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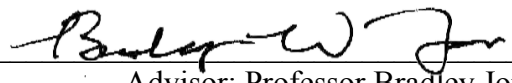
EVALUATION OF THE BAX-VDAC INTERACTION AND THEIR INFLUENCE ON APOPTOSIS IN *DROSOPHILA MELANOGASTER*

by
Frances Marie Gatlin

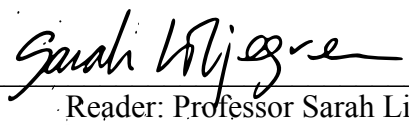
A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2020

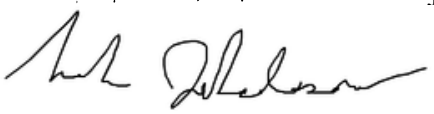
Approved by



Advisor: Professor Bradley Jones



Reader: Professor Sarah Liljegren



Reader: Professor Mika Jekabsons

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ACKNOWLEDGEMENTS

First, I would like to thank Dr. Jones for allowing me to learn in his lab. I have much appreciated the opportunity to be introduced to laboratory research by him. He has patiently demonstrated techniques over the past two years that will be very useful throughout my future career. I would also like to thank Dr. Jekabsons for partnering with us on our research and for participating as a reader. In addition, I would like to thank Dr. Liljegren for also serving as a reader. Finally, I would like to express gratitude to family and friends for their support throughout my educational journey at the University of Mississippi.

ABSTRACT

Apoptosis, also known as programmed cell death, is a cellular process used for development or for when cells undergo injury or stress. The Bcl2 family of proteins includes both pro-apoptotic and anti-apoptotic proteins that control the intrinsic pathway of apoptosis. Understanding the mechanisms and influence these proteins have on apoptosis is an important area of research focused on in Dr. Jones's lab. Evidence shows a homology amongst the Bcl2 family of proteins at the BH3 domain. Dr. Jekabsons' lab has found a potential homology amongst VDAC 1-3 and the Bcl2 family at the BH3 domain.

Specifically, our lab is using *Drosophila melanogaster* to test the interaction between VDACs and Bax by mutating the *Drosophila's* VDAC. We are interested in discovering if mutation of this domain causes a phenotypic change in the *Drosophila melanogaster*, and if the change is harmful or beneficial. We constructed transgenic plasmids using a pUBI-ATTB plasmid as a vector and mouse VDAC cDNA as our gene of interest. We hypothesize that Bax and VDAC interact to prevent the premature assembly of Bax pores in the mitochondrial membrane.

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LIST OF ABBREVIATIONS

Bcl2	B-cell lymphoma 2
cDNA	complementary DNA
MOM	mitochondrial outer membrane
mRNA	messenger RNA
SOC	Super Optimal broth with Catabolite repression
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
UBI	*ubiquitin*
VDAC	voltage-dependent anion channel
wg	*wingless*
WT	wild-type

CHAPTER I:

BACKGROUND AND INTRODUCTION

1.1: Intrinsic Apoptotic Pathway

Apoptosis is a method of programmed cell death, mainly when cells initiate an intracellular death program, killing themselves in a controlled manner (Alberts, et al., 2008). Apoptosis is used for development or for when cells undergo injury or stress (Alberts, et al., 2008). This process takes place in the mitochondria, and the Bcl2 family of proteins is the major regulator in vertebrates (Alberts, et al., 2008). Apoptotic intracellular activation happens by the intrinsic pathway of apoptosis (Alberts, et al., 2008). The pathway operates by regulating the release of cytochrome c in the cytosol (Alberts, et al., 2008).

The Bcl2 family includes both pro-apoptotic and anti-apoptotic proteins (Figure 1). Pro-apoptotic proteins, such as BH123 and BH3-only, initiate apoptosis by promoting the release of cytochrome c (Alberts, et al., 2008). On the other hand, anti-apoptotic proteins, such as the Bcl2 protein, inhibit apoptosis by preventing the release of cytochrome c (Alberts, et al., 2008). There are four unique Bcl2 homology domains BH1, BH2, BH3, and BH4 (Figure 1). BH3-only proteins are different from Bcl2, except for their shared sequence homology at the BH3 domain (Figure 1). Also, BH3-only proteins initiate apoptosis primarily by inhibiting anti-apoptotic Bcl2 proteins (Alberts, et al., 2008). The anti-apoptotic Bcl2 proteins are found on the cytosolic surface of the mitochondrial outer membrane (MOM). They assist in maintaining the integrity of the

membrane (Alberts, et al., 2008). These proteins inhibit apoptosis by binding to and hindering the pro-apoptotic Bcl2 proteins (Alberts, et al., 2008).

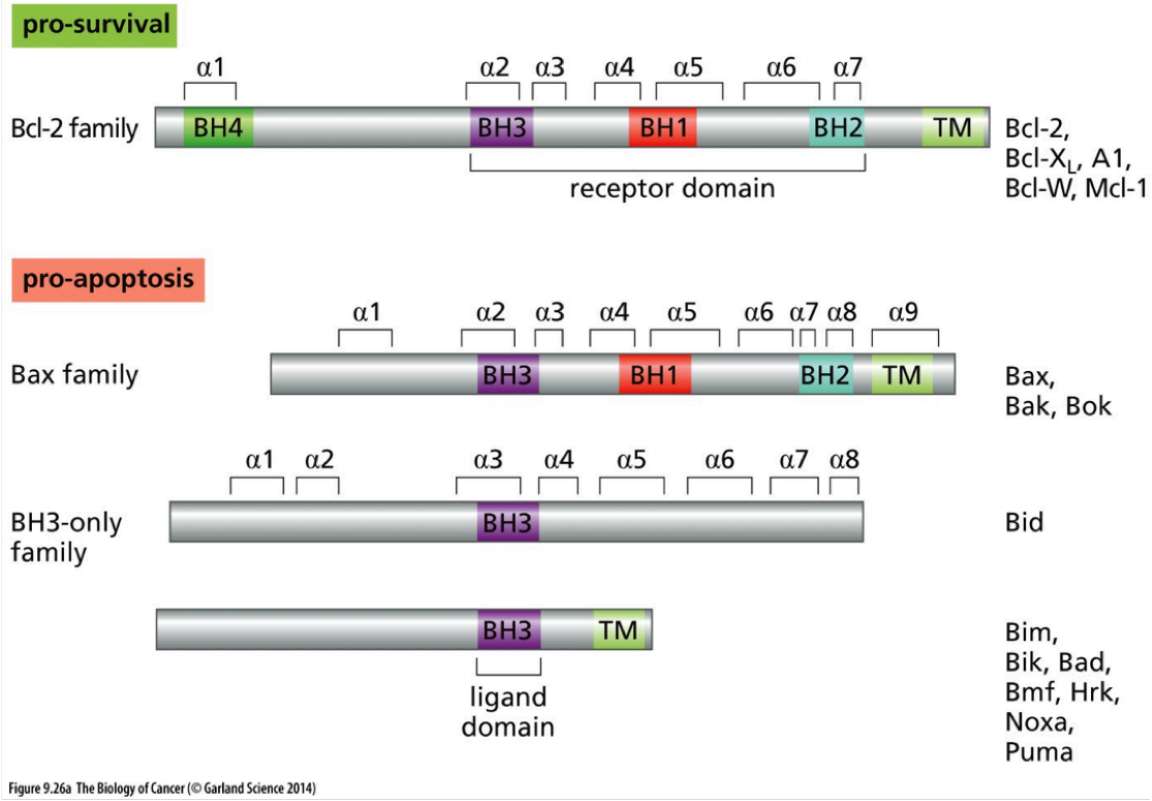

Figure 1: The three classes of the Bcl2 family of proteins. The BH3 domain in purple can be observed as the only domain that is shared by all classes of Bcl2-like proteins (Adapted from Alberts, et al., 2008).

1.2: Bax/Bak regulation with Bcl2 like proteins

BH123 proteins, including Bax and Bak, have similar homologies to Bcl2, except for the BH4 domain (Alberts, et al., 2008). When an apoptotic stimulus initiates the intrinsic pathway, BH123 proteins are activated and aggregate to form oligomers in the MOM (Figure 2). This process causes cytochrome C and other proteins to release into the cytosol (Figure 2). The exact mechanism of this release remains unclear. Bax can be

found in the cytosol and travels to the mitochondria once activated by an apoptotic stimulus (Alberts, et al., 2008). Alternatively, Bak remains tightly bound to the MOM, with or without an apoptotic stimulus present (Alberts, et al., 2008). BH3-only proteins are produced or activated in response to an apoptotic stimulus, and these proteins trigger Bax/Bak oligomers and activation of cytochrome c release. (Alberts, et al., 2008). The BH3 domains of the BH123 proteins bind to a hydrophobic groove on anti-apoptotic Bcl2 proteins (Alberts, et al., 2008). This binding sequesters Bax/Bak in an inactive state with the anti-apoptotic protein and prevents them from forming oligomers (Alberts, et al., 2008).

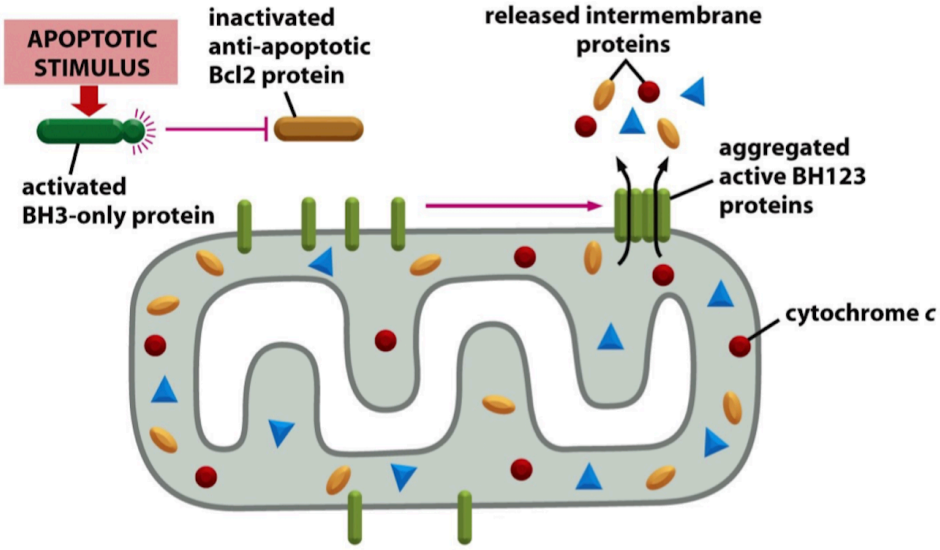

Figure 2: BH3-only and anti-apoptotic Bcl2 proteins regulate intrinsic pathway. BH3-only proteins are activated by an apoptotic stimulus and bind to the anti-apoptotic Bcl2 proteins. These proteins are no longer able to inhibit BH123 proteins, allowing cytochrome c and other intermembrane proteins to release into the cytosol (Adapted from Alberts, et al., 2008)

1.3: Bax/Bak regulation with VDAC

As previously discussed, Bax and Bak are mainly regulated by BH3-only and anti-apoptotic Bcl2 proteins. A voltage-dependent anion channel (VDAC) is a pore forming channel protein found on the MOM. This protein is believed to now play a role in regulating apoptosis as well (Jekabsons and Jones, 2019). VDAC creates a channel for small metabolites such as ATP to exchange between the intermembrane space and the cytosol of the mitochondria (Jekabsons and Jones, 2019). Evidence from Dr. Jekabsons' lab shows a homology amongst the Bcl2 family of proteins at the BH3 domain. A sequence conservation at this domain has been discovered, as seen by the conserved aspartate in red (Figure 3).

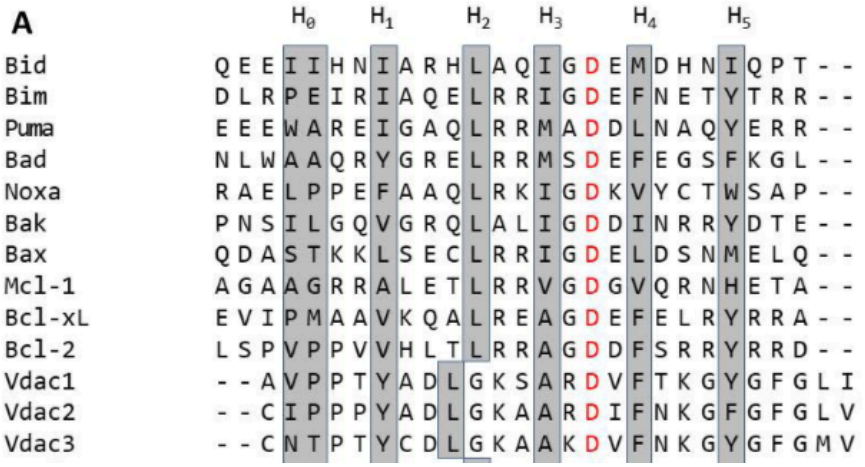

Figure 3: Sequence alignment of mouse BH3 domains of Bcl2 family members. VDAC 1-3 sequences can also be found to demonstrate the homology. The conserved aspartate is highlighted in red (Adapted from Jekabsons and Jones, 2019).

In addition to discovering the sequence homology amongst the Bcl2-family members and VDAC1-3, members of the lab also found protein similarities when using a

computer model to created 3D models of the Bax protein (Figure 4). The models constructed demonstrate that the Bid BH3 domain and the VDAC1 BH3 domain both fit similarly into the hydrophobic groove of Bax, providing further evidence that VDAC could be related to the Bcl2 family of proteins (Figure 4).

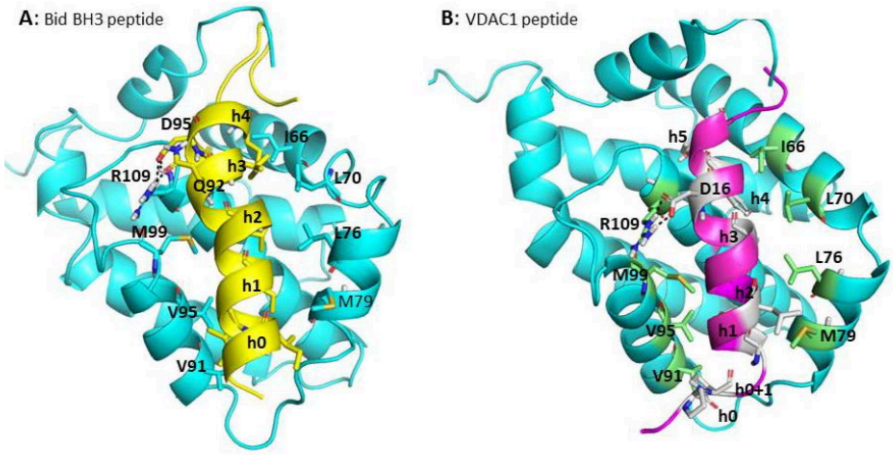

Figure 4: Bid and VDAC1 attached to Bax hydrophobic groove. Bax protein with α 9 deleted is blue. The Bid peptide is yellow on the left, and the VDAC1 N-terminus is pink on the right. The residues important for binding are labeled (Adapted from Jekabsons and Jones, 2019).

Dr. Jones' lab proposes to use *Drosophila melanogaster* to test the interaction between VDACs and Bax by mutating the *Drosophila's* VDAC. I plan to construct DNA plasmids containing mouse VDAC2 and Bax cDNAs to enable the construction of transgenic lines in *Drosophila melanogaster*. These lines will be used to test for genetic interactions between VDAC2 and Bax. The goal of our experiments is to determine if VDAC genetically influences Bax functions in *Drosophila melanogaster*. We hypothesize that Bax and VDAC interact to

prevent the premature assembly of Bax pores in the mitochondrial membrane. Inducing Bax expression in the eyes and wings of *Drosophila melanogaster* causes a change in the wild-type phenotypes (Figure 5). Our lab is taking plasmids containing mouse VDAC 1 and 2 and injecting them into flies to generate transgenic lines, having them expressed highly under control of the ubiquitin promoter, and seeing if the flies with mouse VDAC can influence these wild-type phenotypes induced by Bax expression.

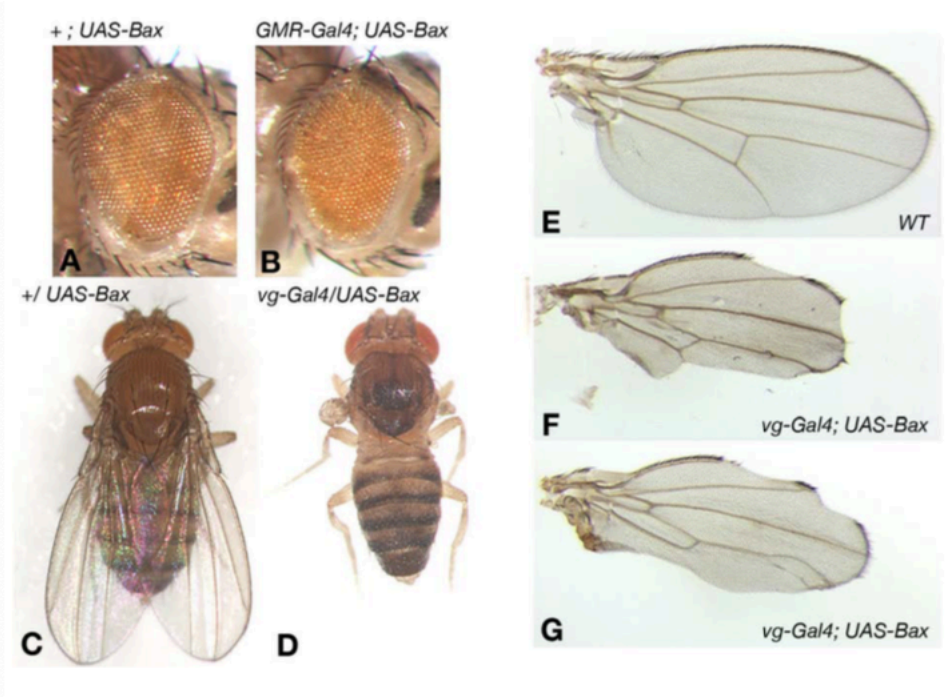

Figure 5: Altered eye and wing phenotypes with induced Bax expression. (A) *+; UAS-Bax* shows an eye exhibiting wild-type morphology from not expressing Bax due to lack of GAL4; a regular arrangement of ommatidia and bristles are shown. (B) *GMR-Gal4; UAS-Bax* expresses Bax in the eye; this crossing causes a disarray of ommatidia and bristles. **(**C) *+; UAS-Bax* shows normal wing morphology, and (D) *vg-Gal4/UAS-Bax* shows wings are nearly absent in *vg-Gal4/UAS-Bax(2)* flies, in which the wing imaginal discs express Bax (Adapted from Jekabsons and Jones, 2019).

CHAPTER II:

MATERIALS AND METHODS

2.1: Restriction Digest of Plasmid DNA

Restriction digests were performed to check the identity of plasmids as well as for restriction cloning. Restriction enzymes were used to cut segments of DNA at specific restriction sites. The digests were carried out using liquid DNA of different plasmids vectors and DNA inserts, appropriate restriction enzymes, as well as a restriction digest buffer. The buffer used in these restriction digests was the CutSmart Buffer. The restriction enzymes used included HindIII, EcoRI-HF, XbaI, and KpnI-HF. pUBI-attB was used as the plasmid vector, and mouse VDAC 2 were used as the insert. In a 1.5 mL tube, 1 µL of DNA, 1 µL of an appropriate restriction enzyme, 2 µL of CutSmart Buffer, and 16 µL of H_2O were added together. The tube was then incubated in 37°C for 30 minutes. Finally, a gel electrophoresis was then performed to visualize the results of the digest.

2.2: Gel Electrophoresis

Gel electrophoresis was used as a standard lab procedure for separating DNA by size for visualization and purification. An electric field was used to move the negatively charged DNA down an agarose gel towards the positive electrode. Shorter DNA fragments were able to migrate further down the agarose gel than larger ones. The estimated length of DNA fragments were found by comparing them to a 1 Kb DNA

ladder. The gel electrophoresis was performed using an electrophoresis chamber, voltage source, prepared sample, TAE buffer, agarose, and ethidium bromide. A 0.8% concentration of agarose gel was measured using 15 mL TAE buffer, 60 mL of 1% agarose solution, and 0.75 µL of 10mg/mL ethidium bromide solution. The agarose gel was poured into the chamber with a well comb in place, and this was left to harden for 20 minutes. 4 µL of 6x loading buffer was added to the prepared sample for visualization. 10 µL of 1 Kb ladder was loaded in the first well, with the prepared samples in the following wells. The gel was left to run for 30 minutes at 120 V. Finally, a gel documentation device with a UV light box and camera was used to visualize the DNA bands in the gel.

2.3: Ligation

Ligations were performed to join two DNA fragments using an enzyme. The plasmid was constructed by connecting the insert DNA into the digested vector backbone. T4 DNA ligase enzyme was used to carry out the reaction. The DNA ligase catalyzed covalent phosphodiester bonds to join the nucleotides together. The restriction digests created overhangs, also known as "sticky end," in the DNA samples. The sticky ends in the vector and insert were compatible, allowing them to bind together in the ligation reaction. Two different enzymes were used to ensure that the insert would bind in the correct orientation and to keep the vector from binding to itself. Controls were also carried out to check for complete digestion. In a 1.5 mL tube, 0.5 µL of pUBI-attB vector, 0.5 µL of VDAC2 insert, 1 µL of T4 DNA ligase enzyme, 1 µL of T4 10x ligase buffer, and 7 µL of H_2O were added together. For the first control sample, 0.5 µL of pUBI-attB vector, 1 µL of T4 DNA ligase enzyme, 1 µL of T4 10x ligase buffer, and 7.5

μL of H_2O were added together in a 1.5 mL tube. Also, for the second control sample, 0.5 μL of VDAC2 insert, 1 μL of T4 DNA ligase enzyme, 1 μL of T4 10x ligase buffer, and 7.5 μL of H_2O were added together in a 1.5 mL tube. The tubes were then incubated at 18°C overnight.

2.4: Transformation

Transformations were conducted to introduce foreign DNA into a cell. First, competent cells were taken out of the freezer and put on ice to thaw. A LB agar plate containing the appropriate antibiotic was placed at 37°C to warm. 1 μL of the sample from the ligation along with 25 μL of max efficiency cells were mixed in a 1.5 mL tube. The mixture was then left on ice for 30 minutes. Next, the sample was heat shocked for 30 seconds at 42°C. Then 250 μL of SOC media was added to the tube. The tube was then placed in a shaking incubator for 1 hour at 37°C. Finally, 25 μL of the sample was plated on the LB algar plate and incubated at 37°C overnight.

2.5: Plasmid DNA Minipreps

Alkaline lysis was performed to isolate the circular plasmid DNA from bacterial cells. First, 2 mL cultures from single colonies of bacteria that contained the plasmid DNA was grown overnight. 1.5 mL of the culture was then poured into a centrifuge tube. The tube was then centrifuged in a microfuge to pellet the cells, and the supernatant was removed. The bacterial pellet was resuspended in 100 μL of Solution 1 and vortexed vigorously. Solution 1 consisted of 25 mM Tris-HCl (pH 8). 200 μL of Solution 2, the P2 Buffer, was then added. The P2 buffer consisted of 0.2 N NaOH and 1.0% SDS. The

contents of the tube were mixed by inverting the tube quickly five times. The tube sat on ice for 5 minutes Then, 150 µL of ice-cold Solution 3, Potassium Acetate, was added, and the tube was gently vortexed for 10 seconds. The tube was then centrifuged at 12,000g for 5 minutes at 4°C in a microfuge. The supernatant was removed, and the tube sat in an inverted position to ensure all fluid drained away. The pellet was then rinsed with 150 µL of 70% ethanol at 4°C, and the supernatant was removed again. Finally, the DNA was redissolved in 50 µL of TE (pH 8.0) and stored at -20°C.

CHAPTER III:

RESULTS

To construct transgenic DNA plasmids containing mouse VDAC2 and Bax cDNAs, we first selected a plasmid vector for experimentation. As seen in Figure 6, Jones designed a plasmid map for the pUBI-attB plasmid.

Figure 6: Plasmid map of the pUBI-attB vector. The blue region from about 4900 bp to 6800 bp represents the *ubiquitin* genomic promoter region. The attB recognition site is in royal blue around 7,600 bp (Adapted from Jones, 2019).

Then, we could use this map to determine which restriction enzymes to use and visualize the lengths of resulting fragments (Figure 6). pUBI-attB was selected as the proper plasmid vector for experimentation due to its *ubiquitin* (UBI) genomic promoter region and its attB recognition site. These elements were necessary because the VDAC2 gene could not be expressed in *Drosophila melanogaster* without them. *Ubiquitin* is a protein that is expressed in all *Drosophila melanogaster* tissues. A DNA sequence inserted into the plasmid after this region will be turned on in all cells. Also, a plasmid with an attB recognition site was chosen because we use a *Drosophila melanogaster* line that expresses phiC31 integrase as well as having an attP site on chromosome 3. This integrase mediates site-specific recombination between its attB and attP recognition sites (Groth, Amy C., et al., 2004). It functions to promote the integration of an attB-containing plasmid into the attP site, creating transgenic offspring (Groth, Amy C., et al., 2004).

The first step in constructing the transgenic plasmids was performing three different restriction digests using various restriction enzymes to confirm the restriction sites of the plasmid. I began by digesting 1 µL of pUBI-attB with 2 µL of Cutsmart buffer, 1 µL of BamHI restriction enzyme, and 16 µL of H_2O (Figure 7). I repeated this process by digesting 1 µL of pUBI-attB with 2 µL of Cutsmart buffer, 1 µL of XhoI restriction enzyme, and 16 µL of H_2O and by digesting 1 µL of pUBI-attB with 2 µL of

Cutsmart buffer, 1 µL of EcoRI restriction enzyme, 1 µL of HindIII restriction enzyme, and 15 µL of H_2O (Figure 7). The resulting fragments in Figure 7 matched with the predicted lengths gathered from Figure 6.

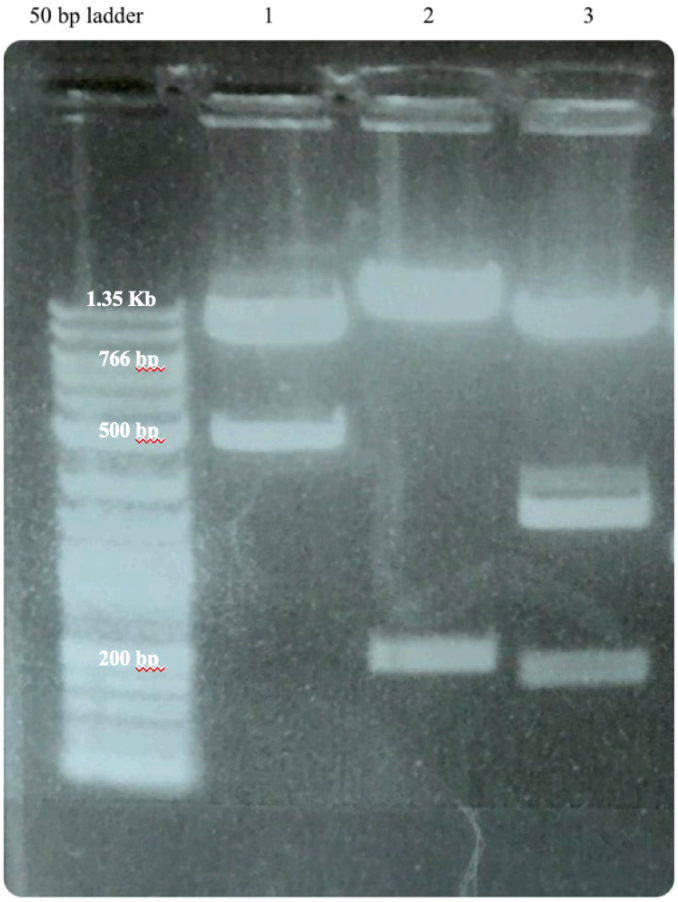

Figure 7: Agarose gel from restriction digest of pUBI-attB. The first lane contains 10 µL of a 50 bp ladder. Lane 1 contains 1 µL of pUBI-attB cut using 1 µL of BamHI restriction enzyme. Lane 2 contains 1 µL of pUBI-attB cut using 1 µL of XhoI restriction enzyme. Finally, Lane 3 contains 1 µL of pUBI-attB cut using 1 µL of EcoRI restriction enzyme and 1 µL of HindIII restriction enzyme.

Next, Jones designed a map for the mouse VDAC2 cDNA insert, as seen in Figure 8. This map was used to determine which restriction enzymes to use and visualize the lengths of resulting fragments.

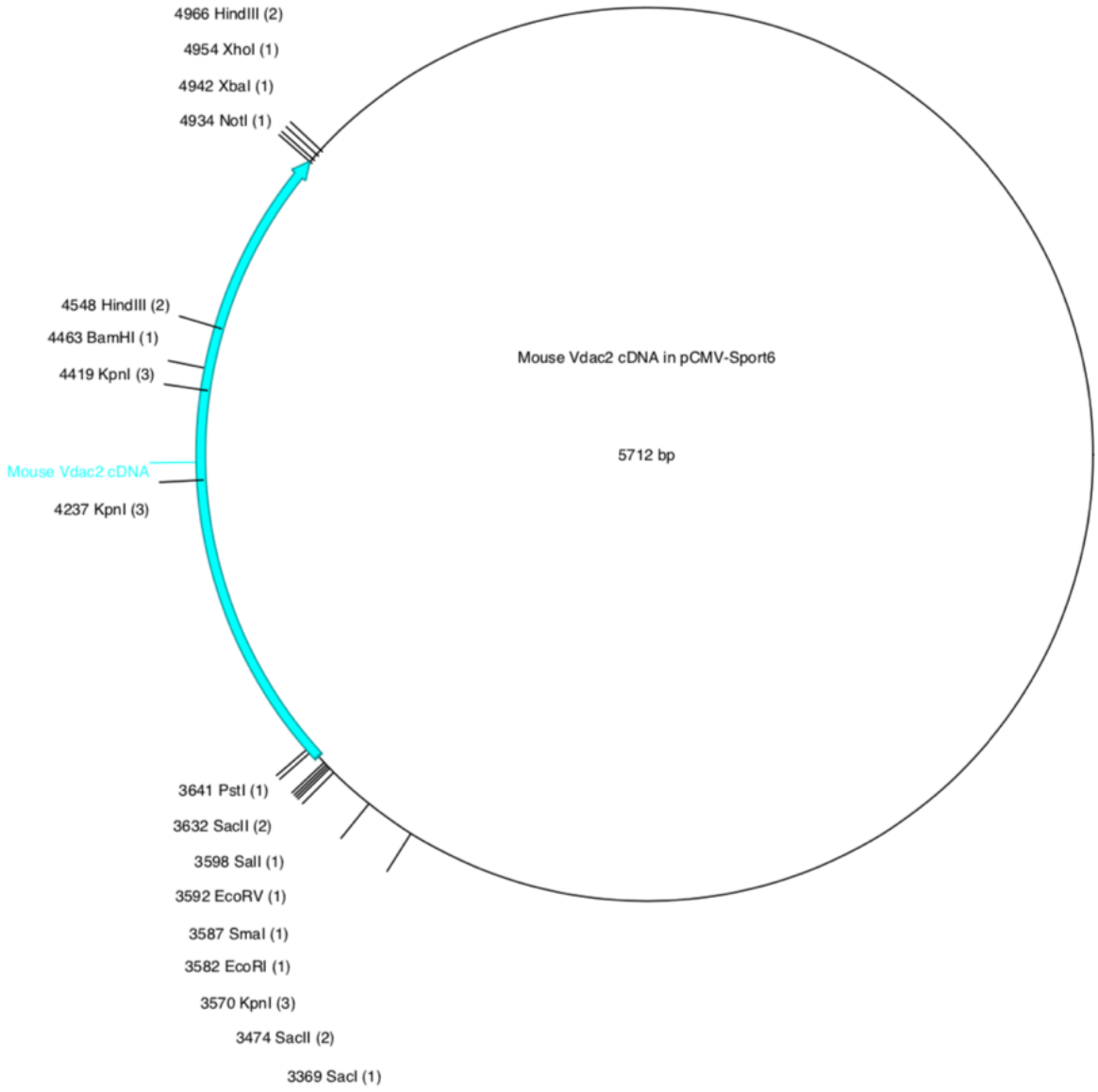

Figure 8: Map of mouse VDAC2 cDNA insert. (Adapted from Jones, 2019).

After analyzing the map of the mouse VDAC2 cDNA insert, we decided to perform a restriction digest on the insert. I first digested 2 µL of DNA insert with 2 µL of

Cutsmart buffer, 1 µL of EcoRI-HF restriction enzyme, 1 µL of XbaI restriction enzyme, and 14 µL of H_2O (Figure 9). I also digested 2 µL of DNA insert with 2 µL of Cutsmart buffer, 1 µL of KpnI restriction enzyme, and 15 µL of H_2O (Figure 9).

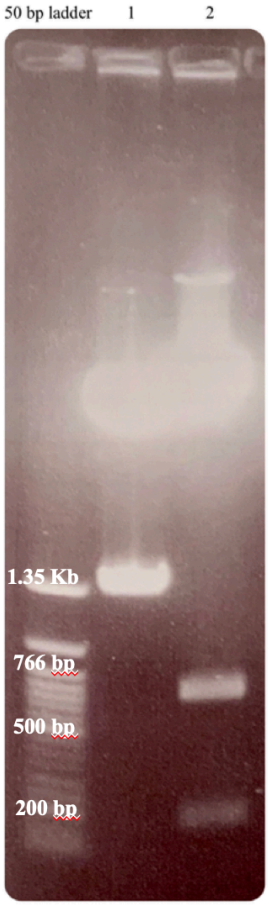

Figure 9: Agarose gel from restriction digest of VDAC2 insert. The first lane contains 10 µL of a 50 bp ladder. Lane 1 contains 2 µL of mouse VDAC2 cDNA insert cut with 1 µL of EcoRI-HF restriction enzyme and 1 µL of XbaI restriction enzyme. Lane 2 contains 2 µL of mouse VDAC2 cDNA insert cut with 1 µL of KpnI restriction enzyme.

Furthermore, Jones constructed a third plasmid map that represented the transgenic plasmid consisting of pUBI-VDAC2-attB (Figure 10). This figure was used to understand the resulting transgenic plasmid from our experiments.

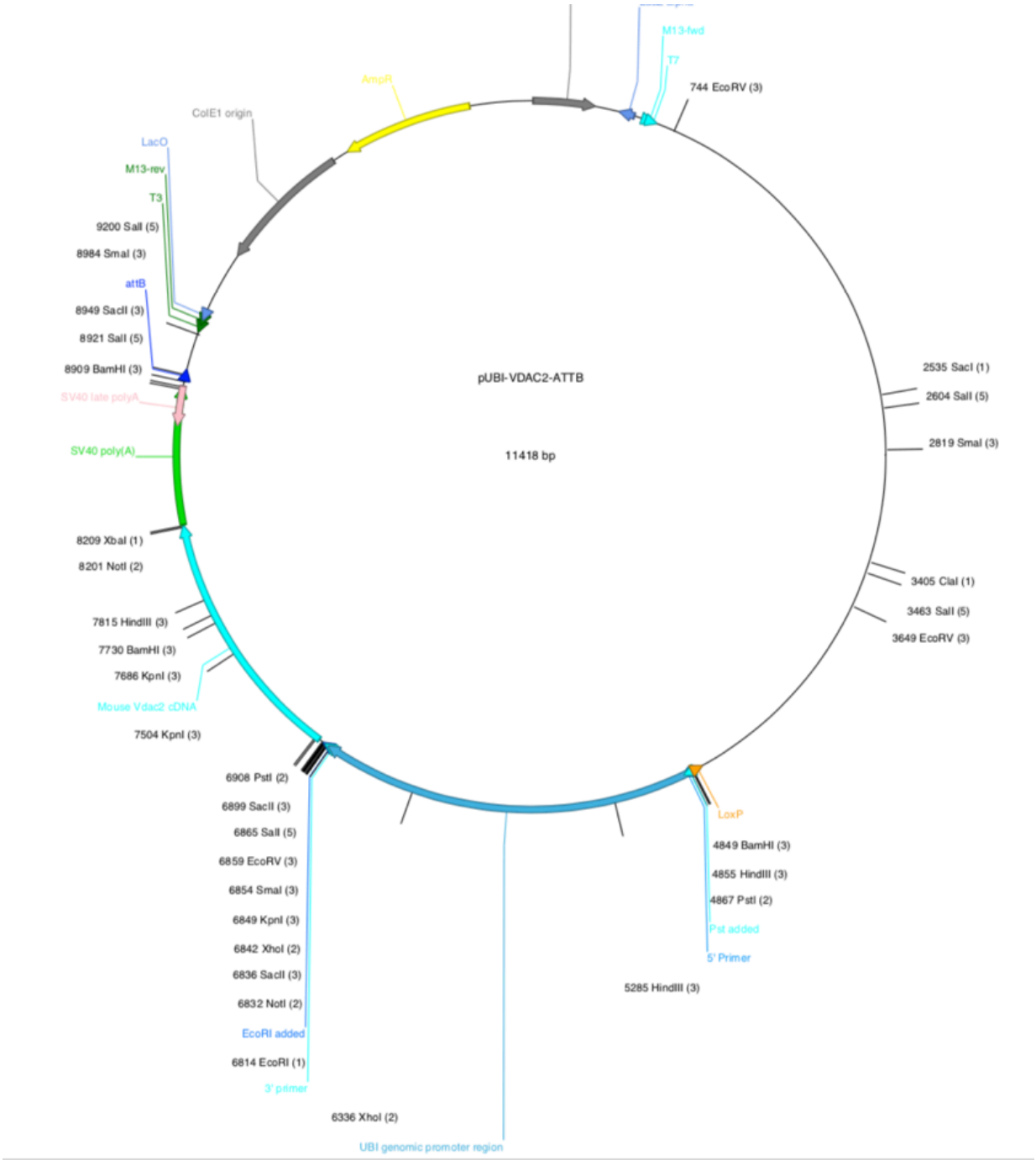

Figure 10: Plasmid map of the pUBI-VDAC2-attB. (Adapted from Jones, 2019).

The next step was to purify the fragments of DNA from the previous restriction digests. Purifying the vector and insert resulted in fragments seen in Figure 11. The size

of the vector fragment was about 10 Kb, and the size of the insert fragment was about 1.4 Kb (Figure 11). These measurements match the predicted measurements made from analyzing the plasmid map of the pUBI-VDAC2-attB (Figure 10).

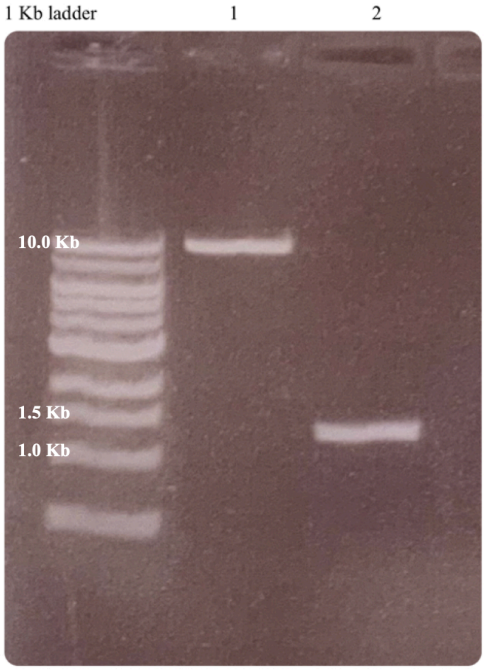

Figure 11: Agarose gel of purified vector and insert. The first lane contains 10 µL of a 1 Kb ladder. Lane 1 contains 1 µL of purified pUBI-attB vector, and Lane 2 contains 1 µL of purified VDAC2 cDNA insert.

Ligations were then performed to join the two DNA fragments using an enzyme. The transgenic plasmid was constructed by connecting the insert DNA into the digested vector backbone. T4 DNA ligase enzyme was used to carry out the reaction. The DNA ligase catalyzed covalent phosphodiester bonds to form to join the nucleotides together. Controls were also performed to check for complete ligation. Transformations on a LB agar plate containing the appropriate antibiotic were also conducted to introduce foreign DNA into a cell. The agar plates incubated at 37°C overnight.

Plasmid DNA Minipreps were then performed to isolate the circular transgenic plasmid DNA from bacterial cells. Figure 12 shows a restriction digest of the Minipreps and that the plasmid DNA was successfully isolated from the bacterial cells.

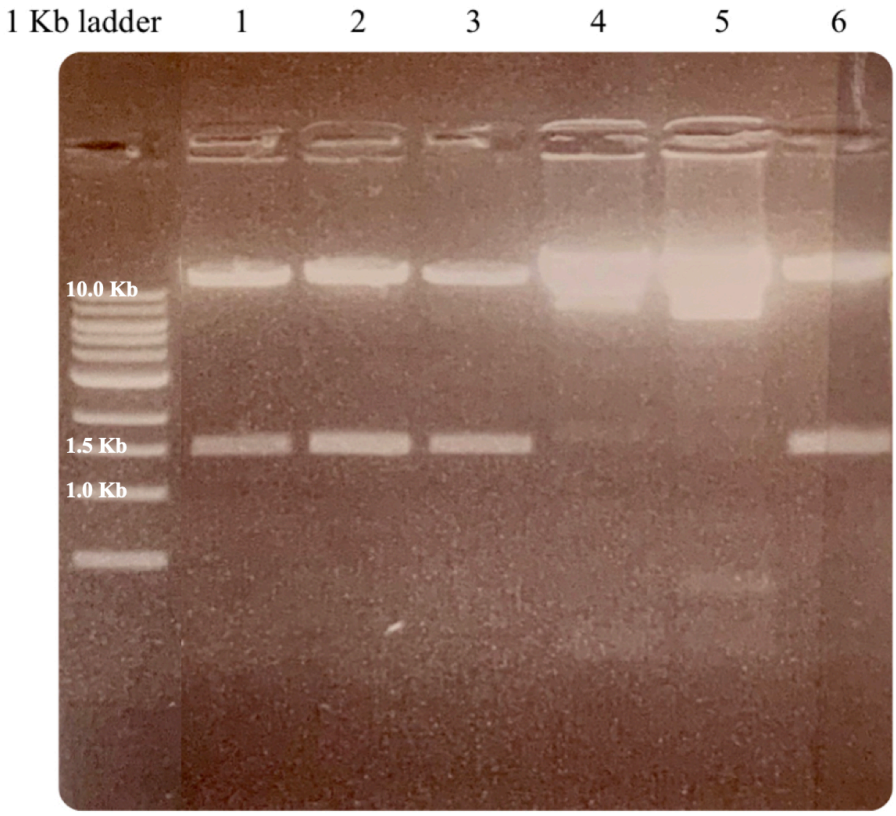

Figure 12: Plasmid DNA Mini preps of pUBI -VDAC2-attB. The first lane contains 10 µL of a 1 Kb ladder. Lanes 1-6 contains 5 µL of DNA, 2 µL of Cutsmart buffer, 1 µL of EcoRI restriction enzyme, 1 µL of XbaI restriction enzyme, and 11 µL of H_2O (Adapted from Jones, 2019).

Finally, a restriction digest was performed on the resulting DNA using 1 µL of pUBI-VDAC2-attB, 2 µL of Cutsmart buffer, 1 µL of EcoRI restriction enzyme, 1 µL of XbaI restriction enzyme, and 15 µL of H_2O (Figure 13). I then performed a DNA gel extraction to isolate the desired DNA.

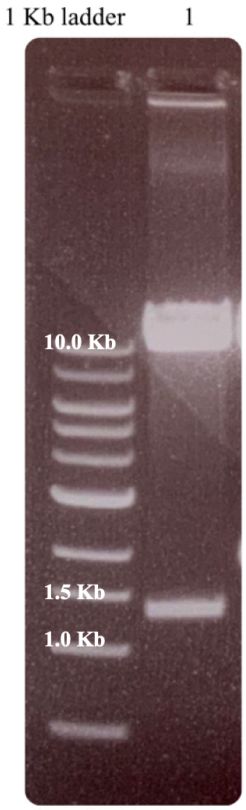

Figure 13: Agarose gel from restriction digest of pUBI-VDAC2-attB. The first lane contains 10 µL of a 1 Kb ladder. Lanes 1 contains 1 µL of pUBI-VDAC2-attB, 2 µL of Cutsmart buffer, 1 µL of EcoRI restriction enzyme, 1 µL of XbaI restriction enzyme, and 15 µL of H_2O.

CHAPTER IV:

DISCUSSION

The results obtained can now be used to create the transgenic lines of *Drosophila melanogaster*. Dr. Jones is currently injecting the pUBI-VDAC2-attB plasmids into hundreds of *Drosophila melanogaster* embryos and integrating it into their genomes. Dr. Jones has obtained one line that has the pUBI-VDAC2-attB plasmid integrated. Lines that have the pUBI-VDAC2-attB plasmid integrated express an altered red eye phenotype compared to the other lines that have white eyes.

We hope to further experimentation by crossing this transgenic line with other lines such as UAS-Bax to examine the phenotypic effect. Once these lines have been crossed, the offspring can be crossed with a line of GMR-Gal4 that will turn on the Bax gene in the eye. The flies containing the pUBI-VDAC2-attB transgene can then be compared to those lacking this alteration.

The goal is to determine whether this genetic change leads to a beneficial or harmful phenotypic morphology in the flies. We predict that expressing VDAC2 in the eye or wing will restrain Bax, making Bax less potent, and therefore cause a less harmful phenotypic morphology. On the otherhand, expressing VDAC2 could cause Bax to become more potent, leading to a more damaging phenotypic morphology. It could also cause no phenotypic change in the flies. The interruption of the academic calendar due to COVID-19 has paused further injecting and screening of the transgenic lines.

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