A novel pathway for Mus301 mediated DNA repair at Replication forks in *Drosophila melanogaster*

An honors thesis for the Department of Biology

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Abstract:

Scientists studying genome integrity are particularly interested in DNA double-stranded break repair. One gene that may be involved in DNA repair in *Drosophila melantogaster* is *mus301*. Previous research suggested a role for *mus301* in homologous recombination (HR), but the mechanism by which Mus301 operates remains largely unknown. Through gene characterization, we report here that *mus301* mutants are sensitive to DNA damaging agents: topotecan and hydroxyurea, which are known for their role in causing stalled and collapsed forks. Sensitivity to these chemicals suggested that Mus301 operates at replication forks to repair damage. In addition we generated *mus301, spn-A* double mutants to study the role of *mus301* in alternative end joining repair. Interestingly, these double mutants were homozygous lethal, suggesting that *mus301* has a role outside of HR. To further understand the lethal phenotype we analyzed double mutant larvae development. The results from this analysis showed that double mutants were able to survive to first instar larvae, but death occurred rapidly after. Furthermore, to visualize damage in these mutants, we created *mus301* homozygous, *spn-A* heterozygous mutants that were able to survive to third instar. Imaginal disc were collected from these mutants and cell death in these tissue was visualized with acridine orange. Results showed increased foci of damage in the mutants and in combination with the results from the double mutant development analysis this suggested that *mus301, spn-A* homozygous flies were unable to survive due to a defect in replication. From our results here, we propose a model in which Mus301 serves to unwind DNA structures at stalled replication forks to allow for replication to bypass the inhibition on the DNA template that originally caused the fork to stall or collapse.

Introduction:

Researchers studying genome integrity are particularly interested in DNA repair. During the course of normal life processes, DNA is constantly damaged and repaired. Without repair, damage to the genome can accumulate and lead to cell death, which can eventually cause organism death. Improper repair can also lead to the formation of new mutations that could cause the loss of function of different genes. This loss of function could give rise to many genetic diseases, including cancer. Studying DNA repair allows us to understand the mechanisms behind maintaining genome stability and the formation of these diseases.

One area in which repair is crucial is during DNA replication. During synthesis, the genome is especially prone to forming DNA breaks since the genetic material is more readily accessible by replication proteins and damaging agents alike. Repair of damage at the replication fork is necessary in order to continue DNA synthesis in a manner such that the genetic content of the cell is not altered. If synthesis cannot proceed, the replication fork can collapse and double stranded breaks (DSBs) can occur. Thus, repair at active replication areas is of particular interest in studying genome integrity.

Drosophila melanogaster, the fruit fly, is an ideal system for studying DNA repair because of a quick reproductive cycle and many genetic similarities with humans. Thus, studies in fruit flies may have clinical significance for humans.

DSB Repair Pathways

During daily life, we are exposed to different types of mutagens that may cause DNA damage in a variety of ways. Thus, there exist multiple pathways in repair of DSBs. These

pathways include end joining (EJ), alternative end joining (alt-EJ), and homologous recombination (HR).

The EJ pathway is prone to gene deletions. During EJ, the broken ends of the chromosome are processed by exonucleases. This processing allows for the formation of 3' overhangs on both sides that can ligate to each other. For ligation to occur, homologous sequences on both sides must be uncovered. End processing to reach the homologous sequences could result in the loss of genetic material when the two ends of the DSB are ligated back together to form the whole chromosome.

Similar to EJ, Alt-EJ is also an error-prone method of DSB repair. The method of repair for Alt-EJ is similar to that of EJ with the difference being Alt-EJ does not utilize the same proteins that EJ utilize. The end results between the two pathways, though, are similar with the potential for gene deletions.

In addition to EJ and alt-EJ, there also exists HR. The HR pathway is the most faithful pathway in that the genetic information is copied with the least amount of error. This pathway utilizes a homologous chromosome or a sister chromatid as a template for synthesis to restore whatever genetic content may have been lost during break formation. When a DSB break occurs, the first step to HR requires processing of the broken ends of the chromosome such that a single stranded 3' overhang is created. Replication Protein A (RPA) binds to the single stranded overhang to stabilize the single stranded structure. RAD51, encoded by the gene *spn-A*, localizes to RPA and displaces the latter protein from the DNA. RAD51 then aids in the invasion of the homologous template. Once the 3' overhang has invaded, it can then synthesize new base pairs using the homologous chromosome or sister chromatid. When the newly synthesized strand has reannealed to the template strand, there remains a single stranded gap where the genetic material

was synthesized off of the homologous template. DNA polymerase will fill in this single stranded gap, after which DNA ligase connect the two ends of the DSB together to reform the whole chromosome (San Filippo et al., 2008).

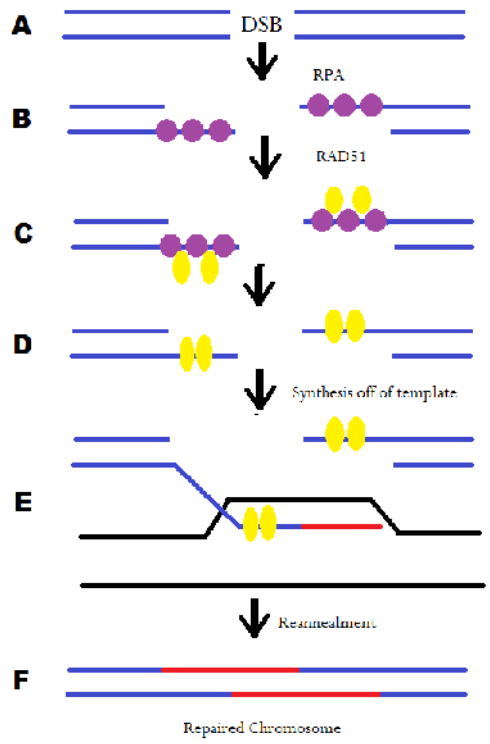

Figure 1. Illustration of HR repair pathway. RPA initially coats the single stranded DNA and localizes RAD51 to the site of DSB. RAD51 displaces RPA and initiates invasion into a DNA template to allow for synthesis to occur. Ligation after synthesis restores the whole chromosome.

There exist a variety of proteins that have a role in DNA repair in any or all of the pathways mentioned above. Currently, these proteins are still being elucidated. Studies of meiotic recombination have revealed roles for many DSB repair genes, including *mus301* in Drosophila. *mus301* could encode for a protein product that may have a role in all three genetic pathways.

Mus301, a gene necessary for repair?

The *mus301* gene was first uncovered through a mutagen sensitivity screen using methyl methane-sulfonate (MMS) and nitrogen mustard (HN2) (Boyd et al., 1981; Laurencon et al., 2004). MMS is known to cause stalled forks by inappropriately methylating DNA that may eventually lead to DSBs while HN2 is known for causing interstrand cross links, both of which require HR for repair. The sensitivity of *mus301* flies to these two mutagens suggests that the flies may be defective in HR.

Indeed, research on fruit flies oogenesis showed similar results. During normal oogenesis, breaks occur to allow for recombination. These breaks are repaired quickly after genetic exchange to preserve the genome. In *mus301*flies though, females undergoing oogenesis, had increased breaks in their oocytes that persisted throughout the development process (McCaffrey et al., 2006). The results from this paper suggested that *mus301* encoded a gene product necessary for DNA repair due to the persistent damage when the gene was mutated.

In addition, Adam Thomas of the McVey laboratory conducted a site-specific DSB repair assay. Preliminary data from the assay showed that a mutation of *mus301* resulted in a decrease in HR-mediated repair and a corresponding increase in EJ repair. This further supports the idea that *mus301* is more specifically necessary for HR in DNA repair.

mus301 shares similarities with other eukaryotic genes

In studying the *mus301* gene, it was discovered that the *mus301* gene exhibited similarities to other drosophila genes, such as *mus308* and *mus309*. In addition, it also contained some protein similarity to Hel308, an archael protein.

The *mus301* encoded gene product was shown to be a part of the *Drosophila mus308* ATP dependent helicase subfamily via strong sequence similarity and conservation of the

helicase domain (McCaffrey et al., 2006). *mus308* is known to have a role in Alt-EJ of DSBs (Chan et al., 2010). *mus301* is 31% similar (11% identical) at the amino acid level compared to *mus308* across the full length of the gene (McCaffrey et al., 2006). These similarities were primarily at the conserved helicase region of *mus308,* which strongly suggested that *mus301* is a part of the *mus308* family and may operate in a manner similar to *mus308*. This similarity to *mus308* suggests that *mus301* may be involved in repair outside of HR.

In addition to having sequence similarities with *mus308*, *mus301* was also shown to have a similar sequence compared to *mus309* at the helicase domain (McVey, 2010). *mus309* is a known ortholog of the human *Blm* gene, which, when mutated, is known to cause an increased risk of cancer as well as UV sensitivity among other severe phenotypes in humans. The human *Blm* gene was shown to have a role in DNA repair and functions after RAD51 in HR (Figure 1) in dissociating structures formed during the repair process (McVey et al., 2004b). Due to the domain similarity between *mus301* and *mus309* this further supported the idea that *mus301* may have a role outside of HR in DSB repair.

In addition to containing sequence similarities with other Drosophila genes, *mus301* is known to contain a helicase domain. Although the domain in fruit flies has not been shown to contain helicase activity directly in a biochemical assay, the region is highly conserved in humans, archaea, and flies, among other species (Figure 2).

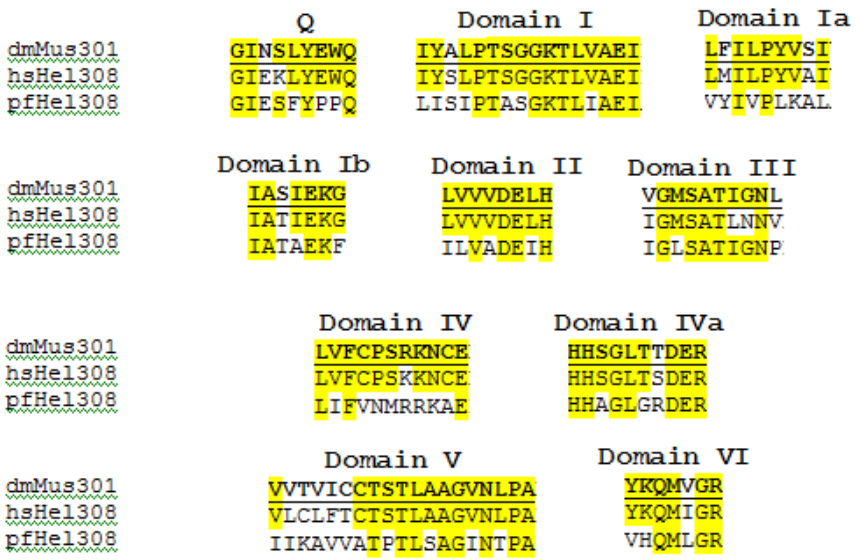

Figure 2. Protein alignment of conserved helicase domains in Drosophila *mus301, Homo sapiens hel308,* and *Pyrococcus furiosus hel308*. Isolated domains Q and I –VI are all regions known to be necessary for helicase function. dmMus301 and hsHel308 contain 81% identical amino acids across the helicase domains shown in the alignment abovewhile dmMus301 and pfHel308 contain 43% identical amino acids across the region. Highlighted letters indicate amino acid conservation between dmMus301, hsHel308, and pfHel308.

Interestingly, *mus301* is the ortholog of human and archaeal *hel308* (Guy and Bolt, 2005; Tafel et al., 2011). The *hel308* encoded protein has *in vitro* and *in vivo* unwinding activity in the 3' to 5' direction. *In vitro* biochemical analysis of Hel308 showed that the encoded gene product has a preference for unwinding the lagging strands at replication fork structures (Guy and Bolt, 2005; Tafel et al., 2011). This result suggested that Hel308, and hence Mus301, may operate more specifically at replication forks. In addition, Hel308 was shown to interact with RPA (Figure 1), a protein necessary for the stabilization of 3' overhangs formed during HR (Woodman et al., 2011). This interaction in addition to its preference for unwinding lagging structures at replication forks suggested that Hel308 might have a specific role in repair by unwinding DNA structures at replication forks to initiate HR or restart the replication fork. Since *mus301* is an

ortholog of *hel308,* it is possible that *mus301* encodes a product that has a similar role to that of the *hel308* encoded protein.

Overall, the *mus301* gene contains many similarities with other eukaryotic genes. These other genes have roles in repair outside of HR repair. The sequence similarities between these genes and *mus301* suggested that *mus301* may share these other roles. *mus301* may be involved in Alt- EJ or DNA structure unwinding at replication forks to reinitiate DNA synthesis.

mus301 and *spn-A* are homozygous lethal

When compiled together, these previous studies strongly suggested that *mus301* is necessary specifically for HR-mediated DNA repair with a potential role outside of HR either in the Atl-EJ pathway or in unwinding DNA structures at replication forks. To test the idea of mus301's involvement outside of HR, we created flies that contained both a *mus301* as well as a *spn-A* mutation. In creating these mutants, we hoped to elucidate (1) whether mus301 is involved in Alt-EJ and (2) whether mus301 acted before or after the *spn-A* encoded protein, Rad51 during HR. Interestingly, we discovered that these double mutant flies were homozygous lethal (unpublished data) suggesting that *mus301* is involved in a significant repair pathway outside of HR. In this thesis, we describe our characterization of this synthetic lethality and report other data suggesting that Mus301 may be important for DNA repair at replication forks.

Materials and Methods:

Generation of *spn-A**, *mus301^{288A}* Double Mutants

In generating the double mutants, stocks containing the *mus301^{288A}* mutation as well as two different *spn-A* mutations (denoted by *spn-A**), *spn-A^{057}* and *spn-A^{093}* were used. The *spn-A^{057}* allele contains a missense mutation that renders the protein product non-functional while the *spn-A^{093}* allele is a nonsense allele that eliminates protein formation (McVey et al., 2004a). Allen Su of the McVey laboratory generated the *mus301^{288A}* via an imprecise excision. This method involves the excision of a P-element, which causes a DSB to form. Repair of the DSB may result in deletion of DNA flanking the location of the P-element. *mus301^{288A}* is one such deletion containing a segment of 2068 bps removed, resulting in a null protein product formed.

To generate double mutants, females heterozygous for the *mus301^{288A}* mutation were crossed to males that were heterozygous for the *spn-A** mutation (Appendix **Figure 1**). From the parents, female *mus301^{288A}/ spn-A** flies were collected and mated to male (*LacZ, w$^{+)}$/TM3* flies. Recombination occurred in the germ line of the females such that *mus301*^{288A} and *spn-A** became located on the same chromosome in the males of the F2 progeny. These males were then crossed to females that were (*Lac Z, w^{+})/ Tm6b.* The F2 cross resulted in progeny flies with both mutations as well as a balancer for sorting as indicated in the F3 generation of the cross scheme (appendix **Figure 1**). Via polymerase chain reaction with primers specific for the *mus301*^{288A} and the *spn-A* mutations, we were able to narrow down the male progeny of the F1 cross to only those that had the recombination event to create the double mutant stock. The F3 progeny were eventually crossed to (*LacZ, w^{+})*/ (*Tm6b, GFP, w^{+}*) to allow for easier sorting of the flies when used in further experiments.

Sensitivity Assays

For the sensitivity assays used to further characterize the *mus301*^{288A} gene, 5 virgin females with the genotype *mus301*^{288A}*/Tm6b, GFP,* w^+ stock were collected and mated to 3 *mus301*^{288A} homozygous males. Flies were allowed to mate and lay eggs for 3 days (treatment vials). After the third day, the parent flies were transferred into new vials (control vials) and allowed to lay eggs for 2 days before being removed.

Topotecan (TPT) and Hydroxyurea (HU) Treatment

Parent flies were crossed as mentioned under "Sensitivity assays". One day after the parental flies were removed from the treatment vials, the food and larvae were treated with the mutagen. For TPT, the experimental vials were treated with 250µL of 5, 10, or 20µM of TPT diluted in water. The control vials were treated with 250µL of water. For HU, experimental vials were treated with 250µL of 10, 40, or 70 mM of HU diluted in water. Control vials were also treated with 250µL of water.

Data analysis for sensitivity assays

The eclosed flies after treatments were counted for 10 days after the first day of eclosure. Each experimental vial tested one dose of the mutagen and each dose consisted of 5 experimental vials and 5 control vials. Only trials that consisted of 100 or more eclosed flies were kept and used in the analysis. For the different crosses sensitivity was analyzed by looking at the number of different progeny that eclosed, as seen in the example cross below:

Parental ♀ $\frac{mus301^{288A}}{Tm6B}$ X $\frac{mus301^{288A}}{mus301^{288A}}$ ♂

↓

F1 progeny $\frac{mus301^{288A}}{mus301^{288A}}$ or $\frac{mus301^{288A}}{Tm6b}$

(homozygous) (heterozygous)

Percent survival was calculated by looking at the number of homozygous flies eclosed in both experimental and control vials. The following equation was used to look at percent survival for the sensitivity assay crosses:

$$\% \text{ survival} = \frac{\% \text{ homozygotes in experimental vials}}{\% \text{ homozygotes in control vials}}$$

Development Analysis

The following fly cross was conducted for the development analysis:

Parental ♀ $\frac{mus301^{288A},\ spn\text{-}A^{093}}{\boldsymbol{Tm6b,\ GFP,\ w^{+}}}$ X $\frac{mus301^{288A},\ spn\text{-}A^{057}}{Tm6b,\ GFP,\ w^{+}}$ ♂

↓

F1 $\frac{mus301^{288A},\ spn\text{-}A^{093}}{mus301^{288A},\ spn\text{-}A^{057}}$, $\frac{mus301^{288A},\ spn\text{-}A^{093}}{Tm6b,\ GFP,\ w^{+}}$, $\frac{mus301^{288A},\ spn\text{-}A^{057}}{Tm6b,\ GFP,\ w^{+}}$

Parent flies were placed into bottles to begin mating. Two days after parents began mating, they were placed into cages to lay eggs on grape agar plates. The progeny from the cross contained larvae that were either heterozygous or transheterozygous for the *mus301^{288A}, spn-A** double mutations. Heterozygous larvae contained only one copy of each of the mutations while

transhtereozygous larvae contained two copies of the *mus301* allele and two different mutated *spn-A* alleles. The transheterzygous larvae behave phenotypically like *mus301, spn-A* homozygous larvae since both genes are mutated in transheterozygotes. One day after the eggs were laid, the larvae hatched and could be sorted by the *GFP* marker. Larvae heterozygous for both mutations were fluorescent while larvae homozygous for both mutations were not. The larvae were then observed at different times points to assess their growth, development, and death. Dead larvae were removed each day to allow for easier observation of live larvae development.

Imaginal disc dissection and damage analysis

The parental flies were crossed in the following manner to generate larvae that were heterozygous for the *spn-A* mutation and homozygous for the *mus301* mutation:

Parental ♀ $\frac{mus301^{288A},\ spn\text{-}A^{093}}{Tm6b,\ GFP,\ w^{+}}$ X $\frac{mus301^{288A}}{mus301^{288A}}$ ♂

↓

F1 $\frac{mus301^{288A},\ spn\text{-}A^{093}}{mus301^{288A}}$, $\frac{mus301^{288A}}{Tm6b,\ GFP,\ w^{+}}$

Larvae at the 3rd instar stage were dissected and their wing imaginal discs were collected. Imaginal discs were stained with 5μM acridine orange for 5 minutes followed by 3 washes of ringer solution (materials in appendix) for 5 minutes each. Discs were mounted on a microscope slide with vecta shield and visualized at 20x magnification. Damage was analyzed via the number of foci present on each wing imaginal disc.

Results:

spn-A, *mus301*^{288A} mutant flies are inviable

In attempting to elucidate whether Mus301 may have a role in repair outside of HR, we generated flies that carried mutations in both *spn-A* and *mus301*^{288A}. *spn-A* is known to encode for the protein Rad51, which is necessary for HR; by mutating *spn-A*, the HR pathway was abolished. These double mutant flies could then be used in site-specific repair assays to analyze what type of repair pathway is favored in mutant backgrounds. When the *mus301*^{288A}, *spn-A* flies were created, it was noted that the stock did not homozygose. Indeed, when different alleles of *spn-A* were used to generate a *mus301*^{288A}, *spn-A* double mutant stock and crossed to each other, flies homozygous for both mutations did not pupate and eclose into adult flies (**Figure 3**). The result indicated that *mus301*^{288A}, *spn-A* homozygous flies were inviable.

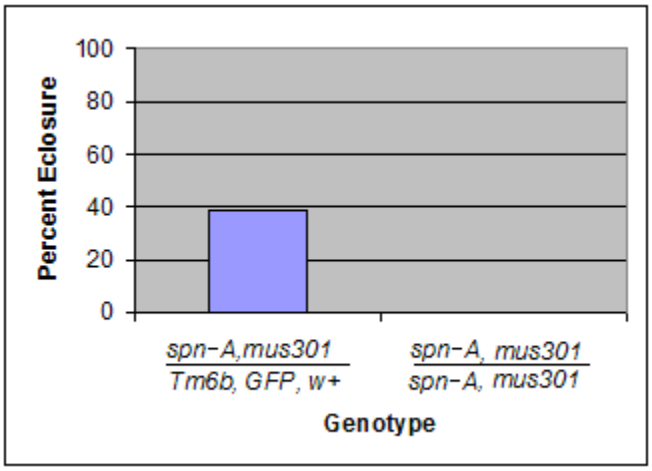

Figure 3. Double mutant developmental survival. Larvae that were heterozygous for the *spn-A* and *mus301*^{288A} mutations exhibited 39 percent eclosure while larvae containing both mutations in transheterozygous form did not survive. N = 124 larvae for both genotypes. Percent eclosure was calculated via the equation: [(# flies eclosed)/(# of initial larvae)] x 100. * indicates either 057 or 093 allele.

spn-A, *mus301*^{288A} mutant larvae do not reach third instar larvae stage

Since *spn-A*, *mus301*^{288A} flies were discovered to be homozygous lethal, we were interested in noting the time point at which death occurred for *spn-A*, *mus301*^{288A} transheterozygous larvae. In order to observe double mutants, we placed parent flies into cages to lay eggs on grape plates for easier observation of progeny. *spn-A*, *mus301*^{288A} transheterozygous larvae were seen to hatch from eggs. Just one day after egg laying (AEL), some deaths were seen to occur. By the end of 5 days AEL, larvae were no longer visible on the transheterozygous larvae plates. During the 5 days of observation, larvae heterozygous for the *spn-A*, *mus301*^{288A} double mutations increased in size throughout development as opposed to homozygous larvae, which seemed to have gained little to no mass throughout the observed days (**Figure 4**).

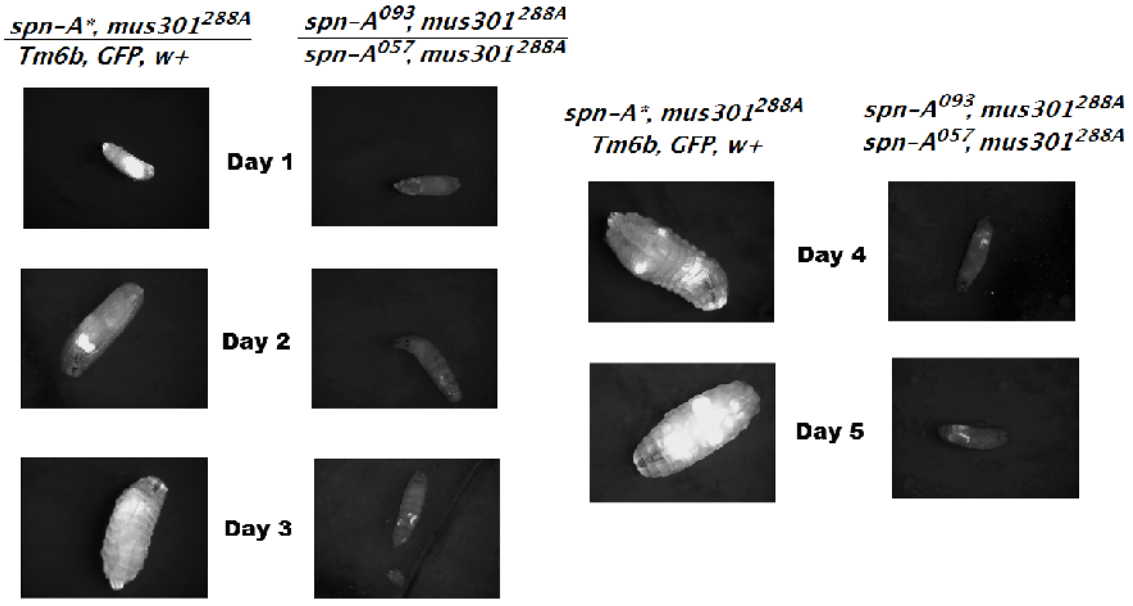

Figure 4. *spn-A, mus301*^{288} transheterozygous and heterozygous larvae development. Heterozygous larvae developed properly, increasing in size as days increased. *spn-A, mus301*^{288A} transheterozygous larvae did not seem to have increased in size throughout the observed days.

Increased apoptosis in *mus301*^{288A} homozygous, *spn-A* heterozygous larvae

Since larvae homozygous for both *spn-A* and *mus301*^{288A} mutations died in early development, we were interested in analyzing DNA damage and cell death in these larvae. One method of doing so is by looking at imaginal discs, which are tissues that undergo rapid development to form the adult fly structures. Unfortunately, since double mutants died during the first instar stage of development, it was difficult to collect imaginal discs to analyze. Thus, we created *spn-A* heterozygous, *mus301*^{288A} homozygous larvae. *spn-A* heterozygous flies are known to be haploinsufficient in repair of DNA breaks via HR (McVey et al., 2004a). *spn-A* heterozygous, *mus301*^{288A} homozygous larvae could reach third instar stage to allow for dissection of discs. Although *spn-A* heterozygous, *mus301*^{288A} homozygous larvae can survive, these larvae, like *spn-A* only heterozygotes, have reduced levels of Rad51. By looking at the imaginal discs of the *spn-A* heterozygous, *mus301*^{288A} homozygous mutants, we can gain insights into damage in *spn-A*, *mus301*^{288A} homozygous larvae. Imaginal discs dissected from *spn-A* heterozygous, *mus301*^{288A} homozygous larvae were stained with acridine orange, which fluoresces at sites of cell death since dead cells do not have the ability to excrete the chemical. As seen in **figure 5**, *mus301* mutant larvae that were also heterozygous for a mutation in *spn-A* had increased foci compared to *mus301* larvae with two wild-type copies of *Spn-A*.

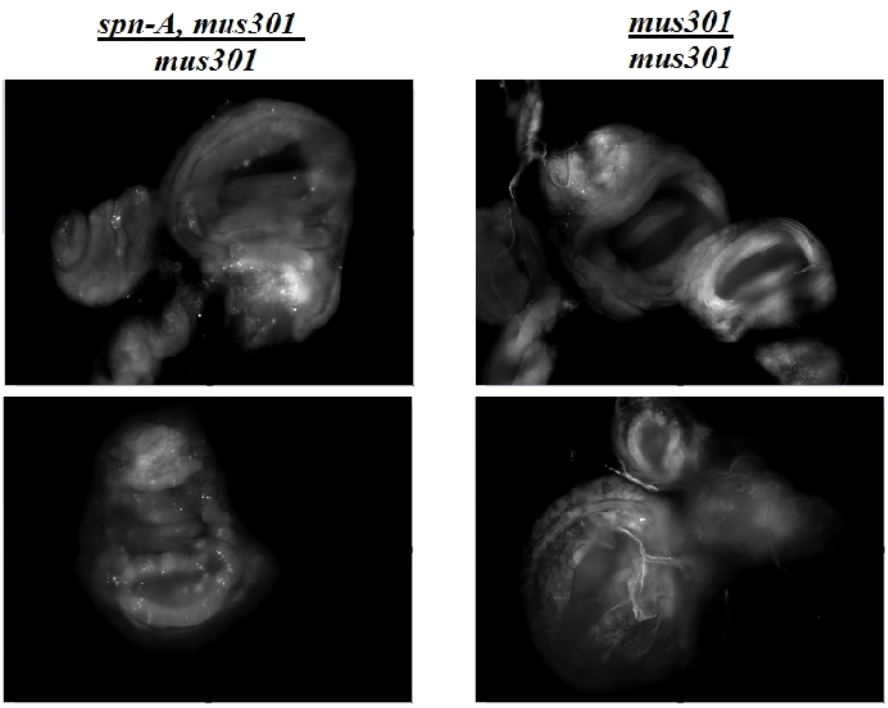

Figure 5. Wing imaginal discs stained with acridine orange from *mus301*^{288A} homozygous; *spn-A* heterozygous and *mus301*^{288A} homozygous larvae. *mus301*^{288A} homozygous; *spn-A* heterozygous larvae discs had increased foci, indicative of cell death. *mus301*^{288A} homozygous larvae discs had minimal foci.

Mus301^{288A} mutants are sensitive to topotecan

Since *mus301*^{288A} mutants were seen to be sensitive to MMS (Laurencon et al., 2004; McCaffrey et al., 2006), we were interested in whether *mus301*^{288A} flies were also sensitive to other damaging agents. One mutagen used during the sensitivity assays, was Topotecan (TPT), which is thought to operate similarly to camptothecin (CPT).

Camptothecin was originally thought to act at replication forks by hindering DNA synthesis. CPT imposes a block at the replication fork via interaction with topoisomerase (TopI), a protein necessary for unwinding DNA super-coiling tension throughout synthesis. Normally, TopI creates a single-stranded cut on the super-coiled DNA to allow it to unwind and relieve

super-coiled tension. In the presence of CPT, though, when TopI makes the cut, CPT inserts into the cut site and prevents TopI from religating the strands back together. This prevents TopI from moving away from the site of the break, and during replication, DNA polymerase would eventually collide with the CPT-TopI structure, causing a DSB to form (Liu, L., *et al*., 2000 and Pommier, 2006). More recently though, it was shown that at extremely low doses replication forks were actually stalled and repair of the stalled forks occurred. Proteins were recruited to the stalled forks to unwind and process the fork past the CPT block to continue synthesis (Ray Chaudhuri et al., 2012). Since TPT is similar in structure to CPT, TPT is thought to operate in a similar mechanism.

mus301^{288A} mutants were treated with varying doses of TPT ranging from 5 to 20μM of the mutagen. Results from the sensitivity assay showed that *mus301*^{288A} homozygous mutant flies were sensitive to the mutagen. As the dose of the mutagen increased, the survival of the mutant flies decreased (**Figure 6**).

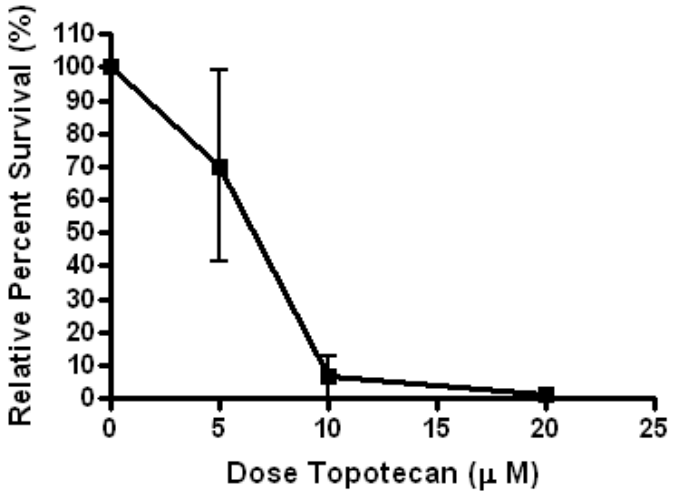

Figure 6. *mus301*^{288A} homozygous flies are sensitive to TPT. *mus301*^{288A} homozygous flies were treated with 5, 10, and 20μM of TPT. As TPT dose increased, percent survival for homozygous flies decreased. Each dose consisted of 3 trials with 5-6 vials per trial.

mus301^{288A} flies are sensitive to hydroxyurea

Since *mus301*^{288A} mutant flies were observed to be sensitive to TPT, we were interested in whether *mus301*^{288A} deficient flies were also sensitive to other mutagens that operated at DNA replication forks, such as hydroxyurea (HU). HU is known to deplete the supply of cellular dNTPs by inhibiting ribonucleotide reductase, the enzyme responsible for generating dNTP for DNA synthesis and repair (Poli et al., 2012). By depleting the supply of dNTPs, DNA polymerase cannot progress further and becomes stalled at the fork. This leaves the fork vulnerable to damage, and DSBs are thought to form. Thus in treating *mus301*^{288A} mutants with HU, we assessed the ability of *mus301*^{288A} deficient flies to fix the stalled forks generated by HU at replication forks.

mus301^{288A} mutant flies were treated with 40, 70, and 100 mM of the mutagen. The trend with the dosages showed that *mus301*^{288A} mutants were sensitive to HU at higher doses (**Figure 7**).

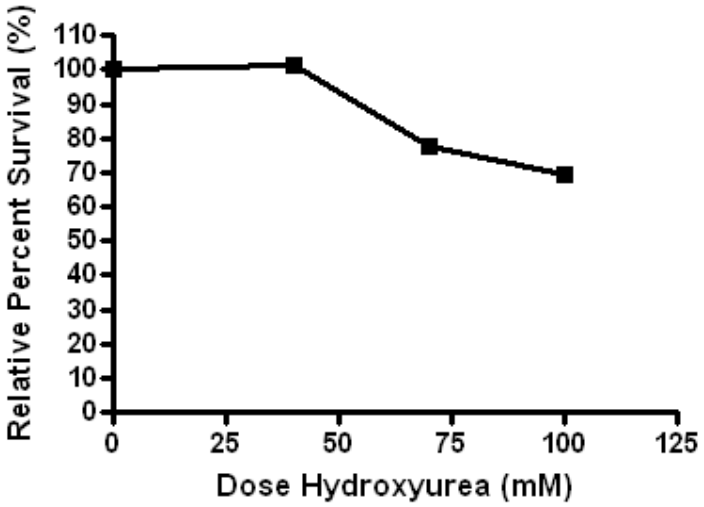

Figure 7. ***mus301*^{288A} homozygous flies are sensitive to HU.** *mus301*^{288A} homozygous flies were treated with 40, 70, and 100 mM of HU. As HU dose increased, percent survival for homozygous flies decreased. Each dose consisted of 2 trials with 5-6 vials per trial.

Discussion:

The study of DNA repair is crucial in order to understand the development of certain genetic diseases, such as cancer. One gene that is of particular interest to us in *Drosophila melanogaster* is *mus301*. The role of *mus301* in DNA repair is poorly understood. Previous research suggested that *mus301* has a role in homologous recombination (HR) repair (McCaffrey et al., 2006). Oddly enough, though, when *mus301* was mutated along with *spn-A*, flies homozygous for both mutations did not eclose (**Figure 3**). Since *spn-A* is known for its role in HR repair, the synthetic lethality suggested that the *mus301* encoded gene product has a role outside of HR. In addition, *mus301* mutants' sensitivity to MMS (Boyd et al., 1981; Laurencon et al., 2004) and camptothecin (CPT) (unpublished data) when mutated suggested that *mus301* may be involved more specifically in DNA repair at replication forks.

In order to further examine this new role of *mus301* in DNA repair at replication forks, we conducted the following: (1) sensitivity assays with TPT and HU, mutagens known to cause damage at replication forks, to further characterize the gene, (2) analysis of the development and death of *mus301, spn-A* homozygous flies and (3) imaginal disc analysis in *mus301, spn-A* larvae to assess cell death during double mutant larvae development.

To further characterize the gene, we treated *mus301* single mutants with different mutagens, including topotecan (TPT). TPT is currently used in clinical treatments of certain cancers. It is similar in structure to camptothecin (CPT) with the exception of a hydroxyl group and a dimethylamino methyl group that give rise to TPT's hydrophilic characteristic. Since the structure of TPT is similar to CPT, it is thought to have a similar mechanism of DNA damage as that of CPT by acting during DNA synthesis to stall the replication and to potentially cause DSBs.

When *mus301* mutant flies were treated with TPT, the mutants exhibited sensitivity to the mutagen at relatively low doses (**Figure 3**). This drastic sensitivity to TPT suggested that *mus301* mutants are particularly sensitive to damage at replication forks, implicating a role for *mus301* in this type of repair. Without *mus301*, mutant flies are unable to either initiate repair of the DSB or efficiently synthesize DNA past the point of damage. This would lead to their sensitivity towards mutagens that target replication forks.

mus301 mutant flies also exhibited sensitivity to HU. HU operates by inhibiting ribonucleotide reductase causing a depletion of nucleotide bases necessary for DNA synthesis. This would case the replication fork to stall since synthesis cannot continue. In this instance the fork would either remain stalled until it can restart or the stall would eventually form a DSB. Since *mus301* mutant homozygous flies were sensitive to HU, the result suggested that the *mus301* encoded protein must be necessary for repair at replication forks.

In addition to further characterizing the *mus301* gene, we originally hoped to study the role of *mus301* outside of HR repair by creating *mus301*, *spn-A* double mutant flies. In the process of our experimentation, we discovered that *mus301* was synthetically lethal with *spn-A* when both genes were mutated (**Figure 5**). This result led to our analysis of the development of fly larvae that contained both mutations. Since adult flies did not eclose, we hoped to address the question of whether fly larvae with both mutations were viable, and if so, at which point did larval death occur. In pinpointing the time of death, we wanted to further understand the mechanism through which *mus301* operates.

To analyze the development of double mutant larvae, we utilized two *spn-A* alleles, *spn-A*^{057} and *spn-A*^{093}, in our crosses as mentioned in methods. By using two different alleles, we eliminated the possibilities that the lethal phenotype we saw could have arisen from a second site

mutation on either one of the *spn-A* alleles and the possibility that *mus301* was only lethal when mutated with a specific type of mutation in the *spn-A* allele. The lethal phenotype observed for double mutants was severe, occurring early in larval development. This result, in combination with the observation that double mutant larvae did not increase in size like their control heterozygous larvae (**Figure 6**), implied that these mutants were unable to undergo replication to increase mass and cell number, suggesting a defect in DNA synthesis. Despite this defect, these eggs were able to survive until hatching and larvae were able to survive into first instar. This ability to hatch may be due to maternal contribution of some functional Rad51 and Mus301 protein that allowed for early survival. Past first instar though, the maternal proteins would not be enough to compensate for the lack of functional Rad51 and Mus301 proteins in the growing larvae, leading to no growth and early death for double mutant larvae. This lethal point in development and lack of growth suggested a role for -*mus301* in replication fork repair

To further analyze the damage that occurred in *mus301*, *spn-A* double mutants, we hoped to collect imaginal discs to visualize cell death. Since *mus301* may have a role in DNA repair at replication forks, a mutated *mus301* would lead to accumulated damage at the forks. *mus301* mutant flies would have increased cell death in the imaginal discs, making visualization easier. Unfortunately, *mus301*, *spn-A* homozygous double mutants were inviable at the third instar stage, the best developmental period to harvest the imaginal discs. Therefore, in order to visualize cell death in double mutant flies, we instead created flies that were homozygous for the *mus301* mutation, but heterozygous for the *spn-A* mutation.

This new mutant was an important tool in studying *mus301*, *spn-A* double mutants. Despite having one wild-type copy of the *spn-A* gene, flies that were heterozygous for the *spn-A* mutations were shown to have a defect in HR (McVey et al., 2004a). This haploinsufficient

phenotype in HR repair would allow us to study larvae that do not have wild-type levels of HR repair. In addition, the *mus301* homozygous, *spn-A* heterozygous larvae were able to survive until adulthood, enabling the harvest of imaginal discs at the third instar stage for visualization.

The results from the staining revealed that in comparison to larvae with only the *mus301* mutation, the *mus301* homozygous, *spn-A* heterozygous mutant larvae had increased cell death in the imaginal discs (**Figure 7**). Since *spn-A* heterozygosity in combination with the *mus301* mutation led to increased cell death, it is likely that even more apoptosis occurs in larvae that were homozygous for both mutations. This increase in apoptosis could explain the lack of growth and lethal phenotype observed in *mus301*, *spn-A* homozygous double mutant larvae. These double mutants are unable to repair damage at replication forks via HR as well as the mechanism by which Mus301 operates. One possibility is that Mus301 operates in a Rad51 independent manner to repair DSBs. Since it was shown that flies with a mutation in the *mus301* gene have increased DNA damage (McCaffrey et al., 2006), it is possible that the *mus301, spn-A homozygous* deaths can be attributed to increased DNA breaks and alludes to *mus301* having a direct role in DSB repair. This may not be the case though since double mutant larvae are able to hatch from eggs, but do not gain any mass throughout development. If the *mus301* product is necessary for repair, it is likely that the larvae would not have hatched from the egg. The ability to survive to first instar larvae suggests it is more likely that *mus301* is involved in repair at replication forks. A deficiency in repair in this area would cause larval cells to be unable to replicate, leading to the lack of growth seen in double mutant larvae. By removing both HR and the Mus301 pathway of repair, DNA damage in the double mutant larvae would accumulate and would eventually led to organism death.

These results, when cumulated, suggested that *mus301* encodes a protein with a specific role in repair at replication forks. The exact mechanism of Mus301 in DNA repair is still unknown, but from our data and from the result of previous research, we propose a model under which Mus301 may operate.

Model of Mus301 Mechanism of Action

Research in archael *hel308*, an ortholog of *mus301*, showed that *hel308* had a preference for unwinding lagging strands at replication forks (Guy and Bolt, 2005; Tafel et al., 2011). Since *mus301* is an ortholog of *hel308*, it is likely that it encodes a protein that has the same preference. In addition, *hel308* was also shown to interact with and bind to Replication Protein A (RPA) (Woodman et al., 2011). RPA, as mentioned before, is necessary for DNA repair by binding to ssDNA (**Figure 1**). This interaction with RPA as well as the ability to unwind lagging strand DNA suggests a model in which the *mus301* protein product is recruited to the sites of stalled forks and acts to either recruit replication proteins to the site of stalling or unwinds DNA structures to help bypass the stall (Woodman et al., 2011).

With previous research and with our experimental results, we provide here a model under which *mus301* may operate in DNA repair at replication forks. When an inhibitor blocks the leading strand template, DNA polymerase will stop synthesis of the leading strand, but lagging strand synthesis may continue uninterrupted for some time. As lagging strand synthesis continues, the leading strand template is exposed in single-stranded (SS) form since the template is unwound, but DNA polymerase cannot access it to continue replication. This exposure of the leading strand template signals RPA to bind the ssDNA to prevent degradation or other processes from damaging the DNA. Once bound, RPA then recruits the *mus301* encoded protein to the site

of ssDNA exposure. The *mus301* protein, when bound to RPA, is then relocated to the lagging strand template and can then help the replication fork bypass the damage by unwinding the lagging strand to allow leading strand synthesis past the inhibition. In this mechanism, Mus301 would unwind the lagging strand to the point that it would then anneal with the newly synthesized leading strand to form a "chicken-foot" structure. Once the two strands have annealed, the leading strand would then use the lagging strand as a template to elongate until the leading strand is long enough to bypass the inhibitor on the leading template. The "chicken-foot" structure then dissociates and the leading strand reanneals with the leading strand template to continue DNA synthesis (Woodman et al., 2011; Ray Chaudhuri et al., 2012) as shown in **figure 8**.

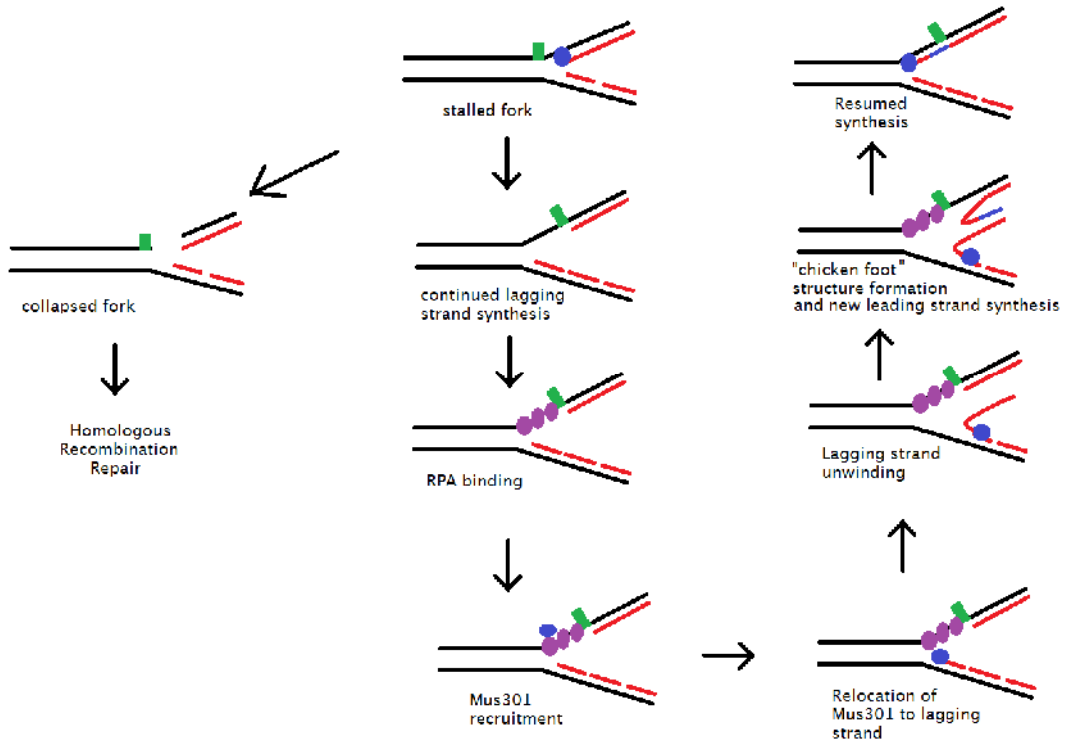

Figure 8. **Mus301 mechanism of action model.** Mus301 is recruited to stalled replication forks by RPA binding to ssDNA. Mus301 is relocated from the leading strand to the lagging strand and unwinds okazaki fragments on the lagging strand. This allows the formation of the "chicken foot" structure by which the leading strand can synthesize past the inhibition on the leading

strand template. The "chicken foot" structure dissociates and allows for normal replication to resume.

This mechanism in which Mus301 unwinds the lagging strand to help bypass the replication block also helps explain why larvae that carried both *mus301* and *spn-A* mutation did not survive to adulthood. Under normal circumstances, blocks can occur at replication forks. In these instances, the Mus301 pathway is likely the primary method of bypassing the inhibition. In the event that Mus301 is not available, though, it is possible that the alternative method is to then use HR to help the leading strand synthesize past the inhibition to continue replication. The leading strand can invade into a homologous chromosome or a sister chromatid, using Rad51 to aid in the invasion step. The leading strand can then use the new template to continue synthesis of the leading strand. Once the synthesis has bypassed the site of inhibition, the leading strand re-anneals with the template strand and normal replication ensues. If both the HR and Mus301 pathways of repair are removed, the flies no longer have a method of bypassing the replication blocks. These forks are then continuously stalled, leading to the increased likelihood of DSB formation. As DSBs increase, cells apoptose, and eventually when enough cell death occurs, organism death will follow.

Results from our sensitivity assay lend support to our proposed model. Since TPT targets replication forks and *mus301* flies are deficient in synthesizing past inhibitors at replication forks, *mus301* mutated flies would not be able to survive increasing amounts of mutagens blocking the replication fork. At low doses of the mutagen though, it is possible to use HR mediated repair to aid leading strand synthesis past the block, but this method is likely not as efficient as the *mus301* pathway of repair. Thus, in increasing amounts of mutagen, the HR pathway is not efficient enough to restart the stalled replication forks. As mentioned before, these persistent, stalled replication forks are likely to form DSBs that can then cause larval death when

accumulated. A similar mechanism in which DSBs are triggered may also be occurring in the HU treatments, but further research needs to be conducted in order to understand the mechanism of HU and its interaction with replication forks.

Future directions

In moving forward with illuminating the role of *mus301* in DNA repair, there remain areas in which more research could help us further understand the mechanism of *mus301*. In looking at *spn-A* heterozygous, *mus301* homozygous flies, we are also interested in whether these mutants would exhibit slower development compared to larvae that are only *mus301* mutant homozygous. Since *spn-A* heterozygotes exhibited more cell death in imaginal discs that *mus301* homozygotes we expect that while the heterozygotes would survive to adulthood, they would develop slower than *mus301* homozygous mutants.

It is also fascinating that *spn-A, mus301* flies are non-viable and a future project that may be of interest would be analyzing survival in *mus301* flies with *spn-A* knocked down via RNAi. It would also be interesting to note whether *mus301, spn-A* knock down larvae had increased cell death in their imaginal discs, indicating more DNA damage.

Another area of interest would be the ability to rescue the synthetic lethality of *mus301, spn-A* mutations. We are currently conducting a rescue cross for *mus301*, *spn-A* double mutants in hopes of further understanding how *mus301* operates in DNA repair. In the rescue cross, we crossed flies with both the *mus301* and *spn-A* mutations to flies with a mutation in *mus81*. *mus81* is thought to have a role in blocked fork repair by cleaving DNA at the site of inhibition (Trowbridge et al., 2007). The breaks that *mus81* induces have to then be processed and repaired. It is possible that *mus81* creates incisions at the site of replication blocks, which allows the

mus301 encoded protein to unwind the DNA and form the chicken-foot structure. As mentioned before, the formation of the structure allows for the leading strand to synthesize past the site of inhibition such that the replication fork can progress normally afterwards.

In our *mus301*, *spn-A* homozygous flies, *mus81* is still functional. It is possible that *mus81* introduces the nicks to the DNA, but these nicks remain unrepaired due to the lack of *mus301* available to process the breaks. These breaks could potentially be repaired through HR, but since *spn-A* is also mutated in the double mutant flies, this pathway is also knocked out. Therefore, with no method of repairing the nicks caused by *mus81*, the damage may accumulate and lead to cell and organism death. By knocking out the function of *mus81*, though, we can prevent the endogenous breaks from forming. In this case, neither *mus301* nor *spn-A* would be necessary for organism survival and the *mus81* mutation could rescue the *mus301*, *spn-A* double mutant lethality observed (cross scheme, appendix **Figure 2**).

Additionally, it would also be interesting to see whether *mus301* and *mus81* operate in the same pathway of repair. To address this question, it would be interesting to conduct sensitivity assays with the mutagens that *mus301* has previously been shown to be sensitive to (TPT, CPT, MMS). In this instance, should *mus301* and *mus81* operate in the same pathway, the sensitivity of *mus301*, *mus81* double mutants should not differ from that of *mus301* single mutants. In conducing this set of sensitivity assay, we can also assess whether *mu81* and *mus301* mutations are also synthetically lethal. *Mus81* and *mus309* were observed to lead to death when both genes were mutated(Trowbridge et al., 2007). Thus, it would be interesting to see the effects of the *mus81* and *mus301* mutations combined.

Aside from conducting further assays to understand the role of mus301 in repair, it would also be important for us to repeat some assays. For instance, it would be valuable to re-generate

the *mus301, spn-A* mutations to see whether the new double mutants also contain the lethal effect. In re-conducting this assay, we can provide further support to our model.

Summary

In conclusion, we found that *mus301*, *spn-A* double mutations are synthetically lethal in the fruit fly. This phenotype could be due to *mus301*'s ability to process DNA at stalled replication forks. Here we proposed a model in which *mus301* unwinds lagging strands at the replication forks to either reload replication proteins past the point of inhibition at stalled forks or to create the "chicken-foot" structure to allow the leading strand to synthesize past the inhibitor. This would restart the DNA replication, allowing for synthesis to complete. There still remains more to be elucidated regarding *mus301*'s role in DNA repair. Comprehending how *mus301* functions normally in fruit flies may offer us insights into how the genome is preserved during replication and repair. In understanding the proteins involved in the important life processes, we may gain a better perspective of how genetic disease may arise and how to potentially treat them in a more efficient manner.

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References:

1. Adams, M.D., McVey, M., and Sekelsky, J.J. (2003). Drosophila BLM in double-strand break repair by synthesis-dependent strand annealing. Science *299*, 265–267.

2. Boyd, J.B., Golino, M.D., Shaw, K.E., Osgood, C.J., and Green, M.M. (1981). Third-chromosome mutagen-sensitive mutants of Drosophila melanogaster. Genetics *97*, 607–623.

3. Chan, S.H., Yu, A.M., and McVey, M. (2010). Dual roles for DNA polymerase theta in alternative end-joining repair of double-strand breaks in Drosophila. PLoS Genet. *6*, e1001005.

4. Fujikane, R., Komori, K., Shinagawa, H., and Ishino, Y. (2005). Identification of a novel helicase activity unwinding branched DNAs from the hyperthermophilic archaeon, Pyrococcus furiosus. J. Biol. Chem. *280*, 12351–12358.

5. Guy, C.P., and Bolt, E.L. (2005). Archaeal Hel308 helicase targets replication forks in vivo and in vitro and unwinds lagging strands. Nucleic Acids Res. *33*, 3678–3690.

6. Heyer, W.-D., Ehmsen, K.T., and Liu, J. (2010). Regulation of homologous recombination in eukaryotes. Annu. Rev. Genet. *44*, 113–139.

7. Kovacic, P. (2011). Hydroxyurea (therapeutics and mechanism): metabolism, carbamoyl nitroso, nitroxyl, radicals, cell signaling and clinical applications. Med. Hypotheses *76*, 24–31.

8. Laurencon, A., Orme, C.M., Peters, H.K., Boulton, C.L., Vladar, E.K., Langley, S.A., Bakis, E.P., Harris, D.T., Harris, N.J., Wayson, S.M., et al. (2004). A large-scale screen for mutagen-sensitive loci in Drosophila. Genetics *167*, 217–231.

9. McCaffrey, R., St Johnston, D., and González-Reyes, A. (2006). Drosophila mus301/spindle-C encodes a helicase with an essential role in double-strand DNA break repair and meiotic progression. Genetics *174*, 1273–1285.

10. McVey, M. (2010). In vivo analysis of Drosophila BLM helicase function during DNA double-strand gap repair. Methods Mol. Biol. *587*, 185–194.

11. McVey, M., Adams, M., Staeva-Vieira, E., and Sekelsky, J.J. (2004a). Evidence for multiple cycles of strand invasion during repair of double-strand gaps in Drosophila. Genetics *167*, 699–705.

12. McVey, M., Larocque, J.R., Adams, M.D., and Sekelsky, J.J. (2004b). Formation of deletions during double-strand break repair in Drosophila DmBlm mutants occurs after strand invasion. Proc. Natl. Acad. Sci. U.S.A. *101*, 15694–15699.

13. Petermann, E., and Helleday, T. (2010). Pathways of mammalian replication fork restart. Nat. Rev. Mol. Cell Biol. *11*, 683–687.

14. Poli, J., Tsaponina, O., Crabbé, L., Keszthelyi, A., Pantesco, V., Chabes, A., Lengronne, A., and Pasero, P. (2012). dNTP pools determine fork progression and origin usage under replication stress. Embo J. *31*, 883–894.

15. Ray Chaudhuri, A., Hashimoto, Y., Herrador, R., Neelsen, K.J., Fachinetti, D., Bermejo, R., Cocito, A., Costanzo, V., and Lopes, M. (2012). Topoisomerase I poisoning results in PARP-mediated replication fork reversal. Nature Structural & Molecular Biology.

16. Richards, J.D., Johnson, K.A., Liu, H., McRobbie, A.-M., McMahon, S., Oke, M., Carter, L., Naismith, J.H., and White, M.F. (2008). Structure of the DNA repair helicase hel308 reveals DNA binding and autoinhibitory domains. J. Biol. Chem. *283*, 5118–5126.

17. San Filippo, J., Sung, P., and Klein, H. (2008). Mechanism of eukaryotic homologous recombination. Annu. Rev. Biochem. *77*, 229–257.

18. Staeva-Vieira, E., Yoo, S., and Lehmann, R. (2003). An essential role of DmRad51/SpnA in DNA repair and meiotic checkpoint control. Embo J. *22*, 5863–5874.

19. Tafel, A.A., Wu, L., and McHugh, P.J. (2011). Human HEL308 localizes to damaged replication forks and unwinds lagging strand structures. J. Biol. Chem. *286*, 15832–15840.

20. Trowbridge, K., McKim, K., Brill, S.J., and Sekelsky, J. (2007). Synthetic lethality of Drosophila in the absence of the MUS81 endonuclease and the DmBlm helicase is associated with elevated apoptosis. Genetics *176*, 1993–2001.

21. Woodman, I.L., Brammer, K., and Bolt, E.L. (2011). Physical interaction between archaeal DNA repair helicase Hel308 and Replication Protein A (RPA). DNA Repair (Amst.) *10*, 306–313.

Appendix:

Ringer solution

The following components were mixed in water and used larvae dissection and staining procedures:

1) 130mM NaCl
2) 5mM KCl
3) 1.5mM MgCl2

Double Mutant Generation cross:

Flies with both the *mus301*^{288A} and *spnA** mutations were generated via the following cross scheme:

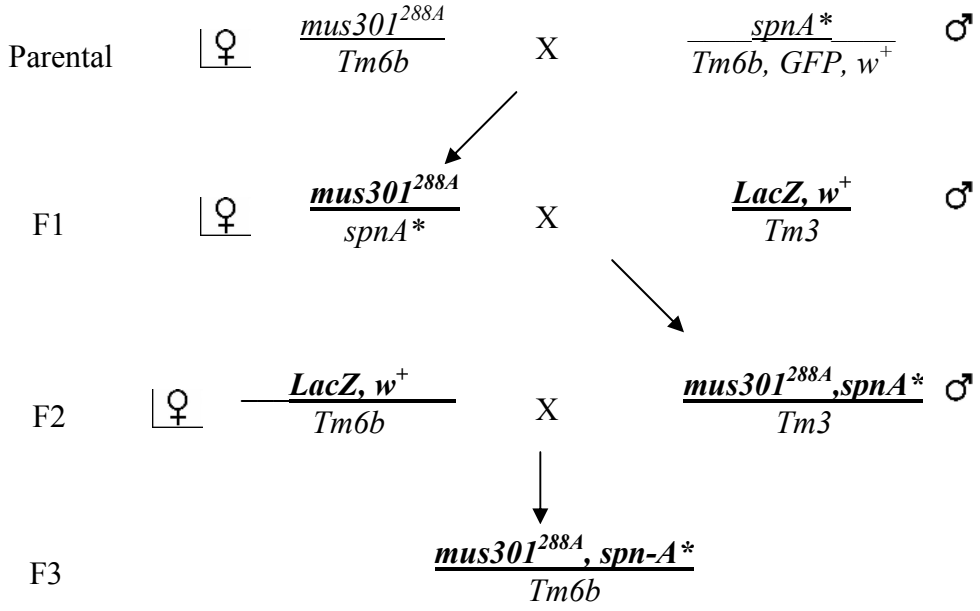

Figure 1. Cross Schematic for Double Mutant generation. This cross scheme was used as describes in "methods".

Rescue Cross

The rescue cross was conducted in the following manner:

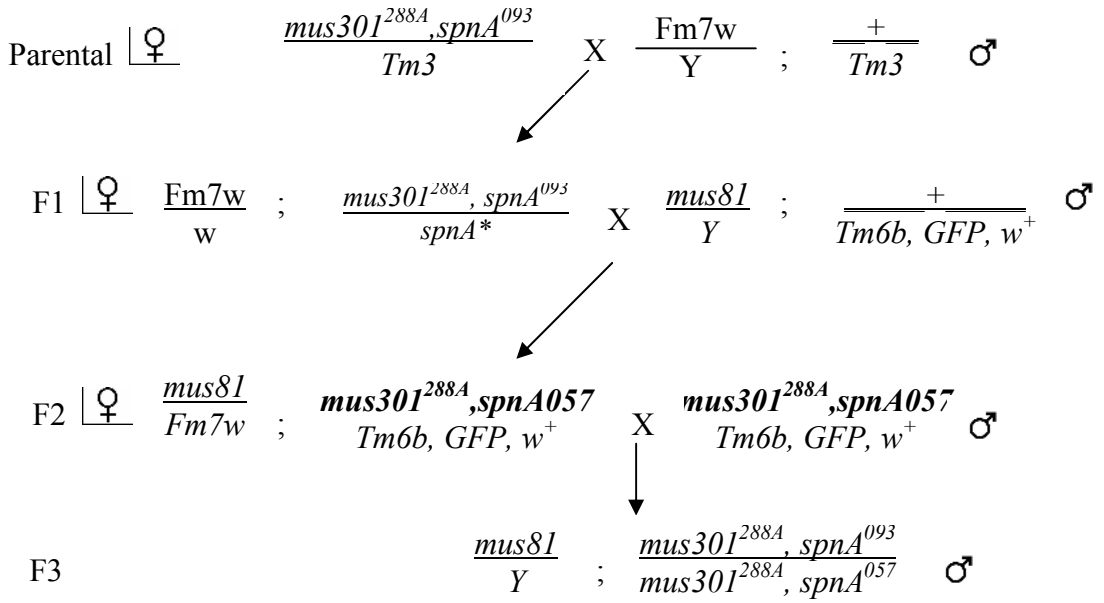

Figure 2. Lethal Phenotype Rescue Cross. Via this cross, a *mus81* mutation will be introduced to *spnA*, *mus301*^{288A} double mutant flies. The F3 progeny of this cross will be observed for survival and/or points of lethality.