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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

MITOCHONDRIAL LOCALIZATION AND INTERACTIONS AT DENDRITIC BRANCH POINTS OF *DROSOPHILA* SENSORY NEURONS

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A thesis
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ABSTRACT

The correct positioning of organelles and proteins in dendrites is a necessity for normal dendrite development and, ultimately, proper neuron functioning. While there is currently an understanding of neuronal growth and development in relation to environmental cues, there is still a lack of understanding in what establishes the specific architecture of a dendrite. Mitochondria, an organelle well known for its energy production and trafficking in neurons, are robustly localized at dendritic branch points, as are other various proteins necessary for neuron development. We have previously determined specific regulators that affect localization of these dendritic branch point proteins. Therefore, we hypothesize that there is an active mechanism that is responsible for targeting mitochondria specifically at dendritic branch points. Using the *Drosophila* dendritic arborization neuron as a model, we have performed candidate RNAi screening on transgenic *Drosophila* with GFP-tagged mitochondria in order to identify the regulators involved in mitochondrial localization at dendritic branch points. The results of these knockdown experiments have shown that mitochondria are strongly targeted at branch points, as genetic screens weakly affect mitochondrial localization.

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Introduction

The Neuron

In many vertebrate and invertebrate animals, cells called neurons are responsible for receiving and responding to environmental stimuli, as well as internal processes that occur within an organism (Silverthorn, 2012). The function of the neuron is highly dependent on its structure, which generally includes three main subcellular compartments: the cell body, or soma, that houses the nucleus and membrane-bound organelles; a single, long axon structure which extends from the soma to a specific target to transmit messages in an outward direction; and the shorter and branched dendrite structure responsible for receiving multiple external signals at distal receptors and propagating these signals back to the cell body for response. A neuron must both develop and maintain this morphology in order to carry out its functions and contribute as a signaling unit to an overall properly functioning nervous system. Within a typical nervous system, there are a variety of classes and types of neurons that are each structured in a unique manner in order to carry out the functions necessary in its environment (Kandel, Schwartz, Jessell, Sigelbaum, & Hudspeth, 2012). The most commonly represented neuron depicts a single cell body containing one long axon and a more complex system of branched dendrites (**Figure 1**).

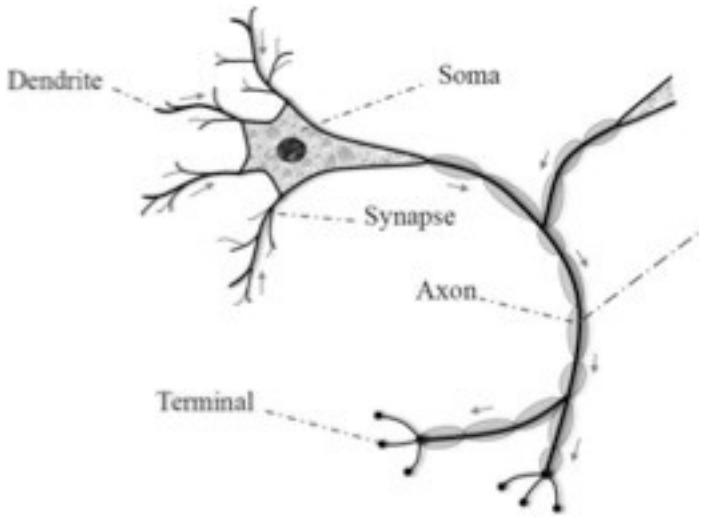


Figure 1. Simple Neuron Structure.

This simple illustration displays the morphological features of a neuron. Adapted from (Haddad, Hui, & Bailey, 2014).

Axonal and Dendritic Development

In order to fully appreciate the difference in information transmission between dendrites and axons, it is important to understand how each part of the neuron develops anatomically to fulfill these functions. This key aspect comes from differences in protein and organelle composition between axons and dendrites. The trafficking of proteins and organelles to these distinct regions is performed by microtubule-based transport. Microtubules are cytoskeletal filaments composed of tubulin subunits. These structures are polarized, where one end of the microtubule is deemed a plus-end and the opposite end is the minus-end. The plus-end tip is home to processes of polymerization and depolymerization of tubulin subunits (Baas & Lin, 2011).

A characteristic of mammalian neurons is that microtubules are oriented with the plus-end distal to the soma while dendrites have mixed microtubule polarity. While this feature exists in mammalian cells, studies performed on *Drosophila* neurons demonstrated that axonal

microtubules are plus-end-out as mammals, but almost all (about 90%) dendritic microtubules are minus-end-out microtubules in dendrites (plus-end-out in dendrites occurs at the tip) that initially had mixed polarity early on in development (Stone, Roegiers, & Rolls, 2008). Microtubules are used as cargo trafficking highways via motor proteins that generate movement from ATP hydrolysis. The two general families of motor proteins are kinesins – moving towards plus-ends – and dyneins – moving towards minus-ends (Cooper, 2000; Guzik & Goldstein, 2004). Because of this difference in microtubule organization, and as a result difference in motor protein trafficking, differential trafficking of biomolecules between axons and dendrites suggests that it is a key driving force in providing axons and dendrites with unique shapes and functions based on protein composition, including those that are involved in processes such as neurotransmission and action potentials. This is strong evidence that microtubule features play a heavy role in determining the morphology of axons and dendrites, which is also an important feature in human diseases. Abnormal dendrite structure due to developmental mutations is a constantly observed structural feature in mental retardation (Kaufmann & Moser, 2000).

Branch Points

Studies have suggested that in order for the correct neuronal morphology to be established, proteins and organelles must be properly localized. For example, the localization of Golgi outposts in the branch points of dendritic arborization neurons regulates the length of the branch that stems from that branch point (Ye et al., 2007). In order to fully expand our understanding of the roles that specific proteins and organelles play during development, it is

important to consider where they are localized in the neuron and how this has an effect on processes that occur during neuron anatomical development.

Dendrites house important proteins and organelles that carry out reactions and processes necessary for establishing correct morphological features. Studies done in mice hippocampal neurons that lacked Microtubule-Associated Protein 2 (MAP2) and MAP1B displayed thinner and shorter dendrites (Harada, Teng, Takei, Oguchi, & Hirokawa, 2002). *Drosophila* neurons with null mutations for *RhoA* had abnormally long dendrite arbors (Lee, Winter, Particke, Lee, & Luo, 2000). The regions within dendrites where arbors project from – termed dendritic branch points – specifically contain key proteins that play organizational roles during development. The protein of interest in this experiment, adenomatosis polyposis coli 2 (Apc2), is a Wnt signaling pathway and tumor-suppressing protein that is localized in the soma, dendrites, and the proximal portion of axons of *Drosophila* arborization neurons (Rolls et al., 2007). Within *Drosophila* Class I dendritic arborization neurons, it is localized at dendritic branch points where it has also been shown to indirectly regulate microtubule activity in dendrites by selectively recruiting proteins that are directly involved in microtubule guidance (Mattie et al., 2010).

Mitochondria in Neurons

Mitochondria play a variety of important roles just within the neuron, ranging from energy production to calcium regulation. Most commonly known as the powerhouse organelle, it contains the necessary enzymes and electron carriers to perform the Citric Acid Cycle and electron transport system that are required for oxidative phosphorylation and production of adenosine triphosphate (ATP). ATP is specifically important in neurons for acting as substrates

for other enzymes and ATP hydrolysis reactions for activating motor proteins involved in cargo trafficking, as well as acting on membrane pumps to reset cellular membrane potentials and regulate synaptic functions after action potentials, which may explain the high mitochondrial density in the (Shepherd & Harris, 1998) as seen in **Figure 2**. Mitochondria are also involved in the cellular intrinsic apoptosis pathway, in which the enzymes within the organelle signal for formation of the apoptosome and ultimately cell death (Lin & Beal, 2006; Wüllner et al., 1999).

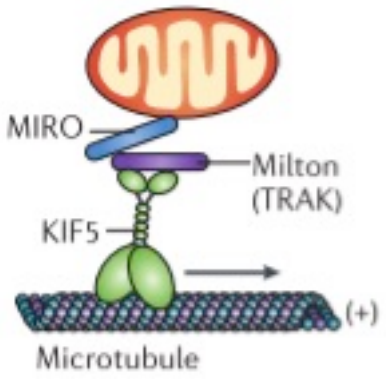


Figure 2. Mitochondrial Trafficking Unit.

The Miro/TRAK complex serves as a connection between mitochondria and kinesin motors (Sheng & Cai, 2012).

Because neurons are cells that can require high local energy demands (Howarth, Gleeson, & Attwell, 2012), mitochondria must be positioned at specific points along axons and dendrites in order to supply energy (Shepherd & Harris, 1998). Whereas the differential trafficking that is associated with different microtubule orientation in axons and dendrites drives polarized trafficking (Rolls et al., 2007), nonpolarized mitochondria are equally distributed throughout axons and dendrites. Mitochondria are trafficked by microtubule-based motors that are active during neuronal development by directly linking to Mitochondria Rho GTPase (Miro), which

acts as an adaptor protein that binds to a family of adaptor proteins known as trafficking protein, kinesin-binding (TRAK, sometimes referred to as Milton). This multi-protein complex is depicted in **Figure 3**. Different types of TRAK proteins are present in axons and dendrites and bind to kinesin and dynein/dynactin motors to affect mitochondrial trafficking in anterograde or retrograde, respectively. At some points, there are halts where mitochondria are fixed (van Spronsen et al., 2013a). This is a case specifically seen at dendritic branch points, as mitochondria have been shown to localize robustly at branch points early on in *Drosophila* development (Hill et al., 2012).

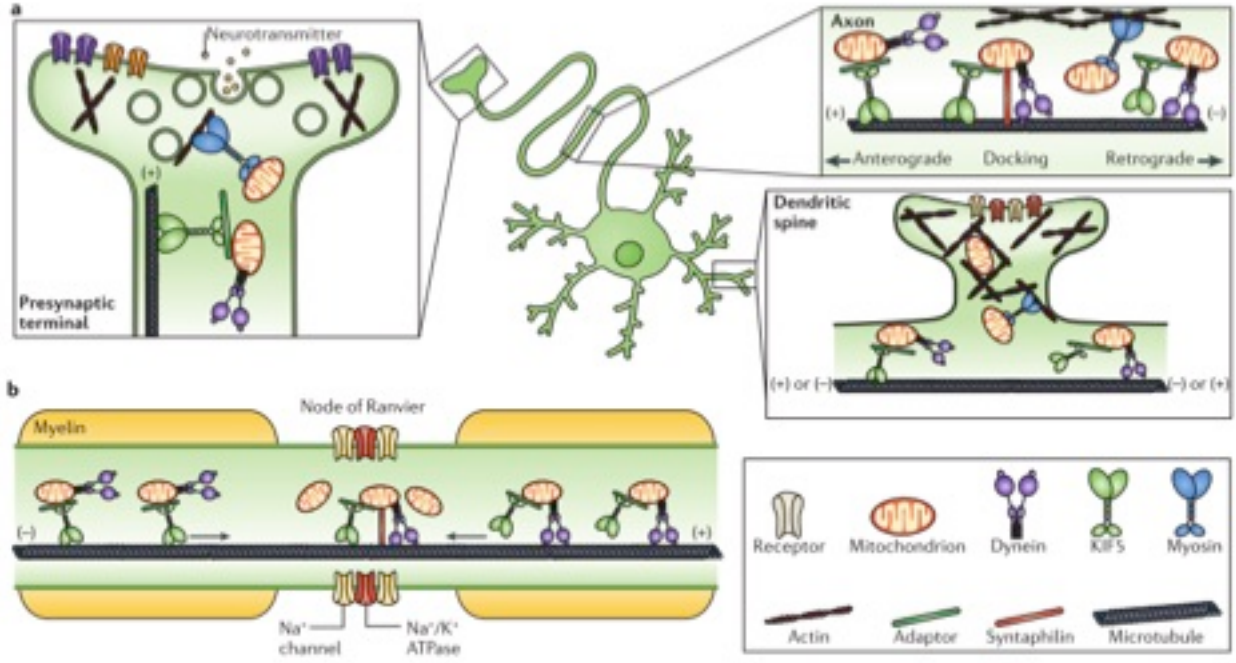


Figure 3. Mitochondrial Trafficking and Functions in Neurons.

The model above provides an understanding what functions mitochondria serves at each subcellular compartment of the neuron, as well as how mitochondria are trafficked to these positions (Sheng & Cai, 2012).

Drosophila, the Model Organism

One of the most efficient systems for studying dendritic structure is the *Drosophila* dendritic arborization neuron model. This model has been used for studying proteins involved in dendritic growth and development (Corty, Matthews, & Grueber, 2009; Hill et al., 2012). This system utilizes the dendritic arborization (da) neurons in the *Drosophila* peripheral nervous system, with the specific dendrites of interest in this study being that of the Class I neuron. There are four classes of da neurons within the *Drosophila* PNS, each of which is unique based on the dendritic branching pattern (**Figure 4**). Class I dendrites, in particular the ddaE neuron, display a dendritic branching network where branches grow almost orthogonally from a main trunk, producing a unique "comb-like" structure (Grueber et al., 2007). This distinctive feature of Class I da neurons is what provided room for understanding how Apc2 regulates microtubule dynamics (Mattie et al., 2010).

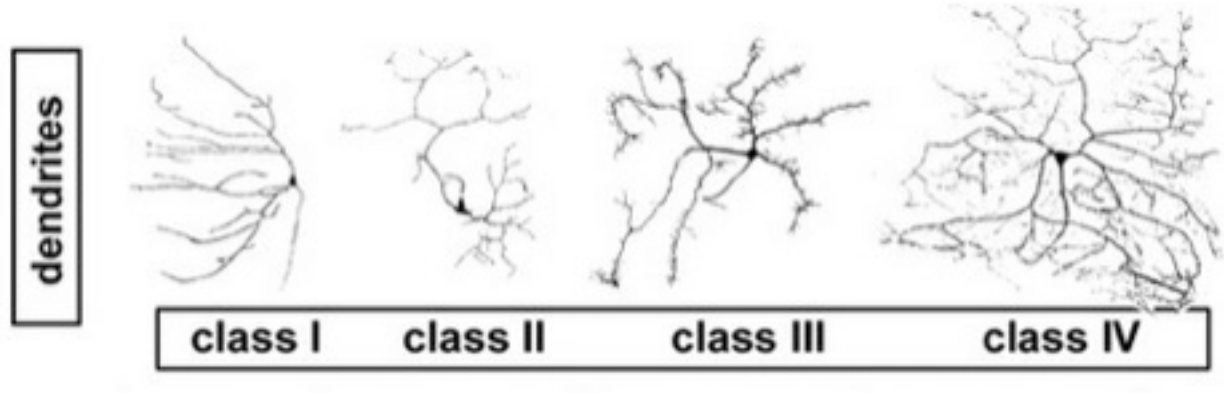


Figure 4. Four Classes of *Drosophila* dendritic arborization (da) neurons.

The four classes of da neurons within the Drosophila PNS each exhibit a distinctive dendrite tree. The neuron of interest for this experiment is the ddaE comb dendrite of Class I. Adapted from (Grueber et al., 2007).

Another important quality about *Drosophila* that makes the model so useful for genetic studies is the ability to create transgenic lines, with particular interest in the Gal4/UAS transcription system. Gal4 is an activating transcription factor derived from yeast that recognizes specific transcriptional regulatory elements – the upstream activating sequence (UAS). The presence of Gal4 in a certain cell is dependent on its regulatory sequence – the driver. Therefore, it is possible to express Gal4 in a specific cell in order to recognize and bind to UAS and activate expression of a gene whose sequence is downstream of UAS. Each type of neuron can be selected for this system by introducing the proper combination of driver-Gal4 (Brand & Perrimon, 1993). In the case of Class I neurons, the driver is 221 that will be used to express Gal4 exclusively in that type of neuron. There are several other drivers that can be used based on the experimental design, such as the elaV driver which is present in all four classes of da neurons (Robinow & White, 1988).

Drosophila can be further manipulated transgenically by altering proteins levels using RNA interference (RNAi). Taking advantage of what is believed to be an antiviral cell defense mechanism, large endogenously produced double-stranded RNA hairpins are processed by the protein *Dicer* into shorter dsRNA molecules. The processed RNA molecule is then cleaved by a protein called Argonaute, which will then associate with one of the single-stranded RNA molecules to detect any free homologous RNA molecules that are then targeted for degradation (Fire et al., 1998). This RNA-protein targeting complex is known as the RNA-induced silencing complex, or RISC (Pratt & MacRae, 2009; Rivas et al., 2005). The RNAi pathway has been used extensively as a powerful genetic interference tool, especially in *Drosophila* models containing the binary UAS/Gal4 expression construct that drives expression of a UAS-RNAi transgene (Dietzl et al., 2007).

Overview of Experimental Study

Candidate screening of Apc2-GFP with RNAi for mitochondrial proteins (involved in trafficking and ATP production) showed a significant knockdown of Apc2 localized at dendritic branch points in Class I *Drosophila* ddaE neurons. This suggests that mitochondria are involved in protein localization at these branch points. In order to determine how exactly mitochondria fits in the developmental map of localized proteins, similar screening techniques were performed to determine what is signaling for or targeting mitochondria at dendritic branch points.

We hypothesize that there is an active mechanism that is responsible for targeting mitochondria at dendritic branch points in *Drosophila* neurons. This was tested for using a forwards genetic approach by mating transgenic flies containing GFP-tagged mitochondria and UAS/Gal4 with flies from a candidate UAS-RNAi line, providing progeny time to develop, then visualizing the effects of this candidate protein knockdown in Class I ddaE neurons using confocal microscopy. Live-cell images were analyzed by quantifying the percent occupancy of mitochondria at dendritic branch points. A Fischer's Exact Test was incorporated to compare candidate RNAi quantification against that of the negative control (rtnl2 RNAi). Candidates screened were based on proteins that are known to localize at dendritic branch points, those that are involved in Apc2 localization (to test if there is a similar pathway), and other proteins involved in mitochondria function. These proteins are categorized as actin branch network members, membranous, microtubule-regulating, Wnt-signaling pathway members, involved in the electron transport chain, or serve other functions (**Figure 5**).

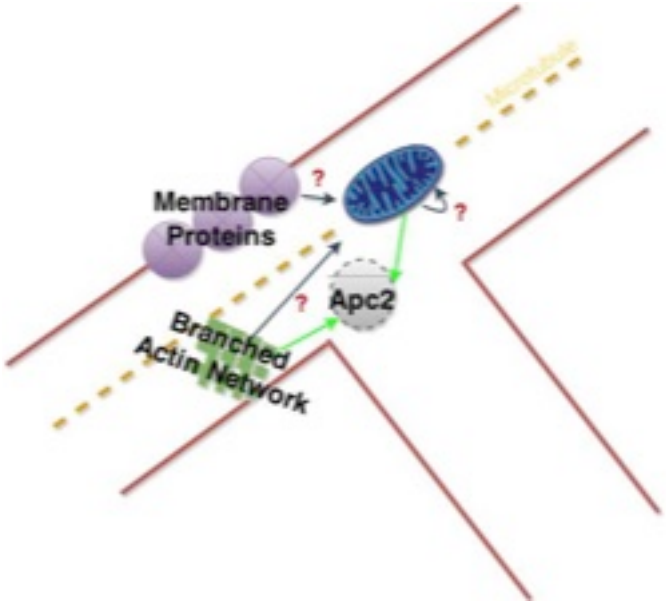


Figure 5. Simple Model of Interacting Branch Point Members.

The illustration above generally depicts what proteins are localized at branch points and how they function to localize Apc2 at dendritic branch points. We are testing to see if this is also true for mitochondria.

Significance to Human Disease and Development

Major mitochondrial roles like energy production and calcium regulation are vital towards eukaryotic cell life. Therefore, it is not surprising to note that dysfunctional mitochondria in neurons are linked to a plethora of commonly known neurological disorders including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), physiological aging, and cognitive defects (Anandatheerthavarada, Biswas, Robin, & Avadhani, 2003; Reddy & Beal, 2008; Wallace, 2005) On a more cellular scale, mitochondrial dysfunctions can lead to excitotoxicity and apoptosis (Ankarcrona et al., 1995; DeVorkin et al., 2014). Particular attention to the mitochondrial density at near synapses has also been made, as dysfunctional mitochondria in this region could negatively impact neurotransmission events (Shepherd & Harris, 1998). Uncovering the underlying signals that properly localize

mitochondria may help to determine if these localizing proteins are involved in the neurological disorders that are linked to mitochondrial dysfunction.

As mentioned before, abnormal dendritic structures are stereotypical of mental retardation, including Down syndrome, Rett syndrome, and Fragile-X syndrome. These abnormal dendrite trees hinder the ability to form functional synapses with axons (Kaufmann & Moser, 2000). Because the positioning of organelles and proteins are related to dendrite anatomy, it is vital to study mitochondrial localization in dendrites to determine if a connection can be made between mitochondrial positioning in neurons and mental retardation.

Methodology

RNAi and Mutant Candidates

Candidates for knockdown were initially selected for based on hypothesized relationships between branch point members and mitochondria. Since mitochondria-trafficking proteins were found to affect Apc2 localization, the candidates selected for screening were based on those that were also screened with a constructed Apc2 tester line. *Drosophila* stocks expressing the UAS-RNAi transgene or mutated genes in this study (**Table 1**) were obtained from the Vienna *Drosophila* RNAi Center (VDRC) or the Bloomington Stock Center at Indiana University.

Table 1. RNAi and Mutant Lines

Symbol	Gene Name	Annotation Symbol	Symbol	Gene Name	Annotation Symbol
Rtnl2	Rtnl2	VDRC 33320	**Axn**	axin	VDRC 7748
GSK3β	shaggy	VDRC 101538	**Rab5**	Rab5	VDRC 103945
Rac.N17	--	BL 6292	**GO-α47a**	G-Protein α O subunit	VDRC 110552
racGAP50c	tumbleweed	VDRC 106850	**Miro**	Mitochondrial Rho	VDRC 106683
Sick	sickie	VDRC 10737	**Rac1**	Rac1	VDRC 49246
Spastin	spastin	VDRC 33110	**limK**	LIM-kinase1	VDRC 25343
ATPSynthaseβ	ATPSynthaseβ	BL 28056	**SesB**	Stress-sensitive B	BL 36661
Klp59C	Kinesin-like protein at 59C	BL 48576	**Fry**	furry	VDRC 103569
Arc-p20	Actin-related protein 2/3 complex, subunit 4	VDRC 41888	**Ank2F^{00508}**	Ankyrin 2 (mutated isoform)	--
Nrg on III	neuroglian	VDRC 27201	**Ank2**	Ankyrin 2	VDRC 107238

Table 1. RNAi and Mutant Lines.

This table reports the UAS-candidates that were screened for mitochondrial localization.

Tester Line

To specifically visualize mitochondria localization in Class I neurons, a tester line was constructed containing transgenes vital for protein visualization and RNAi-pathway efficiency. The tester line, $UAS-Dicer2, UAS-mCD8RFP; \frac{221Gal4, UAS-Mito-GFP}{TM6}$ inserted genes for Dicer2 (endoribonuclease responsible for cleaving double-stranded RNA to activate the RNA-Induced Silencing Complex, or RISC), MCD8 (to outline the neuron and its structures using a membrane marker) tagged with Red Fluorescent Protein (RFP) using Rhodamine Red-X, the 221 Driver to express the yeast transcription factor Gal4 exclusively in *Drosophila* Class I da neurons, and mitochondria tagged with Green Fluorescent Protein (GFP) using Enhanced Green Fluorescent Protein. The Gal4 transcription factor recognizes the Upstream Activation Sequence (UAS) transgenes to increase transcription in *trans*. The TM6 gene is a heterozygous mutation on the third chromosome that acts as a balancer to prevent meiotic homologous recombination from disrupting transgenic constructs. Transgenes were located on the second and third chromosome.

Crossing

About 10 virgin females from the tester line and 5 males from the candidate RNAi line were used to set up crosses. Using virgin females ensured that these flies were not yet inseminated, as females are able to hold sperm for an extended amount of time. Virgin females were collected before reaching sexual maturity. Termination of the pupil stage occurs eight hours at room temperature and 18 hours at 18°C. Gender selection was based on the morphological differences of the poster ends of the *Drosophila*. TM6 acts as a Tb (tubby) larval marker

(Lindsley & Zimm, 1992), so undesirable larvae that displayed the tubby phenotype were selected against.

The samples of *Drosophila* were placed in a container with a 35mm food cap to allow for mating. Food caps were replaced roughly every 24 hours and allowed to incubate for three days at 25°C to obtain third instar larva that had developed dendrites will also allowing enough time for the RISC system to knockdown protein of interest levels. To evaluate the effectiveness of RISC as a genetic tool, mutated candidates were also crossed with the tester line in the same procedure described above.

Confocal Microscopy

Live larvae washed in phosphate buffer solution were mounted with the dorsal side exposed on dry 3% agarose covered slides before gently taping a coverslip on top of the slide. Oiled slides were examined on an Olympus FV1000 microscope controlled by Fluoview software. The lasers used to fluoresce proteins were at wavelengths of 559nm for GFP excitation and 678nm for RFP excitation. Image files captured under 60X objective were composed of second-long picture frames that shifted focus along the neuron in order to later capture a full image on the Class I da neurons, in particular the comb dendrite of the ddaE neuron.

ImageJ

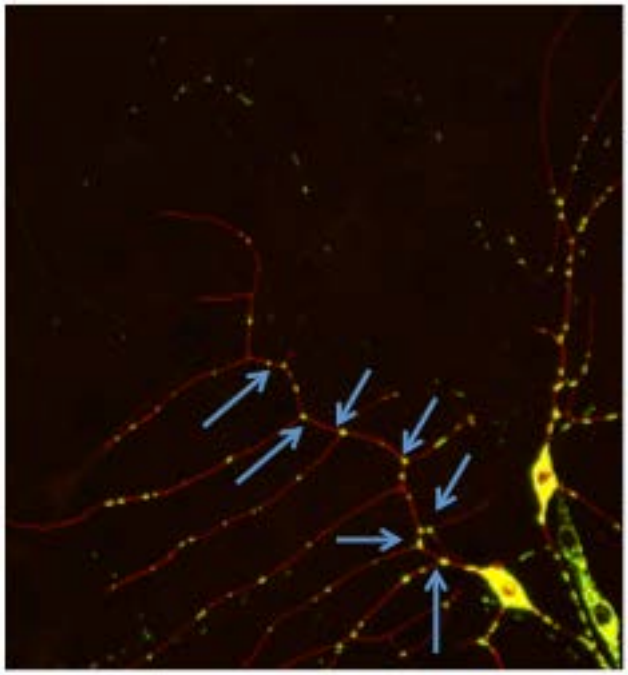
Temporal image files were transferred to the NIH-developed image processing program ImageJ in order to formulate files into one image for analysis. Files were transferred from the Fluoview program and opened up in ImageJ. The initial step in analyzing the images was to

merge the image's different channels (red and green) together. The images contained several frames were truncated to create a time-series with the whole da dendrite in focus by creating a Z-stack that layered all frames into one image. All slices within the Z-stack are then uniform size and bit depth (Hartig, 2013).

Quantification and Statistical Analysis

Each image was analyzed quantitatively by finding the total number of branch points along the main branch of the comb dendrite in the da neuron and the number of those branch points that are occupied by mitochondria localized directly in the branch point. Mitochondria localization was determined by where GFP was exhibited in the image. Counts from candidate RNAi lines were then analyzed statistically by performing a Fischer's Exact Test on a two-by-two contingency table against the *rtnl2* RNAi counts. UAS-*rtnl2* RNAi was used as a negative control, as this transgene was not shown to have a phenotype in microtubule polarity experiments (Mattie et al., 2010). Significance was based on p-value of a two-tailed t-test. The level of significance was based on the resulting p-value that is used to determine the validity of the null hypothesis of this experiment. The knockdown results of candidate screens relative to *rtnl2* RNAi results were significant if the $0.05<p<0.01$, very significant if the $0.01<p<0.001$, and extremely significant if the p-value<0.001.

Results



Total Branch Points	Branch Points Containing Mito-GFP
18	13
17	13
11	7
11	9
11	7
12	9
9	5
11	8
13	8
13	8
126	87

Figure 6. Mitochondria Localize Robustly at Dendritic Branch Points in *Drosophila* Class I Neurons.

The image above represents the control RNAi used throughout the course of this experiment (UAS-Dicer2, UAS-mCD8RFP; 221-Gal-4, Mito-GFP/TM6 x rtnl2 RNAi). The live-cell image (left) represents a Class I ddaE neuron with branch points occupied by Mito-GFP emphasized by blue arrows. The accompanying table (right) quantifies the total number of branch points and number of branch points containing Mito-GFP in the entire control sample. Each row records the results from a single image, with the sum of all images in the bottom row. According to the control sample, mitochondria are localized at about 69.05% (87/126) of Class I ddaE dendrite branch points.

Mitochondrial Localization at Class I Dendritic Branch Points was Affected by Some Proteins Involved in Development

The variety of candidates chosen for screening was based on information regarding protein localization and interactions that could be taking place at branch points. Each screen was quantified in the same manner described in **Figure 7**, and compared to the findings of the control screen with *rtnl2*. Interestingly, these findings do not correlate with the screening results from

Apc2 localization. Apc2 localization at dendritic branch points was decreased when levels of axin, rac, arc-p20, sickie, ank2, nrg, G-Oα47A, spastin, klp59C, miro, ATPSynthaseβ, and sesB were knocked down using RNAi (unpublshed data). From this list, the same effects for mitochondria were only seen in spastin, klp59C, and miro.

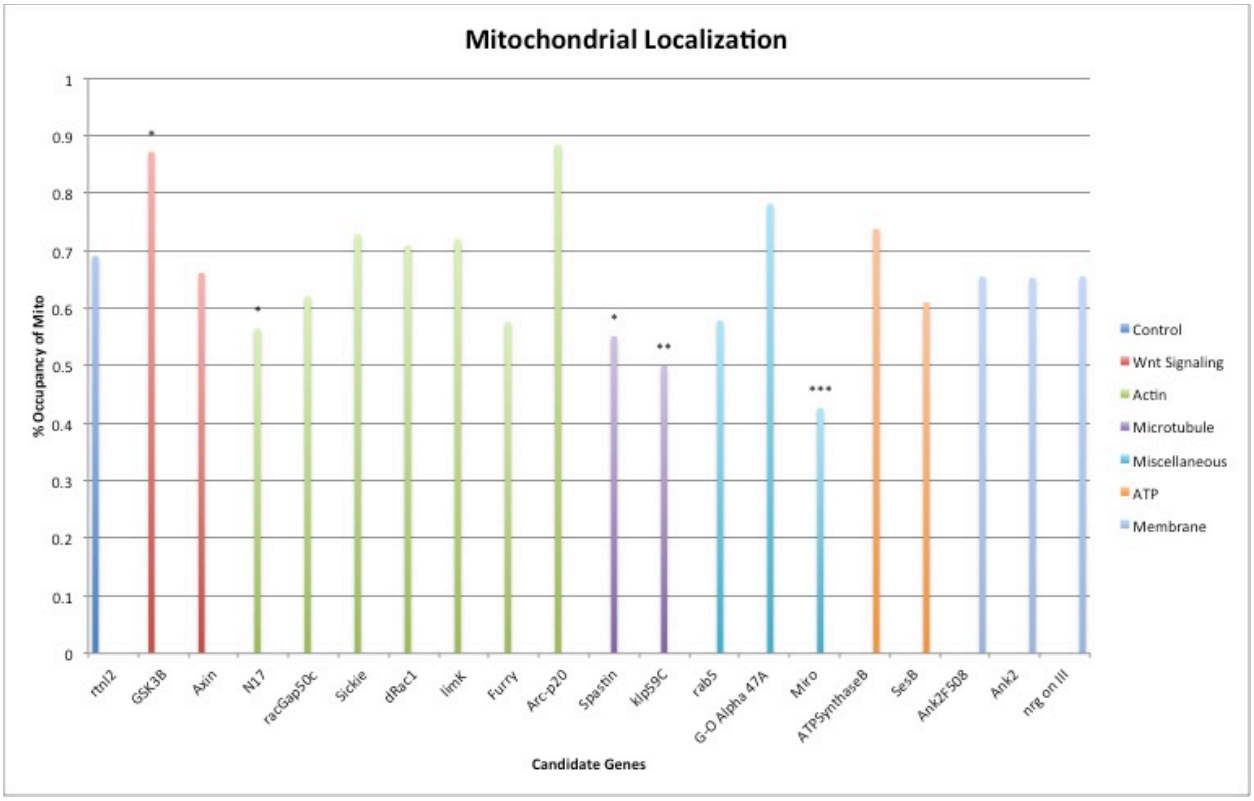


Figure 7. Composite of Screened Candidates.

The results of mitochondrial localization when knocking down candidate proteins is shown above. The proteins that were deemed for candidacy were based off of those that are involved in localization of other branch point members or are involved in mitochondrial functions. Asterisks denote the level of significance based on p-values from two-tailed Fischer's Exact Testing (* $p<0.05$, ** $p<0.01$, *** $p<0.0001$).

Knockdown of Miro Provided a Positive Control Phenotype

Miro is a motor adaptor protein that connects the mitochondrial outer membrane with TRAK/Milton proteins. Mutations in Miro cause a decrease in mitochondrial trafficking activity

(Misko, Jiang, Wegorzewska, Milbrandt, & Baloh, 2010). Therefore, knockdown of Miro resulting in decreased mitochondrial localization is logical as there is a decreased amount of complexes that are even able to traffic mitochondria down the dendrite trunk. Regardless, this finding is important as it validates the experimental design to by drawing upon another mitochondria model. Additionally, this finding because it serves as an example of what the phenotype should be like if a protein that is involved in localization gets knocked down. Therefore, the Miro RNAi helps to provide an example that another potential candidate can phenocopy (**Figure 8A**).

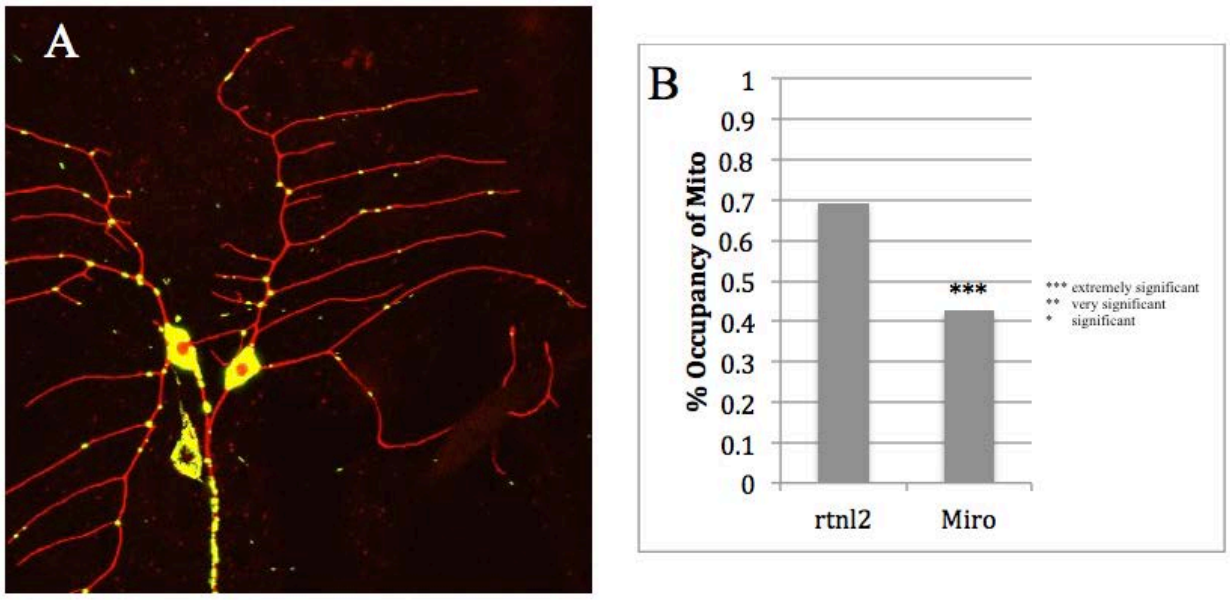


Figure 8. Knockdown of Mitochondrial Rho GTPase (Miro) Causes an Extremely Significant Decrease in Mitochondrial Localization at Dendritic Brach Points.

A. Knockdown of Miro resulted decreased number of branch points containing Mito-GFP. **B.** Quantified 42.59% (46/108) of branch points containing Mito-GFP. Compared to the rtnl2 RNAi control, this finding was extremely significant ($p<0.0001$).

Rac1.N17 Caused a Decrease in Localization of Mitochondria at Dendritic Branch Points

Rac1.N17 is a mutated form of the actin-regulating Rac protein with a mutation where the Amino Acid Asn in position 17 is susbtituted for Thr, creating a protein that is constituately bound to GDP instead of GTP and rendering the Rac1 protein inactive. Technically this is not a knockdown seen in screens with UAS-RNAi lines, but this is a dominant-negative mutation that affects Rac1 functioning. Rac1.N17 has been found to interfere with axonal outgrowth and Rac is involved in actin cytoskeleton (Luo, Liao, Jan, & Jan, 1994a), so it was tested to see if it had an effect on branch point member localization (**Figure 9A**). The Rac1.N17 screen resulted in a significant decrease in localization of mitochondria at branch points (**Figure 9H**).

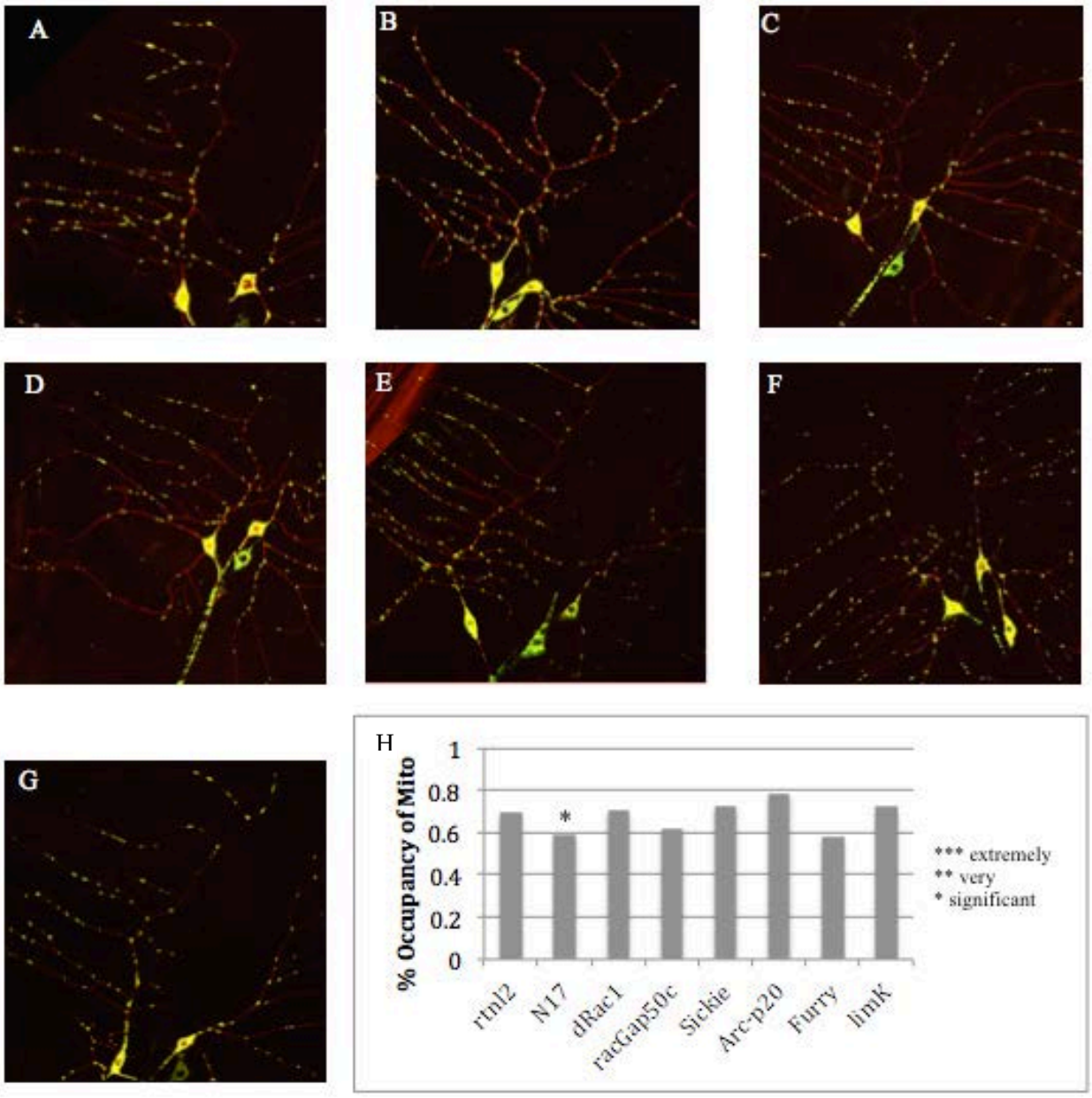


Figure 9. Effects of Knocking Down Members of the Actin Branching Network.

A. Rac1.N17 (66/117, 59.09%) **B.** dRac1 RNAi (85/120, 70.83%) **C.** racGap50c RNAi (67/108, 62.04%) **D.** Sickie RNAi (59/81, 72.84%) **E.** Arc-p20 RNAi (88/112, 78.57%) **F.** Furry RNAi (76/132, 57.58%) **G.** limK RNAi (77/107, 71.96%) **H.** Histogram comparing all actin branching network proteins. Rac1.N17 is the only screened member that caused a significant decrease in mitochondrial localization ($p<0.0467$).

Screened Membranous Proteins Did Not Significantly Affect Mitochondrial Localization

Of the three screens performed to affect membranous proteins, there were no significant changes in mitochondiral localization at branch points. Ankyrin proteins serve to connect the cell membrane to the actin cytoskeleton for stability. The Ank2 RNAi (**Figure 10B**) finding was confirmed by the findings of $Ank2F^{00508}$ (**Figure 10A**), where the function of long isoform of Ank2 is disrupted by a transposon insertion (Pielage et al., 2008). Neuroglian, a cell adhesion molecule that interacts with ankyrin proteins (Bouley et al., 2000), did not influence mitochondrial localization when knocked down with RNAi (**Figure 10C**).

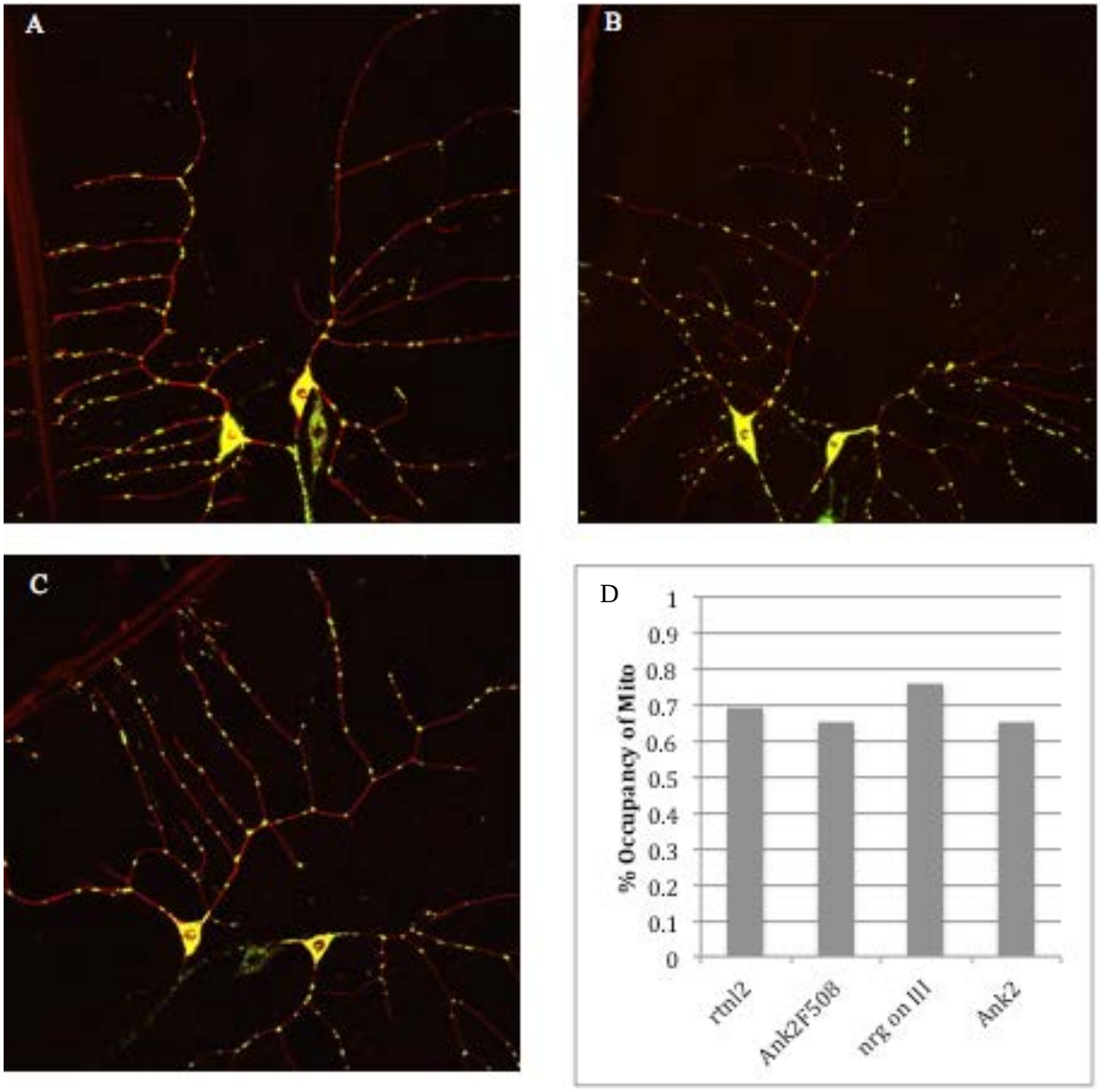


Figure 10. Effects of Knocking Down Levels of Membranous Proteins.

A. Ank2F^{00508} (74/113, 65.49%) **B.** Ank2 RNAi (79/121, 65.29%) **C.** neuroglian on III (85/112, 75.89%) **D.** Histogram comparing the screened membranous proteins.

Knockdown of GSK3β Caused an Increase in Mitochondria Localized at Branch Points

Because Apc and Apc2 plays a role in the Wnt signaling pathway during embryonic development (Bienz & Hamada, 2004), other members of the signaling pathway were screened

to see if they also had localization functions. Regardless, *Drosophila* Apc2 is more involved in actin regulation and microtubule organization rather than Wnt signaling (Zhou, Kunttas-Tatli, Zimmerman, Zhouzheng, & McCartney, 2011). To test of the Wnt-signaling members were involved in mitochondrial localization, members of the "β-catenin destruction complex" were screened. Axin, the Wnt-inhibiting scaffolding protein that binds to Apc2 directly (Y. Chen, Fu, & Ip, 2013; Song, Wang, & Li, 2014), did not have a significant effect on mitochondrial localization when knocked down with RNAi (**Figure 11A**). However, knockdown of GSK3β (glycogen synthase kinase 3) protein somewhow caused in increase in localization of mitochondria at dendritic branch points (**Figure 11B**). At this point there is no direct evidence of GSK3β interacting with mitochondria. However, there is evidence that GSK3 is a negative inhibitor of motor proteins transporting amyloid precursor protein in axons so there is some information regarding GSK3 and trafficking of biomolecules (Weaver et al., 2013).

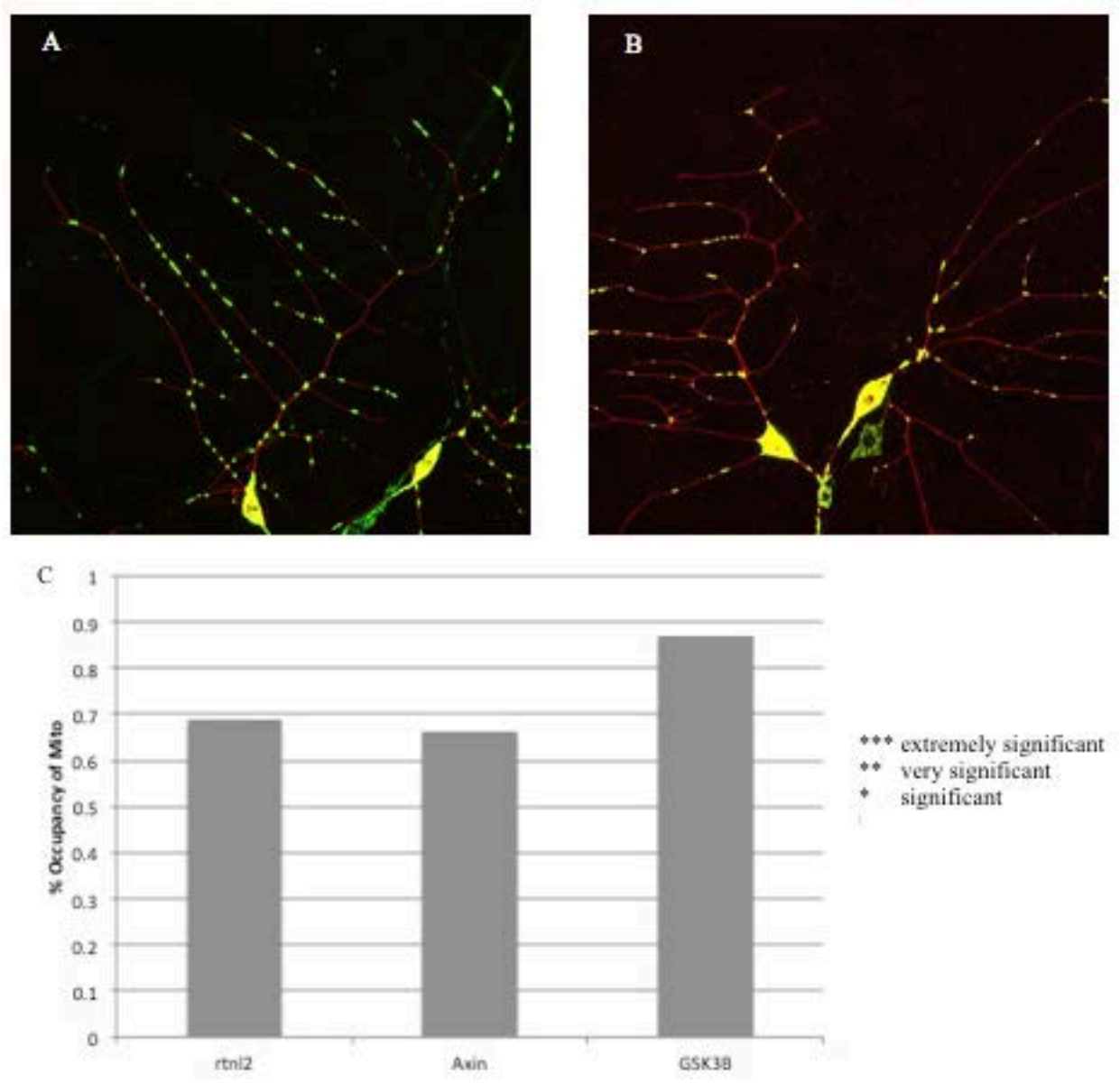


Figure 11. Effects of Knocked Down Levels of Wnt Signaling Proteins.

A. Axin RNAi (80/121, 66.12%) **B.** GSK3β RNAi (61/70, 87.14%) **C.** Histogram comparing the effects of screened Wnt-signaling proteins. GSK3β RNAi displayed very significant results ($p=0.0053$).

Spastin and klp59C Knockdowns Caused a Decrease in Localization of Mitochondria at Dendritic Branch Points

Both of the screened microtubule-regulating proteins caused a decreased localization of mitochondria at dendritic branch points using RNAi. Spastin is a microtubule-severing protein that belongs to the AAA (ATPases Associated with diverse cellular Activities) family, giving it great implications in axon regeneration and in *Drosophila* Class IV da neuron dendritic arbor growth (Jinushi-Nakao et al., 2007; Stone et al., 2012). Interestingly, the spastin loci is close to the miro loci (Guo et al., 2005). Current data on spastin suggests that the transmembrane domain of spastin is important to its microtubule-severing function (Stone et al., 2012). Knockdown of spastin caused a significant decrease in mitochondrial localization where mitochondria occupied 55.1% of branch points in the sample (**Figure 12A**). Kinesin-like protein at 59C (klp59C) belongs to the kinesin family (Goldstein, 1993) and has been identified as required for depolymerization of kinetochore microtubules in mitotic cells (Rogers et al., 2003). Reducing expression levels of klp59C using RNAi resulted in a very significant decrease to 50% mitochondria localized at branch points (**Figure 12B**).

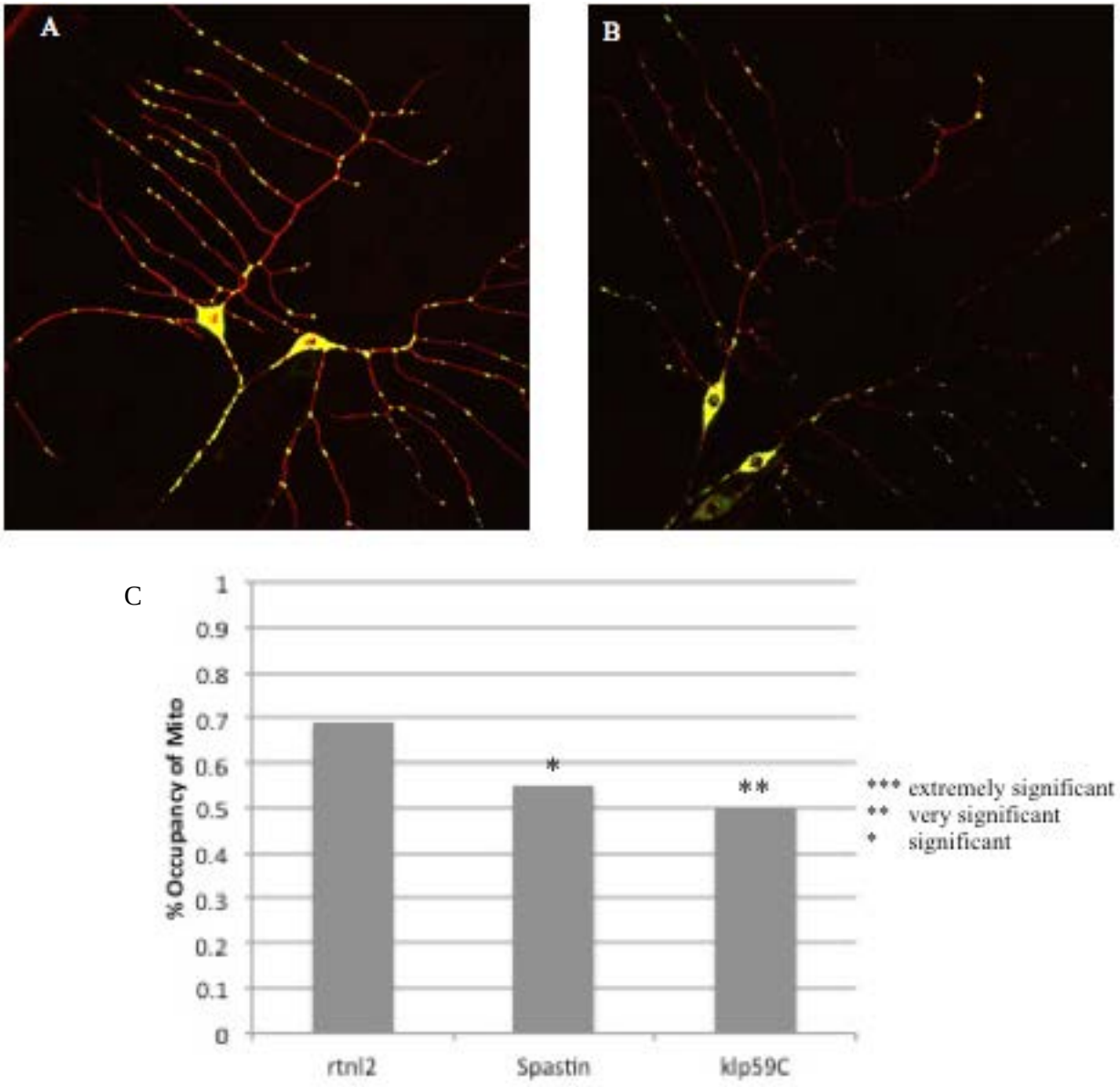


Figure 12. Effects of Knocked Down Levels of Microtubule-Regulating Proteins.

A. Spastin RNAi (54/98, 55.1%) **B.** klp59C RNAi (42/84, 50.0%) **C.** Histogram comparing the knockdown effects of Spastin (p=0.0369) and klp59c RNAi (p=0.0062).

Knockdown of Oxidative Phosphorylation Proteins Did Not Significantly Change Localization of Mitochondria at Dendritic Branch Points

It is important to test for the effects of dysfunctional ATP production in mitochondria on its own localization in order to determine if the role of mitochondia in branch point organization is

directly dependent on its localization. Mitochondria are organelles responsible for ATP production through substrate-level and oxidative phosphorylation, calcium regulation, and phospholipid synthesis. The results reported above test the role of ATP production by knocking down levels of the proteins ATPSynthaseβ (**Figure 13A**) and SesB (**Figure 13B**). ATP synthase is a mitochondrial membrane protein that produces ATP in the presence of an electrochemical proton gradient to oxidatively phosphorylate an ADP substrate. The F-type ATP synthase protein in *Drosophila* is comprised of the F_0 domain that has the membrane proton channel and the F_1 domain that has the extramembranous catalytic unit built up of three alpha and three beta subunits for nucleotide binding (Pena & Garesse, 1993; Senior, 1988). Stress-Sensitive B (sesB) is a mitochondrial translocase responsible for ADP:ATP antitransport across the mitochondrial inner membrane (Rikhy, Ramaswami, & Krishnan, 2003). Neither knockdown of these oxidative phosphorylation proteins caused a significant change in mitochondrial localization (**Figure 13C**). However, knockdowns of ATPSynthaseβ and SesB using RNAi in Apc2-GFP *Drosophila* revealed a decrease in Apc2 localization at branch points.

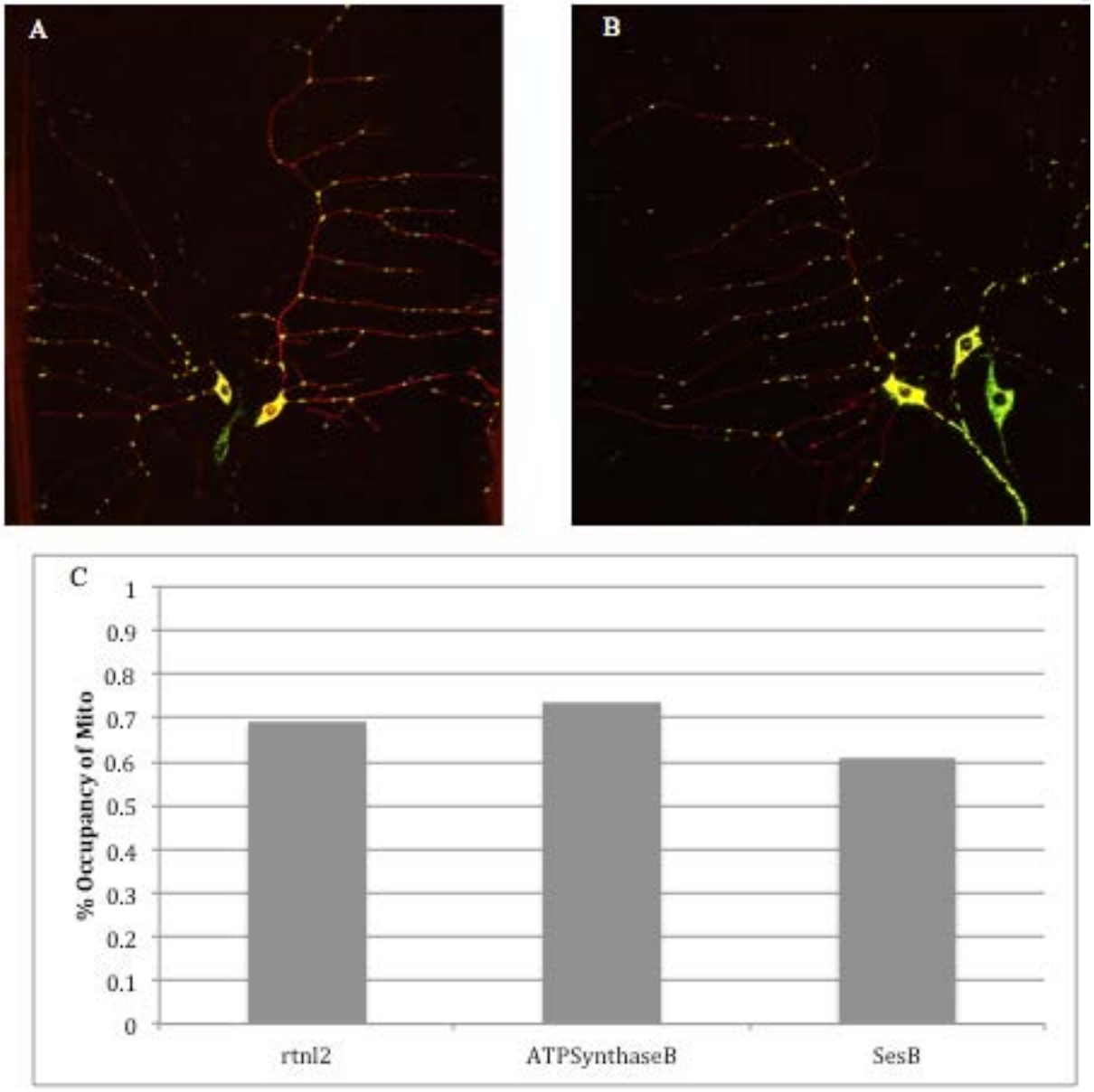


Figure 13. Effects of Knocked Down Levels of Oxidative Phosphorylation Proteins.

A. ATPSynthaseβ RNAi (104/141, 73.76%) **B.** SesB RNAi (72/118, 61.02%) **C.** Histogram displaying the insignificant knockdown effects of proteins involved in oxidative phosphorylation.

Further Candidate Screening was Performed with Miscellaneous Branch Point Members

Organelles are localized in *Drosophila* dendrites early on in development(Hill et al., 2012).

Therefore, it is important to test for colocalization of organelles. Rab5 is a GTPase that is

commonly used as an endosomal marker in early development (Bucci et al., 1992; Horiuchi et al., 1997). It functions to regulate endocytosis and synaptic vessicles (Shimizu, 2003; Wucherpfennig, 2003). Studies done using rab5 in dendritic arbor development have revealed important interactions between endosomes and microtubule transport via kinesin-73 (Huckaba, Gennerich, Wilhelm, Chishti, & Vale, 2010; Satoh et al., 2008). Mitochondrial localization was not significantly affected when knocking down rab5 (**Figure 14A**).

G-protein-coupled receptors are a well-classified family of cell surface transmembrane proteins that serve in cell signaling through either modulation or transduction. The complex (made up of α, β, and γ subunits) acts as a metabotropic receptor that, when activated, disassembles and allows the unbound GTPase α subunit to interact with downstream targets (Cattaneo et al., 2014; Kandel et al., 2012). Because G-oα47A was seen to cause a microtubule polarity phenotype in ddaE neurons, this screen was performed in order to see if there was a link between branch point interactions and microtubule polarity. The α subunit of G-O has been shown to have implications in Wnt-signaling and cytoskeletal components by interacting with Axin (Egger-Adam & Katanaev, 2009; Force, Woulfe, Koch, & Kerkela, 2007), Ank2 (Luchtenborg et al., 2014), and Rab5 (Purvanov, Koval, & Katanaev, 2010). The results of mitochondrial localization when levels of G-oα47A are knocked down are not significant (**Figure 14B**).

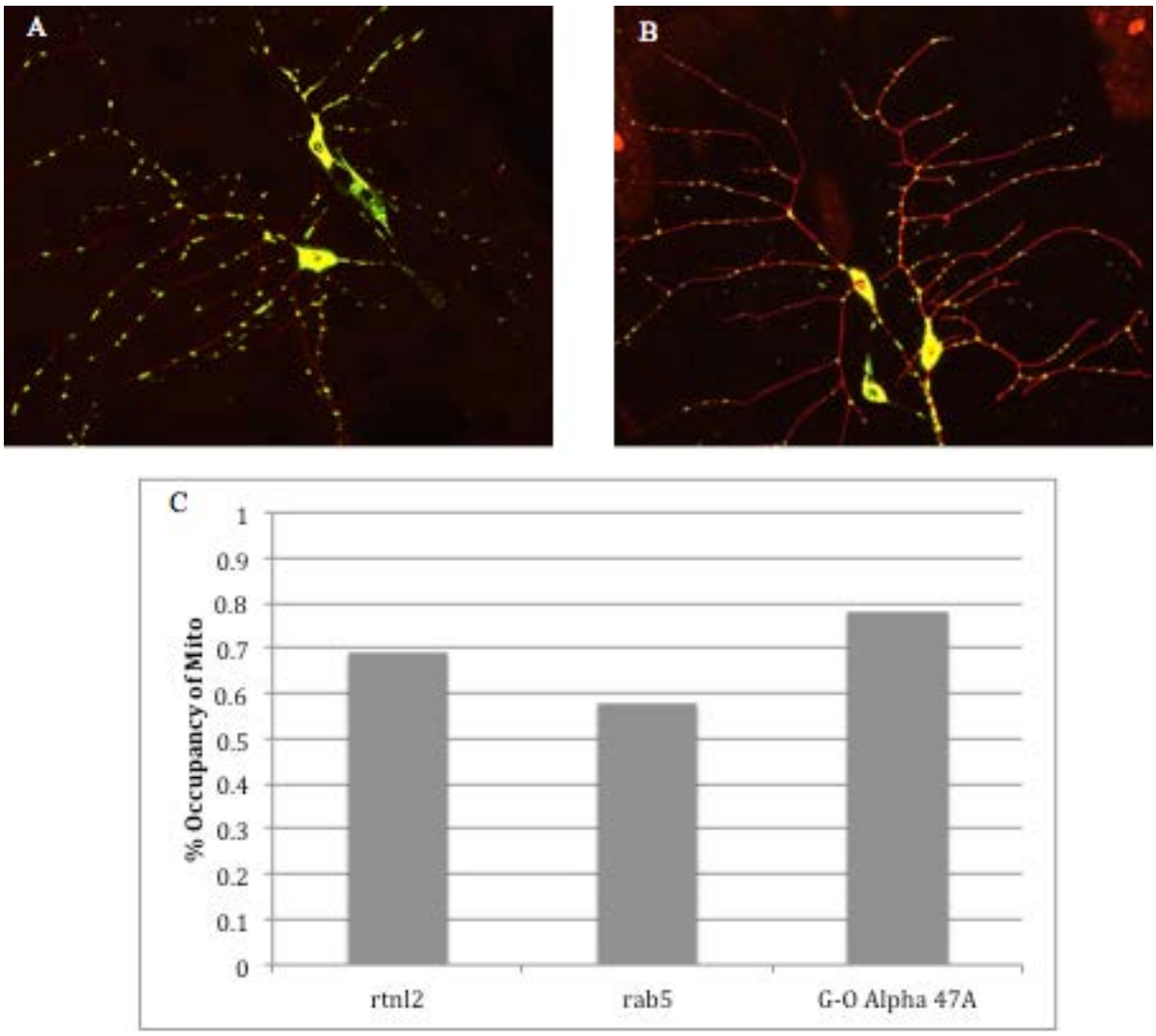


Figure 14. Effects of Knocked Down Levels of Rab5 and G-Oα47A Proteins.

A. Rab5 RNAi (59/102, 57.84%) **B.** G-oα47A RNAi (75/96, 78.13%) **C.** Histogram displaying the insignificant knockdown effects of rab5 RNAi and G-Oα47A.

Discussion

In this study, mitochondria were found to localize robustly at dendritic branch points of Class I ddaE neurons (**Figure 6**). When knocking down levels of mitochondrial proteins in similar Apc2 localization experiments, amounts of Apc2 at dendritic branch points decreased. Because this relationship exists, it is important to understand how mitochondria interact with other proteins during neuronal development. Additionally, because mitochondria are responsible for vital processes such as ATP production and calcium handling (reviewed in (Sheng & Cai, 2012), it is possible that mitochondria may be indirectly responsible for the proper localization of branch point members as opposed to the physical presence of mitochondria being a requirement for localization of branch point members.

At this point, there is no unifying connection between Apc2 and mitochondrial localization experiments, and thus it is not yet possible to thoroughly craft a coherent pathway model that delineates the ordering of proteins i.e. which proteins and organelles are downstream targets of other proteins and organelles. Almost all of the proteins that were suggested to affect Apc2 localization had an insignificant effect on mitochondrial localization, except spastin. It is also possible that because the proteins targeted for knocked down have such vital roles in neuronal development, the extent to which the RNAi-induced silencing complex is targeting mRNA molecules for degradation is less than desired. The use of RNAi results in posttranscriptional knockdown of proteins levels rather than complete knockout of a protein by using an organism with a homozygous recessive genotype for a protein. The UAS-RNAi lines used in this experiment produce large hairpins, so it is also possible that the resulting short-interfering RNA used in the RISC may target degradation of mRNA molecules with similar sequences to that of the desired candidate. This results in a phenotype that could be due to a

knockdown of a group of proteins rather than the single candidate. Therefore, these preliminary results should serve as a primer for further experimentation to increase the sample sizes of these RNAi experiments to truly test for the sensitivity of branch point localization in these knockdown experiments.

The use of RNAi has been put into question in terms of how effective the technique is in experimenting on model organisms (DasGupta et al., 2007; Echeverri et al., 2006; Hernández & Bueno, 2005). It is also difficult to extract the RNA molecules specifically from *Drosophila* Class I neurons via dissection to actually compare the levels of mRNA to a control. However, The localization results from RNAi experiments can verified by crossing the tester line with *Drosophila* with other candidate lines, perhaps containing a loss-of-function mutation, dominant negative mutations, and other mutations that genetically alter the protein of interest. For example, the $Ank2F^{00508}$ mutant experiment used a mutated isoform of Ankyrin2 to confirm the results seen in Ank2 RNAi experiments (**Figure 10**).

The Rac experiments are confusing, as dRac1 RNAi did not cause a significant change in mitochondria localization whereas the Rac1.N17 dominant-negative mutation caused a significant decrease (**Figure 9**). This seems to eliminate the possibility of making the negative conclusion that actin branching is required for mitochondrial localization at branch points. In addition to performing more screening techniques to increase sample sizes and perform stronger statistical analysis, there are more techniques that can be done to test for actin involvement in mitochondrial localization at branch points. There is another mutated form of Rac that is constituently bound to GTP due to a Gly-to-Val substitution at position 12 (Luo, Liao, Jan, & Jan, 1994b). This constantly activated Rac mutant forms ectopic clusters of mitochondria in ddaE neurons near the cell body (example seen in **Figure 15**). The mutant has already been

crossed with the mitochondria tester line but data was excluded, as it is difficult to quantify the number of branch points near the cell body and report reliable results. However, it is important to further study the effects of this mutant.

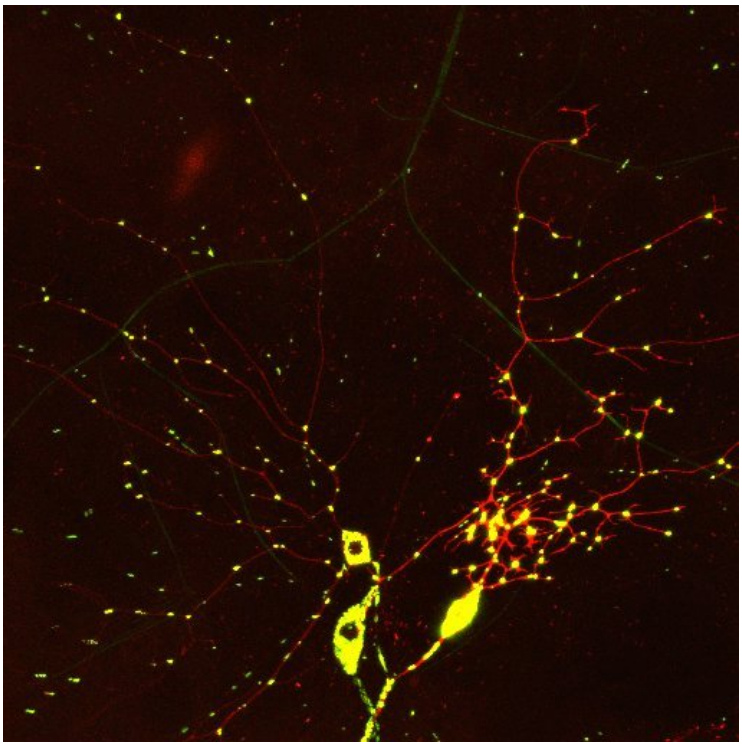


Figure 15. Mito-GFP with Rac1.V12 mutant.

The tester line was crossed with UAS-Rac1.V12 (BL6291) to produce progeny with constitutively active forms of Rac. The example image above shows that the transgene worked, as a key morphological feature is the large ectopic clusters proximal to the ddaE cell body.

Additional experiments can be performed by treating *Drosophila* embryos F-actin-depolymerizing agent such as latrunculin (Akbergenova & Bykhovskaia, 2009; Richards, Rizzoli, & Betz, 2004). In a study that examines the effects of nerve growth factor in attracting mobile mitochondria in axons, signaling was lost when nerves were subjected to latrunculin B (Chada & Hollenbeck, 2004). Lastly, more actin-regulating candidates should be screened with RNAi experiments. WAVE1 is a unique protein that plays a role in mitochondrial mobility and

dendrite development by activating the Arp2/3 complex, of which Arc-p20 is a subunit 4 (Rohn et al., 2011; Sung et al., 2008). All of these will hopefully supply enough evidence to make a stronger conclusion on the effects of actin in mitochondrial localization.

The results from this experiment show that microtubule-associated proteins have the strongest effects on mitochondrial localization when knocked down with RNAi. This may play into the current mitochondrial trafficking model that relies on microtubule dynamics to transport mitochondrial out of the cell body and into the other neuronal subcellular compartments (van Spronsen et al., 2013b). Of particular interest is the *spastin* gene, the meiotic microtubule-severing AAA protein that is associated with hereditary spastic paraplegias (HSP), as it has been shown to associate with other organelles (Roll-Mecak & Vale, 2008; Sherwood, Sun, Xue, Zhang, & Zinn, 2004). One study reports the interaction of spastin with reticulon 1, an endoplasmic reticulum protein (Mannan et al., 2006). This makes room to study the knockdown effects of atlastin, which functions as a tether between microtubules and the endoplasmic reticulum (O'Sullivan, Dräger, & O'Kane, 2013). It has also been shown that spastin mutants cause diffuse mitochondrial localization and abnormal trafficking patterns. Evidence suggests that this is because spastin interacts with kinesin motors (as opposed to directly with mitochondria) (McDermott et al., 2003). It would be interesting to test the branch point localization effects using paraplegin RNAi, as this is an AAA mitochondrial metalloprotease, and paraplegin mutations result in mitochondrial oxidative phosphorylation abnormalities and HSP (G Casari & Rugarli, 2001; Giorgio Casari et al., 1998).

An interesting set of results that came from this study is the insignificant effects of oxidative phosphorylation proteins in mitochondrial localization (**Figure 13**). A similar experiment done with Apc2 showed that knockdowns of ATPSynthaseβ and SesB resulted in a

significant decrease in localization of Apc2 at dendritic branch points. This is strong evidence for the hypothesis that it is the functioning of mitochondria as an organelle being required for localization of branch point members rather than the mitochondria being present at branch points themselves. To test the notion of ATP-sensitive branch points, a project is currently underway to molecularly clone fluorescent biosensors for ATP into *Drosophila*. There are many biosensors available for reported cellular levels of molecules such as ATP, calcium, and hydrogen peroxide (Belousov et al., 2006; Kobayashi, Kikuchi, Ishikawa, Kinuta, & Hashimoto, 1989; Nagai, Sawano, Park, & Miyawaki, 2001). Luciferase is an example of a frequently used reporter assay to measure amounts of ATP:ADP in a system (de Wet, Wood, DeLuca, Helinski, & Subramani, 1987). PercevalHR is being explored as an ATP fluorescent biosensor, as this has been shown to effectively quantitate metabolic flux of neurons *in vivo* (Tantama, Martínez-François, Mongeon, & Yellen, 2013). PercevalHR is an enzymatic construct that consists of the yellow circularly permutated fluorescent protein cpmVenus with both termini bound to the bacterial ammonia transport regulator GlnK1 found in *Methanococcus jannaschii*. This construct relies on the GlnK1 binding of Mg-ATP to induce a conformational change of the protein's characteristic T-loop (encompasses residues Gly37 to Val53), which is then coupled to fluorescent changes in cpmVenus (**Figure 16**). The measurements of PercevalHR can then be normalized by taking the pH of the local environment into account. The pH is quantitatively reported by another construct termed pHRed, coexpressed with PercevalHR (Berg, Hung, & Yellen, 2009). The sequences for these constructs have already been inserted into PUAST vectors and are awaiting successful insertion into *Drosophila* embryos. Hopefully this transgenic line of flies will provide useful information regarding ATP requirements at branch points and supplement the RNAi results for ATPSynthaseβ and SesB.

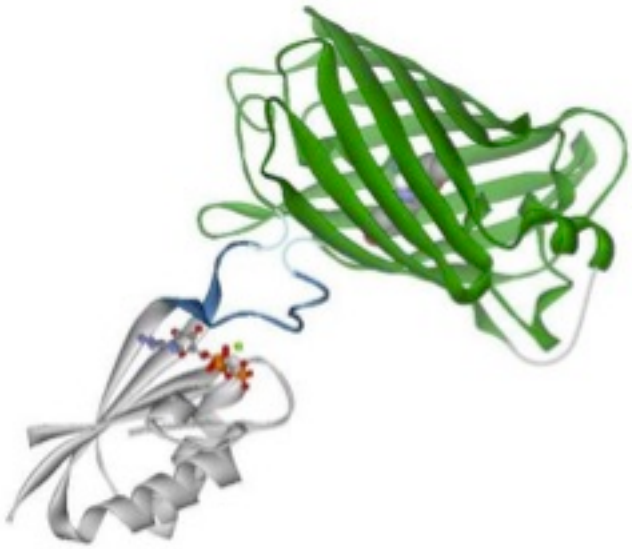


Figure 16. Model of Perceval HR.

The figure above illustrates the binding of PercevalHR to Mg-ATP. The cpmVenus subunit is in green and the T-loop is in dark blue (Berg et al., 2009).

Mitochondrion trafficking in neurons is a highly dynamic process that requires a coordinated system of signals to determine factors such as anterograde or retrograde transport, mitochondrial motility or docking, fission and fusion of mitochondria, and more (reviewed in (Sheng & Cai, 2012). The concept of docking is particularly interesting, as this determines when mitochondria are stationary at a certain position, and this study aims to uncover what proteins are responsible for actively targeting mitochondria at dendritic branch points. Syntaphilin is an example of neuron-specific protein localized in axons that signals for mitochondrial docking from microtubule transport (Kang et al., 2008). This system has been further established by characterizing dynein light chain LC8 as a syntaphilin regulator (Y.-M. Chen, Gerwin, & Sheng, 2009). Additional models suggest that nerve growth factors stimulate mitochondria trafficking complexes to dock with the actin cytoskeleton within the axon (Chada & Hollenbeck, 2004). It

would benefit this study to determine if such a system exists specifically at dendritic branch points and to identify any proteins that may be interacting in this mechanism.

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Academic Vita

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Educational Background

Bachelor of Science in Biology – Genetics and Development *Aug. 2011 – May 2015*
Minor in Bioethics & Medical Humanities
Minor and Honors in Biochemistry & Molecular Biology
Eberly College of Science / Schreyer Honors College (SHC)
The Pennsylvania State University (PSU), University Park, PA

Undergraduate Research Experience

Honors Thesis, Laboratory of Dr. Melissa Rolls, PSU *Oct. 2012 – May 2015*

- Test the hypothesis that there is an active mechanism responsible for protein and organelle targeting in dendritic branch points in *Drosophila* sensory neurons, which may provide insight into the components necessary for proper development of subcellular architecture
- Quantify effects of RNAi candidates related to cell polarity and actin regulators using live-cell images of fluorescently-tagged biomolecules in dendrites
- Awarded a Schreyer Summer Research Grant in Summer 2013, Undergraduate Research Award in Fall 2014

Research Scholar, Lehigh Valley Health Network, Surgery Dept. *June 2014 – Aug 2014*

- Completed a case report for publishing on Transcatheter Aortic Valve Replacement (TAVR) using the transapical approach
- Constructed a database with Microsoft Access to perform retrospective clinical research on outcomes of patients who underwent multiple cardiothoracic surgeries
- Attended surgical training sessions, OB/GYN symposium, and weekly journal clubs
- Shadowed various surgeries in the Department of Surgery, particularly in the open-heart unit at least three times a week for eight weeks

Extracurricular Activities

Schreyer Honors Career Development Mentoring Program *Sept. 2014 – May 2015*

- Served as an Biology mentor to seven honors freshman mentees in the Biology major

State of State 2015 Conference, Content Committee *Sept. 2014 – Feb. 2015*

- Curated the content that is delivered during the inaugural speakers conference State of State that addresses issues and ongoing projects relevant to Penn State, ranging from Engaged Scholarship to Sexual Assault and Mental Health Resources
- Selected and guided the student and faculty speakers for the State of State 2015 conference, sparking meaningful and constructive conversation for the benefit of PSU

Kaplan Test Preparation, Campus Representative *Sept. 2014 – May 2015*

- Connected with and advised students in preparation for pre-professional schools by serving as a Kaplan brand ambassador and introducing them to test prep resources

- Promoted student organizations and leaders to help them achieve scholarships by organizing on-campus tabling and marketing material distribution
- Developed interpersonal skills and marketing experience by working 5-10 hours/week

Penn State IFC/Panhellenic Dance MaraTHON *Aug. 2011 – Feb. 2015*

- Contributed to the university-wide effort of combating pediatric cancer by financially and emotionally supporting the children, families, researchers, and staff of the Four Diamonds Fund at Penn State Hershey Medical Center
- Springfield Club (2011-2012), "OPPerations" Committee Member (2012-2013, 2014-2015), Morale Committee Member (2013-2014)

Science Lion Pride *Jan. 2014 – May 2015*

- Represented the Eberly College of Science through an ambassador organization, providing tours to prospective students, and coordinating educational opportunities for current PSU students and children in the State College area interested in science

New Student Orientation, Premedicine/Science Dept. *May 2013 – Aug. 2013*

- Assisted about 75 incoming Science and Premedicine majors with scheduling first semester classes and preparing their four-year academic plans
- Coordinated courses with Science and Premedicine Departments to accommodate the large incoming class of Science and Premedicine majors

Schreyer Honors College Student Council *Aug. 2011 – May 2013*

- Represented the SHC through governmental representation, volunteered with fundraisers benefiting various philanthropies, and held programs for the benefit of honors students

Leadership Experience

Director of Finance, Penn State Movin' On, PSU *April 2014 – May 2015*

- Organized one of the largest student-run musical festivals in the country, free of charge to the entire Penn State community as a way to celebrate the senior students continuing with their academic and professional lives
- Acted as the Movin' On Treasurer with the responsibilities of all transactions and allocation of funds (through the University Park Allocation Committee – UPAC)
- Oversaw Sponsorship (3), Community Outreach (1), Special Events (1), and Project Development (2) chairs

Hospitality Director, SHC Freshmen Orientation, PSU *Feb. 2013 – Sept. 2015*

- Planed arrival, meals, and lodging for the incoming class of anticipated 300 first-year honors students, 90 orientation mentors, and 20 faculty members involved in August 2014 five-day orientation program
- Led monthly meetings to instruct 20 mentors on the nuances of the SHC and how to prepare the incoming SHC freshman class
- Facilitated the Leadership Workshop and Organizational Meeting to better prepare the 90 orientation mentors

South Halls Residence Association, Treasurer *May 2012 – May 2013*

- Managed all transactions and expenses for events held in South Halls Area, coordinated with Association of Student Activities (ASA) to maintain a stable budget

South Halls Residence Association, Publicity Chair *Aug. 2011 – May 2012*

- Oversaw all public relations of SHRA events, co-managed events, contributed to Stall Stories in the South Halls Area

Teaching Experience

Teaching Assistant, PSU Core Biology Laboratory Courses

Biol 240W: Function and Development of Organisms *Jan. 2015 – May 2015*

- Lectured a two hour weekly laboratory section of 22 underclass science and kinesiology students in topics of plant and animal development using labs ranging from plant miRNA to animal physiology and anatomy
- Crafted, proctored, and graded quizzes, prelaboratory assignments, and in-class assignments
- Personally graded student lab reports in order to provide critical and individualized feedback on writing style and content
- Held weekly office hours to answer student lecture and lab questions
- Received wage payroll and Biol 400 credits, totaling to 10 hrs/week

Biol 110H: Honors Basic Concepts and Biodiversity *Aug. 2014 – Dec. 2014*

- Lectured a two hour laboratory section of 24 Biology first-year students in the Schreyer Honors College in topics of biodiversity, cell reproduction, cell signaling, and bacterial resistance
- Received the same accreditation and income as Biol 240W; same TA duties as above

Biol 220W: Populations and Communities *Jan. 2014 – May 2014*

- Lectured a two hour laboratory section of 21 underclass science, environmental, and biological anthropology students in topics related to ecology and evolution using labs ranging from population genetics to sequencing mitochondrial DNA and haplogroup determination to plant population growth modeling
- Helped students develop proficiency in the analysis programs, including MEGA, BLAST, and Microsoft Excel
- Received the same accreditation and income as Biol 240W; same TA duties as above

Biol 110: Basic Concepts and Biodiversity *Aug. 2013 – Dec. 2013*

- Lectured a two-hour laboratory section of 20 underclass science, engineering, and agricultural students in topics of biodiversity, cell reproduction, cell signaling, and bacterial resistance
- Received the same accreditation and income as Biol 240W; same TA duties as Biol 110H

Assistant TA, Biol 110, PSU *June 2013 – Aug. 2013*

- Served as a communication connection between teaching assistants, laboratory coordinator, and prep crew during laboratory meetings
- Addressed students' questions and concerns about laboratory and lecture content

Community Service

ESL Tutor, Internship with the College of Liberal Arts *Jan. 2014 – May 2014*

- Instructed 37 year old Iranian mechanical engineering student in English vocabulary and grammar for three hours weekly, totaling 40 over the semester

- Promoted literacy in the immediate multicultural State College community through planning and implanting effective advertisements, hosting fundraising events, and working directly with adult learners

Awards & Honors

Academic Excellence Award, Schreyer Honors College
Eberly College of Science Dean's List
Eberly College of Science Undergraduate Research Award
Schreyer Summer Research Grant
Elks National Most Valuable Student Scholarship

Presentations

Follick, B., Wu, J. (2014) *Reoperative Cardiac Surgeries: The Inception of an Institutional Database*, Lehigh Valley Hospital – Cedarcrest, Lehigh Valley Health Network, Research Scholar Reception; Allentown, PA.

Follick, B., Rolls, M. (2015) *Mitochondrial Localization and Interactions at Dendritic Branch Points in Drosophila Sensory Neurons*. Spring 2015 Undergraduate Research Exhibition, Heritage Hall, HUB-Robeson Center; University Park, PA.