Determining the Function of Blm, FancM, and HelQ Helicases in Repair of Double Strand Breaks via Synthesis Dependent Strand Annealing

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Abstract

In *Drosophila*, DNA double-strand breaks that occur in the soma and the pre-meiotic germline are frequently repaired by homologous recombination via synthesis-dependent strand annealing (SDSA). Several different helicases, including Blm, FancM, and HelQ, are thought to be involved in SDSA. Their importance is underscored by the consequences associated with their absence, including genome instability and cancer. However, their exact roles and the ways in which they might interact with one another are not well understood. Here, we report the results of experiments designed to elucidate the *in vivo* roles of these helicases.

We constructed single and double mutants lacking Blm and/or HelQ and tested their ability to carry out SDSA repair using a well-characterized, site-specific gap repair assay. Similar to published reports, we observed that *blm* mutants were defective in SDSA repair, with short repair synthesis tract lengths and a high frequency of deletions flanking the break site. Flies lacking HelQ were also unable to carry out SDSA repair proficiently, though they did not display a deletion-prone phenotype. Interestingly, the absence of HelQ suppressed the deletion-prone repair of the *blm* mutants, suggesting that these two helicases play different roles during SDSA and that a third helicase might be used for this purpose in the absence of Blm.

Because both FancM and Blm can unwind D-loop recombination intermediates *in vitro*, we set out to test whether FancM is involved in SDSA. Indeed, *fancM* mutants showed a decreased ability to complete SDSA repair, though flanking deletions were not observed. Approximately 20% of *fancM* mutant repair events resulted from immediate endjoining, suggesting that FancM may be important for the initiation of SDSA. Repair events from *fancM helQ* double mutant flies exhibited extremely short DNA synthesis tracts, consistent with the hypothesis that these two helicases carry out independent functions during SDSA. Our results

suggest that the Blm and FancM helicases play partially overlapping roles during SDSA repair, but that Blm, HelQ, and FancM also have independent functions in D-loop processing. Together, these data may explain the disparate phenotypes of animal models with mutations in these genes.

Finally, in order to further characterize HelQ's function during the repair of double strand breaks, we used the UAS/GAL4 system to overexpress HelQ in specific fly tissues. Using this system, we were able to detect a tagged version of the protein through western blotting. This system will allow for further experiments to be performed to determine other proteins with which HelQ interacts.

Introduction

Repair of Double Strand Breaks

One of the more dangerous mutations that can damage DNA is a double strand break (DSB), which must be readily repaired to ensure cell survival and prevent mutations. DSBs can arise as a result of exposure to different damaging agents, such as ionizing radiation, or from the presence of reactive oxygen molecules. DSBs can also occur when chromosomes undergo mechanical stress, or during DNA replication, when replication forks stall due to the confrontation of a lesion (Khanna and Jackson 2001). DSBs are produced naturally during meiosis for recombination between homologous chromosomes so as to allow for the exchange information and the creation of genetic diversity.

If DSBs are not repaired, transcription and replication cannot take place and genome integrity is lost. While this damage can lead to cell death, it can also promote tumor growth as these breaks can result in mutations, like genome rearrangements or deletions. This can be most carcinogenic when breaks occur in tumor suppressing regions of the DNA or if regulation of a proto-oncogene is lost. Defects in DNA repair proteins often lead to inherited diseases that are characterized by a predisposition to cancer. In order to better understand diseases, like cancer, it is important to understand the mechanics of how these DSBs are repaired.

When a cell recognizes that damage has occurred, the DNA damage response can arrest or slow cell cycle progression (Rouse et al. 2002). This signaling process is mediated by a phosphatidylinositol 3-kinase-like kinase (PIKK), usually ATM which responds predominately to DSBs (Weterings et al. 2008). ATM, known as Tel1p in yeast, phosphorylates cell cycle regulators such as p53, Chk1, and Chk2 in humans to then in turn arrest cells in G1 or G2. While the division of the damaged cell is prevented, the cell also has several complex ways of repairing

DSBs to prevent the loss of genomic information or the triggering of apoptosis. The mechanism chosen depends on where the cell is in the cell cycle (Brandsma et al. 2012). These repair mechanisms are often divided into two different categories; non-homologous endjoining (NHEJ) and homologous recombination (HR).

NHEJ has frequently been considered to be the error-prone repair pathway because it was observed to generate small insertions and deletions, especially when the broken ends could not be readily re-ligated (reviewed in Pardo et al. 2001). NHEJ has an error rate of about 10^{-3} per joining event between fully compatible DSB ends in yeast (Moore and Haber 1996). This repair pathway is categorized by the proteins recruited in two subcategories: classical NHEJ (C-NHEJ) and alternative-EJ (alt-EJ). In C-NHEJ, the Ku70/80 heterodimer recognizes the break and binds to the DNA ends to prevent degradation (Figure 1, Chan et al. 2010). Polymerases are then able to produce the sequence for repair by filling in missing nucleotides. Finally, the strands can be ligated back together by DNA ligase 4 and XRCC4.

When cells lack one or more of the proteins necessary for C-NHEJ, alternative endjoining occurs. This process can cause larger deletions than those observed in C-NHEJ. Ku80-deficient mice cells showed an increase in chromosomal translocations, which is associated with alt-EJ (Difilippantonio et al. 2000).

In contrast to these two error-prone pathways, homologous recombination (HR) is virtually error-free and, seemingly, a better choice for the cell's survival. HR occurs when the 3' end of the broken strand is able to invade a homologous sequence via Rad51 and produce a product identical to the original sequence before the damage occurred (Figure 1). The mode of repair, whether HR or endjoining, depends on the timing of the cell cycle, as HR is preferable during S and G2 phases when the sister chromatid is present (Brandsma et al. 2012). HR has

been shown to occur in diploid yeast during G1, which suggests that it may occur at other times in the cell cycle and that its regulation depends greatly on which proteins are recruited (Heyer et al. 2010).

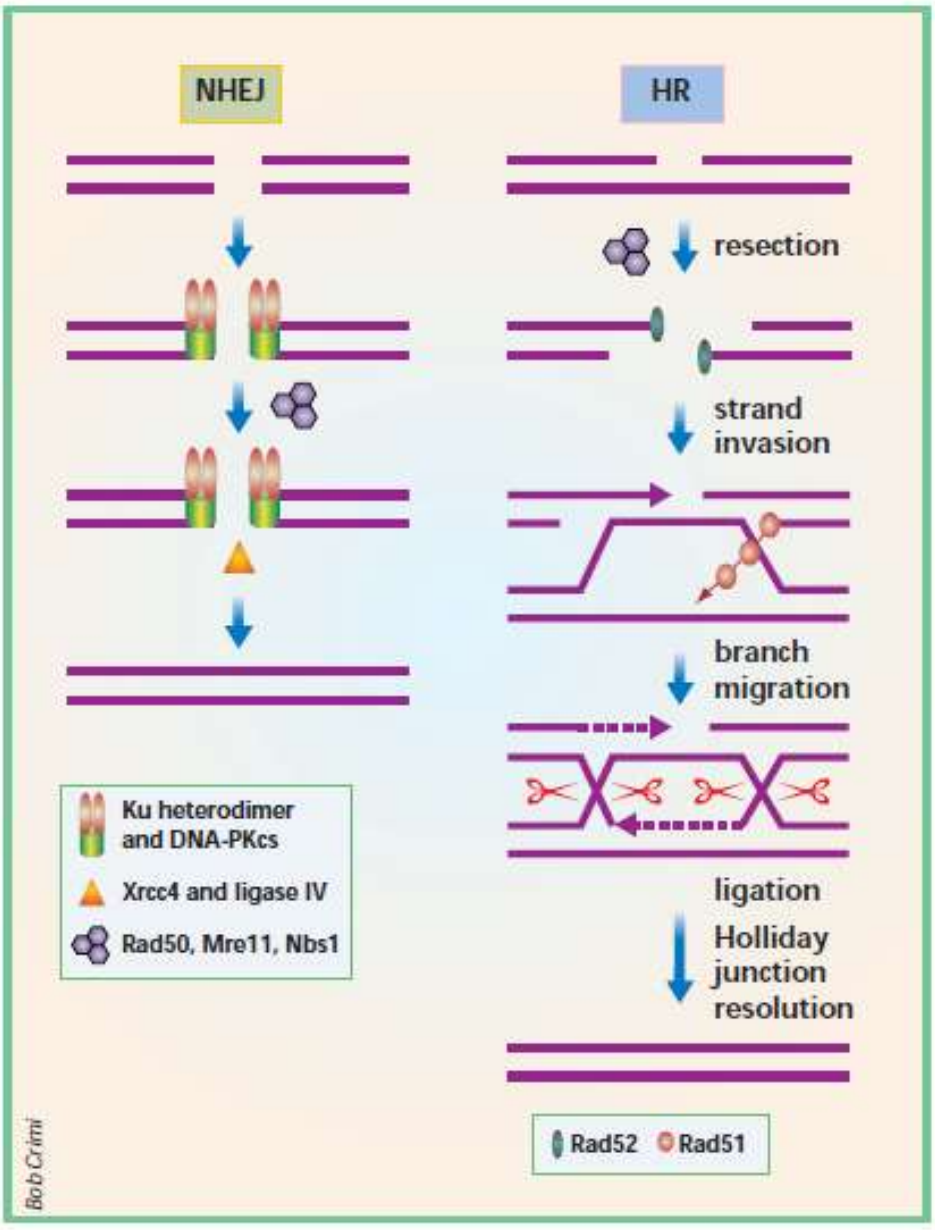

Figure 1. Pathway of Double Strand Break Repair with HR and NHEJ. The two main repair pathways for repair of DSBs. Depending on the point of the cell cycle, one of these two main pathways is chosen. (Adapted from Khanna and Jackson 2001)

At the beginning of HR, several nucleases resect the 5' ends of the DNA, leaving 3' ssDNA overhangs. In yeast, nucleses Exo1, Dna2, and Sae2 (human CtIP), the MRX complex consisting of the proteins Mre11, Rad50, and Xrs2, as well as the helicase Sgs1 (human BLM), are involved in resection (Mimitou and Symington 2008). Mre11, a nuclease in the MRX complex, is necessary for the removal of bulky adducts from DNA ends that arise with IR damage. Mre11 does not perform processive resection and is instead used for an initial endonucleolytic clipping of ends of DNA (Mimitou and Symington 2009). Sae2 is able to regulate Mre11's activity and recruit other downstream factors. Exo1 works in a second step for more extensive resection. It is thought that Sgs1 also works during this step with Dna2, but it is independent and also redundant to Exo1's role (Mimitou and Symington 2008).

Following resection, the ssDNA is bound by RPA (replication protein A) to stop any secondary DNA structures from forming. BRCA2 mediates the exchange of RPA for Rad51 as it acts as a scaffolding protein with binding domains for ssDNA, dsDNA, and Rad51 (Heyer et al. 2010). Once Rad51 binds to the ssDNA to form a nucleoprotein filament, a homology search ensues (Brandsma et al. 2012).

During synapsis, a Rad51 filament invades the homologous template (either a sister chromatid or a homologous chromosome), and the displacement-loop (D-loop), a DNA intermediate, forms as one strand of the dsDNA is displaced while the invading strand anneals to the complementary sequence (Figure 2). This results in heteroduplexDNA (hDNA). Rad54 is also involved in pairing ssDNA to its homologous sequence and may work as a pump to translocate the D-loop structure (Wright et al. 2014). Polymerases extend the sequence off of the homologous template to produce a product identical to the original. While polymerases are synthesizing the repair product, several helicases are likely involved in unwinding the DNA to

enlarge the D-loop to allow for more synthesis and also in disassociating the nascent strand of the D-loop after repair has occurred.

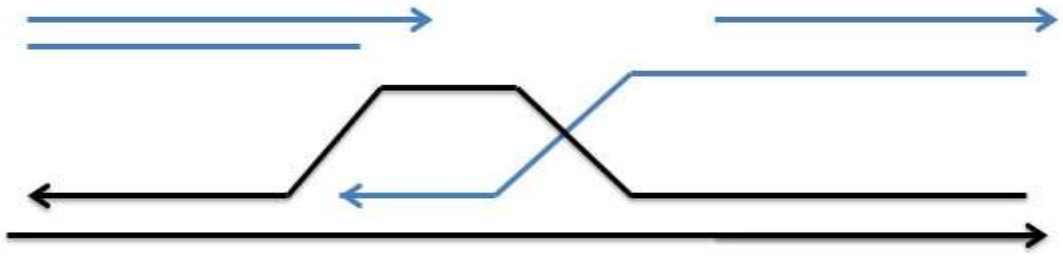

Figure 2. Displacement loop DNA intermediate. DNA structure formed once the 3' ssDNA invades a homologous template via Rad51 filament. Helicases are thought to unwind the D-loop structure during SDSA to dissolve and allow for ligation.

The product produced from the repair event determines the type of HR that occurred. The double Holliday Junction (dHJ) subpathway can often form crossover events. The other repair mechanism is synthesis dependent strand annealing (SDSA), a subpathway of HR in which the second end of the DSB anneals with the extended strand of the first end (Figure 3, Heyer et al. 2010). Two branched DNA structures are formed in dHJ and can lead to crossover or noncrossover products depending on the cleaving. dHJ are often resolved with nucleases, such as Mus81 or Gen, which cleave the structure. In contrast, SDSA appears to involve several helicases that produce a noncrossover product, including Sgs1 (human BLM) and Srs2 in yeast or possibly RecQ family helicases in mammals (Pardo et al. 2010). Crossover events occurring through the cleavage of dHJ can cause a loss of heterozygosity and genome rearrangements, while SDSA avoids crossover products and may reduce genome rearrangements (Bugreev 2007).

The helicase Blm seems to play a role in both mechanisms. With Blm, TopoIIIa, RMI1, and RMI2 form the Blm dissolvasome, a protein complex that can unwind dHJ. Blm has a binding domain for TopoIIIa which allows for it to remove torsional strain in dHJ to prevent forming crossovers (Manthei and Keck 2013). RMI1 interacts with Blm and TopoIIIa to stabilize

the structure and increase its ability to dissolve dHJ by 10-fold (Wu et al. 2006). Blm works to regulate whether crossover events are occurring when these breaks are created in meiosis (McVey et al. 2007). Blm was also shown to promote branch migration of HJs and to be a suppressor of recombination which prevents crossover formation during repair (Karow et al. 2000).

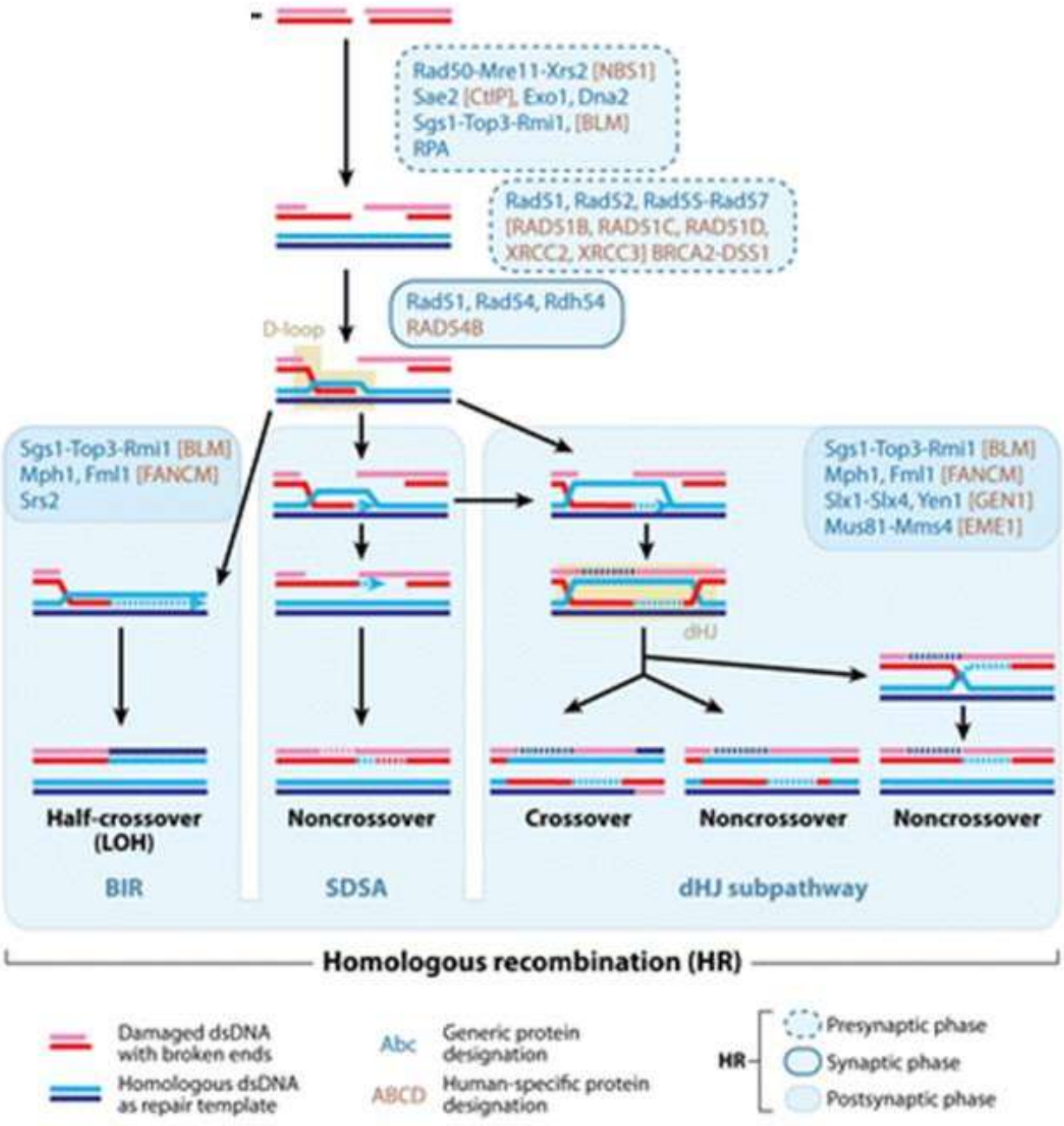

Figure 3. Pathways of DSB repair Utilizing Homologous Templates. The subpathways of HR are shown with the proteins used as well as the DNA products formed from the repair. (Adapted Heyer 2010.)

Helicases Involved in HR

Blm helicase, a RecQ-family helicase encoded by *blm*, has also been seen to play a role in SDSA (reviewed in Manthei and Keck 2013). Mutations in BLM cause Bloom's syndrome, a rare autosomal disease, characterized by aberrant DNA repair and a predisposition to cancer (Deans and West 2009). Patients with Bloom's syndrome (BS) have proportional dwarfism, sterility, and immunodeficiency. This occurs because Blm is necessary for genome stability as BS cells have an increase in chromosomal breaks and rearrangements, as well as an increase in sister chromosome exchange.

Blm by itself, a 3' to 5' ATP dependent helicase, can melt D-loops *in vitro* as it preferentially unwinds D-loops with 3' ssDNA tails (van Brabant 2000). Bugreev showed that not only can Blm dislodge human Rad51 (hRad51) from ssDNA, but that Blm can unwind the displaced strand of the D-loop (2007). This would allow for Blm to promote SDSA by catalyzing D-loop disassociation.

Drosophila melanogaster Blm, the main focus in our study, has been characterized as mutagen-sensitive, as it is sensitive to both nitrogen mustard and MMS as Blm also works in repair of interstrand crosslinks (Boyd et al. 1981). IR sensitivity experiments showed that DmBlm was also involved in the repair of DSBs (Kuo et al. 2014). In flies, Blm could be necessary in resolving converging replication forks during embryogensis, which is what might render the homozygous mutant flies infertile (McVey et al. 2007). DmBlm has also been shown to function in the repair of double strand breaks *in vivo*, through an assay looking specifically at SDSA (Adams et al. 2003). By examining Blm's function *in vivo* through this assay, the specifics of its mechanism during repair were determined. We predict, based on Blm's *in vivo* functions,

that Blm works to unwind the nascent strand of the D-loop to disassociate the intermediate DNA structure during SDSA, similarly to how it works in resolving dHJ.

Another helicase whose role in DSB repair is not well defined is HelQ, which is encoded by the gene *mus301* in *Drosophila*. This helicase has 3' to 5' polarity, similar to Blm. DmHelQ, also known as spn-C, has been characterized for its importance in oogenesis, rendering *mus301* mutants infertile (McCaffery et al. 2006). Spn-C mutants inhibit the formation of dorsal appendages on the eggshell by stopping the localization and translation of gurken, a protein necessary for this process (González-Reyes et al. 1997). *mus301* mutants have ventralized embryos, since persistent meiotic DSBs activate a checkpoint that prevents the correct localization of grk in the embryo. HelQ, like many other DNA repair proteins, is found mostly in the ovaries of the flies, working also in chromosomal segregation in female meiosis (Flybase).

Mice with mutations in HelQ show a higher frequency of tumor formation in ovaries and are rendered infertile (Adelman et al. 2013). In an analysis of a co-precipitation with a tagged HelQ in mice, HelQ was seen to interact with RPA70, components of the BCDX2 complex (required for homologous recombination), and the FancD2-FancI heterodimer (involved in ICL repair) (Adelman et al. 2013). Reciprocal immunoprecipitation with Rad51 showed interaction with HelQ (AdelmanF et al. 2013). In *C. elegans*, accumulation and persistence of Rad51 loci were seen in *helq-1 rfs-1* double mutants, demonstrating HelQ's involvement during HR (Ward et al. 2010). It has also been shown that Hel308, the human ortholog of HelQ, which has 52% similarity to DmHelQ, can interact with RPA and might be recruiting HelQ for the repair of stalled replication forks (McCaffrey et al. 2006; Woodman et al. 2011).

Due to *mus301* mutants' sensitivity to X-rays, interstrand crosslinking agents, and DNA alkylation, HelQ is also likely involved in repair outside of meiosis (McCaffrey et al. 2006).

mus301 mutants are also sensitive to topotecan, a topoisomeraseI inhibitor that creates one-ended DSBs in replicating cells, further supporting a role for HelQ in the repair of DSBs (Thomas and McVey, unpublished data).

To further determine how Blm and HelQ are involved in the repair of DSBs *in vivo,* a gap repair assay was used to test how HelQ may work during. This gap repair assay involved the excision of a *P*-element and the creation of a 14kb gap. The gap can be repaired either through SDSA or failed HR, resulting in endjoining. Repair events that occur in the pre-meiotic germline of males are recovered in the next generation of females and the frequency of the type of repair can be calculated.

In *blm* mutants, there was a significant decrease in the frequency of the SDSA occurring, while there was an increased frequency in endjoining occurring compared to wild type flies (Figure 4, Adams et al. 2003). There was even a decrease in SDSA that occurred when the helicase domain of Blm was present and only part of the N-terminus was missing. This suggests that not only does Blm plays an important role in unwinding the D-loop, but it is also important for the recruitment of other proteins, such as TopoIIIα, to remove torsional strain (McVey et al. 2007). Further molecular analysis of the failed HR events showed that, when endjoining occurred, flanking deletions were created during repair.

mus301 mutants were also used for this assay and, like *blm* mutants, they showed a significant decrease in SDSA and a significant increase in end-joining events (Figure 4, Thomas and McVey, unpublished data). However, the flanking deletions that were observed in the *blm* mutants were not seen in *mus301*mutants. *mus301 blm* double mutants were created for the gap repair assay as well. The frequency of SDSA dropped significantly, even when compared to each of the single mutants, showing an additive effect of the two mutations and suggesting that HelQ

and Blm have different roles during repair. Analysis was performed to examine the repair events that occurred in the double mutants. The flanking deletion phenotype seen in the *blm* mutants was suppressed, and instead the double mutants had a phenotype more like the *mus301* single mutants as they did not have flanking deletions surrounding the repair site (Thomas and McVey unpublished).

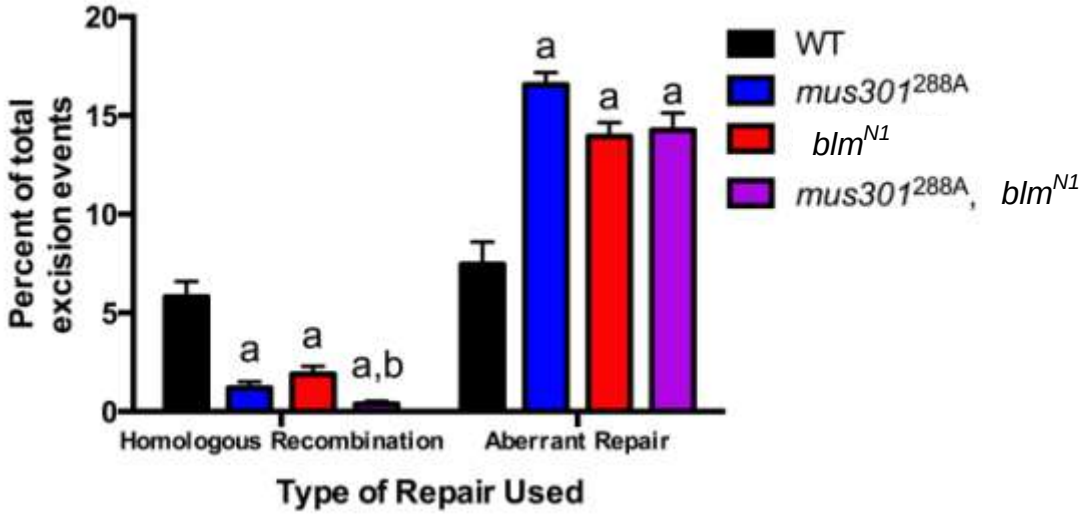

Figure 4. Frequency of repair events occurring in different mutant backgrounds. The homologous recombination shows the amount of SDSA occurring while the aberrant repair classifies the failed HR events that resulted in endjoining. The other percentage not shown is when the P-element did not excise from the genome.

Based on both genetic and biochemical studies, Blm likely acts to unwind the nascent strand on the D-loop during SDSA to produce non-crossover products. Because *mus301* mutants displayed a different phenotype in the gap repair assay, as well as an additive effect in the double mutant background, HelQ must be performing a different function during SDSA. We hypothesize that HelQ works to enlarge the D-loop, allowing for more synthesis. When Blm is not present, the large D-loops cannot be unwound, and nucleases may be working to resolve

these structures, which results in the flanking deletions created during the repair event in the gap repair assay. When HelQ is not present, however, these large D-loop intermediates are not formed and these smaller structures are then unwound by another helicase. Because of the suppression of this phenotype in the double mutant, we propose that another helicase, perhaps FancM, may be involved in unwinding the D-loop when Blm is not present.

Characterization of FancM

FANCM, another highly conserved 3' to 5' helicase, also causes a cancer-prone syndrome, named Fanconi anemia, when mutated (Xue et al. 2014). Fanconi anemia is a disease characterized by fatigue and lowered immune system due to a decrease in the body's ability to produce blood cells. It also results in congenital abnormalities and bone marrow failure (Deans and West 2009). FA patients have shown an increased sensitivity to DNA crosslinking agents such as mitomycin C and cisplatin, as well as an increase in chromosomal abnormalities (Whitby 2009).

FancM works in the Fanconi anemia complex to repair interstrand crosslinks, as it is one of eight Fanc proteins in this core complex. Of these proteins, FancM is the only one that has a DNA binding domain; this helicase also has a binding domain for FancF and Blm (Deans and West 2009). When DNA damage or stalled replication forks occur during S phase, FancM is phosphorylated and binds to the DNA to recruit other proteins. FancD2 and FancI are monoubiquitylated, which is most likely regulated by FancM, as FancD2 shows a decrease in ubiquitylation when FancM is not present. FancM can then independently recruit the Blm complex to the site of the damage (Deans and West 2009).

FancM has also been studied *in vitro* to characterize its helicase activity. Mph1, the *Saccharomyces cerevisiae* ortholog, was examined with different DNA intermediates. Mph1 was

shown to process Holliday Junctions through branch migration in an ATP-dependent manner (Zheng et al. 2011). Mph1 was also shown to preferentially bind and unwind D-loops compared to HJs regardless of the type of ssDNA overhang in vitro (Prakash et al. 2009). Mph1 was also shown to suppress crossover products, like Blm, and was even shown to promote this action independent of Sgs1, the yeast ortholog of Blm (Prakash et al. 2009). Fml1, the *Schizosaccharomyces pombe* ortholog of FancM, was also seen to limit crossover events in the repair of DSBs (Sun et al. 2008). FancM was even shown to disassemble D-loops bound by RPA (Gari et al. 2008).

To determine whether FancM was functioning outside of the Fanconi anemia complex *in vivo*, sensitivity assays were performed on *Drosophila melanogaster*. Mutants lacking FancM and FancL, two proteins working in the FA pathway, are both sensitive to the crosslinking agent nitrogen mustard mechlorethamine (HN2). However, only FancM was sensitive to methyl methanesulfonate (MMS), a damaging agent that generates mono-adducts, and ionizing radiation (IR), which create DSBs (Kuo 2014). This result suggests that FancM plays a role independent of the FA pathway, as it works to repair DSBs. When FancM is absent in *Drosophila*, there is an increase in crossover events, but this percentage is not as severe as the amount of crossovers occurring in a *blm* mutant background (Kuo et al. 2014).

In this study, we attempted to further characterize FancM's role in repair of DSBs in *Drosophila*, specifically in relation to the other helicases already seen to be involved in SDSA repair. *fancM* mutant flies were examined in the gap repair assay. From this experiment, our results showed that FancM does function in SDSA and may be functioning in a role more similar to *Blm*. By looking at *fancM mus301* double mutants, we were able to obtain a better picture of

how these two helicases may be working together. From these data, we were able to suggest a mechanism through which these three helicases interact to unwind D-loops during SDSA.

Methods and Materials

Creation of *Drosophila melanogaster* Stocks

Two mutations crucial for the assay were *fancM* and *mus301*^{288A}. The helicase dead *fancM*^{0693} stock (a gift from J. Sekelsky) was created from the mutagenic agent Ethyl methanesulfonate (EMS) mutagenesis. The *fancM DfED6058,* w^{1118}; Df(3R)ED6058, P{3'.RS5+3.3'}ED6058/TM6C, cu^{1} Sb^{1} allele also had a w^{+} marker. The *mus301*^{288A} mutant was created by imprecise excision of *P{SUPor-P}mus301*^{KG09098}. The excision results in a 2069 bp upstream deletion, removing the second, third and fourth exons entirely as the *P* element had been inserted in the fourth intron (Thomas unpublished). *fancM mus301* and *mus 301 blm* double mutant stocks were created via standard meiotic recombination.

The dominant marker *Drop* was also crossed onto the *fancM* chromosomes with the *Df(3R)ED6058* deficiency to allow us to phenotypically distinguish this chromosome in the gap repair assay (described below).

Verification of mutated genes *mus301*^{288A}, *fancM*^{0693}, *fancMDfED6058* from fly stocks

To confirm that the mutations were present, fly preps were made from male flies of each stock using standard protocol with fly squishing buffer and heated in the thermal cycler. This was prepared with single fly collected in an eppendorf tube and crushed in 50 µL of 10 mM Tris-Cl pH 8.2, 1mM EDTA, 25 mM NaCl, with 1 µL of 10 mg/mL proteinase K added. The sample was then incubated at 25 °C for 30 minutes and then 95 °C for 30 minutes to deactivate proteinase K. The genomic DNA from the flies was then used in PCR in line with standard procedure for Taq polymerase reactions. For 1µl of fly DNA, .5ul of each primer and .5ul of

dNTPs was added as well as 2ul of PCR buffer and .1ul of Taq Polymerase. Finally these PCR products were run on a 1% agarose gel with Ethidum bromide at a constant voltage of 110V for about 40minutes.

For the PCR reactions confirming the presence of *mus301*^{288A} mutation, the program normal PCR was used with an annealing temperature of 56^{o}C for 30seconds and 45 second extensions at 72^{o}C. The primers used in this reaction were P_{out} as the forward primer with the sequence 5'-CCGCGGCCGCGGACCACCTTATGTTGTTTC- 3' and the reverse primer was *mus301* R3586 whose sequence is 5'-TGTGCCGCATCGATCCATTT-3'. *FancM*^{0693} mutation was confirmed using the PCR program touchdown 30 with the primers whose sequence was 5'-CGCAATGAAGGTCTTTCCGT-3', as the forward primer, and 5'-TGTCACGATTTGT GTGATCG-3' as the reverse. To confirm the presence of *fancM DfED6058* the program touchdown 45 was used with the primers with the sequence 5'-TTATGGAGTTAATTCA AACCCCAC-3' and 5'-CACAGTCGCTTCTAAAATATATGGC-3'.

***P{w^{a}}* assay: a gap repair assay**

The *P{w^{a}}* construct is made up of *P* element containing *w^{a}* which is an allele of the *white* gene disrupted by the *copia* retrotransposon (McVey 2010). When female flies are homozygous or males are hemizygous with this construct, they have apricot color eyes. This construct was inserted into the intron of *scalloped* on the *X* chromosome. *Scalloped* is an essential gene and when mutated, the resulting phenotype is scalloped wings (Figure 5). When the construct for *P{w^{a}}* was excised by a transposase, (H{w^{+}, Δ2-3}Hop2,1), a 14kb gap was created with noncomplementary 3' overhangs. These single strand overhangs leave a 17 base pair sequences that are non-complementary. The cell can repair the gap in three ways; complete repair, homologous recombination, or aberrant repair which is failed HR with endjoining. The repair

events that occurs can be determined by the flies' eye color as homologous recombination repair with annealing at the long terminal repeats (LTRs) would produce progeny with red eyes while aberrant repair would result in yellow eyed flies from the single maternal copy. If the flies had apricot color eyes, the transposon either was not excised, the P-element moved to a different part of the genome, or the construct was repaired in full. If the flies had scalloped wings, there was a nonlethal deletion into scalloped. The male flies created for the assay had one *P{w^a}* construct on the X chromosome, a transposase on the second chromosome marked with curly wings, and the desired compound heterozygote *fancM* mutation. These flies that were crossed with females homozygous for the *P{w^a}* construct and female progeny were scored for the repair events. In order to accurately score the flies according to eye color, the female flies with the deficiency mutation were not included in the data and could easily be identified by the *Drop* mutation, which *affects eye shape.* The relative frequency of eye color in a mutant background was compared to wild type flies. Statistical analysis of these data was done using ANOVA and Tukey's multiple comparison posthoc test.

Aberrant repair events were analyzed by PCR and DNA sequencing to determine when HR failed during each repair event. One fly with yellow eyes was taken from each vial and crossed to white eye males. The male progeny that had inherited the failed HR *events* were collected for PCR analysis. Six sets of primers were used in order to determine the proficiency of synthesis that occurred during repair. If the DNA extracted from the flies did not produce any product from the six sets of primers, then the DNA of the altered *scalloped* intron region was sequenced for further analysis.

DNA Sequencing

Primers that annealed to regions in *scalloped* were used to amplify the sequences of the repair events in which HR had failed in the *P{w^a}* assay. The products that formed from using primers Sd5320 forward and Sd5941a reverse were cut out of agarose gels and were purified using a standard PCR purification kit to extract the DNA. These samples of DNA were sent off to Eton Bioscience Inc. for Sanger sequencing. These results were analyzed using the program CLC Main Workbench 7.5.1 to align the sequences.

Cloning FLAG-tagged *mus301*into the pUAST attB vector

Colonies containing the vector pRSET containing *mus301* tagged with a FLAG- and 6HIS-tag was grown on Amp plates. Colonies were selected and grown up in 2ml of LB+Amp overnight. The plasmids were isolated using standard miniprep procedure. PCR was used to obtain *mus301* from the pRSET plasmid using primers with Acc651 and BglII restriction site overhangs. The 15µl of the DNA insert was digested with 1µl of restriction enyzmes BglII and Acc651as well as 2µl of buffer 3.1.

Similarly, minipreps of pUAST attB were created and 10µl of the plasmid was then digested with 0.5µl of each restriction enzyme, BglII and Acc651, and 2µl of buffer 3.1. The vectors ran on an agarose gel and were extracted using a PCR purification kit. A ligation reaction of a 1:5 vector to insert ratio occurred in a 10µl reaction. *mus301* with the restriction sites cut was then inserted into pUAST attB through a ligation reaction. 2.5µl of the ligation reaction was added to 50µl of Mix and Go cells that had ampicillin resistance. This transformation was plated on Amp plates. Colonies grew overnight.

The presence of the insert, tagged *mus301*, was then determined through different digests as well as PCR of the *mus301* gene using the primers mus301 113F and the reverse primer mus301 727R. Of the nine colonies digested to see if the insert was present, only one contained

the insert. The insert which was 3kb and the plasmid without the insert was 8kb which is where the bands appeared on the gel. This plasmid was then sequenced for accuracy, and found that it contained zero mutations.

Injection of transgenic *mus301* into *Drosophila* and overexpression with Gal4 drivers

By using standard midiprep procedure, a large amount of the pUAST attB plasmid containing tagged *mus301*was isolated and sent to BestGene. They injected the plasmid into the embryos for the sequence to be incorporated onto the third chromosome of the fly at the cytological site 86F6. The flies used were FlyC31 strains with the genotype M{3xP3-RFP.attP}ZH-86Fb (with M{vas-int.Dm}ZH-2A).These flies were then crossed into a *mus30* ^{288A} mutant background using standard meiotic crossing on. This stock with the *mus301* transgene was also mated to stocks with GAL4 drivers. Three new stocks were created with the transgene and the GAL4 driver of actin, nanos, or matalpha.

Hatch Rate Assay

To determine if the *mus301* transgene affected the hatching frequencies in the stocks, 20 to 25 fertile females were placed in a cage and allowed to lay on grape agar plates with yeast pellets for 24hours at 25℃. After the eggs had been laid, plates were scored for hatching frequency by counting the number of hatched and under a microscope 3 days after removal from the parental generation. This was repeated for accuracy.

Ovary Dissections

Flies were dissected in 0.7% saline solution. 10 ovaries were collected and put into an Eppendorf tube with 100ul of 0.7% saline solution. These ovaries were frozen at -80C for storage.

Western Blotting

10 flies of 10 ovaries were collected for each sample. 100ul of saline was added to samples with 10 frozen flies. 33ul of 4x Sample buffer was added to each sample. Sample buffer was 10% glycerol, 2% SDS, 0.3ml of 1M Tris-HCl at a pH of 6.8, and 0.1%BPB. Before using 480ul aliquots of sample buffer, 20ul of B-mercaptoethanol was added. After adding the sample buffer, the samples were sonicated for 5 cycles with 30 seconds of sonication and 30 seconds off. Samples were spun down for 10 seconds in a centrifuge and 30ul of the supernatant was aliquoted into several eppendorf tubes and frozen for later use.

These desired samples were boiled in a hot water bath at 95C for 5 minutes. 20ul of each sample was loaded onto a precast 20% acrylamide gel to run for 25 minutes at 240V in 1x Laemmli buffer. 10x Laemmli buffer was made with 30.24g Tris base, 144.2g glycine, 10.0g SDS, and the H2O was added to a liter. The gel was then transferred to a membrane using 1x transfer buffer for 1 hour at 0.4A.

To stain the gel, 4 ml of developer buffer A was put with 36 ml of H2O and poured onto the membrane. This shook at room temperature for 2 minutes. Then the liquid was poured off for 30ml of H2O and 10ml of developer buffer B to be added to the membrane. This was put in the fridge for 10 minutes to develop.

The membrane blocked in 10ml of 5% milk in TBST for an hour while shaking. The membrane was then incubated overnight in primary antibody, anti-FLAG, at 4C. The antibody is diluted 1000x in BSA. The next morning the membrane was rinsed 3 times with TBST for 5 minutes each. Then 1ul of the secondary antibody was added to 10ml of 5% milk in TBST and incubated for an hour. The membrane was then washed again in TBST 3 times for 5 minutes

during each wash. One milliliter of each of the ECL reagents was poured onto the membrane and incubated for one minute. This was then poured off and the membrane was exposed to film for 2 minutes. Once exposed to the membrane, the film was developed.

Results

fancM mutants are defective in double-strand gap repair

A previous member of the McVey lab, Adam Thomas, used a gap repair assay to determine the role of Blm and HelQ helicases in repair of DSBs. By examining flies mutant in this assay for *blm* and *mus301* as well as *blm mus301* double mutants, he determined that there was a significant decrease in the amount of HR occurring in both single mutant backgrounds compared to wild type flies, suggesting that these two helicases play an important role in gap repair. A significant decrease in HR was seen in comparison of the *blm mus301* double mutants to both of the single mutants. This showed that due to this additive phenotype, these helicases serve different functions during the repair even though these proteins are both 3' to 5' helicases (Thomas and McVey unpublished).

Another interesting phenotype seen was flanking deletions surrounding the repair events in the genome of the flies with the *blm* mutant background. When HR failed, nucleases resected back on these gaps to produce a mutated repair product. This flanking deletion phenotype was not seen in the *mus301* mutants, and surprisingly, it was not detected in the *blm mus301* double mutants. This suppressed flanking deletion phenotype in the double mutant phenotype not only further suggests that these two helicases are necessary for different functions, it also suggests that another helicase could be involved in this repair process (Thomas and McVey unpublished).

We hypothesized that the FancM helicase might be this helicase. To test this, we used a gap repair assay, called the *P{w^a}* assay to determine the type of repair that occurred in *fancM*

and *fancM mus301*^{288A} mutant flies. The *P{w*^{a}*}* construct, a *P*-element, was inserted into the intron of scalloped on the *X* chromosome (Figure 5). This construct contains a functional *white* gene that is interrupted by *copia,* a retrotransposon. *Copia* decreases the amount of *white* expression and results in the flies having apricot eyes. In the presence of transpoase, this *P*-element is excised about 20% of the time to produce a 14kb double strand gap in the genome of the flies. Since the efficiency of the excision is low, there is a high chance that repair can take place via HR off of the sister chromatid sequence. The gaps that are created leave 3' single stand DNA overhangs, which lend themselves to repair through homologous recombination via SDSA. Repair events of the gap that occur in the pre-meiotic germline cells of males are recovered in their female progeny.

SDSA that involves synthesis of the *white* gene and annealing of the *copia* LTRs results in progeny with red eyes. If the cell is unable to initiate synthesis or HR fails, aberrant repair events of the *white* gene will occur, and the progeny flies will have yellow eyes. If the *P*-element does not excise from the genome, or if the entire construct is resynthesized during HR repair, then the eye color will be apricot (Figure 6).

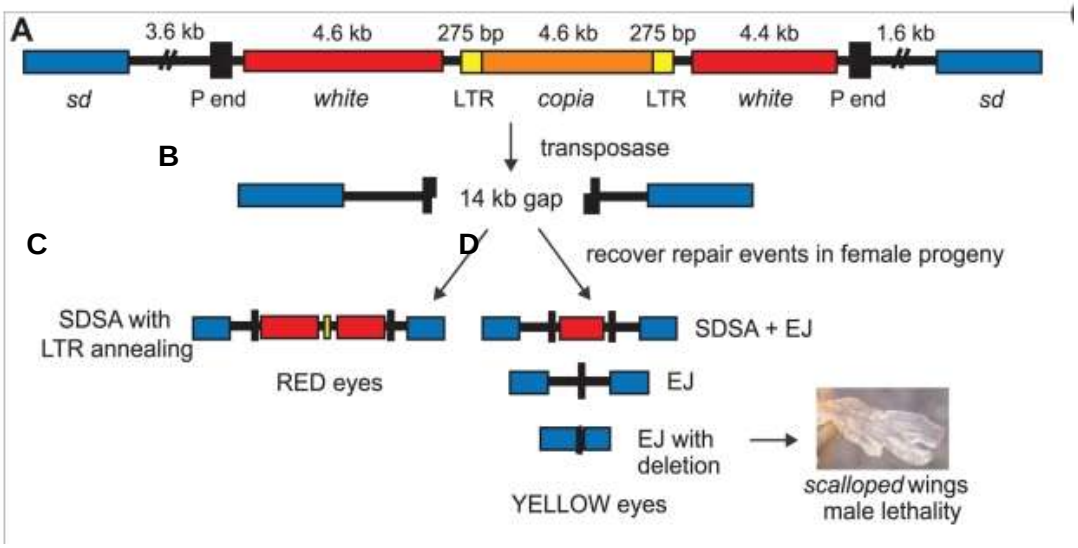

Figure 5. *P{w^a}* assay. (A) Diagram of *P{w^a}* construct inserted into the intron of *scalloped* (*sd*) on the *X* chromosome. (B) The amount of HR occurring in the mutants can be determined by different eye colors in female progeny that inherited the repair event from their fathers and an intact copy of *P{w^a}* from their mothers. A failure to excise the construct results in apricot eyes or complete repair by homologous recombination. (C) HR repair results in red eyes as the white gene is no longer interrupted by *copia*. (D) Aberrant repair in which HR fails to repair the gap results in yellow eyes. (Adapted from Chan, 2010.)

Figure 6. Resulting eye colors of *P{w^a}* assay. Mean percentage of eye color exhibited in the female offspring of males in a mutant background showed type of repair from the excised reporter construct that created double strand breaks. Individual males with *P{w^{a}}* and transposase of the indicated genotypes were crossed with homozygous *P{w^a}* females. The female progeny that had only the *fancM^{0693}* mutation and did not have *drop (Dr)* were scored by their eye color (*fancM* n=91; *fancM mus301* n=103) (Adapted from Adams et al. 2003).

In the *fancM* mutant males, the incidence of red eyes in the female progeny showed a greater than 50% decrease compared to the wild type, while the amount of yellow eyes scored did not increase significantly compared to the wild type progeny (Figure 7). Therefore, it can be

concluded that the repair of SDSA significantly decreased, while the amount of end-joining repair did not significantly increase when compared to wild-type flies. There was a slight increase in the occurrence of endjoining, but this was not statistically significant.

fancM mus301 mutants are defective in double-strand gap repair

mus301 mutants had previously been used in the *P{w^a}* assay and the results showed a significant decrease in the amount of red-eyed flies compared to wild type flies, as well as a significant increase in the amount of yellow-eyed flies (Thomas and McVey unpublished). Since this phenotype was similar to the *fancM* mutant phenotype, we wished to determine if FancM and HelQ might be performing similar or different functions in SDSA. To further characterize FancM's role in SDSA,we created *fancM mus301*^{288A} double mutants were used in the *P{w^a}* assay.

The *fancM mus301*^{288A} flies showed a significant decrease in the red eye class and a significant increase in the yellow eye class in comparison to wild type (Figure 7). This translates to a decrease in the amount of HR occurring and an increase in the amount of aberrant repair. This double mutant showed a more severe phenotype only in the yellow eye class in comparison to the *fancM* single mutants. There was no significant decrease in the amount of HR occurring in the double mutants compared to the single mutants.

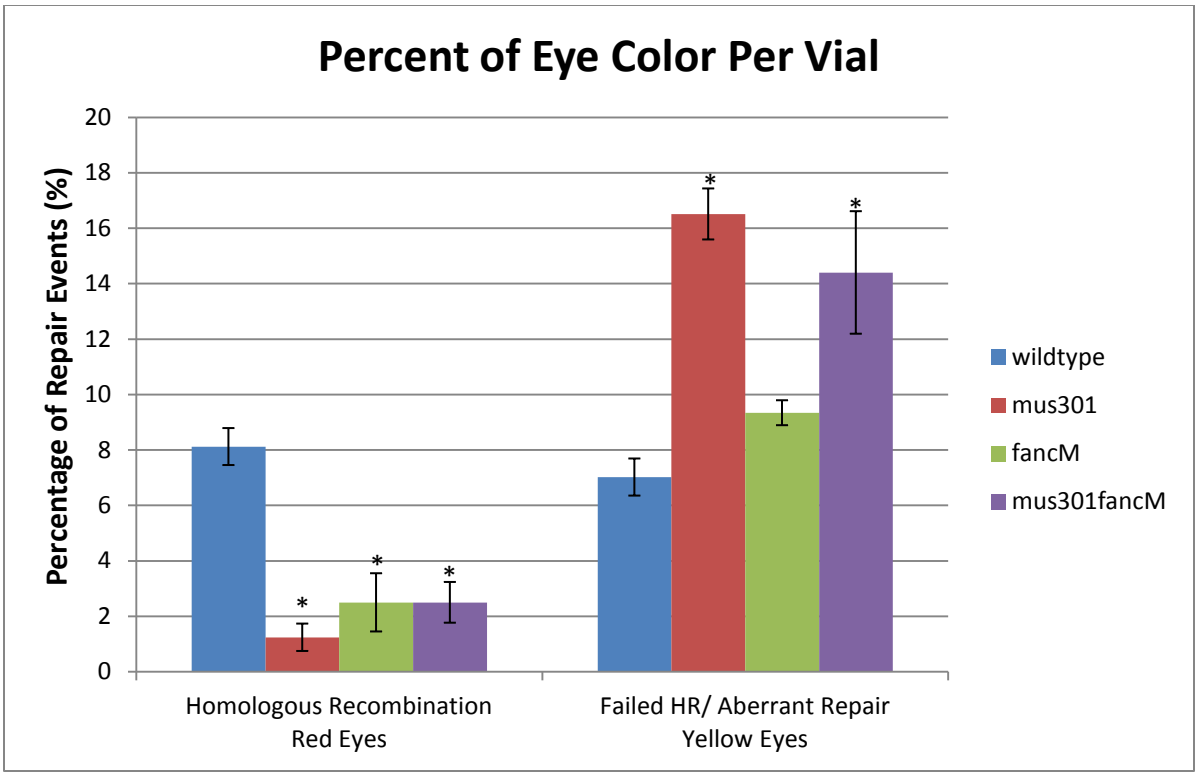

Figure 7. SDSA defects in fancM, mus301, and fancM mus301 mutants. *P{w^a}* assay outcomes in different mutant backgrounds. Percent of eye color of female flies scored in each vial where n = 70, 91, and 71 vials for wild type, *fancM*, and *fancM mus301* mutant flies, respectively. This data was compared to previously acquired results from *mus301* mutant flies. Both the *fancM* and *fancM mus301* mutants have a significant decrease in the number of red-eyed flies seen ($p < 0.05$). Only the *fancM mus301* mutants have a statistically significant increase in yellow-eyed flies in comparison to the wild type control ($p < 0.05$). Error bars are depicted as standard error of the means. (Anova, Tukey's post-hoc test)

fancM and *fancm mus301* mutant flies show a decrease in DNA synthesis during double strand gap repair

To further characterize the role of these helicases during gap repair, molecular analysis of the aberrant repair products was performed. White-eyed males inherited the failed HR event that occurred during the repair of the *X*-chromosome, which was detected in the females with yellow eyes. By using specific primers (Supplementary Data) complementary to sequences at several places along the *P{w^a}* transposon, the extent of repair synthesis could be determined in a PCR

reaction. *mus301*-deficient flies were previously seen to be only slightly defective at synthesis of 250bp or longer compared to wild type flies (Figure 8, Thomas and McVey unpublished). *fancM* mutants did not show a significant decrease in the amount of synthesis when compared to wild type flies until 4.6kbp (Figure 8). HR failed significantly more frequently in the *fancM* mutants when annealing should have been occurring at the LTRs. While the single mutants showed a small amount of decrease in synthesis during failed HR events, there is a statistically significant decrease in synthesis track lengths of the double mutant flies. This significant drop in the percentage of failed HR events observed in the double mutants seems to be additive (Figure 8). In the *fancM mus301* flies, a more severe phenotype is detected as the amount of synthesis track lengths has significantly decreased, especially as sections farther along the construct were analyzed.

About 20% of *fancM* and *fancM mus301* mutant flies failed to initiate any synthesis on either side of the construct. Primers were used to amplify the sequence of the construct for all of these flies. Each PCR product was sequenced and it was determined that end-joining events had occurred with small microhomologies (Table 1). These end-joining sequences appeared to be similar to the end-joining events that occur in Rad51 mutants used the *P{w^a}* gap repair assay (McVey et al., 2004). While endjoining did occur, no flanking deletions were observed in the sequenced repair events, unlike those in a Blm mutant background (Thomas and McVey unpublished).

Table 1 – *P{w[a]}* repair junctions recovered from *fancM* mutants

Type of repair event	Sequence 5' of break [a]	Microhomology / inserted sequence	Sequence 3' of break [a]	Number of isolates
Original sequence	acccagacCATGATGA AATAACATA	-	TATGTTATTTCATC ATGacccagac	-
Long microhomology [b]				
	Acccagac	CATGA	cccagac	3
		ACCCAGAC		2
Short microhomology				
	ACCCAGACCATGA TGAAAT	(AT)	GACCCAGAC	1
	ACCCAGACCATGA	(TGA)	cccagac	1

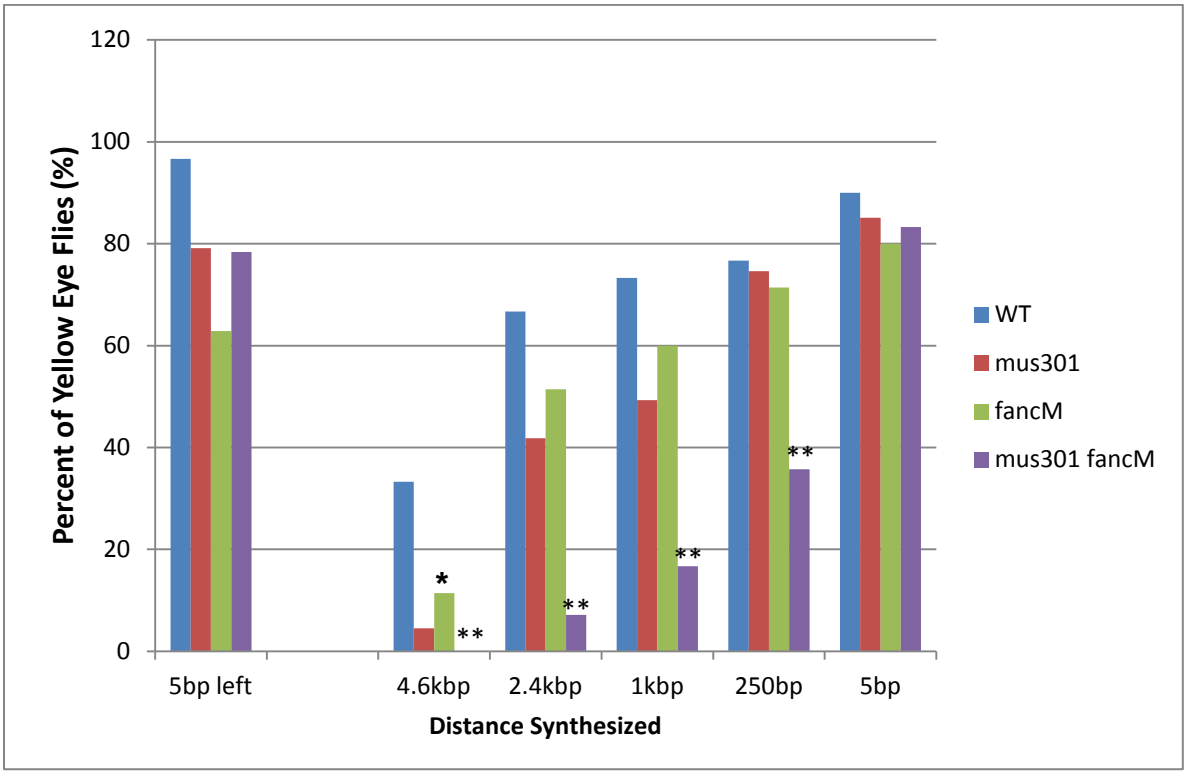

Figure 8: Synthesis tract length analysis of both single mutants and double mutants as compared to WT. By using PCR at specified distances, the percentages out of 44 (WT), 35 (*fancM*), and 42 (*fancM mus301*) of individual males whose DNA produced the correct product along the construct are shown. (*mus301* mutant data from Thomas and McVey unpublished).

***mus301-TG* was inserted in *Drosophila* through Gal4/UAS system**

By creating knockout *mus301* flies, we were able to see a decrease in SDSA occurring during its absence. Another genetic technique we used to determine HelQ's role during repair was to overexpress the protein. By looking at this overexpression in the flies, we could regulate the expression of HelQ in specific tissues with the UAS/Gal4 system. As we have a tagged version of HelQ, we could purify the protein and discover its endogenous interactions in specific tissues.

The reasons for using this UAS/Gal4 system in fruit flies are two-fold. One was because this would allow us to have a tagged version of the protein expressed in flies, which would help to determine with what other proteins it is interacting *in vivo*. By peforming a pull down of the tagged protein in the Western, we could possibly detect other proteins that HelQ binds during HR by using mass spectrometry. Another aspect we investigated was looking at HelQ overexpression in tissues causes any defective phenotypes. Like many repair proteins, HelQ is most highly expressed in the ovaries of the fly so there is a chance that when HelQ is overexpressed in other tissues, it would interfere with other potential repair mechanisms.

The *mus301* gene had been inserted into the pRSET vector with both a 6HIS-tag as well as a FLAG-tag. In order to have *mus301* expressed in flies, the gene was taken out of this vector and put into a pUAST attB plasmid in the multiple cloning site (Figure 9). The pUAST attB vector allowed for control of the expression of the transgene HelQ in tissue specific locations with the upstream activating sequence as well as the hsp70 promoter. By crossing the construct with a Gal4 driver in flies, the transgene could be expressed in all tissues with an Actin driver as well as through the ovaries with a Nanos driver. Gal4 binding to the upstream activating

sequence drove a higher expression of HelQ. This tissue specific expression system worked well to see if the transgene could rescue in a mutant *mus301* background.

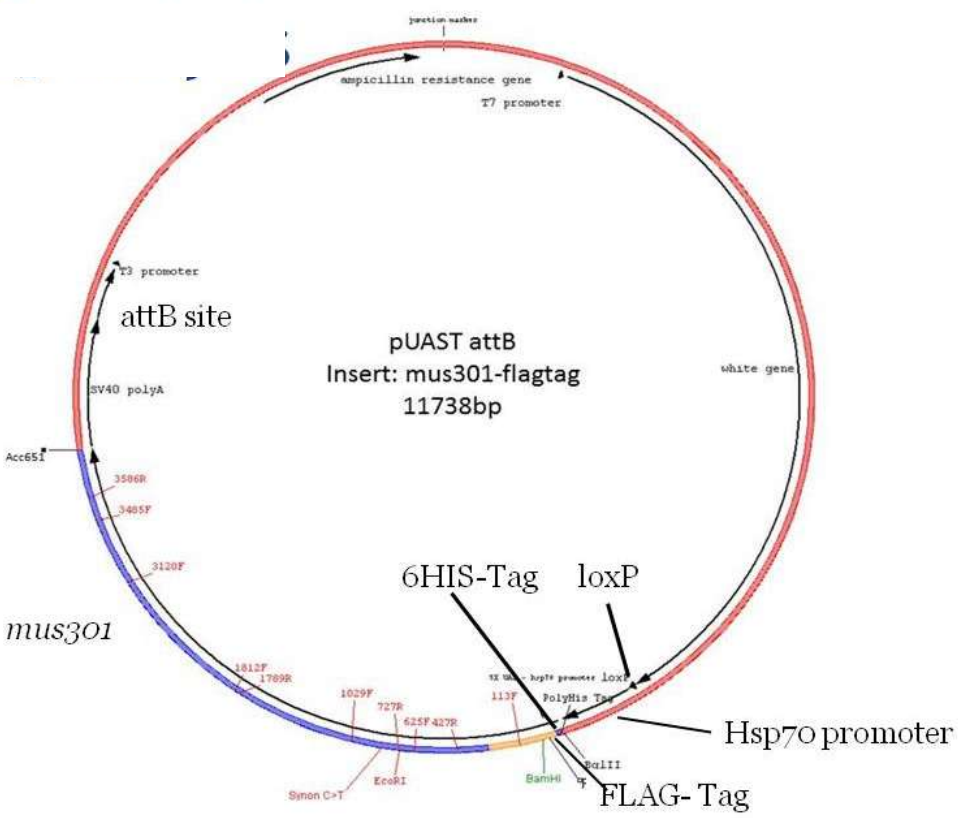

Figure 9. Image of the pUAST attB construct with the mus301 FLAG-tag inserted into the multiple cloning site. The red markers indicate the names of primers used to verify the correct sequence of *mus301* in the vector. The *mus301* gene is in blue.

The plasmid was injected into flies expressing the phiC-31 recombinase. Site-specific recombination between the attB site in the plasmid and an attP site located at 86F8 resulted in the creation of stable transgenic flies with one copy of the tagged *mus301* gene. Under the control of the UAS, the upstream activating sequence, the HelQ-transgene would only be expressed when with a Gal4 driver. We predicted that the control of the UAS would be "leaky" because the UAST vector is under the control of the hsp70 promoter which allows for a low basal level

expression of the mus301 gene during development (Flybase). To test if the transgene would rescue *mus301* female sterility, *mus301*^{288A}*, mus301-TG* homozygotes were created.

mus301-TG did not rescues mutant _mus301_^{288A} infertility and egg shell phenotype

The transgene does not rescue the mutant phenotype as the females with the genotype, $\frac{w}{+};\frac{+}{+};\frac{mus301-TG,mus301^{288A}}{mus301-TG,mus301^{288A}}$ were infertile. The phenotype of fused dorsal appendages and fully ventralized eggshells seen with *mus301*^{288A} homozygous mutants was also detected when the *mus301-TG* had been crossed onto this mutant background (Figure 10). The cell's inability to repair DSBs efficiently in the absence of HelQ activates a meitoic checkpoint. Due to the consequences of misregulation of the cell cycle, there is a lack of the translation of Grk. Grk is needed to establish axial polarity in oogenesis. When Grk cannot properly localize, this causes ventralized eggshell. When *mus301* is not functional in flies, *grk* is not translated and the eggs have difficulty forming dorsal appendages. *mus301* mutant flies have this eggshell due to a defect in meiosis (McCaffery et al. 2006). This phenotype was then seen in the *mus301-TG, mus301*^{288A} homozygotes because there was not an appropriate level for meiotic repair of DSBs being expressed by the transgene in the mutant background.

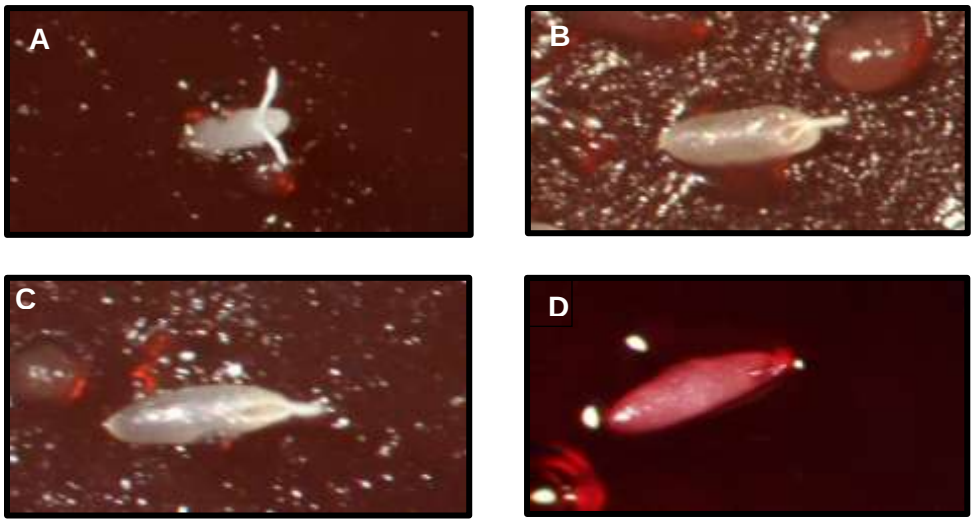

Figure 10. Images of eggshells. A. Wildtype eggshells of w^{118} flies laid eggs with normal dorsal appendages. B. *mus301*^{288A} mutant eggshells have fused dorsal appendages. C. m*us301*^{288A},*mus301-TG* eggshells have fused dorsal appendages. D. Fully ventralized eggshell in *mus301*^{288A}, *mus301-TG* background.

The frequency of wild type eggshells, fused dorsal appendages, and fully ventralized eggshells was determined for *mus301*^{288A} homozygotes and *mus301-TG, mus301*^{288A} homozygotes. By looking at the eggshells, ranging from normal to fully ventralized shells, the severity of the mutant eggshell phenotype could be further characterized. Wild type flies lay eggs with normal dorsal appendages virtually 100% of the time so only the mutant backgrounds were examined. There was a significant decrease in the amount of normal looking eggs for the two genotypes of mutant flies examined also seen by McCaffery *et al.* in 2006 (Figure 11). The McCaffery paper showed ventralized eggshell percentage varied greatly depending on the *mus301* mutation, and did not differentiate when the fully fused eggshell versus fully ventralized eggshell phenotype in their results. We saw there was a slight decrease in the amount of normal eggshells seen in the *mus301-TG,mus301*^{288A} compared to *mus301*^{288A} eggshells, but due to the large standard deviation calculated for the *TG,mus301*^{288A} mutants, this result did not seem

significant (Figure 11). Even though both genotypes occasionally had normal eggshells, they still had a significant decrease in fertility, regardless if the eggs had normal dorsal appendages. Therefore, the HelQ-TG was not being expressed at high enough levels without the promotion of a Gal4 activating sequence.

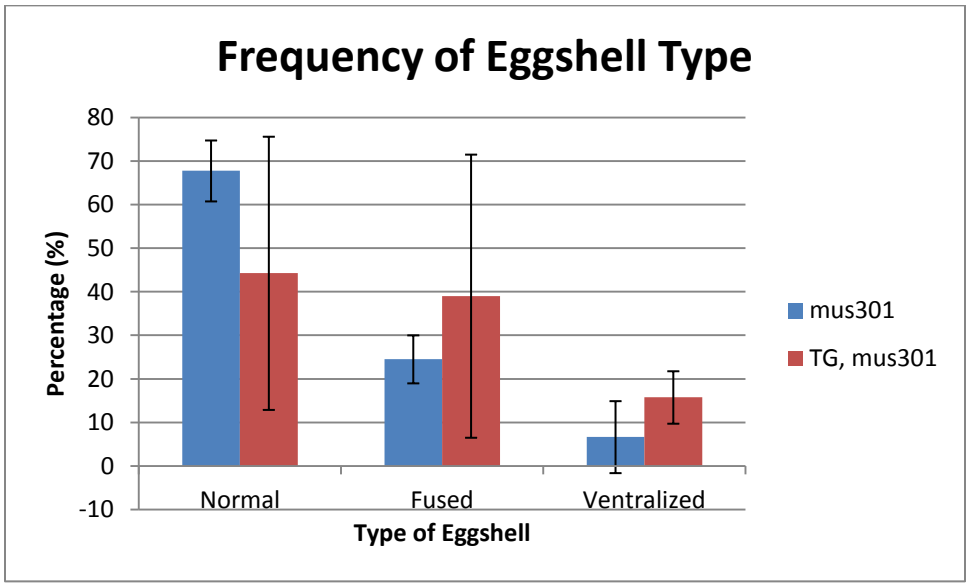

Figure 11. Frequency of eggshell type in *mus301*^{288A} mutant background and *mus301-TG*, *mus301*^{288A} homozygotes. All eggs that had lay after one day were counted and put into one of three categories; normal, fused, or fully ventralized. These phenotypes (shown in TABLE #) were detected and the percentages were calculated. Error bars show the standard deviation of four trials done for each phenotype.

After seeing the ventralized phenotype in the transgene background, the hatching frequencies were examined to confirm that the transgene does not rescue the mutant phenotype due to the very low levels of expression of the hsp70 promoter. The hatching frequency of the *mus301*^{288A} homozygote flies was significantly less than the hatching frequency of wild type flies. While wild type flies have a hatching frequency of about 90%, the mutant *mus301* flies were at 12% (Figure 12). The *mus301*^{288A}, *mus301-TG* homozygotes showed virtually the same frequency as the *mus301*^{288A} mutants as 11% of the eggs hatched (Figure 12). Because these values are so similar and show the same hatching rate phenotype, the *mus301 transgene* cannot

rescue the infertility seen in the mutants because there is not an adequate level of HelQ in the ovaries.

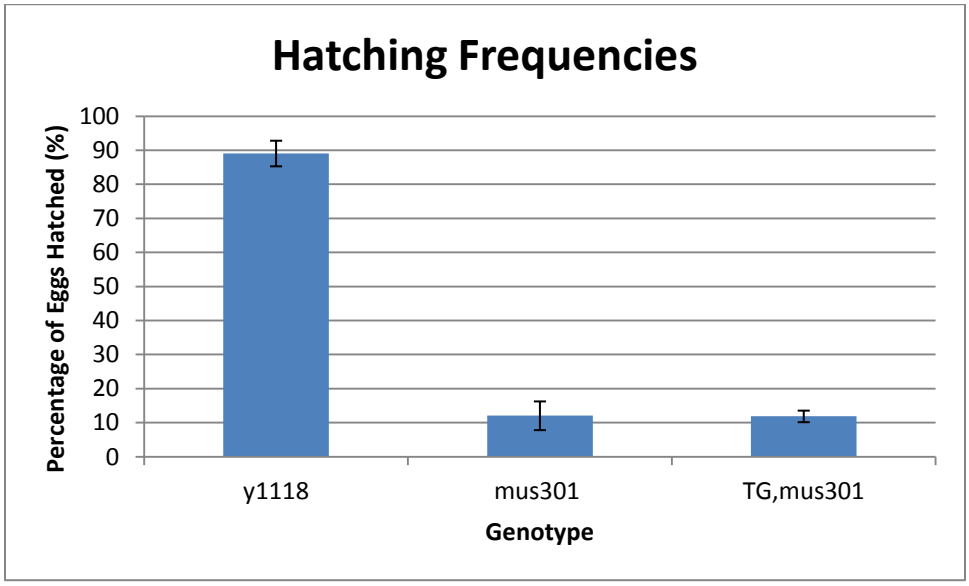

Figure 12. Hatching frequencies of *wild type*, *mus301*^{288A}, and *mus301*^{288A}, *mus301-TG* flies. After looking at the eggshells three days after the flies had lay on grape plates, the percentage of hatched eggs were counted. Three trials were performed for each phenotype. The error bars signify the standard deviation over the three trials.

To determine if overexpression of the *mus301-TG* had a negative effect on the flies, hatching rate assays were performed in a wild type background. While HelQ was being expressed normally in the flies, the Actin5C UAS upregulated the expression of the transgene ubiquitously throughout all tissues in the flies. Another UAS, Nanos, upregulated expression in the ovaries of the fly, the tissue in which *mus301* is most highly expressed (Supplementary Table 3). Normal hatching rates were seen in the flies with the transgene overexpressed with both Actin5C and Nanos Gal4 UAS and neither had rates significantly different from the wild type frequency (Figure 13). It was also detected that the egg shells were not ventralized and had normal looking dorsal appendages when the transgene was overexpressed in wild type flies which shows that there is a proper level of expression of HelQ in the ovaries of the fly.

Overexpression of the transgene did not seem to affect the flies negatively when *mus301* had not been mutated in the flies.

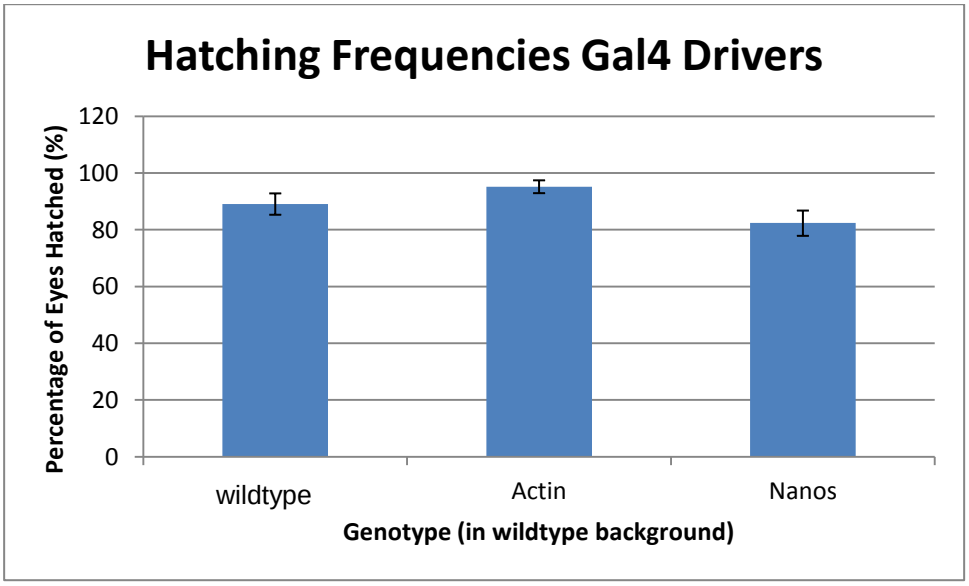

Figure 13. Hatching frequencies of two different Gal4 drivers of the *mus301-transgene* in Actin and in Nanos tissues compared to wild type rates. All flies had a functional HelQ being expressed as well. Two trials of each type of fly were performed three days after the eggs were initially laid. Error bars show the standard deviation.

Tagged version of HelQ was detected through Western blotting in flies overexpressing in specific tissues

HelQ with a FLAG- and 6HIS-tag was overexpressed in the flies with three different tissue specific Gal4 promoters, actin, nanos, and matalpha. While HelQ is only highly expressed in the ovaries of the flies and at lower levels in all other tissues of the fly, the Actin5C Gal4 UAS allowed for HelQ to be expressed ubiquitously throughout. The nanos UAS only drove the expression in the ovaries of the fly and the matalpha UAS allowed for expression in the oocyte (Flybase).

HelQ was detected in both the male and female flies that had Actin5C overexpressing the protein at about 115kD (Figure 14). *mus301* is predicted to encode a 1051-amino-acid (117-kDa)

protein (McCaffrey 2006). HelQ could not be seen in the western with the either the Nanos of Matalpha Gal4 drivers. Now that we know that HelQ is being expressed under the promotion of the Actin Gal4/UAS expression system, further experiments can be performed to determine the other proteins with which HelQ interacts during DNA repair.

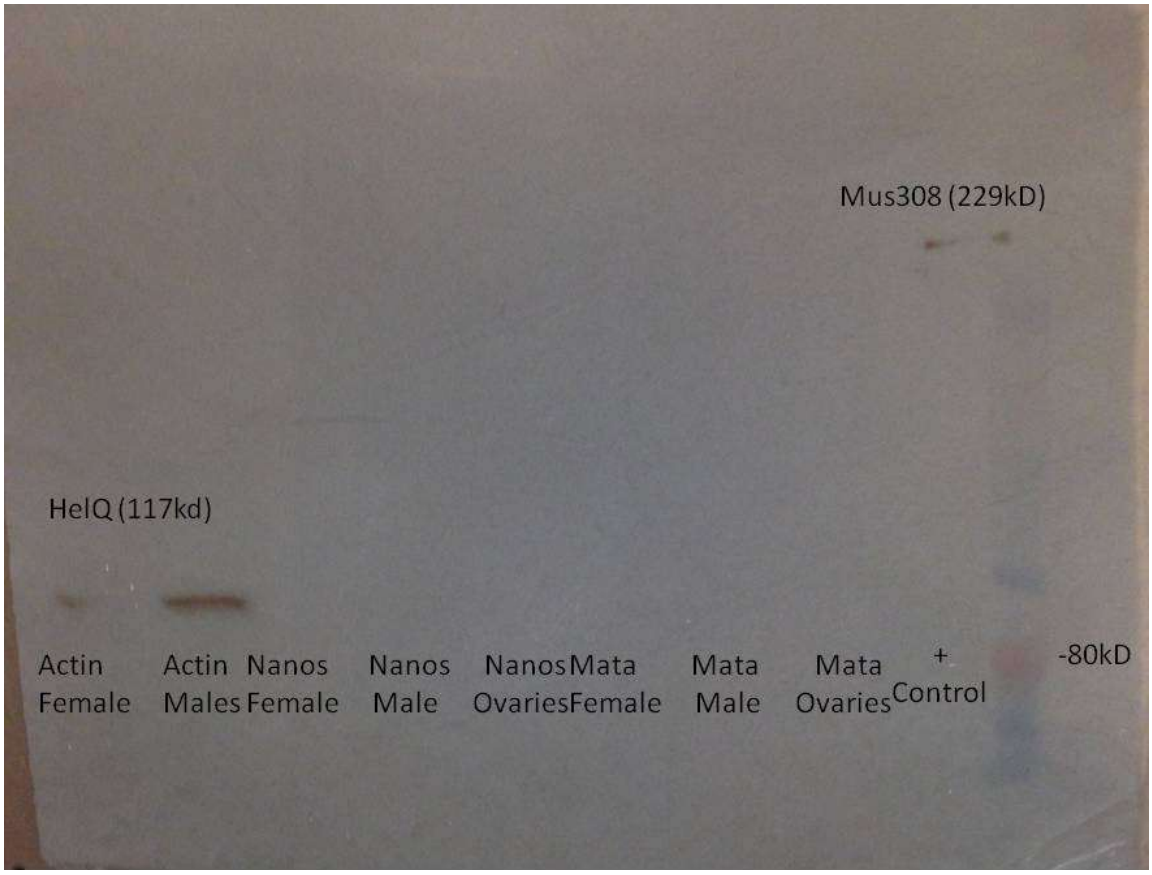

Figure 14. Western blot of HelQ with FLAG- tag expressed in different tissues. HelQ with a FLAG- tag was detected at the expected size on the gel (117kD). In the final lane next to the ladder, the positive control mus308 appeared. No protein was seen in the lanes that had flies with the nanos or matalpha Gal4 drivers.

Discussion

FancM is necessary for Gap Repair *in vivo*

In McVey *et al.* the *P{w^a}* assay results showed that DmBlm is necessary for SDSA during the repair of DSBs (2007). An interesting phenotype seen from *blm* mutants was that when the SDSA pathway failed to occur, there was either a significant decrease in synthesis track lengths or large flanking deletions surrounding the repair event (Thomas and McVey unpublished). In the absence of this helicase, deletions were observed because Blm is thought to be necessary for unwinding D-loops as also suggested by its *in vitro* behavior (Bugreev et al. 2007). When Blm was not present to unwind these D-loop structures, nucleases were thought to be responsible for dissolving this DNA intermediate which resulted in endjoining, thus causing these deletions in the genome. *Drosophila* lacking Blm and Gen are dead at the first instar larvae and those without Slx1 or mus312 are pupae lethal because either Blm or nucleases are used to resolve repair of DSBs (Kuo et al. 2014).

Thomas also showed HelQ's importance in this mechanism as there was a significant decrease in the amount of SDSA occurring and a significant increase in failed HR with *mus301^{288A} Drosophila* (unpublished data). In contrast to Blm mutants, the loss of HelQ did not result in shorter synthesis track lengths nor did it result in large flanking deletions (Thomas and McVey unpublished). Due to the different phenotypes seen from the genetic data, we predict that Blm and HelQ hold very different roles during SDSA.

To further characterize how these two helicases are working during SDSA, double mutants were created. When *blm^{N1}mus301^{288A}* flies were used in the *P{w^a} assay,* an additive effect was seen, as there was a significant decrease in the frequency of the red eye class compared to either of the single mutants. This more severe phenotype detected in the double

mutants verified how these helicases are acting differently during SDSA. Again the additive function of these two helicases was seen when observing the synthesis track lengths because again they were significantly shorter than wild type flies (Thomas and McVey unpublished). An interesting result the double mutants provided was that while there was a decrease in synthesis track lengths, the flanking deletion phenotype, detected in the *blm* mutants, was suppressed.

From this result that found a lack of deletions from the double mutants, we hypothesized that while Blm worked to disassociate the nascent strand of the D-loop, HelQ must be functioning differently and is therefore, working at the front of the D-loop to open it up for more processive synthesis. This prediction is also supported by biochemical evidence that shows HelQ binds with Rad51 in mice and is therefore interacting with the Rad51 filament at the front of the D-loop (Alderson et al. 2013). When HelQ does work to unwind the front of the D-loop, more torsional strain is put on this DNA intermediate and Blm is therefore recruited to unwind these larger structures. It then makes sense that when both HelQ and Blm are not present, the flanking deletion phenotype is not present because Blm is not needed to unwind the nascent strand of the larger D-loops to resolve the larger structures created by HelQ opening up the D-loop. Instead another helicase is working to unwind the nascent strand of smaller D-loops, FancM.

In this work, we showed that *in vivo* FancM plays a role in gap repair through SDSA; when it is not present in the flies, there is a significant decrease in the amount of red eyes scored in the $P\{w^a\}$ assay. This assay allowed us to classify where exactly FancM is working in relation to the other two helicases as a large gap was created in the genome to specifically determine where HR failed. In FancM mutants, HR was seen to fail during initiation. We saw that in the *fancM* mutant background at least 5bp could not be synthesized on either side of the construct, which happened about 20% of the time. These repair events were sequenced, showing that

endjoining was occurring at small microhomologies, as would in a Rad51 background for SDSA repair in this assay (McVey et al. 2004). Because these end-joining events are observed even before the invasion of a homologous template in the *fancM* mutants, it seems that FancM might be needed for an initiation step of SDSA.

If HR could initiate, there was not a significant decrease in the synthesis track lengths compared to wild type flies. The pathway for SDSA is activated and Rad51 allows ssDNA to invade the homologous template on the sister chromatid. When this does occur, there are still long synthesis track lengths in the *fancM* mutants during the failed HR events that, statistically, look like what occurs in wild type flies when examining the yellow eye class. Because there is no significant decrease in the STL beyond the scope of the first initiation step, this evidence further suggests that FancM is acting upstream of Blm to initiate this repair. If SDSA is initiated though, Blm helicase can later come in to disassociate the D-loop and allow for more processive polymerases to produce longer repair products.

No significant increase in yellow eyes in this assay with the *fancM* mutants was seen although this is usually a phenotype detected in other HR deficient backgrounds, such as Rad51 and Blm. This can be explained for two reasons that could easily go unnoticed; this result is due to an increase in apricot eyes or a decrease in survival. When FancM is not present, Blm can later unwind and disassociate the nascent strand of these large D-loop intermediate structures to decrease topological strain, an ability that FancM does not seem to have. Then, the entire construct of the *P*-element could have been resynthesized with the *copia* retrotransposon causing an increase in the apricot class. Another possibility is that the flies could not initiate repair of the large gaps in the genome and were therefore not viable. When FancM is not present, often this

essential repair pathway cannot start, rendering the flies inviable. Unfortunately, we are not able to calculate the frequency of flies that would fall into these two classes.

The *fancM mus301*mutants also had a significant decrease in HR as both helicases are necessary for HR. As the frequency of the red eye class was virtually the same as both single mutants, HelQ and FancM may be working together during the same step of HR. This amount of failed HR occurring contrasts the *fancM* single mutants as there was an increase in yellow eyes for the double mutants. There was a significant increase in the amount of failed HR as HelQ is not present to open up the D-loop for more processive synthesis and HR fails. This would not cause an increase the frequency in apricot eyes or red eyes, so HR fails when smaller D-loops are not properly resolved by FancM.

The decrease in STL was also seen in the *fancM mus301* flies. While the double mutant phenotype was not more severe in the HR frequencies shown in the red eye class, further molecular analysis suggests that these two helicases have different roles, as the result is additive for the STLs of the double mutants. Similarly, *mus301blm* double mutants also showed this additive phenotype in the decreased frequency of HR, providing more evidence that these three helicases serve different functions within this repair process.

Proposed Model of Three Helicases in SDSA

Based on the data from the *P{w^a}* assays conducted in the McVey lab, we propose a model hypothesizing that these three helicases play different roles *in vivo*, although Blm and FancM seem to have redundant roles *in vitro*. FancM works not only as a signaling protein to initiate HR of DSBs, but also serves to unwind the nascent strand of the D-loop of smaller intermediate structures (Figure 15). Early in the pathway, HelQ could be recruited by Rad51 as they have been seen to interact in mice so then HelQ could work at the front of the D-loop to

open it up and allow for more processive polymerases to synthesize repair off of the homologous sequence (Adelman 2013). Opening up the D-loop would increase the torsional strain in both directions on the D-loop and therefore Blm must recruit TopoIIIα and unwind the nascent strand.

Once HelQ creates these larger structures, Blm is recruited to the D-loop (possibly by FancM, which has a Blm binding motif (Deans and West 2009). Blm then unwinds the nascent strand of these larger D-loop structures in a 3' to 5' direction. Blm may also recruit RMI (RMI1/RMI2) and topoisomeraseIIIα to decrease the torsional strain of the larger intermediate D-loops formed when Blm is present. While the Blm dissolvasome has only been shown to unwind double Holliday junctions (dHJ), this could be another important step in SDSA repair TopoIIIα can work to unwind torsional strain (Manthei 2013). In this model, FancM and Blm play similar roles, but act at different stages of the pathway with additional functions, such as recruitment, that were detected *in vivo*.

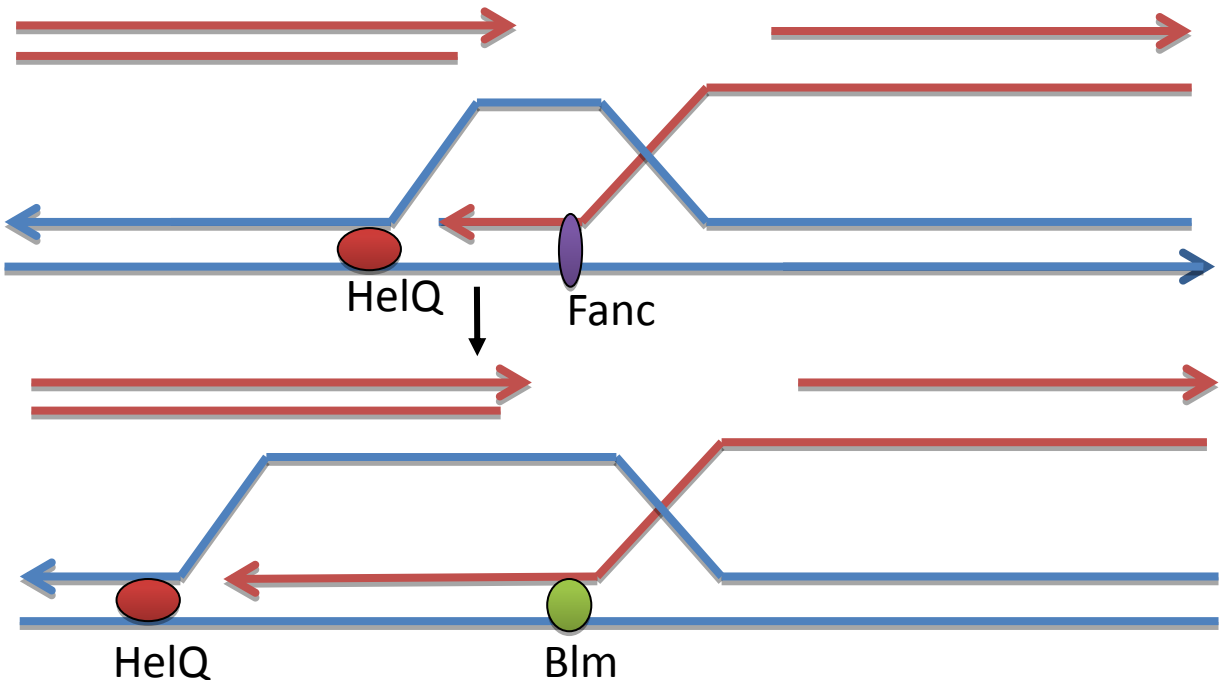

Figure 15. Proposed model for helicases in SDSA. HelQ works at the front of the D-loop to open up the D-loop while FancM initially works to unwind the nascent strand of the D-loop. In the next step Blm works to unwind the disassociated strand of these larger D-loops that HelQ provides to decrease torsional strain.

In the Sekelsky lab, *fancM blm* flies were designed to have the transposon and the transposase for the *P{w^a}* assay. Unfortunately, when the transposon and transposase were both present in the double mutants, these flies were not viable (Kuo et al. 2014). This occurred because repair of these gaps is necessary for survival; it seems that, without these two helicases, the gaps could not be repaired. This fits the genetic model proposing that FancM and Blm have similar roles *in vivo*, unwinding the nascent strand of the D-loop during SDSA. When neither of these helicases is present, the D-loop structure cannot disassociate and repair cannot occur through this pathway. Instead nucleases may cleave this structure to resolve the D-loop, but this would resect back on the genome and create large deletions that could not be repaired. Because FancM and Blm helicases seem to have redundant phenotypes, as they can unwind the nascent

strand of D-loops, their absence for gap repair, results in the inviable flies. Members of the McVey lab are still trying to create double mutants of this nature with a different *blm* allele, the N2 allele, which has shown to have a less severe phenotype in the *P{w^a}* assay and will hopefully be viable as double mutants to complete the story.

If *fancM blm* mutants were made and were viable, according to the model, there would be a significant decrease in SDSA, and possibly an increase in the end-joining phenotype, just as was observed in the Blm single mutants. A large number of the flies would not be viable because FancM would not be present to initiate the repair. If repair did initiate, Blm would not be present to unwind larger D-loops, so the amount of the apricot class would decrease because more processive repair would not be occurring. The scalloped phenotype would also be present, as it was in the *blm* mutants (Thomas and McVey unpublished). The repair events that could initiate SDSA would be resolved with nucleases like in the Blm single mutants as HelQ still present at the front of the D-loop to open it up for more processive polymerases. There would be a significant decrease in the synthesis track lengths, as in the double mutants previously generated. Because the roles of these two helicases are different *in vivo*, this additive function would be shown in a phenotype more severe than that of the Blm single mutants.

HelQ overexpression does not decrease fertility

After looking at the phenotype of flies lacking HelQ, we looked at the affects of overexpressing HelQ *in vivo*. Appropriate levels of HelQ are necessary in the ovaries for the flies to lay eggs that are viable and have proper dorsal appendages, but are also needed at basal levels for DNA repair. Because there was not enough HelQ being expressed without a Gal4 driver to rescue the infertility phenotype in a *mus301* mutant background, HelQ was overexpressed in specific tissue using the UAS/Gal4 system. By mating flies with the drivers to flies with the

transgene, HelQ was produced at higher levels in the progeny. This was determined through a western blot that detected this tagged protein when ubiquitously expressed with the actin Gal4 driver. These flies overexpressing the transgene had normal fertility which showed that the tagged HelQ expression was not negatively effecting the flies when they did not have any other deficiencies.

The tagged protein could only be detected when expressed throughout the fly and could not be detected when the protein was only expressed in the ovaries of the fly. To make sure that native protein was being produced with these drivers, a hatching frequency assays must be performed on flies that have the transgene, the Gal4 driver, as well as a homozygous mutant *mus301* background. This would allow for us to determine not only if HelQ is being expressed with the UAS/Gal4 system in the ovaries, but also if this tagged protein can carry out its normal processes.

Because the western only worked for the Actin5C, RT-PCR would allow us to test if the transgene is being transcribed with all of the Gal4 drivers. Creating cDNA would determine if proper transcription of the gene was occurring to produce mRNA. Although this would not tell us if the protein is folding properly with the tag, it would allow us to bypass the western, that does not seem to be working when HelQ is not ubiquitously expressed. Determining the flies' sensitivity to MMS could help us elucidate the tagged HelQ's ability to work during DNA repair. This experiment could also test if a FLAG-tagged HelQ is folding properly *in vivo*.

Future experiments to determine the role of HelQ *in vivo*

In the future, experiments should be performed to see if FancM and Blm helicases are recognizing the same DNA substrates during the pathway to test if this small D-loop versus large D-loop hypothesis is actually accurate. Additionally, more biochemical analysis to determine

where HelQ is functioning must be conducted to identify if it is recognizing both small and large D-loop and where on the D-loop it is interacting. The processivity of these two helicases should be determined to understand how they play different roles during repair.

Now that we know HelQ can be detected with the FLAG-tag, we could be able to detect what other proteins it interacts with *in vivo*. By purifying HelQ with the FLAG-tag and also through a nickel column for the 6HIS-tag, a sample of the HelQ would be obtained. Hopefully this protein would also pull down other proteins to which it binds. By running this purified sample on a gel, we would hopefully see bands for protein other than HelQ. We could cut out these bands and use mass spectrometry to determine what these proteins are. This result will be especially interesting because with the *in vivo* set up, HelQ can be expressed in different tissues and we can determine how HelQ functions in its roles for DNA repair and embryogenesis.

Although many questions about these helicases still remain, the first steps to classify their *in vivo* function have been taken. Better understanding how these helicases function during repair has lead to the discovery of a possible model for the function of helicases during SDSA. While these helicases were thought to have redundant roles *in vitro*, this *in vivo* evidence shows that they each have distinct roles necessary for SDSA. Future experiments have been planned to characterize these exact function. By determining how these helicases work, we will have a fuller understanding of this important error free repair mechanism, homologous recombination.

Supplementary Data

Table 1. Primers Used

Primer Name	Purpose	Sequence
BglIIm301	Restriction Enzyme cloning with sticky ends to amplify insert	tatata aga tct cat atg cgg ggt tct ca
Acc651m301	Restriction Enzyme cloning with sticky ends to amplify insert	tatata ggt acct aa ttt tta tca gac ggt
Mus301 3586R	Confirmation of mus301^{288A}	tggcagactatt
Pout	Confirmation of mus301^{288A}	CCG CGG CCG CGG ACC ACC TTA TGT TAT TTC
Sd5941a	P{w^{a}}assay walk-in PCR	GCT ACG GAA CTT CAG ACA GGG
Sd5678a	P{w^{a}}assay walk-in PCR	CCC TCG CAG CGT ACT ATT GAT
Pout	P{w^{a}}assay walk-in PCR	CCG CGG CCG CGG ACC ACC TTA TGT TAT TTC
P{w^{a}}248	P{w^{a}}assay walk-in PCR	GTC GAC CTG CAG CCA AGC TTT G
P{w^{a}}997a	P{w^{a}}assay walk-in PCR	GAT GTT GCA ATC GCA GTT C
P{w^{a}}4287	P{w^{a}}assay walk-in PCR	GCA ACG AGC GAC ACA TAC CG
P{w^{a}}4674a	P{w^{a}}assay walk-in PCR	GGA CTG GGC CCA TAA CCT GTT G
P{w^{a}}1487	P{w^{a}}assay walk-in PCR	CGT TGT TTG CAC GTC TCG CTC G
P{w^{a}}2420a	P{w^{a}}assay walk-in PCR	GAG CGA GAT GGC CAT ATG GCT G
P{w^{a}}5320	P{w^{a}}assay walk-in PCR	ACC ATT GCA AGC TAC ATA GCT GAC
fancM0693F	Verification of FancM^{0693}	CGCAATGAAGGTCTTTCCGT
fancM0693R	Verification of FancM^{0693}	TGTCACGATTTGT GTGATCG
fancMDfEDF	Verification of FancMDfED(3058)	TTATGGAGTTAATTCA AACCCCAC
fancMDfEDR	Verification of FancMDfED(3058)	CACAGTCGCTTCTAAAATATATGGC
Mus301 113F	Sequencing mus301 flag-tag	GCG AAG ACG ACG ACA GCT TC
Mus301 652F	Sequencing mus301 flag-tag	GTC AATTGG GAG ACT CAG GC
Mus301 1029F	Sequencing mus301 flag-tag	GAG AAG GTG AGC GCC ATG TCT C
Mus301 3120	Sequencing mus301 flag-tag	GCC ATT CAC AAG GAG CTC AAG C
Mus301 F1	Sequencing mus301 flag-tag	GCC CAG AGC AAA GAC AAT CTG C

Mus301 3485	Sequencing mus301 flag-tag	GAT CAGTCA ATC CAC AGC AGC

Table 2 – *P{w^a}* repair junctions recovered from *fancMmus301* mutants

Genotype	% scalloped Females (F1)	% No white-eyed males (F2)
Wild-type	0.0% (84)	0.0% (44)
mus301^{288A}	0.2% (1061)	2.0% (246)
blm^{N1}	17.3% (623) [a]	55.3% (159) [a]
mus301^{288A}, *blm*^{N1}	3.9% (332) [b]	25.2% (127) [a,b]
fancM	0.0% (147)	0.0% (35)
mus301^{288A}, *fancM*	0.0% (174)	4.0% (42)

Table 3.- Bloomington Stocks used for Gal4 expression system

Common Name	Genotype	Location of Expression
Actin5C	y^1 w*; P{Act5C-GAL4}17bFO1/TM6B, Tb^1	Ubiquitous
Nanos	w^{1118}; P{GAL4::VP16-nos.UTR}CG6325^{MVD1}	Germline cell
Matalpha	w*; P{matα4-GAL-VP16}V37	Oocyte

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