**CHARACTERIZATION OF THE *IN VIVO* FUNCTIONS OF Y-FAMILY DNA POLYMERASES KAPPA AND REV1**

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## DEDICATION

*For my mother, Shelley Kosarek*

"The love of a mother is the fuel that enables a normal human being to do the impossible."  -Marion C. Garretty

# CHARACTERIZATION OF THE *IN VIVO* FUNCTIONS OF Y-FAMILY DNA POLYMERASES KAPPA AND REV1

**By**

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**CHARACTERIZATION OF THE *IN VIVO* FUNCTIONS OF**

**Y-FAMILY DNA POLYMERASES KAPPA AND REV1**

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DNA translesion synthesis (TLS) is a mode of damage tolerance utilized by cells experiencing replicative stress as a result of DNA damage. TLS is characterized by the synthesis of DNA opposite template lesions, a process that requires the function of specialized DNA polymerases. My studies focus on particular aspects of Rev1 and Polymerase kappa (Polκ) function *in vivo*.

One of the main goals of my work was to characterize the conservation of the interaction between the Rev1 C-terminus and other Y-family polymerases (demonstrated in vertebrates) in lower eukaryotic species. I showed that these interactions are not conserved in the yeasts *S. cerevisiae* or *S. pombe*, nor in the nematode *C. elegans*, yet they are conserved in the fruit-fly *Drosophila melanogaster*. Furthermore, I experimentally determined the requirements of the Y-family polymerase interactions in Drosophila and mouse for comparative analysis. The results of this study concluded that special consideration should be exercised when making mechanistic extrapolations regarding translesion DNA synthesis from one eukaryotic system to another.

Another central goal of this work was to identify new Rev1 protein interaction partners in *S. cerevisiae*. I created a yeast two-hybrid library for screening with Rev1 bait. After identifying and verifying interaction partners for Rev1, I further investigated the epistatic relationship of these genes to Rev1 with respect to UV-radiation. The candidate genes investigated do not appear to function in a synonymous pathway to Rev1 in the response to UV-induced stress.

A final goal of this work was to determine the spontaneous mutation frequencies of $Pol\kappa^{-/-}$ mice using a well-validated *in vivo* mutation detection system. I found that somatic spontaneous mutation frequencies are elevated in 9 and 12-month old $Pol\kappa^{-/-}$ kidney and liver, but not wild-type or $Pol\eta^{-/-}$ tissues. Furthermore, I characterized the mutation spectra of these mice and observed a

specific elevation in transversion mutations G:C>C:G and G:C>T:A. These data are consistent with previous observations of *Polκ*$^{-/-}$ mice, and hint at what types of spontaneous damage may be naturally occurring substrates for Polκ *in vivo*.

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## PUBLICATIONS

Kosarek, J.N., Woodruff R.V., Rivera-Begeman, A., Guo, C., D'Souza, S. Koonin, E.V., Walker, G.C., Friedberg, E.C. "Comparative analysis of *in vivo* interactions between Rev1 protein and other Y-family DNA polymerases in animals and yeasts." *DNA Repair* **7**:3 (2008). 439-451.

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## LIST OF ABBREVIATIONS

**a.a.-** Amino acid

**Ade-** Adenine

**AP-** Apurinic or apyrimidinic site

**ATCC-** American Type Tissue Collection

**β-gal-** Beta-galactosidase

**BD-** Binding domain

**BRCT-** BRCA1 C-terminal-like

**CCM3-** Media for culturing insect cells

**cDNA-** Complementary DNA

**CoIP-** Coimmunoprecipitation

**C-terminus-** The carboxy-terminus of a protein

**Δ-** Deletion

**dCMP-** Deoxycytidyl monophosphate

**DDO-** Double drop-out media lacking amino acids leucine or tryptophan

**DTT-** Dithiothreitol

**EDTA-** Ethylene diamine tetraacetic acid

**FL-** Full length

**G193R-** A glycine to arginine amino acid substitution at codon 193

**GST**- Glutathione-S-transferase

**HEPES**- A buffering agent; 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid

**His**- Histidine

**HRP**- Horse radish peroxidase

**J**- Joules

**Kan**- Kanamycin sulfate

**Kc cells**- Drosophila cell line established from Drosophila embryos

**Leu**- Leucine

**MMS**- Methyl-methanesulfonate

**mRNA**- Messenger RNA

**NCBI**- National Center for Biomedical Inventions

**N-terminus**- The amino terminus of a protein

**OD**- Optical density

**ONPG**- Ortho-Nitrophenyl-β-D-galactopyranoside

**ORF**- Open reading frame

**PAD**- Polymerase-associated domain

**PCNA**- Proliferating cell-nuclear antigen

**PCR**- Polymerase chain reaction

**Polζ**- Polymerase zeta

**Polη**- Polymerase eta

**Polι**- Polymerase iota

**Polκ-** Polymerase kappa

**ProA-** Protein A

**ProG-** Protein G

**QDO-** Quadruple drop-out media lacking amino acids histidine, adenine, tryptophan, and leucine

**RCF-** Relative centrifugal force

**RT-PCR-** Reverse transcriptase PCR

**SD media-** Synthetic defined media

**SDS-** Sodium dodecyl sulfate

**SEM-** Standard error of the mean

**snRNA-** Small nuclear RNA

**TBM-** Tandem base mutation

**TEV-** Catalytic domain of the Nuclear Inclusion a protein encoded by the tobacco etch virus

**TLS-** Translesion synthesis

**Trp-** Tryptophan

**UV-** Ultra-violet

**WT-** Wild type

**YFP-** Yellow fluorescent protein

**YPDA-** Media composed of yeast extract (Y), peptone (P), dextrose (D), and adenine (A)

# Chapter I: Introduction

## *DNA Damage Tolerance*

The biological response to DNA damage is orchestrated through a complex series of molecular pathways. Cell survival amidst DNA damage is largely attributed to either the repair/removal of damage, or the tolerance of DNA damage. Although there is a general understanding of what these pathways are and how they are executed, it is less certain how these pathways communicate and work together to restore the stability and integrity of the genome. Several mechanisms have evolved for the removal of damaged DNA, including processes such as nucleotide excision repair, base excision repair, and mismatch repair (Friedberg et al. 2005). These pathways each involve a unique set of proteins which function to remove damage from the genome. However, due to the stochastic nature of DNA damage, it is inevitable that repair will not always precede replication. Unrepaired lesions encountered by the DNA replication machinery during S-phase cause replication fork stalling. Cell death may occur unless DNA synthesis resumes, posing a potentially harmful threat to the survival of the cell. In such a circumstance, the cell can use one of several so-called *damage tolerance* mechanisms (also called post-replicative repair) to overcome the stalling of the replication machinery at the site of a lesion. Implicit in the name, damage tolerance "tolerates" the presence of a lesion on a template DNA

strand actively undergoing replication so that the cell may temporarily escape potential death to repair the lesion at a later time. The recovery of aberrant or blocked replication forks is a major strategy for preserving the integrity of the genome, a phenomenon that is fully distinct from repair (Friedberg 2005).

Several types of DNA damage tolerance have been characterized to date, one of which is daughter strand gap-repair, also know as recombinational repair. A current model for this process suggests that the presence of damage precludes direct replication through the lesion, while DNA synthesis resumes downstream of the lesion (~1000 nucleotides) generating gaps in the damaged daughter strand. These are filled by a recombination event that utilizes the alternative nascent daughter strand as a template (Friedberg 2005). Given the highly accurate nature of recombination, this mechanism of DNA damage tolerance avoids the introduction of mutations in newly synthesized DNA and is thus referred to as *error-free* damage tolerance. This model is well supported with evidence from studies with *E. coli*, in which the observation was first made that DNA synthesized from damaged templates initially contains discontinuities which are eventually restored to full-length daughter DNA molecules by this process (Johnson and McNeill 1978).

Another type of damage tolerance mechanism is known as replication fork regression (or copy-choice DNA replication), a unique modality by which a replication fork reorients the template strand used for DNA synthesis without

employing genetic recombination (Friedberg 2005). Following the detection of a lesion at the replication fork, the replication machinery migrates backwards to allow the original template strands to reanneal so that the strand with the lesion is temporarily synthesized from the other nascent strand. This "copy-choice" replication results in the formation of a variant Holliday junction, called a"chicken foot" structure, which can be restored by reverse regression (Postow, Ullsperger et al. 2001; Grompone, Erlich et al. 2004). Through this process, the original lesion remains in the DNA while an alternative form of replication is performed in an error-free manner.

A third mode of DNA damage tolerance, called DNA translesion synthesis (TLS) employs the replication of DNA directly *across* sites of template-strand base damage. This mechanism involves one or more specialized DNA polymerases that are capable synthesizing daughter DNA from a damaged template. Specialized DNA polymerases are characterized by reduced fidelity on undamaged DNA, weak processivity, and the inability to proofread (Friedberg 2005). Given the nature of these polymerases, DNA translesion synthesis may function in an error-prone fashion, by which new mutations may be introduced into the newly synthesized daughter DNA. However, there is a growing body of evidence to suggest that some (possibly all) specialized polymerases may execute DNA translesion synthesis accurately. Whether or not these polymerases

function mutagenically *in vivo*, it appears that the basis of their evolutionary selection is to promote cell survival by overcoming arrested DNA replication.

The modes of DNA damage tolerance described here are representative of the existing body of research regarding this biologically important phenomenon. Given that proteins required for each of these processes are conserved from prokaryotic to eukaryotic organisms, each mechanism may serve a unique and important function in the tolerance of genotoxic stress. While the benefits of error-free post-replicative repair are generally palpable, the advantages of error-prone damage tolerance (particularly in multi-celled organisms) are not as easily reconciled. At least two rationalizations for why organisms may have evolved an error-prone damage tolerance mechanism(s) should be considered. The first suggests that the ability of a cell to increase its mutation rate in response to DNA damage confers an evolutionary advantage, a hypothesis that is particularly well rationalized in unicellular organisms such as bacteria (Friedberg et al. 2005). A second perspective acknowledges that an error-prone mechanism for damage tolerance may provide a "last line of defense" against the lethal effects of DNA damage, whereby the mutagenic effects are merely a secondary consequence which may bear little evolutionary significance (Lawrence and Maher 2001; Friedberg et al. 2005). Outside of these reasonings, a possible explanation for the utilization of error-prone translesion polymerases in vertebrates is revealed through the involvement of specialized DNA polymerases in the process of

somatic hypermutation, the mutagenic diversification of immunoglobulin genes (Friedberg et al. 2005). However, error-prone damage tolerance likely ensues outside of early B-cell development. Although DNA damage tolerance as a response to genotoxic stress presents many complexities, it is likely that the fine-tuning of these pathways is critical for cell survival and the avoidance of cancer, and may serve as a driving force for evolution and adaptation (Hochegger and Takeda 2006).

The notion of how these pathways are regulated or utilized in relationship to one another is under ongoing investigation. In yeast, the key players for executing error-free and error- prone damage tolerance fall under the same epistasis group of the Rad6 and Rad18 genes. DNA translesion synthesis and error-free damage tolerance by recombination and/or copy choice replication are dependent upon the activities of Rad6 and Rad18 for their functions (Xiao, Chow et al. 2000; Friedberg, Lehmann et al. 2005). A study in *E. coli* has suggested that error free processes involving recombination predominate over mutagenic translesion replication (Berdichevsky, Izhar et al. 2002) . In addition, studies in vertebrate DT40 cells has revealed through genetic analysis that recombinational repair and DNA translesion synthesis may work synergistically, whereas a defect in one pathway results in the elevation of the other function (Hochegger, Sonoda et al. 2004). The molecular mechanisms for how these pathways are uniquely

regulated by the downstream functions of Rad6 and Rad18 proteins are newly emergent in the literature (Friedberg, Lehmann et al. 2005).

## *DNA Translesion Synthesis (TLS)*

The recognition of DNA translesion synthesis as a biologically important process is a somewhat recent event. The general concept of damage-induced mutagenesis stemmed from work done in *E. coli* by Bryn Bridges and Evelyn Witkin in 1967, which gave rise to evidence for a "mutation-prone" mode of survival in response to UV radiation (Witkin 1967). This hypothesis was further elaborated upon with the identification of genes required for UV-damage induced mutagenesis, known as UmuC and UmuD (UV non-mutable), which were isolated from a screen in *E. coli* aimed at identifying mutations associated with reduced UV- induced mutability (Kato and Shinoura 1977). A few years later, a similar screen in the yeast *S. cerevisiae* revealed the presence of "reversionless" mutants, characterized by their UV-reversionless phenotype and sensitivity to UV-radiation (Lemontt 1971). Among these mutants were alleles of the genes designated *REV1* and *REV3* (Lemontt 1971). The newly discovered eukaryotic Rev1 showed considerable homology to the prokaryotic UmuC protein, which resulted in its placement in the UmuC superfamily.

For years, it was predominantly speculated that the protein products found to have a role in damage-induced mutagenesis executed their function by

somehow reducing the fidelity of the replicative polymerases (Yang 2003; Friedberg 2005). However, this hypothesis was disproved by the discovery that replicative DNA polymerases have limited (if any) participation in replication past lesions, and by the revelation that proteins like Rev1 are in fact DNA polymerases *themselves* (Nelson, Lawrence et al. 1996; Friedberg, Wagner et al. 2002; Friedberg 2005). Searches prompted by genome sequencing in recent years have brought forth the discovery of multiple conserved orthologs and paralogs of these "specialized" DNA polymerases, many of which are bona fide DNA polymerases with distinct properties *in vitro* (Friedberg, Wagner et al. 2002). The implications of these findings strongly supported the existence of the process that is now recognized as DNA translesion synthesis.

## *The Y-family of DNA polymerases*

Over 100 homologues of UmuC have been identified in bacteria, archaea and eukarya, forming a family of DNA polymerases called the Y-family (Yang 2003). Prototypic members of this family include DinB and UmuC(PolIV and PolV in *E. coli*), as well as eukaryotic Rev1 and Polη (Rad30). Rev1 and Rad30 have homologues in all eukaryotes examined to date (Yang 2005). Polι and Polκ are also Y-family members, found only in select eukaryotic species including Drosophila and mammals (Figure 1) (Yang 2003).

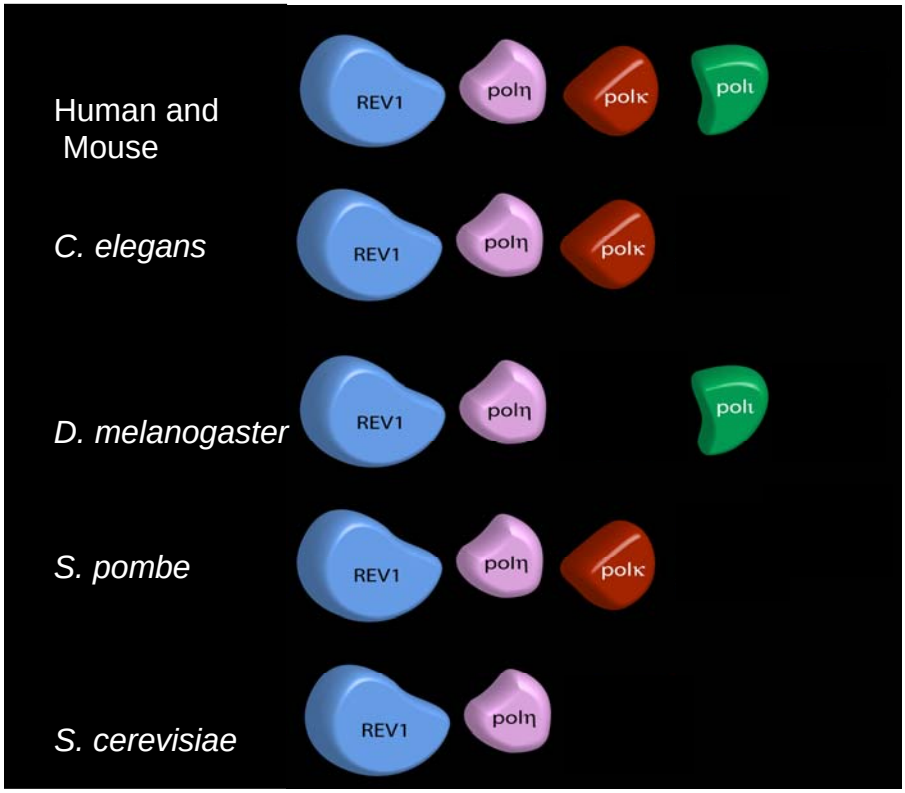


**Figure 1-1: The conservation of the Y-family of DNA polymerases in eukaryotes.**

Of the various processes in which these polymerases participate, including somatic hypermutation or the repair of double-strand breaks by homologous recombination, their functions in DNA translesion synthesis are likely widespread, but not necessarily mechanistically universal (Lawrence 2004).

The Y-family polymerases range in size from 350-800 amino acid residues. They share five conserved sequence motifs located at the N-terminus, which contain the catalytic domains for each polymerase. Although the Y-family DNA polymerases bear little sequence similarity to replicative DNA polymerases of the A, B, C, and X families, their crystal structures reveal a catalytic core consisting of a palm, finger, and thumb domain arranged in a classic right hand-like configuration (Yang 2003; Yang 2005). A unique characteristic of the Y-family polymerases is their spacious active sites which favorably accommodate bulky DNA adducts. Such an open conformation exposes the substrate to surrounding solvent, weakening contacts with the replicating base pair at the active site and thus promoting reduced fidelity and specificity (Yang 2003). An additional feature of Y-family polymerases is a "little finger" domain, a motif encoded by the unique C-terminal region of each protein. This additional DNA binding domain (also called a wrist or polymerase-associated domain) specific to Y-family enzymes determines the catalytic efficiency and mutation spectra for each of the polymerases (Bebenek and Kunkel 2004; Yang 2005).

Y-family polymerases are distinct from high-fidelity replicative polymerases by their inability to proofread. These enzymes lack a 3'-5' exonuclease activity, which prevents them from detecting and reversing incorrectly incorporated nucleotides. Y-family DNA polymerases have error rates of $10^{-2}$-$10^{-4}$ on undamaged DNA, whereas replicative polymerases have error rates

of $10^{-5}$-$10^{-6}$ (Yang 2003). The absence of a proofreading function contributes to the reduced accuracy of DNA synthesis and the potentially mutagenic nature of these specialized polymerases. Furthermore, specialized Y-family DNA polymerases synthesize DNA with weak processivity, replicating DNA largely by a distributive mode of action. For example, Rev1 (a deoxycytidyl transferase) only has the capacity to incorporate one or two dCMP nucleotides opposite a template base before dissociating from the DNA template (Lawrence 2004). This property may be attributed to the underdevelopment of the Y-family finger and thumb domains which weakly bind DNA, as they are notably smaller in specialized polymerases (Yang 2005; Schlacher and Goodman 2007).

Each of these polymerases has been shown *in vitro* to have the capability of inserting a nucleotide base opposite DNA lesions. Additionally, several of these proteins are capable of extending several bases past the template lesion. These two functions of "insertion" and "extension" are both operational definitions of translesion synthesis, and evidence suggests that each polymerase may favor one role over the other (Friedberg et al. 2005). Given the vast array of lesions that can form on DNA, the field has faced a significant challenge in recent years in efforts to identify which type(s) of damage each polymerase is specific for, and in what spacial and temporal context they function in relationship to one another. Data accumulated thus far indicates that, depending on the DNA polymerase, the type of lesion, and the local DNA sequence, DNA translesion

synthesis may either *avoid* or *contribute* to mutagenesis (Bebenek and Kunkel 2004).

## Rev1 Protein

Rev1 is an evolutionarily conserved protein, ubiquitous among eukaryotic species but not represented in prokaryotes (Figure 2). Rev1 is required for UV-induced mutagenesis in yeast together with Polζ, a B-family polymerase comprised of Rev3 and Rev7 subunits. Rev1 protein possesses deoxycytidyl transferase activity, by which it preferentially inserts dCMP opposite a template base or abasic site, as well as other structurally diverse template lesions *in vitro* (Jansen, Tsaalbi-Shtylik et al. 2005). In addition, the presence of an N-terminal BRCT domain (BRCA1 C-terminal-like) distinguishes Rev1 from the other members of the Y-family of DNA polymerases (Figure 2). This domain is often found harbored in proteins important for cell-cycle checkpoint functions in response to DNA damage (Jansen, Tsaalbi-Shtylik et al. 2005). In yeast, a single point mutation in the BRCT domain of Rev1 abolishes its ability to bypass abasic sites or [6-4] UV-photoproducts. Indeed, although in yeast Rev1 is required for the bypass of [6-4] UV- photoproducts *in vivo*, the signature dCMP transferase activity occurs only rarely opposite of a [6-4] photoproduct, suggesting that Rev1 may serve a more indirect role in the bypass of this lesion (Nelson, Gibbs et al. 2000; Lawrence and Maher 2001; Gibbs, McDonald et al. 2005). Furthermore,

notwithstanding the capacity for Rev1 to bypass abasic sites through its dCMP transferase activity, there is no evidence that the enzymatic activity of Rev1 is required for the replicative bypass of this lesion, further implicating an alternative role for Rev1. Studies aimed to identify non-catalytic functions for Rev1 have been enlightened with discoveries of new Rev1 protein interaction partners. Fruits of this effort have revealed that the C-terminus of mouse and human Rev1 maintains an interaction with several other specialized DNA polymerases, including Polη, Polι, Polκ, and the Rev7 subunit of the B-family polymerase Polζ (Guo, Fischhaber et al. 2003; Ohashi, Murakumo et al. 2004; Tissier, Kannouche et al. 2004). The function of these interactions is not known, although it has been demonstrated in yeast that Rev1 enhances the proficiency of the Rev3 subunit of Polζ for the extension of primer termini opposite DNA lesions (Acharya, Johnson et al. 2006). The observation that the C-terminal domain of Rev1 is required for resistance to DNA damaging agents in vertebrates and yeast further emphasizes the necessity of this domain for Rev1 function, although the congruency of the mechanism by which it functions among eukaryotes is still not fully understood. Moreover, the additional observations that PCNA also interacts with the BRCT domain of Rev1, and that Rev1 undergoes monoubiquitination in response to damage, have provided mechanistic details for how Rev1 may function as a key player in the orchestration of DNA translesion synthesis.

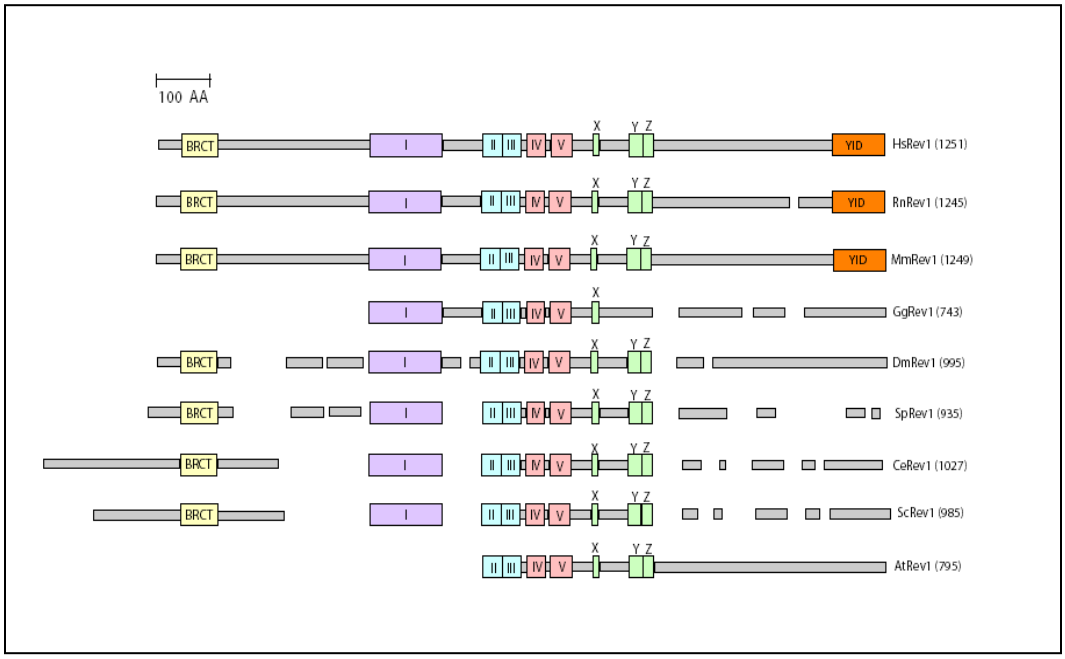


**Figure 1-2: Rev1 is represented as a member of the Y-family of DNA polymerases in all eukaryotes examined (not present in *E. coli*).**
The YID (otherwise referred to as the C-terminal domain) is well conserved among higher eukaryotes. I, N-terminal nucleotidyl transferase domain; II, III, helix-hairpin-helix domains; IV, V, Zn cluster and Zn finger domains. Hs, *Homo sapiens*; Rn, *Rattus norvegicus*; Mm, *Mus musculus*; Gg, *Gallus gallus*; Dm, *Drosophila melanogaster*; Sp, *Schizosaccharomyces pombe*; Ce, *Caenorhabditis elegans*; Sc, *Saccharomyces cerevisiae*; At, *Arabidopsis thaliana.*

## Polκ Protein

DNA polymerase kappa (Polκ/Dinb) is the only member of the Y-family of DNA polymerases conserved in all kingdoms of life, from bacteria to humans (Gerlach, Aravind et al. 1999). Despite its broad conservation, Polκ is not found in all organisms, notably the budding yeast *S. cerevisiae*, and the fruitfly *D.*

*melanogaster* (Figure 1). In living systems, Polκ is ubiquitously expressed, although it is most highly expressed in the testis and adrenal cortex (Gerlach, Aravind et al. 1999; Ogi, Kato et al. 1999; Velasco-Miguel, Richardson et al. 2003). Cell-specific expression of Polκ mRNA in the testis is confined to meiotic spermatocytes and postmeiotic spermatids, suggesting a specific role for Polκ in spermatogenesis (Velasco-Miguel, Richardson et al. 2003). Other observed cell-specific expression profiles include the epithelial cells of small bronchi and large bronchioles in the adult mouse lung, as well as epithelial cells of the stomach and skin (Velasco-Miguel, Richardson et al. 2003).

*Polκ*$^{-/-}$ mouse ES cells exhibit moderate UV-sensitivity (Ogi, Shinkai et al. 2002). Polκ has been shown to bypass a variety of lesions and extend mismatched primers *in vitro*, although this enzyme does not support TLS across the most frequent types of base damage found in DNA exposed to UV-radiation. Therefore, it is not clear what function Polκ performs in response to UV-radiation (Friedberg et al. 2005).

On the other hand, specific insight into Polκ function can be gained from studies investigating the relationship between Polκ and benzo(a)pyrene, a potent carcinogen found in coal tar and cigarette smoke. The extreme sensitivity of *Polκ*$^{-/-}$ mouse ES cells to killing and increased mutagenicity is further reinforced with the observation that Polκ is capable of bypassing different stereoisomers of

dG-$N^2$-BPDE (the major products generated by benzo(a)pyrene) efficiently and accurately (Avkin, Goldsmith et al. 2004). It has also been observed in human cells that BPDE treatment induces the accumulation of GFP-tagged Polκ into nuclear foci, and that recovery from BPDE-induced S-phase checkpoint requires Polκ (Bi, Slater et al. 2005). However, although Polκ exhibits specificity for the bypass of BPDE adducts, it is not likely that this enzyme evolved solely for this purpose; indeed, the prokaryotic Polκ homolog *Dinb* can also bypass BPDE, yet bacteria lack the enzyme required to activate benzo[a]pyrene into BPDE (Ohmori, Ohashi et al. 2004). Additionally, benzo(a)pyrene is largely man-made, arguing against the likelihood that Polκ evolved solely for the bypass dG-$N^2$-BPDE. Given the structural resemblance between planar polycyclic aromatic BPDE and naturally occurring metabolites such as cholesterol derivatives, together with the expression profile of Polκ in steroid-rich tissues such as the adrenal cortex, it is conceivable that Polκ is required for TLS of base damage formed by endogenous reactive species. In support of this notion, Polκ (lacking its C-terminal region) is able to bypass estrogen-derived DNA adducts such as dG-$N^2$-3 MeE with greater magnitude and accuracy than on undamaged DNA, and with efficiency superior to the Y-family DNA Polη (Suzuki, Yasui et al. 2004). In addition, *Polκ*$^{-/-}$ DT40 cells are sensitive to killing by an oestrogen species commonly known as

tamoxifen, and exhibit increased frequencies of chromosomal abnormalities in the presence of oestrogen compounds (Mizutani, Okada et al. 2004).

In conjunction with a growing body of evidence that Polκ may have an important role in error-free bypass of endogenous damage, the literature has reported an increase in the germ line mutation frequency of *Polκ*$^{-/-}$ male mice (Burr, Velasco-Miguel et al. 2006). Along these lines, although no other group has reported abnormalties found in *Polκ*$^{-/-}$ mice, our laboratory has observed a mutator phenotype in multiple generations borne from *Polκ*$^{-/-}$ ancestors (Friedberg lab unpublished results). Together, these observations have begun to further our understanding of how Polκ may function biologically in DNA translesion synthesis.

## Polη Protein

The *Polη* gene (also called Rad30) was originally identified in the yeast *S. cerevisiae* by its sequence homology to the UmuC homologs Dinb and Rev1p. Like *rev1, rev3,* and *rev7*, a *rad30* deletion strain manifests moderate sensitivity to UV-radiation (McDonald, Levine et al. 1997). Epistasis analysis designates Rad30 as a member of the Rad6 epistasis group, although double mutants of *rad30* combined with other Rad6 epistasis group genes *rad5*, *rev1*, *rev3*, or *rev7* exhibit higher sensitivity than either of the single mutants, suggesting that Rad30 functions in a distinct pathway(s) involving these genes (McDonald, Levine et al.

1997). Furthermore, in contrast to the *rev* mutants, the *rad30* deletion mutant has almost no effect on UV-mutability, indicating that Rad30 likely functions in an error-free manner (McDonald, Levine et al. 1997). The unveiling of Rad30 as a DNA polymerase demonstrated that this enzyme is capable of bypassing cyclobutane pyrimidine dimers (CPDs) by efficiently incorporating adenine opposite dimerized thymines with an efficiency similar to that observed when copying undamaged DNA (Lehmann, Niimi et al. 2007). This ability to replicate past the most abundant UV-photoproduct is thought to be the principle function of Polη *in vivo,* although *in vitro* it can bypass other damage-induced lesions as well. In cells over-expressing human Polη, this protein is found constitutively in replication factories within the nucleus during S-phase. Polη co-localizes with both PCNA and Rev1, forming a stable interaction with each of these proteins (Kannouche, Broughton et al. 2001; Tissier, Kannouche et al. 2004). Models for the recruitment of Polη to stalled replication forks implicate both PCNA and Rev1 as key proteins working to coordinate TLS polymerases at sites of stalled replication (Friedberg, Lehmann et al. 2005).

The biological significance of Polη/Rad30 in the bypass of UV-induced lesions is evident from the manifestation of disease in higher eukaryotes such as mice and humans lacking normal Polη protein. Mutations in the *Polη* gene result in a variant form of the human genetic disorder xeroderma pigmentosum (XPV), a

disease characterized by extreme sunlight sensitivity and an early predisposition to skin cancer (McDonald, Rapic-Otrin et al. 1999). This disease arises specifically from the inability of Polη to bypass thymine-thymine dimers during DNA replication in an *error-free* fashion. It is suggested that in the absence of functional Polη, another specialized polymerase bypasses CPDs with reduced efficiency and accuracy, resulting in an increased rate of UV-induced mutagenesis and carcinogenesis in XP-V cells (Lehmann, Niimi et al. 2007). This specific requirement for a specialized DNA polymerase has provided the first biologically significant evidence that a TLS polymerase may possess a "cognate" substrate for which it evolved to bypass accurately.

## Polι Protein

Polι is a Y-family polymerase, often called Rad30b because of its homology to the Rad30 branch of *S. cerevisiae* (Friedberg et al. 2005). Polι is a paralog of Polη, with homologs identified in mice, and fruit flies. Unlike other Y-family polymerases, Polι does not have structural homologs in either bacteria, yeast, or nematodes (Figure 2). Polι is highly error-prone on undamaged templates, incorporating dGMP opposite thymine more frequently than dAMP in a manner that violates Watson-Crick base pairing (Zhang, Yuan et al. 2000). In addition, Polι is capable of performing insertions opposite highly distorting or non-instructional lesions *in vitro*, such as [6-4]-photoproducts and abasic sites,

although Polι has not been shown to possess any capacity for extension beyond these insertion events (Johnson, Washington et al. 2000). A role for Polι in UV-radiation damage bypass has been inferred from the observation that Polι co-localizes with the DNA replication machinery in response to UV-radiation in a Polη-dependent manner (Kannouche, Fernandez de Henestrosa et al. 2002). Much like other TLS polymerases, Polι physically interacts with Rev1 in mice and humans, as well the replication factor PCNA (Ohashi, Murakumo et al. 2004; Tissier, Kannouche et al. 2004; Haracska, Acharya et al. 2005; Guo, Sonoda et al. 2006). *In vitro*, human Polι has been shown to function in DNA damage bypass together with Polζ, whereby Polι performs the insertion event opposite DNA lesions while Polζ acts at the subsequent step of extending from them. These observations suggest a potential functional relationship between these polymerases *in vivo* (Johnson, Washington et al. 2000). The biological significance of these interactions and the mechanism by which they function *in vivo* requires further investigation.

## B-family Polymerase Polζ

Polζ is a B-family DNA polymerase, although it maintains properties similar to the Y-family of polymerases, including poor processivity, low fidelity, and the inability to proofread in the 3'-5' direction (Gan, Wittschieben et al. 2008). *In vitro*, Polζ primarily functions as an extension DNA polymerase. The

enhanced ability of Polζ to extend mismatched primers ranges from a 2-fold to >1000-fold greater efficiency compared to the replicative polymerase Polα(Lawrence and Maher 2001). The Polζ protein is comprised of two essential subunits, Rev3 (the catalytic domain) and Rev7 (the regulatory domain which enhances the catalytic activity) (Gan, Wittschieben et al. 2008). Together these subunits function with Rev1 protein in damage-induced mutagenesis among eukaryotes. Polζ's function in TLS requires the presence of Rev1, although the catalytic activity of Rev1 is entirely dispensable for Polζ-dependent DNA translesion synthesis (Acharya, Johnson et al. 2006). In yeast, Polζ together with Rev1 is required for the generation of ~98% of UV-induced base-pair substitutions, as well as the majority of spontaneous mutations (Lawrence 2004). Recent evidence has shown that in yeast, mice, and humans, the Rev7 subunit of Polζ interacts with Rev1, suggesting that this physical interaction may be evolutionarily conserved for an important function (Guo, Fischhaber et al. 2003; Acharya, Johnson et al. 2006). In addition, recent evidence has revealed that the C-terminus of yeast Rev1 physically interacts with polymerase domain of Rev3, which enhances the efficiency of Polζ to extend mismatched primer-templates and abasic sites (Acharya, Johnson et al. 2006). Furthermore, the deletion of the Rev1 C-terminal residues which interacts with Rev3 confers the same degree of defective UV-induced mutagenesis and sensitivity to killing as a *rev1Δ* mutation

(Acharya, Johnson et al. 2006). Although these experiments require further validation, they hint at significant insights into the relationship between Polζ and Rev1 for mutagenesis.

Aside from the requirement for Rev1, a generally accepted working model for Polζ function involves the activity of an insertion polymerase, coupled with Polζ–catalyzed extension of the mismatched or damaged primer duplex. Evidence in yeast suggests that the extension activity of Polζ is required for the bypass of most [6-4] UV-photoproducts. The bypass of this highly distorting lesion is only 19% efficient in wild- type yeast (compared to 82% bypass of CPDs). (Nelson, Gibbs et al. 2000). Studies have suggested that either Rad30 or the replicative polymerase Polδ has a role in the highly inefficient (and likely mutagenic) insertion event which precedes extension by Polζ (Gibbs, McDonald et al. 2005).

Other unrelated roles for Polζ have been reported for functions independent of DNA translesion synthesis, such as homologous recombination-mediated double-strand break repair, repair of interstrand DNA cross-links, and somatic hypermutation of immunoglobulin genes (Friedberg et al. 2005; Gan, Wittschieben et al. 2008). In addition, unlike in budding yeast, a deletion of the Rev3 gene in mice results in embryonic death between days 8.5 and 14.5. The reason for embryonic lethality is not known, although it may result from a special

requirement for damage bypass and tolerance of endogenous damage in the mouse embryo. Alternatively, an overwhelming number of unrepaired DSBs and sister chromatid exchanges may ensue to an extent incompatible with life (Friedberg et al. 2005). Nevertheless, these results suggest that there may very well be additional functions for Polζ which have yet to be discovered.

## *Model Systems for Studying TLS*

In 1945, Rosenbleuth and Wiener defined a *material model* as the representation of a complex system by a system that is assumed to be simpler and to have some properties similar to those selected for study in the complex real-world system (Massoud, Hademenos et al. 1998). Model organisms have historically served as a fruitful source for gaining insights into scientific concepts. As the plant biologist Paul Williams once said, "Major advances in biology often come when diverse disciplines focus on model organisms."(Williams 1992).

Recent years have witnessed a significant advance in the genetic tools that allow scientists to study hereditary defects and how they manifest biologically. In the 1970s, the prokaryotic bacteria *Escherichia coli* was made popular as a model organism by its ability to accept and process recombinant DNA. Through widespread investigation of the DNA damage response of *E. coli*, the characterization of the *SOS response* provided an important platform for the discovery of specialized polymerases involved in DNA translesion synthesis

(Friedberg et al. 2005). With the identification of specialized polymerase homologs in yeast, our knowledge of specialized polymerases in eukaryotes has been significantly expanded. In addition, the advancement of genetic technologies and the sequencing of multiple genomes has allowed opportunities for experimentation in more complex eukaryotic organisms.

Although very limited attention has been given to DNA translesion synthesis in classic genetic models such as *Drosophila* and *C. elegans*, they offer potentially useful tools for further defining TLS in complex organisms. Studies in mouse models have frequented laboratory experiments at the turn of the twenty-first century in the area of TLS, particularly following the discovery of the human Polη-deficient disease xeroderma pigementosum, variant (XPV). While studies from human cells only provide limited information, mouse models have recapitulated genetic alterations to reflect biological significance in mammals. Model organisms continue to provide powerful and more rapidly available information in the field of TLS that will continue to influence how the process of DNA translesion synthesis is ultimately related in humans.

## *A Model for the Mechanism of DNA Translesion Synthesis in Eukaryotes*

> When we use the term *model* in biomedical research, we do not necessarily mean an actual apparatus whose appearance or properties are similar to the system we are studying. More often, we mean that we think the properties of the entire system derive from the properties of certain defined constituent parts, and that from a knowledge of the functioning of

> these components and of their interactions with each other, we could give an explanation of the functioning of the whole that would be more readily understood than if we tried to describe the whole at once (Pringle 1960).

## The Role of Rad6/Rad18 and PCNA

As previously mentioned, in yeast, all Y-family polymerase homologs are members of the Rad6 epistasis group. Together, these proteins act as gatekeepers of the Y-family polymerase functions in DNA translesion synthesis, regulating the replication of damaged DNA (Friedberg, Lehmann et al. 2005; Parker, Bielen et al. 2007). Rad6 is required for UV-induced mutagenesis in yeast, and exhibits exquisite sensitivity to a variety of DNA damaging agents (Lawrence and Christensen 1976). Rad6 protein functions as an E2 ubiquitin-conjugating enzyme, which interacts with the E3 ligase Rad18. The concerted actions of Rad6 and Rad18 are required for the monoubiquitination of Lys164 of PCNA in response to stalled replication caused by DNA damage (Friedberg, Lehmann et al. 2005).

PCNA, the trimeric processivity clamp required for the normal replication of DNA, also has an important role in DNA translesion synthesis. PCNA functions as a processivity factor for replicative polymerases, but it also serves as a binding platform for several other proteins coordinated at the replication fork (Parker, Bielen et al. 2007). More specifically, PCNA monoubiquitinated by Rad6/Rad18 preferentially interacts with Y-family DNA polymerases including Rev1, Polκ, Polη, and Polι, and in some cases is capable of stimulating their

polymerase activity for more efficient replicative bypass *in vitro* (Haracska, Johnson et al. 2001; Haracska, Johnson et al. 2001; Haracska, Acharya et al. 2005; Parker, Bielen et al. 2007). All Y-family members possess ubiquitin-binding domains (called UBMs or UBZs) located near the C-terminal region of each polymerase. The discovery of these ubiquitin-binding regions, evolutionarily conserved from yeast to humans, has uncovered the molecular basis for the enhanced binding of Y-family polymerases to ubiquitylated PCNA (Parker, Bielen et al. 2007). Futhermore, each Y-family polymerase binds PCNA with a conserved motif, called a PIP for PCNA interacting peptide (Warbrick 1998). This demonstrated relationship between PCNA and the Y-family of DNA polymerases has provided insights into how PCNA may assist in the transition or "switch" from replicative polymerases to the mode of DNA translesion synthesis.

## Polymerase Switching and the "Two-Polymerase" Model

A current model for the polymerase switching that may transpire during TLS involves three DNA polymerase switch events. The first switch involves the displacement of the replicative polymerases Polδ and Polε, followed by replacement with a specialized polymerase(s) presumably through the recruiting action of monoubiquitinated PCNA (Figure 3C). How a given polymerase is chosen for a specific lesion is not known, although some eukaryotic specialized polymerases are specifically suited for the incorporation of nucleotides directly

opposite lesions (Friedberg, Lehmann et al. 2005). These polymerases, such as Polκ or Polι, access the lesion in order to perform the insertion event. The second switch potentially involves the recruitment of a second specialized polymerase which is better suited to extend distorted primer termini *after* an insertion event has taken place (in the instance that the insertion polymerase cannot extend) (Friedberg, Lehmann et al. 2005). This step is critical for efficient lesion bypass, as extension beyond the insertion event is presumably required for productive re-engagement of the replication machinery (Friedberg, Lehmann et al. 2005). Finally, once the primer is sufficiently extended, a third polymerase switch occurs back to replication machinery to resume the synthesis of DNA.

Details of this model demand further attention, such as the issue of how the exchange between the replication machinery and TLS polymerases is executed, how the access of other specialized polymerases is restricted, and how the exchange between insertion and extension polymerases may transpire. One significant clue that may provide insight into the latter question is a unique function of mammalian Rev1: in mice and humans, the C-terminus of Rev1 interacts with Polη, Polκ, Polι, and Rev7 (of Polζ). In addition, these polymerases appear to compete for the C-terminus of Rev1 by mass action. Specifically, competition experiments have shown that the binding of Rev1 to a fixed amount of purified Polκ can be reduced with increasing amounts of Rev7 protein (Guo, Fischhaber et al. 2003). These results imply that Rev1 may

specifically facilitate the switch between the insertion and extension polymerase (Figure 3C) (Friedberg, Lehmann et al. 2005). However, given that lower eukaryotic organisms possess different combinations of Y-family polymerases, it is important to consider the variability that may distinctively define this mechanism in any given organism.

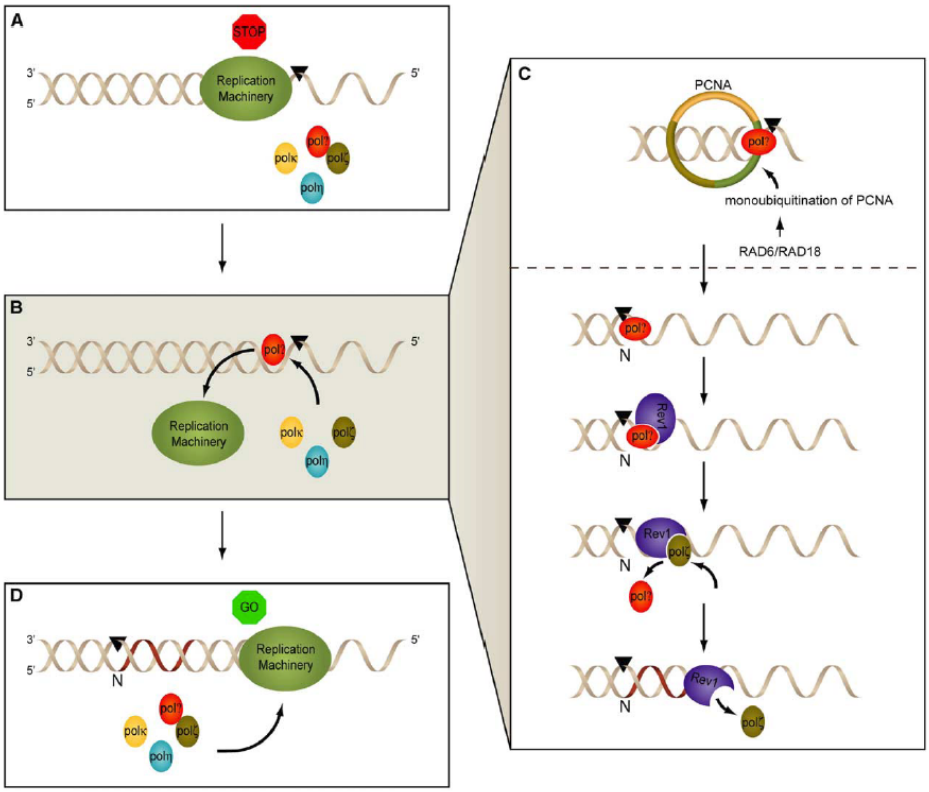


**Figure 1-3: A model of DNA translesion synthesis for mammalian cells, in the cellular response to DNA damage.**
(A) High-fidelity DNA replication (replication machinery) is shown arrested at a generic form of base damage (inverted triangle). Some of the multiple specialized DNA polymerases (Pol $\eta$ , Pol $\kappa$ , Pol $\zeta$ , and one designated generically as "pol?") are depicted in the general proximity of the arrested replication fork.

However, their definitive intranuclear localization in relation to normal and arrested replication is unknown.
(B) A switch between pol δ or polε in the arrested replicative machinery and a selected specialized DNA polymerase (in this case, pol?) is shown.
(C) Top: switching in pol? to bypass the lesion may require monoubiquitination of the trimeric PCNA clamp, supported by the RAD6/RAD18 ubiquitin ligase. (C) Bottom: after a correct residue (N) is incorporated by pol?, Rev1 protein may be involved in a switch between the insertion polymerase (pol?) and an extension polymerase, such as pol ζ . The patch of DNA synthesis generated in this two-polymerase model is shown in red. For clarity, the continued involvement of RNA is not shown in the lower part.
(D) When TLS past the lesion has extended to a suitable position downstream of the lesion, a third polymerase switch transpires during which the replicative machinery is again productively engaged with the primer terminus and high-fidelity DNA replication continues.
Figure taken from (Friedberg, Lehmann et al. 2005) with permission.
(http://www.sciencedirect.com/science/journal/10972765).

## *Significance and Aims of Thesis*

Attempts to assign biological function based exclusively on *in vitro* biochemical characterization can be fraught with misinterpretation (Friedberg EMM 2001). This is a particularly valid cautionary in the study of DNA translesion synthesis, given that *in vitro*, specialized DNA polymerases are capable of bypassing (or influencing the bypass) of lesions in both an error-prone and/or error-free fashion, depending upon the lesion or added components. It is therefore imperative to integrate biochemical studies with cellular and/or genetic investigations that recapitulate the native-cell environment when considering the

biologically significant roles of specialized DNA polymerases (Friedberg EMM 2001).

My work focuses on the investigation of specific Y-family polymerase functions *in vivo*. More specifically, the main goals of this dissertation are to i) characterize protein-interactions involving Rev1 protein in order to distinguish mechanistic properties important for its role in UV-induced mutagenesis and ii) analyze the spontaneous mutability of *Polκ*$^{-/-}$ somatic mice tissues using a highly refined *in vivo* mutation detection system. As reported in chapter II, I compared Rev1/Y-family polymerase interactions between vertebrate and invertebrate species. In chapter III, I identified new yeast Rev1 protein-interaction partners and examined their epistatic relationship to Rev1 with respect to UV-radiation. Chapter IV describes studies that characterize spontaneous mutation frequencies and mutation spectra of *Polκ*$^{-/-}$ mouse somatic tissues with respect to age.

# Chapter II: Comparative Analysis of *in vivo* Interactions Between Rev1 Protein and Other Y-Family DNA Polymerases in Animals and Yeasts.

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Eukaryotes are endowed with multiple specialized DNA polymerases, some (if not all) of which are believed to play important roles in the tolerance of base damage during DNA replication. Among these DNA polymerases, Rev1 protein (a deoxycytidyl transferase) from vertebrates interacts with several other specialized polymerases via a highly conserved C-terminal region. The present studies assessed whether these interactions are retained in more experimentally tractable model systems, including yeasts, flies, and the nematode *C. elegans*. We observed a physical interaction between Rev1 protein and other Y-family polymerases in the fruit fly *Drosophila melanogaster*. However, despite the fact that the C-terminal region of Drosophila and yeast Rev1 are conserved from vertebrates to a similar extent, such interactions were not observed in *S. cerevisiae* or *S. pombe*. With respect to regions in specialized DNA polymerases that are required for interaction with Rev1, we find predicted disorder to be an underlying structural commonality. The results of this study suggest that special consideration should be exercised when making mechanistic extrapolations regarding translesion DNA synthesis from one eukaryotic system to another.

## *Introduction*

The rescue of arrested DNA replication at sites of template base damage is critical for cell survival. Not surprisingly, prokaryotic and eukaryotic cells have evolved multiple strategies for mitigating the lethal effects of arrested DNA

replication without prior removal of the offending DNA damage; so-called DNA damage tolerance (Friedberg 2005). The replicative bypass of base damage by DNA translesion synthesis (TLS) represents a specific mode of damage tolerance that utilizes specialized low-fidelity DNA polymerases to overcome arrested DNA replication, often at the expense of introducing errors and hence generating mutations (Friedberg 2005). To date ten such specialized DNA polymerases have been identified in vertebrates. A newly-discovered subset of these proteins (Rev1, Polη, Polι, and Polκ) is designated the Y-family of DNA polymerases (Friedberg, Walker et al. 1995; Friedberg et al. 2005; Friedberg, Lehmann et al. 2005).

Among the Y-family of DNA polymerases Rev1 protein is highly conserved in eukaryotes, but no archaeal or bacterial Rev1 orthologs have been detected. Structural orthologs of Polη and Polι are also apparently absent in prokaryotes. In contrast, a readily identifiable ortholog of Polκ (DinB protein in *E. coli*) is present in bacteria. Rev1 is unique among the Y-family in that its DNA polymerase activity is restricted to the incorporation of one or two molecules of dCMP regardless of the nature of the template nucleotide. It is thus often referred to as a dCMP transferase (Lawrence 2004). Remarkably, while the catalytic domain of Rev1 protein is required for the replicative bypass of sites of base loss (AP sites), inactivation of this activity does not abrogate a requirement for Rev1 for ultraviolet (UV) radiation-induced mutagenesis in yeast or mammalian cells

(Nelson, Gibbs et al. 2000; Otsuka, Loakes et al. 2002; Ross, Simpson et al. 2005).

Rev1 protein also possesses a conserved N-terminal BRCT domain that is required for TLS in yeast and mammalian cells exposed to UV radiation (Nelson, Gibbs et al. 2000; Guo, Sonoda et al. 2006) and presumably other types of base damage. Indeed, a single amino acid substitution in the BRCT domain of otherwise catalytically active yeast Rev1 abolishes the bypass of [6-4] photoproducts, suggesting a non-catalytic role(s) for Rev1 protein during UV radiation-induced mutagenesis (Nelson, Gibbs et al. 2000). Additional support for the notion that Rev1 has a function(s) in TLS that is independent of its dCMP transferase activity is implicit in the observation that the protein interacts with the Y-family polymerases Polκ, Polη and Polι, and with Rev7 protein [a subunit of a heterodimeric specialized DNA polymerase called Polζ] through a C-terminal 100 amino acid region that is highly conserved among vertebrates (Guo, Fischhaber et al. 2003; Ohashi, Murakumo et al. 2004; Tissier, Kannouche et al. 2004). The functional significance of these interactions is not understood. However, the additional observations that PCNA also interacts with these DNA polymerases and with Rev1 protein (Haracska, Johnson et al. 2001; Haracska, Kondratick et al. 2001; Haracska, Unk et al. 2002; Guo, Sonoda et al. 2006), and that PCNA and some Y-family members (including Rev1 protein) undergo monoubiquitination, has prompted the hypothesis that Rev1 plays a key role in the process of TLS

(Bienko, Green et al. 2005; Friedberg, Lehmann et al. 2005; Guo, Tang et al. 2006).

Several non-vertebrate eukaryotic organisms, such as the yeasts *S. cerevisiae* and *S. pombe*, the fruit fly *Drosophila melanogaster*, and the nematode *C. elegans*, have proven to be informative model systems for various mechanistic studies in vertebrates. In view of the fact that these model organisms are endowed with Rev1 protein as well as one or more other Y-family DNA polymerases, they offer the potential for gaining fundamental insights into the molecular biology of TLS in eukaryotes. In the present studies we have compared interactions between Rev1 protein and other members of the Y-family of DNA polymerases from animals and fungi.

Here we report that Rev1 protein from the fruit fly *Drosophila melanogaster* and the yeasts *S. cerevisiae* and *S. pombe* readily interacts with the Rev7 subunit of the specialized DNA polymerase ζ (Polζ). Additionally, various Y-family DNA polymerases from Drosophila interact with Rev1 protein from this organism. In contrast, members of the Y-family of specialized DNA polymerases from both yeasts and from *C. elegans* do not interact with Rev1 protein from these organisms. Consistent with this observation, the extensive conservation of the C-terminal 100 amino acids of Rev1 protein in vertebrates is not observed in yeasts or nematodes. Remarkably, however, the extent of amino acid conservation

in the C-terminal region of Rev1 protein from Drosophila is not obviously greater than that observed in yeast.

In contrast to the corresponding mouse proteins, the Drosophila Y-family DNA polymerases Polι and Polη utilize two distinct regions to interact with Drosophila Rev1. However, a comparison of the Rev1-interacting domains in Polη, Polι and Polκ from mouse or Drosophila reveals little sequence conservation and does not predict conserved structures. Thus, notwithstanding the presence of Rev1 protein and some specialized DNA polymerases in invertebrates and fungi, interactions between these proteins differ qualitatively among themselves and from the Rev1-DNA polymerase interactions observed in vertebrates. We conclude that no single eukaryotic model system thus far examined can be considered a prototypic model system for generalizing the molecular mechanism of TLS in eukaryotes, and suggest that care must be exercised in making mechanistic extrapolations from one eukaryotic system to another.

## *Results*

### Interactions between Rev1 protein and Rev7 protein, the catalytic subunit of the B-family DNA polymerase Polζ

In addition to its well-documented ability to interact with various Y-family DNA polymerases, the highly conserved C-terminal region of mouse Rev1 protein interacts with the Rev7 subunit of Polζ, a specialized DNA polymerase

from the B-family, which is also implicated in TLS in eukaryotes. Rev1 protein from Drosophila and the yeasts *S. pombe* and *S. cerevisiae* also interact with homologous Rev7 protein (Figs. 2-5). Additionally, mouse Rev1 maintains an interaction with Rev7 from both yeasts and flies (data not shown), suggesting that the region of Rev7 responsible for binding Rev1 is structurally conserved.

## Interactions between Rev1 protein and Y-family DNA polymerases in animals and yeast

As already mentioned, interactions between Rev1and the Y-family of DNA polymerases from humans and mice transpire via the C-terminal 100 amino acids of Rev1, a region of the protein that is highly conserved in vertebrates (Fig. 1). An iterative search of the NCBI non-redundant protein sequence database demonstrated that this region of Rev1 is also conserved in a number of invertebrates, and fungi also reveal homologous sequences (Fig. 1). However, the extent of the amino acid conservation is considerably reduced compared to that in vertebrates (Fig. 1). Furthermore, the C-terminal 100 amino acids of Rev1 are not conserved in nematodes (data not shown). Exhaustive sequence searches failed to reveal sequences homologous to the Rev1 C-terminus of nematodes in other eukaryotes or in prokaryotes. Thus, it appears that the C-terminal domain of Rev1 is an innovation of the animal-fungal lineage that was lost in nematodes.

To explore physical interactions between Rev1 and Y-family DNA polymerases from various non-vertebrate eukaryotes, yeast cells were co-

transformed with Rev1 and a DNA polymerase of interest, and interactions were examined using the yeast two-hybrid system and in some cases by co-immunoprecipitation. In confirmation of previous studies, mouse Rev1 protein interacted with the mouse Y-family DNA polymerases Polη, Polι and Polκ (data not shown, see Fig. 5) (Guo, Fischhaber et al. 2003). Similar results were obtained when yeast cells were transformed with vectors that express Rev1 and either Polη or Polι from *D. melanogaster* (Fig. 2A), an organism not endowed with a Polκ gene. This result was confirmed by immunoprecipitating YFP-tagged Polη from Drosophila cell lysates and detecting Myc-tagged Rev1 on YFP- Polη-bound beads (Fig. 2B).

No interactions were observed between Rev1 and Polη (*eso1*$^{+}$ or Rad30) from the yeasts *S. pombe* or *S. cerevisiae* using the yeast two-hybrid assay (Figs. 3A and 4A). Like Drosophila, the yeast *S. cerevisiae* does not harbor a *Polκ* gene. However, Rev1 protein from *S. pombe* failed to interact with Polκ protein from this organism (Figure 3A). Additionally, Rev1 protein from the nematode *C. elegans* failed to demonstrably interact with either Polη or Polκ from this organism (Figure 3B). In confirmation of these negative results a strain of *S. cerevisiae* modified to express endogenously tagged Rad30 (Polη)-ProA was transformed with a vector expressing yeast Rev1 protein tagged with an HA epitope. Both tagged proteins were functional as evidenced by their ability to complement the sensitivity of *S. cerevisiae rev1Δ* and *rad30Δ* (*polη*) mutants to killing by UV radiation or methyl methane sulfonate (Fig. 4B and 4C). However, in contrast to

the control co-IP observed between *S. cerevisiae* Rev7-Myc and Rev1-HA (Fig. 4D and 4E), when Rad30 (Polη)-ProA was immunoprecipitated from yeast cell extracts (either in the absence or the presence of DNA damage) Rev1-HA failed to co-precipitate (Fig. 4F).

## Drosophila Polη and Polι have different requirements for an interaction with Rev1

The interaction between Drosophila Rev1 and Drosophila Polη or Polι was further examined to determine a requirement for the Rev1 C-terminal region, as previously demonstrated in mice and humans. As shown in Fig. 6A, the C-terminal 117 amino acids of Drosophila Rev1 are necessary and sufficient for an interaction with Drosophila Polη. However, a region adjacent to the C-terminus of Drosophila Rev1 is required for its interaction with Polι (Fig. 6B). Unlike the C-terminal domain, this region of Drosophila Rev1 is poorly conserved, even in orthologs from mosquitoes (data not shown). Additional experiments demonstrated a robust interaction between mouse Rev1 C-terminus and Drosophila Polη, but not between the mouse Rev1 C-terminus and Drosophila Polι (Fig. 6C).

In summary, interactions between Rev1 protein and specialized DNA polymerases from the Y-family (Polη, Polι or Polκ) from mouse or humans are apparently conserved in the fruit fly *D. melanogaster*, but not in the worm *C. elegans* or the yeasts *S. cerevisiae* or *S. pombe*. Furthermore, whereas

Drosophila Polη interacts with the conserved C-terminus of Drosophila Rev1, Drosophila Polι exhibits a different requirement for an interaction with Drosophila Rev1.

## Mapping Rev1-interaction domains in Y-family DNA Polymerases

Having identified a requirement for the C-terminal region of mouse and Drosophila Rev1 protein for their interaction with some Y-family DNA polymerases, we sought to identify and compare the Rev1-binding domains in these DNA polymerases. Truncated cDNAs for mouse Polη, Polι, and Polκ were constructed and tested for their ability to interact with full-length mouse Rev1 in the yeast two-hybrid assay. With respect to the mouse polymerases, regions spanning ~50 amino acids in the C-terminal half of Polη, Polι, and Polκ supported an interaction with Rev1 (Fig. 7). Similar experiments were performed with truncations of Drosophila Polη and Polι. Once again, regions in the C-terminal half of both proteins supported an interaction with Drosophila Rev1 (Fig. 8). Remarkably, interactions with Drosophila Rev1 were also observed in the presence of an N-terminal 280 amino acid peptide from Drosophila Polη and an N-terminal 300 amino acid peptide from Drosophila Polι (Fig. 8). These observations were confirmed using a β-galactosidase reporter assay (Fig. 9).

Amino acid sequences of the Rev1-interacting regions of Polη and Polι from mouse and Drosophila are shown in Fig. 10. The similarly located interaction regions are poorly conserved between mouse and Drosophila (Fig. 10 A, B). In contrast, the N-terminal regions of Drosophila Polη and Polι comprise the polymerase domain proper and are well conserved in mice (Fig. 10 C, D). These findings reveal a paradox. The Rev1-interacting regions that are located adjacent to the C-termini of various Y-family polymerases represent the hinge between the N-terminal polymerase domain and the C-terminal Zn-finger and, as noted above, are poorly conserved, with no reliable alignment observed outside groups of closely related species. For instance, the Rev1-binding regions of mouse Polκ, Polη, and Polι show significant sequence conservation only within the respective sets of mammalian orthologous proteins: neither orthologs from more distant species nor paralogs could be reliably aligned within these regions (Thompson JD 1994; Cuff JA 1998). Although the Rev1-binding regions of the Y-family polymerases lack evidence of evolutionary conservation, these regions are predicted to be enriched in disordered structures (Supplemental Fig. 1) (Linding, Jensen et al. 2003). In contrast, the N-terminal regions of Drosophila Polη and Polι, which also interact with Drosophila Rev1, belong to the polymerase domain proper that is highly conserved in most eukaryotes.

## *Materials and Methods*

### Pair-wise yeast two-hybrid assays and interaction domain mapping

*S. cerevisiae constructs.* Rev1 was PCR amplified from Rev1p-GST-pJN60 (Nelson, Lawrence et al. 1996) and cloned into pACT2 (Clontech) or pGBKT7 (Clontech). Rad30 was PCR amplified from pEGUh6b-Rad30 (Yuan, Zhang et al. 2000) and cloned into pGBKT7 or pGBT9 (Clontech). Rev7 was PCR amplified by colony PCR and cloned into pGADT7 (Clontech).

*C. elegans constructs.* Rev-1 was amplified by RT-PCR of total RNA (prepared by bead disruption and RNAeasy prep of N2 hermaphrodite worms) and cloned into pGADT7. Polη-1 was amplified by RT-PCR and cloned into pGBKT7. Two spliced products were detected, one with a 57 bp deletion in exon 7, as previously reported (Ohkumo, Masutani et al. 2006). Both products were assayed. Polκ-1 was amplified by RT-PCR and cloned into pGBKT7.

*S. pombe constructs*. Rev1 (SPBC1347.01c) was amplified by RT-PCR of total RNA and cloned into pGADT7 or pGBKT7. *Eso1*$^{+}$(Polη), Polκ*(*SPCC553.07c), and Rev*7* were amplified by RT-PCR and cloned into pGBKT7 or pGADT7. Exon boundaries for Rev7 were redefined and annotated accordingly on online databases.

*Drosophila constructs.* Rev1 was amplified by RT-PCR of total RNA prepared by Trizol extraction of Kc cells and cloned into pACT2. Polη and Polι were

amplified from pGEX-dPolη and pGEX-dPolι (Ishikawa, Uematsu et al. 2001) and cloned into pGBKT7. Rev7 was amplified by RT-PCR and cloned into pGBKT7. Truncation constructs were made by PCR cloning.

*Mouse constructs.* As previously described (Guo, Fischhaber et al. 2003). Truncation constructs were made by PCR cloning.

All constructs were sequenced prior to experiments using an automated ABI Prism 3100 Genetic Analyzer.

Pair-wise combinations of yeast two hybrid constructs and corresponding negative controls containing an empty vector were transformed into freshly prepared AH109 competent cells (Clontech) and plated on DDO media (-Trp/-Leu). After 4 days of growth at 30°C, 2-3 colonies were picked, suspended in sterile water, and plated on QDO media (-Trp/-Leu/-Ade/-His) and grown for up to 10 days at 30°C to select for positive interactions. Side by side plating on DDO was performed as a control.

## β-galactosidase assays

Pair-wise combinations of full-length or truncated yeast two-hybrid constructs and corresponding negative controls containing an empty vector were transformed into freshly prepared Y187 competent cells (Clontech) and plated on DDO media (-Trp/-Leu) to grow for 3-4 days at 30°C. Two or three colonies were picked and grown in selective media overnight and log-phase cultures were grown to

$OD_{600}$~0.6 the following day. Three aliquots per culture in Z-buffer were flash frozen. Each sample was subjected to the addition of Z-buffer+β-mercaptoethanol and ONPG substrate (Sigma) and subsequently measured (<24 hours) for their spectrophotometric values with respect to time.

## Immunoprecipitation and immunoblot analysis of dRev1 and dPolη

The full-length ORFs for dRev1 and dPolη were cloned into expression vectors using the Drosophila Gateway system. Kc *Drosophila* cells (40-80% confluency) were co-transfected with dRev1-pAMW(N-terminal Myc) and dPolη-pAWV(C-terminal YFP) or empty pAMW with dPolη-pAWV using Effectene reagent (Quiagen). Transfected cells selected in puromycin (20mg/mL)/CCM-3 reached confluency and were split after 24 hours. Transiently transfected cells were harvested after 48 hours and extracted in lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 0.5% NP-40) spiked with protease inhibitor (Sigma). The lysate was incubated with rabbit anti-GFP serum (Molecular Probes) and added to washed Protein A Sepharose (Amersham), followed by incubation for 3h at 4°C. The beads were washed and the contents bound to the beads were analyzed by Western blot using anti-Myc or anti-GFP.

## Yeast strains

Strains are listed in Table 1. Yeast strains used for the Rev1/Rad30 coIP are derivatives of W1588-4C (*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5*), which is a W303 strain corrected for *RAD5* (Zhao, Muller et al. 1998). Deletion of *REV1* and *RAD30* were constructed by gene replacement by PCR amplification of *rev1::KanMX* and *rad30::KanMX,* respectively, from the Saccharomyces Genome Deletion Project strains 1643 and 4255, respectively. To produce the tagged Rad30 fusion protein, the TEV-ProA-7His tag was PCR amplified from pYM10 (Knop, Siegers et al. 1999) and inserted to replace the stop codon of *RAD30.* Rev1-HA was expressed from pAS311-REV1-HAC, which has been described previously along with YSD5, YLW20 YLW70 (D'Souza and Walker 2006).

## UV radiation survival of yeast

At least three independent cultures of each strain (RWY13, RWY15, and W1588-4C) were used. Cultures were grown to saturation for 3 days at 30°C, diluted in water, plated on SC-H, and immediately irradiated using a G15T8 UV lamp (General Electric) at 254nm, 1 J/m$^2$ per second for varying amounts of time. After irradiation, plates were kept in the dark at 30°C for 3 days before colonies were counted.

## Survival after exposure to methyl methanesulfonate (MMS)

As described previously (D'Souza and Walker 2006). In short, after induction in galactose, appropriate dilutions of yeast cells (W1588-4C plus pAS311; YLW20 with pAS311 or with pAS311-REV1-HAC) were plated on SC-W plates with 2% galactose and the indicated amount of MMS.

## Immunoprecipitation and immunoblot analysis of *S. cerevisiae* Rev1 and Pol η

Yeast cultures were grown in selective media with raffinose for 2 days then subcultured into selective media with galactose to induce protein expression overnight. For UV treatment, cells were spun down and resuspended in water to $OD_{600}$ ~ 0.5, poured into large dishes to form a thin layer, then exposed to 50 J/m2 of UV (resulting in approximately 50% killing of WT). Irradiated cells were then resuspended in selective media with galactose and incubated at 30°C for 110-120 minutes after irradiation before harvesting, because previous work suggests that both Rev1 and Rad30 respond to DNA damage on this time scale (McDonald, Levine et al. 1997; Roush, Suarez et al. 1998; Waters and Walker 2006; Skoneczna, McIntyre et al. 2007). Immunoprecipitations were performed essentially as described previously (Duncker, Shimada et al. 2002; D'Souza and Walker 2006). Cell pellets were washed once in water and resuspended in ice-cold lysis buffer (50 mM HEPES, pH7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton

X-100, 0.1% sodium deoxycholate, 1 mM DTT and Roche complete protease inhibitor cocktail). Cells were lysed either by bead-beating or by French Press. The lysate was centrifuged 13,500 RCF for 7 minutes, and PMSF was added to 1 mM. For the precipitation of ProA tagged proteins, the supernatant was bound to 50 µl IgG Sepharose (Amersham) for 1-2 hours. For Myc or HA tags, the supernatant was mixed with 2 µg of anti-Myc (mouse monoclonal 4A6; Upstate) or anti-HA (mouse monoclonal HA.11 clone 16B12; Covance) antibody and incubated for one hour on ice. 20 µl of ProG-agarose (Sigma) was then added and incubated for 1-2 hours at 4°C. The resin was washed 3 times in 500 µl of lysis buffer, and bound proteins were eluted by boiling the resin in SDS sample buffer.

Several alternate coIP procotols were performed, all yielding similar results. One alternate technique is represented in figure 4C. Yeast cultures were grown as above, butresuspended in alternate lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM $MgSO_4$, 10% glycerol, 0.05%NP40, 1 mM DTT, Roche complete protease inhibitor cocktail). Cell suspension was frozen drop-wise in liquid nitrogen, then lysed by grinding the frozen cells with dry ice in a coffee grinder. Thawed lysates were centrifuged 10,000 RCF for 15 minutes. The supernatant was then incubated with IgG-coupled magnetic beads (Dynabeads M270-Epoxy, Dynal) for 4 hours at 4°C. The beads were collected

and washed three times in alternate lysis buffer lacking glycerol. Bound proteins were eluted by boiling the beads in SDS sample buffer.

For immunoblotting, protein samples were separated on SDS-polyacrylamide gels (Cambrex), transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore), and probed with appropriate antibodies. ProA-tagged proteins were detected using rabbit peroxidase anti-peroxidase (PAP) antibody (Sigma); Myc and HA tags were detected using mouse monoclonal antibody clone 4A6 (Upstate) and mouse monoclonal HA.11 clone 16B12 (Covance), respectively, followed by HRP-conjugated goat anti-mouse secondary antibody (Pierce).

## Protein sequences analysis

Iterative searches of the non-redundant protein sequence database (National center for Biotechnology Information, NIH, Bethesda) were performed using the PSI-BLAST program (Altschul, Madden et al. 1997) with standard parameters and the composition-based statistics applied to eliminate spurious hits emerging as a result of amino acid compositional biases (Schaffer, Aravind et al. 2001). Multiple alignments of protein sequences were generated using the Clustal W program (Thompson, Higgins et al. 1994). Protein secondary structure prediction was performed using the JPred program (Cuff, Clamp et al. 1998). Disordered

protein regions were predicted using the DisEMBL server (Linding, Jensen et al. 2003).

## *Discussion*

Previous studies indicate that Rev1 protein in eukaryotic organisms maintains one or more functions in DNA damage tolerance independent of its dCMP transferase activity (Nelson, Gibbs et al. 2000; Ross, Simpson et al. 2005). In light of the observation that human and mouse Rev1 interact with multiple Y-family DNA polymerases via a highly conserved C-terminal domain (Guo, Fischhaber et al. 2003; Ohashi, Murakumo et al. 2004; Tissier, Kannouche et al. 2004), we inquired whether similar if not identical interactions are conserved in invertebrates and fungi that also possess Y-family homologues. Surprisingly, given that *S. cerevisiae* Rev1 and Polη (Rad30) are both required for the replicative bypass (translesion DNA synthesis) of lesions in DNA generated by exposure of cells to UV radiation, we find no evidence of interaction between *S. cerevisiae* Rev1 and Polη (Rad30 protein), regardless of whether cells were exposed to UV radiation or not. Remarkably, the amino acid sequence of the Rev1 C-terminus of *S. cerevisiae* shows considerable sequence similarity to the corresponding region of Drosophila Rev1, which interacts with both Drosophila Polη and Polι (Fig. 4A, 4B). Thus, the interaction between the C-terminus of Rev1 and Polη appears to be an animal-specific innovation, which is compatible

with the high level of conservation of this portion of the Rev1 sequence in animals as compared to the limited conservation between animals and fungi (Fig. 1). These findings are consistent with the observation that, unlike Rev1 and the Polζ complex (Rev3/Rev7) in *S. cerevisiae*, Rev1 and Rad30 do not exhibit an epistatic interaction with respect to UV radiation sensitivity (McDonald, Levine et al. 1997).

An interaction between the polymerase accessory domain (PAD) of purified Rev1 and Polη (Rad30 protein) *in vitro* was recently reported in *S. cerevisiae* (Acharya, Haracska et al. 2007). This interaction was not documented *in vivo*. However, the authors reported that purified yeast Rev1/Rev7 complex precludes interaction between Rev1 with Polη *in vitro* (Acharya, Haracska et al. 2007). Conceivably, in the native cellular environment of *S. cerevisiae* where Rev7 (the regulatory subunit of DNA polymerase ζ) is abundant, this protein sequesters most, if not all Rev1, thus preventing complex formation between Rev1 and Polη (Rad30 protein).

Recent studies from one of our laboratories (GCW) have documented that Rev1 protein levels are dramatically cell cycle regulated in *S. cerevisiae* (Waters and Walker 2006). To further explore a possible functional relationship between Rev1 and Y-family polymerases in *S. cerevisiae* we performed epistasis analysis between Rev1 and Polη (Rad30 protein) in G1 or G2 arrested cells with respect to

UV radiation exposure, but observed no cell-cycle dependent genetic relationship at low dosage of irradiation. Similar results have been reported for asynchronous cells [24 and R. Woodruff and G. Walker, unpublished results]. The absence of physical and genetic interactions may explain the observation that *S. cerevisiae* Rev1 and Rad30 are not required for the replicative bypass of the same UV radiation-induced cognate lesions (Nelson, Gibbs et al. 2000; Gibbs, McDonald et al. 2005). Indeed, since Rad30 is apparently not required for UV radiation-induced mutagenesis (unlike Rev1 or Polζ), it has been speculated that Polη (Rad30) protein participates in an error-free repair pathway independent of Rev1 protein (McDonald, Levine et al. 1997; Roush, Suarez et al. 1998). In summary, it seems reasonable to suggest that the C-terminus of Rev1 acquired novel functions in more complex eukaryotes. Alternatively, different sets of interactions may execute similar functions, as suggested by the observation that *S. pombe* Rev7 protein interacts with *S. pombe* Rev1, Polκ and Polη (*eso1*$^{+}$*)* [J. N. Kosarek and E. C. Friedberg, unpublished results].

The observation that Drosophila Rev1 protein interacts with both Drosophila Polη and Polι is intriguing. Drosophila is not endowed with an adaptive immune system (Medzhitov and Janeway 2000) suggesting that these interactions did not evolve to support somatic hypermutation, a process in which several Y-family polymerases in higher eukaryotes are implicated (Ross and Sale 2006; Masuda, Ouchida et al. 2007). Remarkably, Drosophila Polη interacts with

the C-terminal 117 amino acids of Drosophila Rev1, just as in mouse and humans. Drosophila Polη also maintains an interaction with the C-terminus of mouse Rev1, suggesting functional analogy between the C-terminal domains of mouse and Drosophila, despite reduced sequence conservation. In contrast, Drosophila Polι does not interact with the C-terminus of Drosophila Rev1, but rather with a distinct domain that does not appear to be conserved in Rev1 protein from the other species examined, nor does it show any sequence similarity to closely related species (data not shown). The minimal conservation of this Polι-binding domain in Drosophila Rev1 suggests that Drosophila Rev1 may have a unique mechanism *in vivo* for switching between Polι and Polη .

Drosophila Polη and Polι each utilize two independent domains for interacting with Drosophila Rev1. In addition to the domain in the C-terminal half of these proteins, (similarly located to the Rev1-interaction domains identified in the mouse homologues of Polη, Polι, and Polκ) we identified a second Drosophila Rev1-interaction domain located at the N-terminus of Drosophila Polη and Polι (Fig. 8A and 8B). The N-terminal motifs of Drosophila Polι and Polη that bind Rev1 contain the five characteristic Y-family motifs, including the catalytic domains of the polymerases, which are well conserved among all species. The N-terminal fragment of Drosophila Polη can also support an interaction with mouse Rev1 protein (data not shown), suggesting there are functional differences

between the N-terminus of mouse Polη and Drosophila Polη, notwithstanding the high degree of amino acid conservation (Figure 10 C). The additional observation that Drosophila Rev1 interacts with the catalytic domains of Drosophila Polη and Polι raises the possibility that these interactions may affect the catalytic properties of these proteins, as has been shown for the interaction between yeast Rev1 and Rev3 (Polζ) (Acharya, Johnson et al. 2006).

The Rev1-interacting regions in the similarly located mouse and Drosophila Y-family polymerases examined in our studies are predicted to be enriched in disordered structures (Supplemental Fig. 1). Disordered interaction domains have been observed among transcription factors (Liu, Perumal et al. 2006) and a variety of other regulatory proteins. A structured protein that interacts with multiple unstructured partners has also been observed (Bustos and Iglesias 2006). Furthermore, functionally analogous domains have been observed which have little sequence similarity but share intrinsic disorder [43], which is predicted to be the case for Rev1-binding partners. UmuD and UmuD' proteins from *E. coli* which are also involved in DNA damage tolerance, have also been shown to be intrinsically disordered (S.M. Simon, F.J.R. Sousa, R.S. Mohana-Borges and G. C. Walker, manuscript in preparation). UmuD and UmuD' are the products of the *umuD* gene; they stably interact with and functionally regulate the activity of the prokaryotic Y-family member UmuC, and interact with many other proteins, including RecA, DinB, and polymerase subunits α, β, and ε (Burckhardt,

Woodgate et al. 1988; Nohmi, Battista et al. 1988; Shinagawa, Iwasaki et al. 1988; Sutton, Opperman et al. 1999; Tang, Shen et al. 1999; Kumar and Thompson 2005; Jarosz, Beuning et al. 2007).

When the ability of the Rev1 C-terminus to interact with Y-family members is related to the phylogeny of species studied here, we observe that this function appears to have been lost outside coelomates, higher metazoans which possess a body cavity (Fig. 11, adapted from (Mushegian, Garey et al. 1998)) (Hedges 2002; Wolf, Rogozin et al. 2004; Rogozin, Wolf et al. 2007; Zheng, Rogozin et al. 2007). In addition, *C. elegans* (a pseudo-coelomate) is a more rapidly evolving species (Hedges 2002), which is supported by our observation that the C-terminal domain of Rev1 was lost in nematodes but retained in yeast.

Interaction between Rev1 and Rev7 (the catalytic subunit of Polζ) is maintained in all organisms studied, suggesting that these proteins co-evolved to maintain an essential function for TLS. Studies in yeast have shown that Polζ is indispensable for DNA damage-induced mutagenesis and that Rev1 is required for the function of Polζ (Baynton, Bresson-Roy et al. 1999; Lawrence 2004). Furthermore, kinetic analyses have shown that Rev1 enhances Polζ function during mismatch extension as well as extension past abasic sites and [6-4] photoproducts (Acharya, Johnson et al. 2006). While the specific role of the Rev1/Rev7 interaction remains to be determined, our results provide evidence that this interaction may underlie a distinctly conserved TLS function.

In conclusion, in our efforts to expand studies of the Rev1/Y-family polymerase interactions to a more tractable model organism, we conclude that no single eukaryote thus far examined can be considered a prototypic model system for generalizing the molecular mechanism of TLS in eukaryotes, and that particular domains of these proteins and their functions are more divergent than originally thought. These studies should advocate special consideration when making mechanistic extrapolations from lower to higher eukaryotes and vice versa.

## *Figures and Tables*

**Table 1**

**Table 1 – Yeast strains used for immunoprecipitation analysis**

| Strain | Genotype |
|---|---|
| W1588-4C | MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 |
| W1588-4A | MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 |
| YSD5 | MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 REV7-13MYC::HIS3MX6 |
| YLW20 | MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 rev1::KanMX |
| YLW70 | MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 |
| RWY13 | MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 RAD30-TEV-ProA-7His::HIS3MX |
| RWY15 | MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 rad30::KanMX |
| RWY254 | MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 RAD30-TEV-ProA-His::HISMX |
| RWY270 | MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 pep4::KanMX |

**Table 2-1: Yeast strains used in this study.**

**Figure 1**


**Figure 2-1: The C-terminus of Rev1 is highly conserved in vertebrates but to a lesser extent among invertebrates.**

The sequences of the C-terminal amino acids of Rev1 protein in vertebrates (top) and invertebrates and fungi (bottom) are shown. Hs, *Homo sapiens*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*; Gg, *Gallus gallus*; Xl, *Xenopus laevis*; Tn, *Tetraodon nigroviridis*; Dm, *Drosophila melanogaster*; Ust, *Ustilago maydis*; Sp, *Schizosaccharomyces pombe*; Asp, *Aspergillus fumigatus*; Sc, *Saccharomyces cerevisiae*. Amino acid identity and similarity are represented by green colored letters, while differences are indicated in grey.

**Figure 2**

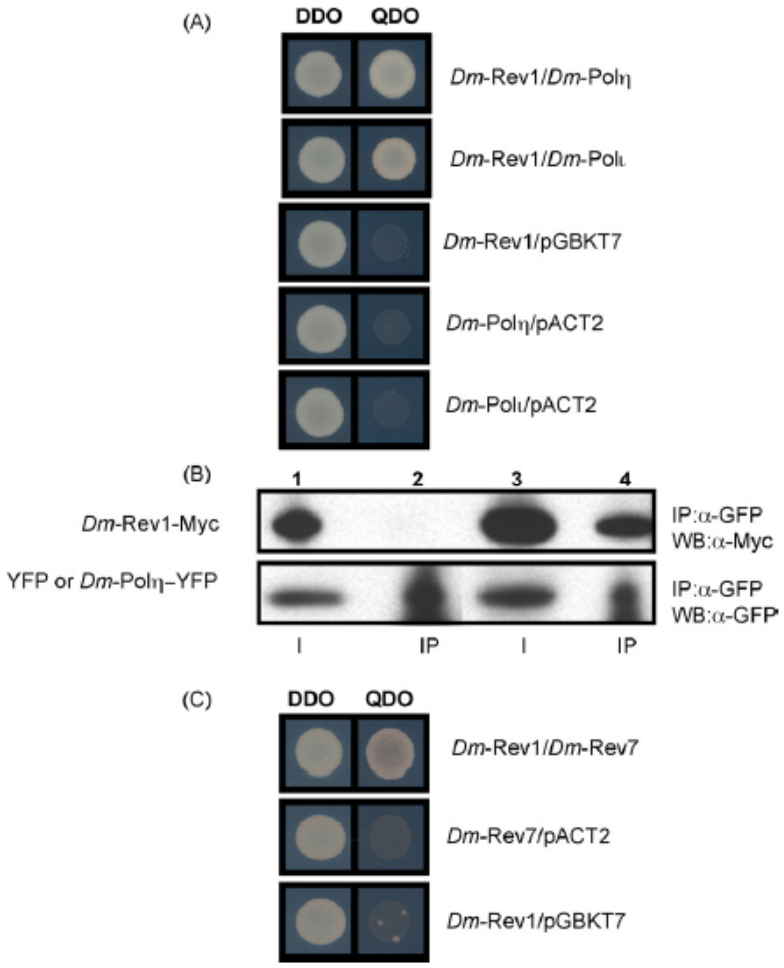


**Figure 2-2: Drosophila Rev1 interacts with Y-family polymerases Polι and Polη, and B-family Rev7 (Polζ).**
(A) Drosophila Rev1 interacts with Drosophila Polη and Polι in the yeast two-hybrid assay **.** Yeast transformants expressing a Drosophila Rev1-activation domain (AD) fusion protein and the designated polymerase (Polι, or Polη)-binding domain (BD) fusion protein are selected on double drop out (DDO) media (*-Trp* or *Leu)*. Positive interactions are indicated by growth on quadruple drop out (QDO) media acids (*-Trp*, *-Leu*, *-Ade*, *-His)*. Growth on QDO media indicates the two proteins physically interact, as their proximity results in the activation of

histidine and adenine protein expression. **(B)** Drosophila Rev1 co-precipitates with Drosophila Polη; Lane 1: input Rev1-Myc + YFP; Lane 2: IP Rev1-Myc + YFP; Lane 3: input Rev1-Myc + Polη-YFP; Lane 4: IP Rev1-Myc + Polη-YFP. **(C)** Drosophila Rev1 interacts with Drosophila Rev7 in the yeast two-hybrid assay.

**Figure 3**

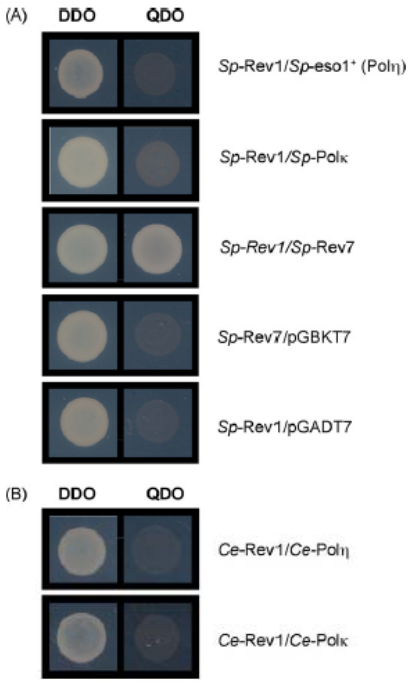


**Figure 2-3: Rev1 interactions in *S. pombe* and *C. elegans*.**
In the yeast two-hybrid assay **(A)** *S. pombe* Rev1 interacts with *S. pombe* Rev7, but not *S. pombe eso1*$^{+}$(Polη) or Polκ. **(B)** *C. elegans* Rev1 does not interact with *C. elegans* Polη or Polκ homologs.

**Figure 4**

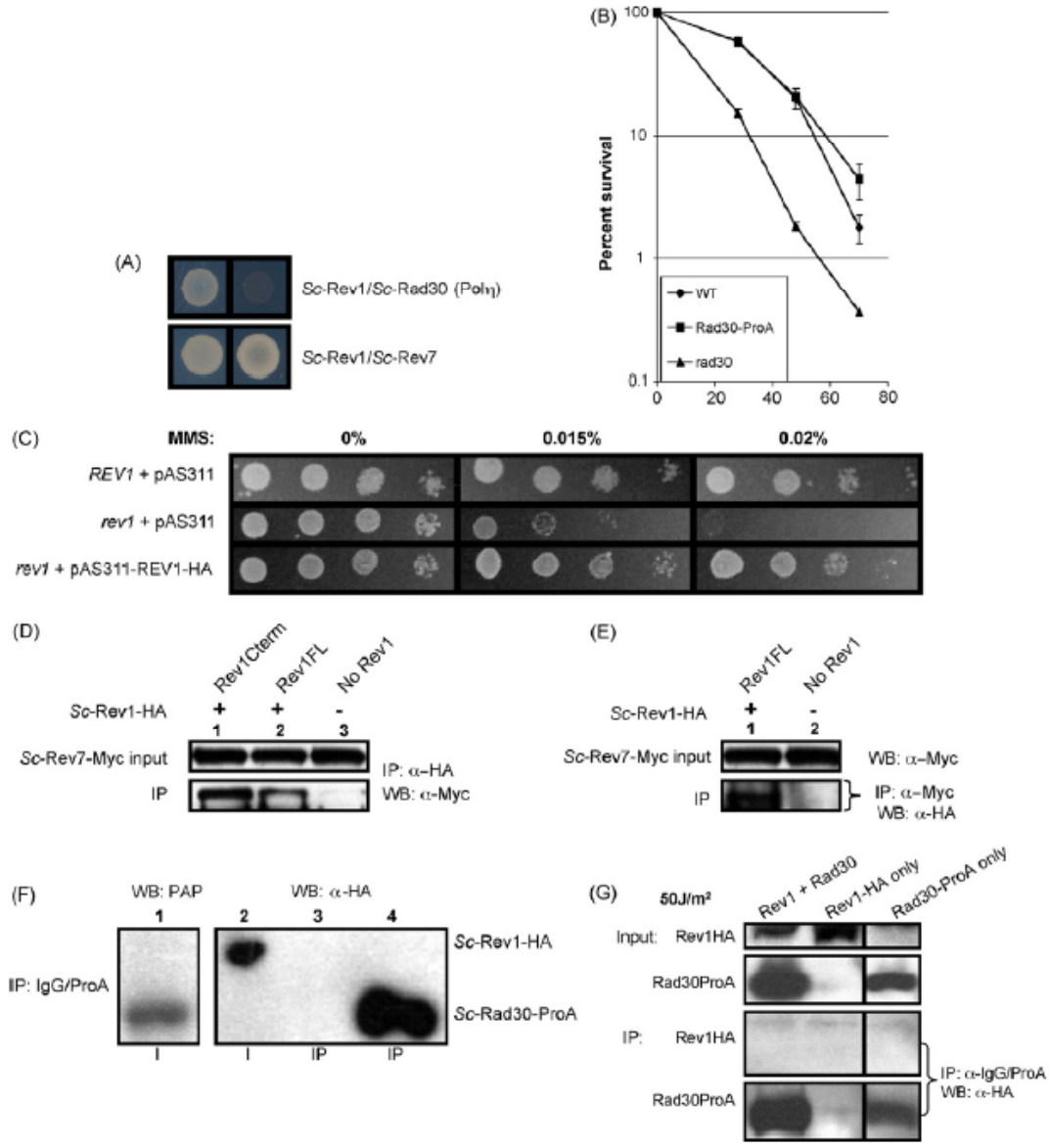


**Figure 2-4: *S. cerevisiae* Rev1 does not interact with Rad30 (Polη) *in vivo*.** **(A)** In the yeast two-hybrid assay, *S. cerevisiae* Rev1 interacts with Rev7, but not Rad30 (Polη). **(B)** In *S. cerevisiae*, *tagged Rad30 (Polη) is fully functional for UV radiation survival*: comparison of WT (W1588-4C), Rad30-TEV-ProA-His (RWY13), and *rad30Δ* (RWY15) strains. Error bars represent standard error. **(C)** *Rev1-HA-pAS311 can rescue the MMS sensitivity of the rev1 null mutant.* Top

row: wildtype (W1588-4A + pAS311), second row: *rev1Δ* (YLW20 + pAS311) bottom row: Rev1-HA (YLW20 + pAS311-REV1-HAC **(D)** *S. cerevisiae Rev1-HA coimmunoprecipitates endogenously tagged Rev7-Myc.* Lane 1: Rev1-Cterm-HA and Rev7-13Myc (YSD5 + pAS311-REV1CT239-HAC); Lane 2: Full length Rev1-HA and Rev7-13Myc (YSD5 + pAS311-Rev1-HAC); Lane 3: Rev7-13Myc alone (YSD5 + pAS311). Full length Rev1 is produced at lower levels than the C-terminal 239 amino acid fragment, resulting in the difference in quantity of Rev7-Myc which coIPs in lane 1 compared with lane 2. **(E)** *In S. cerevisiae, endogenously tagged Rev7 immunoprecipitates Rev1-HA.* Rev7-13Myc immunoprecipitates Rev1 in the presence (+) of Rev1-HA (YSD5 + pAS311-Rev1-HAC) but does not in the absence (-) of Rev1-HA (YSD5 + pAS311). Rev1-HA is undetectable in the input (not shown). **(F)** *S. cerevisiae Rev1 and Rad30 (Polη) do not co-immunoprecipitate in the presence or absence of UV-damage.* IgG was used to precipitate Rad30-TEV-ProA-7His protein using the alternate coIP protocol with strains RWY75 and YSD7. Lane 1: RWY75 input sample probed with PAP for Rad30-TEV-ProA-7His; Lane 2: RWY75 input; Lane 3, YSD7 IP; Lane4, RWY75 IP, showing Rad30 band only. Lanes 2-4 probed with anti-HA antibody, detects Rev1-HA present in the input and also (through the IgG-binding activity of ProA) nonspecifically detects the high concentration of Rad30-ProA in the IP. **(G)** For UV-treated conditions, yeast extracts were made from cells that had been subjected to UV radiation. IgG was used to precipitate Rad30-TEV-ProA-7His protein by the primary coIP protocol. Lane 1: Rev1-HA and Rad30-ProA (RWYRWY254 + pAS311-REV1-HAC); Lane 2: Rev1-HA only (RWY270 + pAS311-REV1-HAC); Lane 3: Rad30-ProA only (RWY254).

**Figure 5**

| | Mouse | Fly | Worm | Budding yeast | Fission yeast |
|---|---|---|---|---|---|
| Polη | *+ | + | - | - | - |
| Polι | *+ | + | | | |
| Polκ | *+ | | - | | - |
| Rev7 | *+ | + | | *+ | + |

**Figure 2-5: The TLS polymerase interactions with Rev1 protein within different species.**

The presence (+) or absence (-) of a DNA polymerase interaction with Rev1 within each species (as determined by the yeast two-hybrid or other methods described here) is indicated. Shaded boxes indicate that the polymerase has not been identified in the species. Asterisks (*) indicate previously published work.

**Figure 6**

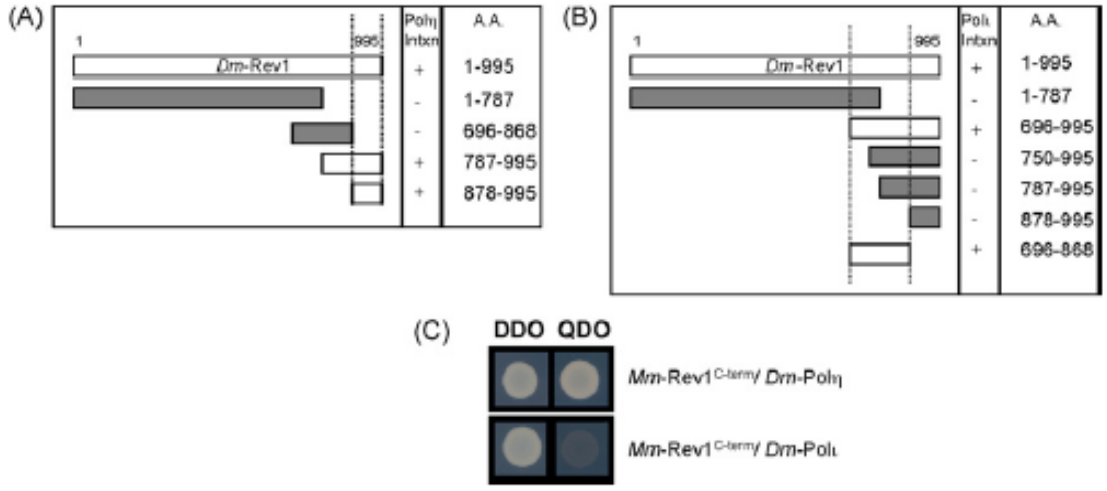


**Figure 2-6: Drosophila Polη and Polι have different requirements for interaction with Rev1.**
**(A)** Drosophila Rev1 interacts with Drosophila Polη through its conserved C-terminal domain (~117a.a.). **(B)** Drosophila Polι requires amino acids upstream of the Drosophila Rev1 C-terminus. **(C)** *Drosophila* Polη interacts with the C-terminus (~120a.a.) of mouse Rev1, while *Drosophila* Polι does not.

**Figure 7**

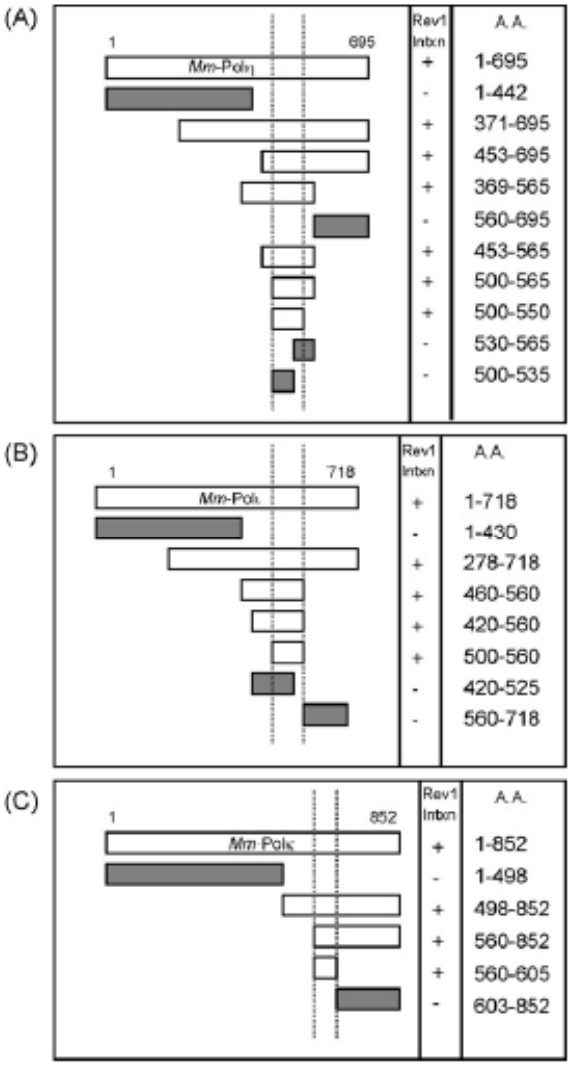


**Figure 2-7: Mapping of mouse Rev1-interaction domains in Y-family DNA polymerases.**
The interaction between mouse Rev1 and the mouse Y-family polymerases requires a region spanning ~50 a.a. in the C-terminal half of **(A)** mouse Polη (500-550), **(B)** mouse Polι (500-560), and **(C)** mouse Polκ (560-605).

**Figure 8**

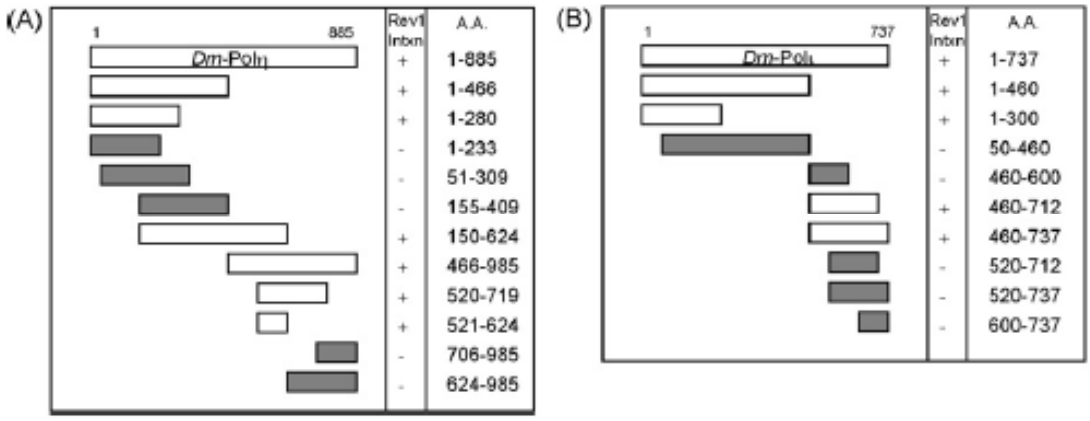


**Figure 2-8: Drosophila Polη and Polι bind Drosophila Rev1 with two independent regions.**
**(A)** Drosophila Polη and **(B)** Drosophila Polι interact with Drosophila Rev1 via an N-terminal peptide as well as a region located in the C-terminal half of each protein.

**Figure 9**

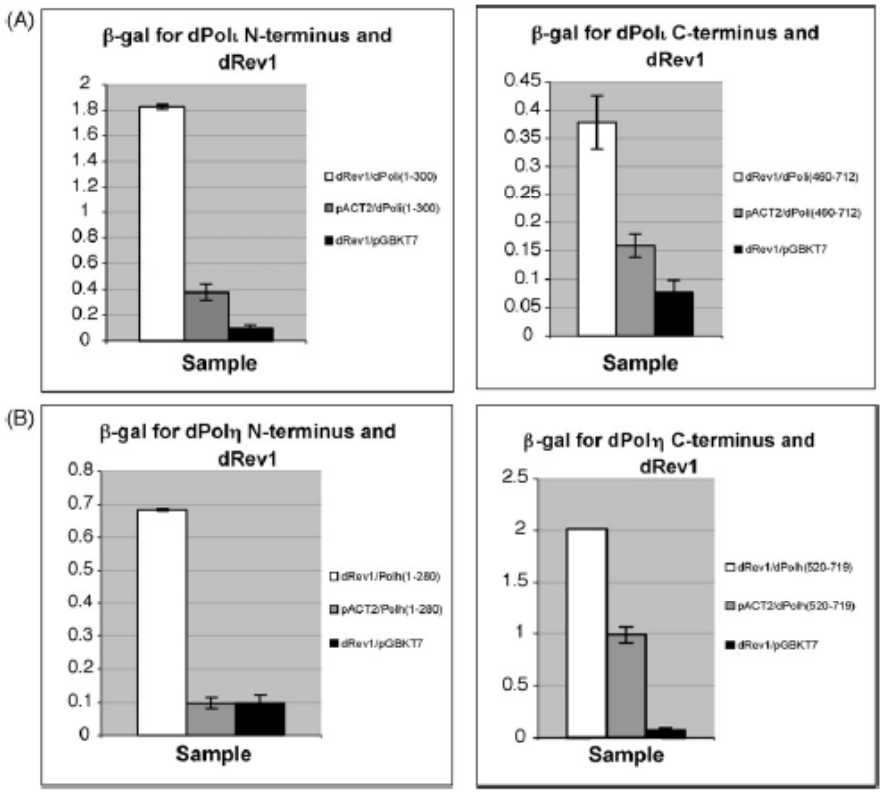


**Figure 2-9: Expression of β-galactosidase confirms two Drosophila Rev1 binding domains in Drosophila Polη and Polι.**
**(A)** Drosophila Polι and **(B)** Drosophila Polη interact with Drosophila Rev1 via an N-terminal peptide as well as a region located in the C-terminal half of each protein. Full-length protein interactions for Polη and Polι are set at a value=1 unit (not shown). All displayed values are normalized to the full-length interaction.

**Figure10**

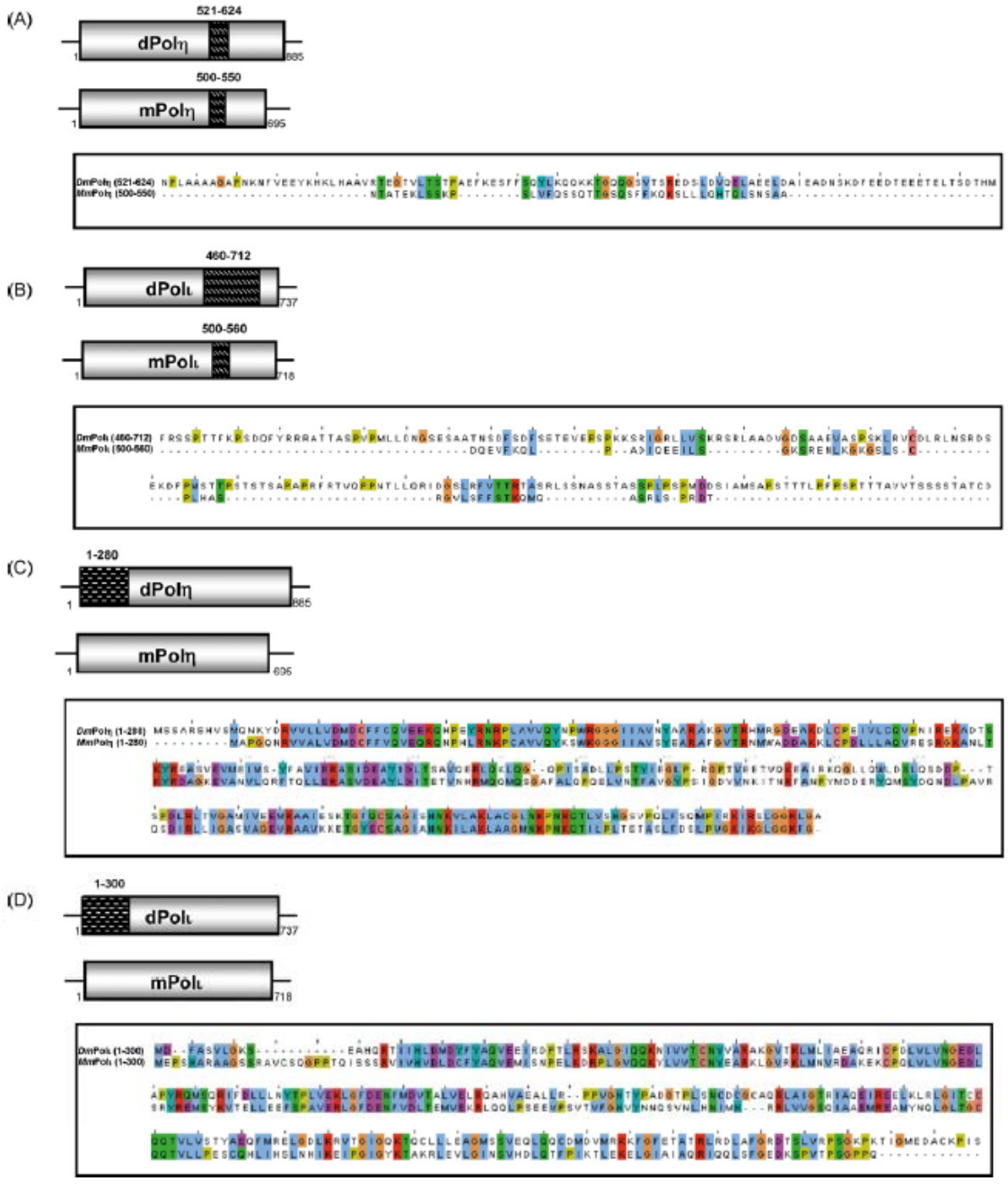
(A)
521-624
dPolη
1
685
500-550
mPolη
1
695
(B)
460-712
dPolι
1
737
500-560
mPolι
1
718
(C)
1-280
dPolη
1
685
mPolη
1
696
(D)
1-300
dPolι
1
737
mPolι
1
718

**Figure 2-10: Amino acid sequence conservation of Rev1-binding regions in Drosophila and mouse.**
(A) An alignment of the experimentally determined, similarly located Rev1-binding region in Drosophila and mouse Polη and (B) Drosophila and mouse Polι. (C) An alignment of the N-terminal Rev1-binding region of Drosphila Polη reveals close homology with the N-terminus of mouse Polη, which does not exhibit an interaction with mouse Rev1.

**Figure 11**

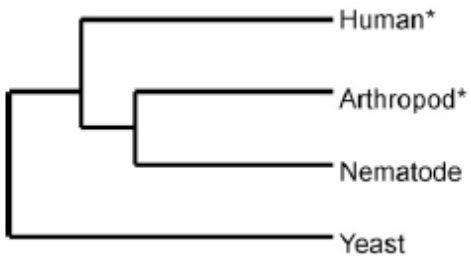


**Figure 2-11: The ability of the Rev1 C-terminus to interact with other Y-family polymerases and its relationship to the phylogeny of species.**
This tree describes a predominant evolutionary relationship between nematodes, arthropods, and humans, known as Coelomata. Here, humans and arthropods are sister taxa, where the nematode sequence is basal to the fly-human clade. *C. elegans* is a 'faster evolving' species of nematode(pseudocoelomate), contributing to its position on the tree. Yeast is an outgroup. (Adapted from (Mushegian, Garey et al. 1998)).
*Indicates the ability of the Rev1 C-terminus to bind other Y-family DNA polymerases.

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Lawrence, Zhigang Wang, and Takeshi Todo for providing polymerase constructs. We also thank Burke Squires for assistance with preliminary sequence alignments and acknowledge the *C. elegans* Genome Consortium for providing N2 hermaphrodite worms. G.C.W. is an American Cancer Society Research Professor and is supported by National Cancer Institute (NCI) Grant CA21615-27. This work was also supported by grant ES11344 from the National Institute of Environmental Health Sciences (ECF) and in part by National Institute of Environmental Health Sciences Grant ESO15818 (GCW).

# Chapter III: The Identification and Characterization of Yeast Rev1 Protein Interaction Partners

In all eukaryotic organisms examined to date, Rev1 and Polζ (Rev3/Rev7) are required for the bypass of UV-induced DNA lesions (Lawrence and Maher 2001; Lawrence 2002; Lawrence 2004; D'Souza and Walker 2006). These proteins (together) represent the "error-prone" branch of the Rad6 epistasis pathway, and are likely regulated in a highly specific manner. In yeast, inactivating mutations in either of these genes results in the sensitizing of cells to DNA damaging agents and a "reversionless" phenotype characterized by reduced damage-induced mutagenesis (Lemontt 1971).

Although Rev1 is strongly implicated in UV-induced mutagenesis, its catalytic activity is dispensable for the replicative bypass of UV-lesions (Nelson, Gibbs et al. 2000; Acharya, Johnson et al. 2006). Given that Rev1 is required for UV-induced mutagenesis for a function other than its catalytic activity, there has been much emphasis on other domains of Rev1 protein, including its BRCT domain and its conserved C-terminal region (Ramos, Hockendorff et al. 1998; Guo, Fischhaber et al. 2003; D'Souza and Walker 2006; Guo, Sonoda et al. 2006). The BRCT domain of Rev1 is located near the N-terminus of the protein, a region required for its role in UV-induced mutagenesis (Nelson, Gibbs et al. 2000; D'Souza and Walker 2006; Guo, Sonoda et al. 2006). A G193R amino acid

substitution in the BRCT domain of yeast Rev1 diminishes the ability of Rev1 to function in UV-induced mutagenesis and confers a mild sensitivity to killing by UV-radiation (Nelson, Gibbs et al. 2000; D'Souza and Walker 2006). BRCT domains are motifs important for the cellular response to DNA damage, and are known to facilitate important protein-protein interactions. Therefore, a standing hypothesis predicts that interactions which may transpire between the BRCT domain of Rev1 and other proteins may be an essential aspect of Rev1 function in TLS.

In addition, the C-terminus of Rev1 has been demonstrated to play a crucial role during Rev1 function in UV-induced mutagenesis. For example, in yeast, deletion of the last 72 amino acids of Rev1 confers UV-sensitivity and a defect in UV-induced mutagenesis comparable to that of *rev1Δ* cells (Acharya, Johnson et al. 2006). Furthermore, over-expressing the C-terminal 426 amino acids of yeast Rev1 confers a strong dominant negative effect on viability and UV-induced mutagenesis (D'Souza and Walker 2006). The C-terminal region of Rev1 in higher eukaryotes such as mice and humans has been shown to facilitate interactions with several other translesion DNA polymerases, including Y-family members Polη, Polκ, and Polι (Guo, Fischhaber et al. 2003; Ohashi, Murakumo et al. 2004; Tissier, Kannouche et al. 2004). However, we have observed that these interactions are not conserved in various lower eukaryotic organisms including the budding yeast *S. cerevisiae*, leaving no satisfactory explanation for

the evolutionarily conserved, non-catalytic role of Rev1 among eukaryotes (Kosarek, Woodruff et al. 2008).

I hypothesized that the crucial C-terminus or BRCT domains of Rev1 are likely involved in one or more protein-protein interactions imperative for Rev1 function in DNA translesion synthesis. By gaining a more broad understanding of which proteins interact with these domains, there is potential for discovering new clues about how Rev1 protein functions in UV-induced mutagenesis. To address this, I performed a yeast two-hybrid screen using baits comprised of the yeast Rev1 C-terminus (a.a. 606-985) or BRCT domain (a.a. 111-321). From the yeast two-hybrid screen, I identified seven candidate proteins that interact with the Rev1 C-terminus in yeast. However, the screen yielded only one interaction with the Rev1 BRCT domain, which I did not further investigate. Of the seven Rev1 C-terminal domain interactions identified, I chose the top two most interesting candidates to further validate, Smc2 (of condensin) and Pup2 (component of the 20S proteasome). Given that each of these proteins is essential for viability in yeast, functional studies presented significant challenges. In place of a *pup-2* mutant, I utilized a viable *ump1Δ* strain deficient for the 20S proteasomal maturase gene to perform epistasis analysis with Rev1. However, I did not observe a genetic relationship between these two genes with respect to UV-sensitivity. Additionally, I investigated the interaction between yeast Rev1 and Jab1 protein, an observation from our laboratory initially identified between the

mammalian homologs of these two proteins. I observed that the BRCT domain of Rev1 is required for this interaction, although epistasis analysis did not reveal a requirement for Jab1 in the response to UV-radiation.

A positive candidate from the Rev1-C terminus yeast two-hybrid screen, *Smc2* (Structural Maintenance of Chromosomes), encodes an essential protein component of condensin (Table 1). Smc2 was of particular interest, given its known association with chromatin. Smc2 protein, together with Smc4, comprise condensin, the factor responsible for driving chromosome condensation required for sister chromatid segregation during mitosis (Strunnikov, Hogan et al. 1995). By sequencing the recovered *Smc2* construct, I found the region of Smc2 that interacts with the Rev1 C-terminus encodes amino acids 782-899. I further validated these interactions in the yeast two-hybrid assay using the full-length open-reading frames (Figure 1A and Figure 1B). Furthermore, a weak interaction was maintained between mouse Rev1 and yeast Smc2 in the yeast two-hybrid assay (data not shown), indicating the possibility of an evolutionarily conserved interaction. *Smc2 i*s an essential gene, and a temperature-sensitive mutant *(smc2-6)* does not survive beyond two cell divisions at the restrictive temperature (Strunnikov, Hogan et al. 1995). This is likely due to the essential role of Smc2 in cell division. Interestingly, condensin is found to associate with DNA at the replication fork barrier site during S-phase, and is reported to localize in locations of converging DNA replication (Wang, Eyre et al. 2005). Condensin also has a

role in re-annealing single-stranded DNA in *S. pombe* (Sakai, Hizume et al. 2003). These are functions that may somehow relate to the DNA structure found at stalled replication forks undergoing DNA translesion synthesis. The possibility remains that Rev1, implicated with a structural role during TLS, may communicate with other structural maintenance proteins during replicative stress. Although I was unable to pursue the significance of this interaction, it is possible that there may be a fundamental role for the interaction between Rev1 and Smc2.

Another positive candidate of interest arising from the Rev1 C-terminus yeast two-hybrid screen was the *Pup2* gene, which encodes the fifth α-subunit of the 20S proteasome (the catalytic core of the 26S proteasome) required for ubiquitin-dependent protein catabolism (Table 1). The region of *Pup2* that interacts with the Rev1 C-terminus encodes amino acids 150-233. I further validated this interaction in the context of the full-length proteins (Figure 2A) and found that removal of the PAD region from the C-terminal fragment of Rev1 ($Rev1^{Cterm\text{-}PAD}$) abolished the interaction between Pup2 and Rev1 (Figure 2B), indicating a requirement for the PAD region of Rev1 in the interaction with Pup2.

The interaction between Rev1 and Pup2 was particularly intriguing given the recent discovery that the 20S proteasome is linked to the post-replicative repair pathway of DNA translesion synthesis (Podlaska, McIntyre et al. 2003; McIntyre, Podlaska et al. 2006). Proteasomal deficiency, as conferred by 20S proteasome mutants, increases the mutagenic effect of UV-irradiation. This

increased mutagenic effect has been connected with the activity of the Rad6/Rad18 DNA damage tolerance pathway, specifically through the activities of the Y-family polymerase Rad30 and the B-family polymerase Polζ (Podlaska, McIntyre et al. 2003; McIntyre, Podlaska et al. 2006). Furthermore, the ubiquitylation of PCNA promotes enhanced mutagenesis caused by proteasome deficiency. Through this work, a model has been developed which implies the role of proteolytic control of the level of an undefined target protein in establishment of the balance between TLS pathways (McIntyre, Podlaska et al. 2006). Given that Rev1 has been shown to be cell-cycle regulated, a property unique from other TLS polymerases, I hypothesized that Rev1 could be the regulatory target of the 20S proteasome (Waters and Walker 2006). Or alternatively, this interaction may be important for some other aspect of UV-induced mutagenesis. Given the link in the literature between 20S proteasome function and DNA translesion synthesis, I further pursued the significance of this interaction in response to UV-radiation.

Like all genes encoding the subunits of the 20S proteasome, the *Pup2* gene is essential for yeast survival, and no viable mutants have been identified or characterized in the literature to date. Alternatively, studies which implicate a role for the 20S proteasome in the Rad6/Rad18 mutagenesis pathway utilize a viable *ump1*Δ mutant which disables the 20S proteasomal maturase protein from organizing various subunits into a functional assembly (Ramos, Hockendorff et al.

1998). I obtained this mutant yeast strain and its isogenic wild-type counterpart to perform epistasis analysis, in order to ask if Rev1 and the 20S proteasome function in the same biological pathway in response to UV-radiation. After the construction of various relevant *rev1Δ* strains, they were assayed by Southern blot to confirm the deletion of genomic copies of Rev1 (Figure 3C). Next, the wild-type, single mutant, and double mutant strains were tested for their sensitivity to killing by UV-radiation. Given that the *rev1Δ* and *ump1Δ* mutants each show different levels of sensitivity to UV-radiation, I expected that if they function in the same pathway that the double mutant would reveal an epistatic relationship. However, the UV-sensitivity of the *rev1Δ ump1Δ* mutant was greater than either of the two single mutants at high doses of UV-radiation, suggesting that Rev1 and the 20S proteasome function independently in response to UV-radiation (Figure 3D).

These results can be interpreted in several alternative ways. A role for Pup2 with Rev1 in UV-induced mutagenesis cannot be excluded based solely on the data presented here. For example, it is possible that the double-mutant *rev1Δump1Δ* may exhibit an altered mutational spectrum, an aspect of UV-induced mutagenesis that was not investigated in this study. Or, if Rev1 is indeed a target for regulation by the 20S proteasome, the defective regulation of Rev1 may not be seen by this assay. Another possible explanation for these results is that Rev1 and Pup2 may function together in *spontaneous* damage-

induced mutagenesis, a pathway in which both proteins function but was not explored in this study. And finally, the possibility remains that the functional purpose of the interaction between Rev1 and Pup2 does not require the context of the properly matured holo-20S complex, the mutant condition used in this work to indirectly investigate Pup2 function.

In addition to the Rev1-interaction partners identified through my yeast two-hybrid screen, I also asked whether the interaction between mouse Rev1 and Jab1 (C. Guo and E.C. Friedberg, unpublished results) was conserved in yeast. It was not until recently that a Jab1 homolog was identified in *S. cerevisiae,* with the help of large-scale genomics and proteomics studies (Schwechheimer 2004). Jab1 (CSN5) is a subunit of the COP9 signalosome, a multi-protein complex of the ubiquitin-proteasome pathway. Jab1(CSN5) possesses isopeptidase activity, which dissolves neddylation of E3 ligases (Ramos, Hockendorff et al. 1998). Jab1 was of particular interest, considering the recent emergence of important post-translational modifications shown to play a role in the regulation of DNA translesion synthesis.

A direct physical interaction was detected between these two proteins by yeast two-hybrid analysis using the full-length ORFs of yeast Rev1 and Jab1 (Figure 3A). In order to determine which region of Rev1 was required for an interaction with Jab1, I performed the yeast two-hybrid assay with Jab1 using the *rev1-1* point mutant ($Rev1^{BRCT\text{-}G193R}$) and a Rev1 BRCT deletion mutant

(Rev1$^{BRCT\Delta}$). No interaction was observed between Jab1 protein and either mutant, suggesting that the BRCT domain of yeast Rev1 is required for a physical interaction with Jab1 (Figure 3A). I further confirmed these findings using the β-gal assay (Figure 3B). Given that the BRCT domain is required for the role of Rev1 in UV-induced mutagenesis, I hypothesized that Jab1 may influence this function.

If Jab1 is required for Rev1 function in UV-induced mutagenesis, a yeast strain deficient in *Jab1* is expected to exhibit sensitivity to killing by UV-radiation. Likewise, the double mutant *rev1Δjab1Δ* should reveal an epistatic relationship between the two genes. Prior to experimentation, I verified that my *jab1Δ* strain (ATCC) was deleted for the Jab1 ORF by PCR (Figure 3C). Isogenic *rev1Δ* and *rev1Δjab1Δ* strains were constructed and confirmed by Southern blot analysis (Figure 3D). Next, I tested the sensitivity of *jab1Δ* and *jab1Δrev1Δ* strains to killing by UV-radiation. In response to various doses of UV-light, I did not observe increased sensitivity of the *jabΔ* strain to UV-radiation with respect to wild-type (Figure 3E). In addition, the *jab1Δrev1Δ* strain did not exhibit sensitivity to killing by UV-radiation different than the *rev1Δ* single mutant (Figure 3E). These results indicated that Jab1 is not likely involved in the essential function of Rev1 in UV-induced mutagenesis.

The possibility remains that the absence of Jab1 may affect the outcome of UV-induced mutagenesis through an altered mutation spectrum, an aspect not investigated in this work. Furthermore, I inquired about the possibility that Jab1 may affect the cell-cycle regulation of Rev1. However, through a collaborative effort, it was found that the absence of Jab1 does not alter the cell-cycle specific regulation of Rev1 protein levels (M.E. Wiltrout and G.C. Walter, unpublished results). Given the diversity of the pathways which Jab1 influences, it seems likely that this interaction is involved in another aspect of Rev1 function not directly tested in these experiments.

My studies broadly focused on yeast Rev1 interaction partners, and concentrated on how these interactions may play a role with Rev1 in response to UV-radiation. Although I was unable to identify novel genes for this pathway, I discovered new areas for further investigation.

## *Materials and Methods*

### Yeast two-hybrid screen for Rev1 interaction partners

#### *Cloning and testing of Rev1 bait*

The two regions of interest for Y2H bait included the C-terminus of Rev1, as well as the BRCT domain. Using RT-PCR and restrictive digests, four unique Rev1 sequences were cloned into pGBKT7 (Clontech) and verified by sequencing. The DNA sequences of the Rev1-BD fusions encoded amino acids (1-256:BRCT), (1-

329:BRCT), (111-321:BRCT), and (606-985:Cterm, which contains the PAD and C-terminal domains) (Prakash 2005). Each of these plasmids was co-transformed with empty vector into AH109 and Y187 cells using a small-scale yeast transformation protocol (Clontech) and plated on various SD media to screen for non-specific growth. Of these baits, the Rev1 C-terminus (606-985) and BRCT domain (111-321) did not exhibit auto-reactive growth when plated on SD/-His/-Trp or SD/-Ade/Trp media, but did form colonies on SD/-Trp media. Additionally, neither of these constructs exhibited toxicity to cells, as determined by the comparison of overnight growth [in 50 mL of SD/-Trp/Kan (20μ/mL)] of bait-transformed Y187 cells with Y187 transformed with empty pGBKT7.

*Construction of an AD-Fusion Library*

Detailed protocols can be found in the MATCHMAKER Library Construction & Screening Kit User Manual (March 2001 version). Briefly, RNA was isolated from W1588-4C yeast cells using the RNAeasy kit (Quiagen). cDNA was synthesized using First-Strand Synthesis (Clontech) with a random primer (CDS III/6, Clontech). Double-stranded cDNA was then amplified by long-distance PCR and purified using CHROMA SPIN+TE 400 columns (1 column per 95μL of cDNA sample). Next, AH109 competent cells were transformed with ds cDNA and pGADT7-Rec (Clontech) and plated on SD/-Leu media (~100 150mm plates). Cells were incubated at 30°C until colonies appeared (~4 days later).

Transformants were harvested and pooled into freezing medium, stored at -80°C in 1 mL aliquots.

### *Library Screening by Yeast Mating*

The library host strain (library cDNA-pGADT7 in AH109) was then mated with the bait strain (Rev1-pGBKT7 in Y187) and selected on QDO media for up to 5 days. Positive colonies were then replica plated onto DDO and QDO media for further validation. From these colonies, plasmid DNA was isolated, sequenced, and retransformed into AH109 competent cells with the Rev1-pGBKT7 bait or empty pGBKT7 to verify the interaction. Positive interaction candidates found to be out of frame with the ATG start site were removed from the pool.

### *Validation of Y2H Positive Interactions*

Full-length ORFs from Pup2 (RT-PCR) and Smc2 (Janet Lindsley) were cloned into pGADT7 (Stray JE and Lindsley JBC 2003). Each clone was tested for an interaction with Rev1-pGBKT7, Rev1-Cterm $^{606\text{-}985}$-pGBKT7 (Cterm+PAD), or Rev1-Cterm$^{765\text{-}985}$-pGBKT7 (Cterm-PAD).

## Functional characterization of Rev1 interaction partners in response to UV-radiation

### *Construction of isogenic rev1Δ, ump1 Δ, and ump1 Δ rev1 Δ strains*

The relevant yeast strains obtained from the Sledziewska-Gojska laboratory are listed in Table 2 (McIntyre, Podlaska et al. 2006). Immediately before use the strains were tested in their original laboratory for UV-sensitivity,

thermosensitivity, and auxotrophies. The strain YAS13 containing the *ump1Δ* is deleted for the Ump1 gene which encodes the proteasomal maturase protein required for the assembly of the various components of the 20S proteasome (Ramos, Hockendorff et al. 1998). In order to obtain isogenic strains deficient in Rev1, the URA3 "gene blaster" fragment (Christopher Lawrence) containing the yeast URA3 gene flanked by the duplicated *Salmonella typhimurium* HisG gene was used to replace the Rev1 gene by means of homologous recombination via complimentary 5' and 3' sequences to Rev1. Knock-out strains were first detected by PCR analysis and then by Southern blot using a probe specific to the N-terminal region of Rev1. For southern blotting, genomic DNA was separated out by electrophoresis and probed with a $P^{32}$ radio-labeled oligonucleotide complimentary to the N-terminus of yeast Rev1.Characterization of the interaction between yeast Rev1 and Jab1

### *Yeast two-hybrid analysis*

Yeast Rev1 and Jab1 were amplified by RT-PCR and cloned into yeast two-hybrid vectors pACT2 or pGBKT7, followed by sequence validation. These constructs together, or in combination with empty vectors, were transformed into AH109 cells and grown on DDO media for 3-4 days. Colonies were then streaked onto QDO to test for an interaction.

### *Determination of Jab1 interaction requirements with Rev1*

In order to determine if the BRCT domain of Rev1 is required for interaction with Jab1, a point mutant Rev1-1$^{G193R}$-pACT2 and a deletion mutant Rev1$^{BRCT\Delta}$-pACT2 were tested in the yeast two-hybrid assay with Jab1-pGBKT7 or empty pGBKT7. To further support this data, these constructs were tested using the β-gal assay. Pair-wise combinations of experimental and control constructs were co-transformed into Y187 cells and assayed according to the Clontech Yeast Protocols Handbook.

### *Construction of isogenic* jab1Δ, rev1Δ *and double mutant* jab1 Δ rev1 Δ *strains*

*Jab1Δ* and an isogenic WT strain were purchased from the ATCC. *Jab1Δ* cells were verified for the deletion of the entire ORF by PCR using flanking primers. To obtain isogenic strains for *rev1Δ* and *rev1Δjab1Δ,* the URA3 "gene blaster" fragment containing the yeast URA3 gene flanked by the duplicated *Salmonella typhimurium* HisG gene (Christopher Lawrence) was inserted in place of the Rev1 gene by means of homologous recombination via complimentary 5' and 3' sequences to Rev1. The Rev1 deletion was detected by PCR analysis and Southern blot analysis using a probe complimentary to the N-terminus of Rev1.

### *Survival of yeast strains in response to UV-radiation*

Cells were grown in YPDA medium to mid-log phase, and counted at a 1000x dilution using a hemocytometer. Serial 10-fold dilutions of each culture were

plated on YPDA, and then irradiated with a germicidal UV lamp at 0.76J/sec/$m^2$ with 0J, 5J, 10J, 20J, and 40J. Following irradiation, the plates were wrapped with aluminum foil and incubated at 30°C (or 24°C) until colonies appeared. Surviving colonies were counted and survival was determined. Duplicates or triplicates were averaged; error-bars represent standard deviations.

## *Figures and Tables*

**Table 1:** ***Yeast two-hybrid screen results***

| Rev1 Bait | Y2H Hits, verified | Known function in yeast |
|---|---|---|
| Cterm | Kss1 | MAP kinase, pheremone response |
| Cterm | Pup2 | α-subunit of the 20S proteasome |
| Cterm | Nrk1 | Kinase, required for cell integrity |
| Cterm | Smc2 | Component of condensin |
| Cterm | Pas8 | Peroxisome assembly |
| Cterm | Pus7 | U2snRNA modification |
| BRCT | Pus7 | U2snRNA modification |

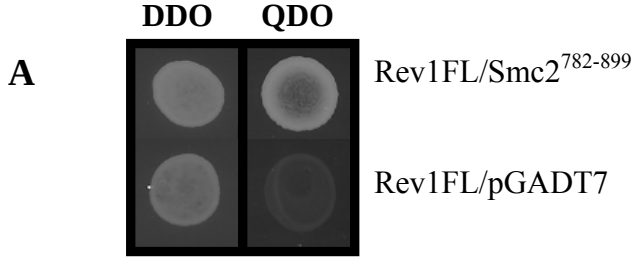
**Table 3-1: List of positive hits from a yeast two-hybrid screen using the C-terminus or BRCT domain of Rev1.**
Functional information is from the SGC database (http://www.yeastgenome.org/)

**Table 2: Yeast strains used for phenotypic analysis**

| Strain | Genotype |
|---|---|
| **WCG4a** (Podlaska, McIntyre et al. 2003) | *MATa ura3 leu2-3, 112 his3-11,15 rad5-G535R* |
| **YAS13** (Podlaska, McIntyre et al. 2003) | *MATa ura3 leu2-3, 112 his3-11,15 rad5-G535R ump1Δ::kanMX4* |
| **WCG4ar*ev1Δ*** This study | *MATa ura3 leu2-3, 112 his3-11,15 rad5-G535R rev1Δ::his-G-URA3* |
| **YAS13*rev1Δ*** This study | *MATa ura3 leu2-3, 112 his3-11,15 rad5-G535R ump1Δ::kanMX4 rev1Δ::his-G-URA3* |
| **BY4741** ATCC | *MATa his3 leu2 met15 ura3* |
| **BY4741*jab1Δ*** ATCC | *MATa his3 leu2 met15 ura3 jab1Δ::KanMX4* |
| **BY4741*rev1Δ*** This study | *MATa his3 leu2 met15 ura3 rev1Δ::his-G-URA3* |
| **BY4741- *jab1Δrev1Δ*** This study | *MATa his3 leu2 met15 ura3 jab1Δ::KanMX4 rev1Δ::his-G-URA3* |

**Table 3-2: Yeast strains used in this study.**

**Figure 1**



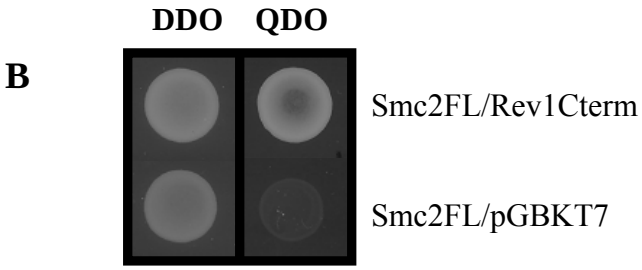
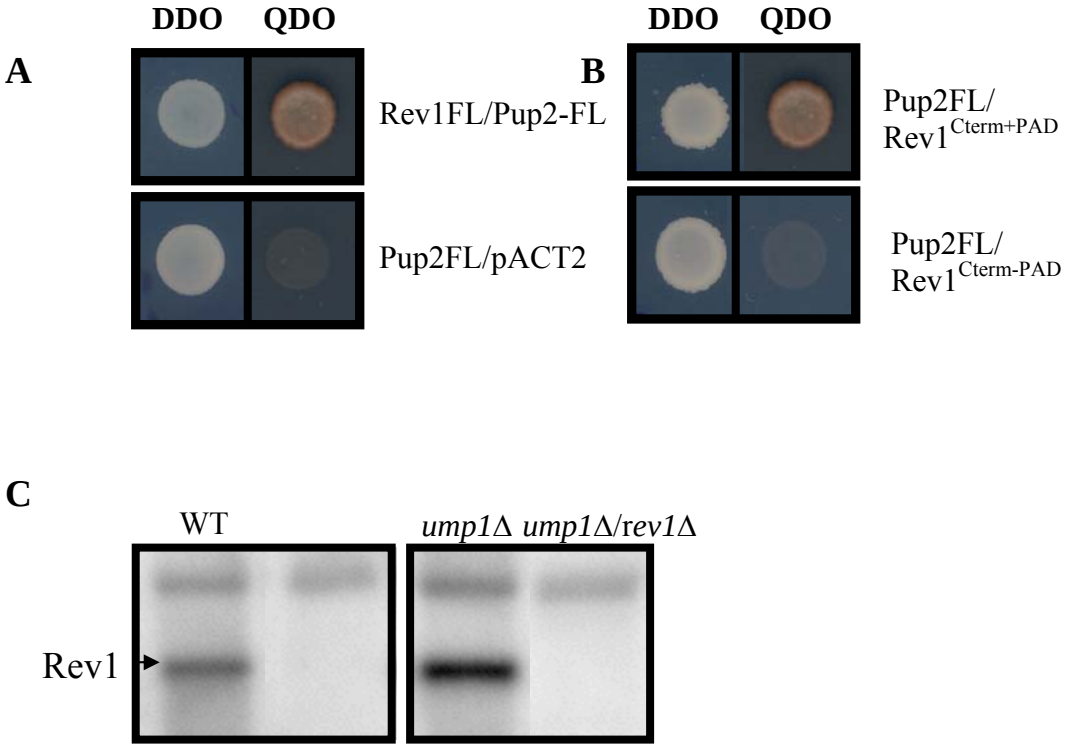



**Figure 3-1: In the yeast two-hybrid system, Smc2 and Rev1 interact.**
A) full length Rev1 protein (Rev1FL) and a peptide encoding amino acids 782-899 of Smc2 (Smc2$^{782-899}$) interact. B) full length Smc2 protein interacts with the C-terminus of Rev1.

**Figure 2**



**D**

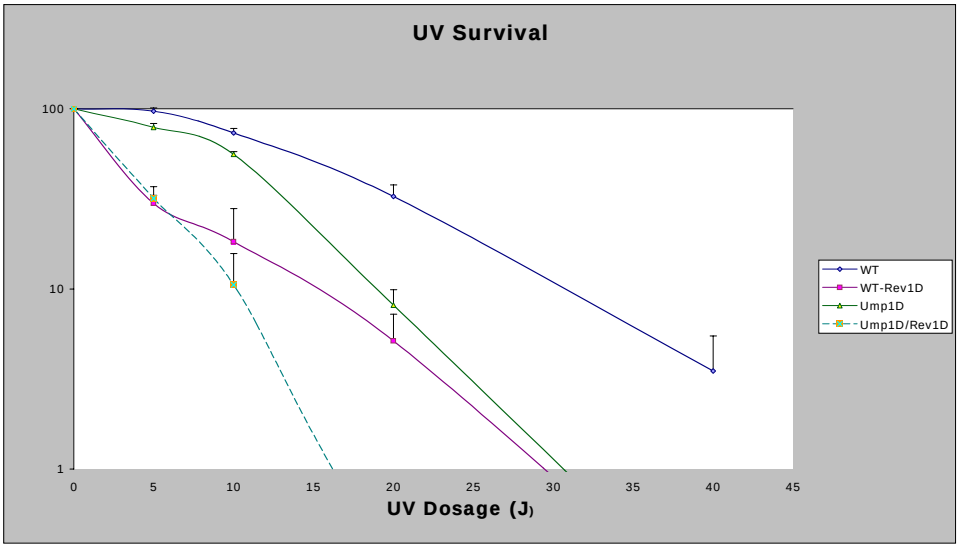
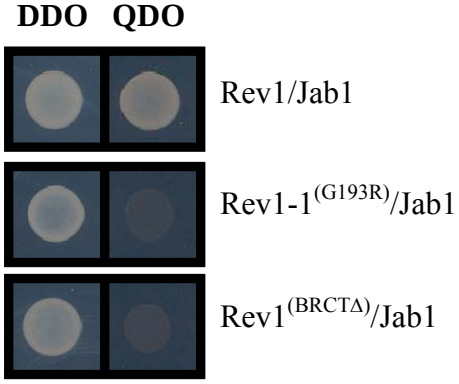


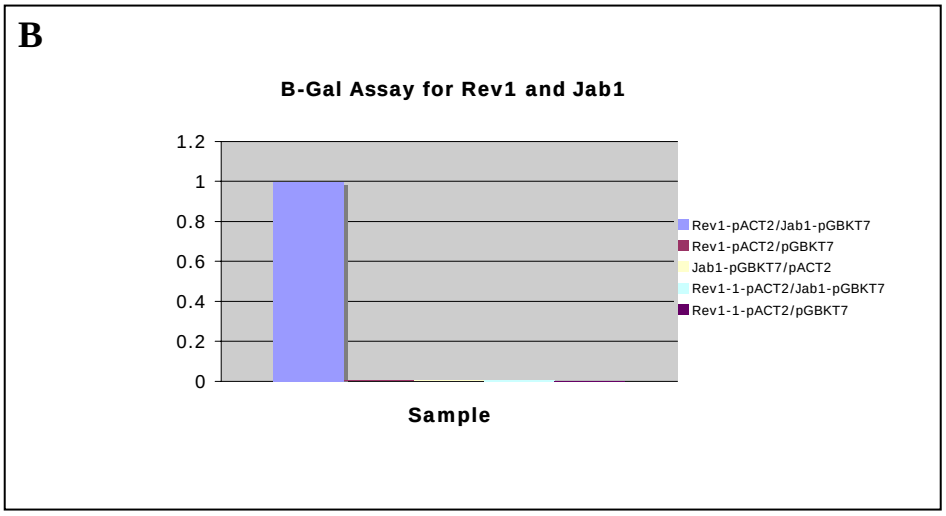

**Figure 3-2: Pup2 and Rev1 proteins interact in the yeast two-hybrid assay.** **A)** Full length Pup2 and Rev1 interact. **B)** Pup2 protein interacts with the C-terminal region of Rev1 which includeds the PAD domain. When the PAD domain is removed, the interaction is no longer observed. **C)** Rev1 deletion strains are verified by Southern blot. **D)** In response to increasing doses of UV-irradiation, *ump1Δ* and *rev1Δ* strains exhibit different sensitivities to killing. The double mutant *ump1Δ rev1Δ* demonstrated an additive sensitivity to killing by UV-radiation.

**Figure 3**

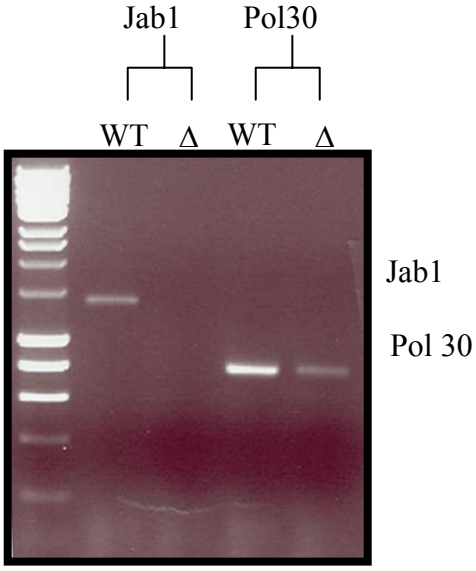
**A**



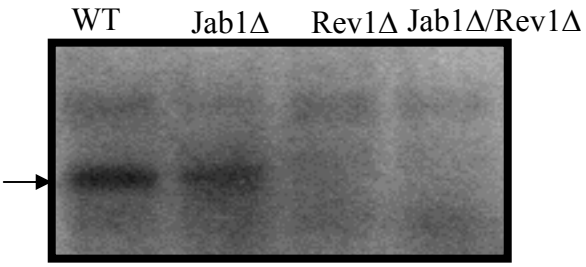
**B**


B-Gal Assay for Rev1 and Jab1
1.2
1
0.8
0.6
0.4
0.2
0
Sample
Rev1-pACT2/Jab1-pGBKT7
Rev1-pACT2/pGBKT7
Jab1-pGBKT7/pACT2
Rev1-1-pACT2/Jab1-pGBKT7
Rev1-1-pACT2/pGBKT7

**C**


Jab1
Pol30
WT
Δ
WT
Δ
Jab1
Pol 30

**D**


WT
Jab1Δ
Rev1Δ
Jab1Δ/Rev1Δ

**E**

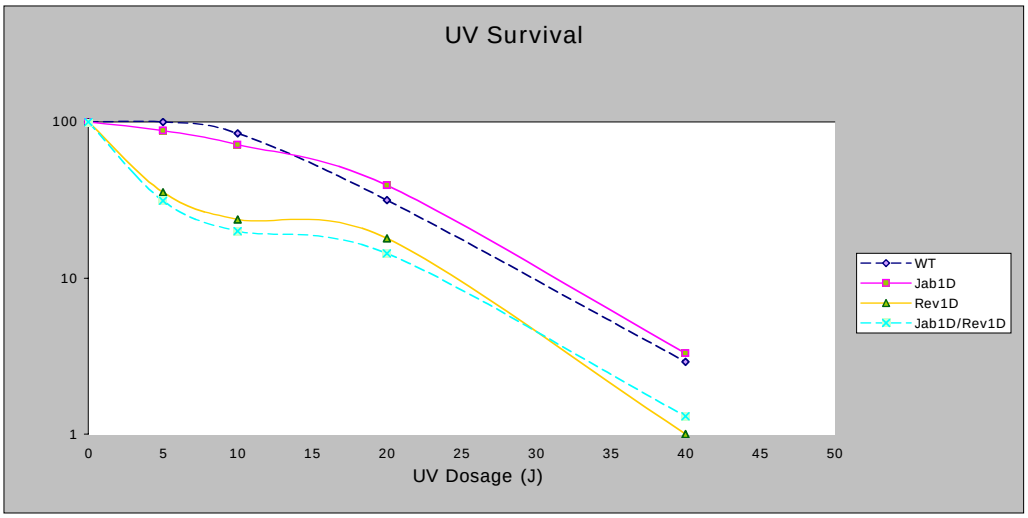



**Figure 3-3: Yeast Rev1 and Jab1 interact, Jab1 mutants do not exhibit UV-sensitivity.**
**A)** In the yeast two-hybrid system, full length yeast Jab1 and Rev1 proteins interact, although a Rev1-1$^{G193R}$ or Rev1$^{BRCT\Delta}$ mutant abolishes the interaction. **B)** Positive or negative interactions between Jab1 and Rev1 or Rev1-1 are confirmed by the β-gal assay. **C)** Full-length Jab1 is confirmed as deleted from the genomic DNA of an ATCC *jab1Δ* strain, with Pol30 (PCNA) as a control. **D)** The deletion of Rev1 from a WT and isogenic *jab1Δ* strain is confirmed by Southern blot analysis. **E)** In response to increasing doses of UV-radiation, the *jab1Δ* mutant does not exhibit sensitivity to killing by UV, nor does the double mutant *rev1Δ jab1Δ* show sensitivity different than the *rev1Δ* strain.

# Chapter IV: Analysis of spontaneous mutagenesis in Big Blue *Polκ*$^{-/-}$ mice

When DNA damage is encountered at the replication fork, high fidelity replicative polymerases are not able to synthesize DNA past structurally obstructive lesions. Specialized DNA polymerases of the Y-family possess the unique ability to replicate DNA through damaged template bases by catalyzing the insertion of a base(s) opposite and/or just beyond the DNA lesion, a process known as DNA translesion synthesis (TLS) (Friedberg, Walker et al. 2005). TLS is an important cellular mechanism for overcoming replication blockage caused by DNA damage, ultimately avoiding cell death.

The engagement of specialized DNA polymerases during TLS can result in either mutagenic or non-mutagenic damage bypass, depending on the type of damage and the repertoire of DNA polymerases available to the cell. Although *in vitro* studies continue to provide insightful analysis into the enzymatic activities of specialized polymerases, they cannot substitute for the *in vivo* experiments that reveal the biology of how specialized polymerases function in complex cellular environments. Lesion specificity, in particular, is a polymerase property that must be validated in a cellular environment.

DNA polymerase kappa (*Polκ*) is a eukaryotic homolog of prokaryotic *Dinb*, conserved in mice, humans, Drosophila, *C. elegans*, and *S. pombe*, but not

in *S. cerevisiae* (Kosarek, Woodruff et al. 2008). The relative multiplicity of Y-family polymerase homologs identified within higher eukaryotes has implies the existence of highly specialized functions for these polymerases in response to different types of DNA damage. Our current understanding of the unique and biologically important role of Polκ in DNA translesion synthesis is limited, calling for further investigation.

Mouse *Polκ* is expressed in most tissues examined, with significantly higher levels observed in the testis (Gerlach, Aravind et al. 1999; Velasco-Miguel, Richardson et al. 2003). Multiple transcripts of the *Polκ* gene are present in the testis, and the cell-specific expression of *Polκ* mRNA in this tissue is confined to the seminferous tubules, observed only in meiotic spermatocytes and post-meiotic spermatids (Velasco-Miguel, Richardson et al. 2003; Guo, Gao et al. 2005). Together these data suggest a role for Polκ in spermatogenesis, although the significance of these findings is not yet understood. In addition, *in situ* hybridization and immunohistochemistry revealed strong *Polκ* staining of the adrenal gland during embryogenesis (Velasco-Miguel, Richardson et al. 2003). *Polκ* is also expressed in mice and human lymphoid tissues, although $Pol\kappa^{-/-}$ mice do not exhibit an altered pattern of somatic hypermutation (a process in which other TLS polymerases have been implicated) (Schenten, Gerlach et al. 2002; Shimizu, Shinkai et al. 2003).

Several phenotypic features of *Polκ*$^{-/-}$ mice and mammalian cell lines have prompted hypotheses for Polκ function *in vivo*. *Polκ*$^{-/-}$ ES cells grow normally and show no obvious defects. In response to UV-radiation, *Polκ*$^{-/-}$ mouse ES cells exhibit modest sensitivity to killing. However, it is not likely that Polκ plays a role in the tolerance of DNA lesions introduced by UV-radiation, given that Polκ is not capable of bypassing the most abundant forms of UV-photoproducts *in vitro* (Ogi, Shinkai et al. 2002; Schenten, Gerlach et al. 2002). A recent study shows that *Polκ*$^{-/-}$ mouse cells have substantially reduced levels of nucleotide excision repair, suggesting that the UV-sensitivity of *Polκ*$^{-/-}$ cells arises from another function of Polκ in NER (Ogi and Lehmann 2006. The possibility remains that Polκ may have a role in bypassing the oxidative damage formed as a byproduct of UV-radiation (Bavoux, Hoffmann et al. 2005).

A similar study has revealed that *Polκ*$^{-/-}$ mouse ES cells are sensitive to both killing and mutagenesis induced by benzo(a)pyrene, which through metabolic activation, forms benzo(a)pyrene diol epoxide (BPDE) (Ogi, Shinkai et al. 2002). *In vitro*, wild-type mammalian cells are 67% more proficient in the bypass of dG-N$^2$-BPDE adducts (BPDE-adducted guanine) than *Polκ*$^{-/-}$ cells (Avkin, Goldsmith et al. 2004). Furthermore, Polκ is able to bypass dG-N$^2$-BPDE adducts with higher efficiency and greater accuracy than other DNA polymerases by correctly inserting dCMP opposite an adducted dG-N$^2$-BPDE

(Avkin, Goldsmith et al. 2004). A biological role for Polκ in the bypass of $N^2$-BPDE-G adducts (or dG-$N^2$-BPDE-*like* adducts) is indirectly supported by the observation that both the mouse and human *Polκ* gene promoters harbor arylhydrocarbon receptors (AhR), which induce *Polκ* gene expression when activated with benzo(a)-pyrene-*like* compounds (Ohmori, Ohashi et al. 2004). Collectively, these data indicate that Polκ has a biologically important role in cellular resistance to benzo(a)pyrene. However, although the ability of Polκ to correctly bypass dG-$N^2$-BPDE is conserved in *E. coli*, it is unlikely that *Polκ* evolved for this role, given that *E. coli* do not have the enzyme required to activate benzo(a)pyrene into BPDE (Ohmori, Ohashi et al. 2004). Evidence that Polκ is also highly efficient in the bypass of lesions such as bulky $N^2$-guanine adducts and estrogen-derived lesions (which resemble the polyaromatic-ringed structure of BPDE) indicates a likelihood that Polκ functions in the bypass of endogenously manufactured lesions generated by naturally occurring metabolites (Suzuki, Yasui et al. 2004; Fischhaber and Friedberg 2005; Choi, Angel et al. 2006).

Aside from these damage-induced phenotypic analyses of *Polκ*-deficient mice, it has been observed that *Polκ*$^{-/-}$ male mice exhibit a modest elevation in germ line spontaneous mutagenesis by ESTR analysis (Burr, Velasco-Miguel et al. 2006). Indications for germ line mutagenesis are further evidenced by our

laboratory's observation of a mutator-like phenotype in *Polκ*$^{-/-}$ offspring. Pups borne of *Polκ*$^{-/-}$ ancestry exhibited diverse phenotypes, including diabetes insipidus, vitiligo, hydro-nephrosis, cytoskeletal defects, and ataxia (E.C. Friedberg, S. Velasco-Miguel, L.D. McDaniel, unpublished observations). From these observations, I hypothesize that Polk maintains a role in protecting germ line and somatic tissues from the otherwise mutagenic bypass of spontaneous DNA damage performed by other "non-cognate" polymerases. Furthermore, given the demonstrated proficiency of Polk in the accurate bypass of adducted guanines, this function is anticipated to be reflected in the spontaneous mutation spectra of *Polκ*-deficient mice.

I set out to experimentally examine somatic spontaneous mutation frequencies in *Polκ*$^{-/-}$ and wild-type mice using the Stratagene Big Blue cII-system. The Big Blue transgenic mouse mutation detection assay provides a powerful tool for examining mutations *in vivo* and has been validated extensively (Monroe, Kort et al. 1998; Zimmer, Harbach et al. 1998; Harbach, Zimmer et al. 1999; Zimmer, Harbach et al. 1999; Swiger 2005). I observed that spontaneous mutation frequencies in *Polκ*$^{-/-}$ tissues (liver and kidney) are elevated almost two-fold at middle-age (9 and 12 months). Furthermore, I determined the mutation spectrum of these tissues and observed a distinct increase in transversion mutations G:C>C:G and C:G>A:T. These results not only suggest that *Polκ* has a

role in accurately bypassing spontaneous DNA damage cumulative with age, but the mutation spectra further provide insight into which base-pair lesions are mutagenic in the absence of *Polκ*.

Liver and kidney DNA from Big-Blue *Polκ*$^{-/-}$ or wild-type mice were subjected to the λ Select-cII mutation detection system. Mutant plaques were isolated and sequenced to obtain corrected mutation frequencies (Table 1). I observed an age-dependent increase in spontaneous mutagenesis most evident at 9 and 12 months of age. However, I did not observe a significant difference at the age of 3 months, suggesting that an age-related accumulation of pre-mutagenic DNA damage is prerequisite for detecting an elevation in *Polκ*$^{-/-}$ spontaneous mutation frequencies.

Based on average values at ages 9 and 12 months of age, *Polκ*$^{-/-}$ kidney had relatively **1.8** and **1.4** fold more mutations than wild-type, while *Polκ*$^{-/-}$ liver had **2.1** and **1.6** fold more mutations than wild-type (Figure 1A and 1B). Mutation frequencies for the liver closely matched those previously reported in the literature (Hill, Buettner et al. 2004). In contrast, when 12 month-old *Polη*$^{-/-}$ kidney and liver DNA were subjected to the λ select-cII mutation detection system, I observed no significant difference between *Polη*$^{-/-}$ and wild-type mutation frequencies in the liver or kidney (Table 1, Figure 2A and 2B). Mice homozygous for a null mutation in *Polη* are viable, fertile, and do not show any

obvious spontaneous defects during the first year of life, observations consistent with these data (Lin, Clark et al. 2006). Thus, an elevation in spontaneous mutagenesis is unique to *Polκ*-deficient mice.

The proposed guidelines for Stratagene's Big Blue system suggest that a total of at least 300,000 pfus should be screened before a mutation frequency is considered significant. In almost all cases presented here, more than 300,00 pfus were screened per animal/tissue. Given that my analysis is limited to a small population of mice, I selected the Wilcoxon rank sum test to determine the statistical significance of the difference observed between mutation frequencies. The Wilcoxon rank-sum test is a nonparametric alternative to the two- sample t-test, which is based solely on the order in which the observations from the two samples fall (given that each dataset exhibits a similar distribution). The Wilcoxon rank-sum test indicated that the difference in mutation frequency observed between *Polκ*$^{-/-}$ mice and WT mice was *marginally significant* in 12-month and 9-month kidney and liver (p=0.028, two-tailed) (Figure 1). However, in 3-month old *Polκ*$^{-/-}$ and WT kidney and liver, there was no statistical difference in mutation frequencies (kidney: p= 0.34, two-tailed; liver: p=0.56, two-tailed). Furthermore, there was not a significant difference between 12-month *Polη*$^{-/-}$ and WT liver or kidney mutation frequencies (liver: p=0.49; kidney: p=0.68) (Figure 2) indicating that statistically significant changes in spontaneous mutation

frequency only occurred in *Polκ*$^{-/-}$ mice at the ages of 9 months and 12 months (Figure 1).

Although the extent to which *Polκ*$^{-/-}$ spontaneous mutation frequencies were elevated appears moderate, it is important to note that the mutation frequencies observed in my 12 month *Polκ*$^{-/-}$ mice are comparable to mutation frequencies observed in 24 month-old wild-type mice (Hill, Buettner et al. 2004). These data correlate with our observation that *Polκ*$^{-/-}$ mice have a lower survival rate than *Polκ*$^{-/+}$ or wild-type mice. For example, at 100 weeks of age (23 month), **62%** (n=40) of wild-type mice are alive, while only **37.5%** (n=56) of our *Polκ*$^{-/-}$ mice remain (L.D. McDaniel, E.C. Friedberg, unpublished observations). The cause of death for these mice is unknown, yet the implications of a prematurely mutated genome may provide some explanation.

Given that the spontaneous mutation frequencies of *Polκ*$^{-/-}$ mice were elevated with respect to age, it was of interest to know if a particular pattern predominated the mutation spectrum of different *Polκ*$^{-/-}$ tissues, and how this may have varied with age. I found that the mutation spectra for *Poλκ*$^{-/-}$ kidney or liver closely resembled each other at 9 and 12 months of age. In addition, *Polk*$^{-/-}$ mutation spectra between kidney and liver were also very similar, regardless of the age of the mouse (Figure 3).

A particularly useful aspect of the Big Blue-cII system is that it enables one to determine *which* mutations are elevated in *Polκ*$^{-/-}$ mice. There were two likely outcomes to be expected from this analysis: i) an overall proportional increase in all types of mutations, or ii) a specific increase in certain types of mutations. I found the latter to be the case in the *Polκ*$^{-/-}$ liver and kidney at 9 and 12 months of age. The *Polκ*$^{-/-}$ kidney and liver mutation spectra exhibited a signature pattern that indicated which base-pair lesions are targets for mutagenesis in the absence of Polκ (Figure 3). The mutations elevated in *Polκ*$^{-/-}$ mice were predominantly C:G>A:T and C:G>G:C base substitutions (Figure 3) with highly significant p-values compared with WT or *Polη*$^{-/-}$ (Table 2). Furthermore, the data very clearly delineated that transversion mutations are predominantly elevated in the *Polκ*$^{-/-}$ liver and kidney, in contrast to transition mutations which were relatively unchanged (Figure 5A and 5B). As expected, mutation spectra for *Polη*$^{-/-}$ mice, were similar to wild-type (Figure 4 and data not shown).

These data provide evidence to support that the absence of functional Polκ results in error-*prone* bypass events that occur most frequently at sites of adducted guanine (or cytosine). Given that these observations are age-dependent, the possibilities remain that damage to guanines or cytosines increases with age, or that repair functions are in decline. These data may also suggest that another polymerase(s) acts in place of *Polκ*, strongly favoring the resulting G:C>T:A or

G:C>C:G transversion events. In the yeast *S. cerevisiae* (not endowed with a *Polκ* gene), Rad30/Polη has been shown to play a role in the mutagenic bypass of dG-$N^2$-BPDE by inserting A or G opposite the adducted G, resulting in G>T and G>C transversion mutations (Zhao, Xie et al. 2004). Similarly, mouse Polη bypasses dG-$N^2$-BPDE by inserting A or G more frequently than the correct C (Ogi *et al.* 2004). With such evidence, it is not unreasonable to hypothesize a mutagenic role for Polη or another specialized polymerase in the bypass of adducted G:Cs of *Polκ*$^{-/-}$ mice.

When considering the nature of mutagenic events in a *Polκ*$^{-/-}$ background, it is important to recognize that the local DNA environment can play a role in determining the efficiency of mutagenesis at a particular DNA position (Friedberg et al. 2005). The cII gene is ~50% G:C, with reported hotspots identified most frequently at CpG sites (Harbach, Zimmer et al. 1999). Thus, I suspected the possibility that *Polκ*$^{-/-}$ tissues may have preferential G:C base pairs mutated more frequently than others. However, analysis of *Polκ*$^{-/-}$ mutation sites compared with wild-type did not reveal obvious consensus sites for *Polκ*$^{-/-}$ mutations within the cII locus. While one or more positions were more frequently mutated in *Polκ*$^{-/-}$ tissues (data not shown), these patterns did not hold consistent between different tissues, or even the same tissues at different ages and thus do not likely comprise hotspot mutations.

Finally, a unique characteristic of *Polκ*$^{-/-}$ mutation spectra was the elevation of tandem base mutations (TBMs). The liver and kidney of 12-month old *Polκ*$^{-/-}$ mice showed a higher frequency of tandem-base substitution mutations. Hill *et al.* previously reported a thorough characterization of spontaneous tandem-base mutations (TBMs) in several different wild-type mouse tissues using the comparable *lacI* Big Blue system, showing that the TBM signature of each tissue and age varies in pattern and spectrum (Hill, Wang et al. 2003). In addition, they observed that TBMs are significantly more frequent in the kidney and liver, as compared to 12 other mouse tissues including skin, brain, heart, and male germ line. TBMs are also more frequent in tissues of older mice between one and two years of age (Hill, Wang et al. 2003).

When comparing frequencies of TBMs in my wild-type and *Polκ*$^{-/-}$ mice with published wild-type frequencies, several observations were made. First, I noted that TBMs were more frequent in 12-month *Polκ*$^{-/-}$ liver and kidney, compared to wild-type and/or published values (Figure 6) (Hill, Wang et al. 2003). In addition, when I compared the frequency of TBMs in 12-month *Polκ*$^{-/-}$ liver and kidney to *Polη*$^{-/-}$ liver and kidney, I found the frequency of TBMs was greatest in *Polκ*$^{-/-}$ tissues (data not shown). All of the TBMs observed from *Polκ*$^{-/-}$ and wild-type tissues were composed of GC base-pairs mutated to AT base pairs, resembling the most common sub-type of spontaneous dinucleotide

mutations previously observed in wild-type tissues (Hill, Wang et al. 2003). Tandem base mutations are speculated to arise from lipid peroxidation, although this notion requires further support (Hill, 2004). This data suggests that *Polκ* may be important for minimizing tandem error during the replicative bypass of endogenously arising DNA lesions.

The results presented here demonstrate that *Polκ*$^{-/-}$ tissues have an elevated frequency of spontaneous mutations in somatic tissues at middle age. The increased mutability of *Polκ*$^{-/-}$ cells is a phenotype reminiscent of the response of *Polη*-deficient XP-V cells to UV-light. These observations provide a different example of the paradoxical situation of an error-prone DNA polymerase actually protecting cells from inducing mutations. These findings are supported by the enzymatic specificity of Polκ protein *in vitro* when presented with different damaged templates. For instance, experiments analyzing the efficiency of Polκ in the bypass of eight different $N^2$-guanyl adducts variable in size showed that, compared to Polι and Polη, Polκ is the most quantitatively efficient enzyme for the correct incorporation of dCTP opposite these adducts (Choi, Angel et al. 2006). Similarly, it has been shown that Polκ is capable of inserting dCTP opposite model estrogen-derived dG-$N^2$-3MeE lesions with a frequency of 13% of the normal dC-dG base pair (Suzuki, Yasui et al. 2004). Furthermore, DT40 *Polκ*$^{-/-}$ cells are sensitive to estrogen-induced chromosomal aberrations, strongly

suggesting that estrogen-adducted guanines are natural substrates for Polκ (Mizutani, Okada et al. 2004; Suzuki, Yasui et al. 2004). Given that Polκ is expressed in steroid-rich metabolic centers such as the adrenal gland, it is feasible that Polκ may have a role in the bypass of steroid adducts. These data, together with the aforementioned relationship between Polκ and the efficient bypass of dG-$N^2$-BPDE, uniformly implicate an important role for Polκ in the error-free bypass of damaged guanine.

The findings demonstrated here are consistent with our laboratory's previously reported observation of elevated spontaneous mutation frequencies in the male germ line of *Polκ*$^{-/-}$ male mice and a potential mutator phenotype (Bavoux, Hoffmann et al. 2005; Burr, Velasco-Miguel et al. 2006). In Figure 7, I outline how these observations may be brought together into a single model. Perhaps the increased somatic mutation rate results in acquired disease states that cause the premature death we have observed in our *Polκ*$^{-/-}$ mice cohorts, while germ line mutations in *Polκ*$^{-/-}$ mice may be a predisposing factor for mutator phenotypes.

These studies support the notion of a function for Polκ in the error-free bypass of spontaneous somatic DNA damage, particularly in the bypass of adducted G:C base-pairs. Given what is know of the biological consequences of mutational

burden, the mechanistic details of how Polκ fulfills this role are of significant future interest.

## *Materials and Methods*

### Generation of Big Blue/*Polκ*$^{-/-}$ mice

Big Blue mice carrying 80 copies of chromosomally integrated λLIZ shuttle vector were obtained from Stratagene (C57B/6 strain background.) The *Polκ*$^{-/-}$ mutant mice used for this study contain a deletion in exon 6 which abolishes the polymerase function of Polκ. Our *Polκ*$^{-/+}$ mice (129/Ola backcrossed twice into C57B/6; ~75% C57B/6) were mated with Big Blue mice to obtain wild-type, heterozygous, and *Polκ*$^{-/-}$ mice carrying 40 or 80 copies of the λLIZ shuttle vector. Relatively equal numbers of male and female mice for each genotype were aged to the appropriate time, and sacrificed at 3 months, 9 months, and 12 months of age.

### Generation of Big Blue/*Polη*$^{-/-}$ mice

Big Blue mice carrying 80 copies of chromosomally integrated λLIZ shuttle vector were obtained from Stratagene (C57B/6 strain background) and mated with *Polη*$^{-/+}$ (129/Ola/C57B/6) mice from the laboratory of Dr. Raju Kucherlapati (Lin, Clark et al. 2006). These mice have a deletion in exon 4 which results in a shift in the open reading frame in exon 5 and generates a stop codon 30 nucleotides

down-stream (Lin, Clark et al. 2006). *Polη*$^{-/-}$ mice carrying 40 or 80 copies of the λLIZ shuttle vector were aged to 12 months and sacrificed. All mice were housed in a pathogen-free facility.

## Genotyping of Big Blue/*Polκ*$^{-/-}$ or Big Blue/*Polη*$^{-/-}$ mice

Genomic DNA was isolated from mouse tails and used as the DNA template for PCR or Q-PCR. For *Polκ* genotyping, primers mpkg-F1 (5'TTGATGAAGAACAATTCAG-CAAAGAC3'), mpkg-b1a (5'GCATTAAAATAGATCACAAAAGCAGAAGAC3'), and mpkg-b1b (5'GAGATGCCTTAGCGGGTAAAGC3') were used in combination at a 1:1:1 concentration at a 58°C annealing temperature to amplify either a 400bp fragment (mutant), 587bp fragment (WT), or both (heterozygous). For *Polη* genotyping, primers XPV-F7 (5'AAGGGACAAGCGAACAGAGA3'), XPV-R14 (5'AGCAATATCACAGGC-CCAAC3'), and XPV-R1 (TCACTTCAACACTAGCTTCCC3') were used in combination at a 1:1:1 concentration at a 58°C annealing temperature to amplify either a 500bp fragment (mutant), a 370bp fragment (WT), or both (heterozygous). For detection of the λLIZ shuttle vector, primers cII-F (5'CCACACCTATGGTGTATG3') and cII-R (5'CCTCTGCCGAAGTTGAGTAT3') were used to PCR-amplify a 432-bp band using a 52°C annealing temperature with 5% DMSO. To further determine whether mice were hemizygous or homozygous for the λLIZ shuttle vector, Q-

PCR was used to quantify relative cII copy numbers using primers CII-F1 (5'CTGCTTGCTGTTCTTGAATGGG3') and CII-R1 (5'CGCTCGGTTGCCGCC3') with Stratagene's Brilliant Q-PCR Mastermix. Primers were used at an optimized concentration of 0.5μM.

## Isolation of DNA and Packaging into λ-Phage

Tissues were harvested at the time of sacrifice, flash frozen, and stored at -80°C. DNA was isolated from the kidney and liver tissues of the designated mice using the Recover-Ease DNA isolation kit (Stratagene). Pelleted cell nuclei were digested with proteinase K and digestion buffer containing RNace-It (Stratagene) at 50°C for 45 minutes. The digested DNA was then dialyzed in 1X TE buffer overnight. 8-12 μL of high molecular weight DNA was recovered the next day and packaged into λ-phage using Transpack packaging extract (Stratagene).

## Transformation into *E. coli*

Packaging extracts were diluted in 966μL of SM Buffer. Triplicates of 100x dilutions were made and 100μL or 20μl of each triplicate was transformed into G1250 *E. coli* culture (in $MgSO_4$, OD=0.5) for titering. The remaining amount of diluted packaged DNA was used to transform G1250 *E. coli* cells for the screening (x10). Transformations were plated onto TB1 plates for titers and screening using heated TB1 top agar cooled to 55°C. Titer plates were grown at

37°C overnight and screening plates were grown at room temperature (24°C) for 48h.

## Verification of putative λ cII⁻ mutants

The putative mutant plaques were cored and transferred to a 96 well plate containing 200μL of SM buffer per well and stored at 4°C overnight. The next day, putative plaques were individually transformed and plated (1μL per transformation) at low density on TB1 media, and grown at the selective temperature (24°C) for 48h. Any visible plaques seen before or at 48h were cored and transferred to a new 96 well plate containing 200μL of SM buffer per well and stored at 4°C indefinitely.

## PCR amplification and sequence analysis of λ CII⁻ mutants

Verified mutant plaques immersed in SM buffer were directly used as PCR template. CII-F and CII-R (Stratagene) primers were used to amplify the cII promotor and open reading frame. Cycling parameters: 94°C 3 min.; 30 cycles of 94°C 30 sec.; 53°C 1 min.; 72°C 1 min.; 72°C extension 10 min. 5μL of each PCR reaction was treated with 2μL of ExoSap-It enzyme (GE Healthcare) and incubated at 37°C for 30 min., followed by a heat-shock at 80°C for 15 min. Each sample was sequenced with the CII-R primer using the ABI Big Dye Terminator Cycle Sequencing Kit on an automated ABI Prism 3100 Genetic Analyzer. The

open-reading frame of the cII gene and the promotor region immediately upstream of the cII gene were analyzed for mutations.

## Mutation frequencies

Raw mutation frequencies were corrected for jackpot mutations. Jackpot mutations are those that arise from clonal expansion, whereby a cell undergoes a cII mutation and subsequently divides to create a clonally expanded population carrying that mutation. Because one cannot distinguish between mutant clonality and duplicate mutation events, I took a conservative approach and counted each mutation once for each tissue analyzed. A corrected mutation frequency represents the total number of independent mutations over the total number of plaque forming units screened (PFUs). Values were averaged to represent inter-animal variations (Figure 1), or totaled values were calculated for each age group/genotype (Table 1). Eleven different types of mutations were encountered, which include C:G>A:T, G:C>A:T, G:C>C:G, T:A>A:T, A:T>G:C, A:T>C:G, large insertions or deletions (n>2 b.p.), frame-shift insertions or deletions (-1 or +1), tandem base mutations (x'x→y'y), or NM (no mutation detected in the region sequenced). Base substitutions are categorized due to the inability to detect in which strand the initial mutation occurred. In order to compare specific mutation-type frequencies, the number of mutations per specific mutation type was divided by the total number of PFUs screened.

## Statistical Analyses of Mutation Frequencies

The standard deviation of the mutation frequency for each age and genotype was calculated by standard methods. The error margin does not take into account the number of mutant plaques screened per sample, but rather merely reflects inter-animal variation for each genotype/age group. In order to determine statistical significance for the differences observed between different genotypes, the non-parametric Wilcoxon rank-sum test was used to obtain a two-tailed *p*-value (http://elegans.swmed.edu/~leon/stats/utest.cgi). In order to determine the statistical significance of the differences observed between specific mutation types, mutation spectra were pooled for each tissue (by age and genotype) and a *p*-value was assigned using a two-tailed Fisher's exact test (Table 2).

## *Tables and Figures*

### Table 1

| ***12 month kidney*** | | | | | |
|---|---|---|---|---|---|
| ***Polκ$^{-/-}$*** | | | | | |
| | **Mouse ID** | **Sex** | **PFUs** | **Jackpots/Corrected** | ***M. Freq.(1E$^{-5}$)** |
| | 5149 | F | 560,000 | 4/28 | 5.00 |
| | 5148 | F | 572,750 | 20/31 | 5.41 |
| | 5156 | M | 490,000 | 9/24 | 4.90 |
| | 5160 | M | 1,206,500 | 28/78 | 6.46 |
| **total:** | | | **2,829,250** | **61/161** | **5.69** |
| **WT** | | | | | |
| | 5137 | F | 510,000 | 6/12 | 2.35 |
| | 6522 | F | 431,666 | 7/19 | 4.40 |
| | 6525 | M | 1,040,000 | 15/22 | 2.12 |
| | 5163 | M | 863,500 | 5/27 | 3.13 |
| **total:** | | | **2,845,166** | **33/80** | **2.81** |

***Polη$^{-/-}$***

| | | | | |
|---|---|---|---|---|
| 6475 | F | 553,333 | 0/10 | 1.81 |
| 6538 | F | 671,667 | 4/16 | 2.38 |
| 6517 | M | 788,333 | 4/18 | 2.28 |
| 6511 | M | 486,667 | 3/15 | 3.08 |
| **total** | | **2,500,000** | **11/59** | **2.36** |

***12 month liver***

***Polκ$^{-/-}$***

| **Mouse ID** | **Sex** | **PFUs** | **Jackpots/Corrected** | ***M. Freq.(1E$^{-5}$)** |
|---|---|---|---|---|
| 5148 | F | 591,500 | 16/44 | 7.44 |
| 5149 | F | 1,256,500 | 32/79 | 6.29 |
| 5156 | M | 679,333 | 12/34 | 5.00 |
| 5160 | M | 346,667 | 1/17 | 4.90 |
| **total** | | **2,874,000** | **61/174** | **6.05** |

**WT**

| | | | | |
|---|---|---|---|---|
| 6522 | F | 380,000 | 2/18 | 4.74 |
| 5137 | F | 596,500 | 36/23 | 3.86 |
| 5163 | M | 781,667 | 7/23 | 2.94 |
| 6525 | M | 297,000 | 2/11 | 3.70 |
| **total** | | **2,055,167** | **47/75** | **3.65** |

***Polη$^{-/-}$***

| | | | | |
|---|---|---|---|---|
| 6475 | F | 391,666 | 8/22 | 5.62 |
| 6538 | F | 725,000 | 24/29 | 4.00 |
| 6517 | M | 567,500 | 10/27 | 4.76 |
| 6511 | M | 475,000 | 22/11 | 2.32 |
| **total** | | **2,159,166** | **64/89** | **4.12** |

***9 month kidney***

***Polκ$^{-/-}$***

| **Mouse ID** | **Sex** | **PFUs** | **Jackpots/Corrected** | ***M. Freq.(1E$^{-5}$)** |
|---|---|---|---|---|
| 5171 | M | 994,000 | 23/47 | 4.73 |
| 5154 | F | 1,375,000 | 19/48 | 3.49 |
| 5155 | F | 930,000 | 6/29 | 3.12 |
| 5191 | M | 666,000 | 13/25 | 3.75 |
| **total** | | **3,965,000** | **61/149** | **3.76** |

**WT**

| | | | | |
|---|---|---|---|---|
| 5153 | M | 920,000 | 18/26 | 2.83 |
| 6540 | M | 505,000 | 6/15 | 2.97 |
| 5150 | F | 460,000 | 0/13 | 2.83 |
| 5165 | F | 876,667 | 6/18 | 2.05 |
| **total** | | **2,761,667** | **30/72** | **2.61** |

***9 month liver***

***Polκ$^{-/-}$***

| | Mouse ID | Sex | PFUs | Jackpots/Corrected | *M. Freq.(1E$^{-5}$) |
|---|---|---|---|---|---|
| | 5191 | M | 258,350 | 8/12 | 4.64 |
| | 5164 | F | 616,667 | 7/24 | 3.89 |
| | 5154 | F | 880,000 | 16/60 | 6.82 |
| | 5155 | F | 341,500 | 2/21 | 6.15 |
| **total** | | | **2,096,517** | **33/117** | **5.58** |

**WT**

| | | | | | |
|---|---|---|---|---|---|
| | 5153 | M | 728,333 | 13/15 | 2.06 |
| | 5165 | F | 573,000 | 4/16 | 2.79 |
| | 5150 | F | 363,000 | 3/10 | 2.75 |
| | 6546 | M | 705,000 | 6/19 | 2.70 |
| **total** | | | **2,369,333** | **26/60** | **2.53** |

***3 month kidney***

***Polκ$^{-/-}$***

| | Mouse ID | Sex | PFUs | Jackpots/Corrected | *M. Freq.(1E$^{-5}$) |
|---|---|---|---|---|---|
| | 5196 | F | 1,113,333 | 46/19 | 1.71 |
| | 5195 | F | 960,000 | 17/15 | 1.56 |
| | 5193 | M | 1,025,000 | 7/24 | 2.34 |
| | 5277 | M | 1,758,333 | 52/42 | 2.39 |
| **total** | | | **4,856,666** | **122/100** | **2.06** |

**WT**

| | | | | | |
|---|---|---|---|---|---|
| | 6575 | M | 1,420,000 | 9/25 | 1.76 |
| | 6573 | M | 618,000 | 10/11 | 1.78 |
| | 6578 | F | 870,000 | 7/8 | 0.92 |
| | 5203 | M | 1,126,666 | 5/14 | 1.24 |
| **total** | | | **4,034,666** | **31/58** | **1.44** |

***3 month liver***

***Polκ$^{-/-}$***

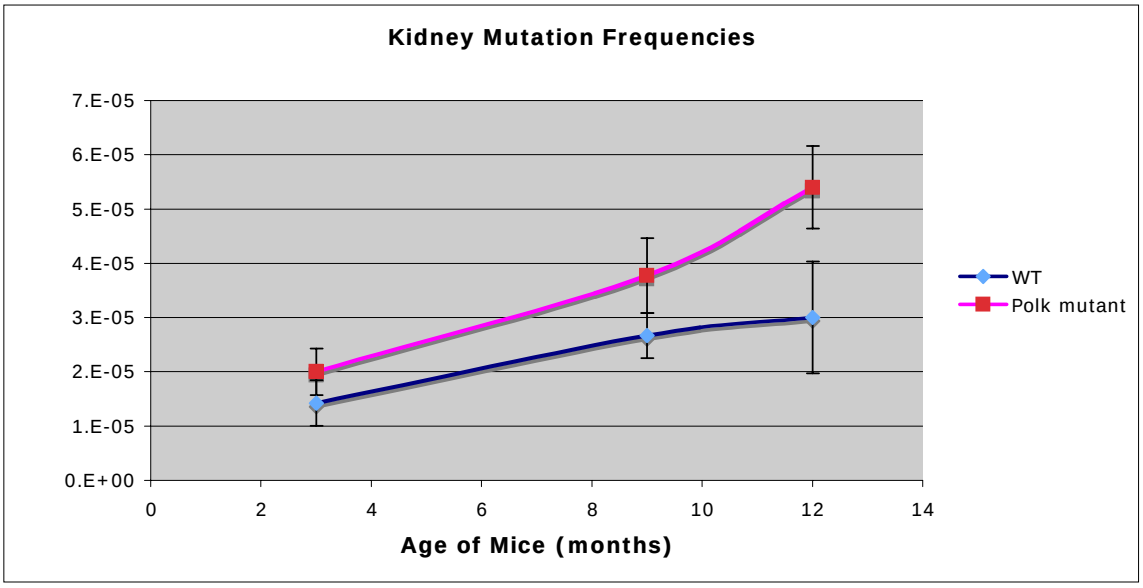
| | Mouse ID | Sex | PFUs | Jackpots/Corrected | *M. Freq.(1E$^{-5}$) |
|---|---|---|---|---|---|
| | 5277 | M | 300,000 | 2/6 | 2.00 |
| | 5196 | F | 1,058,500 | 10/32 | 3.02 |
| | 5193 | M | 550,000 | 2/11 | 2.00 |
| | 5195 | F | 406,667 | 0/13 | 3.20 |
| **total** | | | **2,315,167** | **14/62** | **2.68** |

| | | | | | |
|---|---|---|---|---|---|
| **WT** | | | | | |
| | 6575 | M | 1,200,000 | 16/22 | 1.83 |
| | 6578 | F | 1,206,500 | 11/34 | 2.82 |
| | 5206 | F | 545,000 | 6/15 | 2.75 |
| | 5203 | M | 550,167 | 3/11 | 2.00 |
| **total** | | | **3,501,667** | **36/82** | **2.34** |

**Table 4-1: Data acquired using the Big Blue-cII system for the determination of mutation frequencies.**
Jackpot mutations are indicated. A corrected number of mutations is shown with jackpot mutations removed. The asterisk (*) indicates a mutation frequency corrected for jackpot mutations. Total frequencies are representative additive values for the total number of corrected mutations and total plaque forming units.

**Figure 1**

**A**



**B**

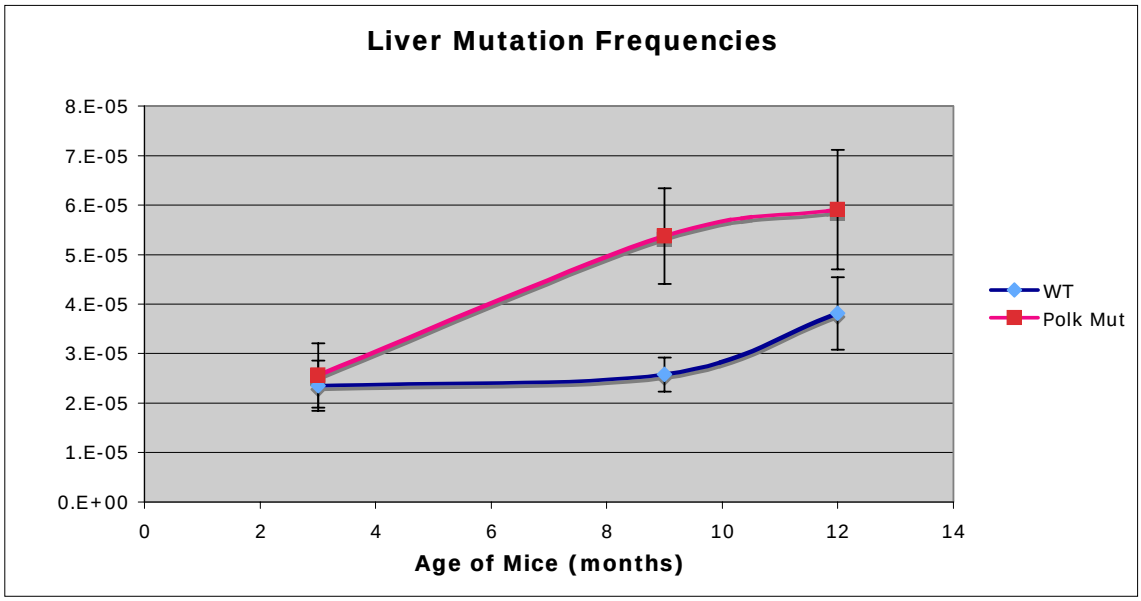
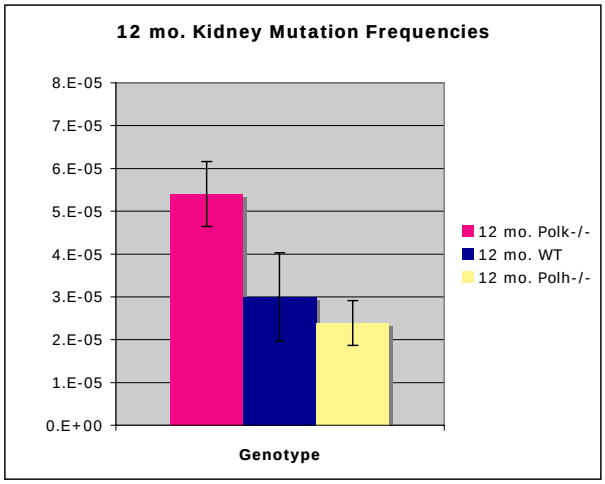



**Figure 4-1: Graphical representation of average mutation frequencies observed in 3, 9, and 12 month-old *Polκ*$^{-/-}$ and WT mice.**
**A)** kidney and **B)** liver. N=4 for each data point shown. Standard deviation is represented by +/- error-bars to demonstrate the degree of inter-animal variation. At 3 months of age, the difference between Polκ$^{-/-}$ and wild-type kidney and liver is not significant (kidney p=0.34; liver p=0.56). At 9 and 12 months of age, the difference between Polκ$^{-/-}$ and wild-type kidney and liver is significant, where p=0.028 (for both), as determined by the Wilcoxon rank-sum test (two-tailed).

**Figure 2**

**A**




**B**

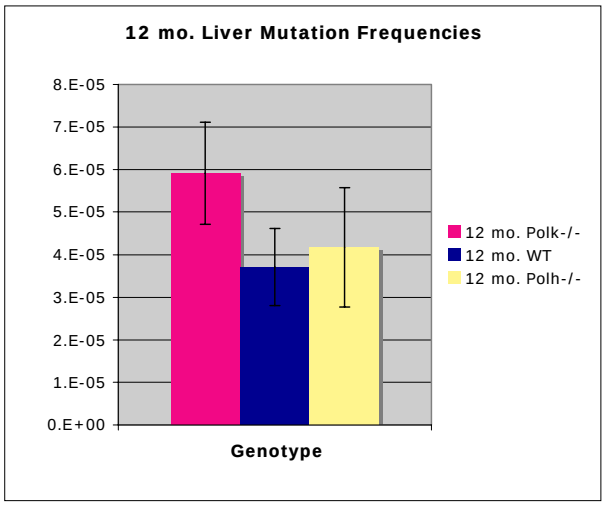
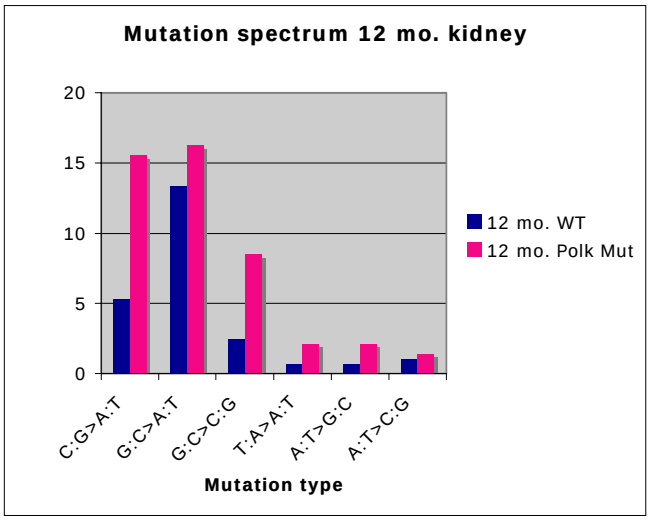


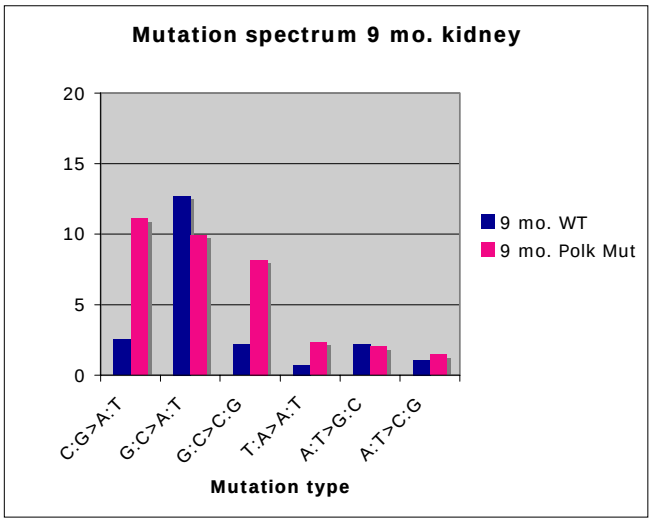

**Figure 4-2: Elevated spontaneous mutation frequencies are not observed in *Pol*$\eta$-deficient mice.**

**A)** kidney or **B)** liver. N=4 for each bar shown. Standard deviation is represented by +/- error-bars to demonstrate the degree of inter-animal variation. At 12 months of age, the difference between *Pol*$\eta^{-/-}$ and wild-type kidney and liver is not significant (kidney p=0.68; liver p=0.49), as determined by the Wilcoxon rank-sum test (two-tailed).

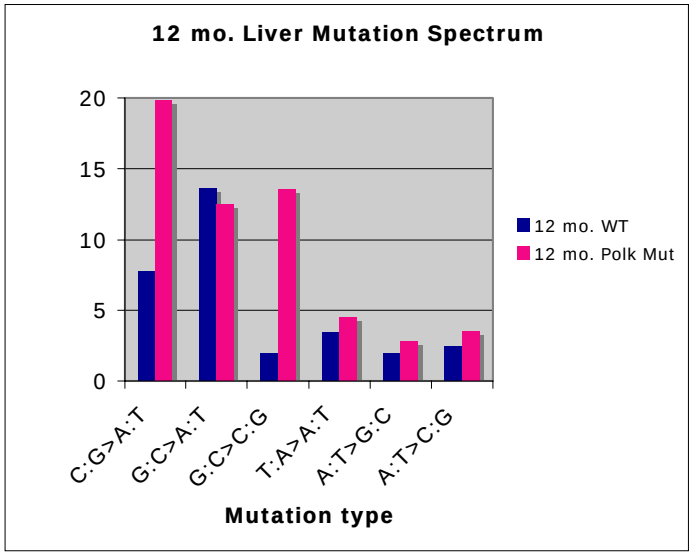
**Figure 3**

**A**



**B**



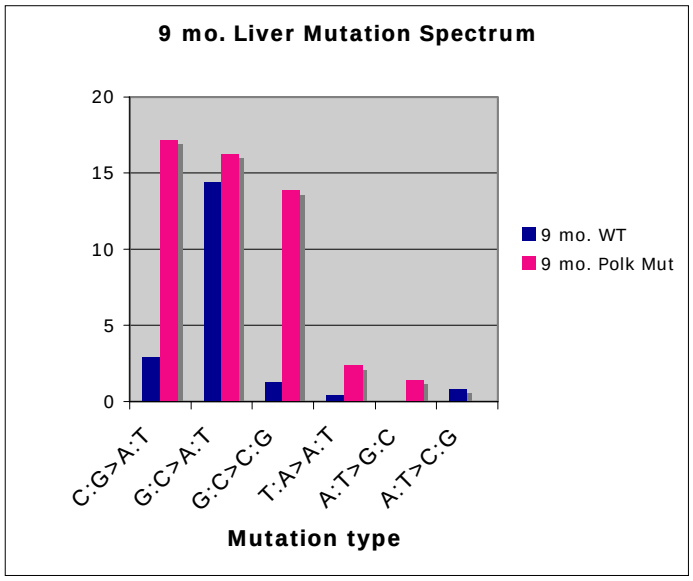
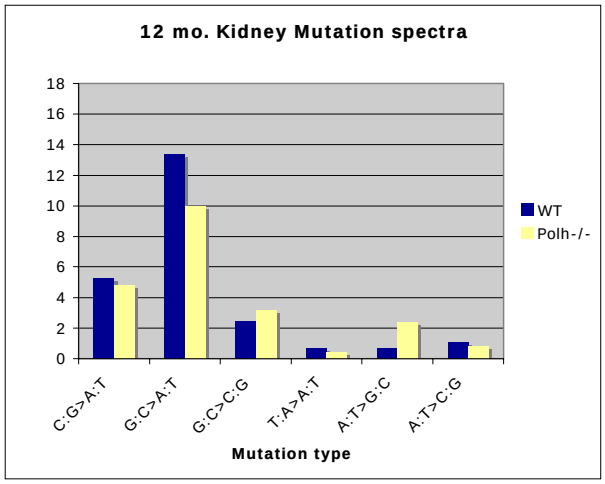




**Figure 4-3: Mutation spectra for *Polκ*$^{-/-}$ and WT tissues.**
**A)** 12 month kidney, 9 month kidney, **B)** 12 month liver, 9 month liver. Each bar represents the number of designated mutation type (for four animals) divided by the total number of plaques screened (pfus).

**Figure 4**

**A**




**B**

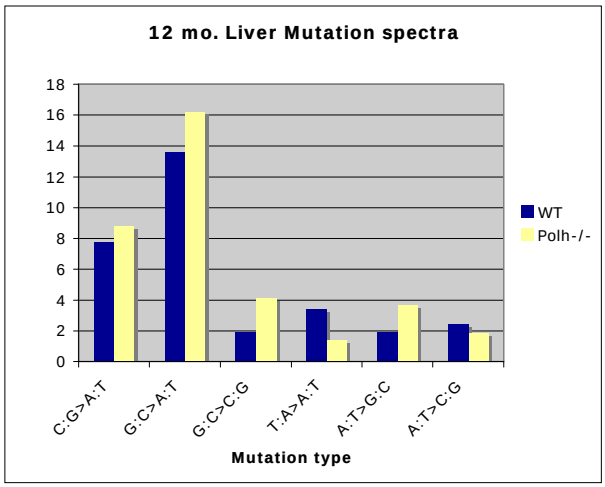



**Figure 4-4: Mutation spectra for *Pol*$\eta^{-/-}$ liver and kidney are similar to wild-type.**
**A)** 12 month-old kidney **B)** 12 month-old liver. Each bar represents the number of designated mutation type (for four animals) divided by the total number of plaques screened (pfus).

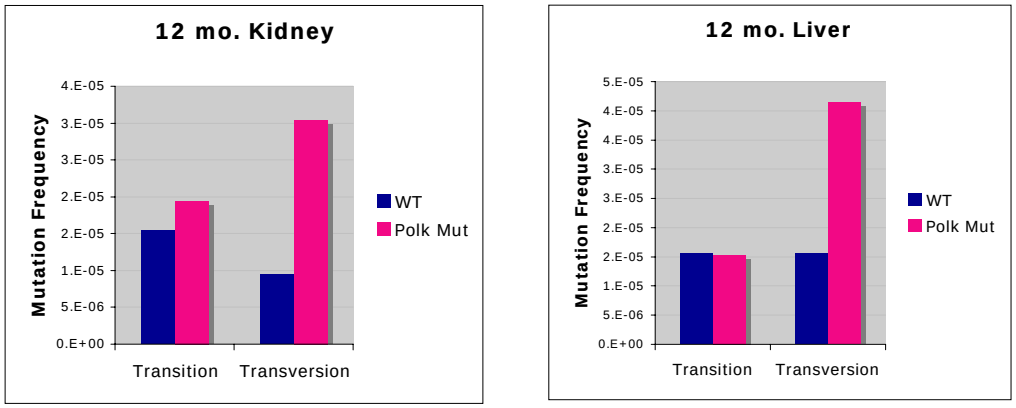
**Table 2**

| *p*-values | 12 mo. kidney | | 12 mo. liver | | 9 mo. kidney | 9 mo. liver |
|---|---|---|---|---|---|---|
| | WT/Polκ$^{-/-}$ | WT/Polη$^{-/-}$ | WT/Polκ$^{-/-}$ | WT/Polη$^{-/-}$ | WT/Polκ$^{-/-}$ | WT/Polκ$^{-/-}$ |
| **G:C>T:A** | **0.00003** | 0.06 | **0.0005** | 0.74 | **0.00006** | **0.00028** |
| **G:C>A:T** | 0.33 | 0.63 | 0.8 | 0.53 | 0.23 | 0.27 |
| **G:C>C:G** | **0.001** | 0.19 | **0.000006** | 0.27 | **0.0013** | **0.000045** |
| **T:A>A:T** | 0.11 | 0.16 | 0.65 | 0.22 | 0.14 | 0.24 |
| **A:T>G:C** | 0.04 | 0.05 | 0.77 | 0.11 | 0.78 | 0.31 |
| **A:T>C:G** | 1 | 0.63 | 0.61 | 1 | 0.75 | 0.19 |

**Table 4-2: *P*-values calculated for mutant (*Polκ*$^{-/-}$ or *Polη*$^{-/-)}$ and wild-type mutation spectra, as determined by the two-tailed Fisher's exact test.** Consistent with the patterns observed in 9 and 12 month old *Polκ*$^{-/-}$ liver and kidney mutation spectra, the most significant differences are observed in the G:C>T:A and G:C>C:G mutation groups (denoted by bold numbers).

**Figure 5**

**A**



**B**

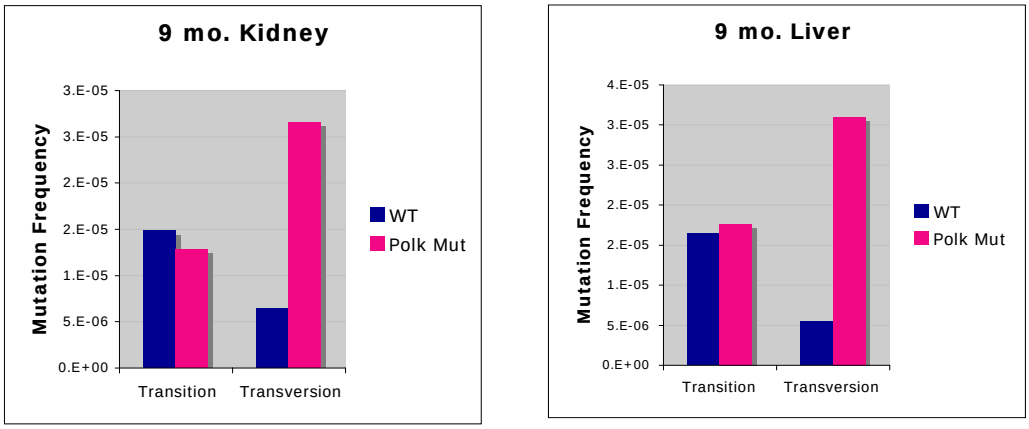



**Figure 4-5: Comparison of transversion and transition mutation distribution in *Polκ*$^{-/-}$ and WT tissues.**
**A)** 12-month old and **B)** 9-month old *Polκ*$^{-/-}$ liver and kidney mutation spectra exhibit elevated transversion mutation frequencies.

**Figure 6**

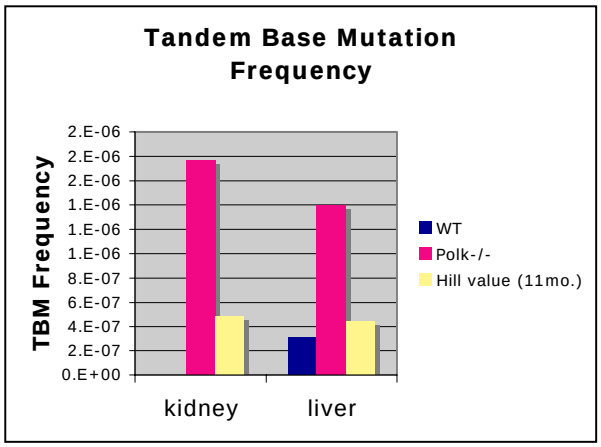



**Figure 4-6: Tandem base mutation frequency analysis.**
TBMs occur more frequently in *Polκ*$^{-/-}$ 12-month kidney and liver than in WT tissues. Hill values are representative of data acquired from untreated wild-type mice at the age of 11 months (Hill, Wang et al. 2003).

**Figure 7**

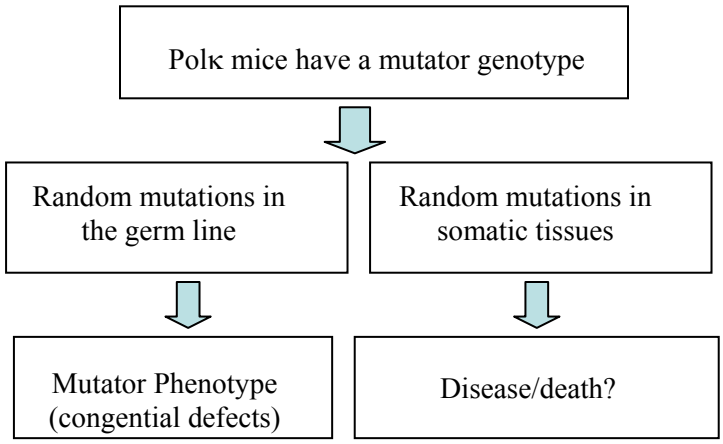



**Figure 4-7: A model for how a *Polκ*$^{-/-}$ mutator genotype may be related to previously reported observations.**
Elevated spontaneous mutation frequencies have been reported in the germ line, and presently in the somatic cells of *Polκ*$^{-/-}$ mice. Mutational burden in these mice may lead to defective offspring and/or acquired diseases states which result in early death ((Burr, Velasco-Miguel et al. 2006), L.D. McDaniel, and E.C. Friedberg, unpublished results).

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