IMPACT OF THE GTP BINDING PROTEIN ARF-6 ON THE BIOGENESIS OF MULTIPLE EXTRACELLULAR VESICLE SUBPOPULATIONS IN C. ELEGANS

by

Katherine Mackenzie Wagner

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the Bachelor's Degree in Biological Sciences

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ABSTRACT

Extracellular vesicles (EVs) are nano-sized, membrane-bound vesicles that play crucial roles in intercellular communication, impacting both physiologic and pathophysiologic pathways. Use of the genetic model organism *C. elegans* allows us to study and track EVs and their cargoes in vivo, to observe EV biogenesis and shedding. *C. elegans* EVs contain various different cargoes, including the calcium homeostasis modulator CLHM-1 and the polycystin PKD-2 ion channels. These cargoes are found to be in two different EV subpopulations that bud from different locations on the cilia of male tail sensory neurons. The small GTPase ARF-6 has been suggested to participate in microvesicle shedding via the phospholipase D pathway of signal transduction based on in vitro studies. However, whether ARF-6 plays this role in the release of EVs from cilia in vivo is unknown. Here, we show, using fluorescent protein tagging and TIRF microscopy, that ARF-6 affects both CLHM-1 and PKD-2-containing EVs, indicating that this protein may act in male-tail sensory neurons to impact biogenesis of all EV subpopulations. Loss of ARF-6 results in an approximate 50% reduction in release of both EV populations, opening the door for several future hypotheses and lines experimentation using the *arf-6* mutant.

INTRODUCTION

Extracellular Vesicles

Extracellular vesicles (EVs) are membrane-enclosed molecules released from nearly all cells. These vesicles are responsible for intercellular transport of biological macromolecules such as RNA, DNA, and proteins. EVs fall into two primary subpopulations including exosomes and ectosomes (Figure 1). Exosomes, which are 30-150 nm in diameter, are formed from the fusion of multivesicular bodies with the plasma membrane, which releases the intraluminal vesicles into the extracellular space. Ectosomes, or microvesicles, are about 100-1000 nm in diameter and bud directly from the plasma membrane of a cell[1,2]. Microvesicles can also bud from the primary cilia, microtubule-based organelles that serve as hubs for intracellular signal transduction and transmission pathways[3]. EVs are heterogeneous; one cell can release several populations of EVs containing different cargoes with different crucial biological functions[4]. However, the mechanisms underlying the biogenesis of specific subpopulations are unknown.

EVs have been involved in the physiology and pathophysiology of different organs. EVs are known to participate in tumor formation, and likely play roles in cell-to-cell communication within the tumor microenvironment, tumor metastasis, and even drug resistance[5]. EVs were shown to contain misfolded proteins involved in several neurodegenerative diseases, such as Alzheimer's, Parkinson's disease, and amyotrophic lateral sclerosis, as well as autoimmune diseases like Systemic Lupus

Erythematosus and Multiple Sclerosis, along with multiple liver diseases[6-8]. EVs also participate in many physiological processes, such as adaptive and innate immunity, stem cell maintenance, bone calcification, embryogenesis, and liver homeostasis [9-11]. Additionally, EVs are involved in reproductive processes, such as gametogenesis, fertilization, and embryo implantation[12].

EVs are currently being considered as promising drug delivery vehicles due to their stability and ability to cross various biological barriers. However, EVs must be studied and tracked *in vivo*, due to their unique nature as transport and signaling molecules[13]. One common practice is when a known cargo is genetically engineered to be tagged with a fluorescent protein, which can be observed under a microscope. For this method of research, it is necessary to have a model organism that has short reproduction cycles with a high volume of progeny, easy maintenance, capacity to reproduce data, and the capacity to generate mutants[14]. Human subjects do not meet these requirements. Thus, a simpler model is needed for proper visualization and analysis of EVs.

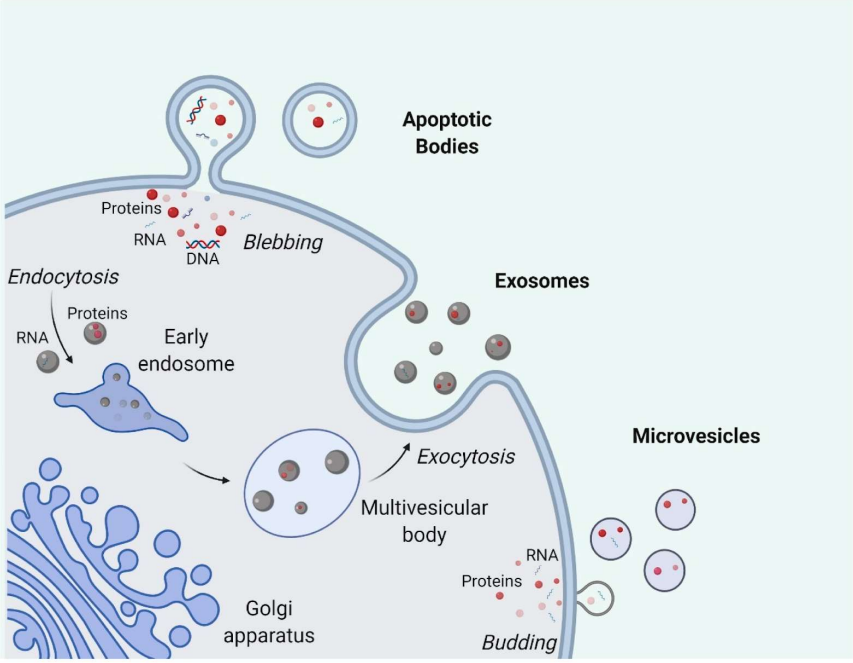

Figure 1: Biogenesis of extracellular vesicle (EV) subtypes, termed exosomes, microvesicles, and apoptotic bodies (Dang et al., 2020).

C. elegans

The nematode *Caenorhabditis elegans,* a multicellular, transparent, non-parasitic organism, is a widely used model organism in genetics research. Its 100Mb genome contains approximately 18,000 genes, with 38% being protein-coding genes sharing human homologs[16,17]. In the wild, *C. elegans* feed on bacteria. In the laboratory setting, *C. elegans* is grown on Nematode Growth Media (NGM) plates and given *Escherichia Coli* as food. By eliminating the variability of food sources*, C. elegans* maintains its vitality without sacrificing necessary controls. *C. elegans* are classified by two sexes: male and hermaphrodite. Males have a single X chromosome, while the hermaphrodites contain 2 X chromosomes. Males are only spontaneously generated in the wild due to rare X chromosome nondisjunction events during hermaphrodite reproduction[18]. Hermaphrodites are able to self-fertilize but will preferentially mate and reproduce with a male if one is present[19].

From hatching, *C. elegans* begins in larval stage 1 (L1), then progresses from L2, to L3, to L4, and finally to adulthood (Figure 2). In hermaphrodites, the transition from L4 to adulthood is marked by fertilization, either from a male or from self-generated sperm. Adult hermaphrodites then lay eggs and continue to live for about two weeks until their death. In the event of self-fertilization, about 300 progeny are produced, and in the event of male fertilization, about 1400 progeny are produced[19].

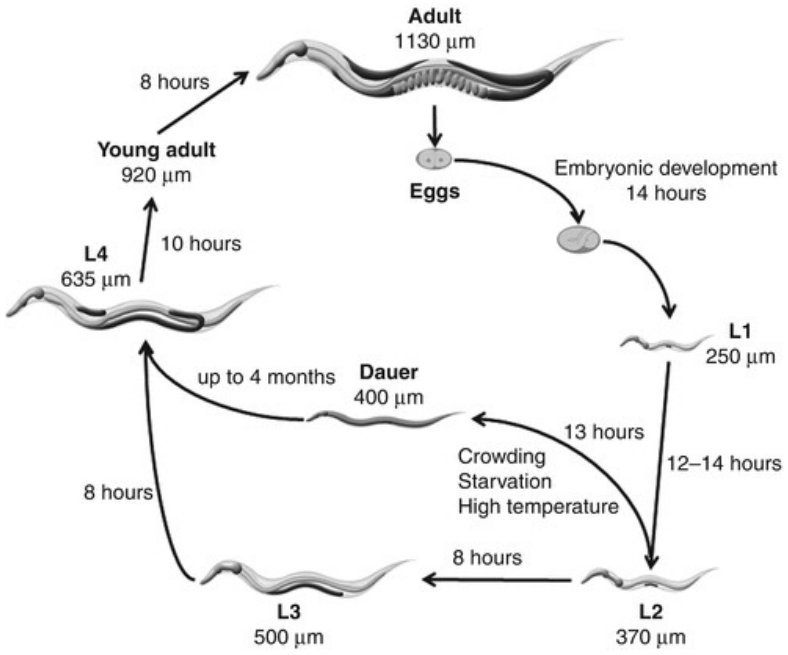

Figure 2: Representation of the life cycle of *C. elegans*. The different stages of growth: egg, L1, L2, L3, L4, and adult are shown (Vergara-Irigaray N. et al., 2012)

Extracellular Vesicles in *C. elegans*

In *C. elegans*, EVs are shed from select sensory neurons including the inner labial type 2 (IL2) and amphid sensory (AS) neurons. In males, they also shed from cephalic male (CEM), ray type B (RnB) and hook B (HOB) neurons[3]. Upon previous analysis of these EVs, it was discovered that the cilia from which they are derived lack the components necessary for exosome formation and are therefore likely microvesicles[3]. In the primary cilia of male-specific tail neurons, EVs accumulate in the lumen near the base of the cilia and then are released into the environment through an opening in the cuticle and enter the hermaphrodite uterus during mating (Figure 3). In males, the release of EVs from the tail neurons is correlated with male tail-chasing behavior,

enabling the male to contact and circle around the hermaphrodite body during mating. Certain cargoes have been identified as conserved in EVs released from male tail sensory neurons. Two known cargoes of EVs in *C. elegans* include the TRPP ion channel PKD-2, and the calcium homeostasis modulator ion channel CLHM-1. When these proteins are tagged with fluorescent proteins GFP and tdTomato, respectively, these cargos are shown to localize specifically in two distinct vesicle subpopulations[23]. These two populations are formed from two different sites, with PKD-2 containing EVs coming from the distal tip of the cilia and CLHM-1 EVs derived from a secondary site within the cilium. However, this does not mean that the subpopulations have distinct methods of biogenesis. In actuality, the specific biogenesis mechanism of the subpopulations is unknown.

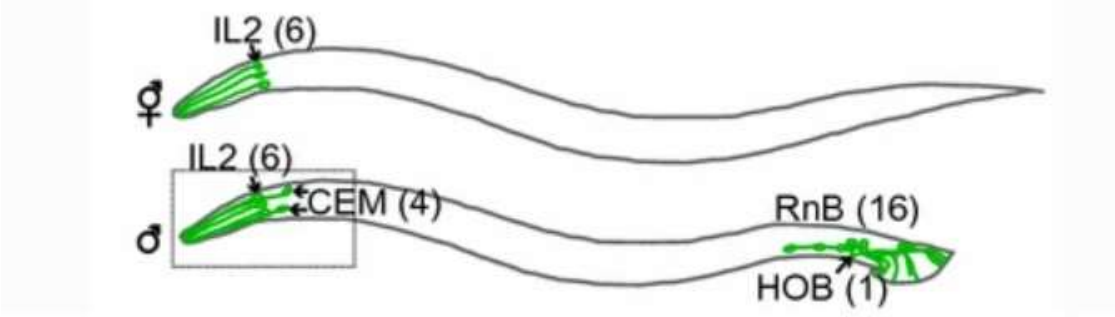

Figure 1: A diagram of EV-releasing ciliated sensory neurons in both male and hermaphrodite *C. elegans* animals (Wang et al., 2016).

GTPases

Small guanosine triphosphatases (small GTPases) are a superfamily of proteins involved in nearly every aspect of cell biology. These enzymes are often used as molecular switches due to their tight regulation by GTP and GDP, along with GTPase activating proteins (GAPs) and guanine nucleotide exchange factor (GEF) accessory proteins (Figure 4). GTP binding allows a conformational change of the GTPase, which allows it to bind other proteins or be recruited to specific places in the cell. GTPases, with the assistance of GAP proteins, then hydrolyze GTP to GDP, inducing a conformational change that often also releases its other binding interactions[26]. To restart the cycle, GDP is replaced with GTP by GEFs, thus reactivating the GTPase. The Ras superfamily of GTPases comprises five families that are conserved across eukaryotes: Ras, Rho, Rab, Arf, and Ran[25].

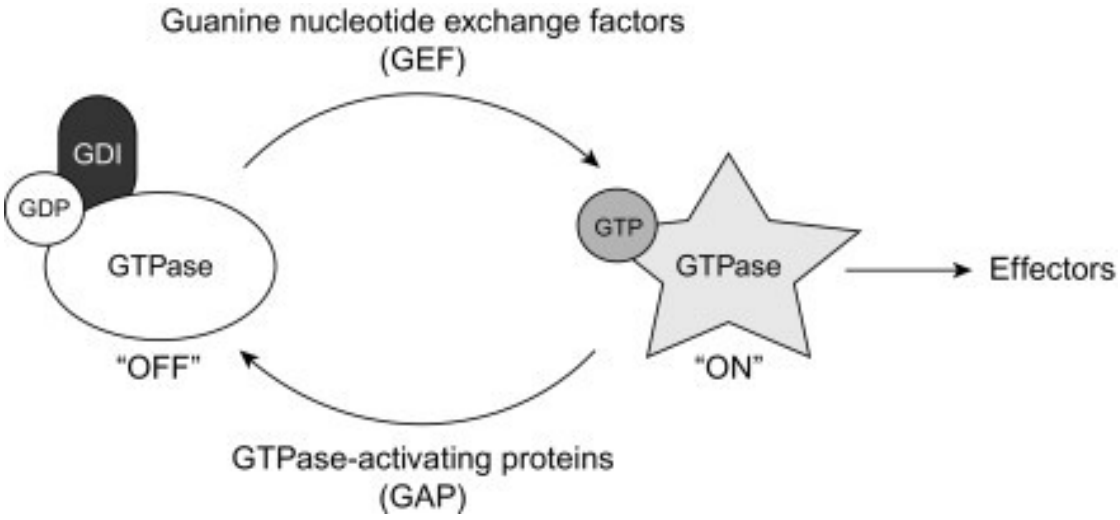

Figure 4: Diagram indicating the GEF/GAP switch in small GTPases (Claing et al., 2013).

ARF6

ARFs (ADP-Ribosylation Factors) are small G proteins that exist in eukaryotic cells. The numerous roles of ARFs in cells are not only related to ADP-ribosylation but instead these proteins are known to participate in the formation of vesicles responsible for membrane traffic, lipid transformation, and reorganization of the actin cytoskeleton[28]. Six isoforms of ARFs have been identified in eukaryotes (ARF1–6), and they are ubiquitously expressed, except for ARF2, which is not found in humans[27]. The best characterized ARFs are ARF1 and ARF6. ARF6 is believed to regulate the recycling of endosomes and receptors to and from the plasma membrane and is also involved in cytoskeleton organization[29].

ARF6 has a direct role in microvesicle biogenesis through the phospholipase D pathway (Figure 5). ARF6-GTP-dependent activation of phospholipase D promotes the recruitment of the extracellular signal-regulated kinase (ERK) to the plasma membrane[30]. ERK then phosphorylates and activates myosin light-chain kinase (MLCK), which mediates MLC phosphorylation, a critical step in MV release. The *C. elegans* ortholog to ARF6, ARF-6, is predicted to have similar functions to its human counterpart, though whether it plays a role in EV biogenesis from the cilia is unknown.

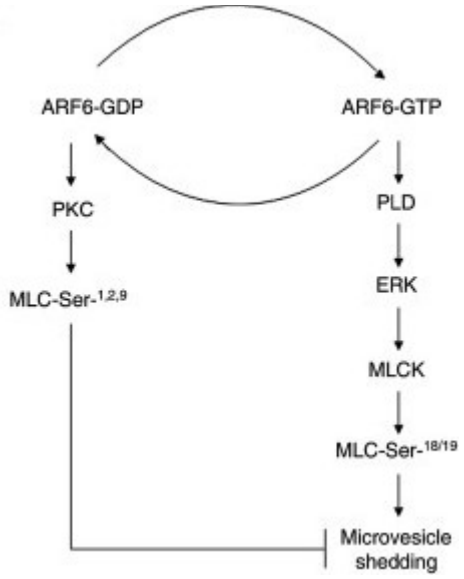

Figure 5: How the ARF-6 GTPase leads to Microvesicle Shedding (Muralidharan-Chari et al., 2009).

col

ARF-6 in *C. elegans*

ARF-6 was confirmed to be enriched in the ciliated sensory neurons which release EVs and this protein was also identified in a large-scale isolation and proteomic profiling of PKD-2::GFP EVs that was performed[32]. The dataset of 2,888 proteins was compared to single-cell transcriptomic data available for *C. elegans*, to show enrichment of identified potential EV cargo candidates in cell types of interest. ARF-6 was amongst these proteins, showing that it could be another cargo associated with PKD-2 containing EVs.

Previous research into the impact of ARF-6 in *C. elegans* on the biogenesis of CLHM-1 containing EVs had been performed by Michael Clupper, a graduate student in the Tanis lab. This research has shown that an elimination of ARF-6 in *C. elegans* results in a 50% reduction in CLHM-1 containing EVs when compared to controls (Figure 6). This data, shown below, supports the theory that ARF-6 plays a role in

biogenesis and/or shedding of CLHM-1 containing vesicles. PKD-2 containing vesicles, however, had yet to be studied in a *C. elegans* strain with an *arf-6* deletion.

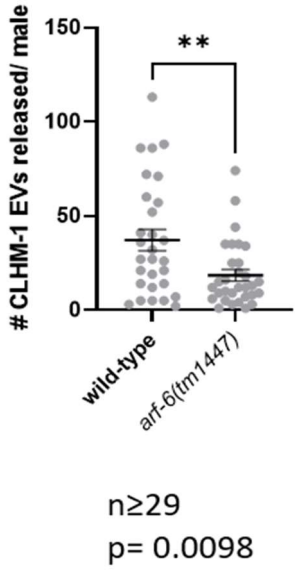

Figure 6: Loss of ARF-6 is associated with a ~50% reduction in CLHM-1 containing EVs; unpublished data collected by Dr. Michael Clupper, graduate student in the Tanis lab.

Objectives and Hypothesis

The purpose of this study is to observe the effects of ARF-6 on extracellular vesicle biogenesis. My goal is to determine if the *C. elegans* ARF-6 GTPase affects the budding of PKD-2 containing EVs, CLHM-1 containing EVs, and/or both EV subpopulations. I hypothesize that GTPases play a crucial role in the biogenesis of multiple microvesicle subpopulations released from *C. elegans* cilia.

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METHODS

Nematode Maintenance

Normal maintenance of *C. elegans* strains occurred on 6 cm NGM petri plates. NGM is made by adding 3 g NaCl, 2.5 g peptone, and 17 g agar into 1 L of distilled water to produce 1 L of media. After autoclaving and allowing the media to cool to ~72°C while stirring at a moderate speed for ~1 hour, the following additives were mixed in: 1 mL cholesterol in ethanol (5 mg/mL), 1 mL 1M $CaCl_2$, 1 mL 1M $MgSO_4$, and 25 mL 1M potassium phosphate buffer (KH_2PO_4; pH 6.0). After drying, the plates were then seeded with 200 µL of the OP50 *E. coli* strain grown up in B broth (1 L of broth: 10 g bactotryptone, 5 g NaCl, and 1 L distilled water; autoclaved). To maintain animals, 3 worms at L4 stage were picked onto each plate with a platinum worm pick and stored in an incubator at 20°C.

Strains List

him-5, or high incidence of males, is a gene in the *C. elegans* genome linked to the presence of sex chromosomes. Mutations in this gene reduce the frequency of crossovers on the X chromosome, meaning that *C. elegans* containing a knockout of this gene are far more likely to be males[32]. By allowing sexual reproduction and meiotic crossover between two different strains of animals, we can greatly reduce the

likelihood of asexual reproduction and allow for the creation of new strains with a specific genetic makeup.

Table 1: Strains List

Strain #	**Genetic Makeup**	**Description**
UDE162	*henSi21* [Ppkd-2::pkd-2::GFP::let858 3' UTR] V; *him-5 (e1490)* V	PKD-2 tagged with GFP, *him-5* deletion
FX01447	*arf-6 (tm1447)* IV	*arf-6* deletion
UDE162 x FX01447	*arf-6 (tm1447)* IV, *henSi21* [Ppkd-2::pkd-2::GFP::let858 3' UTR] V; *him-5 (e1490)* V	PKD-2 tagged with GFP, *him-5* deletion, *arf-6* deletion

PCR Genotyping

To confirm presence of the *arf-6* deletion in the mutant strain, PCR genotyping was necessary. F3 generation worms were first lysed in a centrifuge tube containing 5µL of Proteinase K + 95 µL of worm lysis buffer. After placing these tubes in -80 degrees C for 5 minutes, the Worm Lysis Protocol was run to yield the DNA. For this specific PCR, 3 primers were used: OG658 (shared forward), OG659 (wild type), and OG660 (mutant). Each PCR tube contained: 1µL of template DNA, 1µL of each primer, 1µL of sterile water, and 5µL of GOTAQ (Promega). The primers were annealed at 52 degrees, with an extension time of 35 seconds for 28 cycles.

Genetic Cross

In order to create a strain containing both the *arf-6* deletion and the GFP-tagged PKD-2, a genetic cross was conducted. By using males from the strain containing GFP-tagged PKD-2 and hermaphrodites from the *arf-6* deletion, sexual reproduction can occur. Knowledge of *C. elegans* life cycle as well as fundamental genetics allows us to optimize the ratios to produce animals homozygous for all of the desired mutations. At each stage of the genetic cross, animals were kept on NGM petri plates containing OP50 *E. coli* food. These animals were kept at 20 degrees Celsius for all maintenance and experimentation. Details of the genetic cross are described in the Results section.

Imaging EVs

Using Total Internal Reflection Fluorescence (TIRF) super-resolution microscopy, we are able to image EVs released from male tail sensory neuron cilia. TIRF microscopy allows for critical angle adjustment tailored to each specimen, giving images that have minimal noise. The TIRF angle of incidence was manually adjusted for each animal to achieve critical angle. Each slide is prepared with 4-6 adult males picked from a plate containing both males and hermaphrodites. These males were picked to an unseeded plate (containing no bacterial food source), allowed to crawl for several seconds, picked into 20 mM levamisole on 3% agarose pads, and covered with high-performance cover glass. Each sample is imaged as a Z-Stack of 10 “slices” and the clearest image is used for analysis. All EV images were taken within 30 minutes of animal mounting.

Analysis

Imaris software (Oxford Instruments) was used for quantitative EV analysis. EVs were identified using the "Spot" function, setting approximate object size to 0.350 μm in diameter and a quality threshold of 6. Hot pixels and spots that intersected with the cuticle were manually removed.

Statistical Analysis and Graphing

Dataset normality was determined using the Anderson-Darling normality test. When comparing data sets, the student's t-test was performed. Statistical analyses and graphing were performed with GraphPad Prism 9; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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RESULTS

Strain Creation

To create the target strain, two existing *C. elegans* strains were used. The first, FX01447, contains the *arf-6* deletion. The second, UDE162, contains GFP-tagged PKD-2 in the *him-5(e1490)* background. The *him-5(e1490)* mutation causes increased frequency of X chromosome nondisjunction, resulting in ~33% male offspring in the mutant compared to <1% in the wild type. The *him-5* mutation is in the background of all the strains used for imaging since we are observing release of EVs from the cilia of sensory neurons in the male tail.

Nine UDE162 adult males and three FX01447 L4 hermaphrodites were picked on a 3.5 cm NGM plate using a platinum pick. After incubating for 24 hrs, the FX01447 hermaphrodites were adults. They were separated onto 3 separate 3.5 cm NGM plates, and their progeny observed. By visual inspection, 2 plates contained a high incidence of males, indicating that the mating had successfully occurred. From there, 20 L4 hermaphrodites from the two successful plates were cloned onto NGM plates that had previously been treated with 150 µL of G418 antibiotic. The GFP-tagged PKD-2 transgene contains resistance to this antibiotic, so all living progeny from these plates must be at least heterozygous for PKD-2::GFP. After another 96 hours, the living progeny with a high incidence of males, suggesting the presence of the *him-5* mutation, were lysed and genotyped using PCR. Based on the initial PCR

results, one plate was predicted to be homozygous for the *arf-6* deletion (Figure 7). Five animals from that plate were cloned to new plates, allowed to lay eggs, and then lysed for PCR. These animals were all homozygous for the *arf-6* deletion (Figure 8). Visual inspection of these animals with a Zeiss AxioZoom microscope showed that they were also homozygous for the PKD-2::GFP transgene.

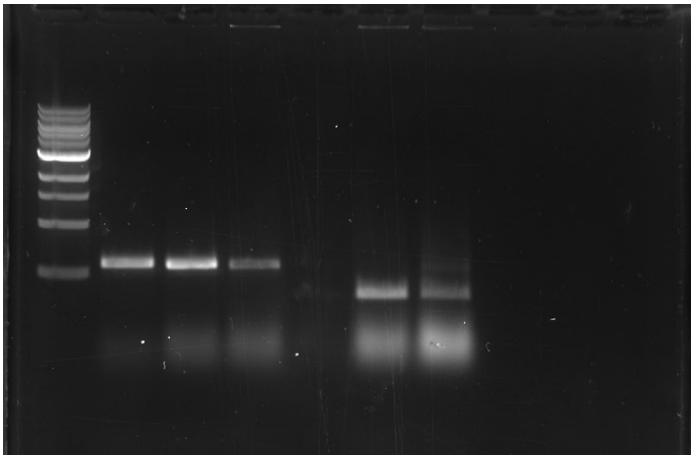

Figure 7: Initial PCR gel depicting 1 well homozygous for the *arf-6* deletion. The first well contains a 1 kb ladder, the sixth well contains the potential homozygote.

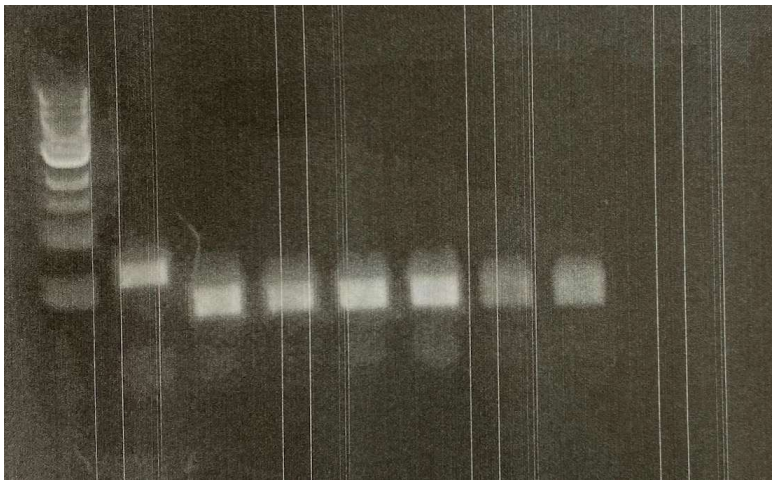

Figure 8: Image of Gel Electrophoresis results of PCR with *arf-6* multiplex primers. All five plates have a single band at the mutant bp length (340 bps) and no bands at the WT bp length (570 bps).

Elimination of ARF-6 Results in Reduction of PKD-2 EVs

To determine if loss of arf-6 impacted release of PKD-2 containing EVs, the adult male tails of *C. elegans* in both the control (Figure 10) and mutant (Figure 9) strains were imaged using TIRF microscopy techniques. Images focused on capturing the most amount of fluorescent EVs in the field, centering around the tail of the animal. A Z-stack of images is taken to ensure as many EVs are captured as possible. To identify EVs, Imaris software was used, utilizing the spot function and a quality threshold of 6.

These images were then manually corrected to eliminate any hot spots or spots that intersected with the cuticle of the animal. After statistical analysis of the Imaris spot data for each strain, a significant correlation was identified. With an n of 10, the average number of EVs in the wild-type strain was 391.4, with a standard deviation of 225.3 (Figure 10). With an n of 17, the average number of EVs in the ARF-6 mutant was 193.4, with a standard deviation of 118.5 (Figure 9). The ratio of the averages is 1:2.02, indicating a 49.4% reduction in the release of PKD-2 containing EVs when ARF-6 is eliminated.

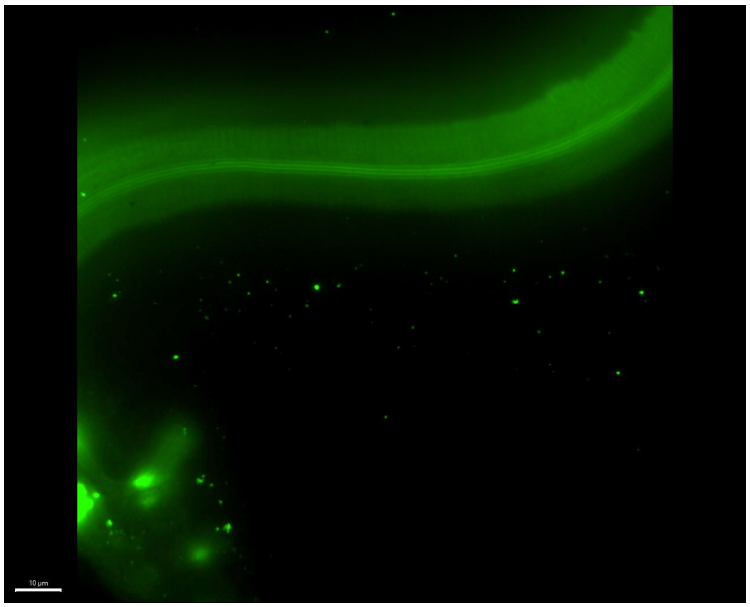

Figure 9: *arf-6* mutant male tail with surrounding PKD::GFP labeled EVs.

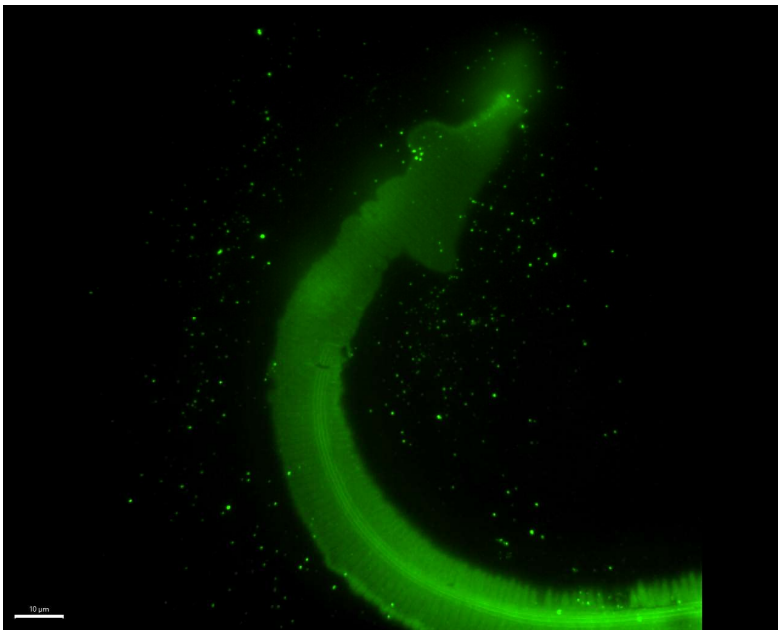

Figure 10: Wild type male tail with surrounding PKD-2::GFP labeled EVs.

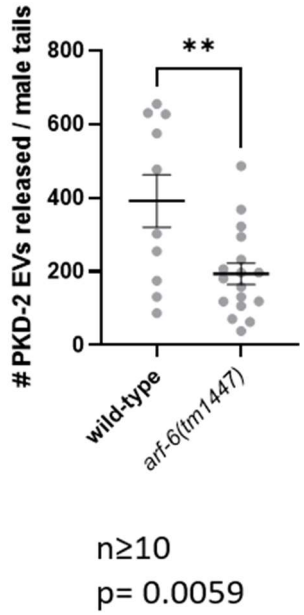

Figure 11: Loss of ARF-6 is correlated with ~50% decrease in PKD-2 containing EVs

4

DISCUSSION

Overview of EVs and Strength of the *C. elegans* In Vivo System

Extracellular vesicles (EVs) are nano-sized lipid bilayer vesicles released by virtually every cell type. These EVs are classified into ectosomes and exosomes, with different methods of biogenesis for each. EVs have diverse biological activities, ranging from regulation of physiological processes to the propagation of pathophysiological conditions. These small vesicles have been considered excellent candidates for biological therapeutics, and it is therefore crucial to study them in vivo. The model organism *C. elegans* provides an in vivo system that allows for the imaging and analysis of EV release in multiple strains with different genetic modifications.

ARF-6 Known Functions in EV Biogenesis

ARF-6 is enriched in *C. elegans* sensory neuron cilia derived, PKD-2-containing EVs, and the mammalian homolog has been shown to play a role in EV biogenesis and release using in vitro systems. The protein CLHM-1, a conserved EV cargo, was tagged with GFP and observed in both control and ARF-6 deletion strains. When ARF-6 is lost, there is about a 50% reduction in CLHM-1 containing EVs. The goal of this thesis was to examine the impact of the ARF-6 protein in *C. elegans* on the number of PKD-2::GFP containing extracellular vesicles released from male tail ciliated sensory neurons.

Data Summary and Interpretation

The data gathered shows a statistically significant, 50% reduction of PKD-2 containing EVs when *arf-6* is eliminated. This was performed using a strain containing PKD-2::GFP, compared to a strain that contains PKD-2::GFP and the deletion of *arf-6*. This data shows that ARF-6 affects the shedding and/or biogenesis of PKD-2-containing EVs, and the reduction of EVs compared to the control indicates that this protein is necessary for proper EV formation from the cilia of male *C. elegans* sensory neurons.

Comparison of CLHM-1 data to PKD-2

When comparing my findings to the previously obtained data for CLHM-1 EVs, the relative reduction in EV release is almost identical in both subpopulations (Figure 12). This suggests that ARF-6 acts ubiquitously in the male tail sensory neurons and has the same impact on release of all EV populations, independent of the specific protein cargo present. Previously obtained data shows that CLHM-1 and PKD-2 are in two distinct vesicle subpopulations, budding from different segments of the cilia. This data suggests that ARF-6 affects EVs budding from different segments of the cilia and isn't specific to one ciliary location.

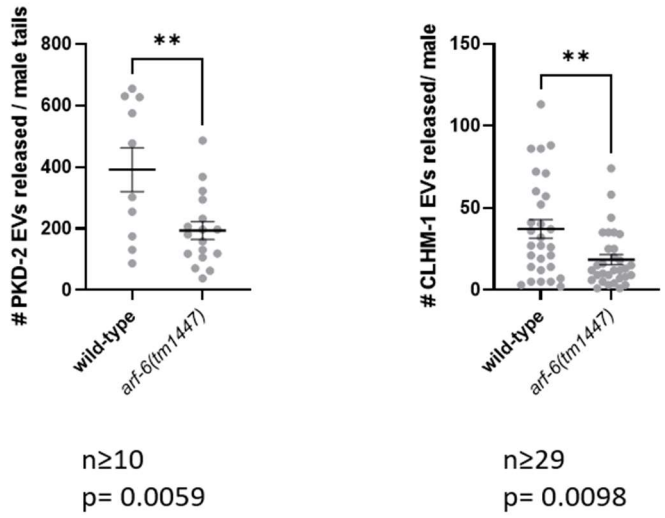

Figure 12: Comparison of known CLHM-1 EV data collected by M. Clupper, a graduate student in the Tanis lab, to the newly obtained PKD-2 EV data generated in this thesis.

Imaging Delays

A large portion of my thesis relied on the Andor Dragonfly microscope to image the EVs. This microscope was originally a system undergoing beta testing at the University of Delaware Bio-Imaging center at Ammon Pinizzoto Biopharmaceutical Innovation Center. The microscope was scheduled to be replaced with a permanent scope on March 7th and be fully functional by March 16th. This allowed us to image up until February 25th, and potentially resume as soon as possible. However, there were unforeseen delays in the shipping, installation, cleaning, and testing of this new microscope. I was not able to resume imaging until April 27th, 2022. Upon imaging, we discovered that our original method of data acquisition, sTIRF, was no longer

functional on the new scope due to dust in the system. Thus, I had to switch to a new method, bTIRF, and collect as many images as possible. This method uses a bundle of lasers instead of a single laser, providing images that look slightly different from the sTIRF images I acquired in the months prior. This difference effectively eliminated all of the sTIRF control images that I had collected, and I was therefore required to retake all of the control images with bTIRF. With multiple labs utilizing the Dragonfly, I was not able to monopolize its use in the 2.5 weeks leading up to the thesis deadline. While my data set did yield statistically significant results and provides preliminary data to suggest that ARF-6 plays a role in PKD-2::GFP EV biogenesis, additional imaging must be done to complete the data set.

Future Directions

For this project, I have focused on a single EV cargo, PKD-2, tagged with a single fluorescent protein, GFP. One possible route for future expansion of this project would be looking at colocalization of multiple proteins within the same EV. One target protein could be CLHM-1, another previously identified EV protein. This would be accomplished by creating a strain that contains the *arf-6* mutation and the GFP-tagged PKD-2, along with a red fluorescent protein-tagged CLHM-1in the *him-5* background. When imaging this strain, images could be taken of both the green PKD-2 and the red CLHM-1, and a merge of these images could be used to measure colocalization. This would allow us to determine if the elimination of *arf-6* affects the colocalization of

different EV proteins. If a difference was observed between the wild type and *arf-6* mutant, it would suggest that ARF-6 affects both EV cargo sorting and biogenesis.

Another potential future direction could be examining EV release in the *arf-6* mutant compared to the wild type without the presence of mating partners. Previous research has shown that PKD-containing EV release in the male tail is relatively decreased in the presence of potential mating partners[21]. In my research, males were in the presence of mating partners from hatching until moments before slide creation. Potential future experimentation could involve adjustment of one of these factors. Males could, for example, be raised in the presence of hermaphrodites but removed from them shortly prior to staging. Alternatively, males could be removed from mating partner presence at the L4 stage, allowed to grow to adults, and imaged thereafter.

This thesis also focused solely on EVs released from the cilia of the male tail neurons. These EVs are known to be mechanosensory, indicating that they respond to mechanical cues. However, EVs are also released from ciliated sensory neurons of both males and hermaphrodites, which are known to be chemosensory. While I have determined that elimination of *arf-6* results in a significant reduction in EV release from male tail neurons, this does not indicate that male head EVs will yield the same result. PKD-2-is known to be a male-specific cargo, so it is unlikely these kinds of EVs will be observed in hermaphrodites[32].

The EVs that I have studied bud from the primary cilia of ciliated sensory neurons in *C. elegans*. The results show that the absence of *arf-6* decreases the amount

of PKD-2::GFP EVs released. Further investigation into the PKD-2 protein concentration in the cilia could potentially provide insights into these results. It is important to understand if the amount of PKD-2 in the cilia from which the EVs are derived is affected by loss of ARF-6. Imaging of the neurons themselves with the fluorescently tagged protein and then quantitation of fluorescence intensity can provide insight into the concentration and location of PKD-2 in the ciliary neurons.

Three ARF-6-GEF proteins are encoded in the genome of *C. elegans*, including GRP-1, EFA-6, and BRIS-1[34]. These are all shown to interact with ARF-6 as part of the GEF/GAP pathway of signal transduction. The PKD-2::GFP transgene could be crossed into mutants and imaging could be done as I have done for this thesis to determine if an elimination of one or more of these proteins also impacts EV release. In theory, if the ARF-6 GEF/GAP switch was rendered inactive, the data from the ARF-6 elimination mutant should look similar to this mutant. Alternatively, examining other *C. elegans* ARFs could provide insight into whether additional small GTPases are required for normal EV shedding.

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