Functional Analysis of Bloom Syndrome Helicase in Development and DNA Repair

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Abstract

Bloom Syndrome is a rare autosomal recessive disorder in humans caused by mutation of the *BLM* gene that leads to increased genome instability and cancer. The *BLM* gene codes for a helicase (BLM) that works together with Topoisomerase 3-alpha (Top3α) in homology-directed repair of DNA. Top3α directly binds to BLM and helps release the torsional stress on DNA as BLM unwinds recombination intermediates. These proteins preserve genome stability and have been shown in many organisms to operate together in the prevention of detrimental mitotic (non-meiotic) crossovers via two main DNA repair pathways, synthesis-dependent strand annealing and double Holliday junction dissolution. In *Drosophila*, *BLM* (known as *Blm*) also has roles in proper meiotic chromosome segregation and rapid cell cycle progression of the developing embryo. Each of these BLM functions are not well characterized and limit development of treatments for *BLM*-related disorders. To investigate the Blm-Top3α interaction in DNA repair, I performed a yeast 2-hybrid (Y2H) assay using the *Drosophila* genes. I found the interaction was specific to certain regions of Blm, with the strongest interaction observed at a C-terminal region conserved among several *Drosophila* species, amino acids (aa) 1381-1487. Based on these data, I created specific deletions of the *Blm* gene via CRISPR/Cas9 editing to characterize the various roles of Blm *in vivo*. First, I assessed the effects of *Blm* deletions on known *Blm* roles in meiotic chromosome segregation via a nondisjunction assay. Both aa 576-720 and N1 produced measurable defects compared to the *wild type* suggesting functional value of aa 576-720. Significance of this Blm region in preventing mitotic crossovers and DNA repair was evaluated by a crossover assay showcasing a lack of significant effect by aa 576-720 relative to the true null allele, N1, but still increased relative to *wild type*. These studies showcase the importance of aa 576-720 and other Blm regions in the roles of meiotic segregation and DNA repair. Blm aa 576-720 will be further assessed by examining the relevance of predicted ATR/ATM phosphorylation sites within the region required for proper Blm function. Additional Blm roles in embryonic development will also be explored via an embryo hatching assay. By characterizing the functions of Blm in *Drosophila*, we will better understand and improve BLM function within humans and the detrimental health effects associated with *BLM* mutations.

I. Introduction

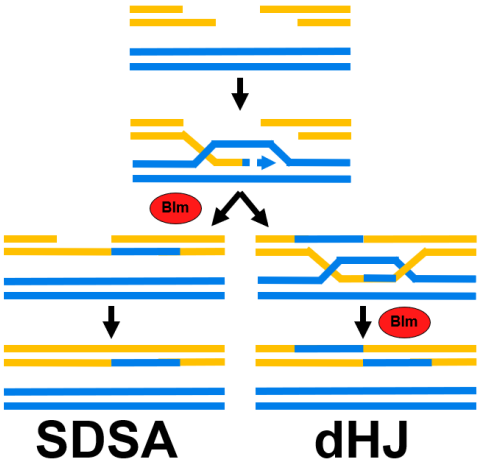

Figure 1*. Mechanism of DNA repair via Synthesis-Dependent Strand Annealing and double Holliday Junction dissolution.*

Homologous recombination DNA repair (HR) is a critical function essential for cell survival throughout growth and development[1]. DNA damage such as double strand breaks or stalled replication forks can cause significant genomic and transcriptional complications for cells affecting their ability to survive and advance[1]. When HR pathways function correctly, detrimental mitotic (non-meiotic) crossovers are prevented by repairing damage via Synthesis Dependent Strand Annealing (SDSA) or double Holliday Junction (dHJ) dissolution or resolution (Figure 1)[1]. However, mutations affecting HR lead to dangerous and possibly lethal chromosomal deletions, rearrangements, and loss of heterozygosity (LOH) within cells[1]. LOH can occur when a functional copy of a gene on one chromosome is replaced via HR with a nonfunctional copy of the gene from the other chromosome, producing homozygosity of the mutant allele and causing an allelic imbalance that can impair development and renewal of cells and tissues[1]. LOH and other chromosomal abnormalities are often observed in numerous conditions affecting HR, such as Bloom syndrome, and are a substantial predisposition for cancer[1].

Bloom syndrome is a rare, autosomal recessive disorder caused by mutation of the *BLM* gene, leading to genomic instability[2]. Symptoms range from retarded growth, sun sensitivity, and immunodeficiency to chromosomal abnormalities and heightened cancer susceptibility (Figure 2)[2]. *BLM* codes for a DNA helicase within the RecQ helicase family[2]. It functions in SDSA and dHJ dissolution through interactions with Topoisomerase 3-alpha (TopIIIa)[3]. TopIIIa aids HR processes by reducing DNA torsional stress, or untangling DNA "knots," when it is being unwound by Blm[4]. The two proteins work together for efficient and successful HR that minimizes crossovers and LOH. If Blm cannot bind to TopIIIa due to a mutation, the cell must instead utilize other pathways that are more likely to lead to LOH, such as dHJ resolution[3].

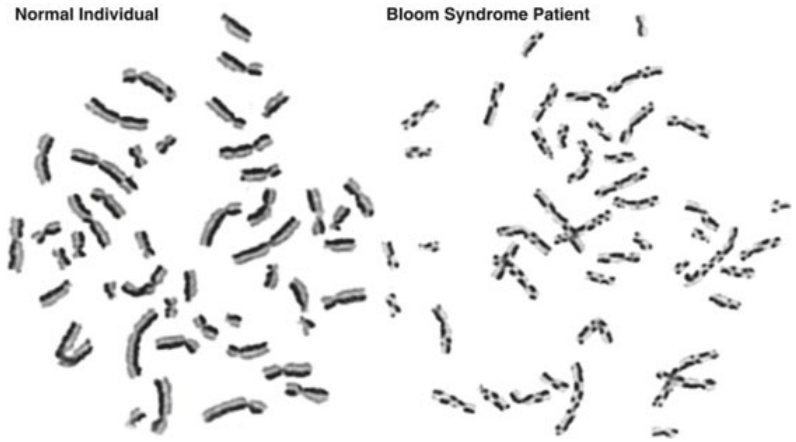

Figure 2. *Comparison of healthy unaffected chromosomes and chromosomal abnormalities of Bloom syndrome patient. Gray and black regions visualize maternal and paternal homologous chromosomes. Checkerboard pattern of Bloom syndrome patient chromosomes indicate spontaneous DNA damage*[11].

Examining the interaction between Blm and TopIIIa can help to understand the defects that may arise. Testing of the *Drosophila* Blm regions required for this interaction can be conducted by a yeast two-hybrid (Y2H) assay[5]. *Saccharomyces cerevisiae* (budding yeast) are small, eukaryotic microorganisms that can be easily manipulated to take up new DNA via transformation[1]. The Y2H assay utilizes a yeast transcription factor normally used in galactose metabolism, *GAL4*, to test the interaction between two proteins of interest. The GAL4 protein has two regions, a binding domain (BD) and an activation domain (AD), that must be present for efficient transcription and activation of metabolic genes in yeast[5]. In a Y2H, the BD is fused to a protein (or protein fragment) and binds the UAS (upstream activating site) promoter of a gene required for yeast growth in a particular restrictive medium[5]. The AD is fused to a different protein (or protein fragment), which will attempt to bind to the first protein (or protein fragment) fused to the BD[5]. Activation of transcription, and therefore an accurate function of a gene necessary for growth on a particular minimal media, will only occur if the two proteins fused to each GAL4 domain are able to interact (Figure 3)[5].

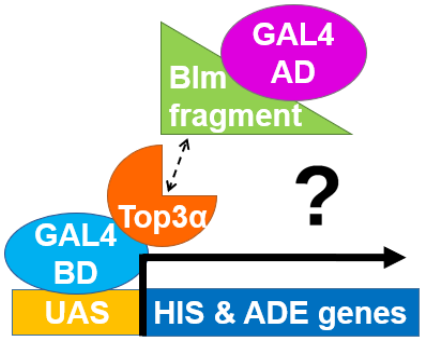

Figure 3. *Diagram of protein-protein interaction with a yeast two-hybrid assay. Gene transcription and growth on restrictive media proceeds when the two proteins interact.*

Blm functional analysis can also be conducted *in vivo* via *Drosophila melanogaster* (fruit flies). These small insects are a great model for human conditions as they share over 75% of human disease-causing genes, including *BLM* (known as *Blm* in *Drosophila*)[6]. By using the

CRISPR/Cas9 gene editing system, these genes can be easily manipulated to test the effects of specific alterations within the fly genome. The Cas9 endonuclease will locate the desired site for editing in germline stem cells of the developing *Drosophila* embryo using an injected guide RNA (gRNA) and will make a double-strand break in the DNA[7]. The DNA will then be repaired using injected template DNA homologous to the area around the cut site and contains the desired genetic changes[7]. This will result in a precisely altered gene that can be analyzed using genetic assays to determine functionality of specific regions of a gene.

This strategy will be applied to *Blm* by examining effects of amino acid deletions on Blm roles, specifically in prevention of mitotic crossovers and in proper segregation of meiotic chromosomes. Overall functionality of the Blm deletions in meiotic chromosome segregation will be evaluated via a nondisjunction (NDJ) assay. NDJ occurs when flies experience an abnormal segregation of chromosomes resulting in genotypes unique from standard male (XY) and female (XX) sex chromosome makeup[8]. A heightened prevalence of NDJ within flies of a specific *Blm* deletion will indicate functional significance of the deleted amino acid region for the successful completion of Blm roles in meiotic chromosome segregation. A similar approach will also be utilized to locate regions of importance to preventing DNA crossover during mitosis. Increased genetic crossover leads to instability of the genome that could influence HR pathways and harm cell growth and development[1].

These experiments will supplement the Y2H results. *Blm* gene fragments potentially involved in Blm-TopIIIα interaction will be deleted via CRISPR/Cas9 *in vivo*. They will be tested for *in vivo* functionality within HR and meiotic chromosome segregation using *Drosophila*. I hypothesize that extreme N-terminal and C-terminal regions of Blm will have the most impact on Blm roles of DNA repair and development and will therefore have the greatest interaction with Top3α and deletion effects, in agreement with prior literature[2]. These studies will obtain useful knowledge about how these proteins interact and how Blm functions in HR and meiotic chromosome segregation. The information will be applicable to diseases caused by improper repair of DNA damage, such as Bloom syndrome and cancer, and could assist in future gene editing and drug production for their treatment and prevention.

II. Materials and Methods

1. Cloning Procedures

PCR Amplification – Drosophila Blm fragment DNA was amplified using a Primestar DNA polymerase PCR protocol (Takara/Clontech). The 20 µL reaction contains 13 µL of ddH_2O, 4 µL of 5x Primestar buffer, 1 µL of DNA template, 0.5 µL of 2.5 mM deoxynucleotide triphosphates (dNTPs), 0.5 µL forward (5') primer, 0.5 µL of reverse (3') primer, and 0.5 µL Primestar GXL DNA polymerase. Primers were designed via SnapGene software. They typically had 15 nucleotides (NTs) complementary to the plasmid DNA vector combined with 18-25 complementary NTs to the target Blm DNA. The reaction was placed in an ABI thermal cycler with an annealing temp of 64°C and extension of one min per 1,000 kb.

Plasmids – pACT2 and pGBD plasmid DNA vectors were used for GAL4 AD and GAL4 BD fusions, respectively. Each plasmid has ampicillin (AMP) resistance for bacterial selection. They also have metabolic genes for yeast selection: (-) Tryptophan ([-]Trp) for pGBD and (-) Leucine ([-]Leu) for pACT2. In this study, three Blm fragments were fused to the GAL4 AD using pACT2 plasmid DNA vector: amino acids (a.a.) 576-720 (N-terminal sequence adjacent to helicase domain), a.a. 1270-1487 (unstructured full C terminus), and a.a. 1337-1487 (conserved C terminus). Full length Blm and two other Blm fragments were previously cloned into pACT2 by other lab members: conserved region 5 (CR5; a.a. 1381-1487) and remaining sequence of the N2 deletion (N2; a.a. 576-1487)[8]. TopIIIa was also previously fused to the GAL4 BD using the pGBD plasmid DNA vector.

Restriction enzyme digest – The vector plasmid was cut through a restriction enzyme digest composition of 9 µL of ddH_2O, 7 µL of uncut, empty plasmid, 2 µL of CutSmart buffer, and 1 µL of each restriction enzyme. The reaction was pipetted up and down to mix before incubating for 1-2 hrs at 37°C.

Gel extraction – The amplified inserts and vector plasmid restriction enzyme digest reactions were mixed with 6x DNA loading dye run with a 1kb DNA ladder (New England Biolabs) on a 1% agarose gel at 115 volts and 400 mA for 40 minutes. The gel was then imaged by a Gel Dox XR+ Documentation machine (BioRad) and visually checked for accuracy based off expected size. DNA bands were excised under a UV lightbox and DNA was extracted from the agarose

gel slices as specified in the QIAquick Gel Extraction protocol (Qiagen) and eluted in 30 µL of water.

In-Fusion – The cut vector plasmid and Blm DNA were fused together (Figure 4) by mixing 3 µL of ddH_2O, 2 µL of cut vector, 2 µL of insert, and 3 µL of 5x In-Fusion enzyme and buffer premix (Takara/Clontech), and incubating at 50°C for 15 min. The protocol was varied to create cut vector and Blm DNA concentrations as high as 150 ng/µL.

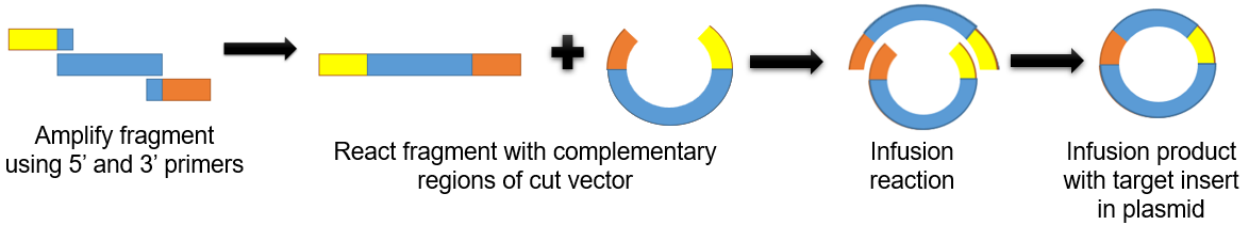

Figure 4. *Diagram of the In-Fusion reaction. PCR obtains the target insert with 15 bases of complementarity to the vector plasmid for overlap. The insert and a cut vector are combined via a specialized In-Fusion enzyme. Sections of complementary bases on either end of both the insert and vector allow fusion of the insert into the vector to create an intact plasmid.*

Bacterial transformation – The fused plasmid DNA was inserted into MACH 1 chemically competent *E. coli* cells by adding 4 µL of the In-Fusion reaction to 50 µL of cells and incubating on ice for 20 min. Cells were heat shocked in a 42°C water bath for 30 sec to induce the *AMP* resistance gene within plasmid DNA for plasmid uptake. Cells were incubated on ice for 2 min and recovered at 37°C with shaking for 1 hr in 500 µL of CIRCLEGROW-rich media. Recovered cells were spread on a LB + AMP agar plate with glass beads, absorbed for 10 min, and incubated overnight at 37°C.

Mini prep – To isolate plasmid DNA, 4-8 colonies of transformed bacteria were grown overnight in 2 mL liquid LB cultures supplemented with AMP and shaking at 37°C. Bacterial cells were pelleted via centrifugation of 1.5 mL of the overnight culture for 1 min at 13,000 rpm, supernatant was removed, and pellets were resuspended in 100 µL of buffer P1 (50 mM Tris-HCl, pH 7.5, 10 mM of EDTA, and 100 µg/mL RNase). Cells were lysed for 5 min at room temperature by gently mixing 100 µL of buffer P2 (0.2 N NaOH and 1% SDS) into the re-suspended cells. Protein and lipids were next precipitated by adding 100 µL of buffer P3 (1.32 M KOAc, pH 4.8) and vigorously mixing. Precipitated protein and lipids were pelleted via

centrifugation at 13,000 rpm for 5 min and the supernatant was added to 750 µl of 95% ethanol. DNA accuracy was verified via Sanger sequencing (Eton Bio).

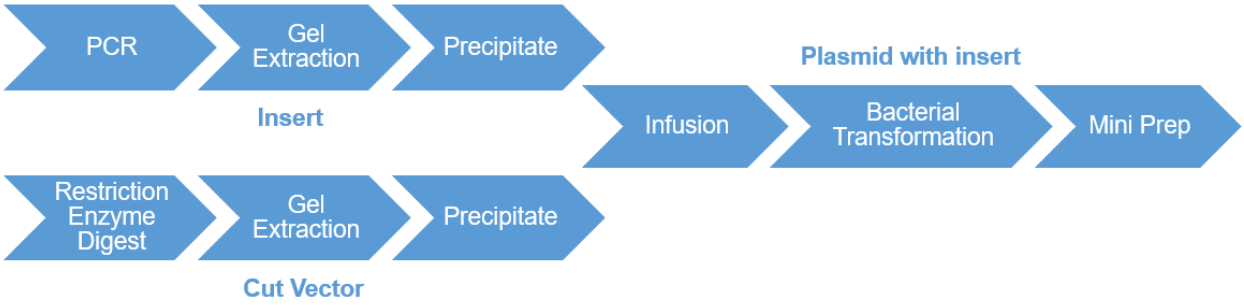

Figure 5. *Chronological visual of cloning procedures. Protocols for the insert and cut vector are completed separately, then combined to produce the desired plasmid.*

2. Yeast Protocols

Yeast transformation – Wild-type yeast were cultured in a 2 mL YPD rich media overnight at 32˚C with shaking. 1 mL of overnight culture was transferred to 1 mL of fresh media and grown for 1-2 hrs to achieve mid-log (exponential growth) phase. The culture was centrifuged for 20 min at 400 x g to pellet the cells, the supernatant was removed, and the pellet was resuspended in 100 µL 1X TE/LiAc (1 mL 10X [1M, each] Tris-HCl + EDTA, 1 mL 10X [1M] Lithium Acetate, 8 mL ddH_2O). A solution of 50 µL of the suspended yeast cells, 1 µL pACT2 *Blm* fragment and pGBD *TopIIIa*, 5 µL of salmon sperm DNA, and 500 µL of PEG solution (8 mL 50% polyethylene glycol 3350, 1 mL 10x TE, 1 mL LiAc) was mixed by inversion and incubated for 30 min at 32°C with shaking. The yeast were heat shocked in a 42°C water bath for 15 min, and then centrifuged at 3,000 rpm for 5 min. The supernatant was removed and the yeast were suspended in 100 µL 1X TE (1 mL 10X [1M, each] Tris-HCl + EDTA, 9 mL ddH_2O). Yeast were plated using glass beads on (-)Leu(-)Trp minimal media plates to select for plasmid uptake and grown for 3 days at 30°C.

Serial dilution – A quantitative methodology of analysis was conducted through Y2H serial dilutions. Isolated colonies for each pACT2 *Blm* fragment + pGBD *TopIIIa* transformant were grown overnight in 5 mL (-)Leu(-)Trp minimal media cultures. Culture optical density at 600 nm (OD_{600}) was measured to create fresh cultures at an OD_{600} of 0.45 units. They were placed back into the 32°C shaker for about 2 hrs. The OD_{600} was then measured from 1:10 dilutions to assess which cultures were in log phase (0.5-1.0 OD_{600} for yeast). Log phase cultures were then diluted

to 0.5 OD_{600} for each sample and 100 µL of the dilution was placed into a 0.2 mL tube. Three other 0.2 mL tubes were filled with 90 µL of water. Starting with the 0.5 OD_{600} solution, 10 µL was added to the next tube, mixed, and drawn up again to add to the next tube to create serial dilutions of 0.05, 0.005, and 0.0005 OD_{600} units for each sample. To plate, 10 µL of each dilution was dispensed on a (-)Leu(-)Trp(-)His(-)Ade experimental plate and a (-)Leu(-)Trp minimal media growth control plate using a multichannel pipettor. The yeast were incubated at 30°C for 24 hours to dry, then inverted and incubated for 2-3 more days. Full yeast growth indicated successful protein interaction; sparse growth suggested a lack of interaction.

3. *Drosophila melanogaster* Protocols

Plasmids – pCFD4 and pSL1180 plasmid DNA vectors were used to make gRNAs and a homology template, respectively, for use in CRISPR/Cas9 gene editing. Cloning procedures followed the PCR, restriction enzyme digest, and In-Fusion protocols above. Forward and reverse primer templates for pCFD4 cloning were designed as described in previous literature and modified with the appropriate gRNA, selected using the CRISPR Optimal Target Finder webpage[12]. The protospacer adjacent motif (PAM) site within the gRNA serves as the trigger for Cas9 endonuclease activity to cut the gene. These sites were altered via infusion primers to prevent any undesired cleavage beyond this site. 5' and 3' homology arms (HA) were cloned sequentially into the pSL1180 plasmid for use as a homology template. Infusion primers were created to clone each *Blm* deletion 5' HA into linearized pSL1180 plasmid vector. Once the 5' HA was successfully inserted into pSL1180, infusion primers for the 3' homology arm amplified and fused the 3' HA into linearized pSL1180 5' HA plasmid to produce a homology template with the target *Blm* deletion. The two plasmids were co-injected into Cas9-expressing fly embryos (via Genetivision, Houston, TX). The gRNA plasmid provided Cas9 with gRNAs to the site where it will make double strand breaks within

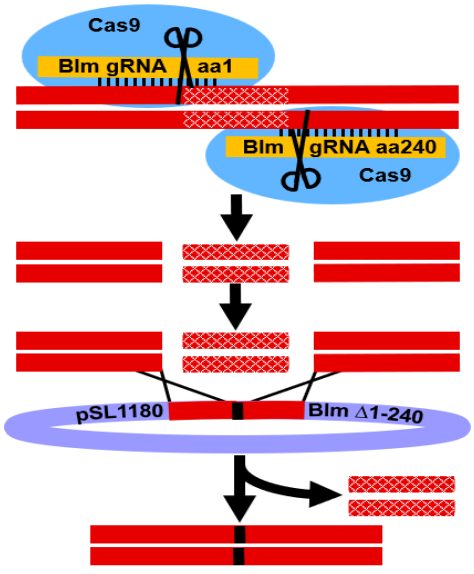

Figure 6. *CRISPR/Cas9 mechanism for in vivo Blm deletion. Cas9/gRNA complex cuts DNA via HDR resulting in deletion based on the given template. Shown here is the deletion of amino acids 1-240.*

the developing germline of *Drosophila* embryos. The damaged DNA was then repaired via HR utilizing the homology template plasmid to create flies that have the specific *Blm* deletion within male germline cells that can be passed onto progeny (Figure 6)[7].

Fly Stocks – In order to recover and create a stock of flies with the desired *Blm* deletion, a series of crosses were set up as depicted in Figure 7. Two balancers, TM3 and TM6B, Hu Tb, were utilized to prevent recombination and potential loss of newly created deletions throughout the process. In step 1, ten balanced females were crossed to one Cas9 male potentially containing the deletion (as indicated by brackets). Male progeny balanced with TM6B, Hu Tb (marked by having more than two shoulder hairs) were collected. In step 2, one of these males was crossed to ten more balanced females. Male and female progeny balanced with TM3 (marked by stubbled back hairs) were collected. Lastly, five females and three males from this collection were crossed to produce a stock of flies with the desired *Blm* deletion balanced over TM3. The presence of the deletion was confirmed using a Fly assay PCR (see below) in the single male crossed in step 2 (after allowing him time to mate), and again after stock creation.

Fly Prep – To extract the DNA of *Drosophila melanogaster* for detection of integrated deletions within the genome, a fly must be anesthetized and frozen for 15 minutes at -20°C in a 0.5 µL tube. Using a pipette tip full of 50 µL of 1:10 proteinase K dilution in Squish Buffer (10 mM Tris-Cl pH 8.2, 1 mM EDTA, 25 mM NaCl), the fly was mashed and ground before expelling the liquid into the tube. The solution was incubated in an ABI thermal cycler at 37˚C for 30 min to activate proteinase K's breakdown of fly proteins, followed by 5 min at 95% to inactivate proteinase K.

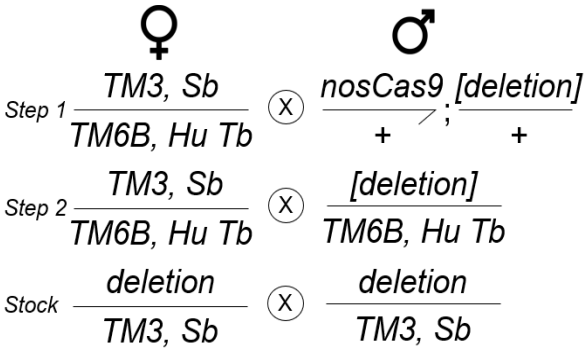

Figure 7. *Drosophila melanogaster crosses to produce stock of desired amino acid deletion of Blm.*

Fly Assay PCR Amplification – Drosophila Blm DNA was amplified using a Taq DNA polymerase PCR protocol (Sekelsky Lab). The reaction contains 15.3 µL of ddH_2O, 2 µL of 20 mM fly buffer, 1 µL of fly prep, 0.5 µL of dNTPs, 0.5 µL forward (5') primer, 0.5 µL of reverse (3') primer, and 0.2 µL Taq DNA polymerase. The primers were designed utilizing SnapGene

and typically consisted of 18-25 nucleotides (NTs) complementary to the *Blm* DNA surrounding the deletion and 40-60% GC. The reaction was placed in an ABI thermal cycler with an annealing temperature of 60°C and extension time of one minute per 1,000 kb. Resulting products were run on a gel and compared to pSL1180 5' + 3' HA (positive) and *wt* (negative) controls to assess the presence or absence of *Blm* deletions.

Nondisjunction Assay – To test Blm function in meiotic chromosome segregation, a two-step process of crosses was created. First, 30 females containing a *Blm* null allele balanced by TM3 were crossed to 15 *Blm* deletion male flies balanced by TM3. Unbalanced progeny indicated a genotype of the *Blm* deletion heterozygous with the *Blm* null allele. Each secondary cross used 10 females of this genotype crossed with 4 male flies with a bar-eyed phenotype (B^sY) on the Y chromosome. (Maternally lethal deletions required higher parental numbers of 30 by 8 to produce equivalent progeny totals.) The resulting progeny were scored based on eye shape, with B^sY males and *wild-type* (*wt*) females indicating proper segregation and *wt* males and B^sY females showing improper segregation. Additional segregation defects that would result in lethality were accounted for by doubling the exceptional progeny for a total NDJ percentage.

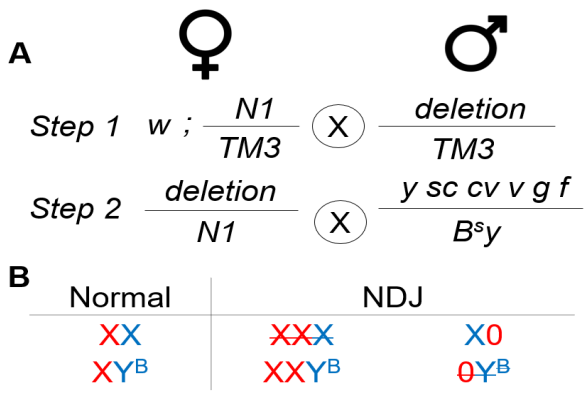

Figure 8. *NDJ assay setup and analysis. (A) Drosophila melanogaster crosses for NDJ assay setup. Progeny of step 2 were scored by eye shape. (B) Genotype categorization for NDJ and normal meiotic segregation. Red and blue indicate maternal and paternal chromosomes, respectively. Genotypes with strikethrough are non-viable.*

Assay Stock creation – In order to create ideal stocks for assay analysis, a series of crosses were conducted according to figure 9. Step 1 used fifteen males from our prior Blm amino acid deletion stocks. They were crossed to thirty balanced females including the markers *net*, *ho*, *dpy*, *b*, *pr*, *cn*, (*net-cn*) balanced over *CyO* on the second chromosome, and various alleles including the dominant lethal *Stubble (Sb)* balanced over TM6B on the third chromosome. Fifteen male progeny of the cross balanced by TM6B were next crossed back to thirty more of the same females. The progeny of this cross were lastly crossed back to each other using five females and

three males to produce a stock of flies with second chromosome markers and the desired *Blm* deletion balanced over TM6B.

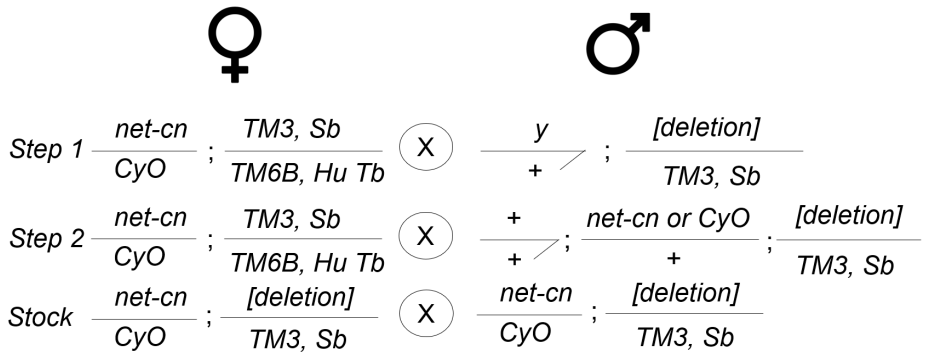

Figure 9. *Drosophila melanogaster crosses to produce stock of desired Blm amino acid deletion balanced with 2nd chromosome markers and TM6B.*

Crossover Assay – The same step 1 as above was utilized for a crossover (CO) assay testing Blm ability to prevent mitotic crossovers in DNA repair. Males of this cross were each placed into separate crosses with 7 homozygous *net-cn* (recessive phenotypic markers) female flies. The resulting progeny were scored into categories of *net-cn*, *wt*, or CO (mix of *wt* and recessive marker phenotypes) to determine the percent of vials and progeny that contained crossovers were unable to be prevented by functional Blm.

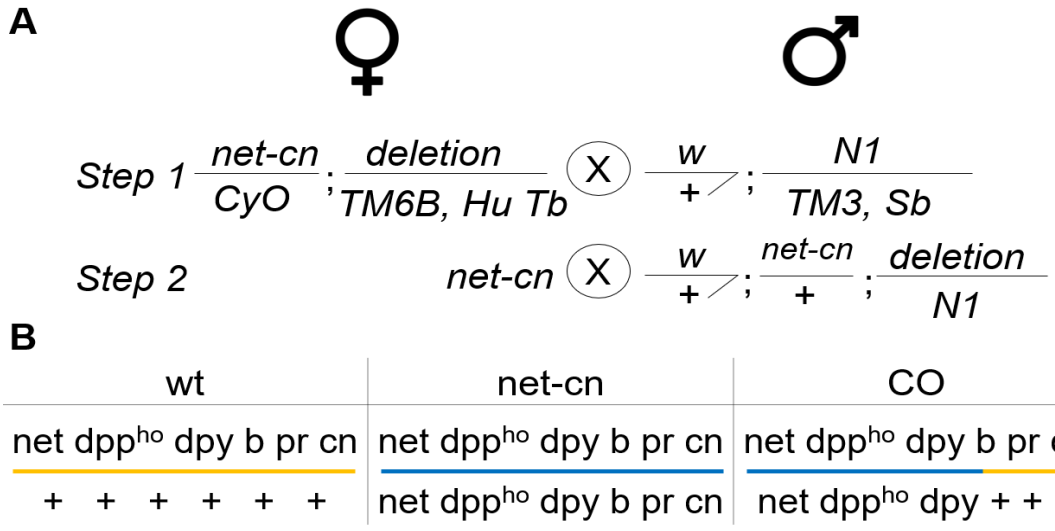

Figure 10. *CO assay setup and analysis. (A) Drosophila melanogaster crosses for CO assay setup. Progeny of step 2 were scored by recessive phenotypic markers. (B) Example genotypes for wt, net-cn, and CO flies. Orange and blue lines represent heterozygosity and homozygosity, respectively.*

III. Results

1.Yeast Cloning and Y2H Analysis

To determine the regions of significance required for proper interaction of Blm and TopIIIa proteins, it was essential that pACT2 vector plasmids were correctly synthesized with the desired *Blm* gene fragment. Figures 14-16 show accurate amplification of each of the *Blm* gene fragment. When comparing to the DNA ladder, each band indicated proper insert size for each fragment (Figures 14-16, appendix). For example, *Blm* fragment 576-720 was localized near the 500 base pair ladder fragment as expected with its size of 432 base pairs. Successful In-Fusion of each *Blm* insert into a plasmid was first tested with restriction enzyme (RE) digests seen in figures 17-19 (appendix). Distinct band patterns would only be present if the insert was integrated properly, adding a new or additional RE site not present in empty pACT2 plasmid (Figures 17-19, appendix). Sanger sequencing accuracy signaled completion of the cloning process and clearance for Y2H testing.

Transformed yeast were utilized in a serial dilution assay to obtain a quantitative test of the Blm-TopIIIa interaction. Three trials from two different yeast transformations were tested and summarized in figure 11A (full results in Figure 20, appendix). The yeast were grown in culture and serially diluted based on their OD_{600} (see methods) to produce growth results indicative of the strength of the proteins' interaction. When compared to the positive control growth of full length Blm, Blm fragment N2 showed the strongest interaction with TopIIIa (Figure 11A). Blm fragment CR5 and full length Blm displayed successful binding, but at a diminished level compared to N2. Weak growth was seen in Blm fragments 1270-1487, 1337-1487, and 576-720. Their growth similarity to the negative control, empty pACT2, indicates a lack of interaction with TopIIIa at these locations in Blm (Figure 11A). The results of the assay produced progress toward determining specific regions of Blm involved in interaction with TopIIIa (Figure 11B).

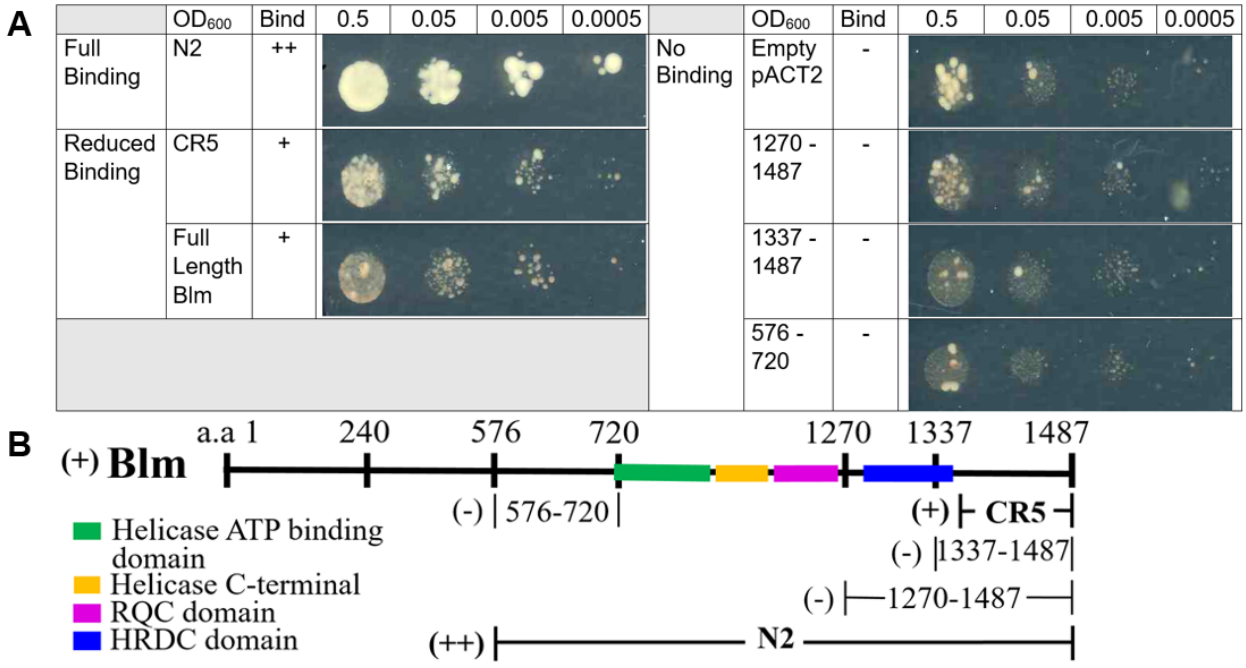

Figure 11. *Y2H experimental analysis of Blm. (A) Experimental serial dilution data for each Blm fragment on (-)His(-)Trp(-)Leu(-)Ade plates categorized by interaction strength. Most representative trial for visualizing the overall experimental trends. (B) Map of studied Blm fragments, alleles, and functional domains (Flybase). Fragments are conserved Blm regions across Drosophila species closely related to D. melanogaster.*

2. CRISPR Cloning and Fly Assay Analysis

To examine the functionality of the *Blm* gene and its roles within DNA repair, it is critical to ensure successful alteration of the *Blm* gene within *Drosophila* and proper synthesis of the gRNA and homology template plasmids. Figures 21A and 21B (appendix) show effective cloning of the pCFD4 gRNA 1-240 and 576-720 plasmids through experimental band sizes identical to the expected for both the insert and vector digest. Another confirmation step was taken by sequencing the In-Fusion product to ensure correct fusion. Similar verification procedures were utilized for the homology template cloning. This two-step process used the first In-Fusion product as the cut vector plasmid for creation of the final desired plasmid. Each of the inserts and digests from both steps displayed accurate band lengths as well as sequencing and restriction enzyme diagnostics showcased a successful In-Fusion result (Figures 22-23, appendix). This was sufficient to send the plasmids for injection into Cas9-expressing embryos.

The success of the CRISPR/Cas9 gene editing was verified within subsequent progeny of the originally injected *Drosophila* embryos by a fly assay PCR confirming the deletion within *Blm.* The assay was conducted after step 2 of the crosses and again after stock creation (see figure 7). The results of the cross proved successful by the distinct band equivalent in size to the positive control band at 817 base pairs for d1-240 (Figure 24A and B, appendix) and 1179 base pairs for d576-720 (Figure 25, appendix). The bands were smaller in size than the negative controls that included the amino acid deletions, totaling to 1534 and 1611 base pairs. This confirms that these stocks of flies have precise deletions of the targeted amino acids within their *BLM* gene.

To evaluate the functional significance of these deletions to Blm roles in meiotic segregation, I conducted a NDJ assay. Figure 12 shows the rates of NDJ seen for each of the tested *Blm* deletions/alleles. *Wt* served as the positive control of fully functional Blm while *Blm* null allele (*D2/N1*) represented a negative control with no production of *Blm* protein. Both the previously characterized N2 allele (which deletes aa 1-576) and d576-720 were statistically significant from *wt* at NDJ rates of 2.79% and 5.90%, respectively[8]. They were more comparable to the *Blm* null allele which indicated abnormal Blm function for these amino acid deletions within meiotic segregation.

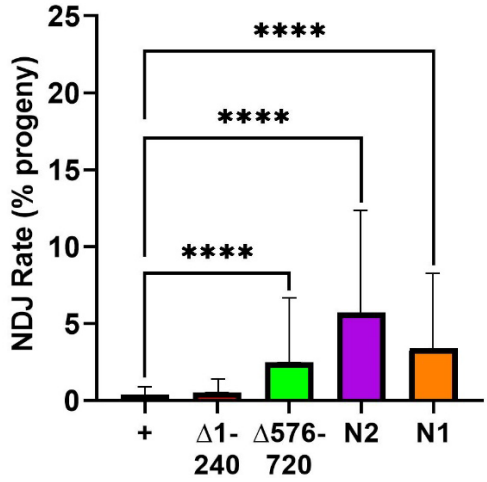

Figure 12. *Experimental results for NDJ prevalence in wt and Blm deletions over Blm null allele. High rates indicate decreased Blm functionality and abnormal chromosome segregation in meiosis. Null data provided via previous literature9. Prevalence evaluated against wildtype for significance (*p<0.05, unpaired t-test). Exact data percentages include: 0.35%, 0.50%, 2.79%, 5.90%, 3.40%.*

An exploration into *Blm* deletion effects on HR was conducted by examining the prevention of mitotic crossovers during HR in a CO assay. In a *wt* fly with fully functional Blm, the CO rate was 0% as depicted in figure 13. *Blm* null allele flies were not able to prevent these crossovers and resulted in a significantly higher rate at 2.34% compared to *wt*. The N2 deletion

was the only significant deletion compared to *wt*, while both d1-240 and d576-720 were not statistically significant from *wt*. The Blm role in preventing mitotic crossovers was most highly affected by the N2 deletion demonstrating abnormal function of the Blm protein without these amino acids.

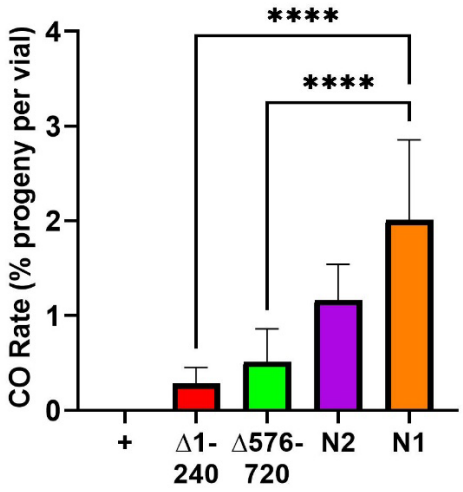

Figure 13. *Male germline mitotic CO rates for wt and Blm deletions over Blm null allele. High percentages indicate a lack of Blm function in preventing mitotic crossovers. Rate evaluated against Blm null allele for significance (*p<0.05, ANOVA with Tukey's post-hoc). Exact data percentages include: 0%, 0.28%, 0.61%, 2.40%, 2.34%.*

IV. Discussion

The quantitative yeast two-hybrid serial dilution assay provided valuable results on the interaction of Blm with TopIIIa. Interactions were seen for N2, CR5, and full length Blm. There was evidence of a lack of interaction for fragments 1270-1487, 1337-1487, 576-720, and empty pACT2. Based on these results, as more amino acids are added to the Blm's C-terminus, binding to TopIIIa diminishes, which is consistent with previous literature[2]. However, the additional N-terminal amino acids within the N2 fragment appear to rescue this function. I hypothesize that the positive control full length Blm is likely weaker in growth than N2 due to autoinhibition within the protein or the requirement for phosphorylation to obtain full growth. These results denote significance through multiple trials across two different yeast transformations that generated similar conclusions. Any error could have arisen through contamination of the very sensitive yeast organisms; however, this is unlikely due to the consistency of the results.

Further testing on the *Blm* gene within *Drosophila melanogaster* provided beneficial information on the functionality and roles of Blm *in vivo*. The NDJ and CO assays characterized different regions of Blm based on their effects on meiotic segregation and mitotic CO rates,

respectively. The N2 allele was the most impactful Blm deletion due to its significant effects of allowing dangerous mitotic crossovers and nondisjunction of chromosomes in meiosis. This is likely attributed to the size of the deletion and removal of the unstructured N-terminal region, possibly leading to weaker interaction with Top3α (refer to figure 11B). The opposite occurred for d1-240 which produced no impact on each of these Blm roles, despite missing many of the same residues as the N2 allele. The remaining tested deletion, d576-720, was the most unique due to the effect on NDJ but not CO rates. Placement of the region within the middle of the Blm protein could influence folding and play a role in specialized function. Future studies should confirm folding of this deletion and the d1-240 deletion via western blot. Significance of the results are shown through consistency with past literature and large sample sizes[8].

Future characterization studies of the Blm protein within *Drosophila* will include an embryo hatching assay to determine deletion effects on embryonic development. Preliminary data for this experiment has already been collected for each of the Blm regions assayed above. Flies homozygous for the *Blm* null allele displayed extremely low hatching rates and evidence of maternal lethality. Fully functional Blm in *wt* flies did not produce this effect. Previous literature and current data for the deletions predict a lack of effect from N2 while d576-720 shows potential for maternal lethality[8]. Larger samples of these data need to be collected for significance but would produce valuable results towards the characterization of Blm regions for roles within DNA repair and development.

Additional avenues of development for the project include further exploration of the d756-720 region and the relevance of its roles to predicted residues of phosphorylation. The region includes 4 residues (T583, T639, S663, and S694) that are phosphorylated by DNA repair regulatory kinases ATM and ATR. Replacement of these residues with phospho-mimetic (aspartic acid) or phospho-dead (alanine) amino acids would evaluate the importance of phosphorylation of this region to the functionality of Blm. Other project explorations will include *in vivo* characterization of the C-terminal regions of Blm using the assays conducted above.

These conclusions surrounding the interaction of Blm and TopIIIα have real-world applications to the cancer field and could assist in research toward finding treatment or prevention methods for Bloom's syndrome and cancer. Mutated sites in these identified regions

of significance within a patient's *BLM* gene could be located and addressed via sequencing and genome editing. Correcting these mutations would encourage the cell to direct HR to less error-prone pathways and prevent detrimental effects such as LOH and genomic instability. Better understanding the interaction between these important proteins could help in restoring normal *BLM* functions through methodologies like drugs and gene editing. There is more to be understood about the roles of *BLM*, but this project has provided beneficial information on the interaction with Top3α and involvement in homologous recombination and development processes.

V. Acknowledgements

I would like to thank Dr. Jeff Sekelsky and Dr. Evan Dewey for their extraordinary support over the course of my project. I am beyond thankful for the privilege to learn from them during my past three years in the lab. I would also like to thank the members of the Sekelsky lab for their constant support and encouragement, especially Mohamed A. Nasr for his help with data collection. Special thanks to Dr. Amy Maddox and Sarah Clinkscales as well for their direction through the creation of my thesis. This work was supported by the Honors Carolina Excellence Senior Thesis Research Award, UNC Office of Undergraduate Research Travel Award, and the University of North Carolina at Chapel Hill.

VI. References

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VII. Appendix

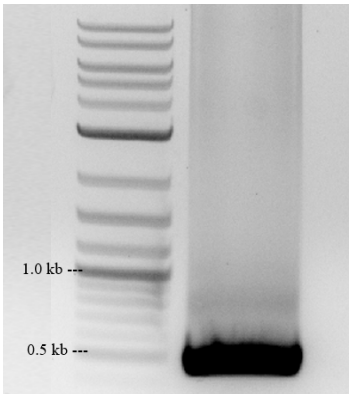


Figure 14. PCR of 576-720 insert with pACT2 In-Fusion overlaps. Bands are at the expected 432 base pair size.

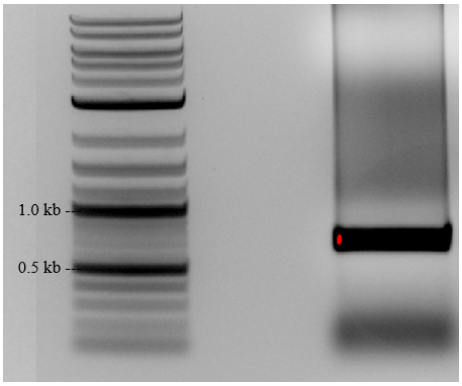


Figure 15. PCR of 1270-1487 insert with pACT2 In-Fusion overlaps. Bands are at the expected 651 base pair size.

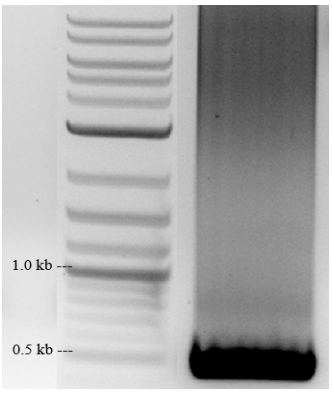


Figure 16. PCR of 1337-1487 insert with pACT2 In-Fusion overlaps. Bands are at the expected 450 base pair size.

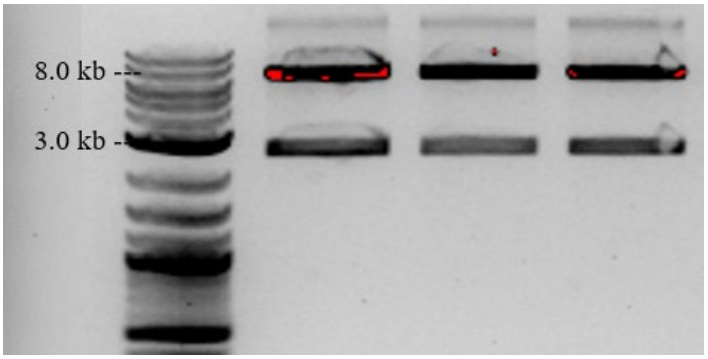


Figure 17. Restriction enzyme diagnostic of fragment 576-720 with BsaI. Lower bands at about 3,000 base pairs display presence of the insert. Each lane presents as accurate. The top bands above ladder represent circular uncut DNA.

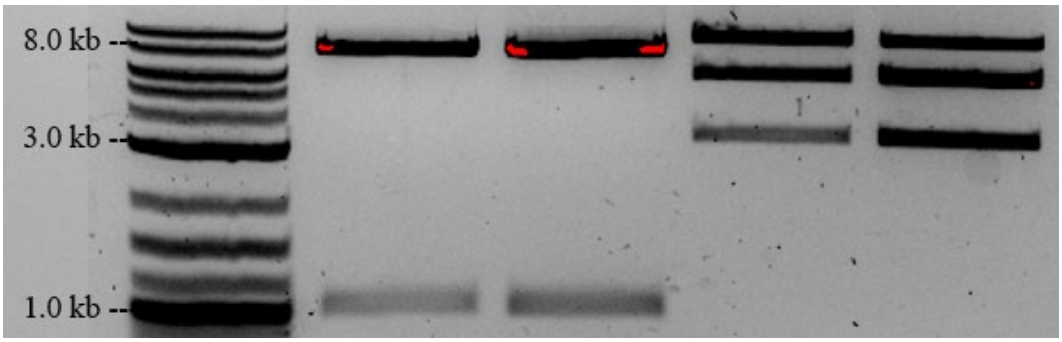


Figure 18. Restriction enzyme diagnostic of fragment 1270-1487 with ScaI. Lower bands on left side at about 1,000 base pairs display presence of the insert. Bands in the right two columns are incorrect and do not display a band at the correct size.

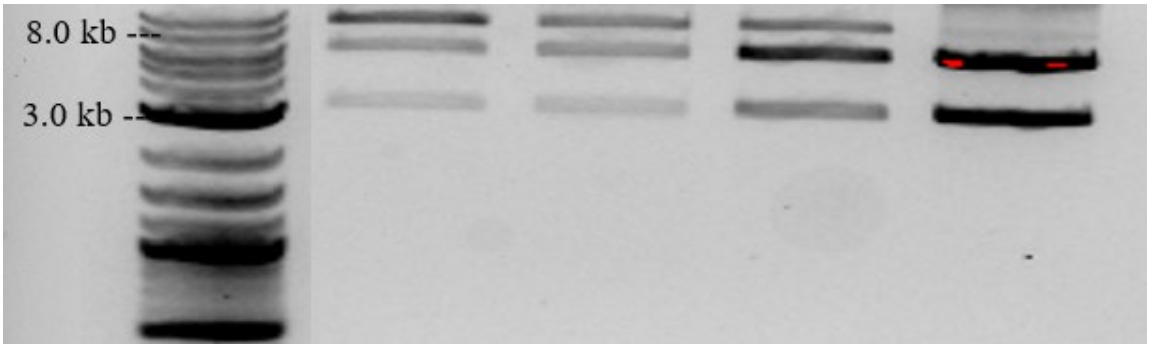


Figure 19. Restriction enzyme diagnostic of fragment 1337-1487 with ScaI. The lowest bands at about 3,000 base pairs display presence of the insert. Each lane is correct, however efficacy varied based off enzyme. The top bands above ladder represent circular uncut DNA.

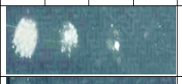
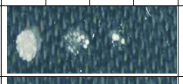
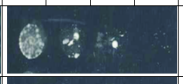
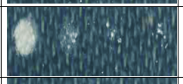
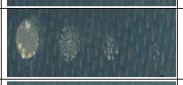
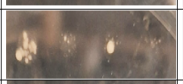
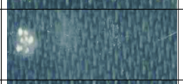
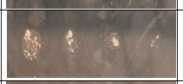
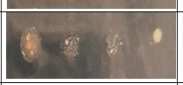
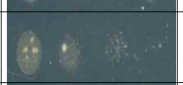
			Plate 1: Trial 1				Plate 1: Trial 2				Plate 1: Trial 3				Plate 2: Trial 1				Plate 2: Trial 2				Plate 2: Trial 3				Plate 1 & 2 Redo			
	OD_{600}	Bind	0.5	0.05	0.005	0.0005	0.5	0.05	0.005	0.0005	0.5	0.05	0.005	0.0005	0.5	0.05	0.005	0.0005	0.5	0.05	0.005	0.0005	0.5	0.05	0.005	0.0005	0.5	0.05	0.005	0.0005
Full Binding	N2	++																												
Reduced Binding	CR5	+																									N/A			
	pGAD Blm	+																												
No Binding	pACT2	-																												
	1270-1487	-																									N/A			
	1337-1487	-																	Insufficient growth											
	576-720	-	Insufficient growth																											

Figure 20. Full yeast data table of every serial dilution trial. Plate 1 and plate 2 consisted of unique yeast transformations.

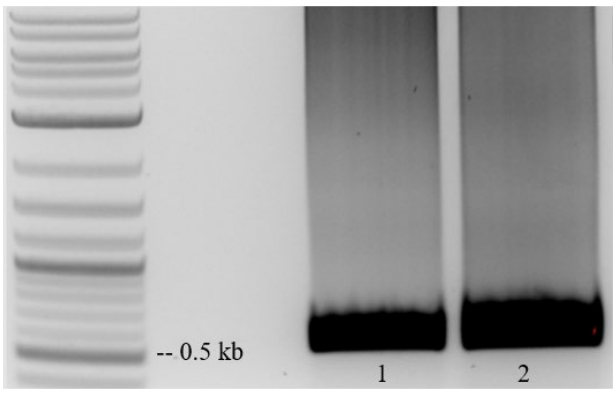


Figure 21a. *Cloning process of pCFD4 1-240 & 576-720 gRNA.* Lane 1 is a PCR amplification of Blm 1-240 gRNAs with pCFD4 In-Fusion overlaps. Successful band at the expected 566 base pair size. Lane 2 is a PCR amplification of Blm 576-720 gRNAs with pCFD4 In-Fusion overlaps. Successful band at the expected 566 base pair size.

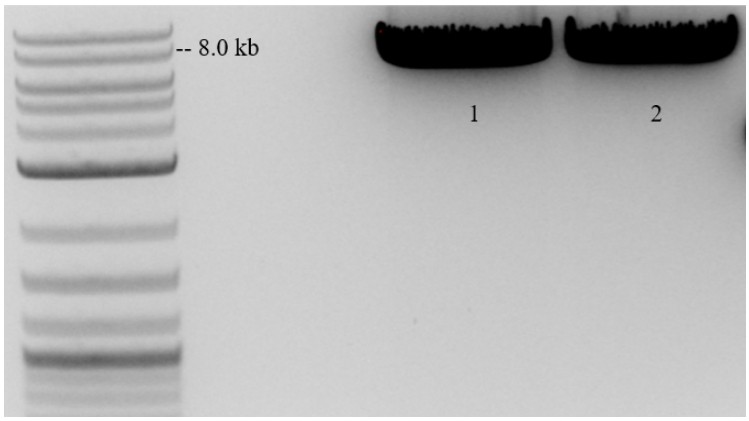


Figure 21b. *Cloning process of pCFD4 1-240 & 576-720 gRNA.* Lane 1 and 2 are vector digests of pCFD4 using BbsI. Successful bands at the expected 7139 base pair size.

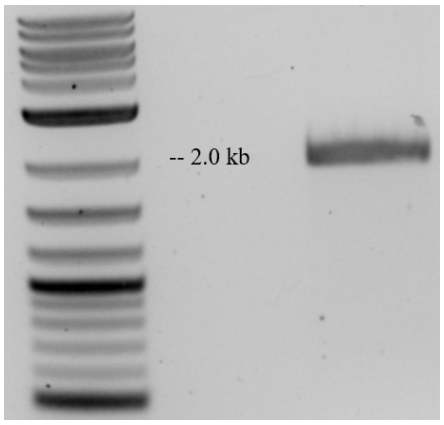


Figure 22a. *Cloning process of pSL1180 Blm d1-240 5'+3' homology template.* PCR of Blm d1-240 5' HA insert with pSL1180 In-Fusion overlaps. Successful band at the expected 2006 base pair size.

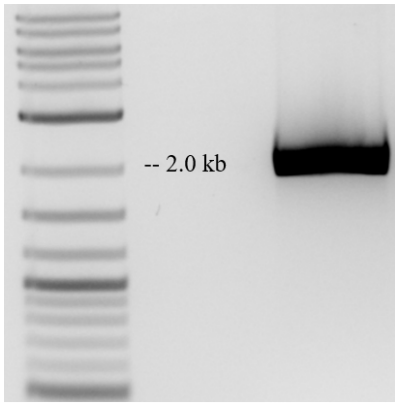


Figure 22b. *Cloning process of pSL1180 Blm d1-240 5'+3' homology template.* PCR of Blm d1-240 5'+3' HA insert with pSL1180 Blm d1-240 5' HA In-Fusion overlaps. Successful band at the expected 2011 base pair size.

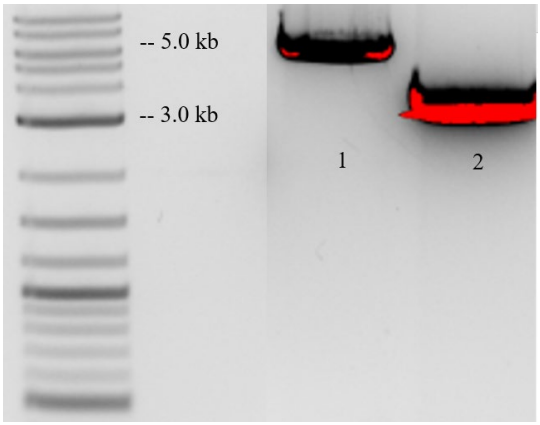


Figure 22c. *Cloning process of pSL1180 Blm d1-240 5'+3' homology template.* Lane 1 is the vector digest of pSL1180 Blm d1-240 5' HA using BamHI and NcoI and will be used in the second In-Fusion step. Successful large band at the expected 5131 base pair size. Lane 2 is the vector digest of pSL1180 using BamHI and XhoI and will be used in the first In-Fusion step. Successful large band at the expected 3207 base pair size. Irrelevant lanes on gel removed for clarity.

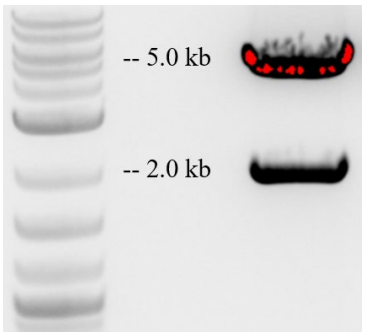


Figure 22d. *Cloning process of pSL1180 Blm d1-240 5'+3' homology template.* Restriction enzyme diagnostic of pSL1180 Blm d1-240 5'+3' HA using BamHI-FD and EcoRI-FD. The two distinct bands prove successful cloning.

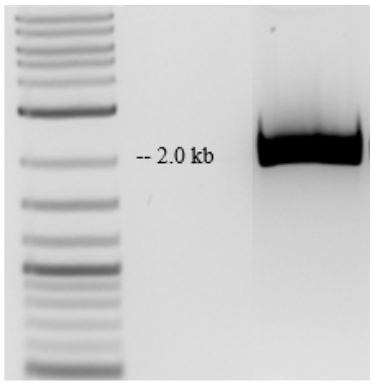


Figure 23a. *Cloning process of pSL1180 Blm d576-720 5'+3' homology template*. PCR of Blm d576-720 5' HA insert with pSL1180 In-Fusion overlaps. Successful band at the expected 1933 base pair size. Irrelevant lanes on gel removed for clarity.

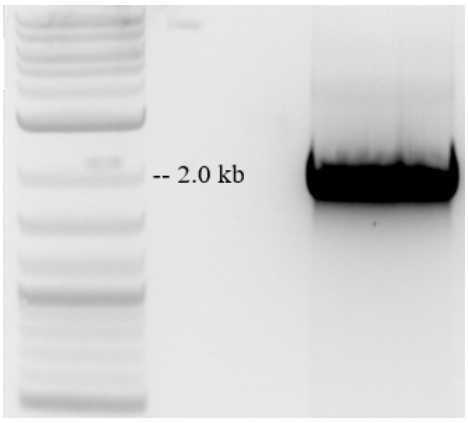


Figure 23b. *Cloning process of pSL1180 Blm d576-720 5'+3' homology template*. PCR of Blm d576-720 5'+3' HA insert with pSL1180 Blm d576-720 5' HA In-Fusion overlaps. Successful band at the expected 1903 base pair size.

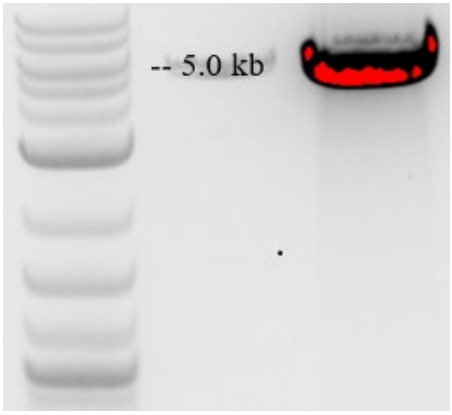


Figure 23c. *Cloning process of pSL1180 Blm d576-720 5'+3' homology template*. Vector digest of pSL1180 Blm d576-720 5' HA using BamHI and NcoI and will be used in the second In-Fusion step. Successful large band at the expected 5048 base pair size. Vector digest of pSL1180 using BamHI and XhoI will be used in the first In-Fusion step and is pictured in figure 19c.

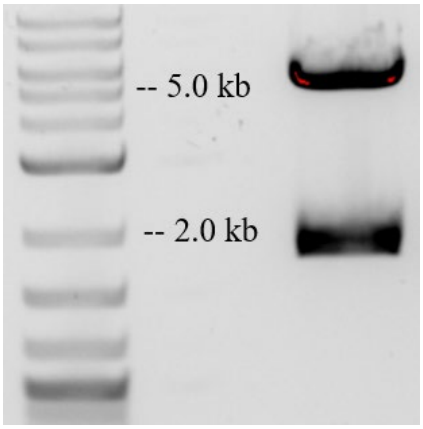


Figure 23d. *Cloning process of pSL1180 Blm d576-720 5'+3' homology template*. Restriction enzyme diagnostic of pSL1180 Blm d576-720 5'+3' HA using EcoHI-FD and PstRI-FD. The two distinct bands prove successful cloning.

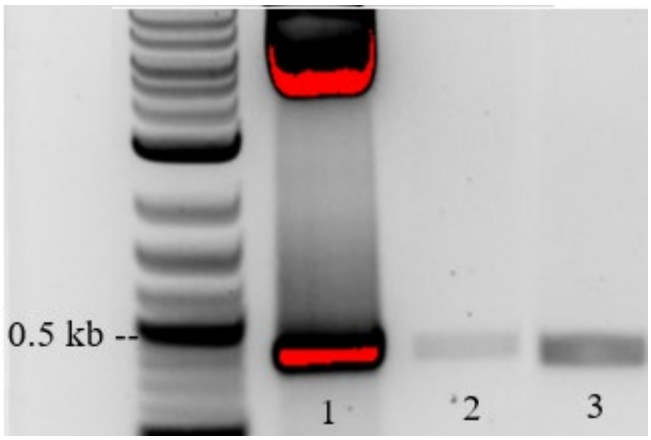


Figure 24a. *Fly assay PCR evaluating presence of Blm 1-240 deletion within the flies' genome.* Lane 1 is positive control using a 1:10 dilution of the pSL1180 Blm d1-240 5'+3' homology template plasmid. Lane 2 is negative control using a *wt* fly. Lane 3 is a deletion confirmation after step 2 of my crosses (Figure 8). Successful band has a dark contrast to the negative control and aligns at the expected 817 base pair size.

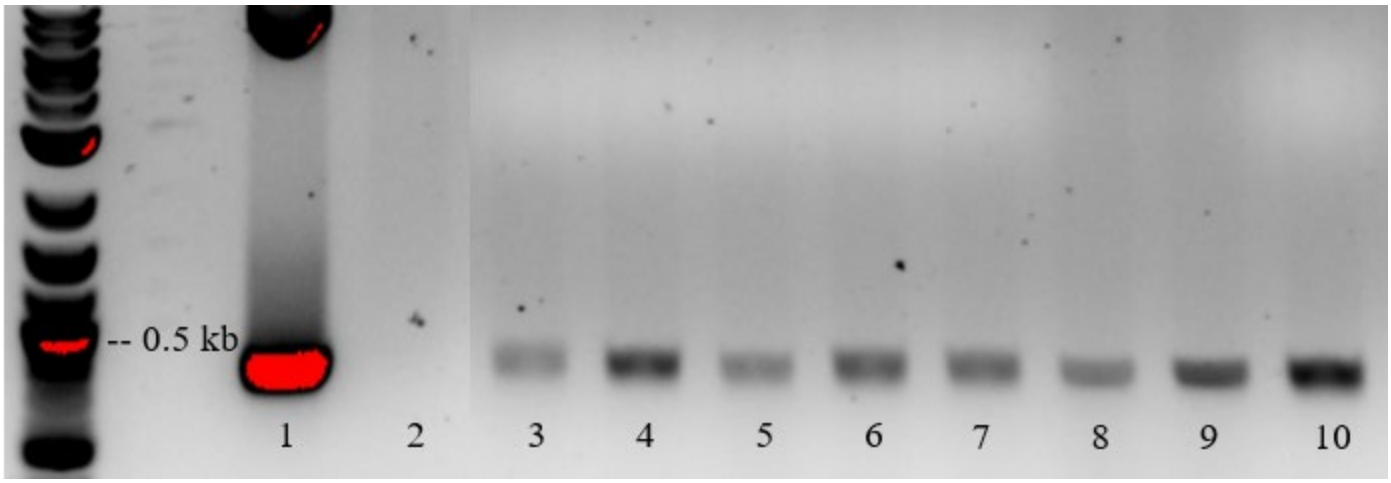


Figure 24b. *Fly assay PCR evaluating presence of Blm 1-240 deletion within the flies' genome.* Lane 1 is positive control using a 1:10 dilution of the pSL1180 Blm d1-240 5'+3' homology template plasmid. Lane 2 is negative control using a *wt* fly. Lanes 3-10 confirm a successful deletion within my stock. Successful bands have a dark contrast to the negative control and align at the expected 817 base pair size. Irrelevant lanes on gel removed for clarity.

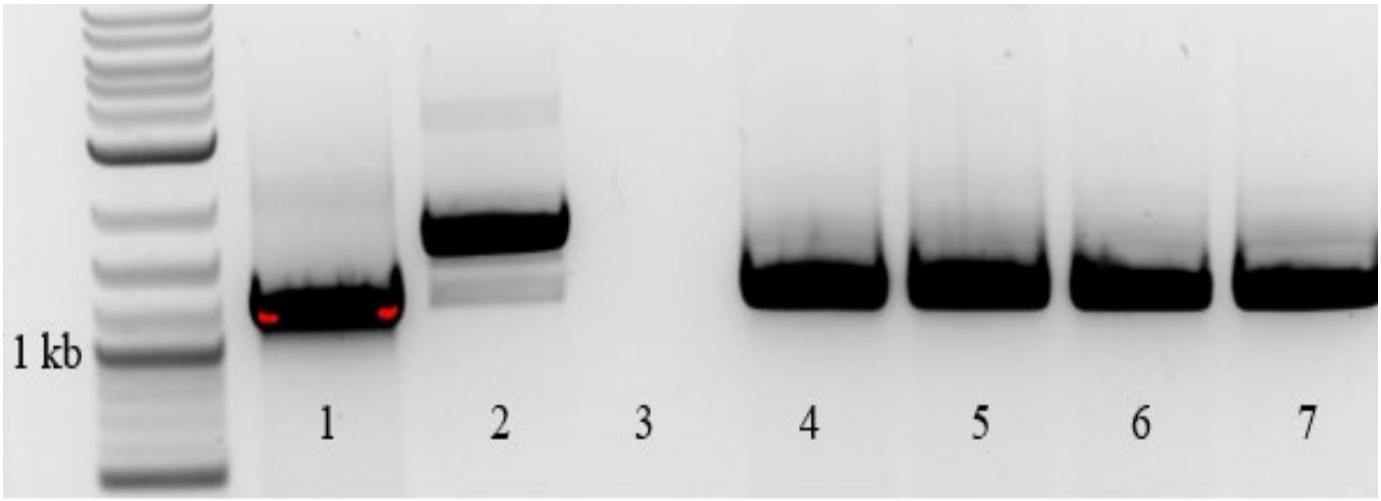


Figure 25. *Fly assay PCR evaluating presence of Blm 576-720 deletion within the flies' genome.* Lane 1 is positive control using a 1:10 dilution of the pSL1180 Blm d576-720 5'+3' homology template plasmid. Lane 2 is negative control using a *wt* fly. Lanes 4-7 confirm a successful deletion within my stock. Successful bands have a dark contrast to the negative control and align at the expected 1179 base pair size.