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LGD-1 regulation of ESCRT-III during multivesicular endosome biogenesis

By

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Chapter 3. ESCRTs, LGD-1, and worms: exploring basic cell biology through biochemistry and *Caenorhabditis elegans*

Introduction

Why I wrote this chapter

Defining themes I have maintained throughout my scientific training are science communication, science literacy, and community building. These themes stem from my experiences with mentors and from the missions of programs which have supported my journey. As a former low-income student and the first in my family to earn bachelor's and doctoral degrees, I have seen firsthand the need for strong science education and critical thinking. I would not be writing this dissertation without support from programs throughout my education aimed at helping disadvantaged students succeed—from the Early College program in high school, to the McNair Scholars program during my undergraduate years, and finally the National Institutes of Health and National Science Foundation training grants that supported me while completing this work. The missions of these programs, which center around increasing diversity in professional settings, have been extremely influential to me as I have progressed in my career and have instilled a deep commitment to promoting a diverse, inclusive, and equitable environment wherever I go. Finally, as a future science educator, I believe it is important to always strive to make information accessible through effective communication. To me, this must start with my own work. To this end, I partnered with staff at the Wisconsin Initiative for Science Literacy (WISL) to write this chapter of my thesis to describe my scientific journey, experience in graduate school, and my thesis work for a non-scientist audience.

My journey into science

I had my first taste of scientific research in high school, when I started working in the Kestler lab at Lorain County Community College. I was attending a dual high school and community college program when my freshman biology teacher offered me the opportunity to work with Dr. Harry Kestler studying HIV after school with a small group of students. We worked collaboratively in groups of high school and traditional community college students, studying mutations in one of the receptors that HIV uses to infect cells. Dr. Kestler taught us how to do experiments and read research articles, but more importantly he instilled a deep appreciation for science communication and the value of using humor to connect with people. I could fill an entire chapter on its own about my time in the Kestler lab. I still think of Dr. Kestler as my science dad, and I am forever grateful that he inspired me to 'persist like a lentivirus'.

As an undergraduate, I originally intended to continue on the path I had started studying viruses by majoring in microbiology. However, after taking Dr. Sina Ghaemmaghami's honors biochemistry class, I became enamored with proteins. Proteins are the building blocks of biology; they make the structure of cells, transform nutrients into energy, protect and replicate DNA, among other essential roles. While DNA and RNA are the instructions, proteins are what those instructions build and they perform nearly all tasks cells and viruses need in order to survive and thrive. I was especially captivated by the elegance of the relationship between protein structure and function. In biochemistry, we can use various tools to map protein structure, and that visualization can reveal an amazing amount of information about how a protein works and interacts with other biological components.

Although I did not end up directly studying protein structure, I did declare my major in biochemistry and worked in Dr. Ghaemmaghami's lab throughout college. In the Ghaemmaghami lab, instead of infectious viruses I studied infectious proteins, called prions. Prion proteins are somewhat unique in that they can change their structure dramatically, called misfolding, in a way that induces other prion proteins to misfold as well. These misfolded proteins stick together to form large aggregates which are often associated with cell death. In humans and many other mammals including cows, sheep, and deer, misfolded prion proteins cause severe neurodegenerative disease. One of my projects in the Ghaemmaghami lab centered on exploring how different pathways for breaking down proteins might impact misfolded aggregate formation.

During one summer in college, I participated in a summer research program at the University of Wisconsin in Madison, where I worked in Dr. Tom Martin's lab. In the Martin lab, my project was less focused on disease than on answering questions about basic cell biology. Namely, I studied how cellular compartments called endosomes are made and how they are moved along with their protein cargoes both inside and outside of the cell. This work combined with my growing interest in the endosome-mediated processes that break down proteins from the Ghaemmaghami lab. Ultimately, these experiences gave me a strong foundation in biochemistry and cellular biology, from which I launched into my thesis work in the Audhya lab.

My thesis

Membranes – the unsung heroes of cell biology

At its most basic level, a cell is a compartment enclosed by a membrane. Within this larger membrane compartment, there are many sub compartments, all with their own specialized roles and with membranes of their own. These sub compartments are organelles, and their specialized roles include essential tasks such as transporting proteins (endosomes), generating energy (mitochondria), protecting DNA (nucleus), and breaking down cellular materials to be recycled (lysosome), among others (Fig. 3.1). For a cell and its organelles to function, it is critical that their membranes are maintained, because they create an inside environment separate from the outside environment. Thus, membranes define an organelle's identity as a distinct compartment. As an example, if the nucleus was no longer closed, DNA could be damaged by cellular components normally kept out of the nucleus, resulting in mutations and possibly cell death.

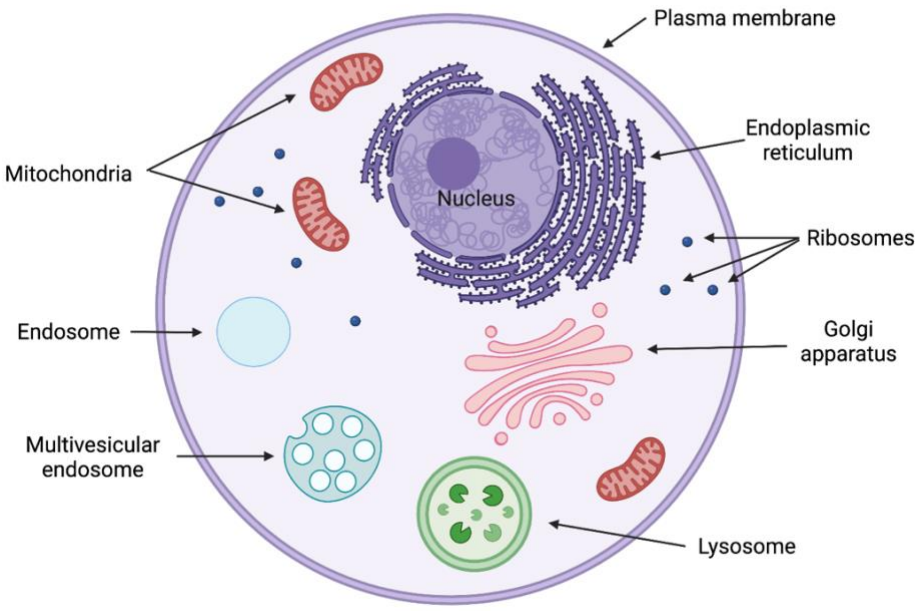

Figure 3.1 Diagram of an animal cell. Cells contain many different types of membrane-bound organelles and other structures which work together to carry out processes necessary for cell survival including energy production (mitochondria), protein transport (endosomes and Golgi apparatus), protein synthesis (endoplasmic reticulum and ribosomes), and protein degradation (lysosomes).

When looking at a diagram of a cell and its organelles, it's easy to assume that scientists for the most part have basic cell biology all worked out. However, these diagrams are an oversimplification. This is in part due to the need to reduce the cell's vast complexity down to an easily representable and digestible cartoon, but also because scientists don't have all the fine details worked out. While it is true that we have a very good general understanding of how the cell works, many gaps in the depth of our knowledge remain. For example, although we know a lot about the proteins that remodel or restructure cell membranes, how those proteins 'know' to go to specific locations in the cell at specific times or how they perform the final steps of membrane remodeling remain unclear.

That we can know so much about basic cell biology without fully understanding fundamental aspects of how cellular membranes are remodeled and maintained is one of the main ideas that initially drew me into the Audhya lab and the world of ESCRT biology.

ESCRTs & LGD-1:

As discussed above, membranes are critical for a cell to maintain its identity, carry out essential functions like metabolism and signaling, and to protect genetic material from damage that could lead to cancerous growth or cell death. My thesis work broadly aims to understand how cellular membranes are remodeled and maintained by a series of protein complexes called the Endosomal Sorting Complexes Required for Transport (ESCRT, pronounced ‘escort’) machinery. The ESCRT machinery is composed of five protein complexes, called ESCRT-0, -I, -II, -III, and VPS4. ESCRT-0, -I, and -II are considered early acting ESCRTs, which are responsible for recruiting ESCRT-III, among other functions. ESCRT-III and VPS4 can be conceptualized as the ‘business end’ of the ESCRT machinery because together they perform the main membrane remodeling steps. The ESCRT-III complex is a spiral filament made up of up to 8 different protein subunits, which assemble on membranes (Figure 3.2). VPS4 uses cellular energy to remodel ESCRT-III filaments in order to constrict membranes. Ultimately, this process leads to membrane separation to either generate a budded vesicle (a small membrane-bound sac) or to seal a membrane bilayer (Figure 3.2).

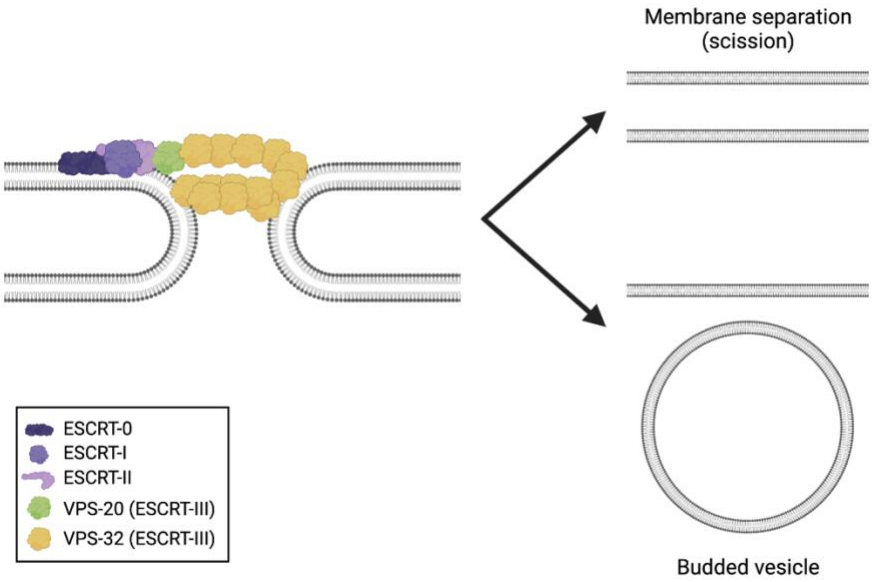

Figure 3.2 ESCRT-mediated membrane remodeling. Early acting ESCRT complexes (purple) recruit ESCRT-III component VPS-20, which recruits VPS-32, the main component of the ESCRT-III spiral filament. The result of ESCRT-mediated constriction of membranes can either be membrane separation (as in nuclear membrane sealing) or a budded vesicle (as in multivesicular endosome formation).

As their name implies, ESCRT proteins were originally discovered and studied for their role in transporting proteins through endosomes, which transport proteins along the endolysosomal pathway (Figure 3.3). In the endolysosomal pathway, membrane proteins are first transported from the plasma membrane to early endosomes, then ESCRT machinery remodels the early endosomal membrane to bud vesicles inside of the endosome to generate an endosome with multiple vesicles inside, also referred to as a multivesicular endosome. Finally, multivesicular endosomes can fuse to lysosomes so that their contents can be broken down and recycled by the cell.

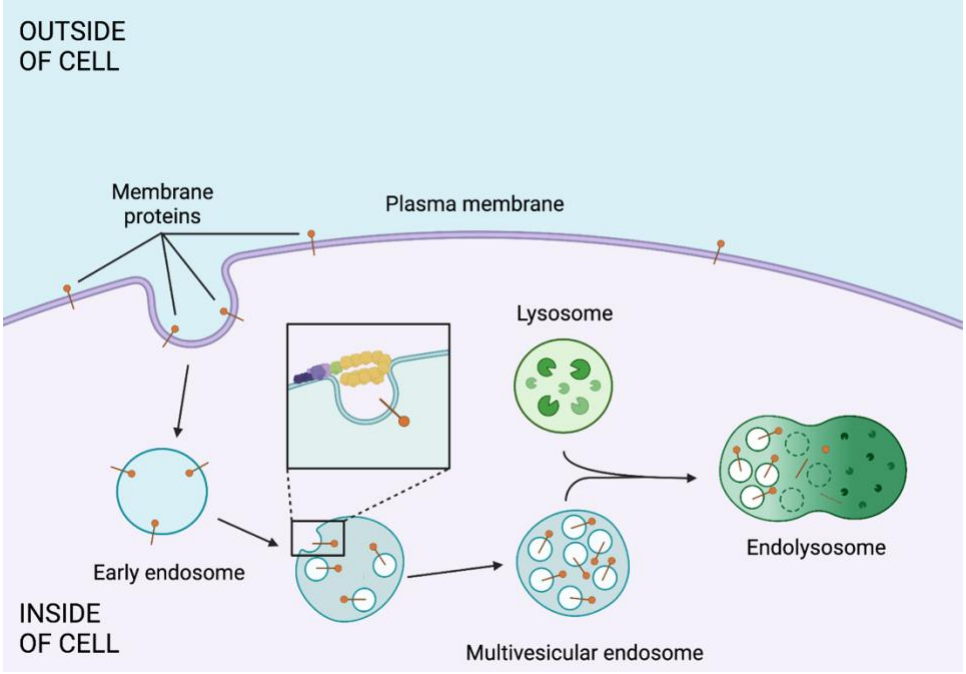

Figure 3.3 The endolysosomal pathway. Membrane proteins in the plasma membrane such as growth receptors are brought into the cell on an early endosome, which matures through ESCRT-mediated remodeling to become a multivesicular endosome. Internalized membrane proteins are budded inside of the endosome on vesicles. Ultimately, multivesicular endosomes can fuse with lysosomes to degrade their contents and recycle cellular nutrients.

The ESCRT machinery is also involved in many other membrane remodeling and repairing processes throughout the cell, including repair of the outer cell membrane (plasma membrane), lysosome, and nucleus. To underline their importance, ESCRT proteins are highly evolutionarily conserved, meaning they are found across many species of organisms, including mammals and other vertebrates, plants, fungi, and other unicellular organisms. For many reasons, ESCRTs have been historically hard to study and therefore the finer details about how they function and how they are regulated have remained elusive.

My thesis work aims to address the question of how ESCRT machinery is targeted to the various membranes it must remodel or repair at the appropriate time.

Membranes within a cell such as the nucleus, endosome, and plasma membrane have similar but distinct compositions, and precise timing of ESCRT recruitment and action is critical to proper remodeling or repair. Specifically, my project explores how ESCRT machinery is regulated by another protein, called LGD-1, using protein biochemistry and the worm model *Caenorhabditis elegans*.

LGD-1 is a member of the Lethal giant discs/Coiled-coil Domain 1 (Lgd/CC2D1) family of proteins found in *C. elegans*. The Lgd/CC2D1 protein family includes Lgd in fruit flies, LGD-1 in worms, and CC2D1A and CC2D1B in humans and other mammals. In fruit flies, mutations in Lgd resulted in developmental defects in the tissue destined to become a fly wing, called wing imaginal discs, hence the name 'lethal giant discs', and CC2D1A/B were named for their predicted protein structure. Early work on Lgd/CC2D1 proteins demonstrated their importance in regulating ESCRT-mediated membrane remodeling specifically along the endolysosomal pathway, although exactly how remained unclear. Lgd/CC2D1 proteins are known to prevent the assembly of the major ESCRT complex, ESCRT-III, through interaction with ESCRT-III component VPS-32/ CHMP4. However, these experiments were carried out with purified proteins outside of the cell, without the presence of membranes or other biologically relevant factors.

C. elegans as an ideal model:

While artificial experimental environments are often crucial to gain a basic understanding of protein function, it remains essential to build upon knowledge gained from such experiments in a biological context. For my work, I chose the nematode worm model *C. elegans. C. elegans* are very small (~1mm in length as adults) transparent

worms that eat bacteria and other microbes in soil and rotting plant matter and are found in many parts of the world. In the lab, worms are grown on agarose plates seeded with *Escherichia coli* bacteria (Figure 3.4). The *C. elegans* lifecycle is relatively short, with a maturation time of 3-4 days. From a general scientific perspective, *C. elegans* are an ideal model for several reasons. First, many techniques are available for genetically modifying worms, and mutant worms are easily maintained. *C. elegans* primarily exist as hermaphrodites, meaning that an adult worm contains both oocytes (eggs) and sperm. This allows for easy maintenance of mutant strains. A small population of male worms also exist, making genetic crosses possible to combine mutations. Second, *C. elegans* transparent body make them practical for imaging experiments, and their regular lifespan and cell division allow for precise timing of cellular events. There are also several reasons *C. elegans* are ideal for my project specifically. *C. elegans* possess many proteins similar to humans. In the case of the ESCRT machinery and Lgd/CC2D1 proteins, worms have single copies of each of the ESCRT protein, and one Lgd/CC2D1 protein, LGD-1. Compared to humans, which possess multiple copies of several ESCRT components and two Lgd/CC2D1 proteins, worms are a simplified system. Additionally, worms naturally undergo a programmed wave of trafficking through the endosomes in the one-cell embryo just after fertilization. This is unlike other popular models such as growing human cells in a petri dish (cell culture) which rely on non-physiological treatments like growth factor stimulation to study ESCRT function in the endolysosomal pathway.

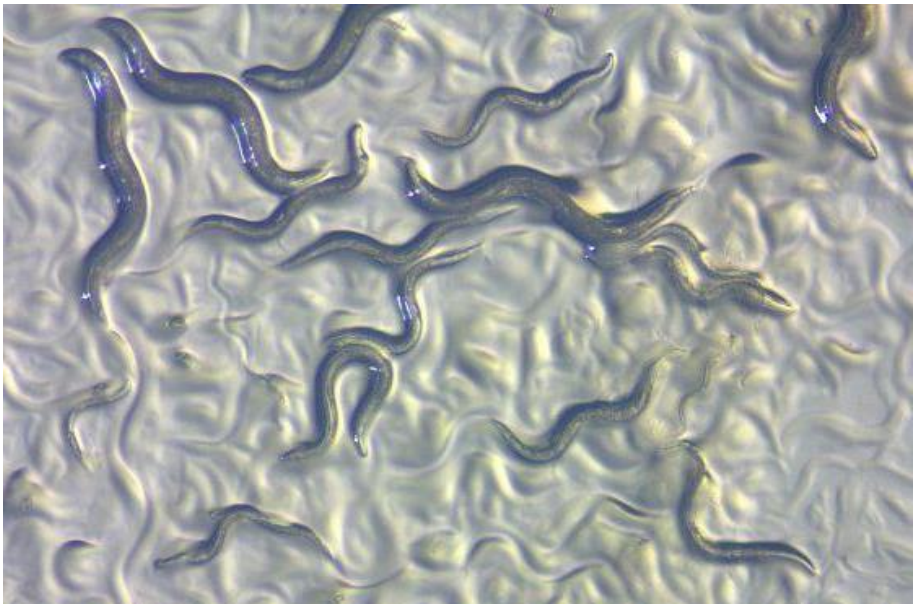

Figure 3.4 *Caenorhabditis elegans* growing on a bacterial plate. Image credit: Carl Zeiss Microscopy, https://bit.ly/3B2FHeq

LGD-1 stabilizes ESCRT-III assembly at the endosome:

Though much of my work was performed in *C. elegans*, it was also important to gain a biochemical understanding of LGD-1 and its interactions with components of the ESCRT machinery. To do this, I purified LGD-1 and ESCRT proteins from bacteria engineered to produce these proteins in large quantities. I used these purified proteins to carry out experiments to test whether LGD-1 could physically interact with subunits of the ESCRT-III complex. I found that out of 7 tested ESCRT-III proteins, LGD-1 only interacts with the major ESCRT-III subunit, VPS-32, and an another ESCRT-III protein, CHMP-7. This agrees with previous studies of fruit fly and mammalian proteins. I also demonstrated LGD-1 can bind to membranes directly, without ESCRT proteins. These results suggested LGD-1 might play a specific role in VPS-32 recruitment or function on membranes.

In parallel to biochemical studies, I also generated several mutant worm strains to study LGD-1 in live *C. elegans* embryos. These included a strain expressing LGD-1 fused to green fluorescent protein and a strain containing a large deletion mutation, resulting in complete loss of LGD-1 protein. I also combined these strains with others available in the lab through genetic crosses to add other fluorescent markers or mutations. Through microscopy experiments using these strains, I found that LGD-1 is present on endosomes during the programmed endolysosomal trafficking occurring in single-cell embryos, similar to ESCRT components. Somewhat surprisingly, I found that the presence of LGD-1 on endosomes is at least in part dependent on early acting ESCRT complexes (ESCRT-0 & ESCRT-I), but not on VPS-32, even though we know LGD-1 directly interacts with VPS-32.On the other hand, loss of LGD-1 results in swollen endosomes containing very few internal vesicles, indicative of ESCRT dysfunction, but not total loss of function (Figure 3.5). I observed VPS-32 did not build up on endosomes in the absence of LGD-1, unlike other ESCRT components, providing an explanation for the abnormal endosome structure. Taken together, my results suggest LGD-1 recruits VPS-32 to the endosome, and potentially stabilizes ESCRT-III as it assembles on the endosomal membrane (Figure 3.5).

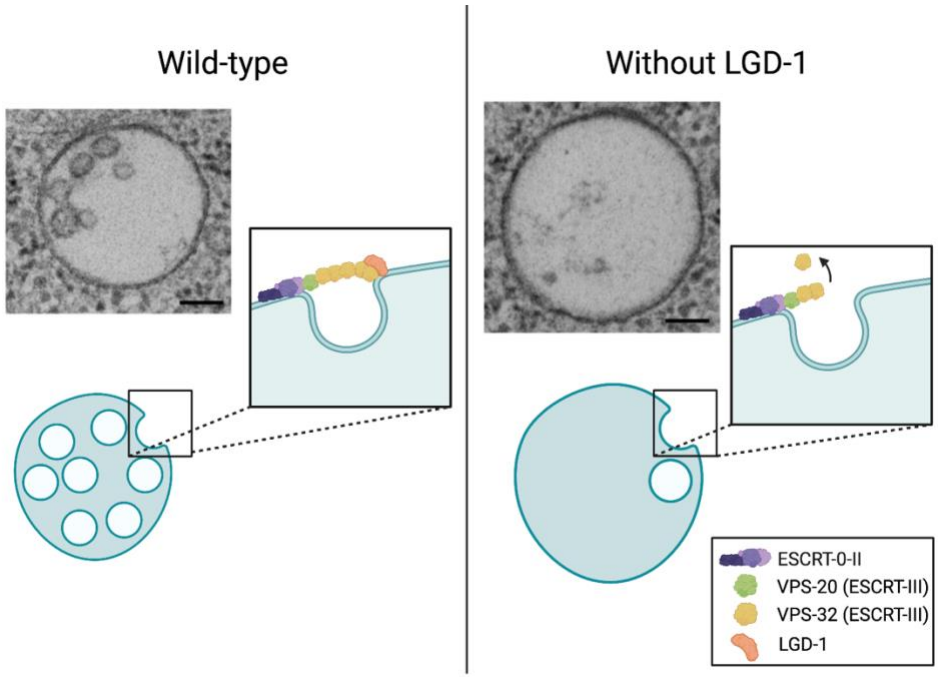

Figure 3.5 Loss of LGD-1 impacts multivesicular endosome formation by destabilizing VPS-32 in ESCRT-III assembly. Electron microscopy images of multivesicular endosomes from either wild-type (control) or LGD-1 loss of function mutant worms. Scale bar = 100 microns. Also shown are cartoon schematics of what I hypothesize is happening at the protein level.

Through my investigation of LGD-1, I have characterized LGD-1 and its interaction with components of the ESCRT machinery biochemically, described the cellular distribution of LGD-1 in the *C. elegans* embryo, and characterized how loss of LGD-1 affects ESCRT function at the endosome. Based on my findings, I have developed a model of LGD-1 regulation of ESCRT assembly where LGD-1 is required for recruiting a critical ESCRT component, VPS-32, and stabilizing ESCRT assembly at the endosome for efficient membrane remodeling (Figure 3.5). My work is the first study of LGD-1's role in ESCRT function using the *C. elegans* model, which is an ideal system to study naturally occurring ESCRT-mediated membrane remodeling. While my thesis focused on ESCRT function at the endosome, because of its interaction with the major

ESCRT-III subunit VPS-32, it is likely that LGD-1 plays a role in other ESCRT-mediated membrane remodeling events such as those occurring at the nucleus.

Reflections

The previous section is a neat summary which might lead you to believe that my thesis work was straightforward. However, I can assure you that much like the oversimplified cell diagram discussed earlier, the reality is much more complicated. Scientists often joke that around 90% of experiments fail, and prospective graduate students are often asked in interviews about their resilience for a reason.

During the first few years in the lab, I generated very little data. In fact, for the first few months after joining the lab, I worked on an entirely different project using traditional cell culture models. After making the switch to studying LGD-1 in worms, it took many months just to learn how to handle worms and the basic techniques to study them. For example, to move worms from one plate to another, or to use worms for experiments, you use a small piece of platinum wire with a scoop fashioned at the end to pick up worms while viewing them under a microscope. Moreover, it is often critical for experiments to pick single worms at specific lifecycle stages. Early on, it was difficult for me to gently pick up worms without squishing them or damaging the agarose plate and I spent hours a day practicing identifying specific larval stage worms or male worms.

Once I was comfortable with the practical skills needed to perform my experiments, I encountered another barrier to progress. Because Lgd/CC2D1 proteins have not been extensively studied by our lab or others, especially not LGD-1 in worms,

there was a lack of ready-made and validated molecular tools to study them. Without this experimental infrastructure, it was up to me to build the tools I needed to study LGD-1 myself. These tools included both LGD-1 worm strains mentioned previously, as well as an antibody against LGD-1 and many, many DNA constructs to make modified proteins for biochemical experiments. Some tools were relatively straightforward to make. Others, like the LGD-1 mutant worm strains and antibody, I struggled with for at least a year, with several mishaps along the way. For example, I nearly lost all of my LGD-1 antibody on my second purification attempt. Antibodies are typically generated by injecting a rabbit or other animal with your protein of interest and collecting the antibodies the animal produces in response from their serum. The process of purifying antibody from serum involves circulating the serum through tubing and over a column bound with the target protein of interest overnight and eluting the antibody off the column the next day. My first attempt to purify LGD-1 antibody from serum failed because I had not bound enough LGD-1 to the column, so very little antibody stuck to the column. The second attempt, I had included much more protein and was feeling confident until I came into the lab the following day to elute only to find the tubing had sprung a leak and my precious rabbit serum was drying in a pool on my bench. Luckily, I had reserved a small amount of serum in the freezer, and eventually was successful.

Along the way, and especially after my tools were built, I did make some exciting discoveries that bolstered my confidence and kept me going. My first major discovery was that LGD-1 and CHMP-7 directly interact using purified proteins. Prior to this experiment, no published data existed to suggest Lgd/CC2D1 proteins interacted with CHMP-7 in other organisms. I was so elated to have data, I texted pictures of the gel to

my partner and family. A few weeks later and the morning before I debuted this result in a lab meeting, Jon forwarded me a newly published paper showing this exact experiment using mammalian proteins, and I was scooped. Although it stung quite a bit, it was also comforting to see another group corroborate my result, and ultimately, CHMP-7 factored much less into my thesis than I originally expected.

A second breakthrough happened just before the COVID-19 pandemic closed the lab. I had successfully made a mutation in *lgd-1* in *C. elegans*, *lgd-1Δ*, but homozygous mutant worms (having two copies of the mutation) died at an early larval stage. I hypothesized a partial depletion of VPS-32 might at least partially rescue these worms. While testing this hypothesis, I accidentally used a different reagent by grabbing the wrong tube out of the freezer. This proved to be a happy accident because this reagent allowed *lgd-1Δ* homozygotes to grow into adult worms. Partial depletion of VPS-32, on the other hand, did not. Without this discovery, I would have been severely limited in my ability to study LGD-1 function without an adult mutant worm to study the consequences of losing LGD-1.

The *lgd-1Δ* worm made the third major success I want to highlight possible. As I was starting out in graduate school, I attended a presentation by a senior student in the Audhya lab. She showed electron microscopy (EM) images of endosomes and their internal vesicles, and I was immediately enamored. After I had joined the lab and needed to find a new project, the promise of doing my own EM experiments enticed me to make the switch to studying LGD-1 in worms. However, it was only years later once I had the *lgd-1Δ* strain, I finally had a reason to do EM for my project. It was so worth the

wait; the first time seeing the abnormal, swollen endosomes in *lgd-1Δ* embryos by EM was a dream come true and this data became the highlight of my publication.

Despite all the ups and downs throughout my graduate school journey, I am thankful for the opportunity to contribute my work to the scientific community and for everything I have learned along the way. Beyond all the science and research-related knowledge I have gained through graduate school, I also learned a lot about myself and what I want to do after graduation, which is to teach. When I was a teenager starting out in science, I was driven by a desire to make a positive impact on my community. At the time, I envisioned this taking place on a grand scale, by making scientific discoveries that helped fight human diseases through treatments and cures. Later, reflecting on my experience in research, I realized I could make a more immediate impact through science communication and education. I was also influenced by the COVID-19 pandemic, and how misinformation and lack of trust in scientists and health officials contributed to the enormous human cost of the pandemic, which is still ongoing today. I used my time out of the lab during pandemic shutdowns to begin training for a career in education through the Delta Program here at UW Madison. I finished earlier this year, completing a teaching certificate through the program. Shortly after submitting this dissertation, I will move into a year-long position as a Lecturer in the Department of Chemistry and Biochemistry at the University of Wisconsin – La Crosse, where I will teach a mix of biochemistry and chemistry lab courses and a biochemistry lecture. Following my year at UW La Crosse, I hope to find a tenure-track position at a primarily undergraduate institution or community college teaching biochemistry and molecular

biology. Although I will be leaving the bench behind for now as I transition into teaching, I will absolutely miss research and the sweetness of generating new data.