DNA DAMAGE RESPONSE TO LESIONS INVOLVING BOTH STRANDS OF THE DOUBLE-HELIX

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology

Chapel Hill
2008

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ABSTRACT

Sushmita Mukherjee: DNA damage response to lesions involving both strands of the double-helix.
(Under the direction of Jeff Sekelsky)

DNA damage response is vital to genome maintenance, cell survival and successful transmission of genetic information to daughter cells. This response is extremely important since DNA is subject to damage daily either by endogenous metabolic errors and byproducts or by exposure to genotoxic agents. Different types of lesions are formed as a result of such insults to the DNA; the most toxic of such lesions are those that affect both strands of the double-helix.

During my dissertation work, I studied cellular response to DNA lesions such as double-strand breaks and interstrand crosslinks using the model system *Drosophila melanogaster*. Double-strand breaks are repaired primarily by two mechanisms: homology mediated repair (HR) and nonhomologous end joining (NHEJ). Here I discuss the importance of homology mediated repair by studying repair defects in mutants defective for either of the two genes: 1) *nbs* gene encodes for the protein Nibrin, which is part of a well characterized protein complex MRN, comprising two other proteins Mre11 and Rad50 2) *okra* encodes the *Drosophila* homolog of the Rad54 protein. While the MRN complex is hypothesized to be required during early steps of HR such as break resection, Rad54 is believed to be involved in chromatin remodeling and facilitating the role of the strand invasion protein, Rad51. I have addressed several questions here about the function of

MRN in responding to double-strand breaks, using mutations in the *nbs* gene. Since the NBS protein is known to target the MRN complex to the nucleus, study of NBS in isolation should be reflective of the nuclear function of the MRN complex. The requirement of MRN for NHEJ and /or HR appears to differ in different organism. I found that *Drosophila* NBS is required for HR and not NHEJ. In addition, I found that in contrast to other studies, MRN may function in late steps of HR, post break resection in *Drosophila*. Study of defects in responding to DNA damage, specifically double-strand breaks (DSBs), in haploinsufficient *nbs* mutant backgrounds provided valuable clues into underlying molecular mechanisms that lead to carcinogenesis in human carriers of *nbs* mutation.

I tested to verify if DmRad54 is functionally conserved. This study showed that not only does DmRad54 facilitate DmRad51 function during first round of strand invasion, but it is also required multiple times while repairing the break, during the several rounds of strand invasion and synthesis that is characteristic of HR in pre-meiotic germline cells in *Drosophila*.

The second type of toxic lesion discussed here are the interstrand crosslinks (ICLs). Multiple repair mechanisms integrate to repair interstrand crosslinks in the bacteria *Escherichia coli* and the budding yeast *Saccharomyces cerevisiae*. Nucleotide excision repair (NER) and HR proteins are required for ICL repair, among others. Also, since DSB intermediates are formed while resolving ICLs, HR proteins seem to be integral in responding to crosslinks. I tested mutants defective in two genes, *mus301* and *mus302*, both of which are hypersensitive to crosslinking agents, for defects in DSB repair. I found that while *mus302* mutants, which have previously been implicated in NER, can repair double-strand breaks normally; *mus301* mutants are severely defective in HR, when the only available homologous template for repair is the sister chromatid.

ACKNOWLEDGEMENTS

At the outset I would like to thank my advisor, Jeff Sekelsky for his constant support and encouragement during the last five years. None of this would be possible without his academic contribution and support during my graduate career. I would also like to thank some of my undergraduate and graduate professors Dr. Rajesh Gokhale, Dr. Pradeep K. Chatterjee, Dr. L.K. Sengupta, Dr. S. Sengupta, Dr. Nwosu and Dr. Harewood, for instilling in me a passion for science. I would like to thank my best critiques, the Sekelsky lab former and current members Susan Cheek, Mitch McVey, Hunter Blanton, Sarah Radford, Jeannine LaRocque, Kirsten Trowbridge, Sabrina Andersen, Matt LaFave, Kathryn Kohl and Dan Bergstralh for contributing to a wonderful working environment conducive for constant growth at both personal and professional level. A special thanks to Paul Brewer-Jensen and the Searles lab for their help and support on the RNA work carried out in *nbs* mutants and also Robert Duronio and his lab for their useful tips and comments on my project during lab meetings. I am grateful to my committee Lillie Searles, Dale Ramsden, Shawn Ahmed and Steve Matson, for their time and input in my work and for helping me move from one step to another in graduate school. I want to thank my friends Abhi, Nino, Avani and Sonali for making my stay in NC memorable. I would like to extend a special thanks to Rakhi and her parents for making me a home away from home. Last but not the least I would like to thank my family: my sister, Supriti who has played a central role in my personal and professional success; my mother for her love and encouragement; my father and my in laws for their support; my nieces, Arushi and Jayoshi for bringing joy into my life; and my loving husband, Pratibhash for constantly supporting me and being by my side through thick and thin.

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1.2 LIST OF ABBREVIATIONS AND *DROSOPHILA* GENE NAMES

IR:	Ionizing radiation
UV:	Ultraviolet
DSB:	Double-strand break
ICL:	Interstrand crosslink
MMR:	mismatch repair
NER:	Nucleotide-excision repair
NHEJ:	Non-homologous end joining
HR:	Homologous recombination
SDSA:	Synthesis-dependent strand annealing
SSA:	Single-strand annealing
NBS:	Nimegan Breakage Syndrome or Nibrin protein
DmBlm:	*Drosophila* Blm homolog (encoded by *mus309*)
DmRad51:	*Drosophila* Rad51 homolog (encoded by *spnA*)

CHAPTER I

GENERAL INTRODUCTION

The most vital component of cellular function is a long but simple polymer made of nucleotides, which resides in the nucleus and mitochondrial compartments of our cells. Structurally, this polymer is held by a backbone made of sugars and phosphate groups that are joined by ester bonds. This polymer, known as 'DNA' or deoxyribose nucleic acid, is compacted with the help of histone proteins, thus enabling packaging as compact units called chromosomes into a tiny nucleus of radius 10^{-5} m. Held as such our genome can retain information for all bodily functions stably over long periods of time. However, even though DNA is vital to survival, it is subject to damage daily by endogenous metabolic process errors and attack by metabolic by-products or exposure to exogenous genotoxic agents.

Sources and Types of DNA damage

Endogenous sources

Myriad metabolic processes occur daily in our cells. By-products of such processes can react with either the nucleotides or the sugar phosphate backbone of DNA: a) Hydrolytic and oxidative attack can lead to spontaneous deamination of nucleotide bases which alters base-pairing (Lindahl,1974) b) Breakage of the N-glycosyl bond can lead to loss of purine and pyrimidine bases from DNA c) Reactive oxygen species (ROS) which are by-products of cellular respiration, are highly toxic and can lead to oxidative damage of bases and ultimately result in ageing and cancer (Reviewed in Friedberg et al., 2006). d) Nitrous acid

produced from dietary nitrites under acidic conditions can lead to formation of DNA cross-links (Kirchner et al., 1991 and Edfeldt et al., 2004). It is believed that for every four deaminations caused by nitrous acid one interstrand crosslink (ICL) is produced (Becker et al., 1964 and Reviewed in Friedberg et al., 2006). Additionally errors during processes such as replication, transcription and translation could lead to DNA damage at low frequency. Such damage is detected by surveillance mechanisms and removed by DNA repair machinery. High frequency of such damage can occur when proteins required for surveillance or repair processes are either non-functional or partially functional.

Exogenous sources:

a) Ionizing radiation (IR) can cause a variety of DNA lesions. Naturally occurring cosmic radiation is a source of IR and can make clusters of hydroxyl radicals which react with bases and leads to damage of the sugar-phosphate backbone and thereby cause double-strand break formation (Hutchinson, 1985, Frankenberg-Schwager, 1990 and Price, 1993). b) Solar UV radiation is toxic to DNA and frequently causes formation of cyclobutane.pyrimidine dimers. Other by-products of UV damage are single-strand breaks, alkali-sensitive lesions and photo-adducts (Peak, 1987). c) Chemotherapeutics and the use of drugs affect DNA integrity; for example: use of nitrogen mustard causes formation of cross-links in the DNA. d) By-products of biomass burning and biological synthesis by microorganisms and micro-algae, produces alkylating agents such as methyl chloride which can be highly toxic.

Stable, inheritable changes in DNA: a boon and a curse

A heritable change in DNA is called “mutation”. Mutation can be both beneficial and detrimental. Dobzhansky defined mutation as “the only known source of new materials of genetic variability, and hence of evolution” (Dobzhansky, 1957). The nobel laureate, John C.

Kendrew described the detrimental aspect of mutation as follows “Just as in a book misprints are more likely to produce nonsense than better sense, so mutations will almost always be deleterious, almost always, in fact, they will kill the organism or the cell, often at so early a stage in its existence that we do not even realize it ever came into being at all” (Kendrew, 1966). Since the majority of mutations are deleterious, cells have evolved multiple ways to preserve genome integrity. Successful genome maintenance ensures high-fidelity transmission of genetic material to daughter cells.

Corrective mechanisms for combating DNA damage and maintaining genome stability

Cells have developed multiple repair systems to respond to chemical changes. Diverse protein networks work independently or in conjunction to carry out various repair mechanisms namely ‘Nucleotide excision repair or NER’ ‘Base-excision repair or BER’ ‘Mistmatch repair or MMR’ ‘Double-strand break repair’ and ‘Inter-strand crosslink repair’ to correct different types of damage. Oxidative damage is detoxified with the help of specialized enzymes. Loss of function of these enzymes or reduced activity leads to cellular senescence, tumorigenesis, or apoptosis (Reviewed in Friedberg et al., 2006). Damage surveillance includes i) sensor proteins that detect the damage and activate downstream transducers ii) activation of checkpoint kinases like ATM and ATR, arrests the cell cycle and allows time for the repair proteins to correct the damage (Weinert,1998) iii) proof-reading polymerases proof-read during replication (Reviewed in Lewin, 2000).

Clinical implications of unrepaired or mis-repaired DNA damage

Failure of this highly regulated genome surveillance and repair system results in diseases which are mostly accompanied by mutagen sensitivity and tumorigenesis (Chu, 1997, Vilenchik, 2003 and Helleday, 2007). Based on the ability to detect DNA damage underlying these diseases, aberrations can be broadly classified in two 1) aberrations that

are detected in karyotypes are at the chromosomal level. These aberrations can be visualized as either an increase or decrease in chromosome number. Inter-chromosomal translocations can also be detected. Several such aberrations have been associated with human diseases. 2) Mutation in a specific genetic locus is difficult to detect. If the mutation is dominant, it manifests itself in the first generation. Recessive mutations are rare and are manifested by chance, if the sperm and egg donors both carry the same mutation or different mutations in the same gene. Such mutations in several cases have been associated with human diseases such as Blooms syndrome, Ataxia telangiectasia and Nijmegan Breakage syndrome among others. Mutations in all the three genetic disorders mentioned here affect genes involved in DNA damage response to double-strand breaks. In this chapter I review mechanisms of repairing double-strand breaks and interstrand crosslinks.

DNA damage response to complex lesions involving both strands of the double-helix

DSBs and ICLs affect both strands of DNA and are thus extremely toxic if unrepaired and detrimental if mis-repaired. While ICL repair is believed to require repair proteins involved in different repair mechanisms such as NER, MMR and HR (Bergstralh et al., 2007, Figure 1.4); work to date has identified two primary mechanisms for repairing DSBs. One mechanism makes use of information from a homologous template (homologous recombination or HR) (Figure 1.1); whereas the other involves joining broken ends with little or no use of homology (non-homologous end joining or NHEJ) (Figure 1.2). Cells compromised for proteins required for either of these repair pathways manifest genome instability, which in mammals can lead to cancer (Chu, 1997, Vilenchik, 2003 and Helleday, 2007).

Double-strand break repair

Double-strand break formation outside of meiosis can be detrimental. During meiosis however, programmed DSBs are induced to facilitate recombination. Evidently most of the proteins required in meiotic recombination are also required to repair DSBs which are formed as a result of endogenous metabolic errors or exposure to mutagens. Here, I discuss mechanisms of repairing such DSBs.

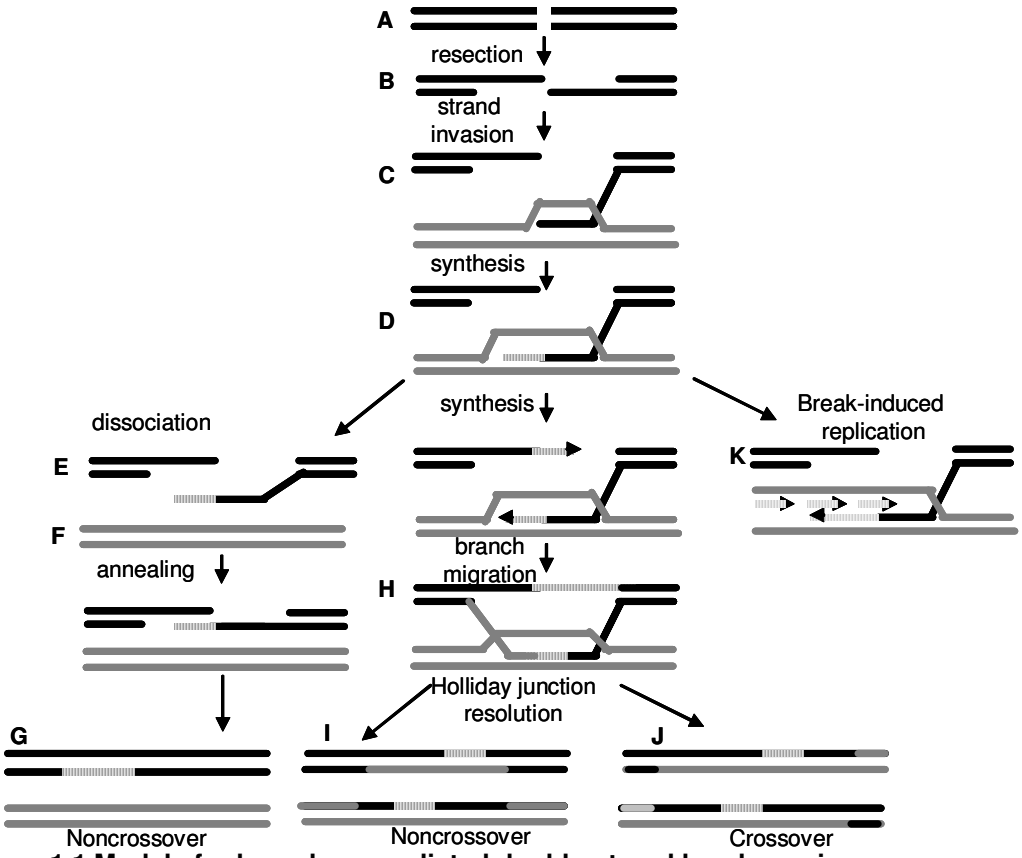


Figure 1.1 Models for homology-mediated double-strand break repair
Double-strand breaks (A) are processed and resected in the 5'-3' direction (B) to generate 3' single-stranded recombinogenic intermediates. Strand invasion proteins bind this single-stranded DNA to initiate homology search and invade a homologous template (C). This step is followed by synthesis using information from the homologous template (D). This intermediate can dissociate from the template (E) and anneal (F) at a complementary region to generate a gene conversion noncrossover product (G). This model is called Synthesis Dependent Strand Annealing (SDSA). The synthesis step (D) can also be followed by second end capture leading to the formation of a double Holliday junction (DHJ) (H). The DHJ can be resolved in two different ways to generate two types of products, noncrossover (I) and crossover (J). This is called the Holliday junction resolution model. The synthesis step (D) can also lead to the formation of a replication fork and synthesis can generate long gene conversion tracts. This model is known as the Break-induced replication (K).

Double-strand break repair via homologous recombination

Double-strand breaks can be repaired accurately by copying sequence from a template carrying homologous sequence. Such a homologous template can be provided by the sister-chromatid, homologous chromosome or an ectopic site on the same or a different chromosome. There are several models proposed to explain homologous recombination (Figure 1.1). All these models are based on an early requirement of 5'-3' resection of DSBs to generate a 3' single-stranded recombinogenic region.

This single-stranded region is coated by strand-invasion proteins to mediate homology search and invasion of a homologous template. In one of the models called Break-Induced Replication (BIR), evidence for which is found in *Saccharomyces cerevisiae* (Haber, 1999), the invading strand establishes a replication fork and synthesis of long stretches of DNA takes place, thus generating long gene conversion tracts (Reviewed in Friedberg et al., 2006). An alternate model, called the Synthesis Dependent Strand Annealing (SDSA) is based on one or two-ended strand invasion, followed by synthesis and annealing at short stretches of complementary sequences leading to shorter gene conversion tracts (Szostak, 1983) than those generated as a result of BIR. The third model called Holliday junction (HJ) resolution model (Holliday, 1964) has been borrowed from the meiotic recombination model. According to this model a two-ended strand invasion is followed by synthesis and ligation to generate a four-stranded junction known as double Holliday junction (DHJ) (Figure 1.1). This intermediate can be resolved by cutting at two junctions. The decision of where this cutting takes place determines whether the repair product will be a gene conversion associated with or without a crossover. In this thesis I discuss the SDSA model extensively.

Early steps of HR: the MRN complex and DmRad54

Early steps of homology mediated DSB repair pathway such as resection (Maryon & Carrol, 1989 and Sugawara and Haber, 1992) to generate 3' single-stranded regions is suggested to require the conserved protein complex MRN, comprising MRE11, RAD50, and NBS/NBN (Tauchi et al., 2002 and van den Bosch et al., 2003). Following resection, the single-stranded DNA is coated by the strand invasion protein Rad51 which mediates homology search. This function is facilitated by the Rad54 protein, which interacts with Rad51 (Clever et al., 1997; Petukhova et al., 1998; Petukhova et al., 1999).

DNA damage response and MRN

Earlier studies have demonstrated that functional knock-outs of the MRN genes cause lethality in metazoans (Kang, 2002, Frappart, 2004 and Ciapponi, 2006). While lethality illustrates the importance of these genes, it also poses challenges for *in vivo* studies of their molecular functions. Nonetheless, substantial progress has been made in the characterization of the MRN complex.

The MRE11 subunit is an exonuclease (Paull, 1998). Studies in *S. cerevisiae* have demonstrated that MRE11 is the only exonuclease required during repair of meiotic DSBs; however, redundancy in the exonuclease function has been found in DSB repair in mitotic cells (Moreau et al., 2001, Liu et al., 2003, Nakada et al., 2004 and Lewis et al., 2004). The RAD50 subunit has important enzymatic and structural functions, including ATPase activity and DNA bridging activity, both of which are required for DSB repair (Hopfner et al., 2000, Hopfner et al., 2001 and Hopfner et al., 2002). NBS/NBN is the major regulator of the complex (Paull et al., 1999, Komatsu, 2007). This protein has a nuclear-localization signal (NLS) and in its absence, Mre11 and Rad50 remain in the cytoplasm even in the presence of DNA damage (Desai-Mehta et al., 2001, Tseng et al., 2005 and Ciapponi et al., 2006).

For this reason, functional studies carried out in *nbs* mutants can be used to understand the nuclear function of the complex as a whole.

In vitro studies have demonstrated an exonuclease activity of the Mre11 protein with 3'-5' polarity (Paull et al., 1998). However, reverse polarity of the exonuclease activity of Mre11 is required *in vivo* to give rise to the hypothesized intermediates with 3' overhangs (Figure1.1). It has been suggested that coupling of endonuclease and exonuclease functions of Mre11 may result in such 3' overhangs *in vivo* (Paull et al., 1998). In yeast at least two other nucleases have been suggested to have redundant functions with Mre11 (Lam et al., 2008). Also, it has been found that in yeast although MRX (Xrs2 is the yeast functional counterpart of NBS) is required for HR, the nuclease function of Mre11 is not essential for this (Bressan et al., 1999).

MRX has also been implicated in NHEJ (Chen et al., 2001; Huang, 2002). The precise contribution of the MRN/MRX complex to HR and/or NHEJ appears to vary in different organisms (Bressan, 1999, Chen, 2001, Huang, 2002, Tauchi, 2002 and Di Virgilio, 2005). Several independent studies in *S. cerevisiae* showed that the MRX complex is required for NHEJ (Moore et al., 1996, Boulton et al., 1998, Chen et al., 2001 and Palmbos et al., 2005). Likewise, studies carried out using HeLa cell extracts demonstrated that the MRN complex is an important NHEJ component (Huang et al., 2002). However, genetic experiments in the fission yeast *Schizosaccharomyces pombe* and using cell-free extracts from *Xenopus laevis* and the DT40 chicken cell lines failed to find a requirement for the MRN complex in NHEJ (Manolis et al., 2001, Tauchi et al., 2002 and Di Virgilio et al., 2005).

With the exception of use of a particular Mre11 mutant allele in yeast, which is defective in repair of DSBs in both meiotic and mitotic cells (Tsubouchi et al., 1998), other studies demonstrated that the yeast MRX complex is required for repair of programmed DSBs by HR in meiotic cells but not in mitotic cells (Ivanov et al., 1994 and Moreau et al., 2001).

Clinical implications of mutation in MRN

Mutations in *MRE11* in humans cause ataxia telangiectasia-like syndrome (ATLD) (Stewart et al., 1999). No genetic disorders have been associated with mutation in *RAD50* in humans, but mouse *rad50* mutants exhibit cancer predisposition and hematopoetic failure (Bender et al., 2002). Specific mutations in human *NBN* that allow expression of a partially functional protein cause Nijmegen Breakage Syndrome (Varon et al., 1998, Carney etal., 1998 and Maser et al., 2001). Clinical symptoms manifested by these patients include microcephaly, immunodeficiency, and lymphorecticular malignancies. Key cellular features of this disease are hypersensitivity to IR, telomere fusions and defects in DNA damage responses. In the Czech Republic, Ukraine, and Poland, the frequency of heterozygous carriers of *NBN* mutations is as high as 1 in 150 to 1 in 190 (Varon et al., 2000). Linkage studies have shown that carriers are predisposed to various cancers, and their cells show gross genome instability (Seemanova 1990, Varon et al., 2000, Dumon-Jones et al., 2003 and Tanzarella et al., 2003). Similarly, $nbn^{+/-}$ heterozygous mice are susceptible to tumor formation (Dumon-Jones et al., 2003). Recently it was demonstrated that activation of the checkpoint protein ATM was limited in heterozygous carrier cells treated with low dose ionizing radiation (IR) (Ebi et al., 2007).

Role of Rad54 in homology mediated repair

Rad54 protein belongs to the family of Swi2/Snf2 family of ATPases (Gorbalenya & Koonin 1993). It has been suggested that proteins Rad54 and Rad51 have interdependent functions. In *Saccharomyces cerevisiae*, it has been demonstrated that Rad54 stimutates the strand invasion function of Rad51 to generate a heterduplex (Clever et al., 1997; Petukhova et al., 1998; Petukhova et al., 1999). In turn, the Rad51 protein bound to the single-stranded DNA enhances DNA unwinding and chromatin remodeling functions of the Rad54 protein (Mazin et al., 2000 and Alexeev et al., 2003). *In vitro* studies showed that

chromatin content enhances strand pairing activity of Rad51 and Rad54 (Alexiadis & Kadonaga 1002). Although most studies point towards Rad54 being a facilitator of Rad51 function, recently it has been suggested that ATP dependent branch migration activity of this protein may be required for DSB repair (Bugreev et al., 2007).

Double-strand break repair via non homologous end joining

Direct end-to-end fusion of DSBs without the need of a homologous template is called non homologous end joining (NHEJ). In most instances processing of the ends is required prior to ligation. For example, breaks formed by reaction with IR have end residues such as phosphoglycolates which are not conducive for ligation (Reviewed in Friedberg et al., 2006). Processing reactions such as single-strand degradation, cleavage of loops or trimming of overhangs takes place prior to ligation. Another requirement for NHEJ is to hold together processed ends. Stable alignment of the broken single-stranded ends enables polymerases to fill in the gaps and ligases to perform the final joining to complete the repair process (Figure 1.2).

While canonical NHEJ in mammalian systems requires proteins such as Ku70-80 heterodimer, XRCC4, Ligase IV, DNA-PKcs and Artemis; error-prone alternative NHEJ can take place without these proteins (Ferguson and Alt, 2001; Lee et al., 2004; reviewed in Roth, 2003).

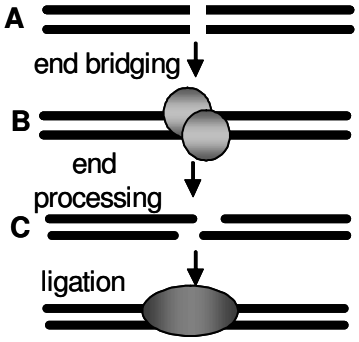


Figure 1.2 Non homologous end joining
Ends of double-strand breaks need to be aligned and processed to generate ends conducive for ligation.

Microhomologies of 5-10 bp may be used to establish the joining junction. Repair using such microhomologies is called Microhomology mediated end joining (MMEJ) (Roth,

1986). Use of homologies longer than 10 bp for repair is called single-strand annealing (SSA). This type of repair is currently classified under HR.

Double-strand break repair by single-strand annealing

Similar to the MMEJ pathway, when resection of DSBs reveals long regions of homology then without the need for strand invasion, annealing at these homologies can take place (Lin et al., 1984). This can be followed by trimming of intervening non-complementary sequences which leads to repair products that are associated with deletions (Figure 1.3). This type of repair is called Single Strand Annealing (SSA) (Lin et al.,1984; Maryon and Carrol, 1991 and Fishman-Lobell et al., 1992).

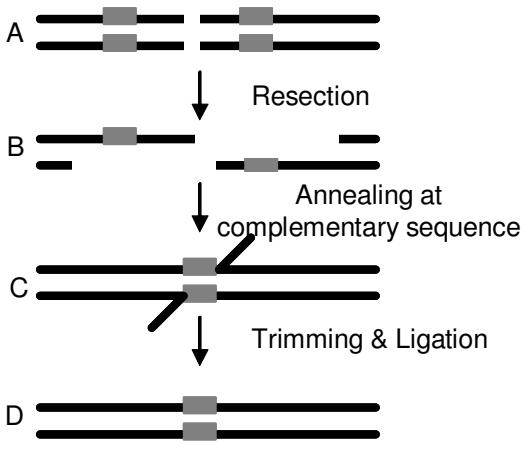


Figure 1.3 Single Strand Annealing
DSB (A) is resected to generate 3' single-stranded overhangs. Complementary sequences flanking the break are shown as grey boxes. Annealing at complementary sequences (B) followed by trimming of intervening sequences and ligation, results in the repair product D.

SSA and SDSA pathways both require Rad52 in yeast; however SSA is independent of the Rad51-Rad54 proteins that are essential in SDSA (Ivanov et al., 1996). It is not clear whether MMEJ and SSA require the same set of proteins or are mechanistically distinct from each other. Recent studies in yeast have shown that MMEJ is not a part of NHEJ but a subset of SSA and certain proteins like Sae2 and Tel1 which promote MMEJ inhibits NHEJ (Decottignies, 2007 and Lee &Lee 2007). More recently MMEJ has been defined as 'micro-SSA' (Decottignies 2007**).**

Interstrand crosslink repair

Interstrand crosslinks (ICLs) are covalent bonds between nucleophilic centers on opposite strands of DNA. Some of the sources of ICLs are alkylating agents such as nitrogen mustard and mitomycin C which are used in chemotherapy (Ali-Osman et al., 1995). Another known crosslinking agent, cisplatin, can form monoadducts, intrastrand crosslink or DNA protein crosslink in addition to interstrand DNA-DNA crosslink (Rudd et al., 1995 and Poklar et al., 1996). DNA Protein Crosslink Aldehydes such as crotonaldehyde found in tobacco smoke or automotive exhaust can also form interstrand crosslinks in DNA. Endogenous sources of ICLs are nitrous acid formed in the stomach by nitrites from dietary sources. Natural compounds found in plants such as Psoralens get activated in the presence of UV - A and can form monoadducts and diadducts or interstrand crosslinks (Wu et al., 2005).

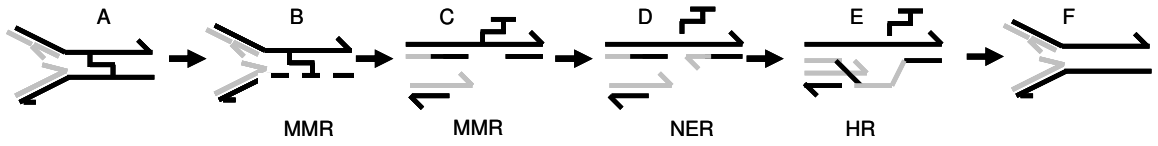


Figure 1.4 Interplay of different repair mechanisms to remove interstrand crosslinks. Interstrand crosslinks (A) blocks progress of replication fork. Mismatch repair (MMR) machinery can mediate unhooking of the crosslink (B,C). Nucleotide excision repair (NER) machinery can remove the unhooked crosslink (D). Homologous repair machinery (HR) may be used to repair the break and restore the replication fork (F). *Adopted from Bergstralh and Sekelsky, 2007.*

Previously it was believed that mechanisms for repairing ICLs have not evolved since spontaneous ICLs do not occur in the cell. Thus ICL agents were employed in chemotherapy to target cancer cells; however, recent evidence shows that spontaneous ICLs can occur in cells and this has been associated with premature ageing phenotypes (Niedernhofer et al., 2006), thus explaining the reason behind increased resistance to chemotherapy by ICL agents (reviewed in Bergstralh et al., 2007).

Integration of various repair mechanisms such as NER, MMR and HR has been suggested to be required to successfully remove ICLs (Reviewed in Friedberg, 2006 and Bergstralh and Sekelsky, 2007, Figure 1.4). Identification of mutants sensitive to crosslinking has lead to several models for ICL repair (Bergstralh and Sekelsky, 2007, Figure 1.4). While the role of HR in ICL repair seems indispensable, the role of certain NER proteins is debatable and has been discussed in details recently (Bergstralh and Sekelsky, 2007). In prokaryotic systems however, there seems to be two separate pathways for repair: one involving NER/HR and the other is a NER/DNA polymerase II dependent pathway (Berardini et al., 1999).

Thus far NER proteins have been implicated in removal of DNA lesions that result from damage induced by UV and IR. The steps required for NER in mammals include: recognition of the damage by proteins RPA, XPA, XPB, XPC-TFIIH, and XPD and incision on either side of the damage by XPF and XPG proteins followed by repair synthesis by DNA Pol δ , DNA Pol ε, PCNA, RPA, and RFC (reviewed in Sancar, 1995). In humans, mutations in the XP protein encoding genes result in diseases such as Xeroderma Pigmentosum (XP) and Trichothiodystrophy (TTD). Clinical manifestations of these diseases include photosensitivity, skin cancer, and neurological abnormalities (reviewed in Bohr et al., 1989; Bootsma, 1993; Robbins, 1988). Proteins required at various steps of HR as well as some diseases associated with mutations in HR genes in humans have been discussed above.

Drosophila melanogaster, a model system

Thomas H. Morgan, the Nobel laureate and father of Fly genetics, started breeding *Drosophila* as early as the 1900s. With the help of aspiring scientists A.H. Sturtevant, C.B. Bridges, and H.J. Muller in his laboratory he developed the chromosome theory of heredity which opened new doors for transmission genetics. Soon geneticists using other model organisms for their studies developed novel genetic tools enabling in-depth research.

Drosophila genetics at the time was lagging. The 1970s was a comeback for *Drosophila* genetics with the advent of novel reverse genetics tool. Currently, with plethora of genetic tools available for use in *Drosophila*, this model organism provides one of the finest systems to work with.

Advantages of using* Drosophila *as a model system

Maturation of *Drosophila* by metamorphosis allows *in vivo* study of this organism at various developmental stages. Mutagen sensitivity assays which allow us to screen for DNA repair related genes, can be carried out *in vivo* with relative ease (Henderson and Grigliatti, 1992). *In vivo* checkpoint assays (Brodsky et al., 2000) and DSB repair assays (Adams et al., 2003 and Rong and Golic 2003) have been developed and can be used in different mutant backgrounds, with the ease of phenotypic readouts which are as simple as nuclear signals on imaginal discs (highly proliferative tissue) in larvae or eye colors in adults. Reverse genetics tools for generating mutations such as by *P* element disruption (Reviewed in Adams and Sekelsky 2002) have been carried out to generate deletions, insertions, etc. These mutants are readily available at several global stock centers. In addition, mutagenesis screens using mutagens such as Ethyl Methane Sulphonate (EMS) have been used to generate point mutations (Laurencon et al., 2004). The mutations have been grouped into complementation groups based on sensitivity to mutagens. Mutants that are hypersensitive to certain mutagens, most likely carry mutations in genes encoding proteins required for DNA damage response to those mutagens. Collections of such mutations are readily available. Recombination mapping of these mutations can be carried out using phenotypic markers with known cytological positions, enlisted systematically in what is popularly known as the “red book” (Lindsley and Zimm, 1992). ‘Deficiency mapping’ based on mutagen sensitivity phenotypes of mutants may be carried out by crossing them to deficiencies or chromosomes carrying large deletions with known or approximately known molecular end-points (Thibault et al., 2004 and Parks et al., 2004). A user-

friendly *in silico* interface known as 'Flybase' (Ashburner, 1993 and Ashburner & Drysdale 1994) is available for sharing, documenting and organizing *Drosophila* research, stocks, resources, gene/RNA/protein sequence information, and references etc.

Study of DNA damage response in Drosophila

DNA damage response encompasses various different responses requiring separate sets of proteins. Study of intricacies of each response including damage repair is complex and beyond the scope of this work. I have attempted to understand DNA damage response to DSBs by studying two aspects of HR: 1) initiation of HR and 2) invasion of a homologous template to borrow information to fill in missing information on the damaged DNA. In addition, I have attempted to understand repair of DSB intermediates formed as a result of interstrand crosslink formation.

Homology mediated double strand break repair in Drosophila

Double-strand break repair has been studied in *Drosophila* by inducing breaks *in vivo* either by excising non-autonomous transposable *P* elements (Figure 1.5) or by generating breaks using the endonuclease, *I-SceI* (Adams et al., 2003, Bellaiche et al., 1999 and Rong and Golic 2003). While the former relies on crossing the *Drosophila* carrying the *P* element to one that carries the enzyme, transposase, required for excision; the latter is an endonuclease encoded by the budding yeast *Saccharomyces cerevisiae*. The *I-SceI* recognition site has been cloned and injected into *Drosophila* embryos to generate *Drosophila* transgenic for the recognition site. These transgenic *Drosophila* can be crossed to transgenic *Drosophila* carrying a heat-inducible *I-SceI* endonuclease. Heat shocking progeny from this cross generates breaks *in vivo*.

Research thus far has shown that DSBs in mitotic cells of the germline in *Drosophila* are primarily repaired by the homology mediated DSB repair pathway, SDSA (Figure 1.5) (Engels,

1990; Gloor, 1991; Flores, 2001 and Adams et al., 2003; Kurkulos et al., 1994 and Nassif et al., 1994).

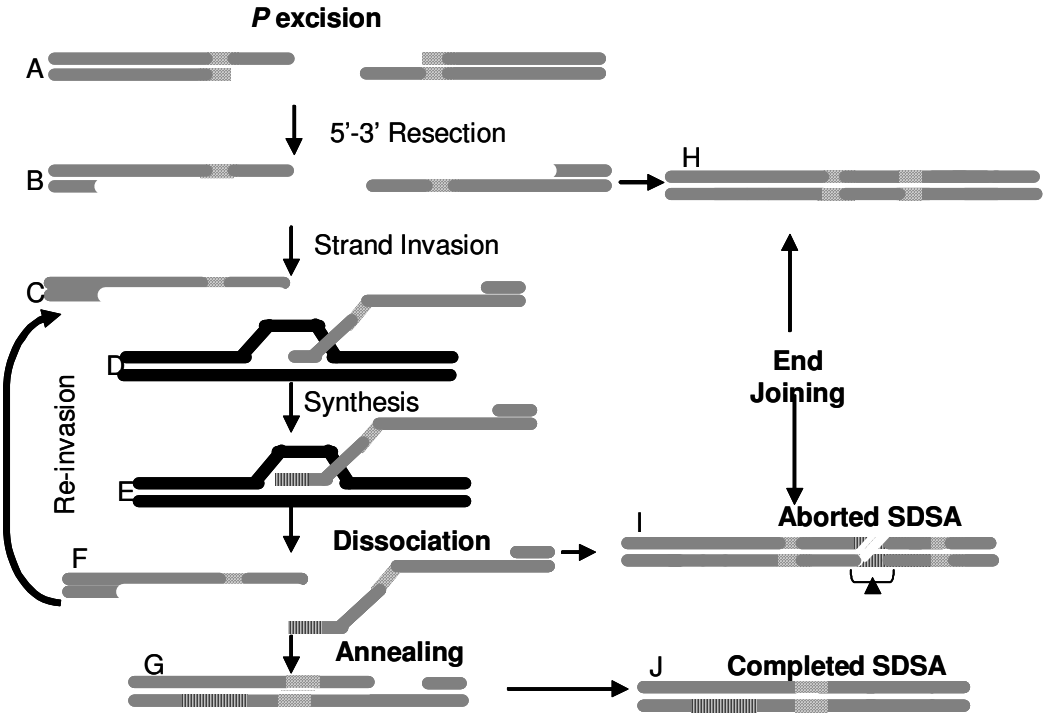


Figure 1.5 Model for repair of a *P*-element induced gap in *Drosophila*
Excision of a *P* element results in generation of breaks with staggered ends with 17 bp overhangs (A). Dotted regions that flank the gap on the double-stranded DNA are 8 bp target site duplication generated by insertion of the *P* element. B) 5'-3' resection results in long single-stranded region that can invade a homologous template to repair the damage by homologous recombination. Alternately, the ends can be joined with little or no use of homology (H). Homologous repair initiates by invading a homologous template (C) to copy sequence. Synthesis follows (D, E). This synthesis is not processive and the invading strand can dissociate (F) to either re-invade and synthesize or anneal (G) at complementary sequence. Failure to go through multiple rounds of synthesis can lead to aborting the SDSA pathway in favor of end-joining (I). Completing repair by SDSA leads to the repair product, J.

Initiation of HR of DSBs

According to DSB repair models discussed above the primary requirement for initiating HR is to resect the breaks. According to studies carried out in other model systems, proteins required for this step are: Mre11, Rad50 and Nbs comprising the MRN complex (discussed in details above). I used mutations in *Drosophila nbs* to verify if *Drosophila* NBS is required for this function as well. In this venture, I also studied other functions of this pleiotropic gene in DNA damage response.

To understand the role of *Drosophila* MRN in DSB repair, I assayed end joining and homologous repair simultaneously in an *in vivo* assay in mutants with reduced NBS function. I sequenced repair junctions to determine if *Drosophila* MRN like the yeast MRX (Paull and Gellert 2000), is required for microhomology-mediated end joining (MMEJ). I also tested if reducing the dosage of NBS causes defects in DNA damage response, to understand what underlies the cancer predisposition in human heterozygous carriers.

Invading a homologous template

The resection step during DSB repair generates single-stranded recombinogenic ends capable of invading a homologous template. Previously it was demonstrated using the *P*-element based assay (Adams et al., 2003) that SPN-A or *Drosophila* Rad51 is required for the strand invasion step during SDSA (McVey et al., 2004b). In yeast and other model organisms it has been demonstrated that Rad54 facilitates the strand invasion function of Rad51 to generate a heterduplex (Clever et al., 1997; Petukhova et al., 1998; Petukhova et al., 1999; Mazin et al., 2000; Alexiadis & Kadonaga, 2002; Alexeev et al., 2003; Kwon et al., 2007).

I tested DmRad54 (OKR) for this function using the same DSB repair assay as was previously used for testing DmRad51. The N-terminus of this protein is well conserved through *Drosophila*, mouse and humans and has been shown to be required for chromatin remodeling and facilitating strand invasion in *Drosophila* (Alexiadis et al., 2004). However, only one of the mutant alleles used in this study (based on the site of mutation) affects this conserved N-terminus (Ghabrial et al., 1998), due to lack of knowledge of this sequence conservation prior to my experiments.

Non homologous end joining in Drosophila

There is evidence for use of the NHEJ pathway for DSB repair in *Drosophila* (McVey et al., 2004a and Weinert et al., 2005) however, this pathway is used at a lesser frequency

than HR (Engels, 1990; Gloor, 1991; Flores, 2001; Adams et al., 2003; McVey et al., 2004b *Genetics* and McVey et al., 2004c), unlike in humans (Karran, 2000; Rothkamm et al., 2003; reviewed in Burma et al., 2006). Players in the NHEJ pathway in *Drosophila* such as Ku70 (Kusano etal., 2001), DmKu80 (Min et al., 2004) and DmLig4 (Gorski et al., 2003) have been identified. Studies have demonstrated that the NHEJ repair complex Ku70-80 is important for maintaining telomere lengths (Melnikova et al., 2005). DmBlm, the homolog of human Blm protein which has been found to play a role in HR (Adams et al., 2003), has been implicated in influencing recognition of breaks during NHEJ in a Ku-dependent way (Min et al., 2004). Several other components of the NHEJ machinery such as Artemis and DNA PKcs are either non-existent in *Drosophila* or have not been identified. NHEJ used for repairing *P* element induced breaks is *ligaseIV* independent (McVey et al., 2004a), unlike the canonical NHEJ in yeast and humans. Currently the protein requirements for this non-canonical NHEJ repair pathway are unknown. It is possible that non-canonical or alternative NHEJ which in humans is error prone (Ferguson and Alt, 2001; Lee et al., 2004; reviewed in Roth, 2003), is the predominant NHEJ pathway in *Drosophila*. Thus, HR being the error-free pathway is the repair pathway of choice in *Drosophila* (Engels et al., 1990; reviewed in Flores, 2001; Preston and Engels, 1996).

DNA damage response to ICLs in *Drosophila*

Another aspect of DNA damage response that I studied during this dissertation work is the response to interstrand crosslinks. I found that in accordance with the ICL repair model, DSBs are an intermediate of ICL repair in *Drosophila* (in collaboration with Kathryn Kohl). I also found that ICLs can activate checkpoint response even when DSB signals are limited. I used mutations in two genes to carry out this study.

In an attempt to identify new alleles of known repair genes or new genes required for DNA repair, mutations recovered from a large-scale mutagenesis project have been tested for mutagen sensitivities and classified into three complementation groups: one sensitive to the alkylating agent methyl methane sulphonate (MMS), the other to the crosslinking agent nitrogen mustard and the third group was sensitive to both (Laurencon et al., 2004). It is possible that mutations sensitive to MMS may identify genes that encode proteins required for DSB repair. This can be used as a starting point to screen for DSB repair defective mutants by assuming that replication past adducts added by this alkylating agent may pose obstacles to replication forks which can result in fork collapse and thus generate breaks. Failure to repair such breaks may render these mutations hypersensitive to MMS.

Since HR of DSBs according to most models is an integral part of ICL repair (Rothfuss et al., 2004 and Shen et al., 2006), mutations that are sensitive to nitrogen mustard in addition to MMS were selected to gain insight into the difference between HR of DSBs intermediates formed during ICL repair versus those that are generated by other sources. Prior to testing the mutations for defects in DSB repair, I screened them for IR sensitivity in a small-scale experiment. This pre-screening was carried out because one of the damage byproducts of IR is DSBs. Thus sensitivity to both MMS and IR may be considered as a stronger clue that the mutant is defective in repair of DSBs.

Genes required for responding to ICLs

One of the candidate genes studied here, *mus301*, also known as *spnC* encodes a protein with a helicase domain reminiscent of the inter-strand crosslink repair protein MUS308 (McCaffrey et al., 2006). The second candidate is the *mutagen-sensitive 302* mutation or *mus302* has been mapped to the third chromosome (Boyd et al., 1981), but has not been localized to a genetic locus. *Drosophila* cell cultures carrying this mutation are

defective in synthesizing normal amounts of DNA after treatment with UV (Brown and Boyd, 1981). *Drosophila* carrying this mutation has been found defective in repairing *P* element induced DSBs (Banga et al., 1991). Both *mus301* and *mus302* are sensitive to MMS and nitrogen mustard. I tested mutations in these genes for IR sensitivity and DSB repair defects. I also employed deficiency mapping techniques to map the *mus302* mutation.

In the following chapters I explain my findings on DNA damage response genes in *Drosophila*. I have tested various mutants for mutagen sensitivity, checkpoint response and DSB repair defects. I have employed both forward and reverse genetic approaches to generate mutations in known repair genes as well as to identify mutants hypersensitive to mutagens such as MMS, IR and nitrogen mustard to study repair of DSBs and ICLs

CHAPTER II

DOUBLE STRAND BREAK REPAIR BY HOMOLOGY MEDIATED REPAIR PATHWAY IN *DROSOPHILA MELANOGASTER*

Introduction

Double-strand breaks in the pre-meiotic germline cells of *Drosophila* are repaired frequently by the homology mediated repair pathway that leads to gene conversions not associated with crossovers (Adams et al., 2003; Kurkulos et al., 1994; McVey et al., 2004b; McVey et al., 2004c; Nassif and Engels, 1993). The model that best fits such repair outcomes is the Synthesis Dependent Strand Annealing (SDSA) model (Adams et al., 2003; Kurkulos et al., 1994; McVey et al., 2004b; McVey et al., 2004c; Nassif and Engels, 1993). Our lab has assayed several mutants for defects in SDSA by inducing *P*-element mediated DSBs *in vivo*. Using this assay proteins required for strand invasion (Figure 1.5, McVey et al., 2004b), dissociation of invading strand from template (Figure 1.5, Adams et al., 2003), and the annealing step downstream of synthesis (Figure 1.5, LaRocque et al., 2007) have been identified. This chapter describes DSB repair defects associated with mutations in two genes, *nbs* and *okra,* with putative functions in resecting DSBs to generate recombinogenic single-strand overhangs and facilitating strand invasion, respectively.

The *nbs* gene encodes for the protein Nibrin which forms a complex with two well conserved proteins: Mre11 and Rad50. The MRN complex has been proposed to function during early steps of DSB repair. Since NBS targets this complex to the nucleus, nuclear function of this complex may be studied using mutations in *nbs*. Models for meiotic recombination and HR repair of DSBs in mitotic cells require that the 5' end of DSBs are

resected to generate intermediates with 3' single-stranded overhangs, which can be bound by strand invasion proteins to mediate homology search (Figure 1.1). Meiotic cells from *S. cerevisiae mre11* mutants show an accumulation of unprocessed DSBs (Moreau et al., 2001), leading to the hypothesis that the MRX complex (Xrs2 is the functional counterpart of NBS/NBN in *S. cerevisiae*) participates in resection. *In vitro* studies, however, have demonstrated an exonuclease activity of the Mre11 protein with 3'-5' polarity (Paull et al., 1998), the opposite of this polarity is required for resection. Mre11 protein also has endonuclease activity, and it has been suggested that resection may be accomplished should coupling of endonuclease and exonuclease activities occur (Paull et al., 1998). At least two other nucleases have been found to have functions partially redundant with those of Mre11 (Lam et al., 2008). Also, it has been demonstrated in yeast that although MRX is required for HR, the nuclease function of Mre11 is not essential for this (Bressan et al., 1999).

Studies in yeast have demonstrated that the yeast MRX complex is required for repair of programmed DSBs by HR in meiotic cells but not in mitotic cells (Moreau et al., 2001 and Ivanov et al., 1994). Thus, I sought to test this function *in vivo* using *Drosophila nbs* mutants, using the same assay which identified other proteins required for repair by SDSA previously.

An additional advantage of using the *P*-element based assay was the ability to simultaneously assay for requirement of NBS in NHEJ or HR; since the MRN/MRX complex has also been implicated in NHEJ. I tested *Drosophila nbs* mutants for defects in HR and NHEJ simultaneously using a *P*-element based assay (Adams et al., 2003). In addition I carried out molecular analysis of the repair events and assayed DSB repair of *I-SceI* endonuclease induced breaks.

Following the formation of single-stranded recombinogenic overhangs, strand invasion proteins are required to bind the single-stranded DNA and search for a homologous

template. Rad51 is a well-conserved strand invasion protein. In yeast, it has been demonstrated that Rad54 facilitates the strand invasion function of Rad51 to generate a heterduplex (Clever et al., 1997; Petukhova et al., 1998; Petukhova et al., 1999). DmRad51 is encoded by *spn-A.* Null mutations in this gene render females sterile and are hypersensitive to IR. These phenotypes suggest defective repair of induced DSBs during meiosis and spontaneous DSBs in mitotic cells in these mutants (Staeva-Vieira et al., 2003). I tested Drosophila *rad54* (*okr*) mutants for defects in DSB repair using the *P*-element assay which previously demonstrated that the DmRad51 protein is required for strand invasion during SDSA. A comparison of results obtained from the *P*-element assay carried out in *okr* (*Drosophila rad54*) mutants and *spnA* (*Drosophila rad51*) mutants allowed me to assess if DmRad54 is essential for DmRad51 function and if it is required during the several rounds of strand invasion hypothesized to occur during repair of *P*-element induced breaks in the pre-meiotic germline cells (McVey et al., 2004). I used two *okr* mutant alleles for this study: the okr^{AG} carries an alteration in first methionine to an isoleucine, and okr^{RU} carries a nonsense mutation (Q-amber) at residue 391 (Ghabrial et al., 1998). These mutant alleles were used because of prior knowledge of sensitivity to mutagens causing DSBs.

Results and Discussion

Hypomorphic and null alleles of nbs *in Drosophila*

The structure of the *Drosophila* NBS protein has been described previously (Ciapponi et al., 2006 and Oikemus et al., 2006). The amino-terminal half of the protein contains a forkhead-associated (FHA) domain and two BRCT domains (Becker et al., 2006) (Figure 2.1). These domains mediate phospho-protein interactions and are required for checkpoint signaling (Durocher et al., 1999 and Yu et al., 2003). At the carboxy terminus there is an MRE11 binding site (Figure 2.1). Vertebrate NBS/NBN has a nuclear localization signal (NLS), and controls entry of the MRN complex into the nucleus (Ciapponi et al., 2006; Desai-Mehta et al., 2001 and

Tseng et al., 2005). Using ScanProsite on the ExPASy database (Hulo et al., 2008), I detected a putative NLS at residues 684-698 of *Drosophila* NBS. Under similar stringency, no NLS motifs were found in MRE11 or RAD50 proteins.

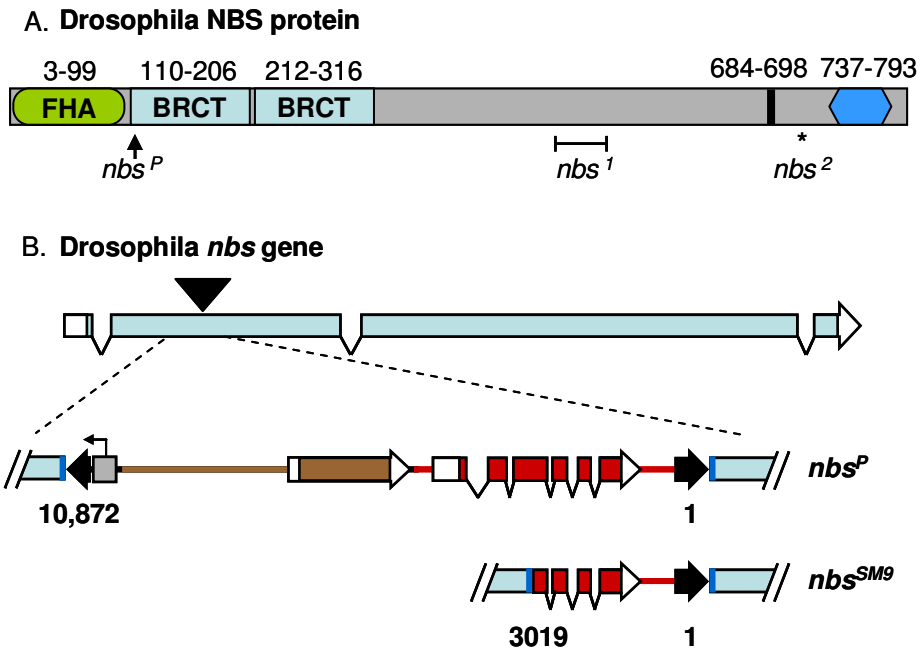


Figure 2.1 Drosophila NBS protein and mutants
A. The predicted Drosophila melanogaster NBS protein is 814 residues
Conserved sequences include an FHA domain (green oval), two BRCT repeats (light blue rectangles), a putative nuclear localization signal (black line), and the MRE11 binding site (dark blue hexagon). The positions of three mutations are indicated: nbs^{P} (insertion of a *P* element), nbs^{1} (238-bp deletion denoted by a bracket), and nbs^{2} (a nonsense mutation; asterisk).

B. Structure of nbs^{P} and nbs^{SM9} mutations.
The nbs^{P} allele is generated by insertion of a 10-kb *P* element construct into coding sequences in the second exon of *nbs*. Dark blue lines represent the 8-bp insertion site sequence that is duplicated at each end of the *P* element. Black arrowheads indicate *P* element sequences, including inverted repeats at the ends that are required *in cis* for transposition. This construct carries a *y+* body color gene and a *w+* eye color gene (brown and red, respectively; rectangles indicate exons; protein-coding sequences filled), and a promoter under the control of a *S. cerevisiae UAS* sequence (gray box; arrow indicates start and direction of transcription). The *P* element is inserted such that the 5' *P* element end is near the 3' end of *nbs*. The nbs^{SM9} derivative has lost approximately 7 kb, including one inverted repeat, all of *y+*, and much of the *w+* gene, retaining nucleotides 1-3019.

Two genetic null mutations in *Drosophila nbs* have been characterized previously (Ciapponi et al., 2006 ; Oikemus et al., 2006 and Leicht et al., 1988). Both cause lethality late in development, at the pharate adult stage. The nbs^{1} allele is a 238-bp deletion and one bp insertion at codon 507, resulting in a frameshift and premature termination (Figure 1a). I did not detect any transcripts in nbs^{1} mutants when I probed an RNA blot with a probe that recognizes the 3' end of *nbs* (Figure 2.2), suggesting that the mutant mRNA is degraded by nonsense-mediated decay.

I obtained a stock with an insertion of a 10 kb *P* element construct into exon two coding sequences of *nbs* (Figure 2.1). I refer to this mutation as nbs^{P}. Although this insertion is predicted to disrupt the first BRCT domain, flies homozygous for nbs^{P} or heteroallelic for nbs^{P} and nbs^{1} are viable as adults. For use in an assay in which DSBs are generated by expressing transposase, I generated a derivative of nbs^{P} that is stable in the presence of transposase (Figure 2.1, part B). This allele was generated by exposure of nbs^{P} to transposase, followed by selection for viability in *trans* to a deletion of *nbs* (to exclude null mutations) and screening for loss of the w^{+} and y^{+} markers on the *P* element.

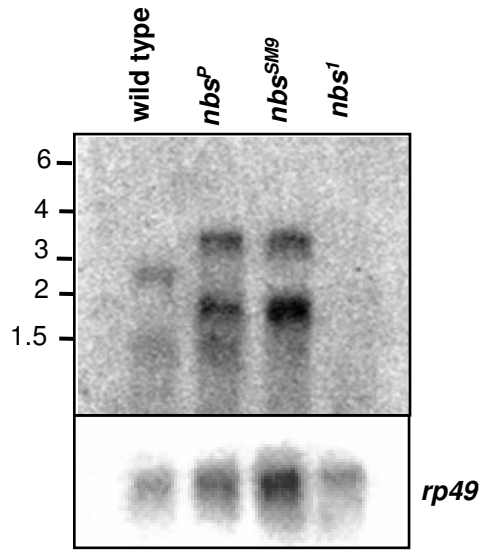


Figure 2.2 Transcripts in *nbs* mutants.
A blot of polyA- selected RNA from wild type and *nbs* mutant L3 larvae was probed with a fragment from the 3' end of *nbs*. Transcripts of about 1200 and 2600 nt are detected in wild type larvae. In nbs^{P} and nbs^{SM9} mutants, larger transcripts (1900 and 3300 nt) are seen; no transcripts are detected in the nbs^{1} mutant. The membrane was stripped and re-probed for *rp49* as a loading control.

I obtained a derivative, nbs^{SM9} that retains about three kb of one end of the *P* element but lacks the other end, including sequences that are essential for transposition.

When blots of RNA from nbs^{P} and nbs^{SM9} were probed with a probe to the 3' half of *nbs*, two transcripts that are 400-500 nt longer than the wild-type transcripts were detected (Figure 2.2). These could arise from use of cryptic or natural splice sites within sequences carried on the *P* element.

In other organisms, *nbs* mutation results in IR hypersensitivity (Bressan et al., 1999). Likewise, *Drosophila* nbs^{P}/nbs^{P} and nbs^{P}/nbs^{1} larvae are hypersensitive to IR relative to wild-type larvae (Figure 2.3). However, nbs^{P}/nbs^{P} larvae are significantly less sensitive than nbs^{P}/nbs^{1} larvae at doses higher than 500 rads ($P < 0.0001$ by Chi square).

Based on the milder sensitivity to IR and adult viability, I conclude that nbs^{P} and nbs^{SM9} are hypomorphic alleles. This could be due to reduced expression or activity or to a separation of function. Also the nbs^{P} mutants showed X-chromosome nondisjunction (Figure 2.4).

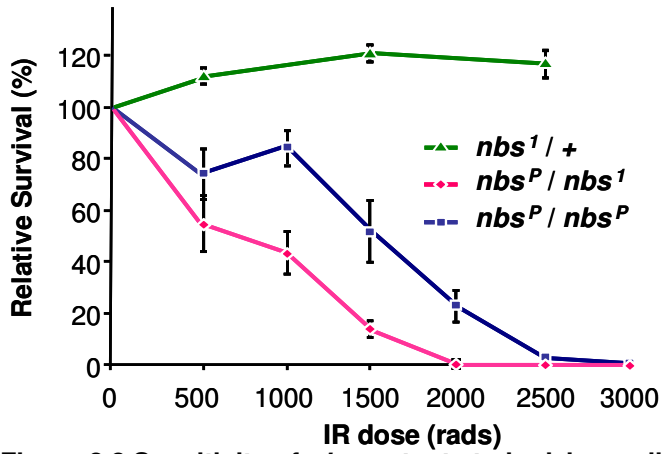


Figure 2.3 Sensitivity of *nbs* mutants to ionizing radiation. An IR sensitivity assay of nbs mutants was carried out at the doses of gamma radiation indicated on the X-axis. Each point is the mean survival of mutants relative to survival of heterozygous siblings (for nbs^{P}/nbs^{P} and nbs^{P}/nbs^{1}) or wild-type siblings (for $nbs^{1}/+$). Error bars indicate standard deviation (n = 6-14 bottles for each point).

More nondisjunction was detected in nbs^{P}/nbs^{1} mutants than in the nbs^{P} mutants (Table 2.1). Fisher's exact test rendered a P value of 0.04 suggesting that this result is significant. This re-confirms that the nbs^{P} mutants are hypomorphic. In the nondisjunction assay used here, males carry X chromosome attached to their Y chromosome, such that they travel together during meiosis (Supplemetal figure 2.1). This attached chromosome is marked with a dominant eye marker gene, *Bar*. Nondisjunction of X chromosomes can be deduced when

exceptional class progeny (*Bar+* daughters and *Bar* sons) are produced from mothers with nullo or diploX eggs (supplemental figure 2.1).

Table 2.1 Nondisjunction in *nbs* mutants.

A. Nondisjunction in nbs^{P} mutants.

	Normal class (mean)	Exceptional class (mean)
Males	335	3
Females	233	5
Total	**568**	**8**

The nondisjunction assay was carried out as described in Supplemental Figure 2.1. Records of exceptional males and females that were scored are shown here. Table A shows results from the nbs^{P} homozygous mutants. Results from nbs^{P}/nbs^{1} mutants are shown in Table B. Nondisjunction is calculated by doubling the exceptional class (to account for inviable progeny as shown in Supplemental Figure 2.1) and dividing by the total # of flies scored to calculate % nondisjunction for each genotype. Table A: % Nondisjunction is 8+8/584 =2.7% Table B: % Nondisjunction is 6+6/146 =8.2%

B. Nondisjunction in nbs^{P}/nbs^{1} mutants.

	Normal class (mean)	Exceptional class (mean)
Males	89	4
Females	51	1
Total	**140**	**6**

***Drosophila* NBS is required for the homology mediated pathway SDSA**

I used a *P* element excision assay to assess the ability of *nbs* mutants to complete HR or NHEJ (Adams et al., 2003 and Figure 2.5). The *P* element, $P\{w^{a}\}$, used in this assay carries the *apricot* allele of the *white* gene (w^{a}), and is inserted into an intron of *sd*, an essential gene on the X chromosome. The w^{a} allele is a *copia* retrotransposon inserted into an intron of *w*; this decreases expression to give apricot-colored eyes instead of red eyes.

Excision of $P\{w^{a}\}$ element was carried out in males by crossing in *P* transposase. This excision generates a DSB that is a 14-kb gap with respect to the HR template, the

sister chromatid. Gap repair in pre-meiotic germline cells is believed to be initiated by synthesis-dependent strand annealing (SDSA, Adams et al., 2003) (Figure 1.5).

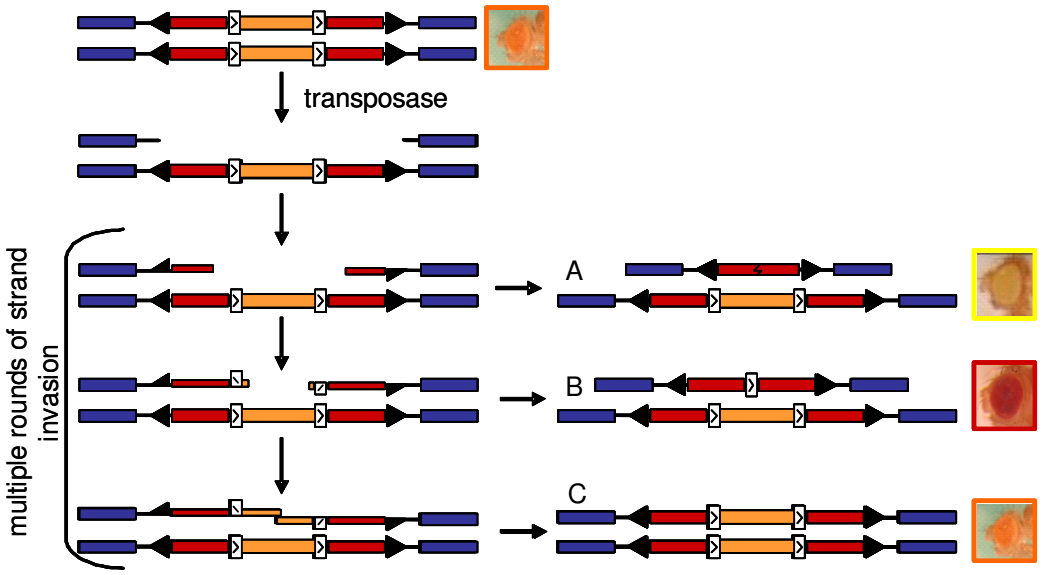


Figure 2.4 *P*-element based DSB repair assay.
Each line represents a sister chromatid. Blue rectangles denote exons of *sd*, an essential gene on the X chromosome. A *P*{*w*^{a}} element is inserted into a *sd* intron. Black arrowheads represent *P* element inverted repeat sequences. Red rectangles are halves of the *w* gene, which is interrupted by insertion into an intron of a *copia* retrotransposon (orange) with LTRs (white rectangles). This transgene confers apricot eye color to otherwise *w*– mutants. Transposase catalyzes excision of the element, leaving a 14-kb gap relative to the sister. This gap is usually repaired by SDSA, with synthesis occurring from both ends independently. In some cases, repair occurs by end joining, with or without synthesis; this leads to loss of *w* expression and yellow-eyed progeny (A). If synthesis from both ends extends past the LTRs, these sequences can anneal as in SDSA, leaving a single LTR in the *w* intron, which allows enough expression of *w* to give red eyes (B). It is also possible for the entire gap to be filled, restoring the *P*{*w*^{a}} element and giving apricot-colored eyes (C).

If synthesis from each end extends through a *copia* long terminal repeat (LTR), the LTRs may anneal to one another to give a repair product that lacks the *copia* insertion, leaving a single LTR. This allows nearly normal expression of *w*, resulting in red eyes (Figure 2.4). This repair type is classified as 'completed SDSA'. It is also possible for HR using the sister chromatid to restore the entire *P*{*w*^{a}}. Since this outcome is indistinguishable from failure to excise, which accounts for the majority of chromosomes recovered (Adams et al., 2003), it is not included in analysis of repair events.

SDSA is sometimes aborted prior to synthesis or annealing of LTRs, and repair is completed by end joining. This process destroys expression of *w*, resulting in yellow eyes when the repaired chromosome is recovered in *trans* with an intact *P*{*w*^{a}} chromosome (Figure 2.4). Yellow eyes can also result from repair by end joining without synthesis. Aborted SDSA and end joining without synthesis are distinguished through molecular analyses (Figure 2.6 and Table 2.2).

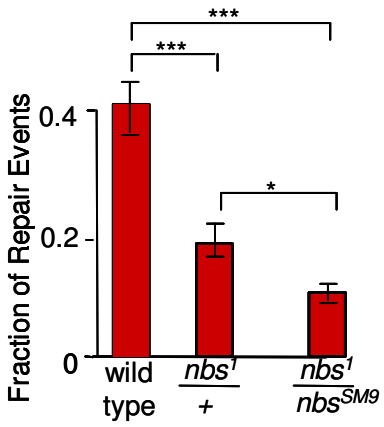


Figure 2.5 NBS is required to complete SDSA. Repair outcomes are scored as completed SDSA (red eyes) or other repair (yellow eyes). Each bar shows the mean fraction of all known repair events (red plus yellow) that were completed SDSA (red). Means were determined by scoring the progeny of each male independently (n=51 for wild type, 67 for *nbs*^{1}/+, and 71 for *nbs*^{1}/*nbs*^{SM9}). Error bars indicate standard error of the mean. P values were calculated for each pairwise comparison by a non-parametric ANOVA (Kruskal-Wallis test). One asterisks denotes P<0.05 and three denotes P<0.0001.

I conducted the *P*{*w*^{a}} assay in *nbs*^{1}/*nbs*^{SM9} mutants (Figure 2.5, Supplemental figure 2.3). The completed SDSA class was significantly less frequent in the mutants than in wild-type or *nbs*^{1}/+ flies (P <0.001), indicating that *nbs*^{1}/*nbs*^{SM9} mutants are defective in SDSA. Notably, *nbs*^{1}/+ mutants also had significantly less SDSA than wild-type flies, revealing haploinsufficiency for *nbs* in gap repair.

Short repair synthesis during SDSA in Drosophila nbs mutants

Consistent with the decrease in completed SDSA, *nbs* mutants showed an in increase in the yellow-eyed class of repair events. I measured the lengths of synthesis tracts among repair products arising from aborted SDSA. The number of repair events that showed any evidence of synthesis was significantly decreased in *nbs* mutants.

This is consistent with the hypothesis that the MRN complex is involved in an early step of HR, such as resection of 5' ends. However, among those repair events in which synthesis was initiated, tracts were significantly shorter in *nbs* mutants than in wild-type flies (Figure 2.6). This finding suggests that *Drosophila* NBS may be required in SDSA steps downstream of end resection. Ends of the aborted SDSA events were sequenced to determine if microhomologies were used preferentially in presence of wild type NBS (Supplemental Table 2.1)

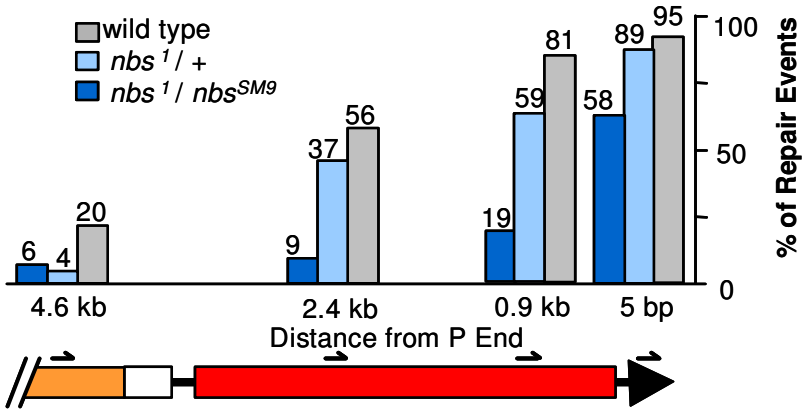


Figure 2.6 Molecular analysis of repair synthesis tract lengths.
Repair events from yellow-eyed progeny were analyzed to determine the extent of repair synthesis, if any. The right end of the *P*{*w*^{a}} element is shown (coloring as in Figure 2.4). Four PCR reactions were carried out to measure synthesis; the position of the innermost primer used in each is indicated. Bars indicate percentage of events analyzed that had synthesis tracts of at least 5 bp, 0.9 kb, 2.4 kb, and 4.6 kb (n=38 for wildtype, 59 for *nbs*^{1}/+, and 81 for *nbs*^{1}/*nbs*^{SM9}). P values were determined by Chi square and Fisher's exact test. P<0.0001 for wild type vs. *nbs*^{1}/*nbs*^{SM9}; P<0.05 for wild type vs. *nbs*^{1}/+, except at 5 bp synthesis (P=0.12). P<0.01 for *nbs*^{1}/*nbs*^{SM9} vs. *nbs*^{1}/+, except at 4.6 kb (P=1).

Single Strand Annealing in nbs *mutants*

To more directly assess the role of NBS in resection during DSB repair, I conducted an assay for single-strand annealing (SSA). In this assay, a DSB is introduced by the *I-Sce*I enzyme (Figure 2.7) (Rong and Golic, 2003). The *I-Sce*I recognition sequence (I-site) is located between two copies of the *w* gene, a partial (3.6 kb) copy to the left and a complete (4.6 kb) copy to the right. If resection extends at least 3.6 kb to the left and 4.6 kb to the

right, the two copies can anneal to produce a product that is diagnostic of SSA repair: loss of one repeat and the sequences between the repeats.

Cutting was induced by crossing in a *P* element carrying *I-Sce*I under the control of a *hsp70* heat shock promoter. Wild type, nbs^{1}/nbs^{SM9}, and $mus309^{N1}/mus309^{D2}$ L2 larvae were heat shocked simultaneously at 38º for one hour. I included *mus309* mutants as a control, since no defect in SSA was found in these mutants previously (Johnson-Schlitz and Engels, 2006 and Li and Rong, 2007). The *mus309* gene encodes the *Drosophila* homolog of the human *BLM* gene (Kusano et al., 2001).

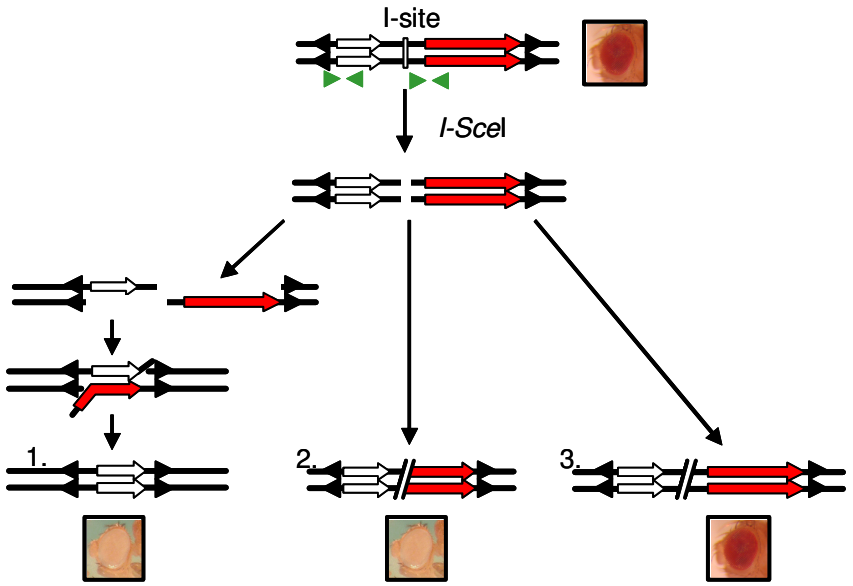


Figure 2.7 Single Strand Annealing Assay.
The *P*{X97-Isite} construct (Rong and Golic, 2003) carries an *I-Sce*I recognition site (I-site, white rectangle) flanked by a wild-type *w* gene of 4.5 kb (red arrow) and a partial *w* gene of 3.5 kb (white arrow); black arrowheads represent *P* element ends. The two horizontal lines represent the two strands of a single chromatid. Expression of *I-Sce*I leads to cutting at the I-site to produce a double-strand break. Repair by SSA requires resection through both copies of the *w* gene, followed by annealing of complementary sequences, trimming, and ligation. The product (1) has only a partial *w* gene, resulting in white eyes. White eyes can also result from repair associated with a deletion into the full-length *w* gene (2). Red eyes result from imprecise NHEJ, in which the I-site is destroyed (3), or precise NHEJ (not shown, because precise NHEJ cannot be distinguished from failure to cut). The green arrows indicate a set of primers that recognize similar sequences on either side of the I-site. These primers help distinguish between white-eyed progeny produced as a result of SSA versus those that are results of deletions associated with repair. Deletions of 1.2 kb or less can be detected using these primers by PCR. While these deletions and the original construct render two PCR bands with the green primer set, SSA products will render only one.

Cutting and repair takes place in the germline of the heat-shocked male larvae, and the results are scored in their progeny. Progeny carrying the uncut construct have red eyes. Repair by SSA as described above leaves only the partial copy of the *w* gene, resulting in white eyes (Figure 2.7 # 1). This is the most frequent repair event (Rong and Golic 2003) but white-eyed progeny can also arise from repair that involves deletion into the full-length *w* gene followed by end joining (Figure 2.7 # 2). I used a PCR assay to detect deletions of up to 1.2 kb (Figure 2.7, green arrows indicate primers). If a deletion was not detected, the event was classified as SSA.

Although the uncut construct produces red eyes, this outcome can also result from repair by precise or imprecise end joining. To measure imprecise end joining, the region containing the I-site was amplified from a subset of the red-eyed progeny, and the product was incubated with *I-Sce*I enzyme. If no cutting was observed, repair was classified as imprecise end joining class (Figure 2.7 # 3). Precise end joining that re-generates the I-site cannot be distinguished from failure to cut (Figure 2.7).

Both *nbs* and *mus309* mutants showed reduced SSA relative to wild-type, but the difference was not significant for *nbs* mutants and only marginally significant for *mus309* mutants (Figure 2.8, Supplemental figure 2.4). Thus, by this assay, I could not detect a function for NBS in resection. The modest decrease in SSA seen in *mus309* mutants could be accounted for by an increase in deletions (Figure 2.9).

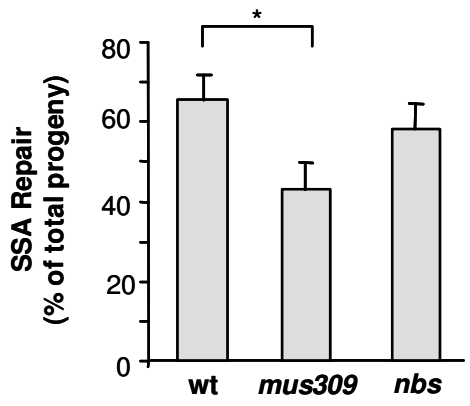


Figure 2.8 Frequency of SSA
The percentage of progeny that inherited a chromosome which was repaired by SSA was calculated for each of the 20 male parent. Bars show the mean percentages, with error bars showing SEM. Asterisk indicates $P<0.05$ by Kruskal-Wallis non-parametric ANOVA.
mus309 = mus309N1/mus309D2.
nbs = nbs1/nbsSM9.

We included *mus309* mutants as a control, since no defect in SSA was found in these mutants previously (Johnson-Schlitz, D. and Engels, 2006).

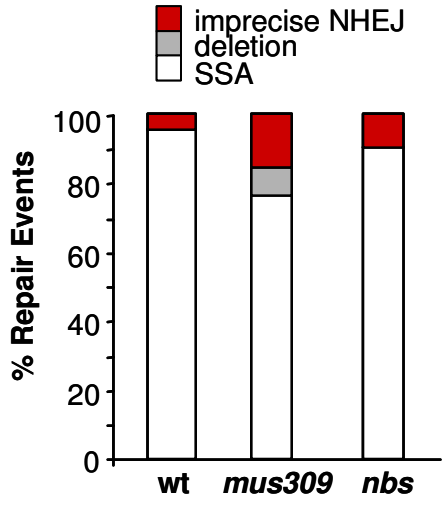


Figure 2.9 Distribution of repair events recovered from SSA assay.
Bars show the distribution of repair events from each genotype indicated. Progeny in classes 1, 2, and 3 only from Figure 2.8 were considered, since precise NHEJ cannot be distinguished from failure to cut.

Deletions have previously been found to be frequent during gap repair in *mus309* mutants (McVey et al., 2004). The deletions seen here could arise from a similar mechanism, if a cut chromatid enters into HR repair with the sister chromatid.

I sequenced junctions resulting from repair by imprecise end joining (Supplemental table 2.2). Most of the junctions had small deletions within the I-site. No differences were apparent between junctions from wild-type flies, *nbs* or *mus309* mutants.

Haploinsufficiency of nbs *in DNA damage response*

I observed mild haploinsufficiency of *nbs* in gap repair. To determine whether any other NBS functions might be sensitive to dosage, I analyzed the DNA damage-dependent G2/M cell cycle checkpoint. I observed a stronger haploinsufficiency in checkpoint response (Figure 2.10). As described previously (Ciaponni et al., 2006 and Oikemus et al., 2006), the G2/M checkpoint induced by IR is completely absent in *nbs* null (nbs^1/nbs^1) mutants (Figure 2.10).

In nbs^1/nbs^P or nbs^P/nbs^P mutants, the checkpoint is strong at 4000 rads, but weak or absent at 1000 rads. Notably, heterozygosity for the null allele nbs^1 also resulted in a significantly reduced checkpoint response at 1000 rads (Figure 2.10). At 4000 rads, a

milder, but statistically significant, defect was also detected. Thus, *Drosophila nbs* shows haploinsufficiency for this checkpoint function.

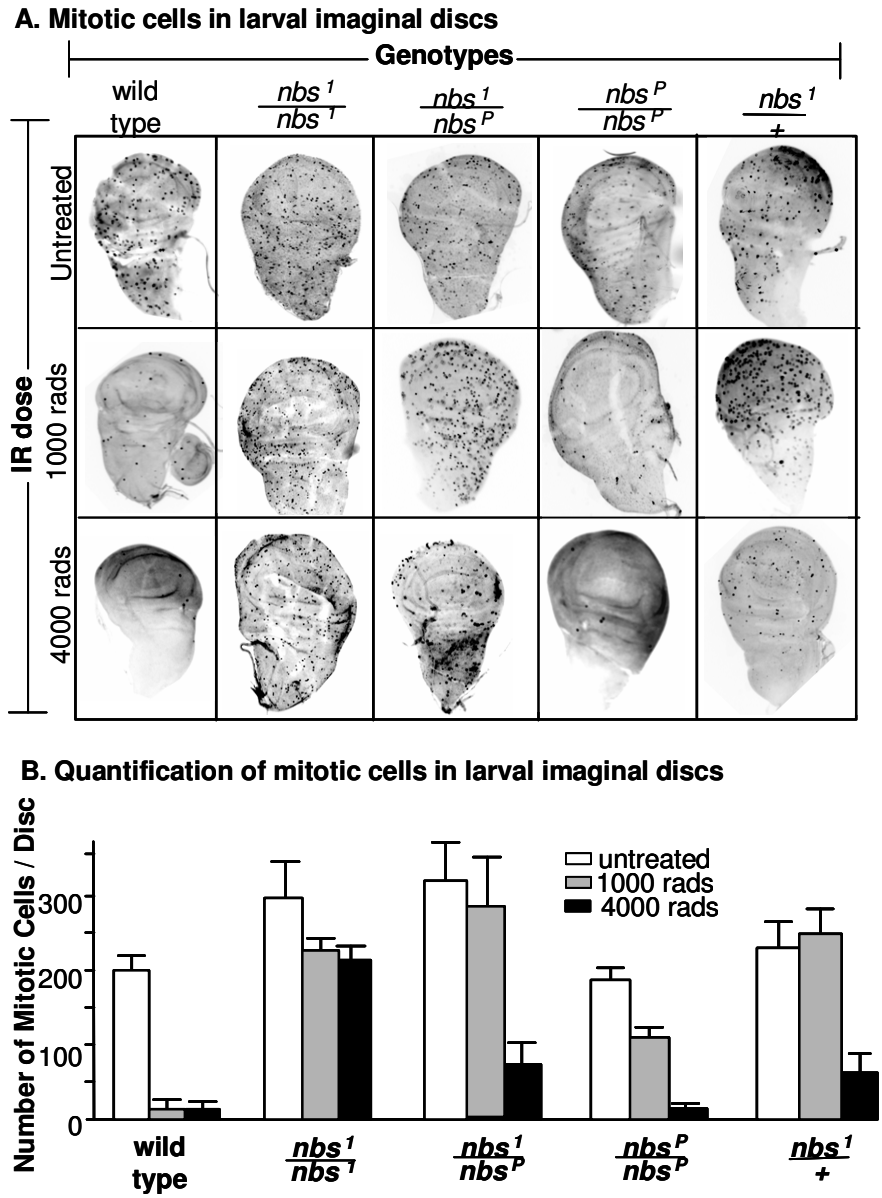


Figure 2.10 The checkpoint response in *nbs* mutants.
Mitotic cells in larval imaginal discs (A). Each panel shows a wing imaginal disc labeled with an antibody to phosph-histone H3 to mark mitotic cells. Discs were dissected out of wandering third instar larvae that were unirradiated or irradiated with 1000 and 4000 rads gamma rays. The number of mitotic cells for the different genotypes and treatments is quantified in (B). Bars represent means from 8-10 discs, and error bars are SEM.

Drosophila NBS is dispensable for NHEJ

I sequenced junctions of repair products recovered from progeny with aborted SDSA events to determine whether *Drosophila* NBS might be required for specific types of end joining. End joining in this assay is effected by a non-canonical, DNA Ligase IV-independent pathway (McVey et al., 2004). In wild-type flies, I observe four types of junctions: insertions (T-nucleotides), junctions without microhomology, junctions with short (1-5 bp) microhomology, and junctions with longer (6-10 bp) microhomology (Adams et al., 2003, Supplemental figure 2.2). I observed all four classes in junctions from *nbs* mutants (Supplemental Table 2.1). The class with short microhomology appeared to be reduced, and the class with long microhomology increased, but the difference was not statistically significant for the sample size available. Repair junctions from *I-SceI* induced breaks have also been analyzed (Supplemental Table 2.2).

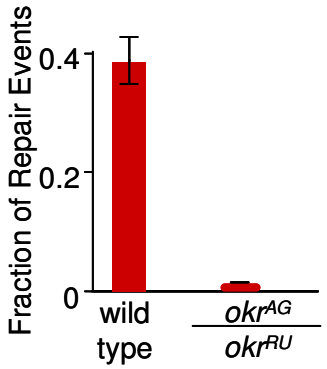


Figure 2.11 OKR is required for SDSA.
Repair outcome is scored as completed SDSA (red eyes) or other repair (yellow eyes). Each bar shows the mean fraction of all known repair events (red plus yellow) that were completed SDSA (red). Third chromosome transposase source was used in this experiment. Means were determined by scoring the progeny of each male independently (n=53 for wild type and 50 for okr^{RU}/okr^{AG}). Error bars indicate standard error of the mean. P values calculated for each pairwise comparison by a non-parametric ANOVA (Kruskal-Wallis test) showed significant difference between wild type and *okr* mutants.

Drosophila Rad54 or OKR is required during SDSA

Previously it has been shown in the lab that DmRad51 or SPN-A, the homolog of human strand invasion protein, Rad51 is required for strand invasion during repair of *P*-element induced breaks (McVey et al., 2004). I tested *okr* or *Drosophila rad54* mutants (Kooistra et al., 1997) using the

same assay as previously used to study DmRad51 function.

Similar to the *spnA* or *Drosophila rad51* mutants (McVey et al., 2004), *okr* mutants showed a severe defect in homology mediated repair. However unlike *rad51* mutants where no HR repair products were detected (Figure 2.11, Supplemental figure 2.3), few HR products were recovered in the *okr* mutant background suggesting that DmRad54 facilitates DmRad51 function but is not absolutely essential for this function.

DmRad54 is required for multiple rounds of strand invasion

It was previously proposed that synthesis following strand invasion is not processive and multiple rounds of strand invasion and synthesis are required to complete repair by SDSA (Figure 2.12, McVey et al., 2004). Molecular analysis of repair synthesis tract lengths in *rad54* mutants in *Drosophila* revealed short synthesis tracts (Figure 2.12) thereby indicating that the DmRad54 protein is required multiple times during the several rounds of strand invasion and synthesis.

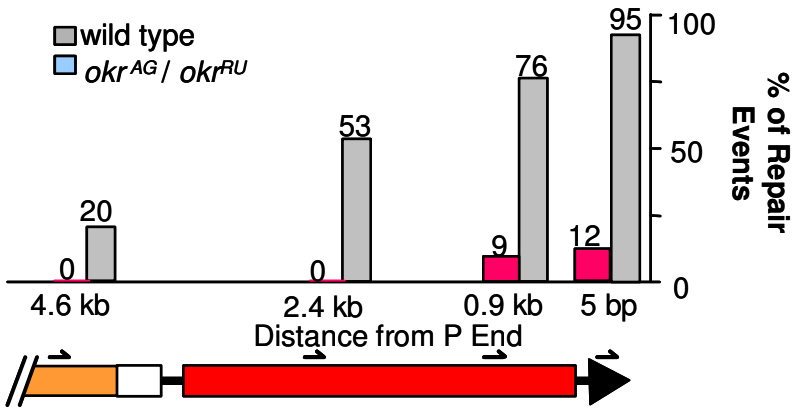


Figure 2.12 Molecular analyses of repair synthesis tract lengths.
Repair events from yellow-eyed progeny were analyzed to determine the extent of repair synthesis, if any. The right end of the *P*{*w*^{a1}} element is shown (coloring as in Figure 2.4). Four PCR reactions were carried out to measure synthesis. The position of the innermost primer used in each is indicated. Bars indicate percentage of events analyzed that had synthesis tracts of at least 5 bp, 0.9 kb, 2.4 kb, and 4.6 kb (n=38 for wildtype, 29 for okr^{RU}/okr^{AG}). P values were determined by Chi square and Fisher's exact test. P<0.0001 for wild type vs. okr^{RU}/okr^{AG} for each PCR.

Discussion

The role of Drosophila NBS in resection of DSBs

I assayed DSB repair in a background with heteroallelic combination of hypomorphic and null alleles of *nbs*. This avoided the lethality issues with complete loss of NBS, and allowed me to assay repair *in vivo*. In yeast and mice, the MRX/MRN complex is required during the early steps of DSB repair, most likely for resection (Tsubouchi and Ogawa, 1998 and Ivanov et al., 1994). The results from my *P* element excision assay are consistent with a similar role for the *Drosophila* MRN complex. In this assay, 97% of repair events from wild-type males showed evidence for synthesis from the right side of the break (the red-eyed progeny, which accounted for 42% of all repair events, plus 95% of the yellow-eyed progeny; this is an underestimate, because it does not include those repair events that regenerated the $P\{w^a\}$, which could not be distinguished from non-excision). In contrast, only 62% of repair events from nbs^{SM9}/nbs^{1} showed evidence of synthesis ($P < 0.0001$). The inability to initiate synthesis could be due to failure to resect the 5' end to produce a 3' overhang that is competent to undergo strand invasion. Still, synthesis was initiated in more than half of the repair products I analyzed from *nbs* mutants. The ability of these mutants to initiate synthesis frequently could be due to partial function retained by the nbs^{SM9} mutation or to maternally-deposited MRN complexes available early in development.

Although reduced initiation of synthesis in the *P* element excision assay is consistent with a role for NBS in resection, I did not detect any requirement for NBS for resection in the SSA assay. I employed this assay because SSA in this case requires >3.5 kb of resection on one side and almost 5 kb on the other. The finding that SSA was as frequent in nbs^{1}/nbs^{SM9} mutants as in wild-type larvae suggests that either NBS is not essential for resection, or that the residual activity of NBS^{SM9} is sufficient for this function.

I expected the SSA assay to be more sensitive to defects in *nbs*, than the *P* excision assay, since a much greater degree of resection is required in the SSA assay. One possible explanation for the contrary result is that DSB formation in the SSA assay occurs after induction of *I-Sce*I expression by heat shock, which was given at the second larval instar stage. There may be enough maternal NBS that persists until this stage to allow nearly normal resection. In contrast, *P* excision may occur throughout development; when excision occurs later in development, maternal NBS may be depleted, resulting in defective resection. An alternative explanation is that the resection required in the SSA assay may be qualitatively different than the resection required in the *P* excision assay. In *S. cerevisiae*, Mre11 has functions that are both distinct from and overlapping with those of the nucleases Exo1 and Pso2 (Lam et al., 2008). Perhaps SSA in the assay I used can be mediated by another nuclease. Finally, resection activity may be intact in the NBS^{SM9} protein, and the defects seen in the *P* excision assay may be due to loss of another activity, as described below.

Drosophila NBS in later steps of SDSA

Reduced initiation of repair synthesis was not the only defect I detected among *nbs* mutants in the *P* excision assay. Among those cases in which synthesis was initiated, tracts were shorter in *nbs* mutants than in wild-type flies. For example, 89% of repair events in wild-type flies had synthesized at least 900 bp (the red-eyed progeny plus 89% of the yellow-eyed progeny). This represents 92% of all events in which synthesis had been initiated. In contrast, in only 44% of repair events from *nbs* mutants in which synthesis had been initiated did the synthesis tract extend for at least 900 bp. If the sole defect in *nbs* mutants is in resection, then I would expect that among those events in which it was possible to initiate synthesis, tracts should be similar in length to those of wild-type flies. I

did not obtain this result, so I conclude that *Drosophila* MRN has some role in addition to resection.

Previous studies have led us to propose that repair of large gaps, as in this assay, requires multiple cycles of strand invasion, synthesis, and displacement of the nascent strand (McVey et al., 2004). Based on this model for repair, I envision two possible downstream requirements for MRN. First, end processing may be a prerequisite for loading strand invasion proteins onto single-stranded DNA prior to each cycle of invasion and synthesis. Reduced availability of NBS in mutants would limit the number of synthesis cycles, resulting in short repair tracts. Second, NBS may be required as a sensor for recognition of an intermediate generated during each round of strand invasion and synthesis. Detection of such intermediates may be required to generate a signal that allows strand invasion proteins to re-load onto the single-stranded region for re-invasion and extension of the synthesis tract. Reduced ability to recognize these intermediates in *nbs* mutants might result in increased use of end joining to complete repair.

Drosophila NBS in end joining

The assays I conducted involve several types of end joining repair. In the *P* excision assay of gap repair, events in which SDSA is initiated but not completed makes use of a DNA Ligase IV-independent end-joining pathway (McVey et al., 2004). End joining was not reduced in nbs^{SM9}/nbs^{1} mutants; indeed, there was an increase in this class compared to wild-type flies, corresponding to the decrease in completed SDSA. Similarly, in the SSA assay, which involves repair of a DSB generated by *I-Sce*I cleavage, the fraction of events in which repair occurred by imprecise end joining was not significantly different between wild-type and *nbs* mutants.

In *S. cerevisiae*, MRX is required for microhomology-mediated end joining (MMEJ), a process characterized by use of microhomologies of five base pairs or longer (Paull and

Gellert, 2000). To determine whether *Drosophila* NBS is required for MMEJ or another type of end joining; I sequenced junctions from both DSB repair assays. I found that all classes observed in wild-type flies were also observed in *nbs* mutants. There were a reduced number of products with short (1-5 bp) microhomologies and a larger number of products with long (6-10 bp) microhomologies. Although the difference was not statistically significant, due to small sample sizes available for sequencing, the results suggest that *Drosophila* NBS may be more important for end joining processes that use short microhomologies, rather than MMEJ.

Haploinsufficiency of *nbs*

Linkage analysis in human populations has shown that heterozygous carriers of *nbs* mutation are predisposed to various types of cancers (Dumon-Jones et al., 2003 and Seemanova 1990) and cells heterozygous for an *NBN* mutation have been found to be defective in activation of the checkpoint protein ATM after low-dose ionizing radiation (Ebi et al., 2007). I found a severe checkpoint defect at low IR doses in *Drosophila* mutants heterozygous for the null mutation nbs^{1}. There was also a defect at higher doses, albeit milder. Previous studies have shown that ATM is essential for checkpoint after low-dose IR, but is not required after high-dose radiation (Gong et al., 2005). It was suggested that ATM is required to amplify the signal due to a low degree of damage. Similarly, when the damage is limited, the cell may need a normal dose of NBS to amplify the signal and establish the checkpoint. At high doses of IR, a lower level of NBS may be sufficient for signalling. The primary checkpoint kinase in *Drosophila* has been suggested to be DmATR or MEI-41 (LaRocque et al., 2007). Studies in other organisms have suggested that activation of ATM by NBS is an important DNA damage response (Wu et al., 2000). It is possible that in *Drosophila,* NBS is required for activating both ATM and ATR. This activation could be

dependent on the damage levels: low damage levels may require activation of ATM or both, whereas excessive damage may require activation of ATR.

I did not detect hypersensitivity to IR in *nbs*^{1}/+ heterozygotes. Similarly, human carriers of *NBN* mutations are not hypersensitive to IR (Neubauer et al., 2002). However, spontaneous chromosomal translocations are elevated in human carriers, and in mice heterozygous for an *Nbn* mutation (Dumon-Jones et al., 2003; Tanzanella et al., 2003 and Neubauer et al., 2002), suggesting defects in DSB repair. Consistent with this interpretation, I found that *nbs*^{1}/+ heterozygous flies had reduced SDSA repair of gaps generated by *P* element excision. This finding is the first report of defects in HR in *nbs/NBN* heterozygotes in a model organism and may have important implications for understanding the cancer predisposition of human carriers of *NBN* mutations.

Role of DmRad54 in facilitating strand invasion

Severe decrease in the red eye class representative of completed SDSA suggests that the DmRad54 protein is required for SDSA. The few red eye progeny could be from maternal contribution of this protein from heterozygous mothers. On the other hand, heterozygous mothers with mutation in *spnA*, the gene encoding DmRad51, when crossed to mutant fathers to generate zygotic nulls resulted in no red-eyed progeny. This may suggest that DmRad54 facilitates DmRad51 function but is not absolutely essential for this function. Mosaicism in the eye with red patches was indicative of repair by SDSA in somatic tissue, was found in *okr* mutants but not in *spnA* mutants.

SPN-A or DmRad51 is required multiple times during the several rounds of strand invasion (McVey et al., 2004). I found a repair synthesis defect associated with okr mutations suggesting that OKR or DmRad54 is also required multiple times to initiate strand invasion. This finding does not corroborate the suggestion of Rad54 is a branch migrase since branch migration is downstream of synthesis.

Rad54 belongs to the Swi2/Snf2 family of ATPases. Site-directed mutagenesis to generate mutants with defective ATPase function could help us understand if the ATPase function of this protein is essential for its role in SDSA. Also, it may be interesting to determine if excision of different *P*-element insertions to generate breaks *in vivo* at different sites, have a differential requirement for DmRad54, with respect to the chromatin environment.

Materials and Methods

Drosophila stocks and genetics

Drosophila stocks were maintained on standard medium at 25º. The *nbs* null mutations used were nbs^{1} and nbs^{2} (Ciapponi et al., 2006 and Oikemus et al., 2006). The hypomorphic allele used here was a *P* element insertion allele generated during the Berkeley *Drosophila* Gene disruption project and is available at the Bloomington stock center (# 21141) $y^{1}\ w^{67c23}$; *P*{EPgy2}$nbs^{EY15506}$. The *P* element is inserted in the 2nd exon of *nbs*, within coding sequences. A derivative of the *P*{EPgy2}$nbs^{EY15506}$ hypomorphic allele was generated for use in assays that involve *P* transposase. Males carrying *P*{EPgy2}$nbs^{EY15506}$ and *P* transposase were generated and crossed to w^{-} females, and progeny that had lost the w^{+} marker from *P*{EPgy2} were screened for the absence of one end of the *P* element. The derivative I recovered, named nbs^{SM9}, lacks the 3' *P* element end and has approximately 3 kb of the original 10 kb *P*{EPgy2} remaining.

The *okra* mutant alleles okr^{AG} and okr^{RU} used here are described as loss of function mutant and an antimorph respectively (Ghabrial et al., 1998).

Northern analysis

Total RNA was isolated from wild type w^{1118}, nbs^{P}, nbs^{SM9} and nbs^{1} homozygous larvae by homogenizing 10 larvae in 1 ml Trizol reagent. Phase separation was carried out

in chloroform to remove nucleoprotein contaminants. RNA was precipitated in isopropanol and washed with 75% ethanol. Air dried RNA was dissolved in 50µl of 0.5% SDS solution prepared in RNase free water. Incubation at 55-60º for 10 minutes ensured that RNA secondary structures were removed but RNA was intact. Total RNA was poly A selected using a kit (Ambion) to remove tRNA and rRNA contaminants. RNA gel electrophoresis was carried out using formaldehyde agarose gels prepared in MOPS buffer. RNA samples prepared with formamide, formaldehyde solution, MOPS [3-(N-morpholino) propanesulfonic acid (MOPS) and 10 mM EDTA, pH 7] and 1 µl ethidium bromide were loaded and run at constant 10 watts power. In a buffer tank containing 20X SSCP (Sodium chloride-Sodium Citrate buffer) the following was assembled bottom to top: whatman paper moistened in SSCP, gel, nylon membrane and 3X whatman paper with a weight on top. This assembly was left overnight at 37 º. The following day the RNA was UV-crosslinked to the membrane by exposing to a UV trans-illuminating source for 2 minutes. The membrane was pre-hybridized with a pre-hybridization buffer [50% formamide; 20X SSCP 5 ml; 100X Denhardts 1.6 ml; Salmon sperm DNA 1 ml (boiled for 5 min); 10% SDS 1ml; yeast tRNA 1.4ml used in concentration 200µl/cm^2]. The membrane with buffer was sealed in a plastic bag and incubated for 4 hrs at 65 º.

The cDNA probe source was a cDNA construct in pOT2 cloning vector available at the *Drosophila* Genome Research Center cDNA collection (LD444 Row D column 2). Maxipreps of DNA from these clones were carried out using the Qiagen maxiprep kit. 10µg of DNA was digested with *EcoRI* restriction endonuclease to render a probe that recognizes 3' end of the *nbs* RNA. Transcription or labeling reaction with 200µC of P-32 labelled CTP was carried out to label the probe. The labeled probe was purified on a Sephadex column, following which 20µl of the labeled probe was added to the pre-hybridized membrane and sealed in a plastic bag and left at 65 º overnight. Exposure of films inserted with an intensifying cassette to the membrane for 48 hrs was carried out for visualizing the blot.

Ionizing radiation sensitivity

Males and females balanced with the third chromosome balancer TM3*, Sb* were crossed in cages and allowed to lay eggs overnight at 25º on grape agar plates; plates were then changed for a second collection. After incubating this second collection for 2 days, the plates were exposed to various doses of gamma irradiation from a ^{137}Cs source; the first brood was used as an un-irradiated control. After irradiation the grape agar with larvae was divided into four sectors and transferred to standard medium in bottles, then incubated at 25º until adults eclosed. Adults were counted to determine the ratio of balanced (heterozygous) to mutant (homozygous or heteroallelic) flies. The expected ratio of balanced to mutant flies was determined from un-irradiated bottles. Relative survival of mutants was calculated from the ratio of observed to expected.

Nondisjunction assay

Nondisjunction of X chromosome was assayed by crossing nbs^{1}/nbs^{P} or nbs^{P} homozygous virgin females to males carrying X attached Y chromosome (X^Y, v f B) marked with a dominant bar or 'B' marker for eye shape phenotype. In backgrounds where nondisjunction does not occur bar-eyed daughters and non-bar eyed sons were produced. In backgrounds where nondisjunction of X took place, exceptional class of non-bar eyed daughters and bar-eyed sons were produced in addition to the normal class. Nondisjunction was also assayed by crossing *nbs* virgins to y cv v f/ B^{S}Y males (where the males have their Y chromosomes marked with B^{S}) produced the same result.

P *excision assay*

The *P*{w^{a}} element used in this assay carries the *apricot* allele of the *white* gene (w^{a}), and is inserted into an intron of *sd*, an essential gene on the X chromosome. The w^{a} allele is a *copia* retrotransposon inserted into an intron of *w*; this decreases expression to give

apricot-colored eyes instead of red eyes. Excision of *P*{*w*^{a}} element was carried out in males by crossing in *P* transposase (*H*{*w*$^+$, Δ*2-3*}*Hop2.1* transposase source located on a the second chromosome marked with *CyO* wings or marked with *Sb* bristle marker on the third chromosome). Excision generates a DSB that is a 14-kb gap with respect to the HR template, the sister chromatid. Gap repair in pre-meiotic germline cells is believed to be initiated by synthesis-dependent strand annealing (SDSA, Adams et al., 2003; Figure 1.5). If synthesis from each end extends through a *copia* long terminal repeat (LTR), the LTRs may anneal to one another to give a repair product that lacks the *copia* insertion, leaving a single LTR. This allows nearly normal expression of *w*, resulting in red eyes (Figure 2.4). This repair type is classified as 'completed SDSA'. It is also possible for HR using the sister chromatid to restore the entire *P*{*w*^{a}}. Since this outcome is indistinguishable from failure to excise, which accounts for the majority of chromosomes recovered (Adams et al., 2003), it is not included in my analysis. SDSA is sometimes aborted prior to synthesis or annealing of LTRs, and repair is completed by end joining. This process destroys expression of *w*, resulting in yellow eyes when the repaired chromosome is recovered in *trans* to an intact *P*{*w*^{a}} (Figure 2.4). Yellow eyes can also result from repair by end joining without synthesis. Aborted SDSA and end joining without synthesis are distinguished through molecular analyses.

Molecular analysis of aborted SDSA events

Repair synthesis tract lengths were determined as described in Adams, *et al.* (2003). Genomic DNA was prepared from single male flies containing the aberrant repair product derived from experiments using the *H* {*w*$^+$, Δ*2-3*} *Hop2.1* transposase source, located on a *CyO* balancer chromosome. The *H* {*w*$^+$, Δ*2-3*} *Hop2.1* transposase source with *Sb* marker, located on the third chromosome was used in experiments with *okr* mutants. PCR reactions

contained 10 mM Tris-HCl pH 9.0, 50 mM KCl, 2.5 mM MgCl2, 0.1% Triton X-100, 1.25 µM of each primer, 250 µM each dNTP, 0.5 - 2 µl of the genomic DNA prep and *Taq* DNA polymerase in a 20 µl volume. PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining. Positive and negative controls were included in each set of reactions.

Sequencing of aborted SDSA events

Junction sequences from repair events were sequenced to understand the mechanism of joining. PCR was carried out with the forward primer 5'-CCCTGTCTGAAGTTCCGTAG-3' and reverse primer 5'-CCCTCGCAGCGTACTATTGAT-3', and products were sequenced with the forward primer.

Single-strand annealing assay

In this assay, a DSB is introduced by the *I-Sce*I enzyme (Figure 2.7) (Rong and Golic, 2003). The *I-Sce*I recognition sequence (I-site) is located between two copies of the *w* gene, a partial (3.6 kb) copy to the left and a complete (4.6 kb) copy to the right. If resection extends at least 3.6 kb to the left and 4.6 kb to the right, the two copies can anneal to produce a product that is diagnostic of SSA repair: loss of one repeat and the sequences between the repeats. Cutting was induced by crossing in a *P* element carrying *I-Sce*I under the control of a *hsp70* heat shock promoter. L2 larvae of different genotypes were heat shocked simultaneously at 38º for one hour.

Sequencing joining junctions of SSA events

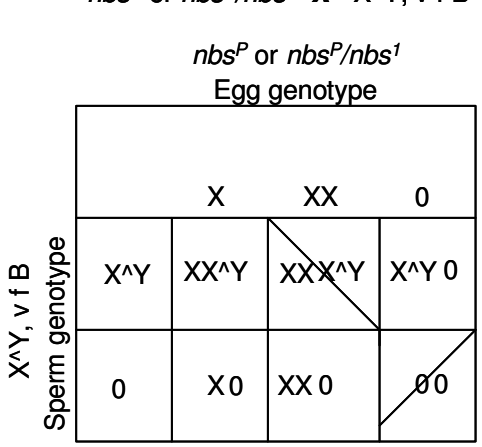
Sequence analysis of the PCR products that did not cut with *I-Sce*I was carried out to determine whether ends were joined with or without the use of microhomologies. PCR was carried out with the forward primer 5'-TGTGTGTTTGGCCGAAGTAT-3' and the reverse

primer 5'-CGCGATGTGTTCACTTTGCT-3', and products were sequenced using the forward primer.

Checkpoint assay

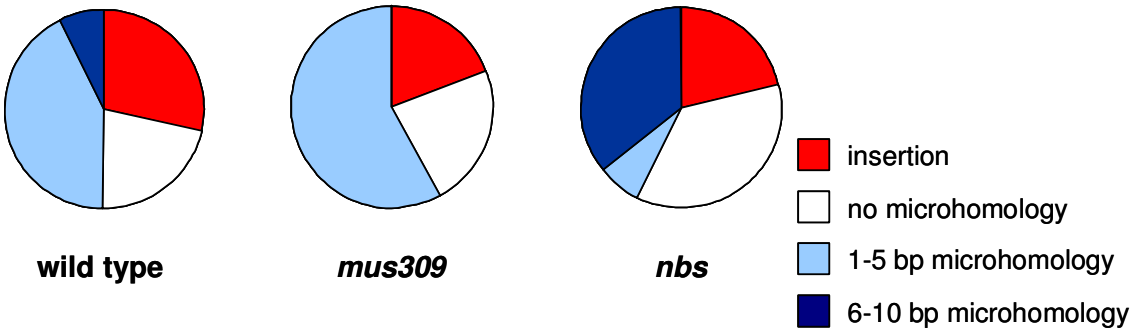
I measured the G2/M DNA damage checkpoint using an assay described previously (Brodsky et al., 2000). Imaginal discs were dissected from L3 larvae. Discs were isolated from wild type and mutant larvae 1 hour after exposure to 1000 and 4000 rads IR from the ^{137}Cs source. Imaginal discs were dissected in Ringer's solution and fixed in 4% formaldehyde and PBS with 0.1% Triton-X (PBT). Discs were washed and blocked in PBT with 5% bovine serum (BSA) and incubated in 1:1000 dilution of rabbit anti-phospho-Histone H3 antibody (Upstate Technologies # 06-570) in PBT overnight at 4^{o}. Following this, discs were incubated for two hours at room temperature with 1:1000 secondary goat anti-rabbit rhodamine-conjugated antibody (Molecular Probes), stained with 10 µg/mL DAPI in PBT and mounted in Flouromount-G (Southern Biotechnology Associates, Inc.). A Nikon Eclipse E800 fluorescent microscope was used to visualize the discs under the TRIT-C filter.

Supplemental Figures and Tables

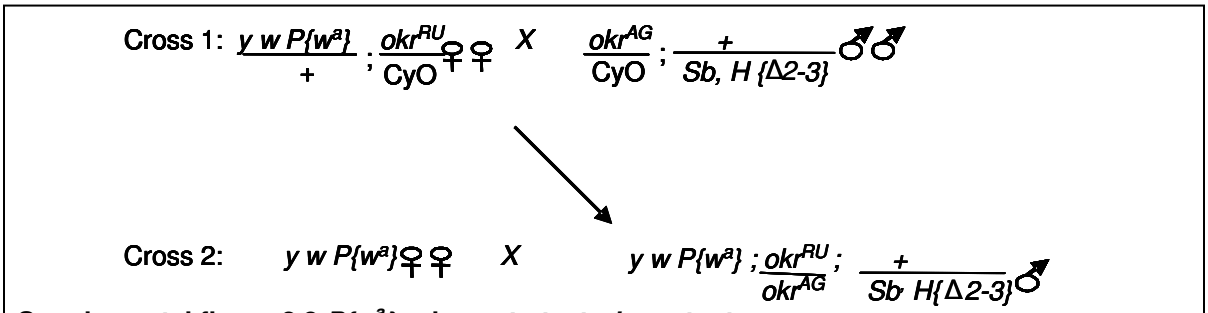


Supplemetal Figure 2.1 Nondisjunction assay in *nbs* mutants

Mutant females of genotype *nbs*^{P} or *nbs*^{P}/*nbs*^{1} were crossed to males with X chromosome attached to Y. These males could produce sperms of two genotypes only X^Y or nullo sperms. The Punnett square shown above shows the various genotypes of progeny, the crossed-out squares are inviable products. Products from XX or nullo females listed are results of nondisjunction. A dominant eye marker B on the X^Y chromosome is used for scoring different types of progeny. B males and non-B females can be produced only when there is nondisjunction of X chromosomes in the females. These exceptional progeny are shown in bold.



Supplemental figure 2.2 Distribution of different types of end joining in *nbs* mutants
The pie charts shown here depict the distribution of types of end joining junctions after *P*{*w*^{a}} excision in wild-type (n=14), *nbs*^{1}/*nbs*^{P} mutant (n=28), and *mus309*^{D2}/*mus309*^{D3} mutant (n=31) flies. Data for wild-type and *mus309* mutants is taken from *Adams et al., 2003*.



Supplemental figure 2.3 *P{w^a}* scheme to test *okr* mutants
Schematic of crosses set up for the *P{w^a}* assay: The non-autonomous *P* element can be mobilized when crossed to a transposase source *H{Δ2-3}*. This results in a gap formation which can be repaired by SDSA. This excision has been carried out in *okr* mutants as shown in cross 2. Daughters from cross 2 receive a repaired X chromosome from the father and a *P{w^a}* from the mother, are scored for eye color representative of repair events that occurred in their fathers.

End sequence after excision CTGCTGACCCAGACCATGATGAAATAACATA-3’ ACCCAGACTCTCAC GACGACTGGGTCTG 3’-ATACAATAAAGTAGTACTGGGTCTGAGAGTG
Junctions with microhomology CTGCTGACCCAGACTCTCACTTGACG (9) CTGCTGACCCAGACCATGACCCAGACTCTCAC (2) CTGCTGACCCAGACCATGATGACCCAGACTCTCAC
Junctions without microhomology CTGCTGACCCAGACCATGATGAAATAACATA:TATGTTATTTCATCATGACCCAGACTCTCAC CTGCTGACCCAGACCATGATGAAATAACATA:TGTTATTTCATCATGACCCAGACTCTCAC (2) CTGCTGACCCAGACCATGATGAAATAACATA:ACCCAGACTCTCAC CTGCTGACCCAGACCATGATGAAATAACATA:GACCCAGACTCTCAC CTGCTGACCCAGACCATGATG:CG CTGCTGACCCAGACCATGA:CG (2) CTGCTGACCCAGACCA:TGACGCG
Junctions with T-nucleotides CTGCTGACCCAGACCATGATGAAATAACATAaTATGTTATTTCATCATGACCCAGACTCTCAC CTGCTGACCCAGACCATGATGAAATAACATgTATGTTATTTCATCATGACCCAGACTCTCAC CTGCTGACCCAGACCATGATGAAATAACATA(at)$_{11}$aac(at)$_4$aac(at)$_3$(gt)$_2$tatACCCAGACTCTCA CTGCTGACCCAGACCATGATGAAATAA(taa)$_3$tataaTATGTTATTTCATCATGACCCAGACTCTC CTGCTGACCCAGACCATGATGAAATAACATgtattacataacATGTTATTTCATCATGACCCAGACTCT CTGCTGACCCAGACCATGATGAAATAtcatgaaatatcataTCATCATGACCCAGACTCTCAC

Supplemental Table 2.1 Junction sequences from repair of P-element induced breaks in *nbs* mutants.
At the top is the double-stranded sequence remaining after *P{w^a}* excision. The 17-nt 3’ overhangs of *P* element inverted repeat sequence are in black and the 8-bp target site duplication is in blue; flanking genomic sequence is in green. Junction sequences are given below (only the top strand is given). For junctions with microhomology, the microhomology is underlined. For junctions without microhomology, a colon separates sequences derived from the left from sequences from the right. T-nucleotides are indicated in lowercase red text. Numbers in parenthesis indicate the number of times we found a particular junction.

End sequence after cutting gggtacATTACCCTGTTAT CCCTAGCggccgc cccatgTAATGGGAC AATAGGGATCGccggcg	
Wild-type gggtacATTACCCTGTTAcattaTCCCTAGCggccgc gggtacATTACCCTGTTAT:CTAGCggccgc gggtacATTACCCTGTTAT:CCTAGCggccgc	 5 bp insertion 2 bp deletion (2) 1 bp deletion
nbs^{1}/*nbs*^{P} gggtacATTACCCTGTTAT:CCTAGCggccgc gggtacATTACCCT:ggccgc	 1 bp deletion (2) 9 bp deletion
mus309^{N1}/*mus309*^{D2} gggtacATTACCCTGTTAT:CCTAGCggccgc gggtacATTACCCTGTTA:CCCTAGCggccgc gggtacATTACCCTGTTAT:CTAGCggccgc gggtacATTACCCTGTTCCCTAGCggccgc gggtacATTACCCTG:CCCTAGCggccgc gggtacATTACCCTGTTAT:GCggccgc gggtacATTACCCCCTAGCggccgc	 1 bp deletion 1 bp deletion 2 bp deletion 2 bp deletion 4 bp deletion 5 bp deletion 7 bp deletion

Supplemental Table 2.2 Junction sequences from repair of *I-Sce1* induced breaks in *nbs* mutants.

Junction sequences after repair of *I-Sce*I-induced breaks from wild-type, *mus309*, and *nbs* mutants are shown. The I-site (uppercase black) and flanking sequences (lowercase green) are shown at the top. *I-Sce*I cuts to give a 4-nt 3' overhang. Inserted sequences are in red. For junctions that had neither an insertion nor a possible microhomology, sequences from the left and right end are separated by a colon. Numbers in parenthesis indicates the number of times we found a particular junction.

Genotype	Wild type	*nbs*^{1}/*nbs*^{SM9}		*nbs*^{1}/+	*okr*^{RU}/*okr*^{AG}	
Experiment	1	1	2	1	1	2
apricot	1716	767	1861	3406	311	333
red	126	5	42	122	0	6
yellow	187	127	322	514	488	422
Total (n)	**2029**	**899**	**2225**	**4042**	**799**	**761**
# of fathers (vials)	53	19	75	67	35	50

Supplemental Table 2.3 Raw number of flies scored for different repair events post *P* excision in *nbs* and *okr* mutant grounds

Eye color of progeny representative of repair events of *P*-element induced breaks generated in the germline in *nbs* and *okr* mutants

				After molecular analysis	
Genotype	Wild type	$\frac{nbs^{1}}{nbs^{SM9}}$	$\frac{mus309^{D2}}{mus309^{N1}}$	$\frac{nbs^{1}}{nbs^{SM9}}$	$\frac{mus309^{D2}}{mus309^{N1}}$
white	928	1830	1632	694	575
red	487	504	591	504	591
Total (n)	**1415**	**2334**	**2223**	**1198**	**1166**
# of fathers (vials)	20	20	20	20	20

Supplemental Table 2.4 Raw number of flies scored for different repair events post *I-Sce1* cutting

Eye color of progeny representative of repair events of *I-Sce1* induced breaks generated in the germline in *nbs* and *mus309* mutants

CHAPTER III

SENSITIVITY TO CROSSLINKING AGENTS, DSB INTERMEDIATE FORMATION & REPAIR

Introduction

Removal of ICLs from DNA requires participation of proteins involved in different repair mechanisms such as NER, MMR and HR (Reviewed in Friedberg, 2006 and Bergstralh and Sekelsky, 2007, Figure 1.4). ICL repair has been well-studied in the budding yeast *Saccharomyces cerevisiae* and to a greater extent in the prokaryotic system *Escherichia coli*. Extensive study of ICL repair in *Drosophila* is however lacking.

Majority of the models suggested for ICL repair are based on participation of HR proteins to repair a DSB intermediate. In an effort to establish that DSB intermediates are formed and repaired by canonical DSB repair pathway during crosslink removal in *Drosophila*, I assayed mutations sensitive to both MMS (DSBs are presumably a byproduct of exposure to this mutagen) and nitrogen mustard (crosslinking agent) (Laurencon et al., 2004) for sensitivity to IR, since majority of byproducts generated by IR are DSBs. Mutations that were sensitive to MMS, nitrogen mustard and IR were tested for defects in DSB repair using the *P*-element based assay discussed in details in Chapter II. Two such mutant alleles of *mus301* and eleven mutant alleles of *mus302* were tested for mutagen sensitivity and DSB repair defects.

One of the mutants carries a mutation in the *mus301* (*spnC*) gene and encodes a protein with a helicase domain reminiscent of the inter-strand crosslink repair protein, MUS308 (Laurencon et al., 2004 and McCaffrey et al., 2006). The name *spnC* comes from the female-sterile mutation *spindle-C*, which renders the mutant's ovary the shape of a spindle (Schüpbach et al., 1991, Gonzalez-Reyes et al., 1994 and Gonzalez-Reyes et al., 1997). This phenotype results from activation of meiotic checkpoint due to disruption of oogenesis as a result of failure to repair meiotic DSBs (Ghabrial, 1999). This gene is the ortholog of the human Hel308 gene, which encodes a protein belonging to the superfamily 2 of helicases and is implicated in recombination steps following replication fork restart in eukaryotes and archae (McCaffrey et al., 2006 and Richards et al., 2008). Mutation in *mus301* is sensitive to IR. I tested *mus301* mutants for defects in DSB repair. I used $mus301^{D1}$ and $mus301^{D2}$ alleles in this study. Both these alleles are sensitive to MMS and nitrogen mustard.

The second mutant discussed in this chapter carries a mutation in *mus302*. This mutation has not been mapped to a genetic locus. Recombination mapping has however localized this mutation to the third chromosome between two markers *st* and *cu* flanking the centromere (Boyd et al., 1981). Six *mus302* alleles were generated in an EMS screen in UC, Davis (numbered D1-D6), and five are part of the Zuker collection from Berkeley (numbered ZIII-1882, ZIII-2530, ZIII-4933, ZIII-5541 and ZIII-6004). Phenotypic analysis of these mutants showed that the mutation causes a viability defect associated with *P*-element transposition (Banga et al., 1991). It was thus suggested that the wild-type *mus302* gene is required for gap repair. Also, *mus302* mutants are defective in synthesis after UV irradiation, suggesting a post-replication repair role (Brown and Boyd, 1981). Consistent with the defective response to UV irradiation, it has also been suggested that MUS302 is required at late stages of nucleotide excision repair, post incision (Harris and Boyd, 1993). Additionally,

these mutants have defective meiotic functions such as meiotic drive in males (McKee et al., 2000).

I tested *mus302^{D2}/mus302^{D4}* for DSB repair defects. All the alleles listed in Table 3.1 were tested for IR sensitivity to determine the strongest allelic combination. I also employed deficiency mapping techniques using mutagen sensitivity phenotype to map this mutation.

Results

mus301 (spnC) *mutants are defective in repairing double-strand breaks when the only available homologous template is the sister chromatid*

I found that both, the *mus301^{D1}* homozygous mutants as well as the *mus301^{D1}/mus301^{D2}* are defective in repairing DSBs by SDSA (Figure 3.1, Supplementaltable 3.4), when the only available homologous template was the sister chromatid. Recently it was published that MUS301 is not required for DSB repair when the homologous chromosome is available as a template (Johnson-Schlitz et al., 2006).

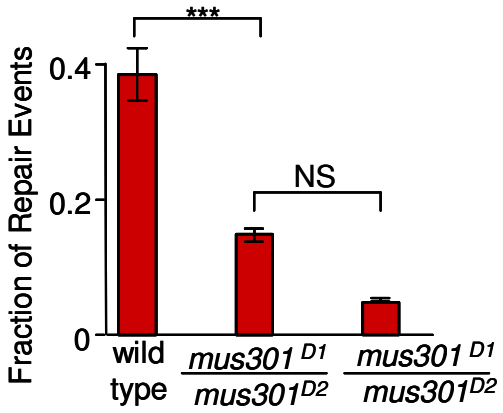


Figure 3.1 The mus301 mutants are defective in SDSA.
Repair outcome is scored as completed SDSA (red eyes) or other repair (yellow eyes). Each bar shows the mean fraction of all known repair events (red plus yellow) that were completed SDSA. Means were determined by scoring the progeny of each male independently (n=51 for wild type, 28 for *mus301^{D1}*, and 60 for *mus301^{D1}/mus301^{D2}*).Error bars indicate standard error of the mean.P values calculated for each pairwise comparison of wild type with both mutants by a non-parametric ANOVA (Kruskal-Wallis) test shows significant difference. No significant difference was found between *mus301^{D1} and mus301^{D1}/ mus301^{D2}.*

This study was based on the use of the same allelic combination as I used here. In addition, this heteroallelic combination also showed defect in SSA (Johnson-Schlitz et al., 2006).

Short repair synthesis tracts in mus301 *mutants*

Molecular analysis of aborted SDSA events from these mutants were carried out. Short repair synthesis tracts were detected (Figure 3.2), suggesting that MUS301 protein may be required during the synthesis step of SDSA. It may be hypothesized that the helicase function of this protein is essential for unwinding during synthesis. It has not been determined whether the helicase function is wild type in these mutants.

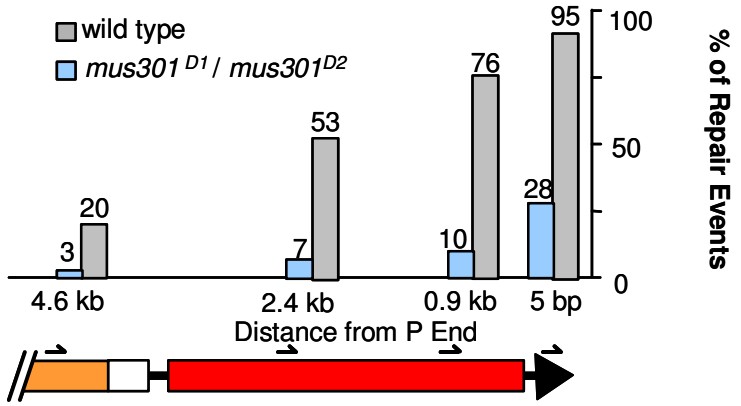


Figure 3.2 Molecular analysis of repair synthesis tract lengths.
Repair events from yellow-eyed progeny were analyzed to determine the extent of repair synthesis,if any. The right end of the *P*{*w*^{a}} element is shown (coloring as in Figure 2.4). Four PCR reactions were carried out to measure synthesis; the position of the innermost primer used in each is indicated. Bars indicate percentage of events analyzed that had synthesis tracts of at least 5 bp, 0.9 kb, 2.4 kb,and 4.6 kb (n=38 for wild type, 31 for *mus301*^{D1}/*mus301*^{D2}). P values were determined by Chi square and Fisher's exact test. Significant difference was found between wild type and *mus301* for all the PCR reactions.

mus302^{D2}/mus302^{D4} *mutants are not defective in repairing double-strand breaks*

It was previously shown that *mei-41* (homolog of the human checkpoint gene *ATR*) and *mus302* were both defective in recovery post *P* element excision (Banga et al., 1991). This viability defect of *mei-41* mutants was recapitulated while carrying out the *P{w*^{a}*}* assay (LaRocque et al., 2007). This assay also detected defects in DSB repair in backgrounds mutant for this checkpoint gene (LaRocque et al., 2007). I carried out the *P{w*^{a}*}* assay in the

mus302^{D2}/*mus302*^{D4} mutant background to verify if there are defects in DSB repair using the same allelic combination which previously showed lethality associated with *P*-element excision (Banga et al., 1991). However, I found that the *P{w*^{a}*}* induced breaks were repaired normally in *mus302*^{D2}/*mus302*^{D4} mutant background (Figure 3.3, Supplementaltable 3.4).

Wild type repair synthesis tract lengths in mus302 *mutants*

I subsequently carried out molecular analysis of the repair products to measure repair synthesis tract lengths and verify if synthesis looks wild type as well. Repair synthesis tract lengths appeared wild type, in these mutants (Supplemental Figure 3.1).

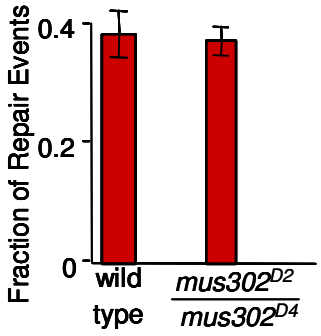


Figure 3.3 *mus302* mutants can carry out SDSA normally
Repair outcome is scored as completed SDSA (red eyes) or other repair (yellow eyes). Each bar shows the mean fraction of all known repair events (red plus yellow) that were completed SDSA (red eyes). Means were determined by scoring the progeny of each male independently (n=53 for wild type, n= 44 *mus302*^{D2}*/mus302*^{D4}). Error bars indicate standard error of the mean. P values calculated for each pairwise comparison of wild type with both mutants by a non-parametric ANOVA (Kruskal-Wallis) test shows significant difference. No significant difference was found between wild type *and mus302*^{D2}*/ mus302*^{D4}*.*

Strong alleles of *mus302*

The *mus302*^{D2}*/mus302*^{D4} mutants were tested for mutagen sensitivity to ensure that the stock still carried the mutation. Although this allelic combination did not show hypersensitivity to IR (Figure 3.4), hypersensitivity to MMS was observed, thereby confirming that the *Drosophila* stock was not contaminated. It is possible that this mutant allelic combination is weak .

Subsequently I tested all 11 alleles (Supplementaltable 3.1) for IR sensitivity at 4000rads, in all possible allelic combinations in a small-scale experiment. This experiment showed that *mus302*^{Z4933}/*mus302*^{Z6004} alleles are the strongest allelic combination, since no homozygous *mus302*^{Z4933} or *mus302*^{Z6004} survivors were observed when irradiated at this dose (Supplementaltable 3.2). A large scale IR sensitivity assay confirmed that the *mus302*^{Z4933}/*mus302*^{Z6004} mutants were hypersensitive to IR (Figure 3.4).

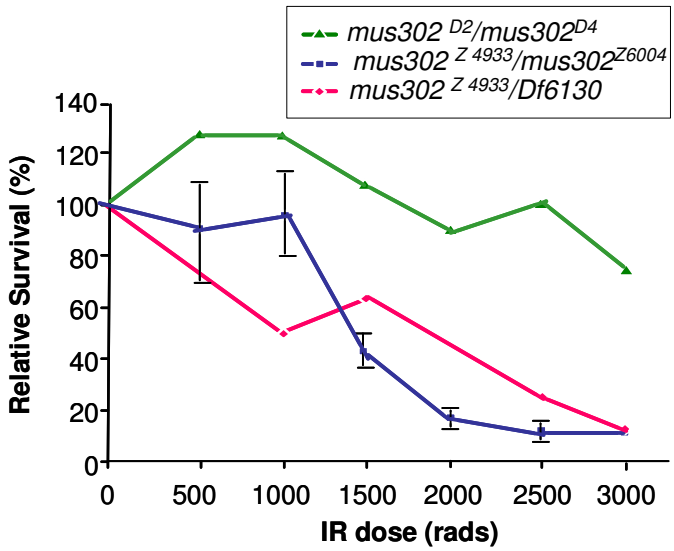


Figure 3.4 Sensitivity of *mus302* mutants to ionizing radiation.
An IR sensitivity assay of *mus302* mutants was carried out at the doses of gamma irradiation indicated on the X axis. Each point is the mean survival of mutants relative to survival of heterozygous siblings. Error bars for *mus302*$^{ZIII-4933}$/*mus302*$^{ZIII-6004}$ indicate standard deviation (n = 3-4 bottles for each point).

Interstrand crosslinks are repaired via a double-strand break intermediate

In other organisms it has already been demonstrated that ICLs are repaired via a DSB intermediate. I tested this in *Drosophila* and found an increase in γHis2av levels (phosphorylated histone variant, marker for DSBs) in larvae treated with the crosslinking agent nitrogen mustard (in collaboration with Kathryn Kohl).

A mutation on the 3rd chromosome, *mus312*, had previously been shown to physically interact with the *Drosophila XPF* homolog, *mei-9*. Mutants for this gene were

defective in meiotic recombination (Yildiz et al., 2002) and hypersensitive to nitrogen mustard. The human homolog of this mutation is defective in generating DSB intermediates after treatment in nitrogen mustard (in personal communication with Dan Bergstralh). Subsequently when this mutation was tested for DSB intermediate formation after treating with nitrogen mustard in *Drosophila* larvae, the same result was obtained (in collaboration with Kathryn Kohl; Figure 3.5). These mutants were used as a control to test if MUS302 is required for formation of DSB intermediate. Western blot with antibody to γHis2av (Figure 3.5) did not show any such defect in DSB formation post nitrogen mustard treatment in the *mus302* mutants.

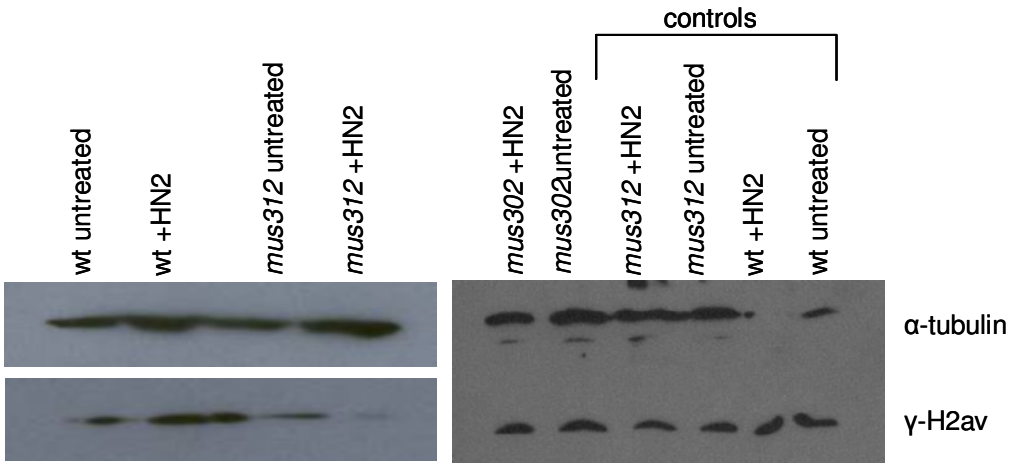


Figure 3.5 Double-strand break intermediate formation after treatment with the interstrand crosslinking agent nitrogen mustard.
The untreated and 0.05% nitrogen mustard treated wild type, *mus312*$^{ZIII\text{-}1973}$/*mus312*$^{ZIII\text{-}3997}$ and *mus302*^{D2}/*mus302*^{D4} larvae (10 larvae for each genotype) has been loaded on a SDS-PAGE gel and blotted with the γ-H2av antibody, marker for double-strand breaks. The α-tubulin marker is used as a loading control.

Interstrand crosslinks activate a checkpoint response

Checkpoint response is integral to DNA damage response. It is not known whether an ICL can activate a G2-M checkpoint or if generating a recognizable DSB intermediate is essential to activate this checkpoint. It has been suggested that the *Drosophila* counterpart

for ATR (DmATR) encoded by the *mei-41* gene is the main checkpoint kinase in *Drosophila*, (LaRocque et al., 2007). The MEI-41 protein is required to arrest cells in mitosis by responding to DSBs during all phases of the cell cycle (Bi et al., 2005; Brodsky et al., 2000; Garner et al., 2001; Hari et al., 1995; Jaklevic and Su, 2004; LaRocque et al., 2007; Sibon et al., 1999). To test if ICLs can activate the checkpoint, I isolated imaginal discs from *mus312* larvae and treated them with the same dose of nitrogen mustard at which these mutants showed low levels of DSB intermediate formation. Checkpoint was activated normally at these doses demonstrating that either ICLs can activate checkpoint response or low levels of DSBs are sufficient to elicit the checkpoint response (Figure 3.6).

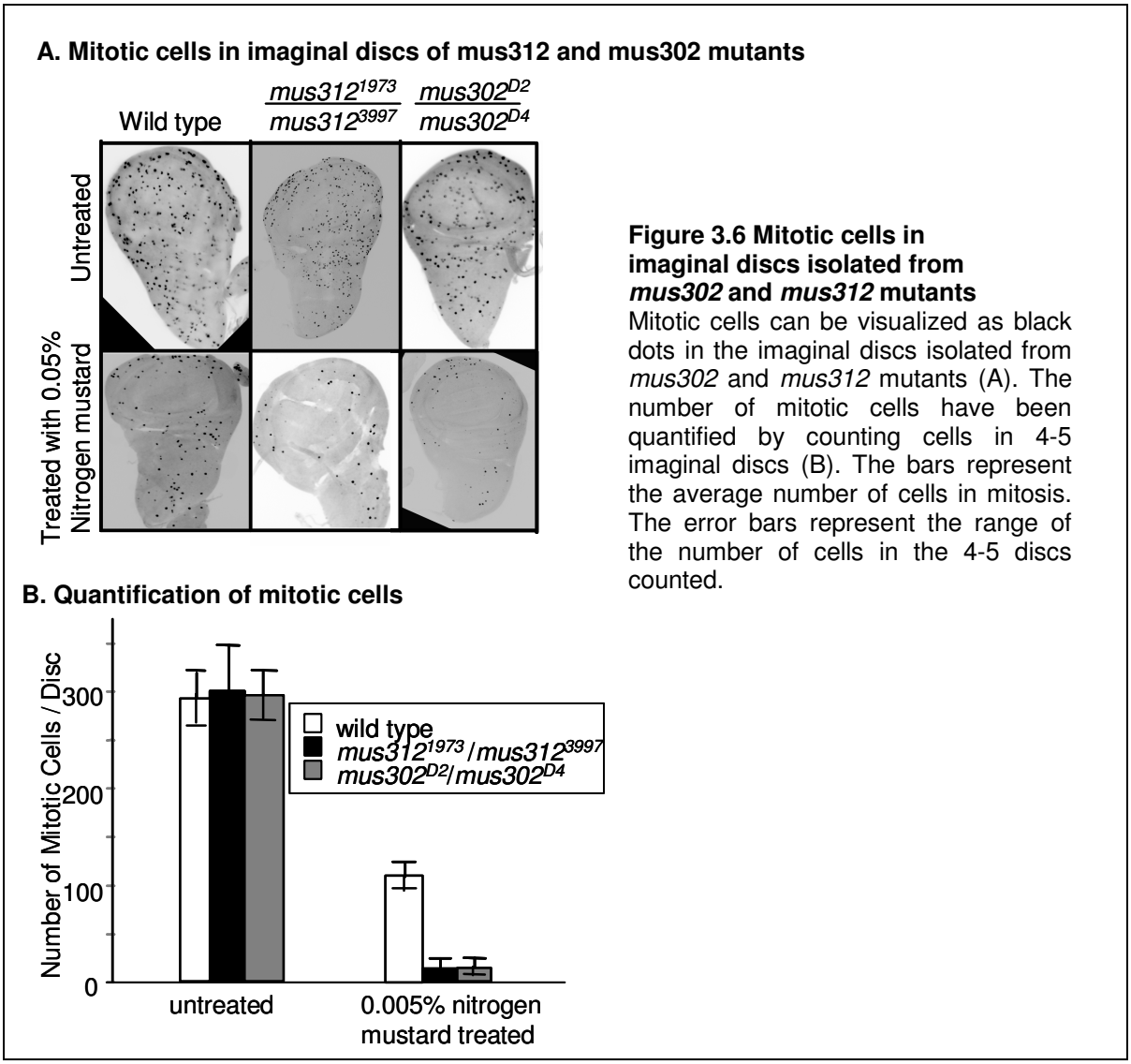


Figure 3.6 Mitotic cells in imaginal discs isolated from *mus302* and *mus312* mutants
Mitotic cells can be visualized as black dots in the imaginal discs isolated from *mus302* and *mus312* mutants (A). The number of mitotic cells have been quantified by counting cells in 4-5 imaginal discs (B). The bars represent the average number of cells in mitosis. The error bars represent the range of the number of cells in the 4-5 discs counted.

Notably, the number of cells in mitosis after treatment with nitrogen mustard in wild type is statistically more than the number of cells in mitosis post nitrogen mustard treatment in *mus312* and *mus302* mutants (Figure 3.6).

Deficiency mapping of mus302 *mutation*

The *mus302* mutation had previously been mapped between *st* and *cu* markers on the 3rd chromosome (Boyd et al., 1981), which is cytologically between 73A3-86D4. Males carrying deficiencies in this region were crossed to *mus302* mutant females and tested for sensitivity to MMS (SupplementalTable 3.3). Representative deficiencies are depicted in Figure 3.7.

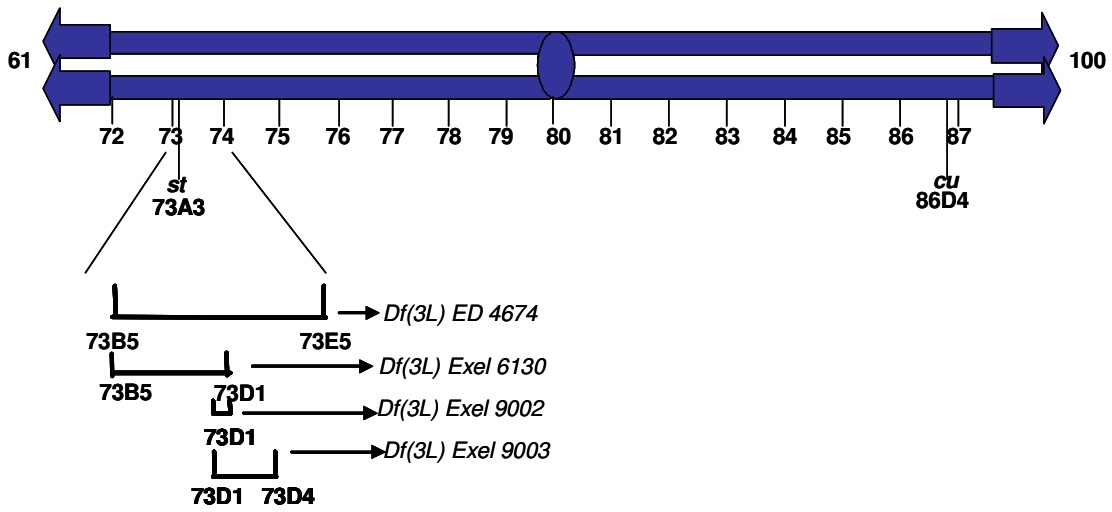


Figure 3.7 Deficiency mapping of *mus302*
The third chromsome starts from cytological location 61 at the telomere to 80 close to the centromere of the left arm of the 3rd chromosome (3L) and from 80 to the telomere at 100 of the right arm (3R).The figure shows regions 72-87, and the region 73-74 has been zoomed in. Recombination mapping mapped the *mus302* mutation between *st* and *cu* (Boyd et al., 1981). Deficiencies shown in the figure were crossed to the $mus302^{D2}$ and $mus302^{D4}$ alleles and tested for mutagen sensitivity. *Df(3L) ED 4674 and Df(3L) Exel 6130* were sensitive in combination with *mus302* mutant alleles. The smaller of the two deficiencies is *Df(3L) Exel 6130* and maps *mus302* within 73B5-73D1. Molecular end points of this deficiency is defined and narrows the region which carries the *mus302* mutation to 3L:16654391-16799748.

The smallest deficiency which is sensitive to these mutagens when in trans with a *mus302* mutation is *Df 6130*, which maps the *mus302* mutation to a smaller region 73B5-73D1 (molecularly 3L:16654391-16799748). There are 22 genes in this region, some of which have unknown function. Based on phenotypes the only likely candidate gene is Dbp73D which belongs to the DNA/RNA Helicase Superfamily 2 and could be important for DNA repair.

Discussion

MUS301 is required for HR repair using a sister chromatid

Repair during different stages of the cell cycle may require different proteins. In yeast, it was found that in G1 phase of the cell cycle, homologous chromosome was used 100% of the time for repair, whereas G2 repair was carried out using the sister chromatid close to 100% of the time (Kadyk and HartWell 1992). Thus it is possible that some mutants that are not defective in repair using a homologous chromosome as a template may be defective in repairing off a sister, because the wild type protein is only available during G1 repair.

In the assay used here, the *P{w^a}* element on the X chromosome was excised in males, thus no homologous chromosome was available as a template. This may be the reason why I see a defect in repair in these mutants unlike previous studies where a homologous chromosome was available (Johnson-Schlitz et al., 2007).

Excision of a somatic insertion of the *P{w^a}* element when both homologous chromosomes carry the *P{w^a}* element may help us address the question whether this protein directs repair off a homologous template, unambiguously. In wild type *Drosophila*, given the choice, both sister chromatid and homologous chromosomes may be used as templates for repair (in personal communication with Sabrina Andersen).

Short repair synthesis tracts in *mus301* mutants

Short repair synthesis tracts in these mutants suggest that MUS301 is required in late stages of SDSA. Targeted mutations to disrupt the helicase domain may help us understand if a helicase activity requirement during SDSA underlies defects seen in *mus301* mutants. Mutations in another helicase, the *Drosophila* homolog of the Blm helicase MUS309, also showed short repair synthesis tracts. The aborted SDSA class of *mus309* mutants were found to be associated with large deletions (McVey et al., 2004). It will be interesting to study if repair byproducts in *mus301* helicase dead mutants are also associated with large deletions indicative of a similar deleterious repair pathway.

Double strand break repair in *mus302* mutants

The $mus302^{D2}/mus302^{D4}$ mutants are sensitive to MMS. However, they are neither sensitive to IR nor did they show a defect in SDSA. Another mutant allelic combination $mus302^{ZIII\text{-}4933}$ / $mus302^{ZIII\text{-}6004}$ is however hypersensitive to IR. The drawback of experiments using the $mus302^{D2}/mus302^{D4}$ alleles may be that they are not reflective of a complete lack of function of MUS302. It is thus important to test the $mus302^{ZIII\text{-}4933}$ / $mus302^{ZIII\text{-}6004}$ allelic combination for DSB repair defects, to further explore possible function of this protein which is required for responding to IR damage.

Activation of checkpoint in response to ICLs

It remains elusive whether the small number of DSB intermediates formed in *mus312* mutants is sufficient to activate the checkpoint and whether this checkpoint is MEI-41 (DmATR) dependent. It is possible that in presence of low levels of DSB intermediate formed post exposure to crosslinking agent, the TEFU (DmATM) protein, which is required to respond to low doses of IR (Bi et al., 2005), is the main checkpoint kinase.

Materials and Methods

Drosophila stocks and genetics

Drosophila stocks were maintained on standard medium at 25º. The *mus301* mutations used were $mus301^{D1}$ *and* $mus301^{D2}$. Both these alleles are available at the Bloomington stock center (# 916 and #917 respectively). The *mus302* mutant alleles $mus302^{D2}$, $mus302D^{D4}$ are also available at the Bloomington stock center (#920 and #101693 respectively). The *mus302* Zuker alleles $mus302^{ZIII-1882}$, $mus302^{ZIII-2530}$, $mus302^{ZIII-4933}$, $mus302^{ZIII-5541}$ and $mus302^{ZIII-6004}$ as well as the *mus312* Zuker alleles $mus312^{ZIII-1973}$ and $mus312^{ZIII-3997}$ were obtained from Berkeley. All these third chromosome mutations are balanced with TM6B*, Tb* or TM3*, Sb.* The TM6B*, Tb* balancer renders larvae tubby shape, and was used for larval studies to distinguish between homozygotes and heterozygotes. The TM3*, Sb* balancer with a stubble bristle phenotype was used to score homozygotes in adults.

Methyl methane sulphonate sensitivity assay

Males and females balanced with the third chromosome balancer TM3*, Sb* were crossed in vials and allowed to lay for two days before being turned over into new vials. After incubating the second vial for 2 days, the flies were dumped. One day after dumping the flies, 125 µl of 0.025%, 0.005% and 0.008% MMS was added to the second set of vials. The first set of vials provided the untreated control. Adults that eclosed from these vials were counted to determine the ratio of balanced (heterozygous) to mutant (homozygous or heteroallelic) to determine the observed ratio. The expected ratio of balanced to mutant flies was determined from un-irradiated bottles. Relative survival of treated mutants was calculated from the ratio of observed to expected. I M Sodium Hydroxide was used to decontaminate all vials, pipette tips and tubes used to make the MMS mutagen solution.

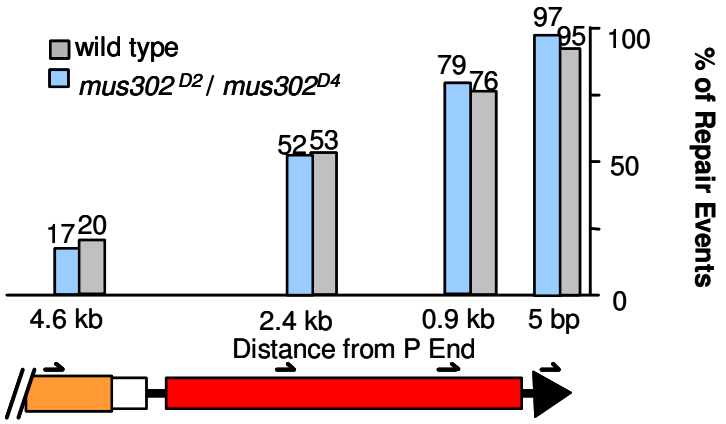
Nitrogen mustard sensitivity assay

This assay was set up in a similar way as the MMS sensitivity assay. Concentration of nitrogen mustard used was 0.025% or 0.005% per vial. Potassium thyoglycolic acid solution was used to decontaminate all vials, pipette tips and tubes used to make the nitrogen mustard mutagen solution.

Deficiency mapping

Deficiency mapping was carried out by crossing Deficiency females to *mus302*^{D2} and *mus302*^{D4} males, and treating them with MMS or nitrogen mustard as described in the mutagen sensitivity assay. Relative survival of *Df/mus302* treated mutants was recorded. The *Df/mus302* combination that showed sensitivity to a similar or greater extent than the *mus302*^{D2} */ mus302*^{D4} was recorded as the *Df* that takes out the region carrying the *mus302* genetic loci.

Supplemental figure



Supplemental Figure 3.1 Molecular analysis of repair synthesis tract lengths.
Repair events from yellow-eyed progeny were analyzed to determine the extent of repair synthesis, if any. The right end of the *P*{*w*^{a}} element is shown (coloring as in Figure 2.4). Four PCR reactions were carried out to measure synthesis; the position of the innermost primer used in each is indicated. Bars indicate percentage of events analyzed that had synthesis tracts of at least 5 bp, 0.9 kb, 2.4 kb, and 4.6 kb (n=38 for wild type, 31 for *mus301*^{D1}/*mus301*^{D2}).

Supplemental Tables

mus302^{D1}	Sensitive to MMS+ nitrogen mustard+ IR
mus302^{D2}	Sensitive to MMS+ nitrogen mustard
mus302^{D3}	Sensitive to MMS+ nitrogen mustard
mus302^{D4}	Sensitive to MMS+ nitrogen mustard
mus302^{D5}	Sensitive to MMS+ nitrogen mustard
mus302^{D6}	Sensitive to MMS+ nitrogen mustard
mus302$^{ZIII-1882}$	Sensitive to MMS
mus302$^{ZIII-2530}$	Sensitive to MMS
mus302$^{ZIII-4933}$	Sensitive to MMS+ nitrogen mustard
mus302$^{ZIII-5541}$	Sensitive to MMS+ nitrogen mustard
mus302$^{ZIII-6003}$	Sensitive to MMS

Supplemental table 3.1 Mutagen sensitivity of various *mus302* alleles.

IR sensitivity (4000 rads)

Ratio of Homozygous/balanced flies					
Genotype	Untreated (expected)	N	Treated (observed)	N	Relative survival (%)
mus302$^{ZIII-2530}$x mus302^{D2}	1.0	111	0.11	35	11
mus302$^{ZIII-1882}$x mus302^{D4}	0.5	205	0.15	30	31
mus302$^{ZIII-4993}$x mus302^{D4}	0.5	117	0.16	45	33
mus302$^{ZIII-2530}$x mus302^{D4}	0.5	137	0.29	28	58
mus302$^{ZIII-4993}$x mus302$^{ZIII-6004}$	0.4	161	0	48	0
mus302$^{ZIII-4993}$x mus302$^{ZIII-2530}$	0.6	185	0.05	43	8
mus302$^{ZIII-2530}$x mus302^{6004}	0.4	142	0.14	23	35

Supplemental Table 3.2 Strongest allelic combination determined by IR sensitivity
This table represents IR sensitivity of different mutant allelic combinations of *mus302.* N represents the number of flies scored. The allelic combinations tested with low 'N' value has not been included in the table.

Deficiency mapping by MMS sensitivity (0.3%)

Ratio of Homozygous/balanced flies					
Genotype	Untreated (expected)	N	Treated (observed)	N	Relative survival (%)
Df 9003 X mus302^{D2}	0.8	156	1.1	58	138
Df 6130 X mus302^{D2}	1.0	107	0	50	0
Df 6130 X mus302^{D4}	0.7	172	0	70	0
Df 9003 X mus302^{D4}	0.8	138	0.7	128	88
mus302^{D2} X mus302^{D4}	0.9	102	0	53	0
Df 9003 X mus302^{D4}	0.9	43	0.8	107	89
Df 9002 X mus302^{D4}	0.4	48	2.3	135	575
Df 6130 X mus302^{D4}	0.9	30	0	83	0
mus302^{D2} X mus302^{D4}	0.7	32	0	81	0
Df 6130 X mus302^{D4}	1.0	104	0	52	0
Df 9003 X mus302^{D2}	0.7	168	0.9	58	128

Supplemental Table 3.3 Deficiency mapping of *mus302* by MMS sensitivity
Deficiencies were crossed to *mus302* mutants and tested for MMS sensitivity at two doses shown above. Relative survival was calculated by determining the homozygous/balanced ratio for untreated and treated crosses. Observed/expected ratio was used to calculate relative survival of *Deficiency/mus302* mutants.

Deficiency mapping by MMS sensitivity (0.5%)

Ratio of Homozygous/balanced flies					
Genotype	Untreated (expected)	N	Treated (observed)	N	Relative survival (%)
Df 9003 X mus302^{D2}	0.6	107	0.6	70	100
Df 9003 X mus302^{D4}	0.5	208	0.6	110	123
Df 9002 X mus302^{D4}	0.4	206	0.7	132	175
Df 6130 X mus302^{D2}	0.8	105	0	26	0
Df 6130 X mus302^{D4}	0.2	295	0	53	0
Df 9002 X mus302^{D4}	0.3	20	1.0	126	333
Df 9003 X mus302^{D4}	0.6	58	0.4	151	67
Df 6130 X mus302^{D4}	0.7	20	0	63	0
mus302^{D2} X mus302^{D4}	0.6	94	0	69	0
Df 9003 X mus302^{D2}	0.6	110	0.7	161	117
Df 6130 X mus302^{D2}	0.7	110	0	48	0
Df 6130 X mus302^{D2}	0.8	79	0	48	0
Df 6130 X mus302^{D2}	0.6	59	0	32	0
Df 6130 X mus302^{D2}	0.8	66	0	39	0
Df (ED4674) X mus302^{D2}	0.7	75	0	36	0
Df (ED4674) X mus302^{D2}	0.8	79	0.04	45	5
Df (ED4674) X mus302^{D2}	0.8	60	0	28	0

Genotype	Wild type	*mus301^{D1}*	$\frac{mus301^{D1}}{mus301^{D2}}$	$\frac{mus302^{D2}}{mus302^{D4}}$
apricot	1716	541	1141	1939
red	126	14	66	76
yellow	187	812	669	160
Total (n)	**2029**	**1367**	**1876**	**2175**
# of fathers (vials)	53	28	60	44

Supplemental Table 3.4 Raw number of flies scored for various repair events post *P* excision

Eye color of progeny representative of repair events of *P*-element induced breaks generated in the germline in *mus301* and *mus302* mutants.

CHAPTER IV

GENERAL DISCUSSION AND FUTURE DIRECTIONS

This study identifies several requirements for DNA damage response, mostly to DSBs and lays the foundation for extensive study to understand the intricacies of every response. In the first half of this thesis I discuss the role of NBS in responding to DSBs. The experiments were carried out in backgrounds that retained partial function of NBS. In this chapter I discuss possible ways in which damage response can be carried out in complete null *nbs* backgrounds in *Drosophila*. In addition I discuss the players in SDSA that have been identified thus far, and the requirements that are yet to be identified.

Study of *nbs* null mutants

Rescue of lethality

Several defects are found associated with mutations in *nbs*. However, it is not clear what makes these mutations lethal. Chromosome end-to-end fusions have been found in these mutants and could be a possible cause of lethality. If Ligase IV is required for this end-to-end fusion then a mutation in *ligase IV* should rescue this lethality. I was unable to rescue the lethality using a mutation in this gene. However, cytological evidence showing the absence of fusions in *nbs Ligase IV* double mutants is required to verify if Ligase IV is actually required for these fusions. Also it is unknown whether the NHEJ machinery is required for chromosome end fusion in *Drosophila*. The other possible cause of lethality could be the defective checkpoint in these mutants, which allows unrepaired damage to be passed on to daughter cells. I attempted to rescue the lethality by slowing down the cell cycle progression by reducing the Cyclin B dose in half using heterozygous *cyclin B* mutants

This mutation did not rescue the lethality. Mutations in *cyclin A* exists in *Drosophila* and heterozygous carriers of both *cyclin A* and *cyclin B* mutations can be used to verify if the defects in cell cycle checkpoint is responsible for lethality in these mutants.

A third possibility is to test if increasing NHEJ can compensate for the decrease in HR and rescue lethality. However, this is the least likely cause of lethality, since in other organisms it has been determined that although expression of the C-terminus of this protein rescues lethality (Kang et al., 2002 and Williams et al., 2002), mutants expressing such truncated versions of the protein are still defective in DNA repair. This suggests that the cause of lethality is independent from the repair defect.

Meiotic recombination in nbs *null mutants*

Nondisjunction was found in *nbs* mutants suggesting that NBS may be required during meiotic recombination. Surprisingly, meiotic crossing over appeared wild type in the hypomorphic mutants. A test of these defects in null mutants will unambiguously help us understand if there are any defects in meiotic crossing over. Mosaic *Drosophila* can be created where cells carrying homozygous lethal mutation can develop in *Drosophila* which is heterozygous for the same mutation and this will allow the study of meiotic defects in the null mutants (Perrimon et al., 1998).

This technique relies on making a female *Drosophila* with a DFS (Dominant Female Sterile) mutation and the mutation of our interest on separate homologous chromosomes which also carry FRT (FLP recombinase target) sites (Figure 4.1). Oogenesis is blocked in females carrying the DFS mutation, thus no eggs will be laid by these females. However, if FLP catalyzes recombination between the FRT sites on homologous chromosomes then following sister chromatid segregation some germline cells will be homozygous for *nbs*

mutation and such cells of the ovaries will be capable of producing functional eggs, unless NBS is essential for oogenesis in which case a requirement of NBS during oogenesis will be confirmed by using a wild-type control.

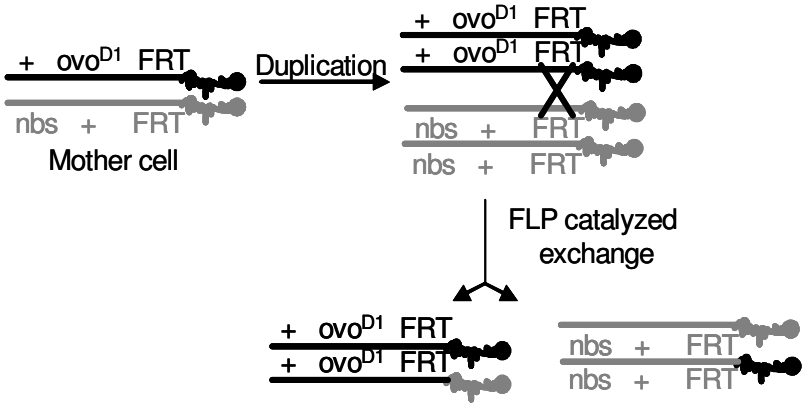


Figure 4.1 Making germline clones of *nbs* null alleles

Ovaries that received the DFS mutation will not produce any functional eggs. Non-disjunction and crossing-over can be assayed in mutants that have no maternal contribution of NBS using this technique.

This experiment can also verify if *Drosophila* NBS plays a role in two highly coordinated processes in meiosis: meiotic recombination and oogenesis. During *Drosophila* oogenesis the development of germcells and overlying follicle cells is highly coordinated. The follicle cells form epithelium around a syncytium of nurse cells and oocyte. Cell-cell communication is orchestrated such that domains of follicle cells are patterned (Dobens et al., 2000) and these follicle cells secrete the egg shell and create specific eggshell structures. Miscommunication between oocyte nucleus and overlying follicle cells can cause mispatterning of egg shells. Such mispatterned egg shells have been observed in mutants defective in meiotic recombination such as *spn-A*, *spn-C or mus301*, *spn-D* and *okra* (Morris et al., 1999). The name *spn-A* comes from 'spindle' referring to the mispatterned eggs in this mutant that appear as spindles on a spinning wheel (Staeva-Vieira, E et al., 2003; Radford et al., 2004). This patterning defect can be rescued by mutation in *mei-41*, which encodes a checkpoint protein. It is believed that defects in meiotic recombination turn on the cell cycle checkpoint, which arrests the cell cycle while oogenesis is still progressing. This disrupts

proper signaling between the oocyte and follicle cells during eggshell patterning and causes patterning defects (Staeva-Vieira et al., 2003). However, since the NBS protein has checkpoint activation functions, mispatterened eggs found in the *spn* mutants may not been found in *nbs* mutant backgrounds.

Epistasis analysis with known meiotic recombination mutants can help decipher what step of this process requires a functional NBS protein. Mutation in *mei-w68*, which encodes for a DSB creating protein which is an ortholog of yeast *spo11* (McKim et al., 1999), results in high levels of non-disjunction and no meiotic crossing-over. This mutation also rescues the eggshell patterning defect in *spnA* mutants. This suggests meiotic recombination is initiated by DSBs made by MEI-W68 and these breaks go through recombination in a SPN-A dependent meiotic recombination pathway.

Possible Phenotypes	**Suggested functions of NBS**
No cross overs, normal egg shell	Acts in a complex with MEI-W68 to make DSBs, high levels of nondisjunction
Persistence of breaks	Required for resecting DSBs, Maybe required for checkpoint
Egg shell patterning defect	Required for resecting DSBs but not for MEI-41-mediated checkpoint
Rescues egg shell patterning defect of *spn-A* mutants	Either it acts upstream of SPN-A or is required for MEI-41 dependent checkpoint or both
Persistence of DSBs and egg shell- patterning defect	Only required for resecting step, not for making DSBs or for checkpoint

Table 4.1: Epistatic analysis
Phenotypes of meiotic recombination mutants suggestive of their role in meiotic recombination, useful in placing NBS at a step of meiotic recombination where it is required, by epistasis analysis

In absence of *spnA,* it has been observed that there is persistence of DSBs. Yeast studies show that the yeast *nbs* homolog, *xrs2* encodes a protein involved in a complex required to make DSBs (Ohta et al., 1998). It can be determined if this role is conserved in *Drosophila*.

Study of mitotic repair in nbs *null mutants*

Assaying repair of breaks in larvae will allow the study of *nbs* null mutants which are viable at larval stages. A novel technique has been developed in the lab (previously proposed and described in details in the dissertation by Jeannine LaRocque, 2007) to study repair in larval tissue. In addition, to giving us the ability to correlate our study on hypomorphic mutations, this assay will also help us correlate results obtained from damage induced in mitotic germline cells to those in somatic cells in imaginal discs of larvae.

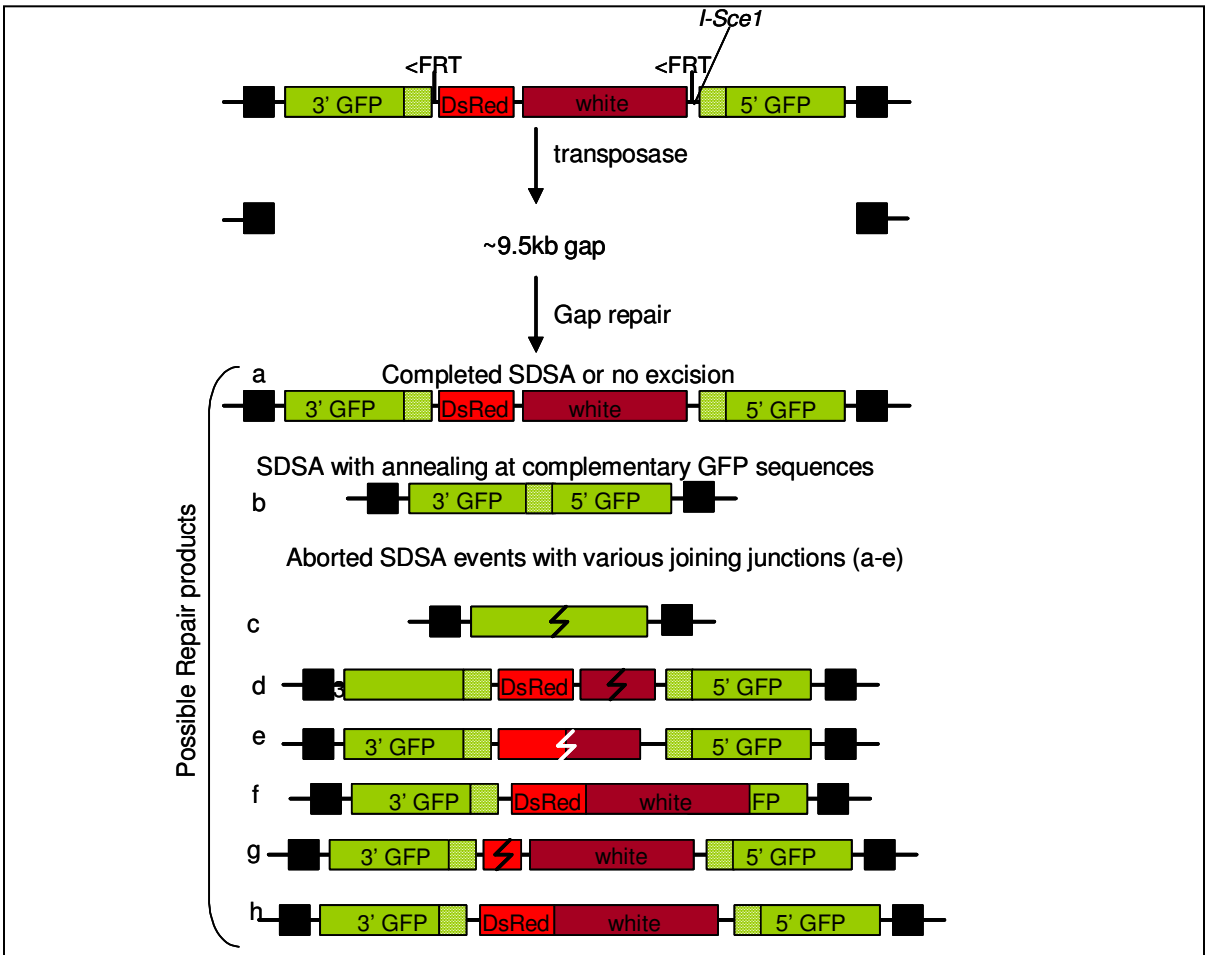


Figure 4.2 SDSA in larval cells
Larvae carrying the construct *P{DsRed, GFP}* have imaginal discs expressing DsRED. In presence of transposase, *P{DsRed, GFP}* excises to generate a 9.5kb gap which can be repaired by SDSA. a) Completed SDSA regenerates the *P{DsRed, GFP}*. b) Synthesis from both ends followed by annealing at complementary GFP sequences gives rise to GFP expression in the discs. Aborted SDSA events may or may not be associated with DsRed expression. No GFP expression can be from aborted SDSA events. Repair products c-h show representative repair junctions from aborted SDSA events.

Similar to the *P{w^a} assay*, this larval assay relies on excision of a *P{GFP, DsRed}* element in presence of a transposase source. DsRed is expressed ubiquitously in imaginal discs of larvae carrying this construct. SDSA followed by annealing at the direct repeats of GFP (Green Florescence Protein) complimentary sequence will result in GFP expression corresponding to the loss of DsRed expression (Figure 4.2). Disadvantages of using this assay over the mitotic germline assay is that aborted SDSA (classes f and h, Figure 4.2), SDSA which copies back the entire *P{GFP, DsRed}* (class a, Figure 4.2), element and no excision event cannot be distinguished. Thus simultaneous assaying of HR and NHEJ in *nbs* nulls cannot be carried out.

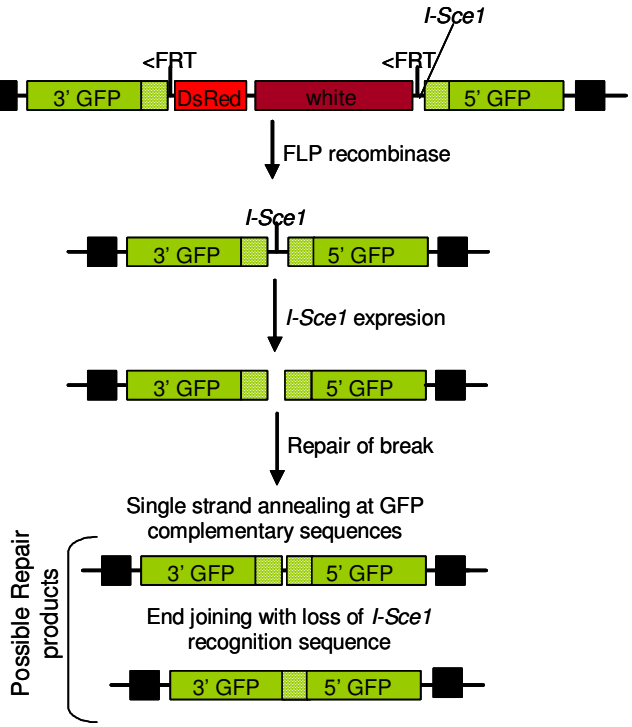


Figure 4.3 SSA in larval cells
Larvae carrying the construct *P{DsRed, GFP}* when crossed to a FLP recombinase source, region between the two FRTs shown in the figure collapses to generate *P {GFP},* where complementary GFP direct repeats flank an I-Sce1 recognition site. Expression of I-Sce1 generates breaks in larval cells carrying this construct. Repair that results in GFP expression could only arise from resecting the breaks and joining by SSA. No GFP expression results from either no cutting events or from end joining events. Precise end joining may regenerate the I-Sce1 recognition sequence whereas imprecise end-joining may result in loss of this sequence.

The *P{GFP, DsRed}* element consists of FRT sequences flanking the DsRed sequence and thus can be collapsed to *P{GFP}* in the presence of FLP recombinase (Figure 4.3). The *P{GFP }* construct can be used to assay SSA in a similar way as described in the mitotic germline cells in Chapter II. SSA at the GFP direct repeat complimentary sequence will lead to expression of GFP (Figure 4.3). This assay in null mutants can help verify if the MRN complex is dispensible for resection or not.

Homology mediated repair in *Drosophila* by SDSA: identification of various players

Homology mediated repair is a complex repair pathway requiring contribution of several proteins to enable accurate repair. Some of the players in this pathway have been determined and some have been found to be functionally conserved from single-cellular to multi-cellular eukaryotes. In *Drosophila*, excising *P* elements in the germline cells creates a gap which is repaired by SDSA. The *P{w^a}* assay described in previous chapters identified several players in this pathway (Adams et al., 2003; McVey et al., 2004a; McVey et al., 2004b and LaRocque et al., 2007).

This study does not rule out the possibility that the MRN complex is required for resecting breaks, however it does not seem to be essential for this function. Additionally, this study suggests novel functions that this complex might be required such as detecting or processing ends of the intermediate generated after dissociation of the invading strand from the template and prior to annealing (Figure 4.4).

Previous studies have demonstrated that DmRad51 seems to be functionally conserved and is required for strand invasion (McVey et al., 2004). In Chapter II here, I discuss that DmRad54 is required for the strand invasion function, but is not absolutely essential for this function. However, requirement of DmRad54 during SDSA seems to be multiple times during the several rounds of strand invasion. It is possible that the DmRad51

and DmRad54 function as a complex to carry out this function. Biochemical studies need to be carried out to determine if there is physical interaction between these two proteins.

The DmBlm protein has been suggested to function at the dissociation step during SDSA (Adams et al., 2003). Here I hypothesize that MUS301 helicase may function prior to the DmBlm during synthesis or in conjunction with it during dissociation (Figure 4.4).

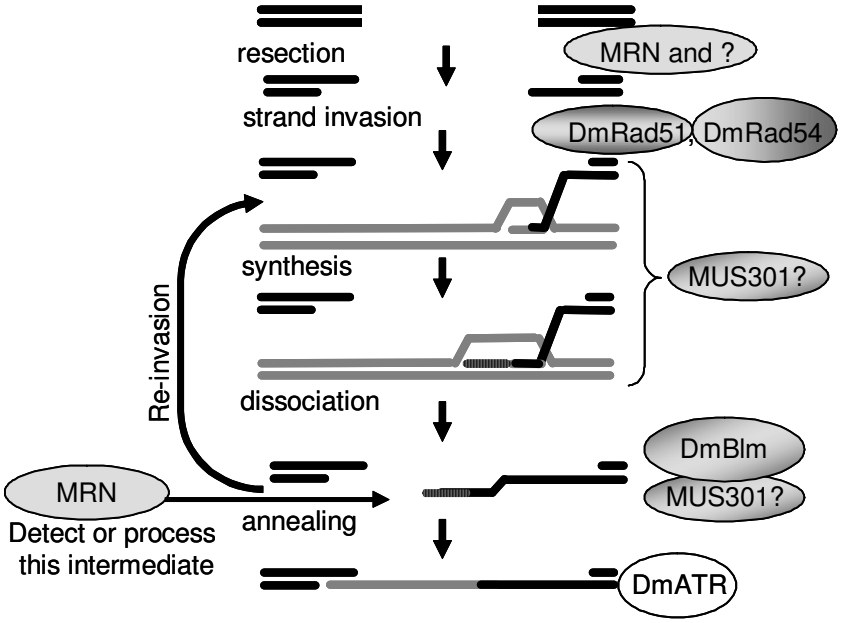


Figure 4.4 Players required for repair of *P*-element induced gaps by SDSA in Drosophila

However, this protein function is probably only required when the available homologous template is the sister chromatid, since previous studies have shown that execution of HR using homologous chromosome as a template is carried out normally in *mus301* mutants (Johnson-Schlitz et al., 2007).

Only one player required during SDSA after the synthesis step, has been identified. The checkpoint kinase DmATR or MEI-41 has been implicated in the annealing step post synthesis during SDSA (Figure 4.4) (LaRocque et al., 2007). There is still potential of identifying new players in the SDSA pathway, to better understand the intricacies of this repair in *Drosophila*. It will be interesting to identify if any proteins are redundant with MRN for the resection function. One of the key players that are thought to participate in SDSA is

the polymerases. Repair synthesis does not seem to be processive (McVey et al., 2004), it will be interesting to identify if any bypass polymerases are required to initiate synthesis during SDSA.

End-joining as a result of aborting the SDSA pathway seems to be Ligase IV independent (McVey et al., 2004). The requirements of this non-canonical end joining pathway still need to be identified.

DNA damage response to complex lesions involving both strands of the double-helix

DNA damage such as ICLs and DSBs pose serious threat to cell integrity if unrepaired or incorrectly repaired. An understanding of several aspects of DNA damage response to ICLs and DSBs is very important since normal metabolic errors can cause ICLs or DSB formation. Thus a thorough study of the requirements of damage response to these complex lesions is necessary to understand corrective measures that cells take when they encounter such damage. This will help in translating normal mechanisms artificially in cells lacking such response to aid therapy.

Also, exogenous sources of such damage are sometimes unavoidable. Early in the 20th century, medical science began using ionizing radiation such as short wavelength X-rays for radiographic procedures. Advantages of the use of this are that these rays can pass through tissues and provide an easy and non-invasive procedure for diagnosis. Extensive exposure to such rays can however causes DSBs in DNA.

Since ICLs block normal metabolic processes in cells, crosslinking agents have been used to destroy cancer cells. However, heterogenous tumor response to crosslinking agents such as Mitomycin C poses challenges for targeting tumor cells. A thorough understanding of what alleviates ICL toxicity as Well as what enhances it is thus essential for either enhancing normal tissue sustenance when exposed to ICLs or enhancing susceptibility of cancer cells to ensure successful chemotherapy.

Since DSBs form an intermediate during repair of ICLs, study of repair of DSBs will help progress in this field of study as Well. Several questions need to be ansWered such as: Are these DSB intermediates repaired in the same way as the DSBs generated by other genotoxic agents?

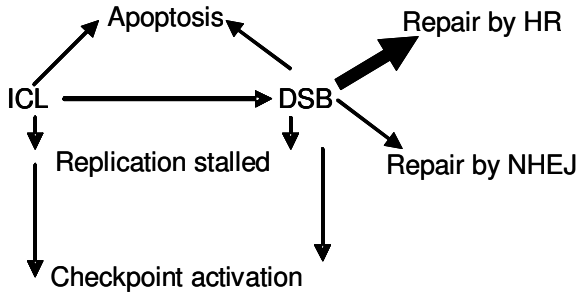


Figure 4.5 Model for repair of complex lesions involving both strands of DNA in Drosophila
ICLs are repaired via a DSB intermediate. Both DSBs and ICLs can activate a checkpoint response. DSBs are primarily repaired by HR in Drosophila. ICL induced DSBs are repairedby HR. Cells with unrepaired ICL and DSB may go down the apoptotic pathway.

What checkpoint proteins are activated during this response? The schematic in Figure 4.5 shows the DNA damage response events that occur in cells as a consequence of formation of complex lesions such as DSBs and ICLs.

This work has contributed to understanding molecular mechanisms that define clinical implications underlying Nijmegan Breakage Syndrome disease as Well as heterozygous carriers for this disease. In addition this work re-establishes the importance of the Rad54 protein during HR in *Drosophila*. Also, a novel player in SDSA, MUS301, has been identified. This work opens new doors as it outlines avenues for future work in better understanding of DNA damage response to DSBs and ICLs.

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