Analyzing Effects of Osiris Gene on *Drosophila* Developing Tracheal Tube Formation

Submitted by

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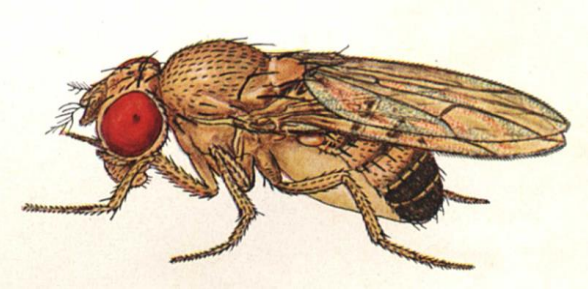
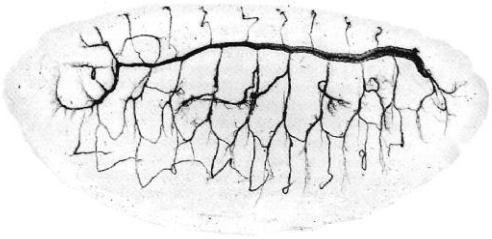
Introduction

This project focuses on specific genes that help with transcription and regulation of transcription and translation of proteins involved in the development of the tracheal tubes and tube morphogenesis. Our research performs tests on Drosophila melanogaster because they are considered a model organism that will develop similar to humans in regard to tubular organs. The tracheal system delivers oxygen to all of the cells in the body, and Drosophila melanogaster's are excellent model organisms to study this tube development process. 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 in stereotypical directions to form distinct tubes. After formation of a continuous tube, the tube expands in both diameter and length to form a liquid-filled trachea lumen. Thereafter, luminal material needs to be cleared and followed by air filling. The mechanisms of tube morphogenesis in this organism are highly conserved, meaning they are consistent steps in order to produce to final fully mature trachea. It also represents a very simple structure in comparison to other organisms. It can easily be studied for its phenotypic effects because of straightforward and easy to understand anatomy.





We are focusing on the phenotypical tube formation differences between a negative control group and combination knockout groups of specific genes within the Osiris gene family, a family of genes seen within the Drosophila and other insects.

The basis behind the research is to look at tube morphogenesis and apical clearing, which is the development of epithelial tubes in order to allow for the tracheal system to form. These tubes can form in many different ways, but one of the things they have in common is how the apical epithelial surface always lines the lumen. Multiple things play a role in tube morphogenesis including vesicle fusion, secretion, and apical and luminal clearing to allow for air filling in the tubes. Our prediction for the role of the Osiris gene family genes is that they play an important role in the development of the tracheal system and also tube morphogenesis as the larvae develops.

Model Organism

The development of the Drosophila trachea is a very branched network of epithelial tubes with apical surfaces facing the lumen and basal surfaces facing surrounding tissues. As the trachea develops, tubes differentiate into four types labelled with roman numerals 1-4. This specification is determined by signaling pathways that involve epithelial growth factor and TFG-β signaling. These signaling pathways guide tube morphogenesis in specific directions for generation of specific branches. All branches start as multicellular and as development continues, some will diverge and become unicellular, and all will have their own destination. The size of the tubes are determined by changes in the apical surface of the tracheal cells. During the time that the tube experiences diametric expansion, which is determined to be embryonic stages 13 to 16, there will be an apical secretory burst initiated that will present luminal proteins required for

membrane growth and a chitin-based apical extracellular matrix. In stage 17, a large wave of endocytic activity will internalize the components and matrix recently composed. The luminal fluid is also cleared, allowing for the entire trachea to fill with air, completing tube dilation during development. Meanwhile all of this is occurring, tube elongation is constantly occurring throughout development to finalize the fully mature tracheal system.

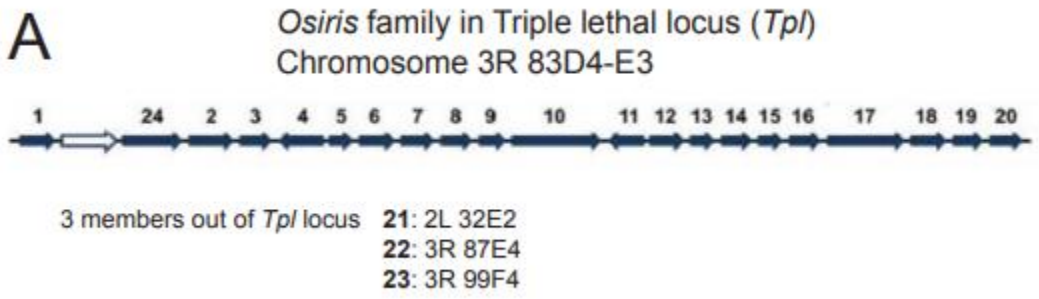
A more specific description of the tube-size regulation associated with tube dilation and apical secretion is the apical secretion pulses drive dilation and allow for components of the matrix to construct its complex formation. This formation is a complex consisting of many proteins including chitin, zona pellucida domain proteins to ensure cell interconnections, and other modifying enzymes. The chitin matrix is the mechanism mostly involved with the diameter and tube dilation of the tracheal tube morphogenesis. Luminal fluid consisting of chitin and other proteins is the outward force driving tube dilation.

After the tube dilation and matrix development within the tubes, the lumen must be cleared in order for air filling to occur, which permits gas to flow through the tracheal system providing necessary molecules to the different locations within the organism.

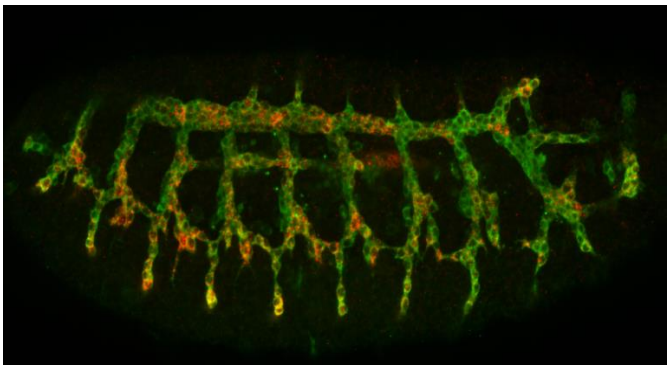
Osiris Gene Family

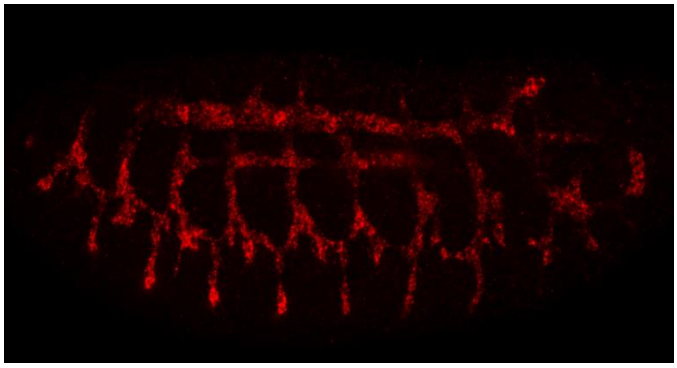
The main gene family associated with the research we conducted is the Osiris gene family. This gene family is heavily expressed phenotypically in the Drosophila trachea. The Osiris gene family is also found in many other organisms and insects that includes, butterflies, mosquitos, honeybees, ants, wasps, and more. No analogs of the Osiris gene family have been found in mammalian species, however, the mechanisms behind tube morphogenesis is believed to be similar to one another. Within the Osiris gene family, there are twenty four genes. Osi,

short for Osiris, proteins are highly expressed in vesicles that are near the apical membrane of tracheal cells. Because apical secretion, cytoskeleton arrangements and clearance, and air-filling processes occur at the apical membrane, Osiris genes were deemed a logical target for insight on their role in tracheal development as well as more defined and exact mechanisms involved in the processes of tube morphogenesis.



This diagram shows the Tpl, or triple lethal focus, which was a specific combination of knockout genes that caused cell death within the trachea and midgut at the larval stage. This is what lead to the initial discovery of the Osiris gene family. Further tests attempted to discover which actual genes within the Osiris gene family were responsible for tracheal development and maintenance in order to further the understanding the of the triple lethal focus and the role of each of those genes. Using a system called UAS-gal4, which will be later described within the paper, we were able to determine exactly which genes were expressed within the trachea.





The use of the gal4 system allowed for the determination that exactly seven of the twenty-four genes available within the Osiris gene family were expressed within the Drosophila trachea. Using fluorescence in situ hybridization, also known as FISH, we were able to detect if each Osiris gene was present by attaching fluorescent molecules to a small amount of DNA specific to each gene and observing under a microscope. The image in the left is the UAS-gal4 system that shows where the tracheal tubes are by using a trachea marker within the system. The image on the right which expresses information in red is actually the location of specific mRNA to different Osi gene family members. The close analysis of the information allowed us to determine that Osi gene members nine, fifteen, seventeen, eighteen, nineteen, and twenty were all expressed within the trachea because we can compare the location of the red fluorescently tagged mRNA to the GFP tagged luminal protein on the right that shows where the trachea is. This also means that seventeen out of the twenty four Osiris genes are not obviously expressed within the trachea.

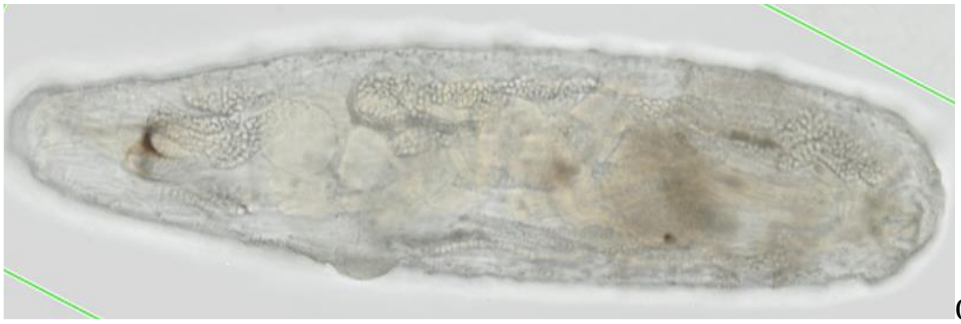
With the introduction of the Osiris gene family, the experiment can now be set up in order to determine which exact genes within the family are associated with what sort of defects in tracheal development. Using the gene editing tool CRISPR-Cas9, loss of function mutants were generated in order to study their phenotypic defects. The first is Osi 54 double mutant, which deletes the function of Osi 15 and Osi 19 genes. The second is the Osi 32 triple mutant consisting of loss of function in Osi 9, Osi 15, and Osi 19. The goal was to then analyze the phenotypic effects associated with air filling of the tracheal tubes compared to the wild type phenotypes.

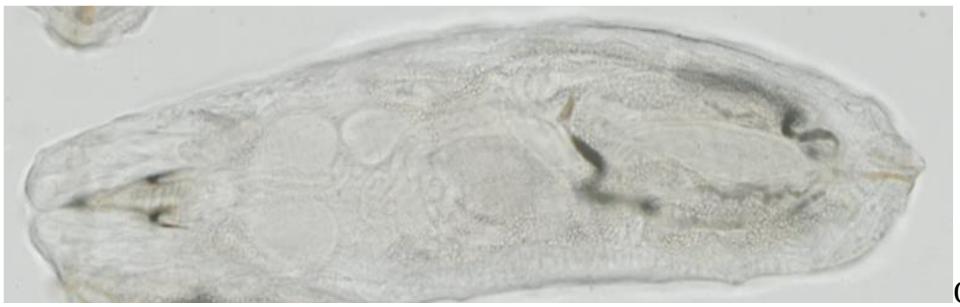
Phenotypic Analysis

After the use of the light microscope to obtain high quality images of Drosophila larvae with the double mutant and triple mutant, comparisons made with the wild type showed a massive difference in the phenotypic presence of air tube filling. Shown in the images below, the wild-type control has obvious air tube filling, evidenced by the black lines that represent the tracheal tube complex throughout the organism. The air filled trachea tube shows dark color and no-air filled tube shows white. We can also use immunohistochemistry to reveal protein localization (apical lumen, cuticle) in wild type and mutant Osi embryos.

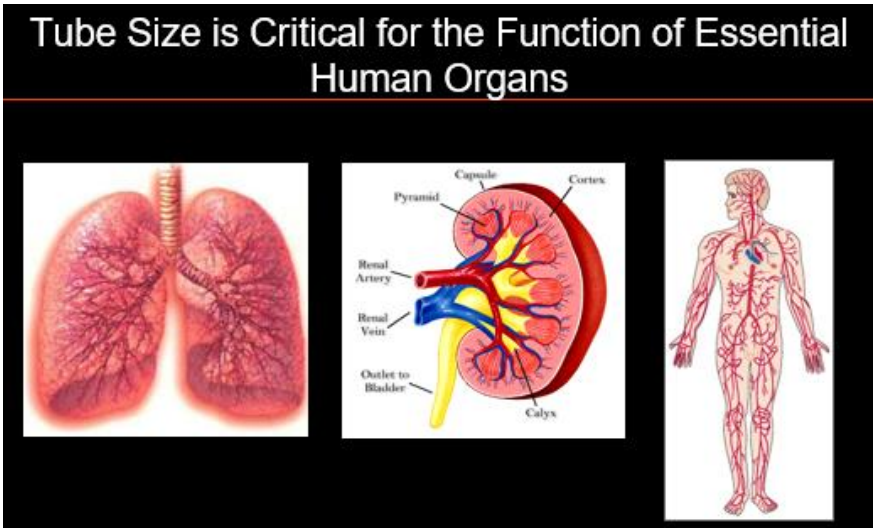
This means that luminal clearing and endocytic activity was successful allowing for air filling to occur through the apical membrane. However, in both Osi 54 double mutant and Osi 32 triple mutant, phenotypic differences can be observed that show that no air filling occurs within the tracheal system. Because of this, the knockout combinations of specific Osiris family genes is detrimental to the luminal clearing and subsequently air filling in later stage developments in the tracheal system.


Wild type control


Osi 54 double mutant

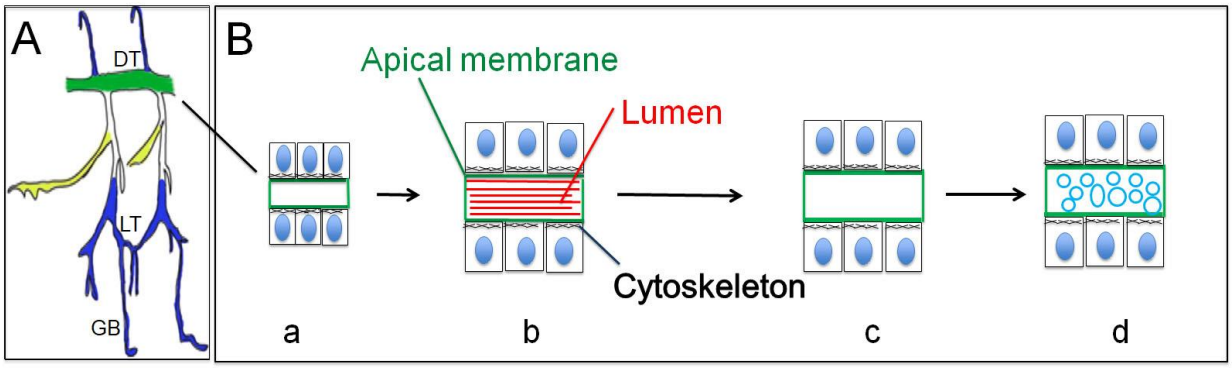

Osi 32 triple mutant

Many major organs are composed of tubes including the lung, kidney, and mass vasculature. Because of this, defects in tube morphogenesis plays a very important role in the longevity and health of an organism. Air filling is critical for the function of human lung. Defective air filling leads to newborn respiratory distress syndrome (NRDS) among many other conditions. Clearance within tubular formations are just as important and can play a role in the initial development of the tracheal systems as well. Some diseases associated with clearance defects are polycystic kidney disease and atherosclerotic heart disease.



Tube Morphogenesis

In order to understand the phenotypic differences associated with the knockout mutant combinations, a closer look is necessary to see where tube morphogenesis development went wrong. There are many stages of tube morphogenesis in this model Drosophila organism that could have caused the lack of air filling because air filling is one of the final developmental stages in tube morphogenesis.

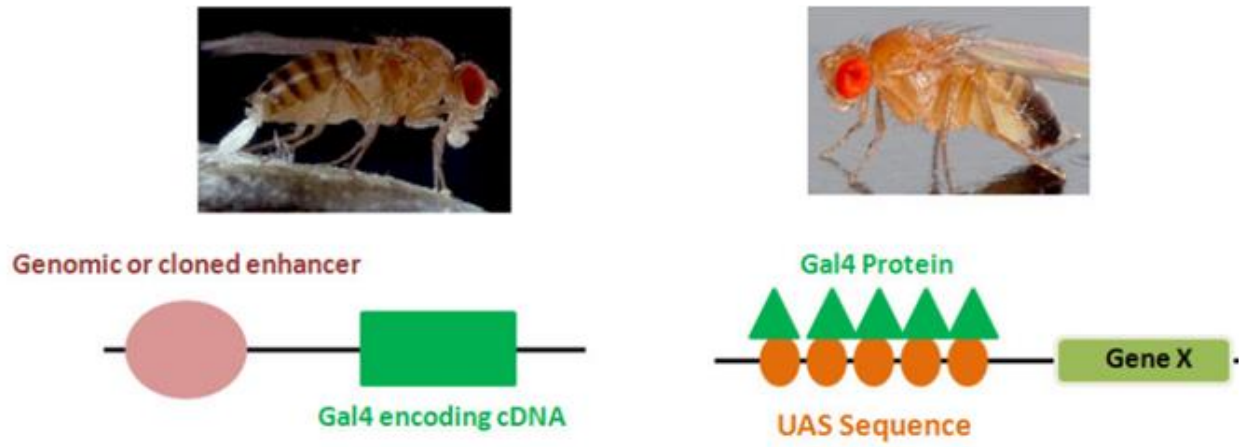


Using this image here, we can group the general development of tube morphogenesis into a few distinct categories. As tube morphogenesis is occurring and the tubes are elongating, tube dilation is occurring at the same time. This is the process that is depicted above that demonstrates the steps taken in order for full dilation and development to finalize. First, we have in image a an early stage cross section and the lumen is clear of protein or other extracellular matrix material. However, in image b, we have cytoskeleton and luminal protein secreted into the lumen to exert a pressure on the epithelial cells pushing outward to initiate tube dilation. This slowly forces the tube to become wider until signaled to stop. In image c, endocytic process are initiated in order to clear the lumen completely of any protein or matrix material that was used to dilate the tube. So, using this information we are able to design an experiment that will be able to tell us where in the process of tube morphogenesis something went wrong, and a defect is occurring. One

method that initially stood out was that of antibody staining. Antibody staining proved to be very difficult, however, because of the shell of the larvae that we were working with in the lab. Proving to break the shell while also maintaining integrity of the components within the larvae and also the luminal matrix of the non-cleared tubes during morphogenesis was too difficult of a task to be done efficiently and replicable to provide clear and accurate results. So, a method using the UAS-gal4 system to drive GFP tagged luminal protein Serp in trachea to allow for imaging proved to be a much more viable method to accurately examine results and phenotypic differences between wild type and mutant combination strains.

UAS-Gal4 System

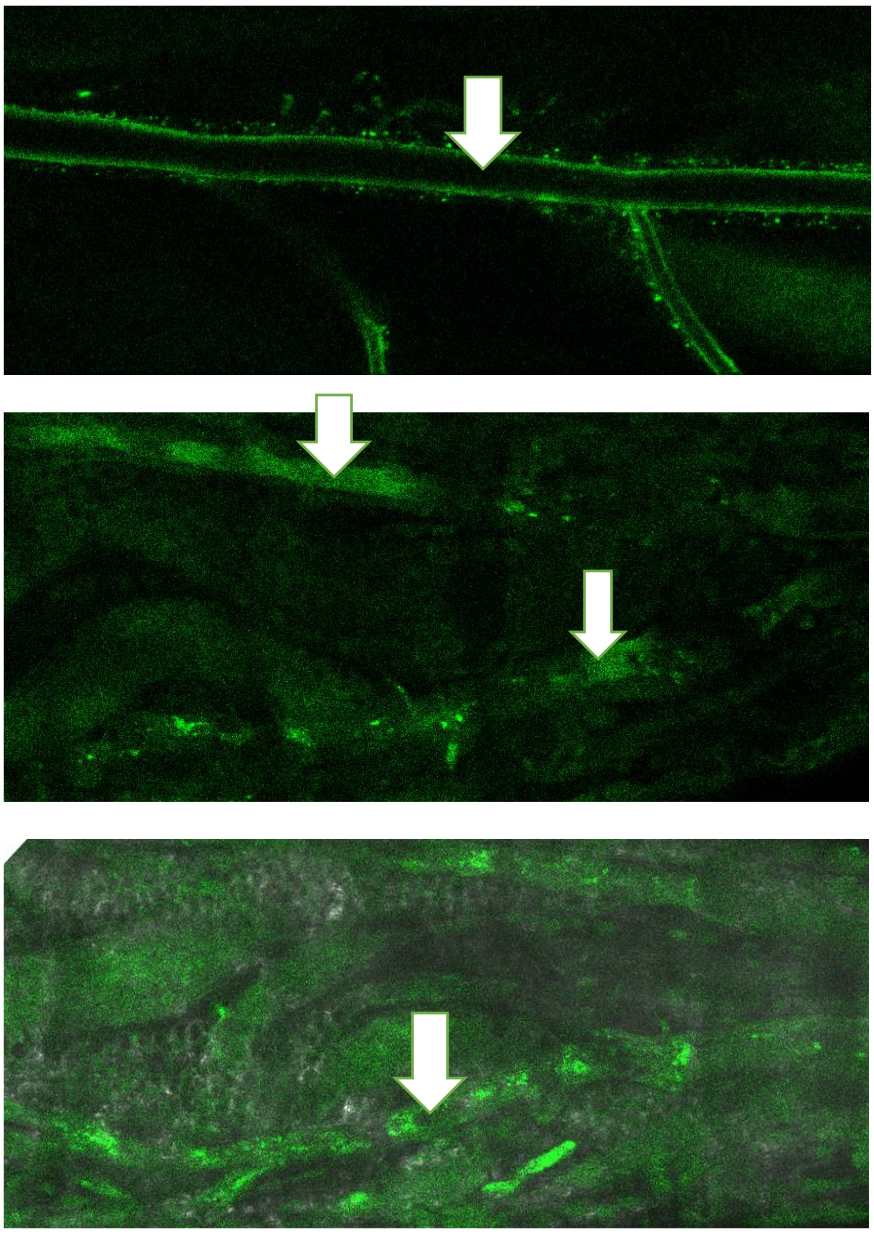
The process of implementing the UAS-gal4 system involves the use of two different flies, one male and one female. In the female fly, a btl promoter is used to enhance the expression of gal4, a gene that is a transcription factor that is only present within the tracheal system of the Drosophila model organism. In the male fly, upstream activating sequence, or UAS, is used to drive expression of a GFP tagged luminal protein called Serp. When these two flies are crossed, UAS enhancer will bind to the gal4 transcription factor, which will subsequently drive expression of the GFP tagged Serp gene which encodes the luminal protein Serp. The key factor in this process is that because the gal4 transcription factor is only found in the trachea, the GFP tagged Serp luminal protein will also only be expressed within the trachea. This allows for images to be taken in order to examine phenotypic differences between wild type and mutant combination strains of the Osiris genes.



This image here is a quick depiction of the process that allows for the UAS system to drive expression of a gene of interest in order to examine the effects of the Osiris gene on tube morphogenesis.

Fluorescence Analysis

After images were taken specifically looking for the fluorescence of the GFP-tagged Serp, phenotypic analysis was completed. The first image is that of the wild type strain, where you can clearly see distinct tube formation as well as full luminal protein clearance to allow for effective air filling in order to finalize the process of fully mature tube formation. However, in both the Osi 54 double mutant in the second image and Osi 32 triple mutant in the third image, you can see obvious lack of clearing of luminal protein Serp. This is a very big and important step to understand so that the Osiris gene family can be comprehended in its function and importance in Drosophila for tube morphogenesis.



The lack of luminal protein clearance from the mutated Osiris gene combinations show that the lack of air filling seen in the phenotypic analysis comparing the wild type to the mutant combinations is due to lack of endocytic processes that clear the luminal protein through the apical membrane to allow for air filling to occur. Without the clearance of luminal protein and matrix, air filling cannot occur and therefore the fully mature final product of tube morphogenesis cannot occur.

Methodology

The main way we do this is by developing a system to continue to provide environments for the flies to mutate so we can observe the effects of specific genes on the effects of tracheal tube development. Using CRISPR Cas9, mutations of the specific targeted Osiris genes specific combinations can be created that we are looking at, specifically the Osi 54 double mutant and the Osi 32 triple mutant. CRISPR Cas9 is a genome editing tool used massively around the world today. It stands for clustered regularly interspaced short palindromic repeats and the CRISPR-associated protein 9. It can be used by attaching a small piece of RNA to a specific target sequence of a target gene of DNA within a genome of an organism. It will also bind to the Cas9 enzyme, which cuts the DNA at the targeted location. Using the cell's own DNA modification systems, we can modify the genetic material in order to measure or research that specific sequence of DNA. This is how the specific mutant combinations of the Osiris gene family were created in order to study their phenotypic effects.

Secondary antibody staining was a technique that was looked at as a potential option to analyze the phenotypic differences between wild type and mutant Osi gene combinations because of its efficiency in immunolabeling, which would allow for the detection of specific enzymes, proteins, and any other molecule that we could potentially look for to determine whether or not luminal clearance is occurring and to see what is or is not clearing if there does happen to be a defect during tube morphogenesis.

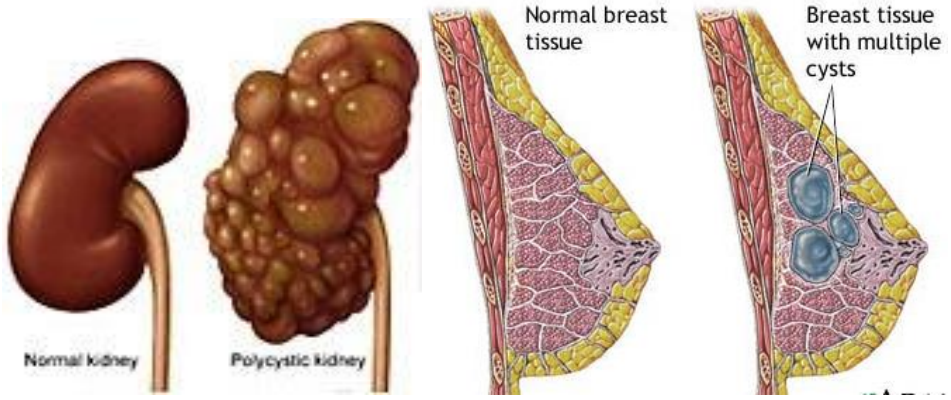
The technique FISH, also known as fluorescence in situ hybridization, was a technique used earlier in our research to determine which Osiris genes were involved within the trachea of Drosophila. This allowed us to target specific genes for knockout mutant variants to study their effects on the tube morphogenesis within the trachea. FISH is a laboratory technique that works

also by using fluorescent dye techniques. Fluorescent dye is attached to DNA, and the DNA is integrated with the original genome of the organism. The fluorescent DNA finds its match within the genome and sticks to it there. This allows for location determination of specific DNA strands used as probes.

In the lab, we develop vials of food for the flies, and once they develop within the food, we extract the embryos and employ a process called embryo fixation to trap the embryo's and isolate them so that we can observe them as they develop. This allows us to see the differences in tracheal tube development and air filling so we can determine how genes directly affect development and also how these genes interact with each other to determine the final product. We can use a compound microscope for visualization of air filling in the trachea in wild type and mutant Osi embryos. The UAS-gal4 system is a system specifically used for gene expression within the model organism Drosophila. It works very well because the UAS enhancer is actually specific towards the gal4 protein, which is what makes the system viable for use to drive a gene of choice. One major advantage of this system is that effects of different genes can be observed during overexpression and under or misexpression due to different amounts of actual promoter available to promote the proliferation of the genes products.

Our research within the lab to closely examine tubular defects in morphogenesis for this model organism can slowly help us reach a deeper understanding of the mechanisms of mammalian tube morphogenesis and therefore create more efficient and effective treatments for tube-related diseases and organs. For example, tube size is extremely important for correct functionality of multiple human organs that include the lungs, kidneys, and all blood vessels that emerge from birth throughout the rest of one's life. Defects in the tube size can lead to human diseases that are difficult to treat and so an insight as to the mechanism behind the disease will

immensely push forward progress to a sustainable treatment option for those affected. A few examples of diseases that are associated with tubular size and function are polycystic kidney disease, fibrocystic breast disease, pancreatic cystic neoplasm, and bile duct cysts.



While the Osiris gene family may not have a current known analog in mammals, the research now provides the insight needed to move forward and to develop the understanding that could guide future research in the right direction to make headway into the understanding of the human tube morphogenesis and its differences from Drosophila tube morphogenesis.

So in conclusion, we discovered that out of the seven of twenty four genes within the Osiris gene family are associated within the trachea of the model organism Drosophila and that by using specific techniques to knockout specific genes, we were able to learn that a lack of functional genes causes no air filling during tube dilation of tube morphogenesis. Using the UAS-gal4 system, we were able to determine where in the process of tube morphogenesis a defect occurs in order to better understand why that Osi genes are important and vital to a fully functional tracheal tube formation. We found that the flaw that occurs with the mutated combinations of Osi genes 54 and 32 is during endocytic processes whose job is to fully clear the lumen through the apical membrane after tube dilation for the last few steps of a fully developing tube to occur and mature. From this, we can infer that because the lack of luminal protein

clearance air filling cannot and will not occur, causing slow oxygen deprivation to different parts of the Drosophila organism and eventually causing death.

Future studies on this topic of Osiris genes and tube morphogenesis can look into how exactly this lack of luminal clearing is caused. Many cases could be made as to what exactly might be the culprit causing the defect but looking closer into exact processes and signaling that maintains and generates the endocytic effects and then observing effects of more variations of knockout genes and combinations of Osiris knockout genes can maybe shed light onto the exact processes occurring and why or why not certain activities are taking place that are critical for proper maturity and development. Being able to identify events that are downstream of one another can reveal the correct order of events that must occur for the next to take place which can also shed light into what exactly goes wrong with the defects seen in our research. Investigation into branch-specific pathways as well as the response to lack of oxygen in terms of signaling would also be relevant to understand why tube morphogenesis cannot recover from the defects caused by the lack of functional Osiris mutant combinations. More research could also look into the potential roles of the other genes within the Osiris gene family. Just because they did not show up as expressed within the trachea does not mean they do not play some sort of miniscule role in tube morphogenesis that was not observed within our research. And finally, far in the future engineering of tracheal cells could become viable as we as humans learn more and understand more of the mechanisms and signaling that all comes together to form the fully functional trachea following tube morphogenesis.

Special thank you to Dr. Jiang for bringing me into the lab to meet and work with the team and for helping me through the process of piecing together this project. Thank you to Aaron Scholl, Istri Ndoja, Clark Brady, and Doria Morante who all helped tremendously with the

workload in the lab and piecing together the information discovered to advocate the conclusions derived for this project.

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Branching Morphogenesis of the *Drosophila* Tracheal System

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