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A Genetic Analysis of Genomic Stability in *Caenorhabditis Elegans*: A Dissertation

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A Dissertation Presented

By

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Gene Function and Expression

A GENETIC ANALYSIS OF GENOMIC STABILITY IN *CAENORHABDITIS ELEGANS*

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DEDICATION

I dedicate my thesis in loving memory of my grandmother Emily Grabowski, my grandfather Bartolo Carreras, my Uncle Edward Grabowski, and my cousins Mary and Dorothy Donnelly.

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ABSTRACT

In humans, Bloom's Syndrome is caused by a mutation of the RecQ helicase BLM. Patients with Bloom's Syndrome exhibit a high amount of genomic instability which results in a high incidence of cancer. Though Bloom's Syndrome has been intensively studied, there are still many questions about the function of BLM which need to be answered. While it is clear that loss of BLM increases genomic instability, the other effects of genomic instability on the organism aside from cancer such as a potential effect on aging, have yet to be elucidated.

In Chapter II, I identify new phenotypes in the *C. elegans* ortholog of BLM, *him-6*. *him-6* mutants have an increased rate of cell death, a mortal germ line phenotype, and an increased rate of mutations. Upon further examination of the mutator phenotype, it was determined that the increased rate of mutations was caused by small insertions and deletions. The mutator phenotype identified in *him-6* mutants closely mimics the cellular phenotype seen in Bloom's Syndrome cells. This indicates that HIM-6 may behave in a similar fashion to BLM. In addition to the mutator phenotype, it was found that loss of *him-6* causes a shortened life span. This may provide evidence that there is a link between genomic stability and aging.

In Chapter III, I identify a new role for the transcription factor DAF-16. DAF-16 in *C. elegans* has been intensively studied and regulates a wide variety of pathways. In this chapter, I demonstrate via the well established *unc-93* assay that loss of *daf-16* causes a subtle mutator phenotype in *C. elegans*. This indicates that DAF-16 may play a role in suppression of spontaneous mutation. When I examined other classic genomic

instability phenotypes, I found at 25°C, the number of progeny in the DAF-16 mutants was significantly reduced compared to wild type worms. Additionally, I demonstrate *daf-16(mu86)* has a cell death defect.

This study identifies several new phenotypes caused by a loss of *him-6*. These phenotypes provide further evidence that loss of *him-6* causes genomic instability. In addition, this study also demonstrates that *him-6* has a shortened life span which may be due to genomic instability. Secondly, this study identifies a new role for DAF-16 in preventing the occurrence of spontaneous mutations. This may indicate a novel function for DAF-16 in maintaining genomic stability.

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STATEMENT OF CONTRIBUTION

In Chapter II, Nenad Svrzikapa, a technician in the Tissenbaum Lab, repeated the brood analysis and the *unc-93* assay experiments. The remainder of the experiments presented in this thesis was conducted solely by me.

CHAPTER I

Genomic Instability and Aging

While there are many theories as to why we age, a long standing theory is that aging may be due, in part, to genomic instability. It is believed that over time, the structure of DNA may be damaged due to both internal and external DNA damaging agents this damage, leads to cellular aging (Lindahl 1993). If the damaged DNA is not repaired, it may accumulate over time thereby causing genomic instability within the cells. When cells with unrepaired mutations undergo division, the damage will be passed on and perhaps result in aging of the organism (Lindahl 1993; Lombard et al. 2005).

This idea furthers the "disposable soma" theory in which somatic cells accumulate DNA damage over time and this damage is not repaired because individually, somatic cells are not crucial for the survival of the organism (Kirkwood and Holliday 1979). As genomic instability increases in the somatic cells, eventually a critical threshold is crossed and the cells begin to die. The accumulation of damage in nuclear DNA is particularly damaging to the cell as there are a limited number of copies of nuclear DNA and this must remain intact during the life time of the cell (Lombard et al. 2005). This increased cell death affects the organism via tissue degeneration and the decline in tissue function (Kirkwood and Holliday 1979; Vijg 2004).

The role of DNA repair and genomic stability in the aging process is also supported by the increase in the number of cells containing mutations such as translocations and insertions, as the organism ages (Vijg 2000; Lombard et al. 2005). While a link between increased mutations in the genome and aging has indeed been demonstrated, it is difficult to determine if the link is causal or correlative. The existence of segmental progeroid syndromes which have both a premature aging phenotype and a

DNA repair defect demonstrate the link to be causal. Additionally, while an argument for a correlative link may be made that a longer life span increases the chances of an increased amount of DNA damage, the damage occurring in the DNA is not repaired indicating that the role of genomic stability in aging may not be clear cut.

It has been shown that the percent of cells having major changes in the structure of the DNA increases six fold in elderly patients when compared to younger patients (Vijg 2000). In addition to the increase in mutations, it has also been demonstrated that gene expression in tissue changes during aging; this change in expression pattern could be due, in part, to the increased rate of mutation in older organisms (Vijg 2004). Genomic instability can be considered to be an important factor in cell and tissue degeneration, in an increase in the occurrence of cancer over time, and finally, in the activation of error prone repair mechanisms. The progressive degeneration of tissue, as well as the increase of age related illnesses, indicates a potential link between genomic stability and aging; however this potential link needs to be better elucidated. Through use of *C. elegans*, a model organism which does not contract age related illnesses and has been invaluable in understanding the role of various factors in longevity, I propose it is possible to determine a link between genomic stability and aging.

Caenorhabditis elegans (C. elegans) **as a model organism**

The small soil dwelling nematode *C. elegans* is a powerful model organism for the study of both the aging process and genomic instability because of the short life cycle and the median life span of *C. elegans* which is between 12 and 14 days (Braeckman and Vanfleteren 2007). The life cycle of *C. elegans* is very simple. It begins as an egg and

proceeds through four larval stages, each ending in a molt, to become an adult hermaphrodite. If faced with unfavorable conditions such as high temperature, overcrowding, or lack of food, the second larval stage proceeds into an alternate third stage known as the dauer stage (Braeckman and Vanfleteren 2007). Dauers are stress resistant, long lived and have a different morphology than a typical larval stage three worm (Braeckman and Vanfleteren 2007). Once conditions become favorable, the worm exits the dauer stage and resumes the life cycle at larval stage four. In addition to the short time it takes for *C. elegans* to develop, *C. elegans* has a short, highly reproducible life span making it possible to identify genes having a 10-20% increase or decrease in life span. There are two approaches to study aging and genomic stability in *C. elegans*.

The first is through screening via classical mutagensis or RNAi libraries to identify genes involved in both aging and maintaining genomic stability. The second approach takes advantage of the sequenced genome of *C. elegans*. The sequenced genome makes it possible to identify human homologs and study the effects of mutating these genes on the life span or genomic stability of the worm.

The assays to study both aging and genomic stability are well defined. To study genomic stability in *C. elegans,* assays include, but are not limited to, brood size analysis and composition, identification of a mortal germ line phenotype, and multiple immunofluorescence assays looking at the chromosomal structures in the germ line. To study aging in *C. elegans*, one of the most powerful tools developed is the life span assay. The life span of a population of worms is measured by tapping an adult worm every 2-3 days with a platinum wire to determine viability (Johnson and Wood 1982).

To better understand the role of genomic stability in aging, I chose to study HIM-6, a gene that when mutated, has been identified as causing genomic instability in multiple model organisms including *C. elegans*. HIM-6 is the *C. elegans* ortholog of the human BLM helicase. When the BLM helicase is mutated in humans, BLM causes Bloom's Syndrome which is characterized by a high incidence of cancer as well as other diseases associated with aging such as Type II Diabetes and Osteoporosis (German 1993). While work has been done in *C. elegans*, flies, mice and cell culture (as outlined in the following sections), there is still much which is unknown about the function of BLM and its role, if any, in the aging process.

Bloom's Syndrome and *Caenorhabditis elegans*

The *C.elegans* gene HIM-6 was identified because mutants of this gene displayed a **H**igh **I**ncidence of **M**ales (HIM) phenotype (Hodgkin et al. 1979). In a wild type population of worms, males typically arise in 0.2% of the population; however, in HIM mutants, this percentage can be increased to up to 30% of the population being male due to non-disjunction of the X chromosome (Hodgkin et al. 1979). The increase in non-disjunction of the X chromosome indicates a potential role for HIM-6 in maintaining genomic stability through prevention of chromosomal non disjunction.

It was determined that HIM-6 encodes the *C. elegans* ortholog of BLM by sequence comparison (Kim et al. 2002; Wicky et al. 2004). The helicase domain of HIM-6 was most similar to the human Bloom's protein and alignment of the full protein sequence demonstrated that HIM-6 was closely related to the human Bloom's protein

(Wicky et al. 2004). Given the Bloom's protein has been demonstrated to play a role in maintaining genomic stability, it is likely HIM-6 plays a similar role to the Bloom's protein in humans.

Multiple alleles of *him-6* have been isolated: *him-6(e1104)* contains a glycine^{561} to glutamine missense mutation at a highly conserved position in the helicase domain (Kim et al. 2002; Wicky et al. 2004), *him-6(e1423)* contains a premature stop codon caused by a T to A transversion midway through the helicase domain (Wicky et al. 2004), and finally *him-6(ok412)* has a 1,687 base pair deletion removing the entire helicase domain; this mutation is considered to be a null alelle (Wicky et al. 2004). While *him-6(e1423)* and *him-6(ok412)* both abolish helicase function, it is unclear if these are null alleles as Western blot analysis has not been conducted on these strains. Original characterization of *him-6(e1423)* and *him-6(e1104)* phenotypes found the strains may have a "sick" phenotype where the worms are smaller, slower, transparent and are less viable than wild type (Hodgkin et al. 1979). *him-6(ok412)* like the other *him-6* alleles has a lower than normal brood size and a high incidence of males (Wicky et al. 2004).

Further examination of the *him-6* phenotypes suggest the mutants have a low brood size due to fertilized eggs not hatching (Hodgkin et al. 1979). It is believed that both the HIM phenotype and the low viability of progeny result from chromosomal non-disjunction (Hodgkin et al. 1979). Upon further examination of *him-6* mutants, it was determined, that unlike other HIM mutants, *him-6* causes non-recombinant chromosomal nondisjunction in both autosomes and the X chromosome (Hodgkin et al. 1979). It was later determined that the increased rate of non-disjunction in *him-6* mutants was caused by a decrease in the number of chiasmata (Wicky et al. 2004). The decreased number in

chiasmata suggests that *him-6* mutants have a meiotic defect and when the crossover rate of *him-6(e1423)* and *him-6(e1104)* was examined, results showed a decrease in the rate of crossovers to less than half of wild type (Zetka and Rose 1995; Wicky et al. 2004). The meiotic defect demonstrated by *him-6* mutants may explain the genomic instability phenotype, it is possible HIM-6 plays a role in regulation of meiosis. Furthermore, the reduction in the rate of crossover events indicates HIM-6 is required at some capacity to facilitate crossing over. Perhaps HIM-6 can facilitate crossing over through its role as a helicase, HIM-6 can unwind the double stranded DNA making it available for cross over events to occur. In a *him-6* mutnat, the DNA is not unwound and cross over events are prohibited. It is also possible that HIM-6 may play a role in regulating and/or facilitating recombination as the cross over rate is decreased in *him-6* mutants. It would be interesting to determine why the increased rate of non disjunction is causing a reduced brood size, it would also be interesting to determine when the progeny of *him-6* mutants die.

In addition to germ line defects, *him-6(ok412)* and *him-6(e1423)* mutants are sensitive to irradiation and have a reduced level of germ cell apoptosis six hours after exposure to irradiation; however after 36 hours post irradiation levels of programmed cell death were comparable to wild type (Wicky et al. 2004). The initial reduction in apoptosis levels may indicate a defect in DNA damage response or perhaps there is a partial DNA checkpoint failure allowing cells which would normally be pushed into programmed cell death to survive. In contrast, *him-6(e1104)* has a cell death phenotype similar to wild type worms. However, the lack of a cell death phenotype could be due to *him-6(e1104)* being a weak *him-6* allele (Kim et al. 2005). Interestingly, it seems that

cell death in *him-6(e1104)* is dependent on *cep-1*, the p53 homolog in *C. elegans* (Kim et al. 2005).

Upon further characterization of *him-6(e1104)*, it was determined when *him-6(e1104)* embryos are irradiated between fertilization and the 50-100 cell stage, the number of embryos which survived was significantly reduced (Kim et al. 2005). However, eggs containing embryos allowed to develop for two hours after being laid did not show any viability defects upon irradiation (Kim et al. 2005). This data suggests HIM-6 plays an important role in defending the early embryo from ionizing radiation (Kim et al. 2005), however, it appears that if the DNA damage occurs early on in development, the worms cannot cope with the damage perhaps because a decision has been made to expend more energy on development rather than repair of the damage. It is also possible that oocytes do not have as much protection as the egg because of the lack of a shell, therefore oocytes are more sensitive to damage. It is interesting that after allowing the eggs to develop for two hours, the damage does not have an effect on viability suggesting there may be secondary DNA repair pathways which can repair the damage. When the chromosomes of the irradiated oocytes at the pachytene stage were examined, it was found that *him-6(e1104)* had an increased number of bivalents compared to wild type (Kim et al. 2005). Additionally, the oocytes appeared to have more abnormally formed chromosomes than wild type (Kim et al. 2005). Interestingly, when the embryos of *him-6(e1104)* exposed to UV damage were examined for viability, it was found that the *e1104* mutant behaved like wild type with similar decreases in viability indicating that *him-6(e1104)* is not sensitive to UV damage (Kim et al. 2005). This data suggests that HIM-6 plays a role in repairing specific types of DNA lesions.

him-6 mutants were exposed to hydroxyurea (HU) to determine if there was a cell cycle defect. HU is a ribonucleotide reductase inhibitor that results in the blocking replication fork progression from early origins of replication; additionally HU activates a checkpoint which inhibits the ability of late origins to fire (Dasika et al. 1999). In *C. elegans,* when wild type worms at the L4 stage are treated with HU, mitotic germ cell arrest occurs; this treatment causes the mitotic germ cell nuclei to swell in size (Ahmed et al. 2001). Upon exposure to HU, *him-6* mutants show an increase number of mitotic germ cells with smaller nuclei than wild type indicating a defect in the S-phase check point in cell cycle (Wicky et al. 2004). This may explain why the cell death levels in *him-6* mutants are lower than wild type worms. There is not an S-phase checkpoint to halt the cell cycle to force the cells to repair the damage to the DNA.

Through genetic analysis, it is possible to identify potential interactors with HIM-6; one interactor identified is TOP3α (Kim et al. 2002). TOP3α in both humans and *C. elegans* is a topoisomerase which is responsible for cutting the DNA to remove knots or unwinding DNA which has become too twisted (Kim et al. 2002). In many systems it has been demonstrated that the BLM ortholog would interact with the TOP3α ortholog; therefore it is not surprising that these two genes would interact in *C. elegans*. In fact, the interaction between TOP3α and HIM-6 strengthens the argument that *C. elegans* can be used as a successful model to study both the function of HIM-6 as well as genomic instability.

In worms, TOP3α is required in germ cell proliferation, oogenesis, and embryogenesis (Kim et al. 2002). When *top-3α* function is reduced by RNAi in a *him-6(e1104)* background, half of the progeny become sterile at adulthood (Kim et al. 2002).

When the chromosome structure of the sterile adults was examined, most chromosomes appeared to be either fragmented or lumped together and additionally, the chromosomes were irregular in both shape and size when compared to the chromosomes in the nuclei of wild type worms (Kim et al. 2002). Furthermore, there is an increase in the number of RAD-51 foci in the *top-3α(RNAi); him-6(e1423)* double mutant which suggests there is a significant increase in the amount of double stranded breaks (Wicky et al. 2004), perhaps this increase in double stranded breaks is correlated to the reduction o f the number of cross over events seen in *him-6* mutants. The increase in double stranded breaks also suggests that TOP3α and HIM-6 play a role in resolving double stranded breaks. In the *top-3α(RNAi); rad-51(lg08701) him-6(e1432)* triple mutant, the phenotypes characteristic of *top-3α(RNAi); him-6(e1423)* are rescued and the nuclei can progress normally through meiotic prophase suggesting that the phenotypes are dependent on RAD-51 function (Wicky et al. 2004).

Another gene that interacts with HIM-6 is DOG-1. While HIM-6 may not interact physically with DOG-1, it has been shown that these two genes function in a synergistic fashion (Youds et al. 2006). DOG-1 is a helicase that was first identified in *C. elegans* and was found to prevent deletions in polyG/polyC tracts as well as to resolve G-quadruplex structures (Cheung et al. 2002). Given that BLM can also unwind G-quadruplex structures (Sun et al. 1998), Youds *et. al.* investigated the possibility that HIM-6 can act in conjunction with DOG-1. When the *dog-1; him-6* double mutant was created, it was found that the worms had reduced viability and could only be maintained for a finite period of time 20° C and 25° C indicating a mortal germ line phenotype (Youds et al. 2006). In addition, the worms produced fewer numbers of progeny and had

a significant increase in the percentage of males (Youds et al. 2006). This indicates a high amount of genomic instability occurs in the double mutants and this genomic instability was partially due to an increase in chromosomal non-disjunction. When the germ line nuclei of the *dog-1; him-6* mutants were examined, it was found that the double mutant had significantly larger nuclei in the germ line than the single mutants alone suggesting *dog-1; him-6* was under more replicative stress than the single mutants (Youds et al. 2006). Larger nuclei indicate a cell has been exposed to genotoxic stress, upon exposure to genotoxic stress, the cell cycle is arrested by a damage checkpoint allowing the cell to repair the damage (Gartner et al. 2000). This data indicates perhaps loss of *him-6* as well as *dog-1* prevent damage from being repaired in the cell resulting in enlarged nuclei. *him-6* mutants have an S-phase checkpoint defect and it would be interesting to determine if loss of DOG-1 can affect cell cycle checkpoint to determine at which checkpoints the cell cycle is being arrested at. Given the nuclei of the *dog-1; him-6* double mutant were larger than either of the single mutant, this data suggests perhaps these genes function similarly but in different pathways.

While some work has been done to further elucidate the function of HIM-6 in *C. elegans*, there are still many unanswered questions. The first and foremost question that remains to be answered is the biological function of HIM-6 similar to that of the Bloom's protein. If the functions are indeed similar, one can hypothesize that the increase in chromosomal non-disjunction may result from an inability of *him-6* mutants to completely unwind DNA during mitosis or meiosis. Additionally, the inability of *him-6* mutants to unwind DNA may also explain why there is a lower number of cross over events, the DNA may not be unwound enough to allow for a crossover event. It is

possible that the DNA damage that is caused by exposure to irradiation cannot be repaired early on because HIM-6 may be required for embryonic development which would also serve to explain the lower brood size that is typical of *him-6* mutants. The phenotypes that are seen in *C. elegans* seem to mimic phenotypes in higher organisms which have lost Bloom's protein function. This demonstrates that *C. elegans* can be a useful model for the study of Bloom's protein function.

Bloom's Syndrome and *Drosophila melanogaster*

Similar to *C. elegans*, in *Drosophila melanogaster* there is one protein which is homologous to human BLM. *Dmblm (**D**rosophila **m**elanogaster **Bl**oom's)* contains a helicase domain with a DExH box and an ATP binding site (Kusano et al. 1999). The location *Dmblm* was mapped and found to correspond with an already identified gene, *mus309* (Boyd et al. 1981; Kusano et al. 2001). The *mus309* phenotypes were rescued by a *Dmblm* transgene, thus confirming *mus309* is *Dmblm* (Kusano et al. 2001). Multiple alleles of *mus309* have been identified. Two of the original alleles, $mus309^{D3}$ and $mus309^{D2}$, disrupt the helicase domain and have become homozygous lethal due to secondary mutations (Kusano et al. 2001). $mus309^{D3}$ has a lysine to glutamic acid substitution in the conserved helicase motif II in addition to another amino acid mutation in the C-terminus (Kusano et al. 2001). This allele is proposed to contain no helicase activity although the protein may still be expressed (McVey et al. 2007). $mus309^{D2}$ has a premature stop codon between helicase motifs III and IV (Kusano et al. 2001); this mutant is considered a complete loss of function allele as no product is seen after RT PCR (McVey et al. 2007). Recently, several other *mus309* alleles ($mus309^{N2}$, $mus309^{N3}$,

and *mus309*^{N4}) have been isolated in which the N-terminal region has been disrupted (McVey et al. 2007). The N-terminus mutations are in the same genetic background and flies homozygous for these mutations are viable (McVey et al. 2007).

Original characterization of *mus309*^{D3} and *mus309*^{D2} mutants before they acquired the secondary lethal mutation, determined that the mutants are sensitive to the DNA alkylating agents MMS (methyl methanesulfonate) and HN2 (nitrogen mustard also know as di(2-chloroethyl) methylamine) (Boyd et al. 1981). This indicates a role for *DmBlm* in repairing DNA damage.

Even more interesting however, was that *mus309*^{D2}*/DF(3R)T-7* males, like human males with Bloom's Syndrome, are sterile (Kauli et al. 1977; Boyd et al. 1981; Kusano et al. 2001). Upon further examination of the male sterility phenotype, *mus309*^{D2} males showed a significant increase in the rate of chromosomal loss and chromosomal nondisjuntion compared to wild type and heterozygous males (Kusano et al. 2001). This indicates that HIM-6 and *DmBlm* may function in similar manners as loss of either of these genes causes an increase in chromosomal non-disjunction. The increase rate of chromosomal loss and nondisjuction is a possible indicator of DNA repair defect (Kusano et al. 1999). The sperm of the *mus309* mutant males also appeared to lose one of the dominant markers on the Y chromosome (Kusano et al. 2001). The loss of the marker may indicate both chromosomal breakage as well as that part of the Y chromosome being lost (Kusano et al. 2001).

Female flies, like male flies, are affected by the loss of *mus309*. It was demonstrated that females mutant for *mus309* would have a lower level of fertility (Boyd et al. 1981; Beall and Rio 1996; Kusano et al. 2001). Females with a *mus309* mutation in

the helicase motif could lay visually normal eggs in the same number as wild type flies; however, there was a high percentage of embryonic lethality (McVey et al. 2007). When proceeding though syncytial nuclear division, these mutant embryos had a significant increase in the number of visible defects such as anaphase bridges and gaps in the monolayer of the nuclei (McVey et al. 2007). This data may suggest a role for *DmBlm* in chromosomal segregation as the chromosomes in the mutants do not separate properly as indicated by the anaphase bridges and other phenotypes. This may also imply a role for *DmBlm* in regulating mitosis. The increase in anaphase bridging may also help to explain the increase in chromosomal non-disjunction seen in *mus309* mutants. In *mus309* female mutants with an N-terminus deletion, a higher percentage of the progeny hatched in comparison to the helicase mutants but the percentage which hatched was still lower than that of wild type flies (McVey et al. 2007). This data suggests a requirement for *mus309* in embryogenesis (McVey et al. 2007). It would be interesting to determine if the embryos which die develop normally or their death is caused by another factor such as chromosome abnormalities.

While a role for *mus309* has been identified in embryogenesis, the extremely low hatch rate of the *mus309* helicase mutants made it difficult to determine if *mus309* played a role in meiosis. The creation of $mus309^{N2}$ allowed for the examination of meiosis in *mus309* mutants. Upon examination of $mus309^{N2}$, it was found that there was a significant increase in the rate of non-disjunction that resulted from faulty meiotic recombination (McVey et al. 2007). Furthermore, the rate of crossovers in $mus309^{N2}$ females was found to be half of the crossover rate seen in wild type females (McVey et al. 2007). This demonstrates a role for *DmBlm* in recombination as well in chromosomal

segregation. Perhaps during recombination events in *mus309* mutants, the DNA does not become fully unwound resulting in aberrant chromosomal structures and like its human homolog, *Dmblm* can unwind these structures. It is also possible that the lower number of crossover events results from an inability to unwind the DNA between the sister chromatids.

In addition to *mus309*'s role in embryogenesis and recombination during meiosis, it has been demonstrated that *mus309* mutants cannot repair double stranded breaks indicating a role for *DmBlm* in both recombination and DNA repair. Double stranded breaks can be induced by P element excision. During P-element excision, large deletions in the sequence occur, and these deletions are not repaired in *mus309* mutants (Beall and Rio 1996). It is possible these large deletions were not repaired because *mus309* mutants could not synthesize long sequences of DNA to repair the sites of damage (Adams et al. 2003). In fact, it was determined that *mus309* is required to synthesize tracts of DNA greater than 14 kb, these stretches of DNA can then be used to repair the gap resulting from P element excision (McVey et al. 2004). If gap repair is not possible due to a lack of *mus309*, large segments of DNA flanking the double stranded break become deleted (Adams et al. 2003; McVey et al. 2004). The inability to synthesize large tracts of DNA, indicates another role for *Dmblm* in which it is involved in DNA synthesis.

In addition to large sequences of DNA being lost during P element excision, small deletions of DNA may also occur (Min et al. 2004). In *Drosophila* cells treated with *mus309* RNAi, there is an increase in the small deletions of 1-49 base pairs (Min et al. 2004). This increase in smaller deletions may be the result of the inability of *mus309* to unwind the DNA at the site of double stranded breaks *Dmblm* may function to initiate

DNA repair via cross over events (Min et al. 2004). However, unlike a complete loss of *mus309*, cells with a reduction in *mus309* can still repair the damage but with a much lower level of fidelity (Min et al. 2004).

It is possible this defect in double stranded break repair may be caused by an inability of *mus309* mutants to use the Synthesis-Dependent Strand-Annealing pathway (SDSA) (Adams et al. 2003; McVey et al. 2007). In fact, it was demonstrated that SDSA typically did not occur without *mus309* with an intact helicase domain present; however, DNA synthesis did occur at least one end of the double stranded break indicating that *mus309* cells do attempt to repair double stranded breaks but the repair is unsuccessful (Adams et al. 2003). The loss of the SDSA pathway may force the *mus309* mutants to repair the DNA damage by using other faulty secondary repair pathways which can generate large deletions such as non-homologous end joining (Gaymes et al. 2002; Adams et al. 2003).

The effect on the SDSA pathway was not limited to only helicase domain mutants. While loss of the N-terminus of *mus309* resulted in a milder embryonic phenotype, the disruption of the SDSA pathway in $mus309^{N2}$strain, is similar to the helicase mutants (McVey et al. 2007). This indicates that the N-terminus of *mus309* is also important in maintaining a functional SDSA pathway (McVey et al. 2007).

Like HIM-6, *mus309* seems to play a role in both DNA recombination, DNA repair and perhaps DNA synthesis; however, like HIM-6 the role of *mus309* as a helicase has not been defined. The first question which should be addressed is what the biochemical role of these genes is. Given that both *him-6* and *mus309* mutants share similar phenotypes; it suggests that the role of the BLM protein is highly conserved

through evolution. This similarity of the phenotypes between both flies and worms suggests that perhaps the increased amount of chromosomal non-disjunction is due to an inability to unwind DNA during recombination events. A second possibility is *Dmblm* may be involved in facilitating chromosome segregation by resolving aberrant chromosomal structures formed during replication. Both of these possibilities could explain the increased amount of anaphase bridging and the aberrant chromosomal structures that is seen in the mutants. Through use of model organisms, it is possible to gain a better understanding of the role of BLM in maintaining genomic stability. It would be interesting to determine which repair pathways *mus309* mutants use to repair DNA lesions as the SDSA pathway is no longer functional in the mutants.

Bloom's Syndrome and Mice

Mouse *BLM* is 81% identical to human BLM at the nucleotide level and 76% identical at the amino acid level (Seki et al. 1998). Further characterization of *mBLM* by Southern blot analysis found that only a single copy of the gene existed in wild type mice (Seki et al. 1998). *mBLM* is expressed in multiple tissue types such as the spleen, thymus, and ovary, and the highest level of expression of *mBLM* is in the testis (Chester et al. 1998; Seki et al. 1998). Like human BLM, *mBLM* is also a 3' to 5' DNA helicase that is ATP dependent (Bahr et al. 1998).

Unlike flies and worms, the *BLM* gene in mice may be required for embryonic viability (Chester et al. 1998; Luo et al. 2000; Goss et al. 2002). When initial attempts were made to create a $Blm^{-/-}$ mouse by targeting exon 8, which is upstream of the *Blm* helicase domain, only mice that were $Blm^{+/-}$ mice were identified; these mice were

phenotypically wild type (Chester et al. 1998). Subsequent attempts by this group did yield a viable $Blm^{-/-}$ mutant; this mutant, $Blm^{tm4Ches}$, is a conditional knockout where exon 8 is flanked by two *loxP* sites (Chester et al. 2006).

The mouse strain $Blm^{m2/m2}$ is a putative null allele as each of the three ORFs have a premature stop codon; if the mBLM protein is produced, it is severely truncated (Luo et al. 2000). A second mouse strain, $Blm^{m3/m3}$, has an extra copy of exon 3 that results in a truncation of the *Blm* protein due to a frameshift mutation (Luo et al. 2000). Like $Blm^{-/-}$ mice, $Blm^{m2/m2}$ mice, were embryonic lethal while $Blm^{m3/m3}$ mice were viable (Luo et al. 2000). Similar to previous mouse models (Chester et al. 1998), mice that were heterozygous for *Blm* were phenotypically normal (Luo et al. 2000).

A fifth mouse model for Bloom's Syndrome was created using a 6 base pair deletion and a 7 base pair insertion to cause a frameshift mutation in exon 10 which introduces a premature stop codon in mBLM; in humans, this mutation typically occurs in Ashkenazi Jews who have a high incidence of this disease (Ellis et al. 1995; Goss et al. 2002). In contrast with the previous groups who had used PGKneo cassette to disrupt *Blm* (Chester et al. 1998; Luo et al. 2000), this group removed exons 10, 11, and 12 to simulate BLM^{Ash} by using a *Hprt* cassette creating the mouse strain $Blm^{cin/cin}$ (Goss et al. 2002). As previously seen in the cases of $Blm^{-/-}$ and $Blm^{m2/m2}$, the $Blm^{cin/cin}$ mouse had an embryonic lethal phenotype (Goss et al. 2002). The $Blm^{cin/+}$ mouse was viable and was found to have a reduction in the amount of wild type *Blm* that was produced. This model allows the consequences of *Blm* haploinsufficiency to be studied (Goss et al. 2002).

To characterize the embryonic lethal phenotype see in $Blm^{-/-}$, 17 day post coitum (dpc) embryos where dissected and examined via Southern blot (Chester et al. 1998).

Out of the nine dissected embryos, only one was *Blm*$^{-/-}$ demonstrating that *Blm*$^{-/-}$ caused an embryonic lethal phenotype (Chester et al. 1998). Upon further examination of the *Blm*$^{-/-}$ embryos, it was determined that *Blm*$^{-/-}$ embryos died at 13.5 dpc; importantly, it was determined that no *Blm* had been transcribed indicating a role for *Blm* in maintaining embryonic viability (Chester et al. 1998). Before death, *Blm*$^{-/-}$ embryos are typically smaller and were developmentally delayed when compared to their heterozygous and wild type litter mates (Chester et al. 1998).

While the embryos may be smaller, their bodies are proportional and all of the hallmarks of development from 9.5 to 13.5 dpc including the number of somites, forming of limb buds, and heart and placenta development are reached (Chester et al. 1998). This indicates that embryonic lethality did not result due to defects in the organs or the tissue as everything formed normally but did so at a slower pace (Chester et al. 1998). At the same time, it appears that the initial rate of apoptosis increases dramatically in the mutant embryo compared to wild type, however at day 9.5, the level of apoptosis decreases to wild type levels (Chester et al. 1998). It should be noted that not all BS mice models develop slowly, the null *Blm*$^{m3/m3}$ viable mouse strain developed at a normal rate suggesting there may be another reason for the slower development of the *Blm*$^{-/-}$ embryos (Luo et al. 2000). One reason for the slow growth may be the mutation which created the *Blm*$^{-/-}$ embryos may result in toxic gene products, while the 4.4 kb *Blm* transcript was not present in *Blm*$^{-/-}$ embryos, there were several transcripts with different mobility present that were not present in wild type (Chester et al. 1998). Additionally, the slow growth phenotype may have resulted from the developmental clock being slowed as the embryos of the *Blm*$^{-/-}$ mice had normal body morphology (Chester et al. 1998).

The *Blm*$^{-/-}$ embryos also seemed to have a lack of blood circulating through vessels in the embryo and the yolk sac; upon dissection the embryos were very pale, indicating anemia (Chester et al. 1998). It was determined that there was a decrease in the number of mature and progenitor red blood cells as well as a decrease in the volume of blood in embryos at 12.5 dpc (Chester et al. 1998). It is possible that the lack of red blood cells is responsible for the embryonic lethality; additionally without the red blood cells to provide oxygen, perhaps the growth of these embryos is slowed. Upon further examination of the erythrocytes, it was found there was a high percentage of cells with micronuclei indicating chromosomal defects (Chester et al. 1998). The increased numbers of micronuclei phenotype was also seen in primary lung fibroblasts of *Blm*$^{cin/+}$ mutants (Goss et al. 2002). The increase in the number of micronuclei were once again seen in mammary tumor cell lines which were derived from *Blm*^{tm4Ches}; in this case, although the numbers of micronuclei in the seven cell lines was variable, all were significantly higher than that seen in the control cells (Chester et al. 2006). This indicates that *mBLM* may play a role in chromosomal segregation. It is also interesting that these cells with gross chromosomal defects still survive. It would be interesting to determine if loss of *mBLM* can also cause a cell death defect. It would also be interesting to determine how much damage is required to push a cell without *mBLM* into apoptosis; given these cells can still replicate, it implies that there is a threshold that is not yet reached.

In addition to the increase in micronuclei in *Blm* mouse embryonic fibroblasts (MEFs), there is a significant increase in the rate of sister chromosome exchanges (SCEs) when compared to wild type (Chester et al. 1998; Luo et al. 2000; Chester et al. 2006). Furthermore, this increase in the number of SCEs was also demonstrated in *Blm*$^{m1/m3}$

embryonic stem cells that were examined at metaphase using a chromosome spread (Luo et al. 2000). The increase in SCE's is interesting as it implies that attempts are being made to repair DNA damage. This may indicate *Blm* null mice are subjected to more genotoxic stress than their wild type litter males. Perhaps this genotoxic stress is the result of defects during chromosome segregation. However, this phenotype was only seen in *Blm* null mice as the $Blm^{cin/+}$ mice did not display an increase in the rate of SCEs (Goss et al. 2002). Based on this data, it is possible to determine that BLM may function to block aberrant recombination.

Interestingly, in $Blm^{m3/m3}$ mutants there was not an affect on the rate of meiotic crossover in male or female meiosis was not affected (Luo et al. 2000). This *Blm* mutation did increase the somatic loss of heterozygosity through an increase in the rate of mitotic recombination (Luo et al. 2000). To determine if there was an effect on mitosis, cells were first treated with nocodazole, to depolymerize microtubules, then the cells were released and the number of anaphase laggards (loose chromosomal pieces between daughter cells which are separating) in the culture were then scored (Chester et al. 2006). It was found that the percent of anaphase laggards was greatly increased in the $Blm^{tm4Ches}$ tumor cell lines (Chester et al. 2006). Like its homologs in worms and flies, *mBLM* plays a role in recombination and chromosomal segregation. It is possible to speculate that *mBLM* may function to resolved junctions formed between the chromosomes. It is also possible that loss of the helicase function of *mBLM* may cause the DNA to not unwind fully and therefore cause the chromosomal abnormalities that are seen in cell culture.

Given the apparent genomic instability that loss of *Blm* causes in cell cultures, it was unsurprising that *Blm* null mice also had a cancer phenotype. In the viable

homozygous null mutants for *Blm*, specifically $Blm^{m3/m3}$, there was an increase in the rate of tumors which developed once the mice reached 20 months of age (Luo et al. 2000). Like BS patients, these mice do display a wide variety of tumors such as lymphomas, sarcomas, and carcinomas (Luo et al. 2000). In addition to the formation of cancerous tumors, the mice also had the potential to be affected by benign tumors (Luo et al. 2000).

When the cells of $Blm^{tm4Ches}$ mice were placed onto soft agar, it was found that they could be transformed into cancer cells at a much higher rate than the control cells (Chester et al. 2006). These tumor cells were analyzed for abnormal chromosome structures such as metacentric end-to-end chromosome translocations, abnormally small chromosomes, and small marker chromosomes. It was found that these cell lines had a higher rate of chromosomal abnormalities than the control cells (Chester et al. 2006). In addition to the increase in abnormal chromosome structures, it was also found that the nuclei of these cell lines also had abnormal morphology (Chester et al. 2006). The nuclei were seen to have lobes, multiple micronuclei, or there were multiple nuclei within the cells (Chester et al. 2006). Furthermore, when exon 8 was deleted in these cell lines via *Cre*, the cell lines generated had a 3.8% chromosome loss per cell compared to control cell lines (Chester et al. 2006). Not only does this demonstrate a role for *Blm* in chromosome segregation; it implies that in *Blm* null mice, there is a cell cycle defect as these cells are allowed to replicate. It would be interesting to determine what the role of BLM plays in cell cycle regulation in mice.

While many of the *Blm* mice phenotypes mimic what has been seen in humans, it is apparent that the study of BLM function in mice is complicated. This is unsurprising as the role of BLM in maintaining genomic stability is complicated. The data from mice

models indicates a role for BLM in recombination, repair, and chromosomal segregation. Perhaps these roles can be traced back to BLM being a helicase. Without the ability to unwind DNA, recombination, repair and chromosomal segregation would be hindered. These processes still seem to occur indicating that there are other helicases which may fill the function of BLM, but may not do the job as well. It would also be interesting to determine why the cell cycle in the *Blm* cells allows the cells with these apparent abnormalities to proceed normally through cell cycle. It would be interesting to determine if there is a cell death defect caused by a loss of BLM.

The role of BLM in recombination and repair serves to explain why loss of BLM causes cancer; however, the research in mice has been limited by the confusion concerning the role of BLM in preventing embryonic lethality. While brood size in worms and flies is reduced in *Blm* mutant strains, it is still possible for these organisms to produced progeny. This indicates that while BLM maybe be important in mice to maintain embryonic viability, this trait is not evolutionarily conserved. It would be interesting to determine if BLM plays a role in embryonic viability or the lethality is a result of increased genomic instability.

Bloom's Syndrome (BS) and Humans

Bloom's Syndrome is a recessive syndrome that, while rare, occurs most prevalently in the Ashkenazi Jewish population where a specific mutation in BLM occurs in 1 out of every 107 individuals (German et al. 1965; German 1993; Ellis and German

1996; Li et al. 1998). Patients born with Bloom's Syndrome have a drastically shortened life span. In 1997, the median age of patients still alive was 21.6 years (German 1997), however it is unclear if patients with Bloom's Syndrome died due to a secondary illness rather than their death being caused directly by BS. It is difficult to determine if BS has an effect on life span. In addition to a shortened life span, physically, patients with Bloom's Syndrome are smaller than average (German 1993). The head of a Bloom's Syndrome patient is usually smaller, and the skull and face are unusually shaped; however, with these two exceptions, the body of those with Bloom's Syndrome is normally proportioned (German 1993). This is interesting as it implies BLM may play a role in growth and development as has been demonstrated in other model organisms. Once again however, it will be need to be determined if the growth and developmental is due to loss of BLM or if the defect is due to a increase in the rate of mutations. Even though the head of the patients is smaller, most patients exhibit normal intelligence along with some form of a learning disorder (German 1993). At birth, the skin of these individuals is normal, however, as the patient ages, an erythema will develop on the cheeks or nose and as the patient becomes exposed to the sun, the erythema can become chronic and spread (German 1993).

In addition to the stature and characteristic facial lesion, patients with Bloom's Syndrome also suffer from fertility issues. Males have smaller testes than normal and the semen contains no sperm causing the males to be infertile; it is thought that male sterility in BS patients is caused by damage to the tubular elements in the testes (Kauli et al. 1977; German 1993). Even though the males are infertile, they still undergo normal sexual development and puberty (Kauli et al. 1977). In the case of female patients, child birth is

possible, however, for most, menstruation is irregular and will halt at a young age (German 1993).

One of the first cellular phenotypes associated with BS is the appearance of broken and rearranged chromosomes (German et al. 1965; German et al. 1974; Wang et al. 2000). In addition to chromosomal abnormalities, there is a high frequency of isochromatid breaks, telocentric chromosomes which were produced by transverse breakage at the centromere, and quadriradial chromosomes (German et al. 1965; German et al. 1974). In fact, it is possible that the persistence of these quadriradial chromosomes may be due to the inability of BS cells to resolve G4 DNA structures (Sun et al. 1998). This implies a defect in chromosome segregation, perhaps this defect is caused by an inability to unwind DNA with a high level of fidelity.

This observation lead to the hypothesis that, in addition to the visible phenotypes associated with BS, there was also a high amount of genomic instability. When BS cells were cultured over a period of 10 days, it was found that the rate of spontaneous mutation increased in both wild type and BS cells using 6-thioguanine-resistance as a marker; however, the increase was more significant in BS cells (Warren et al. 1981). This data suggests that BS causes a significant increase in the amount of spontaneous mutation in cells (Warren et al. 1981; Vijayalaxmi et al. 1983). It is unclear what the nature of these mutations are, it would be interesting to determine what types of mutations are caused as this could potentially shed light on the role of BLM in preventing genomic instability.

The genomic instability seen in BS cells may be due in part to another hallmark of Bloom's Syndrome, an increased rate of Sister Chromatid Exchanges (SCE). While the SCEs can take place at a high frequency without deleterious effects, at times they may

introduce mutations into the cells. When a unique tandem repeat locus on chromosome 1, D1Z2, was examined in BS cells, it was found that in the first generation of cell culture, no mutations were introduced; however, it was determined via southern blot that in the second generation multiple mutations had been introduced into the D1Z2 locus (Groden and German 1992). It is thought that the mutations that are introduced to this particular locus may be caused by short deletions or duplications within the locus (Groden and German 1992). This implies that the cells may be incapable of repairing the damage or it is loss of BLM which causes new mutations to be introduced. The inability to unwind chromosomes during replication or recombination may also prevent the proteins that are responsible for proof reading from sensing the damage and therefore repairing the damage.

The large amount of spontaneous mutation in BS cells lead to the determination that BS causes a mutator phenotype (Warren et al. 1981; Vijayalaxmi et al. 1983; German 1993). It is this apparent genomic instability that leads to another hallmark of Bloom's Syndrome, cancer (German 1993). Cancer in BS patients appears at a very young age with the median age being 24.4 years (German 1993). In the initial 150 patients that were diagnosed with Bloom's Syndrome, the rate of cancer was startling; there were 118 diagnosed neoplasia of which 86 were malignant (German 1993). Interestingly, further studies of the neoplasms that appeared found there was a high amount of diversity in both the types and the sites where the neoplasms occurred (German 1993). While the sites and the types of cancers differ from patient to patient, trends have been identified; for example, during childhood, there is a higher risk of leukemia (German 1993). In both children and adults, there is a risk of lymphomas;

while carcinomas appear during adolescence and are found mostly in adults (German 1993). In addition to the more common types of cancers, patients have also been diagnosed with rarer forms of cancer including osteosarcoma, Wilms tumor, medulloblastoma and meningioma (German 1993). In fact, it is cancer and lung disease that are responsible for one third of the deaths of patients with Bloom's Syndrome (German 1997). The high incidence of cancer caused by mutation of the BLM helicase made BLM an attractive gene to study to better understand genomic instability.

The BLM gene is located on chromosome 15 at 15q26.1. The BLM protein is composed of 1417 amino acids, and has a predicted molecular weight of 159 kDa (Ellis et al. 1995; Ellis and German 1996). A Walker box, which usually correlates with ATPase activity, was first identified by early domain predictions and was shown subsequently to have ATPase activity (Karow et al. 1997). In addition to the Walker Box, the BLM sequence also had a DExH box which is common among all members of the RecQ helicase family (Karow et al. 1997). Further analysis confirmed that like other family members, BLM is a helicase that can unwind DNA in a 3' to 5' manner and is dependent on Mg^{2+} and ATP (Karow et al. 1997).

There are many different mutations in the BLM protein which can cause Bloom's Syndrome. The most common, the *blm*^{ASH} mutation, is comprised of a 6 base pair deletion and a 7 base pair insertion which causes a frameshift mutation that introduces a premature stop codon (Ellis et al. 1995). This mutation reduces the levels of BLM mRNA and when homozygous, it is believed no active BLM is present in the cells of these patients (Ellis et al. 1995). In the *blm*^{ASH} cells, while full length BLM protein is encoded, there is a lack of helicase activity (Neff et al. 1999). In addition to the *blm*^{ASH}

mutation, when the BLM gene of thirteen patients with BS was analyzed in the mid 1990's, seven unique mutations were identified (Ellis et al. 1995). Four of these mutations caused premature stop codons and the remaining three caused amino acid substitutions (Ellis et al. 1995). Further analysis of the four premature stop mutations in *Blm* showed that in each case none of the cell lines contained all of the seven helicase domains and once again, it is believed that patients who are homozygous for this mutation do not produce active BLM (Ellis et al. 1995). In the case of the amino acid substitutions, two occurred in the conserved RecQ helicase domain and the other was found in the C-terminal region, however, further analysis to determine if these amino acids substitutions causes a null mutant in BLM (Ellis et al. 1995). More recently, an additional 64 mutations in BLM have been identified which encompass single base substitutions, small insertions, deletions and duplications (German et al. 2007). When Northern blot analysis was conducted on 52 cell lines with these mutations, 29 of the cell lines showed a significant reduction in BLM mRNA while the remaining lines had either normal or intermediate levels of BLM mRNA indicating perhaps a recombination event or reversion had occurred to restore BLM function, however, patients from whom these cell lines were derived still have Bloom's Syndrome indicating there may be a secondary cause of BS (German et al. 2007).

The helicase activity of BLM is specific; while BLM has a strong affinity for X-junction DNA (similar to what is seen in Holliday Junctions), it cannot unwind short substrates with a 3' tail that are 250 base pairs or less (Karow et al. 2000). In X-junctions, it was found that BLM interacts specifically with the crossover region and not the arms or the blunt ended linear region (Karow et al. 2000). While BLM can disrupt X-junction

DNA, the concentration of BLM required *in vitro* is much higher than for other aberrant DNA structures such as D-loops and G-quadruplex DNA (Bachrati et al. 2006).

BLM has the ability to resolve D-loop structures which are formed early on during homologous pairing between two DNA strands that are going through an exchange (van Brabant et al. 2000; Bachrati et al. 2006). However, in order to resolve these D-loops, a stretch of 3' single stranded DNA is required (van Brabant et al. 2000; Bachrati et al. 2006). In addition to the BLM helicase's ability to unwind X-junctions and resolve D-loops, the BLM helicase can also unwind G4-DNA in the presence of a 3' single stranded region and ATP (Sun et al. 1998). G4-DNA is formed when four strands of DNA containing stretches of guanines become stabilized by Hoogsteen bonding (Sen and Gilbert 1988; Sen and Gilbert 1990). Interestingly, it was demonstrated that BLM is better at unwinding G4 DNA than duplex DNA (Sun et al. 1998). This data supports the hypothesis that BLM function to resolve recombination events in cells. It is possible to hypothesize that without BLM, perhaps a second protein may attempt to unwind these structures. The attempt is unsuccessful and this may introduce additional mutations into the DNA.

In addition to being able to unwind DNA intermediates as well as aberrant DNA structures, BLM is also required for restarting the replication fork when replication has been blocked (Davies et al. 2007; Rao et al. 2007). In BS cells, there is an increased amount of stalled replication forks and the ability to recover from replication fork blockage is severely hindered (Davies et al. 2007; Rao et al. 2007); this increase may be directly correlated to the ability of BLM to resolve aberrant chromosomal structures. In order to cope with the blockage of replication, BS cells have a 4-fold increase in the

number of new DNA replication sites that appear at close proximity to each other when compared to wild type cells (Davies et al. 2007; Rao et al. 2007). Perhaps this increase in replication sites exacerbates the increase in the amount of mutations seen in BS cells. It is thought that the increase in the number of replication sites in BS cells is to ensure that the genome is completely duplicated during DNA replication (Rao et al. 2007). In wild type cells, it has been demonstrated that BLM has the ability to cause regression of an *in vitro* model of a stalled replication fork (Ralf et al. 2006). The stalled replication fork was created by annealing one plasmid with a 68 nucleotide single stranded gap to an almost identical plasmid labeled on the 5' end with a complementary 46 nucleotide single stranded gap; on each of these plasmids there are several restriction endonuclease sites at specific locations (Ralf et al. 2006). The complex was then treated with topoisomerase I to interlink the plasmids (Ralf et al. 2006). The regression of the stalled replication fork is determined by exposing the construct to various restriction enzymes, as the fork regresses the appearance of restriction endonuclease sites will allow for DNA fragments of various lengths to be formed determining how far the replication fork can regress (Ralf et al. 2006). Based on this assay, it was determined regression can move the stalled replication fork backwards a minimum of 250 base pairs (Ralf et al. 2006). It is unclear why BLM functions in this fashion but it is attractive to speculate that BLM may function to keep stalled replication forks intact preventing the creation of double stranded breaks (Ralf et al. 2006).

The BLM protein in human fibroblasts is found in one of three subnuclear distributions: in small foci, in larger diffuse areas, or completely diffused throughout the cell (Ellis et al. 1999; Neff et al. 1999; Yankiwski et al. 2000). Upon further

examination of BLM localization, it was found that BLM would localize to anaphase bridges and during late mitosis BLM would localize to the lagging chromatin (Chan et al. 2007), this indicates that BLm may help to resolved these structures and encourage proper chromosomal segregation. In the cell lines where BLM translation is prematurely terminated, the nuclear localization signal is disrupted and therefore, the mutant protein is not transported to the nucleus thus preventing the protein from functioning (German et al. 2007).

When a cell transfected with EGFP-BLM is exposed to UVA laser irradiation, it was found that the EGFP-BLM would localize to the sites that had under gone irradiation very quickly and remain for a long period of time (Karmakar et al. 2006). This indicates BLM is required during DNA repair, without BLM, the DNA may not be unwound and repair does not occur. The localization pattern of BLM overlaps with many proteins that can respond to DNA damage. When wild type cells were exposed to DNA damaging agents, BLM localizes with γH2AX which accumulates in cells after DNA damage; however, in unchallenged BS cells, there was a high number of γH2AX foci indicating a high number of double stranded breaks (Karmakar et al. 2006; Rao et al. 2007). This may indicate BLM either prevents the occurrence of double stranded breaks or plays a role in the repair of double stranded breaks. It was found that EGFP-BLM co-localizes with ATM (**A**taxia **t**elangiectasia **m**utated) to sites of DNA damage upon exposure to laser-irradiation (Karmakar et al. 2006). This co-localization suggested that BLM accumulates at the sites of double stranded breaks (Karmakar et al. 2006). It was found that BLM localized to Nuclear Domain 10 and a subset of telomeres (Yankiwski et al. 2000). Nuclear Domain 10 gained its name from a dot in the nucleus where nuclear

protein can accumulate when the proteins are inactive or as a dumping ground for excess nuclear protein, there is an increased presence of ND10 in tumor cells and it is believed this increase is caused the overproduction of certain proteins (Maul 1998). It is unclear why Blm would localize to this region and further research is necessary. In spermatocytes, BLM has also been shown to colocalize with RAD51 and DMC1 at the meiotic prophase chromosome cores (Moens et al. 2000). This co-localization once again demonstrates a role for BLM in facilitating DNA repair or preventing the formation of double stranded breaks.

BLM foci and DNA replication foci overlap during S-phase and BLM foci are also localized to the nucleolus of cells during S-phase (Yankiwski et al. 2000). Although BLM is highly expressed during S-phase, there was no S-phase defect detected when BS cells were exposed to HU. In both wild type cells and BS cells treated with HU, the number of cells at the G2/M phase was lower than in the untreated populations indicating that S-phase arrest can be induced by exposure to HU (Ababou et al. 2002). This suggests that BLM does not play a role as an S-phase checkpoint protein. The exposed cells would recover from S-phase arrest normally (Ababou et al. 2002). In BS fibroblasts during S-phase, there is an increase in budding of micronuclei (Yankiwski et al. 2000). The increased number of micronuclei may indicate that there is a high failure of replication in BS cells (Yankiwski et al. 2000). It is thought that the micronuclei may be caused by an excess of anaphase bridging as well as lagging chromosomes that are seen in BS cells (Chan et al. 2007). . In addition to micronuclei budding, chromosomes may also be lost in patients with BS. In one BS patient, the Y chromosome was lost in 10% of the peripheral blood lymphocytes and in 30% of the bone marrow cells (Aktas et al.

2000). It is interesting that the S-phase checkpoint is fully functional in cells, yet cells with increased micronuclei and anaphase bridging are allowed to pass through the cell cycle, perhaps programmed cell death in BS cells is reduced. It is also possible that the mechanisms required for sensing DNA damage in BS cells may not be fully functional.

While the S-phase checkpoint in BS cells may be functional, the G2/M checkpoint may not be fully functional. When mitotic cells, after exposure to UVC, were scored in a wild type and BLM null background, the mitotic index in the BS cells was two to three times higher than that seen in wild type cells indicating that BS cells may partially escape from the G2/M cell cycle checkpoint (Ababou et al. 2002). Additionally, the amount of replicating DNA in BS cells is decreased more than in wild type cells (Ababou et al. 2002). It has also been demonstrated that, when $BLM^{-/-}$ DT40 cells are exposed to varying degrees of UVC, the number of proliferating cells decreases in proportion to the amount of UVC received (Imamura et al. 2001). This indicates that the BS cells are partially escaping the G2/M checkpoint (Ababou et al. 2002).

When $BLM^{-/-}$ cells are grown under normal conditions, it appears that the number of cells in G2/M is slightly increased when compared to wild type (Wang et al. 2000). While the BLM null cells may grow normally, it has been demonstrated that there is a broad distribution of the number of chromosomes in the BS cells when compared to wild type (Leng et al. 2006). This further supports the role of BLM in maintaining proper chromosomal segregation and also suggests this may be when the Y chromosome may be lost. In addition to identifying the broad distribution of chromosomes, it would be interesting to determine if these chromosomes have normal morphology or if there is an increase in the amount of visible chromosomal defects.

In unchallenged $BLM^{-/-}$ DT40 cells, there is a slow growth phenotype which appears to be caused by a slight arrest in the G1 to S phase checkpoint suggesting multiple checkpoints may be affected (Warren et al. 1981; Imamura et al. 2001). Furthermore, it appears that cells derived from patients with Bloom's Syndrome senesce early in cell culture and have a reduced cloning efficiency in comparison to wild type (Warren et al. 1981). In addition to the slow growth phenotype, it has also been demonstrated that DNA replication is slower in BS cells when compared to wild type (Hand and German 1975), this may be due to an increase in stalled replication forks. Other cellular phenotypes can be seen at the G1 to early S-phase when $BLM^{-/-}$ DT40 cells are exposed to UVC irradiation. Like wild type cells, there is an intial increase in the amount of damage seen in the cells, however at 9-12 hours post irradiation, the increased number of chromatid breaks and chromosomal quadriradials is more persistent in BS cells than wild type cells (Imamura et al. 2001), indicating that the cell is unable to resolve aberrant chromosomal structures. Furthermore, this data demonstrates BS cells are sensitive to irradiation and are unable to repair the damage. This also implies that BLM plays a major role in repair as the chromatid breaks persist longer and not attempt is made to repair the damage.

Like mice, cells from patients with BS have a very high amount of SCEs (Chaganti et al. 1974; McDaniel and Schultz 1992; German 1993; Ellis and German 1996). When the number of SCEs was compared between wild type and BS lymphocytes, BS lymphocytes had a 12-fold increase in the amount of SCEs (Chaganti et al. 1974). The high number of SCEs can be reduced when BLM is re-introduced into the cell lines (Ellis et al. 1999). As more BLM is introduced into the BS cell lines, the lower the

number of SCEs becomes (Ellis et al. 1999). In addition to re-introducing BLM into $BLM^{-/-}$ cells, the rate of SCE's can also be reduced by removing RAD54 thus creating a cell line that is $BLM^{-/-}$ /$RAD54^{-/-}$ (Wang et al. 2000). Given that RAD54 plays a role in homologous recombination, it becomes possible to hypothesize that the increase in SCE's in $BLM^{-/-}$ cells is due to homologous recombination (Wang et al. 2000).

There is a caveat however, it has been demonstrated that in certain cell types in multiple individuals, the levels of SCEs are normal while other cell types have the characteristic high level of SCEs (Krepinsky et al. 1979; Ellis and German 1996). It is believed that this discrepancy is due to parents who individually are homozygous for different *BLM* mutations producing an offspring that has two different mutations in *BLM* effectively rendering the gene null yet creating a high SCE/low SCE mosaicism (Ellis and German 1996). For example the mother is homozygous for mutation "a" in *BLM*, the father is homozygous for mutation "b". These two parents produce a child which has both the "a" and "b" mutation, *BLM* will still be null, however, this may create the mosaicism. Further examination of this mosaicism in a patient with both high SCE and low SCE cells demonstrated that it was indeed caused by the inheritance of two different *BLM* alleles (Foucault et al. 1997). In the case of this patient, the mother provided a G3181T mutation and the father had a mutation in *BLM* which affects expression (Foucault et al. 1997).

In addition to the high rate of SCEs in most BS cells, there is also an increase in Non-Homologous End Joining (NHEJ) (Gaymes et al. 2002). NHEJ in BS cells has a low repair fidelity and would introduce various mutations into the genome of these cells (Gaymes et al. 2002). To determine the nature of the genetic lesions that was being

caused, nuclear extracts of BS cells were prepared, these nuclear extracts were then incubated with the *lacZα* plasmid pUC18 which had linearized by *Eco*RI; after incubation, the plasmid was then transformed into bacterial cells (Gaymes et al. 2002). If the double stranded break caused by *Eco*RI was correctly repaired the transformed cells would be blue, however, if faulty repair occurred, the bacteria colonies would be white. When the pUC18 plasmid in the white colonies was sequenced to determine the nature of the misrepair, it was found that there was a significant increase in the number of large deletions (Gaymes et al. 2002). When the nuclear extract of BS cells that had BLM reintroduced to them was examined, it was found that the number of large deletions was reduced to wild type levels (Gaymes et al. 2002). Interestingly in white colonies of both BS cells and BS cells with the additional of functional BLM, sequencing the pUC18 plasmid demonstrated that misrepair was occurring at areas of microhomology (Gaymes et al. 2002). In addition to large deletions, absence of BLM during repair of double stranded breaks can also cause short deletions averaging 6-10 base pairs which do not appear to be caused by microhomology-mediated repair mechanisms (Langland et al. 2002). BS cells seem to have the inability to repair using short repeats of the sequence (Langland et al. 2002). Taken together, this data suggests that BS cells have an error prone method of repairing double stranded breaks in the DNA.

The inability to repair DSBs may explain the sensitivity of BS cells to various DNA damaging agents. When wild type cells are exposed to UVC-irradiation, it was found that the levels of the BLM protein decreased (Ababou et al. 2002). Furthermore, it was found that when wild type cells were exposed to HU or UVC, the BLM protein is phosphorylated in an ATM independent manner (Ababou et al. 2002). It has been

demonstrated that $BLM^{-/-}$ DT40 cells are sensitive to many genotoxic agents such as bleomycin, etoposide (a topoisomerase II inhibitor), and 4-NQO (produces alkalistable bulky DNA lesions) indicating a difficulty with repair (Imamura et al. 2001). BS lymphocytes are also highly sensitive to EMS (ethylmethane sulfonate) damage (Krepinsky et al. 1979). When exposed to EMS, the number is SCEs in the cells increases significantly (Krepinsky et al. 1979). In addition to EMS, $BLM^{-/-}$ cells are more sensitive to MMS than wild type cells (Wang et al. 2000).

Based on the knowledge gained through cell culture, it is obvious BLM plays a role in many processes that a cell must undertake. BLM has been demonstrated to play a role in DNA recombination as well as DNA repair. It has also been demonstrated that loss of BLM results in an inability to repair aberrant chromosomal structures resulting in an increased rate of mutation. It is interesting that loss of BLM results in so many gross chromosomal defects but the cells are still allowed to escape through the majority of the cell cycle checkpoints. Perhaps the cells do not have a way of repairing the damage or perhaps the sensory machinery in the cells is defective when BLM is lost. It is also possible that BLM may act in conjunction with other proteins to maintain a normally functioning cell cycle, however, when BLM is lost, the complex may still be able to partially function and this may explain why the cells with mutations may still be allowed to replicate. It would be interesting to determine if these cells can replicate indefinitely and become cancerous or if the cells will eventually ceases replication. Given the increase in small deletions and insertions, it would also be interesting to determine what is causing these mutations. Perhaps these mutations are due to faulty repair mechanisms such as NHEJ or an inability to resolve various chromosomal structures. Though many

questions have been answered, many still remain and through use of model organisms, it becomes possible to gain an *in vivo* understanding of the role of BLM in DNA recombination and repair.

Summary

Loss of the BLM helicase has long been a model for studying genomic instability. While much is known about the functions of BLM, the exact role of BLM still needs to be elucidated. It is apparent that in each organism, a *BLM* mutation causes genomic instability, however, the long term effects of this genomic instability is unknown. In higher organisms such as mice and humans, loss of functional *BLM* through either null mutations or mutations removing the nuclear localization sequence causes cancer. The appearance of cancer makes it impossible to study the effects of a *BLM* mutation on life span.

In *C. elegans*, it is possible to study genomic instability without cancer arising. I propose the use of *C. elegans* presents a unique opportunity to better understand the function of BLM in a whole organism, and secondly to better understand the role that genomic instability plays in aging. In order to do so, this thesis attempts to establish *C. elegans* as a viable model for Bloom's Syndrome by analyzing the phenotypes associated with loss of *Blm*. Upon establishing that Bloom's Syndrome can be studied in *C. elegans*, it is then possible to determine if loss of *Blm* plays a role in the aging process.

CHAPTER II

I) Abstract

Bloom Syndrome is caused by mutation of the Bloom helicase (BLM), a member of the RecQ helicase family. Loss of BLM function results in genomic instability that causes a high incidence of cancer. It has been demonstrated that BLM is important for maintaining genomic stability by playing a role in DNA recombination and repair; however, the exact function of BLM is not clearly understood. To determine the mechanism by which BLM controls genomic stability *in vivo*, we examined the phenotypes caused by mutation of the *C. elegans* BLM helicase ortholog, HIM-6. We find that the loss of HIM-6 leads to genomic instability as evidenced by an increased number of genomic insertions and deletions, which results in visible random mutant phenotypes. In addition to the mutator phenotype, *him-6* mutants have a low brood size, a high incidence of males, a shortened life span, and an increased amount of germ line apoptosis. Upon exposure to high temperature, *him-6* mutants that are serially passed become sterile demonstrating a mortal germ line phenotype. Our data suggest a model in which loss of HIM-6 results in genomic instability due to an increased number of DNA lesions, which either cannot be repaired and/or are introduced by low fidelity recombination events. The increased level of genomic instability leads to *him-6(ok412)* mutants having a shortened life span.

II) Introduction

The evolutionary conserved family of RecQ helicases play a role in maintaining genomic stability during DNA replication as well as recombination. In humans, there are five RecQ helicases, mutations in three of the five human RecQ helicases (BLM, WRN, and RECQL4) result in Bloom Syndrome, Werner Syndrome, and Rothmund-Thomson Syndrome respectively. Each of these syndromes causes elevated levels of genomic instability that eventually results in cancer.

Patients with Bloom Syndrome exhibit symptoms at an extremely young age. These symptoms include but are not limited to growth retardation, facial erythemas, and impaired fertility (German 1993; Ellis et al. 1995) (Hickson 2003). In cell culture, Bloom Syndrome can be identified by a marked increase in chromosomal abnormalities such as in the occurrence of chromosome breaks and gross rearrangements of chromosomal structure (German et al. 1965; German 1993; Hickson 2003). In addition, there is a significant increase in the amount of sister-chromatid exchanges (SCE) and recombination events (German et al. 1965; German 1993; Hickson 2003). The rise in these chromosomal aberrations is apparently a consequence of the genomic instability which is a hallmark of Bloom Syndrome.

BLM functions in a variety of cellular processes. It is believed BLM maintains genomic stability by inhibiting partially homologous DNA sequences from forming duplexes. These duplexes may cause aberrant structures to form during recombination or repair (Hanada et al. 1997; van Brabant et al. 2000). In addition, BLM can dissociate chromosome junctions such as Holliday junctions, G4 DNA, and D-loops (Sun et al.

1998; Karow et al. 2000; van Brabant et al. 2000). Recently, *Saccharomyces cerevisiae's* only RecQ homolog, SGS1 has been implicated in resolving recombination-dependent cruciform structures that accumulate at damaged DNA replication forks (Liberi et al. 2005). These observations indicate that BLM may function to repair DNA damage by preventing the formation of abnormal recombination intermediates and by resolving abnormal DNA structures. Although BLM is important for maintaining genomic stability, the exact function of BLM is not clearly understood.

To determine the mechanism by which BLM controls genomic stability *in vivo*, we examined the effects of mutations of the *Caenorhabditis elegans* BLM helicase ortholog, HIM-6 (Wicky et al. 2004). It has been previously demonstrated that *him*-6 mutants exhibit many phenotypes that are characteristic of genomic instability in *C. elegans* (Wicky et al. 2004). Through use of the model organism *C. elegans*, we can study the effects of a loss of function of HIM-6 without the complications of a cancer phenotype. Thus, this model system allows us to determine any underlying effects of BLM helicase mutations on genomic stability.

Here, we demonstrate that HIM-6 is required to maintain genomic stability *in vivo*. Mutations in HIM-6 cause a mutator phenotype characterized by an increased number of small genomic deletions and insertions. Mutations of HIM-6 also result in sensitivity to X-rays, indicating an inability to repair DNA damage, specifically double stranded breaks. Additionally, we show that HIM-6 plays a role in preventing germ line apoptosis and in maintaining an immortal germ line. We find that the increase in genomic instability leads to a shortened life span suggesting a potential role for genomic

stability in regulation of longevity. We suggest a model where HIM-6 prevents these random mutations through repair and/or recombination.

III) Results and Discussion

Increased Temperature Further Enhances Brood Size Defects of *him-6(ok412)*

Two parameters indicate genomic instability in *C. elegans*: a high percentage of males in a population and a significantly lower brood size than wild type (Hofmann et al. 2002). When we first examined these phenotypes in our strains, we found that *him-6(ok412)* caused an increase in the incidence of males as well as a low brood size (Table 2.1) similar to that reported in previous studies (Wicky et al. 2004). Interestingly, the low brood size phenotype became more severe at 25ºC, indicating a temperature sensitive phenotype (Table 2.1). The cause of this temperature sensitivity is unknown. It is possible that *him-6(ok412),* which has been previously defined as a null allele is not a null allele. It has not been demonstrated that protein expression has been disrupted in this strain (Wicky et al. 2004). It is also possible that *him-6(ok412)* is temperature sensitive. As the temperature increases, the worm may be unable to produce more progeny as energy reserves are directed towards survival. It is also possible that DNA damage may increase at higher temperatures as more free radicals are being produced. The *him-6* mutant may be unable to deal with the increased amount of DNA damage that is caused by the free radicals.

Table 2.1: The *him-6* Mutant Significantly Reduces Brood Size and Increases Incidence of Males.

Genotype	Eggs Laid*	Brood Size*	Percent Hatched	Percent Males
N2				
20ºC (n=24)	216 ± 7	216 ± 7	100.0	0.0
25ºC (n=12)	171 ± 12	171 ± 12	100.0	0.1
*him-6(ok412)***				
20ºC (n=41)	128 ± 7	71 ± 7	56.5	13.3
25ºC (n=47)	65 ± 4	20 ± 4	30.2	16.2

* Value is number ± Standard Error

* *Statistically different than N2 at both temperatures $p<0.0001$

***him-6(ok412)* Mutants Display Increased Germ-line Apoptosis**

Previous characterization of *him-6* mutants suggested that low brood size and high incidence of males are caused by mis-segregation of chromosomes during meiosis (Wicky et al. 2004). *him-6* mutants cause a reduction in chiasmata formation that correlates with a decrease in levels of meiotic recombination (Wicky et al. 2004); it is not clear, however, how many germ cells are affected by faulty meiotic recombination. Therefore, we examined *him-6* mutants for additional germ line phenotypes. Mutations in BRD-1 and BRC-1, two *C. elegans* proteins that function to maintain genomic stability, lead to an increased amount of cell death in the germ line when the strains with mutations in these proteins are grown under normal conditions (Brenner 1974; Boulton et al. 2004). Using similar methods as those used for characterization of *brd-1* and *brc-1* (Boulton et al. 2004), we crossed a CED-1::GFP strain to *him-6(ok412)* to quantify the amount of apoptosis in the germ line of the *him-6* mutant. CED-1 has been identified as playing a role in the engulfment of apoptotic cells, when fused with a GFP reporter, cells undergoing apoptosis can be visualized as a "circle" (Zhou et al. 2001). When we exposed the CED-1::GFP as well as the CED-1::GFP; *him-6(ok412)* strains to 60 Gy of X-rays, we found that the CED-1::GFP*; him-6(ok412)* strain had a significantly higher number of apoptotic germ cells, 5 ± 1 cells, than the CED-1::GFP strain, which had 1 ± 1 cells ($p<0.0001$)(Figure 2.1A and 2.1C). The reduction in the number of chiasmata caused by decreased meiotic recombination may account for some of the apoptotic cells. Increased germ cell apoptosis may in turn contribute, at least in part, to the reduction of brood size in *him-6* mutants.

The data presented here differs from previous work where *him-6(ok412)* has a reduced level of germ cell apoptosis when compared to wild type worms, 36 hours post irradiation the amount of cell death is comparable to wild type worms (Wicky et al. 2004). There may be several reasons for the discrepancy. The first is the manner in which the experiments were conducted, I would assay worms that were the L4 stage, and the paper is question would assay worms which were 24 hours older, making them a gravid adult. Perhaps the cuticle of the gravid adult is thicker than that of the L4 making the worm more resistant to the effects of X-ray damage. Furthermore, during the L4 stage the gonad arms of *C. elegans* are still forming (Riddle 1997). It is possible the germ line cells are more susceptible to cell death when the gonad is not fully developed. Secondly, the worms in Wicky *et. al*. were scored 2, 4, and 6 hours post irradiation, I chose to look at the worms 12-14 hours later. The rational behind the longer period of time was that the worms were given every available chance to repair the damage caused by irradiation. Finally, in the original work, the apoptosis within the germ line of the worm was scored via Nomarski optics allowing for only one focal plain to be examined (Wicky et al. 2004). In this work, I utilized the CED-1::GFP strain which allows multiple focal plans to be examined for the presence of apoptotic cells. I believe this provides a more accurate means of quantifying germ line apoptosis in *C. elegans.*

Figure 2.1

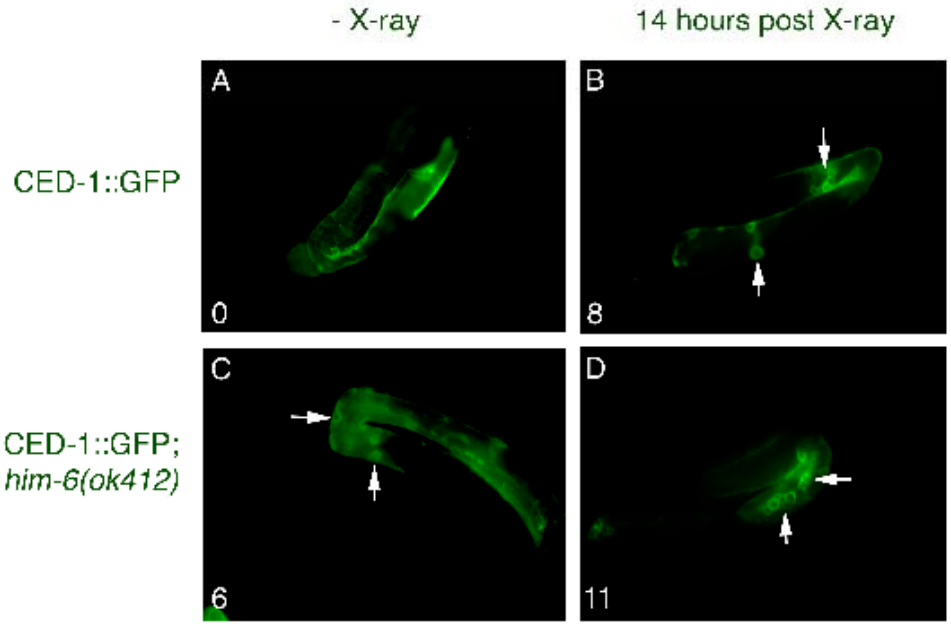
- X-ray
14 hours post X-ray
CED-1::GFP
CED-1::GFP;
him-6(ok412)
A
B
C
D
0
8
6
11

Figure 2.1: *him-6(ok412)* increases the amount of germ line apoptosis in *C. elegans*. (A) CED-1::GFP surrounds the apoptotic cells in the germ line and creates a ring of GFP fluorescence. The image shown is representative of all CED-1::GFP animals. (B) The number of apoptotic cells in CED-1::GFP increases upon exposure to 60 Gy of X-ray. (C) CED-1::GFP; *him-6(ok412)* animals have a higher number of apoptotic cells as demonstrated by the increased number of GFP rings. This image is representative of all CED-1::GFP; *him-6(ok412)* animals. (D) The CED-1::GFP; *him-6(ok412)* strain shows an increase in the number of apoptotic cells upon exposure to 60 Gy of X-ray compared to the control ($p<0.0001$). The number in the lower left corner of the image is the number of apoptotic cells present in the picture.

him-6(ok412) has a Mortal Germ-line Phenotype

Because we observed an apoptotic effect on the germ line of *him-6*mutatns and sterility may occur due to genomic instability (Gartner et al. 2000), we next examined the potential role of HIM-6 in maintaining an immortal germ line. The mortal germ line phenotype (*mrt*) is identified by sterility after a number of consecutive generations (Gartner et al. 2000; Hofmann et al. 2002). *mrt* mutants become sterile between generations F4 and F16, presumably because of an increased level of genomic instability as well as telomere shortening (Gartner et al. 2000). We serially passed wild type and *him-6* mutants at both 20ºC and 25ºC. Examination of *him-6(ok412)* at 25ºC, showed that *him-6* mutants became sterile between generations F2 and F12. At 20ºC, *him-6(ok412)* was maintained indefinitely, similar to other *mrt* strains (Gartner et al. 2000). Therefore, the *him-6(ok412)* mutant at 25ºC defines a new *mrt* strain.

While it is apparent that loss of HIM-6 causes an increased amount of genomic stability, it is also possible that this mortal germ line phenotype can be caused by shortened telomeres. One way to determine if telomeres have are affected by a loss of HIM-6 is to perform a telomere blot. This experiment is interesting, however it is technically impossible because the low brood size in a HIM-6 mutant at 25ºC makes it impossible to generate the number of worms required to perform a telomere blot.

him-6(ok412) Causes a Mutator Phenotype

Loss or reduction of function mutations in many *C. elegans* genes that maintain genomic stability cause a mutator phenotype (Hofmann et al. 2002; Tijsterman et al. 2002). To determine if *him-6(ok412)* mutants have a mutator phenotype, we first

examined the spontaneous mutation rate of *him-6(ok412)* at both 20°C and 25°C by analyzing the progeny of 100 individual worms for visible phenotypes. Worms with visible phenotypes were picked to single plates and their progeny were examined for continued appearance of the mutation. The rate of spontaneous mutations is determined by the percentage of progeny that exhibit a visible phenotype (Tijsterman et al. 2002). In our assay, the percentage of spontaneous mutations in wild type worms and *him-6(ok412)* was determined using the following equation: (number of plates which had a visible mutation)/[(total number of plates examined)(average brood size of strain after two days)]. At 20°C, a population of wild type worms had a mutation rate of 0.001% whereas *him-6(ok412)* had a significantly higher spontaneous mutation rate of 0.78% (Figure 2.2A). At 25°C, the spontaneous mutation rate of *him-6(ok412)* increased to 2.6%, significantly higher than that of wild type (0.003%). Thus, *him-6* mutants displayed a significant increase in the rate of spontaneous mutations at both 20°C and 25°C compared to wild type. Similar results were observed for another independent *him-6* strain, *him-6(e1423)* which had a spontaneous mutation rate of 1.3%.

Next we next examined the *him-6* mutator activity based on a mutation of the *unc-93* gene. *unc-93(e1500)* homozygotes display two visible phenotypes: uncoordinated movement and an egg-laying defect (Greenwald and Horvitz 1980). Over successive generations, *unc-93(e1500)* mutants can spontaneously revert to wild type movement and normal egg laying due to the complete loss of function of *unc-93*(Greenwald and Horvitz 1986). It has been demonstrated that when *unc-93(e1500)* is crossed into the background of a second mutation that induces spontaneous mutations, the reversion rate of *unc-93(e1500)* increases (Hofmann et al. 2002; Tijsterman et al. 2002). To determine the

reversion rate, the number of independent reversion events was compared to the total number of plates examined.

To perform this assay, we crossed *him-6(ok412)* into an *unc-93(e1500)* background and measured the reversion rate of the double mutant strain compared to the reversion rate of *unc-93(e1500)* alone. In each trial, we allowed at least 100 individual worms to grow and have progeny at 20°C until plates were starved. The starved plates were then divided into quarters and these quarters were moved onto fresh plates with food. Two and three days later, the plates were scored for reversion of *unc-93(e1500)* by the appearance of phenotypically wild type worms. We found *unc-93(e1500); him-6(ok412)* had a 5 fold higher rate of reversion than *unc-93(e1500)* (Figure 2.2B). Together with the spontaneous mutation assay results, these data demonstrate that the loss of *him-6* causes a mutator phenotype.

Figure 2.2a

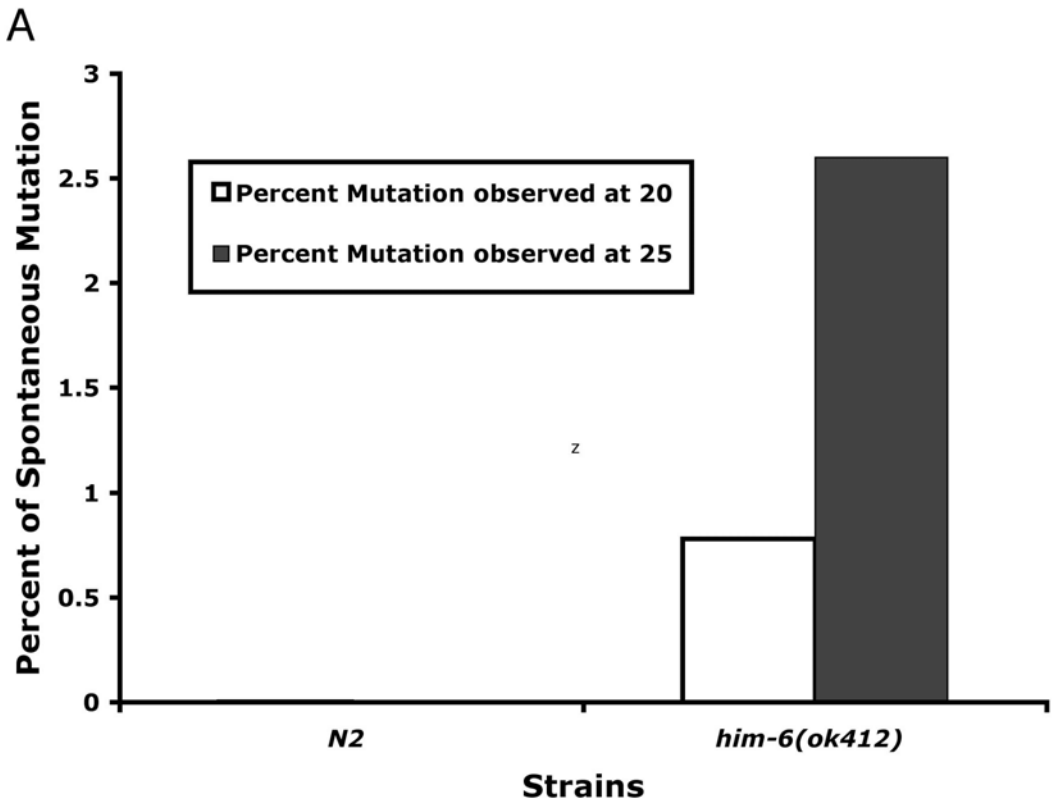
A
Percent of Spontaneous Mutation
3
2.5
2
1.5
1
0.5
0
Percent Mutation observed at 20
Percent Mutation observed at 25
N2
him-6(ok412)
Strains

Figure 2.2a: *him-6(ok412)* causes a mutator phenotype. (A) *him-6(ok412)* exhibits a high spontaneous mutation rate at both 20°C and 25°C compared to wild type. Two hundred plates were examined for each strain.

Figure 2.2b

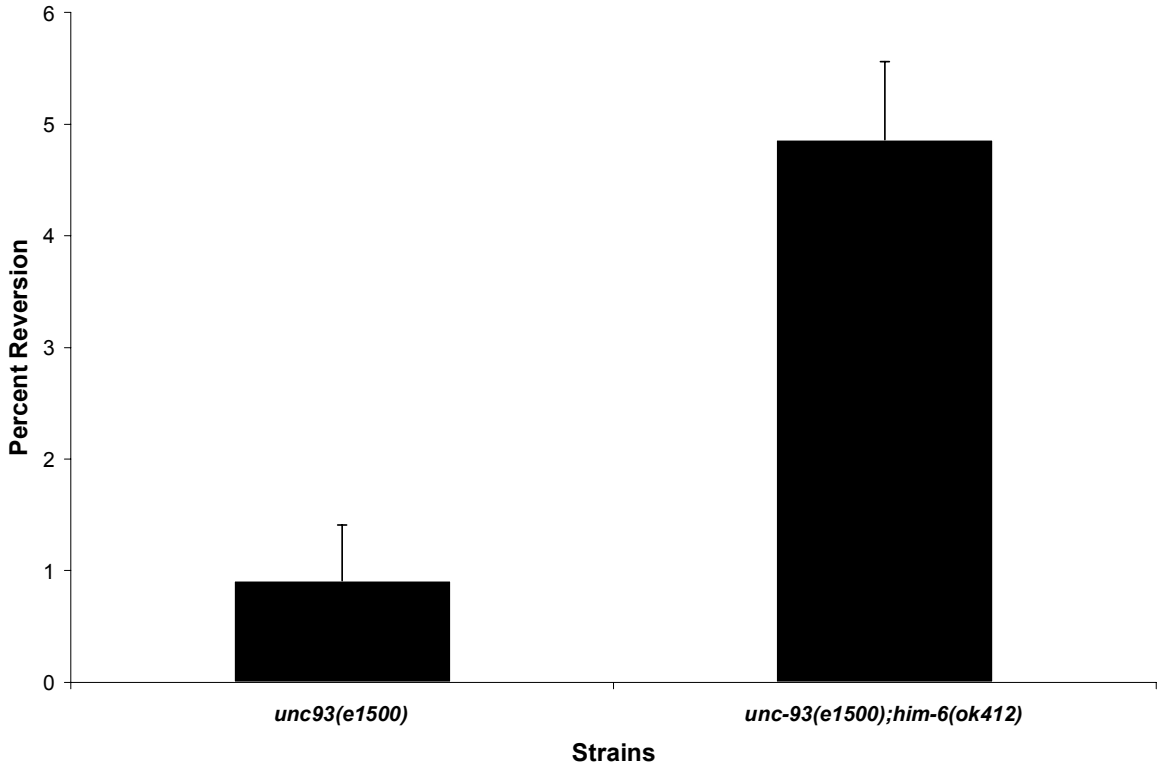
6
5
4
3
2
1
0
Percent Reversion
unc93(e1500)
unc-93(e1500);him-6(ok412)
Strains

Figure 2.2b: *him-6(ok412)* causes a mutator phenotype. (B) *unc-93(e1500); him-6(ok412)* demonstrates a higher level of spontaneous reversion than *unc-93(e1500)* alone ($p=0.0048$), confirming the mutator phenotype. Error bars indicate the standard error (s.e.). For *unc-93(e1500)* 4184 plates were examined and for *unc-93(e1500); him-6(ok412)*, 1808 plates were examined.

him-6(ok412) Causes Small Insertions and Deletions in the Genome

To determine the nature of the genomic lesions caused by a mutation of HIM-6, we first determined if the suppressor of *unc-93(e1500)* was intragenic or extragenic based on data obtained through complementation crosses. There are four known extragenic suppressors (*sup-9, sup-10, sup-11, sup-18)* that, when mutated, cause complete loss of the *unc-93(e1500)* phenotype (Greenwald and Horvitz 1986). Intragenic suppressors of *unc-93(e1500)* can be identified by their failure to complement the previously identified intragenic suppressor *unc-93(e1500 n224)*. Identifying the intragenic suppressors would then allow us to sequence the *unc-93* gene to determine the nature of the mutation causing the intragenic suppression.

Our data indicates that out of 40 independently isolated revertants, 23 isolates were extragenic events that failed to complement *sup-9(n180); unc-93(e1500)* or *sup-10(n403); unc-93(e1500)*. The remaining 17 isolates failed to complement *unc-93(e1500 n224)* indicating an intragenic event. Therefore, to determine the nature of the genomic lesion, we sequenced the UNC-93 gene in these 17 isolates. For 9 isolates, we obtained double strand sequences of the entire UNC-93 gene, this is important as a mutation was only considered to be valid if it could be confirmed in both the forward and the reverse strand of DNA. However for 8 out of the 17 isolates, it was not possible to obtain a PCR product or readable sequence of both DNA strands for all of the UNC-93 exons and therefore we could not determine the exact nature of the reversion event. The 9 reversion events we identified consisted of mutations in the form of small deletions and insertions; interestingly no point mutations were identified (Table 2.2). In total, we identified 7 different types of lesions. With one exception, the insertions and deletions were never

more than 10 base pairs in length. Interestingly, multiple mutations could occur within one isolate and several of these mutations occurred in different isolates; for example, the 8 base pair mutation occurred in two separate isolates. Further examination of the insertions showed that they consisted a tandem repeat of the preceding sequence. The deletions seemed to occur at random with no consensus in the sequence adjacent to the deletion (Table 2.2). Therefore, our data suggest that HIM-6 may function in DNA recombination and/or repair. A loss of HIM-6 could cause lower fidelity of recombination as chromosomal segregation may be hindered. This, in turn, could lead to an accumulation of mutations in the genome. It is equally possible that loss of HIM-6 could lead to the inability to repair damaged DNA, if HIM-6 functions similarly to BLM, perhaps loss of HIM-6 prevents the unwinding of the damaged DNA and thus the damage is passed on in each generation. It is also possible that *him-6* mutants attempt to repair damage using NHEJ, similar to BS cells, and rather than repairing the damage introduce new mutations.

Table 2.2: Types of genomic lesions loss of HIM-6 causes in the *unc-93* reporter gene.

Type of Mutation	Mutation	*unc-93* sequence and position
Insertion*	8 base pair insertion***	(412)gaaaagag<gaagag>cagctgg(426)
	5 base pair insertion	(495)tcgattc<gattc>atctttt(508)
Deletion**	6 base pair deletion	(452)gacagc[ggagc]aagtcg(468)
	7 base pair deletion	(695)ctttcg[agacatg]ggcagg(713)
	7 base pair deletion	(733)ttaa[tgagaag]tggtaag(750)
	5 base pair deletion	(3220)atcaat[ataaa]gttc(3234)
	21 base pair deletion^,***	(2644)[atttttcggatatttttcat]gattgt(2670)

* <> denotes location of insertion as well as sequence
** [] denotes location of sequence that has been deleted
*** Mutation occurred multiple times, see text for details
^ This deletion also extends into the previous intron and deletes 42 base pairs

him-6(ok412) is Sensitive to X-ray Exposure

It is possible that *him-6* mutants have a mutator phenotype because they cannot efficiently repair DNA damage. To explore this possibility, we examined whether *him-6* mutants were sensitive to X-rays, a DNA damaging agent. We exposed L4/young adult worms to varying doses of X-rays and determined the percentage of progeny that hatched. The results shown are normalized against the percent of progeny that hatch when not exposed to X-rays. At 60 Gy, *him-6* mutants had a lower percentage of hatched eggs than wild type indicating sensitivity to X-ray damage (23 ± 3 for *him-6* versus 66 ± 4 for wild type) ($p<0.0001$) (Figure 2.3). It has been well documented that X-ray exposure causes double stranded breaks in the DNA. The sensitivity of *him-6* mutants to this type of damage indicates an inability of *him-6* mutants to repair these breaks. Interestingly, the inability to repair double strand breaks also suggests a recombination defect, as recombination events are typically used to repair double stranded breaks. An interesting experiment to test if recombination is faulty in *him-6* mutants is to make a *him-6*; *rad-51* double mutant. However, this experiment would be very difficult to conduct as both strains have exhibited and embryonic lethality phenotype (Hodgkin et al. 1979; Rinaldo et al. 2002; Wicky et al. 2004). The next step would be to create RNAi constructs of both genes and partially knock down gene function, however, our attempts to create and *him-6* RNAi were unsuccessful as we never recapitulated a HIM phenotype; furthermore, *rad-51* RNAi also causes an embryonic lethality phenotype (Rinaldo et al. 2002). This makes it technically difficult to create the double mutant and given the low number of progeny generated, it would be difficult to derive any meaningful data from this experiment.

Figure 2.3

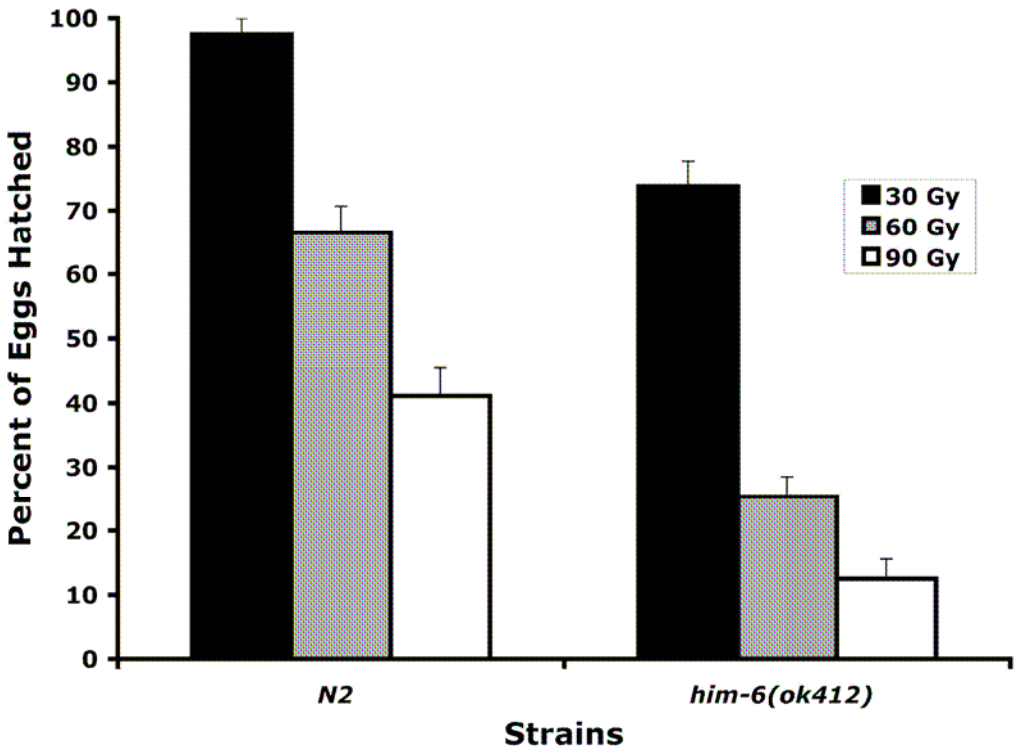
Percent of Eggs Hatched
100
90
80
70
60
50
40
30
20
10
0
30 Gy
60 Gy
90 Gy
N2
him-6(ok412)
Strains

Figure 2.3: *him-6(ok412)* is sensitive to X-ray exposure. The percentage of progeny that hatched is shown for each exposure level which has been normalized to a 100% hatch rate at 0 Gy of exposure. Error bars indicate the standard error (s.e.) for each experiment. Each strain was tested at three times at each level of exposure. The number of worms examined at each level of exposure is as follows: 30 Gy N2 n=17, 30 Gy *him-6(ok412)* n=35, 60 Gy N2 n=23, 30 Gy *him-6(ok412)* n=54, 90 Gy N2 n=21, 90 Gy *him-6(ok412)* n=51.

him-6(ok412) Has a Functional G2/M Checkpoint

Although the brood size of *him-6* mutants is significantly reduced compared to that of wild type, 60% of the progeny survive to adulthood, are fertile, and pass accumulated as well as new mutations to successive generations. The ability to pass on DNA damage such as small insertions and deletions as well as double stranded breaks to subsequent generations indicates a potential checkpoint malfunction. Indeed, it has been previously demonstrated that *him-6* mutants display a partially defective S-phase checkpoint (Wicky et al. 2004). We therefore analyzed additional DNA damage checkpoints by examining the G2/M checkpoint. In *C. elegans*, the G2/M checkpoint is identified by the germ cell response to X-rays. An intact G2/M checkpoint causes an increase in the amount of apoptosis upon exposure to an increasing amount of X-ray dosage (Chin and Villeneuve 2001). To conduct this experiment, we exposed the CED-1::GFP; *him-6(ok412)* strain to increasing dosage of X-rays and determined the amount of apoptosis in the germ line. At each dose we tested, we found that *him-6(ok412)* mutants responded similarly to wild type to increasing amounts of X-rays: both showed a dose dependent increase in cellular apoptosis although *him-6(ok412)* mutants had a higher level of apoptosis compared to wild type (Figure 2.4). This is unsurprising as *him-6* mutants are sensitive to X-ray damage and *him-6(ok412)* has a higher rate of germ line apoptosis than wild type worms. This dose dependent increase in cellular apoptosis indicates a functional G2/M checkpoint. Therefore, the partially defective S-phase checkpoint (Wicky et al. 2004) may be the only cell cycle phenotype of these mutants.

Figure 2.4

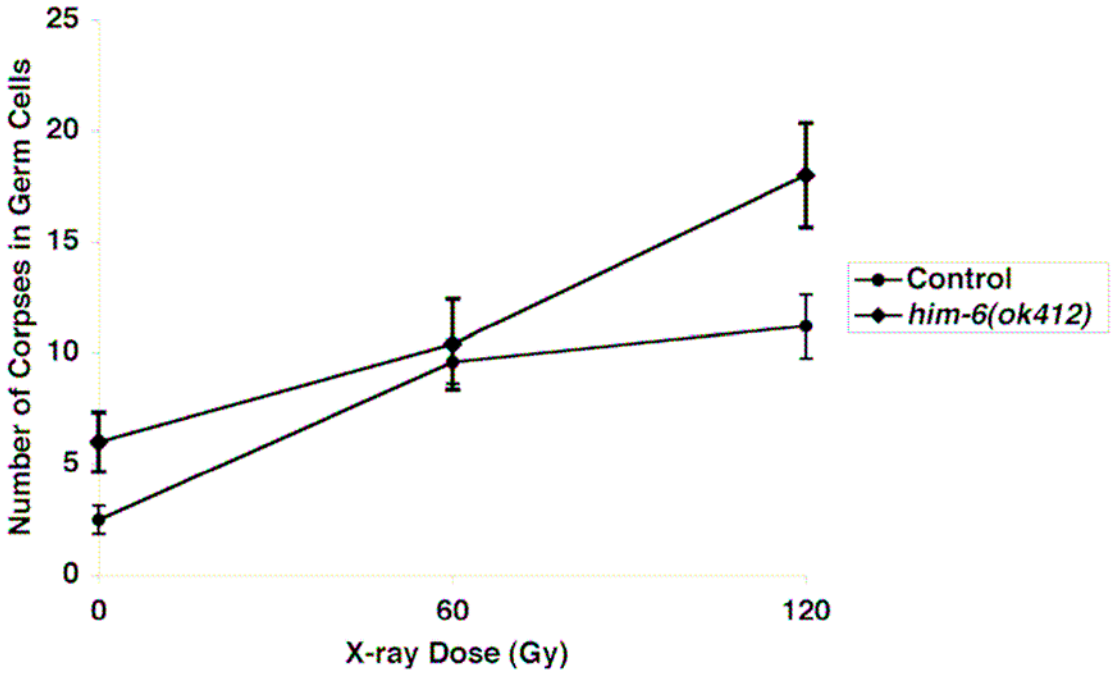
25
20
15
10
5
0
Number of Corpses in Germ Cells
0
60
120
X-ray Dose (Gy)
Control
him-6(ok412)

Figure 2.4: *him-6(ok412)* mutants have a functioning G2/M checkpoint. Using varying dosages of X-ray exposure, we determined the number of apoptotic germ-line cells per strain. *him-6(ok412)* shows a proportional increase in apoptotic cells compared to wild type, indicating a functional G2/M checkpoint. Error bars indicated standard error.

him-6(ok412) Displays a Shortened Life span

A long-standing hypothesis is that the aging process can be correlated to increasing amounts of genomic instability (Arking 1998). Interestingly, two syndromes caused by mutations in the RecQ family of helicases have been characterized as progeroid (premature aging) syndromes: Werner Syndrome and Rothmund-Thomson Syndrome. Therefore, we examined the life span of the *him-6* mutant strain to determine if genomic instability could cause a shortened life span of the worm. We found that at 20ºC, wild type worms had a mean life span of 15.2 ± 0.2 days, while *him-6(ok412)* mutants had a shorter mean life span of 13.5 ± 0.2 days ($p<0.0001$) (Figure 2.5A). Similarly at 25ºC, *him-6* displayed a shortened life span with a mean of 11.4 ± 0.1 days, while wild type worms had a mean life span of 12.1 ± 0.1 days ($p<0.05$) (Figure 2.5B). Although these animals could age prematurely due to a non-specific phenotype of sickness, our data are consistent with the model that elevated levels of genomic instability shorten life span.

Even though the life span of the *him-6* mutant is shortened, it is possible that the shortened life span may be due to a non-specific sickness. Further experiments will need to be conducted to determine if the life span is shortened due to genomic stability or due to a non-specific sickness. There are several ways to differentiate between a shortened life span caused non-specific sickness and a shortened life span caused by the mutation of interest. The first way to determine what is shortening the life span is to look at the curve of the graph. If the curve appears to have a normal sigmoidal shape similar to that of wild type, one can begin to assume that the life span is being shortened due to the presence of the mutation of interest. If the life span curve drops very steeply and suddenly, perhaps

the shortened life span is due to a non-specific sickness. The second way to determine what is causing a shortened life span in mutants is to create double mutant strains with genes that are not part of the pathway of interest, for example, the insulin-like signaling pathway is know to have an effect on life span. If I created a double mutant with *daf-2* (a long live strain) and *him-6* and the life span of *daf-2* was unaffected, then it is possible the *him-6* mutation shortens life span rather than causing a non-specific sickness. If the *daf-2; him-6* life span is slightly shortened compared to *daf-2* alone, this may indicate that HIM-6 does not play a major role in life span regulation it may also indicate loss of HIM-6 causes a generalize sickness in the worm. If the *daf-2; him-6* mutant life span is completely suppressed, there can be two possibilities, one is that loss of *him-6* can suppress *daf-2* function. This possibility is not likely as many screens have been conducted to find suppressors of *daf-2* life span extension and only one has been identified- *daf-16*. The second possibility is *him-6* mutants may cause a non-specific sickness which shortens the life span of these worms. To differentiate between these two possibilities, other double mutants with long lived and short lived strains can be made to determine the effect of loss of HIM-6 on the life span.

Figure 2.5a

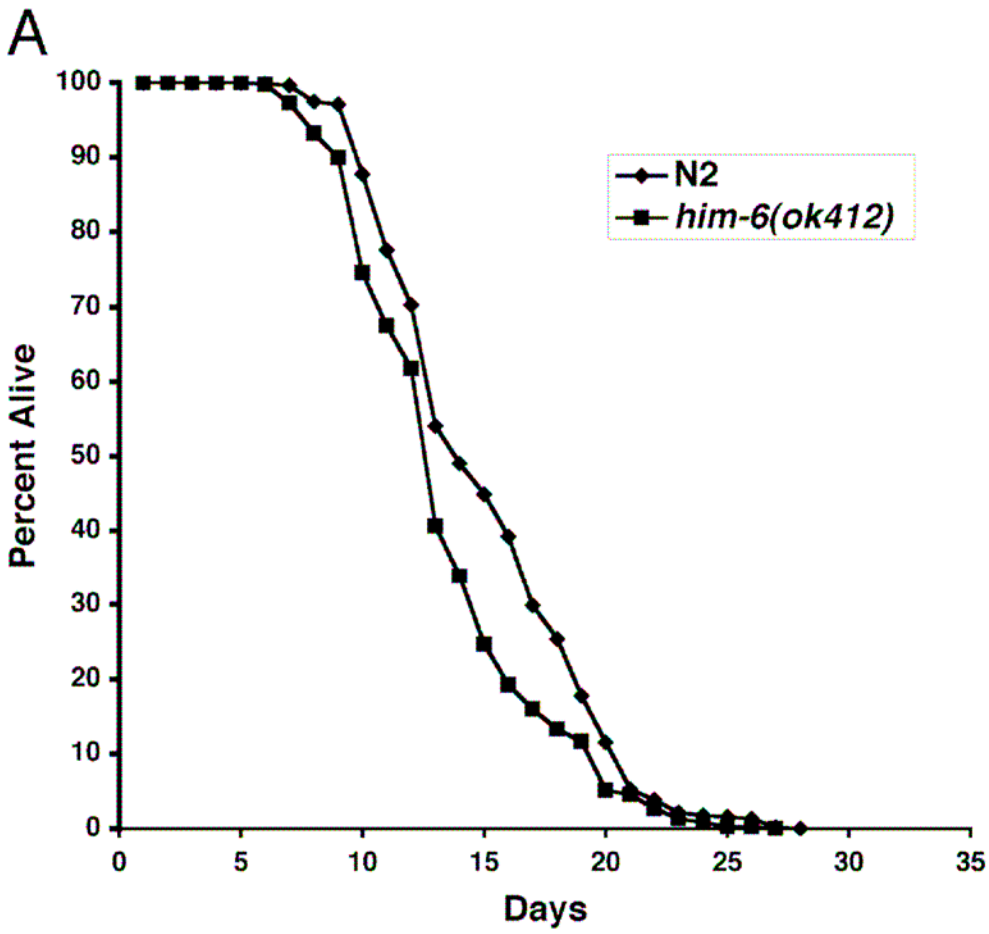
A
Percent Alive
Days
N2
him-6(ok412)
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30
35

Figure 2.5a: Life span effects of loss of the *C. elegans* BLM ortholog, *him-6*.

(A) At 20°C *him-6(ok412)* exhibited a life span of 13.5 ± 0.2 days, compared to wild type (15.2 ± 0.2 days) ($p<0.001$)

Figure 2.5b

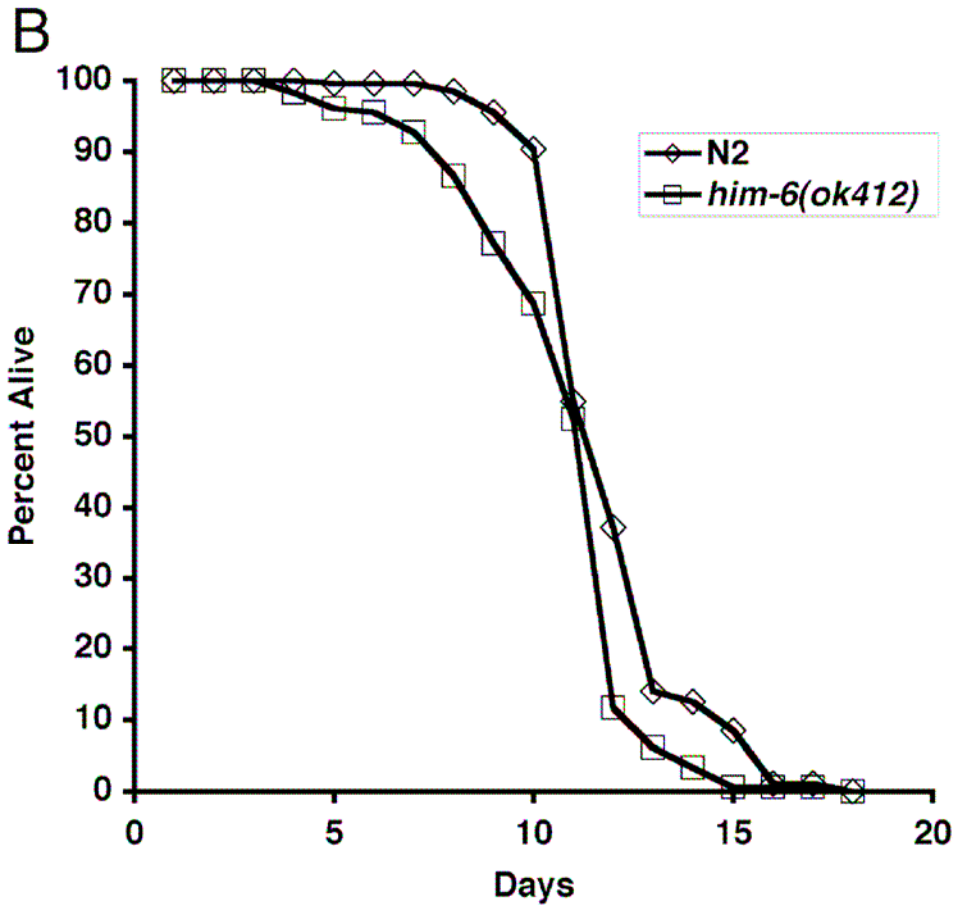
B
100
90
80
70
60
50
40
30
20
10
0
Percent Alive
0
5
10
15
20
Days
N2
him-6(ok412)

Figure 2.5b: Life span effects of loss of the *C. elegans* BLM ortholog, *him-6*.

(B) At 25°C, the results are similar to those seen at 20°C. N2 has a mean life span of 12.1 ± 0.1, while *him-6(ok412)* has a mean life span of 11.4 ± 0.1 All experiments were repeated at least two times with at least 50 worms per trial. Numbers represent mean number of days ± s.e.

IV) Conclusions

Maintenance of genomic stability is central to the ability of an organism to produce viable progeny and sustain continued growth. In *C. elegans him-6* mutants, we found many indications of genomic instability including high incidence of males, low brood size due to a lower percentage of viable eggs, shortened life span, a mortal germ line, sensitivity to X-rays, and, notably, a mutator phenotype.

Evidence of genomic instability in *C. elegans* was observed when *him-6* mutants demonstrated an increase in germ line apoptosis. This is particularly interesting because it may explain two phenotypes: the low brood size, as well as a new phenotype, a mortal germ-line. The mortal germ-line phenotype may arise due to an increased amount of germ cell apoptosis in each generation until the strain becomes sterile. It would be interesting to conduct experiments were the number of apoptotic germ cells is counted through multiple generations to determine if there is an increase in the amount of apoptosis. Also, as many of the *mrt* mutants are only mortal at 25°C (Ahmed and Hodgkin 2000), it would be interesting to determine if the amount of apoptosis is higher at 25°C than at 20°C. Furthermore, it would also be useful to examine the length of the telomeres in cells which are undergoing apoptosis. It is possible that the increased rate of apoptosis in *him-6* mutants is caused by a loss of telomeres.

Loss of HIM-6 could reduce the ability of the cells to resolve abnormal DNA structures because HIM-6 mutants may lack the helicase function to unwind these abnormal structures, thus driving the cells into apoptosis. To determine if this is the case, it would be interesting to look at the chromosomal structures of the cells undergoing apoptosis to determine if there are any gross chromosomal abnormalities. It is unclear

whether the cells in *him-6* mutants are unable to repair the damage to the genome because the recombination pathway is not fully functioning or because HIM-6 has a secondary ability to repair DNA damage through means other than recombination such as NHEJ. It is attractive to speculate that HIM-6 may play a role in non-homologous end joining as BLM does in cell culture, however, non-homologous end joining has not been demonstrated to occur in *C. elegans*.

It has long been hypothesized that genomic instability may adversely affect the life span of an organism (Arking 1998). In humans, both Werner Syndrome and Rothmund-Thomsom Syndrome cause premature aging. Bloom Syndrome has not been classified as a progeriod syndrome; however, patients with the disease die at a young age due to cancer. When we examined the life span of *him-6* mutants, we found a slight but significant shortening of life span. Although it is not clear why *him-6* mutants have a shorter life span, it is attractive to speculate that this may be due to genomic instability. Our data indicates genomic stability may have a minor regulatory role in control of *C. elegans* life span.

While, the life span of the *him-6* mutant is shortened, it is unclear if these animals are aging prematurely. There are several assays which can be conducted to determine if the worms are aging prematurely. One method is to measure the level of autofluorescence in the intestine of the worm. In younger worms, the level of autofluorescence is very low, as the worm ages, the levels being to increase. In a worm which is aging prematurely, the level of autofluorescence would be higher in the mutant worm than the wild type worm at an earlier stage in the life span (Garigan et al. 2002). A second method to determine if a mutant strain is aging prematurely is to examine amount of bacterial packing in the

pharynx of the worm over time (Garigan et al. 2002). As the worm ages, bacteria packing occurs in the pharynx; to determine if *him*-6 mutants age prematurely, the bacteria packing would appear sooner in the mutants than in the wild type worms. A third assay to measure the rate of aging uses movement and muscle structure of the worm over time (Herndon et al. 2002). In an old worm, the muscles begin to break down and the worm will begin to move less frequently. In this assay, I would compare the muscle structure and movement of the *him-6* mutants to that of wild type worms. It should be noted that a shortened life span does not imply a premature aging phenotype since the non-specific phenotype of sickness could shorten life span. However, given that the effect of a loss of function of HIM-6 has on life span is slight and the shape of the life span curve is similar to that of wild type, it seems unlikely that the life span effect is due to a non-specific sickness.

The role of HIM-6 in repairing double strand breaks is suggested by the sensitivity of the *him-6* mutant to X-ray radiation, a known inducer of double strand breaks. The reduction in the rate of meiotic recombination (Wicky et al.) may also indicate an inability to repair the double strand breaks. In such a case, double stranded breaks in the germ line would cause activation of the meiotic recombination pathway to repair the damaged DNA. It is possible that HIM-6 plays a role in the meiotic recombination pathway. Results from cell culture suggest BLM functions to resolve aberrant recombination structures, perhaps HIM-6 functions in a similar manner. If there is a loss of function of HIM-6, the recombination pathways may no longer function because loss of HIM-6 prohibits the unwinding of recombinant DNA structures. It is also possible *him-6* mutants may inhibit DNA recombination because the DNA cannot be

unwound as the helicase function of HIM-6 is lost. This results in the double stranded breaks not being properly repaired, causing a reduction in brood size and an introduction of new mutations. The sensitivity of *him-6* to X-ray damage suggests that meiotic recombination may be the primary means of repairing double strand breaks or, conversely, the double stranded break repair is used in meiosis.

An important indication of genomic instability in *him-6* mutants is the mutator phenotype. We found that *him-6* mutants show a mutator phenotype based on data from two separate assays, the *unc-93* assay and the spontaneous mutation assay. Sequencing the lesions caused by the loss of HIM-6 in the *unc-93* assay revealed that *him-6* mutant have increases the amount of small deletions and insertions. Interestingly, $BLM^{-/-}$ cells also exhibit many small deletions and duplications that occur within a sequence containing multiple tandem repeats (German 1993). In addition, a large number of short deletions occurred in $BLM^{-/-}$ cells in sites prone to double stranded breaks, indicating a lower fidelity of repair (Langland et al. 2002). Therefore, loss of HIM-6 could inhibit the DNA repair pathway responsible for the resolution of double stranded breaks. It is also possible that meiotic recombination may be the primary means of repairing double stranded breaks in *C. elegans*. It has already been established that meiotic recombination is reduced in *him-6* mutants (Wicky et al. 2004). The double stranded breaks may occur when proteins other than HIM-6 attempt to resolved the aberrant recombinant structures; in *him-6* mutants when an attempt to resolve these structures, new mutations may be introduced. A second explanation for the mutator phenotype in $BLM^{-/-}$ cells is an elevated number of sister-chromatid exchanges (Warren et al. 1981; Langlois et al. 1989; Groden and German 1992; German 1993). If similar events are occurring in *C. elegans*

due to low fidelity recombination, then an increase of unequal sister-chromatid exchanges would lead to the small deletions and insertions that are observed in our assay. Finally in cell culture, BLM has been demonstrated to prevent the collapse of the stalled replication forks as well as restarting them (Davies et al. 2007; Rao et al. 2007). If HIM-6 functions similarly to BLM, perhaps the increased amount of DSBs is a result of an increase in stalled replication forks collapsing.

Although it is clear that HIM-6 plays a role in genomic stability, the mechanism by which loss of HIM-6 function causes genomic instability is not clear. There are at least three possibilities. First, HIM-6 may act as a DNA damage sensor; loss of HIM-6 function allows DNA damage to accumulate, thus overwhelming the remaining DNA repair mechanisms. This may be the least likely possibility as there is no evidence in any organism that BLM or any of its orthologs function as a DNA damage sensor. This hypothesis would be difficult to test because to date, there is no protocol to quantitate the amount of damage a worm has accumulated. Second, HIM-6 may be intimately associated with all DNA repair mechanisms and a loss of HIM-6 function could prevent repair of DNA damage. In this case in a *him-6* mutant, the level of DNA damage in the germ line could become catastrophic causing an increase of cell death and a lower brood size. In the case of progeny that survive, there is an increased level of damage in their genome passed from generation to generation until sterility results. To test if this is the case, double mutants with other genes known to be involved with DNA repair can be created. The strains can be assayed for various indicators of genomic instability such as brood size, cell death and mutator phenotypes. Third, HIM-6 may play a role in maintaining recombination fidelity. In a *him-6* mutant, recombination would occur with

a lower level of fidelity, thereby introducing mutations into the genome of the worm or hindering the ability of the worm to repair damage such as double strand breaks. The low level of fidelity could have resulted because of several possibilities. The first is loss of HIM-6 prevents unwinding of the DNA does not allow recombination to occur. There may be a secondary helicase which may only partially unwind the DNA, recombination could occur, but a reduced frequency and the recombination structures would not be properly resolved, this could introduce new mutations. A second reason is HIM-6 may function like BLM and unwind DNA intermediates that are formed during recombination. If these intermediates are not unwound, perhaps a secondary protein will attempt to resolve these structures similar to what has been seen in *Drosophila*. When a secondary pathway becomes involved, perhaps it functions at a lower fidelity and can introduce new mutations.

In summary, we have successfully used *C. elegans* as a model system to uncover the *in vivo* roles of HIM-6 in maintaining genomic stability and provide a possible link between genomic stability and life span. HIM-6 functions to suppress germ cell apoptosis and also plays a role in maintaining an immortal germ line. We suggest that loss of *C. elegans* BLM leads to high levels of genomic instability due to an increased level of random insertions and deletions throughout the genome. Over time, these mutations would accumulate in the organism, ultimately resulting in a premature death. Even though *C. elegans* is a post-mitotic organism, cells can still accumulate damage over time. If this damage is not repaired, or is repaired through a faulty mechanism, it is possible that the gene will no longer produce the proteins which are required for survival of the cell. Additionally, we have found that *him-6* mutants display an inability to repair double

stranded breaks induced by DNA damage as demonstrated by a heightened sensitivity to X-rays. Importantly, we found that *him-6* causes a mutator phenotype similar to that seen in $BLM^{-/-}$ cells. This is the first time that the mutator phenotype has been reconstituted in an *in vivo* system. Given the similarity in phenotypes between *him-6* mutants and $BLM^{-/-}$ cells, *C. elegans* represents a powerful model to study the molecular basis of Bloom Syndrome.

V) Material and Methods

Strains

All strains were maintained at 15°C under standard conditions (Brenner 1974). *him-6(ok412)* was provided by the *Caenorhabditis* Genetics Center (University of Minnesota, St. Paul, Minn.). *him-6(ok412)* was backcrossed five times to N2 and the presence of the deletion was confirmed by PCR. The *Plin7 ced-1::gfp* was a gift from Barbara Conradt at Dartmouth University.

Brood Size Analysis

Five to ten L4 hermaphrodites were picked from individual plates at 20°C. Following this selection, worms were transferred each day to fresh plates until they ceased to lay eggs. The number of L1s and eggs on the plate from the previous day were counted. Three days later, adult worms and males were counted. This experiment was repeated 2 times using at least 10 worms per strain in each analysis. The data shown are an average of several experiments and statistical analysis was determined using the nonparametric Chi Square test.

Germ line Apoptosis Assay

The CED-1::GFP strain was crossed into the *him-6(ok412)* mutant. Ten to twenty L4 CED-1:: GFP and CED-1::GFP ; *him-6(ok412)* hermaphrodites were grown at 20°C for 14-16 hours and then scored for the presence of apoptotic cells in the germ line. Apoptotic cells were identified by the presence of a GFP outline around the cells undergoing apoptosis and were scored using the 40X objective lens of the Zeiss

Axioskop 2 Microscope. At least 50 CED-1::GFP and CED-1::GFP; *him-6(ok412)* worms from each strain were examined. Statistical analysis was determined using the nonparametric Chi Square Test.

G2/M assay

CED-1::GFP; *him-6(ok412)* or CED-1::GFP L4 hermaphrodites were irradiated with 0, 60, and 120 Gy of X-ray. After irradiation, the strains were grown at 20°C for 12 to 14 hours. Then the number of apoptotic cells in the germ line was then counted using GFP microscopy. At least 15 CED-1::GFP and CED-1::GFP; *him-6(ok412)* worms were examined for each X-ray dosage.

Spontaneous Mutation Assay

Plates of *him-6* mutants were grown at 15°C. Ten L4 worms displaying no visible phenotypes were picked and transferred to individual plates. These plates were incubated at the temperature of interest and the worms were allowed to have progeny for two days. From each of these 10 plates, ten L4 healthy looking worms were picked and transferred to individual plates, resulting in a total of 100 worms per genotype. These 100 plates were again incubated at the temperature of interest for 2 days. After 2 days, all 100 plates were examined for visible mutations. Worms with visible mutations were picked to single plates and the progeny were examined for stable inheritance. At the temperature of interest, at least 200 plates per strain were examined per trial. The percentage of spontaneous mutation in wild type worms and *him-6(ok412)* was calculated using the following equation: (number of plates which had a visible mutation)/[(total number of plates examined)(average brood size of strain after two days)].

***unc-93* assay**

Using independent, freshly thawed cultures, 100 worms of each genotype (*unc-93(e1500)* and *unc-93(e1500) ; him-6(ok412)*) were picked and transferred to individual plates. Plates were incubated at 20°C until no food remained on the plates and the animals were starved. The plates were then divided into quarters and these quarters were transferred to fresh plates which had been numbered to ensure independent events were followed up. Two and three days after the transfer to fresh plates, the plates were scored for reversion events that were identified by the presence of phenotypically wild type worms. At least 1800 plates where scored per strain. Statistical analysis was determined using the nonparametric Chi Square Test.

From each independent reversion event, one wild type worm was selected and grown for multiple generations. Extragenic reversion events were identified by their inability to complement the known extragenic suppressors: *sup-9(n180); unc-93(e1500)* and *sup-10(n983); unc-93(e1500)*. Intragenic reversion events were identified by their inability to complement *unc-93(e1500n224)*. In identified intragenic revertants, the UNC-93 gene was sequenced. All additional mutations were confirmed with double strand sequencing. In 8 of the intragenic reversion events, it was not possible to sequence all of the UNC-93 exons due to lack of PCR product or to an incomplete or unreadable sequence.

Life span Assays

Life span assays were conducted at both 20ºC and 25ºC. For these assays, worms were first grown for one generation at 15ºC to ensure that the worms had not starved. Then,

L4 stage animals were placed on plates containing 5-florodeoxyuridine (FUDR). Each assay consisted of 5 plates containing 5-10 worms per plate. Plates were then shifted to either 20ºC or 25ºC and scored every 2-3 days for the ability of the worms to respond to repeated taps with a platinum wire. If the worm had no response to the taps, it was scored as dead. The life span data represents the average adult life span. Each experiment was conducted at least 3 times with at least 50 animals per trial. Statistical analysis was determined using the nonparametric Chi Square Test.

Identification of Mortal Germ line phenotype

Following previously published procedures (Gartner et al. 2000), a total of 25 L4 hermaphrodites from *him-6(ok412)* and N2 were placed on a total of 5 plates at either 20°C or 25°C. With each generation (typically after 3 days), 5 healthy L4 hermaphrodites were transferred to new plates until the brood size was less than 2 progeny per worm and the total brood size of the plate was less than 10 progeny. At this stage the worm was considered to be sterile. This assay was repeated 3 times.

Effects of Exposure to X-ray on Hatching

Each selected group of L4 hermaphrodites was irradiated with 0, 60, and 120 Gy of X-ray. Strains were then grown at 20°C. Each day for three consecutive days, the worms were shifted to new plates and the number of eggs laid in the 24-hour period was counted. After three days, the plates containing eggs were scored for the number of adult progeny that had hatched and matured. This assay was repeated three times with a total 10 worms examined. The percent progeny that hatched was then standardized against the hatch rate

at 0 Gy of X-rays. Statistical analysis was determined using the nonparametric Chi Square Test.

VI) Acknowledgements

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CHAPTER II ADDENDUM

Brood size and composition is unaffected by loss of WRN-1 and RCQ-5

Given the apparent role of HIM-6 in maintaining brood size and incidence of males, I was curious to determine if the other loss of the other RecQ helicases in *C. elegans* also had an impact on brood size and the incidence of males. *C. elegans* has four RecQ helicases, HIM-6, WRN-1, RCQ-5, and K02F3.1. Based on alignment studies, it has been determined that WRN-1 is homolgous to the WRN helicase in humans, which when mutated, is known to cause Werner's Syndrome (Lee et al. 2004). RCQ-5 is homologous human RecQ5 which is not known cause any diseases when mutated (Jeong et al. 2003). In these studies, I used the deletion strains *wrn-1(gk99)* and *rcq-5(fx424)* both of which are thought to be putative null alleles.

When I looked at the brood size and composition of the *wrn-1(gk99)* and *rcq-5(fx424)* mutants at both 20°C and 25°C, I found that like wild type, 100% of the eggs laid by both mutants hatched. Also, there was not an increase in the percentage of males in either *rcq-5(fx424)* or *wrn-1(gk99)*. This data is supported by the previous work using RNAi as knockdown of *wrn-1* and *rcq-5* had no affect on brood size or incidence of males (Jeong et al. 2003; Lee et al. 2004) What was surprising however, was at 20°C, both *wrn-1(gk99)* and *rcq-5(fx424)* had significantly lower numbers of progeny produced (Table 2.3). Wild type worms at 20°C had an average of 216 ± 6 progeny while the *wrn-1(gk99)* mutant had 174 ± 12 progeny ($p=0.0039$); *rcq-5* mutants had on average 185 ± 9 progeny which once again is significantly lower than that of wild type worms ($p=0.0039$) (Table 2.3). This may indicate a role for *wrn-1* and *rcq-5* in egg production, or it is possible loss of *wrn-1* and *rcq-5* limits the amount of sperm the worms can produce. It is important to note that all of the eggs which are laid do hatch, this may indicate that *wrn-1*

and *rcq-5* may not play a role in maintaining genomic stability. The lack of a HIM phenotype also indicates that there is not an increase in the amount of chromosomal non-disjunction further indicating that WRN-1 and RCQ-5 do not play a role in genomic stability

At 25°C, *wrn-1(gk99)* and *rcq-5(fx424)* lay similar numbers of eggs to wild type and 100% of the eggs hatch, additionally these strains do not have a HIM phenotype at 25°C. The lack of a HIM phenotype also suggests that WRN-1 and RCQ-5 do not play a role in the prevention of chromosomal non-disjunction.

This data also indicates that WRN-1 and RCQ-5 mutants are not temperature sensitive. The amount of progeny produced by wild type worms significantly decreases when the worms are grown at 25°C versus being grown at 20°C ($p=0.0008$) (Table 2.3). The decrease in progeny may be a response to the increased stress the worm is placed under at higher temperatures. Given that the number of progeny *wrn-1(gk99)* and *rcq-5(fx424)* mutants produce is unaffected by the temperature shift, this may imply a faulty response to additional stress. Further experiments will need to be conducted to determine if both RCQ-5 and WRN-1 are involved in stress response.

When I created the double mutants between *wrn-1(gk99)*, *rcq-5(fx424)*, and *him-6(ok412)* I found that there was no difference between *rcq-5(fx424); him-6(ok412)* and the *him-6(ok412)* mutant alone; *rcq-5(fx424); him-6(ok412)* had a similar number of eggs laid, percent of eggs which hatched, and total brood size at both 20°C and 25°C (Table 2.3). Interestingly, *wrn-1(gk99); him-6(ok412)* was similar to *him-6* alone in terms of brood size, percent of eggs which hatched, and percent males, however, the number of eggs which were laid was significantly lower than that of *him-6(ok412)* ($p=0.0051$)

(Table 2.3). It is unclear why the brood size *wrn-1(gk99); him-6(ok412)* is comparable to *him-6(ok412)* alone even though the number of eggs laid is lower. While the difference in the percent of eggs which hatch is not statistically significant, in a *him-6(ok412)* mutant 55.8% of the eggs hatch while in a *wrn-1(gk99); him-6(ok412)* double mutant 62.1% of the eggs hatch (Table 2.3). This small difference may be enough to account for the similar brood sizes between the two strains. At 25°C, once again *wrn-1(gk99); him-6(ok412)* lays less eggs than *him-6(ok412)* ($p=0.0005$) and once again, the percent hatch rate is higher in *wrn-1(gk99); him-6(ok412)* versus *him-6(ok412)*; however this difference is not statistically significant (Table 2.3). Even though the difference is not statistically significant, it could account for why the brood sizes between the strains are similar.

Interestingly, at 20°C *wrn-1(gk99); rcq-5(fx424)* is similar to wild type in terms of brood size and the percent of animals which hatch. Additionally, at 20°C the number of eggs laid is similar to wild type (Table 2.3). However at 25°C, the number of eggs that are laid and the brood size are significantly reduced when compared to wild type ($p=0.003$). This may indicate that WRN-1 and RCQ-5 may be able to compensate for each other at higher temperatures. It may also indicate that the double mutant may be more sensitive to stress than either of the single mutants indicating a potential role for WRN-1 or RCQ-5 in stress response.

When I looked at the brood size and composition of a triple mutant of all three helicases, I found that at 20°C the *wrn-1(gk99); rcq-5(fx424); him-6(ok412)* strain behaved similarly to *him-6(ok412)* in terms of number of eggs laid, brood size, percent of eggs which hatched and incidence of males (Table 2.3). At 25°C, only 15.4% of the eggs laid by *wrn-1(gk99); rcq-5(fx424); him-6(ok412)* hatched compared to *him-6(ok412)*

where 29.8% of the eggs hatched (p=0.01) (Table 2.3). Upon further comparison with the double mutants *wrn-1(gk99); him-6(ok412)* and *rcq-5(fx424); him-6(ok412)*, I found that eggs laid by the triple mutant had a significantly lower hatch rate than either of the double mutants (p=0.02 and p=0.05 respectively) (Table 2.3). The lower percentage of eggs which hatch could be due to the temperature sensitivity that is demonstrated by *him-6(ok412)* (Table 2.3).

It is possible that WRN-1, RCQ-5, and HIM-6 are involved in stress response. When all three genes are mutated, the worms become more sensitive to temperature. There are other stress response pathways which are still functional which would explain why the triple mutant is not completely lethal at 25°C. This data also demonstrates that HIM-6 is primarily involved in maintaining genomic stability while WRN-1 and RCQ-5 do not seem to play a role in maintaining genomic stability. It is interesting that the double mutants as well as the triple mutants behave in a similar fashion to *him-6(ok412)* strain. This implies that HIM-6 may be downstream of these two genes; however, it is not possible to conclusively state this as further genetic analysis is needed.

Table 2.3: Brood size and composition of the *C. elegans* Rec Q helicase family

Genotype	Eggs Laid*	Brood Size*	Percent Hatched	Percent Males
N2 20°C (n=24) 25°C (n=12)	 216 ± 6 171 ± 6	 216 ± 6 171 ± 6	 100 100	 0.4 0.5
him-6(ok412) 20°C (n=41) 25°C (n=47)	 127 ± 7 64 ± 5	 70 ± 5 19 ±2	 55.8 29.8	 13.1 16.7
wrn-1(gk99) 20°C (n=15) 25°C (n=10)	 174 ± 12 180 ± 17	 174 ± 12 180 ± 17	 100 100	 0.0 0.05
rcq-5(fx424) 20°C (n=15) 25°C (n=8)	 185 ± 9 183 ± 9	 185 ± 9 183 ± 9	 100 100	 0.0 0.0
wrn-1(gk99); him-6(ok412) 20°C (n=14) 25°C (n=15)	 87 ± 11 30 ± 6	 63 ± 12 12 ± 3	 62.1 40.9	 15.2 21.7
rcq-5(fx424); him-6(ok412) 20°C (n=12) 25°C (n=11)	 102 ± 11 48 ± 7	 50 ± 11 14 ± 3	 57.3 28.8	 9.8 18.7
wrn-1(gk99); rcq-5(fx424) 20°C (n=11) 25°C (n=13)	 243 ± 17 125 ± 12	 243 ± 17 125 ± 12	 100 100	 0.0 0.5
wrn-1(gk99); rcq-5(fx424); him-6(ok412) 20°C (n=11) 25°C (n=12)	 101 ± 10 61 ± 8	 50 ± 9 13 ± 4	 46.8 15.4	 15.7 20.0

***** Value is number ± standard error

Loss of WRN-1 and RCQ-5 does not cause an increase in reversion of *unc-93(e1500)*

Given *him-6(ok412)* has a mutator phenotype, I sought to determine if loss of *wrn-1(gk99)* or *rcq-5(fx424)* also could increase the rate of reversion in *unc-93(e1500)*. Previous work using a strain with a *gfp-LacZ* reporter had identified RCQ-5 as having a role to protect the genome of the worm against mutations (Pothof et al. 2003).

When I looked at the rate of reversion in *wrn-1(gk99); unc-93(e1500)*, I found the percentage of reversions to be similar to that of *unc-93(e1500)* alone; *unc-93(e1500)* has a reversion rate of 0.71 ± 0.3% while *wrn-1(gk99); unc-93(e1500)* has a reversion rate of 1.4 ± 0.8 (Figure 2.6). This indicates that loss of WRN-1 does not increase the rate of mutation within the genome of the worm. Furthermore, this indicates that WRN-1 may not play a role in maintaining genomic stability.

While RCQ-5 had been identified as protecting the genome against mutations (Pothof et al. 2003), I found that *unc-93(e1500); rcq-5(fx424)* had a reversion rate of 1.4 ± 0.7% which is similar to *unc-93(e1500)* alone (Figure 2.6). This indicates that *rcq-5(fx424)* does not increase the rate of mutation in *C. elegans*. This data coupled with the data from the brood analysis indicates that RCQ-5 probably does not play a role in maintaining genomic stability.

There could be multiple reasons for the differences between previously published data and my results. The first is the strain I used, *rcq-5(fx424)*, may not be completely null and if there is still protein formed, the protein still may be functional. In the previously published work, RCQ-5 was identified during an RNAi screen (Pothof et al. 2003), perhaps the RNAi caused a more complete knockdown of RCQ-5 than my mutant. One way to test this is to repeat the *unc-93* assay using *rcq-5* RNAi. A second means

determining if loss of RCQ-5 causes a mutator phenotype is to use another *rcq-5* mutant strain which has become available. It would also be possible to repeat the assay described in Pothof *et. al.* using the RCQ-5 mutants rather than RNAi to determine if the mutants behave the same way as the RNAi.

Figure 2.6

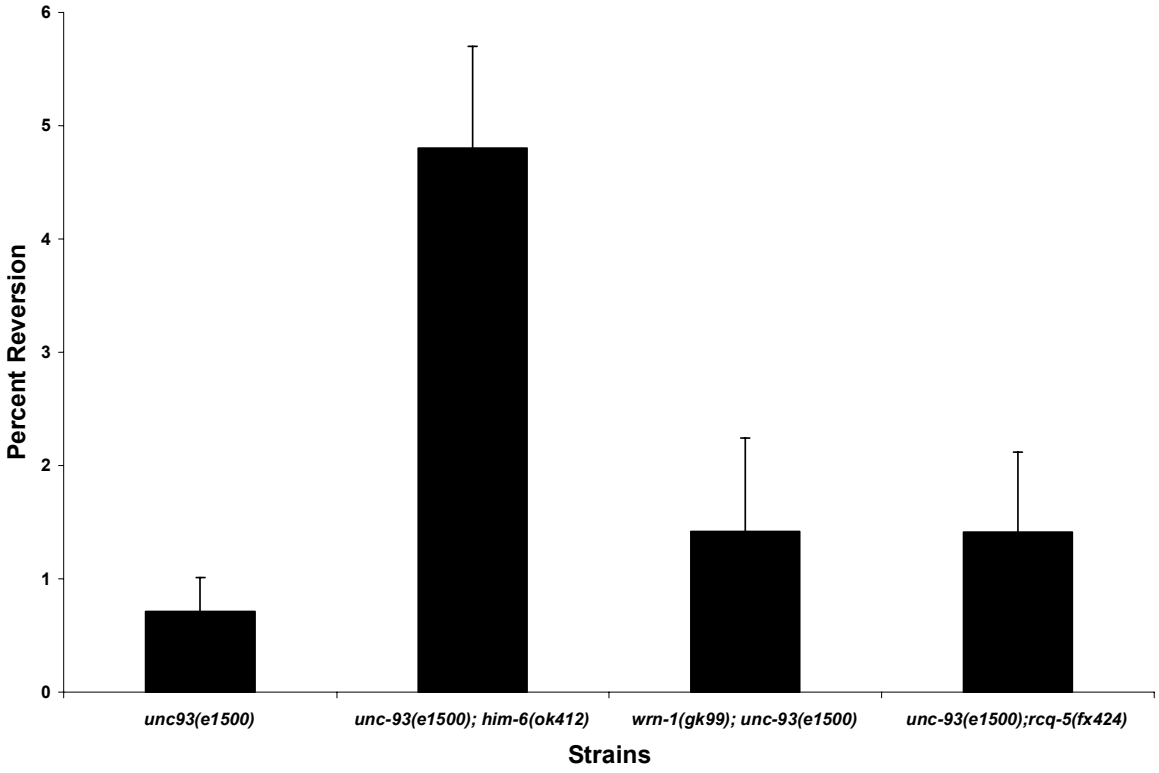
Percent Reversion
0
1
2
3
4
5
6
unc93(e1500)
unc-93(e1500); him-6(ok412)
wrn-1(gk99); unc-93(e1500)
unc-93(e1500);rcq-5(fx424)
Strains

Figure 2.6: Loss of WRN-1 and RCQ-5 does not cause an increased rate of reversion in *unc-93(e1500)*. *unc-93(e1500); rcq-5(fx424)* and *wrn-1(gk99); unc-93(e1500)* do not have a higher level of spontaneous reversion than *unc-93(e1500)*. This demonstrates that loss of WRN-1 and RCQ-5 do not cause a mutator phenotype. Error bars indicate the standard error (s.e.) for *unc-93(e1500)* 4184 plates were examined, for *unc-93(e1500); rcq-5(fx424)*, 1965 plates were examined and for *wrn-1(gk99); unc-93(e1500)* 1169 plates were examined.

Loss of WRN-1 or RCQ-5 does not shorten the life span of *C. elegans*.

When I found that *him-6(ok412)* has a shortened life span when compared to wild type worms, I was curious to determine what the effect of loss of RCQ-5 or WRN-1 would be on life span. When I preformed the life span on *rcq-5(fx424)* and *wrn-1(gk99)* I found that the mean life span was increased over wild type (wild type= 14.9 ± 0.1, *wrn-1(gk99)*= 15.7 ± 0.2, and *rcq-5(fx424)*= 15.4 ± 0.2) (Table 2.4). It is unclear why there is an increase in the life span of these strains. Perhaps *wrn-1(gk99)* and *rcq-5(fx424)* have longer life spans because the strains produce fewer eggs; energy which would have been used for reproduction is redirected towards life span. It is also possible that the life spans are longer because I am working with non-null strains and if there is still protein being produced, perhaps it is beneficial to the worm.

When I conducted life span assays on the double mutants *wrn-1(gk99); him-6(ok412)*, *rcq-5(fx424); him-6(ok412)*, and *wrn-1(gk99); rcq-5(fx424),* I found that both *rcq-5(fx424); him-6(ok412)* and *wrn-1(gk99); rcq-5(fx424)* had life spans similar to that of wild type (Table 2.4). Interestingly, *wrn-1(gk99); him-6(ok412)* had a life span of 14.2 ± 0.2 which is significantly shorter than that of wild type (p=0.003) (Table 2.4). It is unclear why this double mutant has a shortened life span. Perhaps the increased amount of DNA damage caused by loss of HIM-6 is a stress that a *wrn-1* mutant cannot cope with and this results in a shortened life span. Another reason could be that the energy *wrn-1* mutants had saved through decreased egg production and had put towards life span is now redirected towards attempting to repair the damage caused by loss of HIM-6. Finally, it is possible that *wrn-1* mutants suffer from a small amount of DNA damage,

this small amount combined with the mutator phenotype caused by loss of HIM-6 might be responsible for the shortening of life span.

What is especially interesting is the triple mutant has an extremely short mean life span (Table 2.4). There could be multiple reasons for this shortened life span. The first is that *wrn-1(gk99); rcq-5(fx424); him-6(ok412)* causes a non-specific sickness thus reducing the life span of the worm. To test if this if the triple mutant causes a non-specific sickness, it would be interesting to cross this strain into long lived mutants to see if there is an effect on life span. It is also possible that WRN-1, RCQ-5, and HIM-6 all function in different pathways to maintain life span, loss of these pathways could cause a significant shortening of life span; however, to determine if this is the case, further genetic analysis of these mutants would need to be conducted. Finally, it is possible that both WRN-1 and RCQ-5 do play a minor role in maintaining genomic stability, loss of the three RecQ helicases could cause a significant increase in the mutation rate thus shortening life span. This is unlikely as *wrn-1(gk99)* and *rcq-5(fx424)* do not display any classic indicators of genomic instability, however, it would be interesting to determine if *wrn-1(gk99); rcq-5(fx424); him-6(ok412)* has an effect on the reversion rate of *unc-93(e1500)*.

Table 2.4: Life span of *C. elegans* RecQ helicases at 20°C

Genotype	Mean Life Span ± Standard Error	Number of Worms
N2	14.9 ± 0.1	1725
wrn-1(gk99)	15.7 ± 0.2	463
rcq-5(fx424)	15.4 ± 0.2	442
rcq-5(fx424); him-6(ok412)	15.3 ± 0.3	223
wrn-1(gk99); rcq-5(fx424)	14.3 ± 0.3	139
wrn-1(gk99); him-6(ok412)	14.2 ± 0.2	371
wrn-1(gk99); rcq-5(fx424); him-6(ok412)	11.7 ± 0.2	371

Conclusions

Based on the data presented here, it appears that WRN-1 and RCQ-5 do not play a role in maintaining genomic stability. *wrn-1* and *rcq-5* mutant worms do not have a HIM phenotype and all the eggs they lay hatch, indicating that WRN-1 and RCQ-5 do not under go an increased amount of genomic instability. Interestingly enough, it appears however, that loss of WRN-1 and RCQ-5 individually results in fewer eggs being produced. This may imply a role for these genes in development. Given that all of the eggs hatch, it is also possible these genes are involved in sperm production. It would be interesting to determine if the *wrn-1(gk99)* or *rcq-5(fx424)* mutant produced less sperm than wild type.

When the mutant strains of WRN-1 and RCQ-5 were examined to determine if they had a mutator phenotype, I found they did not. This further indicates that WRN-1 and RCQ-5 do not play a role in maintaining genomic stability. It would be interesting to see if there is a mutator phenotype in any of the double mutant or triple mutant strains. This could be done via visual assays or through use of the *unc-93* assay.

Finally, I found that *wrn-1(gk99)* and *rcq-5(fx424)* both had a slightly longer life span than wild type worms. This could be due the limited reproductive capacity both of these mutants demonstrate, however, further analysis will be needed to determine if this is the case. Interestingly *wrn-1(gk99); rcq-5(fx424); him-6(ok412)* was had an extremely short life span. This could be due to non-specific sickness and further experiments are needed to determine if this is the case. It would also be interesting to determine what the life span would be of a strain in which all four of the RecQ helicases are mutated. It may

be possible that there is redundancy between the helicases and upon removal of all four, new phenotypes may be uncovered.

While this data begins to characterize WRN-1 and RCQ-5, more work is needed to understand their role in *C. elegans*. First and foremost, it is important to determine if *wrn-1(gk99)* and *rcq-5(fx424)* are null alleles. Secondly, it would be interesting to determine why their brood size is lower than that of wild type. Additionally, it would be beneficial to determine which pathway WRN-1 and RCQ-5 function, this may provide an indication as to their roles in *C. elegans*. Finally, it would be interesting to determine why these strains appear to live slightly longer and through use of various biomarkers of aging determine if these strains age at the same rate as wild type.

Loss of HIM-6 can slightly shorten the life span of *daf-2(e1370)*

One way to determine if a shortened life span is caused by non-specific sickness or is an effect of loss of gene function is to cross the short lived mutant into a long live strain. To determine if *him-6(ok412)* has a shortened life span because of non-specific sickness or because of genomic instability, I crossed *him-6(ok412)* into *daf-2(e1370)*, a strain known to have a long life span.

At 20°C, *daf-2(e1370)* has a mean life span of 38.2 ± 0.6 days. When I introduced *him-6(ok412)* into a *daf-2* background, the life span was reduced to 35.1 ± 0.6 days ($p=0.0002$) (Figure 2.7). This is especially interesting because almost no genes have been identified which can shorten the life span of *daf-2(e1370)*. Loss of HIM-6 does not cause a completely suppress the life span of *daf-2(e1370)*, but rather causes a 10% reduction in life span; additionally, *him-6(ok412)* has a 10% reduction of life span. Based on this data, perhaps genomic stability only accounts for 10% of life span maintenance. This may imply that multiple factors play a role in the maintenance of a wild type life span and while important, genomic stability is not a major regulator of life span. This would make sense as *C. elegans* is a post mitotic organism and the majority of cells in the worm do not undergo further division.

This data however does not conclusively answer whether or not non-specific sickness is responsible for the shortened life span of *him-6(ok412)*. In order to determine if loss of HIM-6 causes non-specific sickness, *him-6(ok412)* would have to be placed into the background of other long lived strains to determine if loss of HIM-6 can shorten the life span. If all of the double mutants have a shortened life span, then it is likely that loss of HIM-6 causes a non-specific sickness. If loss of HIM-6 only shortens the life span of

daf-2 and does not shorten the life span of any other long lived strains, this may indicate a potential interaction between HIM-6 and DAF-2.

However, this apparent shortening of the *daf-2* life span was interesting to me. I became curious as to what the effect the loss of HIM-6 would have on other members of the insuling signaling pathway such as DAF-16.

Figure 2.7

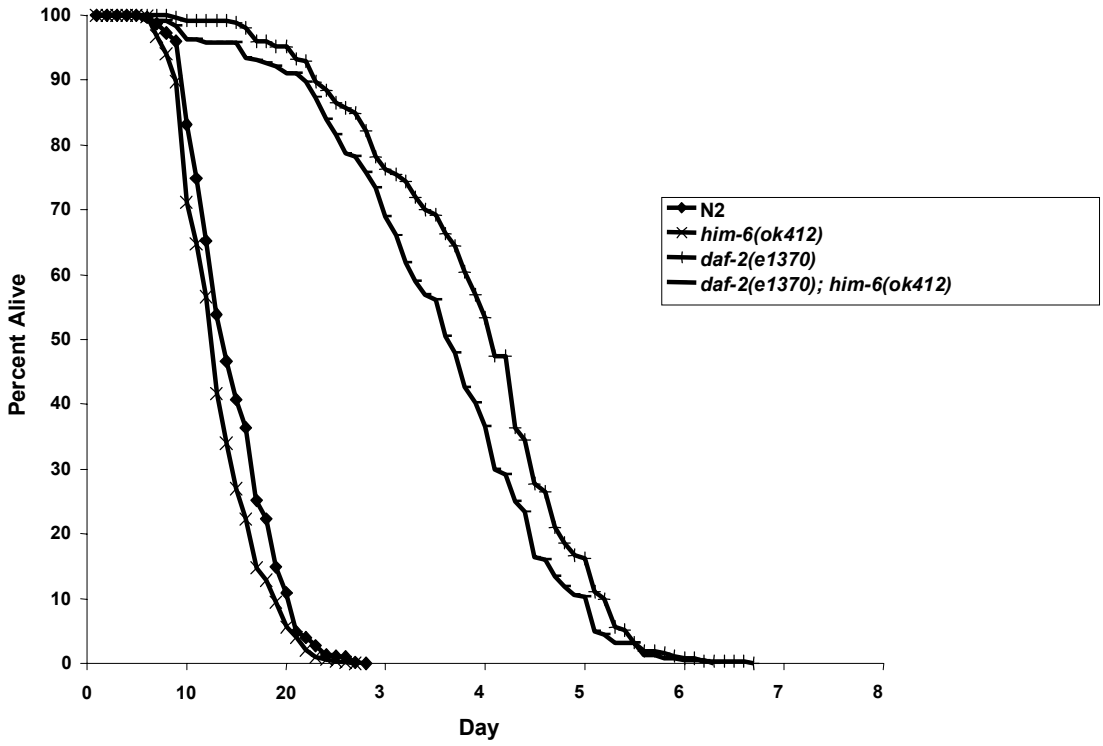
100
90
80
70
60
50
40
30
20
10
0
Percent Alive
0
10
20
3
4
5
6
7
8
Day
N2
him-6(ok412)
daf-2(e1370)
daf-2(e1370); him-6(ok412)

Figure 2.7: *him-6(ok412)* can slightly shorten the life span of *daf-2(e1730)*.

At 20°C *daf-2(e1370)* exhibited a life span of 38.3 ± 0.6 days, compared to *daf-2(e1370); him-6(ok412)* (35.1 ± 0.6 days) (p=0.0002).

CHAPTER III

I) Introduction

FoxO3a (also know as FKHRL1) is a member of the fork head family of transcription factors. FoxO3a can be regulated by several pathways, but its cytosolic sequestration is mainly regulated by the PI 3-kinase pathway (Brunet et al. 1999; Modur et al. 2002). When the PI 3-kinase pathway is functioning normally, FoxO3a is localized to the cytoplasm when it is phosphorylated on multiple thronine and serine residues by Akt/PBK(Brunet et al. 1999; Modur et al. 2002; Birkenkamp and Coffer 2003; Van Der Heide et al. 2004).

When the PI 3-kinase pathway is inhibited by PTEN (Phosphatase and tensin homolog), FoxO3a phosphorylation is reduced and FoxO3a is transported to the nucleus (Nakamura et al. 2000; Modur et al. 2002). The FoxO family can enter the nucleus for multiple reasons, one such as serum starvation and oxidative stress (Martinez-Gac et al. 2004; Lehtinen et al. 2006). Once inside the nucleus, FoxO3a becomes active and binds to FoxO specific binding sequences activating transcription of genes (Brunet et al. 1999; Furuyama et al. 2000). The ability of FoxO3a to activate the transcription of certain genes such as *DAP kinase 1*, *SMAD4*, *Jak1*, and *cyclinG_2* can cause a decrease in cellular proliferation (Modur et al. 2002). Additionally, FoxO3a causes an increase in apoptosis through activation of pro-apoptotic genes such as Fas (Brunet et al. 1999; Modur et al. 2002).

One pro-apoptotic gene which FoxO3a can activate is *Bim* (Gilley et al. 2003). It was found that both *Bim* and FoxO3a are required for apoptosis to occur in the sympathetic neuron (Gilley et al. 2003). Furthermore, it has been demonstrated in gastric

cancers that FoxO3a plays a role in activating *Bim* which in turn activates RUNX3-mediated tumor suppression (Yamamura et al. 2006). It was determined that both RUNX3 as well as FoxO3a are required to induce *Bim* to drive the cells into apoptosis (Yamamura et al. 2006).

Another pro-apoptotic gene that the FoxO3a regulates is TRAIL (TNF-related apoptosis-inducing ligand). TRAIL is a death-inducing ligand in cells (Strasser et al. 2000). When TRAIL is transformed into cell lines, the cells undergo apoptosis (Strasser et al. 2000). Translocation of FoxO3a into the nucleus increases TRAIL expression, when FoxO3a translocation to the nucleus is reduced by PTEN, TRAIL activity is reduced (Modur et al. 2002).

It is the role of FoxO3a in inducing apoptosis that makes it an attractive target for understanding why cancer cells do not undergo apoptosis. It was found that in the absence of cytokines, FoxO3a can induce apoptosis and that inhibition of FoxO3a reduced the level of apoptosis (Dijkers et al. 2002). When FoxO3a is sequestered in the cytoplasm, the genes which are transcribed by FoxO3a such as $p27^{KIP1}$ are also significantly reduced (Dijkers et al. 2002; Lynch et al. 2005). $p27^{KIP1}$ is a cyclin-dependent kinase inhibitor that plays a role in regulating cell proliferation, differentiation, and causes cells to become cancerous (Borriello et al. 2007). Thus, FoxO3a plays a role in the prevention of tumor formation as well as induction of apoptosis.

In addition to activating pro-apoptotic genes such as *Bim* and TRAIL, the FoxO family of proteins also has the ability to bind to *GADD45* promoter (Furukawa-Hibi et al. 2002). *GADD45* has been demonstrated as being important in inhibiting cell growth, G2/M arrest, and DNA repair (Smith et al. 1994; Hollander et al. 1999; Wang et al. 1999;

Zhan et al. 1999; Jin et al. 2000). It was later determined that *GADD45* transcription is FoxO3a dependent, and when cells are exposed to oxidative damage (Furukawa-Hibi et al. 2002), *GADD45* can repair the DNA damage (Tran et al. 2002). In addition, FoxO3a can also regulate cell cycle through binding specifically to the *GADD45* promoter, causing G2/M arrest (Tran et al. 2002).

While the FoxO family can regulate apoptosis and DNA repair, it has also been demonstrated that this family can regulate cell cycle progression through regulation of *cyclin G_2* (Martinez-Gac et al. 2004). When *cyclin G_2* is over-expressed, the cell cycle becomes arrested (Bennin et al. 2002). *cyclin G_2* is highly expressed in the G0 stage before cells enter the cell cycle (Martinez-Gac et al. 2004). As the cell begins the cell cycle, *cyclin G_2* levels decrease, and then increase during late S and G2 phase (Martinez-Gac et al. 2004). In a manner similar to what seen in the regulation of apoptosis, the expression level of *cyclin G_2* is increased when FoxO3a is translocated to the nucleus and becomes transcriptionally active; when FoxO is inactive in the cytoplasm, the level of *cyclin G2* is lowered (Martinez-Gac et al. 2004). Given that FoxO plays a role in the regulation of apoptosis, DNA repair, and cell cycle, it is likely that FoxO may also play a role in maintaining genomic stability.

While the role of FoxO3a in cell cycle, apoptosis and DNA repair continues to be elucidated, the majority of this work has only been conducted in cell culture. It would be beneficial to study the effects of loss on FoxO3a in a model organism. In *C. elegans,* there is one protein, DAF-16, which is homologous to FoxO3a. Sequence homology shows that DAF-16 is a member of the HNF-3/forkhead family of proteins that include FKHR and AFX. Like its human homologs, DAF-16 is regulated by the PI 3-kinase

pathway which includes the insulin receptor like protein DAF-2; additionally in *C. elegans*, the PI3-kinase pathway signaling can be inhibited by PTEN (Lin et al. 1997; Ogg et al. 1997; Gami and Wolkow 2006). The DAF-16 protein in *C. elegans* has been extremely well characterized and is known to function as a transcription factor (Lin et al. 1997; Ogg et al. 1997).

DAF-16 in *C. elegans* was first identified due to its ability to suppress dauer formation (Riddle et al. 1981). *daf-16* mutants have a dauer defective phenotype meaning they can not completely enter the alternate developmental pathway even under unfavorable conditions (Vowels and Thomas 1992; Gottlieb and Ruvkun 1994; Ogg et al. 1997). Instead, when exposed to a high amount of dauer pheromone, *daf-16* mutants form a partial dauer which is identified by a larger pharynx and a low level of refractility (Vowels and Thomas 1992; Gottlieb and Ruvkun 1994; Ogg et al. 1997). Further study of DAF-16 later found that *daf-16* mutants could suppress long life, fat accumulation, and the growth arrest phenotypes of a mutation in DAF-2 (Vowels and Thomas 1992; Kenyon et al. 1993; Gottlieb and Ruvkun 1994; Ogg et al. 1997). In addition to its various phenotypes, it has also been demonstrated that *daf-16* mutants have a life span that is shortened by 20% when compared to wild type worms (Lin et al. 2001).

When transgenic animals with an over-expression of DAF-16 tagged with GFP were studied, it was found that the *daf-16::GFP* construct was expressed in the ectoderm, muscles, intestine, and neurons of the worm (Ogg et al. 1997; Henderson and Johnson 2001; Lin et al. 2001). Expression of *daf-16::GFP* begins in late embryos before hatching and is expressed through the entire life of the worm (Henderson and Johnson 2001). When the *daf-16::GFP* is placed into a *daf-2* mutant background, like FoxO3a,

the expression pattern changes from cytosolic to nuclear indicating that DAF-16 becomes transcriptionally active (Henderson and Johnson 2001; Lin et al. 2001; Gami and Wolkow 2006). *daf-16::GFP* can also become localized to the nucleus during starvation, heat stress and hypoxia, indicating a role for DAF-16 in stress response (Henderson and Johnson 2001; Kondo et al. 2005). Interestingly, when the *daf-16::GFP* strain was exposed to various doses of UV exposure, the localization pattern of *daf-16::GFP* remained cytosolic (Henderson and Johnson 2001).

In several alleles of *daf-16* mutants, there are increased levels of apoptosis in the germ lines of worms exposed to irradiation (Quevedo et al. 2007). This suggests that DAF-16 in *C. elegans* may function in an anti-apoptotic role in the germ line (Quevedo et al. 2007). The role of DAF-16 in apoptosis is unclear as another report demonstrated that functional DAF-16 is required for the increase in germ line apoptosis caused by gamma irradiation (Pinkston et al. 2006). The differences between these two findings could be due to the natures of the alleles selected as well as the manner in which the assays were conducted.

While it is clear DAF-16 plays a major role in regulating many pathways in *C. elegans*, it is unclear why *daf-16* mutants have a shortened life span. It is believed that *daf-16* mutants are "healthy" because these mutants have a normal brood size and composition and are visually indistinguishable from wild type worms. Given that the DAF-16 human homolog FoxO3a does play a role in cell cycle, DNA repair, and apoptosis, I was curious to determine if DAF-16 could also play a role in these processes. Work by the Derry and Kenyon labs have begun to establish a role for DAF-16 in

apoptosis in *C. elegans*, indicating perhaps all of DAF-16's functions in *C. elegans* have not been fully elucidated (Pinkston et al. 2006; Quevedo et al. 2007).

II) Results and Discussion

daf-16(mu86) increases the reversion rate of *unc-93(e1500)*

During the culturing of *daf-16(mu86)*, I noticed the appearance of atypical visible phenotypes that were not associated with known *daf-16* phenotypes. To understand what was causing these visible phenotypes, I conducted a subjective spontaneous mutation assay where I attempted to quantify the number of visible phenotypes which appeared in the strain. The number of visible phenotypes I saw in *daf-16(mu86)* was higher than I expected. I then turned to a well-established, unbiased assay to determine if *daf-16(mu86)* caused a mutator phenotype.

I sought to confirm if *daf-16(mu86)* as well as *daf-16(mu86); him-6(ok412)* had an increased rate of mutation. To do so, I used a more stringent assay based on a mutation in a reporter gene, *unc-93*. Typically, *unc-93(e1500)* is extremely uncoordinated and has an egg laying defect (Greenwald and Horvitz 1980). As *unc-93(e1500)* is grown over multiple generations, mutations can cause the strain to revert back to wild type behavior with normal movement and egg laying abilities (Greenwald and Horvitz 1980). The use of *unc-93* as a means of determining the rate of spontaneous mutation has been established (Hofmann et al. 2002; Tijsterman et al. 2002; Grabowski et al. 2005). Genes which have been known to maintain genomic stability when crossed into the *unc-93(e1500)* background have significantly increased the rate of reversion (Hofmann et al. 2002; Tijsterman et al. 2002; Grabowski et al. 2005).

Upon crossing *daf-16(mu86)* into the *unc-93(e1500)* background; the reversion rate of each strain was compared to *unc-93(e1500)*. I found that *daf-16(mu86); unc-93(e1500)* had a three fold increase over wild type ($p=0.034$) (Figure 3.1). The increased

reversion rate confirms that *daf-16* mutants do have a mutator phenotype. This assay does not identify a potential mechanism through which DAF-16 may function to preserve genomic stability. Although this assay does produce strong evidence that DAF-16 may play a role in preventing spontaneous mutation in the genome from occurring.

In the *daf-16(mu86); unc-93(e1500); him-6(ok412)* mutant, the rate of spontaneous reversion was 7.2 ± 1.3%. Interestingly, the rate of spontaneous reversion in the *daf-16(mu86); unc-93(e1500); him-6(ok412)* was not significantly higher than *unc-93(e1500); him-6(ok412)* which had a spontaneous mutation rate of 4.8 ± 0.9% (p=0.19) (Figure 3.1). This data confirms that the rate of spontaneous mutation is increased when both *daf-16* and *him-6* are lost; thus both genes are required to maintain genomic stability in *C. elegans*. This data however, does not indicate if these genes function in the same pathway or different pathways to maintain genomic stability.

Figure 3.1

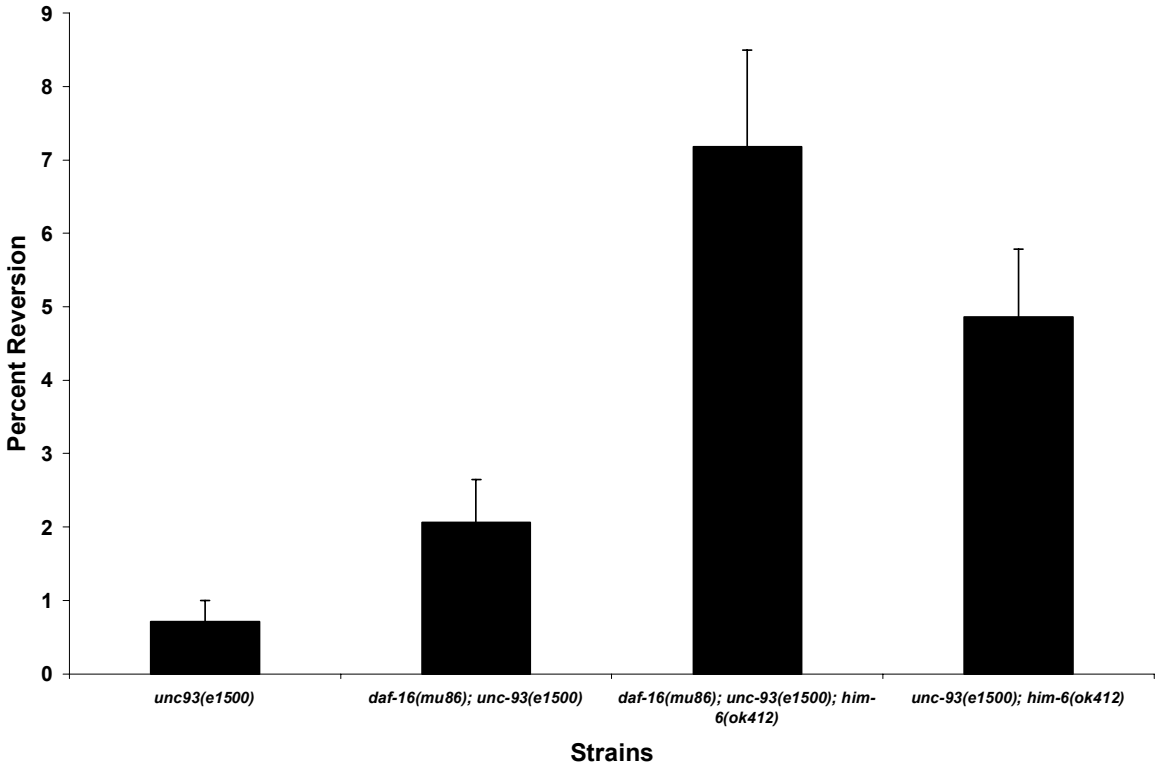
Percent Reversion
9
8
7
6
5
4
3
2
1
0
unc93(e1500)
daf-16(mu86); unc-93(e1500)
daf-16(mu86); unc-93(e1500); him-6(ok412)
unc-93(e1500); him-6(ok412)
Strains

Figure 3.1: *daf-16(mu86)* has a mutator phenotype and *daf-16(mu86); him-6(ok412)* has a high rate of reversion. The rate of spontaneous reversion in *daf-16(mu86); unc-93(e1500)* is significantly higher than *unc-93(e1500)* alone ($p=0.034$). *daf-16(mu86); unc-96(e1500); him-6(ok412)* undergoes significantly more reversion events than *unc-93(e1500)* ($p<0.0001$), however, there is not a significant difference between *unc-93(e1500); him-6(ok412)* and *daf-16(mu86); unc-93(e1500); him-6(ok412)*. This assay was conducted at 20°C and at least 1700 plates per strain were examined. Error bars indicate standard error

DAF-16 has no effect on brood size or composition

In order to better understand the role of DAF-16 in preventing the occurrence of spontaneous mutation, I turned to other well defined assays which correlate with genomic stability. The number and sex of progeny a mutant strain can produce has been defined as a potential indicator of genomic instability. The number of viable progeny each worm produces can identify mutant strains which have a high amount of genomic instability; in this case, a high amount of genomic instability would result in a low brood size (Hofmann et al. 2002). Additionally, the presence of males in the population is an indicator of genomic instability. In a wild type population of *C. elegans*, males typically arise in 0.1% of the population due to a non-disjunction of the X chromosome (Hodgkin et al. 1979). In strains with a high rate of genomic instability, the incidence of males is often increased resulting in a **H**igh **I**ncidence of **M**ales or HIM phenotype (Hodgkin et al. 1979).

In the case of *daf-16(mu86)* the brood size, incidence of males, and percentage of eggs which hatch is comparable to that of wild type (Table 3.1). In this assay we used two null alleles of *daf*-16. *daf-16(mu86)* lacks most of the coding sequence and all of the forkhead domain because of a deletion (Lin et al. 1997), while *daf-16(mgDf50)* has a deletion ranging from exons 1 to 10 (Ogg et al. 1997). This deletion removes all of the conserved domains and nearly all of the coding region (Ogg et al. 1997). I found *daf-16(mgdf50)* does not have a 100% hatch rate, but *daf-16(mgDf50)* it is comparable to both wild type and *daf-16(mu86)* in terms of brood size and incidence of males (Table 3.1). It is unclear why *daf-16(mgDf50)* does not have a 100% hatch rate, the lower hatch rate may be attributed to the size of the deletion and the subsequent severity of the

mutation. Based on this data, at 20°C it appears that *daf-16* mutants may not play a role in maintaining genomic stability. *daf-16* mutants do not display any classic signs of genomic stability such as a low brood size or a high incidence of males.

Upon shifting the strains to 25°C, I found that *daf-16(mu86)* and *daf-16(mgDf50)* were slightly temperature sensitive. At 20°C, *daf-16(mu86)* has a brood size of 246 ± 15 while at 25°C, the brood size is reduced to 136 ± 6 ($p<0.0001$) (Table 3.1). When I repeated the same assay with *daf-16(mgDf50)* I saw the same effect, at 20°C the brood size was 204 ± 22, while at 25°C the brood size was reduced to 95 ± 13 ($p=0.0002$) (Table 3.1). While the difference in brood size of wild type is significantly reduced when grown at 20°C versus 25°C ($p=0.0008$), it is important to note that the brood size of both *daf-16(mu86)* and *daf-16(mgDf50)* is significantly lower than wild type ($p=0.005$ and $p<0.0001$ respectively) (Table 3.1). Interestingly, *daf-16(mu86)* mutants, while having a lower brood size, do not have a HIM phenotype. This implies that if genomic instability is occurring in the worm, it does not cause an increase of chromosomal non-disjunction. If damage is occurring or persisting in the worm, it may not be as severe as the damage seen in *him*-6 mutants. This data implies that DAF-16 is required to deal with mild temperature stress and when challenged, the reproductive capacity of *daf-16* mutants is diminished. Additionally, this data may suggest when *daf-16* mutants are stressed, genomic stability may be harder for *daf-16* mutants to maintain.

Table 3.1

Strain	Percent Hatched[a]	Brood Size	Percent Male
N2 (°C)			
20	100	216	0.04
25	100	171	0.1
daf-16(mu86) (°C)			
20	100	246	0.0
25	100	136	0.2
daf-16(mgDf50) (°C)			
20	95.0 ± 4.9	204	0.03
25	87.5 ± 9.8	95[b]	1.1

Table 3.1: *daf-16* mutants have a normal brood size and composition at 20°C and at 25°C, *daf-16* mutants have a low brood size.

[a] Value is number ± standard error.

[b] statistically different than N2 at 25°C. *daf-16(mu86)* p=0.005, *daf-16(mgDf50)* p=0.0008.

***daf-16* causes an apoptotic defect**

Given previous work on the role of DAF-16 in apoptosis has conflicting results (Pinkston et al. 2006; Quevedo et al. 2007), I attempted to determine the role of DAF-16 in apoptosis. I utilized the CED-1::GFP strain to quantify the amount of germ line apoptosis in *daf-16* mutants. To perform this assay CED-1::GFP; *daf-16(mu86)*, CED-1::GFP; *daf-16(mgDf50)*, or CED-1::GFP L4 hermaphrodites were irradiated with 0, 60, 120, and 150 Gys of X-ray. After irradiation, the strains were grown at 20°C for 14 to 16 hours. The number of apoptotic cells in the germ line was then counted using GFP microscopy.

When I compared the rate of apoptosis of the strains, I found in *daf-16(mu86)* the amount of cell death did not increase in a dose dependent manner (Figure 3.2). In wild type, as the worms are exposed to higher dosages of X-rays, the amount of cell death increases (Figure 3.2). This differs from CED-1::GFP; *daf-16(mu86)* where the amount of cell death remains consistent even though the amount of DNA damage should increase (Figure 3.2). This data is supported by the findings of Pinkston *et. al*. In their work, Pinkston *et. al.* demonstrated that DAF-16 is required for a dose dependent response to X-ray damage (Pinkston et al. 2006). My data differs slightly from Pinkston *et. al.*, I do see an initial increase of apoptosis in *daf-16* mutants when I compare the unexposed mutants to mutants exposed to 60 Gy of X-ray. The differing results could be explained by the differing protocols used to determine the rate of cell death. Pinkston *et al* scored on the distal gonad arm while I scored both a gonad arms in their entirety. Additionally the protocols differ because the germ line apoptosis was identified using SYTO12 labeling and the scoring was performed after 18 hours. To perform this assay, I would

expose the worms to varying amount of X-rays and then let the worms recover for 14-16 hours. I would count the number of apoptotic cells in both gonad arms to get an accurate representation of the amount of apoptosis occurring. I believe that use of the CED-1::GFP may give me more accurate results as I can score the number of apoptotic cells through multiple focal plains rather than just one.

It has been previously reported that other alleles of *daf-16* are sensitive to radiation induced cell death while *daf-16(mu86)* is not (Quevedo et al. 2007). When I examined the effects of X-ray damage on *daf-16(mdDf50)* and quantiated the amount of cell death, I found *mgDf50* had a similar dose dependent cell death response in response to X-ray damage as wild type as both *daf-16(mgDf50)* and wild type had a significant increase in cell death between 60 and 120 Gys of X-ray exposure (Figure 3.2). This is the same result as was demonstrated in Quevedo *et. al*. It is apparent both *daf-16* alleles behave in a different fashions, this could be due to the size and the severity of the deletions in both strains. It is apparent DAF-16 plays a role in apoptosis, however, the role of DAF-16 in apoptosis needs to be further elucidated.

Figure 3.2

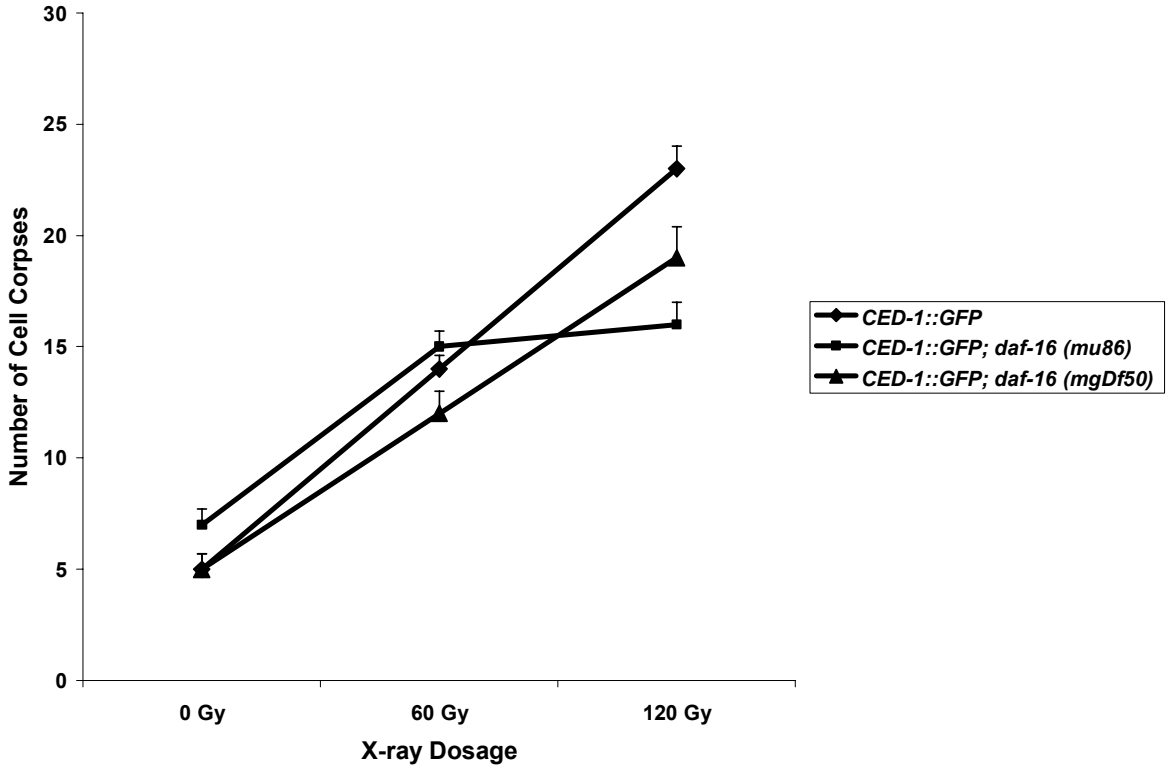
30
25
20
15
10
5
0
Number of Cell Corpses
0 Gy
60 Gy
120 Gy
X-ray Dosage
CED-1::GFP
CED-1::GFP; daf-16 (mu86)
CED-1::GFP; daf-16 (mgDf50)

Figure 3.2: *daf-16* mutants display an allele specific cell death phenotype. Using increases dosages of X-ray exposure, I determined the number of apoptotic germ-line cells compared to wild type worms. *daf-16(mu86)* does not have a proportional increase in the number of cell deaths compared to wild type. *daf-16(mgDf50)* did a have a proportional increase in cell deaths compared to wild type. Error bars indicate standard error.

III) Conclusions

Maintaining genomic stability is a complicated process that involves many different pathways working together. I have identified a novel role for *daf-16* in maintaining genomic stability. While the mechanisms through which DAF-16 maintains genomic stability still remain to be elucidated, a loss of DAF-16 causes a mutator phenotype based on the *unc-93* data. The mutator phenotype is subtle in a *daf-16* mutant. Given how subtle the mutator phenotype is in *daf-16* mutants, it is unsurprising that it has been over looked for so long. This implies that the role of DAF-16 in maintaining genomic stability is minor yet is still important.

To date, I have not identified the pathway(s) through which *daf-16* functions to prohibit this mutator phenotype. It is unclear why *daf-16(mu86); him-6(ok412)* mutants have such a strong mutator effect; however there are several hypotheses which may explain this phenomena. First, DAF-16 and HIM-6 may both operate in separate repair pathways. Secondly, it is possible that DAF-16 is not directly involved with maintaining genomic stability, but instead may transcribe various genes involved in maintaining genomic stability. Therefore, removal of DAF-16 via mutation may affect multiple DNA repair pathways resulting in an increase of spontaneous mutations. Finally, it is possible the effect I see may not be due to DNA repair directly, but in fact, may be due to the inability to cope with oxidative damage. DAF-16 is involved in activating multiple stress response pathways and when these pathways are not activated, the worm may be more susceptible to other stresses that would normally not have as great an effect. This hypothesis is further supported by the effect temperature has on the brood size of *daf-16* mutants. At 25°C, the number of progeny *daf-16* mutants produce is significantly

reduced when compared to wild type worms. The lack of a HIM phenotype indicates if DAF-16 plays a role in genomic stability, loss of DAF-16 does not cause an increase in chromosomal non-disjunction.

The inability to cope with additional stresses is also demonstrated with the apoptosis defect seen in *daf-16(mu86)*. While wild type worms show a dose dependent increase in cell death as the amount of X-ray dosage increases, the level of cell death in *daf-16(mu86)* becomes stable after 60 Gy of exposure. For reasons which are unclear, this is an allele specific effect as *daf-16(mgDf50)* does not have this effect on cell death. It is unclear why the two *daf-16* alleles behave differently, it may be due to the size and nature of the deletions.

The identification of a new role for DAF-16 in maintaining genomic stability introduces a question concerning the role of genomic stability in maintaining a normal life span. It is difficult to make a strong statement concerning genomic stability and its effects on life span. Both *daf-16* and *him-6* mutants have been demonstrated to play a role in maintaining genomic stability and both have a shortened life span. It is unlikely that genomic stability plays a major role in life span maintenance in *C. elegans*. Genomic instability probably accounts for some of the life span shortening see in *daf-16* mutants; however it is more likely that other regulators of life span such as stress response pathways and pathogenic response pathways play a larger role. While much still remains to be understood, based on the data, I have demonstrated a new role for *daf-16* in the suppression of spontaneous mutations in the genome of *C. elegans*.

IV) Materials and Methods

Strains

All strains were maintained at 15°C under standard conditions (Brenner 1974).

unc-93 assay

Using independent, freshly thawed cultures, 100 worms of each genotype *unc-93(e1500)*, *daf-16(mu86); unc-93(e1500)*, and *daf-16(mu86); unc-93(e1500); him-6(ok412)* were picked and transferred to individual plates. Plates were incubated at 20°C until no food remained on the plates and the animals were starved. The plates were then divided into quarters and these quarters were transferred to fresh plates which had been numbered to ensure independent events were followed up. Two and three days after the transfer to fresh plates, the plates were scored for reversion events that were identified by the presence of phenotypically wild type worms. At least 1800 plates where scored per strain. Statistical analysis was determined using the student T test.

Brood Size Analysis

Five to ten L4 hermaphrodites were picked to individual plates at 20°C or 25°C. Following this selection, worms were transferred each day to fresh plates until they ceased to lay eggs. The number of L1s and eggs on the plate from the previous day were counted. Three days later, adult worms and males were counted. This experiment was repeated 2 times using at least 5 worms per strain in each analysis. The data shown are an average of several experiments and statistical analysis was determined using the student T test.

Cell Death Assay

CED-1::GFP; *daf-16(mu86)*, CED-1::GFP; *daf-16(mgDf50)*, and CED-1::GFP L4 hermaphrodites were irradiated with 0, 60, 120 and 150 Gy of X-ray. After irradiation, the strains were grown at 20°C for 14 to 16 hours. Then the number of apoptotic cells in the germ line was then counted using GFP microscopy. At least 15 CED-1::GFP and CED-1::GFP; *daf-16(mu86)* worms were examined for each X-ray dosage.

CHAPTER IV

DISCUSSION

This work identifies several new phenotypes of the BLM ortholog in *C. elegans, him-6*. The phenotypes which have been identified include a cell death phenotype, a mortal germ line phenotype, a shortened life span and a mutator phenotype. Another important finding demonstrated in this thesis is that *him-6* mutants cause a shortened life span. The identification of a mutator phenotype confirms the validity of using *C. elegans* as a model organism for the study of Bloom's Syndrome. In cell culture, loss of BLM results in an increased amount of small deletions and duplications (German 1993; Langland et al. 2002); I have found this damage is similar to the damage that occurs in a *him-6* mutant. All these phenotypes pertain to *him-6* playing a role to maintain genomic stability.

In addition to further characterizing the role of *him-6* in maintaining genomic stability, this work also outlines a new role for DAF-16. DAF-16 has been intensively studied for several reasons including its central role in the *C. elegans* insulin signaling pathway and its role as a transcription factor. DAF-16 is an upstream activator of many pathways that are important for maintaining a normal life span.

While this work provides additional insight into the roles of DAF-16 and HIM-6 in maintaining genomic stability in *C. elegans*, there are still many questions which still need to be addressed. The following section will highlight the major points of interest from this work and address these questions.

HIM-6 and genomic stability

This work and the work of others have demonstrated that HIM-6 plays a role in maintaining genomic stability. *him-6* mutants have a defect in meiosis, the rate of cross over events in the germ line is significantly reduced, and as indicated by the high incidence of males phenotype, *him*-6 mutants undergo a significant amount of chromosomal non-disjunction (Hodgkin et al. 1979; Zetka and Rose 1995; Wicky et al. 2004). In addition to the germ line defects, *him-6* mutants are sensitive to X-ray irradiation, indicating an inability to process DNA damage (McElwee et al. 2003; Wicky et al. 2004; Grabowski et al. 2005; Kim et al. 2005).

In this work, I have demonstrated that *him-6* mutants have an increased rate of cell death in the germ line. It is possible that the increased rate of cell death is due to an inability to repair DNA damage which drives the cells into apoptosis. To better understand why the germ line cells in *him-6* mutants are dying at larger numbers, it would be beneficial to look at the chromosome structure of these cells or other markers that indicate DNA damage.

I have provided further evidence that *him-6* mutants undergo genomic instability because loss of *him-6* results in a mutator phenotype. Upon determining that *him-6* mutants have a mutator phenotype, I have observed that the mutator phenotype may be caused by an increased rate of small deletions and insertions in the genome. The increase of small insertions and deletions mimics the effect of loss of BLM in cell culture (German 1993; Langland et al. 2002). It is unclear however, what is causing this increased rate of small insertions and deletions in *him-6* mutants. There are several

possibilities to explain the increased rate in insertions and deletions. The first is that the mutations could result from the faulty meiotic recombination that is a hallmark of loss of HIM-6. The faulty meiotic recombination could be caused by an inability of *him-6* mutants to resolve recombination intermediates and thus new mutations are introduced as secondary pathways attempt to resolve these intermediates. Another possibility is homologous recombination to repair DNA lesions is no longer possible in *him-6* mutants and secondary pathways such as NHEJ are used to repair the lesions. In this case, if NHEJ occurs in *C. elegans*, it may be responsible for the introduction of mutations as has been previously seen in cell culture (Gaymes et al. 2002).

In cell culture, the small deletions and insertions occur at the sites of double stranded breaks (Langland et al. 2002). Given *him-6* mutants are sensitive to X-ray damage (Wicky et al. 2004; Grabowski et al. 2005; Kim et al. 2005), and suffer from a defect in meiotic recombination (Wicky et al. 2004), perhaps loss of HIM-6 inhibits the pathway responsible for repairing double stranded breaks. This pathway remains to be identified.

It is also possible that HIM-6 may act as a sensor of double stranded breaks. When HIM-6 is functional, double stranded breaks occur and HIM-6 signals to the appropriate repair pathway. It is also possible that repair of the DNA lesions are attempted by a secondary repair pathway such as NHEJ is made to correct the damage. If attempts are made by a secondary pathway to repair the damage, it is possible that the damage may not be repaired correctly and additional mutations may be introduced.

When attempts to repair the double stranded breaks where made in cells derived from patients with Bloom's Syndrome (BS), it was demonstrated that loss of BLM

caused the non-homologous end joining repair pathway to function at a lower level of repair fidelity (Gaymes et al. 2002). In fact, it was demonstrated that repair via the non-homologous end joining pathway causes an increase in the number of both large and small deletions in BS cells (Gaymes et al. 2002; Langland et al. 2002). In *C. elegans*, there is a non-homologous end joining repair pathway; but it is unclear if loss of *him-6* has an effect on this pathway. To determine if *him-6* mutants have an effect on the non-homologous end joining pathway, double mutants could be created between HIM-6 and known members of the pathway. If the mutation rate is decreased, then the mutations were due to faulty non-homologous end joining. It is also possible however, that HIM-6 does not play a role in the non-homologous end joining pathway and that another pathway may be responsible for the rate of increased mutations.

While this work has shed light on a new phenotype caused by a mutation of HIM-6, many questions still remain to be answered. Before the role of HIM-6 in maintaining genomic stability can be determined, the function of HIM-6 protein in *C. elegans* must first be identified. To date, the assumption has been made that the HIM-6 will function in the same manner as its ortholog BLM, as both have a helicase domain. This has not yet been proven, as the biochemical assays needed to identify the helicase activity in *C. elegans* have not been developed. As biochemistry in *C. elegans* becomes accessible, it will be possible to shed more light on the function of HIM-6 as well as identify other proteins which interact with HIM-6; these studies will further explain the role of HIM-6 in maintaining genomic stability.

HIM-6 and life span

In this work, I have demonstrated that *him-6(ok412)* has a slight but significantly shortened life span when compared to wild type worms. This provides evidence for a potential link between genomic stability and aging. While *him-6* causes a shortened life span, there are still questions which need to be addressed. The first is what is causing this shortened life span- is it loss of *him-6*, is it the build up of mutations in the worm or a combination of both. A second question which arises is the shortening of life span due to a non-specific sickness in the worm rather than a direct consequence of loss of *him-6*.

To date, *him-6* has been one of a few genes involved in maintaining genomic stability that also has an effect on life span. Other genes involved in maintaining genomic stability and a shortened life span such as *him-6* are *wrn-1* and *rcq-5* (Jeong et al. 2003; Lee et al. 2004). Like *him-6* mutants, the life span effect of *wrn-1* RNAi and *rcq-5* RNAi on wild type worms was slight but signficant (Jeong et al. 2003; Lee et al. 2004). Interestingly, these three genes are all part of the RecQ family of helicases in *C. elegans* indicating this specific family of genes may also have a role in maintaining life span (Kim et al. 2002; Jeong et al. 2003; Lee et al. 2004; Wicky et al. 2004; Grabowski et al. 2005). Given that the effect of life span in *him-6* mutants as well as the *wrn-1* RNAi and *rcq-5* RNAi is small, the role genomic stability plays in maintaining life span in *C. elegans* may be not be a major role but an important role none the less.

DAF-16 and genomic stability

While the human homolog of DAF-16, FoxO3a, has an established role in maintaining genomic stability; the same role has not yet been identified for DAF-16 in *C. elegans*. While it is unclear if DAF-16 can maintain genomic stability, previous work has

identified a link between functional DAF-16 and cell death (Pinkston et al. 2006; Quevedo et al. 2007). In support of this work, I have found that certain *daf-16* mutants have a cell death defect. This implies DAF-16 plays a role in apoptosis, but this cell death defect is allele specific. It is unclear why there is an allele specific affect, however, the nature of the mutation in the *daf-16* alleles may be responsible. In order to better understand the role of DAF-16 in apoptosis, it would be beneficial to test other alleles of *daf-16* for a cell death phenotype.

In this work, I have demonstrated that loss of DAF-16 causes a mutator phenotype. Upon culturing this strain, I noticed the appearance of visible phenotypes that had not previously been associated with the loss of DAF-16. To confirm that *daf-16* did cause a mutator phenotype, I used the well-established, stringent *unc-93* assay. This assay confirmed that *daf-16* indeed did have an increased rate of mutation. However, this assay does not indicate the types of mutations caused by loss of DAF-16. Attempts to identify the types of mutation caused by *daf-16* through sequencing the *unc-93* gene were unsuccessful. When the exons of the reversions were sent out for sequencing there were multiple problems. The first problem arose during PCR. I used PCR to generate the correct concentration DNA to send for sequencing, to confirm that the correct exons were being generated; I would run the PCR products on a gel. Unlike the reversion events from the *unc-93(e1500); him-6(ok412)* strain, the *daf-16(mu86); unc-93(e1500)* reversion events often generated multiple bands. In order to attempt to remove the bands, I attempted using different primer sets as well as different parameters for the PCR programs, all attempts were unsuccessful. I then purified the PCR products and when the background had been reduced, I would send the samples to be sequenced. The sequence

data itself was inconclusive as there were large tracts of unreadable sequence and mutations that were identified in one strand could not be confirmed in the other strand. If sequencing becomes possible, the sequence data will provide valuable insight into the nature of the mutations that occur in a *daf-16* mutant background and perhaps shed light on the role of DAF-16 in maintaining genomic stability.

There are at least two possible hypotheses to explain this mutator phenotype; the first is that DAF-16 plays a direct role in maintaining genomic stability. When the genome of the worm is damaged, DAF-16 may directly activate a DNA repair pathway. However, it is unclear what may signal to DAF-16 to activate DNA repair pathway(s). Additionally, based on published data, it is unclear if DAF-16 can activate genes responsible for maintaining genomic stability (McElwee et al. 2003; Murphy et al. 2003; Oh et al. 2006). However, it is clear based on these studies that the number of downstream targets may exceed the number of targets which have been identified. As more DAF-16 targets are identified and their functions are studied, it may be possible that DAF-16 directly regulates genomic stability.

The second hypothesis is DAF-16 may play an indirect role in the regulation of genomic stability. DAF-16 may activate specific downstream pathways which in turn influences secondary pathways responsible for DNA repair. This removes the ability of DAF-16 to directly affect DNA repair which may account for the low percentage of spontaneous mutation seen in the *daf-16* mutant. This hypothesis becomes even more likely as DAF-16 has been linked to activating oxidative stress response pathways (McElwee et al. 2003; Murphy et al. 2003; Oh et al. 2006). Perhaps the mutations seen

are not due to genomic instability, but are due to an inability to cope with oxidative damage or additional stress.

The inability to cope with additional stress is supported by the brood size data. At 20°C, the brood size of *daf-16* mutants is comparable to that of wild type worms. When the worms are cultured at 25°C, the brood size of *daf-16* mutants is significantly reduced. This reduction in brood size could be caused by the reproductive capacity of *daf-16* being reduced in order to conserve energy to survive. It is also possible the reproductive capacity of *daf-16* mutants is reduced due to an increased amount of genomic instability. To determine if this is the case, it would be beneficial to look at the germ line of *daf-16* mutants after they had been cultured at a higher temperature to determine if they had chromosomal defects or other indicators of DNA damage. It would also be beneficial to expose the *daf-16* mutants to other stresses such as oxidative or DNA damaging stresses to determine if all types of stress have an effect on brood size.

Although it is unclear why there is an increased rate of mutation in a *daf-16* null mutant, the new phenotype is intriguing as it implies that *daf-16* strains are not as "healthy" as previously thought. Perhaps the shortened life span seen in *daf-16* mutants is due in part to an increased rate of genomic instability, however, significant work will have to be done to prove that this is the case.

Conclusions

The work in this thesis has identified several new mutations caused by a loss of HIM-6 in *C. elegans*. These new phenotypes which include a mutator phenotype, an increased amount of cell death, and a mortal germ line phenotype, indicate a role for

HIM-6 in maintaining genomic stability. Given that loss of HIM-6 causes genomic instability as well as a shortened life span, this work indicates a potential link between genomic stability and maintaining a normal life span.

In addition to identifying new phenotypes in *him-6* mutants, this thesis also outlines a potential new role for DAF-16. Work presented here demonstrates that loss of DAF-16 causes a subtle mutator phenotype. DAF-16 is a very well characterized gene and by identifying a new function in the prevention of spontaneous mutations, this opens new avenues for study and further confirms that DAF-16 in *C. elegans* behaves in a similar fashion to its homolog in humans.

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