THE ROLE OF APC IN KINESIN-II-MEDIATED MICROTUBULE GROWTH AT DENDRITIC BRANCH POINTS AND EXPLORING NOVEL TECHNIQUES IN VIVO

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ABSTRACT

Neurons are highly specialized cells, which contain two major compartments known as axons and dendrites. *Drosophila* dendrites have been shown to have a uniform microtubule polarity with 98% of microtubules growing towards the cell body, unlike the mixed polarity seen in dendrites of cultured mammalian hippocampal neurons. Since *Drosophila* dendrites are branched, it provides a unique opportunity for understanding the mechanisms involved in maintaining this polarity. Kinesin-II has been shown to be an important player in directing microtubule growth at branch points; however, the Kap3 accessory protein of Kinesin-II does not contain a microtubule-binding site that is needed to interact with the growing microtubule.

Since Kap3 is known to interact with APC, a plus tip protein, it seemed to be a good candidate for this mechanism. To explore the role of APC in Kinesin-II mediated microtubule growth, EB1-dynamics was observed in Apc2 and Apc mutants to determine if microtubule polarity changed. Results of the double mutant showed 7% of microtubules growing away from the cell body compared to 3% in heterozygous controls. RNAi of Apc2 was also conducted in *Drosophila* sensory neurons, while observing EB1-GFP comet movement. Apc2 RNAi showed 15% of microtubules turning away from the cell body, compared to 11% in Rtnl2 controls. These results suggest a role for Apc2 in Kinesin-II mediated microtubule growth in *Drosophila* sensory neurons.

In addition to this work, I used the temperature sensitive degron, in vivo, to degrade GFP. It works by unfolding at high temperatures, which provides a signal to
be ubiquinated and degraded by the proteasome. This technique had previously been used in *Drosophila* fillet preps, but not in vivo. By overexpressing the degron in transgenic flies, homozygous and heterozygous, I determined reliable conditions for a 1-hr and a 24-hour heat shock. I also started to explore KillerRed, which locally degrades an attached protein through ROS when activated by green light. This technique has been used successfully in cell culture, but has yet to be used in vivo.
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CHAPTER 1

INTRODUCTION

1.1 Basic neuronal polarity

Neurons are highly specialized, differentiated cells, which contain three distinct structures: the cell body, dendrites, and an axon. The primary function of an axon is to send signals to other neurons or output cells. The dendrite’s function is to receive signals from other neurons or the environment. These specific roles could be related to the distribution of proteins and cellular components, such as the Golgi and ribosomes which are found in dendrites but not axons [1]. This could be a result of the polarized structure of non-centrosomal microtubules located inside each neuron, which allows for polarized trafficking of cellular components [2].

Microtubules contain 13 protofilaments arranged in a helical structure that is made up of alternating alpha and beta tubulin subunit dimers. As a microtubule grows, these subunits are added to the microtubule - this end is referred to as the plus-end or growing end of the microtubule. The minus-end of the microtubule is considered to be much more stable and does not undergo the types of dynamic changes that occur at the plus-end.

Neuronal microtubule polarity was first observed in the early 1980s through the use of electron microscopy. Exogenous tubulin added to the axonal microtubules in vitro formed curved hooks of tubulin when bound to the side of existing microtubules. In cross sections of the axon the direction of the tubulin hooks was observed to have a clockwise hook, which were labeled as the plus end. Axons were
first to be used in observing tubulin attachment to the stable microtubules, from which polarity was determined to be >95% plus-end out of numerous types of vertebrate neurons [1], [3], [4],[5]. More recently, microtubule polarity in axons was observed in cultured hippocampal neurons and Purkinje cells by the use of +TIP proteins [6], which associate with the plus end of growing microtubules. These proteins are observed as moving comets via fluorescence microscopy. By observing the directionality of the +TIP movement, the polarity of the microtubules in axons was determined, which was consistent with the previous electron microscopy work. In addition to these two methods, second harmonic generation microscopy has also confirmed axons have uniform polarity in vivo [7].

With the success of hook labeling and +TIP proteins in axons, the techniques were applied to dendrites in cultured rodent interneurons. Both methods confirmed that dendritic microtubules have a mixed orientation, unlike the uniform polarity observed in axons. In vivo, frog mitral cells appeared to also have a mixed orientation of microtubules in their dendrites [8]. Therefore, the widely accepted model of microtubule polarity is that axons have a uniform plus-end-out microtubule orientation, while proximal dendrites have a mixed orientation of plus-end and minus-end-out microtubules (figure 1A).

1.2 Drosophila neuronal polarity

In 2007/2008, microtubule polarity was observed in Drosophila melanogaster and Rolls et al. [9] and Stone et al. [10] were surprised to find that microtubule polarity displayed a uniform minus –end-out polarity; a polarity that is
different from the mixed polarity observed in vertebrate studies. Polarity in *Drosophila* was observed in proximal dendrites of sensory neurons, interneurons, and motor neurons which all showed a 95% minus-end out. The axons of the same neurons, however, share the conserved uniform plus-end-out polarity previously seen in other species (Figure 1).

**Figure 1**: Overview of microtubule polarity of vertebrates and *Drosophila*. (A) Dendritic polarity in vertebrates has been shown to consist of both minus-end and plus-end out microtubule, while (B) *Drosophila* dendrites have been shown to have 98% minus-end out polarity. Axons in both systems share a conserved uniform polarity of plus-end out. Adapted from Stone et al. [10].

Dendrites, in general, have multiple branches that grow off the main trunk. With these multiple branch points at which growing microtubules can turn, it raises the following question: What are the mechanisms guiding microtubule growth towards the cell body at branch points to maintain dendritic neuronal polarity (Figure 2)?
Figure 2: Microtubules grow towards the cell body at branch points. In the left panel, a growing microtubule (red arrow) is shown turning towards the cell body as it grows through a branch point. This direction of growth maintains minus-end-out microtubule polarity, and is observed 98% of the time when growing microtubules are imaged with EB1-GFP in da neuron dendrites. In the right panel a microtubule is shown growing from one dendrite into the other. This type of growth generates a plus-end-out microtubule and disrupts the overall polarity. [11].

To address this question, Drosophila dendritic arborization (da) sensory neurons were used to study microtubule dynamics. The sensory neurons lie between the cuticle and epithelial cells. When a larva is placed dorsal side up on a slide under a coverslip, the dendrites evenly spread out without becoming crushed or deformed. This provides ideal imaging conditions. The da sensory neurons are divided into 4 classes, which is based on complexity of the dendritic array, which ranges from a simple branching pattern (class I) to a complex branching pattern (class IV) (figure 3).

To determine microtubule polarity in the class 1 ddaE neurons, a plus-tip protein, EB1 (end-binding protein 1) was tagged with a green fluorescent protein (GFP) to observe the direction of microtubule growth in vivo. At branch points, EB1-GFP comets were observed to follow specific tracks, so through the use of a GFP-tagged tau, which label stable microtubules, and red fluorescent protein tagged EB1 (RFP), non-growing microtubules and actively growing microtubules were
simultaneously observed. The EB1-RFP movement coincided with the tau-GFP labeled stable microtubules through branch points, which led to the idea that these stable microtubules could be providing tracks for the growing microtubules. Since kinesins are known to interact with stable microtubules, microtubule polarity was observed in RNAi screens of different substrates of kinesins to determine if uniform microtubule polarity was altered in dendrites. One very strong candidate appeared: Kinesin-II.

Figure 3: Representations of the 4 classes of da sensory neurons. The complexity in branching pattern increases with the classification. Class 1 shows the daE, which is also known as the comb dendrite and is used often in the studies conducted in this paper, class II is the daB, class III is the daF and class IV is the daC of wandering third instar larvae with anterior to the left and dorsal side up [12]

1.3 Kinesin II

Kinesin-II is a heterotrimeric protein consisting of two motor proteins, KLP64D and KLP68D, and an accessory protein, KAP3. It is a plus-end directed motor and has mouse homologues KIF3A, KIF3B, and KIF3C, respectively [13]. Kinesin-II has been studied in axons of Drosophila [14], and was shown to have an important role in transporting choline acetyltransferase along the axon. One of the clearest roles for kinesin-II is in intraflagellar transport [15]. Without kinesin-II, the
components that are needed to construct the motile cilia and flagella are not transported to the tips of the cilium or flagellum. More recently Kinesin-II in Drosophila was found to be important in the development of pupal photoreceptors [16].

To look at the role of Kinesin-II in the dendrites of Drosophila, UAS-hairpin RNAs of KLP64D, KLP68D and KAP3 were expressed using a neuron specific Gal4 driver. The UAS-Dicer2 transgene was also added, as it has been shown to increase the effectiveness of RNAi[17]. In addition to the RNAi, KLP64D mutants were also used to assay microtubule orientation. RNAi targeting Rtnl2, an endoplasmic reticulum protein, was used as a control because previous RNAi studies have not shown a microtubule phenotype, or any other visible neuronal phenotype compared to cells with no RNAi. The results of the RNAi experiments showed an increase in mixed microtubule polarity of class II-IV, observing 20-28% of plus-end-out microtubules between KAP3, KLP68D and KLP64D. The controls where observed to have only 2% of plus-end out microtubules [11].

In the Class II-IV sensory neurons the angle of the branches in which the growing microtubule enters favors turning towards the cell body. However, the class 1 dendrites are observed to have branches that are almost perpendicular to the main branch. This provides about a 90 angle and provides an equal chance of turning to the cell body or away from the cell body. Due to this branch angle, it was hypothesized that there would be a higher need for a turning mechanism in branches that do not favor a specific direction for turning. The results of the class 1 RNAi of KLP64D, KLP68D, and KAP3 each resulted in a stronger polarity phenotype
then the other classes, with the KLP64D RNAi showing the strongest phenotype with a completely mixed polarity, in which about 50% of growing microtubules were plus-end-out and about 50% were plus-end-in microtubules. This data suggests there is a stronger need for kinesin-II-mediated microtubule turning at branch points where the branching angles favor a 50/50 chance of turning.

The kinesin-II accessory protein, KAP3, is known to have various binding partners, however, it does not have a microtubule-binding site. Therefore, there must be an intermediate protein for interaction between the growing microtubule and KAP3. In this paper I explore the role two potential plus-tip proteins as the possible key intermediate protein.

1.4 Exploring in vivo techniques

One technique that has been frequently used in protein manipulation is using mutant alleles of your protein of interest. The use of mutated genes has contributed significantly in identifying the role of proteins involved in a variety of processes, such as the cell cycle, programmed cell death and body patterning during development. However, like all genetic techniques, there are limitations. Genes that are important for development can be homozygous lethal when mutated. This limits the analysis of gene function to a heterozygous state, which can mask the actual phenotype of that gene. Additionally, using mutated genes alters the development of the organism, which could alter the phenotype of the gene you are studying. Mutant proteins can also act as a dominant-negative, which can prevent or alter the
phenotype. Additional work is then required to determine how the protein is functioning and this can be time consuming and costly.

Studies with mutants in *Drosophila* become tricky because embryos contain mRNA passed on from the mother during embryogenesis; these are referred to as the maternal effect genes. The presence of maternal mRNA can be useful when studying proteins that are embryonic lethal, as this allows for the embryo to develop into larval stages before dying. However, it is not clear when the maternal proteins are depleted and when the embryo is relying on its own proteins for development. This can lead to ambiguous data or require other techniques to confirm any true phenotype, which again, costs more time and money.

With the advances in technology and scientific knowledge, a new technique has recently emerged that has become widely used for in vivo protein manipulation in *C. elegans* and *Drosophila*: RNA interference (RNAi). Fire and Mello [18] first developed RNAi in 1998 by using double stranded RNA to target the activity of individual genes. Upon the sequence completion of genomes, such as *C. elegans* and *Drosophila*, many RNAi libraries have been formed and are used regularly in vivo to study proteins, like the experiments described earlier for the kinesin-II constructs.

The high-throughput screens performed with RNAi have significantly advanced research to understand gene functions and more importantly, biological processes [19]. However, RNAi can give off-target effects by binding to non-specific targets, which are usually mRNAs that is similar in sequence to the target RNAi. Without using additional methods, such as antibody staining or using RNAi with a protein of interest that is GFP-tagged, it is difficult to determine the effectiveness of
an RNAi. You can also get different knockdown efficiency based on the cell or environment in which it is being expressed [20]. Additionally, it takes time for mRNA degradation to occur by RNAi, and in *Drosophila*, you must wait for the maternal protein to be depleted before conducting your experiments, and in studies that look at early development, these techniques are not feasible to use.

There are very limited numbers of techniques that can be used successfully, quickly, and reliably in vivo. In this paper I determine the protocol necessary to degrade a protein of interest with an hour, in vivo, without damaging the surrounding environment or altering development.
Chapter 2

APC

2.1 Introduction

Adenomatous polyposis coli (APC) is a microtubule plus-end associated protein that was first identified in 1991 as a frequent culprit in patients that had familial adenomatous polyposis, which is a hereditary colon cancer. Mutations in the APC gene have also been found in more than 70% of sporadic colon cancers. Since then, APC has been widely studied and has been determined to function in Wnt signaling, embryogenesis, and, in mammals, plays an important role in directing axon polarization, outgrowth, and guidance [21]. In vivo studies conducted in Drosophila have implicated APCs in cadherin-based adhesion, spindle structure and chromosome segregation [21].

In Drosophila there are two APC proteins, Apc1 and Apc2. Both Drosophila APC proteins play redundant roles in Wg signaling and Wg independent brain development, however, they have been shown to have separate roles elsewhere[22]. This is due to a basic domain of Apc1 that allows it to bind to centrosomes and microtubules, which is missing in Apc2 so rather then localizing to microtubule structures, Apc2 localizes to the cell cortex [23]. Both proteins contain armadillo repeats in the N-terminus, which also contains a binding site for KAP3, the accessory protein of kinesin-II. It has been previously reported that mammalian KAP3 can interact with EB1 [24] and APC [25], independently. Since the human homolog of Apc1 has an EB1 binding site, it is plausible that EB1 and Apc1 in flies could interact;
however, an EB1 binding site on Drosophila Apc1 has yet to be identified. Based on previous interactions of mammalian APC1 with both kinesin-II and EB1, we believe that the APCs could be acting at the branch points, together or independently, to maintain microtubule polarity in dendrites.

2.2 Results

To explore the role of the two APCs in dendrites, I used mutant alleles of each APC. Since APC has been widely studied, there are well-characterized alleles for both Apc1 and Apc2. In this study I used APC1\(^{Q8}\), which has been described as null [22], and APC2\(^{N175K}\), APC2\(^{ΔS}\), and APC2\(^{d40}\). In all crosses I used elav-GAL4 to drive expression of EB1-GFP in da sensory neurons. Based on EB1-GFP comet movement, I determined the plus end-out polarity in heterozygous single mutant controls, Apc2\(^{d40}\), Apc1\(^{Q8}\), to be 2%. Apc1 homozygous mutants showed only a slight increase to 4% of plus-end out while the homozygous Apc2 mutant polarity was consistent with the control (Figure 4).

I then observed the microtubule polarity in dendrites where both Apc1s were knocked out using a line containing Apc1\(^{Q8}\) and Apc2\(^{N175K}\). In the heterozygote control, which contained each allele, I observed the plus-end-out polarity to be 2.5%, while the homozygous double Apc mutants showed a statistically significant increase of 7% plus-end-out microtubules (p-value=0.01) (Figure 4). Although there was a significant difference in microtubule polarity, it was not as drastic as the KLP64D mutant, which could be a result of maternally loaded Apc proteins still present.
As mentioned before, the different classes of da sensory neurons range in their dendritic branching complexity, but their branching angles differ as well. The comb dendrite of the Class I daE neurons, has the most severe branch angle. The angles of the branch connected to the main trunk resemble a right angle, much like the tines of a comb. When a growing microtubule reaches the main trunk, there is about a 50/50 chance of it turning towards the cell body or away from it (Figure 5). This random turning would then create a mixed polarity of microtubules in the absence of a mechanism to direct growth.
Since the class 1 comb dendrite appeared to be a potentially better environment to study mediated microtubule turning, the 221-GAL4 driver was used to drive EB1-GFP expression in the class 1 neurons. As the larvae for the Apc mutants were very sick and died within late third instar, we wanted to use a different technique to confirm the mild phenotype that was observed in the mutants, without affecting the whole larvae. RNAi, when used with the Gal4/UAS system, allows us to express the RNAi in specific cell types. Similar to the mutant experiments, Apc1 and Apc2 RNAi were tested individually, as well as together.
Rtnl2 was used as a control as described earlier. Only the Apc2 RNAi showed a statistically significant difference in plus-end out microtubule polarity, with polarity shifting from 11% in controls to about 15% in class 1 dendrites (p-value=0.04) (figure 6). Although results were not as dramatic as the RNAi for the kinesin-II components, this does suggest that Apc2 contributes to maintaining microtubule polarity at branch points.

Another approach to understanding Apc2’s role at the branch points is to over-express proteins tagged with fluorophores, and observe localization. Localization data can provide insight to where the protein is most present which can also suggest possible roles not previously known. It can also be used in conjunction
with other fluorescent-tagged proteins to observe possible interaction through co-localization. Apc2-GFP over-expression lines show Apc2 localization at branch points (Figure 7A) in the main trunk of the ddaE class 1 neuron, when driven by 221-Gal4 but Apc1 does not (Figure 7B). It is not until you express Apc2 with Apc1 do you have Apc1 localization (Figure 7C). Since we hypothesize that KAP3 may interact with Apc1, I generated a GFP tagged KAP3 and a RFP tagged KAP3 in transgenic flies. Both constructs show a very diffuse pattern when expressed in the class 1 neurons by the 221-GAL4 driver (Figure 7E and Figure 8A). When KAP3-RFP is expressed with Apc2 and Apc1, localization of Apc2 still occurs at branch points (Figure 7D), however KAP3 localization still remains diffuse throughout the dendrites, soma, and axon (Figure 7E), and does not appear to be co-localizing with Apc2 or Apc1. GFP-KAP3 expressed with EB1-RFP (Figure 8) continues to show diffuse patterning in the soma and axon, but very lightly expresses in the dendrites. While analyzing the videos of this cross, the KAP3 granules moved throughout the soma and axon, but did not correspond with EB1 comet movement.

There are a couple of possible explanations of why I do not see KAP3 localizing with Apc. First, KAP3 may require the other two components of kinesin-II, KLP64D and KLP68D, to interact with Apc or even to localize at branch points. Over-expression with all three components may resolve this issue. Secondly, the KAP-3 tagged GFP may not function properly, due to the attached fluorophore. Expressing the GFP or RFP tagged KAP3 in a mutant KAP3 background to determine if rescue occurs would provide evidence to whether the fluorophore is interfering with KAP3 function.
2.3 Discussion

From my experiments, we can conclude that the Apc proteins do play a role in controlling dendrite microtubule polarity. To determine how this occurs, Floyd
Mattie, a fellow graduate student, conducted further research to better elucidate the role of APC. When further experiments were focused on the class 1 ddaE comb dendrite, a much clearer role for both Apcs appeared. Through the combined results of both his and my results, a proposed model was created to explain the branch point mechanism, which involves Kinesin-II, Apc1, Apc2 and EB1 (Figure 9).

![Figure 9: Schematic of the proposed kinesin-II mediated microtubule turning at dendritic branch points. Apc2 localizes to the branch point membrane to recruit Apc1. In red is a growing microtubule with transient EB1 present. When the growing microtubule enters the branch point the EB1 interacts briefly with Apc, which is also interacting with Kap3 of kinesin-II, to turn the microtubule towards the cell body.]

We believe that Apc2 can localize to branch points through an internal localization signal, and at the branch point it can then recruit Apc1. As the kinesin-II is walking the growing microtubule through the branch point along stable microtubules, Apc1 can interact with both KAP3 of kinesin-II and EB1. This interaction could provide enough force to steer the growing microtubule towards the cell body, therefore maintaining dendritic microtubule polarity of plus-end-in.
Chapter 3
Degron

3.1 Introduction

The use of RNAi and mutated genes has significantly contributed to the hypothesis that kinesin-II plays a role in maintaining non-centrosomal microtubule polarity. However, one of the disadvantages of these techniques is that early stages of larval development may be altered. Arguably, Drosophila have an advantage in these stages of development because they obtain unaltered maternal proteins, which allows larvae to survive longer than usual. On the other hand, this is also a disadvantage because we cannot be sure when the maternal load is fully depleted. Therefore, a technique is needed that can quickly manipulate a protein of interest without disrupting the normal development of the organism. Conditional mutants allow us to do that.

Conditional mutants are used under two different conditions; permissive and non-permissive. These conditions can be controlled, which then allows control of the protein function or expression. One of the most common types of conditional mutants is the temperature-sensitive (ts) mutant. This technique has been used extensively in yeast assays; however, one of the drawbacks to ts mutants is the common occurrence of leaky expression. It is also very time consuming and difficult to find ts mutants. To improve the usefulness of these mutants a new method was created in 1994 [26] that utilized heat-activated degradation, in which degradation of the protein of interest was achieved by utilizing the N-end rule pathway in yeast. This pathway states that the in vivo half-life of a protein is linked to its N-terminal
residue. This residue acts as a degradation signal, prompting ubiquination by the E1 substrate and ultimately degradation by the 26S proteasome. In yeast, arginine was determined to be best N-terminal residue to use, since it has a half-life of about 2 minutes [27]. The construct that was used to create this condition was a ts variant of a 21-kDa mouse dihydrofolate reductase (DHFRts). This construct contained an N-terminal Arg residue. The researchers determined that the Arg- DHFRts was functional at 23°C, the permissive temperature (Figure 10a), but was short-lived at 37°C, the non-permissive temperature (Figure 10b).

Figure 10: Schematic of the conditions for degron construct. Normal protein function occurs at 23°C but 37°C the DHFRts undergoes a conformational change that reveals a Lysine for ubiquination. The DHFRts with the target protein is then degraded by the proteasome. [28]
The temperature sensitivity is due to a missense mutation of a P66L [28]. At non-permissive temperatures the DHFR\textsuperscript{ts} undergoes a conformational change, which reveals a specific Lys residue. The Lys, in conjunction with the Arg, acts as signals to the E1, E2, and E3 substrates to bind and ubiquinate the DHFR\textsuperscript{ts} for degradation. The players involved in protein degradation by the proteasome are very tightly regulated, therefore providing us with a precise method for controlling protein expression in vivo.

3.2 Results

To date, there has only been one use of the degron system in *Drosophila*. In those experiments the researchers used larval fillet preps to look at how the proteasome, through the use of the degron, is involved in controlling the concentrations of important proteins in the synaptic boutons [29]. Although using fillet preps are very useful, it does prevent any further use of the larvae once the experiment is complete. Fillet preps also allow for better exposure to the elements, in this case a 37°C heat shock, and so it raises the question: can the degron construct be activated through heat shock in vivo at the higher temperature that is required for degradation and still retain survival, and if so how long does it take for degradation to occur? Dr. Kendal Broadie was gracious enough to provide the degron construct, tagged with eGFP [29]. Using this line, I created a recombinant line that expresses elav-GAL4 on III with the DHFR\textsubscript{ts}-eGFP, to use in crosses containing other proteins of interest we wish to study.
Since my goal is to keep the larvae alive after a heat shock, I started my heat shock at 30°C, which is above the permissive temperature for the degron construct. This is also a common temperature in which heat shock experiments are conducted in flies since they are able to breed and grow at this temperature. The controls for these experiments were conducted at room temperature, ~about 21°C to 23°C. I first wanted to determine how long it would take for degradation to occur. Since this was being conducted in whole larvae, I decided to heat shock for 1-hour to get a base line of degradation. At room temperature, an average of 85% of GFP fluorescence remained. After heat shock at 30°C, only an average of 65% of GFP fluorescence remained (Figure 12). However, variance in the percent of remaining GFP fluorescence suggests this is not an optimal condition for the degron to reliably work. I further looked at fluorescence after a 24-hour heat shock at 30°C and saw that the average fluorescence decreased to about 38%. This suggests that a 1-hour heat shock at 30°C is not a reliable condition for quick protein degradation but it can reliably sustain protein degradation for at least 24 hours at this temperature.

With advances in imaging capabilities and cloning techniques, many experiments are conducted using two different fluorophores to visualize proteins of interest. To insure that the heat shock does not affect the fluorescence of the additional fluorophore to the degree of the degron, two separate crosses were created. One contained the elavGal4, UAS-degron-GFP with UAS-mcd8-RFP, a membrane marker, and the other contained UAS-degron-GFP with 109(2)80 Gal4, UAS-EB1-RFP. Since I am interested in overall RFP fluorescence that remains and not looking for differences in driver expression, I combined the results of the GFP
and RFP fluorescence. After 1-hour heat shock at 30°C I saw only that 25% of GFP fluorescence remained, while RFP fluorescence remained about 62%. After 24-hours GFP fluorescence was about 34% and RFP fluorescence was about 75% (Figure 12). With the loss of GFP fluorescence, but very little loss of RFP fluorescence, it suggests that the degron construct is unfolding and being degraded, and that the decrease in fluorescence is not a result of bleaching.

Although 30°C heat shock was proving to be a reasonable condition, I wanted to observe the effect of a 35°C heat shock, which is closer to the optimal temperature for the degron construct to unfold. I first started with a 1-hour heat shock with a homozygous elav-Gal4, UAS-degron-GFP and observed about 23% of GFP fluorescence remained. After a 24-hour heat shock I observed about 34% of GFP fluorescence remained (Figure 11), however not all larvae survived to be imaged and those that did were very sick and slow moving.

I next observed the crosses with the RFP constructs present. In these larvae, about 30% of GFP fluorescence remained after 1hr heat shock where as the RFP fluorescence remained about 84%, similar to 30°C. After 24 hours, around 46% and 90% of fluorescence remained for the GFP and RFP, respectively (Figure 12)(Figure 13), however the 24-hour heat shock larvae were very sick and slow moving, same as the homozygotes. Since I have low numbers of larvae for 24-hours at 35°C due to difficulties in survival, statistical analysis cannot be conducted, however the ill effects of a 24-hour heat shock at 35°C suggests this is not a practical condition to use.
Figure 11: Comparison of fluorescence of homozygous degron at 30°C and 35°C for 1-hour and 24-hour exposure. Average fluorescence represents the amount of signal present after temperature exposure relative to signal before exposure. N’s represent the number of larvae used for analysis. At 35°C for 24-hours survival was minimal and statistics could not be conducted*. Error bars represent standard deviation.

Figure 12: Comparison of Fluorescence for Heterozygous Degron-GFP and RFP after 30°C and 35°C Heat-Shock. Average fluorescence represents the amount of signal present after temperature exposure relative to signal before exposure. N’s represent the number of larvae used for analysis. At 35°C for 24-hours survival was minimal and statistics could not be conducted*. Error bars represent standard deviation.
3.3 Discussion

Since temperature sensitive assays are a useful tool to disrupt protein function, it is advantageous to have a system in which we can control when engineered proteins become dysfunctional or degraded. This system also allows you to control the physiological phenotype of your protein during permissive and non-permissive temperatures.
Based on my results, I would suggest using 35°C for 1-hour heat shock to manipulate your protein. This should provide ample time to degrade your protein and show the potential phenotype. If you wish to keep the protein levels degraded for a longer amount of time I would first recommended heat shocking your protein at 35°C for one hour, then after imaging, placing your larva at 30°C for 24 hours to sustain the degradation levels. This should provide reliable and reproducible data for your constructs. In conclusion, I believe the degron can be used, in vivo, to induce degradation by the proteasome.

Now that I have shown the degron system can be used living *Drosophila* larvae to quickly and efficiently degrade GFP, we can now use it to disrupt kinesin-II and Apc proteins. By tagging these proteins with the degron construct and a fluorophore, such as RFP or GFP, I can express them in their respective mutant backgrounds, have normal development and then manipulate the function of the protein through a 1-hour 35°C heat shock. Since we believe the kinesin-II and Apcs are working at the branch point, as stated earlier, I wish to observe EB1 dynamics at the branch points of the class 1 comb dendrite, before and after heat shock to determine if EB1 directional movement becomes more mixed. The microscope contains an objective heat ring, so I could theoretically keep the degron-protein levels sustained at heat shock levels, while imaging EB1 dynamics. However, the temperature at which the ring could be used, since it is in close contact with the larvae, would have to be explored. This use of this technique, in vivo, has proven to be useful tool and has the potential be the next widely used tool for temperature sensitive assays.
Chapter 4

Future Work: KillerRed

4.1 Introduction

Although the degron has shown to be a useful tool in degrading a protein of interest, this technique is limited to how it can be used. It requires a heat shock for activation and the heat could potentially alter the function of other proteins, thereby causing secondary affects. The degron is created as an over-expression line and therefore must be used in a mutant background for proper analysis in whole animals. This led to looking at a different technique that has been used successfully in cell culture to rapidly degrade the attached protein through a fluorescent protein named KillerRed.

KillerRed acts by releasing reactive oxygen species (ROS) upon exposure to green light (a wavelength between 520-590nm) by the use of chromophore-assisted light inactivation (CALI). This damages the protein it is attached to, rendering it inactive. Bulina et al. [30] created KillerRed from a non-fluorescent and non-phototoxic red chromoprotein anm2Cp from the Hydrozoa jellyfish. Two substitutions, T145N and C161A, were found to be crucial in inducing the phototoxic effect. These positions are close enough to the chromophore to cause drastic effects in the fluorescent properties. Although these two substitutions are critical for KillerRed phototoxicity, another key feature of KillerRed that differs from other fluorophores is the presence of a water chamber that comes in contact with the chromophore. Some believe that this direct access promotes the increased toxicity
[31]. Studies previously using KillerRed have shown a limited range of effect for ROS, providing a very specific effect on the attached protein.

4.2 Preliminary work

To date KillerRed has yet to be used in vivo in whole animals. My future goal for this project is to determine the protocol necessary to activate KillerRed in *Drosophila* da sensory neurons. There does not appear to be a specific amount of time needed for CALI application but it is recommended to irradiate for at least twice as long as it takes to significantly photo bleach your protein [32]. To first determine this condition, I have created a transgenic line that expresses UAS-KillerRed. Since EB1 is a potential target to assay dynamics upon KillerRed activation I crossed the UAS- KillerRed with 109(2)80-Gal4, UAS-EB1-GFP. Expression of KillerRed is diffuse through out the cell, as is EB1-GFP, but has low expression in the dendrites (Figure 14). This can make it difficult to activate KillerRed at branch points to monitor EB1 dynamics. I have yet to determine an amount of time that is required to photo-bleach with green light (545nm).
4.3 Future Work

Once I have determined the time needed for proper irradiation, I wish to create constructs that are attached to KillerRed, such as KAP3 or KLP68D, and study EB1-GFP dynamics at branch points after KillerRed activation. I hope to disrupt EB1 dynamics by destroying either of the kinesin-II constructs, to provide additional support for a kinesin-II mediated microtubule turning mechanism. Since this technique does not rely on any internal system in the cell for proper function, such as the degron relies on the proteasome for degradation, it opens up the possibilities to use it anywhere in the organism. If I can determine ideal imaging condition for use in vivo, it could become the next widely used technique for protein manipulation in systems that can be studied by fluorescence microscopy.
Chapter 5

Methods and Materials

*Drosophila stocks*

Many useful stocks were obtained from the Bloomington Drosophila Stock Center, including: flies with gal4 drivers: elav and 109(2)80; flies with transgenes: UASdicer2, and the single FRT8, Apc2d40, Apc1q8 and double mutant flies FRT82B, Apc2N175K, Apc1Q8. 221-Gal4 flies were from Wes Grueber. Most UAS-hairpin RNAi lines were obtained from the Vienna Drosophila RNAi Center (VDRC). They included stocks to target Rtnl2 (33320), Apc1 (51469), Apc2 (22289). We have previously described UAS-EB1-GFP [9]. Several transgenic lines were also generated for this study. UAS-KAP3-GFP, UAS-KAP3-RFP and UAS-KillerRed plasmids were generated from a vector derived from pUAST [33]. Plasmids were injected into embryos and standard procedures were used to generate transgenic flies.

*Collection of larvae for live imaging*

For RNAi experiments in da neurons, RNAi lines were crossed to a UAS-EB1-GFP; UAS-Dicer2, elav-Gal4 line and embryos were collected for 24 hours at 25ºC on food caps, and allowed to age at 25ºC for 72 hours before live imaging. For double APC mutants, embryos were collected for 24 hours at 25ºC on apple juice caps and allowed to age at 25ºC for 24 hours. Larvae were then prescreened for expression of EB1-GFP under a fluorescence-dissecting microscope, and transferred to a food cap. Larvae were aged for 48 more hours at 25ºC before live imaging. Thus, third instar
larvae were imaged between 96 and 120 hours after egg laying.

Live Imaging Assays

Individual larvae were mounted on agarose dried to microscope slides. A cover slip was taped down to hold larvae in place. Live imaging was conducted on either a Zeiss LSM 510 confocal microscope or an Olympus FV1000 confocal microscope. The dynamics of EB1-GFP were assayed at one frame every two seconds as an indication of microtubule array polarity. An EB1-GFP comet must have been present for at least three consecutive frames for directionality to be assigned. For comets that met this criterion we manually determined whether it moved to or away from the cell body. In assays of non-Class I da neurons EB1-GFP comets traveling within all dendrites were assayed. For assays of Class I da neuron dendrites, only the comb-like dendrite of ddaE was used. Only EB1-GFP comets traveling in the dorsal trunk of this dendrite were scored for this assay as many of the peripheral branches are terminal, and are known to have differing microtubule orientation [9].

Degron Imaging Quantification

Quantification of elav-GAL4, R-DHFRts-HA-EGFP were collected on early third instar larvae. Larvae were grown at 25°C until imaging. 50-70 time frames were collected in 4th or 5th hemi-segments using Olympus FV1000 confocal microscope. After imaging larvae were carefully placed on room-temperature food caps and placed either at room temperature (21°C-23°C) for 1-hour and 30°C or 35°C for 1 hour or 24-hours. Larvae were then re-mounted and the same hemi-segment was re-imaged
for EGFP fluorescence. Conditions for re-imaging were the same as the first image. For homozygous lines, 488 laser was used at 10% power, in the heterozygous lines 488 laser was used at 20% and 545 laser was used at 10% power. Alexa fluor 488 and Rhodamine Red-X filters were used, respectively. To quantify GFP and RFP fluorescence before and after temperature conditions, Image J was used. 10 sequential frames, which had the most focus and least amount of movement, was created for a Z-stack at average intensity. In pre and post images a small area around the nucleus in the cell was measured for pixel intensity. To determine how much fluorescence was lost, the mean of pixel intensity for the post treatment was divided by the mean of pixel intensity for the pre-treatment. The percent of loss was averaged together from all larvae and is represented in the bar graphs of Figures 11 and 12.

*KillerRed Activation*

KillerRed construct was given to us by. It was injected into embryos and standard procedures were used to generate transgenic flies. UAS-KillerRed was crossed with 109(2)80-Gal4, UAS-EB1-GFP. Larvae were collected as stated before. Images were collected using an Olympus FV1000 confocal microscope and bleaching was conducted using the programs internal bleach program. To test for proper conditions, bleaching was conducted on EB1-GFP using 100% power of the 488 laser with settings from 10 seconds up to 2 minutes of exposure. In all trials bleaching was achieved. To bleach KillerRed 545 laser is used at 100% power. Trials
from 10 seconds up to 2 minutes have been conducted and fluorescence remains the same (data not shown).
References


