks.[48,68] This observation led the authors to propose the following model to explain duplication of DNA sequences flanked by direct repeats (see also reviews of the Tishkoff paper by Kunkel et al.[69] and Gordenin et al.[70]). When Rad27 is delayed or prevented from cleaving 5′ flaps, the flap may grow longer as polymerases rebind and displace

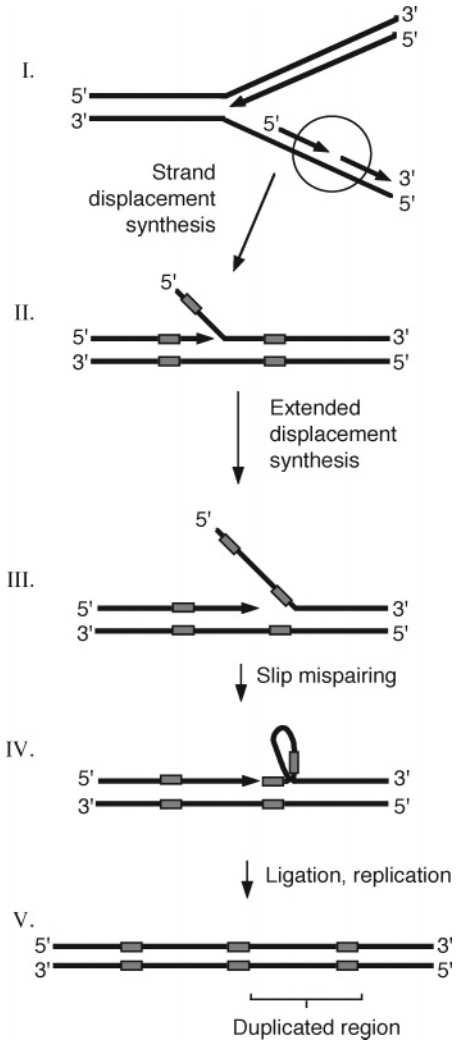

Figure 3. Model for sequence duplication of minisatellite repeats in *rad27* mutants. (I) Lagging strand replication requires the joining of Okazaki fragments, which occurs most efficiently with wild-type FEN1. (II and III) In the absence of FEN1, 5′ flaps remain uncleaved and polymerases may rebind and further lengthen the flap. (IV) If the flap lengthens to include two or more repeats, there is a possibility of slip mispairing of the flap to the template DNA. (V) If the flap reanneals in such a way as to present a ligatable nick, DNA ligase I can seal the loop into the duplex. If the loop is not excised by DNA repair enzymes, it will result in an expanded allele following replication. Adapted from *Cell*, Vol. 88, Tishkoff et al., "A Novel Mutation Avoidance Mechanism Dependent on *S. cerevisiae RAD27* Is Distinct from DNA Mismatch Repair", pp 253−263, 1997, with permission from Elsevier.

more of the downstream primer (Figure 3, II and III). If the displaced flap contains two or more repeats, there is the possibility of slip mispairing between the repeats on the flap and the complementary sequences on the template. This scenario would cause formation of a single stranded loop that if ligated into the daughter strand would lead to duplication of the sequence flanked by the repeats (refer to Figure 3, IV and V). Slip mispairing aside, long unprocessed flaps represent single strand breaks and may lead to double strand breaks requiring repair through homologous recombination or nonhomologous end joining. Either double strand break repair process could also result in sequence duplications when carried out amid repeat regions prone to misalignment.[67]

After the Tishkoff model, Kokoska et al.[18] verified that *rad27* null mutants exhibited higher insertion rates at minisatellite sites that were not interrupted by random sequences. They also reported that the *pol3-t* mutation, which alters the pol δ catalytic domain, led primarily to sequence deletions at the same sites. A plausible reason for the deletions seen in the *pol3-t* mutants is that a decreased synthesis rate leads to accumulation of unannealed lagging strand template DNA, which may form secondary structures such as hairpins. Secondary structure in the template would then necessitate bypass synthesis by DNA polymerases, leading to contraction on the daughter strand.

Other groups have examined the effect of *RAD27* deletion on naturally occurring human minisatellite sequences inserted into the *S. cerevisiae* genome.[71,72] Maleki and colleagues[72] showed that *rad27* null mutant strains destabilize four different minisatellites to differing degrees. The repeating units of the minisatellites chosen for analysis ranged from 10 to 50 base pairs per repeating unit with the repeat arrays showing either strict sequence homogeneity or relatively high sequence variability among repeats. The length of the repeat array constructs ranged from 0.5 to 2.5 kilobases. Not surprisingly, Maleki et al. found that the longer the repeat tract and the more homogeneous the repeat sequence, the higher the degree of instability. This observation correlates with the premise that increases in either tract length or repeat homogeneity increase the possibility of polymerase slipping or slip mispairing forming stable secondary structures.

Tracking the mitotic stability of the notoriously unstable human CEB1 minisatellite, Lopes et al.[71] demonstrated that mutations in *RAD27* and *DNA2* both destabilized CEB1 minisatellite fragments inserted into the *S. cerevisiae* genome. The *rad27* null mutants led to greater CEB1 instability compared to a *dna2* temperature sensitive mutant, consistent with the current model that Dna2 may only be necessary to shorten long 5′ flaps whereas final cleavage at the base of the flap is performed by Rad27 irrespective of flap length. In accordance with the length-dependent instability reported by Maleki et al.[72] noted above, a longer CEB1 fragment with 42 repeats was destabilized to a greater degree by *rad27* mutants than was a fragment containing 14 repeats. Lopes and colleagues suggest this is the result of increased probability that the junction between Okazaki fragments will fall within the repeating region.[71]

3.2. Lagging Strand Enzymes in Telomere Replication

Okazaki fragment processing enzymes have been implicated in maintenance of telomeric structures found at the end of linear chromosomes. Specifically, mutations in *FEN1*, *DNA2*, *WRN*, and *BLM* each affect telomere maintenance and will be discussed below. Telomeric sequences consist of hundreds of nucleotides of tandemly repeated GT-rich repeats. Each time a linear chromosome is replicated, a portion of the telomere is lost because of RNA primer removal and other complexities of lagging strand synthesis at chromosome ends.[73,74] Telomerase, a DNA polymerase with an embedded RNA primer complementary to the telomeric repeats, can add repeats to the 3′ terminated strand to increase the replication potential of a given cell.[75] Elongation of the G strand (the strand that is composed of the GT-rich telomeric repeats) by telomerase in concert with degradation of the complementary C strand results in a 3′ G tail. The G tail is inserted back into the double stranded telomeric DNA, forming a protein-stabilized loop structure called a T-loop that protects the chromosome end from

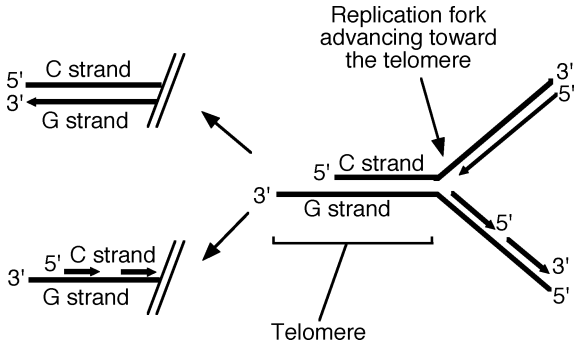

Figure 4. Replication of telomeres. Telomeres consist of hundreds of G-rich repeats and their complementary C-rich repeats, giving rise to the descriptors G strand and C strand, respectively. The single stranded 3′ end of the G strand is the G tail that inserts back into the double stranded telomere (not depicted here) to protect chromosomes from end to end fusions or nuclease degradation. The G strand always serves as the template for lagging strand replication of the telomere, and the C strand is always the leading strand template. The telomere that results from replication of the parental C strand is called the leading strand telomere. The telomere replicated using the parental G strand is referred to as the lagging strand telomere. Adapted with permission from ref 78. Copyright 1999 American Society for Microbiology.

nuclease activity or abnormal chromosome joining.[76] An intriguing aspect of telomere replication is that the G strand always serves as the template for lagging strand synthesis, and the C strand always acts as the leading strand template[77] (Figure 4).

Parenteau and Wellinger[78] studied *S. cerevisiae* telomere processing in cells harboring a temperature sensitive *rad27* mutation and found an accumulation of single stranded G-rich DNA at the restrictive temperature. The G-rich single stranded DNA was not a result of telomeric repeat addition but rather the result of incomplete DNA replication of the lagging, or C, strand. The authors suggest that the G-rich single stranded DNA observed may have been due to gaps between unprocessed Okazaki fragments of the C strand or may have been exposed when entire Okazaki fragments were removed by telomeric helicases.[78] *rad27* mutant strains grown at the restrictive temperature also displayed a wide range of telomere lengths, indicating that expansions and contractions were common among the telomeric repeats due to the propensity of the single stranded DNA to form secondary structures. Overexpression of *EXO1* increased the growth rate of *rad27* cells to near normal at the otherwise restrictive temperature but did not reduce the appearance of single stranded DNA or telomere length heterogeneity. This finding suggests that Exo1 can only partly compensate for the absence of Rad27.

Further studies by the same authors[79] showed that, in the absence of both Rad27 and telomerase, cells reached senescence earlier compared to the case of telomerase single mutant strains, indicating that the absence of Rad27 hastens telomere shortening. In summary, telomeres are processed differently depending on whether they are replicated by leading or lagging strand synthesis, and Rad27 deficiency affects only those telomeres that are replicated by the lagging strand machinery.[79]

The overhanging G tail may be generated by telomerase adding repeats to the G strand or by nuclease resection the C strand.[80,81] In the process of searching for the nuclease responsible for C strand resection in *S. pombe*, Tomita and colleagues[82] reported that Dna2 is involved in producing the G tail necessary for telomere stability. It was already known that Dna2 associates with telomeres at various stages of the cell cycle.[83] Tomita et al. observed that mutating *DNA2* in the *taz1-d* background abrogates C strand resection. Taz1 is the *S. pombe* homologue of TRF2, a double stranded telomere binding protein that participates in telomere stabilization. *Taz1-d* strains were required in order to increase the detection sensitivity of G-rich overhangs in asynchronous cell populations.[84] The authors also indicated that mutating *RAD50*, even in the presence of wild-type Dna2, disables C strand resection. This led them to speculate that Dna2 employs its 5′ to 3′ endonuclease activity to cleave the C strand in a Rad50-dependent fashion.[82]

Bloom syndrome and Werner syndrome patients show signs of early aging, a process also associated with telomere degradation. It is not surprising, therefore, that BLM and WRN helicases have been linked to telomere processing through a variety of mechanisms. For example, the helicase activity of BLM and WRN is active on G tetraplexes,[85] structures that form in vitro amid G-rich sequences such as telomeric repeats.[86] In addition, compounds that bind and stabilize G tetraplex structures inhibit BLM and WRN helicase activity in vitro.[87] When the replication fork reaches the end of a chromosome, the replicative helicase may be unable to resolve tetraplex structures present within the telomere without the aid of either WRN or BLM.[88,89] It is also possible that these helicases are required for dismantling the T-loop structure in advance of the replication fork. In addition, BLM and WRN are both stimulated by a resident telomere binding protein, TRF2,[90] and studies of mice that lack BLM and WRN show severely compromised telomere replication,[91] further validating the idea that they have a role in telomere processing.

Recently, Crabbe et al.[88] reported that human cells expressing mutant forms of WRN also show lagging strand-specific defects in telomere processing. Using chromosome orientation fluorescent in situ hybridization (CO-FISH), they noticed that when WRN helicase activity is defective, the fluorescent signal corresponding to the lagging strand telomere decreased more than 7-fold. Loss of signal indicated that telomere replication by the lagging strand machinery was incomplete. Telomere replication by the leading strand machinery was unaffected by the mutant WRN. As expected, lagging strand telomere loss was relieved by telomerase expression. This result underscores the concept that telomeres replicated by the lagging strand machinery must overcome unique challenges to be processed properly.

Evidence suggests that RecQ helicases, and BLM in particular, promote telomere extension even in the absence of telomerase activity.[92] This novel means of avoiding senescence is used by a subset of tumor cells and some immortalized cell lines. It is referred to as alternate lengthening of telomeres (ALT).[93] The ALT pathway does not occur in *rad52* deficient cells, suggesting that ALT proceeds via some form of double strand break (DSB) repair.[93] Stavropoulos and colleagues[92] report that human BLM localizes to ALT-specific foci in telomerase deficient cell types but not in cell lines with active telomerase. In addition, co-immunoprecipitation and fluorescence resonance energy transfer studies show that BLM interacts with TRF2, a double stranded telomere-binding protein required for formation of the protective T-loop. Overexpression of wild-type BLM leads to rapid increase of telomeric DNA content, whereas a BLM point mutant devoid of helicase activity did not

stimulate ALT. They speculate that the helicase activity of BLM is involved in allowing the rolling circlelike replication of telomeres.[92] In support of the above, Sgs1, the yeast homologue of BLM, is required for ALT in *S. cerevisiae*.[94−96]

3.3. Microsatellite Instability

In addition to minisatellite repeats and telomeric repeats, microsatellite repeats also expose both the vulnerabilities and adaptations of lagging strand enzymes. Microsatellite repeats are arbitrarily defined as repeating units 1−10 nts long.[60] Within this range the GC-rich trinucleotide repeats (TNR) cause unique consequences due to their ability to readily form hairpin structures. Flap sequences capable of forming secondary structures such as hairpins or bubbles are refractory to FEN1 cleavage because FEN1 is unable to track over the structures to gain access to the base of the flap[97,98] (Figure 2, IV and V). The sequence, purity, and length of the TNR,[99−101] together with its orientation in the genome,[102,103] all influence the likelihood that a hairpin will form with a melting temperature higher than physiological temperature. In addition to hairpins, TNRs can form more complex secondary structures including G tetraplexes and various triplex structures.[104]

TNR instability is the cause of a number of human neurodegenerative diseases including fragile X syndrome, Huntington's disease, myotonic dystrophy, and a variety of ataxias.[104−107] In each disease, the sequence and location of the TNR relative to the gene start site differ, but protein expression is inevitably perturbed. For example, TNR expansions are known in individual diseases to aberrantly recruit transcription repressors, inhibit pre-mRNA splicing, disrupt translation initiation, and even introduce repeated amino acids into the gene product.[106,107]

There is evidence that pathological expansion of TNRs occurs during gametogenesis,[108−111] although somatic instability in patients increases through time and varies between tissues.[112−116] These observations imply that errors in DNA metabolism arise in TNR sequences during both meiotic and mitotic processes. It is not currently understood which processes are most important etiologically to TNR human diseases, but as will be discussed below, DNA replication and repair processes are definitely involved. Our review will focus on studies in yeast and human cell lines complemented by in vitro experimentation that suggests how TNR instability is affected by enzymes involved in lagging strand DNA replication and related repair processes. For more information on how meiosis is involved in human TNR disease etiology, see Pearson et al.[109]

3.3.1. Lagging Strand Replication Is Implicated in Triplet Repeat Instability

Early observations in *S. cerevisiae* showed that perturbation of Okazaki fragment processing enzymes, and *RAD27* in particular, exacerbates TNR instability.[18,67,69,97,117−119] Accordingly, consensus has converged around the idea that expression of wild-type FEN1 has a stabilizing effect on TNRs. That said, even in wild-type yeast strains, tracts of CTG repeats are prone to replication-dependent breakage, contraction, and expansion, suggesting that the secondary structures that are likely to form within TNR sequences during lagging strand replication cannot be satisfactorily dealt with even in a wild-type environment.[102,117,120]

Mutations in many lagging strand proteins exhibit TNR related phenotypes. In *S. cerevisiae*, *rad27* null mutants show increased rates of all three hallmarks of chromosome instability—chromosome breakage, sequence expansion, and, to a lesser degree, sequence contraction.[97,102,117,118,120] In addition, biopsied tumors of mice with a heterozygous knockout of *FEN1* and *APC1*, a gene mutated in colorectal cancer, show microsatellite instability, suggesting aberrant Okazaki fragment maturation when FEN1 protein levels are diminished.[50] Both the *pol3−14*[121] mutation isolated by Giot et al.[122] and also mutations in the active site of pol δ (*pol3-t*)[18,120] destabilize TNRs. The most likely reason for the observed destabilization is increased susceptibility to polymerase slippage, leading to contractions.

Schweitzer and Livingston[121] reported that three distinct mutations in *POL30*, the gene that encodes for PCNA, led to varying degrees of TNR destabilization. The PCNA mutants were *pol30−52*, which prevents homotrimerization of PCNA,[123] *pol30−79*, a mutation which disrupts pol δ binding,[124] and *pol30−90*, which inhibits FEN1 binding to PCNA.[124] Interpreting the effect of PCNA mutation is complicated due to the involvement of PCNA in leading and lagging strand synthesis and a variety of repair pathways. However, one plausible explanation is that in the absence of a functional interaction between PCNA and pol δ (i.e. in the *pol30−52* and *pol30−79* strains) the synthesis rate or processivity decreases to a point where polymerase slippage contractions are more likely. Interestingly, the *pol30−90* PCNA mutation, known to disrupt the FEN1/PCNA interaction, led to increased tract expansions reminiscent of the effect of FEN1 mutation on tract stability.[121] In addition, mutations in pol α, Dna2, and DNA ligase I each destabilize TNR sequences slightly.[120,125−127] A number of models have emerged to explain replication-dependent TNR instability. For the most part, the models are not mutually exclusive, and it is possible that any combination of them is in play depending on the repeat tract length, the level of repeat homogeneity, and the presence or absence of mutation in the enzymes involved. Henricksen and co-workers[128] proposed a model for TNR expansion based on characteristics of human proteins in vitro. Their model derives from observation of FEN1 and DNA ligase I activity on a substrate composed of 10 CTG repeats at the 5′ end of a downstream primer and the 3′ end of an upstream primer that can compete for annealing to 10 CAG repeats on a template strand (Figure 5, I). Such a substrate mimics the junction of Okazaki fragments on the lagging strand in that it is capable of equilibrating between different 3′ flaps, 5′ flaps, and a variety of bubble structures arising from slip mispairing.[128,129] The majority of substrate conformations are inert to both FEN1 and DNA ligase I. However, 5′ flaps can be cleaved by FEN1 to produce correct length products, and bubble structures that produce nicks between the upstream and downstream primers can be ligated to form expanded products (Figure 5, II−V). In these experiments there is a balance between FEN1 activity and DNA ligase I activity: the former promoting correct length processing of the lagging strand and the latter enabling expansion of the daughter strand. Henricksen and colleagues[128] suggest that tipping the balance toward FEN1 cleavage would lead to sequence stability whereas tipping the balance in favor of ligation would promote sequence expansion. The balance could be influenced by mutation or modification of FEN1[130] or DNA ligase I as well as by mutations in other proteins involved in Okazaki fragment processing such as PCNA, DNA polymerases, helicases, or other nucleases.

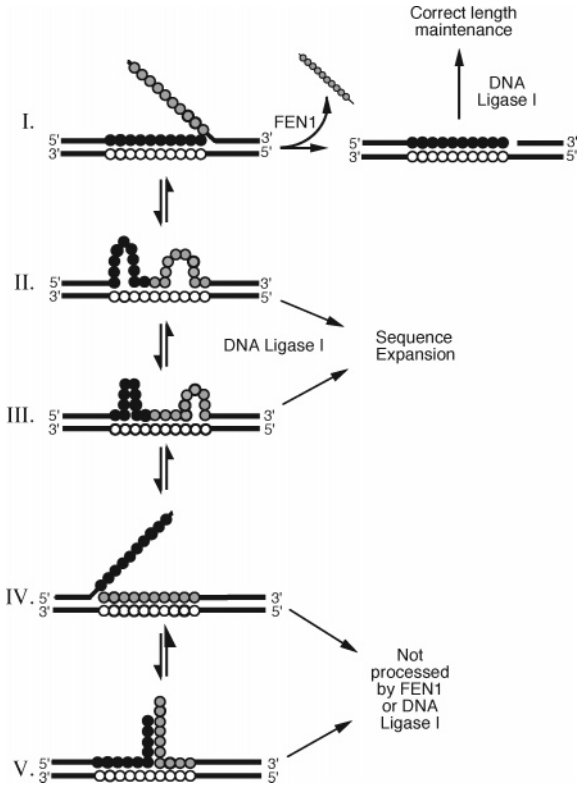

Figure 5. Model for TNR expansion. (I) Schematic of the substrate used by Henricksen et al. Each white circle represents one CAG repeat on the template strand. Gray and black circles represent CTG repeats on the upstream and downstream primers, respectively. Drawings below (I) show a small number of the possible intermediates that might form as the substrate equilibrates. If a cleavable 5′ flap forms, FEN1 can remove the flap (assuming a hairpin has not formed on the flap), leaving a nick that can be ligated leading to preservation of the correct length. (II and III) If bubble structures form that present a nick between the upstream and downstream primers, DNA ligase I can seal the nick, leading to sequence expansion. (IV and V) Many other intermediates can form that are inert to both FEN1 and DNA ligase I but could be substrates for other nucleases or helicases. Substrates IV and V could lead to double strand breaks or illegitimate recombination if not resolved. Adapted with permission from ref 128. Copyright 2002 American Society for Biochemistry and Molecular Biology.

Two recently published works from the Arnheim[127] and Livingston[126] groups highlight the importance of the levels of enzymes involved in flap processing and nick ligation. Subramanian and colleagues[127] showed that overexpression of wild-type DNA ligase I in *S. cerevisiae* destabilizes replication of a $(CTG)_{25}$-containing reporter plasmid. Interestingly, overexpression of catalytically dead ligase also destabilizes CTG repeats. The authors suggest that the latter effect is due to the catalytically dead ligase binding to PCNA and disabling productive interactions between PCNA and its other interacting partners, including FEN1. Refsland and Livingston[126] report that point mutations in the PCNA interaction domains of Rad27 and DNA ligase I both lead to CAG tract instability in yeast single mutants. When the *RAD27* mutant is combined with a PCNA mutant (*pol30−90*) that cannot bind to replication and repair proteins, there is a synergistic effect on CAG tract instability. The data from these two groups strongly suggest that PCNA plays an important role in orchestrating the delicate balance between FEN1 and DNA ligase I activity during Okazaki fragment maturation.

Previous work by researchers in the Livingston group[125] also addressed the effect of DNA ligase I on TNR stability. They showed that two loss of function mutations in *S. cerevisiae* DNA ligase I (*cdc9−1* and *cdc9−2* alleles) actually led to higher TNR expansion.[125] The model they propose to explain their observation involves extended nick half-life due to defective DNA ligase I activity. Increased availability of the unprocessed nick likely allows the DNA polymerase to displace another flap. Consequently, the enhanced need for flap cleavage and ligation leads to the increased possibility that the flap might form a bubble creating a ligatable expansion intermediate. Evidently the absolute and relative levels of DNA ligase I, FEN1, and PCNA relate in a complex manner to the stability of repeat sequences.

Another model for how wild-type FEN1 may protect TNR sequences from expansion was proposed by Liu et al.[131] and involves the tracking requirement of FEN1. Liu and Bambara initially showed[132] that FEN1 utilizes its endonuclease activity to resolve triplet repeats. A hairpin-containing TNR flap is refractory to FEN1 processing but is free to equilibrate into other structures such as 3′ flaps. As this occurs, double flap structures form with 3′ and 5′ flaps of various lengths (refer to Figure 2, III). If the substrate equilibrates in such a way as to present any amount of single stranded 5′ flap, despite the presence of 3′ flap DNA, FEN1 may bind that structure and influence the re-equilibration of the flap into the full length 5′ flap, which is cleavable by FEN1. In such a case, the formation of a hairpin behind the advancing FEN1 would serve to sequester FEN1 on the flap until it recognizes the flap base and cleaves.[131]

3.3.2. Dealing with Substrates That Compromise FEN1

Secondary structures associated with minisatellite and microsatellite repeat sequences impair the cleavage activity of even wild-type FEN1. What backup mechanisms exist to augment correct processing or remedy faulty processing of TNRs? Compensatory mechanisms can be roughly grouped into processes that facilitate correct flap processing and processes which detect expansion intermediates and repair them before subsequent rounds of replication (the latter are discussed in section 3.3.3).

Dna2 may play a role in processing long structure-containing flaps that accumulate amid TNR sequences.[35] Like FEN1, Dna2 must first recognize a free 5′ end.[32] Unlike FEN1, however, Dna2 possesses ATP-dependent helicase activity which allows it to unwind secondary structure concomitant with random cleavage events.[24,35] Therefore, Dna2 seems well suited to unwind and remove segments of hairpin flaps to a point where they are manageable by FEN1. However, the single study that examines the effect of a *DNA2* mutation with partially defective helicase and nuclease activity on CTG repeat tract stability implied only minor involvement of Dna2.[120]

RecQ helicases, such as WRN and BLM, may act on TNR repeat structures to prevent ligation of expansion intermediates. Wang and Bambara[42] showed that, in vitro, BLM helicase activity stimulates FEN1 cleavage of a bubble substrate wherein the 5′ end of the bubble is complementary to the template (Figure 2, V). BLM is proposed to bind to the single stranded bubble and transform the bubble to a flap by unwinding in the 3′ to 5′ direction. Assuming FEN1 interacts with the 5′ flap before it reanneals to its complementary sequence, the repeat-containing sequence could be removed.

Despite the aforementioned in vitro evidence that BLM stabilizes TNR tracts, mutational analysis shows that defective Sgs1, the *S. cerevisiae* RecQ helicase, does not lead to TNR expansion. Surprisingly, compromised Sgs1 either has no effect on TNR stability or stabilizes TNR repeats.[133] In contrast, mutants of the 3′ to 5′ helicase Srs2 strongly destabilize TNR sequences, and purified Srs2 can unwind a CTG hairpin mimic in vitro.[134] In addition, *RAD27* and *SRS2* double knockouts are synthetically lethal,[53] suggesting that they are involved in compensatory pathways. Evidently, subtle differences in helicase specificity are important determinants in genome stability.

3.3.3. Repair of TNR Expansion Intermediates

It seems inevitable that DNA metabolism amid TNR sequences leads to chromosomal instability. Fortunately, a portion of these intermediates can be corrected by the mismatch repair (MMR) machinery (reviewed by Lahue and Slater[135]) and perhaps to some degree by a more recently characterized pathway, large loop repair (LLR).[136]

The canonical substrate of the MMR machinery is a misincorporated base that evades the 3′ to 5′ proofreading activity of pol δ and is recognized by the Msh2/Msh6 heterodimer.[137] In addition, small unpaired loops up to about 15 nts are recognized by the Msh2/Msh3 heterodimer[138] and to a lesser degree by the Msh2/Msh6 heterodimer. If loops are free from intraloop hydrogen bonding, they are efficiently excised, but if secondary structure is present in the loop, the rate of excision and repair decreases dramatically. Indeed a number of groups[139−144] have demonstrated that small TNR insertions and deletions increase when MMR is eliminated. In other words, small TNR loops, most likely caused by polymerase slippage events, do not form stable intraloop structures and are recognized and repaired as long as MMR is operative. On the other hand, loops containing multiple triplet repeats are likely to form hairpins and persist as expansion or contraction precursors.[145] On the basis of the observation that knocking out Msh2 activity stabilizes CAG repeats in Huntington's disease mouse models,[146,147] some have suggested that Msh2 binding to TNR hairpins somehow undermines completion of repair.

Interestingly, tract contractions decrease in *Escherichia coli (E. coli)* when MMR is inhibited.[140,141] Presumably this is because small unstructured loops excised by MMR enzymes are removed together with adjacent DNA, thereby leaving single stranded DNA exposed and able to form hairpins. Hairpins on the template strand are likely to cause polymerase slippage events that lead to tract contractions on the daughter strand. Not surprisingly, the observed contractions are more severe if the template strand contains CTG repeats, which is the most stable hairpin forming TNR.[139] Thus, in the discharge of their duty to stabilize repeat sequences, MMR proteins may under some conditions exacerbate the problems of TNR instability.

LLR is another repair pathway that may take part in controlling expansions at TNR loci. LLR was discovered as an activity capable of repairing heteroduplexes that arise during meiotic recombination in *S. cerevisiae*.[148] Further characterization of LLR revealed that it is active in mitotic cells.[149] LLR is independent of MMR proteins[138,149] but requires pol δ, RFC, and PCNA.[150] A nick is not necessary for resolution of large loops;[151] however, the presence of a nick stimulates LLR machinery to remove the excess DNA from the nicked strand.[136,151] Emergence of the LLR pathway led researchers in the Lahue group[136] to examine LLR proficiency on TNR-containing loops. Reminiscent of the inability of MMR to process secondary structure-containing hairpins, they found that large TNR-containing hairpins are inhibitory to LLR. This result together with similar results showing that palindromic hairpins persist once formed[152,153] underscores the threat of TNR sequences. Additional research is needed to gauge the relationship between LLR and TNR instability.

4. Rescue of Stalled Replication Forks

As discussed in the previous section, efficient replication of the genome is a fundamental process that is critical for the maintenance of genome integrity. The concerted actions of numerous proteins prevent aberrant DNA structures from interfering with lagging strand synthesis. Additionally, many repair mechanisms are in place to ensure efficient repair of DNA lesions prior to encounter with the advancing replication fork. Despite these pathways, the replication fork encounters blocks to replication, such as DNA lesions or frozen protein/DNA complexes that cause the advancing fork to stall. It is estimated that 15−25% of replication forks in *E. coli* require rescue from stalling.[154] Several recent reviews describe the mechanisms involved in replication fork restart in prokaryotes[155,156] and eukaryotes.[157,158] This section highlights the role of lagging strand proteins in mechanisms that rescue a stalled replication fork. These mechanisms are a fundamental component of the arsenal designed to maintain genome integrity, as failure to restart the stalled fork can lead to illegitimate recombination, genome instability, and cell death.

Stalled replication forks can regress (or collapse) to form structures resembling a chicken foot. These structures are versions of Holliday junction (HJ) recombination intermediates. In *E. coli*, regressed replication fork intermediates are processed by RecQ helicase and RecJ exonuclease.[159,160] The mammalian proteins involved in resolving chicken foot structures are unidentified; however, several studies indicate that helicases from the RecQ family play a role.[161] Mutations in RecQ helicase family members WRN, BLM, and RecQ4 have been linked to human diseases that exhibit chromosomal instability.[37] These findings suggest that RecQ helicases help maintain genome integrity during replication, repair, or recombination.

Studies in *S. cerevisiae* support the idea that RecQ helicases unwind aberrant DNA structures that if left unprocessed could lead to deleterious events such as illegitimate recombination. For example, disruption mutation of *SGS1*, the RecQ homologue in *S. cerevisiae*, enhanced illegitimate recombination that occurred via a homologous recombination pathway, suggesting that Sgs1 function suppresses hyper-recombination.[162] Expression of BLM or WRN in these strains suppressed the hyper-recombinogenic effect of *sgs1*, suggesting that WRN and BLM function in human cells suppresses aberrant recombination as well.

4.1. Pathways for Processing Collapsed Forks

Several models explain the pathways by which WRN or another RecQ helicase can restart a stalled replication fork. The intermediates shown in Figure 6 (adapted from ref 41) illustrate the proposed mechanisms by which a replication fork can be rescued following encounter with a lesion on the leading strand template and generation of a regressed

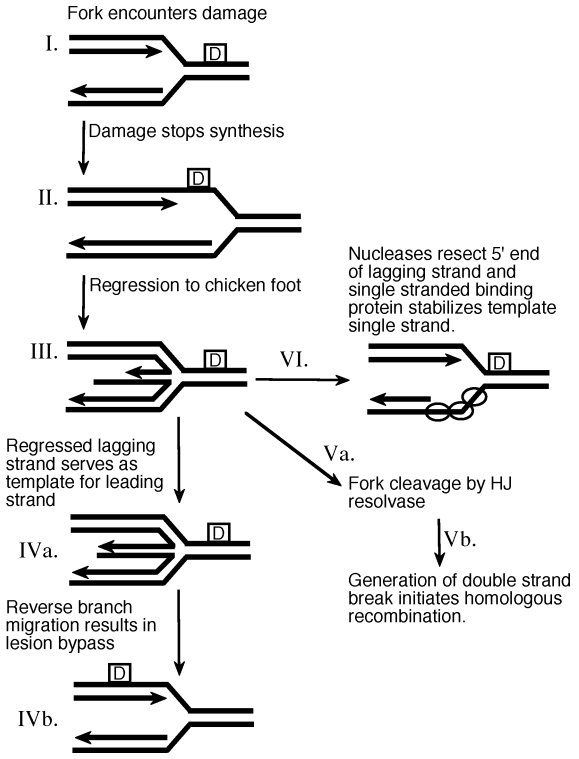

Figure 6. Proposed mechanisms for restart of the replication fork. (I) The advancing replication fork encounters a lesion on the template for the leading strand. (II) Leading and lagging strand synthesis become uncoupled as leading strand synthesis is stalled. (III) The replication fork collapses to form a chicken foot structure by annealing of the leading and lagging strands. (IVa) Leading strand synthesis is resumed using the nascent lagging strand as template. (IVb) Finally, a helicase facilitates reverse branch migration to reset the replication fork past the site of damage. (Va) The regressed fork is cleaved by a resolvase to generate a double strand break. (Vb) This cleavage initiates homologous recombination and allows an undamaged strand to be used for leading strand synthesis. (VI) Helicase unwinds the duplex arm of the chicken foot structure and stimulates cleavage of the nascent lagging strand to create a region of single stranded DNA. This structure can be stabilized by a single strand binding protein while the lesion is repaired. Adapted from Molecular Biology of the Cell (Sharma et al. *Mol. Biol. Cell* **2004**, *15*, 734; published online before print as 10.1091/mbc.E03-08-0567) with permission of the American Society for Cell Biology.

chicken foot structure. In the first pathway (Figure 6, IV), the regressed lagging strand can serve as a template for leading strand synthesis. Following synthesis, a helicase catalyzes reverse branch migration and resets the replication fork beyond the lesion, leaving it to be repaired by other repair pathways. Alternatively, the chicken foot structure is cleaved to generate a double strand break (Figure 6, V). Generation of a double strand break initiates the homologous recombination pathway and allows an undamaged homologous strand to be used as a template for leading strand synthesis. In another mechanism (Figure 6, VI), helicase unwinding of the 5′ end of the lagging strand stimulates cleavage by a nuclease. The resected structure is stabilized by a single stranded DNA binding protein while the lesion is repaired.[41]

Preliminary evidence has highlighted a role for WRN in the processing of collapsed replication forks. Stalled replication forks can be induced by treatment with a DNA damaging agent such as mitomycin C. After treatment of this type, there is evidence that WRN accumulates at foci associated with arrested replication forks.[163] Further studies show that WRN and FEN1 form a complex at these foci.[41] There is a growing body of evidence suggesting that WRN, and WRN stimulation of FEN1 activity, are involved in the rescue of stalled replication forks; however, the mechanism is unclear.

4.2. Competing Models for WRN/FEN1 Roles at Collapsed Forks

Brosh and colleagues[41] have presented one model for FEN1 and WRN interaction at stalled replication forks. In this model, WRN acts to recruit FEN1 to the site of a stalled replication fork that has collapsed into a chicken foot structure. Beginning at the crossover site, the helicase activity of WRN unwinds the HJ and produces a 5′ end onto which FEN1 can load. FEN1 cleavage of the 5′ terminus of the lagging strand produces an intermediate that is stabilized by single stranded DNA binding protein and can await repair of the lesion on the leading strand. Evidence for WRN recruitment of FEN1 to a Holliday junction structure comes from gel shift and immunoprecipitation assays that showed FEN1 binding to HJs only in the presence of WRN. Additionally, experiments performed in vitro showed that structures resembling HJ intermediates can only be cleaved by FEN1 in the presence of WRN and ATP, suggesting that functional WRN helicase activity is required for stimulation of FEN1 cleavage.[41]

Shen and colleagues[164] support an alternative model in which, at a stalled replication fork, WRN and FEN1 initiate the first step in break induced recombination (BIR). This model relies on the ability of FEN1 to generate a double strand break at a replication fork via a novel activity described as gap endonuclease (GEN) activity.[165] The authors showed that FEN1 can cleave the template strand of gapped DNA fork and bubble substrates, providing evidence of the GEN activity and its putative role in fork restart. This type of cleavage at a stalled replication fork would create a double strand break, which is the first step in the recombination pathway that serves to restart the replication fork. In support of the hypothesis that WRN/FEN1 processing of a stalled replication fork initiates homologous recombination, a complex between Rad52 and WRN has been visualized at foci associated with stalled replication forks.[163]

Experiments employing E178A, a FEN1 mutant that has flap endonuclease activity comparable to that of wild type but is deficient in GEN activity, support a role for GEN activity in replication fork restart.[164] Null *rad27* strains of *S. cerevisiae* complemented with either human FEN1 or the E178A mutant showed comparable growth characteristics and spontaneous mutation rates. When the same strains were treated with chemical DNA damaging agents and UV irradiation, the strains complemented with human FEN1 survived at a rate comparable to that of wild-type cells with functioning Rad27. However, cells complemented with the E178A mutant exhibited low survival rates similar to those for the *rad27* null strains. These observations suggest that GEN activity, lacking in the E178A mutant, is required for high level survival following the types of DNA damage that produce stalled replication forks.[164]

In all likelihood, a stalled replication fork can be rescued by several pathways. In fact, the proposed mechanisms by which WRN and FEN1 process a stalled replication fork are not mutually exclusive and both may be relevant. This area of research promises to yield interesting information as further experiments elucidate the mechanisms involved in fork restart.

5. Double Strand Break Repair

Most cells employ two strategies for repair of a double strand break: homologous recombination (HR) and nonhomologous end joining (NHEJ).[166] A number of reviews are available which describe HR in detail;[167−169] however, a brief review of eukaryotic HR is merited here. After damaged chromosome ends are discovered, nucleases degrade the 5′ strand of each broken end, leaving single stranded 3′ tails that are bound by Rad51. After end processing, an identical sequence is sought out on a homologous chromosome or sister chromatid that will act as a bridging template for repair synthesis. Once homology is found, the Rad51-coated 3′ end invades the double helix, allowing the broken strands to anneal to their complements on the donor chromosome. Then, DNA synthesis fills in the resected segments followed by ligation. The processes of strand invasion, DNA synthesis, and ligation lead to Holliday junctions that are resolved by structure-specific resolvases to generate two continuous double helices.

HR is considered relatively error-free in contrast to NHEJ, which by its nature is mutagenic. NHEJ is a pathway that involves the connection of DNA ends possessing only small regions (as little as 1−4 nts) of "adventitious" microhomology.[170,171] In NHEJ, the ends of the DNA are brought together in a process called synapsis. Subsequently, the ends are aligned to exploit any homology. Finally, the aligned ends are processed by nucleases or polymerases to create intermediates that can be ligated.[166,172]

5.1. Rad27 in Nonhomologous End Joining

Lieber and colleagues[170] present data indicating that Rad27 plays a role in NHEJ in *S. cerevisiae*. In this study they examined the frequency of NHEJ in a series of substrates with break ends that were predicted to form a 2 base flap, a blunt end, or a 2 base gap when aligned. Deletion of *RAD27* resulted in a 4.4-fold reduction in NHEJ that was predicted to proceed via generation of a 2 base 5′ flap intermediate. Evidently, alignment of homologous ends results in the production of flaps that are removed by Rad27 prior to completion of joining. In contrast, NHEJ that was predicted to proceed via generation of a blunt end or a 2 base gap was not effected by *rad27* deletion. Considered together, these findings suggest that Rad27 plays a role in a NHEJ pathway that proceeds via flap formation.

In vertebrates, it appears that the dominant nuclease involved in the processing of NHEJ intermediates is the Artemis/DNA-PKcs complex.[173,174] The gene encoding Artemis is mutated in patients with severe combined immunodeficiency (SCID).[175] In higher organisms, V(D)J (variable(diversity)joining) recombination exploits the mutagenic nature of NHEJ to increase immune system diversity. This Artemis complex, present only in vertebrates, can cleave both 3′ and 5′ overhang substrates. It is unclear if another nuclease, such as FEN1, is involved in NHEJ in mammals. However, given the phenotype of Artemis mutants in humans, it is unlikely that other nucleases can fully compensate for lack of this complex.[172]

5.2. Rad27 Suppresses Short Sequence Recombination

A related study by Negritto et al.[176] supports the assignment of a role for Rad27 in maintaining genome stability in *S. cerevisiae*, in this case, by suppression of short sequence recombination (SSR). Null *rad27* mutants displayed an increased level of SSR compared to that for wild-type cells. The authors show that addition of Rad27 or human FEN1 to the *rad27* null cells abrogates SSR. Furthermore, addition of mutant Rad27 or mutant human FEN1 with reduced endonuclease activity and no exonuclease activity partially complemented the *rad27* null phenotype. These studies indicate that the flap endonuclease activity and not exonuclease activity is responsible for the prevention of SSR. The authors explain their observations by the following mechanism. During recombination, Rad27 processes the ends of the recombining fragments. "If unwinding is extensive, Rad27 could remove enough DNA to terminate recombination. Decreasing the length of the sequences shared by the recombination partners would increase the likelihood of complete heteroduplex unwinding, 5′ flap cleavage, or both."[176]

The findings described in the previous section highlight complex roles for Rad27 in recombination. The first example suggests a role for Rad27 in NHEJ, an important repair pathway that increases genome stability. The second example suggests a role for Rad27 in the suppression of short sequence recombination, a pathway that is detrimental to the stability of the genome. Recent work has implicated a role for FEN1 in the processing of divergent sequences at break ends during HR, another pathway that increases genome stability.[177] These findings illustrate the extensive range of functions that proteins involved in lagging strand replication have in genome stability mechanisms.

6. Regulation during the Damage Response

6.1. Initial Damage Signal Cascade

The DNA damage checkpoint exists to preserve genome integrity and halt progression through the cell cycle upon recognition of DNA damage.[169] Recognition is mediated through DNA damage sensors, including ataxia telangiectasia mutated (ATM) protein and the ATM and Rad3 related (ATR) protein, both phosphoinositide 3-kinase related kinases (PIKKs).[178] In response to DNA damage, ATM and ATR are activated, resulting in the phosphorylation of downstream protein targets. This process initiates a signal cascade that leads to arrest of the cell cycle and either repair of damage or apoptosis, if the damage is extensive.[169,178]

Studies have shown that ATM and ATR mediate the damage response to different genotoxic agents and endogenous lesions.[179] While ATM response is activated primarily by ionizing radiation induced damage and double strand breaks,[180−182] the ATR signal cascade is triggered by UV radiation damage and stalled replication forks.[183−187] Upon sensing DNA damage, ATM and ATR phosphorylate checkpoint kinases 2 and 1, respectively, which leads to growth arrest in either G1/S or G2/M phases of the cell cycle.[178] Additionally, it has been shown that RPA stimulates recruitment of ATR and ATR-interacting protein (ATRIP) to damage sites and mediates checkpoint activation.[188] Of particular relevance to this review, it appears that ATR and ATRIP are involved in recognizing lesions resulting from TNR instability. Lahiri et al.[189] demonstrate that *MEC1* and *DDC2*, the homologues of ATR and ATRIP in *S. cerevisiae*, respectively, are important for detecting and mediating repair of TNR related lesions. They hypothesize that ATR mediates upregulation or recruitment of repair proteins to the lesion.

Maintenance of G1/S-phase arrest occurs following ATM- and ATR-mediated phosphorylation and activation of p53, a transcription factor. The activation of p53 leads to p21 induction, which holds the cell in G1/S arrest until damage is repaired.[169] An intra-S-phase arrest can potentially occur by two mechanisms. In one mechanism, ATM and ATR can phosphorylate target proteins that inhibit firing of origins of replication.[169,178] A second mechanism could be mediated through p21 inhibition of replication.[169]

6.2. Effects of p21 on Replication and Repair

It has been shown that the C-terminal region of p21 can bind to PCNA at the interdomain connector loop, the same site that binds pol δ and other replication proteins, including RFC, FEN1, and DNA ligase I.[190−192] Likewise, a C-terminal p21 peptide inhibits replication in vivo[193] and limits synthesis by pol δ in vitro.[194] Still, in vitro, p21 does not appear to affect the loading or sliding of the RFC/PCNA complex on DNA.[195,196] Yet, both in vitro and in vivo evidence reveals a competition between p21 and pol δ for PCNA binding, demonstrating that p21 can disrupt formation of pol δ/PCNA complexes.[191,192,195] Therefore, the inhibition of synthesis by pol δ is most likely due to p21 disruption of the pol δ/PCNA interaction, prohibiting processive synthesis by the polymerase. This is a mechanism for DNA replication to be stalled to allow time for repair of DNA damage.

However, pol δ and PCNA have also been proposed to have roles in repair, specifically nucleotide excision repair (NER)[169,197] and long patch base excision repair (described in section 7), leading to the possibility that p21 inhibition of pol δ has implications for repair. Thus, although the effects of p21 induction on inhibition of replication are fairly well recognized, the effect of p21 on NER remains controversial. The NER pathway is used for excision and repair of damage that distorts the double helix, such as that caused by radiation and chemical genotoxins.[198] NER damage recognition, which involves the lagging strand protein RPA, among other NER-specific proteins, initiates 3′ and 5′ cleavage and removal of a 20−30 nt region surrounding a lesion, followed by gap filling by a DNA polymerase.[169,197,198] Both polymerases δ and ϵ (pol ϵ) have been proposed to fulfill this role.[199]

Consequently, it is possible that p21 sequestration of PCNA alters pol δ and pol ϵ roles in NER because PCNA interacts with both pol δ and pol ϵ.[199] Experiments in vitro utilizing human cell extracts have shown that p21 does not inhibit short gap filling synthesis by pol δ and pol ϵ.[200] Podust et al.[195] demonstrated, via extension of a primer−template substrate, that p21 limited pol δ synthesis of longer products but not short products, consistent with a p21 effect on replication but not repair synthesis by pol δ. It has been proposed, then, that p21 binding to PCNA prevents the reassociation of pol δ with PCNA after a single stretch of synthesis and dissociation from the DNA, inhibiting extended synthesis during replication by the polymerase. Since in NER the gap filling is short synthesis, there may only be one turnover of the pol δ and no need for association with PCNA. This would suggest that NER gap filling is not susceptible to p21 inhibition.[195] Yet, in work by Pan et al.,[201] experiments using full length p21 protein demonstrated inhibition of NER that could be rescued by addition of PCNA. Moreover, Cooper et al.[202] found that both in vitro and in vivo p21 peptides limited NER. The effect of p21 on NER was also observed in vivo using p21 null human fibroblasts.[203] When compared to p21 homozygous or heterozygous fibroblasts, the p21 null cells displayed an increased sensitivity to UV radiation, increased incidence of apoptosis, and a reduction in efficiency of NER. Yet, PCNA recruitment at damage sites was unaltered in the p21 null cells compared to wild type, indicating that a component of p21 inhibition of NER is not directly related to PCNA recruitment to the DNA.[203] In total, these results give a clouded view of the regulation of NER by p21.

In addition, p21 also affects the interaction between PCNA and FEN1. Sequence conservation between species reveals the importance of the interaction site between FEN1 and PCNA.[204] Furthermore, yeast two-hybrid and immunoprecipitation experiments demonstrate a physical interaction between PCNA and FEN1.[205] The interaction is mediated through the C-terminal region of FEN1 and the C-terminal site on PCNA, which also binds p21.[190,205] Both in vitro and in vivo evidence suggests that p21 disrupts the formation of a FEN1/PCNA complex through a competition for the binding site on PCNA.[204,205] In addition, in vitro reconstitution of repair reactions with recombinant human FEN1 and human PCNA shows an inhibition of PCNA-stimulated FEN1 cleavage with increasing amounts of p21.[194] Inhibition of FEN1 stimulation by PCNA during replication does not pose a problem during the damage response because it is important to halt replication. Yet, during the damage response, sequestration of some of the cellular pool of PCNA by p21 could potentially limit stimulation of FEN1, diminishing its activity in damage repair, although the extent of the effect is not clear. It is possible that the lack of stimulation by PCNA only partly attenuates FEN1 activity and that the activity of FEN1 alone is sufficient to support DNA repair.

7. Base Excision Repair

Base excision repair is a major pathway for repair of DNA base damage caused by simple alkylating or oxidizing agents.[206] In the current model of BER, the first step is initiated by recognition of a damaged base by a DNA glycosylase and cleavage of its N-glycosidic bond to form an apurinic/apyrimidinic (AP) site.[207,208] Subsequently, AP endonuclease (APE) cleaves the DNA backbone at the 5′ side of the AP site, creating 3′-hydroxyl and 5′-deoxyribose phosphate (dRP) termini.[209,210] At this point two pathways of BER diverge depending on the oxidation state of the 5′ terminal moiety. If it is unaltered, DNA polymerase β (pol β) inserts a single nucleotide and excises the 5′-dRP via a β-elimination reaction.[211−213] Finally, ligase seals the nick to complete repair.[214] This pathway, referred to as short patch base excision repair (SP-BER), does not involve proteins responsible for lagging strand synthesis. In mammalian systems, this pathway appears to be the major pathway involved in the repair of most lesions corrected by BER.[215]

7.1. Long Patch Base Excision Repair

In contrast to SP-BER, which involves the removal and replacement of a single nucleotide, long patch base excision repair (LP-BER) involves the synthesis of a small segment of DNA typically between 2 and 8 nts in length. LP-BER is necessary in situations where the 5′-dRP residue is oxidized or reduced, so that it cannot be removed by pol β excision. Alternatively, LP-BER may occur if extension of the 3′ terminus takes place before pol β has removed the 5′ terminal dRP. In LP-BER, pol β, pol δ, or pol ϵ incorporates several nucleotides onto the 3′ OH terminus generated by APE

incision. This strand displacement synthesis creates a FEN1 cleavable flap containing the dRP residue at the 5′ terminus.[216] Considering that the mechanism of LP-BER involves strand displacement synthesis and flap cleavage, it is not surprising that several of the proteins involved in lagging strand synthesis play a role. Depending on the proteins involved, LP-BER has been further subdivided into PCNA-dependent and pol β-dependent pathways.

7.2. PCNA-Dependent LP-BER

In 1994, Matsumoto et al.[217] showed that, in extracts of *X. laevis* oocytes, repair of a reduced AP site was PCNA-dependent. Moreover, a series of experiments with Chinese hamster and HeLa cell extracts showed that long patch repair of a normal AP site was completely inhibited by the addition of a polyclonal antibody raised against human PCNA.[218] These experiments indicate that PCNA is a critical protein involved in LP-BER. In the PCNA-dependent pathway, PCNA stimulates pol δ (or pol ϵ) to perform strand displacement synthesis creating a flap structure that is cleaved by the endonuclease activity of FEN1. Ligation of the remaining nick is carried out by DNA ligase I. A role for RPA in this pathway has also been implied by several studies.[219−221] Because PCNA stimulates several proteins in this repair pathway, teasing apart its role is difficult. It may serve to facilitate strand displacement synthesis by pol δ or pol ϵ. Additionally, PCNA stimulation of FEN1 cleavage or the DNA ligase I joining reaction may be critical.[222] In all likelihood, PCNA stimulates multiple steps in the pathway.

7.3. Pol β-Dependent LP-BER

Several studies indicate that pol β plays a central role in a subpathway of LP-BER, often described as pol β-dependent LP-BER. Klungland and Lindahl[215] observed a 20-fold decrease in repair of a reduced AP site upon preincubation with pol β neutralizing antibodies (not cross reactive with pol α, pol δ, pol ϵ, or PCNA) in human cell extracts. In another study with human cell extracts, the LP excision product generated was attributed to the concerted action of pol β and FEN1.[223] Moreover, in cell extracts, pol β was shown to be the major polymerase responsible for initiating LP-BER on a substrate containing a reduced AP site that could not be excised by pol β.[224]

In the pol β-dependent pathway of LP-BER, pol β is the sole polymerase that mediates repair synthesis. In the model,[215] pol β performs strand displacement synthesis, creating a 5′ flap that FEN1 can cleave. Following this action, a DNA ligase can complete the repair. Studies have shown that pol β and FEN1 can stimulate each others' activities and suggest a coordinated interaction between the two proteins that would be relevant during pol β-dependent LP-BER strand displacement and flap cleavage.[225,226] In this situation, pol β could stimulate FEN1 activity and compensate for the absence of PCNA.

7.4. Coordination during LP-BER

The mechanism of BER involves generation of a single strand break that if left unrepaired could be a precursor to more harmful events such as double strand breaks. As a result, coordination of proteins during BER is considered crucial.[227] The appeal of the PCNA-dependent model of LP-BER is that PCNA, which binds and stimulates many BER proteins, such as pol δ, FEN1, and DNA ligase I, likely serves as a platform to coordinate their functions and facilitate efficient repair. However, there is evidence that accessory proteins in the pathway also serve to coordinate the repair components.

For example, WRN has been shown to have functional interactions with many of the proteins involved in BER.[228] WRN helicase activity has been shown to enhance pol β strand displacement.[229] WRN, the only RecQ helicase with exonuclease activity that can remove 3′ mismatches, has been proposed to act as a proofreader for pol β during BER.[228,230,231] WRN also stimulates pol δ synthesis past hairpin and tetraplex sequences that may arise during synthesis through repeat regions.[232] Finally, stimulation of FEN1 cleavage by WRN may also be relevant in BER.[39]

Poly(ADP-ribose) polymerase I (PARP-1), an accessory protein that is activated upon DNA damage, has been suggested to coordinate steps in BER via protein−protein interactions.[233,234] A PARP-1 null mouse fibroblast line exhibits hypersensitivity to DNA alkylating agents and indicates a role for the enzyme in BER.[235] Recently, a physical interaction between WRN and PARP-1 was shown.[236] Further studies[237] indicate that unmodified PARP-1 inhibits WRN activity; however, upon auto-poly(ADP-ribosyl)ation, the inhibition is abrogated. The authors propose a plausible mechanism in which PARP-1 binds to a BER intermediate, and poly(ADP-ribosyl)ation of nuclear proteins signals repair proteins to the site of damage. Upon auto-poly(ADP-ribosyl)-ation, PARP-1 dissociates from the damaged site, having recruited the proteins required for the subsequent steps of repair.

In addition, APE1, has been shown to interact with pol β, FEN1, DNA ligase I, and PCNA.[194,238,239] APE1 slightly stimulates the activities of FEN1 and DNA ligase I in LP-BER.[239,240] Bohr and colleagues[241] proposed a model in which APE1 remains bound following cleavage of an AP site to inhibit "promiscuous" unwinding by WRN. In the presence of pol β, APE1 is displaced, and WRN can stimulate pol β strand displacement synthesis.[229,241] Additionally, APE1, which has exonuclease ability that removes mismatches more efficiently than matched nucleotides,[242,243] has been suggested to act as a proofreader for pol β.[244] Moreover, a recent study indicates that the role of APE1 in BER may be somewhat complex, as its role is modulated by the other BER proteins.[245]

A surprising number of the proteins involved in BER have been shown to participate in physical interactions with each other. Considered together, the impression that emerges is that repair of a lesion may be mediated by a "handing off" of the substrate from one protein to another via interaction.[227,238] In this sequential "passing the baton" mechanism, after completing a required step in BER, a BER protein is displaced by the next protein in the pathway.[227] As a result, following repair initiation, the lesion site is always sequestered by proteins involved in BER. This may serve to enhance efficiency and protect the intermediates from unintended, aberrant processing.

7.5. Consequences of the Damage Response on BER

It is estimated that, under normal physiological conditions, approximately 10,000 AP sites are generated in each mammalian cell per day.[246] Considering the multitude of lesions that need to be repaired, it is understandable that there are redundant pathways. Understanding the dynamics between

the competing pathways of BER is a challenge. At low levels of damage, signaling and relative protein concentrations controlled by the cell cycle stages may dictate a balance between the competing pathways of BER.[247] However, at high levels of damage, induction of the damage response cascade may influence the normal balance between repair pathways.

As discussed in section 6, p21, which inhibits PCNA, is induced in the DNA damage response cascades. It is possible that, in response to DNA damage, the inhibition of PCNA would influence the balance between the PCNA-dependent and pol β-dependent pathways of LP-BER. However, other coordination proteins, such as APE1, may serve to compensate for PCNA inhibition by p21.[194,239] Studies using mouse embryonic fibroblasts indicate that the effects may be more complex. When these cells are treated with plumbagin, which induces oxidative DNA damage, p21 levels are increased; however, pol β and PCNA levels remain unchanged.[248] LP-BER is inhibited in these treated cells although synthesis by pol β is not inhibited by formation of the p21/PCNA complex. Studies with extracts obtained from cells exposed to damaging agents show an accumulation of BER intermediates that require ligation upon p21 induction. This finding suggests that p21 inhibition of PCNA affects PCNA stimulation of DNA ligase I and may be relevant in both pol β-dependent and PCNA-dependent pathways.[248] A p21-mediated disruption of the interaction between DNA ligase I/PCNA complexes is consistent with this hypothesis.[249]

Recent progress in the field of BER has led to the identification of a variety of proteins involved in this pathway. Studies indicate that coordination of repair is likely to take place via a series of protein/protein interactions that serve to sequester the site of damage until repair is complete. In addition, studies are beginning to address the consequences of the damage response on the subpathways of BER. Future work in this area holds the promise of elucidating the roles of the proteins involved in BER and the mechanisms by which they are regulated.

8. Role of Rad9/Rad1/Hus1 in DNA Repair

8.1. Damage Sensor

The Rad9/Rad1/Hus1 (9−1−1) complex acts as a damage sensor in a damage response cascade.[250,251] Following treatment of cells with radiation or chemical genotoxic agents, Rad9 and its partners Rad1 and Hus1 remain more firmly bound in nuclear extracts and associate with chromatin. This indicates that 9−1−1 associates with DNA following damage.[252] Also, several studies utilizing immunofluorescence and microscopy have demonstrated localization of Rad9 to sites of DNA damage, specifically to double strand break foci.[253−256] These results indicate that 9−1−1 is an important component of damage response in the cell.

8.2. Alternative Clamp to PCNA

Initiation of the damage response cascade and induction of p21 serve to limit replication while allowing repair to continue. Although it is possible that the interaction with p21 limits PCNA roles in repair, as discussed previously, recent reports suggest that, in addition to sensing DNA damage, the 9−1−1 complex also serves as an alternative to PCNA that can function during DNA repair.[257]

Rad9, Rad1, and Hus1 are human and *S. pombe* checkpoint proteins found to associate as a heterotrimeric protein complex.[258−261] The 9−1−1 homologue in *S. cerevisiae*, Ddc1/Rad17/Mec3, also associates as a heterotrimer.[262] Although there is little sequence homology between the two, both molecular modeling[262,263] and electron microscopy studies[264,265] reveal that 9−1−1 mimics PCNA in structure. The 9−1−1 complex also has a similar loading molecule, Rad17/RFC (human and *S. pombe*) or Rad24/RFC (*S. cerevisiae*). The alternative clamp loaders Rad17/RFC and Rad24/RFC contain four of the five subunits from RFC and are similar in structure.[264] In a manner analogous to RFC loading of PCNA, Rad17/RFC[266,267] and Rad24/RFC[268,269] have been shown to load their respective heterotrimer clamps onto DNA in an ATP-dependent manner. In addition, RPA interacts with[270] and stimulates loading of 9−1−1 onto 5′ recessed primer−template DNA.[266] Similar to PCNA, 9−1−1 also stimulates FEN1 cleavage in vitro on replication and repair substrates.[257] However, unlike PCNA, 9−1−1 does not stimulate the processive synthesis of pol δ.[257,271] Together, these results further support the proposed role of 9−1−1 as an alternative repair-specific clamp.

8.3. Repair Platform

In addition to its roles in sensing DNA damage, it is possible that 9−1−1, as an alternative clamp, serves a direct role in the repair of damage through its interactions with repair proteins. Both pol β[199] and DNA glycosylase MutY homologue (MYH)[272] play roles in base excision repair. Through immunoprecipitation experiments, a direct interaction between pol β and 9−1−1 was observed. The 9−1−1 complex enhances the efficiency of primer usage and stimulates pol β synthesis.[271] Similarly, immunoprecipitation in *S. pombe* revealed an association between MYH and 9−1−1 that was increased upon cellular exposure to DNA damaging agents.[273] Together, these results suggest that, with DNA damage, 9−1−1 interacts directly to stimulate the activity of repair proteins.

Furthermore, the interactions between 9−1−1 and FEN1[257,274] or DNA ligase I[275] support this idea. Recent in vitro results suggest that 9−1−1 stimulates FEN1 cleavage on oligonucleotide substrates that mimic replication and repair intermediates.[257,274] The stimulation by 9−1−1 does not bypass the need for FEN1 tracking. FEN1 is unable to cleave bubble substrates even in the presence of the 9−1−1 complex.[257] Although stimulation by 9−1−1 is limited on some substrates that have no free ends for 9−1−1 to slide on,[257] FEN1 stimulation by 9−1−1 does not require loading of the 9−1−1 complex onto the DNA (Rossi and Bambara, unpublished data). In addition, in vitro binding of DNA ligase I to a nick substrate is enhanced in the presence of 9−1−1 (Wang and Bambara, unpublished data). Consistent with this observation, 9−1−1 stimulates the nick-sealing activity of DNA ligase I.[275]

In addition, 9−1−1 interaction with translesion polymerases has implications for damage tolerance and repair.[251,276] Translesion polymerases, including polymerases ζ and κ (pol ζ and pol κ, respectively), can replicate through regions of DNA containing base lesions by inserting correct bases opposite damage sites.[277] In *S. cerevisiae*, when pol ζ is mutated in cells having a defect in NER that leads to irreparable DNA damage, the cells display an increased sensitivity to DNA damage by UV radiation, suggesting that pol ζ has a role in damage tolerance by replicating through the damage. The pol ζ-mediated damage tolerance in these cells is dependent on Rad17, Rad24, and Mec3 damage

checkpoint proteins,[278] and pol ζ has been shown to interact with Mec3 and Ddc1 both in vitro and in vivo,[279] indicating that pol ζ is involved in the damage response. Similarly, in *S. pombe*, exposure of cells to a DNA damaging agent leads to an increase in the level of pol κ as a result of damage checkpoint activation. Also, immunoprecipitation studies show that pol κ associates with Hus1 and Rad1, and chromatin binding assays demonstrate that Rad17 mediates pol κ association with chromatin.[280] Together these results suggest that pol ζ and pol κ are involved in tolerance and repair of damage mediated through the activation of damage checkpoint proteins.[251,276,279]

Stimulation of the activity of repair proteins, including pol β,[271] FEN1,[257,274] and DNA ligase I,[275] by 9−1−1, as well as the interaction between 9−1−1 components and MYH[273] and the translesion polymerases,[278,280] suggests that 9−1−1 is directly mediating repair reactions, aside from its role in sensing damage. It is possible, then, that the 9−1−1 complex could serve as a platform onto which repair proteins are recruited, which is comparable to PCNA as a replication platform.[281]

9. Role for FEN1 in Apoptosis

The proteins involved in lagging strand replication play an essential role in genome stability by the mechanisms discussed above. Recent studies also suggest a role for FEN1 in apoptosis. This finding indicates that the role of FEN1 in controlling the integrity of DNA is more complex than previously considered.

CPS6, the *C. elegans* homologue of EndoG, a mitochondrial nuclease in vertebrates, has been implicated in the apoptotic pathway.[282] Recent work by Parrish et al.[165] has shown that CRN1 (cell death related nuclease 1), the *C. elegans* homologue of FEN1, can cooperate with CPS6 to promote apoptotic DNA degradation. CRN1 was initially identified from an RNA interference-based screen designed to identify nucleases involved in apoptotic DNA degradation in *C. elegans*.[283] CRN1 was shown to possess characteristic FEN1 properties such as flap endonuclease and 5′−3′ exonuclease activities. Additionally, this study revealed a preference for an additional substrate-specific activity, GEN activity, not previously described in the literature. An interaction between CRN1 and CPS6 was detected in a GST pull-down assay. In vitro studies showed that CRN1 enhances CPS6 nuclease activity and, similarly, CPS6 enhances CRN1 GEN and exonuclease activities, suggesting that both proteins cooperate to stimulate DNA degradation. Xue and coworkers[165] propose a model by which the two nucleases work in concert to degrade DNA during cell death. The expression of CRN1, CPS6, or NUC1, an additional nuclease implicated in apoptotic degradation, was inhibited by RNAi. Decreased levels of each protein produced the same defect in DNA degradation during cell death, supporting the characterization of CRN-1 as a cofactor involved in apoptotic DNA fragmentation.

The interaction and costimulation of FEN1 and EndoG homologues in *C. elegans* raises the possibility that a similar mechanism is relevant in other organisms. In fact, CPS6 (in *C. elegans*) has 48% identity and 69% similarity with human and mouse EndoG. Moreover, mouse EndoG was shown to rescue the CPS6 phenotype in transgenic nematodes, suggesting that EndoG is a functional homologue of CPS6.[282] Because EndoG has been shown to be a weak endonuclease, there may be a requirement for other nucleases to mediate the degradation.[284] DNase1 and ExoIII were shown to stimulate EndoG degradation; however, FEN1 was not tested. It would be interesting to determine whether a functional interaction between EndoG and FEN1 can be detected in other organisms, such as humans.

An interesting aspect of the putative role of CRN-1 in apoptosis involves regulation. That is, how can this protein play dual, almost contradictory roles in genome maintenance and destruction? In human cells, EndoG is released from mitochondria upon stimulation of the apoptotic pathway. If CPS-6 is only sent to the nucleus upon apoptotic stimulation, this translocation may provide a means by which FEN1 could switch roles from replication and repair to destruction.

10. Modifications of Lagging Strand Proteins

10.1. Modified Polymerases, Clamp, and Clamp Loader

Several of the lagging strand proteins are subject to post-translational modifications that potentially serve as regulation points for roles in replication versus repair. First, immunoprecipitation experiments reveal that phosphorylation of pol α in humans, *S. cerevisiae*, and *S. pombe* is dependent on cell cycle distribution.[285−287] In humans, cyclin-dependent kinase (Cdk)/Cyclin complexes phosphorylate both the p180 large catalytic subunit and the p68 accessory subunit of pol α.[285,288−291] In *S. cerevisiae*, the large catalytic subunit (p165) is a substrate for Cdc7/Dbf4 kinase activity,[292] and the accessory subunit (p86) is phosphorylated in a Cdc28 kinase-dependent manner.[286] In addition, the phosphorylation states of the p180 and p68 subunits of pol α have alternate effects on in vitro initiation of SV40 replication, both inhibiting and stimulating replication.[288−290] Together, these results suggest that, in the pol α complex, the two largest subunits are differentially phosphorylated, leading to regulation of pol α activity.

Furthermore, the large p125 subunit of pol δ is phosphorylated in vivo.[293] Also, the p66 subunit of pol δ is phosphorylated by Cdk/Cyclin complexes in vitro, and phosphospecific antibodies react with p66 in vivo.[191] Interaction between p66 and PCNA inhibits phosphorylation, indicating that the phosphorylation of pol δ may mediate interactions between pol δ and its accessory proteins.[191]

PCNA is acetylated in vivo and immunoprecipitates with p300, a histone-acetyl-transferase, and histone deacetylase (HDAC1). This suggests that PCNA is subject to acetylation by p300 and deacetylation by HDAC1.[294] Analyses show that acetylated PCNA binds more tightly to pol δ and pol β and promotes pol δ and pol β synthesis better than deacetylated PCNA.[294] These results suggest that acetylation of PCNA is a means of coordinating PCNA interaction with other proteins. In addition, recent results demonstrated that PCNA is both mono- and polyubiquitinated in response to DNA damage.[295] Sumoylation, or addition of a small ubiquitin related modifier (SUMO),[296] also occurs on PCNA during the S-phase under normal cellular conditions and in response to treatment with a genotoxic agent to induce large amounts of DNA damage.[295] Moreover, earlier studies demonstrated that PCNA can be phosphorylated, and it associates with Cyclin A and Cyclin D.[297,298] Variations in these modifications of PCNA might serve to mediate many roles of PCNA in replication and repair.

The clamp loader RFC is also subject to phosphorylation. The complex of PCNA/Cdk2/CyclinA phosphorylates RFC

at a PCNA binding domain in vitro,[299] as does the calcium calmodulin-dependent protein kinase (CamKII).[300] Phosphorylation of RFC by CamKII inhibits PCNA binding, and the presence of CamKII in vitro inhibits pol δ and pol ϵ RFC-dependent synthesis. Yet a PCNA/RFC/DNA complex is resistant to CamKII phosphorylation.[300] These results suggest that CamKII may be involved in the inactivation of RFC until it is needed for replication. Similarly, when the p145 subunit of human RFC is phosphorylated, it loses the association with two other subunits, p40 and p37.[301] This implies that the interaction of RFC subunits is mediated through phosphorylation status, which may regulate RFC activity. In addition, when the p145 RFC subunit is phosphorylated, it loses its ability to bind to PCNA.[302] Therefore, the state of phosphorylation is presumably affecting both RFC intersubunit interactions and interactions with other lagging strand replication proteins and may have implications for both replication and repair.

10.2. Modified FEN1

Recent work by Hasan et al.[303] has demonstrated FEN1 acetylation by p300 at C-terminal lysines. The C-terminal lysines appear to be important determinants of FEN1 cleavage efficiency, as mutations in these residues decrease cleavage activity.[12] Immunoprecipitation experiments revealed a physical interaction between FEN1 and p300. Analysis of human embryonic kidney cell extracts for acetylated FEN1 shows an increase in acetylation with overexpression of p300, indicating that p300 is at least in part responsible for FEN1 acetylation. The acetylation inhibits nuclease activity and decreases substrate binding, yet it does not alter FEN1/PCNA binding[303] or inhibit PCNA stimulation of FEN1 cleavage activity.[274] However, p300 acetylation eliminates stimulation of FEN1 by the 9−1−1 damage checkpoint complex, indicating that acetylation may be important for modulating FEN1 interaction with replication and repair proteins.[274] In addition to acetylation, FEN1 is also subject to phosphorylation, and this could regulate its activity. Pull-down assays and immunoprecipitations reveal an interaction with both Cdk1 and Cyclin A. Moreover, in vitro and in vivo analyses show that Cdk1/Cyclin A can phosphorylate FEN1. As cells progress through the S-phase, the level of FEN1 phosphorylation increases. Although the phosphorylation does not alter substrate binding, it inhibits both FEN1 cleavage and association with PCNA.[304] Taken together, these results suggest a possible role for modification in distributing FEN1 activity between replication and repair functions.

10.3. Modified RPA

Although RPA does not have catalytic activity in lagging strand synthesis or in repair, it is present both at the replication fork and at damage sites and is modified by phosphorylation.[21,305] RPA phosphorylation is dependent on cell cycle distribution,[306−309] in response to both interruption of DNA replication[310] and DNA damage.[310−316] Studies in *S. cerevisiae* demonstrate that the large subunit of RPA is phosphorylated conditionally with damage[310] while the middle subunit is phosphorylated normally with the cell cycle and following exposure to ionizing radiation.[311,312] These results indicate that there are different mechanisms for RPA phosphorylation. However, in both *S. cerevisiae* and humans, some RPA phosphorylation is dependent on the PIKKs Mec1[312,317] or ATM/ATR.[313,315,316,318] In addition, both Mec1[317,319] and ATM[315,316] can phosphorylate RPA in vitro. Because Mec1 and ATM/ATR are involved in the damage response cascade, it is possible that phosphorylation plays a role in mediating RPA activity during DNA damage repair. It has also been shown that RPA can be phosphorylated by the DNA-dependent protein kinase (DNA-PK) in vivo[313,318,320] and in vitro[310,318,321,322] and by Cdk/Cyclin complexes in vitro.[322,323] Additionally, in vivo studies with apoptosis-induced human T-lymphocytes revealed that inhibitors of DNA-PK and Cdk's limit RPA phosphorylation.[324] Taken together, the numerous varied mechanisms of RPA phosphorylation suggest that it is an important point of regulation for RPA. In support of this assertion, phosphorylation of RPA results in a disruption of intersubunit binding of RPA[323] and binding with associated proteins, ATM and DNA-PK.[325]

10.4. Modified Helicases and Ligase

The RecQ helicases, BLM and WRN, are also subject to modifications. BLM phosphorylation or dephosphorylation in response to ionizing radiation is dependent on cell cycle distribution.[326,327] Moreover, immunoprecipitation studies demonstrate physical interactions between BLM and the damage sensors ATM[328] and ATR.[329] Both in vitro and in vivo experiments also reveal partial ATM- or ATR-dependent phosphorylation of BLM,[326,328,329] that does not result in loss of BLM helicase activity.[326] These results suggest that BLM is affected in the damage response, and it is likely that the status of phosphorylation plays a role in regulation of BLM during repair.

Phosphorylation also presumably plays a role in the regulation of WRN. Recent results demonstrate a physical interaction between WRN and DNA-PK.[330,331] Both in vitro and in vivo, WRN is phosphorylated by a DNA-PK/Ku protein complex.[330,331] In addition, the phosphorylation of WRN negatively regulates its exonuclease and helicase activities.[331] The association between DNA-PK and WRN inhibits the WRN exonuclease and helicase activities,[330] and removal of WRN phosphorylation removes the inhibition of its helicase activity.[331] Moreover, an association between WRN and the cAbl kinase also results in WRN phosphorylation both in vitro and in vivo.[332] Upon exposure to DNA damaging agents, WRN is phosphorylated and loses the association with cAbl.[332] It has been proposed that the cAbl phosphorylation may target WRN to a repair pathway such that WRN could be recruited to repair sites.[332] In addition, recent studies demonstrate that there is ATM- and ATR-dependent phosphorylation of WRN in response to DNA damage.[333] Besides phosphorylation, experiments revealing WRN acetylation[334] and modification by SUMO[335] indicate a variety of post-translation modifications regulating WRN.

Finally, experimental results show that DNA ligase I is phosphorylated in vivo in a cell cycle-dependent manner.[336,337] A complex of Cdk2/CyclinA can phosphorylate DNA ligase I in vitro.[299,337] The phosphorylation reaction is enhanced in the presence of PCNA, suggesting that PCNA is a link between Cdk2 and its substrates.[299] In addition, DNA ligase I can be phosphorylated by casein kinase II.[338] This phosphorylation does not significantly alter the ligase activity, although it eliminates PCNA stimulation.[13] Furthermore, induction of damage or apoptosis results in a dephosphorylation of DNA ligase I.[339,340] Thus, although the mechanisms of phosphorylation may vary, results suggest that phospho-

rylation is also involved in regulation of DNA ligase I activity.

Altogether, it is likely that a variety of modifications, including phosphorylation,[341] acetylation, ubiquitination, and sumoylation, coordinate lagging strand protein interactions with each other and other cellular proteins to maintain genome stability through replication and repair processes.

11. Summary and Outlook

Maintenance of genome integrity is a crucial component in the survival of an organism. As a result, a myriad of pathways function in concert for this purpose. These pathways can be classified into two distinct areas, ones that exist to prevent the accumulation of mutations during replication of the genome and others that repair damage to the genome. In this review we highlight the role of lagging strand proteins in both of these areas.

Eukaryotic lagging strand synthesis is a complex process that requires the generation and joining of millions of Okazaki fragments. Lagging strand replication of repeat sequences is particularly problematic because of the nature of equilibrating flap intermediates that are generated. However, the lagging strand proteins have evolved multiple mechanisms and pathways to prevent mutation under these circumstances. The rescue of a stalled replication fork is another mechanism that is critical to genome maintenance because, if left unrepaired, it can serve as a precursor to more harmful events. It is not surprising that several lagging strand proteins, already present at the replication fork, participate in the restart process. Lagging strand proteins have also been implicated in DNA damage repair pathways such as BER and NER. These pathways employ mechanisms of end processing and ligation reminiscent of those used in Okazaki fragment maturation. This similarity suggests how these proteins have evolved dual roles in replication and repair. FEN1 is an intriguing example of a lagging strand protein that is implicated in diverse pathways involving genome integrity including replication, repair, and even apoptosis.

The roles of lagging strand proteins in multiple pathways of genome maintenance lead to the question, how are the activities of the proteins regulated to direct their involvement in the various pathways? Under low levels of damage, presumably the pathways of replication and repair work in concert. However, during the damage response, replication is inhibited and repair is promoted. The details of the regulation mechanisms employed during the damage response are now being elucidated. Work in this field promises to fill the gaps in our understanding of the regulation of proteins involved in replication and repair processes that must, at times, coexist or compete.

12. Abbreviations

9−1−1	Rad9/Rad1/Hus1 complex
ALT	alternate lengthening of telomeres
AP	apurinic/apyrimidinic
APE1	apurinic/apyrimidinic endonuclease 1
ATM	ataxia telangiectasia mutated protein
ATR	ATM and Rad3 related protein
ATRIP	ATR interacting protein
BER	base excision repair
BIR	break induced recombination
BLM	Bloom helicase
CamKII	calmodulin-dependent protein kinase
Cdk	cyclin-dependent kinase
CRN1	cell death related nuclease 1
C. elegans	Caenorhabditis elegans
Dna2	5′−3′ endonuclease and helicase
DNA-PK	DNA-dependent protein kinase
dRP	deoxyribose phosphate
DSB	double strand break
E. coli	Escherichia coli
EXO1	exonuclease 1
FEN1	flap endonuclease 1
GEN	gap endonuclease
HDAC1	histone deacetylase 1
HJ	Holliday junction
HR	homologous recombination
LLR	large loop repair
LP-BER	long patch base excision repair
MMR	mismatch repair
NER	nucleotide excision repair
NHEJ	nonhomologous end joining
nt(s)	nucleotide(s)
PCNA	proliferating cell nuclear antigen
PIKKs	phosphoinositide 3-kinase related kinases
pol α	DNA polymerase α
pol β	DNA polymerase β
pol δ	DNA polymerase δ
pol ϵ	DNA polymerase ϵ
pol κ	DNA polymerase κ
pol ζ	DNA polymerase ζ
Rad27	*S. cerevisiae* homologue of FEN1
RFC	replication factor C
RPA	replication protein A
Sgs1	*S. cerevisiae* homologue of RecQ helicases
SP-BER	short patch base excision repair
SSR	short sequence recombination
SUMO	small ubiquitin related modifier
S. cerevisiae	Saccharomyces cerevisiae
S. pombe	Schizosaccharomyces pombe
TNR	trinucleotide repeat
TRF2	telomere repeat binding factor 2
UV	ultraviolet
WRN	Werner helicase
X. laevis	Xenopus laevis

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