Biological tube formation in *Drosophila* tracheal system with loss of function mutant by CRISPR-Cas9 genome editing

Submitted by

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Biomedical Science B.S.

To

The Honors College

Oakland University

In partial fulfilment of the

Requirement to graduate from

The Honors College

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Biological Sciences Department

Oakland University

April 2nd, 2021

Abstract

The *Drosophila melanogaster* trachea is the premier genetic system used to study the fundamental mechanism of the mammalian lung and blood vessel formation process. Seven members of the Osiris (Osi) gene family are highly expressed in the trachea system of *Drosophila*. Protein localization of Osi proteins suggests its potential role in the lumen formation in the *Drosophila* trachea. Tracheal Osi genes are predicted to function redundantly. To reveal the mechanism of Osiris genes in the *Drosophila* trachea, we will generate a triple knockout mutant by removing the function of 3 out of the 7-trachea expressing Osi genes. Specifically, we will generate an Osi9 (CG15592) loss of function mutant in a previously generated double knockout background of Osi15+19. To do this, we will use clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) 9 protein system: an efficient and important genome-editing tool. This technology has been wildly used in cells, insects, and mammals to study the function of novel genes by generating loss of function mutants. The generation of these triple loss of function mutants and phenotypic analysis of these mutants will reveal a novel mechanism of tubular organ formation.

Keywords: *Drosophila*, Osiris (Osi), clustered regularly interspaced short palindromic repeats (CRISPR) Cas9 genome editing tool, tracheal tube development.

Current Research and Knowledge

The mechanisms of tubular organ formation in mammals are currently being researched heavily in *Drosophila melanogaster.* The *Drosophila* trachea is a premier genetic system acting as a good model to obtain information on tube formation, which will be the focus of this study. Due to structure similarity and gene conservation, the knowledge obtained from the *Drosophila* trachea will be applied to understand the formation of mammalian tube-based structures such as the lung, kidney, and blood vessels. Furthermore, it will help us to develop drugs to treat human diseases caused by tube formation defects. Much is known about specific genes and the proteins that these genes encode within the *Drosophila* tracheal system. Determining the functions of new genes and mechanisms of development in *Drosophila* can greatly improve our understanding of tube formation in humans.

The reason that these tubes are important to organisms is due to the fact that they are a fundamental unit of these organs. The tubes transport critical gases, liquids, and cells from one site to another. A detailed mechanistic understanding of tubular genesis is important for medicine and biology. Tube size defects can lead to many human diseases like polycystic kidney disease, fibrocystic breast disease, pancreatic cystic neoplasm, bile duct cysts, and more. So, by looking at the tube formation and what role these genes partake in, we will be able to learn more about the structure and process that can hopefully lead to application in other mammalian systems and help with tubular malfunction diseases. Particularly we will be looking at the cell-to-cell junctions since the Osi9 gene is predicted to be involved in maintaining the structure of the apical membrane.

Currently, the novel CG15592 (*Osi9*) gene is being studied and is known to have some direct relationship to tracheal tube formation and structure of the apical membrane. So, we will be looking specifically at the sub apical region (SAR), apical free region (AFR), adherens junctions, as well as barrier junctions. We know some proteins are important in these regions like DE-Cad in the adherens junction. In fact, the whole Osiris gene family tells us important information for this process.

The Osi gene family is known to have twenty-four members. The Osi gene family is located at a locus that exhibits dosage sensitivity (triplo-lethal and haplo-lethal); these genes are predicted to have functional redundancy due to similar protein structures (Dorer et al. 2003). The predicted Osi protein sequence contains an endolysosome signal that suggests their involvement in protein trafficking. There is also a DUF domain that is known to be there but has an unknown function. The similar protein sequence of the Osi proteins suggest that they have similar function in the *Drosophila* trachea. There are specifically seven Osi genes that are expressed in the *Drosophila* trachea that are of importance for its development. Fluorescence in situ hybridization was used to show Osi mRNA expression (Figure 1). Osi mRNA (red in Fig. 1) was shown to be expressed in trachea, which is outlined by a tracheal marker, Btl mCD8 GFP (green in Fig. 1). What we currently know is that the Osi15 knockout mutant is homozygous viable, the Osi19 mutant is semi lethal with some homozygous adults, and the Osi15 and 19 double mutants (#54) is lethal at the second larval instar stage.

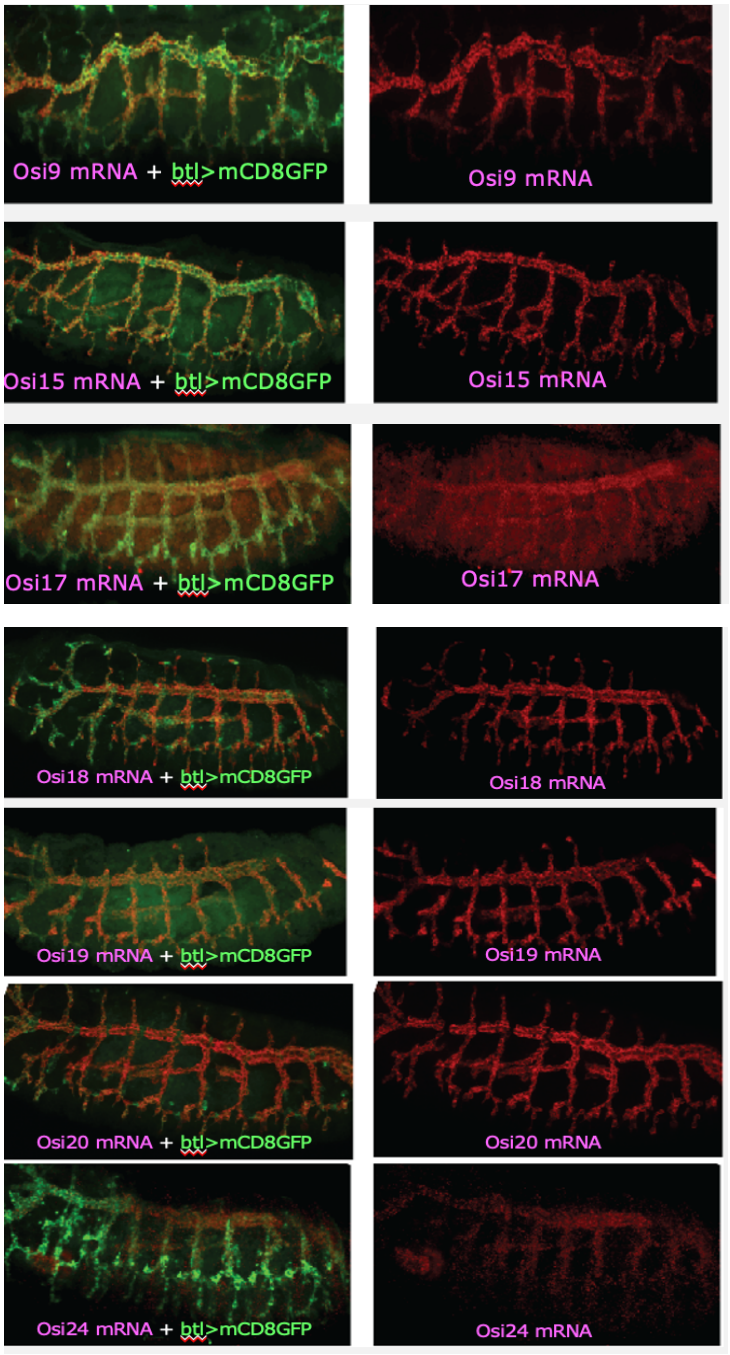


Figure 1. Pictures of the 7 novel Osi genes found in the *Drosophila* trachea. These pictures were taken underneath a confocal microscope. The Green pictures on the left, have Btl mCD8 Green Fluorescent Protein (GFP) marker cells to show the genes importance in the trachea. The red pictures on the right show the Osi mRNA in trachea by fluorescence in situ hybridization.

The Drosophila trachea is a ramifying network of epithelial tubes with a monolayer of tightly adhered polarized cells surrounding a central lumen. Previous research has revealed the mechanisms of the early steps of tube formation, including the specification of branch identities and the migration of tracheal cells. For example, signaling pathways such as Epithelial Growth Factor and Transforming Growth Factor-β signaling that are activated in a given group of cells specify branch identity (Chen et al. 1998, Llimargas and J. Casanova. 1999, Chihara and S. Hayashi. 2000). Thereafter, Fibroblast Growth Factor signaling guides the migration of tracheal cells in typical directions to form distinct branches, including the main multicellular branch, the dorsal trunk (DT), unicellular branches such as the lateral branch (LT) as well as the ganglionic branch (GB), and intracellular tubes, such as in the lateral ganglionic branch (LG), which forms within cells as long cytoplasmic extensions (Figure 2A) (Klambt et al. 1992). Then, branches from neighboring segments will fuse to form an interconnected network (Lee et al. 2003, Jiang and Steve T. Crews. 2003, Steneberg et al. 1999). Finally, tubes mature to acquire proper sizes and are filled with air. Embryonic tracheal maturation is a multistep process: (a) tube formation, (b) apical secretion to form an apical lumen containing chitin-based extracellular matrix (red lines), during which tubes expand in both diameter and length, (c) solute clearance to remove solid material and liquid in the lumen, and (d) air filling (Figure 2B). These changes occur at the ***apical side*** of the tracheal tubes with little or no changes to the basolateral side (Swanson and G. J. Beitel. 2006).

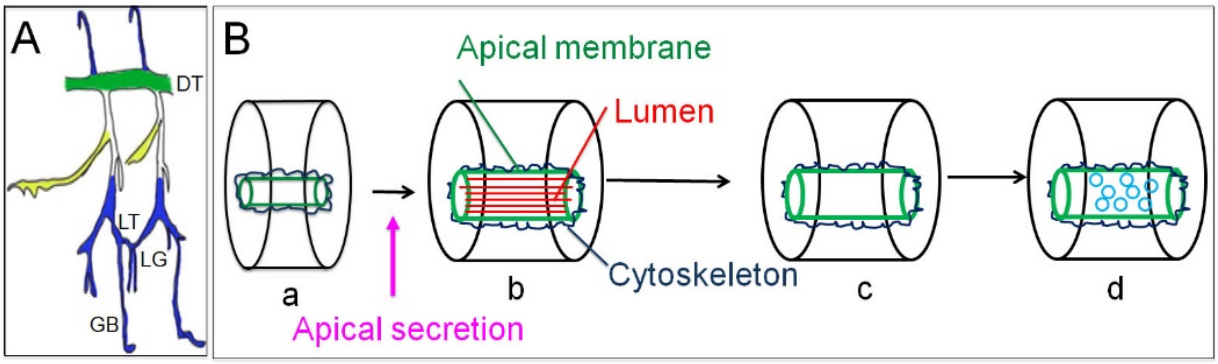


Fig. 2. *Drosophila* tracheal development. A. Branches in *Drosophila* trachea. Multicellular dorsal trunk (DT), unicellular lateral trunk (LT) and intracellular ganglionic branch (GB). B. Tube maturation process in *Drosophila* trachea (cross section). After the formation of a tube (a), the tube expands. Tube expansion is mediated by apical membrane growth (green), transient chitin-based matrix (red) assembly, and reorganization of the apical cytoskeleton (black) (b). When a tube reaches its mature size, the luminal material is cleared (c). Subsequently, air (blue circles) fills the tracheal tube (d).

To study the function of a particular gene, we will generate loss of function mutants and then compare the phenotypes of the mutants to wild type control. These results will provide novel insights into the function of that gene. Observing phenotypes of wild type mutants and loss of function mutants under the microscope, allows us to see what specific protein is actually involved the tracheal development process. Protein localization analysis of Osi9 revealed that Osi9 proteins are highly concentrated in vesicle-like structures near apical membranes, suggesting their potential involvement in apical lumen formation in *Drosophila* trachea which will be a key focus of this study.

Aims and Objectives

Introduction

A lot of background information is known about the early development of the trachea system. However, the later tube maturation is not well known. The *Osiris* family of genes are highly expressed at later stages of tracheal development. Therefore, studying the function of *Osiris* genes will provide novel knowledge about tube maturation. We will generate different single and combinations of double and triple mutants of the Osi genes and compare them to a wild type control group. Doing this provides us with a way to see what role the genes play in the tracheal tube development process. After making and confirming the potential knockout mutants and antibody staining, we can observe under the confocal microscope, which will allow us to see any deformations and key roles of these genes and even specific proteins in the tube formation process.

My research will focus on the generation of an *Osi9* loss of function mutant in the background of an Osi15+19 (*Osi 54b*) knockout using the CRISPR-Cas9 system. The CRISPR-Cas9 system is comprised of a bacterial Cas9 endonuclease that forms a complex with two specific RNA structures: a CRISPR RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA). The two RNA structures can be combined into a single RNA complex called the synthetic guide RNA. The sgRNA contains a 20 bp sequence that is complementary to the sequence of the gene of interest and brings the complex to the target loci in the genome upstream of a specific protospacer adjacent motif (PAM). The Cas9 enzyme then introduces an RNA-guided double-strand break (DSB) in the target gene, which then will be repaired either by homology-directed repair or error-prone mechanisms such as nonhomologous end joining (NHEJ). During NHEJ, loss of function mutations in the gene will be generated.

So, I will use these genome editing tools in order to create mutant strains and compare them to a control group to confirm my mutant formation and see how this loss of function translates to tube formation in the *Drosophila*. My specific goal with this experiment is to actually generate at least 50 potential Osi9+15+19(Osi32) loss of function mutants and then run a PCR (polymerase chain reaction), gel electrophoresis, sequencing, and finally do antibody staining to confirm the generation of these mutants. From there, we will then be able to look at the phenotypic alterations these mutations have on the tracheal system.

Aims:

1. To generate Osi9 gRNA containing vector in the background of *Osi 54b* knockout.
2. Introduce gRNA expressing DNA vector into the *Drosophila* genome to generate a gRNA transgenic line. Then introduce Cas9 into the gRNA transgenic line by crossing these flies to Ca9 containing transgenic line, which is available from the *Drosophila* stock center.
3. Generate 50 potential Osi9+15+19 triple knockout loss of function mutants from the progeny of gRNA and Ca9 crossed flies.
4. Isolate the triple loss of function mutants by PCR and DNA sequencing.
5. Perform antibody staining to confirm the formation of the triple mutant flies.

Objectives:

1. Generate Osi9 gRNA vector in order to generate our mutant strain to compare with the control group and see what differences there may be and if any of those differences applies to the tracheal system and development.

2. Generate gRNA transgenic line in order to have a red eye marker which is present on the vector as a selection marker. This marker will allow us to confirm that we have generated a transgenic line.
3. Introduce Cas9 into gRNA transgenic line by crossing a Cas9 expressing line and our gRNA transgenic line to produce embryos for the next step in our process of generating loss of function mutants.
4. Generate 50 potential Osi9+15+19 loss of function mutants so that we have enough good mutants to pick from and generate mutant stocks to then observe under the confocal microscope.
5. Isolate the triple loss of function mutants by PCR using primers specific for amplifying the Osi9 genomic sequence so that we can analyze our results. The antibody staining will help confirm the formation of the triple mutants.

Methodology

In order to make our mutant Osi9 gRNA vector we must have two oligos: Osi9 target Sense (5' – CTTCGACTTGAACATGGCTCGTGAT – 3') and Osi9 target Antisense (5' – AAACATCACGAGCCATGTTCAAGTC – 3') that are annealed and then inserted into pU6-gRNA vector to generate the Osi9 gRNA vector. Then we must generate our gRNA transgenic line by introducing Osi9 gRNA expressing vector into wild type *Drosophila* line by injecting DNA into germ cells of the *Drosophila* embryos. The Osi9 gRNA transgenic lines will have a red eye marker which is present on the vector as a selection marker. In order to introduce Cas9 into gRNA transgenic line we must pick virgin females from Cas9 containing transgenic line and males from our gRNA transgenic line. We introduce Osi9 gRNA and Cas9 into Osi15+19 double

mutant background. Then, we mate these females and males in a bottle containing food. We want to generate 50 potential Osi9 +15+19 loss of function mutants from the progeny of gRNA and Cas9-crossed flies. Since Osi double mutants (*Osi 54b*) are homozygous lethal, we will cross potential triple mutants to TM3GFP background and keep the mutants as heterozygous. Lastly, we must isolate the homozygous triple loss of function mutants and amplify the Osi9 genomic sequence by PCR using gene specific primers, followed by sequencing analysis. The primers that will be used are the following: Osi9 genomic forward primer (5' - ACAGTTTACCGGTTGCCTT – 3') and Osi9 genomic reverse primer (5'- TCTTTGGGCACCTTGAACT – 3'). After isolating the Osi9+15+19 loss of function mutants by PCR and DNA sequencing, we can confirm the loss of Osi9 in the double mutant background by antibody staining.

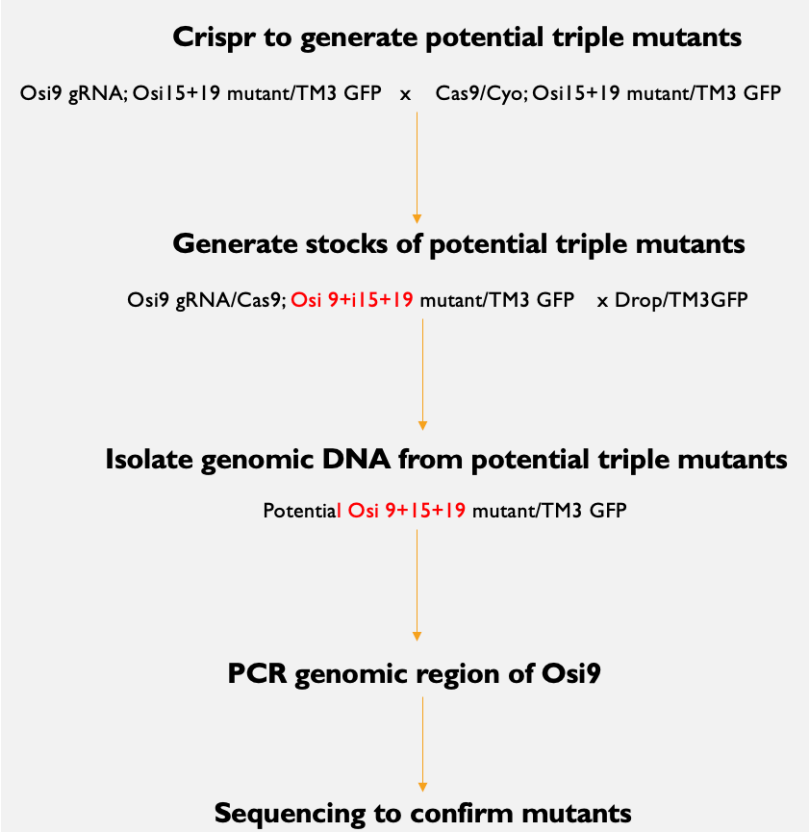


Fig3. Steps to generate Osi triple mutants

Outcomes

Specific outcomes:

We are studying specific genes involved in the tracheal tube formation in *Drosophila melanogaster*, a model organism of the mammalian tubular organ. To study the mechanism of these genes in tubular organ formation, we need to generate loss of function mutants. Specifically, we are studying the novel gene Osi9 that has obvious tracheal expression in *Drosophila*, HA-tagged Osi9 are highly concentrated in vesicle like structures at and near the apical membrane. We are able to observe protein localization of Osi9 by HA tag under the confocal microscope. We know that the localization of Osi9 protein suggest that these proteins are likely involved in tube maturation through vesicular trafficking. By comparing phenotype in wild type control and triple Osi loss of function mutant, we will reveal the function of this novel gene in tubular organ formation. The study of Osi9 will reveal novel mechanisms of tube formation. We will use genome editing tools to make these loss of function mutants to see how this specific gene effects the flies and therefore, hopefully learn the function of this gene in lung and blood vessel development in the near future.

Speculative outcomes:

The research we are doing can be a little tricky because not only is there a lot of information we must unravel, but we are also hoping that it can be applied to humans and help them with certain tracheal tube developmental diseases and cancers. However, if it cannot be applied to humans directly, then other scientists can still take the information we have found and learn more about the *Drosophila melanogaster.* Learning more about model organisms like this, indirectly still helps humans because the more we know about this model human organism, the

more we will be able to apply to humans in the future. With that being said, the information we have found thus far and continue to do so, will at least benefit the scientific community in that sense. If we are able to apply the information of the FGF factors, Osi genes, and more to the tube formation in humans we will be contributing to the scientific community more directly. Other researchers will then have to perform even more research and experiments to see how this is related to humans specifically and if there are any differences, what these differences may be and why they are there. Not only that, but once we know this information, we will more importantly, hopefully be able to find cures and try to reverse or fix these disease processes in humans possibly before they even happen.

Results and Discussion

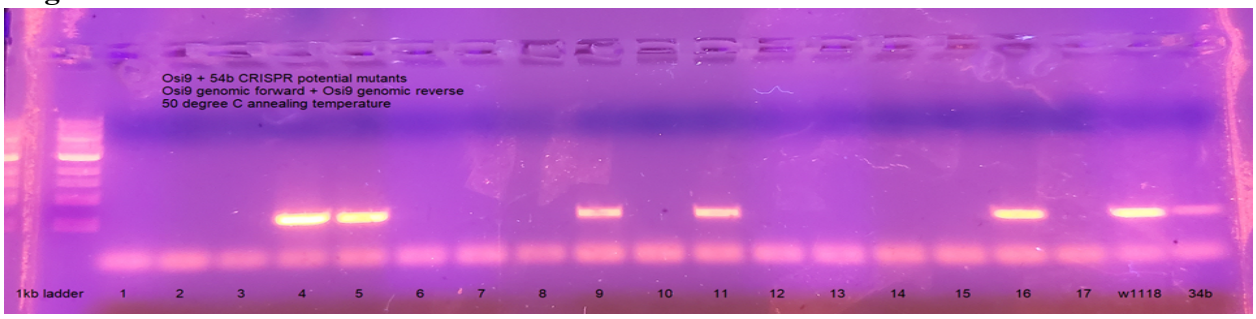
Polymerase Chain Reaction (PCR)

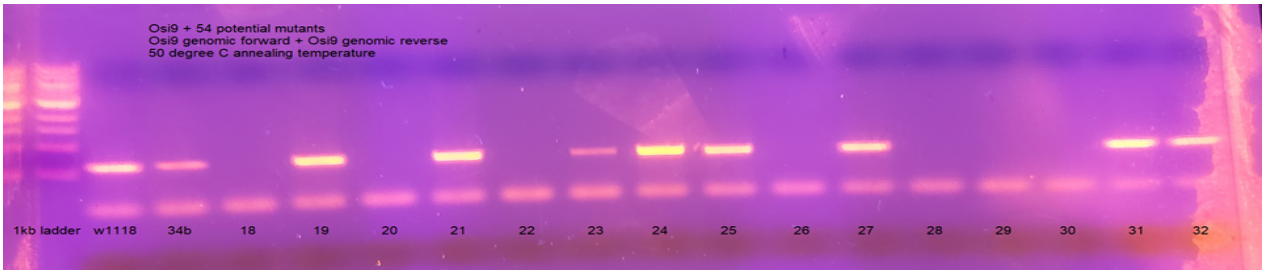
Since the Osi9 is made of DNA because it is a gene, it includes exons and introns, as well as a chosen target sequence that is close to the start site. So, we designed primers: a forward and a reverse primer in order to try to amplify the region and include the target site. Since deletions usually show up close to the target site, we had this in mind. Thus, we amplified a region close to the target site. When we get the DNA sequence results, we are hoping to see this deletion and can compare it to the wildtype, in order to tell us which region is deleted and also be able to predict the specific protein sequence. Since the other Osi15 and 19 knockouts have already been made, we only needed the Osi9 mutant and made a lot of copies of the Osi9 amplified to check and make sure we have deleted the Osi9 gene. Once we made the 50 potential mutants, we were not sure which ones were mutants and which were not. In order to see these results, we needed to run a PCR close to the target site, where the deletion should have occurred. We use the forward

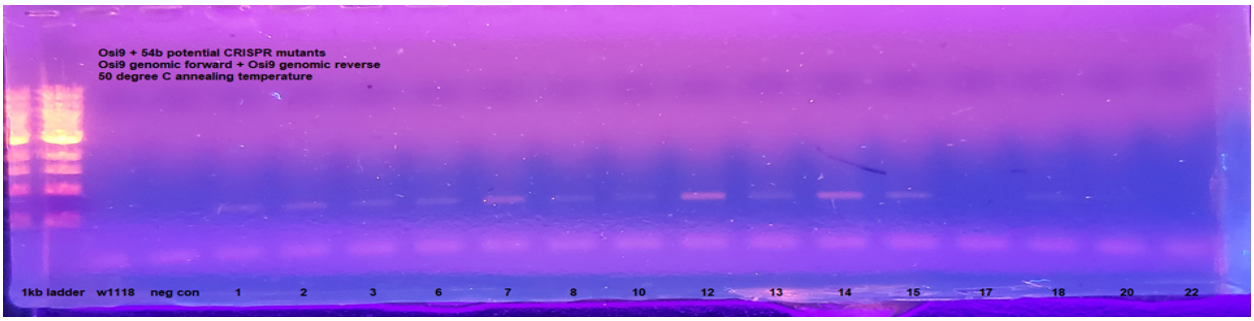
and reverse primer to get regions on either side of the target site. Once we got these PCR fragments, we sent them out for DNA sequencing, which then gave us the nucleotide sequence of our potential mutant. Usually, the PCR product we get is smaller than the original sequence due to the deletion. So, based on this deletion we can get the sequence. After we received our potential mutant sequence results, we compared to the wildtype and this showed us what regions were deleted and if anything was not. From this DNA sequence we then predicted the amino acid sequence.

Gel Electrophoresis

Figure 4:







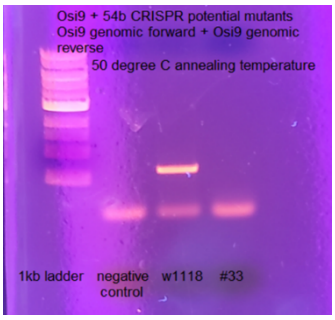


Figure 4: Gel electrophoresis results. These pictures show the amplified region of Osi9 gene region. The DNA fragments will be purified and sequenced.

We had to run multiple PCRs until we got the amplification of the region we wanted and generated those 50-potential loss of function mutants. With these 50-potential mutants, we extracted the DNA from the homozygous embryos, from each potential mutant. We now have 50 of this DNA from the potential mutants and then we run a PCR on this DNA. This will result in 50 PCR products that we can send for sequencing. The bands seen on the gel, seen in Figure 4 are the products we sent out for sequencing. In the gel we expected this mutant to be slightly smaller. However, we cannot tell a difference because the insertion turned out to be only 7 base pairs long. Since this is such a small change, we cannot see this on the gel and are just not able to detect it in the images. In the gel results, some of them indicate no PCR product at all, from that we tried again until we actually got the PCR product we wanted for all 50 potential mutants. We then sent these for sequencing and picked the one that showed the best results with the premature stop codon.

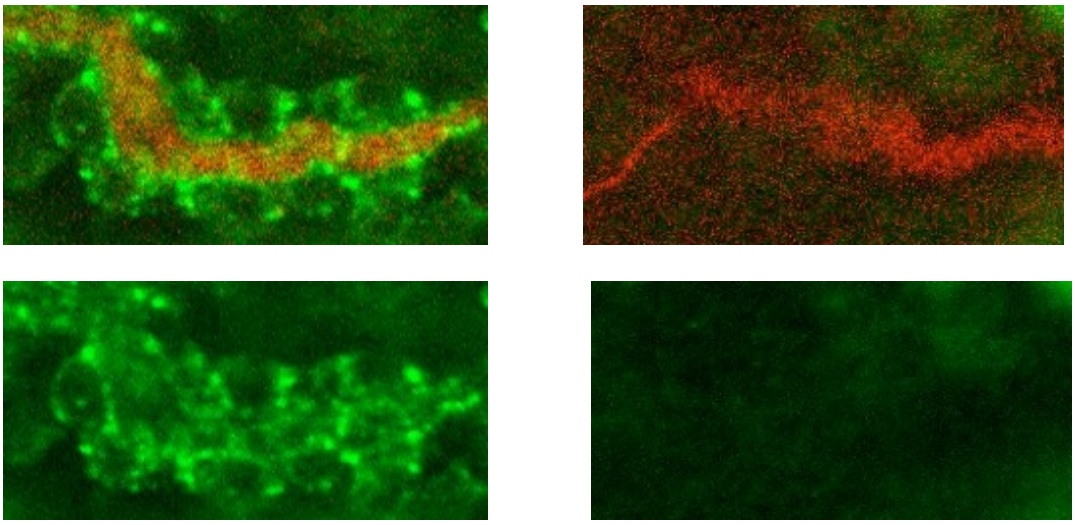
After running a PCR and gel electrophoresis of the potential triple knockout mutants and observing the results, we were able to do an antibody stain and confirm the generation of the triple mutant. The Osi protein results include:

MFKFVCLFALIASTAAATSEADSLLTSALKMVKDCGERSMVLCMKERALHYFDAENGD

VRLTEGIALVKTDEIPVGRSLNEMQLPEEVEAREAEVDSLLVERVARFFGTHTLQFKVPK
DSIQDMQRALEESRGKKKEKKKYLMPLLMLFKLKMAALLPLAIGFLALISFKALVIGKIA
LLLSGIIGLKKLLESKKENYEVVAHPHYEHEHSYGRSLPSDDSQAQQLAYAAYKQ.
While the Osi9 protein in the Osi32 triple knock mutant came out to be:
MFKFVCLFALIASTAAATSEADSLLTSALKMVKDCGERSMVLCI-. This shows a 7 base insertion which led to a frame shift and a premature stop codon. This premature stop leads to a truncated Osi9 protein, essentially a protein null, or a severe loss of function mutation.

Antibody Staining

In order to confirm that we made the triple mutant we did an antibody staining. The red in the staining, seen in Figure 5, represents chitin, a structural polysaccharide component of the lumen. The chitin shows us where the lumen is and the green in the pictures on the left side of Figure 5 represents the Osi protein in wildtype. In the wildtype, we can see the protein pretty well due to the strong green color in the confocal microscope pictures. However, the important takeaway from our antibody staining results can be seen in the lower right-hand picture of Figure 5, where there is no red color seen at all. This indicates that we did in fact generate our Osi32 triple knockout loss of function mutant because there are no Osi proteins seen in our staining results, which indicates that the genes were in fact knocked out. Since the red chitin is showing us the lumen and trachea, we know these structures are still there, however, the pictures do indicate that the actual Osi protein is absent.



Wild Type **Osi32 Mutant**

Figure 5: Antibody staining results. The images on the left show the wildtype and the images on the right show the Osi32 triple mutant. The red in these images indicate chitin is present, which shows the trachea and lumen structure. The Osi32 mutant picture on the bottom right shows no Osi protein present, so this can confirm that the triple knockout was indeed successful. Images were taken using a Nikon C2+ confocal microscope with a 40x objective lens.

Conclusion and Future Directions

After viewing these results and confirming that we had properly made triple Osi9+15+19 knockout mutants, we can now use these mutants to interpret not just the genotypic affects but the phenotypic affects to the *Drosophila* trachea system. Comparing these mutants to the wildtype allows us to see what effects these genes and the proteins that these genes encode for, do in the trachea development process. Knowing the functions of these genes in the tubular formation process expands the knowledge bank of the *Drosophila* model organism system as well as allows us to apply this to future studies in other mammalian organisms. Knowing what a triple mutant can do can also tell us more about what a different variation of these knockout genes does in the tubular formation process. For example, loss of function mutants in other trachea expressing Osi genes. Some of these variations are already known or currently being

studied, but this does open the door for many other opportunities and knowledge about tubular formation in the mammalian system in general.

Acknowledgements

I would like to say a big thank you to my mentor, Dr. Lan Jiang for allowing me the great opportunity to work in her research lab and gain such an amazing experience in this type of setting and expand my biological science knowledge bank. I would also like to extend a thank you to all of my colleagues in Dr. Jiang's laboratory, including Aaron Scholl and more, for helping teach me new techniques and supporting me through this journey. This research was funded by the Honors College of Oakland University.

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