glb-28's role in mating behavior and extracellular vesicle releasing neurons

By

KUMAR LEE TIGER

A thesis submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Masters of Science

Graduate Program in Cell and Developmental Biology

Written under the direction of

Maureen Barr

And approved by

_____

_____

_____

New Brunswick, New Jersey

May 2020

ABSTRACT OF THE THESIS

glb-28's role in mating behavior and extracellular vesicle releasing neurons

By

KUMAR LEE TIGER

Thesis Director

Maureen Barr

Genetic diseases arise from a mutation in a globin protein in humans in hemoglobinopathies, one of the most common diseases that can be inherited. The types of hemoglobinopathy range from structural hemoglobin variants to hemoglobin synthesis disorders. Globins in general are a conserved protein family that is characterized by its 6-8 alpha helices with an oxygen-interacting heme group and exist in all the kingdoms of life. The comprehensive study of globins are critical for more than just hemoglobin; they include myoglobin, neuroglobin, cytoglobin, androglobin, globin E, globin X, and globin Y in vertebrates. Neuroglobin, myoglobin, and cytoglobin have been studied the most out of the vertebrate globins. *Caenorhabditis elegans* globins offer the ability to understand globin protein function at a more fundamental level due to the large number and variety of globins, number of displayed phenotypes, sexual dimorphism, and ease of separating internal and external factors on protein function without inducing lethality. Despite these advantages, only 6 out of 34 globins have been characterized in the nematode *C. elegans*. It is still unknown why a nematode would have so many globins compared to more complex organisms which have fewer globins. I recently discovered that *glb-28*, one of

the uncharacterized 34 globins of *C. elegans*, is endogenously expressed in extracellular vesicle-releasing neurons (EVNs) and oxygen sensing neurons. Currently, *glb-28* is the only globin found in EVNs and it is required in male mating behavior. I will utilize CRISPR/Cas9 mediated genome editing to investigate the function of *glb-28* in mating behavior, oxygen sensing neurons and EVNs. Results from this thesis may a novel connection with *glb-28* between EVNs.

ACKNOWLEDGEMENTS AND DEDICATION

The environment at Rutgers and the worm community was highly conducive to helping me do research and learn as a graduate student. My principal investigator Dr. Maureen Barr and the Barr lab was very insightful mentorship and perseverant in teaching me how to be a better scientist. Special thanks to Jonathon for his help with statistics and helping with crosses, Kade for the confocal microscopy help, Juan, Inna, Deanna, Bob, Shilpa, Yasmin, Gloria, Oliver, Anza for the mentoring and support. Thanks to the biology staff were generous in teaching advance microscopy skills and providing troubleshooting techniques.

Both the Graduate Student Fellowship through the Rutgers University Pipeline (RUP)-Initiative for Maximizing Student Development (IMSD) Program of the National Institute for General Medical Sciences 5R25GM055145-19 (MPI) and NIDDK Diversity Supplement R01 DK0594-19S1 was essential in providing valuable resources and extracurricular activities to give me the resources to be successful. I am also grateful that the IMSD co-directors Dr. Haimovich and Dr. Langer always kept challenging me and giving me advice on how to continue my academic career.

I would like to thank my mother and father for the tremendous support they have provided me over the years. In addition, I'm grateful for my fiancée Makenna May for her encouragement, editing and listening to me practice my science. Without worms we wouldn't have met. I also would like to thank the Church in Piscataway for their prayers and community.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

Introduction

C. elegans are an ideal model for the study of an uncharacterized globin gene

Caenorhabditis elegans (*C. elegans*) are multicellular eukaryotes from the nematode phylum. These animals are transparent and primarily feed on bacteria in rotting organic matter where they thrive in 6% oxygen and they prefer to avoid environments that have lower than 2% oxygen or environments with higher than 12% oxygen (Gray et al., 2004). The animals use conserved microtubule based sensory organelles called cilia on their neurons in order to sense many chemosensory and mechaosensory cues (White et al., 1986). Furthermore, there are other structures in *C. elegans* such as the cuticle, hypodermis, excretory system, neurons, and muscle within their cylindrical body are easily visualized through microscopy due to their transparent bodies. *C. elegans* have an embryonic stage, four larval stages L1, L2 or dauer, L3, L4, and adult where they grow up to roughly 1mm in length in around 3 days at 20°C (Figure 1). Dauer in *C. elegans* is an alternative developmental stage aside from the L2 stage in which the animal responds to the lack of food, high temperature, and or high pheromones by ceasing further development for up to 2 months (Golden and Riddle 1982, 1984) (Figures 1 and 2). Eventually in the presence of food, the animal will develop into a sexually mature reproductive adult male or hermaphrodite. By adulthood, the ciliated neurons on both the male and hermaphrodite will allow a response to a range of mechanosensory and chemosensory cues (White et al., 1986). Using their cilia the male will demonstrate behavior that is more conducive to finding mates more than food while hermaphrodites or larvae will focus on finding food and stay focused on survival (Lipton et al., 2004).

C. elegans are a naturally occurring 99.8% hermaphroditic species with only .2% males, allowing the study of sexual dimorphism, behavioral differences and of mutations that may be lethal in one sex but not the other. One of the key areas of study in *C. elegans* are autosomal dominant polcystic kidney disease gene (ADPKD) and the polycystins *lov-1* and *pkd-2* because they are important for sensory related behaviors such as male mating behavior and cilia function. *lov-1* is the human homolog for PKD-1 and *pkd-2* is the human homolog for PKD-2 (Barr and Sternberg, 1999). ADPKD is a human ciliopathy that is caused by mutations in the polycystins PKD-1 or PKD-2 and it has a 1/1000 chance of occurring in humans (Igarashi, P., & Somlo, S. 2002). *C. elegans* have conserved cilia structure, ciliopathy genes and functions (Chen, N. et al., 2006). The ecological niche of *C. elegans* has likely influenced the animal's ability to use cilia to sense their environment, handle oxygen species, oxygen requirements, stress responses such as dauer, mating behavior and gene function with respect to globins.

Globins are spherical metalloproteins comprised of 6-8 alpha helices with an oxygen-interacting heme group embedded in their hydrophobic region (Vinogradov, S *et al.* 2006). There are 33 globin genes and globin expression may be reduce or increase due to the state of dauer, and some globin genes are responsive to changes in oxygen (Hoogewijs D, et al., 2007). In particular, we are going to focus on the globin-28 (*glb-28*), which is an uncharacterized gene from the globin family and is located on the X chromosome. There is very little known about the *C. elegans* globin genes but one of the most characterized globin gene is *glb-5,* suggests that a globin may have broad functions. *glb-5* is required for regulation of soluble guanylate cyclases (Abergel, Z. et al., 2016), neural information processing (Oda, S. et al., 2017), foraging (Pradhan, S. et al., 2019),

innate immunity to pathogens and aids in the recovery from hypoxia-reoxygenation stress (Zuckerman, B. et al., 2017). We will consider what we know about the characterized globins to guide our approach to understanding *glb-28*.

Many of the 33 *C. elegans* globins remain uncharacterized, but we know that some globin genes are required to perform redox function, reproduction, behavior, and to metabolize gases such as oxygen (De Henau, S *et al.* 2015). The globins in *C. elegans* play diverse roles beyond oxygen sensing such as feeding behavior, worm aggregation, reproduction, enzymatic function, autoxidation, locomotion, and neuroprotection (Tilleman, L *et al.* 2011 and Pesce A *et al.* 2003). Furthermore, in a custom phylogenetic tree we show that human globins and *C. elegans* globins share minimal phylogenetic similarity. The phylogentic tree compared the human globins androglobin, neuroglobin, hemoglobin subunit alpha, hemoglobin subunit beta, cytoglobin, and myoglobin with *C. elegans* globins *glb-28, glb-2, glb-25, glb-32, glb-9, glb-7, glb-10, glb-15, glb-16, glb-19,* and *glb-3*. The phylogenetic tree illustrates that *glb-28* is one of the most distantly related globins out of the globins observed **(Figure 3)**. In addition, the literature shows that GLB-28 has very little protein sequence similarity to other *C. elegans* globins. In order to illustrate this, the data on the globin genes was adapted from supplementary material that used a manual alignment of nematode globins and a similarity matrix based on the alignment (Hoogewijs, D et al., 2008) **(Figure 4)**. Many *C. elegans* globins have low protein sequence similarities with one another with the highest being 35% and the lowest being 6% (Hoogewijs, D et al., 2008). The diversity of protein sequences in the globin family emphasizes that each globin may have a unique function. Almost all of the *C. elegans* globins are expressed in some part of the nervous system throughout the head or

tail. There are 302 neurons in the hermaphrodite and only 8 neurons are specific to the hermaphrodite which serve in egg-laying. In the male, there are 383 neurons with 89 of those neurons specific to mate searching or mating. Between the hermaphrodite and male there are 294 shared neurons that are responsible for feeding, small, taste, touch and movement (Fagan, K. A., & Portman, D. S. 2014). The remaining globins express in non-neuronal cells that make up the muscle, pharynx, or hypodermis (Hoogewijs, D et al., 2008). Globin disorders manifest in humans in the form of hemoglobinopathies such as sickle cell and thalassemia minor (Rosenfeld, L *et al.* 2019). Globin proteins perform in respiratory functions, transporting gases such as O_2 and CO_2 in humans (such as hemoglobin), and they have protective roles in detoxifying nitric oxide within cells such as cytoglobin. Variants of the cytoglobin gene are associated with type 2 diabetes nephropathies (Taira, M *et al.* 2018). Overexpression of globins in tissues protects from oxidative stress (Khan, A *et al. 2006*). In humans there are several types of globins; neuroglobin, alpha globin, beta globin, myoglobin, cyclostome hemoglobin, and gnathostome cytoglobin. 3 of the 6 globin types of globins in humans serve in oxygen transport and are called cyclostome hemoglobin, alpha globin, and beta globin. Despite the broad potential importance of globins and large number of uncharacterized genes, *C. elegans* globins are understudied.

We focused on investigating *glb-28*, one of 33 globins of *C. elegans*, because it is exclusively enriched in a particular subgroup of ciliated neurons, which we call extracellular vesicle-releasing neurons (EVNs) (Wang, J et al., 2015) (Figures 5 & 8). *C. elegans* have non-motile cilia and these cilia function as sensory organelles. The adult male *C. elegans* has 385 neurons, which includes the 60 sex shared ciliated neurons and

an additional 48 ciliated male specific neurons. There are 27 male-specific EVNs that are capable of releasing 100nm extracellular vesicles that contain genetic cargo such as polycystins which can serve in animal communication (Wang, J et al., 2014). The mechanosensory neurons and chemosensory neurons are the only ciliated cells in *C. elegans* (White et al., 1986). *C. elegans* allow us to study EVNs and other ciliated neurons because we can study the function of most mutations without lethality. Ciliopathies are the result of obstructed ciliary proteins and interference of non-ciliary proteins that are involved with ciliary function. Previously, the relationship between ciliary function, polycystins, and gas response has been underappreciated. In response to sensing blood flow in humans and mice, the localized polycystin-1 (Pkd1 or *pkd-1*) and polycystin-2 (Pkd2 or *pkd-2*) on cilia is required for synthesis of nitric oxide gas (NO). The NO produced is responsible for enabling for vasolidation and the regulation of blood pressure through signaling pathways. There is also a possibility that suggests that globins are responsible for the regulation of NO (Tilleman, L *et al.* 2011). Furthermore, this illustrates that the extracellular environment can influence the function of cilia via ciliary localized proteins such as polycystins (AbouAlaiwi, W. A et al., 2009 and Nauli, S. M., et al., 2008). Globins such as *glb-5* can also respond directly to extracellular changes such as oxygen levels and can act through soluble guanylate cyclase genes to influence neuron activity. Soluble guanylate cycases are conserved oxygen binding proteins that generate second messenger molecule cGMP to cause neuron depolarization (Zimmer M et al., 2009). Soluble guanylate cyclases express in oxygen sensing neurons such as AQR, PQR, and URX neurons (Persson, A., et al., 2009). EVNs, polycystins and globins respond to the extracellular cues of chemosensation and mechanosensation. Therefore, it

is worthwhile to elucidate the basic mechanisms in which globins, polycystins, and EVNs may have connecting pathways in response to cilia sensation.

The behaviors and sensations mediated by ciliated EVNs

The male-specific EVNs enable us to use *C. elegans* as a model for ciliopathies and study the function of an uncharacterized gene as it relates to mating behavior. Mating behavior assays (Barr and Sternberg, 1999; Barr et al., 2001) have shown that polycystin genes *pkd-2* and *lov-1* in *C. elegans* are a model for the autosomal dominant polycystic kidney disease. *lov-1* and *pkd-2* encode homologs of the human PKD1 and PKD2 genes, and the PKD gene products that polycystins are localized to cilia in an evolutionarily conserved manner. In addition, *pkd-2* and *lov-1* mutations cause mating behavior defects which is characteristic of disrupted cilia function (Barr et al., 2001). *pkd-2* and *lov-1* are expressed in the 21 male-specific EVNs (Wang, J et al., 2015) (Figure 5). *pkd-2* and *lov-1* are required for male-specific behaviors such as response and vulva location (Barr and Sternberg, 1999) (Figure 6). *pkd-2* and *lov-1* are also involved with mating behaviors such as mate searching or leaving behavior (Figure 7). Male mate searching is another mating behavior believed to involve chemosensation and mechanosensation via cues from hermaphrodites that attract males and decrease the male's desire to wander (Lipton et al., 2004). There are multiple neurons that are involved in mating behaviors and many of them are EVNs. Ray neurons type B (RnB) neurons consist of 9 chemosensory neurons and are paired with the 9 ray neurons type A (RnA) mechanosensory neurons in the male tail's ray structures. Both RnB and RnA are required in contacting the hermaphrodite and generating a response behavior (Liu and Sternberg, 1995). RnB neurons are also required in mate searching behavior (Barrios et

al., 2008). In addition, there is data that suggests that the utilization of both mechanosensation and chemosensation are involved in response and vulva location behaviors based of hermaphrodite cues (Barr and Sternberg, 1999). The HOB neuron is a sensory neuron in the male tail's hook sensilla and is responsible for vulva location (Liu and Sternberg, 1995). The male also has cephalic male neurons (CEM). The CEM enables males to chemotaxis towards hermaphrodites in neuron ablation experiments (Chasnov et al., 2007). Altogether, the male has 27 EVNs which include the sex shared 6 EVNs known as inner labial sensilla (IL2) neurons. Very little is known about IL2 neurons aside from being required for the animal behavior that is conducive to being picked up and dispersed into a new location called nictation (Lee H, et al., 2013). In addition, IL2 can be modified by stress caused by dauer and exhibit reversible dendritic branching after leaving dauer (Schroeder, N. E., et al., 2013). In contrast with previous work, we will show that *glb-28* expresses in more neurons than previously described in (Hoogewijs, D et al., 2008 and Wang, J et al., 2015) and elucidate *glb-28* functions for the first time.

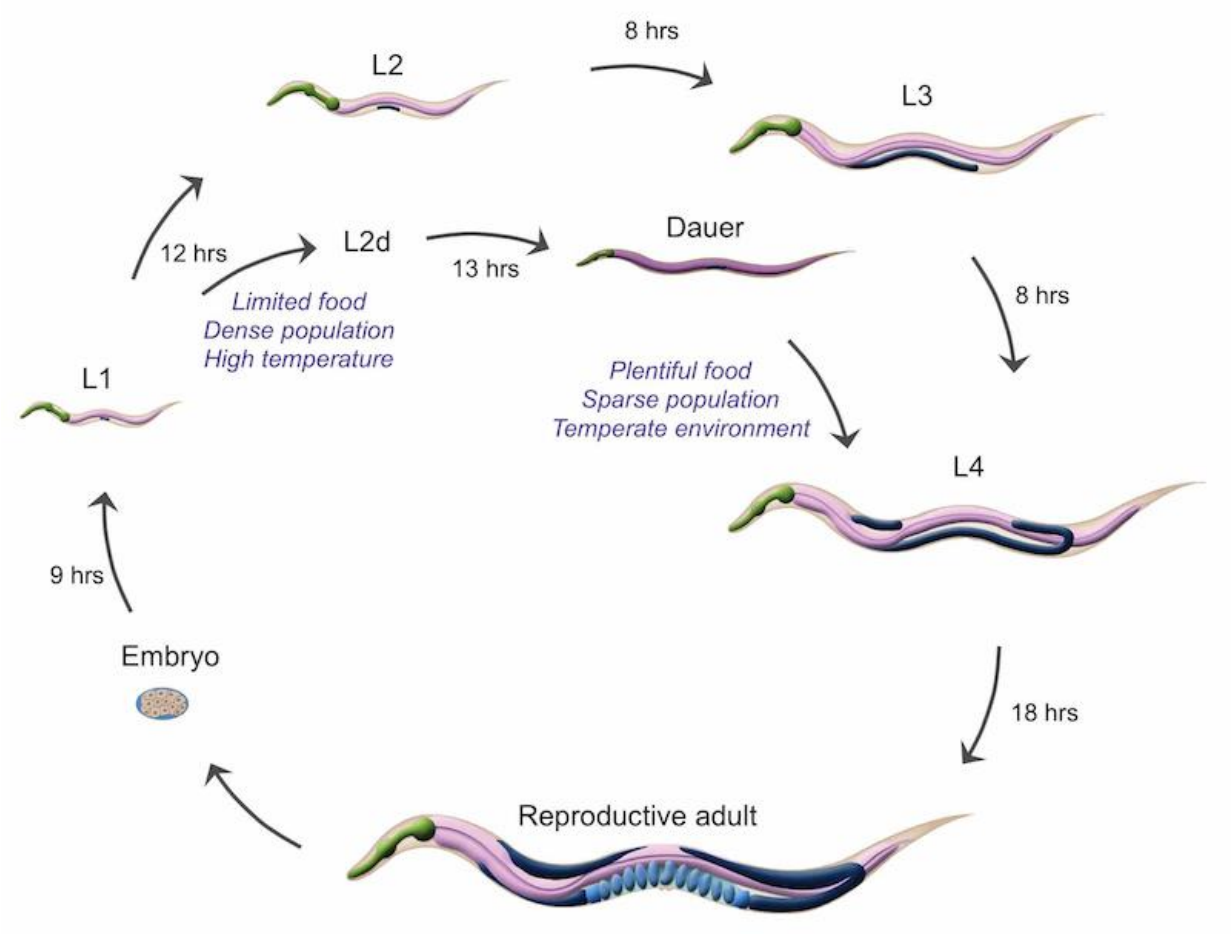


Figure 1: The life cycle of *C. elegans*. *C. elegans* start as an embryo and in 9 hours develop into the L1 larval stage. If the animal is subjected to limited food, dense population, or high temperature as an L1 it will become L2d and become dauer within 13 hours. The animal will not leave dauer until there is food, sparse population, and a temperate environment but once it does will skip L3 and become an L4 and continue normal development. Normally, the L1 animal will molt into an L2 in 12 hours and molt into an L3 in 8 hours. The animal will then molt into an L4 and then within 18 hours finally become a sexually mature reproductive adult male or hermaphrodite (Adapted from WormAtlas Hermaphrodite IntroFIG 6.)

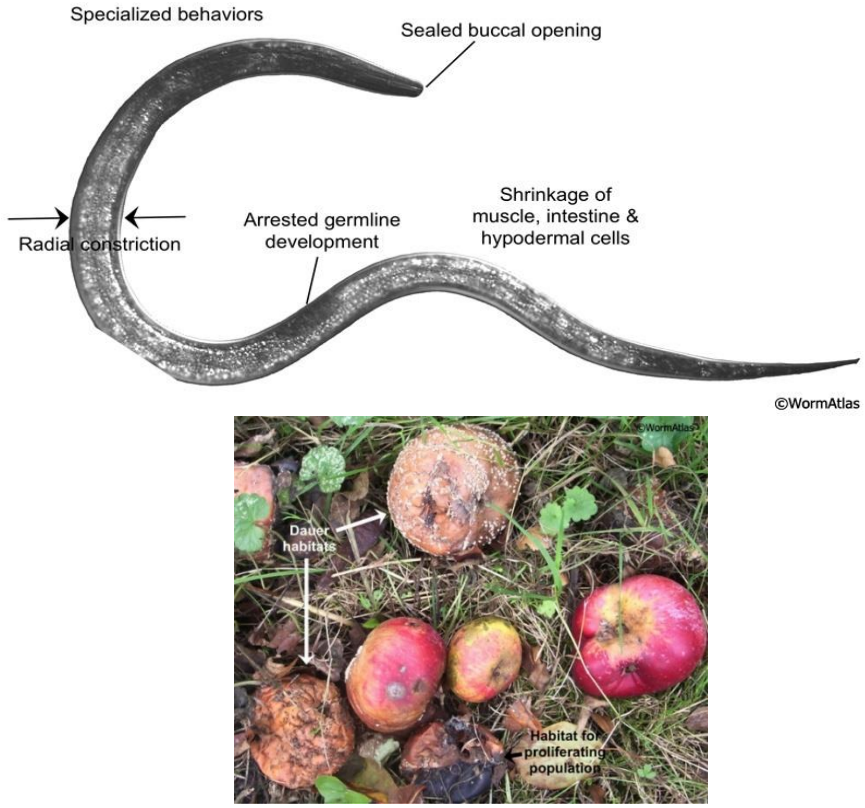


Figure 2: Habitats suitable for dauer and habitats suitable for proliferating populations of *C. elegans*. *C. elegans* will be in the dauer state or continue in development depending on their food source. Dauer animals have arrested germline development and are non-proliferative. Dauer animals are often exploring their habitat in search of greater food sources or exhibit dispersal behavior to reach an area with more food. In the dauer habitat (white arrows) animals are usually found on organic matter that has recently started decomposing and has less bacteria as a result for the animals to eat. In the habitat suited for proliferation (black arrows), the organic matter has decomposed greatly and there are lots of bacteria and thus a greater food source. L1-adult stages of animals will be present because the animals are reproducing. [Image source: S. S. Lee, Cornell University.]

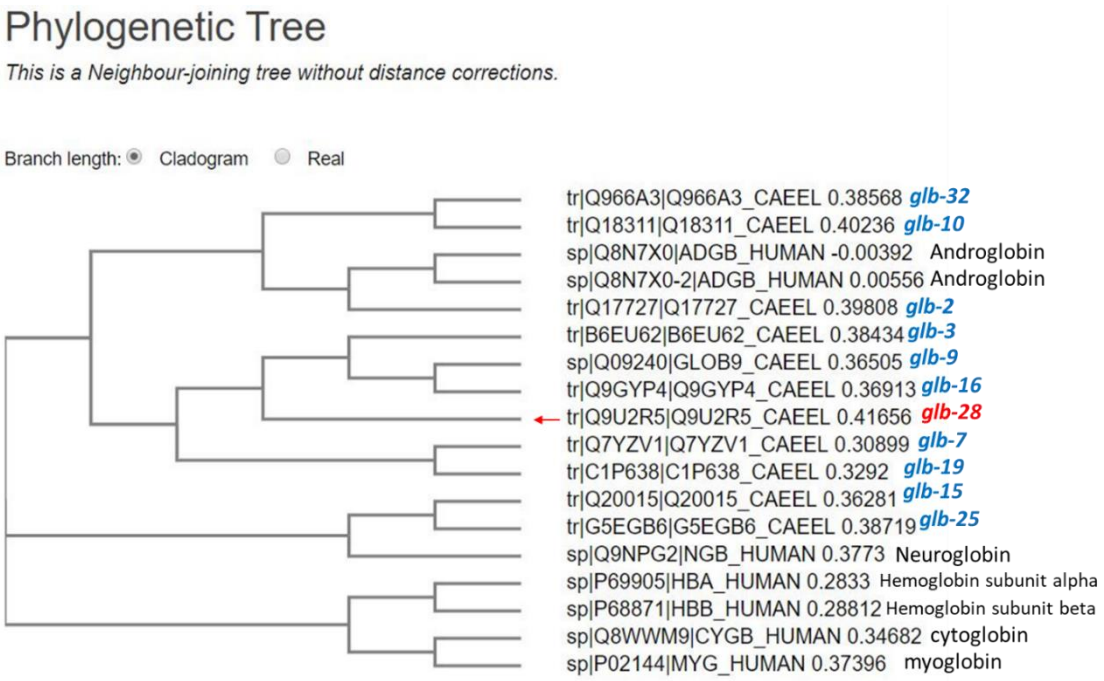


Figure 3: *glb-28* is one of the most distantly related proteins amongst globins. We made a phylogentic tree using several types of human globins and *C. elegans* globins that were associated with *glb-28.* Overall, *C. elegans* globins are phylogenetically distant from human hemoglobin with the most closely related human globins being androglobin and neuroglobin. This illustrates the evolutionary diversity amongst the globin protein family in both species.

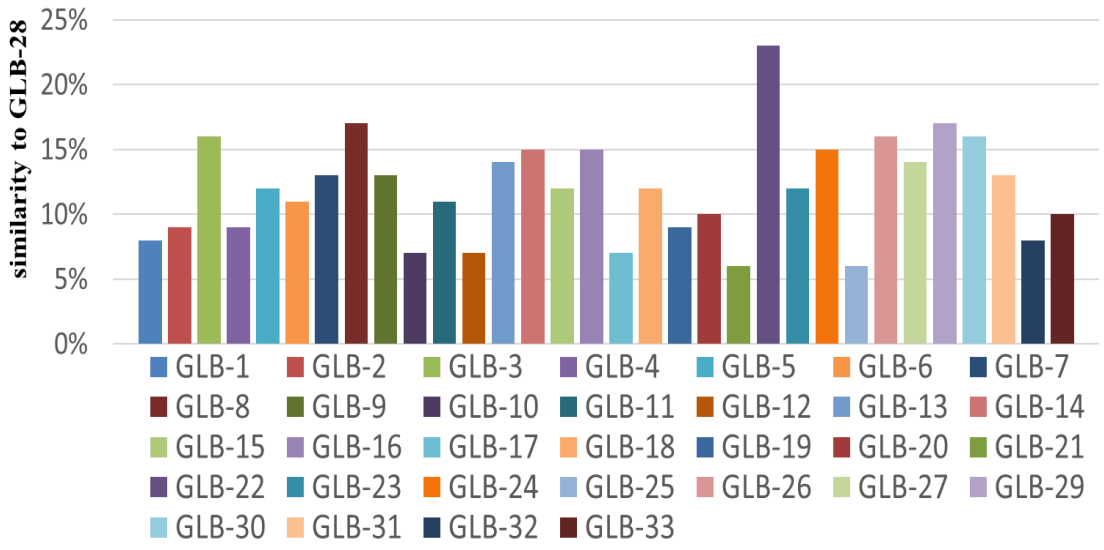


Figure 4: Comparison of *C. elegans* globin protein sequence similarities to GLB-28. This graph illustrates the protein sequence similarity between GLB-28 and each globin gene (globin 1-33). The data on the globin genes was adapted from supplementary material that used a manual alignment of nematode globins and a similarity matrix based on the alignment (Hoogewijs, D et al., 2008). This helps portray the lack of similarity in protein sequence amongst globins despite being from the globin protein family.

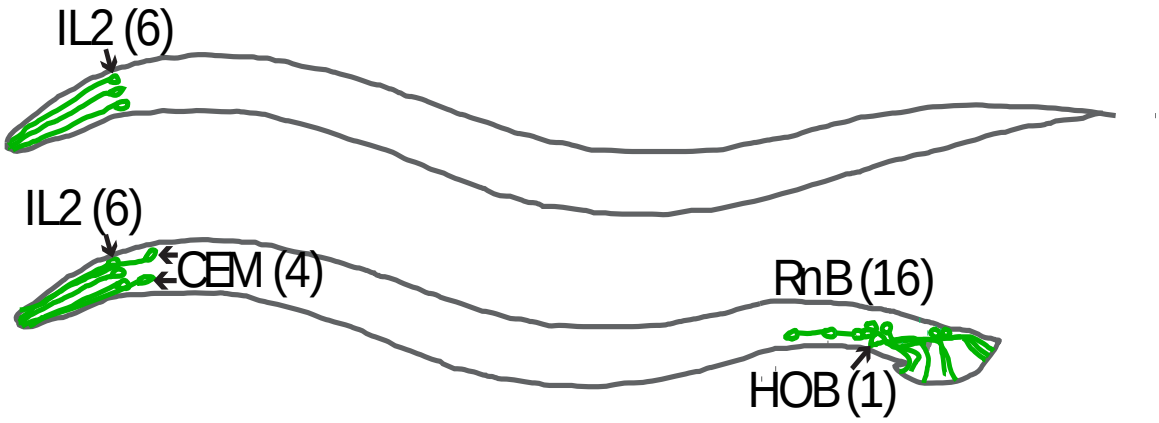


Figure 5: The extracellular vesicle releasing neurons (EVNs) of *C. elegans* in hermaphrodites and in males. In the head, the hermaphrodite has 6 inner labial type 2 (IL2) neurons– 4 quadrant IL2s (IL2Q) and 2 lateral IL2s (IL2L). The male has the IL2 neurons, 4 cephalic male (CEM) neurons in the head, 16 ray type B (RnB) neurons, and 1 HOB neuron in the tail. Altogether, the neurons mentioned previously are what compose the EVNs. Adapted from Wang, J et al., 2015.

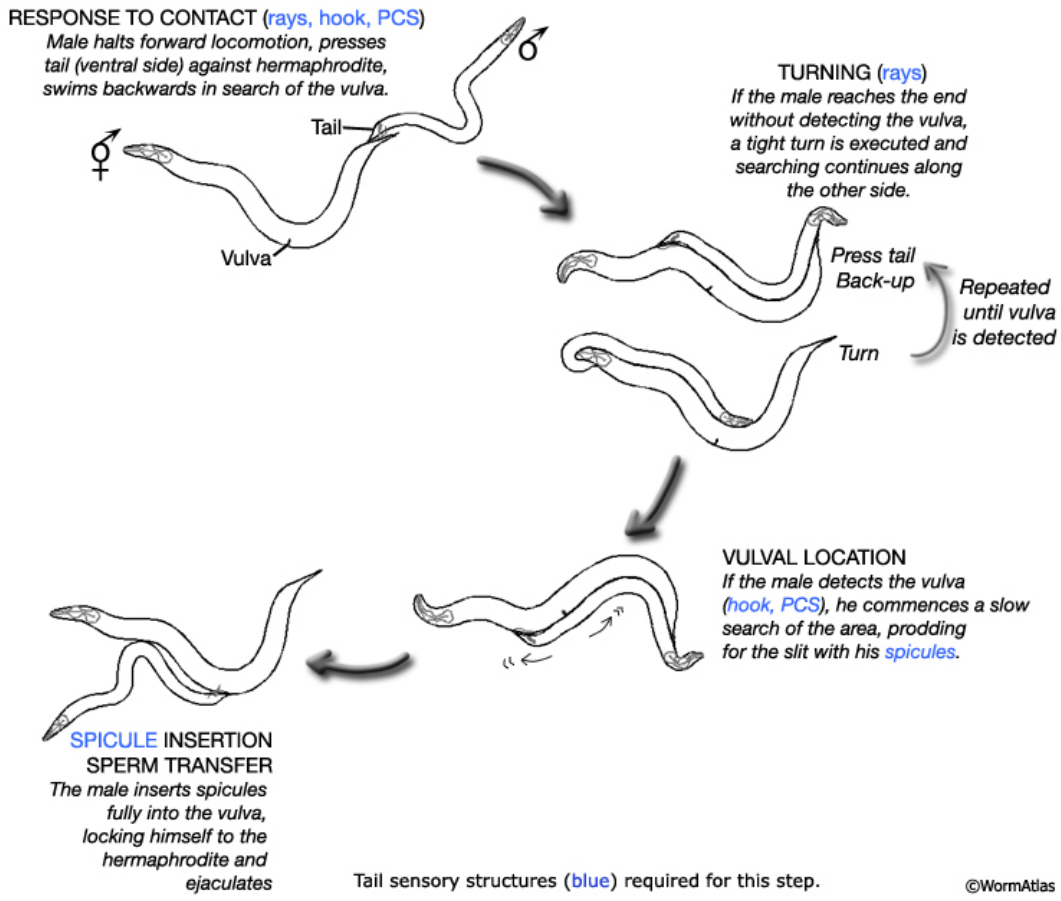


Figure 6: Male mating behavior in *C. elegans*. The positive control (wild-type or WT) or *him-5* will normally perform a response which is when the worm swims forward and make a backwards movement so that the tail is pressed onto the hermaphrodite. The male would then continue its backward movement and make sharp turns as needed on the head or tail until it finds the vulva. Once the male locates the vulva, the male will stop and make fine adjustments backwards and forwards as it tries to insert its spicules. Once the spicules are inserted, sperm transfer will take place. The negative control (mutant or MT) or *pkd-2* mutants have a lower response efficiency and are less likely to make a response to contact. If response is successful, the male takes multiple encounters before it can locate the vulva resulting in low vulva location efficiency (Adapted from Worm Atlas)

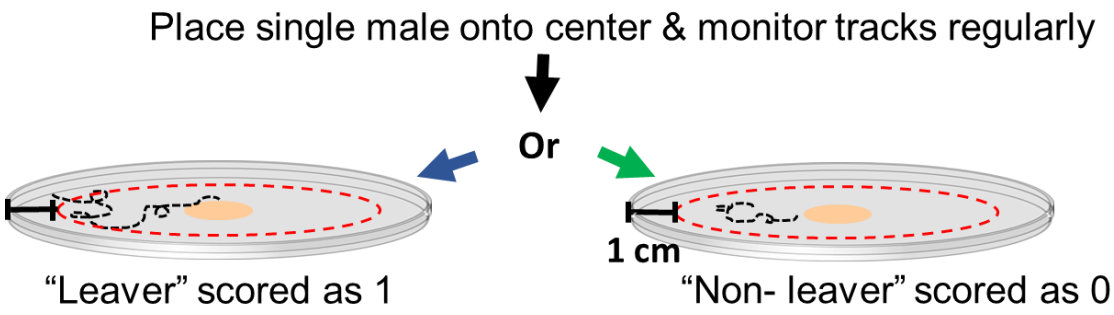


Figure 7: Male-mate searching behavior in *C. elegans*. The isolated adult male positive control (WT) or *him-5* worm is a "leaver" which will eventually leave the restricted food source and search for mates. Adult males of the positive control will only cease mate searching behavior if hermaphrodites are present. The "leaver" behavior is specific to adult males and is not present in hermaphrodites or juveniles. The negative control (MT) or *pkd-2* mutants is a "non-leaver" which will stay in the restricted food source and be less likely to search for mates. Mate searching behavior is scored by the rate in which males leave the perimeter with food and give us the probability of leaving the perimeter with food per hour (Schematic adapted from Anza Rizvi). The code used to analyze this data is listed in Appendix 5.0 and was analyzed accordingly to literature (Barrios, A et al., 2008)

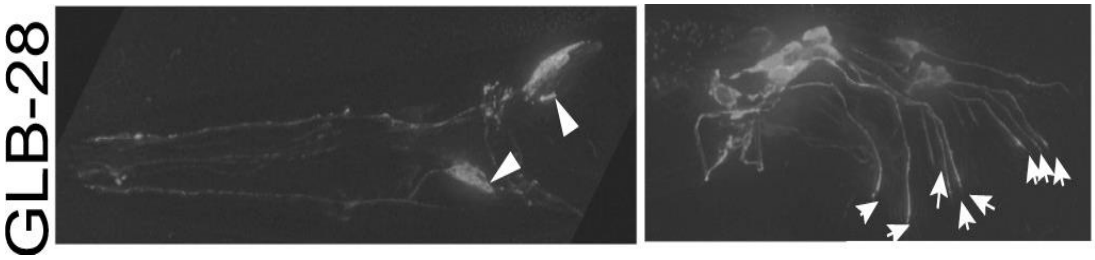


Figure 8: *glb-28* is expressed in male specific head CEMs, tail HOB and RnB neurons with the extrachromosomal reporter strain *pha-1(e2123) III; him-5(e1490) V;myEx857[PCRsoeTGLB-28::GFP(200ng/ul)+pBX(50ng/ul)].* The extrachromosomal translational reporter is a green fluorescent protein tagged with *glb-28* that is localized exclusively in all of the EVNs except IL2s. The white triangles in the image to the left are labeling the cell bodies of the CEMs in the head of the adult male worm. The white arrows in the image to the right are labeling the ray dendrites RnB in the adult male tail. This is published data from Wang, J et al., 2015.

CHAPTER 2

Methods and Materials

Culture of *C. elegans*

C. elegans are cultured at 15-22°C incubators depending on the genotype and temperature sensitive mutations. Within the incubators the nematodes live on the nematode growth media (NGM) agar plates that are supplement with a food lawn of OP50 *E. coli* (Brenner 1974). Well-fed L4 stage hermaphrodites are picked every 3 days to ensure synchronous development for experiments. In all experiments involving males we used *him-5(e1490)* in the background to increase the chance of males in a predominantly hermaphroditic species. *him-5(e1490)* were used as a positive control because it exhibits wild-type mating behavior.

Generation of transgenic CRISPR strains

CRISPR was used for the purpose of tagging a fluorophore on the endogenous C-terminus locus of *glb-28* and creating *glb-28* putative null mutations in our animals. The *glb-28(my70)[glb-28::mscarlet]X)* is a endogenous reporter strain for *glb-28* in the wild-type or N2 background that has been tagged with mscarlet reporter using the CRISPR protocol as described in (Doshkin et al., 2018;Paix et al., 2015). The *glb-28(my56)[stop-in]* is a *glb-28*-specific putative null generated that has a universal 43-nucleotide knock in cassette(STOP-IN) inserted just after the start codon of *glb-28* in the N2 background, thus creating a premature translational stop using the CRISPR protocol as described in (Wang, H et al., 2018).

To make the construct for fusion protein tags in the strain *glb-28(my70)[glb-28::mscarlet]X)* we followed protocol to make double-stranded DNA (dsDNA) donor constructs (Paix et al., 2018) and primers listed in (Table 2). In the generation of the CRISPR transgenic lines we used CRISPOR software to identify the gRNA site (CRISPOR). Large primers are used to generate the homology arms and contain the DNA of interest. Smaller primers that do not contain the homology arms but are homologous to the DNA of interest are generated from the products of the large primers. The products of the large primers and smaller products were mixed and stored as 4 µg total in a 200 ng/µL concentration. The PCR products were allowed to hybridize by heating to 95 ºC and cooling to 4 ºC to re-anneal (95 ºC-2:00 min; 85 ºC-10 sec, 75 ºC-10 sec, 65 ºC-10 sec, 55 ºC-1:00 min, 45 ºC -30 sec, 35 ºC -10 sec, 25 ºC - 10 sec, 4 ºC -forever.) to ensure all PCR products form single-stranded DNA donor sequences. The next step was to make the injection mix. First, mix the tracrRNA – 5µl of 0.4 µg/µl stock and crRNA – 2.8 µl of 0.4 µg/µl stock. Run protocol on PCR machine to create the full sgRNA (Doshkin et al., 2018). The complexed sgRNA is added to an aliquot of Cas9 protein from -80 ºC (0.5 µl of 10 µg/µl stock). The mixture is incubated at 37 °C for 10 minutes, generating the CRISPR RNP complex that targets the sequence complimentary to the crRNA sequence. The PRF4::*rol-6* (*su1006*) plasmid is a co-injection marker used to induce a rolling phenotype and is later used to help us select for successfully injected worms. Single-stranded DNA donor sequences and PRF4::*rol-6* (*su1006*) plasmid – 1.6 µl of 500 ng/µl stock are added last with 4 µg of the partially single stranded DNA from the annealed PCR reactions.

This injection mix was then microinjected into *N2* hermaphrodite germline by research technician Helen Ushakov. The F1 progeny that developed a rolling phenotype from PRF4::*rol-6* (*su1006*) were singled out and allowed to lay eggs for 1-2 days. The F1s were genotyped using mutation specific PCR primers (Table 2). F2 animals that were homozygous for the mutation of interest were confirmed by PCR genotyping using the small, large primers and typWTFGLB-28 or typWTrGLB-28 as controls (Table 1) and Sanger sequencing. If the animals were heterozygous in the F2 generation we would single out 8-10 F2 hermaphrodite worms and allow them to have progeny. We would then select for homozygous animals in the F3 generation that came from the F2 parents using PCR genotyping and sequencing.

To eliminate the chance of off target mutations in the *glb-28* fusion protein tags we outcrossed the transgenic animal with a strain that was *pha-1(e2123) III; him-5(e1490)V ; myEx888[CIL-7::tagRFP(10ng/ul)+pBX1(100 ng/ul)]*. *pha-1(e2123)* is a temperature sensitive mutant that can only produce viable progeny at 15 °C or if it is rescued with the *pBX* co-injection marker. *pha-1(e2123) III* was selected against by doing all the crosses in 20 °C which would eliminate any *pha-1(e2123)* homozygous progeny. The F1 animals that were heterozygotes with tagRFP also had *pBX* and the *glb-28* fusion protein were selected for and singled out. The F2 generation from the F1 animals that did not have tagRFP or *pBX* but had the *glb-28* fusion protein were selected for using microscopy and genotyping. In the F3 generation animals homozygous for *glb-28* fusion protein mutation were confirmed by checking that all animals had the mSc reporter under microscopy.

To make the *glb*-28 putative null mutant using the CRISPR/Cas9 and universal 43bp knock-in cassette (STOP-IN) (*him-5;glb-28(my56)[stop-in])*) we made a single stranded DNA (ssDNA, oligo from IDT). The ssDNA repair template is specific to the mutation that allowed insertion of the STOP-IN right after the start codon of *glb-28* (Wang et al., 2018) and used primers listed in (Table 3). In the generation of the CRISPR transgenic lines we used CRISPOR software to identify the gRNA site (Concordet, J. P., & Haeussler, M., 2018). The next step was to mix the tracrRNA – 5 µl of 0.4 µg/µl stock and crRNA – 2.8 µl of 0.4 µg/µl stock. I used the PCR protocol used to create the full single-stranded DNA donor sequences as described in (Doshkin et al., 2018). The complexed single-stranded DNA donor sequences is added to an aliquot of Cas9 protein from -80 °C (0.5 µl of 10 µg/µl stock). The mixture is incubated at 37 °C for 10 minutes, generating the CRISPR RNP complex that targets the sequence complimentary to the crRNA sequence. ssDNA and PRF4::*rol-6* (*su1006*) plasmid – 1.6 µl of 500 ng/µl stock are added last. This injection mix was then microinjected into the *N2* hermaphrodite germline. The F1 progeny that developed a rolling phenotype from PRF4::*rol-6* (*su1006*) were singled out and allowed to lay eggs for 1-2 days. The F1s were PCR genotyped by PCR reaction with mutation specific primers in (Table 2) called glb-28N-termgenotype F/R. The PCR products were then restriction enzyme digested with Nhe-1. F2 animals that were homozygous for the mutation of interest were confirmed by Sanger sequencing of the PCR product. To eliminate the chance of off target mutations in the *glb-28* null we outcrossed the transgenic animal 3 times with a strain that was *him-5(e1490)V;myIs23[Pcil-7::gCIL-7::GFP_3'UTR+ccRFP]X* . The F1 animals that were

heterozygotes with ccRFP were selected for and singled out. The F2 generation from the F1 animals that did not have ccRFP were homozygous for null mutation.

Imaging

Confocal and fluorescent live imaging was conducted on *C. elegans* to determine *glb-28* expression and GLB-28 localization in neurons. The strain *pha-1(e2123) III; him-5(e1490) V;myEx857[PCRsoeTGLB-28::GFP(200ng/ul)+pBX(50ng/ul)]; glb-28(my70)[glb-28::mSc])X* utilized extrachromosomal *glb-28::GFP* and endogenous GLB-28::mSc. The strain *wzls96; [Prom gcy-32:YC3.60+unc-122::RFP]; glb-28(my70)[glb-28::mSc])X* used to show endogenous GLB-28::mSc and *gcy-32:YC3.60.* Strain *myIs1pkd-2(sy606)IV;him-5(e1490)V; glb-28(my70)[glb-28::mSc])X* show endogenous GLB-28::mSc localization with PKD-2::GFP.

During imaging L4 stage larval males worm cultures are isolated from hermaphrodites the night before. The following day the animals have matured into adults and were immobilized with 10mM levamisole and mounted on 5% agarose pads for imaging. Imaging acquisition via Optical Z-stack projections was done using a 63x/1.4 Oil Plan-Apochromat objective during confocal imaging and fluorescent microscopy. Zeiss Axioplan 2ie Fluorescence microscopy images were taken using Hamatsu digital camera c1140, halogen light excitation, and MetaMorph software version 7.8.13.0. Zeiss LSM 880 confocal microscope with Airyscan super resolution used laser lines for excitation along with a LSM T-PMT detector. The images were taken in Airyscan Fast mode and deconvolved with Airyscan processing. The Zen Black software aided in avoiding oversaturation from the laser intensity and deconvolution. All the images for fluorescent and confocal were then converted to a compatible format with Fiji/ImageJ so

they could be further analyzed using maximum intensity projections. Altogether, the data from imaging will illustrate *glb-28* expression and GLB-28 protein localization as well as genes it may interact with.

Response behavior, location of vulva assay

Mating behavior assays were used to characterize mutant phenotypes in males involving response and location of vulva caused by the *glb-28* deletion. The transgenic strain *him-5(e1490)V; glb-28(tm6910)X* has the deletion allele *tm6910* that eliminates 2/3rds of the globin domain making it a putative null (The C. elegans Deletion Mutant Consortium., 2012) . In particular, response behavior requires the RnB neurons (Liu and Sternberg, 1995) and location of vulva requires HOB neuron (Barr and Sternberg, 1999; Barr et al., 2001). Prior to the mating behavior experiments L4 stage larval males were isolated from hermaphrodites the night before. The control strains were positive control CB1490: *him-5(e1490)* and negative control PT9: *pkd-2(sy606)*. *pkd-2(sy606)* is an effective negative control because it is required for male-specific behaviors such as response and vulva location (Barr and Sternberg, 1999).

The following day the males have matured into adults and single males are placed onto a small food lawn with 20 hermaphrodites from the CB169: *unc-31(e169)IV*. The male initiates the 1st step in mating behavior called response which is when the male touches the hermaphrodite with its tail and starts backing onto the hermaphrodite. If the male successfully responded it was scored as a 1 and if did not respond it was scored as a 0. The percentage of response is calculated by (# responding within 4 minutes/total # worms evaluated) x 100 (Peden, E.M., and Barr, M.M., 2005). Statistical analysis was done using a nonparametric one-way ANOVA (Kruskal–Wallis test) with a Dunn's

multiple comparison test. Similar to the response behavior assay, the location of vulva assay involves isolating L4 stage larval males from worm cultures and placing them on a same sex seeded plate the night before. The following day the males have matured into adults and single males are placed onto a small food lawn with 20 hermaphrodites. Vulva location is the next step in mating behavior in which the male's tail stops at the vulva. During this step the mate can locate by a stop at the vulva for greater than 1 second, pass the vulva or hesitate by pausing at the vulva for less than 1 second. The percentage of vulva location efficiency is calculated as the # of locations/ # of locations + # passes + the number of hesitations x 100 (Peden, E.M., and Barr, M.M., 2005). All of the assays mentioned were performed as described (Barr and Sternberg 1999). The data from these assays could show whether GLB-28 is important for response and vulva location.

Mating Efficiency Assay

This mating behavior assay was used to characterize mutant phenotypes in males that are associated with siring offspring in *glb-28* deletion background. The transgenic strain *him-5(e1490)V; glb-28(tm6910)X* mentioned previously in the response behavior and location of vulva assay was further evaluated. The *dpy-17* hermaphrodites display a short length and their unique phenotype makes it easy to determine self-progeny which would look *dpy-17* and cross progeny which wouldn't display the *dpy-17* phenotype. The mating efficiency experiment was done as described in (Hodgkin 1983) using the quantitative method. Six Late L4 stage larval males and 6 L4 stage larval *dpy-17* hermaphrodites from worm cultures were allowed to mate for 24 hours and then the males were removed. The control strains used for picking males were CB1490: *him-5(e1490)* and PT9: *pkd-2(sy606)* and the hermaphrodites were *dpy-17*. The

hermaphrodites were placed onto new plates every 24 hours including the day they mated. The percentage of cross progeny as shown by a non-*dpy* phenotype over the total progeny x 100% were counted on each plate. The data from the mating efficiency assay allows me to show that GLB-28 may be involved with fertility.

Leaving assay

A behavioral assay focused on the mate searching aspect of male sexual behavior, called a leaving assay, was used as a readout of functional neurons for sensing pheromones as described in (Lipton 2004). This experiment involves isolating 20 L4 stage larval males from worm cultures and placing them on a same sex seeded plate. Once the males have matured into adults, the males are placed individually on assay plates and tracked to see if males leave beyond the 1cm radius from the edge of a 10cm plate. The assay plates are seeded with food as a small circular lawn the 12 hours prior to the experiment. The tracks were observed within time intervals 2, 5, 8, and 24 hours by observing the worm marks on the agar surface of the plates. The leaving rates were calculated as the probability of leaving the perimeter per hour (P_L) (Barrios, A et al., 2008). P_L was estimated using the hazard obtained by fitting an exponential parametric survival model to the censored data using maximum likelihood which utilized the software R **(APPENDIX 5.0)**.

Hypoxia experiments

This hypoxia survival assay was used to characterize mutant phenotypes that are associated with survival in the *glb-28* deletion background. The transgenic strain *him-5(e1490)V; glb-28(tm6910)X* mentioned previously was investigated because it's missing a significant portion of the globin domain which is theorized to serve in gas binding. In

particular, hypoxia survival requires *hif-1* or Hypoxia Inducible Factor homolog. The positive control was N2 wild-type *C. elegans*, negative control was *hif-1(ia4)* with the tested strains being *him-5;glb-28(my56)[stop-in])*. *hif-1(ia4)* is mutation in the *C. elegans* ortholog of human HIF1A(hypoxia inducible factor 1 subunit alpha) and mutants for *hif-1(ia4)* have decreased survival in hypoxia (Jiang et al. 2001). The strains are synchronized worms using alkaline bleach and arrest worms at L1 stage overnight on unseeded plates. Synchronized L1s worms are placed on seeded plates and incubated at 20℃ until L4s. Once the mixed population has reached L4s they are moved onto fresh seeded plates rimmed with garlic extract (50g crushed garlic in 95% ethanol) and place within hypoxia chamber (0.1% O_2) at 25C for 72 hrs. After 72hrs hypoxia treatment the plates are removed from the chamber and allowed to incubate at 20℃ for 24 hrs in ambient oxygen to allow post-hypoxia recovery. Once the recovery period has expired the animals that are alive, dead, censored (burrowed inside agar, desiccated on sides of plates, etc.) and missing are noted. The worm survival is calculated based on percentages of total population and a one-way ANOVA was used to estimate significant differences and the experiment was repeated in 6 independent trials. The data from this away helps determine if *glb-28*'s globin domain is important in hypoxia survival. Dr. Mehul Vorah from the Christopher Rongo laboratory performed these assays.

Strains used or developed

strain name	genotype	reference
N2	*WT*	(Brenner S. 1974).
CB1490	*ali-1(e1934);him-5(e1490)V*	(Hodgkin et al., 1979)

PT9	*pkd-2(sy606) IV; him-5(e1490) V*	(Barr, M.M. et al., 2001)
CB164	*dpy-17(e164) III*	(Hodgkin J.1983)
ZG31	*hif-1(ia4) V*	(Jiang H. et al., 2001)
PT2864	*pha-1(e2123) III; him-5(e1490) V;myEx857[PCRsoeTGLB-28::GFP(200ng/ul)+pBX(50ng/ul)]*	(Wang, J. et al., 2015)
FQ323	*wzls96; [Prom gcy-32:YC3.60+ unc-122::RFP]*	(Smith, E.S.J., et al., 2013)
PT2679	*him-5(e1490)V;myIs23[Pcil-7::gCIL-7::GFP_3'UTR+ccRFP]X*	(Wang, J. et al., 2015)
PT3183	*pha-1(e2123) III; him-5(e1490)V ; myEx888[CIL-7::tagRFP(10ng/ul)+pBX1(100 ng/ul)]*	(Wang, J. et al., 2015)
PT3198	*myIs1[Ppkd-2::GFP + ccGFP]pkd-2(sy606)IV;him-5(e1490) V;glb-28(tm6910)X*	(Wang, J. et al., 2015)
PT443	*myIs1 pkd-2(sy606) IV; him-5(e1490) V*	(Wang, J. et al., 2015)
PT3272	*him-5(e1490)V; glb-28(tm6910)X*	This work
PT3387	*pha-1, him-5; glb-28(tm6910) myEx924[GLB-28:tagRFP+pBX]*	This work
PT3288	*pha-1; him-5; myEx908[glb-28p::GFP+pBX]*	This work
PT3336	*pha-1; him-5; MyEx918 [glb-28p::sfGFP:: GLB-28+pBX]*	This work
PT3477	*him-5(e1490)V;glb-28(my70[glb-28::mSc]X*	This work
PT3399	*glb-28(my56[glb-28KO::STOP-IN])X*	This work
PT3485	*myIs1pkd-2(sy606)IV;him-5(e1490)V; glb-28(my70)[glb-28::mSc])X*	This work
PT3486	*pha-1(e2123) III; him-5(e1490) V;myEx857[PCRsoeTGLB-28::GFP(200ng/ul)+pBX(50ng/ul)] ; glb-28(my70)[glb-28::mSc])X*	This work
PT3487	*wzls96; [Prom gcy-32:YC3.60+unc-122::RFP]; glb-28(my70)[glb-28::mSc])X*	This work

Table 1: Strains acquired or developed

Reagents	Sequence(5'to 3') if Forward or crRNA and (3' to 5') if Reverse
Glb28mSC_LRG_F(Large Forward product) and crRNA site	TCAAAAATTCCGGAATT**TTTATTTTTTAGCGGAAG**GTCCGCGTTCGAGTTCAATAACGGAGGTGG CGGATCTGGAGGTGGAGGCTCTGGAGGAGGTGGATCTATGGTCAGCAAGGGAGAGG
Glb28mSC_LRG_R (Large Reverse product)	ATATGTTATGTTGTTGAAACTGCTTCATTTCGAAGTCACTTGTAGAGCTCGTCCATTCC
Glb28mSC_SML_F (Forward small product)	GTCCGCGTTCGAGTTCAATA
Glb28mSC_SML_R (Reverse small product)	TCACTTGTAGAGCTCGTCCAT
typWTFGLB-28 (wild-type control)	CTGGCTCACCACTCACCAAT
typWTrGLB-28 (wild-type control)	TGTCTTTGTGTTCTCTCCCCA
mScarlet internal reverse primer for genotyping insertion	AGCCGACATCCCAGACTACT

Table 2: Primers, crRNA for *glb-28* mScarlet fusion tag. crRNA site is indicated by bolded black and is located within the homology arm of the Glb28mSC_LRG_F(Large Forward product). There were two silent mutations introduced from A to G indicated by bolded orange and G to C indicated by bolded purple. The light green highlight indicates the flexible linker. The gray highlight indicates the mScarlet primers. The red text highlights stop codon.

Reagents	Sequence(5'to 3') if Forward or crRNA and (3' to 5') if Reverse
crRNA	**CCGGTGGCTGGATTTCATGA**
ssODN donor	CAGAAACAAAGCAATCCAAAAAAAAATGTTCCGTCAGGGAAGTTTGTCCAGAGCAGAGGTGA CTAAGTGATAAGCTAGCTGAAATCCAGCCACCGGAGTTCTCGTTTTCCCCAATATC
glb-28N-termgenotype F (genotyping primer)	GTTGTTGCCAAAGAAATTTAGTCC
glb-28N-termgenotype R (genotyping primer)	TCTCAAACATCACATGCTCTACCA

Table 3: Primers, crRNA for *glb-28* null. crRNA site is indicated by bolded black. Homology arms are indicated by yellow highlight. The light blue highlight indicates an optional built in crRNA site that is not *C. elegans* specific and present for the purpose of making additional edits which we did not use for any experiments. The stop codons are indicated by red highlight. The Nhe1 restriction site is used for genotyping and is indicated by pink highlight.

CHAPTER 3

Results

glb-28 expression and localization

C. elegans glb-28 is expressed in the cephalic male-specific neurons (CEMs), the 6 inner labial neurons type 2 (IL2), URX neurons in the head and Hook B (HOB), B-type ray neurons (RnBs) in the male tail (Figures 9-10). The male specific neurons are CEMs, RnBs, and HOB and previous work has shown them to be essential for male mating behaviors (Barr & Sternberg, 1999; Barr et al., 2001). The IL2s are important in dauer nicitation and dispersal behavior and have been speculated to have a chemosensory role (Lee et al., 2011). The URX neurons function in aerotaxis (Gray et al., 2004), lifespan regulation (Liu and Cai; 2013), suppression of innate immunity (Styer et al., 2008). The strain *wzls96; [Prom gcy-32:YC3.60+unc-122::RFP]; glb-28(my70)[glb-28::mSc])X* show co-expression in the cell bodies of URX neurons (Figure 10). *glb-28* is co-expressed with a soluble guanylate cyclase gene called *gcy-32* (Figure 10). *gcy-32* and *glb-28* are co-expressed in the URX oxygen sensing a neuron. There are also 4 additional neurons that expressed *glb-28* that we were unable to identify that may provide additional clues about GLB-28 function.

We utilized multiple reporter lines for *glb-28* as shown in (Figure 11). Using extrachromosomal reporters for expression of fluorescent proteins under control of the *glb-28* promoter region produced inconsistent results (Appendix 5.1). Specifically, our data indicate that the length of the promoter region that we choose to direct the reporter expression greatly affects its expression pattern. 1.8kb promoter length restricted its expression to EVNs and a few other neurons, whereas longer promoter region length of

4.2kb promotes expression in additional neurons (Appendix 5.1). In order to avoid artifacts produced by regulatory sequences in non-native environment of the gene we employed CRISPR/Cas9 mediated genome editing method to fuse endogenous *glb-28* coding region with fluorescent reporter that was slightly modified from literature (Paix, A et al., 2017) and (Dokshin G.A. et a.l, 2018). We chose to design the editing in a way that places the fluorescent tag on the C-terminus of the *glb-28* gene. The CRISPR-generated *glb-28* fused to the coding region of the scarlet (mSc) fluorescent protein (*him-5;glb-28(my70)[glb-28::mscarlet]X)* reproduces the expression pattern of the extrachromosomal reporter of *glb-28* possessing 1.8kb length of its promoter region (Wang, J et al., 2015): CEM neurons, ray RnB neurons, HOB neuron. However, CRISPR tagged *glb-28::mscarlet* is also expressed in the six IL2 neurons and two oxygen sensing URX neurons in the head (Figures 9-10).

We characterized the GLB-28:mSc localization using PKD-2::GFP. PKD-2 was used as a reference marker because it is expressed in neurons important for mating behavior in males and localizes to cilia, cell bodies, dendrites, and extracellular vesicles (EVs). GLB-28 co-localized with PKD-2 in the CEM neurons, ray RnB neurons, HOB neuron. GLB-28 is localized in more neurons than PKD-2 in both the head and tail but we have not identified most of the neurons. Furthermore, GLB-28 is located in the cell bodies, dendrites, and axons but appears excluded from the cilia and extracellular vesicles (Figure 12). In some cases the GLB-28:mSc appears to be aggregated as puncta within the cell and dendrites. The puncta warrant further investigation as it may be mitochondria in the cells and or mitochondria that are migrating. The fusion protein m Scarlet for *glb-28* is a monomeric structure and is unlike red fluorescent protein which is known to form

aggregates. Furthermore, in some locations *glb-28* and *pkd-2* appear co-localized in the perciliary membrane compartment just below the ciliary base.

glb-28 **regulates male mating behaviors**

glb-28 is expressed in male-specific neurons that are important for several mating behaviors such as response behavior, location of vulva, and sex drive (Liu and Sternberg, 1995; Barrios et al., 2008; Srinivasan, J. et al., 2008; R. O'Hagan et al., 2014**)**. Thus, we used mating behavior assays to assess if the male mating behavior neurons are functioning differently in the *him-5;glb-28 (tm6910)* from the positive control *him-5* and negative control, *pkd-2;him-5*. To understand the function of GLB-28 two mutant strains with disrupted *glb-28* sequence were made (Figure 13). The controls for all the behavior experiments include the positive control *him-5* and negative control *pkd-2; him-5*. The mating behavior assays assessed leaving behavior (sex drive), response behavior, vulva location efficiency, and mating efficiency as described in the methods and materials. *glb-28(tm6910)* mutant males have a response defect (Figure 14). The Response behavior is a step in male mating behavior and it requires the use of the 9 bilateral pairs of tail ray of A-type ray neurons and B-type ray neurons (RnA and RnB) in addition to a structural cell (Liu and Sternberg, 1995). RnB ciliated neurons and *pkd-2* have been shown to be essential in sensory ability of the male cilia and generate a wild-type response efficiency with the hermaphrodite (Barr and Sternberg, 1999). The positive control male would stop moving forward, press the ventral side of its tail against the hermaphrodite, and initiate backward movement as it makes contact with the hermaphrodite. The negative control *pkd-2* had a low response efficiency. The *glb-28* mutant response defect has a greater

statistically significant difference between the mutant and the positive control and a smaller statistically significant difference with the negative control.

In addition to decreased response, the strain *him-5;glb-28 (tm6910)* had defects in turning, what appears to be a new phenotype which I called "missed response". For 15 males, here I provide a representative summary shorthand of my mating behavior observations to illustrate this new phenotype called missed response. The symbols are as follows: r, a response that causes the male's ventral placing of the tail on the hermaphrodite; mr, a missed response in which the male's tail curls away from hermaphrodite body; n, no response; t, turns along the hermaphrodite body's head or tail; p, passed vulva without hesitation; ssb, slow search behavior is when the males tail slowly makes fine backward and forward adjustments around the vulva; l, locate; st, sperm transfer into the vulva. *him-5* positive control animals have the behavioral sequences: r/t/l/st or r/t/t l,/l/st which demonstrated no mating behavioral defects. In the *glb-28* deletion allele *tm6910* animals we mostly observed the two behavioral sequences: n/mr or mr which we scored as response defective. The *pkd-2* negative control animals have the behavioral sequence: n which we scored as response defective. In both the *him-5* and *pkd-2* males the mr phenotype is not displayed. The data in Figure 15 shows that the *glb-28* mutant displays a missed response in preliminary observations because the male ceases forward motion and makes backwards movement and curls its tail as if the mate is attempting respond to a hermaphrodite but there are no hermaphrodites to make contact with that are near. In some of the cases in which *glb-28* mutant males would properly respond to the hermaphrodite, the male would sometimes later turn off the hermaphrodite and start curling its tail repeatedly.

The *him-5;glb-28(tm6910)* mutant males have a vulva location efficiency defect as shown by the location of vulva assay (Figure 16). After a response in the positive control sometimes the male would reach the head or tail of the hermaphrodite without detecting the vulva and in this case the male would have to perform a tight turn in order to search the vulva on the other side. Once the male detects the vulva using the hook and post-cloacal sensillae (PCS) neurons it will slow down and search for the vulva's slit by prodding with the male spicules. The positive control was able to locate the vulva with around 90% efficiency (*him-5(e1490)*). The negative control was unable to locate the vulva despite having multiple encounters (*pkd-2*(*sy606*)). The *glb-28* mutant vulva location efficiency is about 35%, which shows a statistically significant reduction from the positive control but did not show a difference from the negative control. The positive and negative control could perform the turning behavior but the *glb-28* mutant appeared to have trouble with turning which often caused the male to slip off the hermaphrodite before vulva location (data not shown).

The mating efficiency assay showed that the *him-5;glb-28(tm6910)* mutant males have reduced mating efficiency (Figure 17). The mating efficiency assay measures the ability for the male to sire offspring. In addition, mating efficency enables one to understand if the gonad and morphology of the male are normal. The positive control had a mating efficiency of about 43%, the negative control had a mating efficiency of 0%, whereas the *glb-28* mutant had a mating efficiency less than 10% which was statistically significant from the positive control.

The leaving assay displays positive control or wild-type males display a preference for leaving the food in search for mates (Barrios et al., 2008). *pkd-2* mutant

males have a preference to stay in the food then search for mates and we call this a sex drive defect. The probability of leaving food per hour is roughly .1 in the positive control and the probability for negative control is about .02. Males similar to wild-type have an instinctive exploratory behavior and search for mates more often than they search for food. Males use RnA and RnB neurons to aid in their search for hermaphrodites (Lipton et al., 2004). *him-5; glb-28* mutant males have a sex drive defect. The *glb-28* mutants had an extremely statistically significant difference to the positive control. The leaving assay data in (Figure 18) suggests that *glb-28* mutant males have a decreased sex drive and more directly show that the males have a tendency to stay within the food similarly to *pkd-2*. This data supports that there may be a problem with the ability for the *glb-28* mutant males to search for mates. It is also possible that the *glb-28* mutant males have defective CEM or RnB ray neurons that are important for mate searching. This could explain why the *glb-28* mutant males have reduced response efficiency and have a missed response phenotype. In my observations I did note that the worm didn't have any obvious locomotion defects because the worm did leave evidence of movement.

GLB-28 does not appear to be required for dealing with hypoxia. In a 48 hour hypoxia treatment on *glb-28(my56)[stop-in])* the data showed no statistically difference in survival (Figure 19). A hypoxia assay with *him-5*;*glb-28(tm6910)* has not been conducted. The strain *him-5;glb-28(my56)[stop-in])* contains premature stop codons and the *him-5*;*glb-28(tm6910)* strain contains a deletion. However, both mutants have severe molecular lesions that delete more than 2/3 of the GLB-28 protein sequence deleted (Figure 13). The hypoxia survival study *glb-28(my56)[stop-in])* didn't show statistically significant data between the positive or negative controls. The strain *him-5;glb-*

28(my56)[stop-in]) contains premature stop codons and it was thought this mutant would give a distinctive phenotype since the globin protein would theoretically more severely affected. We confirmed that the stop codons were added by genotyping and sequencing. However we did not determine if a *glb-28* mRNA or protein product from *him-5;glb-28(my56)[stop-in])* was still being made. In retrospect, it would have been ideal to use the *glb-28 (tm6910)* in hypoxia studies as the deletion allele has already been characterized and displays male mating defects.

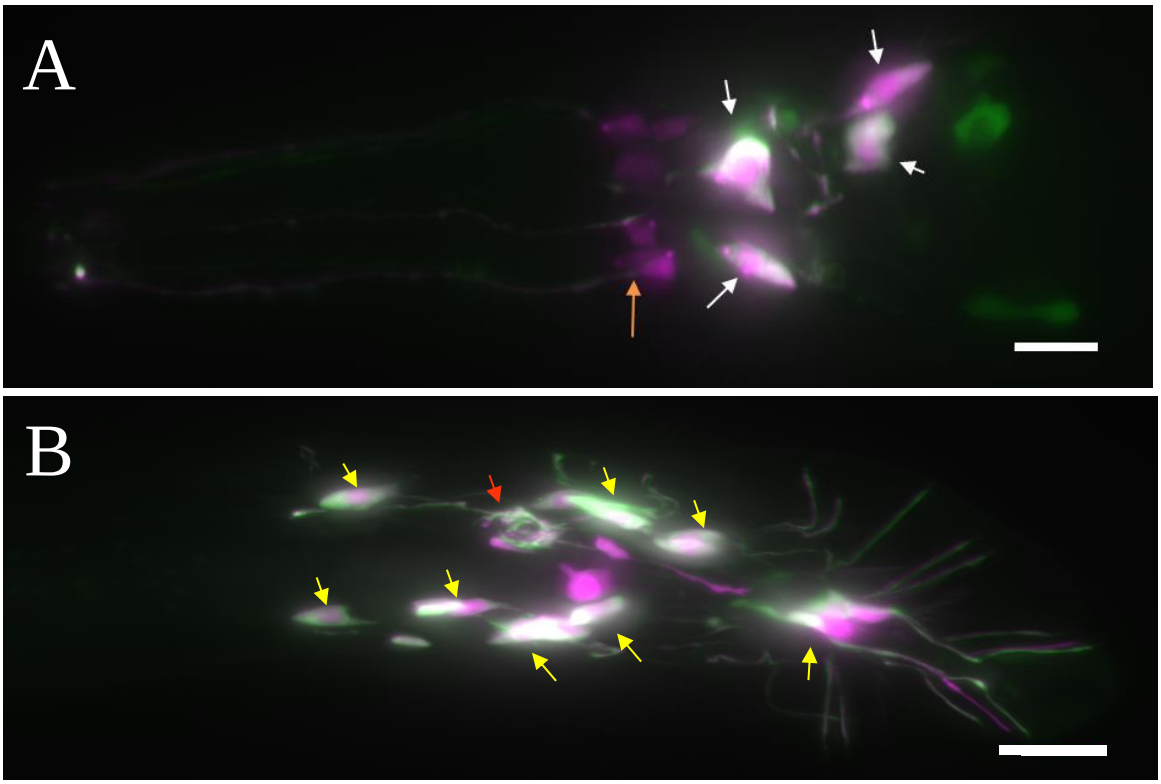


Figure 9 : *pha-1(e2123) III; him-5(e1490) V;myEx857[PCRsoeTGLB-28::GFP(200ng/ul)+pBX(50ng/ul)]; glb-28(my70)[glb-28::mSc])X* CRISPR generated strain shows endogenous *glb-28* is co-expressed with extrachromosomal *glb-28* in CEMs, RnBs, HoB. The extrachromosomal reporter *pha-1(e2123) III; him-5(e1490) V;myEx857[PCRsoeTGLB-28::GFP(200ng/ul)+pBX(50ng/ul)]* strain was crossed into the endogenous reporter *glb-28(my70)[glb-28::mscarlet]X* strain. The magenta reporter is endogenous *glb-28* reporter. In the adult male the extrachromosomal reporter is a green fluorescent protein tagged with *glb-28* known to be in neurons called CEMs, HOB, RnBs. In the panel A the white color indicates co-expression in CEMs in the male head. In the panel B the white color shows co-expression in HoBs and RnBs neurons in the male tail. The white arrows indicate CEMs neurons, yellow arrows indicate RnBs neurons, and the

red arrows indicate HoB neuron. The orange arrow indicates IL2 neurons. The scale bar is 20µm.

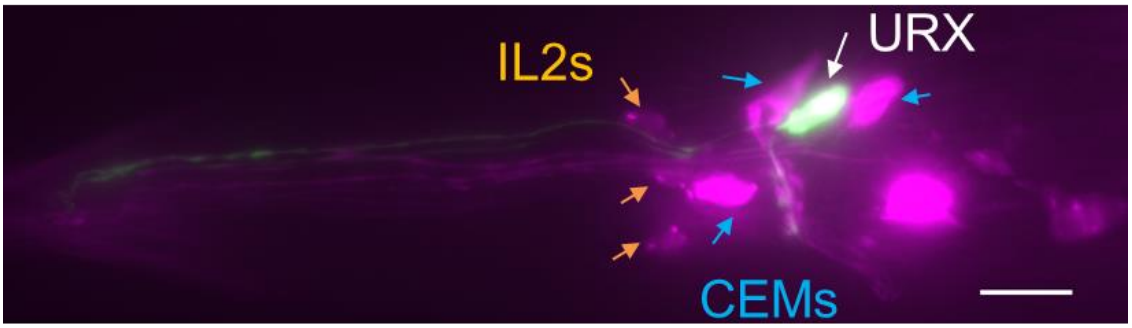


Figure 10: ***wzIs96; [Prom gcy-32:YC3.60+unc-122::RFP]; glb-28(my70)[glb-28::mSc])X*** **shows endogenous** ***glb-28*** **is co-expressed with** ***gcy-32*** **in URX neurons.** The integrated reporter strain *wzIs96[gcy-32p::YC3.60 + unc-122::RFP])* was crossed into *glb-28(my70)[glb-28::mscarlet]X* strain. This is an image of an early L4 male with both markers. The integrated reporter strain shown in green uses the promoter of guanylate cyclase 32 (*gcy-32*) fused with YC3.60. The endogenous *glb-28* reporter is shown in magenta. The white arrow indicates co-expression in the oxygen sensing URX neuron cell body. The orange arrow indicates IL2 neurons and the light blue arrow indicate CEM neurons. The scale bar is 20µm.

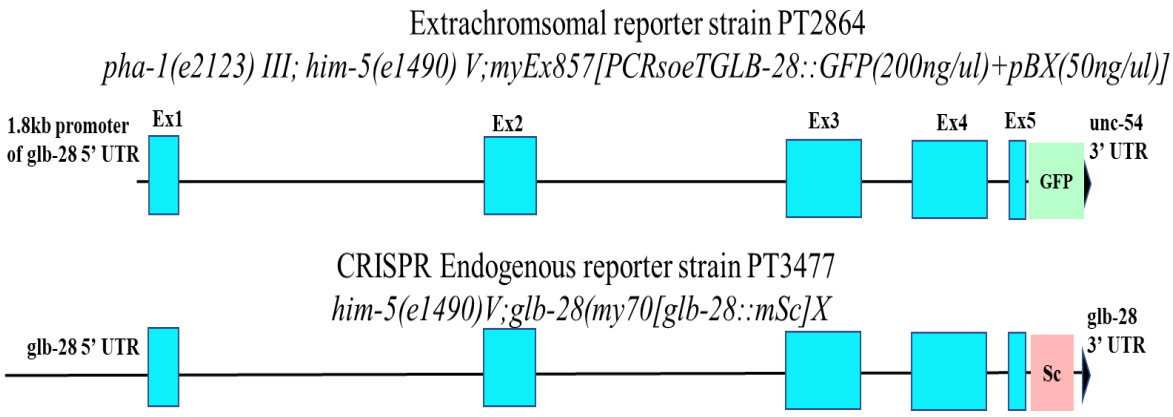


Figure 11: Schematic of fluorescent reporters generated or acquired for the study of *glb-28*. (From top to bottom) The extrachromosomal reporter *pha-1(e2123) III; him-5(e1490) V;myEx857[PCRsoeTGLB-28::GFP(200ng/ul)+pBX(50ng/ul)]* is a plasmid based reporter that has 1.8kb promoter and a GFP fluorescent tag on the C-terminal (Wang, J. et al., 2015). *him-5(e1490)V;glb-28(my70[glb-28::mSc]X* is a CRISPR reporter strain with scarlet fluorescent tag on the C-terminal with endogenous 3' elements. The light blue squares represent the *glb-28* exons (Ex 1-5) and the space in between are introns. The fluorescent tags are indicated by neon green (NG), green fluorescent protein (GFP), and scarlet (Sc). All of these later had a *him-5* mutation added in order to increase the male population.

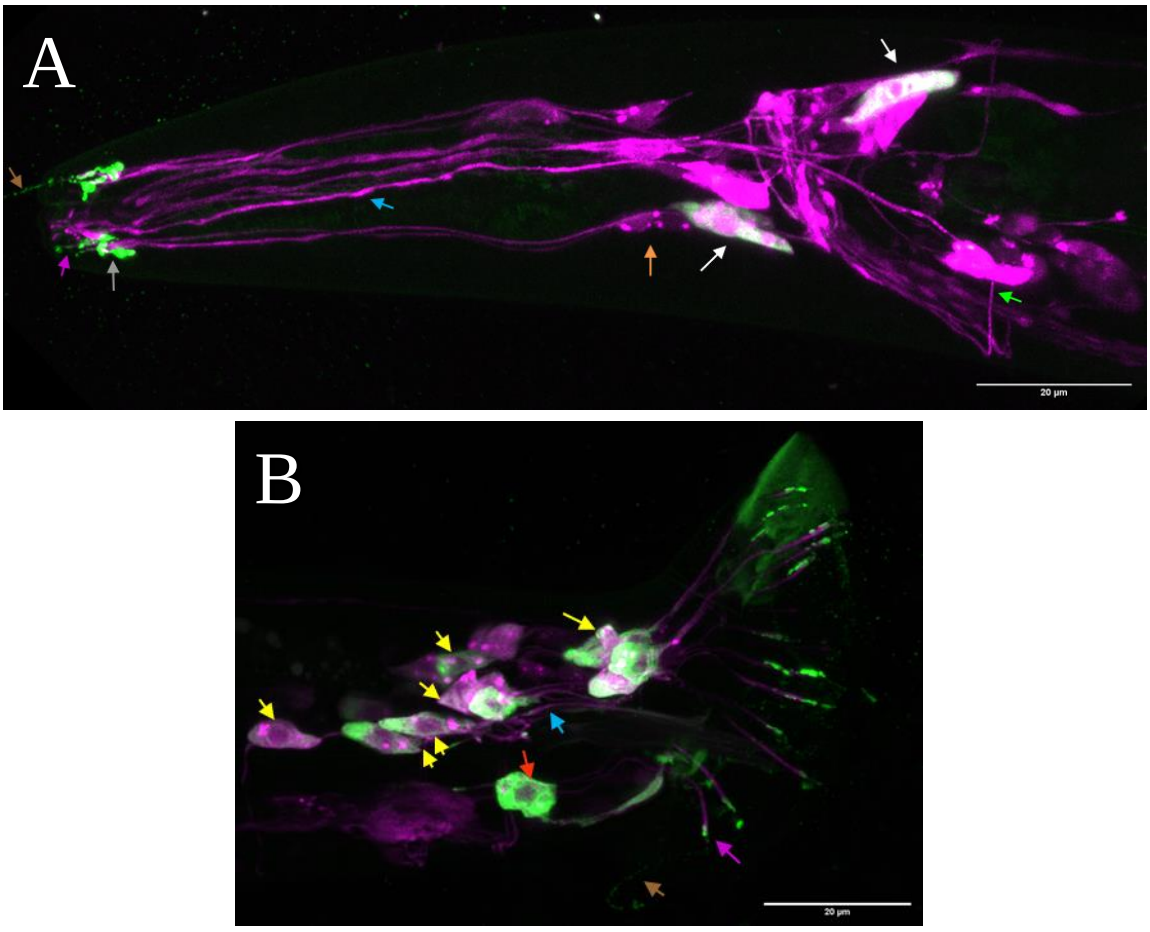


Figure 12: *him-5;glb-28(my70)[glb-28::mscarlet]X* shows *glb-28* is co-expressed with *pkd-2* and localized in dendrites, cell bodies, and axons. The magenta reporter is the endogenous *glb-28* reporter. PKD-2::GFP is localized to CEMs, HOB, RnBs neuronal cell bodies, dendrites, and axons. In the male head on panel A, the white color indicates co-expression in CEMs. In the male tail on panel B, the white color shows co-expression in HOBs and RnBs neurons. The orange arrow indicates IL2 neurons, gray arrows indicate the periciliary membrane compartment (PCMC), magenta arrows are cilia, and brown arrows are EVs. The light blue arrow indicate dendrites, green arrow indicate

axons, white arrows indicate CEMs neurons, yellow arrows indicate RnBs neurons, and the red arrows indicate HOB neuron. The scale bar is 20µm.

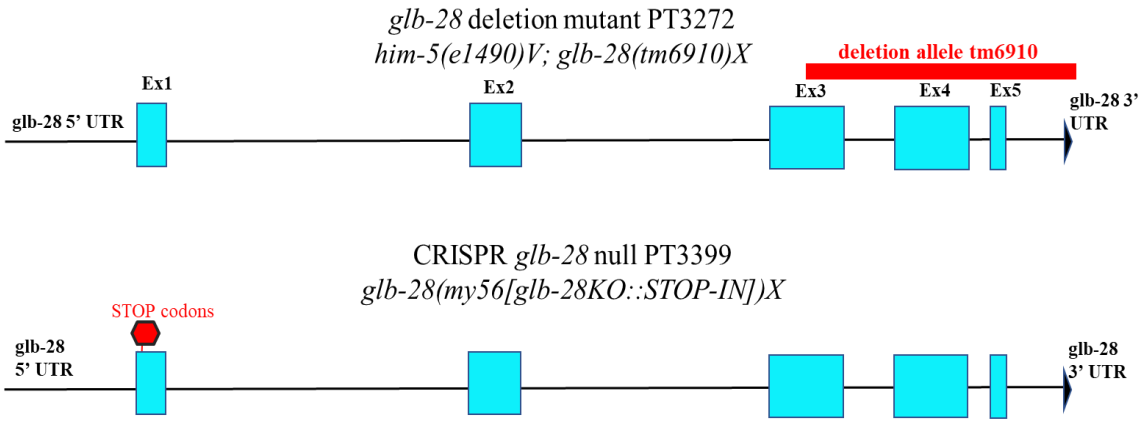


Figure 13: Schematic of mutations generated for the study of *glb-28*. (From top to bottom) *glb-28(tm6910)* deletion allele affects exons (Ex) 3-5 and affects a portion of the globin protein domain in the strain *him-5(e1490)V;glb-28(tm6910)X*. *him-5;glb-28(my56)[stop-in])* is a CRISPR strain that inserted multiple stop codons and should prevent protein product as an alternative to creating a knockout. The light blue squares are representative of the *glb-28* exons (Ex) 1-5 and the space in between are introns. All of these later had a *him-5* mutation added in order to increase the male population.

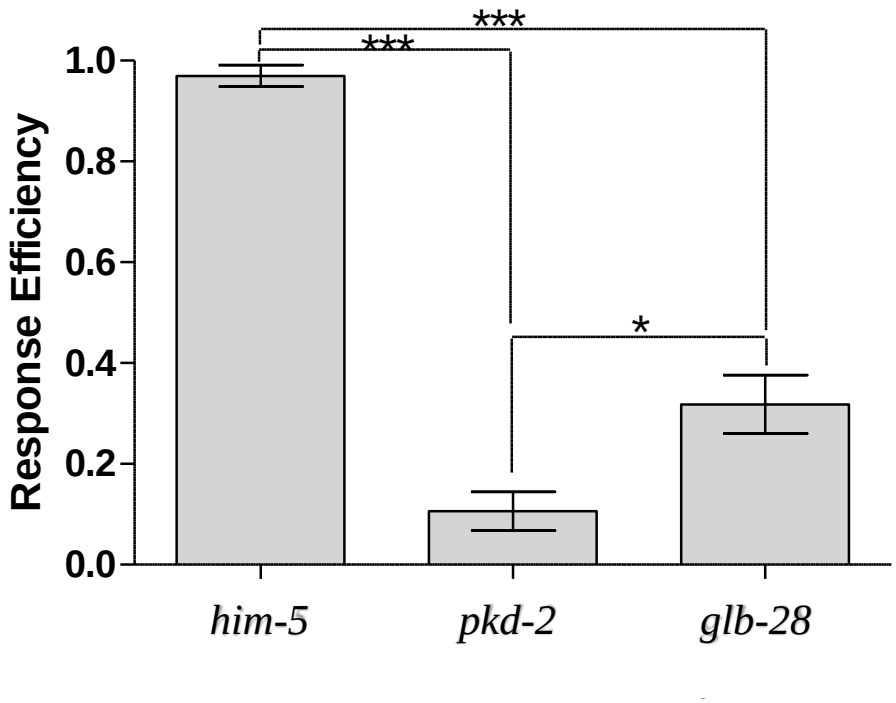


Figure 14: *glb-28(tm6910)* mutant males have a response defect. The mutant *him-5;glb-28 (tm6910)* is the experimental strains, *pkd-2* is a negative control and *him-5* is the positive control. Experiment was repeated in 5 trials with a total n=66 for each control and strain. These are the results of a nonparametric one-way ANOVA (Kruskal–Wallis test) with a Dunn's multiple comparison test. Two tailed bars indicate standard error of mean. Significance is shown as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

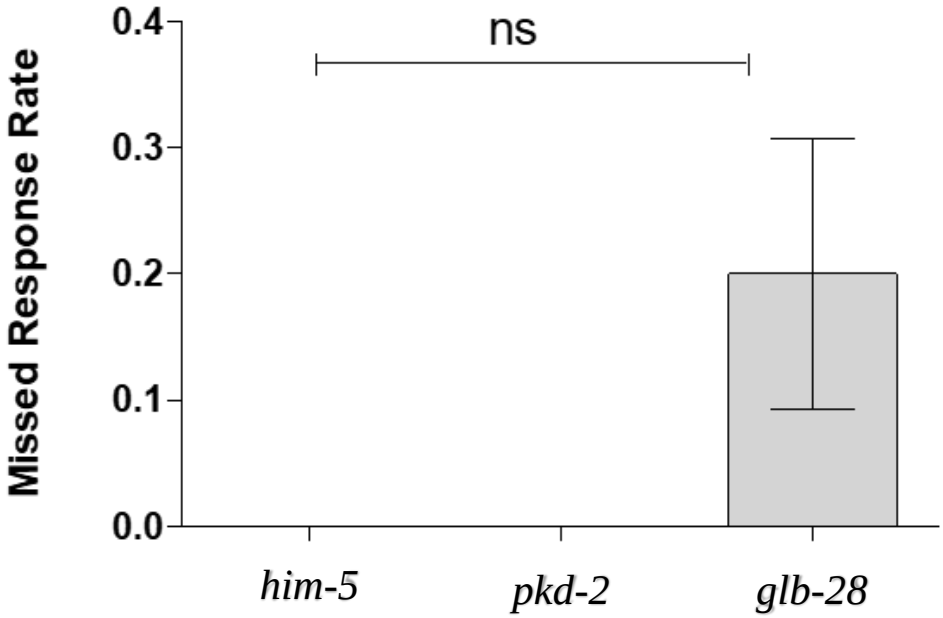


Figure 15: *glb-28(tm6910)* mutants have a novel mating phenotype called "missed response". The mutant *him-5;glb-28 (tm6910)* is the experimental strain, *pkd-2* is the negative control and *him-5* is the positive control. There is not any statistically significant difference amongst the experimental strain, positive control or negative control (n=15) but it's likely due to the low sample size. This experiment was done once. These are the results of a nonparametric one-way ANOVA (Kruskal–Wallis test) with a Dunn's multiple comparison test. Two tailed bars indicate standard error of mean.

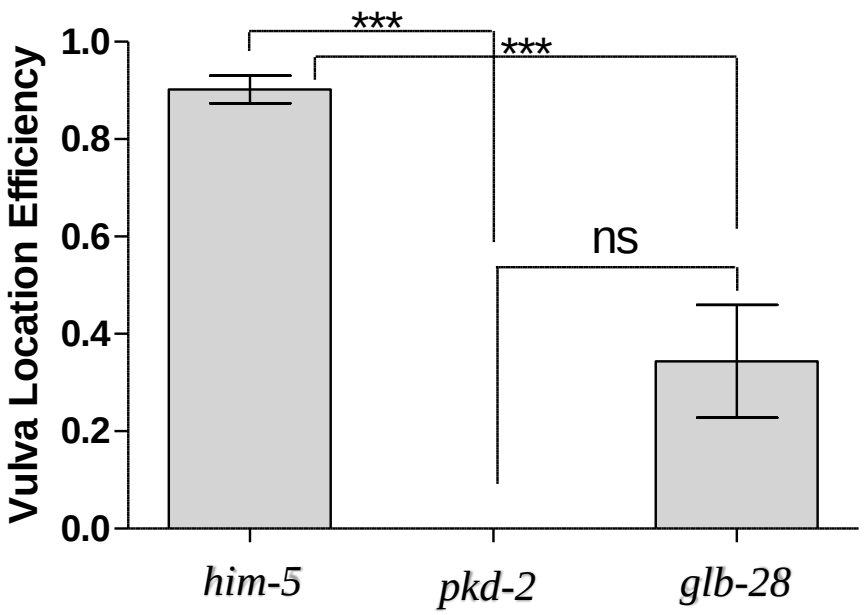


Figure 16: *glb-28(tm6910)* mutant males have a vulva location efficiency defect. Experiments were done in triplicates. The mutant *him-5;glb-28 (tm6910)* is the experimental strain, *pkd-2* is a negative control and *him-5* is the positive control. The experimental strain (n=13), negative control (n=6), and positive control (n=63). These are the results of a nonparametric one-way ANOVA (Kruskal–Wallis test) with a Dunn's multiple comparison test. Two tailed bars indicate standard error of mean. Significance is shown as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

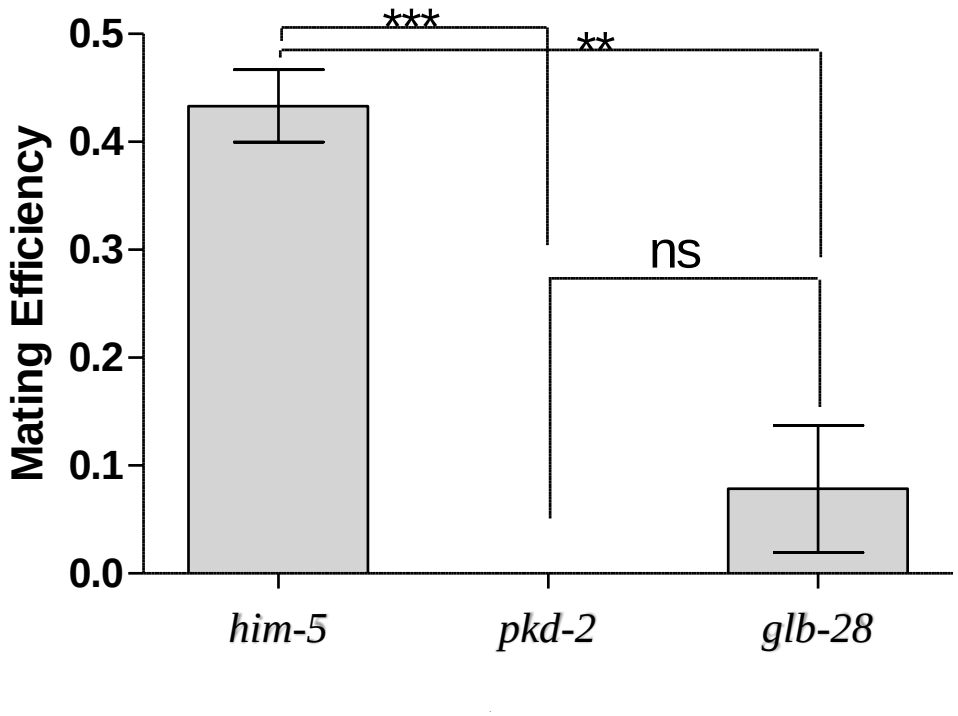


Figure 17: *glb-28(tm6910)* mutants have reduced mating efficiency. The mutant *him-5;glb-28 (tm6910)* is the experimental strain, *pkd-2* is the negative control and *him-5* (WT) is the positive control. Experiment was repeated in biological triplicates (n=18). These are the results of a nonparametric one-way ANOVA with a Tukey's multiple comparison test. Two tailed bars indicate standard error of mean. Significance is shown as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

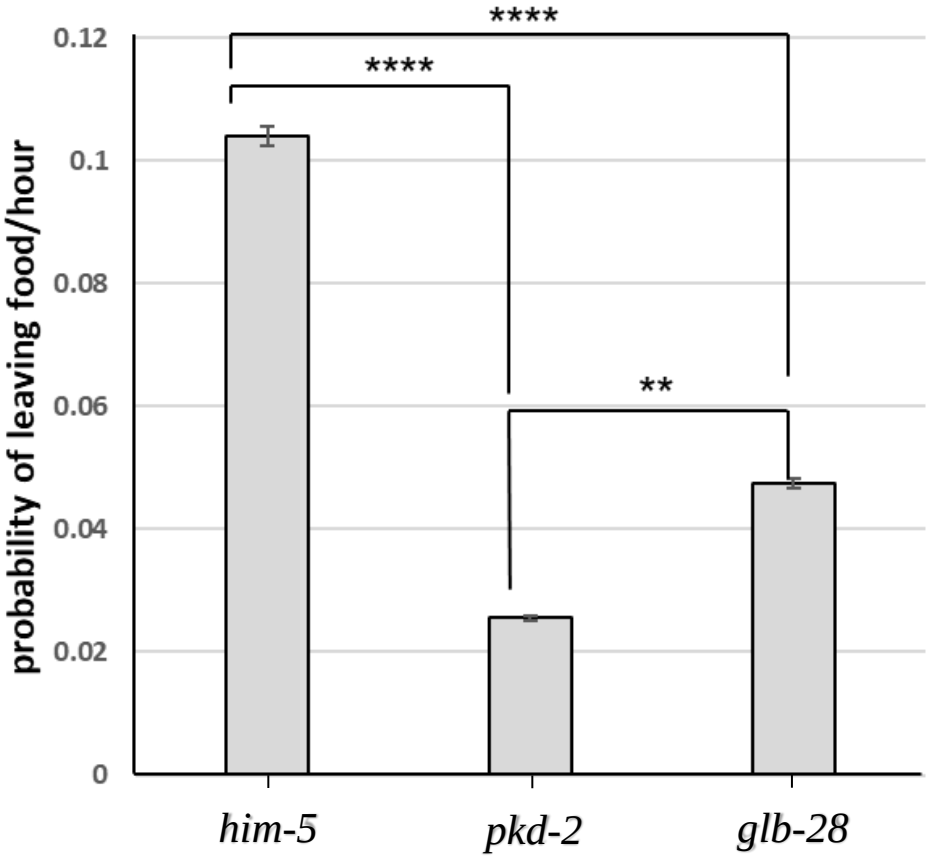


Figure 18: *glb-28(tm6910)* mutant males have a sex drive defect in the leaving assay. The graphs shows P_L (probability of leaving food/hour) across multiple animal strains. The mutant *him-5;glb-28 (tm6910)* is the experimental strain, *pkd-2* is a negative control and wild-type (WT) or *him-5* is the positive control. Experiment was repeated 4 times with an n=80 for the sum of the all experiments. The maximum likelihood statistical analysis was used to compare P_L values across samples. Two tailed bars indicate standard error of mean. Significance is shown as *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001.

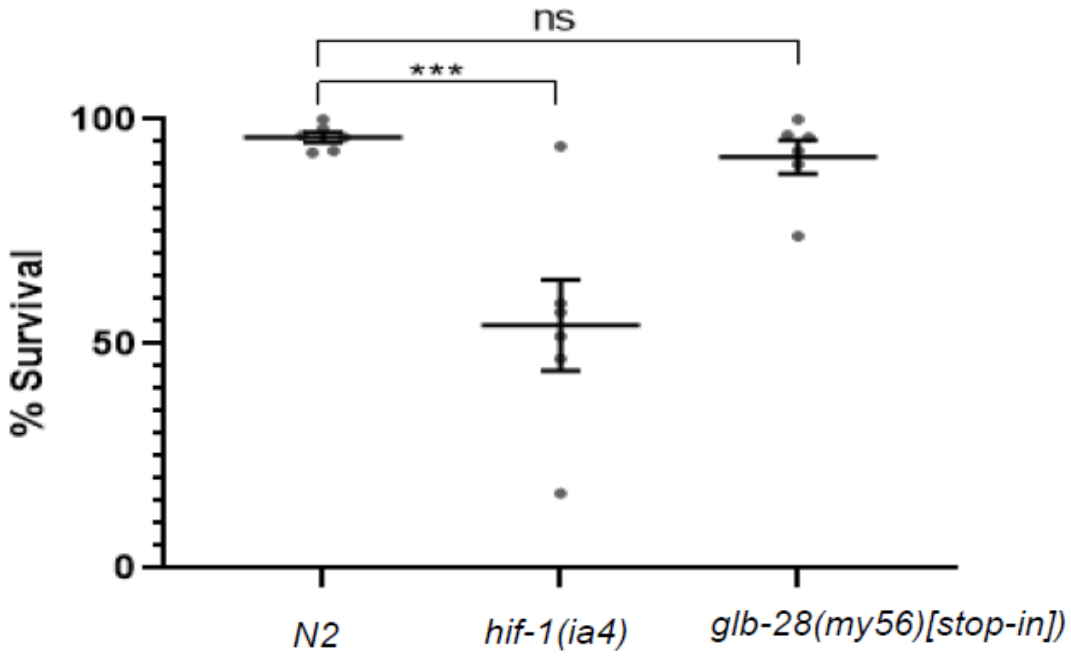


Figure 19: A 48 hour hypoxia survival study on *glb-28(my56)[stop-in])* shows no statistical difference in survival. *N2* is the wild-type control, *hif-1(ia4*) is the negative control, and *him-5;glb-28(my56)[stop-in])* is a *glb-28* putative null. The negative control is extremely statistically significant to the positive control ***$p < 0.001$. The *glb-28* mutant shows no statistical difference.

CHAPTER 4

Conclusions

In this thesis, I show that *glb-28* plays a role in multiple steps of mating behavior, sex drive, and mating efficiency. GLB-28 was shown to be expressed with polycystin PKD-2 and have similar defects as PKD-2 with regards to mating behavior, sex drive, and mating efficiency. However, the defects in response, vulva location and sex drive phenotypes in the *glb-28* mutant is less severe than *pkd-2* mutants. *glb-28* is expressed in male specific neurons and as a result displays sexual dimorphic expression. This is the only work that has shown a globin to be associated with polycystin-mediated male mating phenotypes and have male specific expression patterns. Globins have previously been shown to be involved with oxygen metabolism, enzymatic function, nitric oxide metabolism, reproduction, redox function, and behavior with regards to hypoxia, feeding, and aggregation. The current work presented here indicates that GLB-28 may decrease the ability for ciliated neurons to sense chemosensory and mechanosensory cues.

glb-28's expression and localization patterns

The expression of *glb-28* suggest that the gene is important in the *C. elegans* nervous system as it is expressed in over 28 neurons. In addition, the extrachromosomal reporter for *glb-28* is slightly different from the transgenic endogenous reporter for *glb-28*. The extrachromosomal reporter's expression did not include the 6 IL2 EVNs and 2 unidentified tail neurons. There are also 5 additional unidentified head neurons that are expressed with the extrachromosomal reporter. The differing expression pattern is likely due to differences in the promoter region. Furthermore, additional extrachromosomal reporters of varying lengths showed unique expression patterns (Appendix 5.1). The

differing expression pattern supports the idea that *glb-28* is influenced by the promoter element. In addition, *glb-28* is likely to be developmentally regulated because it has a sexually dimorphic pattern due to its expression in male specific neurons. Although, there are additional unspecified *glb-28* expressing neurons in the hermaphrodite, we were interested mostly in male specific neurons. We were only able to confirm expression of *glb-28* in the URX neurons by examining for an overlapping expression pattern with *gcy-32::YC3.60*.

Furthermore, the co-localization of GLB-28 with PKD-2 suggest that GLB-28 may use a similar signaling pathway to *pkd-2*. The data supports the hypothesis that *glb-28 i*s involved with neurons that are involved with chemosensation and mechanosensation.

***glb-28* has a broad role in behaviors**

Previously there were no phenotypes for *glb-28*, and many globin proteins remained uncharacterized. I revealed that GLB-28 is required for the male-specific neurons to appropriately function. GLB-28 regulates response, vulva location, mating efficiency, and sex drive. It is uncertain as to how GLB-28 can play such a huge role in functions that are normally related to polycystins. It would be interesting to determine the function of *glb-28* in the other neurons it is expressed in. The mechanism for how *glb-28* is affecting chemosensation and mechanosensation needs to be understood.

Future directions

EV biogenesis regulation by *glb-28*

The role of GLB-28 in ciliary extracellular vesicle biogenesis, shedding or release should be examined (Wang, J. et al., 2015). It would be interesting to establish a connection between the biogenesis of EVs and a globin gene using the strain *myIs1[Ppkd-2::GFP + ccGFP]pkd-2(sy606)IV;him-5(e1490) V;glb-28(tm6910)X* . This strain has a marker for EV cargo called *myIs1* and it is a double mutant for *pkd-2* and *glb-28*. It is already established that *pkd-2* is a regulator of EV cargo (Wang, J. et al., 2015).

Determine *glb-28's* role in aerotaxis, life span regulation, suppression of innate immunity

The observation of *glb-28* being expressed in the gas sensing URX neuron warrants the study of *glb-28* in aerotaxis, life span regulation (Liu and Cai; 2013), and suppression of innate immunity (Styer et al., 2008). I also showed GLB-28 to be associated with *gcy-32* an oxygen binding protein. The function of GLB-28 in aerotaxis can be deduced by imaging the strain *wzls96; [Prom gcy-32:YC3.60+unc-122::RFP]; glb-28(my70)[glb-28::mSc])X* while doing calcium imaging in different oxygen parameters (Gray et al., 2004). In addition, the function of GLB-28 in life span regulation (Liu and Cai; 2013), and suppression of innate immunity (Styer et al., 2008) can be studied in the strain *him-5;glb-28(tm6910)* with the assays mentioned in the literature.

Dauer regulation by *glb-28*

Although dauer is called the alternative development, *C. elegans* populations are primarily dauer in the wild (Barrière and Félix, 2005). The role of GLB-28 in dauer

should be examined because GLB-28 is in IL2 neurons which are required for the animal to have nicitation behavior in dauer which is a behavior in which large groups of worms amass together raise their bodies upward. The nicitation behavior is conducive to being picked up and dispersed into a new location (Lee H, et al., 2013). In addition, GLB-28 may have a greater function in dauer than the other developmental stages that I examined. In my preliminary work I observed that a unique *glb-28* expression persisted in dauer worms (Appendix 5.2). We determined a *glb-28* expression pattern that is specific to the dauer stage and specific to animals post-dauer. However, I did not quantitatively analyze an increase in *glb-28* expression post-dauer or observe if the *glb-28* neurons functioned differently after exiting dauer. It would be informative to confirm if *glb-28* is responsive to developmental regulation.

The status of *glb-28*

There are still many unaddressed questions we can ask about *glb-28* and from the data I gathered I think we have even more reason to answer those questions. In summary we showed that *glb-28* is much more widely distributed in neurons than originally reported and we established a function for *glb-28* for the first time. Our data supported *glb-28*'s role in male specific mating behaviors. It is possible that this function was not discovered earlier because many researchers do not study the male worms and many researchers focused emphasized globins as proteins related to gases. Altogether, this thesis gives the most comprehensive report on a function for *glb-28* and illustrates *glb-28* localization in greater detail. More researchers need to take time to study both sexes of worms, study function of a protein beyond what the literature or domain suggests and utilize CRISPR to generate endogenous reporters and mutations.

REFERENCES

Abergel, Z., Chatterjee, A. K., Zuckerman, B., & Gross, E. (2016). Regulation of Neuronal Oxygen Responses in C. elegans Is Mediated through Interactions between Globin 5 and the H-NOX Domains of Soluble Guanylate Cyclases. The Journal of neuroscience : the official journal of the Society for Neuroscience, 36(3), 963–978. https://doi.org/10.1523/JNEUROSCI.3170-15.2016

AbouAlaiwi, W. A., Takahashi, M., Mell, B. R., Jones, T. J., Ratnam, S., Kolb, R. J., & Nauli, S. M. (2009). Ciliary polycystin-2 is a mechanosensitive calcium channel involved in nitric oxide signaling cascades. *Circulation research, 104*(7), 860–869. https://doi.org/10.1161/CIRCRESAHA.108.192765

Barr, M.M., DeModena, J., Braun, D., Nguyen, C.Q., Hall, D.H., and Sternberg, P.W. (2001). The Caenorhabditis elegans autosomal dominant polycystic kidney disease gene homologs lov-1 and pkd-2 act in the same pathway. Curr Biol 11, 1341-1346.

Barr, M. Male mating behavior. (2006). *WormBook* doi:10.1895/wormbook.1.78.1.

Barr, M. M., & Sternberg, P. W. (1999). A polycystic kidney-disease gene homologue required for male mating behaviour in C. elegans. Nature, 401(6751), 386–389. https://doi.org/10.1038/43913

Barrière, A., and Félix, M.-A. (2005). High local genetic diversity and low outcrossing rate in *Caenorhabditis elegans* natural populations. Current Biology *15*, 1176–1184.

Barrios, A., Nurrish, S. & Emmons, S. W. (2008). Sensory Regulation of C. elegans Male Mate-Searching Behavior. *Curr. Biol.* **18**, 1865–1871 (2008).

Blank, M., Wollberg, J., Gerlach, F., Reimann, K., Roesner, A., Hankeln, T., … Burmester, T. (2011). A Membrane-Bound Vertebrate Globin. *PLOS ONE*, *6*(9), e25292. Retrieved from https://doi.org/10.1371/journal.pone.0025292

Brenner S. (1974). The genetics of Caenorhabditis elegans. *Genetics, 77*(1), 71–94.

Cassada, R.C. and Russell, R.L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. Dev. Biol. **46:** 326-342. Abstract

Chang AJ, Chronis N, Karow DS, Marletta MA, Bargmann CI (2006) A Distributed Chemosensory Circuit for Oxygen Preference in C. elegans. PLOS Biology 4(9): e274. https://doi.org/10.1371/journal.pbio.0040274

Chasnov, J. R., So, W. K., Chan, C. M., & Chow, K. L. (2007). The species, sex, and stage specificity of a Caenorhabditis sex pheromone. Proceedings of the National

Academy of Sciences of the United States of America, 104(16), 6730–6735. doi:10.1073/pnas.0608050104

Chen, N., Mah, A., Blacque, O. E., Chu, J., Phgora, K., Bakhoum, M. W., Newbury, C. R., Khattra, J., Chan, S., Go, A., Efimenko, E., Johnsen, R., Phirke, P., Swoboda, P., Marra, M., Moerman, D. G., Leroux, M. R., Baillie, D. L., & Stein, L. D. (2006). Identification of ciliary and ciliopathy genes in Caenorhabditis elegans through comparative genomics. Genome biology, 7(12), R126. https://doi.org/10.1186/gb-2006-7-12-r126

Concordet, J. P., & Haeussler, M. (2018). CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic acids research*, *46*(W1), W242–W245. https://doi.org/10.1093/nar/gky354

De Henau, S., Tilleman, L., Vangheel, M., Luyckx, E., Trashin, S., Pauwels, M., Braeckman, B. P. (2015). A redox signalling globin is essential for reproduction in Caenorhabditis elegans. *Nature Communications*, *6*(1), 8782. https://doi.org/10.1038/ncomms9782

Dokshin, G.A., Ghanta, K.S., Piscopo, K.M. & Mello, C.C. (2018). Robust Genome Editing with Short Single-Stranded and Long, Partially Single-Stranded DNA Donors in Caenorhabditis elegans. Genetics 210, 781

Fagan, K. A., & Portman, D. S. (2014). Sexual modulation of neural circuits and behavior in Caenorhabditis elegans. Seminars in cell & developmental biology, 33, 3–9. https://doi.org/10.1016/j.semcdb.2014.06.007

Fago A, Hundahl C, Malte H, Weber RE (2004). "Functional properties of neuroglobin and cytoglobin. Insights into the ancestral physiological roles of globins". *IUBMB Life*. **56** (11–12): 689–96. doi:10.1080/15216540500037299. PMID 15804833

Geuens E, Brouns I, Flamez D, Dewilde S, Timmermans JP, and Moens L (2003) A globin in the nucleus! *J Biol Chem* **278:** 30417-30420.

Golden JW, Riddle DL. (1982). A pheromone influences larval development in the nematode Caenorhabditis elegans. Science. 218(4572):578–580. doi:10.1126/science.6896933

Golden JW, Riddle DL. (1984). A pheromone-induced developmental switch in Caenorhabditis elegans: Temperature-sensitive mutants reveal a wild-type temperature-dependent process. Proc Natl Acad Sci U S A. 1984;81(3):819–823. doi:10.1073/pnas.81.3.819

Gray, JM; Karow, DS; Lu, H; Chang, AJ; Chang, JS; Ellis, RE; Marletta, MA; Bargmann, CI. (2004) "Oxygen sensation and social feeding mediated by a C-elegans

guanylate cyclase homologue." Nature 430(6997): 317-322. http://hdl.handle.net/2027.42/62676

Hedgecock, E.M., Culotti, J.G., Thomson, J.N., and Perkins, L.A. (1985). Axonal guidance mutants of *Caenorhabditis elegans* identified by filling sensory neurons with fluorescein dyes. Dev. Biol. *111*, 158–170. Abstract Article

Hodgkin, J., H. R. Horvitz and S. Brenner. (1979). Nondisjunction mutants of the nematode Caenorhabditis elegans. Genetics 91: 67-94.

Hodgkin J. (1983). Male Phenotypes and Mating Efficiency in CAENORHABDITIS ELEGANS. Genetics, 103(1), 43–64.

Hoogewijs D, Geuens E, Dewilde S. (2007). Wide diversity in structure and expression profiles among members of the Caenorhabditis elegans globin protein family. BMC Genomics. 2007;8:356. Published 2007 Oct 4. doi:10.1186/1471-2164-8-356

Hoogewijs D, Geuens E, Dewilde S, Moens L, Vierstraete A, Vinogradov S, Vanfleteren J (2004) Genome-wide analysis of the globin gene family of *C. elegans*. IUBMB Life 56:697–702

Hoogewijs, D., De Henau, S., Dewilde, S. (2008). The *Caenorhabditis* globin gene family reveals extensive nematode-specific radiation and diversification. *BMC Evol Biol* **8,** 279. https://doi.org/10.1186/1471-2148-8-279

Hoogewijs, D., Houthoofd, K., Matthijssens, F., Vandesompele, J., & Vanfleteren, J. R. (2008). Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in C. elegans. *BMC molecular biology*, *9*, 9. doi:10.1186/1471-2199-9-9

Igarashi, P., & Somlo, S. (2002). Genetics and pathogenesis of polycystic kidney disease. Journal of the American Society of Nephrology : JASN, 13(9), 2384–2398. https://doi.org/10.1097/01.asn.0000028643.17901.42

Jiang H., Guo R., Powell-Coffman J. A., (2001). The *Caenorhabditis elegans hif-1* gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. Proc. Natl. Acad. Sci. USA 98: 7916–7921.

Khan AA, Wang Y, Sun Y, Mao XO, Xie L, Miles E, Graboski J, Chen S, Ellerby LM, Jin K, Greenberg DA. (2006). Neuroglobin-overexpressing transgenic mice are resistant to cerebral and myocardial ischemia. *Proc Natl Acad Sci U S A*. 2006 Nov 21;103(47):17944-8. doi: 10.1073/pnas.0607497103. Epub 2006 Nov 10. PMID: 17098866; PMCID: PMC1693852.

Larsen, P.L. (1993). Aging and resistance to oxidative damage in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA. **90**: 8905-8909. Article

Lee H, Choi MK, Lee D, (2011). Nictation, a dispersal behavior of the nematode Caenorhabditis elegans, is regulated by IL2 neurons [published correction appears in Nat Neurosci. 2013 Dec;16(12):1906]. Nat Neurosci. 2011;15(1):107–112. Published 2011 Nov 13. doi:10.1038/nn.2975

Lipton, J. (2004). Mate Searching in Caenorhabditis elegans: A Genetic Model for Sex Drive in a Simple Invertebrate. *J. Neurosci.* **24**, 7427–7434

Liu, T., Kim, K., Li, C. & Barr, M. M. (2007). FMRFamide-Like Neuropeptides and Mechanosensory Touch Receptor Neurons Regulate Male Sexual Turning Behavior in Caenorhabditis elegans. *J. Neurosci.* **27**, 7174–7182

Nagai, T., Yamada, S., Tominaga, T., Ichikawa, M., & Miyawaki, A. (2004). Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(29), 10554 LP – 10559. https://doi.org/10.1073/pnas.0400417101

Nauli, S. M., Kawanabe, Y., Kaminski, J. J., Pearce, W. J., Ingber, D. E., & Zhou, J. (2008). Endothelial cilia are fluid shear sensors that regulate calcium signaling and nitric oxide production through polycystin-1. *Circulation*, *117*(9), 1161–1171. https://doi.org/10.1161/CIRCULATIONAHA.107.710111

Oda, S., Toyoshima, Y., & de Bono, M. (2017). Modulation of sensory information processing by a neuroglobin in Caenorhabditis elegans. *Proceedings of the National Academy of Sciences*, *114*(23), E4658 LP-E4665. https://doi.org/10.1073/pnas.1614596114

Paix, A., Folkmann, A., Seydoux, G. (2017). Precision genome editing using CRISPR-Cas9 and linear repair templates in C. elegans, Methods, Volumes 121–122, 2017, Pages 86-93, ISSN 1046-2023,https://doi.org/10.1016/j.ymeth.2017.03.023.

Peden, E.M., and Barr, M.M. (2005). The KLP-6 kinesin is required for male mating behaviors and polycystin localization in *Caenorhabditis elegans*. Curr. Biol. *15*, 394–404. Abstract Article

Persson A, Gross E, Laurent P, Busch KE, Bretes H, de Bono M (2009) Natural variation in a neural globin tunes oxygen sensing in wild Caenorhabditis elegans. Nature 458:1030–1033, doi:10.1038/nature07820, pmid:19262507.

Pesce A, Dewilde S, Nardini M, Moens L, Ascenzi P, Hankeln T, Burmester T, Bolognesi M (2003). "Human brain neuroglobin structure reveals a distinct mode of

controlling oxygen affinity". Structure. 11 (9): 1087–95. doi:10.1016/S0969-2126(03)00166-7. PMID 12962627

Pradhan, S., Quilez, S., Homer, K., & Hendricks, M. (2019). Environmental Programming of Adult Foraging Behavior in C. elegans. Current biology : CB, 29(17), 2867–2879.e4. https://doi.org/10.1016/j.cub.2019.07.045

Rosenfeld, L. G., Bacal, N. S., Cuder, M. A., Silva, A. G., Machado, Í. E., Pereira, C. A., Malta, D. C. (2019). Prevalence of hemoglobinopathies in the Brazilian adult population: National Health Survey 2014-2015. *Revista Brasileira de Epidemiologia*, *22*(Suppl. 2), E190007.SUPL.2. Epub October 07, 2019.https://dx.doi.org/10.1590/1980-549720190007.supl.2

Schroeder, N. E., Androwski, R. J., Rashid, A., Lee, H., Lee, J., & Barr, M. M. (2013). Dauer-specific dendrite arborization in C. elegans is regulated by KPC-1/Furin. *Current biology : CB*, *23*(16), 1527–1535. https://doi.org/10.1016/j.cub.2013.06.058

Srinivasan, J., Kaplan, F., Ajredini, R., Zachariah, C., Alborn, H. T., Teal, P. E., Malik, R. U., Edison, A. S., Sternberg, P. W., & Schroeder, F. C. (2008). A blend of small molecules regulates both mating and development in Caenorhabditis elegans. *Nature*, *454*(7208), 1115–1118. https://doi.org/10.1038/nature07168

Smith, E.S.J., Martinez-Velazquez, L. & Ringstad, N. A Chemoreceptor That Detects Molecular Carbon Dioxide. *Journal of Biological Chemistry* **288**, 37071-37081 (2013).

Taira M, Imamura M, Takahashi A, Kamatani Y, Yamauchi T, Araki S-i, et al. (2018) A variant within the *FTO* confers susceptibility to diabetic nephropathy in Japanese patients with type 2 diabetes. PLoS ONE 13(12): e0208654. https://doi.org/10.1371/journal.pone.0208654

The C. elegans Deletion Mutant Consortium. Large-Scale Screening for Targeted Knockouts in the Caenorhabditis elegans. Genome. *G3: Genes|Genomes|Genetics* **2**, 1415 (2012).

Tilleman, L., Germani, F., De Henau, S., Geuens, E., Hoogewijs, D., Braeckman, B.P., Vanfleteren, J.R., Moens, L., and Dewilde, S. (2011). Globins in *Caenorhabditis elegans*. IUBMB Life *63*, 166-174.

Vinogradov SN, Hoogewijs D, Bailly X, Arredondo-Peter R, Gough J, Dewilde S, Moens L, Vanfleteren JR. (2006). A phylogenomic profile of globins. BMC Evol Biol. 2006 Apr 7;6:31. doi: 10.1186/1471-2148-6-31. PMID: 16600051; PMCID: PMC1457004.

Wang J, Silva M, Haas LA, Morsci NS, Nguyen KC, Hall DH, Barr MM. (2014). C. elegans Ciliated Sensory Neurons Release Extracellular Vesicles that Function in Animal Communication. Curr Biol. 2014;24:519–525

Wang, J., Kaletsky, R., Silva, M., Williams, A., Haas, L. A., Androwski, R. J., Landis, J. N., Patrick, C., Rashid, A., Santiago-Martinez, D., Gravato-Nobre, M., Hodgkin, J., Hall, D. H., Murphy, C. T., & Barr, M. M. (2015). Cell-Specific Transcriptional Profiling of Ciliated Sensory Neurons Reveals Regulators of Behavior and Extracellular Vesicle Biogenesis. Current biology : CB, 25(24), 3232–3238. https://doi.org/10.1016/j.cub.2015.10.057

Wang, H., Park, H., Liu, J. & Sternberg, P.W. (2018). An Efficient Genome Editing Strategy To Generate Putative Null Mutants in Caenorhabditis elegans Using CRISPR/Cas9. *G3: Genes|Genomes|Genetics* **8**, 3607

Wang, J. & Barr, M. M. (2016). Ciliary Extracellular Vesicles: Txt Msg Organelles. *Cellular and Molecular Neurobiology* vol. 36 449–457

White JG, Southgate E, Thomson JN, Brenner S. (1986). The structure of the nervous system of the nematode Caenorhabditis elegans. Philos Trans R Soc Lond B Biol Sci. 1986;314(1165):1–340. doi:10.1098/rstb.1986.0056

Zimmer M, Gray JM, Pokala N, Chang AJ, Karow DS, Marletta MA, Hudson ML, Morton DB, Chronis N, Bargmann CI. (2009). Neurons detect increases and decreases in oxygen levels using distinct guanylate cyclases. Neuron 61:865–879, doi:10.1016/j.neuron.2009.02.013, pmid:19323996

Zuckerman, B., Abergel, Z., Zelmanovich, V., Romero, L., Abergel, R., Livshits, L., Smith, Y., & Gross, E. (2017). Characterization of gene expression associated with the adaptation of the nematode C. elegans to hypoxia and reoxygenation stress reveals an unexpected function of the neuroglobin GLB-5 in innate immunity. Free radical biology & medicine, 108, 858–873. https://doi.org/10.1016/j.freeradbiomed.2017.05.007

APPENDICES (UNPUBLISHED DATA)

5.0 R software code for leaving assay

Jonathon Walsh of the Barr lab and John Favate rewrote some of the code used to calculate the leaving assay (P_L) values and run statistics.

5.1 *glb-28* expression is influenced by promoter length and site of tagging and displays sexual dimorphism

A GLB-28 extrachromosomal translational reporter strain *pha-1; him-5; MyEx918 [glb-28p::sfGFP:: GLB-28+pBX]* was generated with a longer promoter with 4.2kb from with GFP tag in the N terminal, 3' endogenous UTR, and pBX rescue allele. The construct was generated by Gibson assembly fusing a previously generated 4.2kb transcriptional reporter with *glb-28* coding sequence and its endogenous 3' UTR. This GLB-28 extrachromosomal reporter had an expression pattern that was different from the extrachromosomal reporter *pha-1(e2123) III; him-5(e1490) V;myEx857[PCRsoeTGLB-28::GFP(200ng/ul)+pBX(50ng/ul)]*. The males with *pha-1; him-5; myEx918 [glb-28p::sfGFP:: GLB-28+pBX]* reporter or larger extrachromosomal reporter was in 7 unidentified head neurons, 6 unidentified tail neurons and only expressed dimly in the RnBs and HoB EVNs. The hermaphrodites also shared a different expression pattern in *pha-1; him-5; myEx918 [glb-28p::sfGFP:: GLB-28+pBX]* compared to *pha-1(e2123) III; him-5(e1490) V;myEx857[PCRsoeTGLB-28::GFP(200ng/ul)+pBX(50ng/ul)]*. *glb-28* expression is sexual dimorphic in males and hermaphrodites in the *pha-1(e2123) III; him-5(e1490) V;myEx857[PCRsoeTGLB-28::GFP(200ng/ul)+pBX(50ng/ul)]*. Previously the *glb-28* expression was shown to be present in males but it was uncharacterized in hermaphrodites. In the hermaphrodites we show exclusive sexual dimorphic *glb-28*

expression with 6 unidentified head neurons and 1-2 unidentified tail neurons. The male has only 3 head unidentified head neurons that have *glb-28* expression. This suggests that the hermaphrodite has at least 3 head neurons that express *glb-28* that are not expressing in the male. In the tail region it is unclear if the neurons that have *glb-28* expression are also in the male tail.

5.2 *glb-28* expression in *pha-1(e2123) III; him-5(e1490) V;myEx857[PCRsoeTGLB-28::GFP(200ng/ul)+pBX(50ng/ul)]* in dauer shows additional *glb-28* expressing neurons in males

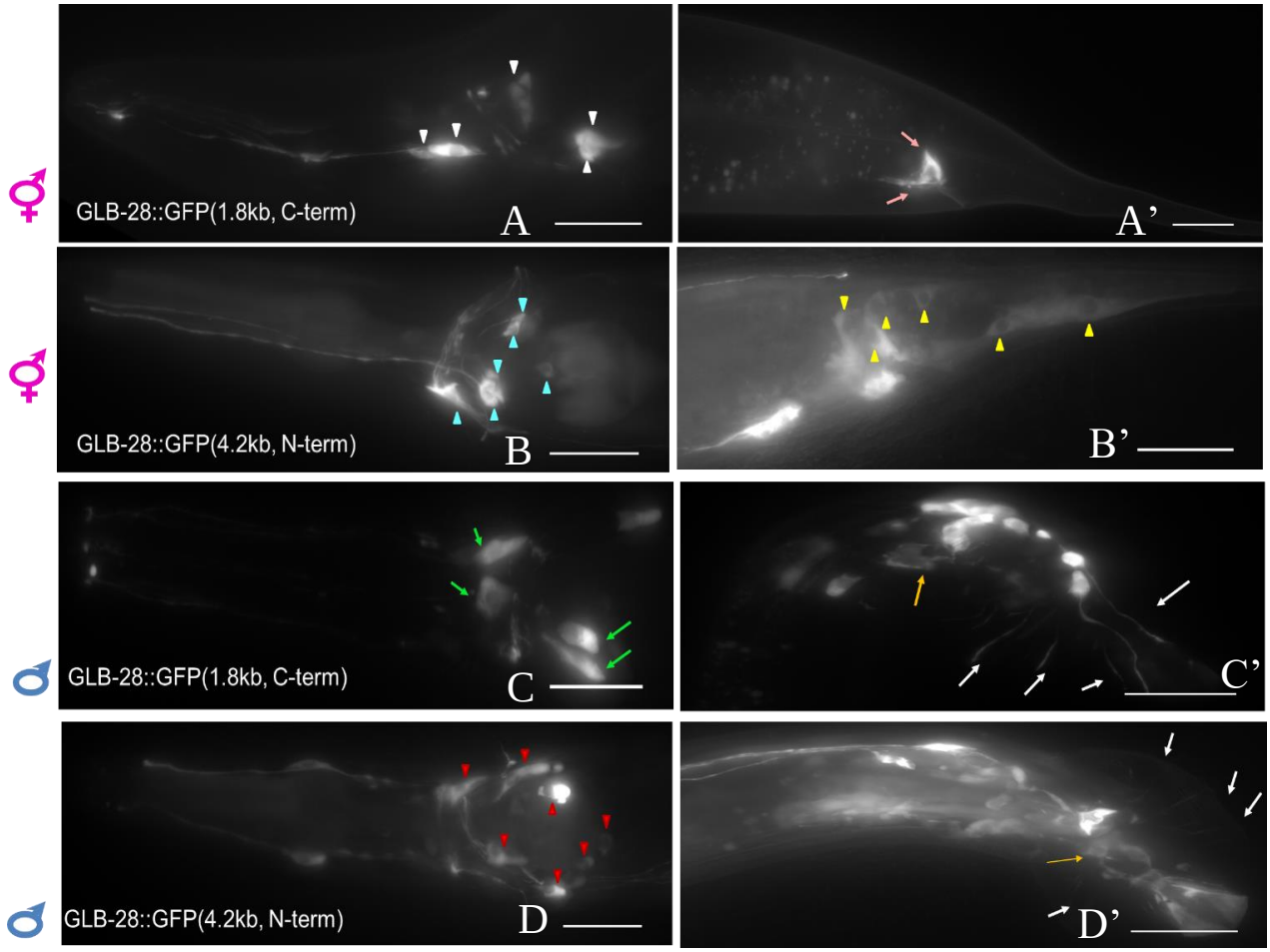
GLB-28 extrachromosomal reporter strain was forced into dauer by starvation and the worms were treated with 1% SDS to further isolate dauer worms as described in (Cassada and Russell, 1975). The 1% SDS treatment will kill any worms that are not dauer because their cuticle is vulnerable to the harsh SDS damage. The worms were imaged in dauer state and then were placed on a seeded plate for roughly 1 day. After the worms had reached come out of dauer and reached the adult stage they were imaged again. I noticed that the post-dauer animals had additional cell bodies that expressed GLB-28 in the males that were not present in animals of the same strain that never went into dauer. This data suggested that GLB-28 is responsive to dauer and has some irreversible expression as a result of dauer that might indicate *glb-28* involvement in stress response.

```
rm(list=ls())

setwd("C:\\Users\\Barrlab\\Documents\\Leaving Assay")

filenames<- c("LASg")

dir()

f<-filenames

library(survival)

cat("\nData:",f,"\n\n")

leaving <- read.table("LASg.txt", header = TRUE)
#leaving<-read.table(paste(f,".txt",sep=""),header=T)
attach(leaving)

genotypes<-names(table(wormType))
dates<-names(table(testDate))

leavtime<-Surv(time,censor)

leavfit<-survfit(leavtime~wormType, conf.type="log-log")

cat("Kaplan-Meier leaving curves\n\n")
print(summary(leavfit))

pdf(file=paste(f,".pdf",sep=""))

plot(leavfit, conf.int=F, log=T, xlab="Time (h)", ylab="Proportion
Remaining", lwd=0.5, lty=2, col=1:5)
legend(2,.13, genotypes, col=1:5, cex=.75, pch=19)

Time<-(0:12)*2

counter<-0
cat("Leaving probability (lambda) estimates\n")

for (i in genotypes)
{

 counter<-counter+1

 leaving.tmp<-leaving[wormType==i,]
```

```
leavtime.tmp<-Surv(leaving.tmp$time,leaving.tmp$censor)
 testDate.tmp<-factor(leaving.tmp$testDate)

 leavfit.tmp<-survfit(leavtime.tmp, conf.type="log-log")
  points(leavfit.tmp,pch=19,col=counter)

 leavmodel.tmp<-survreg(leavtime.tmp~1, dist="exponential")

leavmodel2.tmp<-survreg(leavtime.tmp~testDate.tmp, dist="exponential")
leavmodel3.tmp<-survreg(leavtime.tmp~1, dist="weibull")

lambda.tmp<-exp(-summary(leavmodel.tmp)$table[1])

lambdaU.tmp<-exp(-
(summary(leavmodel.tmp)$table[1]+qnorm(0.025)*summary(leavmodel.tmp)$table[2]))

lambdaL.tmp<-exp(-
(summary(leavmodel.tmp)$table[1]+qnorm(0.975)*summary(leavmodel.tmp)$table[2]))

  pvalue.tmp<-anova(leavmodel.tmp,leavmodel2.tmp)$P[2]

reps.tmp<-anova(leavmodel.tmp,leavmodel2.tmp)$Df[2]+1

 n.tmp<-summary(leavmodel.tmp)$n

cat("\n\nGenotype:", i, "\nPooled estimates:",
     "\n  lambda =", lambda.tmp, "  (",lambdaL.tmp,",", lambdaU.tmp,")",
     "\n  P(hom) =", pvalue.tmp,
     "\n  P(gof) =", anova(leavmodel.tmp,leavmodel3.tmp)$P[2],
     "\n  N =", n.tmp,
     "\n  Based on", reps.tmp, "reps:")

 leavpred.tmp<-1-pexp(Time,rate=lambda.tmp)
  lines(Time,leavpred.tmp,col=counter)
  for (j in dates)
    {
     leaving.tmp2<-leaving.tmp[testDate.tmp==j,]
     if (length(leaving.tmp2[,1])>1)
       {
         leavtime.tmp2<-Surv(leaving.tmp2$time,leaving.tmp2$censor)
         leavmodel.tmp2<-survreg(leavtime.tmp2~1, dist="exponential")
         lambda.tmp2<-exp(-summary(leavmodel.tmp2)$table[1])
         lambdaU.tmp2<-exp(-(summary(leavmodel.tmp2)$table[1]+
                    qnorm(0.025)*summary(leavmodel.tmp2)$table[2]))
```

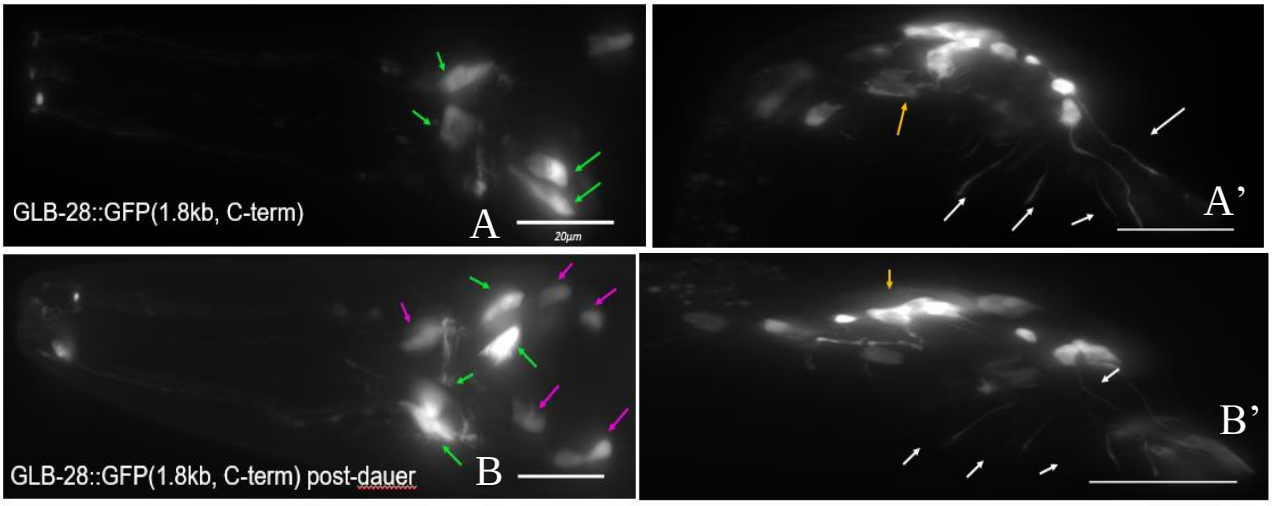
```
        lambdaL.tmp2<-exp(-(summary(leavmodel.tmp2)$table[1]+
                    qnorm(0.975)*summary(leavmodel.tmp2)$table[2]))
        n.tmp2<-summary(leavmodel.tmp2)$n
        cat("\n   ", j,
           "  lambda =", lambda.tmp2, "  (",lambdaL.tmp2,",",
lambdaU.tmp2,")",
           "  N =", n.tmp2)
      }
    }
}
dev.off()
cat("\n\n")
# contrast that specifies numbers of comparisons aka compare all pairs
contrasts<-matrix(
         c(1,2,
         2,3,
         1,3
         ),
           ncol=2,byrow=T)
ncontrasts<-length(contrasts[,1])

cat("\n\nPairwise contrasts\n\n")
for (c in 1:ncontrasts)
{
 contrast<-genotypes[contrasts[c,]]
leaving.con1<-leaving[wormType==contrast[1],]
  leaving.con2<-leaving[wormType==contrast[2],]

  leaving.con12<-rbind(leaving.con1,leaving.con2)
  leavtime.con12<-Surv(leaving.con12$time,leaving.con12$censor)
  testDate.con12<-factor(leaving.con12$testDate)
  wormType.con12<-factor(leaving.con12$wormType)

  leavmodel.con12<-survreg(leavtime.con12~1, dist="exponential")

  leavmodel2.con12<-survreg(leavtime.con12~wormType.con12,
dist="exponential")

  cat("Constrast:", contrast[1], "vs.", contrast[2], "\n",
      "  P =",anova(leavmodel.con12,leavmodel2.con12)$P[2], "\n\n")
}
```

Appendix Figure 5.0 R software code for leaving assay. This script was adapted from Barrios et al., 2008.



Appendix Figure 5.1 *glb-28* expression is influenced by promoter length and site of tagging and displays sexual dimorphism. Expression patterns of *glb-28* using several extrachromosomal reporters show sexual dimorphism. In panel A, the adult hermaphrodite, GLB-28::GFP (1.8kb, C-term) expressed in 5 head neurons (white triangles) and in panel A' there are 2 tail neurons (pink arrows). In panel C, the males with C-terminal GFP tagged GLB-28 with a 1.8kb promoter region (GLB-

28::GFP(1.8kb, C-term)) is expressed in male-specific EVNs: the CEMs (green arrow) in the head. In panel C', the males have expression in HOB (orange arrow) and RnB (white arrow) neurons in the tail. In panel B, GLB-28::GFP (4.2kb, N-term) is expressed in 6 head neurons (blue triangles) in the adult hermaphrodite and 6 tail neurons(yellow triangles) in panel B'. In contrast, the males in panel D with N-terminal GFP tagged GLB-28 with a 4.2kb promoter region (GLB-28::GFP(4.2kb, N-term)) is expressed in 7 neurons in the head (red triangles) and in panel D' there's expression in the tail RnBs and HOB. The scale bar is 20µm.



Appendix Figure 5.2 *glb-28* expression in *pha-1(e2123) III; him-5(e1490) V;myEx857[PCRsoeTGLB-28::GFP(200ng/ul)+pBX(50ng/ul)]* in dauer shows additional *glb-28* expressing neurons in males. GLB-28::GFP (1.8kb, C-term) dauer larvae have different *glb-28* expression patterns compared to adults of the same strain. In panel B, the post-dauer male heads have GLB-28::GFP highly expressed/enriched in CEM neurons compared to panel A males heads that never were exposed to dauer. CEMs (green arrow), HOB (orange arrow), RnBs (white arrow), post-dauer induced neurons (pink). The scale bar is 20µm.