THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF BIOLOGY

EXPLORING MECHANISMS OF MICROTUBULE POLARITY ESTABLISHMENT AND REGULATION IN *DROSOPHILA MELANOGASTER* NEURONS

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ABSTRACT

As cells that must survive an organism's entire lifetime, neurons' distinct microtubule polarity helps maintain structural integrity. Axonal microtubules are oriented such that their plus end points outward towards the cell periphery, while dendritic microtubules in neurons of different animal species are either both plus and minus end out or minus end out only. This polarity of microtubules is critical to ensure proper shipment of cell cargoes throughout the cell through the use of motor proteins. Therefore, understanding mechanisms that maintain and establish microtubule polarity are of utmost importance. Previously discovered mechanisms such as branchpoint steering and nucleation show evidence for how microtubule polarity patterns are maintained in neural dendrites. However, the lack of growing plus ends towards dendritic cell body exit points provides evidence for the existence of checkpoints at cell body exit points. Thus, I hypothesized that there is a relationship between the cell body exit points and microtubule polarity maintenance mechanisms. To examine this hypothesis, several experiments were performed. Manipulation of gene expression by RNA interference and imaging of live neurons by fluorescent microscopy allowed for the screening of candidate proteins possibly involved in positive or negative regulation of microtubule polarity. These experiments provided evidence for a positive regulator at axonal exits as RNAi of Tripartite Motif Containing (TRIM) 9, a microtubule bundling E3 ubiquitin ligase, significantly decreased the amount of plus end microtubules that move past the axonal cell body exit point. Furthermore, neural injury assays were performed by severing the axon from the remainder of the cell, causing the cell to convert a dendrite into a new axon. The percentage of plus end microtubules that exited the new axon, a phenotype of a converted dendrite, was significantly reduced in the TRIM 9 knockdown neurons. Finally, fluorescence microscopy was used to determine that TRIM 9 localizes to the axon of uninjured neurons. Together, these findings suggest that TRIM 9 has a role as a positive regulator of microtubule polarity, and also functions to help maintain microtubule polarity patterns over the course of an organism's life span.

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Chapter 1

Introduction

Neurons and Microtubules

The nervous system is capable of performing many different functions in an organism. Through the central and peripheral nervous systems, organisms use neurons to detect and respond to stimuli from the environment, as well as from within, allowing them to adapt to various conditions in order to survive (Lodish et al., 2000). A typical neuron has three main segments: a singular long slender process known as an axon, several shorter extensions known as dendrites, and a cell body called a soma (Figure 1). Dendrites are responsible for receiving signals from other cells or the outside world, while axons transmit the information to dendrites of other cells (Lodish et al., 2000). Overall, the structure of the neuron helps determine how synaptic inputs are translated into meaningful information that is communicated to other cells.

Most neurons must last an organism's entire lifespan and therefore need specific cell machinery to function properly. Diverse proteins needed to maintain proper neuronal function are made in the cell body and must travel to the axon terminals and peripheral branches of dendrites. The distinct shape of neurons, with their long processes, provides reason for an intricate transportation system that is maintained through cellular "train tracks" of microtubules.

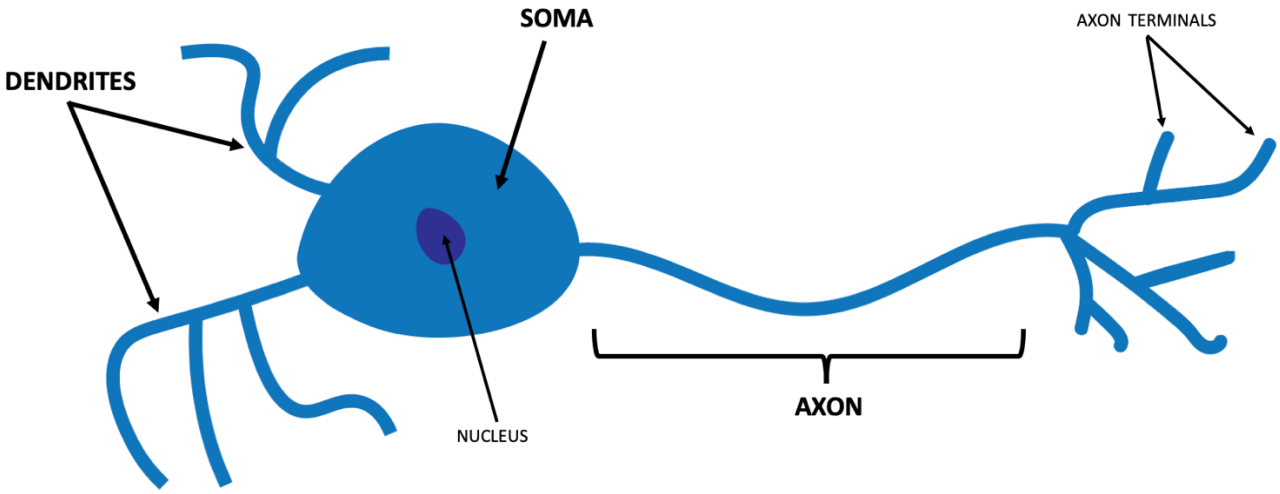

Figure 1: Basic Structure of a Neuron. Dendrites receive electrochemical signals from other cells which ire transmitted through the axon. The soma (cell body) contains a nucleus which stores genetic information. Axon terminals synapse with other cells in order to transmit the message.

As components of the cytoskeleton, microtubules help ship cargoes from the cell body to axon terminals and dendrite periphery (Hirokawa, 1998). Structurally, microtubules are hollow cylindrical polymers made up of 13 protofilaments, including α-, β-, and γ-tubulin. γ-tubulin is important for the initiation of microtubule assembly, as it is part of the base of the microtubule known as the γ-Tubulin Ring Complex (γ-TuRC). The γ-TuRC is formed by several γ-tubulin Small Complexes (γ-TuSCs) and several other subunits. From this base structure, microtubules polymerize with dimers of α- and β-tubulin to elongate (Figure 2) (Teixidó-Travesa, Roig, & Lüders, 2012). Microtubules are constantly polymerizing and depolymerizing with the hydrolysis of GTP bound to β-tubulin, a process known as 'dynamic instability' (Bouissou et al., 2009; Teixidó-Travesa et al., 2012).

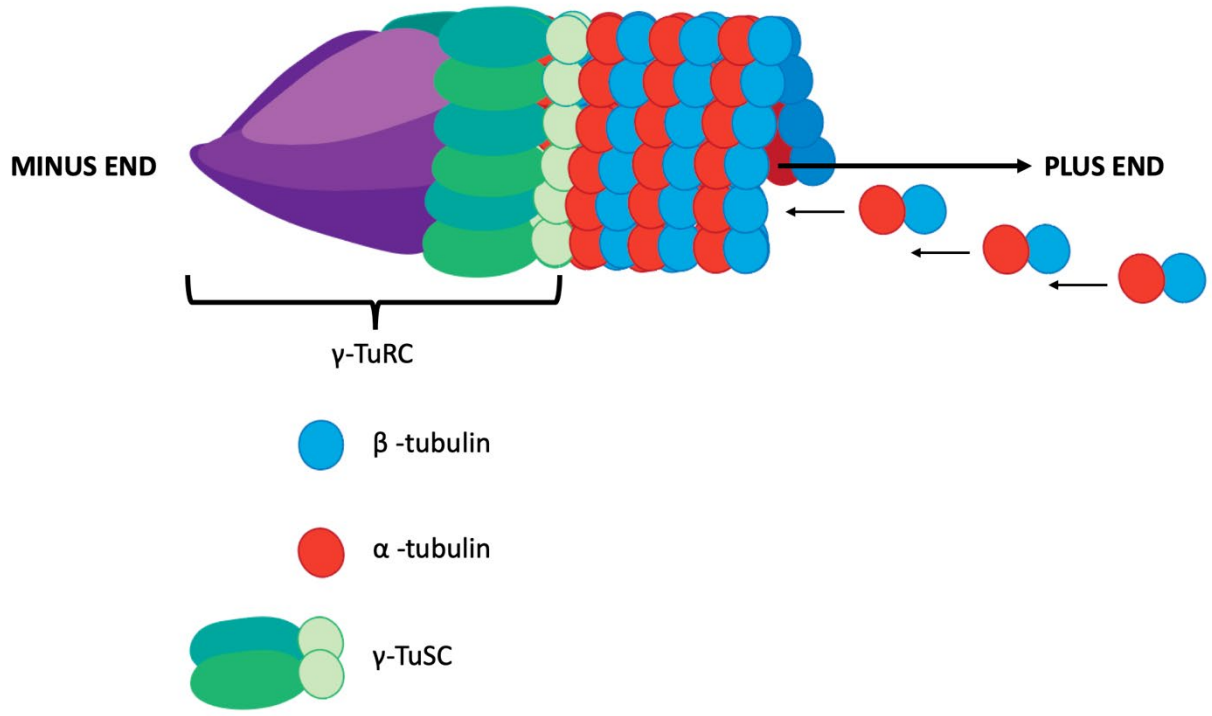

Figure 2: Microtubule Structure (Polymerization). Microtubules are characterized by having a plus and minus end. The minus end is typically capped with the γ-TuRC. From the γ-TuRC, the plus end polymerizes via tubulin dimers, shown as red and blue subunits

Overview of Microtubule Polarity

Microtubules have an inherent polarity, described by their plus- and minus- ends. Plus-ends are faster growing than their minus-end counterparts, and are characterized by exposed β-tubulin, while minus ends are slower growing with exposed α-tubulin and capped with the γ-TuRC (Bouissou et al., 2009; Teixidó-Travesa et al., 2012). Highly polarized cells such as neurons organize microtubules into polarized non-centrosomal arrays, and when looking at neurons across several species, microtubules have a specific orientation to help maintain the proper shipment of cellular cargo.

In vertebrate neurons, axonal microtubules are exclusively arranged with plus-ends pointing away from the cell body ("plus-end out"). Dendrites, have mixed microtubule orientations, with plus- and minus- ends facing both toward or away from the cell body (Rolls & Jegla, 2015). During *Drosophila* embryogenesis, dendritic microtubule polarity is initially mixed during the major period of dendritic outgrowth (Hill et al., 2012). As *Drosophila* continue to develop, the majority of dendritic microtubules (~90%) have their minus-ends oriented away from the cell body ("minus-end out") while axons remain plus-end out (Figure 3) (Stone, Roegiers, & Rolls, 2008). Therefore, the distinct difference in microtubule polarity between axons and dendrites allows for classification of neuronal processes as axons or dendrites by examination of microtubule polarity in a neurite (Rolls & Jegla, 2015).

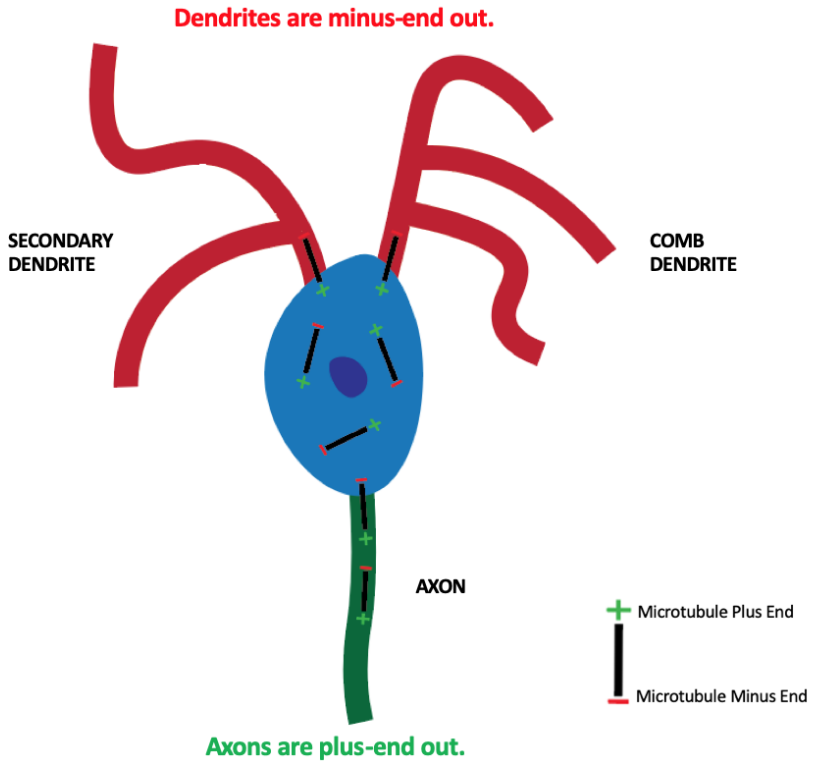

Figure 3: Microtubule Polarity in Drosophila Neurons. In the dendrites, microtubule plus-ends are oriented towards the soma. In the axon, microtubule plus-ends are oriented away from the soma. Therefore, microtubule polarity in drosophila neurons can be simply referred to as "plus-end out" in the axon, and "minus-end out" in dendrites.

This distinct polarity arrangement provides evidence for differences between axons and dendrites in cargo transport (Burton, 1988). In neurons, most of the protein synthetic machinery is located in the cell body. To ship cell cargoes to axons and dendrites, cargoes are transported by motor proteins, such as kinesins and dynein, along microtubules towards the plus or minus end, respectively (Rolls & Jegla, 2015). The kinesin superfamily proteins (KIFs) act as anterograde motors that travel towards the plus-ends of microtubules (Hirokawa & Takemura, 2005). Another class of motor proteins, the dynein superfamily proteins, travel toward microtubule minus-ends (Hirokawa, 1998). Therefore, in axons, kinesins carry cargo from the cell body towards the axon terminal, and away from the axon terminal by dynein. In species with mixed polarity in dendrites, one type of motor protein could move cargo both towards or away from the cell body, depending on the orientation of the microtubule, while in dendrites of Drosophila, dynein acts as a primary anterograde transport protein (Rolls & Jegla, 2015).

Growing microtubules are associated with microtubule plus-end tracking proteins (+TIPS) that bind to microtubule plus ends as they grow and fall off when they shrink (Bieling et al., 2007). One example is End Binding Protein 1 (EB1). EB1 family members accumulate at the growing microtubule ends by recognizing a specific structural feature at the plus end, thus promoting microtubule assembly and growth (Bieling et al., 2007; Jiang & Akhmanova, 2011). Therefore, microtubule polarity patterns can be visualized *in vivo* by tagging proteins like EB1 with Green-Fluorescent Protein (GFP) (Figure 4). With this method, one can assess the overall

polarity by tracking the motion and direction of the EB1-GFP tagged plus-ends of microtubules (also referred to as comets).

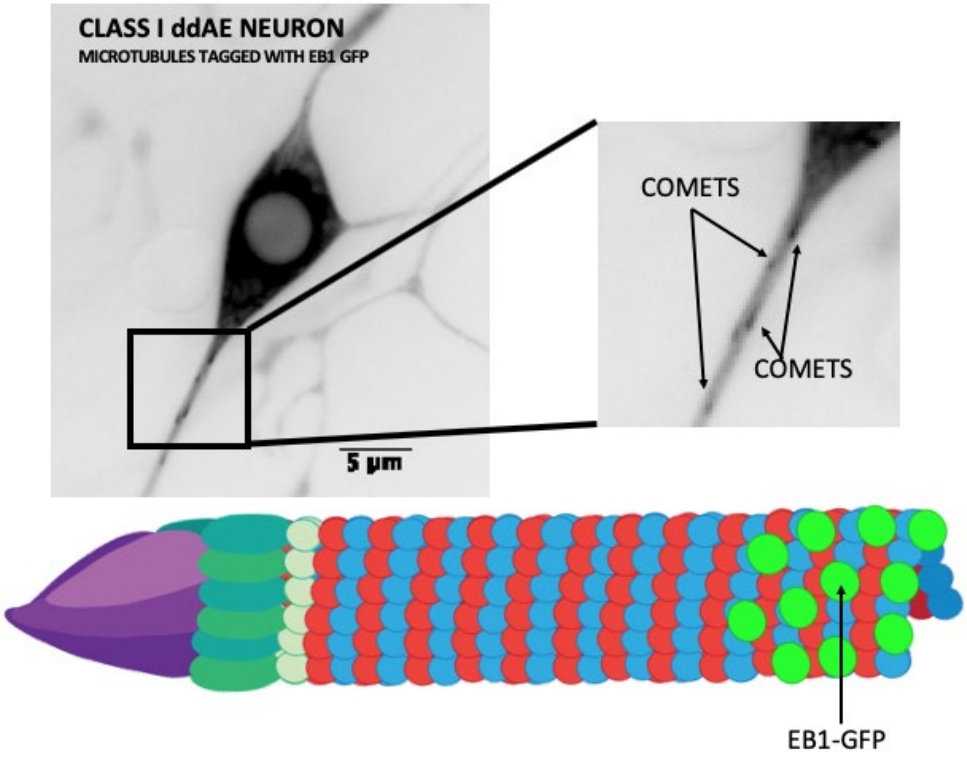

Figure 4: Neuron tagged with EB1 GFP. Tagging neurons with EB1-GFP allows for visualization of microtubule plus-ends *in vivo*. GFP tagged microtubules appear as "comets" under microscopic settings for fluorescent live imaging videos. Movement of comets allows one to determine the orientation of the polarity of microtubules as "plus-end in" or "minus-end out."

Microtubule Polarity Mediators in Neurons

The polarized arrangement of microtubule polarity in neurons alludes to the existence of mechanisms that help mediate their organization, and such processes have been found within the cell periphery. One of the primary means of microtubule polarity maintenance is nucleation, which is typically restricted to the microtubule organizing centers (MTOC). Neurons differ from somatic cells where the centrosome is the principal MTOC (Teixidó-Travesa et al., 2012). In

Drosophila dendritic arborization (da) neurons, the centrosome loses its capacity to organize neuronal microtubules while the organism develops and is not needed to maintain microtubule organization in these cells (Nguyen, Stone, & Rolls, 2011; Teixidó-Travesa et al., 2012). While it has been widely known that microtubule nucleation in *Drosophila* neurons is non-centrosomal, the site of nucleation for *Drosophila* neurons has been only recently been elucidated.

Previous studies have shown that nucleation occurs at dendritic branchpoints (Nguyen et al., 2014). One component needed for microtubule nucleation is γ-tubulin. In *Drosophila*, γ-tubulin localizes to dendritic branchpoints (Nguyen et al., 2014). Further studies have shown that this occurs through the assistance of Wnt family proteins such as Axin and dsh, which also accumulate in those regions in the cell (Weiner et al., 2020). Recent studies have uncovered that Axin and dsh function upstream of microtubule nucleation and that Wnt proteins also localize to Rab5 endosomes. Since endosomes have been found to concentrate at dendrites and their branchpoints, these results provide evidence that endosomes are utilized as a MTOC in dendrites (Weiner et al., 2020).

Dendrites, including those of Drosophila neurons, are highly branched. Drosophila dendrites maintain minus end out polarity, despite their complex dendritic arbors. If microtubules were allowed to grow in any direction, this distinct polarity organization would be compromised. Thus, additional mechanisms must exist in the branchpoints to maintain minus end out polarity. Research shows that Apc and the motor protein Kinesin-2 function to steer microtubules growing into branchpoints and direct them toward the cell body, thus preserving minus end out polarity in dendrites. (Weiner et al., 2016). Specifically, Apc and Kinesin 2 work to resolve microtubule collisions at branchpoints in dendrites, and do not function by mediating lateral interactions.

(Weiner et al., 2016) Furthermore, Kinesin 2 ensures that the colliding microtubule is steered along a pre-existing stable microtubule (Weiner et al., 2016).

The axon is also subject to microtubule organizing mechanisms. Augmin, an 8 subunit protein complex, is known to help nucleate microtubules off of pre-existing microtubules through the recruitment of γ-Tubulin (Tian & Kong, 2019). Studies have shown that disruption of this complex decreases microtubule density and bundling, and impair processes such as axon specification and axonal trafficking (Sánchez-Huertas et al., 2016). Furthermore, it was established that augmin works to orient the direction of nascent microtubules as reduction of augmin did not decrease the amount of microtubules but instead changed the alignment of their nucleation(Sánchez-Huertas et al., 2016). Thus, augmin plays a significant role to maintaining plus end out microtubule polarity in axons through specific orientation of microtubule nucleation.

While mechanisms such as branchpoint steering and nucleation are critical in upholding microtubule polarity in dendritic arbors, little is known about corresponding mechanisms at the cell body exit points. It is possible that positive and negative regulators may exist at each cell body exit point to facilitate microtubule polarity. Positive regulators are those that must be present in the cell to allow microtubules to pass through a region. Without the protein, the plus-end microtubule would be unable to enter a region. Negative regulators, on the other hand, consist of mechanisms whose presence prevent microtubules from entering a region. An example would be a protein localized to the dendritic cell body exit points that inhibits plus-end microtubules from entering the dendrite (Figure 5).

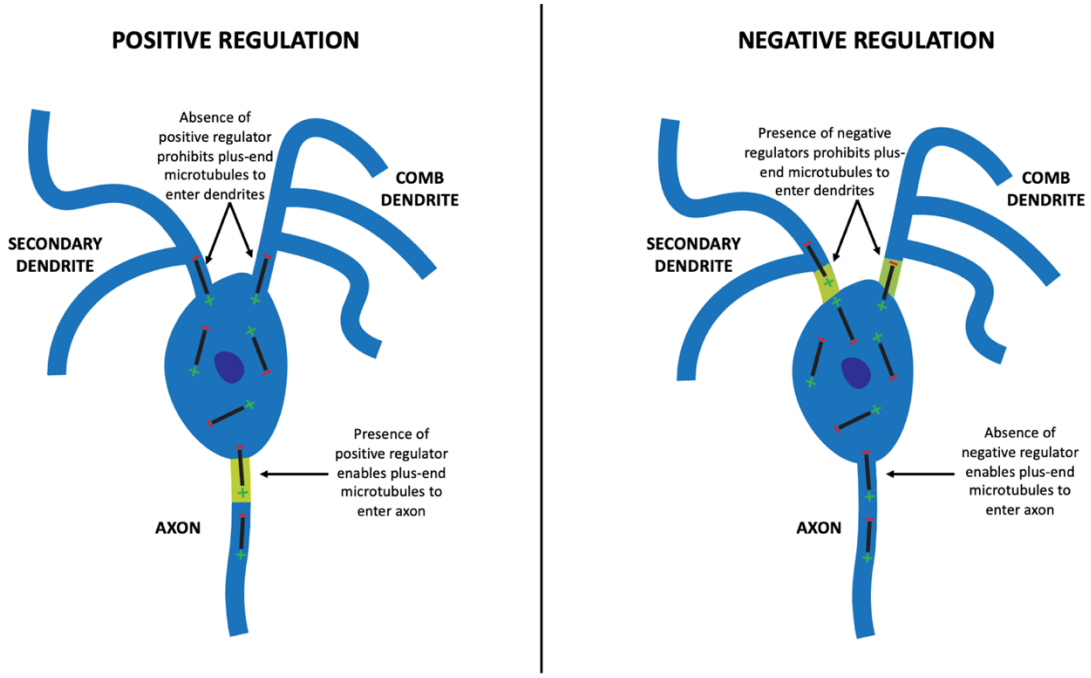

Figure 5: Examples of Positive and Negative Regulation. It is possible that the mechanisms that govern microtubule polarity at the cell body exit points work to enable plus-end microtubules to enter the axon through the presence of a protein at the axonal exit (positive regulation) or by inhibiting microtubules from entering the dendrites through the presence of a protein localized to the dendritic exit points (negative regulation).

The Bipolar Kinesin, Klp61F

A familiar function of microtubule-based motor proteins is formation of the spindle apparatus in mitosis. One of these motor proteins is kinesin-5, which is involved with establishing and maintaining bipolar spindles (Sharp & Rath, 2009a).

The *Drosophila* kinesin5, Kinesin-Like-Protein 61F (Klp61F) is a homotetrameric motor protein that participates in the formation of bipolar mitotic spindles (Sharp et al., 1999). The significance of Klp61F can be seen in mitosis, as disruption of the gene results in a lethal mitotic mutation (Heck et al., 1993). Without Klp61F, spindle poles in mitosis are unable to separate, and instead, monopolar mitotic spindles result (Heck et al., 1993).

Klp61F is a candidate for positive or negative regulation of microtubule polarity in Drosophila neurons due to its ability to crosslink both parallel and antiparallel microtubules (van den Wildenberg et al., 2008). Klp61F has the potential to function as a positive regulator through its ability to detect parallel microtubule arrays, possibly promoting polymerization through the correct exit points. On the other hand, because of Klp61F's preference to detect antiparallel microtubules (Sharp & Rath, 2009a; Tao et al., 2006; van den Wildenberg et al., 2008), it is possible that the protein functions as a negative regulator, possibly by blocking polymerization when antiparallel microtubules attempt to grow into the wrong cell body exit.

TRIM Family Proteins

The Tripartite Motif (TRIM) family of proteins are also candidates for working as positive or negative regulators at the cell body exit points. TRIM proteins are composed of a RING finger domain, B-box domains, as well as a coiled-coil region (Reddy, Etkin, & Freemont, 1992). Collectively, the TRIM family proteins are involved in a broad range of biological functions, including intracellular signaling, development, apoptosis, protein quality control, innate immunity, autophagy, and carcinogenesis (Hatakeyama, 2017). One of the most conserved functions of this the protein family is that they serve as E3 ligases for ubiquitination, a common post-translational modification of proteins (Hatakeyama, 2017).

TRIM46 has been identified as a protein that controls neuronal polarity and is involved in axon formation. TRIM46 localizes to the axon initial segment (AIS) of neurons and helps create parallel microtubule arrays with their plus-ends oriented away from the cell body and toward the axon periphery (Van Beuningen, Will, Harterink, Kapitein, et al., 2015). Reduction of TRIM46

causes microtubule polarity to be mixed in the axon, showing the protein's importance in microtubule polarity organization (Van Beuningen, Will, Harterink, Kapitein, et al., 2015).

While many of the *Drosophila* genes are conserved in vertebrates, there are many more TRIM genes in vertebrates than fruit flies (Vunjak & Versteeg, 2019). Therefore, because there is no paralog of TRIM46 in *Drosophila,* the related TRIM9 homolog was used as it is neuron specific and displays the most homology to TRIM46 (Hatakeyama, 2017). In humans, TRIM9 localizes to cytoplasmic bodies and its mRNA is abundantly expressed in the brain (National Center for Biotechnology Information U.S. National Library of Medicine, 2020).

Strategies to Study Microtubule Polarity Regulation and Establishment

I have hypothesized that there is a relationship between microtubule polarity establishment and/or regulation and cell body exit points. To study this hypothesis, I have conducted experiments utilizing the model organism, *Drosophila melanogaster.* The advantages of using this organism include the fast rate of reproduction, cost efficiency, and potential for a diverse range of experimental procedures. In the variety of fluorescent microscopy experiments outlined in this thesis, I was able to stimulate a variety of conditions to explore microtubule polarity patterns at cell body exit points through RNAi, protein localization, and injury assays.

Chapter 2

Results

Reduction of TRIM 9 and Klp61F prevents microtubules from entering the axon in uninjured cells.

In this thesis, I examine my hypothesis of a relationship between cell body exit points and regulation of microtubule polarity in Class I ddaE *Drosophila* neurons. My first experiment was to determine whether microtubules grow into the axon or the dendrites. This was followed by screening candidate proteins to determine whether any were positive or negative regulators. Reduction of a negative regulator would allow more plus end microtubules to exit the cell body through dendritic exits. Reduction of a positive regulator would decrease the number of plus-end microtubules exiting through the axonal cell body exit.

The first experiment, which also served as a control for baseline cell body exits of microtubules, involved knockdown of γ-Tubulin 37C by RNAi. γ-Tubulin 37C was chosen as a positive control since the protein is only expressed maternally and not in the mature neurons studied in these experiments. Therefore, a significant decrease of γ-Tubulin 37C would have no effect on baseline microtubule polarity patterns in the mature cells studied.

The candidate proteins chosen to screen for positive and negative regulators have known functions related to microtubule polarity or the cytoskeleton of neurons. They include Septin-1, TRIM 9, and Klp61F. Septin proteins belong to a class of GTP-binding proteins and are recognized as part of the cytoskeleton because they interact with cellular membranes, actin, and

microtubules (Mostowy & Cossart, 2012). Septins often localize to specific areas of the neuron such as the base of dendritic spines(Mostowy & Cossart, 2012). However, the overall structural determinants needed to assemble septins remain unclear. In neurons, TRIM proteins aid in microtubule bundling for unidirectional polarity (Van Beuningen, Will, Harterink, Chazeau, et al., 2015). There is also evidence that TRIM9 is involved in axon guidance in *Drosophila* (Song et al., 2011). Thus, it has the potential to be involved with microtubule polarity regulation in the axon. Finally, Klp61F was chosen for its role in the mitotic spindle, as interacts with both parallel and antiparallel microtubule arrays, thereby making it a candidate for both positive and negative regulation of microtubules entering dendrites (Sharp et al., 1999; van den Wildenberg et al., 2008).

To visualize microtubules *in vivo*, I used a tester line of *Drosophila* expressing UAS-Dicer2 ; 221-Gal4 and UAS-EB1-GFP. This enabled visualization of microtubule plus-end "comets" by epi-fluorescent microscopy. Because this experiment was focused on microtubule regulation at cell body exit points, the first 5 μm from the cell body in each of the neurites (comb dendrite and axon), defined to be the region of the exit, were examined for microtubule activity. If plus ends that originated in the cell body grew past the 5 μm region, they were deemed to have "successfully" exited the cell body. Plus ends that did not surpass the 5 μm distance were designated as "unsuccessful" in leaving the cell body (Figure 6).

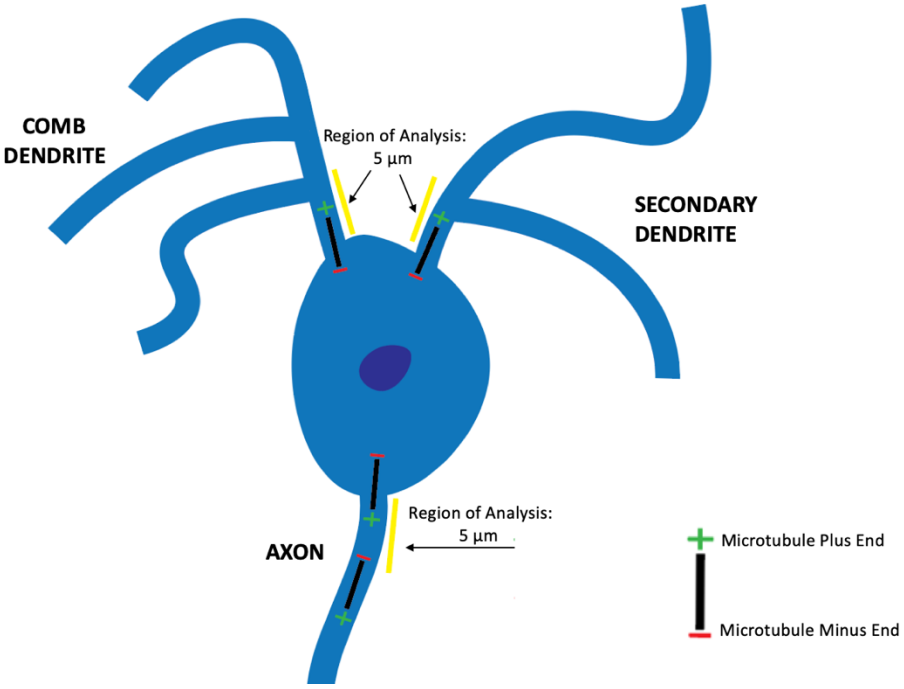

Figure 6: Region of Analysis. Microtubules leaving the cell body were categorized as being "successful" or "unsuccessful" in their exits if the plus end microtubule completely surpassed 5 µm. While in this first experiment, only the comb dendrite was assessed, this region of analysis was consistent for each experiment to follow.

The resulting phenotypes of each fly matings, or crosses, were compared to RNAi of the control cross, γ-Tubulin 37C. In the control cross, plus-end microtubules were 54% successful in exiting the cell body through the axonal exit. However, plus-end exit in the dendritic exit was 7.7%. Moreover, 52 microtubules attempted axonal exit in comparison to the 26 microtubules that attempted dendritic exit (Figure 5). From these results, I concluded that microtubules have a higher percentage of cell body exit success in using the axonal exit over the dendritic exit. There are also more plus-end microtubules that attempt to use the axonal exit compared to dendritic exit thus indicating a bias for the axonal exit. Altogether, these two conclusions indicate that microtubules grow into the axon and not the dendrite, possibly through the use of a positive or negative regulator to help promote or inhibit growth. However, the bias and raw percentage of successful plus-end microtubule exits are likely two different phenomena governed by their own

respective mechanisms.

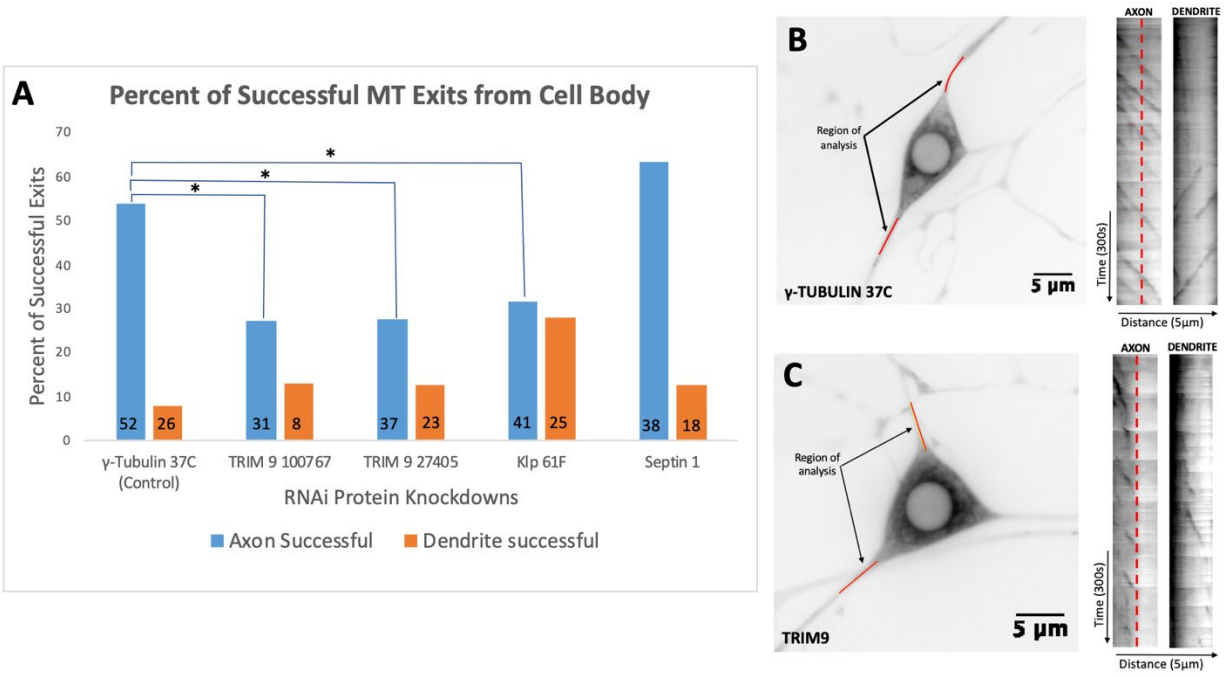

Figure 7: Reduction of TRIM 9 (#100767), TRIM 9 (#27405), and Klp61F (#52549) cause a significant decrease in the percentage of successful axonal plus-end microtubule (MT) exits from the cell body. A. Graph of percentage of successful plus end MTs that exited cell body into axons and dendrites. Using a Fisher's Exact Test, I concluded there is a significant (*$p<0.05$) decrease in the axonal MT exits with TRIM 9 (#100767) ($p=0.0166$, n=31 MT attempts in axons, n=8 MT attempts in dendrites), TRIM 9 (#27405) ($p=0.0213$, n=37 MT attempts in axons, n=23 MT attempts in dendrites), and Klp61F (#52549) ($p=0.0377$, n= 41 MT attempts in axons, n=25 MT attempts in dendrites) RNAi fly lines. The numbers 100767, 27405, and 52549 are the Vienna Drosophila RNAi Center (VDRC) numbers. **B & C.** Kymographs representing time vs position of MTs (dark lines) in γ-Tubulin 37C and TRIM 9 knockdowns. Red line allows for comparison of MT depolymerization between control and TRIM9 crosses.

In examination of the candidate proteins, RNAi of TRIM9 and Klp61F cause a significant decrease in the number of plus-end microtubules that enter the axon from the cell body in comparison to the control (Figure 7). This significant decrease in successful axonal plus-end microtubule exits indicates that these proteins are involved in positive regulation of microtubule polarity. That is, their presence is needed for plus-end microtubules to exit through the axonal exit. However, there was no signficant change in the number of successful exits using the dendritic cell body exit points for these proteins compared to the control. RNAi of Septin1

exhibited no significant change from the control in the number of successful exits from axonal or dendritic cell body exit points.

Reduction of TRIM9 affects re-establishment of microtubule polarity.

Injury assays are a powerful tool to emulate neurodegeneration and the cell's response to stress. Such assays involve injuring the cells with use of a UV-pulse laser to sever a neurite. When severing an axon, the injury causes a global upregulation of microtubule nucleation throughout the entire cell, including the cell body (Stone, Nguyen, Tao, Allender, & Rolls, 2010). Without a fully functioning axon, the cell is forced to convert a pre-existing dendrite into a new axon, noted by the switch of minus end out microtubules to plus end out microtubules (Figure 8) (Stone et al., 2010).

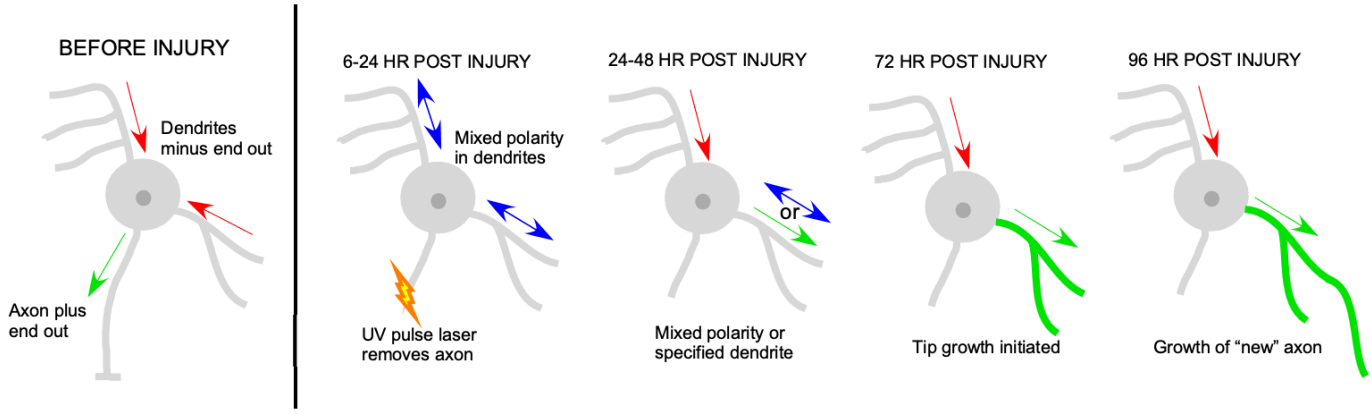

Figure 8: Utilization of injury assays to examine the re-establishment of microtubule polarity *in vivo* (Stone, Nguyen, Tao, Allender, & Rolls, 2010). Axons are severed with a UV-pulse laser force the neuron to trigger a neuroprotective pathway that allows visualization of microtubule polarity re-establishment. 6-24 hours post injury, microtubules are upregulated 10-fold and mixed polarity is seen in dendrites. 24-48 hours post injury, one dendrite may or may not be specified as a new axon as noted by plus end out polarity in the dendrite, or mixed dendritic polarity remains. 72 hours post injury, a dendrite converts to become the new axon and tip growth is initiated and microtubule upregulation dies down. 96 hours post injury, tip growth of the axon continues.

In this experiment, I used injury assays to determine if TRIM9 is involved with the re-establishment of microtubule polarity. I predicted that if TRIM9 was involved in establishing

plus-end out microtubule polarity, there would be a decrease in successful plus-end microtubule exits in the converted dendrite.

To perform this assay, I crossed a TRIM9 RNAi line with a tester line expressing UAS-Dicer2 ; 221-Gal4, UAS-EB1-GFP. Then, I severed the axons of 2nd and 3rd instar larvae and allowed them to incubate for 48 hours. After 48 hours, I imaged the larvae for 5 minutes on a Zeiss Widefield microscope to obtain EB1 videos. These results were quantified using the same 5 μm region of analysis. While the axon was severed, it was still quantified if there was a viable 5 μm region left, thus these results were included in statistical analyses when applicable. The results of the axon, comb dendrite, and secondary dendrite were analyzed and compared to the control cross which used γ-Tubulin 37C RNAi and the same injury assays.

Examination of the γ-Tubulin 37C RNAi EB1 videos showed plus end microtubules had 71% success exiting through the axonal cell body exit point, 50% success exiting through the comb dendrite cell body exit point, and 84% success exiting through the secondary dendrite cell body exit point. In comparison, TRIM9 RNAi EB1 videos showed plus end microtubules had 59% success exiting through the axonal cell body exit point, 53% success exiting through the comb dendrite cell body exit point, and 60% success exiting through the secondary dendrite cell body exit point. The decrease in successful plus-end microtubule exits through the secondary dendrite cell body exit point in TRIM9 RNAi cells was statistically significant in comparison to the control($p=0.0037$) through calculation of a Fischer's Exact Test (Figure 9).

These results were further analyzed by examining if the injured cells had converted one of the pre-existing dendrites to a new axon. While specification of a dendrite into an axon post injury is easier to see at the 72 and 96 hour time points, cells may convert as early as 48 hours (Stone et al., 2010). Once it was determined which dendrites had converted to the new axon,

through examination of which dendrite had the majority of successful plus end microtubule cell body exits compared to the other dendrites, the percent of successful microtubule exits in both comb and secondary dendrite exits were examined again, this time exclusively in dendrites that had converted. The γ-Tubulin 37C RNAi results showed 49% of plus-end microtubules were successful in their exits in converted comb dendrites. However, when the secondary dendrite was converted to the new axon, 92% of plus-end microtubules were successful in their exits. Examination of the TRIM9 RNAi cells showed 61% of plus-end microtubules were successful in their exits in converted comb dendrites. When the secondary dendrite was converted to the new axon, 74% of plus-end microtubules were successful in their exits. Examination of these two conditions together with a Fisher's Exact Test showed a significant decrease in the number of successful exits between the two secondary dendrites under their respective conditions ($p=0.0190$) (Figure 9).

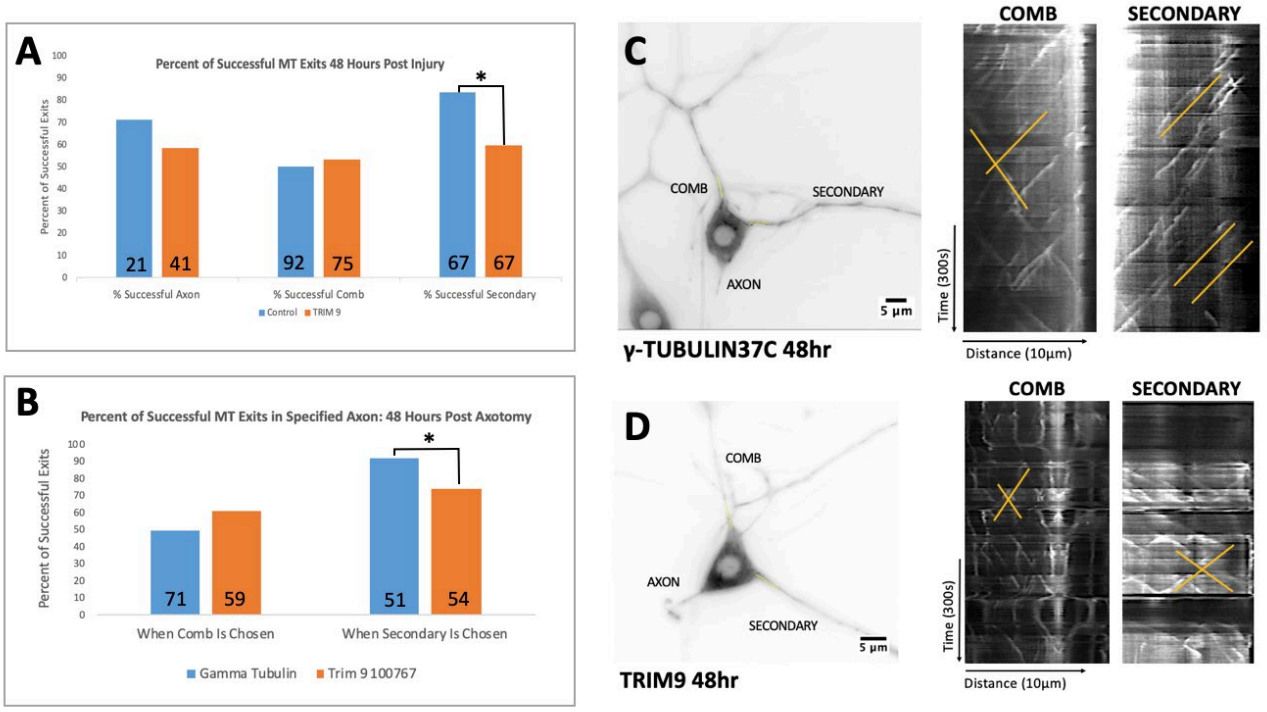

Figure 9: Reduction of TRIM 9 affects re-establishment of microtubule (MT) polarity. Using a Fisher's Exact Test, the following were concluded. **A.** There was a significant (*p<0.05, **p<0.001, and ***p<0.0001) decrease between the percentage of successful MT exits in the secondary dendrite when comparing γ-Tubulin 37C and TRIM 9 (p=0.0037, n=67 MT attempts in γ-Tubulin 37C secondary dendrites, n=67 MT attempts in TRIM9 dendrites). **B.** In secondary dendrites that were converted to axons, the decrease in successful MT exits between TRIM9 and γ-Tubulin 37C is statistically significant (p=0.0190, n=51 MT attempts in γ-Tubulin 37C converted secondary dendrites, n=54 attempts in TRIM9 converted secondary dendrites). **C.** γ-Tubulin 37C cell and kymograph 48 hours post injury showing fully converted secondary dendrite, noted by parallel (yellow parallel lines) microtubule arrays, signifying plus end out polarity. **D.** TRIM9 cell and kymograph 48 hours post injury showing mixed MT polarity in both the comb and secondary dendrites, noted by crossed pattern (yellow crossed lines) on kymograph.

From these results, I concluded that reduction of TRIM9 affects the cell's ability to re-establish microtubule polarity, likely due to a lack of positive regulation for plus end microtubules to enter into a newly converted axon.

TRIM 9 localizes to the axon in an uninjured cell.

Because reduction of TRIM9 caused a significant decrease in successful plus end microtubule exits through the axon in uninjured and injured conditions, and also affected the cell's ability to re-establish plus end out polarity in injured cells, I hypothesized TRIM9

localized to the AIS as a positive regulator. To test this hypothesis, TRIM9-GFP was crossed with UAS-Dicer2 ; 221-Gal4, UAS-mCD8-GFP. This allowed TRIM9 to be clearly visualized in the cell as the GFP would contrast with the remainder of the cell tagged with RFP. To analyze this experiment, 3rd instar larva were examined using fluorescent microscopy. Data was obtained by calculating the average amount of GFP fluorophore that was in the first 5 μm of the axon and dendrites.

To quantify the data, the fluorescence was averaged across all 20 samples for each neurite: the axon, the secondary dendrite, the comb dendrite, and any other neurites, which were classified as "other." Through this quantification, I used a t-test to determine the amount of TRIM9 in the first 5 μm of the axon was significantly higher in comparison to the dendrites ($p < 0.0001$) (Figure 10). Thus, localization of TRIM9 in the axon initial segment further supported the conclusion of TRIM9 acting as a positive regulator.

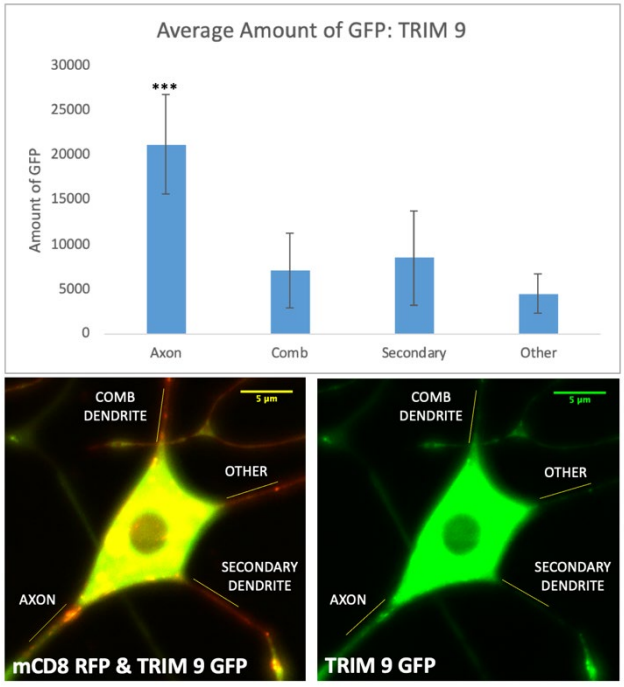

Figure 10: TRIM 9 localizes to the axon in uninjured cells. Top: Using a Simple T-Test, I concluded that there is a significantly higher amount of GFP in the first 5 μm of the axon than compared to that in the dendrites ($p<0.0001$) (***$p<0.0001$). Error bars show standard deviation. **Bottom:** Images of a singular cell showing localization of TRIM 9 GFP (right) alone compared to mCD8 RFP (left).

Chapter 3

Discussion

These experiments were performed collectively to determine what, if any, mechanisms exist at the cell body exit points of Class I ddaE *Drosophila* neurons to establish or maintain microtubule polarity patterns.

The combined results of these experiments show that in *Drosophila*, TRIM9 acts as a positive regulator to enable plus-end microtubules to exit from the cell body into the axon. This is supported by localization, which shows significantly more TRIM9 enriched in the axon than the dendrites. It is also supported by the EB1 comet assays as there is a disruption in microtubule polarity shown via a decrease in successful microtubule exits out of the axonal cell body exit point under uninjured conditions. Moreover, I found that TRIM9 is needed for re-establishment of plus end out polarity in converted dendrites, thus signifying its likely role in axon specification. Therefore, these results parallel the findings of TRIM46 in vertebrate neurons. TRIM46 localizes to the proximal axon, is needed for uniform axonal microtubule orientation, and is required for axon specification (Van Beuningen, Will, Harterink, Chazeau, et al., 2015).

However, these may not be the only functions of TRIM9. Research shows that when a cell is forced to convert a dendrite into an axon, the dendrite that converts is usually the secondary dendrite (Stone et al., 2010). In my examinations of TRIM9 localization, I observed that often, the secondary dendrite is more enriched with TRIM9 in comparison to the comb dendrite in uninjured conditions (Figure 11). This finding suggests that the secondary dendrite

might be predisposed to be more enriched with TRIM9 as an innate neuroprotective mechanism should the cell be subjected to injury and the axon's function is compromised. This would make sense, as neurons must last for the organism's entire life, and robust mechanisms must be in place for the cell to survive if it is subjected to stress or injury. This prediction would also help explain why the secondary dendrite converts more often than the comb when the cell is subjected to injury.

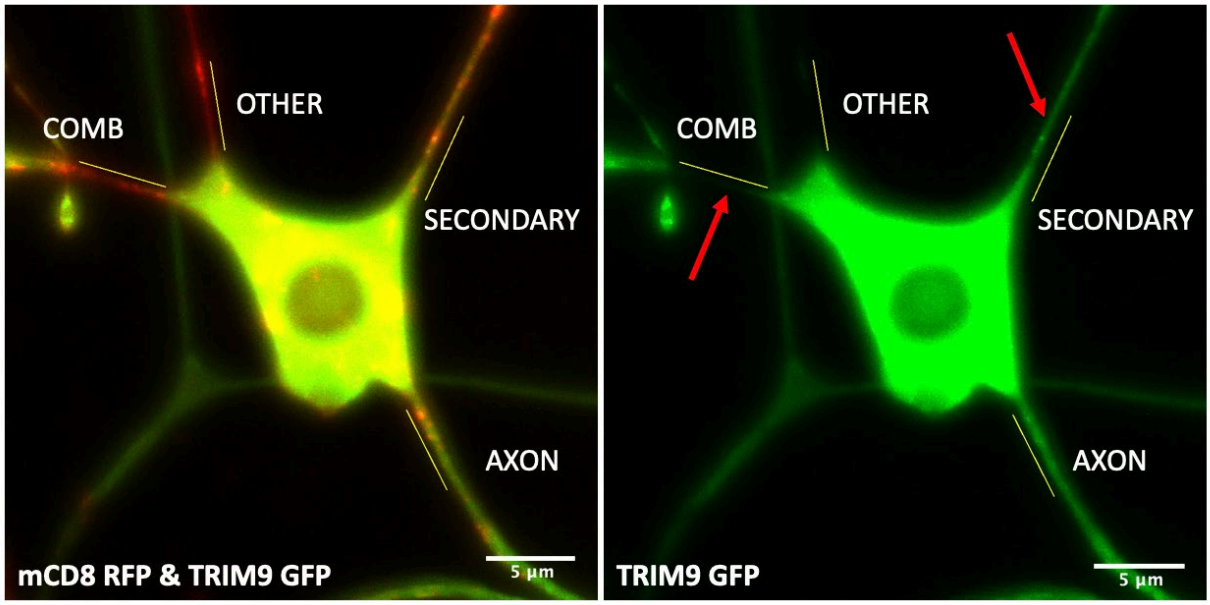

Figure 11: Enrichment of TRIM9 GFP in Secondary Dendrite. It is possible that the secondary dendrite is pre-disposed to have more TRIM9 localized in the secondary dendrite as a potential innate protective mechanism. As neurons must survive an organism's entire lifespan, protective mechanisms are needed should the cell sustain stress or injury. Enrichment of TRIM9 GFP in dendrites in uninjured conditions might serve to ensure the cell has the capability to convert a dendrite into an axon if needed during the organism's lifetime.

However, to confirm these speculations, further experimentation is necessary. One experiment to understand the full function of TRIM9 would be to examine whether TRIM9 relocalizes from the AIS to the converted dendrite post axotomy. To conduct this experiment, one would need to tag TRIM 9 with GFP, cross it with a tester line of Dicer2 ; 221-Gal4, UAS-EB1-GFP, and measure the fluorescence of TRIM9 pre-injury. Then the axon would need to be

severed using a UV pulse laser. Following axotomy, the cell would need to incubate 72-96 hours to allow the cell to convert a dendrite into an axon. Then, one would need to examine which dendrite had converted through examination of plus-end out polarity in the converted dendrite and determine the localization of TRIM9. With this experiment, I would hypothesize that post injury, TRIM9-GFP enrichment moves from the AIS to the cell body exit points of the dendrite that converts, which would be noted by plus-end out microtubule polarity in that neurite. Additionally, with the same experiment, one could also examine the enrichment of TRIM9-GFP in the dendrites pre-injury and determine if the dendrite with more TRIM9 pre-injury is the dendrite that will convert to the new axon post-injury. These results would help to confirm speculations of a neuron's backup mechanism for converting dendrites.

Another area worthy to investigate would be to examine TRIM9's role in tip growth post axotomy. The mechanism for polarity reversal in an injured cell involves tip growth of the converted dendrite approximately 72 to 96 hours post axotomy (Figure 12) (Stone et al., 2010). As this thesis shows evidence for TRIM9's role in microtubule polarity re-establishment 48 hours post injury, it could be possible that significantly reducing the amount of TRIM9 expressed in an uninjured cell via RNAi would significantly decrease the amount of tip growth at the 72- and 96-hour time points compared to a control cross.

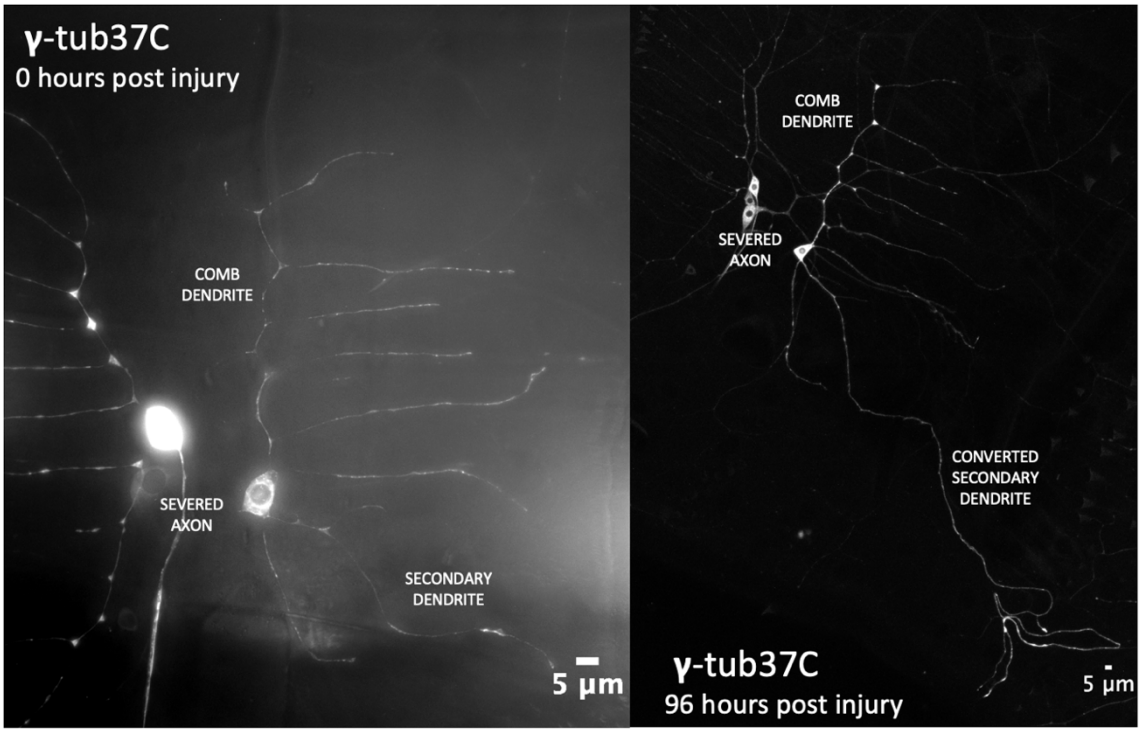

Figure 12: Initiation of tip growth post axotomy. After an axon is severed using a UV-pulse laser, a dendrite will convert into a new axon and extend its process significantly at the 96-hour time point. This image shows an example from our control cross, γ-Tubulin 37C. It is proposed that RNAi of TRIM9 would significantly decrease the amount of tip growth 72-96 hours post injury.

While the current research supplied by this thesis cannot prove the entirety of the function of TRIM9, I believe it is possible for TRIM9 to be a key protein involved in positive regulation of microtubules in both uninjured and injured conditions (Figure 12). However, with the proposed experiments, one would be able to further confirm TRIM9's purpose in *Drosophila* neurons as a positive regulator and innate mechanism for neuroprotection.

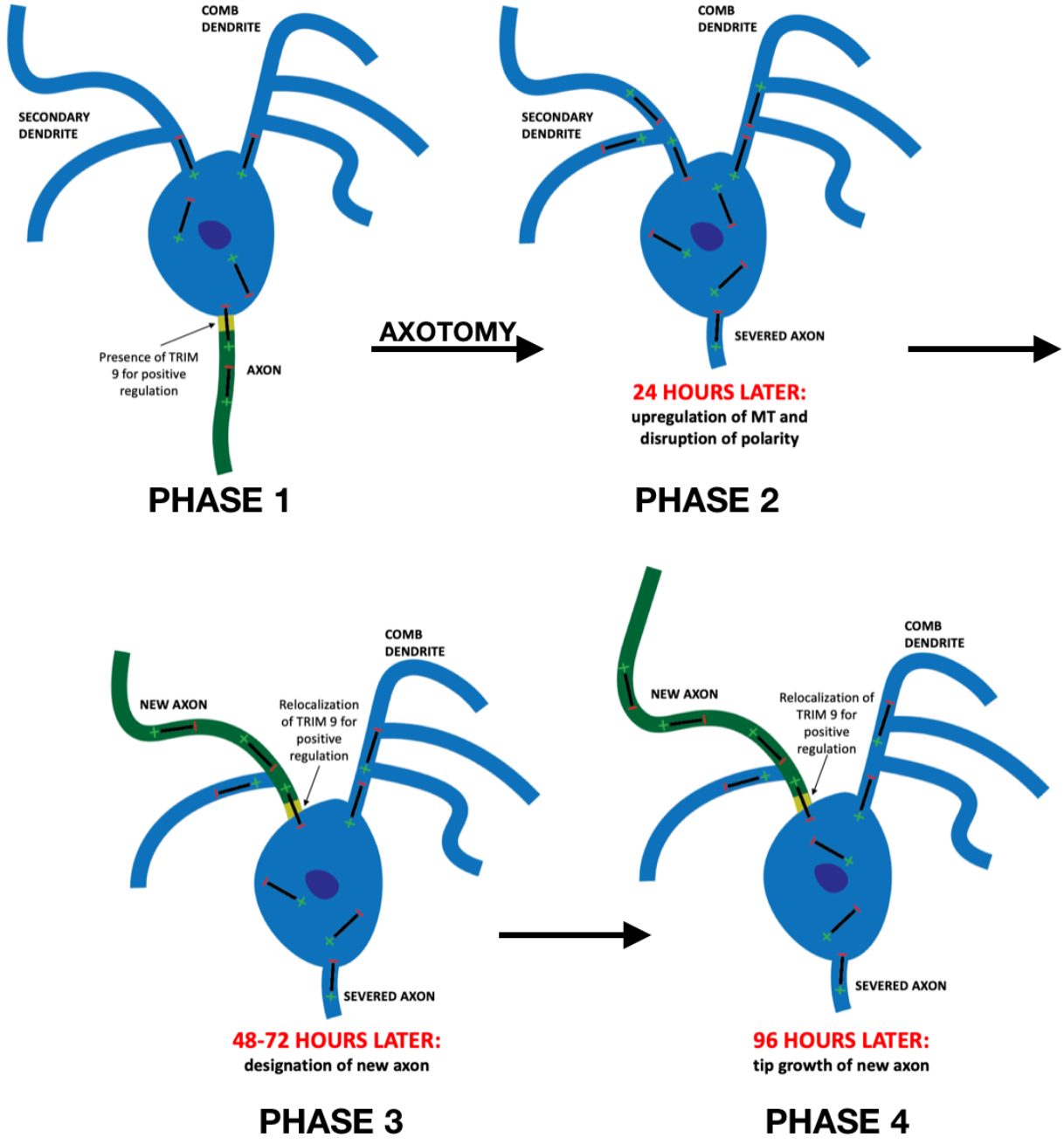

Figure 13: Proposed model of TRIM9's role in microtubule polarity maintenance. TRIM9 is predominantly localized in the AIS to positively regulate plus-end out microtubule polarity in axons. Should the cell be subjected to injury or stress, TRIM9 redistributes to the secondary dendrite to reverse minus-end out polarity to plus-end out polarity and promote tip growth, thus converting the neurite into an axon. It is also possible that a dendrite might be pre-dispositioned with an increased amount of TRIM9 to aid in conversion to an axon should the cell be subjected to stress or injury. This model exhibits TRIM9's role in maintaining uniform microtubule polarity in axons, as well as its role in axon specification.

When originally screening for protein candidates involved in positive and negative regulation, RNAi of Klp61F showed a phenotype analogous to positive regulation. These findings further cement the speculations found in other research studies, stating that Klp61F might work to crosslink parallel microtubule arrays (van den Wildenberg et al., 2008). I predict that as a positive regulator, Klp61F reinforces plus end out microtubule polarity in the axon by cross-linking plus end out microtubules at the cell body exit point. In relation to localization, previous studies have also uncovered Klp61F's function with crosslinking antiparallel microtubules and have proposed its role as a negative regulator (Heck et al., 1993; Sharp et al., 1999; Sharp & Rath, 2009b). Therefore, it might be possible that Klp61F is localized to both the proximal portion of the axon as well as the dendritic cell body exit points but serves different functions at each location: promoting polymerization of plus end microtubules leaving the axon, and depolymerizing plus end microtubules leaving the cell body at the dendrite. Clearly, further studies with Klp61F should be completed to determine if reduction of the protein causes a disruption in the re-establishment of microtubule polarity under injury conditions as well as the localization of Klp61F within the cell. These studies would likely take form under the same protocol used for the TRIM9 experiments, depending on the results uncovered.

Examination of both TRIM9 and Klp61F show the importance of positive regulation of plus end microtubules in the axon. For cell machinery to reach the axon terminal, stable microtubule tracks are needed for motor proteins like kinesin to carry cell cargoes to these areas (Hirokawa, 1998; Rolls & Jegla, 2015). Without stable plus end microtubule arrays, this machinery cannot get to the proper areas of the cell, and cell processes such as neurite outgrowth, axon polarization, pathfinding, and branching (Fong et al., 2017). Clearly, the

mechanisms governing the distinct microtubule polarity plays a pivotal role in maintaining proper neuronal function.

Chapter 4

Methodology

Drosophila Stocks and Expression System

Drosophila were obtained from the Vienna *Drosophila* RNAi Center (VDRC) and Bloomington Drosophila Stock Center (NIH P40OD018537). All flies were kept at a constant temperature of 25°C on a medium of water, agar, yeast, soy flour, yellow cornmeal, light corn syrup, and propionic acid. Flies were flipped approximately every three weeks to maintain stocks' vitality.

For the experiments of this thesis, two tester lines were used. For microtubule dynamics assays, the tester line, dicer2; 221Gal4, UAS-EB1-GFP/TM6 was used. The enzyme, dicer, is used to cleave the dsRNA into short interfering RNAs (siRNA) (Novina & Sharp, 2004). In our tester line, dicer2 was specifically over-expressed to enhance the effects of RNAi. For localization experiments, UAS-mCD8-RFP-221 Gal4 was used. This tester line enabled the entirety of the cell to be visualized with Red Fluorescent Protein (RFP) and provided contrast to observe localization of proteins with Green Fluorescent Proteins (GFP).

To set up a cross, virgin female flies were selected from the respective tester line from stocks that were kept at 18°C. Males with the genotype of interest were put into a chamber with a "food cap" and the previously collected virgins so mating could occur. Each day, food caps were

switched out to collect progeny. Larvae were obtained from the collected food caps in their 2nd or 3rd instar stages for imaging.

With each RNAi cross, a control was used. In our crosses, the VDRC line, γ-tubulin 37C (#25271), was used as a control as the protein is only expressed in embryonic neurons and not the mature neurons I used to study the cellular processes involved in our experiments. The other fly lines that were used for RNAi experiments included VDRC lines, TRIM9 (#100767), TRIM9 (#27405), and Kl61F (#52549), as well as Bloomington line, Septin1 (#27709). These were crossed with the UAS-Dicer2 ; 221-Gal4, UAS-EB1-GFP tester line.

For the localization crosses, TRIM9 RB#4 was used as it labeled TRIM9 with GFP. This was crossed with the UAS-Dicer2, UAS-mCD8-RFP tester line.

Microtubule Polarity Assay

Live 3rd instar larvae were placed on a slide with a circular piece of dried agar. Once larvae bodies were manipulated to be dorsal side up, a coverslip was taped down on top of them. These larvae were viewed through the Zeiss Widefield microscopes. Orientation of the larvae was determined on 10x and neurons were identified using 63x. Class I ddAE neurons in positions 4-6 from the left and right sides of the larvae were chosen to be imaged throughout all experiments.

To collect EB1-GFP movies, larvae were imaged for 300 frames at a rate of one frame per second. After videos were obtained, they were viewed using the Fiji (ImageJ) software. A template matching feature was used to correct for movement. Visible microtubule “comets” leaving the first 5 μm of neuronal processes attached to the cell body were counted. Comets that

surpassed 5 μm outside of the cell body were deemed as "successfully" exiting the cell body. Comets that did not surpass 5 μm were classified as being "unsuccessful" in leaving the cell body. Kymographs were obtained using a built-in plug-in in the Fiji (Image J) software. A percentage of successful and unsuccessful microtubule exits was obtained and statistics were calculated (see Statistical Analyses).

Axon Injury Microtubule Dynamics Assay

Axon injury, or axotomy, was used to emulate establishment of microtubule polarity in mature neurons. Live 2nd or 3rd instar larvae were mounted on slides and neurons were located in using the same methods as the microtubule polarity assay. Neurons that had axons well separated from the ddaD neuron were used to avoid injury of other cells. To sever the proximal portion of the axon from the remainder of the process, a pulsed UV laser was focused through the 63X objective of either a Zeiss confocal microscope or Zeiss Widefield microscope. The laser was triggered through a foot pedal that was paired with the region of the cell body on the microscope. The amount of light from the laser was adjusted so that explosion cuts were avoided.

After axotomy was performed, the larvae were isolated at 20°C for 8, 24, and 48 hours. Once the allotted amount of time had passed, EB1-GFP videos were obtained from a Zeiss Widefield microscope in a manner identical to the uninjured microtubule dynamics assay. Preparation and quantification of EB1-GFP videos was performed using the Fiji (ImageJ) software in the same fashion as the uninjured microtubule dynamics assay. Statistical measures were performed to determine if TRIM9 was involved in the re-establishment of microtubule polarity (see Statistical Analyses).

Fluorescence Live Imaging of *Drosophila*

Live 3rd instar larvae were mounted on slides and neurons were identified through the use of a Zeiss LSM800 Confocal microscope in the same manner as both the microtubule polarity and axon injury assays. Once the cell was brought into focus on the 63X setting, Z-stack and time series images were obtained of both the entire cell and a close-up of the cell body region.

Images were uploaded to Fiji (ImageJ) and examined with the red and green channels overlaid on top of each other. TRIM9RB#4 was measured by using the green channel and calculating the first 5 μm of each neuronal process. Values of fluorescence were calculated by manually tracing the bounds of the neurites within the 5 μm region. These values were averaged to obtain the average values of GFP in the axon, comb dendrite, secondary dendrite, and additional dendrite extending from the cell body (labeled as "other"). Statistical measures were performed to determine if any neurite had significantly more TRIM9 RB#4 than the others (see Statistical Analyses).

Statistical Analyses

Significance of findings was determined though use of GraphPad QuickCalcs software. A Fisher's Exact test was used for both the microtubule polarity and axon injury assays; percentages were calculated from a summation of successful microtubule exits versus unsuccessful microtubule exits, with both of these values compared to the total number of microtubule exit attempts. Thus, no error bars were used. A Simple T-Test was used for average amount of fluorescence in neurites for TRIM9 localization. Error bars show the standard deviation. P values were calculated and categorized by the following notation: *$p<0.05$, **$p<0.001$, and ***$p<0.0001$.

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- Designs independent projects/experiments/grant proposals to study microtubule polarity in *Drosophila* neurons
- Presented data at Undergraduate Research Exhibition at The Pennsylvania State University | Spring 2018, 2019

Global Health Minor Fieldwork | Pennsylvania State University **Dar Es Salaam and Dodoma, Tanzania**
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- Experienced urban and rural settings in Tanzania to learn about and understand healthcare in a low-income country
- Presented lesson about nutrition to students, faculty, and staff at the Dodoma School for the Deaf
- Learned entry level Swahili to converse with Tanzanian colleagues and professors

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Member and Volunteer Fall 2018- May 2020

- Assists in organizing clinics for those who do not have access to proper care in PA
- Volunteered at Gloucester, Virginia and Ashtabula, Ohio clinics to provide $1 million of free health care to 1000+ patients
- Involved in project to change legislation preventing out of state medical professionals from volunteering their services

SHO TIME | Schreyer Honors College **University Park, PA**
Mentor 2017, 2018

- Selected to mentor incoming freshmen in the Schreyer Honors College during orientation sessions
- Provided additional support to mentees after orientation to help with the transition into college
- Completed leadership course, LA 498, and attended SHO-TIME Mentor training sessions

WORK EXPERIENCE

Bedford Regional Urology, PC **Everett, PA**
Medical Assistant Summer 2019

- Assisted surgeon and PAs with medical procedures, including prostate biopsies, cystoscopies, bladder scans, and urinalyses
- Used Intergy EHR to document patient records and procedures; including patient health histories and current medications
- Designed Keynote presentation and Wix websites for marketing of medical services

Omni Bedford Springs Resort **Bedford, PA**
Banquet Server Summer 2018

- Worked on a team to execute high class hospitality events including weddings, banquets, and company retreats
- Nominated for Omni Service Champion Award for showing discipline, improvisation, and leadership skills during events

TEACHING EXPERIENCE

The Pennsylvania State University **University Park, PA**
Teaching Assistant Spring 2020

- BIOL/BBH 470, Functional and Integrative Neuroscience
- NURS 499, Exploring the Healthcare System in Costa Rica

Learning Assistant Position Fall 2019

- PHYS 251, Introductory Physics II

AWARDS AND DISTINCTIONS

- Dean's List | *Summer 2017-Summer 2019*
- Biological Sciences Society Executive Board, Advertising and Relations Director | *2019- 2020*
- Tour Guide and New Member Education Service Chair; Penn State Lion Ambassadors | *2019-2020*
- Alpha Epsilon Delta, Pre-Health Honors Society; National Distinguished Member | *2018-2020*
- Student Engagement Network Grant | *Spring 2019*
- Schreyer upperclassmen advising panel | *2018, 2019*
- Participated in embedded program "Exploring the Healthcare System in Costa Rica" | *Summer 2018*