DmBLM's Functions in DNA Repair and Recombination

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ABSTRACT

Sabrina L. Andersen: DmBLM's Functions in DNA Repair and Recombination

(Under the direction of Jeff Sekelsky)

Maintaining stable chromosomes requires an array of repair and recombination proteins. These proteins ensure that chromosomes are accurately replicated, repaired, and segregated. One such protein is the RecQ-family helicase BLM. In humans, absence of BLM causes Bloom syndrome, which is characterized by proportional dwarfism and the early onset of a broad spectrum of cancers. Cells mutant for BLM are genomically unstable, showing increased chromosome deletions, rearrangements, and sister chromatid exchange. Using *Drosophila melanogaster* as my model, I have characterized the sources and molecular structures of the mitotic crossovers that occur in the absence of BLM. Also, I have studied the synthetic lethality of mutations in *mus309*, the gene that encodes DmBLM, with mutations in genes that encode the structure-specific endonucleases GEN and MUS81, and the endonuclease-interacting protein MUS312. My research on BLM's roles in DNA repair and on the multi-faceted and well-conserved functions of MUS312 and its orthologs has provided insight into the cellular pathways vital for maintaining chromosome integrity, including interstrand crosslink repair and homologous double-strand break repair.

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LIST OF ABBREVIATIONS

CO:	crossover
DSB:	double-strand break
DSBR:	double-strand break repair
DHJ:	double Holliday junction
hDNA:	heteroduplex DNA
HN2:	nitrogen mustard
HJ:	Holliday junction
HR:	homologous repair
ICL:	interstrand crosslink
NCO:	noncrossover
NER:	nucleotide excision repair
SCE:	sister chromatid exchange
SDSA:	synthesis-dependent strand annealing

CHAPTER I

INTRODUCTION

DOUBLE-STRAND BREAK REPAIR

Since the identification of DNA as the bearer of genetic information, geneticists have created a series of models describing how the physical behavior of DNA explains genetic phenomena. Central to these models is the question of how genetic information is exchanged between homologous chromosomes. The models posited by Holliday and subsequent researchers have sought to illustrate the DNA transactions that occur during meiosis and DNA repair that give rise to crossovers (COs), gene conversion, and in some cases, post-meiotic segregation.

Current models for meiotic recombination are based on the double-strand break repair (DSBR) model outlined by Szostak and colleagues in the 1980's (Szostak et al., 1983). The central features of this model are: initiation by a double-strand break (DSB), formation of a double Holliday junction (DHJ) intermediate, and resolution of the DHJ by nicking two strands at each HJ (Fig 1.1). Although several modifications have been made to the original model, these core features are well supported for meiotic recombination in the budding yeast *Saccharomyces cerevisiae*, the organism in which this process has been most extensively studied. The strong evidence for the DSBR model in meiotic recombination in *S. cerevisiae* has frequently led to the assumption that this

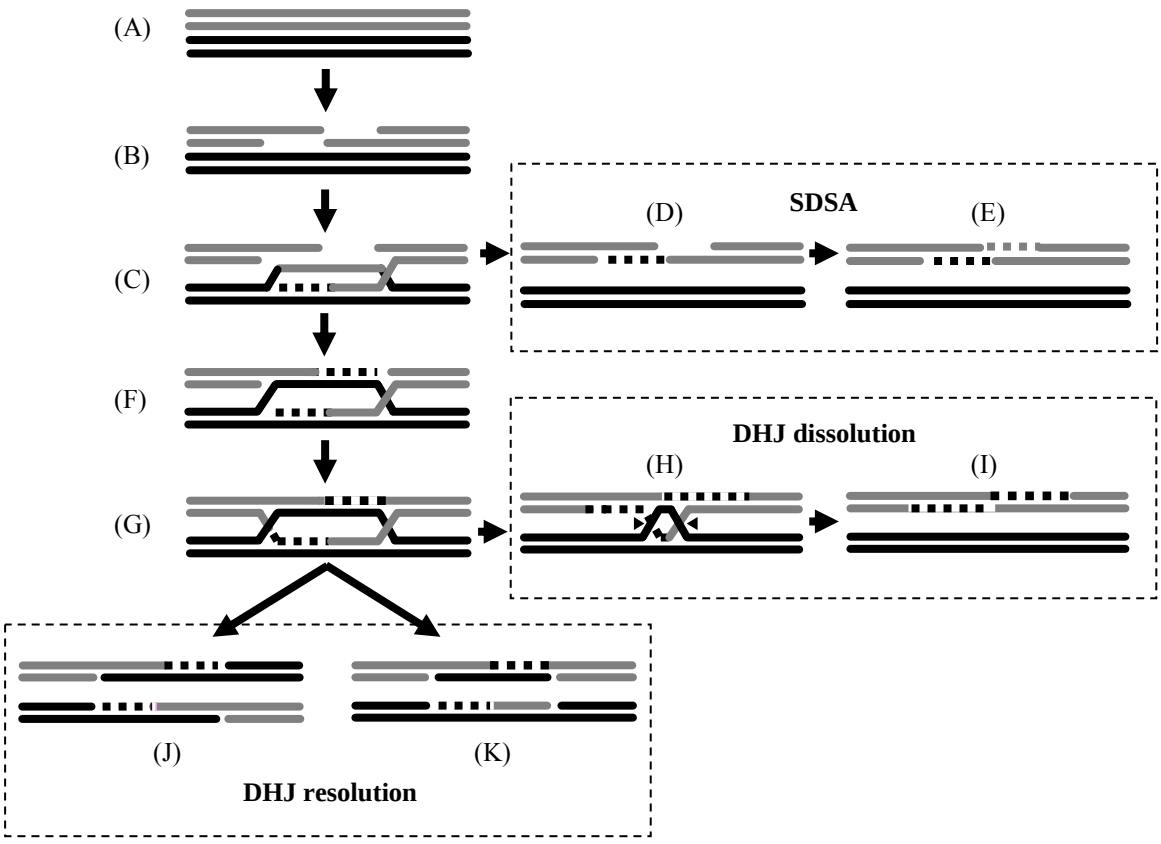

Figure 1.1: Modified double-strand break repair model. Recombination initiates with a double-stranded break (A). (B) The break is resected to leave 3' overhangs, which can strand invade a homologous template chromosome (C). Synthesis-dependent strand annealing (SDSA) is completed by synthesis off the homologous chromosome, disruption of the invading strand, annealing (D), and additional synthesis and ligation (E) to yield a NCO product. Alternatively, a double Holliday junction (G) is produced via second end capture and additional synthesis (F) and ligation. To dissolve the DHJ, the junctions are branch migrated together (H) and decatanated (I) to produce a NCO. DHJ resolution via cutting nicks either each strand once or two strands twice, producing either a CO (J) or a NCO (K).

model also applies to meiotic recombination in other eukaryotes, as well as to mitotic DSB repair. (I use the term "mitotic recombination" to refer to crossing over and/or gene conversion that takes place in cells proliferating mitotically, whether or not the process occurs during the mitotic phase of the cell cycle.) However, there is little evidence to support the direct application of the canonical DSBR model to mitotic DSB repair, and other models may describe mitotic DSB repair more accurately.

Meiotic vs. mitotic recombination

A fundamental difference between meiotic and mitotic recombination is their purpose. Meiotic recombination is actively promoted and highly regulated because it is crucial for accurate chromosome segregation. Meiotic COs create physical links (chiasmata) between homologous chromosomes, thereby facilitating their proper alignment at the metaphase plate and their subsequent segregation during meiosis I; disruption of meiotic COs leads to increased chromosome non-disjunction and sterility. In contrast, mitotic recombination is utilized in the homologous repair (HR) of spontaneous and induced DNA damage. Although there are well-studied examples of programmed mitotic recombination, including mating-type switching in fungi and mammalian V(D)J recombination, the discussion below focuses on spontaneous and induced mitotic recombination.

The different functions of recombination are reflected in the ratios of COs to non-crossovers (NCOs) after DSB repair in meiotic and mitotic cells. In most eukaryotes, meiotic recombination frequently results in COs, consistent with the requirement for COs to direct accurate chromosome segregation. For example, in mouse the ratio of meiotic NCO/CO has been estimated to be ~1:2 (Guillon et al., 2005). In *D. melanogaster* it's been estimated to be 3:1, and in *S. cerevisiae* 1:1 (Martini et al., 2006; Mehrotra and McKim, 2006). In contrast, COs are usually rare in mitotic recombination (Haber and Hearn, 1985; Johnson and Jasin, 2000; Virgin et al., 2001).

The functional differences are also reflected in the differing genetic requirements for meiotic and mitotic recombination, which may stem from the use of different recombination mechanisms in meiosis vs. mitosis, and/or differences in types of initiating

damage, choice of a repair template, or cell cycle stage. In meiosis, a DNA nuclease produces a simple DSB, whereas damage incurred by mitotic cells includes not only simple DSBs, but also single-stranded and double-stranded gaps, breaks ending with damaged bases, one-ended DSBs, and other more complex damage arrangements. Furthermore, DNA damage is not limited to strand breaks, but also includes deleterious structures that can arise during DNA metabolism, such as stalled or blocked replication forks; repair of such structures adds another level of complexity to mitotic recombination.

Meiotic recombination in Saccharomyces cerevisiae

The studies that led to the proposal of the DSBR model, and to its subsequent modification, have been predominantly done in the fungus *S. cerevisiae.* This is because *S. cerevisiae* has traits that facilitate study of the meiotic cell cycle, including hotspots for meiotic recombination, a cell cycle that is easily synchronized across a population, complete recovery of all products of a meiotic event, and a relatively easily manipulated genome. Thus, my introduction to the DSBR model will focus on meiotic recombination research done in *S. cerevisiae.*

The DSBR model (Fig 1.1) was advanced to resolve discrepancies between predictions made by earlier meiotic recombination models and phenomena that were subsequently observed. Experiments performed since the proposal of the DSBR model have likewise required some modifications be made to it, but there is still strong support for the central features of the canonical DSBR model. These central features include initiating DSBs and DHJ intermediates.

DSBs are detected at sites of meiotic recombination and induced DSBs can initiate recombination (Kolodkin et al., 1986); mutants defective in DSB-production are also defective in meiotic recombination. The protein that catalyzes the production of meiotic DSBs, Spo11, is a Type II topoisomerase-like nuclease (Cao et al., 1990; Keeney et al., 1997; Klapholz et al., 1985). Other factors are also important for production of DSBs. This includes the MRX complex, comprising Mre11, Rad50, and Xrs2, and several other proteins that are less well-conserved in primary sequence (Cao et al., 1990; Johzuka and Ogawa, 1995). The exact functions of these additional factors for DSB production are not yet clear.

The ends of the DSB are processed to produce long 3' single-stranded overhangs. This feature of the model is supported by the physical detection of 3' overhangs at meiotic recombination hotspots (Sun et al., 1989; Sun et al., 1991). The identity of the nuclease(s) responsible for resection, however, remains unclear. The best candidate nuclease, Mre11, has the wrong directionality *in vitro*: 3' – 5' (Trujillo and Sung, 2001). The question of how the MRX complex directs resection at DSBs is still unresolved, but possible solutions include Mre11 nicking within the dsDNA and chewing toward the broken end, or unwinding and then nicking at sites of certain secondary structures. Additional factors, such as the Sae2 nuclease, also play a role (Lengsfeld et al., 2007).

After resection, one 3' end invades the homologous template. Again, this intermediate – the single-ended invasion – has been detected in physical assays at recombination hot spots (Hunter and Kleckner, 2001). Mutations in genes in the *rad52* epistasis group, which encode proteins that facilitate strand invasion, ablate meiotic recombination. For example, Rad51, a eukaryotic homolog of bacterial RecA, catalyzes

strand pairing and invasion *in vitro* (Sung, 1994). *rad51* mutants do not produce viable spores due to the inability to properly segregate chromosomes in the absence of meiotic COs. Mutations in genes encoding other proteins that facilitate strand invasion, including Dmc1, have a similar meiotic phenotype (Bishop et al., 1992).

An important byproduct of strand invasion is the creation of heteroduplex DNA (hDNA), which will contain mismatches or insertion/deletion loops between the two strands if the homologous chromosomes have heterologies. Both genetic and physical studies have detected hDNA in *S. cerevisiae* meiosis (Goyon and Lichten, 1993; Nag and Petes, 1993). Heterologies within hDNA are usually repaired, leading to gene conversion or restoration of the original sequence. If hDNA is unrepaired, the heterologies between homologous chromosomes segregate post-meiotically. Post-meiotic segregation (PMS) is increased in mismatch repair (MMR) mutants, supporting a role for MMR proteins in repairing hDNA produced during meiotic recombination. (Kramer et al., 1989).

Other than recombination initiation by a DSB, the most fundamental feature of the DSBR model is the DHJ. Consequently, the strongest support for the DSBR model came with detection and isolation of "joint molecules" formed during recombination in *S. cerevisiae* (Collins and Newlon, 1994; Schwacha and Kleckner, 1994). These joint molecules have the predicted properties of DHJ intermediates: They can be resolved into CO and NCO products by RuvC, an *E. coli* nuclease that cuts HJs (Schwacha and Kleckner, 1995), and all four strands are continuous (*i.e.*, there are no unligated nicks). Additionally, all four strands of the joint molecules have the parental arrangement of flanking markers (Schwacha and Kleckner, 1994). This finding is consistent with an intermediate with two HJs; in an intermediate with one HJ, two of the strands would be

expected to have a parental arrangement of markers, while the other two would have a recombinant arrangement. The *in vitro* RuvC experiment also supports the postulate that DHJs can produce both COs and NCOs, as predicted by the classic DSBR model.

The final step of the DSBR model involves cutting of the DHJ by one or more HJ resolvases. Despite intensive searches, the resolvase has not yet been identified. One candidate is the nuclease Mus81. *In vitro*, Mus81–Mms4/Eme1 can cut HJs, but has higher activity on other branched structures, such as D-loops and nicked HJs (Ciccia et al., 2003; Constantinou et al., 2002; Doe et al., 2002; Gaillard et al., 2003; Osman et al., 2003). *S. cerevisiae mus81* or *mms4* mutants lack the subset of meiotic COs that do not exhibit CO interference. However, Mus81-Mms4 is not required to produce the COs that participate in interference, which represent the majority of COs in *S. cerevisiae*. Instead, they require a set of proteins that includes the mismatch repair-related MutS homologs Msh4 and Msh5 (de los Santos et al., 2003). It is unclear what role Msh4-Msh5 plays in promoting crossing over, but it's been suggested that the heterodimer stabilizes DHJs (Snowden et al., 2004); the enzyme that then cuts these DHJs remains elusive. Recently, an additional candidate HJ resolvase has been characterized. Yen1 has been shown to have strong HJ resolvase activity in vitro, but mutants have not yet been well characterized and thus Yen1's *in vivo* role in meiotic recombination is not yet clear (Ip et al., 2008).

Despite strong support for many features of the DSBR model, additional data inconsistent with the canonical model have required an important modification. In studying the timing of appearance and disappearance of meiotic recombination intermediates in *S. cerevisiae*, Allers and Lichten made the surprising finding that NCOs

appear earlier than DHJs or COs; this challenged the prediction that COs and NCOs are both produced from DHJs (Allers and Lichten, 2001). These authors also cited previous characterization of several mutants, such as *zip1* and *mlh1*, that decrease the frequency of COs but not NCOs, consistent with the possibility that these two types of products do not both arise from the same intermediate. They hypothesized that NCOs arise from a different mechanism, synthesis-dependent strand annealing (SDSA) (Fig 1.1). In SDSA, repair is initiated as in the DSBR model, but before second-end capture the invading strand is dissociated from the D-loop and then anneals to complementary single-strand on the other side of the break. The SDSA model does not involve a DHJ intermediate.

Support for SDSA in meiosis has come from the analysis of hDNA found in recombination products that exhibit PMS. In *S. cerevisiae*, hDNA tracts are frequently restricted to one side of the break, as predicted by the SDSA model (Gilbertson and Stahl, 1996; Merker et al., 2003; Porter et al., 1993). In a recent test of the SDSA model, McMahill *et al.* (McMahill et al., 2007) found that a class of gene conversions best explained by the SDSA model comprised a high percentage of the NCO products recovered.

An additional suggested mechanism for generation of NCOs is DHJ dissolution (Gilbertson and Stahl, 1996; Stahl, 1996). Dissolution occurs when the two HJs are branch migrated toward one another, and the remaining catenation is removed by a type I topoisomerase (Fig1). *In vitro* studies have shown that BLM helicase and TOP3α topoisomerase can catalyze this reaction efficiently (for recent reviews, see (Mankouri and Hickson, 2007; Wu et al., 2006). After DHJ dissolution, the chromatid that received the DSB will have hDNA with sequence from the homologous chromosome on one

strand to one side of the break and on the other strand to the other side of the break – the *trans* configuration (Fig 2C). Trans hDNA has been detected in *S. cerevisiae*, but the frequency is extremely low, suggesting that some or all of the instances noted may actually result from the occurrence of overlapping recombination events at two nearby DSB sites (Gilbertson and Stahl, 1996; Merker et al., 2003; Stahl, 1996). Furthermore, DHJ dissolution cannot account for the appearance of NCOs prior to the appearance of DHJs. Based on these arguments, DHJ dissolution does not seem to be a major source of NCOs in *S. cerevisiae*.

Meiotic recombination in D. melanogaster and other eukaryotes

The major features of the mechanism of meiotic recombination in *S. cerevisiae* are nicely explained by a model that unites the DSBR and SDSA models. The extent that this compound model is applicable to other organisms is still in question. The key initiation event – formation of a DSB – appears to be universal. Orthologs of Spo11 have been found to be essential for meiotic recombination in *S. pombe*, *Arabidopsis*, *C. elegans*, *D. melanogaster*, and mouse; in each of these organisms, mutants lacking Spo11 have elevated nondisjunction or are sterile, and for some of them, irradiation has been shown to partially rescue these phenotypes (Dernburg et al., 1998; Grelon et al., 2001; McKim and Hayashi-Hagihara, 1998; Romanienko and Camerini-Otero, 2000; Sharif et al., 2002). The relationship between Spo11 and initiation of meiotic recombination is so well established that the presence of a gene orthologous to *SPO11* has been taken as evidence for a meiotic cell cycle in a species not known to reproduce sexually (Malik et al., 2007). The removal of covalently bound Spo11 and the production of single-stranded

3’ ends is also well conserved, with the MRE1 RAD50 NBS1 (MRN) complex (Mre11 Rad50 Xrs2 in yeast) important to this process in many organisms (review (Mimitou and Symington, 2009)). The families of proteins required for strand invasion have also been well conserved. Homologs of the canonical strand invasion protein, RecA, are required for fertility in *S. pombe*, *Arabidopsis*, *C. elegans*, *D. melanogaster*, and mouse (Bleuyard and White, 2004; Ghabrial et al., 1998; Muris et al., 1997; Pittman et al., 1998; Rinaldo et al., 2002).

Although these early steps in recombination appear to be very similar across eukaryotic meiosis, conservation of later steps is less certain. As noted above, some COs in *S. cerevisiae* require the Mus81-Mms4 nuclease, whereas others require Msh4-Msh5. This is also true in *Arabidopsis* (Berchowitz et al., 2007). However, in *C. elegans* it appears that the Msh4-Msh5 pathway is responsible for all COs, whereas a Mus81-dependent pathway is required for most COs in *S. pombe*. Recent work in *S. pombe* has revealed that most meiotic recombination in this yeast involves an intermediate with a single HJ (Cromie et al., 2006). This suggests that orthologous proteins may act on different intermediates in different species.

Conversely, a common intermediate, such as a DHJ, may be acted upon by different proteins to produce COs in different species. *D. melanogaster* uses neither Mus81-Mms4 nor Msh4 and Msh5 orthologs, but there is evidence that recombination still goes through a DHJ intermediate (Radford et al., 2007) . Most meiotic COs in *D. melanogaster* require a complex that contains MEI-9-ERCC1 endonuclease, which is orthologous to *S. cerevisiae* Rad1-Rad10 and mammalian XPF-ERCC1, and the proteins MUS312 (Baker and Carpenter, 1972; Carpenter and Sandler, 1974; Sekelsky et al.,

1995; Yildiz et al., 2002) and HDM (Joyce et al., 2009). In the absence of MEI-9 nuclease activity, some recombination events have *trans* hDNA, suggesting that these arose from DHJ dissolution that occurs when MEI-9 is not available to cut Holliday junctions (Radford et al., 2007).

Taken together, the data from different model organisms indicates that the initiation of meiotic recombination is conserved across eukaryotes, but there are multiple ways to turn a DSB into a CO. The DSBR model is an accurate description of CO formation in *S. cerevisiae* and some other organisms, but it doesn't appear to be a universally conserved CO mechanism.

The DSBR model and mitotic DSB repair

Though intended to describe meiotic recombination, the classic DSBR model drew on evidence from studies of mitotic recombination. Prior studies on plasmid-chromosome recombination in bacteria provided evidence that DSBs are recombinogenic, and suggested that gene conversion could be produced by the repair of double-strand gaps (Orr-Weaver and Szostak, 1983; Orr-Weaver et al., 1981; Orr-Weaver et al., 1983). These earlier studies provided the basis for the original description of the DSBR model by Szostak, *et al.* (Szostak et al., 1983). Some of this interchange of ideas between meiotic and mitotic recombination models explains why the DSBR model is frequently co-opted in attempts to describe the mechanism of mitotic recombination. However, the application of the DSBR model to DSB repair in mitotic cells should be cautioned, because, as discussed earlier, meiotically and mitotically dividing cells have very different recombination requirements and outcomes.

While the purpose and environment of recombination is different in mitotic cells, the early steps of the DSBR model are consistent with what is known about mitotic DSBR. There is, for example, no question that DSBs can induce COs in a mitotic context. It has long been known that treatments that produce DSBs, such as X-ray irradiation, can produce breaks and infrequently induce somatic COs (Bauer et al., 1938; Lefevre, 1948). Treating cells with agents that produce other types of damage, such as alkylation, can also yield recombination, but it is generally thought that this is the result of a DSB formed as secondary damage (Kaina, 2004). For instance, the induction of genome rearrangements by crosslinking agents is DNA synthesis-dependent (Akkari et al., 2000; Barber et al., 2005); this observation suggests that the recombinogenic damage ultimately results from secondary damage produced by replication forks encountering the primary interstrand crosslink (ICL) damage. There is also evidence that mitotic recombination can result from single-stranded breaks or gaps in addition to DSBs (Lettier et al., 2006); for comparison to meiotic recombination, we restrict the discussion below to mitotic DSB repair.

Mutations that disrupt the early steps of meiotic DSBR have similar effects on mitotic recombination, which suggests that these steps are the same or similar. A prime example is Rad51: In *S. cerevisiae*, *rad51* mutants are defective in spontaneous and induced mitotic recombination and mating-type switching, in addition to the their defects in meiotic recombination (Shinohara et al., 1992). Conversely, there are some notable differences in the early-acting genetic requirements for meiotic vs. mitotic recombination. Firstly, there are meiosis-specific recombination factors, such as Dmc1, a meiosis-specific Rad51 paralog found in many eukaryotes. Like *rad51* mutants, *dmc1* mutants

are deficient in making meiotic COs (Bishop et al., 1992) and in gene conversion (Fukushima et al., 2000), but *dmc1* mutants are not defective in mitotic recombination (Fukushima et al., 2000). Secondly, there are factors that vary in their relative importance or specific role. The Rad54 paralog Rdh54 (Tid1) seems to be more important for meiotic interaction between homologs, whereas Rad54 is more important for recombinational repair off sister chromatids (Heyer et al., 2006; Shinohara et al., 1997). These differences, however, are indicative of redundancies of function and varying requirements due to differences in cell cycle and type of damage, rather than alternative repair mechanisms.

Although the early steps of mitotic DSB repair are similar to meiotic DSB repair, less is know about the later steps. In principle, mitotic DSB repair may be biased toward NCOs by any combination of the mechanisms described above for meiotic recombination: SDSA, DHJ dissolution, or DHJ resolution that is biased to produce non-crossovers. Some of the earliest evidence for SDSA comes from studies of mitotic gap repair in *D. melanogaster*. In gap repair, the chromosome that receives the break undergoes gene conversion without alteration of the template, and the two ends of the gap can use different templates independently (Nassif et al., 1994). These findings are not compatible with a DHJ intermediate. In experiments in which the gap spans a direct repeat, one of the most common products has collapse of the repeat to a single copy, a result most easily explained by the SDSA model (Adams et al., 2003; Kurkulos et al., 1994).

There is also evidence in favor of SDSA in mitotic DSB repair in *S. cerevisiae*. In a study of repair of a single DSB associated with a repetitive sequence, the repeat

underwent expansion and contraction in the product, but the template was unaltered; there was evidence that more than one donor templates was used, suggesting the repeated rounds of strand invasion and synthesis in SDSA (Paques et al., 1998).

BLM in DSBR

The study of proteins required for DSBR in proliferating cells has provided additional insights into mitotic recombination pathways. One particularly informative repair protein is the RecQ helicase BLM, which is mutated in the hereditary disease Bloom Syndrome.

BLM is an ATP-dependent helicase. *In vitro*, it has both 3'-5' helicase and single-stranded DNA annealing activities (Bennett et al., 1998; Cheok et al., 2005; Karow et al., 1997; Weinert and Rio, 2007). It efficiently acts on complex DNA structures, disrupting D-loops and G-quadruplex DNA, and migrating Holliday junctions and regressing model replication forks (Bachrati et al., 2006; Karow et al., 2000; Machwe et al., 2006; Sun et al., 1998).

BLM forms a core complex with the topoisomerase TOP3a and two OB-fold containing proteins, BLAP75/RMI1 and BLAP18/RMI2. (Singh et al., 2008; Xu et al., 2008; Yin et al., 2005). It also interacts with a slew of other proteins important for replication and repair, including RAD51, RPA, FEN1, and the Fanconi anemia core complex (Brosh et al., 2000; Deans and West, 2009; Meetei et al., 2003; Sharma et al., 2004; Wu et al., 2001; Zhong et al., 1999). BLM localizes to Promyelocytic Leukemia (PML) bodies in mammals, and forms foci at damaged replication forks and other sites of DNA damage (Bischof et al., 2001; Davalos and Campisi, 2003; Sanz et al., 2000).

Cells from Bloom Syndrome patients are characterized by elevated levels of COs between sister chromatids (sister chromatid exchange, SCE) (Chaganti et al., 1974). Likewise, mutations in *S. cerevisiae SGS1*, which encodes the sole RecQ helicase in budding yeast, result in elevation of both spontaneous and induced mitotic COs (Watt et al., 1996). The predominant interpretation of the hyperrecombination phenotype is that BLM is essential for a repair pathway that yields NCOs, and in its absence repair occurs via an alternative pathway that generates COs.

A combination of genetic and biochemical data has driven the hypothesis that the NCO repair function of BLM is, in conjunction with TOP3α, to dissolve DHJ intermediates generated during DSBR. In *S. cerevisiae*, *top3* mutants grow slowly. The slow growth is suppressed by mutations in *SGS1* (slow growth suppressor 1), suggesting that Sgs1 produces an intermediate that is toxic in the absence of Top3 activity (Gangloff et al., 1994). The identity of the toxic intermediate is suggested by the *in vitro* DHJ dissolution activity of BLM and TOP3a. *In vitro*, BLM and TOP3α can carry out DHJ dissolution efficiently, with BLM branch migrating the HJs together so they can be decatenated by TOP3α (Harmon et al., 2003; Harmon et al., 1999; Plank et al., 2006). Thus, unresolved, branch-migrated DHJs may be the toxic intermediates in *top3* mutants. However, there is no direct evidence that DHJ dissolution occurs during mitotic DSB repair *in vivo*. It fact, there is no direct evidence even for the production of DHJs during mitotic recombination.

There is, however, direct evidence for BLM having a role in SDSA. In *D. melanogaster*, a P-element excision assay was used to demonstrate that DmBLM facilitates the SDSA repair of double-strand gaps (Adams et al., 2003). BLM's *in vitro*

D-loop disruption activity suggests that BLM's role in SDSA is to disrupt the invading strand during multiple rounds of strand invasion and synthesis. It is possible that the genetic interaction between BLM and TOP3α reflects a heretofore uncharacterized requirement for TOP3α in SDSA. TOP3α may be required to relax supercoiling produced by D-loop production/migration; this requirement most likely would not be revealed with the short substrates used in biochemical assays.

Conversely, in *S. cerevisiae*, Sgs1 does not appear to facilitate SDSA; rather, this role is carried out by another helicase, Srs2. As in *sgs1* mutants, *srs2* mutants have increased mitotic crossovers and genomic instability (Aboussekhra et al., 1989; Aguilera and Klein, 1988; Rong et al., 1991). Also like BLM, Srs2 is a 3′→5′ DNA helicase and has a D-loop disrupting activity *in vitro*; this activity suggests that it might play a role in SDSA (Dupaigne et al., 2008; Rong and Klein, 1993). *sgs1 srs2* double mutants have extremely slow growth characterized by defects in recombination, indicating they function in alternative recombination pathways (Gangloff et al., 2000; Klein, 2001; McVey et al., 2001). Consistent with Srs2 having a role in SDSA, Srs2 can translocate on ssDNA bound with RPA, and it can disrupt Rad51 filaments (Dupaigne et al., 2008; Krejci et al., 2003; Veaute et al., 2003).

The mitotic phenotypes of *srs2*, *sgs1*, and *top3* mutants and the wild-type ratios of COs to NCOs suggest that NCO mechanisms are strongly promoted in mitotic cells (Haber and Hearn, 1985; Johnson and Jasin, 2000; Virgin et al., 2001). However, though NCO mechanisms are clearly preferred, there are still spontaneous mitotic COs in wild-type organisms. Little is known about the specific genetic requirements of these spontaneous wild-type COs . MUS81 and EME1/MMS4 are, as discussed earlier,

required for a subset of meiotic crossovers in *Arabidopsis*, but AtMUS81 mutants have normal levels of spontaneous mitotic COs (Hartung et al., 2006). In *S. pombe*, Mus81-Eme1 is required for some but not all mitotic COs associated with induced-DSBs (Hope et al., 2007). In *S. cerevisiae mus81* mutants, spontaneous mitotic COs are not reduced and in fact are slightly increased (Robert et al., 2006).

Even less is known about the mechanism that produces COs in BLM-deficient cells. As recombination in BLM-deficient cells is aberrant, with associated deletions and rearrangements, it is possible that the spontaneous COs in *BLM* mutant cells may be created differently than wild-type spontaneous COs. Two main theories have been posited for the source of COs in *BLM* mutant cells. One is that DHJs are made and, without BLM to dissolve them, resolved via nicking by HJ resolvases. The second is that unresolved DHJs are cut by nucleases to create DSBs in both chromosomes, and that the broken chromosomes are repaired via end joining, creating a CO when ends from two different chromosomes are ligated together. In *D. melanogaster*, the detection of deletions in template chromosomes used for HR in DmBLM mutants suggests that HJ resolvases, which make symmetrical nicks, are not responsible for making the COs (Johnson-Schlitz and Engels, 2006a).

Additional clues to BLM's functions come from synthetically lethal interactions with *MUS81* mutations. In the absence of BLM, cells require MUS81 in cerevisiae, *D. melanogaster*, and pombe (Boddy et al., 2000; Fabre et al., 2002; Trowbridge et al., 2007). DmBLM is encoded by the gene *mus309*. *mus81 mus309* double mutant *D. melanogaster* have increased apoptosis and die at the pharate adult stage of development (Trowbridge et al., 2007). It's been proposed that, in the absence of BLM's helicase

activity, DNA structures that arise during repair/replication must be resolved via cutting by a nuclease such as MUS81. *mus81* mutations are viable with an allele of *mus309*, *mus309*^{N2}, that is as defective as null alleles in our DSB repair assays but less severe in maternal effect lethality and IR-sensitivity phenotypes (McVey et al., 2007; Trowbridge et al., 2007). This suggests that DSBs are not the structure requiring either BLM or MUS81 for repair. Since both BLM and MUS81 are known to localize to damaged replication forks, these may be the structures that require either one to properly process and reset the fork (Kaliraman et al., 2001).

INTERSTRAND CROSSLINK REPAIR

Interstrand crosslinks (ICLs) are a particularly cytotoxic form of DNA damage. They consist of the covalent linkage of two strands of a double-stranded DNA molecule, frequently by a bifunctional alkylating agent such as nitrogen mustard (HN2) or cisplatin. As ICLs prevent the separation of DNA strands, they present a block to transcription and replication and thus disrupt rapidly proliferating cells. This trait makes some interstrand crosslinking agents very useful as chemotherapeutics (McHugh et al., 2001).

Despite the clinical relevance of interstrand crosslinking agents, the reigning model for ICL repair in metazoans requires considerable fleshing out. The pathway appears to be something of a Frankenstein, cobbled together with repair proteins drawn from other repair pathways, such as HR and nucleotide excision repair (NER) (McVey).

The current model begins with a replication fork encountering an ICL (Fig 1.2) (Akkari et al., 2000). The stalled replication fork is then cut to form a DSB (Bessho, 2003). The nuclease(s) responsible for creating the DSBs is not identified, although

MUS81, which has an affinity for branched structures like replication forks, and sensitivies to interstrand crosslinking agents, is a good candidate for mammals (Abraham et al., 2003). The ICL is next "unhooked", which involves nicks flanking one of the two cross-linked nucleotides. Mismatch repair proteins have been implicated in the unhooking step (Zhang et al., 2002) (Zhang et al., 2007), as has the structure-specific endonuclease XPF (MEI-9 in *D. melanogaster*). XPF, which nicks 5' to

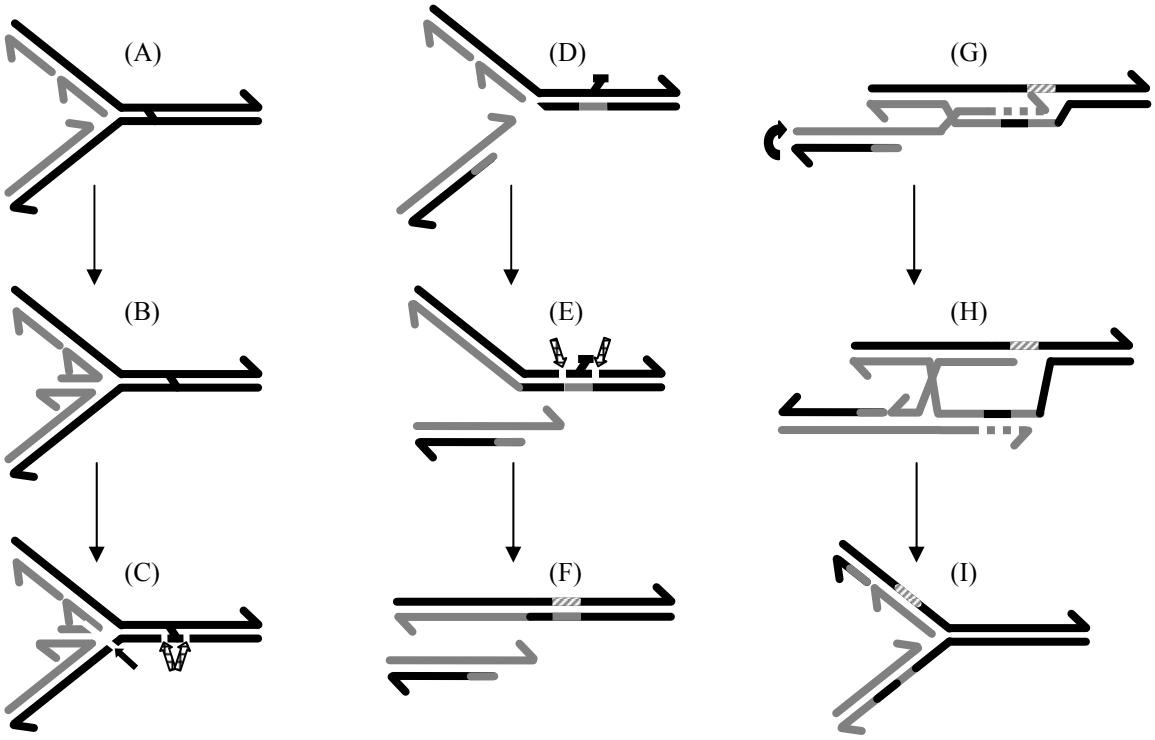

Figure 1.2: Interstrand crosslink repair model. ICL repair initiates when a replication fork encounters an ICL (A). The fork regresses to form a branched intermediate (B) which can be recognized and cut by an endonuclease (C, solid arrow) to produce a single ended DSB (D). Mismatch repair nucleases nick adjacent to one base involved in the ICL, unhooking the ICL (C, hatched arrows). Translesion polymerases synthesize across the resulting gap (D). Nucleotide excision repair machinery excises the unhooked ICL (E, hatched arrows), and additional synthesis and ligation repairs the gap (F). Homologous repair proteins facilitate strand invasion by the DSB (G), creating a junction (H) that can be resolved to produce a reset replication fork (I).

damaged bases in NER, is thought to have an analogous role at the unhooking step of ICL repair (Kuraoka et al., 2000). However, this is mostly based on *in vitro* experiments, and there is evidence that XPF may be important for the RAD51-mediated HR steps that

are thought to complete ICL repair by resetting and restarting the replication fork (Hussain et al., 2003).

The proteins most unique to metazoan, and specifically mammalian, ICL repair are the Fanconi anemia (FA) proteins. Mutation of any of any of the 13 *FA* genes causes Fanconi anemia in humans, which is characterized by increased incidence of cancer and bone marrow defects (Auerbach, 2009). Human FA cells are hypersensitive to interstrand crosslinking agents, as are FA-deficient cells from mouse (Gush et al., 2000). FA proteins are important for ICL repair in both *D. melanogaster* and *C. elegans* as well, even though both lack the full compliment of FA proteins and only have five homologs each (Dequen et al., 2005; Marek and Bale, 2006). Although the exact functions of the FA proteins aren't yet clear, they've been found to be important for directing the activities of other repair proteins. For example, the core FA complex, which comprises eight proteins, has been found to interact with and direct the localization and activity of the BLM complex at sites of replication-associated DNA damage (Deans and West, 2009). Also, MLH1 of the mismatch repair complex Mutlα interacts with FANCJ for ICL repair (Peng et al., 2007), and FA proteins are allelic to, and interact with, BRCA2 and BRCA1, which have roles in HR (D'Andrea and Grompe, 2003).

The modified DSBR model is the best description we have for meiotic recombination in the model organisms in which it has been most extensively studied, including *S. cerevisiae* and *D. melanogaster*. For mitotic recombination, however, we know the following: it can be initiated by a DSB and requires strand invasion activity. While this is consistent with the DSBR model, there is little supporting evidence for the

later steps of the model, including the production of a DHJ intermediate and its resolution/dissolution. Thus, it cannot be concluded whether the observed differences between meiotic and mitotic recombination, including frequency of COs vs NCOs, is the result of disparate repair mechanisms or differential processing of a common intermediate. Differences in genetic requirements raise the possibility that alternative mechanisms are used in meiosis and mitosis, but further work must be done to fully address this question.

My thesis fills in some of the gaps in our understanding of mitotic recombination, and brings us closer to a cohesive and comprehensive model. I've focused my research on the BLM helicase, which plays a central role in metabolizing DNA structures that arise in recombination and repair. By ascertaining the activities of BLM, we simultaneously uncover the underlying repair pathways in which it acts.

I began by helping to characterize novel *mus309* mutations created in a *P*-element excision screen. *mus309* mutants have defects in DSBR, are hypersensitive to various DNA-damaging agents, exhibit maternal effect lethality, and, most central to my work, have increased mitotic COs in both somatic cells and in the male germline. Additionally, the screen yielded a set of novel mutations that appear to be separation-of-function alleles. These alleles have proven incredibly useful in dissecting BLM's multifarious functions.

To determine the mechanisms utilized by cells to produce COs in the absence of DmBLM, I used a candidate approach to determine whether mutations in known DNA repair and recombination proteins altered the levels of COs. I paid particular attention to nucleases and nuclease-associated proteins known to have functions in either meiotic

recombination or DNA repair. I also examined the structures of induced COs by combining the $P\{w^a\}$ gap repair assay with a CO assay. These experiments revealed the complexity of DNA repair events when DmBLM is absent.

In studying the genetic requirements for COs in BLM, my research revealed an unexpected requirement for the protein MUS312 in the absence of DmBLM. This discovery led to the determination that MUS312 complexes with multiple structure-specific endonucleases, including with SLX1 to form a novel HJ resolvase. MUS312 and its associated nucleases act in multiple cellular and developmental contexts, performing a variety of repair and recombination functions. We showed that MUS312's interactions with MEI-9 and SLX1 are conserved from fly to human, and that MUS312 and SLX1 have well-conserved roles in ICL repair, and potentially meiosis. The study of MUS312 and SLX1's functions in ICL repair has the potential to provide great insight into how ICLs are repaired in metazoans.

CHAPTER II

MULTIPLE FUNCTIONS OF DROSOPHILA BLM HELICASE IN MAINTENANCE OF GENOME STABILITY[1]

Bloom Syndrome, a rare human disorder characterized by genomic instability and predisposition to cancer, is caused by mutation of BLM, which encodes a RecQ-family DNA helicase. The Drosophila melanogaster ortholog of BLM, DmBLM, is encoded by *mus309*. Mutations in *mus309* cause hypersensitivity to DNA-damaging agents, female sterility, and defects in repairing double-strand breaks (DSBs). To better understand these phenotypes, we isolated novel *mus309* alleles. We found that female sterility is due to a requirement for DmBLM in early embryonic cell cycles; embryos lacking maternally derived DmBLM have anaphase bridges and other mitotic defects. These defects were less severe for alleles that delete the N terminus of DmBLM, but not the helicase domain. We also found that spontaneous mitotic crossovers are increased by several orders of magnitude in *mus309* mutants. These results demonstrate that DmBLM functions in multiple cellular contexts to promote genome stability.

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Introduction

BLM is an ATP-dependent helicase that belongs to the RecQ family (Ellis et al., 1995). Mutations in BLM cause Bloom Syndrome (BS), a rare, autosomal recessive disorder characterized by proportional dwarfism, sterility, and immunodeficiency. BS patients have an increased incidence of many types of cancers, including leukemias, lymphomas, and carcinomas. BS cell lines are genomically unstable, showing a high rate of chromosome breaks and rearrangements and increased exchange between sister chromatids and homologous chromosomes (Chaganti et al., 1974; German et al., 1977).

In vitro, the human BLM protein acts on structures mimicking those formed during DNA replication and recombination. It promotes branch migration of Holliday junctions (HJs) and unwinds HJs and D-loops (Bachrati and Hickson, 2006; Karow et al., 2000; van Brabant et al., 2000). Biochemical assays have also revealed a strand-annealing activity that may act in conjunction with its helicase activity (Cheok et al., 2005; Machwe et al., 2005). Together, these activities suggest that BLM may function during DNA replication, DNA repair, and/or meiotic recombination. The exact roles that BLM plays in these multiple contexts are currently the subject of intense investigation.

Accumulating evidence suggests that BLM plays an important role in the recovery of damaged and/or stalled replication forks. BLM accumulates at sites of stalled replication forks, where it interacts with repair and checkpoint proteins, including p53, 53BP1, and Chk1 (Sengupta et al., 2003; Sengupta et al., 2004). In addition, *in vitro* studies have shown that BLM can regress a stalled or collapsed replication fork in such a way that the damage or blockage can be bypassed (Ralf et al., 2006).

Other studies suggest that BLM also acts during the repair of DNA double-strand breaks (DSBs). BLM interacts with the homologous recombination repair proteins Rad51, Mlh1, and replication protein A via its N and C termini (Brosh et al., 2000) (Pedrazzi et al., 2001; Wu et al., 2001). These interactions, viewed in light of the increased crossover (CO) phenotype seen both in BS cells and in embryonic stem cells of BLM knockout mice, are consistent with BLM acting within one or more repair pathways that do not result in COs (Chester et al., 1998; Hu et al., 2005).

To learn more about BLM functions, we characterized novel alleles of the Drosophila *mus309* gene, which encodes DmBLM (Kusano et al., 2001). Previous studies have used two alleles, one a nonsense mutation and the other a missense mutation, either in trans to one another or hemizygous. These mutants have reduced fertility, increased sensitivity to alkylating agents and ionizing radiation (IR), and defects in repair of DSBs generated by excision of transposable elements (Adams et al., 2003; Beall and Rio, 1996; Boyd et al., 1981; Kooistra et al., 1999; Kusano et al., 2001; McVey et al., 2004b). We generated deletion alleles predicted to remove either the N terminus or both the N terminus and the helicase domain. Through genetic characterization of these and previously existing mutations, we found important roles for DmBLM in early embryogenesis and meiotic recombination. We also report that *mus309* mutants, like S. cerevisiae sgs1 mutants and human BS cells, have elevated rates of mitotic crossing over associated with DSB repair. We discuss possible functions of DmBLM in these processes.

Results

Isolation of new mus309 *mutant alleles*

The DmBLM protein, encoded by *mus309*, is 1487 amino acid residues and, like human BLM and yeast Sgs1p, contains a DEAH-box helicase domain with seven conserved motifs, a RecQ family C-terminal domain, and a helicaseRNase D C-terminal domain (Figure 1). Three mutant alleles of *mus309* have been described previously (Boyd et al., 1981), two of which, $mus309^{D2}$ and $mus309^{D3}$, are still available. The chromosomes carrying these mutations were homozygous viable when originally isolated, but are now homozygous lethal, presumably due to second-site mutations that arose in the stocks. Consequently, most genetic studies have used heteroallelic ($mus309^{D2}/mus309^{D3}$) or hemizygous genotypes. The $mus309^{D2}$ mutation creates a premature stop codon between the regions encoding helicase motifs III and IV (Kusano et al., 2001). We carried out RT–PCR using primers that span the second intron and RNA isolated from adults hemizygous for $mus309^{D2}$ and were unable to detect a product (data not shown), suggesting that any transcript produced is degraded through nonsense-mediated decay. The $mus309^{D3}$ allele is a missense mutation that changes the glutamic acid residue in the conserved DEAH motif to lysine (Kusano et al., 2001). This motif is critical for nucleotide cofactor binding and hydrolysis, so any DmBLM protein produced by this allele is predicted to lack helicase activity.

To isolate additional alleles of *mus309* in a common genetic background, we conducted a *P*-element excision screen. We used P*{EPgy2}mus309EY03745*, which is an insertion of a *P* element into sequences corresponding to the 5' UTR of *mus309*. After inducing excision, we obtained four new alleles that delete various amounts of the 5' end

Figure 2.1: *mus309* alleles. Boxes indicate exons; untranslated regions are hatched (only the beginning of the 3' UTR is shown) and the region encoding the RecQ core is shaded. Vertical lines mark the positions of the seven conserved motifs of superfamily II helicases. The insertion site of the *P{EPgy2}mus309*^{EY03745} element used to generate deletions is indicated by a solid triangle. The positions of the nonsense mutation in *mus309*^{D2} and the missense mutation in *mus309*^{D3} are given above the schematic, and the regions deleted in *mus309*^{N} alleles are indicated below with dashed lines.

of *mus309*; we named these alleles *mus309*^{N1}, *mus309*^{N2}, *mus309*^{N3}, and *mus309*^{N4} (Fig 2.1). Each of these alleles retains an intact promoter, and RT–PCR using primers that flank the second intron demonstrates that truncated transcripts are present at approximately wild-type levels (data not shown).

The *mus309*^{N1} deletion removes 2480 bp, including the start codon and sequences encoding part of the helicase domain. The first in-frame AUG is at codon 813, so any protein produced by this allele would lack helicase motifs I and Ia. This allele, like the nonsense allele *mus309*^{D2}, appears to be genetically null (see below). The other deletions (*mus309*^{N2}, *mus309*^{N3}, and *mus309*^{N4}) do not extend into sequences encoding the helicase domain. In *mus309*^{N3} and *mus309*^{N4}, the start codon is deleted. Initiation at the first in-frame AUG would yield a protein lacking the N-terminal 236 residues. The deletion in *mus309*^{N2} is unusual in that it begins downstream of the site of the *P{EPgy2}* insertion. We speculate that this deletion arose after an initial transposition of the element to this position in the flies carrying this element and transposase. The deletion begins after the

start codon, but results in a frameshift. There is an AUG in the 5' UTR that is in the correct reading frame for translation through the helicase domain. Initiation at this AUG would produce a protein with 35 residues of novel sequence joined to DmBLM residue 567. Residues 567 and 568 are both methionine, so it is also possible that translation may start at either of these sites or farther downstream. Regardless of the start position, DmBLM produced by the N-terminal truncation alleles lacks at least 236 residues in the case of *mus309*^{N3} and *mus309*^{N4} and at least 566 residues in the case of *mus309*^{N2}.

Female sterility in mus309 *mutants is due to maternal-effect embryonic lethality*

Previous studies have shown that fertility is greatly reduced in *mus309* mutant females (Beall and Rio, 1996; Boyd et al., 1981; Kusano et al., 2001). This could result from defects in meiosis or oogenesis or from a requirement for DmBLM during early embryogenesis. Females mutant for *mus309* laid morphologically normal eggs at a frequency similar to that of wild-type females (data not shown); however, embryos from females carrying mutations that disrupt the helicase domain (*mus309*^{D2}, *mus309*^{D3}, and *mus309*^{N1}) had extremely low hatch rates (Table 2.1). To gain insight into the cause of the embryonic lethality, we examined embryos fixed at various stages of development. In embryos fixed during syncytial nuclear divisions, there were frequent anaphase bridges, asynchronous mitoses, and gaps in the normally uniform monolayer of nuclei; most embryos had at least one visible defect (Figure 2.2). Hatch rates among embryos from females carrying N-terminal deletions that do not include the helicase domain (*mus309*^{N2}, *mus309*^{N3}, and *mus309*^{N4}) were reduced relative to the hatch rate of embryos from wildtype females, but were much higher than for embryos from females carrying

null alleles (Table 2.1). These embryos exhibited phenotypes similar to those described above, but the defects were less severe and less frequent.

Genotype	% Hatched (n)	Cellularized	Gastrulated
+/+	98 (588)	99	96
D2/D3	6.8 (931)	ND	ND
D3/D2	5.9 (593)	ND	ND
N1/N1	2.7 (1057)	ND	ND
N1/D2	4.0 (417)	40	0
N1/D3	9.2 (454)	ND	ND
N2/N2	38 (1120)	76	59
N3/N3	42 (1805)	ND	ND
N4/N4	36(987)	ND	ND

Table 2.1: Hatch rates and staging of embryonic lethality. mus309 alleles of mothers are listed, with the maternal allele at the left of the slash. All values are percentages except those in parentheses, which indicate the number of embryos scored for hatching. For cellularization and gastrulation, n=100 for each genotype. ND, not determined.

To quantify the differences between embryos from the different maternal genotypes and to determine whether the defects that we observed are associated with failure to hatch, we examined other hallmarks of embryonic development. Nearly all embryos from wildtype mothers fixed 4–6 hr after egg laying cellularize and undergo gastrulation (Table 2.1). In contrast, fewer than half of the embryos from $mus309^{N1}/mus309^{D2}$ females had cellularized by this time, and none had gastrulated, suggesting that development either was delayed or had ceased by this time. The discrepancy between the complete lack of gastrulation seen in this assay and the hatch rate of 4% (Table 2.1) may be due to a delay in development or the comparatively low number of embryos scored in this assay (100 total). Rates of cellularization and

gastrulation among embryos from females homozygous for *mus309*^{N2} were much higher but were still reduced relative to those from wild-type females ($P < 0.0001$ for each comparison).

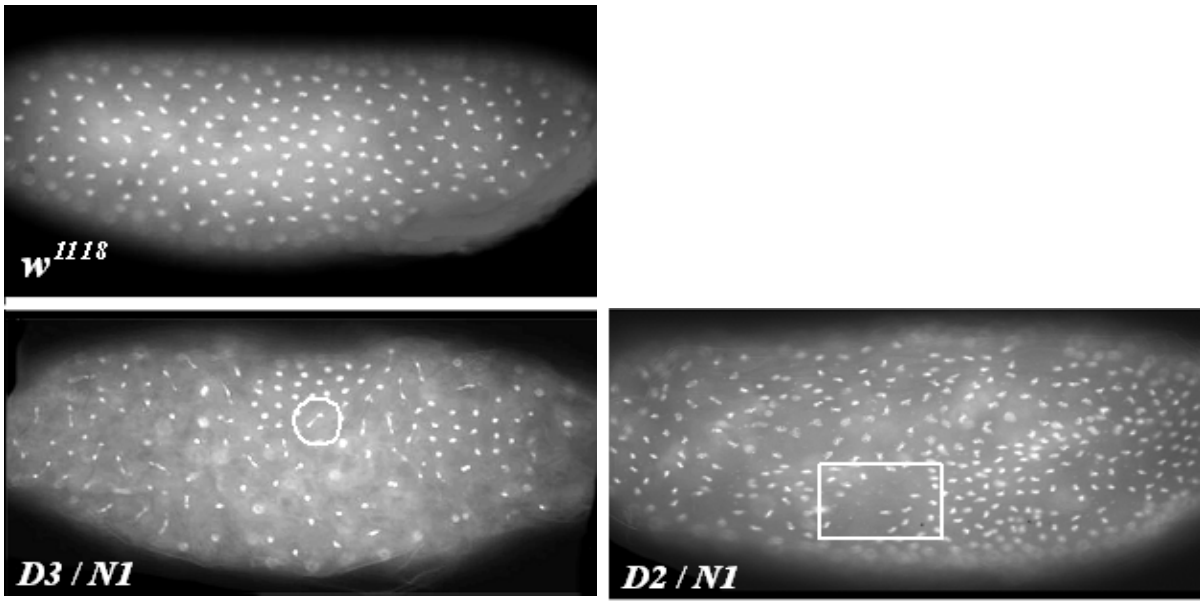

Figure 2.2. Phenotypes of embryos from *mus309* mutant females. Representative DAPI-stained syncytial-stage embryos from wild-type (*w1118*) or *mus309* mutant females are shown. Defects observed frequently include anaphase bridges (circle), gaps in the normally uniform monolayer of nuclei (box), and asynchronous mitoses (middle).

Our observations indicate that the sterility of *mus309* mutant females is due to a requirement for DmBLM in early embryogenesis. This appears to be a strict maternal effect, since zygotic mutants are fully viable (M. McVey and J. Sekelsky, unpublished data). The intermediate severity observed when the N terminus is deleted may indicate that this region is dispensable for at least a subset of early embryonic functions of DmBLM. It is also possible that the RecQ helicase domain of DmBLM is sufficient for the essential embryonic function of DmBLM and that the intermediate phenotype of the N-terminal truncation alleles results from lower levels of the protein due to reduced expression or stability.

Mutants lacking either or both the N terminus and the helicase domains of DmBLM are hypersensitive to gamma radiation

We have shown that embryonic defects are less severe for *mus309* alleles that do not affect the helicase domain than for those predicted to eliminate helicase activity. One possible explanation is that N-terminal truncations reduce protein stability and that the defects that we observed are actually due to a reduction in the amount of DmBlm helicase. Alternatively, or in addition, deletions predicted to cause N-terminal truncations may be separation-of-function alleles. To distinguish between these possibilities, we examined an additional phenotype.

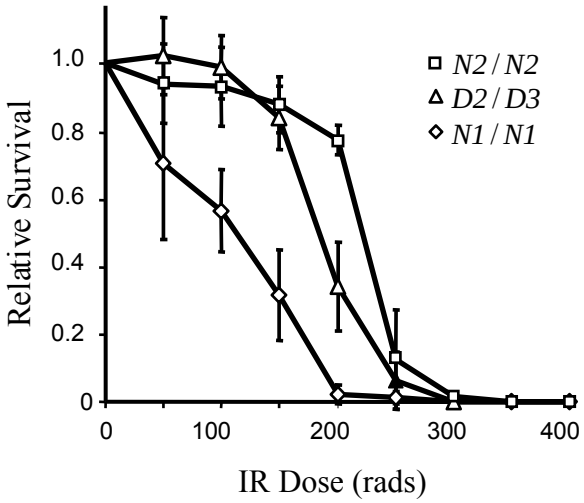

Figure 2.3: Hypersensitivity of *mus309* mutants to ionizing radiation. Survival to adulthood of homozygous or heteroallelic mutants, relative to survival of heterozygous controls, is shown for three mutant genotypes for doses of gamma radiation up to 4000 rad. These doses do not have a large effect on survival of wild-type or heterozygous larvae (data not shown). Error bars indicate standard deviation from three independent experiments.

A previous report showed that $mus309^{D2}/mus309^{D3}$ heteroallelic animals are hypersensitive to ionizing radiation (Kooistra et al., 1999). We tested various *mus309*

allelic combinations to determine whether the different alleles have differences in IR sensitivity. In all cases, *mus309* homozygous or heteroallelic mutants were more sensitive than wild-type flies (Figure 2.3 and data not shown). At an intermediate dose of 2000 rad, mutants carrying alleles predicted to lack helicase activity ($mus309^{N1}$, $mus309^{D2}$) were more sensitive than those carrying the N-terminal deletion alleles $mus309^{N2}$ or $mus309^{N3}$. Interestingly, $mus309^{D2}/mus309^{D3}$ mutants exhibited less sensitivity than $mus309^{N1}/mus309^{N1}$ or $mus309^{N1}/mus309^{D2}$ mutants. These results suggest that the putative helicase dead allele $mus309^{D3}$ and the N-terminal truncation alleles $mus309^{N2}$ and $mus309^{N3}$ retain some function that contributes to resistance to IR.

DmBLM prevents mitotic crossing over during DSB repair

A hallmark of cells from BS patients is increased mitotic crossing over between sister chromatids, homologous chromosomes, and heterologous chromosomes (German et al., 1977). We assayed the rate of mitotic crossing over between homologous chromosomes in *mus309* mutants. We measured CO rates in the germlines of males because males do not have meiotic crossing over (Morgan, 1912), and, unlike *mus309* mutant females, *mus309* mutant males have normal fertility. We tested three heteroallelic combinations: $mus309^{N1}$, $mus309^{D3}$, and $mus309^{N2}$, each in trans to $mus309^{D2}$. The frequency of spontaneous germline crossing over between *scarlet* (*st*) on 3L and *ebony* (*e*) on *3R* was significantly elevated for each of these genotypes relative to wild-type flies, but the mutant genotypes were not significantly different from one another (Fig 2.4).

To determine whether defects in DSB repair in *mus309* mutants can lead to COs, we exposed larvae to various doses of ionizing radiation. IR induced a small number of COs in wild-type males, but even at 1000 rad the frequency was lower than the spontaneous CO frequency in *mus309* mutant males (Fig 2.4). Exposure to IR greatly increased the frequency of germline COs in *mus309* mutant males in a dose-dependent manner. At each dose, the frequency of COs in wild-type males was significantly lower than the frequency in mutant males, but the three different mutant genotypes were not significantly different from one another.

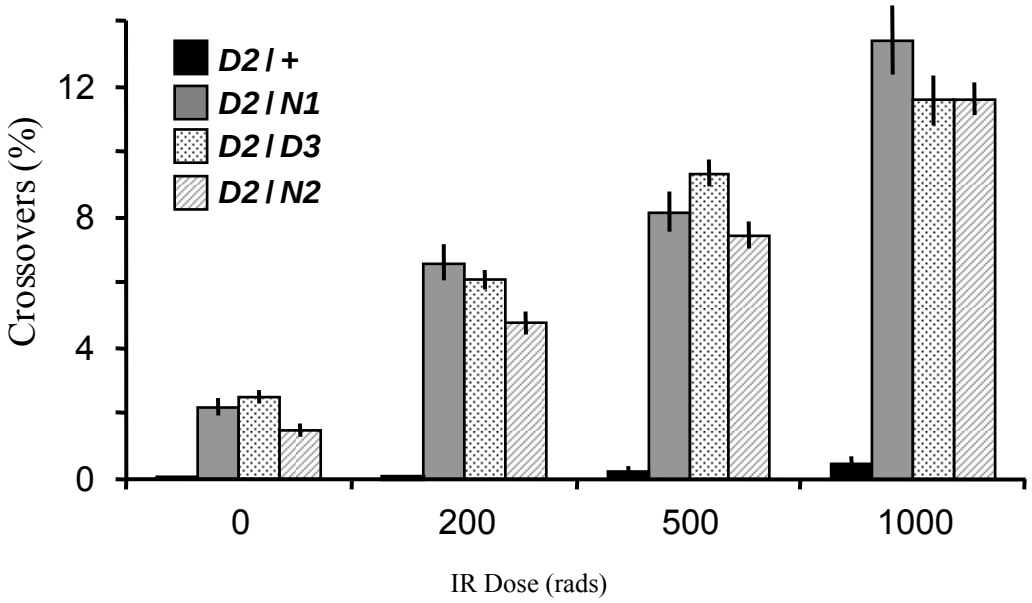

Figure 2.4: Germline crossovers in wild-type and *mus309* mutant males. Bars show the mean percentage of progeny that were recombinant between *st* and *e*, with lines indicating standard error of the mean. Males either were untreated or were exposed to the indicated dose of gamma radiation during larval development. Crossover rates between different mutant genotypes were not significantly different, but at each dose each mutant genotype was significantly different from the wild type ($P<0.0001$ for each comparison).

Discussion

In this article, we describe several phenotypes associated with mutations that affect DmBLM. These phenotypes include defects in double-strand break repair (DSBR) in cycling cells, such as hypersensitivity to ionizing radiation and increased incidence of mitotic crossing over between homologous chromosomes. We observed defects in early embryogenesis, including frequent anaphase bridges, loss of syncytial nuclei, and developmental failure prior to gastrulation. Analysis of the effects of different allelic combinations on some of these phenotypes provides insights into the relationships among the different functions for DmBLM. Each of these functions is discussed below.

DmBLM in embryogenesis

We have shown that the decreased fertility of *mus309* females is due to maternal effect embryonic lethality. The vast majority of embryos obtained from mothers homozygous for null alleles of *mus309* displayed chromosome segregation defects prior to gastrulation (Table 1). Zygotically null mutants are fully viable (M. McVey and J. Sekelsky, unpublished data), suggesting that the essential function for DmBLM is limited to early embryogenesis. This stage of development is characterized by rapid cycles of replication and nuclear division in a syncytium without intervening gap phases. These nuclei are able to achieve replication of the entire genome in 5–6 min at 25° by firing a large number of replication forks (Kriegstein and Hogness, 1974; Zalokar and Erk, 1976). We hypothesize that DmBLM facilitates resolution of converging replication forks during these rapid S phases, when other replication fork repair mechanisms may be unavailable.

This is not unlike the roles proposed for Sgs1 and Rqh1 in decatenating converging replication forks in rDNA (Coulon et al., 2004; Fricke and Brill, 2003).

The defects in embryonic cell cycles were less severe in embryos derived from females homozygous for *mus309* alleles predicted to remove only the N terminus of DmBLM (Table 2.1). This alleviated phenotype may indicate that the N terminus is not essential for DmBLM embryonic function. It is also possible that the N-terminal truncation alleles have decreased maternal protein levels, either because an alternative start codon is used or because protein lacking the amino terminus has lower stability.

DmBLM in DNA repair

We report here that, like cells from BS patients, spontaneous crossing over is elevated in *mus309* mutants. Exposure to ionizing radiation causes a further increase in COs in the male germline. Ira et al. (Ira et al., 2003) previously reported that *S. cerevisiae sgs1* mutants have elevated crossing over when site-specific DSBs are induced, and Johnson-Schlitz and Engels (Johnson-Schlitz and Engels, 2006a) recently reported a similar result in Drosophila *mus309* mutants. These results suggest that defective DSB repair is one source of the mitotic CO elevation seen in the absence of BLM or orthologous proteins.

The dissolvase model (Figure 8A) has been proposed to explain the role of BLM in preventing COs (Ira et al., 2003; Wu and Hickson, 2003). This proposal is based on the meiotic recombination model of Szostak et al. (Szostak et al., 1983), in which a structure with two HJs is a key intermediate in generating COs. Szostak et al. (1983) proposed that this double Holliday junction (DHJ) structure is resolved by nicking two

strands at each HJ. Depending on which strands are nicked, resolution can produce CO or noncrossover (NCO) products. (Thaler et al., 1987) suggested that resolution might also occur without nicking, if the two HJs are branch migrated toward one another and the remaining catenation is removed by a topoisomerase; this process of ‘‘dissolution’’ generates only NCO products. In the dissolvase model, BLM is the HJ branch migrating enzyme and topoisomerase 3a is the decatenating enzyme. Support for the dissolvase model comes from biochemical assays demonstrating that human BLM and TOP3a, as well as the Drosophila orthologs, can carry out this dissolution reaction in vitro (Plank et al., 2006; Wu and Hickson, 2003).

However, the dissolvase model does not easily explain the repair defects that we observe in *mus309* mutants (Adams et al., 2003; McVey et al., 2004b). Gap repair in Drosophila is best explained by a modified version of the SDSA model (Adams et al., 2003; Kurkulos et al., 1994; McVey et al., 2004a; Nassif et al., 1994). In this modified version, a broken 3’ end invades a homologous duplex template, generating a D-loop, and primes repair synthesis. Synthesis is not highly processive, and the nascent strand is dissociated from the template after a few hundred nucleotides of synthesis. Experiments in *S. cerevisiae*, Drosophila, and mammalian cells suggest that repair synthesis is not highly processive and that the nascent strand dissociates from the template frequently (McVey et al., 2004a; Paques et al., 1998; Richardson and Jasin, 2000; Smith et al., 2007). For a simple DSB, the newly synthesized strand can now anneal to the other end of the break, as in the canonical SDSA model.

We proposed that DmBlm acts as a disruptase during SDSA (McVey et al., 2004b). In the disruptase model, DmBlm is the helicase that dissociates the invading and

newly synthesized strand from the template (Figure 8A). This is similar to the activity proposed to explain the ability of Escherichia coli RecQ helicase to prevent illegitimate recombination by reversing unproductive strand invasions (Harmon and Kowalczykowski, 1998), except that it occurs after repair synthesis. Support for a disruptase function comes from biochemical studies that show that BLM efficiently dissociates the invading strand from a D-loop substrate (Bachrati and Hickson, 2006; van Brabant et al., 2000). Weinert and Rio (Weinert and Rio, 2007) recently demonstrated that DmBLM has both strand displacement and strand-annealing activity in vitro; they hypothesize that the combination of these activities promotes SDSA.

Although we proposed the disruptase model to explain the role of DmBLM in gap repair, this activity can also explain the anticrossover function of DmBLM. During repair of a DSB, rather than of a gap, inability to dissociate the invading strand might allow annealing of the strand displaced from the template to the other resected end of the break (Figure 8A). A DHJ intermediate could then be generated and resolved through nicking to produce CO (or NCO) chromatids.

The disruptase model can also explain the finding that repair of the gap generated by excision of *P{wa}* in *mus309* mutants is often accompanied by deletion into adjacent sequences (Adams et al., 2003; McVey et al., 2004b). We hypothesized that when the invading strand cannot be dissociated from the template by DmBLM, it is sometimes cleaved and that the ends of the break are then joined through NHEJ (Figure 8B). Johnson-Schlitz and Engels (Johnson-Schlitz and Engels, 2006a) recently reported the intriguing finding that deletions can occur on the template chromatid used for gap repair, a result that they interpreted as support for the dissolvase model. They suggested that

DHJ intermediates that cannot be dissolved by DmBLM are cut so that both chromatids have DSBs and that these broken chromatids are repaired by NHEJ. If there is branch migration prior to HJ cutting, the DSBs might be located far from the initial break site, yielding repair products with deletions. Depending on which ends are joined, a CO may also result. This hypothesis presumes that DHJ intermediates are formed during repair of large gaps. As described above, we think this is unlikely. The gap generated in the experiments of Johnson-Schlitz and Engels (Johnson-Schlitz and Engels, 2006a) is only 5 kb, compared to the 14-kb gap used in our experiments (or 10-kb gap when LTRs anneal). Johnson-Schlitz and Engels (Johnson-Schlitz and Engels, 2006b) previously showed that a gap of 44 kb or larger is not repaired efficiently in Drosophila, whereas gaps of 11 kb or smaller are repaired. The 14-kb gap generated by excision of P{wa} is within the range that is repaired efficiently (Adams et al., 2003; McVey et al., 2004b). Furthermore, we estimate that a typical repair synthesis event in Drosophila is on the order of a few hundred base pairs (McVey et al., 2004a), so, in both assays, completely filling the gap is likely to involve multiple cycles of strand invasion and synthesis.

It might still be possible to form a DHJ intermediate during gap repair if the entire single-stranded region, which can be thousands of nucleotides in length, undergoes strand invasion into a homologous duplex. If a DHJ intermediate is formed, dissolvase function of DmBLM may still prevent formation of COs. However, there is no reason to believe that failure to dissolve such a DHJ would lead to deletions. Several enzymes are known to resolve HJs, but these enzymes do so by nicking each duplex, not by generating DSBs (Bennett and West, 1995; Boddy et al., 2001; Constantinou et al., 2002; Shah et al., 1997; West and Korner, 1985). On the basis of these considerations, we believe that the

dissolvase model does not easily explain the occurrence of deletions on the template chromatid. We speculate that these deletions could also result from loss of disruptase activity. We previously proposed that the invading strand of a D-loop is cleaved when it cannot be dissociated by DmBLM, but it is also possible that template strands are cut. This would give the result proposed by Johnson-Schlitz and Engels (Johnson-Schlitz and Engels, 2006a): breaks on both chromatids, which can then by repaired by NHEJ. Indeed, it is possible that some of the events that we classified as deletions adjacent to the excision site are actually template deletions. Our experiments involve gap repair on the male X chromosome, so we cannot distinguish the excised chromatid from the sister chromatid that serves as a repair template.

Another argument that has been made in favor of the dissolvase model is that the absence of topoisomerase 3α results in a similar elevated mitotic CO phenotype in *S. cerevisiae* and Drosophila (Ira et al., 2003; Johnson-Schlitz and Engels, 2006a). In biochemical studies, dissolvase activity requires topoisomerase 3α (Plank et al., 2006; Wu and Hickson, 2003), but disruptase activity does not (Bachrati and Hickson, 2006; van Brabant et al., 2000; Weinert and Rio, 2007). However, D-loop substrates used in *in vitro* studies are generated by annealing oligonucleotides, but D-loops generated *in vivo* occur in the context of chromosomes that are orders of magnitude longer than these model substrates. It is reasonable to expect that topoisomerases may be required for disruptase activity in vivo, where one or both ends of the template molecule are essentially immobilized.

The dissolvase and disruptase models are not mutually exclusive, and both may contribute to mechanisms through which BLM prevents crossing over or to other

functions of BLM. We have argued that the disruptase function of DmBLM is more relevant during gap repair and perhaps during DSB repair in proliferating cells. Conversely, meiotic recombination events that occur in the absence of MEI-9, an endonuclease required to generate most meiotic COs in Drosophila, have the structure predicted by DHJ dissolution (Radford et al., 2007). We hypothesized that MEI-9 generates meiotic COs by cutting DHJ intermediates and that, in the absence of MEI-9, these DHJs undergo dissolution (Radford et al., 2007; Yildiz et al., 2004); DmBLM is a strong candidate for such a dissolvase.

It might be possible to distinguish between the disruptase and dissolvase hypotheses with separation-of- function mutations in *mus309*. We note that the gap repair defects and elevated rate of spontaneous mitotic COs are as severe in *mus309*^{N2} mutants as in null mutants. Human BLM lacking the region N-terminal to the helicase domain is proficient in carrying out the dissolution reaction in vitro (Wu et al., 2005). If, like human BLM, N-terminally deleted DmBLM is capable of carrying out dissolution, then the gap repair defects and elevated spontaneous COs that we observed must not result from loss of dissolvase activity. Conversely, the embryonic function for DmBLM may require only the dissolvase function or another function similar to HJ branch migration. In the accompanying study of synthetic lethality between mutations in *mus81*and mutations in *mus309* (Trowbridge et al., 2007), we argue that the viability of *mus81*; *mus309*^{N2} mutants suggests that the *mus309*^{N2} mutation destroys the disruptase activity of DmBLM, but does not eliminate the ability to catalyze branch migration of HJs. The results reported therein, together with the findings described above, are

consistent with the hypothesis that the disruptase activity of DmBLM is critical for DSB repair.

In conclusion, we have illustrated multiple functions for DmBLM in genome maintenance. Our results demonstrate that DmBLM is important for normal embryonic development. We have also shown that DmBLM maintains genomic integrity in proliferating cells by inhibiting crossing over and by promoting accurate repair of double-stranded gaps. These findings highlight the multifunctional nature of DmBLM in the prevention of genomic instability.

Materials and methods

Deletion alleles of mus309

Deletion alleles of *mus309*, which is located on chromosome *3* in cytological region 86E17, were generated by *P*-element excision (reviewed in (Adams and Sekelsky, 2002). *P{EPgy2}mus309*^{EY03745} harbors a *P* element just upstream of the ATG corresponding to the initiator codon (Bellen et al., 2004). Flies homozygous for *P{EPgy2}mus309*^{EY03745} are viable and fertile, and we did not detect any defects like those of *mus309* mutations in these homozygotes (data not shown). A total of 759 excisions were screened by PCR to detect any that created deletions in *mus309* protein-coding sequences but not in the other direction. Final structures were determined by DNA sequencing. The four deletion alleles that we recovered are named *mus309*^{N1}, *mus309*^{N2}, *mus309*^{N3}, and *mus309*^{N4}.

Ionizing radiation sensitivity assay

Balanced, heterozygous parents were crossed and allowed to lay eggs on grape-juice agar plates for 12 hr. Embryos were allowed to develop at 25° until larvae reached third instar stage. Plates were then irradiated in a Gammator 50 irradiator at a dose rate of 225 rad/min, after which larvae were transferred to bottles. Relative survival was calculated as the number of mutant adults (homozygous or heteroallelic) divided by the total number of adults (mutant and heterozygous) that eclosed within 10 days of irradiation. Ratios were normalized to an unirradiated control. In experiments with crosses of heterozygous females to wild-type males, there was no difference in survival of heterozygous progeny relative to wild-type progeny (data not shown), indicating that *mus309* is completely recessive for IR sensitivity.

Crossover assay

To measure premeiotic crossovers in the male germline, virgins of the genotype *st* *mus309*^{D2} *e*/*TM6B* were mated to males that were wild type or that carried another allele of *mus309*. Embryos were collected and allowed to hatch, and third instar larvae were irradiated and transferred to bottles. Adult males were collected after eclosion and crossed to *ru h st ry e* virgin females. Progeny were scored as parental or recombinant with respect to *st* and *e*. Each vial was considered a different experiment.

Studies of embryonic development

Virgin females of various genotypes were mated to w^{1118} males. Eggs were collected on grape-juice agar plates for 12 hr and scored for hatching 48 hr later. To

analyze syncytial-stage nuclear divisions, embryos were collected for 2 hr on grape-juice plates, dechorionated with 50% bleach, devitellenized with heptane, and fixed in 3.7% formaldehyde (Fisher F79-500). Fixed embryos were stained with 1 μg/ml DAPI and mounted with Fluoromount-G (SouthernBiotech). Images were taken with WinView/32 software (Roper Scientific) on a Nikon Eclipse E800 fluorescence microscope. To score cellularization and gastrulation (germ-band extension), embryos were collected for 2 hr, aged for 4 hr, and processed in the same way.

CHAPTER III

HYPERRECOMBINATION IN *MUS309* MUTANTS[1]

Introduction

There are large gaps in our knowledge about how recombination arises in cells outside of meiosis. The well-tested meiotic double-strand break repair (DSBR) model of recombination largely serves as the framework for the less well-proven model of mitotic recombination. The identities of many of the proteins whose activities are predicted by the current mitotic model remain unidentified, but a key protein known to be important for mitotic recombination is the RecQ helicase BLM.

The most striking phenotype of cells mutant for BLM is extreme hyperrecombination; they have increased sister chromatid exchange and crossovers (COs) between homologous chromosomes (Chaganti et al., 1974). In wild-type cells COs are suppressed, as they can result in loss of heterozygosity (LOH), loss of sequence, and chromosomal rearrangements (Lorenz and Whitby, 2006). BLM is thought to repress COs via the promotion of DNA repair mechanisms that yield noncrossover (NCO) repair events instead of CO repair events (Hanada and Hickson, 2007).

BLM has several biochemical activities which suggest how it blocks COs.

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[1]Kathryn Kohl contributed to measuring drug-induced COs in *mus309* mutants. Lena Hyatt did the *spn-A mus309* CO analysis.

Firstly, it's been shown *in vitro* that BLM can effectively dissociate D-loops, which are created early in homologous repair when the single-stranded 3' end of a processed DSB invades a homologous template chromosome (Bachrati et al., 2006) (Fig 1.1 This *in vitro* activity, coupled with genetic data from our lab and others, suggests that BLM may disrupt the invading strand during synthesis-dependent strand annealing (SDSA) (Adams et al., 2003; Kikuchi et al., 2009) (Fig 1.1). Completed SDSA precludes the creation of an intermediate, such as a double Holliday junction (DHJ), that could be cut to create COs. However, if a DHJ is formed, BLM has a second anti-crossover activity. BLM can resolve DHJs *in vitro* by branch migrating the junctions together, where they can then be decatenated by the topoisomerase TOP3α to produce a NCO product (Plank et al., 2006; Wu and Hickson, 2003).

DHJs, formed and left unresolved in the absence of BLM, are thought to be CO precursors (Szostak et al., 1983). The nuclease that cuts DHJs could be a structure-specific HJ resolvase that nicks each strand to produce both COs and NCOs, or strictly COs if there is a directional bias. Alternatively, the DHJ could be cut by other, nonspecific nucleases to produce double-strand breaks (DSBs) in both chromosomes. The ends could then be processed and ligated together; ligating together the ends of different chromosomes would result in a CO.

To test and refine the current model of mitotic recombination, we determined the effects of DNA-damaging agents and mutations in candidate genes on the mitotic COs produced in *mus309* mutants. I tested candidates with known or speculated roles in meiotic recombination and DNA repair, including candidates that act early in recombination, such as recombinases, and those thought to act late in recombination,

such as structure-specific endonucleases. To shed light on the mechanism that produces COs, I examined at the sequence level COs produced during the repair of induced double-strand gaps in *mus309* mutants. Finally, I determined whether the increased COs in *mus309* mutants can be exploited as a tool to increase the rate of gene targeting events in Drosophila.

Results

CO induction by DNA damage

Previously, we determined that *mus309* mutants spontaneously have increased COs in the male germline, and that treatment with gamma radiation further induces the rate of COs (McVey et al., 2007). However, little is known about the sources of the spontaneous COs, including whether they are DSBs and/or other types of damage. To gain further insight on what kinds of DNA damage can generate COs, we tested the following treatments for their ability to further induce COs in *mus309* mutants: camptothecin (CPT), a topoisomerase inhibitor that creates DNA nicks that can become DSBs during replication; methyl methanesulfonate (MMS), an alkylating agent; ultraviolet radiation (UV), which makes photoproducts such as cyclobutane pyrimidine dimers; bifunctional nitrogen mustard (HN2), which creates monoadducts and interstrand crosslinks (ICLs); monofunctional nitrogen mustard (HN1), which creates monoadducts; and hydroxy urea (HU), which deplates the available pool of nucleotides and consequently stalls replications machinery. All of the above treatments, except HU, increased COs in *mus309* mutants to varying degrees (Fig 3.1). At the levels we used, HU kills mutants extremely hypersensitive to DNA damage (e.g. mutant for the

checkpoint kinase MEI-41), but the lack of a positive control to show that the drug reached the germline prevents an interpretation of this result.

These data underscore the importance of BLM in maintaining genomic stability in multiple DNA damage contexts, even though the ultimate DNA lesion that is repaired to create a CO is not known for any of these agents. For example, nicks created by CPT may be the initiating lesion, but DSBs produced by replication forks encountering the nicks could also be the initiating lesion. However, the increased COs in *mus309* mutants treated with HN2 as compared with HN1 does support BLM having a role in promoting the NCO repair of ICLs.

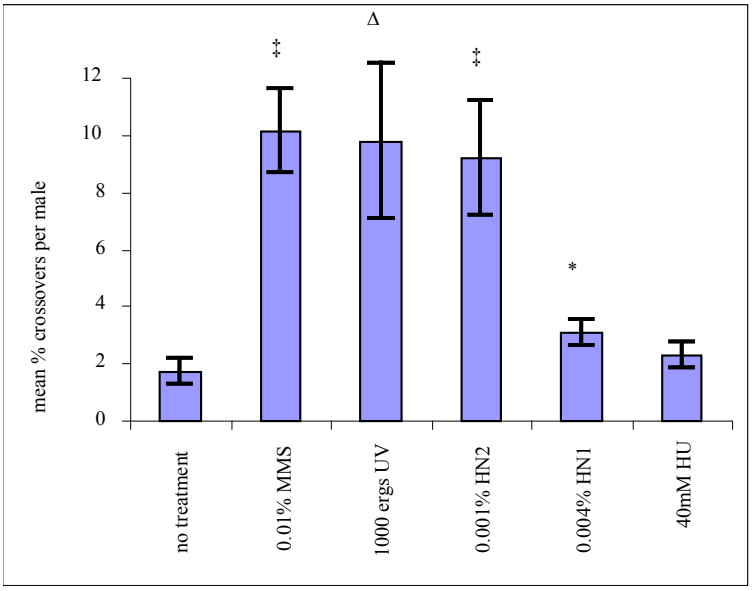

Figure 3.1: Crossover induction in *mus309* mutants. *mus309*^{N1}/ *st mus309*^{D2} *e* mutants males were treated as larvae as indicated. Adult males were then crossed out to *st e* females and their offspring were scored for COs. Error bars represent standard error. As compared to untreated males, P values calculated with the Mann-Whitney test are as follows: * <0.05, Δ <0.01, ‡ <.001.

Blocking early steps of homologous recombination inhibits COs

Strand invasion is an essential step in all current models of recombinational repair. Therefore, we anticipated that loss of strand invasion would eliminate COs in *mus309* mutants. To eliminate or reduce strand invasion in Drosophila mutant for DmBLM, we used mutations in genes encoding the recombinases SPN-A (the Rad51 ortholog) and OKR (the Rad54 ortholog). *okr* and *spnA* mutants are deficient in repairing meiotic DSBs, and are hypersensitive to radiation (Kooistra et al., 1997; Staeva-Vieira et al., 2003).

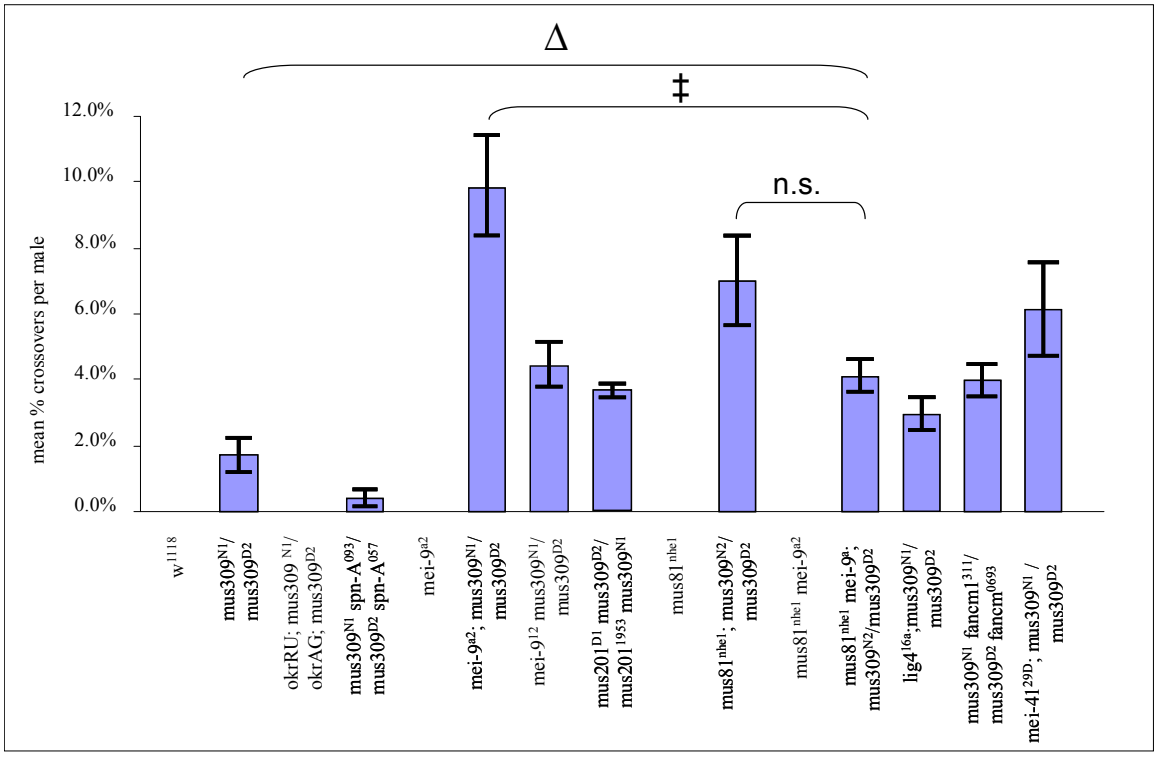

Figure 3.2: Male germline crossovers. Males of the indicated genotype and heterozygous for the markers *st* and *e* were crossed out to *st e* females and their offspring were scored for COs. Error bars represent standard error. P values calculated with the Mann-Whitney test are as follows: Δ <0.01, ‡ <.001, n.s. not significant. All genotypes are statistically significantly different from $mus309^{N1}$ / $mus309^{D2}$ males.

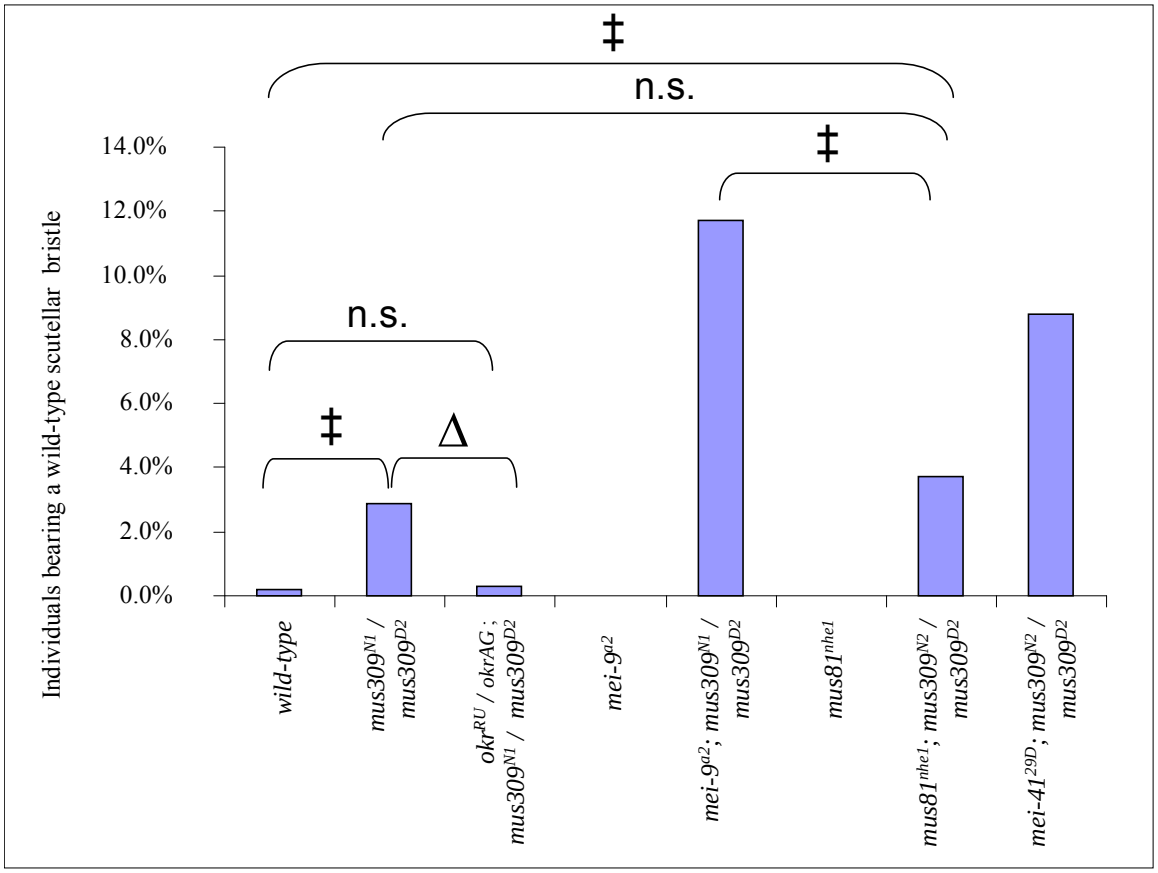

Figure 3.3: Somatic crossovers. Adult Drosophila of the indicated genotype and heterozygous for a dominant allele of *Sb, Sb^1,* were scored for somatic COs that produced at least one wild-type scutellar bristle. Scutellar bristles are a pair of bristles on the dorsal side of the thorax. Cells that lose Sb^1 due to chromosome loss, or that become Sb^1/Sb^1, are inviable. P values calculated with the Fisher's Exact test are as follows: Δ <0.01, ‡ <.001, n.s. not significant

Germline COs were completely eliminated in *okr mus309* mutants, and severely reduced in *spn-a mus309* mutants (Fig 3.2). Similarly, somatic COs scored via the bristle assay were also reduced to wild-type levels in *okr mus309* double mutants (Fig 3.3). While the germlines of wild-type males never produce COs, wild-type somatic cells do experience some COs. These COs don't require OKR-dependent strand invasion activity, and may represent recombination events that don't require strand invasion, such as those initiating with a regressed replication fork. The residual crossovers in *spn-a mus309* double mutants may result from a redundant recombinase activity, or may have been

created early during development in the presence of maternally contributed protein or mRNA. Collectively, these data support a model in which COs in *mus309* mutants are initiated by strand invasion of a homologous template chromosome.

Candidate structure-specific endonucleases are not required for COs

The core feature of the DSBR model is a DHJ intermediate that can be cut to produce a CO. I tested candidate nucleases to determine whether they were required for COs in Drosophila, focusing on those nucleases with known HJ-cutting activities. The best candidate for a HJ resolvase in Drosophila is MEI-9, a structure-specific endonuclease that has a HJ-cutting activity *in vitro* and is required for >90% of meiotic COs in Drosophila (Baker and Carpenter, 1972; Yildiz et al., 2002). Instead of reducing CO levels, mutation of *mei-9* in a *mus309* mutant background increased COs in both the germline and somatic assays (Figs 3.2 and 3.3). MEI-9 has roles in nucleotide excision repair (NER) and ICL repair, and it's possible that in the absence of MEI-9 lesions left unrepaired by these pathways are alternately repaired by the pathway that creates COs (Boyd et al., 1976b). I tested this hypothesis by looking at CO levels in *mus201 mus309* double-mutants (Boyd et al., 1982). Whereas MEI-9 cuts 5' to the lesion in NER, MUS201 cuts 3' to the lesion. If the increased COs come from unrepaired damage, we would expect *mus201 mus309* double mutants to have increased COs, and, in fact, they do. The level of COs in *mus201 mus309* mutants is less than that in *mei-9 mus309* mutants, which may be reflective of MEI-9 having more roles, or more central roles, in multiple repair pathways.

In meiosis, MEI-9 must pair with the nuclease-binding protein MUS312 to make most meiotic COs in Drosophila (Yildiz et al., 2002). The separation-of-function allele *mei-9*^{12} encodes a MEI-9 protein which, due to a single amino acid change, cannot bind MUS312. Therefore, *mei-9*^{12} mutants are defective in making meiotic COs. *mei-9*^{12} mutants are not hypersensitive to HN2 or MMS, which was interpreted as MEI-9 not needing to bind MUS312 for its roles in ICL repair and NER (Yildiz et al., 2002). Also, *mei-9 mus312* mutants have the same hypersensitivity to HN2 as *mus312* single mutants, which is greater than the hypersensitivity of *mei-9* mutants (Yildiz et al., 2002). The epistatis analysis suggests that a subset of MUS312's ICL repair functions require MEI-9, although the interaction is not dependent on a direct MEI-9, MUS312 interaction. *mei-9*^{12} *mus309* mutants have increased germline COs compared to *mus309* single mutants, but less COs compared to double-mutants carrying null alleles of *mei-9* and *mus309* (Fig 3.2). This indicates that MEI-9^{12} retains some level of DNA repair function, but still lacks complete wild-type function. This may be due to altered protein levels stemming from the single amino acid change affecting stability, perhaps via altered binding to stabilizing binding partners. More interestingly, it could reflect a requirement for a MEI-9-MUS312 repair function that only is utilized when DmBLM is absent.

MUS81-EME1/MMS4 was the first nuclear eukaryotic endonuclease that was presented as a potential HJ resolvase (Boddy et al., 2001). MUS81 cuts HJs *in vitro* and is required for subsets of meiotic COs in multiple organisms (Berchowitz et al., 2007; Boddy et al., 2001; Boddy et al., 2000; de los Santos et al., 2003). However, it doesn't have a canonical HJ resolvase activity, and prefers structures such as nicked HJs over fully-ligated HJs (Ciccia et al., 2003; Doe et al., 2002). MUS81-MMS4 doesn't have an

obvious meiotic CO role in Drosophila (Trowbridge et al., 2007). As with *mei-9 mus309*, *mus81 mus309* double mutants have more germline COs than *mus309* mutants (Fig 3.2). Again, the increases may result from MUS81's absence disrupting other repair pathways, causing increased damage that then feeds into the pathway that produces COs. MUS81 is thought to act at blocked and damaged replication forks, so these may be the source of the additional COs (Doe et al., 2002). In contrast to the germline assay, there was a small but not significant increase in the somatic assay (Fig 3.3). This may indicate that MUS81 is not as important for repair in somatic tissues as it is in the germline. This is consistent with previously published sensitivity assays, in which Drosophila *mus81* mutants are not particularly sensitive to UV, MMS, or IR, are only mildly hypersensitive to HN2 and CPT, and are actually resistant to HU as compared to wild-type (Johnson-Schlitz and Engels, 2006a; Trowbridge et al., 2007). Thus, MUS81 appears not to be critically important for repairing DNA in somatic Drosophila tissues.

One complication to the interpretation of the *mus81* results is that I used a separation-of-function allele of *mus309*, *mus039*^{N2}, to make the *mus81 mus309* double-mutant. *mus81* mutations are synthetically lethal with null *mus309* mutations (Johnson-Schlitz and Engels, 2006a; Trowbridge et al., 2007). Given that *mus309*^{N2} mutants have germline and somatic CO levels equal to those of null alleles, and are equally defective in DSBR, it's unlikely that the use of *mus309*^{N2} appreciably impacted the results of the assays.

Given the importance of HR, and the apparent toxicity of unresolved recombination intermediates, it would not be unexpected if multiple nucleases can produce COs. Therefore, we tested germline CO levels in *mus81 mei-9 mus309* triple

mutants to determine if MUS81 and MEI-9 are redundant for CO production. *mus81 mei-9 mus309* triple mutants have increased COs compared to *mus309* single mutants, indicating that COs are not solely dependent on either MUS81 or MEI-9 (Fig 3.2). Interestingly, the triple mutant CO level is not significantly different from the CO level in *mus81 mus309* mutants, but it is significantly less than that in *mei-9 mus309* mutants. The lack of an additive increase suggests that the nucleases are both required for repair of the same initiating DNA lesions. Further, the reduction in COs in the triple mutant as compared to *mei-9 mus309* mutants implies that MUS81 and its repair functions are epistatic to MEI-9 and its repair functions in the germline.

Mutations in DNA repair-associated genes alter CO levels

One of the models for the source of COs in cells lacking BLM function suggests that DNA intermediates are not symmetrically cut by HJ resolvases, but are rather cut by non-specific nucleases, or mechanically torn apart during anaphase to yield two chromosomes with a DSB; ligation of broken ends from two different homologous chromosomes would produce a CO (Johnson-Schlitz and Engels, 2006a). To see whether end joining was important for producing COs, as would be expected for the latter model, I made *mus309 lig4* double mutants. The double mutants had the same CO level as *mus309* mutants alone, suggesting that canonical LIG4-dependent end joining is not required to make the COs (Fig 3.2). However, there is evidence for non-LIG4-dependent end joining in Drosophila, and this may still contribute to CO formation (McVey et al., 2004c).

While BLM has been classically defined by its role in DSBR, BLM also functions alongside Fanconi anemia (FA) proteins in the repair of ICLs and the resolution of fragile site-associated bridges during anaphase (Chan et al., 2009; Pichierri et al., 2004). In mammalian cells, BLM and the FA core complex physically interact to form the mega complex BRAFT (Deans and West, 2009; Meetei et al., 2003). The FA-BLM interaction is important for localizing BLM to site of damage during ICL repair, but is not important for BLM's roles in repairing other kinds of damage (Deans and West, 2009). The FA-BLM interaction is mediated by the helicase FANCM, a member of the FA core complex (Deans and West, 2009). *in vitro*, FANCM can branch migrate both HJs and model replication forks (Gari et al., 2008; Poot and Hoehn, 1993). Recently, an elevation in SCE was measured in FANCM-deficient cells (Deans and West, 2009; Rosado et al., 2009). This result prompted us to question whether the COs detected in FANCM-deficient cells are due to the loss of BLM function, and whether epistasis anaylsis of COs in double and single *fancm* and *mus309* mutants might reveal something of the functions of these proteins in repair.

In the germline assay *fancm mus309* mutants have increased COs compared to *mus309* alone (Fig 3.2). This is in marked contrast to what is seen in *fancc/blm* deficient DT40 cells, which have the same level of COs as *blm* deficient cells (Hirano et al., 2005). The CO increase indicates that FANCM has repair functions aside from those that it might partner with BLM for, such as ICL repair, or that BLM is not as vital to repairing ICLs as FANCM. As Drosophila do not have the full complement of thirteen FA proteins, only having five orthologs, FA proteins in Drosophila likely function differently

than the core FA complexes of vertebrates. Consequently, it's not unforeseen that the relationship between BLM and FA proteins may be different in Drosophila.

Poor fertility of the deficiency stock used in the assay of single *fancm* mutants resulted in difficulty scoring COs from *fancm* single mutant males; no COs were recovered, but the low number of offspring (n=200) prevents this result from being significant and interpretable.

MEI-41, the Drosophila ATR checkpoint kinase ortholog, directs damage-dependent checkpoints throughout the cell cycle, including during G1-S, S, and G2-M phases (LaRocque et al., 2007). It also ensures that replication is completed before entering mitosis. We predicted that entering S phase without detecting and repairing existing damage could result in more severe or additional damage. For example, unrepaired ssDNA encountered by a replication fork could result in a one-ended DSB that would require recombination to repair the break and reset the replication fork. Consistent with this, *mei-41 mus309* double mutants have increased germline and somatic COs as compared to *mus309* single mutants (Fig 3.2).

To find novel players in mitotic recombination mechanisms, we tested *mus* genes on the X chromosome for roles in *mus309* CO production. MUS101 is homologous to topoisomerase DNA II binding protein 1 (TopBP1), a multiple-BRCT domain containing protein with functions in the replication checkpoint (Yamamoto et al., 2000). *mus101 mus309* double mutants had no change in CO levels as compared to *mus309* single mutants. *mus106* and *mus108* are only roughly mapped. *mus106* mutants are slightly

hypersensitive to MMS and IR, and *mus108* mutants are somewhat sensitive to MMS and HN2 and highly hypersensitive to IR (Boyd et al., 1976a; Smith et al., 1980). Both *mus106 mus309* and *mus108 mus309* double mutants exhibit increased CO rates. Mapping and characterizing these mutations may lead to unexplored functions in mitotic recombination.

Molecular analysis of induced gap repair associated with COs

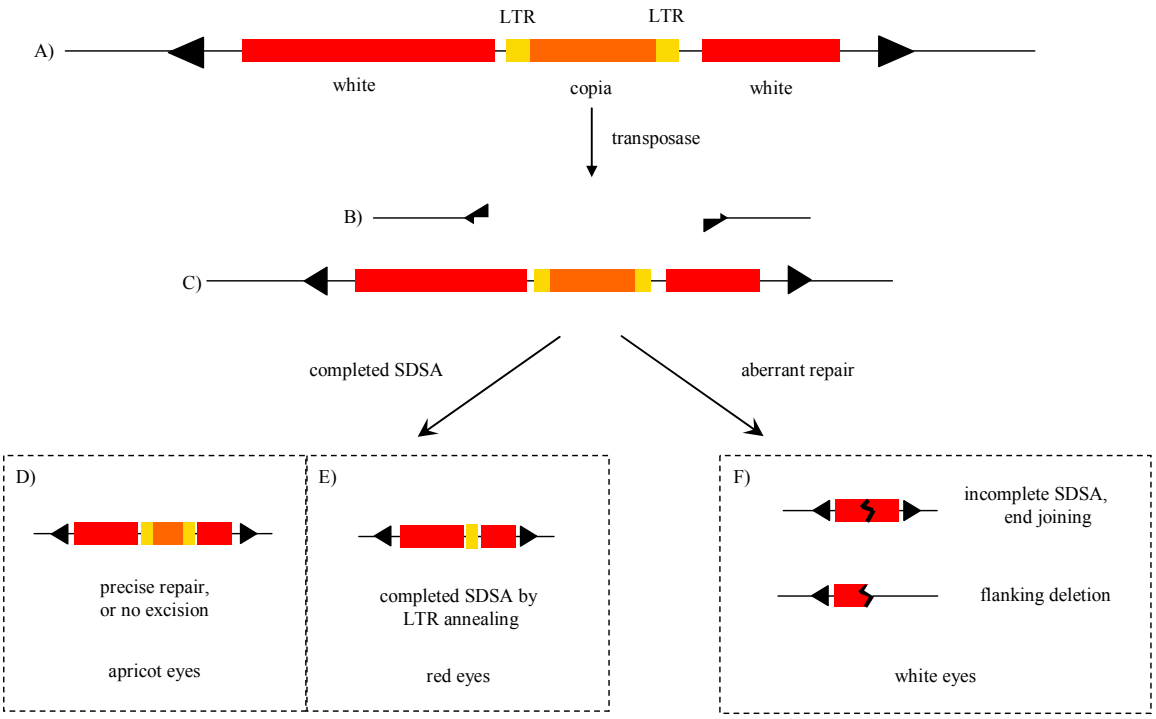

Figure 3.4: *P*{*w*^{a}} gap repair assay. A) *P*{*w*^{a}} construct. Black triangles are P element ends. Red boxes are the gene *w*, which generates red eye color when wild-type. However, *w* is interrupted by a *copia* retrotransposon (orange), creating the allele, *w*^{a}, which causes apricot-colored eyes. Yellow boxes are *copia*'s long terminal repeats (LTRs). In the presence of transposase, the P element is excised (B), leaving short 3' overhangs. When repairing off the sister chromatid (C), SDSA can copy back the entire *P* element (D), which is indistinguishable from an unexcised element. SDSA can also be completed via annealing of the complimentary LTRs (E). This restores function to *w* and produces red eye color. F) Aberrant repair, which disrupts *w* and causes unpigmented white eyes, can yield several products, including flanking deletions in one or both directions, and aborted SDSA with incomplete synthesis followed by end joining. Another source of white eyes is repair using the homolog, which lacks *P*{*w*^{a}}.

To determine if the structures of mitotic COs in *mus309* mutants reveal anything of the CO mechanism, I analyzed CO repair events recovered from the *P{w^a}* excision gap repair assay (Fig 3.4) (Adams et al., 2003). The *P*{*w*^{a}} element carries a *w* allele called *w*^{a}, which is the gene *w* with a *copia* retrotransposon inserted. Flies carrying *w*$^+$ have red eyes, while those carrying *w*^{a} have apricot colored eyes due to *w* being disrupted by *copia*. In the presence of transposase, the P element is excised to produce a double-strand gap relative to a sister chromatid or a break relative to the homologous chromosome. Repair is completed either homologously via SDSA off the sister chromatid or the homologous chromosome, or repaired via a nonhomologous pathway such as end joining. Repair using the sister chromatid can yield several repair events: completed SDSA, producing a *P*{*w*^{a}} element indistinguishable from an unexcised event; completed SDSA that completes with the annealing of the complimentary *copia* LTR's to produce a functional *w* gene and thus red eyes; or incomplete SDSA where synthesis is started from one or both ends, culminating in an end joining event that disrupts *w* and thus creates white eyes. White-eyed flies can also come from repair events that used the homologous chromosome, which lacks *P*{*w*^{a}}, as a repair template.

The repair events listed above are easily explained by the SDSA model of HR. Of a less certain origin are other aberrant repair events that result in white eyes. These are events with deletions into flanking sequence; deletions are more frequently recovered from *mus309* mutants (McVey et al., 2004b). McVey et al hypothesized that flanking deletions occur during SDSA when, without BLM to unwind it, the invading strand becomes trapped in a D-loop which is then cut to resolve the structure (McVey et al., 2004b). This is based on the supposition that BLM's dominate role is in SDSA, and not

in HJ dissolution. Others have suggested that the deletions stem from a HJ intermediate that, unresolved BLM's dissolvase activity, is cut by non-specific nucleases (Johnson-Schlitz and Engels, 2006a). This would create DSBs in the homologous chromosomes, and COs could then occur when homologous chromosomes are endjoined together (Johnson-Schlitz and Engels, 2006a). The uncertainty of what DNA intermediates are created in cells lacking BLM that form deletions and COs led to my examining repair events from the $P\{w^a\}$ assay that had associated COs. If the COs were produced via DHJ resolution by a Holliday junction resolvase, then a single patch of GC flanking the point of CO, with no loss of sequence, would be predicted (Fig 1.1, J and K). If the COs were produced via DHJ shearing, then COs would be predicted to be associated with loss of sequence near the point of crossing over.

Thus, I set up the $P\{w^a\}$ assay used previously in our lab, with the addition of dominant markers flanking the $P\{w^a\}$ loci to allow for identification of CO events associated with P element excision and repair (Adams et al., 2003). The markers were *Ly*, located 2.7 Mb upstream of $P\{w^a\}$, and *Sb*, located 20 Mb downstream. The P element is at the cytological location 72D. Germline repair events were recovered in the offspring of males that had a transposase source on a second chromosome and, for the third chromosomes, a chromosome carrying $P\{w^a\}$(72D) flanked by *Ly* (both with and without a mutant copy of *mus309*) in trans to a chromosome carrying the dominant marker *Sb* and a mutant copy of *mus309*. White-eyed, CO offspring were outcrossed to a deficiency to simplify analysis of the events. Polymorphisms between the two paternal chromosomes were scored via allele-specific PCR and sequencing.

Control males, which were transheterozygous for mutant *mus309* but lacked a transposase source, produced 0.7% offspring with a CO between *Ly* and *Sb*, while *mus309* mutant males with transposase had a CO rate of 3.9% (Table 3.1). Thus, roughly a fourth of CO events recovered were unassociated with P element excision. Consistent with previous studies (Adams et al., 2003), very few completed SDSA events (red-eyed offspring) were recovered from *mus309* mutant males; as predicted by the SDSA model, no events of completed SDSA were associated with COs (Table 3.1).

The COs associated with the P element excision can be placed in two general categories, the first being simple exchanges that appear either directly at, or up to 10kb away from, the site of P element excision. Within the limits of the spacing of the polymorphism markers, these events appear to represent a single CO, with no other exchange of sequence information. Two of the three rare CO events that came from my *mus309*/+ control fell into the first category. The second category includes more complex events with multiple regions of sequence exchange, representing either synthesis tracts from gene conversion or multiple COs. The P element excision caused sequence alterations up to at least 18 kb distant, which is consistent with data seen for mitotic COs in *S. cerevisiae* (Lee et al., 2009).

The structures of both *mus309* and *mus309*/+ events are clearly complex, and don't clearly resemble the structures predicted by the two main hypotheses for CO production. Further, it is unfortunately difficult to determine what kind of intermediate structures yielded them. The exchanges of sequence may stem from multiple COs, or may be the result of GC tracts associated with the CO. They may be both. In the absence

mus309^{D2}/+	+		+	
mus309^{D2}/mus309^{N1}		+		+
transposase			+	+
TOTAL FLIES	1082 (16)	943 (22)	481 (15)	465 (13)
apricot	39.3%	50.2%	12.1%	23.7%
red	0.0%	0.0%	5.8%	0.6%
yellow	60.7%	49.7%	81.5%	74.2%
orange	0.0%	0.1%	0.6%	1.5%
red/red and yellow	0.0%	0.0%	6.7%	0.9%
CO				
total CO	0.0%	0.7% (7)	0.6% (3)	3.9% (18)
apricot CO	0.0%	42.9%	0.0%	11.1%
red	0.0%	0.0%	0.0%	0.0%
yellow	0.0%	57.1%	100.0%	88.9%
orange	0.0%	0.0%	0.0%	0.0%

Table 3.1: Percentages of crossovers and repair events. Paternal genotype is indicated above. The total number of offspring that were scored for crossovers is indicated next to "total flies", and the number of males whose offspring were scored is indicated in parentheses. "red/red and yellow" indicates the rate of completed SDSA. Some yellow offspring don't carry aberrant repair events, but rather are offspring that inherited the parental chromosome that lacks *P*{*w*^{a}}.

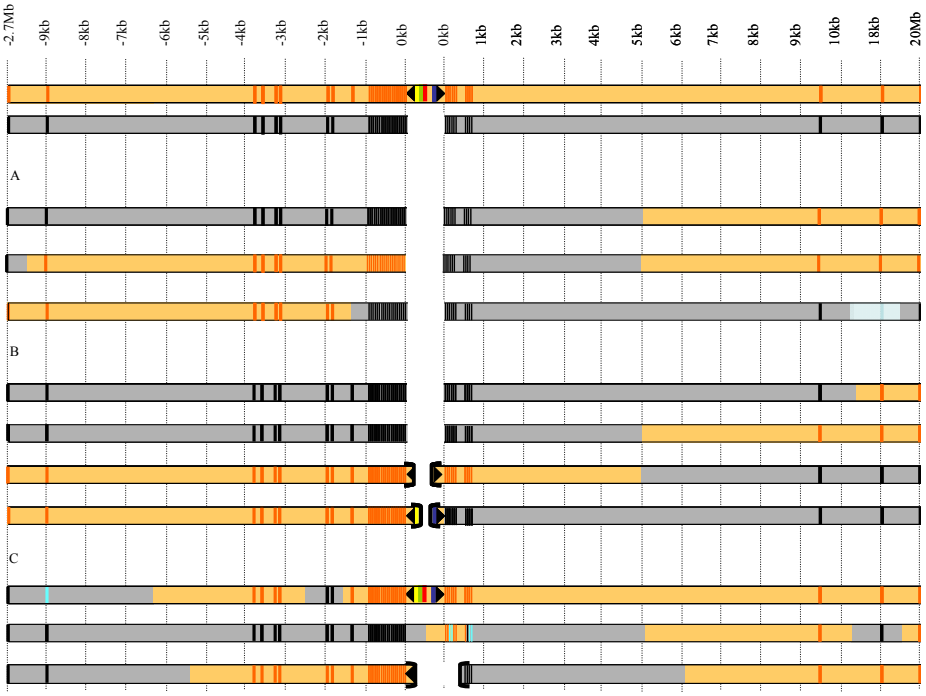

Figure 3.5: Crossovers associated with *P*{*w*^{a}} excision. Vertical hatches represent polymorphisms between the parental chromosomes, which are at the top. Orange represents the *Ly P*{*w*^{a}} (*mus309*^{N1}) chromosome, while gray represents the *mus309*^{D2} *Sb* chromosome. Black triangles represent *P* element ends, and colored boxes between the *P* element ends represent regions of *P*{*w*^{a}}. Blue hatches are unscored. A) Events from *Ly P*{*w*^{a}} / *mus309*^{D2} *Sb* males. B + C) Events from *Ly P*{*w*^{a}} *mus309*^{N1} / *mus309*^{D2} *Sb* males. B) Simple, one point exchanges. C) More complex events involving multiple exchanges.

of the favored, BLM-mediated repair pathway, cells may choose a variety of repair mechanisms in their attempt to compensate for the loss of the preferred mechanism.

Use of mus309 *hyperrecombination as a tool in gene targeting*

Gene targeting is an exceedingly powerful genetic tool, as demonstrated by the research renaissance following the development of gene targeting in mouse (Abbott, 2007). Unfortunately, not all organisms have efficient methods for gene targeting, and many, such as Drosophila and *C. elegans*, are primarily reliant on knockdown techniques such as RNAi. In Drosophila, targeted gene replacement is a labor-intensive process, requiring the screening of tens of thousands of flies for rare targeted events.

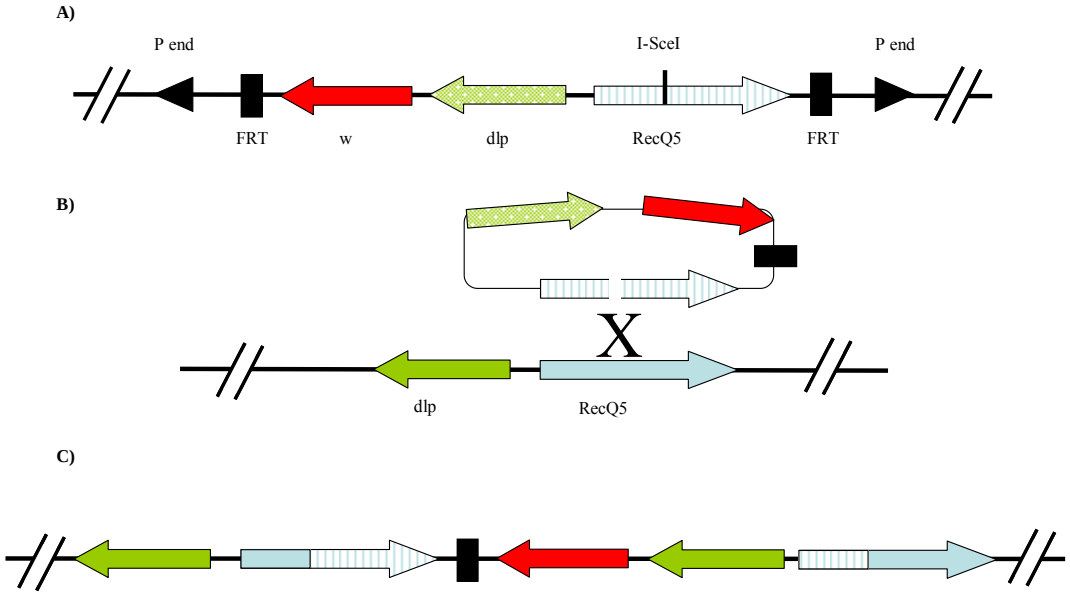

Figure 3.6: ***RecQ5*** **ends-in targeting scheme.** A) *P* element targeting construct inserted on the second chromosome. B) Heat shock-induced FLP recombinase circularizes the contruct at the FRT sites, and I-SceI linearizes it. The construct then homologously recombines with the endogenous RecQ5 locus. C) The targeted *RecQ5* locus.

Previously, our lab successfully used targeted gene replacement to knock out the gene *RecQ5*, and I utilized that ends-in targeting scheme (Fig 3.6). Using the stocks and

method used previously, I tested whether the hyperrecombinational background of a *mus309* mutant increases the levels of targeted events recovered and thereby simplifies the gene targeting screening process. In a wild-type background, 1 out of 1 potential events were correctly targeted out of 10,064 offspring from 86 males, giving a success rate of 1.2% correct events/male. In the *mus309* mutant background, 2 events out of 7 potential events were correctly targeted out of 8,562 offspring from 73 males, giving a success rate of 2.7% correct events/male. While the *mus309* mutant background increased insertions of the construct, the majority of the insertions were incorrectly integrated into other locations, and the rate of correct events recovered is not statistically significantly different from wild-type. The increase in misdirected events may stem from the increased homeologous recombination reported in cells mutant for BLM, or may stem from increased integration sites in the form of unrepaired damage in the absence of DmBLM (Myung et al., 2001). Thus, utilization of a *mus309* mutant background for directed targeting is not only not beneficial, but in fact impairs the screening process.

Discussion

Mechanisms that yield NCOs are clearly preferred in wild-type cells, likely because COs can be detrimental by generating LOH, and are associated with deletions and rearrangements. It's just as clear that BLM plays a key role in important NCO pathways, and is crucial for maintaining genomic integrity in the face of diverse insults from both endogenous and exogenous sources. We've shown DmBLM function is required to inhibit mitotic COs induced by alkylating, radiomimetic, and base-altering agents.

Mitotic COs are absolutely repressed in the Drosophila male germline, but wild-type somatic cells do experience rare mitotic COs. Our data suggest that wild-type somatic COs are fundamentally different from the increased somatic COs in *mus309* mutants in their initiating lesions and/or their generating mechanism(s). Removing the strand invasion protein OKR from *mus309* mutants only reduces somatic CO levels to that of wild-type flies. This suggests that wild-type COs do not require strand invasion for their production, whereas *mus309* mutant COs do. This gives a clue as to what kind of lesions may be resolving into COs. For example, a regressed replication fork may not require strand invasion to produce a recombination substrate, whereas DSBs do.

The fact that somatic COs occur regularly if rarely in wild-type cells suggests that there may be a regulated, designated mechanism for making them. The current mitotic recombination model predicts that COs are made by the symmetrical cutting of a DHJ by a HJ resolvase. I've not yet recovered a spontaneous somatic CO from flies mutant for MEI-9 or MUS81, leaving open the possibility that one or both of these nucleases are required for producing the COs. However, the rarity of somatic CO events necessitates higher numbers be scored to show if MEI-9 or MUS81 are required. If COs are recovered, it will not rule out other, uncharacterized HJ resolvases playing a role in either wild-type or *mus309* mutant CO production. Our lab is currently studying whether the novel HJ resolvases GEN and MUS312-SLX1 are important for mitotic recombination in Drosophila.

MUS81 and MEI-9 aren't solely required for the COs that occur in the germlines of *mus309* mutants. Unexpectedly, *mus81 mei-9 mus309* triple mutants have the same level of germline COs as *mus81 mus309* double mutants, which is less than that seen in

mei-9 mus309 mutants. This suggests that MUS81 and MEI-9 are operating in the same repair pathway, with MUS81 operating upstream of MEI-9. However, *mus81* mutants in Drosophila aren't particularly hypersensitive to HN2, MMS, or UV, which suggests that MUS81 isn't important for the NER or ICL repair pathways that MEI-9 functions in (Trowbridge et al., 2007). It is possible that loss of DmBLM creates a compensating role for MUS81 in one or both of these repair pathways. If this is the case, the data is consistent with what is known of the ICL repair pathway. MUS81 has been implicated in cutting replication forks stalled by ICLs to produce the single-ended DSB intermediate (Hanada et al., 2006). MEI-9 has been implicated in the later steps of unhooking and in the homologous repair needed to repair the MUS81-induced DSB and reset the replication fork (Bergstralh and Sekelsky, 2008).

As illustrated by the differing requirements for MUS81 in germline and somatic cells when DmBLM is gone, there also seems to be a difference in COs in the germline as opposed to somatic tissues. This may stem from different types of lesions occurring more commonly in one developmental context than the other, or more likely may reflect differences in the availability and activities of repair proteins.

My data also suggest that MEI-9 and MUS312 have a heretofore unrecognized DNA repair function that requires their physical interaction when BLM is absent. While MEI-9 is hypothesized to act as a HJ resolvase in meiotic cells when complexed with its partners MUS312, HDM and ERCC1, its repair functions in NER and ICL repair were previously thought to not require a physical interaction with MUS312 (Yildiz et al., 2002). However, *mei-9*^{12} *mus309* mutants have increased COs compared to single *mus309* mutants, indicating that MEI-9^{12} lacks completely wild-type MEI-9 repair

function. The CO level in *mei-9*^{12} *mus309* is less than that of null *mei-9 mus309*, so MEI-9 ^{12} must maintain some MUS312-independent repair function. Given that *mus309* mutants have lower COs than null *mei-9 mus309* mutants, it's unlikely that the MUS312-MEI-9 nuclease is responsible for cutting DHJs to produce COs in the absence of DmBLM. It's more likely that it plays an earlier role in repair, such as in the cutting of regressed replication forks to initiate homologous repair.

A well-supported and well-tested model for mitotic recombination has not yet been developed, but the findings in this paper will contribute to its development. Mapping *mus106* and *mus108* and determining their roles in recombination may identify novel, informative participants in repair/recombination. Characterizing the function of MUS312-MEI-9 in the absence of DmBLM will help clarify DmBLM's roles, as will further work establishing whether other putative HJ resolvases are required for the COs in *mus309* mutants.

Materials and methods

Pre-meiotic mitotic germline CO assay

Females of the indicated genotype were crossed in vials to males of the indicated genotype. After three days of laying, the adults were removed and the larvae were administered the treatments described. Once the treated larvae had developed and eclosed, individual adult males heterozygous for mutations of the genes *st* and *e* were outcrossed to homozygous mutant *st e* females. The offspring of the individual males were then scored for COs between *st* and *e*.

Somatic bristle assay

Adult flies of the indicated genotype and heterozygous for a dominant mutation in the gene *Sb* (Sb^1/Sb^+) were scored for mitotic COs that yielded at least one wild-type (Sb^+/Sb^+) scutellar bristle.

P{w^a} *CO assay*

The $P\{w^a\}$ assay was done as described previously (Adams et al., 2003), only with a $P\{w^a\}$ insertion on 3L in the cytological location 72D. Repair events were recovered from the germlines of males that carried either a *Ly* $P\{w^a\}$ chromosome or a *Ly* $P\{w^a\}$ $mus309^{N1}$ chromosome in trans to a $mus309^{D2}$ *Sb* chromosome. COs were scored between the dominant markers *Ly* and *Sb*. Repair events were analyzed over deficiency.

Gene targeting

Virgins carrying the targeting construct *P*f and heterozygous for the $mus309^{N1}$ mutation were crossed to males expressing sources of flippase and I-SceI and homozygous for $mus309^{N1}$. From that cross, individual males either heterozygous or homozygous for $mus309^{N1}$ and carrying $P\{Q5\}$ in trans to FLP and I-SceI were crossed to FLP carrying females. Offspring carrying potential targeted events (identified by the *w*+ carried on the targeting construct, and lacking mosaicism that would indicate the *w*+ source was actually just an unexcised targeting construct) were then crossed to create balanced stocks and the events were checked for correct targeting via PCR.

CHAPTER IV

DROSOPHILA MUS312 AND THE VERTEBRATE ORTHOLOG BTBD12 INTERACT WITH DNA STRUCTURE-SPECIFIC ENDONUCLEASES IN DNA REPAIR AND RECOMBINATION[1]

DNA recombination and repair pathways require structure-specific endonucleases to process DNA structures that include forks, flaps, and Holliday junctions. Previously, we determined that the Drosophila MEI-9-ERCC1 endonuclease interacts with the MUS312 protein to produce meiotic crossovers, and that MUS312 has a MEI-9-independent role in interstrand crosslink (ICL) repair. The importance of MUS312 to pathways crucial formaintaining genomic stability in Drosophila prompted us to search for orthologs in other organisms. Based on sequence, expression pattern, conserved protein-protein interactions, and ICL repair function, we determined that the mammalian ortholog of MUS312 is BTBD12. Orthology between these proteins and S. cerevisiae Slx4 helped identify a conserved interaction with a second structure-specific endonuclease, SLX1. Genetic and biochemical evidence described here and in related

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[1]Previously published as *Drosophila MUS312 and the vertebrate ortholog BTfBD12 interact with DNA structure-specific endonucleases in DNA repair and recombination*. Andersen SL, Bergstralh DT, Kohl KP, LaRocque JR, Moore CB, Sekelsky J. Mol Cell. 2009 Jul 10;35(1):128-35.

papers suggest that MUS312 and BTBD12 direct Holliday junction resolution by at leasttwo distinct endonucleases in different recombination and repair contexts.

Introduction

Specialized endonucleases execute important steps in DNA repair and recombination pathways by recognizing and cleaving specific DNA structures, including 50 and 30 flaps, bubbles, forks, and Holliday junctions (HJs). Some such endonucleases can cut different structures in different pathways. An example is vertebrate XPF-ERCC1 and its orthologs Rad1–Rad10 in S. cerevisiae and MEI-9-ERCC1 in D. melanogaster (Ciccia et al., 2008). These enzymes were first identified for their roles in nucleotide excisionrepair (NER), a pathway responsible for removal of UV-damaged bases. In NER, these enzymes nick the damaged strand at the 5' end of a bubble (Bardwell et al., 1994; Park et al., 1995). They also function in repair of double-strand breaks (DSBs) (Bergstralh and Sekelsky, 2008). It is likely that they function in multiple DSB repair pathways; one role is in single-strand annealing (SSA), where Rad1–Rad10 and XPF-ERCC1 cleave 3' ended flaps (Al-Minawi et al., 2008; Fishman-Lobell and Haber, 1992). MEI-9-ERCC1 is important for meiotic DSB repair, where it is thought to cut double Holliday junction (DHJ) intermediates to generate crossovers (Radford et al., 2007). Finally, these enzymes are critical for repair of DNA interstrand crosslinks (ICLs), though their exact functions in ICL repair are not well understood (Bergstralh and Sekelsky, 2008).

The ability of enzymes like XPF-ERCC1 and orthologs to recognize different substrates in different pathways is likely dependent on specific protein-protein

interactions. Such interactions may recruit the nuclease to the site of damage. For example, an interaction with the damage-binding protein XPA recruits XPF-ERCC1 for NER (Park and Sancar, 1994). Alternatively, interaction of a nuclease with another protein might directly modulate substrate specificity. A possible example is seen with Drosophila MEI-9-ERCC1, where the meiotic function, but not the DNA repair functions, requires physical interaction with the MUS312 protein (Yildiz et al., 2002).

MUS312, like MEI-9-ERCC1, functions in ICL repair. Relative sensitivities of mutants to crosslinking agents indicate that MUS312 has a more crucial function and acts independently of MEI-9-ERCC1 (Yildiz et al., 2002), raising the possibility that MUS312 partners with a different nuclease in ICL repair. Given the central importance ofMUS312 to meiotic recombination and ICL repair, we hypothesized that homologs would have similar functions in other eukaryotes. We report here the identification of BTBD12 as the vertebrate ortholog of MUS312. Expression patterns and knockdown studies suggest that BTBD12 has functions similar to MUS312. Both are orthologous to yeast Slx4 and, like Slx4, complex with at least two different endonucleases.

Results and discussion

MUS312 is orthologous to BTBD12 and Slx4

MUS312 lacks known functional domains and is poorly conserved even within arthropods. We therefore conducted sequence analyses to detect conserved structural characteristics. MUS312's predicted architecture includes a short coiled-coil domain and a C terminus with seven a helices (Fig 4.1). The final two predicted helices are separated by a conserved glycine. This structure is similar to the SAP domain, a DNA-binding

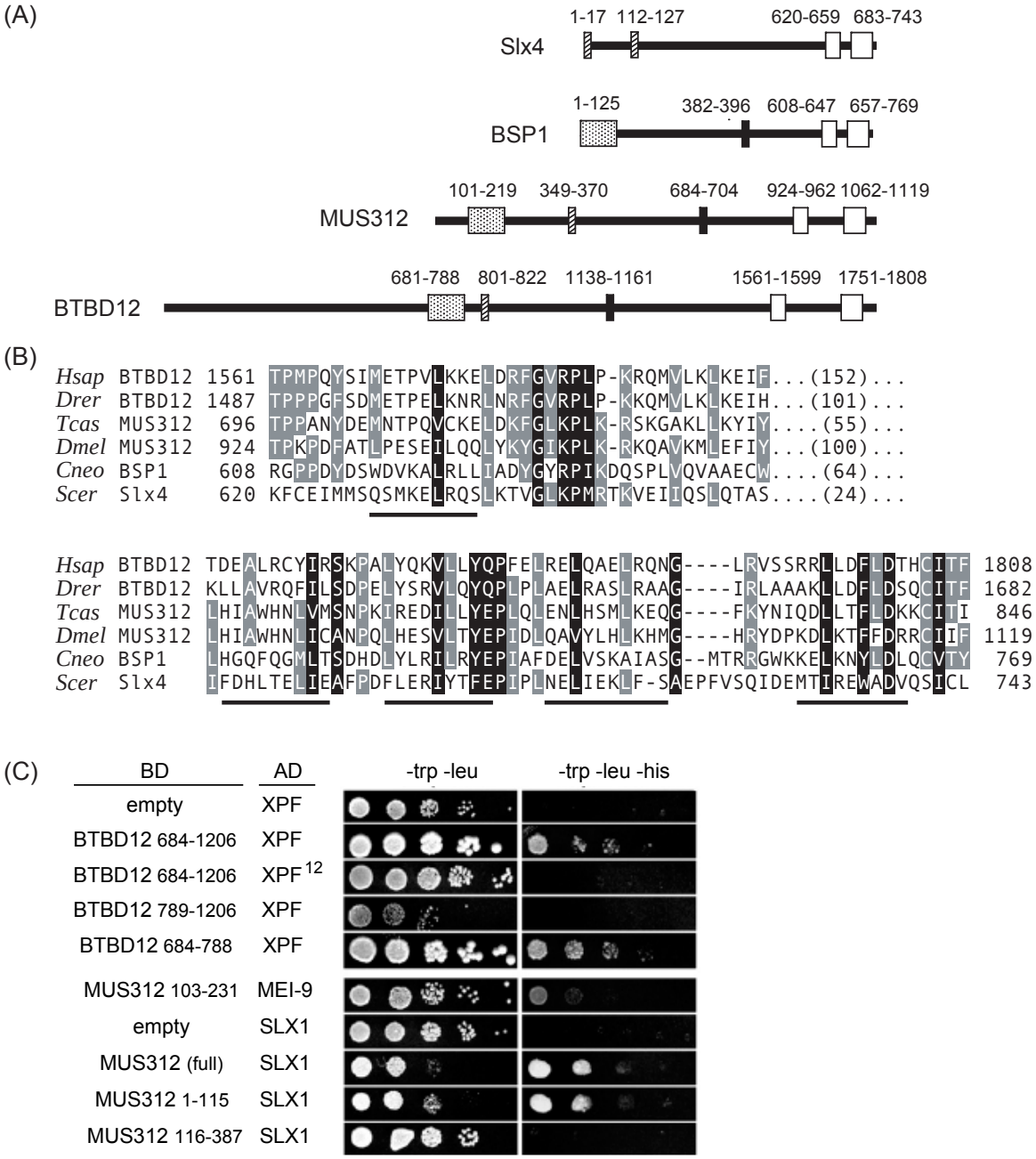


Figure 4.1: MUS312 is orthologous to BTBD12 and Slx4. (A) Domain architecture of *S. cerevisiae* Slx4, *C. neoformans* BSP1, *D. melanogaster* MUS312, and *H. sapiens* BTBD12. Open boxes, conserved C-terminal domain; filled boxes, internal motif; hatched boxes, predicted coiled coils; stippled boxes, BTB domain of BTBD12 and the regions on MUS312 and BSP1 that have sequence similarity. (B) Alignments of C termini. Two divergent representatives from vertebrates, arthropods, and fungi are shown. Hsap, *H. sapiens*; Drer, *Danio rerio*; Tcas, *Tribolium castaneum*; Dm, *D. melanogaster*; Cneo, *C. neoformans*; Scer, *S. cerevisiae*. Predicted alpha helices are underlined. (C) Yeast two-hybrid interactions. Serial dilutions of cells expressing the indicated fusions to the Gal4 DNA-binding domain (BD) or activating domain (AD) were plated on -leu -trp or -leu -trp -his dropout plates; growth on the former requires the presence of both the BD and the AD plasmid, and growth on the latter indicates a physical interaction. Top half: human proteins; bottom half: fly proteins.

domain found in many repair proteins (Aravind and Koonin, 2000). PSI-BLAST searches using the C-terminal sequence identified proteins with similar C termini, including one in each vertebrate genome (Fig 4.1). The mammalian protein BTBD12 has a predicted coiled-coil domain like MUS312, and a BTB (Broad-complex, Tramtrack, Bric-a-brac) domain. BTB domains mediate hetero and homotypic protein interactions and are commonly located N-terminal to other conserved domains (Stogios et al., 2005).

MUS312 interacts physically with MEI-9, the catalytic subunit of the MEI-9-ERCC1 endonuclease (Yildiz et al., 2002). By yeast two-hybrid (Y2H) assay, we found that human BTBD12 interacts with XPF, the ortholog of MEI-9 (Fig 4.1). The interacting region mapped to the BTB domain. Although MUS312 does not have a BTB domain, we detected weak sequence similarity in residues 101–219. This region interacts with MEI-9 (Fig 4.1). Conservation between these interacting regions indicates the biological relevance of the Y2H interactions. Additional support comes from our finding that the same single-amino-acid substitution in MEI-9 or XPF abolishes the interaction. The mei-912 mutation G349E abolishes interaction with MUS312 (Yildiz et al., 2002). We made the equivalent substitution in XPF (G325E), and it abolishes interaction with the BTB domain of BTBD12 (Fig 4.1).

Elements of the MUS312/BTBD12 architecture are also recognizable in BSP1, a protein from bipolar mating species of the Cryptococcus genus of fungal pathogens. SP1 has sequence similarity with the C termini of MUS312 and BTBD12 and with the N-terminal MEI-9/XPF-interaction region (Fig 4.1). These three proteins also share another short motif (Fig 4.1).

Others recently identified MUS312 and BTBD12 when searching for orthologs of *S. cerevisiae* Slx4 (Fekairi et al., 2009; Munoz et al., 2009). The previous finding that Slx4 interacts with Rad1–Rad10 supports this identification (Fricke and Brill, 2003). Slx4 also interacts with a second structure- specific endonuclease, Slx1 (Flott et al., 2007). In a Y2H assay, Drosophila MUS312 and SLX1 also interact (Figure 1C); a similar interaction has been shown for human BTBD12 and SLX1 (Fekairi et al., 2009; Svendsen et al., 2009). We conclude that Drosophila MUS312, vertebrate BTBD12, and yeast Slx4 are orthologous proteins whose functions involve physical interactions with at least two different structure-specific DNA repair endonucleases.

BTBD12 expression suggests conservation of the meiotic recombination function

MUS312 is important for meiotic recombination in Drosophila; in *mus312* mutants, meiotic crossovers (COs) are decreased by about 95% (Green, 1981; Yildiz et al., 2002). It has been unclear whether the MUS312 CO pathway is unique to Drosophila, but mined expression data suggest a meiotic function for Btbd12 in mice. Murine *Btbd12* mRNA is most highly expressed in testes and oocytes (Fig 4.2). Testis expression increases as the animal approaches sexual maturity (Schultz et al., 2003). The increase begins when spermatocytes first enter pachytene, the stage at which meiotic recombination takes place, and expression is much higher in pachytene spermatocytes than mitotically dividing premeiotic spermatagonia (Fig 4.2) (Namekawa et al., 2006). This expression pattern suggests that mammalian BTBD12 might also have a role in generating meiotic COs.

Orthology to Cryptococcus BSP1 raises intriguing evolutionary implications for the importance of these proteins to meiosis. BSP1 is one of 26 genes at the mating type (MAT) loci of *C. neoformans* and *C. gattii*. The MAT loci of these organisms are unlike

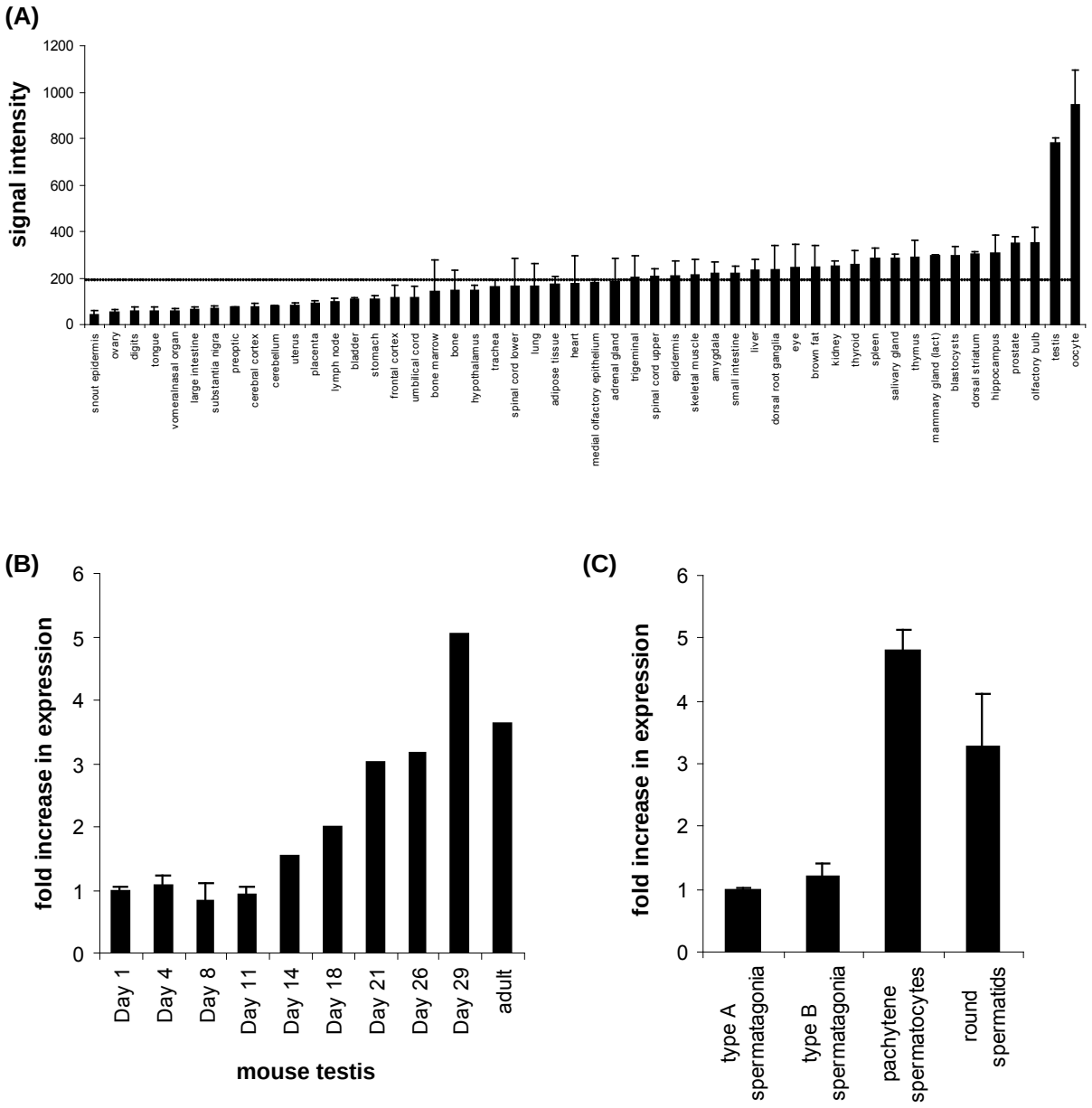


Figure 4.2: *Btbd12* expression is increased in cells undergoing meiotic recombination. (A) Relative expression levels across multiple tissues. *Btbd12* mRNA is most highly expressed in mouse testis and oocytes (http://biogps.gnf.org/). (B) Postnatal testis expression. *Btbd12* expression increases during development, peaking at sexual maturity (Schultz et al., 2003). (C) Stage-specific testis expression. *Btbd12* expression peaks during pachytene, which is when meiotic recombination occurs (Namekawa et al., 2006). Error bars are standard deviations for tissues or stages for which replicates were reported.

those of other fungi but share features with animal sex chromosomes (Fraser et al., 2004). BSP1 is part of the ‘‘intermediate II’’ class of genes, which were incorporated into the mating locus at a period in evolution thought to be important for the genesis of bipolar mating. Three of the six genes incorporated into the MAT loci during this period have been functionally characterized, and two of these (SPO14 and RUM1) are orthologous to meiotic genes from other fungi (Honigberg et al., 1992; Quadbeck-Seeger et al., 2000). We speculate that Cryptococcus BSP1 has meiotic functions similar to those of MUS312.

MUS312 interacts with MEI-9-ERCC1 to generate meiotic COs (Yildiz et al., 2002). However, COs are decreased by >95% in *mus312* mutants, but by only 85%–90% in *mei-9* mutants (Yildiz et al., 2004; Yildiz et al., 2002), suggesting that some COs generated by MUS312 are independent of MEI-9. It is possible that a small percentage of COs require MUS312 and another endonuclease, such as SLX1. It will be interesting to see whether, as suggested by its expression pattern, BTBD12 has a meiotic recombination function and the extent to which this requires XPF-ERCC1, SLX1, or other nucleases.

MUS312 and BTBD12 have important functions in ICL repair

Repair *mus312* mutants were first recovered in screens for hypersensitivity to DNA-damaging agents (Boyd et al., 1981). *mus312* mutants are mildly hypersensitive to the alkylating agent methyl methanesulfonate (MMS) but are highly hypersensitive to the nitrogen mustard mechloramine (HN2), a bifunctional agent that can cause DNA interstrand crosslinks (Boyd et al., 1981). A role for BTBD12 in responding to DNA damage is suggested by the identification of BTBD12 in proteomic screens for substrates ofthe DNA damage checkpoint kinases ATM and ATR (Matsuoka et al., 2007; Mu et al.,

2007). *S. cerevisiae* Slx4 is a target of the orthologous kinases Mec1 and Tel1 (Flott and Rouse, 2005). To determine whether BTBD12 has a role in ICL repair, we used siRNA to knock down BTBD12, XPF, and SLX1 in HeLa cells. Reduction of the tetrazolium reagent XTT was measured as an indicator of cell respiration before and after exposure to DNA-damaging agents (see Materials and methods). Transfection with control or XPF siRNA had no effect on XTT reduction, but transfection with siRNAs for BTBD12 or SLX1 caused a 25% decrease, suggesting that BTBD12-SLX1 plays a role in cell proliferation or survival in the absence of exogenous damage (Fig 4.3).

Knockdown of BTBD12 or SLX1 caused a significant increase in sensitivity to HN2, but not to MMS (Fig 4.3). Since ICL repair occurs primarily during replication (Niedernhofer et al., 2004), ICLs should slow progression through S phase, and a repair defect should arrest cells in S phase. Cell-cycle profiles of cells treated with HN2 are consistent with this hypothesis: cells transfected with control siRNA and then treated with HN2 show a broader distribution through S phase than untreated cells (Fig 4.3). In contrast, cells knocked down for BTBD12 or SLX1 accumulate in early S phase after treatment with HN2 (Fig 4.3), suggesting that these cells arrest in S phase due to failure to repair ICLs.

Evidence that BTBD12 is required for ICL repair has also been obtained by others using different siRNAs, different sensitivity assays, and different crosslinking agents (Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009). Thus, the previously identified role of MUS312 in ICL repair appears to be broadly conserved in animals.

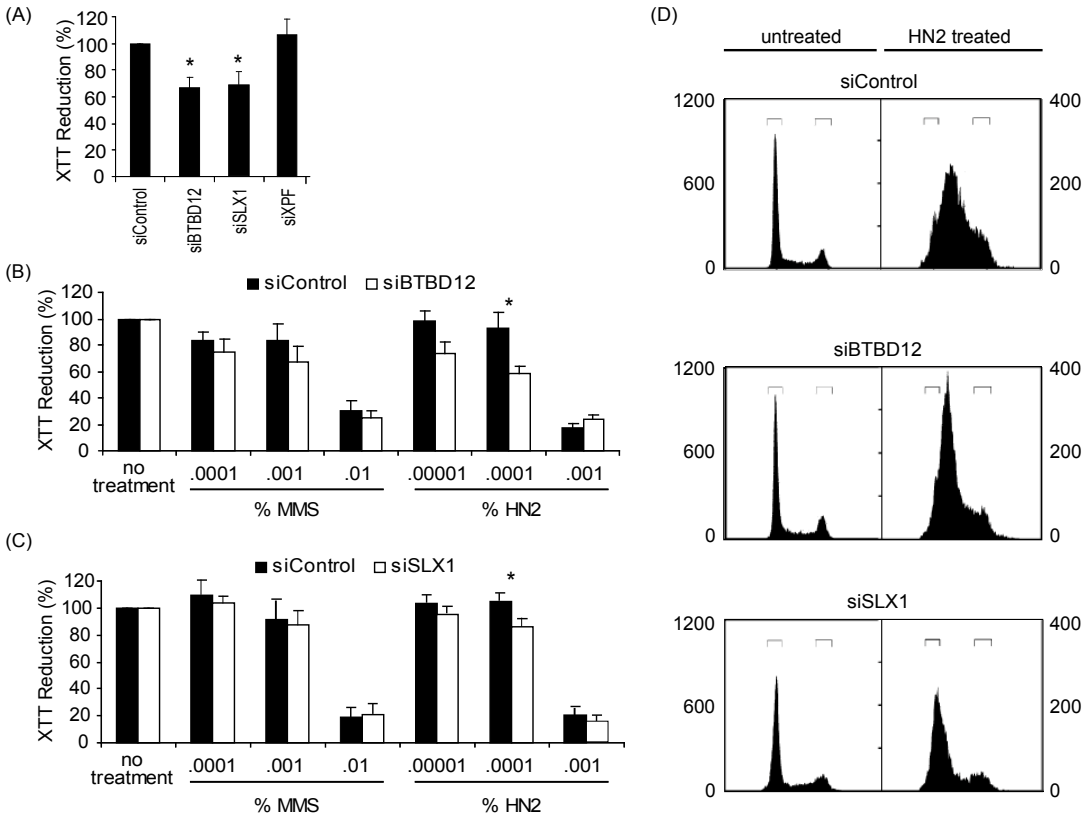


Figure 4.3: BTBD12 acts in ICL repair. (A) Depletion of BTBD12 or SLX1 affects cell proliferation. XTT reduction was measured 4 days after transfection of HeLa cells with siBTBD12, siSLX1, siXPF, or siControl. siBTBD12 and siSLX1 caused identical decreases in XTT reduction, indicating slowed proliferation or cell death; siXPF and siControl had no effect. Bars indicate mean of at least five experiments, and error bars denote SEM. Asterisks indicate $p < 0.05$ by paired Student's t test. (B and C) Transfection with siRNA to deplete BTBD12 or SLX1 causes hypersensitivity to HN2. Three days after transfection with the indicated siRNA, cells were exposed to the indicated concentration of HN2 or MMS for 24 hr. Relative cell respiration was measured with the XTT assay, normalizing to decreases caused by siRNA treatment alone (A). Each bar represents the mean from five separate experiments, with error bars indicating SEM. Sensitivity was not detected at the lowest dose, and the highest dose caused extensive cell death in both control and experimental; the difference was significant at the intermediate dose, however. Asterisks indicate $p < 0.05$ by paired Student's t test. (D) HN2 causes early S phase accumulation after knockdown of BTBD12 or SLX1. Cell number is plotted as a function of DNA content. Cells were transfected with siRNA and then 3 days later were mock treated (left) or treated with HN2 (right). Bars indicate cells with 2C (G1) and 4C (G2) DNA content.

Synthetic lethality between mus312 and mus309 reveals an important function in cell proliferation

SLX4 was first identified in a screen for mutations that are lethal in the absence of Sgs1, the *S. cerevisiae* ortholog of BLM (Mullen et al., 2001). The Drosophila ortholog DmBLM is encoded by *mus309*. We found that *mus312 mus309* double mutants are

inviable, dying after pupariation (Fig 4.4). Third-instar larvae have melanotic tumors, suggestive of elevated cell death, and lack the highly proliferative larval imaginal discs. Furthermore, larval brains of double mutants were greatly reduced in size (Fig 4.4). These phenotypes indicate a severe proliferation defect and reveal an important function for MUS312 even in the absence of induced DNA damage.

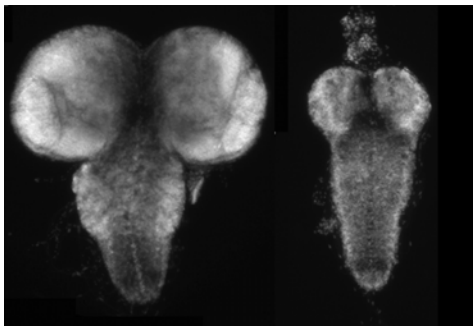
mus81; mus309 double mutants, which lack DmBLM and the structure-specific endonuclease MUS81-MMS4, also have melanotic tumors; imaginal discs are present,

(A)

$$\frac{mus312^{1973}\ mus309^{D2}\ Sb}{TM6B} \times \frac{mus312^{D1}\ mus309^{N1}}{TM3,\ Sb}$$

Genotype	Eclosed
$\frac{mus312^{1973}\ mus309^{D2}\ Sb}{mus312^{D1}\ mus309^{N1}}$	0
$\frac{mus312^{D1}\ mus309^{N1}}{TM6B}$	200

(B)



wild-type *mus312 mus309*

(C)

$$\frac{mus312^{1973}\ mus309^{N1}\ spn\text{-}A^{057}}{TM3,\ Sb} \times \frac{mus312^{D1}\ mus309^{D2}\ spn\text{-}A^{093}}{TM3,\ Sb}$$

Genotype	Eclosed
$\frac{mus312^{1973}\ mus309^{N1}\ spn\text{-}A^{057}}{mus312^{D1}\ mus309^{D2}\ spn\text{-}A^{093}}$	0
$\frac{mus312\quad mus309\quad spn\text{-}A}{TM3,\ Sb}$	404

Figure 4.4: Synthetic lethality between *mus312* and *mus309*. (A) Cross scheme to detect synthetic lethality. This cross generates progeny doubly mutant for *mus312* and *mus309*. No double mutant adults (top genotype) eclosed, though there were expected to be as frequent as the lower genotype (the two genotypes not listed are inviable). (B) *mus312 mus309* double mutants have small brains. Brains were dissected from wandering L3 larvae and stained with DAPI. Brains from *mus312* or *mus309* single mutants are indistinguishable from wild-type brains (data not shown), but brains from double mutants are severely underdeveloped. (C) *mus312 mus309* synthetic lethality is not suppressed by mutation of *spn-A*. This cross generates progeny triply mutant for *mus312*, *mus309*, and *spn-A*. No triple mutant adults (top genotype) eclosed, though they were expected to be half as frequent as the lower genotype (either triple mutant chromosome over the TM3 balancer; TM3/TM3 is inviable).

but apoptosis is highly elevated (Trowbridge et al., 2007). This synthetic lethality is suppressed by mutations in *spn-A*, which encodes the ortholog of the strand invasion protein Rad51 (Trowbridge et al., 2007). In contrast, *spn-A* mutations do not suppress the lethality of *mus312 mus309* double mutants (Fig 4.4), suggesting that *mus312 mus309* lethality is not due to defects in processing a repair intermediate that requires strand invasion. This result is similar to the case in S. cerevisiae, where *rad51* mutations suppress *mus81 sgs1* lethality but not *slx4 sgs1* lethality (Bastin-Shanower et al., 2003). It is likely that the function of MUS312 that is essential in the absence of DmBLM requires SLX1, as in *S. cerevisiae* (Mullen et al., 2001). Consistent with this suggestion, *mei-9; mus309* double mutants are viable as adults (data not shown); unfortunately, there are currently no *slx1* mutations available to directly test this hypothesis.

Roles of MUS312/BTBD12 complexes in DNA repair and recombination

MUS312 and its orthologs clearly have multiple functions in DNA repair and recombination. Since these proteins interact with at least two different structure-specific endonucleases, it is important to consider what structures are cleaved in different pathways. *In vitro*, *S. cerevisiae* and *S. pombe* Slx4-Slx1 are most active on 5' flaps and structures that mimic replication forks (Coulon et al., 2004; Fricke and Brill, 2003). These activities could explain the known function for Slx4-Slx1 in maintaining rDNA stability in the absence of Sgs1 (Coulon et al., 2004; Fricke and Brill, 2003). It is thought that replication forks that stall at natural barriers in rDNA are normally processed by Sgs1 but are cut by Slx4-Slx1 if Sgs1 is not available. Slx4-Rad1–Rad10 is thought to cut 3' flaps in its role in SSA (Li et al., 2008).

The function of MUS312-MEI-9-ERCC1 in generating meiotic COs is not easily explained by cleavage of flaps or forks. Rather, the genetic and molecular defects in meiotic recombination seen in *mei-9* mutants suggest that MEI-9-ERCC1 cuts a DHJ intermediate to generate COs (Radford et al., 2007; Yildiz et al., 2004). Although biochemical activities of MUS312-MEI-9-ERCC1 have not been reported, human BTBD12-SLX1 is reported to have HJ resolvase activity (Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009). It seems reasonable to propose that MUS312 and BTBD12 share an ability to confer HJ resolution activity on associated nucleases. *S. cerevisiae* Slx4-Slx1 can cut HJs *in vitro*, though Fricke and Brill (Fricke and Brill, 2003) concluded that it is not a true resolvase. This could be due to reaction conditions, a missing cofactor, or some divergence in activities of the yeast and metazoan enzymes.

HJ resolution could also account for the roles of MUS312/BTBD12-SLX1 in ICL repair. ICL repair is known to involve formation of a DSB, and HJs are predicted to be generated during processing of this DSB for replication restart (Bergstralh and Sekelsky, 2008; Li and Heyer, 2008). HJs might also be generated prior to DSB formation, by regression of the blocked fork to form a ‘‘chicken foot’’ structure. It is possible that MUS312/BTBD12-SLX1 cuts this structure to initiate ICL repair. However, ICL repair is poorly understood, and some models do not involve fork regression (Raschle et al., 2008).

The synthetic lethal interaction between *mus312* and *mus309* may also reflect an inability to process HJs. BLM helicases have HJ branch migration activity that is believed to function in several mechanisms of replication fork repair, including reversing

regressed forks and dissolving DHJ intermediates (Wu, 2007). Loss of BLM might therefore leave HJs that must be processed by other mechanisms. We speculate that, in some cases, perhaps depending on the type or location of blockage, HJs that cannot be acted upon by DmBLM must be cut by MUS312-SLX1 to allow replication to proceed. Our data indicate that the intermediate that requires either MUS312 or DmBLM is not generated during Rad51-mediated recombination, but HJs may be generated in other ways (e.g., replication fork regression).

Several eukaryotic proteins cut HJs in vitro have now been identified, including Mus81-Eme1 (Boddy et al., 2001), Yen1/GEN1 (Ip et al., 2008), and BTBD12-SLX4 (Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009). Although it is unknown whether these all cut HJs in vivo, their identification raises the question of whether cells need more than one resolvase enzyme. HJs are thought to be formed during meiotic recombination, regression of blocked replication forks, and some types of replication fork restart, and perhaps during DSB repair. These different processes may well employ different HJ cutting enzymes. It is also evident that the same function may use different enzymes in different species. For example, most meiotic COs in S. pombe require Mus81-Eme1, but the orthologous enzyme generates only a subset of COs in S. cerevisiae, and none in Drosophila (Trowbridge et al., 2007; Whitby, 2005).

In summary, MUS312, BTBD12, and Slx4 are orthologous proteins that each interact with at least two different structure-specific endonucleases. Based on previous studies, the work presented here, and recent work from other groups, we propose that MUS312 and BTBD12 are key noncatalytic subunits of Holliday junction resolvases.

Materials and methods

Sequence analysis

Coiled-coil domain prediction used COILS2 (http://www.ch.embnet.org/software/COILS_form.html). Structural analysis was performed with PHYRE (Bennett-Lovsey et al., 2008). The H. sapiens BTB domain position was determined using Pfam (http://pfam.sanger.ac.uk/search/).

Yeast two-hybrid analysis

Yeast two-hybrid analysis was performed as described previously (Radford et al., 2005).

Expression data

Cross-tissue mRNA expression of mouse BTBD12 was mined from BioGPS (http://biogps.gnf.org/). Mouse testis (GDS410) and mouse sperm (GDS2390) expression patterns were mined from the NCBI GEO database (Barrett et al., 2009).

Cell lines and reagents

HeLa cells were cultured in DMEM (GIBCO) with 8% FBS, 10 units/ml penicillin, and 100 mg/ml streptomycin. Cells were maintained in 5% CO2 at 37ºC. DharmaFECT 1 transfection reagent and Control, BTBD12, and XPF SMARTpool siRNA oligonucleotides were purchased from Dharmacon.

XTT assay

HeLa cells were plated into 96-well plates at _20% confluency. siRNA was transfected according to the manufacturer's instructions. Transfection medium was replaced with 100 ml of complete DMEM after 2 days. On day three, 100 ml treatment medium was added to each well. After 24 hr, 50 ml serum-free medium with 25 mM phenazine methosulfate and 1 mg/ml XTT (2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2Htetrazolium hydroxide) was added to each well. Optical density at 450 nM was measured 4 hr later.

Cell-cycle analysis

Cell-cycle analysis was performed as described previously (Bergstralh et al., 2004).

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

In this thesis, I've profiled DmBLM as a key guardian of the genome in both somatic and germline tissues. The mutant phenotypes we've characterized clearly illustrate the importance of DmBLM for maintaining undamaged, properly segregating chromosomes. We determined that Drosophila mutant for *mus309* have maternal effect lethality, with embryos from *mus309* mutant females exhibiting defective mitotic divisions that result in damaged, inviable nuclei. *mus309* mutants are also hypersensitive to DNA-damaging agents such as gamma radiation and HN2, and display the classic BS phenotype of hyperrecombination both in somatic cells and in the male germline.

I also discovered that *mus309* mutations are synthetically lethal with mutations in *mus312*. Until this discovery, MUS312 was only known to have an undefined role in ICL repair, and to partner with the nuclease MEI-9 to make meiotic COs in Drosophila (Yildiz et al., 2002). Research done by us and others has determined that MUS312 and its orthologs not only interact with MEI-9 and its orthologs, but in fact interact with several different structure-specific nucleases, including SLX1 and, in human cells, MUS81 (Andersen et al., 2009; Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009). Further, BTBD12-SLX1 has been identified as a novel HJ resolvase (Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009). The discovery of these

well-conserved MUS312-nuclease interactions has implications for ICL repair, DSBR repair, and meiotic recombination.

Roles for MUS312, SLX1, and DmBLM in ICL repair

ICL repair is one of the least well understood DNA repair pathways in multicellular eukaryotes (McVey). It is of particular interest because many commonly used chemotherapeutics create ICLs, which are extremely cytotoxic to proliferative cells (McHugh et al., 2001). Unfortunately, upregulation of DNA repair pathways can make cancerous cells resistant to subsequent rounds of chemotherapy (Longley and Johnston, 2005). Consequently, targeting DNA repair pathways to enhance the effectiveness of chemotherapeutics is a hot topic of research (Helleday et al., 2008). My research provides insights into ICL repair, particularly into the ICL repair functions of DmBLM, SLX1, and MUS312.

MUS312/BTBD12 and SLX1 in ICL repair

Yildiz et al previously showed that Drosophila mutant for MUS312 are hypersensitive to the interstrand crosslinking agent HN2, but not to ionizing radiation or the alkylating agent MMS (Yildiz et al., 2002). We and others determined that MUS312's role in ICL repair is well-conserved by detecting hypersensitivity to HN2, mitomycin C, and cisplatin in human cells treated with siRNA targeting *BTBD12*, the mammalian ortholog of *mus312.* (Andersen et al., 2009; Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009). Additionally, we determined that *BTBD12* knockdown

cells treated with HN2 accumulate in early S phase, which we interpret as unrepaired ICLs blocking replication progression (Andersen et al., 2009).

Human cells with *BTBD12* knockdown don't show a defect in producing the DSB intermediate, as detected by phosphorylated H2AX, so another role for MUS312/ BTBD12 must be conjectured (Fekairi et al., 2009; Munoz et al., 2009). It's possible that, during the HR stage of ICL repair, a DNA intermediate such as a DHJ is created that requires resolution by a nuclease directed by BTBD12. A good candidate for BTBD12's partner nuclease is SLX1. Human cells treated with siRNA targeting *SLX1* (previously GIY-YIG domain-containing protein 1) are hypersensitive to HN2 and, when treated with HN2, accumulate in early S phase (Andersen et al., 2009). Munoz et al also showed that depletion of BTBD12 or SLX1 causes defects in the repair of DSB intermediates during ICL repair (Munoz et al., 2009). Conflictingly, Fekairi et al detected similar ICL repair defects in BTBD12 knockdown cells but not SLX1 knockdown cells (Fekairi et al., 2009). Overall, however, the sensitivity data, the cell cycle data, and the physical interaction between MUS312 and SLX1 in human and Drosophila suggests that MUS312/BTBD12 and SLX1 may complex for their roles in ICL repair. In Drosophila, the full testing of this hypothesis awaits the creation of null *slx1* mutations. We are in the process of making *slx1* mutants to determine whether they have similar DNA damage sensitivities to *mus312*, and if so, whether the *mus312 slx1* double mutants have equivalent or additive sensitivities.

DmBLM in ICL repair

A growing body of evidence supports a role for BLM in ICL repair, especially as facilitated by an interaction (in vertebrates at least) with fanconi anemia (FA) proteins. The defining characteristics of FA-deficient cells are genomic instability and hypersensitivity to interstrand crosslinking agents. Mammals have thirteen *FA* genes, of which eight code for proteins that form a core FA complex (McVey, 2010). BLM and FA core complexes associate in cells treated with interstrand crosslinking agents, forming a mega-complex dubbed BRAFT that localizes to sites of replication damage (Meetei et al., 2003). BLM's localization to ICLs is dependent on FA proteins (Deans and West, 2009; Hirano et al., 2005). However, the combined functions of FA proteins and BLM do not appear to be strictly limited to repair of ICLs, as they're also found to localize to common fragile sites during normal mitoses (Chan et al., 2009).

My CO data are consistent with DmBLM having a role in ICL repair as well. Germline CO levels in Drosophila mutant for *mus309* increased with treatments for both HN1, a monofunctional nitrogen mustard that makes single-stranded adducts, and HN2, a bifunctional nitrogen mustard that can make both single-stranded adducts and ICLs. However, HN2, at comparable doses, induces COs considerably more than HN1, which supports DmBLM having a role in ICL repair. Knowing that DmBLM has roles in the HR of DBSs, we theorize that DmBLM's ICL repair role may lie in the HR of the DSB intermediate (Fig 1.2).

The strong evidence that FA proteins and BLM cooperate in ICL repair in vertebrates prompted us to examine CO rates in *fanc* mutants and *mus309 fanc* double mutants. Since Drosophila has only five of the thirteen mammalian FA proteins, it's

likely that Drosophila FA proteins function very differently, both in their ICL repair functions and in their relationship with BLM, compared to mammalian FA proteins.

We chose to examine DmBLM's genetic interactions with the Drosophila ortholog of FANCM. FANCM is a member of the core FA complex, and *in vitro* is capable of branch migrating both HJs and structures mimicking replication forks (Gari et al., 2008). Early studies of human FANC-deficient cells showed no spontaneous increase in sister chromatid exchange (SCE), but did show an increase in drug-induced SCE (Hayashi and Schmid, 1975; Sperling et al., 1975). However, a recent study showed that FANCM- deficient cells have a modest increase in spontaneous SCE that was attributed to a loss of interaction between the FA and BLM complexes (Deans and West, 2009). Similarly, chicken DT40 cells show increased spontaneous and induced SCE in FA-deficient cells (Hirano et al., 2005; Rosado et al., 2009). *fancc/blm* mutant chicken cells also have the same level of COs as *blm* mutants, supporting the CO increase stemming from a loss of Blm function (Hirano et al., 2005).

My CO data are not consistent with the recent FANCM studies, as we have not recovered COs from the germlines of *fancm* mutant Drosophila males. However, low numbers of progeny due to fertility defects stemming from a particular stock limit the strength of the result. If, upon further testing, *fancm* mutants prove to not have germline COs, it indicates that the FA proteins aren't important for targeting DmBLM. This would suggest that Drosophila FA proteins have altered functions as compared to mammalian or avian cells. In further support of this, *mus309 fancm* double mutants have increased COs and not the same level of COs as *mus309* mutants. The increase in COs suggests that

ICLs are not the sole source of spontaneous COs, and that, as shown for the human orthologs, not all of BLM's repair functions are facilitated by FA proteins.

The ICL repair pathway continues to be a difficult nut to crack. The discovery that the endonuclease nuclease SLX1 may partner with MUS312 and its orthologs in multiple organisms is a huge step forward in our understanding of ICL repair. Additionally, further study of the genetic interactions of FA proteins and DmBLM will illuminate differences in ICL repair between organisms.

Requirements for nucleases and a nuclease-binding protein in the absence of DmBLM

It was known previously that MUS81 is required for viability in the absence of BLM in *S. cerevisiae*, *S. pombe*, and Drosophila (Boddy et al., 2000; Johnson-Schlitz and Engels, 2006a; Mullen et al., 2001). This led to the development of a model in which BLM unwinds or branch migrates DNA structures that arise during replication/repair, and in the absence of BLM, MUS81 resolves the structures via cutting. In Drosophila, *mus81 mus309* mutants have increased apoptosis relative to an apoptosis increase found for *mus309* alone (Trowbridge et al., 2007). The apoptosis elevation in the double mutant suggests there are two categories of damage: one which can only be repaired by DmBLM, and a second which can be repaired by either DmBLM or MUS81. Additionally, Trowbridge et al provided evidence from a separation-of-function allele of *mus309* that supports the former damage being double-strand breaks and the latter damage being damaged or blocked replication forks (Trowbridge et al., 2007).

During the course of my graduate research I've discovered two additional synthetic lethal mutations: in the gene the encodes the structure-specific endonuclease-binding protein MUS312, as I've discussed previously in this work, and most recently in the gene that encodes the putative HJ resolvase GEN1. Yen1/GEN1 were isolated via their HJ-cutting activities from, respectively, budding yeast and HeLa cells, and are classical HJ resolvases *in vitro* (Ip et al., 2008). GEN can rescue meiotic phenotypes in *mus81* mutants of *S. pombe*, which utilizes Mus81 for meiotic COs (Lorenz et al., 2009). Also, while mutation of *yen1* appears to have little appreciable mutant phenotype in *S. cerevisiae*, evidence from *mus81 yen1* double mutants suggests that Yen1 is partially redundant with Mus81 (Blanco et al.). Otherwise, little genetic data has been reported, leaving the biological relevance of GEN1 and Yen1 unclear.

We hypothesize that *mus312* and *mus309* mutations are synthetically lethal because loss of DmBLM creates a requirement for one or more of the nucleases that partner with MUS312. The requirement may be for a single nuclease, such as MUS312-SLX1, or for several nucleases. We do know that the *mus312 mus309* synthetic lethality doesn't stem from the loss of MUS312-MEI-9 alone, as *mei-9 mus309* double mutants are viable. Unlike with the human proteins (Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009), we don't have evidence for a MUS312, MUS81 interaction in Drosophila, and in fact by yeast two-hybrid they appear to not interact. However, the increased severity of the lethal phenotype for *mus312 mus309* mutants as compared to *mus81 mus309* mutants is formally consistent with the loss of MUS81 function contributing to the overall phenotype observed in *mus312 mus309* mutants. Our lab is currently creating *slx1* mutations, and will be testing whether they are synthetically lethal

with *mus309* alone, or when combined with mutations in other nucleases. Other combinations, such as *Gen* and *mus81*, will also be tested.

As discussed above, the primary somatic phenotype of *mus312* mutants is hypersensitivity to interstrand crosslinking agents. Thus, one key unanswered question originating from my research is whether unrepaired ICLs are the cause of the severe proliferation defects observed in *mus309 mus312* mutants: these phenotypes include small larval brains and imaginal discs and severe polyploidy in neuroblasts. There are two broad explanations for the double mutant phenotypes. The first explanation is that MUS312 has roles outside of ICL repair, perhaps directing nucleases that can rescue some of DmBLM's repair functions. This is supported by my CO data, which implies that there is a repair role produced for MUS312-MEI-9 when DmBLM is absent. The second explanation is that the sole defect is in ICL repair, and that spontaneous ICLs do happen with enough frequency and severity to cause the mitotic catastrophe observed in *mus312 mus309* mutants. The second explanation seems less likely, as we cannot easily account for the polyploid phenotype with a loss of DNA repair function. However, it is still a possibility, as a satisfactory explanation for the extreme polyploidy has yet to be proposed.

In order to glean information on the relative functions of MUS312, GEN, and MUS81 when DmBLM is absent, I've begun comparing and contrasting the relative phenotypes of *mus312 mus309*, *mus81 mus309*, and *Gen mus309* mutants (Table 5.1). *mus81 mus309* mutants die as pharate adults, and *mus312 mus309* mutants die at pupal stage (Andersen et al., 2009; Trowbridge et al., 2007). *mus309 Gen* double mutants die earliest in development, as first instar larvae. This may reflect the stability and the

abundance of the maternal protein or mRNA contribution (as compared to MUS312 and not as compared to MUS81, as *mus81* mutants have no maternal wild-type MUS81 in our assays), and/or may reflect a more important role for GEN in development.

Additional mutation of *spn-A* partially rescues *mus81 mus309* lethality, potentially by blocking the early steps of a recombination pathway that would otherwise produce a DNA intermediate requiring resolution by either DmBLM or MUS81 (Trowbridge et al., 2007). *spn-A* mutation doesn't rescue *mus312 mus309* lethality, nor does it rescue *Gen mus309* lethality (Andersen et al., 2009). It does, however, alleviate the severity of the *Gen mus309* phenotype and, instead of dying at first instar, the double mutants die at pupal stage. *Gen mus309 spn-A* mutants still cannot reach the pharate adult stage, as, like *mus312 mus309* and *Gen mus309* mutants, they have a proliferative defect and lack imaginal discs. The alleviation of the *Gen mus309* mutant phenotypes by *spn-A* mutation denotes a novel, important, and as yet undefined role for GEN in somatic DNA recombination.

As previously mentioned, *mus81* mutations are viable with a particularly informative allele of *mus309*, *mus309*^{N2}. We believe that *mus309*^{N2} is a separation-of-function allele as *mus309*^{N2} behaves like a null allele in our double-strand gap repair assay, yet embryos from *mus309*^{N2} females lack the severe nuclear damage and bridging detected in offspring of *mus309* null females; this suggests that DmBLM^{N2} is deficient in DSBR, but is proficient in the resolution of replication fork structures that arise during the rapid syncytial cycles of early Drosophila development (McVey et al., 2007). Additionally, there is evidence from Trowbridge et al that suggests that DmBLM^{N2} can repair the DNA lesions left unrepaired in the absence of MUS81, but that it is still

Genotype	mus81^{nhe1}; mus309^{N1} / mus309^{D2}	mus312^{1973} mus309^{D2} / mus312^{D1} mus309^{N1}	Gen^{5997} mus309^{N1} / Gen^{4325} mus309^{D2}	mus81^{nhe1}; mus309^{N2} / mus309^{N1}	mus312^{1973} mus309^{N2} / mus312^{1973} mus309^{N2}	Gen^{4325} mus309^{N2} / Gen^{4325} mus309^{N2}	mus81^{nhe1}; mus309^{N1} spn-A^{057} / mus309^{D2} spn-A^{093}	mus312^{1973} mus309^{N1} spn-A^{057} / mus312^{D1} mus309^{D2} spn-A^{093}	Gen^{5997} mus309^{D2} spn-A^{057} / Gen^{4325} mus309^{N1} spn-A^{093}
A) Stage of lethality	pharate adult	pupal	1st instar	80% adult survival	Pupal	ND	60% adult survival	pupal	pupal
B) Imaginal discs	+	-	ND	+	- occasional rudimentary	ND	+	-	- occasional rudimentary
C) Salivary imaginal ring	wild-type	↓nuclei ↑nuclear size	ND	wild-type	wild-type	ND	ND	ND	↓nuclei ↑nuclear size
D) Brain size	wild-type	↓	ND	wild-type	↓	ND	ND	↓	↓
E) Aberrant neuroblast metaphase	29%	100%	ND	9%	11%	ND	ND	ND	ND
F) Mitotic index	0.02	0.01	ND	0.10	0.06	ND	ND	ND	ND

Table 5.1. Comparison of synthetic lethal phenotypes. Phenotypes for mutants of the indicated genotypes are given. ND=not determined. A) lists the furthest developmental stage the mutants can attain. B) indicates whether or not the highly proliferative imaginal disc tissues are present at the 3rd instar larval stage. C) describes the nuclear phenotypes of imaginal ring cells in the larval salivary glands. D) indicates larval brain size as compared to wild-type. E) percentage of apparently aberrant metaphase neuroblast cells as scored in a larval brain squash assay. Aberrant metaphases includes increased ploidy, breaks, and gross rearrangements. F) shows the rate of scored nuclei that are in metaphase.

defective for other types of repair (Trowbridge et al., 2007). As MUS81 is implicated in repair at damaged replication forks (Osman and Whitby, 2007), this is also supportive of DmBLM^{N2} being functional at the repair or resolution of replication forks but not the repair of DSBs. A portion of the N-terminus of DmBLM^{N2} is deleted, but it's predicted to retain its helicase domain. Thus, the separation of function may result from a loss of protein-protein interaction as the N-terminus is predicted to mediate many of BLM's protein interactions (Bachrati and Hickson, 2003).

mus312 mutations aren't viable with *mus309*^{N2}, but the null *mus312 mus309* phenotypes of polyploid neuroblasts, increased nuclear size/decreased nuclear number in salivary gland imaginal cells, and low mitotic index, are ameliorated. The remaining aberrant phenotype of damaged chromosomes is consistent with a defect in DSBR and with what is known of DmBLM^{N2} function. This suggests that MUS312 - and perhaps one or more of its nuclease partners- are important for compensating for the loss of DmBLM's replication-associated repair functions. This also presents the intriguing suggestion that a replication defect may underlie the puzzling polyploidy phenotype.

Further analysis of the relative phenotypes of the *mus309* synthetic lethalities will help illuminate the complex, dynamic interactions of homologous repair (HR) proteins. Outlining the differing requirements for the nucleases MUS81 and GEN, and for MUS312 and its partner nucleases, will help to define their individual roles in recombinational repair, as well as further clarify BLM's multifarious functions. It also will distinguish whether MUS312's sole function is the partnering and direction of multiple nucleases, or whether MUS312 has other unique functions as well.

MUS312/BTBD12-SLX1, a novel HJ resolvase

Since the development of the current DSBR model, which features a DHJ as the central CO intermediate, researchers have hunted for elusive HJ resolvases. The prokaryotic nuclease RuvC was the first HJ resolvase discovered, almost ten years after Szostak et al proposed the DSBR model of recombination (Iwasaki et al., 1991; Szostak et al., 1983). RuvC is the classic, canonical HJ resolvase, and its biochemical activity of symmetrical cutting to produce religatable products defines what is considered a legitimate HJ resolvase. It took another ten years after the characterization of RuvC for the first potential eukaryotic HJ resolvase, Mus81-Eme1, to be described (Boddy et al., 2001) However, Mus81-Eme1 has proven to be a poor HJ resolvase (Heyer, 2004). Following this, Yen1/GEN1 was proposed to be an eukaryotic HJ resolvase, but the mutant phenotypes of *yen1* single mutants have not yet indicated important primary roles in DNA repair or meiosis (Blanco et al.). Thus, the discovery of the novel HJ resolvase BTBD12-SLX1 has had a significant impact on the fields of recombination and repair. The evidence that BTBD12/MUS312-SLX1 may have roles in both DNA repair and in meiosis makes the find even more exciting (Andersen et al., 2009; Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009; Yildiz et al., 2002).

MUS312/BTBD12-SLX1 in ICL repair

Individually, both MUS312/BTBD12 and SLX1 are important for ICL repair. The next step is to determine whether they partner with each other for ICL repair *in vivo*, or whether they work independently. Knowing what we now do about MUS312's

affinity for nuclease binding, the latter result would still leave the possibility that MUS312 interacts with another nuclease, perhaps not yet identified, for its role in ICL repair. Our lab has undertaken steps to identify other MUS312-interacting proteins via pull-downs from Drosophila carrying HA-tagged MUS312. Knowing that MUS312 interacts and directs the activities of multiple structure-specific endonucleases crucial for repair and recombination makes us hopeful we'll find important repair proteins interacting with MUS312.

MUS312/BTBD12-SLX1 in meiosis

Until recently, the roles for MEI-9 and MUS312 in meiosis were considered a species-specific exception, something which is not unusual in meiosis (Shaw and Moore, 1998). MUS312 is very poorly conserved at the sequence level among organisms, and the dominant organism for meiotic studies, *S. cerevisiae*, didn't have a meiotic defect in mutants for *slx4*, the ortholog of *mus312*. However, we've since show that *Btbd12* is expressed in mouse tissues in a pattern consistent with it having a role in mammalian meiosis (Andersen et al., 2009). Further, the *C. elegans* ortholog of MUS312, HIM-18, has recently been shown to have a crucial role in meiosis, seemingly through the resolution of meiotic CO intermediates (Saito et al., 2009). Whether SLX1 has a meiotic function is yet to be fully determined for any organism. We are eager to determine whether SLX1, perhaps partnering with MUS312, is important for generating a subset of the residual COs in MEI-9 mutants.

HJ resolvases and the DSBR model

Our contribution to the discovery and characterization of a novel HJ resolvase opens the doors to much new research on recombination in meiosis and mitotic repair. Perhaps, most importantly, it may prompt important revisions of the current DSBR model. Central questions remain for those studying recombination: is the DSBR model an accurate representation of recombination in meiosis for most organisms? In mitosis? Is the difficulty in isolating biologically relevant HJ resolvases due to technical limitations, or does what we're looking for truly exist at all? Is it realistic to expect the activity of the bacterial protein RuvC to be mimicked by eukaryotic nucleases, especially given the variability in genetic requirements for meiotic COs among different organisms? Even in the model organisms in which meiotic recombination has been most studied, COs have never been completely eliminated, telling us that we've never determined the full compliment of proteins that can/do create COs. Less is even known about requirements for COs in a mitotic context.

The novel protein interactions and functions I've characterized in my graduate work have important implications for the field of DNA repair and recombination. Further, they provide key insights not only into the mechanisms underlying homologous recombination in mitosis and meiosis, but also into the pathways and proteins that comprise the ICL repair pathway.

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