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SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

DETERMINING THE ROLE OF APC2 AND INTERACTING PROTEINS IN DENDRITE ORGANIZATION OF DROSOPHILA NEURONS

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ABSTRACT

Microtubules are extremely important in defining cell shape and structure, but also provide polarized tracks in the cell to assist in long-range transport in neurons. Understanding the cellular processes controlling this polarity may give insight into understanding various neurological diseases. It has been shown that adenomatous polyposis coli protein 2 (APC2), a plus end binding protein, plays a role in maintaining the polarity of these microtubules in dendrites of neurons. More importantly, a recent study has shown that APC2 is specifically localized to dendrite branch points to maintain polarity at these sharp junctions. Understanding what controls the localization of APC2 to these distinct locations within the cell is crucial to understanding how the organization of these microtubules is maintained. Using RNA interference to knock down the expression of different interacting partners of APC2, we determined that microtubule severing proteins, GTPases and members of the Wnt signaling pathway were all responsible for disrupting localization. In other studies in the lab, additional organelles and proteins have been found to localize to branch points to help maintain the complex cellular organization. We hypothesize that the same machinery may be required to localize all branch point components and several mechanisms may establish the localization at this distinct cellular site. We can create a hierarchy of the candidates involved in upstream or downstream pathways to help determine the order necessary to control branch point localization and microtubule polarity.

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Introduction

Neuronal Function

Of all cell types found in vertebrate and invertebrate organisms, neurons are one of the most specialized cells whose function depends directly on their morphology and intrinsic polarity (Baas 2010). The typical neuron can be divided into three distinct subcellular compartments each responsible for a different function. The cell body, the major site of protein synthesis, has two major processes that extend away from it: axons and dendrites. Axons are extremely long structures that span long distances over the organism's body to transmit messages to other neurons or target cells. The part most proximal to the cell body is a very simple, unbranched structure until it reaches the synapses with other cells. At the synapses, the axons become increasingly complex with its branching in order to increase the efficiency of messages sent. Dendrites on the other hand, are meant to receive messages from other neurons or their surroundings to send back to the soma for processing and so they typically have a much shorter and complex branching structure as to increase the number of receptive fields for messages (Baas 2010). While the exact structures of neurons may vary between different neuron types and organisms, the most studied structure involves a cell body with one axon and multiple dendrites (Figure 1).

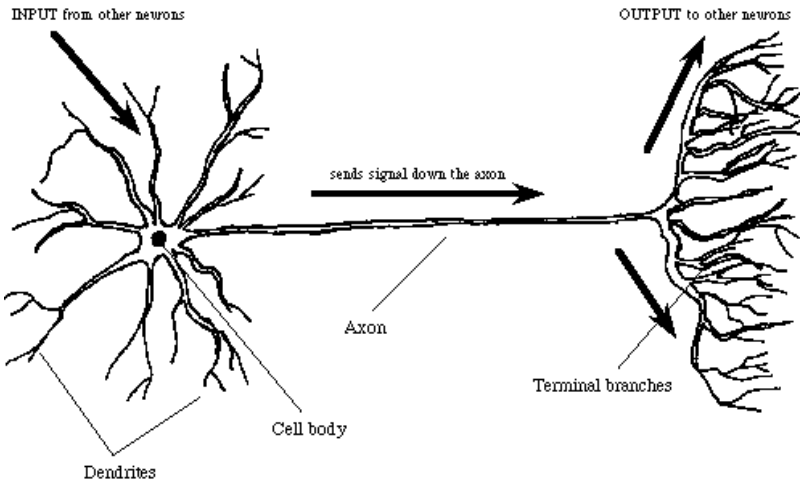

Figure 1: Schematic of neuron demonstrating the morphology and orientation of all processes.

Microtubule Polarity

The highly directionalized transport in neurons relies heavily on its intracellular cytoskeletal organization. Every neuron contains polarized tracks called microtubules. These tracks allow for the transport of cargo to different regions within the cell. Microtubules are long polymers composed of α and β tubulin dimers that arrange themselves in a "head to tail" manner which gives the long filaments an inherent polarity (Jiang 2011). The long filaments then arrange themselves laterally into a hollow tube composed of 13 protofilaments to form a single microtubule necessary for the long-range transport (Figure 2).

These structures are highly dynamic in cells as they are responsible for some of the most important cellular processes including cell division, cell migration and transport of molecules to their intended destinations. Microtubules are nucleated at their α, or "minus" ends and grow with the constant association or dissociation of αβ tubulin dimers at their "plus" ends (Figure 2). In most undifferentiated interphase cells, nucleation occurs in a stable centrosomal microtubule organizing center (MTOC) and the plus ends grow away from the structure (Bartolini 2006). Neurons are very different in that their nucleation is not fully understood however it is known

that microtubules are not nucleated from a centrosomal MTOC (Bartolini 2006). This is evident from a particular study in *Drosophila* neurons that have found that microtubules are not nucleated from the centrosome (Nguyen 2011)

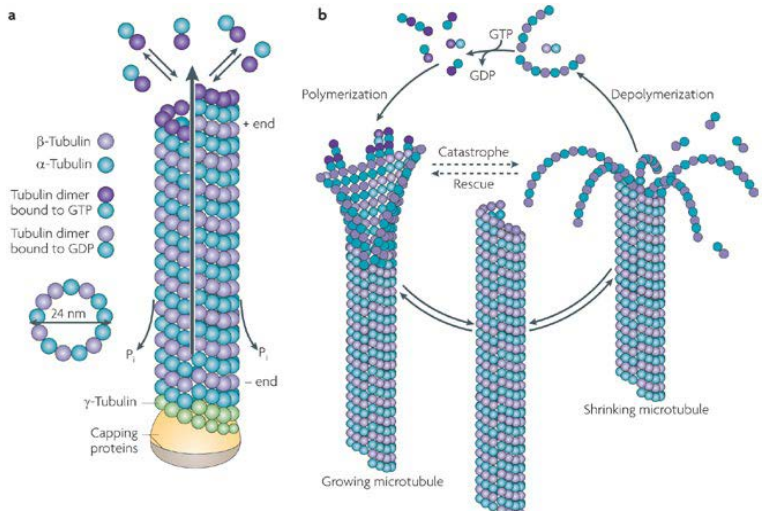

Figure 2: Diagram of the polar microtubule structure composed of α and β tubulin subunits. Demonstration of the dynamic structure of microtubules responsible for cell migration and division etc (Conde 2009).

The fundamental differences between axons and dendrites have led some to study whether the basic cytoskeleton structure plays a role in determining their functional differences. While it has been difficult to show that microtubules are responsible for the functional differences, studies have supported the idea that axons and dendrites are fundamentally different in their microtubule polarities (Kollins 2009). It used to be understood that the "default" organization of microtubules was always plus end out in each of these extremities. Studies in cultured rat hippocampal neurons however demonstrated that while axons have a consistent "plus"-end out orientation, dendrites have a non-uniformly oriented microtubule network with the polarity that is closer to a mixed or 50:50 (plus vs. minus end out) orientation (Baas 2010). Studies with an invertebrate *Drosophila melanogaster* system shows that microtubule polarity in

axons are representative of that found in a vertebrate system with close to 100% being plus end away from the cell body (Stone 2008). Dendrites however are different from vertebrates in that they have a more uniform polarity with close to 90% of the microtubules being oriented minus end distal to the cell body (Stone 2008).

The differences in microtubule polarity within the cell provide a sense of directionality for transport of molecules to different ends of the neuron. Molecular motors are proteins that are able to move along microtubules in a specific direction depending on their affinity for either plus or minus ends while carrying cargo with them (Craig 1994). Kinesins are molecular motors that will typically carry their cargo towards the plus end of microtubules while dyneins are responsible for movement towards the minus ends (Stone 2008).

Subcellular Localization is Critical for Neuronal Function

The bulk of protein synthesis occurs in the cell body of neurons but the products must be transported to their final location in either the axons or dendrites via molecular motors. The differences in function and microtubule organization suggest that the molecular composition of each extremity will be different as well. Membrane proteins, neurotransmitter receptors, and ion channels are a few of the members that are targeted to axons and dendrites differently depending on their roles (Rolls 2007). Some organelles such as endosomes and Golgi are only found in the proximal dendrites and not in axons while others such as ribosomes and mitochondria are seen extended throughout the entire cell (Craig 1994). Protein localization is also very different between axons and dendrites as well. A specific microtubule associating protein, MAP2 is distinctly found in dendrites while axons are typically found with elevated levels of dephosphorylated tau (Avila 2004).

While the exact mechanisms for this subcellular localization are still not understood, an interesting study has shown how protein localization in *Drosophila* is responsible for maintaining microtubule polarity at unique sites within specific sensory neurons (Mattie 2010). The "directed growth machinery" is thought to have a critical role at branch points in class I dorsal dendritic arborization neurons in order to assist with maintaining a uniform microtubule polarity throughout the branch points (Mattie 2010).

The dendritic arborization (da) neurons in the peripheral nervous system of *Drosophila* are characterized into different classes based on the branching patterns of their dendritic trees (Figure 3). The class I dendrite has a unique shape in that the main branch forms a comb-like structure. The branch points along the main trunk form angles that are close to 90 degrees. This has critical implications with growing microtubules because it would be expected that as growing microtubules reach this branch point, there is a 50% chance that it can turn either away from or towards the cell (Figure 4). As studies suggest however, there is a consistent 90% minus end distal to the cell body in all classes of neurons (Stone 2008).

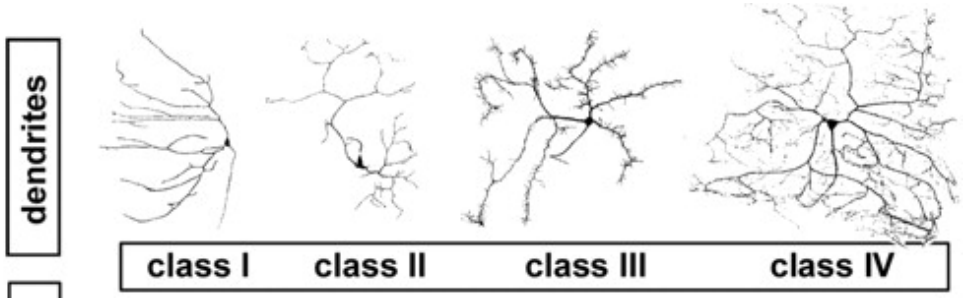

Figure 3: Dendritic Arborization Neurons in Drosophila Larvae. The complexity of the dendritic tree increases with class number. The class I neuron has a unique comb-like dendrite appearance (Grueber 2007).

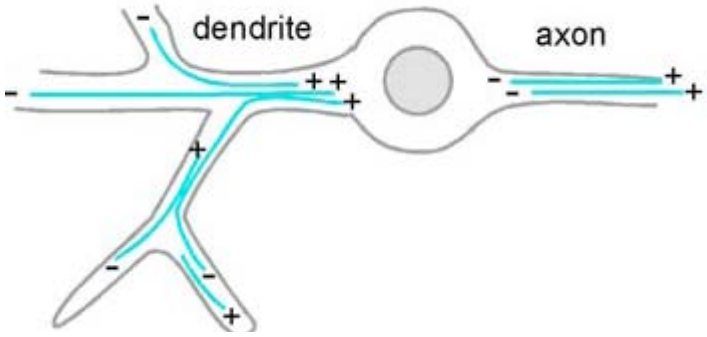

Figure 4: Schematic of how microtubules grow through branch point in class I ddaE neurons. Branch points in dendrites have a 90 degree angle that should statistically mean microtubules will end up with a 50:50 mixing in polarity. Data suggests however that close to 90% ends up with a minus end out phenotype (Stone 2008).

The protein adenomatous polyposis coli2 (APC2) is a known plus end tracking tip (+TIP) protein that has been shown to be a part of the directed growth machinery in the comb dendrite of class I dda neurons (Mattie 2010). Reduction in levels of APC2 has indicated a change in this 90% minus end out to a more mixed polarity phenotype (Mattie 2010). There is also reason to believe that APC2 has a critical function in maintaining polarity at the branch points because this is where the protein shows a specific localization within the cell (Figure 6), even from early on in development before microtubule polarity is established (Mattie 2010 and unpublished data). While the specific mechanism of how APC2 functions in these dendrites is still unclear, it has been shown that APC2 is involved in organizing polarized microtubules.

Physiological function of APC2

APC2 is a member of the *Drosophila* APC family of proteins that are most conventionally known for their role as tumor suppressor genes (Aoki 2007). APC is a multi domain protein that contains binding sites for numerous molecules including microtubules, and proteins from the Wnt signaling pathway including β-catenin, axin as well as cytoskeletal regulators such as EB1 and the Rac GEF, ASEF 1 (Aoki 2007). APC2 is a truncated version of APC that still contains many domains for protein binding, but it lacks the C-terminal microtubule binding domain and EB1 binding domain of APC (Figure 5). One of the major differences between the APC and APC2 proteins is their known localization and function. While both are expressed in the central nervous system, they localize to very different places in the brain (Akong 2002). APC is found mainly in centrosomes and microtubules while APC2 localizes to the cell cortex. While APC is critical in suppressing the canonical Wnt pathway that controls proliferation and differentiation of cells through the inhibition of β-catenin, studies have also

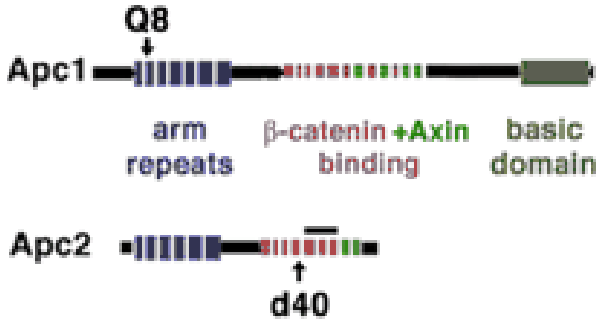

Figure 5: The APC1 and APC2 genes in Drosophila. The APC2 gene shows a truncated C-terminal end that lack the β-catenin and Axin binding sites (Ahmed 2002).

found that the APC proteins may also have cytoskeletal or adhesive roles especially during development. (Aoki 2007 and Akong 2002). The role of APC2 has been well characterized in the central nervous system during development and embryogenesis, but very few studies have actually looked at its role during a later stage in development. APC2 function is certainly not well characterized for its function in dendrites other than we have already seen with its role in maintaining microtubule polarity. This is why we wish to further characterize its expression and hope to get a better understanding of its role in neurons during a later stage in development (Rusan 2008 and Mattie 2010).

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Overview of Study

While the precise localization of APC2 and its role in maintaining microtubule polarity in *Drosophila* da dendrites is understood, the mechanism for how it is localized there is still not well characterized (Figure 5). It is hypothesized that interacting partners with APC2 may be responsible for the trafficking of this protein to branch points in the dendrites of class I da neurons to assist with maintaining microtubule polarity. Using a *Drosophila* model system and through the reduction of protein levels of interacting partners with APC2, I examined the APC2 expression in class I dendrites to observe any significant changes. In these experiments APC2 was tagged with a green fluorescent protein (GFP) and a membrane marker, MCD8 was tagged in a red fluorescent protein (RFP) in order to observe the localization of APC2 within the class I dendrite of *Drosophila* larvae *in vivo*. RNA interference was used to knock down interacting partners of APC2 and the resulting APC2 expression was monitored. If these proteins play a role in trafficking APC2 to the branch points, I would expect that reduction in their protein levels will decrease APC2 expression at branch points.

Because APC2 has a known role in maintaining microtubule polarity, we expect that those proteins that decreased APC2 expression in dendrites may also disrupt the established microtubule polarity. I tracked microtubule polarity by observing the direction of movement of EB1 comets, a +TIP protein that associates with the growing plus ends of microtubules. EB1 was tagged with GFP and through RNA interference, the candidate protein levels were reduced. I will expect that those proteins that disrupt APC2 localization are also important for microtubule orientation in dendrites.

APC2 is not the first molecule to show a distinct expression pattern in neurons. Other proteins and organelles also have been seen in either dendrites or axons (Rolls 2007). It would be expected that those proteins that interfere with APC2 expression at branch points may themselves also have a role at branch points. A number of different proteins and organelles such as axin and mitochondria were tagged with GFP and the membrane marker MCD8 was tagged with RFP in order to observe the specific localization within the cells. For those members that were found to have similar expression patterns as APC2 were further observed when interacting partners were knocked down using RNA interference.

I hypothesize that there may be a general mechanism that controls branch point localization in class I da dendrites and ultimately microtubule polarity. With gaining an understanding of this branch point localization mechanism, we can further hope to understand how microtubule polarity is maintained in these cells and how regional specializations are established in neurons.

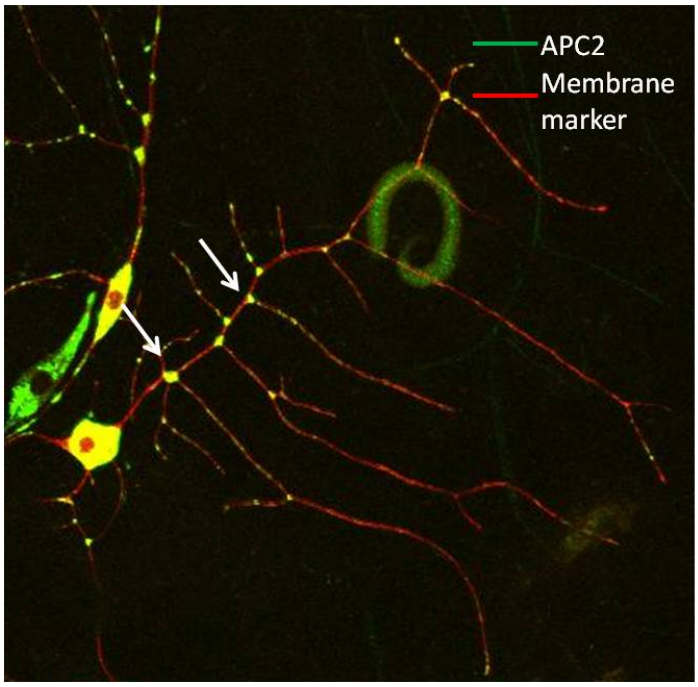

Figure 6: APC2 Localization in comb dendrite of class 1 ddaE neuron. APC2 is localized to branch points along the main trunk of the dendrite as indicated by arrows.

Methodology

Experimental Set up and Crosses

All *Drosophila* stocks were maintained at 25°C and kept in appropriate media. For all crosses that were set up, embryos were collected and raised for 3 days at 25°C until they reached third instar, their final developmental stage before adulthood. Several lines were used in order to visualize APC2, mitochondria, Axin, and ankyrin2 expression within the cells. Males from the line UAS-MCD8-RFP; 221-Gal4 were crossed with virgins from the following lines in order to observe expression of APC2, mitochondria, Axin, AxinM3 (a mutant form of axin) and Ank2 under the 221 Gal4 promoter to drive expression in class I dda neurons respectively: UAS-APC2-GFP/TM3 on chromosome III, UAS-mito-GFP on III, UAS-Axin-GFP/TM3 on III, UAS-AxinM3-GFP on III and UAS-venus-ank2-L8-GFP on III.

To examine APC2 expression after selective knockdown of interactive partners, virgins from the tester line: UAS-dicer2, UAS-MCD8RFP; 221Gal4, UAS-APC2-GFP/TM6 were crossed with males of various RNAi lines that were obtained from the Vienna Drosophila RNAi Center (VDRC). These stocks included members to target those proteins that are listed in Table 1. Mitochondria localization was also observed by using virgins from dicer2, UAS-MCD8-RFP; elavGal4, UAS-mito-GFP/TM6 and crossing them to the same RNAi lines previously mentioned. Axin localization was observed in a similar manner by using UAS-dicer-2, UAS-MCD8RFP; 221Gal4, UAS-Axin-GFP/TM6 as the tester line. In order to observe microtubule polarity, virgins from the tester line dicer2; 221-Gal4, UAS-EB1-GFP/TM6 were collected and crossed to various RNA lines (see above). The same experimental set up was used for each of these crosses as before, larvae were allowed to age for three days before visualizing.

Confocal Microscopy and Analysis

Live imaging was performed on three day larvae that would be mounted onto a dried agarose glass slide under a cover slip and observed using a confocal microscope. An Olympus FV1000 microscope was used for examining all localization data while EB1 data was analyzed using a Zeiss Axio Imager M2 confocal microscope. All videos were taken under a 63X oil objective and analyzed using the ImageJ software.

The localization of APC2, mitochondria, Axin, AxinM3 and Ank2 (without RNAi analysis), were all analyzed by creating a z-stack image in ImageJ and qualitatively analyzing where expression was seen throughout the cell.

For analysis of APC2, mitochondria and Axin expression following RNAi knockdown of different genes, a z-stack image was created in ImageJ and the branch points were analyzed for expression. The total number of branch points along the main comb were analyzed and the expression of the various proteins or organelles were counted along the branch point to determine an overall percentage of branch points that contained the protein or organelle of interest. Only the comb dendrite was analyzed because this is the only branch that contains branch points that are close to 90° and of are considerable interest to us.

EB1-analysis was analyzed one frame at a time in order to assay polarity. In order for a comet to be assigned with directionality, it must be in focus for at least three frames. Only the main trunk of the class I ddaE neuron was analyzed as the peripheral branches have a much more mixed polarity and do not exhibit the classical “minus-end out” model for other *Drosophila* neurons. The comets were then scored for their direction either towards or away from the cell body. Comets that moved along the main trunk towards the cell body, or from a peripheral trunk onto the main trunk towards the cell body were considered to have a minus end out polarity. On

the contrary, comets that moved away from the cell body on the main trunk or came in from a peripheral branch but then moved away from the cell body as it entered the main trunk, were considered to have a plus end out orientation. The total percentage of minus-end out dots were then calculated to assay microtubule polarity for that cross.

Results

APC2 expression at branch points is disrupted by a variety of players

APC2 was found to be localized distinctly at branch points along the main trunk of the comb dendrite in the ddaE neuron (Figure 6). In order to determine what members are responsible for establishing this localization, I did a screen to knock out different known interacting partners of APC2 and observed what happened to the subsequent APC2 localization (Figure 7). I chose potential candidates that were known to have some kind of physical interaction with the APC2 protein as indicated by FlyBase (Table 1). Any candidate that had caused a change in APC2 localization, I further examined any other interacting partners to see if they also changed APC2 expression. I then selected males from the candidate RNAi lines and crossed them with females from the tester line: UAS-dicer2, UAS-MCD8RFP; 221Gal4, UAS-APC2-GFP/TM6 to observe APC2 localization. Localization was scored based off of the number of branch points that had APC2 at them. Any branch point that had two secondary branches that extended away from the main trunk was counted as two branch points. I then took a total ratio by comparing the number of branches with APC2 to the total number of branch points along the main trunk in order to get an average percentage of branch points with APC2 localization. A summary of all results can be seen in Table 2.

Total List of Candidates Screened for Change in APC2 Localization [(VDRC)genotype]

(45373) Klp64D	(45400) Kap3	(19182) αctn	(103914) Dia
(107344) Arm	(27406) Sif	(17344) Sep1	(26413) Sep2
(105374) HERC2	(24451) EB1	(100794) cdc42	(49246) dRac1
(101538) Gsk-3β	(107502) Rho1	(107238/107369) ank2	(7748) Axin
(21995) capulet	(33110) Spastin	(109274) dlg	(17766) Katanin 80
(38368) Katanin-60	(51469) APC	(105408) Pontin	(24746) Fidgetin
(31718) RhoGEF3	(32038) Esn	(31598) Katanin 60L1	(21982) msps
(106683) dmiro	(103569) fry		

Table 1: List of candidates that were screened for a change in APC2 localization. All candidates have a (VDRC) identification number

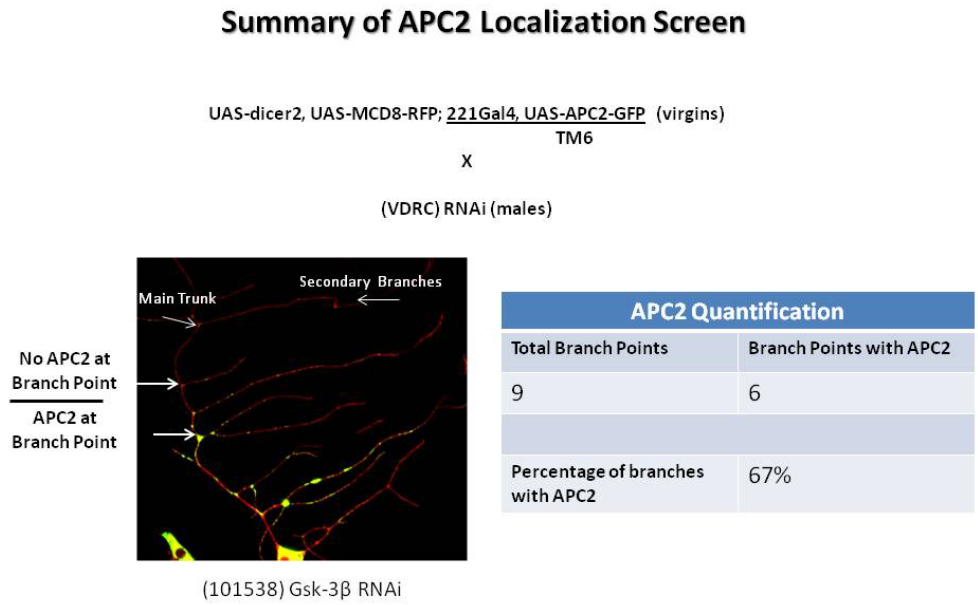

Figure 7: Summary of APC2 Screen Crosses and Quantification. Virgin females from the tester line were crossed with males from RNAi candidates. The resulting three day larvae were examined for APC2 expression and results were quantified by comparing the number of branches with APC2 to the total number of branches along the main trunk.

Summary of APC2 Screen

Decreased APC2 Localization at Branch Points	Increased APC2 Expression (delocalized)	No Change in APC2 Expression
Axin	cdc42	Capulet
Gsk-3β	Rho1	Kap3
dRac1	Katanin-60	Klp64D
Ank2	Katanin-80	Dia
dmiro	Kat-60L1	αctn
	RhoGEF3	arm
	Spastin	Sif
		Sep1/sep2
		HERC2
		EB1
		APC
		dlg
		msps
		Fidgetin
		Fry
		pontin
		esn

Table 2. Summary of findings from APC2 Screen: Decreased APC2 localization (column 1) refers to a decreased in the percentage of branch points occupied by APC2 compared to the rtnl2 control. Increased APC2 expression (Column 2) refers to extra APC2 at the tips of branches or a delocalization of APC2 from its normal branch point localization. No change in APC2 expression (column 3) refers to no observed phenotype when expression of these candidates were reduced.

While the expression of APC2 changed for a number of different candidates, the most notable and most relevant changed occurred with a decreased localization at branch points of APC2 (Figure 8). The rtnl2 RNAi was used as my negative control as there has been no observed phenotype with this genotype (Mattie 2010). Quantifying APC2 expression at branch points with

the rtnl2 RNAi yielded approximately 95% of branch points that were occupied by APC2. When the expression of proteins such as ankyrin2, dRac1, GSK3β, and Axin were knocked down, I saw the numbers of APC2 occupied branch points drop to close to 70-75%. While these proteins are very different in their physiologic functions, they suggest the different elements or pathways that may have a role in controlling this APC2 localization. The most notable is that the ank2 and dRac1 are both cytoskeletal regulators and may suggest a role in actin cytoskeletal elements being necessary to localize APC2 to branch points.

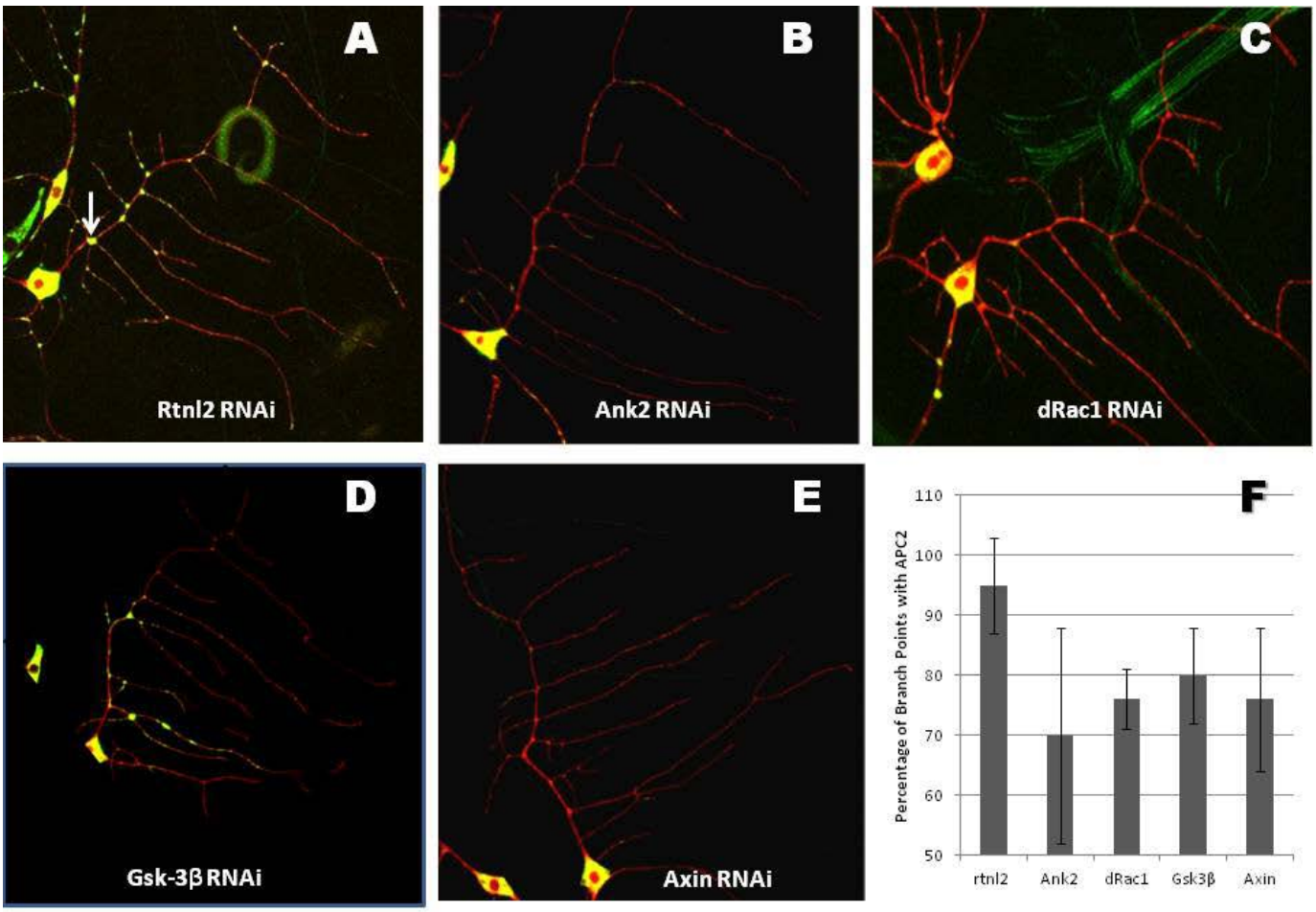

Figure 8. APC2 Localization in Dendrites after Knockdown of Interacting Partners: A. Rtnl2 RNAi control shows that APC2 is found at the branch points along the main trunk of the ddaE dendrite. **B.** Ank2 RNAi knockdown shows that APC2 is drastically reduced at branch points. **C.** dRac1 RNAi knockdown also exhibits much fewer branch points with APC2 expression. **D.** GSK3β shows a reduction of the number of branch points occupied with APC2. **E.** Axin RNAi exhibits fewer APC2 at branch points. **F.** Percentage of branch points occupied with APC2 compared between the different proteins. All show a significant reduction compared to the control.

Normal microtubule dynamics are not required for APC2 localization

While not conclusive, there is evidence that actin regulators may be necessary for APC2 localization. Therefore, I wanted to see whether or not the other major cytoskeletal filament in neurons, microtubules, also played a role. Mini spindles is an important protein that associates with microtubules *in vitro (*Cullen 1999). Knock down of this protein disrupts the dynamic (growing) microtubules in neurons and can give rise to whether or not microtubules are important for APC2 localization (Figure 9). APC2 expression at branch points does not change with msps RNAi. When compared to the rtnl2 control, the number of branch points that contain APC2 are both approximately 95%. This suggests that dynamic microtubules may not have a direct role in locating APC2 to the branch points, whereas the actin cytoskeleton may be slightly more involved with this process.

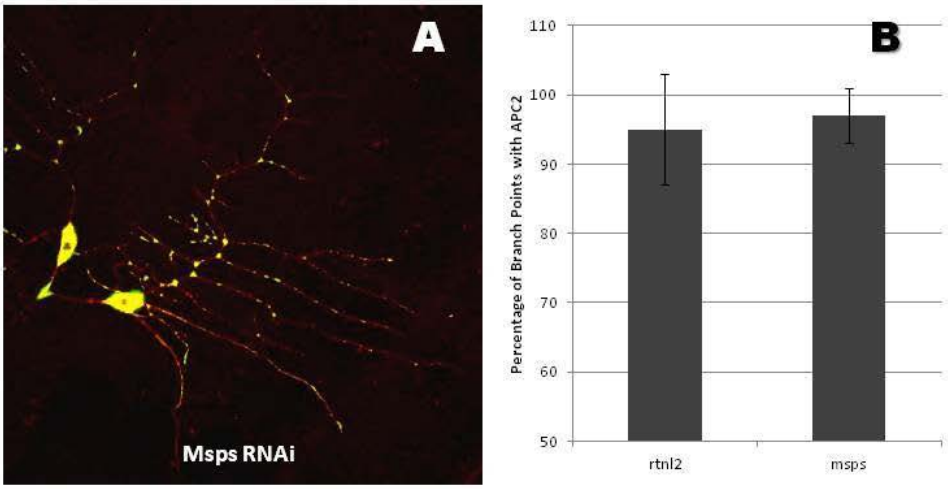

Figure 9: MSPS RNAi does not alter APC2 expression. A. When mini spindles is knocked down using RNA interference, APC2 expression does not change and still occupies approximately 97% of the branch points along the main comb dendrite. **B.** These results are not significantly from the rtnl2 control.

Localization of other branch point members

I have found that a number of different proteins are important for establishing the localization of APC2 in dendrites however there is no clear indication of what they do there. Therefore, I screened through the list of candidates that disrupted APC2 localization in order to see where in the cell they localized so that we could gain insight to what their function may be. Of the proteins I screened, I found that axin and a specific construct of the ank2 protein (ank2 L8) had the same distinct localization as APC2 in dendrites (Figure 10). From previous work, I understand that mitochondria localize to the same branch points and so I looked at its localization in order to compare the ratio of branch points that these branch point members occupy to the APC2 expression (unpublished findings).

The ankyrin 2 L8 splice form, mitochondria and axin protein were all tagged with GFP, while the membrane marker MCD8 was tagged with RFP, and these can be seen in Figure 10. The ank2-L8-GFP and Axin-GFP were expressed using the 221Gal4 driver so that expression in the class I neuron is the brightest making it easy to observe the localization pattern. The mito-GFP in the class I neuron is a little more difficult to see because it was expressed using the elavGal4 driver. This driver expresses mainly in class IV neurons; however I can still observe the class I ddaE dendrite in the background. When comparing the percentages of branch points that were occupied with what I saw with APC2 expression, I found that the numbers were all relatively similar with about 95% of branch points occupied.

Other splice forms of the ank2 protein were analyzed, however only this specific splice form showed branch point localization. Other forms with variation in their splice sites at the C-terminus showed only diffuse expression throughout the cell.

These results clearly suggest that the unique branch point localization is not so unique to the APC2 protein. Instead, this may indicate that branch points may act as central “hubs” that require the coordination of multiple proteins and/or organelles in order to maintain microtubule and overall dendrite function. In fact, axin is a very large scaffolding protein that may act to tether other proteins to the branch points in order to function.

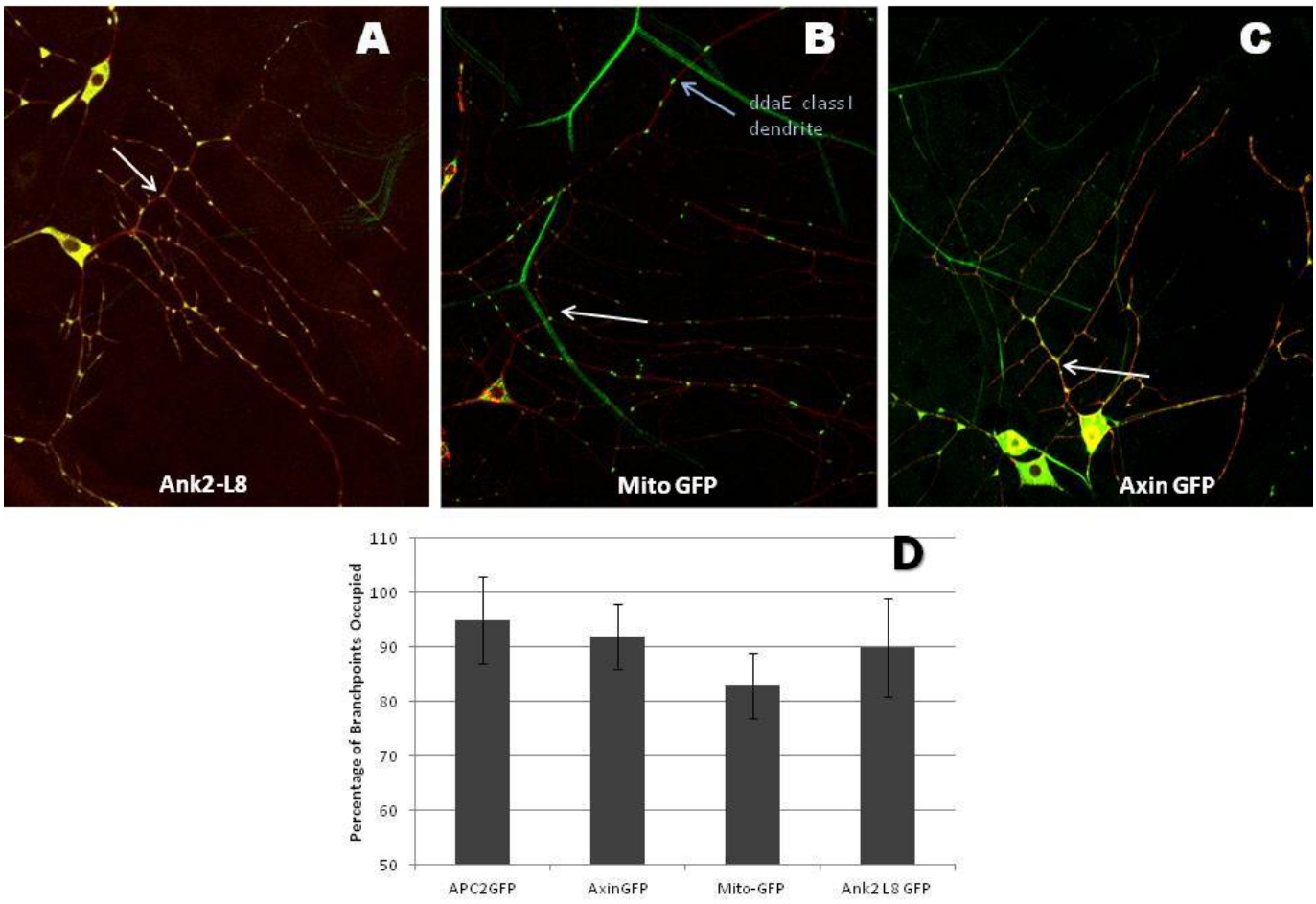

Figure 10: Localization of other Branch Point Members. A. Ank2 L8 Splice form shows Ank2 L8 GFP localized to branch points along the main dendrite. **B.** Mitochondria are labeled with mito-GFP under the elavGal4 driver. The class I ddaE neuron is located with its dendrite tree oriented upwards Again, mitochondria are also localized to branch points. **C.** Axin-GFP indicates that this large scaffolding protein is found to be localized to branch points. **D.** Comparison of the number of branch points that are occupied with different branch point members with the APC2 given as a comparison.

Polymerization of Axin is not required for its localization to branch points

Axin is a large scaffolding protein with a polymerization domain that allows it to form multi-protein complexes (Mendoza 2011). I decided to test the theory that Axin can serve as a part of a major protein complex that is responsible for recruiting other members to branch points. Axin requires its polymerization domain to be activated in order to form multiprotein complexes (Mendoza 2011). Therefore, I took a mutant form of the protein, Axin-M3 (Mendoza 2011) that cannot polymerize and observed its localization within dendrites. Presumably, we would not see this distinct localization as seen with the Axin-GFP, however there was no difference in localization from the axin that can polymerize normally (Figure 11). Both Axin and AxinM3 were able to localize to approximately 92-95% of branch points within dendrites.

The idea that axin does not need to polymerize in order to localize to branch points can suggest different things. First, this may show that axin may be upstream of all other members and can localize on its own and then recruit other proteins and/or organelles to the branch points

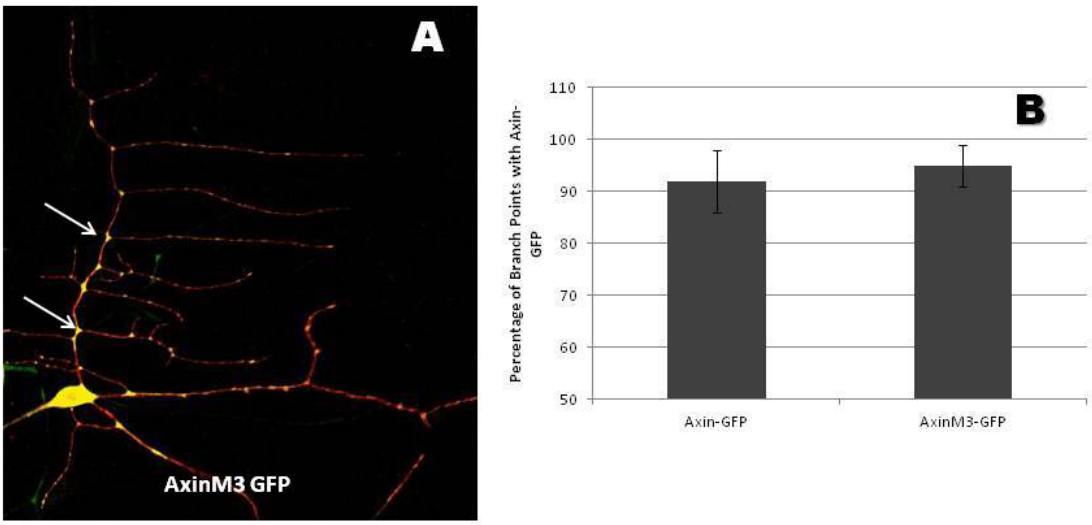

Figure 11: AxinM3-GFP Localization in Neurons: A. Axin-M3 is tagged with GFP and localized to branch points along the main comb dendrite showing that its polymerization is not required for localization. **B.** The number of branch points with AxinM3 is essentially the same as with Axin.

to function. Or, axin can act completely on its own and may be independent of other members at the branch points. This situation seems unlikely however in that we have shown that axin is required for APC2 localization to branch points. Another possibility is that it may bind something else that is abundant at branch points which therefore localizes itself at this site.

Mitochondria localization is not controlled by all of the same players as for APC2 localization

To further assess whether or not these branch points act like central "hubs" that function as a unit, I hypothesized that the localization of all proteins or organelles to the branch points would depend on the same machinery. I started testing this by looking at the mitochondria localization after knockdown with the same candidates that changed APC2 expression. Most of the candidates that were tested showed no change in mitochondria localization (Figure 12 C-D) however when the kinase GSK3β, expression was reduced, there was a change in mitochondria localization from the rtnl2 control (Figure 13). While mitochondria typically only localized to approximately 85% of branch points with the negative control, the GSK-3β knockdown showed a much different localization with occupying only 60% of branch points in the comb dendrite.

It is also interesting to note that when we examined APC2 localization after miro knockdown, a protein that reduces the number of mitochondria *in vivo*, I saw a significant reduction in the number of branch points with APC2 in them (Figure 12A,B). There was significant variation with these results however as seen by Figure 12B. The variation may be due to the fact that RNAi is not a complete knock-out of a gene, but instead only a knock down. Mitochondria are very stable structures and therefore hard to completely get rid of and the remaining organelles may be enough to allow for APC2 localization.

This data may shed some light into a potential pathway of localization at branch points. The fact that the same machinery that is important for APC2 localization does not control mitochondria in the same way may suggest that mitochondria could be further upstream of APC2 and is important for recruiting it to branch points. This is further supported by the fact that when the number of mitochondria are reduced, APC2 no longer localizes to branch points in the same way.

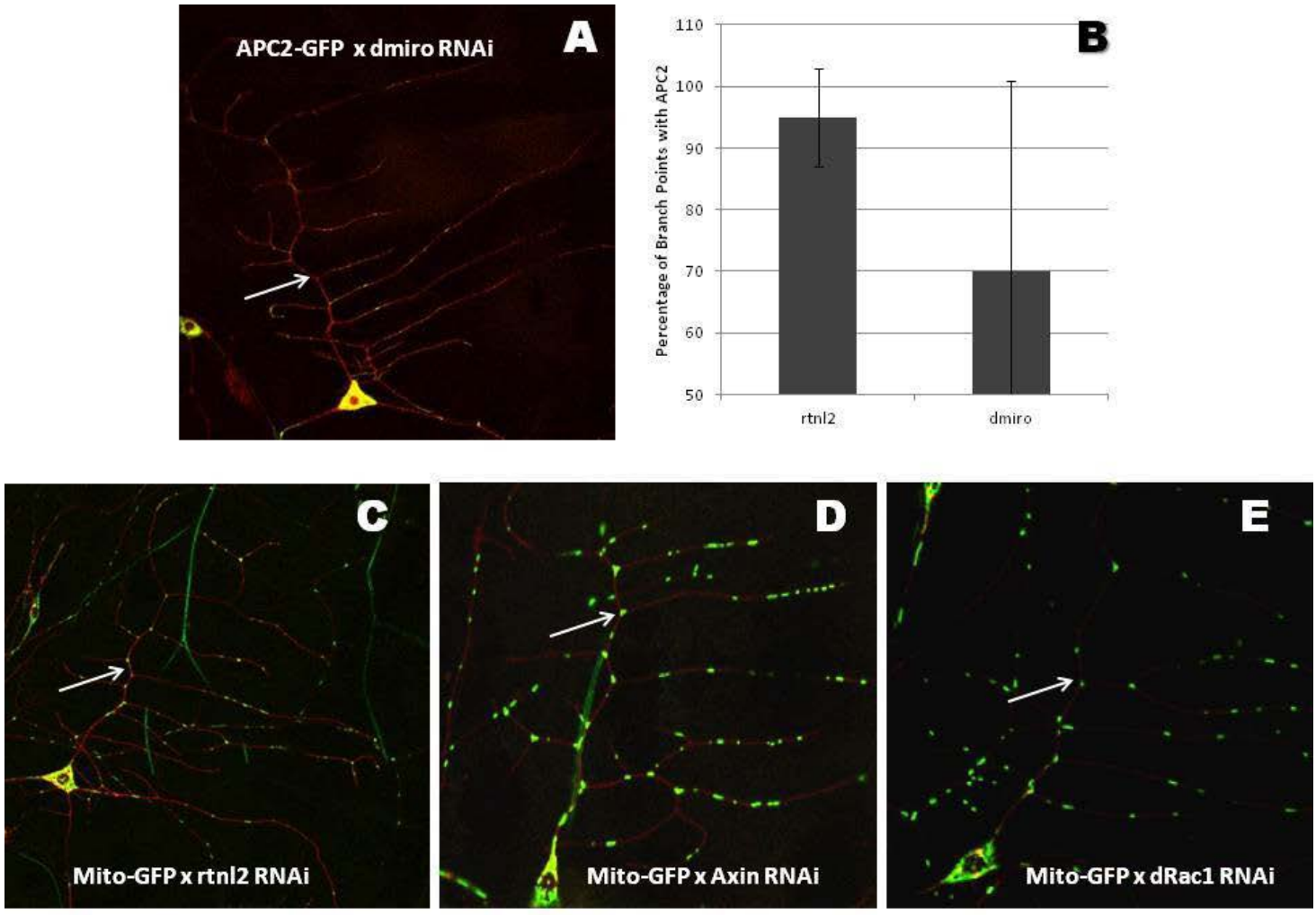

Figure 12. Mitochondria localization to branch points: A. APC2-GFP expression is greatly reduced after knockdown of miroRNAi indicating that mitochondria may be responsible for recruiting APC2 to branch points. **B.** Comparison in the percentage of branch points with APC2 between the negative control and miroRNAi. There is a large standard deviation due to the fact that it may be difficult to get rid of all mitochondria. **C.** Mitochondria localization (mito-GFP) after RNAi knockdown of rtnl2 (negative control). **D/E.** Mitochondrial localization after knockdown with Axin and dRac RNAi respectively. No changes in mitochondrial localization

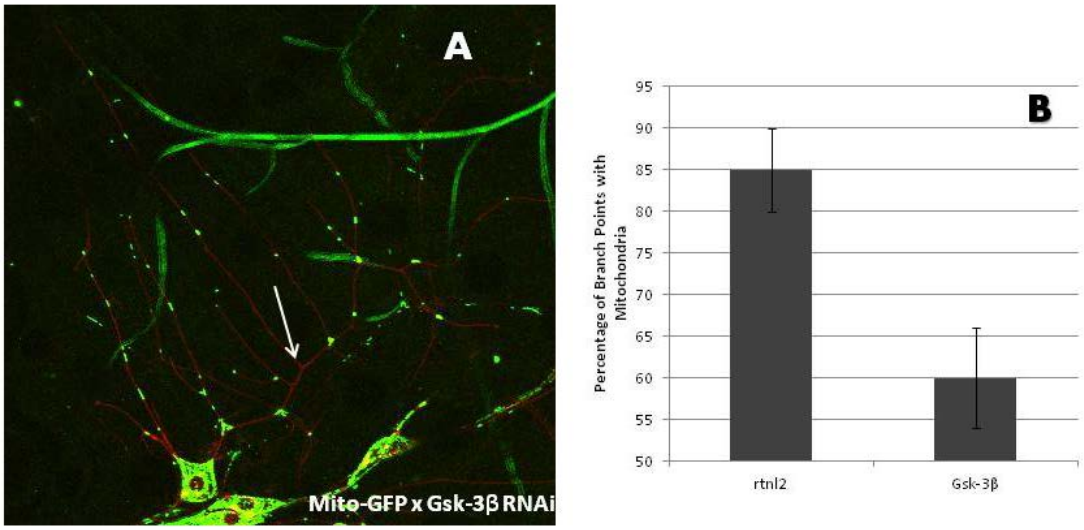

Figure 13: A. Mitochondria localization in comb dendrite after knockdown of GSK-3β. Mitochondria only localized to 60% of branch points. **B**. Comparison between mitochondria localization with negative control and GSK-3β.

Microtubule polarity does not change with APC2 candidates

An important goal for me was to determine whether or not the machinery required for the recruitment of APC2 and other proteins/organelles to branch points is required for maintaining microtubule polarity. All candidates that were found to disrupt APC2 localization were then screened to assay microtubule polarity (Figure 14). More candidates were screened through than what was shown to disrupt APC2 localization. The negative control yielded approximately a 92% minus end out phenotype however all other candidates had no notable changes except for dRac 1which had a polarity that was close to a full mixing of microtubules. While not terribly exciting, this data is key for understanding that same mechanisms that control branch point localization may not directly be involved in microtubule polarity.

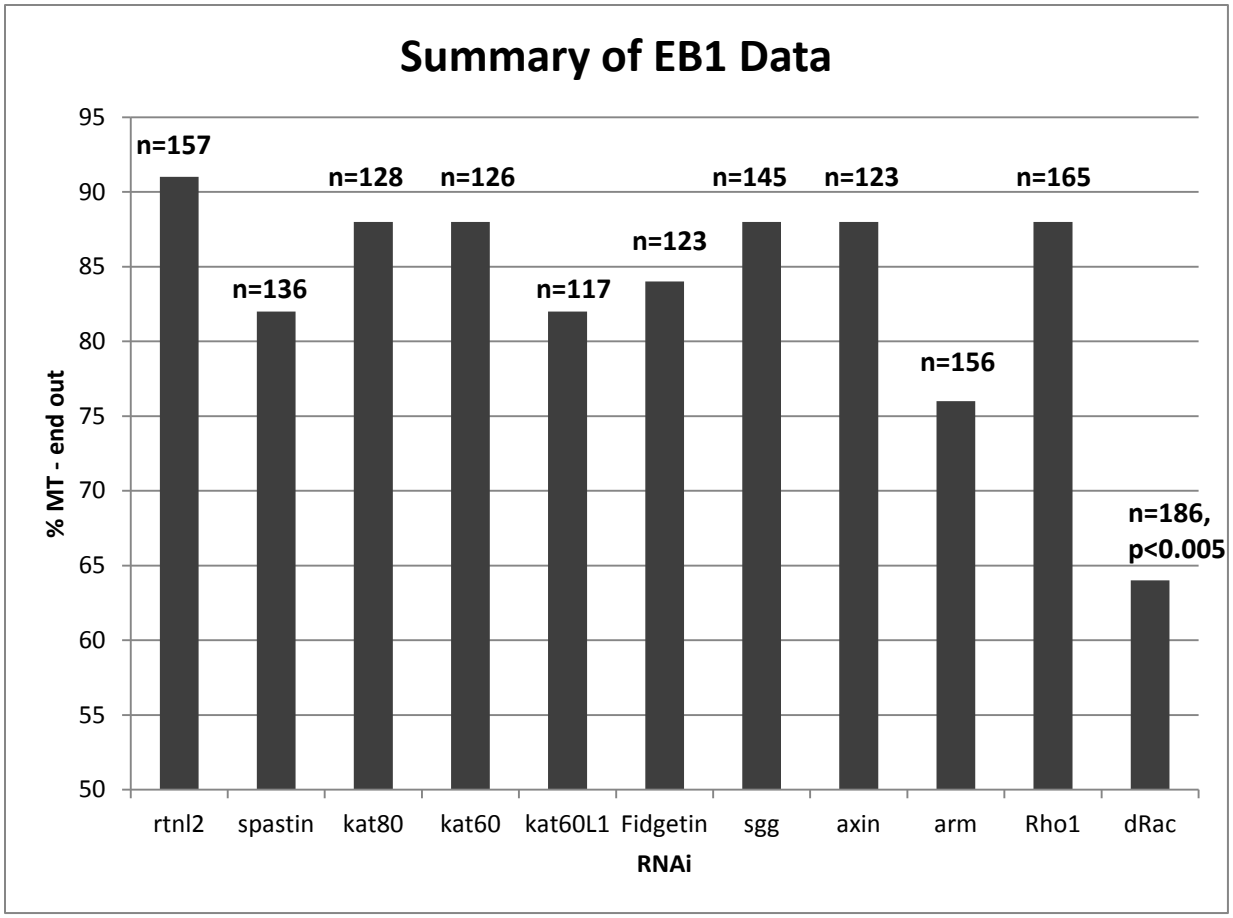

Figure 14: Summary of EB1 dynamics in dendrites after RNAi knockdown. More candidates were screened than we saw disrupt APC2 localization however there are no notable changes in microtubule polarity except for dRac1 RNAi. “n” values were recorded as the number of EB1 dots counted and statistical analysis was performed using a t-tes

Discussion

Other than axon development, there have only been a few roles for APC2 established in the nervous system including the part it plays in maintaining microtubule polarity in dendrites of peripheral neurons in *Drosophila* (Mattie 2010).Even more interesting, this protein has a very distinct localization in dendrites further suggesting that is has a critical role in establishing dendrite function. It is still not clear however, how such an important protein with an important role gets to this precise location within a cell. In this study, I have hoped to find a way to understand the mechanism behind APC2 localization in dendrites and whether that mechanism is also responsible for controlling microtubule polarity.

When screening through interacting proteins of APC2 to determine which one(s) had roles in maintaining the localization at branch points, I found a number of different players that had some kind of part. Microtubule severing proteins, actin cytoskeletal regulators and members of the Wnt signaling pathway are just a few classes that disturbed APC2 localization (Table 2). I decided to focus my efforts on those that only reduced the number of branch points that exhibited APC2 localization because these may give the best insight into a potential pathway that controls this localization. I narrowed down the candidates to the structural protein ankyrin 2, cytoskeletal regulatory protein Rac1, and members of the Wnt signaling pathway GSK3β and Axin. Each of these proteins have very different physiological roles which makes it very different to determine a coherent story that involves all of the players. Each player however does give clues into a potential overall picture.

The Rac1member of the Rho family of GTPases was the only regulator of cytoskeletal filaments that showed a direct loss of APC2 at branch points. This may suggest that APC2

localization could be mediated more by actin based mechanisms rather than microtubules. While microtubule associated proteins did change localization of APC2, they generally caused an overexpression in dendrite tips rather than a loss of APC2 at branch points. By disrupting microtubule stabilization, APC2 remained at branch points, which further suggests that its localization is a more actin-based mechanism. Because the Rac signaling pathways are so complex however, there is still a great deal of work that must be done in order to fully confirm the idea that a specific actin structure organized by Rac1 is involved in recruiting APC2 to branch points.

The logical next step in this process of determining a branch point localization mechanism was to understand where these proteins localize themselves. These critical experiments found that a specific splice form of anyrkin 2, and axin both localized to branch points in a similar fashion as APC2. We have also known from previous experiments that organelles such as mitochondria also localize to branch points indicating that due to the number of things that localize to branch points there may be central hubs that are important for coordinating dendrite function.

I further examined mitochondria for its role in APC2 localization as well as other proteins roles in maintaining mitochondria localization to branch points. By reducing the number of mitochondria, I found that APC2 was drastically reduced at branch points. However, when we knocked down the expression of other protein candidates to examine what happened with mitochondria localization, there was little to no observed effect except for GSK3β. One hypothesis I can generate based on this data is that the other proteins, including axin may act downstream of mitochondria to control APC2 localization. This begins to provide us with some order of how branch point members are localized. While mitochondria is upstream of APC2

localization and other members including Axin, dRac1 and ankyrin, it is not as far upstream as GSK3β. GSK3β is best known for its role as a regulatory kinase in the Wnt signaling pathway to activate the destruction complex for degradation of β-catenin (Mecalfe 2011). It may be acting in a similar fashion in this situation for branch point localization in terms of regulating downstream targets for further processing. Again, this is an extremely complicated situation that requires much more investigation in order to understand the true mechanism behind this kinase. This data does go to show that while not all branch point members are regulated by the same set of machinery, they may function in a pathway to control the overall localization to the intended target.

Axin, another member of the destruction complex in the Wnt signaling pathway was also shown to have a unique branch point localization as well as playing a role in APC2 localization. Axin is a very large scaffolding protein that can only form multi-protein complexes if its polymerization domain is active (Mendoza 2011). I tested this hypothesis of whether polymerization was required for its localization to branch points and found that it was not necessary. This may indicate that Axin is able to independently localize to branch points without interacting in multi-protein complexes. We cannot be sure of this however, until we determine whether the same machinery that controls APC2 localization also controls Axin localization. Current studies are being performed to help identify the role of Axin in this pathway. I was also not able to test whether the polymerization domain of Axin was required for the recruitment of other proteins, including APC2.

The ank2-L8-GFP that we used in this experiment is a long form of the ankyrin 2 gene found in *Drosophila* that has been shown to be a link between microtubule stability and the membrane stability in neurons, especially at the neuromuscular junction (Pielage 2008). Other

forms of ank2 did not show as significant a phenotype which fits with our study of how this was the only splice form that had a significant phenotype in branch point localization. It is important to note that it is likely that only the terminal end of this construct was required for localization and not necessarily the entire protein as this construct is the “long” form of the protein while others have the terminal end truncated (Pielage 2008). This may suggest a critical role for ank2 in the cytoskeletal involvement of APC2 localization.

Although there are still many questions as to the exact mechanism of branch point localization, I have at least identified some of the key players in this process. I have also determined that the same mechanism that controls branch point localization is not the same mechanism that controls microtubule polarity as there was no disruption of EB1 comets when interacting partners were knocked down.

I have begun to identify the key players involved in the mechanism of branch point localization. There is strong evidence to suggest that an actin based mechanism may be responsible for this localization as I have identified a few critical players involved such as dRac and a specific ankyrin construct. There may also be reason to believe however that these branch points may serve as mini-hubs in which multiple proteins and organelles localize under potentially the same mechanism. It is difficult to make any definite conclusions about a specific order to this organization; however we have a good start to making a great story that defines branch point localization.

References

Ahmed, Y., Nouri, A., Wieschaus, E. *Drosophila* APC and APC2 regulate wingless transduction through development. *Development*. 2002. 129: 1751-1762.

Akong, K., McCartney, B. M. and Peifer, M. Drosophila APC2 and APC1 have overlapping roles in the larval brain despite their distinct intracellular localizations. *Dev. Bio*. 2002. 250: 71-90.

Aoki,K., Taketo, M. Adenomatous polyposis coli (APC): a multi-functional tumor suppressor gene. Journal of Cell Science. 2007. 120:3327-3335.

Avila, J., Lucas, J., Perez, M., Hernandez, F. Role of tau protein in both physiological and pathological conditions. *Physiological Reviews.* 2004. 84 (2): 361-384.

Baas, P., Qiang, L. Neuronal microtubules: when the MAP is the roadblock. Trends in Cell Biology. 2005. 15: 183-187.

Baas, P., Lin, S. Hooks and comets: the story of microtubule polarity in the neuron. Developmental Biology. 2010. 71:403-418.

Bartolini, F., Gundersen, G.. Generation of noncentrosomal microtubule arrays. *J. Cell Sci.* 2006. 119, 4155–4163.

Conde, C., Caceres, A. Microtubule assembly, organization and dynamics in axons and dendrites. *Nature Reviews Neuroscience*. 2009. 10:319-332.

Craig, A., Banker, G. Neuronal Polarity. Annual Review of Neuroscience. 1994. 17:267-310.

Cullen,C.F., Deak, P., Glover, D., Ohkura,H. A gene encoding a conserved microtubule-associated protein required for the integrity of mitotic spindle in *Drosophila*. *Journal of Cell Biology.* 1999. 146(5): 1005-1018.

Grueber, W., Ye, B., Yang, C.H., Younger, S., Borden, K., Jan, L., Jan, Y.N. Projections of *Drosophila* multidendritic neurons in the central nervous system: links to peripheral dendrite morphology. *Development*. 2007. 134: 55-64.

Jiang, K., Akhmanova, A. Microtubule tip-interacting proteins: a view from both ends. Current Opinions in Cell Biology. 2011. 23:94-101.

Kollins, K., Bell, R., Butts, M., Withers, G. Dendrites differ from axons in patterns of microtubule stability and polymerization during development. *Neural Development*. 2009. 4:26.

Mattie, F., Stackpole, M., Stone, M., Clippard, J., Rudnick, D., Qiu, Y., Tao, J., Allender, D., Parmar, M., Rolls, M. Directed microtubule growth, +Tips, and kinesin-2 are required for uniform microtubule polarity in dendrites. Current Biology. 2010. 20:2169-2177.

Mendoza-Topaz, C., Mieszczanek, J., Bienz, M. The adenomatous polyposis coli tumour suppressor is essential for Axin complex assembly and function and opposes Axin's interaction with disheveled. *Open Biology*. 2011. 10: 1098-1113.

Metcalfe, C., Bienz, M. Inhibition of GSK3 by Wnt signaling-two contrasting models. *J. Cell Science.* 2011. 124 (21): 3537-3544.

Nguyen, M., Stone, M., Rolls, M. Microtubules are organized independently of the centrosome in *Drosophila* neurons. *Neural Develaopment*. 2011. 6(38).

Pielage, J., Cheng, L., Fetter, R., Carlton, P., Sedat, J., Davis, G. A presynaptic giant ankyrin stabilizes the NMJ through regulation of presynaptic microtubules and transsynaptic adhesion. *Neuron*. 2008. 55(2): 195-209.

Rolls, M., Satoh, D., Clyne, P., Henner, A., Uemura, T., Doe, C. Polarity and intracellular compartmentalization of Drosophila neurons. 2007. 2(7):

Rusan, N., Akong, K., Peifer, M. Putting the model to the test: Are APC proteins essential for neuronal polarity, axon outgrowth and axon targeting? The Journal of Cell Biology. 2008. 183:203-212.

Shintani, T., Ihara, M., Tani, S., Sakuraba, J., Sakuta, H., Noda, M. APC2 plays an essential role in axonal projections through the regulation of microtubule stability. The Journal of Neuroscience. 2009. 29(37): 11628-11640.

Shintani, T., Takeuchi, Y., Fujikawa, A., Masaharu, N. Directional neuronal migration is impaired in mice lacking adenomatous polyposis coli 2. The Journal of Neuroscience. 2012. 32(19):6468-6484.

Stone, M., Roegiers, F., Rolls, M. Microtubules have opposite orientation in axons and dendrites of Drosophila neurons. Molecular Biology of the Cell. 2008. 19: 4122-4129.

Yamanaka, H., Hashimoto, N., Koyama, K., Nakagawa H., Nakamura, Y., Noguchi, Y. Expression of APC2 during mouse development. Brain Res Gene Expr Patterns. 2002. 1: 107-114.

Academic Vita

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EDUCATION:

The Pennsylvania State University, University Park, PA
Schreyer Honors College
Bachelor of Science in Biochemistry and Molecular Biology
Bachelor of Science in Toxicology
Minor in Mathematics
Anticipated Date of Graduation: August 2012
Served as Student Marshal for the College of Agricultural Sciences during the summer commencement ceremony
Maintained Deans List every semester attended
*Thesis: "*Discovering the role of APC2 and interacting proteins in dendrite organization of *Drosophila* neurons"
Advisor: Dr. Melissa Rolls, Assistant Professor of Molecular Biology

RESEARCH EXPERIENCE:

The Pennsylvania State University, University Park, PA
Research Assistant, Undergraduate Research September 2009-Present

- Designed an individual project that characterized the cellular machinery responsible for localization of different proteins that maintain microtubule polarity in neurons.
- Collaborated with graduate students and research professors to understand the mechanisms of neuronal development in *Drosophila melanogastor*.
- Proficient with a variety of confocal and wide-field microscopes to produce videos from live imaging samples.
- Developed a senior thesis project in order to graduate with honors in Biochemistry.

United States Army Medical Research Institute of Chemical Defense, Aberdeen, MD
Student Intern, Pharmacology Branch Summers 2008/2009/2010

- Utilized immunohistochemical techniques to characterize the inflammatory expression of cytokines and chemokines in the rat brain following organophosphate exposure.
- Published work in the Journal of Neuroinflammation:
  Johnson E, Dao T, Guignet M, Geddes C, Koemeter-Cox A, Kan R. Increased expression of the chemokines CXCL1 and MIP-1α by resident brain cells precedes neutrophil infiltration in the brain following prolonged soman-induced status epilepticus in rats. *Journal of Neuroinflammation.* 2011; 8(1):41-50.
- Generated the standard operating procedure for the first confocal microscope in the lab.
- Proficient in fluorescent and light microscopy.
- Experienced in laboratory animal handling (mice, rats, and guinea pigs) and dissections.

S*tudent Intern* September 2007-May 2008

- Studied apoptotic markers in rat brains following exposure to the chemical warfare agent soman.
- Presented final work to a group of students, faculty members and professionals in a conference-like setting.

- Skilled in techniques including Western Blots, gel electrophoresis, EZQ protein quantitation and ELISA assays.

LEADERSHIP EXPERIENCE

The Pennsylvania State University, University Park, PA
Undergraduate Teaching Assistant January 2011-Present

- Worked as a laboratory teaching assistant for undergraduate Microbiology and Biochemistry labs.
- Responsible for teaching material, instructing students on proper laboratory techniques, and grading assignments on a weekly basis.

The Pre-Veterinary Club at The Pennsylvania State University, University Park, PA
Vice President May 2011-Present

- Planned general meetings for the club by inviting professionals from around the area to speak about their career choices.
- Organized trips for students to visit different veterinary schools.
- Collaborated with an executive board to make important decisions on the club's behalf.

Stay-Over Chair September 2010-March 2011

- Coordinated an event in which high school students who were accepted to the university could come and visit for three days to experience the college lifestyle.
- Oversaw a committee of students involved in planning a successful event.

Volunteers in Public Schools, State College, PA
Tutor November 2010- Present

- Assisted high school students in their understanding of algebra and chemistry for two hours each week.

HONORS/AWARDS

Schreyer Scholarship for Academic Excellence

- Academic scholarship on the basis of maintaining a cumulative 3.4 GPA and meeting all honors requirements of the Schreyer Honors College.

Jacqueline Hemming Whitfield Student Research Endowment Summer 2011

- Research fellowship in the Biochemistry and Molecular Biology department to fund undergraduate research over the summer.

Facings Award August 2010-May 2011

- Biochemistry departmental scholarship for academic excellence awarded to junior-year students in the top 10% of the class