Genetic analysis of homologous recombination repair of DNA double-strand breaks in *Drosophila melanogaster*

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Abstract

Eukaryotic cells employ a number of pathways to ensure that their genomes are faithfully duplicated and accurately repaired when damaged. One critical pathway for the repair of DNA double strand breaks (DSBs) is homologous recombination (HR). HR involves copying DNA sequence from an intact template, often a sister chromatid, to restore genetic information lost at a DSB site. The HR pathway is dependent on many proteins, including BRCA2, RAD51, HELQ and BLM. Mutations in these and other repair proteins result in an increased risk for various cancers, but their molecular mechanisms are not completely understood.

We have used *Drosophila melanogaster* to investigate various aspects of HR using a genetic approach. First, we confirmed that Drosophila Brca2 and Rad51 are equally important in the repair of DSBs induced by transposon excision, mutagenic chemicals or radiation. This is consistent with the established role for Brca2 in recruiting Rad51 to the sites of DSBs. Unlike its mammalian homologs, DmBrca2 does not have a Rad51-independent role in mitotic DSB repair.

We went on to show that *brca2* mutants in flies are sensitive to the topoisomerase inhibitor, camptothecin. This sensitivity is exacerbated by mutations in the cytochrome P450 gene, *Cyp6d2*. We showed that *cyp6d2* mutations are widespread in Drosophila stocks, which can skew results from experiments using this drug to investigate DNA repair mechanisms. We rescued the sensitivity phenotype using a wild type copy of *Cyp6d2*.

We also looked at the steps of HR downstream of Rad51 recruitment. We showed that DmHelQ and DmBlm are both involved in promoting accurate HR repair of a DSB. In the absence of either or both of these enzymes, we see a decrease in the frequency and efficiency of accurate repair along with a compensatory increase in inaccurate repair processes. We suggest a model to explain these observations.

Together, our data have clarified several intermediate steps of homologous recombination repair of a DSB. This dissertation illustrates the effectiveness of using *Drosophila melanogaster* to study DNA repair mechanisms.

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Mechanisms/Structures/Types of damage	
BIR	break induced replication
D-loop	displacement loop
DSB	double-strand break
DSBR	DSB repair
FA/FANC	Fanconi anemia pathway
HJ	Holliday junction
HR	homologous recombination
ICL	interstrand crosslink
IR	ionizing radiation
NHEJ	non-homologous end-joining
SDSA	synthesis-dependent strand annealing
SSA	single-strand annealing
SSB	single-strand break
Genes/Proteins	
BLM	Bloom's helicase
BRCA1/2	Breast cancer susceptibility gene/protein 1/2
Cyp	cytochrome P450
cyp6d2^{NT}	*cyp6d2* allele: no transcript
cyp6d2^{SD}	*cyp6d2* allele: splicing defective
Dm (prefix)	*Drosophila melanogaster*
Hel (prefix)	helicase
mus301	DmHelQ/DmHel308
mus308	DmPolθ
mus309	DmBlm
Pol (prefix)	polymerase
P{*w*^{a}}	P white apricot
RPA	replication protein A
spn-A	DmRad51
Top (prefix)	topoisomerase
TDP1	tyrosyl-DNA phosphodiesterase 1
w	white
Other	
LTR	long terminal repeat
scpt	sensitive to camptothecin (mutation)
STL	synthesis tract length
UTR	untranslated region

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Chapter 1
Introduction: Homologous recombination repair of DNA double-strand breaks

DNA Damage and Repair

Every adult human is the result of successive near-perfect duplications of a single cell. Each duplication, in turn, requires faithful replication of the complete genome, encompassing some 3 billion base pairs. Failure to perform these duplications accurately can have disastrous, sometimes fatal, consequences. Understandably, the cell has numerous mechanisms in place to ensure that its duplication is both complete and correct. This is critical, as the cell must not only ensure replication accuracy, but it must do so among constant endogenous and exogenous insults that could potentially derail cell division by altering the structure of DNA.

Hazardous changes to the structure of DNA include bulky additions, chemical modifications and breaks (Figure 1-1). Each type of DNA damage, whether it was generated from an internal or external source, requires a particular repair response to restore the DNA molecule to its original state (Reviewed in Hoeijmakers 2001). Many of these processes use the intrinsic complementarity of DNA to repair the damage. Pathways such as base excision repair (BER) and nucleotide excision repair (NER) remove a single damaged nucleotide or many nucleotides, which are then replaced using the complementary strand as a template. When sequences on both strands are missing, as in a double strand break (DSB), homologous recombination can restore the sequence using a homologous template. Conversely, non-homologous end joining can repair DSBs with no template at all (Reviewed in Hartlerode and Scully 2009).

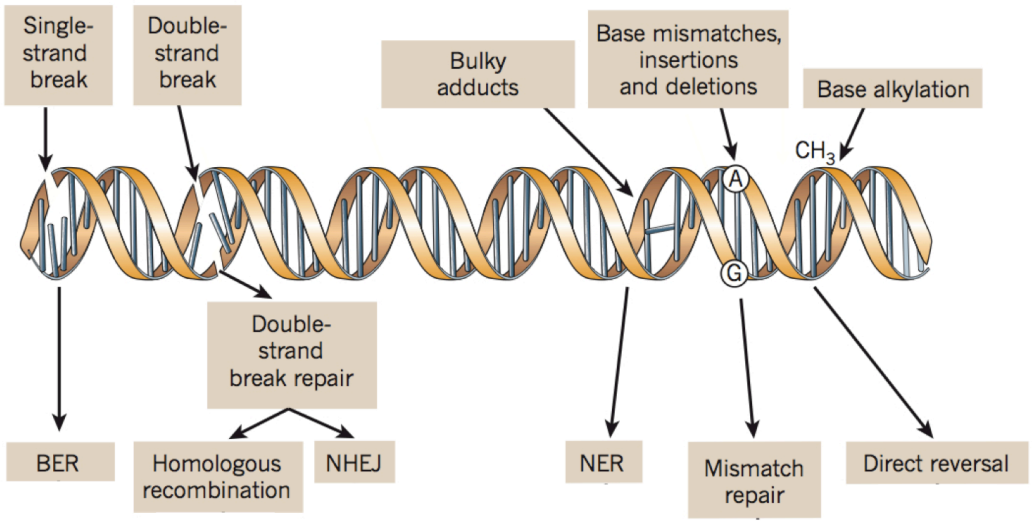

Figure 1-1: Multiple mechanisms of repairing DNA damage. DNA damage can be attributed to multiple endogenous and exogenous sources. Different types of damage elicit different specialized pathways, as depicted above. BER: base excision repair, NHEJ: non-homologous end joining, NER: nucleotide excision repair. Figure modified from (Lord and Ashworth 2012).

Double-strand break repair overview: The double-strand break (DSB) is the most toxic type of DNA damage, as a single DSB can be lethal to a cell if not properly repaired (Bennett et al. 1993; 1996). Fortunately, numerous pathways have evolved to repair this damage and prevent the disastrous consequences of a DSB.

The two main pathways of DSB repair are non-homologous end joining and homologous recombination. Though the focus of this dissertation will be on homologous recombination, it is important to consider alternate pathways of DSB repair, as these error prone pathways may be favored in the absence of critical HR proteins. Non-homologous end joining (NHEJ) is typically divided into canonical (C-NHEJ) and alternative (A-NHEJ) pathways, primarily on the basis of the

presence or absence of the canonical factors, Ligase IV or the Ku70/80 proteins (Reviewed in Mladenov and Iliakis 2011; Hartlerode and Scully 2009).

DSB recognition and response: DSBs are recognized by the MR(X)N complex, which consists of the proteins Mre11, Rad50 and Nbs1 (Xrs2 in yeast) (Reviewed in Pardo et al. 2009). MR(X)N also plays crucial roles in many aspects of DSB repair, including activation of the PIKK (PI3K-like protein kinase) family members (Reviewed in Williams et al. 2010). This family includes ATM (*A*taxia *T*elangiectasia *M*utated) and ATR (*AT*M and Rad3 *R*elated). Ataxia telangiectasia (A-T) is a neurodegenerative disease marked by genome instability and cancer predisposition (Reviewed in McKinnon 2012). Much of the downstream signaling at DSBs is initiated by these two kinases, explaining the phenotype of this disease.

Under normal conditions, ATM is unphosphorylated and dimerized. Recruitment of ATM to the sites of DNA damage by the MRN complex results in its activation via monomerization and autophosphorylation (Bakkenist and Kastan 2003; Carson et al. 2003). ATR, coordinated with its binding partner ATRIP (*ATR* *I*nteracting *P*rotein), is recruited to ssDNA, which is exposed during replication and repair. This is mediated by an interaction between ATRIP and RPA (*R*eplication *P*rotein *A*) (Zou and Elledge 2003), which, like prokaryotic SSB (*s*ingle *s*tranded DNA *b*inding protein), binds and protects ssDNA from degradation (Alani et al. 1992). ATR can be recruited to breaks because resection at the sites of DSBs (see below) exposes ssDNA, which is quickly bound by RPA. Therefore, there are multiple mechanisms by which ATM and ATR can respond

to DSBs. Their activation is followed by the recruitment of additional DNA damage response proteins.

Activated ATM and ATR have multiple downstream targets. One of these is the tumor suppressor p53 (Siliciano et al. 1997; Canman et al. 1998; Banin et al. 1998; Khanna et al. 1998), as well as the protein that mediates p53 degradation, MDM2 (Maya et al. 2001). The phosphorylation of p53 and MDM2 prevent them from interacting, resulting in increased p53 concentration (Su 2006). p53 then promotes transcription of DNA repair and pro-apoptotic genes (Brodsky et al. 2000; 2004), which leads to either reversal of the damage or cell death.

Additional targets of ATM and ATR include the checkpoint kinases, Chk1 and Chk2. Both Chk1 and Chk2 are implicated in phosphorylation of Cdc25a. Unphosphorylated Cdc25a activates Cdk2, leading to cell cycle progression (Jin et al. 2003; Falck et al. 2001). Phosphorylated Cdc25a is degraded or otherwise prevented from promoting Cdk2 activity and, thus, cell cycle progression (Sancar et al. 2004). Therefore, via activation of the checkpoint kinases, ATM and ATR can halt cell cycle progression, preventing replication or mitosis in the presence of DNA damage.

Lastly, ATM and ATR are responsible for phosphorylation of the histone variant H2AX (Burma et al. 2001; Ward and Chen 2001), a critical marker of DSBs (Rogakou et al. 1998). Phosphorylated H2AX (γH2AX) may spread megabases away from the actual break in mammals (Pilch et al. 2003). In yeast, the distance is approximately 50 kb (Shroff et al. 2004). γH2AX is believed to signal the presence of the DSB in order to recruit additional repair proteins. Mice

lacking H2AX show increased levels of chromosomal aberrations but are checkpoint proficient in response to ionizing radiation (Celeste et al. 2002). In humans, γH2AX is bound by the BRCT (*BR*CA1 *C-t*erminus) domain of MDC1 (*M*ediator of DNA *d*amage *c*heckpoint 1), which serves as a scaffold for the recruitment of other proteins (Stucki and Jackson 2006), including BRCA1 and 53BP1 (Thompson 2012), discussed in the next section.

Repair pathway choice: The decision to repair a DSB by HR or NHEJ is an important one, and there are many factors that influence pathway choice, such as cell cycle stage. At the most basic level, the decision is essentially between protection of DSB ends (promoting NHEJ) and resection of DSB ends (promoting HR) (Reviewed in Symington and Gautier 2011).

One of the proteins involved in DSB repair pathway choice in mammals is CtIP (*Ct*BP *I*nteracting *P*rotein), a functional homolog of the yeast protein Sae2 (Sartori et al. 2007). When phosphorylated by CDK, CtIP promotes resection of DSB ends, which favors HR repair of the break (Huertas and Jackson 2009). Since HR requires a homologous template, it tends to be favored during S and G2 stages of the cell cycle, as the sister chromatid is in close proximity (Moynahan and Jasin 2010). Mre11 (a subunit of the MRN complex) complexed with CDK2 coordinates phosphorylation of CtIP and BRCA1, which leads to the formation of a BRCA1-CtIP-MRN complex. It is this complex that initiates the resection at DSBs that precedes HR repair in mammals (Chen et al. 2008). CDK2 activity requires Cyclin A, which is highest during S and G2 phases of the cell cycle (Buis et al. 2012). Thus, phosphorylation of CtIP and BRCA1 regulate their association

and activity such that resection is permitted only when a sister chromatid is available for repair (Buis et al. 2012). In yeast, Mre11 and Sae2 introduce nicks upstream of the 5'-termini on both sides of the break. Mre11 then degrades DNA in a 3'→5' direction, moving towards the break, while the exonuclease Exo1 resects in a 5'→3' direction, moving away from the break (Garcia et al. 2011).

The Ku70/80 proteins and 53BP1 (p*53 b*inding *p*rotein 1) antagonize resection and promote NHEJ by blocking access to the DSB end and surrounding area (Chapman et al. 2012). Expression of 53BP1 is reduced in some breast cancers, and this reduced expression is correlated with triple-negative phenotype, *BRCA1/2* mutations, and poorer clinical outcome (Bouwman et al. 2010). Mice with large deletions in *BRCA1* show extensive genomic instability following mutagen treatment, presumably because of error-prone end-joining repair in the absence of this protein. Deletion of 53BP1 rescues this phenotype, suggesting that 53BP1 is responsible for shuttling breaks toward these error prone pathways (Bunting et al. 2010). Similarly, murine cells with *BRCA1* deletions show reduced RAD51 foci formation, suggesting an HR defect. Removing 53BP1 via shRNA (*s*mall *h*airpin *RNA*) rescues this defect (Bouwman et al. 2010). These data support the hypothesis that 53BP1 promotes end joining repair of DSBs, while BRCA1 inhibits this pathway and promotes HR instead (Chapman et al. 2012).

Homologous Recombination

Homologous recombination (HR) is generally believed to be an error free mechanism of DSB repair. This is because it uses an intact template, usually a sister chromatid, to restore sequences lost at the break or gap. In order for repair

to occur, however, the RPA that is coating the ssDNA must be replaced by the recombinase, Rad51. Rad51, along with its prokaryotic homolog RecA, is conserved throughout evolution (Shinohara et al. 1993). In budding yeast, Rad52 mediates the replacement of RPA by Rad51 (Sung 1997). In metazoans, Rad51 is recruited by the early onset breast cancer susceptibility gene product, BRCA2 (Chen et al. 1998).

The role of BRCA2 in HR: Individuals inheriting a mutant copy of either of the two breast cancer susceptibility genes (*BRCA1* or *BRCA2*) have an 80% chance of developing breast cancer during their lifetime, presumably due to a defect in DNA repair. In addition, there also appears to be a significant risk of ovarian cancer in these carriers (Boulton 2006). Because of this dramatic phenotype, there is a great deal of interest in these two genes. The fact that loss of *BRCA1* or *BRCA2* confers such a high risk of carcinogenesis suggests that the gene products are vital to preserving genome stability.

The mammalian BRCA2 protein (Figure 1-2) contains eight repeated domains known as BRC motifs or BRC repeats (Boulton 2006). These motifs are thought to mimic the oligomerization domain of Rad51, thereby enabling BRCA2 to bind multiple Rad51 monomers (Pellegrini et al. 2002). Homologs of *BRCA2*, including that of *Drosophila*, were identified largely based on sequence similarity at these BRC motifs, as the rest of the protein has diverged significantly throughout evolution (Lo et al. 2003). Until the full length BRCA2 protein was purified (Jensen et al. 2010; Liu et al. 2010; Thorslund et al. 2010), all

biochemical research on the protein used fragments, typically one or more of the BRC repeats (Thorslund and West 2007).

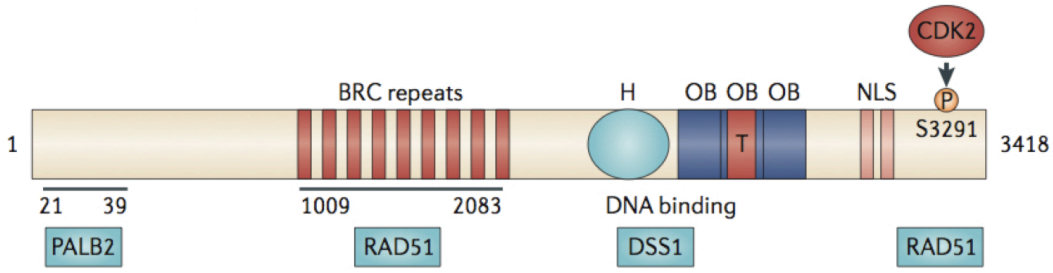

Figure 1-2: Human BRCA2 domains and interaction sites. The human BRCA2 protein is 3418 amino acids (385 kDa). Its most important binding partner is RAD51, for which there are two separate interaction sites. The first includes the 8 BRC repeats that interact with RAD51 monomers, and the second is the C-terminal domain that interacts with RAD51 filaments in a cell-cycle dependent manner. The DNA binding region includes a helical (H) domain, an oligonucleotide binding (OB) domain and a tower (T) domain. Binding sites for PALB2 and DSS1 are also shown. NLS: nuclear localization signal. Figure modified from (Roy et al. 2012)

Besides the BRC repeats, human BRCA2 contains multiple interaction domains that play a role in its repair function. The DNA binding region of the protein contains a helical domain, an oligonucleotide binding domain and a tower domain (Figure 1-2). These domains allow the protein to bind specifically at ssDNA/dsDNA junctions, which facilitates the loading of RAD51 monomers on to the 3' overhangs flanking DSBs (Yang et al. 2002). The DNA binding domain also interacts with the protein DSS1 (*d*eleted in *s*plit hand/*s*plit foot 1) (Marston et al. 1999). While DSS1 appears to promote the HR activity (Kristensen et al. 2010) and stability (Li et al. 2005) of BRCA2, its precise role is not completely clear (Boulton 2006). In the fungus, *Ustilago maydis*, Dss1 protein enhances DNA binding ability of Brh2, a BRCA2 homolog (Zhou et al. 2009).

A third BRCA2-binding protein was recently identified as PALB2 (*p*artner *a*nd *l*ocalizer of *B*RCA*2*) (Xia et al. 2006), also identified as FANCN (Reid et al. 2006). Researchers showed that PALB2 enhanced the function of BRCA2, and that numerous previously unexplained cancer-susceptibility mutations in the N-terminus of BRCA2 are in the PALB2 binding site (Figure 1-2). Further work established that BRCA1 interacts with PALB2, physically linking the two BRCA proteins in HR repair (Sy et al. 2009; Zhang et al. 2009). More recently, PALB2 and BRCA2 were shown to have a role in checkpoint signaling. Equally important to checkpoint activation is checkpoint inactivation, since the cell cycle cannot progress while checkpoints are activated. Phosphorylated PLK1 (*p*olo-*l*ike *k*inase 1) leads to inactivation of the checkpoint and subsequent cell cycle progression. BRCA2 and PALB2 prevent premature inactivation of the checkpoint by suppressing phosphorylation of PLK1 in the presence of DNA damage (Menzel et al. 2011).

While the BRC repeats are thought to bring RAD51 monomers to the sites of DSBs, they are not the only domains of the protein that interact with RAD51. The C-terminus, or TR2 domain, is believed to interact specifically with RAD51 multimers (Figure 1-2). The first indications that this domain played an important role in HR repair came when research revealed that C-terminal truncations in mouse (Moynahan et al. 2001) and human (Howlett et al. 2002) BRCA2 resulted in repair defects. It was later shown that the C-terminal (S3291) phosphorylation blocked the interaction with RAD51, and that this residue was dephosphorylated after IR treatment and during S-phase (Esashi et al. 2005). The same group also

showed that this domain interacted only with RAD51 multimers, and not monomers (Esashi et al. 2007). The current model proposes that when HR is activated, S3291 is dephosphorylated, allowing BRCA2 to stabilize the RAD51-ssDNA nucleoprotein filaments (Thorslund and West 2007). The BRC repeats, on the other hand, actually disrupt RAD51 filaments by binding to individual monomers (Galkin et al. 2005). Another group provided evidence that the C-terminal interaction with RAD51 filaments served to disassemble the complexes in preparation for mitosis. Interestingly, this work (in chicken DT-40 cells) suggested that mutations in the C-terminus did not affect HR efficiency, only the rate of mitotic entry (Ayoub et al. 2009).

Drosophila presents a convenient model system for the study of *BRCA2* and its protein product. Although homozygous *brca2* female fruit flies are sterile, presumably due to defects in repair of DSBs during meiotic recombination (Klovstad et al. 2008), homozygous males and females are viable. The first functional analysis of the Drosophila Brca2 homolog showed that it colocalizes with Rad51, and that it is necessary for inter-homolog gene conversion (Brough et al. 2008). Additional work indicated a role in the meiotic checkpoint response and an interaction with the checkpoint protein Rad9 (Klovstad et al. 2008). These studies also showed that *brca2* and *rad51* deficiencies resulted in equivalent sensitivity to DNA damage caused by ionizing radiation, hydroxyurea and methylmethanesulfonate (Brough et al. 2008; Klovstad et al. 2008). These results are consistent with the idea that Drosophila Brca2 is a functional homolog of human BRCA2.

Although the primary function of BRCA2 is thought to be the recruitment of Rad51 to the site of DSBs, its dramatic mutant phenotype in humans suggests that it may have multiple roles in DNA repair. Thus, there is a great deal of interest in finding Rad51-independent functions of BRCA2. Recently, mammalian BRCA2 was shown to protect stalled replication forks from MRE11-mediated degradation, and that this function required the S3291 residue implicated in RAD51 filament binding (Schlacher et al. 2011). MRE11-mediated degradation of the forks led to aberrant repair processes, suggesting that this may be a critical tumor suppressive role for BRCA2 (Schlacher et al. 2011). We explore potential Rad51-independent roles of Drosophila Brca2 in Chapter 2.

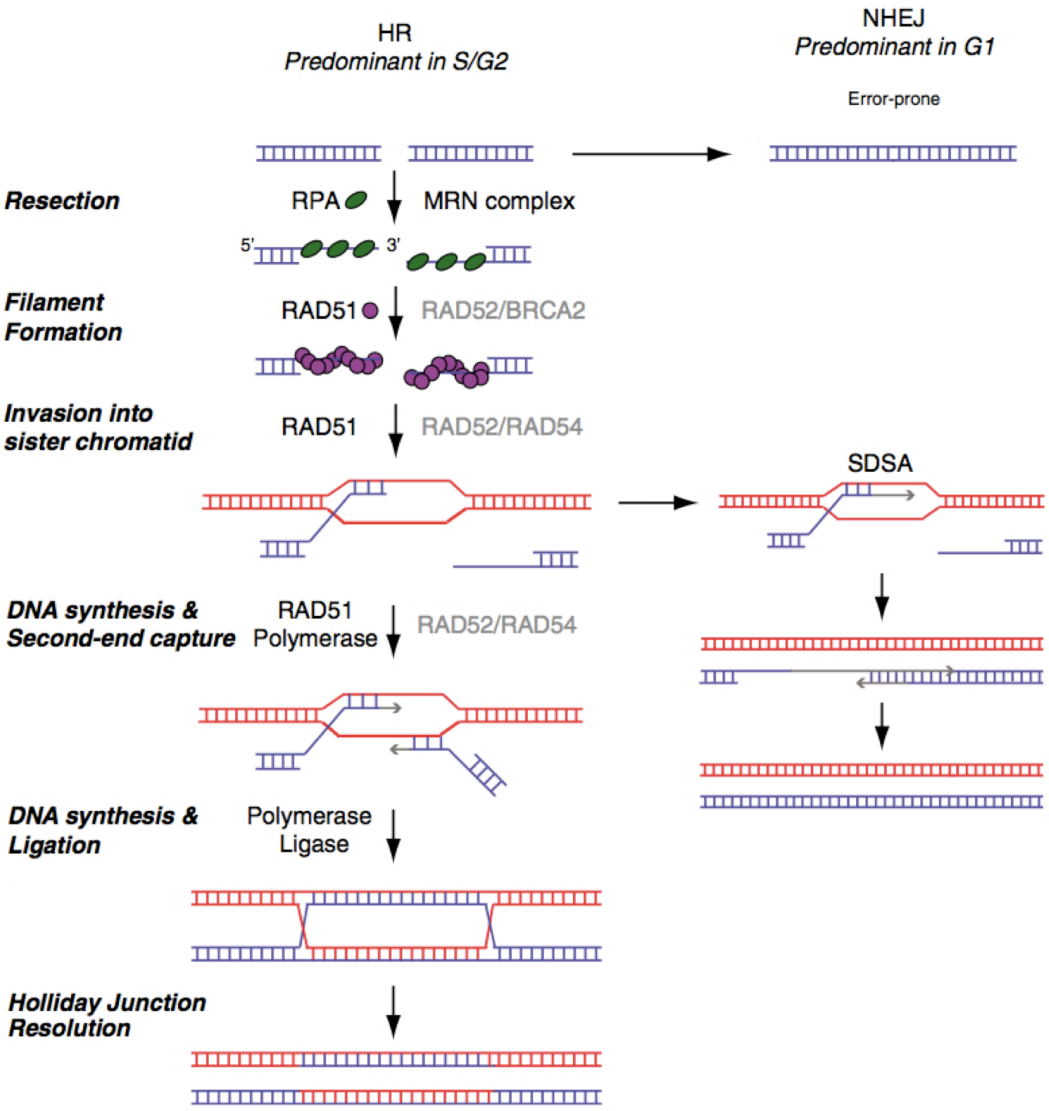

Figure 1-3: Homologous recombination repair of a DSB. A broken DNA duplex (blue) is repaired using an intact homologous template (red). Two possible outcomes are shown: (left) DSBR model of repair with second end capture, double Holliday junction formation, and crossover products; (right) SDSA model of repair with D-loop displacement, reannealing and noncrossover products. Before strand invasion, HR can abort and the DSB can be repaired by NHEJ (top right). Figure modified from (Thorslund and West 2007).

Rad51 filament assembly and homology search: Single molecule studies showed that Rad51 nucleation occurs rapidly, while extension is slow and discontinuous, producing a gapped nucleoprotein filament (Hilario et al. 2009).

The gaps in the filament are proposed to allow for flexibility during recombination (Holthausen et al. 2010). Conversely, the bacterial homolog RecA is nucleated slowly, followed by a rapid extension, leading to a saturated filament (Galletto et al. 2006). The absence of chromatin in prokaryotes may mean that there is less requirement for flexibility (Holthausen et al. 2010). The strand exchange reaction catalyzed by Rad51 is dependent on ATP (Sung 1994), although only ATP binding is required, not ATP hydrolysis (Sung and Stratton 1996). ATP binding facilitates substrate binding and strand exchange (Sung and Stratton 1996), while ATP hydrolysis appears to be more important for the dissociation of Rad51 from bound DNA (Chi et al. 2006).

Once RAD51 has formed a nucleoprotein filament with the 3' single strand overhang, the complex can now search for its homolog (Figure 1-3). To facilitate homology search, the DNA is stretched from 3.4 Å per base to 5.1 Å per base, and untwisted from 10.4 bases per helical turn to 18.6 bases per helical turn (Ogawa et al. 1993). The nucleoprotein filament now searches for a homologous sequence. This search is biased towards the sister chromatid by the cohesin complex (Ström et al. 2004). Rad51 has two separate DNA binding domains, one for the invading strand and one for the target. The proximity of the two facilitates the strand exchange reaction (Sung et al. 2003).

Biochemical evidence suggests that a transient three-stranded structure is formed during strand exchange, with some base pairing between the target duplex DNA strands, and some base pairing between the complementary template and the invading strand (Stasiak 1992; Gupta et al. 1999). The strand exchange

reaction itself moves 5' to 3' along the template strand (Symington 2002). Complete base pairing between the invading strand and the template strand displaces the third strand, creating a displacement loop (D-loop). The RAD51 nucleoprotein filament now contains double-stranded heteroduplex DNA (Adelman and Boulton 2010). Base pairing stringency for RAD51 is much higher than that of RecA (Holmes et al. 2001), likely due to the increased frequency of repetitive sequences in eukaryotic genomes, which increase the chance of incorrect template choice (Holthausen et al. 2010).

Repair synthesis: Once the correct template is found, synthesis can initiate from the 3' end of the invaded strand. However, whether synthesis occurs before, during or after complete removal of Rad51 is unclear. It does appear that at least partial clearance of Rad51 is occurring. In prokaryotes, RecA must be cleared from the 3' end in order for synthesis to take place (Xu and Marians 2002). This is also the case in budding yeast, and the protein responsible for Rad51 filament displacement is Rad54 (Li and Heyer 2009). However, in human cells, Rad54 is proposed to disrupt D-loops by branch migration, not direct removal of Rad51 monomers (Bugreev et al. 2007). Rad54 is known to function at multiple points during homologous recombination, though, so it is difficult to separate its specific functions at a particular step (Heyer et al. 2006).

Regardless of the presence of Rad51, repair synthesis requires a polymerase to extend the invaded strand. In budding yeast, the data suggest that components of the replicative polymerase δ are critical for repair synthesis (Lydeard et al. 2007). In Drosophila, there is *in vivo* evidence that both replicative

and translesion polymerases can function during HR synthesis (Kane et al. 2012). In humans, polymerase η, but not δ, was shown to extend from an *in vitro* D-loop substrate (McIlwraith et al. 2005). More recently, however, polymerase δ was shown to extend from D-loops with high processivity, provided the replication clamp, PCNA (*p*roliferating *c*ell *n*uclear *a*ntigen), was present (Sneeden et al. 2013).

Crossovers vs. non-crossovers: The end stages of homologous recombination determine whether or not a large-scale exchange of sequence (crossover) takes place. In meiotic recombination, such exchanges are thought to foster genetic diversity, and, more practically, to ensure proper alignment and segregation of the chromosomes (Youds and Boulton 2011). In somatic HR repair, however, crossovers and the large scale gene conversion that can accompany them can lead to loss of heterozygosity (LOH) if a homologous chromosome is chosen (Chapman et al. 2012). Thus, non-crossover products are favored in somatic DSB repair. To understand the different outcomes, it is important to discuss the mechanisms that drive them.

The classical DSB repair mechanism was proposed thirty years ago to explain the phenomena of gene conversion and crossing over (Szostak et al. 1983). In this model, both ends of a DSB are engaged with the intact template (Figure 1-3, lower left), an event referred to as second-end capture. Synthesis and ligation of strands result in the creation of a double Holliday junction. These junctions can be migrated, potentially generating more heteroduplex DNA, and are eventually resolved. The mechanism of resolution determines whether or not a

crossover occurs (Pardo et al. 2009). Alternatively, before second-end capture can occur, the invading strand can be displaced and reannealed to the other end of the break (Figure 1-3, lower right). This mechanism, identified as synthesis-dependent strand annealing (SDSA) produces no Holliday junctions and results in only non-crossover products (Nassif et al. 1994). This dissertation will focus mainly on the SDSA pathway of HR repair and the proteins that promote this non-crossover favoring mechanism. Chapter 4 discusses the roles of DmHelQ and DmBlm in promoting SDSA repair of DSBs.

If second-end capture does occur and two Holliday junctions form, there are mechanisms in place to drive the resolution of these structures toward non-crossover, small tract length gene conversion products. The Bloom dissolvasome, also known as the BTR complex, consists of Bloom's helicase, topoisomerase IIIα, RMI1 and RMI2 (*R*ecQ *m*ediated genome *i*nstability 1 and 2). This complex is believed to promote migration of two Holliday junctions toward one another, followed by cleavage and decatenation (Reviewed in Manthei and Keck 2013; 2013). *In vitro*, Bloom has been shown to migrate Holliday junctions (Karow et al. 2000) and, when combined with TopoIIIα, could also dissolve these structures resulting in non-crossover products (Wu and Hickson 2003). Double Holliday junctions can also be resolved by nucleolytic cleavage, which can be executed by SLX1-SLX4, MUS81-EME1 or GEN1 (Wechsler et al. 2012). However, this type of resolution can produce both crossover and non-crossover products, and the extent of gene conversion can vary, depending on migration of the junctions before cleavage.

Processing of HR Intermediates

For HR to progress and eventually resolve a DSB, Rad51 must be removed from the DNA. Several proteins have been implicated in this process in different systems. The budding yeast protein Srs2 has been shown to prevent HR by dissolving Rad51-ssDNA filaments (Veaute et al. 2003; Krejci et al. 2003). Srs2 could unwind dsDNA with less efficiency (Van Komen et al. 2003), and it was also shown to disrupt D-loop structures, promoting SDSA (Dupaigne et al. 2008). Originally identified as a regulator of telomeres in mice (Ding et al. 2004), the helicase RTEL1 has also been shown to effectively disrupt D-loop intermediates in *C. elegans* (Youds et al. 2010; Barber et al. 2008). Disruption of D-loops likely prevents second-end capture from occurring, thereby channeling repair toward non-crossover products (Chapman et al. 2012). In flies, the Bloom helicase is proposed to promote SDSA by unwinding D-loops during HR repair (Adams et al. 2003).

A component of the Fanconi Anemia pathway (see below), FANCM, has also been shown to unwind D-loops (Gari et al. 2008a), as has its budding yeast homolog, Fml1 (Sun et al. 2008). The authors propose that Fml1 may serve to suppress crossing over by promoting SDSA (Sun et al. 2008). FANCM/Fml1 seems to be important for HR repair at stalled replication forks (Sun et al. 2008), where it may promote fork regression after DNA damage is encountered (Gari et al. 2008a). FANCM is also implicated in the unwinding of triplex DNA (Meetei et al. 2005) and the migration of Holliday junctions (Gari et al. 2008b). An

important consideration of these data regarding D-loops is whether or not *in vitro* unwinding activity accurately represents *in vivo* function.

HelQ: Another enzyme with a role in homologous recombination repair is HelQ/Hel308. Originally recognized by its homology to the helicase domain of Drosophila *mus308*, Hel308 homologs have been identified in mammals (Marini and Wood 2002), archaea (Guy and Bolt 2005) and *C. elegans* (Muzzini et al. 2008). Confusingly, its closest homolog in fruit flies was later identified as the gene product of *mus301* (McCaffrey et al. 2006), not the dual helicase-polymerase product of *mus308*.

The lack of a HelQ ortholog in yeast has prevented an in-depth analysis that has elucidated many characteristics of other repair proteins in this system. Some of the recent characterization of HelQ has been done in *C. elegans*. An initial characterization identified both the helicase HEL-308 and POLQ-1, a true homolog to the entire *mus308* protein product (Muzzini et al. 2008). Mutating both *hel-308* and *polq-1*, or *polq-1* and *fcd-2* (*C. elegans* FANCD2) resulted in synergistic sensitivity to interstrand crosslinks. Mutating *hel-308* and *brc-1* was synthetically lethal. In contrast, the *hel-308*, *fcd-2* double mutants showed sensitivity to crosslinks identical to each single mutant (Muzzini et al. 2008). These data suggested that HEL-308 functioned in the Fanconi anemia (FA) pathway of ICL repair (see below), while POLQ-1 functioned in a BRC-1 (*C. elegans* BRCA1) dependent pathway of ICL repair (Muzzini et al. 2008).

More recently, it was shown that *C. elegans* HELQ-1 (HEL-308) and RFS-1 (a Rad51 paralog) are both involved in removal of RAD-51 from dsDNA-

RAD-51 filaments (Ward et al. 2010). Mutations in both genes were synthetically lethal, with only a small percentage of double mutants surviving. Both proteins could interact with RAD-51 and remove it from dsDNA (but not ssDNA) *in vitro*. Interestingly, HELQ-1-mediated removal of RAD-51 did not require ATPase activity or even the N-terminal half of the protein. The researchers proposed that a C-terminal region of HELQ-1 induces conformational changes in bound RAD-51 that result in its displacement (Ward et al. 2010).

Human HEL308 is proposed to unwind the lagging strand at stalled replication forks. Its activity is stimulated by RPA and the presence of a gap on the leading strand (Tafel et al. 2011). Such a structure is expected to form when the nascent leading strand encounters a lesion, stalling leading strand synthesis but not lagging strand synthesis (Pagès and Fuchs 2003). HEL308 could unwind the nascent lagging strand, allowing additional repair proteins to bind or facilitating fork reversal (Tafel et al. 2011). Since archaeal HEL308 homologs have been shown to unwind lagging strands (Guy and Bolt 2005), interact with RPA (Woodman et al. 2011), and promote fork reversal (Li et al. 2008), such functions may be evolutionarily conserved. Other work on archaeal HEL308 homologs has shown that the protein can unwind both proteins and DNA at a stalled fork, supporting a model whereby HEL308 clears the site for repair proteins and synthesis (Richards et al. 2008).

Other mechanisms of HR repair: Two additional pathways exist for the resolution of DSBs. The first, single-strand annealing (SSA), does not require an intact template and is Rad51-independent (Hartlerode and Scully 2009).

Consequently, it is distinct from homologous recombination, but it is often discussed in conjunction with HR because it requires resection at the site of a DSB and is Rad52-dependent (Sugawara and Haber 1992). If the resection uncovers homologous sequences flanking the break, these sequences can be annealed (Figure 1-4). Importantly, though, this results in deletion of one copy of the repeated sequence along with any sequence between the repeats (Krejci et al. 2012). Human RAD52 is also known to play a critical role in the SSA pathway (Singleton et al. 2002).

A second alternative pathway in HR is break-induced replication (BIR), which is specific to one-ended DSBs (Figure 1-4). In BIR, a D-loop converts to a replication bubble, and replication proceeds to the end of the chromosome (Bosco and Haber 1998). This leads to extensive gene conversion when initiated between homologous chromosomes (Pardo et al. 2009). BIR is mechanistically different from replication, however, because it is highly mutagenic. The rate of frameshift mutations is nearly 3,000 times as high during BIR (Deem et al. 2011) and it is characterized by multiple invasion cycles (Sakofsky et al. 2012) and frequent template switching (Smith et al. 2007). Paradoxically, BIR can also act in the absence of Rad51, though at a much less efficient level (Malkova et al. 1996).

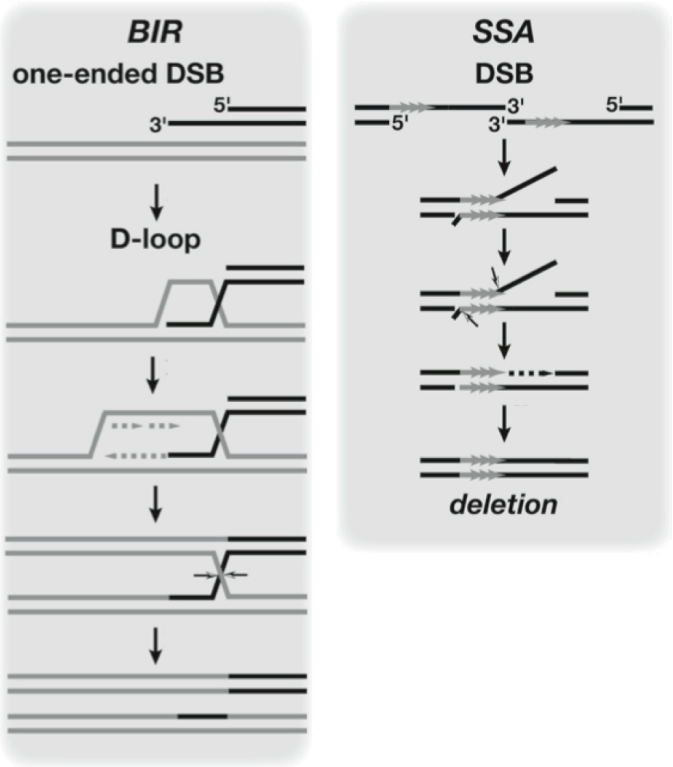

Figure 1-4: BIR and SSA. In break-induced replication (BIR), a DSB leads to the recreation of a replication fork, and synthesis continues to the end of the chromosome. In single-strand annealing (SSA), two homologous sequences on the same DNA strand are annealed, deleting one of the repeats and any intervening sequence. Both pathways are mutagenic, since they lead to loss of heterozygosity or deletions. Figure modified from (Pardo et al. 2009).

Studying DNA repair mechanisms in *Drosophila melanogaster*: Much of the research on DNA repair mechanisms is done in single-celled organisms such as yeast and bacteria, complex mammalian systems such as mice, or isolated mammalian cells in culture. The Drosophila system is a unique option in which to study these mechanisms for several reasons. First, flies develop much quicker than mice, transitioning from a fertilized egg to an adult in 10-11 days at 25°C. This facilitates the analysis of multiple generations in a relatively short amount of time. Second, within a much shorter lifespan, the number of offspring from a single female fly vastly outnumbers that of a single female mouse, allowing for larger sample sizes and, therefore, increased statistical power. Third, and perhaps

most importantly, these repair mechanisms can be analyzed in the context of a multicellular organism with many different tissue types and multiple developmental stages. This is not possible in yeast, bacteria or mammalian cell culture. One criticism of the Drosophila model system is that the evolutionary distance between flies and humans is too great to derive any clinical relevance from studies in Drosophila. However, the complete sequencing of the Drosophila genome (Adams et al. 2000) revealed many homologs of human disease related genes (Fortini et al. 2000) and DNA repair genes (Sekelsky et al. 2000), lists which have both grown since the original genome release.

Additionally, Drosophila has helped clarify several aspects of DNA repair and cancer over the last few decades. For example, the model of synthesis-dependent strand annealing (SDSA) was originally proposed using data from flies (Nassif et al. 1994). Cancer research has benefited greatly from fruit fly research, particularly the genetic aspect of carcinogenesis. Unlike mice, flies do not reject tumor transplants (Gateff 1978; Gateff and Schneiderman 1974). This is important for studying tumors because a characteristic of malignancy is the capacity for lethal growth when transplanted (Gateff 1978). Drosophila mutations that were initially shown to result in neoplastic growth, such as lethal(2) giant larvae (*lgl*) and discs large (*dlg*), were later shown to have human homologs that act as tumor suppressors (Humbert et al. 2003).

Mutagen-induced DNA Damage

One strategy for understanding the molecular functions of a particular DNA repair gene or pathway is to induce DNA damage through mutagenic

chemicals or radiation. Many chemotherapeutic drugs are designed to induce DNA damage, such as double-strand breaks or interstrand crosslinks, in rapidly dividing cancer cells (Ferguson and Pearson 1996; Lawley and Phillips 1996). Thus, the benefits of using these drugs to research DNA repair mechanisms are twofold: it serves to clarify the proteins involved in repairing mutagen-induced damage and it furthers our understanding of how these drugs function in a clinical setting.

The first cancer chemotherapeutic was discovered accidentally during the Second World War. Victims of mustard gas exposure showed dramatically lowered white blood cell counts, suggesting that this chemical warfare agent could have antineoplastic properties (Deans and West 2011). After the war ended, clinical trials using nitrogen mustard for leukemia began (Goodman et al. 1946). Along with additional chemotherapeutics such as mitomycin C and cisplatin, nitrogen mustard induces covalent crosslinks between DNA strands. When these crosslinks join two strands of DNA, an interstrand crosslink (ICL) results (Reviewed in Deans and West 2011). ICLs are a particularly dangerous type of DNA damage for which there is a dedicated pathway, known as the Fanconi Anemia pathway (Reviewed in Kim and D'Andrea 2012). Since Fanconi Anemia (FA) is a disease resulting in severe sensitivity to ICLs, many facets of the pathway and its multiple protein components have been elucidated using ICL-inducing drugs.

Bleomycin, originally identified as an antibiotic, is a mutagenic chemical that induces DNA damage similar to that of ionizing radiation (Reviewed in Chen

and Stubbe 2005). Like ionizing radiation (Thompson 2012), bleomycin induces a wide variety of DNA damage, including SSBs, DSBs, oxidative damage and irregular ends like 3'-phosphoglycolates (Chen and Stubbe 2005). Because of the end modifications common to bleomycin-induced damage (Mimitou and Symington 2009), the drug is useful in clarifying the enzymes responsible for end processing during repair. Additionally, bleomycin was used in the characterization of γH2AX (Rogakou et al. 1998; Burma et al. 2001) and components of the Fanconi Anemia pathway (Carreau et al. 1999).

Camptothecin overview: Of particular interest in this dissertation is the topoisomerase poison, camptothecin, and its derivatives. Camptothecin was isolated from a Chinese tree in the 1960s and shown to have antitumor activity in a preliminary screen (Wall et al. 1966). Unfortunately, the promising drug had to be abandoned when it was shown to be too toxic in clinical trials (Wall and Wani 1995; Legarza and Yang 2006). However, the drug regained interest when its target was identified as topoisomerase I (TopI) (Hsiang and Liu 1988; Hsiang et al. 1985). The water-soluble derivatives, irinotecan (Kunimoto et al. 1987) and topotecan (Mattern et al. 1991) were developed soon after, and they are currently the only two camptothecin derivatives approved by the FDA. Topotecan is prescribed for ovarian cancer and lung cancer, while irinotecan is prescribed for colorectal cancers (Pommier 2006). Several camptothecin derivatives as well as non-camptothecin topoisomerase inhibitors are currently in development (Pommier 2013; Yves Pommier, personal communication).

The mechanism of action of camptothecin and its derivatives is rather unique in that they poison rather than inhibit their target (Pommier 2013). During replication and other DNA metabolic processes, the double helix becomes torsionally strained. This stress is relieved by the nicking of one or both strands by enzymes known as topoisomerases. This transient break allows the DNA to partially unwind, reducing the strain on the molecule. Importantly, the creation of the break requires a covalent link between a tyrosine residue in topoisomerase, and, in the case of Type IB topoisomerases (the target of camptothecin), a 3'-phosphate group in the DNA backbone. The covalent complex is resolved by a religation reaction between the free 5'-hydroxyl group and the 3'-phosphate group. This reaction restores the continuous DNA backbone and displaces topoisomerase, freeing it to react again elsewhere on the molecule (Pommier 2006).

It is this transient covalent link (Top1 cleavage complex, or Top1cc) between Top1 and the DNA backbone that is the target of camptothecin. The planar molecule is stabilized between the bases flanking the single stranded break, both by base stacking interactions with DNA and hydrogen bonds with the enzyme (Pommier et al. 2006). Should a collision occur between the Top1cc and a replication fork, the normally transient single-strand break (SSB) will be converted into a stable, one-ended DSB (Hsiang et al. 1989). The standard mechanism of removing Top1 from the DNA is no longer possible if the 3'-phosphate and 5'-hydroxyl groups are not in close proximity (Figure 1-5).

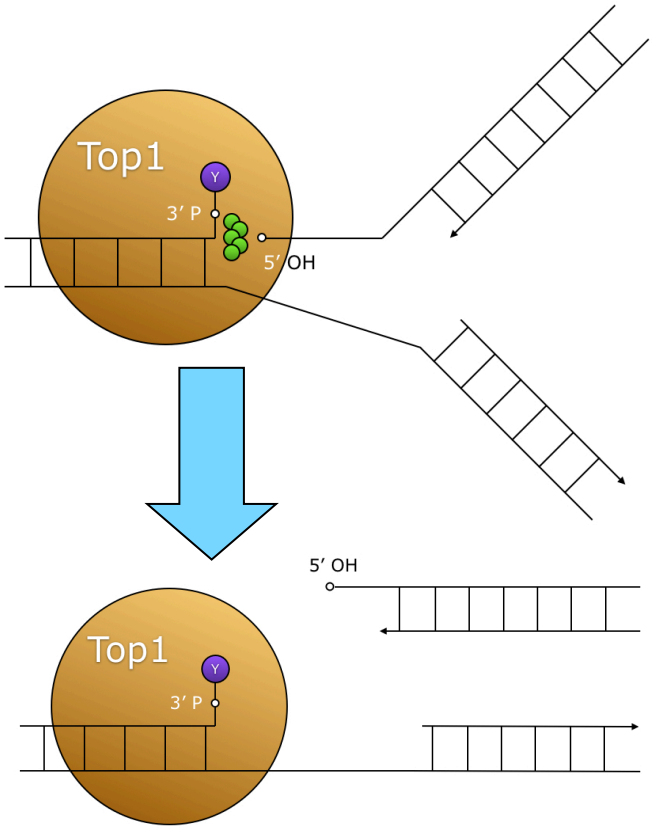

Figure 1-5: Camptothecin-induced DSBs. Topoisomerase I (Top1) introduces a single-stranded break through a 3'-phosphotyrosyl linkage (Y-3'P). Camptothecin (green) is stabilized in the gap between the 3'-phosphate and 5'-hydroxyl group, preventing the religation reaction from occurring. Collision with a replication fork (moving from the right) converts the SSB to a one-ended DSB with a covalent protein linkage. This model has been challenged by some data.

The toxicity of this naturally occurring compound begged the question of how the cells of the tree that produces it (*Camptotheca acuminata*) are unaffected. Recent research showed that not only does the tree have mutations in Top1 that destabilize the interaction with camptothecin, but cancer patients treated with camptothecins that develop resistance to the drugs sometimes carry the exact same mutations in resistant tumor cells (Sirikantaramas et al. 2008; Chrencik et al. 2004; Fujimori et al. 1995).

Some research suggests that the replication fork collision model may not be entirely correct. For example, single-molecule techniques and *in vivo* yeast

research suggest that the accumulation of positive supercoiling due to trapped topoisomerases causes DSBs (Koster et al. 2007). More recently, an analysis of multiple systems suggested that replication forks stall and reverse in the presence of camptothecin. An inability to process the reversed forks can then lead to DSBs (Ray Chaudhuri et al. 2012).

Another possibility is that camptothecin interferes with transcription. Camptothecin treatment was found to inhibit transcription of rRNA in HeLa cells, and this inhibition was associated with Top1-linked breaks in the DNA (Zhang et al. 1988). The observation of cell death induction in post-mitotic neurons treated with camptothecin also suggests a mechanism independent of DNA replication (Morris and Geller 1996). Only Top1-DNA links on the template strand were problematic for transcription elongation, consistent with a model whereby the transcription machinery collides with a Top1 cleavage complex on that strand, but can bypass those on the non-template strand (Wu and Liu 1997).

Repair of camptothecin-induced damage: Two major problems result from collision between a replication fork and a Top1 cleavage complex (Figure 1-5). First, because the 5'-hydroxyl group and 3'-phosphate are no longer in close proximity, the large topoisomerase enzyme cannot be displaced, leaving a covalently linked protein on the DNA backbone. Second, the collision itself transforms a single-stranded break into a double-stranded break. Importantly, this DSB is one-ended, which is a poor substrate for end-joining repair (Moynahan and Jasin 2010). In fact, cells defective for end-joining are resistant to the

cytotoxicity of camptothecin (Adachi et al. 2004), perhaps because the damage is channeled away from repair pathways that could induce translocations.

One-ended DSBs are likely to be treated differently than two ended DSBs. For example, Mre11 is known to adopt two different structural conformations depending on whether the break is one-ended or two-ended (Williams et al. 2010). The absence of a second end for ligation precludes the use of non-homologous end-joining (NHEJ; Moynahan and Jasin 2010), meaning recombinational repair is likely the only choice to restore the DNA. One model proposes that the Ku heterodimer (Ku70/Ku80), important for NHEJ (Reviewed in Weterings and Chen 2008), prevents binding of HR proteins at two-ended breaks, but cannot do so at one-ended breaks (Pierce et al. 2001). Break-induced replication (BIR, see above) is a potential candidate for repair of one-ended breaks (Lydeard et al. 2010) formed after camptothecin treatment. Whether camptothecin treatment results in collapsed (Pommier et al. 2003) or regressed forks (Ray Chaudhuri et al. 2012), mechanisms of replication fork restart (Reviewed in Petermann and Helleday 2010) may also be critical.

Top1ccs are not exclusive to camptothecin-induced damage, and the cell has evolved multiple mechanisms for repairing them (Vance and Wilson 2002). One enzyme in particular, TDP1 (*t*yrosyl-*D*NA *p*hosphodiesterase 1) is critical for the repair of these complexes (Pouliot et al. 1999), and its specificity for 3'-phospho-linked proteins supports the hypothesis that Top1ccs are common occurrences in the absence of camptothecins (Yang et al. 1996). Furthermore, mutations in the human TDP1 gene can cause spinocerebellar ataxia with axonal

neuropathy (SCAN1), a neurodegenerative disease (Takashima et al. 2002). TDP1, along with PNKP (*p*oly*n*ucleotide *k*inase *p*hosphatase), is thought to restore a 3'-phospotyrosyl linkage to a 3'-hydroxyl group that can be used to initiate repair synthesis (Plo et al. 2003). Because TDP1 is critical for camptothecin-induced damage repair, there is great interest in developing TDP1 inhibitors that can be combined with topoisomerase poisons for more effective chemotherapy (Pommier 2013; Yves Pommier, personal communication).

Dissertation Outline

This dissertation focuses on the intermediate steps of homologous recombination (HR), with a particular focus on the repair of DSBs induced by camptothecin and its derivatives. The dissertation is divided into five chapters. The remaining four chapters are summarized as follows:

In **Chapter 2**, we discuss the role of Drosophila Brca2 in recruiting Rad51 to the sites of DSBs created by mutagens, specifically the camptothecins. We were interested in finding Rad51-independent roles for DmBrca2, based on recent research that suggested such roles may exist for mammalian BRCA2.

In **Chapter 3**, we explore the genes involved in camptothecin-induced damage repair and camptothecin detoxification. We found that Drosophila *Cyp6d2* is particularly important for preventing camptothecin toxicity, and that many Drosophila stocks carry one of two mutations that inactivate this protein.

In **Chapter 4**, we look at the role of DmHelQ in the repair of DSBs by HR. We found that HelQ, along with DmBlm, promotes accurate repair by the

SDSA pathway of HR. We discuss possible mechanisms for these two proteins based on our data and previously published results.

In **Chapter 5**, we provide some concluding remarks about questions that remain to be answered and the potential experiments to answer them. We focus largely on the function of DmHelQ and its relationship to DmRad51 and DmBlm. Ideas for experiments to test the hypotheses in Chapter 4 are discussed.

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Chapter 2
Drosophila melanogaster Brca2 and Rad51 coordinate in homologous recombination repair of DNA double-strand breaks

Author Contributions

Creation of *brca2*^{47} mutant: Adam South

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Abstract

DNA double strand breaks (DSBs) are repaired through different pathways, each of which requires the coordination of many protein factors. In homologous recombination (HR) repair, the recombinase RAD51 promotes a strand exchange reaction through which the DNA sequences lost at the break site can be restored. In metazoans, RAD51 is recruited by BRCA2, the breast cancer susceptibility gene product. While its canonical function in recruiting RAD51 has been well characterized, several studies have uncovered RAD51-independent functions of BRCA2, including checkpoint roles and stabilization of stalled replication forks. Using model systems to study BRCA2 homologs has provided much of what is currently known about the protein. We studied Drosophila *brca2* and *spn-A* (DmRad51) null mutants to identify any Rad51-independent functions of DmBrca2. We used multiple DSB-induction mechanisms including transposable elements, radiation and chemical mutagens to characterize these mutants. We found that, unlike *spn-A* heterozygotes, *brca2* heterozygous mutants were proficient in repair of a single double-stranded gap by HR. Homozygous null mutations in either gene, however, resulted in complete removal of HR repair. Furthermore, *brca2* and *spn-A* mutants were equally sensitive to ionizing radiation, bleomycin, nitrogen mustard and topotecan. We found no evidence of a Rad51-independent function of DmBrca2, which may be because the Drosophila homolog lacks many of the critical domains found in human BRCA2.

Introduction

Eukaryotic cells employ a number of mechanisms to faithfully repair DNA damage from both endogenous and exogenous sources. The most harmful type of DNA damage is the double strand break (DSB), and as such, it requires immediate attention by the cellular repair mechanisms (Reviewed in Pardo et al. 2009). The two canonical repair pathways that target DSBs are non-homologous end-joining (NHEJ) and homologous recombination (HR). In NHEJ, the two broken pieces of DNA are processed and then joined together, potentially with the loss of some genetic material (Reviewed in Weterings and Chen 2008; Weterings and van Gent 2004). In HR, an intact template, either a homologous chromosome or more often a sister chromatid, is used as a model for repair. No genetic material is lost, assuming the correct template is used (Reviewed in Krejci et al. 2012).

BRCA2 and homologous recombination: For metazoans, one of the most crucial proteins involved in homologous recombination (HR) is BRCA2, the product of the breast cancer susceptibility or early onset gene, *BRCA2* (Reviewed in Thorslund and West 2007; Boulton 2006). Individuals inheriting a mutant copy of either of the two breast cancer susceptibility genes (*BRCA1* or *BRCA2*) have an 80% chance of developing breast cancer at some point during their lifetimes, presumably due to a defect in DNA repair. In addition, there appears to be a significant risk of ovarian cancer in those carrying a mutant copy of one of these genes (Boulton 2006). These phenotypes are particularly striking considering that HR involves many additional proteins, mutations in which do not cause such an exceptional cancer risk (Klovstad et al. 2008). The implication of these

phenotypes is that the BRCA proteins may have additional uncharacterized roles, and the mutations inactivate multiple options for repair of DNA damage.

Recently, human BRCA2 was shown to protect stalled replication forks from degradation by MRE11, a function completely distinct from its well defined role in HR repair (Schlacher et al. 2011). Research has also shown that CHK2 phosphorylates the N-terminus of human BRCA2, suggesting a checkpoint role for the protein (Kim et al. 2008). Characterization of mammalian BRCA2 protein is hindered because of its large size and the embryonic lethality of null mutants. Until the recent purification of human BRCA2 (Jensen et al. 2010; Thorslund et al. 2010; Liu et al. 2010), most studies used fragments of the protein (Pellegrini et al. 2002; Shin et al. 2003; Davies et al. 2001). Studying smaller BRCA2 homologs in other eukaryotes (Kojic et al. 2002; Brough et al. 2008; Bork et al. 1996; Lo et al. 2003) allows the analysis of a full length protein within a whole organism, from which inferences on the function of human BRCA2 can be made.

Research on the *C. elegans BRCA2* homolog, *CeBrc-2*, suggested a RAD-51 independent function in single strand annealing (SSA) (Petalcorin et al. 2006; Martin et al. 2005). Researchers proposed that CeBRC-2 fulfills the role of Rad52, which mediates SSA in yeast (Mortensen et al. 1996), but is not present in *C. elegans* (Martin et al. 2005). However, *brca2* mutants do not show an SSA defect in Drosophila (Klovstad et al. 2008), which also lacks a Rad52 homolog (Sekelsky et al. 2000).

The Drosophila homolog of *BRCA2* was first identified by sequence similarity (Lo et al. 2003) and then later confirmed by functional analysis in cell

culture (Brough et al. 2008). Evidence for a meiotic Rad51-independent function of DmBrca2 came from analysis of its role in checkpoint function (Klovstad et al. 2008). *brca2* mutations suppressed the phenotypes of other DNA repair deficiencies during meiosis, suggesting it has a role in transducing the checkpoint signal. Consistent with a meiosis-specific checkpoint role, Brca2 co-immunoprecipitated with Rad9, a component of the 9-1-1 checkpoint complex, in Drosophila ovaries. No mitotic checkpoint defects were observed following ionizing radiation treatment, however (Klovstad et al. 2008).

Given the RAD51/Rad51-independent roles of human BRCA2 at stalled forks (Schlacher et al. 2011) and Drosophila Brca2 during meiosis (Klovstad et al. 2008), we sought to find a mitotic Rad51-independent function of DmBrca2. We tested *brca2* and *spn-A* (DmRad51) null mutants for their response to different types of DNA damage, including DSBs induced by transposon movement, mutagen treatment and radiation. We were particularly interested in the role of DmBrca2 during repair of camptothecin-induced damage, since low doses of camptothecin lead to stalled forks (Ray Chaudhuri et al. 2012) that may be stabilized by BRCA2 (Schlacher et al. 2011). Though DmBrca2 was shown to be critical in the repair of several types of DNA damage, no Rad51-independent function was found.

Materials and Methods

Drosophila stocks: The *brca2*^{KO} mutant, created by ends-out homologous recombination, deletes 3321 bp of the 3417 bp gene, leaving behind a small amount of the untranslated regions (Klovstad et al. 2008). The *brca2*^{47} mutant was generated via an imprecise excision screen using *P{SUPor-P}KG02287*, 87 bp upstream of the transcription start site of *Brca2*. It deletes from that point through the first 2169 bp of the gene. Non-quantitative RT-PCR showed that both *Brca2* and the upstream gene, *CG4612*, produce no transcript in the *brca2*^{47} mutant. The *brca2*^{56E} mutant was generated via an imprecise excision of *P{SUPor-P}Brca2*^{KG03961}, and removes the majority of *BRCA2* coding sequence (Klovstad et al. 2008). The progenitor stocks of *brca2*^{47} and *brca2*^{56E}, *P{SUPor-P}KG02287* and *P{SUPor-P}Brca2*^{KG03961}, respectively, carry a recessive mutant allele of *Cyp6d2* that results in camptothecin hypersensitivity (Thomas et al. 2013).

The two *spn-A* (DmRad51) alleles, *spn-A*^{057} and *spn-A*^{093}, were generated by mutagenesis screens and are null alleles. *spn-A*^{057} is an alanine to valine (A205V) mutation while *spn-A*^{093} is a glutamine to stop codon (Q70Stop) mutation in the 336 amino acid protein (Staeva-Vieira et al. 2003).

P{w*^{a}*} assay: The *P{w*^{a}*}* assay was carried out as described in (McVey 2010). Briefly, homozygous or heterozygous *brca2*^{KO} single males were bred to carry the *P{w*^{a}*}* (*P-white*-apricot) transposon on the *X* chromosome and the *P{ry*$^{+}$*, Δ2-3}* transposase on chromosome *3*. The *P{w*^{a}*}* construct consists of a *white* gene interrupted by the retrotransposon *copia* (Kurkulos et al. 1994). The inserted

retrotransposon reduces expression of *white* considerably, so that it produces a faint yellow eye color instead of red. The entire construct is inserted in an intron of *scalloped*, an essential gene on the *X* chromosome. The single males (P generation) were mated to homozygous $P\{w^a\}$ females and repair events in the pre-meiotic male germline were recovered in female progeny (F_1 generation). These females inherited a complete maternal copy of $P\{w^a\}$ as well as a potentially cut and repaired paternal copy. Excision events occur less than 15% of the time, so the majority of the F_1 females inherit two complete copies of $P\{w^a\}$. The combined expression of the two copies results in the apricot eye color that gives the assay its name.

In the 10-15% of F_1 females that inherited a repaired excision event, the type and extent of repair can be measured by several methods. If homologous recombination initiates and synthesis of the *copia* long terminal repeats (LTRs) occurs, these homologous LTRs can anneal, resulting in deletion of *copia* and full expression of *white*. The resulting female progeny have red eyes. Alternatively, if synthesis fails to initiate or fails to reach the LTRs, and instead an aberrant end joining event connects the two ends, the repaired $P\{w^a\}$ construct will not produce eye pigment. Only the faint yellow pigment derived from the maternal copy of $P\{w^a\}$ will be observed in these F_1 females. We analyzed the percentage of events that utilized homologous recombination followed by LTR annealing vs. those using an aberrant end joining mechanism in each mutant background and compared these to a wild-type control. Statistical analysis of these data was done using Kruskal-Wallis test with Dunn's multiple comparisons post-test.

Additionally, since the aberrant repair events may have involved some synthesis prior to end joining, we analyzed the extent of synthesis by PCR. F_1 females were mated to sibling males and then isolated to recover white-eyed male progeny (F_2). Synthesis tract lengths were determined for each individual male using primers along the right end of the $P\{w^a\}$ transposon. We calculated the percentage of aberrant events that reached specific distances by PCR analysis of the F_2 males and determined statistical significance using Fisher's exact test. Similarly, junctions in which little to no synthesis occurred were sequenced to analyze the use of microhomologies during aberrant repair of the construct.

Mutagen and IR sensitivity assays*:* Sensitivity assays consisted of at least 5 vials of flies per dose. In each vial, 5-8 virgin female flies heterozygous for the mutation of interest were crossed to 3 heterozygous males. The parents were left in vials for 3 days before being transferred to new vials. They were then removed after 2 days. For both sets of vials, treatment was administered on to food 1 day after the parental flies were removed. The first set of vials received 250 µl of mutagen, while the second set received 250 µl of vehicle. Camptothecin (Sigma) was dissolved in DMSO, and further diluted in Tween20/Ethanol for application. Mechlorethamine, bleomycin (Sigma) and topotecan (Enzo Life Sciences) were diluted in water.

All eclosing flies were counted until 18 days after initial cross setup. The fraction of homozygotes was calculated for both experimental and control sets. Untreated vials produced roughly a 2:1 ratio of heterozygotes to homozygotes. Percent survival was calculated for each set of 5-8 vials as follows:

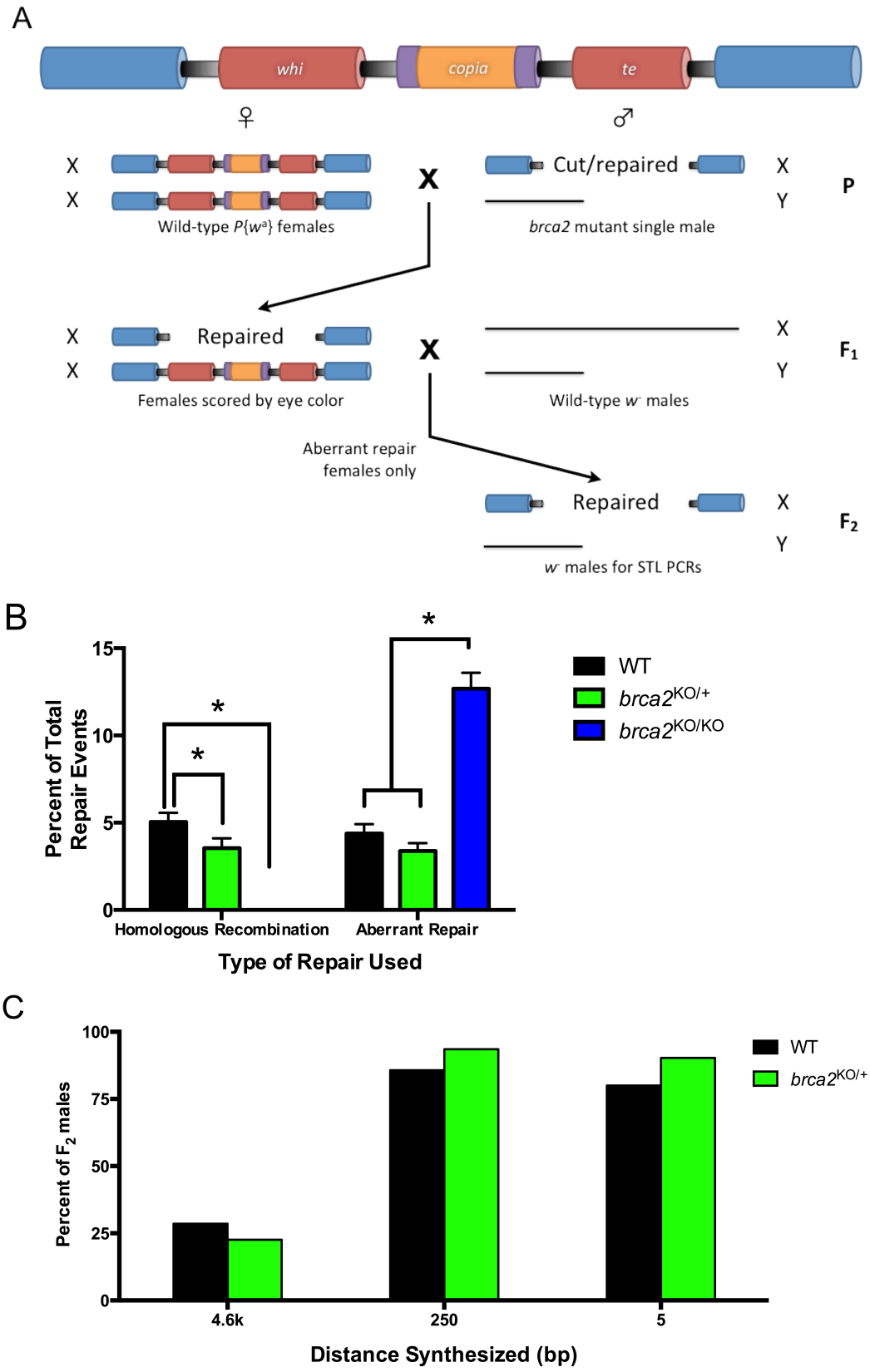
$$\text{Relative percent survival} = \frac{\text{percent of homozygous flies in treated vials}}{\text{percent of homozygous flies in control vials}} \times 100\%$$

Ionizing radiation sensitivity was calculated in a similar way. Heterozygous parents were mated in grape agar cages. Grape plates with 3rd instar larvae were irradiated at a rate of 800 rads/min with a Gammator 1000 irradiator. The larvae were transferred to bottles, where eclosing adults were counted for 18-20 days after initial mating. All irradiated larvae were compared to a matched unirradiated control to calculate percent survival of adult homozygotes.

Results

BRCA2 is critical for homologous recombination (HR) repair of DSBs because of its well established role in recruiting the recombinase RAD51 to the break site (Reviewed in Roy et al. 2012). Recent research has suggested, however, that human BRCA2 may have additional roles outside of this function (Schlacher et al. 2011). In order to investigate potential Rad51-independent roles of Drosophila Brca2, we first tested *brca2*^{KO} mutants using the *P*{*w*^{a}} assay, which measures the use of different DSB repair pathways *in vivo* following the creation of a 14 kb gap on the *X* chromosome (Figure 2-1A). As expected, homozygous *brca2*^{KO} mutant flies did not repair any DSBs by HR, and there was a large increase in the use of aberrant repair mechanisms (Figure 2-1B). To better understand the aberrant repair processes occuring in the absence of DmBrca2, we isolated aberrant repair events (F_2 males) and sequenced the repair junctions. We observed frequent insertions and deletions at the break site, as well as increased reliance on the use of microhomology-mediated repair (Table 2-1). These data are consistent with previous results obtained with *spn-A* (DmRad51) homozygous

null mutant flies (McVey et al. 2004a), which suggests that DmBrca2-mediated DSB repair is dependent on Rad51.


A
whi
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♀
♂
X
X
Wild-type P{w^a} females
X
Cut/repaired
X
Y
brca2 mutant single male
P
X
X
Repaired
Females scored by eye color
X
X
Y
Wild-type w- males
F1
Aberrant repair females only
Repaired
X
Y
w- males for STL PCRs
F2
B
Percent of Total Repair Events
15
10
5
0
*
*
*
WT
brca2KO/+
brca2KO/KO
Homologous Recombination
Aberrant Repair
Type of Repair Used
C
Percent of F2 males
100
75
50
25
0
WT
brca2KO/+
4.6k
250
5
Distance Synthesized (bp)

Figure 2-1: Homozygous, but not heterozygous, *brca2*^{KO} flies are defective for HR repair. (A) Diagram of fly generations used for analysis. (Top) A complete diagram of *P*{*w*^{a}} construct, including *white* gene (red) split by *copia* retrotransposon (orange with purple long terminal repeats), inserted into an intron of *scalloped* (blue). F_1 females (middle left) were scored by eye color, shown in B. *w*$^{-}$ F_2 males (bottom right) produced by aberrant repair females (yellow eyes) used for PCR analysis of STLs in C. (B) Frequency of repair pathway used in different genetic backgrounds. Mean frequencies of 93 (WT), 106 (*brca2*$^{KO/+}$) and 94 (*brca2*$^{KO/KO}$) individual vials shown with standard error of the mean. (*) $P < 0.001$, Kruskal-Wallis test with Dunn's multiple comparisons post-test. (C) Synthesis tract lengths for wild-type and *brca2* heterozygotes. Percent of F_2 males with successful PCR at specified distance (from right end of *P*{*w*^{a}}) shown. 35 WT and 31 *brca2*$^{KO/+}$ F_2 males were tested for both genotypes. No significant differences between genotypes were observed using Fisher's exact test.

Sequence 5' of break [a]	Microhomology/ inserted sequence	Sequence 3' of break [a]	Number of isolates
Original sequence acccagacCATGATGAAATAACATA	-	**Original sequence** TATGTTATTTCATCATGacccagac	-
Long microhomology [b]			
acccagac	(CATgATGA) [c]	cccagac	3
acccagac	(CATGA)	cccagac	2
none [d]	(TGACCCAGAC)	-	4
Short microhomology [b]			
acccagacCATGATGAAATA	(A)	TGTTATTTCATCATGacccagac	1
acccagacCATGATGAAATAAC	(AT)	GTTATTTCATCATGacccagac	1
acccagacCATGATGAAATAACA	(TA)	TGTTATTTCATCATGacccagac	2
acccagac… (784 nt) …*AATGG*	(A)	TGTTATTTCATCATGacccagac	1
acccagacCATGATGAAAT	(AACA)	*AATGA*…(312 nt)…acccagac	1
Insertion [e]			
acccagacCATGATGAAATAACAT	GAA	ATGTTATTTCATCATGacccagac	1
acccagacCATGATGAAATAACAT	GATGAAA	ATGTTATTTCATCATGacccagac	1
acccagacCATGATGAAATAACAT	GT	TATGTTATTTCATCATGacccagac	1
acccagacCATGATGAAATAACATA	TGTTATATGTTAT GTTATCA	TATGTTATTTCATCATGacccagac	1
acccagacCATGATGAAATAACATA	CA	TATGTTATTTCATCATGacccagac	1
acccagacCATGATGAAATAACATA	CC	ATGTTATTTCATCATGacccagac	1
acccagacCATGATGAAATAACATA	TAACATATAAAC	ATGTTATTTCATCATGacccagac	1
acccagacCATGATGAAATAACATA	TATGTTATATAAT AATAATTATATA ATATATGTTACAT ATAACAAA	TATGTTATTTCATCATGacccagac	1
acccagacCATGATGAAATAACAT	GAAATAACATGA TGAAA	TTATTTCATCATGacccagac	1
acccagacCATGATGAAATAACATA	ACA	TATGTTATTTCATCATGacccagac	1
acccagac…(225 nt)…*AGAGA*	CCCATGTGCTATT CATGTGC	TATTTCATCATGacccagac	1

Table 2-1: *P*{*w*[a]} repair junctions recovered from *brca2*[47] mutants show evidence of aberrant repair processes. Aberrant repair events from *brca2*[47] mutant flies were isolated and sequenced. Only top strand (5'→3') shown. 26 total events were sequenced. Frequency of each event shown in right-hand column. a: Uppercase letters represent the 17-nucleotide 3' single-stranded tails that remain following transposase action, lowercase letters correspond to the 8 base pair target sequence duplicated upon *P* element insertion. Italicized capital letters (along with parenthetical nucleotide numbers) indicate *P*-element sequence synthesized at either side of the break. b: Microhomologies (in parentheses) are sequences that could have been derived from either side of the break site. Long microhomologies were five or more nucleotides, while short microhomologies were four or fewer nucleotides. c: represents an 8-nucleotide imperfect microhomology. d: indicates a deletion that extends past the 8 base pair target sequence. e: Insertions were identified as any sequence not present at the original break site.

Interestingly, previous results also showed that while heterozygous *spn-A* mutants could initiate HR repair, the pathway was only about 50% as effective as in wild-type flies (McVey et al. 2004a). To see if *brca2*^{KO} heterozygotes

displayed a similar haploinsufficiency, we also tested these flies in the $P\{w^a\}$ assay. For *brca2*^{KO} heterozygotes, there was a significant decrease in HR but not a significant increase in the use of aberrant repair (Figure 2-1B).

Heterozygous null *spn-A* flies are also deficient in the extent of synthesis completed during repair of the $P\{w^a\}$ construct (McVey et al. 2004a). To further investigate haploinsufficiency in *brca2*^{KO} heterozygous flies, we analyzed synthesis tract lengths (STLs) of the aberrant repair events recovered from *brca2*^{KO} heterozygotes. *brca2*^{KO} heterozygotes were not defective in repair synthesis when compared to a wild-type control at the three distances analyzed (Figure 2-1C). Therefore, we concluded that the decrease in HR repair by *brca2* heterozygotes is not biologically relevant. Thus, unlike *spn-A* heterozygotes, *brca2* heterozygotes are proficient at HR repair. The disparity between *brca2*^{KO} and *spn-A*^{093} heterozygotes is consistent with a role for DmBrca2 in recruiting large amounts of Rad51 protein to the site of a DSB.

Previously, it was shown that RNAi knockdown of Brca2 or Rad51 in Drosophila Kc and S2-R+ cells resulted in equivalent sensitivity to ionizing radiation and hydroxyurea (Brough et al. 2008). In fact, this identical sensitivity was used to confirm that a previously unidentified gene (*CG30169*) was the *Drosophila* homolog of *BRCA2* (Brough et al. 2008). We were interested to see if the sensitivity phenotypes were also similar in the whole organism. We first tested the sensitivity of *brca2* and *spn-A* null mutants to ionizing radiation (IR), which causes single and double strand breaks in the genome. The predominant pathway for repair of IR-induced damage in flies is thought to be Rad51-dependent HR

(Chan et al. 2010; McVey et al. 2004b), so any Rad51-independent functions of IR-induced damage repair by DmBrca2 should result in greater sensitivity in *brca2* mutants. We tested multiple allelic combinations for the *brca2* null mutants. The *brca2*^{47} and *brca2*^{56E} alleles were created through imprecise excision screens from our lab and the Schüpbach lab, respectively. All allelic combinations of *brca2* null mutants behaved similarly to the *spn-A* transheterozygous mutant (Figure 2-2A), consistent with the hypothesis that DmBrca2 and DmRad51 coordinate to repair IR-induced DNA damage by HR.

We next tested *rad51* and *brca2* null mutants with bleomycin, a chemotherapeutic thought to mimic radiation induced DNA damage (Chen and Stubbe 2005). As with IR, bleomycin sensitivity was equal in both null mutants (Figure 2-2B), suggesting that bleomycin-induced damage repair requires Rad51-dependent HR.

Repair of interstrand crosslinks (ICLs) is thought to involve the coordination of multiple pathways, including homologous recombination (HR) (Reviewed in Deans and West 2011; 2011). Therefore, we were also interested in investigating how DmBrca2 and DmRad51 functioned in the repair of ICLs. We tested mutants for their sensitivity to the DNA crosslinking agent, nitrogen mustard (mechlorethamine). *spn-A* and *brca2* null mutants were equally sensitive to nitrogen mustard, suggesting mutations in either protein equally compromise the HR component of ICL repair (Figure 2-2C).

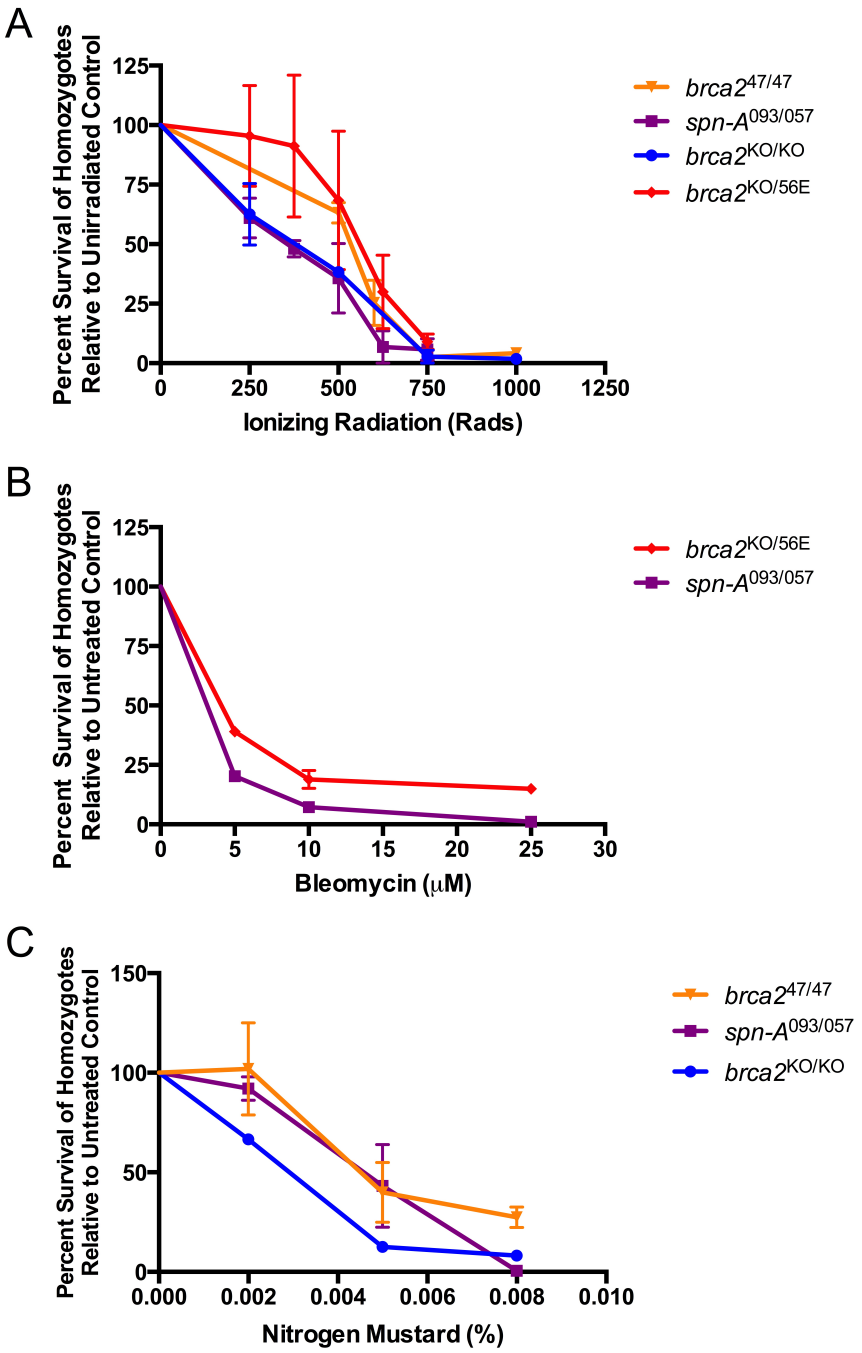

Figure 2-2: *brca2* and *spn-A* mutants are equally sensitive to multiple DNA damaging agents. (A) Ionizing radiation sensitivity. Data points represent independent trials of between one and five bottles with average percent survival and standard deviation shown. (B) Bleomycin sensitivity. Data points represent average of three independent trials of six vials each with standard deviation shown. (C) Nitrogen mustard sensitivity. Data points represent average of one to three independent trials of five to eight vials each with standard deviation shown.

The camptothecins are a family of drugs that inhibit topoisomerase I (Top1) by targeting the transient single stranded break formed by Top1-DNA linkage (Hsiang and Liu 1988). These stabilized transient complexes might lead to one-ended DSBs following collision with a replication fork (Hsiang et al. 1989), or they may cause replication forks to stall and regress (Ray Chaudhuri et al. 2012). To investigate a Rad51-independent function of DmBrca2 in either of these two situations, we tested *brca2* and *spn-A* null mutants for sensitivity to camptothecin. Interestingly, multiple allele combinations of *brca2* null mutants showed greater sensitivity to camptothecin than *spn-A* mutants (Figure 2-3A), suggesting a Rad51-independent role for DmBrca2 specific for the repair of one-ended DSBs or stabilization of stalled forks. The extreme sensitivity of the $brca2^{47}$ mutant was later shown to be a result of a mutation in *Cyp6d2* (Thomas et al. 2013), but the other *brca2* null mutants also appeared to be more sensitive than *spn-A* null mutants. This discrepancy was not observed with IR, nitrogen mustard or bleomycin (Figure 2-2A-C). Since IR and bleomycin generate two-ended DSBs and the response to one- and two-ended DSBs is thought to be different (Williams et al. 2010), one possible explanation is that DmBrca2 recruits a one-ended DSB specific protein or proteins to the site of the break. Alternatively, if camptothecin causes fork stalling, DmBrca2 may be required to stabilize replication forks, similar to its human homolog (Schlacher et al. 2011). To further investigate a role for DmBrca2 in repair of camptothecin-induced damage, we tested *brca2* and *spn-A* mutants with the water-soluble camptothecin analog, topotecan. Camptothecin and topotecan are expected to function identically and differ only in their

solubility. We observed no difference in sensitivity for these two mutants (Figure 2-3B), which is inconsistent with a one-ended DSB or fork stabilization specific role for DmBrca2.

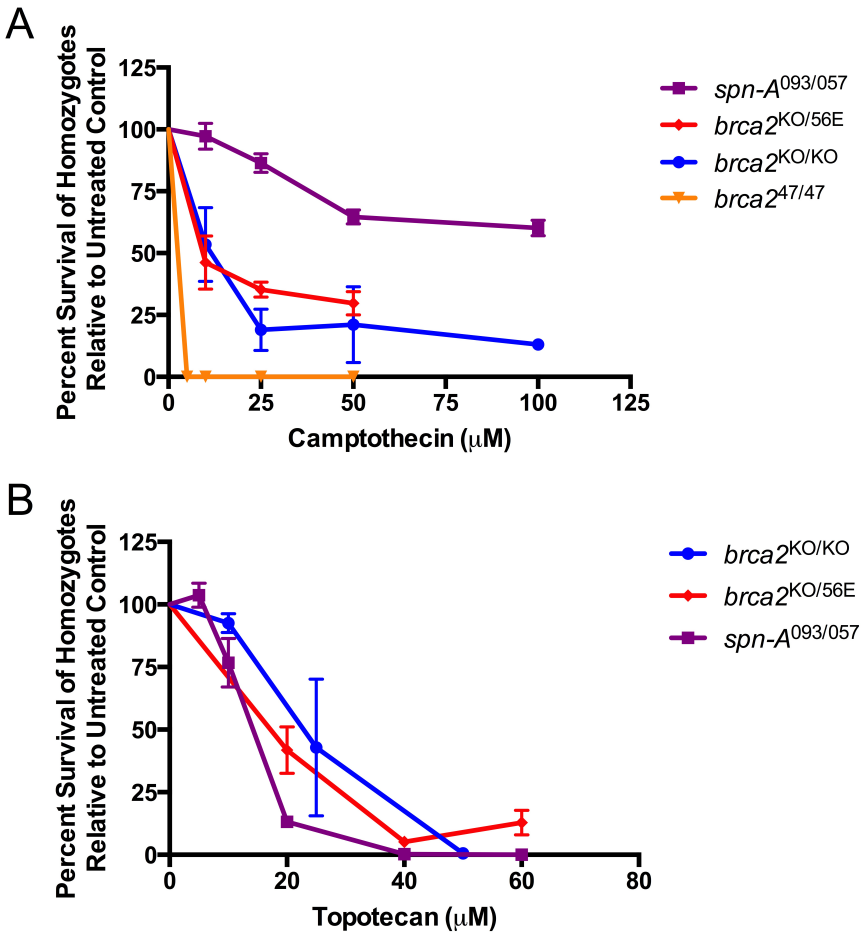

Figure 2-3: *brca2* and *spn-A* mutants show different sensitivities to camptothecin but equivalent sensitivities to topotecan. (A) Camptothecin sensitivity. Data points represent average of three to five independent trials of five to eight vials each with standard deviation shown. (B) Topotecan sensitivity. Data points represent average of three independent trials of six vials each with standard deviation shown.

Discussion

The recent purification of human BRCA2 (Jensen et al. 2010; Liu et al. 2010; Thorslund et al. 2010) has greatly facilitated biochemical research on the protein. However, genetic research is limited to case studies of patients and

families. Fortunately, several model organisms share functional homologs of *BRCA2*, allowing researchers to answer questions about the gene in less complex systems (Lo et al. 2003). Drosophila *BRCA2* shares DNA repair functions with its mammalian homologs (Brough et al. 2008; Klovstad et al. 2008). We further characterized DmBrca2 by testing mutants for sensitivity to several types of double strand breaks (DSBs), and showed that *brca2* null mutants behave very similarly to *spn-A* (DmRad51) null mutants in these assays. This suggests that DmBrca2 does not have a mitotic DNA repair role outside of recruiting Rad51 to DSB sites, regardless of the type of DSB formed.

The best characterized function of the human BRCA2 homolog as well as of those in other organisms is the recruitment of RAD51 to the sites of DSBs (Yang et al. 2002). While this function appears to be conserved in Drosophila (Brough et al. 2008), there are additional functions of mammalian BRCA2 that are likely not conserved in flies. We propose that this is largely because the Drosophila homolog is a smaller protein (971 amino acids vs. 3418 for human BRCA2), and lacks many of the domains identified in mammalian BRCA2 proteins (Lo et al. 2003).

DmBrca2, like the homologs in *C. elegans* and *P. falciparum*, lacks the complex DNA binding domain of mammalian BRCA2 (Lo et al. 2003). This domain consists of three oligonucleotide binding (OB) folds and a helix-turn-helix (HTH) motif (Yang et al. 2002). Since the OB folds bind ssDNA and the HTH motif binds dsDNA, it is hypothesized that the combination allows BRCA2 to target the ssDNA-dsDNA junction found at resected DSBs, where it can deposit

RAD51 for the initiation of strand invasion (Yang et al. 2002). The lack of such a domain in Drosophila suggests that an adaptor protein must be required for localization of DmBrca2 to DSBs. One candidate for this adaptor function is Dss1, which regulates DNA binding of Brh2, the *Ustilago maydis* homolog of BRCA2 (Zhou et al. 2009), and also appears to be critical for stabilization of mammalian BRCA2 (Li et al. 2005). Although a Drosophila Dss1 homolog does exist, recombinant DmBrca2 and DmDss1 do not interact (Brough et al. 2008). How exactly DmBrca2 is localizing to DSBs and binding to the DNA remains to be clarified.

Our $P\{w^a\}$ data show that homozygous *brca2* mutant flies are defective in HR repair. These flies are unable to synthesize the *white* gene removed after excision of $P\{w^a\}$ (Figure 2-1B). Additionally, repair junctions include sequences characteristic of aberrant repair: insertions, deletions and use of microhomologies flanking the gap (Table 2-1). These repair junction sequences in *brca2* homozygotes were very similar to those in *spn-A* homozygotes (McVey et al. 2004b), consistent with the idea that aberrant end-joining repair pathways are acting in the absence of either Brca2 or Rad51.

In contrast, flies heterozygous for *brca2* null mutations are not defective in HR repair (Figure 2-1B-C). This differs from previous results showing that *spn-A* heterozygotes had decreased use of HR repair as well as decreased synthesis tract lengths (McVey et al. 2004a). We believe that the reason for this difference is that small amounts of DmBrca2 are needed to recruit larger quantities of DmRad51 monomers. Cutting the availability of Rad51 in half

results in less effective repair because multiple invasions (each requiring a sufficient amount of Rad51 to coat the ssDNA at resected ends) are needed to synthesize the complete *white* gene in the $P\{w^a\}$ construct (McVey et al. 2004a). There is a statistically significant decrease in HR repair for *brca2* heterozygotes (Figure 2-1B), but the lack of a compensatory increase in aberrant repair or difference in synthesis tract lengths (Figure 2-1C) suggests that this decrease is not biologically relevant. Therefore, a 50% reduction in DmBrca2 is unlikely to inhibit its recruitment function during HR repair. Most likely, a single Brca2 protein can recruit Rad51 to the break, deposit it, and then continue to recruit more Rad51 until a threshold of nucleoprotein filament is formed. While Rad51 levels must be high to reach that threshold, only a small amount of Brca2 is required for HR. Thus, *spn-A* heterozygotes, but not *brca2* heterozygotes, show defects during gap repair.

In addition to the BRC repeats that bind Rad51 monomers (Chen et al. 1998), mammalian BRCA2 also contains a C-terminal domain that has been proposed to have several critical functions. At least by sequence, there does not appear to be a homologous domain in DmBrca2. The C-terminal interaction with RAD51 was first discovered in mice (Mizuta et al. 1997). Later, relatively small truncations at the C-terminus of human BRCA2 were shown to result in HR defects (Moynahan et al. 2001), but the reason was unclear. It was later shown that phosphorylation of Ser 3291 prevented C-terminal interaction with Rad51, and that this residue was dephosphorylated during S-phase or following DNA damage (Esashi et al. 2005). Soon after, researchers discovered that the C-

terminus was important for stabilizing RAD51 filaments (Esashi et al. 2007). Further research in chicken DT40 cells showed that the C-terminal-dependent disassembly of Rad51 filaments was a necessary step prior to mitotic entry, linking BRCA2 with cell cycle progression (Ayoub et al. 2009).

A completely RAD51-independent role for BRCA2 was discovered more recently, when it was shown that human BRCA2 prevents degradation of stalled replication forks by the nuclease MRE11 (Schlacher et al. 2011). This work showed that it was also the C-terminal domain of BRCA2 that was responsible for this function. While the study used hydroxyurea, a dNTP-depleting drug, to stall forks, another recent study showed that camptothecin and its derivatives also induce fork stalling (Ray Chaudhuri et al. 2012). This study proposed that stabilization of stalled and reversed forks is critical for camptothecin-induced damage repair.

Therefore, if BRCA2 is required for preventing degradation of stalled forks by MRE11, *brca2* mutations would result in exceptional sensitivity to camptothecin. Our initial results in a Drosophila model suggested this might be the case (Figure 2-3A), but this was refuted by our topotecan data (Figure 2-3B). An important consideration when analyzing this result is that fork stalling and regression were only seen at clinical (nanomolar) levels of camptothecin, while higher (micromolar) levels were predicted to result in collapsed forks and DSBs (Ray Chaudhuri et al. 2012). Therefore, the doses of camptothecin required to observe effects (lethality) in our sensitivity assays may be beyond the levels at which fork stabilization can facilitate cell survival. Consequently, we also tested

the sensitivity of *brca2* and *spn-A* mutants to hydroxyurea, the fork stalling effects of which are less controversial (Lopes et al. 2001; Sogo et al. 2002). No difference between the two mutants was observed (data not shown). However, since Drosophila lack the C-terminal domain of mammalian BRCA2, which is required for stalled fork protection (Schlacher et al. 2011), flies may not be a useful model for studying this mechanism.

Clinical studies on the efficacy of topoisomerase inhibitors with BRCA-deficient cancers show mixed results. An analysis of ovarian cancers with or without *BRCA1* or *BRCA2* mutations treated with topotecan did not show a difference in response between the two genotype categories (Hyman et al. 2011), though the patients were not separated by which *BRCA* gene was mutated. An earlier case study showed a remarkable complete recovery of a patient with metastatic pancreatic cancer treated with irinotecan (James et al. 2009). This patient carried a 6174delT frameshift mutation in *BRCA2*, common in Ashkenazi Jewish populations (Neuhausen et al. 1996). Admittedly, this case study included only a single patient, but the authors propose that topoisomerase inhibitors could be very effective in *BRCA2* deficient cancers.

Interestingly, poly(ADP-ribose) polymerase (PARP) inhibitors, which synergize well with topoisomerase inhibitors (Patel et al. 2012), are particularly effective in cancers with *BRCA* mutations (Chalmers 2009). Consistent with this observation, depleting both Brca2 and PARP in Drosophila cell culture results in a 50% reduction in survival as compared to only depleting Brca2 (Brough et al. 2008). Since topoisomerase inhibitors and PARP inhibitors have a similar

mechanism of action and result in similar protein-linked DNA structures (Murai et al. 2012), this would suggest that topoisomerase inhibitors would also be effective in *BRCA2* mutant backgrounds. More clinical research on this possibility is necessary.

In summary, we investigated potential Rad51-independent roles of DmBrca2 in mitotic DNA repair, including stabilization of stalled replication forks. We did not find evidence for any such function. Since DmBrca2 lacks the C-terminal domain involved in additional functions of mammalian BRCA2 (Ayoub et al. 2009; Schlacher et al. 2011), we conclude that its sole function in mitotic DNA repair is recruitment of DmRad51 to DSBs. We did observe one major difference between the two null mutants, which was that *brca2* heterozygous mutants are proficient at HR repair, while *spn-A* heterozygotes are not (McVey et al. 2004a). We believe the reason for this is that only minimal amounts of Brca2 are sufficient to recruit the large quantity of Rad51 required during HR.

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Chapter 3
Common variants of *Drosophila melanogaster* Cyp6d2 cause camptothecin sensitivity and synergize with loss of Brca2

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Abstract

Many chemotherapeutic agents selectively target rapidly dividing cells, including cancer cells, by causing DNA damage that leads to genome instability and cell death. We utilize *Drosophila melanogaster* to study how mutations in key DNA repair genes affect an organism's response to chemotherapeutic drugs. In this study, we focused on camptothecin and its derivatives, topotecan and irinotecan, which are type I topoisomerase inhibitors that create DNA double strand breaks in rapidly dividing cells. Here, we describe two polymorphisms in Drosophila *Cyp6d2* that result in sensitivity to camptothecin, but not topotecan or irinotecan. We confirmed that the sensitivity was due to mutations in *Cyp6d2* by rescuing the defect with a wild-type copy of *Cyp6d2*. Additionally, we showed that combining a *Cyp6d2* mutation with mutations in Drosophila *Brca2* results in extreme sensitivity to camptothecin. Given the frequency of the Cyp6d2 polymorphisms in publicly available Drosophila stocks, our study demonstrates the need for caution when interpreting results from drug sensitivity screens in Drosophila and other model organisms. Furthermore, our findings illustrate how genetic background effects can be important when determining the efficacy of chemotherapeutic agents in the background of various DNA repair mutations.

Introduction

The complexities of human genetic disorders often require model systems to provide a better understanding of the disease mechanism. *Drosophila melanogaster* provides an excellent system for human disease research because of its genetic tractability and the presence of many homologues of human disease genes in the fly genome (Rubin 2000). As such, it has been used to the study the genetic mechanisms of cancer for nearly forty years (Gateff 1978; Gateff and Schneiderman 1974) and multiple facets of carcinogenesis have been investigated in that time (reviewed in Rudrapatna et al. 2012). Drosophila has also proven to be an invaluable tool to research the effects of chemotherapeutic drugs (Jaklevic et al. 2006; Radcliffe et al. 2002; Boyd and Setlow 1976; Edwards et al. 2011; Gladstone and Su 2011) and the effects of mutations in key DNA repair genes (reviewed in Su 2011).

We have used mutagenic chemicals and radiation to better understand the functions of critical DNA repair proteins (Kane et al. 2012; Chan et al. 2010). Of particular interest to us is the topoisomerase I (Top1) poison, camptothecin, from which the chemotherapeutic drugs topotecan and irinotecan are derived (reviewed in Pommier 2006; Legarza and Yang 2006). Camptothecin and its derivatives stabilize the normally transient covalent link between DNA and Top1, thereby interfering with the relaxation of supercoiling that occurs during events requiring DNA unwinding, such as replication or transcription (Hsiang et al. 1985; Hsiang and Liu 1988; Koster et al. 2007). The classical model proposes that single strand breaks at the sites of camptothecin-induced Top1-DNA links are converted into

DSBs following collision with a replication fork (Holm et al. 1989; Hsiang et al. 1989). Recent research has challenged this theory, however, proposing instead that the accumulation of regressed forks or supercoiled DNA is responsible for the toxic effects (Ray Chaudhuri et al. 2012).

Although cancer frequently involves defects in multiple genes or pathways, there are specific examples where mutations in a single gene are associated with significant cancer risk. Perhaps one of the most well known examples is the breast cancer susceptibility gene, *BRCA2*. Individuals inheriting a mutant copy of the gene exhibit a significant increase in breast and ovarian cancer risk (Wooster et al. 1995). The Brca2 protein functions in homologous recombination (HR) repair of DNA double strand breaks (DSBs) (reviewed in Thorslund and West 2007; Boulton 2006). HR utilizes an intact DNA template to synthesize nucleotides lost on a broken homologous chromosome or sister chromatid. It is mediated by the recombinase, Rad51 (Sung 1994), which is recruited to the sites of DSBs by Brca2 (Davies et al. 2001). Similar to its mammalian homolog, Drosophila Brca2 interacts with DmRad51 (Brough et al. 2008), and plays a critical role in both mitotic and meiotic HR repair of DSBs *in vivo* (Klovstad et al. 2008).

We were interested in examining the role of Drosophila Brca2 in the repair of camptothecin-induced DNA damage. To do this, we treated two stocks of *brca2* mutant flies with camptothecin. We were surprised to discover that one line of *brca2* mutants was exceptionally sensitive to the drug. Further investigation revealed that these flies carried a second mutation in *Cyp6d2*, a cytochrome P450

gene, which, when combined with the *brca2* mutation, resulted in synergistic hypersensitivity to camptothecin. We now report that many publicly available Drosophila stocks carry this mutation or a second, independent mutation in *Cyp6d2* that also causes extreme sensitivity to camptothecin.

Materials And Methods

Fly culture conditions and stocks: Flies were kept at 25° with an alternating 12 hour light:12 hour dark cycle and fed a standard cornmeal agar diet. Fly stocks were acquired from the Bloomington Stock Center, with the exception of $brca2^{KO}$ (see below). For our mapping and sequencing studies, we used the sequence available on Flybase as our wild-type standard (Adams et al. 2000).

Creation and Isolation of Mutants: The $brca2^{47}$ mutation was created via an imprecise excision of *P{SUPor-P}KG02287*, located between *BRCA2* and *CG3746*. The excision resulted in a deletion removing the first 2169 bp of *BRCA2*. The $brca2^{KO}$ mutant was a donation from Trudi Schüpbach's laboratory, and was created by ends-out homologous recombination (Klovstad et al. 2008).

The original *scpt* mutant was found in the same stock used to create $brca2^{47}$, but was created via a precise excision of the P-element. This mutant was later renamed $cyp6d2^{SD}$. The other *Cyp6d2* mutation, $cyp6d2^{NT}$, was found in the $P\{GT1\}CG42565^{BG02301}$ stock.

Sensitivity Assays: Sensitivity assays were set up using 5-8 virgin female flies and 3 male flies. The females were heterozygous for the mutation of interest, while males were either heterozygous or homozygous. Heterozygous flies were balanced by the *CyO* chromosome.

Parental flies were kept in vials for 3 days to lay eggs and were then transferred to new vials. The flies were then discarded after 2-3 additional days. Each set of vials was treated with mutagen or vehicle one day after the parents were removed. Camptothecin (Sigma) was dissolved in DMSO and then diluted in Tween 20/EtOH (5% ethanol, 1% Tween 20). Mechlorethamine, methyl methanesulfonate, hydroxyurea (Sigma) topotecan, irinotecan, and bleomycin (Enzo Life Sciences) were dissolved in water. Each sensitivity trial consisted of 5-8 vials. Heterozygous and homozygous offspring were counted periodically until 18-20 days after the crosses were established. Percent survival was calculated by the following equation:

$$\text{Relative percent survival} = \frac{\text{percent of homozygous flies in treated vials}}{\text{percent of homozygous flies in control vials}} \times 100\%$$

Sensitivity to ionizing radiation was characterized in a similar way. Parental flies (40-60 virgin females and 10-20 males) were allowed to lay eggs on grape agar plates for several days, with plates replaced every 10-14 hours. The plates were then supplemented with yeast paste and placed at 25°. Once larvae reached third instar stage, the grape agar plates were irradiated at a rate of 800 rads/min in a Gammator 1000 irradiator. After irradiation, the larvae were moved into fly bottles for further development. Adult flies were counted and sorted as above, and irradiated flies were compared to an unirradiated control.

Rescue sensitivity assays consisted of 5-8 virgin female flies that were heterozygous for the mutation of interest (balanced by *CyO*) as well as one copy of the *Cyp6d2* rescue construct (see below). They were crossed to 3 males that were heterozygous or homozygous for the mutation tested, with no rescue

construct. Vials were treated as above, and the progeny were sorted by wing type and eye color. Percent survival was calculated as above, but separately for rescued and non-rescued flies.

Mapping: For complementation mapping, flies heterozygous for a defined deletion or insertion (over 2nd chromosome balancer *CyO*) were crossed to *scpt* homozygous flies in vials. Vials were treated as above for sensitivity assays, using the *scpt* lethal dose of 25 μM camptothecin. Flies were sorted by wing type to determine complementation.

Meiotic mapping used non-complementing stocks carrying *white*$^{+}$ markers at defined locations. Female flies heterozygous for the non-complementing marked deletion or insertion (over a wild type 2nd chromosome to allow for meiotic recombination) were crossed to homozygous *scpt* mutant males in vials. Vials were treated with 25 μM camptothecin. Offspring were sorted by eye color, and the percent of red-eyed progeny was calculated.

Construction of the Cyp6d2 rescue stock: The full-length *Cyp6d2* gene, including 402 bp upstream of the transcription start site and 285 bp downstream of the transcription termination site, was amplified by PCR using Phusion polymerase (New England Biolabs) and the primers 5'-TCTAGAGGTACCGCGCTGACAATCCTACAAGC-3' and 5'-AGATCTGCGGCCGCGATTCCGCAAGGTGGAGAAG-3'. The PCR product was digested with *Acc*65I and *Not*I and directionally cloned into *pattB* (gift of K. Basler). Purified plasmid (500 ng/μL) was injected into fly embryos < 2h AEL (after egg laying) containing an *attP* site on 3R: *y*^{1}*,M{vas-int}ZH2A,w**; *M{3xP3-*

RFP,attP}ZH96E. Injected embryos were allowed to develop into adult flies at 25°. These adults were crossed to w^{1118} males or females, and the progeny sorted by eye color. We recovered at least one red-eyed fly in the progeny of about 6% of all surviving injected flies. The presence of the construct was confirmed by PCR and sequencing. Flies carrying the rescue construct were mated to mutant flies to generate stocks carrying both the mutation of interest and the rescue construct. These flies were used in rescue sensitivity assays as described above.

Allele-specific PCR: To identify stocks with specific Cyp6d2 mutations, allele-specific primers were each paired with the genomic primer downstream of *Cyp6d2*, 5'-ctctcgaattcagaacgagc-3'. The allele-specific primer, 5'-GGGTCCTAGGCACTGCAGAC-3' was used to detect $cyp6d2^{SD}$, with an annealing temperature of 60°. The allele-specific primer, 5'-CCCATCGCTTCGATTCAGAGAC-3' was used to detect $cyp6d2^{NT}$, with an annealing temperature of 56°. These pairs yield 391 bp and 452 bp products, respectively, when amplifying template DNA from the appropriate mutant but produce no product with a wild-type template. Phusion polymerase (New England Biolabs) was used for amplification.

Reverse-transcriptase PCR: To determine expression levels of *Cyp6d2* in various backgrounds, 15 wandering 3rd instar larvae (5-6 days AEL) were isolated and frozen at -80º. RNA was purified using RNAqueous-4PCR (Ambion) and cDNA was synthesized using RETROscript (Ambion). Random decamers were used as primers for cDNA synthesis. *Cyp6d2* cDNA was then amplified to detect transcripts in each genetic background. The following primers were used:

XInt1 5’-CATTAGCTTAGCAATCGGTGG-3’; XInt2 5’-GGACATCTGCATCATGGAAACC-3’; XInt3 5’-CTTTTCCCATGCGAAGAGCTATGC-3’; F1 5’-CTCGCCAAATCATGACCAGC-3’; F2 5’-GCTAAGCTAATGAACCGCTTGG-3’; R1 5’-GCGGCATCGAAACGGAACTC-3’; rp49F 5’-CCATCCGCCCAGCATACAGG-3’; rp49R 5’-CTCGTTCTCTTGAGAACGCAG-3’.

Results

Identification of a genetic modifier of camptothecin sensitivity:

Drosophila Brca2 has been shown to play a critical role in homologous recombination (HR) repair of DNA double-strand breaks (DSBs) (Klovstad et al. 2008; Brough et al. 2008). Along with its clinically approved analogs topotecan and irinotecan, camptothecin is traditionally thought to create DSBs in rapidly dividing cells via replication run-off (Strumberg et al. 2000), leaving a one-ended DSB (Tsao et al. 1993). Brca2-dependent homologous recombination (HR) repair could then be required to restart replication.

To test this hypothesis, we treated two independently derived *brca2* mutants with camptothecin and quantified survival to adulthood. The first was a null allele ($brca2^{KO}$) created by ends-out homologous recombination (Klovstad et al. 2008). The second ($brca2^{47}$) was created via an imprecise excision of a P-element 90 bp upstream of *BRCA2* (*P{SUPor-P}KG02287*) and deletes the 5’ half of *BRCA2*. Both alleles were sensitive to camptothecin, suggesting that BRCA2-

mediated homologous recombination repair is important for repair of one-ended DSBs. Surprisingly, the *brca2*^{47} mutant was significantly more sensitive to camptothecin than the *brca2*^{KO} allele (Figure 3-1A). We suspected that the *brca2*^{47} stock contained a second-site mutation that made it more sensitive than the *brca2*^{KO} mutation. Such mutations are common in imprecise excision screens, and often occur near the site of the inserted element.

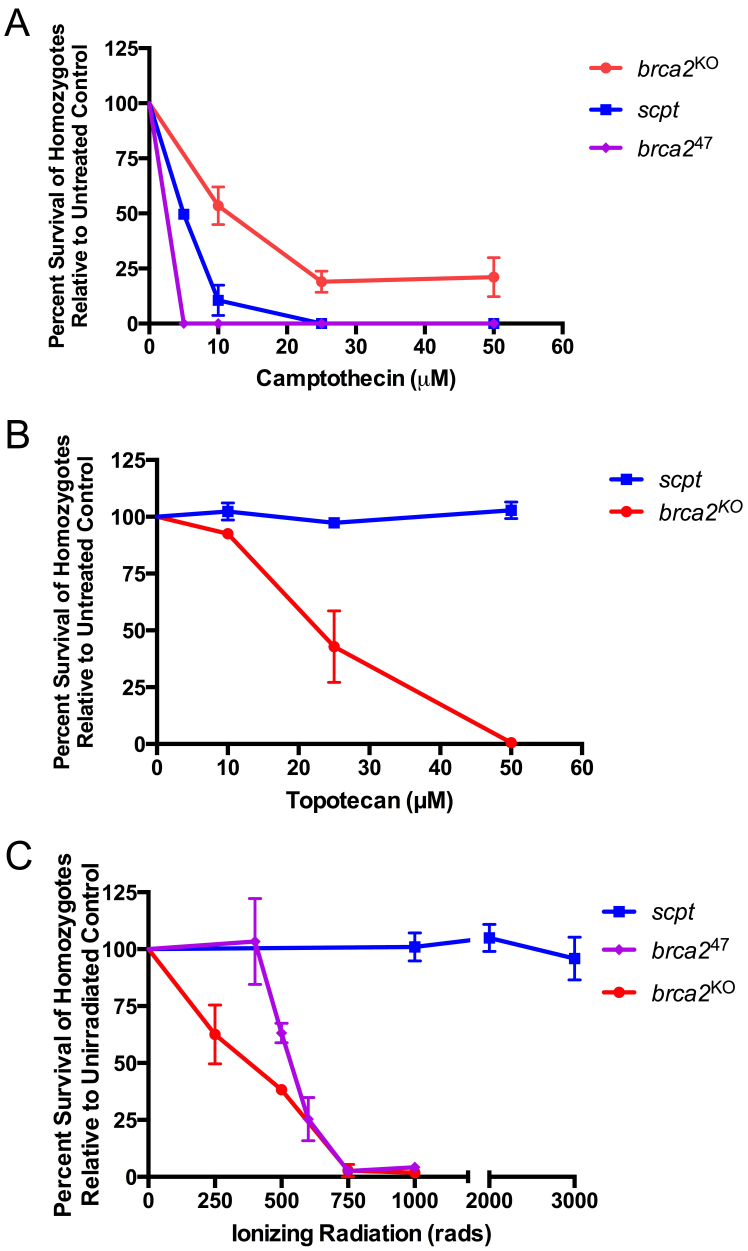

Figure 3-1: The *scpt* mutant is sensitive to camptothecin, but not to topotecan or ionizing radiation. (A) Camptothecin sensitivity of the *scpt* mutant as well as two null alleles of *brca2*. All data points represent 3 independent trials of 5-6 vials each. (B) Topotecan sensitivity of the *scpt* mutant and *brca2*^{KO} mutant. All data points represent 3 independent trials of 5-6 vials each. (C) Ionizing radiation sensitivity of the *scpt* mutant as well as two *brca2* mutant alleles. All data points represent 2-4 independent trials of 1 bottle each. Standard deviations are shown as error bars.

To confirm this, we created a precise excision of *P{SUPor-P}KG02287*. Sequencing of DNA from this stock revealed that the *BRCA2* coding sequence was identical to wild-type sequence. Surprisingly, this precise excision stock also displayed high sensitivity to camptothecin, although not to the level of $brca2^{47}$ flies (Figure 3-1A). This suggested that the second-site mutation did not arise during the imprecise excision of the P-element, but rather was present in the original stock used to create $brca2^{47}$. We named this mutation *scpt* (sensitive to camptothecin). Unlike *brca2* mutants, *scpt* mutants were not sensitive to the camptothecin analog topotecan (Figure 3-1B) or to ionizing radiation (Figure 3-1C). Furthermore, *scpt* mutants were not sensitive to irinotecan, bleomycin, mechlorethamine (nitrogen mustard), methyl methanesulfonate, or hydroxyurea (data not shown).

The *scpt* mutation is present in multiple stocks: In order to understand the unusual specificity of the *scpt* mutation, we sought to identify the gene(s) mutated in this stock. Since *scpt* exhibited Mendelian segregation with the 2nd chromosome balancer *CyO*, we assumed the mutation was located on the 2nd chromosome. To map the mutation, we began by performing complementation tests using chromosome *2* deficiency stocks. We crossed deficiency stocks to *scpt* mutants and treated the offspring with 25 μM camptothecin, a lethal dose for *scpt* homozygotes. Our initial tests used the DrosDel (ED) Deficiency Collection (Ryder et al. 2004), available from the Bloomington Stock Center.

We were surprised to find that none of the 2nd chromosome fly stocks we used from this collection were able to complement the *scpt* mutation when treated

with a lethal dose of camptothecin, regardless of the location of the deletion (Table 3-1). However, multiple stocks from the Exelixis (Exel) and Bloomington Stock Center (BSC) deficiency collections did complement the mutation at this dose. Therefore, we concluded that the progenitor stocks of the DrosDel collection as well as *P{SUPor-P}KG02287* carry the *scpt* mutation.

Since multiple DrosDel stocks were carriers, we wanted to see if other chromosomes containing *P{SUPor-P}* elements also carried *scpt*. We performed complementation tests as before, using *P{SUPor-P}* stocks. Roughly two-thirds of the *P{SUPor-P}* stocks tested did not complement the *scpt* mutation (Table 3-1). Location of the *P{SUPor-P}* element did not correlate with complementation of *scpt*. Thus, we hypothesize that the progenitor stock used to create the *P{SUPor-P}* collection (Roseman et al. 1995) carried the mutation, although it appears to have been lost in a subset of these stocks.

TABLE 3-1. Cyp6d2 status of various stocks.

Stock [a]	Cytological Location	*Cyp6d2* allele[b]	Complementation Test[c]
	P{SUPor-P} Insertions		
KG00490 (*CG34370*)	58B1	WT	Complements
KG01596 (*whd*)	47A11	WT	Complements
KG04872 (*CG13322*)	49E1	WT	Complements
KG06046	60F5	WT	Complements
KG06805 (RabX1)	59E2	WT	ND[d]
KG07568 (*CG15704*)	53A4	WT	Complements
KG00006 (*CycB*)	59B2	SD	Does not complement
KG02089 (*Hrb87F*)	87F7	SD	ND
KG02287 (*CG4612*, *BRCA2*)	60D4	SD	Does not complement
KG02463 (gce, *Top1*)	13B6	SD	ND
KG02566 (*CG10880*)	40F1	SD	Does not complement
KG05061 (*Babos*)	58D4	SD	Does not complement
KG06675 (*CG9896*)	59C1	SD	Does not complement
KG07633 (*Egfr*, *CG30286*)	57E9	SD	Does not complement
KG07401 (*CG13511*)	58F4	SD	Does not complement
KG07430 (*Tim17b2*)	35D2	SD	Does not complement
KG06763	35B1	SD	Does not complement
KG07930 (*Jheh3*)	55F8	SD	Does not complement
	Mi{MIC} Insertions		
MI02105 (*grp*)	36A10	NT	ND
MI02462 (*Mad*)	23D3	NT	ND
MI00056 (*jbug*)	59A3	WT	ND
MI02085 (*Cyp6d2*)	58F4	WT	ND
	Mi{ET1} Insertions		
MB00453 (*dp*)	25A1	NT	ND
MB01292 (*CG3746*)	58F4	NT	Does not complement
MB05269 (*Cyp6d2*)	58F4	NT	Does not complement
MB05513 (*CG13579*)	60D1	NT	ND
	P{GT1} Insertions		
BG02301 (*CG42565*)	58F4	NT	Does not complement
BG02743 (*Hrb87F*)	87F7	NT	ND
	DrosDel Deficiencies		
Df(2L)ED284	25F2-26A3	NT	Does not complement
Df(2L)ED1303	37E5-38C6	NT	Does not complement
Df(2R)ED3683	55C2-56C4	NT	Does not complement
Df(2R)ED3728	56D10-56E2	NT	Does not complement
Df(2R)ED3923	57F6-57F10	NT	Does not complement
Df(2R)ED3952	58B10-58E5	NT	Does not complement
Df(2R)ED4061	60C8-60D13	NT	Does not complement
Df(2R)ED4065	60C8-60E8	NT	Does not complement

Df(2R)ED4071	60C8-60E8	NT	Does not complement
Df(3R)ED4408	66A22-66C5	NT	ND
Df(3R)ED10257	83A7-83B4	NT	ND
Bloomington Stock Center Deficiencies			
Df(2R)BSC597	58A2-58F1	WT	Complements
Df(2R)BSC598 (*Cyp6d2*)	58F3-59A1	Deleted	Does not complement
Df(2R)BSC599	59B1-60F5	WT	Complements
Df(2R)BSC602	60C8-60E5	WT	Complements
Df(2R)BSC603	60C7-60D1	ND	Complements
Df(2R)BSC604	60D4-60E11	ND	Complements
Df(2R)BSC605	60D8-60E8	ND	Complements
Df(2R)BSC606	60D10-60E1	WT	Complements
Df(2R)BSC607	60E4-60E8	WT	Complements
Df(2R)BSC769	59B7-59D9	WT	Complements
Df(2R)BSC784	59B4-59B6	ND	Complements
Df(2R)BSC787	58F4-59B1	ND	Complements
Exelixis Deficiencies			
Df(2R)Exel6044	37F2-38E3	ND	Complements
Df(2R)Exel6079	59A3-59B1	WT	Complements
Df(3R)Exel6178	90F4-91A5	WT	ND

[a] Genes potentially affected by transposon insertions are shown
[b] WT: sequence matches Flybase at two loci of interest; SD: stock carries *cyp6d2*^{SD} allele; NT: stock carries *cyp6d2*^{NT} allele.
[c] Stock tested for complementation (survival) with original *scpt* allele (precise excision of *P{SUPorP}KG02287*) at a dose of 25 μM camptothecin
[d] ND= no data

Stocks carrying *scpt* can be used to map the mutation: Because the DrosDel stocks and many *P{SUPor-P}* stocks carry the *scpt* mutation at an unknown location and a red eye marker (*white*$^{+}$) at a defined location, we used traditional meiotic mapping to determine the location of *scpt* (Figure 3-2A). Unbalanced female flies heterozygous for the *scpt*, *white*$^{+}$ chromosome (from either *P{SUPor-P}* or DrosDel stocks) were crossed to white-eyed homozygous *scpt* males and the progeny were treated with 25 μM camptothecin. Lower than expected survival rates of red-eyed flies (<50%) indicated linkage of the *white*$^{+}$ marker to *scpt*. We did this using multiple DrosDel and *P{SUPor-P}* stocks near

the right end of chromosome 2 (Figure 3-2B). These tests indicated a location of interest near the cytological bands 58B-59C.

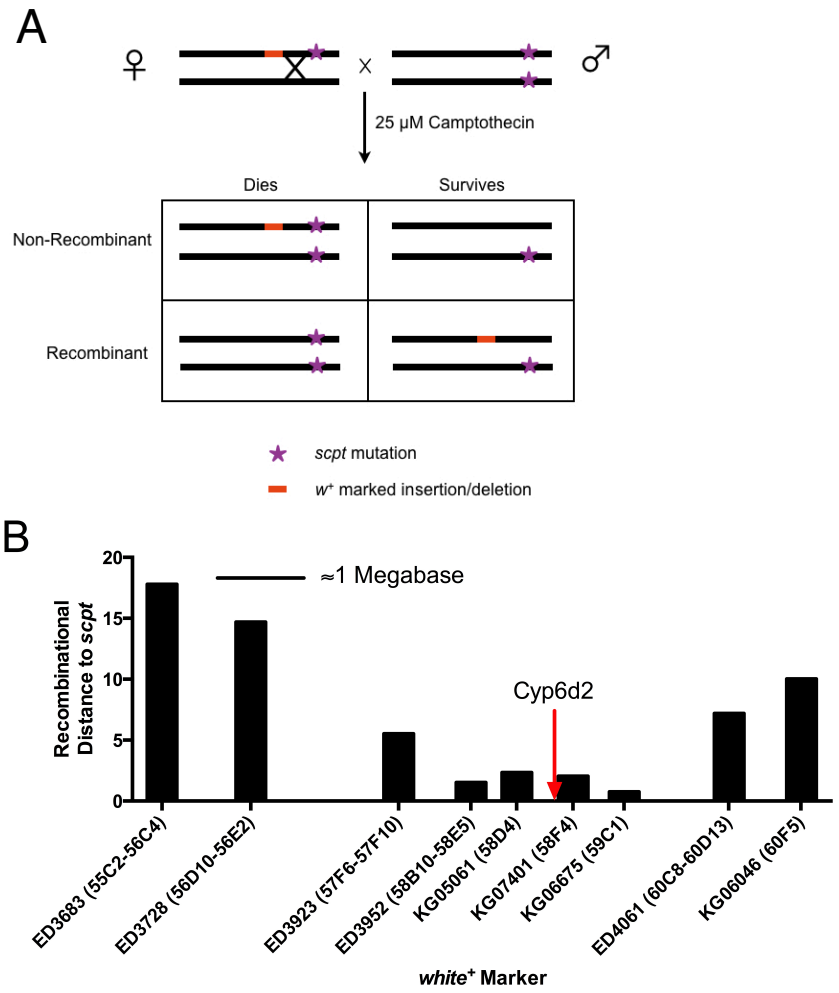

Figure 3-2: Meiotic mapping of the *scpt* mutation. (A) Cross scheme for meiotic mapping. Flies that inherit the *white*$^{+}$ marker have red eyes. Flies that inherit two mutant copies of the *scpt* mutation do not survive treatment with 25 µM camptothecin. Percentage of surviving flies with red eyes should be equal to the recombination distance between the *white*$^{+}$ marker and *scpt*. (B) Multiple meiotic mapping crosses identified a region containing the *scpt* mutation. Both DrosDel deficiencies (ED) and *P{SUPor-P}* elements (KG) were used. Distances on the x-axis are approximations of the physical distance between these markers. The region shown spans from cytological band 55C to 60F (the terminal quarter of chromosome 2R, or about 7 megabases).

We further refined the position of *scpt* with complementation tests using deficiencies from the Exelixis (Exel) and Bloomington Stock Center (BSC)

collections. Based on our complementation tests (Table 3-1), we assumed that these deficiency collections did not carry *scpt*, and therefore non-complementation indicated that the deficiency deleted the gene(s) mutated in *scpt* flies. Of the five deficiencies near the region of lowest recombination distance, only *Df(2R)BSC598* did not complement the *scpt* mutation. This suggested that *scpt* was located in the 36.9 kb region deleted in this deficiency. Furthermore, we found that an overlapping deletion in *Df(2R)BSC787* complemented *scpt*, allowing us to narrow down the *scpt*-containing region to approximately 20 kb. This region contains 13 genes, one of which is the cytochrome P450 gene, *Cyp6d2*.

We sequenced the coding regions of all 13 of these genes using flies from complementing *P{SUPor-P}* stocks (*KG06046* and *KG01596*) and non-complementing *P{SUPor-P}* stocks (*KG02287* and *KG06675*). Amino acid changes we identified in the non-complementing stocks are shown in Table 3-2. The complementing *P{SUPor-P}* stocks matched the published *Drosophila* sequence found on FlyBase at almost all locations. Importantly, the Flybase sequenced stock, y^1; $Gr22b^1$ $Gr22d^1$ cn^1 $CG33964^{R4.2}$ bw^1 sp^1; $LysC^1$ $MstProx^1$ $GstD5^1$ $Rh6^1$ (Adams et al. 2000) was not sensitive to camptothecin (data not shown).

Table 3-2: Amino acid changes found in non-complementing region

Gene	Mutation
CG30195	I12S
CG34445	D26V
	E55K
	K128Q
CG3746	E20G
	S44A
Cyp6d2	A459P/R
CG13511	A49T

The *scpt* mutation alters splicing of the Cyp6d2 transcript: Two of the polymorphisms detected by sequencing were located near the exon three / intron three junction in *Cyp6d2* (Figure 3-3A-B). The first (G→C) is located at the terminal nucleotide of exon three and is also the first nucleotide of an alanine codon. The second (G→A) is located five nucleotides downstream of the exon-intron junction. If the two SNPs did not affect splicing, the first SNP would change the alanine (A458) to a proline. Alignment of *Drosophila* Cyp6d2 and other P450 enzymes indicated that this change is in a highly conserved region identified as the heme-binding domain (Tijet et al. 2001). Alternatively, if the two SNPs combined to disrupt splicing of intron three, the alanine would become an arginine and a frameshift would result in a premature stop codon 17 amino acids downstream.

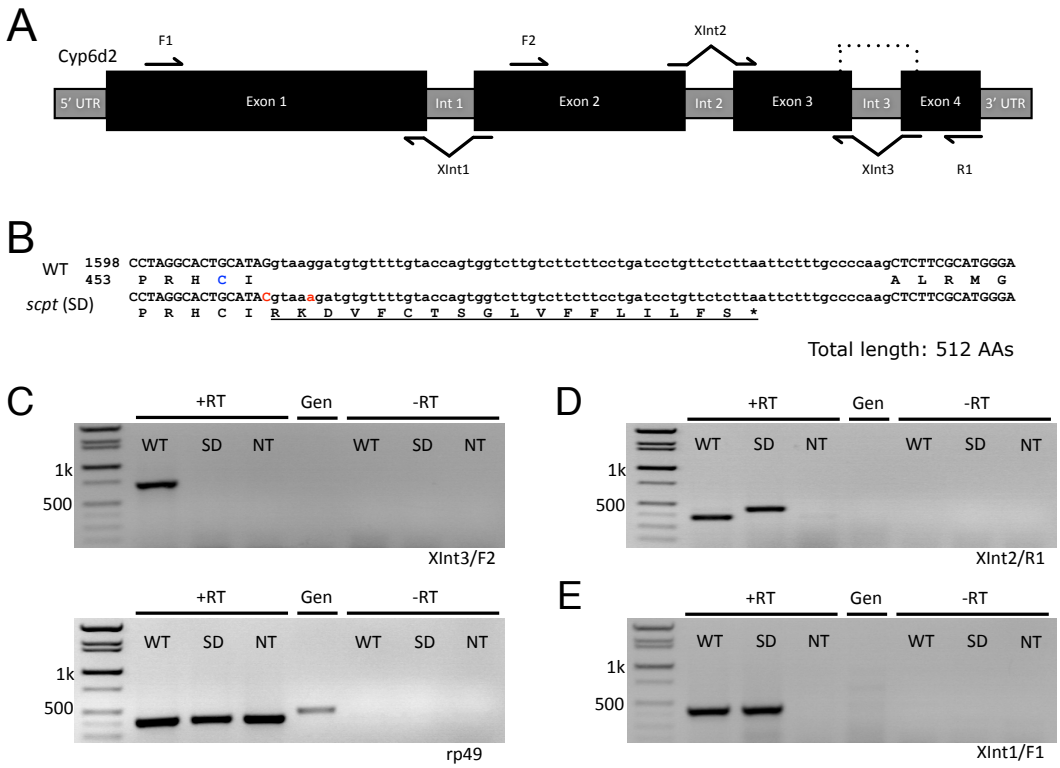

Figure 3-3: Mutations in *Cyp6d2* affect transcription and RNA processing. (A) Diagram of the *Cyp6d2* gene structure. Protein coding regions are represented by black boxes, while gray bars represent introns and UTRs. Dotted line indicates the region shown in B. Primers shown were used in RT-PCR reactions in C-E (B) Nucleotide and amino acid alignment of wild-type and *scpt* mutant Cyp6d2. *scpt* SNPs in this region are shown in red. Heme-binding cysteine is shown in blue. Failure to splice intron three (sequence in lower-case letters) results in a frameshift mutation and a premature stop codon (*). (C-E) Semi-quantitative RT-PCR of wild-type and mutant larvae. Primers indicated below gels refer to those shown in A. RT-PCR with (+RT) and without (-RT) reverse transcriptase was performed. Genomic (Gen) template is also shown for comparison. Sizes of molecular weight markers are indicated. (C) The XInt3/F2 primer pair yields a PCR product of 709 bp if splicing of intron 3 is correct. The rp49 primer pair (control reaction) yields PCR products of 398 bp and a genomic product of 460 bp. (D) The XInt2/R1 primer pair yields a PCR product of 375 bp if introns 2 and 3 are properly spliced. Intron 3 length is 70 bp. (E) XInt1/F1 yields a PCR product of 440 bp if intron 1 is correctly spliced.

To distinguish between these two possibilities, we performed semi-quantitative RT-PCR to analyze *Cyp6d2* transcripts in wild-type and *scpt* mutant larvae. We synthesized total cDNA from RNA purifications and amplified DNA using primers that will anneal only to correctly spliced exons (Figure 3-3A).

Pairing a reverse primer that spans intron three with a forward primer in exon two resulted in no product for *scpt* mutant larvae, while both mutants showed normal transcript levels of *rp49* control cDNA (Figure 3-3C). In addition, pairing a forward primer spanning intron two with a reverse primer in exon four produced a larger than expected product for *scpt* larvae (Figure 3-3D). These results are consistent with a splicing defect in *scpt* mutants and suggest that intron three sequence is included with the final transcript. Sequencing confirmed that the cDNA templates isolated from the *scpt* mutant included intron three (data not shown). To demonstrate that splicing of other Cyp6d2 introns is normal in the *scpt* mutant, we paired a reverse primer that spans intron one with a forward primer in exon one. This produced equal length products for wild-type and *scpt* mutant larvae (Figure 3-3E). Based on these findings, we reasoned that the *cyp6d2*^{SD} (splicing defective) allele was the best candidate for the *scpt* mutation.

In order to confirm that *cyp6d2*^{SD} was the *scpt* mutation, we sought to rescue the mutant flies with a wild-type copy of *Cyp6d2* using phiC31-mediated transgenesis (Bischof et al. 2007). To do this, we cloned and inserted wild-type *Cyp6d2*, along with several hundred base pairs of flanking sequence, into the vector *pattB*. The *pattB-Cyp6d2* plasmid was injected into fly embryos carrying an *attP* site at cytological band 96E. Successful germline integration of the plasmid was detected in the following generation by screening for *white*$^{+}$ flies.

We crossed the *attP-Cyp6d2* transgenic stock to our *cyp6d2*^{SD} mutant flies to generate flies that carried both the mutation and the rescue construct and conducted sensitivity assays with these flies. *cyp6d2*^{SD} homozygotes carrying one

copy of the rescue construct showed nearly 100% survival at all doses tested (Figure 3-4A). We concluded that *cyp6d2*^{SD} is the *scpt* mutation.

A second, naturally occurring Cyp6d2 variant also sensitizes flies to camptothecin: During the course of examining *Cyp6d2* sequences in other fly stocks, we discovered a second mutation that produced a nearly identical camptothecin sensitivity phenotype (Figure 3-4B). In these stocks, consecutive asparagine residues (N438 and N439) were mutated to aspartic acid and threonine respectively. Interestingly, semi-quantitative RT-PCR revealed that this mutant produced little to no *Cyp6d2* transcript (Figure 3-3C-E). We named this mutant *cyp6d2*^{NT} (no transcript). Sequence analysis showed that *Mi{ET1}* and *P{GT1}* insertion stocks, as well as DrosDel collection deficiencies carried the *cyp6d2*^{NT} mutation (Table 3-1). The mutant phenotype of these flies was also completely rescued by the wild-type construct (Figure 3-4B).

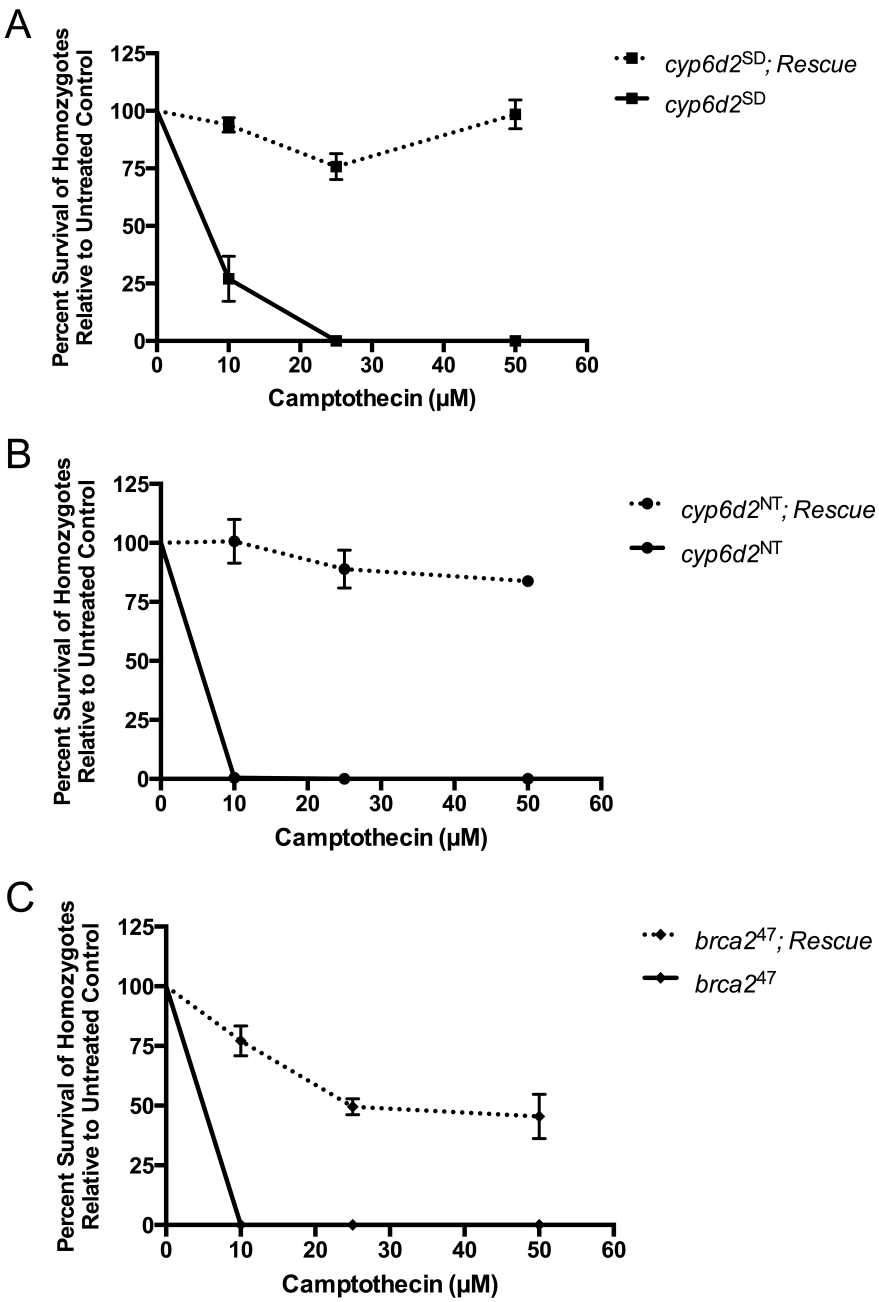

Figure 3-4: Rescue of camptothecin sensitivity by a wild-type Cyp6d2 transgene. All data points represent 3-4 independent trials of 5-6 vials each. Error bars represent standard deviations. (A-B) Rescue of both *cyp6d2* mutants. A precise excision of *P{GT1}CG42565* was used as the *cyp6d2*^{NT} mutant in B. (C) Partial rescue of the *brca2*^{47} allele.

We developed allele-specific primers to rapidly screen additional stocks for either of the *scpt* mutations (Figure 3-5, see Materials and Methods). For the

cyp6d2^{NT} allele, we used allele-specific primers specific to the N438D and N439T polymorphisms. Table 3-1 lists all insertions and deletions we have examined via complementation tests and/or PCR. Notably, multiple stocks with insertions or deletions on the X and 3rd chromosomes were also shown to carry the point mutations in *Cyp6d2*.

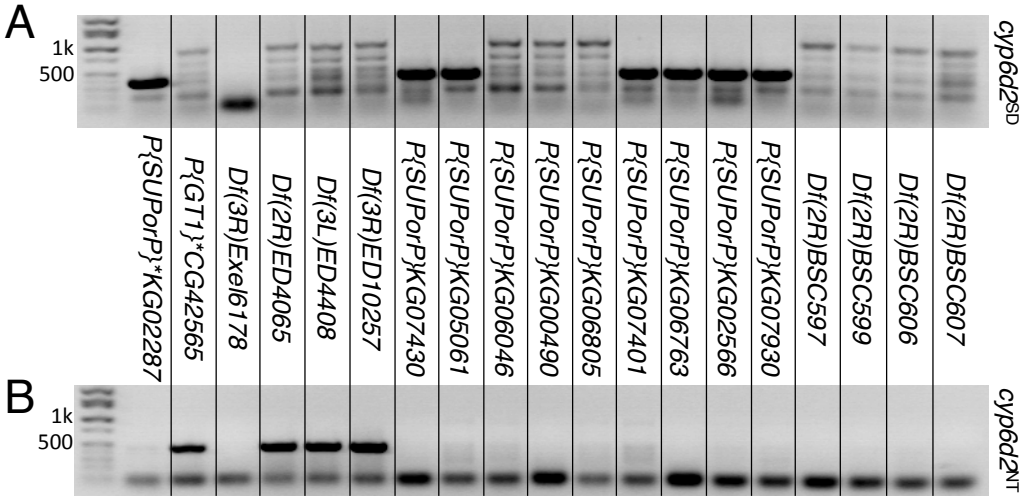

Figure 3-5: Allele-specific PCR to detect mutations. (*) indicates a precise excision of the insertion shown. Both PCR products are around 400 bp (see Materials and Methods). Sizes of molecular weight markers are indicated. (A) Allele-specific PCR gel for *cyp6d2*^{SD} mutation. *P{SUPor-P}*KG02287* serves as a positive control. (B) Allele-specific PCR gel for *cyp6d2*^{NT} mutation. *P{GT1}*CG42565* serves as a positive control.

The amino acid changes in *cyp6d2*^{NT} must be closely linked to a non-coding change that affects the regulation of *Cyp6d2*. Since the camptothecin sensitivity in *cyp6d2*^{NT} was successfully rescued by the transgene, such a change would have to be within the region contained in the rescue construct. Although there are no obvious changes in the promoter sequence that would be predicted to affect transcription initiation, the 3' UTR of *cyp6d2*^{NT} is significantly different from wild-type sequence (Figure 3-6).

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WT    -277        AAGAACCACATTCTATTTGGGGTCAACATATTGAAAGCATAATGATATGCTGTTGATATC
SD                AAGAACCACATTCTATTTGGGGTCAACATATTGAAAGCATAATGATATGCTGTTGATATC
NT                AAGAACCACATTCTATTTGGGGTCAACATATTGAAAGCATAATGATATGCTGTTGAAATC
                  ********************************************************* ***

WT    -217        ATATACCTTTGAAATTAGTTGTGCATTTCTGTAATGTGCTCAACAGGTAGTCTCTTTGCT
SD                ATATACCTTTGAAATTAGTTGTGCATTTCTGTAATGTGCTCAACAGGTAGTCTCTTTGCT
NT                ATATACCTTTGAAATTAGTTGTGCATTTCTGTCATGGGCTCAACAGGTAGTCTCTTTGCT
                  ******************************** *** **********************

WT    -157        TTTTGCCGCCCTGCTTCCCCTGAAGAGTCGAAAGCTTTTTCCAGCTGAGAAGTCACTTGT
SD                TTTTGCCGCCCTGCT--CCCTGAAGAGTCGAAAGCTTTTTCCAGCTGAGAAGTCACTTGT
NT                TTTTGCCGCCCTGCTTCCCCTGAAGAGTCGAAAGCTTTTTCCAGCTGAGAAGTCACTTGT
                  ***************  ****************************************

WT     -97        TGATTTGCTGGAAGAGCAGCAGAGTCCAGAGAAAAAGACTCGGTTGTTTGTAGCGCTTAT
SD                TGATTTGCTGGAAGAGCAGCAGAGTCCAGAGAAAAAGACTCGGTTGTTTGTAGCGCTTAT
NT                TGATTTGCTGGAAGAGCAGCAGAGTCCAGAGAAAAAGACTCGGTTGTTTGTAGCGCTTAT
                  ************************************************************

WT     -37        CGCGGGTTCTACGTCTATATAAGACCGATGCTCATGCAGTCGAATTGTACAGTTTGGAAG
SD                CGCGGGTTCTACGTCTATATAAGACCGATGCTCATGCAGTCGAATTGTACAGTTTGGAAG
NT                CGCGGGTTCTACGTCTATATAAGACCGATGCTCATGCAGTCGAATTGTACAGTTTGGAAG
                  ************************************************************

WT      24        GTGTGCATCGAGTAGTTCGCAAATATTTCACCACTTTTAGAAATTGTCGATTTTCAATAC
SD                GTGTGCATCGAGTAGTTCGCAAATATTTCACCACTTTTAGAAATTGTCGATTTTCAATAC
NT                GTGTGCATCGAGTAGTTCGCAAATATTTCACCACTTTTAGAAATTGTCGATTTTCAATAC
                  ************************************************************

WT      84        CAAAAGTTAGACTAGAGATTCTCAATATGTGGACCATTCTCCTGACGATCCTAATCGCGG
SD                CAAAAGTTAGACTAGAGATTCTCGATATGTGGACCATTCTCCTGACGATCCTAATCGCGG
NT                CAAAAGTTAGACTAGAGATTCTCAATATGTGGACCATTCTCCTGACGATCCTAATCGCGG
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WT     144        GTCTGCTGTACAGATATGTGAAGCGGCACTACACTCACTGGCAGCGATTGGGAGTCGATG
SD                GTCTGCTGTACAGATATGTGAAGCGGCACTACACTCACTGGCAGCGATTGGGAGTCGATG
NT                GTCTGCTGTACAGATATGTGAAGCGGCACTACACTCACTGGCAGCGATTGGGAGTCGATG
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WT     204        AGGAGCCGGCAAAGATTCCCTTTGGCGTAATGGATACTGTCATGAAGCAGGAACGAAGTT
SD                AGGAGCCGGCAAAGATTCCCTTTGGCGTAATGGATACTGTCATGAAGCAGGAACGAAGTT
NT                AGGAGCCGGCAAAGATTCCCTTTGGCGTAATGGATACTGTCATGAAGCAGGAACGAAGTT
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WT     264        TGGGCATGGCTCTGGCTGATATATACGCCCGGCACGAGGGCAAAATCGTTGGCATATACA
SD                TGGGCATGGCTCTGGCTGATATATACGCCCGGCACGAGGGCAAAATCGTTGGCATATACA
NT                TGGGCATGGCTCTGGCTGATATATACGCCCGGCACGAGGGCAAAATCGTTGGCATATACA
                  ************************************************************

WT     324        TGTTGAACAAACGAAGTATCCTGATCCGAGATGCCCAATTGGCTCGCCAAATCATGACCA
SD                TGTTGAACAAACGAAGTATCCTGATCCGAGATGCCCAATTGGCTCGCCAAATCATGACCA
NT                TGTTGAACAAACGAAGTATCCTGATCCGAGATGCCCAATTGGCTCGCCAAATCATGACCA
                  ************************************************************

WT     384        GCGATTTCGCCAGTTTCCACGATCGCGGGGTGTACGTGGACGAGGATAAGGACCCGTTGT
SD                GCGATTTCGCCAGTTTCCACGATCGCGGGGTGTACGTGGACGAGGATAAGGACCCGTTGT
NT                GCGATTTCGCCAGTTTCCACGATCGCGGGGTGTACGTGGACGAGGATAAGGACCCGTTGT
                  ************************************************************

WT     444        CGGCCAACCTTTTTAACCTAAGAGGTGCTTCATGGCGAAATCTACGTCAGAAACTTACGC
SD                CGGCCAACCTTTTTAACCTAAGAGGTGCTTCATGGCGAAATCTACGTCAGAAACTTACGC
NT                CGGCCAACCTTTTTAACCTAAGAGGTGCTTCATGGCGAAATCTACGTCAGAAACTTACGC
                  ************************************************************

WT     504        CGTCGTTCTCCTCCGGCAAAATTAAGGGCATGTTCGGCACCATCGACGACGTGGGCGATA
SD                CGTCGTTCTCCTCCGGCAAAATTAAGGGCATGTTCGGCACCATCGACGACGTGGGCGATA
NT                CGTCGTTCTCCTCCGGCAAAATTAAGGGCATGTTCGGCACCATCGACGACGTGGGCGATA
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WT      564          AGTTGGTGCAGCATTTGGAGGGGGCCCTTGACCAAAGCGATGAAGTGGAGATCAAGGATG
SD                   AGTTGGTGCAGCATTTGGAGGGGGCCCTTGACCAAAGCGATGAAGTGGAGATCAAGGATG
NT                   AGTTGGTGCAGCATTTGGAGGGGGCCCTTGACCAAAGCGATGAAGTGGAGATCAAGGATG
                     ************************************************************

WT      624          TGATGACCACGTATGCGGTGGACATCATTGGGTCAGTCATTTTTGGCTTGGAGATAGACA
SD                   TGATGACCACGTATGCGGTGGACATCATTGGGTCAGTCATTTTTGGCTTGGAGATAGACA
NT                   TGATGACCACGTATGCGGTGGACATCATTGGGTCAGTCATTTTTGGCTTGGAGATAGACA
                     ************************************************************

WT      684          GCTTCCGAAATCCAAAAAACGAGTTCCGCGAGATCAGCAGCTCTACGTCTAGGGATGAAT
SD                   GCTTCCGAAATCCAAAAAACGAGTTCCGCGAGATCAGCAGCTCTACGTCTAGGGATGAAT
NT                   GCTTCCGAAATCCAAAAAACGAGTTCCGCGAGATCAGCAGCTCTACGTCTAGGGATGAAT
                     ************************************************************

WT      744          CTCTGCTGCTGAAGATTCACAATATGTCCATGTTCATTTGTCCACCgtgagttcaaaaat
SD                   CTCTGCTGCTGAAGATTCACAATATGTCCATGTTCATTTGTCCACCgtgagttcaaaaat
NT                   CTCTGCTGCTGAAGATTCACAATATGTCCATGTTCATTTGTCCACCgtgagttcaaaaat
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WT      804          aacccagggtatatatttgggaatctttatttctatatttataactaaattttgtacagG
SD                   aacccagggtatatatttgggaatctttatttctatatttataactaaattttgtacagG
NT                   aacccagggtatatatttgggaatctttatttctatatttataactaaattttgtacagG
                     ************************************************************

WT      864          ATTGCTAAGCTAATGAACCGCTTGGGCTACGAAAGCCGCATTCTGACATCCCTGCGAGAC
SD                   ATTGCTAAGCTAATGAACCGCTTGGGCTACGAAAGCCGCATTCTGACATCCCTGCGAGAC
NT                   ATTGCTAAGCTAATGAACCGCTTGGGCTACGAAAGCCGCATTCTGACATCCCTGCGAGAC
                     ************************************************************

WT      924          ATGATGAAGCGCACAATCGAGTTCCGGGAGGAGCACAACGTGGTGCGAAAGGACATGCTG
SD                   ATGATGAAGCGCACAATCGAGTTCCGGGAGGAGCACAACGTGGTGCGAAAGGACATGCTG
NT                   ATGATGAAGCGCACAATCGAGTTCCGGGAGGAGCACAACGTGGTGCGAAAGGACATGCTG
                     ************************************************************

WT      984          CAGCTGCTGATCCGCCTCCGAAATACGGGCAAGATTGGCGAGGACGACGACCAGGTGTGG
SD                   CAGCTGCTGATCCGCCTCCGAAATACGGGCAAGATTGGCGAGGACGACGACCAGGTGTGG
NT                   CAGCTGCTGATCCGCCTCCGAAATACGGGCAAGATTGGCGAGGACGACGACCAGGTGTGG
                     ************************************************************

WT     1044          GACATGGAAACCGCACAGGAGCAGCTGAAGTCTATGTCGATCGAGAAGATTGCCGCCCAG
SD                   GACATGGAAACCGCACAGGAGCAGCTGAAGTCTATGTCGATCGAGAAGATTGCCGCCCAG
NT                   GACATGGAAACCGCACAGGAGCAGCTGAAGTCTATGTCGATCGAGAAGATTGCCGCCCAG
                     ************************************************************

WT     1104          GCATTCCTGTTCTACGTGGCGGGCTCGGAATCCACAGCTGCGGCGTCTGCCTTCACTCTC
SD                   GCATTCCTGTTCTACGTGGCGGGCTCGGAATCCACAGCTGCGGCGTCTGCCTTCACTCTC
NT                   GCATTCCTGTTCTACGTGGCGGGCTCGGAATCCACAGCTGCGGCGTCTGCCTTCACTCTC
                     ************************************************************

WT     1164          TACGAGCTGTCCATGTATCCGGAACTGCTGAAGGAGGCGCAAGAAGAAGTGGATGCTGTG
SD                   TACGAGCTGTCCATGTATCCGGAACTGCTGAAGGAGGCGCAAGAAGAAGTGGATGCTGTG
NT                   TACGAGCTGTCCATGTATCCGGAACTGCTGAAGGAGGCGCAAGAAGAAGTGGATGCTGTG
                     ************************************************************

WT     1224          CTGATGAAGCACAATCTGAAGCCCAAGGATAGGTTCACCTACGAGGCGGTGCAGGACTTA
SD                   CTGATGAAGCACAATCTGAAGCCCAAGGATAGGTTCACCTACGAGGCGGTGCAGGACTTA
NT                   CTGATGAAGCACAATCTGAAGCCCAAGGATAGGTTCACCTACGAGGCGGTGCAGGACTTA
                     ************************************************************

WT     1284          AAGTTTCTGGACATCTGCATCATGGgtgggtttcacatagcattcagtttcaacgatcct
SD                   AAGTTTCTGGACATCTGCATCATGGgtgggtttcacatagcattcagtttcaacgatcct
NT                   AAGTTTCTGGACATCTGCATCATGGgtgggtttcacatagcattcagttttaacgatcct
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WT     1344          tagttaaacgtatttttgcagAAACCATTCGCAAATACCCCGGGCTACCATTCCTCAATC
SD                   tagttaaacgtatttttgcagAAACCATTCGCAAATACCCCGGGCTACCATTCCTCAATC
NT                   tagttaaacgtatatttgcagAAACCATTCGCAAATACCCCGGGCTACCATTCCTCAATC
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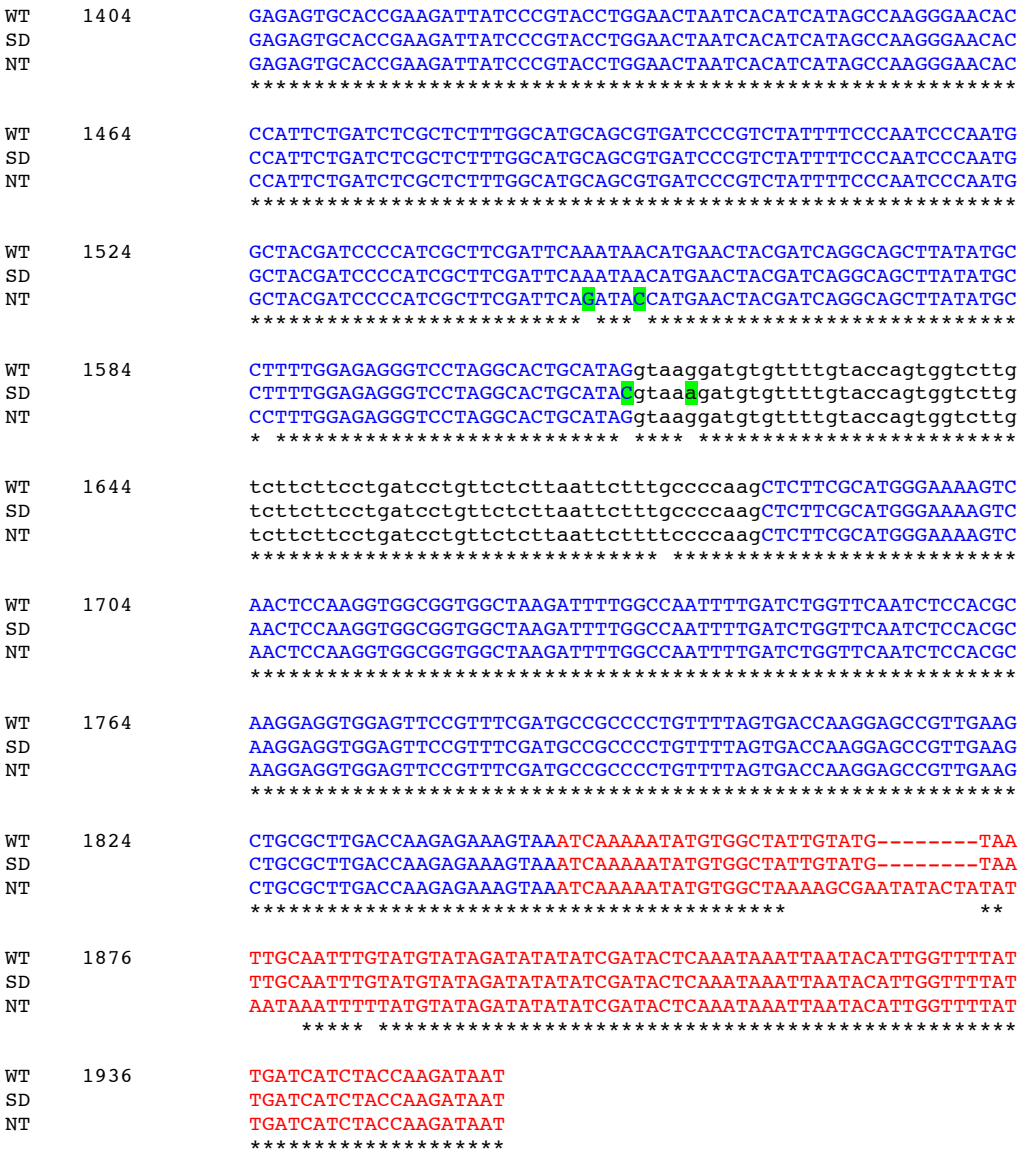

Figure 3-6: Nucleotide sequence of *Cyp6d2* alleles. Nucleotide positions relative to *Cyp6d2* transcription start site are indicated. Red: untranslated regions, Blue: protein coding regions, Black uppercase: upstream intergenic region, Black lowercase: introns. Asterisk below sequence indicates all three alleles are identical at this position. Highlighted SNPs predicted to change amino acid sequence. Two stocks for each allele were sequenced to confirm changes: WT (*P{SUPor-P}KG06046*, *P{SUPor-P}KG01596*), SD (*P{SUPor-P}KG02287*, *P{SUPor-P}KG06675*), NT (*Mi{ET1}CG3746*, *P{GT1}CG42565*). Alignment performed using Clustal Omega (Sievers et al. 2011) and boundaries are as defined by FlyBase.

Cyp6d2 polymorphisms in wild-type Drosophila populations:

Interestingly, the amino acid changes in the *cyp6d2*^{NT} mutant are also found in many other Drosophila species. When we aligned the *D. melanogaster* Cyp6d2 protein with its closest homologs in ten other Drosophila species (Clark et al. 2007), we found that all ten had the first asparagine changed to an aspartic acid, and three others also had the second asparagine changed to a threonine (Figure 3-7). The rest of the protein appeared to be very well conserved among these eleven species. We tested *D. simulans* for sensitivity to camptothecin but found that they were not unusually sensitive to the drug (data not shown).

D. melanogaster	421	PVYFPNPNGYDPHRFDSNNMNYDQAAYMPFGEGPRHCIALRMGKVNSKVAVAKILANFDL	480
D. erectus	421	PGYFPNPNGYDPHRFDADTMNYDQAAYMPFGEGPRHCIALRMGKVNSKVAVAKVLANFDL	480
D. simulans	408	PVYFPNPNGYDPHRFDSDTMNYDQAAYMPFGEGPRHCIALRMGKVNSKVAVAKILANFDL	467
D. ananassae	421	PIYFPNPNDYDPHRFDADIMNYNPAAYMPFGEGPRHCIALRMGKINSKVAVAKVLANFNV	480
D. persimilis	425	PVYFPNPEGYDPHRFDADNMNYDQAAYMPFGEGPRHCIALRMGEVNTKVAVAKILANFDL	484
D. pseudoobscura	425	PVYFPNPEGYDPHRFDADKMNYDQAAYMPFGEGPRHCIALRMGEVNTKVAVAKILANFDL	484
D. yakuba	349	PAYFPNPNGYDPHRFDADTMNYDQAAYMPFGEGPRHCIALRMGKVNSKVAVAKVLANFDL	408
D. virilis	423	PSYFPNPEGYDPHRFDADVMNYDQTAYMPFGEGPRHCIALRMGKVNAKVAVAKILANFNL	482
D. willistoni	423	PAYFPNPVDYDPHRFDVDNMNYDQAAYMPFGEGPRHCIALRMGKVNSKVAVAKILANFDV	482
D. grimshawi	423	PAYFPNPMDYDPHRYDADNMNYDPTAYMPFGEGPRHCIALRMGKVNAKVAVAKVLANFNL	482
D. mojavensis	423	AKYFPNPEDYGPHRFDADNMNYNQTAYMPFGEGPRHCIAIRMGKVNAKVAVAKVLLNFDL	482

Figure 3-7: Amino acid sequences of Cyp6d2 homologs in ten other Drosophila species. Amino acid changes shown in red match those associated with the *cyp6d2*^{NT} allele.

We also surveyed *Cyp6d2* sequences from wild-type *D. melanogaster* flies from the Drosophila Genetic Reference Panel (Mackay et al. 2012). 24% of the sequences we scanned carried the N438D, N439T polymorphisms associated with *cyp6d2*^{NT}, while the rest matched the *D. melanogaster* sequence on Flybase at this locus. We did not find the *cyp6d2*^{SD} polymorphisms in other *Drosophila* species or Reference Panel strains of *D. melanogaster*.

The *brca2*^{47} phenotype results from defects in both homologous recombination and Cyp6d2: We reasoned that the *brca2*^{47} mutant could be especially sensitive to camptothecin for two reasons: (1) it is unable to repair

DSBs by homologous recombination and (2) it carries the *cyp6d2*^{SD} mutation. To test this, we attempted to rescue the sensitivity phenotype of the *brca2*^{47} mutant with the wild-type *Cyp6d2* construct. Importantly, the construct provided only a partial rescue of the sensitivity (Figure 3-4C). Interestingly, the sensitivity of the Cyp6d2-rescued *brca2*^{47} mutants is similar to the *brca2*^{KO} stock (Figure 3-1A). These findings demonstrate that the *brca2*^{47} mutant phenotype results from two mutations and strongly suggest that the rescue of the *cyp6d2*^{SD} and *cyp6d2*^{NT} mutant phenotypes by a wild-type copy of Cyp6d2 is not simply the result of overexpressing a detoxification gene.

Discussion

In this study, we have shown that many Drosophila stocks carry mutations in *Cyp6d2* that render the flies hypersensitive to the drug camptothecin. We hypothesize that Cyp6d2 is a critical enzyme required for the breakdown and/or removal of camptothecin. In its absence, camptothecin levels remain high and cause significant cell death, most likely through the creation of DSBs. When combined with a mutation in the double strand break (DSB) repair gene, *Brca2*, we observed a synergistic effect. Therefore, we hypothesize that at low doses of camptothecin, the resulting DSBs can be repaired through Brca2-dependent homologous recombination (Figure 3-1A).

These variants of *Cyp6d2* illustrate a potential pitfall in the study of DNA repair genes in Drosophila. It is likely that many genes involved in detoxification or removal of harmful compounds are polymorphic. Such polymorphisms may skew drug sensitivity screens such that the results are misinterpreted. In an

attempt to understand the role of HR proteins in one-ended DSB repair, we used camptothecin sensitivity assays to characterize different DNA repair mutants. We were initially misled to believe that *brca2* mutants were exceptionally sensitive to camptothecin, more so than *rad51* mutants (Figure 2-3A), suggesting a function for Drosophila Brca2 in camptothecin-induced damage repair outside of its well-defined role in homologous recombination. However, our results here, along with the observation that *rad51* and *brca2* mutants display similar sensitivities to topotecan (Figure 2-3B), suggest that this is not the case.

Collectively, the sequences from the Drosophila Genetic Reference Panel (DGRC; Mackay et al. 2012) and other Drosophila species (Clark et al. 2007) suggest that the N438D, N439T polymorphisms in the *cyp6d2*^{NT} mutant do not cause the phenotype we observe. Rather, they are closely linked to a mutation that results in a defective transcript, and the subsequent absence of Cyp6d2 protein results in camptothecin sensitivity. We speculate that the altered 3' UTR in the *cyp6d2*^{NT} mutant results in an unstable transcript. Notably, the 8 bp insertion in the 3' UTR of the *cyp6d2*^{NT} mutant (Figure 3-6) is not present in the DGRC stocks that have the N438D, N439T polymorphisms.

Cytochrome P450s and detoxification of xenobiotics: The cytochrome P450 superfamily, of which Drosophila *Cyp6d2* is a member, is a group of metabolic enzymes with an unusually wide range of substrates (Coon et al. 1992). They are conserved throughout evolution (Coon et al. 1996) and are involved in the metabolism of many endogenous and exogenous compounds. P450 enzymes catalyze the addition of oxygen to a substrate via a heme cofactor (Coon et al.

1992; Hollenberg 1992). The additional oxygen atom may alter the stability of the substrate, leading to other molecular rearrangements (Bergé et al. 1998; Coon et al. 1996), or it may trigger conjugation by enzymes such as glutathione-S-transferases (reviewed in Tu and Akgül 2005). These processes lead to the detoxification and/or excretion of harmful compounds.

P450 activity is critically dependent on the heme ligand, which mediates the electron transfer reactions that ultimately lead to a modified substrate (Hollenberg 1992). The heme group is bound to the protein via a cysteine residue near its C-terminus and surrounded by conserved sequence (Tijet et al. 2001). Notably, the *cyp6d2*^{SD} mutation is located very close to the heme-binding cysteine in Cyp6d2 (Figure 3-3B). In addition to altering the C-terminus of the protein sequence, this mutation also results in structural changes very close to the heme-binding cysteine. Sequence analysis of P450 enzymes from many different organisms shows that a glycine or alanine is almost always found in the mutated coding position. Since the *cyp6d2*^{SD} mutant appears slightly more resistant to camptothecin than the *cyp6d2*^{NT} mutant (Figure 3-4A-B), we hypothesize that *cyp6d2*^{SD}, which still produces transcript, is a severe hypomorph.

Potential roles of Drosophila Cyp6d2: In Drosophila, chemical detoxification occurs in the Malpighian tubules, midgut and larval fat body. Consistent with a role in detoxification, the organ with the highest level of expression of *Cyp6d2* is the larval fat body (Yang et al. 2007; Chintapalli et al. 2007). Temporally, the highest expression levels of Cyp6d2 occur during the larval and prepupal stages (Chintapalli et al. 2007), which coincide with the days

immediately following treatment in our sensitivity assays. *Cyp6d2* expression was very high in hyperoxic conditions (Gruenewald et al. 2009), but it was not identified in a survey of multiple xenobiotic and insecticide screens (Giraudo et al. 2010). Therefore, its preferred substrates are unknown.

There are 83 functional P450 genes in the Drosophila genome, including 22 from the insect-specific *CYP6* family (Tijet et al. 2001). Considering that P450 enzymes frequently have overlapping substrate specificity (Hollenberg 1992; Coon et al. 1992), it is reasonable to expect that another P450 enzyme could detoxify camptothecin in the absence of Cyp6d2. However, our data suggest that Cyp6d2 provides a non-redundant function in camptothecin detoxification. It is possible that camptothecin removal or breakdown could also involve multiple steps, with additional enzymes mediating other reactions. In this case, at least one of the reactions must require Cyp6d2.

The fact that *scpt* mutants are sensitive to camptothecin but not topotecan was an unexpected result. We propose three hypotheses to explain this. First, camptothecin may have alternate cytotoxicity separate from its inhibition of Top1. The initial discovery and chemical characterization of camptothecin was promising, but clinical trials were abandoned after issues of toxicity arose (Wall and Wani 1995; Legarza and Yang 2006). Later work prompted the development of topotecan and irinotecan (Kunimoto et al. 1987; Mattern et al. 1991). The camptothecin sensitivity we observed in fruit flies could be caused by the same mechanisms that resulted in the side effects observed in human clinical trials. This

would suggest that Cyp6d2-mediated breakdown or removal of camptothecin prevents this toxicity from reaching lethal levels in flies.

Alternatively, a second hypothesis is that the active site of Cyp6d2 can accommodate camptothecin but not topotecan. The most likely explanation for such specificity would be the chemical properties of the two drugs. Camptothecin is a lipophilic molecule, while topotecan is hydrophilic. The human P450 enzymes most critical for drug detoxification, including Cyp3a4, tend to favor lipophilic substrates (Smith et al. 1997). Despite being structurally similar, the water soluble topotecan (Pommier 2009) may not be a suitable substrate for Cyp6d2, which would explain why *cyp6d2* mutants are not sensitive to the drug.

Our third hypothesis posits that lipophilic camptothecin may accumulate in the fat body (Zijlstra and Vogel 1988), which functions analogously to the liver in fruit flies and other insects (Buchon et al. 2009; Yang et al. 2007). In contrast, topotecan, being hydrophilic, may be more easily excreted, resulting in a shorter time of exposure to the drug. The observation that Cyp6d2 is highly expressed in the fat body (Yang et al. 2007), supports this model.

All of these hypotheses assume that Cyp6d2 is involved in camptothecin detoxification, but other possible explanations may exist. Recently, Cyp6d2 was shown to be upregulated 18 hours after ionizing radiation treatment, independent of p53 (van Bergeijk et al. 2012). This suggests that Cyp6d2 may have a role in p53-independent apoptosis. A defective apoptotic pathway could impair imaginal disc development in larvae, leading to the adult lethality that we observe.

Conclusions and future implications: Drosophila provides an excellent *in vivo* system to study the effects of DNA repair mutations and mutagens on genome stability and survival. Nevertheless, our studies suggest caution when using Drosophila as a tool for drug screening. Care must be taken in any analysis of phenotypes because of the possibility that genetic background effects, such as the *cyp6d2* mutation, could be influencing the results.

Our study also has implications for the development of cancer chemotherapeutics. P450 enzymes and the polymorphisms that exist within them pose significant challenges in drug development (Guengerich 1999; Maekawa et al. 2010; Plant 2007). Point mutations in Drosophila P450 genes have previously been shown to dramatically affect drug detoxification (Amichot et al. 2004), suggesting fruit flies may be a valuable model for this research. Since many cancer chemotherapeutics damage DNA or target defective DNA repair mechanisms (Ferguson and Pearson 1996; Lawley and Phillips 1996), studying these drugs in Drosophila allows us to evaluate the influences of both detoxification and repair enzymes simultaneously.

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Chapter 4
Drosophila melanogaster HelQ functions in homologous recombination repair of mitotic DNA double-strand breaks

Author Contributions

Generation of *mus301*^{288A} mutant: Mitch McVey

***mus301*^{288A}, *spn-B*^{1} double mutant viability; nitrogen mustard sensitivity of *mus301*^{288A} and *mus308*^{2003} single mutants and *mus301*^{288A}, *mus308*^{2003} double mutants (Figure 4-1A-B): Brenda French**

Topotecan sensitivity of *mus301*^{288A} single mutants (Figure 4-2): Carrie Hui

Abstract

Homologous recombination (HR) is a template driven mechanism of repairing DNA double strand breaks (DSBs). Invasion into homologous templates requires the recombinase Rad51/RecA and the action of multiple helicases. While the early and late stages of HR are well characterized, the exact mechanisms of the intermediate steps are less clear. A subset of the HR pathway that favors noncrossover products is known as synthesis-dependent strand annealing (SDSA). One protein that may act to promote SDSA during the intermediate steps of HR is HelQ. Here, we show that Drosophila HelQ, a helicase homologous to archaeal and mammalian HEL308, is involved in somatic HR repair of DSBs and interstrand crosslinks. We found that *mus301* (DmHelQ) mutants were phenotypically similar to *spn-A* (DmRad51) mutants when treated with chemical mutagens, suggesting both are critical for HR repair. Additionally, *mus301* and *mus309* (DmBlm) mutants behave similarly in a double stranded gap repair assay. An important exception to this is the lack of flanking deletions in *mus301* mutants. Together, our data are consistent with a model by which DmBlm and DmHelQ promote accurate SDSA repair of DSBs via different mechanisms.

Introduction

The genome is continuously assaulted by various endogenous and exogenous sources of damage. A wide variety of proteins have evolved to repair or bypass this damage, and defects in these proteins are linked to many genetic diseases, including cancer. Understanding the mechanisms and genetic

relationships in DNA repair can lead to the development of promising therapeutics for these diseases.

The most dangerous type of DNA damage is the double strand break (DSB), because it can lead to genomic rearrangements or large-scale loss of genetic information, both of which are carcinogenic. DSB repair is usually divided into two main classes: homologous recombination (HR) and end joining (EJ) (Reviewed in Pardo et al. 2009). HR is traditionally believed to be error-free, while EJ is more error-prone, especially the non-canonical types such as microhomology-mediated end joining (Reviewed in Weterings and Chen 2008; Mladenov and Iliakis 2011). HR repair derives its accuracy from the use of an intact homologous template, such as a homologous chromosome or, more often, a sister chromatid (Reviewed in Krejci et al. 2012).

Synthesis-dependent strand annealing: One mechanism of HR repair is synthesis-dependent strand annealing (SDSA) (Nassif et al. 1994), which differs from the canonical double-strand break repair (DSBR) model (Szostak et al. 1983) because it suppresses the formation of crossovers. While SDSA can result in extensive gene conversion (Pâques and Haber 1999; Mladenov and Iliakis 2011), it avoids crossover events because the invading 3' end is unwound before the displaced template strand is engaged by the opposite 3' end. Both 3' ends flanking a DSB can initiate invasion at separate locations, displacing opposite strands. If both ends simultaneously engage the same unwound template for repair, however, two Holliday Junctions (HJs) are formed, which can lead to crossover formation (Reviewed in Krejci et al. 2012; Hartlerode and Scully 2009).

To suppress crossing over in somatic cells, therefore, several proteins, including multiple helicases, can promote SDSA. One such helicase in yeast is Srs2, which was shown to counteract Rad51-mediated recombination (Milne et al. 1995). Srs2 can disassemble Rad51-bound ssDNA (Veaute et al. 2003; Krejci et al. 2003), which suppresses inappropriate recombination events prior to D-loop formation. Srs2 has also been shown to unwind D-loops *in vitro*, which promotes SDSA (Dupaigne et al. 2008).

Other proteins are proposed to act specifically downstream of strand invasion. RTEL/RTEL-1, originally identified as a *r*egulator of *te*lomere *l*ength in mice (Ding et al. 2004), was later shown to function in suppression of crossovers during HR in *C. elegans* (Barber et al. 2008; Youds et al. 2010). Also involved in D-loop unwinding is the Bloom helicase (BLM), mutations in which cause Bloom's syndrome in humans (Karow et al. 2000). In Drosophila, Blm was shown to be critical for SDSA repair of DSBs (Adams et al. 2003), where it displaces the invading DNA strand (McVey et al. 2004b). This suppresses mitotic crossovers during homologous recombination repair by favoring SDSA (McVey et al. 2007; Johnson-Schlitz and Engels 2006).

BLM is also known to function further downstream during HR by suppressing crossovers during Holliday junction (HJ) resolution. Cleavage of HJs can lead to crossover events (Karpenshif and Bernstein 2012). BLM can suppress these crossovers as part of the Bloom dissolvasome, or BTR complex, which also includes topoisomerase IIIα (Reviewed in Manthei and Keck 2013). The proposed model for the suppression of crossovers entails migration of two HJs toward one

another by Bloom, and subsequent decatenation by topoisomerase IIIα (Cheok et al. 2005). Metaphase spreads of Bloom's syndrome patients show severely intertwined chromosomes (Wechsler et al. 2012), suggestive of this role in crossover suppression.

The Drosophila homologs of BLM and other helicases that function during repair enable genetic analysis in this model system. Several proteins with helicase domains, including DmBlm, are encoded by a series of genes initially characterized in a screen of Drosophila 3rd chromosome mutagen sensitive (*mus*) mutations (Boyd et al. 1981). While DmBlm (*mus309*) has been well characterized (Kusano et al. 1999; McVey et al. 2004b; 2007; Johnson-Schlitz and Engels 2006), the role of DmHelQ (*mus301*) is less defined.

HelQ (Hel308): Human HEL308 was originally identified after scanning the human genome for homologs of Drosophila *mus308*, which contains a helicase-like and polymerase domain (Marini and Wood 2002; Harris et al. 1996). Because of its homology to the helicase domain of human POLQ, it is also known as HelQ. The *C. elegans* HELQ-1/HEL-308 protein was shown to function in a pathway of ICL repair functionally separate from POLQ-1, the *C. elegans* homolog of Drosophila Polθ (Muzzini et al. 2008). These data suggested that HELQ-1 functioned in the Fanconi Anemia (FA) pathway of ICL repair, while POLQ-1 operated in a pathway dependent on BRC-1 (*C. elegans* BRCA1). Further work showed that purified HELQ-1 or RFS-1 (a Rad51 paralog), is sufficient to remove Rad51 protein from dsDNA, but not ssDNA (Ward et al. 2010).

Several archaeal Hel308 homologs have been identified and have provided insight into the activity of the eukaryotic protein. In the archaeon, *Methanothermobacter thermautotrophicus*, Hel308 interacts directly with Replication Protein A (RPA) (Woodman et al. 2011), which binds free ssDNA. This suggested that RPA recruits Hel308 to stalled replication forks. Similarly, the unwinding activity of human HEL308 is stimulated by human RPA, but not bacterial SSB (the prokaryotic homolog of RPA), implying a specific protein interaction rather than the protection of unwound ssDNA. Furthermore, the protein prefers stalled fork-like structures with a nascent lagging strand and no nascent leading strand. This suggests a model whereby stalled forks with uncoupled polymerases recruit HEL308 for unwinding, facilitating the binding of additional repair proteins (Tafel et al. 2011).

Drosophila Hel308/HelQ is encoded by the gene *mus301*/*spn-C*, mutations in which result in ventralized embryos (González-Reyes et al. 1997). *mus301* point mutants were shown to have specific defects during meiotic recombination (McCaffrey et al. 2006). This explains the ventralized embryos, since persistent meiotic DSBs activate a checkpoint that prevents proper dorsoventral patterning (Ghabrial and Schupbach 1999). These *mus301* point mutants are also sensitive to X-rays (Oliveri et al. 1990), interstrand crosslinks (Boyd et al. 1981) and DNA alkylation (McCaffrey et al. 2006; Boyd et al. 1981), indicative of a general DSB repair defect and not one specific to meiosis.

In vitro studies of HelQ suggest a role in removing DNA and/or proteins at stalled replication forks, but it is unclear whether this represents an *in vivo*

function. *In vivo* studies in Drosophila provided additional clues, but these experiments used EMS-generated point mutants that may have retained some functionality. We isolated a large deletion in *mus301*, which we identify as *mus301*^{288A}. We expect that this allele is a functional null mutant because all of the canonical helicase domains are absent. We characterized the *in vivo* repair defects of *mus301*^{288A} mutants through mutagen sensitivity assays and a gap repair assay. Our results suggest that DmHelQ facilitates error-free HR repair of DSBs induced by chemical mutagens or transposon excision. Furthermore, it shares some aspects of this role with DmBlm.

Materials and Methods

Generation of mutants: The *mus301*^{288A} mutant was created by imprecise excision of *P{SUPor-P}mus301*^{KG09098}, a *P* element inserted in the fourth intron of *mus301*. The excision results in a 2069 bp upstream deletion, removing the second, third and fourth exons entirely, as well as part of the first. Although the promoter, transcription and translation start sites are unaffected, all of the conserved helicase motifs are deleted. The first 202 amino acids (of total 1051) are intact.

The *mus309*^{N1} mutant is a null mutation created by imprecise excision of *P{EPgy2}Blm*^{EY03745} (McVey et al. 2007). The *mus308*^{2003} mutant is a null mutation generated by a premature stop codon (Koundakjian et al. 2004; Chan et al. 2010). The *spn-A* (DmRad51) mutants used in the mutagen sensitivity assays are also null mutations. *spn-A*^{093} is a nonsense mutation in the N-terminal portion

of the protein, while *spn-A*^{057} is a missense mutation in the central region of the protein (Staeva-Vieira et al. 2003).

P{w*^{a}*}* assay*: The *P{w*^{a}*}* construct contains a functional copy of the *white* gene disrupted by the *copia* retrotransposon (Kurkulos et al. 1994). Flies homozygous for the construct have apricot colored eyes as a result of the *copia* insertion, which impairs transcription of *white*. The entire *P{w*^{a}*}* construct is inserted into an intron of *scalloped*, an essential gene located on the *X* chromosome. Excision of *P{w*^{a}*}* leaves a 14 kb gap with non-complementary 17 nucleotide 3' overhangs. Repair events fall into three categories: complete (entire *P{w*^{a}*}* construct restored), homologous recombination (complete synthesis of *white*, annealing at *copia* LTRs), and aberrant repair (failure to initiate synthesis or synthesis aborted before reaching *copia* LTRs).

Mutant male flies carried (1) a single copy of the *P{w*^{a}*}* construct on the *X* chromosome, (2) a transposase (*H{w*$^{+}$*, Δ2-3}Hop2.1*) on chromosome *2,* and (3) deletions in *mus301*, *mus309* or both genes on chromosome *3*. They were mated to females that were homozygous for *P{w*^{a}*}* but otherwise wild-type. Repair events from the pre-meiotic male germline were recovered in the following generation of female flies. All female offspring inherited one wild-type maternal copy of *P{w*^{a}*}* and one paternal copy of *P{w*^{a}*}*. If the paternal copy was not excised or was repaired completely, the female offspring have two complete copies of *P{w*^{a}*}* and apricot eyes. The overwhelming majority of female offspring fell into this category, most likely because the transposon was not excised in these events. If the paternal copy was repaired with full synthesis of *white* and

annealing at the LTRs ("homologous recombination"), the female offspring have red eyes. If repair from either end of the break did not initiate or did not reach the LTRs ("aberrant repair"), the female offspring have faint yellow eyes resulting from the single maternal copy of $P\{w^a\}$. In this way, we could determine the relative frequency of homologous recombination and aberrant repair in the different mutant backgrounds. All mutant backgrounds were compared to a wild-type control, where males carried only the $P\{w^a\}$ transposon and $H\{w^+, \Delta 2\text{-}3\}Hop2.1$ transposase. Statistical analysis of these data was done using the Kruskal-Wallis test with Dunn's multiple comparisons post-test.

In addition to the relative frequencies of repair pathways, we also determined the extent of synthesis in the aberrant repair categories. We did this by recovering *white*$^-$ male progeny of the F_1 yellow-eyed females. These males must have inherited the aberrantly repaired $P\{w^a\}$ construct, and subsequent PCR analysis was used to determine the extent of repair synthesis for each event. Statistical significance was determined using Fisher's exact test.

Repair events occasionally resulted in flanking deletions, which included both lethal and non-lethal varieties. Deletions that extended into the coding region of *scalloped* resulted in female progeny with misshapen wings and/or an absence of *white*$^-$ male progeny in the following generation. Either of these phenotypes indicated a lethal flanking deletion during repair. Additionally, non-lethal deletions could remove some *scalloped* intron sequence but not extend into an exon. Analysis of small deletions was performed using a series of primers that

anneal to *scalloped* intron sequence. Statistical analysis of deletion frequencies was performed using Fisher's exact test.

Mutagen sensitivity assays: Sensitivity assays tested the ability of different fly mutants to survive various doses of mutagenic chemicals. Five to eight female flies heterozygous for the mutation of interest were crossed to three heterozygous mutant males. The females were allowed to lay eggs for three days and the parents were then moved into new vials for two additional days. The first set of vials was treated with mechlorethamine (Sigma) or topotecan (Enzo Life Sciences), while the second set was treated with vehicle (water). Between two and six replicates (each with five to ten individual vials) for each dose of mutagen were conducted. Percent survival to adulthood was calculated by comparing the percentage of homozygous adult offspring in treated vials to the percentage of homozygous adult offspring in untreated vials.

Results

mus301 mutants are sensitive to nitrogen mustard and topotecan:

Previous work showed that point mutations in *mus301* resulted in sensitivity to alkylating damage (McCaffrey et al. 2006; Boyd et al. 1981), interstrand crosslinks (ICLs) (Boyd et al. 1981) and X-rays (Oliveri et al. 1990). To better understand the function of DmHelQ in repair of mutagen-induced DNA damage, we generated a complete null allele of *mus301* via an imprecise excision screen. This allele, which we designated $mus301^{288A}$, removes all of the conserved helicase domains of the protein. We first sought to confirm the sensitivity of $mus301^{288A}$ flies to nitrogen mustard (mechlorethamine). The results showed that

our *mus301* null mutant larvae were sensitive to ICLs (Figure 4-1A), which corroborates previous work with *mus301* point mutants (Boyd et al. 1981). The sensitivity was similar to that of *spn-A* (DmRad51) mutants (Figure 4-1A), which suggests an epistatic relationship between the two genes.

HelQ is also known as Hel308 because its helicase domain was originally identified as similar to that of DmPolθ, encoded by *mus308* (Marini and Wood 2002). Drosophila Polθ is a large protein that contains a helicase-like domain and a polymerase domain joined by a flexible linker domain (Harris et al. 1996; Chan et al. 2010). Mutations in *mus308* result in sensitivity to interstrand crosslinking agents, but not to other chemical mutagens or ionizing radiation (Boyd et al. 1981; 1990; Chan et al. 2010). Since both *mus301* and *mus308* mutant flies are sensitive to interstrand crosslinking agents, and these genes code for related proteins, we investigated potential overlaps in their function. Flies with a null mutation in *mus308* (*mus308*^{2003}) are exceptionally sensitive to nitrogen mustard, and *mus301*^{288A}, *mus308*^{2003} double mutants show a synergistic effect when treated with this drug (Figure 4-1B). These data imply that DmPolθ and DmHelQ have distinct functions during ICL repair.

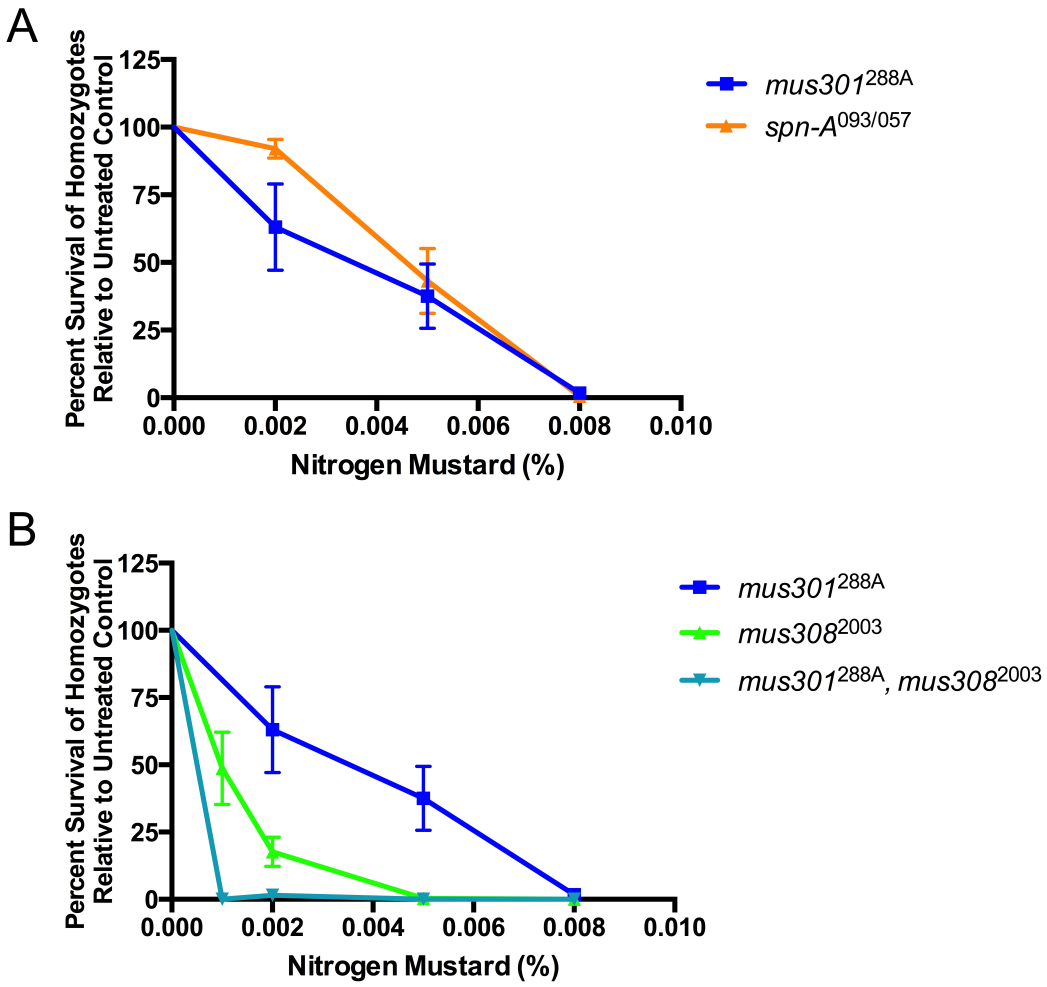


Figure 4-1: *mus301* mutants are sensitive to nitrogen mustard. (A) Nitrogen mustard sensitivity of *mus301* and *spn-A* (DmRad51) mutant flies. Data points consist of two to three trials of five to eight vials with average sensitivity and standard deviation shown. (B) Nitrogen mustard sensitivity of *mus301* and *mus308* single mutants, along with *mus301*, *mus308* double mutants. Data points consist of three to six trials of five to ten vials with average sensitivity and standard deviation shown.

We next tested the sensitivity of $mus301^{288A}$ flies to topotecan, a topoisomerase I inhibitor that generates one-ended DSBs in replicating cells. As such, these breaks cannot be repaired by an end-joining mechanism. This allowed us to look specifically at HR repair defects. $mus301^{288A}$ mutants showed sensitivity to topotecan comparable to or slightly greater than HR-deficient *spn-A*

mutant flies (Figure 4-2). This again suggests an epistatic relationship between the two genes for the repair of DSBs.

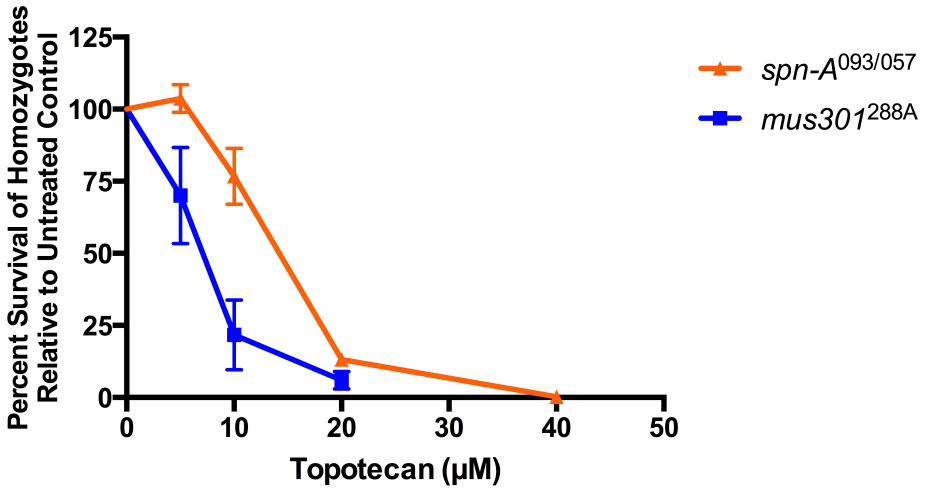
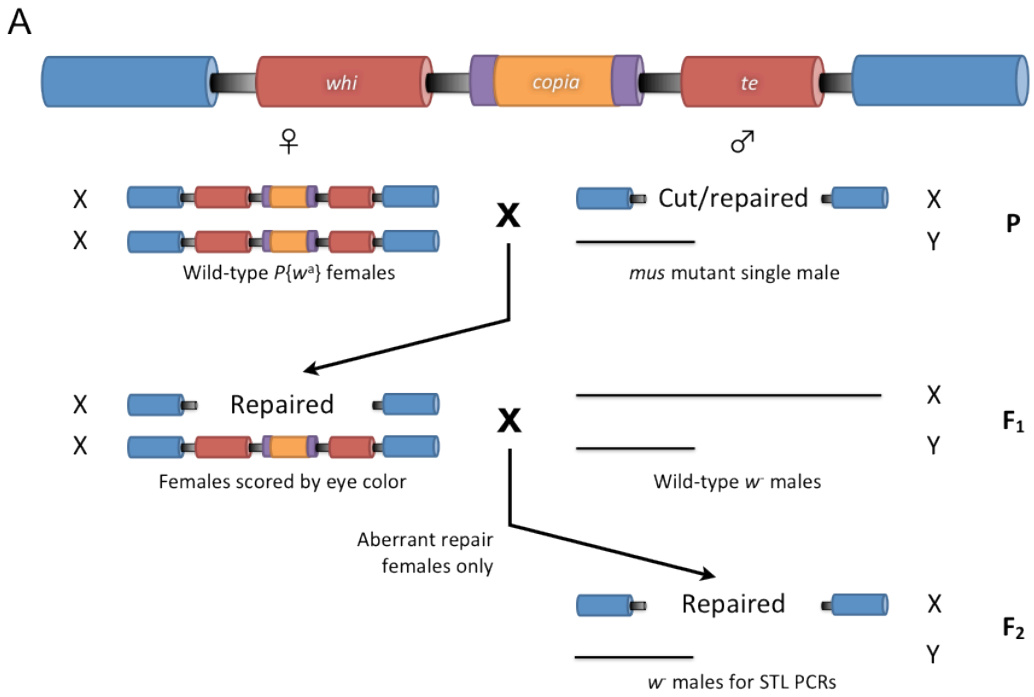
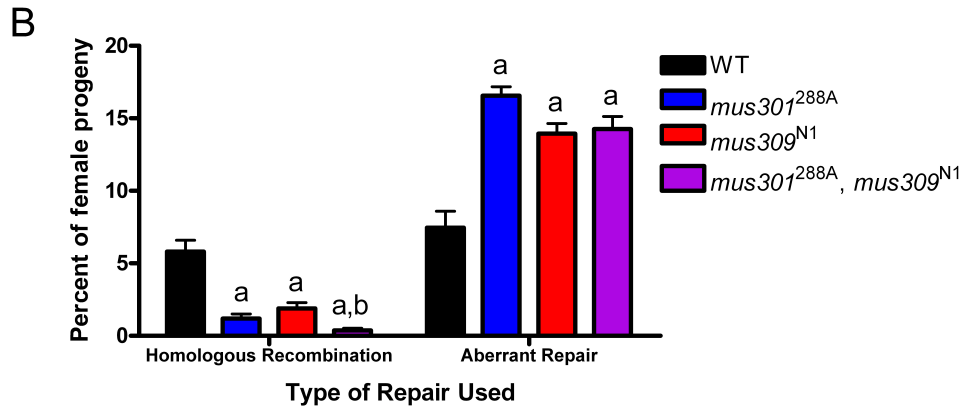


Figure 4-2: *mus301* and *spn-A* mutants are sensitive to topotecan. Data points consist of two to three trials of five to eight vials with average sensitivity and standard deviation shown.

***mus301* and *mus309* mutants are defective in double-strand gap repair:** Since the damage incurred from topotecan is purportedly occurring only during S-phase of the cell cycle, we wanted to see if DmHelQ was also important for repairing DSBs outside of replication. We tested *mus301* mutants using the $P\{w^a\}$ assay, previously described in (McVey 2010). This assay creates a 14 kb double stranded gap in the pre-meiotic male germline that must be repaired in G2 phase to ensure survival of progeny. The construct includes the complete *white* gene interrupted by the *copia* retrotransposon (Figure 4-3A, top). The presence of *copia* greatly reduces the expression of *white*, resulting in faint yellow eye pigment produced from one copy of $P\{w^a\}$. *P*-element excision followed by HR repair can result in deletion of *copia*, resulting in full expression of *white*, and red eye color. Frequency of homologous recombination repair and aberrant end joining repair can be measured via eye color of offspring.

With *mus301* deficient single males, repair by homologous recombination was significantly decreased and aberrant end joining repair significantly increased compared to wild-type (Figure 4-3B). To further understand the function of DmHelQ in double strand break repair, we analyzed the offspring of the female aberrant repair progeny (Figure 4-3A, bottom). White-eyed males from this generation inherited the aberrantly repaired X-chromosome from the yellow-eyed F_1 females. We analyzed the extent of repair synthesis by PCR at specific points along the $P\{w^a\}$ transposon. *mus301* deficient flies were defective at synthesis of 250 bp or longer, as compared to a wild-type control (Figure 4-3C).


A
whi
copia
te
♀
♂
X
X
Wild-type P{w^a} females
X
Cut/repaired
X
Y
mus mutant single male
P
X
Repaired
X
Females scored by eye color
X
X
Y
Wild-type w- males
F1
Aberrant repair females only
Repaired
X
Y
w- males for STL PCRs
F2


B
Percent of female progeny
20
15
10
5
0
a
a
a,b
a
a
a
Homologous Recombination
Aberrant Repair
Type of Repair Used
WT
mus301288A
mus309N1
mus301288A, mus309N1

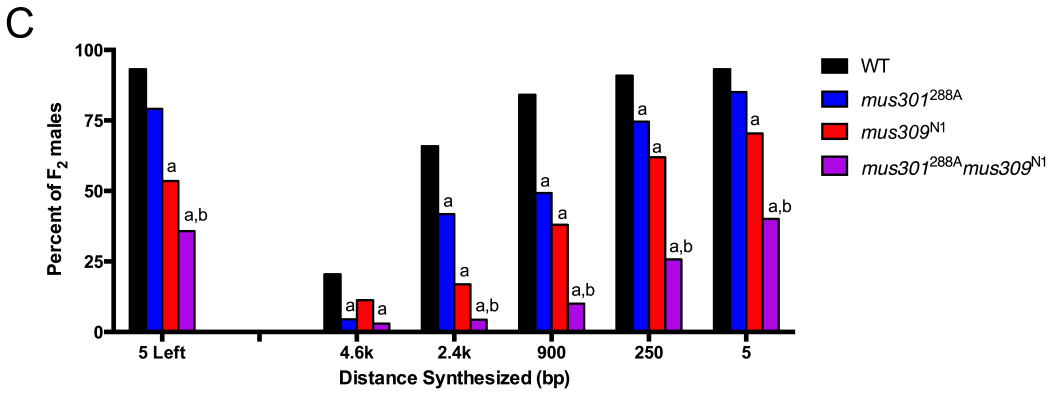

C
Percent of F2 males
100
75
50
25
0
a
a,b
a
a
a
a
a
a,b
a
a
a,b
a
a
a,b
a
a
a,b
5 Left
4.6k
2.4k
900
250
5
Distance Synthesized (bp)
WT
mus301288A
mus309N1
mus301288Amus309N1

Figure 4-3: *mus301* and *mus309* mutants are defective in HR repair of a double-stranded gap. (A) Diagram of fly generations used for analysis. (Top) A complete diagram of $P\{w^a\}$ construct, including *white* gene (red) split by *copia* retrotransposon (orange with purple long terminal repeats), inserted into an intron of *scalloped* (blue). F_1 females (middle left) were scored by eye color, shown in B. w^- F_2 males (bottom right) produced by aberrant repair females (yellow eyes) used for PCR analysis of STLs in C. (B) Frequency of repair pathway used in different genetic backgrounds. Mean frequencies of WT (n=50), $mus301^{288A}$ (n=188), $mus309^{N1}$ (n=166) and $mus301^{288A}$, $mus309^{N1}$ (n=102) shown with standard error of the mean. a: significantly different from WT, $P < 0.001$, b: significantly decreased from $mus309^{N1}$, $P < 0.05$; Kruskal-Wallis test with Dunn's multiple comparisons post-test. (C) Synthesis tract length analysis of both single mutants and the double mutant as compared to WT, using successful PCR at specified distance as a positive event. Percent out of 44 (WT), 67 ($mus301^{288A}$), 71 ($mus309^{N1}$) and 70 ($mus301^{288A}$, $mus309^{N1}$) individual males with successful PCR at specified distance shown. a: significantly decreased ($P < 0.05$) from wild-type, b: significantly decreased from both single mutants ($P < 0.05$); Fisher's exact test.

The decreased use of HR repair and shorter STLs observed for *mus301* mutants were similar to those previously published for multiple *mus309* (DmBlm) mutant allele combinations (McVey et al. 2007). However, in *mus309* mutants, aberrant repair events are frequently accompanied by lethal flanking deletions (McVey et al. 2004b). Therefore, we were also interested in analyzing how often lethal flanking deletions were occurring during DSB repair in *mus301* mutants. To analyze the frequency of these flanking deletions during repair of the $P\{w^a\}$ construct in *mus301* deficient flies, we divided the deletion events into two separate categories: *scalloped* F_1 females and F_2 male lethal deletions. In contrast to *mus309* mutants, we observed very few lethal deletions in *mus301* mutants (Table 4-1).

To confirm that *mus301* and *mus309* mutants showed different deletion phenotypes, we tested $mus309^{N1}$ mutants using the $P\{w^a\}$ assay. $mus309^{N1}$ flies showed decreased use of HR and increased use of aberrant repair (Figure 4-3B)

and decreased repair synthesis tract lengths (Figure 4-3C). Unlike the *mus301*^{288A} mutants, however, there was an increase in lethal flanking deletions for *mus309*^{N1} mutants (Table 4-1). These data were consistent with previous results using other *mus309* mutant alleles (McVey et al. 2007; 2004b; Adams et al. 2003).

Genotype	% scalloped Females (F1)	% No white-eyed males (F2)
Wild-type	0.0% (84)	0.0% (44)
mus301^{288A}	0.2% (1061)	2.0% (246)
mus309^{N1}	17.3% (623) [a]	55.3% (159) [a]
mus301^{288A}, *mus309*^{N1}	3.9% (332) [b]	25.2% (127) [a,b]

Table 4-1: Lethal flanking deletions increase in *mus309* mutants, but not in *mus301* mutants. The *P*{*w*^{a}} construct is inserted in an intron of *scalloped* (*sd*), an X-chromosome gene required for viability. Females carrying one *sd*$^{-}$ mutation can have malformed wings (left), while males inheriting *sd*$^{-}$ mutations do not survive (right), allowing two levels of analysis for lethal deletions. Parenthetical numbers indicate total number of events analyzed: left, total number of yellow-eyed F_1 females counted; right, total number of independent yellow-eyed F_1 females from which F_2 males were counted. a: significantly increased from WT ($P < 0.0001$); b: significantly different from both single mutants ($P < 0.0001$); Fisher's exact test

To better understand the genetic relationship between these two genes, we generated *mus301*^{288A}, *mus309*^{N1} double mutants and again conducted the *P*{*w*^{a}} assay. We saw similar levels of end joining repair compared to the single mutants and a significant decrease in homologous recombination repair compared to *mus309*^{N1} mutants (Figure 4-3B). Synthesis tract lengths were also significantly shorter than those of single mutants at all but the longest distance (Figure 4-3C). Interestingly, there was a suppression of lethal deletions in the double mutant (Table 4-1).

Smaller flanking deletions can extend into *scalloped* intron sequence, but not into exon sequence. If such deletions do not affect *scalloped* expression, they may be non-lethal and remain undetected by our analysis of deletions in Table 4-

1. A negative result for STL analysis at 5 bp on either end of $P\{w^a\}$ (Figure 4-3C, left and right sides) could indicate either a lack of synthesis from that end or a non-lethal flanking deletion. To discern between these two possibilities, we analyzed these events using primers that anneal to *scalloped* intron sequence, either to the left or the right of the insertion site. Similar to the lethal deletions, *mus301* mutants showed very few small flanking deletions, while *mus309* mutants showed frequent small flanking deletions. In the double mutant, we again saw suppression of the *mus309* phenotype (Table 4-2). Together, these data suggest that the flanking deletions occurring in the absence of DmBlm are partially dependent on the activity of DmHelQ.

Genotype	Left deletion >100 bp	Right deletion >100 bp	Right deletion >400 bp	Right deletion >1200 bp
mus301^{288A}	14.3% (14)	0% (10)	0% (10)	0% (10)
mus309^{N1}	84.8% (33) [a]	52.4% (21) [a]	38.1% (21) [a]	23.8% (21)
mus301^{288A}, *mus309*^{N1}	48.8% (43) [a,b]	23.8% (42) [b]	21.4% (42)	4.8% (42) [b]

Table 4-2: Non-lethal flanking deletions also increase in *mus309* mutants, but not in *mus301* mutants. Aberrant repair events negative for synthesis from the left or right end of $P\{w^a\}$ were analyzed for small flanking deletions into *scalloped* intron sequence on that end. a: significantly increased from *mus301* mutants ($P < 0.05$); b: significantly decreased from *mus309* mutants ($P < 0.05$); Fisher's exact test

Discussion

We have shown that Drosophila HelQ is involved in the repair of one-ended DSBs, interstrand crosslinks, and transposase-mediated DSBs. In ICL repair, HelQ participates in a pathway distinct from Polθ, most likely HR. Our results from the $P\{w^a\}$ assay provide clues as to how DmHelQ and DmBlm may be acting during homologous recombination. Clearly, both are involved in HR, as loss of either protein results in decreased HR and increased aberrant repair (Figure

4-3B). DmHelQ and DmBlm appear to have some functional overlap, and both proteins promote accurate repair by synthesis dependent strand annealing (SDSA).

Models of SDSA in yeast and flies propose that there can be multiple invasion attempts during repair (McVey et al. 2004a; Smith et al. 2007; Pâques et al. 1998). Each attempt consists of strand invasion and D-loop formation, templated DNA synthesis, unwinding of the D-loop, and an attempt to resolve the DSB, either by reannealing at the opposite end of the break or an end joining event. Removal of DmBlm results in large flanking deletions during DSB repair, possibly because D-loops are cleaved by nucleases to free the invading strand (Table 1; McVey et al. 2004b). After nucleolytic cleavage, either a second invasion attempt takes place, or the DSB is resolved. If a second invasion initiates, the DNA must be cleaved again, further deleting flanking sequences. This cycle likely continues until the two DSB ends are joined, resulting in an extensive loss of sequence.

The function of DmBlm has previously been characterized genetically (Adams et al. 2003; McVey et al. 2007; 2004b), and our data agree with published results (Figure 4-3B-C, Table 4-1, Table 4-2). The fact that both *mus301* and *mus309* mutants display similar phenotypes in the $P\{w^a\}$ assay would suggest that their protein products have similar roles during SDSA. However, we do not believe this is the case because of the difference in the deletion frequency observed in the two mutant backgrounds. *mus301* mutants do not show the increased deletions typically found in *mus309* mutants, and the double mutant

shows a suppression of deletions (Table 4-1, 4-2). These data suggest a potential model for independent functions of DmBlm and DmHelQ during SDSA.

Potential roles for DmHelQ and DmBlm during SDSA: While the strand invasion step of HR is well characterized, there are conflicting data concerning the fate of RecA/Rad51 filaments post-synapsis. Data from bacteria suggest that RecA is cleared from the 3' end of an invading strand prior to synthesis (Xu and Marians 2002). In yeast, the motor protein Rad54 is proposed to dissociate Rad51 from dsDNA (Solinger et al. 2002), allowing D-loop extension by polymerases that can now access the 3' end (Li and Heyer 2009). An alternative explanation is that Rad54 activity is primarily responsible for branch migration, which then permits extension of the invading strand (Bugreev et al. 2007). It is difficult to separate these two possibilities, especially considering Rad54 has been shown to be involved in numerous steps of HR (Mazin et al. 2010; Heyer et al. 2006). Another candidate for Rad51 removal is Srs2, which is known to disassemble ssDNA-Rad51 filaments (Veaute et al. 2003; Krejci et al. 2003) and disrupt D-loops (Dupaigne et al. 2008). However, no Srs2 ortholog has been identified in flies. Therefore, the question of how Rad51 is cleared from homologous recombination intermediates, specifically in Drosophila, remains open. In *C. elegans*, HELQ-1 has been shown to remove Rad51 from dsDNA (Ward et al. 2010). Possibly, DmHelQ could perform a similar function. If post-synaptic clearance of Rad51 from dsDNA is a prerequisite for extensive repair synthesis, this would explain the phenotype we observe in *mus301* mutants (Figure 4-3B,C).

A recent model of HR repair proposes that D-loop extension induces torsional strain that eventually obstructs repair synthesis (Sneeden et al. 2013). Therefore, the authors suggest that the D-loop maintains a constant size during repair, migrating in the direction of synthesis (Sneeden et al. 2013). This migrating, constant sized D-loop would require extrusion of the 5' end of the invading strand as synthesis proceeds. If the 5' end of the invading strand cannot be extruded, the D-loop would increase in size rather than migrate in the direction of synthesis, eventually reaching a limit of topological constraint. As a result, the D-loop would dissociate, and the break may be resolved by end-joining. Perhaps DmHelQ-dependent clearance of Rad51 from dsDNA is required for the extrusion of the 5' end, and therefore the establishment of a migrating versus extending D-loop (Figure 4-4A). We propose that the helicase loads onto the template strand used during repair synthesis and proceeds in a 3'→5' direction. The extensive ssDNA at the repair site is likely coated in RPA, which may serve to recruit DmHelQ and/or stimulate DmHelQ activity, as it does in archaea (Woodman et al. 2011) and humans (Tafel et al. 2011). Meanwhile, DmBlm could be responsible for migrating the Holliday junction (HJ) behind the D-loop (Wu and Hickson 2003), which results in the extrusion of the 5' end (Figure 4-4A).

In *mus301* mutants, however, an inability to remove Rad51 from dsDNA prevents the formation of a migrating D-loop (Figure 4-4B). DmBlm cannot migrate the HJ forward. Synthesis can proceed for a limited distance, but the torsional strain induced by an extending D-loop eventually becomes prohibitive. The D-loop is dissociated, and the gap may be repaired via end joining processes.

As a result, synthesis tracts are shorter (Figure 4-3C), and fewer repair events are successful in complete synthesis of *white* (Figure 4-3B).

In a wild-type fly, DmBlm may facilitate migration of the HJ behind the D-loop, solely in the direction of synthesis (Figure 4-4A). In the absence of DmBlm, there is an increase in flanking deletions during gap repair (Table 4-1, 4-2). This may be a result of stochastic migration of the HJ (Figure 4-4C). Migration in the opposite direction of synthesis would draw flanking sequences into the junction and into the D-loop itself. In the absence of DmBlm, the D-loop is more likely to be resolved by cleavage rather than unwinding (McVey et al. 2004b). Endonucleolytic cleavage of the HJ after stochastic migration in the opposite direction of synthesis would result in flanking deletions (Figure 4-4C). In *mus301* mutants, however, the presence of Bloom prevents stochastic migration in the opposite direction (Figure 4-4B). As a result, there are very few flanking deletions in these mutants (Table 4-1, 4-2).

When both *mus301* and *mus309* are mutated, the deletions associated with DmBlm deficiency are largely suppressed (Table 4-1, 4-2). We propose that this is because the stochastic migration of the HJ is limited by the topological constraints of persistent Rad51-dsDNA filaments (Figure 4-4D). Just as repair synthesis is inhibited by the torsional strain induced by an extending D-loop, so too is the migration of the HJ into flanking sequences. The absence of DmBlm permits some migration, but less than what occurs in the *mus309* single mutant. Consequently, the double mutant phenotype includes short synthesis (Figure 4-3C) and fewer flanking deletions than in *mus309* mutants (Table 4-1, 4-2).

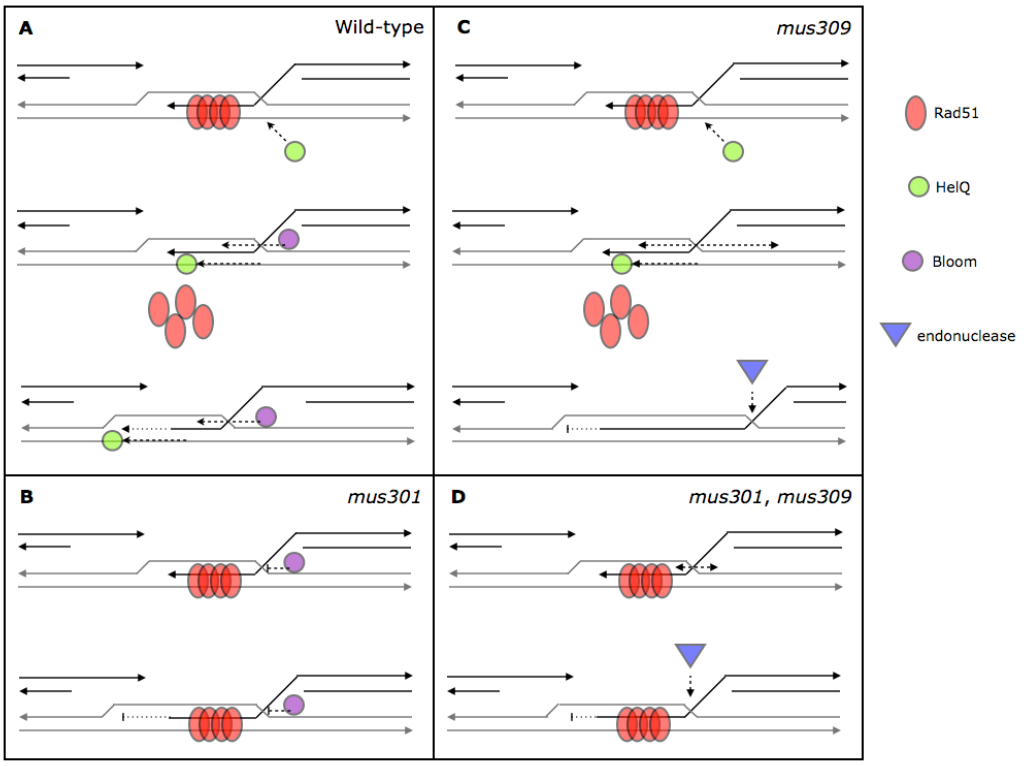


Figure 4-4: Proposed post-synaptic activities of Bloom and HelQ proteins in promoting SDSA repair of a double-stranded gap. (A) In wild-type flies, HelQ loads onto the template strand used for repair synthesis. It proceeds in a 3'→5' direction, which displaces Rad51 filaments from dsDNA. It may continue along the template strand to unwind the D-loop ahead of repair synthesis. Meanwhile, Bloom migrates the Holliday junction in the direction of repair synthesis to maintain the size of the D-loop. (B) In *mus301* mutants, the absence of HelQ results in persistent Rad51-dsDNA filaments. Repair synthesis and D-loop extension generates topological strain. Bloom prevents junction migration away from the invasion site, preventing flanking deletions. However, the inability to migrate the D-loop results in decreased synthesis. (C) In *mus309* mutants, HelQ can remove Rad51 from dsDNA, but the absence of Bloom allows the Holliday junction to migrate stochastically. In some cases, the junction migrates away from the initial invasion site. Bloom-independent resolution of the D-loop requires endonucleases, resulting in flanking deletions. (D) In *mus301*, *mus309* double mutants, persistent Rad51-dsDNA induces topological strain that has two important consequences: repair synthesis is minimal and the junction cannot migrate extensively away from the invasion site. Consequently, synthesis tracts are short, but the flanking deletions observed in *mus309* mutants are suppressed.

The loading of DmHelQ on the template strand would also allow the protein to facilitate repair synthesis progression. Following removal of Rad51, further movement of the helicase would unwind the template duplex, thereby

allowing repair synthesis to proceed (Figure 4-4A). The combination of reduced unwinding ahead of the fork (HelQ) and reduced HJ migration behind the fork (Blm) would explain the synergistic decrease in repair synthesis observed in the double mutants (Figure 4-3C).

In *C. elegans*, either HELQ-1 or RFS-1 (a Rad51 paralog) were sufficient to remove Rad51 from dsDNA (Ward et al. 2010). Synthetic lethality between *helq-1* and *rfs-1* mutations suggested that one of these proteins is required for the resolution of HR repair (Ward et al. 2010). The Drosophila Rad51 paralog with the highest level of expression is *spn-B* (DmXRCC3). We see that adding a mutation in *spn-B* to *mus301* deficient flies does not affect viability (data not shown). We believe this is because of the existence of multiple Rad51 paralogs in Drosophila, encoded by *spn-B*, *spn-D*, *Rad51C* and *Rad51D* (Staeva-Vieira et al. 2003). In the absence of *spn-B*, one or more of the other paralogs can fulfill the role of Rad51 disassembly. The implication is that removing all of the Rad51 paralogs and HelQ would result in a lethal phenotype. However, since the Rad51 paralogs cannot compensate for the loss of DmHelQ, we suggest that they are much less efficient at Rad51 removal if this function is conserved in flies.

Single strand annealing: Some conflicting data have been published regarding the role of DmHelQ in single strand annealing (SSA), the process by which homologous sequences flanking a DSB are annealed without the use of an intact template. One group (Engels) reports that *mus301* deficient flies show a statistically significant decrease in SSA (Johnson-Schlitz et al. 2007). However, another group (Rong) reported no change in SSA capability for *mus301* mutants

(Wei and Rong 2007). The two groups used different assays to determine SSA function, which may explain the disparate results. If the Engels group is correct in stating that *mus301* is important for SSA repair, a role in clearance of Rad51 may explain the decreased SSA efficiency. For the cell to resort to SSA repair, it must be able to clear Rad51 monomers from a broken substrate. This is supported by the fact that both groups showed an increase in SSA repair for *spn-A* (Rad51) mutants in their respective assays (Wei and Rong 2007; Johnson-Schlitz et al. 2007). Therefore, in the absence of DmHelQ, we would expect a decrease in SSA repair, which is observed by the Engels group (Johnson-Schlitz et al. 2007). Importantly, though, this clearance would likely be from ssDNA, an activity that CeHELQ-1 was incapable of *in vitro* (Ward et al. 2010). One possibility is that some Rad51-dsDNA intermediates are cleared by DmHelQ, unwound by DmBlm and then shunted toward an SSA resolution. This would imply that presynaptic Rad51-ssDNA intermediates destined for SSA are disassembled by another mechanism.

Unlike DmHelQ, mutations in DmRad54 result in increased SSA via the Engels assay (Johnson-Schlitz et al. 2007; Klovstad et al. 2008). This may be explained by the role of Rad54 in stabilizing Rad51 filaments (Mazin et al. 2003). Less stable filaments would lead to more exposed ssDNA, and, thus, more opportunity for SSA repair. This could mean that, at least in Drosophila, Rad54 is more critical for the early stages of HR, and that Rad51 filament disassembly is relegated to HelQ. Alternatively, these data could simply reflect temporal

separation of Rad54 function: SSA increases in *okra* (DmRad54) mutants because the filament stabilization function occurs prior to filament disassembly.

DmHelQ and interstrand crosslink repair: Central to the repair of ICLs is the Fanconi anemia (FA) pathway, a continually expanding set of proteins that coordinate the response to DNA crosslinks (FANCA, FANCB, FANCC, etc.) (Kitao and Takata 2011). Briefly, the mechanism involves recognition of the crosslink, monoubiquitination of FANCD2 and FANCI, incisions flanking the crosslink, and HR repair of the resulting gap (Cybulski and Howlett 2011). Interestingly, *C. elegans* HEL-308/HELQ-1 is proposed to function in the FA pathway, based on an epistatic relationship with FCD-2 (CeFANCD2) in ICL repair (Muzzini et al. 2008). Our data show that there is a synergistic effect when flies treated with nitrogen mustard are deficient in HelQ and Polθ (Figure 4-1B). An identical phenotype is observed in *C. elegans helq-1*, *polq-1* double mutants treated with nitrogen mustard (Muzzini et al. 2008). Additionally, our data are consistent with the fact that Drosophila Polθ-mediated repair of ICLs is separate from Rad51-mediated HR (Chan et al. 2010), which we propose requires HelQ for proper function. The epistatic relationship between *spn-A* and *mus301* after nitrogen mustard and topotecan treatment (Figure 4-1A, 4-2) also suggests that DmHelQ is involved in HR. Combined, these data suggest a role for Drosophila and *C. elegans* HelQ/HELQ-1 in HR-mediated repair of ICLs and one-ended DSBs. ICL repair is particularly dependent on Polθ (Figure 1B; Boyd et al. 1981), but it appears that HelQ can compensate for the loss of Polθ at low doses of crosslinking agents (Figure 4-1B).

We have shown that Drosophila HelQ, like its archaeal (Richards et al. 2008; Woodman et al. 2011; Guy and Bolt 2005), roundworm (Ward et al. 2010; Muzzini et al. 2008) and mammalian homologs (Tafel et al. 2011; Marini and Wood 2002), plays an important role in DNA repair. For repair of mutagen-induced damage, *mus301* (DmHelQ) mutants behave similarly to *spn-A* (DmRad51) mutants, suggestive of a role in HR for DmHelQ. During gap repair of an excised transposon, DmHelQ and DmBlm independently promote synthesis dependent strand annealing (SDSA). A biochemical analysis of DmHelQ and DmBlm will help clarify the respective roles of these proteins post-synapsis.

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Chapter 5
Future Directions

Introduction

We have used several genetic analyses to characterize some of the critical proteins involved in homologous recombination (HR) repair of DSBs, along with the P450 enzyme Cyp6d2. Several questions still remain, however. In many cases, biochemical experiments clarifying *in vitro* functions of these proteins may provide additional clues about their *in vivo* roles. Here, we discuss future experiments that could build upon the data presented in this dissertation.

DmBrca2 and DmRad51

An initial analysis of *brca2* and *spn-A* mutant using sensitivity assays suggested a Rad51-independent role for DmBrca2 in the repair of camptothecin-induced DNA damage (Figure 2-3A). After showing that both mutants were equally sensitive to the camptothecin analog topotecan (Figure 2-3B), we concluded that such a role was unlikely to exist. Given that camptothecin-induced damage is likely repaired via homologous recombination, which is dependent on Rad51, the important question is not why the *brca2* mutants are so sensitive to the drug, but why *spn-A* mutants are not very sensitive. We have suggested that camptothecin is a substrate for Cyp6d2 in Drosophila, and that potentially multiple cytochrome P450 enzymes could be involved in its detoxification (Thomas et al. 2013). If this is true, one potential explanation for the camptothecin resistance observed in *spn-A* mutants is that a separate P450 mutation enhances detoxification of camptothecin. Such a mutation would result in camptothecin resistance but not affect topotecan sensitivity. This hypothesis

assumes that there are other P450 enzymes involved in camptothecin detoxification.

There are 83 functional cytochrome P450 enzymes in Drosophila, 26 of which are on the 3rd chromosome (along with *spn-A*) (Tijet et al. 2001), so it would be difficult to identify a specific background mutation on the *spn-A* mutant chromosome that causes the phenotype. An easier approach would be to attempt to cross off second-site mutations from the $spn\text{-}A^{057}$ and/or $spn\text{-}A^{093}$ alleles. This is typically accomplished by crossing the mutant chromosome to a wild-type fly for 3 or more generations. If a second-site mutation is causing the resistance we see in *spn-A* mutants, retesting the sensitivity of these flies after these crosses might show a phenotype similar to those of *brca2* mutants.

Cyp6d2 and Camptothecin

Sensitivity limits: An interesting phenomenon we observed with increasing doses of camptothecin is that, except in *cyp6d2* mutants, survival decreases but does not reach 0%. Survival for either $brca2^{KO/KO}$ or $spn\text{-}A^{093/057}$ mutants is equivalent at 50 and 100 μM camptothecin (Figure 2-3A). At doses greater than 100 μM camptothecin, overall survival decreases to a point that precludes accurate analysis of sensitivity (data not shown). We believe that this supports our model that Cyp6d2 is detoxifying camptothecin in these HR-deficient backgrounds. Only a small amount of camptothecin is entering the nucleus and causing DNA damage. Even in these HR mutants, this damage is not sufficient to cause complete lethality. When a *cyp6d2* mutation is introduced, however, the survival does reach 0% at relatively low doses. In this case, all of the

drug can access the DNA, and it is sufficient to cause lethality at a dose of 25 μM even in the presence of HR repair proteins (Figure 3-1A). Interestingly, additional data in our lab show that this phenotype is consistent in other repair deficient backgrounds (data not shown). With some exceptions, only in *cyp6d2* deficient backgrounds do we observe 0% survival at any dose of camptothecin. Though interesting, this observation, along with the prevalence of *cyp6d2* mutations (Thomas et al. 2013), means that the water soluble derivative, topotecan, is a better choice for analyzing sensitivity to DSBs in flies.

***In vitro* experiments with DmCyp6d2:** In Chapter 3, we proposed that DmCyp6d2 could detoxify camptothecin. This is based on genetic data, as well as previously published results on cytochrome P450 enzymes. However, it does not conclusively show how such detoxification occurs. Some of this uncertainty could be clarified with an *in vitro* characterization of the enzyme. A crystal structure of human CYP3A4 bound to one of its many substrates, progesterone, has been published (Williams et al. 2004). Crystallization of DmCyp6d2 bound to camptothecin would conclusively show that it is, in fact, a substrate. Such an experiment would require collaboration with a lab that has experience and equipment for crystallization.

Instead of crystal structures, there may be other experiments that could clarify the role of DmCyp6d2. For example, one possibility is incubating DmCyp6d2 with camptothecin, along with necessary cofactors, like the heme group required for catalysis. Mass spectrometry could be used to identify byproducts of the reaction. This could be compared with a similar reaction using

whole cell extracts, which would contain any additional P450 enzymes required for full detoxification. Different camptothecin byproducts in these two experiments would suggest additional steps in camptothecin detoxification that require an enzyme other than Cyp6d2. If there are additional P450 enzymes that are needed to detoxify camptothecin, this experiment could also identify whether they function upstream or downstream of Cyp6d2. For example, if camptothecin incubated with Cyp6d2 alone is unchanged, but camptothecin incubated with whole cell extracts is modified, it is likely that Cyp6d2 binds the byproduct of another enzyme that can interact with camptothecin. If, on the other hand, both reactions result in modified camptothecin molecules, but the products are different, another enzyme may function downstream of Cyp6d2. Such an enzyme could be a member of the glutathione-S-transferase family, which is known to function downstream of P450 enzymes during drug detoxification (Chahine and O'Donnell 2011).

Two other interesting questions could be answered by these *in vitro* experiments. First, does DmCyp6d2 bind and/or modify topotecan? If it does not, this is consistent with the genetic data showing *cyp6d2* mutants are resistant to topotecan. However, there is another explanation that we put forward in Chapter 3. The highest expression levels of *Cyp6d2* are in the larval fat body (Yang et al. 2007). Hydrophobic camptothecin may be sequestered in the fat body, where Cyp6d2 and potentially other enzymes break it down. The water soluble topotecan, on the other hand, may not remain in the fat body. Therefore, Cyp6d2

could still bind topotecan *in vitro*, but the drug and the enzyme are spatially separated *in vivo*.

A second question to ask is whether the *cyp6d2*^{SD} mutant can bind camptothecin. Our hypothesis is that, even though this mutant does have the critical heme-binding cysteine and 90% of the protein, structural changes near the heme-binding cysteine prevent detoxification of its substrate. Potentially, the mutant form of the protein can bind camptothecin, but not detoxify it, because the heme interaction is impaired. Additionally, developing an antibody that recognizes any residues in the first 90% of the protein could be used to definitively show that a truncated protein is produced. The antibody could also be used to detect any Cyp6d2 protein in the *cyp6d2*^{NT} mutant, which does not appear to produce any transcript (Figure 3-3C-E). Our genetic data cannot rule out that a small amount of Cyp6d2 is translated in this mutant background.

DmHelQ and Homologous Recombination

How Rad51 is removed from HR intermediates is not completely clear. A separate but equally important question is *when* Rad51 is removed. Is the entire filament removed before synthesis begins? Or is there clearance at the 3'-end only, followed by a separate clearance from the rest of the heteroduplex DNA? If they are separate events, do separate proteins control them? The Boulton group has proposed that *C. elegans* RAD-54 removes RAD-51 from the 3'-end of the invading strand, which allows synthesis to begin (Ward et al. 2010). HELQ-1 and/or RFS-1 then remove RAD-51 from the remainder of the heteroduplex DNA (Ward et al. 2010). This is consistent with work in yeast showing that Rad54

preferentially associates with the Rad51-dsDNA filament terminus (Kiianitsa et al. 2006). It is clear that Rad51 does not dissociate from dsDNA on its own, and therefore, one or more proteins are probably involved in its removal (Miné et al. 2007). We propose that DmHelQ has some function in this process, though we are not certain of its exact role. There are several experiments that could potentially answer some of our remaining questions and clarify the function of DmHelQ in DSB repair.

Purification of DmHelQ: We have recently cloned *mus301* into BL21(DE3)pLysE *E. coli* cells in preparation for DmHelQ purification. We confirmed that the protein could be induced by IPTG (isopropyl β-D-1-thiogalactopyranoside) in these cells (data not shown). Once we have purified a substantial amount of the protein, we will collaborate with biochemistry experts to test multiple potential functions of DmHelQ. The Boulton group used gel shift assays to show that HELQ-1 and RFS-1 could bind to RAD-51. Additionally, both proteins could remove RAD-51 from dsDNA but not ssDNA (Ward et al. 2010). If similar results were seen with DmHelQ, this would be very convincing evidence supporting our model. The Opresko and Sung groups have used *in vitro* assays to show that helicases can unwind D-loop structures (Prakash et al. 2009; Opresko et al. 2009). Such activity has been observed *in vitro* for other helicases, including yeast Mph1 (Prakash et al. 2009) and human Bloom (Bachrati et al. 2006). It is unclear, however, if these results necessarily imply an *in vivo* unwinding function. Thus, we must be careful interpreting any unwinding activity observed for purified DmHelQ. Since DmBlm is proposed to unwind D-loop

structures (Adams et al. 2003), and we propose that DmHelQ has a separate role in Rad51 clearance, we would expect that DmHelQ does not unwind D-loops *in vivo*.

Because of the ATP-independent function of HELQ-1 in removing RAD-51 from dsDNA (Ward et al. 2010), efforts to purify the DmHelQ will include the purification of a helicase dead mutant as well. The *helq-1* mutant used by Ward et al. was K197R, and the corresponding DmHelQ mutation would be K293R. This mutation can be achieved using *in vitro* mutagenesis, modifying the lysine codon AAA to AGA for arginine (Figure 5-1).

```
CeHELQ-1   191  LPTGAGKTLIAEVLMLRE  208
                LPT  GKTL+AE+LMLRE
DmHelQ     287  LPTSGGKTLVAEILMLRE  304

  DNA: CTGCCCACAAGTGGTGGAAAAACTCTAGTAGCCGAAATCCTTATGCTGCGTGAA
K293R: CTGCCCACAAGTGGTGGAAGAACTCTAGTAGCCGAAATCCTTATGCTGCGTGAA
```

Figure 5-1: Proposed mutagenesis to generate helicase-dead DmHelQ. (Top) Partial protein alignment of *C. elegans* HELQ-1 (923 aa) and Drosophila HelQ (1051 aa), showing conserved ATPase domain. Catalytic lysines are underlined. (Bottom) DNA sequence that codes for the DmHelQ region shown above. Catalytic lysine codon underlined. Single nucleotide change required to generate K293R mutant shown in bold below.

A role for HelQ in post-synaptic removal of Rad51: While many aspects of HR repair are well characterized, the timing of Rad51 dissociation and repair synthesis is unclear. *In vitro* data using a single-molecule approach and magnetic tweezers showed that human RAD51 depolymerization was 150X slower on dsDNA than ssDNA, suggesting that other proteins must be required for its dissociation from the dsDNA-RAD51 complex at D-loops (Miné et al. 2007). A more recent model of recombinational repair developed through an *in vitro* system with human proteins suggests that RAD51 is cleared from the

invaded strand before synthesis begins (Sneeden et al. 2013). The mechanism of this removal, however, was not specified. This model also proposed that a migrating D-loop is required to avoid torsional strain during HR repair synthesis (Sneeden et al. 2013). We propose that the inability to remove Rad51 from dsDNA prevents transformation from an extending to a migrating D-loop, and therefore leads to synthesis stalling and end-joining repair of a double-stranded gap (Figure 4-4).

Several important questions may be answered using biochemical experiments. For example, can DmHelQ interact with Rad51? More importantly, can it remove Rad51 from dsDNA? The Boulton group was able to identify a Rad51-interacting sequence on the Rad51 homolog RFS-1, but was unable to do so for HELQ-1 (Ward et al. 2010). Thus, we cannot determine if that interaction is conserved using only the amino acid sequences of the two proteins. An additional important question is the type of substrate that is targeted by DmHelQ. Whether the protein can load on nicked, single-stranded, 3'-tailed or blunt ends of DNA can suggest potential roles for the protein. We propose that DmHelQ loads onto a single-stranded region of the template DNA, behind the invading strand. Proceeding from that point in a 3'→5' manner, the proposed directional activity of the human protein (Marini and Wood 2002), this may (1) dislodge Rad51 from dsDNA and (2) facilitate unwinding of the duplex template ahead of repair synthesis (Figure 4-4A). Such activity would require DmHelQ to be able to load onto a nicked circle substrate, which can be demonstrated biochemically.

The role of DmRad54: The Rad54 protein has been shown to promote Rad51-mediated strand exchange (Petukhova et al. 1998), but also promote dissociation of Rad51 from dsDNA (Solinger et al. 2002). These seemingly contradictory functions are explained by a model that proposes that separate domains of Rad54 facilitate different interactions with Rad51 (Mazin et al. 2010). This is supported by the observations that stabilization of Rad51 filaments does not require ATPase activity of Rad54 (Mazin et al. 2003), whereas the removal of Rad51 does require Rad54 ATPase activity (Li et al. 2007). In yeast, it is known that Rad54 can remove Rad51 from the 3' end of invading strands during HR (Solinger et al. 2002), thereby allowing the initiation of DNA synthesis (Li and Heyer 2009). Similar disassembly of RecA is necessary in bacteria prior to synthesis, though this disassembly is proposed to be inherent to the dynamic nature of RecA, not a specific adaptor protein (Xu and Marians 2002).

A screen for mutations causing oogenesis defects and female sterility in Drosophila uncovered the gene *okra* (Schupbach and Wieschaus 1991). Later, *okra* was shown to be the Drosophila homolog of Rad54 (Ghabrial et al. 1998). The connection between DNA repair and oogenesis is that unrepaired DSBs activate a meiotic checkpoint that prevents proper dorsal-ventral patterning, leading to nonviable eggs (Ghabrial and Schupbach 1999). The same problem is observed during oogenesis in *spn-A* mutants (Staeva-Vieira et al. 2003) and *mus301* mutants (McCaffrey et al. 2006). The similar phenotypes suggest that meiotic DSBs are persisting in *okra*, *spn-A* and *mus301* mutants. We suggest that this is due to partially overlapping function between DmHelQ and DmRad54 in

Rad51 clearance. However, mutations in several DNA repair genes result in this female sterility phenotype. Further genetic analysis using *okra* mutants in our assays, like $P\{w^a\}$, is therefore necessary to confirm this hypothesis.

The Boulton group proposed that, at least in *C. elegans*, RAD-54 clears RAD-51 from the 3' end of the invading strand, after which it switches to a branch migration role. Meanwhile, HELQ-1 or RFS-1 remove the remainder of the RAD-51 filament from the dsDNA (Ward et al. 2010). This sequential mechanism would explain why both *rad-54* mutants and *helq-1*, *rfs-1* double mutants show persistent RAD-51 foci during meiosis (Ward et al. 2010). If the mechanism is conserved in Drosophila, it would explain why we observe a defect in *mus301* mutants, despite the presence of a Rad54 homolog. Rad54 could be clearing Rad51 from the 3' end of the invading strand, allowing repair synthesis to initiate. The inability to clear Rad51 from the back of a D-loop in *mus301* mutants may lead to an inability to relieve topological stress generated by D-loop extension. This topological stress causes polymerase stalling (Sneeden et al. 2013) and decreased synthesis.

Functions of DmHelQ and DmRad54: *RAD-54* is essential in *C. elegans* (Ward et al. 2010), but *okra* is not essential in Drosophila (Ghabrial and Schupbach 1999), suggesting that Rad54/RAD-54 may be performing different functions in these organisms. Therefore, an alternate explanation for our data is that DmRad54 and DmHelQ have partially overlapping functions. Since a helicase-dead HELQ-1 mutant protein can remove RAD-51 (Ward et al. 2010), and Rad54 requires ATP to remove Rad51 (Li et al. 2007), these may represent

two distinct mechanisms of Rad51 removal, either in the presence (Rad54) or absence (HelQ) of ATP.

Since yeast Rad54 has previously been implicated in the removal of Rad51 from dsDNA (Solinger et al. 2002; Kiianitsa et al. 2006), it is important to determine if this role is conserved in Drosophila. Mutations in DmRad54 lead to sensitivity to ionizing radiation and methyl-methanesulfonate (MMS) (Kooistra et al. 1997; Klovstad et al. 2008), as well as DSB repair defects following P-element excision (Kooistra et al. 1999; Romeijn et al. 2005). Furthermore, DmRad54 deletions result in chromatin remodeling defects (Alexiadis et al. 2004), consistent with the established role for Rad54 in chromatin remodeling (Reviewed in Heyer et al. 2006; Mazin et al. 2010). However, a role in removal of Rad51 has not been established in Drosophila.

To test the involvement of DmRad54 in removal of Rad51, we can start by running the $P\{w^a\}$ assay with *okra* mutants. If DmRad54 functions in a sequential mechanism with DmHelQ, we would expect to see results similar to those of the *mus301* mutants: decreased use of HR, decreased synthesis tract lengths, but no increase in flanking deletions. A solely sequential function would also suggest that an *okra*, *mus301* double mutant would behave similarly to the single mutants. However, since Rad54 has multiple functions during HR, including chromatin remodeling and presynaptic stabilization of Rad51 (Reviewed in Heyer et al. 2006; Mazin et al. 2010), we may see a synergistic effect with the double mutant. An *okra*, *mus301* double mutant could also be synthetically lethal, which would show that the proteins perform non-overlapping functions. Additionally, like

DmHelQ, a purified DmRad54 may help to answer some questions on the exact function of the protein.

DmHelQ and mitotic crossovers: One question remaining for the Drosophila HelQ homolog is whether or not the helicase prevents mitotic crossovers. Such a function does exist for DmBlm, as it was shown that *mus309* mutant larvae exposed to ionizing radiation show an increase in mitotic crossovers between recessive markers on the third chromosome (McVey et al. 2007). This is consistent with the activity of human Bloom protein, which prevents such crossovers from occurring during repair of a DSB (Wu and Hickson 2003). Our model posits that DmHelQ is not suppressing crossovers during HR, so we would not expect to see an increase in crossovers with this assay. Still, these genetic data would help to clarify the function of this protein. To test this, we will cross the recessive markers *st* (scarlet) and *e* (ebony) on to a *mus301*^{288A} mutant chromosome (Figure 5-2). *mus301*^{288A} *e st* / *mus301*^{288A} larvae will be irradiated, raised to adulthood and crossed to *e st* mutant flies. An increase in mitotic crossovers during DSB repair will result in an increase in recombinant (+ *st* or *e* +) offspring. Since *mus309* mutations do increase the frequency of mitotic crossovers (McVey et al. 2007), it would be interesting to see if adding a *mus301* mutation suppresses this defect, as it does with large flanking deletions in the *P*{*w*^{a}} assay (Table 4-1). Our prediction is that no suppression would occur, since the mitotic crossover phenotype in *mus309* mutants is due to a defect in Holliday junction resolution, not D-loop unwinding. An alternative approach to this experiment is to use P-element excision to create a site-specific double stranded

gap rather than the breaks of random type and location generated by IR.

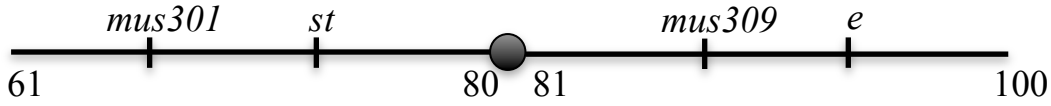

Figure 5-2: Testing for mitotic crossovers in *mus301* mutants. Approximate cytological locations shown for *mus301* (66), *st* (73), *mus309* (86) and *e* (93) on chromosome *3*. Mitotic recombination assays would test for crossovers between *st* and *e* following ionizing radiation induction of DSBs. Cytological locations for Drosophila chromosomes: 1-20 (*X*), 21-40 (*2L*), 41-60 (*2R*), 61-80 (*3L*), 81-100 (*3R*), 101-102 (*4*).

Interacting proteins: In addition to investigating the *in vitro* function of the protein, it will be interesting to see if DmHelQ has any interactions with other repair proteins *in vivo*. Such an experiment is ongoing in the lab. We are in the process of cloning a FLAG-tagged *mus301* gene into the plasmid pattB. Once the pattB-FLAG-*mus301* construct is completed, we can use a similar strategy to the one employed for the *Cyp6d2* rescue described in Chapter 3. Following successful integration into the germline and screening for transformants, we will have a fly stock that expresses FLAG-tagged HelQ. This will allow us to see, *in vivo*, which proteins interact with DmHelQ. We can isolate the FLAG-tagged protein with an anti-FLAG antibody, and use mass spectrometry to identify any proteins bound to DmHelQ. The experiment can be conducted in the presence or absence of DNA damaging agents, such as ionizing radiation. Potential interacting proteins include RPA (Woodman et al. 2011) or Rad51. An interaction with Rad51, especially if it is found only in the presence of DNA damage, would support our hypothesis that DmHelQ is removing Rad51 from D-loop structures. A similar approach using purified Rad51 paralogs could be used to test whether the overlapping functions

of *C. elegans* HELQ-1 and RFS-1 in removal of Rad51 (Ward et al. 2010) are conserved in Drosophila.

Conclusions

In this dissertation, we have presented a series of genetic experiments that have helped to clarify the roles of several Drosophila proteins involved in the repair or prevention of DNA damage. We have shown that DmBrca2 and DmRad51 are involved in homologous recombination (HR) repair of DSBs induced by transposon excision, chemical mutagens and radiation. We have shown that repair of camptothecin-induced damage requires DmBrca2, and that the drug itself may be broken down by a mechanism that requires DmCyp6d2. Furthermore, mutations in *Cyp6d2* are unexpectedly widespread in Drosophila stocks. Lastly, we showed that DmHelQ and DmBlm promote accurate repair of DSBs via synthesis-dependent strand annealing and that the two proteins have distinct functions during HR. In this final chapter, we have proposed a number of experiments that could complement the genetic data presented in previous chapters or serve as starting points to investigating other roles of these proteins.

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