Characterization of the role of *Drosophila melanogaster* Pol32 in Homologous Recombination and DNA Repair

An honors thesis for the Department of Biology, in supplement to the requirements for a Degree of Bachelors of Science.

Michael Shusterman

Tufts University

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ABSTRACT

Double strand breaks (DSBs) in DNA represent one of the most toxic and dangerous classes of DNA lesions. DSB repair (DSBR) occurs through two canonical pathways, homologous recombination (HR) or nonhomologous end joining (NHEJ). In *Drosophila melanogaster*, DSBR of P-element induced breaks is believed to occur through the HR pathway known as synthesis dependent strand annealing (SDSA). A variety of proteins have been implicated in SDSA, but the polymerases involved in initiating and extending repair products have not been fully characterized. The Pol32 subunit of polymerase δ in *S. cerevisiae* has been found to be necessary for break induced replication and various DNA repair pathways. We hypothesize that the *pol32 Drosophila* ortholog may also play a role in processive synthesis during HR repair. Two deletion mutations in *pol32* utilized in this study were generated by an imprecise P-element excision (*L2* and *L30)*. Mutagen sensitivity assays and a site specific DSBR assay were employed to study the mutants. The *L2* null mutation was found to be sensitive to a range of mutagens, including MMS and IR, indicating a role for *pol32* in base excision repair, nucleotide excision repair, replication restart, and HR. Analysis of the repair assay revealed a significant defect in *pol32* mutants for HR repair and processive synthesis. These results implicate *Pol32* as an important player in DSBR and in the processive synthesis of polymerase δ.

CHAPTER 1: INTRODUCTION

1.1 Induction of DNA Damage

The faithful propagation of genetic information during cellular division is critical for ensuring the integrity of the genome in progeny. However, cells are constantly exposed to both exogenous and endogenous agents that can cause aberrant DNA lesions. Mutations resulting from replication errors or damage inducing agents can jeopardize the regulation of cellular proliferation and transcriptional processes. Multiple environmental, metabolic, and stochastic processes can result in the induction of genomic damage in cells (Hoeijmakers 2001; Figure 1A). Spontaneous hydrolysis of DNA bases, alkylation, and reactive radical oxygen species can introduce incorrect bases like uracil, lead to deamination, or produce abasic sites (Friedberg et al. 2005). Hydrolytic damage has been estimated to lead as many as 10^4 depurinations per human cell which must be repaired by the cell (Lindahl 1993). Replication errors can result in mismatches and insertion/deletions that can distort crucial coding sequences. Exposure to various forms of electromagnetic radiation will also results in lesions. Ultraviolet radiation (UV) can result in thymine dimers, while X-rays (ionizing radiation, IR) and γ radiation will cause breaks in the phosphodiester backbone of the DNA double-helix. The accumulation of multiple single strand breaks (SSBs) in close proximity resulting from damage to the DNA backbone can generate particularly toxic lesions known as double strand breaks (DSBs).

1.2 General Cellular Responses to Damage

Cellular responses to DNA damage will vary depending upon the type of damage inflicted. Damage responses in proliferating cells will often involve cell cycle arrest during various checkpoints activated during the G1, S, G2 and M cell phases. Signaling checkpoints

may lead to the activation of multiple repair mechanisms. Many of these mechanisms serve complementary and overlapping roles in recognizing and repairing damage (Figure 2). Severe damage that cannot be handled by repair mechanisms and that hinders transcription and replication can cause the cell to undergo apoptosis. Cells which escape apoptosis and that suffer gross chromosomal rearrangements, mutations, and various other distortions of the genome may become malignant. The loss of tumor-suppressor genes, the activation of oncogenes, and damage to vital repair and damage sensory pathways may lead to oncogenesis, aging, or the development of a genetic disorder (Figure 1B; Hoeijmakers 2009).

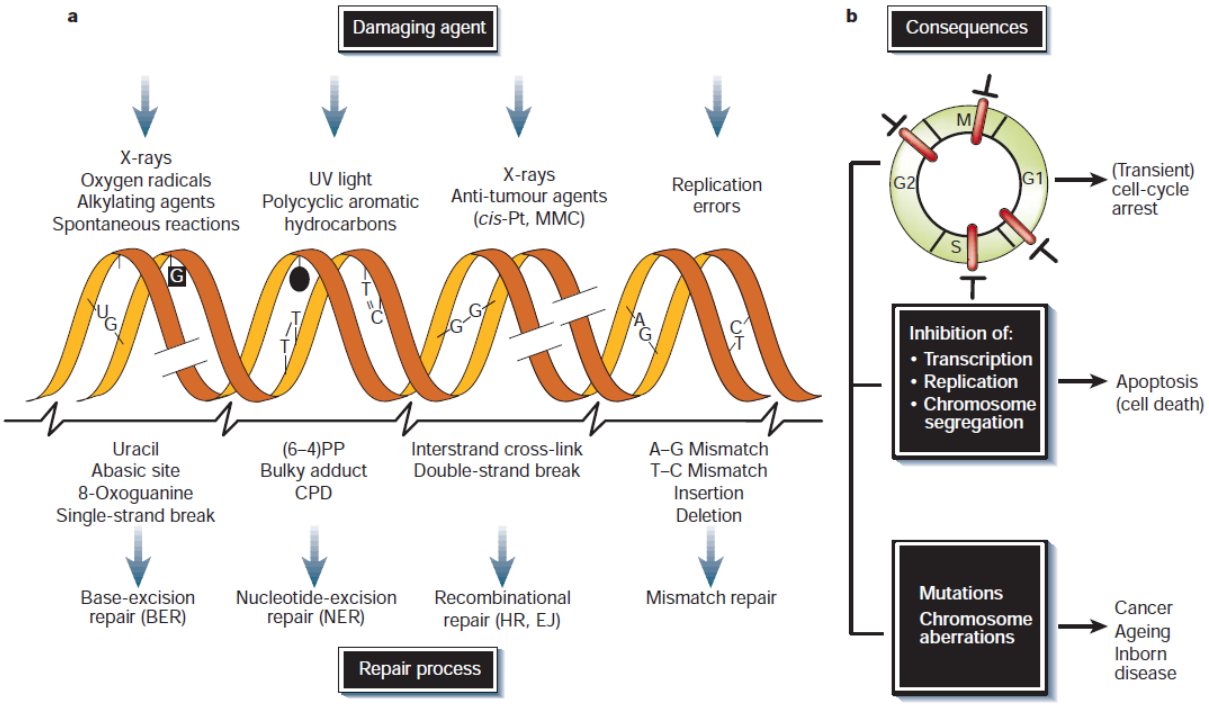

Figure 1. Cellular consequences of DNA damage and ensuing repair mechanisms. (A) Introduction of DNA damage by various endogenous and exogenous agents (top) results in a variety of lesions (middle) which can be repaired by multiple mechanisms (bottom). (B) DNA damage may result in activation of cell cycle checkpoints during G1, S, G2 and M phases and initiate cell cycle arrest (top). Severe damage to metabolic pathways may result in cell death (middle), while accumulated mutations and aberrations in the genome may lead to malignancies and various pathologies (bottom). (Adapted from Hoeijmakers 2001)

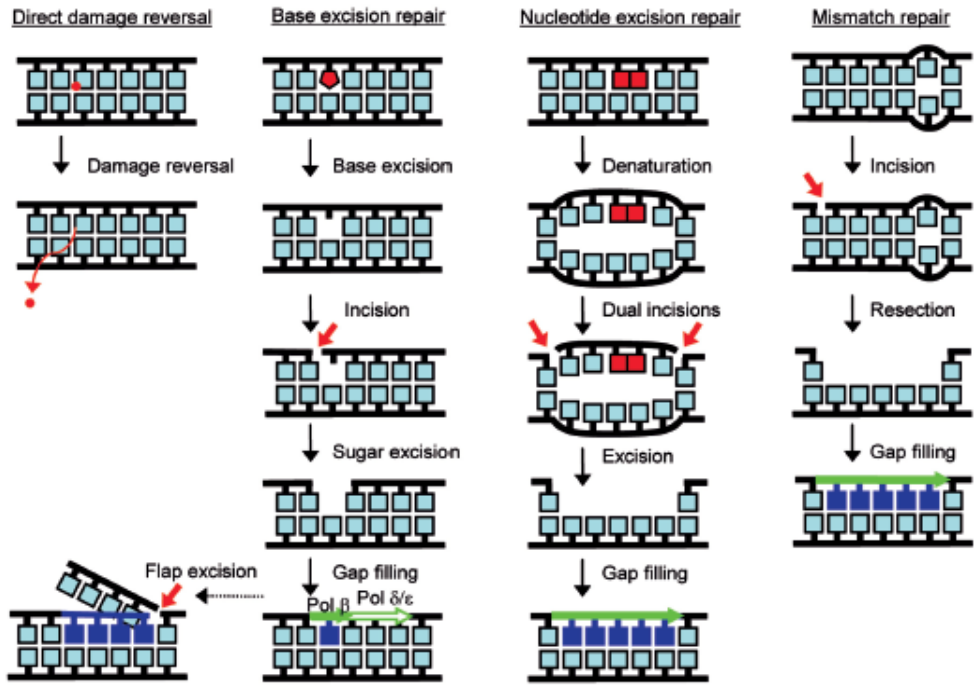

Figure 2. Standard pathways for DNA repair in cells. From left to right: (1) Direct damage reversal (DDR) is an elegant pathway allowing for the reversion of chemical defects in DNA through enzymatic restoration of the original structure. Unfortunately, DDR is limited to the removal of several types of specific alkylated bases and dipyrimidine crosslinks (reviewed in Ecker et al. 2009). (2) Hydrolytic damage that results in the introduction of uracil, apurinic or apyrimidinic sites, SSBs, or oxidative lesions can be repaired through base excision repair (BER). BER requires the recognition of damaged bases by a specialized glycosylase and the excision of the lesion. The abasic site is then excised by an endonuclease and repair can occur via either short patch or long patch mechanisms (reviewed in Wilson and Bohr 2007). (3) Lesions which distort the double helix are repaired by a flexible pathway known as nucleotide excision repair (NER). Bulky DNA adducts formed by chemicals and carcinogens, inter and intrastrand crosslinks, and photoadducts can all be handled by NER. NER follows a complex pathway in eukaryotes in which recognition of helical distortions is accompanied by excision of the damaged strand and repair synthesis by processive polymerases delta or epsilon (reviewed in Nouspikel 2009b). (4) Finally, replication or recombination induced base mismatches are repaired through a pathway known as mismatch repair (MMR) in which mismatches are recognized and excised (reviewed in Iyer et al. 2003). (Adapted from Nouspikel 2009a)

The most toxic genetic lesions, DSBs, direct the activation of a checkpoint signaling cascade, resulting in recruitment of factors leading to initiation of various repair mechanisms (reviewed in Su 2006). This class of lesions is dangerous enough to be lethal to cells if not repaired and can lead to the loss of as much as 100 Mb of information (Helleday et al. 2007). DSBs may arise in cells either through endogenously or exogenously induced damage (described above) or through controlled cellular pathways. Both meiotic recombination and immune receptor diversity depend upon the controlled creation of DSBs in specific locations. During meiotic recombination DSBs are generated by the highly conserved enzyme Spo11 to allow for the exchange of genetic information between homologous chromosomes (Keeney and Neale 2006). In the immune system the coding sequences of immunoglobulins and T-cell receptors found on B- and T-lymphocytes are assembled via V(D)J recombination. Here variable (V), diversity (D), and joining (J) segments are rearranged through induced DSBs, allowing for the production of vast numbers of receptors for the immune system (Soulas-Sprauel et al. 2007).

However, no matter their origin – whether through damage or carefully regulated pathways DSBs must be promptly repaired by the cell to avoid the introduction of significant gross chromosomal rearrangements into the genome. Failure to repair DSBs may result in rearrangements, including translocations, duplications, inversions and deletions, and lead to genome instability and malignancies (Aguilera and Gómez-González 2008). DSB repair defects have been linked to a variety of hereditary diseases and the aging process. Defective repair of DSBs has been associated with multiple conditions including ataxia-telangiectasia, Bloom's syndrome, breast cancer, and Fanconi anemia (McKinnon and Caldecott 2007). Double strand break repair (DSBR) is therefore critical for both insuring the integrity of genetic information and the regulation of critical cellular processes.

1.3 Mechanisms for Repair of Double Strand Breaks

Two broad classes of repair mechanisms exist to handle DSBR: non-homologous end joining (NHEJ or EJ) and homologous recombination (HR). Both pathways are initiated through the binding of the MR(X)N complex to the ends of the DSB (MRN in vertebrates and MRX in yeast). This complex aids in tethering the DSB ends to allow for further processing (Figure 3A). Subsequently, repair may be funneled into one of the two major repair pathways. NHEJ involves the religation of the broken ends of the DNA and has often been referred to as a less accurate repair mechanism due to the potential loss of nucleotides during repair. At the same time, NHEJ is a robust pathway capable of handling many types of breaks without the need for homology and is the dominant pathway during the G1 and M phases of the cell cycle when no easily accessible homologous templates are present (Weterings and Chen 2008). During NHEJ the Ku70/80 heterodimer is formed in conjunction with the MR(X)N complex to stabilize the DSB ends (Figure 3B). Ku70/80 is part of the larger DNA-dependent protein kinase (DNA-PK) complex in vertebrates. The Ku/DNA-PK complex recruits Ligase 4/XRCC4 to the break site and ligation and processing following to reseal the DSB (Figure 3C, 3D).

In the presence of a sister chromatid, during the G2 and S phases of the cell cycle, HR is the preferred pathway. Cyclin-dependent kinases (CDKs) regulate crucial resection steps during DSBR to limit access to HR when the cell is not in G2 or S phase (Aylon et al. 2004). Multiple pathways exist for HR including synthesis-dependent strand annealing (SDSA), the classical DSBR model, break-induced replication (BIR), and single-strand annealing (SSA). All of these pathways share the common 5' to 3' resection of DNA at the DSB involving nucleases in the MR(X)N complex and other enzymes (Figure 3E). Following resection the exposed single strand is coated by RPA, a heterotrimeric complex with high affinity for DNA (Figure 3F). RPA

binding is followed by binding of Rad52, which interacts with RPA and facilitates the loading of Rad51 and the subsequent displacement of RPA (Figure 3G). The Rad 55/57 and Rad54 complexes are also believed to bind the coated single strand to assist in stabilization of the nucleoprotein filament (Figure 3G). The assembled nucleoprotein filament will then invade other DNA duplexes in search of homologous sequences through a poorly characterized mechanism that likely involves random searches along sister chromatids and homologous chromosomes (Pardo et al. 2009). Discovery of a homologous sequence will result in the creation of a displacement loop (D-loop) and the initiation of one of multiple repair pathways depending on the type of DSB (Figure 3H).

The repair of two-ended breaks is believed to be primarily handled through the SDSA pathway. For instance, in *Drosophila melanogaster*, repair of gaps following the excision of transposable P-elements is believed to be repaired predominantly by this pathway (Engels et al 1990; Nassif et al. 1994). During SDSA the sister chromatid often serves as the preferred template for repair. Following resection and nucleofilament formation, the protein coated filament invades a sister chromatid forming a D-loop (Figure 4A – 4C). The D-loop is extended by synthesis from the break site and missing sequence is restored (Figure 4D). Migration of the D-loop in the direction of synthesis (Figure 4E) will result in synthesis of DNA that will be homologous to the sequence at the original break site. Upon encountering this sequence the nascent strand can be released and will anneal to the other end of the DSB (Figure 4F). Processing of the extraneous flaps, gap filling, and ligation of nicks completes repair (Figure 4G). The SDSA mechanism differs from the classical DSBR model proposed by Szostak *et al*., in that the nascent strand being synthesized within the homologous duplex does not capture the other end of the DSB. If the other end of the DSB is also extended by synthesis, a structure

known as a Holliday junction (HJ) will result (Szostak et al. 1983). Differential resolution of HJs can lead to both crossover products and noncrossover products.

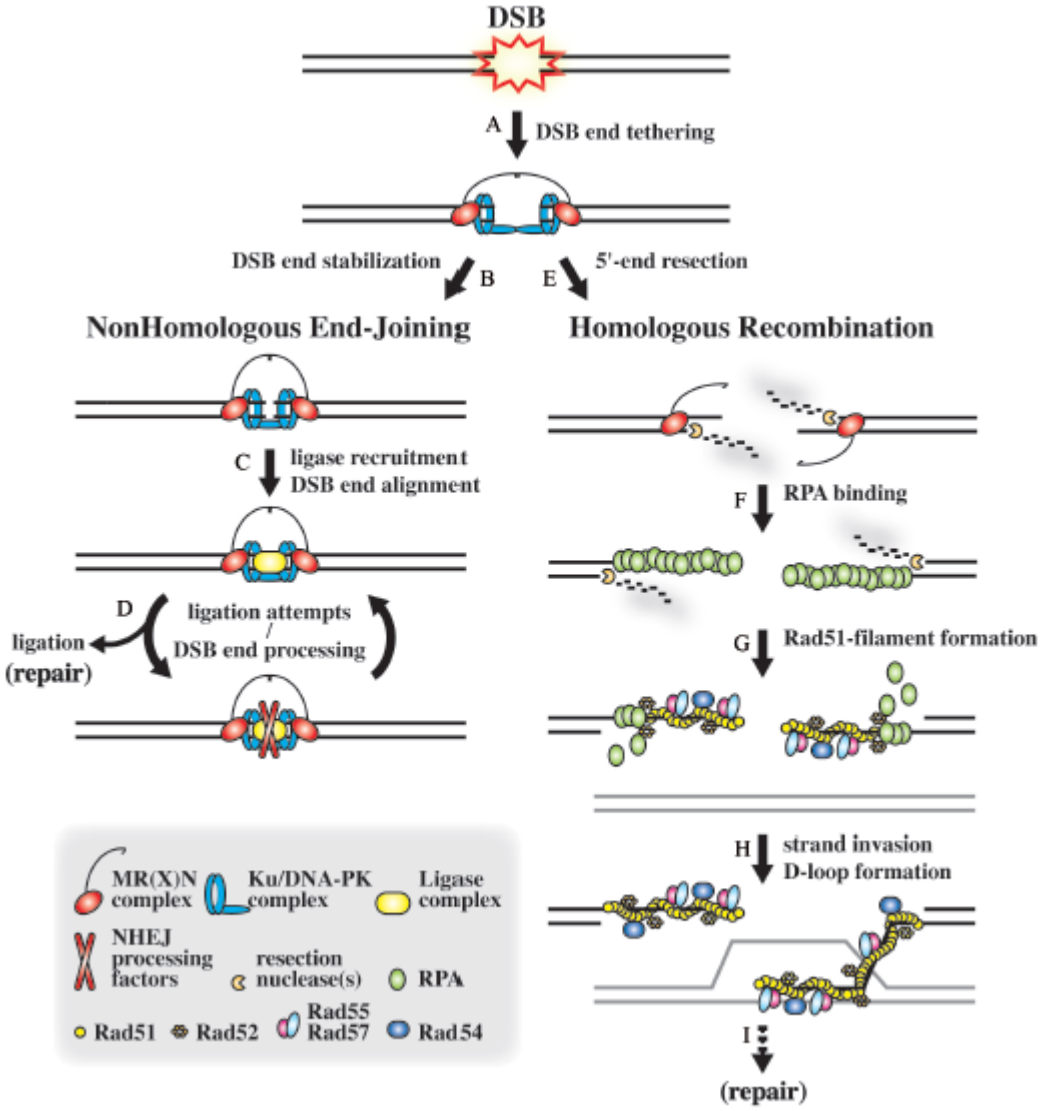

Figure 3. DSBR pathways through NHEJ and HR. (A) The DSB is tethered by the MR(X)N complex. (B) Progression into the NHEJ pathway results in recruitment of the Ku/DNA-PK complex. (C) Ku/DNA-PK recruits ligases and additional processing factors to seal the break. (D) Ligation and processing will occur to repair the damage. (E) Homologous recombination repair will involve nucleases degrading DNA 5' to 3' to expose single strands. (F) RPA binds with high affinity to single stranded DNA. (G) Rad51 and paralogs bind to the exposed DNA and displace RPA. (H) The nucleoprotein filament invades a homologous duplex and forms a D-loop (I) Repair synthesis ensues by one of multiple pathways. (Figure adapted from Pardo et al. 2009)

In the event that only one end of the DSB is available for repair due to loss of the other end, a mechanism known as BIR is used to repair the break (Llorente et al. 2008). During BIR unidirectional synthesis proceeds from the D-loop formed by invasion of the nucleofilament. Multiple BIR models have been proposed including continuous synthesis of entire chromosome arms, multiple strand invasions and disassociations, or even resolution of the D-loop into a full replication fork. Finally, if no template exists for the DSB to repair off of, resection of the DSBs may ensue for hundreds of base pairs until homologous sequences are located at both ends of the break and annealed to one another through the SSA pathway.

1.4 Homologous Recombination Repair Synthesis and Polymerase Delta

Many of the proteins involved in the various pathways involved in DSBR have been characterized. However, the role of polymerases during HR repair synthesis remains an area of continuing investigation. Recent *in vitro* studies with translesion polymerase eta (Polη), showed that Polη was able to extend synthesis from a synthetic D-loop (McIlwraith et al. 2005). McIlwraith and colleagues speculated that a more processive polymerase like polymerase delta (Polδ) could take over synthesis once polη had initiated synthesis within the D-loop. Though this has not been confirmed by *in vivo* studies, Polδ has been proposed as a candidate polymerase for HR synthesis.

Polδ is an essential replicative polymerase that is currently believed to be the lagging strand polymerase at the replication fork and is involved in synthesis during mismatch repair, base excision repair, and potentially homologous recombination (McElhinny et al. 2008; Longley et al. 1997; Blank et al. 1994). In alternative replication models in cells lacking the

main leading strand polymerase pol epsilon (pol ε), Polδ is also believed to take over leading strand synthesis (Burgers 2009).

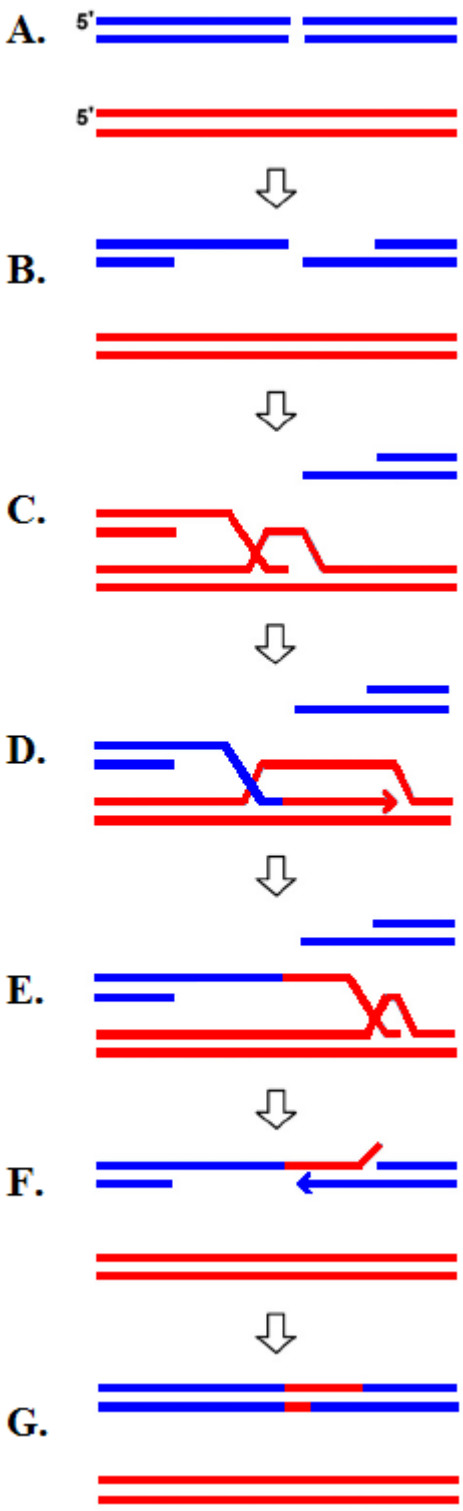

Figure 4. Synthesis dependent strand annealing mechanism. The SDSA model is believed to be the predominant mechanism by which homologous recombination directed repair occurs in *Drosophila* following a double strand break induced by P-element excision (A). In SDSA the 5' end of both sides of the break site is resected (B) and the resulting 3' overhangs are employed in conjunction with RAD51 to invade a homologous chromosome or sister chromatid and form a displacement loop (D-loop) between the invading and template strands. (C) As the D-loop migrates, repair synthesis will then proceed along the template (D) and as the nascent DNA encounters a complementary sequence in the original chromosome at the other end of the break (E), it is released (F) and gaps are filled in, completing repair (G). (Adapted from Helleday et al. 2007)

Recent analysis of a nonlethal mutation in the catalytic subunit of Polδ mutants has also suggested that Polδ may be the preferred polymerase for repair synthesis during HR (Maloisel et al. 2008). In *Saccharomyces cerevisiae*, Polδ had been characterized as consisting of three subunits: Pol3 (125 KDa), Pol31 (58 KDa), and Pol32a (55 KD; Gerik et al. 1998) (Figure 5A). Orthologs for the *S. cerevisiae* subunits exist in *Schizosaccharomyces pombe*. In *S. pombe* Polδ is constituted by four subunits, Pol3, Cdc1 (ortholog of Pol31), Cdc27 (ortholog of Pol32), and Cdm1 (Zuo et al. 2000). Cdm1 has no defined homolog in *S. cerevisiae* and is a nonessential subunit for cellular viability and division (Reynolds et al. 1998). In mammalian cells Polδ has also been found to consist of four subunits; PolD1 (p125), PolD2 (p50), PolD3 (p66), and PolD4 (p12; Podust et al. 2002). PolD4 appears to be the ortholog of Cdm1, while the mammalian p66, *S. pombe* Cdc27, and *S. cerevisiae* Pol32 subunits also share homology.

Pol3 is the catalytic subunit of the holoenzyme and has both polymerase activity and a 3' to 5' proofreading exonuclease (Boulet et al. 1989; Simon et al. 1991). Pol3 interacts with Pol31 to form the incomplete, but stable, Polδ* complex (Burgers and Gerik 1998). Pol32 is attached to Pol31 by its N-terminal domain and in conjunction with the Polδ* complex reconstitutes the full holoenzyme (Burgers and Gerik 1998). Both Pol3 and Pol31 are essential for cellular viability and null mutations in either gene leads to cell death (Hashimoto et al. 1998; Gerik et al. 1998). While in S. *pombe* Cdc27 is an essential gene and interacts with PCNA through a C-terminal domain, in *S. cerevisiae* Pol32 is a nonessential subunit that plays an important role in DNA replication, DNA repair, and mutagenesis (Reynolds et al. 2000; Gerik et al. 1998; Johansson et al. 2004). Early biochemical analyses proposed an interaction model for the three common subunits of S. *pombe*, *S. cerevisiae*, and mammalian cells (Figure 5A). Recent analysis of *S. cerevisiae* Polδ_T consisting of Pol3, Pol31, and Pol32N (amino acids 1 – 103) with small-

angle X-ray scattering (SAXS) revealed that Polδ$_T$ adopts an elongated conformation (Figure 5B, Jain et al. 2009). Jain and colleagues (2009) confirmed earlier biochemical analyses by demonstrating that Pol3 and Pol32N do not interact. SAXS analysis also revealed that the elongated structure of Pol32 may allow for the protein to interact with other complexes and proteins during replication or synthesis (Jain et al. 2009).

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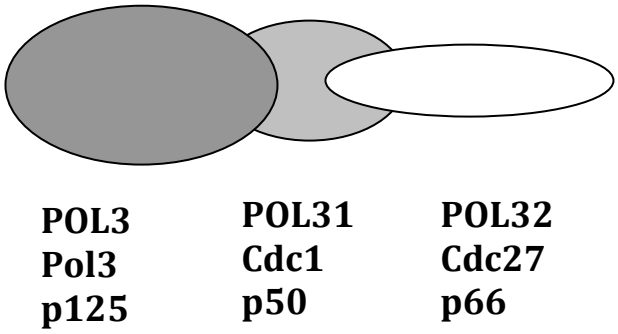

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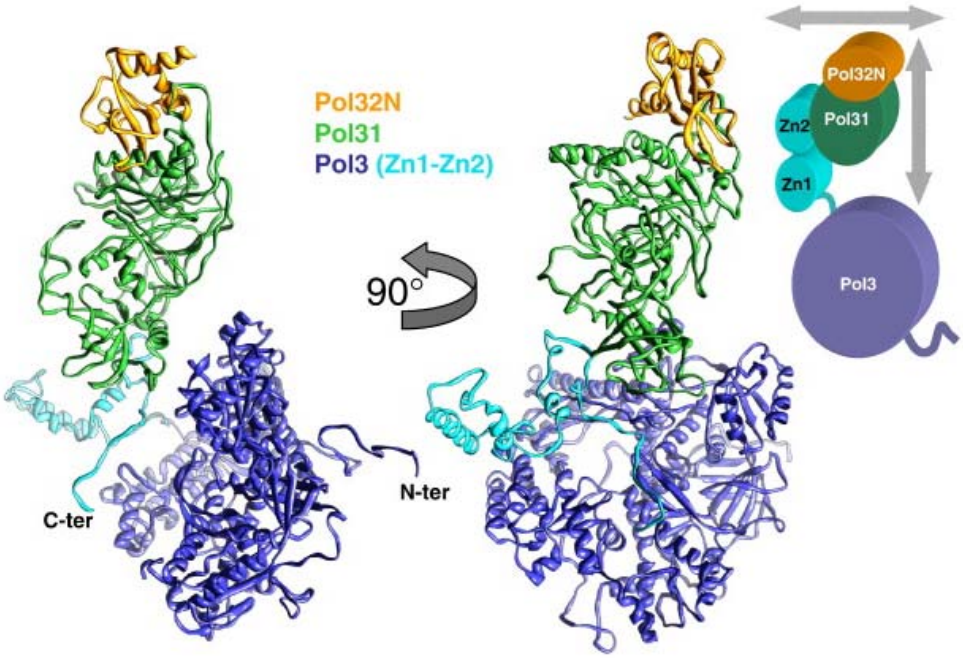

Figure 5. Models of interaction of subunits comprising the replicative polymerase Polδ. (A) Proposed model for interactions of Polδ subunits. Interactions are noted as Pol3 –^{N}Pol31^{C}-^{N}Pol32^{C}. Orthologs are provided for *S. cerevisiae* (POL3, POL31, POL32), *S. pombe* (Pol3, Cdc1, Cdc27), and humans (p125, p50, p66). (Adapted from Johansson et al. 2001) (B) Small-angle X-ray scattering model for Polδ$_T$ consisting of Pol3, Pol31, and Pol32N (amino acids 1 – 103). (Adapted from Jain et al. 2009)

1.5 Pol32 Subunit of Polymerase Delta

Pol32 is a nonessential subunit whose deletion is known to impact the function of Polδ. *S. cerevisiae* deletion mutants for Pol32 exhibit cold sensitivity for growth and sensitivity to various DNA damaging agents. Pol32 mutants have been found to be sensitive to both UV damage and methyl methanesulfonate (Gerik et al. 1998). Yeast-2-hybrid analysis has revealed that at minimum the first 92 amino acids of POL32 are necessary for interaction with POL31. In fact, the full Polδ holoenzyme can be reconstituted in the presence of the first 102 amino acids of POL32. The PCNA consensus binding domain for Pol32 lies at the extreme carboxy-terminal end of the protein, while interactions between Pol32 and Pol1 (Pol α-primase) are mediated through a domain upstream of the PCNA binding domain (Johansson et al. 2003). *In vitro* studies using plasmid replication assays demonstrate that in the presence of PCNA the PCNA binding domain on Pol32 plays a less crucial role than the Pol31 interaction domain in the processivity of Polδ (Johansson et al. 2003).

Additional studies have explored the role that Pol32 plays in DNA repair. Pol32 has been implicated in mediating translesion synthesis by polymerase zeta and in the pathways resulting in chromosomal translocations and segmental duplications (Hanna et al. 2007; Ruiz et al. 2009; Payen et al. 2008). Furthermore, Pol32 is an essential component of BIR. Lydeard and colleagues (2007) determined that Pol32 plays a key role in BIR, but not in gene conversion, through a site specific HO endonuclease system that induces DSBs in *S. cerevisiae* and requires repair by BIR. The role of Pol32 in HR synthesis, however, remains controversial. Jain and colleagues (2009) have published work in which they argued that Pol32 may play a role in break repair initiation during HR. Meanwhile, Smith and colleagues (2009) found evidence to suggest

that strand invasion and initiation of synthesis was not defective in Pol32 mutants, but that extension of the 3' end of the nascent strand was.

1.6 Experimental System and Focus

We turned to *Drosophila melanogaster* to investigate the potential role Pol32 in HR DSBR and repair synthesis. *Drosophila* has proven to be a versatile model organism, allowing research into eukaryotic genetics, developmental biology, and chromosomal theory (Sekelsky et al. 2000). In addition, a wide range of genetics and molecular assays exist to study DSBR in *Drosophila*. In particular, P-element (transposable element) based repair assays allow for the analysis of site specific DSBs. Breaks and mutations in specific genes can be induced by crossing flies with a non-autonomous P-element to flies containing a transposase source (Ryder and Russell 2003).

The Pol32 ortholog CG3975 in *Drosophila* was discovered through a bioinformatics search of the *Drosophila* genome. *pol32* mutants were generated via imprecise P-element excision by the laboratory of Yikang Rong. Here we describe the characterization of two mutants, $pol32^{L2}$ and $pol32^{L30}$. Sequence analysis revealed that $pol32^{L30}$ mutants retain the majority of the N-terminal domain of the protein, while $pol32^{L2}$ mutants eliminate most amino acids present in the wild type protein. The $pol32^{L2}$ homozygotes exhibit a short bristle phenotype characteristic of defects in DNA replication and $pol32^{L2}$ homozygous females are infertile. We found $pol32^{L2}$ mutants to be sensitive to a range of mutagens including MMS, hydroxyurea, ionizing radiation, and high doses of nitrogen mustard. Sequence analysis and mutagen sensitivities strongly suggest that $pol32^{L2}$is a null mutation, while $pol32^{L30}$ is a hypomorphic mutation retaining most of the crucial N-terminal domain required for Pol31 interaction.

To study the role of Pol32 in DSBR we employed the site specific *P{w^a}* DSBR assay. We found *pol32^{L2}* mutants to be deficient in SDSA repair and long distance synthesis from the right end of the P-element. Furthermore, processive repair from the left end of the P-element was impaired in *pol32^{L2}*mutants due to what we believe are sequence barriers to the Pol32 deficient Polδ complex in *pol32^{L2}*mutants. Our findings reveal a role for *Drosophila melanogaster* Pol32 during DNA repair and particularly HR synthesis.

CHAPTER 2: MATERIALS AND METHODS

2.1 Drosophila Stocks and Genetics

Flies were maintained on standard cornmeal agar medium at 25 ºC, supplemented as necessary with dry yeast pellets. The *L2* and *L30* alleles of *pol32* were provided by the laboratory of Yikang S. Rong. *L2* and *L30* flies were maintained over a *CyO*, w^{+}, *GFP* balancer. Information regarding all other genetic stocks and balancers can be found on Flybase (2010). The X-chromosome *P{w^{a}}* transgene used in this study has been previously described in Adams et al. 2003. The transposase source used in the X-chromosome *P{w^{a}}* was *Sb{Δ2-3}*. The *L2* allele *P{w^{a}}* cross can be found in the Supplementary Information.

2.2 Phenotypic Analysis and Viability Studies

Viability studies for *L2* homozygous females were performed by mating *L2* homozygous females to heterozygous *L2* males. *L2* and *L30* bristle defects were visualized in a stereoscopic microscope.

2.3 Mapping of Pol32 Deletions

Primers -306F (5'-TAGCGGCAAGTAGATGTTATCG-3') and reverse primers 1712R (5'-CGATAGCG GAGATAACGAGTTTCG-3'), 1909R (5'-TAGGAATCAAACGCAATCACTG C-3'), and 2469R (5'-GTCA AGGCAAATATCG TGCTAGAG-3') were designed around the expected site of deletion, after excision of the P{EPgy2} element. The polymerase chain reaction (PCR) was used to amplify the sequence between the primer sets -306F/1712R, -306F/1909R, and -306F/2469R, as described in LaRocque *et al.* (2006). PCR conditions used were: 94 °C for 5 min, 16 cycles of 94 °C for 30 sec, 62.0 to 54.0 °C for 30 sec each (-0.5 °C/cycle), 72 °C for 90 sec, 20 cycles of 94 °C for 30

sec, 58 °C for 30 sec, 72 °C for 90 sec, and finally, 72 °C for 5 min. The PCR products were run on a 1% agarose gel and visualized by staining with ethidium bromide. The 500 bp band for *L2* (-306F/1712R) and the 1400 bp band for *L30* (-306F/2469R) were extracted from the gel, purified using a QuickClean 5M Gel Extraction Kit according to the manufacturer's recommendations (GenScript Corporation 2006), and sequenced at the Tufts Core Facility.

2.4 Sequence Alignment

Amino acid multiple sequence alignment was performed using the CLUSTAL-W software as described in the instruction manual (Larkin et al. 2007). Sequence comparisons were made between Pol32 othologs for *S. pombe* Cdc27, *Drosophila* CG3975, *S. cerevisiae* POL32, and human p66.

2.5 Reverse Transcriptase PCR

The reverse transcription polymerase chain reaction (RT-PCR) was performed to amplify cDNA derived from RNA extracted from *L2*, *L30*, and wild type (w^{1118}) *pol32* alleles. RNA was isolated using the RNAqueous®-PCR Kit and RT-PCR performed using the RETROscript® Kit (Ambion Inc.). Briefly, 30 flies were squished and RNA eluted from the preparation. DNase-I was used to eliminate contaminating DNA and was subsequently eliminated from the RNA solution to prevent degradation of DNA created during PCR. The extracted RNA was assessed by spectrophotometer to evaluate purity and concentration of the sample. Extracted RNA was then used in the RT reaction to produce cDNA.

PCR was used to amplify the sequence between two primers, as described in LaRocque et al. (2006). Primers 29F (5'-GCATGATCGATTTCGATCGCTGTG-3'), 29F*L2* (5'-GCATGATCGATTTCGCCTGGCCTA-3'), 562R (5'- GAGAGGACTTGCTGGCCACTG-3'),

and 2469R (5'-GTCAAGGCAAATATCGTGCTAGAG-3') were designed within the *pol32* gene. RP-49 primers 386F (5'-ATCCGCCGAGCATACAGG-3') and 843R (5'-CTCGTTCTCTTGAGAACGCAG-3') were used as controls to ensure the presence of cDNA and analyze possible DNA contamination. Genomic DNA was prepared from an *L2/CyO* and a w^{1118} as described in Gloor et al. (1993). Primer set 29F/562R was optimized for a 68.9 °C annealing temperature. Primer set 29F*L2*/2469R was run at 58.1 °C annealing temperature and primer set 386F/843R was run at 58.8 °C annealing temperature. PCR program conditions for all three primer sets using the respective annealing temperatures were: 30 cycles of 94 °C for 30 sec, 69.9 or 58.8 or 58.1°C for 30 sec, and finally, 72 °C for 5 min. PCR products were run on a 1% agarose gel and visualized by staining with ethidium bromide.

2.6 Sensitivity Assays

Sensitivity assays were performed in the below manner for *L2* and *L30* flies. Heterozygous males and females were sorted into vials and allowed to mate and lay eggs for 3 days before being turned over into new vials. They were then again allowed to mate and lay eggs for 2 days before being removed from the vials. Each first set of vials was aged for one day post-parental removal to ensure treatment of hatched larvae. Both the experimental and control treatments consisted of 5 vials for each dosage. Progeny were allowed to develop at 25° C and scored for 10 days after the first progeny eclosed. Survival rates were calculated as the ratio of the number of treated surviving mutant homozygotes to the expected number of homozygotes to survive based on the observed survival rate in the control treatment. Sensitivity assays described below followed the above procedure, except where noted.

Methyl Methanesulfonate: Experimental vials were treated with 250 µL/vial of 0.03%, 0.05%, or 0.08% of freshly prepared methyl methanesulfonate (MMS) solution in ddH_2O (distilled deionized). The second set of vials, employed as the control, was treated with 250 µL/vial of ddH_2O.

Camptothecin: Experimental vials were treated with 0.1 mM and 0.05 mM camptothecin (CPT) solution. CPT was prepared by dissolving a 5mg/mL stock in dimethyl sulfoxide (DMSO). All concentrations of CPT were prepared by dilution in 2% Tween/10% Ethanol. The control vehicle was DMSO dissolved in 2% Tween/10% Ethanol.

Nitrogen Mustard: Experimental vials were treated with 0.003%, 0.005%, and 0.008% nitrogen mustard (H(2)N) in ddH_2O. The second set of vials, employed as the control, was treated with 250 µL/vial of ddH_2O.

Hydroxyurea: Experimental vials were treated with 60 mM and 100 mM hydroxyurea (HU) in ddH_2O. The second set of vials, employed as the control, was treated with 250 µL/vial of ddH_2O.

Gamma Rays (Ionizing Radiation, IR): Embryos were collected during the morning and at night from grape agar supplemented with yeast paste and allowed to age until the larvae were in the late second and early third instar. Plates were then irradiated at the Tufts Medical School facility in a Gammator 1000 and irradiated larvae were transferred to fresh bottles of food and allowed to develop. Experimental plates were exposed to 500, 1000, 1500, or 2000 rads of radiation.

Control plates for each cross were allowed to develop without irradiation. Adult progeny were scored for 10 days after the first progeny eclosed.

2.7 *P{w^a}* Assay for Double Strand Break Repair

P{w^a} is a transposable element inserted within an intron of the X-linked *scalloped* gene. The P-element is comprised of a *copia* retrotransposon flanked by 276-bp long terminal repeats (LTRs) embedded within an intron of the w^+ gene, which confers red eye color. This interrupted expression of the white gene results in homozygote female *P{w^a}* flies with apricot eyes and heterozygote female *P{w^a}* flies with yellow eyes (Adams et al. 2003; McVey et al. 2004b). Females with *P{w^a}* and the mutation of choice were crossed to males with a Δ2-3 transposase source and the mutation of choice (*L2*/*L2*). Progeny males containing the transposase source and *P{w^a}* experience excision of the transgene, resulting in a 14 Kb deletion. Individual excision events can repair by EJ or through HR off a sister chromatid in the males' premeiotic germ cells. These repair events were characterized by mating individual males to *P{w^a}* females and observing phenotypes in the female progeny. Unsuccessful excision of the *P{w^a}* element will generate the apricot eye color. Homologous recombination followed by annealing of the 276 bp LTRs of the retrotransposon will result in red eye color. Alternatively, EJ or aborted HR will result in yellow eye color. To determine whether events are EJ or aborted HR, each yellow eyed female is crossed to white *FM7* males.

The resulting white eyed males containing only the repaired X-chromosome were then assayed via PCR to determine synthesis track lengths. Genomic DNA was prepared as described in Gloor *et al.* (1993). The polymerase chain reaction (PCR) was used to amplify the sequence between the two primers, as described in LaRocque et al. (2006). Synthesis track lengths were determined using *P{w^a}* specific primers and touchdown thermocycler programs for the 5 bp,

250 bp, 0.9 Kb, 2.4 Kb, and 4.6 Kb distances from the right end of the P-element as described in Adams et al. (2003). Additional primers were specifically designed for distances at 3.5 Kb, 4.3 Kb, and 5.5 Kb on the right end of the P-element and for the 5 bp, 300 bp, 1 Kb, 2.5 Kb, 3.5 Kb, 4.3 4.5 Kb, 4.8 Kb distances on the left end of the P-element.

2.8 Statistics

A Mann-Whitney statistical test was used to compare HR and EJ events between mutant and wild type flies in the *P{w^a}* assay. Fisher's exact test was used to determine significance for synthesis tract lengths in the same assay between wild type and mutant tract lengths. All nonparametric statistical tests were performed with Graphpad Instat software.

CHAPTER 3: RESULTS

3.1. Generation and analysis of mutations in *pol32* yields two deletions alleles.

In order to understand the role of Pol32 in DSBR, genetic studies were undertaken by creating mutations in the *pol32* locus. Previous bioinformatic analyses comparing known *pol32* orthologs with the *Drosophila* genome had identified the CG3975 locus as *pol32* in *Drosophila melanogaster*. Mutations in the putative *pol32* gene were generated by Yikang Rong and colleagues through an imprecise excision screen utilizing the *mus309* background that has been shown to increase deletion size (Witsell et al. 2009). Deletions were generated by mobilizing the *P{EPgy2}* P-element located 1622 bases into *pol32* (Figure 7A). Two mutations, $pol32^{L2}$ (referred to as *L2*) and $pol32^{L30}$ (referred to as *L30*) were provided for this study. Mutant flies carrying the *L2* and *L30* alleles were found to be viable, although homozygous *L2* females were found to be sterile. Phenotypic characterization of *L2* and *L30* flies revealed defects in bristle morphology with shortened bristles evident as compared to wild type. A severe phenotype was present throughout the *L2* stock in homozygous flies (Figure 6A and 6E). *L30* flies exhibited a range in bristle phenotype severity, from wild type to intermediate loss of one bristle, to *L2*-like severe loss of all bristles (Figure 6B – 6C). These defects may be characteristic of a defect in DNA synthesis during development.

PCR mapping employing a set of nested primers was used to determine the extent of the deletions in the mutant alleles. Both deletions based upon the PCR were estimated to be approximately 1500 – 1700 bp in length. Sequencing of the PCR products revealed a deletion from +43 to +1614 (+1 marks transcription start site, 1571 bp) in *L2* mutant flies and a deletion from +656 to +2304 (1648 bp) in *L30* mutant flies (Figure 7A). As it was unclear whether either of the alleles was a null mutation, RT-PCR was performed to analyze if *L2* or *L30* mutants

produced a transcript. Primers were designed for potential *L2* and *L30* transcript (Figure 8A). RT-PCR of *L30* transcript using primers to the 5' end of the gene revealed a band size of slightly over 500 bp (Figure 8B, Lane 5). A faint band was observed for *L2* transcript of approximately 850 bp (Figure 8B, Lane 9). All RT negative and water control reactions showed no DNA contamination. Primers for transcript from the *rp49* gene were used to confirm the efficacy of the RT reaction on mRNA extracted from *L2*, *L30*, and w^{1118} flies (Figure 8B, Lanes 11 – 18). Transcript levels were not quantified, although the detected transcript band for *L2* was visibly lighter than the *L30* band and both were lighter in comparison to the control *rp49* band.

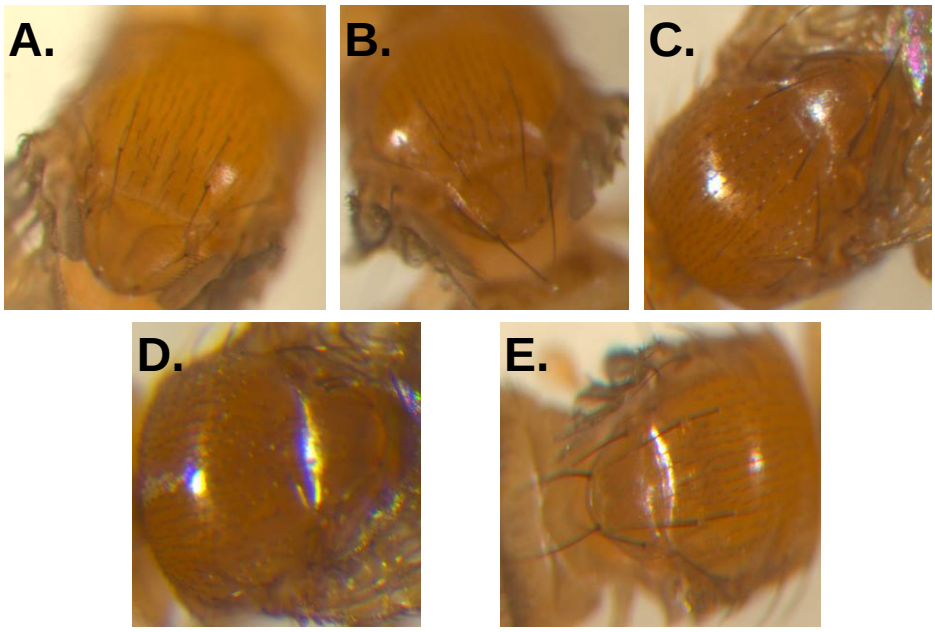

Figure 6. Dorsal view of *L2*, *L30*, and w^{1118} scutellar bristles. (A) *L2* homozygote severe bristle phenotype. (B) *L30* homozygote wild type-like phenotype. (C) *L30* homozygote intermediate bristle phenotype. (D) *L30* homozygote severe bristle phenotype. (E) Wild type w^{1118} bristle phenotype.

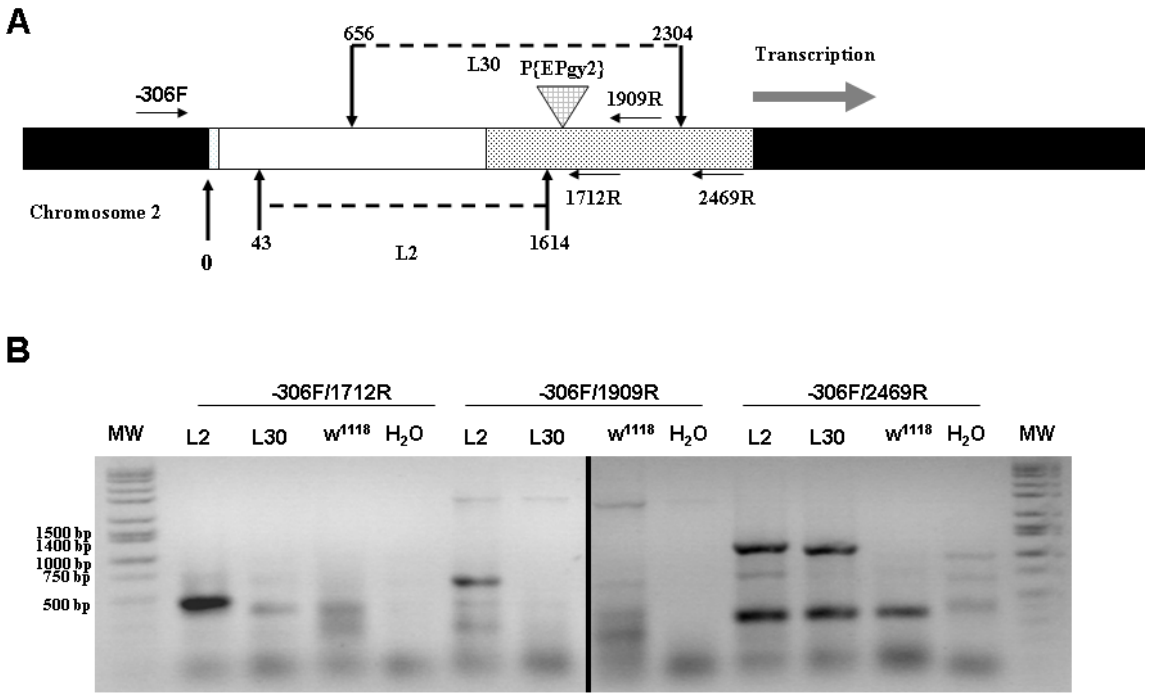

Figure 7. Mapping of *pol32 L2* and *L30* alleles. (A) Schematic of *Drosophila pol32* with dotted areas indicating transcribed regions and clear areas indicating translated regions. Arrows below and above the gene connected by dash lines indicate deletions locations for *L2* and *L30* mutants with the base locations denoted numerically. Primers (-306F, 1712R, 1909R, and 2469R) are indicated by the number of base pairs from the zero point, defined as the start of the gene. The P-element *P{EPgy2}* (triangle) is located 1622 bases into the gene. (B) Determination of *pol32* deletion sizes in *L2* and *L30* mutants, wild-type (w^{1118}) and a no-DNA control (H_2O) using primers in *pol32*. DNA ladder indicated as MW.

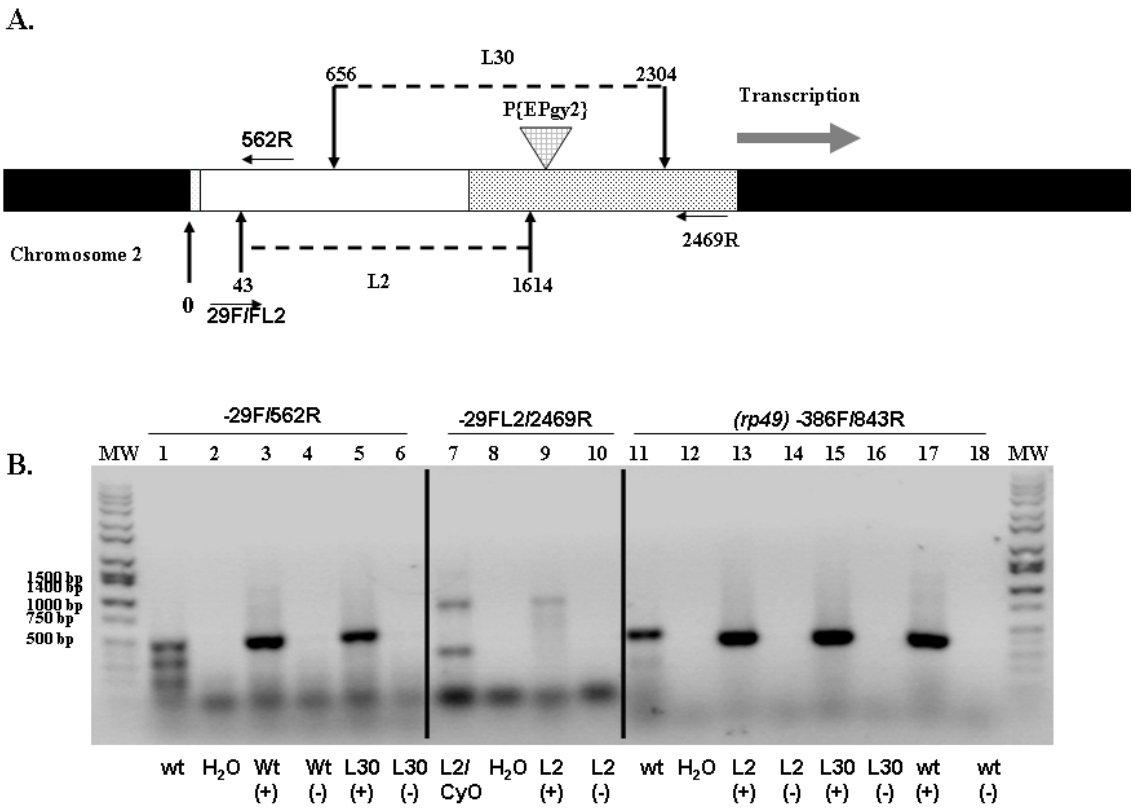

Figure 8. RT-PCR Analysis of *L2* and *L30* alleles. (A) Schematic of *Drosophila pol32* with dotted areas indicating transcribed regions and clear areas indicating translated regions. Primers 29F, 29F*L2*, 562R, and 2469R are indicated by the number of base pairs from the zero point, defined as the start of the gene. The P-element *P{EPgy2}* (triangle) used to generate the deletion is located 1622 bases into the gene. Arrows below and above the gene connected by dashed lines indicate deletion coordinates for *L2* and *L30* mutants with the base locations denoted numerically. (B) RT-PCR for determination of *pol32* transcription products in *L2* and *L30* mutants, and wild-type (w^{1118}). DNA ladder indicated as MW. Primer sets used in each set of reactions indicated above the wells. Primers -29F and 562R were specific to *L30* transcript (lanes 1 – 6, separate by division line). Primers -29FL2 and 2469R were specific to transcript from *L2* (lanes 7 – 10, separate by division line). Primers 386F and 843R were specific to transcript from the *rp49* gene and were used as positive controls (lanes 11 – 18, separate by division line). Labeling below the gel refers to specific genotypes of flies or types of control reaction (plus sign indicates reverse transcriptase reaction, minus sign denotes no reverse transcriptase present, no sign indicates standard PCR from genomic DNA). The numbering scheme above is as such: Lanes 1 – 6 are w^{1118} genomic DNA, H_2O, w^{1118} RT (+), w^{1118} RT (-), *L30*/*L30* RT (+), and *L30*/*L30* RT (-). Lanes 7 – 10 are *L2*/*CyO* genomic DNA, H_2O, *L2*/*L2* RT (+), and *L2*/*L2* RT (-). Lanes 11 – 18 are w^{1118} genomic DNA, water, *L2*/*L2* RT (+), *L2*/*L2* RT (-), *L30*/*L30* RT (+), *L30*/*L30* RT (-), w^{1118} RT (+), and w^{1118} RT (-).

3.2. Sequence analysis of *L2* and *L30* mutations reveals putative Pol31, Pol1, and PCNA interaction domains in *pol32*.

Both *pol32* deletions removed sequence in the translated region of the *pol32* gene (Figure 6A). Bioinformatics analysis was done to determine the extent of the deletions within the protein products of the mutant alleles. The predicted amino acid sequences of *Drosophila* wild type *pol32*, *L2*, and *L30* were compared. *L30* mutants maintained 219 of the 431 amino acids from the wild type *pol32* protein, while *L2* preserved only 14 amino acids from wild type (Figure S2). Cross-species multiple sequence alignment analysis using CLUSTAL-W was performed between orthologs of pol32: *S. pombe* Cdc27 (372 amino acids), *Drosophila* CG3975 (431 amino acids), S. *cerevisiae* POL32 (350 amino acids), and human p66 (466 amino acids; Figure 9).

Functional analysis of *S. cerevisiae* POL32 revealed that a minimum of 92 amino acids from the N-terminal domain of POL32 were required for interaction with POL31 (Johansson et al. 2004). The C-terminal domain of *S. cerevisiae* POL32 contained the PCNA-consensus motif Q*XX*(L/I)*XX*FF (underlined amino acids are conserved, Johansson et al. 2004) which was necessary for POL32 interaction with PCNA. Previously, it was reported that the initial 160 amino acids of Cdc27 were essential for Cdc1 (POL31 ortholog) interaction, amino acids 293 – 332 for Pol1 interaction, and 362 – 372 for PCNA interaction (Gray et al. 2004; Reynolds et al. 2000). Furthermore, the human p66 subunit of polymerase δ contains a C-terminal PCNA binding motif, a Pol1 interaction motif, and the first 144 amino acids are required for p50 (POL31, Cdc1) interaction (Pohler et al. 2005). The *L30* deletion preserves a significant portion of a putative *Pol31* interaction motif in *Drosophila* and lack the putative PCNA interaction motif (bolded sequence, Figure 8). We therefore hypothesize that that *L30* allows for interaction with *Drosophila* Pol31 protein.

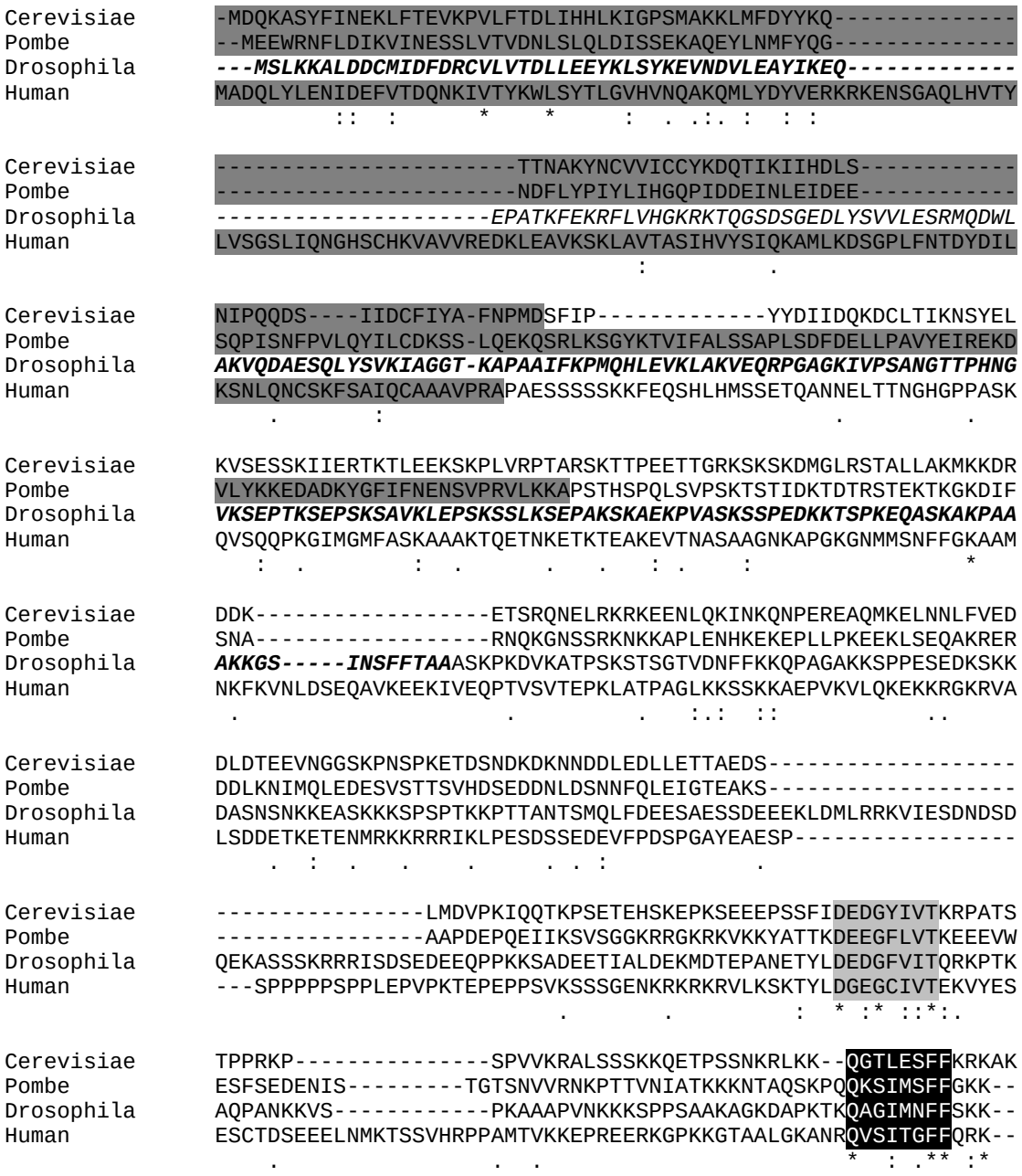

Figure 9. Multiple sequence alignment of Pol32 orthologs in *S. cerevisiae*, *S. pombe*, *Drosophila melanogaster*, and humans. An '*' indicates identical residues are found within the column, a ':' indicates that conserved residue substitutions were observed, and a "." notes semi-conserved substitutions. The POL31 (and associated orthologs) binding domain is highlighted in dark grey at the N-terminal sequence for all species. The Pol1 (Polα) binding domain is noted by a highlight light gray box, and the PCNA-interaction motif is highlighted in black at the C-terminal sequence for all species. The bolded sequence denotes amino acid sequence remaining in the *L30* allele.

3.3. *L2* mutants exhibit high sensitive to MMS and IR and are moderately sensitive to H(2)N and HU.

The ortholog of Pol32 in *S. cerevisiae* has been reported to exhibit sensitivity to a range of DNA damaging agents including UV light and MMS. To examine the potential role of *Drosophila* Pol32 in DNA damage repair pathways a series of mutagen sensitivity assays were performed on *L2* and *L30* flies using MMS, camptothecin, hydroxyurea (HU), nitrogen mustard, and IR. MMS damages DNA by inducing non-bulky adducts, which can be repaired by nucleotide excision repair or base excision repair. HU inhibits the deoxynucleotide pool and stalls replication, while camptothecin is a topoisomerase-I inhibitor. Both HU and camptothecin have the potential to introduce DSBs into the DNA. Nitrogen mustard forms interstrand crosslinks that impair the replication fork. Finally, IR induces a wide range of damage in cells, including DSBs. In the mutagen sensitivity assays fly larvae are treated with mutagens and survival of homozygotes to heterozygotes is calculated by comparing homozygouys survival to adulthood to expected ratios obtained relative to heterozygote control survival rates.

We observed *L2* homozygous mutants to be exceptionally sensitive to all doses of MMS and IR (Figure 10A and 10D). Even at the lowest dosage of 0.03% MMS *L2* homozygotes were completely eliminated, while a comparable effect was seen at 1000 rads of IR. In contrast, *L30* homozygotes show a progressive decrease in survival when exposed to MMS and IR and demonstrate a wide range of variability in survival percentages (Figure 10A and 10D). *L2 in trans* to a deficiency (*Df-24112*) that removed Pol32 and several surrounding genes resulted in similar extreme sensitivity to MMS and IR, indicating that *L2* sensitivity was not the result of second site mutations introduced during initial mutant generation (data not shown). Similarly, *L30 in trans* to the same deficiency exhibited comparable survival to MMS as *L30* homozygotes.

L2 sensitivity to MMS and IR indicates a potential role for Pol32 in base excision repair, nucleotide excision repair, or homologous recombination.

L2 homozygotes showed moderate sensitivity to HU, whereas *L30* homozygotes showed increased survival to HU over *L2* homozygotes. *L30* homozygotes showed no sensitivity to nitrogen mustard, but for *L2* homozygotes a dosage between 0.005% and 0.008% H(2)N was lethal (Figure 10B and 10C). No sensitivity to camptothecin was observed in *L2* homozygotes (Figure 10E). These results suggest additional potential roles for Pol32 in replication fork restart and interstrand crosslink repair, pathways that can involve homologous recombination, NER, or BER.

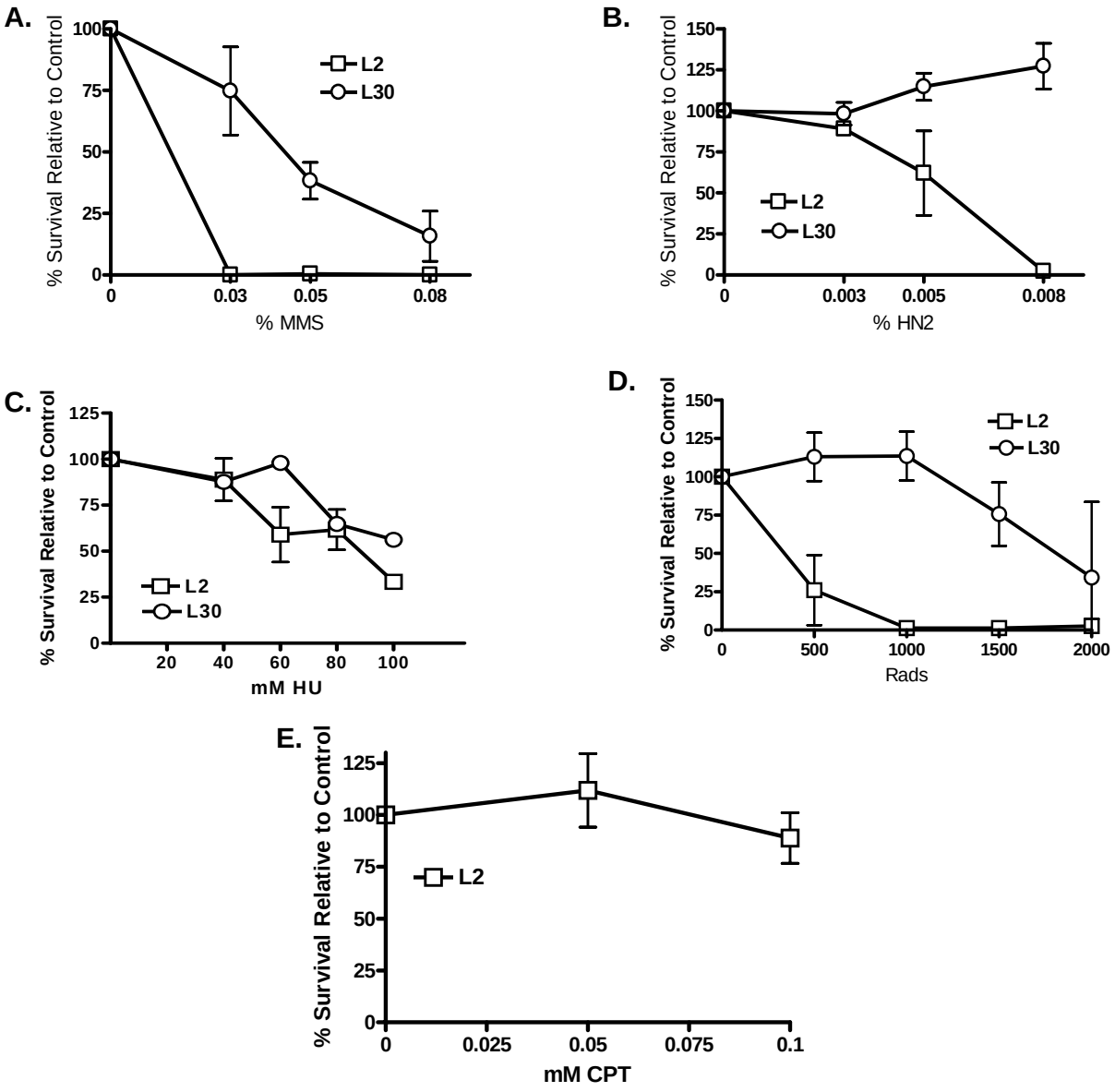

Figure 10. Sensitivity of *pol32 L2* and *L30* mutants to MMS, HN2, HU, and IR. (A) Percent survival of *L2*/*L2* and *L30/L30* mutants when exposed to varying concentrations of MMS. (B) Percent survival of *L2*/*L2* and *L30*/*L30* mutants (relative to heterozygous controls) when exposed to varying concentrations of HN2. (C) Percent survival of *L2*/*L2* mutants when exposed to varying concentrations of HU. (D) Percent survival of *L2/L2* and *L30/L30* mutants when exposed to varying concentrations of IR**.** (E) Percent survival of *L2/L2* mutants when exposed to varying concentrations of camptothecin. Percentages are survival rates of homozygous mutants compared to expected ratios relative to the control survival rates. Errors bars represent SD and each point represents the average of three experiments, except data depicted for *L30*/*L30* for HU represent only one experiment.

3.4 *L2* is proposed as a null mutation and *L30* as a hypomorphic mutation.

Based on phenotypic, mutagen sensitivity, and sequence analysis data, we propose that *L2* is a null mutation that drastically impairs or eliminates Pol32 function and *L30* is a hypomorphic mutation that retains the semi-functional Pol32 protein. *L30* preserves the bulk of the N-terminal Pol31 interaction domain, while deleting the Pol1 and PCNA interaction motifs. At the same time, *L30* homozygotes show elevated levels of survival to all mutagens to which *L2* homozygotes are sensitive. This indicates the retention of the Pol31 interaction domain is important for full Polδ function. We posit that *Drosophila* Pol32 plays an equivalent role to its orthologs in other species by acting as an accessory subunit allowing for reconstitution of the complete Polδ holoenzyme. In such a scenario, the *L2* mutation would impede Polδ function more severely than the *L30* mutation by eliminating the ability of Pol32 to interact with Pol31. This does not preclude the possibility that the *L30* mutation may adversely affect other aspects of Polδ function.

3.5 SDSA is significantly reduced and long distance repair synthesis is impaired in *L2* homozygotes.

In order to examine the role of Pol32 in DSBR and repair synthesis we utilized the site specific *P{w^a}* assay (Figure 11). The *P{w^a}* assay involves mobilization of a P-element construct known as *P{w^a}* that is embedded in the *scalloped* gene on the X-chromosome. Repair of the DSB induced by mobilization has been found to occur through the SDSA pathway (Adams et al. 2003). Repair by HR or EJ can be determined through phenotypic assessment of eye color in females and EJ products can be further assayed in male progeny of EJ females to determine the extent of repair synthesis prior to abortion of HR. Assuming *L2* to be a null mutation we

performed the *P{w^a}* assay using *L2* homozygotes. *L2* homozygotes showed a significant decrease in HR (Figure 12A, two tailed $p < 0.001$, Mann-Whitney Test, $n_{\text{wild type}} = 60$; $n_{L2/L2} = 130$). We saw no difference in the EJ classes between *L2* homozygotes and wild type flies.

To classify the effect of the decrease in HR on synthesis we quantified the extent of synthesis in flies in which HR had been aborted in favor of EJ (yellow eye class) using the right end of the P-element. A significant decrease in synthesis was observed at 920-bp *L2* as compared to wild type (Figure 12C, two tailed $p < 0.05$, Fisher's Exact Test, $n_{\text{wild type}} = 48/55$; $n_{L2/L2} = 103/151$). Interestingly, we also found that at 4675 bp repair synthesis in *L2* was significantly impaired (Figure 12C, two tailed $p < 0.05$, Fisher's Exact Test; $n_{\text{wild type}} = 10/55$; $n_{L2/L2} = 10/151$). In order to synthesize 4.6–kb, it is necessary to pass the LTRs. To determine if distances immediately prior to the LTRs also showed a defect we analyzed the 3500 bp and 4300 bp distances prior to the LTRs. However, we found no significant defect in synthesis prior to the 4.6-kb distance (Figure 12C).

To clarify if LTR specific sequence was impeding synthesis at 4.6-kb in *L2* flies we examined synthesis from the left end of the P-element. Surprisingly we saw significant defects in repair synthesis at the 5-bp, 2500 bp, 3500 bp, 4300 bp, and 4800 bp (located after the LTR) distances (Figure 12B). The combination of multiple distances at which synthesis was impaired on the left end and the significant impairment at 4.6-kb on the right *L2* homozygotes appeared to be the result of sequence specific secondary structures present on the left end of the P-element that affected Polδ processivity and a defect in long distance synthesis on the right.

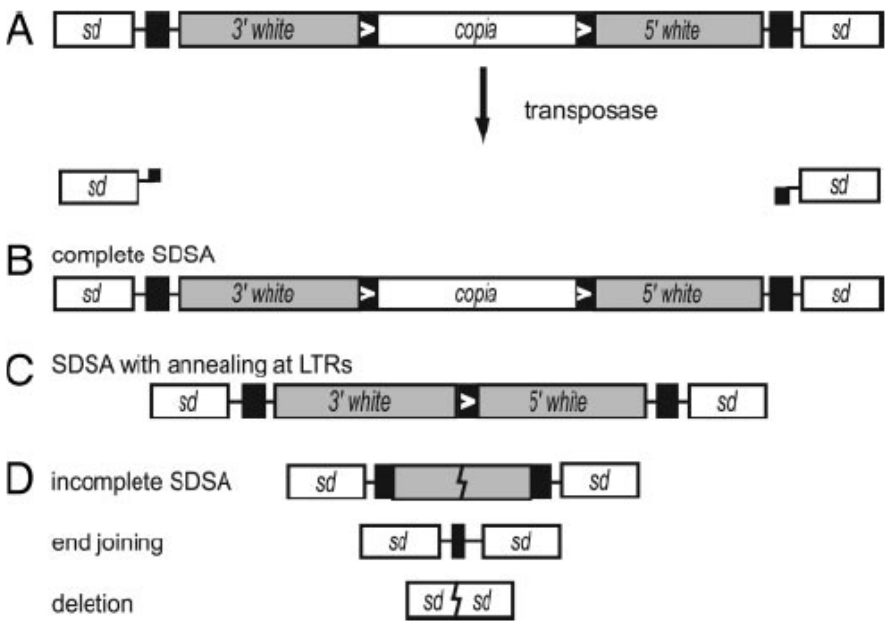

Figure 11. The *P{w^a}* assay for double strand break repair. *P{w^a}* is inserted into the essential *scalloped* gene (*sd*) in a white mutant background [A]. The inverted repeats of the P-element (solid black) flank the white gene (gray), into which the *copia* retrotransposon (white) is inserted. The 276 bp long terminal repeats (LTRs) of the retrotransposon are depicted flanking *copia* (black with white arrows). After excision of $P\{w^a\}$ in males, 17 nucleotide noncomplementary overhangs remain. Repair in males from the sister chromatid that results in complete SDSA will produce apricot eyes in female progeny with a second $P\{w^a\}$ [B]. Homologous recombination followed by annealing of the LTRs results in red eyed progeny [C]. Homologous recombination aborted for end joining (aborted SDSA), joining of the inverted repeats, and deletions into the flanking *sd* gene all result in yellow eyed progeny [D]. (Adapted from McVey et. al 2004b.)

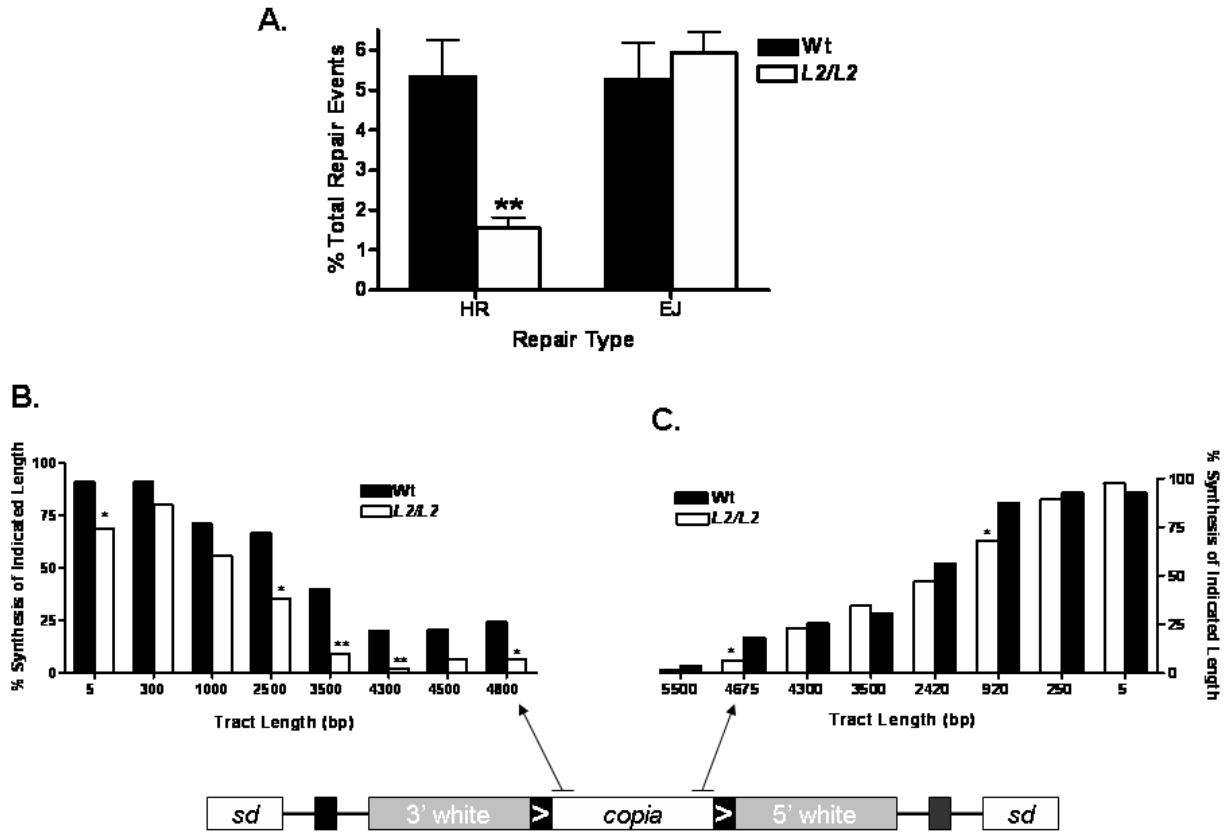

Figure 12. *L2* mutants exhibit defects in SDSA repair and processivity during repair synthesis. (A) Percent of end joining (EJ) and homologous recombination (HR) repair events after *P{w^a}* excision in *L2*/*L2* mutants (**p<0.001, Mann-Whitney Test**)**. (B) Percent of yellow eyed repair events that had repair synthesis of a certain distance from the left end of the P-element (*p < 0.05; **p < 0.001). (C) Percent of yellow eyed repair events that had repair synthesis of a certain distance from the right end of the P-element by homologous recombination before HR was aborted in favor of end joining. (*p<0.05, Fisher's Exact Test**)**. *P{w^a}* notation and coloring as in Figure 11. Distances after LTRs are denoted by arrows pointing to distance locations on the *P{w^a}* diagram located below tract lengths. Data for left end tract lengths provided courtesy of Daniel Kane.

CHAPTER 4: DISCUSSION

Double strand breaks are a toxic class of lesions in DNA that can prove lethal to cells if not repaired. Homologous recombination is an error-free pathway by which DSBs can be accurately repaired and is the most common mechanism utilized during the S1 and G2 cell cycle phases. HR can precisely restore lost sequence when a sister chromatid is used as a repair template (Kadyk et al. 1992). Many of the key factors involved in signaling and initiation of DSBR via HR have been previously explored (Krogh and Symington 2004; Pardo et al. 2009). However, the polymerases necessary for the initiation and elongation of repair products following invasion by the 3' end of the broken chromosome into a template remain unknown. In *S. cerevisiae* Polα is not necessary for repair synthesis during DSBR, while Polδ and Polε may serve redundant roles during repair (Wang et al. 2004). Recent research has concentrated on the replicative polymerase Polδ as a key player in the initiation and elongation of repair synthesis during HR (Maloisel et al. 2004).

The lack of viable mutations in the catalytic and essential subunits of Polδ has shifted interest towards the nonessential subunit Pol32 as a candidate for genetic studies of Polδ function. Orthologs of Pol32 exist in multiple species including *S. cerevisiae* (POL32)*, S. pombe* (Cdc27), and humans (p66). Surprisingly Pol32 is a nonessential subunit in *S. cerevisiae*, whereas its orthologs Cdc27 in *S. pombe* and p66 in humans are essential for Polδ during cellular replication and PCNA interaction (Pohler et al. 2005; Reynolds et al. 2000). In our study we sought to characterize the *Drosophila melanogaster* ortholog of Pol32 and investigate its potential role in DSBR.

4.1 Pol32 mutants are viable and Pol32 contains putative protein interaction domains.

To explore the role of Pol32 in DNA repair we undertook a genetic study of the Pol32 subunit by performing an imprecise excision screen to generate mutants in the *pol32* locus. Two unique mutations, *L2* and *L30* were used from the excision screen. Our studies revealed that similar to *S. cerevisiae* POL32 and unlike Cdc27 in S. *pombe*, *Drosophila pol32* is a nonessential gene. Both *L2* and *L30* homozygotes are marked by a shortened bristle phenotype, a finding consistent with possible defects in DNA repair, although the bristle phenotype is most severe in *L2* flies. Furthermore, *L2* homozygous females are sterile, indicating that Pol32 may be required during synthesis in early mitotic divisions in the embryo. Although both *L2* and *L30* produce transcripts, the expected protein that would be translated from the *L2* transcript would consist largely of amino acids absent in the wild type protein (Figure S1).

Johansson and colleagues (2004) reported that the first 92 amino acids of POL32 are necessary for interaction with POL31, amino acids 292 – 310 are required for POL1 (Polα) interaction, and the extreme C-terminus is for required for PCNA (PO*L30*) interaction. The *L2* deletion removes the majority of wild type coding sequence, while *L30* preserves a putative Pol31 N-terminal interaction domain. We believe that this domain allows for reconstitution of a partially functional Polδ complex that is more processive than the Pol3-Pol31 subunits by themselves. We also found that wild type Pol32 in Drosophila maintains the Polα (Pol1) and PCNA interaction motifs found in orthologs, but both the *L2* and *L30* mutants lack these domains and most likely do not interact with either of these proteins.

4.2 Pol32 is likely involved in several DNA repair pathways.

Previous studies of Pol32 have shown that *S. cerevisiae* cells lacking the protein exhibit a cold-sensitive growth phenotype and are defective in damage-induced mutagenesis (Gerik et al. 1998). Pol32 mutants are sensitive to multiple DNA damaging agents including HU, MMS, and HN2 (Johansson et al. 2004; Hanna et al. 2007; Sarkar et al. 2006). Recent *in vitro* studies have confirmed that Pol32 recruits Pol zeta (ζ) through Rev1, a finding consistent with a role for Pol32 in recruiting the Polζ translesion polymerase to adducts encountered by Polδ at the replication fork (Acharya et al. 2009).

In this study we found *L2* homozygous mutants to be hypersensitive to low levels of MMS and IR and moderately sensitive to HU and HN2. In contrast, *L30* homozygotes exhibit moderate sensitivity to MMS, IR and HU and no sensitivity to HN2. MMS is an alkylating agent that converts guanine to 7-methylguanine and adenine to 3-methlyladenine (Beranek 1990). Repair of MMS induced lesions is primarily handled through base excision repair (BER) and Polδ is believed to be involved in long-patch BER (Wilson and Bohr 2004; Blank et al. 1994). Ionizing radiation (IR) contributes to a wide spectrum of damage to DNA including DSBs and SSBs, which are frequently repaired by BER or HR. HN2 causes interstrand cross-links that can be repaired by nucleotide excision repair (NER), while exposure to HU may result in replication fork stalling due to depletion of the available deoxynucleotide pool (Nouspikel 2009; Koç et al. 2004). The extreme sensitivity of *L2* homozygous mutants to MMS and IR implicates Pol32 as an important part of Polδ dependent BER and HR in *Drosophila*. The moderate sensitivity of *L2* homozygotes to HN2 (with hypersensitivity at the highest dose) and HU implicate Pol32 in the NER and replication fork restart pathways. Interestingly *L30* mutants are only moderately sensitive to these agents, and not sensitive at all to nitrogen mustard.

This differential sensitivity between the alleles may imply that only the N-terminal Pol31 interaction domain of Pol32 is important for restoring a Polδ capable of completing successful repair. If this is the case, more precise domain mapping through sensitivity analysis of various other deletions mutations will be necessary to ascertain the specific domain requirements of Pol32 for different DNA repair pathways.

4.3 We propose that *L2* is a null mutation and *L30* is a hypomorph.

Although both the *L2* and *L30* deletions show mRNA transcript via RT-PCR, *L2* putatively shows reduced transcript as compared to *L30*. Sequence analysis of the potential protein produced by *L2* indicates that the protein would not be sufficient to interact with Pol31 and reconstitute a fully functional Polδ holoenzyme. The hypersensitivity of *L2* flies to multiple DNA damaging agents and the relatively moderate sensitivity of *L30* flies to the same agents further denotes that *L2* eliminates functional Pol32 protein interaction. Finally, the observed difference in bristle phenotype severity between *L2* and *L30* imply a stronger effect of the *L2* deletion on early mitotic synthesis. *L2* is therefore most likely a null mutation in *pol32*. *L30*, however, preserves the putative Pol31 interaction domain and eliminates the PCNA interaction domain. We hypothesize that the *L30* mutation based on observed sensitivities and data is a hypomorph. Whether this is a result of a reduced level of protein produced or due to a loss of the PCNA interaction domain is unclear.

4.4 Pol32 is important for SDSA and processivity of polymerase δ.

The role of Pol32 in DSBR by HR remains controversial. Pol32 is known to be essential for BIR, but is unlikely to be involved in gene conversion involving short tract lengths (Lydeard

et al. 2007). There is evidence to suggest that repair of gaps of as small as 1.2-kb is defective in *pol32Δ* mutants (Jain et al. 2009). However, other research has shown that repair of a gap of 238 bp in a plasmid is reduced, but not eliminated, in *pol32Δ* mutants (Smith et al. 2009). These findings may show that *pol32* mutants are capable of initial strand invasion and synthesis, but are unable to carry out synthesis across longer distances due to a loss of Polδ processivity. To investigate these findings in *Drosophila melanogaster* we used a *P{w^a}* excision assay.

P-element excision repair in *Drosophila* occurs through the SDSA pathway. As we believe *L2* to be a null mutation we used *L2* homozygotes and observed a highly significant 72% decrease in red eye females in *L2* flies as compared to wild type. However, HR was not completely abolished in *L2* homozygotes. There are several possible reasons for this. HR could be handled by the remaining essential subunits of Polδ. If this is the case, it will be difficult to produce additional mutations in Polδ, since the remaining subunits cannot be deleted without producing nonviable flies. Furthermore, other polymerases could also be recruited to the break site for repair synthesis and engage in repair synthesis. We plan to explore this possibility by creating double mutants combining *pol32 L2* with other DNA polymerases deletions.

The yellow eyed class of females in the *P{w^a}* assay can be further investigated by recovering the aberrant repair event in progeny males and studying synthesis tract lengths (Figure S2). We quantified tract lengths from both the right and left end of the P-element. Our findings showed the surprising result that synthesis from the right end in *L2* homozygotes was affected at the 920-bp and 4.6-kb distances. Since the 4.6-k distance was past the LTR we suspected that LTR specific sequence might be the reason that Polδ lacking Pol32 falls off at the LTR on the template. However, when looking at the left end of the P-element we found that synthesis was affected at multiple distances prior to the LTR. We see two potential explanations

for this left-right discrepancy. First, the left end of the P-element may invade first and Polδ loads prior to the loading of accessory replication factors. Without Pol32 present to achieve processivity, Polδ function will be impaired at multiple time points. In contrast, the right end of the P-element may initiate synthesis later when Polδ will be more securely tethered to the DNA template by multiple replication factors. An alternative and more likely hypothesis is that sequence on the left end of the P-element contains tracts of secondary structure that the impaired Polδ consisting of only Pol3 and Pol31 cannot navigate. In this case the 920-bp defect may possibly be indicative of sequence on the right that also prevents processive synthesis. Further, the LTR sequence would not be the reason for falloff at 4.6-kb distance, but rather processivity could be impeded at this distance or sequence specific secondary structure issues at this point could prevent further synthesis. To confirm the validity of these findings a second $P\{w^a\}$ is being carried out in an *L2* background.

Although it may not be possible to determine the precise physical reason for Polδ displacement from the template strand, these findings clearly show that the absence of Pol32 results in processivity defects in Polδ, results that confirm previous analyses in yeast.

4.3 Functional analyses and additional repair assays are necessary to clarify the role of Pol32 in DNA repair.

Our initial characterization of Pol32 reveals a role for it in multiple DNA repair pathways and indicates its importance for processivity of Polδ during SDSA repair synthesis. We characterized two viable Pol32 mutants, a null mutation *L2* and a hypomorphic mutation *L30*. Initial sequence analysis revealed three interaction domains for Pol31, Pol1, and PCNA in *Drosophila* Pol32. Pol32 *L2* mutants are hypersensitive to MMS and IR, indicating roles in BER

and HR and moderately sensitive to HU and H2N indicating a role in NER/interstrand-crosslink repair and replication fork restart. These findings have led us to believe that the Pol31 interaction domain of Pol32 is necessary for mediating successful Polδ navigation of repair pathway processes.

Furthermore, through the $P\{w^a\}$ assay we found that Pol32 null mutant *L2* results in a significant decrease in SDSA repair. Unexpectedly we also saw differential defects in repair synthesis in *L2* flies on the left and right ends of the P-element. These initial results offer tantalizing clues to Pol32 function in *Drosophila*. In order to further clarify Pol32 domain interaction and function in repair synthesis we are currently undertaking a yeast two hybrid assay and an additional set of DSB repair assays. In order to establish the Pol31, Pol1, and PCNA interaction domains of Pol32 we will perform a yeast-2-hybrid screen. Using wild type Pol32, *L2*, and *L30* sequences we hope to establish the Drosophila interaction domains for Pol32. The yeast-2-hybrid technique is a powerful assay that measures protein-protein interactions by quantifying the product of a reporter gene activated by the two proteins. Our system involves the use of the GAL4 promoter to drive expression of a HIS3 reporter. By separating the activation and binding domains of GAL4 and linking them separately to proteins of interest the GAL4 promoter will become active in cases of the two proteins of interest interacting in the nucleus.

To gain additional insight into the role of Pol32 in DSBR we are examining the potential role of *L30* in SDSA through a $P\{w^a\}$ assay. Lydeard and colleagues observed a decrease in BIR in pol32 mutants lacking the PCNA interaction motif (2007) and we suspect that the absence of Pol32 to provide an additional tether for Polδ will affect SDSA, though we are uncertain as to what effect this may have on repair synthesis. Another area of ongoing interest is the controversial role of Pol32 in synthesis initiation. We have recently attempted to resolve this

question in Drosophila by using a *P{w^a}* on the second chromosome that would induce a double strand break within the second chromosome that may be repaired either by gene conversion off of the homolog or gap repair (HR or EJ) off of the sister chromatid. Although we found no role for Pol32 in gene conversion, low excision levels prevented the assay from yielding definitive results (data not shown). We are therefore utilizing a customized I-SceI DSBR assay that uses the I-SceI endonuclease to induce breaks in a target sequence. This will allow us to analyze the role of Pol32 in gene conversion and synthesis initiation. Finally, we remain interested in the possibility of additional polymerase involvement in SDSA synthesis and will continue to seek polymerases that may play a critical role in both HR and NHEJ repair synthesis.

REFERENCES

Acharya N, Johnson RE, Pagès V, Prakash L, Prakash S. 2009. Yeast Rev1 protein promotes complex formation of DNA polymerase ζ with Pol32 subunit of DNA polymerase δ. Proceedings of the National Academy of Sciences 106(24):9631-6.

Adams MD, McVey M, Sekelsky JJ. 2003. Drosophila BLM in double-strand break repair by synthesis-dependent strand annealing. Science 299(5604):265-7.

Aguilera A and Gomez-Gonzalez B. 2008. Genome instability: A mechanistic view of its causes and consequences. Nat Rev Genet 9(3):204-17.

Aylon Y, Liefshitz B, Kupiec M. 2004. The CDK regulates repair of double-strand breaks by homologous recombination during the cell cycle. EMBO J 23(24):4868-75.

Beranek DT. 1990. Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 231(1):11-30.

Blank A, Kim B, Loeb LA. 1994. DNA polymerase delta is required for base excision repair of DNA methylation damage in *Saccharomyces cerevisiae*. Proceedings of the National Academy of Sciences of the United States of America 91(19):9047-51.

Boulet A, Simon M, Faye G, Bauer GA, and Burgers PM.. 1989. Structure and function of the *Saccharomyces cerevisiae* CDC2 gene encoding the large subunit of DNA polymerase III. EMBO J. 8**:**1849–1854

Burgers PMJ. 2009. Polymerase dynamics at the eukaryotic DNA replication fork. Journal of Biological Chemistry 284(7):4041-5.

Burgers PMJ and Gerik KJ. 1998. Structure and processivity of two forms of *Saccharomyces cerevisiae* DNA polymerase δ. Journal of Biological Chemistry 273(31):19756-62.

Eker A, Quayle C, Chaves I, van dH. 2009. DNA repair in mammalian cells. Cellular and Molecular Life Sciences 66(6):968-80.

Engels WR, Johnson-Schlitz DM, Eggleston WB, Sved J. 1990. High-frequency P element loss in *Drosophila* is homolog dependent. Cell 62(3):515-25.

Friedberg EC and Friedberg EC. 2006. DNA repair and mutagenesis. 2nd ed. Washington, D.C.: ASM Press.

Gerik KJ, Li X, Pautz A, Burgers PMJ. 1998. Characterization of the two small subunits of *Saccharomyces cerevisiae* DNA polymerase δ. Journal of Biological Chemistry 273(31):19747-55.

Gray F, Pohler JR, Warbrick E, MacNeill S. 2004. Mapping and mutation of the conserved DNA polymerase interaction motif (DPIM) located in the C-terminal domain of fission yeast DNA polymerase delta subunit Cdc27. BMC Molecular Biology 5(1):21.

Hanna M, Ball LG, Tong AH, Boone C, Xiao W. 2007. Pol32 is required for polζ-dependent translesion synthesis and prevents double-strand breaks at the replication fork. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 625(1-2):164-76.

Hashimoto K, Nakashima N, Ohara T, Maki S, Sugino A. 1998. The second subunit of DNA polymerase III (delta) is encoded by the HYS2 gene in *Saccharomyces cerevisiae*. Nucl Acids Res 26(2):477-85.

Helleday T, Lo J, van Gent DC, Engelward BP. 2007. DNA double-strand break repair: From mechanistic understanding to cancer treatment. DNA Repair 6(7):923-35.

Hoeijmakers JHJ. 2009. DNA damage, aging, and cancer. N Engl J Med 361(15):1475-85.

Hoeijmakers JHJ. 2001. Genome maintenance mechanisms for preventing cancer. Nature 411(6835):366-74.

Iyer RR, Pluciennik A, Burdett V, Modrich PL. 2006. DNA mismatch repair: functions and mechanisms. Chem Rev 106(2):302-23.

Jain R, Hammel M, Johnson RE, Prakash L, Prakash S, Aggarwal AK. 2009. Structural insights into yeast DNA polymerase δ by small angle X-ray scattering. J Mol Biol 394(3):377-82.

Johansson E, Garg P, Burgers PMJ. 2004. The Pol32 subunit of DNA polymerase δ contains separable domains for processive replication and proliferating cell nuclear antigen (PCNA) binding. Journal of Biological Chemistry 279(3):1907-15.

Johansson E, Majka J, Burgers PMJ. 2001. Structure of DNA polymerase δ from *Saccharomyces cerevisiae*. Journal of Biological Chemistry 276(47):43824-8.

Kadyk LC and Hartwell LH. 1992. Sister chromatids are preferred over homologs as substrates for recombinational repair in *Saccharomyces cerevisiae*. Genetics 132(2):387-402.

Koç A, Wheeler LJ, Mathews CK, Merrill GF. 2004. Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools. Journal of Biological Chemistry 279(1):223-30.

Krogh BO and Symington LS. 2004. Recombination Proteins in Yeast. Annu Rev Genet 38(1):233-71.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al. 2007. Clustal W and clustal X version 2.0. Bioinformatics 23(21):2947-8.

LaRocque JR, Jaklevic B, Su TT, Sekelsky J. 2007. *Drosophila* ATR in double-strand break repair. Genetics 175(3):1023-33.

Lindahl T. 1993. Instability and decay of the primary structure of DNA. Nature 362(6422):709-15.

Llorente B, Smith CE, Symington LS. 2008. Break-induced replication: What is it and what is it for? Cell Cycle 7:859–864.

Longley MJ, Pierce AJ, Modrich P. 1997. DNA polymerase δ is required for human mismatch repair in vitro. Journal of Biological Chemistry 272(16):10917-21.

Lydeard JR, Jain S, Yamaguchi M, Haber JE. 2007. Break-induced replication and telomerase-independent telomere maintenance require Pol32. Nature 448(7155):820-3.

Maloisel L, Fabre F, Gangloff S. 2008. DNA polymerase {delta} is preferentially recruited during homologous recombination to promote heteroduplex DNA extension. Mol Cell Biol 28(4):1373-82.

Maloisel L, Bhargava J, Roeder GS. 2004. A role for DNA polymerase {delta} in gene conversion and crossing over during meiosis in *Saccharomyces cerevisiae*. Genetics 167(3):1133-42.

McIlwraith MJ, Vaisman A, Liu Y, Fanning E, Woodgate R, West SC. 2005. Human DNA polymerase η promotes DNA synthesis from strand invasion intermediates of homologous recombination. Mol Cell 20(5):783-92.

McKinnon PJ and Caldecott KW. 2007. DNA strand break repair and human genetic disease. Annual Review of Genomics and Human Genetics 8(1):37-55.

McVey M, Adams M, Staeva-Vieira E, Sekelsky JJ. 2004. Evidence for multiple cycles of strand invasion during repair of double-strand gaps in *Drosophila*. Genetics 167(2):699-705.

Nassif N, Penney J, Pal S, Engels WR, Gloor GB. 1994. Efficient copying of nonhomologous sequences from ectopic sites via P-element-induced gap repair. Mol Cell Biol 14(3):1613-25.

McElhinny Nick SA, Gordenin DA, Stith CM, Burgers PMJ, Kunkel TA. 2008. Division of labor at the eukaryotic replication fork. Mol Cell 30(2):137-44.

Nouspikel T. 2009. DNA repair in mammalian cells: So DNA repair really is that important?. Cellular and Molecular Life Sciences 66(6):963-7.

Nouspikel T. 2009. DNA repair in mammalian cells: Nucleotide excision repair: variations on versatility. Cellular and Molecular Life Sciences 66(6):994-1009.

Pardo B, Gomez-Gonzalez B, Aguilera A. 2009. DNA repair in mammalian cells: how to fix a broken relationship. Cellular and Molecular Life Sciences 66(6):1039-56.

Payen C, Koszul R, Dujon B, Fischer G. 2008. Segmental duplications arise from Pol32-dependent repair of broken forks through two alternative replication-based mechanisms. PLoS Genet 4(9):e1000175.

Podust VN, Chang L, Ott R, Dianov GL, Fanning E. 2002. Reconstitution of human DNA polymerase δ using recombinant baculoviruses. Journal of Biological Chemistry 277(6):3894-901.

Pohler JR, Otterlei M, Warbrick E. 2005. An in vivo analysis of the localisation and interactions of human p66 DNA polymerase delta subunit. BMC Molecular Biology 6(1):17.

Reynolds N, Watt A, Fantes PA, MacNeill SA. 1998. Cdm1, the smallest subunit of DNA polymerase d in the fission yeast *Schizosaccharomyces pombe*, is non-essential for growth and division. Curr Genet. 34(4):250-8.

Reynolds, N., Warbrick E., Fantes PA, MacNeill SA. 2000. Essential interaction between the fission yeast DNA polymerase delta subunit Cdc27 and Pcn1 (PCNA) mediated through a C-terminal p21(Cip1)-like PCNA binding motif. EMBO J. 19*:*1108–1118.

Ruiz JF, Gomez-Gonzalez B, Aguilera A. 2009. Chromosomal translocations caused by either Pol32-dependent or Pol32-independent triparental break-induced replication. Mol Cell Biol 29(20):5441-54.

Ryder E and Russell S. 2003. Transposable elements as tools for genomics and genetics in drosophila. Briefings in Functional Genomics and Proteomics 2(1):57-71.

Sarkar S, Davies AA, Ulrich HD, McHugh PJ. 2006. DNA interstrand crosslink repair during G1 involves nucleotide excision repair and DNA polymerase [zeta]. EMBO J 25(6):1285-94.

Sekelsky JJ, Brodsky MH, Burtis KC. 2000. DNA repair in drosophila: Insights from the *drosophila* genome sequence. The Journal of Cell Biology 150(2):F31-6.

Simon M, Giot L, Faye G.. 1991. The 3′ to 5′ exonuclease activity located in the DNA polymerase delta subunit of *Saccharomyces cerevisiae* is required for accurate replication, *EMBO J.* 10: 2165–2170.

Soulas-Sprauel P, Rivera-Munoz P, Malivert L, Le Guyader G, Abramowski V, Revy P, de Villartay J. 0000. V(D)J and immunoglobulin class switch recombinations: A paradigm to study the regulation of DNA end-joining. Oncogene 26(56):7780-91.

Su TT. 2006. Cellular responses to DNA damage: One signal, multiple choices. Annu Rev Genet 40(1):187-208.

Szostak JW, Orr-Weaver TL, Rothstein RJ, Stahl FW. 1983. The double-strand-break repair model for recombination. Cell 33(1):25-35.

Wang X, Ira G, Tercero JA, Holmes AM, Diffley JFX, Haber JE. 2004. Role of DNA replication proteins in double-strand break-induced recombination in *Saccharomyces cerevisiae*. Mol Cell Biol 24(16):6891-9.

Weterings E and Chen DJ. 2008. The endless tale of non-homologous end-joining. Cell Res 18(1):114-24.

Wilson III DM and Bohr VA. 2007. The mechanics of base excision repair, and its relationship to aging and disease. DNA Repair 6(4):544-59.

Witsell A, Kane DP, Rubin S, McVey M. 2009. Removal of the bloom syndrome DNA helicase extends the utility of imprecise transposon excision for making null mutations in Drosophila. Genetics 183: 1187-1193.

Zuo S, Bermudez V, Zhang G, Kelman Z, Hurwitz J. 2000. Structure and activity associated with multiple forms of *Schizosaccharomyces pombe* DNA polymerase δ. Journal of Biological Chemistry 275(7):5153-62.

SUPPLEMENTARY INFORMATION

Figure S1. Amino acid sequence of *L2*, *L30*, and wild type *pol32* alleles. Amino acid sequences are given in N-terminus to C-terminus arrangement. Underlined amino acids in the *L2* and *L30* sequences indicate the extent of wild type sequence present in each mutant. Italics denote divergence from the wild type sequence. The approximate number of base pairs (bp) corresponding to the translated sequence is provided in parentheses.

***L2* Predicted Protein Sequence (**162 bp translated)

M S L K K A L D D C M I D F *A W P M L Y Q C V F*
L F L V L Y L L F S I S I R D R N S L S P L S L
S Y F K T L

***L30* Predicted Protein Sequence (**696 bp translated)

M S L K K A L D D C M I D F D R C V L V T D L L
E E Y K L S Y K E V N D V L E A Y I K E Q E P A
T K F E K R F L V H G K R K T Q G S D S G E D L
Y S V V L E S R M Q D W L A K V Q D A E S Q L Y
S V K I A G G T K A P A A I F K P M Q H L E V K
L A K V E Q R P G A G K I V P S A N G T T P H N
G V K S E P T K S E P S K S A V K L E P S K S S
L K S E P A K S K A E K P V A S K S S P E D K K
T S P K E Q A S K A K P A A A K K G S I N S F F
T A A *Y C S Q C K M K K S F A I*

Wild Type (Pol32) Protein Sequence (1293 bp translated)

M S L K K A L D D C M I D F D R C V L V T D L L
E E Y K L S Y K E V N D V L E A Y I K E Q E P A
T K F E K R F L V H G K R K T Q G S D S G E D L
Y S V V L E S R M Q D W L A K V Q D A E S Q L Y
S V K I A G G T K A P A A I F K P M Q H L E V K
L A K V E Q R P G A G K I V P S A N G T T P H N
G V K S E P T K S E P S K S A V K L E P S K S S
L K S E P A K S K A E K P V A S K S S P E D K K
T S P K E Q A S K A K P A A A K K G S I N S F F
T A A A S K P K D V K A T P S K S T S G T V D N
F F K K Q P A G A K K S P P E S E D K S K K D A
S N S N K K E A S K K K S P S P T K K P T T A N
T S M Q L F D E E S A E S S D E E E K L D M L R
R K V I E S D N D S D Q E K A S S S K R R R I S
D S E D E E Q P P K K S A D E E T I A L D E K M
D T E P A N E T Y L D E D G F V I T Q R K P T K
A Q P A N K K V S P K A A A P V N K K K S P P S
A A K A G K D A P K T K Q A G I M N F F S K K

Figure S2. *P{w^a}* Assay Cross Scheme. The following cross scheme was employed in the *P{w^a}* assay. The scheme detailed below employs *L2*/*L2* homozygotes. All crosses are given as ♀ (left) ⊗♂ (right), even in cases where the Y chromosome is not explicitly depicted (*FM7w* males).