C-terminal flap endonuclease (*rad27*) mutations: lethal interactions with a DNA ligase I mutation (*cdc9-p*) and suppression by Proliferating Cell Nuclear Antigen (*POL30*) in Saccharomyces cerevisiae

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Last but not least, much thanks to my family in Kenya and here in the US for their support and encouragement. In particular I would like to thank my friend and spouse, Diana, for putting up with my long hours in the lab and for helping me on many a weekend.

Dedication

This dissertation is dedicated to my parents, Sammy and Hellen Karanja and to my siblings, Priscilla, Netta, Nelly and Stella.

Abstract

We recovered the flap endonuclease mutation *rad27-K325** in a synthetic lethal screen with *cdc9-p*, a DNA ligase mutation with two substitutions (F43A/F44A) in its proliferating cell nuclear antigen (PCNA) interaction domain. We created two additional *rad27* alleles, *rad27-A358** with a stop codon at residue 358 and *rad27-pX8* with substitutions of all eight residues of the PCNA interaction domain. Tests of mutation avoidance and DNA repair showed that *rad27-K325** confers severe phenotypes similar to *rad27Δ*, *rad27-A358** confers mild phenotypes and *rad27-pX8* confers phenotypes intermediate between the other two alleles. *rad27-K325** behaves similarly to *rad27Δ* in being lethal with *exo1Δ* and *rad51Δ* and not with *rad2Δ*. Interestingly, *rad27-pX8* is lethal with *rad51Δ*, while *rad27-A358** is lethal with *rad51Δ* at an elevated temperature. High copy expression of *POL30* (PCNA) suppresses the canavanine mutation rate of all the *rad27* alleles, including *rad27Δ*. *rad27-K325** has an absolute lethality with the PCNA mutation *pol30-90* that is not possessed by *rad27Δ*. These studies show the importance of the C-terminus of the flap endonuclease in mutation avoidance, and, by virtue of the initial screen, the role that PCNA plays in coordinating the entry of DNA ligase and the flap endonuclease in replication and repair.

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CHAPTER 1

Introduction

Maintenance of genomic integrity is essential for the viability of all organisms. DNA insults have to be repaired and the fidelity of DNA replication maintained to ensure that deleterious mutations are not passed on to progeny or cause cancer. Organisms have evolved an integrated mechanism that utilizes shared proteins involved in DNA replication, repair and recombination to maintain genome stability. Because these mechanisms produce similar DNA intermediates such as single-stranded DNA, 5' or 3' flaps, nicks and gaps, several proteins can have multiple roles in DNA metabolism.

The flap endonuclease in *Saccharomyces cerevisiae* is involved in DNA replication, repair and recombination mechanisms, is a key player in ensuring that these processes function efficiently. The flap endonuclease is a 43 kDa multifunctional, structure specific nuclease that has important roles in DNA replication, repair and recombination. It is the product of the *RAD27* and FEN1 genes in yeast and humans, respectively. The flap endonuclease is a member of the rad2-family of nucleases, which have either exo- or endonuclease activity or both (LIEBER 1997). Additionally, they are characterized by the presence of two evolutionarily conserved Mg^{2+} metal coordinating domains, N-terminal (N), and Intermediate (I). These domains are crucial for the catalytic activity of these enzymes since the metal ions are required for substrate binding (HARRINGTON and LIEBER 1994a) and the hydrolysis of the phosphodiester linkage (LEE and WILSON 1999; TOCK *et al.* 2003). The C-terminal portion of some members of this family of proteins also contains a conserved sequence motif of the amino

acids QxxL/I/MxxFF/Y. This motif is important for those nucleases that interact with proliferating cell nuclear antigen (PCNA) (FRANK *et al.* 2001; HARRINGTON and LIEBER 1994b). The C-terminal end has a series of lysines and arginines that are important for posttranslational regulation (HASAN *et al.* 2001; HENNEKE *et al.* 2003) as well as for nuclear localization (QIU *et al.* 2001).

Across species, the flap endonuclease is structurally and functionally well conserved. In eukaryotes, sequence similarity is between 80% and 96% within the N and I domains as well as in the PIP box (SHEN *et al.* 1998; SHEN *et al.* 2005). Indeed, human FEN1 can complement the deletion of *RAD27* in yeast (GREENE *et al.* 1999).

Cellular expression of the flap endonuclease is consistent with its role during DNA repair and replication. It is up-regulated during the G1 phase of the cell cycle in preparation for its function during S-phase (REAGAN *et al.* 1995). Additionally, protein levels are increased after exposure to the DNA-damaging agent methyl methanesulfonate (MMS), a clear indication of its requirement for DNA repair (VALLEN and CROSS 1995).

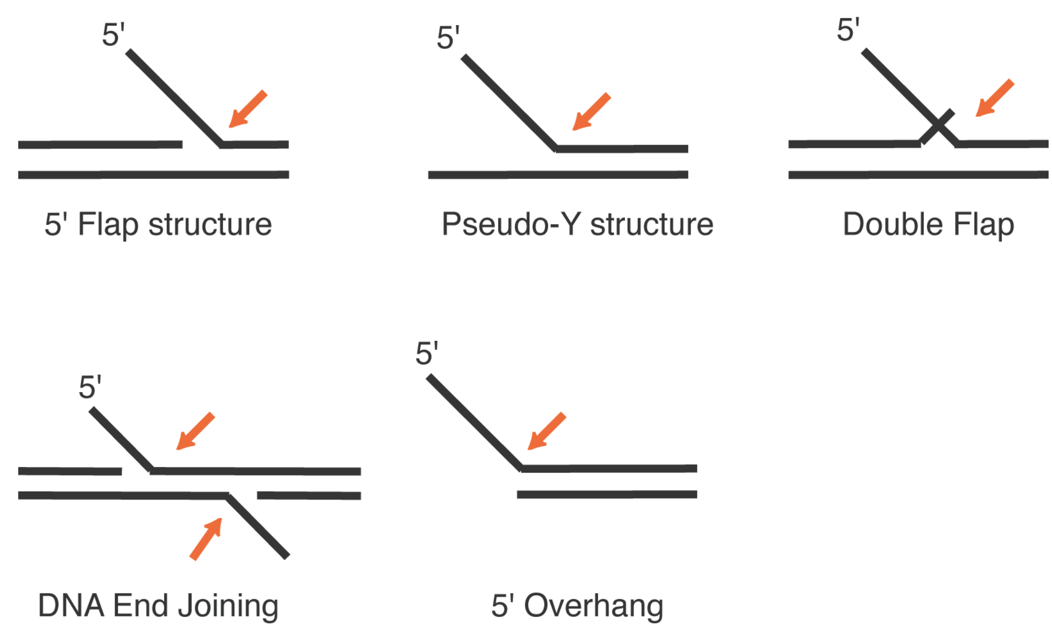
The flap endonuclease is versatile in its choice of substrates (Figure 1). The enzyme's endonuclease activity can cleave 5'-flaps on DNA or RNA, pseudo-Y structures, double flap structures, and 5'-flap DNA assembled into nucleosomes (HUGGINS *et al.* 2002; LIEBER 1997; SHEN *et al.* 2005). The substrates for the 5'-3' exonuclease activity include nicks, gaps and recessed 5'-ends DNA (LIEBER 1997; SHEN *et al.* 2005). Finally the substrates for the

more recently described gap endonuclease may recognize gaps in the replication fork and bubble structures (ZHENG *et al.* 2005). This repertoire of substrates illustrates the multiple reactions the flap endonuclease is capable of catalyzing during DNA replication, repair and recombination.

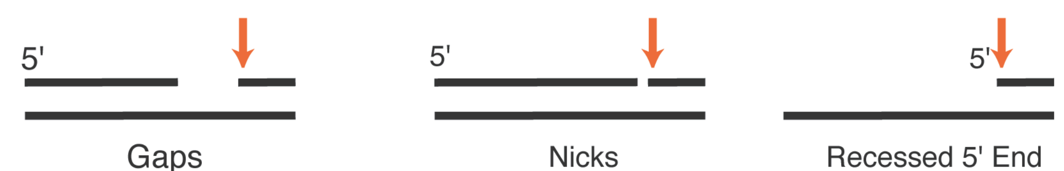
Figure 1. DNA structures cleaved by the flap endonuclease

(A) The flap endonuclease endonucleolitically cleaves single stranded 5'-flap structures consisting of DNA or RNA. The ideal structure for this cleavage is the double flap with a 1 nt 3'-flap. (B) The exonuclease activity of the flap endonuclease is less efficient than the endonuclease activity. It excises single bases at a nick, gap or recessed 5'-end on double stranded DNA. (C) The recently described gap endonuclease function cleaves DNA bubble structures and single stranded DNA gaps that may be generated by stalled replication forks.

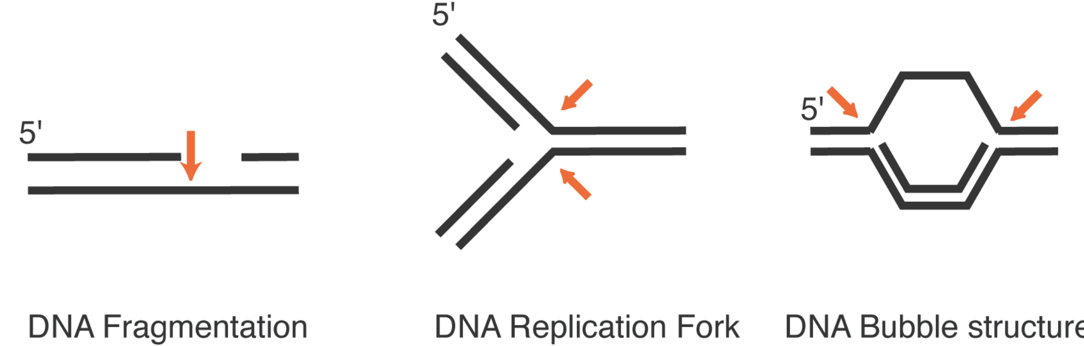
A. 5' flap endonuclease activities



B. 5' exonuclease activites



C. Gap endonuclease activities



The flap-independent endonuclease activity

Apart from the well-defined endonuclease activity requiring a 5'-flap structure, the flap endonuclease has been reported to have flap-independent endonuclease activity. Zheng *et al*., (2005) reported that the human flap endonuclease possesses gap-dependent endonuclease (GEN) activity required during DNA replication for the repair of stalled replication forks. Utilizing GEN activity, the flap endonuclease cleaves the single stranded DNA sections of stalled replication forks or gapped DNA duplexes to yield double stranded breaks that are substrates for break induced repair. This gap endonuclease activity is distinct from the flap endonuclease activity in both substrate specificity and enzyme catalytic site (SINGH *et al.* 2007; ZHENG *et al.* 2005). Separation of function mutations in the human flap endonuclease has mapped the GEN activity to E178 (ZHENG *et al.* 2005) and flap endonuclease activity to D34, D86 and D181 (LIU *et al.* 2004).

The abrogation of the GEN and EXO (exonuclease) functions of the flap endonuclease has severe consequences. A sampling of human cancers found point mutations in the flap endonuclease that resulted in defective GEN and EXO activity (ZHENG *et al.* 2007). The same authors reported that mice harboring a specific mutation in FEN1, E160D, that eliminates both the GEN and EXO activities, were more sensitive to MMS, had increased DNA damage-induced apoptosis and were highly susceptible to cancer. Biochemical studies have also suggested a role for GEN and EXO activities in maintaining triplet

repeat stability. Singh *et al*., (2007) used a yeast flap endonuclease mutant deficient in EXO and GEN activity and showed that were defective in cleaving various triplet repeat intermediates.

Characterization of *rad27* deletion mutants

The cellular role of the flap endonuclease has been revealed by genetic and biochemical studies characterizing various alleles of *RAD27* in yeast. Although *RAD27* is non-essential, its dispensability is not trivial to the cells as can be inferred from its array of phenotypes. *rad27Δ* mutants are viable at 30°C but are temperature sensitive at 35°C where cells arrest in S-phase (REAGAN *et al.* 1995; SOMMERS *et al.* 1995), an indication of the requirement for *RAD27* during replication at elevated temperatures. These replication defects also extend to plasmid maintenance where strains lacking the flap endonuclease have elevated levels of plasmid loss under non-selective conditions and have a high rate of spontaneous mutations (REAGAN *et al.* 1995; TISHKOFF *et al.* 1997b). The ability of *rad27* null strains to repair DNA is also compromised since these strains are sensitive to DNA-damaging agents MMS and ultraviolet irradiation (REAGAN *et al.* 1995).

The high rate of spontaneous mutations implicates *RAD27* in mutation avoidance. Tishkoff *et al*. (1997b) examined the role of the flap endonuclease in mutation avoidance and found that it is required to prevent duplication of DNA. When they determined the spectrum of mutations in the *LYS2* and *CAN1* locus of *rad27Δ* strains, they found that most are duplications of 32 nt in the former

and between 3-12 nts in the latter. These duplicated DNA sequences are flanked by direct repeats suggesting that the mutants have increased rates of replication slippage of unprocessed Okazaki fragments or that strand breakage results in recombination events (KOKOSKA *et al.* 1998; TISHKOFF *et al.* 1997b; XIE *et al.* 2001). Indeed, *rad27Δ* strains are hyperrecombinogenic and exhibit elevated levels of mitotic recombination (TISHKOFF *et al.* 1997b; VALLEN and CROSS 1995).

Some lesions generated in the absence of the flap endonuclease are processed by homologous recombination (HR) and not by the non-homogous end-joining (NHEJ) pathway (SYMINGTON 1998; TISHKOFF *et al.* 1997a). This observation is reported in studies showing *rad27Δ* to be synthetically lethal with *rad51Δ* and *rad52Δ*, two proteins that are important in HR (SYMINGTON 1998; TISHKOFF *et al.* 1997a). In examining whether the NHEJ pathway also participated in fixing *rad27Δ* lesions, the Symington group (SYMINGTON 1998) generated *rad27Δ hdf1* double mutants. *HDF1* encodes Ku70, an essential component of the NHEJ pathway. The double mutants are viable suggesting that *rad27Δ* lesions are not fixed by this pathway.

The ability of *rad27* null strains to survive at lower temperatures is attributed to the presence of compensatory nucleases such as Exo1. This makes *rad27* essential when combined with *exo1*, a double-stranded DNA-specific 5'-3' exonuclease involved in the mismatch repair pathway. Tishkoff *et al.* (1997a) reported that *exo1Δ rad27Δ* double mutants are inviable, suggesting

that Exo1 compensates for loss of the flap endonuclease during DNA replication. In support of this observation, overexpression of *EXO1* suppresses both the temperature sensitivity and mutator phenotype of *rad27* mutants (SUN *et al.* 2003; TISHKOFF *et al.* 1997a).

The role of the flap endonuclease in lagging strand replication

Due to the polarity of the DNA double helix, DNA polymerases δ/ε synthesize DNA continuously on the leading strand and discontinuously on the lagging strand. Replication of the lagging strand necessitates the formation of numerous Okazaki fragments that have to be joined to produce a continuous strand of nascent DNA. Aberrant Okazaki fragment maturation can led to sequence duplication, deletions, double and single-stranded DNA breaks and other injurious lesions (ROSSI *et al.* 2006). With this in mind, the most well studied function of the flap endonuclease is its role during lagging strand DNA replication. Proficient lagging strand replication requires the concerted effort of the flap endonuclease, DNA ligase I and PCNA. In yeast, DNA ligase I and PCNA are the products of the *CDC9* and *POL30* genes respectively. In particular, these three proteins are instrumental for proper Okazaki fragment processing thus maintaining replication fidelity.

The flap endonuclease has both an endonuclease and 5'-3' exonuclease activity postulated to play a role during lagging strand synthesis (HARRINGTON and LIEBER 1994a; MURANTE *et al.* 1994). In the final stages of Okazaki fragment processing, the flap endonuclease removes 5'-flaps generated by

polymerase δ (Pol δ). When a newly synthesized Okazaki fragment encounters a downstream Okazaki fragment, Pol δ displaces a short single stranded 5'-flap containing the initiating RNA-DNA primer (BAE *et al.* 2001a). The exposed 5'-end is threaded through the core domain of the flap endonuclease (LIEBER 1997; STEWART *et al.* 2006). The enzyme then tracks along this flap to the junction with duplex DNA where it cleaves the base of the flap to produce a nick (HARRINGTON and LIEBER 1994a; HARRINGTON and LIEBER 1995; MURANTE *et al.* 1995). This tracking requirement is illustrated by the inability of the flap endonuclease to cleave a flap whose 5'-end is blocked by streptavidin (STEWART *et al.* 2006) or annealed with a complementary oligonucleotide (MURANTE *et al.* 1996).

Biochemically, the optimal flap substrate for FEN1 cleavage is a short flap about 10 nt long (AYYAGARI *et al.* 2003; ROSSI *et al.* 2006). Physiologically, the short flaps are generated by the exonuclease activity of Pol δ since a mutant Pol δ lacking the 3'- 5' exonuclease (*pol3-01*) activity generates longer flaps (GARG *et al.* 2004; JIN *et al.* 2003) (Figure 2). The importance of these short flap substrates is underscored by the synthetic lethality between *rad27-p* (an allele of the flap endonuclease which weakly interacts with PCNA) and *pol3-01* (GARY *et al.* 1999; JIN *et al.* 2001; KOKOSKA *et al.* 1998).

When Pol δ strand displacement generates a long flap that is not a suitable substrate for the flap endonuclease, as occurs in the *pol3-01* mutant, an alternative mechanism involving the Dna2-nuclease and the flap

endonuclease takes over. Bae *et al*. (2001) postulated that flaps longer than 27 nt are coated by the single-strand-binding protein replication protein A (RPA) which prevents intra-molecular base pairing, facilitates the recruitment of, and stimulates, Dna2 nuclease/helicase to its DNA substrate. The RPA coated flap is resistant to cleavage by the flap endonuclease (BAE *et al.* 2001a; KAO *et al.* 2004b). Dna2 tracks along the flap but does not cleave the flap at the base (BAE and SEO 2000; KAO *et al.* 2004a; KAO *et al.* 2004b). Consequently, Dna2 interacts with Rad27 and the remaining short flap serves as the substrate for the endonucleolytic cleavage by the flap endonuclease (BAE *et al.* 2001b; LIU *et al.* 2004). The nick that is generated is sealed by DNA ligase, thus joining two adjacent Okazaki fragments (Figure 2).

Long flaps have a propensity to form secondary structures that cannot be cleaved by the flap endonuclease. Dna2-helicase can resolve such structures to facilitate flap endonuclease cleavage (BAE *et al.* 2002; KAO *et al.* 2004b). Another yeast helicase, *SGS1*, is also essential in resolving DNA secondary structure intermediates caused by the absence of *rad27*. The importance of these interactions is evidenced by the synthetic lethality between *dna2-1 rad27Δ* and *sgs1Δ rad27Δ* double mutants (OOI *et al.* 2003; TONG *et al.* 2004). These strains are incapable of processing Okazaki fragments because of their inability to resolve secondary structures. The interdependency of Dna2-nuclease/helicase and the flap endonuclease is highlighted by the results that show that the overexpression of *RAD27* partially compensates for the

temperature sensitive phenotype of *dna2-1* (BUDD and CAMPBELL 1997; BUDD *et al.* 2000). Conversely, overexpression of *DNA2* compensates for the loss of *RAD27* and for the synthetic lethality of the *pol3-01 rad27-p* double mutant (BUDD and CAMPBELL 1997; JIN *et al.* 2003).

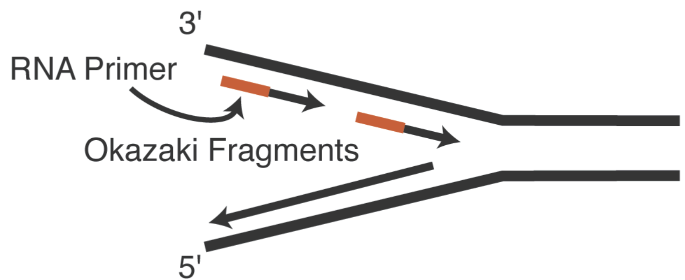
Alternatively, another mechanism of Okazaki processing has been postulated whereby the flap endonuclease utilizes its 5'-3' exonucleolytic activity to excise primer DNA from the 5'-end of a downstream Okazaki fragment. In this model, Pol δ extends nascent DNA up to the downstream Okazaki fragment creating a nicked end. Nicked DNA is the optimal substrate for the exonuclease activity of the flap endonuclease and as such it removes 1 nucleotide from the 5'-end of the downstream primer. Subsequently, Pol δ then adds a nucleotide to the 3'-end to regenerate a nick. This process is reiterated until the primer is removed and DNA ligase seals the nick to produce a continuous strand of nascent DNA (MURANTE *et al.* 1994).

Figure 2. The proposed mechanisms of Okazaki fragment maturation.

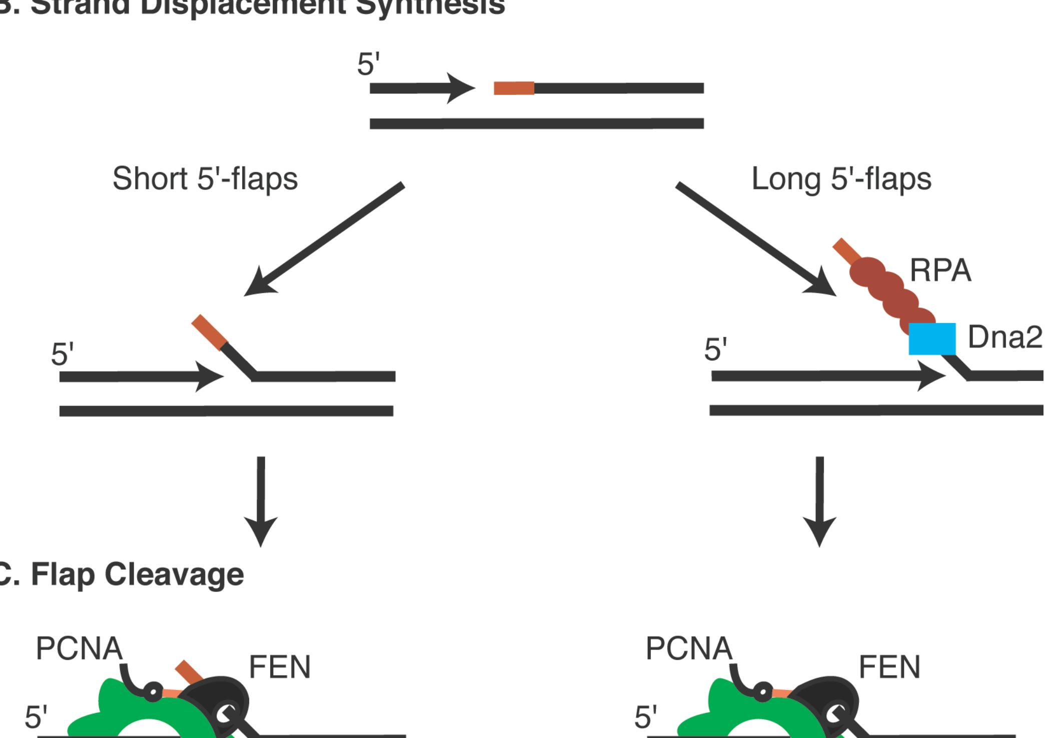
(A) Lagging strand synthesis proceeds after the polymerization of a short RNA-DNA primer made by the Polymerase α/primase complex. (B) When a newly synthesized Okazaki fragment reaches the previously synthesized fragment, DNA Pol δ initiates strand displacement. The resulting 5'-single stranded flap can be long (>10 nt) or short (< 10 nt). Long flaps are coated with RPA, which serves to recruit the Dna2-nuclease. Dna2 cleaves most of the flap leaving about 2 nt. (C) The flap endonuclease interacts with Dna2 and PCNA to hew the 2 nt at the base leaving a nick. At the short flaps, PCNA tethers the flap endonuclease to the DNA substrate where the flap is removed endonucleolytically (D) Subsequently, DNA ligase I interacts with PCNA to catalyze a phosphodiester linkage producing a continuous strand of duplex DNA.

FEN - Flap endonuclease, RPA – Replication Protein A, Dna2 – Dna2 nuclease, PCNA – Proliferating Cell Nuclear Antigen

A. Lagging Strand Synthesis

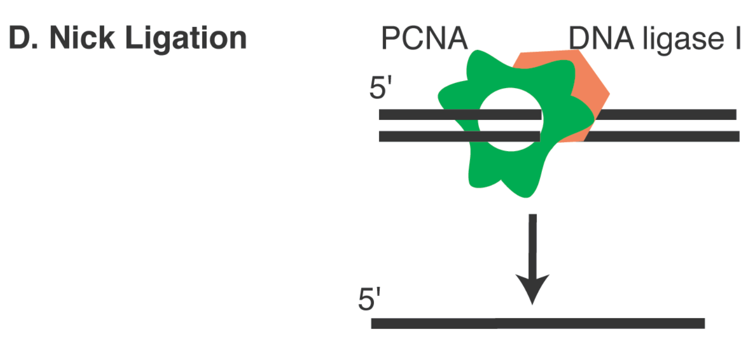


B. Strand Displacement Synthesis



C. Flap Cleavage

D. Nick Ligation



The flap endonuclease is required for repeat sequence stability

The activity of the flap endonuclease during Okazaki fragment maturation is critical to the stability of repetitive DNA within the genome. Numerous studies have shown that proper function of this enzyme is required to prevent repeat sequence instability and fragility. For example the rate of di- and trinucleotide repeat instability is significantly higher in *rad27* mutant strains compared to the wildtype (FREUDENREICH *et al.* 1998; JOHNSON *et al.* 1998; KOKOSKA *et al.* 1998; REFSLAND and LIVINGSTON 2005; SCHWEITZER and LIVINGSTON 1998; XIE *et al.* 2001). When the Prakash group determined the rate of stability of a poly(GT) tract in a *rad27Δ* strain, they found that the rate of tract instability was 80-fold higher than in the wildtype. Further examination found the instability to be caused by sequence duplication (JOHNSON *et al.* 1998).

Schweitzer *et al*. (1998) and Fruedenreich *et al.* (1998) reported that frequent expansion of CAG/CTG repeat tracts occurs in yeast *rad27Δ* mutants, while Callahan *et al*. (2003) (CALLAHAN *et al.* 2003) reported that these trinucleotide (TNR) expansions are susceptible to breakage. The expansion of the CAG/CTG tract is greatest when the CAG sequence is the lagging strand template, a direct indication of faulty Okazaki fragment maturation where the flap endonuclease is an important participant (IRELAND *et al.* 2000; REFSLAND and LIVINGSTON 2005). The working model for these trinucleotide repeat expansions involves a failure by the flap endonuclease to remove 5'-flaps, the

un-excised DNA is incorporated into duplex DNA resulting in expansions (IRELAND *et al.* 2000; SCHWEITZER and LIVINGSTON 1998).

In diploid yeast cells heterozygous for the *rad27Δ* mutation, the trinucleotide repeat expansion was found to be length dependent. CAG repeat tracts that are 85-130 repeats long show an increase in expansion frequency (YANG and FREUDENREICH 2007). These results are reminiscent of the similar length dependence of expansions in human diseases such as fragile X, myotonic dystrophy and Huntington's disease.

The flap endonuclease is needed for telomere stability

Together with the replication of chromosomal DNA, the flap endonuclease has been linked to the maintenance of telomeric DNA. Telomeres are specialized structures that cap the ends of linear chromosomes and ensure that these ends are not degraded or recognized as sites of double stranded breaks. Proper replication of telomeres is important because the transformation of cells to tumors often involves aberrant telomere maintenance. The flap endonuclease is required for the proper replication of telomeric DNA on the lagging strand template, thus ensuring proper telomere stability. In yeast, *rad27Δ* strains have shortened telomeres due to faulty telomere replication (PARENTEAU and WELLINGER 1999).

Saharia and colleagues (2008, 2009) (SAHARIA *et al.* 2008; SAHARIA and STEWART 2009) showed that human FEN1 is localized at the telomere where it interacts with TRF2, a component of the telomeric Shelterin protein complex.

More directly, the depletion of human FEN1 in somatic cells leads to the loss of telomeres that are located on the lagging strand. This telomere dysfunction results in the accumulation of γH2AX, suggesting that un-replicated telomeres are recognized as sites of DNA damage. In another publication Saharia and Stewart (2009) also showed that the flap endonuclease is required for telomere stability in tumor cells that utilize the alternative lengthening of telomeres (ALT) to maintain telomere stability. Loss of FEN1 in these cells results in telomere loss and chromosome end-to-end fusions, again underscoring the importance of the flap endonuclease to telomere maintenance.

The flap endonuclease is a vital component of DNA repair mechanisms

The flap endonuclease, DNA ligase and PCNA are also required for the repair of damaged DNA during long patch base excision repair (LP-BER). In particular, the flap endonuclease participates in LP-BER to remove 5'-flaps generated during the repair process. When an oxidized or reduced base is formed in the DNA, DNA glycosylase cleaves off the base leaving an abasic site. Subsequently, an apyrimidinic/apurinic (AP) endonuclease generates a nick at the 5'-end of the abasic site. DNA polymerases then synthesize new DNA while displacing downstream DNA into a 5'-flap. This flap is then removed by the flap endonuclease prior to ligation by DNA ligase in a process similar to that of lagging strand DNA replication (LIU *et al.* 2004). *In vitro*, the flap endonuclease and AP-endonuclease physically interact with the latter stimulating FEN1 cleavage activity. Both the flap endonuclease and the AP-

endonuclease also interact with PCNA, which positions the proteins at the repair site (DIANOVA *et al.* 2001).

Genetically in yeast, *RAD27* and *APN1*, the gene that encodes for the AP endonuclease, also interact. Wu and Wang (1999) showed that the requirement for the flap endonuclease during LP-BER depends on an active AP-endonuclease generating 5'-flaps. In their study they showed that *rad27Δ apn1Δ* double mutant is more resistant to the DNA damaging agent methyl methane-sulfonate (MMS) than the *rad27Δ* single mutant. They concluded that in the absence of the AP-endonuclease, 5'-flaps are not generated and therefore the flap endonuclease is not required (WU and WANG 1999).

The flap endonuclease has been reported to have a role in double stranded break repair. Wu *et al.* (1999) (WU *et al.* 1999) showed that deletion of *rad27* in yeast results in a significant decrease in the rate of NHEJ in a pathway that utilizes a 5'-flap intermediate. However, mutation of *rad27* does not affect other types of NHEJ events that did not require generation of a flap intermediate. Tseng and Tomkinson (2004) (TSENG and TOMKINSON 2004)adduced further evidence for the role of the flap endonuclease in NHEJ. They demonstrated that Rad27 physically and functionally interacts with Pol4 and Dnl4, a polymerase and ligase respectively, that are vital for NHEJ. The complex formed by these proteins is required for the processing and joining of DNA breaks with incompatible 5'-ends. Additionally, Kikuchi *et al.* (2005) reported that the flap endonuclease is required for homologous recombination

between divergent sequences. In Fen1 deficient DT40 cells, the rate of HR was reduced 20-fold because efficient HR required the flap endonuclease to remove the heterologous regions before recombination (KIKUCHI *et al.* 2005).

The flap endonuclease interacts with PCNA for its activity

PCNA, encoded by *POL30* in yeast, is the replicative clamp that acts as a coordinating scaffold to facilitate the access of various proteins including DNA ligase and the flap endonuclease to DNA. PCNA is essential for cell viability indicative of its central role in DNA metabolism. During the final stages of Okazaki fragment processing and during DNA repair, PCNA is required to stimulate both the endonuclease and exonuclease activity of the flap endonuclease (FRANK *et al.* 2001; GOMES and BURGERS 2000; STUCKI *et al.* 2001; TOM *et al.* 2000) and to tether the ligase to its DNA substrate to ensure efficient and robust joining of Okazaki fragments (GOMES and BURGERS 2000; SAKURAI *et al.* 2005). The ability of DNA ligase to efficiently catalyze the formation of a phosphodiester bond on the DNA backbone depends on it's binding to PCNA. Tom *et al*. (2001) showed that *in vitr*o, PCNA enhances the ligation reaction 5-fold and that the stable association of DNA ligase with nicked duplex DNA requires PCNA. Both DNA ligase and the flap endonuclease interact with PCNA via the PIP box, a conserved sequence motif of the amino acids QXXLXXFF.

The interaction of human FEN1, DNA ligase and PCNA has been structurally elucidated. Sakurai *et al*. (2005) and Bruning and Shamoo (2004)

reported that the flap endonuclease forms extensive contacts with PCNA, which involve the enzyme's core domains, the PIP-box and residues located at the C-terminus. In these studies, the PIP box forms a 3_{10} helix that allows the hydrophobic residues to jut out and form extensive contacts with the PCNA binding pocket. Other residues located C-terminal to the PIP box also interact with the inter-domain connector loop (IDCL) of PCNA to provide an extensive protein-protein interaction surface (BRUNING and SHAMOO 2004; GOMES and BURGERS 2000; SAKURAI *et al.* 2005).

The region immediately N-terminal to the PIP box of the flap endonuclease has also been implicated in crucial interactions with PCNA that allow for the proper functioning of the nuclease. This region consists of the conserved amino acids QGST in humans, mice, rats and xenopus and SG in yeast and worms and is postulated to form a hinge region (SAKURAI *et al.* 2005). This hinge is proposed to allow the flap endonuclease to rotate and interact with the C-terminal portion of PCNA. This rotation positions the flap endonuclease near the 5'-flap substrate for cleavage (GOMES and BURGERS 2000; SAKURAI *et al.* 2005, Chapados, 2004 #375).

Studies with human DNA ligase and PCNA illustrate a similar protein-protein interaction mechanism. The DNA ligase PIP box also forms a 3_{10} helix that allows it to interact with PCNA (PASCAL *et al.* 2006). Outside of the PIP box interactions, DNA ligase assumes a toroidal conformation that allows for

extensive contacts with the face of PCNA (PASCAL *et al.* 2006). This arrangement potentially excludes other proteins from interacting with PCNA.

Consequences of impaired PCNA-flap endonuclease interaction

Perturbation of the interaction between the flap endonuclease, DNA ligase and PCNA has severe consequences for genome stability. *rad27* mutants that have amino acid changes in the PIP box and *pol30* mutants that have amino acid changes in the IDCL and the C-terminal have replication and repair defects indicative of the need for proper interaction between these two proteins. In yeast the PCNA mutants *pol30-79* (IL126,128AA) and *pol30-90* (PK252,253AA), that have mutations in the IDCL and C-terminal, respectively, have been well characterized (EISSENBERG *et al.* 1997; GOMES and BURGERS 2000). These alleles have growth defects, high rate of spontaneous mutations and elevated sensitivity to MMS. When these mutants are combined with a null allele of *RAD27,* the double mutants are viable but showed poor growth at 23°C and fail to grow at 14°C (EISSENBERG *et al.* 1997).

Gomes and Burgers (2000) separated the functions of the *pol30-79* and *pol30-90* alleles with regard to their interaction with *rad27* alleles. Using purified proteins, they found that *pcna-79* could not interact with the flap endonuclease but it is still able to stimulate its flap cleavage activity. In contrast, *pcna-90* was able to bind to the flap endonuclease but fails to stimulate its activity. *In vivo*, they examined the phenotype of strains harboring a combination of *pol30-79* or *pol30-90* with *rad27-ga*, an allele of *rad27* where the two-phenylalanine

residues in the conserved PIP-box were mutated to a glycine and alanine (F346G/F347A). They reported that both double mutants are viable but show elevated sensitivity to MMS. *In vitro* pcna-90 fen1-ga is completely inactive, however, this is not the case *in vivo*. This suggested that in the cells the mutant flap endonuclease has residual interaction with PCNA. Taken together these data suggests that the flap endonuclease first binds PCNA at the IDCL, and then using the hinge domain, shifts to the C-terminal in order to cleave its 5'-flap substrate (Figure 3).

Refsland and Livingston (2005) examined the phenotypes of *rad27-p* (FF346,347AA) and *cdc9-p* (FF43,44AA) alleles of the flap endonuclease and DNA ligase I which had the conserved phenyalanines of the PIP box mutated to alanines in combination with *pol30-90*. They reported that *pol30-90 rad27-p* double mutant has elevated susceptibility to DNA damaging agents and has synergistic levels of CAG tract instability when compared to the single mutants. Triple mutants between *pol30-90, rad27-p cdc9-p* have severely impaired growth suggesting that these three proteins need to interact to ensure normal functioning of DNA metabolic processes.

The importance of this interaction between the flap endonuclease and PCNA to the viability of an organism cannot be gainsaid. Zheng *et al*. (2007) generated a mouse model containing the *rad27-p* mutation and showed that the disruption of the PCNA interaction results in DNA replication defects, growth

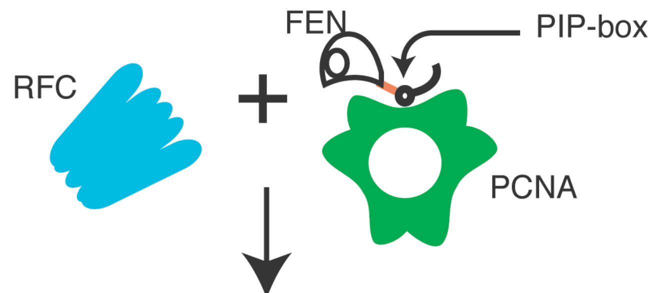
retardation and neo-natal lethality. The pups succumb to gross organ failure due pulmonary hypoplasia and pancytopenia.

Figure 3. Proposed mechanism for the loading and cleavage of the 5'-flap substrate by the flap endonuclease.

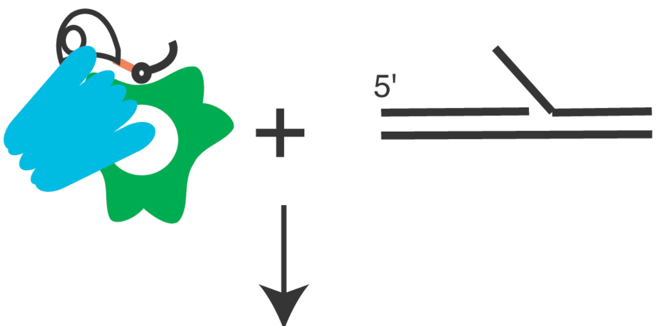
(A) The flap endonuclease binds to PCNA at the IDCL using its PIP-box independent of its substrate. (B) RFC then binds to this complex loads it onto the DNA. (C) The flap endonuclease swivels using it hinge domain causing the C-terminal of the enzyme and PCNA to interact. This movement also allows the enzyme to access the 5'-end of the flap. The flap is then threaded through an aperture in the flap endonuclease core domain. (D) While tethered to PCNA, the flap endonuclease tracks along the flap to the junction between single-stranded DNA and duplex DNA. The enzyme cleaves at the junction to generate a nicked end.

FEN - Flap endonuclease, RFC – Replication Factor C, PCNA – Proliferating Cell Nuclear Antigen, ssDNA – Single stranded DNA

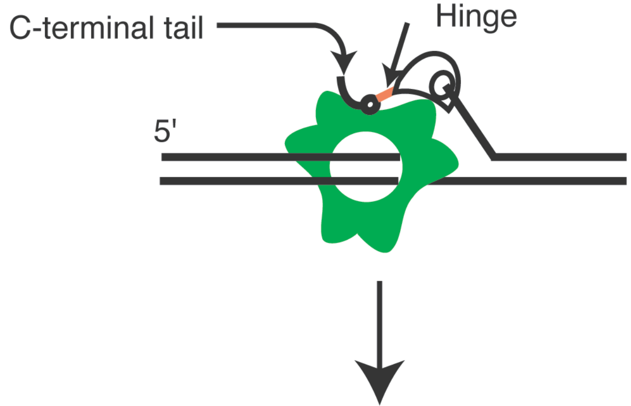
A. FEN, PCNA and RFC interact



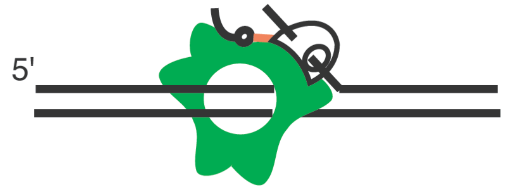
B. RFC loads the complex onto DNA



C. FEN swivels on PCNA to access the 5' end of the flap



D. FEN tracks and cleaves ssDNA



Coordination of the interactions between PCNA, DNA ligase and the flap endonuclease

The precise nature of how PCNA coordinates the entry of the flap endonuclease and DNA ligase into the replication fork is not well understood. Biochemical and structural studies have begun to elucidate a possible ordering of these PCNA-mediated interactions. The possibility of such an ordering is underscored by the observation that DNA ligase adopts a toroidal conformation by completely encircling duplex DNA while interacting with PCNA (PASCAL *et al.* 2004). Moreover, both PCNA and DNA ligase or PCNA and FEN1 may be loaded onto the DNA in a mechanism utilizing the replication clamp loader RFC (CHO *et al.* 2009; LEVIN *et al.* 2004), again suggesting a complete encirclement of DNA (Figure 3). PCNA and DNA ligase are similar in size and their interaction extends along the face of PCNA such that it would prevent other proteins such as the flap endonuclease from binding to the IDCL (PASCAL *et al.* 2004; PASCAL *et al.* 2006). These data argues for a coordinated sequential interaction between PCNA, DNA ligase and the flap endonuclease.

Alternatively, both the flap endonuclease and DNA ligase may bind to the same molecule of PCNA. Since PCNA is a homotrimer, DNA ligase can potentially bind to one monomer while the flap endonuclease binds to another using its extended C-terminal tail in a conformation allowing it to be tethered to PCNA concurrently with DNA ligase (GOMES and BURGERS 2000; SAKURAI *et al.* 2005). DNA ligase could also bind to PCNA in an extended conformation while

the flap endonuclease cleaves the DNA. *Sulfolobus solfataricus* DNA ligase has been shown to have an open, extended conformation while binding to PCNA. Presumably, once the flap endonuclease has removed the 5'-flap, DNA ligase acquires a closed, ring-shaped conformation to catalyze the joining of the Okazaki fragments (PASCAL *et al.* 2006).

Despite this body of knowledge, the precise mechanisms at play during these last steps of the Okazaki fragment maturation pathway remain enigmatic. Further elucidation of the mechanism will greatly advance our understanding of how aberrant functions lead to genomic instability and hence disease.

SUMMARY OF THE THESIS

The role of flap endonuclease, DNA ligase and PCNA in maintaining genome integrity is well documented. However, exactly how PCNA coordinates the entry of the flap endonuclease and DNA ligase into the replication fork, whether singly or concurrently, is not well understood. To further elucidate how DNA ligase interaction with PCNA is ordered, we performed a genetic screen to identify genes that are synthetically lethal with *cdc9-p*, an allele of DNA ligase that has impaired binding to PCNA. We postulated that genes recovered from this screen would function in DNA repair, replication, and recombination or would be involved in ordering the *cdc9-p* –PCNA interaction. From the screen we recovered an allele of *RAD27*, which we named *rad27-K325**. This allele encoded a protein that lacked the PIP box and the entire C-terminal domain.

To better understand the role played by the C-terminal of the flap endonuclease during PCNA binding, we made two additional mutants, *rad27-A358** which retains the PIP box but lacks all amino acid residues C-terminal to it and *rad27-pX8* which has the entire 8 amino acids of the PIP box replaced with a polyalanine tract. We proceeded to characterize these mutants in comparison with the wildtype and the *rad27* null allele. Phenotypically, all mutants grow well at 25°C, however, *rad27-K325*,* like *rad27Δ* is temperature sensitive at 35°C. When tested for viability after exposure to the DNA-damaging agent MMS, we observed that *rad27-A358** is not sensitive, *rad27-pX8* is moderately sensitive, and *rad27-K325** is highly sensitive although not as

sensitive as *rad27Δ*. This result suggests that *rad27-K325** retains some biological activity. The rate of spontaneous forward mutations is highest in *rad27Δ* and *rad27-K325** followed by *rad27-pX8*, while *rad27-A358** has the lowest mutation rate. Surprisingly, the temperature sensitivity and hypermutagenic phenotypes are partially complemented by overexpression of PCNA. These data suggests that the C-terminal end of the flap endonuclease that is required for interaction with PCNA is important for proper DNA replication, repair and mutation avoidance.

Finally we combined the *rad27* mutant alleles with the PCNA alleles, *pol30-79* and *pol30-90. rad27Δ* and *rad27-K325** are synthetically lethal with *pol30-79* while *rad27-A358** and *rad27-pX8* in combination with *pol30-79* have severe synergistic growth defects. However, when double mutants were made with *pol30-90,* only the *pol30-90 rad27-K325** is lethal. This implies that *rad27-K325** is dominant negative in the presence of *pol30-90* because of its residual biological activity. The growth phenotype at 25°C of the other double mutants is graduated such that *pol30-90 rad27-A358** grows better than *pol30-90 rad27-pX8,* which grows better than *pol30-90 rad27Δ.* This graduated growth corresponds to the length of the C-terminal truncation of the flap endonuclease, for instance, the enzyme with the least truncation grows the best and the one without the entire protein grows the least. Taken together this data supports the seminal two-step model of PCNA-flap endonuclease interaction postulated by Gomes and Burgers (2000). Briefly, the flap endonuclease first interacts with

PCNA at the inter-domain connector loop (mutated in *pol30-79*). Subsequently, the C-terminal tail of the flap endonuclease swivels to contact the C-terminal end of PCNA (mutated in *pol30-90*) as evidenced by the graduated growth phenotype of the double mutant.

CHAPTER 2

C-terminal flap endonuclease (*rad27*) mutations: lethal interactions with a DNA ligase I mutation (*cdc9-p*) and suppression by Proliferating Cell Nuclear Antigen (*POL30*) in Saccharomyces cerevisiae

INTRODUCTION

Maintenance of genomic integrity is essential for the viability of all organisms. The fidelity of DNA replication has to be maintained and DNA insults have to be repaired to ensure that deleterious mutations are not passed on to progeny or cause cancerous growth. A number of cellular proteins have multiple roles in DNA replication, mutation avoidance and repair. In *S. cerevisiae*, the flap endonuclease, proliferation cell nuclear antigen (PCNA) and DNA ligase I encoded by *RAD27, POL30* and *CDC9*, respectively, are all required for proper replication and also function to avoid mutation and participate in repair.

The flap endonuclease, FEN-1 in humans, is a highly conserved structure specific nuclease that has both endonuclease and 5'-3' exonuclease activity. During lagging strand replication these activities facilitate removal of primers from Okazaki fragments, either by endonucleolytic cleavage of a flap made by strand displacement (LIU *et al.* 2004) or by sequential exonucleolytic removal of single nucleotides at the 5'-end of the primer (MURANTE *et al.* 1994).

While deletion of *RAD27* is not lethal to yeast cells, the *rad27Δ* mutant exhibits temperature sensitive growth, is a mutator and undergoes genomic instability (CHEN and KOLODNER 1999; JOHNSON *et al.* 1995; REAGAN *et al.* 1995; TISHKOFF *et al.* 1997b). In addition, its sensitivity to low doses of the methylating agent methylmethane sulfonate (MMS) implicates the participation of the enzyme in base excision repair (BER) (REAGAN *et al.* 1995; WU and WANG 1999). *rad27Δ* mutants have been reported to be either mildly sensitive to UV

light or not sensitive to UV light (REAGAN *et al.* 1995; SOMMERS *et al.* 1995). In the strain background that the mutant is mildly sensitive, its combination with *rad2Δ* yields a double mutant more sensitive than each single mutant, implying that the enzyme does not participate in *RAD2*-mediated nucleotide excision repair (NER) (REAGAN *et al.* 1995). The flap endonuclease has also been implicated in double strand break (DSB) repair by virtue of the incompatibility of *rad27Δ* with mutations of the DSB repair pathways (SYMINGTON 1998; TISHKOFF *et al.* 1997b). In addition, either the yeast enzyme or its human ortholog has been shown to participate in reactions of homologous recombination, nonhomologous end joining and telomere maintenance (KIKUCHI *et al.* 2005; PARENTEAU and WELLINGER 1999; PARENTEAU and WELLINGER 2002; WANG *et al.* 2004; WU *et al.* 1999). Curiously, the *rad27Δ* mutant is not sensitive to gamma radiation but is sensitive to high doses of MMS that are thought to act as a radiomimetic agent (REAGAN *et al.* 1995; SOMMERS *et al.* 1995).

PCNA is the replicative clamp that acts as a scaffold to facilitate the loading of DNA replication and repair proteins including DNA ligase I and the flap endonuclease to DNA. PCNA (*POL30*) is essential for cell viability, which is indicative of its central role in DNA metabolism. Biochemical characterization of its effect on the flap endonuclease shows that it stimulates its activity, suggesting the productive nature of the interaction (FRANK *et al.* 2001; GOMES and BURGERS 2000; STUCKI *et al.* 2001; TOM *et al.* 2000). The ability of DNA ligase to efficiently catalyze the formation of phosphodiester bonds in the DNA

backbone may also be facilitated by its binding to PCNA. Tom *et al*. (2001) (TOM *et al.* 2001) showed that, *in vitro*, PCNA enhances the ligation reaction 5-fold, and that the stable association of DNA ligase with nicked duplex DNA requires PCNA.

Both DNA ligase and the flap endonuclease bind to PCNA via their respective PCNA interactive peptide domain (PIP box). The PIP box is a conserved sequence motif of the amino acids QXXLXXFF. The PIP box fits into the interdomain connector loop (IDCL) of PCNA to provide a protein-protein interaction surface (CHAPADOS *et al.* 2004; GOMES and BURGERS 2000; PASCAL *et al.* 2006; SAKURAI *et al.* 2005). Mutations in the PIP box or the IDCL that impair the interaction of DNA ligase and the flap endonuclease to PCNA lead to genomic instability (AMIN and HOLM 1996; EISSENBERG *et al.* 1997; GARY *et al.* 1999; REFSLAND and LIVINGSTON 2005; SCHWEITZER and LIVINGSTON 1999; SUBRAMANIAN *et al.* 2005). We have reported that the double mutants made by combinations of *cdc9-p*, *rad27-p* and *pol30-90* —mutations with alterations of the PIP box or the IDCL in the respective proteins— have synergistic phenotypes with respect to MMS sensitivity and to trinucleotide repeat instability (REFSLAND and LIVINGSTON 2005). These results suggest that the two enzymes function in a concerted manner that is facilitated by PCNA.

The precise nature of how PCNA coordinates the entry of the flap endonuclease and DNA ligase into the replication fork is not well understood. Biochemical and structural studies have begun to elucidate a possible ordering

of these PCNA-mediated interactions. The possibility of such an ordering is underscored by the observation that DNA ligase adopts a toroidal conformation by completely encircling duplex DNA while interacting with PCNA (PASCAL *et al.* 2004). Moreover, both PCNA and DNA ligase may be loaded onto the DNA in a mechanism utilizing the replication clamp loader RFC (replication factor C)(LEVIN *et al.* 2004), again suggesting a complete encirclement of the DNA by DNA ligase as well as by PCNA. PCNA and DNA ligase are similar in size and their interaction is likely to extend along the face of PCNA in a manner that would prevent other proteins such as the flap endonuclease from binding to the IDCL (PASCAL *et al.* 2004; PASCAL *et al.* 2006). A biochemical study with purified yeast proteins showed that the two enzymes cannot bind simultaneously to PCNA (SUBRAMANIAN *et al.* 2005). These studies suggest that a coordinated sequential interaction among PCNA, DNA ligase and the flap endonuclease is important for replication and repair.

Alternatively, both the flap endonuclease and DNA ligase may bind to the same molecule of PCNA. Since PCNA is a homotrimer, DNA ligase can potentially bind to one monomer while the flap endonuclease binds to another using its extended C-terminal tail in a conformation allowing it to be tethered to PCNA concurrently with DNA ligase (GOMES and BURGERS 2000; SAKURAI *et al.* 2005). DNA ligase could also bind to PCNA in an extended conformation while the flap endonuclease cleaves the DNA. *Sulfolobus solfataricus* DNA ligase has been shown to have an open, extended conformation while binding to PCNA

(PASCAL *et al.* 2006). Presumably, once the flap endonuclease has removed the 5'-flap, DNA ligase acquires a closed, ring-shaped conformation to catalyze the joining of Okazaki fragments (PASCAL *et al.* 2006).

Exactly how the interaction of these enzymes with PCNA is coordinated *in vivo*, whether singly or concurrently, is not well understood. To further elucidate how the interaction of DNA ligase with PCNA is ordered, we performed a genetic screen to identify mutations that are synthetically lethal with *cdc9-p* (F44A/F35A), an allele of DNA ligase that has impaired binding to PCNA (REFSLAND and LIVINGSTON 2005; SUBRAMANIAN *et al.* 2005). We postulated that genes recovered from this screen would function in DNA repair, replication, and recombination or would be involved in ordering the DNA ligase–PCNA interaction. From the screen we recovered a truncated allele of *RAD27*, *rad27-K325**. This allele encodes a protein that lacks the PIP box and the entire C-terminal domain of the enzyme but retains the N-terminus containing the nuclease activities. We have characterized this allele and compared it to two other *rad27* alleles in which we have created different alterations of the C-terminal end of the flap endonuclease.

MATERIALS AND METHODS

Yeast strains: All *S. cerevisiae* strains used in this study, with the exception of SSL938, are isogenic to SSL204 (*ura3 ade2 his3 trp1 leu2*) and are listed in Table 1 (DORNFELD and LIVINGSTON 1991). Only differences between this strain and the derived strains are noted. Deletion strains were created in this background by single step disruption (ROTHSTEIN 1983) and allelic substitutions were made using two step transplacement (SCHERER and DAVIS 1979). In some cases mutations vital to the synthetic-lethal screen, e.g., *ura3* and *ade2*, are shown for clarity. Strain SSL938 was derived from *ura3*, *his3*, *trp1* and *CAN1* strains in our collection that did not contribute to the SSL204 lineage and was made *rad27Δ* by single step replacement.

TABLE 1

Yeast Strains

Strain	Genotype	Source
SSL204	*his3Δ200 trp1 leu2 ade2-101 ura3-52*	(DORNFELD and LIVINGSTON 1991)
SSL254	*MAT*a *lys5Δ::HIS3*	This study
SSL533	*MAT*a *rad27Δ::HIS3*	(SCHWEITZER and LIVINGSTON 1998)
SSL534	*MAT*α *rad27Δ::HIS3*	(SCHWEITZER and LIVINGSTON 1998)
SSL535	*MAT*a *rad2Δ::URA3*	This study
SSL539	*MAT*a *exo1Δ::URA3*	This study
SSL543	*MAT*a *pol30-79*	(SCHWEITZER and LIVINGSTON 1999)
SSL544	*MAT*a *pol30-90*	(SCHWEITZER and LIVINGSTON 1999)
SSL545	*MAT*α *pol30-90*	(SCHWEITZER and LIVINGSTON 1999)
SSL612	*MAT*a *cdc9-1*	(IRELAND *et al.* 2000)
SSL615	*MAT*a *dna2-1*	(IRELAND *et al.* 2000)
SSL630	*MAT*a *cdc9Δ::KanMx {pRS316.CDC9}*	This study

SSL631	*MATα cdc9Δ::KanMx {pRS316.CDC9}*	This study
SSL632	*MAT*a *cdc9-p*	(REFSLAND and LIVINGSTON 2005)
SSL633	*MATα ade3Δ::hisG*	This study
SSL634	*MAT*a *ade3Δ::hisG cdc9-p*	This study
SSL635	*MATα ade3Δ::hisG cdc9-p*	This study
SSL636	*MAT*a *ade3Δ::hisG cdc9-p lys5Δ::HIS3*	This study
SSL637	*MATα ade3Δ::hisG cdc9-p lys5Δ::HIS3*	This study
SSL638	*MAT*a *ade3Δ::hisG cdc9Δ::KanMx {pRS316.CDC9} lys5Δ::HIS3*	This study
SSL639	*MATα ade3Δ::hisG cdc9Δ::KanMx {pRS316.CDC9} lys5Δ::HIS3*	This study
SSL642	*MAT*a *FUR1::HIS3::FUR1*	This study
SSL643	*MATα FUR1::HIS3::FUR1*	This study
SSL644	*MAT*a *ade3Δ::hisG cdc9-p FUR1::HIS3::FUR1*	This study
SSL651	*MATα ade3Δ::hisG cdc9-p FUR1::HIS3::FUR1*	This study

SSL649	*MAT*a *rad27-p*	(REFSLAND and LIVINGSTON 2005)
SSL650	*MAT*α *rad27-p*	(REFSLAND and LIVINGSTON 2005)
SSL667	*MAT*α *cdc9-p rad27-p*	(REFSLAND and LIVINGSTON 2005)
SSL826	*MATa rad51Δ::ADE2*	(ASLESON and LIVINGSTON 2003)
SSL927	*MAT*a *rad27-A358*-isolate1*	This study
SSL928	*MAT*a *rad27-A358*-isolate2*	This study
SSL929	*MAT*a *rad27-pX8-isolate1*	This study
SSL930	*MAT*a *rad27-pX8-isolate2*	This study
SSL931	*MATa cdc9-p rad27-pX8*	This study
SSL933	*MATa cdc9-p rad27-A358**	This study
SSL935	*MAT*a *rad27-K325*-isolate1*	This study
SSL936	*MAT*α *rad27-K325*-isolate2*	This study
SSL938	*MATa rad27Δ::HIS3*	This study
SSL944	*MATa exo1Δ::URA3 rad27-pX8*	This study
SSL955	*MATa exo1Δ::URA3 rad27p*	This study
SSL957	*MATa exo1Δ::URA3 rad27-358**	This study

Media: Media was prepared according to standard recipes (BURKE *et al.* 2000) except that synthetic media was buffered with succinic acid-NaOH as previously described (HARTWELL 1970). In particular, the omission media in these studies were made from a single batch of nutrients that were mixed from solid ingredients in the proportion previously described (BURKE *et al.* 2000) but lacked uracil and tryptophan. When necessary, these were added in the amount previously specified (BURKE *et al.* 2000).

Synthetic Lethal Screen: To screen for mutations that are synthetically lethal in combination with *cdc9-p*, a *cdc9-p* strain was modified to make it both *ade3* and *FUR1::HIS3::FUR1*. Tester strains were also constructed to test for dominance (*cdc9-p ade3Δ::hisG lys5::HIS3*) and to test for inactivating mutations in *CDC9* (*cdc9Δ::KanMx* {pRS316.*CDC9.ADE3*} *ade3Δ::hisG lys5::HIS*).

The synthetic lethal screen was carried out using a red/white colony-sectoring assay in an *ade2 ade3* background (KRANZ and HOLM 1990). The strain was modified by duplicating *FUR1* to eliminate false positives mutations (*fur1*) in uracil utilization (KOREN *et al.* 2003). The modified *cdc9-p* strain was transformed with the plasmid pRS316.*ADE3.CDC9*. Approximately 300 transformed cells were spread on YPD media and mutagenized by exposure to 3.4 mW/cm^2 of UV light (253.7 nm UV-C) to 60% survival. Cells that grew at 30° and did not appear to contain white sectors were streaked onto agar containing

5-FOA. Those that proved to require pRS316.*ADE3.CDC9* for survival were tested for dominance and for the absence of inactivating mutations of *CDC9*. The tester strain for dominance could also be used to test the segregation pattern of the synthetic lethal mutation. At this point candidates that were recessive and segregated as a single mutation were outcrossed to the wild-type parent to recover the synthetic lethal mutation in a *CDC9* background. Of the candidates that were recessive and segregated as a single mutation, we found two that also conferred temperature sensitive growth at 35°. Because mutations in *POL30* (PCNA) might have caused a synthetic lethal phenotype and temperature sensitive growth, we transformed the two candidates with pRS316.*POL30*. One candidate grew better when transformed, and we chose this candidate for further study.

Identification of the Synthetic Lethal Mutation: To identify the mutation causing synthetic lethality with *cdc9-p*, we transformed the outcrossed mutant with a yeast genomic library (a gift from D. T. Kirkpatrick, (JAUERT *et al.* 2005)). We recovered plasmids with the genomic fragment containing *APN1, RAD27* and *ABF1*. We took a candidate gene approach and transformed the isolate with pRS316.*RAD27* which rescued the temperature lethality. The *rad27* allele from the candidate was recovered by PCR and subjected to DNA sequencing. The copy had a stop codon at amino acid residue 325 in *RAD27*.

***rad27* alleles:** We recreated the *rad27* mutation, *rad27-K325**, that we had recovered, in our wild-type strain along with a flanking restriction enzyme recognition sequence that did not further alter the gene. We also made two additional alleles, *rad27-A358** and *rad27-pX8* (339QGRLDGFF346 to 339AAAAKAAA346) by the same methods. Because of the propensity of *rad27* mutations to undergo genetic changes, each of our mutations as well as *rad27Δ* was backcrossed to our wild-type parental strain. The resulting diploids were sporulated, and only strains that yielded a majority of complete tetrads were used. Mutant isolates were confirmed by PCR and restriction digestion.

Detection of wild-type and mutant flap endonuclease: Epitope tagged *RAD27* and *rad27-K325** were constructed by inserting three flag-epitopes (3X-LDDYKDKKF) into the coding sequence such that the tag appeared after the start codon and the gene was under the control of the *RAD27* promoter. The constructs were placed into pRS316.

To detect epitope tagged flap endonuclease, SSL533 (*rad27Δ*) was transformed with plasmids harboring flag-tagged *RAD27*, flag-tagged *rad27-K325**, and an empty vector control. The transformants were grown in selective media to log phase (OD_{600} ~ 0.6 - 1) at 25° and proteins were extracted by the TCA method. The proteins were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and visualized by immunoblotting with M2 monoclonal anti-Flag antibodies (Sigma-Adrich, St. Louis, MO). A loading control was

performed by concurrently running another SDS-PAGE gel, which was used for Commassie staining.

Mutation Avoidance Assays: The rate of spontaneous forward mutations to canavanine resistance was measured in the *rad27* mutants with or without the PCNA overexpression plasmid, pRS424.*POL30*. The canavanine mutagenesis assay was modified from methods described previously (MARSISCHKY *et al.* 1996; SIA *et al.* 1997). Briefly, strains were streaked on appropriate media for single colonies and incubated for 3 days at 25°. After their emergence, 5 individual colonies were inoculated into 3 ml of appropriate liquid media. The cultures were grown at 25° in a roller drum until they reached stationary phase (~ 2 X 10^8 cells/ml in YPD and ~ 2 X 10^7 in selective media). Cell density was determined by optical density (OD_{600}) and 1 X 10^7 cells were harvested and washed in sterile distilled water and suspended in 1 ml of water. Serial dilutions were made to place ~1 X 10^6 cells on appropriate selective agar lacking arginine and containing 60 μg/ml L-canavanine (Sigma-Aldrich, St. Louis, MO). Concurrently, approximately 100 cells were plated on appropriate media to determine the viable cell count. Cultures were incubated at 25° for 4 days and the canavanine resistant colonies were enumerated. At least 3 independent experiments were performed for each strain. Mutation rates were estimated using the method of the median (LEA and COULSON 1949). Statistical differences

were determined by averaging three or more rate estimations and comparing values using Students t test.

To measure the rate of dinucleotide repeat instability, plasmid pSH44 was introduced into our strains, grown in media selective for tryptophan and plated to medium containing 5'-FOA as previously described (HENDERSON and PETES 1992; KOKOSKA *et al.* 1998). Mutation rates were estimated using the method of the median (LEA and COULSON 1949). Statistical differences were determined by averaging three or more rate estimations and comparing values using Students t test.

Methylmethane sulfate (MMS) sensitivity: All *rad27* strains with or without pRS424.*POL30* and *cdc9-p rad27-p* with or without pRS426.*rad27-K325** were tested for MMS resistance as previously described (BOUNDY-MILLS and LIVINGSTON 1993). Briefly, strains were grown in 3 ml of appropriate media at 25° to stationary phase (~ 2 X 10^8 cells/ml in YPD and ~2 X 10^7 cells/ml in selective media). Enough volume of culture was harvested to yield 2 X 10^8 cells, which were then washed twice in sterile distilled water. The pellet was suspended in 6 ml of 50 mM potassium monohydrogen phosphate. To determine the initial cell viability (at time 0 min), a 1 ml sample was taken and quenched in an equal volume of freshly made 10% sodium thiosulfate. A 100 μl sample of an appropriate dilution was plated on YPD or selective media and incubated at 25° for 4 days. The remaining 5 ml were mixed with 25 μl of MMS

(Acros Organics, NJ) and incubated at room temperature in the dark. At 15 minutes intervals, 1 ml samples were taken, quenched in 10% sodium thiosulfate, diluted and plated on appropriate media. All strains were incubated at 25° for 4 days before enumeration of viable colonies. The means of at least three independent experiments were used to generate survival curves.

Growth capability, doubling time and plating efficiency: The temperature sensitivity of the *rad27, cdc9-1* and *dna2-1* strains with or without pRS424.*POL30* or pRS426.*rad27-K325** was examined by performing spotting assays. Log phase cultures (OD_{600} ~ 0.6 - 1) were grown in appropriate media and serially diluted in a 96-well micro-titer dish. Spots were made on media such that the highest density spot contained 15,000 cells with 10-fold decreases in the following 3 consecutive spots. The plates were incubated at 25° and 35° for 4 days unless otherwise specified.

To determine the doubling time, *rad27* strains with or without pRS424.*POL30* were grown to mid-log phase (OD_{600} ~ 0.6 – 0.8) in selective media at 25° with shaking. Cultures were then diluted to OD_{600} ~ 0.01 and grown for 12 hrs at 30° or 36°. Samples were taken every hour and the cell density determined by optical density. These experiments were repeated thrice. Mean doubling times were calculated from the log-transformation of the growth curves.

To determine plating, strains with or without pRS424.*POL30* were grown to mid-log phase (OD_{600} ~ 0.6 –0.8) in selective media. Serial dilutions were made and approximately 200 cells were plated on selective media in triplicate for each strain and temperature. One set of plates was incubated at 25° and the other at 35° for 3 days. The number of viable colonies was enumerated and the means of 3 independent experiments were used to determine the percent plating efficiency.

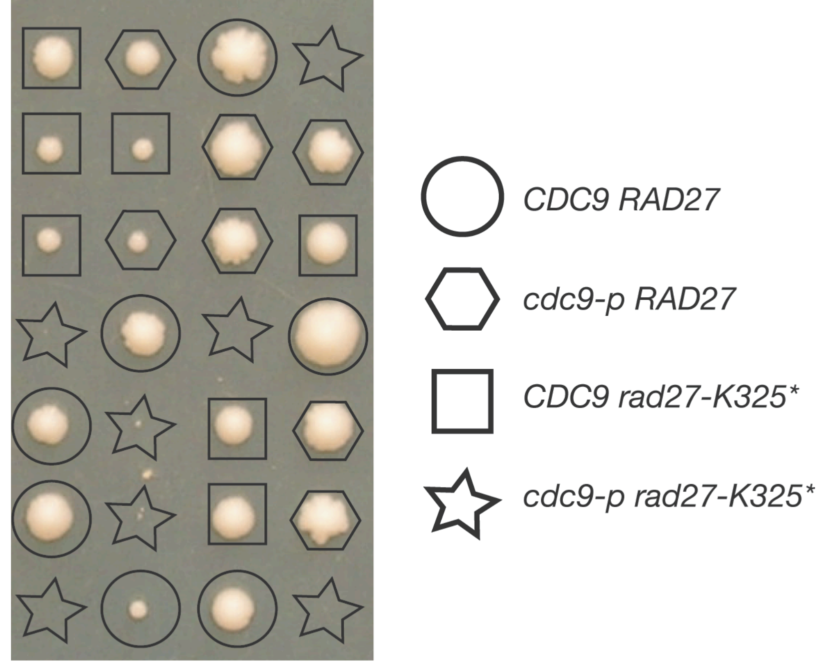
RESULTS

Identification of *rad27-K325 in a synthetic lethal screen with *cdc9-p*:** We screened for mutations that confer synthetic lethality with the DNA ligase I mutation *cdc9-p* that has substitutions of the two aromatic residues of the enzyme's PIP box (F43A/F44A). The *cdc9-p* mutant does not exhibit growth defects, but the mutated copy of DNA ligase I binds weakly to PCNA and causes instability of CAG repeat tracts (REFSLAND and LIVINGSTON 2005; SUBRAMANIAN *et al.* 2005). To recover mutations that are synthetically lethal with *cdc9-p*, we introduced a wild-type copy of *CDC9* on a *URA3 ADE3* containing plasmid into the *cdc9-p* strain, mutagenized it and screened for colonies that could not sector or grow on agar containing 5'-FOA. We next screened the isolates for additional phenotypes that might suggest an involvement in DNA replication or repair. One isolate was temperature-sensitive for growth and partially suppressible by high-level expression of PCNA (*POL30*) on YPD. Rescue of the temperature sensitivity with a yeast genomic library identified clones containing *RAD27* encoding the flap endonuclease. DNA sequencing of the *RAD27* and *POL30* alleles in the isolate revealed a stop codon at position 325 in *RAD27* and no mutations in *POL30*. The *rad27* mutation *rad27-K325** was recreated and used to replace the wild-type copy in our parental wild-type strain. To authenticate that *rad27-K325** is sufficient to yield the synthetic lethal phenotype with *cdc9-p*, the *rad27-K325** allele was mated to the parental *cdc9-p* strain and the diploids were sporulated. The spore colonies (Figure 1) show

that each single mutant is able to germinate and form colonies, but the *rad27-K325* cdc9-p* double mutant cannot.

Figure 1.—Synthetic lethality of *rad27-K325 combined with *cdc9-p* (F43A/F44A).**

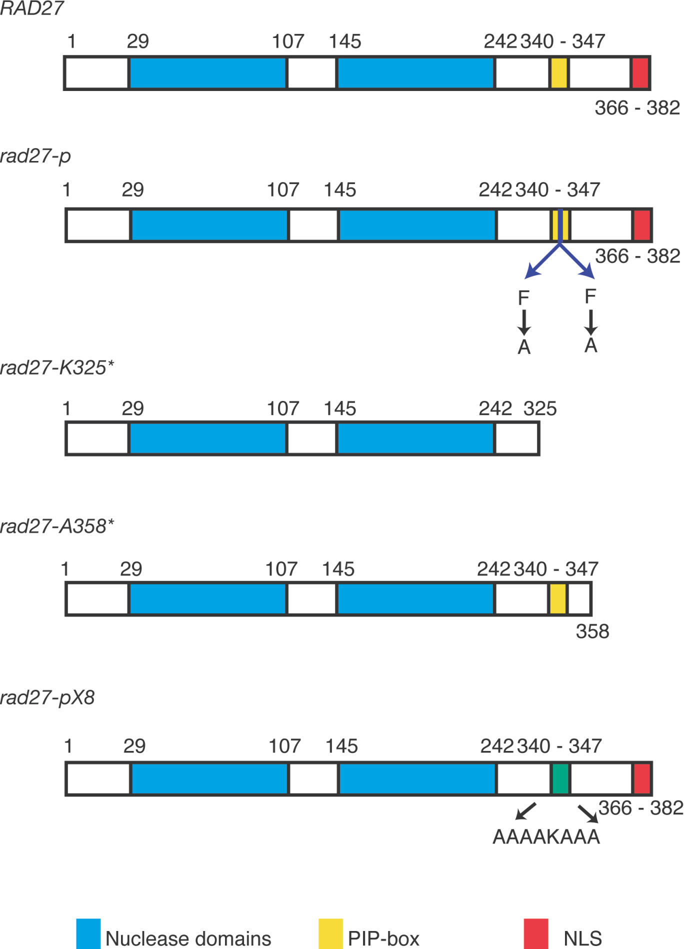
A diploid heterozygous for both mutations was sporulated, and the spore colonies were germinated on YPD agar at 25°. The genotypes of viable spore colonies were determined by restriction enzyme digestion of PCR products of the *RAD27* and *CDC9* alleles.


CDC9 RAD27
cdc9-p RAD27
CDC9 rad27-K325*
cdc9-p rad27-K325*

Having already found a genetic interaction between *cdc9-p* and *rad27-p* (REFSLAND and LIVINGSTON 2005), the corresponding PIP box mutations of the aromatic residues of the flap endonuclease (F346A/F347A) (GARY *et al.* 1999)), the identification of an additional *rad27* allele that disturbs the axis among the flap endonuclease, DNA ligase and PCNA warranted further examination. The position of the stop codon in *rad27-K325** leaves the nuclease domains of the flap endonuclease intact but removes other domains. The deleted domains include (1) a hinge region that permits rotation of PCNA-bound flap endonuclease to reach the DNA, (2) the PIP box, (3) residues C-terminal to the PIP box that bind to PCNA and (4) additional C-terminal domains that may be necessary for nuclear import, protein-protein interactions and post-translational modifications (LIU *et al.* 2004) (Figure 2). Cocrystalization of the human flap endonuclease with PCNA showed that the enzyme makes contacts with PCNA through a number of its domains. The flap endonuclease makes contacts with PCNA through its core domain, through residues N-terminal to the PIP box, through the PIP box, and through residues C-terminal of the PIP box (SAKURAI *et al.* 2005). The most extensive interactions are between the PIP box of the flap endonuclease and the hydrophobic pocket of the IDCL of PCNA and between C-terminal residues of the flap endonuclease and additional residues of the IDCL. To better understand how these regions of the flap endonuclease contribute to its interaction with PCNA, we made two additional *rad27* alleles. One allele substitutes alpha helical forming residues for the eight amino acids of

the PIP box (*rad27-339AAAAKAAA346* named *rad27-pX8*) and the other truncates the enzyme beyond the C-terminal residues that interact with the IDCL of PCNA (*rad27-A358**) (Figure 2). These alleles, as well as the complete deletion allele *rad27Δ* and the flap endonuclease PIP box allele *rad27-p* were used for subsequent characterizations and comparisons.

Figure 2.—*rad27* alleles.


RAD27
1
29
107
145
242
340 - 347
366 - 382
rad27-p
1
29
107
145
242
340 - 347
366 - 382
F
A
F
A
rad27-K325*
1
29
107
145
242
325
rad27-A358*
1
29
107
145
242
340 - 347
358
rad27-pX8
1
29
107
145
242
340 - 347
366 - 382
AAAAKAAA
Nuclease domains
PIP-box
NLS

Phenotypic characterizations of *rad27*alleles: To learn whether these *rad27* mutations possess unique phenotypes and to distinguish one from another and from the null mutation, we have carried out assays for growth, mutagenesis and DNA repair. Because *rad27* mutants are genetically unstable, most subsequent characterizations were carried out with two isolates of each mutant, although both are not always shown. In all tests the two isolates had the same phenotype.

To begin, we determined whether the strains are temperature sensitive for growth at 35°. Both *rad27Δ* and *rad27-K325** exhibit temperature-sensitive growth whereas *rad27-A358** and *rad27-pX8* grow robustly at 35° on YPD (Figure 3A). Thus, the loss of the C-terminus that eliminates all PCNA interacting residues (save for the few interactions between the core of the enzyme and PCNA) is more deleterious than the other mutations that retain either the PIP box (*rad27-A358**) or the residues that interact with the hydrophobic pocket of the IDCL (*rad27-pX8*).

While neither *rad27-pX8* nor *rad27-A358** confer temperature sensitive growth as single mutations, *rad27-pX8* exhibits temperature-sensitive growth as a double mutant with *cdc9-p*, and *rad27-A358** confers slow growth at the restrictive temperature when combined with *cdc9-p* (Figure 3B, Table 2). This also contrasts them with the *rad27Δ* and *rad27-K325** mutants that have an absolute lethality with *cdc9-p* (Table 2). The temperature-sensitive lethality of *rad27-pX8* and the slow growth phenotype of *rad27-A358** with *cdc9-p* are

another indication of the balance that must be achieved among the flap endonuclease and DNA ligase I in binding to PCNA.

TABLE 2

The viability of *rad27* double mutants[a]

Genotype	25°	35°
cdc9-p rad27-p[b]	Viable	Viable
cdc9-p rad27Δ	Inviable	-
*cdc9-p rad27-K325**	Inviable	-
*cdc9-p rad27-A358**	Viable	Slow Growth
cdc9-p rad27-pX8	Viable	Inviable
exo1Δ rad27-p	Viable	Viable
exo1Δ rad27Δ	Inviable	-
*exo1Δ rad27-K325**	Inviable	-
*exo1Δ rad27-A358**	Viable	Viable
exo1Δ rad27-pX8	Viable	Inviable
rad2Δ rad27-p	Viable	Viable
rad2Δ rad27Δ	Viable	Inviable
*rad2Δ rad27-K325**	Viable	Inviable
*rad2Δ rad27-A358**	Viable	Viable
rad2Δ rad27-pX8	Viable	Viable

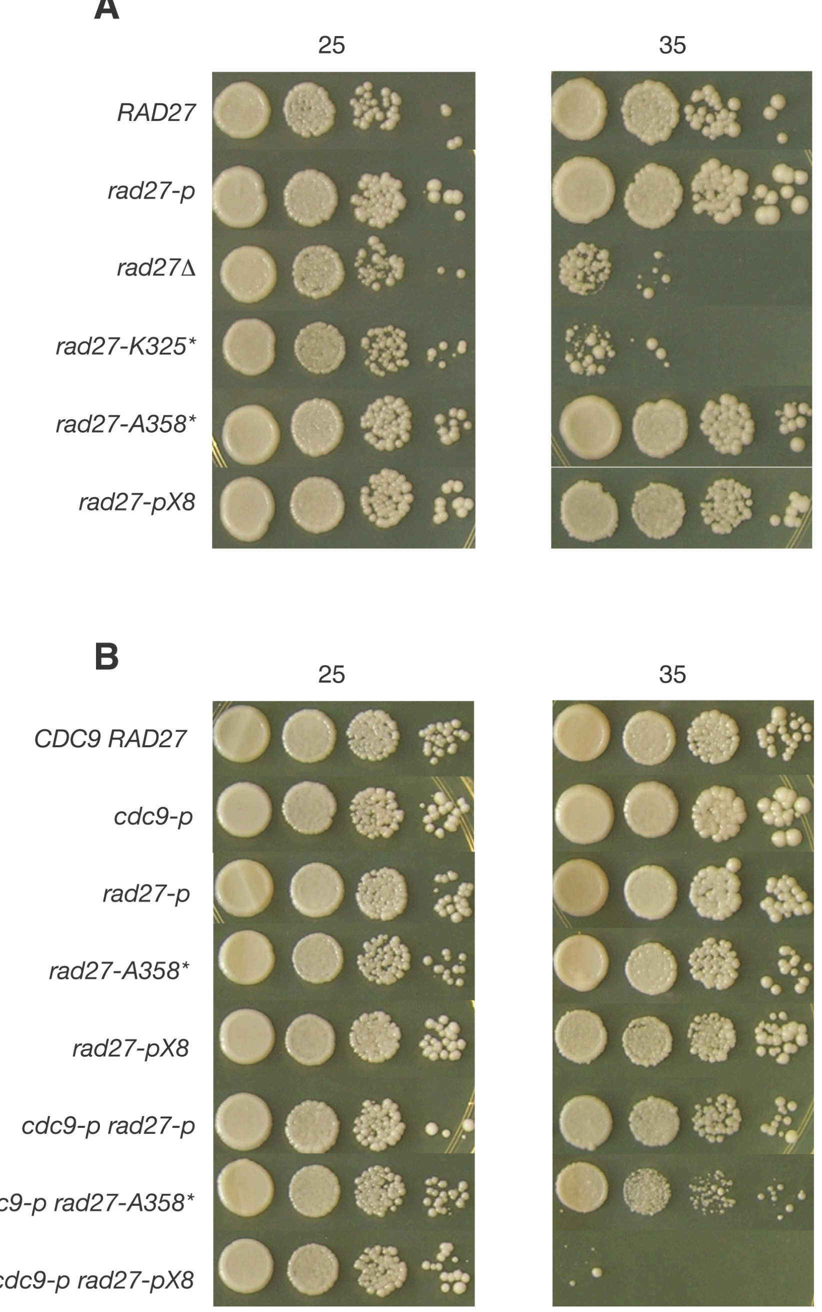
rad51Δ rad27-p[b]	Viable	Viable
rad51Δ rad27Δ	Inviable	-
*rad51Δ rad27-K325**	Inviable	-
*rad51Δ rad27-A358**	Viable	Inviable
rad51Δ rad27-pX8	Inviable	-

[a]Diploid strains were generated and sporulated to recover double mutants as described in Materials and Methods. Tetrads were germinated at 25° and viable spores were subsequently streaked on YPD media and grown at 25° and 35° for 3 days. Growth on YPD media was recorded as follows: spore colonies that are inviable at 25° were not tested at 35°.

[b]This double mutant had previously been characterized in REFSLAND and LIVINGSTON 2005.

Figure 3.—Temperature-sensitive growth of *rad27* mutants.

(A) Growth of mutants at 25° and 35° on YPD agar (B) Growth of *cdc9-p rad27* double mutants at 25° and 35° on YPD agar


A
25
35
RAD27
rad27-p
rad27Δ
rad27-K325*
rad27-A358*
rad27-pX8
B
25
35
CDC9 RAD27
cdc9-p
rad27-p
rad27-A358*
rad27-pX8
cdc9-p rad27-p
cdc9-p rad27-A358*
cdc9-p rad27-pX8

Mutation to canavanine resistance and repeat tract instability: As part of our initial characterization of the *rad27* alleles, we determined the rate of spontaneous forward mutation to canavanine resistance. The rate of mutagenesis is significantly higher in all the *rad27* alleles tested, apart from *rad27-p,* when compared to the wildtype ($P<0.05$) (Figure 4). The *rad27Δ* and *rad27-K325** alleles have a mutation rate greater than 120-fold that of the wild type. The other two *rad27* alleles*, rad27-A358** and *rad27-pX8*, have 2-fold and 17-fold higher mutation rates than that of the wild type, respectively. These results show the relative importance of the C-terminal domains of the flap endonuclease in mutation avoidance. In particular, the mutation rate of the *rad27-pX8* mutant shows the necessity of a complete PIP box in mutation avoidance. The mutation rate of *rad27-K325** is greater than the product of the mutation rates of *rad27-A358** and *rad27-pX8*. The *rad27-K325** mutation eliminates the PIP box and all residues C-terminal to it. In particular, it eliminates a set of residues C-terminal to the PIP box that are retained by the *rad27-A358** mutation. These residues contact the IDCL of PCNA and their elimination in *rad27-K325** may account for the mutant's synergistic mutation rate.

We also made another test of mutation avoidance using a standard assay for dinucleotide repeat instability. We introduced a plasmid containing a run of GT repeats embedded in frame in *URA3* into the *rad27* mutants (HENDERSON and PETES 1992). As in the canavanine resistance assay, the

*rad27-K325** mutation confers a mutation rate to Ura$^-$ that is as great, if not greater, than *rad27Δ* (Table 3). The *rad27-pX8* and *rad27-A358** mutations confer rates proportional to their mutation rate to canavanine resistance. While the product of the mutation rates to Ura$^-$ conferred by these two mutations is greater than the rate found for the *rad27-K325** mutation, the sum of the two is less than the *rad27-K325** rate, again suggesting that the extra portion of the flap endonuclease between residues 347 and 358 is important for mutation avoidance.

TABLE 3

Dinucleotide repeat instability in *rad27* alleles[a]

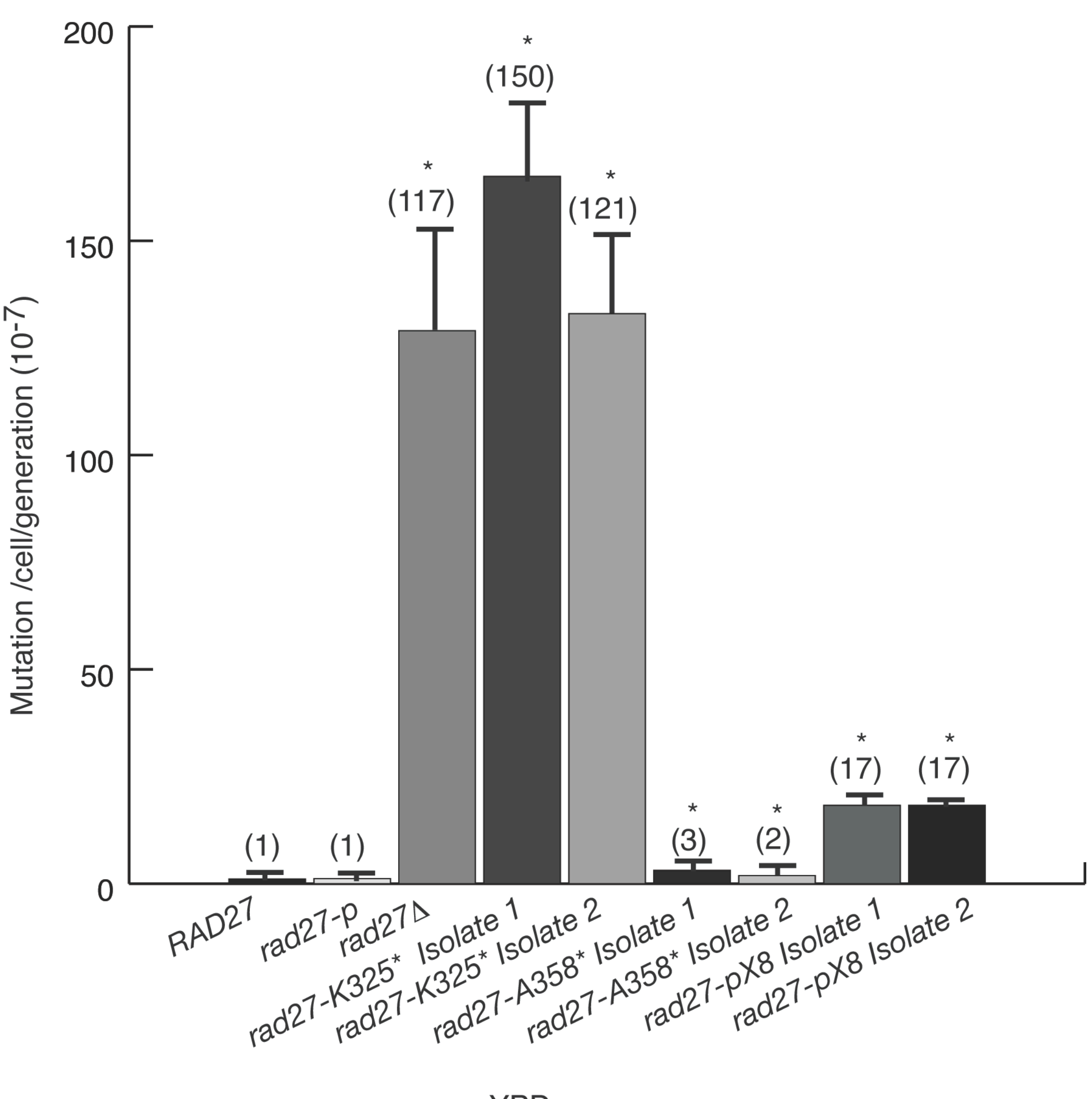
Strain	Mutation Rate ($x10^{-6}$)[b]
RAD27	0.9
rad27-p	2
rad27Δ	604
rad27-K325-isolate1*	810
rad27-K325-isolate2*	614
rad27-A358-isolate1*	22
rad27-A358-isolate2*	16
rad27-pX8-isolate1	272
rad27-pX8-isolate2	339

[a]Strains were transformed with a reporter plasmid pSH44 containing a poly(GT) tract in-frame with a *URA3* gene. The mutation rate was determined by the method of the media LEA and COULSON (1949).

[b]All the *rad27* mutants, apart from *rad27-p*, have mutation rates significantly higher (P<0.05) than the wild-type.

Figure 4.—Mutation rates to canavanine resistance.

The median mutation rates from multiple trials were averaged and compared to the values for the wild type by Student's t-test. * $P < 0.05$ The error bars represent the standard deviation of the determined means.


200
150
100
50
0
Mutation /cell/generation (10^{-7})
*
(150)
*
(117)
*
(121)
(1)
(1)
*
(3)
*
(2)
*
(17)
*
(17)
RAD27
rad27-p
rad27Δ
rad27-K325* Isolate 1
rad27-K325* Isolate 2
rad27-A358* Isolate 1
rad27-A358* Isolate 2
rad27-pX8 Isolate 1
rad27-pX8 Isolate 2
YPD

Characterization of *rad27* alleles in combination with *exo1Δ*, *rad2Δ* and *rad51Δ*: The involvement of the flap endonuclease in multiple DNA transactions is evidenced by the genetic interaction of *rad27Δ* with mutations of the repair and recombination genes *EXO1*, *RAD2* and *RAD51*. To characterize and to distinguish among our *rad27* alleles, we mated them to *exo1Δ*, *rad2Δ* and *rad51Δ*. We sporulated the resulting diploids and geminated the spores at 25° on YPD. If the spores germinated, we then tested their growth at 35° (Table 2).

EXO1 encodes exonuclease I, a 5'-3' exonuclease (TISHKOFF *et al.* 1997a). *rad27Δ* is synthetically lethal in combination with *exo1Δ* (TISHKOFF *et al.* 1997a). This lethality is thought to occur because Exo1 has 5'-flap cleavage capability that is redundant with the flap endonuclease activity of the enzyme (TRAN *et al.* 2002). In addition, high-level expression of the nucleases encoded by *EXO1* suppresses the hypermutation phenotype of *rad27Δ* (SUN *et al.* 2003). Like the *rad27Δ exo1Δ* double mutant, the *rad27-K325* exo1Δ* double mutant is inviable (Table 2). *rad27-pX8 exo1Δ* is viable at 25° but exhibits a temperature-sensitive phenotype at 35°. *rad27-A358**, the mutant with the least severe phenotype, is viable in combination with *exo1Δ* at both temperatures. These data show that the *rad27Δ* and *rad27-K325** mutants, that are temperature sensitive and have high mutation rates, have an absolute requirement for *EXO1*. The *EXO1* requirement for growth of *rad27-pX8* at 35° shows the need

for this nuclease to support viability of the flap endonuclease mutant lacking all the residues of its PIP box.

RAD2 encodes an endonuclease homologous to the flap endonuclease and the *EXO1* encoded nuclease that is required for nucleotide excision repair. In contrast to the inviability of the *exo1Δ rad27Δ* double mutant, the *rad2Δ rad27Δ* double mutant is viable indicating that other nucleases can support DNA replication and repair (REAGAN *et al.* 1995; SOMMERS *et al.* 1995). Like the suppression of *rad27Δ* by high-level expression of *EXO1*, so too can high-level expression of *RAD2* suppress the mutation avoidance phenotype of *rad27Δ* (SUN *et al.* 2003). When we combined *rad2Δ* with our *rad27* alleles, all the double mutants are viable at 25°. However at 35°, the *rad2Δ* double mutants with *rad27Δ* and *rad27-K325** are temperature sensitive. The double mutants of the less severe mutations, *rad27-pX8* and *rad27-A358**, are viable at 35° (Table 2). Because both *rad27Δ* and *rad27-K325** are tempterature sensitive as single mutants, the iniviablilty of the *rad2Δ* double mutants do not yield information on the contribution of *RAD2* to their inviability.

RAD51 encodes the strand exchange protein that facilitates double strand break repair. Previous studies have shown that the lesions caused by the absence of the flap endonuclease are repaired via the recombinational repair pathway (SYMINGTON 1998). When we attempted make double mutants of each of our *rad27* alleles with *rad51Δ* we found that, with the exception of the two alleles with the least severe phenotypes, *rad27-p* and *rad27-A358**, all are

inviable at 25° (Table 2 and Refsland and Livingston 2005). The *rad27-A358* rad51Δ* double mutant is temperature sensitive at 35° (Table 2). This temperature-induced lethality is interesting because neither the *rad51Δ* nor the *rad27-A358** single mutant is temperature sensitive. Furthermore, the *rad27-A358** mutant exhibits slight sensitivity to MMS, and has a slightly elevated mutation rate. The synthetic lethality of the *rad27-A358* rad51Δ* double mutant at 35° suggests that the DSB repair pathway is essential to repair the few lesions generated in the *rad27-A358** mutant.

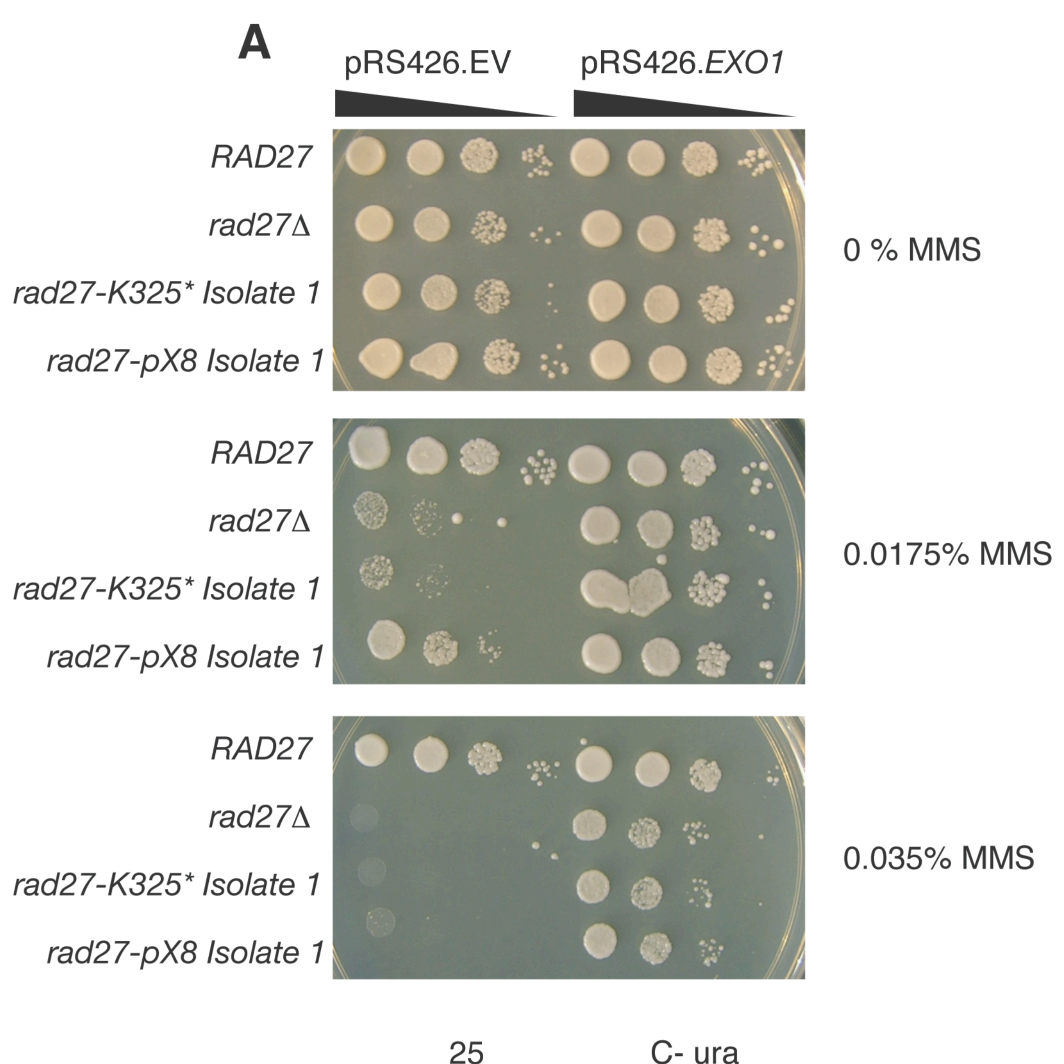
MMS sensitivity of *rad27* mutants: We have also examined the sensitivity of the *rad27-K325** and *rad27-pX8* mutants to two tests of MMS sensitivity, one at relatively low concentrations in agar that reports base excision repair and one at a high concentration where the chemical acts as a radiomimetic agent. *rad27-K325** has a sensitivity much like *rad27Δ* at concentrations of MMS of 0.0175% and 0.035% (Figure 5). The *rad27-pX8* mutant is partially resistant at the lower of these two concentrations (Figure 5). The sensitivity can be suppressed by high-copy expression of *EXO1* (Figure 5A), and to a lesser extent by high-copy expression of *RAD2* (Figure 5B).

We have also incubated mutants in a higher concentration of MMS that produces double strand breaks in the DNA. All of the *rad27* mutants are susceptible to DNA damage induced by this concentration of MMS (Figure 6). The mutants exhibit a gradient in their sensitivity. *rad27-p* is nearly as resistant

to MMS as the wild-type parent as previously observed (GARY *et al.* 1999; REFSLAND and LIVINGSTON 2005). *rad27-A358** is sensitive but more resistant than either *rad27-pX8* or *rad27-K325**. The *rad27-pX8* mutant retains partial resistance. Interestingly, both *rad27-pX8* isolates yielded a biphasic curve not seen for the other mutants. Of greater interest to us is the partial resistance of the *rad27-K325** mutant in comparison to the null mutant *rad27Δ*. This is an indication that the truncated protein product of the *rad27-K325** allele retains partial biological function.

Figure 5.—Growth of *rad27* mutants on MMS agar.

The mutants were spotted on selective agar containing MMS at 25°. (A) Strains were transformed with either pRS246 (EV empty vector) or pRS426.*EXO1*. (B) Strains were transformed with either pRS246 or pRS426.*RAD2*.


A
pRS426.EV
pRS426.EXO1
RAD27
rad27Δ
rad27-K325* Isolate 1
rad27-pX8 Isolate 1
0 % MMS
0.0175% MMS
0.035% MMS
25
C- ura

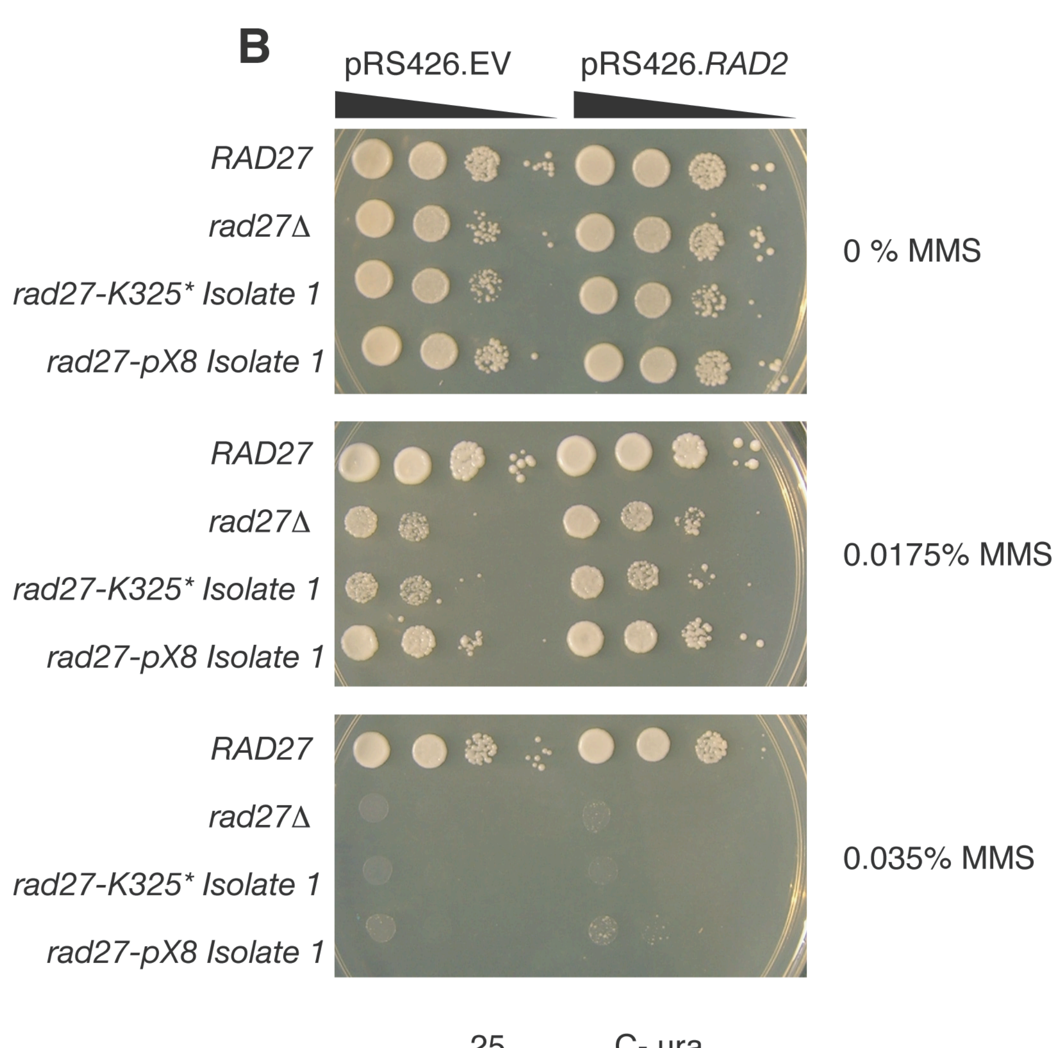
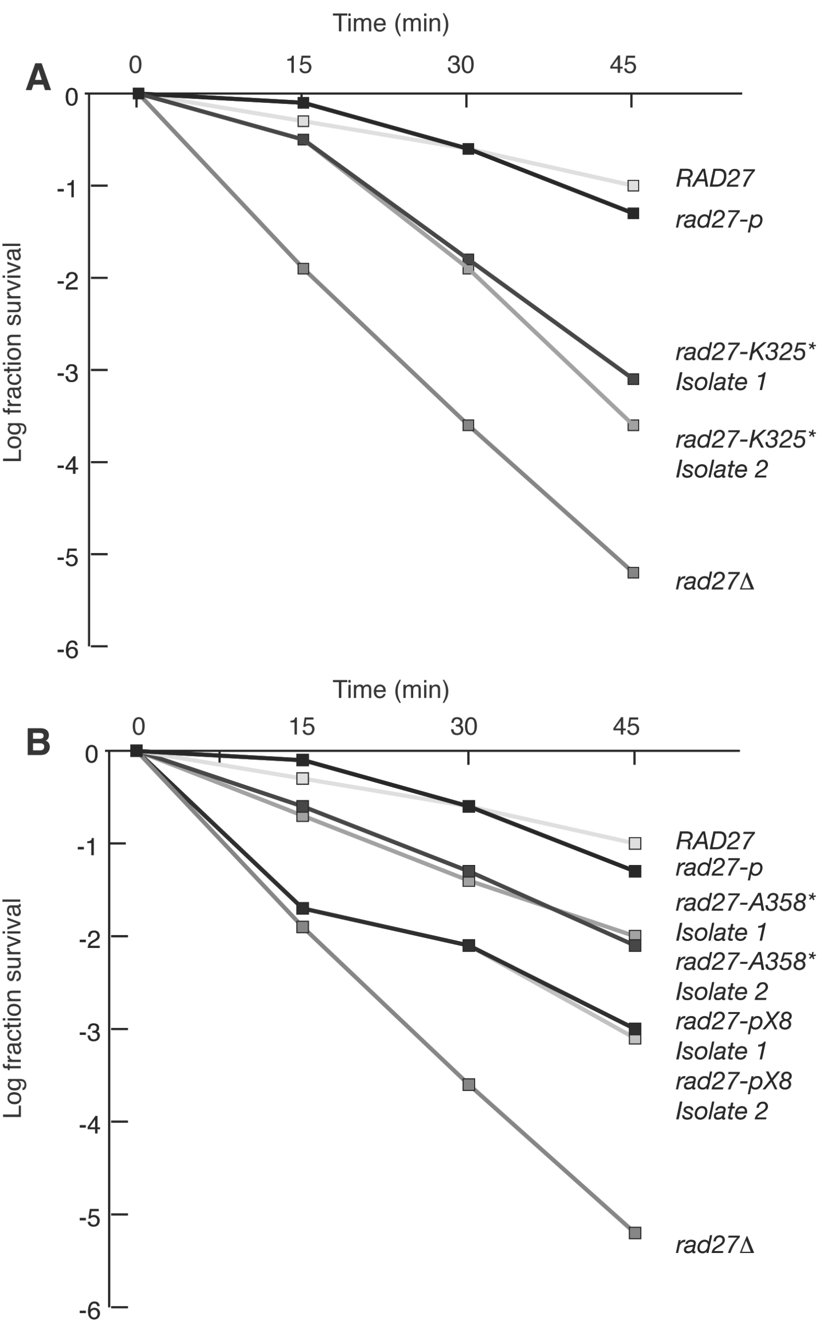

B
pRS426.EV
pRS426.RAD2
RAD27
rad27Δ
rad27-K325* Isolate 1
rad27-pX8 Isolate 1
0 % MMS
RAD27
rad27Δ
rad27-K325* Isolate 1
rad27-pX8 Isolate 1
0.0175% MMS
RAD27
rad27Δ
rad27-K325* Isolate 1
rad27-pX8 Isolate 1
0.035% MMS
25
C- ura

Figure 6.—Survival of *rad27* mutants to exposure of 0.5% MMS. Multiple survival studies were carried out for the strains contemporaneously, and the results were averaged. For clarity, the error for each time point is not shown and the results are divided between two graphs.


Time (min)
A
0
15
30
45
0
-1
-2
-3
-4
-5
-6
Log fraction survival
RAD27
rad27-p
rad27-K325*
Isolate 1
rad27-K325*
Isolate 2
rad27Δ
Time (min)
B
0
15
30
45
0
-1
-2
-3
-4
-5
-6
Log fraction survival
RAD27
rad27-p
rad27-A358*
Isolate 1
rad27-A358*
Isolate 2
rad27-pX8
Isolate 1
rad27-pX8
Isolate 2
rad27Δ

High-level expression of *rad27-K325 suppresses the temperature and MMS sensitivity phenotypes of *cdc9-p rad27-p* mutant:** While the temperature sensitivity of its growth on YPD, its synthetic lethality in combination with *cdc9-p, exo1Δ, rad2Δ and rad51Δ,* and its mutation rate do not distinguish the *rad27-K325** mutant from the null, its partial resistance to MMS suggests that the mutant protein retains partial biological function. The truncated enzyme retains its nuclease domain, and other studies have shown that mutation of its PCNA binding motifs does not destroy its nuclease activity (FRANK *et al.* 2001).

An immediate question in probing for retained biological function of the *rad27-K325** mutant protein was to learn whether the protein product is as abundant as the wild-type protein. Consequently, we cloned epitope tags at the start of *RAD27* and *rad27-K325** to place the tags at the N-terminus of the protein products and expressed them from their own promoter, in low copy, in a *rad27Δ* strain. Using immunoblotting, we observed that both the wild-type and the mutant proteins are expressed at similar levels (Figure 7). Thus, partial activities that are retained by the *rad27-K325** mutant are not likely the result of an unstable protein.

As another indicator of the biological activity remaining in the *rad27-K325** mutant, we discovered that this mutant is capable of growth at 35° when plated on selective media (C-Ura) (Figure 8). Its ability to grow on selective media contrasts it to the null, which is unable to grow at on this media at 35°. In

addition, when *rad27-K325** is expressed in the *rad27Δ* strain, it confers growth. Thus, the *rad27-K325** mutant enzyme retains partial function.

Finally, we transformed the *rad27-K238** allele expressed from a high-copy plasmid into a strain with partial resistance to MMS to ask whether it could enhance or reduce the partial resistance. The strain we chose was the *cdc9-p rad27-p* double mutant that we had already characterized to have partial MMS resistance (REFSLAND and LIVINGSTON 2005). We tested the transformed strain for temperature and MMS sensitivity. The *cdc9-p rad27-p* double mutant grows weakly at 35°, and high-level expression of *rad27-K325** suppresses the temperature-sensitive growth defect although the growth is not as robust as the wildtype (Figure 9). In addition, high-level expression of *rad27-K325** increases the MMS resistance of the double mutant nearly to wild-type resistance (Figure 10). These results suggest that the *rad27-K325** enzyme, despite having its entire C-terminal truncated, retains partial biological function. Furthermore, while the product of *rad27-K325** might have interfered with the weakened binding of the *rad27-p* product to reduce the MMS resistance of the double mutant, it did not do so. While we cannot rule out that a physical interaction between the products of *rad27-K325** and *rad27-p* accounts for the increase in function, the most straightforward conclusion is that the *rad27-K325** enzyme retains partial activity.

Figure 7.—Expression of epitope-tagged proteins.

RAD27 and *rad27-K325** were tagged with the flag epitope and expressed from the native promoter on *RAD27* in a *rad27Δ* strain. After electrophoresis, blotting and immunological identification, proteins specific to either the full-length flap endonuclease or a truncated protein of the size predicted from the *rad27-K325** were detected. (A minor nonspecific band appears at the same mobility as full length product.) A portion the Coomassie stained gel is shown as an indication of the equal loading.

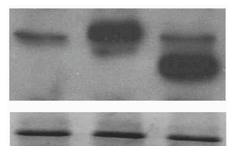

rad27Δ
Empty Vector
FL-RAD27
FL-rad27-K325*
IB: anti-Flag
Coomassie

Figure 8.—Growth of *rad27Δ* and *rad27-K325 mutants on selective agar.**

Dilutions of mutants transformed with either pRS426 or pRS426.*rad27-K325**

were spotted on agar lacking uracil and incubated at 25° and 35°.

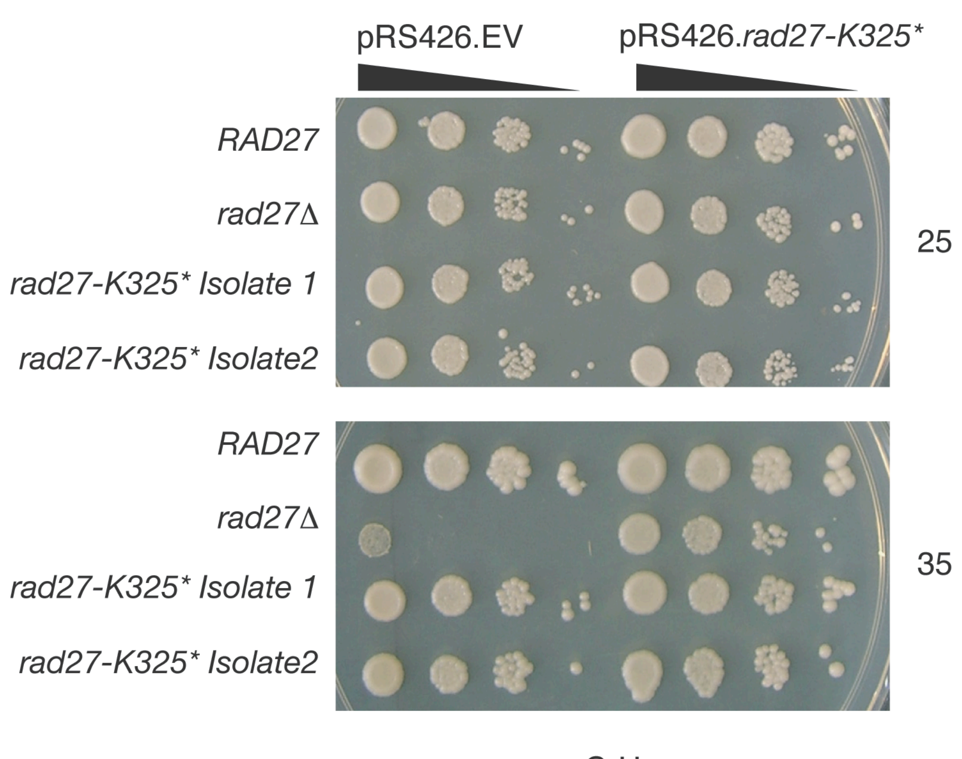

pRS426.EV
pRS426.*rad27-K325**
RAD27
rad27Δ
rad27-K325 Isolate 1*
rad27-K325 Isolate2*
25
RAD27
rad27Δ
rad27-K325 Isolate 1*
rad27-K325 Isolate2*
35
C-Ura

Figure 9.—Growth of the *cdc9-p rad27-p* double mutants.

Dilutions of mutants transformed with either pRS426 or pRS426.*rad27-K325** were spotted on agar lacking uracil and incubated at 25° and 35°. (Note that the *rad27-p* mutant was spotted on a different agar dish and subjected to identical manipulation.)

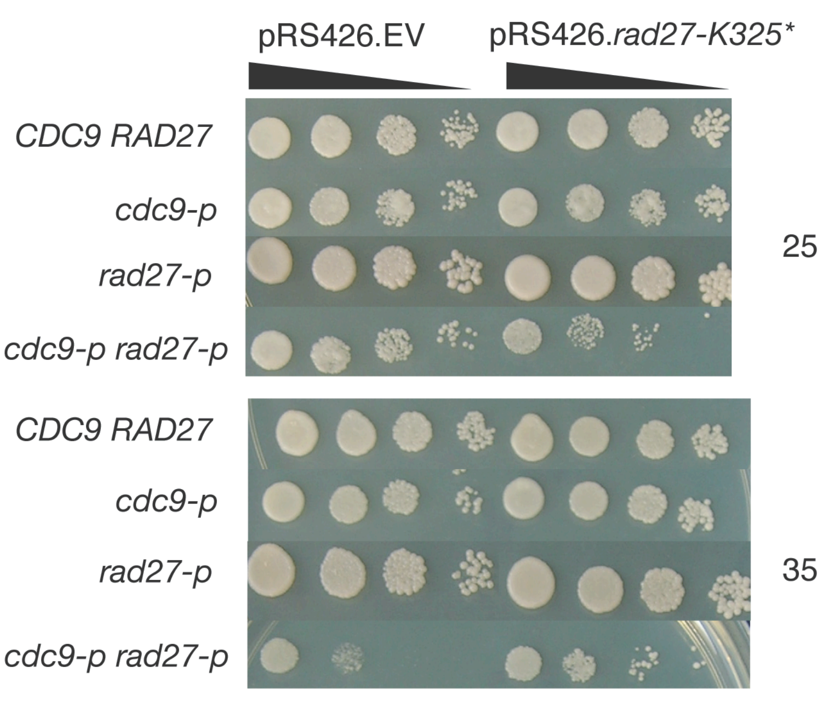
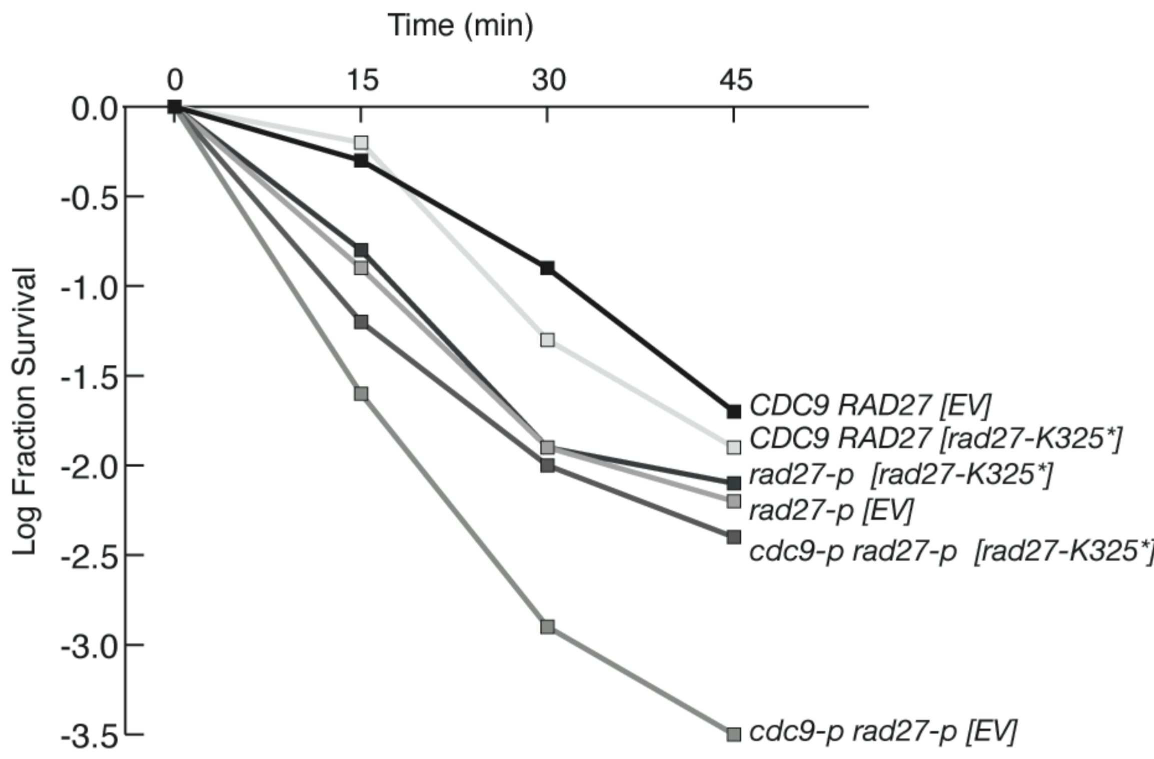

pRS426.EV
pRS426.rad27-K325*
CDC9 RAD27
cdc9-p
rad27-p
cdc9-p rad27-p
25
CDC9 RAD27
cdc9-p
rad27-p
cdc9-p rad27-p
35
C-Ura

Figure 10.— Survival of the *cdc9-p rad27-p* double mutant to exposure of 0.5% MMS.

Multiple survival studies were carried out for the strains contemporaneously, and the results were averaged. For clarity, the error for each time point is not shown. For these studies, the strains were grown in selective media, exposed to MMS and plated on selective agar at 25°.


Time (min)
0
15
30
45
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
Log Fraction Survival
CDC9 RAD27 [EV]
CDC9 RAD27 [rad27-K325*]
rad27-p [rad27-K325*]
rad27-p [EV]
cdc9-p rad27-p [rad27-K325*]
cdc9-p rad27-p [EV]

High-level expression of PCNA suppresses the temperature and hypermutagenic phenotypes of *rad27-K325 and *rad27Δ* mutants:** Next, we revisited our initial observation that high copy expression of *POL30* (PCNA) partially suppresses the temperature-sensitive growth of the *rad27-K325** mutant on YPD. As our initial observation was done using the mutagenized strain from which we identified the *rad27-K235** mutation, we transformed the reconstituted *rad27-K235** mutant with a high-copy plasmid expressing *POL30* and assayed for growth at 25° and 35°. On YPD agar, overexpression of *POL30* partially suppresses the temperature sensitive growth defect of the *rad27-K325** mutant (Figure 11A). When the analyses were conducted on selective media, we observed that neither the null mutant nor the *rad27-K325** mutant that had been transformed with the empty vector exhibited as profound a temperature growth deficit as they did on YPD agar at 35° (Figure 11B). (We note the variation in growth of *rad27* mutants on rich and synthetic media in this and previous figures. This variation among different media is reproducible.) When transformed with the plasmid that expresses PCNA, each mutant grows better as seen by the larger colonies that form (Figure 11B). Thus, high-level of PCNA has a suppressive effect on both mutations.

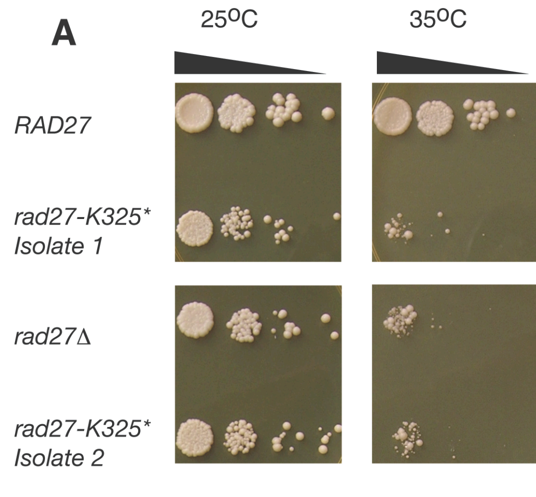
In an attempt to provide a quantitative demonstration of the suppressive effects of *POL30* on the two alleles, we measured doubling times and plating efficiencies. High-level expression of *POL30* reduced significantly ($P < 0.05$) the doubling time of the two *rad27-K325** isolates by 51 minutes and 22 minutes

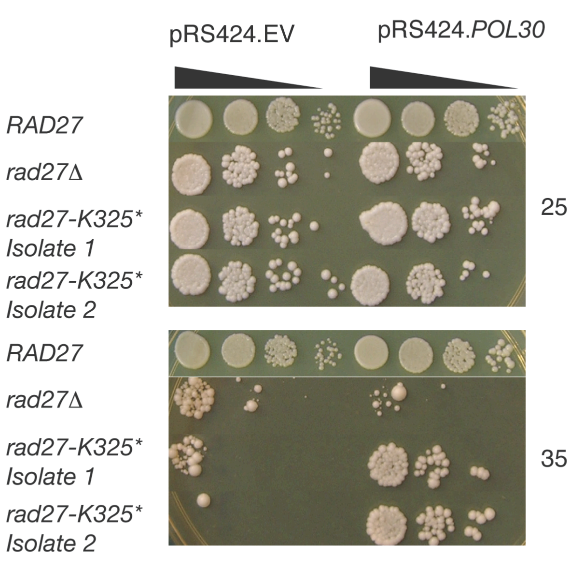
when grown at 36° in liquid culture (Table 4). To buttress these doubling time results, we simultaneously tested the strains for plating efficiency by removing samples of the log phase culture grown at 25° and spreading equal amounts on selective media at 25° and 35°. The plating efficiency of the *rad27-K325** isolates is above 80%, commensurate with temperature tolerance observed in the plating of serial dilutions on C-Trp (Figure 11B). Because the plating efficiency is below 100% we were able to observe a small but significant increase in viability in cells expressing PCNA compared to control cells with empty vector ($P < 0.05$) (Table 4). The two isolates have 39% and 28% better viability at 35°, respectively.

In the case of the null allele, we did not measure a reduction in doubling time when PCNA was overexpressed (Table 4). Furthermore, the plating efficiency of the null mutant was lower than that of the *rad27-K325** mutant and appeared to worsen when transformed with the PCNA expressing plasmid. The results show that suppression of *rad27-K325** by high level expression of PCNA is more pronounced than suppression of *rad27Δ*. Thus, while high-level expression of PCNA ameliorates the growth phenotypes of both *rad27-K325** and *rad27Δ*, the differences in the degree of suppression suggests that more than one mechanism of suppression may be operating in the *POL30* suppression of *rad27-K235**.

Figure 11.—PCNA suppression of *rad27* mutant growth defects on YPD and selective agar.

(A) YPD agar with pRS424 or pRS424.*POL30* (B) selective agar with pRS424 or pRS424.*POL30.*


A
25oC
35oC
RAD27
rad27-K325*
Isolate 1
rad27Δ
rad27-K325*
Isolate 2
YPD


pRS424.EV
pRS424.POL30
RAD27
rad27Δ
rad27-K325*
Isolate 1
rad27-K325*
Isolate 2
25
RAD27
rad27Δ
rad27-K325*
Isolate 1
rad27-K325*
Isolate 2
35
YPD

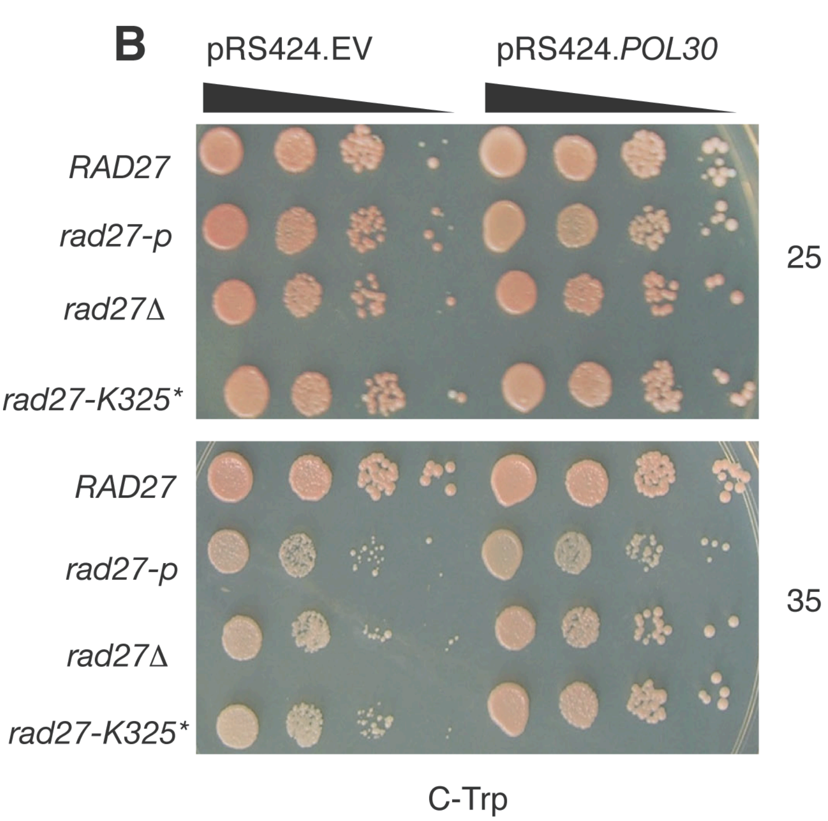

B
pRS424.EV
pRS424.POL30
RAD27
rad27-p
rad27Δ
rad27-K325*
25
RAD27
rad27-p
rad27Δ
rad27-K325*
35
C-Trp

TABLE 4

PCNA (*POL30*) overexpression affects on doubling time and plating efficiency

Strain	[a]Doubling Time (min)	[b]Plating Efficiency (%)
RAD27 + pRS424.EV	163	96
RAD27 + pRS424.*POL30*	187	103
rad27Δ + pRS424.EV	209	33[d]
rad27Δ + pRS424.*POL30*	207	17[e]
rad27-K325-isolate1* + pRS424.EV	238	98 [d]
rad27-K325-isolate1* + pRS424.*POL30*	187 [c]	87 [e]
rad27-K325-isolate2* + pRS424.EV	191	95 [d]
rad27-K325-isolate2* + pRS424.*POL30*	169[c]	87 [e]

[a]Strains were grown to mid-log phase at 25°, diluted and grown at 36°

[b]Percent viable cells grown at 35° were compared to those grown at 25°

[C]*P*<0.05 when compared to their respective empty vector controls.

[d]*P*<0.05 when compared to the empty vector control in *rad27Δ*

[e]*P*<0.05 when compared to *POL30* overexpression in *rad27Δ*

We next tested whether high copy expression of *POL30* can suppress the canavanine mutation rate of the *rad27* alleles. We found that overexpressing PCNA significantly ($P < 0.05$) decreases the rates of spontaneous mutation to canavanine resistance in all the *rad27* mutants tested including the null (Figure 12). (*rad27-A358** and *rad27-p* were not tested because they have a low rate of mutagenesis.) Overexpression of PCNA reduces the spontaneous mutation rate of canavanine resistance 5- and 6-fold in the two *rad27-K325** isolates. Similarly, overexpression of PCNA reduces the spontaneous mutation rate 4- and 7-fold in the two *rad27-pX8* isolates.

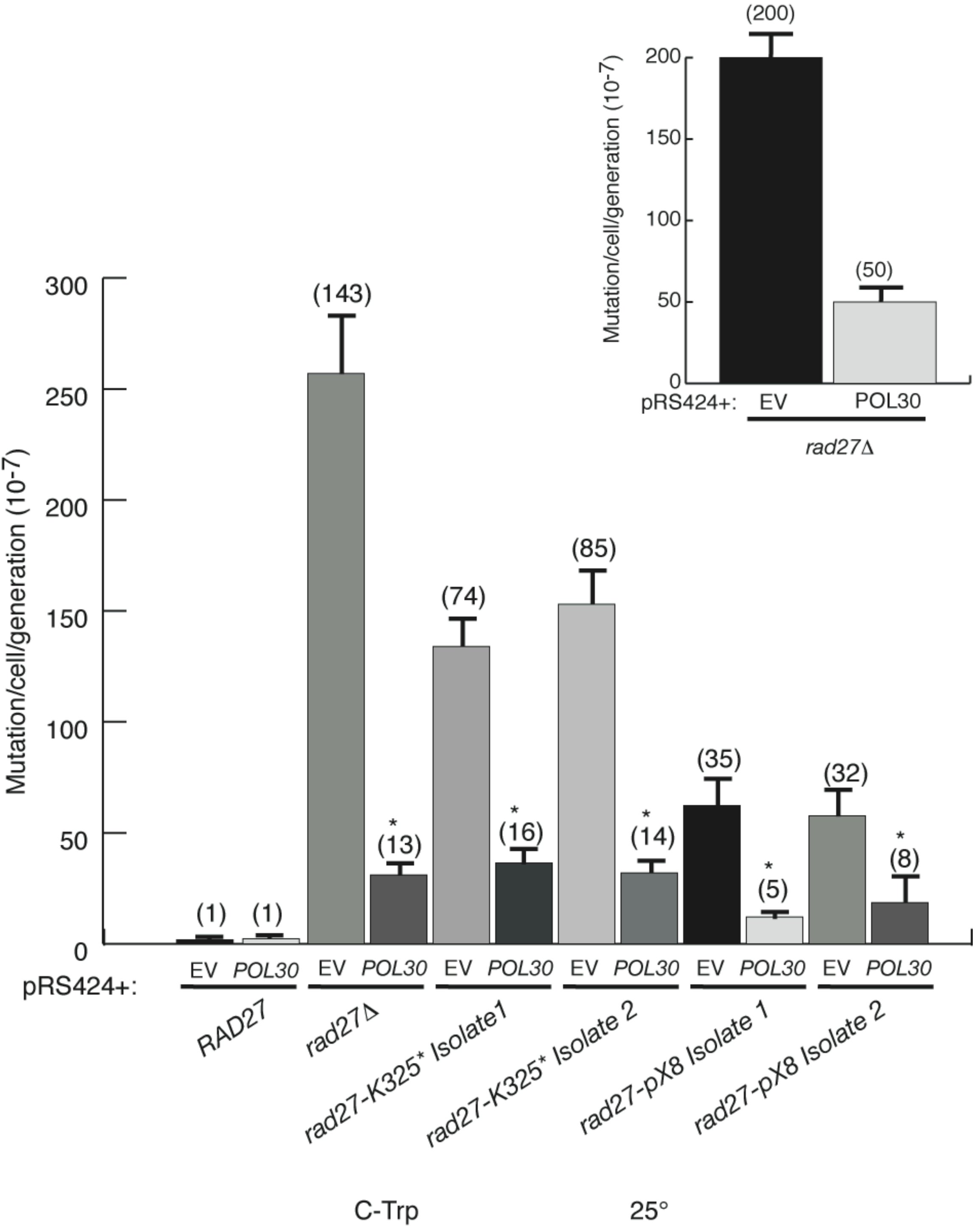
In accord with the suppressive effects of PCNA on colony growth, high levels of PCNA also significantly reduce the mutation rate in the null mutant (Figure 12). To exclude the possibility that this PCNA suppression results from complementation of a hypomorphic *pol30* mutation in our parental strain, we recovered and sequenced the endogenous *POL30* gene including its upstream region. Sequencing showed that our parental strain does not harbor any *pol30* mutations. As further assurance, we repeated this study in a *rad27Δ* strain derived from a different strain background. The suppressive effect of high-level expression of PCNA on canavanine resistance was also seen in strain SSL 939, a *rad27Δ* strain that is not isogenic to our *rad27* alleles (Figure 12 insert). The ability of PCNA to suppress both hypomorphic and null *rad27* alleles suggests that additional PCNA helps mutant cells either by increasing flap endonuclease function in the hypomorphic mutants, *rad27-K325** and *rad27-*

pX8, or by providing an opportunity for another nuclease to take the place of the flap endonuclease or both.

The high-copy suppression of *rad27-pX8* afforded the opportunity of examining the behavior of the *rad27-pX8 exo1Δ* double mutant. Both mutations have a mild mutator phenotype in comparison to *rad27Δ*. When combined they produce a synergistic mutation rate that is noteworthy because of the low mutation rate of the *exo1Δ* mutant (Figure 13). This genetic interaction is consistent with the temperature lethality of the double mutant although the mutation rates were determined at the permissive temperature (25°). Next, we introduced *POL30* into these strains to determine the extent of suppression. High-copy expression of *POL30* has previously been reported to suppress *exo1Δ* although the results show very slight suppression (AMIN *et al.* 2001). Because the mutation rate to canavanine resistance is only slightly elevated in our *exo1Δ* mutant over the wild-type rate, we were unable to detect *POL30* suppression. *POL30* is able to suppress the *rad27-pX8 exo1Δ* double mutant, but it is unable to bring it down to the level of the most severe single mutant (*rad27-pX8*). The results suggest that the synergism underlies a necessity for each enzyme to act on the other's deficiency, and that the suppression by *POL30* cannot make up for this necessity.

Figure 12.—PCNA suppression of the rate of mutation to canavanine resistance in *rad27* mutants.

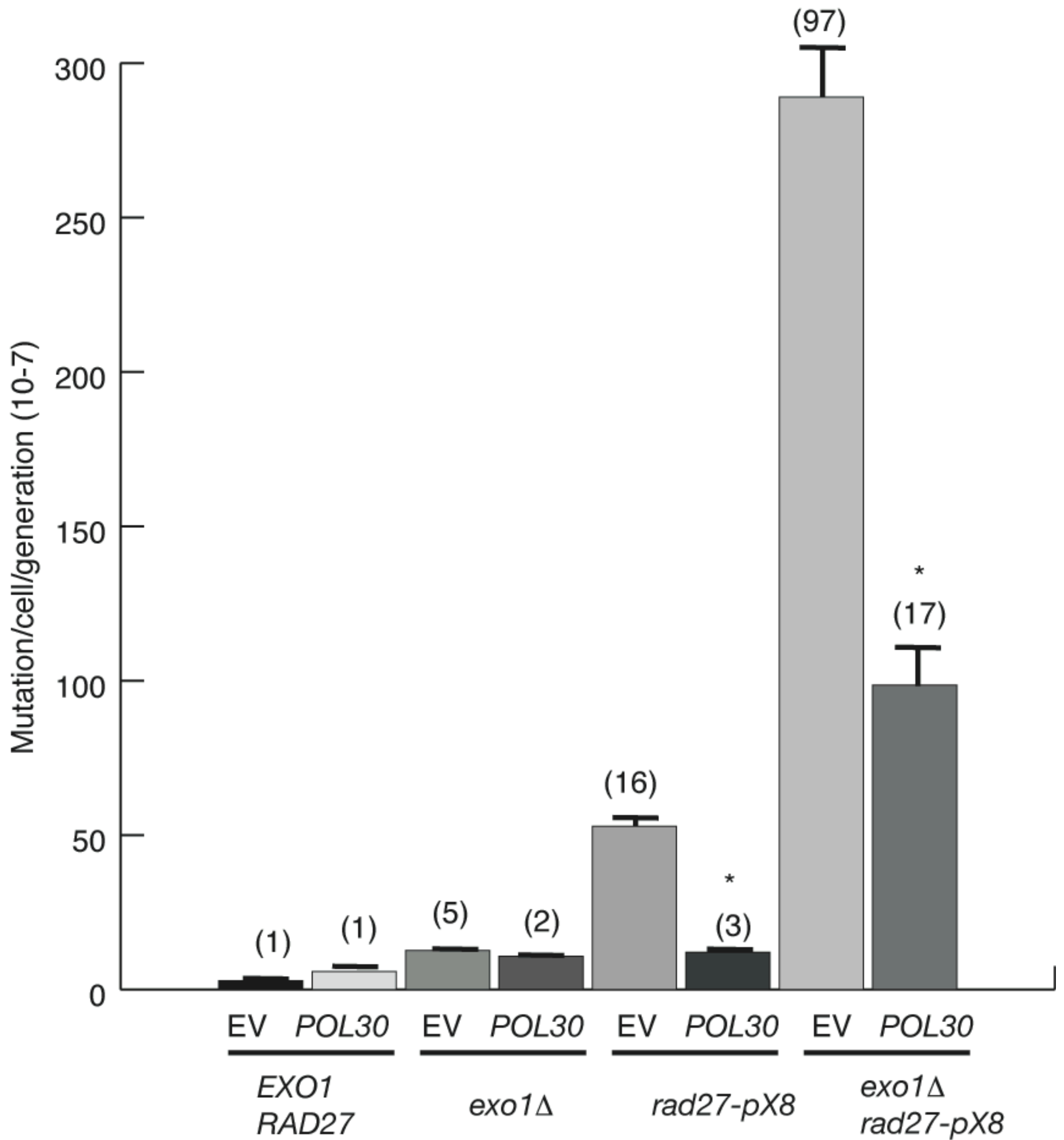
The analysis follows the methods described under Figure 4 except the mutants were grown in selective media and plated to selective agar containing canavine.



Mutation/cell/generation (10-7)
300
250
200
150
100
50
0
(143)
(13)
(74)
(16)
(85)
(14)
(35)
(5)
(32)
(8)
(1)
(1)
pRS424+:
EV POL30 EV POL30 EV POL30 EV POL30 EV POL30 EV POL30
RAD27
rad27Δ
rad27-K325* Isolate1
rad27-K325* Isolate 2
rad27-pX8 Isolate 1
rad27-pX8 Isolate 2
C-Trp
25°
(200)
(50)
EV
POL30
rad27Δ

Figure 13.—PCNA suppression of the rate of mutation to canavanine in the *rad27-pX8 exo1Δ* double mutant.

The analysis follows the methods described under Figure 4 except the mutants were grown in selective media and plated to selective agar containing canavine.



Mutation/cell/generation (10-7)
300
250
200
150
100
50
0
(1)
(1)
(5)
(2)
(16)
*
(3)
(97)
*
(17)
EV
POL30
EV
POL30
EV
POL30
EV
POL30
EXO1
RAD27
exo1Δ
rad27-pX8
exo1Δ
rad27-pX8
C-Trp

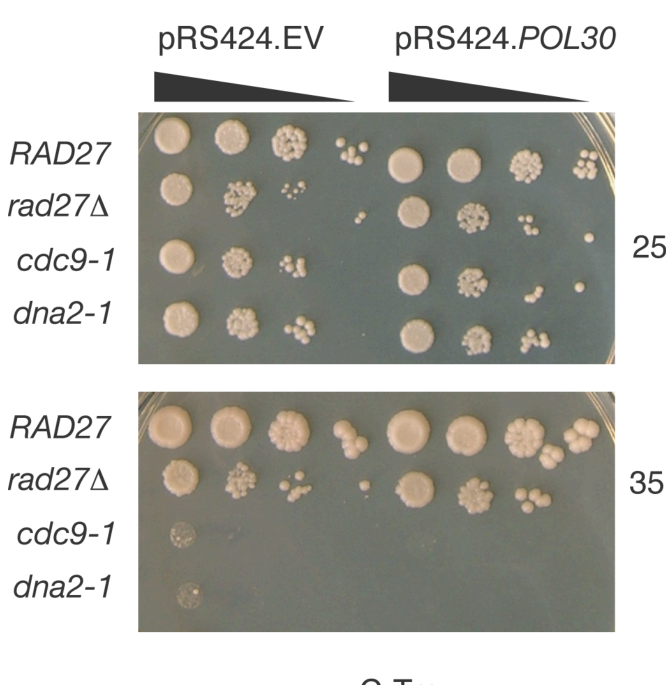
High-level expression of PCNA does not suppress the temperature-sensitive phenotypes of the DNA ligase I mutant *cdc9-1* or the helicase-endonuclease mutant *dna2-1*: To examine whether suppression by high-level of expression of PCNA is specific to *rad27* mutations, we tested two additional replication mutations. We chose mutations of the enzymes most closely associated with the activity of the flap endonuclease in DNA replication. During DNA replication, the flap endonuclease is thought to bring the *DNA2* helicase-nuclease to the replication fork because the helicase-nuclease binds to the flap endonuclease (BUDD and CAMPBELL 1997; STEWART *et al.* 2006). However, PCNA is not known to bind to the *DNA2*-encoded helicase-nuclease. The *DNA2* nuclease may participate in primer removal along with the flap endonuclease (BAE *et al.* 1998; BAE *et al.* 2002; BUDD *et al.* 2000; STEWART *et al.* 2006). The role of the *DNA2* helicase activity remains enigmatic, although it, along with the nuclease activity, is essential to the yeast cell (BUDD *et al.* 1995; BUDD *et al.* 2000). High-level expression of *DNA2* suppresses *rad27Δ* (BUDD and CAMPBELL 1997). For our studies on PCNA suppression chose to test the *dna2-1* mutant that has an alteration in the enzyme's helicase domain and is temperature-sensitive for growth (BUDD and CAMPBELL 1995). This *dna2* mutation is synthetically lethal with *rad27Δ* (BUDD and CAMPBELL 1997). We also tested the DNA ligase I mutation *cdc9-1* that is temperature-sensitive for growth.

Each mutant was transformed with a high copy plasmid harboring *POL30* and grown at 25° and 35°. High-copy expression of *POL30* does not suppress

temperature-sensitive lethality of either mutant (Supplemental Figure 1). Comparisons among temperature-sensitive mutations of multiple genes are difficult because of potential differences in phenotypic characteristics. Nevertheless, these results show that the suppressive effect of PCNA is specific to the flap endonuclease mutants rather than a generic, ameliorating effect that high levels of PCNA might have on other DNA replication proteins.

Supplemental Figure 1.- PCNA does not suppress the temperature sensitivity of *cdc9-1* or *dna2-1*.

Dilutions of mutants transformed with either pRS424 or pRS424.*POL30* were spotted on agar lacking tryptophan and incubated at 25° and 35°.


pRS424.EV
pRS424.*POL30*
RAD27
rad27∆
cdc9-1
dna2-1
25
RAD27
rad27∆
cdc9-1
dna2-1
35
C-Trp

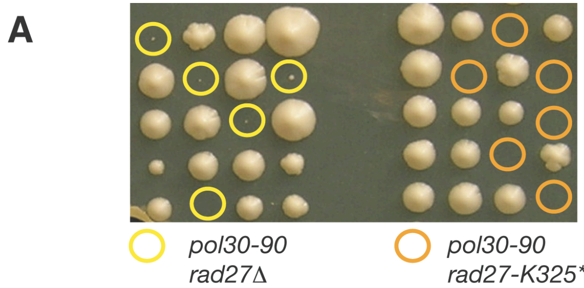
rad27-K325* **is synthetically lethal with** ***pol30-79*** **and** ***pol30-90*****:** Finally, because our mutations have altered the PCNA binding capacity of the flap endonuclease, we were interested in whether they could be combined with *pol30* mutations that are known to affect the flap endonuclease. Mutational analysis of *POL30* has identified two mutations of PCNA that have profound effects on flap endonuclease binding and activation (EISSENBERG *et al.* 1997). *pol30-79* (L126A/I128A) and *pol30-90* (P252A/K253A) are mutations in the hydrophobic pocket of the IDCL and the C-terminal end of PCNA, respectively. *In vitro*, the *pol30-79* mutant protein fails to interact with the flap endonuclease but manages to stimulate the nuclease activity. In contrast, the *pol30-90* mutant protein binds to the flap endonuclease but fails to stimulate its nuclease activity (GOMES and BURGERS 2000). In genetic studies, Eissenberg *et al*., (1997) reported that the *pol30-79 rad27Δ* and *pol30-90 rad27Δ* double mutants are viable but exhibit poor growth at 23°.

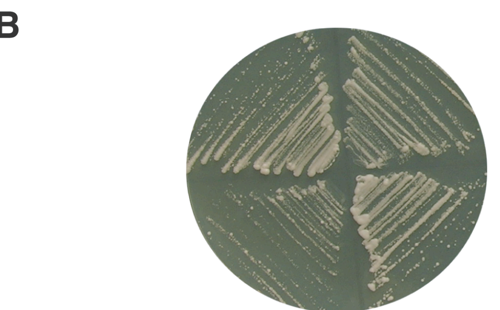
To determine whether our *rad27* alleles are viable in combination with these PCNA mutations, we mated *pol30-79* and *pol30-90* with the *rad27* alleles. The diploids were sporulated and dissected onto YPD at 25°. In our strain background both *rad27Δ* and *rad27-K325** proved inviable with *pol30-79*, while the *rad27-A358* pol30-79* and *rad27-pX8 pol30-79* double mutants were viable but grew slower than either single mutant (Supplemental Figure 2). More interesting to us was the observation that the *rad27Δ pol30-90* double mutant is viable and grows slowly at 25° (as previously observed (EISSENBERG *et al.*

1997)), whereas the combination of *rad27-K325** with *pol30-90* is inviable (Figure 14A). We were able to streak out the *rad27Δ pol30-90* spore colonies onto rich media where they formed micro-colonies after ten days at 25° (Figure 14B). As expected, the combinations of *rad27-A358** and *rad27-pX8* with *pol30-90* yielded viable spores. When grown on rich media these double mutants exhibit growth defects that are commensurate with the severity of their mutator and repair phenotypes (Figure 14C). For example, *pol30-90 rad27-pX8* grows slower than does *pol30-90 rad27-A358**. Thus, *rad27-K325** is anomalous in that it exhibits synthetic lethality with *pol30-90* that is not shown by the null mutation.

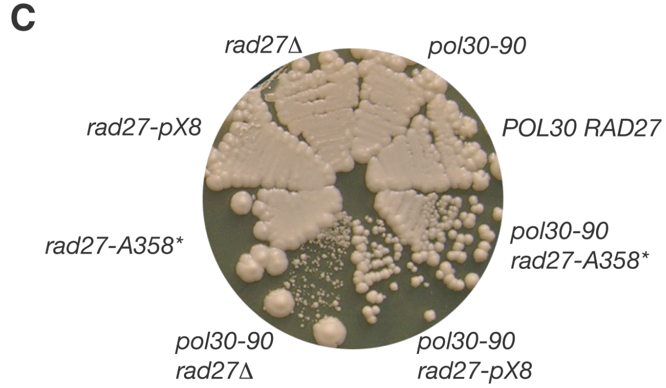
Figure 14.—Interaction between *rad27* mutations and the *pol30-90* mutation.

(A) Germination of spore colonies that have the genotype *rad27Δ pol30-90* or *rad27-K325* pol30-90*. (B) The agar dish shows four spores with the genotype *rad27Δ pol30-90* that are able to form micro-colonies at 25° after 10 days of incubation. (C) The difference in colony size among the *rad27 pol30-90* double mutants after 10 days of incubation at 25°.





pol30-90 rad27Δ
10 days at 25 on YPD



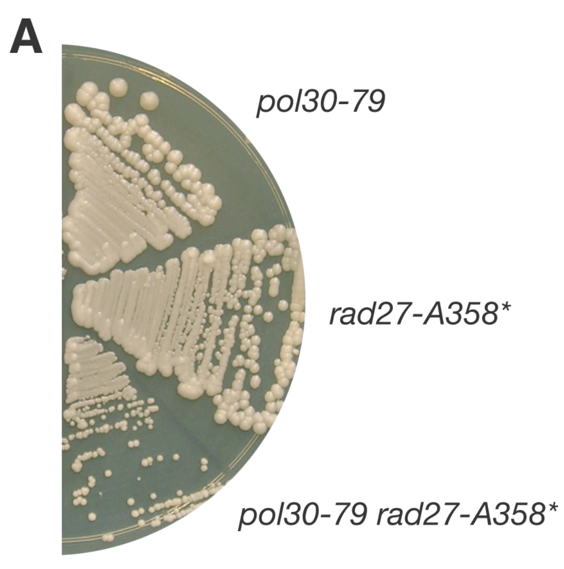
10 days at 25 on YPD

Supplemental Figure 2.- The *rad27-A358* pol30-79* and *rad27-pX8 pol30-79* double mutants were viable but grew slower than either single mutant.

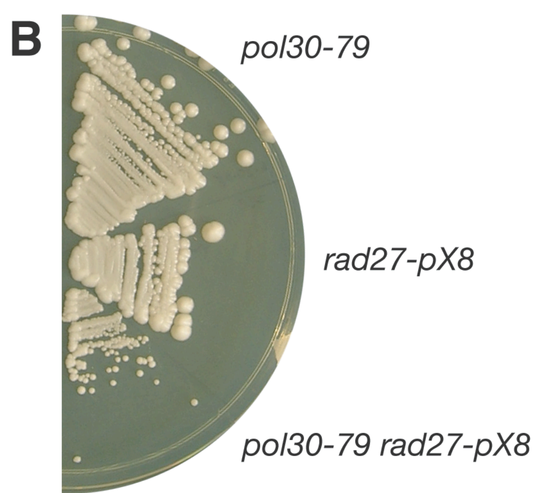
(A) Germinated spores of *rad27-A358* pol30-79* double mutant and *rad27-A358** or *pol30-79* single mutants were streaked on YPD and grown for 6 days.

(B) Germinated spores of *rad27-pX8 pol30-79* double mutant and *rad27-pX8 or pol30-79* single mutants were streaked on YPD and grown for 6 days.

A



B



DISCUSSION

PCNA is responsible for bringing the flap endonuclease and DNA ligase I into lagging strand synthesis during DNA replication. How PCNA orders these final steps in lagging strand synthesis is not well understood. The recovery of a C-terminal truncated mutation of the flap endonuclease missing its PCNA-interacting domains that is synthetically lethal with a mutation in DNA ligase I that weakens its interaction with PCNA contributes to our understanding of how the interactions among PCNA, the flap endonuclease and DNA ligase I occur. The recovery of the *rad27-K325** allele in a synthetic lethal screen with an allele of DNA ligase I, *cdc9-p*, and the mutant's genetic interactions with *pol30-90* might not have been unexpected. Prior studies have shown a genetic interaction between mutations of the flap endonuclease and DNA ligase that weaken each enzyme's binding to PCNA, *rad27-p* and *cdc9-p*, (REFSLAND and LIVINGSTON 2005) and between *rad27Δ* and various *pol30* alleles (EISSENBERG *et al.* 1997).

The *rad27* allele we recovered from our screen, *rad27-K325**, and the ones we created, *rad27-A358** and *rad27-pX8*, have a spectrum of phenotypes that are consistent among most of the tests of growth, mutation avoidance and DNA repair. In general, they fall within a gradient that extends from the null mutant with the most severe display of a phenotype to the wild type. In between, the *rad27-K325** mutant is closest to the null mutant and the *rad27-A358** mutant, along with the *rad27-p* mutant, are closest to the wild type in

their phenotypes. The *rad27-pX8* mutant occupies a middle ground. The combinations of these *rad27* alleles with *rad2Δ*, *exo1Δ*, *cdc9-p* and *rad51Δ* exemplify a novel example of this gradient. While all *rad27* mutations are viable in combination with *rad2Δ* at 25°, the most severe ones, *rad27Δ* and *rad27-K325** are temperature sensitive for growth, while the two other, *rad27-pX8* nor *rad27-A358**, are not. This again suggests that *rad2Δ* does not exacerbate the mutant phenotypes of *rad27* mutations. Next, while the most severe mutations are inviable when combined with *exo1Δ*, the *rad27-pX8* double mutant is temperature sensitive for growth and the *rad27-A358** mutant grows at the restrictive temperature. When the alleles are combined with *cdc9-p*, now, not only does the *rad27-pX8* double mutant become inviable at 35° but the *rad27-A358** double mutant exhibits a growth defect at that temperature. Finally, while all mutations except *rad27-A358** are inviable in combination with *rad51Δ*, this weakest mutation becomes temperature sensitive. The consistency among tests suggests that progressive loss or mutational substitution in the C-terminal tail plays evenly among the myriad of functions that the flap endonuclease fulfills in the cell.

While the *rad27* mutations fall into a pattern, each has properties that are unique. First, the *rad27-K325 ** mutant, while it shares a number of phenotypes with *rad27Δ*, is different from *rad27Δ*. Like *rad27Δ*, *rad27-K325** is lethal in combination with *cdc9-p*, confers temperature-sensitive growth and is as mutagenic as *rad27Δ*. Similarly, both are partially suppressible by high-level

expression of PCNA. Furthermore, when we combined *rad27Δ* and *rad27K325** with mutations of genes required for DNA repair, *exo1Δ* and *rad2Δ,* and recombination, *rad51Δ*, they proved to behave similarly. Both are synthetically lethal when combined with *exo1Δ* and *rad51Δ* and temperature sensitive with *rad2Δ*.

*rad27-K325** can be distinguished from *rad27Δ* in tests that show that the truncated protein is likely to retain some biological activity and in other tests that show that the mutant protein may confer a degree of toxicity to the cell. First, the *rad27-K325** mutant has a greater plating efficiency than the null, suggesting that it does not undergo cell cycle arrest or incur lethal mutations as does the null. Furthermore, the *rad27-K325** mutant is more resistant to MMS exposure than is the null mutant. The partial resistance suggests that the mutant protein is partially capable in the repair of the damage caused by the radiomimetic agent. Retention of partial activity might be expected because the mutant retains all the nuclease domains of the enzyme and the mutant protein appears as stable as the wild-type protein. Retention of partial activity in one assay (MMS resistance) and not in another (mutation avoidance) is likely a reflection on the multiple activities that the enzyme possesses. Depending on the substrate presented to the purified enzyme, it acts as a flap endonuclease, as an exonuclease or as a gap endonuclease (SINGH *et al.* 2007; ZHENG *et al.* 2005). Furthermore, flap endonuclease mutations differentially affect the multiple activities (SINGH *et al.* 2007; XIE *et al.* 2001; ZHENG *et al.* 2005). Thus,

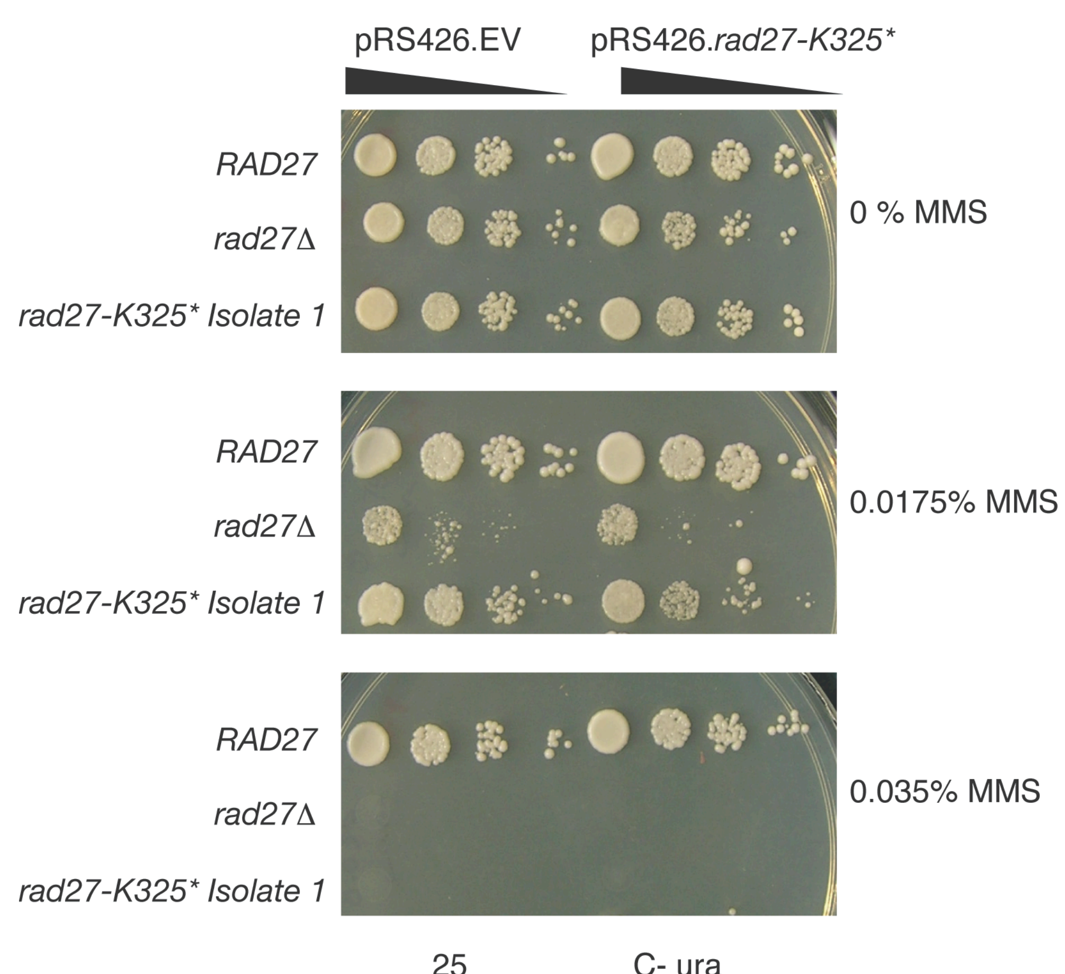
the difference in phenotypic penetrance conferred by *rad27-K325** may reflect the possibility that the mutant protein can act on some chromosomal substrates but not on others.

We have found that while high-level expression of *rad27-K325** did not increase the level of MMS resistance of the null mutant beyond the level seen in the *rad27-K325** strain (Supplemental Figure 3), high-level expression of *rad27-K325** increases the resistance of the *cdc9-p rad27-p* double mutant to MMS exposure. Why the product of the *rad27-K325** allele increases the MMS resistance of the *cdc9-p rad27-p* double mutant, where the PIP box of both enzymes have been mutated, is not obvious. Such results might be accounted for by physical interactions between the two mutated types of the flap endonuclease or by a partition of function between the two mutants types. The first explanation does not appear to be plausible because the flap endonuclease is not known to form multimers, the product of the *rad27-K325** allele appears to be stable and the product of the *rad27-p* allele is presumed to be stable by virtue of its near wild-type MMS resistance. The second explanation of a partition of function cannot be ruled out, but what function one mutant form could confer to the other is not apparent. We suggest that complementation may result from the manner in which the two mutant forms of the flap endonuclease bind to PCNA. Binding studies show that removal of the PIP box, as created in the *rad27-K325** allele, increases the affinity of the flap endonuclease for PCNA (GUO *et al.* 2008). Possibly, in the suppressed double

mutant, the product of *rad27-K325** binds first to PCNA and alters the conformation of PCNA so that the product of *rad27-p* is able to bind in an active conformation by a mechanism first suggested by Gomes and Burgers (2000).

Supplemental Figure 3.—Growth of *rad27* mutants on MMS agar.

The mutants were spotted on selective agar containing MMS at 25°. Strains were transformed with either pRS426 (EV empty vector) or pRS426.*rad27-K325*.*


pRS426.EV
pRS426.rad27-K325*
RAD27
rad27Δ
rad27-K325* Isolate 1
0 % MMS
RAD27
rad27Δ
rad27-K325* Isolate 1
0.0175% MMS
RAD27
rad27Δ
rad27-K325* Isolate 1
0.035% MMS
25
C- ura

The other assays that show the difference between the *rad27-K325** allele and the null allele is the lethality that the former has with *pol30-90*. While *rad27Δ pol30-90* grows slowly, it is not lethal as is *rad27-K325* pol30-90*. This not only shows a difference between the two alleles but also shows that *rad27-K325** may confer a slight toxicity to the cell when PCNA is mutated. We also note that in measures of mutation avoidance, the *rad27-K325** mutant exhibits slightly greater promiscuity than the null mutant. Toxicity is not unprecedented, as a *rad27* allele with a mutation of the nuclease catalytic site is toxic to the yeast cell. The deleterious allele is detoxified by mutating the PIP box (GARY *et al.* 1999). Thus, some mutant forms of the flap endonuclease appear to bind to PCNA in a manner that inhibits the binding of other enzymes, in turn inhibiting DNA replication.

The *rad27-pX8* mutation that substitutes alpha helical forming residues for the eight amino acids of the PIP box is interesting because of the severity of its phenotypes in comparison to *rad27-p*, a mutation of two of the eight PIP box residues. It is distinctly more sensitive to MMS than *rad27-p* and lethal in combination *rad51Δ*. These phenotypes suggest a strong need for interaction between the flap endonuclease and PCNA in the incidence of DSBs or recovery from DSBs. Furthermore, it becomes temperature sensitive when combined with *exo1Δ*. In the case of the *exo1Δ rad27-pX8* double mutant, we note that its synergistic mutation rate at 25° places this *rad27* mutation in a class of mutations uncovered in a screen for enhancers of the weak *exo1Δ* mutator

phenotype (AMIN *et al.* 2001). The simplest explanation for the temperature sensitivity of the *exo1D rad27-pX8* double mutant is that the mutant enzyme becomes inactive, and the synthetic lethality reflects the synthetic lethality of the *exo1Δ rad27Δ* double mutant. Considering that the *rad27-pX8* mutant is viable at 35°, and, consequently, likely to retain partial function, the synthetic lethality may reflect the sensitivity of the *exo1Δ* mutant to diminished *RAD27* function.

The *rad27-A358** mutant is interesting because of its seemingly mild phenotypes in combination with its absolute requirement for *CDC9* and *RAD51* at 35°. In particular, its requirement for *RAD51* implies that the mutant protein introduces DNA lesions that have an absolute requirement for the DSB repair system.

One result that comments strongly on the role of PCNA in mediating the events of DNA replication and repair is that overexpression of PCNA reduces the rates of spontaneous mutations not only in the two partial hypomorphic mutants *rad27-K325** and *rad27-pX8* but also in the null mutant. A possible explanation for suppression of *rad27Δ* is that the large quantity of PCNA recruits a novel nuclease that suppresses the mutant phenotype. Potential candidates for suppression of the null are the nucleases encoded by *EXO1* and *RAD2* and the nuclease activity of the *DNA2* helicase-nuclease. High levels of ExoI compensate for the loss of the flap endonuclease in *rad27Δ* mutants by suppressing the temperature-induced lethality and mutation avoidance phenotypes (SUN *et al.* 2003; TISHKOFF *et al.* 1997a). High level expression of

RAD2 also compensates (SUN *et al.* 2003). While both Rad2-family members suppress the temperature-sensitive growth and mutator activity of *rad27Δ*, only *RAD2* high-level expression suppresses its MMS sensitivity (SUN *et al.* 2003). In addition, high-level expression of *DNA2* partially compensates for the absence of the flap endonuclease (BUDD and CAMPBELL 1997). While all three activities might compensate, we note that none of the three have been shown to directly interact with PCNA.

Suppression of *rad27-K325** and *rad27-pX8* by overexpression of PCNA may occur by a second mechanism that does not solely involve compensation by another nuclease. Instead, suppression may occur, either in part or in whole, by increased binding of the mutant nucleases to PCNA by mass action. In discussing this possibility, we take into account the many contacts that the human flap endonuclease ortholog makes with PCNA and the mechanism by which the nuclease is thought to reach DNA substrates. Cocrystalization of human FEN-1 with PCNA revealed that the flap endonuclease makes contacts throughout its length including the core nuclease domain that remains in our *rad27-K325** mutant (SAKURAI *et al.* 2005). The multiple contacts also include segments of FEN-1 that extend on either side its PIP box. In addition, a truncated mutant of the human flap endonuclease missing its PIP box is capable of binding to PCNA (GUO *et al.* 2008). Thus, all mutant forms that we have created should be capable of binding to PCNA with reduced affinity.

Increasing the amount of PCNA should increase the amount of binding, and that may be partially responsible for suppression of the mutant phenotypes.

The activity of the flap endonuclease is greatly increased by its binding to PCNA (FRANK *et al.* 2001; GOMES and BURGERS 2000; JONSSON *et al.* 1998; LI *et al.* 1995; TOM *et al.* 2000). The mechanism that accounts for this activation suggests that the enzyme first binds to PCNA through its PIP box in a manner that precludes its binding to DNA substrates and then rotates through a hinge region between its core and PIP box to allow the enzyme to meet its substrates (GOMES and BURGERS 2000; SAKURAI *et al.* 2005). This two-step mechanism was first proposed by the difference between the binding affinity and nuclease activation of the *pol30-79* and *pol30-90* mutant copies of PCNA (GOMES and BURGERS 2000). The *pol30-79* mutation lies in the hydrophobic pocket of the IDCL and interacts directly with the PIP box, while the *pol30-90* mutation is adjacent to the hinge domain and may be important for rotation. The *pol30-79* mutant protein does not bind strongly to the flap endonuclease but can still stimulate its activity, while the *pol30-90* mutant protein binds more strongly but does not stimulate the nuclease activity. The two modes of flap endonuclease binding to PCNA are supported by the cocrystalization results that demonstrate a hinge permitting rotation of the nuclease across the front face into the PCNA-encircled DNA (SAKURAI *et al.* 2005). The difference in behavior between the two *pol30* mutations suggests that while initial binding is important, it not

necessary for stimulation, while rotation through the hinge is necessary to achieve stimulation.

The behavior of our mutations can be interpreted based on the proposed mechanism. We suggest that the product of *rad27-K325** retains partial biological activity and binds weakly to PCNA. Because it is missing the hinge, it cannot reach most DNA substrates. The extra toxicity of *rad27-K325** when combined with *pol30-90* in comparison to the viability of the *rad27Δ pol30-90* double mutant supports our view that the mutant protein binds to PCNA through it core domain and excludes binding by other PCNA binding proteins. In essence, the product of *rad27-K325** may act as a competitive inhibitor, and this effect is exacerbated in the *pol30-90* mutant that weakens the interaction between PCNA and its other partners. This competitive effect may also operate to prevent the product of *cdc9-p* from binding leading to the synthetic lethality. Based on the rotation model, we interpret our results to suggest that the product of *rad27-pX8* has difficulty with the initial steps of binding to PCNA but possesses the hinge region and C-terminal residues so it is able to rotate and complete catalysis if binding occurs. Combining *rad27-pX8* with *pol30-90* diminishes both the initial binding and possibly the rotation event as the substitutions in *rad27-pX8* change the glutamine residue that interacts with the amino acid residues of PCNA that are mutated in *pol30-90*. These additive changes make the double mutant grow slowly. Finally, the product of *rad27-A358** mutant possesses all the binding regions and is nearly completely

functional. Thus, the behavior of our flap endonuclease alleles is consistent with the binding studies and the proposed rotation model.

Finally, we return to the hypothesis that propelled these studies. Based on our studies of CAG repeat tract expansions, we suggested that PCNA mediates the ordered entry of the flap endonuclease and DNA ligase I into replication (REFSLAND and LIVINGSTON 2005). We began this study with a synthetic lethal screen. As befitting a synthetic lethal screen, we used a *cdc9* allele that can best be characterized as having a mild phenotype, particularly as it grows throughout a range of temperatures and does not exhibit a severe sensitivity to MMS exposure that is seen for other *cdc9* mutations (HARTWELL *et al.* 1973; MONTELONE *et al.* 1981; REFSLAND and LIVINGSTON 2005). Our synthetic lethal screen with *cdc9-p* yielded a *rad27* mutation. Considering that *rad27Δ* is synthetically lethal with many yeast genes, especially those involved in DNA transactions, the appearance of a flap endonuclease mutation with seemingly little activity might not have been unexpected (LOEILLET *et al.* 2005; PAN *et al.* 2006; TONG *et al.* 2004). While we have not systematically screened the collection of yeast gene deletions or combined *cdc9-p* with a panel of other replication and repair mutations, the finding of a new *rad27* allele might not have been chance alone. Previously, we had found that we could combine *rad27Δ* with *cdc9-1* at a permissive temperature (IRELAND *et al.* 2000). Thus, while direct comparisons between *cdc9-1* and *cdc9-p* have not been made, the absolute lethality *rad27-K325** with *cdc9-p* is novel. The novelty is that the

combination of the *cdc9* and *rad27* alleles with the less severe phenotypes (*cdc9-p* and *rad27-K325**) is lethal while the combination of the alleles with more severe phenotypes (*cdc9-1* and *rad27Δ*) is not.

More importantly, the absolute lethality of *rad27Δ* and *rad27-K325**, the temperature-sensitive lethality of *rad27-pX8*, and the temperature-impaired growth of *rad27-A358** and *rad27-p* when combined with *cdc9-p* suggest a strong cellular requirement that both enzymes need to be able to interact with PCNA to complete DNA replication and repair. Thus, the inability of the double mutants to grow is best described as a synergistic effect. The relationship is clearly not one of epistasis. We argue that the effect is greater than additive, making it synergistic. We might have expected the double mutants to grow weakly if their combination were additive. In support of our contention, we note that the triple mutant of *cdc9-p rad27-p pol30-90* is viable at 30° (REFSLAND and LIVINGSTON 2005), whereas the *cdc9-p rad27-K325** double mutant is not. By this view, the triple mutant shows an additive effect and the *cdc9-p rad27-K325** double mutant is synergistic. While our arguments may be circumstantial, our results warrant further examination of the role PCNA plays in ordering the entry of the flap endonuclease and DNA ligase I into replication and repair.

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CHAPTER 3

Future Directions

The current studies have been instructive in furthering our understanding of how the C-terminal tail of flap endonuclease interacts with PCNA and the role of this interaction in maintaining cellular homeostasis. Furthermore, the ability of PCNA to suppress the aberrant growth and DNA damage phenotypes of *rad27-K325**, *rad27-A358** and *rad27-pX8* is a reminder of the versatility of this protein in ensuring cell survival through its many binding partners.

The mutants described in this study can be used to further examine some of the cellular roles played by the flap endonuclease. As such, several questions remain outstanding, for example i) how do the *rad27* alleles contribute to mitotic recombination, ii) does PCNA also play a role in recombination, iii) how does the truncation of the C-terminal tail impact the interaction between the flap endonuclease and Dna2 nuclease during replication and iv) how is the PCNA interaction with the flap endonuclease impaired in *rad27-K325**, *rad27-A358** and *rad27-pX8* mutants? Here, I propose some further studies that will address these questions.

The contribution of mutant flap endonuclease and PCNA to recombination

Yeast *rad27* null strains have a significantly higher rate of mitotic recombination than the wildtype. Symington (1998)(SYMINGTON 1998) reported that *rad27Δ* strains have a 21-fold higher mitotic recombination rate than the wild type but do not have appreciable increase in meiotic recombination. Similarly, Tishkoff *et al.*, (1997) (TISHKOFF *et al.* 1997) reported that *rad27Δ* strains had a 7-fold, 30-fold and 25-fold higher rate of gene conversion, single-

site conversion and crossover events respectively when compared to the wild type. It has been suggested that this high rate of recombination is necessary to repair some of the DNA lesions generated in the absence of the flap endonuclease. The importance of recombination is supported by a previous observation that *rad27Δ rad51Δ* double mutant is synthetic lethal (SYMINGTON 1998) and our observation that double mutants of *rad27-K325** and *rad27-pX8* in combination with *rad51Δ* are also synthetic lethal. In our studies we did not examine the rate of mitotic recombination in the *rad27* alleles or whether high levels of PCNA suppress the elevated recombination rate of *rad27*Δ. I postulate that *rad27-K325**, *rad27-A358** and *rad27-pX8* are hyperrecombinogenic and that overexpression of PCNA suppresses the formation of lesions. Consequently high-levels of PCNA lower the rate of recombination to levels that allow for viability of *rad27-K325**, *rad27-A358** and *rad27-pX8* in combination with *rad51Δ*. I will test these hypotheses by performing the following experiments.

First, the rate of mitotic recombination will be determined in *rad27-K325**, *rad27-A358** and *rad27-pX8* with or without a high-copy plasmid harboring *POL30*. Each of the *rad27* alleles, and a wild type strain, will contain a *his3* reporter construct developed by Kaytor and Livingston (1994) (KAYTOR and LIVINGSTON 1994). Briefly, *rad27* alleles containing a *his3* heteroallele in the *CAN1* locus will be used to perform fluctuation assays. Cells that undergo mitotic recombination will generate a functional *HIS3* gene that allows the cells

to grow on media lacking histidine. Colonies growing on media lacking histidine will be enumerated and the rate of recombination estimated by the method of the median (LEA and COULSON 1949).

Second, I would expect that if high-levels of PCNA sufficiently suppresses the rate of the recombination in the *rad27-K325**, *rad27-A358** and *rad27-pX8* strains, then these mutants would be viable in combination with *rad51Δ*. Another possibility is that the magnitude of PCNA suppression may not be sufficient to prevent the co-lethality of the *rad27* alleles with *rad51Δ*. To test which of these outcomes is true, a heterozygous diploid containing *rad27-K325**, *rad27-A358** and *rad27-pX8* each in combination with *rad51Δ* will be generated. This diploid will be transformed with a high-copy vector containing *POL30* and subsequently sporulated. Tetrad analysis will be performed to establish if PCNA overexpession is sufficient to prevent synthetic lethality between the *rad27* alleles and *rad51Δ*.

The impact of truncating the C-terminal of the flap endonuclease on Dna2 interaction

The flap endonuclease and the Dna2 nuclease are important for the processing of long 5'-flaps during lagging strand replication. The Dna2 nuclease and the flap endonuclease have been shown to physically and genetically interact (BUDD and CAMPBELL 1997). Stewart *et al.*, (2006) (STEWART *et al.* 2006) showed that the flap endonuclease and Dna2 act sequentially to cleave Okazaki fragments and they physically interact during this process. Moreover,

high-levels of *DNA2* compensate for the loss of *RAD27* and for the synthetic lethality of the *pol3-01 rad27-p* double mutant (BUDD and CAMPBELL 1997; JIN *et al.* 2003). The *pol3-01* mutant produces long 5'-flap that requires both *DNA2* and *RAD27* for processing. The flap endonuclease protein-protein interaction domains have been mapped to the C-terminal (Liu *et al*., 2004) (LIU *et al.* 2004). Guo *et al*., (2008) (GUO *et al.* 2008 546) showed that *in vitro* the human flap endonuclease interacts with the AP-endonuclease, Werner helicase and the 9-1-1 complex using its C-terminus. Presently, the contribution of this C-terminal to the interaction between Dna2 nuclease and the flap endonuclease has not been closely examined. The *rad27* mutants we generated for this study lack portions of the C-terminal and would provided valuable information pertaining to how they interact with *DNA2*. Thus, I propose studies to examine how well *rad27-K325**, *rad27-A358** and *rad27-pX8* interact with Dna2 both biochemically and genetically. I postulate that the ability of each of these mutant flap endonucleases to bind to Dna2 correlates to length of the truncated C-terminal.

Genetically, the extent to which the mutant flap endonuclease can survive in the presence of a temperature sensitive allele of *DNA2* will be evaluated. Double mutants would be generated by mating each of the *rad27* alleles with *dna2-1*, a temperature sensitive allele of *Dna2*. The resulting diploid will be sporulated and tetrad analysis conducted to determine their viability. Double mutants would be conformed by genotyping and any viable mutants

would be further characterized. My expectation is that *rad27-K325* dna2-1* is lethal, but *rad27-A358** or *rad27-pX8* in combination with *dna2-1* maybe viable, have severe growth defects or maybe lethal.

Biochemical studies would be conducted using the methods described by Budd and Campbell (1997) (BUDD and CAMPBELL 1997). Briefly, each of the endogenous *rad27* mutants and *Dna2* would be tagged with a different epitope. The proteins would be immunoprecipited from actively growing cultures and probed for physical interaction using the appropriate antibodies and western blot techniques. If the interaction of the flap endonuclease and the Dna2 nuclease is dependent on the length of the C-terminal tail then these biochemical studies would reveal such a requirement. Presumably, this would mean that *rad27-K325** has the least binding affinity followed by *rad27-A358** and finally *rad27-pX8* has the highest affinity.

Taken together, results from these experiments would be instructive in establishing the role played by the C-terminal tail of the flap endonuclease in binding Dna2 and whether this binding is similar to that seen between PCNA and the flap endonuclease.

The C-terminal tail of the flap endonuclease is important for PCNA binding

Finally, we have shown that *rad27-A358** and *rad27-K325** have a graduated growth phenotype when combined with *pol30-79*. We interpret these results to suggest that *rad27-A358** and *rad27-pX8* would have different binding capabilities to PCNA. Gary *et al*., (1999) (GARY *et al.* 1999) showed that rad27-

p and PCNA interact weakly in comparison to the wild-type flap endonuclease. This weak binding was attributed to the mutation of the PIP box. The PIP box is essential in mediating PCNA interactions with various proteins. Additionally, Sakurai *et al*., (2005) (SAKURAI *et al.* 2005) showed that amino acid residues located C-terminal to the PIP box are also involved in binding PCNA. Therefore, I would expect that the protein products of *rad27-A358** and *rad27-pX8* have weaker binding to PCNA and that between these two, *rad27-pX8* would have the weakest binding because we completely abrogated its PIP box.

Utilizing biochemical techniques, each of the *rad27* alleles will be epitope tagged and used in immunoprecipitation experiments. The precipitated proteins will be probed using PCNA specific antibodies to determine how well PCNA interacts with each mutant. This would be an indication of the contribution of the different portions of the C-terminal tail to interaction with PCNA *in vivo*.

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