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Neurons are highly specialized cells that transmit information over long distances by way of polarized processes known as axons and dendrites. One of the major cell biological differences between axons and dendrites is the arrangement of microtubules. In Drosophila dendritic arborization (da) neurons, which are multipolar neurons with a single axon and many dendrites, dendritic microtubules are minus-end-out and axonal microtubules are plus-end-out (Stone 2008). While microtubule polarity is likely to play a key role in maintaining neuronal polarity, it is not known how a single cell can establish two compartments with completely different arrangements of microtubules.

In a screen to identify regulators of microtubule polarity in da neurons, we identified kinesin-13s, which are known to be important for spindle organization in mitosis, as regulators of neuronal microtubules. Kinesin-13s are a family of non-canonical microtubule-based motors that are known to be microtubule depolymerases in mitotic and interphase cells (Mennella 2005); however, there have been very few studies of their roles in neurons.

In Drosophila, there are three kinesin-13s. We found that two of these, Klp59C and Klp10A, play important roles in da neurons. Reduction of Klp59C caused severe mixing of microtubule polarity in dendrites as well as dendrite branching defects. No non-microtubule functions were observed. Reduction of Klp10A had a comparably mild effect in dendrites, and instead showed the greatest phenotype in axons, changing both microtubule orientation and overall number of polymerizing microtubule plus ends.

We therefore conclude that Klp59C and Klp10A act differentially on dendritic and axonal microtubules; Klp59C is a major regulator of dendritic microtubules and Klp10A is a major regulator of axonal microtubules. Their localized function may be one mechanism that allows neurons to establish different arrangements of microtubules within axons and dendrites.

Interestingly, neurons were observed to be especially sensitive to early developmental loss of Klp59C. With genomic deletion of the kinesin-13, microtubule polarity is restored but the number of dynamic plus ends are disrupted. We suggest that, upon loss of Klp59C, neurons use a kinesin-13 independent “bypass pathway” to maintain normal polarized microtubule growth. JNK signaling may play a key role in this bypass pathway. Additional factors, such as microtubule minus end regulators, may also play a role. In the future, we seek to elucidate the molecular players that help to control microtubule polarity in the kinesin-13 bypass pathway.
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Chapter 1. INTRODUCTION

Neurons are specialized cells that rely on a unique shape for proper function. These cells are responsible for receiving, transmitting, and interpreting information from across the body, communicating with both other neurons and tissues such as muscle. While there are a variety of neuronal shapes, multipolar neurons are among the most commonly studied. Multipolar neurons receive information through highly branched and tapered dendrites. Multipolar neurons send information via a single long, thin process called an axon.

In order to efficiently communicate and maintain cell homeostasis at distant cell edges, neurons, in part, use polarized Microtubule (MT) arrays. MTs are hollow, circular filaments made up of α and β tubulin dimers. Ends of MTs are dynamic and highly regulated in vivo. MT plus ends tend to be sites of rapid depolymerization/growth and the target of a large group of factors called +TIPs (Zhu 2009). EB1/EB3 is considered to be a core +TIP, responsible for recruiting many proteins to the plus end (Sayas 2014). MT minus ends are usually more stable through association with capping proteins such as γTubulin and Patronin, which prevent depolymerization (Wiese 2000, Hendershott 2014).

MTs form long, dynamic tracks in both dendrites and axons. As shown in Figure 1, polarity of MT tracks in axons is set up early in development with >95% MT plus ends away from the cell body, referred to as “plus end out”, a property conserved from mammals to C. elegans (Rolls 2011). Axonal MTs tend to be fairly stable, although dynamic plus ends are observed. Dendritic MT polarity is established later in development (Hill 2012). In in vivo Drosophila and C. elegans systems, MTs in dendrites resolve to >90% MT plus ends towards the cell body (Figure 1), referred to as “plus end in” (Stone 2008, Goodwin 2012). In cultured (in vitro) mammalian hippocampal cells, MT polarity in dendrites remains mixed (50% plus end in)
(Baas 1988). However, due to microscopy and model constraints, it is unknown if MT polarity resolves to plus end in in mammalian dendrites \textit{in vivo}. In all systems, dendritic MTs are much more dynamic than those in the axon (Kollins 2009).

Two families of motor proteins, dyneins and kinesins, move down axonal and dendritic MTs in an ATP-dependent manner, transporting organelles, vesicles, and other factors produced in the cell body. In axons, dyneins are responsible for axon-cell body retrograde transport as the motor protein moves towards MT minus ends (Zheng 2008). Kinesins move towards the plus ends, creating anterograde transport from the cell body into the axon (Lodish 2008). In the Drosophila neurons, where dendrites are mostly plus end in, kinesin and dynein roles are reversed: Dynein establishes anterograde transport and kinesin establishes retrograde transport (Lodish 2008). It is hypothesized that highly polarized MT arrays, and resultant motor protein movement, help neurons efficiently sort cargoes into either axons and/or dendrites and maintain compartment specificity (Horton 2003). It also has been shown that disruption of Dynein causes a change in MT polarity (Zheng 2008), suggesting that the activity of motor proteins and regulation of MT polarity are intertwined.

To test if kinesins had an effect on neuronal MT polarity and/or neuronal development, we performed a candidate RNAi screen in the Drosophila model system. Drosophila is an excellent system in which to answer basic cellular biology questions in part due to the availability of genetic tools such as genome-wide RNAi libraries, UAS controlled expression constructs, and a variety of cell-type specific-Gal4 promoters. Drosophila larvae are also advantageous because they are translucent, permitting live imaging of fluorescent proteins in whole animals \textit{(in vivo)}. Neuron types are well characterized in Drosophila and studies in the lab
focus on the dda cluster of sensory neurons, multipolar neurons that lie under the outer cuticle in larvae.

Results from our initial knockdown screen are described in further detail in Chapter 2.1. Two of the most intriguing candidates, Klp10A and Klp59C, belong to the kinesin-13 family. Results were somewhat surprising; these non-canonical kinesins are primarily known to be important for correct chromosome segregation in mitosis (Cross 2014). Based on our novel findings, we sought to further explore the role of Klp59C and Klp10A, within a neuronal context. The kinesin-13s are an interesting family of motor proteins. Unlike most kinesins, the motor domain lies between the neck and microtubule-binding domains (Moores 2006) (Figure 2A, B). However, motor function still relies upon ATP. Kinesin-13s use the motor domain to catalyze MT depolymerization, peeling back MT filaments and dissociating α,β tubulin subunits.

As shown in Figure 2C, the kinesin-13 family is conserved from mammals to C. elegans (Walczak 2013). In Drosophila, there are three well-studied kinesin-13 family members: Klp10A, Klp59C, and Klp59D. Klp59D has not been found to affect MT dynamics (Sharp 2005). There is also a fair amount of literature on the three members of the mammalian kinesin-13 family: Kif2a, Kif2b, and Kif2c/MCAK. Based on alignment and phylogeny studies, the Drosophila kinesin-13 family members are more related to each other than to any mammalian kinesin-13 (Dagenbach 2004) (Figure 2C). However, Klp59C and MCAK have been suggested to be orthologs due to similarities in function (Wordeman 2007).

The kinesin-13 family has been found to be important for regulating MT polarity in both mitotic and interphase cells. In mitotic systems, Klp10A has been found to associate with MT minus ends at centromeres, creating chromatid poleward flux (Rogers 2004). Conversely, Klp59C associates with the MT plus end at the kinetochore, moving chromatin outward via a
“Pac-Man” mechanism (Rogers 2004). Together, the two kinesin-13s help pull chromatids, through MT depolymerization, away from the midline of the cell (Figure 3A). In S2 interphase cells (in vitro), Klp59C and Klp10a have been shown to regulate MT polarity differently (Mennella 2005). In interphase, depletion of Klp10a causes a reduction in MT catastrophe events suggesting Klp10A is necessary for initiating depolymerization. In contrast, Klp59C knockdown causes an increase in the frequency of MT rescue with no change of number of catastrophe events as compared to the control. This suggests that Klp59C is primarily responsible for sustained MT depolymerization. Echoing these results, Klp10A and Klp59C GFP/RFP constructs have been found to localize to the MT plus ends (Figure 3B) (Mannella 2005). Klp59C however, also binds along the length of the MT, potentially enabling long-term MT depolymerization (Tan 2006).

While the activity of the kinesin-13s has been well established in in vitro systems, there remains a dearth of information about their activity in in vivo interphase cells, especially for specialized cell types such as neurons. Currently, there are only two published peer-reviewed articles that examine kinesin-13 protein function in neurons. Homma et al, suggest mammalian Kif2a is important during axonal development in cultured hippocampal cells, regulating axonal branching (Homma 2003). Santama et al, suggest that mammalian Kif2b is important for lysosomal trafficking (Santama 1998). However, both articles do not assay for any effects the kinesin-13s may have on MT dynamics.

We sought to understand what role the kinesin-13 family is playing in in vivo neurons, an interphase cell type that establishes and maintains highly polarized MT arrays. We report that Klp10A and Klp59C are important regulators of neuronal MT dynamics, most likely acting as microtubule depolymerases. Knockdown produces non-overlapping phenotypes, suggesting that
Klp10A and Klp59C are regulating MT dynamics differently. Loss of Klp59C mainly affects MT dynamics in the dendrites. Interestingly, neurons seem to be extremely sensitive to the timing and/or amount of Klp59C depletion. Klp10A knockdown causes changes in MT dynamics in both the axonal and dendritic compartments. Differing phenotypes for the depolymerases may be due to compartment-specific regulation.

Through our findings we suggest that the Klp10A and Klp59C are critical regulators of neuronal development and homeostasis, functioning to regulate MT dynamics in a compartment-specific manner. We further suggest that loss of kinesin-13 activity activates kinesin-13 bypass pathway, which functions to restore MT polarity.
Chapter 2. RESULTS

2.1 Kinesin-13 knockdown alters neuronal MT dynamics

We initially sought to determine which, if any, kinesin motor proteins had an affect on MT dynamics. We performed a candidate RNAi screen, specifically targeting kinesin motors and assaying for changes in MT dynamics. GFP-tagged EB1 and kinesin RNAi (or control, pav) constructs were expressed under the control of a 221-Gal4 promoter, which expresses specifically in the ddaE and ddaD neurons of the dda cluster. As a +TIP, UAS-EB1-GFP is used to visualize polymerizing MT plus tips. MT minus ends were assumed to follow behind UAS-EB1-GFP-labelled plus tips (referred to as EB1 “comets”). EB1 comet polarity was analyzed within the main dendritic branch of the ddaE neuron.

In the initial RNAi knockdown screen of kinesins, only four showed a change in EB1 comet polarity: Klp64D, Unc-104, Klpl0A, and Klpl59C (data not shown). More information about Klp64D and Unc-104’s effects on neuronal MT polarity can be found at the following citations: Mattie 2010, Chen 2012.

Encouraged by the initial screen, we sought to fully characterize the effects of kinesin-13 loss within Drosophila sensory neurons. We hypothesized that if the kinesin-13s were MT depolymerases, located primarily at the plus end as previously suggested (Mannella 2005), neurons may employ the kinesin-13s to control aberrant plus end growth. Furthermore, there may be two ways in which the kinesin-13s may be used control plus end dynamics. First, the kinesin-13s may regulate the number of polymerizing plus ends, maintaining a ratio of long, stable MT to short, dynamic MTs. It has previously been shown that stable MTs are important for axonal specification (Chen 2013) and suggested that both stable and dynamic MTs may be
important to neuronal function (Dent 2014). Second, the kinesin-13s may control the polarity of MT plus ends, depolymerizing wrong-way nucleation events. It was previously demonstrated neuronal MT nucleation is non-centrosomal and γTubulin (located at dendritic branch points, the cell body, and axon termini) is primarily responsible for new MT growth (Nguyen 2014). Kinesin-13s may control the polarity of these non-centrosomal nucleation events, maintaining the directionality of neuronal MT arrays. The possible activities of kinesin-13s are not exclusionary.

In order to elucidate the effect of kinesin-13s on neuronal MT dynamics, we expressed UAS-Dicer2, UAS-EB1-GFP, and UAS-RNAi constructs under the control of the 221-Gal4 driver. The 221 promoter drives UAS-transgene within the ddaE and ddaD neurons. Dicer2 is expressed in order to drive RNAi hairpin cleavage and target mRNA degradation. In this experiment and following RNAi experiments, Rtnl2 (VDRC 33320) was used as a negative control. Rtnl2 is an ER associated protein with no known effect on MT plus end dynamics in dda sensory neurons (Mattie 2010). MT plus end dynamics were tracked in both axons and dendrites. Only the primary dendritic comb of the dendrites (past at least the first branch point up from the cell body) and the proximal region of the axon (at least 10µm from the cell body, but no more than 100µm) were analyzed.

In agreement with the initial screen, changes in EB1 comet dynamics were observed with reduction of Klp59C and Klp10A. No discernable phenotypes were observed for Klp59D knockdown. As shown in Figure 4A, in Klp59C RNAi (VDRC 48576) knockdown animals, MT polarity was mixed in dendrites with 66% of EB1 comets moving in toward the cell body. This polarity defect is significant as compared to the Rtnl2 control, which had 91% of EB1 comets heading in towards the cell body. No change in EB1 comet number was observed (Figure 4C).
However, “stuck” EB1 comets were observed in Klp59C knockdown cells (Figure 4F). Small, stagnant accumulations of EB1 were observed in both the axon and the dendrites. These “stuck” comets are strange as EB1 is a very kinetically active protein, quickly dissociating and binding to MTs (Zhu 2009). Although we are not able to fully examine what these dots are, they may the product of one of two possible options. First, it may be possible the Klp59C knockdown is causing dissociation of EB1 from the MT, creating small, free accumulations of the protein. Secondly, the loss of Klp59C may completely block MT depolymerization creating a stagnant MT ends, which are actively bound by EB1. At this time we are unable to discern which of these two possibilities is more likely.

Surprisingly, no effect was seen with use of an alternate Klp59C RNAi (VDRC 109829) (data not shown). However RNAi constructs do not always produce effective hairpins, limiting knockdown efficiency and creating false negative phenotypes.

To test the efficiency of the VDRC 48576 RNAi we extracted brains from larvae expressing the target and control constructs under the control of an elav promoter (a pan neuronal driver expressed within brain neurons). We then isolated RNA and created cDNA via a reverse transcriptase (RT) reaction. We used a primer set from within and outside (Primer Set 1 and Primer Set 2, respectively) the described hairpin region, as shown in Figure 5A. Using Primer Set 1, 48576 showed almost complete knockdown of Klp59C as compared to the control (Figure 5B). Primer Set 2 showed an increase in signal as compared to the control (Figure 5B). Although surprising, BioRad, a biotechnology company that produces qPCR machines and reagents, previously published similar results (Van Maerken 2007). BioRad determined that RNAi knockdown specifically targets the hairpin region. Regions outside the hairpin show higher levels of amplification in qPCR experiments indicating an increase in relative abundance.
Our results suggest that VDRC 48576 RNAi hairpin is on-target and the phenotype is caused by almost complete knockdown of Klp59C expression.

To look for knockdown effects for Klp10A we used two different RNAi constructs, VDRC 41534 and Bloomington 33963. Phenotypes from both RNAi constructs were consistent, suggesting that the phenotypes observed were a true positive and a result of on-target knockdown of Klp10A expression.

As illustrated in Figure 4B, Klp10A knockdown causes a change in MT polarity in the axon with 35% (VDRC 41534) and 19% (VDRC 33963) of EB1 comets moving in towards the cell body. Both were significant as compared to the Rtnl2 control, which only had 2% of EB1 comets moving in towards the cell body. Klp10A knockdown additionally produced an increase in EB1 comet number in the axon (Figure 4D). Klp10A knockdown cells had an average of 9 EB1 comets pass through a 10 µm region over 300 seconds. This was significant in comparison to the Rtnl2 control, which has an average of 4 EB1 comets pass through a 10 µm region in 300 seconds.

Klp10A knockdown phenotypes were milder in the dendrites. Only the VDRC 41534 RNAi construct significantly changed MT polarity in dendrites, with 86% of EB1 comets moving in towards the cell body (Figure 4A). Only VDRC 33963 showed a significant increase in the number of MT comets in dendrites, with 7 EB1 comets moving through a 10 µm region over 300 seconds (as compared to ~5 in the Rtnl2 control) (Figure 4C).

In order to fully capture dendritic MT phenotypes, we assayed MT polarity within ddaC neurons after kinesin-13 knockdown. ddaC neurons are a complex, highly branched neuron within the dda cluster of sensory neurons. It has been shown that MT polarity in ddaC neurons is
more consistent than in ddaE, possibly due to steep branch angles that partially correct for defects in kinesin 2/APC mediated MT steering at branch points (Mattie 2010).

Under control of a ddaC specific, 477-Gal4 promoter we expressed UAS-Dicer2, UAS-EB1-GFP, and UAS-RNAi constructs. We housed larvae at 30°C, the optimum temperature for the bacterial Gal4-UAS system, in order to drive transgene expression and complete RNAi knockdown. Within ddaC neurons only Klp59C caused a change in MT polarity, with 78% of EB1 comets moving in towards the cell body as compared to 97% in the Rtnl2 control (Figure 4E). This result suggests that, as in ddaE neurons, Klp59C functions to control MT polarity in dendrites.

When considered together, it is apparent that Klp10A and Klp59C knockdown phenotypes are significant yet dissimilar. Klp59C knockdown causes a reversal of MT polarity in the dendrites, but no change in comet number or axonal MT dynamics. Loss of Klp10A results in an accumulation of axonal phenotypes, altering MT polarity and the number of MT plus ends. Dendritic phenotypes in Klp10A knockdown cells was comparatively mild.

Klp59D knockdown was not observed to alter MT dynamics, suggesting that it does not play a major role in controlling MT dynamics. Therefore the following experiments will focus mainly on elucidating the role of the kinesin-13s, Klp10A and Klp59C.

2.2 Kinesin-13 knockdown alters neuronal dendritic morphology

We hypothesized that a change in the underlying MT dynamics in dendrites, caused by Klp10A or Klp59C RNAi-mediated knockdown, may cause a change in overall dendrite structure and/or outgrowth. dda sensory neurons are an optimal model in which to survey for branching defects, as each cell type has a well characterized branching pattern. ddaE neurons
establish a relatively simple branch structure early in larval life (Grueber 2002). As demonstrated by the control in Figure 6A, These neurons are characterized by a main dendrite comb extending from the cell body with secondary branches extending out at approximately right angles. While ddaE neurons expand with larval growth, dendrite branching remains the same and no novel outgrowth events occur.

In order to visualize ddaE dendrites, we expressed UAS-mCD8-RFP, UAS-EB1-GFP under control of the 221-Gal4 promoter. mCD8 is a well-characterized membrane marker that labels the entire neuron. As observed with MT dynamics, loss of Klp10A and Klp59C created significant, yet dissimilar, phenotypes in dendrite branching. Klp59C knockdown creates the most pronounced effect, causing a variety of notable phenotypes including short secondary branches, bent backbones, increased proximal branching, and clipped dendrites (Figure 6B). Due to the diversity of the dendritic phenotypes observed with loss of Klp59C, phenotypes other than branch length were not formally quantified. Klp10A knockdown caused a milder change in dendrite phenotypes including curled branches and bent backbones (Figure 6C). 30% to 50% of Klp10A knockdown cells exhibited no change in dendritic phenotype, as demonstrated in Figure 6C. Due to this inconsistency, phenotypes were not quantified. We expect that dendritic phenotypes in Klp10A knockdown cells are not significant as compared to the control.

In an attempt to capture any overall change in dendrite length caused by Klp59C knockdown, we measured the growth of the secondary dendrites over larval life. In order to characterize individual cell growth individual ddaE neurons were consistently followed over a total of 96 hours (imaged every 24 hours). Neurons that underwent dendrite clipping were excluded from the assay. Klp59C knockdown cells had significantly shorter dendrites at 24, 48, and 72 hours than the Rtnl2 control (Figure 6D). The defect in dendrite length is especially
noticeable at 72 hours, when control cell dendrites have reached their maximum length (*Figure 6D*). With loss of Klp59C, dendrites still grow an average of 247.59 µm between 72 and 96 hours. However, dendrites in Klp59C knockdown neurons eventually reach an average size that is similar to control cells.

The severity of dendritic phenotypes observed parallel the changes in MT dynamics caused by loss of Klp59C and Klp10A. Klp59C knockdown, which causes a dramatic change in dendritic MT polarity, produced a diverse set of dendritic phenotypes including clipping and significantly slowed growth. Klp10A knockdown, which only exhibits a small change in dendritic MT polarity, causes a much milder set of dendritic phenotypes, and does not seem to affect outgrowth (not formally measured).

### 2.3 Kinesin-13 localization within neurons

Due to the dendritic and axonal phenotypes created by Klp59C and Klp10A knockdown, we sought to characterize where the MT regulators localized within neurons. We hypothesized that the both kinesin-13s would localize to the axonal and dendritic compartments. While we report that Klp59C knockdown causes dendritic phenotypes, concurrent studies in the lab have determined that Klp59C is responsible for fast MT depolymerization in axons following injury (Tao unpublished). Therefore we expect Klp59C may also be present axon, but compartmentally silenced in the axons of uninjured cells. Loss of Klp10A causes both axonal and dendritic phenotypes suggesting that it is in both compartments.

We first tried to visualize Klp59C and Klp10A localization via GFP or YFP-tagged constructs. UAS-Klp10A-GFP was obtained from Bloomington Stock Center (Indiana University) while UAS-Klp59C-YFP was made in the lab. Both constructs were easily visualized
in the muscle under a muscle-specific Gal4 driver (*data not shown*). However, only Klp59C was able to be visualized under control of the 221-Gal4 promoter (*Figure 7A*). We suggest that this may indicate that neurons are especially sensitive to Klp10A over-expression, driving down expression of the GFP tagged construct in order to maintain normal MT dynamics. Klp59C-YFP localized to the axon, cell body, and dendrites (*Figure 7A*). Klp59C was observed along the length of the axon (*Figure 7A*). In dendrites, Klp59C seemed to localize as puncta along the main branch and in branch points.

We next sought to characterize endogenous kinesin-13 localization using immunofluorescence. Dr. David Sharp created and tested antibodies for Klp10A and Klp59C in order to examine kinesin-13 activity in interphase S2 cells (Mennella 2005). Unfortunately, the antibody reactive for Klp59C has been lost. There are no other known antibodies for the Drosophila kinesin-13 family.

Using the anti-Klp10A antibody (supplied by Dr. Sharp) we sought to characterize the localization of Klp10A within neurons. Wild-type larval fillet preps were fixed and then stained with anti-Klp10A followed by a green fluorophore-conjugated secondary antibody. Staining revealed that neurons had large concentrations of Klp10A in the cell bodies and axons (*Figure 7B*). Muscle and epithelial tissues also showed high levels of Klp10A, creating a large amount of background signal (*Figure 7B*). No Klp10A staining was discernable in the dendrites. However, dendrites are fine processes that are not bundled as neuronal cell bodies and axons are. It is possible that the high levels of background signal in surrounding tissues may mask Klp10A staining in dendrites.

In order to discern the placement of the dendrites, UAS-EB1-GFP was expressed under the control of the 221-Gal4 promoter. EB1-GFP was used to label sensory neuron clusters and
visualize dendrites in ddaE, ddaD, and ddaC cells. Filet preps were fixed and stained with anti-Klp10A. Anti-Klp10A was labeled via a red secondary antibody. To reduce bleed-through signal, green and red channels were captured alternately and then merged for analysis. Staining again revealed high levels of Klp10A in the neuronal cell body, axonal bundle, and surrounding tissues (Figure 7C). However, a low level of Klp10A staining was visible within dendrites (Figure 7C).

Localization results for Klp59C and Klp10A seem to fit well with previous functional studies. Based on Klp59C-YFP, we suggest that Klp59C localizes to both neuronal dendrites and axons (Figure 7A). This result complements our findings that Klp59C regulates MT dynamics in axons and dendrites in injured and non-injured cells, respectively. Results from endogenous Klp10A staining suggest that the protein localizes to neuronal axons, dendrites, and cell body; although levels of Klp10A are much lower in the dendrites. These results complement results from earlier studies that demonstrate that loss of Klp10A causes only mild dendritic phenotypes yet a large disruption in axonal MT dynamics (Figure 4).

2.4 Kinesin-13s control the level of stable MTs within neurons

While it is apparent that Klp59C and Klp10A are regulators of neuronal MTs, the mechanism by which kinesin-13 act remained unresolved. In both mitotic and interphase in vitro systems, the kinesin-13s have been found to act as MT depolymerases (Rogers 2004, Mannella 2005). Only a single previous publication has suggested that kinesin-13s may have an alternate function trafficking lysosomes (Santama 1998). Since the function of the kinesin-13s within neurons in vivo has not been directly examined, we sought to characterize their mechanism of activity. We hypothesized that if the kinesin-13s are acting as MT depolymerases in in vivo
neurons, then loss of Klp59C or Klp10A may cause an increase in the amount of stable MTs within the cell.

To determine if kinesin-13s affected levels of stable MTs, we fixed larval fillets expressing UAS-RNAi under the control of the 477-Gal4 promoter (Figure 8B). We then stained larvae preps using an anti-acetylated tubulin antibody. Acetylated tubulin is commonly associated with stable MTs. Although the acetyl group itself is not stabilizing, the modification helps to recruit stabilizing MAPs to the MT (Palazzo 2003, Tala 2014). Primary antibody was counterstained using a red-fluorophore-conjugated secondary. Since acetylated tubulin is enriched in neurons, dda clusters were easily distinguished from background staining (Figure 8B).

In order to account for staining efficiency, staining intensities in RNAi expressing ddaC cells were normalized to non-RNAi-expressing ddaE cells from within the same cluster, as outlined in Figure 8A. In Klp10A knockdown cells we observed a non-significant increase in stable MTs within the cell body as compared to the Rntl2 control (Figure 8C). Klp59C knockdown cells showed a significant decrease in stable MTs within the cell body (Figure 8C) but an increase in stable MTs within the dendrites (Figure 8D) as compared to the Rtnl2 control. Levels of stable MTs within dendrites of Klp10A knockdown cells have not been assayed at this time. We were unable to test for changes the level of stable MT in the axons. ddaE and ddaC axons, along with the axons of the entire dda group of sensory neurons, are bundled making individual measurements impossible.

To rule out the possibility that Klp59C is responsible for lysosomal trafficking, live larvae fillets, expressing UAS-RNAi under the control of the 221-Gal4 promoter, were stained with Lysotracker Red. Sold by Life Technologies, Lysotracker Red consists of a fluorophore
conjugated to a weak base. At neutral pH, the probe can easily permeate live cells and is selective for acidic organelles, such as lysosomes (Life Technologies 2013). Within acidic compartments the fluorophore is active, creating a strong signal without the use of a secondary antibody (Wang 2012) (Figure 9A).

As a kinesin, Klp59C would be expected to move towards the MT plus ends carrying any cargo towards the cell body in dendrites and out of the cell body towards axonal termini. However, because Klp59C knockdown causes MT polarity mixing in the dendrites, directional transport by both dyneins and kinesins may be broadly disrupted. This may make any possible defects in organelle trafficking upon loss of Klp59C difficult to decipher. Axonal MT polarity in Klp59C knockdown cells is unaffected, presumably leaving normal directional transport intact. We hypothesize that if Klp59C is responsible for moving lysosomes towards the MT plus end, knockdown would cause an accumulation of lysosomes in the cell body (near axonal MT minus ends). Staining in Klp59C knockdown cells revealed that the number of lysosomes within the cell body, an average of about 4 lysosomes per cell body, was consistent with the Rtnl2 control (Figure 9B).

Results suggest that the Klp59C controls the amount of stable MTs in the cell body and dendrites while Klp10A controls the level of stable MTs within the cell body. We were unfortunately unable to determine the effect that either Klp10A or Klp59C had on axonal MT stability. Loss of Klp10A causes an increase in stable MTs within the cell body. Klp59C knockdown caused both an increase in dendritic MT stability and a decrease in MT stability within the cell body. Lysosomal trafficking is unaffected upon loss of Klp59C.

2.5 Klp59C knockout affects MT dynamics in an unexpected manner
RNAi is a powerful tool that allows for cell-specific knockdown of key regulators of cell dynamics. However, there are drawbacks. First, RNAi constructs may not produce effective hairpins, causing incomplete target mRNA degradation and false negative phenotypes. Secondly, RNAi expression is controlled by a cell-specific promoter. While this allows for knockdown of essential proteins, it means that hairpin expression is temporally limited. By the time RNAi expression is turns on, there may already be substantial amount of target protein within the cell. RNAi knockdown is then limited by the rate of target protein turnover, which may also lead to false negative phenotypes. However, as demonstrated in Figure 5B, expression of VDRC 48576 Klp59C RNAi almost completely knocks out Klp59C mRNA expression in developed neurons. We hypothesized that a genomic deletion of Klp59C would create a phenotype similar to that of the RNAi.

We obtained a Klp59C deletion mutant from Dr. Oren Schuldiner. The mutant line was generated as part of a piggyBac-based instertional mutation screen, meant to isolate genes important for axonal pruning (Schuldiner 2008). In the screen, the authors used a modified piggyBac construct, which did not rely on P-element insertion sites. The construct was transposed into the genome with high efficiency, disrupting over 2000 genes. One of the identified mutant lines deleted the genomic region containing both Klp59C and Klp59D (Schuldiner unpublished). We validated the deletion mutant using qPCR. As shown in Figure 5C, homozygous mutants demonstrate an almost complete loss of Klp59C mRNA suggesting that the deletion is on-target. Interestingly, homozygous Klp59C deletion flies are able to live into adulthood but are sterile.

To characterize the effects of whole animal Klp59C depletion within neurons, we expressed UAS-EB1-GFP under the control of a 221-Gal4 promoter in a Klp59C deletion mutant
or control background. As seen in Figure 10A, heterozygous Klp59C deletion (Klp59C Het) causes a significant change in MT polarity within dendrites, with 89% of EB1 comets moving in toward the cell body as compared to 94% in the Yellow-White (YW) control. Klp59C Het also produced a significant increase in the number of EB1 dots in dendrites. An average of 8 EB1 comets moved through a 10 µm region over 300 seconds in Klp59C Hets (compared to an average of 5 EB1 comets moving through a 10 µm region over 300 seconds in the YW control) (Figure 10C). No changes in MT dynamics were observed within the axons of Klp59C Het animals (Figure 10B, D).

Complete loss of Klp59C in homozygous Klp59C deletion (Klp59C Mutant) animals causes no significant change in dendritic or axonal MT polarity, as compared to the control (Figure 10A). However, EB1 comet number is significantly changed within dendrites. Klp59C Mut dendrites had an average of 7 EB1 comets move through a 10 µm region over 300 seconds (Figure 10C). Axonal EB1 comet number is unaffected in Klp59C Mut cells (Figure 10B, D).

Results from the Klp59C deletion mutant were unexpected. Both Klp59C Het and Klp59C Mut cells show a significant change in MT comet number in dendrites, a phenotype not observed in Klp59C RNAi expressing cells. While changes in MT polarity were consistent between Klp59C RNAi knockdown and Klp59C Het cells, MT polarity returned to normal in homozygous deletion flies. However, no axonal phenotypes were observed; neither Klp59C knockdown or knockout caused a change in axonal MT dynamics. Results from deletion animals suggest that Klp59C is an important regulator of dendritic MT dynamics within uninjured neurons, regulating the number of MT plus ends within dendrites.

2.6 Klp10A does not control rescue of MT dynamics in Klp59C knockout
We predicted that kinesin-13 family member Klp10A may compensate for the loss of Klp59C in deletion mutants. As Klp10A and Klp59C has been previously suggested to have a similar mechanism of MT depolymerization during interphase (Menella 2005), Klp10A may be up-regulated in response to loss of Klp59C early in development. From this hypothesis we would expect that loss of Klp59C may cause an increase in Klp10A expression. Levels of Klp10A would be expected to drastically increase within dendrites, where Klp59C is important for regulating cell dynamics.

To characterize any changes in Klp10A levels, RNA was isolated from whole larvae. Control, Klp59C Het, and Klp59C Mut animals were used. Klp59C and Klp10A were amplified via qPCR. Levels of Klp59C were analyzed to verify larval genotypes. Results matched previous studies (Figure 5C) with levels of Klp59C RNA partially and almost completely depleted in heterozygous and homozygous animals, respectively. Levels of Klp10A also changed in Klp59C Het and Mut larvae (Figure 11A). Upon heterozygous loss of Klp59C, the expression of Klp10A RNA dropped to about half of what was observed in the control. Interestingly, the level of Klp10A did not drop further with homozygous loss of Klp59C as compared to the Klp59C Het.

To ensure the change in Klp10A expression was not cell-type specific, we characterized the levels of Klp10A within Klp59C Het and Mut neurons. Larval fillet preps expressing UAS-EB1-GFP under control of a 221-Gal4 promoter, were fixed and then stained with anti-Klp10A followed by a red fluorophore conjugated secondary antibody. Staining revealed that neurons had continuous concentrations of Klp10A in the cell bodies and axons. Klp10A staining in dendrites, beyond the first branch point, was not visible. These results closely match previous findings (Figure 7C). Fluorescence intensities within the ddaE cell body, proximal axon, and
proximal main dendritic comb were recorded and normalized to background (staining within surrounding epithelial tissue).

Results in the cell body were consistent with results from the qPCR; Klp10A levels are significantly reduced in Klp59C Het and Mut, as compared to the control (Figure 11B). The level of Klp10A remains unchanged between heterozygous and homozygous loss of Klp59C. Surprisingly, within the proximal axon, levels of Klp10A remain unchanged between control, Klp59C Het, and Klp59C Mut (Figure 11D). Within the proximal dendrite, the level of Klp10A was only significantly changed from the control in Klp59C Het neurons (Figure 11C).

While whole animal and neuronal-specific results differ, data from both assays demonstrate that Klp10A is not up-regulated in order to compensate for Klp59C knockout. However, there is some feedback between the level and regulation of the two kinesin-13s. In the majority of tissues and neuronal cell bodies, heterozygous or homozygous knockdown of Klp59C leads to about a 50% loss of Klp10A. It is interesting to note that the levels of Klp10A in the axon are unaffected by Klp59C knockdown. Differences in Klp10A expression within dendrites and axons of Klp59C Het and Mut animals suggest that expression of Klp10A is tightly regulated in a compartment-specific manner within neurons.

In order to further test if Klp10A was responsible for controlling MT dynamics following Klp59C knockdown, we sought to characterize the effect of simultaneous Klp10A and Klp59C knockdown. We expressed five transgenes, including UAS-Dicer2, UAS-EB1-GFP, and UAS-RNAi under the control of the 221-Gal4 driver. In order to control for the dual expression of the Klp59C and Klp10A RNAi constructs, we additionally expressed UAS-mCD8-RFP in single Klp10A and Klp59C knockdown larvae. MT plus end dynamics were tracked in both axons and dendrites.
As shown in Figure 12A, in dual Klp10A, Klp59C knockdown neurons, MT polarity was only slightly mixed in dendrites with 92% of EB1 comets moving in toward the cell body. This polarity defect is significant as compared to the single Klp59C knockdown (80% of EB1 comets heading in towards the cell body) but not the single Klp10A knockdown (90% of EB1 comets heading in towards the cell body). In axons, MT polarity was mixed with 13% of EB1 comets moving in toward the cell body (Figure 12B). As in the dendrites, the change in MT polarity is significant as compared to the single Klp59C knockdown (2% of EB1 comets heading in towards the cell body) but not the single Klp10A knockdown (19% of EB1 comets heading in towards the cell body).

Dual Klp10A, Klp59C knockdown causes a significant change in the number of EB1 comets in the dendrites with an average of 4 EB1 comets passing through a 10 µm region over 300 seconds, as compared the individual Klp10A and Klp59C knockdown (Figure 12C). Single Klp59C knockdown cells have an average of 5 EB1 comets passing through a 10 µm dendritic region over 300 seconds. In single Klp10A knockdown neuron dendrites, an average of 7 EB1 comets pass through a 10 µm region in 300 seconds. No changes in EB1 comet number were observed in the axons of Klp59C, Klp10A knockdown cells, as compared to single Klp59C or Klp10A knockdown (Figure 12D).

Results for dual Klp59C, Klp10A knockdown are somewhat difficult to interpret. Results for MT polarity seem to suggest that dual kinesin-13 knockdown phenocopies Klp10A RNAi cells, establishing Klp10A as an upstream regulator of Klp59C (Figure 12A, B). However, the decrease in dendritic EB1 comet number observed in dual kinesin-13 knockdown cells, a significant change from both Klp10A and Klp59C (Figure 12C), negates this possibility. In addition, dendritic EB1 comet number is increased in both Klp59C Het and Mut animals.
However, the level of Klp10A staining is lowest in Klp59C Het animals’ dendrites, as compared to control and Klp59C mut animals. This suggests that the rise in EB1 comet number with Klp59C loss is not correlated with the level of Klp10A within dendrites.

When considered with Klp10A staining results, it can be concluded that the level of Klp59C expression affects Klp10A expression. However, we suggest Klp10A is not primarily responsible for correcting dendritic MT dynamics following Klp59C knockdown.

2.7 JNK signaling is activated following Klp59C loss.

Although studies in the lab have not elucidated all the factors that work to control neuronal MT dynamics, we have extensively studied a variety of pathways that are important for establishing and regulating MT arrays. One of these pathways includes JNK-dependent regulation of MT dynamics following injury.

Previous studies in the lab have found that JNK is an important regulator of the neuronal injury response (Chen 2012). Although JNK (Drosophila Bsk) is not a direct regulator of MT dynamics, it is responsible for up-regulating a microtubule based neuroprotective program in neurons following axonal injury. Following proximal axotomy, MT spawning is drastically increased via the activity of γTubulin 23C (Chen 2012). Dendrite outgrowth following axotomy is dependent on increased MT comet number (Chen 2012). After injury, EB1 comet number in dendrites increases to an average of 7 EB1 comets over 10 µM in 200 seconds, from an average of 4 EB1 comets over 10 µM in 200 seconds in uninjured cells.

Although not as severe, Klp59C Mut animals also have an increase in EB1 comet number within dendrites. This up-regulation in EB1 comet number may be caused by a change in JNK-
regulated γTubulin 23C activity, as seen after injury. We hypothesize that JNK-dependent MT
dynamics may also be used to help restore MT polarity as outlined in Figure 13A.

To test for activation of the JNK pathway in uninjured, Klp59C knockdown cells we
expressed Puckered-GFP, under control of its endogenous promoter, concomitantly with UAS-
mCD8-RFP, UAS-RNAi under control of the 221-Gal4 promoter. Puckered (puc) is a JNK
phosphatase, activated by JNK signaling through the activity of the transcription factor Jun
(Tanigutchi 2007). Studies in the lab have determined that activation of the JNK pathway causes
puc-GFP expression within the neuron nucleus (Stone 2014). To assay for a change in puc
expression, GFP signal within the nucleus of ddaE is recorded and then normalized to
background signal.

In Klp59C RNAi-mediated knockdown cells, levels of puc-GFP expression were
increased 2 fold as compared to the γTubulin 37C control (Figure 13B). This significant increase
over control suggests that JNK signaling is activated within Klp59C knockdown cells as outlined
in the pathway in Figure 13A.

To determine if JNK was important for maintaining MT polarity following Klp59C
knockdown, we sought to characterize the effect of Klp59C knockdown in a mutant Bsk
background. We expressed five transgenes, including UAS-BskDN, UAS-Dicer2, UAS-EB1-
GFP, and UAS-RNAi under the control of the 221-Gal4 driver. BskDN is a dominant negative
mutant previously shown to obstruct the JNK signaling pathway and prevent the up-regulation of
MT dynamics (Chen 2012). In order to control for the dual expression of the Klp59C RNAi and
BskDN, we additionally expressed UAS-mCD8-RFP in single Klp59C RNAi expressing larvae.
Changes in dendritic and axonal MT polarity were recorded.
In co-expressing Klp59C RNAi, BskDN neurons, MT polarity was significantly mixed in dendrites with 56% of EB1 comets moving in toward the cell body. This polarity defect is significant as compared to the single Klp59C knockdown (79% of EB1 comets heading in towards the cell body) (Figure 13C). In axons, no change in MT polarity was observed (data not shown). Previous findings in the lab have determined that BskDN does not cause a change in dendritic MT polarity within the primary dendrite of uninjured cells as compared to a wild type control (Stone 2010) (data not shown). These results suggest that active JNK signaling helps to control MT polarity following Klp59C knockdown.

Although not previously suggested to interact, results indicate that the JNK signaling pathway, formerly characterized to regulate MT dynamics following injury, is activated in a Klp59C-dependent manner in uninjured cells. Knockdown of Klp59C causes an up-regulation of JNK signaling, as indicated by an increase in puc-GFP (Figure 13B). This suggests that JNK signaling is active following the loss of Klp59C activity. Knockdown of both Klp59C and JNK signaling significantly affects MT polarity within dendrites but not axonal MT polarity (Figure 13C). This suggests that JNK signaling may play an important role in preserving at least partial MT polarity in the dendrites following Klp59C knockdown.
Chapter 3. DISCUSSION

Previously published studies characterize kinesin-13s as MT depolymerases, facilitating MT end bending and dissociating α,βTubulin subunits (Tan 2006). In mitosis, Klp10A and Klp59C have been shown to bind opposite sides of the MTs, which make up the mitotic spindle (Figure 1). Depolymerization from both ends of the MT enables correct chromosome separation and segregation to the centrosome (Rogers 2004). In Interphase, Klp10A and Klp59C maintain correct MT dynamics by promoting depolymerization (Mannella 2005) (Figure 3B). While Klp59C has been reported to coat the length of MTs (Tan 2006), both kinesin-13s have been demonstrated to promote depolymerization from the MT plus end (Moores 2006). However, the bulk of previous studies were performed in in vitro, non-differentiated cells.

We sought to characterize the role of the kinesin-13s in neurons, a differentiated cell type that maintains highly polarized MT arrays (Stone 2008). In a literature search, we found that all previous literature examined the role of kinesin-13s in vitro (Homma 2003, Santama 1998). In order to study the neuronal function of the kinesin-13s within a live, in vivo system, we used model system Drosophila melanogaster. Along with the wide variety of genetic tools available for use in the system, D. Melanogaster larvae are transparent, facilitating the visualization of fluorescently tagged proteins within a whole animal.

In an initial phenotypic assay, the expression of each kinesin-13 was individually knocked down via cell-specific RNA hairpin expression (Figure 4). Neuron types examined included the ddaE and ddaC neurons of the da sensory bundle, which lie just under the cuticle. In order to determine if the kinesin-13s change MT plus tip growth, a GFP-tagged EB1 was co-expressed under control of the same promoter. Interestingly, Klp59C and Klp10A were observed to independently control MT dynamics in a compartment specific manner. Klp59C knockdown
created a 60% polarity reversal within dendrites, but had no effect on the number of MT plus ends or axonal MT dynamics (Figure 4A-D). Klp10A knockdown creates about a 30% reversal of MT polarity in axons; it also significantly increased the number of MT plus ends in axons (Figure 4B,D). In dendrites, the effect of Klp10A knockdown was milder, with VDRC 41534 altering MT polarity and VDRC 33963 altering the number of MT plus ends (Figure 4A,C). Klp59D was observed to have no effect and was omitted from following experiments.

We suggest that differences in Klp59C and Klp10A knockdown phenotypes indicate that the two kinesin-13 family members are regulated or function separately within neurons. Klp59C seems to only be active within dendrites, controlling MT polarity but not the number of polymerizing plus ends. Most of Klp10A’s activity seems to be focused within the axon, controlling both MT polarity and the number of MT plus ends. However, Klp10A does have a mild effect on dendritic MT dynamics. We hypothesize this independent, compartment-specific activity may be controlled through two different mechanisms, specific localization of each kinesin-13 or compartment specific regulation of activity by a third party factor. These two possibilities are not exclusive. We test this hypothesis in Chapter 2.3, with discussion to follow.

Although not formally linked, correct regulation of the MT cytoskeleton may be important for establishing proper dendrite outgrowth and branching (Homma 2003). We hypothesized that dendritic phenotypes may be an important readout of any changes in underlying MT dynamics. To test this prediction, we examined kinesin-13 knockdown animals co-expressing a mCD8-RFP membrane marker, which clearly labeled dendrites. Klp59C, which was observed to have the strongest effect on MT polarity in dendrites, also causes the strongest dendritic phenotypes (Figure 6B). In addition, Klp59C knockdown significantly slows dendrite outgrowth (Figure 6D). Klp10A knockdown, which was only observed to cause mild changes in
dendritic MT dynamics, produces mild dendritic phenotypes, although not all neurons tested were observed to have any observable change from the control (Rtnl2) (Figure 6C). Loss of Klp10A also does not seem to change the rate of dendrite outgrowth, although this has not been quantified.

Results establish Klp59C as an important regulator of dendrite development and maintenance. Klp59C knockdown is seen to strongly affect both dendritic development and the underlying polarity of MT arrays. This effect is pronounced as compared to Klp10A knockdown dendrite phenotypes. This evidence reinforces the hypothesis that Klp10A and Klp59C differentially affect neuronal development and maintenance. We additionally suggest that changes in dendrite phenotype are caused by changes in MT regulation, establishing a link between cell morphology and the maintenance of supporting MT arrays. Data suggests that normal dendritic growth and structure may rely on the correct regulation of MT polarity, carried out mainly by Klp59C.

We next sought to test our earlier hypothesis, in which we suggested that Klp10A and Klp59C may achieve compartment specific phenotypes through discrete localization to either the dendritic or axonal compartment. Alternatively, kinesin-13 localization may be continuous and individual activity determined by a currently unknown third party.

We first used fluorophore labeled constructs to characterize kinesin-13 localization. Klp59C-YFP could be seen within dendrites, the cell body, and axon (Figure 7A). This localization matches well with current functional data. While this report presents evidence that Klp59C is important for controlling MT dynamics in dendrites (Figure 4A,C), concurrent research in the lab has found that Klp59C is necessary for axonal MT depolymerization following axon severing (Tao, unpublished). A lack of axonal phenotype in non-injured cells
may suggest that Klp59C is differentially regulated in the dendritic and axonal compartments.

A GFP-tagged Klp10A construct could be visualized in muscle tissue but not in neurons (data not shown). Based on this initial observation, we suggest that Klp10A levels may be tightly controlled in neurons. Over-expression of Klp10A may be inhibited at either the transcriptional or translational level, preventing visualization of the GFP-tagged construct.

In order to visualize endogenous populations of Klp10A, we stained larvae preps using an anti-Klp10A antibody developed by Dr. David Sharp (Mennella 2005). Within neurons, the highest level of staining was found within cell bodies and axons (Figure 7C). Lower levels of Klp10A staining were observed within dendrites (Figure 7C). This suggests Klp10A is mainly localized to outside the dendrites. This conclusion matches earlier phenotypic data well; Klp10A is observed to primarily control MT dynamics in axons in cells (Figure 4A-D), only producing mild dendritic phenotypes (Figure 6C). Results suggest that neurons control Klp10A activity by mainly focusing Klp10A localization to the cell body and axon where it functions to control MT dynamics.

Klp59C and Klp10A localization results suggest that neurons control the kinesin-13s’ activity through different, possibly independent, mechanisms. In uninjured cells, we suggest axonal Klp59C is post-translationally inactivated while dendritic Klp59C is active and acts as a key regulator of MT dynamics. At this time, it is not known what third party factor may be responsible for silencing axonal Klp59C activity. Conversely, we suggest that compartment-specific Klp10A activity is determined by discrete localization. The majority of Klp10A was observed to localize to the neuronal axon and cell body (Figure 7B), where Klp10A has also
been observed to have the largest functional impact. At this time, the mechanism behind the specific localization of Klp10A remains unknown.

Although initial knockdown phenotypes show Klp10A and Klp59C to be important regulators of neuronal MT dynamics, it remained unknown by what mechanism the kinesin-13s regulated MT dynamics. We hypothesized that, as seen in mitotic and interphase in vitro systems, the kinesin-13s controlled MT dynamics through depolymerization (Rogers 2004, Mannella 2005). However, we also had to consider the possibility that Klp59C was a key player in neuronal lysosomal transport, as suggested in Santama 2008.

First, we sought to determine if the kinesin-13s acted as depolymerases within in vivo neurons. We hypothesized that loss of their depolymerase activity, mediated by RNAi knockdown, would result in an increase in stable MTs. We additionally hypothesized that the change in stable MTs within each compartment would be dependent on the presence on the kinesin-13s; for example, Klp10A is abundant through the cell body and axon, therefore the abundance of stable MTs would be expected to increase within the cell body and axons of Klp10A knockdown cells as compared to wild type.

To test this hypothesis, we stained larvae preps using an anti-acetylated Tubulin antibody. Acetylated Tubulin is considered to be a hallmark of stable MTs (Palazzo 2003). To account for sample variability, signal from each RNAi expressing ddaC cell was normalized both to background signal and to the non-expressing ddaE cell from within the same da cluster. Methods for normalization are detailed in Figure 8A. Unfortunately, we were unable to assay for changes in axonal staining. The axons of da neurons are bundled, making individual signal measurement impossible.
In Klp10A knockdown cells, levels of stable MTs were increased within the cell body, although not significantly (Figure 8C). This result may suggest that Klp10A is acting as a depolymerase in the cell body, controlling MT dynamics and the number of stable MTs. However, further testing may be needed to strengthen these conclusions.

In Klp59C knockdown cells, levels of stable MTs were significantly increased within dendrites (Figure 8D). Loss of Klp59C also caused a decrease in the level of stable MTs within the cell body (Figure 8C). Although unexpected, these results suggest that Klp59C is an important regulator of MT homeostasis within multiple neuronal compartments of un-injured neurons. Klp59C may function as a depolymerase in dendrites, controlling MT polarity and the level of stable MTs. The effect of Klp59C knockdown upon stable MTs in the cell body may suggest two possibilities. First, Klp59C mediated regulation of dendritic MTs may be important for maintaining normal polarized transport into and out of the dendrite. Klp59C knockdown may cause a disruption in cell body homeostasis and a loss of MT stability. Second, loss of Klp59C may cause up-regulation of a currently unknown regulator of MT growth/stability. Upregulation of this currently unknown third party may cause a change in MT dynamics that also affects the cell body. These two possibilities are not exclusive. At this time we have only begun to look into the second hypothesis, searching for a possible MT regulator and/or bypass pathway that is up-regulated in response to Klp59C depletion. We term this possible pathway the “kinesin-13 bypass pathway”. Preliminary results are described in Chapter 2.6 and Chapter 2.7 with discussion to follow.

As hypothesized, localization of activity fits well with the localization of both kinesin-13s; Klp59C localizes to and was observed to impact the level of stable MTs in both the cell body and dendrites (Figure 8C,D). Klp10A localizes to and was seen to affect the level of stable
MTs in the cell body (Figure 8C). However, it is interesting to note that the two kinesin-13s have the opposite affect upon stable MTs within the cell body. This discrepancy further suggests that Klp10A and Klp59C are regulated and/or function independently within neurons.

Based on the 1998 peer-reviewed publication “KIF2beta, a New Kinesin Superfamily Protein in Non-Neuronal Cells, In Associated with Lysosomes and May be Implicated in Their Centrifugal Translocation”, by Santama et al., we sought to determine if Klp59C functions as a lysosomal transporter. The article’s proposed mechanism of action of Klp59C was possible, although unlikely. While Santama et al suggest that Klp59C localizes to LAMP-1 coated organelles (Santama 1998), Klp59C’s motor domain has been previously shown to be non-processive (Moores 2006) and specialized to disassembling MTs (Sharp 2005). Additionally, kinesin-13 family members have been previously suggested to move to the MT plus end via diffusion (Tan 2006). We suggest that is unlikely that neurons transport lysosomes via diffusion. The transport of many large organelles in neurons has been shown to be mediated by the step-wise action of processive MT motors, including dynein or other kinesin family members (Dent 2014). Building on this previously published data, we hypothesized that Klp59C plays no direct role in neuronal lysosomal transport.

We stained for live, dissected animals with Lysotracker, a red fluorescent marker that that is activated by the low pH within lysosomes (Figure 9A). The number of lysosomes within the cell body was then counted. We reasoned that, in Klp59C knockdown cells, axonal MT growth is unaffected. This suggests axonal MT minus ends are situated toward the cell body as in wild type. Therefore, if Klp59C is responsible for lysosomal transport, knockdown should cause lysosomes originally destined for the axon to accumulate within the cell body. However, upon
knockdown of Klp59C, there was no observed increase of lysosomes within the cell body as compared to wild type (Figure 9B).

Based on these results, we suggest that Klp59C does not play a role in lysosomal transport within neurons. Instead, we believe Klp59C to function mainly as a depolymerase in uninjured neurons, controlling the level of stable MTs and MT plus end growth within dendrites. This neuronal role closely matches the function previously suggested in mitotic and interphase in vivo systems (Sharp 2005).

In order to fully characterize the role of Klp59C in neurons, we turned to a deletion mutant. A limitation of RNAi knockdown is that hairpin production is limited both temporally and in efficiency. Hairpin production is determined by the promoter that it is expressed under, either 221 or 477 for this report. Both 221 and 477 are promoters activated somewhat late in development, after cell fate has been determined (Grueber 2002). This may allow for wild type-like primary establishment of cellular structures, such as MT arrays, and the establishment of compartment identity. After activation, RNAi is only able to prevent the production of new mRNA/protein. RNAi targeting can also suffer from low target efficiency. However, we previously demonstrated that Klp59C mRNA levels are drastically reduced in RNAi hairpin expressing cells (Figure 5B). Even with high knockdown efficiency, hairpin expression may only cause a slow run-down of Klp59C protein levels, especially if the protein has an extended half-life. Due to the relative scarcity of neurons in larvae, we were unable to assay for lingering Klp59C protein levels in neurons following RNAi knockdown. However, any level of residual Klp59C protein may muddle observed phenotypes.

To circumvent any artifacts created by RNAi targeting, we used a Klp59C/D deletion mutant (Schuldiner 2008). We first noted that heterozygous deletion animals are viable and able
to produce offspring. Homozygous deletion animals are able to live until adulthood but are sterile. This suggests that gamete production and possibly meiosis, is Klp59C dependent. The survival of homozygous deletion animals through adulthood suggests that somatic cell mitosis and interphase are able to progress in a Klp59C-independent manner. This observation is surprising given that Klp59C has previously been shown to be an important regulator of mitotic division in vitro(). One possibility is that Klp59C may be maternally loaded and facilitates early embryo or larval development. At this time, we have not determined if Klp59C is a maternally contributed protein.

We sought to determine how a genetic deletion knockout of Klp59C affected neuronal MT dynamics. Based on our previous findings, we hypothesized that Klp59C knockout animals would present mainly dendritic phenotypes, with an increase in mixed MT polarity. EB1-GFP was expressed in a Klp59C Het, Klp59C Mut, or wild type background under a 221 promoter as described in Chapter 2.1, and EB1 comet polarity and number was counted. In heterozygous deletion animals, dendrite EB1 comet polarity was significantly changed from wild type (Figure 10A). No change was observed in axons (Figure 10B). This result closely fit our hypothesis; comet polarity is mixed in Klp59C RNAi animals (Figure 4A). Surprisingly, Klp59C Het neurons had a significant increase in dendritic EB1 comet number as compared to the control (Figure 10C). Klp59C RNAi had not previously been observed to have an effect on EB1 comet number (Figure 4C).

MT dynamics observed in Klp59C Mut animals were completely unexpected. In homozygous deletion animals, MT polarity was unchanged from that of the control in both dendrites (Figure 10A). This lack of phenotype is a sharp contrast to both Klp59C RNAi (Figure 4A) and Klp59C Het animals (Figure 10A), which showed a significant change in dendritic MT
polarity. Klp59C Mut animals also had an increase in EB1 comets in dendrites. This phenotype matches results seen in Klp59C Het animals (*Figure 10C*), but is dissimilar to Klp59C RNAi results (*Figure 4C*). The sole similarity between Klp59C Mut, Klp59C Het, and Klp59C RNAi was a lack of any observed axonal phenotypes as compared to wild type.

Results primarily confirm that Klp59C does not play a role in regulating axonal MT dynamics in uninjured neurons. Even upon full genetic knockout of Klp59C, axonal MT plus end number and directionality remained unchanged from wild type. Interestingly, this was the only consistent phenotype between RNAi knockdown and heterozygous or homozygous deletion animals. Results additionally suggest that neurons are sensitive to both the temporal depletion of and level of Klp59C. Developmentally early loss of Klp59C, with either one or two copies of the deletion mutation, causes an increase in MT plus end number within dendrites (*Figure 10C*). The level of protein depletion, via RNAi mediated knockdown or number of knockout alleles, affects MT polarity in dendrites. We note that level and timing of Klp59C expression may have a compounding effect, creating a more severe phenotypes than either parameter alone.

Developmentally early, complete knockout of Klp59C may force the neuron to control MT polarity through a completely independent pathway or currently unknown third party. We discuss this hypothesis in more detail below. Lastly, results confirm that Klp59C is important for regulating dendritic MT dynamics in uninjured neurons.

Differences observed when comparing the effects of RNAi-mediated Klp59C knockdown and homozygous Klp59C deletion suggest two possible explanations. First, the VDRC 48576 Klp59C RNAi has off-target effects, knocking down mRNA expression of an unknown regulator of MT polarity. To test this hypothesis we are currently performing genetics, generating fly lines that will allow UAS-Klp59C RNAi expression within a Klp59C Mut background. If Klp59C
Mut, Klp59C RNAi expressing neurons show a change in dendritic MT polarity, then the phenotype observed for VDRC 49576 may be an artifact of off-target effects. The second possibility is that cells are temporally sensitive to the loss of Klp59C; early developmental loss causes the activation of a kinesin-13 bypass pathway that controls MT dynamics independently of Klp59C. This hypothesis is closely aligned with the prediction that levels of stable MTs in the cell body are reduced in Klp59C knockdown cells due to the activation of an unknown regulator of MT dynamics, termed the “kinesin-13 bypass pathway”, within the cell body. All remaining studies, described in Chapter 2.6 and 2.7, focused on elucidating a possible kinesin-13 bypass pathway, which may control MT dynamics in neurons following loss of Klp59C.

We first investigated the possibility that Klp10A, a fellow member of the kinesin-13 family, was responsible for regulating dendritic MT dynamics following loss of Klp59C. Although our previous research suggests that Klp10A was primarily responsible for regulating axonal MT dynamics, we hypothesized that the depolymerase be relocated to or be activated within dendrites following Klp59C deletion knockout. If true, we would expect that Klp10A levels would increase following heterozygous or homozygous deletion of Klp59C.

In order to test this hypothesis, we quantified the level of Klp10A expression in Klp59C Het or Mut animals. qPCR analysis of whole larvae extract and found that Klp10A levels decreased following genetic deletion of Klp59C. In both homozygous and heterozygous deletion animals, Klp10A expression was reduced to about half of that of wild type controls (Figure 11A). To verify that Klp10A expression was also reduced in a Klp59C dependent manner in neurons, we stained for Klp10A as previously described in Chapter 2.3. Within cell bodies, the level Klp10A was significantly reduced as compared the control (Figure 11B). However, within dendrites, the level of Klp10A was significantly reduced only in Klp59C Het animals. Klp10A
levels in Klp59C Mut animals were unchanged from the control *(Figure 11C)*. The level of Klp10A was not altered by the presence of absence of Klp59C *(Figure 11D)*.

qPCR and staining results suggest that Klp10A expression is not up-regulated in response to the absence of Klp59C. Contrary to our hypothesis, Klp10A levels in the whole animal and neuronal cell body are reduced following loss of Klp59C. Interestingly, Klp10A levels were not further depleted in homozygous animals as compared to heterozygous animals. Results additionally suggest that neurons tightly control protein expression in a manner independent of the whole animal. Neurons can additionally achieve compartment-specific regulation of protein localization and/or expression. The level of Klp10A in axons is independent of Klp59C expression *(Figure 11D)*. This observation suggests that Klp10A is tightly regulated and plays a key role within the axonal cell compartment. When considered with our previous results, we suggest that Klp10A is a critical regulator of MT dynamics within the axon. Within dendrites, the level of Klp10A expression is only reduced following partial loss of Klp59C. While this result suggests that dendrites also tightly control protein localization, it additionally suggests that Klp10A may not be as important for regulating dendritic MT dynamics. However, this fits well with previous results that demonstrate that Klp10A knockdown only has a mild effect on MT polarity and plus end number in dendrites.

These results suggest two possibilities. First, Klp10A is a negative regulator of Klp59C. Loss of Klp59C reduces the cell’s need for Klp10A. No previous literature can be found to substantiate this hypothesis. No direct interaction of Klp59C and Klp10A has been previously reported. Results from staining in the proximal dendrite additionally seem to contradict this first possibility. Additionally, if Klp10A was a negative regulator of Klp59C, we would expect the level of Klp10A expression to be low within dendrites following homozygous deletion of
Klp59C. Loss of Klp59C would reduce the cell’s need for its antagonist. With heterozygous loss of Klp59C, the level of Klp10A is reduced (Figure 11C). However, the level of Klp10A is unchanged from the control in Klp59C Mut. This suggests that the level of Klp10A within dendrites is not dependent on Klp59C expression and Klp10A is unlikely to be a key regulator of Klp59C activity. Second, Klp10A is not part of the kinesin-13 bypass pathway. Early loss of Klp59C activity causes the activation of the kinesin-13 bypass pathway, which controls MT dynamics in the cell body and dendrites. In support of this hypothesis, we observed that EB1 comet number in dendrites is increased in both Klp59C Het and Mut animals (Figure 10C). However, the level of Klp10A within the proximal dendrite of Klp59C Mut animals is similar to control (Figure 11C). This discrepancy between Klp10A levels and EB1 comet phenotypes suggests that Klp10A is not singularly responsible for controlling MT dynamics following Klp59C loss. Instead, we suggest that another factor, possibly the kinesin-13 bypass pathway, may be responsible. Activation of the kinesin-13 bypass pathway may also reduce the cell’s need for Klp10A.

In order to further investigate the possibility that Klp10A regulates Klp59C activity, we assayed MT dynamics within a double knockdown animal. We hypothesized that if Klp59C function was dependent on Klp10A, the two proteins were likely to control dendritic MT dynamics in a single pathway. Therefore, we could carry out a simple genetic analysis. If the two kinesins operated within the same pathway, MT polarity and plus end number in a double knockdown animal would be expected to phenocopy the one of the kinesins. However, if the two kinesins functioned separately within neurons, the phenotype would be additive and different than the knockdown of either kinesin individually.
We expressed Klp10A and Klp59C RNAi singularly and in combination and assayed EB1 comet polarity and number as described in Chapter 2.1. To control for transgene number, we additionally expressed a control protein (mCD8-RFP) as needed. In dendrites, dual Klp59C, Klp10A knockdown caused a significant decrease in comet number as compared to both single Klp59C and Klp10A knockdown animals (Figure 12C). Dendritic MT polarity was restored to over 90% plus end in Klp59C, Klp10A RNAi cells, which was significant as compared to single Klp59C RNAi (Figure 12A). Axonal EB1 polarity in dual kinesin-13 knockdown cells was also significantly changed as compared to Klp59C knockdown animals (Figure 12B).

At first glance, MT polarity results seem to indicate that double Klp59C, Klp10A RNAi neurons phenocopy single Klp10A knockdown. However, dendritic EB1 comet number in dual kinesin-13 knockdown cells is dissimilar from either single knockdown, suggesting that Klp59C and Klp10A do not function within the same pathway. Instead, we hypothesize that complete knockdown of Klp10A and Klp59C activates the kinesin-13 bypass pathway in dendrites, helping to restore MT polarity. MT polarity in double Klp59C, Klp10A RNAi dendrites closely matches the MT polarity reported in Klp59C Mut cells (Figure 10A). This similarity suggests that the restoration of normal dendritic MT polarity may be due to the use of the kinesin-13 bypass pathway. No changes in axonal MT dynamics were observed in Klp59C Mut animals (Figure 10B, D), suggesting that the bypass pathway may not affect the regulation of axonal MTs. As a result, axonal MT polarity may be similarly affected by the loss of Klp10A in single and double knockdown animals.

In an attempt to select new potential candidates for the kinesin-13 bypass pathway, we turned to previous research in the lab. Through extensive candidate RNAi screens the lab has
determined and characterized a variety of pathways that affect neuronal MT dynamics. These pathways include JNK-dependent regulation of MT dynamics following injury, kinesin 2-mediated MT steering, γTubulin and Patronin-mediated regulation of MT minus end stability and growth, and Katanin and Katanin 60-Like mediated MT severing. We hypothesized that a possible kinesin-13 bypass pathway may utilize one or more of these previously identified MT regulation pathways to help restore normal dendritic MT polarity following homozygous deletion of Klp59C.

We first examined the possibility that JNK signaling plays a role in the kinesin-13 bypass pathway. Previous studies in the lab demonstrated that, following axonal injury, an increase of dynamic MT plus ends is observed in both the cell body, dendrites, and axon (Stone 2010). This increase in MT dynamics is JNK dependent (Chen 2012). JNK signaling activates γTubulin 23C, a protein that promotes MT growth by protecting the minus end and acts as a platform for polymerization (Nguyen 2011). We noted that JNK-dependent increase of MT dynamics following injury was similar to the increase of EB1 comets observed in Klp59C homozygous deletion animals (Figure 10C). Based on these similarities, we hypothesized that JNK signaling may be activated following Klp59C loss, helping to regulate MT dynamics as part of the kinesin-13 bypass pathway.

To test for JNK signaling activation following loss of Klp59C, we first assayed for puc accumulation within the nucleus. Puc is a transcription factor previously found to be activated in a JNK dependent manner (Chen 2012). In order to accurately assay for an increase in JNK signaling, we used a GFP-tagged puc construct that exists under control of its endogenous promoter (Taniguchi 2007). In uninjured Klp59C RNAi knockdown neurons, we saw an about a
two fold increase of puc-GFP within the nucleus as compared to the control (Figure 13B). This suggests that JNK signaling is active following loss of Klp59C.

We next sought to determine if JNK plays a role in regulating MT polarity following Klp59C knockdown. Klp59C RNAi and EB1-GFP were expressed in a Bsk dominant negative (DN) background. Bsk is the Drosophila homolog of JNK (Stone 2010). Results were compared to cells expressing a control transgene in addition to Klp59C RNAi. Dendritic EB1 polarity was significantly more mixed in Klp59C knockdown, BskDN cells (55% plus end in) as compared to single Klp59C knockdown (78% plus end in) (Figure 13C). Single BskDN expression was previously reported to not cause a significant change in MT polarity in uninjured cells (Stone 2010). No significant change in EB1 polarity was observed in axons (data not shown). These results suggest that, following loss of Klp59C, JNK signaling acts to regulate dendritic MT dynamics in uninjured cells.

To our knowledge, Klp59C activity in uninjured cells has not previously been associated with JNK signaling. However, our results suggest that JNK may play an important role in the kinesin-13 bypass pathway. First, JNK signaling is activated following a knockdown of Klp59C. Secondly, we suggest that JNK may help to control MT polarity following loss of Klp59C. In co-Klp59C knockdown, BskDN MT polarity in dendrites is more severe than Klp59C knockdown alone (Figure 13C). This result suggests that JNK activation following Klp59C loss may help to preserve at least a basal level of MT directionality within dendrites. A lack of axonal phenotype suggests that Klp59C and JNK signaling do not play a role in regulating MT dynamics in the axons of uninjured cells. Considered together, results suggest that JNK may play a key role in the kinesin-13 bypass pathway. This JNK-dependent control of MT dynamics may be mediated through γTubulin 23C, as seen in injury. We have not yet investigated this hypothesis.
As described in Mattie 2010, Klp64D (kinesin-2) knockdown results in severe dendritic MT polarity phenotype, similar to that of Klp59C. Knockdown of Klp64D in ddaE neurons results in 48% of EB1 comets moving toward the cell body while knockdown of Klp59C exhibits 66% of EB1 comets moving toward the cell body (Mattie 2010). Although Klp64D and Klp59C have not been previously suggested to work together (kinesin-2 controls MT polarity by helping to correctly guide polymerizing MT ends through branch points) due to their similar MT polarity phenotypes, we tested the possibility that the two kinesins controlled MT polarity through the same pathway, as outlined in Figure 14A.

Using the 447-Gal4 promoter, we expressed UAS-Dicer2, UAS-EB1-GFP, and either the UAS-Klp64D RNAi or UAS-Klp59C RNAi constructs. At 25C, Klp64D or Klp59C do not cause a significant change in dendritic MT polarity as compared to the control (data not shown). An increase in the number of EB1 comets was observed in Klp59C knockdown dendrites, with an average of 6 EB1 comets passing through a 10µm region over 300 seconds (as compared to an average of 4 for the Rtnl2 control) (Figure 14C).

However, when the Klp59C and Klp64D RNAi constructs are expressed together, dendritic MT polarity is severely affected with 54% of EB1 comets moving toward the cell body (Figure 14B). Dual Klp59C/Klp64D knockdown cells also show a significant reversal in axonal MT polarity, with 14% of EB1 comets moving toward the cell body (Figure 14D). In addition, dual Klp59C/Klp64D knockdown cells show a significant increase in the number of EB1 comets in both dendrites and axons as compared to the control (Figure 14C,E). The axonal phenotypes observed in the dual knockdown cells were surprising, as individual knockdown of either kinesin does not change MT dynamics in the axon. However, the severity of the MT polarity phenotype
within both dendrites and axons following knockdown of both kinesins suggests that Klp59C and Klp64D are controlling MT polarity through different pathways.

As described in Nguyen 2014, γTubulin 23C is important for establishing golgi-independent, non-centrosomal MT nucleation events throughout the neuron. This factor is thought to provide a platform for new MT growth and also stabilize MT minus ends, protecting them from depolymerization (Merdes 2007). Patronin is also important for regulation of the MT minus end dynamics. Patronin has been suggested to protect the minus end from kinesin-13 mediated depolymerization and regulate minus end dynamics (Hendershott 2014, Goodwin 2010). Although results demonstrate that loss of kinesin-13 activity causes a change in MT plus end dynamics, we hypothesized that the dynamics of minus ends may also be affected. As regulators of MT minus ends, γTubulin 23C and/or Patronin may be important for balancing MT plus end and minus end dynamics, especially following kinesin-13 depletion.

Interestingly, γTubulin 23C<sup>bmps1</sup>, a suggested overactive variant (Nguyen 2014), causes a similar change in MT polarity in axons and dendrites as VDRC 41534 Klp10A knockdown (Figure 15C, Figure 4A,B). γTubulin 23C<sup>bmps1</sup> additionally causes an increase in MT “spawning” (the number of new MT polymerization events) (data not shown). The number of MT spawning events may be directly linked to the number of EB1 comets. More spawning creates an increase in dynamic MT ends, and thus EB1 comets. In γTubulin 23C<sup>bmps1</sup> neurons, rates of spawning are increased 2.5 to 4 times as compared to the control (Nguyen 2014). A significant increase in MT comet number is also observed in dendrites in VDRC 33963 knockdown neurons (Figure 4C). These similarities may indicate that Klp10A may be regulating the MTs that result from γTubulin-mediated MT nucleation events, possibly within both dendritic and axonal compartments.
Similar to Klp59C, RNAi mediated knockdown of Patronin also produces a severe dendritic phenotype. Using the 221-Gal4 promoter, we expressed UAS-Dicer2, UAS-EB1-GFP, and UAS- RNAi constructs as before. As shown in Figure 14B, Patronin RNAi (VDRC 36695) causes a significant change in dendritic MT polarity as compared to the control. Patronin dendrites had 50% of EB1 comets moving in towards the cell body. 84% of EB1 comets moved in toward the cell body within dendrites of the control RNAi, γTubulin 37C (VDRC 25271). γTubulin 37C is a maternally loaded protein that has been shown to have no effect on neuronal MT dynamics in larvae (Nguyen 2011). The Patronin dendritic phenotype is similar to what was observed in Klp59C knockdown cells (Figure 4A). These results suggest that Patronin is an important regulator of MT dynamics within dendrites. Similarities between Klp59C and Patronin knockdown, may additionally suggest that Klp59C may act with Patronin to regulate dendritic MT polarity.

Although we have not tested for direct interaction between the kinesin-13s and MT minus end regulators, preliminary results are intriguing. Similarities between Klp10A and γTubulin 23C phenotypes in both the axon and dendrite compartments suggest that they might be acting in the same pathway to control neuronal MT dynamics. Similarities between Klp59C and Patronin phenotypes suggest that these two factors may be acting together to control MT polarity in the dendrites. These possible pathways are outlined in Figure 15A. At this time, we cannot conclude if the MT minus end regulators, γTubulin 23C and Patronin, compensate for the complete loss of kinesin-13 activity (in Klp59C Mut and/or double Klp10A, Klp59C RNAi knockdown animals). However, we hypothesize that these MT minus end proteins may be important for regulating MT dynamics upon loss of kinesin-13 activity, potentially playing an important role in the kinesin-13 bypass pathway.
The last group of factors we hypothesize may be playing a role in the kinesin-13 bypass pathway are the MT severing proteins. P60-Katanin, a MT severing protein, has been previously found to be important for regulating MT dynamics in *in vitro* neuronal and Interphase systems (Yu 2008, Ghosh 2012). Within *in vivo* ddaC sensory neurons, Katanin P60-Like has been shown to affect MT dynamics, reducing EB1 comet number and regulating ddaC dendrite outgrowth/complexity (Stewart 2012). At this time, we have not assayed for a change in MT polarity in ddaE in Katanin P60-Like knockdown cells. However, we intend to pursue the hypothesis that Katanin-mediated MT severing may play a role in controlling MT polarity and possibly help control dendritic MT polarity in the kinesin-13 bypass pathway.

Based on initial results, we hypothesize that neurons have a kinesin-13 bypass pathway. Additionally, this bypass pathway may involve a number of factors, which have been previously characterized to control neuronal MT dynamics. JNK signaling, activated by loss of Klp59C (*Figure 13*), may act to correct dendritic MT polarity and cause an up-regulation of EB1 comets in kinesin-13 depleted cells (Klp59C Mut and Klp10A, Klp59C knockdown). Patronin and/or γTubulin 23C may also play an important role in the kinesin-13 bypass pathway. Although we have not yet examined the MT minus end regulators within the context of the bypass pathway, Patronin and γTubulin 23C may be working with Klp59C and Klp10A to control MT dynamics (*Figure 15*). We hypothesize that loss of kinesin-13 activity at MT plus ends may be balanced by a change in MT minus end regulation. In the kinesin-13 bypass pathway, Patronin and/or γTubulin 23C may help to restore MT polarity through an up-regulation of new MT growth or stabilization of the minus end. These two possible activities are not exclusionary. We further hypothesize that Katanin P60-Like, a MT severing protein that has been found to control EB1 comet number in ddaC neurons, also may contribute to restoring dendritic MT polarity in the
kinesin-13 bypass pathway. Although initial results for the kinesin-13 bypass pathway are exciting, further studies must be done to fully characterize the pathway and its critical players.
Chapter 4. MATERIALS AND METHODS

4.1 Drosophila Stocks

All Gal4 driver lines, UAS-BskDN, Klp10A RNAi 33963, and fluorescently tagged mCD8 and Klp10A lines were provided by the Bloomington Drosophila Stock Center (Bloomington, IN). All other UAS-RNAi lines were provided by the Vienna Drosophila Stock Center (Vienna, Austria). Klp59C/D Deletion lines were obtained from Dr. Oren Schuldiner (Weizmann Institute of Science, Rehovot, Israel) and re-balanced over CyO, Actin-GFP. The generation of UAS-EB1-GFP lines were previously described (Rolls 2007). The puc-GFP reporter line (G00462) was obtained from the FlyTrap Project (http://flytrap.med.yale.edu/) and was rebalanced over Tm6.

4.2 Generation of UAS-Klp59C-YFP transgenic fly lines

The full-length coding sequence of Klp59C was placed into fly vector pUAST::3YNT. The DNA template was a gift from Dr. Lai (The Pennsylvania State University, University Park, PA). Correct gene insertion into the plasmid was verified via DNA sequencing (Nucleic Acid Facility, The Pennsylvania State University). The verified plasmid was then sent to BestGene Inc. (Chino Hills, CA) for injection and the generation of stable fly lines. Resultant transgenic lines were balanced over CyO.

4.3 Analysis of EB1 comet dynamics

The polarity of EB1 comets was assessed as previously described (Nguyen 2011). Live third-instar larvae were imaged using a Zeiss Imager.M2 AxioVision wide-field microscope (Carl Zeiss, Jena, Germany). Larvae were live imaged over 300 seconds at a 1 second frame rate. The stage was moved as necessary to keep the neuron in focus for the length of the video. Videos were analyzed using Image J (a NIH open source image processing software, http://imagej.nih.gov/ij/). In dendrites, the directionality of all visible dots within the main trunk was recorded. In axons, the directionality of all visible dots within the axon, at least 10µm from the cell body, was recorded. To calculate the percentage of EB1 comets towards the cell body, we divided the total number of dots traveling in by the total number of EB1 comets. The number of EB1 comets was assessed using the same videos. In dendrites and axons, the number of EB1 comets traveling through a 10µm region was recorded. The number of EB1 comets per video was averaged for each neuronal compartment and genotype.

4.4 Analysis of mRNA levels via qPCR

Larval brains or whole larvae were frozen at -80°C. RNA was extracted using Qiagen RNeasy Plus Mini Kit, per the manufacturer’s instructions. A reverse transcriptase reaction was performed using Quanta Biosciences qScript cDNA Super Mix, per the manufacturer’s instructions. cDNA was amplified using QuantBio PerfeCTa SYBR Green Super Mix, in a 96-well format using a Applied Biosystems StepOnePlus Real-Time PCR System. Thresholds were manually set for each sample set. At least three biological replicates were used for each sample set. Ct values were averaged for each sample set. ΔCt for each sample set was calculated using the following equation, ΔCt=Ct(target)−Ct(endogenous control), where Rpl32 was used as the endogenous
control. Sample ΔCt values were normalized using the following equation, relative fluorescence=\(2^{-(\Delta Ct_{control}-\Delta Ct_{sample})}\) where YW was used as the control.

### 4.5 Analysis of dendrite morphology and outgrowth

mCD8-RFP expressing ddaE neurons of live third-instar larvae were imaged in vivo. An Olympus FluoView FV1000 confocal microscope was used to capture the morphology of the proximal axon, cell body, and entire dendrite comb. A time series was taken that consisted of at least 200 stacks. During the time series the stage was moved incrementally in an attempt to capture the entire dendrite comb. Appropriate stacks were merged to create composite images. Dendrite length was measured using the Image J plug-in, NeuronJ (created by Dr. Erik Meijering, http://www.imagescience.org/meijering/software/neuronj/). Each 24 hours, the lengths of all secondary dendrites were measured. Measurements were then combined to calculate total dendrite length. Average dendrite length was compared.

### 4.6 Larval Immunostaining

Third-instar larvae were prepared and stained as described in Nguyen 2011. An Olympus FluoView FV1000 confocal microscope was used to image stained fillet preps. Image stacks consisted of at least 200 slices. Relevant slices were stacked in Image J to create composite images. The following primary antibodies were used: rabbit anti-Klp10A (a gift from Dr. David Sharp, Albert Einstein College of Medicine, Bronx, New York) and mouse monoclonal anti-acetylated Tubulin (Sigma-Aldrich, T6793). Secondary fluorescent antibodies were procured from Jackson ImmunoResearch.

### 4.7 Lysotracker Red Staining

Fillet preps of third-instar larvae were prepared as described in Nguyen 2011. However, larvae were kept live and not fixed using 4%PFA. Any larvae fillet preps displaying necrotized tissue were discarded. Larvae were stained using Lysotracker Red DND 99, supplied by the Life Technologies. Following dissection, larvae were washed in Schneider’s Media to remove debris. Larvae were incubated in a 50nM solution of Lysotracker, covered, for 5 minutes. Larvae were subsequently washed twice with saline. A Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany) was used to live-image stained fillet preps. Image stacks consisted of at least 200 slices. Relevant slices were stacked in Image J to create composite images. Stained lysosomes in cell bodies were counted and an average was calculated.

### 4.7 Analysis of Puckered expression

Puckered expression was assessed as previously described (Stone 2014). Live third-instar larvae were imaged using an Olympus FluoView FV1000 confocal microscope. Live larvae were imaged, capturing the entire cell body. Relevant slices were then merged and images were analyzed via Image J. Florescence of Puc-GFP was measured in ddaE neurons. Change in GFP expression was calculating by creating a ratio of ddaE to background signal.
Figure 1. MT Orientation in Multipolar Sensory Neurons.
Neurons within the dda cluster of Drosophila Sensory Neurons are Bipolar neurons with a single thin axonal process and many tapered dendrites. MTs in axons are >95% “plus end out” with dynamic plus ends polymerizing away from the cell body. MTs in dendrites are >90% “plus end in”, with dynamic plus end polymerizing towards the cell body.
Figure 2. Kinesin-13 structure and phylogeny

As shown in A, the kinesin-13s consist of a non-canonical family of kinesins with an internal motor domain. Although not processive, kinesin-13s bind to MTs using MT binding domains at the N-Terminus. The internal motor domain, responsible for MT depolymerization, functions in an ATP dependent manner. Kinesin-13 activity additionally depends on dimerization, facilitated by a dimerization domain at the C-terminus. This is compared to the conical kinesin shown in B. Homodimeric kinesin-2 is a processive motor protein that binds MTs via the motor domain, situated at the N-Terminus (Scholey 2013). The ATP-dependent motor domains progress towards the MT plus end in stepwise manner. A flexible coiled-coil domain links the C-Terminus. At the C-Term, kinesin-2 binds its cargo via cargo binding domains (Scholey 2013). The structure/function of kinesin-13s is well conserved between plants (A. thaliana), protozoa (P. falciparum), and both vertebrate (H. sapiens, as indicated) and non-vertebrate (D. melanogaster, as indicated) animals (C). Phylogeny tree thanks to the Department of Cell Biology at Duke University (http://labs.cellbio.duke.edu/kinesin/BE6b_MCAK.html).
Figure 3. Kinesin-13s differentially regulate MT depolymerization in Mitotic and Interphase cells.

In Mitosis (A), Klp10A is recruited to the minus end of spindle MTs. Depolymerization from the minus end facilitates poleward flux. Klp59C is recruited to the plus end of spindle MTs, generating a “Pac-Man” flux. Through both the activity of Klp59C and Klp10A, a sister chromatid is moved from the cell midline towards centrioles at the cell periphery. During Interphase, the Kinesin-13s are used to control the activity of MTs, preventing MT overgrowth (Dawson 2007). As demonstrated in B, Klp10A and Klp59C are mainly recruited to the plus end of MTs, depolymerizing towards the minus ends. Klp10A and Klp59C are recruited to MT plus tips by regulatory and +TIPs proteins, including EB1 (Mannella 2005, Braun 2014). Klp59C has additionally been suggested to coat the length of MTs, facilitating long-range depolymerization.
Figure 4. Kinesin-13 knockdown changes MT dynamics in neuronal dendrites and axons

Klp59C, Klp59D, and Klp10A affect MT polarity in a compartment specific manner in ddaE (Class I) neurons. As shown in A, Klp59C causes a significant change in MT polarity in dendrites. However, only one Klp10A RNAi construct (VDRC 41534) causes a significant change in dendritic MT polarity. In B, two distinct RNAi constructs of Klp10A significantly change MT polarity in axons. Klp59D does not change MT polarity in dendrites or axons. In A and B, n represents the total number of EB1 comets counted. The x-axis represents the percent of EB1 comets polymerizing towards the cell body. Significance was calculated using a Fisher’s exact test (* p<0.05, ** p<0.005).

EB1 comet number is only affected by Klp10A knockdown, as demonstrated in C and D. As seen in D, both Klp10A RNAi constructs significantly increase the number of EB1 comets observed in axons. However, only Klp10A VDRC 33963 knockdown causes an increase in dendritic EB1 comet number (C). Klp59C and Klp59D knockdown do not change the number of EB1 comets observed in either dendrites or axons (C and D). n represents the total number of larvae examined. The x-axis represents the percent of the number of EB1 comets observed in a 10µm region over 300 seconds. Significance was calculated using an unpaired t test (* p<0.05, ** p<0.005). In ddaC dendrites, represented in E, Klp59C knockdown significantly changes MT polarity. Klp59D and Klp10A knockdown do not change MT polarity in ddaC (Class IV) dendrites. n, x-axis, and significance as described in A and B. An additional interesting phenotype observed was “stuck” comets in ddaE axons (shown in F). Open arrow heads represent dynamic MT ends. Closed arrow heads represent “stuck” dots. Stuck dots are only observed in axons of Klp59C knockdown cells. Klp10A knockdown showed an increase in dynamic MT comets.
Figure 5. RNAi Klp59C VDRC 48576 and the genomic Klp59C/D Del causes Klp59C mRNA depletion.

As diagrammed in A, the Klp59C gene consists of a 5' and 3' UTR and a single long exon. The Klp59C gene has introns. The VDRC 48576 hairpin lies within the first half of the gene (approximate scale). The genomic Klp59C/D Deletion spans the entire length of the gene. Primer Set 1 and Primer Set 2 both amplify 50 nucleotide spans (approximate scale), with Primer Set 1 within the 48576 hairpin region. The genomic Klp59C/D Deletion spans the entire length of the gene. Primer Set 1 and Primer Set 2 both amplify 50 nucleotide spans (approximate scale), with Primer Set 1 within the 48576 hairpin region. We used both primer sets to examine Klp59C mRNA levels via qPCR. All Klp59C mRNA levels are normalized to control Rpl23 mRNA levels, a ribosomal protein. Shown in B, with 48576 hairpin expression, the level of Klp59C mRNA amplification via Primer Set 1 drops to negligible levels as compared to the Elav-Gal4 control. With dual hairpin expression (co-expressed with Klp10A RNAi VDRC 41534), the level of Klp59C mRNA amplification is increased. This result indicates that RNAi vector expression, and resultant knockdown, is sensitive to the number of UAS-controlled transgenes expressed. Primer Set 2, which is set outside the hairpin region, demonstrates an increase in levels of Klp59C mRNA. This effect is similar to what is described by BioRad (Maerken 2007). Both Primer Set 1 and Primer Set 2 lie within the range of the genomic Klp59C/D Deletion. As shown in C, with heterozygous loss of Klp59C (Klp59C Del/YW), both Primer Set 1 and 2 show about a 50% decrease in Klp59C mRNA levels as compared to the YW control. With homozygous loss of Klp59C (Klp59C Del), Primer Set 1 and 2 show about a 75% decrease in Klp59C mRNA levels. Depletion of Klp59C mRNA in Klp59C Mut animals acts as a validation of the deletion construct. Remaining low levels of Klp59C mRNA may indicate a small amount of maternally loaded mRNA.
Figure 6. Klp59C and Klp10A knockdown cause defects in dendritic morphology.
As demonstrated in A, in control (Rtnl2) cells, wild type ddaE neurons have straight main dendritic combs extending from the cell body (marked by an asterisk). Secondary dendrites then extend out at about a 90 degree angle from the main dendritic comb. In all pictures, the main dendritic comb extends up while the secondary dendrites extend to the right. Axons extend down out of the image frame. Shown in B, Klp59C knockdown causes an accumulation of morphological defects including short branches, a bent primary backbone, and increased branching near the cell body. Interestingly, Klp59C knockdown cells also show clipped secondary dendrites, a phenotype not previously observed. We have not observed a dendrite clipping in real time, we hypothesize this phenotype is the result of the cell separating a secondary dendrite from the main dendrite comb (possibly the result of cellular stress). Klp10A knockdown cells have a milder set of dendritic phenotypes that includes a bent primary backbone and curled branches (C). However, some cells seem unaffected. In D, growth of secondary dendrites is quantified. Dendrite growth was measured via ImageJ. At each time point, secondary branch length was measured and then summed. Total secondary branch length was then averaged for each genotype. In control (Rtnl2) cells, secondary dendrites grow to their full size by 72 hours. In Klp59C knockdown cells, secondary dendrite growth is significantly stunted at 24, 48, and 72 hours. However, at 96 hours, Klp59C cells have fully elongated dendrites. This demonstrates that Klp59C knockdown is coincident with a slower rate of secondary dendrite outgrowth.
Figure 7. Klp59C and Klp10A localize to multiple compartments within neurons.
As demonstrated in A, UAS-Klp59C-YFP was expressed under a 221-Gal4 promoter, allowing for visualization in the ddaE cell. Indicated by white arrowheads, Klp59C localized to the axon, primarily as puncta. Yellow arrows indicate cell body populations of Klp59C. Levels within the cell body appear to be high, although this may be an artifact of Klp59C-YFP over-expression. Blue arrowheads indicate Klp59C-YFP within the dendrites. Klp59C is seen to localize mainly to branch points, primarily as puncta. Endogenous staining of Klp10A in a wild type (YW background, shown in B, shows Klp10A mainly within cell bodies (yellow arrows) and the axon bundle (white arrow head). However, high levels of background staining may mask Klp10A placement within dendrites. In C, we labeled ddaE, ddaD, and ddaC cells by expressing EB1-GFP under a 221-Gal4 promoter. This allowed for better visualization of thin dendrites. We once again observed Klp10A staining within cell bodies (yellow arrows) and axons (white arrow heads). However, as indicated by blue arrowheads, we also were able to visualize endogenous Klp10A populations within dendrites, with higher Klp10A levels closer to the cell body. UAS-Klp59C-YFP flies were created and screened by Chengye Feng and Daniel Goetschius.
Figure 8. Klp59C and Klp10A alter the levels of stable MTs within neurons.
Larvae expressing mCD8-GFP (a GFP labeled membrane marker) and RNAi vectors under a 477-Gal4 promoter were stained for acetylated Tubulin, a post-translation mark of stable neuronal MTs. As seen in B, and indicated by a white arrow, ddaC cells are clearly labeled by GFP. ddaE cells, with their distinctive structure and off-set from the sensory cluster, are also clearly visible (labeled by a white arrowhead). In order to internally control acetylated Tubulin staining, total change in fluorescence is calculated by the equation in A. Background signal, measured just outside to the region assayed (either cell body or dendrite), is subtracted from both ddaC and ddaE values. Adjusted signal from ddaC cells are then normalized to wild type ddaE cells. Total signal from cell bodies and dendrites is compared in C and D. The Y-axis represents the relative fluorescence of acetylated Tubulin in cells. n equals the number animals assayed. Significance calculated using an unpaired t test (* p<0.05, ** p<0.005). In cell bodies (C), Klp59C RNAi expressing ddaC cells had lower acetylated Tubulin staining in comparison to control ddaE cells, indicating a reduction in stable MTs. In dendrites (D), Klp59C knockdown caused an increase of acetylated Tubulin staining in comparison to control ddaE cells, indicating an increased level of stable MTs. Signal from acetylated Tubulin staining in Klp10A knockdown ddaC cell bodies as compared to control ddaE cells, indicating an increase in the level of stable MTs. Analysis of dendritic stable MTs performed by Juan Tao. Staining images courtesy of Juan Tao.
Figure 9. Klp59C knockdown does not change lysosomal localization to the cell body.
As shown in A, we stained live larvae fillet preps with Lysotracker Red, a fluorescent lysosomal stain. For easy cell localization, ddaE cells were labeled with mCD8-GFP, a membrane marker. Lysosomes, marked by white arrowheads, were found within the cell body and dendrites. We counted lysosomes within the cell body of control (Rtnl2) and Klp59C knockdown neurons. Demonstrated in B, we found no significant change in the number of lysosomes within the cell body in Klp59C knockdown as compared to control (Rtnl2) neurons. The Y-axis represents the number of lysosomes counted within the cell body. n indicates the number of animals assayed. Significance was calculated using an unpaired t test.
Figure 10. Klp59C Deletion causes a change in dendritic but not axonal MT dynamics.
As in Klp59C knockdown, heterozygous and homozygous loss of Klp59C affects MT dynamics in a compartment specific manner in ddaE neurons. Comet polarity and number were counted as in Figure 4. As shown in A, heterozygous loss of Klp59C causes a significant change in MT polarity in dendrites. However, homozygous loss of Klp59C is similar to control (221-Gal4, EB1-GFP/YW). No significant change was observed between Het and Mut animals. In addition, no significant change in polarity was observed as compared to the control in axons (B). n represents the total number of EB1 comets counted. The Y-axis represents the percent of EB1 comets polymerizing towards the cell body. Significance was calculated using a Fisher’s exact test (* p<0.05, ** p<0.005).
EB1 comet number in dendrites is changed by both heterozygous and homozygous loss of Klp59C, as demonstrated in C. Klp59C loss does not significantly change the number of EB1 comets observed in axons (D). n represents the total number of larvae examined. The Y-axis represents the percent of the number of EB1 comets observed in a 10µm region over 300 seconds. Statistical significance was calculated using an unpaired t test (* p<0.05, ** p<0.005).
Figure 11. The level of Klp10A expression decreases with lowered Klp59C expression

We assayed levels of Klp10A within the entire control, Klp59C Het, and Klp59C Mut animals, as shown in A. We performed qPCR, using primers specific to Klp10A. In Klp59C Het and Mut animals, levels of Klp10A mRNA were reduced to about 50% that of YW animals. The Y-axis represents the relative amount of Klp10A mRNA, normalized to the control (Rpl23). We additionally sought to assay levels of Klp10A specifically within neurons following Klp59C knockdown. Relative fluorescence (represented by the Y-axis of B-D) was measured in expressing ddaE cells. Signal taken from within the neurons was normalized to nearby background. As seen in B, levels of Klp10A within the cell body were similar to levels observed in whole animal extracts. In Klp59C Het and Mut animals, the level of Klp10A is reduced by about 50% as compared to the control. In dendrites (C), the level of Klp10A was significantly reduced in Klp59C Het animals as compared to the control. However, the level of dendritic Klp10A remained unchanged in Klp59C Mut animals. No significance was discovered between Het and Mut animals. The level of Klp10A also did not change in the axons of Klp59C Het or Mut neurons (D). n represents the number of animals assayed. Significance was calculated using an unpaired t test (*p<0.05, ** p<0.005).
Figure 12. Depletion of Klp10A and Klp59C causes an accumulation of neuronal MT dynamics defects

Loss of Klp59C and Klp10A affects MT dynamics in a compartment specific manner in ddaE neurons. n represents the total number of EB1 comets counted. The Y-axis represents the percent of EB1 comets polymerizing towards the cell body. In dual Klp10A and Klp59C knockdown cells, MT polarity echoes Klp10A knockdown, but is significantly different than Klp59C (A and B). Dendritic MT polarity in dual Klp59C, Klp10A knockdown cells is close to WT and Klp59C Mut values (<90% in dendrites, 4A and 10A respectively), although not formally compared. Mixing in axonal MT polarity is significantly increased from the single Klp59C knockdown (B). Significance was calculated using a Fisher’s exact test (* p<0.05, ** p<0.005). Changes in EB1 comet number are demonstrated in C and D. n represents the total number of larvae examined. The Y-axis represents the number of EB1 comets observed in a 10µm region over 300 seconds. In dendrites, dual loss of Klp59C and Klp10A significantly reduces EB1 comet number as compared to either single knockdown. No significant change in EB1 comet number was observed in axons. Significance was calculated using an unpaired t test (* p<0.05, ** p<0.005).
Figure 13. JNK signaling may play a role in the kinesin-13 bypass pathway.

We examined a possible role for JNK signaling in the Kinesin-13 bypass pathway. JNK signaling was previously shown to be important for the neuronal injury response (Chen 2012). In B, we examined Puckered (puc) expression, which is downstream of active JNK signaling. In the graphs, the Y-axis represents the relative fluorescence (caused by puc-GFP expression) within neuronal nuclei. Fluorescence in RNAi-expressing ddaE cells was normalized to both background and non-expressing ddaC cells. n represents the number of animals assayed. Klp59C RNAi knockdown cells had a significant increase in nuclear puc expression as compared to the control (γTubulin 37C). Analysis of Puckered expression performed by Gary Teeters. Significance was calculated using an unpaired t test (* p<0.05, ** p<0.005).

In C, the Y-axis represents the percent of EB1 comets moving in towards the cell body. n represents the total number of EB1 comets counted. Co-depletion of JNK signaling (via BskDN, a dominant negative mutant) and Klp59C (via RNAi-mediated knockdown) caused a significant increase in dendritic MT mixing as compared to the single Klp59C RNAi. Axonal MT polarity was unaffected (data not shown). Significance was calculated using a Fisher’s exact test (* p<0.05, ** p<0.005). We suggest that JNK signaling may be activated following Klp59C knockdown, working to control MT polarity within dendrites (as diagrammed in the flow chart in A). Previous publications (Chen 2012), suggest that γTubulin 23C is important for controlling MT polarity following activation of JNK signaling.
Figure 14. Klp64D and Klp59C likely control MT polarity through parallel pathways.
We examined Klp64D, a kinesin responsible for directing MT steering at dendrite branch points, as a potential player in the kinesin-13 bypass pathway. As shown B and D, RNAi-mediated knockdown of both Klp59C and Klp59C causes a significant increase in MT mixing in both dendrites and axons as compared to single knockdown of either kinesin. In the graphs, the Y-axis represents the percent of EB1 comets moving in towards the cell body. n represents the total number of EB1 comets counted. Significance was calculated using a Fisher’s exact test (* p<0.05, ** p<0.005). EB1 comet number in dendrites in Klp59C, Klp64D knockdown animals is significantly increased as compared the control (Rtnl2) (C). Comet number is also significantly increased in the dendrites of Klp59C knockdown cells (C). As seen in E, dual Klp64D, Klp59C knockdown also causes an increase of EB1 comets in axons, as compared to Rtnl2 control. EB1 comet number was not found to be significant for either single Klp59C or Klp64D knockdown as compared to double knockdown animals. n represents the total number of larvae examined. The Y-axis represents the percent of the number of EB1 comets observed in a 10µm region over 300 seconds. Statistical significance was calculated using an unpaired t test (* p<0.05, ** p<0.005). The accumulation of dendritic and axonal phenotypes in dual knockdown cells suggests that Klp64D and Klp59C control MT polarity through distinct, parallel pathways as outlined in the flow chart (A). Analysis of dual Klp59C, Klp64D knockdown performed by Emily Skonecki.
Figure 15. Klp10A and Klp59C may regulate different subsets of minus end protected MTs.

We examined MT minus end regulators, Patronin and γTubulin, as potential players in the kinesin-13 bypass pathway. In the graphs, the Y-axis represents the percent of EB1 comets moving in towards the cell body. n represents the total number of EB1 comets counted. For all experiments, significance was calculated using a Fisher’s exact test (* p<0.05, ** p<0.005). Patronin RNAi knockdown, shown in B, caused a severe mixing phenotype in dendrites as compared to the control (γTubulin 37C). This phenotype is similar to that observed for Klp59C knockdown (4A). In C and D, the effects of γTubulin 23Cbmps1, a proposed overactive mutant, are examined. This data has been previously published in Nguyen 2014. γTubulin 23Cbmps1 causes mild, yet significant MT mixing in the dendrites as compared to the control (C). In axons, γTubulin 23Cbmps1 shows severe MT mixing as compared to the control (D). These dendritic and axonal MT polarity phenotypes are similar to what is observed in Klp10A knockdown cells (4A). We propose that Klp59C and Klp10A may be controlling different sets of minus end regulated MTs as laid out in the flow chart in A. Analysis of Patronin RNAi performed by Alex Weiner.
REFERENCES


Schuldiner, Oren, Daniela Berdnik, Jonathan Ma Levy, Joy Sing-Yi Wu, David Luginbuhl, Allison Camille Gontang, and Liqun Luo. “piggyBac-Based Mosaic Screen Identifies a
Postmitotic Function for Cohesin in Regulating Developmental Axon Pruning.”  
doi:10.1016/j.devcel.2007.11.001.

doi:10.4161/cc.4.11.2116.


Stone, Michelle C., Michelle M. Nguyen, Juan Tao, Dana L. Allender, and Melissa M. Rolls. “Global Up-Regulation of Microtubule Dynamics and Polarity Reversal during Regeneration of an Axon from a Dendrite.”  


Tala, Xiaou Sun, Jie Chen, Linlin Zhang, Ningning Liu, Jun Zhou, Dengwen Li, and Min Liu. “Microtubule Stabilization by Mdp3 Is Partially Attributed to Its Modulation of HDAC6 in Addition to Its Association with Tubulin and Microtubules.”  
*PLoS ONE* 9, no. 3 (March 10, 2014).  
doi:10.1371/journal.pone.0090932.

Tan, Dongyan, Ana B. Asenjo, Vito Mennella, David J. Sharp, and Hernando Sosa. “Kinesin-13s Form Rings around Microtubules.”  
doi:10.1083/jcb.200605194.

doi:10.1016/j.ydbio.2007.08.048.

Van Maerken, Tom, Pieter Mestdagh, Sarah De Clercq, Filip Pattyn, Nurten Yigit, Anne De Paepe, Jean-Christophe Marine, Frank Speleman, and Jo Vandesompele. “Using Real-time qPCR to Evaluate RNAi-mediated Gene Silencing.”  
doi: 10.2144/000113006


