Microtubule Severing Protein Regulation of Sensory Neuron Form and Function in

*Drosophila melanogaster*

by

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Amy Bejsovec

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Dendrite shape is a defining component of neuronal function. Yet, the mechanisms specifying diverse dendritic morphologies, and the extent to which their functioning depends on these morphologies, remain unclear. Here, we demonstrate a dendrite-specific requirement for the microtubule severing protein Katanin p60-like 1 (Kat-60L1) in regulating the elaborate branch morphology and nocifensive functions of Drosophila melanogaster larval class IV dendritic arborization (da) neuron dendrites. Through genetic loss of function analysis we show that loss of kat-60L1 reduced dendrite branching and process length, particularly during a period of normally extensive growth. This morphological defect was paralleled by a reduction in nocifensive responsiveness mediated by these neurons, indicating a tight correlation between neuronal function and the full extent of the dendritic arbor. To understand the mechanism underlying Kat-60L1’s effects, we used in vivo imaging of the microtubule plus-end binding protein EB1, and found fewer polymerizing microtubules within mutant dendrites. Kat-60L1 thus promotes microtubule growth within class IV dendrites to establish the full arbor complexity and nocifensive functions of these neurons.

Although reduction of the related microtubule severing protein Spastin also compromised class IV dendrite arbor outgrowth and nocifensive responses, microtubule polymerization in dendrites was unchanged in spastin mutants, and behavioral defects
arose from generally compromised neuronal excitation. Kat-60L1 and Spastin thus function in distinct neuronal compartments to establish the complex dendritic morphology and sensory functions of class IV da neurons via distinct mechanisms of microtubule regulation. Whereas Spastin regulates stable microtubules affecting both pre- and post-synaptic compartments of these neurons, Kat-60L1 function is required specifically in dendrites to promote their complex arbor outgrowth through the addition of growing microtubule numbers. Double mutant analysis demonstrated that Kat-60L1 and Spastin function antagonistically to promote dendritic outgrowth, likely involving other molecular players involved in regulating the microtubule cytoskeleton. Lastly, we identified Mi-2 as a transcriptional regulator of both kat-60L1 and spastin and show a genetic interaction between mi-2 and kat-60L1 in the class IV dendritic arbor, demonstrating that Mi-2 antagonizes Kat-60L1 function, possibly through the parallel upregulation of spastin. These data support a key role for the differential utilization of microtubule severing in generating distinct neuronal morphologies and subsequent function.
Contents

Abstract ........................................................................................................................................... i

Contents......................................................................................................................................... iii

List of Figures ................................................................................................................................... vii

Acknowledgements ...................................................................................................................... x

Chapter 1....................................................................................................................................... 1

Introduction ................................................................................................................................... 1

1.1 The Microtubule Cytoskeleton Underlies Crucial Aspects of Neuronal Morphology and Function ................................................................................................................................. 2

1.1.1 Microtubule Severing Proteins Belong to the Superfamily of AAA ATPases .... 3

1.1.2 A Model for Microtubule Severing ........................................................................... 5

1.1.3 Microtubule Interacting Proteins and Modifications ............................................. 6

1.1.4 Evidence For Microtubule Severing in vivo .............................................................. 8

1.1.5 Microtubule Severing in Neurons........................................................................... 10

1.2 Drosophila Dendritic Arborization Neurons ............................................................... 11

1.2.1 Thermal and Mechanical Nociception in Drosophila ............................................. 13

1.2.2 Molecules and Mechanisms of Dendrite Morphology in Drosophila ................. 16

1.2.3 Microtubule Severing Proteins in Dendritic Arborization Neurons .................. 23

1.2.3.1 Spastin is Regulated by Knot and Required for Dendritic Arborization ... 23

1.2.3.2 Katanin p60-like 1 is Required for Dendritic Pruning of a Subset of Dendritic Arborization Neurons During Metamorphosis ......................................................... 26

1.3 Proposal of Research...................................................................................................... 29
Chapter 2 ..................................................................................................................................... 31

Specific Regulation of Dendrite Morphology and Function by the Microtubule Seving
Protein Katanin p60-like 1 (Kat-60L1) in Drosophila ................................................................. 31

2.1 Introduction/Rationale ........................................................................................................... 31

2.2 The Molecular Characterization of Kat-60L1 and Mutant Alleles ...................................... 32

2.2.1 *Kat-60L1* Short and Long Isoforms are Expressed Differentially Throughout
Development in the Nervous System ......................................................................................... 32

2.2.2 Characterization of *Kat-60L1* Alleles, Including a New Deletion, Created by
Excision of a Nearby Transposable Element .......................................................................... 34

2.3 Kat-60L1-short is Necessary in the Dendrite for Proper Nocifensive Responses
Mediated by Multidendritic Sensory Neurons .......................................................................... 37

2.4 Kat-60L1 is Required for the Complex Morphology of Class IV Dendritic
Arborization Neurons ................................................................................................................ 43

2.5 Kat-60L1 is Required During Late Larval Stages for Class IV Dendrite Arbor
Outgrowth .................................................................................................................................. 48

2.6 Overexpression of Kat-60L1 in Class IV Da Neurons Leads to a Reduction in
Dendritic Branching .................................................................................................................. 51

2.7 Kat-60L1 Promotes Dynamic Microtubule Growth in Class IV Dendritic Arbors 55

2.8 Discussion and Conclusions .............................................................................................. 60

2.9 Future Directions ................................................................................................................ 63

2.10 Materials and Methods ..................................................................................................... 64

Chapter 3 ..................................................................................................................................... 73

Katanin p60-like 1 (Kat-60L1) and Spastin Regulate Dendritic Microtubules in Class IV
Neurons Through Distinct Mechanisms ...................................................................................... 73

3.1 Introduction/Rationale .......................................................................................................... 73
3.2 Spastin is Necessary for Proper Nocifensive Responses Mediated by Multidendritic Sensory Neurons

3.3 Spastin is Necessary for the General Excitability of Class IV neurons, While Kat-60L1 is Required Upstream at the Level of the Dendrite

3.4 Spastin and Kat-60L1 Function Antagonistically to One Another to Sculpt the Class IV Dendritic Arbor

3.5 Spastin, Unlike Kat-60L1, Does Not Promote Dynamic Microtubule Growth in Class IV Arbors

3.6 Spastin Regulates Stabilized, Futsch-labeled Neuronal Microtubules in Class IV Arbors

3.7 Investigating Post-translational Modifications and Tau localization in a Katanin p60-like 1 Loss of Function Background

3.8 Discussion and Conclusions

3.9 Future Directions

3.10 Materials and Methods

Chapter 4

A Screen for Genetic Interactors in the Drosophila Eye Reveals Mi-2 as a Transcriptional Regulator of Katanin p60-like 1

4.1 Introduction/Rationale

4.2 An Overexpression Screen in the Drosophila Eye Identifies Six Genetic Interactors of Kat-60L1

4.3 The Identification of Mi-2 as a Potential Transcriptional Regulator of Kat-60L1

4.4 Molecular Characterization of the Mi-2\textsuperscript{\textit{bD4}} Allele

4.5 Mi-2 Regulates Transcription Levels of Kat-60L1
4.6 Loss of Mi-2 Suppresses the Kat-60L1 Loss of Function Phenotype in the Class IV Dendritic Arbor................................................................................................................... 108

4.7 Mi-2 Regulates the Expression of Mep1, a component of the dMec complex...... 112

4.8 Mi-2 Regulates the Expression of Microtubule Severing Genes, Spastin and Katanin-60 ........................................................................................................................................ 113

4.9 Discussion and Conclusions ............................................................................... 115

4.10 Future Directions ................................................................................................ 119

4.11 Materials and Methods ...................................................................................... 120

Appendix.......................................................................................................................... 125

Loss of Katanin p60-like 1 Does Not Result in Defective Chordotonal Organs........... 125

References ...................................................................................................................... 128

Biography ........................................................................................................................ 135
List of Figures

Figure 1: Amino Acid Comparison of Kat-60L1 and Spastin Relative to Katanin-60 ....... 3
Figure 2: Proposed Mechanism of Severing by Spastin. .......................................................... 6
Figure 3: Dendritic Arborization Neurons are Categorized by Class-Specific Dendrite Arbor Shape ......................................................................................................................... 12
Figure 4: Noxious Heat Produces a Stereotypical Behavioral Output in a Drosophila Larva ............................................................................................................................................. 15
Figure 5: Diversity of Dendritic Arborization Neuron Morphology and Transcription Factor Expression ........................................................................................................................................ 18
Figure 6: Differential Regulation of Rac1 Activity by Cut and Collier/Knot may Lead to Filopodia and/or Branch Formation ........................................................................................................ 22
Figure 7: Spastin is Required In Class IV Da Neurons for Proper Outgrowth ..................... 24
Figure 8: Upregulation of the Microtubule Severing Protein Spastin in Dar1 Mutant Neurons May be Responsible for the Dendrite Defects .................................................................................. 26
Figure 9: Kat-60L1 Contributes to Microtubule Disassembly in Class IV Neurons During Pruning Events in Pupal Metamorphosis .................................................................................... 28
Figure 10: Kat-60L1 is Expressed in the Embryonic Peripheral and Central Nervous System ............................................................................................................................................ 33
Figure 11: Molecular Characterization of Kat-60L1 .................................................................. 36
Figure 12: Reduced Lifespan in Kat-60L1 Mutant Adults ...................................................... 37
Figure 13: Kat-60L1 is Necessary for Proper Nocifensive Responses Mediated by Class IV Neurons ................................................................................................................................. 40
Figure 14: Kat-60L1 is Required in the Dendritic Compartment For Proper Nocifensive Behavior ........................................................................................................................................ 43
Figure 15: Kat-60L1 is Required for Dendrite Complexity and is Enriched in Class IV Neurons ........................................................................................................................................... 47
Figure 16: Kat-60L1 is Required for Late Larval Development of Class IV Neuron Dendrites.................................................................50

Figure 17: Cytoplasmic Distribution of Venus-Kat-60L1 ......................................................52

Figure 18: Ectopic Kat-60L1 Expression in Class IV Neurons, but not Class I Neurons Leads to Reduced Branch Length.................................................................54

Figure 19: Kat-60L1 Mutants Exhibit No Change in the Distribution of Futsch-labeled Microtubules in Class IV Dendrites. .................................................................56

Figure 20: Kat-60L1 Mutants Exhibit No Change in the Distribution of Acetylated-tubulin in Class IV Dendrites.................................................................57

Figure 21: Microtubule Dynamics are Reduced in Kat-60L1 Mutant Dendritic Arbors....60

Figure 22: Spastin is Necessary for Proper Mechanical and Thermal Nocifensive Responses.........................................................................................74

Figure 23: Nocifensive Behavior Defects are Bypassed Through Optogenetic Activation of Class IV Neurons in Kat-60L1, but not Spastin Mutants..................76

Figure 24: Spastin and Kat-60L1 Function Antagonistically to Sculpt the Class IV Dendritic Arbor .........................................................................................78

Figure 25: Kat-60L1 and Spastin Interact Indirectly in Class IV Neurons .......................80

Figure 26: Loss of Spastin Does Not Perturb Microtubule Growth in Class IV Arbors....81

Figure 27: Kat-60L1 and Spastin Regulate Distinct Subsets of Microtubules in the Class IV Dendritic Arbor .................................................................83

Figure 28: No Change in Tyrosinated-tubulin is Detected in Class IV Dendritic Arbors Lacking or Overexpressing Kat-60L1 .................................................................87

Figure 29: No Change in Tau-GFP is Detected in Class IV Dendritic Arbors Lacking Kat-60L1 .........................................................................................89

Figure 30: Overexpression of Microtubule Severing Proteins in the Drosophila Eye.......101
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Chapter 1. Introduction

This body of work investigates the role of the microtubule severing protein, Katanin p60-like 1 (Kat-60L1) in the Drosophila nervous system. We demonstrate a role for Kat-60L1 in promoting complex dendritic arbors in Drosophila sensory neurons and mediating their nocifensive function. Additionally, we show that Kat-60L1 and Spastin function through distinct mechanisms of microtubule regulation to sculpt complex dendritic arbors. Finally, we address the regulation of Kat-60L1 by post-translational modifications (PTMs), Tau, a microtubule-associated protein, and a transcription factor, Mi-2, which was identified in a screen for genetic interactors of kat-60L1. In the Introduction, I highlight relevant background information regarding the regulation of the microtubule cytoskeleton by microtubule severing proteins, as well as MAPs and PTMs. I then describe the subset of highly branched sensory neurons in which Kat-60L1 functions, the Drosophila dendritic arborization (da) neurons, and their nocifensive sensory functions. Lastly, I highlight the molecules that govern dendritic specification and the growth and morphology of da neurons, including how Kat-60L1 and Spastin have thus far in the literature been reported to regulate da neuron dendritic arborization.
1.1 The Microtubule Cytoskeleton Underlies Crucial Aspects of Neuronal Morphology and Function

The remarkable diversity found in the morphology of neuronal dendrite arbors plays a key role in the specialized functions of specific neurons. The structure and branching of a neuron’s dendrites strongly influences how it receives sensory input and integrates signals from other neurons. Dendrite structures vary dramatically from simple projections to elaborate space-filling arbors, such as those of purkinje neurons in the cerebellar cortex. Identification of the molecules and mechanisms conferring these varied and distinctive morphologies is thus essential for understanding the diverse activities of the nervous system.

The microtubule cytoskeleton underlies crucial aspects of neuronal morphology and function, including neuronal polarization, process outgrowth and stabilization, synapse formation, and transport of essential proteins to distant pre-, post- or extrasynaptic domains. Microtubule dynamics must be tightly regulated to ensure cellular processes are carried out with precision. One point of regulation is that of growth or nucleation, as well as shrinkage or disassembly. Microtubule disassembly can occur by two distinct processes. The best understood, termed catastrophe, occurs when a growing microtubule depolymerizes and shrinks by the dissociation of end-terminal α/β tubulin dimers that have hydrolyzed bound GTP to GDP (Mitchison and Kirschner, 1984). Disassembly also occurs through the less-understood process of severing,
which microtubule polymers are broken along their lengths by a family of enzymes known as the microtubule severing proteins (MTSPs) (Roll-Mecak and McNally, 2010). Unlike depolymerization from microtubule ends, severing does not occur spontaneously but requires an ATP-dependent catalytic reaction.

1.1.1 Microtubule Severing Proteins Belong to the Superfamily of AAA ATPases

Three microtubule severing proteins (MTSPs), Katanin-60, Spastin, and Katanin p60-like 1 (Kat-60L1) are expressed in the *Drosophila* nervous system and share high amino acid sequence conservation, particularly in their AAA ATPase domains (Figure 1) (Sherwood et al., 2004).

![Amino Acid Comparison of Kat-60L1 and Spastin Relative to Katanin-60.](image)

**Figure 1: Amino Acid Comparison of Kat-60L1 and Spastin Relative to Katanin-60.**

Red dashed region represents the AAA ATPase domain. Both short (PA) and long (PB) isoforms of Kat-60L1 (Kat-like) are more highly conserved to Katanin-60 than Spastin in the AAA domain.
Microtubule severing enzymes belong to the “AAA” (ATPases Associated with various cellular Activities) superfamily of proteins, which use ATP hydrolysis to catalyze kinetically unfavorable association or dissociation of protein complexes (Erzberger and Berger, 2006). Studies of Katanin and Spastin indicate that each of these enzymes assembles into hexameric rings to disrupt tubulin-tubulin contacts along the microtubule polymer, thereby severing microtubules into smaller pieces (McNally and Vale, 1993; Roll-Mecak and Vale, 2008). Katanin and Spastin are monomeric when bound to ADP and only form hexamers in the presence of ATP; only in this hexameric form can MTSPs bind microtubules (Hartman and Vale, 1999). The ATP is hydrolyzed in their highly conserved C-terminal AAA ATPase domains. X-ray crystallographic studies of Spastin revealed that this domain contains a canonical α/β nucleotide binding domain (Roll-Mecak and Vale, 2008). Recent structural studies have elucidated a Microtubule Interacting and Trafficking (MIT) domain in the N-terminal region of Spastin and Katanin, capable of binding microtubules with low affinity (Yang et al., 2008). The MIT and AAA ATPase domains are sufficient for both Spastin and Katanin microtubule severing.

Severed microtubule pieces can be further broken down into tubulin monomers and dimers, as observed in isolated microtubule preparations in vitro or in cell lines overexpressing these proteins (see for example (McNally and Vale, 1993; Yu et al., 2008;
Zhang et al., 2007). However, given a sufficient cohort of stabilizing factors, severed polymers can also provide a source of more easily transported or nucleating pieces, thus promoting new polymer formation (Roll-Mecak and McNally, 2010).

### 1.1.2 A Model for Microtubule Severing

Microtubule severing proteins (MTSPs) must break strong lateral and longitudinal contacts in the microtubule lattice, which seems like an incredible molecular task. Many AAA proteins remodel their substrates by threading a polypeptide through a central pore in their AAA ATPase rings (Sauer et al., 2004). Microtubule severing activity by Katanin and Spastin depends on the disordered and negatively charged C-terminal tails of tubulin (McNally and Vale, 1993). Structural analysis revealed that one side of the central pore of the Spastin AAA ATPase ring is positively charged and the lumen is lined with three highly conserved loops that are required for severing (Roll-Mecak and Vale, 2008). These results suggest a model in which MTSPs use their pore loops to tug on the C-terminal tail of tubulin, generating a mechanical force that may unfold tubulin or destabilize tubulin-tubulin interactions in the microtubule lattice, leading to catastrophic breakdown (Figure 2). Interesting questions pertaining to this model remain unknown, such as, how many tubulin dimers need to be removed for severing to occur? How many MTSP hexamers are required at a severing site? How do MTSPs recognize the C-terminal tails?
Figure 2: Proposed Mechanism of Severing by Spastin.

The Spastin AAA core is shown in cyan with pore loops 1, 2 and 3 highlighted in red and numbered in the figure. The MIT domains are shown as gold ovals. The valency of the interaction of the MIT domains with the microtubule is unknown. On the basis of affinity measurements, it is likely that not all MIT domains are engaged with the microtubule (the potentially unengaged MIT domain is shown hatched). The tubulin heterodimers forming the microtubule are shown in green as a ribbon representation, whereas the C-terminal tubulin tails are shown in red cartoon representation. (Figure modified from Roll-Mecak A, Vale RD. Structural basis of microtubule severing by the hereditary spastic paraplegia protein spastin. Nature. (2008) 451(7176):363-7.)

1.1.3 Microtubule Interacting Proteins and Modifications

In addition to microtubule severing proteins (MTSPs), numerous interacting proteins that favor microtubule maintenance, assembly or disassembly provide a potential mechanism for microtubule functional specialization. This mechanism relies on highly regulated interactions between microtubules and a variety of binding partners, such as motor proteins, stabilizing microtubule associated proteins (MAP1B and Tau), MTSPs, and plus-end tracking proteins (+TIPs). Additionally, diverse post-translational
modifications (PTMs) are proposed to form a tubulin ‘code’ that can be ‘read’ by microtubule interacting proteins. Tubulin PTMs that occur on microtubules include acetylation, detyrosination, glycylation, and glutamylation.

Tubulin detyrosination involves the proteolytic removal of a C-terminal tyrosine residue of α-tubulin, a reaction that is reversible by an enzyme, Tubulin Tyrosine Ligase. Detyrosination occurs after tubulin subunits have been added to the lattice on diverse microtubules (Kumar and Flavin, 1981). In mammalian migrating cells, microtubules that orient toward the leading edge are more stable and enriched in acetylation and detyrosination (Palazzo et al., 2004). Tubulin detyrosination also affects microtubule plus-ends by inhibiting the binding of +TIPs, many of which have a Cytoskeleton-Associated Protein Glycine-rich cap (CAP-Gly) that interacts with the Tyr-residue of α-tubulin (Peris et al., 2006). In neurons, microtubules enriched for tyrosinated tubulin are expected to favor functions such as those in the growth cone (i.e. dynamic microtubule processes for rapid remodeling of cytoplasm), whereas microtubules enriched in detyrosinated tubulin appear to favor functions in the shafts of neurites (i.e. microtubule stability and efficient cargo transport).

PTMs on microtubules may render them differentially susceptible to MTSP regulation depending upon how the modifications affect the presence of MAPs such as Tau, which is thought to protect microtubules from severing by Katanin (Qiang et al.,
2006). As studies on the molecular and cellular mechanisms of PTMs emerge, advancements will also progress in how these regulatory cues diversify neuronal microtubule activities.

1.1.4 Evidence For Microtubule Severing in vivo

The first insights into microtubule severing in vivo came from Caenorhabditis elegans (C. elegans) studies that identified two genes, MEI-1 and MEI-2 (C. elegans homologs for Katanin catalytic (Katanin-60) and regulatory (Katanin-80) subunits, respectively) as essential for the assembly of the acentriolar female meiotic spindle (Mains et al., 1990). EM tomographic analysis revealed fewer and longer microtubules in the spindle of MEI mutants compared to wild-type embryos, as well as a large decrease in the total microtubule mass (McNally et al., 2006). These studies suggest that microtubule severing in C. elegans meiotic spindle assembly produces shorter microtubules capable of acting as seeds to produce a net increase in microtubule mass (Ribbeck and Mitchison, 2006).

One of the most conserved roles of Katanin is thought to be in the assembly and disassembly of cilia and flagella. Loss of Katanin results in flagella or cilia without a central microtubule pair in Chlamydomonas (Dymek et al., 2004) and Tetrahymena (Sharma et al., 2007).
Direct evidence for the role of severing in promoting microtubule nucleation was discovered in plants. Cortical microtubules in plants are arranged in parallel arrays and control the direction of cellulose deposition in the cell wall, thereby contributing to cell shape. New microtubules track along preexisting polymers and branch off at discrete angles by treadmilling, which allows the formation of parallel arrays (Shaw et al., 2003). However, in katanin mutants, microtubules from branch points fail to release, resulting in disorganized microtubule arrays, decreased cellulose deposition and a defective cell shape (Murata et al., 2005).

In Drosophila S2 spindles, the MTSPs Spastin and Fidgetin localize to the centrosome and promote microtubule minus-end depolymerization, while Katanin localizes to kinetochores and is required for plus-end depolymerization (Zhang et al., 2011; Zhang et al., 2007). Zhang et al. have shown that the overexpression of Spastin and Katanin-60 lead to disrupted/lost microtubule arrays in interphase S2 cells. Overexpressing Katanin p60-L1 (Kat-60L1) in Drosophila S2 cells, however, caused an increase in MT polymers, indicating Kat-60L1 functions distinctly from Spastin and Katanin-60 in these cells. Data from the Sharp lab support a role for Kat-60L1 in severing and depolymerizing microtubule ends, much as they have shown for Katanin-60 in vitro (D. Sharp laboratory, personal communication).
1.1.5 Microtubule Severing in Neurons

Our understanding of severing is less clear in other subcellular structures such as dendrites, axons, cilia, and spindles, which have bundled microtubules with less than 200 nm spacing, thus precluding the direct observation of individual microtubules by light microscopy. However, in addition to forming dense microtubule arrays in spindles and cilia, Spastin and Katanin have been implicated in axon elongation and branch formation in neurons.

Spastin functions to promote microtubule growth by severing in developing Drosophila motoneurons during synaptogenesis. Loss of Spastin leads to sparse and disorganized microtubules at boutons (synaptic terminals) of the Drosophila neuromuscular junction (Sherwood et al., 2004). Spastin is one of the most commonly mutated genes in a disorder called Autosomal Dominant Hereditary Spastic Paraplegia, a human neurodegenerative disease characterized by lower limb weakness due to axonopathy of the cortico-spinal neurons (Hazan et al., 1999). In Drosophila, loss of Spastin also results in defects of dendritic arbor outgrowth in a subset of highly branched neurons called class IV dendritic arborization (da) neurons (Jinushi-Nakao et al., 2007); see section 1.2.3.1. In zebrafish, Spastin loss leads to disorganized and sparse microtubule arrays and impaired axonal outgrowth (Wood et al., 2006).
Work in hippocampal neurons suggests a role for Katanin in axonal elongation, possibly through the release of centrosomal microtubules (Ahmad et al., 1999). Little work has been carried out to investigate the role of Kat-60L1 in the nervous system. However, Kat-60L1 was recently implicated in dendritic pruning of the Drosophila nervous system during pupal metamorphosis (Lee et al., 2009); see section 1.2.3.2. Further characterization of these three MTSPs should help address the need for multiply expressed proteins in the nervous system.

1.2 Drosophila Dendritic Arborization Neurons

The results described in this study implicate a dendrite-specific role for Drosophila Kat-60L1 in promoting complex dendrite morphology and function in a subset of larval sensory neurons called dendritic arborization (da) neurons. Although the morphology of presynaptic terminals can clearly affect neuronal function, considerably greater diversity is displayed by the morphology of dendritic arbors, reflecting their specialized sensory requirements. An excellent example of this is seen in the Drosophila da neurons, a subset of the multidendritic (md) sensory neurons that innervate the overlying larval epidermis in a stereotyped pattern, providing nearly complete coverage. Four classes of da neurons are distinguished according to their respective dendritic branching patterns and complexity, ranging from the class I neurons with simply constructed dendritic arbors, to the class IV neurons, which exhibit
the most highly branched dendritic trees (Figure 3) (Grueber et al., 2002). Knot (Kt), a transcription factor expressed specifically in class IV neurons promotes complex microtubule-dependent arborization, while Cut (Ct) is expressed most highly in class III neurons and promotes actin-dependent branching of the characteristic dendritic spines of class III neurons (Figure 3) (Jinushi-Nakao et al., 2007). Class IV neurons, with their complex arbors, mediate larval mechanical and thermal nociception, while class I neurons are thought to provide proprioceptive feedback to motor neurons and are required for the peristaltic movements of normal larval locomotion (Hughes and Thomas, 2007; Hwang et al., 2007). These nociceptive neurons thus provide a valuable system in which to study the correlation between dendritic form and function.

![Figure 3: Dendritic Arborization Neurons are Categorized by Class-Specific Dendrite Arbor Shape.](image)

Tracings to illustrate the dendrite arbor morphology of the four classes of da neuron: class I ddaE, class II ddaB, class III ddaF and class IV ddaC at wandering third instar.
stage. Anterior is to the left, dorsal is up, the scale bar is 75 µm. The expression of the
different class-specific transcription factors is shown. For Cut: − no protein expression, +
low, ++ intermediate, +++ high. This figure is reproduced with modifications from
Jinushi-Nakao et al. (2007). (Figure modified from Moore AW. Intrinsic mechanisms to
define neuron class-specific dendrite arbor morphology. Cell Adhesion & Migration. (2008) 2:2,
81-82.)

1.2.1 Thermal and Mechanical Nociception in Drosophila

In addition to demonstrating a role for Kat-60L1 in promoting complex dendritic
branching, we show that Kat-60L1 is required in class IV sensory neurons for their
proper nocifensive responses. The highly branched class IV da neurons are necessary
and sufficient for triggering the stereotyped Drosophila Nocifensive Escape Locomotion
(NEL) in response to noxious mechanical or thermal sensory input (Hwang et al., 2007).
In humans and invertebrates, mechanical, thermal and chemical noxious stimuli are
detected by nociceptors (pain sensing neurons), which have free nerve endings and are
typically found in the skin. The class IV nociceptors in Drosophila morphologically
resemble vertebrate nociceptive neurons due to the presence of multiply branched,
naked nerve endings attached to epidermal cells (Bodmer et al., 1989; Merritt and
Whittington, 1995). Another line of evidence in support of md neurons functioning as
nociceptors is that larvae with genetically silenced md neurons are insensitive to
noxious stimulation (Tracey et al., 2003; Williams and Truman, 2005). Lastly, Painless, a
transient receptor potential (TRP) channel is required for nociception and is expressed in
md neurons (Tracey et al., 2003). The signals are received by channels Ppk and dTRPA1 in the nociceptors and processed by the central nervous system and perceived as pain, which serves to protect animals from harmful stimuli as well as a warning signal of pathological states, such as nerve damage, inflammation, or cancer.

The *Drosophila* TRP channel Painless was discovered in a forward genetic screen for mutants defective in nociception (Tracey et al., 2003). A paradigm for *Drosophila* nociception was designed, in which a probe heated above 46°C and held to the side of a larva produces a stereotyped NEL, characterized by rotation around the long body axis, unlike the normal forward, rhythmic motion (Figure 4) (Tracey et al., 2003). *Drosophila* larvae with a mutation in *painless* exhibit increased sensory thresholds to elicit NEL to both noxious mechanical and thermal stimuli. The mammalian homologue of Painless is TRPA1 and Painless is expressed in multidendritic (md) and in chordotonal sensory neurons, the internally located stretch sensors (Tracey et al., 2003). Understanding the molecular mechanisms by which nociceptors are able to sense distinct stimuli continues to be of great interest to the scientific community.
Figure 4: Noxious Heat Produces a Stereotypical Behavioral Output in a Drosophila Larva

Images extracted from digital video recordings. (A) Normal larval movement. (B) In response to a light touch, the larva pauses. (C) When touched with a probe heated to a noxious temperature (left), the larva responds by rolling laterally in a corkscrew-like motion. (D) The behavioral output quantitatively analyzed by measuring the delay in response time. (Figure from Tracey DW et al. *painless, a Drosophila Gene Essential for Nociception*. Cell. (2003) 113:261-273.)

In addition to TRP channels, some members of the degenerin (DEG/ENaC) family can be activated by mechanical force. DEG/ENaCs play a critical role in detecting gentle touch stimuli in *C. elegans* and have been implicated in harsh touch sensation in mammals. *Drosophila pickpocket (ppk)*, which encodes a DEG/ENaC subunit, is expressed in md neurons and is required for mechanical nociception but not thermal nociception in
class IV neurons. Larvae mutant for \textit{ppk} show greatly reduced nociception behaviors in response to harsh mechanical stimuli (>30mN) (Zhong et al., 2010).

In this work we show that Kat-60L1 is required in class IV neurons for proper NEL in response to both mechanical and thermal nociception. This is the first study to implicate a microtubule regulator in the nocifensive function of class IV sensory neurons. This work should instigate further investigation into the role of the microtubule cytoskeleton in nocifensive function in neurons.

\textbf{1.2.2 Molecules and Mechanisms of Dendrite Morphology in \textit{Drosophila}}

Transcription factor codes contribute to type-specific dendrite patterns. The \textit{Drosophila} da neurons are an excellent system to study the effects of transcription factors since they can easily be divided into four classes based on dendritic arbor complexity (Grueber et al., 2002). The zinc-finger transcription factor 	extit{Hamlet} distinguishes the fate between an external sensory (es) neuron and a multidendritic (md) sensory neuron. \textit{Hamlet} is expressed in the immediate precursors of es neurons, where it suppresses dendritic branching. Lack of Hamlet in the immediate precursors of md neurons allows these neurons to form elaborate branches (Moore et al., 2002). Subsequently, an RNA interference screen identified more than 70 transcription factors affecting dendrite morphogenesis of class I da neurons (Parrish et al., 2006). The BTB-zinc finger protein \textit{Abrupt}, is expressed in class I neurons and when ectopically expressed in class II, III or
IV da neurons, it reduces their size and complexity, suggesting a role for Abrupt in ensuring the simple morphology of class I neurons (Li et al., 2004; Sugimura et al., 2004).

Cut, a homeodomain-containing transcription factor, is expressed at undetectable, low, medium and high levels in the class I, II, IV and III da neurons, respectively. Loss of function and class-specific ectopic expression studies have demonstrated that the level of Cut expression determines the distinct patterns of dendrite branching through the promotion of actin-dependent branching (Grueber et al., 2002).

The expression of Collier/Knot distinguishes class IV from class III da neurons by promoting microtubule-dependent branching. Collier/Knot is only expressed in class IV da neurons where it suppresses Cut-induced F-actin-rich protrusions, known as dendritic spikes, normally characteristic of class III da neurons. The combinatorial co-expression of Collier/Knot and Cut in class IV neurons ensures their correct complex dendritic morphology without dendritic spikes; class III neurons express high levels of Cut but not Collier/Knot, which favors the formation of dendritic spikes (Figure 5) (Crozatier and Vincent, 2008; Jinushi-Nakao et al., 2007). Overexpression experiments support the model whereby Cut promotes F-actin-based dendrite extensions and Knot/Collier promotes the growth of a microtubule-based arbor (Jinushi-Nakao et al.,
Whether Knot promotes *kat-60L1* expression remains to be determined and is discussed in the Future Directions section 3.9.

**Figure 5: Diversity of Dendritic Arborization Neuron Morphology and Transcription Factor Expression.**

Dendritic arbors of class I, II, III and IV da neurons (left to right). Arrowheads indicate regions of arbors that exemplify class-specific branching complexity. Cells are classified according to increasing arbor complexity. The expression status of transcription factors Cut, Knot, Abrupt and Spineless is listed below each morphological class. Filled boxes indicate expression, white boxes indicate no detectable expression. Progressively higher levels of Cut expression are indicated by progressively darker shadings (the degree of shading is not intended to indicate relative levels among the different transcription factors). Images in A-C reproduced with permission from Matthews et al. (Matthews et al., 2007). (Figure modified from Corty MM, Matthews BJ, Grueber WB. *Molecules and mechanisms of dendrite development in Drosophila.* Development. (2009) 136, 1049-1061.)

Dendrites function as specialized information receiving units for a neuron and the particular dendrite shapes are important to neuronal function and circuit assembly. The growth of dendritic arbors can require large amounts of plasma membrane and specialized proteins for development, and hence, the polarized trafficking of cargoes and the addition of new membrane are essential for branching and growth. Golgi
outposts, a component of the secretory pathway found in dendrites of some vertebrate and invertebrate neurons, have been shown to be a conserved feature of growing dendrites (Horton and Ehlers, 2003; Ye et al., 2007). A forward genetic screen for mutations that affect dendrite and axon morphology using Drosophila class IV da neurons, recovered mutations in several genes encoding proteins involving ER-to-Golgi transport, such as sar1, sec23 and Rab1 (Ye et al., 2007). Mutations in the vesicle trafficking gene sar1 showed reduced dendrite growth and diffuse Golgi outposts, while axons did not appear to be as strongly affected (Ye et al., 2007). This finding provides insight as to how polarized dendritic and axonal growth is maintained through differential golgi trafficking.

The size of a dendritic arbor increases in proportion to the whole animal, a phenomenon known as dendritic scaling. The final size of an arbor is then maintained throughout the lifetime of a neuron. Dendrites of Drosophila da neurons exhibit a rapid period of ‘catch up’ growth in mid-embryogenesis, followed by dendritic scaling in later larval stages, during which the body length triples over only three days (Parrish et al., 2009). Recently, loss of function analysis of the microRNA bantam demonstrated that signaling from the overlying epithelial cells was crucial for proper dendritic scaling (Parrish et al., 2009). Bantam inhibits the phosphoinositide 3-kinase (PI3K)-mammalian target of rapamycin (mTOR) kinase pathway in epithelial cells overlying the da neurons,
limiting dendritic growth. The PI3K-mTOR kinase pathway, in addition to the mitogen-activated protein kinase (MAPK) kinase cascade, regulate dendrite complexity and branching, and both pathways are activated by brain-derived neurotrophic factor (BDNF) (Dijkhuizen and Ghosh, 2005; Kumar et al., 2005).

In order to cover the correct target field, many neurons need to form extensive dendritic branches. The predominant mechanism in physiological states is the emergence of side branches from established dendritic shafts, called interstitial branching. Studies in live rat pyramidal neurons showed that new branches form in this way, first forming a single filopodia, some of which form stable branches (Dailey and Smith, 1996). A similar process is thought to occur in Drosophila md sensory neurons, whereby embryonic dorsal dendrite extensions precede the formation of lateral branches that transiently extend and retract, and finally are either lost or stabilized to become the final lateral branches (Gao et al., 1999). Little is known about the mechanisms of interstitial branching in dendrites; however, studies of axonal branching have offered insights. Local cues are thought to act on the actin cytoskeleton and membrane to form protrusions. Filopodia can act as precursors of transient branches that can be stabilized by fragmented microtubules from the axonal trunk (Qiang et al., 2010; Zakharenko and Popov, 1998). Recent molecular studies of dendritic branching are consistent with this hypothesis. The Rho family of small GTPases are important for controlling neuronal
morphogenesis and dendritic development; (for reviews, see Luo, 2000; Redmond and Ghosh, 2001). Knot/Collier and Cut interact differently with Rac1. When Rac1 was co-expressed with Cut, it enhanced the formation of filopodia in dendrites. However, when Rac1 was co-expressed with Knot, together they increased dendrite branch formation (Jinushi-Nakao et al., 2007). From these results, one model Jinushi-Nakao et al. propose is that after the Rac1-mediated filopodial extension step, Knot mediates microtubule invasion by upregulating the expression of Spastin to create new fragmented microtubules to promote branch formation (Figure 6).
Figure 6: Differential Regulation of Rac1 Activity by Cut and Collier/Knot may Lead to Filopodia and/or Branch Formation.

Differential Modulation of Rac1 activity by Cut and Knot May lead to Filopodia or Branch Formation. Rac1 mediates local F-actin rearrangement at the dendrite cortex. Rac1 activity may provide a local site (green arrows) for the action of factors controlled by either the Knot or Cut mediated pathways (purple arrows). Cut controls a pathway leading to filopodia formation from this local site. Knot upregulates Spastin expression. Spastin then causes localized microtubule (MT) severing at the region where Rac1 has promoted protrusion formation. Rac1 at the cortex mediates microtubule capture. Invasion of microtubules into this protrusion causes its stabilization and results in dendrite branch formation. (Figure modified from Moore AW. Intrinsic mechanisms to define neuron class-specific dendrite arbor morphology. Cell Adhesion & Migration. (2008) 2:2, 81-82.)
1.2.3 Microtubule Severing Proteins in Dendritic Arborization Neurons

1.2.3.1 Spastin is Regulated by Knot and Required for Dendritic Arborization

A role for microtubule severing in the regulation of class IV da neuron dendrites is supported by studies demonstrating that spastin mutants, whether loss or gain of function, result in dendritic arbors of reduced complexity (Jinushi-Nakao et al., 2007; Ye et al., 2011). Jinushi-Nakao et al. showed that the transcription factor, Knot/Collier promotes the formation of a dendritic arbor that contains microtubules, at least in part by upregulating the expression of Spastin in class IV da neurons. Loss of Knot resulted in a reduction of dendrite outgrowth and branching, and occasional tufts of short terminal branches. Spastin expression was shown to be upregulated in cells expressing ectopic Knot by RT-PCR. Consistent with Spastin being regulated by Knot activity, loss of Spastin in class IV da neurons resulted in gaps within a dendritic arbor and between neighboring arbors, reminiscent of the knot mutant phenotype, as well as a reduction in the number of dendrite termini and dendrite length (Figure 7).
Figure 7: Spastin is Required In Class IV Da Neurons for Proper Outgrowth

(M and N) Comparison of the dendritic arbor morphology in class IV ddaC neurons of wandering third-instar larvae. (M) Wild-type: ppk-Gal4, UAS-mCD8::GFP. (N) ppk-Gal4, UAS-mCD8::GFP, spastin5.75/+. In spastin mutant neurons large gaps are seen between adjacent neurons (red asterisk) and within the arbor of a single neuron (blue asterisks). Scale bar, 75 µm. (O) A bar chart illustrating that spastinRNAi, spastin5.75/+, and kn1/knKN2 all have more empty area within the class IV dendritic arbor as compared with wild-type. (P and Q) Bar charts showing the number of dendrite termini (P) and overall dendrite length (Q) for the class I ddaE neuron visualized using Gal42-21, UAS-mCD8::GFP. Expression of a UAS-spastinRNAi construct does not alter dendrite termini number or length in a wild-type background. However, expression of a UAS-spastinRNAi construct strongly represses both dendrite branching and total length in an ectopic knot (UAS-kn; Gal42-21, UAS-mCD8::GFP) expression background. (Figure modified from Jinushi-Nakao S, et al. Knot/Collier and Cut Control Different Aspects of Dendrite Cytoskeleton and Synergize to Define Final Arbor Shape. Neuron. (2007) 56, 963-978.)
In a forward genetic screen designed to identify mutations in genes affecting dendritic outgrowth, the *dendritic arbor reduction 1 (dar1)* mutant was identified, which displayed defects mainly in dendritic growth and not axonal growth (Ye et al., 2011). The *dar1* gene encodes a novel transcription factor in the Kruppel-like factor family. Ye et al. demonstrated that loss of Dar1 resulted in a severe reduction in growth of microtubule- but not actin-based dendritic branches, while overexpression of *dar1* resulted in increased growth of these branches. They asked if Dar1 could regulate *Spastin* expression since Dar1 preferentially regulates microtubule-based dendritic growth. Levels of *Spastin* transcripts were elevated in *dar1* mutant neurons, suggesting that Dar1 negatively regulates *Spastin* levels and Spastin function can antagonize dendritic outgrowth. Consistent with the *dar1* mutant phenotype, overexpression of *spastin* using the T32 EP insertion (*Spa^{T32})*, led to a dramatic reduction in dendritic length and branch number (Figure 8). Their results suggest that Dar1 restricts the expression of Spastin either directly or indirectly through transcriptional repression, allowing for dendrite growth. Unlike *dar1* mutants, *spastin* upregulation also resulted in a reduction in axonal growth.
Figure 8: Upregulation of the Microtubule Severing Protein Spastin in Dar1 Mutant Neurons May be Responsible for the Dendrite Defects

(A) Representative images showing that upregulation of Spastin by overexpressing the SpaT32 reduced both total dendritic length and branch numbers in class IV da neurons. (Figure modified from Ye B. et al. Differential regulation of dendritic and axonal development by the novel Krüppel-like factor Dar1. J. Neurosci. (2011). 31(9): 3309-3319.)

1.2.3.2 Katanin p60-like 1 is Required for Dendritic Pruning of a Subset of Dendritic Arborization Neurons During Metamorphosis

While Spastin functions in synaptic boutons and in sensory neuron dendrites (Jinushi-Nakao et al., 2007; Sherwood et al., 2004), the functions of Kat-60L1 and Katanin-60 in the Drosophila nervous system are less understood. Kat-60L1 has been suggested to function in dendrite pruning of these neurons during metamorphosis, inducing microtubule destruction during dendrite degeneration (Lee et al., 2009).

In Drosophila, extensive neuronal remodeling occurs during metamorphosis, as some larval neurons prune their processes, while others die and new neurons are generated (Truman, 1990). Pruned processes are eliminated before the formation of
adult-specific connections. Most of the larval peripheral neurons die during metamorphosis, but a subset including some of the class IV da neurons survive and undergo dendritic pruning, starting with severing of proximal dendrites, followed by blebbing, and degeneration of disconnected dendrites (Kuo et al., 2005; Williams and Truman, 2005). Ecdysone signaling, the ubiquitin-protease system, as well as matrix metalloproteases and caspase activity regulate dendrite pruning. Lee et al. demonstrated a role for kat-60L1 in severing microtubules in pruning processes at the early step of dendrite pruning. Dendrite breakage was delayed in pupae expressing tissue-specific kat-60L1 RNAi and in homozygous kat-60L1^Plac loss of function mutants. Additionally, GFP-tagged microtubules remained intact for much longer in kat-60L1 loss of function mutants, suggesting an in vivo role for Kat-60L1 in microtubule disassembly during dendritic pruning events in pupae (Figure 9). However, in situ data indicates that kat-60L1 is already expressed in the embryonic nervous system, suggesting Kat-60L1 might be required prior to metamorphosis (Tomancak et al., 2002).
Figure 9: Kat-60L1 Contributes to Microtubule Disassembly in Class IV Neurons During Pruning Events in Pupal Metamorphosis.

Kat-60L1 is involved in dendrite severing of ddaC neurons during dendrite pruning. (A) Schematic diagram of the kat-60L1 gene structure and the insertion site of 2 piggyBac lines, kat-60L1c01236 and kat-60L1f00564. The start sites of the ORF (in black and gray) for 2 splicing variants are indicated by arrows and the exons encoding the AAA ATPase domain are in gray. (B–E) The ddaC neurons were labeled by UAS-mCD8GFP and ppk-GAL4, and their morphology at 16 h APF was shown. Arrowheads mark the axons, and arrows indicate the proximal dendrites. Dendrite severing was prevented in a pupa with kat-60L1-RNAi expression (C) and in homozygous kat-60L1c01236 mutants (D). (E) The ddaC neurons in a revertant line of kat-60L1c01236 showed normal dendrite pruning as in wild type (B). At 5 h APF, the microtubules marked by anti-GFP antibody staining showed breakage in the proximal dendrites (arrows) of wild-type ddaC neurons (F), but remained intact in those of kat-60L1c01236 mutants (G). (H) Quantitative analysis of dendrite-severing phenotypes in ddaC neurons. The percentage of cells was determined by dividing the number of ddaC neurons, whose primary dendrites were connected to
soma at 16 h APF by the total number of cells examined for each genotype. The precise excision of piggyBac enables normal dendrite pruning in revertants. kat-60L1: piggyBac line kat-60L1c01236; Df: deficiency line Df(3R)Exel6145, which deletes the kat-60L1 gene. (Scale bars, 50 µm.) (Figure from Lee HH et al. Drosophila IKK-related kinase Ik2 and Katanin p60-like 1 regulate dendrite pruning of sensory neuron during metamorphosis. PNAS. (2009) (15):6363-8.)

1.3 Proposal of Research

Prior to this study, little was known about the function of Kat-60L1. Given that there are three highly conserved microtubule severing proteins expressed in the Drosophila nervous system, we sought to determine the role of Kat-60L1 in the nervous system and investigate whether Kat-60L1 functions uniquely from Spastin and Katanin-60.

Aim 1: To investigate the unique or redundant role(s) of Kat-60L1 in the Drosophila nervous system. In this study, we determine a unique role for Kat-60L1 in promoting complex dendritic arborization and function of class IV neurons in the larva through a mechanism involving the upregulation of growing microtubules. We also investigate the interaction between Kat-60L1 and Spastin in class IV neurons and show evidence for their antagonistic relationship and unique roles in sculpting the dendritic arbor.

Aim 2: To identify genetic regulators of Kat-60L1 in the nervous system. We designed an overexpression screen in the Drosophila eye to identify genetic interactors of
kat-60L1. We identify the transcription factor Mi-2 as a positive transcriptional regulator of Kat-60L1, as well as Spastin and Katanin-60. We show a genetic interaction between kat-60L1 and mi-2 in the class IV sensory neurons that suggests Mi-2 negatively regulates Kat-60L1 function to promote arborization through a parallel pathway, likely involving the antagonistic function of Spastin.
Chapter 2. Specific Regulation of Dendrite Morphology and Function by the Microtubule Severing Protein Katanin p60-like 1 (Kat-60L1) in *Drosophila*

2.1 Introduction/Rationale

The Sherwood laboratory is interested in the roles of microtubule severing proteins (MTSPs) in the *Drosophila* nervous system. There are three MTSPs expressed in the fly nervous system: Spastin, Katanin-60 and Katanin p60-Like 1 (Kat-60L1). As described earlier in chapter 1, section 1.1, Katanin-60 has been well studied in neuronal cell culture and implicated in axon outgrowth. Spastin has been studied the most extensively of the three and is required for proper synapse formation at the *Drosophila* neuromuscular junction. Interestingly, Spastin is the most commonly mutated gene in the human neurodegenerative disease, Autosomal Dominant Hereditary Spastic Paraplegia. Kat-60L1 is the least studied of these; therefore, we sought to determine the function of Kat-60L1 in the *Drosophila* nervous system and to address the need for three distinct, yet highly conserved MTSPs in the fly nervous system. With the identification of the first MTSP occurring only twenty years ago (Vale, 1991), in vitro studies over the last ten years suggest that these proteins have evolved unique functions and do not simply act redundantly (Yu et al., 2008; Zhang et al., 2007). Our goals for this study were to create a *kat-60L1* genetic null allele for loss of function analysis in order to understand the function of Kat-60L1. Through genetic, behavioral, cell biological and
live imaging techniques, we demonstrate that Kat-60L1 functions as a dendrite-specific MTSP that promotes microtubule polymerization to enable proper branch arborization and sensory neuron nocifensive function.

2.2 The Molecular Characterization of Kat-60L1 and Mutant Alleles

2.2.1 Kat-60L1 Short and Long Isoforms are Expressed Differentially Throughout Development in the Nervous System

Several lines of evidence support a role for Kat-60L1 in the Drosophila nervous system. Embryonic in situ made available by the Berkeley Drosophila Genome Project show kat-60L1 mRNA expression localized to sensory organs and neurons, the brain and the ventral nerve cord (Figure 10) (Tomancak et al., 2002). Fly Atlas, the Drosophila adult gene expression database also reveals kat-60L1 transcript is enriched four-fold in the adult brain and ventral nerve cord, and eight-fold in adult testis, relative to the whole animal (Chintapalli et al., 2007). The role of Kat-60L1 in these adult tissues has not been investigated.
Figure 10: Kat-60L1 is Expressed in the Embryonic Peripheral and Central Nervous System

Embryonic expression pattern of kat-60L1 by BDGP. (A) Lateral view of kat-60L1 in situ hybridization pattern shows expression in the peripheral sensory neurons (black arrow) and the ventral nerve cord (red arrow) of the central nervous system. (B) Dorsal view shows kat-60L1 expression in sensory neurons (black arrows) and the supraesophogeal ganglion (blue arrow). Anterior is to the left.

To further understand the expression of Kat-60L1 we performed Reverse Transcription PCR (RT-PCR) on various tissues. Kat-60L1 encodes a short (605 amino acids) and long (669 amino acids) isoform: the 5' UTR of the long isoform initiates from within the second intron of the short, while exons 2 through 5, which include the AAA ATPase catalytic domain, are identical (Figure 11A). We found that the two kat-60L1 isoforms are expressed differentially across the tissues examined and therefore, likely play distinct roles (Figure 11B). Transcript for the short isoform was highly enriched in
third-instar larval brains, at intermediate levels in adult testes, and at low levels in stage
11-17 embryos and adult heads (Figure 11B). Neither isoform was expressed in the adult
ovary, consistent with adult Fly Atlas results. In contrast, the long isoform was highly
enriched in adult testes but expressed only at low levels in third instar larval brains, and
was not detectable in embryos or adult brains (Figure 11B). Kat-60L1-short is thus the
predominant isoform in the larval nervous system and throughout nervous system
development, albeit at lower levels in embryos and adults.

2.2.2 Characterization of Kat-60L1 Alleles, Including a New Deletion,
Created by Excision of a Nearby Transposable Element

We next sought to identify mutant alleles for kat-60L1 with the goal of
investigating Kat-60L1 in vivo functions. We began with an available piggyBac insertion,
katanin p60-Like 1\textsuperscript{PBac00564}, hereon referred to as \textit{kat-60L1}\textsubscript{PBac} (Thibault et al., 2004). This
insertion is located in the second intron of \textit{kat-60L1}, which is common to both isoforms
(Figure 11A). Notably, this allele showed significantly reduced, but still detectable
transcript levels, suggesting that it is a strong hypomorph (Figure 11C). Homozygous
adults for the mutation appeared healthy and, unlike \textit{spastin} mutants (Sherwood et al.,
2004), did not exhibit defects at the larval NMJ (data not shown).

Since small amounts of transcript may be sufficient for Kat-60L1 function, we
reasoned that the above alleles might not uncover all of the endogenous requirements
for Kat-60L1. We therefore generated a genetic deletion of \textit{kat-60L1} by imprecise excision
of the P-element EY21697 which is located in the 5' UTR of CG2051, an uncharacterized gene that is immediately distal to kat-60L1 on the right arm of chromosome 3 (Figure 11A). Out of the approximately 300 lines screened by PCR, we recovered one excision, denoted Df(3R)kat-60L1BE6, or BE6, that deletes from the second intron of kat-60L1 to the 5' UTR of CG2051, removing the majority of kat-60L1 and the entire coding sequence of CG2051 (Figure 11A). RT-PCR using primers spanning exons 3 through 5 confirmed that this region of kat-60L1 transcript is absent in a homozygous BE6 background (Figure 11C). As transcript was also undetectable in the brains of trans-heterozygous BE6/PBac animals (Figure 11C), we preferentially used this background throughout the study. The use of the transheterozygous allelic combination allowed us to reduce the potential effects of second-site mutations in the genetic background of each unique allele, and to also cover the CG2051 deletion in Df(3R)kat-60L1BE6.

We also characterized kat-60L1 RNAi lines made available by the Vienna Drosophila RNAi Center by crossing UAS-RNAi animals to a GAL4 driver with ubiquitous promoter expression, e22c-GAL4, sqh-GAL4 (Franke et al., 2010). RT-PCR of third-instar larval brains showed a significant, albeit incomplete reduction of kat-60L1 transcript levels in all RNAi samples compared to wild-type controls (Figure 11D).
Figure 11: Molecular Characterization of Kat-60L1.

(A) Kat-60L1 encodes two transcripts, short (top) and long (bottom) that differ at the 5’ UTR and first exon. PBac{PB}kat-60L1\textsuperscript{PBacc00564} (red triangle) is inserted in the shared second intron. P{EPgy2}CG2051\textsubscript{YE21697} (blue triangle) inserted in the neighboring gene was imprecisely excised to generate Df(3R)kat-60L1\textsuperscript{BE6} (dashed line), which removes kat-60L1 exons 2-5, the region encoding the AAA ATPase domain. Primers used to amplify a cDNA-specific kat-60L1 688 bp product are denoted by black arrows spanning exon 3 and 4, and in exon5. Primers used to amplify cDNA and isoform-specific products are denoted as red arrows in exon 1 of both isoforms and in exon 3. (B) Isoform-specific RT-PCR on isolated tissues reveals strong enrichment of the short isoform in larval brains and adult testes, while the long isoform is predominantly expressed in testes, suggesting isoform-specific roles. Primers for the short isoform amplify a 344 bp product and those for the long isoform amplify a 245 bp product. (C) Transcript levels are strongly reduced in homozygous kat-60L1\textsuperscript{PBacc00564} adult heads, suggesting that the PBac{PB}kat-60L1\textsuperscript{PBacc00564} allele is a strong hypomorph, while transcript is absent from adult heads homozygous for the Df(3R)kat-60L1\textsuperscript{BE6} allele. (D) Transcript levels are markedly reduced in larval brains expressing kat-60L1 RNAi transgenes driven ubiquitously by e22c-GAL4, sqh-GAL4.
Animals homozygous for the $BE6$ deletion, as well as $kat-60L1^{BE6/PBac}$ transheterozygotes, survived to adulthood but exhibited a reduced lifespan, with 49% of the $kat-60L1^{BE6/PBac}$ population dying by two weeks after eclosion. This was dramatically different from the 2.5% mortality seen in wild-type controls by two weeks after eclosion (Figure 12).

**Figure 12: Reduced Lifespan in Kat-60L1 Mutant Adults**

Graph comparing adult lifespan between WCS control and $kat-60L1^{BE6/PBac}$ mutant animals. 50% of $kat-60L1$ adults die by 11-15 days after eclosion, compared to only 3% of WCS controls.

### 2.3 Kat-60L1-short is Necessary in the Dendrite for Proper Nocifensive Responses Mediated by Multidendritic Sensory Neurons

Given the established role of the class IV da neurons in mediating mechanical and thermal nocifensive responses (Hwang et al., 2007), we used a mechanical
nociception assay to determine whether kat-60L1 mutations affect class IV neuron function. We also carried out a light-touch assay and explored a role for Kat-60L1 in chordotonal organ function in the Appendix. In the mechanical nociception assay, larvae are delivered a harsh touch by depressing the dorsal body wall with a 50mN Von Frey fiber (Tracey et al., 2003). Wild-type larvae typically (70% of animals) responded to this stimulus with nocifensive escape locomotion (NEL), in which the animal rotates around its long body axis. In contrast, kat-60L1BE6/PBac larvae were less sensitive to the noxious mechanical stimulus, exhibiting NEL only 40% of the time (Figure 13A).

To pinpoint whether this mechanical nociceptive defect originated in the class IV da neurons, we utilized the GAL4/UAS system (Brand and Perrimon, 1993) to drive tissue-specific expression of kat-60L1-RNAi, as well as Dicer2 (Dietzl et al., 2007), under the control of ppk1.9-GAL4, which drives expression uniquely in the class IV neurons (Ainsley et al., 2003). Notably, this manipulation phenocopied the kat-60L1BE6/PBac mutants, indicating that loss of Kat-60L1 solely in class IV da neurons reduces sensitivity to mechanical nociception to levels equivalent to the genetic loss of function mutant (Figure 13B).

We then assayed kat-60L1BE6/PBac mutant larvae for their response to noxious heat, also mediated by the class IV da neurons (Hwang et al., 2007). Wild-type third-instar larvae touched with a high temperature probe of 46°C initiated NEL within the first 2 to
3 seconds of stimulation. In contrast, $kat-60L1^{BE6/PBac}$ mutant larvae were severely delayed in their response, often requiring up to 10 seconds (Figure 13C). This insensitivity was maintained at a lower threshold temperature of 42°C, indicating an overall failure to respond to noxious temperatures (Figure 13D). Class IV neuron-specific RNAi knockdown of $kat-60L1$ expression again phenocopied the $kat-60L1$ genetic mutant results, providing further evidence that the site of action for Kat60-L1 in regulating this behavior is localized to this neuronal subset (Figure 13E).

To further confirm that the mutations in $kat-60L1$ were responsible for the observed nociceptive phenotypes, we generated an N-terminal Venus-tagged $UAS-kat-60L1$ rescue transgene. $kat-60L1^{BE6/PBac}$ larvae that also expressed the short isoform cDNA transgene specifically in class IV neurons were assayed for rescue of the $kat-60L1$ mutant behavior phenotypes. In both thermal and mechanical nociceptive assays, these larvae displayed nocifensive responses indistinguishable from controls (Figure 13A, 13F). Together, these data demonstrated that the mutation in $kat-60L1$ was indeed responsible for the defective nociception phenotypes observed in the mutant animals. Furthermore, the results showed that the site of action requiring $kat-60L1$ for nociception was the class IV da neurons.
Figure 13: Kat-60L1 is Necessary for Proper Nocifensive Responses Mediated by Class IV Neurons
(A-B) Mechanical nociception assay. (A) Control larvae subjected to a noxious mechanical force typically display an escape roll 70% of the time, whereas kat-60L1BE6/PBac mutant larvae roll only 40% of the time (p<0.5). Rescue animals display a completely wild-type avoidance response, similar to controls (p<1x10^-4). (B) Expressing kat-60L1 RNAi specifically in class IV da neurons phenocopies kat-60L1BE6/PBac insensitivity to mechanical nociception compared to ppk1.9-GAL4 controls (p<1x10^-4). (C-F) Thermal nociception assay. (C) kat-60L1BE6/PBac mutant larvae subjected to a high temperature thermal probe of 46°C are significantly delayed in their response compared to WCS controls (p<1x10^-4). (D) When subjected to a thermal probe at the threshold temperature of 42°C, kat-60L1BE6/PBac mutant larvae take significantly more time to exhibit an escape response compared to WCS controls as seen by the rightward-shifted distribution of mutant animals (p<1x10^-4). (E) Specific expression of kat-60L1 RNAi in class IV da neurons phenocopies kat-60L1BE6/PBac insensitivity to noxious heat compared to ppk1.9-GAL4; Dicer2 controls (p<1x10^-4). (F) Specific expression of Venus-kat-60L114 in class IV da neurons in the kat-60L1BE6/PBac mutant background rescues the thermal insensitivity phenotype to control levels. In this and all subsequent figures, error bars indicate the standard error of mean (SEM); p values were calculated using a Student’s t test except for thermal nociception experiments, which were calculated using a nonparametric rank-sum Wilcoxon test, and *p<0.05, **p<0.01, ***p<0.001.

To test whether Kat-60L1 is broadly required for neuronal excitation or functions in the dendritic compartment, we triggered NEL optogenetically rather than mechanically in kat-60L1 mutants, thus bypassing the dendritic requirement for sensory function in this behavior. We utilized optogenetically triggered NEL using Channelrhodopsin-2YFP (ChR2::eYFP) under the control of ppk1.9-GAL4. Exposure of these animals to blue light activates class IV da neurons and triggers NEL (Hwang et al., 2007). The optogenetically activated NEL response bypasses normal mechanical transduction mechanisms but still requires factors downstream of transduction such as the voltage-gated sodium channel Para (Siddiqi and Benzer, 1976; Wu and Ganetzky,
In control larvae expressing ChR2::eYFP in class IV nociceptors, the NEL behavior was seen in 69% of animals in response to blue light illumination. This was reduced to 21% when para RNAi and ChR2::eYFP were simultaneously expressed in class IV neurons (Figure 14). Knockdown of kat-60L1 by RNAi within these sensory neurons did not affect ChR2::eYFP-triggered escape behavior (Figure 14), indicating that Kat-60L1 activity is not necessary for steps downstream of transduction but functions at the level of the dendrite for the class IV neuron-mediated nocifensive response. Thus, the general excitability and presynaptic function of these sensory neurons are intact and our data support a dendrite-specific role for Kat-60L1 function.
RNAi knockdown of kat60-L1 does not impair blue-light ChR2-triggered activation of nociception behavior, which was seen in 72% of larvae versus 69% of controls (p>0.6) (control genotype ppk1.9-GAL4 UAS-ChR2::eYFP/+; UAS-dicer-2/+). In contrast, knockdown of para by RNAi dramatically reduced the response to blue light stimulation (p<1x10^-4).

2.4 Kat-60L1 is Required for the Complex Morphology of Class IV Dendritic Arborization Neurons

To understand the cell biological defects underlying compromised class IV neuron function in kat-60L1 mutants, we examined the dorsal cluster dendritic arbors marked with ppk1.9-GAL4 UAS-mCD8::GFP in the kat-60L1^{BL6/PBac} background (Figure 15A-C). Class IV dendrite arbors normally exhibit complex branching and extensive coverage of the overlying epidermal wall. Lack of kat-60L1 resulted in large gaps, both
between neighboring class IV dendritic arbors and within the arbor of an individual neuron. We measured dendrite arbor coverage of each neuron by quantifying the area or “white space” that lacked any portion of a dendrite branch using an overlaid grid of 250 μm² squares (Jinushi-Nakao et al., 2007). \textit{kat-60L1} loss of function larvae showed a 26% reduction in the grid squares containing dendrite segments as compared to control neurons, which showed dendrites in 98% of the squares in the grid (Figure 15D). To determine the source of the reduced coverage we counted the number of dendrite termini in the class IV da neuron arbors and observed a 22% reduction in \textit{kat-60L1}^\text{BE6/PBac} mutants compared to controls (Figure 15E). In addition, average lengths of dendrite termini exhibited a 29% decrease in \textit{kat-60L1}^\text{BE6/PBac} mutants compared to controls (Figure 15F and Figure 15A-C insets).

Although terminal branches appeared most affected, closer analysis of branching patterns using Strahler analysis (Grueber et al., 2002) revealed an overall reduction of branches in more proximal regions as well. \textit{Kat-60L1}^\text{BE6/PBac} mutants exhibited a significant reduction of branches in all orders except for those most proximal (near the cell body), compared to controls (Figure 15G). Kat-60L1 thus regulates both branch number and length of class IV dendrites to establish complete coverage of the overlying epidermis. Expressing a single copy of \textit{UAS-Venus-kat-60L1^14} specifically in class IV neurons of \textit{kat-60L1}^\text{BE6/PBac} larvae completely restored dendritic arbor morphology (Figure
15C-G), confirming that loss of *kat-60L1* within these neurons is responsible for the observed mutant phenotypes.

To determine whether the morphological defects were specific to the dendritic compartment or affected the overall development of the neuron, we observed class IV axonal projections. Dorsal and ventral class IV axons extend from the cell body near the larval epithelium to the ventral nerve cord (VNC) in the central nervous system. In the VNC, class IV axons form a ladder-like pattern due to projections within the longitudinal and contralateral tracts that cross the midline via commissures (Grueber et al., 2007). *ppk1.9-GAL4 UAS-mCD8::GFP*-labeled axonal projections within the VNC in *kat-60L1BE6/PBac* mutant animals showed no gross alterations in the appearance of commissures or the longitudinal axon tracts compared to controls (Figure 15H), suggesting that the *kat-60L1* loss of function defects are specific to the regulation of the dendritic arbors. From these results, we conclude that Kat-60L1 function is not required for the axonal projections of larval class IV da neurons but it is required to establish their highly branched dendritic arbor and nocifensive responses.

To investigate the possibility that Kat-60L1 plays a direct role in specifying the distinctive complexity of the class IV arbors, we used laser-capture microdissection and microarray analysis to compare the genetic profile of class IV da neurons to the very simply branched class I da neurons. The isolated RNAs from each set of samples were
amplified and then used to probe Affymetrix microarrays. These experiments revealed that class IV da neurons abundantly express kat-60L1 transcript. Indeed, kat-60L1 was among the most highly enriched transcripts, expressed at 79-fold greater levels in class IV neurons relative to class I neurons (Fig. 15I). Genes known to be expressed at elevated levels within the class IV neuronal subset such as pickpocket, gustatory receptor 28b, cut and knot/collie were also found to be enriched, providing validation of these microarray results (Fig. 15I). The dramatic enrichment of Kat60-L1 transcript in this subset of da neurons suggests that kat-60L1 gene function is crucial to their complex branching morphology.
Figure 15: Kat-60L1 is Required for Dendrite Complexity and is Enriched in Class IV Neurons.
(A-C) Comparison of kat-60L1 mutant and wild-type dorsal cluster class IV da neurons from wandering third-instar larvae in a ppk1.9-GAL4, UAS-mCD8::GFP/+ background. (A) Representative arbors from control, (B) kat60-L1BE6/PB mutant and (C) kat60-L1 genetic rescue larvae. Scale bar, 50 µm. (D-G) Comparison of (D) dendritic field coverage (p<1x10^-4), (E) total terminal branch number (p<0.02), (F) terminal branch length (p<1x10^-4), and (G) dendritic arbor complexity as measured by Strahler analysis between control, mutant and rescue animals (p<0.02 or less for orders 1-3). (H) Representative images of ppk1.9-GAL4 UAS-mCD8::GFP-labeled axons in third-instar larval ventral nerve cords from wild-type (top) and kat60-L1BE6/PB mutant (bottom) larvae appear identical. Scale bar, 50 µm. (I) kat-60L1 is highly enriched in class IV da neurons relative to class I neurons (p<1x10^-3, 1-way ANOVA Welch t-test). Genes known to be expressed in class IV neurons, pickpocket1, gustatory receptor28b, cut and collier, are shown for reference (p<0.02).

2.5 Kat-60L1 is Required During Late Larval Stages for Class IV Dendrite Arbor Outgrowth

Kat-60L1 is expressed in the embryo, suggesting a possible early requirement during the initial developmental branching of class IV dendrite arbors. To determine when Kat-60L1 regulates class IV dendrite formation, we conducted a developmental time course. By analyzing mutant and control arbors at discrete time points, we sought to distinguish whether Kat-60L1 is required for arborization throughout dendritic development, functions only at a discrete developmental time point, or if it regulates the maintenance of formed branches rather than their growth. Branching of class IV neuron dendrites begins ~16 hrs after egg lay (AEL) during mid-embryogenesis. Class IV arbors then undergo a rapid growth stage to tile and provide proper field coverage of the body wall early in larval development. Between 48 hr AEL (the second-instar stage) and 120
hr AEL (prior to metamorphosis), a larva more than triples in body length and the dorsal area covered by class IV arbors expands more than 6-fold, a process referred to as dendritic scaling that requires rapid branching (Parrish et al., 2009).

We compared dendritic arbors of kat-60L1 mutants to wild-type controls from early second-instar larvae (48 hrs AEL), early third-instar larvae (72 hrs AEL) and late wandering third-instar larvae (120 AEL). Boxes of the grids that were overlaid on the dendrite arbors at the different stages were scaled in size relative to the entire arbor so that each box represented approximately 0.12% of the total area. At 48 hrs AEL, kat-60L1 mutants showed a slightly lower average dendritic coverage compared to controls (89% versus 93%) (Figure 16A, 16A’, 16D). Twenty-four hours later during the first day of the third-instar stage, no significant difference in dendritic coverage was observed in mutant larvae compared to controls (Figure 16B, 16B’, 16D). However, by the late-wandering third-instar stage, morphological differences were equivalent to the kat-60L1\textsuperscript{BE6/PBac} mutant dendrite arbors reported above, with kat-60L1 mutants displaying on average only 69% coverage, compared to 93% coverage in wild-type controls (Figure 16C, 16C’, 16D). These data indicate that Kat-60L1 is not required for early dendritic branch formation in the larva, nor does it play a role in late-stage maintenance of the class IV arbor. Rather, Kat-60L1 is required for dendritic outgrowth late in larval
development, during the third-instar stages when arbors are rapidly increasing in complexity in order to scale to a rapidly growing larva (Parrish et al., 2009).

Figure 16: Kat-60L1 is Required for Late Larval Development of Class IV Neuron Dendrites.

(A-C) Confocal images of class IV dendritic arbors from control (left) and kat-60L1 BE6/PBac mutant larvae. Representative arbors from (A) second-instar, (B) day one third-instar, and (C) day three third-instar larvae. (D) Dendritic coverage measured from 48-120 hr AEL for kat-60L1 BE6/PBac mutant and control larvae shows a strong reduction late in larval development (p<0.05, p>0.05, p<1x10^-4 for 48hr, 72 hr and 120 hr AEL, respectively). Scale bars, 50µm.
2.6 Overexpression of Kat-60L1 in Class IV Da Neurons Leads to a Reduction in Dendritic Branching

Given that loss of Kat-60L1 reduced branch number and terminal branch length in class IV dendritic arbors, we tested whether Kat-60L1 overexpression is sufficient to perturb branch number and/or branch length. We reasoned if the loss of function phenotype is due to a lack of microtubule severing activity, then one might expect that too much severing would also have deleterious effects on dendritic branching, as is the case with the overexpression of spastin, which causes a reduction in class IV arborization (Ye et al., 2011). Expressing one copy of Venus-kat-60L1 in class IV arbors revealed a cytoplasmic distribution of the protein throughout main branches and a faint distribution in some terminal branches of the dendritic arbor (Figure 17). This subcellular expression of a microtubule severing protein is consistent with that of Spastin cytoplasmic expression (Du et al., 2010). The majority of microtubule severing proteins is believed to exist in monomeric form, while only a small fraction of ATP-bound hexamers bind microtubules.
Figure 17: Cytoplasmic Distribution of Venus-Kat-60L1

(A-C) A representative class IV dendritic arbor from a larvae expressing Venus-kat-60L1<sup>14</sup> under the control of the multidendritic neuron driver, MD-GAL4 immunostained for (A) GFP to reveal Venus-Kat-60L1 expression and (B) Futsch to reveal stable neuronal microtubules. (C) A merged image of channels in A,B reveals Kat-60L1 is cytoplasmic in distribution and does not specifically colocalize with stable microtubules.

Expressing two copies of Venus-kat-60L1<sup>14</sup> in class IV neurons often resulted in tufted terminal arbors with short branches (compare Figure 18B to 18A). White space measurement revealed reduced dendritic coverage, similar to arbors lacking Kat-60L1 (Figure 18E); however, this did not arise from a change in terminal branch numbers (Figure 18F). Rather, reduced terminal branch length, as well as their distally clustered distribution, accounted for the loss in coverage of these branches (Figure 18G). To determine whether this was a general effect of Kat-60L1 overexpression, we ectopically expressed the gene instead within the more simply branched class I ddaE neurons and found dendritic morphology grossly unaffected (compare Figure 18D to 18C). Branch number was unchanged relative to wild-type class I arbors (Figure 18H); however, a
slight increase in branch length was observed (Figure 18I). Therefore, while high levels of Kat-60L1 decreased dendritic coverage and terminal branch length in class IV arbors, ectopic Kat-60L1 induced longer branches in class I arbors, demonstrating that Kat-60L1 activity affects branching in a cell–specific manner. Whether Kat-60L1 acts intracellularly in class IV dendrites at preexisting branch points to induce process growth and control branch length, more proximally to the cell body, or throughout the arbor remains to be determined.
Figure 18: Ectopic Kat-60L1 Expression in Class IV Neurons, but not Class I Neurons Leads to Reduced Branch Length
(A-B) Comparison of dorsal cluster class IV ddaC neurons from wandering third-instar larvae expressing wild-type and ectopic levels of kat60-L1. (A) Representative wild-type class IV arbor. (B) Representative class IV arbor from a larva with ectopic kat-60L1 in class IV neurons driven by ppk1.9-GAL4. (C-D) Comparison of dorsal cluster class I neurons from wandering third-instar larvae expressing wild-type and ectopic levels of kat60-L1. (C) Representative wild-type class I arbor and (D) representative class I arbor from a larva with ectopic kat-60L1 in class I neurons driven by 2-21-GAL4. (E-G) Bar graphs comparing (E) dendritic coverage (p<0.03), (F) the total terminal branch number (p>0.5), and (G) terminal branch length (p<1x10^-4) between wild-type control larvae and those expressing ectopic kat-60L1 in class IV neurons. (H-I) Bar graphs comparing (H) the total terminal branch number (p>0.5) and (I) terminal branch length (p<1x10^-4) between wild-type control larvae and those expressing ectopic kat-60L1 in class I neurons.

2.7 Kat-60L1 Promotes Dynamic Microtubule Growth in Class IV Dendritic Arbors

Given the requirement of Kat-60L1 in the microtubule-rich class IV arbors for proper outgrowth and its proposed function as a microtubule-severing protein, we examined whether Kat-60L1 regulates microtubules within the larval class IV dendrites. Using an antibody directed against the Drosophila MAP1B ortholog Futsch, a neuron-specific microtubule binding protein and a marker of stabilized microtubules, we observed no difference in the intensity or distribution of this microtubule population between kat-60L1 mutants and wild-type controls (compare Figure 19B1-B3 to 19A1-A3). However, Futsch immunostaining extends only to proximal branches near the cell body (Figure 19A2) and is not detected in distal branches where the most striking kat-60L1 morphological phenotype is observed.
Figure 19: Kat-60L1 Mutants Exhibit No Change in the Distribution of Futsch-labeled Microtubules in Class IV Dendrites.

(A-B) Comparison of Futsch-positive microtubule distribution in class IV neurons from wild-type control larvae (A1-A3) and kat-60L1^{BE6/PBac} larvae (B1-B3) in a ppk1.9-GAL4 UAS-mCD8::GFP/+ background. (A1-A3) Representative arbor from a control third-instar wandering larva. (B1-B3) Representative arbor from a kat-60L1^{BE6/PBac} third-instar wandering larva. (A3, B3) Merged images of GFP and Futsch staining reveal no significant change in the levels within dendrites between control and kat-60L1^{BE6/PBac} mutant arbors. Scale bar, 50 µm.

Immunostaining of the stable, acetylated microtubule population labeled somewhat more distal branches in comparison, but also failed to reveal any difference between genotypes (compare Figure 20D-F to 20A-C). These results suggest that
stabilized microtubules within da neurons are localized proximally in the cell and are not grossly affected by loss of Kat-60L1.

![Figure 20: Kat-60L1 Mutants Exhibit No Change in the Distribution of Acetylated-tubulin in Class IV Dendrites.](image)

(A-B) Comparison of acetylated-tubulin-positive microtubule distribution in class IV neurons from wild-type control larvae (A-C) and kat-60L1<sup>BE6/PBac</sup> larvae (D-F) in a ppk1.9-GAL4 UAS-mCD8::GFP/+ background. (A-C) Representative arbor from a control third-instar wandering larva. (D-F) Representative arbor from a kat-60L1<sup>BE6/PBac</sup> third-instar wandering larva. (C, F) Merged images of GFP and acetylated-tubulin staining reveal no significant change in levels within dendrites between control and kat-60L1<sup>BE6/PBac</sup> mutant arbors. Scale bar, 50 µm.

We next examined the dynamic population of microtubules within the class IV arbors. To achieve this we imaged the microtubule plus-end tracking protein (+TIP) EB1
(Stone et al., 2008) in living whole-mounted animals. EB1 proteins exchange rapidly at growing microtubule tips (Zanic et al., 2009), thus providing an ideal marker of growing filaments. The concentrated pool of exchanging EB1-GFP at growing microtubule tips, often described as a “comet,” can be visualized and tracked along polymerizing microtubules. \textit{UAS-EB1-GFP} was expressed using the class IV specific driver \textit{477-GAL4}, which drives expression at a lower level compared to \textit{ppk1.9-GAL4} (Williams et al., 2006). Moderately low expression of EB1-GFP is required to distinguish individual comets among the background of EB1-GFP monomer in branches. Importantly, \textit{kat-60L1\textsuperscript{BE6/PBac}} mutants exhibited fewer than half as many EB1 plus-end “comets” compared to controls (Figure 21A-C). Average comet speed did not significantly differ, moving 0.1 \textmu m/sec in both genotypes (p>0.1), values consistent with speeds observed elsewhere (Mattie et al., 2010). Furthermore, there was no difference between genotypes in the number of EB1-GFP comets in the axons of these neurons, which averaged \textasciitilde1/120\textmu s (p>0.8), consistent with our data thus supporting a dendrite-specific role for Kat-60L1.

We propose a model whereby Kat-60L1 promotes dendritic branching by upregulating dynamic microtubule growth in class IV arbors. Therefore, we wanted to test if increased levels of Kat-60L1 lead to changes in EB1 comet number. We hypothesized that increased Kat-60L1 levels would lead to increased severing, possibly increasing the number of polymerizing microtubules, evidenced by elevated numbers of
EB1 comets. Alternatively, increased Kat-60L1 levels, if too high, could result in a reduction of microtubules due to excessive severing, leading to a decrease in growing microtubules and a reduction in EB1 numbers. To address this, we imaged EB1 comets in animals expressing Venus-kat-60L1 in class IV neurons under the control of 477-GAL4, compared to wild-type controls. We saw a slight increase in EB1 comet number in dendritic arbors overexpressing kat-60L1 (0.37 comets/µm) compared to controls (0.31 comets/µm); however, this increase was not statistically significant (Figure 21D). There are several possible explanations why increased Kat-60L1 did not lead to a change in EB1 comet number. The class IV driver, 477-GAL4 is known to drive expression of genes at much lower levels compared to the ppk1.9-GAL4 driver, levels possibly too low to invoke a robust kat-60L1 overexpression phenotype. Alternatively, there could be other rate-limiting factors to microtubule polymerization superseding elevated levels of Kat-60L1. Despite our inability to invoke an overexpression phenotype with labeled EB1, our loss of function data support a novel mechanism for Kat-60L1 in upregulating the number, but not the rate, of growing dendritic microtubules in late stages of class IV arbor development.
Figure 21: Microtubule Dynamics are Reduced in Kat-60L1 Mutant Dendritic Arbors.

(A-B) Representative time-lapse images of EB1::GFP comets (arrowheads) in class IV dendrites of (A) control, and (B) kat60-L1BE6/PBac third-instar larvae. Images were collected at 63x magnification over consecutive 5-second intervals. Arrow depicts the direction of the cell body, towards which most EB1 comets travel. Scale bar, 5 µm. (C) kat60-L1BE6/PBac mutants had fewer than half as many EB1 comets/µm compared to controls (p<1x10^-4).

(D) Arbors overexpressing Venus-kat-60L1 under the control of 477-GAL4 exhibited no change from wild-type controls in the number of EB1 comets in class IV arbors (p>0.2).

2.8 Discussion and Conclusions

We have shown a dendrite-specific requirement for the microtubule severing protein Kat-60L1 in the establishment of the complex morphology of class IV da
neurons. Loss of Kat-60L1 results in fewer and shorter dendrite branches. Furthermore, its activity is critical only during late larval stages, when rapid outgrowth of the higher order branches that make up the distal dendritic arbor occurs. Together with the striking enhancement of Kat-60L1 expression in class IV neurons, our data provide strong evidence for Kat-60L1 as a defining component of complex class IV dendrite morphology.

Since the class IV da neurons mediate mechanical and thermal nociception in larvae (Hwang et al., 2007), the morphological consequences of the kat60-L1 mutation also allowed us to analyze the degree of correlation between dendritic form and function in these neurons. Reduced NEL behavioral responses paralleled the reduced arbor complexity in mutants, but could be bypassed using optogenetic stimulation, demonstrating that axon function was normal. Functional defects in mutant class IV neurons thus arose specifically from the dendritic compartment, where the morphological reduction in arborization was observed. Dendrite form and function are therefore tightly correlated in class IV neurons, with full nocifensive function requiring complete dendritic coverage by terminal branch outgrowth.

Given that Kat-60L1 is a microtubule severing protein, we hypothesized that microtubule distribution in dendrites might be susceptible to its loss. Although differences in total or subpopulations of stable microtubules were not detectable by
immunostaining, we observed a robust reduction in the growing microtubule population by live imaging of individual polymers. *Spastin* loss yields a similar reduced microtubule phenotype in the presynaptic terminals of the larval neuromuscular junction, supporting the proposal that Spastin severs microtubules to generate new fragments from which additional plus-end polymerization can occur (Sherwood et al., 2004; Roll-Mecak and Vale, 2006). Similarly, our EB1-GFP data support a model in which Kat-60L1 generates additional growing microtubule polymers by creating severed fragments, and that this in turn promotes new dendrite outgrowth. The requirement for Kat-60L1 during late-stage larval dendritic growth further suggests that Kat-60L1-mediated microtubule severing may be utilized during development to enable rapid expansion of the microtubule population during periods of extensive branch arborization, when the slow transport of microtubule polymers to distant branch sites may be insufficient to support the required rate of growth.

Interestingly, Kat-60L1 appears to play opposing roles at two different stages of development within the same cells, promoting net microtubule gain during dendritic outgrowth in larvae as shown here, but net microtubule loss during dendritic pruning in pupae (Lee et al., 2009). Such differences may arise from regulation of Kat-60L1 itself, or from differences between microtubules and their associated proteins at each developmental stage. A reasonable prediction would be that in comparison to larval
dendrites, pupal dendrites are rich in microtubules susceptible to Kat-60L1 severing, and lacking in molecules required for fragment stabilization and subsequent polymerization. The microtubule associated protein Tau is thought to regulate the susceptibility of microtubules to severing by Katanin-60 (Qiang et al., 2006; Yu et al., 2008); other MAPs, or post-translational modifications of microtubules, may serve to differentiate microtubule susceptibility to net polymerization or depolymerization.

The differential consequences of *kat-60L1* expression at different stages provide evidence that the microtubule-severing protein family evolved to allow for unique and precise regulation of the microtubule cytoskeleton. The identification of Kat-60L1 as a specific regulator of dendrite arbor complexity adds to our understanding of the diverse ways in which this family of proteins is deployed, and supports a model in which microtubule severing is necessary in contexts necessitating rapid changes in microtubule growth or shrinkage across multiple developmental contexts.

### 2.9 Future Directions

We have shown that Kat-60L1 promotes microtubule growth and subsequent dendrite branching in class IV neurons and we propose a model whereby Kat-60L1-severing creates new short microtubule pieces that serve as templates for polymerization. We do not know if Kat-60L1 acts locally near growing branches or proximal to the cell body, which would require transport of severed polymers to sites of
branching. One way to address this question is to determine Kat-60L1 subcellular localization. We recently ordered a Kat-60L1 monoclonal antibody from Abmart Inc. We plan on using this antibody to conduct immunostaining on endogenous Kat-60L1 in larval class IV neurons. Observing where Kat-60L1 localizes at a subcellular level in the neuron will provide insight into where it mainly functions. However, if the majority of Kat-60L1 protein exists in a monomeric, unbound state as is predicted for Spastin, then we may see Kat-60L1 immunostaining generally within the class IV arbor, similar to what we observe when Venus-Kat-60L1 is expressed in these neurons (Figure 17, section 2.6). If only a small amount of active and microtubule-bound Kat-60L1 acts at branch sites then we may not be able to detect this protein population by immunostaining and confocal imaging. The subcellular localization pattern of Kat-60L1 would help us better understand its molecular function to promote dendritic branches and allow us to make more accurate predictions and hypotheses about interactions with other proteins expressed in the arbor.

2.10 Materials and Methods

*Drosophila Stocks and Crosses:* The genetic deletion, *Df(3R)kat-60L1*BE6 was created by imprecise excision of the neighboring EP, *EY21697* in the gene, *CG2051*. Approximately 350 potential deletion lines were screened by PCR to uncover one deletion in *kat-60L1*, denoted *BE6*. A combination of primer walking and sequencing was used to delineate
the breakpoints of Df(3R)kat-60L1BE6 to a 498 bp region, 3R:1609500-1609997 in the second intron of kat-60L1 and 3R:1,614,857 where part of the EP remains in the genomic DNA of CG2051.

For kat-60L1 loss of function analysis, we crossed pppk1.9-GAL4 UAS-mCD8::GFP;; kat-60L1BE6/TM6b to kat-60L1P{Bac} c00564/TM6b.

VDRC kat-60L1 RNAi lines: 31599, 31598, 108168. For kat-60L1 RNAi experiments we crossed pppk1.9-GAL4 UAS-mCD8::GFP to a double RNAi stock, kat-60L131599 RNAi; kat-60L131598 RNAi (III) or to kat-60L1108168 RNAi.

For rescue experiments, we crossed pppk1.9-GAL4 UAS-mCD8::GFP;; Df(3R)kat-60L1BE6/TM6b to kat-60L1P{Bac} c00564 UAS-Venus-kat-60L114/TM6b to obtain genetic rescue animals of the genotype, pppk1.9-GAL4 UAS-mCD8::GFP/+;; Df(3R)kat-60L1BE6/kat-60L1P{Bac} c0056 UAS-Venus-kat-60L114 (III).

For live EB1-GFP analysis, we crossed 477-GAL4 UAS-EB1-GFP/CyO;; kat-60L1P{Bac} c00564/TM6b to Df(3R)kat-60L1BE6/TM3 Actin5c-GAL4 UAS::eGFP to obtain larvae of the genotype, 477-GAL4 UAS-EB1-GFP;; kat-60L1P{Bac} c00564/ Df(3R)kat-60L1BE6. Control larvae were obtained from crossing 477-GAL4 UAS-EB1-GFP/CyO to WCS.

Drosophila stocks were provided by the Bloomington Stock Center (Bloomington, IN) and the Vienna Drosophila RNAi Center (Vienna, Austria).
**RT-PCR for Developmental Analysis of *kat-60L1* Isoforms:** Primers were designed to amplify cDNA of both the *kat-60L1* short and long isoforms. Forward *kat-60L1-short* primer in exon 1: AACGACTGGTGAGCAAAAT. Forward *kat-60L1-long* primer in exon 1: CGGCACACTCCATACCACT. Each was used with the shared reverse primer in the second exon: GCATTAGAGCCCAGGTTG. The short isoform primers amplify a predicted cDNA-specific 344 bp product and the long isoform primers amplify a predicted cDNA-specific 245 bp product.

RNA was isolated by Trizol extraction from the following tissues: stage 11-17 embryos, third instar larval brains, adult heads, adult testes and adult ovaries; cDNA was generated using the Bioline cDNA kit (Bioline USA Inc.Tauton, MA 02780).

**RT-PCR to Characterize *kat-60L1* Loss of Function Alleles and RNAi:** A forward primer specific to *kat-60L1* cDNA, spanning exon 3 and 4, excluding intronic sequence (illustrated in Figure 11A), GACAATACTCCAGGAAGCAGT and a reverse primer in exon 5, ACGTTGCTTTATGTCGGATCC were used for all RT-PCR experiments to characterize expression levels of *kat-60L1* loss of function mutant and RNAi backgrounds. RT-PCR was performed on samples of adult heads from WCS controls and *kat-60L1* mutant animals and from larval brains of control and animals with either *kat-60L1* or spastin RNAi driven by the ubiquitous driver, e22c-GAL4, sqh-GAL4 and UAS-dicer2. RNA was isolated and cDNA was generated as above.
Laser Capture Microdissection and Microarray Analysis (experimentation by Jason Caldwell and W. Dan Tracey): Laser capture microdissection and RNA isolation was performed by the Duke University Laser Capture Microdissection core facility under the operation of the NIH Microarray Consortium. Four independently generated RNA samples were generated for both class I and class IV neurons (approximately 50-60 neurons/per sample). The isolated RNA samples were then sent to the NIH Neuroscience Microarray Facility at the Translational Genomics Research Institute (TGEN). The RNA was amplified and labeled and then used to probe Affymetrix Drosophila Genome 2.0 Microarrays. In order to compensate for differences in washing and staining, and to allow for comparison across arrays, global scaling was used in Affymetrix GCOS v1.4 software to bring the overall intensity of each array to a value of 150. Using the Affymetrix presence and absence call algorithm the Kat60-L1 RNA was scored as present in all four class IV da neuron samples with raw intensity scores ranging from 193-700. In contrast, Kat60-L1 was scored as absent in three of the class I samples (intensity 0.9-2.7). In the fourth class I sample, Kat60-L1 was scored as present with an intensity score of 44. Agilent Gene Spring Software was used for statistical analysis. Microarray processing and analysis was performed by Dr. Jill Gerber of TGEN.
Behavioral Analysis

To perform the nociception assays, approximately 6-10 female flies were allowed to lay eggs for 4 days at 25°C and 75% humidity. On approximately the sixth day, wandering third-instar larvae were rinsed out of the vial and into a plastic 60 mm Petri dish. Excess water in the dish was aspirated such that the larvae remained moist but were not floating.

For thermal nociception experiments, larvae were treated and tested using a noxious heat probe, according to (Caldwell and Tracey, 2010; Hwang et al., 2007). Briefly, the stimulus was delivered by touching the larvae laterally, in abdominal segments four, five, or six. Each larva was tested only once and discarded. The response latency was measured as the time interval from the point at which the larva was first contacted by the probe until it initiated the rolling movement (the beginning of the first, complete 360° roll). Statistical significance was determined using the non-rank sum Wilcoxon test.

Mechanical nociception assays were similarly carried out according to Hwang R.Y. and Zhong L., et al. 2010. Briefly, wandering third-instar larvae were stimulated with a 50 mN calibrated Von Frey filament. Von Frey filaments were made from Omniflex monofilament fishing line (6 lb test, diameter 0.009 inch [0.23 mm]). Fibers were cut to a length of 18 mm and attached to a pulled glass pipette such that 8 mm of the fiber protruded from the end and 10 mm anchored the fiber. Noxious stimuli were delivered
by the rapid depression and release of the fiber on the dorsal side of a larva. A positive response was scored if at least one nocifensive roll occurred after the first stimulus. For thermal and mechanical, each genotype underwent 3 trials where at least 5 vials of crosses were established to obtain a sample size of approximately 40 larvae for a given trial. The averaged results from the trials for a given genotype were used to obtain a behavioral score and to generate the standard error of the mean (SEM), and statistical analyses were performed using the Student’s T test.

**Optogenetic Activation**

In cages, approximately six virgin female flies of the genotype, **ppk1.9-GAL4, UAS-ChR2::eYFP;; UAS-dicer-2/K87** were crossed to approximately 10-15 males of the desired RNAi line. VDRC lines used were: **para (6131)** and **kat-60L1 (108168)**. Females were allowed to lay eggs for 24 hr with a dollop of yeast paste containing either 500 µM all trans-Retinol (atR) or no atR for control experiments. The larval progeny were allowed to develop and feed on the yeast paste for an additional 72 hr in the dark. Larvae were then stimulated with blue light (460-500nm) with the Hg light source of a Leica MZ16 FA stereomicroscope. Blue light pulses were manually controlled and lasted several seconds. A positive nocifensive roll was scored if the larva completed at least one revolution (360°) in response to a blue light pulse. The averaged results from three trials for a given genotype were used to obtain a behavioral score and to generate the
standard error of the mean (SEM), and statistical analyses were performed using the Student’s T test.

Gateway Cloning of UAS-Venus-kat-60L1

The kat-60L1-long (RA) cDNA clone (accession number, AY051591) was obtained from the Drosophila Genomics Resource Center and was amplified by PCR and gel extracted for purification. The forward primer included the ATG start site: CACCATGCGGGTCGAGGA; the reverse primer included the 5’ UTR: GGGTGCTGGGCCAGCAGCCCGTAC, and amplified a 1.84 kb product. This product was cloned into a pENTRM™/D-TOPO® entry vector (Invitrogen) and verified by sequencing. Recombination into the pTVW Gateway vector (Invitrogen) was carried out according to the manufacturer’s instructions. The final N-terminally Venus-tagged kat-60L1-long vector, UAS-Venus-kat-60L1 was sent to Duke University Model Systems Genomics for injection and fly transformation services (Science Dr. Durham, NC 27708).

Immunohistochemistry

Antibodies used: mouse anti-GFP (1:500) (from Invitrogen), mouse anti-Futsch/22C10 (1:500) and mouse anti-Acetylated tubulin (1:500) (from Developmental Hybridoma Studies Hybridoma Bank, University of Iowa, IA). Alexa-fluor 488 or 568 fluorescent secondary antibodies (1:200) were from Invitrogen. Whole-mount immunohistochemistry on embryos was performed according to standard techniques.
Larval preparations were as follows: larvae were filleted by opening the ventral side in HL3 Ringers solution, followed by fixation for 40 minutes in 4% paraformaldehyde. The larvae were then rinsed 3 times and washed 3 x 15 minutes in PBST (0.2% Triton-X) and blocked with PBTNA (10% natural goat serum, 1% BSA and 0.01% Azide in 0.2% PBST) for 1 hr. Antibody solutions were prepared in PBTNA and the larvae were incubated with primary antibody for either 3 hrs at room temperature or overnight at 4 deg C, rinsed 3 times and washed 3 x 15 minutes in PTST. Secondary antibodies were incubated with samples for either 2 hours at room temperature or overnight at 4° C and then washed in the same fashion as for the primary antibodies. Samples were then mounted in Vectashield (Vector Laboratories, Inc. Burlingame, CA) for imaging.

**Neuron Visualization and Analysis**

Visualization of neuron morphology was carried out as described in Grueber et al. (2003) then analyzed either manually or by NeuronJ, a plugin of ImageJ software (Meijering et al., 2004). Images were collected on a Zeiss 510 confocal microscope and converted to maximum intensity projections of Z stack images using LSM Image Browser software, and brightness and contrast were adjusted using Adobe Photoshop. One to two neurons per larva from abdominal segments 3 or 4 were used for visualization and analysis and at least 5 individual larvae per genotype were used for each set of analyses. The total number of terminal dendritic branches was assessed by
manual counts to address the degree of branching. Terminal branch length was measured using the trace function of the NeuronJ. Dendritic coverage was estimated by overlaying a grid of boxes approximately 0.12% the total average arbor size, created in Image J over the arbor and calculating the number of boxes containing only white space as a percentage of total area. Strahler analysis was conducted to assess branching complexity across orders of branches. Branches were ordered and quantified, with the most distal branches from the cell body (terminal) denoted 1 and those approaching the cell body (primary branches) ordered consecutively, up to 6, terminating at the cell body. For the developmental timecourse, embryos were collected on grape juice plates from overnight collections and newly hatched first-instar larvae of both genotypes were synchronized for staging at 25° C. Beginning at the second-instar stage, larvae were collected at the appropriate time point and imaged by confocal microscopy.

Live Imaging of EB1-labeled Microtubules

All imaging of da neurons was performed on intact third-instar larvae mounted on a dried agarose pad under a coverslip. Neurons were imaged on a Zeiss Axiovision confocal microscope or a Zeiss 510 confocal microscope and images were recorded every second for 200 cycles. Movies were analyzed using ImageJ software. An EB1-labeled comet was counted only if it was detectable and tracked in consecutive frames for at least 7 seconds.
Chapter 3. Katanin p60-like 1 (Kat-60L1) and Spastin Regulate Dendritic Microtubules in Class IV Neurons Through Distinct Mechanisms

3.1 Introduction/Rationale

In *Drosophila*, lack of Spastin results in sparse microtubule bundles at distal synaptic boutons at the neuromuscular junction (NMJ), consistent with the hypothesis that severed microtubules generate free ends available for additional polymer synthesis (Sherwood et al., 2004). Interestingly, partial loss (heterozygous for a genetic deletion, *spastin*\(^{5.75/}-\)) or gain of the microtubule-severing protein Spastin also results in larval class IV dendritic arbors with reduced complexity (Figure 7 and Figure 8, respectively) (Jinushi-Nakao et al., 2007; Ye et al., 2011). Since loss of function phenotypes appeared to be very similar in class IV sensory dendritic arbors for both *kat-60L1* and *spastin*, we sought to understand the regulation for these two highly related microtubule severing proteins within the same neurons. To address this question we characterized Spastin function in class IV neurons and created *kat-60L1, spastin* double mutants for genetic analysis in class IV neurons.

3.2 Spastin is Necessary for Proper Nocifensive Responses Mediated by Multidendritic Sensory Neurons

Based on the similar morphological phenotypes of *kat-60L1* and *spastin* mutants, reduced arborization in class IV neuron dendrites, we hypothesized that similar to *kat-
mutants, reduced Spastin levels should lead to nocifensive defects. Indeed, animals deleted for one copy of fly spastin, spastin$^{5.75/+}$ larvae, exhibited comparably reduced sensitivity to both noxious mechanical and thermal stimuli (Figure 22A, 22B). This correlation between a reduction in dendritic arbor complexity and compromised nocifensive responses, also seen for kat-60L1 mutants (chapter 2, section 2.4), provides further support that the full dendritic complexity of class IV arbors, including the function of Spastin and Kat-60L1 in the class IV neurons is required for proper nocifensive responses.

Figure 22: Spastin is Necessary for Proper Mechanical and Thermal Nocifensive Responses

(A) When subjected to a constant mechanical force, WCS control larvae display an escape roll 73\% of the time, whereas spastin$^{5.75/+}$ mutant larvae roll 58\% of the time (p<1x10$^{-4}$). (B) Spastin$^{5.75/+}$ mutant larvae require significantly longer to roll in response to a 46°C thermal probe compared to controls (p<1x10$^{-4}$).
3.3 Spastin is Necessary for the General Excitability of Class IV neurons, While Kat-60L1 is Required Upstream at the Level of the Dendrite

Spastin is required not only for complete dendrite arborization (Jinushi-Nakao et al., 2007), but also for da neuron axon elaboration in the VNC (Ye et al., 2011). Thus, the behavioral defects in spastin mutants could result from the dendritic morphology perturbations, the axonal defects, or both. In contrast, loss of kat-60L1 affected dendrites without altering the central projections of the class IV da neurons (described in chapter 2, section 2.4).

To further tease apart the spastin and kat-60L1 loss of function phenotypes we tested the effects of the mutations on optogenetically triggered nocifensive escape locomotion (NEL) using Channelrhodopsin-2YFP (ChR2::eYFP) under the control of ppk1.9-GAL4 (as described in chapter 2, section 2.3 and Figure 14). Briefly, exposure of these animals to blue light activates class IV da neurons, bypassing normal transduction mechanisms and triggers NEL (Hwang et al., 2007). In control larvae expressing ChR2::eYFP in class IV nociceptors, the NEL behavior was seen in 69% of animals in response to blue light illumination. This was reduced to 21% when para RNAi and ChR2::eYFP were simultaneously expressed in class IV neurons (Figure 23). Knockdown of spastin by RNAi within these sensory neurons reduced ChR2::eYFP-triggered escape comparably, as did genetic removal of one copy of spastin (Figure 23). Thus, loss of
*spastin* causes neuronal defects that cannot be overcome by bypassing the normal upstream transduction mechanisms in the nociceptors, in contrast to loss of *kat-60L1* (detailed in chapter 2, section 2.3). This result for *spastin* is consistent with a functional requirement for Spastin not only in dendrite outgrowth, but also for normal axon development and the excitation of the class IV sensory neurons.

**Figure 23: Nocifensive Behavior Defects are Bypassed Through Optogenetic Activation of Class IV Neurons in *Kat-60L1*, but not *Spastin* Mutants.**

RNAi knockdown of *kat60-L1* does not impair blue-light ChR2-triggered activation of nociception behavior, which was seen in 72% of larvae versus 69% of controls (control genotype *ppk1.9-GAL4 UAS-ChR2:eYFP/+; UAS-dicer-2/+; p>0.6*). In contrast, knockdown of either *spastin* or *para* by RNAi dramatically reduced the response to blue light stimulation (*p<1x10^-4*).
3.4 Spastin and Kat-60L1 Function Antagonistically to One Another to Sculpt the Class IV Dendritic Arbor

Kat-60L1 and Spastin are both required for proper dendritic outgrowth of larval class IV sensory neurons, as well as proper nocifensive behavior mediated by these neurons. We therefore asked whether these two related microtubule severing proteins function redundantly or additively in the class IV neurons. To investigate redundancy or possible independent roles by Kat-60L1 and Spastin we carried out double mutant analysis. Animals that had recombined loss of function alleles for both spastin and kat-60L1 on the same third chromosome were generated and built into the ppk1.9-Gal4 UAS-mCD8::GFP background for cell biological analysis of the double mutant phenotype.

As reported previously, class IV dendritic arbors from kat-60L1 (chapter 2, section 2.4) and spastin (Jinushi-Nakao et al., 2007) loss of function animals resulted in reduced arborization and dendritic coverage (Figure 24, compare B and C to A).

Interestingly, arbors from double mutant animals, containing both the kat-60L1BE6/PBac and spastin5.75/+ mutant alleles appeared normal with full dendritic coverage, compared to control arbors (Figure 24, compare D to A). Dendritic coverage was quantified by measuring the area of white space not occupied by dendrites (as described in section 2.4) and demonstrated that the double mutant arbors displayed 91.8% coverage, comparable to 90.2% coverage in control arbors (p>0.23), as opposed to arbors from either mutant
alone, which were significantly reduced (76.9% for kat-60L1 mutants and 82.4% in spastin mutants; p<1x10^-4) (Figure 24E).

Figure 24: Spastin and Kat-60L1 Function Antagonistically to Sculpt the Class IV Dendritic Arbor

Comparison of dorsal cluster class IV da neurons from wandering third-instar larvae in a ppk1.9-GAL4, UAS-mCD8::GFP/+ background. (A) Representative arbors from control, (B) kat60-L1^{BE6/PB} mutant (C) spastin^{5.75/+} mutant, and (D) double mutant kat60-L1^{BE6/PB},

78
*spastin* 

(E) Bar graph comparing dendritic field coverage across genotypes in A-D illustrates *spastin*, *kat-60L1* double mutants exhibited normal dendritic coverage (p>0.23), while either *spastin* or *kat-60L1* single mutants exhibited reduced coverage (p<1x10^-4).

If *kat-60L1* and *spastin* function redundantly to one another we would have expected a phenotype in the double mutants no worse than either single mutant alone. In contrast, if they function similarly to one another we would have expected an additive phenotype resulting in a more severely reduced dendritic arbor in double mutants than the single mutant arbors. However, our results instead suggest that Kat-60L1 and Spastin do not simply play redundant or similar roles, but function antagonistically to one another, which is why when both genes are reduced the arbor appears restored to a wild-type morphology.

We wanted to test the possibility of a direct antagonistic interaction between Kat-60L1 and Spastin. One hypothesis is that Kat-60L1 could oligomerize with Spastin preventing normal Spastin assembly into an active, hexameric conformation. Alternatively, Kat-60L1 and Spastin could function antagonistically through indirect interactions with other molecules. To test the former hypothesis we overexpressed *spastin* simultaneously with *kat-60L1* in class IV neurons using *ppk1.9-GAL4 UAS-mCD8::GFP*. The overexpression of *spastin* leads to a dramatically reduced dendritic arbor (Figure 25B). We predicted that overexpressing *kat-60L1* in this background would
suppress the *spastin* phenotype if Kat-60L1 was directly binding to Spastin and inhibiting normal Spastin function. However, we observed no change in the *spastin* overexpression phenotype when simultaneously overexpressing *kat-60L1*, indicating that their antagonistic relationship is not direct (compare Figure 25C to B).

![Figure 25: Kat-60L1 and Spastin Interact Indirectly in Class IV Neurons](image)

(A-C) Dendritic arbors of class IV neurons from wandering third-instar larvae in a *ppk1.9-GAL4, UAS-mCD8::GFP/+* background from control animals overexpressing (OE) *kat-60L1* (A), overexpressing *spastin* (B) and simultaneously overexpressing both genes (C). We observed no modification of the spastin overexpression phenotype when overexpressing both genes. Scale bar, 20 µm.

### 3.5 Spastin, Unlike Kat-60L1, Does Not Promote Dynamic Microtubule Growth in Class IV Arbors

To better understand the differences between *spastin* and *kat-60L1* function and gain insight into their antagonistic roles in class IV neurons, we examined the effects of *spastin* loss of function on EB1-GFP comets. In striking contrast to *kat-60L1* mutants (chapter 2, section 2.7, Figure 21), *spastin*5.75+ larvae showed no significant change in the
number of comets compared to controls (Figure 26), suggesting that Spastin does not promote microtubule growth in dendrites and must regulate microtubules in a distinct manner from Kat-60L1. Consistent with the dendrite morphology results in the double mutants that demonstrate their independent function, this result provides evidence at the cytoskeletal level that Spastin functions distinctly from Kat-60L1 since spastin mutant arbors do not phenocopy the kat-60L1 mutant phenotype of reduced EB1 comets, i.e. a reduction in polymerizing microtubules.

Figure 26: Loss of Spastin Does Not Perturb Microtubule Growth in Class IV Arbors

Bar graph depicting the average number of EB1-labeled comets over branch length in spastin mutants compared to wild-type controls. No significant difference was observed between the two genotypes (p>0.42).
3.6 Spastin Regulates Stabilized, Futsch-labeled Neuronal Microtubules in Class IV Arbors

If Spastin does not promote microtubule growth as does Kat-60L1 then what role is Spastin playing on the microtubule cytoskeleton in class IV neurons? We further investigated the activities of Spastin and Kat-60L1 on microtubules by overexpressing each in class IV neurons. We observed a dramatic reduction of Futsch-labeled microtubules in spastin-overexpressing cells relative to wild-type controls (compare branches near arrows in Figure 27C3 to A3). Neighboring neurons not overexpressing spastin served as internal controls and were consistently Futsch-positive. In contrast to spastin, when kat-60L1 was ectopically expressed within these cells, we saw no change in the level of Futsch-labeled microtubules compared to controls (compare branches near arrows in Figure 27B3 to A3).
Figure 27: Kat-60L1 and Spastin Regulate Distinct Subsets of Microtubules in the Class IV Dendritic Arbor.

(A-C) Comparison of Futsch-positive microtubules in class IV neurons from larvae expressing wild-type (A1-A3) or ectopic levels of kat60-L1 (B1-B3) or spastin (C1-C3).

(A1-C1) Representative class IV arbors from each genotype expressing ppk1.9-Gal4, UAS-mCD8::GFP (GFP staining in magenta). Arbors expressing high levels of spastin are strongly reduced in branch complexity (compare C1 to A1). (A2-C2) Futsch-positive neuronal microtubules are reduced in arbors expressing high levels of spastin compared to wild-type or ectopic kat-60L1 expression (compare C2 to A2 and B2). (A3-C3) Merged images reveal that the class IV cell body (arrow, insets) and proximal branches in control and kat-60L1 overexpressing cells are microtubule-rich, in contrast to spastin-overexpressing neurons. Scale bars, 20µm.
These results suggest that Spastin regulates a stable (Futsch-positive) population of microtubules, while Kat-60L1 regulates a dynamic, growing population of microtubules and each microtubule severing protein acts exclusively on a distinct microtubule subset. Whether Spastin is capable of recognizing Futsch-bound microtubules or some other marker of stabilized microtubules, such as a MAP or PTM is not yet known. Based on their antagonistic function and the results described here as well as in section 2.7, we hypothesize that Kat-60L1 and Spastin regulate the microtubule architecture and promote the complex arborization of larval class IV dendrites through distinct mechanisms of severing different microtubule populations.

3.7 Investigating Post-translational Modifications and Tau localization in a Katanin p60-like 1 Loss of Function Background

Our observations that Spastin and Kat-60L1 have clear differences in their effects on class IV dendritic microtubules and axons raises the possibility that they could sever distinct populations of microtubules. As described in chapter 1, section 1.1.5, numerous interacting proteins, such as Tau, as well as post-translational modifications (PTMs) provide a potential mechanism for microtubule functional specialization. PTMs and/or microtubule-associated proteins (MAPs) could mediate the differential regulation of microtubules between Kat-60L1 and Spastin.

We have demonstrated that Spastin destabilizes stable (MAP1B/Futsch-positive) class IV neuronal microtubules, while Kat-60L1 does not; and distinct from Spastin, Kat-
60L1 regulates a subset of dynamic EB1-labeled microtubules in these cells. Since tubulin modifications have been shown to differentially affect Spastin and Katanin-60 severing (Yu et al., 2008), we sought to understand how Kat-60L1 acts on distinct microtubule populations. We observed the effects of reduced or ectopic levels of Kat-60L1 on distinct populations of post-translationally modified microtubules and the microtubule-associated protein, Tau in class IV dendritic arbors.

As described in section 1.1.5, neuronal microtubules enriched for Tyrosinated tubulin are expected to favor functions such as those in the growth cone (i.e. dynamic microtubule processes for rapid remodeling of cytoplasm), whereas microtubules enriched in detyrosinated tubulin appear to favor functions in the shafts of neurites (i.e. microtubule stability and efficient cargo transport). We observed no changes in the distribution of PTM markers of stable microtubules (MAP1B/Futsch or acetylated-tubulin, chapter 2, section 2.7, Figures 17 and 18, respectively) in kat-60L1 loss of function arbors. This data combined with our observations that EB1-positive, dynamic microtubules are crucial for class IV dendritic complexity and are regulated by Kat-60L1 (chapter 2, section 2.7), led us to hypothesize that tyrosinated microtubules would be enriched in dendritic branches of larval class IV neurons and that we might observe a change in their distribution in the presence or absence of Kat-60L1. We dissected and fixed wild-type control third-instar larvae in the background of ppk1.9-GAL4 UAS-
mCD8::GFP, and those that were mutant for kat-60L1 or overexpressed kat-60L1 in class IV neurons. We immunostained with an antibody specifically directed against tyrosinated-tubulin and a GFP antibody to label class IV neurons and saw no differences among genotypes (Figure 28). Control arbors displayed very little Tyr-tubulin staining within class IV arbors (Figure 28A2), and neither arbors that lacked Kat-60L1 or overexpressed Kat-60L1 showed any differences from controls (compare Figure 28B2, B3 and 28C2, C3 to 28A2, A3). These results suggest that tubulin in class IV dendritic arbors is not tyrosinated at high enough levels to detect with immunostaining. Modifying Kat-60L1 levels within these neurons had no effect on Tyr-tubulin as far as we could detect with our immunostaining methods.
Figure 28: No Change in Tyrosinated-tubulin is Detected in Class IV Dendritic Arbors Lacking or Overexpressing Kat-60L1

(A-C) Comparison of Tyr-positive microtubules in class IV ddaC neurons from larvae expressing wild-type kat-60L1 (A1-A3), lacking kat60-L1 (B1-B3), or overexpressing (OE) kat-60L1 (C1-C3). (A1-C1) Representative class IV arbors from each genotype expressing ppk1.9-Gal4, UAS-mCD8::GFP (GFP staining green). Control arbors do not exhibit strong Tyr-tubulin staining in dendritic arbors (A1-A3). Arbors lacking kat-60L1 do not show changes in Tyr-tubulin compared to controls (B1-B3). Arbors overexpressing kat-60L1 do not show changes in Tyr-tubulin compared to controls (C1-C3). Scale bars, 20µm.
The microtubule-associated protein Tau is thought to regulate the susceptibility of microtubules to severing by Katanin-60 (Qiang et al., 2006; Yu et al., 2008), and therefore could differentially regulate the severing activity between Kat-60L1 and Spastin in class IV neurons. To address whether Kat-60L1 disassembles microtubule-associated Tau, we analyzed Tau::GFP distribution in a *kat-60L1* loss of function background.

The insertion *P{Wee-P.un1}tau*304 is a GFP protein trap in the *Drosophila* Tau protein (Stone et al., 2008). This allele was recombined with both loss of function alleles for *kat-60L1*, the *PBac* insertion and the deletion, *BE6*. Both recombined alleles were built into the *ppk1.9-GAL4 UAS-mCD8::cherryRFP* background to visualize Tau localization in the green and class IV neurons in the red channel. Third-instar larvae were dissected, fixed and immunostained with GFP and dsRed antibodies for confocal imaging. Tau was observed in the cell bodies (arrowheads) and main branches (white arrows) of class IV arbors, but not in terminal branches (yellow arrows) (Figure 29). No difference was observed between *kat-60L1* mutant and wild-type control class IV neurons in any region of the neuron (Figure 29).
Figure 29: No Change in Tau-GFP is Detected in Class IV Dendritic Arbors Lacking Kat-60L1

(A-C) Comparison of Tau-GFP in class IV ddaC neurons from larvae expressing wild-type kat-60L1 (A1-A3) and lacking kat60-L1 (B1-B3). (A1, B1) Representative class IV arbors from each genotype expressing ppk1.9-Gal4, UAS-mCD8::chRFP. (A2, B2) Tau-GFP signal in all neurons including the class IV (chRFP-labeled). (C1, C2) Merged images from the red and green channel to show overlap. Scale bars, 20µm.

These negative data do not support a role for Tau in the regulation of Kat-60L1 function in the main dendritic branches of class IV neurons. Obtaining loss of function mutations in tau and conducting double mutant analysis could provide a more definitive
analysis of whether Tau is important for the regulation of Kat-60L1 or Spastin microtubule severing in class IV neurons.

3.8 Discussion and Conclusions

Studies in the *Drosophila* sensory nervous system have begun to reveal how the diverse morphologies of neuronal dendrites are generated (Corty et al., 2009; Jan and Jan, 2010). Transcription factors Knot and Cut are differentially expressed across the different da neuron classes, reflecting each class’ distinctive morphologies. Together with the transcription factor Dar1, these proteins influence the dendritic arbors of the da neurons at least in part through their regulation of the actin and microtubule cytoskeletons (Jinushi-Nakao et al., 2007; Ye et al., 2011). The microtubule severing protein Spastin has been identified as a downstream target of both Knot and Dar1, regulating overall class IV neuron dendrite and axon morphology (Jinushi-Nakao et al., 2007; Ye et al., 2011). Other proteins effecting these cell-specific changes in cytoskeletal dynamics remain to be identified.

With the identification of the first microtubule-severing protein occurring only twenty years ago (Vale, 1991), *in vitro* studies over the last ten years have revealed that these proteins have evolved unique functions and do not simply act redundantly (Yu et al., 2008; Zhang et al., 2007). Our work provides confirmation of this concept in neurons *in vivo*. Our studies reveal not only similarities but also important differences in the
mutant phenotypes for these two closely related proteins. Both \textit{kat-60L1} and \textit{spastin} mutant animals display reduced branching and reduced nociception responses. Yet, these similar phenotypes arise by distinct mechanisms of microtubule regulation. Mechanistically Kat-60L1 promotes microtubule polymerization in the dendrites, visualized by tracking EB1-GFP comets, while Spastin does not. In addition, Spastin is required for axon and dendrite integrity of the class IV neurons while Kat-60L1’s role is specific to the dendritic arbors. We propose that these phenotypes arise from distinct activities of the two severing proteins on the microtubule cytoskeleton, and these functions differentially affect the dendritic and axonal compartments. Alternatively, \textit{spastin} dendrite defects could be a secondary effect of impaired axon morphology and function since we show a role for Spastin function in the general excitability of the neuron.

What is the cell biological mechanism by which Kat-60L1 promotes dendrite outgrowth? To date there is no published account demonstrating that Kat-60L1 severs purified microtubules, and exogenous overexpression in class IV neurons, as well as \textit{Drosophila} eye or muscle (Figure 28D; data not shown for muscle), fail to elicit detectable effects on tissue integrity or microtubule distribution. This is in stark contrast to the microtubule severing proteins Spastin and Katanin-60, both of which induce dramatic breakdown of microtubules \textit{in vitro} and \textit{in vivo}. Nevertheless, we favor a role for Kat-
Kat-60L1 in microtubule severing based on the following observations. First, the 67% amino acid sequence conservation between the catalytic domains of Kat-60L1 and Katanin-60 is considerably greater than that between Katanin-60 and Spastin, consistent with Kat-60L1 sharing a common enzymatic function with these two proteins. Second, data from the Sharp lab support a role for Kat-60L1 in severing and depolymerizing microtubule ends, much as they have shown for Katanin-60 in vitro (D.A. Sharp, personal communication, and (Zhang et al., 2011)). Finally, our EB1 analysis revealed reduced numbers of growing microtubules in kat-60L1 mutant dendritic arbors, reminiscent of the net microtubule loss observed in neuromuscular junction boutons of spastin mutants (Jinushi-Nakao et al., 2007; Sherwood et al., 2004). Together, these data support a model in which Kat-60L1 severs dynamic microtubules to generate new microtubule fragments from which additional plus-end polymerization can then occur, promoting new neurite outgrowth.

The parallels between the requirement for Kat-60L1 in promoting late-stage larval dendritic growth, and for Spastin in synaptic bouton formation at the larval neuromuscular junction, suggest that microtubule severing is utilized during neuronal development to enable rapid expansion of the microtubule population during periods of extensive arborization, when the slow transport of microtubule polymers to distant branch sites may be insufficient to support the required rate of growth. Our observations
that Spastin and Kat-60L1 have clear differences in their effects on class IV dendritic microtubules and axons further indicate that they regulate distinct populations of microtubules, much as overexpressed Spastin and Katanin-60 do in cultured vertebrate neurons (Yu et al., 2008), thereby yielding distinct effects. The dramatic reduction in microtubule distribution caused by spastin overexpression in class IV neurons and elsewhere is consistent with it severing microtubules broadly, while Kat-60L1 likely targets a very specialized dendritic subset of dynamic microtubules that we detected only by live imaging of individual polymers.

The microtubule associated protein Tau is thought to regulate the susceptibility of microtubules to severing by Katanin-60 (Qiang et al., 2006; Yu et al., 2008); other MAPs, or post-translational modifications of microtubules, may serve to differentiate microtubule susceptibility to net polymerization or depolymerization, possibly through differential severing by Kat-60L1 and Spastin. Overexpressed Spastin, but not Kat-60L1, is sufficient to destroy the Futsch-positive, stable microtubule cytoskeleton, but we were unable to detect differences in other populations of post-translationally modified microtubules, including acetylated and tyrosinated. Regardless of mechanism, the numerous contrasts between Kat-60L1 and Spastin function in class IV neurons provide evidence that the microtubule severing protein family evolved to allow for unique and precise regulation of the microtubule cytoskeleton. The unique activities of different
microtubule severing proteins thus provide mechanistic insight into generating the differential morphology of dendrites and axons within neurons.

### 3.9 Future Directions

We currently do not know the mechanism underlying the antagonistic relationship between Kat-60L1 and Spastin in class IV sensory neurons. As we continue to screen for and investigate genetic regulators of Kat-60L1 and Spastin function, we hope to elucidate the molecular components that mediate their antagonistic interaction. We have shown that reducing a genetic copy of *spastin* in a *kat-60L1* loss of function background suppresses the dendritic phenotype caused by loss of either gene alone, indicating the activity of either protein could lead to the repression of the other’s function. Since the overexpression of *kat-60L1* fails to modify the overexpression phenotype of Spastin, these proteins are likely interacting indirectly (Figure 25). Yet, it would be interesting to test if either Kat-60L1 or Spastin were capable of regulating the expression of one another, even if indirectly. We think this scenario is unlikely, but perhaps if the upregulation of *spastin* by Knot is carefully controlled by a levels-dependent feedback mechanism during larval development and Knot simultaneously regulates *kat-60L1* levels in a coordinated fashion then we could see evidence of their coregulation. We could test this hypothesis by isolating RNA and generating cDNA from *kat-60L1* and *spastin* loss of function tissue and conducting RT-PCR with cDNA-
specific primers for either gene on each sample. If either Spastin or Kat-60L1 repressed
the other’s expression, we would expect to see an increase in transcript levels by RT-PCR
in the loss of function sample. Conversely, if either enhanced the other’s expression, we
would expect to see a decrease in transcript levels by RT-PCR in the loss of function
sample. If we were to find that the expression levels of neither gene are modified by a
reduction in the other, we could conclude that Kat-60L1 and Spastin do not regulate the
expression of each other and their antagonistic relationship is indirect, likely involving
other downstream molecular players involved in regulating the microtubule
cytoskeleton.

We would also like to know more about how Kat-60L1 is regulated in the class
IV neurons. We identified six candidate genetic interactors through an enhancer screen
in the Drosophila eye and have begun to characterize the role of a transcription factor,
Mi-2 in regulating Kat-60L1 (chapter 4). As discussed earlier, Knot and Dar1 are recently
identified transcription factors that promote dendritic complexity in class IV neurons
(Jinushi-Nakao et al., 2007; Ye et al., 2011). Interestingly, Spastin was identified as a
downstream target for both transcription factors. We would like to test whether Kat-
60L1 is regulated by either of these proteins.

We have obtained both overexpression and loss of function alleles for both genes.
To test if Knot or Dar1 is regulating the transcription levels of kat-60L1 we will conduct
RT-PCR with primers specific to kat-60L1 cDNA on larval brains overexpressing either gene under the pan-neuronal driver, Elav-GAL4. Spastin transcript levels were shown to be increased in a knot overexpression background and decreased in a dar1 overexpression background (Jinushi-Nakao et al., 2007; Ye et al., 2011). Their results imply that Knot upregulates spastin levels, while Dar1 represses spastin levels. Since both Knot and Dar1 were shown to promote class IV dendritic arborization, the placement of Spastin in both pathways is somewhat conflicting and suggests that Spastin levels must be tightly regulated in order for Spastin to promote class IV branching. However, overexpressing transcription factors can often result in large-scale changes in other genes’ expression levels and cause non-specific effects. This caveat was not taken into account in either study and could have resulted in the misinterpretation of data. It will be instructive to test whether kat-60L1 is similarly or differently regulated by these transcription factors.

We can also test whether kat-60L1 is regulated by either Knot or Dar1 genetically in the class IV neurons. To test their interactions we can overexpress either Knot or Dar1 while reducing Kat-60L1 by RNAi in class IV neurons. However, we must acknowledge the caveat of non-specific effects due to overexpressing transcription factors, as discussed above and should therefore validate these results in a knot or dar1 loss of function background as well. If either transcription factor regulates kat-60L1 levels in
these neurons we would predict that overexpressing a positive regulator would suppress the *kat-60L1* loss of function phenotype and conversely, a negative regulator would exacerbate the phenotype. These experiments should provide more information about how Kat-60L1 is regulated in the context of known transcription factors that promote dendritic arborization and regulate Spastin. Determining how Kat-60L1 fits in these two pathways will help resolve our question as to why two highly conserved microtubule severing proteins are expressed in the same neurons, and provide insight into how different microtubule severing proteins are differentially controlled in sensory neurons to establish a complex dendritic arbor.

### 3.10 Materials and Methods

**Drosophila Stocks and Crosses:** The genetic deletion, *Df(3R)kat-60L1*BE6 was created by imprecise excision and described in section 2.10. For *kat-60L1* loss of function analysis, we crossed *ppk1.9-GAL4 UAS-mCD8::GFP;; kat-60L1*BE6/TM6b to *kat-60L1*Bac c00564/TM6b. VDRC *spastin* RNAi line: #108739.

For live EB1-GFP analysis, we crossed *477-GAL4 UAS-EB1-GFP/CyO;; spastin*5.75/TM3, *actin-GAL4, UAS-GFP* to WCS to obtain larvae of the genotype, *477-GAL4 UAS-EB1-GFP;; spastin*5.75+. Control larvae were obtained from crossing *477-GAL4 UAS-EB1-GFP/CyO* to WCS.
Drosophila stocks were provided by the Bloomington Stock Center (Bloomington, IN) and the Vienna Drosophila RNAi Center (Vienna, Austria).

**Behavioral Analysis**

Nociception assays and optogenetic experiments are described in section 2.10.

**Immunohistochemistry**

Antibodies used: mouse anti-GFP (1:500) (from Invitrogen), mouse anti-Futsch 22C10 (1:500) (from Developmental Hybridoma Studies Hybridoma Bank, University of Iowa, IA) and rat anti-Tyr-tubulin (1:50) (from Millipore). Alexa-fluor 488 or 568 fluorescent secondary antibodies (1:200) were from Invitrogen. Larval preparations, confocal imaging and analysis are described in section 2.10.

**Live Imaging of EB1-labeled Microtubules**

All imaging of da neurons was performed on intact third-instar larvae mounted on a dried agarose pad under a coverslip. Neurons were imaged on a Zeiss Axiovision confocal microscope or a Zeiss 510 confocal microscope and images were recorded every 1 second for 200 cycles. Movies were analyzed using ImageJ software. An EB1-labeled comet was counted only if it was detectable and tracked in consecutive frames for at least 7 seconds.
Chapter 4. A Screen for Genetic Interactors in the Drosophila Eye Reveals Mi-2 as a Transcriptional Regulator of Katanin p60-like 1

4.1 Introduction/Rationale

Little is known about how the microtubule severing proteins are regulated at the level of transcription or catalytically at the protein level within cells. Recent insights into the transcriptional regulation of spastin were described in chapter 1. Briefly, transcription factors Knot and Dar1 have been shown to regulate levels of spastin in the class IV dendritic arborization (da) neurons (Jinushi-Nakao et al., 2007; Ye et al., 2011). Knot and Dar1 have been described to promote microtubule-based dendritic arborization in class IV neurons through independent pathways (Ye et al., 2011). The role of spastin regulation by these transcription factors is somewhat controversial since Knot is thought to upregulate spastin levels while Dar1 represses spastin levels. Their results imply that excessive or insufficient Spastin activity impairs dendritic arborization and therefore, spastin expression must be tightly regulated to ensure proper branching.

The factors that regulate kat-60L1 at the transcriptional and protein level were unknown at the onset of this study. We sought to identify transcriptional regulators of kat-60L1 and/or factors that modify Kat-60L1 protein activity to gain a better understanding of how Kat-60L1 functions and is regulated in class IV neurons. To
address how Kat-60L1 might be regulated in vivo, we designed an overexpression screen in the Drosophila eye to identify genetic interactors of kat-60L1.

**4.2 An Overexpression Screen in the *Drosophila* Eye Identifies Six Genetic Interactors of *Kat-60L1***

Eye screens can be very useful when ectopic expression of proteins specifically in the eye produces an observable phenotype due to disruption of cellular processes, such as ommatidial development or cell division (Johnston and Gallant, 2002). Modification of an eye phenotype by simultaneous perturbation or loss of another gene can indicate a potential interaction. In addition to the eye being a fast and simple readout of genetic modification, another key advantage to screens localized in the eye is that genes whose global overexpression or knock-down is lethal can be studied, since a defect in the eye does not affect viability of the animal.

Overexpressing *spastin* alone in the eye causes a smooth and reduced eye phenotype that has proven very useful in uncovering genetic modifiers by either enhancement or suppression (Figure 30A, B) (Ozdowski et al.). Similarly, when *katanin-60* together with its regulatory binding partner, *katanin-80* is overexpressed in the eye, a smooth and reduced eye phenotype is observed (Figure 30A, C). Overexpressing *katanin-60* alone in the eye does not produce this effect, indicating that the regulatory subunit of Katanin is required for its activity in the eye and to produce a reduced eye phenotype. Overexpressing *kat-60L1* in the eye alone produces no effect (Figure 30A, D).
Figure 30: Overexpression of Microtubule Severing Proteins in the *Drosophila* Eye

(A) A wild-type eye of normal size and texture. (B) Overexpression of *spastin* in the eye causes a smooth eye of reduced size. (C) Overexpression of both *katanin-60* and *katanin-80* in the eye causes a similarly smooth eye of reduced size. (D) Overexpression of *kat-60L1* in the eye does not cause a reduced eye phenotype.

The absence of an eye phenotype when *kat-60L1* is overexpressed in the eye suggests two possible explanations. First, Kat-60L1 may not sever microtubules. Given the high degree of sequence similarity between Katanin-60 and Kat-60L1, however, a more likely scenario is that additional protein(s) required for Kat-60L1-dependent severing are not present in the eye. We hypothesized that similar to Katanin-60, Kat-60L1 has a co-activator, analogous to Katanin-80 and both are required to produce an eye phenotype. The overexpression of *kat-60L1* with either *katanin-60* or *katanin-80* failed to elicit variations in the eye (data not shown). Searching the *Drosophila* genome for proteins similar to Katanin-80 yielded only low homology hits. Therefore, we employed an EP screen using the eye as a readout for genetic interactions by overexpressing *kat-
60L1 in the eye concomitantly with a library of EP (UAS-containing) insertion lines on the third chromosome (a gift from Amy Bejsovec). The lines were originally obtained from the Berkeley Drosophila Genome Project (BDGP) Disruption Kit (Bellen et al., 2004). Each EP line has GAL4 binding sites, so when inserted in the 5’ end of a gene and crossed with an eye-specific GMR (Glass Multiple Reporter)-GAL4 promoter line, the gene directly downstream of the EP insert is overexpressed in the eye.

We screened approximately 300 lines overexpressing genes on the third chromosome. Adult eyes expressing both the candidate gene and kat-60L1 were compared to eyes of sibling control animals of the candidate gene alone. Our screen did not find evidence of a Kat-60L1 regulatory subunit, but did reveal several potential genetic interactors with Kat-60L1. We recovered six lines that gave a genetic interaction in the eye: mi-2, sprouty, papss, roq, aef1, and CG1275. All six overexpression lines produced a smooth eye phenotype when overexpressed individually; however, when coexpressed in the eye with kat-60L1, the eye phenotype of each was suppressed and the eye was rescued to normal morphology (for representative example with mi-2, Figure 31).
Figure 31: Overexpression of Kat-60L1 Suppresses the Overexpression Phenotype of Mi-2 in the Eye.

(A) An eye overexpressing kat-60L1 with normal size and texture. (B) Overexpression of mi-2 in the eye causes a rough eye phenotype. (C) Overexpression of kat-60L1 in the eye simultaneously with mi-2 suppresses the rough eye phenotype of mi-2 overexpression.

Sprouty is a tyrosine kinase signaling receptor, well-studied for its role in tracheal branching. It is also expressed in the peripheral nervous system and therefore has potential to have an interesting interaction with Kat-60L1 in dendritic arborization (da) neurons. Aef1 is a transcription factor; Papss has sulfate adenylyltransferase activity; Roq is an uncharacterized zinc ion binding transcription factor; and CG1275 is an uncharacterized gene with possible electron carrier activity; Mi-2 is a transcription factor, described below.

4.3 The Identification of Mi-2 as a Potential Transcriptional Regulator of Kat-60L1

Mi-2 has been implicated in dendrite morphogenesis of class I neurons and is required for proper branch outgrowth of these simply branched dendrites, (Figure 32)
(Parrish et al., 2006). Of the six genes identified in the EP screen, we decided to first focus on mi-2, with the rationale that it was likely expressed in the da sensory neurons and it exhibited a similar loss of function phenotype to kat-60L1 and therefore was an excellent candidate regulator of Kat-60L1 function in class IV neurons.

**Figure 32: Mi-2\(^{3D4}\) Homozygous Mutant L2 Larvae Exhibit Reduced Dendrite Branching in Class I Neurons.**

Live image of GFP-expressing class I dendrites in wild-type (A) and a mi-2\(^{3D4}\) homozygous mutant (B) second instar larva (48 hours after egg laying). (Figure modified from Parrish JZ, et al. Genome-wide analyses identify transcription factors required for proper morphogenesis of Drosophila sensory neuron dendrites. Genes Dev. (2006) 1;20(7):820-35.)

Mi-2 is a chromatin remodelling factor with ATPase activity and has been shown to be involved in transcriptional repression. *Drosophila* Mi-2 exists in various forms: as a monomer, in a Nucleosome Remodeling and Deacetylase (NuRD) complex with several other proteins, and bound to the MEP-1 protein in a *Drosophila* MEP-1 Containing (dMec) Complex (Kunert et al., 2009). Recently, it was shown that the majority of *Drosophila* Mi-2 is contained in the dMec complex, which is present in embryos and in
the larval nervous system (Kunert et al., 2009). Interestingly, in a Katanin-60 eye screen, similar to the one performed for Kat-60L1, our lab identified the MEP-1 gene (Lisa Pang and Nina Sherwood, unpublished data). The interaction between MEP-1 and Katanin-60 has yet to be elucidated, but the fact that MEP-1 and Mi-2 were both identified in genetic interaction screens with microtubule severing proteins provides evidence that the dMec complex may act as a transcriptional regulator of multiple microtubule severing proteins.

The mi-2 overexpression EP line we used, P{EPgy2}Mi-2EY08138, is inserted in the 5’ end of mi-2 and the 3’ end of Su(Tpl). We obtained a null allele, P{lacW}Mi-2^{j3D4} Su(Tpl)^{j3D4} that was used by Parrish et al. to analyze the possible loss of function phenotypes in class IV da neurons. Mutants that are homozygous for the mi-2^{j3D4} allele die by the second-instar stage of larval development, making observation of the arbors during third-instar larval stages impossible without clonal analysis. As reported in chapter 2, Kat-60L1 promotes dendrite arborization during late larval stages and therefore, we are currently identifying mi-2 hypomorphic alleles that are viable at the third-instar stage for loss of function analysis in these neurons.

4.4 Molecular Characterization of the Mi-2^{j3D4} Allele

The mi-2 mutant allele j3D4, which contains a P{LacW} insertion in the 5’ end of the mi-2 gene, was obtained from the Bloomington Drosophila Stock Center (Figure 33)
(Bellen et al., 2004). The inserted construct is a P-element transposon that contains a LacZ reporter and a mini-white marker. The P-element insertion is also in the Su(Tpl) gene, which is within mi-2.

Figure 33: Mi-2 Gene Region and the Mi-2 j3D4 Allele.

Mi-2 is located on chromosome 3L and encodes one isoform. The P[LacW] j3D4 insertion is in the first intron of mi-2 and Su(Tpl) (white triangle). The EP, P[EPgy2]EY8138 is inserted in the 5’ region of mi-2 (grey triangle) (Figure, courtesy of Natalie Como).

To characterize the j3D4 allele, we measured mRNA expression using Reverse-Transcriptase PCR (RT-PCR) on homozygous first-instar larvae. RT-PCR revealed highly reduced mi-2 mRNA transcripts in the homozygous mi-2 j3D4 background (lanes 1, 2, Figure 34).
Figure 34: Mi-2\textsuperscript{3D4} Mutant Larvae Exhibit Reduced Transcript Levels of Mi-2 and Kat-60L1.

Gel image from RT-PCR using \textit{w\textsuperscript{1118}} or \textit{mi-2\textsuperscript{3D4}} cDNA from first-instar larvae. Lanes were alternately loaded \textit{w\textsuperscript{1118}} (control) and \textit{mi-2\textsuperscript{3D4}} homozygous mutant cDNA so that odd numbered lanes correspond to control cDNA samples and even numbered lanes correspond to \textit{mi-2\textsuperscript{3D4}} homozygous mutant cDNA. Bands highlighted in red correspond to the predicted cDNA band. Lanes 1, 2 show a 195 bp \textit{mi-2} cDNA product; lanes 3, 4 show a 609 bp \textit{kat-60L1} product, not specific to either isoform; lanes 5, 6 show a 344 bp \textit{kat-60L1}-short isoform product; lanes 7, 8 show \textit{GAPDH} loading controls. In all gels, 100 bp ladder was used.

4.5 Mi-2 Regulates Transcription Levels of Kat-60L1

Since Mi-2 has been shown to regulate expression levels of other genes, we hypothesized, based on the genetic interaction we observed in the eye, that Mi-2 could be regulating transcript levels of \textit{kat-60L1}. To test this, we isolated RNA from homozygous null \textit{mi-2\textsuperscript{3D4}} larvae to examine the effects of Mi-2 loss on \textit{kat-60L1} transcript levels. \textit{Mi-2} mutant larvae had decreased levels of \textit{kat-60L1} mRNA transcript, suggesting that Mi-2 directly or indirectly upregulates transcription of \textit{kat-60L1} (lanes 3,4 and lanes 5,6 Figure 34). The interaction is likely indirect, since Mi-2 has typically been shown to
act as a transcriptional repressor (Kunert et al., 2009; Passannante et al.; Unhavaithaya et al., 2002).

We also tested whether the \textit{mi-2}\textsuperscript{3D4} allele affected transcript levels of \textit{Su(Tpl)} in homozygous \textit{mi-2}\textsuperscript{3D4} mutants and found that \textit{Su(Tpl)} levels were unperturbed by this mutation (Figure 35). This result indicated the utility of the \textit{j3D4} allele for investigation of Mi-2 function, since it specifically knocks down the transcription of \textit{mi-2} without affecting \textit{Su(Tpl)} levels. Therefore, the phenotypes we observed in \textit{j3D4} mutants are due specifically to loss of \textit{mi-2}.

![Figure 35: Mi-2\textsuperscript{3D4} Mutant Larvae Exhibit No Reduction in Su(Tpl) Transcripts.](image)

Gel image from RT-PCR using \textit{mi-2}\textsuperscript{3D4} or \textit{w}\textsuperscript{1118} cDNA from first-instar larvae. Lanes were alternately loaded \textit{w}\textsuperscript{1118} (control) and \textit{mi-2}\textsuperscript{3D4} homozygous mutant cDNA. Lanes 1, 2 show \textit{GAPDH} loading controls; lanes 3, 4 show the \textit{Su(Tpl)} 710 bp band cDNA product. No change in \textit{Su(Tpl)} transcript levels were detected in the \textit{mi-2} mutant background.

### 4.6 Loss of Mi-2 Suppresses the Kat-60L1 Loss of Function Phenotype in the Class IV Dendritic Arbor

Because the genetic interaction of \textit{mi-2} with ectopic \textit{kat-60L1} in the eye and whole animal RT-PCR interactions suggested a role for Mi-2 in the regulation of \textit{kat-60L1}, we
wanted to directly test for a genetic interaction with *kat-60L1* mutants in the biologically relevant site of class IV neurons where we know Kat-60L1 functions. We hypothesized that removing one functional copy of *mi-2* in the *kat-60L1* loss of function background would exacerbate the *kat-60L1* mutant phenotype if Mi-2 primarily promotes *kat-60L1* expression.

In order to carry out this double mutant analysis we recombined the *mi-2*<sup>j3D4</sup> allele with the *kat-60L1*<sup>PBac</sup> allele onto the same chromosome and confirmed recombinant animals by PCR analysis of both alleles. These animals were then crossed into the *ppk1.9-GAL4 UAS-mCD8::GFP* (II) background to visualize class IV neurons. For the analysis, crosses described in Materials and Methods were carried out to generate animals that were transheterozygous mutant for *kat-60L1* and contained one mutant allele of *mi-2*. The transheterozygous *kat-60L1* loss of function background used in all experiments consisted of one *kat-60L1*<sup>PBac</sup> allele and a deletion, *kat-60L1*<sup>BE6</sup> (described in section 2.2.2). These experimental animals were compared to control animals that were either transheterozygous mutant for *kat-60L1*, heterozygous mutant for *mi-2* or wild-type, containing only *ppk1.9-GAL4 UAS-mCD8::GFP*. Surprisingly, we observed that genetically reducing levels of *mi-2* suppressed the reduced branching phenotype of *kat-60L1* mutant arbors (compare Figure 36D to C). Removal of one copy of *mi-2* alone had no effect on branching (compare Figure 36B to A).
Figure 36: Loss of Mi-2 Suppresses the Kat-60L1 Loss of Function Phenotype in Class IV Neurons

(A-C) Comparison of wild-type, mi-2 heterozygous, kat-60L1, and double mutant dorsal cluster class IV da neurons from wandering third-instar larvae in a ppk1.9-GAL4, UAS-mCD8::GFP/+ background. (A) Representative arbors from wild-type control, (B) mi-2\textsuperscript{β3D4/+}, (C) kat60-L1\textsuperscript{BE6/PB} mutant and (D) mi-2\textsuperscript{β3D4}, kat60-L1\textsuperscript{PB/kat60-L1\textsuperscript{BE6}} double mutant larvae. Scale bar, 50µm.

We measured dendrite arbor coverage of each neuron by quantifying the area or “white space” that lacked any portion of a dendrite branch using an overlaid grid of 250
µm² squares, as in section 2.4 for *kat-60L1* analysis (Jinushi-Nakao et al., 2007). We have consistently observed that *kat-60L1* loss of function mutants exhibit approximately 75-80% dendritic coverage (Figure 37). Reducing Mi-2 function with one copy of the *mi-2j3D4* allele mutation in the *kat-60L1* mutant background restored dendritic coverage to approximately 93% coverage, similar to the 94% coverage seen in wild-type control and *mi-2* heterozygous animals (Figure 37).

**Figure 37: Genetic Reduction of Mi-2 Suppresses the Reduced Branching Phenotype of *Kat-60L1* Mutant Dendrites in Class IV Neurons.**

Bar graph displaying the percent of dendritic coverage in wild-type control, *mi-2*, *kat-60L1* double mutant, *mi-2* heterozygous, and *kat-60L1* mutant dorsal cluster class IV da neurons from wandering third-instar larvae in a *ppk1.9-GAL4, UAS-mCD8::GFP/+* background. Dendritic coverage was significantly reduced in *kat-60L1* mutant arbors compared to wild-type controls (p<0.02), while double mutants were not significantly different from wild-type controls (p>0.9).
This genetic suppression was somewhat counterintuitive to our expected results. Since loss of \textit{mi-2} causes a reduction in \textit{kat-60L1} transcript levels (Figure 34), we anticipated a reduction in \textit{mi-2} levels should worsen the \textit{kat-60L1} mutant phenotype. Therefore, Mi-2 must antagonize Kat-60L1 function in the sensory neurons indirectly through other genetic interactions (discussed below in the Discussion, section 4.9).

4.7 Mi-2 Regulates the Expression of \textit{Mep1}, a component of the dMec complex

The majority of the \textit{Drosophila} Mi-2 protein is contained within a recently discovered complex called the dMec complex (Kunert et al., 2009), which consists of just two proteins, Mi-2 and MEP-1. No work has been done to examine the dendritic phenotype of \textit{MEP-1} mutants. However, we investigated whether \textit{mi-2} mutants exhibited reduced levels of \textit{MEP-1} mRNA transcripts, since previous work has suggested their mutual dependence in terms of protein stability (Kunert et al., 2009). We performed RT-PCR using \textit{MEP-1} cDNA-specific primers on cDNA generated from RNA isolated from \textit{w\textsuperscript{1118}} (control) and homozygous \textit{mi-2\textsuperscript{j3D4}} mutant first-instar larvae. Mi-2 mutants had reduced levels of MEP-1 transcripts compared to controls (Figure 38A), suggesting that in \textit{Drosophila} larvae, MEP-1 expression is dependent upon Mi-2. Whether MEP-1 along with Mi-2 in the dMec complex regulates \textit{kat-60L1} expression remains to be determined.
4.8 Mi-2 Regulates the Expression of Microtubule Severing Genes, Spastin and Katanin-60

Our results in section 4.5 show that Mi-2 is required to promote transcription of kat-60L1, most likely through indirect interactions since the removal of mi-2 suppresses the kat-60L1 mutant phenotype in class IV arbors. Given that removing one copy of spastin similarly suppresses the kat-60L1 loss of function phenotype in class IV dendritic arbors (chapter 3, section 3.4), we hypothesized that Mi-2 also regulates spastin expression, which could underlie the suppression of kat-60L1 by the loss of mi-2. We
tested whether Mi-2 regulated *kat-60L1* exclusively among the neuronally expressed microtubule severing genes or if it also regulated *spastin* and/or *katanin-60*.

To investigate the potential of Mi-2 to regulate levels of *spastin* and *katanin-60*, we performed RT-PCR on *w*¹¹¹⁸ (control) and homozygous *mi-2*³D⁴ mutant first-instar larvae. Using primers to amplify *spastin* and *katanin-60* mRNA transcripts, we found that *mi-2* mutants had moderately reduced levels of both *spastin* and *katanin-60* (Figure 38B, C). A role for *katanin-60* in da neurons has so far not been shown. Our RT-PCR data suggests that Mi-2 may play a role in generally regulating microtubule severing genes and promoting their transcription, which suggests that their coordinated coregulation may be important for the proper development and maintenance of nervous system structures, such as the da sensory neurons.

Since all genes tested thus far, with the exception of *GAPDH* controls, showed reduced levels in *mi-2* mutants, we wanted to ensure this was not an artifact and identify a gene whose transcription was not regulated by Mi-2. We performed RT-PCR with cDNA-specific primers in an unrelated gene called *stall* on *w*¹¹¹⁸ (control) and *mi-2*³D⁴ cDNA samples. *Stall* is an ADAMTS metalloprotease required for ovarian follicle formation (Ozdowski et al., 2009); additionally, we have shown that *kat-60L1* is expressed in the ovary and Fly Atlas reports that *mi-2* expression is enriched in the ovary relative to the whole animal (Chintapalli et al., 2007). Results showed that *mi-2*
homozygous mutants had the same levels of stall transcript levels as controls (Figure 39), supporting our results that show the downregulation of kat-60L1, spastin, katanin-60, and MEP-1 seen in mi-2 mutants is somewhat specific to these genes. Mi-2 mutants do not simply exhibit globally downregulated gene transcription. However, we intend to analyze the expression of other genes expressed in the nervous system to ensure that Mi-2 does not control general transcription in neurons, but more specifically promotes microtubule severing gene transcription to affect dendrite outgrowth and branching.

**Figure 39: Mi-2<sup>3D4</sup> Mutants do Not Show Reduced Levels of Stall Transcripts**

Gel image from RT-PCR amplifying stall cDNA. Lane 1 was loaded with wild-type <i>w<sup>1118</sup></i> cDNA and lane 2 was loaded with homozygous <i>mi-2<sup>3D4</sup></i> cDNA. Bands highlighted in red correspond to a 380 bp stall cDNA band.

### 4.9 Discussion and Conclusions

We have identified a genetic interaction between the transcription factor Mi-2 and the microtubule severing protein Kat-60L1 in Drosophila class IV neurons. Based on its effects in class I dendrite morphogenesis (Parrish et al., 2006), <i>mi-2</i> was the strongest
candidate gene out of the six identified in our overexpression eye screen for potential interactors with \textit{kat60-L1}.

We observed an interaction between \textit{mi-2} and \textit{kat-60L1} in the adult eye, as well as at the transcription level by whole-animal RT-PCR. Our data show that \textit{mi-2} mutants have reduced \textit{kat-60L1} transcript levels, suggesting that Mi-2 promotes of \textit{kat-60L1} transcription or that Mi-2 represses a transcriptional repressor of \textit{kat-60L1}. While our current experiments do not discriminate between these two scenarios, the latter seems more likely based on previous work on Mi-2 in a variety of organisms, which have consistently identified Mi-2 as a transcriptional repressor during development (Kunert et al., 2009; Passannante et al.; Unhavaithaya et al., 2002).

Based on our RT-PCR data, we hypothesized that genetically reducing the amount of Mi-2 in a \textit{kat-60L1} loss of function background would increase the severity of the \textit{kat-60L1} mutant phenotype due to even less Kat60-L1 protein in the double mutants. However, we observed the opposite: reducing the amount of Mi-2 suppressed the \textit{kat-60L1} mutant phenotype. As an established transcriptional repressor in many systems, the suppression of the \textit{kat-60L1} phenotype by a reduction of Mi-2 is most likely due to the modification of other Mi-2-regulated genes that normally antagonize Kat-60L1 function in class IV neurons. The dendritic phenotype observed in \textit{mi-2}, \textit{kat-60L1} double mutants is likely due to the modification of a separate dendritic branching pathway not
involving Kat-60L1. One example of such a pathway could involve other microtubule severing proteins, since we have shown that mi-2 mutants exhibit reduced levels of \textit{spastin} and \textit{katanin-60}. Interestingly, we also found that Spastin and Kat-60L1 function antagonistically to one another in class IV neurons (chapter 3, section 3.4); the suppression of the \textit{kat-60L1} dendritic phenotype by loss of Mi-2 mimics a similar suppression by loss of Spastin. One explanation for the suppression we observe with loss of Mi-2 could be the following: Mi-2 promotes the expression of \textit{spastin} (in addition to \textit{kat-60L1}), which antagonizes Kat-60L1 function (Figure 40). Therefore, loss of Mi-2 would result in a simultaneous reduction in Spastin, which normally antagonizes Kat-60L1 function, leading to genetic suppression. We propose Mi-2 functions in regulating the crucial levels of these two microtubule severing proteins, which we have shown can regulate distinct populations of microtubules within class IV da neurons (chapter 3).
Mi-2, possibly along with MEP-1 in the dMec complex, is a positive transcriptional regulator of *kat-60L1*, likely by indirectly repressing a repressor. In a parallel pathway, Mi-2 promotes *spastin* transcription, which negatively regulates Kat-60L1 function in the class IV neuron by an unknown mechanism (section 3.4).

We have identified Mi-2 as a transcriptional regulator of *kat-60L1*, as well as *spastin* and *katanin-60* and shown a novel role for Mi-2 function in the regulation of microtubule severing genes within a highly branched class IV sensory neurons. While Spastin and Kat-60L1 are both expressed in class IV neurons and are required for their complex dendritic arborization, we have shown that they function antagonistically to one another. Our data demonstrate a novel role for Mi-2 in regulating the expression levels of these two microtubule severing genes to achieve the proper dendritic morphology in the larval class IV da neurons. Our results are consistent with the results from Parrish et al., demonstrating that Mi-2 is an important transcriptional regulator for dendrite development.
4.10 Future Directions

To better understand the relationship between Mi-2 and Kat-60L1, it will be useful to investigate the effect of overexpressing Mi-2 in a \textit{kat-60L1} loss of function background. If Mi-2 represses a repressor of \textit{kat60-L1}, overexpression of Mi-2 in an otherwise wild-type background should increase levels of \textit{kat60-L1} and may phenocopy \textit{kat60-L1} overexpression, resulting in altered branching and dendritic coverage. It will be interesting to overexpress Mi-2 in a partial \textit{kat-60L1} loss of function background to test what the effects on the \textit{kat-60L1} mutant phenotype will be. If Mi-2 represses a \textit{kat-60L1} repressor, we may see suppression of the \textit{kat-60L1} dendritic phenotype due to restored levels of Kat-60L1. However, given that we observe \textit{kat-60L1} suppression when reducing Mi-2 function (similar to reducing Spastin function), we may see a worsening of the \textit{kat-60L1} phenotype if Mi-2 is primarily acting in a parallel pathway (such as through promoting \textit{spastin} expression), in which Mi-2 primarily antagonizes Kat-60L1 function.

Our RT-PCR results with MEP-1 in a \textit{mi-2} mutant background and the identification of MEP-1 as an interactor with \textit{katanin-60} in an independent screen suggests a possible joint role for both Mi-2 and MEP-1 in regulating the expression of microtubule severing proteins in the nervous system. Previous work has suggested the mutual stability of Mi-2 and MEP-1 in a complex through their co-localization in cell nuclei, as well as shown a role for a Mi-2/MEP-1 protein complex called dMec in the
repression of proneural genes (Kunert et al., 2009). In the future, it will be interesting to
determine if dMec is the Mi-2-containing complex responsible for mediating the genetic
interaction between mi-2 and kat60-L1 observed in the class IV sensory da neurons. One
approach to this question would be to test whether mep-1 mutants phenocopy the mi-2
suppression of the kat-60L1 mutant phenotype in the class IV neurons. Microarray
experiments on mi-2 mutants could provide a more global view of the genes regulated
by Mi-2 that may function in dendrite morphogenesis in concert with kat-60L1 and
spastin.

To further our understanding of the regulation of Kat60-L1, the Sherwood
laboratory can continue to follow up on other potential kat-60L1 interactors identified
from the initial eye screen. Discovery of additional points of regulation of Kat60-L1 at
the transcriptional, translational, and protein-protein interaction/modification level, will
help shed light on how proper levels of this microtubule severing protein and its activity
are maintained for proper developmental dendritic outgrowth and branching to occur.

4.11 Materials and Methods

Drosophila stocks and crosses: The P[LacW] insertion line mi-23D4 was obtained from the
Bloomingston Drosophila Stock Center. The pBac insertion line kat-60L1co564 was obtained
from the Exelixis Collection at Harvard Medical School, and the kat-60L1^{ble} deletion is
described in section 2.10. All animals were kept on yeast-sugar-agar media at room
temperature (approximately 22 degrees Celsius) or in a humidified incubator during crosses at approximately 25 degrees Celsius.

For the double mutant analysis, the recombinant animals, *ppk1.9-GAL4 UAS-mCD8::GFP;; mi-2* /3D4, *kat-60L1* /3D4, *Actin-GAL4 UAS-mCD8::GFP* were crossed to *kat-60L1* /BE6/TM3, *Actin-GAL4 UAS-mCD8::GFP* animals to generate larvae of the following genotype: *ppk1.9-GAL4 UAS-mCD8::GFP;; mi-2* /3D4, *kat-60L1* /3D4, *kat-60L1* /BE6. The transheterozygous *kat-60L1* loss of function background used in all experiments consisted of one *kat-60L1* allele and *kat-60L1* BE6. The three other control genotypes were the following: wild-type controls, *ppk1.9-GAL4 UAS-mCD8::GFP;; kat-60L1* loss of function animals, *ppk1.9-GAL4 UAS-mCD8::GFP;; kat-60L1* /3D4, *kat-60L1* /BE6; and *mi-2* /3D4 heterozygous animals, *ppk1.9-GAL4 UAS-mCD8::GFP;; mi-2* /3D4/+

**Overexpression eye screen:** *GMR-Gal4/CyO; UAS-Kat-60L1 /TM6b* flies were crossed to individual EP insertion lines on chromosome 3, a kind gift from Amy Bejsovec, who originally obtained them from the Bloomington Drosophila Stock Center as part of the disruption kit created in the BDGP Gene Disruption Project. Progeny heterozygous for the EP insertion, the *GMR-Gal4* driver, and the *UAS-Kat-60L1* were compared to their sibling controls, which were heterozygous for *GMR-Gal4* and *EP* alone. Eye size, texture, and necrosis were evaluated to determine the interaction between Kat-60L1 and the gene represented by each EP line. For potential interactors, heads were imaged at 500X with a
Fujifilm E550 camera at 4X optical zoom.

**PCR and Reverse Transcription-PCR:** 40-50 first-instar larvae of each genotype were flash frozen in liquid N$_2$, and RNA was isolated using the RNAspin Mini/Nucleospin kit from GE Healthcare/Clontech. RNA concentration was measured using UV Spectrophotometry and 1µg RNA was used for cDNA synthesis with Bioline reverse transcriptase. PCR amplification of the loading control, *GAPDH2*, was performed with the following primers: GAPDH2 RTForward: (5’-GCA AGC AAG CCG ATA GAT AAA C-3’) and GAPDH2 RTReverse: (5’-TCG ATG AAG GGA TCG TTG AC-3’). *GAPDH2* RTForward primer sequence spans the exon-intron border so that only cDNA is amplified. PCR amplification of *mi-2* cDNA and genomic DNA was performed with the following primers: Mi2x3For: (5’-AAG CGC AAG AAG AAC GAG AG-3’) and Mi2x4Rev: (5’-CTC CAC ATT GCA GAC GCT AA-3’). The former is within exon 3 and the latter in exon 4. This primer set produces a 195 base pair cDNA band and a 322 base pair genomic band. Primers for the amplification of *kat-60L1* cDNA (both the long and short isoforms) are described in section 2.10. PCR amplification of *Su(Tpl)* cDNA was performed with the following primers: Su(Tpl) x1_2For (5’-ATT CAA GCG TTC CTT CCA TAT T-3’) and Su(Tpl) x1_2Rev (5’-CAA CCA AAA AAA CCA ACA ATA AC-3’). The primers spanned exons 1 and 2, so they were cDNA specific and produced a 710 base pair band. PCR amplification of Katanin-60 cDNA was performed with the
following primers: 6242-64rkat60 (5’-CTC CTA CAA TCG CTA GCC ATT CG-3’) and 5703kat60 (5’-AGA GGA ACA GCC CAA GGT G-3’). The primer set produced a 550 base pair genomic band and a 450 base pair cDNA band. PCR amplification of spastin was performed with the primers, spas F8: GAACCATCCTTCCGGAGCTGAAC and spas B9: CCTGGTCTTCTGGCTTTG. This primer set produced a ~500 base pair cDNA band and an 800 base pair genomic band. PCR amplification of MEP-1 cDNA was performed using the MEP-1 5’_exon 5 and MEP-1-3’_exon7 primers. PCR amplification of Stall was performed using 3622 F1C/3622 RTBR primers.

**mi-2**<sup>3D4</sup> and **kat-60L1**<sup>Pbac</sup> Recombination: Mi-2 and kat-60L1 genes are both on the third chromosome (3R) and we recombined the **mi-2**<sup>3D4</sup> mutation onto the chromosome containing the **kat-60L1**<sup>Pbac</sup> allele for use in double mutant analysis and confirmed recombinants using PCR. To amplify the genomic region containing the **P{LacW}** insertion in mi-2, we used Mi-2 3’ Rev (5’-TGT GCA TTG GCG TAG GAA TA-3’) and PlacW 3’ For-1 (5’-CTC GCA CTT ATT GCA AGC AT-3’) primers. One primer was in the Mi-2 gene and the other was in the insertion, so the primer set produced a 500 bp band only when the insertion was present in the genome. To amplify the genomic region containing the piggy-Bac insertion in Kat-60L1, we used primers pB-3Seq:

CGATAAAACACATGCGTCAATT and KLintLPB 3’ For:

TCCCCAATTTTCTCCCCAAA. Since one primer was within a Kat60-L1 intron and the
other was within the piggy-Bac insertion, the primer set produced a 150 base pair band only when the insertion was present in the genome.

**Immunostaining:** Wandering third-instar larvae were dissected, fixed and immunostained as described in section 2.8. The primary antibody used was rabbit anti-GFP (1:500 dilution in PBTNA, from Invitrogen). Secondary antibody, goat anti-rabbit Alexa-488 was obtained from Invitrogen (1:200 in PBTNA). After immunostaining, samples were mounted in Vectashield from Vector Laboratories, Inc., Burlingame, CA.

**Neuron Visualization:** Dendritic images were obtained using a Zeiss LSM 510 inverted confocal microscope and converted to projections of Z stack images using LSM Image Browser software. One to two neurons per larva from abdominal segments 3 or 4 were used for analysis. Four to six larvae of each genotype were used for white space analysis. For white space or dendritic coverage analysis, a grid of 500μm² boxes created in Image J was layered over the dendritic arbor. The number of boxes containing only white space was subtracted from the total number of boxes to obtain a value for the number of boxes containing dendritic coverage. The number of boxes with dendritic coverage was divided by the total number of boxes to contain a value for percent coverage.
Appendix

Loss of Katanin p60-like 1 Does Not Result in Defective Chordotonal Organs

A reduced light touch behavioral response was observed in a \textit{kat-60L1} loss of function background, in addition to the reported nocifensive defects, suggesting a possible role for Kat-60L1 in mechanosensation. The light touch behavioral assay was designed by Kernan M et al. for a screen to identify genes involved in mechanosensation, which led to the identification of several \textit{Drosophila unc} (uncoordinated) genes involved in mechanosensory bristle function (Kernan et al., 1994). The simple assay involves stroking a crawling larva across the anterior region with an eyelash and scoring the larva’s response on a scale from 0-4, 0 indicating no response and 4 indicating a robust wild-type response of multiple contractile waves backward (Figure 41A). A larva is subjected to four trials, which are averaged for a maximum score of 16. In this assay, \textit{kat-60L1} loss of function backgrounds exhibited significantly reduced light touch responses compared to WCS controls, as well as animals bearing a precise excision event from the P-element excision screen (denoted \textit{AY3}) (Figure 41B).

Chordotonal organs are ciliated stretch-activated mechanosensors in the fly that have been implicated in the light touch response. They are internal stretch organs located in the subepidermal layer, one of the major sensory organs in the peripheral nervous system. Each scolopidium is composed of support cells: the ligament and
scolopale cell; a neuron, and a cap cell near the cuticle. Chordotonal organs in \textit{kat-60L1} mutant larvae were examined using immunohistochemistry and confocal imaging; however, microtubule distribution of the cilia and cell morphology of the scolipidium in the chordotonal organs appeared normal compared to controls (Figure 41C-F). Therefore, Kat-60L1 does not affect the gross composition of the chordotonal organs and further analysis using electron microscopy would be required to detect a phenotype at the ultrastructural level.
Figure 41: *Kat-60L1* Mutant Larvae Exhibit a Reduced Light-Touch Response, but Normal Chordotonal Organ Morphology

A) Schematic of the light-touch assay (from Kernan M et. al, 1994) where a larva elicits a single response to gentle touch, designated 0 (least responsive) through 4 (most responsive). (B) When subjected to a light-touch, *kat-60L1* BE6/PBac mutant larvae exhibit reduced sensitivity compared to control larvae (*wcs*) and animals containing a precise excision event (*AY3*) (*WCS* n = 11, *AY3* n = 20, *PBac/PBac* n = 20, *BE6/BE6* n = 30, *BE6/PBac* n = 11; p<0.004 or less for all *kat-60L* mutant backgrounds when compared to controls). (C) A wild-type and (D) *kat-60L1* BE6/PBac mutant chordotonal organ immunostained for HRP and Futsch exhibit similar morphology of ciliated neurons and scolopidium. (E) A wild-type and (F) *kat-60L1* BE6/PBac mutant chordotonal organ immunostained for α-tubulin and Futsch exhibit similar morphology of ciliated neurons and scolopidium.
References


Biography

Andrea Stewart was born in 1979 in California, USA. From 1997-2001 she attended the University of California, Davis where she obtained her Bachelor of Science degree in Genetics, with honors. She then worked in the laboratory of Mark Krasnow at Stanford University as a research assistant from 2001-2004 and conducted independent research on the Drosophila tracheal system. She attended the Physiology Summer Course at the Marine Biological Laboratory at Woods Hole in 2005. In 2006 she was awarded a National Science Foundation research fellowship for her Ph.D. studies at Duke University, where she carried out her dissertation work in the lab of Nina Sherwood. Her manuscript entitled “The microtubule severing protein Katanin p60-like1 specifically promotes dendrite complexity and function” is in the submission process for publication. She was awarded her Ph.D. in December of 2011.