# On the role of Rev1 in the repair of DNA double strand breaks through homologous recombination in *Drosophila*

An honors thesis for the department of Biology

Under the advisement of Professor Mitch McVey

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**Abstract**

Throughout the lifecycle of an organism, DNA is damaged due to both endogenous processes and exogenous damaging agents. Failure to properly repair DNA can lead to potentially oncogenic mutations as the result of base changes, chromosomal translocations, or deletions. One of the most dangerous types of lesion is a DNA double strand break (DSB); the oncogenic potential of DSBs is evidenced in the cancer-prone phenotypes of mutations in DSB repair genes such as BRCA1 and BRCA2. It has long been believed that the error-free approach to DSB repair is a pathway known as homologous recombination (HR), where the DNA surrounding the break is removed by exonucleases and then resynthesized using the homologous chromosome as a template. However, recent research has suggested that error-prone translesion synthesis (TLS) polymerases may be responsible for synthesizing new DNA during HR; calling this supposition of error-free repair into question. In fact, it has been observed in budding yeast that HR is far more mutagenic than normal replication; although TLS polymerases were not found to contribute to HR associated mutagenesis in that system.

Far less is known about polymerases involvement in HR higher eukaryotes. In this paper we discuss the creation and characterization of *Drosophila* deficient in the Rev1 TLS polymerase. Using 2 different reporter assays to probe the role of Rev1 in repair of large double strand gaps and simple breaks, we present evidence for a role for Rev1 in recruiting and coordinating the activity of other TLS polymerases during homologous recombination repair. Interestingly, we found that in the absence of Rev1 HR can still proceed, albeit at a lower efficiency - likely due to the use of a more complex replication-like pathway which depends on highly processive polymerases.

## Introduction

DNA damage, repair and mutagenesis

Our cells are constantly faced with a variety of DNA damaging agents. DNA damage can be caused by endogenous stressors such as free oxygen radicals that are produced during aerobic respiration as well as exogenous agents such as ultraviolet light, X-rays and environmental carcinogens [1]. The induction of DNA damage poses a serious problem for cells; which depend on an undamaged DNA template in order to faithfully replicate their genome as well as transcribe essential genes. Failure to properly replicate the genome can lead to spontaneous point mutations, insertions, deletions, translocations and aneuploidies; all of which are potentially cancer driving mutations [2,3].

Human cancers are generally caused by the simultaneous dysregulation of several classes of genes; which can be caused by a mutation in the gene itself or in regulatory regions of DNA. The primary driver of cancers is the upregulation of a class of genes known as oncogenes. Classical examples of oncogenes include RAS, a key part of cell proliferation signaling pathways; and MYC, a transcription factor promoting cell growth and proliferation. In order for dysregulated oncogenes to be truly dangerous, they must be combined with the inactivation of protective “tumor suppressor” genes, such as p53 and Retinoblastoma protein. Mutations in genes controlling apoptosis, angiogenesis, telomerase activity, and cell mobility are also known to drive cancer progression [4].

In order to protect themselves against cancer driving mutations, higher eukaryotes have evolved a complex and tightly controlled DNA damage response which acts to repair DNA damage while minimizing the introduction of genomic mutations. Upon induction of DNA

damage the ATM and/or ATR kinases are activated, triggering a signaling cascade that may ultimately result in cell cycle arrest -- or in cases of irreparable damage -- apoptosis [5]. Repair pathways such as base excision repair and nucleotide excision repair leave breaks in the phosphodiester backbone of the DNA helix. While these single strand breaks (SSBs) are generally relatively innocuous lesions, they are dangerous during replication as they can cause fork collapse -- resulting in a much more dangerous double strand break (DSB).

Translesion DNA synthesis

Because of the danger posed by repair during replication, the cell will often forgo repair of certain types of lesions and instead use DNA damage tolerance pathways. The most well understood DNA damage tolerance pathway involves the use of a specialized class of DNA polymerases known as translesion synthesis (TLS) polymerases. DNA polymerases are a class of enzyme that can synthesize new DNA using a DNA template. The standard leading and lagging strand polymerases, pol ε and pol δ respectively, are specialized for high fidelity and high processivity, which is essential in order to quickly and faithfully replicate the genome. This high fidelity is largely due to rigid conformation of the active site, which will only accommodate a canonical Watson-Crick base pair. Additionally, these polymerases have a 3'-5' "proofreading" exonuclease activity which allows them to remove and replace an incorrectly paired nucleotide [6]. Due to this rigid specificity, however, replicative polymerases are unable to accommodate damaged DNA structures or bases. For this purpose, cells have evolved TLS polymerases, which can replicate from a damaged template, albeit at an increased error rate, due largely to their more flexible active sites and lack of proofreading activity.

Some form of translesion is synthesis is present in all domains of life. *E. coli* polymerases II, IV and V have all been shown to play a role in lesion bypass during replication. Eukaryotes have a much more extensive group of TLS polymerases, with some organisms possessing as many as ten [7]. Aside from their evolutionary conservation, genetic data suggests that TLS polymerases are essential for normal development and cellular function in higher eukaryotes. Humans with mutations in the gene encoding DNA polymerase η have a variant form of the cancer prone disorder xeroderma pigmentosum (XP-V) [8]. Additionally, a mutation in the rev3 gene, which encodes the catalytic subunit of DNA polymerase ζ, in mice is embryonic lethal [9].

Despite their requirement for viability, TLS polymerases pose a serious danger to an organism if their activity is not carefully controlled. TLS polymerases Rev1 and Rev3 (the catalytic subunit of DNA polymerase ζ) were initially identified in budding yeast in a screen for UV-reversionless mutants. A mutant is considered reversionless when it is deficient in acquiring a reversion mutation which converts the nonfunctional *arg4-17* allele to a functional allele [10]. Practically, this suggests that Rev1 and Rev3 are primarily responsible for mutations acquired during exposure to UV light. More recent research has shown that, together, Rev1 and Rev3 are responsible for the majority of both spontaneous and damage-induced mutagenesis [11]. Due to this inherent danger of mutagenesis, conventional wisdom dictates their use is restricted as much as possible in order to minimize potentially carcinogenic mutations. This intuition has made recent discoveries of the involvement of TLS polymerases in “error-free” processes such as normal replication and homologous recombination [12,13] puzzling for researchers.

DNA double strand break repair

Homologous recombination is one of the major pathways that act to repair DNA double strand breaks (DSB). DSB's can arise both during replication, when a fork collapses, and independently of replication, as the result of exogenous damaging agents such as ionizing radiation. Additionally, certain cell types purposefully induce DSB's; such as B and T-cell lymphocytes during V(D)J recombination and gametocytes during meiosis. An unrepaired DSB is one of the most dangerous types of DNA lesion, as it completely blocks replication of the chromosome as well as transcription of nearby genes. Additionally, failure to properly repair a DSB can lead to chromosomal translocations or loss of significant amounts of genetic information [14].

To combat this threat, cells have evolved an intricate DSB response that acts to recognize and repair these breaks in the least damaging way possible. One of the first responders to a DSB is the Mre11-Rad50-Nbs1 (MRN) complex, which acts as a scaffold, tethering the two ends of the break together in preparation for repair. The MRN complex additionally activates the ATM signaling kinase, which ultimately leads to checkpoint induction and H2AX (H2Av in *Drosophila*) phosphorylation surrounding the break [15]. From this point the break can be processed through one of two classes of repair pathways: homologous recombination (HR) or non-homologous end-joining (NHEJ).

In NHEJ the DNA ends are first bound by the Ku 70/80 heterodimer, which together with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and the DNA itself forms the DNA-PK holoenzyme. DNA-PK phosphorylates several protein targets including itself; activating a signaling cascade which ultimately leads to the loading of XRCC4 and XLF onto the

DNA surrounding the break -- creating a rigid filament which may assist in holding the DNA ends together [16]. In the final steps of classical NHEJ, DNA Ligase IV ligates the two ends back together, repairing the break. In addition to classical NHEJ, the cell may also use a more error-prone end-joining pathway known as alternative end-joining (Alt-EJ). Relatively little is known about the Alt-EJ pathway, although it is known to be dependent on DNA Ligase III and may take advantage of microhomologies adjacent to the break [17]. Alt-EJ is an inherently error-prone process which generally results in small deletions and/or insertions. Classical NHEJ has the potential to be an error free process so long as the break was "clean" and no information was lost adjacent to the break. Because of its accuracy under most situations classical NHEJ is the preferred DSB repair pathway during G1 and G0 phases of the cell cycle, when no sister chromatid is available for HR [18].

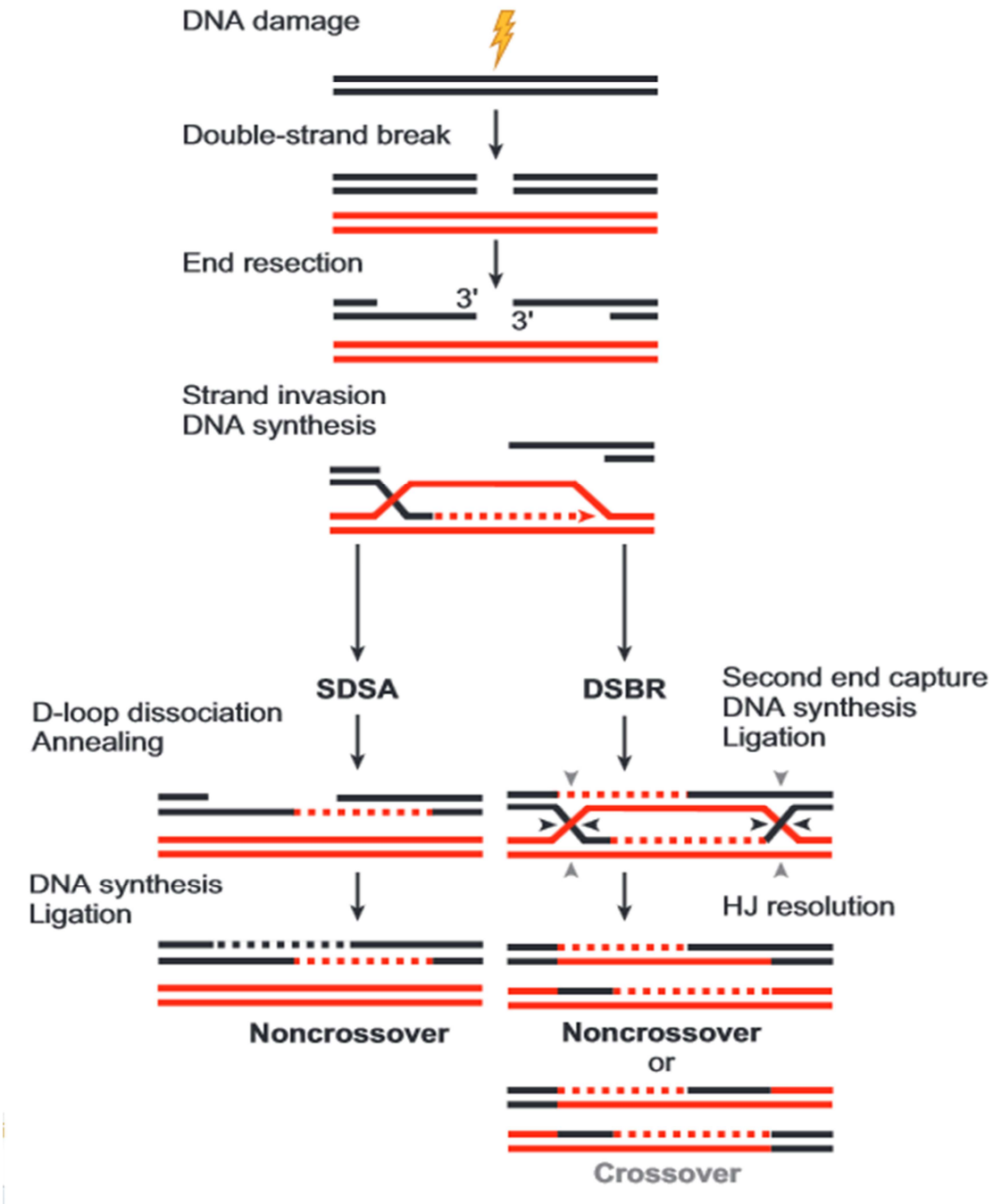
Homologous recombination

While NHEJ has the potential to be error-prone, HR has long been viewed as the "error-free" pathway of double strand break repair, as it uses an undamaged homologous template to restore the sequence surrounding the break. The initial steps of HR involve 5' - 3' resection of the DNA ends leaving long single stranded 3' overhangs. The initial resection is promoted by the MRN complex in conjunction with CtIP; more extensive resection is then carried out by Exo1 and/or the combined activities of BLM and Dna2 [19]. These 3'-overhangs are immediately coated by RPA which prevents the formation of secondary structures and prevents annealing at microhomologies which could lead to inaccurate Alt-EJ [20]. Current evidence suggests that these RPA coated overhangs are next acted on by Rad52 and/or BRCA2 which mediate the removal of RPA and coating of the single strand with the Rad51 recombinase [21]. Rad51 then mediates a search for homologous DNA sequence, likely through a mechanism

similar to the bacterial RecA recombinase, which randomly collides with and dissociates from double stranded DNA until homology is found [22].

Once a homologous sequence is found, Rad51 and other enzymes catalyze a strand invasion reaction, where the Rad51 coated filament displaces one strand of the double stranded homologous sequence and base pairs with the complementary strand, forming a displacement loop (D-loop, figure i). Once the D-loop is formed, homologous recombination can proceed through several different mechanisms. The first step in every HR mechanism is extension of the invading strand by a DNA polymerase; after this step the pathway can proceed in several different ways. In the classical double strand break repair (DSBR) model of HR, the newly synthesized invading strand captures the second end of the break and is ligated to it, forming a structure known as a Holliday junction (HJ). The presynaptic filament from the other end of the break is also extended and ligated to its second end forming what's known as the double HJ intermediate [23]. This double HJ intermediate must then be resolved through the action of various HJ resolving endonucleases, which cut 2 strands of DNA. Depending on which strands are cut, DSBR can result in either crossover or non-crossover products. Because of the potential to generate crossovers, the DSBR pathway is essential for meiotic recombination.

**Figure i: Homologous recombination can proceed through several mechanisms-From [21]**




Homologous recombination can proceed through two major pathways. In SDSA the invading strand (black) synthesizes from the homologous template (red) and is then dissociated through the action of D-loop dissolving helicases. This newly synthesized DNA can then anneal to the other end of the break, producing a noncrossover repair product. In DSBR, the invading strand captures the second end of the break forming a double Holliday junction, these Holliday junctions can be resolved by the action of nicking endonucleases which cut at the positions indicated by arrows above, producing either crossover or noncrossover repair products.

During mitotic homologous recombination, crossovers are generally suppressed in order to prevent erroneous translocations and maintain genomic stability. This is thought to be due to the use of a distinct HR mechanism when a non-crossover product is favored, known as synthesis dependent strand annealing (SDSA). The initial steps of SDSA are similar to DSBR up until the formation of the D-loop and extension of the invading strand. In SDSA, instead of capturing the second end of the break and forming a double HJ, the invading strand is dissociated from the D-loop, likely through the action of the BLM helicase [24]. This strand can then anneal to the 3'-overhang of the second end of the break - or if necessary - reinvade the homologous template and synthesize further. After re-annealing of the original double stranded molecule, gap-filling and ligation is all that is necessary to complete repair of the break, forming a non-crossover product. The requirement for SDSA in order to form non-crossover products is supported by the hyperrecombination phenotype in *Drosophila* lacking functional BLM [25].

Polymerase involvement in homologous recombination

Although the early and late stages of the various HR mechanisms have been the subject of intensive study, the DNA synthesis stage of HR has been poorly characterized in higher eukaryotes. Early studies in *S. cerivisiae* using temperature sensitive alleles of the genes encoding the leading and lagging strand polymerases (pols ε and δ respectively), suggested that they both play a role in recombination during mating type switching [26]. Additionally, the finding that the mutation frequency is increased up to 100-fold adjacent to a double strand break led researchers to ask whether more error-prone TLS polymerases may be involved [27]; and in fact, several years later it was noted that in the absence of the catalytic subunit of pol ζ, this mutation frequency was significantly decreased [28]. However, more recent research has suggested that gene conversion associated with mating type switching in yeast is mutagenic

independent of polymerase ζ; and that polymerase η acts to decrease mutation frequency during HR [29].

Until recently, very little was known about polymerase involvement in HR in higher eukaryotes. Some of the earliest indications that TLS polymerases may be involved came from *in vitro* experiments using HeLa cell extracts and an artificial D-Loop substrate. Researchers found that after several purification steps, the fraction active for D-Loop extension contained pol η. They were additionally able to show that purified pol η is capable of D-Loop extension *in vitro* in the absence of any other replication factors, whereas purified pol δ and pol ι were incapable of extending the D-Loop substrate [30]. Around the same time another group showed that DT40 cells lacking pol η were deficient in Ig gene conversion as well as HR mediated DSB repair, and that these deficiencies were rescued by complementation with human pol η [31]. More recently, researchers have found that HeLa cells lacking TLS polymerases ζ or Rev1 are hypersensitive to IR and deficient in HR repair of an I-SceI induced break [32].

Recent research in our lab has taken advantage of a system known as $P\{w^a\}$, which allows us to monitor the repair of 14 kb, transposase induced double stranded gap (DSG) in *Drosophila* using an eye-color reporter gene. Research has shown that this gap is repaired primarily through the SDSA pathway of HR [24], making it an ideal system to study HR in *Drosophila*. Using this system, earlier studies in our lab have demonstrated that the pol32 subunit of pol δ is needed for efficient HR. Levels of efficient HR were similarly decreased in the absence of TLS polymerases η or ζ, suggesting that all 3 polymerases play some role in DSG repair through HR. Interestingly, when polymerase η and ζ were both absent, the rate of gap repair through HR returned to wild type levels and the extent of synthesis in aborted HR events increased significantly over wild-type [13]. These unexpected results led us to suggest that pol δ

competes with less processive TLS polymerases, including pols η and ζ during HR. This explains the surprising result in the double mutant, because in the absence of both pols η and ζ, only the much more processive pol δ would be able to extend from the D-Loop.

The TLS polymerase Rev1 is known to interact physically with polymerase η and ζ and is also known to be essential for some pol η and ζ dependent TLS processes [33]. This, combined with the genetic evidence of polymerase competition during HR, suggested that Rev1 may play a role in recruiting TLS polymerases during D-loop extension, and mediating the interaction between different polymerase types. In this paper, we discuss several genetic studies on the role of Rev1 in HR in *Drosophila*, suggesting that while it is important for efficient DSB and DSG repair, a backup pathway exists; lending support to the competition model of polymerase recruitment.

**Materials and Methods**

Rev1 Imprecise Excision Screen

Fly stocks containing a P-element insertion upstream of Rev1 (P{EP}Rev1[G18538], Bloomington Stock #28417 abbreviated PRev1) were obtained from the Bloomington Stock Center. Flies were reared at 25° C on standard cornmeal agar medium. PRev1 virgin females were crossed with males carrying a P{ry$^{+}$, Δ2-3} transposase on the third chromosome to induce excision of P-elements. The transposase was crossed out before further experimentation.

Fly DNA Preparation

For large scale DNA preparation 30 flies were collected in an eppendorf tube and frozen at -20 °C. The flies were then ground until only cuticles remained in 400 μL of 100 mM Tris-

HCl pH 7.5, 100 mM EDTA, 100 mM NaCl, 0.5% SDS solution. This solution was then incubated at 65 °C for 30 minutes. After incubation, 800 μL of 1.4 M KAc, 4.3 M LiCl was added, and the solution was incubated on ice for 10 minutes. The sample was then centrifuged for 5 minutes, the pellet was discarded and the supernatant was centrifuged again for 5 minutes. The pellet was again discarded and the supernatant was transferred to a fresh tube. 0.6 volumes of isopropanol was then added to the tube causing the DNA to precipitate. The sample was then centrifuged for 15 minutes, and the supernatant was discarded. The pellet was washed with 70% ethanol, dried, and resuspended in distilled, deionized water.

For lower yield DNA preparations, a single fly was 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.

Hatching Frequency Assays

In order to assay hatching frequencies, flies were reared on standard cornmeal agar medium at 25 °C. Virgin females, homozygous for the mutation of interest, were collected at 8 hour intervals when kept at 25 °C. Flies were moved to 18 °C overnight to which allows for the collection of virgin females at 16 hour intervals. These virgin females were crossed to males carrying the mutation of interest in bottles containing dried yeast pellets in addition to the standard cornmeal agar medium. After 24 hours these flies were transferred to cages and allowed to lay eggs for 24 hours on grape agar plates at 25 °C. After 24 hours the grape plate was exchanged for a fresh plate - this was repeated for 3 days. Plates were scored for hatching frequency by counting under a microscope 3 days after removal from the parental generation.

*P{w^a}* Assay

Flies were reared on standard cornmeal agar medium at 25 °C. Crosses were performed to create a stock of flies that were homozygous *rev1Δ* and also carried a copy of *P{w^a}* on the X-chromosome and the CyoΔ2-3 transposase on chromosome 2. In order to isolate unique repair events, males from this stock were crossed with virgin wild type females, carrying an intact copy of *P{w^a}*. The progeny of this cross were then scored for eye-color. Only female progeny that did not carry the transposase gene were scored. Vials containing less than 10 scoreable flies in total were omitted from results. Statistical analysis was performed using the unpaired Mann-Whitney test.

DR-White Assay

Two different stocks were constructed: one heterozygous for the DR-White.mu construct on chromosome 2 and heterozygous for the Rev1 null allele on chromosome 3, and the other heterozygous for a heat-shock inducible I-SceI transgene on chromosome 2 and heterozygous for the Rev1 null allele on chromosome 3. Males from the stock carrying I-SceI were crossed with virgin females from the stock carrying DR-White.mu. These flies were moved to new vials every 2 days and discared on day 8. Each vial was heat-shocked at 37 °C for one hour on the day following transfer of the parental generation. Once the flies eclosed, male progeny carrying I-SceI and DR-White were isolated. Flies homozygous for Rev1 were separated from the heterozygotes which were used as a wild type control. Each individual male was crossed to 4 virgin females, and the progeny were scored for eye and body color. From each vial up to 3 red-eyed males were isolated and used for molecular analysis.

To perform molecular analysis, we purified DNA from red-eyed males as discussed above. We performed PCR using primers surrounding the repaired copy of *white*, giving us a product of approximately 2 kb. The PCR products were then purified and sequenced using primers internal to the PCR primers. Sequences were aligned and analyzed for polymorphisms indicative of gene conversion using the CLC Main Workbench 7 software suite.

## Results

### *Generation of a Rev1 null mutation in Drosophila*

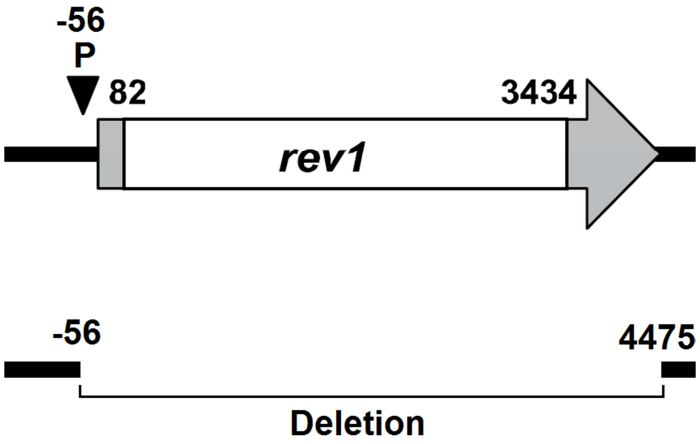
In order to investigate the role of Rev1 in homologous recombination (HR), we first generated a line of flies that were deficient for Rev1 gene expression. To do this we utilized a technique known as imprecise *P*-element excision to generate a genomic deletion at the endogenous Rev1 locus, abrogating all gene expression. A *P*-element is a type of transposable element that is common throughout the genomes of wild *Drosophila*. Additionally, there are numerous laboratory stocks available with *P*-elements in well-defined locations in the genome, making them useful for genome engineering. Many wild type *P*-elements carry a gene encoding a transposase; a class of enzyme that catalyzes the excision of the transposable element from the genome. Early research on *P*-elements has been able to separate the transposase function from the P-element, allowing us to precisely control *P*-element excision by expressing the transposase in *trans*.

*P*-elements are useful for genome engineering because they have a tendency to generate deletions of DNA sequence adjacent to the site of excision, due to error-prone repair of the break left behind after transposition. In this case we utilized a stock of flies carrying a *P*-element upstream of the Rev1 coding sequence, referred to as P{Rev1}. We then crossed these flies to a

line carrying a transposase, and collected males carrying the both P{Rev1} and the transposase. In order to isolate single germ-line excision events, these males were crossed to wild type flies. Flies in which the *P*-element was no longer present were selected based on their loss of the *white* phenotypic marker, which is manifested as loss of their red eye color.

In order to screen for imprecise excisions, we utilized several sets of PCR primers adjacent to and spanning the site of *P*-element excision. Using this method, we identified a single candidate isolate that contained a deletion of approximately 4.5 kb downstream of the *P*-element; a region which includes the entire coding region of Rev1, but no other genes. The precise deletion boundaries were confirmed by sequencing the PCR reaction, and are illustrated in Figure 1 below. Additionally we identified and maintained a strain of precise excision flies as a control. As precise excision is any excision event where repair of the excised *P*-element does not affect the surrounding sequence; this serves as a good control as its genetic background is identical to the Rev1 deletion line aside from the actual deletion.

**Figure 1: The *Rev1* null allele contains a deletion of the entire *REV1* coding sequence**

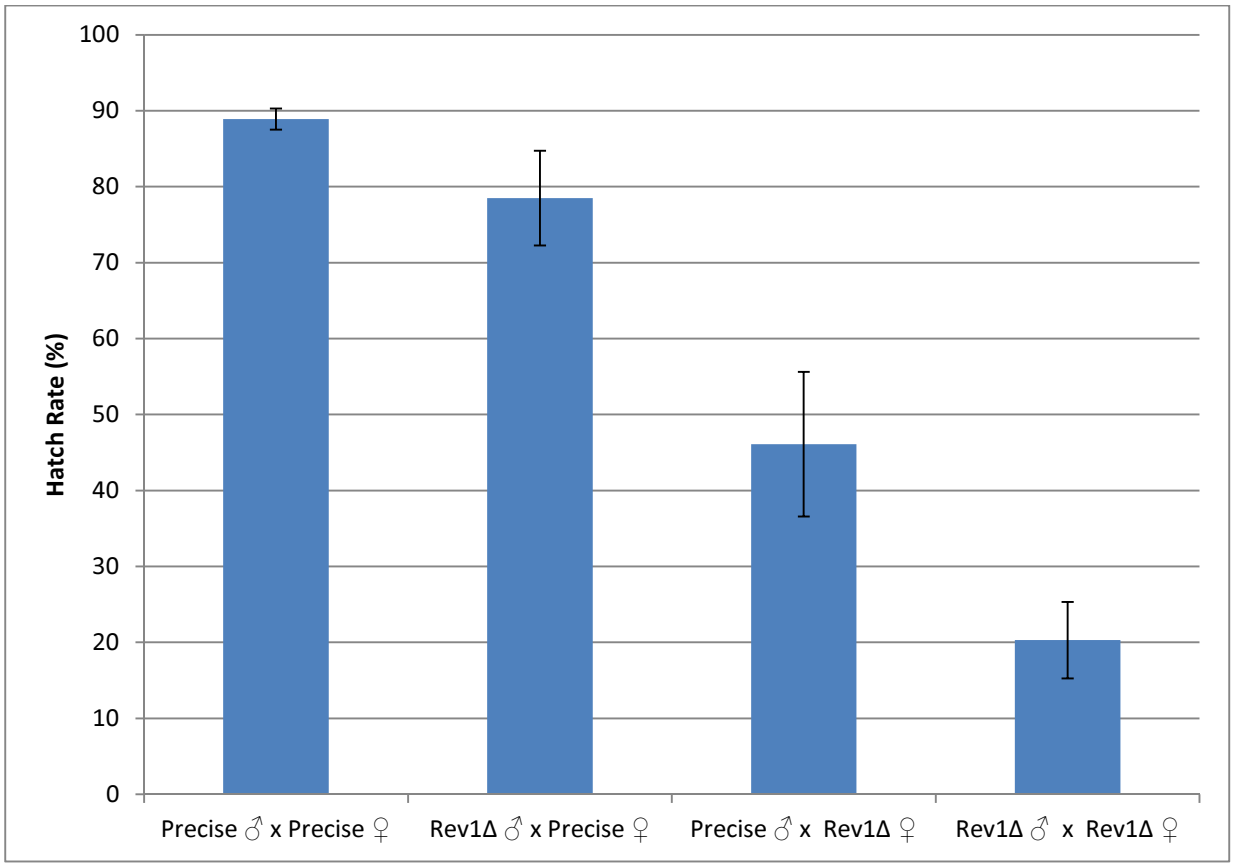


*Initial characterization of the Rev1 null allele*

After creation of the Rev1 null allele, Rev1 null homozygotes were collected by crossing heterozygous males with females. The Rev1 null flies were first characterized by monitoring their hatching frequency, which serves as a general indicator of overall fertility and viability of the strain. Hatching frequency is determined by mating homozygous males with virgin homozygous females and allowing them to lay eggs for three days. The hatching frequency is then scored three days after the eggs are laid, which allows more than enough time for healthy embryos to develop and hatch. As is illustrated in figure 2 below, flies homozygous for the Rev1 null mutation hatch at a significantly lower frequency than the precise excision flies, indicating that Rev1 plays an important role in either fertility or embryogenesis.

Mutations in genes encoding homologous recombination proteins such as the Rad51 orthologue, Spn-A, are known to lead to female fertility defects due to their inability to repair breaks induced during meiotic recombination [34]. To determine if this was a female fertility issue we crossed homozygous Rev1 null females with precise excision males. The resultant offspring all express wild type Rev1; therefore any hatching defect is most likely due to a female fertility issue. As is shown in figure 2 below, the hatching frequency of Rev1 null females mated with precise excision males is still significantly lower than the precise excision; although the defect is not as severe as for homozygous Rev1 null embryos. Additionally, the reciprocal cross (Rev1 null males mated with wild type females) has no significant hatch rate defect, demonstrating that there is no haploinsufficiency of Rev1 with regards to hatching frequency. While these results seem to indicate that the hatching defect is due to a defect in oogenesis in Rev1 mutants, we are unable to rule out the possibility that the defect is due to the lack of maternally loaded Rev1 within the first 2 hours of embryogenesis.

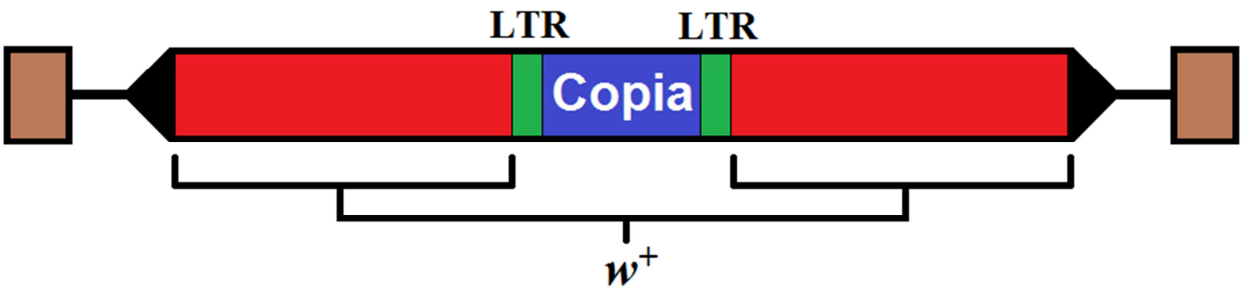
**Figure 2 - *Rev1* null mutants have a severely decreased hatch rate**




*Rev1 null flies show an increase in DNA synthesis during double strand gap repair*

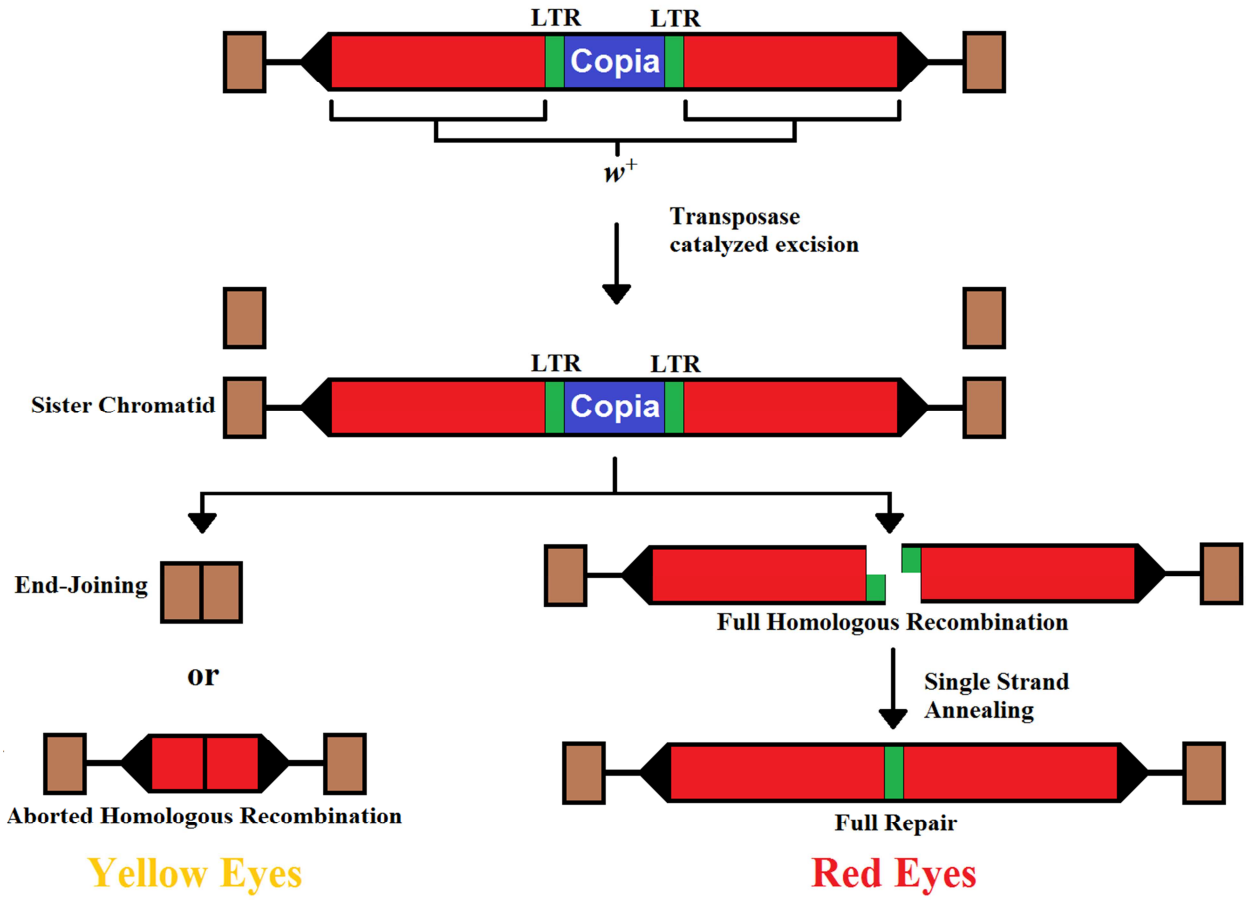
In order to look at the effect of Rev1 on homologous recombination we utilized the *P{w$^a$}* (P-white-apricot) double strand gap repair (DSGR) reporter assay. The basis of the *P{w$^a$}* assay is the *P{w$^a$}* construct, a *P*-transposable element with several unique features (See figure 3 below). The *P{w$^a$}* construct carries the *w$^a$* (white-apricot) allele of the *white* gene. The wild type *white* gene produces a gene product that gives flies their red eye color; the *w$^a$* allele is expressed at a lower level than the wild type allele owing to the presence of the *copia* retrotransposon in one of the introns, which significantly reduces the splicing efficiency of *white.* The result is an apricot eye color in flies carrying the *w$^a$* allele.

**Figure 3: The *P{w$^a$}* construct**




The power of the *P{w$^a$}* construct is that, like any P-element, it can be excised when a transposase is expressed in *trans*. When a P-element is excised it leaves behind a 14 kb DSG which must be repaired through homologous recombination (HR), non-homologous end joining (NHEJ) or some combination of the two pathways. If the gap is repaired through HR, then it is possible to restore all or part of the construct, resulting in restoration of the eye-color phenotype. On the other hand, if NHEJ is used, the construct cannot be restored. A common event during HR repair is annealing of the long terminal repeats (LTR, Figure 3 in green), and premature termination of HR. This significantly shortens the intronic size, resulting in near wild-type splicing efficiency, and higher expression levels of *white*, giving a darker eye color.

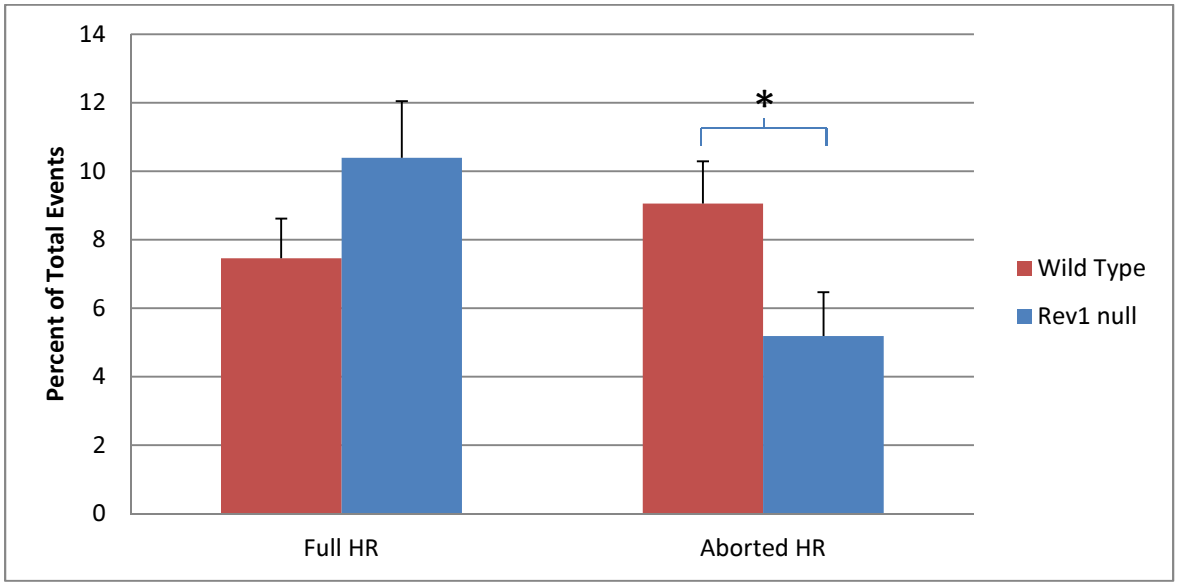
**Figure 4: The repair of *P{w$^a$}* excision can be occur through multiple pathways, producing different eye-color phenotypes**




In order to characterize the ability of Rev1 mutants to carry out DSG repair, we first crossed females that were heterozygous for the Rev1 null allele and that carried the *P{w$^a$}* transgene on their X-chromosome, to males that were also heterozygous for the Rev1 null allele and that carried the P-transposase gene. From this cross, single males were isolated that were homozygous for the Rev1 null allele and that carried both the *P{w$^a$}* transgene as well as the *P*-transposase source. Flies that were heterozygous for the Rev1 null allele were also collected and used as a wild type control. As has been previously reported [24], these males displayed significant eye-color mosaicism, indicative of excision and repair throughout the somatic cells.

In order to score the assay, we next crossed these mosaic eyed males, to females homozygous for the *P{w^a}* transgene on the X-chromosome. The female progeny of the next generation will carry one intact copy of *P{w^a}* and one repaired copy. This can manifest itself as three unique eye colors: red eyes if repair utilized HR to synthesize up to the LTRs before annealing, yellow eyes if repair utilized NHEJ or partial HR followed by premature EJ, or apricot eyes if the construct was never excised (Figure 4). While it is possible that apricot eyes may be the result of HR repair past the LTRs; this is unlikely based on evidence that flies lacking the Rad51 orthologue have apricot eyes at a similar frequency to wild-type, but no red-eyed events [35].

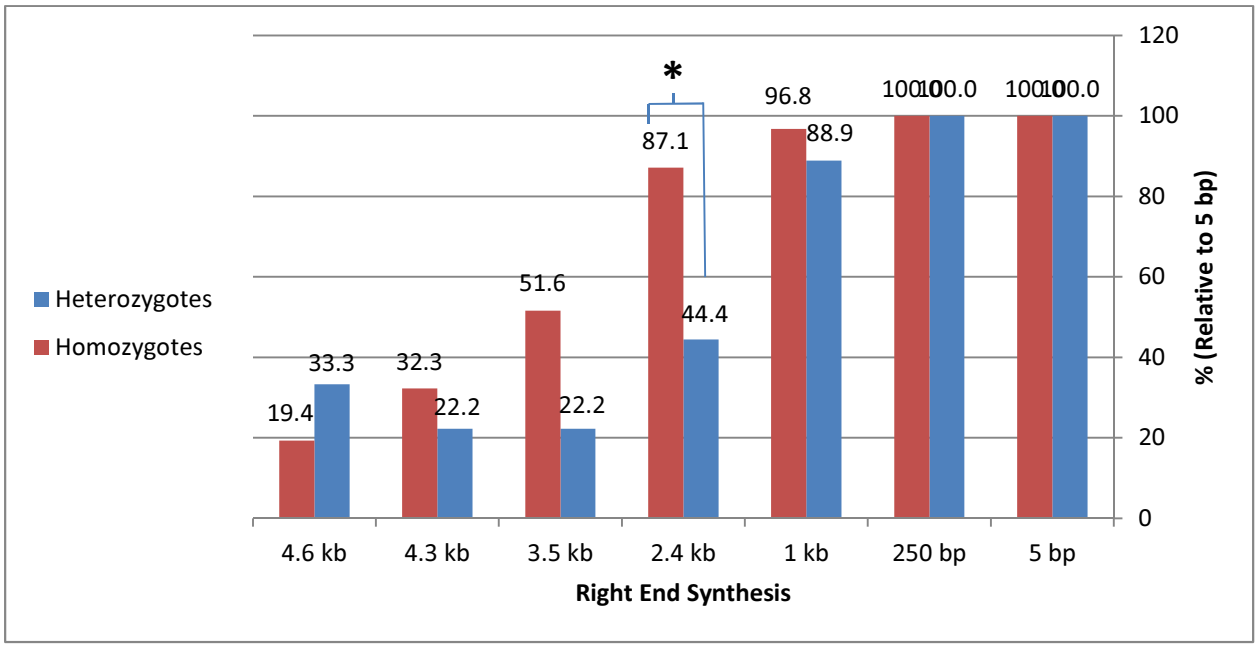
**Figure 5: Rev1 null flies show a significant decrease in aborted HR in the *P{w^a}* assay**




When we performed the *P{w^a}* assay we found that Rev1 null flies display a significant decrease in yellow-eyed events, indicative of a decrease in aborted HR or an increase in NHEJ (Figure 5). In order to more specifically determine the repair outcome of the yellow-eyed events, we mated each yellow-eyed female and recovered white-eyed male offspring - as they carry only the repaired copy of *P{w^a}*. We then isolated DNA from these males and performed PCR using

primers within the right half of the *P{w^a}* construct. The primer sets were designed such that each pair would only give a product if the repair from the right end had proceeded a certain distance before aborting. We found that in wild type flies as well as Rev1 null flies, the vast majority of yellow-eyed repair events involved some amount of synthesis off of the homologous template prior to end joining, suggesting that HR is the predominant pathway used to repair the transposase induced gap. Additionally we found that in the absence of Rev1, a significantly greater proportion of repair events synthesized at least 2.4 kb of sequence from the homologous template (Figure 6). This result suggests that in the absence of Rev1, DNA synthesis from the D-loop is more processive than when Rev1 in present.

**Figure 6: Rev1 null flies show significantly longer DNA synthesis off of a homologous template during repair of a transposase induced DNA gap**



*Rev1 is required for efficient HR mediated repair of an endonuclease induced break*

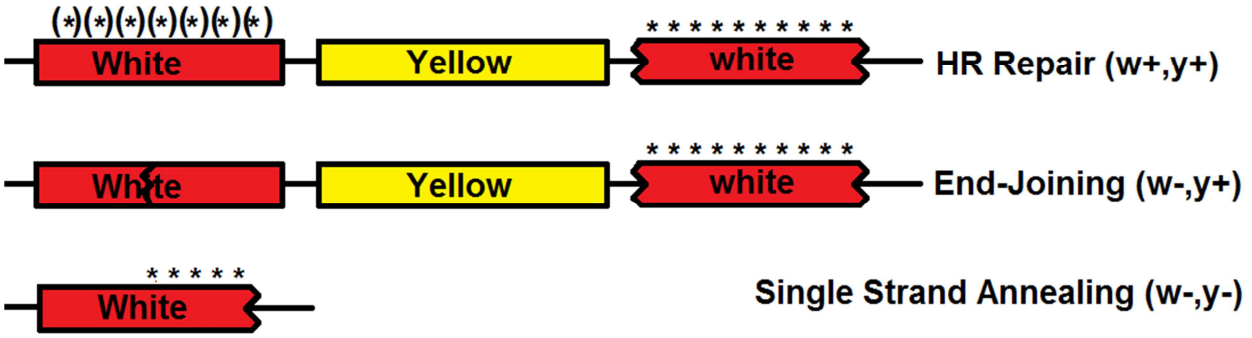
Although the *P{w$^a$}* construct is a great tool to study the genetic components of DSGR, there are some downfalls to the assay. First of all, the efficiency of transposon excision is only about 20%; additionally a large double strand gap is not a very physiologically relevant DNA lesion. Most breaks induced by exogenous agents or cellular processes do not require kilobases of DNA synthesis for proper repair. On the contrary, physiological DSBs often require limited, if any, synthesis to repair the break; making end-joining a much more viable repair pathway choice.

To address the question of whether Rev1 plays a similar role in the repair of a small break requiring limited synthesis, we took advantage of the DR-White (Direct Repeat White) DSB repair reporter system developed by the LaRocque group [36]. This reporter construct (illustrated in Figure 6 below) consists of 2 nonfunctional copies of the *white* gene. The first copy of the *white* gene is interrupted by an I-SceI cut site which introduces a premature stop codon, resulting in a non-functional protein product. The second copy of *white* is nonfunctional due to 5' and 3' truncations which remove the promoter, 5' untranslated region (UTR), start codon and 3' UTR. Between the 2 copies of white is a functional copy of the *yellow* gene, which gives the flies a normal pigmented body color.

**Figure 7: The DR-White.mu construct**

Using this system we can induce a double strand break in the I-SceI interrupted copy of *white* using a heat shock inducible I-SceI transgene. This break can be repaired through one of three processes, each producing a unique phenotype in the progeny. If repaired through homologous recombination using the second copy of *white* as a template, a functional white gene will be restored giving progeny that express both *yellow* and *white* (dark body and red eyes). If the break is repaired through end-joining, the *white* gene will maintain the premature stop codon, producing progeny that express functional *yellow*, but not *white* (dark body and white eyes). Lastly, if there is extensive resection through the *yellow* transgene, and into the second copy of *white*, the break can be repaired through single strand annealing (SSA) resulting in loss of *yellow* and a nonfunctional *white* gene (yellow body and white eyes).

**Figure 8: DR-White.mu Repair outcomes**

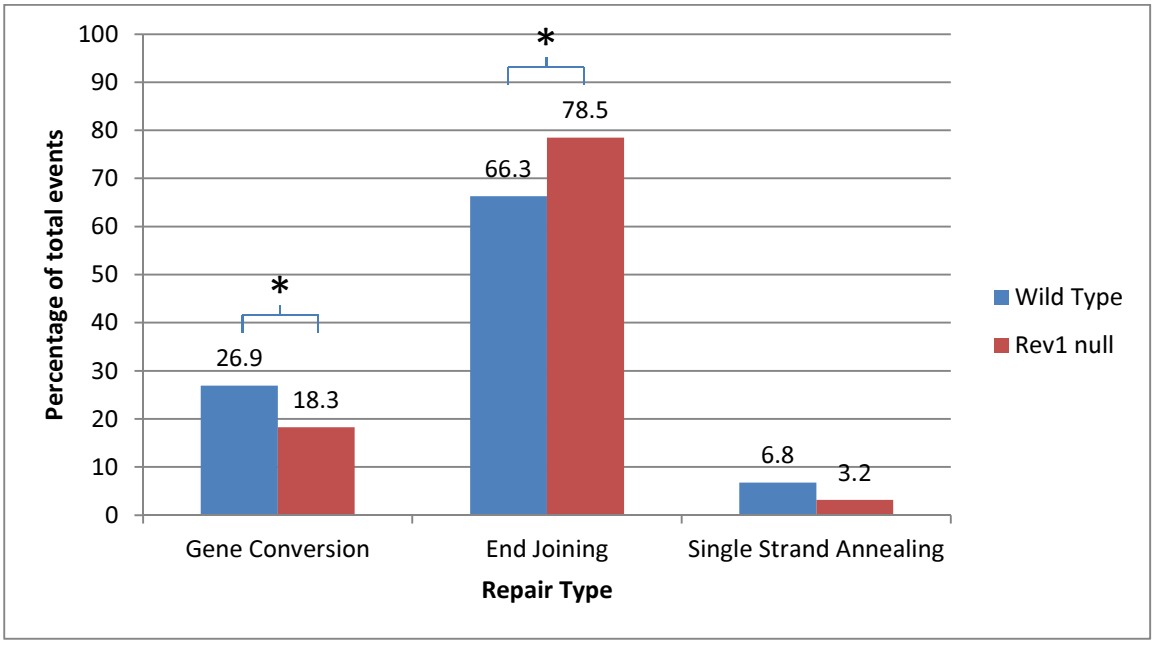



DR-White Repair outcomes: After a break is induced by the heat-shock inducible I-SceI, repair can proceed through 3 classes of repair pathways. Homologous recombination repair will result in active white and yellow expression. Additionally, the first white gene may now carry some or all of the silent polymorphisms present on the second copy of white (indicated by *) through gene conversion. End-joining repair that leaves the I-SceI site intact or that abolishes I-SceI, but does not restore the correct reading frame in white will result in active yellow expression, but no white expression. Lastly, repair through single-strand annealing will result in deletion of the yellow transgene and formation of a 3'- truncated white gene -- leading to expression of neither yellow nor white.

We performed the DR-White assay on Rev1 null flies by first setting up crosses to generate a line of flies carrying heterozygous Rev1 null alleles and either the DR-White construct or a heat shock inducible I-SceI transgene. We then crossed these flies to each other

and collected flies homozygous for the Rev1 null allele that carried both the reporter construct and the heat-shock inducible I-SceI. By heat shocking these flies as larvae we were able to generate mosaic adults, and isolate unique germline events in the following generation, similar to the strategy used for the *P{w^a}* assay. As is illustrated in Figure 8 below, Rev1 null flies show a significant increase in end-joining repair and a significant decrease proportionally in repair through HR. Although there is a noticeable decrease in the frequency of SSA in the Rev1 mutants, this was not found to be statistically significant with this sample size. While this may seem to contradict the results of the *P{w^a}* assay, where we observed an increase in the efficiency of HR; it is important to note that the nature of this break is very different than the DSG induced by transposon excision. Whereas the I-SceI induced break can be efficiently repaired through end-joining, the transposon induced gap cannot be -- forcing the cell to use HR even if the pathway is compromised.

**Figure 9: Rev1 null flies show decreased rated of gene conversion and increased rates of end-joining**



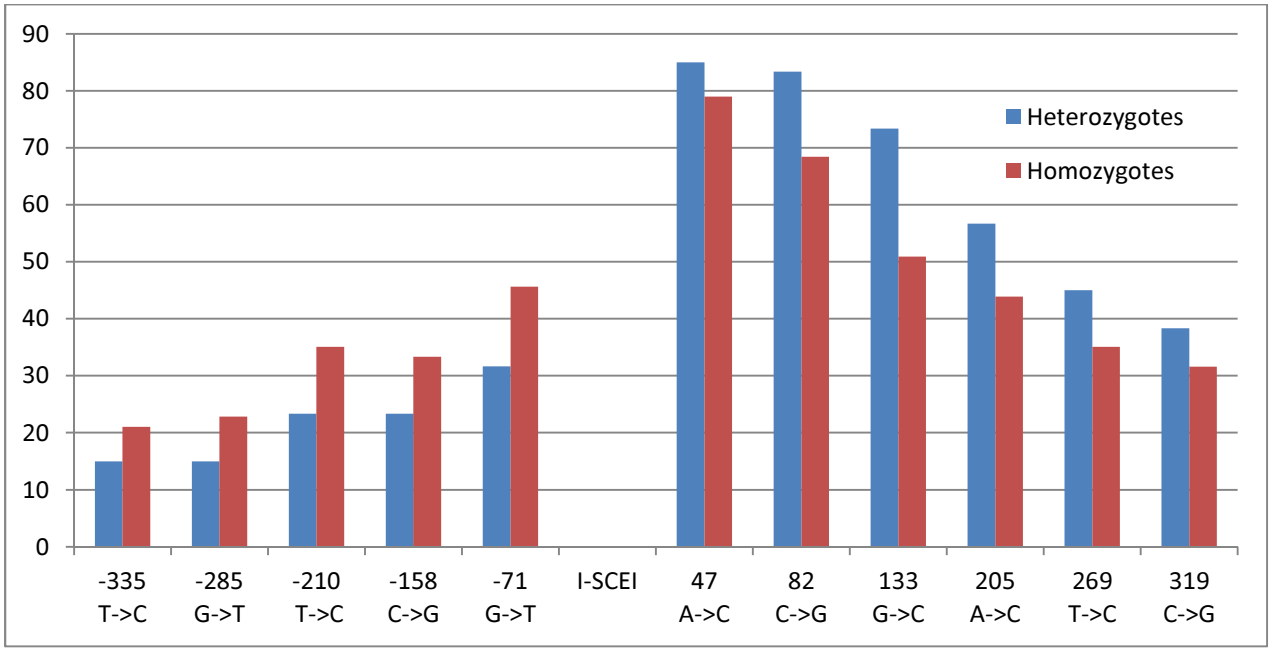
*HR Repair of the DR-White.mu construct favors gene conversion to the right of the break*

Using the DR-White.mu construct we can look at DSB repair in greater detail by monitoring the extent of gene conversion on either side of the break. The truncated copy of the white gene in DR-White.mu contains a series of silent polymorphisms throughout (indicated by * in Figure 8). When the I-SceI induced break is repaired by homologous recombination the repaired product may contain some of the polymorphisms from the second copy of white depending on the extent of gene conversion. By specifically amplifying the repaired copy of white through PCR we can determine the extent of gene conversion through DNA sequencing. The exact locations of the polymorphisms examined in this experiment are indicated in Figure 10 below.

As expected we found a wide range of different gene conversion events. We observed both unidirectional gene conversion, which is likely the result of single strand invasion and synthesis followed by annealing, as well as bidirectional gene conversion most likely resulting from double stranded invasion. We also observed a wide range of gene conversion tract lengths - ranging from less than 70 bp on each side to the limits of experimental detection (~300 bp on each side). The most immediately striking trend to this data is the apparent preference for gene conversion to the right side of the break rather than the left. To assay this quantitatively, we compared the number events reaching a certain polymorphism on the left to the number reaching an equidistant polymorphism on the right. We found that for all 5 sets of equidistant polymorphisms (-71/82, -158/133, -210/205, -285/269, -335/319), there was a significantly greater number of repair events reaching that point to the right of the break than to the left.

This observation may be due to the geometry of the DR-White construct. In order to get gene conversion to the right, the left end of the break must invade the truncated white gene which is to the left of the break on the same chromosome. On the other hand, in order to get gene conversion to the left, the right end of the break would have to fold back and invade the truncated white gene on the same side of the break, which may a less probable event. If repair occurs during G2 or S-phase it is also possible that the sister chromatid is used for repair; however the preference for gene conversion to the right suggests that intrachromosomal HR is responsible for at least a significant portion of the observed HR events.

**Figure 10: Gene conversion tract lengths during repair of an I-SceI induced double strand break in the presence and absence of Rev1**




*The impact of loss of Rev1 function on gene conversion tract lengths*

Due to the increase in synthesis tract lengths observed in the absence of Rev1 in the *P{w$^a$}* assay we asked whether there would be a similar increase in gene conversion tract (GCT)

lengths during repair of the I-SceI induced break. As is illustrated in Figure 10 there is no clear increase in GCT lengths in the Rev1 mutants. There are, however, some interesting trends to take note of. Although there is no statistically significant difference between Rev1 heterozygotes and homozygotes to the left of the break, there appears to be a trend towards increased synthesis in the Rev1 mutants, consistent with *P{w$^a$}* results. On the right side of the break, however, the trend appears to be the opposite with Rev1 mutants showing a decrease in GCT length relative to wild type. It's important to note, however, that with the current sample size the only statistically significant difference is the G->C polymorphism 133 bp to the right of the break ($P < 0.05$, Fisher's exact test). Additionally, about 50% of the GCT tract extended to at least the limit of detection of this assay, making these results difficult to interpret.

**Discussion**

Rev1 knockout causes a severe defect in oogenesis or embryogenesis

Homologous recombination has been the subject of intensive study for several decades due to its essential function in DNA repair and maintenance of genomic stability. Although the general mechanism of HR has been fairly well characterized over the past several years, the specific proteins that are involved are not fully known to date. Considering that certain defects in homologous recombination proteins are known to lead to cancer susceptibility - such as defects in the breast cancer susceptibility genes, BRCA1 and BRCA2 [37,38] - research on HR has potential implications in both the treatment and diagnosis of human cancers.

The data presented in this paper strongly supports a novel role for the Rev1 translesion polymerase in HR; however, due to the involvement of Rev1 in other cellular processes like TLS, some of the genetic data is difficult to interpret. When we first knocked out Rev1 we

observed a severe decrease in hatching frequency when homozygous males were crossed with homozygous females. This hatching defect was partially rescued by crossing precise excision males to Rev1 null females suggesting that the defect may be partially due to impaired oogenesis. Considering that meiotic recombination only occurs in female *Drosophila*, an attractive explanation is that in the absence of Rev1 meiotic recombination is impaired, resulting in a depressed hatching frequency. However, it is equally possible that the defect is not due to oogenesis, but rather a requirement for maternally loaded Rev1 during the first 2 hours of embryogenesis. One genetic approach to answering this question would be to knock out Mei-W68, the *Drosophila* orthologue of Spo11, in conjunction with Rev1. In the absence of Mei-W68 there can be no meiotic recombination, it should therefore rescue the Rev1 dependent defect if the issue is with meiotic recombination. Work is currently underway to construct and characterize Rev1, Mei-W68 double mutants.

If a defect in meiotic recombination is not responsible for the depressed hatching frequency observed in the Rev1 mutants, then it is not clear what is responsible. There are several possibilities, including a requirement for either DSB repair or translesion synthesis during early embryogenesis. It's difficult to gain much mechanistic insight using a purely genetic approach due to the fact that Rev1 is involved in both TLS and HR. One approach would be to knock out Spn-A, the *Drosophila* orthologue of Rad51, which knocks out HR entirely [34]. If it were Rev1's HR function that was causing the defect we would expect to see epistasis with Rad51, whereas we would expect an increase in the severity of the phenotype if the TLS role was responsible. Unfortunately, Spn-A knockout leads to female infertility due to defective meiotic recombination, however female infertility due to HR defects can be rescued by inactivation of Mei-W68 [39]. Taking the inverse approach of inactivating TLS and looking for

epistasis would be more difficult as there are no components of the TLS pathway that couldn't feasibly be involved in HR.

The efficiency of gap repair through HR is increased in the absence of Rev1

The first indications of the Rev1's involvement in HR came from the $P\{w^a\}$ assay where we observed a significant decrease in aborted homologous recombination as well as an increase in DNA synthesis in cases where homologous recombination did not proceed to completion. In conjunction with the results of Kane et al. [13], who found similar changes in HR in a DNA polymerase η and ζ double mutant as well as in a mutant carrying a transposon interrupted Rev1 allele, initially led us to propose a model where Rev1 was one of the first enzymes to bind the 3' terminus of the invading strand where it then recruits polymerases η and/or ζ. Importantly, we propose that when Rev1 binds it prevents extension by non-TLS polymerases such as pol δ. This explains the increase in DNA synthesis in the absence of Rev1, as pol δ would be able to bind more readily resulting in synthesis that is generally more processive.

This of course, raises the question of why Rev1 and TLS polymerases would ever be involved in HR. Polymerase δ is not only more processive; it also synthesizes DNA with higher fidelity resulting in a lower rate of mutagenesis. Although we didn't look at mutagenesis during HR in this study we would expect a decreased rate of HR associated mutagenesis in the Rev1 mutants. A future goal of the lab is to develop an assay that will allow us to quantify rates of mutagenesis during homologous recombination - which could give us more insight into the mechanisms of HR. An intuitive explanation for the involvement of error prone TLS polymerases is to deal with damaged bases surrounding the break. This is especially appealing because polymerase ζ is specialized to extend from damaged or mismatched primer termini

which would be necessary if the invading strand was damaged. Although there is presumably no base damage in our system, it's possible that TLS polymerases are recruited preemptively and used regardless of whether base damage is present. Recent work has also shown that Rev1 and pol ζ are necessary for bypassing not only damaged bases, but also unusual DNA structures such as hairpins [12]. It's possible that these structures form more readily in a D-loop due to the strand exchange reaction, or that the D-loop structure itself is less amenable to replicative polymerases due to its 3 stranded nature.

Another interesting possibility has to do with the ease of assembling the required protein components at the D-loop. *In vitro* experiments have shown that TLS polymerase η is capable of extending from an artificial D-loop substrate in the absence of any additional factors, whereas polymerase δ and ι cannot. More recent experiments using human proteins have found that pols δ, ι, and κ are also capable of extending an artificial D-loop substrate, however they require the sliding clamp, PCNA [40,41]. PCNA works by encircling the DNA strand and binding to the polymerase, tethering it to its template. In order for PCNA to encircle the DNA strand it needs to be loaded by a clamp loading complex - making it a rather complex and energy intensive process. In cases where high processivity is not required, such as DSB repair, it's possible that the cell opts for the simpler non-PCNA dependent synthesis pathway. *In vitro* D-loop extension studies have not yet been performed using Rev1 and pol ζ, so it is unknown whether or not they depend on PCNA for synthesis. This has been due to the difficulty in purifying these large, multi-subunit proteins; however, purification of the 4 subunit DNA polymerase ζ complex has just recently been reported, making *in vitro* studies feasible for the first time [42].

Loss of Rev1 results in a decrease in HR repair of a simple endonuclease induced break

There are several features of the *P{w$^a$}* assay that make it a less than ideal system to study double strand break repair. First of all, excision of the P transposable element leaves a very large gap of approximately 14 kilobases; which is not a type of damage a cell has to deal with routinely. Additionally, it's unclear how often gametocytes opt for apoptosis upon failure to properly repair the break, making it difficult to use the results quantitatively. The DR-White assay provides an approach that somewhat addresses these concerns. First of all, I-SceI induces a simple DSB by cutting both strands of the DNA without deleting any sequence. Unlike the *P{w$^a$}* induced break which requires extensive homology directed DNA synthesis to avoid a large deletion -- the DR-White break can be efficiently repaired through either HR or NHEJ allowing us to observe the relative contribution of both pathways. Due to the ease of repairing a single endonuclease induced break, we would not expect damage induced apoptosis to occur at any significant frequency.

We expected that the DR-White assay would give us results that reflected the *P{w$^a$}* data, in accordance with our model of polymerase competition at the D-loop. In that regard, the results were somewhat surprising in that we observed an increase in white-eyed events and a decrease in red eyed events in Rev1 null mutants; indicative of increased rates of NHEJ and decreased HR. At first this may seem to contradict the apparent increase in efficiency of HR observed in the *P{w$^a$}* assay, however there are several critical considerations to take into account. First of all, in order for a repair event to be scored as a successful in the *P{w$^a$}* assay, extensive DNA synthesis, of around 4.5 kb is required; in the DR-White assay a repair event can be scored as successful HR without extensive synthesis. Additionally, although error-free end-joining is impossible following resection and commitment to HR; error prone Alt-EJ is still

possible and may be more favorable in the DR-White system where it would not lead to such an extensive deletion.

With these considerations in mind, the DR-White results are consistent with the proposed model of polymerase competition. In the absence of Rev1, TLS polymerases such as η and ζ cannot be efficiently recruited, leaving polymerase δ and potentially other PCNA dependent polymerases to extend the invaded strand. Due to extra time and energy required to load PCNA, there is more time for the D-loop to be dissolved before processive synthesis can begin, leaving more opportunities for alternative end-joining to occur. This model is relatively straightforward to test, as we would expect an increase in alternative end-joining, but no change in classical end-joining; which would be easily observable by sequencing repair junctions from a representative sample of white-eyed flies.

Repair of an I-SceI induced break in the DR-White system favors gene conversion to the right and this bias is diminished in Rev1 mutants

Another prediction from our model is that in cases where we do get repair through HR, the gene conversion tract lengths should be longer on average; due to the use of more processive polymerases. When we used the DR-White.mu construct to monitor gene conversion tract lengths we did not see the straightforward trend that we expected - in fact we observed a significant decrease in gene conversion at 133 basepairs to the right of the break. The most obvious trend, however, is the very large increase in GCT lengths to the right of the break as opposed to the left. This is most likely due to the geometry of the DNA molecule during intramolecular strand invasion and D-loop formation. In the case of left strand invasion, resulting in GC to the right, the invading molecule is not covalently connected to the strand

containing the homologous sequence due to the I-SceI induced break. For all intents and purposes, left-stranded invasion is an intermolecular invasion event. This is not the case for right-strand invasion; first of all, a much greater degree of DNA bending is required for the right strand to invade the downstream homology. Additionally, the invading strand is topologically linked to the homologous template; therefore negative supercoils generated behind the D-loop will be transferred to the invading strand. Both the increase in DNA bending as well as the increase in negative supercoiling would act to destabilize the D-loop; explaining the decrease in GCT length to the left.

The more interesting trend is the fact that the right-side bias seems to be less severe in the Rev1 null mutants. In fact, aside from the closest pair of equidistant polymorphisms (-71 G->T & 82 C->G) there is no significant bias for GC on the right in Rev1 mutants. This makes sense given the proposed model where processive PCNA dependent synthesis occurs more often in the absence of Rev1. Although it would take more time and energy to load PCNA, once PCNA has been loaded and processive synthesis begins, it is expected to form a much more stable structure that is more resistant to passive D-loop unwinding due to forces generated by DNA structures. On the other hand, the processive D-loop would not necessarily be more resistant to active D-loop unwinding, catalyzed by enzymes such as the Bloom syndrome helicase, explaining why there is no general increase in GCT lengths in the Rev1 mutants.

It would be interesting to look at BLM mutants in this assay both in isolation and in conjunction with Rev1 mutation; if this model were to hold true we would expect an increase in GCT length in the BLM single mutant, but an even greater increase in GCT length in the double mutant. It's also possible that we are simply unable to observe an increase in overall GCT length at these short distances. In the *P{w$^a$}* assay we observed no significant difference in synthesis

tract lengths until 2.4 kb of synthesis. Although we cannot observe GC at that length due to the length covered by our polymorphisms, we can observe up to ~1000 bp of GC on either side of the I-SceI break and plan to do so in the near future.

Future directions and implications of this research

There are several other experiments which could provide evidence to either support or refute our model. A critical component of our model is that in the absence of Rev1, extension of the invaded strand is dependent on PCNA. Genetic studies of PCNA are difficult, due to the fact that it is absolutely essential for DNA replication. In *S. cerevisiae* there exist several temperature sensitive alleles of PCNA as well as of the RFC clamp loader, including the originally *cdc44-1* cold sensitive allele, which encodes the large subunit of RFC [43]. It's unclear what role, if any, TLS polymerases play in HR in *S. cerevisiae*; however, assuming that a similar system is at play, these temperature sensitive alleles may be useful to test our model. Perhaps a more promising way to look at PCNA involvement would be to overexpress the *Drosophila* RFC1 orthologue, Gnf1. In mice it was shown that overexpressing this subunit of RFC accelerated cellular proliferation, likely due to an increased rate of PCNA loading during replication [44]. Our model predicts that with an increased rate of PCNA loading, the decrease in HR repair observed in Rev1 mutants in the DR-White assay should be less severe.

Aside from investigating the role of PCNA in our observations, it will be very interesting to further investigate the mechanism of action of Rev1 during normal HR. In order to address this we are currently in the process of creating several different lines of flies with various mutations in the Rev1 gene. One of our main hypotheses is that the catalytic activity of Rev1 is not essential for its role in HR, and that its principle role is in recruiting other TLS polymerases;

to that end we have constructed a catalytic dead Rev1 mutant that we will assay using the techniques discussed in this paper. We are also investigating the roles of the BRCT domain and ubiquitin binding motif (UBM) of Rev1, which are critical for its TLS role by mediating the interaction with PCNA in its unmodified and monoubiquitylated form, respectively [45,46]. As discussed earlier, the role of PCNA and PCNA modifications in HR is a largely unexplored field; however, in accordance with our model we would predict no effect on HR in either of these mutants.

Although there are many questions that remain to be answered about the specific mechanism of Rev1's involvement, we have presented here several experiments that support a novel role for Rev1 in HR. In the absence of Rev1, both large double strand DNA gaps as well as simple endonuclease induced breaks are repaired differently. In the case of simple DSBs, which can be caused physiologically by ionizing radiation, replication fork collapse and other damaging agents, repair through HR is impaired in the absence of Rev1. Rev1 has been studied as a potential therapeutic target due to its role in tolerating chemotherapy induced damage [47]. If the results presented in this paper translate to human DSB repair, which is likely considering HR is a very conserved process between *Drosophila* and humans, we would expect Rev1 inhibition to combat resistance to both base damaging therapeutics and DSB inducing agents. Additionally, although it's not clear what impact Rev1 mutation would have on human patients, a Rev1 deficient tumor may be specifically targetable by DSB inducing agents such as radiation and topotecan. Identification and characterization of genes that similarly sensitize cells to specific types of damage will contribute to the development of genome based personalized medicine.

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